

# **Microbiological and Food Safety Aspects of Tempeh Production in Indonesia**

Dissertation

to obtain the Ph.D. degree

in the International Ph.D. Program for Agricultural Sciences in Göttingen (IPAG)

at the Faculty of Agricultural Sciences,

Georg-August-University Göttingen, Germany

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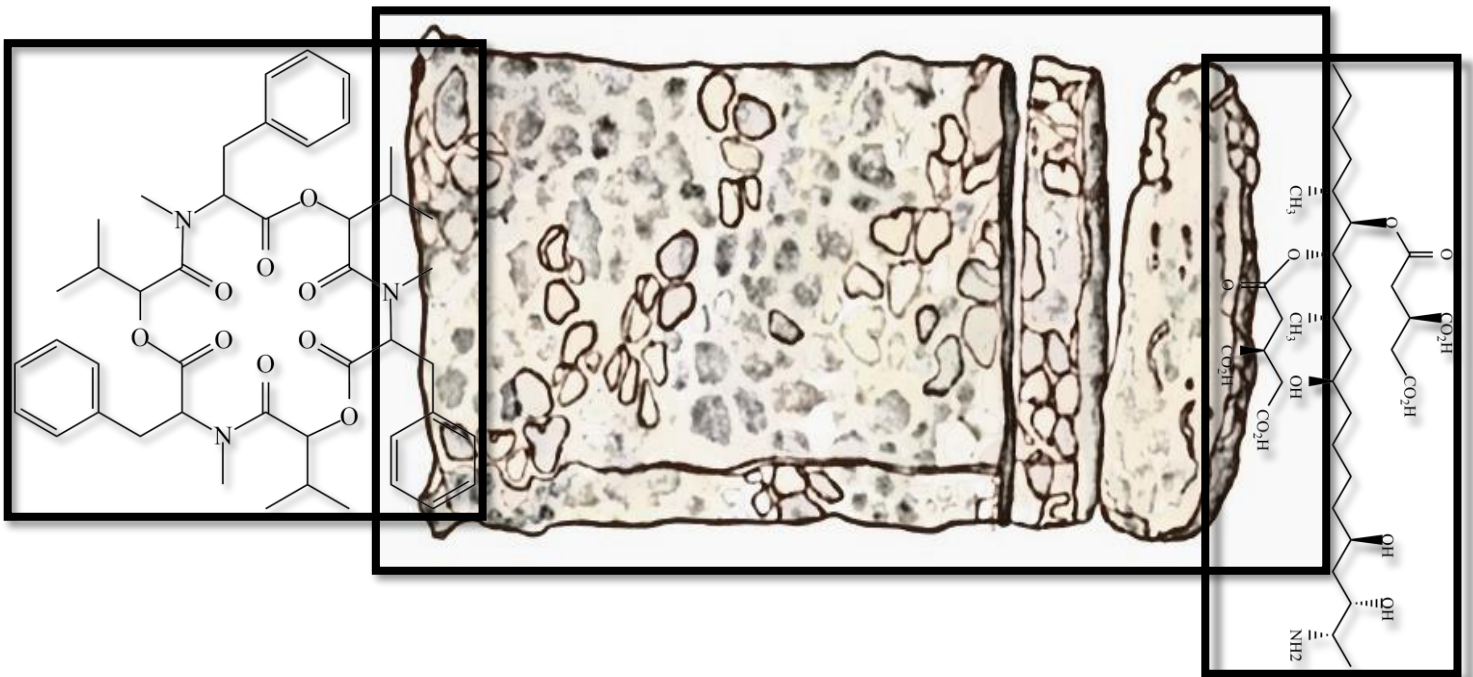
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Date of dissertation: January 25<sup>th</sup> 2018

”Food can not be cheap, local, green, safe and veried all at the same time”  
(The Economist, March 2001)

“Food for thought and thinking for food”  
(Crowther, J.R.)

“Knowing is not enough; we must apply. Willing is not enough; we must do”  
(Goethe)



## Contents

### Chapter 1

<b>General Background.....</b>	<b>1</b>
1. Tempeh and Its Benefits .....	1
2. Tempeh Production .....	2
3. The Role of Microorganism in Tempeh Production .....	3
4. Potential Biosafety Issues in Tempeh Production .....	6
5. Could Masked Zearalenone be Generated during Food Fermentation ? .....	9
6. Current Food Safety Conditions in Indonesia.....	11
7. Aim of the Work .....	15
8. References.....	16

### Chapter 2

<b>REVIEW ARTICLE 01: Mycotoxins in Indonesian Foodstuffs: Occurrence, Prevention, and Remedial Methods .....</b>	<b>21</b>
Abstract .....	21
1. Introduction.....	21
2. Factors Promoting Mycotoxins Contamination in Indonesian Foods.....	22
3. Occurrence of Mycotoxins in Indonesia .....	23
4. Health Impact of Mycotoxins in Indonesia.....	31
5. Updated Risk Assessment of Maize and Peanut Consumption in Indonesia.....	32
6. Mycotoxin Regulation in Indonesia.....	35
7. Mycotoxin Control in Indonesian Foodstuffs .....	38
8. Challenges and Future Perspectives.....	48
9. References.....	49

### Chapter 3

<b>RESEARCH ARTICLE 01: Determination of Fungal Diversity in Indonesian Tempeh, and Update on Their Safety Status .....</b>	<b>56</b>
Abstract .....	56
1. Introduction.....	56
2. Materials and Methods.....	59
3. Results and Discussion .....	67
3.1. Results of Part I (Fungal Diversity and Identification).....	67
3.2. Discussion of Part I (Fungal Diversity and Identification).....	85
3.3. Results of Part II (Safety Status of Tempeh Fungi).....	89
3.4. Discussion of Part II (Safety Status of Tempeh Fungi) .....	96



3.5. Results of Part III (Zearalenone Biotransformation by Tempeh Fungi).....	99
3.6. Discussion of Part III (Zearalenone Biotransformation by Tempeh Fungi)....	102
4. Conclusion .....	105
5. Acknowledgement .....	105
6. References.....	106
7. Appendix.....	110

## Chapter 4

<b>RESEARCH ARTICLE 02: Mycotoxin Producing <i>Fusarium</i> Species in Indonesian Traditional Tempeh and Starter (<i>Usar</i>).....</b>	<b>120</b>
Abstract .....	120
1. Introduction.....	121
2. Materials and Methods.....	123
3. Results and Discussion .....	136
3.1. Results of Part I ( <i>Fusarium</i> Isolates Identification and Their Mycotoxin Screening) .....	136
3.2. Discussion of Part I ( <i>Fusarium</i> Isolates Identification and Their Mycotoxin Screening) .....	144
3.3. Results of Part II (Detection of <i>Fusarium</i> and Mycotoxin in Traditional Tempeh) .....	147
3.4. Discussion of Part II (Detection of <i>Fusarium</i> and Mycotoxin in Traditional Tempeh) .....	151
3.5. Results and Discussion of Part III (Traditional Tempeh Quality and Risk Assessment) .....	155
4. Conclusion .....	158
5. Acknowledgement .....	158
6. References.....	159
7. Appendix.....	162

## Chapter 5

<b>REVIEW ARTICLE 02: Fighting Toxigenic Fungi in Food Industry by Applying Lactic Acid Bacteria: A Review of Established and Recent Research.....</b>	<b>164</b>
Abstract .....	164
1. Introduction.....	164
2. Overview of Lactic Acid Bacteria .....	166
3. Lactic Acid Bacteria as Antifungal Agent.....	167
4. Antifungal Compounds Produced by Lactic Acid Bacteria.....	169

5. Genetic Engineering to Improve Lactic Acid Bacteria Antifungal Properties .....	185
6. Application of Antifungal Lactic Acid Bacteria and Prospect in Food Industry....	188
7. Future Research Trends .....	190
8. References.....	191

## Chapter 6

<b>RESEARCH ARTICLE 03: Diversity and Biological Activity of Yeast and Lactic Acid Bacteria from Soak Water in Tempeh Production.....</b>	<b>201</b>
Abstract.....	201
1. Introduction.....	201
2. Materials and Methods.....	204
3. Results and Discussion .....	214
3.1. Results of Part I (Identification and Characterization of Tempeh Yeast and Lactic Acid Bacteria) .....	214
3.2. Discussion of Part I (Identification and Characterization of Tempeh Yeast and Lactic Acid Bacteria) .....	221
3.3. Results of Part II (Antifungal Activity of Tempeh Yeast and Lactic Acid Bacteria).....	223
3.4. Discussion of Part II (Antifungal Activity of Tempeh Yeast and Lactic Acid Bacteria).....	228
3.5. Results of Part III (Zearalenone Biotransformation by Tempeh Yeast and Lactic Acid Bacteria) .....	230
3.6. Discussion of Part III (Zearalenone Biotransformation by Tempeh Yeast and Lactic Acid Bacteria) .....	233
4. Conclusion .....	235
5. Acknowledgements.....	235
6. References.....	235
<b>General Discussion.....</b>	<b>239</b>
<b>Summary.....</b>	<b>244</b>
<b>Acknowledgements .....</b>	<b>247</b>
<b><i>Curriculum vitae</i> .....</b>	<b>249</b>

## List of Abbreviations

BEA	Beauvericin
BMDL	Benchmark dose lower limit
bw	Body weight
CFS	Cell free supernatant
CFU	Colony forming unit
CTAB	Cetyl trimethylammonium bromide
ENN	Enniatin
ESI	Electrospray interface
FUM	Fumonisin
GAP	Good agricultural practices
GMP	Good manufacturing practices
GRAS	Generally recognized as safe
HPLC-MS/MS	High performance liquid chromatography-tandem mass spectrometry
ITS	Internal transcribed spacer
LA	Lactic acid
LAB	Lactic acid bacteria
LD	Lethal dose
LOD	Limit of detection
LOQ	Limit of quantification
MEA	Malt extract agar
MRS	De Man, Rogosa and Sharpe
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PDB	Potato dextrose broth
PDI	Probable daily intake
PLA	Phenyllactic acid
RAPD	Random amplified polymorphic DNA
RH	Room humidity
RPM	Revolutions per minute
SEM	Scanning electron microscopy
SPC	Standard plate count
TDI	Tolerable daily intake
TEF	Translation elongation factor
TTA	Titrateable acidity
UPLC-MS/MS	Ultra performance liquid chromatography-tandem mass spectrometry
UV	Ultraviolet
v/v	Volume per volume
w/v	Weight per volume
ZEN	Zearalenone

## Chapter 1: General Background

### 1. Tempeh and Its Benefits

Tempeh is traditional food from Indonesia made by fermenting soybeans with mold *Rhizopus* spp. It has a solid form, distinctive aroma and is white and slightly gray in color (INCS, 2009). Besides of the Indonesian National Council for Standardization (INCS), the standard of tempeh is also defined in the Codex Alimentarius (CAC, 2013) as compact, white, cake-form product, prepared from dehulled-boiled soybeans by solid state fermentation with *Rhizopus* spp. Moreover, Codex Alimentarius also assesses the organoleptic quality of tempeh, among others: (1) compact texture and not easily disintegrated when cut 2) white color growth of *Rhizopus* spp., 3) nutty, meaty, and mushroom-like flavor, 4) fresh tempeh should not smell of ammonia.

Historical records reveal tempeh originates from ancient *Javanese* as mentioned in *Serat Centhini* vol. 3 (1814), depicting tempeh as royal menu of Sunan Giri served in Java during the 17<sup>th</sup> century (Astuti, 1999a, Purwadaria et al., 2016) as follows: “ *Jangan menir ulur - pitik, brambang kunci sambel sinantenan, brambang jae santen Tempe, ....* ” (Chicken eggs sauteed with spinach, hot sauce with coconut milk, shallot and ginger root, Tempe in coconut milk with shallot and ginger ...). In 1950, research on the changes of the chemical, microbial and nutritional value during fermentation was conducted by Dutch researchers. Thus tempeh gained popularity in Europe. Tempeh was also known in the United States since 1946. American scientists have conducted research on tempeh since 1960, including Steinkraus, Hesseltine, and Wang who started their commercial tempeh company (Karyadi, 1996).

Indonesia is known for the largest tempeh production in the world and has the largest soyfood market in Asia. In 2012, approx. 60% of soybean produced in Indonesia was for self-sufficiency as tempeh at a national level, with the annual consumption amounting to 8.5 kgs/person/year (BPS, 2012). About 2.4 million tons of tempeh produced a year by more than 81 thousand tempeh producers. Only 600 thousand of the 2.2 million tons soybeans used each year is grown in Indonesia, the rest is imported from USA to meet the demand (INCS, 2012).

Tempeh is beneficial, compared to other healthy food. Its raw materials, soybeans, contain on average 40% protein, 20% fat, 35% carbohydrates, and 5% ash (Liu, 1997). The soybean processing into tempeh degrades macromolecules into smaller units, so that it can easily be digested and utilized by the body (Nout and Kiers, 2005). The concentrations of some vitamins of the B-group (B12, riboflavin, pyridoxine, niacin, biotin, and folic acid) are higher in tempeh than in soybeans (Denter et al., 1998). Besides, tempeh also heals chronic diarrhea in children quickly because of antibacterial compounds inhibiting the growth of *Salmonella typhi*, *Shigella flexneri*, and *Escherichia coli* 0125 K70 (Hermana, 1995; Roubos-van den Hil and Nout, 2011). According to Murata (1985), FAO considers tempeh as one of the best sources of protein.

Antioxidants are essential for protecting the body from free radicals that can cause carcinogenesis as well as other age-related diseases (Cutlar, 1992). 6,7,4-Trihydroxyisoflavone (factor 2) (Klus et al., 1993) and 3-hydroxyanthranilic acid (HAA) (Esaki et al., 1996) are antioxidants found in tempeh. Beta-carotene produced by *Rhizopus* strains during tempeh fermentation provide an alternative solution for those who suffer from vitamin A deficiency (Denter et al., 1998). Moreover,

tempeh with iron fortification is able to overcome Anemia (Astuti, 1999b). Isoflavone aglycone (daidzein and genistein) and gamma amino butyric acid produced during tempeh fermentation are also beneficial for health (Nakajima et al., 2005; Handoyo and Morita 2006). A recent study in Malaysia showed that tempeh can be used as a non-dairy source of calcium (Haron et al., 2010). Intervention studies in humans also showed that tempeh provides hypolipidemic effects for hyperlipidemic patient (Roubos-van den Hil and Nout, 2011; Utari, 2011) and hypoglycemic activity for diabetic mellitus patient (Aitoman, 2011).

## 2. Tempeh Production

Tempeh-producing technology has been handed down through generations and was changed based on experience. It is diverse, but basic similarities are shared among producers, namely the boiling, dehulling, acidification, washing, inoculation, bagging, and incubation of soybeans (Figure 1). However, alterations in the processing is made in accordance means and resources which are available (Karyadi, 1996).

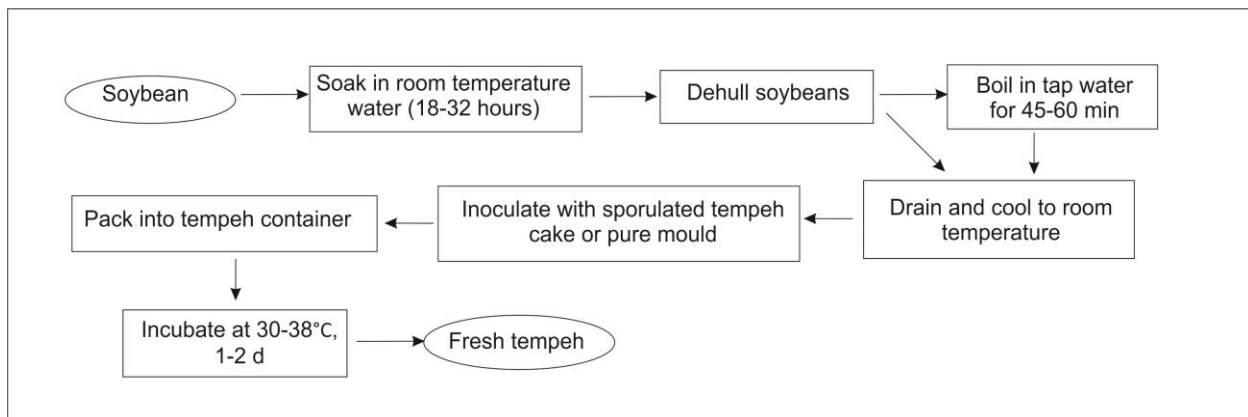


Fig 1. Production process of soybean tempeh in general.

During the first stage soybeans are boiled, dehulled, soaked, and washed. All producers use the same steps. The second stage may vary. Some drain the soybeans processed in the first stage directly and mix them with the mold inoculum, bag them, and go on to incubation. Others, reboil the soybeans, cool them, mix them with inoculum, bag them, and then go to the third stage, incubation. Other producers mix the inoculum together with soaked soybeans, after which the soybeans are drained, bagged, and incubated (Hermana, 1996). Incubation is the main stage of tempeh production (Kasmidjo, 1995). It varies not only in terms of stage but also the approach. Producers modify, for example, the soaking time, boiling time, inoculum type and the way inoculum is added, packaging materials, the methods of packaging, and methods of incubation (Hermana, 1996).

The procedure prior to fungal fermentation can support the growth of the mold on soybeans substrate. Boiling and soaking will hydrate soybeans, which in turn increase tenderisation of the cotyledons. Soybeans hydration results in the increasing water activity ( $a_w$ ) to 0.99 in environment, in which molds grow best. Boiling soybeans will also kill most microorganisms,

tenderize the soybean tissue so that mycelia penetrate easily, and increase water (moisture) content of the soybeans in order to make soybean dehulling easier. During the soaking period, acidification by lactic acid bacteria (LAB) occurs, thus the pH drops to less than 5. Washing is done to dehull the beans, eliminate the mucilage and lower the acidity during the soaking period. After inoculation with the starter culture, *Rhizopus spp.* is the main microorganism responsible for tempeh fermentation. Meanwhile, packaging does not only serve as an oxygen barrier but also provides optimum conditions for mold growth. When incubation temperature is between 20° to 37°C mold growth is optimal (Hermana, 1996; Karyadi, 1996). During the incubation (fermentation), the mold mycelium grows and binds with bean cotyledons (Nout and Kiers, 2005). The fermentation may take 18 to 48 hours (Mulyowidarso et al., 1989). Sudarmadji (1977) divides the fermentation process into three phases:

- 1). Rapid growth phase (0-30 hours of fermentation) which is the initial phase. At this stage free fatty acids increase, the temperature increases, the mold grows rapidly and mycelia form on the surface of soybean.
- 2). Transition phase (30-50 hours of fermentation) is the optimal fermentation phase where fresh tempeh is formed that is ready to be consumed. In this step, the temperature decreases, some fatty acids are released and mold growth is almost constant or increases slightly, the specific tempeh flavor is formed, and the tempeh texture is more compact.
- 3). Further phases of fermentation (50-90 hours of fermentation). In this phase, there is a rise in the number of bacteria and the amount of free fatty acids, a reduction in mold growth, the mold stops growing at a certain water content and the flavor changes due to protein degradation into ammonia.

### **3. The Role of Microorganisms in Tempeh Production**

Complex of microbial communities colonize in soybean tempeh, their development start during the soaking of the raw ingredients (Nout and Kiers, 2005). These microorganisms are involved in several stages. They are responsible for the acidification during the soaking stage, there may be cross-contamination through the handling during hand drying and packaging, during the incubation and in the composition of inoculum. The essential microorganisms involved in fermentation of tempeh comprise molds and bacteria. Mold mycelium is essential for tempeh production since tempeh is considered excellent when soybean cotyledons are bound together by the mycelium. Meanwhile, bacteria are required for the acidification of soybeans.

Tempeh is typically produced by home industry on a small-scale basis with poorly controlled fermentation. The fermentation is inadequately performed under aseptic conditions although the starter culture containing fungi is added at the beginning of the fermentation. Various organism contribute to tempeh fermentation (Barus et al., 2008; Seumahu et al., 2013). This causes variations in flavor and quality of tempeh in Indonesia.

Molds that play the most important role in fermentation include the genera *Rhizopus* belonging to the family *Mucoraceae*, of the order *Mucorales*, in the subclass *Zygomycotina* of the class *Zygomycetes*. This genus is classified into several species (Hesseltine, 1985). The most

important ones are *Rhizopus oligosporus*, *R. arrhizus*, *R. stolonifer*, *R. oryzae*, *Mucor* sp. and *Aspergillus* sp. (Pawiroharsono, 1994). During tempeh production, *Rhizopus* is responsible for the increase in soluble proteins, fats, and carbohydrates in soybeans (Jutono, 1985). However, each species produces tempeh of varying quality. The highest concentration of free amino acids is found in *R. oligosporus* tempeh, compared to *R. stolonifer* and *R. oryzae* (Keuth and Bisping, 1994). *Rhizopus* sp. produce enzymes that are found to be able to degrade soybean substrates, such as carbohydrases (polygalacturonase, endocellulase, xylase, and arabinase), besides lipases and proteases (Nout and Kiers, 2005). During tempeh fermentation by a single culture *R. oligosporus*, raffinose content is reduced by 60% and stachyose content by 10%; sugar content increased by 700mg/100g; free amino acids by 70 mg/100g (Egounlety and Aworh, 2003).

Bacteria are important for soybeans acidification during tempeh production. However, it is not known whether bacteria are important in other processes during tempeh production. In subtropical regions, natural acidification of soybeans during soaking proceeds either very slowly or does not occur at all (Liu, 1997). During chilly weather, lactic acid fermentation does not work well. To obtain desired pH, the dehulled beans are hydrated in water or 0.85% lactic acid solution for 2 hours at 25°C or 30 minutes at 100°C (Nout and Kiers, 2005),  $\leq 0.5\%$  lactic acid or  $\leq 0.25\%$  acetic acid is added. In the tropics the rapid growth of bacteria starts during soaking, with a bacteria plate count of up to  $4.4 \times 10^9$  CFU/g (Nuraida et al., 2008). The soak water is rich in simple sugars such as glucose, fructose, and galactose. Glucose serves as primary substrate for microbial growth in soak water. Invertases and  $\alpha$ -galactosidases on soybean produce simple sugars in the soak water. Bacteria and yeasts are found in the soak water (Mulyowidarso et al., 1991).

Moreno et al. (2002) investigated the growth of lactic acid bacteria during tempeh production in Malaysia. Count of LAB, bacteria on raw beans were low ( $<10^2$  CFU/g). The number of LAB on the soak water increased to 7.9-9.3 log CFU/mL at the end of the soaking process and 7.8-9.2 log CFU/g in the soaked soybean. Second boiling of the beans results in reductions of LAB populations to less than 4 log CFU/g. The number increased sharply to 6.8- 9.9 log CFU/g in final product (tempeh) (Moreno et al. 2002). Nuraida et al. (2008) and Efriwati et al. (2013) reported change in LAB number during tempeh preparation at a lab scale. Nuraida et al. (2008) reported that LAB population on soaked soybeans reached 9 log CFU/g. However, the number decreased to 6 log CFU/g on tempeh. Efriwati et al. (2013) reported two methods applied in producing LAB in tempeh preparation, namely one- and two-time boiling steps. At the end of soaking process, number of LAB produced from these two methods reached 6 log CFU/g. When soybeans were boiled once (one time boiling step), LAB number increased to 8 log CFU/g, greater than the population results in two-time boiling steps i.e. 6.5 log CFU/g.

According to Ashenafi and Busse (1991b), LAB have been shown to contribute to the safety of tempeh through the decrease in pH, the production of organic acids, and metabolites that act as inhibiting agents against pathogens. The lower pH can only inhibit contamination of spoilage bacteria, but not the growth of *Rhizopus* sp. If the natural acidification does not occur, tempeh production is susceptible to undesirable contaminants, pathogens and spoilage microorganisms (Mulyowidarso et al., 1991b; Hidayat et al., 2006). During the soaking process,

LAB will produce lactic acid as a major fermentation product besides of other organic acids (Table 1) (Nout and Kiers, 2005). Acidification can also provide a good environment for spore germination and result in a reduction of the lag time of *R. oligosporus* (Nour and Kiers, 2005). Moreno et al. (2002) reported that the LAB *Enterococcus faecium* isolated from overripe tempeh also produce of bacteriocins inhibiting the growth of *Listeria monocytogenes*, *Bacillus pumilus* and *Clostridium sporogenes* on growth medium. Further reports exist on the interaction between LAB and pathogenic bacteria in tempeh. For example, *Lactobacillus plantarum* and *L. brevis* that were isolated from soak water inhibited the growth of *S. aureus* and the production of *Staphylococcus* toxins (Tuncel and Goktan, 1990); co-cultures of *L. plantarum* (1) reduced the number of *B. cereus* by 1 log CFU/g (Ashenafi and Busse, 1991); (2) inhibits the growth of *Listeria monocytogenes* significantly (Ashenafi, 1991a); and (3) also inhibits *Salmonella infantis* and *E. coli* in soybean (Ashenafi, 1991b) but the origin of the isolates of *L. plantarum* has not been revealed.

Table 1. Organic acid accumulation (% w/v) on the soak water with a natural fermentation, pure-culture inoculation and back-slopping<sup>a</sup>.

Fermentation <sup>b</sup>	pH	Lactic acid	Malic acid	Acetic acid
Spontaneous	4.5	0.6	0.2	0.07
<i>Enterococcus faecium</i>	-	1	0.02	0.15
<i>Lactobacillus acidophilus</i>	4.2	1.61	-	0.14
<i>L. casei</i>	-	1.1	0.2	0.05
<i>L. plantarum</i>	4.15	1.78	-	0.14
<i>Pediococcus pentosaceus</i>	4.24	1.56	-	0.16
Back-slopping	4.12	2.14	-	0.29

<sup>a</sup>source: Nout and Kiers (2005), Soaking was carried out with 300 g soybeans in 900–1000 mL tapwater during 24 hours at 30°C, without inoculum (natural), with addition of 10<sup>4</sup> CFU/mL soak water of pure cultures of lactic acid bacteria, or with addition of 3% v/v previously fermented soak water (back-slopping).

Lactic acid bacteria contribute positively to the safety of tempeh because the fermentation results in the formation of organic acids that inhibit the growth of pathogens and spoilage microorganisms in soybean. Microorganisms that are commonly found during the production process are from the genus *Enterobacillus* such as *Lactobacillus* spp., and *L. plantarum* (Pawiroharsono, 1994). Lactic acid bacteria which dominate during the soaking stage cause a significant increase of organic acids (Nout and Kiers 2005). The main organic acids contained in soybeans soak is lactic acid (Sparringa and Owens, 1999; Nout and Kiers, 2005). The main microorganisms for tempeh production are from *Rhizopus* species. However, fresh tempeh also contains mesophilic aerobic bacteria, enterobacteria, staphylococci, and yeasts. The presence of bacteria generally does not inhibit the fermentation process (Ko, 1985). In Indonesian tempeh, the levels of lactic acid bacteria were 10<sup>9</sup>-10<sup>10</sup> CFU/g, and *Enterobacteriaceae*, bacterial spores and total aerobic mesophilic bacteria (except for LAB) were 10<sup>8</sup>-10<sup>9</sup>, 10-10<sup>2</sup> and 10<sup>8</sup>-10<sup>9</sup> CFU/g respectively (Han et al., 1999).

Commonly, research on LAB and yeast at some stages of tempeh production process is conducted in the laboratory, not in the tempeh industry. The presence of these microbes in a sample



of tempeh has been reported, but only from the final product (tempeh from a market). It was not reported during production process comprehensively, especially the soybean soaking process. Generally, research on LAB and yeast are based on conventional approach which takes time, expense much effort, and usually is limited on microbial assessment (Aslam et al., 2010). Up until now, LAB study of tempeh production is limited to the value of colony forming units (CFU). Lactic acid bacteria populations for tempeh production has been screened for tempeh producer in Malaysia (Moreno et al., 2002) and only laboratory research level in Indonesia (Nuraida et al., 2008). Neither Moreno et al. (2002) and Nuraida et al. (2008) did not specifically study about yeast population, which was included in the value of CFU fungi. Moreover, the study about the diversity of LAB and yeast during the soaking of mixture material (such as soybean-corn, soybean-rice) has never been investigated, except for a list of some types of LAB and yeast which ever found in tempeh and only characterized phenotypically (Samson et al., 1987; Mulyowidarso et al., 1990; Ashenafi, 1991; Ashenafi and Busse, 1991).

#### **4. Potential Biosafety Issues in Tempeh Production: Food, Toxication or Infection?**

Like many other traditional foods in Indonesia, tempeh is produced by a small scale home industry with poorly controlled fermentation that is carried out without sufficient hygienic precautions. Therefore, various types of microorganisms can participate in the process of fermentation, and natural contamination is always possible (Winarno, 1985; Barus et al., 2008; Seumahu et al., 2013). Moreover, there is no standard on making tempeh and its starter. This is the cause of many variations in the manufacture of tempeh in some areas (Astuti et al., 2000) which lead to nonhomogeneous quality and safety of traditional tempeh.

Although tempeh is characterized as safe for consumption, recently there have been cases of intoxication in Indonesia as a result of tempeh consumption. From 1998 until 2015 a dozen of cases were reported in the mass media, but complete data are still lacking. Many speculations may arise in line with any new discoveries such as that some *Rhizopus* strains could be agents of mucormycosis, and some strains are also capable of producing toxins that are dangerous to human health.

In recent years the clinical range of the infections caused by zygomycetes has changed dramatically because of the increase in the number of immune-system deficiencies (Meis and Chakrabarti, 2009). Among Mucorales, *Rhizopus* species, are the most common agent of mucormycosis (Ibrahim et al. 2012). Autopsy study showed that fungal infections as those caused by *Aspergillus* and *Candida*. They are 10-50 times more common in the inpatient population (Yamazaki et al., 1999) with 50% or greater mortality of mucormycosis pneumonia (Quan et al., 2010) acute infection caused by Mucoralean Fungi. This arouses concern in clinical mycology. Clinical associated-strains often differ from food associated-strains (Jennessen et al., 2005), or are at least perceived as such because the latter has undergone domestication for centuries on rich substrates such as soybeans (Feng, 2006), also possibly as a consequence of selection and mutation mechanism. However, according to Dolatabadi et al. (2014), the clinical associated-strain has an identical genetic character to food and condiment associated-strains.

Rhizoxin and rhizonin pose another threat to food safety associated with *Rhizopus* is posed by (Jennessen et al., 2005) (Figure 2). Rhizoxins are a family of macrolactones firstly isolated from a plant-borne isolate of *R. microsporus* by Iwasaki et al. (1984). These metabolites (mycotoxins) exhibit potent antitumor activity. The fungi can attack rice plants and trigger rice seedling blight. Symptoms of the infection include abnormal swelling of the roots possibly due to the inhibition of cell division (Lackner et al., 2011). *Burkholderia rhizoxinica* produces rhizoxin during soybeans fermentation by *Rhizopus* sp. on *sufu* preparation (Rohm et al., 2010). The rhizoxin derivatives are known as very strong antimycotic agents (Scherlach et al., 2006), also causing health hazards for human.

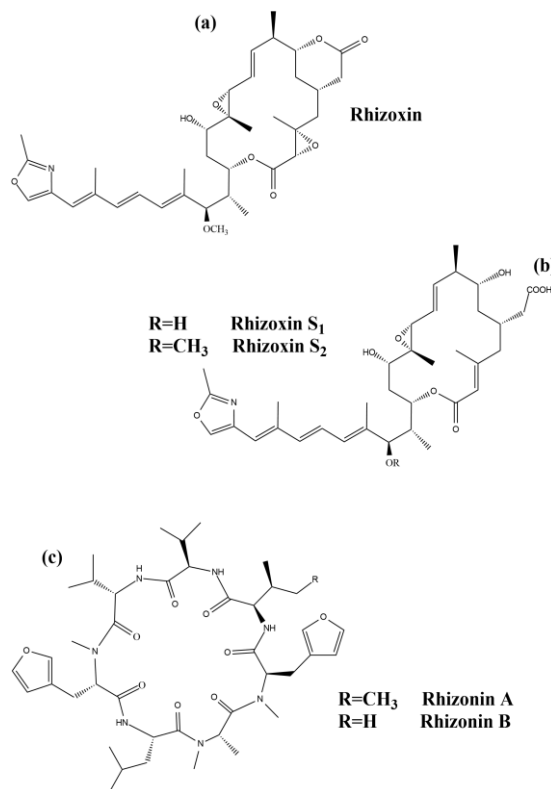


Fig 2. a) Rhizoxin; b) Rhizoxin S1 and S2; c) Rhizonin A and B.

Rhizonin A is acutely toxic for ducklings and rats (Wilson et al. 1984) and affects mainly the liver and kidneys causing 100 % mortality (Rabie et al. 1985 ). It was initially reported as the first " mycotoxin " from lower fungi, but they are actually biosynthesized by the endosymbiotic bacteria *Burkholderia rhizoxinica* or *Burkholderia endofungorum* (Partida-Martinez et al. 2007) residing within the cytosol (Partida-Martinez et al., 2007, Scherlach et al., 2006). Strains carrying endosymbionts can be found as wild types as well as those that are used for the production of fermented foods (Dolatabadi et al., 2015). Because only some subspecies of *R. microsporus* are used for fermenting soybeans (such as for *sufu* and tempeh production), the potential risk to human health derives from this toxinogenic symbiosis. The cyclic peptide is highly toxic to mammals as they severely affect the liver (Wilson et al., 1984). For the sake of public health, attention should be given to toxins produced by *R. microsporus* in order to prevent any misuse. These results

underline the urgent necessity to consider potential detrimental effects resulting from bacteria-fungi interactions during food production to warrant food safety (Lackner and Hertweck, 2011). In order to increase food safety and for modernized tempeh industrial-scale processing, thus toxicological and clinical screening of *Rhizopus* strains from tempeh manufactures in Indonesia is needed.

The safety aspect of traditional tempeh is also threatened by possible contamination by the use of the traditional starter (*usar*). *Usar* is the microbial inoculum for traditional tempeh fermentation. It is prepared by placing pieces of matured tempeh between two *hibiscus* (or *Tectona*) leaves, which are then piled up and allowed to stand for a few days at room temperature (30°-34°C) (Figure 3). The leaves are then separated from the pieces of seed tempeh and dried. For the inoculation of the microorganism from *usar*, the leaves are rubbed on the material to be inoculated.

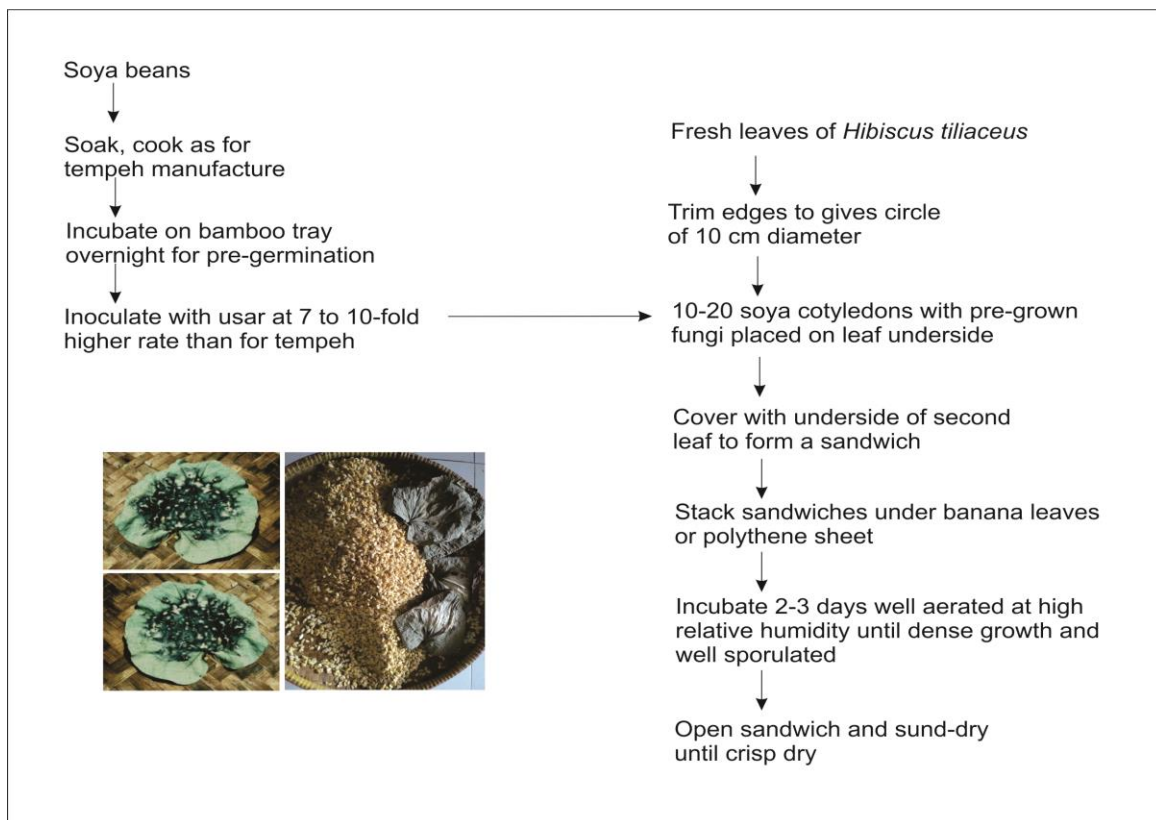


Fig 3. Flow-chart of *usar* manufacture as practised at traditional tempeh producer in Indonesia.

Standard of hygiene is not applied in *usar* production due to the lack of knowledge about good sanitation by the producers. *Usar* is produced from *Rhizopus*, which as Nout et al. (1992) report, may result in mycotoxins formed by fungi such as *Fusarium* spp. and *Aspergillus flavus* on *waru lengis* leaves, and which might act as contaminants during the fermentation process. As much as 35% of tempeh production in Indonesia still uses *usar*. The big question is whether the 800 thousand metric tons of traditional tempeh produced a year are safe for human consumption? So

far, there has been no further research on the contamination from toxins produced by molds in *usar* as well as traditional tempeh.

Another safety problem in tempeh production is the use of corn as substitute for soybean. Producers involve corn substitution for 10-30 percent to reduce cost production because the price of soybean is high nowadays. This generates a health issue for thus produced tempeh because most Indonesian corn is contaminated by toxigenic fungi such as *Aspergillus* spp., *Fusarium* spp., *Penicillium citrinum* and *P. chrysogenum* (Purwoko et al., 1991; Ahmad et al., 1996; Gholib et al., 2004; Ahmad et al., 2009; Kusumaningrum et al., 2010; Rahmawati et al., 2013). Rahmawati et al. (2013) reported that toxigenic fungi are found in soaking corn, such as *Fusarium* spp. Consequently, food safety aspect of tempeh can not be guaranteed anymore.

### 5. Could Masked Zearalenone (ZEN) be Generated during Food Fermentation?

Masked mycotoxins are plant or fungal metabolites that contaminate food and feed (Dellafiora et al., 2017). Gareis et al. (1990) referred to a zearalenone-glycoside as a masked mycotoxin to emphasize the fact that it was not detected by routine analysis of food because it has different chemical behaviors than the origin but possibly contributed to total mycotoxin and subsequent effect. This masked mycotoxin can be hydrolyzed to their precursors in the animal digestive tract, thus showing similar toxicity than free mycotoxins (Gareis et al. 1990; De Saeger and van Egmond, 2012; Gratz et al., 2016). The mycotoxin conjugate could be generated by infected host plant (Engelhardt et al., 1988; Kovalsky et al., 2014) and some fungal species such as *Fusarium graminearum*, *Rhizopus arrhizus*, *Aspergillus niger*, *Rhizopus* spp. and *A. oryzae* (El-Sharkawy et al., 1991; Plasencia and Mirocha, 1991; Berthiller et al. 2005; Jard et al., 2010; Brodehl et al., 2014) by transforming ZEN into different conjugated form. In infected plant, ZEN is transformed into more polar derivatives by conjugation with sugars, amino acids, or sulfate groups, which are then stored in the vacuoles (Gratz, 2017) (Figure 4).

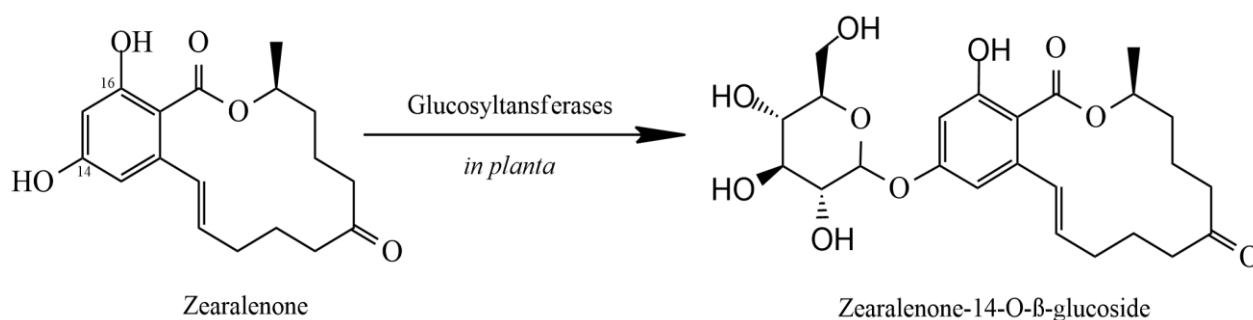


Fig 4. Conversion of *Fusarium* mycotoxin ZEN into ZEN-14-β-glucoside by plants.

Conjugated ZEN derivatives have been detected in various cereal (foods) and animal feeds (Berthiller et al., 2005; Vendl et al., 2010; De Boevre et al., 2012; Kovalsky et al., 2016). ZEN14Glc and ZEN16Glc as β linked glucose conjugates are found in food, and ZEN14S as sulfo-conjugated form is found in naturally contaminated animal feed. ZEN14Glc can contribute to the

total amount of mycotoxin up to 30% (Berthiller et al., 2013). ZEN14Glc has been reported in bread and breakfast cereals at relative proportions of 20-100% of free ZEN (Wallace et al., 2016) and ZEN16Glc has recently been identified as a novel masked ZEN metabolite (Kovalsky et al., 2014). ZEN14Glc has been tested *in vitro* and found to be non-cytotoxic to MCF-7 human breast cancer cells (1  $\mu$ M, 6 h) (Dellafiora et al. 2016), and Caco-2 cells (20 and 40  $\mu$ M, 6 h) (Cirlini et al., 2016) and to exhibit reduced estrogen receptor binding capacity compared to ZEN (Poppenberger et al., 2006). ZEN16Glc is also non-cytotoxic in Caco-2 cells (Cirlini et al., 2016). ZEN14S is also non-estrogenic in MCF-7 cells (Drzymala et al., 2015) and 40% less potent than ZEN in a rat uterus enlargement bioassay *in vivo* (Plasensica and Mirocha, 2006). Besides conjugated ZEN products, reductive metabolites are generated in plant and fungal metabolism, for example,  $\alpha$ - and  $\beta$ -ZEL (zearalenol).  $\beta$ -ZEL is less toxic than ZEN,  $\alpha$ -ZEL possesses an about 10-fold higher estrogenicity than ZEN (Metzler et al., 2010).

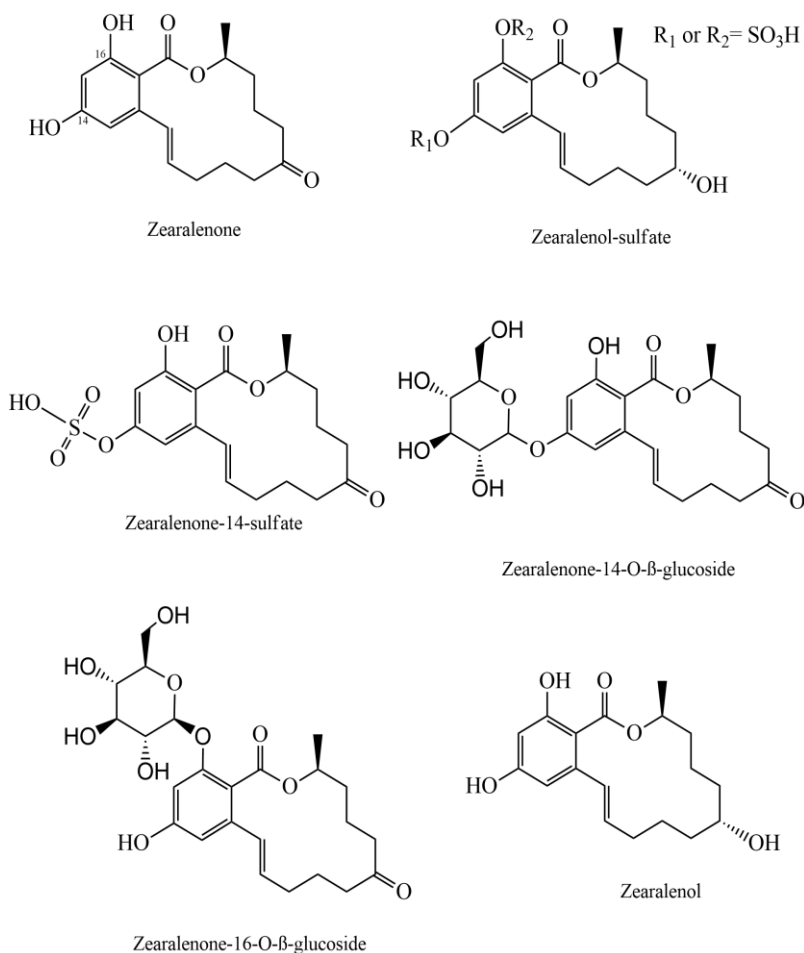


Fig 5. ZEN and masked-ZEN structures

Food fermentation (e.g. tempeh, *sufu* or soy sauce) may also contribute to the production of masked mycotoxins because the process involved ZEN transforming fungi. Recent research by Brodehl et al. (2014) examined capability of some *Rhizopus* and *Aspergillus* strains to transform

ZEN into some masked form e.g.  $\alpha$ -ZENS, ZEN14S, ZEN14Glc, ZEN16Glc, and  $\alpha$ -ZEL (Figure 5). Two *Aspergillus oryzae* strains and all seven *Rhizopus* species were able to convert ZEN into various metabolites, including ZEN14S as well as ZEN14Glc and ZEN16Glc. However, *in vitro* studies done so far are still very limited for fungal strains, especially strains derived from food fermentation. Further studies are needed by using more strains isolated from fermentation industry, for example, industrial tempeh. *In situ* trials involving fermentation process also needs to be done to see the extent of its influence on the masked-ZEN formation.

## 6. Current Food Safety Conditions in Indonesia

Food is of fundamental importance to life (De Vries et al., 1997) and the most essential for maintaining continued survival. The quality of human resources is determined by food safety. Given the importance of food safety, government issues Law of the Republic of Indonesia no. 23/1992 concerning Health, Law No. 23 of 1992 on health and Act No. 7 of 1996 on Food and Government Regulation No. 28 of 2004 on Food Safety, Quality and Nutrition. Food safety deals with requirements should be met in order to preserve food from contamination or food-borne illnesses.

There is no sense in talking about quality, nutritional value, sensory or functional properties if the food product is not safe for consumption. Act No. 7 of 1996 article 1 paragraph 4 stipulates food safety includes the condition and pursuits needed to prevent food from possible biological and chemical contamination as well as contamination by other objects which may disturb, harm, and endanger the human health. It also mentioned that safe conditions and efforts are needed to prevent food from possible contamination of biological, chemical and other substances that can disturb, harm and danger to people. Safe food is defined as food which does not have (1) biological or microbiological hazard, (2) chemical hazards, and (3) physical danger (Ardiansyah, 2006; Fardiaz, 2008).

Biological hazards include cockroaches and ants, and microbiological hazards by pathogens, microbial toxins, viruses and microbial spoilage. Organism growing and developing in food can be infectious or toxic to humans (Anggrahini, 1990; Anggrahini, 1992). Intoxication is a condition in which toxin is formed in foods. It is harmful to human health. Although food or ingredients can be treated with heat to minimize microbial activities, already formed toxins still could remain active (Ardiansyah, 2006; Tannenbaum, 1979).

As we enter the 21st century, food safety faces a rapidly changing paradigm. As a result of the projected global demand for food source due to the increase in populations and expanding international travel and trade, consumers are shifting from regional commerce to global environment. Emerging infections and foodborne illness will continue to have a major impact. Negative health effects have a broad impact (Paige and Tollefson, 2003). Johnson (2003) cited a report WHO, globally, WHO has estimated approximately 1.5 billion cases of foodborne illness and more than 3 million deaths annually, with this number is likely to increase. Lund and O'Brien (2003) estimate foodborne illnesses are ranging 100–300 times, compared to the actual occurrence.

Food industry in Indonesia consists of informal and formal sector. Informal sector includes small industries, domestic industry, street vendor, etc. Home food industry (IRT) is potentially vulnerable to food safety (Nda, 2004; Apriliana, 2006). Based on National Agency of Drug and Food Control (NA-DFC) data, only 54% out of 1835 home industry (IRT), which already have permit number of home food industry (PIRT) in 2001-2013 (survey in 2011). The figure rose slightly to 59% in 2012, and to 67% in 2013. This means about that 33% still have not been able to implement good manufacturing process. This condition arouses serious concern since structure of food industry in Indonesian is dominated by small and medium-sized enterprises (SME), in which 99% of them are small industries, and rest is large industry.

NA-DFC reports that most of food poisoning is caused by the food that is produced by the domestic industry (39%), followed by catering services (caterers) (20%), comfort food industry (restaurant) (21%), and processed food industry (13%) (Figure 6). The data also indicates that most food poisoning is caused by a microbiological agent (46%), followed by chemical agents (18%) (Figure 7). The data provide important information, strongly indicating that food safety issues are more common in small and medium-sized enterprises (SMEs). The reason is that the most SMEs have not met the standards of sanitation and hygiene and good manufacturing practices (GMP) or good food processing method (GFPM). Food poisoning is an iceberg phenomenon, meaning that not all cases or incidents is reported. WHO states that only 1% out of 100% cases reported (NA-DFC, 2012).

The condition domestic food safety is reflected from the data of food poisoning (Table 2). Table 2 records food poisoning cases and a number of people who fall sick or are poisoned increased since 2004. It might be not associated with the food safety situation getting worse, but because of the increasing of survey intensity done by NA-DFC.

The condition of food safety in Indonesia is also reflected by the export rejection of Indonesian food products. This is because food safety has become an absolute prerequisites for international trade, and therefore the food safety will also directly affect the export performance of the country. Based on data collected from USFDA (US Food and Drug Administration) in 2011-2014, the number of export rejection cases due to food safety reasons are 1.451 cases, or about 30 cases per month (Figure 8).

Table 2. Food poisoning case data in 2001-2006 by NA-DFC.

<b>Year</b>	<b>Poisoning case number</b>	<b>Number of food</b>	<b>Severe illness</b>	<b>Death number</b>
2001	26	1965	1183	16
2002	43	6543	3635	10
2003	34	8651	1843	12
2004	164	22297	7366	51
2005	184	23864	8949	49
2006	159	21282	8747	38

The reasons are slightly different for refusal concerning the export of Indonesian food to the European Union (EU). The trade with the EU is not as intensive as with the US. There are 64 border rejections on Indonesia's exports of food products based on data collected from the portal Rapid Alert System for Food and Feed (RASFF Portal, 2015) during 2011-2014 for several different reasons. The main difference was the level of mycotoxin contamination (28%). In this case, the EU is very strict to determine maximum limit of mycotoxins in comparison with US and other countries. The next one is the level of pathogenic microbial contamination (26%) followed by non-pathogenic microbial contamination (16%) (Figure 9).

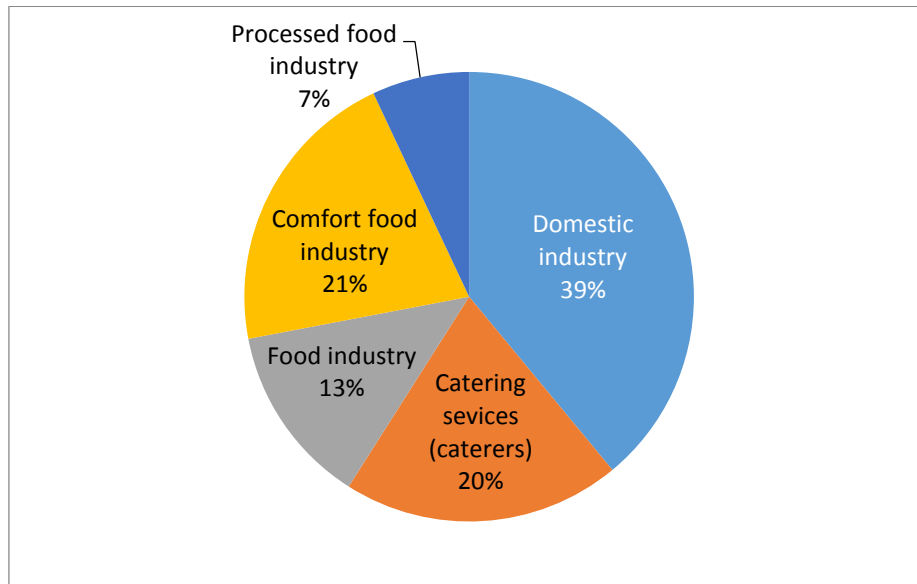


Fig 6. The source of food poisoning outbreaks in Indonesia by NA-DFC in 2011-2013.

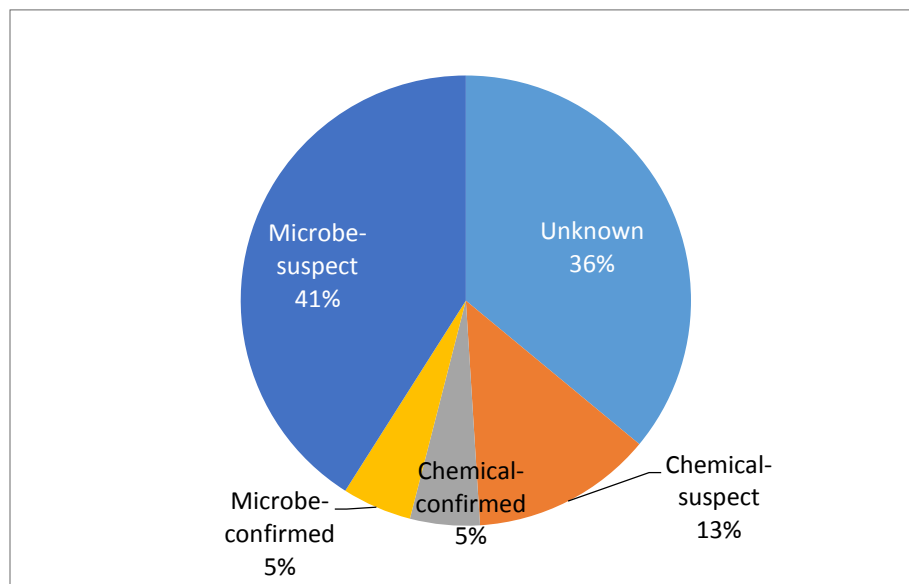


Fig 7. Causative agent profile of food poisoning outbreaks in Indonesia by NA-DFC in 2011-2013.



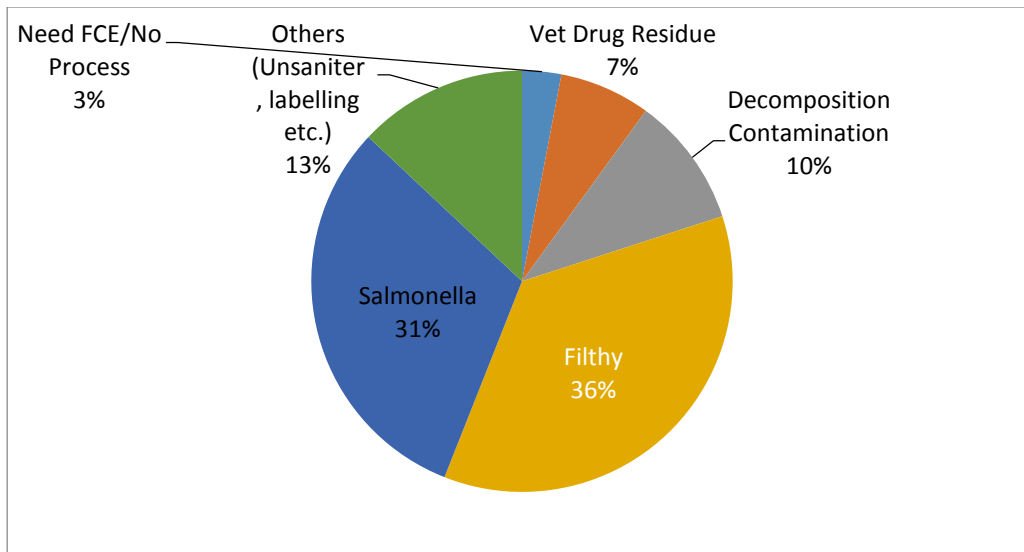


Fig 8. The reason for rejection of Indonesian food exports by USFDA in 2011-2014 (total: 1451 rejection cases).

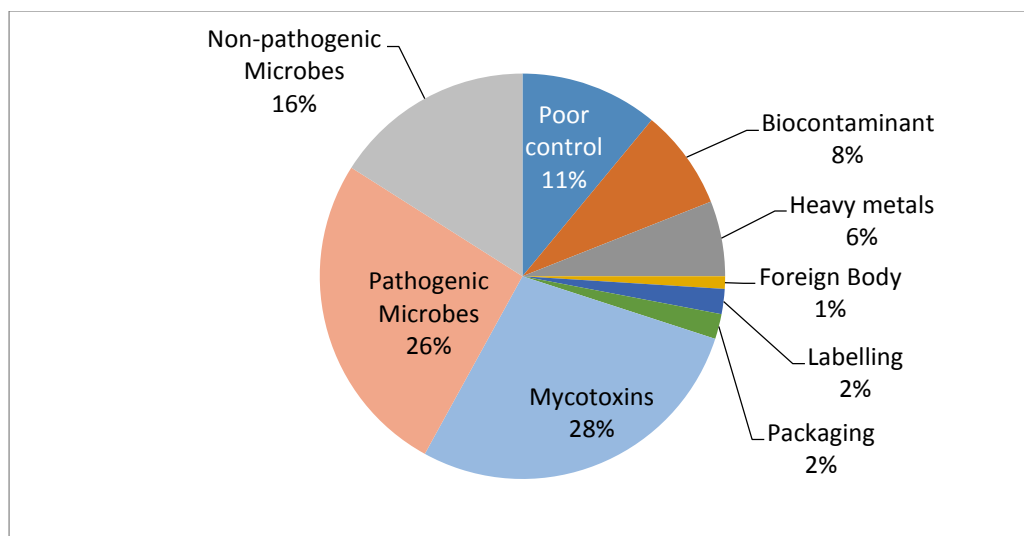


Fig 9. The reason for rejection of Indonesian food exports by the European Union in 2011-2014 (total: 64 rejection cases).

The high frequency of the export rejection because of microbial contamination, both pathogenic and non-pathogenic, indicates that food processing in Indonesia is still not appropriate to comply with good sanitation and hygiene. In conclusion at the outset that despite SMEs are major manufacturers of processed food in Indonesia, most of these SMEs have difficulties to implement good manufacturing practice (GMP). This condition requires stakeholders, especially the government to focus on SME food safety development.

## 7. Aim of the Work

Food safety is a prerequisite for a food product. There is no sense in talking about quality, nutritional value, sensory or functional properties if the food product is not safe enough for consumption. Indonesia suffers a double burden of food safety problem. The first is related to fundamental issues of food safety; especially about the insufficient practice of good manufacturing practices (GMP) principles. The second is related explicitly to export-oriented food industries; they must face a variety of new food safety issues which always appear and change from time to time, also vary from one country to another (Hariyadi, 2008). One of the food products that need attention for its safety in Indonesia is tempeh.

Tempeh is produced mostly by a small scale home industry with poorly controlled fermentation procedures that in addition carried out without sufficient hygienic precautions. Therefore, there are various types of microorganisms that can participate during the process of fermentation, and natural contamination is always possible (Winarno, 1985; Barus et al., 2008; Seumahu et al., 2013). Moreover, there is no standard on making tempeh and its starter. This cause many variations in the manufacture of tempeh in many areas (Astuti et al., 2000) and lead to inconsistencies in quality and safety of tempeh. Tempeh, as important food is massively produced and consumed by Indonesian people. As a new export commodity, it really needs standardized starter and controlled processing to fulfill safety aspect.

The scope of this thesis was to address microbiological and food safety aspect of tempeh production in Indonesia. It was divided into 3 topics:

### **Topic 1:**

This topic deals with the exploration of mucoralean fungi involved during tempeh production as well as solid fermentation process. *Zygomycetes* strains derived from Indonesian tempeh and starter are screened toxicologically and clinically to evaluate possible health risks for human consumption. In this part we also study the role of tempeh fungi on zearalenone (ZEN) biotransformation.

### **Topic 2:**

To study about toxigenic fungal contamination on *usar* (traditional tempeh inoculum). We characterize the contaminants and detect their mycotoxin production. Mycotoxin contamination in traditional tempeh is also observed. We also study the effect of contamination on tempeh quality, and how to distinguish contaminated tempeh. The exposure levels of Indonesian infants, children, and adult to mycotoxin as a consequence traditional tempeh consumption also studied.

### **Topic 3:**

To characterize and investigate the diversity of lactic acid bacteria (LAB) and yeast by analyzing soak water samples collected from different tempe manufactures in Java. We also screen and investigate their potential antifungal activities against *Fusarium proliferatum*. Their contribution to ZEN biotransformation also observed.

**Hypotheses** were defined as follows:

**Topic 1:**

Diverse species possibly found in Indonesian tempeh and starter. Some *zygomycetes* strains used for tempeh manufacture possibly contribute to toxication or infection. Tempeh fungi also have activity to transform ZEN.

**Topic 2:**

*Usar* possibly contains microbial contaminant that could produce mycotoxins. Mycotoxins are also possibly found in traditional tempeh. Contamination will give effect to quality of traditional tempeh.

**Topic 3:**

Diverse microorganism is found during soaking process of tempeh manufacturing. Selected yeast and LAB have role to increase safety level in tempeh production. Lactic acid bacteria have potential application to produce antifungal compounds which could be used to protect traditional tempeh from the contaminant. Yeast and LAB from soak water also have ability to transform ZEN.

**Research outcome:**

Safe and defined starter for tempeh production could be selected and formulated afterward. In the near future, starter could be produced in mass scale and distributed to tempeh manufactures, thus tempeh production would be controlled from the beginning process and indeed increase its safety level.

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## Review Article 01

# Mycotoxins in Indonesian Foodstuffs: Occurrence, Prevention and Remedial Methods

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### Abstract

Mycotoxins are secondary metabolites produced by toxigenic fungi which often contaminate various foods at any food chain level. Mycotoxin food contamination is a problem because of the negative impact, it causes not only on the health of humans or animals but also on the global economy. Contamination of agricultural products with mycotoxins in Indonesia is quite intensive because of the heavy rainfall throughout the year and high temperatures which are favorable for the growth and proliferation of mycotoxin-producing fungi on the field plantation or crops. Agricultural commodities such as corn and peanuts have been reported to be heavily contaminated with mycotoxins. In this present paper, we reviewed the data published since 1971 concerning the contamination of food with single or combinations of mycotoxins in Indonesia. Associated health risks, as well as stringent prevention and remedial strategies with decontamination or detoxification, are also discussed in detail. This comprehensive review gives a real insight into the progress that has been achieved and an outlook to further research required in Indonesia.

**Keywords:** Mycotoxins, foods, food safety, food chain, decontamination, detoxification

### Introduction

Food safety is a prerequisite for a food product, which should be addressed in an integrated manner, involving various stakeholders; from the government, industry, and consumers. Food safety has priority over quality, nutritional value, sensory or functional properties of the food. Indonesia suffers a double burden of food safety problem. Food quality is related to the fundamental issues of food safety; it is particularly important to comply to the standards of Good Manufacturing Practices (GMP) to reduce the risk of food poisoning incidences. Indonesian food poisonings are commonly reported in the mass media. From 1998 until 2015 a dozen of cases were reported, but the cause was unknown, and these cases are presumably underreported. Based on the National Agency of Drug and Food Control (NA-DFC) data (2011-2013), the causative agent of food poisoning outbreaks in Indonesia is dominated by microbiological agents (41%) and chemical agents (13%). Export-oriented food industries must face a variety of new food safety issues which arise constantly, dynamically and vary from one country to another (Hariyadi, 2008). Based on data collected from the US Food and Drug Administration (US-FDA) during 2011-2014, there were many export rejections of Indonesian food products for food safety reasons, 1451 cases in the total period or about 30 refusal cases per month (Hariyadi, 2015). In the same period, Indonesia



also had 64 border rejections from the European Union (EU) (RASSF portal, 2015). The data reflects the poor control and handling of food safety in Indonesia. One of the most significant food safety problems in Indonesia that should be solved and monitored is mycotoxin contamination.

Mycotoxin contamination on diet and feedstuff not only endangers the human health but also effects the economic situation in terms of international trade causing trade revenue loses up to hundreds of million dollars per year in many countries due to the rejection of their peanuts export by EU (Zanelli, 2000; Wu, 2006; Schmaile and Munkvold, 2009; Amaike and Keller, 2011). For Indonesia itself, mycotoxin contamination in economically important crops also have an adverse impact on the export rate. Acceptable mycotoxin limits are applied strictly in many of the export destination countries causing Indonesia lost revenue as a result of the detention on the exported commodities. For example, between 2009-2012 Indonesia lost revenue due to the refusal of nutmeg export to EU. Indonesia has received notifications about contamination up to 21 times from EU RASFF (Rapid Alert System for Food and Feed) (ITPC Milan, 2015). Therefore, more intensive studies on mycotoxins occurrence and control are necessary to improve Indonesian food safety conditions. So far, mycotoxins studies carried out in Indonesia from 1971 up to the present, focused primarily on surveillance and monitoring studies of aflatoxin (AF) contamination in foodstuffs and their products. Recently, mycotoxins studies in Indonesia are focused not only on the existence of mycotoxin problems but also searching ways of how to control the problem. This paper describes the published studies and reports on the different mycotoxins in Indonesia and discusses the occurrence and the associated health risks as well as strategies for prevention and decontamination.

### **Factors Promoting Mycotoxins Contamination in Indonesian Foods**

Mycotoxin contamination rate in Indonesia is quite high and difficult to avoid because of the tropical climate in Indonesia which is very favorable for the growth and proliferation of mycotoxin-producing fungi (Benneth and Klich 2003; Mary et al., 2007). Moreover, Indonesia is one of the countries influenced by extreme climate conditions during the last decade. In August 2015, the temperature in Indonesia ranged from 22°-38°C, and RH (room humidity) ranged from 45-98% (Indonesian Agency for Meteorological, Climatological, and Geophysical, 2015). Increasing global temperatures are predicted to occur in the future due to climate change which could lead to more mycotoxin-producing fungi contamination in foodstuffs during storage especially in developing countries. Some agricultural commodities in Indonesia such as rice, corn, peanuts, and soybeans are contaminated with mycotoxins. In Indonesian agriculture AF, ochratoxin (OCA), and fumonisin (FUM) are the most important mycotoxins (Dharmaputra, 2004).

The main factors that determine the fungal infestation and mycotoxin production are temperature and high humidity (Agag, 2004). Also, other factors that affect the growth of molds on foodstuffs are oxygen, moisture, time, degree of mold invasion, damage to the substrate, insects, and ticks (Medion, 1995; Dharmaputra, 1999). Dharmaputra (2004) stated that the presence of mycotoxins in grains in Indonesia is influenced by several factors, among others are: 1) biological

factors, including grains contaminated with fungi and fungal toxins, 2) environmental factors, including temperature, humidity, and damage to the pods by insects, 3) harvesting, including the level of pods maturity, temperature, and humidity, 4) storage, including temperature and humidity of disk space, screening and separation of contaminated grains, and 5) processing, such as drying and sorting. Mold contamination in grains led to a decrease in viability, discoloration, and weight loss. These accumulated damages influence the levels of mycotoxins in food commodities.

According to Miller (1995), AF-producing fungi ecology in the tropics is different from other geographic areas. *Aspergillus* spores are widespread in the soil, creating high contamination level of AF in agricultural products in tropical countries. Also, the food products sold in the open market in the tropics are usually displayed under inappropriate conditions. Long term exposure to mold spores, dust and pollution from the environment for the, increase the mycotoxin production of fungi in contaminated food.

Dharmaputra et al. (2003) reported that 45% and 58% of AF is produced from 113 and 90 isolates of *A. flavus* respectively were found in the soil of peanut plantation in Wonogiri. Soil is the main source of inoculum for aflatoxigenic *Aspergillus* species and since peanut pods grow underground, they are in direct contact with the fungal population in the soil. In Indonesia, peanuts are often infected during pre-harvest, and the contamination becomes more intensive due to poor farming practice. According to Kasno (2004), pods filling phase is highly affected by temperature stress and drought. These factors are optimal for invasion fungal invasion. The optimum temperature in the field for *A. flavus* growth is between 25.70°C to 31.30°C, and the plants that experience drought stress 4-6 weeks before harvest at this temperature are more susceptible to the fungal contamination. AF contamination occurs at a temperature of 26.30°C, AF content will continue to increase in line by rising temperature until 31.20°C.

According to Avivi (2005), peanut farmers and traders in Indonesia are still not paying attention to proper post-harvest handling. Farmers dry peanuts on cement floors with the help of sunlight. After that peanuts are stored at room temperature for days or even weeks with poor sanitation before reaching the consumers, during this time *A. flavus* can grow and produce AF intensively. Poor distribution handling will also increase the growth of mold on peanuts. The level of contamination is also increased during rainy season though the higher water content in the material (Dharmaputra et al., 1993). Therefore, it is necessary to improve postharvest handling which can be implemented easily by farmers and traders in Indonesia to suppress the growth of toxigenic fungi.

## **Occurrence of Mycotoxins in Indonesian Foods**

From 1971 to 2015, 54 surveillance research projects have been conducted in Indonesia (Table 1). Most of the of mycotoxin analysis were done using HPLC, followed respectively by TLC and ELISA (Figure 1a). The limited budget, high throughput and user-friendly character of ELISA make this method also chosen among laboratories in Indonesia. Most of the data collected were from maize and peanut (either processed or raw material), followed by milk and other food

products (Figure 1b). Most of the samples from the report were obtained by purchasing products from local markets.

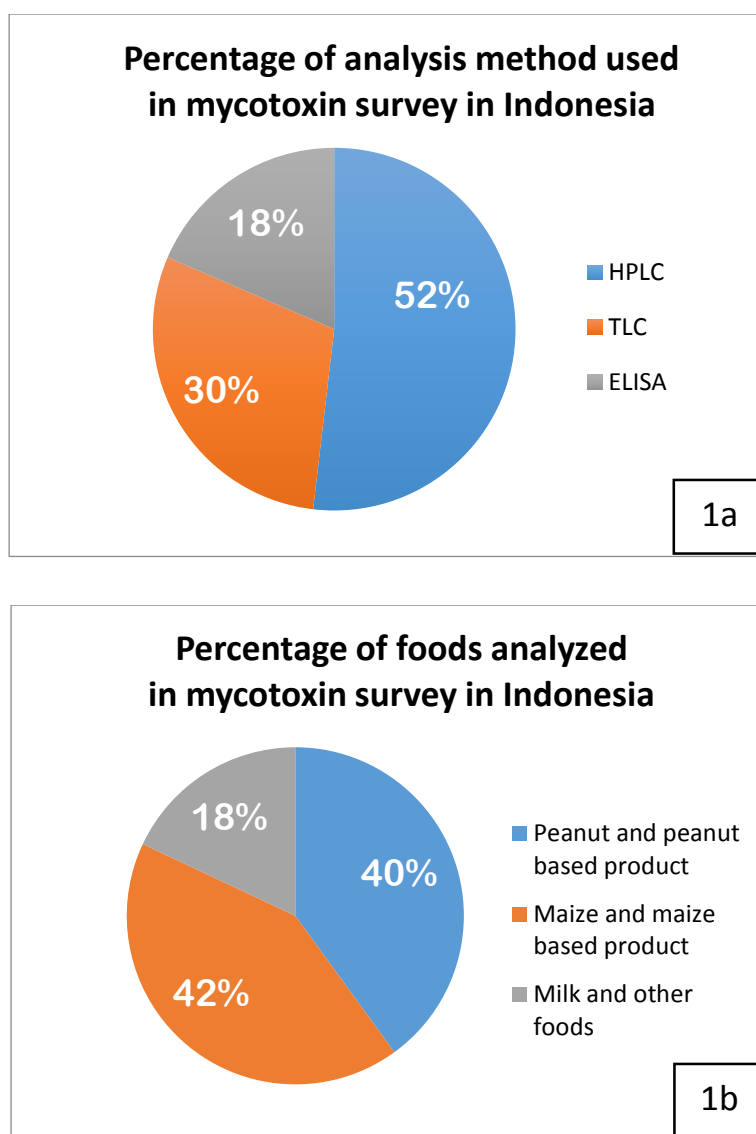


Figure 1. a) Percentage of analysis method used for mycotoxin detection in Indonesia; b) Percentage of analyzed foods related with mycotoxins in Indonesia.

Up to now, other important food products in Indonesia such as fruits, *jamu* (Indonesian traditional medicine), drinks (coffee, tea, and chocolate), fermented foods and animal-derived products (such as milk, sausage, meat products, etc.) have not been well studied. Moreover, the research so far is limited to AF, although other mycotoxins may also be present in many food commodities, for example patulin in fruits (Miskiyah, 2010). Therefore, more surveillance and monitoring are required.

Table 1. Mycotoxins occurrence in Indonesian foods.

## A. Peanut and peanut-based products

Origin of sample	Reference	Crop year or survey year	Mycotoxins	Commodity or food product	Positive/Total samples	Moisture content	Concentration (range or average)	Analytical technique	LOD
West Java	Muhilal et al. (1971)	1971	AFs	Peanut	9/15	-	42-4500 µg/kg	TLC	NM
West Java	Roedjito et al. (1972)	1972	AFB1	Fried peanut	13/13	-	332 µg/kg	TLC	NM
			AFs	Peanut oilcake	13/13	-	119 µg/kg		
			AFs	<i>Oncom</i>	13/13	-	36 µg/kg		
Bogor	Muhilal and Karyadi (1984)	1984	AFs	Fried <i>oncom</i>	11/35	11.4%	7-2000 µg/kg	TLC	NM
Bogor	Muhilal (1986)	1984	AFs	<i>Bumbu pecel</i>	25/30	-	68-83 µg/kg	TLC	NM
Bogor	Dharmaputra et al. (1991)	1988	AFs	Peanut	23/30	-	126 µg/kg	TLC	20 µg/kg
			AFs	Peanut	35/35	3.6-11%	1154 µg/kg	TLC	NM
Bogor	Roedjito et al. (1994)	1994	AFB1	<i>Bumbu pecel</i>	23/45	-	68.5 µg/kg	TLC	NM
Semarang	Haryadi and Setiastury (1994)	1994	AFB2		23/45	-	18.5 µg/kg		
			AFs		23/45	-	>15 µg/kg		
Bogor and Denpasar	Haryadi and Setiastury (1994)	1994	AFB1	Raw and processed peanut	8/15 (rainy season)	-	>20 µg/kg	TLC	NM
Yogyakarta	Pitt and Hocking (1996)	1996	AFs		5/15 (dry season)	-	>20 µg/kg		
			AFs	Peanut	97/216	-	>50 µg/kg	HPLC	5 µg/kg
			AFs		71/216	-	>300 µg/kg		
Bogor	Fardiaz (1996)	1996	AFs		48/216	-	>1000 µg/kg		
			AFs	Peanut crisp and peanut oil	23/50	-	>1000 µg/kg	TLC	NM
Central Java	Johnson (1997)	1997	AFs	Peanut (importer)	33/33	-	4.6-6.4 µg/kg	HPLC	1 µg/kg
			AFs	Peanut (wholesalers)	33/33	-	<3.6-330.2 µg/kg		
			AFs	Peanut (retailers)	33/33	-	<3.6- 330.2 µg/kg		

Chapter 2: Review Article (Mycotoxins in Indonesian Foodstuffs)

Bogor	Arifiana (1999)	1998	AFs	<i>Bumbu pecel</i>	9/9		0.2-3 µg/kg	TLC	NM
Bogor, Malang, Pati, and Yogyakarta	Lilieanny (2002)	2002	AFs	Crunchy peanut Peanut atom Oven nuts	88/88 88/88 88/88	-	1.8 µg/kg 5.2 µg/kg 41.6 µg/kg	TLC	NM
				<i>Bumbu pecel</i> <i>Enting-enting</i>	88/88 88/88		20.8 µg/kg undetectable		
Wonogiri	Junita (2003)	2003	AFs	Peanut (farmers)	50/50	-	3.6-93.9 µg/kg	HPLC	1 µg/kg
				Peanut (collectors)	50/50		3.6-1336.9 µg/kg		
Wonogiri	Dharmaputra et al. (2005)	2003	AFs	Peanut	37/113	-	>15 µg/kg	ELISA	NM
					68/90		>15 µg/kg		
Cianjur	Akbar (2005)	2004	AFs	Peanut (farmer)	3/13	-	>15-50 µg/kg	ELISA	3 µg/kg
				Peanut (collector)	40/40		<5-1000 µg/kg		
Wonogiri	Dharmaputra et al. (2005)	2004	AFs	Peanut	72/90	-	3.6-6073 µg/kg	HPLC	NM
					(wholesaler)				
Bogor	Razzai-Fazelli et al. (2004)	2004	AFs	Peanut products	30/82	-	5-870 µg/kg	HPLC	≤1.7 µg/kg
Yogyakarta	Lilieanny et al. (2005)	2004	AFs	<i>Bumbu pecel</i>	12/12	-	41.6 µg/kg	HPLC	1 µg/kg
Yogyakarta	Mahardikarani (2007)	2006	AFs	<i>Bumbu pecel</i>	16/16	-	121.3 µg/kg	HPLC	NM
Rembang	Hakim (2008)	2007	AFs	Peanut	5/20	-	>20 µg/kg	HPLC	4 µg/kg
Bogor	Dharmaputra et al. (2013)	2012	AFB1	Peanut	4/26	-	>15 µg/kg	TLC	NM

## B. Maize and maize-based products

Origin of sample	Reference	Survey year	Mycotoxins	Commodity	Positive/Total samples	Moisture content	Concentration (range or average)	Analytical technique	LOD
Various regions	Widyastuti et al. (1988)	1988	OCA	Maize	1/26	-	3 µg/kg	TLC	NM
			CA		21/26	2-1005 µg/kg	≤1 µg/kg		
Jakarta and Bogor	Purwoko et al. (1991)	1990	AFB1	Maize	24/26	-	150-9220 µg/kg		NM
			AF		30/34	22-6171 µg/kg	TLC	NM	
			AFB1		30/34	22-4074 µg/kg			
			AFB2		24/34	11-3021 µg/kg			
			AFG1		2/34	101.5 µg/kg			
			AFG2		1/34	144 µg/kg			
Lampung	Dharmaputra et al. (1995)	1992	AFB1	Maize	35/35	-	25.4-367 µg/kg	HPLC	5 µg/kg
			AFB2		10/35	11.9-278.7 µg/kg		5 µg/kg	
Lampung, Kediri	Dharmaputra et al. (1996)	1993	AFB1	Maize	108/108	-	<5-291 µg/kg	HPLC	5 µg/kg
West Java	Maryam and Zahari (1994)	1994	DON	Maize	54/96 (highland)	-	3.87 mg/kg	HPLC	25 µg/kg
			ZEN		85/96 (lowland)	5.66 mg/kg			
					54/96 (highland)	5.73 mg/kg			
					85/96 (lowland)	4.50 mg/kg			
Bogor	Maryam (1994)	1994	Moniliformin	Maize-based food	54/96	-	1.0-12.1 mg/kg		
			AFB1		24/32	1.1-63.7 µg/kg	HPLC	NM	
			AFB2		24/32	0.5-12.6 µg/kg			
			AFG1		6/32	1.2-51.7 µg/kg			
			AFG2		6/32	0.6-4.2 µg/kg			
			FUM B1		7/12	226-1780 µg/kg	HPLC,	50 µg/kg	
Indonesia	Yamashita et al. (1995)	1995	FUM B2	Maize	3/12	-	231-556 µg/kg	GC-MS	50 µg/kg
			NIV		0/12	-	10 µg/kg		
			ZEA		0/12	-	10 µg/kg		
			AFB1		10/12	1-3300 µg/kg		1 µg/kg	
			AFB2		8/12	1-680 µg/kg		1 µg/kg	

Chapter 2: Review Article (Mycotoxins in Indonesian Foodstuffs)

Surakarta,	Ali et al. (1998)	1995	FUM B1	Maize	16/16	-	668-2440µg/kg	HPLC	50 µg/kg
Purworejo,			FUM B2		7/16		62-376 µg/kg		50 µg/kg
Yogyakarta,			AF B1		11/16		9-59 µg/kg		1 µg/kg
Central Java			AF B2		9/16		12-109 µg/kg		1 µg/kg
Indonesia	Bahri (1998)	1997	AF B1	Maize	26/193	-	>200 µg/kg	HPLC	5 µg/kg
Bandung,	Maryam	1999	FUM B1	Maize	100	-	514.2-	HPLC	50 µg/kg
Cianjur, Sukabumi,	(2000)						28380µg/kg		
Bogor									
Yogyakarta, Java	Nuryono et al. (2002)	2001	FUMs	Maize based food	76/105	-	10.0-3307 µg/kg	HPLC	10 µg/kg
					13/19 (industry)		22.8 – 105 µg/kg		
					19/20 (home-made)		12.9-234 µg/kg		
Malang, Tuban,	Rahayu et al. (2003)	2003	AFs	Maize	88/115	12.6-	<20-350 µg/kg	HPLC	1 µg/kg
Kediri, and						20.8%			
Sumenep									
Yogyakarta	Nuryono et al. (2004)	2003	FUMs	Maize based food	80/101	-	12.9-3306 µg/kg	ELISA	8.7 µg/kg
Indonesia	Razzai-Fazelli et al. (2004)	2004	AFs	Maize based food	2/11	-	6-12 µg/kg	HPLC, ELISA	1.7 µg/kg
Lampung and East Java	Bahri et al. (2005)	2004	AFs	Maize	58/65	-	31.5 µg/kg	HPLC	NM
Bogor	Kusumaningrum (2008)	2008	AFs	Maize based food	20/50	-	10-30 µg/kg	HPLC	1 µg/kg
West Java	NA-DFC (2007)	2007	AF B1	Maize	30/60	-	12-125.7 µg/kg	HPLC	5 µg/kg
South	Tandiabang (2010)	2009	AF B1	Maize	61/61	16-30%	4-665 µg/kg	HPLC	1 µg/kg
Kalimantan, South Sulawesi									
Malang, Tuban,	Rahayu et al. (2010)	2008	AFs	Maize	48/115	12-14%	<20 µg/kg	HPLC	5 µg/kg
Kediri, Madura					26/115		20-100 µg/kg		
					14/115		>100 µg/kg		

## Chapter 2: Review Article (Mycotoxins in Indonesian Foodstuffs)

Bogor, Boyolali, and Bojonegoro	Kusumaningrum et al. (2010)	2008	AF B1	Maize	22/25	12.7-34.7%	<4-137.5 µg/kg	TLC	4 µg/kg
Bogor	Yusrini (2010)	2008	AFs	Maize	26/28	-	0.3-75.7 µg/kg	ELISA	<0.3 µg/kg
Kupang	Rubak (2011)	2010	AF B1	Maize based food	6/6	-	<15 µg/kg	ELISA	NM
Yogyakarta	Setyabudi et al. (2012)	2011	DON	Maize based food	50/50	-	47-348 µg/kg	HPLC	20 µg/kg
Yogyakarta	Widyaswari (2015)	2014	AFs	Maize based food	3/20	11.8-35.7%	4-89.8 µg/kg	HPLC	1 µg/kg

### C. Milk and other foods

Origin of sample	Reference	Survey year	Mycotoxin	Commodity	Positive/total samples	Concentration (range)	Analytical technique	LOD
West Java	Bahri et al. (1991)	1990	AF M1	Milk	73/97	0.04-5.91 ng/L	HPLC	NM
West Java	Bahri et al. (1994)	1993	AF M1	Milk	12/12	0.04-0.18 ng/L	HPLC	5 ng/L
West Java	Widiastuti et al. (2006)	2006	AF M1	Milk	29/37	0.001-1.2 µg/L	HPLC	1 ng/L
Yogyakarta	Nuryono et al. (2009)	2009	AF M1	Milk	48/113	6-25 ng/L	ELISA	<5 ng/L
Flores	Wangge et al. (2012)	2011	AF B1	Cocoa beans	16/16	2.2-3.6 µg/kg	ELISA	<0.1 µg/kg
Many regions	Ramadhani (2003)	2003	OCA	Baby food	15/16	0.1-0.3 µg/kg	ELISA	<0.1 µg/kg
Bogor, Semarang	Roedjito et al. (1994)	1993	AFs	Rice	31/51	0.1-1.6 µg/kg	ELISA	NM
Many regions	Dharmaputra (1997)	1997	AFs	Soybean	11/51	18.8-375.4 µg/kg	TLC	NM
Lampung	Dharmaputra (1999)	1998	OCA	Coffee	20/35	10.2-16.9 µg/kg	TLC	NM
					5/13	6.7-8.2 µg/kg	ELISA	NM
					70/100 (local trader)	33-178 µg/kg	ELISA	NM
					45/100 (exporter)	21-127 µg/kg	ELISA	NM
						7.9-34 µg/kg	ELISA	NM
						0.3-39.8 µg/kg	ELISA	NM
						0.3-27.5 µg/kg	ELISA	NM

Note: *Marning* (traditional snack from corn), *enting-enting* (candy-like snack made from peanut), *bumbu pece* (fermented food made from peanut presscake. AFs: Total Aflatoxins, AF B1: Aflatoxin B1, AF B2: Aflatoxin B2, Aflatoxin G1, Aflatoxin G2, AF M1: Aflatoxin M1, CA: Cyclopirozonic acid DON: Deoxynivalenol, FUMs: Total fumonisin, FUM B1: Fumonisin B1, FUM B2: Fumonisin B2, OCA: Ochratoxin A, ZEN: Zearalenone, LOD: Limit of detection, NM: Not mentioned.



Although rice is the main product and staple food in Indonesia, research about this commodity was very limited and only conducted by Roedjito 24 years ago. Most Indonesian researchers assume rice is already safe for consumption, because there is no intoxication case in this commodity. Most data on AF contamination in Indonesia deal with two other commodities, namely maize and peanut (Table 1). The data showed a decrease in mycotoxin contamination level from year to year; this suggests that government already gave effort to address AFs contamination problem, but it has not been optimal since the contamination level is still above the regulatory limit.

Peanut is the most popular bean in Indonesia. They are consumed as dietary supplements and processed into various snack food. The rate of peanuts consumption in Indonesia is about 7.3 gram per day (BPS, 2016). According to studies in the last decade, range of AFs contamination level is 2-1337  $\mu\text{g}/\text{kg}$ . About 42% of total sample, its level of AF contamination were above the regulatory limits, mainly in *bumbu pecel* paste. It should get attentions since the paste is always served as dressing for vegetable salad that is consumed by most Indonesian people, mainly in Java. It also arises attention in many researchers on how to reduce the AF level during processing stage.

In Indonesia, AF contamination of maize, according to data, have the same tendency to peanuts. The data shows the contamination level is relatively high. From last decade reports, AF contaminations were in the range of 10-1375  $\mu\text{g}/\text{kg}$ . About 45% of studied samples exceeded the acceptable limit, mainly for corn kernel. The contamination level varies widely because postharvest treatment and storage condition in each region also varies from farmers to distributors level. Studies also suggest that improper storage, mainly provided by local stores, thrive on the growth of aflatoxigenic molds.

Besides peanut and maize, AF or AFM1 level of other commodities such milk, soybeans, and chocolate, were also reported. However, the contamination level was lower than the former ones. Chocolate and dairy products are considered safe because the mycotoxin level is much lower than permissible limit.

In addition to the information presented before (Table 1), several other dangerous mold contaminated Indonesian foods are listed below:

1. Dharmaputra et al. (1999) reported that 18 out of 20 black pepper samples obtained from markets and supermarkets in Bogor, and 16 out of 20 samples of white pepper from exporters or collectors in Bangka Island and Lampung province were infected with *A. flavus*. Seventeen toxigenic fungal species were isolated from black pepper.
2. Aminah and Supraptini (2003) reported that some fruits from the traditional and modern markets were infected by mycotoxigenic fungi. *Fusarium*, *Penicilium*, and *Aspergillus* were the dominant fungal genus.
3. Yani (2004) reported that 15 species of toxigenic fungal species were found in coffee bean from Bengkulu province.
4. Yani (2008) reported that 17, 13 and 15 fungal species had been isolated from coffee beans obtained from farmers, collectors in Bengkulu Province. The dominant fungal species was *A.*

*niger*. The percentage of samples infected by *A. ochraceous* (OCA-producing fungus) in farmers, collectors in district and regency level were 23.3, 46.7 and 46.7%, respectively.

5. Rukmi (2009) reported the presence of *Aspergillus* sp. in herbs commonly consumed by *Javanese* people as a traditional medicine. Isolates found in this study were *A. parasiticus*, *A. flavus*, *A. oryzae*, *A. tamari*, and *A. clavato-flavus*.
6. Rahmadi and Graham (2010) reported that all cocoa beans sampled from Indonesia were contaminated by mycotoxin producing fungi. The main species were *Aspergillus flavus*, *A.niger*, *A. wentii*, *A. clavatus*, *Penicillium citrinum*, and *P. spinolosum*.
7. Anggriawan et al. (2017) discovered the fungus *Fusarium* spp. which can produce mycotoxins in the traditional tempeh starter in Central Java and Yogyakarta provinces.

