Biological Detoxification of Enniatins

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Chapter 1

General background

1. Introduction

Enniatins are secondary metabolites produced by several *Fusarium* species such as *F. orthocerar* (Gäumann *et al*., 1947) *F. tricinctum, F. lateritium, F. oxysporum*, *F. avenaceum* (Blais *et al*., 1992; Herrmann *et al*., 1996; Wang *et al*., 2013) and a few other genera of fungi such as *Alternaria* (Mckee *et al*., 1997), *Verticillium* (Nilanonta *et al*., 2003) and *Halosarpheia* (Lin *et al*., 2002). More recently, Zobel *et al.* (2005) also demonstrated the production of enniatin B by a recombinant *Bacillus subtillis* and Richter *et al*. (2014) who engineered *Aspergillus niger* for the production of several variants of enniatin. According to Jestoi *et al*. (2008) the main enniatin producers are *F. subglutinans, F. proliferatum* and *F tricinctum.*

Enniatins are a group of cyclic hexadepsipepdides which have alternated residues of three N-methyl amino acids in their structure, often valine, leucine and isoleucine and three hydroxyisovaleric acids. The branched chain amino acids are linked by peptide and ester bonds. Sy-Cordero *et al*. (2012) reported that 29 variants of enniatins have been isolated and characterized, they differ from each other in their amino acid and hydroxyacid composition, but more variants could be found due to the multiple possibilities of incorporation of amino acids and the hydroxyl acids by the enzyme enniatin synthetase and D-2-hydroxyisovalerate dehydrogenase. Beauvericin and destruxin mycotoxins are structurally similar to enniatins. Destruxins contain only one hydroxy acid and five amino acid residues in their structure (Pedras *et al.*, 2002), while beauvericin is composed of alternating N-methylphenylalanine and hydroxy acid residues (Hamill *et al*. 1969).

High concentrations of enniatins in food commodities have been reported in several countries mainly in northern Europe and the Mediterranean. The main tested foods were cereal and cereal-based products. 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, containing wheat as

main cereal. 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. In cereal (wheat, barley, maize, sorghum) and cereal- derived products (mainly pasta and couscous) originating from Tunisia, high levels of enniatins A, A1, B and B1 were found. A maximum concentration of the four enniatin variants of 683.9 mg/kg was detected in sorghum. Among the tested enniatin variants, enniatin A1 had the highest prevalence with concentrations ranging from 11.1 to 480 mg/kg (Oueslati *et al.*, 2011). A survey carried out by Uhlig *et al*. (2006) in Norwegian oat, barley and wheat grain samples from 2000 to 2002 revealed that concentrations of enniatin B above 1000 µg/kg are common and similarly, enniatin B occurred with the highest prevalence and a maximum concentration of 5800 µg/kg was found in wheat. Finnish barley and wheat were also investigated by Yli-Mattila *et al*. (2004, 2008, 2009), Jestoi *et al*. (2004) and Hietaniemi *et al*. (2016). Besides the studies made on the occurrence of enniatins in cereal and cereal-based products, enniatins have been also been found in egg and wine from Finland and China respectively. Enniatin B and B1 were found in whole eggs and yolk egg in trace-levels (< limit of quantification) of samples from 2004 (Jestoi *et al*., 2009). Enniatin B (Wang *et al*., 2011) was detected in wine samples made out of grapes harvested in a vineyard contaminated by *Fusarium tricinctum* (Corda). 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.

Various biological activities of enniatins have been described. Some damaging effects on plants have been associated to enniatins, for instance they affect water uptake by cells in tomato shoots, leading to toxic wilt and necrosis (Gäumann *et al*., 1960). An *in vitro* toxicity

of a mixture of enniatins A, A1, B and B1 characterized by necrosis was also observed in potato tubers, depending on the incubation time and the amount of enniatins applied (Herrmann *et al.,* 1996). Moreover, enniatins (mainly B and B1) concentrations ranging from 10-80µg/mL have been directly associated to a growth reduction of the wheat seed during germination, characterized by a higher inhibition of the root elongation compared to the leaf development (Burmeister and Plattner, 1987). Logrieco *et al.* (1998) associated some insecticidal activities and apoptosis in mammalian cells to beauvericin exposure in mammals. The toxicity of enniatins is assumed to derive from their ionophoric properties and to their action as uncouplers of oxidative phosphorylation (Shemyakin *et al.*, 1969). Individual and combined toxicity of enniatins A, A1, B and B1 were evaluated with CHO-K1. The highest half maximal inhibition concentration (IC_{50}) was attributable to enniatin B. Furthermore, the mixture of enniatin A+B1, A1+B, B+B1 led to an increase of the observed effect (Lu *et al.*, 2013)

Besides the above mentioned harmful effects of enniatins, they also possess some antibiotic properties which have been exploited in the production of an antibiotic known as fusafungine -a mixture of enniatin A, B and C-produced by *Fusarium lateritium* (Moffat, 1986). More recently, some researchers confirmed the role of enniatins as antibiotic. Enniatins B, B1 and G were effective against some bacterial strains (*Bacillus subtilis, Candida albicans, Trychosporom cutaneum* and *Cryptococcus neoformans*) in agar diffusion test by lowering the activity of the 12- Lipoxygenase and therefore proved an antibiotic activity (Firákova *et al.*, 2008). Another study highlights the fact that enniatins J_1 and J_3 could be potentially effective antibacterial agents against several pathogenic and lactic acid bacteria (Sebastià *et al.*, 2011). Enniatin B induced the inhibition of the microorganisms of some normal intestinal tract pathogens such as *Escherichia coli*, *Salmonella enterica*, *Clostridium perfringens*, *Pseudomonas aeruginosa* (Meca *et al.*, 2011). The antibiotic properties conferred by fusafungine make it the active compound of a medicine commercially available in Germany under the name Locabiosol. Locabiosol which is used for the treatment of bacterial upper respiratory infections for example for a sore throat or a blocked nose (German-Fattal and Mösges, 2004).

The broad range of biological activity of enniatins is ascribed to their ionophoric properties. It was demonstrated that a mixture of enniatin A, A1, B and B1 is easily incorporated into the

cell membrane through passive channels and form cation selective pores. Enniatins induce changes in intracellular ion concentration and disrupt cell function (Kamyar *et al*., 2014).

2. In vitro production of enniatins

Early in 1974, Audhya and Russell produced enniatins using *Fusarium sambucinum in* chemically defined liquid media. The enniatin production was favored by various factors, such as supplementation with lactose and tryptone, moderate temperatures and light exposure. Quantitative paper chromatography or spectrophotometry was used to quantify enniatin A with a yield of 1.6 g/L. Madry *et al*. (1983) carried out a similar study where the impact of various media components were studied for the production of enniatins using *F. oxysporum* in liquid culture. The production of enniatins was promoted by the addition of sucrose, glucose and amino acids (valine, leucine and isoleucine) leading to a yield up to 5g/L of several enniatins after analysis of the chloroform extract using spectrophotometry methods. The first study about the production of enniatin by *F. tricinctum* was reported by Burmeister and Plattner (1987). *F. tricinctum* was incubated on a semi solid medium of corn grit for 16 days at ambient temperature and cultures were extracted using methanol and analyzed using mass spectrometry/ mass spectrometry (MS/MS) to give a yield of 3-3.270 µg/g of enniatin B and B1.

2.1. Fermentation substrate

Initially the main substrates used for enniatin productions were chemically defined liquid media (Audhya and Russell, 1974; Madry *et al*., 1983; Strongman *et al*., 1988; Visconti *et al*., 1992; Herrmann *et al*. 1996), and semi solid media such as corn grit (Burmeister and Plattner 1987). More recently, several authors have reported the production of high amounts of enniatins using solid matrices such as rice (Moretti *et al*. 2007), corn (Meca *et al*. 2010), or several solid substrates including white beans, soybeans, radish, potato, wheat, corn and rice (Wang *et al*. 2013). According to Wang *et al*. (2013) enniatins production was highest in the white bean media without any supplementation of amino acids (1.365mg/L enniatins A, A1, B1 and B after 18 days).

2.2. Extraction, detection and quantification

Enniatins are strong hydrophobic compounds and their extraction from fungal extracts have been done using mild to strong hydrophobic solvents such as water/methanol and ethyl acetate (Cuomo *et al*., 2013), methanol (Burmeister and Plattner 1987), acetonitrile (Serrano *et al*., 2013), hexane (Firáková *et al*., 2008), chloroform media (Audhya and Russell, 1974; Madry *et al*., 1983), ethyl acetate (Wang *et al*., 2013). Among the techniques used so far for the detection and quantification of enniatins from extracts are quantitative paper chromatography or spectrophotometry (Audhya and Russell, 1974; Madry *et al*., 1983), mass spectrometry/mass spectrometry (Burmeister and Plattner 1987), HPLC-UV (Firáková *et al*., 2008 Wang *et al*., 2013), LC-MS-MS (Sørensen *et al*. 2008), LC-MS-LIT (Serrano *et al*., 2013).

2.3. Purification

Generally preparative chromatographic techniques have been used for the purification of enniatins from fungal extract. Visconti *et al*. (1992) reported the purification of enniatins A, A1, B, B1, B2, B3 and B4 from liquid culture of *Fusarium acuminatum* strains, these authors made a liquid-liquid extraction of the culture broth using hexane, then separated enniatins using two-step preparative reverse phase HPLC and acetonitrile water as mobile phase to get pure enniatins in the range of 2 to 30 mg from 2 L liquid culture. Enniatins A, A1, B and B1 were purified from *Fusarium tricinctum* grown on solid corn medium using low pressure liquid chromatography, followed by semipreparative liquid chromatography both on reverse stationary phase to obtain enniatins with a yield varying from 20 to 56 mg from 100 g solid corn medium (Meca *et al*., 2010). Similarly, The purification of enniatins A, A1, B, B1, B4 and J produced by *Fusarium tricinctum* in solid corn culture has been reported by Cuomo *et al*. (2013), the fermented solid culture was extracted first using water/methanol (50/50, v/v), then by ethyl acetate and the extract was separated using a two steps process including low pressure liquid chromatography (normal phase silica gel and an isocratic gradient of chloroform/isopropylalcohol 80/20 v/v) and semi-preparative HPLC (a Gemini phenomenex column and a gradient elution with water and acetonitrile) to get mixtures of six enniatins with yields of 30-300 mg per compound.

3. Mycotoxin decontamination strategies

In order to limit the exposure of human and animals to mycotoxins in food and feed, methods for mitigating the mycotoxin content have been developed. The techniques used for detoxification of mycotoxins in food can be classified into three categories: physical, chemical and biological methods (He and Zhou, 2010).

3.1. Physical detoxification

Among the physical methods used to remove mycotoxins a method based on the separation of zearalenone-deoxynivalenol- infected wheat, corn and sorghum grains was studied by Huff *et al*., (1982) and Babadoost *et al*., (1987). Milling provides a partial removal of mycotoxins as a higher amount will be found in the bran than in the flour, but the process does not destroy mycotoxins. It was shown that during dry milling of corn, fumonisin B1 was found in highest amounts in the bran fraction that is used as animal feed followed by the germ fraction, which may be used as animal feed or for oil extraction (Katta *et al*., 1997). Aflatoxin B1 and Ochratoxin A were relatively stable at boiling temperatures of the mash cooking step, but were more sensitive to mash malting (protein hydrolysis) with removal of 12-27% (Chu *et al*., 1975). A decrease of the enniatin (A, A1, B and B1) content of 30% was observed during bread baking, while no enniatins were found in final beers probably due to their high hydrophobic character (Vaclavikova *et al*., 2013). In a study of the variation of the enniatin content in naturally contaminated wheat and rye grains during milling, the distribution of enniatins B and B1 was about 70- 82% in the bran, while the rest was present in the flour. Similarly, 24 hour sourdough fermentation at 40°C led to 17-19% and 10% reduction of enniatins and beauvericin respectively compared to 7-10% enniatins reduction at 30°C. Generally, enniatins and beauvericin concentrations were 25-41% lower in the bread compared to unprocessed material (Hu *et al*., 2014). Food processing techniques can reduce the amount of some mycotoxins in food, by diluting and distributing mycotoxins into certain fractions, but this might be used for animal feed and then still enter the human food chain. The process of reducing mycotoxins in food using heat treatments is temperature and time dependant. Most mycotoxins are moderately stable in most food processing systems (Bullerman and Bianchini, 2007).

3.2. Chemical detoxification

Chemical detoxification involving alkalis and acids to deactivate aflatoxins was developed in raw material intended for animal and human food products including cottonseed, peanuts, coconuts and corn grains. Treatment of one fraction of the raw material was made with a base and the other fraction was treated with an acid, then the two fractions are combined to bring the pH back to neutral (Emetco, 1995).

3.3. Biological detoxification

Biological detoxification can be defined as the enzymatic degradation or transformation of toxins that leads to less toxic products (Bhatnagar, 1991). Methods of detoxification of mycotoxins using microorganisms generally used enrichment cultures based on a single substrate. Fumonisins B1 and B2 were detoxified as unique carbon source by fungi *Exophilia spinifera* (ATCC 74269) and *Rhinocladiella atrovirens* (ATCC 74270) and the bacterium ATCC 55552, the microorganisms were isolated from field-grown maize kernels and stalks (Duvick and Rood, 1998). In the scientific literature a US patent on the biological detoxification of beauvericin is available. A method allowing the reduction of beauvericin *in vitro* and *in vivo* was found and beauvericin contaminated corn was detoxified employing strains of *Norocardia glubera* (Duvick and Rood, 2000). The detoxifying action of fungus *C. rosea* was demonstrated to be due to the enzyme zearalenone lactonohydrolase that cleaves the lactone rings from the backbone and transforms zearalenone to a far less potent compound (Kakeya *et al*., 2002). Two *Pseudomonas* strains were able to degrade zearalenone by about 57- 68% using an enrichment technique with zearalenone as the sole carbon source (Tan *et al*., 2014). A bacterium isolated from a soil sample from a wheat field classified as *Nocardioides* was able to completely remove 1000 µg/mL of DON after 10 incubation days from a liquid culture medium (Ikunaga *et al*., 2011). The use of *Saccharomyces* strains have led to a reduction of enniatins A, A1, B and B1 *in vitro* or in food system, LC-MS-LIT was used to find three degradation products (Roig *et al*., 2013). A reduction of enniatins A, A1, B and B1 was induced by six *Bacillus subtilis* strains in tryptic soy broth after incubation for 48 hours at 37°C under aerobic conditions. Two degradation products were identified using LC-MS resulting from the loss of hydroxyisovaleric acid in the structures of enniatin B and B1 (Meca *et al*., 2014).

Food processing techniques such as sorting, trimming, cleaning, milling, brewing, cooking, baking, frying, roasting, canning, flaking, alkaline cooking, nixtamalization and extrusion may have an impact of the mycotoxin content in food (Bullerman and Bianchini, 2007). But these methods present some disadvantages like possible losses of important nutrients. From the opinion of many scientists, biological detoxification is a promising technique enabling the removal of mycotoxins under mild conditions and without the use of harmful chemicals and significant losses in nutritive value and palatability of the decontaminated feed and food (Karlovsky, 1999; Bata and Lásztity, 1999; Kabak *et al*., 2006; Wu *et al*., 2009). Furthermore, the genes responsible for some of the detoxification activities can be cloned and expressed in heterologous hosts.

4. The complex of problems and objectives

Due to the relatively high price of enniatins as raw products on the market (and in our case the large quantity involved in the detoxification assay), it has been common for many authors to purify the enniatins that were required for further use such as for toxicity tests for instance. Moreover, some authors have attempted to optimize the *in vitro* conditions for the production of enniatins.

The first objective of this study will be to optimize the enniatin production by examining two strains of *F. tricinctum* and to find the best harvesting time point after incubation of the strains on solid white bean media. Then enniatins will be purified from the fungal crude extract.

The data reported in the literature about enniatins contents in food products are obtained using different extraction techniques as well as analytical instruments and thus might vary from each other in terms on sensitivity and precision. However, the values in general are very high and should raise concern about the long term effect of those metabolites on consumer health, data about the chronic exposure of humans through diet to such high concentrations of enniatins are still lacking. Maximum levels of Enniatins concentrations in food has not been set by the European Union suggesting the ignorance related to the possible risks to which the population may be exposed. This has led to some investigations targeting the degradation of enniatin in food by some microorganisms.

In the second part of this thesis, microorganisms (bacteria and/or yeast and/or fungi) able to degrade enniatins into less toxic compounds will be screened, the degradation products will be identified and the degradation reaction will be deduced.

Since random screening of unknown microorganisms will be used as a strategy to isolate the microorganisms with the ability to degrade enniatins into less toxic compounds, the third part of this work will consist in the taxonomical identification of the active microorganisms with the aid of phenotypic and molecular means.

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Chapter 2

Enniatins production by *F. tricinctum* on white bean medium and purification using flash chromatography

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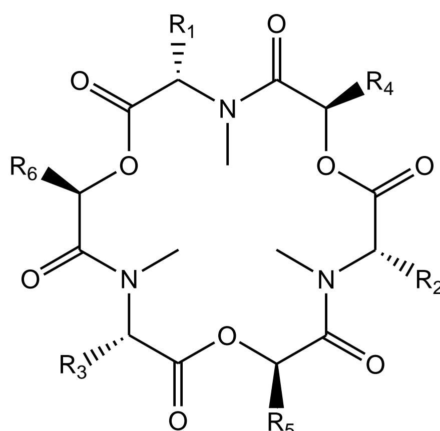
Abstract

Strains *F. tricinctum* DSM 23357 and *F. tricinctum* O32 were examined for the production of enniatins A, A1, B and B1 on solid white bean (*Phaseolus vulgaris*) (Wang *et al*., 2013) medium at 25°C for 30 days. A method was established for the quantification of enniatins content using HPLC UV-visible photodiode array detector (HPLC-DAD) with a reverse phase column, elution with a methanol-water gradient and detection by light absorption at 210 nm. The enniatin content increased up to day 24. Total enniatin content of *F. tricinctum* O32 culture reached 614 mg/kg, which was about two times more as compared to enniatin produced by *F. tricinctum* DSM 23357. *F. tricinctum* O32 was therefore used for the production of enniatins in white bean solid medium. Enniatins were extracted with ethyl acetate and the extract was fractionated using flash chromatography on a reverse phase column with a step gradient consisting of water-methanol mixtures. In total 92 fractions were collected and enniatins were detected using thin-layer chromatography (TLC) and HPLC-DAD in fractions 50-74. Fractions containing enniatins (about 4.75 g per kg culture) were combined and further purified using recrystallization from methanol and water. 3.18 g enniatins containing about 77% enniatin B, 17% enniatin B1, 6% enniatin A1 and 0.3% enniatin A were obtained per 1 kg culture. The mixture of enniatin B, B1, A1 and A had a purity of 96.25%.

Keywords: Enniatins, *F. tricinctum*, production, HPLC-DAD, flash chromatography.

1. Introduction

The enniatin first discovered and known as enniatin A was isolated from the fungus *Fusarium orthoceras* by Gäumann *et al.* (1947). Enniatins are a group of cyclohexadepsipeptides composed of alternating residues of three N-methyl amino acids, mostly valine, leucine and isoleucine depending on the variant, as well as three hydroxy acids, typically hydroxyisovaleric acid. The branched chain amino acids are linked by peptide and ester bonds. Enniatins are produced by several *Fusarium* species such as *F. sambucinum* (Audhya and Russell, 1974), *F. avenaceum* (Blais *et al.*, 1992), *F. oxysporum, F. acuminatum, F. arthrosporiodes, F. merismoides, F. tricinctum* (Herrmann *et al.*, 1996) and a few other genera like *Verticillium hemipterigenum* (Nilalonta *et al.*, 2003). Twenty-nine variants of enniatins have been isolated and characterized; they differ from each other in their amino acid and hydroxy acid composition (Sy-Cordero *et al.*, 2012). Figure 1 shows the general structure of enniatins as summarized by Sy-Cordero *et al.*, 2012.



Compound	Radicals
Enniatin A	R1=R2= R3= $-CH(CH_3)CH_2CH_3$; R4= R5= R6= $-CH(CH_3)_2$
Enniatin A1	R1=R2= $-CH(CH_3)CH_2CH_3$; R3= $-CH(CH_3)_2$; R4= R5= R6= $-CH(CH_3)_2$
Enniatin B	R1=R2= R3= $-CH(CH_3)_2$; R4= R5= R6= $-CH(CH_3)_2$
Enniatin B1	R1= R2= $-CH(CH_3)_2$; R3= $-CH(CH_3)CH_2CH_3$; R4= R5= R6= $-CH(CH_3)_2$

Figure 1: General structure of enniatins, R_1 to R_6 could be sec-butyl, isobutyl, isopropyl, hydroxy-sec-butyl, ethyl or methyl radicals depending on the variant.

In vitro production of enniatins using fungi has been reported in several studies using a variety of media including solid and chemically defined liquid media. Mainly analytical chromatographic and preparative chromatographic methods have been used for the detection, quantification and purification of enniatins. Early in 1974, Audhya and Russell demonstrated the production of enniatins in a semi-defined liquid culture by *F. sambucinum*, the process was light and temperature dependant, also the enrichment of cultures with carbon and nitrogen sources such as lactose, glycerol and tryptone supported enniatin production after detection and quantification with paper chromatography and spectrometry. The fermentation of *F. avenaceum* DAOM in liquid media resulted in the production of a mixture of enniatins containing predominantly enniatin A, followed by enniatins A1 and B1 (Blais *et al*., 1991). Wang *et al*. 2013 examined the production of *F. tricinctum* in seven solid media and in one liquid medium with variation in the supplementation of peptone or amino acid precursors. It was demonstrated that white bean solid media led to the highest enniatins yield in the growing condition chosen, after analysis using HPLC with UV-visible photodiode array detector. Solid corn was also used as substrate for enniatin production using *F. tricinctum*, the separation of the crude extract produced enniatins A, A1, B, B1, B4 and J (Cuomo *et al*., 2013). Furthermore, extraction of enniatins from crude liquid or solid media has been carried out using non polar solvents such as hexane and dichloromethane (Blais *et al*., 1991), ethyl acetate (Wang *et al*., 2013), chloroform (Madry *et al*., 1983) or moderate non polar solvent mixtures of water and methanol (Cuomo *et al*., 1991).

Biological activities of enniatins are mostly due to their ionophoric properties enabling them to translocate cations through bilayer membranes without the formation of membrane pores (Jestoi, 2008). Enniatins have shown antibiotic, insecticidal, anthelmintic and herbicidal activity in biological systems (Tomoda *et al*., 1992). A severe risk for humans associated with consumption of enniatins has been suggested by Cuomo *et al*. (2013) due to the induction of mitochondrial respiration resulting in the uncoupling of the oxidative phosphorylation.

Recently high enniatin concentrations in the range of several mg have been detected in food commodities with total enniatin (A, A1, B and B1) content up to 683.9 mg/kg in Tunisian sorghum (Oueslati *et al*., 2011), up to 1.1 mg/kg in commercial baby food samples (Juan *et al*., 2014), up to 0.10 mg/kg enniatins in Italian cereal and cereal products (Serrano *et al*., 2013), up to 1.8 mg/kg enniatins in nuts, dried fruits and dates from Spanish markets (Tolosa *et al*., 2013).

Due to the multiple effects of enniatins encompassing the possible medical uses and the toxic effects to human resulting from high concentration in food commodities, we intended to purify enniatins for the purpose of searching some potential detoxifying microorganisms.

This study was carried out with two general objectives. First we intended to examine two *F. tricinctum* strains for enniatins (A, A1, B and B1) production on solid white bean media. Our specific objectives were: to establish a HPLC-DAD protocol for the detection and quantification of enniatin from fungal crude extract matrices after fermentation on solid white bean media; to quantify enniatins A, A1, B and B1 in the crude extract of fungi and establish a kinetics of enniatin production by the two strains over 30 days in order to find the best enniatins (A, A1, B and B1) producer strain. Secondly we intended to purify enniatins from solid white bean culture using the best enniatin (A, A1, B and B1) producer strain found from the previous part of the study. Enniatins were purified for further use in microorganisms screening with the capacity of detoxifying enniatins (as described in chapter 3 of this thesis). For the second part of this study, the specific objectives involved: the separations of enniatins in the crude extract using flash chromatography, the identification of positive fractions using TLC and HPLC-DAD; further purification of enniatins using recrystallization and finally the determination of the level of purity and the percentage of each enniatin variant in the purified mixture.

2. Material and methods

Part I

2.1. Examination of two *Fusarium tricinctum* strains for enniatin production

2.1.1. Microorganisms and solid culture

F. tricinctum strains DSM 23357 and O32 used in this study originated from the Institute of Sugar Beet Research (Göttingen) and were isolated from sugar beet. To examine the production of enniatins (A, A1, B1 and A) by the two above mentioned *Fusarium* strains, three agar plugs (5 mm of diameter) of each of the two *F. tricinctum* strains grown on potato dextrose agar (PDA, from Merck, Darmstadt, Germany) plates for 5 days at 25°C were inoculated on white bean (*Phaseolus vulgaris*, Bio Bohnen, weiß, Ökologische Landwirtschaft DE-ÖKO- 001 Nicht EU- Landwirtschaft) solid medium. White bean solid medium was prepared by autoclaving (120°C, 15 psi, 15 min; Tuttnauer- Systec/ 3870 EL) 30 g white beans and 30 mL distilled water in 300 mL Erlenmeyer flasks. The cultures were incubated at 25°C (Schütt Lichtthermostat) for 30 days. Every 3 days, 3 flasks were collected and frozen at -20°C until the extraction date. Three repetitions were made for each *Fusarium* strain and each time point.

2.1.2. Extraction of enniatins and High Performance Liquid Chromatography analysis

The total culture of each flask was ground in a mortar; two extractions were performed using 75 mL of EtOAc each by shaking using a shaker (Adolf Kühner AG Basel, Switzerland) at 175 RPM at room temperature for14 hours and 6 hours respectively. The cultures were filtered under vacuum, the two extracts were mixed together and the 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 oily extract.

High Performance Liquid Chromatography (HPLC) analysis of the ethyl acetate extracts was performed using a HPLC system equipped with a diode array detector (DAD) at 210 nm. The HPLC instrument consisted of: a pump (PU-2085 Plus Semi-Micro HPLC Pump, Jasco Inc.), a detector (Varian Prostar PDA Detector), a sample injecting system (AS-2059 Autosampler, Jasco), a proportioning valve (LG-2080-04, 4-solvents), a degasser (DG-2080-54, 4-solvents), a hardware interface between the computer and the system components (Jasco LC

Net II / ADC), a C18 reversed phase column (Polaris C18-Ether, HPLC column, 180Å, 5 µm, 100 x 2.0 mm, Agilent) in an oven (set at 40°C). The separation was achieved using a gradient elution with distilled water (solvent A) and methanol (solvent B) at a flow rate of 0.2 ml/min. The composition of mobile phase was: solvent A: H_2O + 7mM acetic acid + 5% ACN; solvent B: MeOH + 7mM acetic acid. The mobile phase gradient was as follows: 0-4 min 70% B-98% B; 4-13 min 98% B-98% B; 13-14 min 98% B- 70% B 14-20 min 70% B-70% B.

Each extract was dissolved in 15 mL methanol; diluted 1:100 in methanol HPLC grade, filtered using micro-filters (25 mm, 0.20 µm, WICOM PERFECT-FLOW®) and 10 µL were injected into the column. The enniatin variants (A, A1, B, B1) were identified according to comparison of the retention times and mass spectra with the standards, the concentrations were calculated with the use of the calibration curve of enniatin A, A1, B and B1 standards (from *Gnomonia errabunda*; ≥95% HPLC; Sigma-Aldrich Chemie GmbH Riedstr. 2D-89555 Steinheim). The quantification of enniatins was carried out on the basis of a comparison of peak areas with the calibration curves of standards.

2.1.3. HPLC-MS confirmation of produced enniatins

Confirmation of enniatin presence was carried out using a mass spectrometer system made of solvent delivery module (Varian Prostar 210), an autosampler (Varian Prostar 410), an ion trap 800 MS, an oven (Varian Model 510 Prostar). A C18 reversed phase column (Polaris C18-Ether, HPLC column, 180Å, 5 µm, 100 x 2.0 mm, Agilent) in an oven (temperature set at 40 °C) was used. Liquid chromatography conditions were set up using a constant flow rate at 0.2 ml/min and the injection volume was 10 µL. Methanol and water (7 mM acetic acid + 0.01 mM sodium acetate) were used as mobile phase in gradient elution consisting of a linear increase of methanol 70% to 98% for 4 minutes, then the methanol was maintained at 98% for 9 minutes, and decreased to 70% for 2 minutes and finally hold for 5 minutes at 70%. The instrument was configured in the positive ionization mode. The injection volume was 10 µL. The sodium adduct ions 662.6; 676.6; 690.6; 704.5 were used as precursor ions for enniatin B, B1, A1 and A respectively. The daughter ions were: 549.4, 449.4 and 336.4 for enniatin B; 563.4, 463.4 and 336.4 for enniatin B1; 563.4, 463.4 and 350.5 for enniatin A1; 577.5 and 350.3 for enniatin A.

2.1.4. Statistical analysis

Evaluation of the enniatins A, A1, B, B1 production by two *F. Tricinctum* strains DSM 23357 and O32 was done in three replicates for each fungi and each time point (3 days). The data was analyzed statistically using the software IBM SPSS for Macintosh version 22.0 (2013). Analysis of Variance (ANOVA) and post-hoc Tukey test in a completely randomized design were performed for the comparison of means of dependent variables, two tailed P values were considered as statistically significant on the level 0.05.

Part II

2.2. Large scale production of enniatins

2.2.1. Microorganism and culture

According to the results obtained in the screening test, *F. tricinctum* O32 was retained for the large scale production of enniatin. The solid culture medium was prepared by autoclaving (120°C, 15 psi, 15 min; Tuttnauer- Systec/ 3870 EL) 200 g white beans (*Phaseolus vulgaris*) (Bio Bohnen, weiß, Ökologische Landwirtschaft DE-ÖKO- 001 Nicht EU-Landwirtschaft) and 200 mL distilled water in 1 L Erlenmeyer flasks. Five flasks were prepared. Twenty-five agar pieces (5 cm diameter) of *F. tricinctum* O32 grown for 5 days on PDA (Merck, Darmstadt, Germany) plates at 25°C were inoculated under sterile conditions in one Erlenmeyer flask containing the solid white bean medium. The fungus was grown at 25°C for 30 days followed by extraction.

2.2.2. Extraction and quantification of enniatins

At time of harvest, the culture media containing mycelia were cut into small pieces with a knife inside the flask and 400 mL of ethyl acetate (EtOAc) were added to each flask. Enniatins were extracted on a shaker (Adolf Kühner AG Basel, Switzerland) at 175 RPM, at room temperature for 4 days and filtered under vacuum, the extraction was repeated a second time with the same volume of EtOAc for 3 days. The two filtrates were mixed together and the solvent was concentrated in a 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 finally dried overnight inside the SpeedVac (Christ RVC 2-25 CD plus, 35°C,

30 mbar) to remove the residual solvent from the extract. The solvent evaporation resulted in an oily extract of 15 mL which was diluted with 2.5 mL methanol. A serial dilution was performed on a fraction of the extract and 10 µL were injected in HPLC for the quantification of the enniatins in the crude extract as described previously (subheading 2.1.2).

2.2.3. Preliminary purification of enniatins using flash chromatography

The crude extract was purified with flash chromatography (Sepacore® Flash system X10/X50) using a constant flow rate of 25 ml/min. The flash chromatography device consisted of: a pump Module C-601/C-605, disposable plastic cartridge of 31×194 mm (Diameter×Length, Buchi Sepacore®) (loaded with C18, average pore size 60 Å, pore volume 0.8 mL/g, specific surface area 500 m^2/g, particle size 40-63 µm), a UV-Visible Detector C-640, a fraction collector C-660 and a computer with the SepacoreControl software. The mobile phase was composed of methanol and distilled water. The elution was performed with a step gradient starting with 100% water for 15 min, then changed to 75% in 5 min, held for 25 min at 75% and changed to 40% in 5 min, the gradient was maintained at 40% MeOH for 35 min before being reduced to 20% in 5 min and kept for 50 min at this concentration, methanol percentage was changed again to 0% for 5 min and kept at this concentration for 35 min. The pressure was constant between 2-6 bars during the whole separation. The injection volume was 10 mL and the detection wavelength was 210 nm. The collection time was 2 minutes. In total, 92 fractions were collected and aliquots of 1 mL of each was collected and transferred in a 1.5 mL Eppendorf tube, the elution solvent was evaporated in the SpeedVac (Christ RVC 2-25 CD plus, 35°C, 30 mbar) and the content of each Eppendorf tube was re-suspended in 1 mL methanol (HPLC grade) then diluted 1/10 and 1/1000 for checking the positive fractions using TLC and HPLC with UV-visible photodiode array detector (HPLC-DAD) respectively.

2.2.4. Screening of flash chromatography fractions

TLC

The fractions eluted from the flash chromatography separation were monitored by TLC on normal phase silica gel plates (40×80 mm, ALUGRAM® Xtra SIL G/UV_{254}) using DCM:MeOH (10:1; v/v) as mobile phase. The crude extract was diluted 1/100 and 1 µL was spotted on the TLC plate, for the mixture of enniatin A, A1, B and B1 standards (1 mg/mL) and the flash fractions, 3 µL and 1 µL were spotted respectively. After development, the TLC plate was placed in a tank with iodine granules. The spots were visualized as brown spot and photographed under UV light at 254 nm. The retention factor (Rf) of each fraction was calculated according to the formula below. In order to find the positive fractions, Rf value of the flash chromatography fractions was compared with that of enniatin standards. The weight of each positive fraction was recorded and they were mixed together.

$$Rf = \frac{Distance\ travelled\ by\ the\ compound}{Distance\ travelled\ by\ the\ solvent\ front}$$

HPLC-DAD

HPLC-DAD analysis for the detection of positive fraction resulting from flash chromatography separation was performed using the protocol described previously (subheading 2.1.2). Enniatins identification was performed by comparing retention times and UV spectra of compounds present in each fraction and the pure standards.

2.2.5. Purification of enniatin using recrystallization

For purification of enniatins using recrystallization, 500 mg of positive fractions were mixed with 3mL heated methanol (about 50°C) and allowed to cool down for 10 minutes at room temperature, then 1.5 mL distilled water was added. The enniatins contained in the mixtures were allowed to recrystallize at room temperature overnight and then kept on ice for 6 hours. The crystals were filtered under vacuum using a filter paper (Ederol quality, Nr. 75, Ø11cm) and rinsed with a cold mixture of methanol:water (v/v 2:1), then dried in a desiccator for 3 days. Recrystallization was carried out as described above to obtain a mixture of pure enniatins A, A1, B1 and B. The proportion of each enniatin variant in the recrystallized fractions was determined using HPLC-DAD as described previously (subheading 2.1.2).

Purity check of the purified enniatin mixture was done using HPLC-DAD with the same conditions as mentioned previously (subheading 2.1.2) except that the mobile phase gradient used was as follows: 0-100 min 2% B-98% B; 100-115 min 98% B-98% B; 115-120 min 98% B-2% B; 120-130 min 2%B-2% B. Solvent B was MeOH + 7 mM acetic acid. The level of purity was calculated by quantification of enniatins present in the mixture and comparison to the mass of the mixture used.

3. Results-Part I

3.1. Detection of enniatin standards and crude extract using HPLC

Typical chromatograms of enniatin B, B1, A1 and A are presented in Figure 2, showing a clear separation of enniatin variants using HPLC with UV-visible detector. A separation and detection method was successfully developed for the separation of enniatins. The retention times were 8.13 min; 8.45 min; 8.85 min; and 9.04 min for enniatins B, B1, A1 and A respectively. The optimal detection wavelength was found to be 210 nm.