## Health Impact of Mycotoxins in Indonesia

High level of AF contamination in agricultural products has a significant impact to food safety and human health in Indonesia. In 1991, Cipto Mangunkusumo Hospital (RSCM) in Jakarta reported that 20% of liver cancer cases were not correlated with hepatitis B or hepatitis C virus infection but was thought to be caused by AF B<sub>1</sub> (Noer, 2002). Sudjadi et al. (1999) reported that 80 out of 81 patients (66 men and 15 women) suffered liver cancer because of the consumption of contaminated *oncom*, tempeh, roasted peanuts, peanut sauce, soy sauce and salted fish. AF B<sub>1</sub>, AF G<sub>1</sub>, and AF M<sub>1</sub> were detected in the liver of 58% of these patients with concentrations above 400 µg/kg. Pitt and Hocking (1996) mentioned that the level of AFs in foodstuffs in some tropical countries exceed the tolerance limits. Estimation number of deaths due to liver cancer caused by AF in Indonesia is more than 20000 people per year. Recent study under ASEAN (The Association of Southeast Asian Nations)-Australia Development Cooperation Program Stream in 2007 showed that Indonesia has the fourth highest estimated cancer cases in ASEAN (0.27-mean exposure for hepatitis B population and 0.009-mean exposure for non-hepatitis B, as shown in Table 2).

Table 2. Estimated cancer number due to AF exposure in ASEAN (Rahayu, 2010).

Country	Estimated cancer number due to AF exposure (cancer/year/100000 population)	
	Hepatitis B	Non-Hepatitis B
Brunei Darussalam	0.03-mean exposure	0.001-mean exposure
Cambodia	0.18-mean exposure	0.006-mean exposure
Indonesia	0.27-mean exposure	0.009-mean exposure
Laos	NA	NA
Malaysia	1.5-mean exposure	0.05-mean exposure
Myanmar	0.36-mean exposure	0.012-mean exposure
Philippines	3.8-mean exposure	0.13-mean exposure
Singapore	0.0264-mean exposure	0.0009-mean exposure
	0.0798-high exposure	0.0027-high exposure
Thailand	0.006-mean exposure	0.0002-mean exposure
	0.6-high exposure	0.02-high exposure
Vietnam	0.12-mean exposure	0.004-mean exposure
	10.3-high exposure	0.01-high exposure

Note: NA (not available).

Rahayu (2010) estimated that about 227 new cases of cancer per year due to peanut and peanut sauce consumption. Furthermore, Pitt and Hocking (1997) suggested that from the epidemiological data from several African countries and Thailand, there is a logarithmic relationship between AF intake and the incidence of liver cancer.

### **Updated Risk Assessment of Maize and Peanut Consumption in Indonesia**

Risk assessment is the process of quantifying the magnitude and exposure, or probability, of a harmful effect on individuals or populations from specific agents or activities (Kuiper-Goodman, 2004). Until now, literature is lacking information on exposure and risk assessment of mycotoxins in Indonesia. Here, we update risk assessment of peanut and maize consumption in Indonesia. We used secondary data of mycotoxin contamination taken from last decade literature or reports. We determined the exposure levels of Indonesian infants, children, and adult to aflatoxin through maize and peanut consumption and assessed the associated risks as a result of its consumption.

Table 3 shows the exposure and risk assessment data for AFs among the different population groups that consume peanut in Indonesia. The results showed quite high exposure levels to aflatoxins among Infants, children and adult with PDI (national) of 51.2, 20.5 and 8.5  $\text{ng.kg}^{-1}\text{bw.day}^{-1}$  respectively. The exposure levels of all categories of maize consumers to AFs exceeded the 0.017  $\text{ng.kg}^{-1}\text{bw.day}^{-1}$  permissible exposure threshold by more than 531 fold. This is indicating a public health risk even though it is much less than PDI in African countries and other developing countries.

Table 4 shows the exposure and risk assessment data for aflatoxins among the different population groups that consume maize in Indonesia. The results also showed quite high exposure levels to aflatoxins among infants, children, and adult with PDI (national) of 21.1, 8.4 and 8.1  $\text{ng.kg}^{-1}\text{bw.day}^{-1}$  respectively. The exposure levels of all categories of maize consumers to AFs exceeded the 0.017  $\text{ng.kg}^{-1}\text{bw.day}^{-1}$  permissible exposure threshold by more than 506 fold. This is also indicating a public health risk even though it is much less than PDI in African countries.

Risk exposure level for maize consumption is less than peanut. This in line on the data population risk for primary liver cancer. This also indicates that peanut is more susceptible to AF contamination than maize. The PDI data across the population showed a similar trend of infants > children > adults for both foods. This explains that infants are the most susceptible to be exposed to the effect such as stunting (impaired growth), and immune suppression.

By doing comparison data, Indonesia has higher PDI compared with Korea (5.79  $\text{ng.kg}^{-1}\text{bw.day}^{-1}$ ) (Park et al., 2004), France (0.345.79  $\text{ng.kg}^{-1}\text{bw.day}^{-1}$ ) (Leblanc et al., 2005), Australia and USA (0.15 and 0.26  $\text{ng.kg}^{-1}\text{bw.day}^{-1}$ , respectively) (WHO, 1998), but has almost same level with Brazil (59.1  $\text{ng.kg}^{-1}\text{bw.day}^{-1}$ ) and lower level compare to China (91  $\text{ng.kg}^{-1}\text{bw.day}^{-1}$ ) (WHO, 1998).

Table 3. Exposure assessment and estimated liver cancer risk due to consumption of peanut in Indonesia.

Areas	<sup>1)</sup> Total Aflatoxins (ng/g)	<sup>2)</sup> PDI (ng.kg <sup>-1</sup> bw.day <sup>-1</sup> )			<sup>3)</sup> MOE			<sup>4)</sup> PRPLC (cancer/Year/100000 population)		
		Infant	Children	Adult	Infant	Children	Adult	Infant	Children	Adult
Bogor	67.3	49.1	19.7	8.2	3.5	8.6	20.8	4.1	1.6	0.7
Wonogiri	151	110.2	44.1	18.4	1.5	3.8	9.3	9.1	3.6	1.5
Cianjur	98.2	71.7	28.7	11.9	2.4	5.9	14.2	5.9	2.4	0.9
Yogyakarta	81.4	59.5	23.8	9.9	2.6	7.1	17.1	4.9	1.9	0.8
Rembang	23	16.8	6.7	2.8	10.1	25.3	60.8	1.4	0.5	0.2
West Java	45	32.8	13.1	5.5	5.2	12.9	31.1	2.7	1.1	0.5
Bali	27	19.7	7.9	3.3	8.6	21.6	51.8	1.6	0.6	0.3
Purworejo	49	35.7	14.3	5.9	4.7	11.9	28.5	2.9	1.2	0.5
National	70.2	51.2	20.5	8.5	3.3	8.3	19.9	4.2	1.7	0.7

Note:

<sup>1)</sup> Summation of mean of means of total aflatoxins as reported from literatures (last decade reports)

<sup>2)</sup> Probable Daily Intake (PDI) (Assumed 10 kg, 25 kg (Rodriguez-Carasco et al., 2013) and 60 kg (Liu and Wu, 2010) body weight for infants, children and adults, respectively) calculated by multiplying the aflatoxin concentration in peanut by consumption rates of peanut in Indonesia (7.3 g/person/day) as estimated by the Statistics Indonesia (BPS, 2015) and divided by body weight of consumers

<sup>3)</sup> Margin of Exposure (MOE), calculated by dividing the benchmark dose lower limit (BMDL) for aflatoxins (170 ng.kg<sup>-1</sup> bw.day<sup>-1</sup>) by AFB1 exposure.

<sup>4)</sup> Population risk for primary liver cancer (PRPLC) = Exposure x Average potency (0.0825) (Shephard, 2008).

Table 4. Exposure assessment and estimated liver cancer risk due to consumption of maize in Indonesia.

Areas	1) Total Aflatoxins (ng/g)	2) PDI (ng.kg <sup>-1</sup> bw.day <sup>-1</sup> )			3) MOE			4) PRPLC (cancer/Year/100000 population)		
		Infant	Children	Adult	Infant	Children	Adult	Infant	Children	Adult
West Java	34.3	17.1	6.9	2.9	9.9	24.8	59.5	1.4	0.6	0.2
Lampung	28.1	14.1	5.6	2.3	12.1	30.2	72.6	1.2	0.5	0.2
South Kalimantan	148.8	74.4	29.8	12.4	2.3	5.7	13.7	6.1	2.5	1
South Sulawesi	64.3	32.5	12.9	5.4	5.3	13.2	31.7	2.7	1.1	0.4
Malang	34	17	6.8	2.8	10	25	60	1.4	0.6	0.2
Yogyakarta	15.2	7.6	3	1.3	22.4	55.9	134.2	0.6	0.3	0.1
Bogor	11	5.5	2.2	0.9	30.9	77.3	185.4	0.4	0.2	0.07
Kupang	32.4	16.2	6.5	2.7	10.5	26.2	62.9	1.3	0.5	0.2
Gorontalo	45	22.5	9	3.7	7.5	18.9	45.3	1.8	0.7	0.3
Taban	11	5.5	2.2	0.9	30.9	77.3	185.5	0.5	0.2	0.07
Kediri	40	20	8	3.3	8.5	21.3	51	1.7	0.7	0.3
National	42.1	21.1	8.4	8.1	8.1	20.1	48.3	1.7	0.7	0.3

Note:

1) Summation of mean of total aflatoxins as reported from literatures (last decade reports)

2) Probable Daily Intake (PDI) (Assumed 10 kg, 25 kg (Rodriguez-Carasco et al., 2013) and 60 kg (Liu and Wu, 2010) body weight for infants, children and adults, respectively) calculated by multiplying the aflatoxin concentration in maize grains by consumption rates of maize in Indonesia 5 g/person/day) as estimated by the Statistics Indonesia (BPS, 2016) and divided by body weight of consumers

3) Margin of Exposure (MOE), calculated by dividing the benchmark dose lower limit (BMDL) for aflatoxins (170 ng.kg<sup>-1</sup> bw.day<sup>-1</sup>) by AFB1 exposure.

4) Population risk for primary liver cancer (PRPLC) = Exposure x Average potency (0.0825) (Shephard, 2008).

## Mycotoxin Regulation in Indonesia

Mycotoxin regulation in Indonesia is still very limited. Mycotoxins were first mentioned in the Food Act (No. 7, 1996) and improved in 2004 by Government Regulation (No. 28, 2004). The regulation was developed because of the increase in awareness about the relationship between food safety and health. In 2004, NA-DFC issued regulations on the maximum limit of AFs in food products. The regulation stated that the maximum contamination of AF B1 and total AF in peanut-based food products and corn are 15 µg/kg and 20 µg/kg respectively. This limit is similar to the regulations that apply in Hong Kong, Thailand and the Philippines. In the meantime, Indonesia has more stringent regulation of the AF limit compared to other Asian countries like India (30 µg/kg), Malaysia (35 µg/kg) and Sri Lanka (30 µg/kg). Indonesian dairy products have a maximum limit of AF M1 of 0.5 µg/kg which is more stringent than in Malaysian and Sri Lankan regulations. In 2009, Indonesian National Council for Standardization (INCS) launched a comprehensive regulation (SNI 7385-2009) about the limits of mycotoxin contamination in food products (Table 3, details chemical structure in Figure 2). This new regulation includes 5 types of mycotoxins (AF, DON, FUM B<sub>1</sub>+B<sub>2</sub>, OCA, and patulin). The maximum limit established for AF in various food products are the same as in the regulations issued by NA-DFC in 2004. Unfortunately in the newest regulations, zearalenone is still not included.

The maximum limit of AF content in foods:

No.	Food Products	Type	Maximum Level (ppb or µg/kg)
1	Milk and milk drink products	M1	0.5
2	Fermented milk and rennin hydrolyzed milk products	M1	0.5
3	Concentrated milk and analog products	M1	0.5
4	Cream and related products	M1	0.5
5	Milk powder and analog products	M1	5
6	Cheese and analog products	M1	05
7	Desserts made from milk (pudding, yogurt)	M1	0.5
8	Whey and its processed products	M1	0.5
9	Peanuts and its processed products	B1	15
		Total	20
10	Corn and its processed products	B1	15
		Total	20
11	Spice powder	B1	15
		Total	20

The maximum limit of DON content in foods:

No.	Food Products	Maximum Level (ppb or µg/kg)
1	Wheat	1750
2	Corn	1750
3	Corn processed product as a raw material	1000
4	Wheat processed product as a raw material	1000
5	Wheat processed products ready for consumption (pastry, bread, biscuits, snacks)	500
6	Pasta and noodles and similar products	750
7	MP-ASI (complementary food for infant)-wheat flour based	200

The maximum limit of FUM B<sub>1</sub>+B<sub>2</sub> content in foods:

No.	Food Products	Maximum Level (ppb or µg/kg)
1	Corn	2000
2	Corn processed product as a raw material	2000
3	Corn processed products ready for consumption	1000

The maximum limit of OCA content in foods:

No.	Food Products	Maximum Level (ppb or µg/kg)
1	Cereals (rice, maize, sorghum, wheat)	5
2	Processed cereal products as raw materials	5
3	Processed cereal products ready for consumption	3
4	MP-ASI (complementary food for infant)- cereal based	0.5
5	Dried grapes, including raisins	10
6	Grape juice	2
7	Roasted coffee, coffee powder	5
8	Instant coffee	10
9	Beer	0,2

The maximum limit of patulin content in foods:

No.	Food Products	Maximum Level (ppb or µg/kg)
1	Apple	50
2	Canned apple	50
3	Apple puree	25
4	Extract apple	50
5	Nectar apple	50
6	Apple purees for babies and children	10
7	Alcoholic beverages-apple based	50

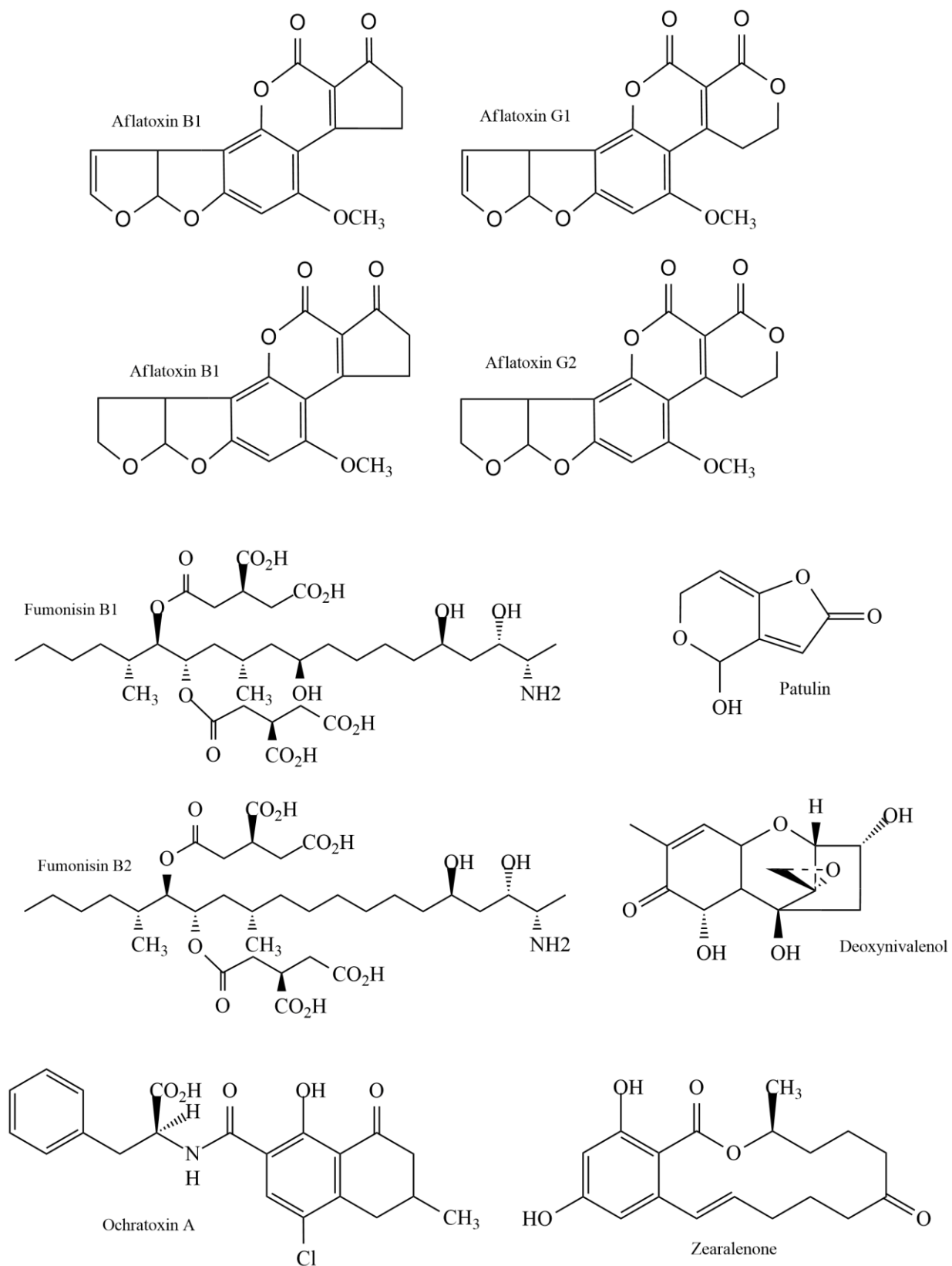


Figure 2. Chemical structure of important mycotoxins in Indonesia.



## Mycotoxin Control in Indonesian Foodstuffs

Considering the danger resulting through mycotoxin contamination, it is necessary to establish effective preventative steps from pre-harvest to storage, or remedial actions including decontamination or detoxification. AF control will be the main focus of attention since Indonesian research has focused most on this.

### A. Preventive Strategies (Pre-harvest, Harvest, and Post-harvest)

#### 1. Pre-harvest

The critical point of mycotoxin contamination is in the pre-harvest stage when crops are in the pods or fruit filling phase. Seed filling phase is a phase of plant growth which is very sensitive, mainly to temperature stress and drought. The optimum soil temperature for *Aspergillus flavus* ranges from 25.7°C to 31.3°C. Plants under drought stress are easily exposed to such high temperature during the maturation stage which is about 4-6 weeks before harvest (Kasno, 2004). At a temperature of 25.7°C, the intact peanut seeds infection with *A. flavus* is low, since *A. flavus* favors a temperature of 26.3°C, furthermore the fungal growth proliferates with increased temperature up to 31.2°C. Soil temperatures over 31.2°C dramatically inhibit the growth of *A. flavus* (Ginting and Beti, 1996). In Indonesia, peanuts are usually planted during dry season and suffering drought stress in addition to high temperature. Therefore, the chances for plant infection or contamination with *A. flavus* are very common. During the seed filling phase, plants need a sufficient supply of water and calcium (Ca) (Taufik, 2000). Plants which are exposed to water and Ca deficiencies usually produce small pods with seeds wrinkles, where the latter might encourage a successful *A. flavus* infection. Approximately 60% of Indonesian peanut plants are grown on dry land. Though this land is alfisol, alkaline and calciumrich, it has low concentrations of iron nutrients which results in chlorosis which is very similar to chlorosis resulting from Ca deficiency (Taufik 2000; Sudaryono 2001).

Abiotic stress (e.g. drought, lack of nutrients) and biotic stress (e.g. herbivores or insect attack, and fungal contamination) in addition to harvest-timing have a direct effect on mold growth in plant tissues and subsequently on mycotoxin production (Kasno, 2004). Good Agricultural Practices (GAP) can minimize the fungal occurrence and plant infection in the field as follows:

*Crop rotation*: to break the cycle of toxigenic fungi proliferation in the soil. This is a very effective method to prevent the spread of toxigenic fungi in the field. For instance, a crop rotation of corn-soybean can reduce *Fusarium* spp. attack compared to regular corn planting (Lopez-Garcia and Park, 1998; Kasno, 2004).

*Selection of resistant varieties*: is an effective strategy to avoid toxigenic fungi attack on commodities. Kasno (2004) discovered the existence of chromosomes related to the resistant to *A. flavus* in some agricultural commodities such as corn and peanuts. The peanut varieties of *Jerapah*, *Sima*, and *Turangga* were more resistant to *A. flavus* infection than *Tigers* and *J11* varieties. These three resistant varieties have been introduced in Indonesia through local varieties and derivatives which have been examined for resistance

to *A. flavus* (Kasno, 2004). However, there are not enough resistant seeds available at the farm level (Kasno, 1994).

*Pests, diseases and weed control:* Insect infestations cause damage to the plant and make it susceptible to fungal infection. Control strategy using insecticides and fungicides will help to prevent mold growth and mycotoxin production. In susceptible varieties, disease control with spraying fungicides Topsin M, Daconil, and Baycor 4-6 times to avoid production loss of up to 55.1%. Control of leaf disease with chemicals increased the yield of dry pods (2.48-2.70 t/ha) as compared to non-control (1.30-1.60 t/ha) (Hardaningsih et al. 1992). Kasno (1993) reported that the use of tolerant varieties can prevent yield losses up to 20%. Leaf diseases can also increase the intensity of *A. flavus* contamination. According to Kasno (2002a), control of foliar diseases can reduce the intensity of *A. flavus* from 13% to 7%. Meanwhile, weeds can also be a vector for toxigenic fungal infections, especially soil-borne pathogens, such as *Fusarium graminearum* and *F. moniliforme*. Weeds should be removed mechanically or by using herbicides or other safer means (Noor, 1997).

*Soil and irrigation management:* Drought and soil fertility influence intensity of fungal infection in the soil (Kasno, 2004). Temperature and soil moisture control play an important role in mycotoxin management. Peanut crop grown under drought stress during the reproductive stage is very sensitive to *A. flavus* infection (Kasno, 2004). Moreover, drought stress and high levels of humidity are ideal conditions for the proliferation of *F. moniliforme* and fumonisins production in corn plants (Lopez-Garcia and Park, 1998). The deficiency of soil nutrients like iron was found to increase invasion of *A. flavus* to peanut plants (Kasno, 2004).

Mehan (1989) made a selection of resistant peanut plants on sandy soil under drought stress during the seed filling stage. Peanuts selection was also carried out in Indonesia by providing optimum and sub-optimum irrigation conditions at the reproductive stage. The results showed that the lack of irrigation significantly increased the rate of *A. flavus* infection. Optimum irrigation during the reproductive stage can suppress fungal attack intensity from 28% (without irrigation) to 3% (with irrigation) (Kasno et al., 2002).

*Biological control:* Prevention of toxigenic mold infestation in plants can also be done using biological methods by spreading non-toxigenic *Aspergillus* sp. which will compete with toxigenic *A. flavus* and *A. parasiticus* so that the toxigenic fungal growth is hampered. This method showed satisfactory results on peanut plants, where AF contamination can be reduced by up to 90% (Cole and Dorner, 1999). To ensure this success, Pitt (1999) suggested that the spore ratio of non-toxigenic to toxigenic fungi in the field should be 100:1. However, the challenge is to achieve this ratio practically in the field. The United States of America has commercial bio-control products to control pathogens such as *A. flavus* in particular plants. The product is "AF36" which is a sterile wheat seed colonized with *A. flavus* AF36 to cope up *A. flavus* attack in cotton plants. Product "afla-

guard" that is a granola with 0.01% *A. flavus* NRRL 21 882 which is applied to peanut plants (Gardener, 2005; Dharmaputra et al., 2005).

Research about *A. flavus* antagonistic fungi was carried out in Indonesia by Dharmaputra et al. in 2002. *Aspergillus niger* was the most promising antagonistic mold because growth of *A. flavus* was inhibited and showed 80% of AF reduction. Non-toxicogenic isolates of *A. flavus* 6I2 reduced AF production by 61%, while using *A. tamarii* and non-toxicogenic *A. flavus* 361 showed a reduction of AF by 60% and 59% respectively. Dharmaputra et al. (2003) used *Trichoderma harzianum* in soil to control *A. flavus*. The results showed that *T. harzianum* is able to prevent *A. flavus* attack, thus preventing AF contamination.

The use of biological control agents was tested to prevent *F. oxysporum* contamination. *Fusarium oxysporum* is able to produce fusaric acid, which is also toxic for animals. This pathogen is one of the main problems of plant diseases, especially banana plants in Indonesia. Effendi (2013) conducted experiments using some *Trichoderma* species showing antagonistic effects. All the strains had direct and indirect inhibitory effects (with producing volatile compounds) in the antagonistic test to *F. oxysporum* f.sp. cubense. The highest inhibition was shown by *T. laugher* IPBCC 13.1031 (85.63%). In the volatile test, the three best strains of *T. harzianum* LIPIMC namely 0572, *T. ovalisporum* LIPIMC 0571, and *Trichoderma* sp. LIPIMC 0570 showed antagonistic activity due to volatile compounds showing inhibition values of 45.25%, 45.65%, and 45.38% respectively. Nurbailis (2008) demonstrated that *Trichoderma* strains from the center of the banana production in West Sumatra were able to overcome Panama wilt disease. Sudantha et al. (2011) also described several *Trichoderma* strains that effectively inhibit the growth of *F. oxysporum in vitro*. Despite the fact that the use of biocontrol agents are successful in the laboratory, their commercialization depends on whether they can consistently control the mycotoxigenic fungus in different field conditions.

## 2. Harvest

At the harvest stage, there are several issues to be considered in order to prevent mycotoxin contamination: (i) Harvesting has to be done at the optimal maturity level of grain followed by rapid drying (1-2 days) so that the moisture content of grain quickly reaches 14% to prevent the development of toxigenic fungi; (ii) separation of damaged grains; (iii) the moisture content and surrounding temperature should be maintained in the appropriate range; (iv) insect and rodent control (Rahayu, 2009).

Harvesting at the optimum stage of grain maturity can reduce or prevent infection of *A. flavus*. The maturity level of peanut can be recognized visually from the slightly darker skin color of the pods when they are peeled, the black skin sketch will appear with a white background. Quantitatively, the optimum maturity level is achieved when the seed ratio (seed-hull) is at its maximum level (Trustinah 1987). Earlier harvest will produce many young pods and dried seeds as the pods shrivel because the drying time is too long for the small seeds. The unripe seed is more susceptible to *A. flavus* infection compared to

the mature seed (Rucher et al. 1994). Conversely, at late harvest, skin pods are attached tightly to endosperm which results in many broken seed when peeled (Trustinah and Kasno 1992). Wrinkled and damaged seeds are susceptible to *A. flavus* infection (Ginting dan Beti 1996). If the damaged and wrinkled pods are kept at low as possible at this stage, further contamination can be reduced significantly.

Harvesting should be done in the dry season and when seeds are ready to be harvested. Unripe seeds or grains (containing large amounts of water) are beneficial for the mold infection. The water content at harvest time should be set in a certain range, such as 23-25% for maize grain, in sorghum this should be between 12-17%, 11-15% in soybeans and 35-50% in peanuts (Kasno, 2004). Harvesting at the wrong stage leads to fungal infection in farming products. Tools used during harvesting, transport, drying and storage should be clean, free from insects and mold. Mechanical damage and contact with the infected plant parts should be avoided during harvest.

### **3. Post-harvest**

Prevention through pre-harvest management is the best method to control mycotoxin contamination; however, if contamination has occurred at that stage, the potential dangers associated with the toxin must be monitored during post-harvest procedures, especially if the product is intended for human consumption. Sorting, drying, storage and processing of grains after crop harvesting are necessary steps to prevent contamination. Drying should be done under good conditions, sufficient light, cleanliness, dust protection (because dust can increase the spread of fungal spores), insect- and rodent-free (Rahayu, 2009).

A delay in drying after harvest can lead to *A. flavus* growth. Food industries are only willing to buy wet peanut which has been stored for less than 24 hours after harvesting because this storage process or period will produce crunchy peanut with a rancid flavor due to oil oxidation. AF contamination can occur if the crop is not dried within 48 hours after harvest (Cardona et al. 1989). In Indonesia, traditional drying is held with smoke or fogging at rainy season. Drying should reduce the moisture content of grain by 35-40% after harvest (depending on the age of maturity) which is a safer moisture content. Grain with moisture content about 15-20% is very conducive for *A. flavus* AF production, and on moisture content of 5-8%, grains were contaminated with AF after being stored for three months (Cardona et al. 1989; Ginting and Beti 1996). Therefore, drying must be performed until the moisture content is less than 5%. Although this can be done, temperature and relative air humidity in Indonesia is the limiting factor and usually a balanced grain moisture content in the range of 7-9% is achieved. This suggests that storage is a critical stage in preventing infection of *A. flavus* as grains should be kept at very low levels of absolute water content, but this requires sophisticated storage facilities with relatively expensive costs. Practical solutions that can be considered are to shorten the time of distribution from farmers to consumers in Indonesia which usually takes 40-110 days (Machmud, 1989; Kasno 2004).

Furthermore, storage at the farm, collectors, retailers or merchants is critical. The lack of storage facilities, and improper packaging of the product can cause an increase in mycotoxin contamination. The accumulation of moisture, heat and physical damage to plant materials can trigger the growth of fungi which then produce mycotoxins. To prevent fungal growth maize, it must be ensured that the stored grains remain dry (water content <12%). Moreover, rodents and insects should not be present around the storage area. The hygiene procedures inside packing houses and the use of pesticides can minimize fungal contamination. The temperature and humidity must be measured regularly throughout the storage period. An increase in temperature of 2°-3°C may indicate the presence of mold or insect infestation. Packaging containers which have pores are recommended for air circulation, and the packages should be stored on pedestal boards (Rahayu, 2009).

Besides temperature regulation, air circulation and proper moisture inside store rooms, chemicals or natural materials can also be applied for mycotoxin control of agricultural products during storage. The use of chemicals is primarily designed to protect animal feed (Muller and Thaler, 1981), but not for foodstuffs intended for human consumption. The use of natural preservatives to inhibit fungal growth are preferred to protect foods. Gowda et.al. (2004) used spices to inhibit AF biosynthesis by *A. parasiticus*. The study showed that clove oil was the best compound to inhibit the growth of *A. parasiticus* and AF production was inhibited by 100%, followed by turmeric, garlic, onion oil each with respective concentrations of 0.5-1.0% provided 77-85%, 73-77%, and 80-84% inhibition respectively. Juglaletal (2002) also mentions that clove oil provided the highest inhibition activity against the growth of *A. parasiticus* and *F. moniliforme*, followed by cinnamon, oregano, and nutmeg flower oil. Ethanolic extract from China onion (*Welsh Onion*) could prevent the growth of *A. parasiticus* at 10 mg/mL (Chen, 1999). Essential oil extracted from thyme species show MIC (minimum inhibitory concentration) on solubility level to 1/8 and MFC (minimum fungicidal concentration) at the rate of 1/4 (Rasooli and Abyaneh, 2004).

To the present, there are few studies in Indonesia that use natural substances and biocontrol agents to prevent fungal contamination during storage. In 1984, the use of spices was evaluated for mycotoxin control. Clove was the most effective compound against mold growth inhibition of *A. flavus*. At a concentration of 0.4% (w/v) of cloves, mold growth was inhibited by extending the adaptation phase another three days contact time. With the use of powdered nutmeg, AF produced ranged between 0-10.8 µg/kg, which is lower than in controls. The addition of garlic powder in medium could reduce AF levels 0-6.7 µg/kg. The contact with cinnamon for seven days reduced AF to 0-5.4 µg/kg, while ginger reduced AF production to 0-3.3 µg/kg. Turmeric and cloves delayed AF production until seven days of contact time.

In 2015, Hidayah used chitosan to prevent mycotoxin contamination during storage. Chitosan is a cationic polymer, non-toxic and biodegradable, that has potential as an antifungal agent. Chitosan has been used during the storage of peanuts to inhibit growth



of *A. flavus*. The lowest content of AF B1 was obtained with 1% chitosan soaking treatment on day 30. In addition, Lisangan (2014) also examined the use of extracted *kebar* leaves. *Kebar* grass, leaves-extract were extracted using the solvents hexane, ethyl acetate, methanol, and distilled water. The results showed that the hexane-ethyl acetate-methanol (HEM) extracts of *kebar* grass leaf which were added at a concentration of 20 mg/mL in a rich media with carbohydrates, fat and protein caused growth inhibition of *A. flavus* of 96.2, 100, and 96.1% respectively. AF B1 reduction in media with a concentration of corn 100% ranging from 7.0-91.3%, while in the media with a concentration of peanut 100% were at 19.3-59.4%. Further Anggriawan et al. (2014) reported the use of brown seaweed to inhibit the growth of *A. flavus*. The nonpolar extract produced by nonpolar extraction (solvent-solvent extraction) showed the highest inhibition. In contrast, sulfate polysaccharide produced using a water boiling extraction method promoted the fungal growth. The most effective brown seaweed species for *A. flavus* inhibition were *Turbinaria decurrens* and *T. conoides*.

Biocontrol methods use microorganism to compete with toxigenic fungi. Rahayu (2014) reported the use of yeast to inhibit contamination combined with temperature control and storage humidity. At 40 °C with a relative humidity of 70, 80 and 90% *F. verticillioides* BIO 957 was unable to grow, thus the production of FUM B1 can be avoided. *F. verticillioides* BIO 957 growth can be inhibited by *S. cerevisiae* ATCC 9376 by 44, 44 and 38% on CDA, corn, and soybeans media respectively. The following year, Pratiwi used the same yeast to reduce *A. flavus* contamination. The growth of *A. flavus* BIO 2237 and BCC F0213 were also controlled by *S. cerevisiae* ATCC 9376. The reduction of growth of *A. flavus* BIO 2237 by *S. cerevisiae* ATCC 9376 on PDA, corn, and soybean were 46, 47, and 45% respectively, while for *A. flavus* BCC F0213 it was 52, 52 and 38% respectively. The ability of *S. cerevisiae* to act as an antimicrobial agent is due to the secretion of a proteinaceous compound called killer protein (Ray, 2001). Selitrennikoff (2001) mentioned that *S. cerevisiae*, *Hanseniaspora uvarum*, *Z. Bailii*, *Pichia rhodozyma*, *Kluyveromyces lactis* and *Pichia* species secrete these killer proteins. Twenty killer toxins have been successfully identified with molecular weights ranging from 10.7 to 156.5 kDa. Ray (2001) mentioned that yeast can attach strongly on mycelia and produce the enzyme glucanase to degrade part of the fungal cell wall.

The use of microbes as a competitor for *A. flavus* have also been reported by Edi (1988) and Fardiaz et al. (1993) using the fungus *Neurospora sitophila* and *Rhizopus oligosporus*. The use of a competitor mold inhibited the growth of *A. flavus* and decreased AF production by 77 and 41.1% respectively in corn media. Until now, there are some microbes that have also been described to reduce the growth of *A. flavus* among of them are *R. oligosporus* and *Candida* sp. which were isolated from tempeh. The yeast inhibits AF production by 37.21 and 99.96% respectively (Purwijatiningsih et al., 2005). *Aspergillus flavus* can be controlled by *S. cerevisiae* not only in synthetic media but also on the feed (Kusumaningtyas et al. 2006). Dewanti-Hariyadi et al. (2008) reduced the

amount of AF from *A. parasiticus* using *Mucor rouxii* and *Saccharomyces* sp. by 99.7 and 98.1% respectively.

Lactic acid bacteria has also been studied and has been proven to inhibit the growth of mycotoxigenic fungus, this also has potential for application in grains storage. *Lactobacillus plantarum* sa28k reduced the growth of *A. flavus* (Hidayat, 2001). *Lactobacillus plantarum* pi28a inhibited *Penicillium citrinum* growth (Handy, 2001) and *L. plantarum* reduced the growth of *Fusarium graminearum* (Pramasari, 2001). Rohmawati (2001) reported the ability of *L. plantarum* and *Leuconostoc* sp. to inhibit mold growth and AF production due to the decrease in pH media. Suspension of *L. coryneformis* was better for the inhibition of *A. flavus* growth, while suspensions of *L. plantarum* isolates were useful to inhibit *P. citrinum* (Ismail, 2002). Magnusson and Schnurer (2001) reported protein compounds that are antimycotic with a broad spectrum produced by *L. coryneformis* subsp. *coryneformis* strain Si3. Ridawati (2003) found the results of ultrafiltration fractions of *L. coryneformis* to be the most effective in reducing the levels of AFs in peanuts. The fraction had <1 kDa molecular weight with the ability to reduce AF by 87.5%.

## B. Detoxification Strategies

Food that has been contaminated by mycotoxins need to be detoxified before consumption. Detoxification or decontamination not only reduces the toxins concentration to safe levels but can also inactivate the toxins by transformation. Fungal spores and mycelia should also be totally destroyed to avoid new toxins production. The nutritional value, taste and physical properties must be maintained during this process, and the process must be economically feasible (Park, 1993). Moreover, the process has to be simple, and its implementation can be carried out using existing technology. Detoxification can be done in three ways: physical, chemical, and biological (Smith et al., 1994).

### 1. Physical Detoxification

Detoxification can physically take place during food processing, by milling, heating, and irradiation.

#### a. Milling and sorting

Wet milling begins with cleansing followed by soaking grains in warm water plus SO<sub>2</sub> for 24-36 hours. Grains are separated from the endosperm dried and separated from the oil, gluten and endosperm are dried and milled. Starch and oil are the main products of the wet milling process. The results of laboratory studies indicate that most of the AF B1 is in the water immersion (39-42%) and fiber (30-38%), with some in gluten (13-17%), germ (6-10%), and starch (1%) (Wood et al., 1982). Wet milling does not change AF much. While the dry milling will divide AF into factions. When dry milling corn, the highest concentration of AF B1 is in the germ and the skin, and about 6-10% of the initial contamination ends up on the major products (low-fat flour, grain maize). The same happens to the rice contaminated with AF, most of the toxin (> 95%) will be on the skin or bran fraction after grinding (Achroder et al., 1986).

There are several important notes to be considered during grinding and sorting for mycotoxins decontamination: (i) Peeling the skin, because the food surface can be a source of contaminants; (ii) Separation of defect grains, because they are more likely to be contaminated and can act as source of contamination; (iii) Sorting by color or appearance; (iv) Separation based on density of grains, because the defect grains have lower density; and (v) Desired component (e.g., oil or starch) have to be separated for further processing (Rahayu, 2009).

#### b. Heating

Heating treatment during food processing can be done by autoclaving, drying, boiling and roasting. Some studies reported that baking (roasting) is a good method to reduce AFs in certain commodities. Conway et al. (1978) reported that when maize is baked at a temperature of 145°-165°C there is a reduction 40-80% of AF B1. Rice cooking can eliminate 40% of AF B1, with pressure cooking or cooking with excess water will more eliminate AFs by 73% and 82% respectively. These results prove the influence of water on the stability of AF during heating. This method is generally applicable to agricultural products and processed products. Heating effectiveness depends on the type of mycotoxin, heating time, temperature, and moisture content of the material. AFs, zearalenone, and tricothecene are very stable at high temperatures, but citrinin and ergot alkaloids are relatively easily degraded by heating (Mobiuddin, 2000).

Cooking methods also largely determine the degradation level of mycotoxin. Kaimura (1999) suggest frying at temperatures between 150°-180°C decompose mycotoxins. Direct roasting on the fire with temperatures around 210°C is more helpful to degrade mycotoxins such as nivalenol which was reduced by 90% with baking for 15 minutes. AFs are stable to heat even above 100°C (Makfoeld, 1993). AF B1 was removed from peanut or corn oil by heating to a temperature of 250°C. However, a combination factors between water content and heat have a role in AF degradation Heat degradation of nuts for AF degradation is more efficient when there is a high water content. With 30% water content and heating for 2.5 hours at 100°C could reduce AF up to 85% (Davis et al., 1966).

Based on the research results of Roedjito et al. (1994), washing the corn with water at 50°C can reduce AF content by 47% and soaking for 24 hours can reduce AF content by 75%. Steaming heat treatment and boiling can reduce AFs by 35% and 63% respectively. The effect of wet heating conditions to decreased levels of AF was also reported by Novia (1994) who determined the effect of steaming (95°C), boiling (100°C) and the heating pressure (autoclave 121°C) against AF content. Pressurized heating is the fastest way to reduce AF. According to Novia (1994) steaming for 125 minutes can reduce AF to 37.38%, 50 minutes boiling to 68.25% and heating pressure for 25 minutes to 42.74%. While, according to Fardiaz (1992) roasting at 160°C for 15 minutes could reduce AF B1 to about 21.9-39.2%. For AF effective degradation, the heating temperature should be below its melting point (237°-289°C) and sufficient moisture content is required.



### c. Irradiation

The effect of irradiation on most mycotoxins is not well known and due to its high-cost facility, thus this technology is difficult to apply in most food industries (Irawati, 2013). Fuel (1966) reported that gamma irradiation (2.5 M rad) did not affect AF content in peanut products. However, the treatment of peanut oil with short wave and ultraviolet light was able to reduce levels of AF.

Irradiation with sunlight or light was reported be effective in degrading AF in unpurified peanut oil. When peanut flakes with a thickness of 0.5 mm were exposed to sunlight for 14 hours 77-90% of AF was degraded. While with the same treatment on the nuts (0.5 mm thick) exposed for 14 hours AF was reduced by only 50% (Samarajeewa et al., 1990). Irradiation by the sun rays can damage AF in just a few hours, but this has not been tested on an industrial scale. The mechanism of decontamination by irradiation showed that AF is very sensitive to oxidation reactions induced by light. One possibility is that AF is oxidized by singlet oxygen formed by the light, sensitizer, and triplet oxygen. However further research in this topic is required.

Ultraviolet rays and radiation can also degrade mycotoxins, including lowering the content of AF M1 in milk (Yousef and Marth, 1985). However, ultraviolet light and radiation can damage the nutrients compounds in food (Mobiuddin, 2000). Radiation and ground nuts heating in the microwave for 3 and 5 minutes can show a reduction in AF content of respectively up to 25 and 49.25% (Chinaputi, 1999).

## 2. Chemical Detoxification

Chemical AF degradation has been widely studied. Some of the chemical compounds which are able to detoxify AF includes chlorine (sodium hypochlorite, chlorine gas), hydrogen peroxide, ozone, sodium bisulfite, calcium hydroxide, acetic acid, ammonium or ammonia gas (Suttajit, 1989).

Detoxification with ammonia is efficient and can degrade AF up to 95%. The effectiveness of AF degradation using ammonia is influenced by the levels of given ammonia, the moisture levels of the product, temperature, contact duration with ammonia, and the level of AF contamination (Smith et al., 1994). Detoxification with ammonium hydroxide (NH<sub>4</sub>OH) at high temperatures and pressure opens the lactone ring in the AF. At high pH, the lactone ring in the AF molecule is hydrolyzed. The use of ammonia to detoxify AF has been applied in various countries including USA, France, Senegal, Sudan, Brazil, Mexico, and South Africa. Based on research results, ammonium treatment can be used to reduce the potential hazards of AF mainly when treated together with high temperature or pressure.

Sodium chloride also has an effect on the reduction of AF contamination of unskinned peanuts, followed by boiling with pressure for 30 minutes; the reduction of AF concentration by 80-100% with 5% sodium chloride, but only 35% reduction reached if without salt (Scott, 1984). Sodium bisulfite also reacts with AF B1, G1, M1, and aflatoxicol at different temperatures and using different concentrations, to form a water-soluble product

(Hagler et al., 1982). Treatment of AF B<sub>1</sub> for 24 hours with hydrochloric acid at pH of 2.0 can degrade AF by 19.3%, while at pH 3.0 only 6.4% AF degradation was observed (Doyle et al., 1981). Tabata et al. (1994) reported the ability of some food additives on AF degradation. Food additives were grouped into acidic materials such as sodium bicarbonate, sodium carbonate, sodium hydroxide, sodium sulfite and sodium hypochlorite; food additives which are neutral as potassium metabisulfite, sodium bisulfite, sodium hydrosulfite, hydrogen peroxide, sodium chloride and ammonium ferodisulfat. The ability of these food additives in degrading AFs was observed under different temperature conditions, incubation times and concentrations. The result showed that 0.25% sodium chloride at pH 4 after a 48 hour incubation was most effective in degrading AF.

Research conducted by Nugroho (2005), indicate that a combination of physical and chemical methods, such as the use of lime water during boiling could further increase the AF B<sub>1</sub> reduction efficiency in the processing of contaminated corn chips. Based on the logarithmic equation, to reduce 90% of AF B<sub>1</sub> contamination, 1.52% concentration of lime water is needed. Chemical detoxification can also be accomplished by the addition of acid, in this case AF B<sub>1</sub> will be converted into AF B<sub>2a</sub> and showing a lower toxicity than the original (Suttajit, 1989). During acid fermentation (pH $\leq$ 4.0), AF B<sub>1</sub> also can be converted into AF B<sub>2a</sub>.

### 3. Biological Detoxification

Biological detoxification is regarded as the biotransformation or degradation of the toxin by microorganisms or enzymes to produce metabolites that are either non-toxic or less toxic than the parent toxin molecule when ingested by animals (Karlovsky, 1999; Boudergue et al., 2009). Biological detoxification is seen as a better alternative way than the physical and chemical detoxification with regards to the presence of nutrients in a food product as well as the impact on human health.

The study of biological detoxification of mycotoxins is still very limited in Indonesia. A preliminary study on degradation of AFB<sub>1</sub> by proteolytic fungi *Aspergillus oryzae* isolated from *Koji* has done by Mashuri and Sardjono (2001). Results showed the formation of several compounds that fluoresce under UV light with different R<sub>f</sub> values. The formation of the new compound proves that AF B<sub>1</sub> was converted into other compounds. In liquid glucose medium of ammonium nitrate (GAN) the reduction levels of AF B<sub>1</sub> in line with the growth of the mycelia was demonstrated (Sardjono et al., 1992). Sardjono et al. (2004) proved that the extracellular enzymes produced by *A. oryzae* KKB4 are able to detoxify AF B<sub>1</sub>. Based on the analysis of IR spectra they demonstrated the ability of these enzymes to degrade the lactone ring, the reduction of cyclopentanone, and open the difuran ring of AF molecule.

Setyabudi (2002) also found that the degradation of AF B<sub>1</sub> in *oncom* fermentation occurred enzymatically and involves the fungus *Neurospora* sp. Sandhi (2005) reported the ability of isolates of *Rhizopus oligosporus* MK-1 to reduce AF content in liquid media and the manufacture of soy tempeh. During tempeh production, reduction of AF B<sub>1</sub> by these

strains reached 41.47%. Visual decrease or reduction of AF B<sub>1</sub> appeared to be related with the growth of *R. oligosporus* MK-1. The longer the fermentation, the more bushy the growth was and the greater was the reduction of AF B<sub>1</sub>. AF degradation mechanisms by *R. oligosporus* MK-1 happened enzymatically through the transformation of cyclopentanone bond forming aflatoxicol. Jensen et al. (1997) suggested that the presence of *Rhizopus* spp. can change 87% of AF B<sub>1</sub> into compounds that are non-fluorescent and further explained that the process of fermentation using *R. oryzae* and *R. oligosporus* could transform AF B<sub>1</sub> into aflatoxicol A, which is ten times less toxic than AF B<sub>1</sub>.

## Challenges and Future Perspectives

To protect consumers from the risk of mycotoxin contamination in Indonesia, the government should launch regulations on more diverse food products. So far, there is a lack of studies on fruits, *jamu* (Indonesian traditional medicine), drinks (coffee, tea, and chocolate), fermented foods and the animal-derived products (such as milk, sausage, meat products, etc.), all of which are products that are heavily consumed by Indonesian people. Moreover, the research so far is limited to AF, although it is extremely likely that other mycotoxins can also be found in many food commodities, for example patulin in fruits. Therefore, more surveillance and monitoring is required in more diverse foods and in a larger mycotoxin spectrum.

The government also needs to do an economic analysis of the annual cost of mycotoxin contamination regarding products spoilage, losses in livestock and plant productivity, and human health effects (mortality, morbidity, hospitalization and other variables). With the analysis, government could observe the progression of the implementation program in every year.

Although many attempts to prevent the formation of mycotoxins have been made, the contamination still occur and found in many agricultural commodities. Therefore, it is necessary to develop practical, efficient and safe decontamination methods that can be widely applied to the contaminated food products on a large scale in cluster areas which are prone to mycotoxins contamination.

The emergence of the phenomenon of "chasing zero" in which lower permissible limits of mycotoxin contamination is demanded, is a big challenge for Indonesia as a food exporter. Indonesia needs to conduct internal restructuring to create a system to prevent the contamination of mycotoxins at different levels of the food production chain with a supportive integrated system and with regular monitoring in various areas. The poor sanitary conditions for the storage of raw materials in farmer, trader and food industries show the difficulties in implementing GAP or GHP (Good Handling Practices) or GMP or HACCP (Hazard Analysis Critical Control Point) principles. The government needs to focus more on the development of food safety methods at all food production chain levels by mentoring programs and intensive education. Various stakeholders; from government, industry and consumers, should sit together in one focus group discussion establishing how to implement effectively proper pre- and post-harvest mycotoxins management strategies.

Every stage of the pre-harvest, harvest and post-harvest has to be monitored and the implementation of GAP and GMP has to be controlled. This concept could be applied if there is a proper coordination and cooperation among farmers, governments, and food producers. The active role of food industry is important to provide appropriate incentives so that farmers will have more motivation to produce good raw materials. The government has to supervise the safety aspect of each manufactured food product through easy, quick and inexpensive mycotoxins detection methods. Methods of monitoring and mitigation of mycotoxins on storage in shops and houses should also be developed. Coordination of analytical techniques for each mycotoxin analysis laboratory owned by the government is necessary, to reduce the data deviation as well as to facilitate comparison of analysis results obtained from different places and from different researchers. Indonesia also should be prepared to deal with various updated mycotoxin research topics such as masked mycotoxin, epidemiology research relating to risk assessment based on hazard and exposure evaluation, as well as the development of multi-mycotoxins analysis methods. Government authorities involved in policy making should recognize research work as a necessity for transforming research results into practical applications.

## Acknowledgement

This paper was supported financially by Indonesia Endowment Fund for Education, Ministry of Finance, The Republic of Indonesia (Ref:1064/LPDP/2013).

## Conflict of Interest

The authors declare no conflict of interest.

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## Research Article 01

# Determination of Fungal Diversity in Indonesian Tempeh and Update on Their Safety Status\*

\*<sup>1</sup>This research has been presented partly in 37<sup>th</sup> Mycotoxin Workshop 2015, Bratislava, Slovakia.

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## Abstract

Tempeh is a popular food in Indonesia that is prepared by fermenting soybean. Fungi play an important role in the making of tempeh. In this study, fungi were isolated from tempeh and subjected to diversity analysis using DNA fingerprinting and identified with sequencing analysis. A total of 557 pure fungi isolates were obtained from 247 samples fresh tempeh and inocula in 16 different provinces in Indonesia. Diverse fungal species found in Indonesian tempeh and inocula. RAPD typing was capable of producing discriminating DNA fingerprints of *Rhizopus* isolates and indicated there was genetic differences among them. Many distinct patterns were observed indicating that tempeh and inocula from different location in Indonesia composed of a quite wide number of strains. Combination of origin or geographical data and molecular identification showed that isolates (identified as *Rhizopus oryzae*, *R. delemar*, and *R. stolonifer*) dominantly used by tempeh producer from outside Java, whereas isolates (*Rhizopus microsporus* complex isolates, identified as *R. microsporus* var. *chinensis*, *R. microsporus* var. *microsporus* and *R. microsporus* var. *oligosporus*) were mostly used by tempeh producer from Java. Tempeh fungi isolates are safe for human consumption since they are not mucormycosis or toxication agent, but food safety supervision has to be given to some tempeh producer that applied unhygienic practice in tempeh production because we found few sample contaminated with bacteria classified as a human pathogen causing gastroenteritis. Fungal strains used in production of tempeh in Indonesia also could convert ZEN to conjugated ZEN and ZEL derivatives *in vitro*. More attention needs to be given to tempeh made with mix soybean-maize. If the unprocessed grains for tempeh fermentation like mix soybeans-maize are contaminated with ZEN, upon analysis ZEN content of the final tempeh product may be underestimated, because ZEN has been conjugated during fermentation.

**Keywords:** tempeh, *Rhizopus*, DNA fingerprinting, ACT sequencing, morphology, physiology, taxonomy, virulence, ZEN biotransformation

## 1. Introduction

Zygomycetes surround us in our daily life, not only as agents of human disease and a toxin producer, but also as starters in the production of oriental fermented foodstuffs (in Indonesia, China, Japan, and Korea), biocatalysts in food industry and as pioneer degraders in food spoilage (Martens et al., 2006; Voigt et al., 2013; Doggett and Wong 2014; Dolatabadi et al., 2015). They are well spread and can be encountered in several different niches from the warm and moist Southern Asia to the colder Northern Europe. Like other fungi, zygomycetes are heterotrophic and grow inside the substrate, dissolving it by excreting extracellular enzymes and taking up degraded

substrate as nutrients by absorption. The zygomycetes are characterized by a fast growth on high sugar content substrates in the early stage of decay. Some zygomycetes are known in industry and biotechnology as biocatalysts in the production of steroids, carotenoid compounds (Hesseltine 1965; Certik and Shimizu, 1999), organic acids (citric and gluconic) and for ethanol, biodiesel and lipase production (Khare et al., 2000; Hiol et al., 2000; Ghosh and Ray, 2011), and also as biodegradable plastics decomposer (Oda et al., 2000).

Since ancient times fungi also have played a significant role, especially in Asian countries where many foods processed via fungal fermentation, mostly based on ground soybeans (Steinkraus et al., 1960; Hesseltine and Ellis, 1973). One of popular fermented food involved the fungi in its process is tempeh. There are 120 varieties and species of moulds could producing tempeh according to Shurtleff and Aoyagi (1979). The principal genus important for the manufacture of tempeh is *R. microsporus*, with *microsporus* varieties, *oligosporus*, *rhizopodiformis*, and *chinensis* (Nout and Rombouts, 1990). An additional variety *tuberosus* is also described (Zheng and Chen 1998). Some others are also produced by genus *Mucor*. However, the primary one is *R. microspores* var. *oligosporus*. Dwidjoseputro dan Wolf (1970) discovered mold growth on tempeh are vary in by the region. Unfortunately, not all species are completely identified, and sampling number was also insufficient.

In Indonesia, *Rhizopus* spp. have been known as one of most economically important mold due to their role as inoculum source for making tempeh, but their information on diversity has been confusing due to limited information, and outdated determination approach previously applied by Indonesian mycologists. As example, *Rhizopus oligosporus* has still commonly been regarded as inoculant name of tempeh in Indonesia until now (Prihatna and Suwanto, 2007; Dewi and Aziz, 2011), although, this name was, a long time ago, transferred as var. *oligosporus* within *R. microsporus* group by Schipper and Stalpers (1984). Moreover, the taxonomy and natural systematics within the Mucorales is extremely problematic due to insufficient in distinguishing morphological characters and by the high intra-specific variability of those that do exist. Their characters are also dependent upon physiological growth parameters (Schipper, 1973; Voigt and Wöstemeyer, 2001). Benny (1995) pointed out the limitations of the morphological features in the species delimitation within certain zygomycetes, suggesting the use of molecular tools for solving existing taxonomic controversies. Combination morphological, physiological and molecular features would be the best way and very useful to address the problem.

To develop a standardization of tempeh quality in Indonesia, it is important to have an accurate name and valid identification of *Rhizopus* species. This research deals with the role of mucoralean fungi in tempeh production as well as solid fermentation process. In this research, sample of fresh tempeh and its inoculums from various places in Indonesia are isolated, its diversity is tested using DNA fingerprinting technique and identified, using phenotypic and genotypic approaches to gain valid and accurate information about *Rhizopus* spp. taxonomy of fresh Indonesian tempeh and its inoculum. The re-identification of the *Rhizopus* producing tempeh and its inoculum is required to set tempeh quality standard, namely modern inoculum with a

distinctive identity. The strains of the research are also expected to be part of the long-term *ex-situ* conservation effort of fungi.

Although tempeh is characterized as safe for consumption by FDA, recently there have been cases of intoxication in Indonesia as a result of tempeh consumption. From 1998 until 2015 a dozen of cases was reported in mass media, but complete data regarding the cause is still lacking, and the cases are probably underreported. Many speculations may arise in line the recent findings that *Rhizopus* strain isolated from food could be agents of toxin producer and possibly also as mucormycosis agent. According to Dolatabadi et al. (2014), the clinical associated-strain also has an identical genetic character to food and condiment associated-strain. Some species like *Mucor circinelloides*, *Rhizopus oryzae*, and *Rhizopus stolonifer* are able to infect both immunocompromised and immunocompetent people (Roden *et al.*, 2005). Some strains are also capable of producing toxins that are dangerous to human health. Some *Rhizopus* species harbor endosymbiont bacteria of the genus *Burkholderia* which are able to produce several extracellular metabolites. Rhizoxin and rhizonin resulting from *Burkholderia rhizoxinica* are produced during soybeans fermentation by *Rhizopus* sp. strain CBS 111563, on *sufu* preparation (Rohm et al., 2010). The rhizoxin derivatives are known as powerful antimycotic agents (Scherlach et al., 2006), a compound that prevents the formation of new blood vessels (angiogenesis). None of these metabolites should be permitted to occur in foods, even at pico- or femtogram amounts, as they cause health hazards for human (Rohm et al. 2010). In this study, zygomycetes strains derived from Indonesian tempeh fermentation and starter are screened toxicologically and clinically to evaluate possible health risks for human consumption. This research is important to answer questions and to clarify about the safety status of Indonesian tempeh and inocula.

Another safety problem in tempeh production is the use of corn as a substitute for soybean. Producers involve corn substitution for 10-30 percent to reduce cost production because the price of soybean is high nowadays. This generates a healthiness issue of produced tempeh because most Indonesian corn has been contaminated by toxigenic fungi such as *Alternaria spp.*, *Aspergillus flavus*, *A. fumigatus*, *A. parasiticus*, *A. candidus*, *A. glaucus*, *A. tamari*, *A. terreus*, *A. niger*, *A. nomius*, *Fusarium spp.*, *Penicillium citrinum* and *P. chrysogenum* (Purwoko et al., 1991; Ahmad et al., 1996; Gholib et al., 2004; Ahmad et al., 2009; Kusumaningrum et al., 2010; Rahmawati et al., 2013). Rahmawati et al. (2013) also reported that toxigenic fungi are found in soaking corn, such as *Fusarium spp.* It is high possibly happens that those fungi produce mycotoxins during soaking process, then the toxins absorbed into the material that is used to produce tempeh. Moreover mycotoxins content in raw material itself also endangers consumer health. Fumonisin and zearalenone were found in some cereals in Indonesia especially corn (Maryam and Zahari, 1994; Yamashita et al. 1995; Ramahani 2003). Therefore safety aspect of tempeh cannot be guaranteed anymore. In this research, we also study tempeh fungi's contribution to biotransformation of zearalenone.

## 2. Materials and Methods

### Part I (Fungal diversity and identification)

#### 2.1.1. Sampling, isolation and purification

Fresh tempeh and inocula were collected from many markets (traditional, semi-traditional and modern markets) and centre of tempeh producers in different areas in Indonesia (on 2013-2015). Samples were transported in sterile plastic container to nearby laboratory in each location (provinces). Samples were maintained at 10°C until direct plating done. Details information of sampling can be seen on Table 1 and Figure 1. Locations were coloured in the map.

Fresh tempeh size was reduced into small particle then transferred with sterile forceps onto Potato Dextrose Agar (PDA) medium and incubated for five days in incubator (Thermofisher) at 30°C. For inocula samples, 5 gram of each sample (powder) was suspended in 10 mL sterilized tap water in a sterile falcon tube, homogenized with vortex (VM-300), then one mL of each solution was spread onto PDA medium, and incubated in the same condition.

Purification of isolates: A spore suspension is prepared by transferring a loopful of the fungal mass from PDA slant into 10 mL sterile Tween®20 (Applichem, Darmstadt, Germany) (0.05% v/v) kept in a sterilized falcon tube followed by vigorous shaking with vortex for a few minutes in order to disperse the spores from the spore-bearing structures, continued with single spore isolation method (modification of Zhang et al., 2013). All the procedures were done in sterile condition inside clean bench. About 578 pure isolates were collected. Isolates were stored as spore suspension in 25% (v/v) glycerol (Carl Roth, Karlsruhe, Germany) at -60°C. All isolates were grown in PDA at 30°C for a week and maintained in malt extract agar (MEA) (Oxoid). One-week-old cultures on MEA were used for morphology, culture, and metabolic examinations.

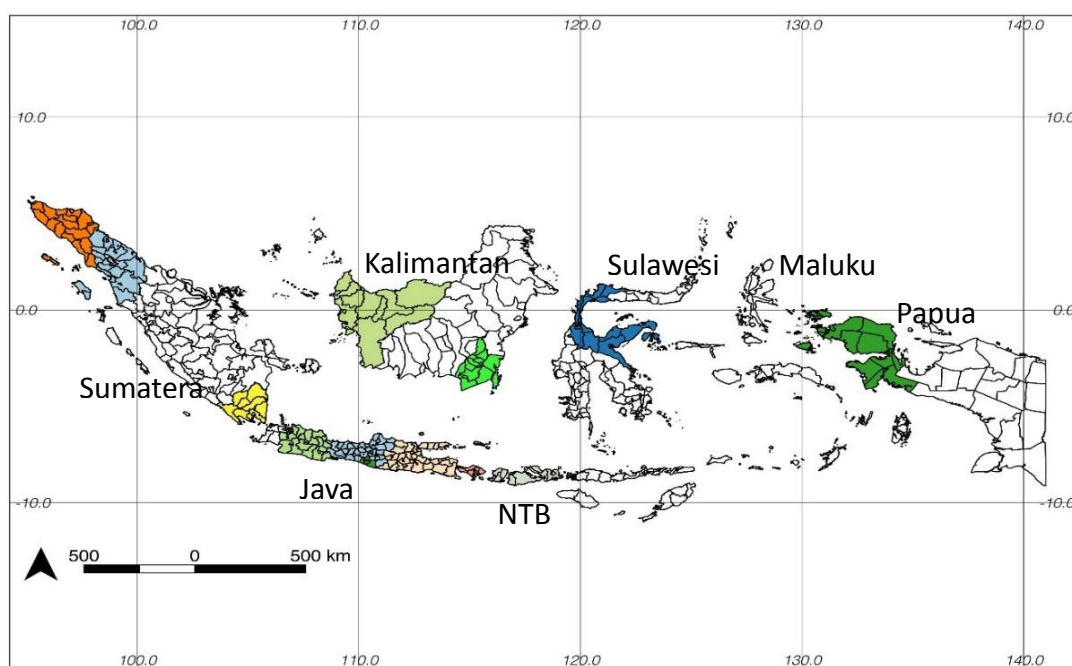


Figure 1. Sampling map (Indonesia)

Table 1. Information of sampling locations

Location	Source	Sample amount	Year
Lhokseumawe; Aceh	Fresh tempeh, and inocula	7	2015
Medan, North Sumatera	Fresh tempeh, and inocula	8	2015
Jambi, Central Sumatera	Fresh tempeh, and inocula	7	2015
Lampung Metro, Lampung	Fresh tempeh, and inocula	9	2015
South Jakarta, DKI Jakarta	Fresh tempeh, and inocula	7	2015
Bogor, West Java	Fresh tempeh, and inocula	13	2014, 2015
Bekasi, West Java	Fresh tempeh, and inocula	12	2015
Sukabumi, West Java	Fresh tempeh, and inocula	12	2015
Banuyumas, Central Java	Fresh tempeh, and inocula	26	2013, 2014, 2015
Gombong, Central Java	Fresh tempeh, and inocula	11	2015
Magelang, Central Java	Fresh tempeh, and inocula	11	2015
Kutoarjo, Central Java	Fresh tempeh, and inocula	9	2015
Cilacap, Central Java	Fresh tempeh, and inocula	9	2015
Kudus, Central Java	Fresh tempeh, and inocula	9	2015
Yogyakarta, DIY	Fresh tempeh, and inocula	15	2013, 2014, 2015
Malang, East Java	Fresh tempeh, and inocula	15	2014, 2015
Surabaya, East Java	Fresh tempeh, and inocula	5	2015
Jember, East Java	Fresh tempeh, and inocula	9	2015
Denpasar, Bali	Fresh tempeh, and inocula	5	2015
Mataram, NTB	Fresh tempeh, and inocula	5	2015
Pontianak, West Kalimantan	Fresh tempeh, and inocula	7	2015
Banjarmasin, South Kalimantan	Fresh tempeh, and inocula	7	2015
Palu, Central Sulawesi	Fresh tempeh, and inocula	11	2015
Ambon, Maluku	Fresh tempeh, and inocula	7	2015
Manokwari, Papua	Fresh tempeh, and inocula	9	2015
Total of sample		247	

### 2.1.2. Morphological and physiological analysis

#### Morphological characteristics

The key characteristics, such as sporangiophore, sporangium (conidia), sporangiospore, columellae, zygospore, rhizoid type and colony appearance were examined according to an established taxonomic system of *Rhizopus* was used (Schipper 1973; Schipper & Stalpers 1984; Liou, et al., 1990; Zheng et al., 2007; Samson, 2010). Measurements of sporangiophore length and sporangio- spore size were made in 15 replications. The strains were cultured on MEA at 25°C. Slide mounts were prepared and examined under a light microscope (Leica Leitz DMRB) equipped with a color camera (Leica DFC420). Response to temperature was determined by measuring the diameters of colonies on agar plates incubated at temperatures of 25°C, 30°C, 33°C, and 35°C with vernier caliper or ruler. Sporulation was determined in cultures on 5 % MEA (Oxoid) derived from inocula as mycelial parts of growing colonies, as well as from fresh spores from 3 days old cultures,

incubated at 30 °C for 4 days. Levels of sporulation were graded in five grades varying from 0 (no spores) to 4 (fully covered plate with spores). It is defined macroscopically according to the color on the plate.

### **Physiology test**

For selected strains, tests for enzyme activities, including gelatin liquefaction, presence of urease, siderophores, lipase (Maas et al., 2008), amylase (Caldwell et al., 2000), cellulose (Caldwell et al., 2000), laccase (Nawange et al., 2012), and tyrosinase (Mandyam et al., 2010), were performed.

### **Determination of carbon assimilation profiles**

To obtain sufficient sporulation, all strains were cultured for 7 days on Sabouraud agar slants at 30°C. Stock suspensions were prepared in 1 mL 0.9% NaCl and were counted in a Thoma Chamber (0.0025 mm<sup>2</sup>). Spore suspensions were kept on ice and homogenized well before using.

A commercial identification kits were used, ID32C (bioMe´rieux, Marcy l’Etoile, France). With ID32C strips, the assimilation of 29 carbon sources are evaluated. For ID32C strips, an adequate volume of spore stock suspension was transferred to 7 mL of API C medium (bioMe´rieux) to achieve a final spore concentration of  $5 \times 10^5$  spores/mL. The suspension was vigorously vortexed, and 135 µL was distributed into each well of the strips and incubated for 72 h at 28°C. After incubation, the strips were read visually and growth or lack of growth was noted. Weak growth was considered a positive result. Duplicate experiments were carried out for 50% of the tested isolates. Duplicate testing was performed on randomly chosen isolates.

### **2.1.3. Molecular analysis**

Identification of zygomycetes isolates were performed according to the combination of morphological, physiology and molecular characters. Morphological and physiological observation were accomplished as described above. Then DNA fingerprinting was employed to distinguish identical and non identical isolates. Screened isolated then confirmed at species level by the results of DNA sequence of the Actin gene, which is taxonomically most informative part in this fungal genus. ACT is known as a housekeeping gene, as its expression is relatively stable under variable conditions. ACT encodes actin, a protein that is abundant in all eukaryotic cells, in which it is the major component of cytoplasmic microfilaments. The ACT gene has been used extensively to infer interspecies relationships across broad evolutionary distances, as the ACT gene of many species contains highly conserved (i.e. exon) and less well-conserved (i.e. intron) sequences, which are grouped as nuclear pre-mRNA introns (Donnelly et al., 1999).

### **DNA extraction and gel electrophoresis**

Fungal strains used in this study are listed in Table 2. The cultures were grown on potato dextrose broth (PDB) (Roth, Karlsruhe, Germany) in Erlenmeyer flasks in darkness at 25°C for 5 days. Mycelium was harvested by filtration onto sterile paper disks, froze in -70°C, freeze-dried in a freeze drier (Christ, Beta 1-8) and stored at room temperature till extracting genomic DNA. Fifty milligrams of lyophilized mycelium were ground in 2 mL Eppendorf tubes with round bottom containing 4-5 wolfram carbide spheres (diameter 3 mm, Retsch, Haan, Germany) in a reciprocal



mill (Mixer Mill MM 200, Retsch, Haan, Germany). Genomic DNA was extracted by a CTAB method with little modification (Brandfass and Karlovsky, 2008), as described detail below.

One mL of the CTAB-buffer, 1  $\mu$ L Proteinase K (AppliChem Bio Cemica, Darmstadt) and 2  $\mu$ L Mercaptoethanol (SERVA Electrophoresis GmbH) were added to the sample and the mixture was vortexed until everything was well mixed. The samples were incubated first for 10 min in a 42°C water bath and afterwards for another ten min in a 65°C water bath (GFL). The samples were mixed every three minutes during the two 10-minutes incubation periods. 0.8 mL of chloroform /isoamylalcohol (24:1) was added to each sample, the samples were shaken thoroughly and incubated on ice for ten min. Then the samples were centrifuged for ten min at 10000 RPM until no cell material was left in the upper phase. About 600  $\mu$ L of the upper phase was transferred to a 1.5 mL Eppendorf tube. Precipitation was completed for all samples with PEG. About 200  $\mu$ L PEG 6000 (30 %) and 100  $\mu$ L 5M NaCl (Carl Roth GmbH & Co) was added and mixed thoroughly. In a second separate extraction, the precipitation was done with isopropanol instead of PEG.

Afterwards the samples were incubated for 20 min at room temperature again, and then centrifuged for 15 min at 14800 RPM. Supernatant was carefully poured off and 500  $\mu$ L Ethanol (80 %) was added without disturbing the pellet. The samples were centrifuged again for five min at 14800 RPM and the washing step was repeated twice. After the cleaning the pellets were dried for ten to fifteen min at 30°C in the vacuum drier (Eppendorf AG). The last step for the DNA extraction was to add 50  $\mu$ L of TE-buffer and shake the tube until the pellet was released from the bottom and the DNA was dissolved. Samples were then incubated at room temperature about 30 min. The tubes were kept in a refrigerator until the next day to control the quality of the DNA extraction by gel electrophoresis. The DNA of every sample was diluted 1:20, 1:50 and 1:100. For further usage the DNA was kept in a freezer (-20°C).

In order to check the efficiency of DNA extraction and the purity of extracted DNA, a 5  $\mu$ L DNA sample were mixed with 2  $\mu$ L Blue juice and loaded on a 0.8% (w/v) agarose gel (Cambrex, Rockland, ME, USA), prepared in TAE buffer (40 mM Tris, 1mM EDTA (ethylene diamine tetra acetic acid), pH set to 8.5) (both substances were obtained from Carl Roth, Karlsruhe, Germany). The electrophoresis was carried out at 60V for 60 min. After staining with ethidium bromide (0.5  $\mu$ g mL<sup>-1</sup>) and destaining in demineralized water (each for 10 min), DNA bands were visualized in UV light using a CCD camera (Vilber Lourmat, Marne La Vallee, France).

### **DNA fingerprinting**

In the preliminary experiments, four arbitrary primers were tested with Random Amplified Polymorphic DNA (RAPD) with DNA extracted from pure cultures. List of primers can be seen on Table 2. Preliminary test determined the optimal concentration of the component in the PCR reaction mixture, amplification conditions and primer selection. Strong, unique, sharp and clearly reproducible bands were produced by primer C6.2\_introns\_2\_R and C7\_1\_seq\_f2. For further analysis C7\_1\_seq\_f2 was used.

Table 2. Primer and their sequences tested in preliminary RAPD analysis

Primers	Sequence 5'-3'
C6.2_introns_2_R (C6_2_int_R3)	TGGCTCACGCAAGGGTGTAG
C2_1++inv_f	TGACGTCCTAGTTGATATCCTCG
C2_1++inv_r	ACCGTTATGATCAATAAATATTTGACCA
C7_1_seq_f2	AGGTTAGCTCGCGACTATCGGATC

In brief, the optimal condition of PCR as described below:

The amplification reaction was carried out in a volume of 25  $\mu\text{L}$  containing 18.45  $\mu\text{L}$  water, 2.5  $\mu\text{L}$  10x reaction buffer (Bioline, Luckenwalde, Germany), 1.5  $\mu\text{L}$  dNTPs 150  $\mu\text{M}$  (Amersham Biosciences), 0.75  $\mu\text{L}$  of primer 0.3  $\mu\text{M}$  (GenXpress), 0.05  $\mu\text{L}$  of Taq DNA polymerase 0.25 U (New England BioLabs, Ipswich, MA, USA) and 1  $\mu\text{L}$  of DNA sample. For detection of any other kinds of DNA contaminants, a negative control of PCR-mix without any template DNA was used. The PCR program had initial denaturation at 94°C for 2 min, 2x45 cycles of: 20 s at 94°C, 20 s at 37°C, 20 s at 72°C and followed by final extension at 72°C for 5 min, storage 15°C for 5 min (peQ STAR Thermocycler). Eight microliters of the PCR products was mixed with 2  $\mu\text{L}$  of blue juice and pipetted into the slots. The electrophoresis is carried out at 60 V for 90 min. After staining with ethidium bromide (0.5  $\mu\text{g mL}^{-1}$ ) and destaining in demineralized water (each for 10 min), DNA bands were visualized in UV light using a CCD camera (Vilber Lourmat, Marne La Vallee, France).

The RAPD banding patterns were compared and a matrix based on the presence or absence of amplification products observed. From this, Jaccard (SJ) coefficients were calculated and a dendrogram was produced with UPGMA linkage (Sneath and Sokal, 1973).

#### **Polymerase chain reaction and phylogenetic analysis**

Identification at species level was confirmed by the results of DNA sequence of Actin gene, which is taxonomically most informative part in this fungal genus. Hot start PCR protocol was used to amplify the ACT gene region using the fungal specific primer set: Act-1 (5'-TGGGACGATATGGAAIAAIATCTGGCA-3') and Act-4ra (5'-TCITCGTATTCTTGCTTIGAIATCCACAT-3') described by Voigt et al. (2000). The reaction mixture consisted of reaction buffer (16 mM  $(\text{NH}_4)_2\text{SO}_4$ ; 67 mM Tris-HCl; 0.01% (v/v) Tween-20, pH: 8.8 at 25°C), 0.1 mM concentration of each of the four deoxynucleoside triphosphates (Bioline, Lükenwalde, Germany), 2 mM of  $\text{MgCl}_4$  (Bioline), 1.75 U of hot-start DNA polymerase (New England BioLabs, Ipswich, MA, USA) and), 0.3  $\mu\text{M}$  concentration of each primer (Invitrogen by Thermo Fisher Scientific). Every reaction contained 1  $\mu\text{L}$  template DNA or 1  $\mu\text{L}$  sterile water as negative control. The PCR reactions were performed in a total volume of 25  $\mu\text{L}$  reactions with a peQ STAR Thermocycler (96 Universal Gradient). The cycling conditions were as follows: 1 cycle of 10 min at 95°C, 30 cycles of 60 s at 94°C (denaturalization), 45 s at 58.5°C (annealing), 60 s at 72°C (extension) and followed by a final extension cycle at 72°C for 5 min.

Lambda DNA was digested with the restriction enzyme PstI and used as a size standard. For agarose gel electrophoresis of the PCR products, 2  $\mu\text{L}$  blue juice were mixed with 2  $\mu\text{L}$  digested lambda DNA (25 ng/mL). The checking of the PCR product was performed on agarose gel

electrophoresis as described above. The PCR products were purified using isopropanol precipitation and 5  $\mu$ L of the PCR product were sent to LCG Genomics GmbH (Ostendstr. 25. TGS Haus 8, 12459 Berlin, Germany) for sequencing. Similarity searches in the sequences of the sequenced regions were done using BLAST in NCBI database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Alignments and phylogenetic analysis of the above mentioned sequences were performed and phylogenetic trees were constructed using the software MEGA 7 (Kumar *et al.*, 2016).

## **Part II (Safety status of tempeh fungi strains)**

### **2.2.1. Virulence test**

#### **Ethic statement**

All experiments were performed in compliance with European and German animal protection laws. No ethic statement is necessary for insect models.

#### **Virulence test**

Method of virulence test followed Fallon *et al.* (2012) and Kaerger *et al.* (2015) with small modification. *Galleria mellonella* (wax moth) were acquired in the larval stage. *G. mellonella* received were a mixture of larvae with and without grey color markings on the cuticle. *G. mellonella* larva used for experiments were selected to be similar in size (approx. 275–330 mg) and absent of any grey markings. We have found that we achieve the best results by using only larva that were light-colored; therefore we select larva without grey markings to be used for experiments. The selected larva were inoculated with the pathogen of interest.

Our preferred delivery route for microbial pathogens is delivery of a known quantity of pathogen directly to the hemocoel. With this delivery method, *G. mellonella* larva were infected by injecting (Myjector syringe, Terumo Europe) the inoculum into the hemocoel.

Larva of *G. mellonella* were stored in the dark at 18°C prior to use (Schütt Lichtthermostat). Ten of healthy larvae were placed on Whatman filter paper in sterile 9-cm petri dishes. Enterobacter cloacae was grown to stationary phase ( $1-2 \times 10^9$  /mL) in nutrient broth (Merck, Darmstadt, Germany) at 30°C, with shaking at 200 RPM (Innova44, New Brunswick Scientific Co). Bacterial cells were harvested by centrifugation (Rotixa 120R centrifuge, Andreas Hettich Co), washed in phosphate buffered saline (PBS) (Carl Roth Co) and re-suspend in PBS with cell density at  $1 \times 10^6$  per 20  $\mu$ L. Zygomycetes strains were grown in PDA (Merck, Darmstadt, Germany) for 5 days, spores were harvested by centrifugation, washed with PBS and re-suspend in PBS with  $1 \times 10^6$  spores per 20  $\mu$ L. Larva were inoculated by injecting 20  $\mu$ L through the last left proleg into the haemocoel using a Myjector syringe and place at 30°C in the dark for up to 96 h. Untouched larva and larva were injected with 20  $\mu$ L of water or PBS should be included as controls. Assessing larva at regular intervals (every 24 h for 4 days) for viability and disease progression. For assessment of viability, larvae should be gently probed with a needle, and if no response is observed, the larvae may be considered to be dead. Changes in cuticle melanisation can also be used to monitor the severity of an infection. Significance of mortality rates was evaluated by using Kaplan-Meier survival curves with the PRISM statistics software (Mantel-Cox log rank test) using pooled data. All experiments were performed three times, each time with duplicates.

### 2.2.2. Detection of bacteria producing rhizonin

Fungal mycelia were transferred to LB (Luria broth; Oxoid) bottles and were incubated at 30°C for 4 d under 200 RPM rotation. Using mechanical stress (pipetting) and physical stress (freezing-thawing), fungal mycelia were broken and submitted to centrifugation (30 min, 3000 RPM, 10°C). Five hundred µl of supernatant was added on 25 mL sterilized nutrient broth and incubated at 30°C for 3 d with shaking mode. After 3 days, sample then checked with plating in NA medium and checked with light microscopy. Only sample had bacterial colonies were then extracted its DNA (Ausubel et al., 1986) and followed with amplification partial sequence 16S rRNA genes using the bacterial universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG) (Lane, 1991) and bacterial primer 1100R (5'-AGGGTTGCGCTCGTTG) (Turner et al., 1999). with cycling conditions of 94°C 30 sec, 10× (94°C 30 sec; 62°C-53°C -1°C/cycle 40min; 72°C 1 min), close loop 30× (30x 94°C 30 sec; 56°C 40 min; 72°C 1 min), close loop 72°C 5 min. The DNA was diluted 1/100 and 1/1000. PCR was carried out with a total volume of 25µL composed of 18.45 µL water, 2.5 µL 10x reaction buffer (New England BioLabs, Ipswich, MA, USA), 1.5 µL dNTPs 150 µM (Bioline, Luckenwalde, Germany), 0.75 µL of each primer 0.3 µM (Invitrogen by Thermo Fisher Scientific), 0.05 µL of Taq DNA polymerase 0.25 U (New England BioLabs, Ipswich, MA, USA) and 1 µL of DNA sample. A control PCR without DNA was set up to check for nonspecific amplification. The expected PCR products' size was 1000 bp.

A marker of 1000 bp (Forever 100 bp Ladder Personalizer, Seegene) was used as a size standard. 2 µL blue juice were mixed with 2 µL marker (25 ng/mL). The PCR product was checked on a gel, prepwared for sequencing and sequenced as described above. Similarity searches in the sequences of the sequenced 16S rRNA gene, alignments and phylogenic analysis were performed as described before.

## Part III (Zearalenone biotransformation by tempeh fungi)

### 2.3.1. Microorganism and *in vitro* test (in liquid culture)

Seventy strains of tempeh fungi used for *in vitro* test could be seen on Supplemental Table 3. In brief, Stock cultures were grown in PDA (Merck, Darmstadt, Germany) for 5 days at 30°C (Schütt Lichtthermostat). A spore suspension was prepared by transferring a loopful of the fungal mass from PDA slant into 10 mL sterile Tween®20 (Applichem, Darmstadt, Germany) (0.05% v/v) kept in a sterilized falcon tube followed by vigorous shaking with vortex for 3 min in order to disperse the spores from the spore-bearing structures. Spores were harvested by centrifugation (Rotixa 120R centrifuge, Andreas Hettich Co). Spores of fungi previously isolated were counted using a Thoma chamber (0.1mm depth, 0.0025mm<sup>2</sup> square area) and the suspension was diluted to certain concentration with sterile tap water.

Potato Dextrose Broth (PDB) was used as media in *in vitro* screening. Fifty milliliters of PDB was poured into the 250 mL Erlenmeyer flask, the flask was closed with a cellulose plug and then autoclaved (120°C, 15 psi, 15 min; Autoclave 3870 EL). The sterile liquid media was inoculated with 2 mL spore solution (10<sup>6</sup>-10<sup>7</sup> spores/mL) and then incubated at 30°C for 5 days in a rotary shaker (Innova44, New Brunswick Scientific Co) at 150 RPM. For the treatment, 1 mL working

solution of zearalenone (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) ( $c = 5 \mu\text{g/mL}$ ; methanolic solution) was added to the liquid culture of the fungal strains R1 – R70. Medium without culture was added with 1 mL working solution of zearalenone as positive control. Instead of zearalenone, 1 mL methanol was added to the culture as negative control. The incubation was continued in the rotary shaker for 2 days. Five hundred microliters of the liquid media was transferred into a 1.5-mL Eppendorf tube and 500  $\mu\text{L}$  ice-cold acetonitrile was added for protein precipitation. Samples were then stored overnight at 4 °C and centrifuged at 9800 RPM for 5 min. The supernatant was transferred to a HPLC (high performance liquid chromatography) vial and analyzed by HPLC hyphenated to tandem mass spectrometry (HPLC-MS/MS).

### **2.3.2. HPLC-MS/MS analysis**

Mycotoxin separation and analysis was carried out by high pressure liquid chromatography coupled with electrospray ionization and tandem mass spectrometry detection. HPLC-MS/MS was performed on an API 4000 mass spectrometer (AB Sciex, MA) connected to an Agilent 1100 series HPLC (Agilent Technologies GmbH, Böblingen, Germany). The analytical column was a ProntoSIL 120-5-C18 AQ (150 mm x 3 mm, 5  $\mu\text{m}$ ; Bischoff Chromatography, Leonberg, Germany), preceded by a ProntoSIL 120-5-C18 AQ guard column (10 mm x 3 mm, 5  $\mu\text{m}$ ). Mobile phase A was water with 5 mM ammonium acetate and as mobile phase B acetonitrile/water (99 : 1 v/v) with 5 mM ammonium acetate was used. Detailed protocol was according to Brodehl et al. (2014).

### 3.1. Results of Part I (Fungal diversity and identification)

Identification of zygomycetes isolates were performed according to the combination of morphology, physiology and molecular characters.

#### Distribution of mould genera from Indonesian tempeh

As shown in Figure 2, the moulds isolated from tempeh and inocula were broadly represented by 9 genera. In total, 557 fungi and 5 fungi-like isolates were obtained from 247 samples. *Rhizopus* was the most frequently detected genera, found in 84% of total samples, followed by *Mucor* (5.5%), *Neurospora* (4.1%) and *Syncephalastrum* (3%). Other very minor genera that found as contaminants were *Chaetomium*, *Phoma*, *Acremonium*, *Aspergillus* and *Geotrichum* (less than 1%).

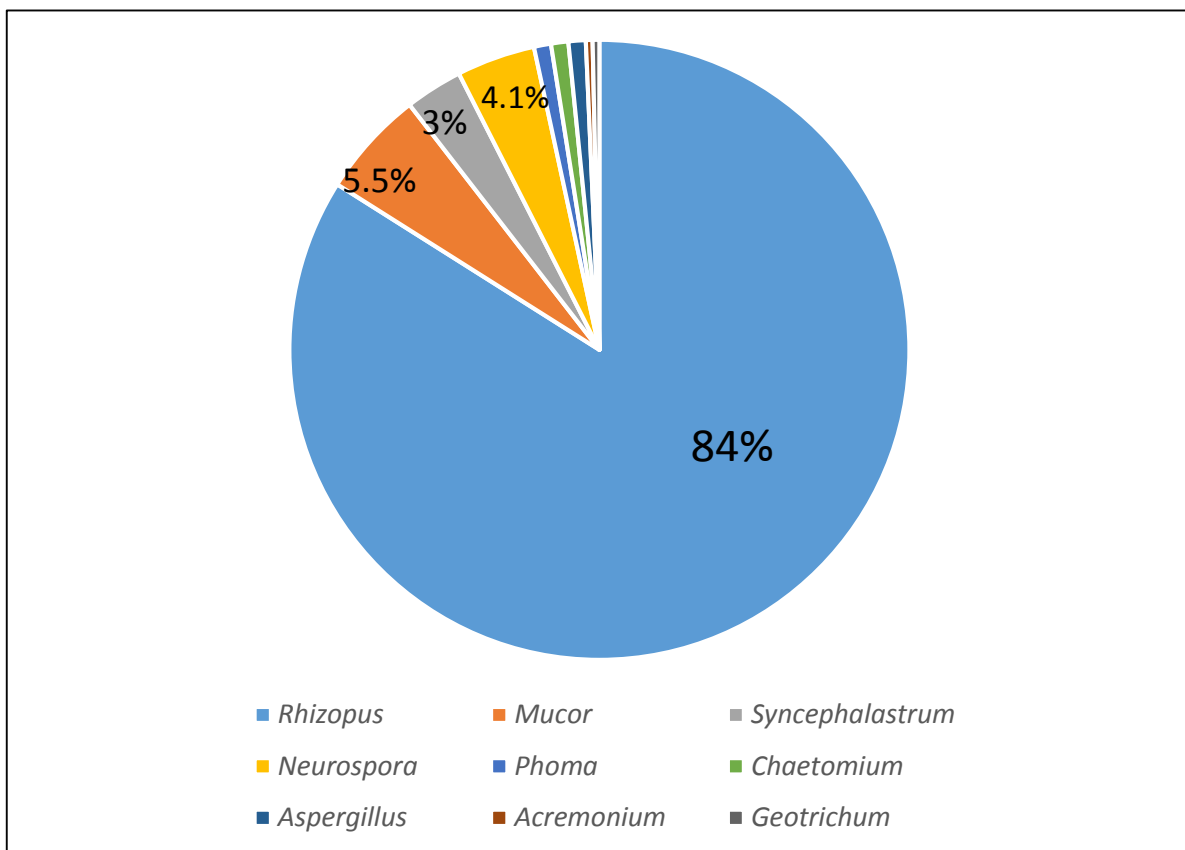


Figure 2. Distribution of mould genera isolated from Indonesian tempeh.

#### Morphological and physiological features

Identification of zygomycetes isolates from tempeh based on morphology and physiology characteristics supported the results from the phylogenetic analyses. The key characteristics, such as sporangiophore, sporangium, sporangiospore, columellae, zygospore, rhizoid type and colony appearance were examined according to an established taxonomic system of *Rhizopus* was used

(Schipper 1973; Schipper and Stalpers 1984; Liou et al. 1990; Zheng et al., 2007; Samson, 2010). In total, 562 samples were checked via microscopy approach. Measurements of sporangiophore length and sporangio-spore size were made in 15 replications. We also observed non-zygomycetes fungi found in this study. Detail of morphological result can be seen on Table 3. Main fungal species isolated from tempeh are also described below. Detail morphological structures is shown in Figure 3.

#### *Rhizopus microsporus* complex

Sporangiophores borne singly or in groups of two to three from clusters of poorly formed rhizoids, stipes unbranched, relatively short, commonly 150-300  $\mu\text{m}$  long, with brown walls; sporangia black, 50–150  $\mu\text{m}$  diameter, usually spherical; columellae 25–30  $\mu\text{m}$  in diameter, spherical, some remaining so in age, others collapsing downwards, to form umbrella shapes; sporangiospores ellipsoidal, 3-5  $\mu\text{m}$  long, with thin, striate walls. Distinctive features from this species are: *Rhizopus microsporus* differs from *R. stolonifer* by shorter stipes and smaller columellae, by the absence of white immature sporangia and by strong growth at 33°C. It is distinguished from *R. oryzae* by production of smaller columellae and smaller spores.

#### *Rhizopus oryzae*

Sporangiophores borne in clusters of one to three from rhizoids, with stipes up to 1300  $\mu\text{m}$  long, usually unbranched; sporangia spherical, up to 190  $\mu\text{m}$  in diameter, white at first then becoming greyish black at maturity; columellae usually spherical, up to 100  $\mu\text{m}$  in diameter, pale brown, in age often collapsing downwards to form umbrella shapes; sporangiospores brown, of variable shape, ellipsoidal to broadly fusiform or irregularly angular, commonly 5–8  $\mu\text{m}$  long, with striate walls. Distinctive features. This species is distinguished from *Rhizopus stolonifer* by its smaller sporangia and spores and by much faster growth at 30°C. It differs from *R. microsporus* by longer stipes, larger collumellae and larger spores. It differs from *R. delemar* because could produce lactic acid.

#### *Rhizopus delemar*

Colonies on MEA at young are white, becoming grey to blackish grey when mature, covering the plate about 3-4 days at room temperature. Stolons well-developed, subhyaline to grayish brown, septate or not septate. Rhizoids sometimes absent, or fingerelike or branched when present, unequal in length, grayish brown, paler at the tip. Sporangiophores arising from stolon and opposite rhizoids, sometimes arising directly from mycelia and without rhizoids, solitary or simple, straight to slightly curved, light brown to dark brown, aseptate, smooth to verruculose, sometimes forked or trifurcate at the apex and swollen at the middle apophyses conspicuous. Sporangia globose to subglobose yellowish to dark brown, without collar. Columellae ovoid, smooth, light brown. Sporangiospores angular, subglobose to ellipsoidal, with ridges on the

Table 3. Morphological and physiological features of tempeh fungi.

Characteristics		Isolates suspected as									
		Major isolates					Minor isolates				
		<i>R. oryzae</i>	<i>R. microsporus</i>	<i>R. delemar</i>	<i>R. stolonifer</i>	<i>Mucor</i> spp	<i>S. racemosum</i>	<i>Neurospora</i> spp	<i>Phoma</i> spp	<i>C. globosum</i>	<i>Aspergillus</i> spp
Morphological	Colony	White to brownish-grey	Pale brownish-grey	White to brownish-grey	White to brownish	Greyish-green to slight orange	White to Greyish	White to slight orange	Yellow to brownish	Yellow to brownish	Pale greenish yellow
	Complexity	Rather complex	Very short, simple	Rather complex	Complex, branched-rhizoid	Greyish-green to slight orange	Very short, simple				
Sporangiospores	Shape	Globose, ovoid, irregular shape (7-15)	Irregular shape, globose, ellipsoidal (5-10)	Globose, ovoid, irregular shape (7-15)	Irregular in shape, often polygonal, ovoid (9-23)	Sporangia (60-90 µm), columella obovoid to ellipsoidal (35-50 x 25-44 µm). Sporangiospores ellipsoidal	Morphology description: Vesicles globose to subglobose, terminal vesicles (35-91 µm), lateral (13-35 µm) without a septum. Merosporengia cylindrical (11-35 µm) in length. Merospores globose or ovoid (3-7 µm) in diam. Chlamydo-spore-like less spherical, (10-15 µm) in diam.	Morphology description: Conidia are one-celled, produced in chain and connected by a narrow hyaline strand. They are ellipsoidal or cylindrical, often irregular in shape and size (10-16 x 5-12 µm).	Morphology description: Pycnidia superficial in agar, sometimes with one ostiole, globose (25-230 x 43-240 µm). Conidia one-celled mostly ovoid to ellipsoidal, slightly curved, (6-9 x 2-4 µm).	Morphology description: Ascomata globose to ovoid (150-255 µm) in diam. Ascospore lemon-shaped (9-13 x 7-9 µm) with an apical germ-pore.	Morphology description: Conical heads globose to radiate (330-600 µm) in diam, conidiophores subglobose (50-70 µm). Phialides cylindrical (10-16 x 4-5 µm), conidia ellipsoidal when young, globose to subglobose when mature (5-9 µm).
	Size (µm)	Both recurved and erect (350-1300)	Both recurved and erect (140-160)	Both recurved and erect (550-1127)	Erect (900-1567)	(5-7 x 4-5 µm), sporangiophore (11-21 µm) in diam. Zygosporangia globose to somewhat compressed (25-40 µm) in diam.					
Sporangiophores	Shape	Globose or subglobose with spinulose wall (73-189)	Globose (50-135)	Globose or subglobose (65-210)	Globose to subglobose (89-276) in diam.						
	Size (µm)	Ovoid to or globose, slightly rough-walled (39-123)	Globose to subglobose with funnel-shaped (25-29)	Ovoid to or globose (39-145)	Globose to subglobose (70-234)						
Chlamydo-spore	Abundant, single	Abundant, single	Abundant, single, short chain	Abundant single	Absent in stolon						
	+	+	+	-							
Lactic acid production <sup>2)</sup>	Optimal growth temperature	30°C	33°C	33°C	35°C	30°C	30°C	25°C	25°C	25°C	30°C
	Colony diameter (mm) <sup>1)</sup>	77-85	70-85	70-80	85-90	45-67	73-80	40-55	37-45	44-51	48-60
Physiological <sup>3)</sup>	Number of isolates examined	89	259	98	25	31	17	23	5	5	5
	Gelatin liquefaction	+ (90%)	+ (80%)	+ (80%)	+ (90%)	+ (100%)	+ (100%)	+ (100%)	+ (100%)	+ (100%)	+ (100%)
Enzymes	Urease	+ (90%)	+ (100%)	+ (100%)	+ (100%)	+ (100%)	+ (100%)	+ (100%)	+ (100%)	+ (100%)	+ (100%)
	Lipase	+ (100%)	+ (90%)	+ (100%)	+ (100%)	+ (100%)	+ (100%)	+ (100%)	+ (100%)	+ (100%)	+ (100%)
Cellulose	Amylase	+ (60%)	+ (50%)	+ (60%)	+ (80%)	+ (80%)	+ (100%)	+ (80%)	-	-	+ (100%)
	Cellulose	-	-	-	-	-	-	-	-	-	-
Tyrosinase	Laccase	-	+ (50%)	-	-	+ (70%)	-	+ (70%)	+ (80%)	-	-
	Tyrosinase	-	-	-	-	-	-	-	-	-	-
Siderophores	Siderophores	+ (20%)	-	+ (50%)	-	-	-	-	-	-	-
	Number of isolates examined	10	10	10	10	10	10	10	5	5	5

Note:

Abbreviation: R= *Rhizopus*, S= *Syncephalastrum*, C= *Chaetomium*

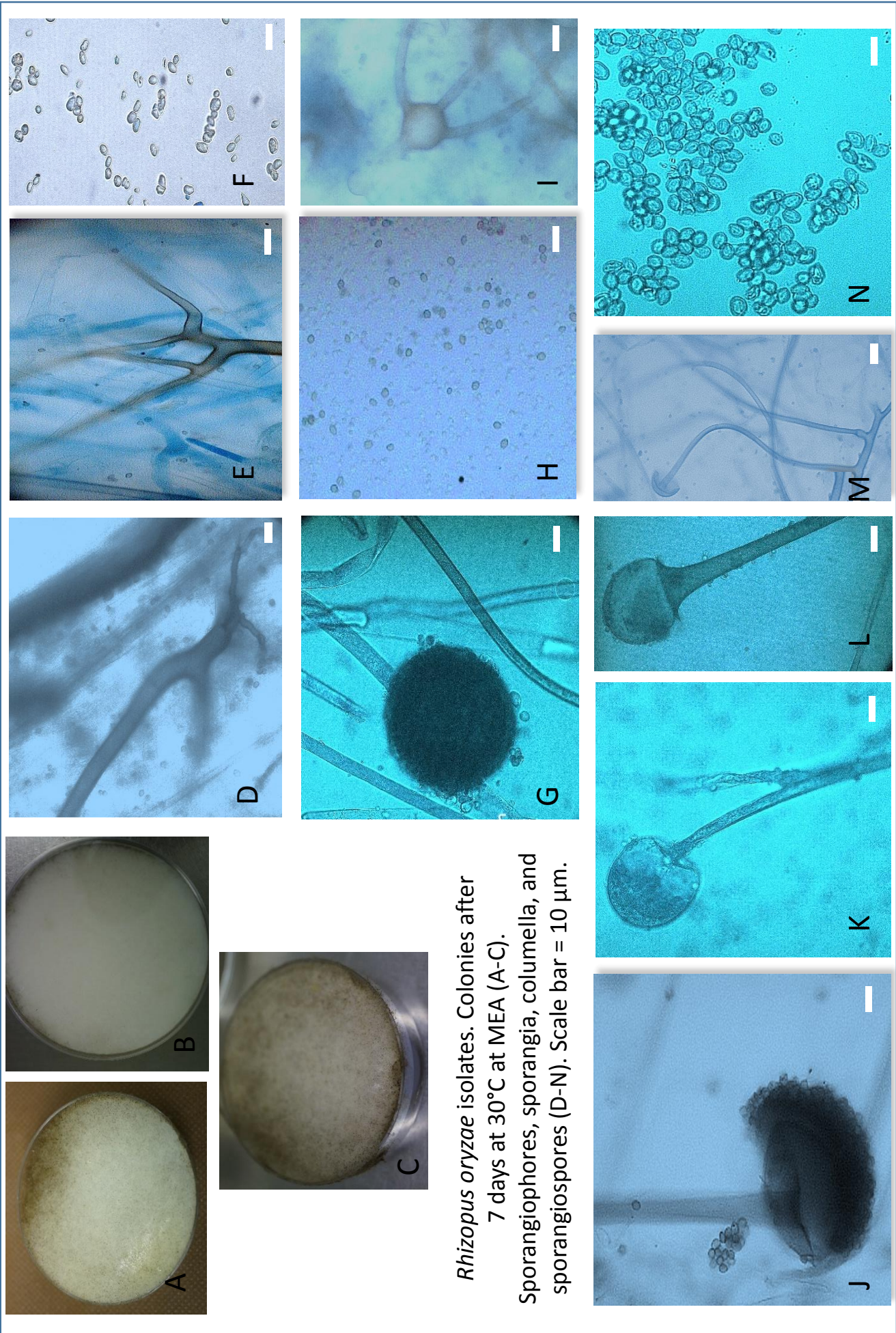
1) Diameter of mycelial growth was measured after 2 days of incubation on MEA at the optimal temperatures.

2) Lactic acid production tested only to distinguish between *R. oryzae* and *R. delemar* isolates

3) Result expressed as positive (+) and negative (-). Positive result accompanied with percentage of total positive samples in bracket.

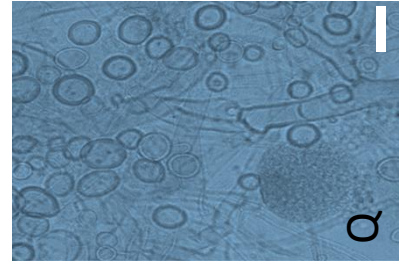
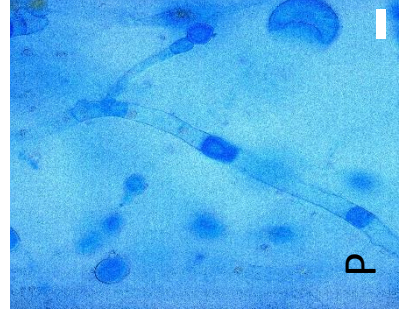
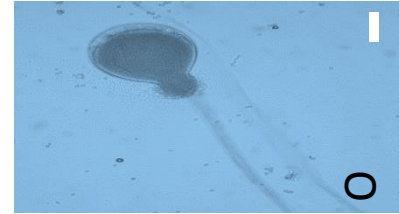
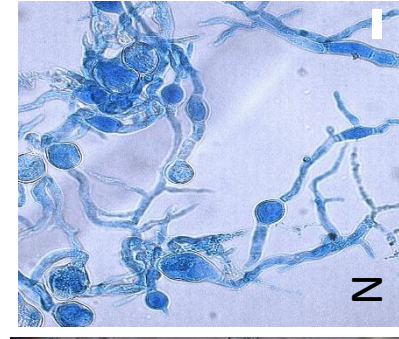
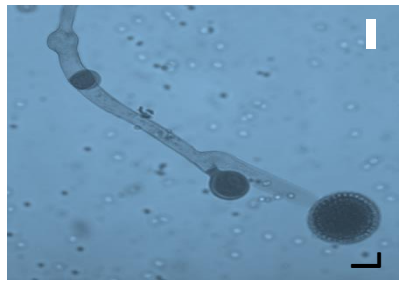
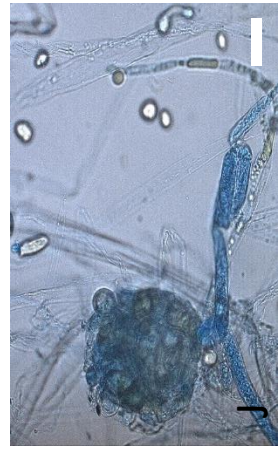
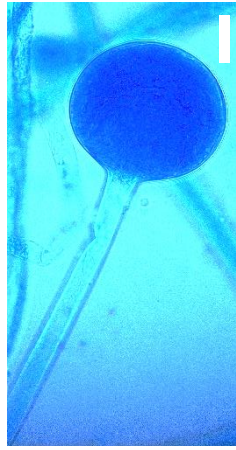
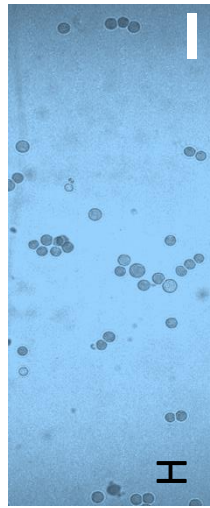
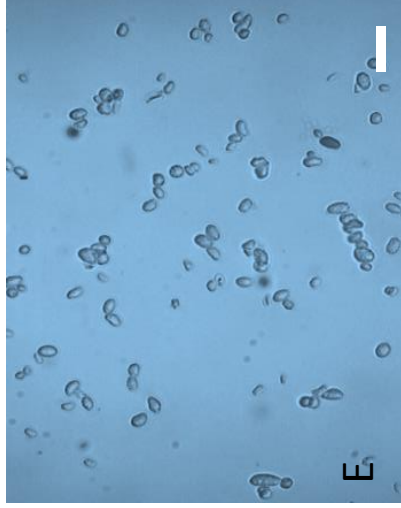
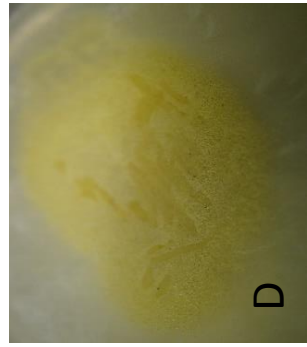
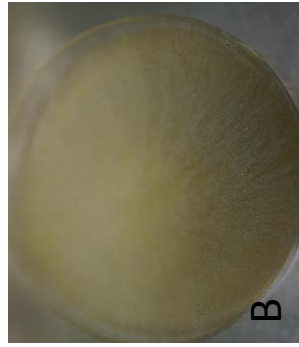
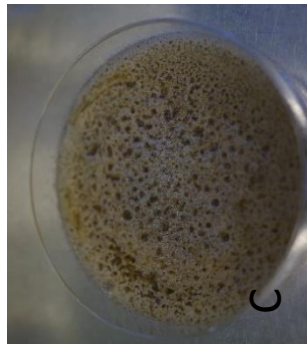
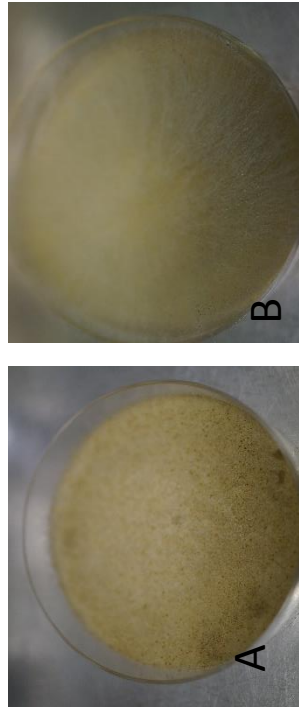


Figure 3. Various morphological structures in the tempeh fungi and fungi-like isolates (major isolates).



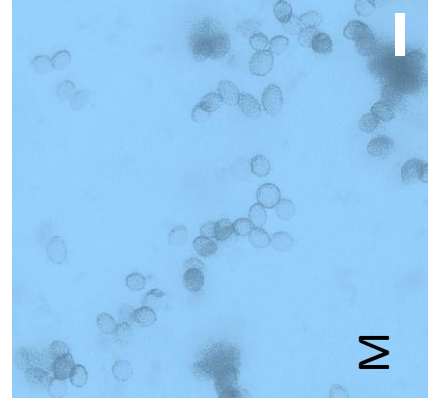
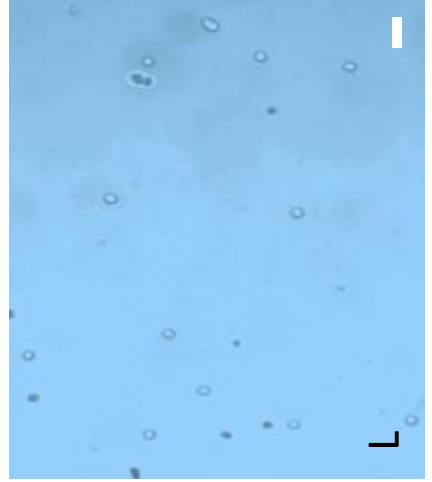
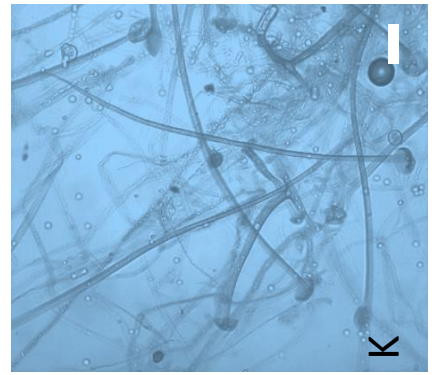
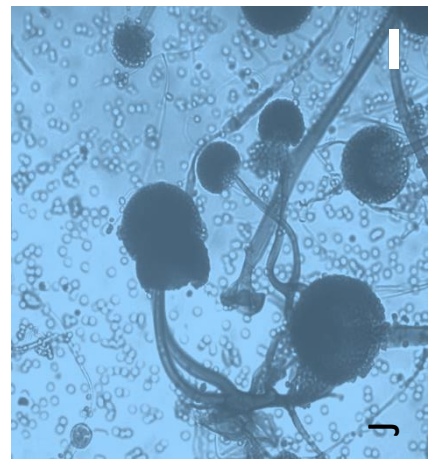
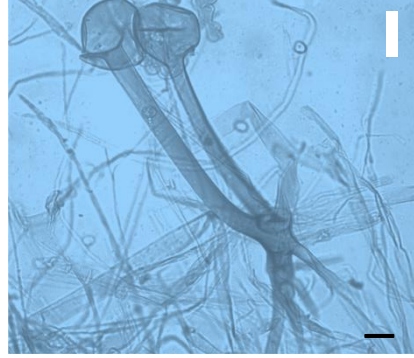
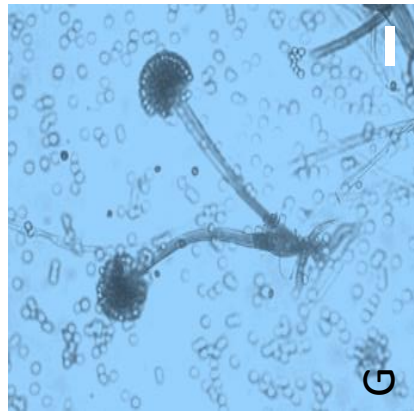
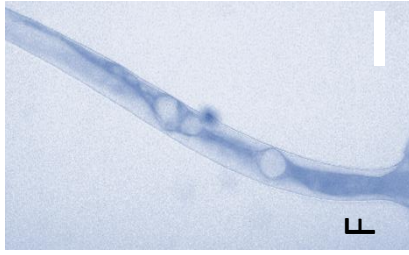
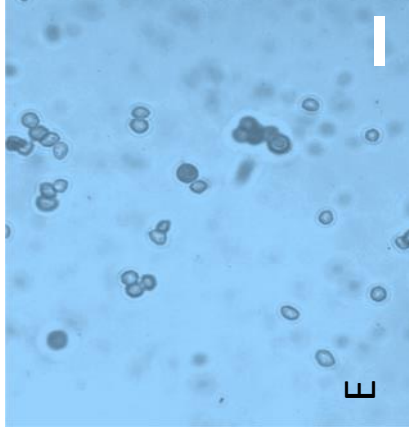
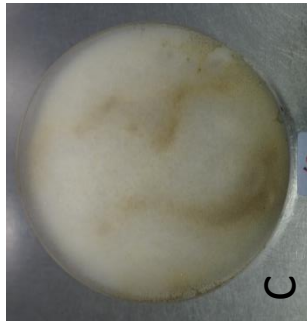
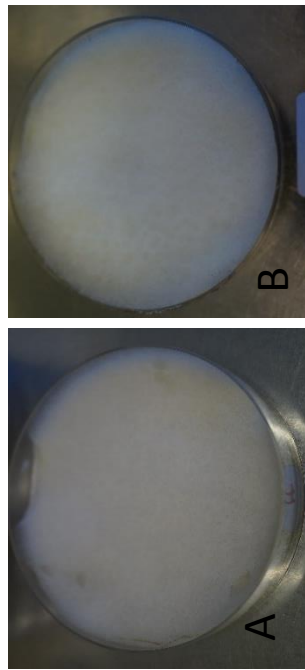
*Rhizopus oryzae* isolates. Colonies after 7 days at 30°C at MEA (A-C). Sporangioophores, sporangia, columella, and sporangiospores (D-N). Scale bar = 10  $\mu$ m.



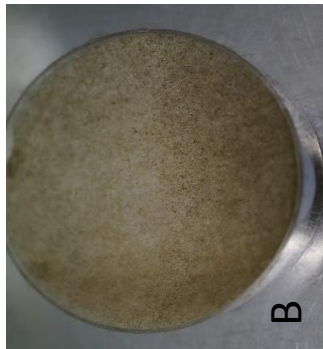
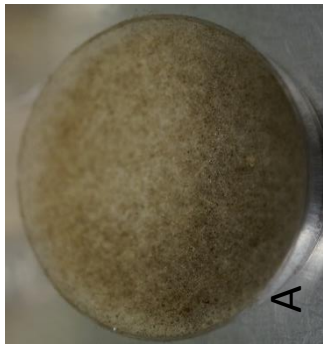


*Mucor* spp. isolates. Colonies after 7 days at 30°C at MEA (A-C). Sporangioophores, sporangia, columella, chlamydospores and sporangiospores (E-R). Scale bar = 10 µm.

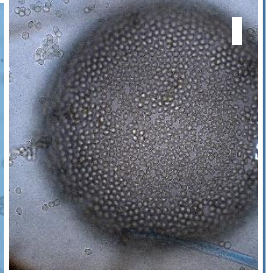
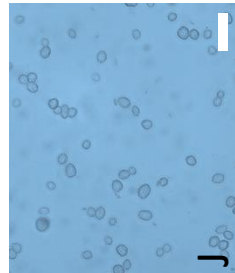
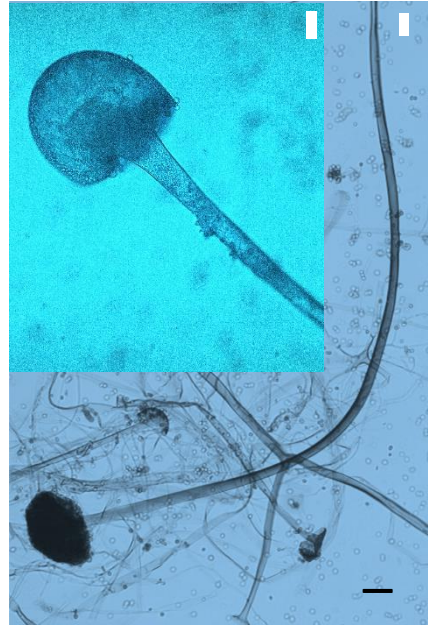
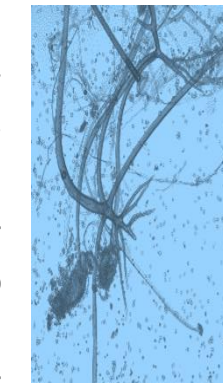
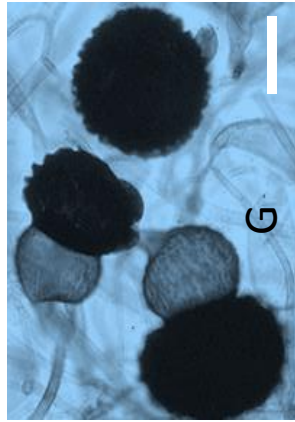
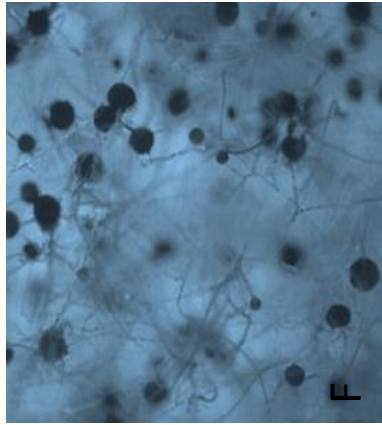
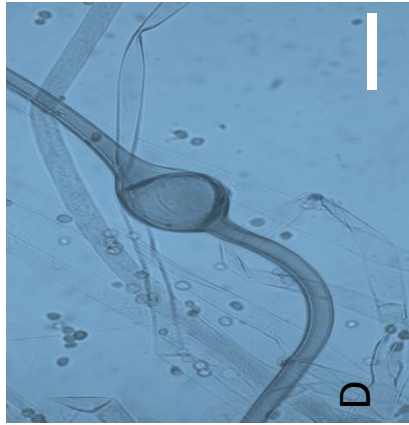
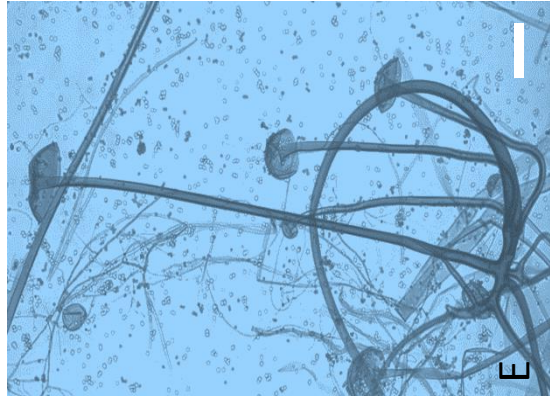




*Rhizopus microsporus* isolates. Colonies after 7 days at 33°C at MEA (A-C). Sporangiospores, sporangia, columella, chlamydospores and sporangiospores (D-M). Scale bar = 10 µm.

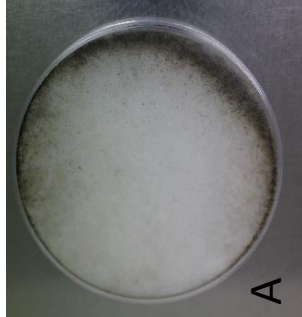


*Rhizopus stolonifer* isolates. Colonies after 7 days at 35°C at MEA (A-C). Sporangioophores, sporangia, columella, zygospores and sporangiospores (D-J). Scale bar = 25 µm.



H

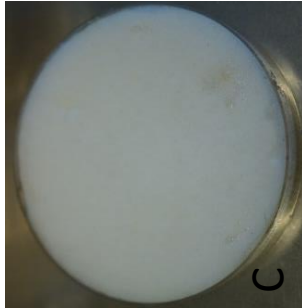




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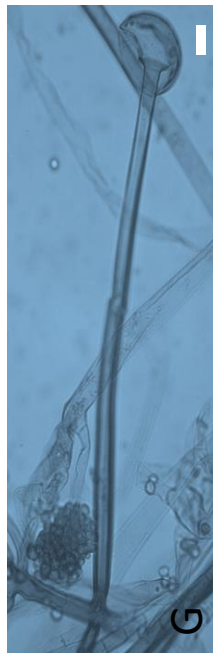


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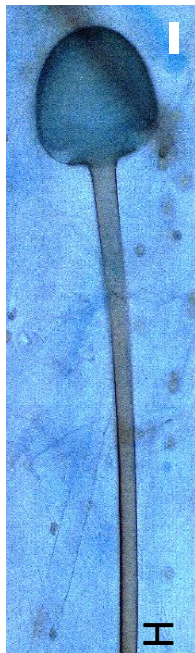


C

*Rhizopus delemar* isolates. Colonies after 7 days at 33°C at MEA (A-C). Sporangioophores, sporangia, columella, and sporangiospores (D-O). Scale bar = 10 µm.



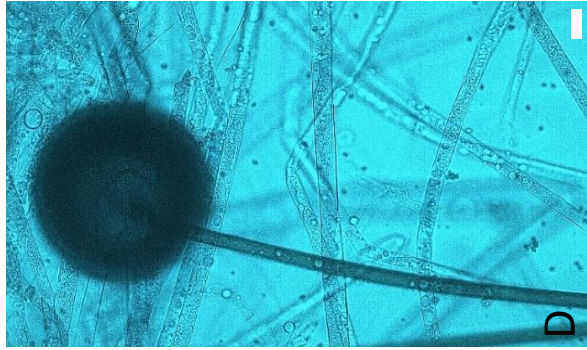
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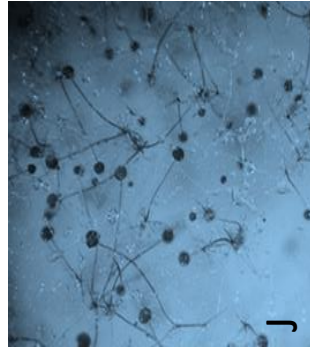
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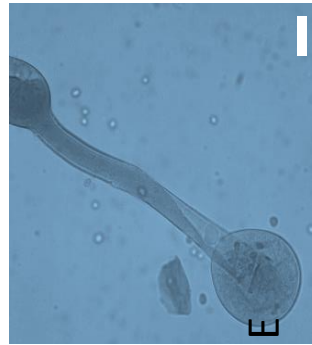
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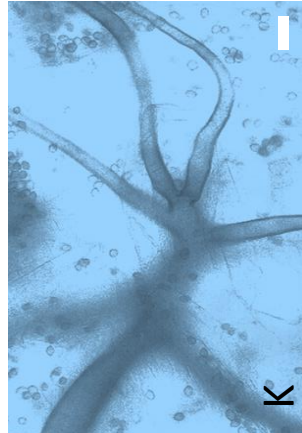
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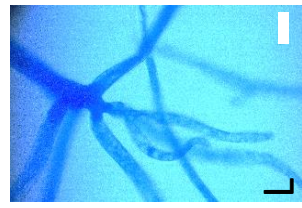
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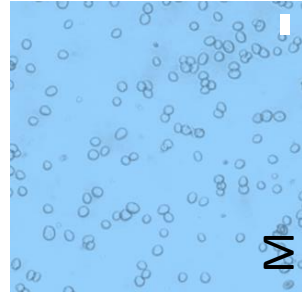
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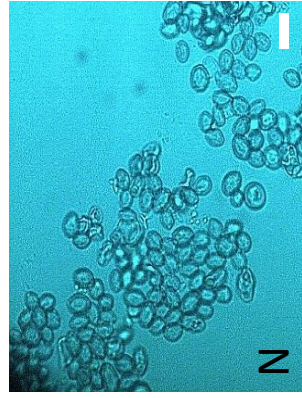
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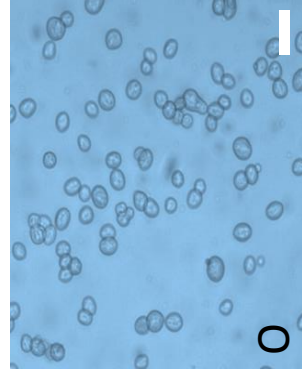
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M

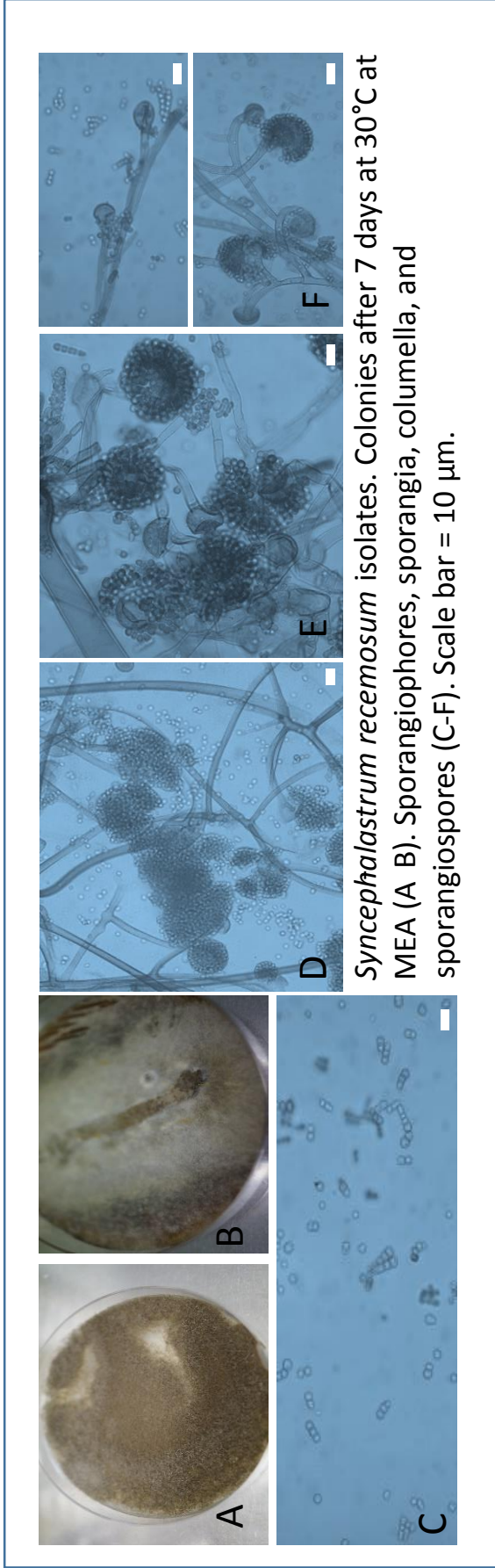


N

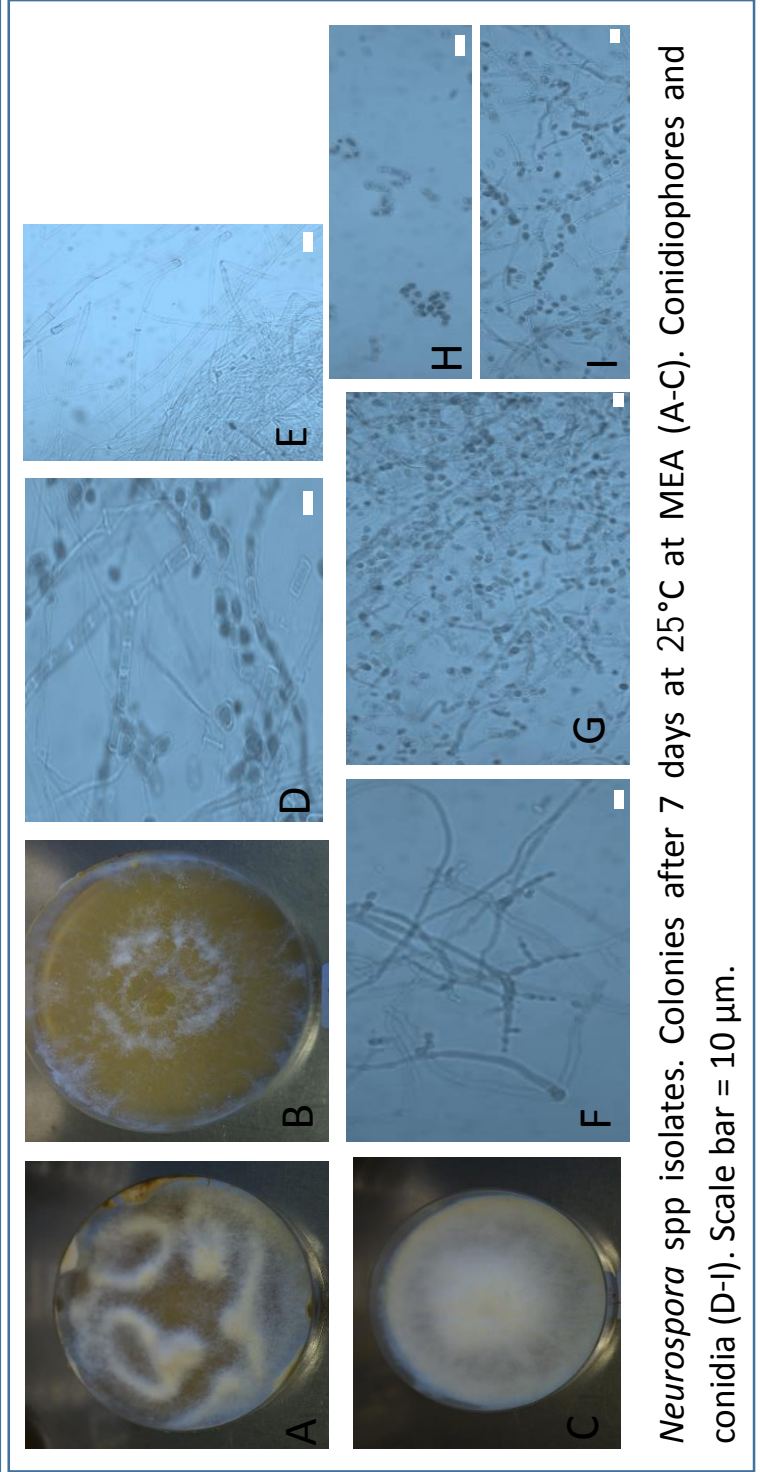


O

Figure 3. Various morphological structures in the tempeh fungi and fungi-like isolates (minor isolates).

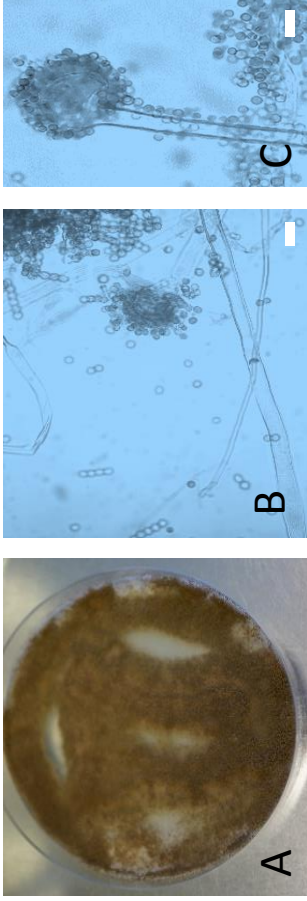


*Syncephalastrum recemosum* isolates. Colonies after 7 days at 30°C at MEA (A B). Sporangiophores, sporangia, columella, and sporangiospores (C-F). Scale bar = 10 µm.

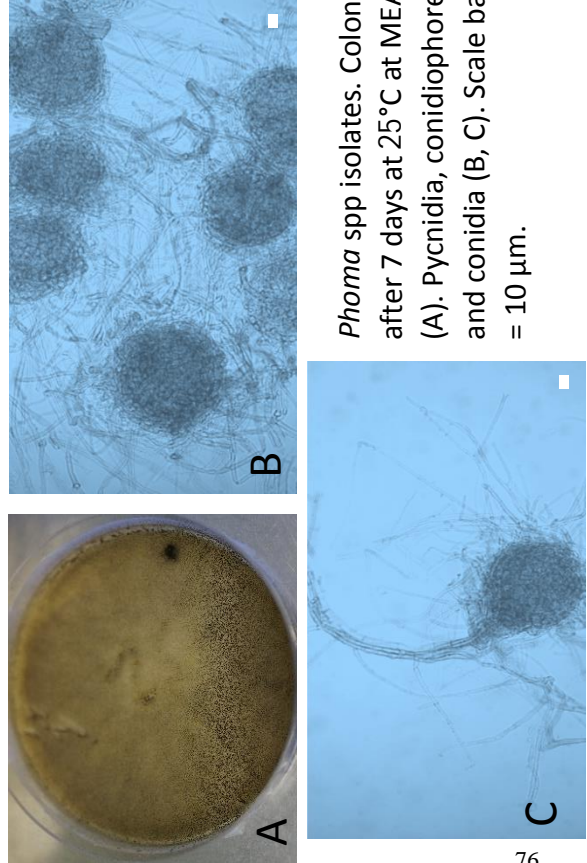


*Neurospora* spp isolates. Colonies after 7 days at 25°C at MEA (A-C). Conidiophores and conidia (D-I). Scale bar = 10 µm.

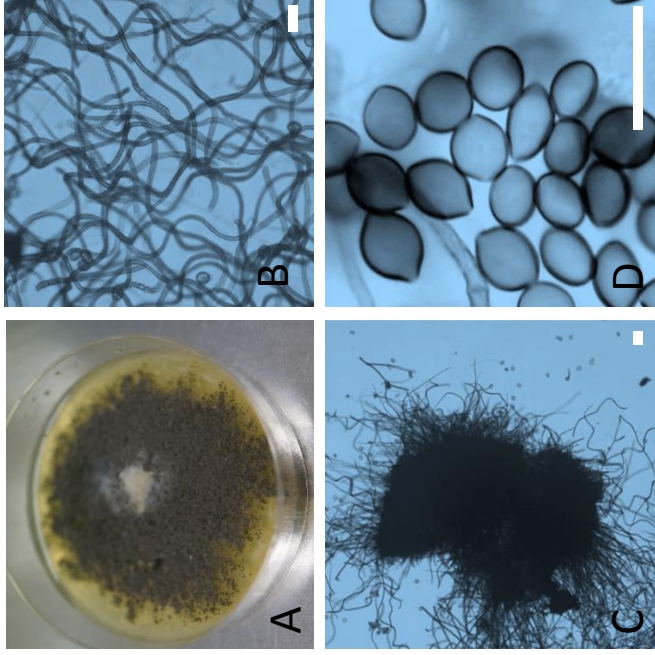




*Aspergillus* spp isolates. Colonies after 7 days at 30°C at MEA (A). Conidiophores and conidia (B-E). Scale bar = 10 µm.



*Phoma* spp isolates. Colonies after 7 days at 25°C at MEA (A). Pycnidia, conidiophores and conidia (B, C). Scale bar = 10 µm.



*Chaetomium globosum* isolates. Colonies after 7 days at 25°C at MEA (A). Fruiting bodies, asci and ascospores (B-D). Scale bar = 10 µm.



(Fungi-like strains) *Geotrichum candidum* isolates. Colonies after 7 days at 30°C at MEA (A). Hyphae and arthrospores (B-D). Scale bar = 10 µm.



surface, up to 12  $\mu\text{m}$  long. No growth at 45°C, growth at 33°C. Almost in all morphological aspect this species looks similar with *Rhizopus oryzae*, but this species could not produce lactic acid.

#### *Rhizopus stolonifer*

Sporangiophores borne in groups of three to five from clusters of rhizoids, stipes unbranched, robust and up to 1600  $\mu\text{m}$  long, with brown walls; sporangia 100–300  $\mu\text{m}$  diameter, usually spherical; columellae roughly spherical, up to 200  $\mu\text{m}$  diam, in age often collapsing downwards and outwards to produce umbrella shapes; sporangiospores commonly 8–20  $\mu\text{m}$  in long axis, pale brownish, with striate walls. Distinctive features from this species are:

*Rhizopus stolonifer* is distinguished by its habit, with coarse hyphae and rampant growth at 30–35°C, and by sporangia which are white when first formed but which change to black with maturity. Sporangiospores are large, with striate walls. This species has most complex and branched rhizoid form.

Some strains also tested for enzymes activities including gelatin liquefaction, presence of urease, siderophores, lipase, amylase, cellulose, laccase and tyrosinase. Results of physiological tests are shown also in Table 3. All strains analyzed were positive for lipase production. Most strains were able to liquefy gelatin. They also could produce urease, lipase and amylase. Some strains were able to produce laccase. All strains were negative for tyrosinase and cellulase. Only few strains (assumed as *R. oryzae* and *R. delemar*) showed siderophores.

Carbon assimilation profiles of the different species of zygomycetes and from other fungal division identified with ID32C strips are presented in Table 4. All species assimilated D-galactose, N-acetylglucosamine, D-maltose, D-trehalose, and D-glucose and were esculin positive. None of the tested species was able to use methyl D-glucoside, L-rhamnose, erythritol, glucuronate, levulinate, inositol, or L-sorbose as a sole source of carbon. DL-Lactate were assimilated only by some isolates of *Aspergillus spp.* Every species has different pattern in assimilation indicate their specific capability in using some carbon as source of energy. Those various pattern also show diversity especially among zygomycetes strains. The difference between zygomycetes and non-zygomycetes isolates could be seen obviously from the ability to assimilate D-cellobiose.



Table 4. Carbon assimilation profiles of selected fungi isolates with ID32C.

Carbon source	Carbon assimilation profiles of fungi isolates with ID32C strips									
	Isolates suspected as									
	<i>R. oryzae</i>	<i>R. microsporus</i>	<i>R. delemar</i>	<i>R. stolonifer</i>	<i>Mucor</i> spp	<i>S. racemosum</i>	<i>Neurospora</i> spp	<i>Phoma</i> spp	<i>C. globosum</i>	<i>Aspergillus</i> spp
D-galactose	+ (100%)	+ (100%)	+ (100%)	+ (100%)	+ (100%)	+ (100%)	+ (100%)	+ (100%)	+ (100%)	+ (100%)
Actidione	+ (60%)	-	+ (70%)	-	+ (100%)	+ (100%)	+ (100%)	-	-	+ (100%)
D-saccharose	+ (50%)	-	+ (70%)	-	-	+ (70%)	+ (100%)	-	-	+ (100%)
<i>N</i> -acetyl-glucosamine	+ (100%)	+ (100%)	+ (100%)	+ (100%)	+ (100%)	+ (100%)	+ (100%)	+ (100%)	+ (100%)	+ (100%)
DL-lactate	-	-	-	-	-	-	-	-	-	+ (50%)
L-arabinose	+ (100%)	-	+ (100%)	-	+ (100%)	+ (100%)	-	-	-	+ (100%)
D-cellobiose	+ (100%)	+ (100%)	+ (100%)	+ (100%)	+ (100%)	+ (100%)	-	-	-	-
D-raffinose	+ (70%)	-	+ (50%)	-	-	+ (100%)	-	-	-	+ (100%)
D-maltose	+ (60%)	+ (100%)	+ (50%)	+ (100%)	+ (100%)	+ (100%)	+ (100%)	+ (100%)	+ (100%)	+ (100%)
D-trehalose	+ (100%)	+ (100%)	+ (100%)	+ (100%)	+ (100%)	+ (100%)	+ (100%)	+ (100%)	+ (100%)	+ (100%)
Potassium 2-keto-gluconate	-	-	-	-	+ (100%)	-	+ (100%)	-	-	-
<i>methyl-<math>\alpha</math></i> -D-glucopyranoside	-	-	-	-	-	-	-	-	-	-
D-sorbitol	+ (100%)	+ (100%)	+ (100%)	+ (100%)	+ (100%)	+ (100%)	-	+ (100%)	-	+ (100%)
D-xylose	+ (100%)	+ (100%)	+ (100%)	+ (100%)	+ (100%)	+ (100%)	+ (100%)	+ (100%)	+ (100%)	+ (100%)
D-ribose	+ (100%)	+ (100%)	+ (100%)	+ (100%)	+ (100%)	+ (100%)	+ (100%)	-	+ (100%)	-
Glycerol	+ (100%)	+ (60%)	+ (100%)	+ (60%)	-	-	-	-	-	-
L-rhamnose	-	-	-	-	-	-	-	-	-	-
Palatinose	-	-	-	-	+ (100%)	+ (100%)	+ (100%)	+ (100%)	+ (100%)	+ (100%)
Erythritol	-	-	-	-	-	-	-	-	-	-
D-melibiose	-	-	-	-	-	+ (100%)	-	-	-	+ (100%)
sodium glucuronate	-	-	-	-	-	-	-	-	-	-
D-melezitose	+ (70%)	-	+ (100%)	+ (100%)	+ (100%)	-	+ (100%)	+ (100%)	+ (100%)	+ (100%)
Potassium gluconate	-	+ (100%)	-	+ (100%)	+ (100%)	-	-	-	-	-
Levulinate	-	-	-	-	-	-	-	-	-	-
D-mannitol	+ (100%)	+ (100%)	+ (100%)	+ (100%)	+ (40%)	+ (100%)	+ (100%)	+ (100%)	+ (100%)	+ (100%)
D-lactose	-	-	-	-	-	-	-	-	-	+ (100%)
Inositol	-	-	-	-	-	-	-	-	-	-
D-glucose	+ (100%)	+ (100%)	+ (100%)	+ (100%)	+ (100%)	+ (100%)	+ (100%)	+ (100%)	+ (100%)	+ (100%)
L-sorbose	-	-	-	-	-	-	-	-	-	-
Glucosamine	+ (100%)	+ (80%)	+ (80%)	+ (50%)	+ (100%)	-	-	-	-	-
Esculin	+ (100%)	+ (100%)	+ (100%)	+ (100%)	+ (100%)	+ (100%)	+ (100%)	+ (100%)	+ (100%)	+ (100%)
Number of isolates examined	10	10	10	10	10	10	10	5	5	5

Note:  
Abbreviation: R= *Rhizopus*, S= *Syncephalastrum*, C= *Chaetomium*  
Strips were read visually. Weak growth was considered to be positive.  
Result expressed as positive (+) and negative (-). Positive result accompanied with percentage of total positive samples in bracket.

## Molecular results

In this study, identification of zygomycetes isolates were performed according to the combination of morphological, physiology and molecular characters. To confirm morphological identification, representative isolates were observed with molecular approach. First, DNA fingerprinting was employed to distinguish identical and non identical isolates. Second, non identical isolates with distinct band were selected and confirmed at species level by the results of DNA sequence of the Actin gene, which is taxonomically most informative part in this fungal genus.

ACT is known as a housekeeping gene, as its expression is relatively stable under variable conditions. ACT encodes actin, a protein that is abundant in all eukaryotic cells, in which it is the major component of cytoplasmic microfilaments. The ACT gene has been used extensively to infer interspecies relationships across broad evolutionary distances, as the ACT gene of many species contains highly conserved (i.e. exon) and less well-conserved (i.e. intron) sequences, which are grouped as nuclear pre-mRNA introns (Donnelly et al., 1999).

## DNA fingerprinting

In the preliminary experiments, four arbitrary primers were tested with Random Amplified Polymorphic DNA (RAPD) with DNA extracted from pure cultures. Preliminary test determined the optimal concentration of the component in the PCR reaction mixture, amplification conditions and primer selection. Strong, unique, sharp and clearly reproducible bands were produced by primer C6.2\_introns\_2\_R and C7\_1seq (Figure 4). For further analysis C7\_1seq was used. The number of DNA bands in these profiles ranged from six to 10 with sizes ranging from 250 to 850 bp. Although the primer generated some common bands, sufficient numbers of polymorphic bands were obtained to detect diversity among the test strains and most of them can be easily distinguished. The experimental procedure was repeated at least three times with the same condition to confirm the reproducibility of RAPD analysis. No significant differences were observed in the patterns, although some bands varied in intensity.

The RAPD patterns of 471 *Rhizopus* isolates obtained with the primer are shown partly in Figure 5. Forty distinct RAPD patterns were obtained and clustered. A dendrogram was generated by using UPGMA (unweighted pair-group method using arithmetic averages) linkage clustering of the Jaccard coefficients. Cluster analysis of the banding patterns revealed six different clustered groups (in Figure 6). Cluster 1, 2 and 3 were *Rhizopus* isolates dominantly from Java, whereas cluster 4, 5 and 6 were dominantly from outside Java. This means *Rhizopus* isolates used for tempeh fermentation in Java and outside Java are different from genetic perspective.

Comparison between clustering and morphological data showed that cluster 1, 2 and 3 were isolates from *Rhizopus microsporus*. This species has 3 main groups, they are var. *microsporus*, var. *oligosporus* and var. *chinensis*. High possibility that those 3 cluster are representative from the groups. Based on morphological data, cluster 4, 5 and 6 are possibly as representative of *R. delemar*, *R. oryzae*, and *R. stolonifer* isolates, respectively). This result would be further confirmed with ACT sequencing result.

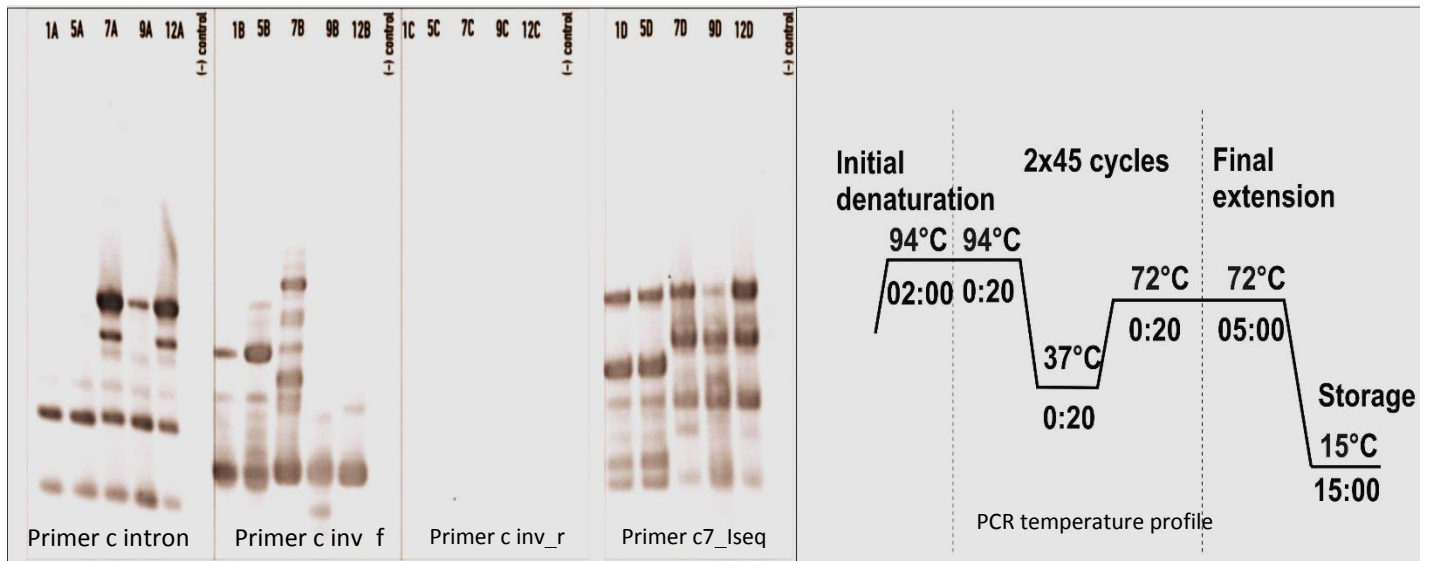


Figure 4. Preliminary result (primers selection) and its PCR temperature profile.

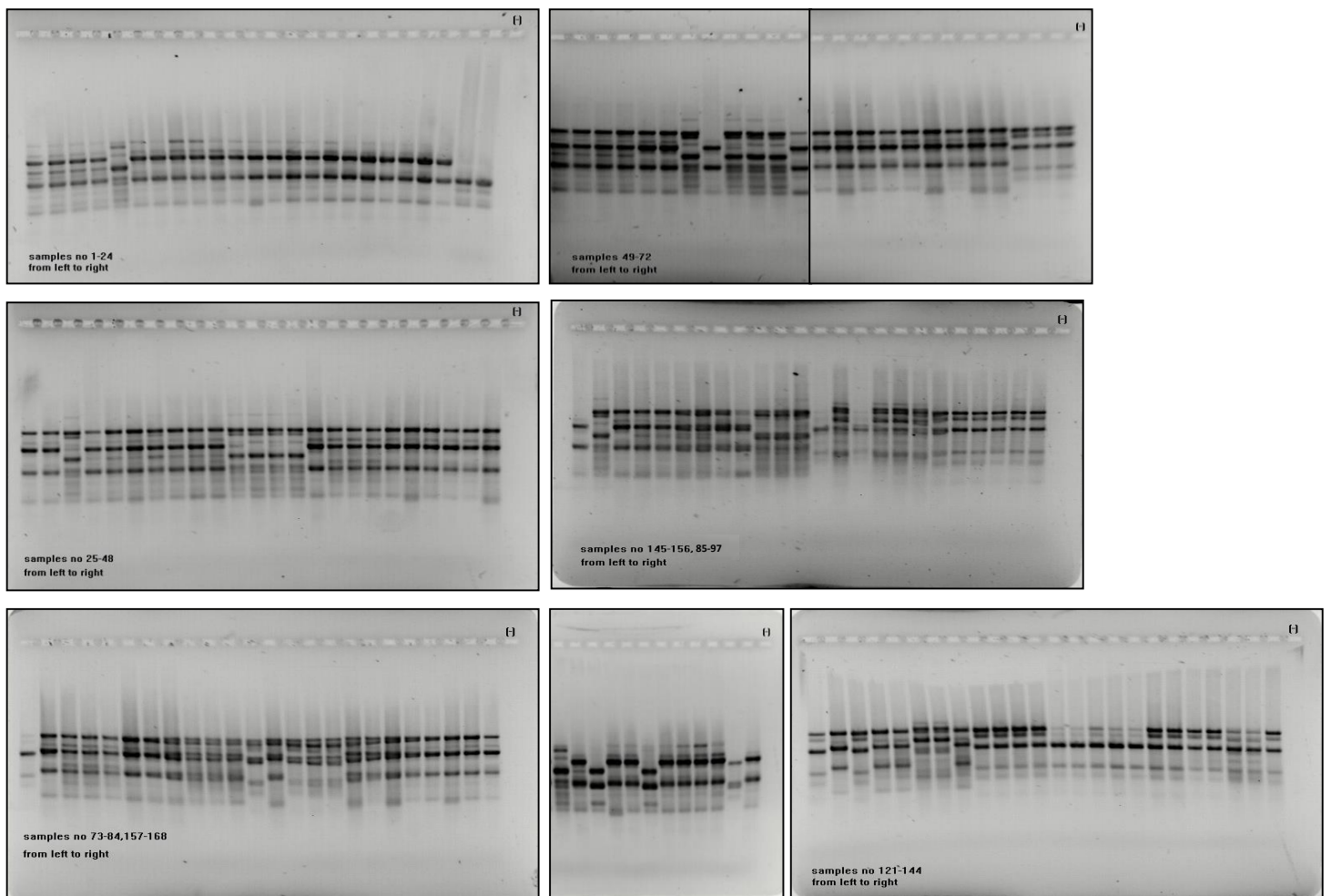


Figure 5. Some results of DNA fingerprinting from tempeh fungi isolates (isolate no 1-168, part 1)

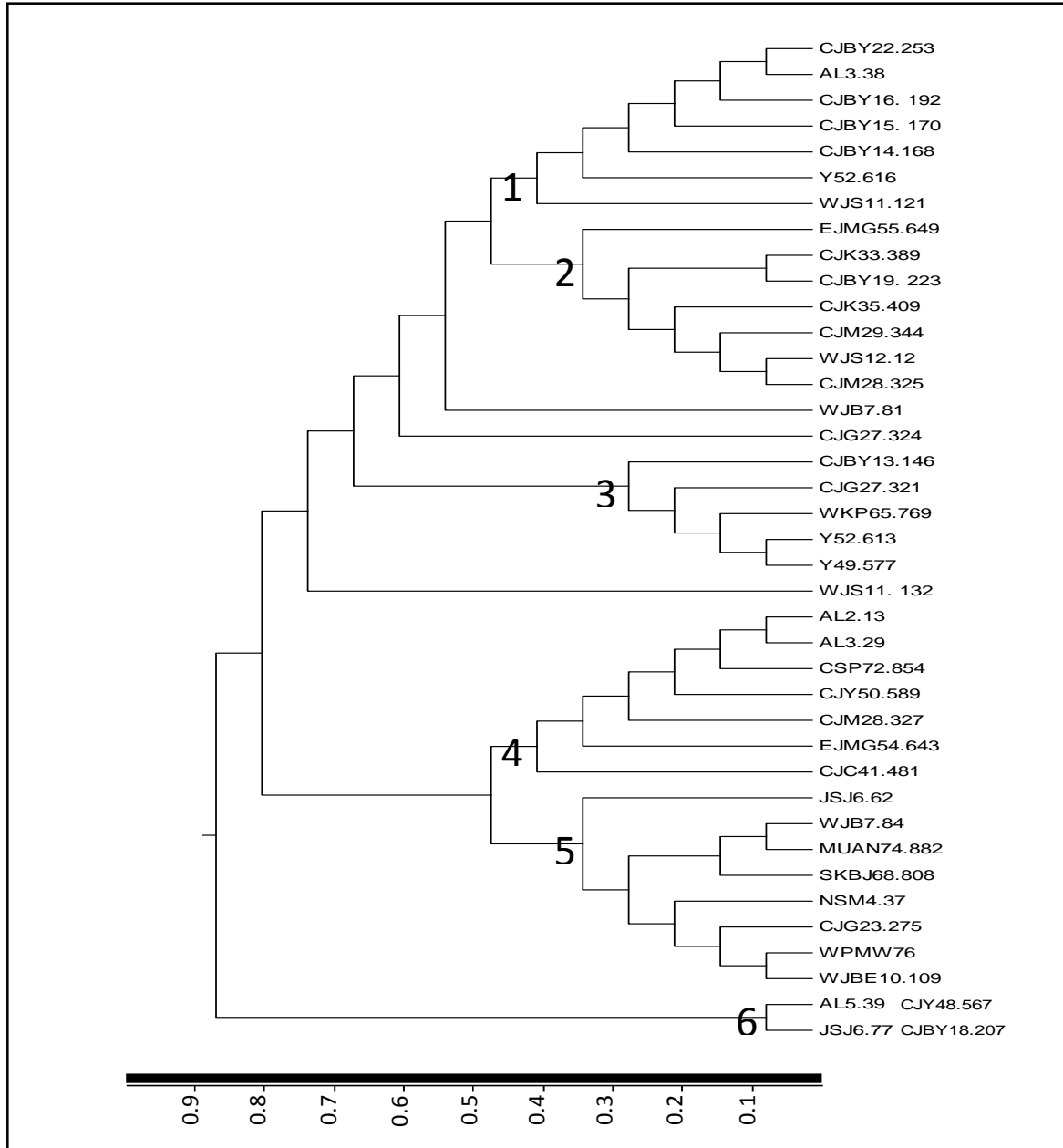


Figure 6. Dendrogram created by UPGMA linkage with clustering of Jaccard coefficients calculated from RAPD data on 42 *Rhizopus* isolates.

RAPD typing was capable of producing discriminating DNA fingerprints of *Rhizopus* isolates and indicated there were genetic differences among them. Forty distinct patterns were observed indicating that tempeh and inocula from different locations in Indonesia composed of quite a wide number of strains. Genetic diversity was measured as the Shannon-Wiener  $H'$  index and the average HS index. HS was estimated to be 0.23 and Shannon's information index was maximum, 1.5. Our results suggest the existence of significant genetic variation among these *Rhizopus* isolates.

### DNA sequencing of tempeh fungi isolates

To confirm morphological identification, 42 representative isolates from RAPD selection were sequenced based on the ACT gene which has been widely used for species identification. Thermal PCR conditions suggested by Geiser et al. (2004), lead to a mixture of specific and non-specific products. The situations were achieved by running a gradient PCR in the range of 53-63°C under “Hot start PCR”. Hot start PCR protocol was used to amplify the ACT gene region using the fungal specific primer set: Act-1 (5'-TGGGA CGATATGGAI AAIATCTGGCA-3') and Act-4ra (5'-TCITCGTATTCTTGCTTIGAIATCCAC AT-3') described by Voigt et al. (2000).

At the annealing temperature of 58.5°C, a single band in the size range of 810 to 853 bp was successfully amplified from all isolates. After DNA sequencing, each unknown *Rhizopus* strain was identified according to the deposited sequences in NCBI database which had a high homology (= 99%) with them. The first three hits for the sequence of each isolate have been assigned in Supplemental Table 1. The studied sequences were, then, aligned with ACT sequences of *R. microsporus* var. *chinensis*, *R. oryzae*, *R. delemar*, *R. stolonifer*, *R. microsporus* var. *microsporus* and *R. microsporus* var. *oligosporus* retrieved from sequence database (Supplemental Table 2) and exposed to phylogenetic analysis (Fig. 7 and 8).

PCR amplification and DNA sequencing using the primer were successful, the blast of the sequenced regions gave conclusive results concerning the molecular taxonomy of the fungi. The UPGMA dendrogram clearly clustered distinct clades regarding to *Rhizopus* spp. studied. Identification was well supported with bootstrap values. The studied strains, therefore, were placed into groups matching with those determined by NCBI database.

UPGMA dendrogram showed that *Rhizopus* spp. from Indonesian tempeh and inocula were placed into *R. stolonifer* clade (4 strains), *R. delemar* clade (7 strains), *R. oryzae* clade (8 strains), *R. microsporus* var. *microsporus* clade (10 strains), *R. microsporus* var. *oligosporus* clade (8 isolates) and *R. microsporus* var. *chinensis* clade (4 isolates). The *R. delemar* clade was sister to *R. oryzae* clade with 99% BS. The *R. microsporus* var. *chinensis* clade was sister to *R. microsporus* var. *microsporus*, and *R. microsporus* var. *oligosporus* clade with 100% BS. The data are also in line with morphological and physiological characters which previously described. As example, the strain MUAN74.882 which was identified morphologically as *R. delemar*, obviously identified NCBI database also as *R. delemar*. Similarly, isolates identified morphologically as *R. stolonifer* placed in *stolonifer* clade. The phylogenetic tree (Figure 7) clearly showed that the resolution of sequence from ACT region was sufficient in determining *Rhizopus* species into variety level, particularly in *R. microsporus* complex, dividing into var. *chinensis*, var. *microsporus* (often not written in dendrogram) and var. *oligosporus*, whereas morphological and physiological data only recognize until species level (*R. microsporus*). This prove that the DNA sequencing gave better taxonomy information until level of variety than morphological approach in this genus.

Combination of origin or geographical data and molecular identification (Figure 8) showed that isolates (identified as *Rhizopus oryzae*, *R. delemar*, and *R. stolonifer*) dominantly used by tempeh producer from outside Java, whereas isolates (*Rhizopus microsporus* complex isolates, identified as *R. microsporus* var. *chinensis*, *R. microsporus* var. *microsporus* and *R. microsporus*

var. oligosporus) were mostly used by tempeh producer from Java. This suggest that's why *Rhizopus microsporus* strain was the main and most important species for tempeh industry in Java where best tempeh quality produced.

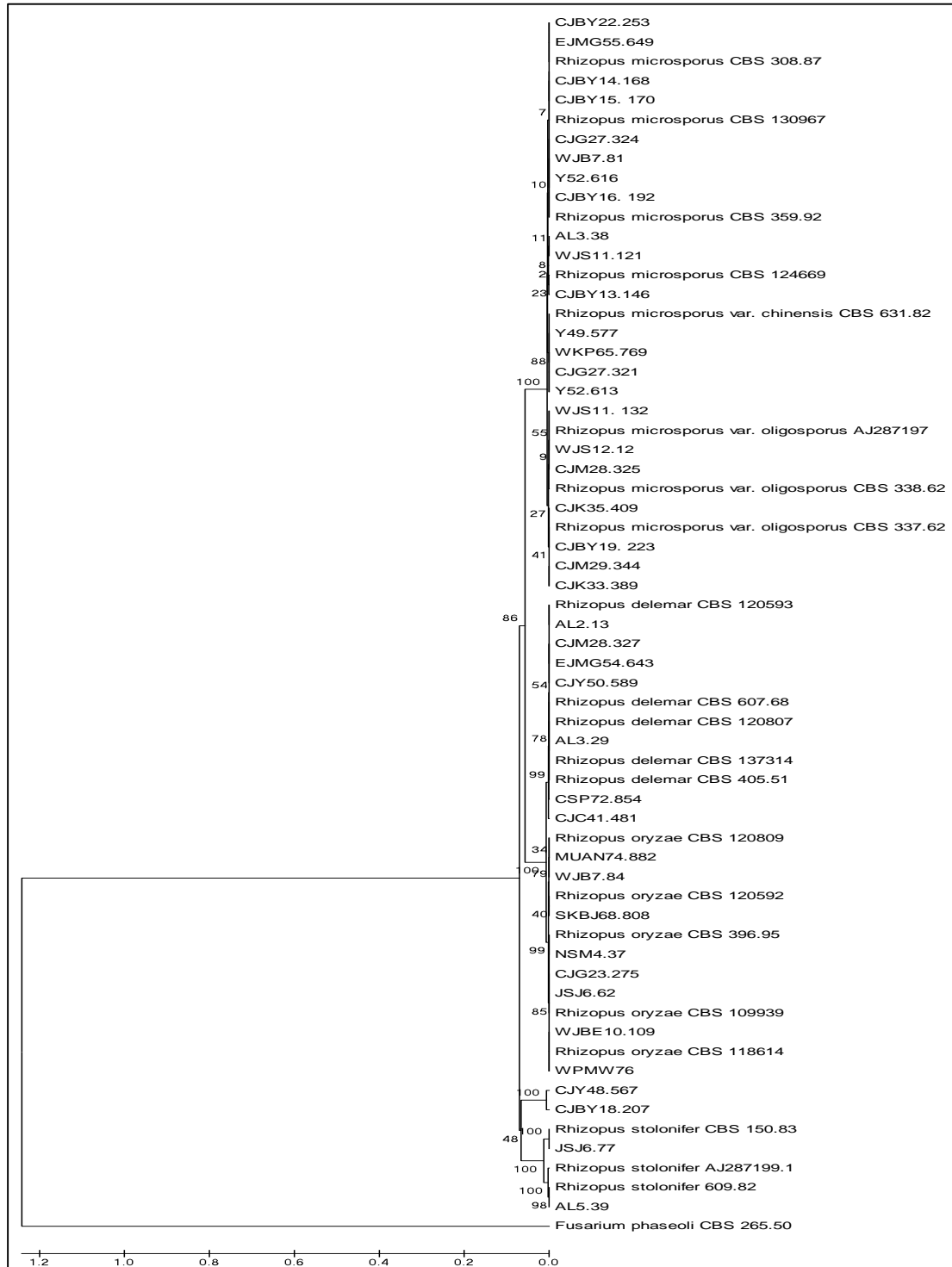


Figure 7. The tree was inferred from Actin sequence and constructed using UPGMA method. Numbers at nodes indicate levels of bootstrap support calculated from 1000 trees.

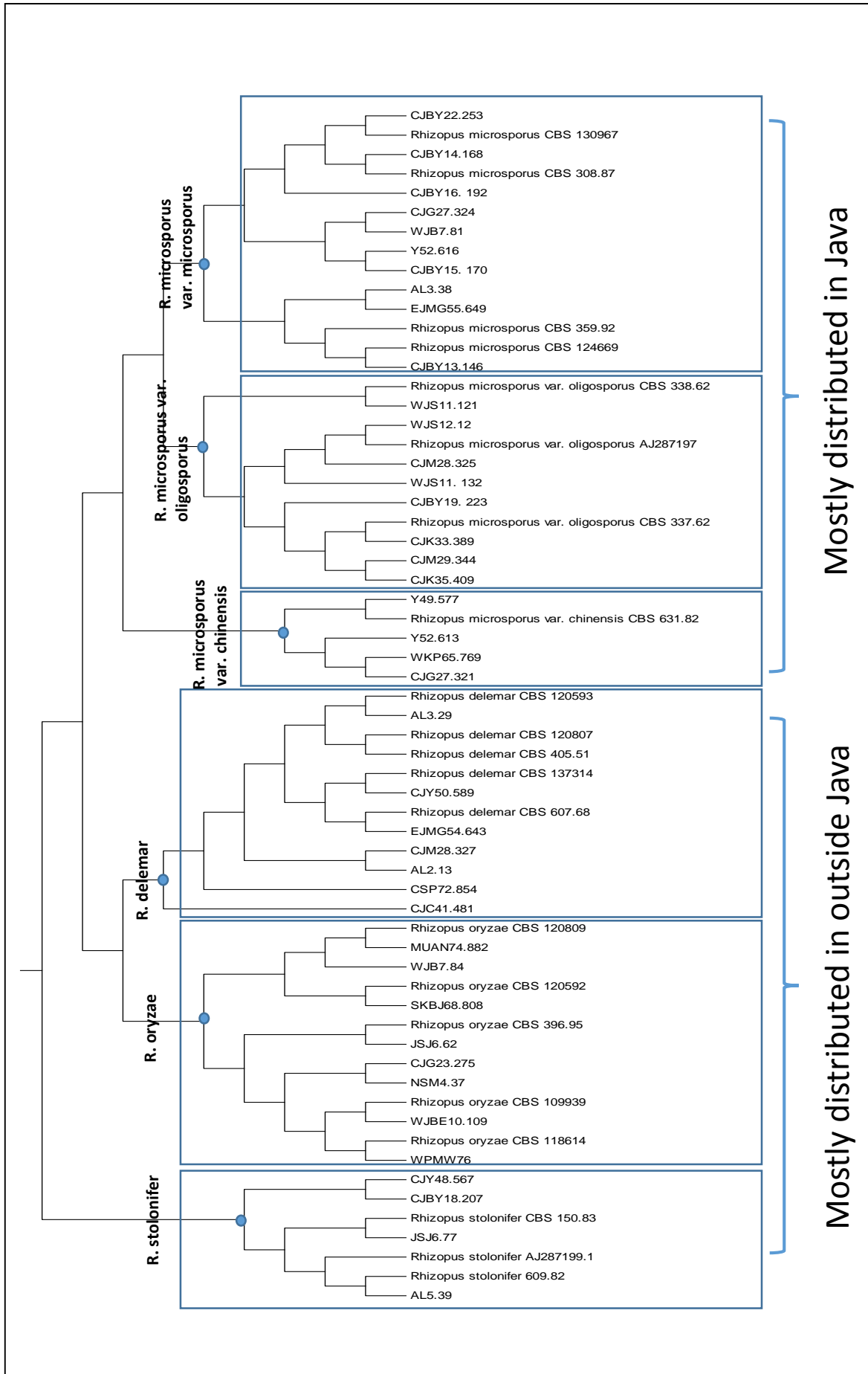


Figure 8. Grouping of tempeh fungi species and their distribution in Indonesia (with bootstrap consensus tree UPGMA, ACT gene).

### 3.2. Discussion of Part I (Fungal diversity and identification)

In Indonesia, *Rhizopus* spp. have been known as one of most economically important mold due to their role as inoculum source for making tempeh, but their information on diversity has been confusing due to limited information, and outdated determination approach previously applied by Indonesian mycologists. As example, *Rhizopus oligosporus* has still commonly been regarded as inoculant name of tempeh in Indonesia until now (Prihatna and Suwanto, 2007; Dewi and Aziz, 2011), although, this name was, a long time ago, transferred as *var. oligosporus* within *R. microsporus* group by Schipper and Stalpers (1984). Moreover, the taxonomy and natural systematics within the Mucorales are extremely problematic due to insufficient in distinguishing morphological characters and by the high intra-specific variability of those that do exist. Their characters are also dependent upon physiological growth parameters (Schipper, 1973; Voigt and Wöstemeyer, 2001). Benny (1995) pointed out the limitations of the morphological features in the species delimitation within certain zygomycetes, suggesting the use of molecular tools for solving existing taxonomic controversies. Combination morphological, physiological and molecular features would be the best way and very beneficial to address the problem

In this study, identification of zygomycetes isolates was performed according to the combination of morphological, physiology and molecular characters. To confirm morphological identification, representative isolates were observed with molecular approach. First, DNA fingerprinting was employed to distinguish identical and non-identical isolates. Second, non identical isolates with distinct band were isolated and confirmed at species level by the results of DNA sequence of the Actin gene, which is taxonomically most informative part in this fungal genus.

The moulds isolated from tempeh and inocula in this study were broadly represented by nine genera. In total, 557 fungi and five fungi-like isolates were obtained from 247 samples. *Rhizopus* was the most frequently detected genera, found in 84% of total samples, followed by *Mucor* (5.5%), *Neurospora* (4.1%) and *Syncephalastrum* (3%). Other very minor genera that found as contaminants were *Chaetomium*, *Phoma*, *Acremonium*, *Aspergillus* and *Geotrichum* (less than 1%). Nout and Kiers mentioned that there is a complex of microorganisms in soybean tempeh, their development start during the soaking of the raw ingredients. Those contaminant might be from raw material itself, environment of local factories, tools or cross contamination from human. However, to distinguish between tempeh fungi and contaminant could be done by quick checking their assimilation pattern. We found that the difference between zygomycetes and contaminant isolates could be seen obviously from the ability to assimilate D-cellobiose. Mostly all the contaminant could not assimilate D-cellobiose.

Classical work on the circumscription and identification of *Rhizopus* species has primarily been based on morphological criteria (Inui et al., 1965). The identification by traditional methods, however, is not clear-cut as there are few character suites to use to classify taxa. This has resulted in an excessive number of species, with few character differences, having been described. There is a need for an alternative approach in the precise naming of fungal strains. Schipper and Stalpers



(1984) suggested that a useful approach to *Rhizopus* taxonomy was to classify species based on the combination of maximum growth temperature rates and sporangiospores characteristics. This approach is very effective for initial species identification. It enables the division of the genus *Rhizopus* into several large groups. The followings are the characteristics adopted by most scientist: branching pattern and abundance of rhizoid, length, width and straightness of sporangiophores, diameter and color of sporangia, size and striation of sporangiospores, size and color of zygosporangia and equality of the two suspensor also taken into consideration. Those criteria well observed also in this study. With detail morphological, growth and physiological criteria, we successfully identified tempeh fungi into four main groups, but we failed to identify *R. microsporus*-group into variety level. Therefore RAPD and DNA sequencing approach were then used.

The RAPD patterns of 471 *Rhizopus* isolates obtained with the selected primer. Forty two distinct RAPD patterns were obtained and clustered. A dendrogram was generated by using UPGMA (unweighted pair-group method using arithmetic averages) linkage clustering of the Jaccard coefficients. In general, *Rhizopus* isolates form several clusters with a medium level of genetic variability. Dissimilarity values higher than 0.6 were detected between these six groups, indicating species-level differences. These results indicate a genetic heterogeneity within the species *Rhizopus*. Cluster analysis of the banding patterns revealed six different clustered groups. Cluster 1, 2 and 3 were *Rhizopus* isolates dominantly from Java, whereas cluster 4, 5 and 6 were dominantly from outside Java. This means *Rhizopus* isolates used for tempeh fermentation in Java and outside Java are different from genetic perspective. A combination of clustering and morphological data showed that cluster 1, 2 and 3 were isolates from *Rhizopus microsporus*. This species has three main varieties, they are var. *microsporus*, var. *oligosporus* and var. *chinensis*. High possibility that those 3 cluster are representative from the variety. Based on morphological data, cluster 4, 5 and 6 are possibly as representative of *R. delemar*, *R. oryzae*, and *R. stolonifer* isolates, respectively. The difference in the RAPD patterns reveals also a high, practically interspecific distance between the clusters of the *R. stolonifer* with another cluster of *Rhizopus* species. This suggests that *R. stolonifer* is most different compared with other *Rhizopus* species from Indonesian tempeh. This supported also with morphological data which reveal that *R. stolonifer* has most distinct features than the other *Rhizopus* species, for example complex-branched rhizoid, big sporangiospores, black sporangia in mature and longest sporangiophores.

RAPD typing was capable of producing discriminating DNA fingerprints of *Rhizopus* isolates and indicated there was genetic differences among them. Many distinct patterns were observed indicating that tempeh and inocula from different location in Indonesia composed of a quite wide number of strains. Genetic diversity was measured as the Shannon-Wiener H' index and the average HS index. HS was estimated to be 0.23 and Shannon's information index was maximum, 1.5. Our result suggest existence of significant genetic variation among these *Rhizopus* isolates. These result were in line with several previous works related to mucoralean diversity. RAPD analysis has proved to be an effective method for the revelation of genetic variations in various species of mucorales (Williams et al., 1990). The investigation of seven strains of the

facultative mycoparasite *Parasitella parasitica* by means of RAPD, restriction fragment length polymorphism RFLP analysis and electrophoretic karyotyping showed that they are highly diverse, creating two intraspecific groups (Burmester and Wöstemeyer, 1994). RAPD markers correlating with the mating competency have been described in the post-harvest pathogens *Mucor piriformis* (Papp et al., 1997) and *Gilbertella persicaria* (Papp et al., 2001). RAPD analysis was used successfully to characterize the genetic variability in the mainly heterothallic *Rhizomucor pusillus* and the homothallic *Rhizomucor miehei*: the latter revealed substantially lower intraspecific polymorphism (Vastag et al., 2000).

DNA sequences of genes such as ACT have been widely used for supporting morphological traits of mucoralean species (Voigt and Wöstemeyer, 2001; Abe et al., 2010; Dolatabadi et al., 2014). The results of sequence alignment by BLAST and phylogenetic analysis have proven that ACT sequencing is suitable for accurate identification of tempeh fungal isolates. All the phylogenetic trees with high bootstrap supports have revealed that the isolates are successfully identified at species level and further differentiated as particular strains within the same species. The UPGMA dendrogram clearly clustered distinct clades regarding to *Rhizopus* spp. studied. The dendrogram showed that *Rhizopus* spp. from Indonesian tempeh and inocula were placed into *R. stolonifer* clade (4 strains), *R. delemar* clade (7 strains), *R. oryzae* clade (8 strains), *R. microsporus* var. *microsporus* clade (10 strains), *R. microsporus* var. *oligosporus* clade (8 isolates) and *R. microsporus* var. *chinensis* clade (4 isolates). The *R. delemar* clade was sister to *R. oryzae* clade with 99% BS. The *R. microsporus* var. *chinensis* clade was sister to *R. microsporus* var. *microsporus*, and *R. microsporus* var. *oligosporus* clade with 100% BS. The data are also in line with morphological and physiological characters which previously described. The result clearly showed that the resolution of sequence from ACT region was sufficient in determining *Rhizopus* species into variety level, particularly in *R. microsporus* complex, dividing into var. *chinensis*, var. *microsporus* (often not written in dendrogram) and var. *oligosporus*, whereas morphological and physiological data only recognize until species level (*R. microsporus*). This prove that the DNA sequencing with ACT gene gave better taxonomy information until level of variety than morphological approach in this genus. ACT is known as a housekeeping gene, as its expression is relatively stable under variable conditions. ACT encodes actin, a protein that is abundant in all eukaryotic cells, in which it is the major component of cytoplasmic microfilaments. The ACT gene has been used extensively to infer interspecies relationships across broad evolutionary distances, as the ACT gene of many species contains highly conserved (i.e. exon) and less well-conserved sequences, which are grouped as nuclear pre-mRNA introns (Donnelly et al., 1999).

Combination of origin or geographical data and molecular identification showed that isolates (identified as *Rhizopus oryzae*, *R. delemar*, and *R. stolonifer*) dominantly used by tempeh producer from outside Java, whereas isolates (*Rhizopus microsporus* complex isolates, identified as *R. microsporus* var. *chinensis*, *R. microsporus* var. *microsporus* and *R. microsporus* var. *oligosporus*) were mostly used by tempeh producer from Java. Indeed, long time ago many members of *Rhizopus* and *Mucor* genera were previously reported in Java from inocula of tempeh

(ragi) and fresh tempeh (Dwidjoseputro and Frederick, 1970; Saono et al., 1974). However, to verify whether some species (*R. sexualis*, *R. arrhizus*, *R. microsporus* var. *azygosporus* etc.) have lost from tempeh in Indonesia, it is necessary to examine more samples in more other regions of Indonesia. This finding indicates that commercialization of tempeh inocula by using certain *Rhizopus* species in Java possibly threatens the genetic diversity of *Rhizopus* associated with this traditionally soybean-based fermented foods. The impact of heavy commercialization of tempeh inocula using particular species of *Rhizopus* has shifted the diversity of *Rhizopus* species associated with tempeh in Java. Interestingly, in our result, we also found many different strains of some variety of *R. microsporus*. This is probably because many producers in Java also develop their starter strains which composed of those variety. Therefore genetic variation of Java's isolates expressed intraspecies (*R. microsporus*). In contrast, commercialization of tempeh inocula is limited in outside Java. Therefore, many species of *Rhizopus* still could be found, and genetic variation of non-Java's isolates expressed in interspecies (*R. stolonifer*, *R. delemar*, *R. oryzae* and *R. microsporus* complex) level. Differences in the use of fungi species by producer in outside Java have more significant impact on various (heterogenous) nutritional and sensory quality of the tempeh than in Java's tempeh at least, various in the texture, aroma and the color of tempeh.

### 3.3. Results of Part II (Safety status of tempeh fungi strains)

Food safety is a prerequisite for a food product. There is no sense in talking about quality, nutritional value, sensory or functional properties if the food product is not safe for consumption. There is two possible hazards from *Rhizopus* species, first related to their role as mucormycosis agent and second as toxication agent. In this study, we tried to answer about current safety status of tempeh fungi from Indonesia. Tempeh fungi isolates were tested for their virulence potency and also checked for their possibility role as host for bacteria producing rhizoxin or rhizonin.

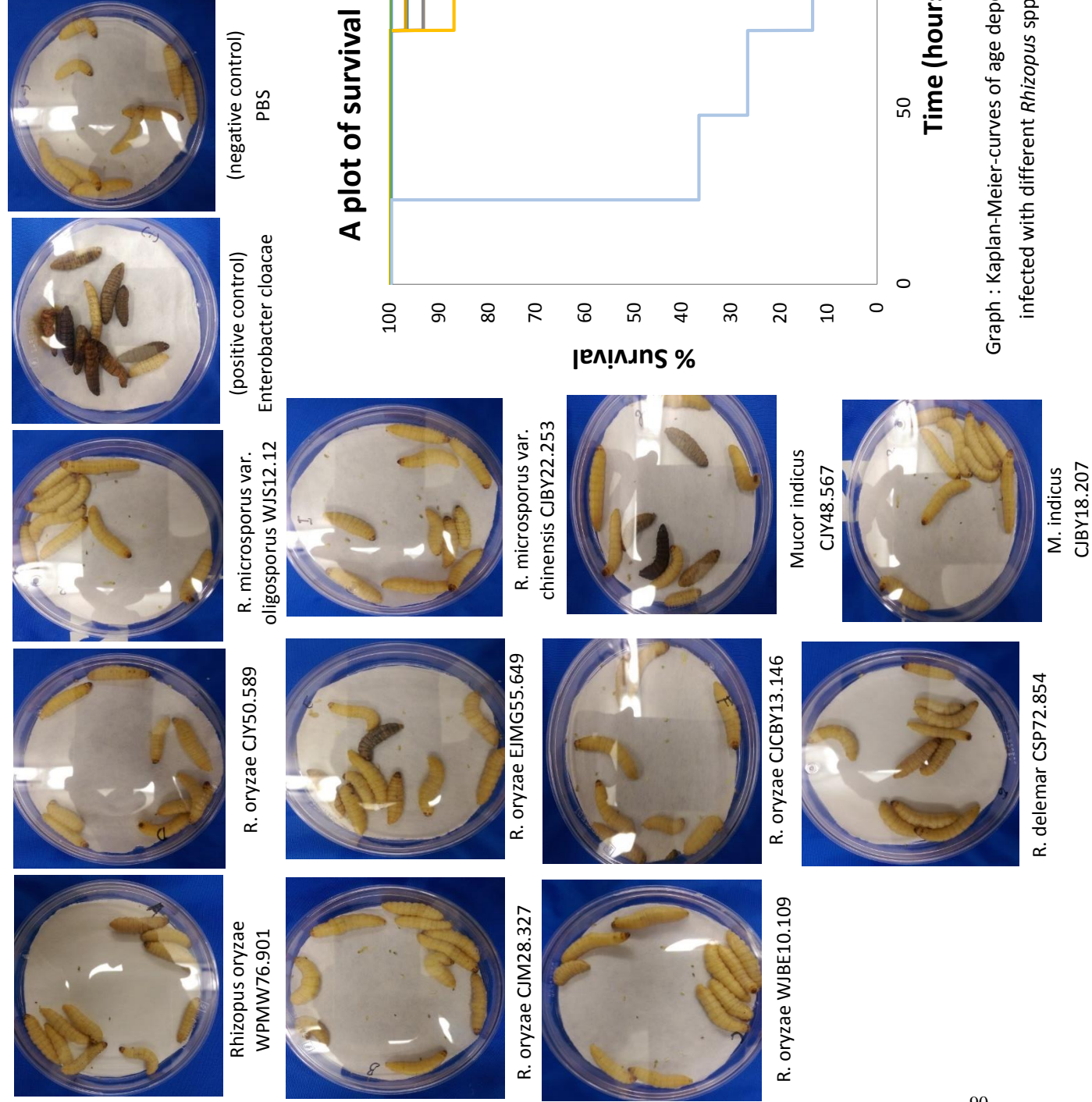
#### Virulence test

Tempeh is important traditional Indonesian fermented food made from soybeans employing *Rhizopus species*. However, *Rhizopus species* also known as opportunistic pathogens causing mucormycosis in human. *Rhizopus oryzae* (*arrhizus*) and *R. microsporus* predominantly described as potential agents of severe mucormycosis (Ribes et al., 2000; Roden et al., 2005). In this study, the virulence potential of the different *Rhizopus* species from tempeh was determined in an invertebrate model host, *Galleria mellonella*. This organism has advantages as it is economical, ethically expedient and easy to handle. For assessment of viability, larvae should be gently probed with a needle, and if no response is observed, the larvae may be considered to be dead. Changes in cuticle melanisation can also be used to monitor the severity of an infection. Significance of mortality rates was evaluated by using Kaplan-Meier survival curves. Mortality rates at four days were found as 100% for the larvae infected with *E. cloacae* (positive control), 0-20% for the larvae infected with *Rhizopus* strains (Figure 9). As a result of this study, potential evidence provided that tempeh fungi isolates are not virulent for *G. mellonella* larvae model.