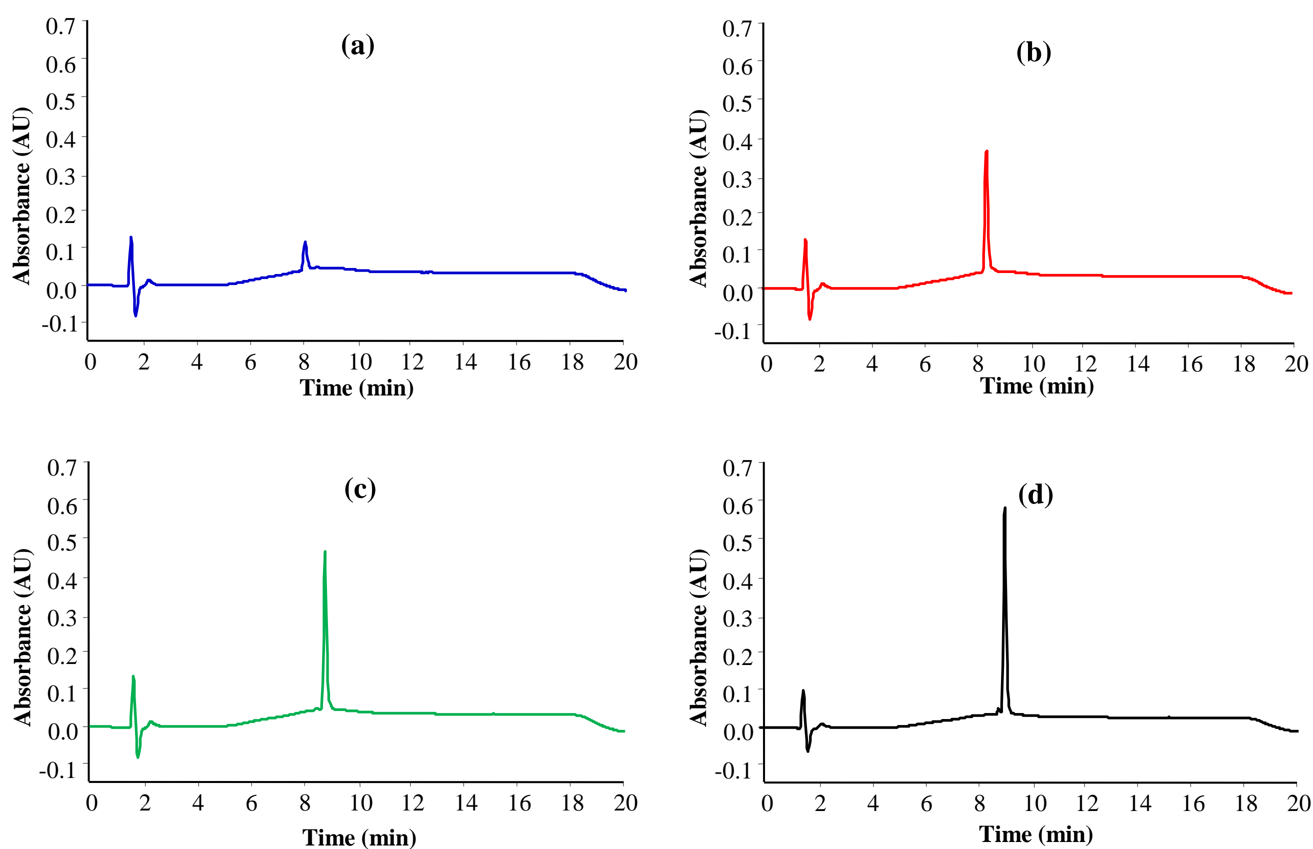


Figure 2: HPLC-UV Chromatograms of enniatin B (a), B1 (b), A1 (c) and A (d) standards at concentration 5 ppm.

Figure 3 below shows a typical HPLC-UV chromatogram of ethyl acetate crude extract originating from fermentation of *F. tricinctum* O32 on solid white bean medium. The chromatogram patterns of crude extracts from both *F. tricinctum* strains used were similar. The fermentation of the two *Fusarium* strains on solid white bean medium produced enniatin B, B1, A1 and A. The peaks were attributed to enniatin variants according to the retention times and UV spectra. The retention times were 8.13 min; 8.53 min; 8.85 min; and 9.12 min for enniatin B, B1, A1 and A respectively.

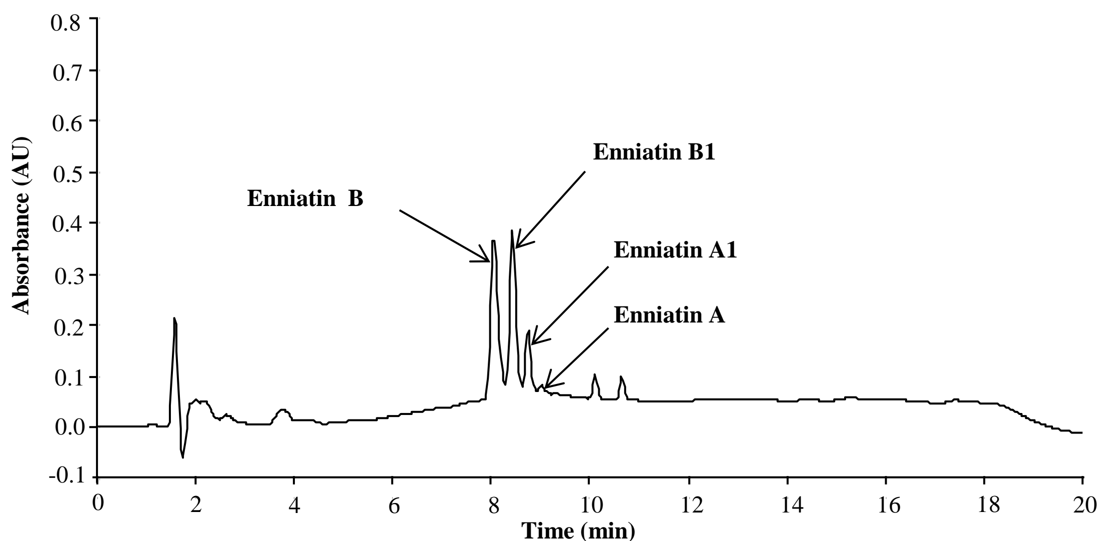


Figure 3: HPLC-UV chromatogram of a sample of *F. tricinctum* O32 after an incubation of 24 days grown on white beans and showing enniatins B, B1, A1 and A.

3.2. Calibration curves

Calibration curves of enniatin B, B1, A1 and A are presented in Figure 4. Correlation coefficients ranging from 0.992 to 0.999 indicated linearity within the used concentration ranges.

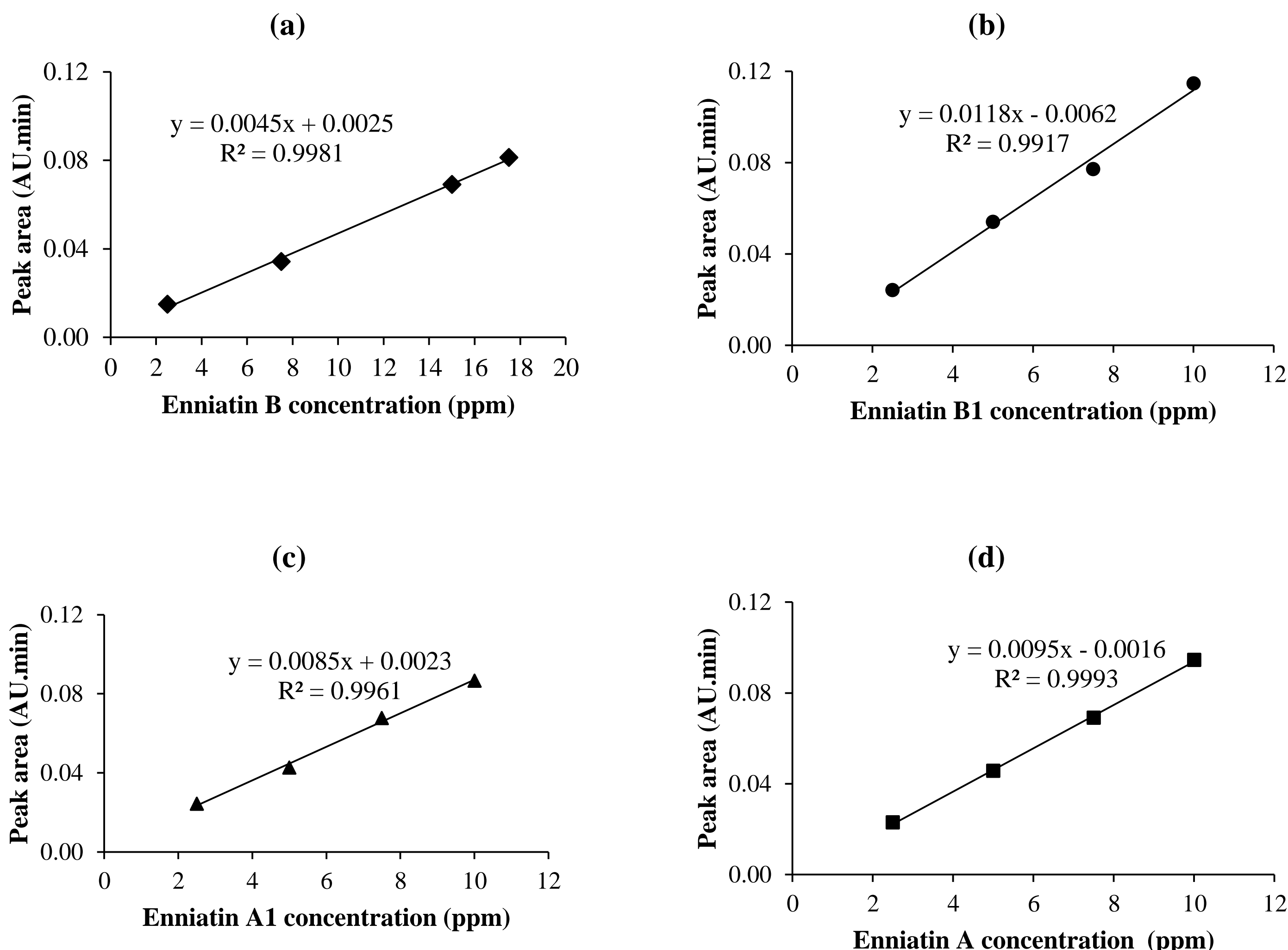


Figure 4: Calibration curves of enniatin standards B (a), B1 (b), A1 (c) and A (d).

3.3. Assay validation

Limit of detection and limit of quantification for enniatin A, A1, B1 and B were determined according to a signal/noise ratio of 3:1 and 10:1 respectively. The limit of detection was 1.23 ppm; 3.98 ppm; 1.78 ppm; 0.78 ppm for enniatins B, B1, A1 and A respectively. The limit of quantification was 4.091 ppm; 13.26 ppm; 5.94 ppm; 2.61 ppm for enniatins B, B1, A1 and A respectively. The recoveries were calculated by diluting enniatin B, B1, A1 and A standards

in pure methanol and quantifying them using HPLC-DAD. The mean recovery value from four replicates in concentrations ranging from 2.5 to 10 ppm for enniatins B1, A1 and A and 2.5- 17.5 for enniatin B was 100.47%; 100.35%; 99.97%; 93.70% for enniatins B, B1, A1 and A respectively. All the samples were pretreated and the enniatin concentrations were within the measureable range. Values of the data analysis of calibration curve are summarized in Table 1.

Table 1: Construction of calibration curve and analysis of the data

Parameters	Enniatin B	Enniatin B1	Enniatin A1	Enniatin A
Accuracy (%)	100.47±6.62	100.35±4.11	99.97±3.95	93.70 ±3.46
Slope	0.005	0.012	0.009	0.010
Intercept	0.003	-0.006	0.001	-0.002
Correlation of determination R^2	0.998	0.992	0.996	0.999
Correlation coefficient R	0.999	0.996	0.999	1.000
Linearity range	2.5-17.5	2.5-10	2.5-10	2.5-10
Standard error of intercept	0.002	0.005	0.003	0.001
Standard deviation of intercept	0.003	0.010	0.005	0.002
LOD (ppm)	2.211	2.661	1.772	0.776
LOQ (ppm)	7.369	8.870	5.906	2.585

3.4. Enniatin production by *F. tricinctum* strains O32 and DSM 23357

Production of enniatin A, A1, B1 and B by *Fusarium* strains O32 and DSM 23357 was monitored over a period of 30 days (Figure 5). In Appendix, Figure i shows the time course of enniatin production by two studied *Fusarium* strains expressed in ppm. The concentrations of enniatin B and A1 after 3 culture days were below the limit of detection and therefore were not considered in these calculations, for the same reason the concentrations of enniatin A were not considered at any time point. Total enniatin content mentioned refers to enniatin B, B1 and A1. The amount of total enniatin content produced by strain O32 over 30 days was significantly different ($P<0.05$) from the total enniatin content produced by strain DSM 23357 (statistics not shown). For *Fusarium* strains O32 and DSM 23357, the total enniatin concentration reached a maximum of 1228 mg/L and 959 mg/L respectively after 24 culture

days corresponding to 614 g/kg and 479g/kg white bean used respectively. Total enniatin production increased gradually until day 18 amounting 950 mg/L and 479 mg/L for strains O32 and DSM 23357 respectively and declined at day 21. For both strains, no significant difference (P>0.05) was detected among the values of the total enniatin concentrations at day 18 and day 21. The total enniatin concentrations peaked at day 24 (1228 mg/mL, 959 mg/mL for strains O32 and DSM 23357 respectively) and declined to reach about 1095 mg/L and 674 mg/L for strains O32 and DSM 23357 respectively at day 30. However, no significant (P>0.05) difference was observed among total enniatin contents at day 24, day 27 and day 30 for strain O32. While no significant difference (P>0.05) was observed among the mean values of total enniatin content produced by strain O32 at day 24 and day 30, the mean values of total enniatin content produced by strain DSM 23357 at day 30 and day 24 were significantly different (P<0.05). Under the culture conditions set for this study and the *Fusarium* strains used, the optimal harvest time for enniatin A1, B and B1 production is between days 24 and 30. In general, throughout the 30 days, *F. tricinctum* strain O32 produced about two times more enniatins (B, B1 and A1) than the strain DSM 23357; therefore it was used for the large scale production of enniatins in the next step of this study.

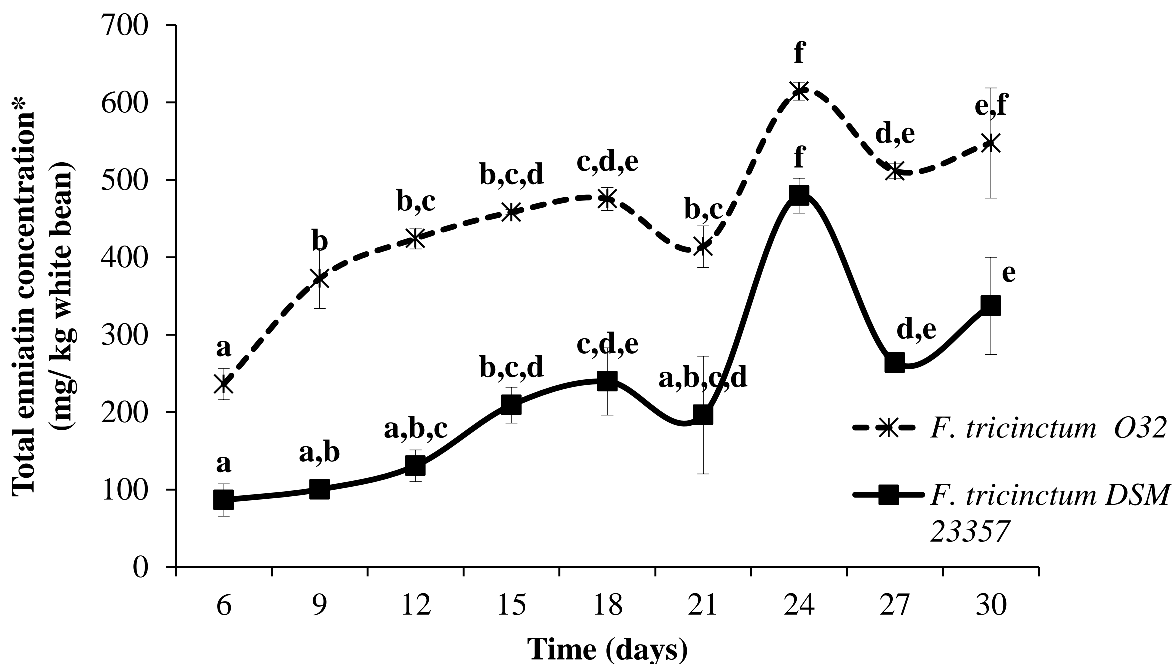


Figure 5: Time course of mean values of total enniatin (* refers to enniatins B, B1, and A) content in mg/kg medium produced by *F. tricinctum* strains O32 and DSM 23357 on solid white bean medium from day 6 to day 30. *a,b,c,d,e,f: Markers carrying the same letter within the same the line chart represent mean ± Standard deviation statistically not different P>0.05; n=3.*

3.5. HPLC-MS confirmation of produced enniatins

The analysis of the ions seen in HPLC-MS showed $[M+Na]^+$ ions at m/z 662.6, 676.6, 690.6 and 704.5. The daughter ions obtained after fragmentation of each precursor ion are summarized in Table 2. Chromatograms of the above mentioned precursor ions and the mass spectra of daughter ions are shown in Figure 6. The retention times of the analyzed precursor ions were 4.31 min, 4.73 min, 5.02 min and 5.20 respectively.

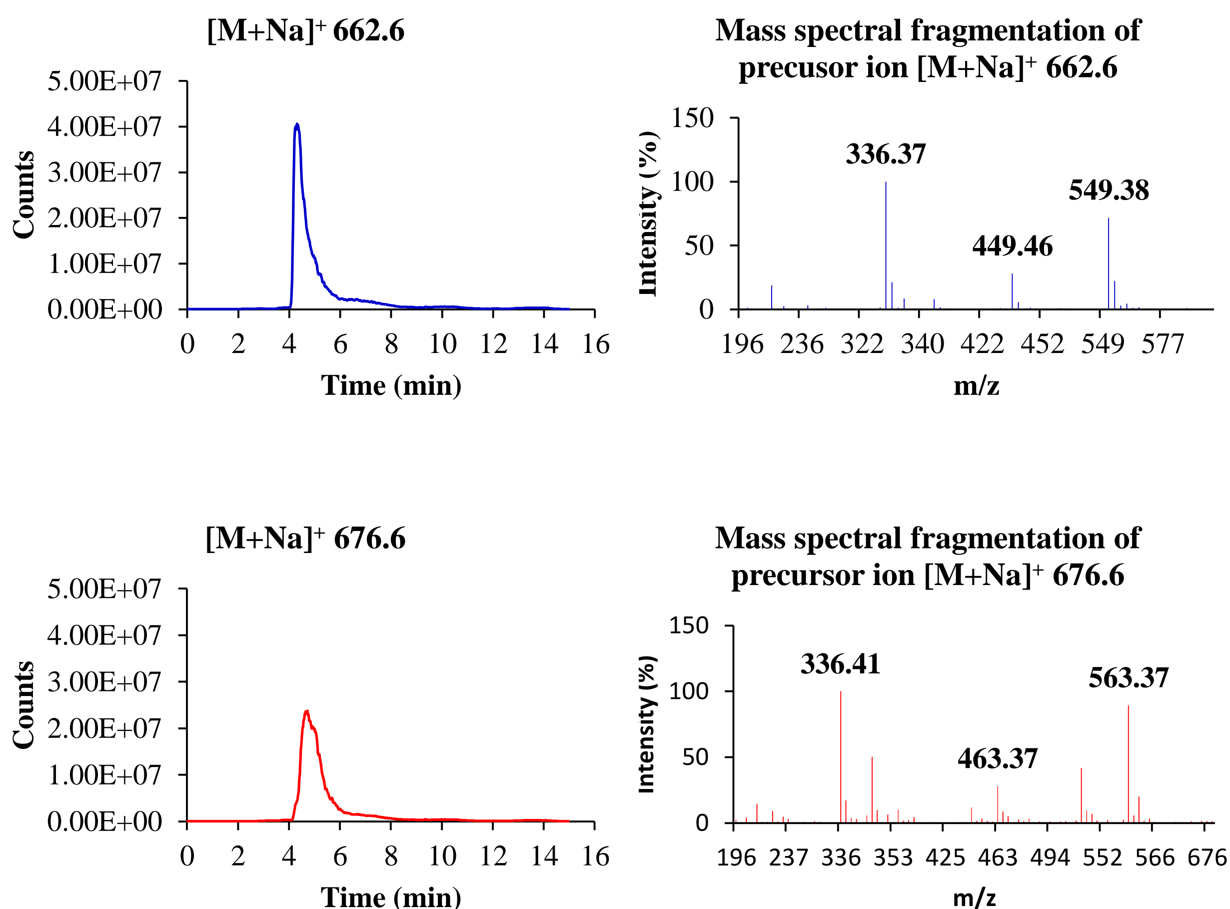


Figure 6: Precursor ions chromatograms and mass spectra of enniatins B, B1, A1 and A from the fungal extract of *F. tricinctum* O32 grown on solid white bean medium.

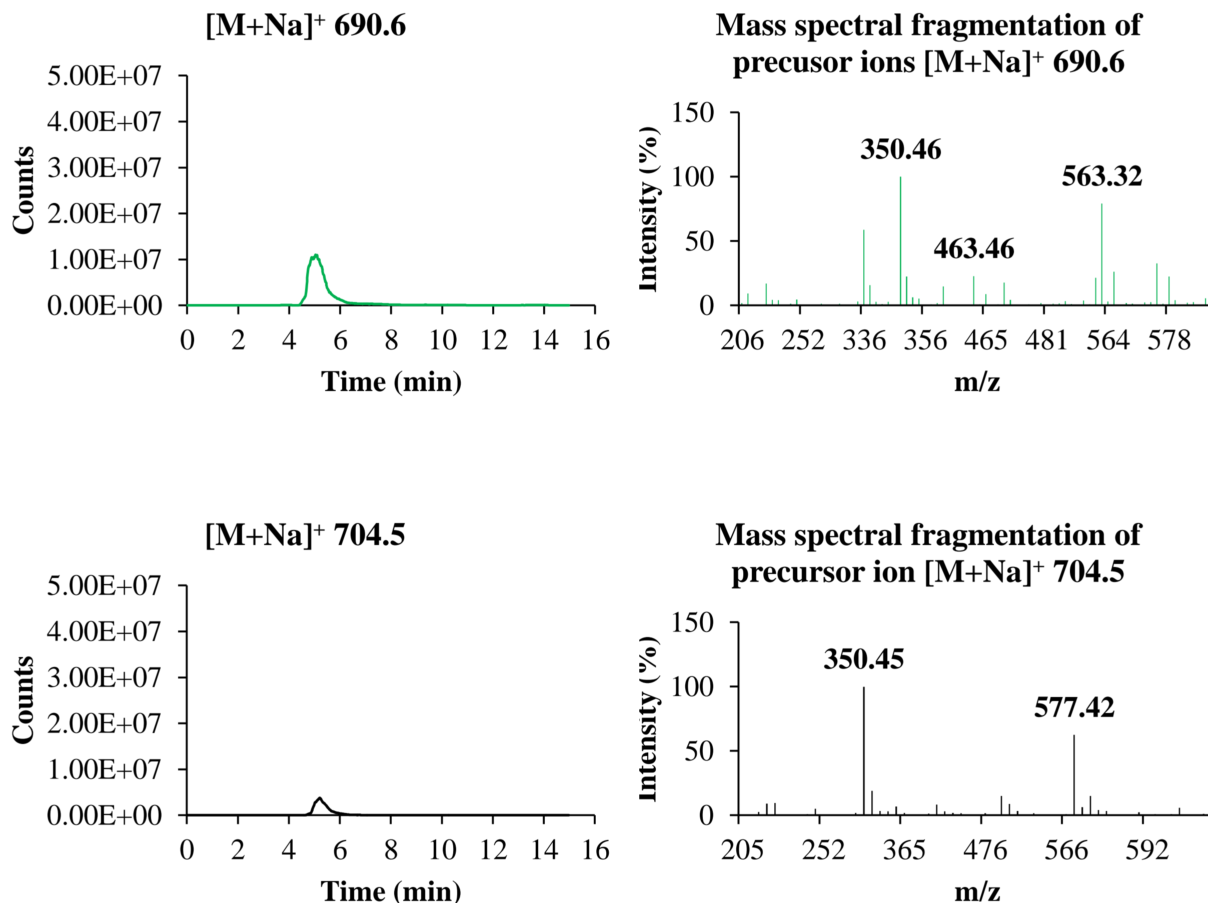


Figure 6 (cont.): Precursor ions chromatograms and mass spectra of enniatins B, B1, A1 and A from the fungal extract of *F. tricinctum* O32 grown on solid white bean medium.

Table 2: Daughter ions of the analyzed precursor ions

Precursor ions $[M+Na]^+$	**Daughter ions $[M+Na]^+$**		
662.6	549.38	449.46	336.37
676.6	563.37	463.37	336.41
690.6	563.32	463.46	350.46
704.5	577.42	350.45	

3.6. Relative proportion of enniatin variants

In Figure 7 the mean of relative proportion of enniatin B, B1 and A1 content measured every three days from the 6th to 30th culture day is presented. Both *F. tricinctum* strains O32 and DSM 23357 show a similar pattern with regards to proportion of each enniatin variant in the cultures. The major variant was enniatin B accounting for more than 65% of the total enniatin content in the extract, followed by enniatin B1 and A1 accounting for more than 25% and less than 10% of the total enniatin content respectively.

The variation of percentage of each enniatin variant over the time was consistent in the crude extract of *F. tricinctum* O32. For enniatin B for example the percentage was between 63% and 66% during the study. In contrast to strain O32, the relative percentage of enniatin B produced by *F. tricinctum* DSM 23357 was not consistent and varied from 54% to 79% during the study (data not shown).

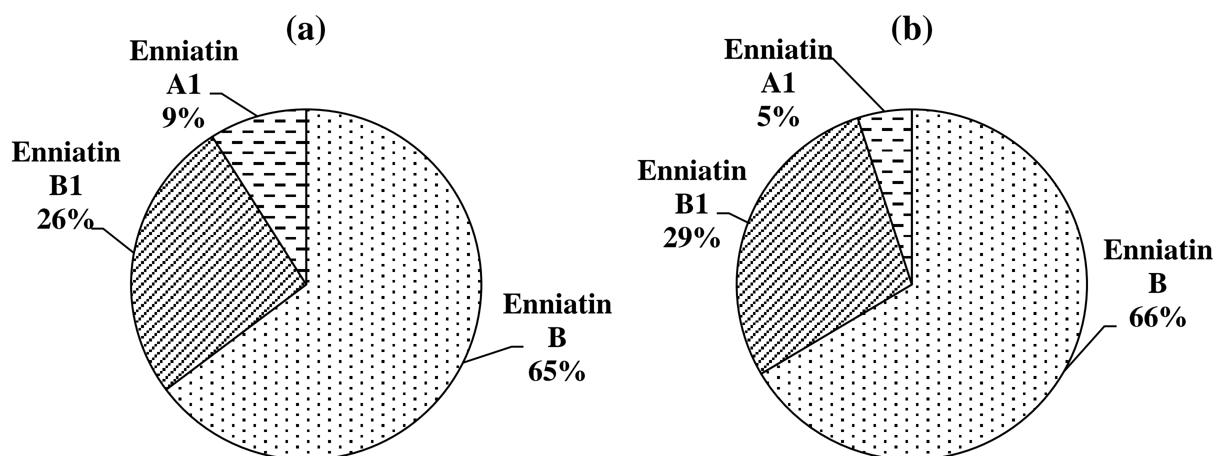


Figure 7: Relative percentage of enniatins (A1, B and B1) produced by strains *F. tricinctum* O32 (a) and *F. tricinctum* DSM 23357 (b) after 30 days on solid white bean medium.

4. Discussion-Part I

4.1. Enniatin production by *F. tricinctum* strains O32 and DSM 23357

This part of the study was carried out in order to compare *F. tricinctum* strains O32 and DSM 23357 regarding their enniatin production on white bean medium over 30 days at 25°C. Total enniatin produced during this study is comparable with data reported in other studies. For instance, Wang *et al*. (2013) reported enniatin A, A1, B1 and B yield of 1365 mg/L by *F. tricinctum* AB 470859.1 cultured on white bean medium during 18 days, while in the present study a yield of enniatins A1, B1 and B reached 1228 mg/L and 959 mg/L after 24 days on solid white bean medium by *F. tricinctum* strains O32 and DSM 23357 respectively. Likewise, Meca *et al*. 2010 cultured *F. tricinctum* CECT 20150 on white corn solid medium during 30 days and recorded a yield of 1320 mg/L of enniatin A, A1, B, B1. Some earlier studies reported higher enniatin yields than the yield obtained during our study. For instance, Audhya and Russel (1973) reported a yield of 2000-3000 mg/L of enniatin A, B and C after 28 days fermentation of *F. sambucinum* Fuckel HLX316 in liquid medium supplemented with lactose and tryptone. In a study carried out by Madry *et al*. (1983), 5000 mg/L of enniatin A, A1, B, B1 were obtained after incubation of *F. oxysporum* ETH 1536/9 in a chemically defined liquid medium supplemented with amino acids for four days. In a further study, Herrmann *et al*. (1996) characterized thirteen *Fusarium* strains capable of producing enniatins in diverse media; of thirty-six tested strains, *F. merismoides* BBA 64329, *F. lateririum* BBA 65090 and *F. sambucinum* BBA 63933 exhibited the highest enniatin concentration of 500 mg/L, 400 mg/L and 350 mg/L respectively after being cultured in solid chemically defined medium FDM containing fructose for the first two strains and in solid FCM for the third strain during 6 days. These authors assessed enniatins as a group of compounds with the aid of photometric methods, the lack of specificity in the method could be the reason why higher yields were obtained during their study. Besides, Firáková *et al*. (2008) described a total enniatin B, B1 and G of 350 mg/L after culturing *F. dimerum* in sabouraud maltose broth for 4 days.

The present study shows a divergence of the total enniatin content produced by two *F. tricinctum* strain O32 and DSM 23357 under the same conditions. In general, the strain O32 produced about two times more enniatins than strain DSM 23357. Similar findings have also been reported by Burmeister and Plattner (1987), after analyzing the production of enniatins by 13 strains of *F. tricinctum* grown on solid maize grit medium. A higher level of

polimorphism of fifty-eight collection strains from twenty *Fusarium* strains were detected in a study reported by Stepien *et al*. (2013). This findings support the idea that, the enniatin synthetase (ESyn1) gene divergence might result in differences in toxin biosynthesis among strains of *Fusarium* of the same species.

Solid state fermentation (SSF) provides greater advantages than submerged fermentation, therefore is an alternative to submerged fermentation for the production of biologically relevant compounds such as antibiotics, proteins or enzymes (Mojsov, 2010). The technique of SSF is defined as the fermentation involving solids in absence (or near absence) of water, however, the substrate must possess enough moisture to support growth and metabolism of microorganisms (Pandey *et al*., 2001). This is the case during this study, several authors have reported high yield of enniatins from solid media such as solid corn or white bean (Burmeister and Plattner,1987; Meca *et al.,* 2010; Wang *et al*., 2013).

4.1.1. HPLC-MS confirmation of produced enniatins

The presence of enniatin was confirmed by the presence of $[M+Na]^+$ ions at m/z 662, 676.6, 690 and 704.5 After HPLC-MS analysis of the samples. These compounds corresponded to $[M+H]^+$ ions at m/z 640, 654, 668 and 682 also reported in several previous studies (Monti *et al*., 2000; Uhlig and Ivanova, 2004, Sørensen *et al*., 2008, Meca *et al*., 2010) which were identified as enniatin B, B1, A1 and A respectively. Also, the daughter ions $[M+Na]^+$ for example at m/z 549.38, 563.37, 563.32 and 577.42 corresponded to daughter ions $[M+H]^+$ at m/z 527.3, 541.5, 541.2 and 55.4 reported by Cuomo *et al*. (2013).

4.2. Relative proportion of enniatin variants produced by *F. tricinctum* O32 and DSM 23357

In our study, enniatin B was the major constituent in the extracts of the two *F. tricinctum* strains, accounting for more than 65% of the total enniatin content followed by enniatin B1 and enniatin A1 present in about 25% and 10% respectively. The amount of enniatin A was not considered since the amounts were below the limit of detection. The high proportion of the other enniatin variants such as enniatin B would have been a risk for the HPLC equipment if the samples were concentrated to a greater degree.

Several studies report enniatin B as the highest concentration followed by enniatin B1, A1 and A in food commodities such as wheat grains (Jestoi *et al*., 2004). The contamination level of the wheat correlated with the presence of *F. arthrosporoides* and *F. tricinctum* (Jestoi *et al*., 2004). Uhlig *et al*. (2006) investigated norwegian oat, barley and wheat samples from 2000 to 2002, enniatin B, B1, A1 and A percentages were respectively about 64%, 28% 8% in oat, 70%, 24%, 5% and 1% in barley and finally 79%, 18%, 2% and 1% in wheat. More recently, Wang *et al.* (2013) demonstrated a similar trend in samples of *F. tricinctum* AB 470859.1 cultured in solid white bean medium for 18 days. The proportions obtained were 43%, 38%, 12% and 7% for enniatin B, B1, A1 and A respectively. Although the proportions are different from ours, the same order of prevalence of enniatin B, B1, A1, A is reported in several studies. No clear explanation about the factors commanding the distribution of enniatin variants produced by *Fusarium* species are available in the literature at present.

5. Results-Part II

5.1. Enniatin purification

Enniatins were purified at an earlier stage using flash chromatography and later on using recrystallization.

5.1.1. Screening of positive fractions after flash chromatography separation

TLC

In total 92 fractions of 50 mL each were collected. Results of the TLC of each individual fraction using a mobile phase DCM:MeOH (10:1; v/v) and normal phase silica gel plates are presented in Figure 8. The retention factor of enniatin standards was Rf= 0.52. Fractions 50 to 74 contained principally compounds having the same retention factor as the enniatin standards as it can be seen on plates 5, 6 and 7. The positive fractions (50-74) resulting from the flash chromatography separation yielded 3.17 g product corresponding to 4.75 g product/kg white bean used. Figure ii in Appendix shows the positive fractions after solvent evaporation.

HPLC-DAD

Fractions containing enniatins resulting from the analysis of all 92 fractions collected from the flash chromatography separation were analyzed using HPLC-DAD; Figure 9 shows HPLC-UV chromatograms of some fractions namely fractions 50, 54, 55, 58, 61, 64, 70 and 73. Peaks with retention times of 8.08 min, 8.64 min, 9.09 min, 9.81 min corresponding to enniatin B, B1, A1 and A respectively can be observed. This confirmed a preliminary separation of enniatins from impurities in the fungal extract.