To distinguish between mucormycosis and non- mucormycosis agent, some more test must be tried. They are thermotolerance, sporulation level, and genetic comparison test. Thermotolerant character, is a prerequisite for colonization of warmblooded hosts by opportunistic agent. Thermotolerance of Mucorales has classically been considered as a prime virulence factor in human infection (Scholer, 1970). Most of mycormycosis agent proved to be thermotolerant and only produce high sporulation at high temperature (50-55°C). Mucormycosis agent has low ability to produce spores at moderate temperature (30°C). For measuring thermotolerance, MEA plates were inoculated with small blocks taken from the edge of 3 days-old colonies. Plates were incubated at the following temperatures: 30°C, 37°C, 40°C, 45°C, 50°C, 52°C, and 55°C. Diameters were measured twice a day for three days. The growth rate, measured in millimeters per hour, was calculated for each strain and each temperature.

We have checked tempeh fungi isolates with mentioned test above. The results can be seen in Table 5 and Figure 10. None of the tempeh fungi isolates has thermotolerant character. All of them can not grow in temperature over 40°C. Five distinguishable levels of sporulation were checked among strains. The results showed that all tempeh fungi strains have high sporulation character in moderate temperature setting. The isolates have significant higher sporulation level than mucormycosis agent. The results indicated that tempeh isolates are not mucormycosis agent.

Figure 9. Virulence test result on some tempeh fungi isolates.



Graph : Kaplan-Meier-curves of age dependent survival of *Galleria mellonella* larvae infected with different *Rhizopus* spp. and *Mucor* spp. isolated from tempeh.

Those result also supported by genetic comparison data. The clustering dendrogram showed two separate clusters representing tempeh fungi isolates from tempeh or inocula and the medical isolates. The dendrogram showed unambiguously that tempeh isolates were genetically different from medical isolates (Figure 10). That figure showed that medical isolates were clearly separated in a different group. Our results suggested that tempeh fungi isolates could be a distinctive nonpathogenic group of this species. This is also in line with the facts that tempeh production and consumption have been practiced in Indonesia for centuries and, to our knowledge, there is no single report on infectious disease associated with tempeh.

Table 5. Comparison of physiology character between some isolated zygomycetes from tempeh (present study) with mucormycosis agents studied by Dolatabadi et al. (2014).

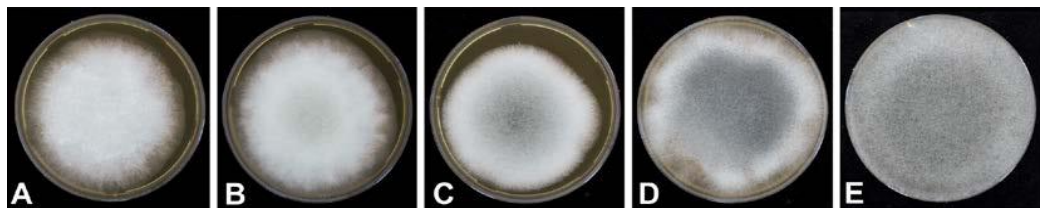
Obsolete name	Geography	Source	Thermotolerance	Optimal growth (°C)	Sporulation
<i>Rhizopus oryzae</i>					
Mucormycosis agent*					
CBS 109939	Ontario	Dermal lesion of black	+	55	B
CBS 118614	Turkey	Palate	+	50	B
CBS 120591	France	Sinus	+	55	B
CBS 120592	France	Sinus	+	49	B
CBS 120594	France	Skin	+	55	B
CBS 120806	France	Rhino-cerebral	+	55	B
CBS 120809	France	Sputum	+	55	B
<b>Present study</b>					
MUAN74.882	Indonesia	Tempeh inoculum	-	30	E
WJB7.84	Indonesia	Tempeh inoculum	-	30	E
SKBJ68.808	Indonesia	Tempeh inoculum	-	30	E
JSJ6.62	Indonesia	Tempeh inoculum	-	30	D
CJG23.275	Indonesia	Tempeh inoculum	-	30	D
NSM4.37	Indonesia	Tempeh inoculum	-	30	D
WJBE10.109	Indonesia	Tempeh inoculum	-	30	E
WPMW76	Indonesia	Tempeh inoculum	-	30	D
<i>R. delemar</i>					
Mucormycosis agent*					
CBS 395.54	Georgia	Diabetic patient	+	55	B
CBS 120593	France	Lung	+	50	B
CBS 120807	France	Sputum	+	55	B
<b>Present study</b>					
AL3.29	Indonesia	Tempeh inoculum	-	33	E
CJBY50.559	Indonesia	Fresh tempeh	-	33	E
EJMG54.643	Indonesia	Tempeh inoculum	-	33	E

### Chapter 3: Determination of fungal diversity in Indonesian tempeh

CJM28.327	Indonesia	Fresh tempeh	-	33	E
AL2.13	Indonesia	Tempeh inoculum	-	33	E
CSP72.854	Indonesia	Fresh tempeh	-	33	E
CJC41.481	Indonesia	Tempeh inoculum	-	33	E
<i>R. microsporus</i>					
Mucormycosis agent*					
CBS 118987	France	Human	+	50	C
CBS 124669	Greece	Human, soft palate	+	55	B
CBS 359.92	Australia	Liver, premature infant	+	50	B
CBS 357.92	Australia	Human	+	55	B
CBS 308.87	Australia	Human, cutaneous necrosis	+	50	B
CBS 712.73	Netherland	Skin	+	55	C
CBS 102277	Unknown	Human rhinocerebral	+	55	B
<b>Present study</b>					
CJBY22.253	Indonesia	Fresh tempeh	-	33	E
CJBY14.168	Indonesia	Tempeh inoculum	-	33	E
CJBY16.192	Indonesia	Tempeh inoculum	-	33	E
CJG27.324	Indonesia	Tempeh inoculum	-	33	E
WJB7.81	Indonesia	Tempeh inoculum	-	33	E
Y52.616	Indonesia	Tempeh inoculum	-	33	E
CJBY15.170	Indonesia	Fresh tempeh	-	33	E
AL3.38	Indonesia	Fresh tempeh	-	33	E
EJMG55.69	Indonesia	Fresh tempeh	-	33	E
CJBY.13.146	Indonesia	Fresh tempeh	-	33	E
WJS11.121	Indonesia	Tempeh inoculum	-	33	E
WJS12.12	Indonesia	Fresh tempeh	-	33	E
CJM28.325	Indonesia	Fresh tempeh	-	33	E
WJS11.132	Indonesia	Fresh tempeh	-	33	E
CJBY19.223	Indonesia	Tempeh inoculum	-	33	E
CJK33.389	Indonesia	Tempeh inoculum	-	33	E
CJM29.344	Indonesia	Tempeh inoculum	-	33	E
CJK35.409	Indonesia	Fresh tempeh	-	33	E
Y49.577	Indonesia	Tempeh inoculum	-	33	E
Y52.613	Indonesia	Tempeh inoculum	-	33	E
CJG27.321	Indonesia	Tempeh inoculum	-	33	E

Note:

Sporulation criteria (Based on Dolatabadi et al. 2014): Level (A-E) of sporulation.



**Comparison between tempeh and clinical isolates by molecular similarity**

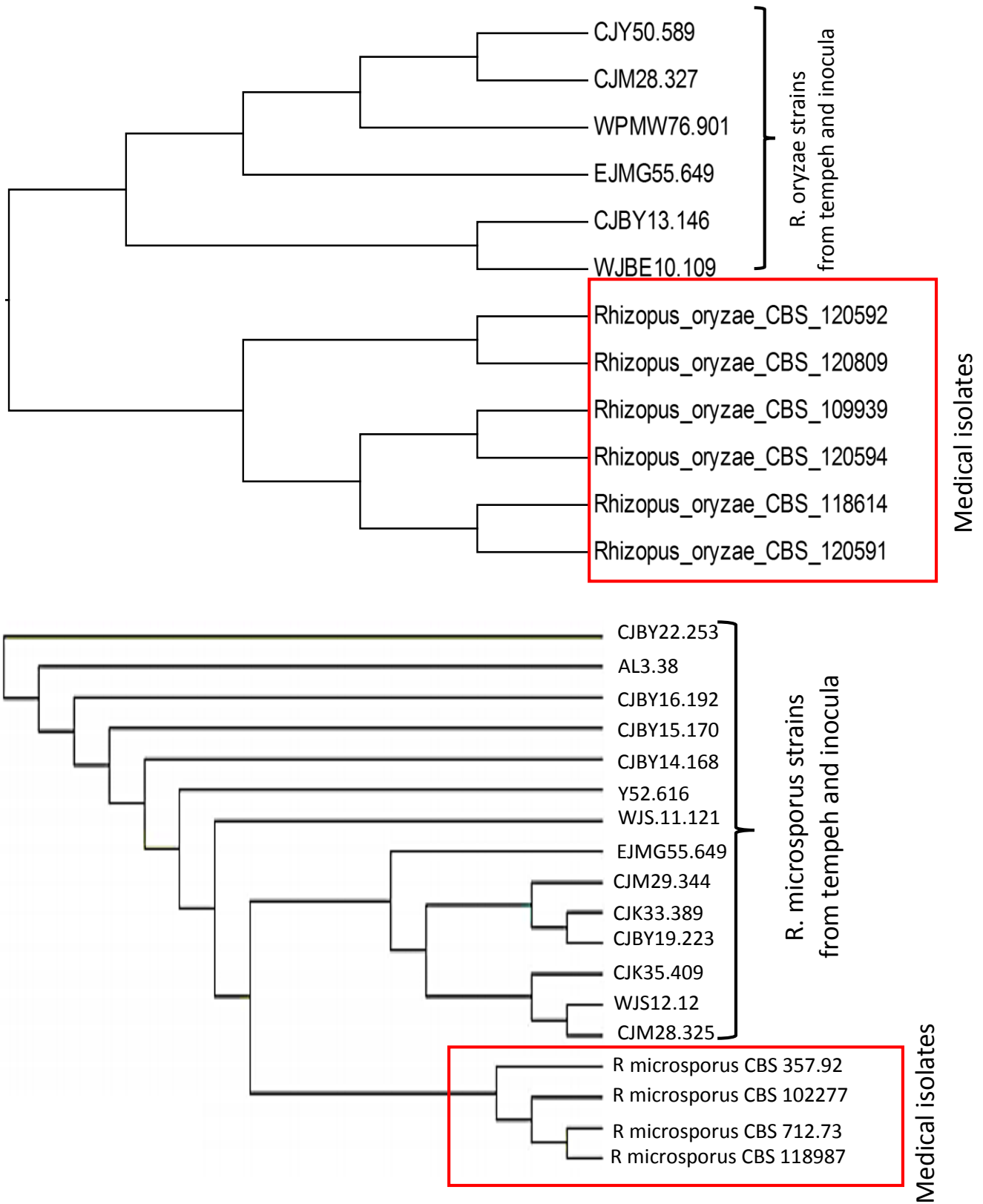


Figure 10. The tree was inferred from Actin sequence and constructed using maximum likelihood method. Numbers at nodes indicate levels of bootstrap support calculated from 1000 trees.



### Detection of bacteria producing rhizonin

Another threat to food safety associated with *Rhizopus* is posed by rhizoxin and rhizonin. None of these metabolites should be permitted to occur in foods, even at pico- or femtogram amounts, as they are toxic for human consumption. The compounds are however not produced by the fungus itself, but by symbiotic bacteria (*Burkholderia rhizoxinica*) that reside within the fungal cytosol (Lackner et al., 2009). *Burkholderia* is gram-negative, a rod-shaped endosymbiotic bacterium (Partida Martinez et al., 2007).

In this study, we used mechanical stress (pipetting) and physical stress (freezing-thawing) to break fungal mycelia and isolate any possible symbiotic bacteria. Samples were checked with plating in NA medium and checked with light microscopy. Only sample had bacterial colonies were then extracted its DNA and followed with amplification partial sequence 16S rRNA genes using the bacterial universal primers 27F (5'-AGAGTTTG ATCMTGGCTCAG) (Lane, 1991) and bacterial primer 1100R (5'-AGGGTTGCGCTCGTTG) (Turner et al., 1999). The identification result was shown in Table 7 and Figure 11. The physiological and biochemical characterization of the bacterium (including Gram staining, starch hydrolysis, Sulfide-Indole-Motility, nutrient gelatin, citrate, urease and catalase tests, growth on different media) are also tested and summarized in Table 6. All bacteria detected as gram-negative, their cell were rod-shaped (Length:  $3.21 \pm 0.40 \mu\text{m}$  and Width:  $0.55 \pm 0.12 \mu\text{m}$ ), arranged singly or in pairs.

Table 6. Biochemical characteristics of isolated bacteria from tempeh fungi.

Biochemical tests	BMSR12, BASC02	BKSM1	BMSR10, BBSC01
Gram staining	-	-	-
Starch hydrolysis	+	+	+
Sulfide	-	-	+
Indole	-	-	-
Motility	-	-	-
Nutrient gelatin stab	-	-	-
Citrate	+	-	+
Urease	-	-	-
Catalase 3%	+	+	+
Growth on			
6.5% NaCl	+	+	+
Nutrient broth	+	+	+
Starch agar	+	+	+
Pectine agar	+	+	+
Casein agar	+	+	+

Table 7. Isolation of bacteria from tempeh fungi (zygomycetes strains)

No	Fungal species	Amount of tested strains	Postive fungal containing bacteria	Result of 16S rRNA sequence	Additional Information
1	<i>Rhizopus oryzae</i>	30	1 (3.3%)	<i>Enterobacter cloacae</i>	Non-rhizonin producing bacteria
2	<i>R. microsporus</i>	30	1 (3.3%)	<i>E cloacae</i> , and <i>C. testosteroni</i>	Non-rhizonin producing bacteria
3	<i>R. delemar</i>	30	1 (3.3%)	<i>Ochrobacterum intermedium</i>	Non-rhizonin producing bacteria
4	<i>R. stolonifer</i>	10	-	-	Not found any bacteria
5	<i>Mucor indicus</i>	5	-	-	Not found any bacteria

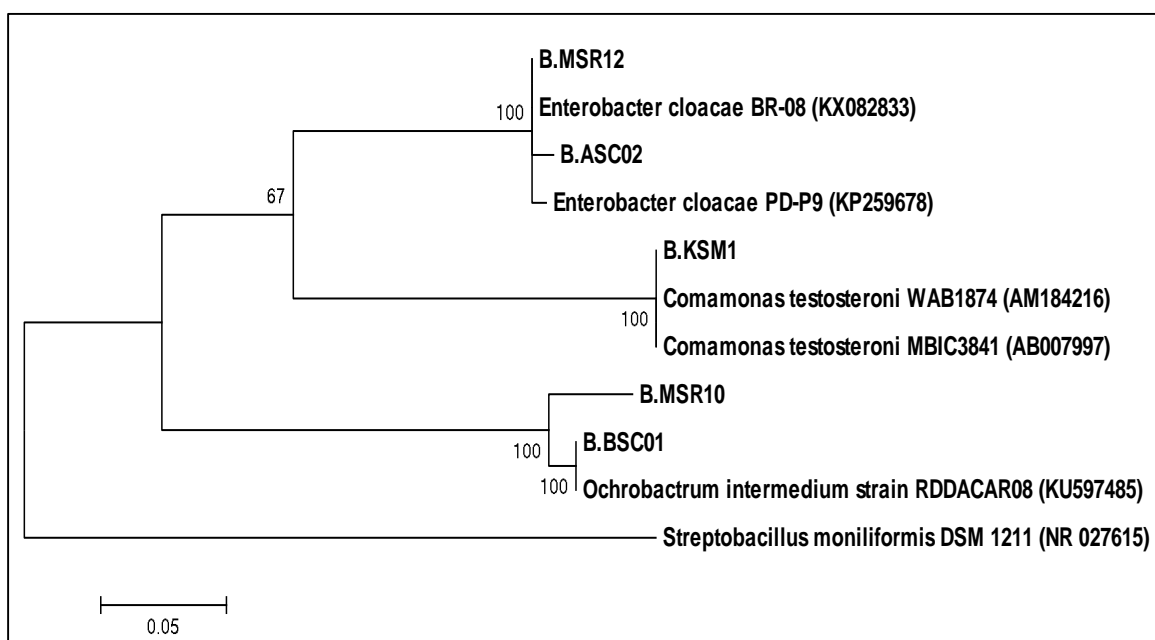


Figure 11. The tree was inferred from 16s RNA sequence and constructed using maximum likelihood method. Numbers at nodes indicate levels of bootstrap support calculated from 1000 trees.

The phylogenetic tree of the isolated bacteria is shown in Figure 11. Bacterial strains (BMSR12, BASC02), revealed 100% identity with *Enterobacter cloacae* strain BR-08 and strain PD- P9. Bacterial strain (BKSM1), revealed 100% identity with *Comamonas testosteroni* strain WA B1874 and MBIC3841. Bacterial strains (BMS10, BBSC01) showed 100% identity with *Ochrobactrum intermedium* strain RDDACAR08. In this study, we could not find any *Burkholderia* species. Most of the tempeh fungi isolates are clean from any contamination of bacteria. Only three fungal samples were contaminated, those samples were isolated from fresh tempeh where produced at non-hygienic workshop in Java. Those bacteria known do not produce any harmful toxin.

### 3.4. Discussion of Part II (Safety status of tempeh fungi strains)

In recent years the clinical range of the infections caused by zygomycetes has changed dramatically, because of the increase in the number of the immunocompromised patients (Meis and Chakrabarti, 2009). Most of the clinically important zygomycetes are in the order Mucorales and comprise approximately 60 genera. The genus *Rhizopus* exhibits the largest impact on mankind, being important in agriculture and industry and is furthermore the main causing agent of mucormycosis, followed by *Lichtheimia* and *Mucor*. The three genera are responsible for 70 to 80 % of the reported infections, predominantly as rhinocerebral, pulmonary or disseminated manifestations, and associated with high mortality rates (Skiada et al., 2011; Gomes et al., 2011). Roden et al. (2005) established that the most prevalent species were *Rhizopus oryzae* (nearly half of all isolates), followed by *R. microsporus*, *Mucor circinelloides*, *Absidia* (= *Lichtheimia*) *corymbifera*, *Rhizomucor pusillus*, *Cunninghamella bertholletiae*, *Mucor indicus*, *Cunninghamella echinulata*, and *Apophysomyces elegans*. Three recent retrospective studies carried out in the USA (Kontoyiannis et al., 2000, 2005; Sims et al., 2007) included a significant number of cases (24, 27 and 26, respectively). However, one of these (Sims et al., 2007) distinguished only between *Mucor* spp. and *Rhizopus* spp., and in the others two (Kontoyiannis et al., 2000), only the genera were mentioned (*Cunninghamella*, *Mucor*, *Rhizopus*, and *Syncephalastrum*).

The agents of mucormycosis are unable to penetrate through unbroken skin to produce cutaneous infection; however, burns, traumatic disruptions or macerations of the skin (Ledgard et al., 2008; Vega et al., 2006), insect bites or stings, (Lechevalier et al., 2008; Saravia-Flores et al., 2010), birds pecks (Wilson, 2008), traumatic inoculations (Andresen et al., 2005; Snell and Tavakoli, 2007), and any process of skin disruption can facilitate the implantation and colonization of tissues by the spores. Cutaneous infections can be very invasive, also affecting subcutaneous tissues and the adjacent fat, fascia, muscle, and finally bone. Besides, the fungus can affect deep solid organs by hematogenous dissemination. Gastrointestinal mucormycosis is very uncommon. The ingestion of food or fermented drinks contaminated with spores may play a role in promoting gastric infections and, although it is rare in humans, it can significantly affect animals, i.e. amphibians, bovines and rodents (Creepers et al., 1998; Jensen et al., 1994; Speare et al., 1997).

*Rhizopus* species also are among the mucoralean fungi that are most often involved in the production of Asian fermented foods, both as a natural invader of soybean-based condiments (Hong et al., 2012) and as starter culture (Nout and Rombouts, 1990). This study tried to answer current safety status of tempeh fungi from Indonesia. Tempeh fungi isolates were tested for their virulence potency and also checked for their possibility role as host for bacteria producing rhizoxin or rhizonin. Although nearly all patients with mucormycosis have severe underlying metabolic or innate immune disorders, this contrasting behavior and usage of *Rhizopus* species necessitate the development of methods to determine the intrinsic virulence of individual strains and comparative virulence between species. We used *G. melonella* as infection model. Larvae are bred commercially as live food for captive reptiles and amphibians. The larvae are the caterpillar stage

of the moth, 1–2 cm in length and survive as supplied for up to 3 weeks before pupating. During this time they do not need feeding and require minimal maintenance. Infection is accompanied by the generation of melanin, which is available in the haemocoel and becomes deposited in tissues (Nappi and Christensen, 2005). Infected *G. mellonella* larvae change from their normal cream color to pale or dark brown.

Summary of overall test was shown in Table 8. Our result showed that *Rhizopus* isolates from tempeh did not display a virulence potential. Mortality rates at four days were found very low in the range 0-20% at *G. mellonella* model. Many reports explained that mucormycosis agent from *Rhizopus* genera causes high mortality rate in range 80-100% (Ribes et al., 2000; Dolatabadi et al., 2014), but this behavior not found in any tempeh fungal isolates. Tempeh fungal isolates are also non-thermotolerant and genetically distinct from medical isolates. They have high sporulation character in moderate temperature setting. The isolates have significant higher sporulation level than mucormycosis agent. These result indicated that tempeh fungal isolates are not mucormycosis agent. This is also in line with the facts that tempeh production and consumption have been practiced in Indonesia for centuries and, to our knowledge, there is no single report on mucormycosis case associated with tempeh.

Ribes et al. (2000) mentioned that from the mesophilic species of the genus *Rhizopus*, only *R. stolonifer* can be found in clinical settings, but is rarely seen; mostly as agents of allergic alveolitis or superficial infections but being predominantly non-invasive. Only thermotolerant species of *R. arrhizus* and *R. microsporus* are reported more frequently in severe infections than any other species from the Rhizopodaceae (Wimander et al. 1980, Scholer et al. 1983).

Table 8. Summary of safety status from tempeh fungi strains (zygomycetes isolates).

No	Isolates	Amount	Classified as thermotolerance strains*	Virulence test*	Rhizonin producing bacteria	Status (food safety perspective)
1	<i>Rhizopus oryzae</i>	30	None	Negative	Not found	Safe
2	<i>R. microsporus</i>	30	None	Negative	Not found	Safe
3	<i>R. delemar</i>	30	None	Negative	Not found	Safe
4	<i>R. stolonifer</i>	10	None	Negative	Not found	Safe
5	<i>Mucor indicus</i>	5	None	Negative	Not found	Safe

Note:

\*Thermotolerance and virulence test used to check any potency of mucormycosis.

Another threat to food safety associated with *Rhizopus* is posed by rhizoxin and rhizonin. None of these metabolites should be permitted to occur in foods, even at pico- or femtogram amounts, as they are toxic for human consumption. The compounds are however not produced by the fungus itself, but by symbiotic bacteria (*Burkholderia rhizoxinica*) that reside within the fungal cytosol (Lackner et al., 2009). *Burkholderia* is a gram-negative, rod-shaped endosymbiotic

bacterium (Partida Martinez et al., 2007). In this study, we used mechanical stress (pipetting) and physical stress (freezing-thawing) to break fungal mycelia and isolate any possible symbiotic bacteria from tempeh fungi isolates. Samples were checked with plating in NA medium and checked with light microscopy. Only sample had bacterial colonies were then extracted its DNA and followed with amplification partial sequence 16S rRNA genes using the bacterial universal primers 27F (5'-AGAGTTTG ATCMTGGCTCAG) (Lane, 1991) and bacterial primer 1100R (5'-AGGGTTGCGCTCGTTG) (Turner et al., 1999).

We could not find any *Burkholderia* species from Indonesian tempeh fungi isolates. Most of them are clean from any contamination of bacteria. Only three fungal samples were contaminated, those samples were isolated from fresh tempeh where produced at non-hygienic workshop. Those bacteria known do not produce any harmful toxin, but they are classified as human pathogen causing gastroenteritis. They were identified as *Enterobacter cloacae*, *Comamonas testosteron*, and *Ochrobactrum intermedium*. More attention should be given since there were several gastroenteritis outbreaks regarding unpasteurized tempeh consumption reported recently in Indonesia.

In conclusion, tempeh fungi isolates are safe for human consumption since they are not mucormycosis agent or toxication agent. Indeed, there is no doubt to consume soybean-tempeh anymore. Attention only needs to be given to some tempeh-soybean producer that applied unhygienic practice in the production because we found few sample contaminated with bacteria classified as a human pathogen causing gastroenteritis.

### 3.5. Results of Part III (Zearalenone biotransformation by tempeh fungi)

In this study, a total of 70 strains of fungal species from Part 1 that commonly found in tempeh fermentation were screened for Zearalenone (ZEN) metabolite formation *in vitro* (list of strains could be seen in supplemental Table 3). Nine different species were presented from the total strains; they are *Rhizopus oryzae*, *R. delemar*, *R. microsporus*, *R. microsporus* var. *oligosporus*, *R. microsporus* var. *chinensis*, *Syncephalastrum racemosum*, *Aspergillus oryzae*, *Neurospora sitophila*, and *Neurospora crassa*. The fungal strains were isolated from inocula or fresh tempeh from different tempeh producers and market areas in Indonesia.

ZEN (c = 0.3  $\mu$ M) was added to liquid cultures of the fungi. After two days of incubation, the media were analyzed for metabolite formation by HPLC-MS/MS. Semi-quantitative measurements were conducted for ZEN-14-S,  $\alpha$ -ZEL-S, ZEN-14-Glc, and ZEN-16-Glc. ZEN-14-S and  $\alpha$ -ZEL-S were determined using relative response factors of ZEN-14-S to ZEN and  $\alpha$ -ZEL-S to  $\alpha$ -ZEL. Response factors were estimated by comparing the MS/MS peak area before and after quantitative sulfate ester cleavage. ZEN and  $\alpha$ -ZEL were determined by external calibration using the commercially available standard substances. Matrix-matched calibration was applied for semi-quantification of ZEN-14-Glc and ZEN-16-Glc. Detail semi-quantification data can be seen in Supplemental Table 4.

The conjugate formation varied with the fungal strain. Interestingly, we found four main groups of fungi based on their ZEN metabolization type (Table 9). Their extracted ion chromatogram could be seen in Figure 13. Most of the samples belong to group 1 (36%), followed by group 3 (27%), group 4 (24%), and group 2 (13%). In group 1, strong decrease of ZEN, no (or only very small) formation of ZEN metabolites were observed. Unknown transformation products of ZEN may be formed because some fungal isolates rapidly reduced ZEN content without producing known metabolites. Seven different species from two genera (*Rhizopus* and *Neurospora*) gave contribution in this group. Reduction of ZEN was in range 69 to 96%. In group 2, strong decrease of ZEN (95-100%) and diverse formation of ZEN metabolites were found. Only species from *Rhizopus* genera belong to this group. In group 3, strong decrease of ZEN were detected (86-100% reduction), ZEN-14-sulfate is the only formed metabolite (others in very small amounts). *R. microsporus* var. *chinensis*, *Syncephalastrum racemosum*, and *Aspergillus oryzae* strains contributed to this group. Five different species from two genera (*Rhizopus* and *Syncephalastrum*) gave contribution in this group. This result also showed that *Rhizopus* strains have ability to produce diverse ZEN transformation. This genera is more active on ZEN transformation compared to other genera tested in this study. Combination with geographical data revealed that all groups can be found in west area of Indonesia, and only group 1 can be found in central and east area of Indonesia as shown in Figure 12.

In most cultures ZEN-14-sulfate was the major metabolite accompanied (64% of total samples) by rapid disappearance of ZEN; also, formation of  $\alpha$ - and  $\beta$ -ZEL, ZEN-16-glucoside and two ZEL-sulfates has been observed. The *in vitro*-screening showed that fungal strains used in tempeh fermentation have the potential for ZEN conversion leading to ZEN and ZEL conjugate formation.

Table 9. Categorization of tempeh fungi isolates based on ZEN metabolism types.

No	Characteristics	Type of transformed ZEN	Number of strains/total strains (%) (n=70)	Remaining ZEN	Fungal species
1	<b>GROUP 1:</b> Strong decrease of ZEN, no (or only very small) formation of ZEN metabolites	-	25/70 (36%)	4-31%	RO, RD, RMC, RMO, RM, NC, NS
2	<b>GROUP 2:</b> Strong decrease of ZEN, diverse formation of ZEN metabolites	ZEN14-S, ZEN-16-Glc, $\alpha$ - and $\beta$ -ZEL, two ZEL-S	9/70 (13%)	0-5%	RO, RM, RMO, RMC
3	<b>GROUP 3:</b> Strong decrease of ZEN, ZEN-14-sulfate is the only formed metabolite (others in very small amounts)	ZEN-14-S	19/70 (27%)	0-14%	RMC, SR, AO,
4	<b>GROUP 4:</b> Strong decrease of ZEN, sulfate conjugates of ZEN and ZEL are the major metabolites	ZEN-14-S, two ZEL-S	17/70 (24%)	0-21%	RMO, RMC, RD, RO, SR

Note: abbreviation= RO (*R. oryzae*), RD (*R. delemar*), RMC (*R. microsporus* var. *chinensis*), RMO (*R. microsporus* var. *oligosporus*), RM (*R. microsporus*), NC (*Neurospora crassa*), NS (*N. sitophila*), SR (*Syncephalastrum racemosum*), AO (*Aspergillus oryzae*)

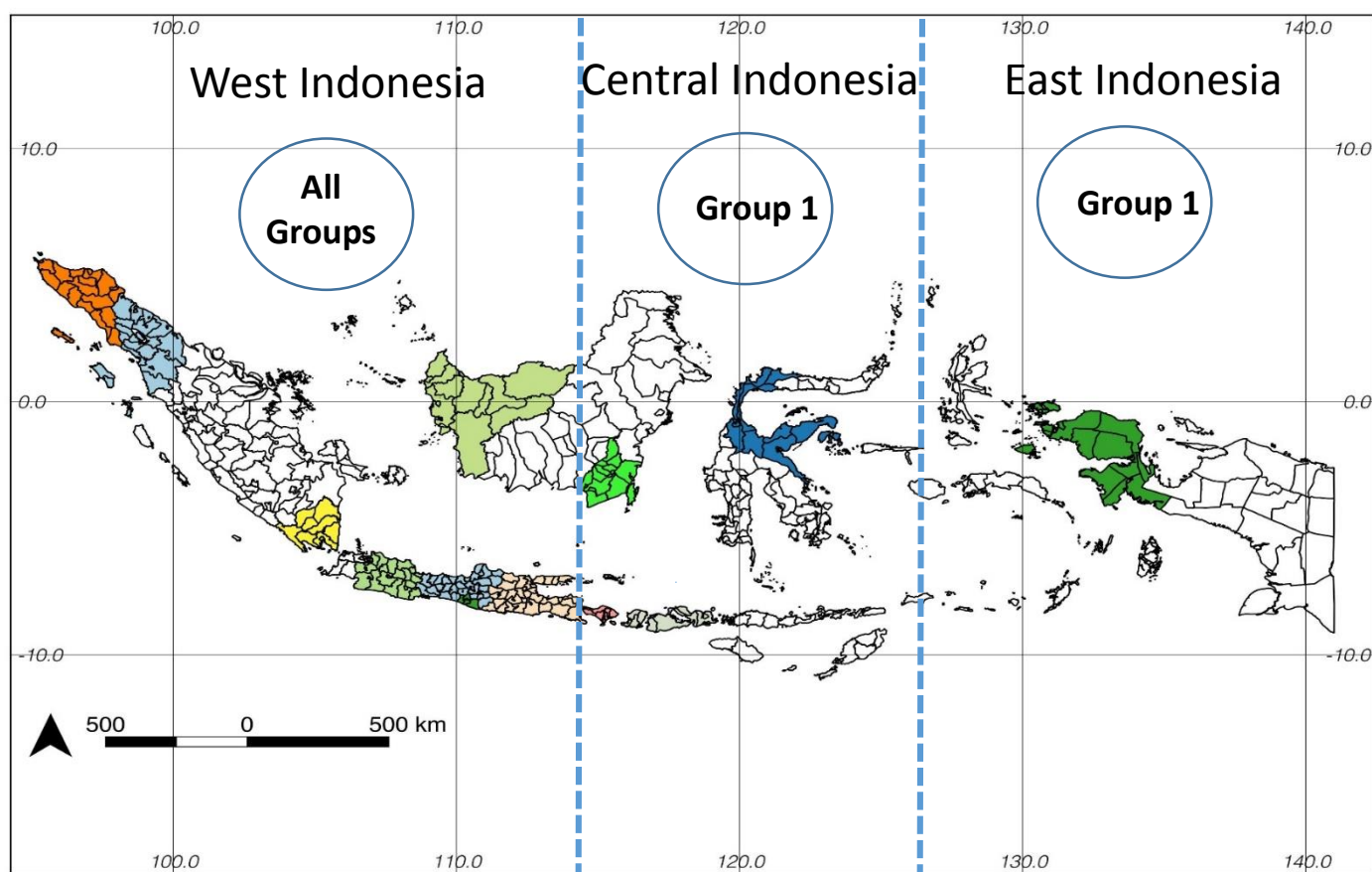
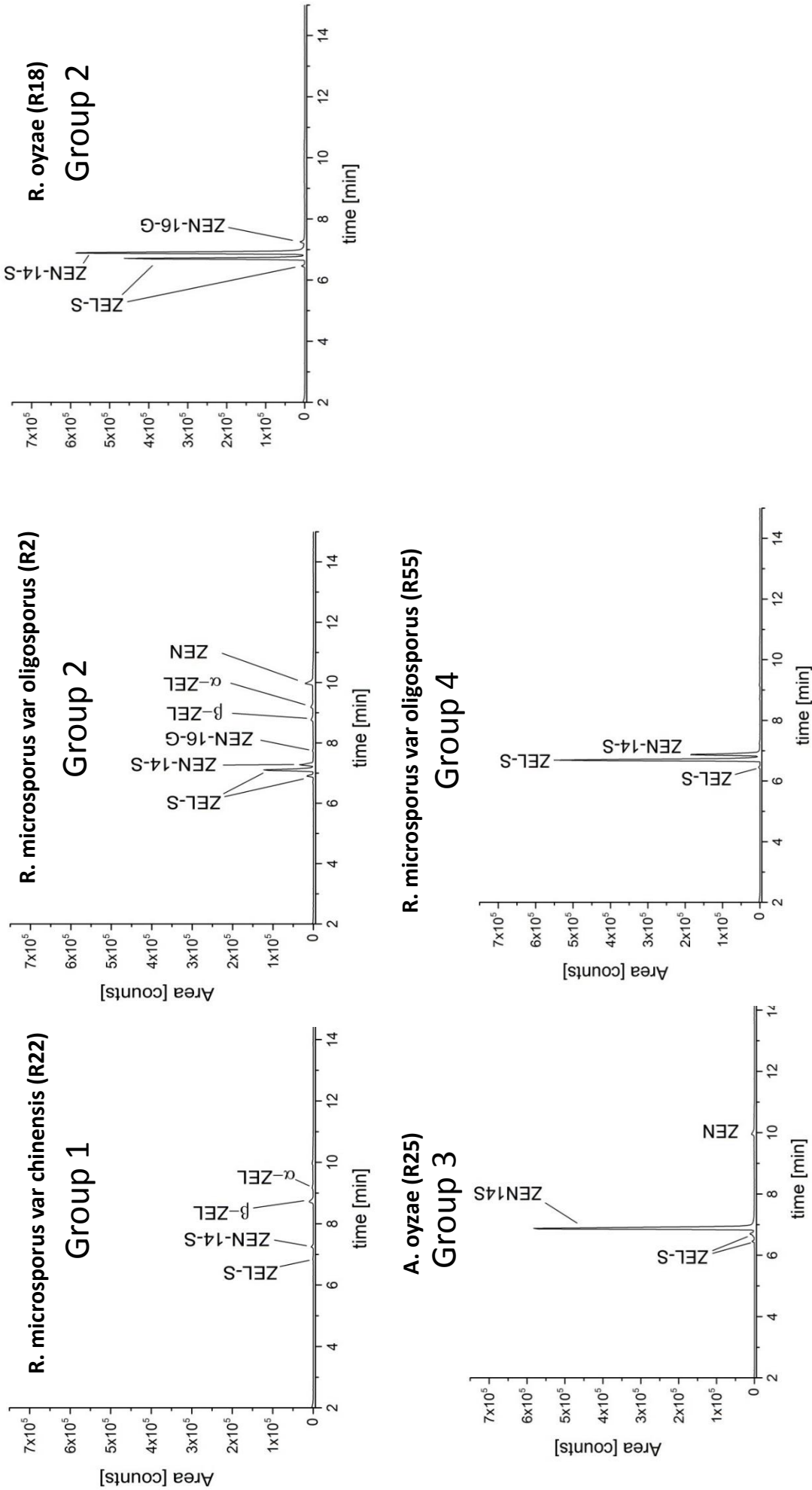


Figure 12. Distribution of tempeh fungi based on ZEN transformation ability

Figure 13. HPLC-MS/MS chromatogram based on ZEN transformation types.





### 3.6. Discussion of Part III (ZEN biotransformation by tempeh fungi isolates)

The term “masked mycotoxin” was used for the first time in 1990, referring to a glycosidic derivative of zearalenone found in corn and cleaved to its parent compound in the gastrointestinal tract. Recently, masked mycotoxins defined as plant or fungal metabolites of mycotoxins that contaminate food and feed (Dellafiora et al., 2017). Gareis et al. (1990) referred to a zearalenone-glycoside as a masked mycotoxin to emphasize the fact that it was not detected by routine analysis of food because it has different chemical behaviors than the origin but possibly contributed to total mycotoxin and subsequent effect. This masked mycotoxin can be hydrolyzed to their precursors in the animal digestive tract, thus showing similar toxicity than free mycotoxins (Gareis et al. 1990; De Saeger and van Egmond, 2012; Gratz et al., 2016). This mycotoxin conjugate could be generated by infected host plant (Engelhardt et al., 1988; Kovalsky et al., 2014) and some fungal species such as *Fusarium graminearum*, *Rhizopus arrhizus*, *Aspergillus niger*, *Rhizopus spp.* and *A. oryzae* (El-Sharkawy et al., 1991; Plasencia and Mirocha, 1991; Berthiller et al. 2005; Jard et al., 2010; Brodehl et al., 2014) by transforming ZEN into different conjugated form. A variety of microorganisms like *Penicillium sp.*, *Rhodoturula sp.*, *Arthrobacter sp.*, *Saccharomyces sp.*, *Candida sp.* were also reported to be able to transform ZEA to  $\alpha$ -zearalenol and  $\beta$ -zearalenol (McMullen, 1977). In infected plant, ZEN is transformed into more polar derivatives by conjugation with sugars, amino acids, or sulfate groups, which are then isolated into the vacuoles (Gratz, 2017).

Due to the fact that conjugated ZEN derivatives can be efficiently hydrolyzed into their parent compound by human colonic microbiota (Dall’Erta et al., 2013; Kovalsky Paris et al., 2014), the exposure of ZEN might be underestimated. Because of its estrogenic effects that may lead to severe hormonal disorder and infertility, a tolerable daily intake (TDI) for ZEN of  $0.25 \mu\text{g kg}^{-1}$  body weight has been established (Shier et al., 2001; EFSA, 2011). Besides conjugated ZEN products, reductive metabolites are formed in plant and fungal metabolism, for example,  $\alpha$ - and  $\beta$ -ZEL. Whereas  $\beta$ -ZEL is less toxic than ZEN,  $\alpha$ -ZEL possesses an about 10-fold higher estrogenicity than ZEN (Metzler et al., 2010). For further toxicological investigations of ZEN and ZEL conjugates, the availability of pure standard substances is required. Very recently, the EU-CONTAM Panel found it appropriate to set a group tolerable daily intake (TDI) expressed as ZEN equivalents for ZEN and its modified forms (phase I and phase II metabolites).

Zearalenone (ZEN) and its metabolites such as the phase II sulfate and glucoside conjugates have been detected in food and feed commodities (Berthiller et al., 2005; Vendl et al., 2010; De Boevre et al., 2012; Kovalsky et al., 2016). In addition to these naturally occurring ZEN conjugates, food fermentation by fungi may also lead to conjugate formation. Fungal strains of the genera *Rhizopus* and *Aspergillus* are used in tempeh fermentation, and it is known that some strains can conjugate ZEN. Moreover, during this process the reductive phase I metabolites  $\alpha$ - and  $\beta$ -zearalenol ( $\alpha$ -/ $\beta$ -ZEL) and their conjugates are formed by the same microorganisms *in vitro*. In present study, a total of 70 food-grade strains of fungal species (partly from Part I) that commonly found in tempeh fermentation were screened for Zearalenone (ZEN) metabolite formation *in vitro*

(list of strains could be seen in supplemental Table 3). Nine different species were presented from the total strains; they are *Rhizopus oryzae*, *R. delemar*, *R. microsporus*, *R. microsporus* var. *oligosporus*, *R. microsporus* var. *chinensis*, *Syncephalastrum racemosum*, *Aspergillus oryzae*, *Neurospora sitophila*, and *Neurospora crassa*. The fungal strains were isolated from inocula or fresh tempeh from different tempeh producers and market areas in Indonesia.

ZEN ( $c = 0.3 \mu\text{M}$ ) was added to liquid cultures of the fungi. After two days of incubation the media were analyzed for metabolite formation by HPLC-MS/MS. An assessment of the formed metabolite quantity is hampered by the lack of analytical standards of sufficient purity. To get an approximate idea of the quantitative dimensions of ZEN conversion, a semi-quantification of the formed ZEN metabolites was carried out. Semi-quantitative measurements were conducted for ZEN-14-S,  $\alpha$ -ZEL-S, ZEN-14-Glc, and ZEN-16-Glc. ZEN-14-S and  $\alpha$ -ZEL-S were determined using relative response factors of ZEN-14-S to ZEN and  $\alpha$ -ZEL-S to  $\alpha$ -ZEL. Response factors were estimated by comparing the MS/MS peak area before and after quantitative sulfate ester cleavage. The resulting response factors (i.e., peak area of conjugate divided by peak area of the free form) are 11 for ZEN-14-S compared to ZEN and 16 for  $\alpha$ -ZEL-S compared to  $\alpha$ -ZEL. These data are in accordance with previously published findings, stating that for ESI-MS analysis, ZEN-sulfate is detectable even at very low levels caused by its high ionization efficiency (Vendl et al., 2010). ZEN and  $\alpha$ -ZEL were determined by external calibration using the commercially available standard substances. Matrix-matched calibration was applied for semi-quantification of ZEN-14-Glc and ZEN-16-Glc.

The conjugate formation varied with the fungal strain. Interestingly, we found four main groups of fungi based on their ZEN metabolization type (Table 10). Their extracted ion chromatogram could be seen in Figure 13. Most of the samples belong to group 1 (36%), followed by group 3 (27%), group 4 (24%), and group 2 (13%). In group 1, strong decrease of ZEN, no (or only very small) formation of ZEN metabolites were observed. Unknown transformation products of ZEN may be formed, because some fungal isolates rapidly reduced ZEN content without producing known metabolites. Seven different species from two genera (*Rhizopus* and *Neurospora*) gave contribution in this group. Reduction of ZEN was in range 69 to 96%. In group 2, strong decrease of ZEN (95-100%) and diverse formation of ZEN metabolites were found. Only species from *Rhizopus* genera belong to this group. In group 3, strong decrease of ZEN was detected (86-100% reduction), ZEN-14-sulfate is the only formed metabolite (others in very small amounts). *R. microsporus* var. *chinensis*, *Syncephalastrum racemosum*, and *Aspergillus oryzae* strains contributed to this group. Five different species from two genera (*Rhizopus* and *Syncephalastrum*) gave contribution in this group. This result also showed that *Rhizopus* strains have ability to produce diverse ZEN transformation. This genera is more active in ZEN transformation compared to other genera tested in this study. The results are in accordance with Varga et al. (2005) who observed a decrease in ZEN concentration in liquid culture of numerous strains of the species *R. stolonifer*, *R. oryzae*, and *R. microsporus* (Varga et al., 2005). Combination with geographical data revealed that all groups can be found in west area of Indonesia, and only group 1 can be found in central and east part of Indonesia.

The glycosylation of ZEN could be observed from the strains especially in group 2. ZEN-14-Glc is known to be a natural metabolite formed by *Rhizopus* species (Kamimura, 1986). Also, the formation of ZEN-16-Glc, a substance discovered by Berthiller and coworkers (Kovalsky Paris et al., 2014). In most cultures ZEN-14-sulfate was the major metabolite accompanied (64% of total samples) by rapid disappearance of ZEN; also, formation of  $\alpha$ - and  $\beta$ -ZEL, ZEN-16-glucoside and two ZEL-sulfates has been observed. A recent study by Kovalsky Paris et al. (2014) has reported the conversion of ZEN into a mixture of ZEN-14-O-b-glucoside (ZEN-14-Glc) and ZEN-16-O-b-glucoside (ZEN-16-Glc) in barley. Studies on the metabolism of ZEN by *Rhizopus* and *Thamnidium* species have shown the formation of ZEN-14-Glc and ZEN-14,16-O-b-diglucoside (Kamimura, 1986; El-Sharkawy, 1989). ZEN-14-sulfate (ZEN-14-S) was found to be a natural metabolite of *Fusarium graminearum*, *Rhizopus arrhizus*, and *Aspergillus niger* (El-Sharkawy et al., 1991; Plasencia and Mirocha, 1991; Jard et al., 2010). To date,  $\alpha$ -ZEL-S has not been described as a microbial metabolite. As the cleavage of ZEN from its sulfate and glucose conjugates was shown recently (Dall'Erta et al., 2013; Kovalsky Paris et al., 2014). All these derivatives are not covered by most routine analysis due to the lack of the pure compounds. The development of reference standards is required to allow for the quantitative measurement of ZEN metabolites. We consider the exploitation of the metabolic capabilities of the fungi demonstrated in our study as an option for providing such standards.

In conclusion, fungal strains used in production of tempeh in Indonesia could convert ZEN to conjugated ZEN and ZEL derivatives *in vitro*. This study showed that the investigated fungal strains have a high potential for ZEN conjugation by metabolic processes. These transformations may also occur during fermentation of tempeh. As the conjugates can be hydrolyzed by human intestinal microbiota, also sulfates and glucosides represent a relevant source for the human exposure to ZEN. If the unprocessed grains for tempeh fermentation like mix soybeans-maize are contaminated with ZEN, upon analysis ZEN content of the final tempeh product may be underestimated, because ZEN has been conjugated during food processing. Thus, transformation products of ZEN in authentic tempeh should also be analyzed. Reference substances of ZEN conjugates are urgently needed for monitoring food safety of tempeh and other fermented products manufactured from grains that can be contaminated with ZEN. It must be taken into account that the estrogenic potency of ZEN derivatives differ. Potency factors assigned to these derivatives by EFSA CONTAM Panel are 0.2 for  $\beta$ -ZEL and  $\beta$ -ZEL-sulfate but 60 for  $\alpha$ -ZEL and  $\alpha$ -ZEL-sulfate.

#### 4. Conclusion

The present study revealed the following points:

- The moulds isolated from tempeh and inocula in this study were broadly represented by nine genera. In total, 557 fungi and five fungi-like isolates were obtained from 247 samples. *Rhizopus* was the most frequently detected genera, found in 84% of total samples, followed by *Mucor* (5.5%), *Neurospora* (4.1%) and *Syncephalastrum* (3%). Other very minor genera that found as contaminants were *Chaetomium*, *Phoma*, *Acremonium*, *Aspergillus*, and *Geotrichum* (less than 1%). With detail morphological, growth and physiological criteria, we successfully identified tempeh fungi into four main groups, but we failed to identify *R. microsporus*-group into variety level with only those criteria.
- RAPD typing was capable of producing discriminating DNA fingerprints of *Rhizopus* isolates and indicated there was genetic differences among them. Many distinct patterns were observed indicating that tempeh and inocula from different location in Indonesia composed of a quite wide number of strains.
- Combination of origin or geographical data and molecular identification showed that isolates (identified as *Rhizopus oryzae*, *R. delemar*, and *R. stolonifer*) dominantly used by tempeh producer from outside Java, whereas isolates (*Rhizopus microsporus* complex isolates, identified as *R. microsporus* var. *chinensis*, *R. microsporus* var. *microsporus* and *R. microsporus* var. *oligosporus*) were mostly used by tempeh producer from Java.
- Tempeh fungi isolates are safe for human consumption since they are not mucormycosis agent or toxication agent. Indeed, there is no doubt to consume soybean-tempeh anymore. Attention just needs to be given to some tempeh producer that applied unhygienic practice in tempeh production because we found few sample contaminated with bacteria classified as human pathogen causing gastroenteritis.
- Fungal strains used in production of tempeh in Indonesia could convert ZEN to conjugated ZEN and ZEL derivatives *in vitro*. These transformations may also occur during fermentation of tempeh. If the unprocessed grains for tempeh fermentation like mix soybeans-maize are contaminated with ZEN, upon analysis ZEN content of the final tempeh product may be underestimated, because ZEN has been conjugated during food processing.

#### 5. Acknowledgement

This research was supported financially by Indonesia Endowment Fund for Education, Ministry of Finance, The Republic of Indonesia (Ref:1064/LPDP/2013).

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## 7. Appendix

Supplemental Table 1. Species identity of 42 *Rhizopus* isolates based on morphological characters and ACT sequences.

Isolate	<i>Rhizopus</i> spp.	Sources	Locations in Indonesia	Year	Accession for the first three matched hits in NCBI	Related <i>Rhizopus</i> spp.	Similarity
CJBY22.253	<i>Rhizopus microsporus</i> var. <i>microsporus</i>	Fresh tempeh	Banyumas	2014	KC822394	<i>Rhizopus microsporus</i>	99
					KC822395	<i>Rhizopus microsporus</i>	99
					KC822393	<i>Rhizopus microsporus</i>	99
CJBY14.168	<i>Rhizopus microsporus</i> var. <i>microsporus</i>	Inoculum	Banyumas	2015	KC822393	<i>R. microsporus</i>	100
					KC822394	<i>R. microsporus</i>	99
					KC822395	<i>R. microsporus</i>	99
CJBY16.192	<i>Rhizopus microsporus</i> var. <i>microsporus</i>	Inoculum	Banyumas	2015	AB512247	<i>R. microsporus</i>	99
					KC822393	<i>R. microsporus</i>	99
					KC822394	<i>R. microsporus</i>	99
CJG27.324	<i>Rhizopus microsporus</i> var. <i>microsporus</i>	Inoculum	Gombong	2015	KC822422	<i>R. microsporus</i>	100
					KC822393	<i>R. microsporus</i>	99
					KC822426	<i>R. microsporus</i>	99
WJB7.81	<i>Rhizopus microsporus</i> var. <i>microsporus</i>	Inoculum	Bogor	2015	KC822422	<i>R. microsporus</i>	100
					KC822393	<i>R. microsporus</i>	99
					KC822426	<i>R. microsporus</i>	99
Y52.616	<i>Rhizopus microsporus</i> var. <i>microsporus</i>	Inoculum	Yogyakarta	2015	KC822422	<i>R. microsporus</i>	99
					KC822393	<i>R. microsporus</i>	99
					KC822426	<i>R. microsporus</i>	99
CJBY15.170	<i>Rhizopus microsporus</i> var. <i>microsporus</i>	Fresh tempeh	Banyumas	2014	AB512232	<i>R. microsporus</i>	100
					KC822393	<i>R. microsporus</i>	99
					KC822394	<i>R. microsporus</i>	99
AL3.38	<i>Rhizopus microsporus</i> var. <i>microsporus</i>	Fresh tempeh	Lhokseumawe	2014	KC822397	<i>R. microsporus</i>	100
					KC822394	<i>R. microsporus</i>	100
					KC822395	<i>R. microsporus</i>	100

Chapter 3: Determination of fungal diversity in Indonesian tempeh

EJMG55.69	<i>Rhizopus microsporus</i> var. <i>microsporus</i>	Fresh tempeh	Malang	2013	KC822423	<i>Rhizopus microsporus</i>	99
					KC822397	<i>Rhizopus microsporus</i>	98
CJBY.13.146	<i>Rhizopus microsporus</i> var. <i>microsporus</i>	Fresh tempeh	Banyumas	2013	KC822394	<i>Rhizopus microsporus</i>	98
					KC822399	<i>Rhizopus microsporus</i>	99
					KC822420	<i>Rhizopus microsporus</i>	99
					KC822401	<i>Rhizopus microsporus</i>	99
WJS11.121	<i>Rhizopus microsporus</i> var. <i>oligosporus</i>	Inoculum	Sukabumi	2015	AJ287197	<i>R. microsporus</i> var. <i>oligosporus</i>	99
					AB512238	<i>R. microsporus</i> var. <i>oligosporus</i>	99
					AB512237	<i>R. microsporus</i> var. <i>oligosporus</i>	99
WJS12.12	<i>Rhizopus microsporus</i> var. <i>oligosporus</i>	Fresh tempeh	Sukabumi	2015	AJ287197	<i>R. microsporus</i> var. <i>oligosporus</i>	100
					AB512238	<i>R. microsporus</i> var. <i>oligosporus</i>	99
					AB512237	<i>R. microsporus</i> var. <i>oligosporus</i>	99
CJM28.325	<i>Rhizopus microsporus</i> var. <i>oligosporus</i>	Fresh tempeh	Magelang	2014	AJ287197	<i>R. microsporus</i> var. <i>oligosporus</i>	100
					AB512238	<i>R. microsporus</i> var. <i>oligosporus</i>	99
					AB512237	<i>R. microsporus</i> var. <i>oligosporus</i>	99
WJS11.132	<i>Rhizopus microsporus</i> var. <i>oligosporus</i>	Fresh tempeh	Sukabumi	2015	AJ287197	<i>R. microsporus</i> var. <i>oligosporus</i>	100
					AB512238	<i>R. microsporus</i> var. <i>oligosporus</i>	99
					AB512237	<i>R. microsporus</i> var. <i>oligosporus</i>	99
CJBY19.223	<i>Rhizopus microsporus</i> var. <i>oligosporus</i>	Inoculum	Banyumas	2014	AJ287197	<i>R. microsporus</i> var. <i>oligosporus</i>	100
					AB512238	<i>R. microsporus</i> var. <i>oligosporus</i>	99
					AB512237	<i>R. microsporus</i> var. <i>oligosporus</i>	98
CJK33.389	<i>Rhizopus microsporus</i> var. <i>oligosporus</i>	Inoculum	Kutoarjo	2014	AB512237	<i>R. microsporus</i> var. <i>oligosporus</i>	99
					AB512238	<i>R. microsporus</i> var. <i>oligosporus</i>	99
					AJ287197	<i>R. microsporus</i> var. <i>oligosporus</i>	99
CJM29.344	<i>Rhizopus microsporus</i> var. <i>oligosporus</i>	Inoculum	Magelang	2014	AB512237	<i>R. microsporus</i> var. <i>oligosporus</i>	99
					AB512238	<i>R. microsporus</i> var. <i>oligosporus</i>	99
					AJ287197	<i>R. microsporus</i> var. <i>oligosporus</i>	99
CJK35.409	<i>Rhizopus microsporus</i> var. <i>oligosporus</i>	Fresh tempeh	Kutoarjo	2014	AB512237	<i>R. microsporus</i> var. <i>oligosporus</i>	100
					AB512238	<i>R. microsporus</i> var. <i>oligosporus</i>	99
					AJ287197	<i>R. microsporus</i> var. <i>oligosporus</i>	99
Y49.577	<i>Rhizopus microsporus</i> var. <i>chinensis</i>	Inoculum	Yogyakarta	2015	AB512246	<i>R. microsporus</i> var. <i>chinensis</i>	100
					AB512232	<i>R. microsporus</i> var. <i>chinensis</i>	99

Chapter 3: Determination of fungal diversity in Indonesian tempeh

Y52.613	<i>Rhizopus microsporus</i> var. <i>chinensis</i>	inoculum	Yogyakarta	2015	AB512246	<i>R. microsporus</i> var. <i>chinensis</i>	100
					AB512232	<i>R. microsporus</i> var. <i>chinensis</i>	99
CJG27.321	<i>Rhizopus microsporus</i> var. <i>chinensis</i>	Inoculum	Gombong	2015	AJ287198	<i>R. microsporus</i> var. <i>chinensis</i>	99
					AB512246	<i>R. microsporus</i> var. <i>chinensis</i>	100
					AB512232	<i>R. microsporus</i> var. <i>chinensis</i>	99
AL3.29	<i>Rhizopus delemar</i>	Inoculum	Lhokseumawe	2015	AJ287198	<i>R. microsporus</i> var. <i>chinensis</i>	99
					KJ551428	<i>R. delemar</i>	99
					KJ551450	<i>R. delemar</i>	99
					KJ551446	<i>R. delemar</i>	99
CJBY50.559	<i>Rhizopus delemar</i>	Fresh tempeh	Banyumas	2015	KJ551428	<i>R. delemar</i>	99
					KJ551450	<i>R. delemar</i>	99
					KJ551446	<i>R. delemar</i>	98
EJMG54.643	<i>Rhizopus delemar</i>	Inoculum	Malang	2015	KJ551428	<i>R. delemar</i>	100
					KJ551450	<i>R. delemar</i>	99
					KJ551446	<i>R. delemar</i>	99
CJM28.327	<i>Rhizopus delemar</i>	Fresh tempeh	Magelang	2015	KJ551428	<i>R. delemar</i>	100
					KJ551450	<i>R. delemar</i>	99
AL2.13	<i>Rhizopus delemar</i>	Inoculum	Lhokseumawe	2015	KJ551446	<i>R. delemar</i>	99
					KJ551428	<i>R. delemar</i>	99
CSP72.854	<i>Rhizopus delemar</i>	Fresh tempeh	Jambi	2015	KJ551450	<i>R. delemar</i>	99
					KJ551446	<i>R. delemar</i>	99
CJC41.481	<i>Rhizopus delemar</i>	Inoculum	Cilacap	2015	KJ551428	<i>R. delemar</i>	99
					KJ551450	<i>R. delemar</i>	99
					KJ551446	<i>R. delemar</i>	99
MUAN74.882	<i>Rhizopus oryzae</i>	Inoculum	Ambon	2015	KJ551431	<i>R. oryzae</i>	98
					KJ551427	<i>R. oryzae</i>	97
WJB7.84	<i>Rhizopus oryzae</i>	Inoculum	Bogor	2015	KJ551454	<i>R. oryzae</i>	97
					KJ551431	<i>R. oryzae</i>	100
					KJ551427	<i>R. oryzae</i>	99

Chapter 3: Determination of fungal diversity in Indonesian tempeh

SKBJ68.808	<i>Rhizopus oryzae</i>	Inoculum	Banjarmasin	2015	KJ551427	<i>R. oryzae</i>	100
					KJ551462	<i>R. oryzae</i>	99
					KJ551454	<i>R. oryzae</i>	99
JSJ6.62	<i>Rhizopus oryzae</i>	Inoculum	Jakarta	2015	KJ551453	<i>R. oryzae</i>	100
					KJ551444	<i>R. oryzae</i>	100
					KJ551423	<i>R. oryzae</i>	100
CJG23.275	<i>Rhizopus oryzae</i>	Inoculum	Gombong	2015	KJ551453	<i>R. oryzae</i>	100
					KJ551444	<i>R. oryzae</i>	100
					KJ551441	<i>R. oryzae</i>	100
NSM4.37	<i>Rhizopus oryzae</i>	Inoculum	Medan	2015	KJ551453	<i>R. oryzae</i>	99
					KJ551444	<i>R. oryzae</i>	99
					KJ551441	<i>R. oryzae</i>	99
WJBE10.109	<i>Rhizopus oryzae</i>	Inoculum	Bekasi	2015	KJ551423	<i>R. oryzae</i>	100
					AB281498	<i>R. oryzae</i>	99
WPMW76	<i>Rhizopus oryzae</i>	Inoculum	Manokwari	2015	KJ551422	<i>R. oryzae</i>	99
					KJ551423	<i>R. oryzae</i>	99
					AB281498	<i>R. oryzae</i>	98
					KJ551422	<i>R. oryzae</i>	98
CJY48.567	<i>Rhizopus stolonifer</i>	Fresh tempeh	Banyumas	2014	AB512233	<i>R. stolonifer</i>	98
					AB512234	<i>R. stolonifer</i>	98
CJBY18.207	<i>Rhizopus stolonifer</i>	Fresh tempeh	Banyumas	2014	AB512233	<i>R. stolonifer</i>	98
					AB512234	<i>R. stolonifer</i>	97
JSJ6.77	<i>Rhizopus stolonifer</i>	Fresh tempeh	Jakarta	2014	AB512231	<i>R. stolonifer</i>	99
					AB512245	<i>R. stolonifer</i>	98
					AJ287199	<i>R. stolonifer</i>	97
AL5.39	<i>Rhizopus stolonifer</i>	Fesh tempeh	Lhokseumawe	2015	AB512245	<i>R. stolonifer</i>	100
					AJ287199	<i>R. stolonifer</i>	99
					HM117700	<i>R. stolonifer</i>	99

Supplemental Table 2. Discription of *Rhizopus* accessions for ACT sequence used in this study

Accession No	Strain	Species	Reference
KJ551424	CBS 109939	<i>Rhizopus oryzae</i>	Dolatabadi et al. (2014a)
KJ551425	CBS 118614	<i>Rhizopus oryzae</i>	Dolatabadi et al. (2014a)
KJ551427	CBS 120592	<i>Rhizopus oryzae</i>	Dolatabadi et al. (2014a)
KJ551453	CBS 396.95	<i>Rhizopus oryzae</i>	Dolatabadi et al. (2014a)
KJ551431	CBS 120806	<i>Rhizopus oryzae</i>	Dolatabadi et al. (2014a)
KJ551459	CBS 137314	<i>Rhizopus delemar</i>	Dolatabadi et al. (2014a)
KJ551460	CBS 607.68	<i>Rhizopus delemar</i>	Dolatabadi et al. (2014a)
KJ551463	CBS 405.51	<i>Rhizopus delemar</i>	Dolatabadi et al. (2014a)
KJ551428	CBS 120593	<i>Rhizopus delemar</i>	Dolatabadi et al. (2014a)
KJ551432	CBS 120807	<i>Rhizopus delemar</i>	Dolatabadi et al. (2014a)
KC822395	CBS 130967	<i>Rhizopus microsporus</i>	Dolatabadi et al. (2014b)
KC822399	CBS 124669	<i>Rhizopus microsporus</i>	Dolatabadi et al. (2014b)
KC822423	CBS 359.92	<i>Rhizopus microsporus</i>	Dolatabadi et al. (2014b)
KC822396	CBS 308.87	<i>Rhizopus microsporus</i>	Dolatabadi et al. (2014b)
KC822415	CBS 712.73	<i>Rhizopus microsporus</i>	Dolatabadi et al. (2014b)
AJ287197	PLN 01	<i>Rhizopus microsporus</i> var. <i>oligosporus</i>	Voight and Wostemeyer (2001)
AB512238	CBS 338.62	<i>Rhizopus microsporus</i> var. <i>oligosporus</i>	Abe et al. (2010)
AB512237	CBS 337.62	<i>Rhizopus microsporus</i> var. <i>oligosporus</i>	Abe et al. (2010)
AB512232	CBS 262.28	<i>Rhizopus microsporus</i> var. <i>chinensis</i>	Abe et al. (2010)
AB512246	CBS 631.82	<i>Rhizopus microsporus</i> var. <i>chinensis</i>	Abe et al. (2010)
AB512231	CBS 150.83	<i>Rhizopus stolonifer</i>	Abe et al. (2010)
AJ287199	PLN 02	<i>Rhizopus stolonifer</i>	Voight and Wostemeyer (2001)
AB512245	CBS 609.82	<i>Rhizopus stolonifer</i>	Abe et al. (2010)

Supplemental Table 3. List of tempeh fungi R1-R70 used for ZEN transformation test.

	<b>Fungi name</b>	<b>Original source</b>	<b>Year sampling</b>
R1	<i>R. microsporus</i> WJB7.81	Tempeh starter, West Java, Bogor	2015
R2	<i>R. microsporus</i> var. oligosporus CJG27.324	Tempeh starter, Central Java, Gombong	2015
R3	<i>R. microsporus</i> var. oligosporus CJM28.325	Tempeh starter, Central Java, Magelang	2015
R4	<i>R. microsporus</i> var. oligosporus WJS11.121	Tempeh starter, West Java, Sukabumi	2015
R5	<i>R. microsporus</i> var. oligosporus CJB13.145	Tempeh starter, Central Java, Banyumas	2015
R6	<i>R. microsporus</i> var. oligosporus WJS11.132	Tempeh starter, West Java, Sukabumi	2015
R7	<i>R. microsporus</i> var. oligosporus WJBE10.114	Tempeh starter, West Java, Bekasi	2015
R8	<i>R. oryzae</i> WJBE8.85	Tempeh starter, West Java, Bekasi	2015
R9	<i>R. microsporus</i> var. chinensis WJBE8.89	Tempeh starter, West Java, Bekasi	2015
R10	<i>R. oryzae</i> WJBE11.120	Tempeh starter, West Java, Bekasi	2015
R11	<i>R. oryzae</i> WJB7.77	Tempeh, West Java, Bogor	2015
R12	<i>R. microsporus</i> var. chinensis CJK33.385	Tempeh starter, Central Java, Kutoarjo	2015
R13	<i>R. microsporus</i> var. chinensis CJK37.444	Tempeh starter, Central Java, Kutoarjo	2015
R14	<i>R. microsporus</i> var. oligosporus WJBE8.87	Tempeh starter, West Java, Bekasi	2015
R15	<i>R. microsporus</i> var. oligosporus WJBE9.103	Tempeh starter, West Java, Bekasi	2015
R16	<i>R. delemar</i> WJB7.73	Tempeh, West Java, Bogor	2015
R17	<i>R. oryzae</i> WJBE10.109	Tempeh starter, West Java, Bekasi	2015
R18	<i>R. oryzae</i> WJB7.84	Tempeh, West Java, Bogor	2015
R19	<i>R. microsporus</i> var. oligosporus CJB13.154	Tempeh starter, Central Java, Banyumas	2015
R20	<i>R. microsporus</i> var. chinensis CJB14.168	Tempeh starter, Central Java, Banyumas	2015
R21	<i>R. microsporus</i> var. chinensis CJB15.170	Tempeh starter, Central Java, Banyumas	2015
R22	<i>R. microsporus</i> var. chinensis CJB16.192	Tempeh starter, Central Java, Banyumas	2015
R23	<i>R. oryzae</i> WJS11.121	Tempeh starter, West Java, Sukabumi	2015
R24	<i>Syncephalastrum racemosum</i> CJG23.273	Tempeh starter, Central Java, Gombong	2015
R25	<i>Aspergillus oryzae</i> CJB12.260	Tempeh starter, Central Java, Banyumas	2015
R26	<i>A. oryzae</i> CJM28.329	Tempeh starter, Central Java, Magelang	2015
R27	<i>Neurospora sitophila</i> CJM30.353	Tempeh starter, Central Java, Magelang	2015
R28	<i>N. sitophila</i> WJB7.75	Tempeh starter, West Java, Bogor	2015
R29	<i>R. oryzae</i> CSP71.841	Tempeh starter, Central Sulawesi, Palu	2015
R30	<i>R. microsporus</i> var. chinensis WKP65.769	Tempeh starter, West Kalimantan, Pontianak	2015
R31	<i>R. oryzae</i> BDP59.700	Tempeh, Bali, Denpasar	2015
R32	<i>R. oryzae</i> MUAN74.882	Tempeh, Maluku, Ambon	2015
R33	<i>R. oryzae</i> WPMW76.901	Tempeh, West Papua, Manokwari	2015
R34	<i>R. oryzae</i> SKBJ68.808	Tempeh starter, South Kalimantan, Banjarmasin	2015

Chapter 3: Determination of fungal diversity in Indonesian tempeh

R35	<i>R. microsporus</i> CJBR43.505	Tempeh starter, Central Java, Brebes	2015
R36	<i>R. microsporus</i> CJM30.349	Tempeh starter, Central Java, Magelang	2015
R37	<i>R. oryzae</i> LLM5.50	Tempeh starter, Lampung, Lampung Metro	2015
R38	<i>R. microsporus</i> var. <i>chinensis</i> LLM5.56	Tempeh starter, Lampung, Lampung Metro	2015
R39	<i>R. oryzae</i> NSM4.37	Tempeh starter, North Sumatera, Medan	2015
R40	<i>R. delemar</i> AL2.13	Tempeh starter, Aceh, Lhokseumawe	2015
R41	<i>R. delemar</i> AL3.29	Tempeh starter, Aceh, Lhokseumawe	2015
R42	<i>R. oryzae</i> NSM4.48	Tempeh starter, North Sumatera, Medan	2015
R43	<i>R. oryzae</i> JSJ6.62	Tempeh starter, South Jakarta, Jakarta	2015
R44	<i>R. oryzae</i> CJG23.275	Tempeh starter, Central Java, Gombong	2015
R45	<i>R. microsporus</i> var. <i>chinensis</i> CJG27.321	Tempeh starter, Central Java, Gombong	2015
R46	<i>R. delemar</i> CJBR44.517	Tempeh starter, Central Java, Brebes	2015
R47	<i>R. microsporus</i> var. <i>chinensis</i> Y52.613	Tempeh starter, Yogyakarta	2015
R48	<i>R. microsporus</i> var. <i>chinensis</i> Y51.603	Tempeh starter, Yogyakarta	2015
R49	<i>R. oryzae</i> Y50.590	Tempeh starter, Yogyakarta	2015
R50	<i>R. microsporus</i> var. <i>chinensis</i> Y49.577	Tempeh starter, Yogyakarta	2015
R51	<i>R. oryzae</i> EJSU56.661	Tempeh starter, East Java, Surabaya	2015
R52	<i>R. oryzae</i> EJSU57.673	Tempeh starter, East Java, Surabaya	2015
R53	<i>R. oryzae</i> EJMG53.638	Tempeh starter, East Java, Malang	2015
R54	<i>R. microsporus</i> var. <i>oligosporus</i> BDP60.710	Tempeh, Bali, Denpasar	2015
R55	<i>R. microsporus</i> var. <i>oligosporus</i> CSP71.850	Tempeh starter, Central Sulawesi, Palu	2015
R56	<i>R. microsporus</i> Y52.616	Tempeh starter, Yogyakarta	2015
R57	<i>R. delemar</i> EJMG54.643	Tempeh starter, East Java, Malang	2015
R58	<i>R. delemar</i> AL3.32	Tempeh starter, Aceh, Leuksomawe	2015
R59	<i>R. oryzae</i> AL2.23	Tempeh starter, Aceh, Leuksomawe	2015
R60	<i>R. oryzae</i> NSM4.41	Tempeh starter, North Sumatera, Medan	2015
R61	<i>R. delemar</i> CJC41.481	Tempeh starter, Central Java, Cilacap	2015
R62	<i>Syncephalastrum racemosum</i> CJC42.499	Tempeh starter, Central Java, Cilacap	2015
R63	<i>S. racemosum</i> CJK34.400	Tempeh starter, Central Java, Kutoarjo	2015
R64	<i>S. racemosum</i> CJG23.272	Tempeh starter, Central Java, Gombong	2015
R65	<i>S. racemosum</i> CJBY13.148	Tempeh starter, Central Java, Banyumas	2015
R66	<i>Aspergillus oryzae</i> LLM5.53	Tempeh starter, Lampung, Lampung Metro	2015
R67	<i>A. oryzae</i> AL3.35	Tempeh starter, Aceh, Lhokseumawe	2015
R68	<i>Neurospora crassa</i> WJBE10.111	Tempeh starter, West Java, Bekasi	2015
R69	<i>N. sitophila</i> WJS11. 137	Tempeh starter, West Java, Sukabumi	2015
R70	<i>N. sitophila</i> WJS11. 144	Tempeh starter, West Java, Sukabumi	2015

Supplemental Table 4. HPLC-MS/MS result of ZEN transformation by tempeh fungi (semi-quantitative data).

Isolates	Masked ZEN							
	Area (count) after adjusted with response							
	ZEN	$\alpha$ -ZEL	$\beta$ -ZEL	ZEN14S	ZELS 1	ZELS 2	ZEN14G	ZEN16G
R1	7,16x10 <sup>3</sup>	11,36x10 <sup>3</sup>	1,47x10 <sup>3</sup>	1,14x10 <sup>4</sup>	0	1,14x10 <sup>4</sup>	0	1,53x10 <sup>4</sup>
R2	0	0	8,98x10 <sup>2</sup>	5,65x10 <sup>5</sup>	1,33x10 <sup>2</sup>	1,34x10 <sup>5</sup>	0	1,45x10 <sup>4</sup>
R3	9,84x10 <sup>2</sup>	0	1,64x10 <sup>3</sup>	4,14x10 <sup>5</sup>	4,61x10 <sup>3</sup>	7,81x10 <sup>4</sup>	0	1,34x10 <sup>4</sup>
R4	1,25x10 <sup>5</sup>	0	1,81x10 <sup>3</sup>	1,08x10 <sup>5</sup>	1,37x10 <sup>2</sup>	1,03x10 <sup>3</sup>	0	0
R5	1,20x10 <sup>5</sup>	0	0	1,66x10 <sup>4</sup>	0	1,5x10 <sup>2</sup>	0	0
R6	1,88x10 <sup>4</sup>	6,53x10 <sup>2</sup>	0	6x10 <sup>5</sup>	8x10 <sup>2</sup>	8,0x10 <sup>4</sup>	0	0
R7	0	0	0	4,25x10 <sup>5</sup>	6,27x10 <sup>3</sup>	1,22x10 <sup>5</sup>	0	1,83x10 <sup>4</sup>
R8	0	0	7,14x10 <sup>2</sup>	5,30x10 <sup>5</sup>	6,73x10 <sup>3</sup>	8,84x10 <sup>4</sup>	0	0
R9	0	0	5,35x10 <sup>2</sup>	4,39x10 <sup>5</sup>	6,70x10 <sup>3</sup>	1,44x10 <sup>5</sup>	0	9,36x10 <sup>3</sup>
R10	8,05x10 <sup>3</sup>	1,11x10 <sup>4</sup>	1,47x10 <sup>3</sup>	5,22x10 <sup>4</sup>	2,16x10 <sup>2</sup>	3,85x10 <sup>4</sup>	0	3,93x10 <sup>4</sup>
R11	5,17x10 <sup>3</sup>	1,65x10 <sup>3</sup>	1,23x10 <sup>3</sup>	5,65x10 <sup>5</sup>	2,78x10 <sup>3</sup>	7,03x10 <sup>4</sup>	0	0
R12	0	0	4,49x10 <sup>2</sup>	4,19x10 <sup>5</sup>	8,66x10 <sup>3</sup>	1,33x10 <sup>5</sup>	0	1,30x10 <sup>4</sup>
R13	0	0	1,28x10 <sup>3</sup>	4,77x10 <sup>5</sup>	7x10 <sup>3</sup>	1,06x10 <sup>5</sup>	0	2,49x10 <sup>3</sup>
R14	0	0	6,28x10 <sup>2</sup>	4,40x10 <sup>5</sup>	1,01x10 <sup>4</sup>	9,74x10 <sup>4</sup>	0	0
R15	1,33x10 <sup>5</sup>	0	0	1,77x10 <sup>4</sup>	0	1,66x10 <sup>2</sup>	0	0
R16	0	0	1,42x10 <sup>3</sup>	3,74x10 <sup>5</sup>	3,96x10 <sup>3</sup>	7,42x10 <sup>4</sup>	0	8,55x10 <sup>3</sup>
R17	1,16x10 <sup>3</sup>	0	1,34x10 <sup>3</sup>	3,59x10 <sup>5</sup>	4,63x10 <sup>3</sup>	9,29x10 <sup>4</sup>	4,77x10 <sup>2</sup>	1,09x10 <sup>4</sup>
R18	5,98x10 <sup>3</sup>	3,68x10 <sup>3</sup>	5,07x10 <sup>2</sup>	2,67x10 <sup>5</sup>	2,75x10 <sup>2</sup>	4,05x10 <sup>2</sup>	2,42x10 <sup>3</sup>	5,74x10 <sup>4</sup>
R19	0	0	1,87x10 <sup>3</sup>	3,87x10 <sup>5</sup>	3,16x10 <sup>4</sup>	1,85x10 <sup>5</sup>	0	0
R20	0	0	0	5,34x10 <sup>5</sup>	1,02x10 <sup>4</sup>	1,25x10 <sup>5</sup>	0	0
R21	0	0	2,0x10 <sup>3</sup>	3,01x10 <sup>5</sup>	1,67x10 <sup>4</sup>	1,86x10 <sup>5</sup>	0	0
R22	4,76x10 <sup>4</sup>	1,24x10 <sup>3</sup>	1,49x10 <sup>4</sup>	0	0	0	0	2,78x10 <sup>3</sup>
R23	0	0	1,46x10 <sup>3</sup>	6,92x10 <sup>5</sup>	3,42x10 <sup>4</sup>	9,35x10 <sup>3</sup>	0	0
R24	1,61x10 <sup>3</sup>	3,31x10 <sup>2</sup>	0	4,63x10 <sup>5</sup>	3,23x10 <sup>3</sup>	1,09x10 <sup>5</sup>	0	0
R25	2,64x10 <sup>3</sup>	0	0	7,02x10 <sup>5</sup>	6,01x10 <sup>3</sup>	1,4x10 <sup>4</sup>	0	0
R26	2,18x10 <sup>3</sup>	0	0	7,09x10 <sup>5</sup>	7,26x10 <sup>3</sup>	1,29x10 <sup>4</sup>	0	0
R27	4,29x10 <sup>4</sup>	1,88x10 <sup>3</sup>	7,33x10 <sup>2</sup>	0	0	0	0	0



Chapter 3: Determination of fungal diversity in Indonesian tempeh

R28	2.88x10 <sup>4</sup>	3.34x10 <sup>3</sup>	0	4.35x10 <sup>2</sup>	0	0	0	0
R29	3.82x10 <sup>4</sup>	0	0	1.79x10 <sup>3</sup>	0	0	0	0
R30	1.05x10 <sup>4</sup>	0	0	7.14x10 <sup>3</sup>	0	1.12x10 <sup>2</sup>	0	0
R31	4.27x10 <sup>4</sup>	0	0	3.29x10 <sup>3</sup>	0	0	0	0
R32	2.91x10 <sup>4</sup>	0	0	5.95x10 <sup>4</sup>	0	6.11x10 <sup>2</sup>	0	0
R33	3.63x10 <sup>4</sup>	0	0	3.60x10 <sup>3</sup>	0	0	0	0
R34	2.50x10 <sup>4</sup>	0	4.80x10 <sup>2</sup>	8.05x10 <sup>2</sup>	0	0	0	0
R35	3.42x10 <sup>4</sup>	0	0	2.23x10 <sup>3</sup>	0	3.46x10	0	0
R36	1.23x10 <sup>3</sup>	3.28x10 <sup>3</sup>	2.94x10 <sup>2</sup>	3.86x10 <sup>4</sup>	2.23x10 <sup>2</sup>	5.56x10 <sup>4</sup>	0	1.41x10 <sup>4</sup>
R37	0	0	0	3.13x10 <sup>5</sup>	1.12x10 <sup>4</sup>	7.61x10 <sup>4</sup>	0	0
R38	1.01x10 <sup>4</sup>	0	0	3.05x10 <sup>5</sup>	3.64x10 <sup>2</sup>	1.43x10 <sup>4</sup>	0	0
R39	9.76x10 <sup>3</sup>	5.81x10 <sup>2</sup>	6.25x10 <sup>2</sup>	2.22x10 <sup>5</sup>	2.31x10 <sup>2</sup>	1.4x10 <sup>4</sup>	0	0
R40	3.79x10 <sup>4</sup>	0	5.08x10 <sup>2</sup>	1.02x10 <sup>4</sup>	0	2.76x10 <sup>2</sup>	0	0
R41	3.47x10 <sup>4</sup>	0	0	0	0	0	0	0
R42	3.02x10 <sup>4</sup>	0	0	4.59x10 <sup>4</sup>	0	7.70x10 <sup>2</sup>	0	0
R43	7.20x10 <sup>3</sup>	1.10x10 <sup>3</sup>	8.88x10 <sup>2</sup>	2.82x10 <sup>5</sup>	6.29x10 <sup>2</sup>	1.38x10 <sup>4</sup>	0	2.49x10 <sup>3</sup>
R44	2.53x10 <sup>4</sup>	0	0	0	0	0	0	0
R45	2.64x10 <sup>4</sup>	0	0	7.95x10 <sup>4</sup>	5.62x10	4.95x10 <sup>3</sup>	0	0
R46	0	0	3.19x10 <sup>2</sup>	2.06x10 <sup>5</sup>	1.16x10 <sup>4</sup>	9.52x10 <sup>4</sup>	0	6.01x10 <sup>3</sup>
R47	2.85x10 <sup>4</sup>	0	0	2.47x10 <sup>4</sup>	0	2.01x10 <sup>2</sup>	0	0
R48	8.52x10 <sup>2</sup>	1.33x10 <sup>3</sup>	2.94x10 <sup>2</sup>	7.85x10 <sup>4</sup>	6.59x10 <sup>2</sup>	8.83x10 <sup>4</sup>	0	0
R49	1.28x10 <sup>4</sup>	1.05x10 <sup>3</sup>	3.18x10 <sup>2</sup>	8.61x10 <sup>4</sup>	1.74x10 <sup>2</sup>	1.31x10 <sup>4</sup>	0	0
R50	1.94x10 <sup>4</sup>	0	0	5.38x10 <sup>4</sup>	0	4.91x10 <sup>3</sup>	0	0
R51	2.09x10 <sup>4</sup>	0	0	2.76x10 <sup>3</sup>	0	0	0	0
R52	3.80x10 <sup>3</sup>	0	0	2.38x10 <sup>5</sup>	1.23x10 <sup>2</sup>	1.07x10 <sup>4</sup>	0	5.45x10 <sup>3</sup>
R53	0	0	0	1.80x10 <sup>5</sup>	4.90x10 <sup>3</sup>	1.18x10 <sup>5</sup>	0	0
R54	2.73x10 <sup>4</sup>	0	0	6.05x10 <sup>3</sup>	0	0	0	0
R55	0	0	2.11x10 <sup>2</sup>	2.55x10 <sup>5</sup>	3.04x10 <sup>4</sup>	2.37x10 <sup>5</sup>	0	0
R56	3.25x10 <sup>3</sup>	1.59x10 <sup>3</sup>	7.13x10 <sup>3</sup>	3.4x10 <sup>2</sup>	0	0	0	0
R57	1.39x10 <sup>4</sup>	0	2.68x10 <sup>2</sup>	0	0	0	0	0
R58	1.28x10 <sup>4</sup>	2.47x10 <sup>2</sup>	4.99x10 <sup>3</sup>	0	0	0	0	0
R59	5.37x10 <sup>3</sup>	7.28x10 <sup>3</sup>	0	0	0	0	0	0

### Chapter 3: Determination of fungal diversity in Indonesian tempeh

R60	9.59x10 <sup>3</sup>	9.51x10 <sup>2</sup>	5.01x10 <sup>3</sup>	6.54x10 <sup>3</sup>	3.82x10 <sup>2</sup>	3.88x10 <sup>2</sup>	0	0
R61	1.64x10 <sup>4</sup>	0	0	0	0	0	0	0
R62	0	0	0	4.22x10 <sup>5</sup>	2.93x10 <sup>3</sup>	4.95x10 <sup>2</sup>	0	0
R63	8.48x10 <sup>2</sup>	0	0	3.30x10 <sup>5</sup>	1.45x10 <sup>3</sup>	1.95x10 <sup>2</sup>	0	0
R64	0	0	0	4.05x10 <sup>5</sup>	2.85x10 <sup>3</sup>	1.76x10 <sup>3</sup>	0	0
R65	0	0	0	3.94x10 <sup>5</sup>	2.34x10 <sup>3</sup>	2.06x10 <sup>2</sup>	0	0
R66	0	0	0	4.27x10 <sup>5</sup>	2.92x10 <sup>3</sup>	4.63x10 <sup>3</sup>	0	0
R67	0	0	0	3.15x10 <sup>5</sup>	1.80x10 <sup>3</sup>	8.09x10 <sup>2</sup>	0	0
R68	6.83x10 <sup>3</sup>	4.28x10 <sup>2</sup>	1.33x10 <sup>3</sup>	0	0	0	0	0
R69	9.36x10 <sup>3</sup>	0	1.36x10 <sup>3</sup>	0	0	0	0	0
R70	1.53x10 <sup>4</sup>	9.30x10 <sup>2</sup>	2.22x10 <sup>3</sup>	0	0	0	0	0
(+) control	1,37x10 <sup>5</sup>	0	0	0	0	0	0	0
(-) control	0	0	0	0	0	0	0	0

Abbreviation:

ZEN: Zearalenone,  $\alpha$ -ZEL: alpha-Zearalenone,  $\beta$ -ZEL: beta-Zearalenone, ZEN14S: Zearalenone 14-Sulfate, ZELS 1: Zearalenol sulfate type 1, ZELS 2: Zearalenol sulfate type 2, ZEN14G: Zearalenone-14-Glucoside, ZEN16G: Zearalenone-16-Glucoside.

## Research Article 02

# Mycotoxin Producing *Fusarium* Species in Indonesian Traditional Tempeh and Starter (*Usar*)\*

\*<sup>1</sup>This research has been presented in 39<sup>th</sup> Mycotoxin Workshop 2017, Bydgoszcz, Poland.

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## Abstract

Like many other traditional foods in Indonesia, tempeh is mainly produced as households or cottage industries with poorly controlled fermentation which is often carried out without sufficient hygienic precautions. Therefore, there are various types of microorganisms that can participate during the process of fermentation, and presence of natural contamination is always possible. In this study, *usar* and traditional tempeh were investigated for the presence of *Fusarium* spp and mycotoxin contamination. In total, 650 fungal isolates recovered from traditional tempeh inocula (*usar*) in Java. Traditional inocula (*usar*) were collected from many markets and center of tempeh producers in 9 different areas in Central Java and Yogyakarta provinces, Indonesia. Six clusters (Banjarnegara, Banyumas, Gombong, Kebumen, Kudus, and Yogyakarta) showed a *Fusarium* contamination in *usar*, while no contamination was found in other remaining clusters. About 8.4% of total fungal isolates recognized belongs to *Fusarium* genera. Fifty-five pure isolates of *Fusarium* sp were collected, then observed via microscopy and molecular approach. We found two main species of *Fusarium*, identified as *F. solani* and *F. proliferatum*.

All *F. proliferatum* isolates included in our study lacked the TRI5 gene and possessed the FUM1 gene, indicating that they are presumably trichothecene non-producers and potentially fumonisin producers. This result was confirmed by different set of primers (FUM8, and FUM12) with identical result. In other hands, *F. solani* isolates lacked in both kind of genes, indicating they are not trichothecene or fumonisin producers. LC-MS/MS screening showed *F. solani* isolates were not able to produce any important mycotoxins, whereas *F. proliferatum* positively produced BEA, FB1, and Enns. *Fusarium proliferatum* extracts showed about 10-fold stronger activity as toxic agent toward *F. solani* extracts based on their value of LD<sub>50</sub>.

About 214 traditional tempeh samples from the six clusters mentioned above were screened for *F. proliferatum* and *F. solani* contamination by real-time PCR. Contamination of traditional tempeh samples with *F. proliferatum* was found in 12% of the samples with amounts of 10-175 ng/mg fungal DNA. In these samples, the amount of FB1 and BEA ranged between 170-2682 µg/kg and 20-50 µg/kg, respectively. About 7 % of the traditional tempeh samples were contaminated with *F. solani* in amounts of 20-129 ng/mg fungal DNA. All samples had lower FB1 contents than legal limit in Indonesia (2000 µg/kg; SNI 7385:2009), except for one sample from Yogyakarta area which contained FB1 above the allowed limit. Although mycotoxin contamination was detected below the legal limit, the presence of mycotoxin should be controlled in traditional tempeh production.

There were no differences between normal and contaminated tempeh on the compactness and physical characters (texture, pH, and color). Interestingly we found that chemical contents were significantly different especially in protein and amino acid content. We suggest that consumer could distinguish contaminated tempeh by checking the taste of fresh tempeh before cooked. Contaminated tempeh is less umami than normal tempeh because of less L-aspartic acid and L-glutamic acid content, in other words, contaminated-tempeh is less delicious compared to the normal.

**Keywords:** traditional tempeh, *usar*, contamination, mycotoxin, *Fusarium* spp.

## 1. Introduction

Among Asian fungal fermented foods, tempeh is a fermented soybean of high nutritional value, originally and traditionally produced in cottage industries in Indonesia. It has solid form, distinctive aroma and white and slightly gray (INCS, 2009). Tempeh-producing technology has been handed down through generations and changes with experience. Alterations in the processing are usually made by the means and resources which are available (Karyadi, 1996). It is a household art in Indonesia, and therefore its quality varies from place to place (Purwadaria et al., 2013).

The production of tempeh requires inoculum or starter containing sporangiospore to initiate the fermentation. Several types of tempeh inocula can be distinguished. Natural inoculum made with plant leaves and soybeans, known as *usar* are still widely used in Indonesia (Thanh, 2004). *Usar* is manufactured at the village scale (a few thousand leaves may be processed daily) and is obtained by cultivating *Rhizopus spp.* on a few inoculated soybeans sandwiched between two leaves. At the beginning, *usar* was made with leaves of *Hibiscus similis*, *H. tiliaceus*, *Tectona grandis*, *Bambusa spp.* or *Musa paradisiaca* (Jutono 1985). Whereas *Tectona spp.*, *Musa paradisiaca*, and *Bambusa spp.* are regarded as unsuitable for *usar* making because they are brittle after drying, produce bitter taste and their surface are smooth. *Hibiscus* is more suitable and commonly leaves used for making *usar*, and some are specifically used for *usar* manufacture. The leaf structure of these plants are such that, enabling sufficient oxygen in the micro-environment are provided for the fermentation process. The rough surface of the underside of the leaves allows mycelial attachment. *Hibiscus* leaf possibly acts as one of its natural reservoirs. Jutono (1985) and Arbianto (1990) stated the leaf environment would stimulate the chlamydospores and zygosporangia formation. These features shall contribute to the viability and biological stability of the mixed population of *Rhizopus spp.* on *usar*. The natural occurrence of wild strains of *Rhizopus* species on the leaves should in any case increase the chance of zygosporangia formation. In 2004, Japanese research team, Yoshio Ogawa and his colleagues, suggested *Rhizopus oryzae* dwelling in those leaves would have fermented soybeans into an easily digestible and good-tasting food, producing the original form of tempeh. Those fresh leaves might be the original habitat of *Rhizopus spp.* or several other fungi which can penetrate into living plant tissue (Ogawa et al., 2004).

*Usar* is prepared by placing pieces of matured tempeh between two *Hibiscus* leaves. The leaves are turned over and piled one on top of the other, afterwards covered with banana leaves or cloth and left to undergo the first stage of fermentation for about 24–36 hours room temperature (30°–34°C). In the second stage of fermentation, the leaves now facing upwards, are separated individually and kept so for 3–7 days, partly to let the *usar* dry at room temperature. The leaves are then separated from the pieces of seed tempeh and dried. Sun drying may be applied at the end of the second stage of fermentation to ensure that *usar* reaches a sufficiently low moisture content that will secure its shelf life about seven days. For the inoculation of the microorganisms from *usar*, the leaves are rubbed on the material to be inoculated. Tempeh producers prefer to use freshly harvested *usar* for their own tempeh production process; they consider fresh *usar* to be of better quality than the dried one.

Like many other traditional foods in Indonesia, traditional tempeh is mostly made with poorly controlled fermentation which is carried out without sufficient hygienic precautions. Therefore, there are various types of microorganisms that can participate in the process of fermentation, and natural contamination is always possible (Winarno, 1985; Barus et al., 2008; Seumahu et al., 2013). Standard of hygiene is also not applied in *usar* production due to the lack of knowledge about good sanitation by the producers. Until now traditional tempeh producers still involve *waru lengis* leaves (*Hibiscus tiliaceus*) for *usar* manufacture, even though as reported Nout et al., (1992) and Suliantari (1996) these leaves may serve as source of *Fusarium spp.* and *Aspergillus spp.* About 2.4 million tons of tempeh produced a year by more than 81 thousand tempeh producers. As much as 35% of tempeh production in Indonesia still uses *usar*. To date, only little attention has been given to microbiological and safety research of *usar*. The big question is whether about 800 thousand metric tons of traditional tempeh produced a year are still safe for human consumption? So far, there has been no further research on the contamination from toxins produced by molds in *usar* as well as traditional tempeh. Moreover, there is no standard for making tempeh and its starter. This is the cause of many variations in the manufacture of tempeh in some areas which lead to nonhomogeneous quality and safety of traditional tempeh.

Another safety problem in tempeh production is the use of corn as a substitute for soybean. Producers involve corn substitution for 10-30 percent to reduce cost production because the price of soybean is high nowadays. This generates a healthiness issue of produced tempeh because most Indonesian corn has been contaminated by toxigenic fungi such as *Fusarium spp* (Purwoko et al., 1991; Ahmad et al., 1996; Gholib et al., 2004; Ahmad et al., 2009; Kusumaningrum et al., 2010; Rahmawati et al., 2013). Rahmawati et al. (2013) also reported that toxigenic fungi are found in soaking corn, such as *Fusarium spp.* It is high possibly happens that those fungi produce mycotoxins during soaking process, then the toxins absorbed into the material that is used to produce tempeh. Moreover mycotoxin content in raw material itself also endangers consumer health. Therefore safety aspect of tempeh cannot be guaranteed anymore.

This study is conducted to explore and examine the innocuousness of *usar* for tempeh manufactures as well as the traditional method handed down through generation. The salient point in tempeh production is also needed to discuss to increase the safety of traditional tempeh production.

## 2. Materials and Methods

### Part I (*Fusarium* identification from traditional inocula)

#### 2.1.1. Sampling, isolation and purification

Traditional inocula (*usar*) were collected from many markets and centre of tempeh producers in 9 different areas in Central Java and Yogyakarta provinces, Indonesia (on 2015). Samples were transported in sterile plastic container to nearby microbiological laboratory in each area. Samples were then freeze dried (Supermodulyo, Japan), powdered and maintained at 4 °C until analysis in refrigerator (Sanyo, Japan). Details information of sampling can be seen on Table 1.

Five gram of each sample (powder) was suspended in 10 mL sterilized tap water in a sterile falcon tube, homogenized with vortex (VM-300), then one mL of each solution was spread onto Potato Dextrose Agar (PDA) medium (Roth, Karlsruhe, Germany), and incubated in the same condition. Purification of isolates: A spore suspension is prepared by transferring a loopful of the fungal mass from PDA plate into 10 mL sterile Tween®20 (Applichem, Darmstadt, Germany) (0.05% v/v), kept in a sterilized falcon tube followed by vigorous shaking with vortex for a few minutes in order to disperse the spores from the spore-bearing structures, and continued with single spore isolation method (modification of Zhang et al., 2013). All the procedures were done in sterile condition inside clean bench. Distinct fungal colonies temporarily identified as *Fusarium* were transferred on PDA and recorded for colony morphology, radial growth and pigmentation based on the visual inspection of the plates. About 55 pure isolates of *Fusarium* sp were collected.

Table 1. Information of sampling locations.

Areas	Samples	Amount	Year
Banyumas (B)	Traditional inocula ( <i>usar</i> )	52	2015
Banjarnegara (BJ)	Traditional inocula ( <i>usar</i> )	42	2015
Cilacap (CL)	Traditional inocula ( <i>usar</i> )	37	2015
Kebumen (KE)	Traditional inocula ( <i>usar</i> )	41	2015
Kudus (KU)	Traditional inocula ( <i>usar</i> )	34	2015
Purbalingga (PU)	Traditional inocula ( <i>usar</i> )	32	2015
Tegal (TG)	Traditional inocula ( <i>usar</i> )	30	2015
Gombong (G)	Traditional inocula ( <i>usar</i> )	32	2015
Yogyakarta	Traditional inocula ( <i>usar</i> )	45	2015
Total of sample		345	

#### 2.1.2. Morphological observation

##### Morphological characteristics

Since *Fusarium* isolates produce uniform spores on SNA-Synthetic Nutrient Agar medium (1 g KH<sub>2</sub>PO<sub>4</sub>, 1 g KNO<sub>3</sub>, 0.5 g MgSO<sub>4</sub> .7H<sub>2</sub>O, 0.5 g KCl, 0.2 g glucose, 0.2 g sucrose and 15 g agar/liter distilled water) (Nirenberg, 1976), all single spores isolates were transferred to SNA plates and incubated at 25°C under near UV light for 5 days. Small pieces of sterile filter paper were placed on the surface of cooled agar to induce fungal sporulation. Microscopic analysis was used to observe the fungi. Lactophenol cotton blue and oil immersion were applied on preparing slide mounts. Observation was done using a light microscope (Leica Leitz DMRB) equipped with

a color camera (Leica DFC420). Identification was carried out at species level based on the morphological descriptions with several parameters (colony, conidia and conidiophores, conidiogenous cells, perithecia, and chlamydospores) according to Booth (1971), Gerlach and Nirenberg (1982), Nelson *et al.* (1983), Burgess *et al.* (1994), and Leslie and Summerell (2006). At least 20 microconidia and macroconidia of each isolate were selected randomly for measurement. *Fusarium* strains were stored as spore suspension in 25% (v/v) glycerol (Carl Roth, Karlsruhe, Germany) at -60°C.

### **2.1.3. Molecular analysis**

Identification of *Fusarium* isolates was performed according to the combination of morphological and molecular characters. Microscopy observation were employed for morphological identification as described before. Afterward, DNA fingerprinting was employed to distinguish identical and non identical isolates. Screened isolated then confirmed at species level by the results of DNA sequence of the “Translation Elongation Factor 1-alpha” (TEF-1 $\alpha$ ) gene, which is taxonomically most informative part in this fungal genus.

#### **DNA extraction and gel electrophoresis**

The cultures were grown on potato dextrose broth (PDB) (Roth, Karlsruhe, Germany) in Erlenmeyer flasks in darkness at 25°C for 5 days. Mycelium was harvested by filtration onto sterile paper disks, froze in -70°C, freeze-dried in a freeze drier (Christ, Beta 1-8) and stored at room temperature till extracting genomic DNA. Fifty milligrams of lyophilized mycelium were ground in 2 mL Eppendorf tubes with round bottom containing 4-5 wolfram carbide spheres (diameter 3 mm, Retsch, Haan, Germany) in a reciprocal mill (Mixer Mill MM 200, Retsch, Haan, Germany). Genomic DNA was extracted by a CTAB method (Brandfass and Karlovsky, 2008). In order to check the efficiency of DNA extraction and the purity of extracted DNA, a 5  $\mu$ L DNA sample were mixed with 2  $\mu$ L Blue juice and loaded on a 0.8% (w/v) agarose gel (Cambrex, Rockland, ME, USA), prepared in TAE buffer (40 mM Tris, 1mM EDTA (ethylene diamine tetra acetic acid), pH set to 8.5) (both substances were obtained from Carl Roth, Karlsruhe, Germany). The electrophoresis was carried out at 60V for 60 min. After staining with ethidium bromide (0.5  $\mu$ g mL<sup>-1</sup>) and destaining in demineralized water (each for 10 min), DNA bands were visualized in UV light using a CCD camera (Vilber Lourmat, Marne La Vallee, France).

#### **DNA fingerprinting**

In the preliminary experiments, four arbitrary primers were tested with Random Amplified Polymorphic DNA (RAPD) with DNA extracted from pure cultures of *Fusarium*. List of primers can be seen on Table 2. Preliminary test determined the optimal concentration of the component in the PCR reaction mixture, amplification conditions and primer selection. Strong, unique, sharp and clearly reproducible bands were produced by primer C6.2\_introns\_2\_R and C7\_1\_seq\_f2. For further analysis C7\_1\_seq\_f2 was used.

Table 2. Primer and their sequences tested in preliminary RAPD analysis.

Primers	Sequence 5'-3'
C6.2_introns_2_R (C6_2_int_R3)	TGGCTCACGCAAGGGTGTAG
C2_1++inv_f	TGACGTCCTAGTTGATATCCTCG
C2_1++inv_r	ACCGTTATGATCAATAAATATTTGACCA
C7_1_seq_f2	AGGTTAGCTCGCGACTATCGGATC

In brief, the optimal condition of PCR as described below:

The amplification reaction was carried out in a volume of 25  $\mu$ L containing 18.45  $\mu$ L water, 2.5  $\mu$ L 10x reaction buffer (Bioline, Luckenwalde, Germany), 1.5  $\mu$ L dNTPs 150  $\mu$ M (Amersham Biosciences), 0.75  $\mu$ L of primer 0.3  $\mu$ M (GenXpress), 0.05  $\mu$ L of Taq DNA polymerase 0.25 U (New England BioLabs, Ipswich, MA, USA) and 1  $\mu$ L of DNA sample. For detection of any other kinds of DNA contaminants, a negative control of PCR-mix without any template DNA was used. The PCR program had initial denaturation at 94°C for 2 min, 2x45 cycles of: 20 s at 94°C, 37 s at 37°C, 20 s at 72°C and followed by final extension at 72°C for 5 min, storage 15°C for 5 min (peQ STAR Thermocycler). Eight  $\mu$ L of the PCR products, and 0.5  $\mu$ L of size marker (100bp), respectively, is mixed with 2  $\mu$ L of blue juice and pipetted into the slots. The electrophoresis is carried out at 60 V for 90 min. The EtBr-stained DNA fragments are visualized under a UV-transilluminator.

#### **Polymerase chain reaction and phylogenetic analysis**

Identification at species level was confirmed by the results of DNA sequence of (TEF-1 $\alpha$ ) gene, which is taxonomically most informative part in this fungal genus. Hot start PCR protocol was used to amplify the TEF-1 $\alpha$  gene region using the fungal specific primer set: ef1 (5'-ATGGGTAAGGA(A/G)GACAAGAC-3') and ef2 (5'GA(G/A)GTACCAGT(G/C)ATCATGTT-3') described by O'Donnell et al. (1998). The reaction mixture consisted of reaction buffer (16 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>; 67 mM Tris-HCl; 0.01% (v/v) Tween-20, pH: 8.8 at 25°C), 0.1 mM concentration of each of the four deoxynucleoside triphosphates (Bioline, Lükenwalde, Germany), 2 mM of MgCl<sub>4</sub>, 1.75 U of hot-start DNA polymerase (Immolase DNA Pol, Lükenwalde, Germany), 0.3  $\mu$ M concentration of each primer. Every reaction contained 1  $\mu$ L template DNA or 1  $\mu$ L sterile water as negative control. The PCR reactions were performed in a total volume of 25  $\mu$ L reactions with a peQ STAR Thermocycler (96 Universal Gradient). The cycling conditions were as follows: 1 cycle of 10 min at 95°C, 30 cycles of 60 s at 94°C (denaturalization), 45 s at 58.5°C (annealing), 60 s at 72°C (extension) and followed by a final extension cycle at 72°C for 5 min. The checking of the PCR product was performed on agarose gel electrophoresis as described above. The PCR products were purified using isopropanol precipitation and 5  $\mu$ L of the PCR product were sent to LCG Genomics GmbH (Ostendstr. 25. TGS Haus 8, 12459 Berlin, Germany) for sequencing. The sequences were, then, used as queries for *Fusarium*-ID v. 1.0 database (Geiser et al., 2004). In order to construct a phylogenetic tree sequences with the highest similarity to the query, together with some sequences retrieved from the GenBank database were aligned and constructed using the software MEGA 7 (Kumar *et al.*, 2016).



### 2.1.4. Mycotoxin screening from isolates

#### Molecular approach

PCR amplification of the FUM1, FUM8, FUM12 and TRI5 genes. To assess the ability of obtained *Fusarium* isolates to produce fumonisins and trichothecenes, the presence of the FUM and TRI genes was checked by PCR amplification using different primer sets as described below.

Table 3. Primers used for PCR amplification FUM1, FUM8, FUM12 and TRI5 genes.

Primer name	Primer sequence (5' - 3')	Source
FUM 1F	ACAAGTGTCTTGGGGTCCAGG	Proctor et al. (2004)
FUM 1R	GATGCTCTTTGGAAGTGGCCTACG	
FUM 8R	TGAGAAGGATGTTTCATGACGCC	
FUM 8F	TTGGAACCCAGCTCTGCCA	
FUM 12R	GATAAGCCAGATTCTGTCCGATGC	
FUM 12F	GCAGCGTACTACTCCGACCTTG	
TRI 5F	TCTATGGCCCAAGGACCTGTTTGA	Hallen-Adams et al. (2004)
TRI 5R	TGACCCAAACCATCCAGTTCTCCA	

PCR reaction components were the same as in the previous section. PCR cycling conditions were as follows: 95°C for 3 min, then 35 cycles of 94°C for 10 s, 57°C for 15 s, 72°C for 40 s. After electrophoresis, the presence or absence of PCR fragments at expected sizes was checked using a UV-transilluminator.

#### Mycotoxin screening with HPLC-MS/MS

##### Microorganisms and solid culture

*Fusarium* strains used in this study isolated from traditional tempeh inocula as described above. To examine their mycotoxin production, three agar plugs (5 mm of diameter) of each strains grown on potato dextrose agar (PDA, from Merck, Darmstadt, Germany) plates for 7 days at 25°C were inoculated on rice solid medium. Rice solid medium was prepared by autoclaving (120°C, 15 psi, 15 min; Tuttnauer- Systec/ 3870 EL) 3 and 5 mL tap water in 50 mL falcon tube (water content around 40 - 50%). The cultures were incubated at 21 °C (Schütt Lichtthermostat) for 25 days. Samples froze in -20°C, dried in a freeze drier (Christ, Beta 1-8), powdered in a reciprocal mill (Mixer Mill MM 200, Retsch, Haan, Germany) and stored in freezer at -20°C.

##### Mycotoxin extraction and HPLC-MS/MS analysis

Two g of each ground sample was weighed into falcons (50 mL) and 20 mL of acetonitrile, water and acetic acid (84:15:1) were added. The falcons were thoroughly vortexed until the powdered sample was completely wet. Then it was shaken overnight using a shaker (Adolf Kühner AG Basel, Switzerland) at 175 RPM at room temperature. The samples were centrifuged for ten minutes at 4500 RPM. After that, 1 mL of the supernatant was transferred into 2 mL Eppendorf tubes and evaporated in the vacum drier (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) at a temperature 40°C. For storage the residues were kept in the freezer at -20 °C. To re-dissolve the evaporated samples, 1 mL methanol/water (1:1) was added. After two hours at room temperature the samples were vortexed and sonicated for approximately ten seconds. Half of each sample (500 µL) was transferred to a new Eppendorf tube and stored at -20 °C until analysis. Those were not defatted due to the loss of BEA and ENNs during the defatting step and

were used to analyze beauvericin, fumonisin B1 and enniatin A, A1, B and B1. On the day of analysis the samples were mixed, centrifuged for five minutes at high speed (14800 RPM) and transferred to 200  $\mu$ L vials.

Mycotoxin separation and analysis was carried out by high pressure liquid chromatography coupled with electrospray ionization and tandem mass spectrometry detection. HPLC separation was carried out on a reverse-phase on a reverse phase Kinetex C18 column (50.0  $\times$  2.1 mm, 2.6  $\mu$ m particle size) coupled with a C18 security guard cartridge (4 mm  $\times$  2 mm i.d., both from Phenomenex, Aschaffenburg, Germany) maintained at a temperature 40°C with a methanol-water gradient, followed by electrospray ionization essentially as described (Ratzinger et al., 2009), and detected using ion trap 500 MS (Varian, Darmstadt, Germany) as described by Nutz et al. (2011). The mobile phase consisted of water with 5% acetonitrile (A) and methanol (B), both containing 7 mM of acetic acid. The injection volume was 10  $\mu$ L and the flow rate was set at 0.2 mL min<sup>-1</sup>. The binary gradient was from 40 – 98 % B. Pure analytical standards (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) in methanol/water (1:1) were used to construct calibration curves. The evaluation of the chromatogram were done with the MS Workstation version 6.9.1.

Table 4. MS/MS parameters for the determination of mycotoxin.

Mycotoxin	Ionisation modus	Precursor-Ion (m/z)	Product-Ion (m/z)
Zearalenone (ZEN)	ESI-	317	175 130.9
Fusarenone X (Fus X)	ESI-	353	204.8 262.9
Nivalenol (NIV)	ESI-	371	311 281
15-ADON (15-acetyldeoxynivalenol)	ESI-	337	172.9 150
3-ADON (3-acetyldeoxynivalenol)	ESI-	337	307 246.8
Deoxynivalenol (DON)	ESI-	355	295 265
Neosolaniol (NEO)	ESI+	400.5	215.3 185.3
Diacetoxyscirpenol (DAS)	ESI+	384.5	229.2 307.2
HT2-toxin (Tricothecene)	ESI+	442	215 263
T2-toxin (Tricothecene)	ESI+	484	185 215
Beauvericin (BEA)	ESI+	806.4	645.5 545.3 384.4
Fumonisin B1 (FB1)	ESI+	722.5	686.4 528.4 352.5
Enniatin A	ESI+	704.5	577.5 477.4 350.3

Enniatin A1	ESI+	690.6	563.4
			463.4
			350.5
Enniatin B	ESI+	662.6	549.4
			449.4
			336.4
Enniatin B1	ESI+	676.6	563.4
			463.4
			336.4

The limit of quantification (LOQ) values were 10 ng/g for ZEN, 1000 ng/g for Fus X, 200 ng/g for NIV, 250 ng/g for DON, 250 ng/g for 3-ADON, 1000 ng/g for 5-ADON, 100 ng/g for HT-2 toxin and T-2 toxin, 50 ng/g for DAS, 100 ng/g for NEO, 30 ng/g for FB1, 10 ng/g for BEA, 20 ng/g for enniatin A, 10 ng/g for enniatin A1, 36 ng/g for enniatin B and 110 ng/g for enniatin B1. Limit of detection (LOD) values were 5 ng/g for ZEN, 500 ng/g for Fus X, 100 ng/g for NIV, 700 ng/g for 15-ADON, 30 ng/g for DON, 100 ng/g for 3-ADON, 30 ng/g for HT-2 toxin and T-2 toxin, 10 ng/g for DAS, 30 ng/g for NEO, 30 ng/g for FB1, 3 ng/g for BEA, 8 ng/g for enniatin A, 3 ng/g for enniatin A1, 20 ng/g for enniatin B and 88 ng/g for enniatin B1.

### 2.1.5. Toxicity test (brine shrimp)

Growing brine shrimps: Three spoons of the ready-to-use shrimp egg/salt-mixture were dissolved into in a big glass petridish contained 500 mL tap-water. Then, stirred gently with a spatula to dissolve the salt. Petri-dish was placed under light at room temperature and incubate the eggs for 48 hours.

Preparation of crude mycotoxin solutions: Supernatants (20 mL) from each isolates (from part mycotoxin screening above) were used for this test. Solvent was initially evaporated in the rotary evaporator (Büchi 011, Switzerland, at 35°C) equipped with a vacuum pump V-700 and vacuum controller V-855 (Büchi, Switzerland, at 150 mbar) and then in the SpeedVac (Christ RVC 2-25 CD plus, 35°C, 30 mbar) to obtain an concentrated crude extract and weighed.

Crude toxins extract were prepared on concentrations of 20, 40, 60, 80 and 100 ppm in 100% methanol. One hundred microliter of each crude mycotoxin solution was transferred with micropipette into cavities of the 96-well microplate microplate (Sarstedt AG & Co., Nuembrecht, Germany) in five repetitions, methanol without mycotoxins was used as negative control and the methanol was evaporated under a fumehood.

Toxicity assay: The brine shrimp (*Artemia salina*) bioassay procedure followed the microwell plate method. The brine shrimp culture was poured into a breaker and illuminate the glass from one side with a flashlight. Light source was aimed for attracting the shrimps to one side of the breaker. Two hundred microliter of the solution with 10-20 brine shrimps was transferred into cavities of microplate using 1000 µL tips. Dead brine shrimps were counted with stereo microscope for 0, 24 and 48 hours observations. The surviving shrimps were killed by freezing at -20°C for at least 12 hours and count the total number of brine shrimps with stereo microscope.

LD<sub>50</sub> is the amount of a material, given all at once, which causes the death of 50% (one half) of a group of test animals. The LD<sub>50</sub> is one way to measure the short-term poisoning potential (acute

toxicity) of a material. Mean of mortality percentage was calculated in excel program. The LD<sub>50</sub> values as concentrations at which half of the animal survived was estimated with 4 Parameter Logistic (4PL) nonlinear regression model using Sigmaplot ver11 program.

## Part II (Detection of *Fusarium* and mycotoxins in traditional tempeh)

### 2.2.1. Traditional tempeh sampling

Traditional tempeh samples were collected in centre of tempeh producer where *Fusarium* found positively before in their traditional inocula (on end of 2015). Samples were transported in sterile plastic container to nearby laboratory in each location. Samples were then freeze dried (Supermodulyo, Japan), powdered and maintained at 4°C until analysis in refrigerator (Sanyo, Japan). To determinate the water content of the different samples 5 g were weighed in falcons and analyzed with water content analyzer (Ohaus MB45, Germany). Details information of sampling can be seen on Table 5.

Table 5. Information of traditional tempeh sampling.

Areas	Samples	Amount	Year
Banyumas	Traditional fresh tempeh	39	2015
Kebumen	Traditional fresh tempeh	37	2015
Yogyakarta	Traditional fresh tempeh	41	2015
Banjarnegara	Traditional fresh tempeh	33	2015
Gombong	Traditional fresh tempeh	33	2015
Kudus	Traditional fresh tempeh	31	2015
Total of sample		214	

### 2.2.2. Detection and quantification of *Fusarium* with qPCR from traditional tempeh

This method is based on the classical Polymerase Chain Reaction (PCR) and the amplification of DNA. The difference of Real Time PCR (qPCR) is the possibility to quantify the DNA. The procedure runs through up to 40 cycles and each cycle passes through three different steps: Denaturation, Annealing and Elongation (Dorak, 2006). Detection of two *Fusarium* species (*F. proliferatum* and *F. solani*) were the main focus in this analysis.

#### Preparation of DNA standards for qPCR

DNA quantification was carried out by densitometry of DNA bands after electrophoretic separation; because calculation of DNA concentration from absorbance of the solution in UV light is error-prone (Wilfinger et al., 1997). For this purpose, a range of dilutions of genomic DNA was separated electrophoretically on 1.2% (w/v) agarose gels prepared in TAE buffer (40 mM Tris, 1 mM EDTA, pH set to 8.5 with acetic acid, Riedel-de Haen, Hanover, Germany) along with a dilution series of lambda phage DNA of known concentration (methylated, from *Escherichia coli* host strain W3110). The electrophoresis was carried out at 60 V for 60 min. After staining with ethidium bromide (0.5 µg mL<sup>-1</sup>) and destaining in demineralized water, DNA bands were visualized in UV light using a CCD camera (Vilber Lourmat, Marne La Vallee, France). The electrophoretic bands within the linear range of the densitometry were used for quantification using

Multi Analyst-Software (BioRad, Hercules, CA, USA). Standards for qPCR were prepared in a range of 0.056 pg  $\mu\text{L}^{-1}$  to 1111 pg  $\mu\text{L}^{-1}$  by consecutive 1:3 dilutions, starting with the highest standard, in sterile distilled water.

#### DNA extraction of samples

DNA was extracted from 60 mg samples following a protocol of Brandfass and Karlovsky (2008). Extracted samples were diluted to approximately 10-15 ng  $\mu\text{L}^{-1}$  of total DNA based on comparison of the intensity of DNA bands after agarose electrophoresis with DNA standards, the dilutions were recorded and DNA was subjected to qPCR. A thermocycler (CFX384TM Real-Time System, C1000TM Thermal Cycler, BioRad, USA) with 384-well microplates (Kisker Biotech GmbH, Steinfurt, Germany) was used for the qPCR analysis.

#### Inhibition test

To find out if there is a natural inhibition from tempeh material, one test with the real time PCR was done with *Verticillium longisporum*. For that purpose the forward and reverse primer OLG 70 (CAGCGAAACGCGATATGTAG) and OLG 71 (GGCTTGTAGGGGGTTTAGA) with 261 bp were used. Those were developed by Eynck et al. (2007). For that test some samples were used with a dilution of each 1:10; 1:20, 1:50, and 1:100 and three replications each. Fifty pg of the fungal DNA *V. longisporum* was added. Furthermore there was a negative control with bi-distilled water. The result showed there is no natural inhibition from tempeh.

#### Detection of *F. solani*

Two kind of primers were tested their specificity. First primer was Fsol1 (CTCATCAACCC TGTGAACATACC) and Fsol2 (ATGCCAGAGCCAAGAGATCC) based on Bernal-martinez et al. (2012), second primer was TEF-Fs4f (ATCGGCCACGTCGACTCT) and TEF-FS4r (GG CGTCTGTTGATTGTTAGC) based on Arif et al. (2012). To check specificity of the primer, 63 DNA of *Fusarium* from different species were used (can be seen in Table 6, those DNA were kindly provided by Dr. Katharina Pfohl, and Maria Vinas). The result showed that the primer TEF-Fs4f has better specificity than Fsol primer. The melting temperature for the species specific evaluation is at  $87 \pm 0.5$  °C. But there was unspecific peak especially in low concentrations of DNA, then optimization was continued on annealing temperature (50-63°C),  $\text{MgCl}_2$  concentrations (2, 2.5, 3 and 3.5 mM), BSA, dNTP and primer concentration (BSA 2, 3 and 4 mg  $\text{mL}^{-1}$  (dNTP 300 mM and primer 0.4  $\mu\text{M}$ ) and BSA 2, 3 and 4 mg  $\text{mL}^{-1}$  (dNTP 400 mM and primer 0.5  $\mu\text{M}$ )). Initial experiment showed that that 63°C of annealing temperature, 2.5 mM  $\text{MgCl}_2$  and BSA 2 mg/mL (dNTP 300 mM and primer 0.4  $\mu\text{M}$ ) found as the optimal qPCR conditions.

Table 6. Fungal strains used in the specificity test.

<i>Fusarium</i> species	Source
<i>F. avenaceum</i> DSM 62161 <sup>1)</sup>	Maria Vinas
<i>F. oxysporum</i> DSM 62296 <sup>1)</sup>	Maria Vinas
<i>F. tricinctum</i> 063	Maria Vinas
<i>F. verticilioides</i> Fv. Ita 1	Maria Vinas
<i>F. proliferatum</i> DSM 62261 <sup>1)</sup>	Maria Vinas
<i>F. subglutinans</i> CBS 215.76 <sup>3)</sup>	Maria Vinas

<i>F. fujikuroi</i> IMI 58289	Maria Vinas
<i>F. oxysporum</i> fsp asp BBA 62286 <sup>2)</sup>	Maria Vinas
<i>F. oxysporum</i> fsp lycop CBS 16730 <sup>3)</sup>	Maria Vinas
<i>F. avenaceum</i> 649 11P	Maria Vinas
<i>F. oxysporum</i> fsp longl Foc 5a <sup>4)</sup>	Maria Vinas
<i>F. oxysporum</i> F11.1 <sup>4)</sup>	Maria Vinas
<i>F. avenaceum</i> DSM 21724 <sup>1)</sup>	Maria Vinas
<i>F. avenaceum</i> CBS 121.73 <sup>3)</sup>	Maria Vinas
<i>F. subglutinans</i> CBS 215.76 <sup>3)</sup>	Maria Vinas
<i>F. avenaceum</i> Fa1.2	Dr. Katharina Pfohl
<i>F. avenaceum</i> Fa9	Dr. Katharina Pfohl
<i>F. avenaceum</i> Fa3	Dr. Katharina Pfohl
<i>F. avenaceum</i> Fa1.3	Dr. Katharina Pfohl
<i>F. oxysporum</i> Foxy 5 <sup>4)</sup>	Dr. Katharina Pfohl
<i>F. oxysporum</i> F oxy 6 <sup>4)</sup>	Dr. Katharina Pfohl
<i>F. oxysporum</i> F oxy 119S <sup>4)</sup>	Dr. Katharina Pfohl
<i>F. subglutinans</i> F sub7 <sup>4)</sup>	Dr. Katharina Pfohl
<i>F. subglutinans</i> F sub 220 <sup>4)</sup>	Dr. Katharina Pfohl
<i>F. subglutinans</i> F sub 6 <sup>4)</sup>	Dr. Katharina Pfohl
<i>F. subglutinans</i> F sub 2215 <sup>4)</sup>	Dr. Katharina Pfohl
<i>F. subglutinans</i> F sub 2202 <sup>4)</sup>	Dr. Katharina Pfohl
<i>Microdochium nivale</i> 10	Dr. Katharina Pfohl
<i>M. nivale</i> GF-B2	Dr. Katharina Pfohl
<i>M. nivale</i> GF-B3	Dr. Katharina Pfohl
<i>M. nivale</i> GF-B8	Dr. Katharina Pfohl
<i>M. nivale</i> GF-B11	Dr. Katharina Pfohl
<i>F. culmorum</i> Fc 21	Dr. Katharina Pfohl
<i>F. culmorum</i> Fc 40	Dr. Katharina Pfohl
<i>F. culmorum</i> Fc 11	Dr. Katharina Pfohl
<i>F. culmorum</i> Fc 31	Dr. Katharina Pfohl
<i>F. culmorum</i> Fc 22	Dr. Katharina Pfohl
<i>Microdochiae nivae</i> GN 35	Dr. Katharina Pfohl
<i>M. nivale</i> GN 7	Dr. Katharina Pfohl
<i>M. nivale</i> GN 25	Dr. Katharina Pfohl
<i>M. nivale</i> GN 36	Dr. Katharina Pfohl
<i>M. nivale</i> GN 12	Dr. Katharina Pfohl
<i>F. proliferatum</i> Fpro 7	Dr. Katharina Pfohl
<i>F. proliferatum</i> Fpro 12	Dr. Katharina Pfohl
<i>F. proliferatum</i> Fpro11	Dr. Katharina Pfohl
<i>F. proliferatum</i> Fpro2	Dr. Katharina Pfohl
<i>F. proliferatum</i> Fpro5	Dr. Katharina Pfohl
<i>F. verticillioides</i> Fv2	Dr. Katharina Pfohl
<i>F. verticillioides</i> Fv3	Dr. Katharina Pfohl
<i>F. verticillioides</i> Fverti 1:10	Dr. Katharina Pfohl
<i>F. verticillioides</i> Fv IFA 420	Dr. Katharina Pfohl
<i>F. verticillioides</i> Fv3	Dr. Katharina Pfohl
<i>F. verticillioides</i> Fv IFA 429	Dr. Katharina Pfohl
<i>F. graminearum</i> FG 62722	Dr. Katharina Pfohl
<i>F. graminearum</i> FG 62217	Dr. Katharina Pfohl
<i>F. graminearum</i> FG 83699	Dr. Katharina Pfohl
<i>F. graminearum</i> FG 67638	Dr. Katharina Pfohl

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<i>F. avenaceum</i> FA5	Dr. Katharina Pfohl
<i>F. avenaceum</i> FA 118	Dr. Katharina Pfohl
<i>F. avenaceum</i> FA 7	Dr. Katharina Pfohl
<i>F. avenaceum</i> FA 2	Dr. Katharina Pfohl
<i>F. avenaceum</i> FA 39	Dr. Katharina Pfohl
<i>F. solani</i> type 1 FS GS21.02 <sup>5)</sup>	Riyan Anggriawan
<i>F. solani</i> type 2 FS B25.07 <sup>5)</sup>	Riyan Anggriawan
<i>F. solani</i> type 1 FS GS21.02 <sup>5)</sup>	Riyan Anggriawan
<i>F. solani</i> type 2 FS B25.07 <sup>5)</sup>	Riyan Anggriawan

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Note:

<sup>1)</sup>Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany, via E. Moller.

<sup>2)</sup>Nirenberg (BBA Berlin, Germany) via E. Moller.

<sup>3)</sup>Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.

<sup>4)</sup>Department of Crop Sciences, University of Gottingen, Germany

<sup>5)</sup>This present research.

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In brief, optimal assay described as follows:

The reaction mixture contained reaction buffer (16 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>; 67 mM Tris-HCl; 0.01% (v/v) Tween-20, pH 8.8 at 25°C, Bioline, Lünenwalde, Germany), 0.1 mM of each of the four deoxynucleoside triphosphates (dNTPs; Bioline, Lünenwalde, Germany), 2.5 mM MgCl<sub>2</sub>, 0.025U of *Taq* DNA polymerase (BIOTaq, Bioline, Lünenwalde, Germany), 0.4 μM of each primer, 0.1x SYBR Green I (Invitrogen, Karlsruhe, Germany). The cycling conditions were performed according to following protocol: 1 cycle of 2 min at 95°C (initial denaturation), 39 cycles of 30 s at 94°C (denaturation), 30 s at 72°C (annealing), 40 s at 72°C (extension) and followed by a final extension cycle at 72°C for 5 min. Fluorescent data were obtained during the annealing phase to construct a melting curve at the end of assay. The qPCR was completed by running a melting curve analysis.

#### **Detection of *F. proliferatum***

To quantitate the DNA of *F. proliferatum* the forward and reverse primer Fp3-F (CGGCCACCAGAGGATGTG) and Fp4-R (CAACACGAATCGCTTCCTGAC) with a size of 230 bp were used. They were developed by Jurado et al. (2006). *F. proliferatum* strain DSM 62267 was used to generate the standard curve in a dilution series. The melting temperature for the species specific evaluation is at 89 ± 0.5 °C. The species specific real time assay based on the method from Nutz et al. (2011).

In brief, the assay described as follows:

The reaction mixture contained reaction buffer (16 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>; 67 mM Tris-HCl; 0.01% (v/v) Tween-20, pH 8.8 at 25°C, Bioline, Lünenwalde, Germany), 0.1 mM of each of the four deoxynucleoside triphosphates (dNTPs; Bioline, Lünenwalde, Germany), 2.5 mM MgCl<sub>2</sub>, 0.4 U of *Taq* DNA polymerase (BIOTaq, Bioline, Lünenwalde, Germany), 0.6 μM of each primer, 0.1x SYBR Green I (Invitrogen, Karlsruhe, Germany). The cycling conditions were performed according to following protocol: initial denaturation for 1.5 min at 95 °C; followed by 35 cycles with 35 s at 95 °C, 30 s at 64 °C, and 30 s at 72 °C, with fluorescence measurement during the annealing step of each cycle; and a final elongation of 5 min at 72 °C. The qPCR was completed by running a melting curve analysis.

### 2.2.3. Extraction of tempeh and HPLC analysis (Fumonisin, Beauvericin and Enniatins)

In the beginning, inhibition test was done before extracting the whole samples. Inhibition test was aimed to know the effect of tempeh material to detection of mycotoxin. The result showed there was no inhibition effect from the samples.

For the extraction of the mycotoxins from tempeh, 2 g of each ground sample was weighed into falcons (50 mL) and 20 mL of acetonitrile and water (84:16) were added. The falcons were thoroughly vortexed until the powdered tempeh was completely wet. Then it was shaken overnight using a shaker (Adolf Kühner AG Basel, Switzerland) at 175 RPM at room temperature. The samples were centrifuged for ten minutes at 4500 RPM. After that, 1 mL of the supernatant was transferred into 2 mL Eppendorf tubes and evaporated in the vacuum drier (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) at a temperature 40°C. For storage the residues were kept in the freezer at -20 °C. To re-dissolve the evaporated samples, 1 mL methanol/water (1:1) was added. After two hours at room temperature the samples were vortexed and sonicated for approximately ten seconds. Half of each sample (500 µL) was transferred to a new Eppendorf tube and stored at -20 °C until analysis. On the day of analysis the samples were mixed, centrifuged for five minutes at high speed (14800 RPM) and transferred to 200 µL vials.

Mycotoxin separation and analysis was carried out by high pressure liquid chromatography coupled with electrospray ionization and tandem mass spectrometry detection. HPLC separation was carried out on a reverse-phase on a reverse phase Kinetex C18 column (50.0 × 2.1 mm, 2.6 µm particle size) coupled with a C18 security guard cartridge (4 mm × 2 mm i.d., both from Phenomenex, Aschaffenburg, Germany) maintained at a temperature 40°C with a methanol-water gradient, followed by electrospray ionization essentially as described (Ratzinger et al., 2009), and detected using ion trap 500MS (Varian, Darmstadt, Germany) as described by Nutz et al. (2011). The mobile phase consisted of water with 5% acetonitrile (A) and methanol (B), both containing 7 mM of acetic acid. The injection volume was 10 µL and the flow rate was set at 0.2 mL min<sup>-1</sup>. The binary gradient was from 40 – 98 % B. ESI was configured in the positive ionization mode. The mass transitions of FB1 were described by Bartók et al. (2006) and for BEA Döll (2013) and the Enniatins by Adejumo et al (2007). Pure analytical standards (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) in methanol/water (1:1) were used to construct calibration curves. The limit of quantification (LOQ) was : 30 ng/g for FB1, 10 ng/g for BEA, 20 ng/g for enniatin A, 10 ng/g for enniatin A1, 36 ng/g for enniatin B and 110 ng/g for enniatin B1. Limit of detection (LOD) values were 30 ng/g for FB1, 3 ng/g for BEA, 8 ng/g for enniatin A, 3 ng/g for enniatin A1, 20 ng/g for enniatin B and 88 ng/g for enniatin B1.



### Part III (Risk assessment part)

#### 2.3.1. Tempeh quality assessment

Tempe making was done as follows: Sixty gram of soybeans as raw materials were washed and boiled for 30 minutes (ratio tap water:raw materials = 5:1), then dehulled manually. The raw materials were soaked in tap water overnight (18 hours) at room temperature (ratio tap water:raw material = 3:1). Furthermore, soybeans were boiled again 30 minutes (ratio tap water:raw material = 5:1), drained and dried-aired until ready at room temperature and inoculated with 3 mL of spore suspension ( $10^6$  spores/mL) *R. microsporus* var. *oligosporus* WJBE8.87. After inoculation, raw materials were placed on petridish and incubated at a temperature 30°C for 48 hours (incubator ThermoFisher). Fresh tempeh directly used for all quality test except for amino acid composition analysis. For amino acid composition, samples then froze at -20°C (Sanyo, Japan) and dried with freeze dryer (Supermodulyo, Japan). Samples were powdered (LG blender) and kept in -20°C until extraction for UPLC-MS/MS analysis.

Treatment consists of: negative control (tempeh made only with pure *R. microsporus* var. *oligosporus* WJBE8.87), and tempeh made with *R. microsporus* var. *oligosporus* plus contamination of *F. proliferatum* BJ09.05.

To test whether the consumer can recognize contaminated tempeh, some physico-chemical and organoleptic test were done (control (normal tempeh/non-contaminated) and contaminated tempeh). Tests carried out include compactness observation, texture, color, pH, protein content and content of amino acid determinants. Detailed testing can be seen in the following Table 7.

Table 7. Quality test on tempeh.

No	Quality test	Method
1	Compactness observation	Stereo-microscopy analysis
2	Texture (with texture analyzer Rheometer)	Rubenthaler et al., (1990)
3	Color ( $L^*$ , $a^*$ , and $b^*$ ) (with Minolta Chromameter)	Minolta, Inc. (2013)
4	pH and protein content	AOAC (2001)
5	Amino acid composition (with Waters UPLC-MS/MS)	AOAC (2005)

#### 2.3.2. Risk assessment traditional tempeh consumption in Indonesia

Risk assessment is the process of quantifying the magnitude and exposure, or probability, of a harmful effect on individuals or populations from specific agents or activities (Kuiper-Goodman, 2004). Data of mycotoxin contamination taken from research part II. We determined the exposure levels of Indonesian infants, children, and adult to fumonisin B1 through traditional tempeh consumption.

$$PDI_m = (\mu c \times Cc) / Bw$$

PDI<sub>m</sub> : Probable daily intake for mycotoxin ( $\mu\text{g} \cdot \text{Kg}^{-1} \text{ bw} \cdot \text{day}^{-1}$ )

$\mu c$  : Mean of mycotoxin

Cc : Average consumption of traditional tempeh in Indonesia

Bw : Body weight for the population groups (infants, children, and adults)

The risk characterization for non-genotoxic and non-carcinogenic mycotoxins (%Tolerable Daily Intake (TDI)) was determined by dividing the PDI<sub>m</sub> with the TDI<sub>m</sub> and multiplying the product with 100 as shown in the equation below.

$$\%TDI = (PDI_m/TDI_m) \times 100$$

Probable Daily Intake (PDI<sub>m</sub>) (Assumed 10 kg, 25 kg (Rodriguez-Carasco et al., 2013) and 60 kg (Liu and Wu, 2010) body weight for infants, children and adults, respectively) calculated by multiplying the fumonisin B1 concentration (mean) in traditional tempeh by consumption rates of traditional tempeh in Indonesia (20.7 g/person/day) as estimated by the Statistics Indonesia (BPS, 2014) and divided by body weight of consumers.

### 3.1. Results of Part I (*Fusarium* isolates identification and their mycotoxin screening)

Identification of *Fusarium* isolates was performed according to the combination of morphology, and molecular characters. There are limits on the use of morphological characters for identification of *Fusarium* spp. These limitations increase when differentiation is within members belonging to a species complex with highly similar morphology. In this study, molecular techniques based mostly on DNA sequencing of genes have supported morphological identification of *Fusarium* species. Nucleotide sequencing of genes is performed particularly in informative parts of genome such as translation elongation factor 1-alpha (TEF-1 $\alpha$ ). In this work, typical *Fusarium* structures were utilized to identify the recovered isolates based on the morphological descriptions with several parameters (colony, conidia and conidiophores, conidiogenous cells, perithecia, and chlamydo spores) according to Booth (1971), Gerlach and Nirenberg (1982), Nelson *et al.* (1983), Burgess *et al.* (1994), and Leslie and Summerell (2006). At least 20 microconidia and macroconidia of each isolate were selected randomly for measurement. Molecular information obtained from the DNA sequence of TEF-1 $\alpha$  and phylogenetic analysis was then employed to confirm the morphological identification and probably distinguish closely related strains.

#### ***Fusarium* identification from traditional tempeh inocula**

##### **Morphological characteristic**

In total, 650 fungal isolates recovered from 345 traditional tempeh inocula (*usar*). Traditional inocula (*usar*) were collected from many markets and center of tempeh producers in 9 different areas in Central Java and Yogyakarta provinces, Indonesia (on 2015). *Rhizopus* was the most frequently detected genera, found in 90 % of total samples. We also found contamination of *Fusarium* and this finding become our focus in present study concerning food safety problem. Distinct fungal colonies temporarily identified as *Fusarium* were transferred on PDA and recorded for colony morphology, radial growth, and pigmentation based on the visual inspection of the plates. About 55 pure isolates of *Fusarium* sp. were collected and observed via microscopy approach. Detail of morphological result can be seen in Table 8. We found two main species of *Fusarium*, they are suspected as *F. solani* and *F. proliferatum*. Morphological descriptions of these isolates are also described below. Morphological structures are shown in Figure 1.

##### *F. solani*

##### Macroscopic characteristics:

Colonies on PDA at 25°C reach a diameter of 31-39 mm 4 days. Aerial mycelium sares or dense and floccose, sometimes leathery, greyish-white, cream to buff, conidial slime formed in sporodochia or pionnotes. Colony pigment on the reverse side of the plate was yellowish white to olive yellow or pale yellow and turning brownish yellow in aged cultures. Odor was not perceptible from the cultures.

##### Microscopic characteristics:

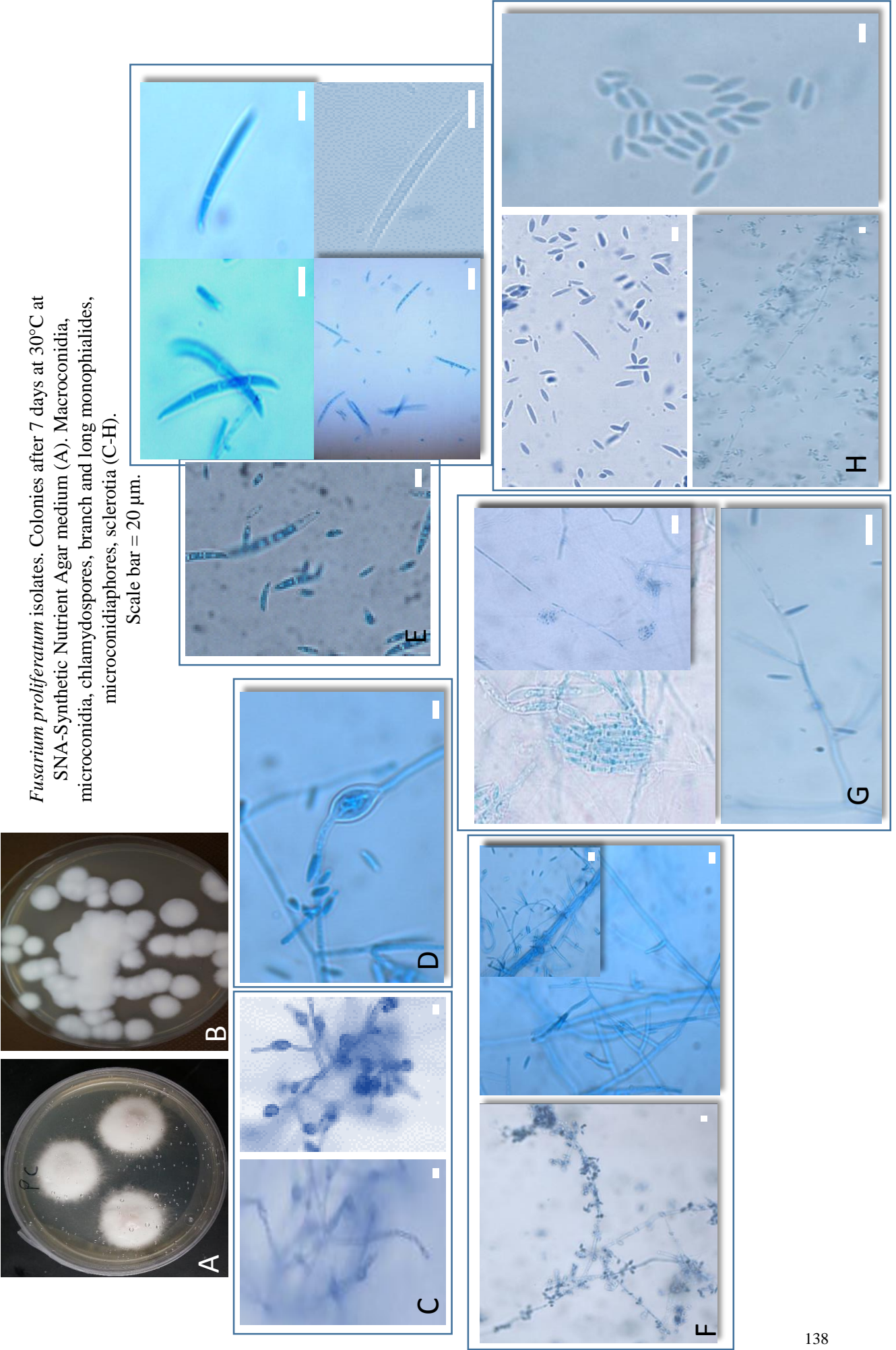
Conidiophores on SNA were unbranched or branched bearing monophialides. Macroconidia formed after 3-5 days from shorty branched conidiophores which may form

sporodochia, usually three septates (1-4 septates), relatively wide, straight, stout and robust. Cream, blue or green sporodochia are common (cream sporodochia usually contain more macroconidia than do the blue or green ones). Apical cells were blunt and rounded. Basal cells have a distinct foot shape or be poorly developed, straight to almost cylindrical, usually with a notched or a rounded end. Microconidia were abundant, oval, ellipsoid, reniform and fusiform with 0 or 1 to occasionally 2 septates. Conidiogenous cells were monophialides, often quite long. Chlamydospores were abundant, hyaline, smooth or rough-walled, globose to ovoid, in hyphae or conidial cells, either terminal, on lateral branches, intercalary or in chains.

Table 8. Morphological characteristics of *Fusarium* spp. isolated from traditional tempeh inocula.

Characters		Isolates suspected as	
		<i>Fusarium solani</i>	<i>Fusarium proliferatum</i>
Macroscopic characteristics			
Colony diameter, average (range)		3.4 cm (3.1-3.9 cm)	3.7 cm (3.5-4.1 cm)
Pigmentation on PDA		White to yellow or green	White to purple violet
Aerial mycelium		Present	Present
Radial growth rate, average (range)		8 mm (8.1-8.7 mm)	8.5 mm (8.3-8.9 mm)
Optimal growth temperature		25°C	25°C
Microscopic characteristics			
Sporodochia	Shape	Cream, or green sporodochia are common in the agar and contain numerous macroconidia	Tan to pale orange sporodochia often are produced infrequently
Microconidia	Shape	Oval, ellipsoid, reniform and fusiform	Long chain, false head
	Septa	0-1	0
	Length (µm)	14-18	15-22
	Width (µm)	4.5-9	3.5-8
Macroconidia	General morphology	Relatively wide, straight, stout	Slender, thin-walled, relatively straight
	Apical cell	Blunt and rounded	Curved
	Basal cell	Have a distinct foot shape or be poorly developed, straight to almost cylindrical	Poorly developed
	Septa	1-4	2-5
	Length (µm)	21-55	19-69
	Width (µm)	6-12	5-10
Chlamydospores		Present	Present only in few isolates
Sclerotia		Absent	Present, blue-black color
Conidiogenous cell		Long monophialides	Long and branched mono or polyphialides
Number of isolates examined		30	25
Note: Colonies on PDA at 25°C in intermittent light after 4 days. Colony color were determined by observing the upper surface of the colony. Pigmentation were determined by observing the lower surface of the colony.			

Figure 1. Various morphological structures in *Fusarium* spp isolated from traditional tempoh inocula (*usar*).







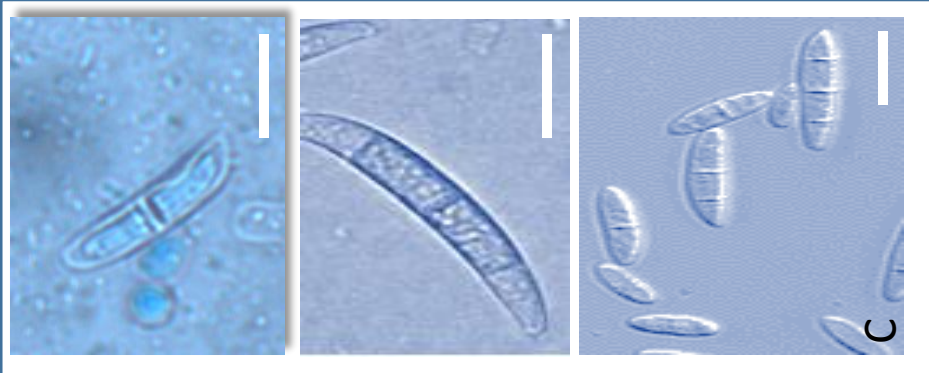
A



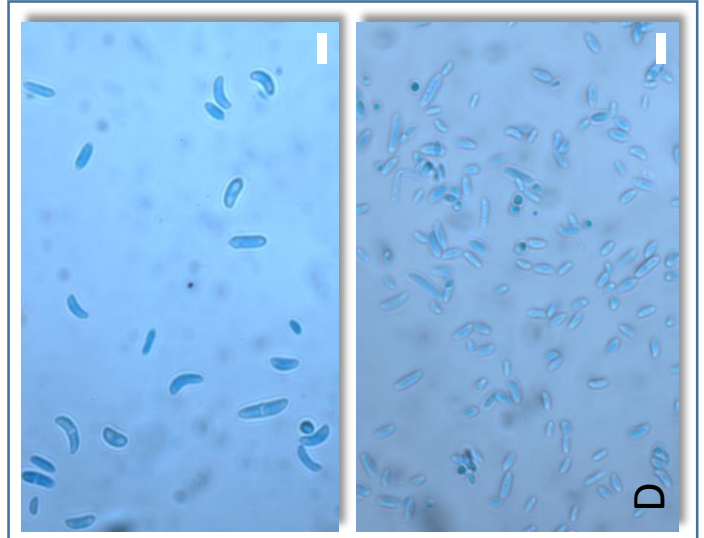
B

*Fusarium solani* isolates. Colonies after 5 days at 25°C at SNA-Synthetic Nutrient Agar medium (A-B). Macroconidia, microconidia, chlamydospores, long monophialides (C-F).

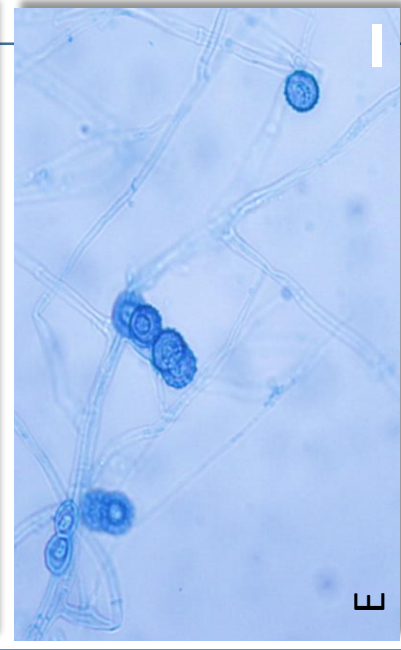
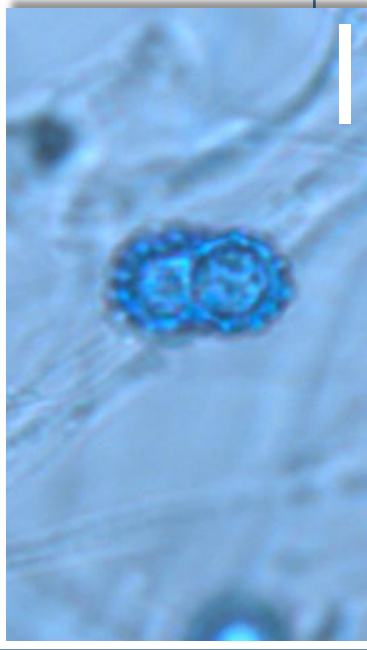
Scale bar = 20 µm.



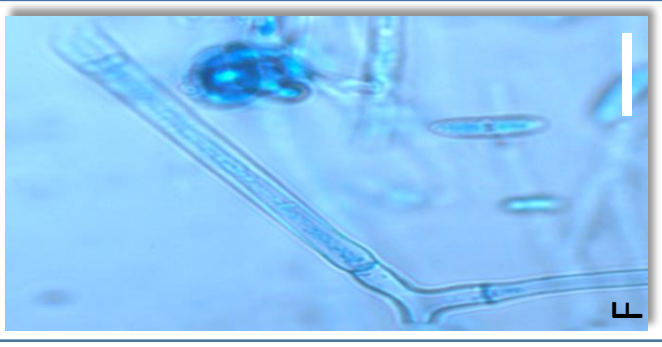
C



D



E



F



*F. proliferatum*

Macroscopic characteristics:

Colonies on PDA at 25°C reach a diameter of 35-41 mm 4 days. The abundant aerial mycelium initially was white but may become purple-violet with age, with or without concentric rings. Sporodochia were present as discrete entities or nearly confluent over portions of the colony. Violet pigments usually were produced in the agar, but with overall pigmentation varying in intensity from nearly colorless to almost black. Blue-black sclerotia develop in some isolates.

Microscopic characteristics:

Conidiophores on SNA were branched bearing mono- and polyphialides. Macroconidia were hyaline, delicate, slightly sickle-shaped, slender, relatively straight, thin-walled, 2-5 septates, with curved apical cell and poorly developed basal cell. Fresh cultures usually produce large numbers of macroconidia in sporodochia.

Microconidia abundant, clavate shaped with a flattened base; 0- septate. The abundance of macroconidia varies since this character can be lost in this species following repeated subcultures. Pyriform microconidia also may occur but generally were rare. Microconidia found in chains of varying, but usually moderate, length or false heads, from mono- and polyphialidic conidiogenous cells. Club-shaped with a flattened base and 0-septate. Microconidia were produced mostly from polyphialides. Chlamydospores were big globose to ovoid, very rare and only found in few isolates.

**Molecular result**

To confirm morphological identification, representative isolates were observed with molecular approach. First, DNA fingerprinting was employed to distinguish identical and non identical isolates. Second, nonidentical isolates with distinct band were selected and confirmed at species level by the results of DNA sequence of the TEF gene, which is taxonomically most informative part in this fungal genus.

**DNA fingerprinting and sequencing of *Fusarium* isolates**

In the preliminary experiments, four arbitrary primers were tested with Random Amplified Polymorphic DNA (RAPD) with DNA extracted from pure cultures. A preliminary test determined the optimal concentration of the component in the PCR reaction mixture, amplification conditions and primer selection. Strong, unique, sharp and clearly reproducible bands were produced by primer C6.2\_introns\_2\_R and C7\_1seq. For further analysis, C7\_1seq was used. In brief, the RAPD patterns of 55 pure *Fusarium* isolates obtained with the primer, and 12 distinct RAPD patterns were acquired. RAPD typing was capable of producing discriminating DNA fingerprints of *Fusarium* isolates and indicated there were genetic differences among them. The RAPD patterns of *Fusarium* isolates obtained with the primer are shown partly in Figure 2.

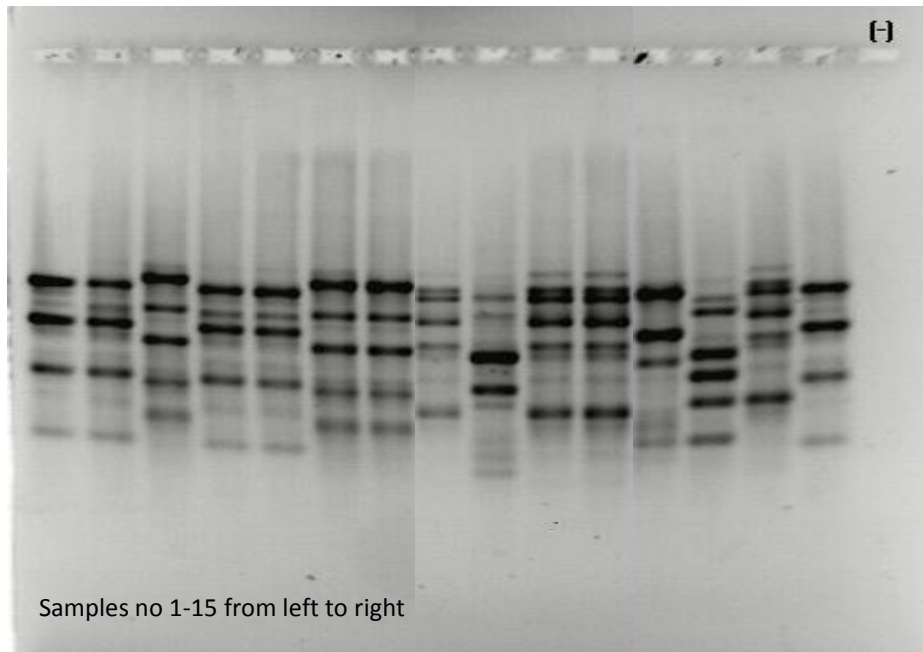


Figure 2. Some results of DNA fingerprinting from *Fusarium* isolates.

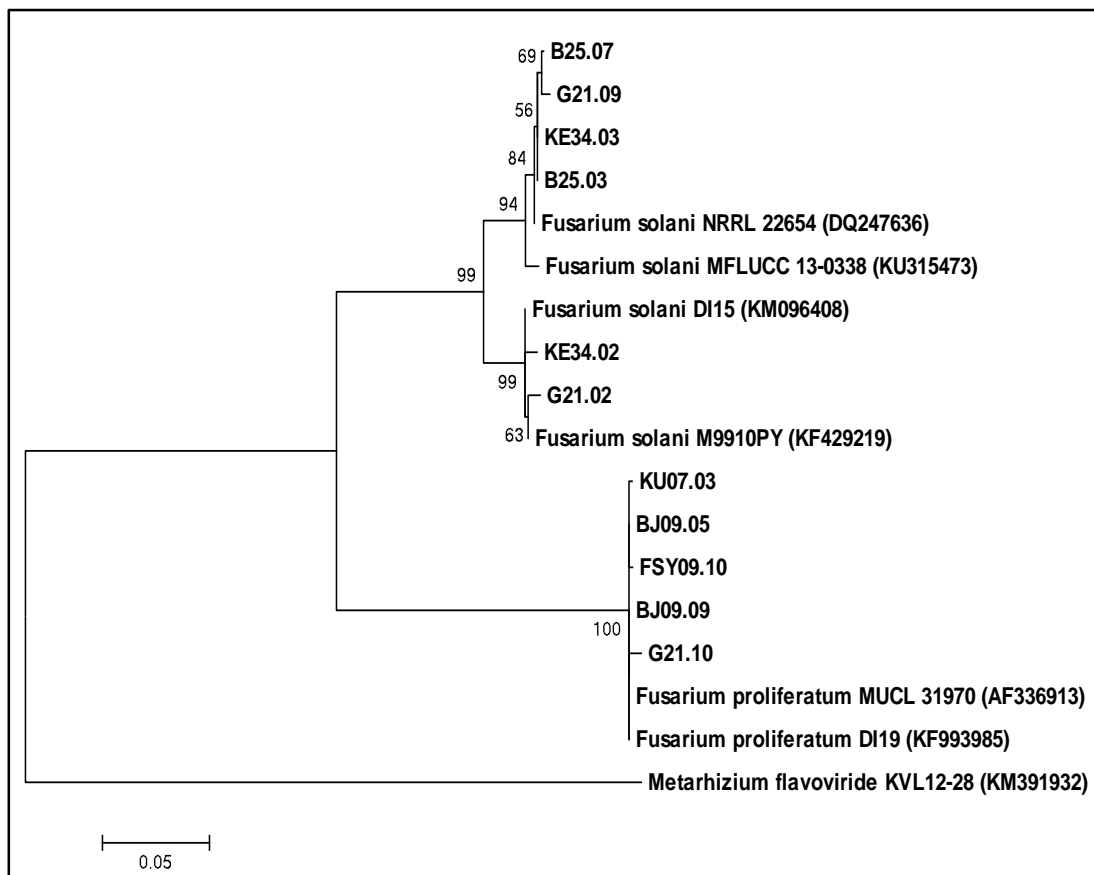


Figure 3. The tree was inferred from TEF-1 $\alpha$  sequence and constructed using maximum likelihood method. Numbers at nodes indicate levels of bootstrap support calculated from 1000 trees.



Twelve representative isolates from RAPD selection were sequenced based on the TEF gene which has been widely used for species identification. Thermal PCR conditions suggested by Geiser et al. (2004) and Paul et al. (2010), lead to a mixture of specific and non-specific products. The situations were achieved by running a gradient PCR in the range of 53-63°C under “Hot start PCR”. Hot start PCR protocol was used to amplify the TEF-1a gene region using the fungal-specific primer set: ef1 (5'-ATGGGTAAGGA(A/G)GACAAGAC-3') and ef2 (5'GA(G/A)GTACCAGT(G/C)ATCATGTT-3') described by O'Donnell et al. (1998). At the annealing temperature of 58.5°C, a single band in size range of 600 to 650 bp was successfully amplified from all isolates. After DNA sequencing, each unknown *Fusarium* strain was identified according to the deposited sequences in Fusarium-ID and NCBI database which had a high homology ( $\geq 99\%$ ) with them. The phylogenetic tree (Figure 3) clearly showed that the resolution of sequence from TEF region was sufficient in determining *Fusarium* species. Six isolates were identified as *F. solani* and five other isolates as *F. proliferatum*. The first three hits for the sequence of each isolate have been assigned in Supplemental Table 1.

#### **LD<sub>50</sub> and mycotoxin screening (molecular and LC-MS/MS analysis) of *Fusarium* isolated from traditional tempeh inocula (*usar*)**

All five *F. proliferatum* isolates included in our study lacked the TRI5 gene and possessed the FUM1 gene, indicating that they are presumably trichothecene non-producers and potentially fumonisin producers. This result was confirmed by different set of primers (FUM8 and FUM12) with identical result. *Fusarium solani* isolates lacked in both kind of genes, indicating they are not trichothecene or fumonisin producers. Screening of mycotoxin production showed that *Fusarium proliferatum* positively produced BEA, FB1, and Enns with range 23-41 µg/g, 215-288 µg/g, and 2-5 µg/g respectively. These isolates did not produce other important mycotoxin except three mycotoxins mentioned before. *Fusarium solani* isolates were not able to produce any important mycotoxins.

Since there is no information about how toxic *Fusarium solani* isolates so far, then toxicity test done by brine shrimp. In our study, the brine shrimp assay has proven to be a convenient system, less time consuming and more cost-efficient bioassay for monitoring toxicity of mycotoxins. Analysis of LD<sub>50</sub> can be done after 48 h incubation. We found that crude toxin extract from *F. proliferatum* is much more toxic than *F. solani* to brine shrimp. *Fusarium proliferatum* extract showed about 10-fold stronger activity as toxic agent toward *F. solani* extract based on their value of LD<sub>50</sub>. All screening result could be seen in Table 9.

Table 9. LD<sub>50</sub> and mycotoxin screening (molecular and LC-MS/MS analysis) of *Fusarium* isolated from traditional tempeh inocula (*usar*).

Characteristics	<i>Fusarium solani</i>	<i>Fusarium proliferatum</i>
Molecular screening		
FUM1	-	+ (100%)
FUM8	-	+ (100%)
FUM12	-	+ (100%)
TRI5	-	-
Screening of mycotoxin production by HPLC-MS/MS		
ZEN	-	-
Fus X	-	-
NIV	-	-
15-ADON	-	-
3-ADON	-	-
DON	-	-
NEO	-	-
DAS	-	-
HT2-toxin	-	-
T2-toxin	-	-
BEA	-	+ (100%)
FB1	-	+ (100%)
Enn A1	-	+ (60%)
Enn B	-	+ (60%)
Enn B1	-	+ (60%)
Toxicity of crude mycotoxin		
LD <sub>50</sub> average (in range, µg/mL)*	134.8 (125-141)	14.8 (12-18)
Number of strains examined	6	5
Origin of isolates	Banyumas, Banjarnegara, Gombong	Banyumas, Kebumen, Kudus, Banjarnegara, Gombong
(-) means negative result (+) means positive result Positive result accompanied with percentage of total positive samples in bracket. *LD <sub>50</sub> was obtained from crude extract of each isolates, tested by brine shrimp test. BEA concentration (range): 23-41 µg/g FB1 concentration (range): 215-288 µg/g Enns concentration (range): 2-5 µg/g		

### 3.2. Discussion of Part I (*Fusarium* isolates identification and their mycotoxin screening)

Like many other traditional foods in Indonesia, tempeh is produced as a small scale home industry with poorly controlled fermentation which is carried out without sufficient hygienic precautions even though the starter culture containing fungi are added at the beginning of fermentation. Therefore, there are various types of microorganisms that can participate during the process of fermentation, and natural contamination is always possible (Winarno, 1985; Barus et al., 2008; Seumahu et al., 2013). Moreover, there is no standard on making tempeh and its starter. This is the cause of many variations in the manufacture of tempeh in some areas (Astuti et al., 2000) which lead to nonhomogeneous quality and safety of traditional tempeh.

Several types of tempeh fermentation starters can be distinguished. Natural starters made with plant leaves (e.g., *Hibiscus* spp.), and soybeans, known as “*usar*” are still widely used in Indonesia. *Usar* is prepared by placing pieces of matured tempeh between two *Hibiscus* leaves. The leaves are turned over and piled one on top of the other, afterward covered with banana leaves or cloth and left to undergo the first stage of fermentation for about 24–36 hours room temperature (30°–34°C). In the second stage of fermentation, the leaves now facing upwards, are separated individually and kept so for 3–7 days, partly to let the *usar* dry at room temperature. Standard of hygiene is also not applied in *usar* production due to the lack of knowledge about good sanitation by the producers. Until now traditional tempeh producers still involve *waru lengis* leaves (*Hibiscus tiliaceus*) for *usar* manufacture, even though as reported Nout et al., (1992) and Suliantari (1996) these leaves may serve as a source of *Fusarium* spp.

Identification of *Fusarium* isolates was performed according to the combination of morphology, and molecular characters. There are limits on the use of morphological characters for identification of *Fusarium* spp. These limitations increase when differentiation is within members belonging to a species complex with highly similar morphology. In this study, molecular techniques based mostly on DNA sequencing of genes have supported morphological identification of *Fusarium* species. In total, 650 fungal isolates recovered from traditional tempeh inocula (*usar*). Traditional inocula (*usar*) were collected from many markets and center of tempeh producers in 9 different areas in Central Java and Yogyakarta provinces, Indonesia (on 2015). About 8.4% of total fungal isolates recognized belongs to *Fusarium* genera. Fifty-five pure isolates of *Fusarium* sp were collected and observed via microscopy approach. We found two main species of *Fusarium*, suspected as *F. solani* and *F. proliferatum*. To confirm morphological identification, representative isolates were observed with molecular approach. DNA fingerprinting was employed to distinguish identical and non-identical isolates. Non-identical isolates with distinct band were selected and confirmed at species level by the results of DNA sequence of the TEF gene, which is taxonomically most informative part in this fungal genus. DNA sequences of genes such as TEF-1 $\alpha$  have been widely used for supporting morphological traits of *Fusarium* species (Yli-Mattila et al., 2002; Harrow et al., 2010; Hsuan et al., 2011). In the present study, molecular investigation based on the TEF-1 $\alpha$  gene led to more precise identification of *Fusarium* isolates. After DNA sequencing, each unknown *Fusarium* strain was identified according to the deposited sequences in Fusarium-ID and NCBI database which had a high homology ( $\geq 99\%$ ) with them. The

phylogenetic tree clearly showed that the resolution of sequence from TEF region was sufficient in determining *Fusarium* species. Six isolates were identified as *F. solani* and five other isolates as *F. proliferatum*.

All *F. proliferatum* isolates included in our study lacked the TRI5 gene and possessed the FUM1 gene, indicating that they are presumably trichothecene non-producers and potentially fumonisin producers. This result was confirmed by different set of primers (FUM8 and FUM12) with identical result. In other hands, *F. solani* isolates lacked in both kind of genes, indicating they are not trichothecene or fumonisin producers. Molecular screening for mycotoxin is limited with the primers that can be used, therefore study expanded with LC-MS/MS screening for many mycotoxins. The results showed *F. solani* isolates were not able to produce any important mycotoxins, whereas *F. proliferatum* positively produced BEA, FB1 and Enns with range 23-41 µg/g, 215-288 µg/g, and 2-5 µg/g respectively. These isolates did not produce other important mycotoxins except three mycotoxins mentioned before. This is in line with report from Jestoi et al. (2008) that mentioned *F. proliferatum* mainly produce BEA, fumonisins, and Enns.

FB1 is well known harmful for human. It is proven carcinogens in rodents and suspected carcinogens in humans. So far, there are no reports about the association of BEA and Enns with mycotoxicoses (Jestoi et al., 2004) and because of this, it may be considered that they are not as relevant for human and animal health. However, recent research reported that these compound might also have adverse effect to human. Meca et al. (2010) found cytotoxic effects on human cells for enniatin A1 and B1 dependent on the concentration. The cell growth was stronger inhibited by enniatin A1 then enniatin B1. Also, Wätjen et al. (2009) found cytotoxic effects of the tested enniatins on human cells and induction of apoptotic cell death.

Since there is no information about how toxic *F. solani* isolates so far, then toxicity test done by brine shrimp. Toxicological tests conducted using crude extracts from the isolates. In our study, the brine shrimp assay has proven to be a convenient system, less time consuming and more cost-efficient bioassay for monitoring toxicity of mycotoxins. The brine shrimp are used in the laboratory bioassay of toxicity through estimation of lethal dosage (LD<sub>50</sub>). LD<sub>50</sub> is the amount of material, given all at once, which causes the death of 50% (one half) of a group of test animals. The LD<sub>50</sub> is one way to measure the short-term poisoning potential (acute toxicity) of material. The brine shrimp assay consist of hatching and growing brine shrimps, incubation with mycotoxins at different concentrations and calculation of LD<sub>50</sub>. Analysis of LD<sub>50</sub> can be done after 48 h incubation. We found that crude toxin extract from *F. proliferatum* is much more toxic than *F. solani* to brine shrimp. *Fusarium proliferatum* extract showed about 10-fold stronger activity as toxic agent toward *F. solani* extract based on their value of LD<sub>50</sub>. This result possibly related to mycotoxins content in each crude extract. Based on characterization of mycotoxins production, *F. proliferatum* isolates produce FB1, BEA and Enns; whereas *F. solani* isolates were not able to produce any important mycotoxins. Most of *F. proliferatum* isolates showed a high level of toxin production that might also related to their ability to be toxic toward brine shrimp larvae. Previous studies also found toxicity towards brine shrimp was significantly correlated with fumonisins

content (Hlywka et al., 1997; Hartl and Humpf, 2000), BEA content (Moretti et al., 2007) and Enns content (Tan et al., 2011) of some *Fusarium* spp. extracts.

In conclusion, this study found important information about *Fusarium* contamination in traditional tempeh inocula (*usar*). More safety attention needs to be given to traditional tempeh made with *usar*. Harmfull metabolites produced by *F. proliferatum* might also present in the traditional tempeh. Producers should be aware of this food safety matter to improve their hygiene on producing of inocula, and also to not use anymore *Hibiscus* leaves. Contamination of traditional inocula by fumonisin producing fungi is therefore highly relevant for food safety concern.

### 3.3. Results of Part II (Detection of *Fusarium* and mycotoxins in traditional tempeh)

Real-time PCR (qPCR) has become the standard method for species-specific quantification of fungal biomass in many kind of samples, including food product. The qPCR assays for major plant pathogens and decay fungi have been established and used extensively in the last decade. The majority of qPCR assays carried out in research laboratories and plant diagnostic services relied on real-time thermocyclers in 96-well or 384-well format; both SYBR Green-based detection of PCR products and hybridization probes (e.g., TaqMan) were extensively used.

In this study, quantification of two *Fusarium* species (*F. proliferatum* and *F. solani*) were the main focus. This screening was done before mycotoxin analysis step. The DNA extraction and the following gel electrophoresis resulted in a good amount of DNA for the traditional tempeh samples. The yield of the DNA was so high that a dilution was needed to achieve a clean band for the gel electrophoresis. The inhibition test for the traditional tempeh samples for the real time PCR showed no natural inhibition of the plant material. Therefore, pure fungal DNA of each species in TE buffer without plant material was used as standards. Table 10 lists the results of the qPCR for the detection of fungal DNA of *F. proliferatum*, and *F. solani* in the samples. Results are shown for a dilution of 1:50.

The occurrence of *F. proliferatum* in Banyumas was the highest (20.5%) followed with Kebumen (16.2%), Yogyakarta (14.6%), Kudus (12.9%), Banjarnegara (12.1%), and Gombong (12.1%). The highest occurrence of *F. solani* was found in Gombong (18.2%), followed by Kebumen (13.5%) and Banyumas (12.8%). Another three regencies are not found *F. solani* in their traditional tempeh. Contamination of traditional tempeh samples with *F. proliferatum* was found in 12% of the total samples with amounts of 10-175 ng/mg fungal DNA. Seven percent of total samples were contaminated with *F. solani* in amounts of 20-129 ng/mg fungal DNA. Since *F. solani* was found not harmful (about 10-13 times less harmful than *F. proliferatum*, and did not produce any important mycotoxins), then, we only checked mycotoxins content on the samples containing *F. proliferatum*.

The results of the mycotoxin detection in the traditional tempeh samples are given in Table 11. HPLC-MS/MS analysis only applied to check samples were positively contaminated by *F. proliferatum* (based on previous qPCR data). Contamination of traditional tempeh samples with *F. proliferatum* was found in 12% of total samples with amounts of 10-175 ng/mg fungal DNA. In these samples, the amount of FB1, BEA, EnnB, EnnB1 and EnnA1 were in range 170-2682 µg/kg, 20-45 µg/kg, 41-456 µg/kg, 16-67 µg/kg, and 8-28 µg/kg respectively. Most of the positives samples showed Fumonisin B1 in quantifiable amount. Many of positive samples contained BEA, and Enniatins in low or unquantifiable amount (< LOQ). All samples had lower FB1 contents than legal limit in Indonesia (2000 µg/kg; SNI 7385:2009), except for one sample from Yogyakarta (samples area Yogyakarta 22) which contained FB1 above the allowed limit. This sample also had highest amount of *F. proliferatum* DNA. We found positive linear relationship ( $R^2=0.8$ ) between the amount of *F. proliferatum* DNA with amount of FB1. The presence of enniatins maybe from the raw material such as soybean or corn which were contaminated during storage.

Table 10. Results of qPCR for *Fusarium solani* and *F. proliferatum*, in ng/mg in the traditional tempeh samples. Dilution of DNA is 1:50. Positive samples are shown in red and LOQ = Limit of Quantification is shown yellow; n.d. = no detection.

Area	<i>F. solani</i>	<i>F. proliferatum</i>
Banyumas		
1	n.d	25
2	n.d	n.d
3	n.d	n.d
4	<LOQ	n.d
5	<LOQ	144
6	n.d	n.d
7	n.d	n.d
8	n.d	n.d
9	n.d	n.d
10	n.d	53
11	25	n.d
12	n.d	n.d
13	n.d	<LOQ
14	n.d	n.d
15	n.d	n.d
16	n.d	n.d
17	n.d	n.d
18	n.d	n.d
19	40	n.d
20	n.d	<LOQ
21	n.d	n.d
22	n.d	n.d
23	n.d	n.d
24	n.d	27
25	n.d	n.d
26	n.d	113
27	129	n.d
28	n.d	n.d
29	n.d	n.d
30	n.d	n.d
31	n.d	n.d
32	n.d	n.d
33	n.d	n.d
34	n.d	n.d
35	n.d	32
36	66	n.d
37	n.d	n.d
38	n.d	n.d
39	n.d	n.d
% Occurrence	12.8	20.5

Area	<i>F. solani</i>	<i>F. proliferatum</i>
Kebumen		
1	<LOQ	n.d
2	n.d	n.d
3	n.d	n.d
4	n.d	n.d
5	n.d	n.d
6	n.d	n.d
7	n.d	n.d
8	n.d	43
9	<LOQ	n.d
10	n.d	n.d
11	n.d	n.d
12	n.d	n.d
13	n.d	n.d
14	n.d	n.d
15	n.d	n.d
16	n.d	n.d
17	35	n.d
18	n.d	n.d
19	n.d	n.d
20	n.d	<LOQ
21	n.d	n.d
22	n.d	n.d
23	n.d	n.d
24	n.d	n.d
25	n.d	45
26	n.d	n.d
27	47	n.d
28	n.d	n.d
29	n.d	n.d
30	n.d	n.d
31	n.d	22
32	n.d	<LOQ
33	n.d	n.d
34	89	n.d
35	n.d	n.d
36	n.d	n.d
37	n.d	<LOQ
% Occurrence	13.5	16.2

Area	<i>F. solani</i>	<i>F. proliferatum</i>
Yogyakarta		
1	n.d	n.d
2	n.d	n.d
3	n.d	n.d
4	n.d	n.d
5	n.d	n.d
6	n.d	n.d
7	n.d	n.d
8	n.d	n.d
9	n.d	n.d
10	n.d	n.d
11	n.d	n.d
12	n.d	54
13	n.d	n.d
14	n.d	35
15	n.d	30
16	n.d	n.d
17	n.d	n.d
18	n.d	n.d
19	n.d	n.d
20	n.d	n.d
21	n.d	n.d
22	n.d	175
23	n.d	n.d
24	n.d	n.d
25	n.d	n.d
26	n.d	n.d
27	n.d	n.d
28	n.d	n.d
29	n.d	<LOQ
30	n.d	n.d
31	n.d	n.d
32	n.d	n.d
33	n.d	n.d
34	n.d	n.d
35	n.d	n.d
36	n.d	n.d
37	n.d	36
38	n.d	n.d
39	n.d	n.d
40	n.d	n.d
41	n.d	n.d
% Occurrence	0	14.6

Table 10. Results of qPCR for *Fusarium solani* and *F. proliferatum*, in ng/mg in the traditional tempeh samples. Dilution of DNA is 1:50. Positive samples are shown in red and LOQ = Limit of Quantification is shown yellow; n.d. = no detection. (Continued)

Area	<i>F. solani</i>	<i>F. proliferatum</i>	Area	<i>F. solani</i>	<i>F. proliferatum</i>	Area	<i>F. solani</i>	<i>F. proliferatum</i>
Banjarnegara			Gombong			Kudus		
1	n.d	n.d	1	<LOQ	n.d	1	n.d	n.d
2	n.d	n.d	2	n.d	n.d	2	n.d	n.d
3	n.d	<LOQ	3	n.d	n.d	3	n.d	20
4	n.d	n.d	4	35	n.d	4	n.d	<LOQ
5	n.d	n.d	5	n.d	n.d	5	n.d	25
6	n.d	n.d	6	n.d	n.d	6	n.d	n.d
7	n.d	n.d	7	n.d	n.d	7	n.d	n.d
8	n.d	n.d	8	n.d	n.d	8	n.d	n.d
9	n.d	n.d	9	n.d	n.d	9	n.d	n.d
10	n.d	<LOQ	10	43	n.d	10	n.d	n.d
11	n.d	n.d	11	n.d	n.d	11	n.d	n.d
12	n.d	n.d	12	n.d	n.d	12	n.d	43
13	n.d	n.d	13	n.d	37	13	n.d	n.d
14	n.d	n.d	14	n.d	n.d	14	n.d	n.d
15	n.d	n.d	15	n.d	n.d	15	n.d	n.d
16	n.d	n.d	16	n.d	n.d	16	n.d	n.d
17	n.d	n.d	17	21	33	17	n.d	33
18	n.d	n.d	18	n.d	29	18	n.d	n.d
19	n.d	n.d	19	n.d	20	19	n.d	n.d
20	n.d	n.d	20	n.d	n.d	20	n.d	n.d
21	n.d	n.d	21	n.d	n.d	21	n.d	n.d
22	n.d	n.d	22	n.d	n.d	22	n.d	n.d
23	n.d	n.d	23	112	n.d	23	n.d	n.d
24	n.d	n.d	24	n.d	n.d	24	n.d	n.d
25	n.d	31	25	n.d	n.d	25	n.d	n.d
26	n.d	n.d	26	<LOQ	n.d	26	n.d	n.d
27	n.d	n.d	27	n.d	n.d	27	n.d	n.d
28	n.d	n.d	28	n.d	n.d	28	n.d	n.d
29	n.d	n.d	29	n.d	n.d	29	n.d	n.d
30	n.d	n.d	30	n.d	n.d	30	n.d	n.d
31	n.d	21	31	n.d	n.d	31	n.d	n.d
32	n.d	n.d	32	n.d	n.d	32	n.d	n.d
33	n.d	n.d	33	n.d	n.d	33	n.d	n.d
% Occurrence	0	12.1	% Occurrence	18.2	12.1	% Occurrence	0	12.9



Table 11. HPLC-MS/MS result of BEA, FB1, and the Enniatins A1, B and B1 for positive samples (*F. proliferatum* present). Sample contained FB1 content higher than legal limit in Indonesia (2000 µg/kg; SNI 7385:2009) is shown in red.

Locations	<i>F. proliferatum</i> (DNA ng/mg)	BEA (µg/kg)	FB1 (µg/kg)	EnnB (µg/kg)	EnnB1 (µg/kg)	EnnA1 (µg/kg)
Kebumen 08	43	<LOQ	320	<LOQ	<LOQ	<LOQ
Kebumen 25	45	17	361	367	51	19
Kebumen 31	22	<LOQ	212	<LOQ	<LOQ	<LOQ
Banyumas 01	25	<LOQ	337	41	<LOQ	<LOQ
Banyumas 05	144	45	1407	95	16	<LOQ
Banyumas 06	45	<LOQ	362	<LOQ	<LOQ	<LOQ
Banyumas 10	53	37	566	<LOQ	<LOQ	<LOQ
Banyumas 24	27	<LOQ	170	164	19	12
Banyumas 26	113	34	776	174	34	14
Banyumas 35	32	<LOQ	381	<LOQ	<LOQ	<LOQ
Kudus 03	20	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Kudus 05	25	17	183	456	67	28
Kudus 12	43	25	437	<LOQ	<LOQ	<LOQ
Kudus 17	33	<LOQ	284	<LOQ	<LOQ	<LOQ
Yogyakarta 12	54	<LOQ	526	<LOQ	<LOQ	<LOQ
Yogyakarta 14	35	20	217	131	19	<LOQ
Yogyakarta 15	30	<LOQ	214	<LOQ	<LOQ	<LOQ
<b>Yogyakarta 22</b>	175	163	<b>2682</b>	<LOQ	<LOQ	<LOQ
Yogyakarta 37	36	37	323	220	36	14
Banjarnegara 25	31	28	253	190	34	16
Banjarnegara 31	21	<LOQ	197	107	16	<LOQ
Gombong 13	37	25	474	<LOQ	<LOQ	<LOQ
Gombong 17	33	20	220	<LOQ	<LOQ	<LOQ
Gombong 18	29	<LOQ	255	160	18	8
Gombong 19	20	<LOQ	179	154	27	10

### 3.4. Discussion of Part II (Detection of *Fusarium* and mycotoxins in traditional tempeh)

Fumonisin (FBs) are important naturally present in environment mycotoxins formed mainly by *Fusarium proliferatum* and *F. verticillioides*. The occurrence, frequency, level of the contamination and implications of mycotoxins entering the food and feed chain have gained global attention over the last decade. There are at least 28 fumonisin analogues that have been identified and these have been classified into series A, B, F and P based on their chemical structures (Musser and Plattner, 1995), of which fumonisin B1 (FB1) is the most abundant of the group (Scott, 1993). FB1 has been shown to be hepatocarcinogenic and nephrocarcinogenic in the male rat and hepatocarcinogenic in the female mouse (Howard et al., 2001) and on the basis of available evidence, the International Agency for Research on Cancer (IARC) has declared the 'toxins produced by *F. moniliforme*' to be possibly carcinogenic to humans (Class 2B carcinogens). Present study is the first report about *Fusarium* and mycotoxin contamination in traditional tempeh. Many people believe that contamination never happen in the fermentation product like tempeh. In fact, there are a complex of microorganisms in soybean tempeh, their development start during the soaking of the raw ingredients (Nout and Kiers, 2005). Many microorganisms are involved in several stages of making tempeh, some of them are responsible for the acidification during the soaking stage and forming good taste-odor; there may be cross-contamination through the handling during hand drying and packaging, during the incubation and in the composition of inoculum. Therefore, contamination is always possible in this product.