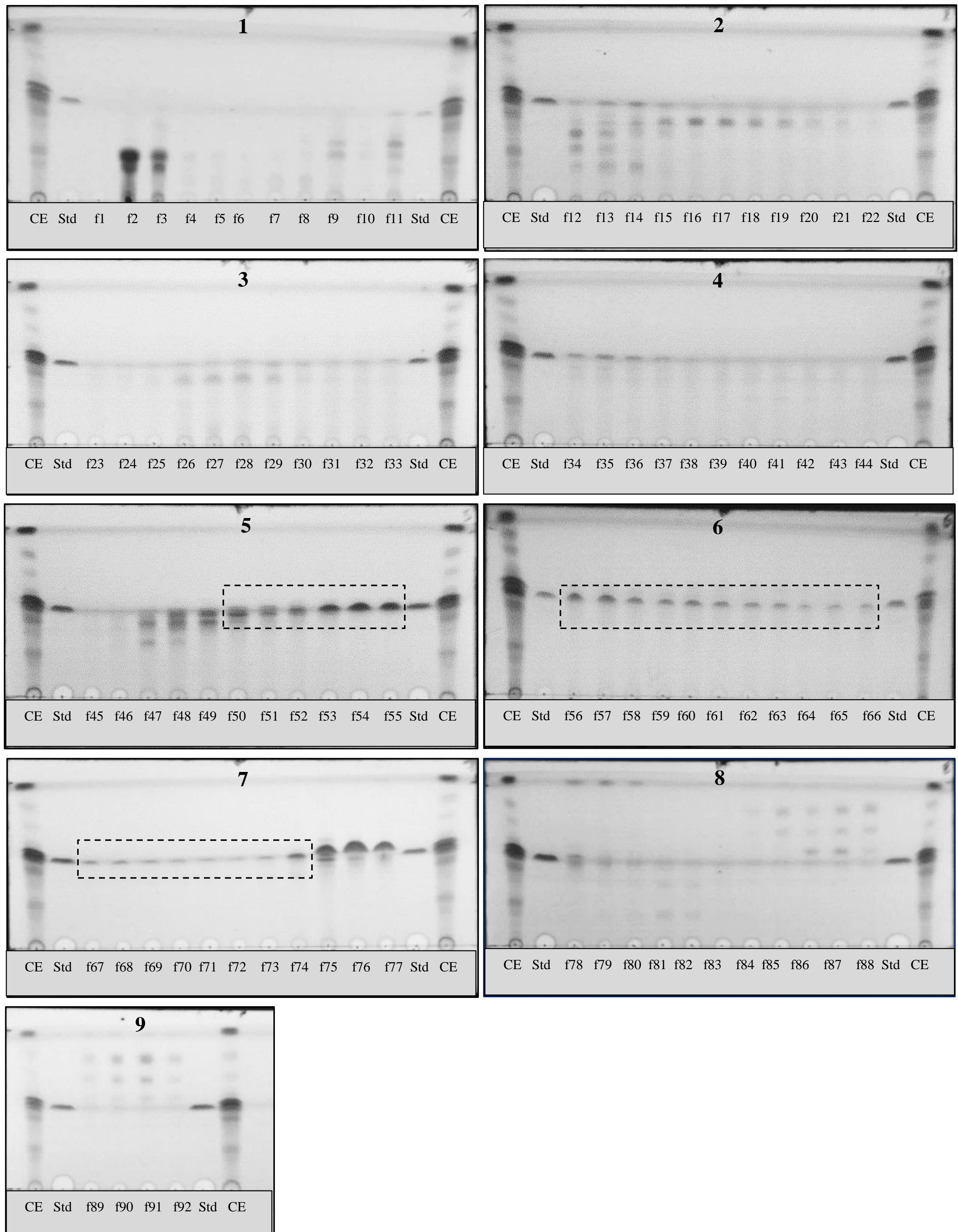


Figure 8: TLC plates of individual fractions resulting from flash chromatography separation; *CE: crude extract, Std: enniatin standards, f: fraction.*

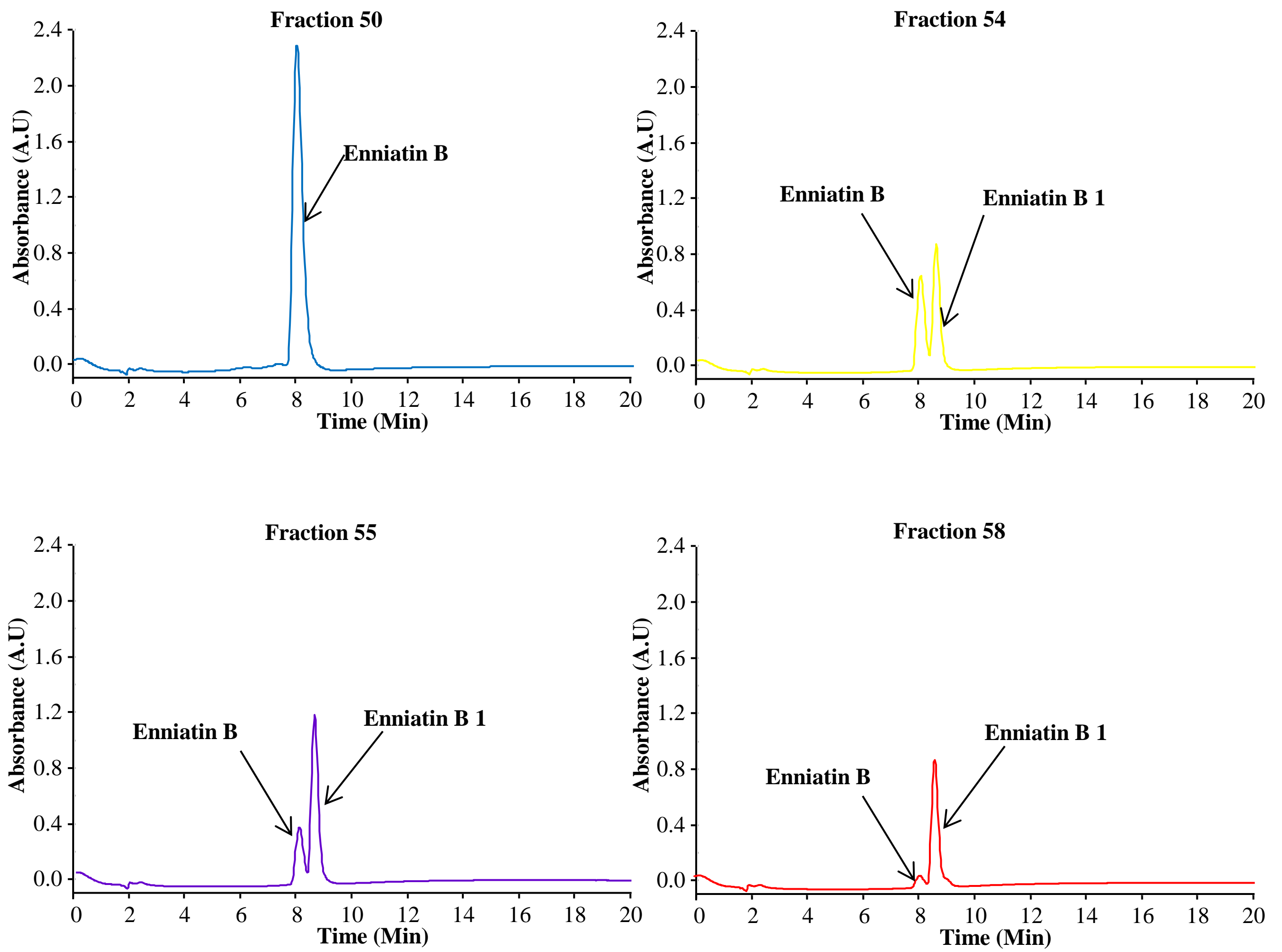


Figure 9: HPLC-UV chromatograms of fractions 50, 54, 55, 58, 61, 64, 70 and 73 resulting from the flash chromatography separation of enniatins in the ethyl acetate crude extract showing peaks corresponding to enniatin B, B1, A1 and A.

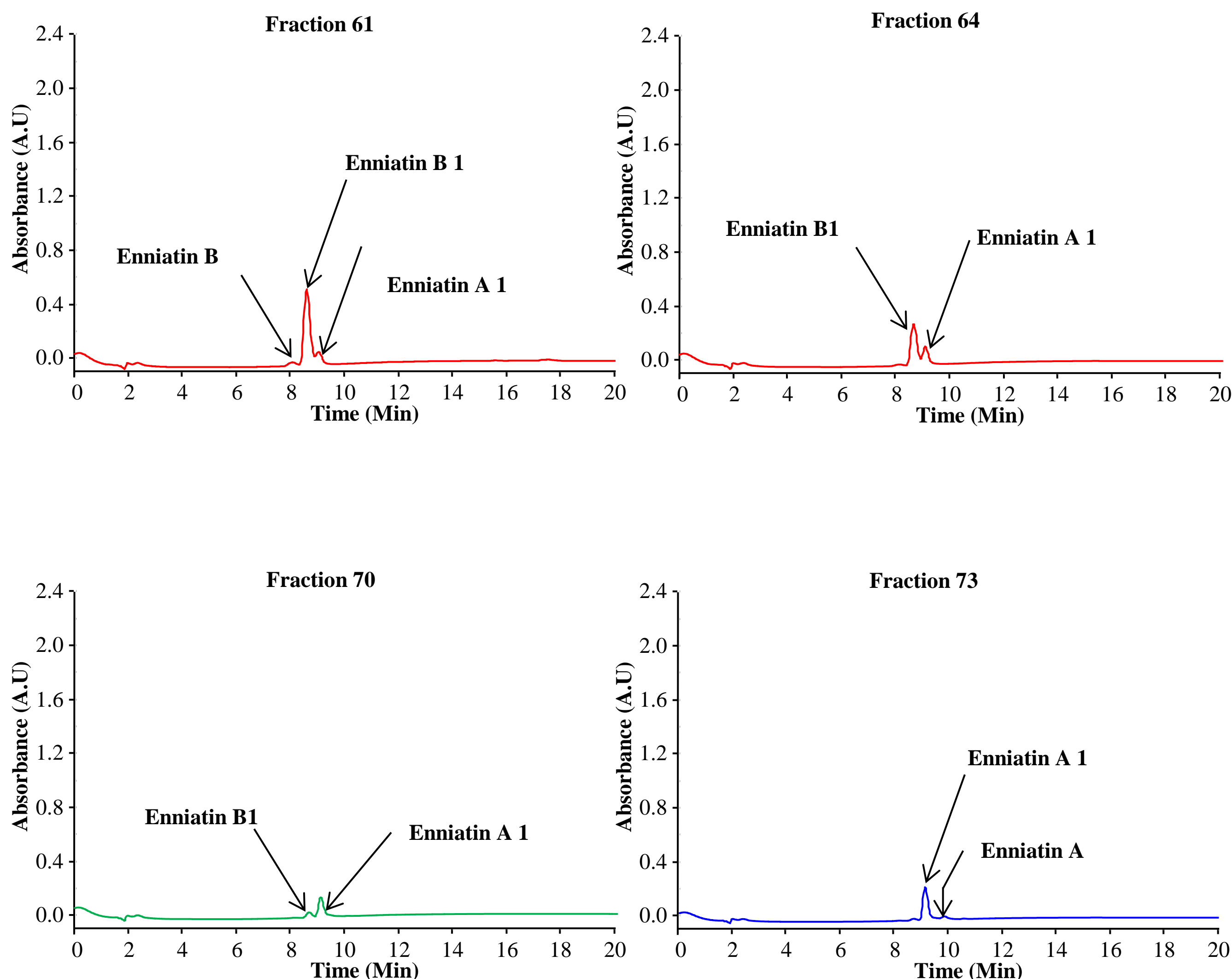


Figure 9 (cont.): HPLC-UV chromatograms of fractions 50, 54, 55, 58, 61, 64, 70 and 73 resulting from the flash chromatography separation of enniatins in the ethyl acetate crude extract showing peaks corresponding to enniatin B, B1, A1 and A.

5.1.2. Recrystallization

When subjected to recrystallization, the positive fractions yielded 2.12 g enniatins corresponding to 67% yield (Figure iii in Appendix) containing about 77% enniatin B, 17% enniatin B1, 6% enniatin A1 and 0.3% enniatin A. This corresponds to 3.18 g enniatins/kg white bean culture. The purity of enniatins B, B1, A1 and A in the recrystallized fraction was 96.25%. Figure 10 shows HPLC-UV chromatograms of the crude extract and that of enniatins after recrystallization. The retention times of enniatins B, B1, A1 and A were

73.81min, 75.97 min, 78.00 min and 79.81 min respectively. As it can be observed in Figure 10, chromatogram of recrystallized enniatins shows a more or less pure compound compared to that of the crude extract. Several peaks of compounds more hydrophilic than enniatins such as those eluting at 24.37 min, 40.55 min, 54.45 min, and other compounds more hydrophobic than enniatins with the retention times 85.41 min, 88.80 min, 102.55 min, 113.07 min and 115.37 min were no longer present in the purified fraction.

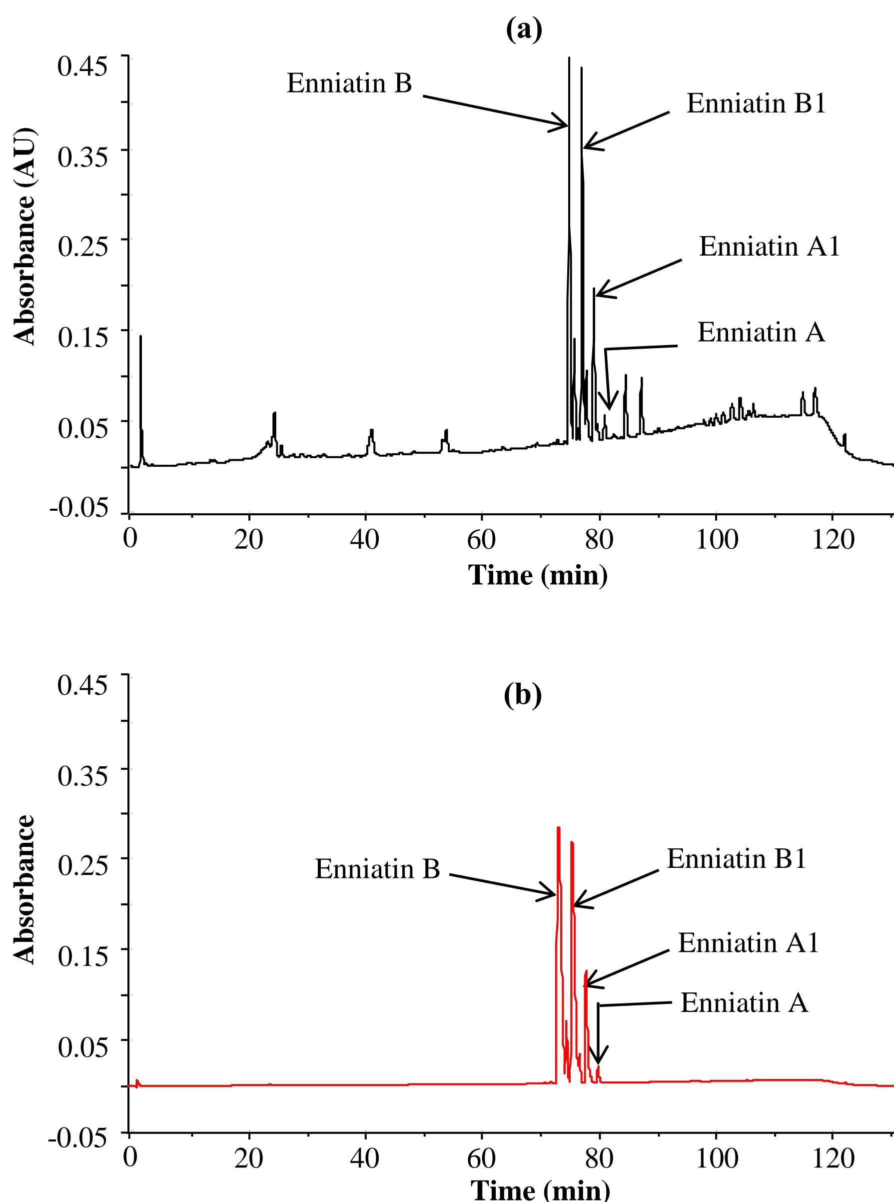


Figure 10: HPLC-UV chromatograms of the ethyl acetate crude extract of *F. tricinctum* O32 grown on solid white bean medium for 30 days (a) and that of enniatins purified by flash chromatography and recrystallization (b).

6. Discussion-Part II

F. tricinctum O32 was grown on solid white bean medium for 30 days, then extracted using ethyl acetate, the crude extract was submitted to flash chromatography, followed by recrystallization in order to obtain a pure mixture of enniatins A, A1, B and B1. During this study our aim was to obtain a pure enniatin mixture and not individual enniatin variants for further use in detoxification as reported in chapter 3 of this thesis. This was done so as to get a scenario in which the relative percentage of enniatin variants is more or less similar to what is encountered in nature.

Previous studies have reported the purification of individual enniatin variants using combination of various chromatographic technics. For instance, Meca *et al.*, 2010 purified enniatins B, B1, A and A1 from a fungal extract obtained after fermentation of *F. tricinctum* CECT 20150 on solid culture of corn during 30 days. The purification was achieved by low pressure liquid chromatography (LPLC) coupled to semi-preparative column liquid chromatography. Enniatins A (20 mg), A1 (46 mg), B1 (26 mg) and B (40 mg) were obtained from 100 g solid corn media. The fractions collected by the authors were analyzed using LC-MS/MS. According to the report of Meca *et al*., 2010, during enniatin purification, enniatin A eluted before enniatin A1 using LC-UV, regarding this fact the results obtained by those authors were different to the one obtained during our study and also during studies carried out by several other authors (Sørensen *et al*., 2008; Wang *et al*., 2013). The quantity of total enniatins (including enniatin A, A1, B1 and B) purified by techniques used by Meca *et al*., (2010) corresponded to 0.132 g/kg medium, whereas the yield obtained with the present method (using *F. tricinctum* O32, solid white bean medium, flash chromatography and recrystallization) was 3.2 g/kg medium. Flash chromatography is a faster preparative chromatographic technique for the routine purification of complex mixtures ranging in several grams (Still *et al*., 1978). By comparison of the results obtained during the present study and those of Meca *et al*. (2010), flash chromatography is a fast and automated technique that is appropriate for the large scale purification of enniatins, the flash cartridge of 31×194 mm (Diameter × Length, Buchi Sepacore®, loaded with C18, average pore size 60 Å, pore volume 0.8 mL/g, specific surface area 500 m^2/g, particle size 40-63 µm) used in our case was recommended for the separation of compounds ranging from 0.1-8 g. Thin Layer Chromatography was an easy and cheap method to find fractions containing mainly enniatins after flash chromatography separation. Moreover, precise insight on the composition of flash chromatography fractions was provided by HPLC-DAD showing that a separation of

individual enniatin variants from each other and from other contaminants in the fungal extract was possible (although the fractions were intentionally mixed together). This again highlighted the efficacy of flash chromatography in purification of enniatins.

Recrystallization is a procedure for purifying compounds involving the separation of a small amount of contaminant from desired solid compound (Harwood and Moody, 1989), provided the desired compound has the capability to form crystals. During the present study, a double solvent recrystallization with methanol and water was used as a second step for the purification of enniatins. A mixture of enniatins B, B1, A1 and A crystals composed of about 77% enniatin B, 17% enniatin B1, 6% enniatin A1 and 0.3% was obtained. Burmeister and Plattner (1987) reported the production of enniatins B and B1 by 11 *F. tricinct*um strains using MS/MS out of 13 tested, enniatins were purified using a combination of processes including methanol extract of the fungi cultured on white corn grit medium, a partitioning between chloroform and water, then a liquid extraction of the dried chloroform fraction using hexane and acetonitrile. Enniatins were then recrystallized by dissolution in boiling acetonitrile, followed by filtration. The compounds in the filtrate were allowed to recrystallize at -18°C during 16 hours, after this the crystals were rinsed with hexane and acetonitrile. Enniatin were confirmed by MS, MS/MS and NMR. About 1-3 g/kg enniatin B and B1 were prurified from the culture of *F. tricinctum* NRRL 26430. In contratry to our study, no enniatin A and A1 was purified by these authors probably due to the many hydrophilic extractions performed in the purification process, given that *F. tricinctum* produced relatively low amount of enniatins A and A1 compared to enniatin B and B1.

7. Conclusion

From this study, the following conclusions can be drawn:

The total enniatin B, B1 and A1 concentrations peaked at day 24 with 1228±23 mg/mL, 959±45 mg/mL equivalent to 614±12 and 479±23 mg/kg medium used for *F. tricinctum* strains O32 and DSM 23357 respectively.

The total enniatin B, B1 and A1 concentrations after 30 days were 1095±142 mg/mL, 674±126 mg/mL equivalent to 547±71 and 337±63 mg/kg medium used for *F. tricinctum* strains O32 and DSM 23357 respectively.

F. tricinctum strain O32 produced about two times more enniatin B, B1 and A1 than *F. tricinctum* strain DSM 23357 under same conditions. Enniatin production under same conditions is therefore highly dependent on the strains.

According to the culture conditions set for this study and the *Fusarium* strains used, the optimal harvest time for enniatin A1, B and B1 production is between days 24 and 30.

For both *F. tricinctum* strains used in our study, enniatin B was predominant in the fungal extract accounting of about 65% followed by enniatin B1 and A1 with relative percentages about 27% and 7% respectively.

The enniatin yield in the present study in comparison to previous studies shows that enniatin production varies according to species, culture condition and quantification method used.

The technique of purification of the fungal extract by flash chromatography coupled to recrystallization enabled a separation of enniatin A, A1, B and B1 from the impurities present in the extract, giving a mean purity of 96.25% of enniatin mixture. Criteria for determining the purity of the isolated enniatins were the quantification of enniatin variants using HPLC-UV related to the mass of compound analyzed. Flash chromatography also enabled the separation of individual enniatin variants. This is a fast method to achieve large scale purification of enniatins from the complex fungal extracts.

The purified enniatin mixture contained about 77% of enniatin B, 17% of enniatin B1, 6% of enniatin A1 and 0.3% of enniatin A.

A yield of about 3 g of enniatin mixture containing enniatin B, B1, A1 and A was obtained from 1 kg of white bean medium.

In this study an easy and efficient method for production and purification of enniatins A, A1, B and B1 with high yield and purity has been proposed.

8. References

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9. Appendix

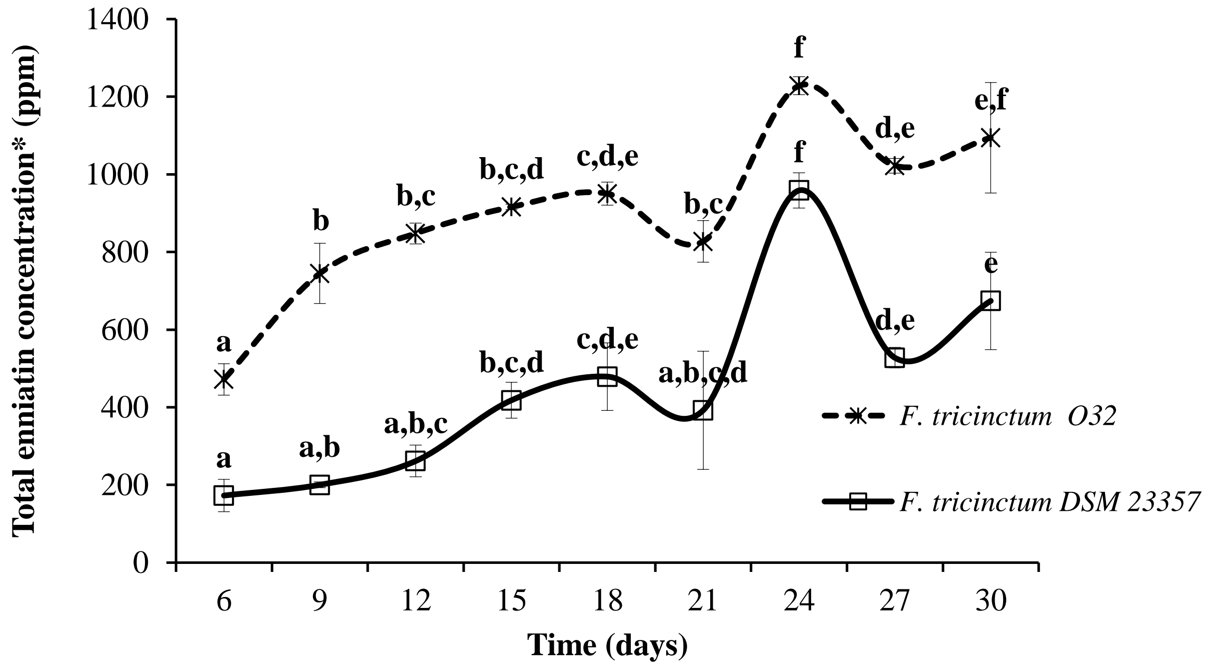

Figure i: Time course of means of total enniatin (B, B1 and A) content in ppm produced by *F. tricinctum* strains O32 and DSM 23357 on solid white bean medium from day 6 to day 30.

a,b,c,d,e,f: Markers carrying the same letter within the same line chart represent mean ± Standard deviation statistically not different P>0.05; n=3.

**Total enniatin concentration refers to enniatin B, B1 and A1 content.*

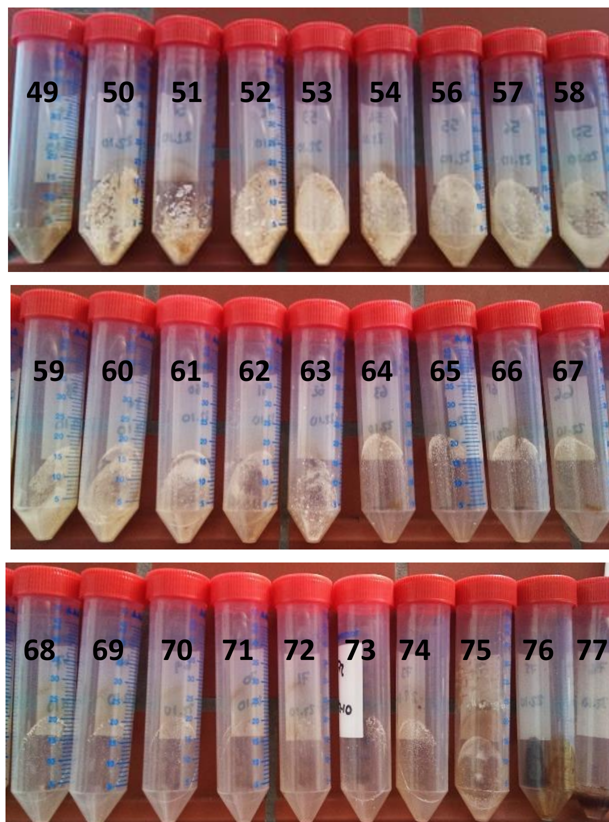

Figure ii: Fractions (including positive fractions, 50-74) resulting from flash chromatography separation after solvent evaporation in the SpeedVac.

The numbers of each fraction is written on the tubes.

Figure iii: Dried enniatin crystals after recrystallization.

Chapter 3

Biological detoxification of enniatins and identification of degradation products using HPLC-MS

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Abstract

This study was conducted to isolate enniatin-degrading microorganisms (fungi, yeast or bacteria) from sources including soil, water, grains, cereal-based food and fruits. Wash fluid from these materials was mixed with a minimal medium containing enniatins (final concentration 1 mg/ml) as unique carbon source and incubated for 7 days at 20°C. The cultures were diluted 25-times in a minimal medium with enniatins and incubated for further 21 days. The cultures were plated on full agar media. A total of 114 isolates of bacteria and/or yeast-like microorganisms and 34 fungal isolates were obtained. These strains were inoculated individually into the minimal media and a minimal medium containing a limited amount of glucose and tryptone with enniatins as sole carbon source as described above. After 3 days (bacteria) or 5 days (fungi) enniatins were extracted from culture supernatants and analyzed for enniatin content by HPLC-DAD (RP chromatography, detection at 210 nm). One bacterial strain and two fungal strains were able to transform enniatins into new products. Although enniatin content was reduced in most other cultures, new products were identified only in these three cultures using HPLC-DAD. Analysis including HPLC with full-scan-MS detection, differential metabolic profiling (noise reduction, chromatogram alignment, normalization) enabled the distinction of new signals in culture supernatants. Four enniatin degradation products resulting from the hydrolysis of presumably an ester bond in enniatin ring were identified.

Keywords: Enniatins, degradation, degradation products, HPLC-MS.

1. Introduction

Enniatins are cyclic hexadepsipeptides composed of alternating D-α- hydroxy- isovaleric acid (HyLv) and several N-methylamino acid residues such as valine (Val), leucine (Leu) and isoleucine (Ile) (Jestoi, 2008). Some damaging effects on plants have been associated to enniatins, for example the water uptake of cells in tomato shoots is affected by enniatins, which leads to toxic wilt and necrosis (Gäumann *et al*., 1960). In an *in vitro* toxicity test a mixture of enniatins A, A1, B and B1 characterised by necrosis was also observed in potatoe tubers depending on the incubation time and the concentration of enniatins applied (Hermann *et al.,* 1996). Moreover, a precise concentration of enniatins has been directly associated to a growth reduction of the wheat seed during germination, characterized by a higher inhibition of the root elongation compared to the leaf development (Burmeister and Plattner, 1987). The toxicity of enniatins is assumed to derive from their ionophoric properties and because they uncouple oxidative phosphorylation (Shemyakin *et al.*, 1969). More recently, (Cuomo *et al*., 2013) showed an increase in the mitochondrial respiration leading to an uncoupling of the oxidative phosphorylation as a result of the flux of K^+ ions into the mitochondrial matrix was caused by enniatins A, A1, B, B1 and J. The increase of the mitochondrial respiration suggested a severe risk for humans associated with consumption of enniatins. Besides these harmful effects of enniatins, they also possess some antibiotic properties which have been exploited in the production of an antibiotic known as fusafungine - a mixture of enniatin A, B and C - by *Fusarium lateritium* (Moffat, 1986). Furthermore, enniatins B, B1 and G were effective against some bacterial strains (*Bacillus subtilis, Candida albicans, Trychosporom cutaneum* and *Cryptococcus neoformans*) in agar diffusion tests by lowering the activity of the 12-lipoxygenase and therefore proved an antibiotic activity (Firákova *et al*., 2008). Another study highlights the fact that enniatins J_1 and J_3 could be potentially effective antibacterial agents against several pathogenic and lactic acid bacteria (Sebastià e*t al*., 2011). Likewise, enniatin B induced the inhibition of microorganisms of some normally intestinal tract pathogens such as *Escherichia coli*, *Salmonella enterica*, *Clostridium perfringens*, *Pseudomonas aeruginosa* (Meca *et al*., 2011).

High concentrations of enniatins reaching mg/kg have been detected in many food products including many cereals and cereal based products, but also in nuts and dried fruits (Jestoi *et al*., 2004; Yli-Mattila *et al*., 2008; Oueslati *et al.*, 2011; Meca *et al*., 2011; Zinedine *et al*., 2011; Mahnine *et al*., 2011; Serrano *et al*., 2013; Tolosa *et al*., 2013; Juan *et al*., 2014). Due

to lack of *in vivo* data of continual exposure to enniatins through food products, the chronic toxic effects of these compounds to humans are unknown (EFSA panel on contaminants in the food chain, 2014).

Biological detoxification is defined as the enzymatic degradation or transformation of toxins that lead to less toxic products (Bhatnagar, 1991). The enzymes involved in enzymatic degradation can originate from microorganisms, plants and animals. Unlike chemical and physical detoxification methods, biological detoxification has been poorly studied until the great advances in molecular and biotechnology techniques were developed. As mentioned by Karlovsky (1999) with the aid of such techniques it is possible to define the organisms, enzymes and genes responsible for the detoxification under specific conditions for further applications in the detoxification of agricultural systems, animal feed and food production.

Typical examples of the use of microorganisms for the biological detoxification of mycotoxins are reported in the literature. Early in 1966, Ciegler *et al*. demonstrated the ability of *Flavobacterium aurantiacum* to detoxify aflatoxin. Fusaric acid has been transformed by *Penicillium* spp., *Aspergillus fumigatus* and other fungi (Braun, 1960). The hydrolysis of the peptide bond of ochratoxin has been demonstrated by the activity of symbiotic microorganisms in the stomach of ruminant, leading to less toxicity of ochratoxin in ruminants (Hult *et al*., 1976). Duvick and Rood (2000) isolated bacterial strains *Nocardia globulera* or *Rhodococcus fascians* and *Bacillus sphaericus* leading to a reduction of beauvericin (structurally similar to enniatins) *in vitro* and *in vivo*. Deoxynivalenol (DON) - the major toxin produced by *Fusarium* in grains- has been enzymatically detoxified in several experiments, among others *Bacillus subtilis* was demonstrated to remove 80 % of DON after incubation with wheat and maize grains under anaerobic conditions (Cheng *et al*., 2010). *Bacillus licheniformis* CK1 reduced Zearalenone by 98% in Lysogeny broth and in corn kernels (Yi *et al*., 2011). A reduction of zearalenone of about 68% and 57% by *Pseudomonas alcaliphila* TH-C1 and *Pseudomonas plecoglossicida* TH-L1 respectively has been reported by Tan *et al*. (2014).

Considering that less research related to the biological detoxification of enniatins has been reported, the main aims of the present study were to screen microorganisms (fungi, bacteria or yeast) for the ablility to degrade enniatins into less toxic compounds and secondly to identify the degradation products. The specific objectives were to screen microorganisms based on their ability to grow on media containing enniatins as unique carbon source, to

select microorganisms able to degrade enniatins.Then to find new products from the culture media in which the microorganisms have been incubated in comparison with non inoculated media by looking for new signals after HPLC-MS and then identify the masses of new compounds. Finally our objective was to elucidate the molecular formula of the degradation compounds and to predict the nature of the degradation reaction.

2. Material and methods

2.1. Selection of microorganisms for enniatin detoxification

2.1.1. Microorganism source

The selection of microorganisms capable of growing on media containing enniatins as unique carbon source was performed using mixed cultures of several samples comprising foodstuff, soil and water from the local environment as summarized in Table 1. Grains and pasta material were crushed in Tissuelyser (Retsch MM300) with 2 wolfram carbide balls for 30 seconds; 0.3 g of each material was mixed with 2 mL sterile tap water in a 15 mL falcon tube. The same amount was taken for soil and fruit; the fruit was sliced into thin pieces, and mixed with 2 mL sterile tap water. No pretreatment was applied on water and juice samples. The samples used were grown on potato dextrose agar (PDA, Merck, Darmstadt, Germany) at 25°C for 7 days, an agar plug was cut out from each agar plate using a cork borer (5 mm diameter) and mixed with 2 mL of sterile tap water. The tubes containing each material mixed with water were shaken at room temperature for two hours at 175 RPM, and then left for one hour to allow the material to settle, the fresh supernatant from each tube was collected inside sterile Eppendorf tubes. The mixed cultures were prepared in three replicates each.

Table 1: Overview of the sources from which microorganisms were obtained.

	Microorganisms sources (S)	Origin
Grain	S1: Amaranth	Bio aus Liebe, Austria
	S2: Summer wheat (var. Taifum)	KWS (Einbeck)
	S3: Maize (var. Ronaldino)	KWS (Einbeck)
	S4: Yellow soybean (var. Kanada)	Ewert, Göttingen
	S5: Weed (*Balium elarive*)	Botanical gardens, Göttingen
	S6: Mini maize (var. Gaspe flint)	KWS (Einbeck)
	S7: Sorghum bicolor (var. “Tall polish”)	Leibniz Institute of plant Genetics and Crop Plant Research in Gatersleben, Germany
	S8: Rice genotype “Taichung Sen 10 (TCS 10)”	Africa Rice Center, Cotonou, Benin
	S9: White beans	Bio Bohnen, weiß, Ökologische Landwirtschaft DE-ÖKO- 001 Nicht EU-Landwirtschaft
	S10: Summer rape *Brassica napus* var. napus Genom ACaacc	Department of crop science, section plant pathology and crop protection (University of Göttingen, Germany)

Table 1 (cont.): Overview of the sources from which microorganisms were obtained.

	Microorganisms sources (S)	**Origin**
Nut	S11: Hazelnut (intact)	Trader Joe's Haselnusskerne ganz (Märsch Importhandels-GmbH, Ulm)
	S12: Almond	Trader Joe's Hazelnusskerne ganz (Märsch Importhandels-GmbH, Ulm)
	S13: Mung beans	Uzbekistan (Mardin Food, Denmark)
	S14: Peanuts	Cameroon
Water	S15: Water from lake Wendebachstausee	Göttingen
	S16: Water from river Leine Kanal	Göttingen
	S17: Water from lake Kiessee	Göttingen
Soil	S18: Soil from a wheat field	Wendebachstausee Göttingen
	S19: Garden soil	Geismar Göttingen
	S20: Soil from a wheat Field	Weende Nord Göttingen
	S21: Soil from a maize field	Geismar Göttingen
Juice	S22: Apple juice	Apfelsaft,Vitafit, Moers, Deutschland
	S23: Orange juice	Hohes C Eckes- Granini Deutschland GmbH
	S24: Tomato juice	A. Dohrn & A. Timm GmbH & Co. KG, Diedersdorf, Deutschland
Pasta	S25: Chinese noddle	Kridsada Food CO., LTD, Bangkok, Thailand
	S26: Noodle Penne	Erfurter Teigwaren GmbH, Erfurt, Deutschland
	S27: Vegetarian Noodle Vietnam	Hoang Tuan Food PTE, Vietnam
	S28: Noodle Echt Schwäbische Frischei-Nudeln	Schätzle, Gesellschaft für Nahrungsmittel-Herstellung und Vertrieb mgH, Waiblingen, Deutschland
Strains	S29: 5 Fungal strains from forest	Göttingen
Fruits	30: Plums	Göttingen
	S31: Raisins	Tafeltrauben, Thompson Seedless, Labros Giorgos A.S. Griechenland

2.1.2. Selection of microorganisms from mixed culture

To select microorganisms able to grow on media containing enniatins as the unique carbon source, the following procedure was used. 10 % (v/v) solution of minimal medium (MM) (pH 5.5 and pH 7.2) containing enniatins was prepared using a stock solution of enniatins (10 mg/mL) in order to get a culture medium containing 1 mg/mL of enniatins. 100 µL of the mixture MM/enniatins were put in each well of a microtiter plate (96 well plates). To each well 4 µL of fresh supernatant from each source of microorganisms was added, 4 µL sterile water was added in the control wells as represented in Table 2. The microtiter plates were incubated at 20° C for one week. A 10 times dilution was made from the content of each well and 4 µL of each dilution was transferred to a new well containing 100 µL of MM/enniatins (1 mg/mL). The new microtiter plates were incubated for three weeks at 20°C. Every week, the wells were checked for the disappearance of enniatin crystals and the presence of a colloidal suspension or mycelium using a binocular microscope. Agar plates containing a full medium (FM) having the same pH either pH 5.5 or 7.2 (depending on the pH of the MM used for microorganism screening) were used. After incubation, the content of each well was streaked onto the agar plates to obtain single bacterial colonies. Fungi were isolated using the dilution series and the dilution plating method.
The enniatins originated from the process described in chapter 2 of this thesis, the mixture contained about 77% enniatin B, 17% enniatin B1, 6% enniatin A1 and 0.3% enniatin A had a purity of 96.25%.

The composition of MM is as follows: 0.8 g K_2HPO_4, 0.2 g KH_2PO_4, 0.2 g $MgSO_4$, 1.5 g NH_4Cl, 0.001 g $CaCl_2$, 0.001 g $FeCl_3$, and 2.0 ml trace element solution (TS2) per liter medium (Völkl *et al.*, 2004). The TS2 contained per liter medium: 100 mg $ZnSO_4.7H_2O$, 20 mg $Fe\text{-}SO_4.4H_2O$ **,** 30 mg $MnCl_2.4H_2O$ **,** 10 mg $CuCl_2.2H2O$ **,** 300 mg H_3BO_3**,** 200 mg CoCl2.6H2O, 900 mg $Na_2MoO_4.2H2O$, 20 mg $NiCl_2.6H2O$, 20 mg Na_2SeO_3, 1000 mL distilled water (Meyer and Sch1egel , 1983)**.** The pH was adjusted to pH 5.5 or pH 7.2 using HCl 8 M and NaOH 1M respectively.

The medium FM contained: 5 g yeast extract, 10 g nutrient broth, 1 g glucose, 1.75 g K_2HPO_4, 0.75 g KH_2PO_4, 1000 mL distilled water (Völkl *et al.*, 2004). The pH was adjusted to 5.5 and 7.2 using HCl 8 M and NaOH 1 M respectively and the medium was solidified with 20 g agar/L.

Table 2: Pipetting scheme on microtiter plate for the microorganisms screening

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	C	C	C	S1	S1	S1	S2	S2	S2	S3	S3	S3
B	S4	S4	S4	S5	S5	S5	S6	S6	S6	S7	S7	S7
C	S8	S8	S8	S9	S9	S9	S10	S10	S10	S11	S11	S11
D	S12	S12	S12	S13	S13	S13	S14	S14	S14	S15	S15	S15
E	S16	S16	S16	S17	S17	S17	S18	S18	S18	S19	S19	S19
F	S20	S20	S20	S21	S21	S21	S22	S22	S22	S23	S23	S23
G	S24	S24	S24	S25	S25	S25	S26	S26	S26	S27	S27	S27
H	S28	S28	S28	S29	S29	S29	S30	S30	S30	S31	S31	S31

C: Control
S: Microorganisms source

2.1.3. Single culture selection of microorganisms

A second screening of the bacterial and fungal pure culture that are able to grow on media containing enniatin as unique carbon source was made by incubating single cultures obtained from the initial screening. The bacteria were grown on liquid FM for 24 hours after which the wells of a microtiter plate were filled with 100 µL of MM containing enniatins at the concentration 1 mg/mL (v/v) and 10 µL of bacteria suspension (OD600= 0.6) were added to the corresponding wells. Fungi were grown on FM agar plate for 5 days at 25°C, a 5 cm agar plug was mixed in 2 mL sterile tap water and diluted 1/100 to get a spore suspension, 10 µL of spores suspension were added to wells containing 100 µL MM/enniatins (1 mg/mL). The plates were incubated at 20°C for 3 and 5 days for bacteria and fungi respectively. The microorganisms obtained from the first selection with mixed cultures were inoculated on the MM/enniatins having the same pH as the minimal media used for the first selection. After incubation, the content of each well was streaked on FM agar plates and the plates were incubated at 25 °C. The number of microorganisms that remained alive was recorded. The control wells contained 100 µL of MM/enniatins (1mg/mL) and 10 µL of sterile tap water. Three replicates were made for each microorganism and each medium.

2.2. Enniatin degradation

2.2.1. Enniatin degradation in the microtiter plates

In order to check which of the microorganisms among those previously isolated was able to degrade enniatins, a minimal medium containing a limited amount of glucose and protein (MMGP) was used in addition to the previously used minimal medium. The composition of MMGP is as follows: MM with 5% terrific broth (TB) (v/v) and 50 mg glucose per liter medium (Völkl *et al.,* 2004), the pH was adjusted to 7.2 using NaOH 1M. TB consisted of 12.0 g tryptone, 24.0 g yeast extract and 2 ml glycerin (Tartof and Hobbs, 1987). MMGP and MM (pH 5.5 or pH 7.2) containing enniatins were prepared to a final enniatin concentration of 1 mg/mL.

Most of the microorganisms isolated were growing faster at 28°C than at 20°C on FM agar plates. Bacteria and fungi were pretreated as follows: bacteria were streaked on FM agar plates and incubated at 25°C for 24 hours. A single colony was mixed with sterile tap water, then the optical density (OD600) was measured and adjusted to 0.6, 10 µL of each bacteria suspension was added to a well of a 96-microtiter plate containing 100 mL of MM/enniatin or MMGP/enniatins. Spores of fungi previously isolated were counted using a Thoma chamber (0.1mm depth, 0.0025mm^2 square area) and the suspension was diluted in order to have a final spore concentration of 10000 spores/mL. A 10 µL of spores' suspension (100 spores) was mixed with 100 mL MM/enniatins or MMGP/enniatins in microtiter plates. The cultures were incubated at 28°C, but no significant growth of microorganisms was observed in the wells after two days. So the experiment was suspended and repeated using 10 µL bacterial suspension (OD600= 1)/10 µL fungal spores suspension (200 spores) mixed with 100 µL MM/enniatins or MMGP/enniatins (1 mg/mL) followed by incubation at 25°C during 3 and 5 days for bacteria and fungi respectively. The control wells contained 100 µL of MM/enniatins (1mg/mL) and 10 µL of sterile tap water. Three replicates were made for each microorganism and each medium.

2.2.2. Bacterial and Fungal enniatin degradation under shaking conditions

Since bacteria and fungi grow faster in liquid media when shaken, this step was carried out in order to see if the degradation process improves. 250 µL Bacteria suspension (OD600= 1) or 250 µL spore suspension (50000 spores) were mixed with 2500 µL MM/enniatins or

MMGP/enniatins (final enniatin concentration: 1 mg/mL), the cultures were shaken and incubated at 25°C during 3 and 5 days for bacteria and fungi respectively. Three replicates were made for each microorganism and each medium. The negative control was made by mixing 250 µL of sterile tap water with 2500 µL minimal medium containing enniatins at the final concentration 1 mg/mL.

2.3. Enniatin extraction and HPLC-UV analysis

2.3.1. Enniatin extraction

The liquid culture of each microtiter plate well was collected and transferred into a 1.5 mL Eppendorf tube for the extraction of enniatins; the wells were rinsed using 100 µL MeOH. Methanol was evaporated in the SpeedVac (Christ RVC 2-25 CD plus, 35°C, 30 mbar). The content of each Eppendorf tube was diluted in 200 µL ethyl acetate and 200 µL water were added, each mixture was vortexed during 1 min and left for 2 hours at room temperature, the upper organic phase was carefully collected using a pipette and put into a new tube. Enniatin extraction was repeated twice using the same ethyl acetate volume, the organic phases were mixed and the solvent was evaporated in the SpeedVac as above. The dried samples containing enniatins were resuspended in 200 µL methanol HPLC grade then transferred into HPLC vessels which were frozen at -20 °C until enniatin analysis by HPLC-DAD.

The cultures from the test-tubes (as mentioned above) were centrifuged at 4 °C (5 min, 4000 RPM), 600 µL of the supernatant was collected and transferred to a new tube, enniatins and degradation products were extracted twice using 600 µL EtOAc. For extraction, the tubes were vortexed during 2 min and left at room temperature for 30 min. The upper ethyl acetate phase was carefully collected using a pipette and put in a new tube. The extraction was done twice, then the organic phases were mixed together and the solvent was evaporated in the SpeedVac as above. The dried samples were resuspended in 600 µL methanol HPLC grade then transferred into HPLC vessels which were frozen at -20 °C until enniatin analysis by HPLC-UV.

2.3.2. HPLC-UV analysis

The method used to analyze the ethyl acetate extract from the cultures of microorganisms is identical to the method developed and described under the subtitle "2.1.2. Extraction of enniatins and High Performance Liquid Chromatography analysis" of the previous chapter of this thesis. After HPLC-UV analysis, the percentage of reduction of enniatins by each microorganism for three repetitions was calculated.

2.4. Identification of degradation products

Analysis of enniatin degradation products was achieved using the following procedure: first a characterization of the compound isolated using HPLC-MS with the modality full scan was done using m/z range from 50 to 800 Da to obtain general spectra of the degradation compounds. Then the results were processed using the script Perl in order to generate a heatmap of the fragments, and new signals present in the treated samples were sorted out. Finally the mass spectra of the potential product were checked and their possible molecular formula was deduced using ChemCalc (2013).

2.4.1. HPLC-MS analysis

The separation was carried out using a mass spectrometer system made of solvent delivery module (Varian Prostar 210), an autosampler (Varian Prostar 410), an ion trap 800 MS, an oven (Varian Model 510 Prostar). A C18 reversed phase column (Polaris C18-Ether, HPLC column, 180Å, 5 µm, 100 x 2.0 mm, Agilent) in an oven (temperature set at 40 °C) was used. Liquid chromatography conditions were set up using a constant flow rate at 0.2 ml/min. Methanol and water (7 mM acetic acid + 0.01 mM sodium acetate) were used as mobile phase in gradient elution consisting of a linear increase of methanol from 2 % to 98% for 40 minutes, methanol was then maintained at 98% for 5 minutes, decreased to 2% for 2 minutes and finally held for 8 minutes at 2%. The injection volume was 10 µL. The instrument was configured in the positive ionization mode. The protonated ions 640.4; 654.4; 668.8; and 682.4 were used as precursor ions for enniatins B, B1, A1 and A respectively. The sodium adduct ions 662.4; 676.4; 690.8; 704.4 were used as precursor ions for enniatins B, B1, A1 and A respectively.

3. Results

3.1. Selection of microorganisms from mixed cultures- Binocular microscopic observation of the microtiter plates

The pictures below show some wells of the microtiter plates containing microorganisms from various sources inoculated in a minimal media (MM) containing enniatin as sole carbon source (1mg/mL). The wells present differences relative to color and consistency depending on the origin of the microorganism. The control well remained colorless and translucent, while some other wells showed a colloidal aspect or a change in color varying from creamy, reddish, greenish or blackish. Furthermore, green/grey or white mycelium could be observed in some wells. After incubation of each microorganism in the medium containing enniatin, dilution 1/10 and re-incubation for 21 days, some wells were observed under a binocular microscope. The wells of the control (containing medium and enniatins) (Figure 2-A, B and C) showed aggregated enniatin crystals mostly planning inside liquid medium or at its surface. The wells of some samples under the binocular microscope are shown on Figure 3. In contrary to control wells, a change in texture and turbidity of cultures in some wells was observed containing sample supernatants. Mycelium was present in some wells (example: Figure 3, B, C, D), while in others the turbidity of medium increased or the combination of both changes was observed (example: Figure 3, E, F, D). Also the enniatin crystals in wells containing sample supernatants were dislocated into smaller pieces and scattered all around the medium (example: Figure 3, A, D, E, F). However, the observations on the binocular microscope showed some wells presenting a colloidal solution or a disappearance of enniatin crystals, but no microorganisms were isolated from those wells, some microorganisms were able to grow in the minimal medium containing enniatin as unique carbon source but did not grow on the agar plates used, thus they could not be isolated during these experiments.

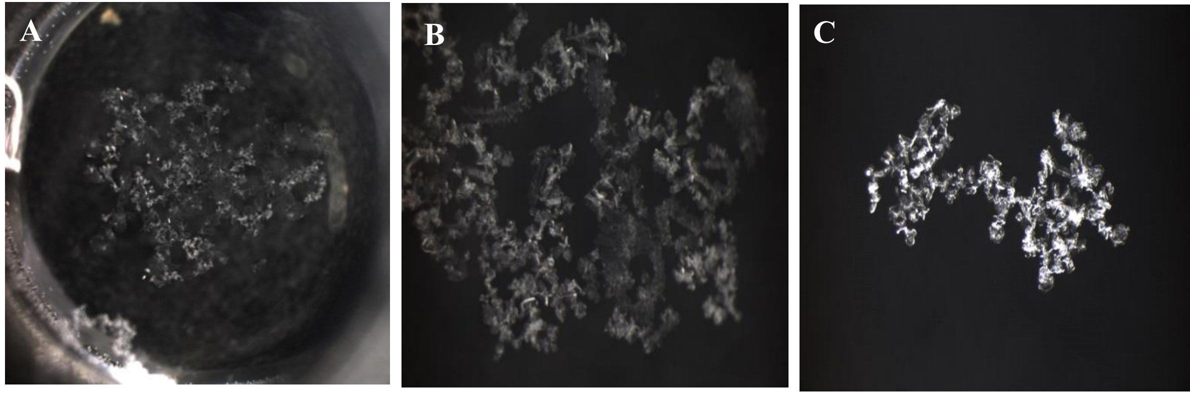

Figure 2: Microtiter plate wells of control under binocular microscope after 1/10 dilution of the cultures and 20 incubation days at 20 °C. A: resolution 20x, B and C: resolution 80x.

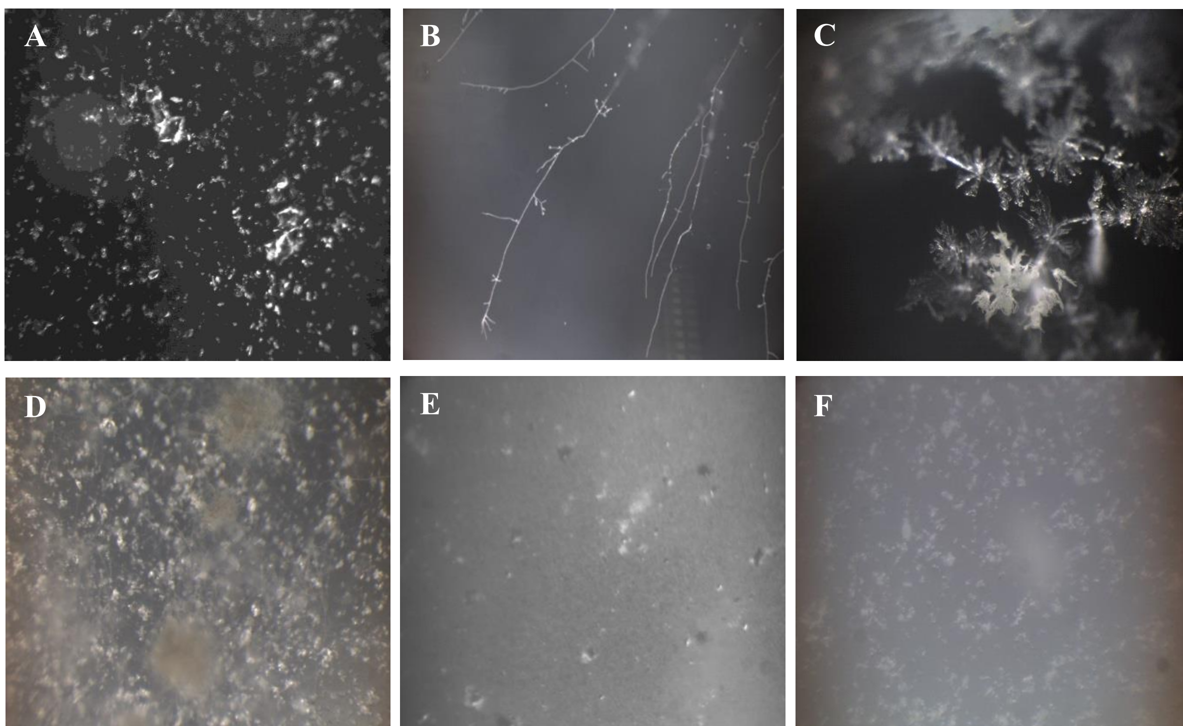

Figure 3: Microtiter plate wells of some samples under the binocular microscope (resolution 80x) after 1/10 dilution of the cultures and 21 incubation days at 20 °C. A & B: maize sample; C: almond sample, D: peanut sample, E: Water Lake Kiessee sample and F: amaranth sample.

3.2. Microorganisms isolated from mixed cultures

Microorganisms isolated on the basis of their ability to grow on MM containing enniatins as unique carbon source are summed up in Table 3.

Table 3: Number and kind of microorganisms isolated from each source used.

Microorganism sources (S)		Bacteria and Yeast-like		Fungi	
		PH 7.2	PH 5.5	PH 7.2	PH 5.5
Grains	S1: Amaranth	3	1	0	0
	S2: Summer wheat	0	0	0	2
	S3: Maize	5	4	0	2
	S4: Yellow soybean	0	0	0	0
	S5: *Balium elarive*	0	0	1	0
	S6: Mini maize	0	0	1	1
	S7: Sorghum	2	3	0	0
	S8: Rice	2	0	0	0
	S9: White beans	0	0	0	0
	S10: Summer rape	0	0	1	1
Nuts	S11: Hazelnut	2	0	0	0
	S12: Almond	0	0	2	3
	S13: Mung bean	2	0	0	1
	S14: Peanuts	3	0	2	1
Water	S15: Lake Wendebachstausee	4	8	0	0
	S16: River Leine Kanal	6	6	0	0
	S17: Lake Kiessee	5	6	0	0
Soil	S18:Wheat field soil (around Wendebachstausee)	5	2	1	0
	S19: Garden soil	8	4	0	0
	S20: Wheat field soil (Weende Nord)	6	0	0	0
	S21: Maize field soil	11	2	1	0
Juices	S22: Apple juice	6	6	0	0
	S23: Orange juice	0		0	0
	S24: Tomato juice	0	2	0	0
Noodle	S25: Chinese noodle	0	0	0	0
	S26: Noodle Penne	0	0	0	1
	S27: Vegetarian Noodle Vietnam	0	0	0	0
	S28: Noodle Echt Schwäbische Frischei-Nudeln	0	0	0	0
Strains	S29: 5 fungal strains from forest				
Fruits	S30: Plums	0	0	0	1
	S31: Raisin	0	0	0	0
Total		**70**	**44**	**10**	**24**

Bacteria and Yeast-like microorganisms

In total 114 bacteria and yeast-like microorganisms were isolated from different sources after incubation in minimal medium (MM) containing enniatin as sole carbon source at pH 7.2. Bacteria and yeast-like microorganisms were differentiated depending on their colony characteristics on full medium (FM) agar plates. The major sources of bacteria and yeast-like microorganisms were: soil from maize field, followed by garden soil from which respectively 11 and 8 sorts of bacteria and yeast-like microorganisms were isolated. In general, most bacteria and yeast-like microorganisms were isolated from soil samples (38) and water samples (35). The number of bacteria and yeast- like microorganisms isolated from the MM with pH 7.2 (70 bacteria) was higher than those isolated from MM with pH 5.5 (44). No bacteria and yeast-like microorganisms were isolated from noodle and plums and raisin samples.

Fungi

In total, 34 fungi were isolated on their ability to grow on MM containing enniatin as sole carbon source. The distinction among fungi was made essentially based on colony visual characteristics on FM agar plates. The main sources were nuts (hazelnuts, almonds and peanuts) and grains (summer wheat and maize) from which 15 and 5 fungi were isolated respectively. A majority of fungi were isolated from MM with pH value 5.5.

3.3. Single culture selection of microorganisms

Out of the 148 microorganisms previously isolated 20 bacteria and 10 fungi could not be cultivated. When the pure cultures previously isolated were incubated as single cultures in MM/enniatin (enniatin final concentration: 1 mg/mL), 6 bacteria and 1 fungus did not grow on the MM medium containing enniatin as sole carbon source. The sources of these bacteria were garden soil (2 bacteria), wheat field from Weende Nord in Göttingen (1 bacterium), apple juice (1 bacterium) and the tomato juice (1 bacterium) and wheat field around Wendebachstausee in Göttingen (1 bacterium). The fungus which didn’t grow on MM/enniatin as single culture was isolated from the noodle sample. This reduced the number of microorganisms isolated to 88 and 23 for bacteria and fungi respectively.

2.3. HPLC-UV analysis of culture media

Enniatin concentrations were generally reduced in all samples compared to the control (data not shown). The reduction was variable depending on the enniatin variant and the microorganism used. The reduction rate was comparable in both media used (MM and MMGP) for bacteria, whereas with fungi there seemed to be a greater enniatin reduction, when incubated in MMGP compared to MM. The concentration of enniatin A was reduced most. With the exception of one bacterium (K4) and two fungi (F18 and F20) the pattern of the chromatograms peaks and the UV spectra were similar to that of the control sample.

For bacterium K4, the retention times of the peaks observed after HPLC-UV analysis of the cultures are summarized in Table 4. In the control samples, the retention times of enniatins B, B1, A1 and A were 7.68 min; 8.11 min; 8.48 min and 8.8 min respectively. In the chromatograms of samples of the bacterium K4 a new peak was observed with retention time of 7.28 min which is present neither in the control nor in the chromatogram of the bacterium K4 incubated in the media without enniatin. HPLC-UV chromatograms of control and cultures of bacterium K4 incubated in MM or in MMGP are presented in Figure 4.

Table 4: Retention times of peaks observed on the HPLC-UV chromatograms resulting from the incubation of the control sample and the fungus K4 in MM and MMPG containing enniatins (1mg/mL).

Peaks	**Retention times**		
	Control	**K4 in MM**	**K4 in MMGP**
New peak		7.28	7.28
Enniatin B	7.68	7.68	7.73
Enniatin B1	8.11	8.11	8.13
Enniatin A1	8.48	8.48	8.48
Enniatin A	8.80	8.85	8.85

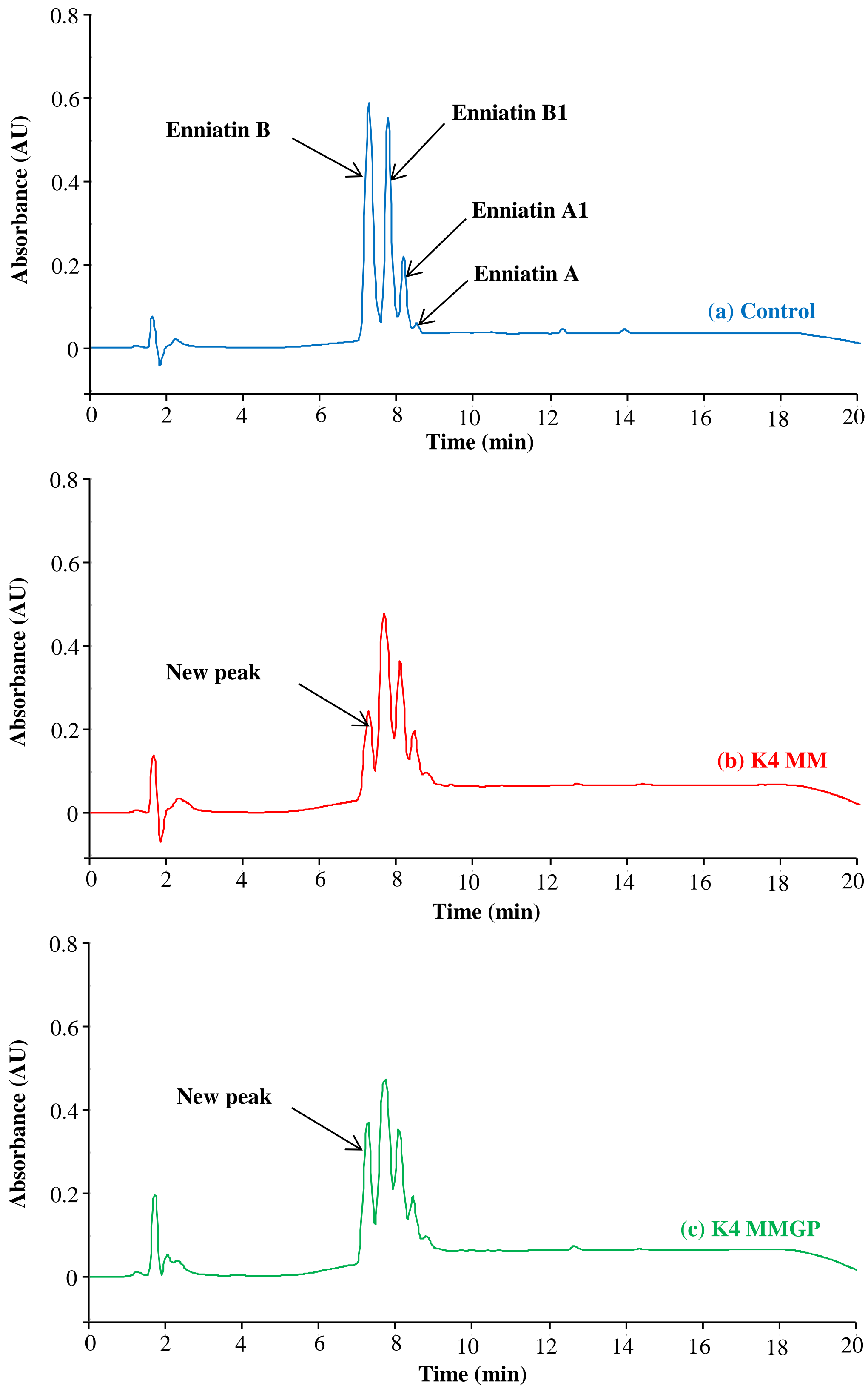

Figure 4: HPLC-UV chromatograms of enniatins and related degradation products present in the growth medium after incubation with bacteria K4 for 3 days at 25°C.

The chromatograms of samples containing the fungi F18 and F20 showed a different pattern when the fungus was incubated in the medium MM compared to the medium MMGP. Table 5 (1 and 2) summarizes the retention times of peaks observed on the chromatograms resulting from the incubation of fungi F18 and F20 in MM and MMPG containing enniatins and the control sample. New peaks eluting before the four enniatin variants were observed. In general it was not simple to match the peaks in the chromatograms of the treated samples and those in the control sample. HPLC-UV chromatograms of treated cultures of fungi F18 and F20 are shown in Figure 5 and Figure 6 respectively.

Table 5: Retention times of peaks observed on the HPLC-UV chromatograms resulting from the incubation of the control sample and the fungus F20 (Table 5.1) and fungus F18 (Table 5.2) in MM and MMPG containing enniatins (1 mg/mL).

1.

Peaks	**Retention times**		
	Control	**F20 in MM**	**F20 in MMGP**
New peak			4.67
		6.19	5.28
		6.96	6.56
Enniatin B	7.68	7.57	7.23
Enniatin B1	8.11	8.03	8.03
Enniatin A1	8.48	8.40	8.51
Enniatin A	8.80	8.72	8.85

2.

Peaks	**Retention times**		
	Control	**F18 in MM**	**F18 in MMGP**
New peak			3.47
			3.87
			4.91
			5.68
		6.96	6.64
Enniatin B	7.31	7.31	7.28
Enniatin B1	7.79	7.81	7.79
Enniatin A1	8.19	8.21	8.19
Enniatin A	8.53	8.56	-

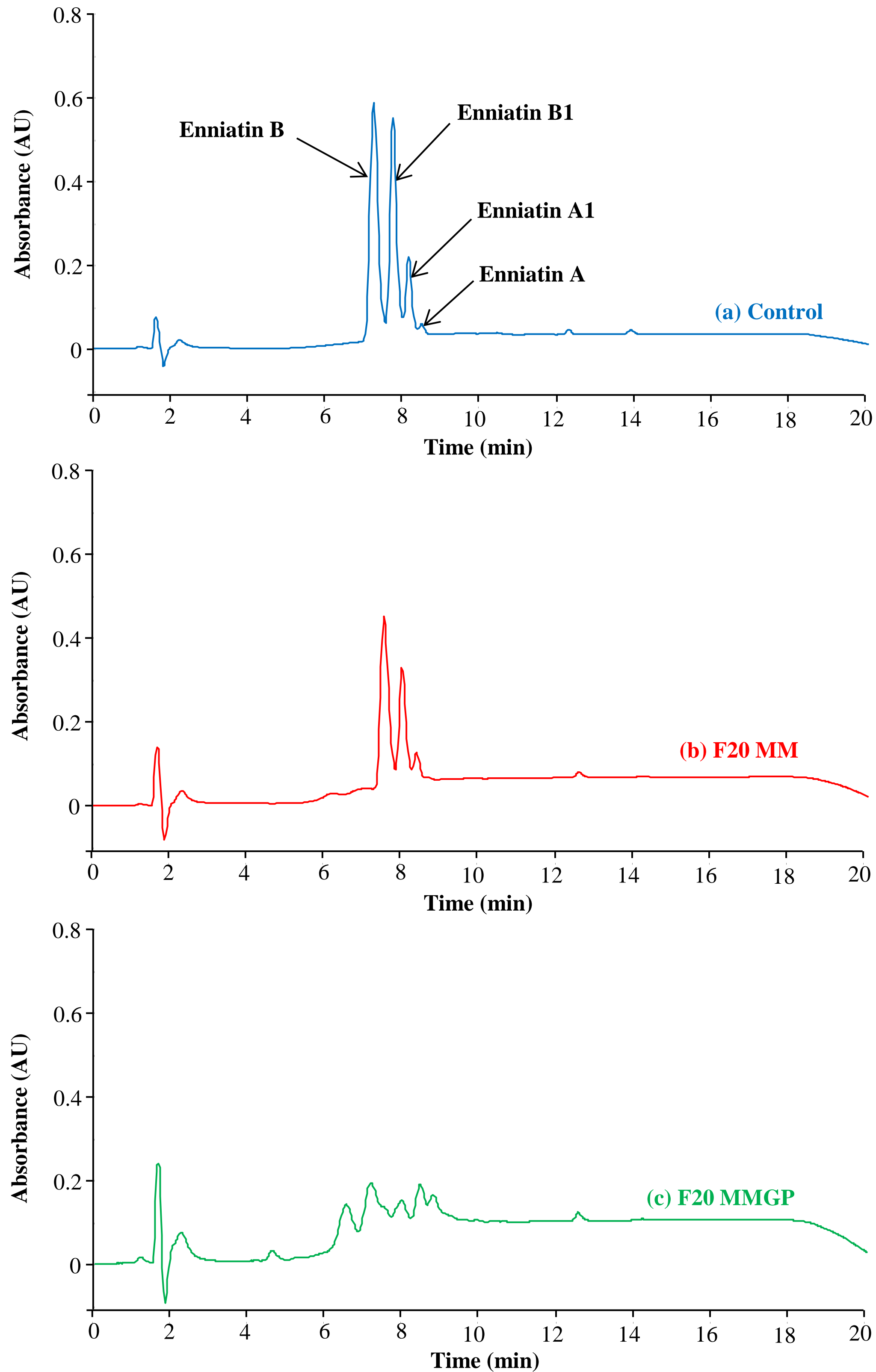

Figure 5: HPLC-UV chromatograms of enniatins and related degradation products present in the growth medium after incubation with fungi F20 for 5 days at 25°C.