The biomass of *Fusarium* spp. in traditional tempeh was estimated by species-specific real-time PCR analysis of DNA extracted from representative samples of traditional tempeh. The analysis was carried out for the two major *Fusarium* contaminant (*F. proliferatum* and *F. solani*) which previously found in traditional tempeh inocula. This screening was done before mycotoxin analysis step. The DNA extraction and the following gel electrophoresis resulted in a good amount of DNA for the traditional tempeh samples. The yield of the DNA was so high that a dilution was needed to achieve a clean band for the gel electrophoresis. The inhibition test for the traditional tempeh samples for the real time PCR showed no natural inhibition of the food material. Therefore, pure fungal DNA of each species in TE buffer without food material was used as standards. Table 10 lists the results of the qPCR for the detection of fungal DNA of *F. proliferatum*, and *F. solani* in the samples. Results are shown for a dilution of 1:50.

The occurrence of *F. proliferatum* in Banyumas was the highest (20.5%) followed with Kebumen (16.2%), Yogyakarta (14.6%), Kudus (12.9%), Banjarnegara (12.1%), and Gombong (12.1%). The highest occurrence of *F. solani* was found in Gombong (18.2%), followed by Kebumen (13.5%) and Banyumas (12.8%). Another three regencies are not found *F. solani* in their traditional tempeh. Contamination of traditional tempeh samples with *F. proliferatum* was found in 12% of the total samples with amounts of 10-175 ng/mg fungal DNA. Seven percent of total samples were contaminated with *F. solani* in amounts of 20-129 ng/mg fungal DNA.

Founding DNA of *F. proliferatum* in the total DNA extracted from traditional tempeh motivated us to extend the analysis of mycotoxin content to fumonisin and beauvericin, which are both produced by *F. proliferatum*. We also analyzed the content of enniatins, because according

to Jestoi et al. (2008) *F. proliferatum* also the main enniatins producer. Since *F. solani* was found not harmful (about 10-times less harmful than *F. proliferatum* based on artemia test, and did not produce any important mycotoxins based on HPLC-MS/MS analysis), then, we only checked mycotoxins content on the samples containing *F. proliferatum*.

The results of the mycotoxin detection in the traditional tempeh samples are given in Table 11. HPLC-MS/MS analysis only applied to check samples were positively contaminated by *F. proliferatum* (based on previous qPCR data). Contamination of traditional tempeh samples with *F. proliferatum* was found in 12% of total samples with amounts of 10-175 ng/mg fungal DNA. In these samples, the amount of FB1, BEA, EnnB, EnnB1 and EnnA1 were in range 170-2682 µg/kg, 20-45 µg/kg, 41-456 µg/kg, 16-67 µg/kg, and 8-28 µg/kg respectively. Most of the positives samples (*F. proliferatum* present) showed Fumonisin B1 in quantifiable amount. Daughter ions generated from the molecular ion possessed the same m/z ratios and occurred in similar relative abundances as daughter ions of authentic fumonisin B1 standard. Together with the retention time and m/z value of the molecular ion, these results proved convincingly that fumonisin B1 was present in traditional tempeh. The presence of beauvericin and enniatins A1, B and B1 was supported by their retention times, the molecular ions and three daughter ions for each analyte. Many of positive samples contained BEA, and enniatins in low or unquantifiable amount (< LOQ). Since tempeh is an important food in Indonesia these results represent an important human health issue that needs further monitoring.

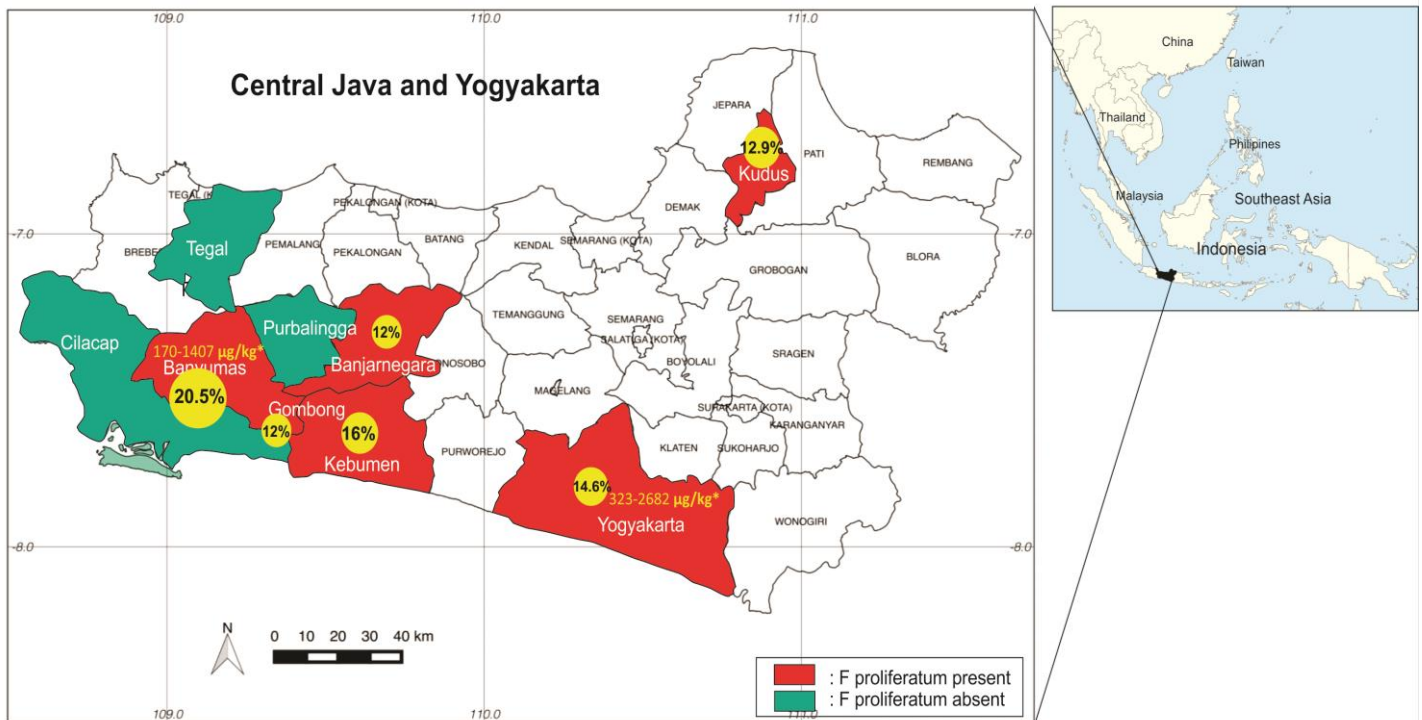


Figure 4. Contamination of *F. proliferatum* and Fumonisin B1 on traditional tempeh from central production of traditional tempeh at Central Java and Yogyakarta provinces.

All samples had lower FB1 contents than legal limit in Indonesia (2000 µg/kg; SNI 7385:2009), except for one sample from Yogyakarta (samples area Yogyakarta 22) which contained FB1 above the allowed limit. Two samples contained FB1 higher than the recommended EFSA level 1000 µg/kg. These samples were from Banyumas and Yogyakarta. These sample also had highest amount of *F. proliferatum* DNA. We found positive linear relationship ( $R^2=0.8$ ) between the amount of *F. proliferatum* DNA with amount of FB1, but we found low positive correlation between *F. proliferatum* DNA with amount of Enns. The presence of Enns maybe from the raw material such as soybean /corn which were contaminated during storage or because of other *Fusarium* species contamination. Although mycotoxin contamination was detected below the legal limit, the presence of mycotoxin should be controlled in traditional tempeh production.

Figure 4 shows contamination of *F. proliferatum* and mycotoxins was more prevalent in east area compare to west part of Central Java. East areas have more rainfall and warmer temperature based on Indonesian Meteorological Organization (BMKG). There are two possibilities of contamination source, first comes from contaminated traditional inocula which were also found in those area (as described in result part I), second maybe the use of corn as substitute in tempeh production. Producers involve corn substitution for 10-30 percent to reduce cost production because the price of soybean is high nowadays. This generates a healthiness issue of produced tempeh because most Indonesian corn has been contaminated by toxigenic fungi such as *Fusarium spp.* (Purwoko et al., 1991; Ahmad et al., 1996; Gholib et al., 2004; Ahmad et al., 2009; Kusumaningrum et al., 2010; Rahmawati et al., 2013). Rahmawati et al. (2013) also reported that toxigenic fungi such as *Fusarium spp.* are found in soaking corn during corn flour production.

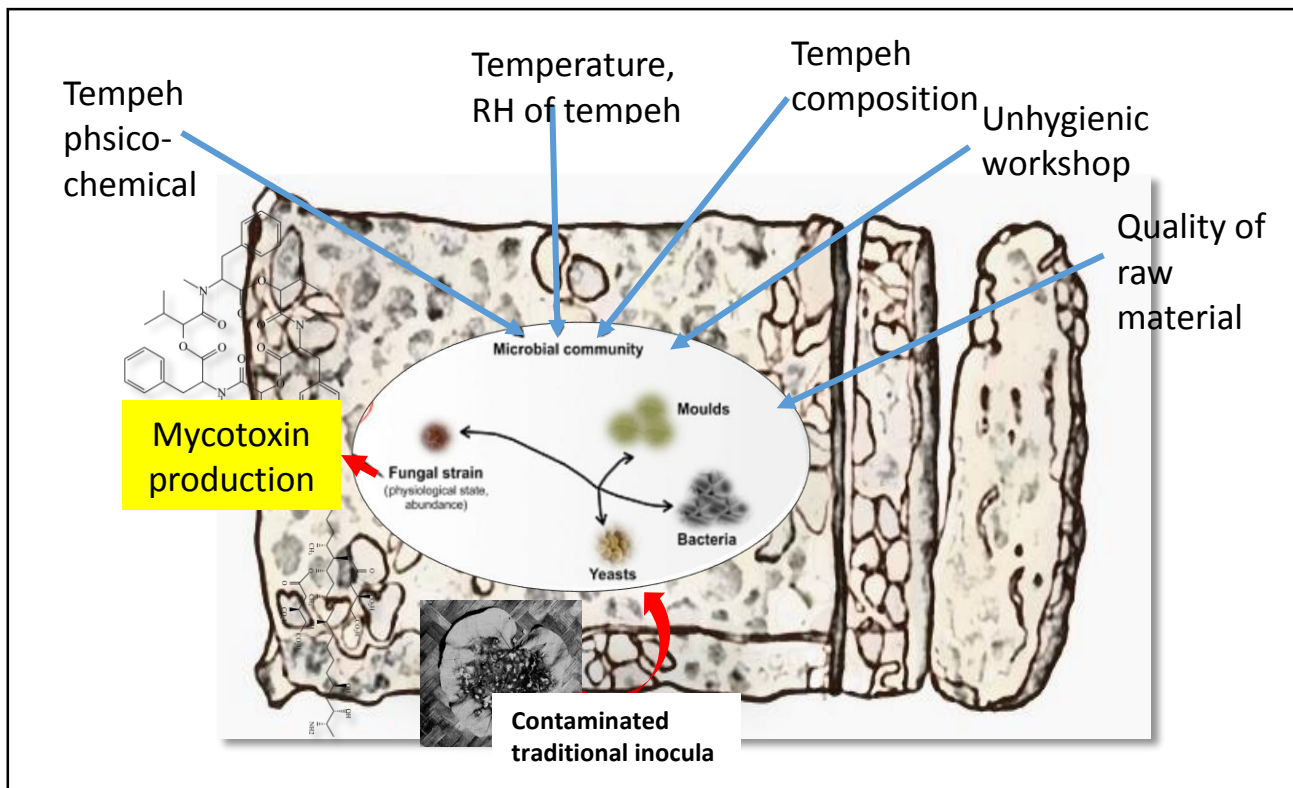


Figure 5. Mycotoxin production and possible factors might play role in the tempeh system.

Furthermore, abiotic and biotic factors also may influence fungal and mycotoxin contamination in tempeh (Figure 5). Abiotic factors, such as normal room temperature, neutral pH and humid condition supported many microorganism to grow optimal. Biotic factors modulating mycotoxin production include the intrinsic capacity of contaminant to produce mycotoxins, its physiological state and the interactions that may occur between this organism and the other members of the tempeh microbiota. Our observation found that there is no antagonism interaction between main tempeh fungal (*Rhizopus* spp.) with *F. proliferatum*. They could grow together. Unhygienic environment of the workshop may also contribute to contamination problem. Most of the tempeh samples were positive in *Fusarium* and mycotoxin contamination isolated from unhygienic tempeh workshop. These condition make contamination is always possible in this product.

As mentioned in the beginning that FB1 is harmful for human. It is proven carcinogens in rodents and suspected carcinogens in humans. Contamination of traditional tempeh with FB1 is therefore highly relevant for food safety. BEA and Enns were also detected in the samples. So far, there are no reports about the association of BEA and Enns with mycotoxicoses (Jestoi et al., 2004) and because of this, it might be considered that they are not as relevant for human and animal health. However, recent research reported that these compound may also have adverse effect to human. Meca et al. (2010) found cytotoxic effects on human cells for enniatin A1 and B1 dependent on the concentration. The cell growth was stronger inhibited through enniatin A1 then enniatin B1. Also Wätjen et al. (2009) found cytotoxic effects of the tested enniatins on human cells and induction of apoptotic cell death.

Occurrence of enniatins in food commodities have been reported in several countries. Enniatins B, B1, B4, A and A1 were quantified in baby food including infant formula milks, dairy products (cheese and yogurt), cereal based baby food, fruits and vegetables compotes, fruits and vegetable puree from the Italian market. The detected concentrations ranged from 11.8 to 832 µg/kg in cereal-based baby food. The highest value was enniatin B (Juan *et al.*, 2014). According to a study carried out by Serrano *et al.* (2013), organic pasta from Spain was more contaminated with enniatin (A, A1, B1 and B) than the conventional counterpart. Although the majority of the enniatin concentrations found in organic and conventional dry or fresh samples in Spain were below 25 µg/kg, concentrations up to 979.56 µg/kg of enniatin A, A1 B1 and B were detected in conventional dry pasta. Nuts and dried fruits from Spain were screened for the occurrence of enniatins by Tolosa *et al.* (2013), the samples included peanuts fruit and shell, almonds fruit and shell, Pistachios fruits and shell, walnuts fruit and shell, fried maize, hazelnuts fruit and shell, sunflower seeds fruit and shell, dried fruits (dried apricots, dried figs, dried raisins, dried plums, blueberries and dates). Enniatin A and B were the most prevalent in the analyzed samples. The percentages of total enniatin contamination of samples analyzed ranged were 35.7%, 50% and 83.3% for dried fruits, nuts and dates respectively. The highest concentration of enniatins (A, A1, B and B1) was found in peanuts, almonds and dates reaching a maximum of 1.855 mg/kg, 1.323 mg/kg and 1.494 mg/kg respectively.

### 3.5. Results and Discussion of Part III (Tempeh quality and risk assessment)

#### Tempeh quality assessment

To test whether the consumer can recognize contaminated tempeh, some physico-chemical tests were done (control (normal tempeh/non-contaminated) and contaminated tempeh). Tests carried out include compactness observation, texture, pH, color, and content of protein and amino acid determinants. Result of quality test was summarized in Table 12.

Table 12. Summary of quality result between normal and contaminated tempeh.

No	Quality test	Result
1	Compactness observation	Not different
2	Texture (with texture analyzer Rheometer)*	Not different
3	pH*	Not different
3	Color ( <i>L</i> , <i>a</i> , and <i>b</i> ) (with Minolta Chromameter)*	Not different
4	Protein content*	Significantly different
5	Amino acid composition (with Waters UPLC-MS/MS)*	Significantly different

\*Significant differences was checked by t-student test ( $P < 0.05$ ).

There is no differences between normal and contaminated tempeh on the compactness and physical characters (texture, pH and color). Both kind of tempeh showed good compactness and strong ramification of *Rhizopus* mold. Their texture are in range (weakness: 8.21-9.15 N; modulus elasticity:  $2.12 \times 10^9$ - $2.23 \times 10^9$  Pa; surrender value:  $1.14 \times 10^6$ - $3.27 \times 10^6$  Pa), pH (6.4-6.7) and color in range (*L*:83.1-84.2; *a*:2.3-2.5; *b*:16.2-17.1, white color). Interestingly we found that chemical contents were significantly different especially in protein and amino acid content (Figure 6 and Table 13). This result suggest that *Fusarium proliferatum* contamination affects chemical content of tempeh (reduction of protein content, L-aspartic acid and L-glutamic acid content) but not changes any physical appearance and characteristics.

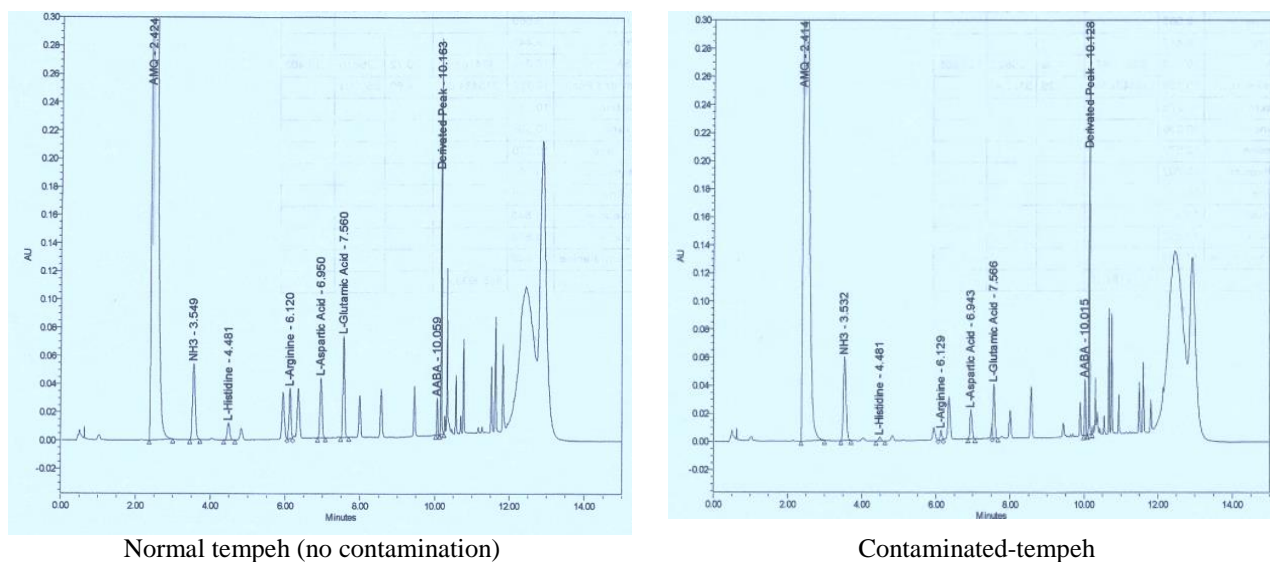


Figure 6. UPLC-MS/MS chromatogram of amino acid between normal and contaminated-tempeh.



Table 13. Protein, L-Aspartic acid and L-Glutamic acid content between normal and contaminated tempeh.

Samples	Protein content (g /100g dw)	Amino acids		Interpretation for taste
		L-Aspartic acid (g/100g dw)	L-Glutamic acid (g/100g dw)	
Normal tempeh	42.2 ± 0.42a	5.23 ± 0.45a	8.55 ± 0.79a	Umami
Contaminated tempeh	35.34 ± 1.27b	1.01 ± 0.21b	2.17 ± 0.32b	Less umami

dw means dry weight.

Data are mean ±SD (standard deviation) of n=3.

Significant by t-student test ( $P < 0.05$ ; n:3) are indicated by different letter.

In normal tempeh, color changed from white to black when stored for more than 8 days, the color changing is because of *Rhizopus* spores accumulation. In contrast, many white spots obviously appeared in contaminated tempeh, because of white color from *Fusarium* colonies. This indicator can be used by producer to check quality of their product monthly, to know whether their tempeh product are safe or not for consumption. Producer can do this small test before start to produce traditional tempeh in mass production.

We suggest that consumer could distinguish contaminated tempeh by checking the taste of fresh tempeh before cooked. Contaminated tempeh is less umami than normal tempeh because less L-aspartic acid and L-glutamic acid content, in other words contaminated-tempeh is less delicious compare to the normal. Umami or savory taste, is one of the five basic tastes (together with sweetness, sourness, bitterness, and saltiness). It has been described as brothy or meaty. People taste *umami* through taste receptors that typically respond to glutamate and aspartic acid.

### Risk Assessment

Risk assessment is the process of quantifying the magnitude and exposure, or probability, of a harmful effect on individuals or populations from specific agents or activities (Kuiper-Goodman, 2004). The degree of exposure to chemical or toxin compounds is one of the most important parameters in the evaluation of risk (Moreno et al., 2009). In this study, we determined the exposure levels of Indonesian infants, children, and adult to fumonisin B1 through traditional tempeh consumption (Table 14). Data of mycotoxin contamination taken from research part II.

Table 14 reveals the risk of exposure of infant, children and adult to fumonisins with their corresponding %TDIs. The risk of exposure of infants in the Banyumas regency to fumonisin was the highest (%TDI: 10.2) compared to the risk in other regencies (%TDI: 9.6 in Yogyakarta, 3.4 in Gombong, 2.9 in Kudus, 1.6 in Kebumen, and 1.4 in Banjarnegara respectively). These values are ten to sixty-fold lower than the 2000 ng·kg<sup>-1</sup>bw provisional maximum TDI (PMTDI) established for fumonisin by JECFA (2011). Based on this study consumers in those regency had low risk of exposure to fumonisin. Although risk of the exposure was detected very low, fumonisin control still needed to guarantee safety on traditional tempeh consumption.

Table 14. Risk characterization of fumonisin based on probable daily intake.

Areas	Daily Intake	<sup>1)</sup> Fumonisin B1 (ng/g)	<sup>2)</sup> PDI (ng.kg <sup>-1</sup> bw.day <sup>-1</sup> )		
			Infant	Children	Adult
Banyumas	PDI	102.3	204.6	81.8	34.1
	%TDI		10.2	4.1	1.7
Kebumen	PDI	16.3	32.6	13	5.4
	%TDI		1.6	0.7	0.3
Yogyakarta	PDI	96.6	193.3	77.3	32.2
	%TDI		9.6	3.9	1.6
Banjarnegara	PDI	13.6	27.3	10.9	4.5
	%TDI		1.4	0.5	0.2
Gombong	PDI	34.2	68.4	27.3	11.4
	%TDI		3.4	1.4	0.6
Kudus	PDI	29.2	58.3	23.3	9.7
	%TDI		2.9	1.2	0.5

Note:  
<sup>1)</sup> Summation of mean of means of total FB1 in each area from research part II  
<sup>2)</sup> Probable Daily Intake (PDI) (Assumed 10 kg, 25 kg (Rodriguez-Carasco et al., 2013) and 60 kg (Liu and Wu, 2010) body weight (bw) for infants, children and adults, respectively) calculated by multiplying the fumonisin concentration in traditional tempeh by consumption rates of tempeh in Indonesia 20 g/person/day) as estimated by the Statistics Indonesia (BPS, 2016) and divided by body weight of consumers.  
 \*Tolerable daily intake (2000 ng.kg<sup>-1</sup> bw.day<sup>-1</sup>) by Join FAO/WHO Expert Committee on Food Additives/ JEFCA (2011).

In this regard, risk analysis with its components of risk assessment, risk management and risk communication, is an important tool in dealing with tempeh safety issues. Reduction of the risk could be done in integrated manner. Many stakeholders should be involved to increase safety of traditional tempeh production and consumption. Diagram of quality and safety assessment is proposed to improve quality and safety of traditional tempeh (as seen in Figure 7).

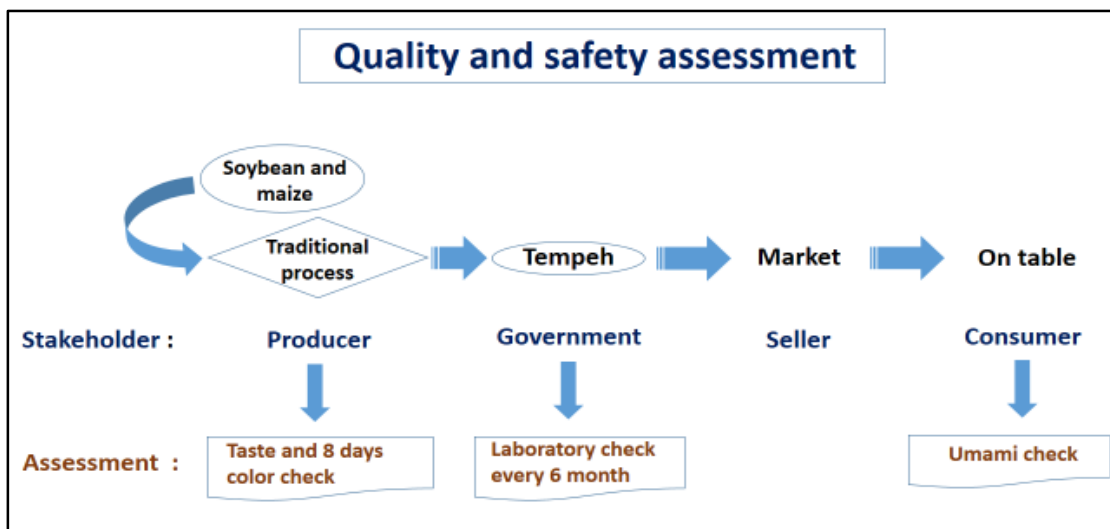


Figure 7. Proposed diagram of quality and safety assessment for traditional tempeh at various stakeholders.



## 4. Conclusion

From this study, the following conclusion can be drawn:

- In total, 650 fungal isolates recovered from 345 traditional tempeh inocula (*usar*) which collected from many markets and center of tempeh producers in 9 different areas in Central Java and Yogyakarta provinces, Indonesia. Six clusters (Banjarnegara, Banyumas, Gombong, Kebumen, Kudus, and Yogyakarta) showed a *Fusarium* contamination in *usar*, while no contamination was found in other remaining clusters. About 8.4% of total fungal isolates recognized belongs to *Fusarium* genera. Fifty-five pure isolates of *Fusarium* sp were collected, then observed via microscopy and molecular approach. We found two main species of *Fusarium*, identified as *F. solani* and *F. proliferatum*.
- All *F. proliferatum* isolates included in our study lacked the TRI5 gene and possessed the FUM1 gene, indicating that they are presumably trichothecene non-producers and potentially fumonisin producers. This result was confirmed by different set of primers (FUM8, and FUM12) with identical result. In other hands, *F. solani* isolates lacked in both kind of genes, indicating they are not trichothecene or fumonisin producers. LC-MS/MS screening showed *F. solani* isolates were not able to produce any important mycotoxins, whereas *F. proliferatum* positively produced BEA, FB1, and Enns. *Fusarium proliferatum* extracts showed about 10-fold stronger activity as toxic agent toward *F. solani* extracts based on their value of LD<sub>50</sub>.
- About 214 traditional tempeh samples from the six clusters mentioned above were screened for *F. proliferatum* and *F. solani* contamination by real-time PCR. Contamination of traditional tempeh samples with *F. proliferatum* was found in 12% of the samples with amounts of 10-175 ng/mg fungal DNA. In these samples, the amount of FB1 and BEA ranged between 170-2682 µg/kg and 20-50 µg/kg, respectively. About 7 % of the traditional tempeh samples were contaminated with *F. solani* in amounts of 20-129 ng/mg fungal DNA. All samples had lower FB1 contents than legal limit in Indonesia (2000 µg/kg; SNI 7385:2009), except for one sample from Yogyakarta area which contained FB1 above the allowed limit. Although mycotoxin contamination was detected below the legal limit, the presence of mycotoxin should be controlled in traditional tempeh production.
- There were no differences between normal and contaminated-tempeh on the compactness and physical characters (texture, pH, and color). Interestingly we found that chemical contents were significantly different especially in protein and amino acid content. We suggest that consumer could distinguish contaminated tempeh by checking the taste of fresh tempeh before cooked. Contaminated tempeh is less umami than normal tempeh because of less L-aspartic acid and L-glutamic acid content, in other words, contaminated-tempeh is less delicious compare to the normal.
- The exposure levels of Indonesian infants, children, and adult to fumonisin B1 through traditional tempeh consumption are low. The risk of exposure of infants in the Banyumas regency to fumonisin was the highest (%TDI: 10.2) compared to the risk in other regencies (%TDI: 9.6 in Yogyakarta, 3.4 in Gombong, 2.9 in Kudus, 1.6 in Kebumen, and 1.4 in Banjarnegara respectively). These values are ten to sixty-fold lower than the 2000 ng·kg<sup>-1</sup>·bw provisional maximum TDI (PMTDI) established for fumonisin by JECFA (2011).

## 5. Acknowledgement

This research was supported financially by Indonesia Endowment Fund for Education, Ministry of Finance, The Republic of Indonesia (Ref:1064/LPDP/2013).

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## 7. Appendix

Supplemental Table 1. Species identity of 11 *Fusarium* isolates based on morphological characters and TEF-1 $\alpha$  sequences.

Isolate	<i>Fusarium</i> spp.	Sources	Locations in Indonesia	Year	Accession for the first three matched hits in Fusarium-ID database	Related <i>Fusarium</i> spp.	Similarity (%)
BJ09.05	<i>Fusarium proliferatum</i>	Traditional tempeh starter ( <i>usar</i> )	Banjarnegara	2015	Zm-62	<i>F. proliferatum</i>	99.55
B25.03	<i>F. solani</i>	Traditional tempeh starter ( <i>usar</i> )	Banyumas	2015	Zm-16 Br-1 NRRL37640	<i>F. proliferatum</i> <i>F. proliferatum</i> <i>F. solani</i> species complex	99.54 99.39 98.81
KU07.03	<i>F. proliferatum</i>	Traditional tempeh starter ( <i>usar</i> )	Kudus	2015	NRRL32849 NRRL32785	<i>F. solani</i> species complex <i>F. solani</i> species complex	98.67 98.67
G21.02	<i>F. solani</i>	Traditional tempeh starter ( <i>usar</i> )	Gombong	2015	Zm-16 Br-1 Zm-54	<i>F. proliferatum</i> <i>F. proliferatum</i> <i>F. proliferatum</i>	99.54 99.4 99.39
B25.07	<i>F. solani</i>	Traditional tempeh starter ( <i>usar</i> )	Banyumas	2015	NRRL43433 NRRL32751 NRRL32710	<i>F. solani</i> species complex <i>F. solani</i> species complex <i>F. solani</i> species complex	100 100 100
KE34.03	<i>F. solani</i>	Traditional tempeh starter ( <i>usar</i> )	Kebumen	2015	NRRL37640 NRRL32849	<i>F. solani</i> species complex <i>F. solani</i> species complex	98.66 98.51
KE34.02	<i>F. solani</i>	Traditional tempeh starter ( <i>usar</i> )	Kebumen	2015	NRRL32785 NRRL37640 NRRL32849	<i>F. solani</i> species complex <i>F. solani</i> species complex <i>F. solani</i> species complex	98.51 98.81 98.67
G21.09	<i>F. solani</i>	Traditional tempeh starter ( <i>usar</i> )	Gombong	2015	NRRL22792 NRRL32838 NRRL22640 NRRL32707 NRRL37640	<i>F. solani</i> species complex <i>F. solani</i> species complex <i>F. solani</i> species complex <i>F. solani</i> species complex <i>F. solani</i> species complex	98.67 99.7 99.7 99.55 99.02
					NRRL32849 NRRL32785	<i>F. solani</i> species complex <i>F. solani</i> species complex	99.02 99.02

Chapter 4: Mycotoxin-producing *Fusarium* in traditional tempeh and *usar*

G21.10	<i>F. proliferatum</i>	Traditional tempeh starter ( <i>usar</i> )	Gombong	2015	Zm-62	<i>F. proliferatum</i>	99.4
					Br-1	<i>F. proliferatum</i>	99.25
					Zm-54	<i>F. proliferatum</i>	99.25
FSY09.10	<i>F. proliferatum</i>	Traditional tempeh starter ( <i>usar</i> )	Yogyakarta	2015	Zm-62	<i>F. proliferatum</i>	99.36
					Zm-16	<i>F. proliferatum</i>	99.35
					Zm-54	<i>F. proliferatum</i>	99.35
BJ09.09	<i>F. proliferatum</i>	Traditional tempeh starter ( <i>usar</i> )	Gombong	2015	Zm-62	<i>F. proliferatum</i>	99.55
					Zm-54	<i>F. proliferatum</i>	99.4
					Br-1	<i>F. proliferatum</i>	99.39

## Review Article 02

# Fighting Toxigenic Fungi in Food Industry by Applying Lactic Acid Bacteria: A Review of Established and Recent Research

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### Abstract

Food spoilage by molds and the occurrence of mycotoxins produced by these molds represent a major concern for all stakeholders, not least researchers, and research and development divisions of the food manufacturing industry. Mycotoxins produced by fungi, e.g. aflatoxin, trichothecene, fumonisin, ochratoxin A, and patulin can pose a health risk due to their carcinogenic, immunotoxic, teratogenic, neurotoxic, nephrotoxic and hepatotoxic effects. Moreover, trends enforced by the customer, demand on natural labels, friendly preservatives have over the last few years stimulated research on antifungal lactic acid bacteria (LAB) as a bio-preservative. This article reviews current knowledge on antifungal LAB, their bioactive metabolites, genetic engineering applications to increase production of antifungal compounds and their application in the food system.

**Keywords:** lactic acid bacteria, antifungal metabolites, mycotoxins, food safety

### Introduction

The history of the food industry is in part, also the history of the battle with toxigenic fungi. Mycotoxigenic molds such as *Aspergillus*, *Fusarium*, and *Penicillium* play an unavoidable role in the decline of the value, quality and hygiene of foodstuffs by the synthesis of highly toxic metabolites known as mycotoxins. They can penetrate into the food chain throughout the food production system - in the field, during shipping, handling, storage - and they are be found in various food products all over the world. *Penicillium* and *Aspergillus* are recognized as food contaminants introduced during storage, whereas *Fusarium* species are destructive plant pathogens producing mycotoxins before or immediately post-harvest (Rahayu, 2007; Rahayu 2009). Food mycotoxin contamination is becoming a serious problem, especially in tropical countries.

Six classes of mycotoxins are often found in food products: aflatoxins, fumonisins, ochratoxins, patulin, trichothecenes and zearalenone (Bhat et al., 2010). Mycotoxins cause a toxic response, called mycotoxicosis. When ingested by humans, mycotoxins can cause damage to kidney function (Fuchs and Peraica, 2005), disturb the development of fetus (Pitt, 2000), induce many kinds of cancer (IARC, 2002; Schwartz, 2002; Hamid et al., 2013), cause Kashin-Beck disease (Stone, 2009), alimentary toxic aleukia (Peraica et al., 1999), premature thelarche (Rodriguez et al., 1985), and human ergotism (Belser-Ehrlich et al., 2012). Some mycotoxins are neurotoxins (Leese-Haley, 2003), while others act by disrupting protein synthesis causing effects ranging from necrosis to immunodeficiency (Jiang et al., 2008; Meissonier et al., 2008; Jolly et al., 2011).

Mycotoxin contamination of food and feedstuff is important also from an economic point of view. Apart from the obvious food and feed losses, they can have a negative affect on productivity, foreign exchange earnings, increase cost inspections before and after shipments, compensation payments in case of claims, research, and training program costs, detoxification costs, etc. When combined, these costs are certainly extremely high (Bhat and Miller, 1991; Wu, 2006). Many countries must spend money up to hundreds of million dollars per year to deal with this problem (Zanelli, 2000; Wu, 2006; Schmaile and Munkvold, 2009; Amaike and Keller, 2011).

Physical and chemical methods have been used to prevent toxigenic fungal growth in food commodities. The physical methods, i.e., drying, freezing, freeze-drying, modified atmosphere packaging, UV, microwave and infrared radiation usually require expensive multi-faceted approaches that limit their applicability. Chemical preservatives have been used as an additional constraint to restrict the number of microorganisms capable of growing within the food. However, the decline in consumer acceptance of the classical preservation methods and the undesirable allergenic potential of many additives have led researchers to look for natural inhibitors from plant or microbial sources. Some preservatives may be metabolized in the foodstuff or *in vivo* to produce toxic substances (Classen et al., 1987). Additionally, some fungi have acquired the ability to tolerate chemical preservatives. For example, some *Penicillium* can grow in the presence of potassium sorbate (Davidson, 2001), benzoic acids (Schnurer and Magnusson, 2005) while other molds have the ability to break down sorbate (Nielsen and de Boer, 2000).

The trend for using natural-label friendly preservatives has drastically increased over the last years and has been replacing the traditional preservation system. Biopreservation is an interesting alternative approach to physical and chemical preservation methods commonly applied in food. Biopreservation refers to the use of natural microflora or controlled microbiota or antibacterial products to extend shelf life and increase food safety (Stiles, 1996). Among natural biological antagonist, lactic acid bacteria (LAB) have potential applications as bio-preservative. LAB can play a vital role as preservatives since they have a long history in fermented food production, are generally recognized as safe (GRAS status) and they have a Qualified Presumption of Safety (QPS) status in the EU, consequently the scientific exploration of their potential as biocontrol agents have received a consistent and growing interest. These microorganisms occur naturally in many food systems and are also part of the intestinal microflora. Careful selection of specific strains of LAB with antifungal properties can be used as food preservatives reducing mold contamination, therefore, improving the shelf-life of many fermented food products (Corsetti et al., 2000; Clarke et al., 2002; Crowley et al., 2002; Arendt et al., 2007) and reducing health risks due to mycotoxins exposure (Gourama and Bullerman, 1995). Many research reports also indicate that LAB has beneficial health effects in humans (Perdigon, 2001; Masood et al., 2011; Florou-Paneri, 2013; Fernández et al., 2015; Nuraida et al., 2015). Moreover, due to certain specific metabolic properties, LAB are of particular interest in the food industry, since they can improve the sensory and nutritional value of food products (Crowley et. al., 2002; Arendt et al., 2007).



In recent years, literature has accumulated on this topic, but so far there are only a limited number of comprehensive reviews. The focus of this review is to summarize the new findings in this research topic including new insights into new methods of improving antifungal properties such as genetic engineering. Application of antifungal LAB in the food system is also taken into account.

## Overview of LAB

Lactic acid bacteria belong to the phylum *Firmicutes*. According to Hofvendahl and Hahn-Hagerdal (2000), LAB are Gram-positive, microaerophilic, non-spore forming, and acid-tolerant bacteria, with DNA base composition of less than 50 mol% (G + C), they can survive at pH 5 or lower and its broad optimal growth temperatures range from 20°C to 45°C. They are generally catalase negative but sometimes pseudo-catalase has been detected when they grow on low sugar concentrations. The most important LAB property is the capacity to convert hexoses into lactic acid (LA) (Södergård and Stolt, 2002).

Lactic acid bacteria were initially classified into four classes, namely *Lactobacillus*, *Leuconostoc*, *Streptococcus* and *Pediococcus* (Wessels et al., 2004). The classification considers morphological characteristics, fermentation patterns, capability to grow at different temperatures, stereospecificity (D or L-lactic), as well as acid and base-tolerance. Nowadays, each class includes several genera. Genus *Lactobacillus* comprises *Lactobacillus* and *Carnobacterium*. Genus *Streptococcus* consists of *Streptococcus*, *Lactococcus*, *Vagococcus*, and *Enterococcus*. Genus *Pediococcus* includes *Pedicoccus*, *Tetragenococcus* and *Aerococcus*. While, genus *Leuconostoc* remains the same (Ruas-Madiedo et al., 2012). The new classification is based on composition of fatty acid in cell membranes, motility and rRNA sequences, as well as guanine and cytosine percentage in DNA. For identification, sequencing of 16S rRNA gene is an important guideline in the classification of LAB (Wood and Holzapfel, 2012).

According to the fermentation patterns, LAB are grouped into two, homofermentative and heterofermentative (Prescott and Dunn, 1959; Davidson et al., 1983). During the cell metabolism, the homofermentative LAB produces LA from hexoses via glycolysis (Embden-Meyerhof-Parnas pathway). This LAB does not ferment pentoses and gluconate. They also do not produce gas from glucose and nor do they possess phosphoketolase (Surono, 2004). Homofermentative LAB is often used for food preservation because the high amount of LA produced, which inhibits the growth of other bacteria. Heterofermentative bacteria, ferments glucose to produce LA, CO<sub>2</sub>, ethanol, acetaldehyde, diacetyl and other compounds (Mitsuoka, 1990). Aldolase enzyme is absent in this LAB. However, phosphoketolase is present in long and short rods type (Surono, 2004). In hetero-fermentation, pentose is converted into LA and acetic acid, via pentose phosphate pathway (Figure 1).

LAB can be found in many fermented foods such as meat, dairy products, vegetables, beverages, and bread. They are also present in soil, water, animal manure, waste, and plants. These bacteria are also members of microbiotas of the mucous membrane in the human, and animal digestive tract, mouth, skin, urinary tract and genital organs, and can have a beneficial impact on

these tracts and organs. The bacteria can enter food via the environment, as naturally occurring microorganisms, or can be added into the food as cultures.

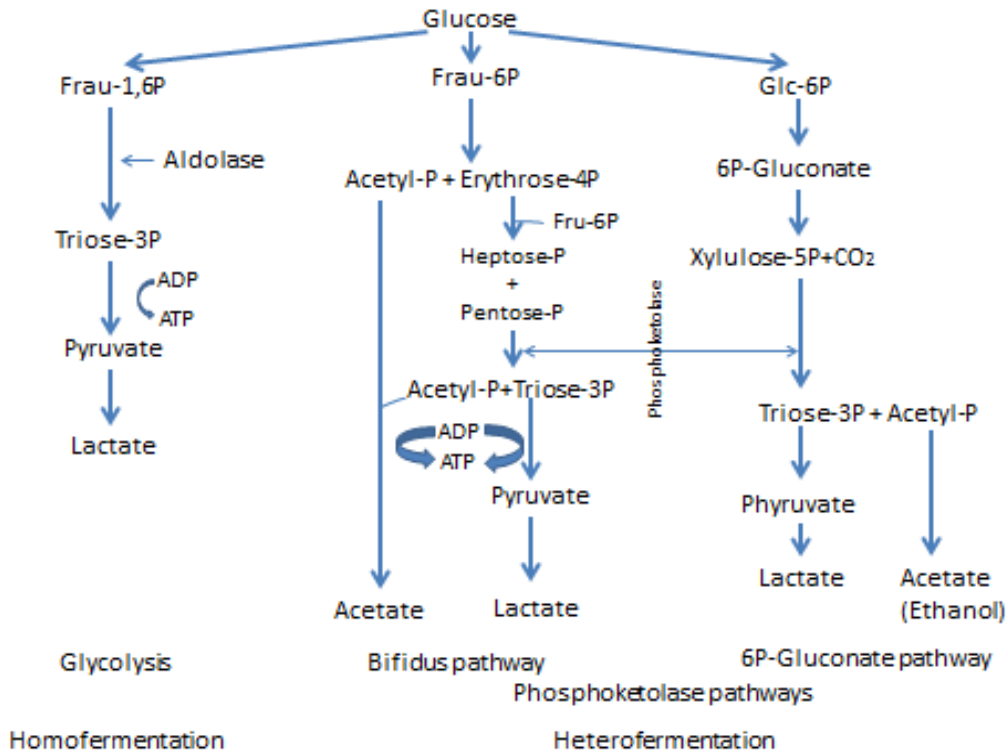


Fig 1. The main routes of hexose fermentation in LAB (Modified of Wood and Holzapfel, 2012)

LAB has gained attention because of the practical application for starter cultures in fermented food and feed, medicines, probiotics, and as a biological control agent. In food industry, LAB is widely utilized as a starter to make a pleasant change in the texture, aroma, flavour and acidity of foods (Harzallah and Belhadj, 2013). LAB is used in various ways such as a probiotic, bacteriocin producer and as a starter culture in fermented foods.

## LAB as Antifungal Agent

An antifungal agent is an organism capable of inhibiting biodeterioration and preventing fungal disease. It can be produced during microbial fermentations (Carlile et al., 2001). In general, there are four mechanisms of antifungal action: inhibition of cell wall synthesis, perturbation of membrane function, inhibition of protein synthesis, and inhibition of cell division (Odds et al., 2003). The inhibition of  $\beta$ -glucans synthesis and chitin synthetase is the main mechanism in which antifungal compounds inhibit cell wall synthesis on mold. While, antimicrobial compounds can also disrupt cell membranes in several ways, for example by binding sterols to destroy membrane function or inhibiting sterol biosynthesis. Protein synthesis can also be disrupted since blasticidin (antimicrobial peptide) inhibits protein synthesis by binding ribosomes. Inhibition also occurs during cell division by interfering with microtubules (Ström, 2005).

Bioactive compounds are secondary metabolites produced by microorganisms which are not essential for the growth of the microorganism. The production of secondary metabolites can act antimicrobial (Carlile et al., 2001). Over 80 years ago, LAB was described to produce antimicrobial compounds. The antifungal strains were isolated from different environments such as sourdough culture (Corsetti et al., 1996), *salami* (Coloretti et al., 2007), grass silage (Magnusson and Schnurer, 2001), cheeses and milk (Roy et al., 1996) and vegetable products (Sathe et al., 2007).

LAB antagonism refers to the inhibition of other microorganism, caused by competition for nutrients, and by the production of antimicrobial metabolites (Holzapfel et al., 1995). Extensive research has been done on the antifungal ability of LAB, and fungal inhibitory components have been isolated and identified. So far, there are 114 research papers regarding antifungal LAB and, as shown in Figure 2, the genus *Lactobacillus* is the most frequently isolated and researched. Among *Lactobacillus* species, *Lact. plantarum* is the most important one regarding antifungal activity against toxigenic fungus. The identification of these LAB antifungal compounds started in 2007 and some of them are described below (Table 1, Figure 3). The antifungal compounds produced by LAB vary. The majority of the antifungal substances identified are low molecular weight compounds including organic acids, hydrogen peroxide, proteinaceous compounds, hydroxyl fatty acids and phenolic compounds.

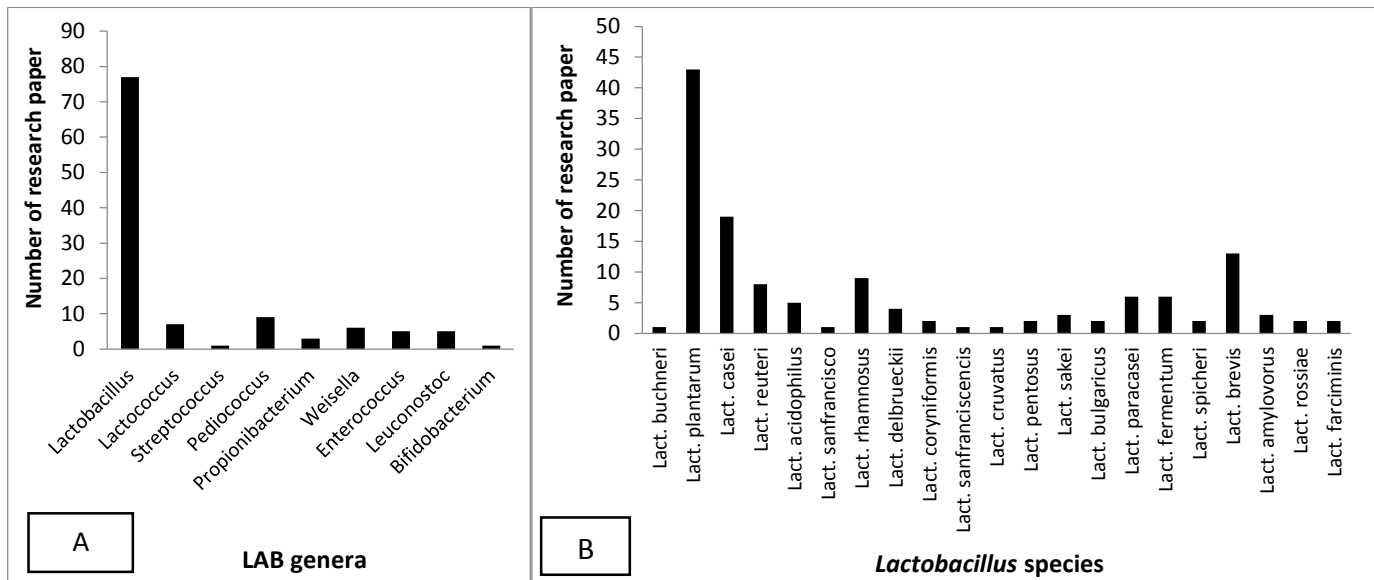


Fig 2. Number of research papers since 1981 based on antifungal properties of:  
A) LAB genera, B) *Lactobacillus* species.

## Antifungal Compounds Produced by LAB

### Organic acids

The antimicrobial effect of organic acids lies in the decrease of pH (Ammor et al., 2006). De Vyust and Vandamme in 1994 stated that LAB has long been known to produce organic acids, such as acetic acid, lactic acid, etc. Accumulation of acidic end-products and the concomitant low pH, results in a wide inhibitory spectrum against microbes. Both the dissociation constant and the acid concentration, determine the inhibitory activity level. In their undissociated form, lipophilic acids, such as lactic and acetic acid can penetrate the microbial cell, and dissociate to produce hydrogen ions at the higher intracellular pH, reducing the intracellular pH, thus interfering in essential metabolic functions such as substrate translocations and oxidative phosphorylation (Baird and Parker, 1980; Lindgren and Dobrogosz, 1990). Organic acids can only penetrate the microbial cell wall if their pH is beneath their  $pK_a$ . The  $pK_a$  value of the most common acids produced by LAB are below 5.0. The  $pK_a$  of lactic, acetic, 3-phenyl-L-lactic and caproic acids are 3.8, 4.7, 3.5 and 4.9, respectively (De Muynck et al., 2004). Inhibition of fungal pathogens by organic acids may involve inhibition of enzymes activity, and cell membrane disruption caused by undissociated active fractions of decomposed organic acids (Gerez et al., 2010).

Synthesis of LA and acetic acid are the main factors responsible for LAB use as biopreservatives (Trias et al. 2008a). Lactic acid is the main acid component produced by LAB. It could reduce pH in fermentation media; however when citrate is present in the medium together with glucose during the initial fermentation, the LA produced is degraded. Citrate is concomitantly degraded, resulting in the accumulation of formic, acetic and succinic acids along with  $CO_2$  (Lindgren et al., 1990). Acid production and the reduction of pH prolong the lag phase of the organism. A recent study reported that LA is the primary antifungal agent produced by *Lact. plantarum* UFG 108 and *Lact. plantarum* UFG 121, it inhibits the growth of various fungi, i.e. *Aspergillus niger*, *A. flavus*, *Fusarium culmorum*, *Penicillium expansum* and *P. chrysogenum* (Russo et al., 2016). Baek et al. (2012) and Lan et al. (2012) also described that a mixture of organic acids produced by *Weissella sp.* and *Leuconostoc sp.* can prevent the growth of mold spoilage.

Acetic acid is reported to be more inhibitory than LA against yeasts and molds (Eklund, 1989). This can be explained by its dissociation properties since acetic acid has between two to four times more of the acid in the undissociated state at a pH interval between 4.0 and 4.6 compared to LA (Lindgren and Dobrogosz, 1990; Batish et al., 1997). Heterofermentative LAB also produces propionic acid in very small amounts. Propionic acid can inhibit cell membranes by neutralizing the electrochemical proton gradient, it can cause bacteriostatic and fungistatic effects. However the effect of acetic and propionic acids depend on the pH decrease caused by LA (Lind et al., 2005; Le Lay et al., 2016). Propionic acid is known to be more inhibitory than acetic or lactic acid. Moon (1983) reported that a mixture of lactic, acetic and propionic acid results in a drastic reduction in yeast growth, while the yeast tends to grow well at relatively high acetic or lactic acids concentrations (100 mmol/L).

Another organic acid that shows antifungal activity is phenyllactic acid (PLA) produced by *Lact. plantarum* with a broad spectrum antifungal activity (Lavermicocca et al., 2000, Magnusson and Schnürer 2001; Lavermicocca et al., 2003, Ström et al., 2002). Some LAB can synthesize PLA and 4-hydroxy-acid phenyllactic (Valerio et al., 2004). 3-PLA is derived from the metabolism of phenylalanine. As follows, phenylalanine is transaminated to phenylpyruvic acid (PPA) (Li et al., 2007) and then PPA is converted to 3-phenyllactic acid (Mu et al., 2009). Supernatant of *Lact. plantarum* 21B inoculated with wheat starch hydrolysates reliably prevents the growth of several toxigenic fungi, namely *A. niger*, *A. flavus*, *Monilia sitophila*, *F. graminearum*, *P. roqueforti*, and *P. expansum*, (Lavermicocca et al., 2000; Lavermicocca et al., 2003). Broberg et al. (2007) reported that *Pichia anomala* and *P. roqueforti* are inhibited by *Lact. plantarum* strain MiLAB14 and *Lact. coryniformis* MiLAB 393 through their antifungal compounds, 3-PLA. Lavermicocca et al. (2000) reported that PLA was contained at the highest concentration in the bacterial culture filtrate and had the highest activity and inhibited all the tested fungi at a concentration of 50 mg except for *P. roqueforti* IBT 18.687 and *P. corylophilum* IBT 6978 (166 mg/mL). PLA also inhibits the growth of *Candida pulcherrima*, *C. parapsilosis* and *Rhodotorula mucilaginosa* (Schwenninger et al., 2008). Prema et al. (2010) identified PLA in the culture supernatant of *Lact. plantarum* using NMR and IR spectroscopy, showing inhibitory effects against *A. fumigatus* and *Rhizopus stolonifer*. Ndagano et al. (2011) found PLA mixed with other organic acids and free fatty acids in the supernatant of *Lact. plantarum* VE56 and *W. paramesenteroides* LC11 inhibiting fungal growth. Antifungal compounds from culture supernatant of *Lact. plantarum* 1.7 FST was isolated by Dal Bello et al. (2007), they were identified as cyclo (L-Leu-L-Pro), cyclo (L-Phe-L-Pro), and PLA. The main constituent of antifungal compounds were PLA and lactic acid. Gerez et al. (2010) revealed that PLA is the most effective organic acid.

Benzoic acid is one of the oldest chemical preservatives used in drug, cosmetic and food industries. This organic acid was isolated and identified in the culture filtrate of *Lact. plantarum* VTT E-78076 by Niku-Paavola group in 1999 and it was active against *Fusarium avenaceum* VTT D-80 147 (Niku-Paavola et al., 1999). The elimination of the electrochemical gradient across the cell membrane caused by benzoate is the reason for the microbial growth inhibition (Eklund, 1989).

Wang et al. (2012) managed to purify a new antifungal compound, benzeneacetic acid produced by *Lact. plantarum* IMAU10014, which was active against *Botrytis cinerea*, *F. oxysporum*, *P. citrinum* and *P. digitatum*. LAB also produce a mixture of organic acids during fermentation, including caproic acid, formic acid, butyric acid and n-valeric acid. Corsetti et al. (1998) identified a mixture of organic acids produced by *Lact. sanfrancisco* CB1 and found that the organic acids act in a synergic way with caproic acid during molds growth inhibition.

### Free fatty acids

Antifungal mechanism of free fatty acids is caused by the alteration of the membrane fluidity by partitioning the lipid bilayer of cells or by inclusion into fatty acyl chains of membrane phospholipids (Avis and Belanger, 2001). This increases membrane permeability and the release of intracellular electrolytes and proteins eventually, leading to the cytoplasmic disintegration of fungal cells (Sjögren et al., 2003). Some LAB can produce hydroxy fatty acids with potent antifungal activity. Sjögren et al. (2003) identified and characterized four types of antifungal substances namely 3-(R)-hydroxy decanoic acid, 3-hydroxy-5-cis-dodecenoic acid, 3-(R)-hydroxy dodecanoic acid and 3-(R)-hydroxy tetradecanoic acid, from *Lact. plantarum* MiLAB 14. Ryu et al. (2014) also have purified the antifungal 3-hydroxy decanoic acid, 5-oxododecanoic acid and 3hydroxy-5-dodecenoic acid from *Lact. plantarum* isolated from *kimchi* which are active against a broad fungal spectrum. Corsetti et al. (1998) reported that *Lact. sanfrancisco* CB1 can produce butyric acid, caproic, propionic and valeric acid which actively inhibits the growth of *Aspergillus* sp., *Fusarium* sp., *Monilia* sp., and *Penicillium* sp. Among these fatty acids, caproic acid was the key compound of the antifungal mixture. Ndagano et al. (2011) detected the presence of the antifungal 2-hydroxy-4-methylpentanoic acid in the culture supernatants of *Lact. plantarum* VE56 and *W. paramesenteroides* LC11.

### Peptides and Protein

There is no clear explanation of the mechanism of proteins during the inhibition of the fungal growth. Many researchers reported that the antifungal metabolites are sensitive to protease. Initial research conducted by Roy et al. (1996) showed that antifungal supernatants are no longer able to inhibit fungi after being treated with protease. One year later Gourama and Bullerman (1997) also found something similar, the ability of culture supernatants from *Lact. casei* ssp. pseudoplantarum was decreased following treatment with trypsin or chymotrypsin. This antifungal protein has a molecular weight less than 1 kDa. Corsetti et al. (1998) isolated bacteriocin-like inhibitory substance from *Lact. sanfrancisco* C57 and found the antimicrobial compounds produced from strains of different species contained a protein moiety and showed varying sensitivity to proteases. Niku-Paavola et al. (1999) found the compound cyclo-(Gly-L-Leu) produced by *Lact. plantarum* and active against *F. avenaceum*. Antifungal protein compounds were also isolated by Magnusson and Schnurer (2001) from *Lact. coryniformis* ssp. coryniformis. This active compound has a molecular mass of approximately 3 kDa. In 2002, Ström et al. reported the first isolation of cyclic (Phe-Pro) and cyclic (Phe-trans-4-OH-Pro) from *Lact. plantarum* MiLAB 393, their chemical structures were composed of both L and D forms, in a 9/1 ratio. The following year Magnusson et al. (2003) found a cyclic dipeptide produced by *Lact. coryniformis*, i.e., cyclic (Phe-Pro) and cyclo (Phe- 4-OH-Pro).

Coloretti et al. (2007) found that the antifungal compounds of lactobacilli culture from *salami* is sensitive to proteinase K. Coda et al. (2008) identified eight peptide mixtures from the water-soluble extract of sourdough *Lact. brevis* AM7, and five of these exhibited inhibitory activity with the range of MICs of 3.5 to 8.2 mg/mL against *Aspergillus* and *Penicillium*. Roy et al. (2009) isolated an antifungal protein with molecular mass of 11 kDa from *En. faecalis* CHD



28.3 using ultrafiltration and chromatographic techniques. Recent research from Muhialdin et al. (2015a) reported that five peptides from *Leuconostoc mesenteroides* are able to inhibit the growth of *A. niger*. Antifungal peptides have also been purified from MRS medium culture supernatant of *Lact. plantarum* IS10 strain. Most research suggests that antifungal peptides and proteins are purified from culture supernatants of *Lact. plantarum*.

### Hydrogen Peroxide

Lactic acid bacteria have flavoprotein oxidases capable of producing hydrogen peroxide ( $H_2O_2$ ) in the presence of oxygen. The formation of hydrogen peroxide is catalyzed by a very active cytoplasmic flavoprotein (FAD) NADH oxidase which remove excess electrons from NADH thus competing with lactate dehydrogenase for available NADH (formed during glucose breakdown) (Condon, 1987). Antimicrobial effect due to the production of hydrogen peroxide led to hyperbaric oxygen toxicity, peroxidation of membrane lipids, and a strong oxidizing effect on the cells, which cause the destruction of basic molecular structures, such as nucleic acids and cell proteins (Dahl et al., 1989). Hydrogen peroxide can also react with another compound showing antimicrobial effects known as lactoperoxidase system (LP System). Raw milk thiocyanate at concentrations of 1-10 mg/L is oxidized producing antibacterial hypothiocyanite compounds by the enzyme lactoperoxidase in the presence of hydrogen peroxide at a concentration of about 10 mmol/L (Reiter and Harnulv, 1984; Condon, 1987; Schnurer and Magnusson, 2005). Hypothiocyanite is an antimicrobial compound which is lethal or inhibitory to microorganisms. Venturini et al. (2002) studied in vitro antimicrobial activity and the MIC (minimum inhibitory concentration) of hydrogen peroxide activity against *P. expansum* using agar diffusion method, volatility and agar method. The effectiveness of 5 and 10% hydrogen peroxide on growth inhibition of *P. expansum* in the agar diffusion assay was total, and its MIC as determined by the agar and broth dilution assays was less than 0.025%.

### Reuterin

Reuterin produced by *Lact. reuterii* is part of the human and animal digestive system. Its molecular weight is less than 200 Da and is resistant to protease activity. Reuterin is an equilibrium mixture of monomeric, hydrated monomeric, and cyclic dimeric  $\beta$ -hydroxypropionaldehyde formed during anaerobic metabolism of glycerol and glyceraldehyde (Talarico et al., 1988; Talarico and Dobrogosz, 1989). Reuterin is a broad-spectrum antimicrobial that is effective primarily against Gram-negative bacteria, yeast, molds, protozoa and viruses. It can inhibit the growth of *Aspergillus* spp. and *Fusarium* spp. (Axelsson et al., 1989). This compound inhibits sulfhydryl enzymes such as ribonucleotide reductase, an enzyme involved in DNA biosynthesis (Talarico and Dobrogosz, 1989).

Table 1. LAB antifungal activity against toxigenic fungi.

LAB isolates	Source of isolation	Activity spectrum	Antifungal compounds	References	Patent number
<i>Streptococcus lactis</i> C10	Stock cultures	<i>A. parasiticus</i>	ND	Wiseman and Marth (1981)	
<i>Lact. casei</i> ATCC 393	Stock cultures	<i>A. parasiticus</i>	ND	El-Gendy and Marth (1981)	
<i>L. lactis</i>	Stock cultures	<i>A. flavus</i>	A heat stable -low molecular weight-compound	Coallier-Ascah and Idziak (1985)	
<i>Lact. casei</i> ssp. rhamnosus	Unknown	<i>Penicillium oxalicum</i>	ND	King et al. (1987, 1990)	EP 0221499 A2 US 4956177
<i>Lact. reuteri</i>	Stock cultures	Broad spectrum	Reuterin	Talarico et al. (1988)	
<i>Lact. reuteri</i>	Swine intestine	<i>A. flavus</i> , <i>Fusarium sanfucienum</i> , and <i>Trypanosoma cruzi</i>	Reuterin	Chung et al. (1989)	
<i>Pediococcus acidilactici</i>	Unknown	<i>P. oxalicum</i>	Proteinaceous	Vandenbergh and Kunka (1989, 1990a, 1990b)	US 4877615 US 4906611 US 4942032
<i>Lact. casei</i> var. rhamnosus	Stock cultures	Broad spectrum	<1Kda	Vandenburgh and Kanka (1989)	
<i>L. lactis</i>	Centraalbureau voor Schimmelcultures	<i>A. parasiticus</i>	ND	Luchese and Harrigan (1990)	
<i>L. lactis</i> subsp. diacetylactis DRCl, <i>Lact. acidophilus</i> R	Unknown	<i>A. fumigatus</i> , and <i>A. parasiticus</i>	ND	Batish et al. (1989, 1990)	
<i>Lact. plantarum</i> , Ln. <i>mesentroides</i> , <i>Lact. casei</i> subsp. rhamnosus	Unknown	<i>Penicillium spp.</i> , and <i>Aspergillus spp.</i>	ND	Suzuki et al (1991)	
<i>Lact. casei</i> subsp. rhamnosus LC.705	Unknown	<i>A. niger</i> , <i>Fusarium spp.</i> , and <i>Penicillium spp.</i>	ND	Mayra-Makinen et al. (1994), Mayra-Makinen and Soumalainen (1995)	EP 0576780 A2 US 5378458
<i>L. lactis</i> ssp. lactis CHD 28.3	Cheese	<i>A. parasiticus</i> , <i>A. flavus</i> , and <i>Fusarium sp.</i>	Possibly proteinaceous	Roy et al. (1996)	
<i>Lact. casei</i> ssp. pseudoplantarum	Silage	<i>A. flavus</i>	Possibly proteinaceous	Gourama and Bullerman (1997)	
<i>Lact. casei</i>		<i>Penicillium sp.</i>	Possibly low molecular weight peptide	Gourama (1997)	
<i>Lact. sanfrancisco</i> CB1	Sourdough	<i>Fusarium sp.</i> , <i>Penicillium sp.</i> , <i>Aspergillus sp.</i>	Caproic, propionic, butyric and valeric acid	Corsetti et al. (1998)	



Chapter 5: Review Article (Antifungal Lactic Acid Bacteria)

<i>Lact. plantarum</i> VTT E-78076	Beer	<i>F. avenaceum</i>	Benzoic acid, methylhydantoin, mevalonolactone, cyclo (Gly-L-Leu)	Niku-Paavola et al. (1999)	US 5695414 US 6277374
<i>Lact. casei</i> ssp. <i>rhamnosus</i>	Unknown	<i>Penicillium oxalicum</i>	ND	Vandenberg and King (1999, 2001)	
<i>Lact. plantarum</i> strain 21B	Sourdough	<i>Eu. repens</i> , <i>Eu. rubrum</i> , <i>P. corylophilum</i> , <i>P. roqueforti</i> , <i>P. expansum</i> , <i>Endomyces fibuliger</i> , <i>A. niger</i> , <i>A. flavus</i> , <i>Monilia sitophila</i> , and <i>F. graminearum</i>	PLA and 4-hydroxyphenylacetic acid	Lavermicocca et al. (2000); Lavermicocca et al. (2003)	
<i>Lact. rhamnosus</i> VT1, and <i>Lact. reuteri</i> CCM 3625	Unknown	<i>Fusarium</i> sp., <i>Aspergillus</i> sp., and <i>Penicillium</i> sp.	Lactic acid and other substances	Plockova et al. (2001)	
<i>L. lactis</i> subsp. <i>cremoris</i> , <i>Lact. casei</i> , <i>Lact. delbrueckii</i> subsp. <i>bulgaricus</i>	Culture collection	<i>P. expansum</i>	ND	Florianowicz (2001)	
<i>Lact. coryniformis</i> ssp. <i>coryniformis</i> Si3	Grass	<i>A. fumigatus</i> , <i>A. nidulans</i> , <i>P. roqueforti</i> , <i>Mucor hiemalis</i> , <i>Talaromyces flavus</i> , <i>F. poae</i> , <i>F. graminearum</i> , <i>F. culmorum</i> , <i>F. sporotrichioides</i> and <i>Rhodotorula mucilaginosa</i>	PLA, cyclo(Phe-Pro), cyclo (Phe-4-OH-Pro)	Magnusson and Schnurer (2001); Magnusson et al. (2003)	
<i>Lact. rhamnosus</i>	Unknown	<i>Fusarium</i> sp., <i>Penicillium</i> sp., <i>Aspergillus</i> sp. and <i>Alternaria</i> sp.	Sodium acetate	Stiles et al. (2002)	
<i>Lact. plantarum</i> MiLAB 393	Grass silage	<i>F. sporotrichioides</i> , <i>A. fumigatus</i> , <i>A. nidulans</i> and <i>Kluyveromyces marxianus</i>	PLA, cyclo(Phe-Pro), cyclo (Phe-OH-Pro)	Ström et al. (2002); Ström et al. (2005)	
<i>Lact. plantarum</i> VTT E-78076 (E76), and VTT E-79098 (E98)	Beer and pickled cabbage	<i>F. avenaceum</i> , <i>F. culmorum</i> , <i>F. graminearum</i> and <i>F. oxysporum</i>	ND	Laitila et al. (2002)	

Chapter 5: Review Article (Antifungal Lactic Acid Bacteria)

<i>Lact. sanfranciscensis</i> FERM P-18244, FERM P-18245, FERM P-18246	Unknown	<i>A. niger</i> <i>A. oryzae</i> <i>Mucor hiemalis</i> <i>P. citrium</i> <i>P. funiculosus</i> , and <i>Rhizopus oryzae</i>	Proteinaceous	Ando et al. (2003, 2004)	US 2003/0035860 A1 US 6827952 B2				
<i>Lact. plantarum</i> MiLAB 14	Lilac flowers	<i>P. roqueforti</i> , <i>P. commune</i> , <i>A. nidulans</i> , and <i>A. fumigatus</i>	3-(R) hydroxyl decanoic acid, 3-hydroxy-5-cis-dodecanoic acid, 3-(R)hydroxydodecanoic acid ND	Sjögren et al. (2003)					
<i>Lact. acidophilus</i> LMG 9433, <i>Lact. amylovorus</i> DSM 20532, <i>Lact. brevis</i> LMG 6906, <i>Lact. coryniformis</i> ssp. <i>coryniformis</i> LMG 9196, and <i>Lact. plantarum</i> LMG 6907	Dairy products	<i>A. flavus</i> , <i>Endomyces fibuligera</i> , <i>Eu. repens</i> , <i>P. paneum</i> , <i>P. roqueforti</i> , <i>R. oryzae</i> , <i>Cerinosterus</i> sp., and <i>Cladosporium</i> sp.	ND	De Muynck et al. (2004)					
<i>Pr. acidipropionici</i> , <i>Pr. jensenii</i> , <i>Pr. thoenii</i> , <i>Pr. freudenreichii</i> ssp. <i>Freudenreichii</i> , and <i>Pr. freudenreichii</i> ssp. <i>shermanii</i>	Dairy	<i>P. roqueforti</i> , and <i>A. fumigatus</i>	Propionic acid, acetic acid, and lactic acid	Lind et al. (2005)					
<i>Lact. casei</i> ssp. <i>rhamnosus</i> , and <i>Lact. acidophilus</i>	Unknown	<i>P. chrysogenum</i> , and <i>Aureobasidium pullulans</i>	Unknown compounds with MW < 1 KDa ND	Yang and Clausen (2005) Kim (2005)					
<i>Lact. cruvatus</i> , <i>L. lactis</i> ssp. <i>lactis</i> , <i>Lact. casei</i> , <i>Lact. pentosus</i> , and <i>Lact. sakei</i> <i>Lact. delbrueckii</i>	Kimchi Unknown	<i>A. flavus</i> , <i>F. moniliforme</i> , <i>P. commune</i> , and <i>R. oryzae</i> <i>P. olsonii</i>	Unknown	Miyamoto and Naito (2006, 2008, 2011)	EP 1726638A1 US 2008/0286406 A1 US 8029779 B2				
<i>Lact. plantarum</i>	<i>Salami</i>	<i>A. candidus</i> , and <i>P. nalgiovense</i>	Phenyl-lactate, hydroxyl-phenyl-lactate and peptidic Phenolic compound	Coloretto et al. (2007)					
<i>Ped. acidilactici</i> LAB 5	Fermented meat	<i>A. fumigatus</i> , <i>A. parasiticus</i> , <i>F. oxysporum</i> , and <i>Penicillium</i> sp.		Mandal et al. (2007)					

<i>Pr. freudenreichii</i> ssp. shermanii, <i>Pr. thoenii</i> , <i>Pr. acidipropionici</i> , <i>Pr. freudenreichii</i>	Dairy origin	<i>A. fumigatus</i> , and <i>R. mucilaginosa</i>	Diketopiperazines, cyclo(L-Phe-L-Pro), cyclo(L-Ile-L-Pro)	Lind et al. (2007)
<i>Lact. paracasei</i> ST 68, <i>Lact. fermentum</i> ST 40, and ST 41 Genus <i>Lactobacillus</i>	Edam cheese Unknown	<i>F. proliferatum</i> , <i>Penicillium</i> sp., and <i>A. niger</i> <i>F. latericum</i> , <i>Mucor hiemalis</i> , <i>A. niger</i> , <i>A. ochraceus</i> , <i>Candida mycoderma</i> , <i>Alternaria alternata</i> , <i>Geotrichum candidum</i>	ND ND	Tuma et al. (2007) Klewicka (2007)
<i>Lact. plantarum</i>	Vegetables	<i>F. graminearum</i> , <i>R. stolonifer</i> , <i>Sclerotium oryzae</i> , <i>Sclerotinia minor</i> , <i>Rhizoctonia solani</i> and <i>B. cinerea</i>	ND	Sathe et al. (2007)
<i>Lact. plantarum</i> FST 1.7	Malted barley	<i>A. niger</i> , <i>F. culmorum</i> , <i>F. graminearum</i> and <i>P. roqueforti</i>	Lactic acid, PLA and cyclic dipeptides cyclo (L-Leu-L-Pro) and cyclo (L-Phe-L Pro)	Dal Bello et al. (2007)
<i>Ped. pentosaceus</i> <i>Lact. plantarum</i> 1A7 (S1A7) <i>Ln. mesenteroides</i> , <i>Ln. citreum</i> , <i>Lact. plantarum</i> , <i>L. lactis</i> , <i>W. cibaria</i> , and <i>En. mudntii</i> <i>Lact. paracasei</i> ssp.	Malted cereals Sourdough Fruits and vegetables Sourdough	<i>P. expansum</i> <i>Broad spectrum</i> <i>P. expansum</i> , <i>Monilinia laxa</i> , and <i>B. cinerea</i> <i>F. proliferatum</i> , and <i>F. graminearum</i>	Possibly peptides Peptides ND ND	Rouse et al. (2008) Coda et al. (2008) Trias et al. (2008b) Hassan and Bullerman (2008)
<i>Lact. fermentum</i> , and <i>Ln. mesenteroides</i>	Cassava	<i>A. niger</i> , <i>A. flavus</i> , <i>Penicillium</i> sp., and <i>F. oxysporum</i>	Diacetyl and hydrogen peroxide	Ogunbanwo et al. (2008)
<i>Lact. plantarum</i> CRL 778, <i>Lact. reuteri</i> CRL 1100, and <i>Lact. brevis</i> CRL 772, CRL 796, <i>Lact. amylovorus</i> FST 2.11	Sourdough Sourdough	<i>Aspergillus</i> , <i>Fusarium</i> , and <i>Penicillium</i> <i>A. niger</i> , <i>F. culmorum</i> , <i>P. expansum</i> , and <i>P. roqueforti</i>	Acetic acid and PLA Cytidine, Deoxycytidine, cyclo(Pro-Pro), cyclo(Leu-Pro), cyclo(Tyr-Pro), cyclo(Met-Pro),	Gerez et al.(2009) Arend et al. (2009, 2011) EP 2009/056229 US 2011/0200569

Chapter 5: Review Article (Antifungal Lactic Acid Bacteria)

	Wheat semolina	<i>A. niger</i> , <i>P. roqueforti</i> , and <i>Endomyces fibuliger</i>	Lactic and acetic acid	Valerio et al. (2009)
<i>W. confusa</i> , <i>W. cibaria</i> , <i>Ln. citreum</i> , <i>Ln. mesenteroides</i> , <i>L. lactis</i> , <i>Lact. rossiae</i> , and <i>Lact. plantarum</i>				
<i>E. faecalis</i> CHD 28.3	Cheddar cheese	<i>Broad spectrum</i>	Protein	Roy et al. (2009)
<i>Lact. brevis</i> PSI	Brewing barley	<i>F. culmorum</i>	Organic acids and proteinaceous compounds	Mauch et al. (2010)
<i>Lact. plantarum</i> (CRL 1081, CRL 778, and CRL 1073), <i>Lact. casei</i> (CRL 76, and CRL 208), <i>Lact. acidophilus</i> CRL 1063, <i>Lact. paracasei</i> CRL 1501, and <i>Ped. pentosaceus</i> CRL 908	Dairy products	<i>P. digitatum</i> , and <i>Geotrichum citriauranti</i>	Lactic acid, acetic acid and PLA	Gerez et al. (2010)
<i>Lact. rhamnosus</i> O236	Sheep milk	<i>A. nomius</i>	ND	Munoz et al. (2010)
<i>Lact. casei</i> FF1, and <i>Lact. brevis</i> SG1	Indigenous Nigerian fermented foods	<i>P. citrinum</i>	ND	Adebayo and Aderiye (2010)
<i>Lact. plantarum</i>	Grass silage	<i>A. fumigatus</i> , and <i>R. stolonifer</i>	PLA	Prema et al. (2010)
<i>Ped. pentosaceus</i> L006	Maize	<i>F. verticillitoides</i> , and <i>F. proliferatum</i>	ND	Dalie et al. (2010a)
<i>Lact. plantarum</i> PCS 20, and <i>Ln. pseudomesenteroides</i> PCK 18	Probiotic	<i>P. nordicum</i>	ND	Schillinger and Villarreal (2010)
<i>Lact. plantarum</i> AF1	<i>Kimchi</i>	<i>A. flavus</i>	cyclo(Leu-Leu)	Yang and Chang (2010)
<i>Lact. plantarum</i> LB1, and <i>Lact. rossiae</i> LB5	Sourdough	<i>P. roqueforti</i>	Four peptides and organic acid mixture	Rizzello et al. (2011)
<i>Lact plantarum</i> , <i>Ped. pentosaceus</i>	Dairy products, vegetables and fermented pickles	<i>Penicilim sp.</i>	ND	Zhao (2011)
<i>Lact. casei</i> ssp. <i>paracasei</i>	Unknown	<i>Mucor circinelloides</i> <i>M. plumbeus</i> <i>P. brevicompactum</i> <i>P. expansum</i> <i>P. roqueforti</i>	ND	Perrier et al. (2011)
<i>Lact. fermentum</i> Te007, <i>Ped. pentosaceus</i> Te010, <i>Lact. pentosus</i> G004, and <i>Lact. paracasei</i> D5	Malaysian fermented foods	<i>A. niger</i> , and <i>A. oryzae</i>	ND	Muhalidin et al. (2011)

Chapter 5: Review Article (Antifungal Lactic Acid Bacteria)

<i>Lact. amylovorus</i> DSM 19280	Unknown	<i>A. niger</i> , <i>A. fumigatus</i> , <i>F. culmorum</i> , <i>P. expansum</i> , and <i>P. roqueforti</i>	Carboxylic acids, nucleosides, sodium decanoate and five cyclic dipeptides	Ryan et al. (2011)
<i>Lact. plantarum</i> , <i>W. cibaria</i> and <i>W. paramesenteroides</i>	Mill flour and fermented cassava	<i>A. niger</i> , <i>A. tubingensis</i> , <i>P. crustosum</i> , and <i>C. albicans</i>	2-hydroxy-4-methylpentanoic acid, PLA and other organic acids	Ndagano et al. (2011)
<i>Lact. plantarum</i> IMAU10014	Xinjiang koumiss	<i>B. cinerea</i> , <i>Alternaria solani</i> , <i>Phytophthora drechsleri</i> , <i>F. oxysporum</i> , and <i>Glomerella cingulata</i>	Proteinaceous and other unidentified compounds	Wang et al. (2011)
<i>Lact. casei</i> AST18	Cheese	<i>Penicillium</i> sp.	Cyclo-(Leu-Pro), 2,6-diphenyl-piperidine, and 5,10-diethoxy 2,3,7,8-tetrahydro-1H,6H-dipyrolo[1,2-a:10,20-d]pyrazine	Li et al. (2012)
<i>Lact. plantarum</i> IMAU10014	Koumiss	<i>B. cinerea</i> , <i>Glomerella cingulata</i> , <i>Ph. drechsleri</i> , <i>P. citrinum</i> , <i>P. digitatum</i> , and <i>F. oxysporum</i>	Benzeneacetic acid, 2-propenyl ester	Wang et al. (2012)
<i>Ln. citreum</i> and <i>W. confusa</i>	Rice cakes	<i>Cladosporium</i> sp., <i>Neurospora</i> sp., and <i>P. crustosum</i>	Lactic and acetic acid	Baek et al. (2012)
<i>W. cibaria</i> 861006, and <i>W. paramesenteroides</i> 860509	Wax gourd	<i>P. oxalicum</i> , <i>A. flavus</i> , <i>A. sydowii</i> , and <i>Mucor racemosus</i>	Organic acids	Lan et al. (2012)
<i>Lact. rhammosus</i> , and <i>Lact. fermentum</i>	Unknown	<i>Aspergillus</i> section Flavi strains	ND	Gerbaldo et al. (2012)
<i>Lact. brevis</i> , <i>L. lactis</i> ssp. cremoris, <i>L. lactis</i> ssp. lactis, and <i>En. faecium</i>	Pomegranate (flowers, leaves, buds, and fruits)	<i>F. graminearum</i>	ND	Gajbhiye et al. (2012)
<i>Lact. plantarum</i> 16, and 62	Steep water, Sauerkraut	<i>P. expansum</i> , <i>P. digitatum</i> , <i>P. notatum</i> , <i>P. roqueforti</i> , <i>R. stolonifer</i> , <i>F. culmorum</i> , <i>A. fumigatus</i> , and <i>Rhodotorula mucilaginosa</i>	ND	Crowley et al. (2013)

<i>Lact. plantarum</i> LB54, LB51, LB52, LB20, LB24, <i>Lact. farciminis</i> LB53	Silage, carrot and camel milk	<i>Aspergillus spp.</i>	ND	Laref et al. (2013)
Genus <i>Lactococcus</i> , <i>Pediococcus</i> , <i>Lactobacillus</i> , <i>Weissella</i> , and <i>Enterococcus</i>	Rhizosphere samples of olive trees and desert truffles	<i>P. expansum</i> , and <i>A. niger</i>	ND	Fhoula et al. (2013)
<i>Lact. sakei</i> KTU05-6, <i>Ped. acidilactici</i> KTU05-7, <i>Ped. pentosaceus</i> KTU05-8, KTU05-9, and KTU05-10	Lithuanian rye sourdoughs	<i>F. culmorum</i> , <i>P. chrysogenum</i> , <i>A. fumigatus</i> , <i>A. versicolor</i> , <i>P. expansum</i> , <i>A. niger</i> , <i>Debaryomyces hansenii</i> , and <i>C. parapsilosis</i>	Organic acids and bacteriocin-like substance	Cizeikiene et al. (2013)
<i>Lact. plantarum</i> YML007	Korean kimchi	<i>A. niger</i> , <i>A. oryzae</i> , <i>A. flavus</i> , and <i>F. oxysporum</i>	Protein	Rather et al. (2013)
Genus <i>Lactobacillus</i> , <i>Lact. plantarum</i> , and <i>farciminis</i> strains	Carrot, silage and camel milk	<i>Aspergillus spp.</i> , <i>Fusarium roseum</i> , <i>Trichoderma spp.</i> , <i>Penicillium spp.</i> , and <i>Stemphylium spp</i>	ND	Laref and Guessas (2013)
<i>Lact. plantarum</i>	Fresh herbs, fruits and vegetables	<i>P. solitum</i> , <i>A. versicolor</i> and <i>Cladosporium herbarum</i>	ND	Cheong et al. (2014)
<i>Lact. casei</i> AST18	Unknown	<i>P. chrysogenum</i>	ND	Li et al. (2014)
<i>Lact. plantarum</i> LR/14	Unknown	<i>A. niger</i> , <i>R. stolonifer</i> , <i>Mucor racemosus</i> , and <i>P. chrysogenum</i>	Peptides	Gupta and Srivastava (2014)
<i>Lact. plantarum</i> B4496, <i>Lact. brevis</i> 207, and <i>Lact. sanfranciscensis</i> BB12	Fermenting cocoa	<i>A. carbonarius</i> , <i>A. niger</i> , and <i>A. ochraceus</i>	Possibly proteinaceous	Ngang et al. (2014)
<i>Lact. plantarum</i> HD1	Kimchi	Food- and feed-borne filamentous fungi, and yeasts	5-oxododecanoic acid, 3-hydroxy decanoic acid, and 3-hydroxy-5-dodecenoic acid	Ryu et al. (2014)
<i>Enterococcus durans</i> F2.1	Tarhana	<i>P. griseofulvum</i> , <i>P. chrysogenum</i> , <i>A. fumigatus</i> , and <i>A. parasiticus</i>	ND	Kivanc et al. (2014)
<i>Lact. brevis</i> KR3, KR4, KR51, and KR53	Dair product ( <i>katak</i> )	<i>P. claviforme</i> , <i>A. awamori</i> , and <i>A. niger</i>	ND	Tropcheva et al. (2014)
Genus <i>Lactobacillus</i>	Plant materials or some Romanian traditional foods	<i>A. flavus</i> , <i>A. ochraceus</i> , and <i>A. niger</i>	ND	Sicuia et al. (2014)
<i>L. plantarum</i> strains e Ts1, Ts2, Ts3, Ts4, and Ts5, and <i>L. helveticus</i> Ts6	National Bank for Industrial Microorganisms and Cell Cultures	<i>A. niger</i> and <i>P. claviforme</i>	ND	Sevgi and Tsyetelava (2015)

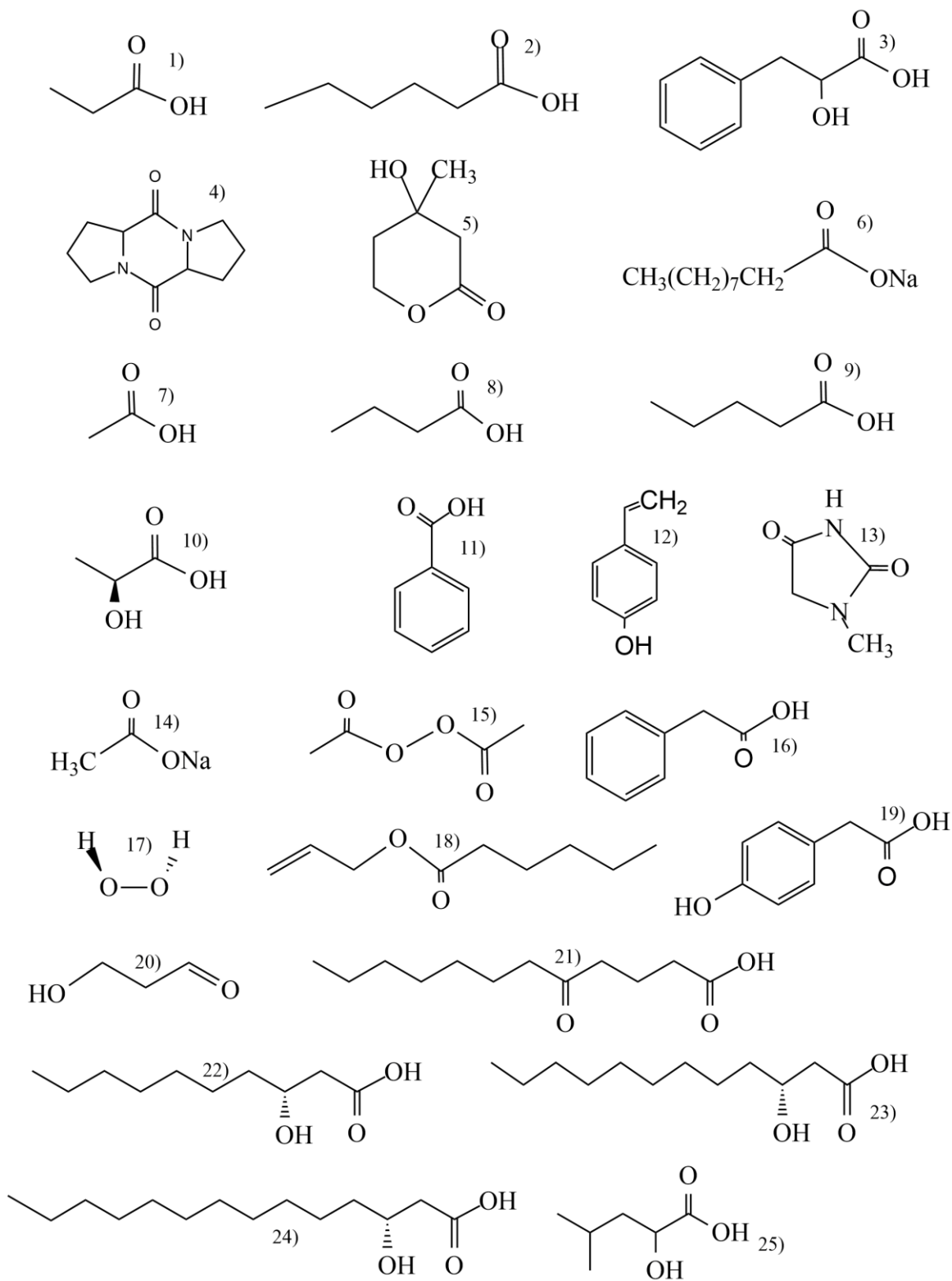
<i>Lactobacillus</i> VJC6, VJC11, VJC13, VJC23, VJI16, and VJI17	Crop, intestine, gizzard and ceca of seven weeks old broiler chicken	<i>A. niger</i> , <i>A. flavus</i> , <i>P. expansum</i> , and <i>P. roqueforti</i>	ND	Nallala and Jeevaratnam (2015)
<i>Lact. plantarum</i> KCC-25, KCC-26, KCC-27, and KCC-28	Animal manure	<i>A. fumigatus</i> , <i>P. chrysogenum</i> , <i>P. roqueforti</i> , <i>Botrytis elliptica</i> , and <i>F. oxysporum</i>	Extracellular proteolytic and glycolytic enzymes	Ilavenil et al. (2015)
<i>E. faecium</i> SFD	Stock cultures	<i>F. proliferatum</i> , <i>F. culmorum</i> , <i>F. moniliforme</i> , and <i>F. oxysporum</i>	ND	Deraz et al. (2015)
<i>Lact. acidophilus</i> , <i>Lact. plantarum</i> , <i>Lact. delbrueckii</i> , and <i>Lact. bulgaricus</i>	Yoghurt	<i>A. niger</i> , and <i>Penicillium spp.</i>	ND	Yasmin et al. (2015)
<i>Ln. mesenteroides</i> DU15	Unknown	<i>A. niger</i>	Peptides	Muhalidin et al. (2015a)
<i>Lact. plantarum</i> IS10	Unknown	<i>A. flavus</i> , <i>P. roqueforti</i> , and <i>Eu. rubrum</i>	Peptides	Muhalidin et al. (2015b)
<i>Lact. plantarum</i> FST1.7 and <i>Lact. brevis</i> R2	Unknown	<i>F. culmorum</i>	Organic and phenolic acids	Peyer et al. (2016)
<i>Lact. reuteri</i> R29 and <i>Lact. brevis</i> R2	UCC culture collection	<i>Broad spectrum</i>	Carboxylic acids	Axel et al. (2016)
<i>Lact. delbrueckii</i> ssp. <i>lactis</i> , <i>Lact. brevis</i>	Unknown	<i>A. flavus</i> , and <i>A. parasiticus</i>	ND	Prathivadi Bayankaram and Sellamuthu (2016)
<i>Lact. plantarum</i> UFG 108 and <i>Lact. plantarum</i> UFG 121	Unknown	<i>A. niger</i> , <i>A. flavus</i> , <i>F. culmorum</i> , <i>P. roqueforti</i> , <i>P. expansum</i> , <i>P. chrysogenum</i> , and <i>Cladosporium sp.</i>	Lactic acid and PLA	Russo et al. (2016)
<i>Lact. paracasei</i> LOCK 0921	Human and plant	<i>Alternaria brassicicola</i> , <i>A. niger</i> , and <i>F. latericum</i>	ND	Lipińska et al. (2016)
<i>Lact. brevis</i> , <i>Lact. buchneri</i> , <i>Lact. cellobiosus</i> , and <i>Lact. fermentum</i>	Corn and Fermented corn paste	<i>Aspergillus spp.</i> , <i>Fusarium spp.</i> , and <i>Penicillium spp.</i>	ND	Tatsadjieu et al. (2016)
<i>Lact. plantarum</i> MYS6	Fermented pomegranate wine	<i>F. proliferatum</i>	10-Octadecenoic acid, methyl ester; palmitic acid, methyl ester; heptadecanoic acid, 16-methyl ester; stearic acid and lauric acid	Deepti et al. (2016)
<i>Lact. plantarum</i> FT723	Milk and cheese	<i>P. expansum</i>	ND	Tulini et al. (2016)

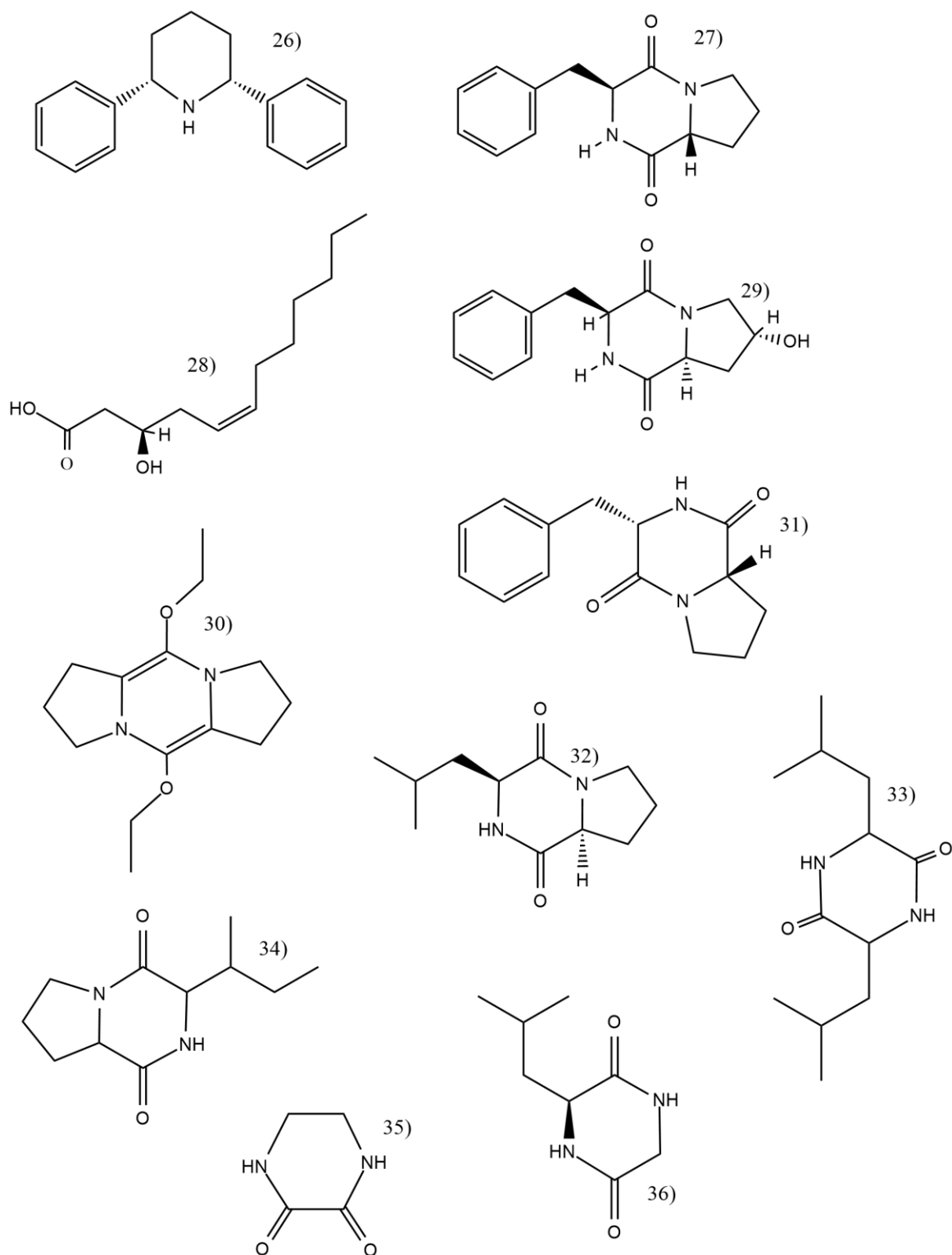
Chapter 5: Review Article (Antifungal Lactic Acid Bacteria)

<i>Ped. pentosaceus</i>	Sourdough	<i>A. flavus</i> , and <i>A. niger</i>	ND	Sadeghi et al. (2016)
<i>B. bifidum</i> PTCC 1644, <i>Lact. fermentum</i> PTCC 1744	Stock culture (IROST)	<i>A. parasiticus</i>	ND	Ghazvini et al. (2016)
<i>Lact. plantarum</i> , <i>Lact. casei</i> , <i>Lact. reuteri</i>	Raw milk	<i>P. expansum</i>	NB	Delavenne et al. (2016)
<i>Lact. plantarum</i> , and <i>Ped. acidilactici</i>	Milk	<i>A. flavus</i>	ND	Samaneh et al. (2016)
<i>Ln citreum</i> , <i>Lact. sakei</i> , <i>Lact. plantarum</i> , <i>Lac. spicheri</i> , <i>L. reuteri</i> , and <i>Lact. brevis</i>	Unknown	<i>Aspergillus spp.</i> , <i>Eurotium spp.</i> , <i>Penicillium spp.</i> , <i>Cladosporium spp.</i> , and <i>Wallemia spp.</i>	ND	Le lay et al. (2016)
<i>Ln citreum</i> L123, <i>Lact. brevis</i> Lu35, <i>Lact. reuteri</i> 5529, and <i>Lact. spicheri</i> O15, and <i>Pr. freudenreichii</i> LSaci68	Culture collection	<i>A. niger</i> , <i>P. corylophilum</i> , and <i>Eurotium repens</i>	Lactic, acetic and propionic acids, ethanol, and hydrogen peroxidephenyllactic, hydroxyphenyllactic, azelaic, and caproic acids.	Le lay et al. (2016)

Note : *B*: Bifidobacterium, *E*: Enterococcus, *L*: Lactococcus, *Lact*: Lactobacillus, *Ln*: Leuconostoc, *Ped*: Pediococcus, *Pr*: Propionibacterium, *W*: Weissella, *UCC*: University College Cork, *IROST*: Iranian Research Organization for Science and Technology, *ND*: not determined.