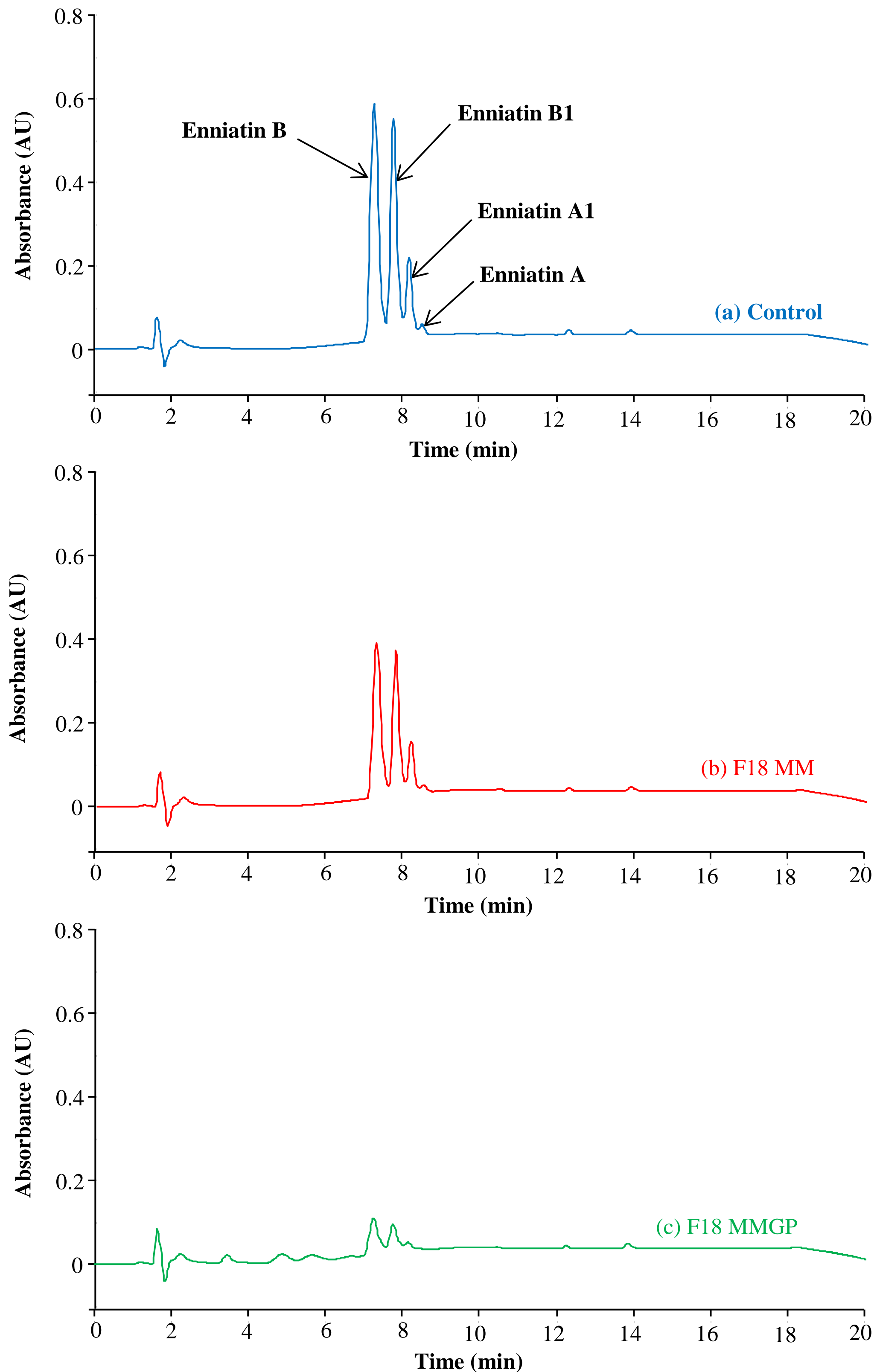

Figure 6: HPLC-UV chromatograms of enniatins and related degradation products present in the growth medium after incubation with fungi F18 for 5 days at 25°C.

In contrast to the HPLC-UV chromatogram of the bacterium K4, the chromatogram patterns of the fungi F20 and F18 were different when the fungi were cultured in MM or in MMGP. When the fungi F18 was incubated in MM containing enniatins, no new peak was observed, while in MMGP, new peaks were detected having lower retention times than those of enniatin variants (Table 5.2). Two and three new peaks (eluted before enniatin peaks) were detected on the HPLC-UV chromatogram of the fungi F20 after incubation in MM and MMGP respectively (Table 5.1). But in general, the matching of the peaks with enniatin peaks in the control was not conclusive.

2.4. Enniatin degradation under shaking conditions

When microorganisms isolated on the basis of their property to grow on media containing enniatins as unique carbon source were incubated on MM and on MMGP, growth (turbidity for bacteria/ mycelium for fungi) was generally observed in all the samples of bacteria and fungi. After visual observation, mycelium was more abundant in MMGP than in MM, while the turbidity of bacteria cultures was unaffected by the medium used as shown in Figure 7.

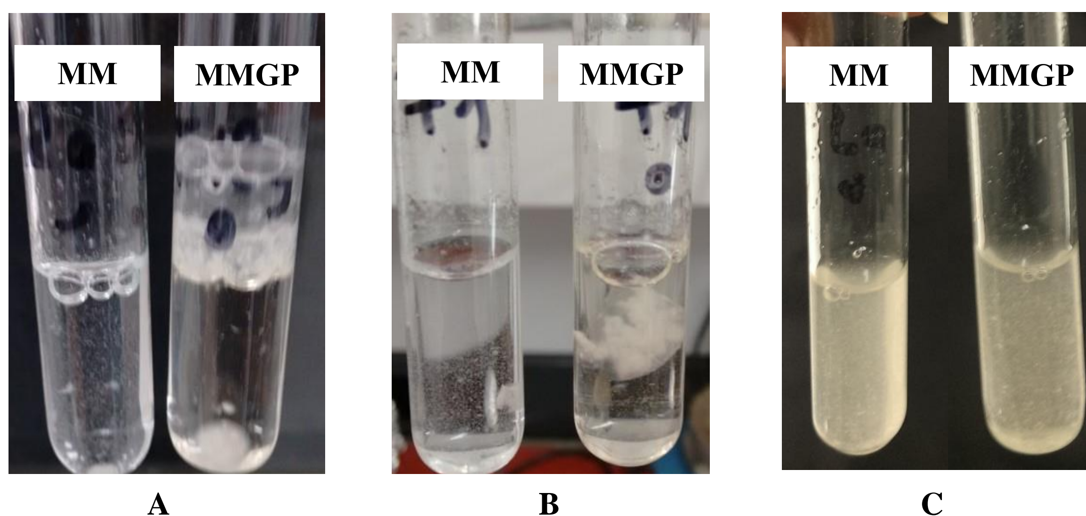

Figure 7: Cultures of two fungi (A and B) and one bacterium (C) in MM or in MMGP both containing enniatins after 5 days (fungi) and 3 days (bacterium) incubation at 25°C.

The HPLC-UV analysis of the pure cultures of the bacterium and the fungi incubated with MM or MMGP while shaking did not show any difference regarding the chromatogram pattern. The chromatograms of bacterium K4 and fungi F18 and F20 were identical to those obtained when the microorganisms were incubated in MM and MMGP in the microtiter plate without shaking during the incubation.

2.5. Identification of degradation products using HPLC-MS

The culture media of fungi F18, F20 and bacterium K4 incubated with enniatins in MMGP for 3 days (bacterium) or 5 days (fungi) were analyzed using LC-MS in full scan mode and a positive ionization mode using the m/z range from 50-800 Da. The control was made of the medium MMGP containing enniatins. The total ion chromatograms (TIC) of culture supernatants are presented in Figures 8. The TIC of microorganisms grown in MMGP medium without enniatins is presented in Figure i in Appendix. Figure 8 shows the presence of enniatins B, B1, A1 and A in the control sample with the retention times 35.379 min; 36.338 min; 37.308 min; 38.113 min respectively.

ENN: enniatin

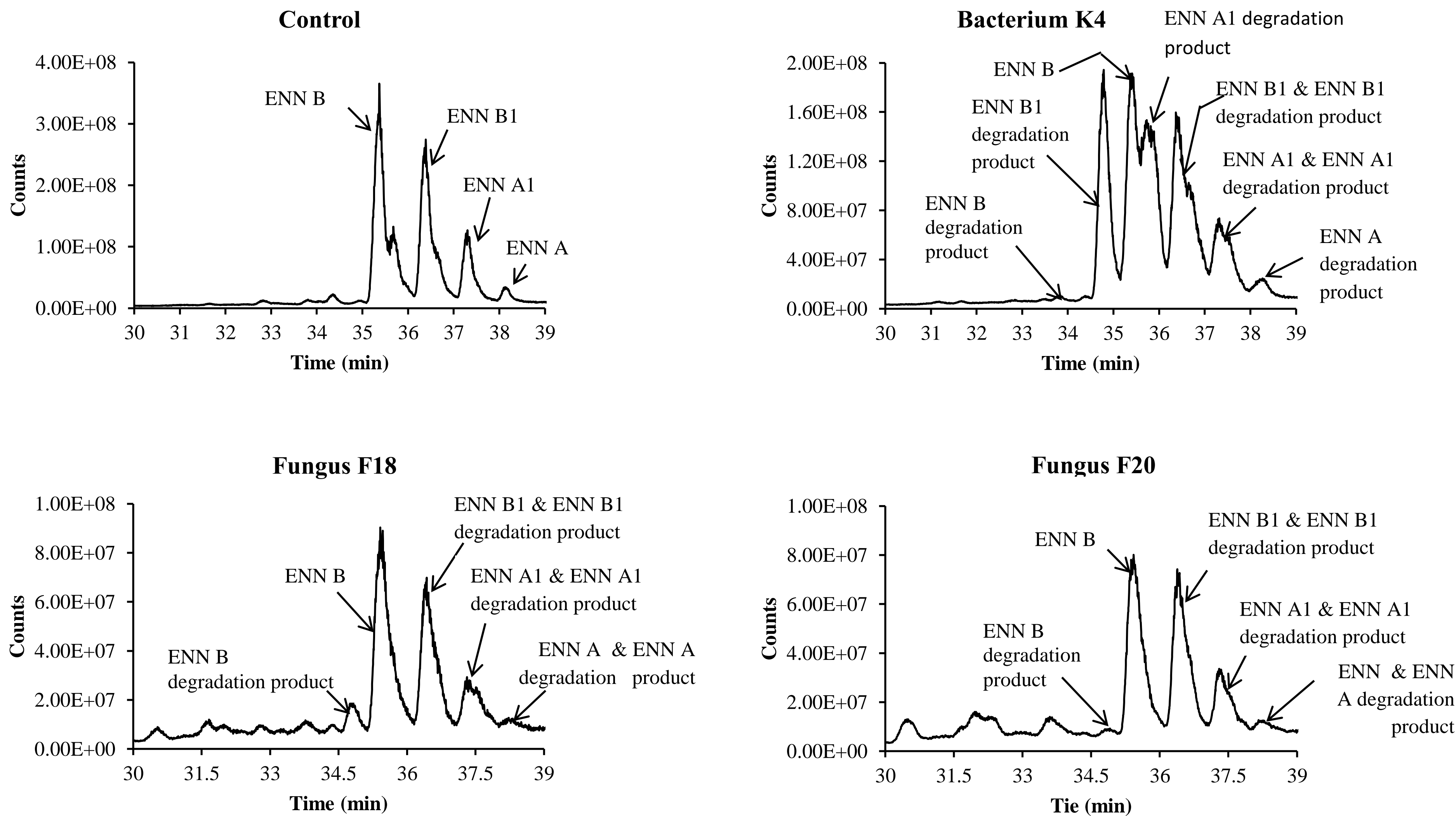

Figure 8: Total ion chromatograms of culture supernatants of control, bacterium K4, fungi F18 and F20 incubated in MMGP for 3 days (K4) and 5 days (fungi) at 25°C.

3.1.1. Degradation product of enniatin B

The Figures 9, 10 and 11 show the extracted ion chromatograms (EIC) of the degradation product of enniatin B in the culture supernatant of bacterium K4 and fungi F18 and F20 respectively. The mass spectra related to the enniatin B degradation compound identified with $[M+H]^+$ and $[M+Na]^+$ at m/z 658.08 and 680.4 respectively are also presented. In the EIC of the culture supernatant of bacterium K4 (Figure 9) the enniatin B degradation product appears as a double peak with retention times 33.5 min and 33.8 min, while the residual enniatin B in culture is present as a peak with the retention time 35.4 min. The product of enniatin B degradation in the culture supernatant of the fungi F18 and F20 appears as a double peak eluted at 33.3 min and 34.9 min respectively (Figure 10 and 11). In the EIC of enniatin B degradation product of the sample K4, both peaks representing enniatin B degradation product were dominated by the protonated ion compared to the sodium adduct ion, whereas the opposite was observed for both fungi F18 and F20. The molecular formula of the protonated ion of enniatin B degradation product is $C_{33}H_{60}N_3O_{10}$.

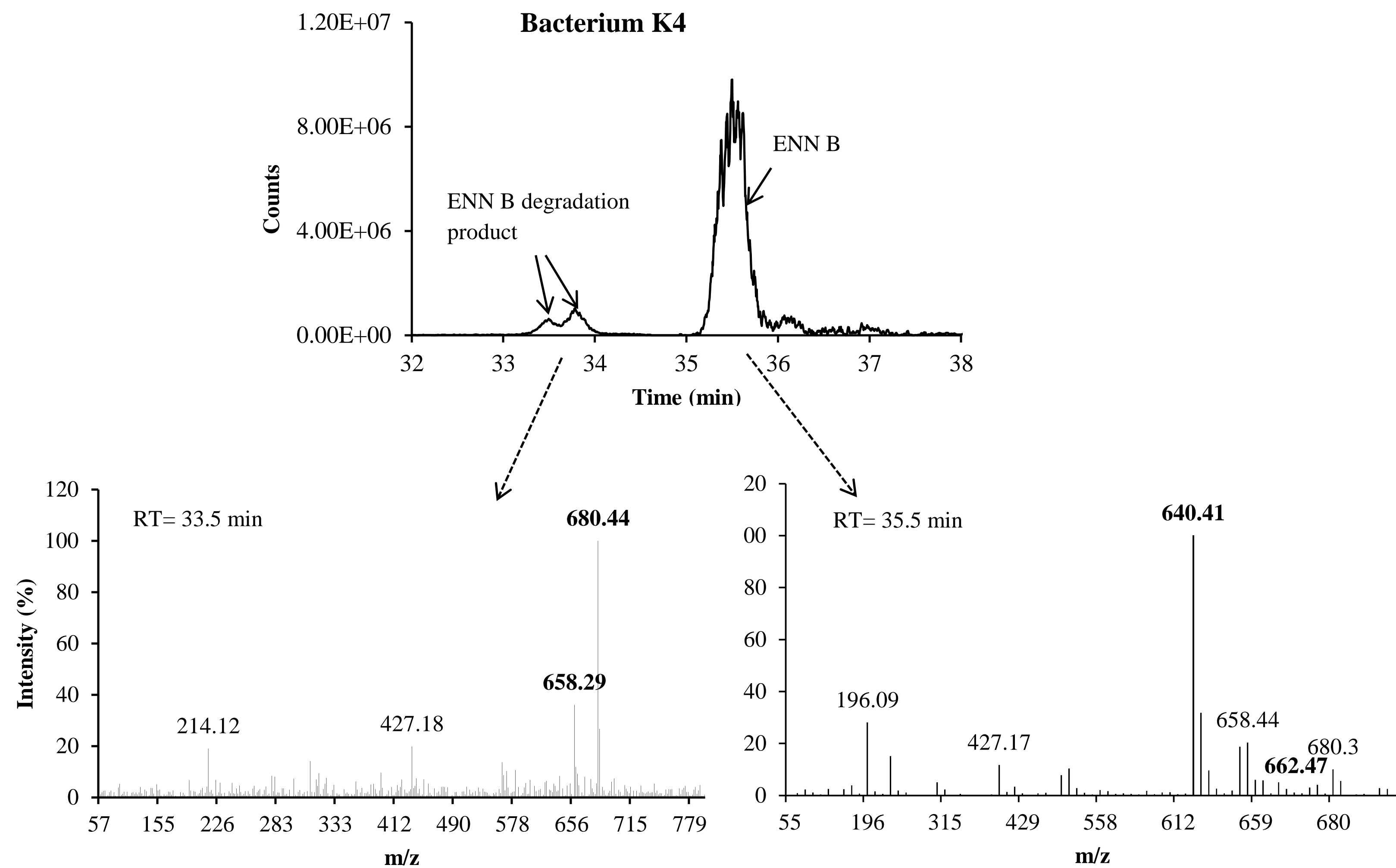

Figure 9: EIC and mass spectra of enniatin B degradation product by bacterium K4 with $[M+H]^+$ 658.29.

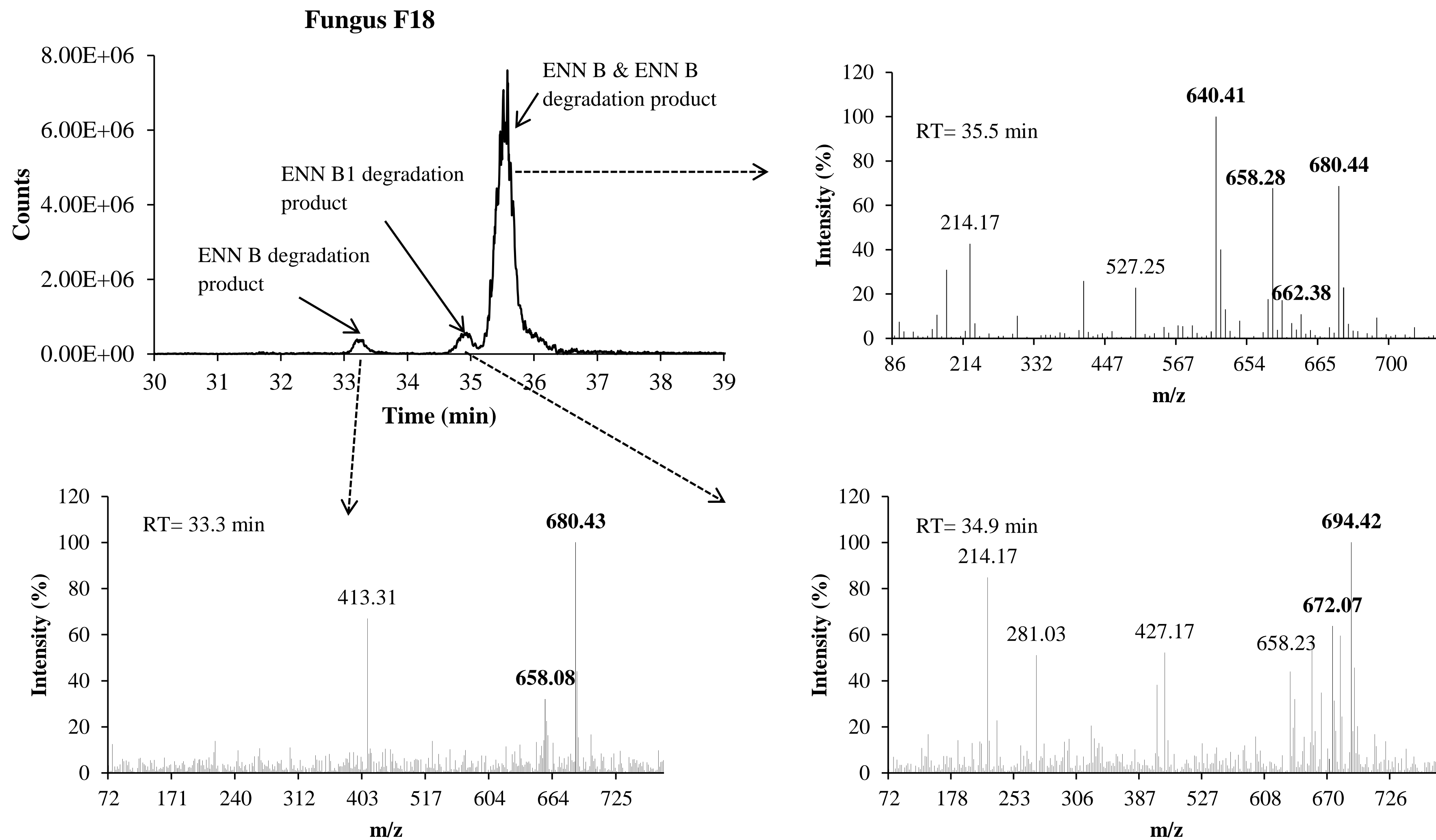

Figure 10: EIC and mass spectra of enniatin B degradation product by fungus F18 with $[M+H]^+$ 658.08 or 658.28.

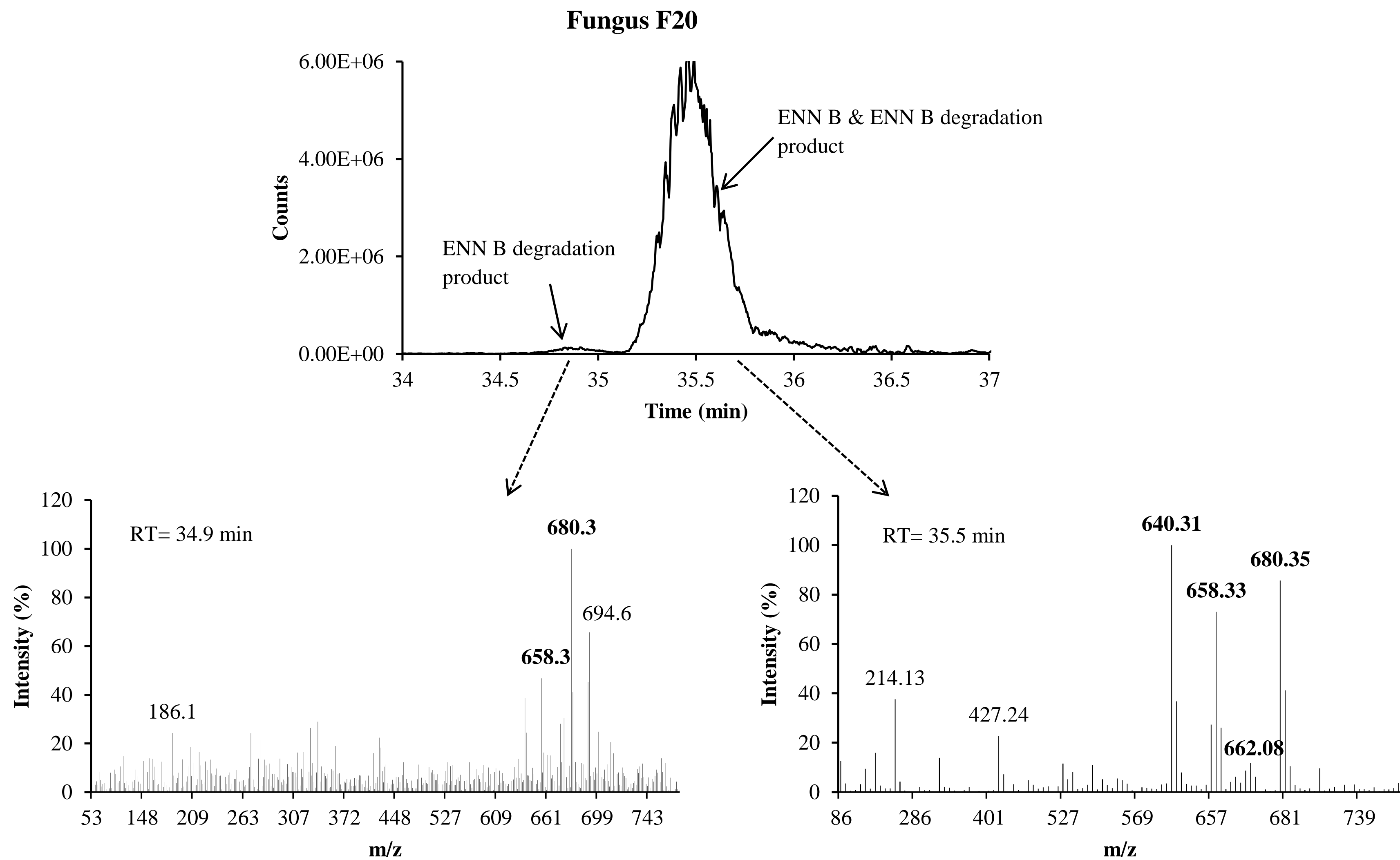

Figure 11: EIC and mass spectra of enniatin B degradation product by fungus F20 with $[M+H]^+$ 658.3.

3.1.2. Degradation product of enniatin B1

The EIC of the degradation product of enniatin B1 and the related mass spectra of the peaks observed are shown in Figures 12, 13 and 14. The enniatin B1 degradation product resulting from the incubation of bacterium K4 (Figure 12) and both fungi F18 and F20 (Figure 13 and 14 respectively) in MMGP containing a mixture of enniatins A, A1, B1 and B was demonstrated by the extracted ion chromatogram (EIC) of the compound with m/z 672.3 for the $[M+H]^+$ and 694.4 for $[M+Na]^+$ showing two peaks with identical mass spectrum. The retention time of enniatin B1 in the culture medium was 36.5 min. The first peak of the enniatin B degradation product corresponding to the m/z 672.3 $[M+H]^+$ eluted before enniatin B1 with a retention time of 34.8 min indicating that the compound is more hydrophilic than the parent compound, while the second peak had a retention time of 36.5 min and overlapped with enniatin B1. In the EIC of enniatin B1 degradation product of the sample K4, both peaks representing enniatin B1 degradation product were dominated by the protonated ion compared to the sodium adduct ion, whereas the opposite was observed for both fungi F18 and F20. A molecular formula of the protonated ion of enniatin B1 degradation product could be $C_{34}H_{62}N_3O_{10}$.

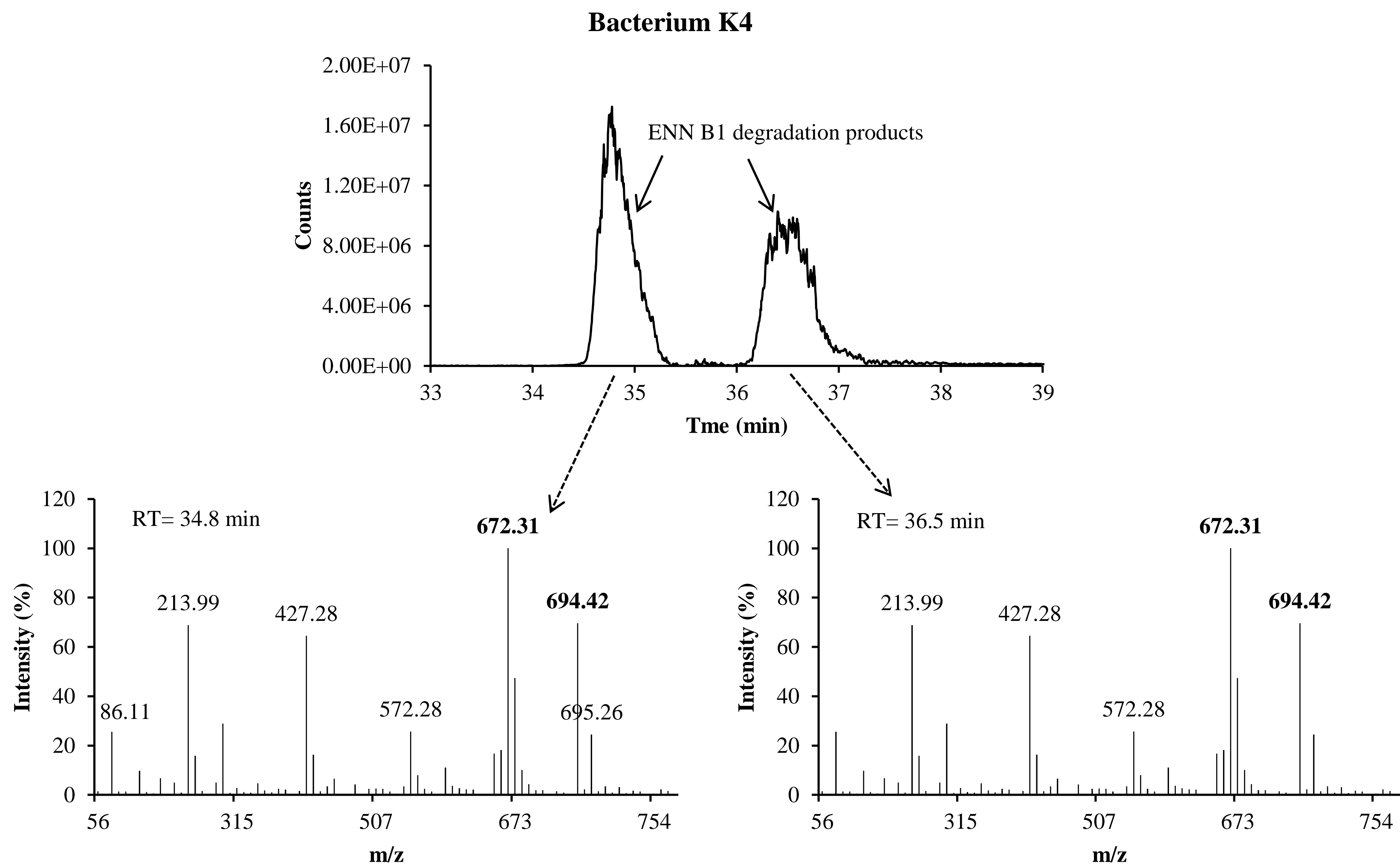

Figure 12: EIC and mass spectra of enniatin B1 degradation product by bacterium K4 with $[M+H]^+$ 672.31.

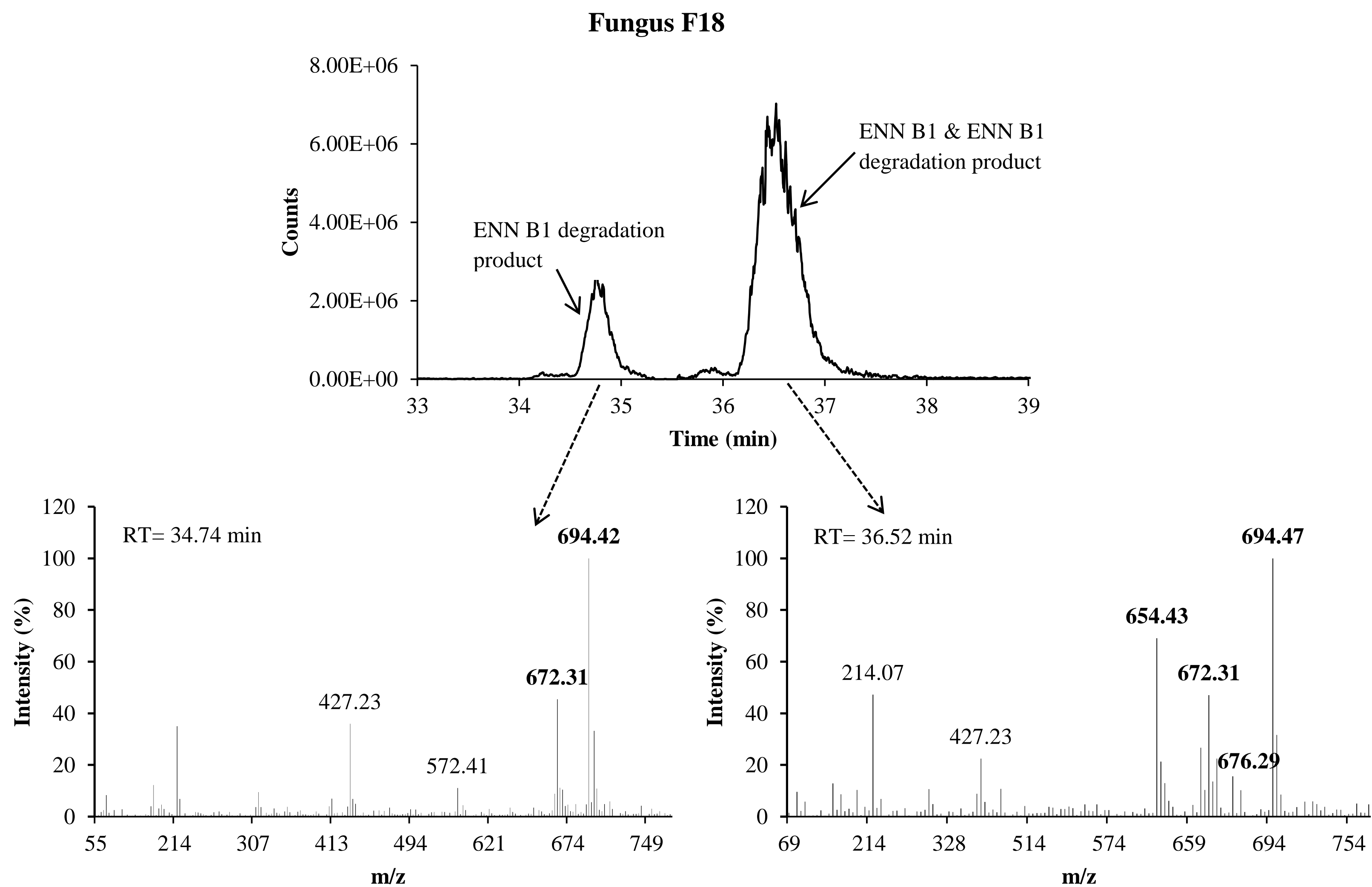

Figure 13: EIC and mass spectra of enniatin B1 degradation product by fungus F18 with $[M+H]^+$ 672.31.

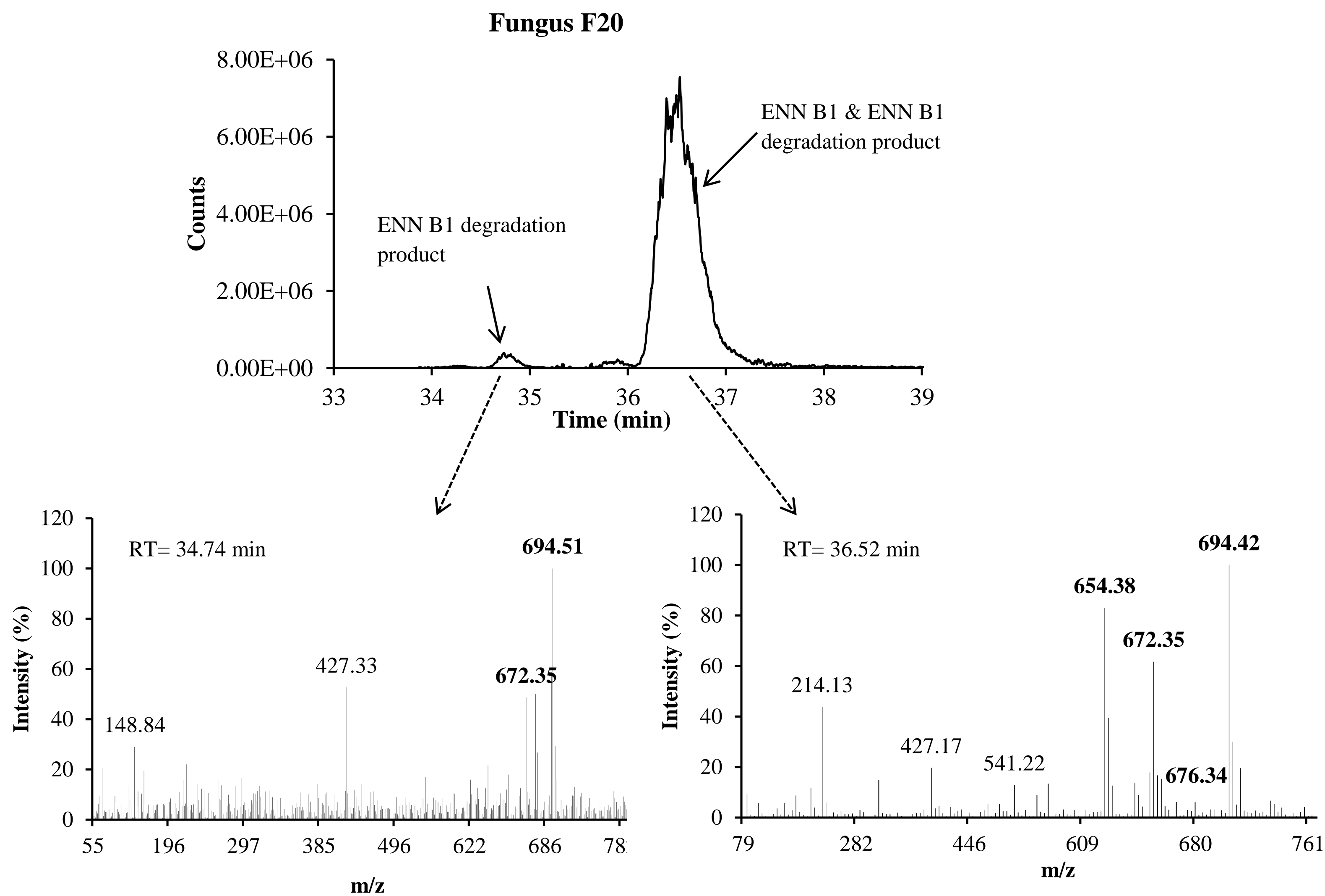

Figure 14: EIC and mass spectra of enniatin B1 degradation product by fungus F20 with $[M+H]^+$ 672.35.

3.1.3. Degradation product of enniatin A1

A product of degradation of enniatin A1 was identified in the culture supernatant of all the 3 microorganisms (K4, F18 and F20). The fragment with a m/z 686.3 corresponded to the molecular weight of the protonated ion of enniatin A1 degradation product and was represented by enniatin A1 with the addition of one molecule of water. The fragment with the m/z 708.5 represented the sodium adduct of enniatin A1 degradation product. The presence of this compound was confirmed by the extracted ion chromatogram (EIC) presented in Figures 15, 16 and 17. The EIC obtained after the LC-MS showed two peaks for bacterium K4 (Figure 15) and fungus F18 (Figure 16), having the same mass spectrum, while only one peak is observed for the fungus F20 (Figure 17). For K4 and F18, the first peak was more hydrophilic than enniatin A1 due to a lower retention time (35.9 min) compared to that of enniatin A1 (37.4 min). The second peak had a retention time of 37.4 min which is comparable to that of enniatin A1. The mass spectrum of the second peak was dominated by the fragments with $[M+H]^+$ and $[M+Na]^+$ at m/z 686.3 and 708.5 respectively but there were also some fragments with $[M+H]^+$ and $[M+Na]^+$ at m/z 668.4 and 690.4 respectively corresponding to the protonated ion and the sodium adduct ion of enniatin A1. For both fungi F18 and F20, the sodium adduct ion of the enniatin A1 degradation product was dominant compared to the protonated ion. While for the bacterium K4, the first peak representing enniatin A1 degradation product was dominated with the protonated ion compared to the sodium adduct ion. The molecular formula of the protonated ion corresponding to the enniatin A1 degradation product is $C_{35}H_{64}N_3O_{10}$.

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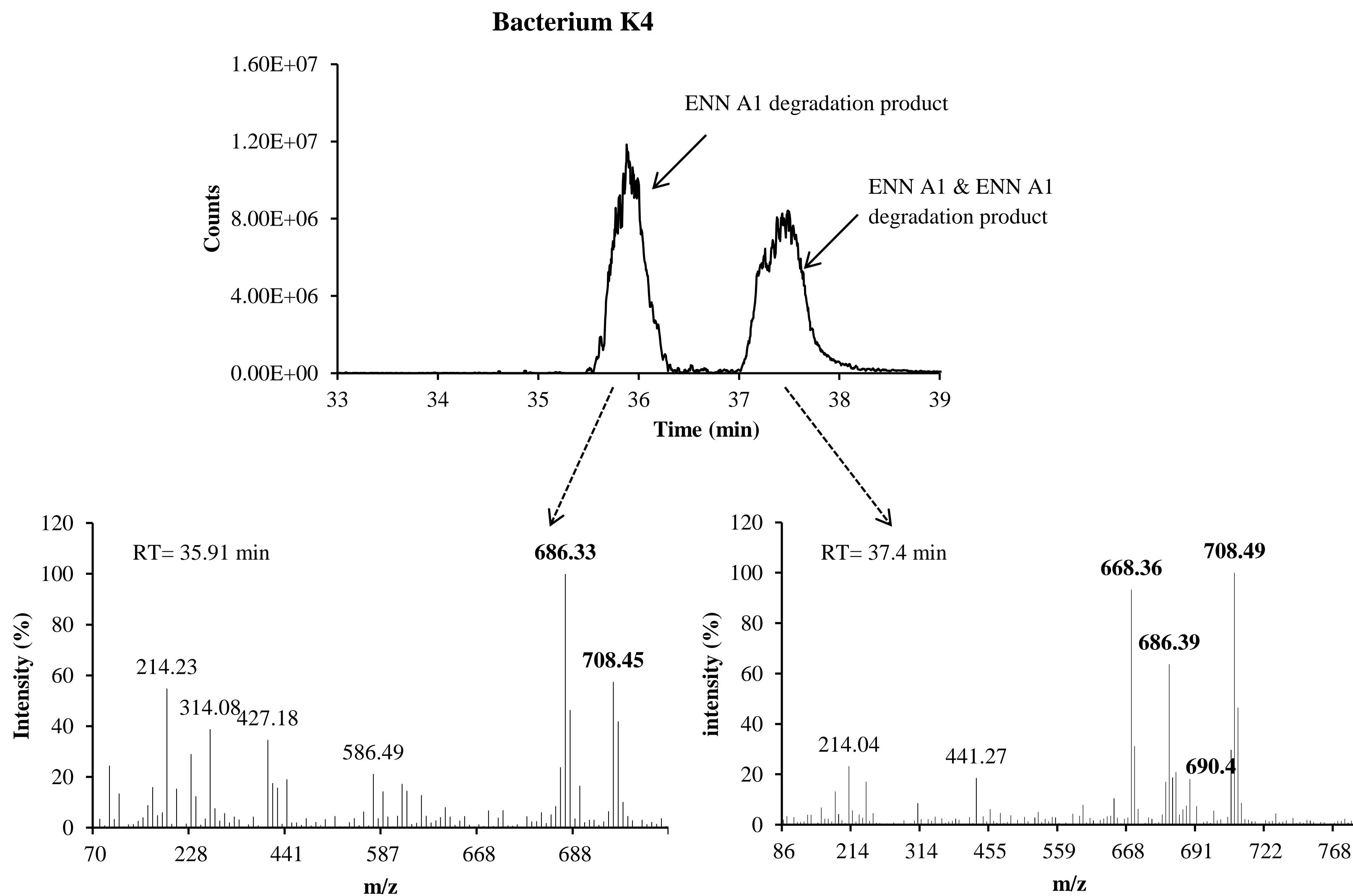


Figure 15: EIC and mass spectra of enniatin A1 degradation product by bacterium K4 with $[M+H]^+$ 686.33 and 686.39.

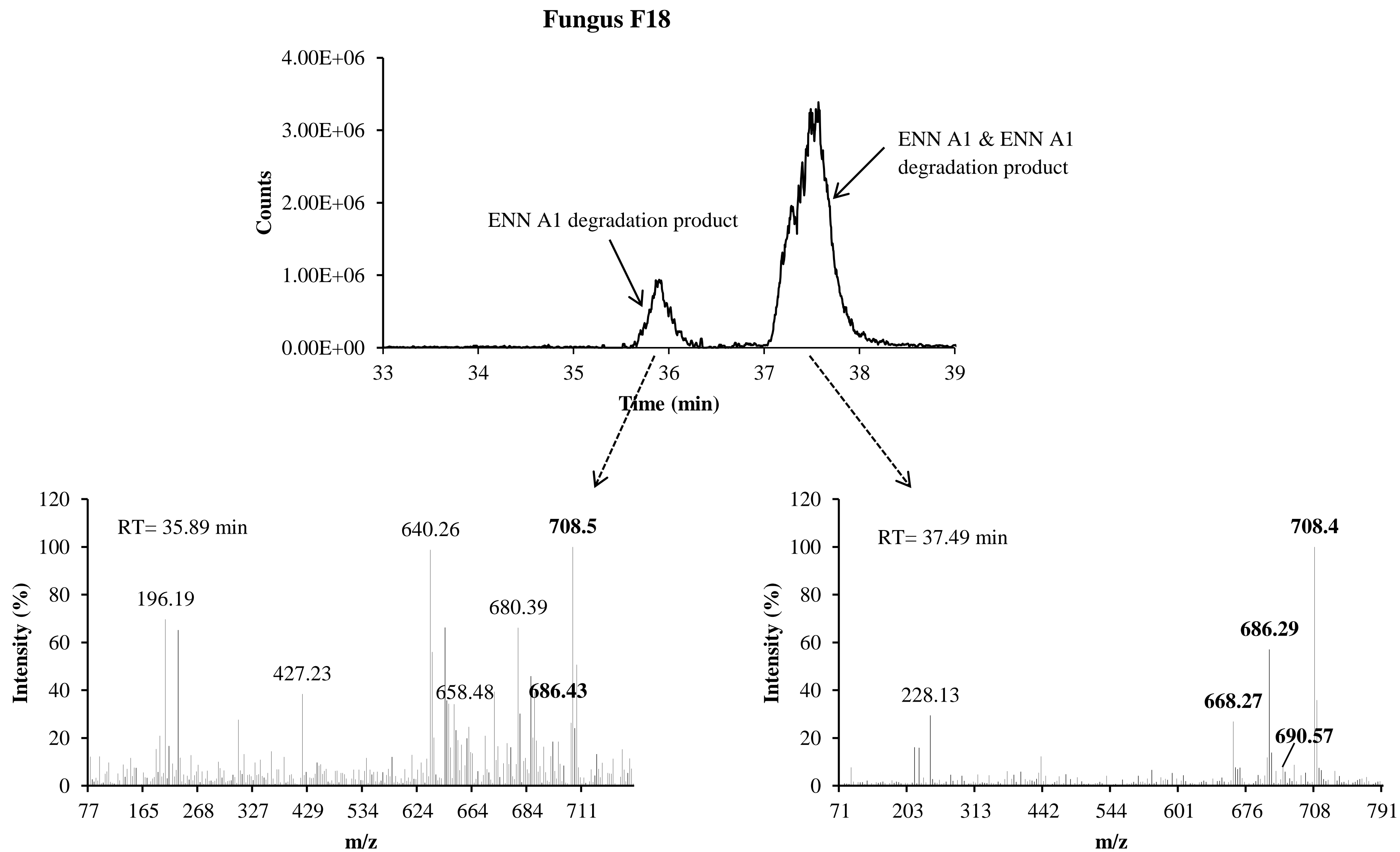


Figure 16: EIC and mass spectra of enniatin A1 degradation product by fungus F18 with $[M+H]^+$ 686.29 or 686.43.

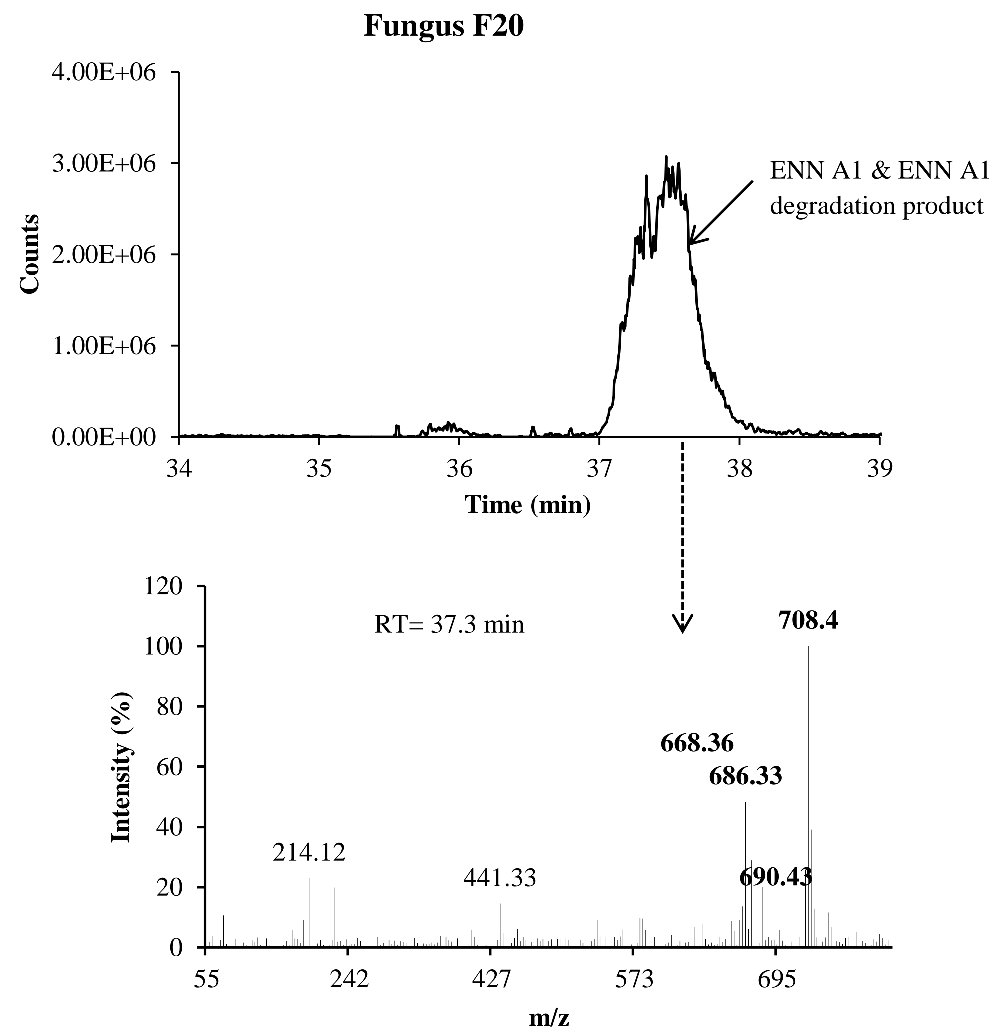


Figure 17: EIC and mass spectra of enniatin A1 degradation product by fungus F20 with $[M+H]^+$ 686.32 or 686.33.

3.1.4. Degradation product of enniatin A

Fragments were found corresponding to the enniatin A degradation products with a protonated ion at m/z 700.3 and sodium adduct ion at m/z 722.4, the EIC and the mass spectra corresponding to the degradation products are presented in Figure 18, 19 and 20. For bacterium K4 (Figure 18), two peaks were observed eluting at 36.6 min and 38.2 min respectively and with the same mass spectrum. The second peak overlapped with enniatin A. Enniatin A was recognized by the presence of fragments with $[M+H]^+$ and $[M+Na]^+$ at 682.3 and 704.5 with a retention time of 38.4 min. In the culture supernatant of the fungus F20, enniatin A degradation product was present as a single peak overlapping that of enniatin A on the EIC (Figure 20). Contrary to the bacterium K4 and fungus F20, no residual fragment corresponding to enniatin A was observed on the EIC after analysis with LC-MS for the fungus F18, the fragment of the enniatin A degradation product eluted at 38.2 min (Figure 19). For the 3 microorganisms tested, the sodium adduct ion of the enniatin A degradation product was dominant compared to the protonated ion. The molecular formula of the protonated ion corresponding to the enniatin A degradation product is $C_{36}H_{66}N_3O_{10}$.

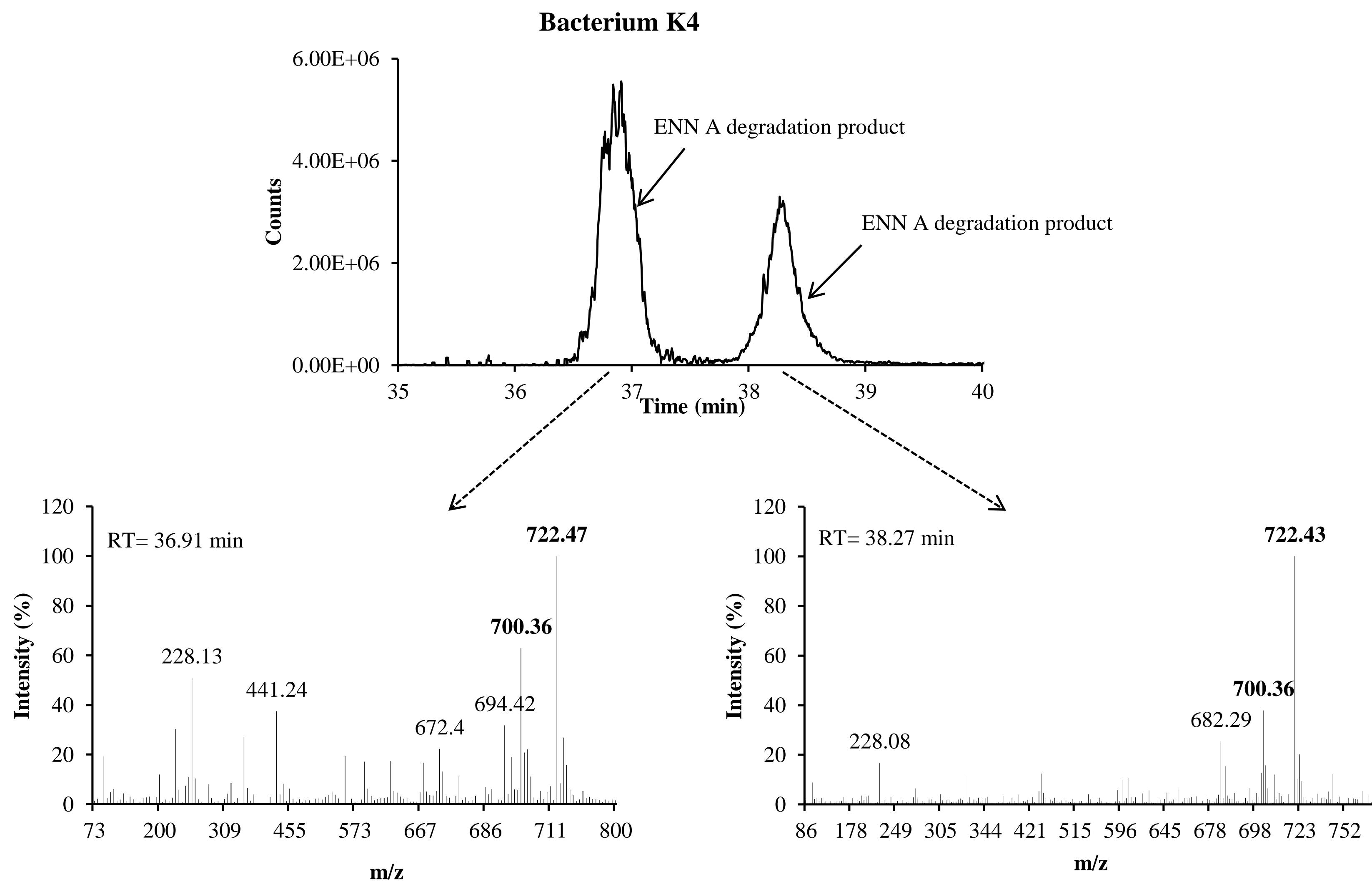
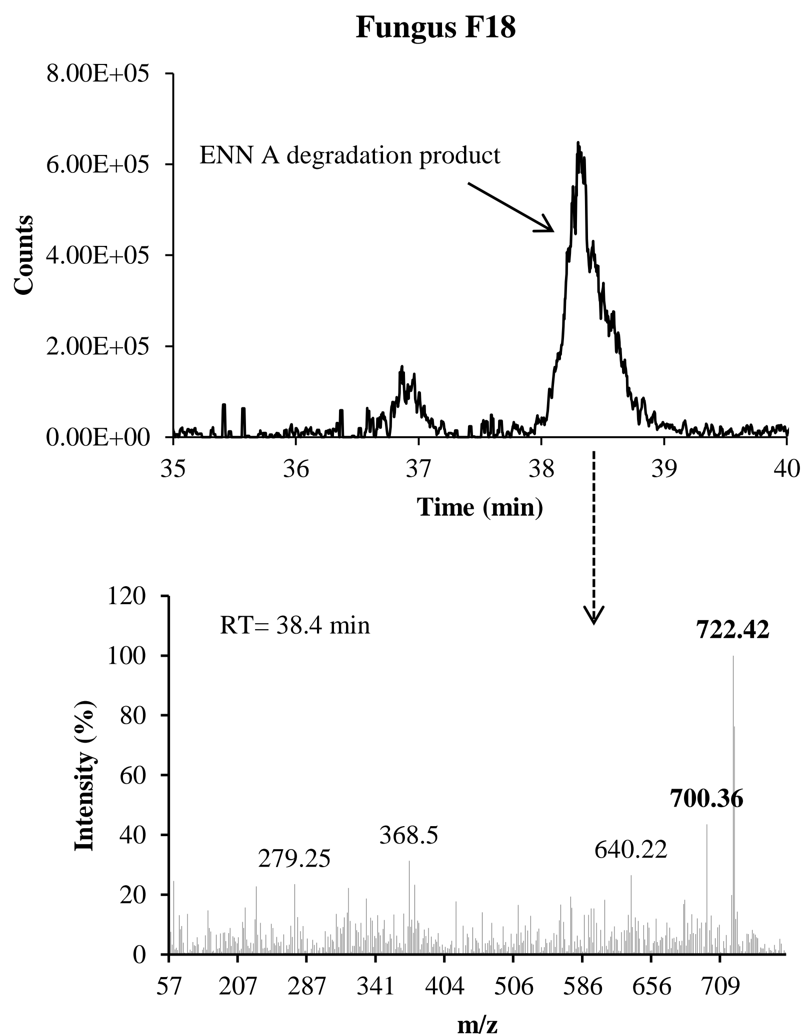


Figure 11: EIC and mass spectra of enniatin A degradation product by bacterium K4 with $[M+H]^+$ 700.36.



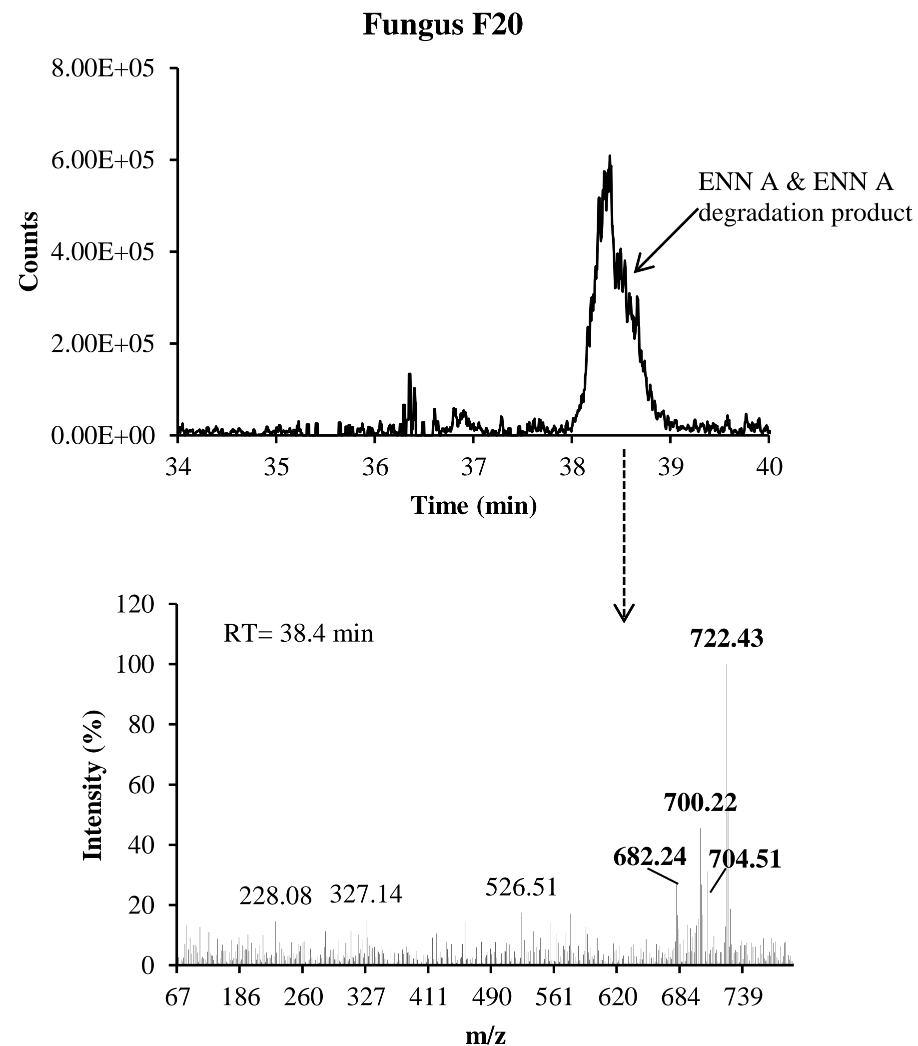


Figure 14: EIC and mass spectra of enniatin A degradation product by fungi F18 and F20 with $[M+H]^+$ 700.36 or 700.22.

4. Discussion

4.1. Selection of microorganisms from mixed cultures

This section of the study was conducted in order to isolate some microorganisms capable of utilizing enniatin as unique carbon source, by incubating a mixed culture from diverse sources in a minimal medium containing enniatin (final concentration 1 mg/mL). After observation of the wells of the microtiter plate under binocular microscopes, signs of growth of the microorganisms through the presence of mycelia or turbidity and colloidal solution were noted, the disappearance of enniatin crystals was also observed. Enniatins are a class of compounds which form crystals as shown in the previous chapter of this thesis and also shown by Burmeister and Plattner (1987), Strongman *et al*. (1988), Blais *et al*. (1991), Richter *et al*. (2014). The property of beauvericin to form crystals has been exploited by Duvick and Rood (2000) in the detoxification process of the mycotoxin beauvericin which is structurally similar to enniatin. Beauvericin was incubated with a mineral salt suspension medium and the disappearance of beauvericin crystals was monitored during the process. During our assay, although enniatin crystals disappeared in some wells, no microorganism was isolated. This may be because some microorganisms which are able to grow in the minimal medium containing enniatin as unique carbon source may not grow on the agar plates and the conditions used here, what might have prevented their isolation during these experiments.

4.2. Sources of isolated microorganisms

During our study, the strategy used to isolate enniatin-metabolizing microorganisms included first the isolation from mixed cultures of microorganisms able to utilize enniatin as unique carbon and energy source then the isolated microorganisms were screened for enniatin transformation by monitoring the enniatin disappearance and the appearance of new compounds in the culture media by HPLC-UV.

In the literature available, many materials have been used in the search of mycotoxin detoxifying microorganisms. In the case of the present study, the major elements providing bacteria with the ability to grow on media containing enniatin as a unique carbon source were soil, water, grains and nuts in a decreasing order. These results conform to other findings in the literature (Ikunaga *et al.*, 2011; Yi *et al.*, 2011; Tan *et al*., 2014) which confirms the richness of soil in terms of the microorganisms possessing mycotoxin degrading properties.

The use of grains, nuts and cereal based products as sources of microorganisms for the detoxification of enniatin was based on the idea that, microorganisms having the ability to degrade enniatins might be present in the same compound from which high enniatin concentrations have been found. This was verified by the high number of microorganisms isolated from these sources. A typical example of this hypothesis is the use of wheat kernels infested with *F. graminearum* to isolate *Nocardia globulera* strains able to detoxify beauvericin (Duvick and Rood, 2000).

Water samples also provided several bacteria able to grow on media containing enniatin as a unique carbon source. Although no further activity was detected using HPLC in this study, water constitutes a suitable source of bacteria and yeast in terms of mycotoxin degradation. For example Zhu *et al.* (2015) demonstrated the ability of the yeast *Rhodosporidium paludigenum* isolated from South East China Sea to degrade patulin into a less toxic compound.

4.3. HPLC-UV chromatograms and enniatin reduction in culture media

In general a reduction of enniatin concentrations in the cultures of all isolated microorganisms was observed, but no new signal was observed for most microorganisms after analysis with HPLC-UV of those cultures. Völkl *et al.* (2004) have described an adsorption of deoxynivalenol into the cell wall of the microorganism; this was verified by lysing the cell wall with proteinase K and SDS. Although this was not tested in the present study, this might be a plausible explanation of the fact that enniatin concentrations were reduced in some cases but no new signal of metabolization of enniatin by the microorganism was observed.

The order of reduction of enniatin in the culture media of tested microorganisms was in general enniatin A, A1, B1 and B in a decreasing order, this might be due to the initial proportion of enniatin used (the mixture of enniatin contained about 77% enniatin B, 17% enniatin B1, 6% enniatin A1 and 0.3% enniatin A); there is no other obvious explanation of this trend where enniatin A is utilized most compared to enniatin B.

The chromatograms of the samples containing the bacterium K4 or the fungi F18 and F20 showed different patterns compared to those of the control samples, also new peaks having a lower retention time were observed. After comparison of the HPLC-UV chromatograms of bacterium K4 and fungi F18 and F20 when incubated in medium containing enniatin and those when incubated in medium without enniatin, they were assumed to transform enniatins

into new products. The new peaks suggested the presence in the culture media of new less apolar compounds resulting from the degradation of enniatins used by the microorganisms present in the media. Since the chromatogram pattern of bacterium K4 was identical when it was incubated either in MM or in MMGP, the degradation of enniatins by bacterium K4 seemed not to be affected by the composition of the media used and was independent of the presence of another carbon source such as glucose and tryptone present in the medium MMGP and absent in the medium MM. On the contrary, fungus F20 and F18 seemed to manifest a higher change when enniatins are present in the media when incubated in MMGP compared to MM, the presence of glucose and peptone in MMGP seemed to favor the ability of the fungi to transform enniatins. Due to the limitation in terms of precision of the HPLC-UV, no further information such as the possible compound corresponding to those new signals could be extracted from the results; therefore the samples were analyzed using HPLC-MS.

4.4. Identification of degradation products using HPLC-MS

Experiments in this section were conducted to find the enniatin degradation products resulting from the action of the bacterium K4 and fungi F18 and F20 incubated in MMGP with enniatin A, A1, B1 and B using HPLC-MS and to deduce the degradation reactions and the possible formula of the products. The procedure to identify the degradation products during our study involved the analysis of the cultures of the supernatants of the microorganisms incubated in MMGP containing enniatin, compared to the microorganisms incubated in MMGP without enniatin. When new signals in the treated samples were found, the mass to charge was determined and the detoxification reaction was postulated.

Four degradation products were confirmed, resulting from the hydrolysis of enniatins B, B1, A1 and A. All the four degradation products found in the culture supernatant of bacterium K4 appeared as double peaks in the EIC, the two peaks possessed the identical mass spectrum. This led to the idea that the hydrolysis occurred at 2 different sites in ring of cyclic structure of enniatin, leading to two compounds having the same molecular mass and formula but different chemical structures. In EIC of the HPLC-MS analysis the samples of the fungus F18, the degradation products of enniatins B, B1 and A1 showed also two peaks having the same mass spectrum, while the degradation product of enniatin A was present as a single peak. As for the fungus F20, the degradation products of enniatins A and A1 were single peaks in the EIC while the ones of enniatins B and B1 were present as double peaks with the same mass

spectra. This highlights the divergence of the action of the bacterium K4 and fungi F18 and F20 towards enniatin degradation.

Few references are available in the scientific literature concerning enniatin degradation by microorganisms and their degradation products. Roig *et al*. (2013) identified three enniatin degradation products after incubating several strains of *Lactobacillus* and *Saccharomyces* in the liquid medium De Man Rogosa Sharpe (MRS) and potato dextrose broth respectively for 48 hours. The degradation products were characterized using liquid chromatography coupled to the mass spectrometry detector in tandem (LC-MS/MS). They were found as enniatin B with the loss of a structural component represented by the hydroxyisovaleric acid, the enniatin B with the loss of two molecules of water and also of a methyl group and the enniatin B1 with the loss of a structural component as the valine unit. The degradation of enniatins B and B1 was reported by Meca *et al*. (2014). In this case, the degradation was done by a *Bacillus subtilis* strain incubated in tryptic soy broth for 48 hours, and then the bioactive compounds were analyzed using liquid chromatography coupled to mass spectrometry detector (LC-MS). The degradation products resulted from the loss of hydroxyisovaleric acid unit in the structure of enniatins B and B1.

The process of hydrolysis of enniatins demonstrated in this study is similar to the process of detoxification of the mycotoxin zearalenone (ZEN) mechanisms demonstrated by Kakeya *et al.* (2002), due to the cleavage of the lactone rings from the backbone and therefore transforming ZEN into a far less potent compound.

5. Conclusion

The present study revealed the following points:

- In total 114 bacteria and 34 fungi isolated from various sources were able to grow on a minimal medium containing enniatins as unique carbon source.
- Soil, water, grains and nuts were the main sources of bacteria, while nuts and grains were the main sources of fungi able to grow on a minimal medium containing enniatins as unique carbon source.
- A reduction of enniatins was observed when the above mentioned microorganisms were incubated in MM and MMGP, the tendency of reduction varied depending on the microorganism and the media used. Enniatin reduction was higher in MMGP compared to MM for fungi, while bacteria showed a comparable rate of reduction in both media.
- Out of the 88 bacteria and 23 fungi tested as single culture for enniatin degradation, one bacterium and two fungi were able to metabolize enniatins when incubated in MMGP and/or MM leading to the formation of new compounds. Reduction of enniatins in the culture media of other microorganisms was presumably due to adsorption into the cell wall.
- The bacterium K4 and one fungus F18 able to degrade enniatins were isolated from a wheat field soil sample, while the other fungus F20 was isolated from hazel nut kernels.
- Four enniatin degradation products were identified using HPLC-MS.
- Enniatin degradation products had protonated ions with m/z 658, 672, 686 and 700 for enniatin B, B1, A1 and A respectively. It was assumed that enniatin degradation products resulted from the hydrolysis of an ester or an amide bond in the cyclic ring of enniatins.

This is the first report which demonstrates the capacity of a bacterium K4 and fungi F18 and F20 to degrade enniatin B, B1, A1 and A forming four degradation products.

The deep characterization of the degradation compounds in a kinetics assay is ongoing using Ultra Performance Liquid Chromatography (UPLC). Further investigations will focus on the incubation of single enniatin variants with the detoxifying microorganisms, the monitoring of the degradation process under different parameters and the purification of the degradation compounds. The structure of the detoxification product should be confirmed using nuclear magnetic resonance (NMR). Moreover, the toxicological parameters of the purified degradation compounds should be evaluated.

6. Acknowledgement

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5. Appendix

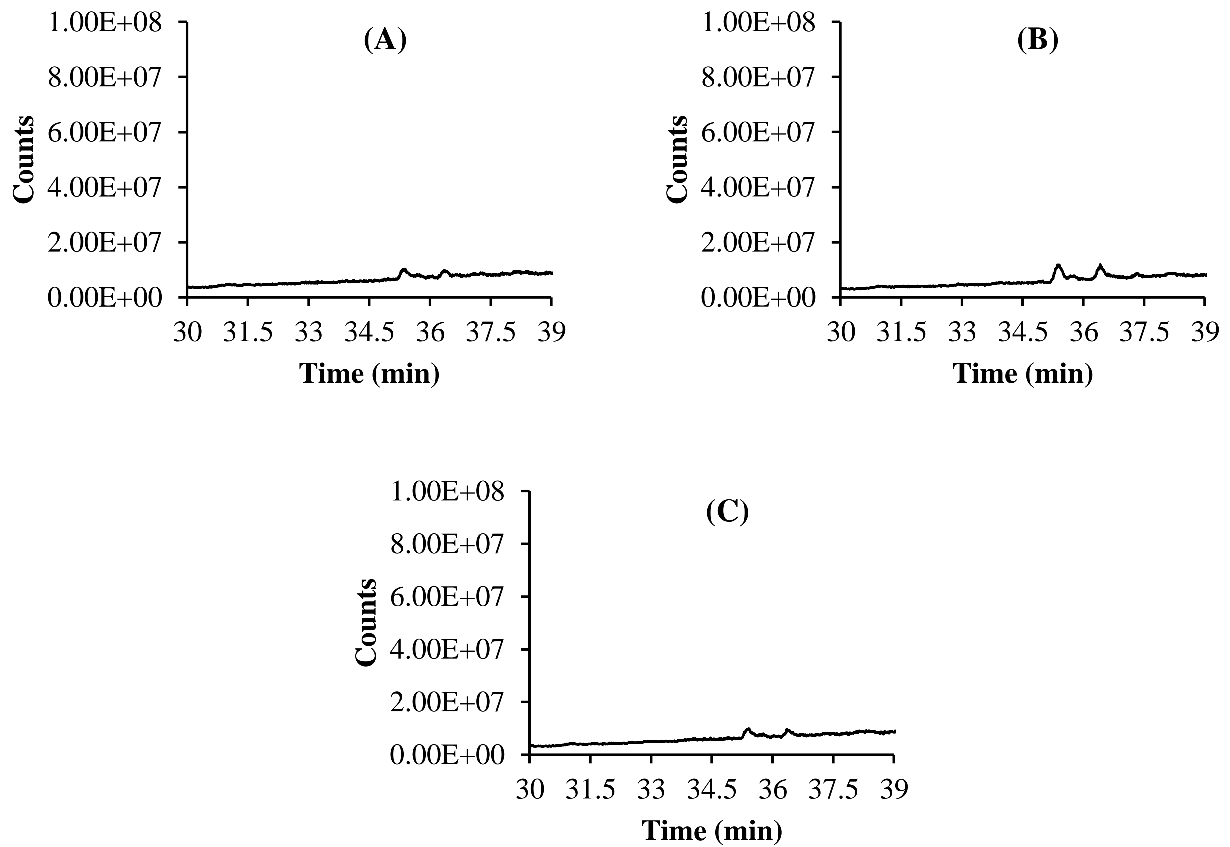

Figure i: Total ion chromatograms of bacterium K4 (A), fungus F18 (B) and fungus F20 (C) incubated in MMGP for 3 days (K4) and 5 days (F18, F20).