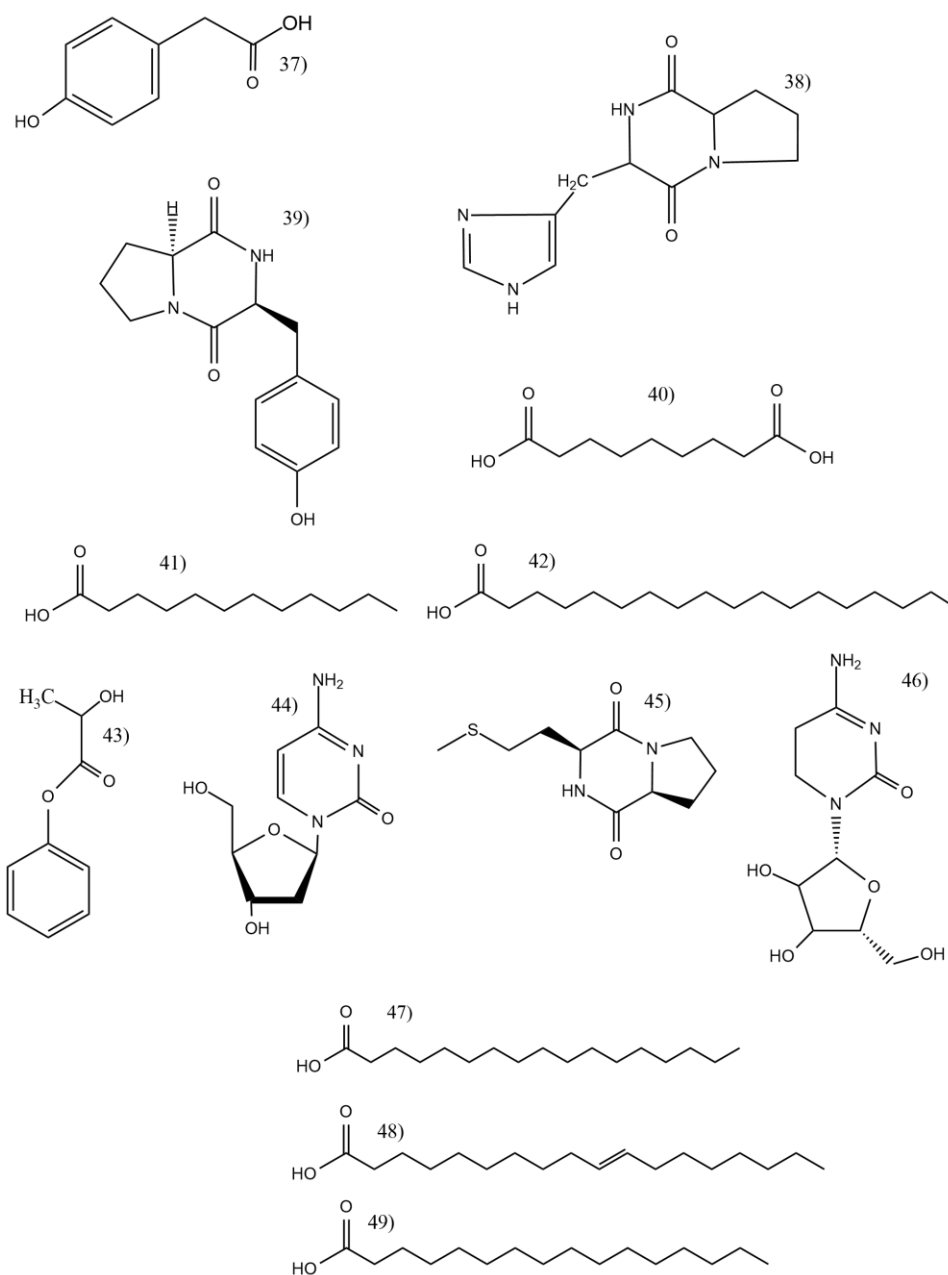


Figure 3. Broad spectrum of antifungal metabolites produced by LAB.

1) propionic acid, 2) caproic acid, 3) phenyllactic acid, 4) cyclo (Pro-Pro), 5) mevalonolactone, 6) sodiumdecanoate, 7) acetic acid, 8) butyric acid, 9) valeric acid, 10) lactic acid, 11) benzoic acid, 12) 4-vinyl phenol, 13) 1-methylhydantoin, 14) sodium acetate, 15) diacetyl peroxide, 16) benzeneacetic acid, 17) hydrogen peroxide, 18) 2-propenyl ester, 19) phenylacetic acid, 20) reuterin, 21) 5-oxododecanoic acid, 22) 3-hydroxydecanoic acid, 23) 3-hydroxydodecanoic acid, 24) 3-hydroxytetradecanoic acid, 25) 2-hydroxy-4-methylpentanoic acid, 26) 2,6 diphenyl piperidine, 27) cyclo (Phe-Pro), 28) 3-hydroxy-5-dodecanoic acid, 29) cyclo (Phe-4-OH-Pro), 30) 5,10-diethoxy-2,3,7,8-tetrahydro-1H, 31) cyclo (Phe-Pro), 32) cyclo (Leu-Pro), 33) cyclo (Leu-Leu), 34) cyclo (Ile-Pro), 35) diketopiperazines, 36) cyclo (Gly-Leu), 37) 4-hydroxyphenyllactic acid, 38) cyclo(His-Pro), 39) cyclo(Tyr-Pro), 40) azelaic acid, 41) lauric acid , 42) stearic acid , 43) phenyl-lactate, 44) deoxycytidine, 45) cyclo(Met-Pro), 46) cytidine, 47) heptadecanoic acid, 48) 10-octadecenoic acid, 49) palmitic acid.

## Genetic Engineering to Improve LAB Antifungal Properties

The biosynthesis of some LAB compounds have been improved by means of genetic engineering. Genome shuffling, recombinant DNA technology and whole cells transformation using LAB vectors are some of the methods used to increase the yield and the purity of LAB-compounds. In Table 2 some of the methods are partially described for the most important LAB: *Lact. helveticus*, *Lact. lactis*, *Lact. pentosus*, *Lact. plantarum*, *Lact. rhamnosus* and *L. lactis*.

Research on LAB genetic engineering focus mainly on the production of LA for use as either a food additive (bio-preservatives) or as a bio based-chemical compound useful in chemistry, pharmacy and agriculture (Gaffoor et al., 2005; Dusselier et al., 2013; Hayek and Ibrahim 2013; Ghaffar et al., 2014; Dedenaro et al., 2016). It is important to highlight the great contribution that the latter is generating in the area of LAB genetic engineering, some methods generated for this purpose could be useful for the improvement of LAB antifungal strains for use in the food industry. Examples of this bio-based chemical contribution are the heterologous expression of xylose assimilation-related genes in *Lact. plantarum* to produce cost effective D-LA (Zhang et al., 2016) and the integration of LA biosynthesis-related genes in yeast (Ilmén et al., 2013) or in cyanobacteria (Angermayr et al., 2012) to improve LA production.

Although most of the research focus is on LA, some attempts have been made to improve PLA production. Using genome shuffling, Wang et al. (2013), produced a modified *Lact. plantarum* strain which can produce both, LA and PLA, showing an increase in antifungal activity compared to the wild-type. Zheng et al. (2015) increased PLA purity and yields by heterologous expression of *ldhL* and formate dehydrogenase genes from *Bacillus coagulans* in *E. coli* and, similarly, Zhu et al. (2015) increased PLA yields in *E. coli* using the gene *ldhD* from *Lact. pentosus*.

Genetic engineering methods generated for LA and PLA production could also be used to improve other LAB antifungal compounds listed in the present review. However, knowledge of the biosynthetic pathway is necessary for most purposes and, up to now, it is only available for a few antifungal compounds. Genome sequence information present will help to increase the use of genetic engineering tools to expand these applications in this area. Many LAB genomes have been already sequenced (Klaenhammer and Dairy, 2002; Makarova et al., 2006; Wegmann et al., 2007), and the genes involved in the biosynthetic pathway of reutericyclin were described recently by Lin et al. (2015), while the gene cluster of nisin was described in 1993 (Kuipers et al., 1993).

Genome shuffling is an interesting method to improve LAB antifungal characteristics and does not require genome information. This method is based on the induction of genetic diversity (by mutagenesis for example), followed by recombination of the selected strains. This is repeated several times until there is a useful set of mutations in the population (Biot-Pelletier and Martin, 2014). For example, after chemical or physical mutagenesis, bacteria showing the highest antifungal activity can be selected and can be used for further recombination by protoplast fusion using another starter bacteria which exhibits a better growth rate, low pH tolerance, etc. In this way, the creation of a bacterial population showing an improved antifungal activity can be obtained. Using this method, Patnaik et al. (2002) improved the LA production in *Lactobacillus*

sp. and Wang et al. (2013) increased the antifungal activity by up to 200% after three rounds of genome shuffling in *Lact. Plantarum*, mainly against *Penicillium digitatum* and *P. citrinum*.

Once the biosynthetic pathway is known then other methods could be used. The yield and the purity of LA were improved in *E. coli* by blocking the synthesis of ethanol, acetate, formate, and succinate thus redirecting the pathway to the desired LA route (Mazumdar et al. 2010; Chen et al. 2014). By homologous recombination, Jia et al. (2011) disrupted *ldhL* gene and integrated *ldhD* gene into *Corynebacterium glutamicum* genome to increase the purity of D(-)-LA. While Angermayr et al. (2012) integrated *ldhL* gene from *Bacillus subtilis* into a cyanobacteria genome (*Synechocystis* sp.) to improve the yield of L(+)-LA. Another promising method is LAB whole cells transformation using constructed shuttle vectors that carry the desired genes. Those expression vectors have all machinery to allow gene expression, they are easily transformed, have to be cryptic (no effect in host phenotype) and stable in LAB (Landete, 2016). Some promising vectors have been constructed for LAB, such as pUL6erm, pLC494, pGID052, pRS4, pAMb1, pEL5.6, pEL5.7, pCU and pWV01 (Pérez-Arellano et al., 2001; Beltramo et al., 2004; Alegre et al., 2005; An and Miyamoto, 2006; Okano et al., 2007; Chang and Yan, 2014; Chen et al., 2014). Using a pCU vector carrying the *xylRAB* genes for xylose assimilation, Shinkawa et al., (2011) improved the purity and yield of L(+)-LA from xylose.

Table 2. Description of some LAB genetic engineering methods.

LAB	Compound	Improvement*	Method	Reference
<i>Lact. helveticus</i>	L(+)-LA	Increase in L-LDH activity (53 to 93%) and LA yield (76.2-91.6%)	Removal of promoter in <i>ldhD</i> gene to increase L(+)-LA production. Replacement of <i>ldhD</i> with an additional copy of <i>ldhL</i> .	Kylä-Nikkilä et al. (2000)
<i>Lactobacillus</i> sp.	LA	Acid tolerance (3-fold LA increase at pH 4)	Genome shuffling: Selection of a population with pH tolerance and protoplast fusion with a poorly characterize <i>Lactobacillus</i> sp.	Patnaik et al. (2002)
<i>Lact. rhamnosus</i>	LA	Acid tolerance (2.6 and 3.1 fold increase at pH 3.8)	Genome shuffling: UV or nitrosoguanidine as mutagens, selection and protoplast fusion	Wang et al. (2007)
<i>Lact. rhamnosus</i>	L(+)-LA	Increase yield (70%)	Genome shuffling: UV or nitrosoguanidine as mutagens, selection and protoplast fusion	Yu et al. (2008)
<i>L. lactis</i>	D(-)-LA	Increase yield (73%)	UV mutagenesis and selection	Joshi et al. (2010)
<i>Sporolactobacillus inulinus</i>	D(-)-LA	Acid tolerance (119% D-LA increase at pH 5)	Genome shuffling: UV and diethyl sulphate as mutagens, pH gradient filter, selection and protoplast fusion	Zheng et al. (2010)
<i>L. lactis</i>	LA	Acid tolerance (5-10-fold survival increase at pH 3)	Increase in trehalose production using a plasmid carrying <i>trePP</i> and <i>pgmB</i> genes from <i>L. lactis</i> (trehalose operon) and the <i>otsB</i> gene from <i>P. freudenreichii</i>	Carvalho et al. (2011)
<i>L. lactis</i>	LA	Increase in glucose uptake and LA production	Expression of gene encoding phosphofructokinase ( <i>pfkA</i> ) from <i>Aspergillus niger</i> using recombinant DNA technology	Papagianni and Avramidis (2011)

<i>L. lactis</i>	L(+)-LA	Increase in optical purity (99.6%) and yield (1.58 moles/mol xylose)	Insertion of a vector for xylose assimilation and modification of the phosphoketolase pathway to produce only LA	Shinkawa et al. (2011)
<i>Lact. plantarum</i>	LA	Acid tolerance (64% increase at pH 4)	Genome shuffling: two starting populations, one generated using gradual low pH adaptation and the other one using UV as mutagen; followed by selection and protoplast fusion	Triratna et al., (2011)
<i>L. lactis</i>	LA	Acid tolerance (10-fold survival increase at pH 2.5)	Histidine decarboxylation pathway of <i>Streptococcus thermophilus</i> was expressed in <i>L. lactis</i> using a plasmid which contains the <i>hdcAPB</i> operon under the control of the nisin-inducible promoter ( <i>NisA</i> )	Trip et al. (2012)
<i>Lact. plantarum</i>	LA and PLA	Increase antifungal activity to 192-200%	Genome shuffling: UV and diethyl sulphate as mutagens, selection and protoplast fusion	Wang et al. (2013)
<i>Lact. pentosus</i>	LA	Acid tolerance (95% LA increase at pH 3.8)	Error-prone whole genome amplification: electroporation of PCR products (10-2000bp) to generate random mutations in genome	Ye et al. (2013)
<i>L. lactis</i>	nisin	Increase yield (6-fold)	Insertion of a plasmid carrying complete genes related with nisin biosynthesis	Kong and Lu, (2014)
<i>Lact. plantarum</i>	D(-)-LA	Increase in optical purity (99%). Production of D-LA from xylose	Insertion of a plasmid carrying genes for xylose assimilation into a deficient L-LA <i>Lact. plantarum</i>	Zhang et al.(2016)
<i>L. lactis</i>	LA and nisin	Increase in acid tolerance and nisin titer (74.84%)	Transformation with a plasmid carrying combinations of acid-tolerant encoding genes from <i>Lact. casei</i> , <i>L. lactis</i> or <i>E. coli</i> and LA encoding genes from <i>Lact. casei</i> and <i>L. lactis</i> .	Zhang et al. (2016)

Notes: *ldhD*: gene encoding D-lactate dehydrogenase. *ldhL*: gene encoding L-lactate dehydrogenase.

\*increase is always compared to the wild type.

In addition to the methods already mentioned and described briefly in Table 2, specific controlled gene expression systems can be used to improve the antifungal production. A good example could be the nisin-induced controlled gene expression system (NICE) derived from the *L. lactis nis* operon. In this system the induction of a gene of interest is achieved by inserting the genes involved in nisin signal transduction (*nisRK*) into a bacterial plasmid or the chromosome, and the inducible gene *PnisA* is carried on a plasmid upstream of the gene of interest, the addition of a certain amount of nisin will activate or inactivate the transcription of the gene (Kuipers et al. 1998). This system has two big advantages, one is that the concentration of the compound of interest can be regulated based on the amount of nisin added and NICE can also be used to control the whole operon which makes it useful for complex metabolic pathways (Boels et al., 2004; Mierau and Kleerebezem, 2005). Using the NICE system Trip et al. (2012) increased the acid tolerance of *L. lactis* by expressing the *hdcAPB* operon for the histidine decarboxylation pathway.

Other systems based on specific controlled gene expression are already available (Axelsson et al., 2003; Hickey et al., 2003; Mathiesen et al., 2004; Miyoshi et al., 2004; Linares et al., 2015).

One of the main challenges to increase the production of compounds by LAB is the pH range in which the bacteria, in spite of its metabolic activity (which causes pH decrease), can continue growing. Some research has been already done to increase LAB acid tolerance. Using genome shuffling, the ability of several *Lactobacillus* sp. to grow at low pH was enhanced, thus the production of LA was improved (Patnaik et al., 2002; Wang et al., 2007; Zheng et al., 2010; Triratna et al., 2011). The acid tolerance of *Lact. pentosus* was also enhanced by error-prone whole PCR amplification (Ye et al., 2013), while *L. lactis* was modified for the same purpose by heterologous expression of genes related with histidine decarboxylation (Trip et al., 2012), trehalose production (Carvalho et al., 2011) and acid-tolerance (Zhang et al., 2016).

Based on the fact that LAB antifungal activity is usually attributed to more than one compound, techniques such as genome shuffling, in which several traits can be improved in the same strain, is a promising method to ensure good results. However, the stability of the improved traits after several generations must be taken into account. Methods including DNA modifications such as gene replacement, insertions and disruptions to redirect pathways are promising. However, the use of genetically modified LAB as a food additive is always of debate. On the other hand, food-grade shuttle expression vectors could be an interesting way to express desired antifungal-related genes in LAB for use in the food industry and does not involve genome modifications. Moreover, improved LAB antifungal producers could be obtained using a combination of techniques, for example, an acid-tolerant LAB generated using genome shuffling, can be used further as a host strain for expression vectors including genes related to fungistatic agents produced by other bacteria, such as benzoic or propionic acids.

Regardless of the method, genetic engineering to improve the production of antifungal compounds by LAB could help to reduce the risk of fungal-mycotoxins food contamination and therefore increase food quality. LAB improvements could focus on the large-scale production of promising antifungal compounds in biofabrics for the direct addition into food or the modified bacteria showing enhance antifungal activity could be used directly as a food additive.

## **Application of Antifungal LAB and Prospect in Food Industry**

Various types of LAB have emerged and been used as natural bio-preservatives on food products because their secondary metabolite substances are innocuous and possess inhibitory effects on harmful bacteria and fungi (Theron and Lues, 2011). *In situ* testing is essential to substantiate the potential application of these organisms, generally regarded as safe (GRAS) as bio-preservatives against toxigenic fungal and spoilage, as well their sensory and safety aspects.

LAB usage is still limited to the fermentation products, using bacteria strains that have been deemed as safe, for example, LAB has been applied as starter cultures for many years in the brewing industry. The addition of *Lact. plantarum* VTTE-78076, and *Ped. pentosaceus* VTTE-90390 in the soaking water clearly proved that it could inhibit the growth of the fungus *Fusarium* during brewing, resulting in a potentially limited accumulation of trichothecene (Laitila et al.,

2002). LAB principle application can be seen in the fermentation of wine which controls *Penicillium* and *Aspergillus* that can cause ochratoxin contamination. Nevertheless, the introduction of large-scale food biopreservation systems in the food industry requires careful safety assessment including detailed risk analysis. Besides the possible influence of LAB culture and its metabolites towards sensory quality of food products must also be taken into account. Indeed, various studies have demonstrated the successful application of LAB alleviates fungal contamination in different foods conferring attributes similar to chemical preservatives substitutes or complements (Table 3).

Gautam and Sharma (2009) define the term bio-preservative:

- a. The bio-preservative should not be toxic.
- b. It should be accepted by recognized authorities.
- c. It should be economical to the industries using it.
- d. The product in which the bio-preservative is being used should not be affected by it and should not show any deleterious effect toward the organoleptic properties of that product.
- e. The effect of the bio-preservative should be observed at relatively low concentrations.
- f. The bio-preservative should be sufficiently stable when stored.
- g. It should not have any medicinal use.

Table 3. Application of antifungal LAB in food system.

LAB	Method	Food product	Result	Literature
<i>Lact. plantarum</i> and <i>Ped. pentosaceus</i>	Culture adding	Beverage	Inhibiting the growth of <i>Fusarium</i> molds to prevent gushing	Haikira et al. (1993)
<i>Lact. plantarum</i> 21B	Culture adding, co- fermentation with <i>S.</i> <i>cerevisiae</i>	Bakery	Retarding the growth of <i>A. niger</i> FTDC3227 over a seven day storage period	Lavermicocca et al. (2000)
<i>Lactobacillus</i> genus	Culture adding	Beverage	Protect quality of malt and wort when <i>Fusarium</i> spores present	Lowe et al. (2004)
<i>Lact. paracasei</i> subsp. <i>paracasei</i> and <i>Ped.</i> <i>Jensenii</i>	Culture adding	Dairy product	Retarding growth of various <i>Candida</i> species in an <i>in situ</i> yoghurt model as well as on cheese surface	Schwenninger and Meile (2004)
<i>Lact. plantarum</i> CUK501	Culture adding	Fruit and vegetables	Inhibit growth of four different fungi on cucumbers for up to eight days compared to an untreated control	Sathe et al. (2007)
<i>Ped. acidilactici</i> KTU05-7, and <i>Ped.</i> <i>pentosaceus</i> KTU05-8 and KTU05-10	Spraying on surface of bread	Bakery	Effective against a number of food related fungi such as <i>F. culmorum</i> AI-2 and <i>C. parapsilosis</i> C.7.2.	Zhang et al. (2010)
<i>Lact. buchneri</i> FUA 3525 and <i>Lact.</i> <i>diolovorans</i> DSM 14421	Culture adding	Bakery	Inhibiting growth of a number of bread- spoilage fungi, including <i>A. clavatus</i> and <i>Cladosporium</i> spp., through the accumulation of acetate and propionate	Zhang et al. (2010)
<i>Lact. acidilophilus</i> NCDC 291	Culture adding	Raw poultry meat	A reduction in viable numbers of <i>A.</i> <i>alternata</i> when inoculated into raw poultry meat	Garcha and Natt, (2011)



<i>Lact. fermentum</i> Te007, <i>Pediococcus pentosaceus</i> Te010, <i>Lact. pentosus</i> G004, and <i>Lact. paracasei</i> D5	Culture adding	Tomato puree	Demonstrating the antagonistic effects against <i>A. niger</i> and <i>A. oryzae</i>	Muhaladin et al. (2011)
<i>Lact. fermentum</i> Te007, <i>Pediococcus pentosaceus</i> Te010, <i>Lact. pentosus</i> G004, and <i>Lact. paracasei</i> D5	CFS adding	Dairy product	Extent shelf-life of cheese slices and cheese	Muhaladin et al. (2011)
<i>Lact. amylovorus</i> DSM 19280	Culture adding	Bakery	Extent shelf-life of wheat bread. inhibition observed against <i>Aspergillus</i> , <i>Fusarium</i> and <i>Penicillium</i> molds	Ryan et al. (2011)
<i>Lact. plantarum</i> isolates	Culture adding	Dairy product	Demonstrating anti-mold capabilities when used as adjuncts during cheddar cheese production	Zhao (2011)
<i>Pediococcus</i> and <i>Weissella</i> isolates	Culture adding	Fruit and vegetables	<i>Penicillium</i> spoilage was delayed on apples, pears, plums and grapes	Crowley et al. (2012); Lan et al. (2012); Rouse et al. (2008)
<i>Lact. harbinensis</i> K.V9.3.1Np, and <i>Lact. rhamnosus</i> K.C8.3.1I	Culture adding	Dairy product	Exhibiting protective properties against a number of fungi including <i>D. hansenii</i> and <i>R. mucilaginosa</i> in yoghurts, while they did not alter the growth or acidification rates of the yoghurt starters, nor did they affect the pH, lactic or acetic acid levels	Delavenne et al. (2012).
<i>Lact. rossiae</i> LD108 and <i>Lact. paralimentarius</i> PB127	Culture adding	Bakery	Preventing growth of <i>A. japonicus</i> with shelf lives ranging from 11-32 days as compared to bread prepared with baker's yeast dough	Garofalo et al. (2012)
<i>Lact. plantarum</i> IMAU10014 (mutant strain)	CFS adding	Fruit and vegetables	Elimination growth of <i>Penicillium digitatum</i> KM08 on the surface of kumquats compared to the wild type.	Wang et al. (2013)
<i>Lact. plantarum</i> B.BS08	Culture adding	Tempeh	Preventing growth of <i>F. proliferatum</i> BJ09.05	Anggriawan et al. (unpublished)

Note: CFS (cell free supernatant)

## Future Research Trends

Efforts to improve quality and food safety by replacing the traditional food preservation system have been increasing during the last decade. One of the most popular methods of food biopreservation is fermentation, a process based on the growth of microorganisms in food, either naturally or with the addition of starters (Ross et al., 2002; Ananouet al., 2007). About 25% of dietary adjustments menu in Europe and 60% in many developing countries consists of fermented food (Stiles, 1996; Adebayo et al., 2013). LAB plays a major role in the fermentation process, it can be found in products such as cheese, yogurt, meat and vegetable fermentation, *kefir*, *whhey* (buttermilk) and bread (Hammeset al., 1990; Caplice and Fitzgerald, 1999; Carr et al., 2002) and they are important for food preservation. Due to the nature of certain specific metabolites, LAB is of interest to the food industry because it can improve the taste, texture and nutritional value of food products (Guldfeldtet al., 2001; Arendt et al., 2007).

Clearly, as stated in the present review, some LAB have antifungal properties and could potentially be used as safe bio-preservative agents for food processing and also effectively address yeast and mold fermentationsafety issues. However, until now there is no clear standardization of LAB isolation. A standardization process for LAB isolation and purification is indispensable to improve procedures and make them cost-effective. In-depth research on the nature and characteristics of LAB antifungal compounds is necessary before assessing the further potential of LAB antifungal properties.

Future research has to be oriented towards expanding our knowledge of the metabolic pathways of antifungal compounds and their genetic background. Genomic and transcriptomic information will contribute towards better descriptions, but also to determine the molecular targets of the antifungal metabolites, to discover new promising antifungal-related compounds and to improve, the antifungal properties of LAB through genetic engineering. Efforts have already been made to improve antifungal properties using genetic engineering; some methods are feasible and are already available which will facilitate a wider use of this technology in the near future. Undoubtedly, some methods including food-grade shuttle expression vectors carrying the genes of interest and controlled gene expression systems will be taken into account.

## Acknowledgement

This paper was supported financially by Indonesia Endowment Fund for Education, Ministry of Finance, The Republic of Indonesia (Ref:1064/LPDP/2013).

## Conflict of Interest

The authors declare no conflict of interest.

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## Research Article 03

# Diversity and Biological Activity of Yeast and Lactic Acid Bacteria Isolates from Soak Water in Tempeh Production\*

\*Diversity part in this research has been presented partly in 4th International Conference on Biological Science ICBS 2015 Yogyakarta, Indonesia.

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## Abstract

Soaking is a spontaneous microbiological process. It is considered as an important step in the manufacture of high-quality tempeh. The objectives of this study were, 1) to characterize and investigate the diversity of dominant lactic acid bacteria (LAB) and yeast by analyzing soak water samples collected from different producers in Java, 2) to screen and investigate their potential antifungal activity against *F. proliferatum*, 3) to check their contribution to zearalenone (ZEN) biotransformation. The LAB counts in these samples varied between 5.21 and 8.88 log CFU/mL whereas yeasts counts varied from 4.31 and 4.89 log CFU/mL, APC counts varied between 7.33 and 9.10 log CFU/mL. In total, 165 isolates were obtained from these samples using selective media. Our results revealed a certain variety of culturable LAB and yeast in soak water. The principal yeast found to be responsible for fermentation were *Candida tropicalis*, *Kluyveromyces marxianus*, and *Saccharomyces cerevisiae*, while the dominant LAB were *Lactobacillus plantarum*, *L. paracasei* and *Pediococcus pentosaceus*. We found LAB species were less diverse than yeast species. Cell free supernatants (CFS) of 20 LAB strains possessed antifungal properties against *F. proliferatum* BSM17, while none of the yeast isolates showed the activity. It was found that phenyl lactic acid (PLA) might has main role for the activity. The use of those selected LAB strains as part of starter culture has been proven to positively increase tempeh safety. The results obtained from screening 20 isolates belonging to 10 different species revealed that nearly all the tested organisms were able to reduce ZEN in range 10 to 96.9%. In general, yeast and LAB isolates could only transform ZEN into two major transformation product  $\alpha$ -ZEL and  $\beta$ -ZEL. In all cases no conjugation product detected.

**Keywords:** soak water, tempeh, natural fermentation, lactic acid bacteria, yeast, antifungal activity, ZEN transformation

## 1. Introduction

Indonesian food *tempe*, or known as tempeh in foreign language, kind of beans that is fermented by fungi (such as *Rhizopus* sp.), which become solid and compact form and can be sliced. Besides beans or grains as its raw ingredients, tempeh also can be produced by other ingredients such other food processing byproduct, for example, *tofu* dregs and another. Yellow soybean is the most favoured source to produce tempeh (Nout and Kiers, 2005).

Tempeh is a substantial food in Indonesia. As traditional food, tempeh has been consumed since long ago; even its consumption is increasing every year. It happens because tempeh has a delicious taste, contains high nutrition, cheap, and easy to be cooked (Pawiroharsono, 1994). Besides, tempeh is a functional healthy food, which contains B12 vitamin, and bioactive compounds (Keuth and Bisping, 1994; Astuti et al., 2000; Nout and Kiers, 2005; Nakajima et al., 2005; Shurtleff and Aoyagi, 2011). As a popular and healthy food, tempeh is not only consumed by Indonesian people, but also by foreign people, such as Dutch, Japanese, and American people (Naidoo, 1996; Shurtleff and Aoyagi, 2011).

Tempeh-producing technology is handed down through generations and changes with experience. The manufacture process is quite complex, which divided into two stages. First, it is fermented by microorganisms (microbial consortia) process that occurs while soybeans are soaking. Then, the second is solid state fermentation process occurs by fungi after inoculation. There are some different process in producing tempeh in every region, and also depends on the producers (Astuti et al., 2000; Shurtleff and Aoyagi, 2011). However, there are some other similarities in producing tempeh, such as the process of soybean production, soybean peeling and soybean acidification, washing, inoculum mixing, packaging, and incubation (Hermana, 1996).

Like many other traditional foods in Indonesia, tempeh is produced as a small scale home industry with poorly controlled fermentation which is carried out without sufficient hygienic precautions even though the starter culture containing fungi are added at the beginning of fermentation. Therefore, there are various types of microorganisms that can participate during the process of fermentation, and natural contamination is always possible (Winarno, 1985; Barus et al., 2008; Seumahu et al., 2013). Moreover, there is no standard on making tempeh and its starter. This is the cause of many variations in the manufacture of tempeh in some areas (Astuti et al., 2000) which lead to nonhomogeneous quality and safety of traditional tempeh.

In 2015 we investigated possible contamination of toxigenic *Fusarium* in traditional tempeh. About 214 traditional tempeh samples from the six different regions were screened for *F. proliferatum* and *F. solani* contamination by real-time PCR. Contamination of traditional tempeh samples with *F. proliferatum* was found in 12% of the samples with amounts of 10-175 ng/mg fungal DNA. In these samples, the amount of FB1 and BEA ranged between 170-2680 µg/kg and 20-50 µg/kg, respectively. About 7 % of the traditional tempeh samples were contaminated with *F. solani* in amounts of 20-129 ng/mg fungal DNA. This is allegedly caused by unhygienic process of making tempeh and the use of contaminated traditional starter.

The use of biological agents, antifungal microbes, has been researched and expected to overcome fungal contaminant in food production. Generally, microorganisms commonly used for prevention of mycotoxin contamination are lactic acid bacteria (LAB) because of their ability to inhibit growth of toxigenic fungi (Laitila et al., 2002; Hathout et al., 2011). *Lactobacillus plantarum* AF1 can produce a compound C<sub>12</sub>H<sub>22</sub>N<sub>2</sub>O<sub>2</sub>, 3,6-bis (2-methylpropyl)-2,5, piperazinedon with 226 Da of molecular weight. The compounds effectively inhibit *Aspergillus flavus* ATCC 22546 and *A. fumigatus* ATCC 96 918 (Yang and Chang, 2010). Störm et al. (2002) have isolated *L. plantarum* from the antifungal silage. The antifungal substance of *L. plantarum*

MiLAB 393 was isolated, and its structure was determined. The compound was identified as a 3-phenyllactic acid with 9/1 ratio of L and D isomers, two cyclic dipeptides cyclo (L-Phe-L-Pro) and cyclo (L-Phe-trans-4-OH-L-Pro). Lavermicocca et al. (2000) also have isolated antifungal compounds from *L. plantarum*, such as phenyllactic acid and 4-hydroxy acid phenyllactic. Besides, yeast were also found to inhibit the growth of toxigenic fungi. Ramos et al. (2010) reported that some isolates of *Debaryomyces hansenii* (UFLACF 889 and UFLACF 847) and *Pichia anomala* (UFLACF 710 and UFLACF 951) were able to inhibit the growth of *A. ochraceous*, *A. parasiticus*, and *Penicillium roqueforti*. Other researchers have also found the role of *Saccharomyces cerevisiae* isolates as an inhibitor of toxigenic fungi (Munoz et al., 2010; Persons et al., 2013, and El-Mohamady et al., 2014).

Careful selection of specific strains of LAB and yeast with antifungal properties can be used as food preservatives reducing mold contamination, therefore, improving the shelf-life of many fermented food products (Corsetti et al., 2000; Clarke et al., 2002; Crowley et al., 2002; Arendt et al., 2007) and reducing health risks due to mycotoxins exposure (Gourama and Bullerman, 1995). Many research reports also indicate that LAB has beneficial health effects in humans (Masood et al., 2011; Florou-Paneri, 2013; Fernández et al., 2015; Nuraida et al., 2015). Moreover, due to certain specific metabolic properties, LAB are of particular interest in the food industry, since they can improve the sensory and nutritional value of food products (Crowley et al., 2002; Arendt et al., 2007).

*Rhizopus oligosporus* is a major tempeh fermentation agent, but other involved microbial fermentation also grow especially since in the soaking process (Nout and Rombouts, 1990; Nout and Kiers, 2005). The presence of LAB (Nout et al., 1985) and yeast (Samson et al., 1987) in tempeh have been reported. Afterward, the LAB and yeast were also studied and reported by other researchers. Commonly, research of LAB and yeast at some stages of tempeh production process is conducted in laboratory, not in the tempeh industry. The presence of these microbes in a sample of tempeh has been reported, but only based on the final product (tempeh from a market). It was not reported comprehensively during production process, especially soaking process. Research of LAB and yeast are based on conventional approach which takes time, expense much effort, and limited on microbial analysis (Aslam et al., 2010). Up until now, LAB study of tempeh production is limited to the value of colony forming units (CFU). LAB population for tempeh production has been researched for tempeh producer in Malaysia (Moreno et al., 2002) and only laboratory research in Indonesia (Nuraida et al., 2008). Neither Moreno et al. (2002) and Nuraida et al. (2008) did not specifically study about yeast population, which was included in the value of CFU fungi. The study about the diversity of LAB and yeast during the soaking of mixture soybean-corn has never been researched, except for a list of some types of LAB and yeast which ever found in tempeh and only characterized phenotypically (Samson et al., 1987; Mulyowidarso et al., 1990; Ashenafi and Busse, 1991).

In the present study, we isolated and characterized lactic acid bacteria (LAB) and yeast by analyzing soak water samples collected from different producers and regions in Java. Screening and evaluating anti-*F. proliferatum* activity from the isolates was also conducted. Nature of the

antifungal activity was also investigated. In addition, their potential protective application together with *R. oligosporus* as the main starter was studied in tempeh model.

Another safety problem in tempeh production is the use of corn as a substitute for soybean. Producers involve corn substitution for 10-30 percent to reduce cost production because the price of soybean is high nowadays. This generates a healthiness issue of produced tempeh because most Indonesian corn has been contaminated by toxigenic fungi such as *Fusarium spp.*, *Penicillium citrinum* and *P. chrysogenum* (Ahmad et al., 2009; Kusumaningrum et al., 2010; Rahmawati et al., 2013). Rahmawati et al. (2013) also reported that toxigenic fungi are found in soaking corn, such as *Fusarium spp.* It is high possibly happens that those fungi produce mycotoxins during soaking process, then the toxins absorbed into the material that is used to produce tempeh. Moreover mycotoxins content in raw material itself also endangers consumer health. Fumonisin and zearalenone were found in some cereals in Indonesia especially maize (Maryam and Zahari, 1994; Yamashita et al. 1995; Ramahani 2003). Therefore safety aspect of tempeh cannot be guaranteed anymore. In this research we also study tempeh microorganisms's contribution to biotransformation of zearalenone.

## **2. Materials and Methods**

### **Part I (Diversity of LAB and yeast)**

#### **2.1.1. Tempeh manufacturing in local producers**

Dry abrasion-dehulled yellow var. soybeans: local maize, the ratio depends on tempeh manufacture (10 kg) were rinsed twice with 50 L tap water of 50°C, drained and soaked either for spontaneous fermentation in 30 L tapwater in a big container at room temperature for 18-32 h (some manufactures use recycled soak water). After draining the soak water, the beans were rinsed twice with 50 L tap water and boiled in 30 L fresh tap water for 60-90 min. After draining and discarding the boiling water, the hot mixture beans were immediately spread in a layer of approx 1 cm thickness on a wire-mesh and turned twice to facilitate the evaporation of adhering water. After approx. 20 min, the beans were hand-dried and cooled to 20-25°C and were transferred into a container and mixed with spore suspension. After inoculation, 50-200 g beans were stiffly packed into a perforated plastic or dried banana leaf and incubated at room temperature for 36-48 h, yielding fresh tempeh.

#### **2.1.2. Sampling**

Samples (soak water) were collected from 6 tempeh manufactures from different regions in Java and approved by local government (sampling map can be seen in Figure 1). Samples of about 100 mL soak water were taken aseptically within 15 min at ambient temperatures into sterile glass bottles after thorough mixing in the bulk samples container. The samples were transported to nearby microbiology laboratory in a cooling box (<10°C) containing ice packs. Microbiological and chemical analysis was carried out immediately after arrival. Each ten mL of sample were used to microbial count analysis and isolation. All experiments were conducted in triplicate.

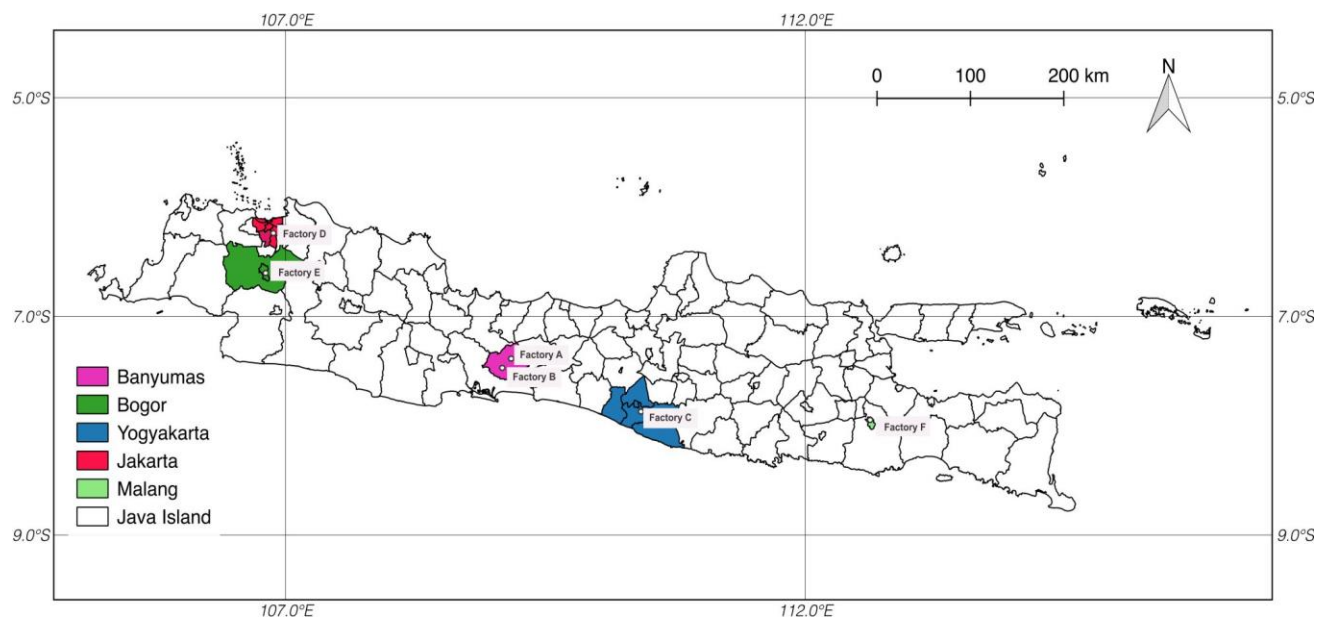


Figure 1. Sampling map (Java, Indonesia)

### 2.1.3. Determination of pH, titratable acidity (TTA), temperature and humidity in local factories

**pH of soak water.** The pH value of the soak-water was determined according to Association of Official Analytical Chemists (AOAC, 2000) and read with a digital pH meter (Hanna).

**TTA content of soak water.** The amount of TTA produced in the fermenting soak-water was determined according to Lonner (1988). With 9 mL of sterile distilled water, 1 mL of the soak-water was diluted. Of the resultant mixture, 10 mL was then titrated against 1 N NaOH with two drops of phenolphthalein as indicator. The TTA content was thus calculated from the formula.

$$\text{Lactic acid (mg/mL)} = \frac{\text{Average titre value} \times 90}{\text{Volume of sample}}$$

90 is the lactic acid equivalent factor.

**Temperature and humidity in workshops.** Temperature was checked by portable infrared thermometer (OS-VIR50). Humidity was measured by portable digital hygrometer (Amarell).

### 2.1.4. Microbiological analysis of the soak water

The samples were homogenized using a vortex before plating.

Ten (10) mL of soak water were homogenized with 90 mL of 0.85% (w/v) sterile saline water to make an initial dilution ( $10^{-1}$ ). The suspension was used for making suitable serial dilution up to  $10^{-7}$  by incorporating one mL into nine mL sterile saline water in sterile tubes. The suspension was homogenized using a vortex before plating. Each 0.1 mL of appropriate dilutions were spread-plated in specific media.

Enumeration of LAB was determined with surface plating onto Mann Rogosa Sharp agar (MRS) and incubated anaerobically at 30°C for 48 h.



Yeast counts were performed by plating onto chloramphenicol bromophenol blue agar consisting of [(g/l in distilled water): yeast extract, 5 g; glucose, 20 g; chloramphenicol, 0.1g; bromophenol blue, 0.01g; agar, 15 g; pH, 6-6.5) and incubated aerobically at 25°C for five days.

Aerobic mesophilic bacteria enumeration was done with Plate Count Agar. Aerobic incubation at 37°C for 48 h.

Coliforms enumeration was done with Violet Red Bile Agar plates. The plates were incubated at 37°C for 24 h. Purple-red colonies surrounded by a reddish zone of precipitated bile were counted as coliforms.

The total number of colonies that developed on the plates was recorded as colony forming units (CFU) per mL of samples. The calculation was based on standard plate count (SPC). All analyses were performed in triplicate, and the means  $\pm$  standard deviations were used as data.

### **2.1.5. Isolation of culturable yeast and LAB**

After counting, single colonies with distinct morphological differences (color, shape, and size) were randomly picked from plates 30-300 colonies ( $10^{-5}$  dilution for LAB and  $10^{-4}$  dilution for yeasts), and transferred in 10 mL of corresponding sterile broth media. The selected colonies were purified by repeated streaking on the specific agar media before being subjected for characterization. For long term storage, purified culture was stored -80°C with 25% (v/v) glycerol as cryoprotectant.

### **2.1.6. Phenotypic characterization of culturable yeast and LAB**

**LAB:** Strains were characterized after 24 h of incubation at 30°C on MRS agar. Microscopic observation was done to examine cell shape. The preparations were analyzed using oil immersion and a light microscope (Leica Leitz DMRB) equipped with a color camera (Leica DFC420). All strains were subjected to Gram staining, catalase activity, hydrolysis of arginine, lactic acid isomer test, and gas production from glucose, growth test at different temperatures (10, 15, 45 and 50°C) for 7 days, tolerance of different salt levels (2, 3, 4, 6, 6.5, 8, 10% NaCl w/v) at 30°C for 2 days and pH (3, 3.5, 4, 4.5, 5, 9, 9.6 adjusted with glacial acetic acid) for 3 days (Harrigan and McCance, 1976; Schillinger and Lücke, 1987; Kozaki et al., 1992; MacFaddin, 2000; Yu et al., 2011). Carbohydrate fermentation test was conducted with API 50CHL strips (API system, BioMerieux). The characterization results of LAB isolates were compared with reference to Bergey's Manual of Determinative Bacteriology (Holt et al., 1986).

**Yeast:** All the yeast isolates were characterized by the conventional method described by several authors (Deak and Beuchat, 1996; Kurtzman and Fell, 2000; Kurtzman et al., 2011). The isolates were cultured for 2 days in yeast extract–glucose–chloramphenicol–agar (YGCA) at 25°C and observed their colony characteristics (color, texture and topography of the surface and edges). Isolates were stained with lactophenol blue and observed under microscope after 24 h incubation on YGC broth. Yeasts were observed for cell shape, sporulation, and vegetative reproduction. All yeast isolates were also examined on urea hydrolysis, growth in presence of 0.01% (w/v) cycloheximide, growth at 37°C, vitamin requirement test and assimilation of KNO<sub>3</sub>.

Yeast isolates were tested for their ability to assimilate various compounds by using the API ID 32C kits (API system, BioMerieux). The characterization results were compared with yeast identification keys of Barnett et al. (1990) and Kurtzman and Fell (2000).

### 2.1.7. Molecular identification

#### DNA extraction of yeast and LAB isolates

DNA extraction was achieved according to a modified method of Ausubel *et al.*, (1987). 1.8 mL of 48 h LAB culture on MRS broth (or 3 days yeast culture on YGC broth) was put into a 2 mL sterile tube and centrifuged during 10 min at 20°C and 14000 RPM. The supernatant was carefully drawn off, the pellet was resuspended in 576 µL TE-buffer and 30 µL of 10% SDS were added followed by 3 µL proteinase K (20 mg/mL), the mixtures were incubated for 1 h in a 37°C preheated water bath. 100 µL NaCl 5 M and 80 µL 10% CTAB (in 1×TE, 0.7 M NaCl) were respectively added to the mixture and incubated in a 65°C preheated water bath for 10 min. After this incubation, 700 µL of chloroform/isoamyl alcohol 24:1 (v/v) were added and the whole sample was thoroughly mixed and kept on ice for 10 min. The tubes were then centrifuged at 14000 RPM during 10 min in a 4°C precooled centrifuge. The aqueous viscous phase was recovered, and 30% PEG was added to a final concentration of 6.5% (divide volume of the aqueous phase by 3.6). The content of the tubes was cautiously mixed by tilting and left for precipitation for 20-30 min at room temperature. The samples were centrifuged for 10 min at 4°C and 14000 RPM. After centrifugation, the supernatant was discarded and the pellet washed twice with cold EtOH (70%) with the precaution not to loose the pellet. After the second washing, the rest of EtOH was spinned down and removed as much as possible using a pipette. The pellet was dried at 30°C in the SpeedVac (Eppendorf concentrator 5301, 1400 RPM) for 10 min and resuspended in 50 µL of TE-buffer overnight. To check for the success of the DNA extraction step, 2 µL DNA solution, 1 µL blue juice and 2 µL of the standard Lambda-DNA (25 ng/mL Lambda DNA) were run on 0.8% agarose gel.

#### DNA fingerprinting

In the preliminary experiments, four arbitrary primers were tested with Random Amplified Polymorphic DNA (RAPD) with DNA extracted from pure cultures (yeast and LAB). List of primers can be seen on Table 1. Preliminary test determined the optimal concentration of the component in the PCR reaction mixture, amplification conditions and primer selection. Strong, unique, sharp and clearly reproducible bands were produced by primer C2\_1++inv\_f and C7\_1\_seq\_f2. For further analysis C2\_1++inv\_f was used.

Table 1. Primer and their sequences tested in preliminary RAPD analysis

Primers	Sequence 5'-3'
C6.2_introns_2_R (C6_2_int_R3)	TGGCTCACGCAAGGGTGTAG
C2_1++inv_f	TGACGTCCTAGTTGATATCCTCG
C2_1++inv_r	ACCGTTATGATCAATAAATATTTGACCA
C7_1_seq_f2	AGGTTAGCTCGCGACTATCGGATC

In brief, the optimal condition of PCR as described below:

The amplification reaction was carried out in a volume of 25  $\mu\text{L}$  containing 18.45  $\mu\text{L}$  water, 2.5  $\mu\text{L}$  10x reaction buffer, 1.5  $\mu\text{L}$  dNTPs 150  $\mu\text{M}$  (Amersham Biosciences), 0.75  $\mu\text{L}$  of primer 0.3  $\mu\text{M}$  (GenXpress), 0.05  $\mu\text{L}$  of Taq DNA polymerase 0.25 U, and 1  $\mu\text{L}$  of DNA sample. For detection of any other kinds of DNA contaminants, a negative control of PCR-mix without any template DNA was used. The PCR program had initial denaturation at 94°C for 2 min, 2x45 cycles of: 20 s at 94°C, 37 s at 37°C, 20 s at 72°C and followed by final extension at 72°C for 5 min, storage 15°C for 5 min (peQ STAR Thermocycler). Eight  $\mu\text{L}$  of the PCR products, and 0.5  $\mu\text{L}$  of size marker (100bp), respectively, is mixed with 2  $\mu\text{L}$  of blue juice and pipetted into the slots. The electrophoresis is carried out at 60 V for 90 min. The EtBr-stained DNA fragments are visualized under a UV-transilluminator.

### **Polymerase chain reaction and phylogenetic analysis**

#### **16s rRNA gene sequences analysis for LAB**

Molecular identification of the LAB isolates was achieved with the amplification partial sequence 16S rRNA genes using the bacterial universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG) (Lane, 1991) and bacterial primer 1100R (5'-AGGGTTGCGCTCGTTG) (Turner *et al.*, 1999) with cycling conditions of 94°C 30 sec, 10 $\times$  (94°C 30 sec; 62°C-53°C -1°C/cycle 40min; 72°C 1 min), close loop 30 $\times$  (30 $\times$  94°C 30 sec; 56°C 40min; 72°C 1min), close loop 72°C 5 min. The DNA was diluted 1/100 and 1/1000. PCR was carried out with a total volume of 25 $\mu\text{L}$  composed of 18.45  $\mu\text{L}$  water, 2.5  $\mu\text{L}$  10x reaction buffer (New England BioLabs, Ipswich, MA, USA), 1.5  $\mu\text{L}$  dNTPs 150  $\mu\text{M}$  (Bioline, Luckenwalde, Germany), 0.75  $\mu\text{L}$  of each primer 0.3  $\mu\text{M}$  (Invitrogen by Thermo Fisher Scientific), 0.05  $\mu\text{L}$  of Taq DNA polymerase 0.25 U (New England BioLabs, Ipswich, MA, USA) and 1  $\mu\text{L}$  of DNA sample. A control PCR without DNA was set up to check for nonspecific amplification. The PCR product was checked on a gel, prepared for sequencing and sequenced. To check for the success of the DNA extraction step, 2  $\mu\text{L}$  DNA solution, 1  $\mu\text{L}$  and 2  $\mu\text{L}$  of the standard Lambda-DNA (25 ng/mL Lambda DNA) were run on 0.8% agarose gel as described above. Similarity searches in the sequences of the sequenced 16S rRNA gene, alignments and phylogenetic analysis were performed.

#### **ITS/5.8S ribosomal DNA (rDNA) gene sequences analysis for yeast**

A touch down program was used for the amplification of the internal transcribed spacer region with the primers pairs (Invitrogen) ITS 4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS 5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') (White *et al.*, 1990). The cycling conditions included 94°C 30 sec, 10  $\times$  94°C 30 sec, 62°C-53°C -1°C/cycle 40 sec, 72°C 1 min, close loop 30 $\times$  94°C 30 sec, 56°C 40 sec, 72°C 1 min, close loop 72°C 5 min. The DNA was diluted 1/100. A total volume of 25 $\mu\text{L}$  mixtures composed of 13.45  $\mu\text{L}$  water, 2.5  $\mu\text{L}$  10x reaction buffer, 0.2  $\mu\text{L}$  MgCl<sub>2</sub> (Bioline), 1.5  $\mu\text{L}$  dNTPs 150  $\mu\text{M}$  (Bioline, Luckenwalde, Germany), 0.75  $\mu\text{L}$  of each primer 0.3  $\mu\text{M}$  (Invitrogen by Thermo Fisher Scientific), 0.05  $\mu\text{L}$  of DNA polymerase 0.25 U and 6  $\mu\text{L}$  of DNA sample was used for PCR. A control PCR without DNA was set up to check for nonspecific amplification. The expected PCR products' size was 600-750 bp. For agarose gel electrophoresis of the PCR products, 2  $\mu\text{L}$  blue juice were mixed with 2  $\mu\text{L}$  digested lambda DNA (25 ng/mL). The PCR products were checked on agarose gel electrophoresis and purified using isopropanol precipitation and sent for sequencing as described above. The checking of the PCR product was performed on agarose gel electrophoresis as described above. The PCR products were purified using isopropanol precipitation and 5  $\mu\text{L}$  of the PCR product were sent to LCG Genomics GmbH (Berlin, Germany) for sequencing.

## Part II (Screening and characterization of antifungal activity)

### 2.2.1. Antifungal activity assay

#### Fungal spore suspension preparation

The fungus was grown on PDA petridishes for 7 days at 25°C until sporulation formed. Spores were collected in a sterile phosphate buffer solution containing 0.05% of Tween 20 and diluted to obtain final concentration containing  $2 \times 10^6$  spores/mL determined using hemocytometer.

#### Cell free supernatant (CFS) preparation

The selected LAB were cultured in 100 mL Erlenmeyer with an initial concentration of  $10^6$  CFU/mL in 50 mL of MRS broth (pH 6.5) and was incubated at 30°C for 72 h. Afterwards, CFS was prepared by centrifugation at 12000 RPM for 5 min at 4°C (to remove the cells) and sterile filtration (0.45 µm pore size filter; Millipore). CFS was concentrated by ammonium sulfate precipitation method and dialyzed with phosphate buffered saline pH 7.4 (PBS) and stored at -20°C for further use. CFS obtained was used for determination of the antifungal activity.

#### Screening of antifungal activity

In the preliminary screening, modification of microtitre plate well method was used. In this method, 60 µL of SCF, 40 µL PDB and 20 µL of a spore suspension ( $2 \times 10^6$  spores/mL) were added to each well of a sterile 96-well microplate (Sarstedt AG & Co., Nuembrecht, Germany). Microcultures containing 60 µL of sterile tap water instead of SCF were used as a negative control. The commercial fungicide cycloheximide at 0.5 mg/mL was used as a positive control. All samples were placed in triplicate on the plates. Plates were kept in sterile glass petridish to avoid contamination from outside during incubation. Observation was done after 5 days of incubation at 25°C.

Selected strains from pre-screening were tested again with well diffusion agar technique. Total of 10 mL of soft agar at 40°C containing  $10^6$  spores/mL were surface spread on the PDA (2% agar) plates. Wells of 8 mm diameter were cork bored in the soft agar. Aliquots (100 µL) of the CFS were poured into the wells, and the plates were incubated at 25°C for 72 h. Un-inoculated MRS broth acidified at pH 4 (HCl 1M) was used as negative control. After incubation, the plates were examined for the presence of a growth inhibition halo around each well. Experiments were performed in triplicates. The LAB isolates showing potent antifungal activity were selected for further study (Fungal biomass inhibition).

To determine the characteristics of the antifungal compounds, the CFS showing antifungal effect were subjected to heating (100°C for 15 min), neutralization (adjust to pH 7) with 3 N NaOH, and proteinase K (Sigma-Aldrich) treatment (1500 U/mL CFS) at pH 7, 40°C for 60 min. The CFS treated with proteinase K were adjusted to pH 4 before antifungal assay. Well diffusion agar technique used as described earlier.

#### Fungal biomass inhibition

Test was carried out by using selected CFS from screening step. Ten percent of CFS were prepared in 50 mL MRS medium and inoculated with a 8 mm diameter fungal disc. The flasks were incubated at 25°C for 12 days. Flask without CFS served as control. After incubation, fungal mat was harvested, filtered (Whatman No. 4 filter papers), washed twice thoroughly with distilled water and dried with freeze dryer. Fungal biomass of each treatment was weighed and compared with the control. The

experiment was conducted in triplicate. Fungal growth inhibition (%) was calculated according to the following formula:

$$\text{Fungal growth inhibition (\%)} = \left[ \frac{(\text{Total control weight} - \text{Total sample weight})}{\text{Total control weight}} \right] \times 100$$

### Scanning Electron Microscopy (SEM) analysis

SEM was employed to visualize the morphology and structure of *F. proliferatum* BJ09.05 and to know the possible mechanism or interaction between *L. plantarum* B.BS08 and *F. proliferatum* BJ09.05. SEM method was according to method of Alves et al. (2013) with modification. The samples were transferred to silica coverslip with suspension method and fixed with 2.5% (v/v) glutaraldehyde and 0.1% (v/v) tannic acid overnight at fridge (4°C) on aluminium cover. Post-fixing with 1% osmium tetroxide for 45 min. Rinsing three times in distilled water and then followed by dehydration in crescent series of ethanol (30, 50, 70, 80, 90 and 95%), continue dehydration with 99% ethanol for three times and 100% ethanol for three times. Afterwards, the samples were transferred to a critical point dryer to complete the drying process with carbon dioxide as a transition fluid. The samples were air dried, mounted on aluminium stub using double sided carbon tape and liquid silver on two edge, and kept in vacuum dessicator until observing. Observation was done with a Hitachi H-7600, equipped with a MegaView 3 CCD camera (SIS, Soft Imaging Solutions, Münster, Germany) in Max Planck Institute Biophysical and Chemistry, Göttingen Germany.

### 2.2.2. Antifungal Compounds Analysis

#### PCR amplification of LDH encoding gene.

PCR procedure has been described previously by Zhang et al. (2014) with little modification.

Forward primer: CATCAAAAAGTTGTGTTAGTCGGCG

Backward primer: TCAGCTAAACCGTCGTTAAGCACTT

PCR was performed in a final volume of 50 µL of PCR Master Mix containing 4 µL primers for the LDH gene, 5.0 µL dNTP, 19.0 µL ddH<sub>2</sub>O, 2.0 µL DNA, and 25.0 µL MIX high fidelity DNA Polymerase. Amplifications were carried out in peQ STAR Thermocycler (96 Universal Gradient) under the following programs: initial denaturation for 3 min at 94 °C, followed by 30 cycles consisting of pre-denaturation 94 °C for 45 s, annealing at 52 °C for 1 min, and elongation at 72 °C for 1 min. Amplification fragments were determined using electrophoresis on 0.8% agarose gel. After staining with ethidium bromide (0.5 µg mL<sup>-1</sup>) and destaining in demineralized water (each for 10 min), DNA bands were visualized in UV light using a CCD camera (Vilber Lourmat, Marne La Vallee, France).

#### Titrate acidity (organic acid content)

Procedure (Lonner, 1988) as described earlier.

#### Phenylactic acid (PLA) extraction and determination with HPLC-MS/MS

CFS are extracted using ethyl acetate (1:1, v/v) in falcon tube. Two fraction were collected (polar and non polar fraction). Polar fractions were concentrated under reduced pressure (Büchi, Rotary Evaporator). The dried polar extracts were dissolved in 500 µL (MeOH:H<sub>2</sub>O, 1:1, v/v), and stored at -20°C until analysis commenced.

PLA separation and analysis were carried out using a high pressure liquid chromatography system equipped with an autosampler (ProStar 410, Varian, Darmstadt, Germany), a binary pump system, a

degasser and a column oven (Jetstream 2 plus, Techlab, Germany) coupled to an electrospray ionization (ESI) source followed by an ion trap mass spectrometer (500 MS, Varian, Darmstadt, Germany). PLA separation was carried out by high pressure liquid chromatography using a reverse phase column Kinetex C18 (50.0 x 2.1 mm, particle 2.6  $\mu\text{m}$ ) coupled with a C18 security guard cartridge (4 mm x 2 mm i.d., both from Phenomenex, Aschaffenburg, Germany) maintained at a temperature of 40°C. An aliquot of 10  $\mu\text{L}$  of the sample was used for injection. The mobile phase consisted of water with 5% acetonitrile (A) and methanol (B), both containing 0.01 mM sodium acetate and 7 mM acetic acid. The binary gradient used was: 0-3 min from 40 to 80% B, 3-8 min from 80 to 98% B, 8- 11 min at 98% B, 11-11.5 min from 98 to 40% B and finally from 11.5-16.5 at 40% B. The flow rate was set to 0.2 mL min<sup>-1</sup>. ESI was operated in positive mode for all analytes with the following settings: spray chamber temperature 50 °C, nebulizing gas (nitrogen) 50 psi, drying gas (nitrogen) 25 psi at 350 °C, shield voltage 600 V, needle voltage 5000 V, trap damping gas (helium) 0.8 mL min<sup>-1</sup>. The detector was operated in standard mode with 15.000 Da sec<sup>-1</sup>.

### 2.2.3. New tempeh starter production and its application

#### Modern tempeh starter production

LAB dry starter production: Growing LAB starter on MRS medium at 37°C for 24 h with shaker. The production of dry starter for selected isolates was done using spray dryer with tapioca acid matrix as coating medium. A 500 mL sample was prepared by mixing 400 mL of a 1% (w / v) sterile matrix solution with 100 mL of LAB culture at the optimum growth stage, then dried with a spray dryer at an inlet temperature of 170°C and an outlet of 70°C. Dry starter then kept in plastic container at fridge temperature 4°C.

*R. microsporus* var. oligosporus dry starter production: Cassava was peeled, washed and then shredded roughly. Cassava was then steamed for 30 min then cooled (aerated). Inoculation with pure *R. oligosporus* culture done by adding 20 mL spore suspension ( $2 \times 10^6$  spores/mL) into 500 gram steamed cassava and incubation for 60 h in 30°C. Samples were dried by cabinet dryer in 40-45°C for 48 h and powdered in 120 mesh size. Dry starter then kept in plastic container at fridge temperature 4°C.

Mixing starter: *R. microsporus* var. oligosporus and LAB starter powders were mixed with a ratio of 75:25 (w/w) to further use in tempeh making.

#### Application in tempeh model

Tempe making was done as follows: 60 g of soybeans as raw material were washed and boiled for 30 min (ratio tap water:raw materials = 5:1), then dehulled manually. The raw materials were soaked in tap water for 18 h at room temperature (ratio tap water : raw material = 3:1). Furthermore, materials were boiled again 30 min with the same ratio before, drained and dried-aired until ready at room temperature and inoculated with 5 g of dry starter. After inoculation, samples were placed on petridish and incubated at a temperature 30°C for 48 h. Samples then frozen -20°C and dried with freeze dryer. Samples were powdered and kept in -20°C for analysis.

Treatment consists of: negative control (tempe made only with pure *R. microsporus* var. oligosporus starter), tempeh made with modern starter plus contamination of *F. proliferatum*, and positive control (tempe made only with *F. proliferatum* inoculation). Contamination was made by adding 3 mL of spore suspension *F. proliferatum* ( $10^6$  spores/mL) to the raw materials. Analysis of *F. proliferatum* biomass done by qPCR technique.

### Part III (Zearalenone biotransformation by tempeh yeast and LAB)

#### 2.4.1. Microorganism and *in vitro* test (in liquid culture)

Yeast and lactic acid bacteria (LAB) strains used for *in vitro* test could be seen on Table 2. In brief, Stock cultures were grown in specific liquid medium (Yeast Glucose Broth for yeast and de Man, Rogosa and Sharpe broth for LAB) for 3 days at 30°C 150 RPM (Innova44, New Brunswick Scientific Co). Cell suspension of each isolates were arranged (OD 600 = 0.8) using a spectrofotometer.

Liquid medium was used as media in *in vitro* screening. Fifty milliliters of the liquid medium was poured into the 250-mL Erlenmeyer flask, the flask was closed with a cellulose plug and then autoclaved (120°C, 15 psi, 15 min; Autoclave 3870 EL). The sterile liquid media was inoculated with 2 mL cell suspension (OD 600 = 0.8) and then incubated at 30°C for 2 days in a rotary shaker (Innova44, New Brunswick Scientific Co) at 150 RPM.

For the treatment, 1 mL working solution of zearalenone (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) ( $c = 5 \mu\text{g/mL}$ ; methanolic solution) was added to the liquid culture. Medium without culture was added with 1 mL working solution of zearalenone as positive control. Instead of zearalenone, 1 mL methanol was added to the culture as negative control. The incubation was continued in the rotary shaker for 1 day. Five hundred microliters of the liquid media was transferred into a 1.5-mL Eppendorf tube and 500  $\mu\text{L}$  of ice-cold acetonitrile (Th. Geyer Co) was added for protein precipitation. Samples were then stored overnight at 4 °C and centrifuged at 9800 RPM for 5 minutes. The supernatant was transferred to a HPLC (high performance liquid chromatography) vial and analyzed by HPLC hyphenated to tandem mass spectrometry (HPLC-MS/MS).

Table 2. Some isolates of yeast and lactic acid bacteria (LAB) used for *in vitro* test.

Microbes name	Original source	Year sampling
<i>Saccaromycopsis fibuligera</i> Y.BS01 (Y1)	Soak water (natural acidification step), Manufacture Banyumas	2015
<i>Saccaromycopsis fibuligera</i> Y.ASC06 (Y2)	Soak water (natural acidification step), Manufacture Bogor	2015
<i>Pichia kudriavzevii</i> Y.BS09 (Y3)	Soak water (natural acidification step), Manufacture Banyumas	2015
<i>Candida tropicalis</i> Y.BS07 (Y4)	Soak water (natural acidification step), Manufacture Banyumas	2015
<i>Kluyveromyces marxianus</i> Y.BS08 (Y5)	Soak water (natural acidification step), Manufacture Banyumas	2015
<i>Schizosaccaromyces pombe</i> Y.MSR02 (Y6)	Soak water (natural acidification step), Manufacture Malang	2015
<i>Meyerozyma guilliermondi</i> Y.KSM11 (Y7)	Soak water (natural acidification step), Manufacture Jogja	2015
<i>Schizosaccaromyces pombe</i> Y.MSR02 (Y8)	Soak water (natural acidification step), Manufacture Malang	2015
<i>Saccaromyces cerevisiae</i> Y.BSC09 (Y9)	Soak water (natural acidification step), Manufacture Banyumas	2015

<i>Saccaromyces cerevisiae</i> Y. BSC10 (Y10)	Soak water (natural acidification step), Manufacture Banyumas	2015
<i>Meyerozyma guilliermondi</i> Y.MSR11 (Y11)	Soak water (natural acidification step), Manufacture Malang	2015
<i>Meyerozyma guilliermondi</i> Y.BS02 (Y12)	Soak water (natural acidification step), Manufacture Banyumas	2015
(+) control	Yeast Glucose Broth without culture was added with 1 mL working solution of ZEN	
(-) control	1 mL methanol was added to the culture	
<i>Lactobacillus plantarum</i> B.BS10 (L1)	Soak water (natural acidification step), Manufacture Banyumas	2015
<i>Pediococcus pentosaceus</i> B.BSC07 (L2)	Soak water (natural acidification step), Manufacture Banyumas	2015
<i>Lactobacillus paracasei</i> B. KSM06 (L3)	Soak water (natural acidification step), Manufacture Jogja	2015
<i>Lactobacillus paracasei</i> B. KSM11 (L4)	Soak water (natural acidification step), Manufacture Jogja	2015
<i>P. pentosaceus</i> B.MSR10 (L5)	Soak water (natural acidification step), Manufacture Malang	2015
<i>L. plantarum</i> B.BSC09 (L6)	Soak water (natural acidification step), Manufacture Banyumas	2015
<i>L. plantarum</i> B.BS03 (L7)	Soak water (natural acidification step), Manufacture Banyumas	2015
<i>L. plantarum</i> B.BS01 (L8)	Soak water (natural acidification step), Manufacture Banyumas	2015
(+) control	MRS broth without culture was added with 1 mL working solution of ZEN	
(-) control	1 mL methanol was added to the culture	

#### 2.4.2. HPLC-MS/MS analysis

Mycotoxin separation and analysis was carried out by high pressure liquid chromatography coupled with electrospray ionization and tandem mass spectrometry detection. HPLC-MS/MS was performed on an API 4000 mass spectrometer (AB Sciex, MA) connected to an Agilent 1100 series HPLC (Agilent Technologies GmbH, Böblingen, Germany). The analytical column was a ProntoSIL 120-5-C18 AQ (150 mm x 3 mm, 5 µm; Bischoff Chromatography, Leonberg, Germany), preceded by a ProntoSIL 120-5-C18 AQ guard column (10 mm x 3 mm, 5 µm). Mobile phase A was water with 5 mM ammonium acetate and as mobile phase B acetonitrile/water (99 : 1 v/v) with 5 mM ammonium acetate was used. Detailed protocol was according to Brodehl et al. (2014).



### 3.1. Results of Part I (Identification and characterization of tempeh yeast and LAB)

#### Chemical and microbiological observation on soak water from different local tempeh factories

The measured pH and other parameter of the soak water taken from the local factories are shown in Table 3. The temperature and relative humidity in the workshop of all the six factories were around 26-29° C and 80–85%, respectively. The temperature of supernatant in fermentation sinks was in the range of 28-30° C, a little higher than the ambient temperature. The pH of the soak water in all factories decreased sharply from 7.3-7.5 to around 4.5-5.2 after 24 h of soaking time, while the TTA increased from 0.50 to around 6.01-6.77mg/mL indicating that the medium was barely acidic. Organic acids of the supernatants mainly included lactic acid and acetic acid (data not shown), indicating that some of the LAB involved are heterofermentative and lactic acid was predominant among the two acids. The quite low acidity observed in the present work may be due to the fact that soak water contained sugar or starch that can be metabolized by LAB isolates. Warm temperature in the workshop might also support indigenous LAB and yeast to grow fast.

Table 3. Parameters obtained from local tempe factories.

Factories (Locations)	Material:water ratio*	Spontaneous fermentation (Soaking time)	pH value of soak water	Titrateable acidity/TTA (mg/mL)	Temperature of soak water (°C)	Temperature (°C) and humidity of workshop (%)
Factory A (Banyumas)	S:C:W (5:2:8)	24	5.2	6.13	28.3	27; 85
Factory B (Banyumas)	S:C:W (5:2:7)	24	5.0	6.22	28.2	27; 85
Factory C (Yogyakarta)	S:C:W (5:2:8)	24	5.0	6.01	28.4	28; 86
Factory D (Jakarta)	S:C:W (5:3:8)	24	4.5	6.77	30.0	29; 85
Factory E (Bogor)	S:C:W (5:2:8)	24	4.8	6.25	28.3	26; 80
Factory F (Malang)	S:C:W (5:2:9)	24	4.7	6.58	30.0	27; 86

Note: \*S: soybean, C: corn, W: tap water

The pH of tap water in all six factories was around 7.3-7.5

Table 4 shows the distribution of microorganisms in the soak water in the factories. The microbial numbers during fermentation differed among factories. LAB counts in soakwater varied between 5.21 and 8.88 log CFU/mL whereas yeasts counts varied from 4.31 and 4.89 log CFU/mL whereas APC counts varied between 7.33 and 9.10 log CFU/mL. This result showed that the number of LAB is larger than yeast. Enterobacteriaceae was not detected in soak water with high LAB number. This seems dominant LAB could suppress the present of Enterobacteriaceae. The use of recycle soak water which applied by Factory D, E and F gave significant different microbial properties in their soak water, compared to Factory A, B and C (using only fresh soak water).

Using previous soak water gave impact to higher number of LAB and avoid contamination Enterobacteriaceae. Daily addition of previous soakwater (backslopping) is a powerful method of obtaining a vigorous lactic fermentation. This ‘accelerated natural lactic fermentation’ results from the gradual selection of ecologically adapted acidifying organisms.

Table 4. Microbial counts (log CFU/mL) of the soak water in six local factories

Factories	LAB	Yeast	APC	Enterobacteriaceae
Factory A*	5.36 ±0.33	4.51 ±0.88	7.45 ±0.41	1.23 ± 0.17
Factory B*	5.21 ±0.51	4.31 ±0.98	7.33 ±0.42	1.45 ± 0.34
Factory C*	7.11 ±0.77	3.98 ±0.31	7.50 ±0.63	2.22 ± 0.65
Factory D**	8.88 ±0.35	5.21 ±0.23	9.10 ±0.13	ND
Factory E**	8.31 ±0.34	4.45 ±0.27	8.80 ±0.55	ND
Factory F**	8.22 ±0.29	4.89 ±0.31	8.50 ±0.23	ND

Note: LAB: lactic acid bacteria, APC: Aerobic plate count

Result given as means ± SD of triplicate determination

ND refers to below detection level

\* Using fresh soak water

\*\* Use recycle/previous soak water (50% total volume of soak water)

### Characterization and identification of LAB and yeast isolates from soak-water

The species of LAB and yeast were identified according to phenotypic, and biochemical characterization. Identification into species level mainly on the basis of their sugar fermentation profiles in the API 50 CH test for LAB. The isolated yeast strains were identified based on their physiologic and morphology characteristics as described in several authors (Kurtzman and Fell, 2000; Kurtzman et al., 2011). The identification into species level was performed based on API ID 32C test kit results, and also by the following tests: fermentation of sugars, assimilation of KNO<sub>3</sub> and carbon compounds, assimilation of nitrogen compounds, growth in vitamin-free medium, growth at 37°C, growth on 50% (w/v) glucose yeast extract agar, resistance to 0.01% cyclo-heximide, and carbohydrate assimilation. To confirm the identification result we also check with DNA sequencing technique, by only choosing 1-4 representative strains from each species group that previously identified with API method.

One hundred yeast strains were isolated and identified as *Schizosaccharomyces pombe* (9 strains), *Saccharomycopsis fibuligera* (13 strains), *Kluyveromyces marxianus* (19 strains), *Candida tropicalis* (22 strains), *Saccharomyces cerevisiae* (22 strains), *Meyerozyma guilliermondi* (15 strains). Their physiologic, morphology, and API 32C identification are shown in Table 5 and Figure 2.

We isolated 22 *S. cerevisiae* species strains, oblong/eclipse, ovoid and (2-8) x (0.5-3.0) µm that were detected after incubation for five days at 25 °C. They produced ascospores but did not form pseudohyphae. Among them, 19 strains fermented glucose, galactose, sucrose, maltose, and raffinose, but not lactose. They could not assimilate cellobiose, lactose or inositol, but grew in a vitamin-free medium and also on 50% (w/w) glucose yeast extract agar. Twenty-two smooth ovoid strains at sizes (1.5-3) x (5-7) µm were identified as *C. tropicalis*. Abundant pseudohyphae often radiating with clusters of blastoconidia at the center; variable appearance. Pseudohyphae is filament formed by the

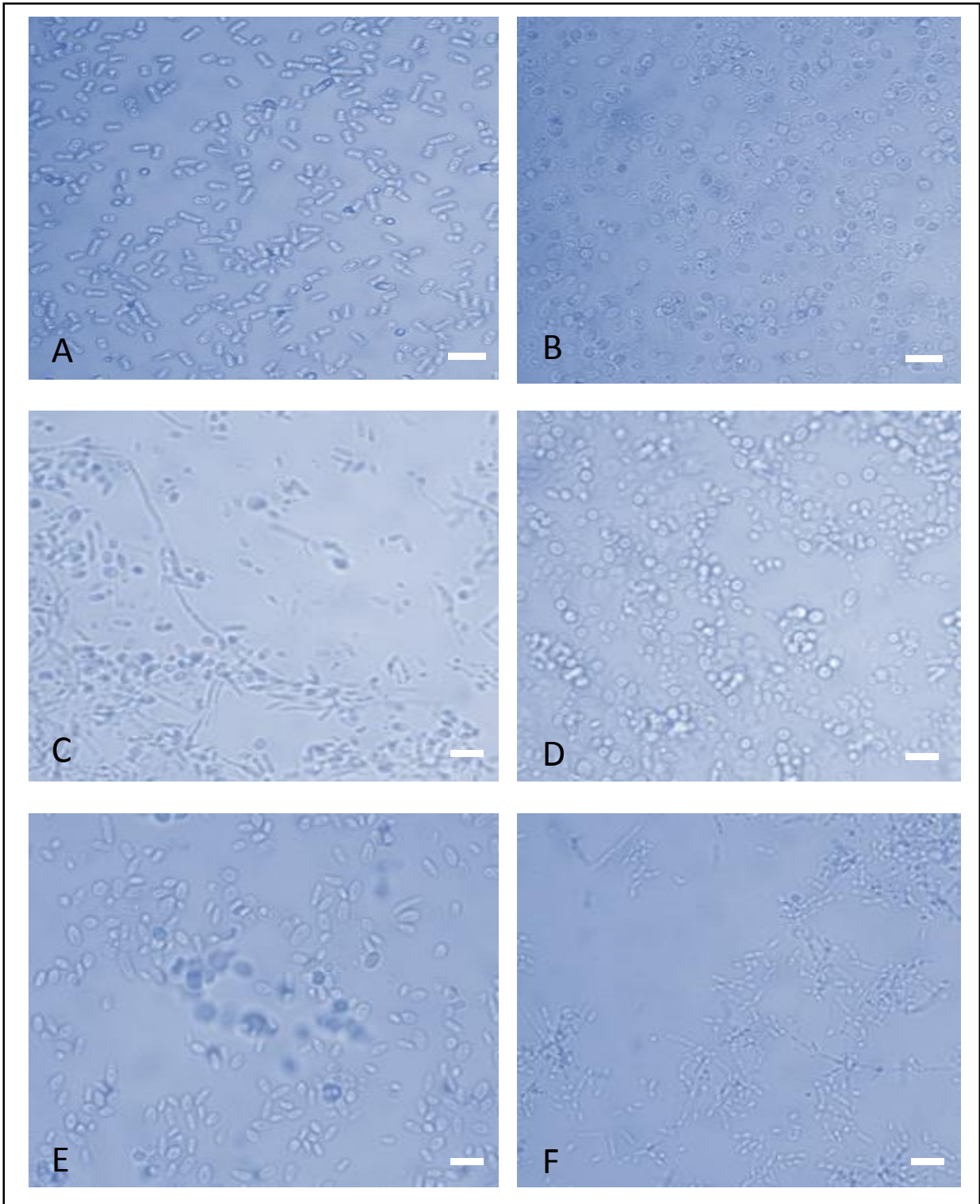


Figure 2. Yeast isolated from soak water (tempeh factories). A. *Schizosaccharomyces pombe*, B. *Saccharomycopsis fibuligera*, C. *Kluyveromyces marxianus*, D. *Candida tropicalis*, E. *Saccharomyces cerevisiae*, F. *Meyerozyma guilliermondi*. Scale bar = 10  $\mu$ m.

budding of elongated cells which do not separate. The tip cell is short and there are constriction at the septa where the cells are connected. Nineteen spheroidal, ovoid or cylindrical strains at sizes (1.5-3) x (5-7)  $\mu\text{m}$  were identified as *K. marxianus*. They fermented glucose, galactose and lactose, and could assimilate glucose, galactose, sucrose, lactose, cellobiose, and trehalose. Fifteen ovoid and elongated yeasts strains at sizes of (3-11) x (2-4)  $\mu\text{m}$  were identified as *Meyerozyma guilliermondi*. They produced ascospores after incubation for five days at 25°C and formed pseudohyphae. They are most active in assimilating many kind of sugars. We found the three first yeast species group mentioned as dominant yeast in soak water. Detail explanation for other two yeast species group was also described in Table 5.

Sixty-five LAB strains were isolated and identified into three big group species. They are *Lactobacillus plantarum* (29 strains), *L. paracasei* (15 strains) and *Pediococcus pentosaceus* (21 strains). Their physiologic, morphology, and API 50 CH test are shown in Table 6. We found *L. plantarum* as dominant LAB in the soak water, followed by *L. paracasei* and *P. pentosaceus*. They are recognized as heterofermentative LAB.

To confirm the identification result from API system then sequencing assignment based on DNA were applied, by only choosing representative 2-4 strains from each species group of yeast and LAB. In addition to the restriction patterns, some of the yeast groups were also characterized by RAPD-PCR analysis. DNA fingerprinting were also used to see which strains identical and non-identical from each species group (Figure 3). RAPD-PCR analysis has been reported to be a useful method for the differentiation of the yeast strains belonging to the same species (Dlauchy et al., 1999). Molecular identification of the LAB isolates was achieved with the amplification partial sequence 16S rRNA genes using the bacterial universal primers 27F (5'-AGA GTTTGATCMTGGCTCAG) (Lane, 1991) and bacterial primer 1100R (5'-AGGGTTGCG CTCGTTG) (Turner et al., 1999). For selected yeast, a touch down program was used for the amplification of the internal transcribed spacer region with the primers pairs (Invitrogen) ITS 4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS 5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') (White et al., 1990). PCR amplification and DNA sequencing using the primer were successful, the blast of the sequenced regions gave conclusive results concerning the molecular taxonomy of the yeast and LAB. The phylogenetic tree can be seen in the Figure 4 and Figure 5.