Chapter 4

Identification of a bacterium and two fungi capable of degrading enniatins

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Abstract

Within the framework of finding some microorganisms able to degrade enniatins as reported in the previous chapter, this study was conducted in order to identify pure unknown cultures of a bacterium and two fungi isolated previously based on their ability to degrade enniatins. The identification process was made initially based on the cultural, morphological and biochemical (for the bacterium) characteristics of the microorganisms. The species of the microorganisms were confirmed based on the 16S rRNA gene sequence for the bacterium and the sequences of the Internal Transcribed Spacer 1 (ITS1), 5.8S subunit of the ribosomal RNA and Internal Transcribed Spacer 1 (ITS2) regions for the fungi. The bacterium isolated from a soil sample collected in a wheat field was identified as *Bacillus licheniformis*, while the fungi isolated from Hazel nuts and a wheat field soil sample (same source as the bacterium) were identified as *Clonostachys rosea* and *Acremonium strictum* respectively.

Keywords: Enniatin, *Bacillus licheniformis, Clonostachys rosea, Acremonium strictum*, morphological and molecular characterization, ITS1, ITS2, 16S rRNA.

1. Introduction

Bacteria, yeast and fungi have recently been used for degradation of mycotoxins by acting as biotransforming agents. These microorganisms are active in the degradation of particular mycotoxins or class of mycotoxins by transforming them into less toxic compounds (Boudergue *et al*., 2009). The isolation of microorganisms which have mycotoxin detoxification properties generally involves the use of enrichment culturing and/or screening strategies to obtain mixed or pure cultures (Karlovsky, 1999). Generally random testing of unknown cultures is used and the taxonomy of active microorganisms is made after detecting any activity. For instance strains of *Nocardia globulera* have shown activity in the detoxification of beauvericin -an enniatin structurally identical mycotoxin- both *in vitro* and in beauvericin contaminated corn, the bacteria were isolated from wheat kernel infested with *F. graminearum* (Duvick and Rood, 2000). According to several studies, soil is a source that has provided numerous microorganisms able to degrade mycotoxins. Bacterium *Nocardioides* sp. strain WSN05-2 isolated from a wheat field soil was able to degrade deoxynivalenol into an intermediate 3-epi- deoxynivalenol (Ikunaga *et al*., 2011). Four different soil types including sandy, loamy, gyttja and moldy soil were used as a source of microorganisms for the degradation of zearalenone (ZEN). *In vitro* degradation of ZEN was efficiently achieved by two *Pseudomonas* strains namely *Pseudomonas alcaliphilia* TH-C1 and *Pseudomonas plecoglossicida* TH-L1 (Tan *et al*., 2014). Likewise, Yi *et al.* (2011) isolated and characterized a *Bacillus licheniformis* strain able to degrade zearalenone out of 168 bacterial strains isolated from soil samples. In the three previously mentioned studies, the active bacteria were taxonomically characterized after discovering their detoxification properties, with the combination of biochemical and molecular techniques involving the sequence of the 16S rRNA gene.

On the other hand, some authors have tested taxonomically known microorganisms thought to degrade mycotoxins. Spores, mycelium and culture filtrate of *Aspergillus niger* strain FS10 removed ZEN in potato dextrose broth, with fluctuating efficiency of 89.56%, 43.10%, and 68.16% respectively (Sun *et al*., 2014). Nine taxonomically known bacteria (*Bifidobacterium longum, Bifidobacterium bifidum, Bifidobacterium breve, Bifidobacterium adolescentes, Lactobacillus rhamnosus, Lactobacillus casei-casei, Lactobacillus ruminis, Lactobacillus casei, Streptococcus thermofilus*) and twenty-two strains of *Saccharomyces cerevisiae* showed a significant reduction of enniatins *in vitro* (in liquid medium of De Man Rogosa

Sharpe or in potato dextrose broth) ranging from 5 to 99% (Roig *et al*., 2013). Beauvericin reduction in the range of 66 to 100% has been demonstrated in model solution and in corn flour treated with intracellular raw enzymes of four *Saccharomyces cerevisiae* (named LO9, YES, A34 and A17) (Meca *et al*., 2013). Moreover, Meca *et al*. (2014) tested six known *Bacillus subtilis* strains in tryptic soy broth contaminated with enniatins (A, A1, B and B1) and recorded a mean reduction of enniatin content of 64 to 99%.

In the previous chapter of this thesis, a bacterium K4, two fungi F18 and F20 were isolated from wheat field soil (K4 and F18) and hazel nut (F20) samples, and showed positive results regarding the degradation of enniatins A, A1, B1 and B . In the present study, bacterium K4, fungi F18 and F20 were phenotypically and genotypically characterized.

For bacterium K4, more specifically we intended to identify:

- The morphological characteristics of the bacterium K4 including: colony size, form, elevation, margin/edges, color, pigment production, opacity, surface, consistency and odor.
- The biochemical characteristics of the bacterium including: Gram stain, catalase test (3%, 15%), starch hydrolysis, SIM (indole, motility and sulfide production), nutrient gelatin stab, citrate test and urease test, the bacterial growth at different temperatures and in different media (nutrient broth containing 6.5% NaCl, sabouraud dextrose agar, nutrient broth, starch agar, pectine agar and casein agar).
- The species of bacterium K4 through the sequence of the 16S rRNA gene.

For fungi F18 and F20 our specific objectives were to determine:

- The optimal growth temperature and pH.
- The morphological characteristics of fungi after visual observation of factors such as colony margin, structure, surface, color (bottom and top), excretion into the medium and presence of oil droplets. And after microscopic observation of fungal structures such as conidia, macroconidia, hyphae, conidiophores, phialides, presence of chlamydospores, septae and clamps connections.
- The species of the fungi using the ITS1, 5.8S RNA ribosomal units and ITS2 regions of the DNA, and translation elongation factor 1-alpha (TEF-1α) gene sequence.

2. Material and methods

2.1. Identification of enniatin-detoxifying fungi

2.1.1 Optimal growth temperature and pH

To examine the optimal growth temperature of the isolated fungi, one agar plug (5 mm diameter) of a 5 days old fungus was inoculated on potato dextrose agar (PDA, from Merck, Darmstadt, Germany) and the plates were incubated at 12°C, 16°C, 20°C, 24°C, 28°C and 32°C respectively during a period of 10 days. The four radial lengths were measured every 24 hours. Each experiment was done in three replicates.
In order to find out the most suitable growth pH of the isolated fungi, potato dextrose broth (PDB) was prepared and the pH adjusted to 2.4; 3; 3.6; 4.4; 5.6; 7; 7.5; and 8 respectively. One agar plug of 5 days old fungi (5 mm diameter) was inoculated into each flask containing 25 mL autoclaved PDB. The flasks were incubated for 10 days (160 RPM, at optimal growth temperature of each fungus). After 10 days, the cultures were vacuum filtered and the pH-values of the filtrates were recorded. Before filtration, filter papers were dried in an oven overnight at 70°C, cooled down in a desiccator for 2 hours and weighed prior to filtration. The mycelium on the filter paper was dried in an oven for 24 hours at 70°C, cooled down in a desiccator for 2 hours and weighed. The weight of mycelium was calculated by deducting the final weight of the mycelium on filter paper from the weight of the filter paper prior to filtration. Each experiment was performed in four replicates.

2.1.2. Morphological characterization

Visual description of colony characteristics

Fungal isolates were grown on PDA (Merck, Darmstadt, Germany) for 7 and 14 days respectively and visual characteristics including colony features (margin, structure, surface, color-bottom and top, excretion into the media and presence of oil droplets were recorded.

Microscopic characteristics of the fungal isolates

A modified Riddel's simple method of slide culturing (Riddell, 1950) was used for the *in situ* microscopic observation of the fungi F18 and F20. A glass petri dish containing a filter paper, a U-shaped glass rod and a glass microscope slide were autoclaved at 120°C for 20 minutes.

A block of sabouraud agar of about 7×7 mm was placed on the microscope slide and the four sides were aseptically inoculated with spores or mycelium fragments from a 7 days old fungus. A sterile cover-slip was placed on the upper surface of the agar block and the filter paper in the petri dish was moisturized with about 3 mL sterile tap water. The petri dish was incubated at 25°C for 48 and 72 hours. After incubation, a drop of lactophenol cotton blue was applied on a clean microscopic slide and the cover-slip was placed mold side down on the lactophenol cotton blue stain slide. The preparations were analyzed using oil immersion and a light microscope (Leica Leitz DMRB) equipped with a color camera (Leica DFC420).

2.1.3. Molecular characterization of fungi isolates

The molecular characterization of active fungi was performed using the ITS1 and ITS4 primers to amplify the ITS1 region, 5.8S ribosomal RNA subunit and ITS2 region of the DNA, as well as the amplification a 700 bp region of the translation elongation factor 1-alpha (TEF-1α) gene sequence. The DNA of the fungi was extracted, amplified using polymerase chain reaction (PCR) and sequenced.

DNA extraction

Fungi were grown in PDB for 7 days (120 RPM, 25°C) and vacuum filtered. Fungal mycelium was freeze dried in a freeze drier (Christ, Beta 1-8) and stored at -20°C. DNA extraction was carried out according to CTAB method (Brandfass and Karlovsky, 2008).

Gel electrophoresis

In order to check the efficiency of DNA extraction and the purity of extracted DNA, a 3µL DNA sample were mixed with 2 µL Blue juice and loaded on a 0.8% agarose gel and run for 1 hour at 60 V. The gel was stained with ethidium bromide for 10 minutes, then distained in water for 10 minutes and visualized under UV light.

Polymerase chain reaction

Elongation factors (EF)

Primers pairs for elongation factor 1 EF1-forwards (5’-ATGGGTAAGGARGACAAGAC-3’) and for elongation factor 2 EF2-reverse (5’-GGARGTACCAGTSATCATGTT-3’) (O’Donnell *et al.*, 1998) were used for PCR amplification of a part of the translation

elongation factor 1-α gene. The cycling conditions included 95°C 30 sec, 30 × 94°C 30 sec, 59°C 30 sec, 72°C 1 min, close loop 72°C 5 min. The extracted DNA was diluted 1/100. A final volume of 25µL was used for PCR and was composed of 10.75 µL water, 5 µL 5x reaction buffer (New England BioLabs), 0.2 µL $MgCl_2$ (Bioline), 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 One Taq Hotstart DNA polymerase 0.25 U (New England BioLabs, Ipswich, MA, USA) and 6 µL of DNA sample. A control PCR without DNA was set up to check for nonspecific amplification. The expected PCR products' size was 700 bp.
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 µL of the PCR product (144 ng/µL) were sent to LCG Genomics GmbH (Ostendstr. 25. TGS Haus 8, 12459 Berlin, Germany) for sequencing.

Internal transcribed spacer (ITS)

A touch down program was used for the amplification of the internal transcribed spacer region with the primers pairs ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White *et al*., 1990). The cycling conditions included 94°C 30 sec, 10 × 94°C 30 sec, 62°C-53°C -1°C/cycle 40 sec, 72°C 1 min, close loop 30× 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 25µL mixtures composed of 13.45 µL water, 2.5 µL 10x reaction buffer (New England BioLabs, Ipswich, MA, USA), 0.2 µL $MgCl_2$ (Bioline), 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 DNA polymerase 0.25 U (New England BioLabs, Ipswich, MA, USA) and 6 µL 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.
Lambda DNA was digested with the restriction enzyme PstI and used as a size standard. For agarose gel electrophoresis of the PCR products, 2 µL blue juice were mixed with 2 µL 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.

Phylogenetic analysis

Similarity searches in the sequences of the sequenced ITS1, 5.8S ribosomal subunit and ITS2 regions were done using BLAST in NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Alignments and phylogenic analysis of the above mentioned sequences were performed and phylogenetic trees were constructed using the software MEGA.7 (Kumar *et al*., 2015).

2.1.4. Statistical analysis

Significance of data on radial mycelium growth according to growth temperature and mycelium weight according to pH of the fungi F18 and F20 was analyzed using the software IBM SPSS for Macintosh version 22.0 (2013). Analysis of Variance (ANOVA) and post-hoc Tukey test in a completely randomized design were performed for the comparison of means of dependent variables and two tailed P values were considered as statistically significant on the level 0.05.

2.2. Identification of enniatin-detoxifying bacterium

The identification of enniatin-degrading bacterium was done initially according to morphological examination and some biochemical characterization. Morphological parameters investigated included colony characteristics such as size, form, elevation, margin/edges, color, pigment production, opacity, surface, consistency and odor. Moreover, the biochemical characteristics including Gram stain, catalase test (3%, 15%), starch hydrolysis, SIM (indole, motility and sulfide production), nutrient gelatin stab, methyl red, citrate test and urease test were investigated. The bacterial growth at different temperatures and in different media (nutrient broth containing 6.5% NaCl, sabouraud dextrose agar, nutrient broth, starch agar, pectine agar and casein agar) was tested. A final step involving the molecular characterization of the microorganism was carried out involving DNA extraction, PCR and sequencing of the 16S rRNA genes.

2.2.1. Morphological characterization and optimal growing temperature of the bacterium K4

The bacterium K4 was streaked onto nutrient agar plates using T-streaking method and grown for 24 hours at 25°C. The features of the colonies such as size, form, elevation, margin/edges, color, pigment production, opacity, surface, consistency and odor were recorded.
To evaluate the optimal growth temperature in liquid medium, bacterium K4 was grown in full media (FM) broth at 25°C for 24 hours. The OD600 of the culture was measured and

adjusted to 0.6. A volume of 500 µL of bacteria suspension was inoculated in 5 mL of tryptic soy broth and the tubes were incubated at 25°C, 28°C, 30°C, 35°C and 37°C respectively during a period of 24 hours. Each experiment was performed in 3 repetitions. After 24 hours, the bacterial cultures were diluted ½ and the OD600 was measured.

A light smear of the bacterium K4 from a young isolated colony was inoculated onto nutrient agar containing 6.5% NaCl, nutrient broth, sabouraud dextrose agar, starch agar, pectin agar and casein agar. After 24 hours incubation at 30°C, growth was recorded.

2.2.2. Biochemical characterization of the bacterium K4

Gram Stain

Gram Stain was performed according to Hucker's Modification of Gram's Stain method as described by Gephardt *et al*. (1981). Reagent crystal violet, Gram's Iodine, decolorizing agent and the counterstain were prepared as described by Gephardt *et al*. (1981).

Catalase test

The slide or drop catalase test was performed to detect the catalase production and activity according to Duke and Jarvis (1972), MacFaddin (2000) and Forbes *et al*. (2007).

Starch Hydrolysis

The starch agar media used were prepared according to Zimbro *et al*. (2009) and the method used for the starch hydrolysis test was the method described by Collin *et al*. (1995).

Sulfide Indole Motility (SIM) test

SIM test was performed using the SIM media (Difco, 1998; Green *et al*., 1951; Hiss, 1920). Motility test was carried out as described by Sulkin and Willett (1940), Difco (1998) and Green *et al*. (1951). Sulfur reduction test (Sulkin and Willett, 1940; Difco, 1998) and the indole test were performed according to Baron *et al*. (1990), Harley (2005) and MacFaddin (2000).

Nutrient gelatin stab

Gelatin stab test was performed as described by Difco Laboratories (2009).

Citrate test

The procedure used was as described by Difco (1998), MacFaddin (2000), Harley (2005) and Reddy (2007). The Simmons citrate medium was prepared as described by Difco & BBL Manual (2009).

Urease test

Urease test was conducted according to the Christensen's Method (Christensen, 1946).

2.2.3. Molecular identification of bacterial isolate

16S rRNA gene sequencing was performed for the molecular characterization of the active bacterium. The procedure encompasses three major steps: DNA extraction, PCR and sequencing. Then the phylogenic analysis was carried out.

DNA extraction

DNA extraction from enniatin detoxifying bacterium K4 was achieved according to a modified method of Ausubel *et al.,* (1987). 1.8 mL of 24 hours bacterial culture on full media (FM) broth was put into a 2 mL sterile tube and centrifuged during 10 minutes 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 hour 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 minutes. 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 minutes. The tubes were then centrifuged at 14000 RPM during 10 minutes 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 minutes at room temperature. The samples were centrifuged for 10 minutes 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 minutes and resuspended in 50 µL of TE-buffer overnight.

Electrophoresis

To check for the success of the DNA extraction step, 2 µL DNA solution, 1 µL and 2 µL of the standard Lambda-DNA (25 ng/mL Lambda DNA) were run on 0.8% agarose gel as described above.

PCR

Molecular identification of the bacterial isolate was achieved through 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× (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 40min; 72°C 1min), close loop 72°C 5minutes. 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, prepared for sequencing and sequenced as described above.

Phylogenetic analysis

Similarity searches in the sequences of the sequenced 16S rRNA gene, alignments and phylogenic analysis were performed as described above.

2.2.4. Statistical analysis

Significance of OD600 data related to growth of bacterium K4 at different temperatures was analyzed using the software IBM SPSS for Macintosh version 22.0 (2013). Analysis of Variance (ANOVA) and post-hoc Tukey test in a completely randomized design were performed for the comparison of means of dependent variables, two tailed P values were considered as statistically significant on the level 0.05.

3. Results

3.1. Identification of enniatin-detoxifying fungi

3.1.1. Optimal growth temperature and pH

In general, the growth of fungus F18 was slower compared to fungus F20 when grown on PDA (growth expressed as radial growth) or on PDB (growth expressed as dried mycelium weight) at various tested temperatures and pH values.

The fungus F20 grew slower at temperatures lower than 20°C and faster at temperatures above 24°C. The fastest growth of fungus F20 was recorded at 28°C, where the radial growth reached about 40 mm after 10 days and was significantly different ($P<0.05$) from other values, while the lowest growth was observed when the fungus was incubated at 12°C (Figure 1, A). For fungus F20, the optimal pH growth in PDB was between 3.6 and 8 where the values of the dried mycelium reached about 0.25 g, while the lowest growth was observed in PDB pH 2.4 and a moderate growth at pH 3 (Figure 2, A).

When the fungus F18 was grown on PDA, a maximum radial growth of about 16 mm at 24°C was recorded after 10 days. The lowest radial growth was recorded when the fungi was incubated at 32°C (almost no growth) and 12°C (about 6 mm) (Figure 1, B). No significant difference ($P>0.05$) between the values of the dried mycelium weight of fungus F18 was observed among the tested pH values. According to the results obtained, the fungus F18 can tolerate a wide range of pH in liquid media such as PDB. However, the optimal pH growth in PDB was between 3 and 3.6, where the dried mycelium weight reached about 0.2 g, while the lowest growth was observed at higher pH values from 5.6 to 8 in PDB (Figure 2, B).

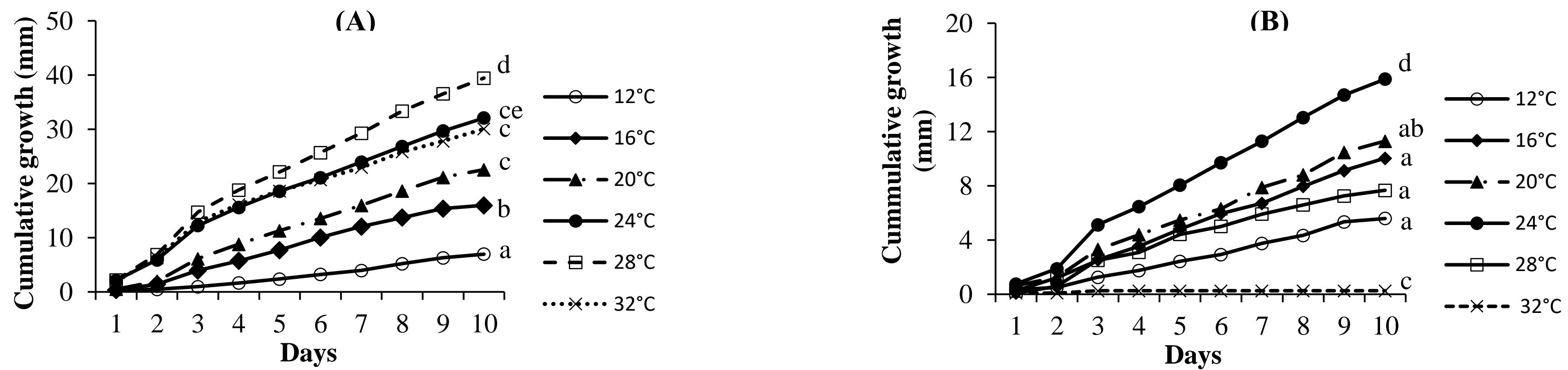

Figure 1: Mean values of the cumulative radial growth of fungi F20 (A) and F18 (B) grown on PDA plates at different temperatures.

(A), (B): a, b, c, d, e Lines (mean ± Standard deviation) carrying the same letter represent values (after 10 days) statistically not different P>0.05, n=3.

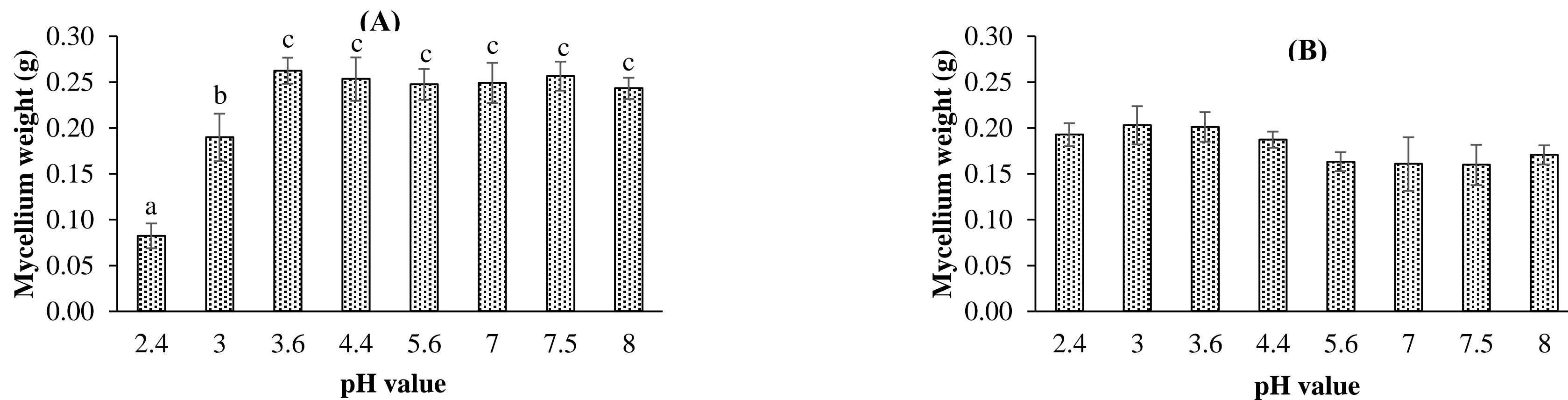

Figure 2: Mean values of the dried mycelium weight of fungi F20 (A) and F18 (B) after culture in PDB with different pH values for 10 days at 28°C for F20 and 24°C for F18. *(A): a, b, c Histograms (mean ± Standard deviation) carrying the same letter represent values statistically not different P>0.05. (B): No statistically difference among the values P>0.05, n=4.*

3.1.2. Morphological characterization of fungal isolates

Visual description of the colony characteristics

Morphological characteristics of both fungi F18 and F20 are recorded in Table 1. The colonies of fungus F20 were circular with irregular margin. The mycelium color was white on the top and cream on the reverse plate. When observed from the side, colonies presented abundant air mycelium. For fungus F18, the colonies were flat and slightly raised in the center. On the surface of the agar, the colonies were membrane-like and embedded in the agar (forming few lines from the center to the margin of the colony). The colonies were white in color, while the reverse plate was yellowish to orange in color. Fungi F20 and F18 grown on PDA for 14 days at 25°C are shown in the Figure 3.

Table 1: Description of the colony characteristics of fungal strains F18 and F20 after growth for 7 days on PDA at 24°C and 28°C for F18 and F20 respectively.

Parameters	F 18	F20
Culture medium, age, growth temperature	PDA, 7 days, 24°C	PDA, 7 days, 28°C
Growth rate*	1.61	4.18
Colony margin and structure	Circular and regular	Circular and irregular
Colony surface and color on agar surface	Smooth, orange	Smooth, white-cream
Colony color from the top	White	Cream
Colony color from underneath	Orange	Cream
Colony appearance from the side	Little aerial hyphae	Abundant aerial hyphae
Excretion into the media	No	No
Oil droplets and aerial mycelium floccose**	No	Yes

** Radial colony expansion in mm/day during 7 days*
*** After 14 culture days*

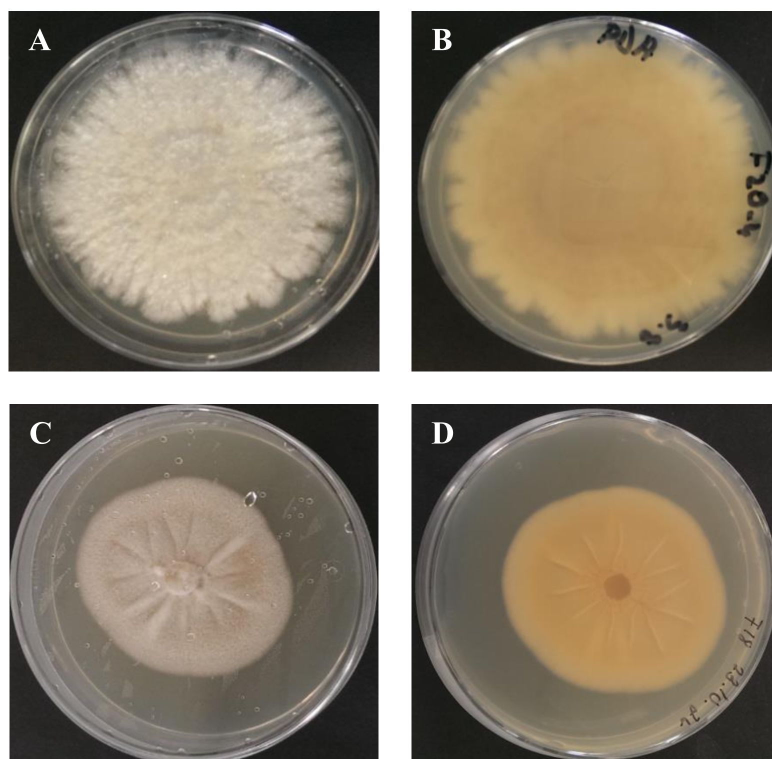

Figure 3: Colony of fungi F20 (A: top view; B: reverse plate view) and F18 (C: top view; D: reverse plate view) grown on PDA for 14 days at 25°C.

Microscopic description of the fungal isolates

Fungus F20

Morphological features of fungus F20 under light microscope (100×) are shown in Figure 4. The conidia (Figure 4-A & B) of the fungus F20 had variable sizes corresponding to different developmental stages and possess an oblong shape. The sizes are as follows: length: 5.2 µm and width: 2.2 µm for microconidia; length: 11.63 µm and width: 3.7µm for macroconidia. Conidia were produced singly and presented pigmentation but no septae. The conidiophores (Figure 4-C & D) were long and frequently branched. The phialides (Figure 4-C & D) were medium sized, elongate-ampulliform in shape and possessed a basal septum. Moreover, the hyphae were pigmented and septate (Figure 4-E & F). Branchings of hyphae to the conidiophores were regular. Clamp connections and chlamydospores were also observed.

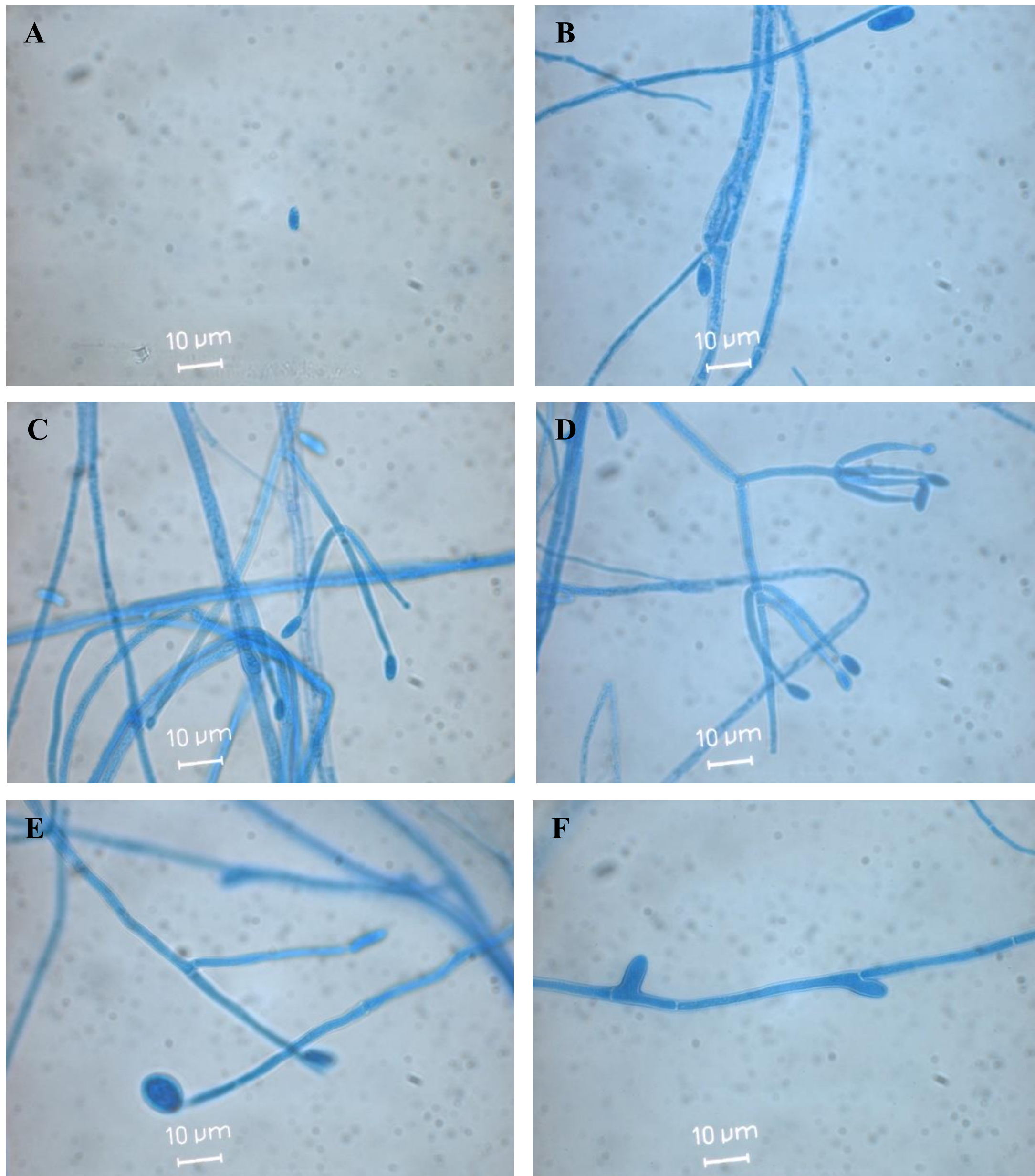

Figure 4: Microscopic structures of fungus F20 under light microscope (100×). (A) Conidia, (B) Macroconidia & hyphae, (C) & (D) Conidiophores, phialides, conidia & hyphae, (E) Chlamydospore & hyphae, (F) Clamps connection & hyphae.

Fungus F18

Morphological features of fungus F18 under light microscope (100×) are shown in Figure 5. The conidia (Figure 5-A & B) were fast developing during microscopic observation and have an oblong-elliptical shape. Their sizes were variable, length: 1.4 µm and width 1.1 µm for microconidia, length: 2.6 µm-4.5 µm and width: 1.1 µm for intermediate conidia; length: 6.0 µm and width 1.6 µm for macroconidia. Conidia were pigmented and aseptate. Fungus F18

showed individual phialides that were long sized, elongated-ampulliform in shape; they were regularly branched to the hyphae and possessed a basal septae (Figure 5-C & D). Hyphae (Figure 5-E & F) of fungus F18 were pigmented and aseptate.

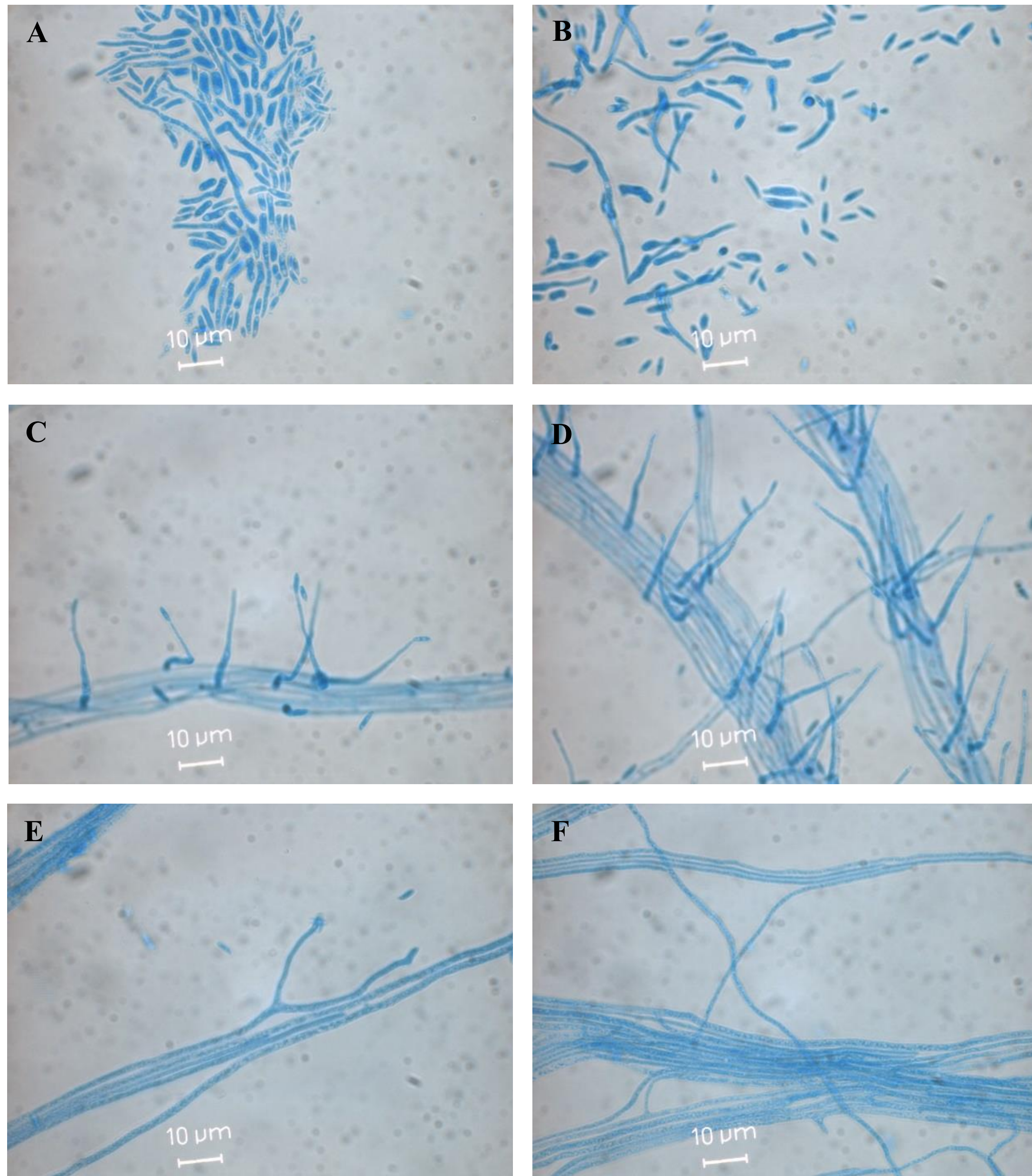

Figure 5: Microscopic structures of fungus F18 under light microscope (100×), (A) and (B) Micro- and macroconidia, (C) and (D) Conidiophores, phialides and hyphae, (E) and (F) Hyphae.