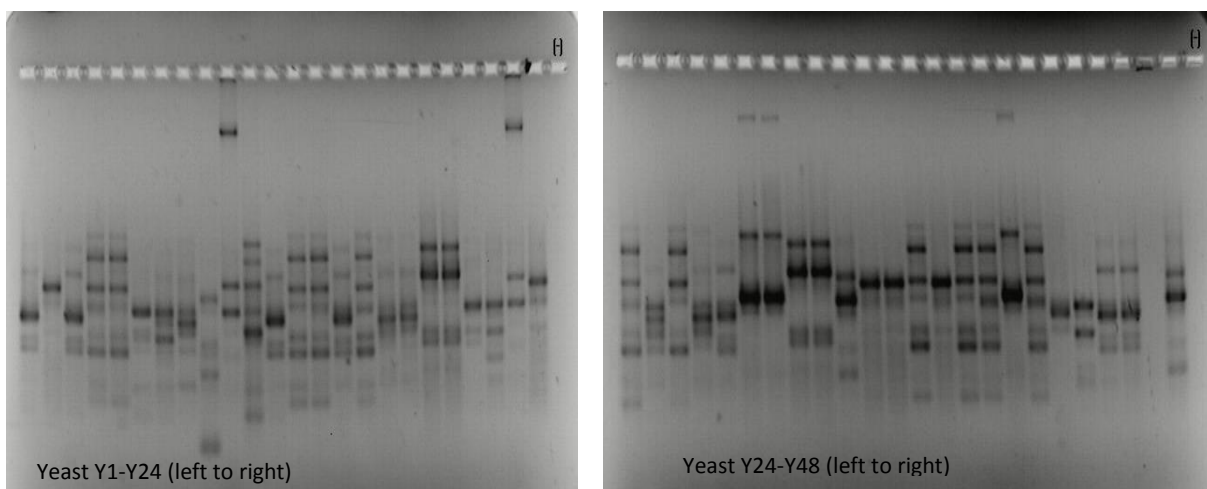


Figure 3. Some result of DNA fingerprinting from selected yeast isolates

## Chapter 6: Yeast and LAB from tempeh production

**Table 5. Phenotypic characterization of yeast isolated from soak water of tempeh production in Java.**

Characteristics	Groups					
	1 <i>M. guilliermondii</i>	2 <i>S. fibuligera</i>	3 <i>C. tropicalis</i>	4 <i>K. marxianus</i>	5 <i>S. cerevisiae</i>	6 <i>Sch. pombe</i>
Number of isolates	15	13	22	19	22	9
Colony color	White	Creamy, white	Creamy, white	Creamy, yellowish	Creamy white	Yellowish
Vegetative cell	Long ovoid, spheroidal	Ovoid	Flat, smooth, ovoid	Raised smooth clustered, spheroidal, ovoid or cylindrical	Oblong/ellipse, ovoid	Ovoid or long ovoid
Shape	(3-11) x (2-4)	(2-6) x (1-4)	(1.5-3) x (5-7)	(5-10) x (2-5)	(2-8) x (0.5-3.0)	(2-6) x (1-2)
Size (µm)						
Budding cell	+	+	+	+	+	+
Fragmenting	-	-	-	-	-	-
Pellicle	+	-	V	V	-	-
Pseudomycelium	-	-	-	-	-	-
Mycelium	-	-	-	-	-	-
Ascospore	-	-	-	+	+	+
Assimilation of KNO <sub>3</sub>	-	-	-	-	-	-
Vitamin-free medium	+	+	+	-	+	-
50% glucose	+	+	+	-	+	-
Growth at 37°C	+	V	+	+	V	+
Cycloheximide	-	-	-	-	+	+
<b>Sugar fermentation (API 32C test)</b>						
N-acetylglucosamine	+	-	+	-	-	-
Galactose	V	-	+	+	+	-
Sucrose	+	+	V	+	+	+
Lactic acid	+	-	-	-	-	-
L-Arabinose	+	-	-	-	-	-
D-Cellobiose	+	-	-	-	-	-
Raffinose	+	V	V	+	V	V
D-Maltose	-	+	+	-	V	V
Trehalose	+	-	+	+	+	-
2-Ketogluconic calcium	+	-	-	-	-	-
α-Methyl-D-glucoside	+	V	V	-	+	-
Mannitol	+	V	+	-	+	-
Lactose	-	-	+	+	-	-
Inositol	-	-	-	-	-	-
D-Sorbitol	-	+	-	-	-	-
D-Xylose	+	-	+	-	-	-
D-Ribose	+	-	V	-	-	-
Glycerol	+	+	V	-	V	V
L-Rhamnose	V	-	-	-	-	-
Parathinose	-	-	-	-	-	-
Erythritol	-	V	-	-	-	-
D-Melibiose	+	-	-	-	V	-
Glucuronic acid	+	V	-	-	V	V
D-Melezitose	+	V	V	-	-	-
Potassium gluconate	V	+	V	-	-	V
Levulinic acid	-	-	-	-	-	-
Glucose	+	+	+	+	+	+
L-Sorbose	V	-	V	-	-	-
Aesculin	+	+	+	+	+	+

Symbols: +: positive, -: negative, v: variable

Table 6. Phenotypic characteristic of LAB isolated from soak water of tempeh production in Java.

Characteristics	Groups		
	1: <i>Pediococcus pentosaceus</i>	2: <i>Lactobacillus paracasei</i>	3: <i>Lactobacillus plantarum</i>
Number of isolates	21	15	29
Shape	Coccus	Rod	Rod
Gas from glucose	-	-	-
Lactic acid isomer	DL	L	DL
NH <sub>3</sub> production from arginine	-	-	-
Growth at pH value			
3.0	-	-	-
3.5	V	V	+
4.0	+	+	+
4.5	+	+	+
5.0	+	+	+
9.0	-	-	-
9.6	-	-	-
Growth in NaCl (w/v)			
2.0%	+	+	+
3.0%	+	+	+
4.0%	+	+	+
6.0%	-	+	+
6.5%	-	+	+
8.0%	-	V	V
10.0%	-	-	-
Growth at temperature (°C)			
10	V	+	+
15	+	+	+
45	+	V	V
50	-	-	-
<b>Sugar fermentation</b>			
<b>(API 50CH test)</b>			
N-acetylglucosamine	+	+	+
Amygdalin	+	+	+
D-cellobiose	+	+	+
Esculin	+	+	+
D-Fructose	+	+	+
D-Galactose	+	+	+
D-Glucose	+	V	+
Glycerol	+	+	+
D-Lactose	V	V	+
D-Maltose	-	+	+
D-Mannitol	-	V	+
D-Mannose	-	+	V
D-Melibiose	-	-	V
D-Raffinose	-	-	+
D-Ribose	-	+	+
Salicin	+	+	+
D-Sucrose	+	+	+
D-Trehalose	+	+	+

Symbols: +: positive, -: negative, v: variable

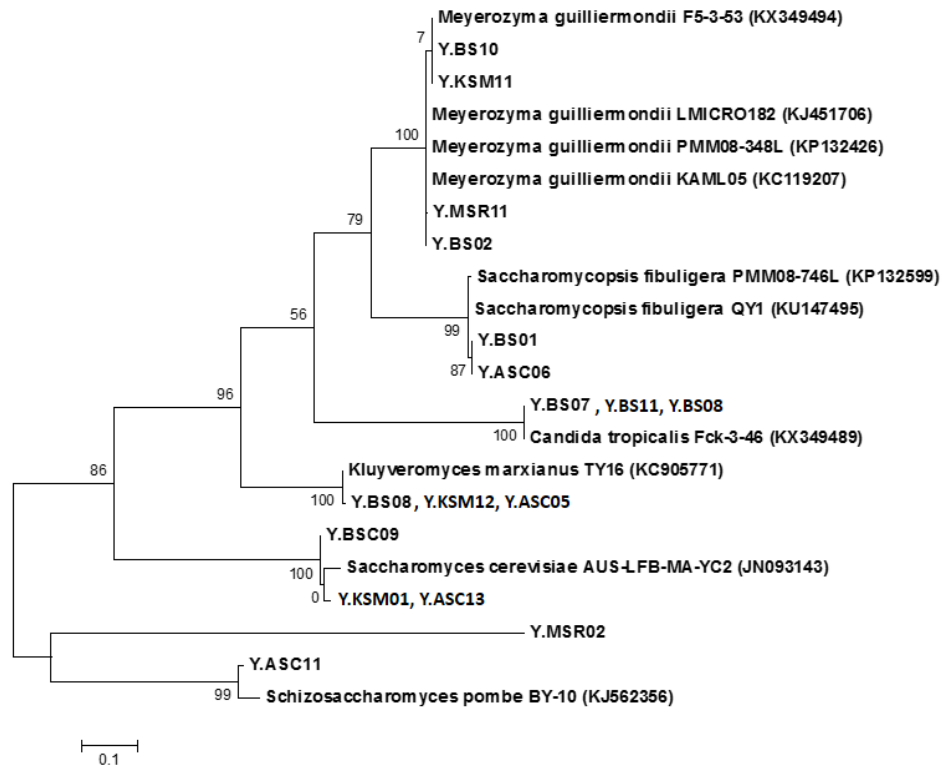


Figure 4. The tree was inferred from ITS sequence and constructed using maximum likelihood method (Yeast isolates). Numbers at nodes indicate levels of bootstrap support calculated from 1000 trees.

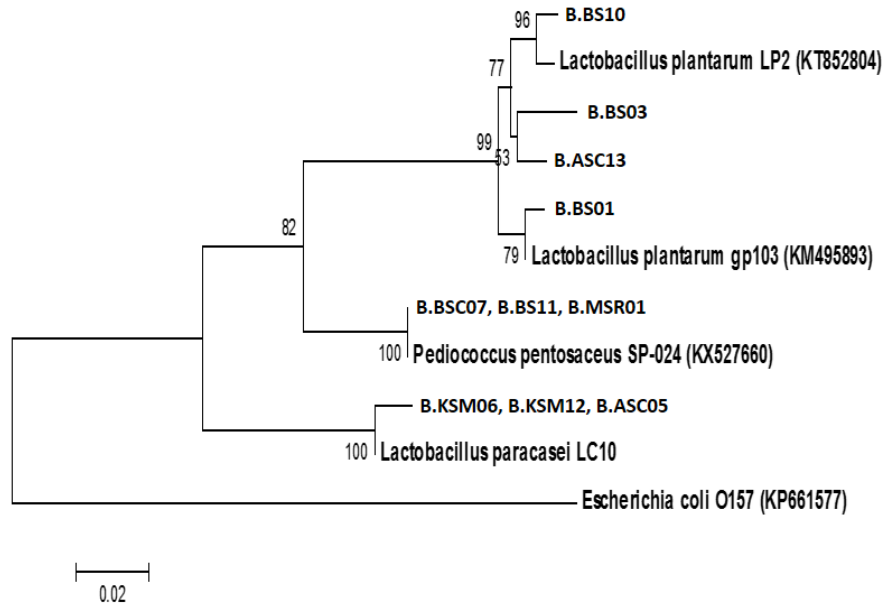


Figure 5. The tree was inferred from 16s RNA sequence and constructed using maximum likelihood method (LAB isolates). Numbers at nodes indicate levels of bootstrap support calculated from 1000 trees.

### 3.2. Discussion of Part I (Identification and characterization of tempeh yeast and LAB)

The manufacture process of tempe was comprised two main fermentation stages, including a fermentation dominated by a natural bacterial microbiota during the preparatory soaking of soybeans and a fungal solid state fermentation stage, usually with *Rhizopus microsporus* var. *oligosporus* (Han and Nout 2000; Nout and Kiers 2005). There are a complex of microorganisms in soybean tempeh, their development start during the soaking of the raw ingredients (Nout and Kiers, 2005). This unique method preserves beneficial microorganisms and that indigenous microbiota plays a major role in the beginning tempeh production process, that may effect to end quality of the product especially the flavor and taste. In this study, we determined the composition of LAB and yeast, we also described their phenotypic, morphological and genotypic characteristics present in soak water from tempeh manufactures in Java.

The number of LAB and yeasts differed among manufactures and increased during soaking time. Steinkraus (1996) proposed that LAB create an acidic environment conducive to yeast proliferation while the yeasts provide vitamins and other growth factors such as amino acids for the lactic acid bacteria. The simultaneous increase in numbers of both LAB and yeasts may therefore be attributed to their symbiotic association. LAB counts in soak water varied between 5.21 and 8.88 log CFU/mL whereas yeasts counts varied from 4.31 and 4.89 log CFU/mL whereas APC counts varied between 7.33 and 9.10 log CFU/mL. This result showed that the number of LAB is larger than yeast. Enterobacteriaceae was not detected in soak water with high LAB number. This seems dominant LAB could suppress the present of Enterobacteriaceae. The use of recycle soak water which applied by Factory D, E and F gave significant different microbial properties in their soak water, compared to Factory A, B and C (using only fresh soak water). Using previous soak water gave impact to higher number of LAB and avoid contamination Enterobacteriaceae. Coliforms are known to be acid intolerant (Steinkraus, 1996). Similar results have also been reported for other fermented products (Mbugua, 1984; Nche et al., 1994). Other antimicrobial substances produced by the dominating lactic acid bacteria (Helander et al., 1997) in addition to lactic acid may also contribute to inhibition of Enterobacteriaceae (Nche et al., 1994). Daily addition of previous soakwater (backslopping) is a powerful method of obtaining a vigorous lactic fermentation. This 'accelerated natural lactic fermentation' results from the gradual selection of ecologically adapted acidifying organisms.

Moreno et al. (2002) reported the growth of lactic acid bacteria during in tempeh production in Malaysia. Count of LAB, bacteria on raw beans were low ( $<10^2$  CFU/g). The number of LAB on the soaking water increased to 7.9-9.3 log CFU/mL at the end of the soaking process and 7.8-9.2 log CFU/g in the soaked soybean. Second boiling of the beans results in reductions of LAB populations to less than 4 log CFU/g. The number increased sharply to 6.8- 9.9 log CFU/g in final product (tempeh) (Moreno et al. 2002). Nuraida et al. (2008) and Efriwati et al. (2013) reported change in LAB number during tempeh preparation at a laboratory level. Nuraida et al. (2008) reported LAB population on soaked soybeans reached 9 log CFU/g. However, the number decreased to 6 log CFU/g on tempeh. Efriwati et al. (2013) reported two methods applied in producing LAB in tempeh preparation, namely one- and two-time boiling steps. At the end of



soaking process, number of LAB produced from these two methods reach 6 log CFU/g. When soybeans are boiled once (one time boiling step), LAB number increases to 8 log CFU/g, greater than the population results in two-time boiling steps i.e. 6.5 log CFU/g.

The species of LAB and yeast were identified according to phenotypic, and biochemical characterization. Identification into species level mainly on the basis of their sugar fermentation profiles in the API 50 CH test for LAB and API ID 32C test for yeast. One hundred yeast strains were isolated and identified as *Schizosaccharomyces pombe* (9 strains), *Saccharomycopsis fibuligera* (13 strains), *Kluyveromyces marxianus* (19 strains), *Candida tropicalis* (22 strains), *Saccharomyces cerevisiae* (22 strains), *Meyerozyma guilliermondi* (15 strains). Sixty-five LAB strains were isolated and identified into three big group species. They are *Lactobacillus plantarum* (29 strains), *L. paracasei* (15 strains) and *Pediococcus pentosaceus* (21 strains). We found *L. plantarum* as dominant LAB in the soak water, followed by *L. paracasei* and *P. pentosaceus*. They are recognized as heterofermentative LAB. *L. plantarum* has been shown to be the dominant organism at the end of several natural cereal fermentations (Olasupo et al., 1997) as for instance in maize-derived products like ogi (Steinkraus, 1996). *L. plantarum* has also been identified as the predominant species in most vegetable fermentations (Oyewole and Odunfa, 1990). The dominance of *L. plantarum* at the late stages of fermentation has been attributed to its high acid tolerance (Hounhouigan et al., 1993).

To confirm the identification result from API system then sequencing assignment based on DNA were applied, by only choosing representative 2-4 strains from each species group of yeast and LAB. In addition to the restriction patterns, some of the yeast groups were also characterized by RAPD-PCR analysis. DNA fingerprinting were also used to see which strains identical and non-identical from each species group. RAPD typing was capable of producing discriminating DNA fingerprints of yeast isolates and indicated there were genetic differences among them. RAPD-PCR analysis has been reported to be a useful method for the differentiation of the yeast strains belonging to the same species (Dlauchy et al., 1999). Molecular identification of the LAB isolates was achieved with the amplification partial sequence 16S rRNA genes using the bacterial universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG) (Lane, 1991) and bacterial primer 1100R (5'-AGGGTTGCGCTCGTTG) (Turner *et al.*, 1999). For selected yeast, a touch down program was used for the amplification of the internal transcribed spacer region with the primers pairs ITS 4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS 5 (5'-GGAAGTAAAAGTC GTAACAAGG-3') (White *et al.*, 1990). PCR amplification and DNA sequencing using the primer were successful, the blast of the sequenced regions gave conclusive results concerning the molecular taxonomy of the yeast and LAB. The sequencing results are similar with API.

In conclusion, tempe is not the product of a pure culture fermentation with *R. microsporus* var. *oligosporus*, bacterial and yeast species are also essential components of the fermentation. The acidification of soak water was reported to be an important process step enabling the control of the growth of food-borne pathogens and ensuring the microbiological safety of the final tempe. In this study, culturable yeast and LAB isolates have been successfully identified from soak water in tempeh production from six different manufactures.

### 3.3. Results of Part II (Antifungal activity from tempeh yeast and LAB)

#### Screening and characterization of antifungal activity from tempeh yeast and LAB isolates

Lactic acid bacteria (LAB) and yeast have been exploited for decades for their antibacterial activity. More recently, they have also received scientific attention because of their antifungal potential (Magnusson et al., 2003; Dal Bello et al., 2007). In this study, we searched LAB or yeast isolates that have antifungal activity against harmful fungus *F. proliferatum* that found as contaminant in traditional tempeh.

In a first screening, out of 165 isolates tempeh yeast and LAB from natural acidification process (tempeh industry) were screened with 96 plates method using their CFS extract (cell-free supernatant). About 12% of total isolates showed antifungal activity against *F. proliferatum*. Of these, 20 LAB strains have consistent antifungal activity. No antifungal activity was observed in all yeast isolates. Both the mycelium growth and sporulation mostly were inhibited by active CFS LAB (Figure 4).

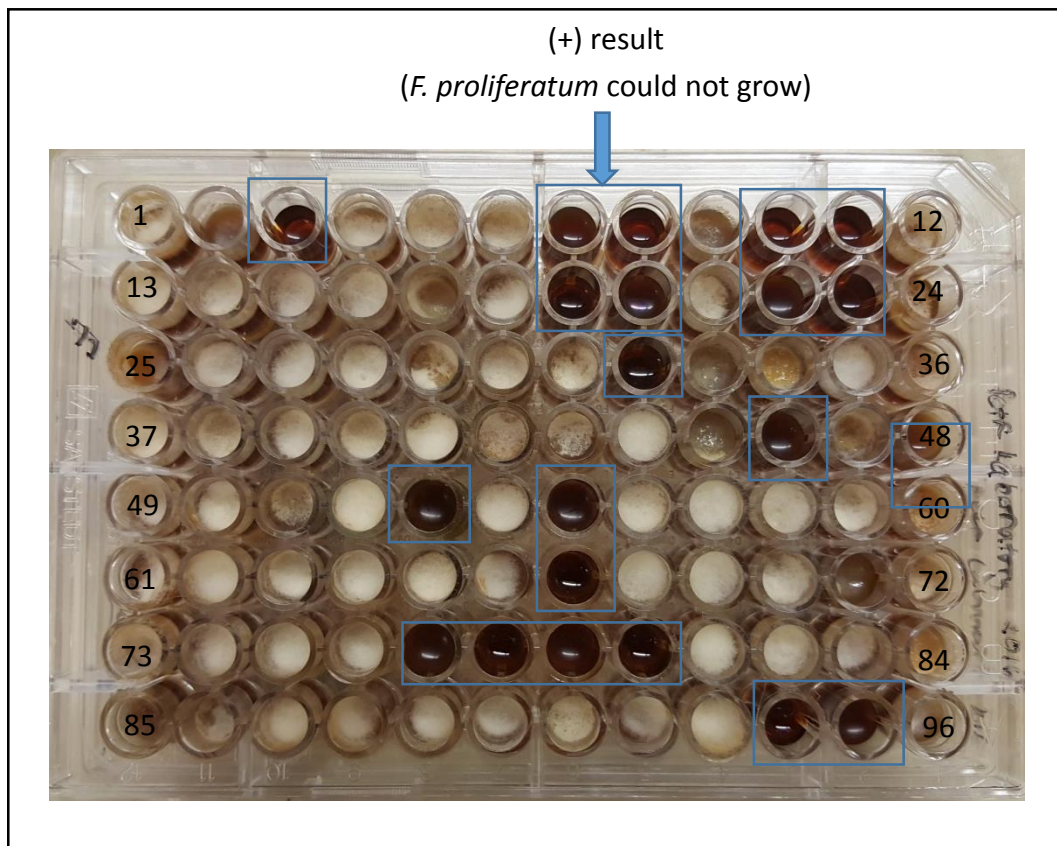


Figure 4. Screening of antifungal activity from tempeh yeast and LAB against *F. proliferatum* using their CFS (cell free supernatant) in 5 days incubation. (First 96 isolates screening)

Positive screening results were checked again with well test method and biomass reduction test. The result confirmed that there is variation in inhibition of *F. proliferatum* (Figure 5 & 6, Table 7). In brief, three best strains were obtained, they are *Lactobacillus plantarum* B.BS08, *L. plantarum* B.BS10 and *L. paracasei* B.KSM06 with strong inhibitory activity and great reduction in fungal biomass production.

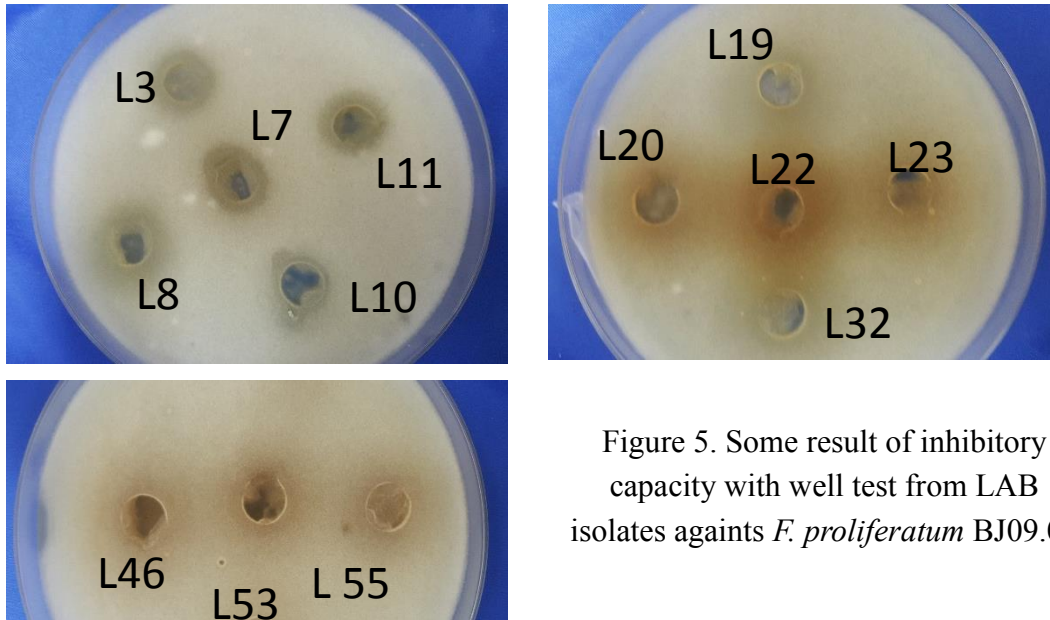


Figure 5. Some result of inhibitory capacity with well test from LAB isolates against *F. proliferatum* BJ09.05

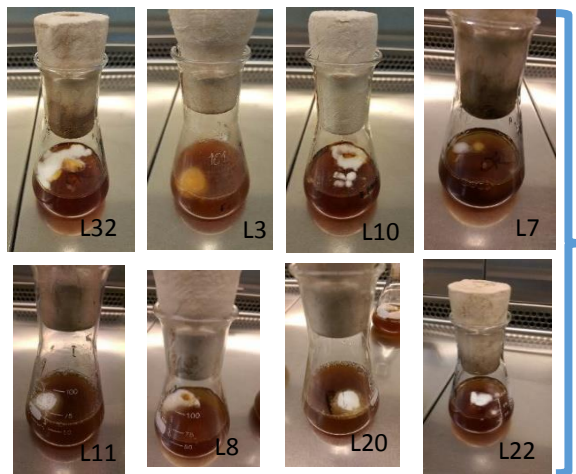
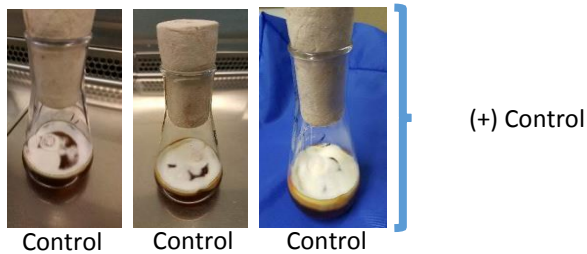


Figure 6. Some result of biomass reduction of *Fusarium proliferatum* BJ09.05 using selected CFS from tempeh LAB isolates (14 days incubation).

Table 7. Inhibiton and biomass reduction of *Fusarium proliferatum* by selected LAB isolates.

Selected LAB species	Strains	Inhibition againts		% Reduction of <i>Fusarium</i> biomass (in range)
		<i>F. proliferatum</i> BJ09.05	<i>F. proliferatum</i> KU07.03	
<i>L. plantarum</i>	B.BS01, B.BS02, B.BS03, B.ASC13, B.KSM05, B.MSR11	++	++	(50-60%)
<i>L. plantarum</i>	B.BS08, B.BS10	+++	+++	(75-87%)
<i>L. paracasei</i>	B.MSR04, B.KSM03, B.BS13, B. KSM07, B.BS12, B. KSM02	++	++	(45-60%)
<i>L. paracasei</i>	B.KSM06, B.ASC05, B.KSM12	+++	++	(65-73%)
<i>P. pentosaceus</i>	B.MSR01, B.BS11, B.BSC07	++	++	(44-58%)

Note:  
The inhibitory capacity was scored as follows: -, no inhibiton; +, inhibiton halo up to 8 mm; ++, halo between 9 and 15 mm; +++, halo larger than 15 mm.

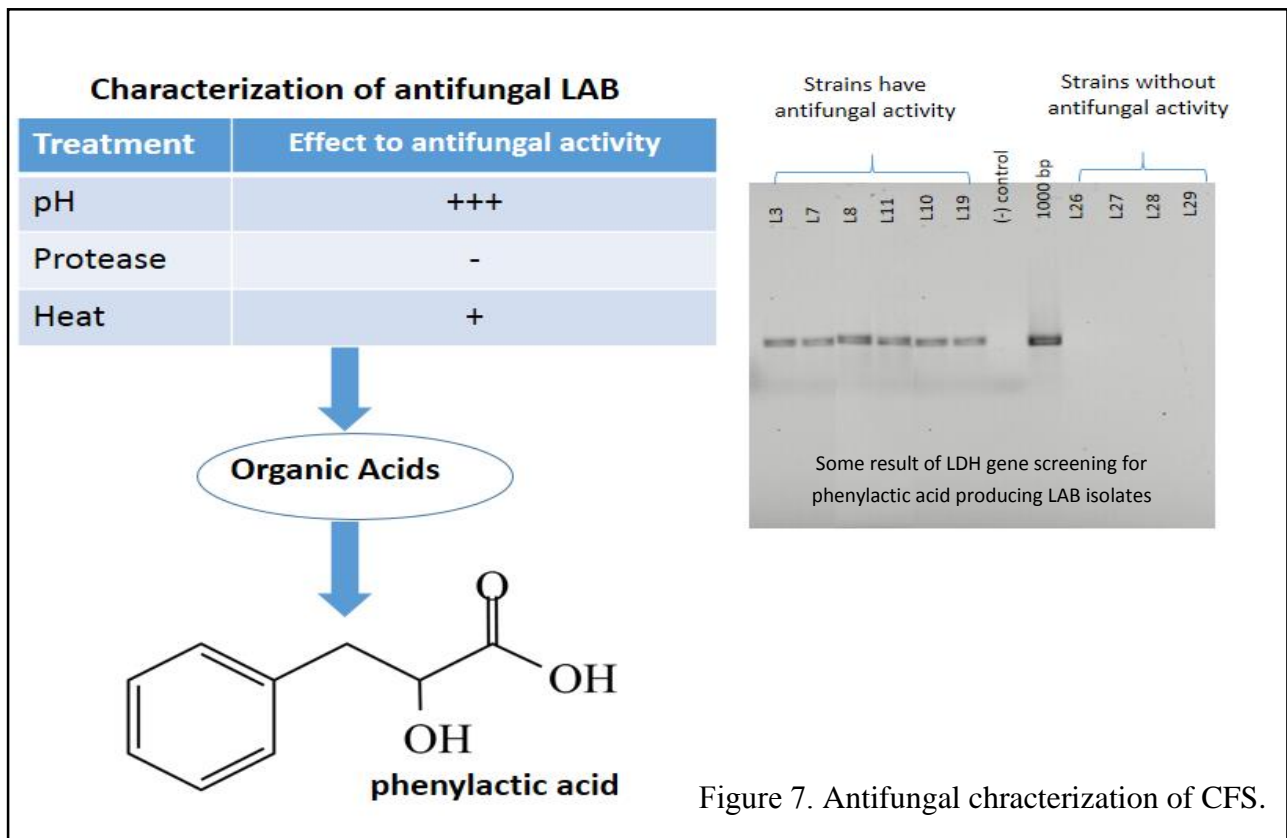


Figure 7. Antifungal chracterization of CFS.

Following heat treatment, no reduction in the zone of inhibition against *F. proliferatum* was observed compared to untreated control showing that the antifungal CFSs are heat resistant and thus, non-volatile. Proteolytic enzymes presented no reduction in zone size compared to an untreated control. In contrast pH (neutralized pH) gave significant effect to the activity. This suggests that organic acid may play a role in the antifungal activity (Figure 7). In this study we also found that active LAB isolates possessed LDH gene, indicating they are phenylacetic acid (PLA) producer. In contrast, all non-active LAB isolates lacked this gene. We found an interesting finding that only active-LAB (with antifungal activity) have the ability to produce PLA. This suggests that PLA might be the major antifungal compound. The active-LAB isolates produced PLA in the range 45-221 µg/g based on LC-MS/MS analysis.

Beside CFS effect, we also studied the interaction of LAB and *F. proliferatum*. The study on the linear growth of 3-days-old fungal mycelia in the paired culture with 3-days-old lactic acid bacteria in PDA medium is shown in Figure 9. The lowest increase of fungal biomass was shown at the presence of *Lactobacillus plantarum* B.BS08. The activity of the bacteria against the fungus *F. proliferatum* was dependent on their growth phase. The investigated LAB in paired culture showed the capacity for fungal mycelial growth inhibition in liquid MRS and solid PDA media. Their antagonistic interaction was also observed with SEM (as shown in Figure 8). From the scanning electron micrographs, we found some changes in *F. proliferatum* morphology, some damage was also visible (holes in hyphae structures). The LAB secreted something that may damage spores and hyphae. The LAB also sticks into hyphae and might inject them into the fungal cell.

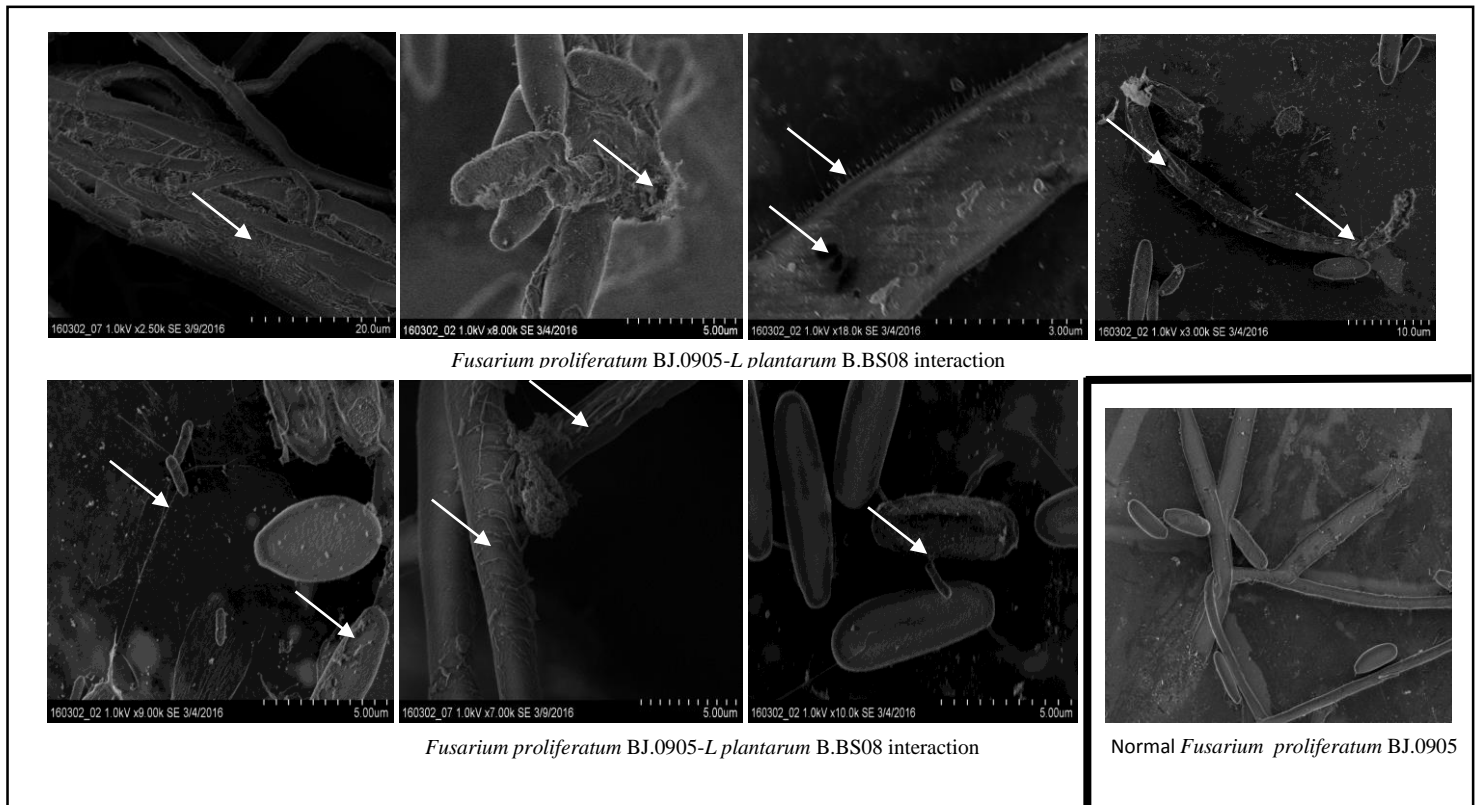


Figure 8. Antagonistic interaction between *Fusarium proliferatum* BJ.09.05 and *Lactobacillus plantarum* B.BS08 under SEM observation.



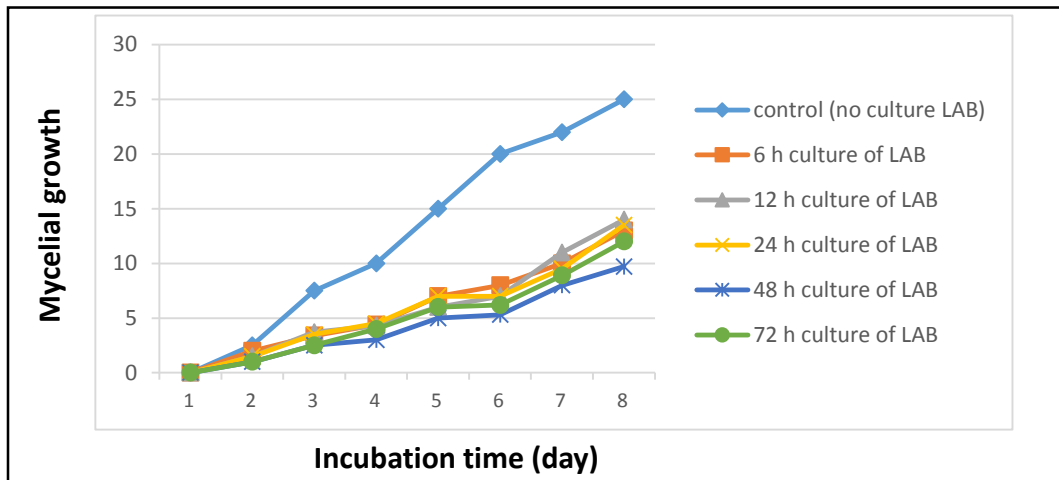


Figure 9. Mycelial growth of *F. proliferatum* BJ09.05 on PDA in mixed culture with *L. plantarum* B.BS08.

### Application of antifungal LAB in tempeh model

The three best isolates obtained then tested again in tempeh-making (laboratory level). The qPCR result showed that three best isolates from *L. plantarum* could inhibit more than 70% growth of *F. proliferatum*. *Lactobacillus plantarum* B.BS10 was found as best *F. proliferatum* inhibitor that can be applied to the tempeh production (Figure 10). The result also showed that *R. microsporus* var. oligosporus could still grow well together with LAB isolates (Figure 11). However, this LAB application made pH of tempeh become lower (pH in range 5.1-5.5) than normal tempeh. This additional LAB culture into fermentation might change sensory characteristic of tempeh. Further research is necessary to cope this application problem.

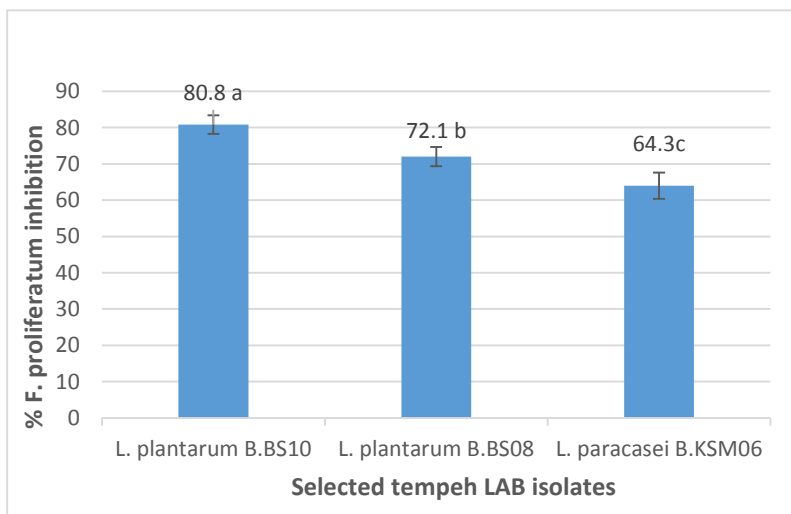


Figure 10. Graphic of *Fusarium proliferatum* BJ09.05 growth inhibition by selected LAB tempeh isolates. Markers carrying the same letter represent mean  $\pm$  standard deviation statistically not different  $P > 0.05$ ;  $n = 3$ .

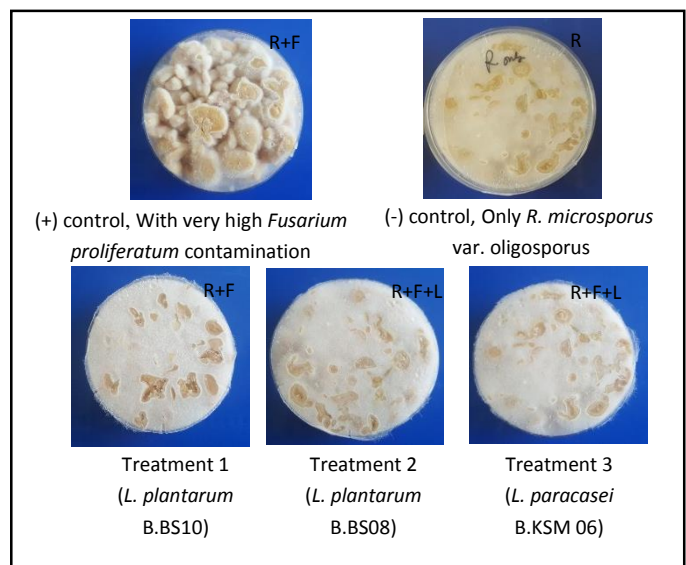


Figure 11. Application of selected three LAB isolates to inhibit *F. proliferatum* contamination during tempeh making.

### 3.4. Discussion of Part II (Antifungal activity from tempeh yeast and LAB)

The trend for using natural-label friendly preservatives has drastically increased over the last years and has been replacing traditional preservation system. Biopreservation is an interesting alternative approach to physical and chemical preservation methods commonly applied in food. Biopreservation refers to the use of natural microflora or controlled microbiota or antibacterial products to extend shelf life and increase food safety (Stiles, 1996). Among natural biological antagonist, lactic acid bacteria (LAB) and yeast have potential applications as bio-preservative. LAB can play a vital role as preservatives since they have a long history in fermented food production, are generally recognized as safe (GRAS status), and they have a Qualified Presumption of Safety (QPS) status in the EU, consequently the scientific exploration of their potential as biocontrol agents have received a consistent and growing interest. These microorganisms occur naturally in many food systems and are also part of the intestinal microflora. In this study, we searched LAB or yeast isolates that have antifungal activity against harmful fungus *F. proliferatum* that found as contaminant in traditional tempeh.

In a first screening, out of 294 isolates tempeh yeast and LAB from natural acidification process (tempeh industry) were screened with 96 plates method using their CFS extract (cell-free supernatant). Very few (12 %) of them possess antifungal activity against *F. proliferatum*. Of these, 20 LAB strains have consistent antifungal activity. No antifungal activity was observed in yeast isolates. Both the mycelium growth and sporulation mostly were inhibited by active CFS LAB. Positive screening results were checked again with well test method and biomass reduction test. The result confirmed that there is variation in inhibition of *F. proliferatum*. In brief, three best strains were obtained, they are *Lactobacillus plantarum* B.BS08, *L. plantarum* B.BS10 and *L. paracasei* B.KSM06 with strong inhibitory activity and great reduction in fungal biomass production. Inhibitory activity of *L. plantarum* against antagonistic fungal species is in agreement with previous studies (Niku-Paavola et al. 1999; Lavermicocca et al. 2000; Magnusson et al.2003).

Beside CFS effect, we also studied the interaction of LAB and *F. proliferatum*. The study on the linear growth of 3-days-old fungal mycelia in the paired culture with 3-days-old lactic acid bacteria in PDA medium. The lowest increase of fungal biomass was shown at the presence of *Lactobacillus plantarum* B.BS08. The activity of the LAB against the fungus *F. proliferatum* was dependent on their growth phase. The investigated LAB in paired culture showed the capacity for fungal mycelial growth inhibition in liquid MRS and solid PDA media. Their antagonistic interaction was also observed with SEM. From the scanning electron micrographs, we found some changes in *F. proliferatum* morphology, some damage was also visible (holes in hyphae structures). The LAB secreted something that may damage spores and hyphae. The LAB also stick into hyphae and might inject secreted compounds into the fungal cell. Our results also indicate that *L. plantarum* B.BS08 and its secreted antifungal metabolites changes the structural integrity of fumonisin producing *F. proliferatum* BJ09.05 and might interfere with fungal cell development.

Sensibility of inhibitory CFS extract was also observed. Heat treatment showed that no reduction in the zone of inhibition against *F. proliferatum* compared to untreated control showing that the antifungal CFSs are heat resistant and thus, non-volatile. Proteolytic enzymes presented

no reduction in zone size compared to an untreated control. This indicates that they are not proteinaceous substances. In contrast pH (neutralized pH) gave significant effect to the activity. This suggests that organic acid may play main role in the antifungal activity. In this study we also found that active LAB isolates possessed LDH gene, indicating they are phenylactic acid (PLA) producer. In contrast, all non-active LAB isolates lacked this gene. We found interesting finding that only active-LAB (with antifungal activity) have ability to produce PLA. This suggests that PLA might be the major antifungal compound. The active-LAB isolates produced PLA in range 45-221 µg/g based on LC-MS/MS analysis. However, purification of the compound continued with a single compound test for antifungal activity is still necessary to confirm whether PLA as main antifungal agent or not.

The role PLA and other organic acid as antifungal substances also observed by several authors. PLA mostly produced by *L. plantarum* with a broad spectrum antifungal activity (Lavermicocca et al., 2000, Magnusson and Schnürer 2001; Ström et al., 2002). Some other LABs can synthesize PLA and 4-hydroxy-acid phenyllactic (Valerio et al., 2004). 3-PLA is derived from the metabolism of phenylalanine. As follows, phenylalanine is transaminated to phenylpyruvic acid (PPA) (Li et al., 2007) and then PPA is converted to 3-phenyllactic acid (Mu et al., 2009). Supernatant of *L. plantarum* 21B inoculated with wheat starch hydrolysates reliably prevents the growth of several toxigenic fungi, namely *A. niger*, *A. flavus*, *Monilia sitophila*, *F. graminearum*, *P. roqueforti*, and *P. expansum*, (Lavermicocca et al., 2000; Lavermicocca et al., 2003). Broberg et al. (2007) reported that *Pichia anomala* and *P. roqueforti* are inhibited by *L. plantarum* strain MiLAB14 and *L. coryniformis* MiLAB 393 through their antifungal compounds, 3-PLA. Lavermicocca et al. (2000) reported that PLA was contained at the highest concentration in the bacterial culture filtrate and had the highest activity and inhibited all the tested fungi at a concentration of 50 mg except for *P. roqueforti* IBT 18.687 and *P. corylophilum* IBT 6978 (166 mg/mL). PLA also inhibits the growth of *Candida pulcherrima*, *C. parapsilosis* and *Rhodotorula mucilaginosa* (Schwenninger et al., 2008). Prema et al. (2010) identify PLA in the culture supernatant of *L. plantarum* using NMR and IR spectroscopy, showing inhibitory effects against *A. fumigatus*. Ndagano et al. (2011) found PLA mixed with other organic acids and free fatty acids in the supernatant of *L. plantarum* VE56 and *W. paramesenteroides* LC11 inhibiting fungal growth. Antifungal compounds from culture supernatant of *L. plantarum* 1.7 FST was isolated by Dal Bello et al. (2007), they were identified as cyclo (L-Leu-L-Pro), cyclo (L-Phe-L-Pro), and PLA. The main constituent of antifungal compounds were PLA and lactic acid. Gerez et al. (2010) revealed that PLA is the most effective organic acid.

The three best isolates obtained then applied in tempeh-making (laboratory level). The qPCR result showed that three best isolates from *L. plantarum* could inhibit more than 60% growth of *F. proliferatum*. *Lactobacillus plantarum* B.BS10 was found as best *F. proliferatum* inhibitor that can be applied to the tempeh production. The result also showed that *R. microsporus* var. oligosporus could still grow well together with LAB isolates. However, this LAB application made pH of tempeh become lower (pH in range 5.1-5.5) than normal tempeh (6.5-6.7). This additional LAB culture into fermentation might impart undesirable sensory characteristic of tempeh. Further research is necessary to cope this application problem before applied into large-scale production systems in traditional tempeh industry.



### 3.5. Results of Part III (Zearalenone biotransformation by tempeh yeast and LAB)

In the present study, a total of 20 strains of yeast and lactic acid bacteria (LAB) species mostly from Part I that commonly found in natural acidification process (part of tempeh production) were screened for zearalenone (ZEN) metabolite formation *in vitro* (list of strains could be seen in supplemental Table 3). Twelve isolates yeast (seven different species) were used; they are *Saccharomycopsis fibuligera*, *Pichia kudriavzevii*, *Candida tropicalis*, *Kluyveromyces marxianus*, *Schizosaccharomyces pombe*, *Meyerozyma guilliermondi*, and *Saccharomyces cerevisiae*. Eight isolates LAB (three different species) were used, they are *Lactobacillus plantarum*, *Pediococcus pentosaceus*, and *Lactobacillus paracasei*. Isolates mentioned were investigated whether they can reduce ZEN and which metabolites they might produce preferentially.

ZEN ( $c = 0.3 \mu\text{M}$ ) was added to liquid cultures of the fungi. After two days of incubation, the media were analyzed for metabolite formation by HPLC-MS/MS. Semi-quantitative measurements were conducted for ZEN-14-S,  $\alpha$ -ZEL-S, ZEN-14-Glc, and ZEN-16-Glc. ZEN- 14- S and  $\alpha$ -ZEL-S were determined using relative response factors of ZEN-14-S to ZEN and  $\alpha$ -ZELS to  $\alpha$ -ZEL. Response factors were estimated by comparing the MS/MS peak area before and after quantitative sulfate ester cleavage. ZEN and  $\alpha$ -ZEL were determined by external calibration using the commercially available standard substances. Matrix-matched calibration was applied for semi-quantification of ZEN-14-Glc and ZEN-16-Glc. Detail semi-quantification data can be seen in Table 8.

The results obtained from screening 20 isolates belonging to 10 different species revealed that nearly all the tested organisms were able to reduce ZEN in range 10 to 96.9% (Figure 12). In general, yeast and LAB isolates could only transform ZEN into two major transformation product  $\alpha$ -ZEL and  $\beta$ -ZEL. In all cases no conjugation product detected. Yeast isolates were more active than LAB isolates based on ZEN reduction and transformation point of view. Yeast isolates transformed ZEN primarily to the  $\beta$ -isomer, whereas  $\alpha$ -ZEL being the predominating product from LAB isolates. From yeast isolates, we found *C. tropicalis*, *M. guilliermondi*, and *Saccharomyces cerevisiae* are potential species to be used as biotransformation agent, because they could reduce significant amount of ZEN (>70% reduction) and mostly transformed to  $\beta$ -ZEL (known less estrogenic). In LAB isolates, we found *L. plantarum* B.BS01 is most potential isolate because could reduce more than 90% ZEN, almost without  $\alpha$ -ZEL formed. An example of the chromatograms obtained for potential isolates is also depicted in Figure 4. The *in vitro*-screening showed that complex microorganism involved in tempeh production have potential for ZEN reduction. However to know are they agent of biotransformation, further toxicity test with animal needed to confirm it.

## Chapter 6: Yeast and LAB from tempeh production

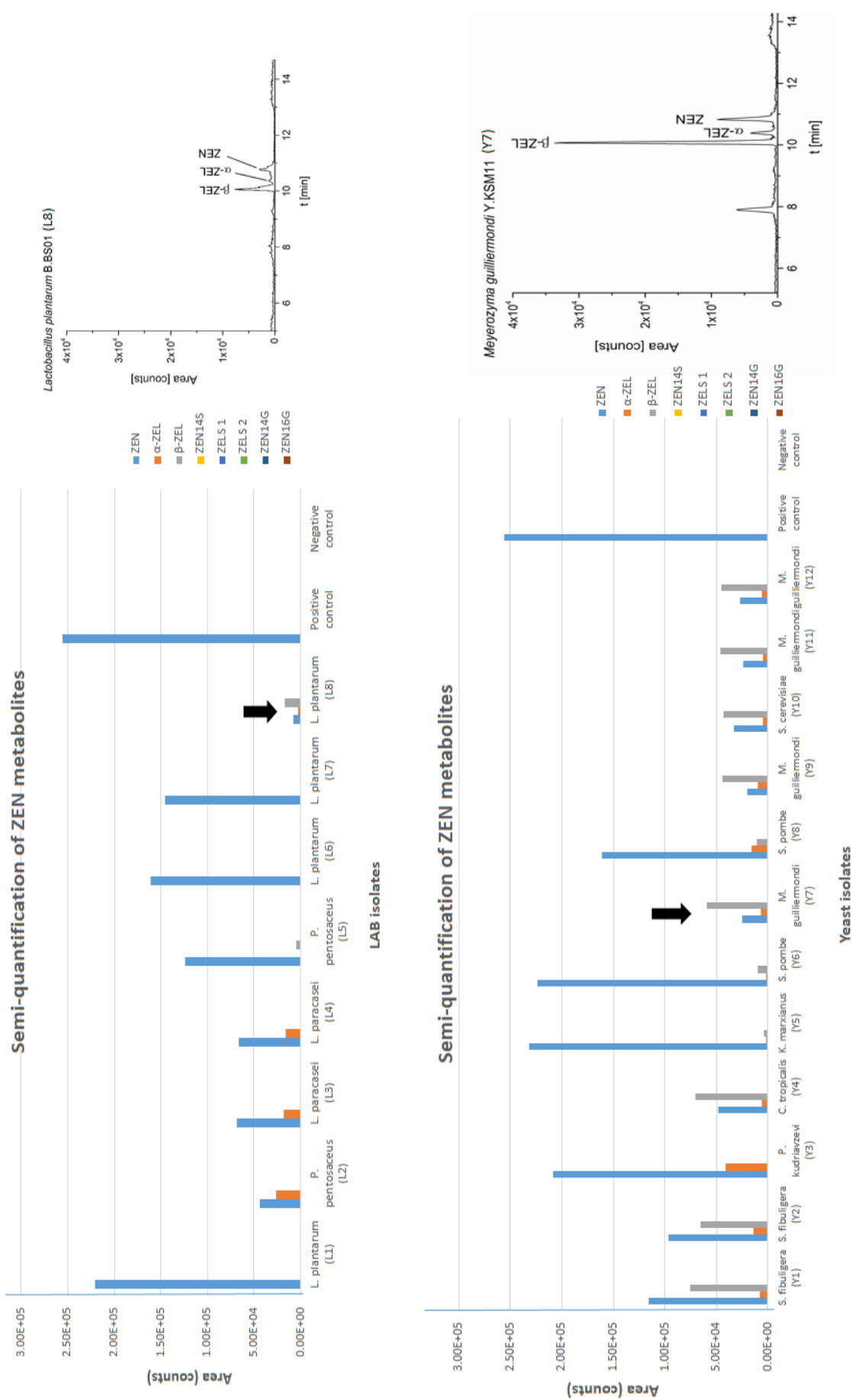


Figure 12. Graph of semi-quantification of ZEN metabolites (Yeast and LAB isolates *in vitro* test)

Table 8. HPLC-MS/MS result of ZEN transformation by tempeh yeast and LAB.

		Masked ZEN						
		Area (count) after adjusted with response						
	ZEN	$\alpha$ -ZEL	$\beta$ -ZEL	ZEN14S	ZELS 1	ZELS 2	ZEN14G	ZEN16G
Y1	1.15E+05	8.48E+03	7.54E+04	0	0	0	0	0
Y2	9.67E+04	1.38E+04	6.59E+04	0	0	0	0	0
Y3	2.08E+05	4.07E+04	0	0	0	0	0	0
Y4	4.79E+04	6.25E+03	7.01E+04	0	0	0	0	0
Y5	2.32E+05	1.87E+03	3.64E+03	0	0	0	0	0
Y6	2.24E+05	1.68E+03	1.00E+04	0	0	0	0	0
Y7	2.55E+04	6.82E+03	5.93E+04	0	0	0	0	0
Y8	1.61E+05	1.62E+04	1.15E+04	0	0	0	0	0
Y9	2.04E+04	1.04E+04	4.39E+04	0	0	0	0	0
Y10	3.36E+04	5.34E+03	4.33E+04	0	0	0	0	0
Y11	2.36E+04	5.33E+03	4.62E+04	0	0	0	0	0
Y12	2.68E+04	5.52E+03	4.51E+04	0	0	0	0	0
L1	2.21E+05	0	0	0	0	0	0	0
L2	4.38E+04	2.66E+04	0	0	0	0	0	0
L3	6.81E+04	1.76E+04	0	0	0	0	0	0
L4	6.60E+04	1.58E+04	0	0	0	0	0	0
L5	1.24E+05	0	0	0	0	0	0	0
L6	1.61E+05	0	0	0	0	0	0	0
L7	1.45E+05	0	0	0	0	0	0	0
L8	7.94E+03	2.24E+03	1.67E+04	0	0	0	0	0
Positive control	2.56E+05	0	0	0	0	0	0	0
Negative control	0	0	0	0	0	0	0	0

### 3.6. Discussion of Part III (Zearalenone biotransformation by tempeh yeast and LAB)

Specific strains of lactic acid bacteria (LAB) and yeast may be able to remove mutagenic contaminants including mycotoxins in foods (Haskard et al., 2001; Peltonen et al., 2001; Shetty et al., 2007). LAB are a group of related bacteria (e.g., *Streptococcus* spp., *Lactobacillus* spp., *Lactococcus* spp., and *Leuconostoc* spp.) that produce lactic acid as a result of carbohydrate fermentation. LAB and yeast occur naturally in foods and are considered either harmless or possibly supportive of human health. However, when added to foods, these microorganisms are termed “starter culture.”

In tempeh processing, complex microorganism consist of bacteria and yeast are important for soybeans acidification and tendering. However, it is not known whether bacteria are important in other processes during tempeh production, including about their role in food safety of this product. The soak water is rich in simple sugars such as glucose, fructose, and galactose. Glucose serves as primary substrate for microbial growth in soak water. The activity of the invertase enzyme and  $\alpha$ -galactosidase enzymes on soybean produce simple sugars in the soak water. Bacteria and yeasts are microbes found in that soak water (Mulyowidarso et al., 1991). In the present study, a total of 20 strains of yeast and lactic acid bacteria (LAB) species used from Part I, that commonly found in natural acidification process (part of tempeh production) were screened for zearalenone (ZEN) metabolite formation *in vitro*. Twelve isolates yeast (seven different species) were used; they are *Saccharomycopsis fibuligera*, *Pichia kudriavzevii*, *Candida tropicalis*, *Kluyveromyces marxianus*, *Schizosaccharomyces pombe*, *Meyerozyma guilliermondi*, and *Saccharomyces cerevisiae*. Eight isolates LAB (three different species) were used, they are *Lactobacillus plantarum*, *Pediococcus pentosaceus*, and *Lactobacillus paracasei*. Isolates mentioned were investigated whether they can reduce ZEN and which metabolites they might produce preferentially.

ZEN ( $c = 0.3 \mu\text{M}$ ) was added to liquid cultures of the fungi. After two days of incubation, the media were analyzed for metabolite formation by HPLC-MS/MS. Semi-quantitative measurements were conducted for ZEN-14-S,  $\alpha$ -ZEL-S, ZEN-14-Glc, and ZEN-16-Glc. ZEN-14-S and  $\alpha$ -ZEL-S were determined using relative response factors of ZEN-14-S to ZEN and  $\alpha$ -ZELS to  $\alpha$ -ZEL. Response factors were estimated by comparing the MS/MS peak area before and after quantitative sulfate ester cleavage. ZEN and  $\alpha$ -ZEL were determined by external calibration using the commercially available standard substances. Matrix-matched calibration was applied for semi-quantification of ZEN-14-Glc and ZEN-16-Glc.

The results obtained from screening 20 isolates belonging to 10 different species revealed that nearly all the tested organisms were able to reduce ZEN in range 10 to 96.9%. In general, yeast and LAB isolates could only transform ZEN into two major transformation product  $\alpha$ -ZEL and  $\beta$ -ZEL. In all cases no conjugation product detected. Yeast isolates were more active than LAB isolates based on ZEN reduction and transformation point of view. Yeast isolates transformed ZEN primarily to the  $\beta$ -isomer, whereas  $\alpha$ -ZEL being the predominating product from LAB isolates. From yeast isolates, we found *C. tropicalis*, *M. guilliermondi*, and *Saccharomyces cerevisiae* are potential species to be used as biotransformation agent because they could reduce

significant amount of ZEN (>70% reduction) and mostly transformed to  $\beta$ -ZEL (known less estrogenic). According to Shier et al. (2001), *in vitro* breast cancer cell stimulation experiments indicate that the  $\alpha$ -ZEL exhibits 92-fold higher estrogenicity, compared to ZEN. On the other hand,  $\beta$ -ZEL is less estrogenic, which only exhibits half of the ZEN activity. In LAB isolates, we found *L. plantarum* B.BS01 is most potential isolate because could reduce more than 90% ZEN, almost without  $\alpha$ -ZEL formed. An example of the chromatograms obtained for potential isolates is also depicted in Figure 4. The *in vitro*-screening showed that complex microorganism involved in tempeh-making process have potential for ZEN decontamination. However to know are they really agent of biotransformation, further toxicity test with animal needed to confirm it.

The novel result of this study is not about tempeh LAB and yeast activity to reduce ZEN amount but their capability to transform ZEN by reducing the keto group into two major transformation product  $\alpha$ - and  $\beta$ -ZEL. More important information is none of isolates could transform ZEN into ZEN conjugates, thus this microorganism will not contribute in producing masked-ZEN. Most of research in this area so far only highlight role of LAB and yeast as adsorbent (binding) agent of mycotoxins. El-Nezami et al. (2004) only demonstrated that, after co-incubation of ZEN and *Lactobacillus*, a considerable proportion (38.0%–46.0%) of these mycotoxins were recovered from bacterial pellets. Bacteria showed capacity to adsorb toxins and, as expected, results demonstrated that the binding depended on bacterial concentration. Co-incubation of ZEN and -ZEL with bacteria significantly affected the percentage of toxin bound, suggesting that these toxins may share the same binding site. Tinyiro et al. (2011) using some *Bacillus* strains, showed that the adsorption percentage was high (78.0% and 95.0%) and variation depended on the strain used. Haskard et al. (2001) reported reversible binding of aflatoxins to probiotic bacteria. Other authors reported partially reversible AFB1 binding with *L. casei*, *L. rhamnosus* and *L. amylovorus* (Hernandez-Mendoza et al., 2009; Peltonen et al., 2001). Pure yeast as well as yeast cell wall products is also known could adsorb ZEN (Yiannikouris et al., 2004; Fruhauf et al., 2012).

In biotechnology, yeast and LAB is used to catalyze the asymmetric reductive transformation of a variety of substrates containing a carbonyl group or a carbon-carbon double bond (Utaka et al., 1991). The advantages using microorganisms instead of classical synthesis are mainly the relatively broad substrate specificity, the regiospecific and stereospecific reactions, and the high purity of the products. A number of the strains investigated here might be considered for biotechnological applications. The potent  $\beta$ -isomer producing strains *Saccharomyces fibuligera* Y.BS01 or the potent  $\alpha$ -isomer-producing *P. pentosaceus* B.BSC07 may be particularly suitable for biotechnological syntheses.

In conclusion, LAB and yeast fermentation is a recommended method for reducing ZEN in contaminated raw material such as corn that usually used also in tempeh production, although safety of decontaminated food is not guaranteed because mode of action may not only involve transformation process but also reversible binding. We are planning to establish the mode of ZEN reduction in natural fermentation in our future studies.

## 4. Conclusion

The present study revealed the following points:

- The LAB counts in these samples varied between 5.21 and 8.88 log CFU/mL whereas yeasts counts varied from 4.31 and 4.89 log CFU/mL, APC counts varied between 7.33 and 9.10 log CFU/mL. In total, 165 isolates were obtained from these samples using selective media. Our results revealed a certain variety of culturable LAB and yeast in soak water. The principal yeast found to be responsible for fermentation were *Candida tropicalis*, *Kluyveromyces marxianus*, and *Saccharomyces cerevisiae*, while the dominant LAB were *Lactobacillus plantarum*, *L. paracasei* and *Pediococcus pentosaceus*. We found LAB species were less diverse than yeast species.
- Cell free supernatants (CFS) of 20 LAB strains possessed antifungal properties against *F. proliferatum* BSM17, while none of the yeast isolates showed the activity. It was found that phenyl lactic acid (PLA) might has main role for the activity. The use of those selected LAB strains as part of starter culture has been proven to positively increase tempeh safety. However, the additional LAB culture into fermentation might impart undesirable sensory characteristic of tempeh. Further research is necessary to cope this application problem before applied into large-scale production systems in traditional tempeh industry.
- All the tested yeast and LAB isolates were able to reduce ZEN in range 10 to 96.9%. They could only transform ZEN into two major transformation product  $\alpha$ -ZEL and  $\beta$ -ZEL. In all cases no conjugation product detected. LAB and yeast fermentation is a recommended method for reducing ZEN in contaminated raw material such as corn that usually used also in tempeh production, although safety of decontaminated food is not guaranteed because mode of action may not only involve transformation process but also reversible binding. Further research to establish the mode of ZEN reduction in natural fermentation is needed.

## 5. Acknowledgements

I acknowledge Dr. Volker Cordes and his team (Max Planck Institute for Biophysical Chemistry) for helpful advice on using scanning electron microscope.

This research was supported financially by Indonesia Endowment Fund for Education, Ministry of Finance, The Republic of Indonesia (Ref:1064/LPDP/2013).

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## General Discussion

Food is fundamental importance to life and the most essential for maintaining continued survival (De Vries et al., 1997). There is no sense in talking about food nutritional value, sensory or functional properties if the food product is not safe enough for consumption. Indonesia suffers a double burden of food safety problem. The first, related to fundamental issues of food safety; about the insufficient practice of Good Manufacturing Practices (GMP) principles that may lead to food poisoning for the consumer. The second, related explicitly to export-oriented food industries; Indonesia must face a variety of new food safety. One of the food product that needs attention for its safety is tempeh, a commonly consumed dish in Indonesia.

Tempeh is produced mostly on a small scale home industry with poorly controlled fermentation that carried out without sufficient hygienic precautions. Various types of microorganisms can participate during the process of fermentation, natural contamination is always possible (Winarno, 1985; Barus et al., 2008; Seumahu et al., 2013). Moreover, there is no standard on making tempeh and its starter. This causes many variations in the manufacture of tempeh in many areas and lead to inconsistencies in quality and safety of tempeh (Astuti et al., 2000). Tempeh, as important food is massively produced and consumed by Indonesian people. As a new export commodity, it really needs standardized starter and controlled processing to fulfill safety aspects.

The scope of this study was to address questions about microbiological and food safety in tempeh production in Indonesia. Researchs were divided into three main topics. **Topic 1 (Chapter 3)** dealt with the exploration of mucoralean fungi involved during tempeh production as well as solid fermentation process. *Zygomycetes* strains were isolated from Indonesian tempeh and starter and screened toxicologically and clinically to evaluate possible health risks for human consumption. In this topic, we also studied the role of tempeh fungi on zearalenone (ZEN) biotransformation. **In Topic 2 (Chapter 4)**, we studied the toxigenic fungal contamination of *usar* (traditional tempeh inoculum). We characterized the contaminant strains and detect mycotoxins they produced. Mycotoxins contamination in traditional tempeh was also screened. **In Topic 3 (Chapter 6)**, we characterized and investigated the diversity of lactic acid bacteria (LAB) and yeast by analyzing soak water samples collected from different tempe manufactures in Java. We also screened and investigated their potential antifungal activity against *Fusarium proliferatum*, and observed their contribution to ZEN biotransformation.

DNA sequences of genes such as ACT (actin) have been widely used for supporting morphological traits of mucoralean species (Voigt and Wonstemeyer, 2001; Abe et al., 2010; Dolatabadi et al., 2014). The results of sequence alignment by BLAST and phylogenetic analysis proved that ACT sequencing is suitable for accurate identification of tempeh fungal isolates. All the phylogenetic trees with high bootstrap supports revealed that the isolates had been successfully identified at the species level and further differentiated as particular strains within the same species. The dendrogram clearly showed distinct. The dendrogram indicated that *Rhizopus* spp. from Indonesian tempeh and inocula were placed into *R. stolonifer* clade (4 strains), *R. delemar*

clade (7 strains), *R. oryzae* clade (8 strains), *R. microsporus* var. *microsporus* clade (10 strains), *R. microsporus* var. *oligosporus* clade (8 isolates) and *R. microsporus* var. *chinensis* clade (4 isolates). The *R. delemar* clade was sister to *R. oryzae* clade with 99% BS. The *R. microsporus* var. *chinensis* clade was sister to *R. microsporus* var. *microsporus*, and *R. microsporus* var. *oligosporus* clade with 100% BS. The result clearly showed that the resolution of sequence from ACT region was sufficient in determining *Rhizopus* species into variety level, particularly in *R. microsporus* complex. This proved that DNA sequencing with ACT gene gave better taxonomy information until level of variety than morphological analysis in this genus. ACT is known as a housekeeping gene. Its expression is relatively stable under variable conditions. ACT encodes actin, a protein that is abundant in all eukaryotic cells, in which it is the major component of cytoplasmic microfilaments. The ACT gene has been used extensively to infer interspecies relationships across broad evolutionary distances, as the ACT gene of many species contains highly conserved (i.e. exon) and less well-conserved sequences, which are grouped as nuclear pre-mRNA introns (Donnelly et al., 1999).

There are three kind of threats to food safety associated with *Rhizopus*. **First threat** is about clinical infection. Most of the clinically important zygomycetes are in the order Mucorales and comprise approximately 60 genera. The genus *Rhizopus* exhibits the largest impact on mankind, being the main causing agent of mucormycosis, followed by *Lichtheimia* and *Mucor* (Skiada et al., 2011; Gomes et al., 2011). Our result showed that *Rhizopus* isolates from tempeh did not display a virulence potential. Mortality rates at four days after infection were found very low in the range 0-20% at *G. mellonella* model. Many reports explained that mucormycosis causing *Rhizopus* strains induce high mortality rates (80-100%) (Ribes et al., 2000; Dolatabadi et al., 2014). Those results also supported by genetic data. Consequently, the tempeh fungi isolates probably are nonpathogenic isolates. This is also in line with the fact that tempeh production and consumption have been practiced in Indonesia for centuries and, to our knowledge, there is no single report on infectious disease associated with tempeh. **The second threat** is rhizonin and rhizoxin produced by symbiotic bacteria (*Burkholderia rhizoxinica*) that reside within *Rhizopus microsporus* cytosol (Partida-Martinez et al., 2007; Lackner et al., 2009). Our result indicated that no *Burkholderia* species occurs in fungal isolates from Indonesian tempeh. Only three fungal samples were contaminated. Those samples were isolated from fresh tempeh that was produced in non-hygienic workshops. They were identified as *Enterobacter cloacae*, *Comamonas testosteron*, and *Ochrobactrum intermedium*, classified as human pathogen causing gastroenteritis. More attention should be given since there were several gastroenteritis outbreaks regarding unpasteurized tempeh consumption reported recently in Indonesia. **The third threat** is about possibility of masked-ZEN produced by the fungal. Our result revealed that authentic fungal strains used in production of tempeh in Indonesia could convert ZEN to conjugated ZEN and ZEL derivatives *in vitro*. This study showed that the investigated fungal strains have a high potential for ZEN conjugation by metabolic processes. These transformations may also occur during fermentation of tempeh. As the conjugates can be hydrolyzed by human intestinal microbiota, also sulfates and glucosides represent a relevant source for the human exposure to ZEN. If the

unprocessed grains for tempeh fermentation like mix soybeans-maize are contaminated with ZEN, upon analysis ZEN content of the final tempeh product may be underestimated, because ZEN has been conjugated during food processing.

Several types of tempeh fermentation starters can be distinguished. Natural starters made with plant leaves (e.g., *Hibiscus* spp.), and soybeans, known as “usar” are still widely used in Indonesia. *Usar* is prepared by placing pieces of matured tempeh between two *Hibiscus* leaves. Until now traditional tempeh producers still involve *waru lengis* leaves (*Hibiscus tiliaceus*) for *usar* manufacture, even though these leaves may serve as a source of *Fusarium* spp (Nout et al., 1999; Suliantari 1996) We successfully identified *Fusarium* from traditional tempeh inocula (*usar*) which were collected from various markets and center of tempeh producers in 9 different areas. DNA sequences of genes such as TEF-1 $\alpha$  have been widely used for supporting morphological traits of *Fusarium* species (Yli-Mattila et al., 2002; Hsuan et al., 2011). In the present study, molecular investigation based on the TEF-1 $\alpha$  gene led to more precise identification of *Fusarium* isolates. We found two main species of *Fusarium*, identified as *F. solani* and *F. proliferatum*. We not only study their presence in *usar* but also in traditional tempeh product. Out of 214 traditional tempeh samples from the six clusters mentioned above were screened for *F. proliferatum* and *F. solani* contamination by real-time PCR. Contamination of traditional tempeh samples with *F. proliferatum* was found in 12% of the samples with amounts of 10-175 ng/mg fungal DNA. In these samples, the amount of fumonisin B1 (FB1) and beauvericin (BEA) ranged between 170-2682  $\mu\text{g}/\text{kg}$  and 20-50  $\mu\text{g}/\text{kg}$ , respectively. About 7 % of the traditional tempeh samples were contaminated with *F. solani* in amounts of 20-129 ng/mg fungal DNA. All samples had lower FB1 contents than legal limit in Indonesia (2000  $\mu\text{g}/\text{kg}$ ; SNI 7385:2009), except for one sample from Yogyakarta area that contained FB1 above the allowed limit. Although mycotoxin contamination was detected below the legal limit, the presence of mycotoxin should be controlled in traditional tempeh production. To help consumer distinguish between contaminated and non-contaminated tempeh, we assessed quality aspect of both tempeh. There were no differences between normal and contaminated tempeh on the compactness and physical characters (texture, pH, and color). Interestingly we found that chemical contents were significantly different especially in protein and amino acid content. We suggest that consumer could distinguish contaminated tempeh by checking the taste of fresh tempeh before cooked. Contaminated tempeh is less umami than normal tempeh because of less L-aspartic acid and L-glutamic acid content, in other words, contaminated-tempeh is less delicious compared to the normal.

Our study was continued on finding the way to protect traditional tempeh from *Fusarium* contamination and also to reduce any possibility of ZEN or masked-ZEN contamination. Among natural biological antagonist, lactic acid bacteria (LAB) and yeasts have potential as bio-preservative. LAB can play a vital role as preservatives since they have a long history in fermented food production, are generally recognized as safe (GRAS status), and they have a Qualified Presumption of Safety (QPS) status. In our study, we used soak water as source of LAB and yeast. The soak water is side product of natural acidification process in tempeh production. In total, 165 isolates were obtained from soak water samples using selective media. Our results revealed a

certain variety of culturable LAB and yeast in soak water. The principal yeast found to be responsible for fermentation were *Candida tropicalis*, *Kluyveromyces marxianus*, and *Saccharomyces cerevisiae*, while the dominant LAB were *Lactobacillus plantarum*, *L. paracasei* and *Pediococcus pentosaceus*. We found that LAB species were less diverse than yeast species. Cell free supernatants (CFS) of 20 LAB strains possessed antifungal properties against *F. proliferatum* BJ0905, while none of the yeast isolates showed the activity. It was found that phenyllactic acid (PLA) might be involved in activity. The role of PLA and other organic acids as antifungal substances also observed by several authors. PLA is mostly produced by *L. plantarum* with a broad spectrum antifungal activity (Lavermicocca et al., 2000, Ström et al., 2002; Ndagano et al., 2011). Some other LABs can synthesize PLA and 4-hydroxy-acid phenyllactic (Valerio et al., 2004). The use of those selected LAB strains as part of starter culture has been proven to positively increase tempeh safety. However, this LAB addition made pH of tempeh become lower (pH in range 5.1-5.5) than normal tempeh (6.5-6.7). The LAB culture addition might impart undesirable sensory characteristic of tempeh. Further research is necessary to cope with this application problem before it can be applied into large-scale production systems in traditional tempeh industry.

The results obtained from screening 20 yeast and LAB isolates belonging to 10 different species revealed that nearly all the tested organisms were able to reduce ZEN in range 10 to 96.9%. In general, yeast and LAB isolates could only transform ZEN into two major transformation products  $\alpha$ -ZEL and  $\beta$ -ZEL. In all cases no conjugation product was detected. The novel result of the study is not about tempeh LAB and yeast activity to reduce ZEN amount but their capability to transform ZEN by reducing the keto group into two major transformation product  $\alpha$ - and  $\beta$ -ZEL. More importantly, none of isolates could transform ZEN into ZEN conjugates, thus this microorganism will not contribute to producing of masked-ZEN. Most of research in this area so far only highlight the role of LAB and yeast as adsorbent (binding) agent of mycotoxins (El-Nezami et al., 2004; Haskard et al., 2001). LAB and yeast fermentation is a recommended method for reducing ZEN in contaminated raw materials such as corn that usually used in tempeh production, although safety of decontaminated food is not guaranteed because mode of action may not only involve transformation process but also reversible binding. Further research is necessary to establish the mode of ZEN reduction in natural fermentation.

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## Summary

Food safety is a prerequisite for a food product. There is no sense in talking about quality, nutritional value, sensory or functional properties if the food product is not safe for consumption. Indonesia suffers a double burden of food safety problem. The first, related to fundamental issues of food safety; especially about the insufficient practice of Good Manufacturing Practices (GMP) principles that may lead to food poisoning for the consumer. The second, related explicitly to export-oriented food industries; Indonesia must face a variety of new food safety issues. One of the food product that needs attention for its safety matter in Indonesia is tempeh.

Tempeh is traditional food from Indonesia made by fermenting soybeans with mold *Rhizopus* spp. It has solid form, distinctive aroma and white and slightly gray. Indonesia is known for the largest tempeh production in the world and has the largest soyfood market in Asia. About 2.4 million tons of tempeh produced a year by more than 81 thousand tempeh producers. Tempeh is produced mostly on a small scale home industry with poorly controlled fermentation and carried out without sufficient hygienic precautions. Therefore, there are various types of microorganisms that can participate during the process of fermentation, and natural contamination is always possible. Moreover, there is no standard on making tempeh and its starter. This causes many variations in the manufacture of tempeh in many areas and lead to inconsistencies in quality and safety of tempeh. Tempeh, as important food is massively produced and consumed by Indonesian people. As a new export commodity, it really needs standardized starter and controlled processing to fulfill safety aspect.

The scope of this study was to address questions about microbiological and food safety in tempeh production in Indonesia. Researchs were divided into three main topics. **Topic 1 (Chapter 3)** dealt with the exploration of mucoralean fungi involved during tempeh production as well as solid fermentation process. *Zygomycetes* strains were isolated from Indonesian tempeh and starter and screened toxicologically and clinically to evaluate possible health risks for human consumption. In this part we also studied the role of tempeh on zearalenone (ZEN) biotransformation. **In Topic 2 (Chapter 4)**, we studied the toxigenic fungal contamination of *usar* (traditional tempeh inoculum). We characterized the contaminant strains and detect mycotoxins they produced. Mycotoxins contamination in traditional tempeh was also screened. **In Topic 3 (Chapter 6)**, we characterized and investigated the diversity of lactic acid bacteria (LAB) and yeast by analyzing soak water samples collected from different tempe manufactures in Java. We also screened and investigated their potential antifungal activity against *Fusarium proliferatum*, and observed their contribution to ZEN biotransformation.

**Result of Topic 1:** A total of 557 pure fungi isolates were obtained from 247 samples fresh tempeh and inocula in 16 different provinces in Indonesia. Diverse fungal species found in Indonesian tempeh and inocula. RAPD typing was capable of producing discriminating DNA fingerprints of *Rhizopus* isolates and indicated there was genetic differences among them. Many distinct patterns were observed indicating that tempeh and inocula from different location in Indonesia composed

of a quite wide number of strains. Combination of origin or geographical data and molecular identification showed that isolates (identified as *Rhizopus oryzae*, *R. delemar*, and *R. stolonifer*) dominantly used by tempeh producer from outside Java, whereas isolates (*Rhizopus microsporus* complex isolates, identified as *R. microsporus* var. *chinensis*, *R. microsporus* var. *microsporus* and *R. microsporus* var. *oligosporus*) were mostly used by tempeh producer from Java. Tempeh fungi isolates are safe for human consumption since they are not mucormycosis or toxication agent, but food safety supervision has to be given to some tempeh producer that applied unhygienic practice in tempeh production because we found few sample contaminated with bacteria classified as a human pathogen causing gastroenteritis. Fungal strains used in production of tempeh in Indonesia also could convert ZEN to conjugated ZEN and ZEL derivatives *in vitro*. More attention needs to be given to tempeh made with mix soybean-maize. If the unprocessed grains for tempeh fermentation like mix soybean-maize are contaminated with ZEN, upon analysis ZEN content of the final tempeh product may be underestimated, because ZEN has been conjugated during fermentation.

**Result of Topic 2:** In total, 650 fungal isolates recovered from traditional tempeh inocula (*usar*) in Java. Traditional inocula (*usar*) were collected from many markets and center of tempeh producers in 9 different areas in Central Java and Yogyakarta provinces, Indonesia. Six clusters (Banjarnegara, Banyumas, Gombong, Kebumen, Kudus, and Yogyakarta) showed a *Fusarium* contamination in *usar*, while no contamination was found in other remaining clusters. About 8.4% of total fungal isolates recognized belongs to *Fusarium* genera. Fifty-five pure isolates of *Fusarium* sp were collected, then observed via microscopy and molecular approach. We found two main species of *Fusarium*, identified as *F. solani* and *F. proliferatum*. All *F. poliferatum* isolates included in our study lacked the TRI5 gene and possessed the FUM1 gene, indicating that they are presumably trichothecene non-producers and potentially fumonisin producers. This result was confirmed by different set of primers (FUM8, and FUM12) with identical result. In other hands, *F. solani* isolates lacked in both kind of genes, indicating they are not trichothecene or fumonisin producers. LC-MS/MS screening showed *F. solani* isolates were not able to produce any important mycotoxins, whereas *F. proliferatum* positively produced beauvericin (BEA), fumonisin B1 (FB1), and enniatins. *Fusarium proliferatum* extracts showed about 10-fold stronger activity as toxic agent toward *F. solani* extracts based on their value of LD50. About 214 traditional tempeh samples from the six clusters mentioned above were screened for *F. proliferatum* and *F. solani* contamination by real-time PCR. Contamination of traditional tempeh samples with *F. proliferatum* was found in 12% of the samples with amounts of 10-175 ng/mg fungal DNA. In these samples, the amount of FB1 and BEA ranged between 170-2682 µg/kg and 20-50 µg/kg, respectively. About 7 % of the traditional tempeh samples were contaminated with *F. solani* in amounts of 20-129 ng/mg fungal DNA. All samples had lower FB1 contents than legal limit in Indonesia (2000 µg/kg; SNI 7385:2009), except for one sample from Yogyakarta area which contained FB1 above the allowed limit. Although mycotoxin contamination was detected below the legal limit, the presence of mycotoxin should be controlled in traditional tempeh production. There were no differences between normal and contaminated tempeh on the compactness and



physical characters (texture, pH, and color). Interestingly, we found that chemical contents were significantly different especially in protein and amino acid content. This suggest that consumer could distinguish contaminated tempeh by checking the taste of fresh tempeh before cooked. Contaminated tempeh is less umami than normal tempeh because of less L-aspartic acid and L-glutamic acid content, in other words, contaminated-tempeh is less delicious compare to the normal.

**Result of Topic 3:** The LAB counts in soak water samples varied between 5.21 and 8.88 log CFU/mL whereas yeasts counts varied from 4.31 and 4.89 log CFU/mL, APC counts varied between 7.33 and 9.10 log CFU/mL. In total, 165 isolates were obtained from these samples using selective media. Our results revealed a certain variety of culturable LAB and yeast in soak water. The principal yeast found to be responsible for fermentation were *Candida tropicalis*, *Kluyveromyces marxianus*, and *Saccharomyces cerevisiae*, while the dominant LAB were *Lactobacillus plantarum*, *L. paracasei* and *Pediococcus pentosaceus*. We found LAB species were less diverse than yeast species. Cell free supernatants (CFS) of 20 LAB strains possessed antifungal properties against *F. proliferatum* B.J0905, while none of the yeast isolates showed the activity. It was found that phenyllactic acid (PLA) might has main role for the activity. The use of those selected LAB strains as part of starter culture has been proven to positively increase tempeh safety. The results obtained from screening 20 isolates belonging to 10 different species revealed that nearly all the tested organisms were able to reduce ZEN in range 10 to 96.9%. In general, yeast and LAB isolates could only transform ZEN into two major transformation product  $\alpha$ -ZEL and  $\beta$ -ZEL. In all cases no conjugation product detected.

Research outcome for society: Safe and defined strains for modern inoculum and tempeh production have been selected and formulated. In the near future, safe and high quality inoculum could be produced on a mass scale and distributed to tempeh manufactures, thus tempeh production would be controlled from the beginning process and indeed increase its safety.

## Acknowledgements

First of all, it is my great honor and pleasure to express my deepest gratitude and heartiest thanks to my principal supervisor Prof. Dr. Petr Karlovsky for his enthusiastic guidance, creative supervision, advice and encouragement during the whole period of the study. This study would not have been possible to complete unless his continuous and excellent guidance in exploring, generalizing and broadening new findings. I thank him also for the motivation and good discussion during whole period of the study. I am so grateful for the time and discussions that we had enriching my knowledge, skills and experiences. I learnt from him to be critical and to obtain insight in scientific research.

Furthermore, I would like to express my deepest thank Dr. Katharina Pfohl, who support me especially in HPLC-MS/MS analysis and give excellent advice during the study.

I am very grateful to have PD Dr. Franz Hadacek and Prof. Dr. Richard Splivallo as my Ph.D. committee members.

My thank goes to LPDP Ministry of Finance, The Republic of Indonesia for the doctoral scholarship funding.

I acknowledge former Rector of Jenderal Soedirman University Prof. Edi Yuwono Ph.D. for the help and motivated me to continue my study.

I acknowledge Dr. Antje Borzekowski, Maryeni Auliyati, and Prof. Dr. Ronald Maul for innovative and nice research collaboration about zearalenone transformation.

I am thankful to all members of the Karlovsky lab, in particular, Ruth Pilot and Heike Rollwage, for their constant advice and prompt technical support, especially during the first year. Apart from this, I thank Ruth Pilot for checking partly my thesis. I thank also our apprentices Christian Kolodziel and Jonas Waterott for their technical assistance.

I also acknowledge Dr. Israr Arbar from Ministry of Environment The Republic of Indonesia, providing me GIS (Geographic Information System) for my sampling area and making professional sampling map.

I acknowledge Dr. Volker Cordes and his team (Max Planck Institute for Biophysical Chemistry) for teaching and the helpful advice for using scanning electron microscopy (SEM).

I acknowledge Dr. Daniel, Prof. Dr. Loekas Soesanto, Prof. Emeritus Rubiyanto Misman, Dr. Narongrit Muangmai, Dr. Nur Aini, for the support, encouragement and good scientific discussion.

I thank my colleagues Muhammad Sherif, Pervin Akter, Rosine G. Fotso, Olaf Henke, Samal, Maria Vinas, Yang Xu, Muhammad Al Hussein, Rassoul Abousaedi, Zana J. Kareem, Vincent Ayugi, Albatol Alsarrag, Mareen and Ling Su for the nice working atmosphere.

I thank friends in Germany and abroad for all the fun and good scientific discussion.

My sincere thanks go to the whole division for the good working atmosphere and support, especially to Mr. Thomas Oesterreich, Mrs. Kistner, Mrs. Thinggaard and Mrs. Christine Schachtebeck for administrative support and patience.

I thank Mr. Subekti and his wife in Jakarta, for support, motivation and help.

Last but not least, I am thankful to my family for the endless support, love and motivation during the last years.

Danke Schön!

Thank you!

Terima kasih!

Göttingen, December 2017

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## Curriculum Vitae



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### Recent International Seminar or Symposium (as main or co-author)

- Antje Borzekowski, Maryeni Auliyati, Riyan Anggriawan, Hans-Jörg Kunte, Sascha Rohn, Matthias Koch, Petr Karlovsky, and Ronald Maul. 2017. Fungi isolated from Indonesian tempeh production capable of zearalenone conjugation. 39<sup>th</sup> Mycotoxin Workshop, Bydgoszcz, Poland.
- Riyan Anggriawan, Katharina Pfohl, and Petr Karlovsky. 2017. Mycotoxin-producing *Fusarium* species in Indonesian traditional tempeh and starter (*usar*). 39<sup>th</sup> Mycotoxin Workshop, Bydgoszcz, Poland.
- Riyan Anggriawan, and Petr Karlovsky. 2015. Genome shuffling for microbes from natural fermentation of tempeh against mycotoxin: constructing parent strains library. 2<sup>nd</sup> ICBS, Yogyakarta, Indonesia.

- Riyan Anggriawan, and Petr Karlovsky. 2015. Genome shuffling for zygomyceteus fungi from tempeh against mycotoxins: constructing parent strains library. 37<sup>th</sup> Mycotoxin Workshop, Bratislava, Slovakia.
- Riyan Anggriawan, A. Ilalqisny Insan, Jantana Praiboon, Anong Chirapart, Loekas Soesanto, Aisyah Tri Septiana, and Karseno. 2015. Antioxidant activity and phytochemicals of lipophilic extract obtained from selected Indonesian brown seaweeds. 2<sup>nd</sup> ISAPPROSH, Semarang, Indonesia.
- Riyan Anggriawan, Anong Chirapart, Jantana Praiboon, Karseno, and Loekas Soesanto. 2014. Assessment of phytochemical, antifungal activity and toxicity of selected brown seaweeds extract. 36<sup>th</sup> Mycotoxin Workshop, Göttingen, Germany.

#### **Other Recent International Symposium (as observer)**

- 7<sup>th</sup> European Conference on Prokaryotic and Fungal Genomics, Göttingen, Germany (19-22, September 2017)
- GlobalFood Symposium, Göttingen, Germany (28-29, April 2017)
- 2<sup>nd</sup> ICONIC Industrial Revival, Hamburg, Germany (30<sup>th</sup> October 2016)

#### **Other Academic Experiences**

- Coordinator of reviewer for Food Industry field in ICONIC symposium, Hannover. (2018)
- Research for submitting in partial fulfillment of requirement of Master Degree with the research title “Assessment of phytochemical, antifungal, antioxidant activities and toxicity of selected brown seaweeds extract” (Funding No: 62875/A2.4/LN/2011). (2011-2012)
- Training of ISO 9001-2008 Quality Management System, held by PT. Jireh and Training Consultant. (2011)
- Assistant researcher for research “FeSO<sub>4</sub> fortification in arrowroot biscuit enriched by protein from tempeh-fish flour to avoid anemia on pregnancy period” led by Dr. V. Prihananto. STRANAS Project (National Strategic Project)-LPPM UNSOED. (2010)
- Guest Speaker for workshop/dissemination “Improvement Training of Local Food Technology and Food Consumption Diversity”, held by Badan Ketahanan Pangan and UNSOED. (2010)
- Research for submitting in partial fulfillment of requirement of Bachelor Degree “Effect of milling method on physical, chemical and functional properties of hybrid corn flour”. (2009)
- 3<sup>rd</sup> winner on Local Food Product Development, held by Badan Ketahanan Pangan and Agriculture Faculty UNSOED. (2009)
- Guest Speaker for workshop/dissemination “Training for Small-Medium Food Company in Banyumas”. Held by Badan Ketahanan Pangan and UNSOED. (2009)
- Assistant researcher for research “Formulation of instant breakfast food product based on talas flour (*C. esculenta*) and supplemented by fish-tempeh flour” led by Dr. Hidayah Dwiyantri, Coordination between Balitbang Jateng and LPPM UNSOED. (2008)
- Field Work Practice (PKL) in PTP Nusantara IX (Persero) Kebun Krumpit- Banyumas (Ribbed Sheed Smoked Factory), supervised by Dr. Tri Yanto. (2008)