3.1.3. Molecular characterization of fungal isolates

Gel electrophoresis of extracted DNA and PCR products

The agarose gel electrophoresis of total DNA extracted of fungi F18 and F20 is shown in Figure 6-A. With comparison of the sample to the lambda DNA (slot 1), a successful DNA extraction was achieved. The PCR amplification of the extracted DNA using primers EF1, EF2, ITS1 and ITS4 was successful as shown in Figure 6-B and as expected, fragments having about 600-750 base pairs were generated according to comparison with the molecular marker used (Lambda DNA digested with PstI). The products are indicated using an arrow in Figure 6-B.

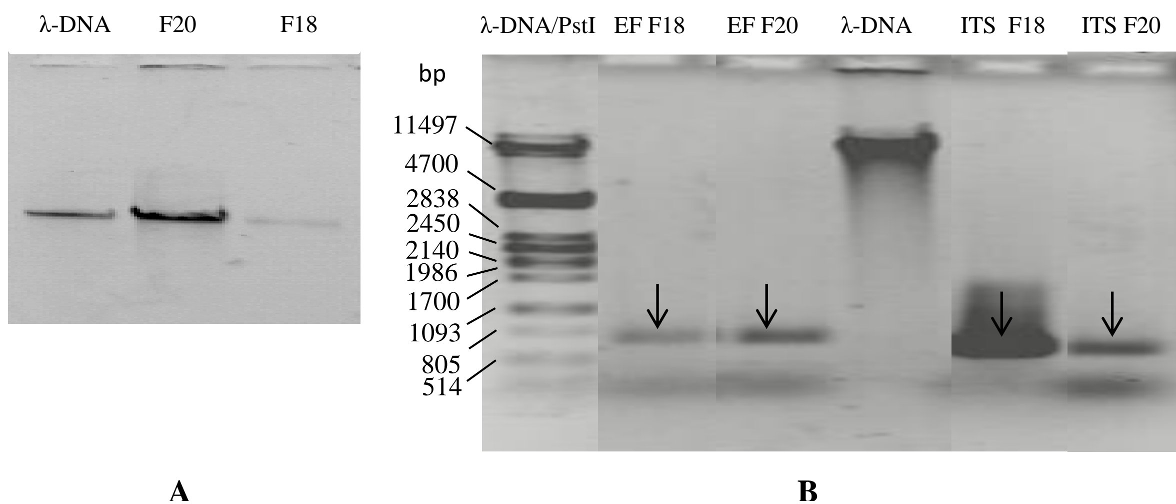

Figure 6: A: Gel electrophoresis of DNA extraction (from left to right: Lambda DNA, extracted DNA of fungus F20, extracted DNA of fungus F18); B: Gel electrophoresis of PCR products of EF1 & 2 and ITS 1 & 4 (from left to right: molecular marker digested with PstI, PCR products using EF1 and EF2 for fungus F18, PCR products using EF1 and EF2 primers for fungus F20, Lambda DNA, PCR products using ITS1 and ITS4 primers for fungus F18 and fungus F20).

Phylogenetic analysis of fungi F20 and F18

Although the PCR amplification and DNA sequencing using the elongation factors 1 and 2 primers were successful, the blast of the sequenced regions did not give conclusive results concerning the molecular taxonomy of the microorganisms. Since a highly mutated area makes species classification possible, probably in this case there was not enough variation in the region of the DNA to distinguish the two species. So the sequence of the translation

elongation factor 1-alpha (TEF-1α) gene was not suitable for the molecular identification of the two fungal species in this study.

The phylogenetic tree obtained using primers pairs ITS1 and ITS4 (including ITS1 and ITS2 regions, and 5.8S ribosomal RNA sequences of the DNA) of fungal strain F20 and C*lonostachys* species is shown in Figure 7. The fungal strain F20 revealed 100% similarity with several *C. rosea* strains (BBA68698, R2-4, 10066) and *Bionectria ochroleuca* SBBEB. *C. rosea* was formerly known as *Gliocladium roseum*, teleomorph: *Bionectria ochroleuca* (Schroers *et al*., 1999).

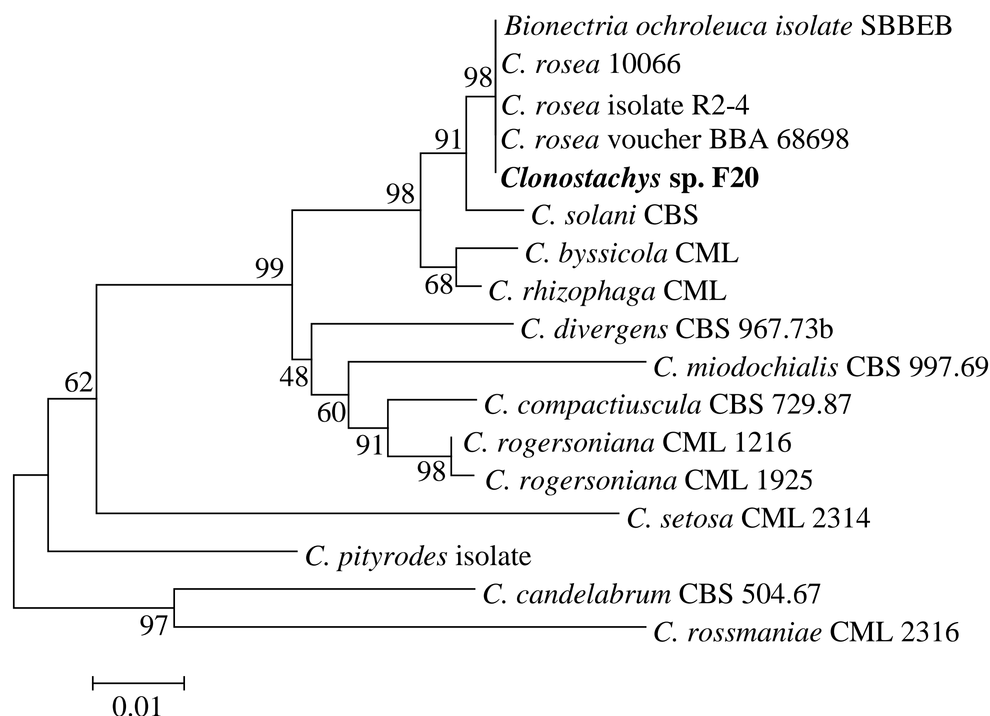

Figure 7: Neighbor-joining phylogenic tree based on the DNA sequences using ITS1 and ITS4 primers of fungus F20 and related fungi genera. At major nodes, bootstrap percentages for 1000 resamplings are shown. The scale bar represents 0.01 nucleotide substitutions per nucleotide position.

Clonostachys species include: *Bionectria ochroleuca* isolate SBBEB (GenBank accession number: EU484311.1), *C. rosea* 10066 (KC819614.1), *C. rosea* isolate R2-4 (KT876552.1), *C. rosea* voucher BBA 68698 (KT215192.1), *C. solani* strain CBS 228.74 (AF358243.1), *C. byssicola* strain CML 2404 (KC806271.1), *C. rhizophaga* strain CML 2312 (KC806275.1), *C. divergens* strain CBS 967.73b (AF210677.1), *C. miodochialis* strain CBS 997.69 (AF210674.1), *C. compactiuscula* strain CBS 729.87 (AF358242.1), *C. rogersoniana* strain CML 1216 (KC806287.1), *C. rogersoniana* strain CML 1925 (KC806292.1), C. *setosa* strain CML 2314 (KC806294.1), *C. pityrodes* isolate C40376SNA1CC1089 (JQ411387.1), *C. candelabrum* strain CBS 504.67 (AF210668.1), *C. rossmaniae* strain CML 2316 (KC806299.1).

According to the results of phenotypic and microscopic observations and the phylogenetic analysis based on the DNA sequences using ITS1 and ITS4 primers, the fungal strain F20

showed 100% similarities with strains *Bionectria ochroleuca* (former name of *C. rosea*), *C. rosea* 10066, *C. rosea* R2-4, *C. rosea* BBA 68698 .

Figure 8 below shows results of the phylogenetic analysis of DNA sequences of the 5.8S ribosomal RNA subunit and the ITS1 and ITS2 regions. Sequence comparison with other fungi of the same species showed high sequence identity. Fungal strains *S. strictum* NRRL 47828, NRRL 47825, T66M were grouped together with the fungus F18, therefore the fungus F18 was identified as *Sarocladium strictum* commonly known as *Acremonium strictum*.

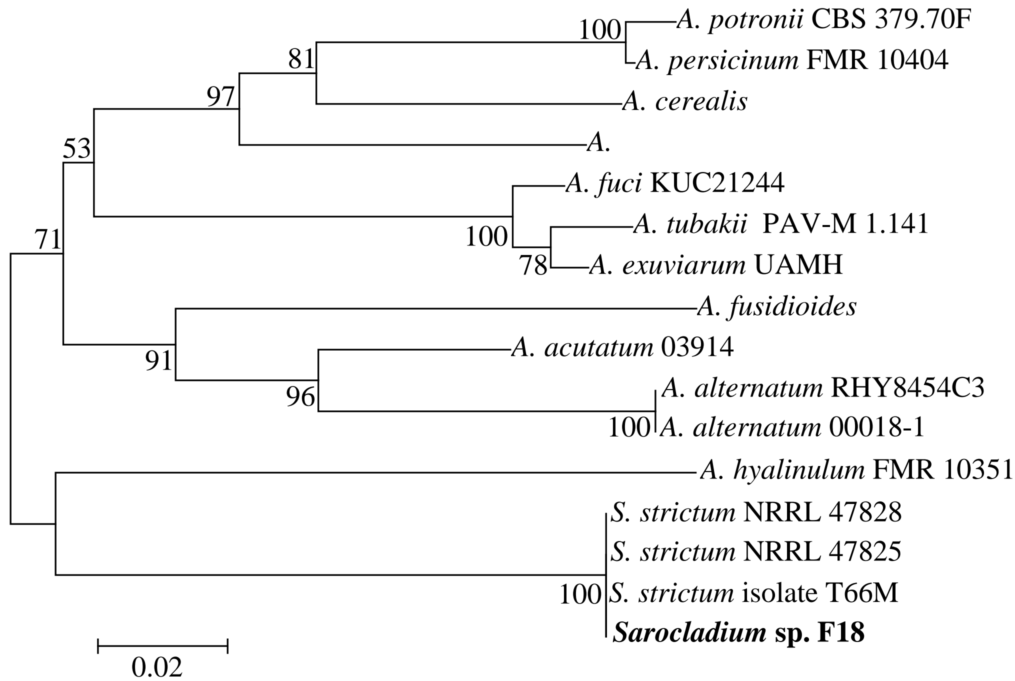

Figure 8: Neighbor-joining phylogenic tree based on the DNA sequences using ITS1 and ITS4 primers of fungus F18 and related fungi genera. At major nodes, bootstrap percentages for 1000 resamplings are shown. The scale bar represents 0.01 nucleotide substitutions per nucleotide position.

Acremonium species include: *A. potronii* CBS 379.70 (GenBank accession number: AY632655.1), *A. persicinum* FMR 10404 (F KP131529.1), *A. cerealis* HSAUP063188 (FJ914717.1), *A. cyanophagus* (DQ393594.1), *A. fuci* KUC21244 (KT207762.1) , *A. tubakii* isolate PAV-M 1.141 (KF915990.1), *A. exuviarum* UAMH 9995 (AY882946.1), *A. fusidioides* (FN706544.1), *A. acutatum* 03914 (KT878339.1), *A. alternatum* RHY8454C3 (KM268869.1), *A. alternatum* 00018-1 (KT192193.1), *A. hyalinulum* FMR 10351 (NR 131321.1), *S. strictum* NRRL 47828 (GU183127.1), *S. strictum* NRRL 47825 (GU183126.1), *S. strictum* isolate T66M (HM052811.1), *Sarocladium* sp. F18.

3.2. Identification of enniatin detoxifying bacterium

3.2.1. Morphological characterization and optimal growing temperature of the bacterium K4

The colonies of bacterium K4 were rough with irregular margin; the overall evaluated characteristics are summarized in the Table 2 below. On nutrient agar, the colonies of bacterium K4 had a cream color (lighter as on FM agar). At the same temperature, growth of the studied bacterium was slower on nutrient agar than on FM agar. Figure 9 shows the bacterium K4 grown on nutrient agar and on FM agar.

Table 2: Phenotypic characteristics of the bacterium K4 observed on FM agar after 24 hours at 28°C.

Colony characteristics	Bacterium K4
Size	Medium (variable)
Form	Irregular
Elevation	Flat
Margin/edge	Undulate
Color	Cream
Pigment production	No
Opacity	Opaque
Surface	Rough/ dull
Consistency	Buttery
Odor	Putrid

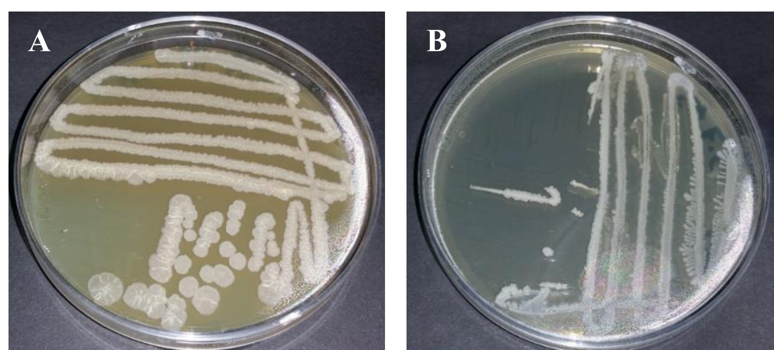

Figure 9: Bacterium K4 grown on FM agar pH 7.2 (A) and on nutrient agar (B) both for 24 hours at 28°C.

The optimal growth temperature of bacterium K4 in tryptic soy broth was between 28°C and 30°C (Figure 10). At those temperatures, the values of the OD600 were significantly different ($P<0.05$) from values obtained at 25, 35 and 37°C. The growth was expressed by the values of OD600 of ½ diluted cultures after 24 hours culture. Bacterium K4 showed lowest growth at 25°C, while its growth was moderate at 35°C in tryptic soy broth. In contrary to cultures at optimal growth temperatures, the cultures at 25°C or 37°C were clearer and thinner showing a low cell number.

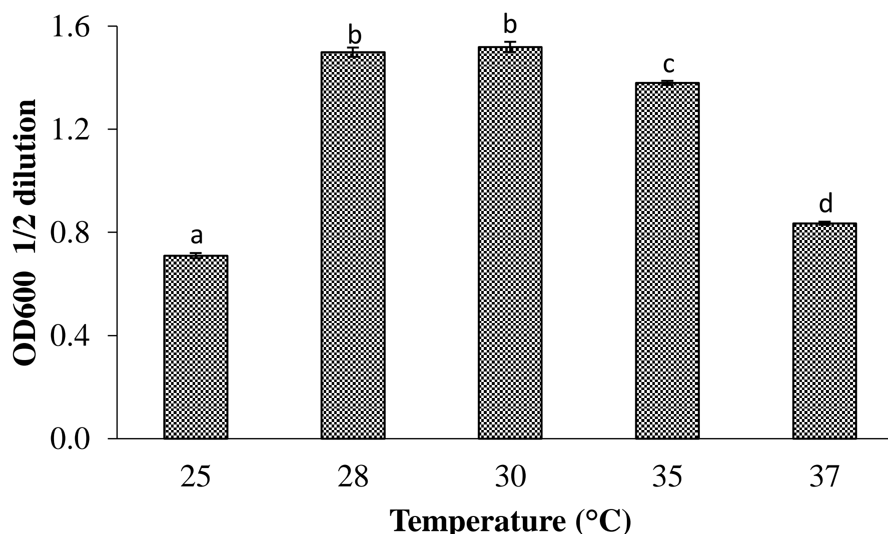

Figure 10: Optical density (OD600) of bacterium K4 grown in tryptic soy broth during 24 hours for the determination of optimal temperature growth of bacterium K4.

a, b, c: Histograms (mean ± Standard deviation) carrying the same letter represent values statistically not significant $P>0.05$, $n=3$.

3.2.2. Biochemical characterization of the bacterium K4

The physiological and biochemical characterization of the bacterium K4 (including Gram staining, starch hydrolysis, Sulfide-Indole-Motility, nutrient gelatin, citrate, urease and catalase tests, growth on different media) are summarized in Table 3. Bacterium K4 is a gram positive bacterium, the cells of bacterium K4 were rod-shaped (Length: 2.85 ± 0.39 µm and Width: 0.58 ± 0.12 µm), arranged singly or in pairs (Figure 11). The features of K4 were consistent with the description of *Bacillus sp.* in Bergey's Manual of Systematic Bacteriology (Claus and Berkeley, 1986).

Table 3: Biochemical characteristics of bacterium K4

Biochemical tests	Bacterium K4
Gram staining	+
Starch hydrolysis	+
Sulfide	-
Indole	-
Motility	+/ -
Nutrient gelatin stab	-
Citrate	+
Urease	-
Catalase 3%	+
Catalase 15%	+
Growth on	
6.5% NaCl	+
Sabouraud dextrose agar	+
Nutrient broth	+
Starch agar	+
Pectine agar	+
Casein agar	+

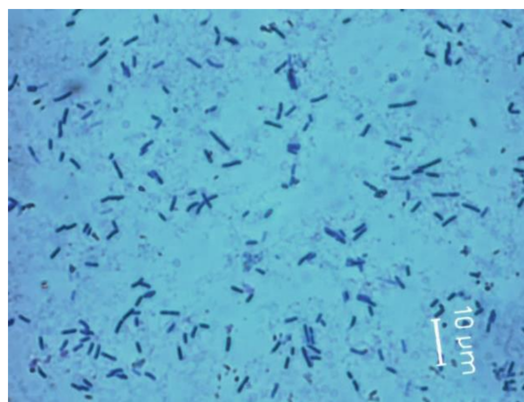

Figure 11: Microscopic examination of bacterium K4, Rod-shaped cells observed after Gram stain under light microscope (100×).

3.2.3. Molecular identification of bacterial isolate

Gel electrophoresis of extracted DNA and PCR product

Figure 12-A shows the agarose gel electrophoresis of DNA extracted of bacterium K4. With comparison of the sample to the lambda DNA, a successful DNA extraction was achieved. The PCR amplification of the partial sequence of 16S rRNA gene resulted in a product of about 1000 bp as expected (Figure 12-B).

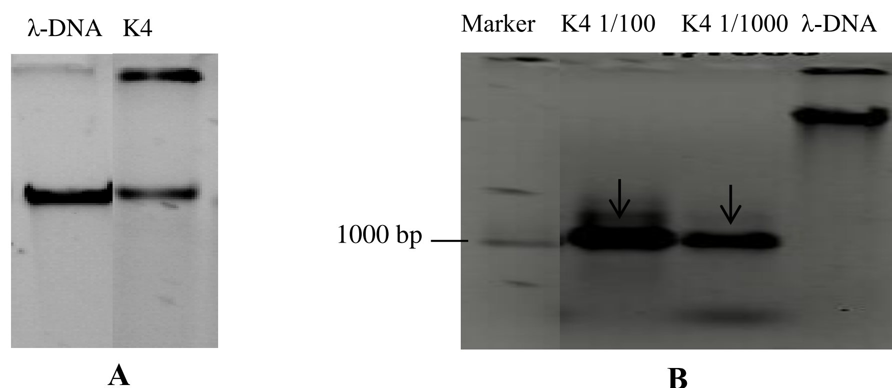

Figure 12: A: Gel electrophoresis of DNA extraction (from left to right: Lambda DNA, extracted DNA of bacterium K4); B: PCR products of the amplification of the 16S rRNA gene of bacterium K4 (from left to right: 1000 bp marker, PCR product of the partial sequence of 16S rRNA gene of bacterium K4 dilution 1/100; PCR product of the partial sequence of 16S rRNA gene of bacterium K4 dilution 1/1000; Lambda DNA).

Phylogenetic analysis of bacterium K4

The phylogenetic tree of the bacterium K4 and related bacillus species is shown in Figure 13, the bacterial strain K4 revealed 100% identity with *B. licheniformis* strain CY-012, and very high similarity with *B. licheniformis* RPk and *B. licheniformis* SB 3131.

According to the results of phenotypic and microscopic observations, physiological and biochemical tests, and the phylogenetic analysis based on the partial sequence of the 16S rRNA gene, the strain K4 was identified as *B. licheniformis*.

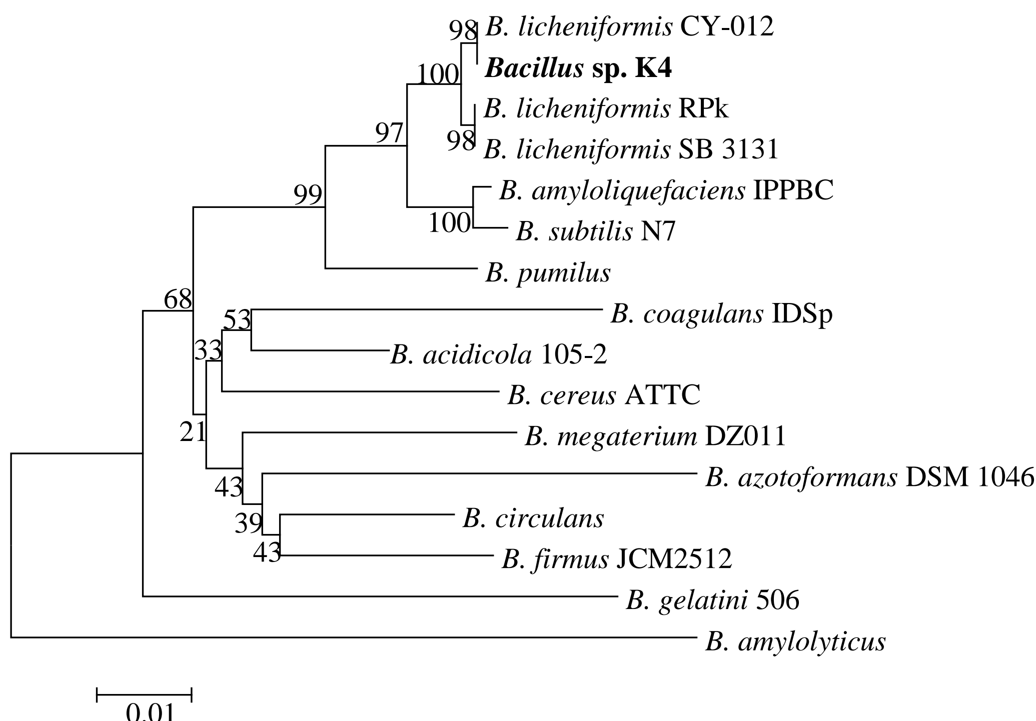

Figure 13: Neighbor-joining phylogenic tree based on the partial sequence of the 16S rRNA gene of bacterium K4 and related bacteria. At major nodes, bootstrap percentages for 1000 resamplings are shown. The scale bar represents 0.01 nucleotide substitutions per nucleotide position.

B. licheniformis CY-012 (GenBank accession number: KR349358.1), *B. licheniformis* RPk (EU445292.1) , *B. licheniformis* SB 3131 (GU191917.1), *B. amyloliquefaciens* IPPBC 10A (HQ2843041), *B. subtilis* N7 (JQ317780.1), *B. pumilus* ST277 (EU350371.1), *B. cereus* ATTC 14579 (NR_074540.1), *B. coagulans* IDSp (AF466695.1), *B. acidicola* 105-2 (NR_041942.1), *B. megaterium* DZ011 (QD408589.1), *B. azotoformans* DSM 1046 (NR 115576.1), *B. circulans* WZ12 (EF100968), *B. firmus* JCM2512 (NR_115581.1), *B. gelatini* 506 (DQ350818.1), *B. amylolyticus* (X60606.1).

4. Discussion

This work was performed with the aim of identifying three microorganisms (one bacterium and two fungi) isolated as described in the previous chapter due to their property to degrade enniatins. The identification was made with the aid of morphological and molecular parameters of the microorganisms. Based on the results obtained during this study, the microorganisms capable of degrading enniatins previously labelled as K4, F20 and F18, it was revealed that K4, F20 and F18 were *Bacillus licheniformis*, *Clonostachys rosea* and *Acremonium strictum* respectively. The analysis of the translation elongation factor 1-alpha (TEF-1α) gene for the identification of both fungi did not give any conclusive results and therefore was not appropriate in this case. Since ribosomal genes are highly conserved, sequence variation between strains belonging to different species from distinct genera is less evident with rDNA gene sequence (Wayne *et al*., 1987).

4.1. Bacterium K4: *Bacillus licheniformis*

The 16S rDNA gene sequencing has allowed the identification of the bacterium K4 species. This technique is a great tool and by far the single most common molecular technique currently used for the purpose of bacterial species identification (Wilson, 1995). The technique of 16S rDNA gene sequencing for bacteria identification has been successfully used in several studies (Yi *et al*., 2011; Ikunaga *et al*., 2011; Tan *et al*., 2014). This technique relies on the sequence of the DNA that encodes for the 16S rRNA subunit and examines only a small portion of the microbial genome.

In the previous chapter, the ability of *B. licheniformis* to degrade enniatin A, A1 and B1 was demonstrated. This result joined an already published report on the effect of *B. licheniformis* in the reduction of the mycotoxin zearalenone (Yi *et al*., 2011). Although Yi *et al*. (2011) did not investigate the presence of degradation products, the adsorption of zearalenone by the bacterium cell wall was excluded due the absence of zearalenone in the *B. licheniformis* cell wall extract. Moreover, in a study carried out by Petchkongkaew *et al*. (2008), *B. licheniformis* was able to decrease the amount of aflatoxin B1 and ochratoxin A at a rate of 74% to 92.5% in liquid and solid media respectively.

Regarding the use of microorganisms in food and feed, the character of not to produce harmful toxins is a prerequisite in order to avoid damaging health effects. In recent studies, the capacity of *B. licheniformis* strains CK1 and ATCC to produce enterotoxins was tested (Yi *et al*., 2011; Liu *et al*., 2013). It was found that both strains CK1 and ATCC are non-enterotoxin producing *B. licheniformis*. In addition, Liu *et al*. (2013) also showed that *B. licheniformis* CK1 is non-hemolytic and displayed high levels of extracellular xylanase, cellulase and proteinase activity.

Among the beneficial effects of *B. licheniformis* is the ability to produce proticin, a phosphorus-containing triene which has a strong activity against *proteus* bacteria (Präve *et al*., 1972, Katz and Demain, 1977), as well as against *Mycobacterium tuberculosis* (Callow *et al*., 1947).

According to the evidence of bio-transformation of enniatins A, A1 and B1 demonstrated in the previous chapter of this thesis, its potential use as a probiotic demonstrated previously and after further investigations concerning enterotoxins nonproduction by *Bacillus licheniformis* (in studies carried out by other authors), the possibility of applying bacterium K4 as a food and feed supplement for bio-detoxification of enniatins should be considered.

4.2. Fungus F20: *Clonostachys rosea*

Based on morphological features with comparison of those reported by Schroers (2001) and Toledo *et al*. (2006), the fungus F20 could belong to the genus *Clonostachys.* According to the results of phenotypic and microscopic observations and the phylogenetic analysis based on the DNA sequence using the ITS1 and ITS4 primers, the fungal strains F20 was identified as *Clonostachys rosea*.

Clonostachys rosea is a common soil fungus and mycoparasite (Schroers *et al*., 1999) from temperate and tropical regions (Schroers, 2001). The mycoparasitic effects of *C. rosea* have been demonstrated against several fungal plant pathogens such as *Botrytis cinerea* in strawberry (Cota *et al*., 2009), *S. sclerotorium* (Rodríguez *et al*., 2011) and *F. graminearum* (Kosawang *et al*., 2014). *C. rosea* has also been reported to be an entomopathogenic fungus of two leafhoppers pest namely *Oncometopia tucumana* and *Sonesimia grossa* (Toledo *et al*., 2006).

The microbial activity of *C. rosea* on zearalenone (ZEN) was demonstrated several decades ago. El-Sharkawy and Abul-Hajj (1988) demonstrated an *in vitro* reduction of ZEN in 80-90% yields by *G. roseum* (early name of *C. rosea*). The enzyme zearalenone lactonohydrolase catalyzing the reaction has been purified and cloned (Takahashi *et al*., 2002). Other authors demonstrated a ZEN dose-dependent induction of the zearalenone lactonohydrolase and suggested a specificity of the enzyme towards ZEN due to the absence of the induction enzyme expression by the known inducers of the Polyketide Synthase pathway (Kosawang *et al*., 2014). The degradation of ZEN by *C. rosea* as reported by Kakeya *et al.* (2002) involved the hydrolysis of the ester bond followed by a decarboxylation to produce 1-(3,5 dihydroxyphenyl)-10'-hydroxy-1'E-undecene-6'-one. The same authors demonstrated that the estrogenic activity of the degradation product did not show a potent estrogenic activity like that of ZEN and 17 b-estradiol in the human breast cancer MCF-7 cell proliferation assay. Although *C. rosea* does not degrade fumonisins, it was shown that it suppresses the synthesis of fumonisnins by *F. verticillioides* in co cultures (Chatterjee *et al*., 2016).

The ability of *C. rosea* to degrade enniatins as described in our study highlights the importance of *C. rosea* for mitigation of toxins in foodstuffs.

4.3. Fungus F18: *Acremonium strictum*

Based on morphological features with comparison of those reported by Domsch *et al*., 2007, the fungus F18 could be classified as a member of the genus *Acremonium*. Further analysis of DNA sequences of 5.8S rRNA subunit, ITS 1 and ITS 2 regions followed by phylogenetic analysis helped to identify the fungal strain F18 as *Acremonium strictum*.

Acremonium strictum and other *Acremonium* species are not known to produce mycotoxins in food (Gams, 1971). *A. strictum* is a saprobe generally found in soil, plant debris, and rotting mushrooms (Guarro *et al.,* 1997). Choi *et al*., (2008) reported the mycoparasitism of *A. strictum* against several fungi including A*lternaria alternata, Bipolaris maydis, Magnaporthe grisea, Botrytis cinerea, Botrytis allii, Colletotrichum orbiculare, Fusarium oxysporum, Phytophthora capsici* and *Pythium ultimum.* A previous study by Kim *et al*., (2002) attributed the mycoparasitic effect of *A. strictum* to verlamelin, an antifungal compound. This highlighted the possible use of *A. strictum* as a biological control agent for plant diseases caused by *Botrytis cineria*.

The production of cellulosic enzymes by *Acremonium strictum* was investigated by Goldbeck *et al*., (2013). CMCase, FPase, cellobiase and β-glucosidase activity were determined on

several substrates including microcrystalline cellulose, carboxymethylcellulose and sugarcane bagasse. The results of the study suggested the use of *A. strictum* as a potential fungus for the production of new enzymes with potential biotechnological applications.
Acremostrictin has been isolated from liquid culture of *A. strictum* collected from choristida sponge off the coast of Korea. Acremostrictin exhibits a weak antibacterial activity against *Micrococcus luteus, Salmonella typhimurium and Proteus vulgaris,* as well as a moderate antioxidant activity leading to protection against oxidative stress induced cell death (Julianti *et al*., 2011).

In the scientific literature, few references are available regarding enniatin degrading bacteria. Nine bacteria of the gastrointestinal tract: *Bb. longum, Bb. bifidum, Bb breve, Bb adolescents, Lb. rhamnosus, Lb. casei-casei, S. termofilus, Lb. ruminis, Lb. casei* and 22 strains of *S. cerevisiae* were reported to significantly reduce enniatins in an *in vitro* fermentation system and three degradation products deriving from enniatin A, B and B1 were identified (Roig *et al*., 2013). The degradation of enniatins by six *Bacillus subtilis* strains (CECT 35, CECT 39, CECT 371, CECT 497, CECT 498, CECT 4522) was studied by Meca *et al*., (2014). All the tested strains were able to degrade enniatin A, A1, B and B1 in TSB. The mean reductions were 88.1%; 83.1%; 84.5% and 92.2% for enniatin A, A1, B and B1 respectively.

To the best of our knowledge and the thorough coverage of the available literature about the biological detoxification of enniatins, this is the first report of enniatin degradation by the microorganisms *Bacillus licheniformis, Clonostachys rosea* and *Acremonium strictum*.

5. Conclusion

In conclusion:

- One enniatin-degrading bacterium (K4) and two enniatin-degrading fungi (F18 and F20) were phenotypically and genotypically characterized.
- Visual and microscopic observations enabled the genus identification of fungus F18 and fungus F20 as *Acremonium* and *Clonostachys* respectively.
- The amplification of the translation elongation factor 1-alpha (TEF-1α) gene sequence were not appropriate for the molecular identification of the two fungal species examined in this study.
- The sequence of 5.8S ribosomal subunit and the ITS1 and IT2 regions of the DNA enabled the identification of the fungi F18 and F20.
- Fungi F18 and F20 isolated respectively from wheat field soil (Göttingen) and hazel nut (Trader Joe's Haselnusskerne ganz, Märsch Importhandels-GmbH, Ulm) samples were identified as *Acremonium strictum* and *Clonostachys rosea* respectively.
- An initial characterization of the bacterium K4 using visual and microscopic observations, followed by some biochemical tests led to the classification of the bacterium K4 within the *Bacillus* genus.
- The analysis of the 16S rRNA gene sequence was required for the characterization of bacterium K4 species.
- The bacterium K4 isolated from a wheat field soil (Göttingen) was identified as *Bacillus licheniformis*.

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Chapter 5

General discussion

This study was conducted with the major objective of degrading enniatins (A, A1, B1 and B) into less toxic compounds by means of microorganisms (bacteria, yeast or fungi). The specific objectives consisted of purifying enniatins from fungal extract, then finding some microorganisms with the ability of breaking down enniatins into less toxic compounds and finally, identifying these active microorganisms. To achieve the first specific objective, we examined two *Fusarium tricinctum* strains for enniatins production on solid white bean medium, and then the best enniatins producing strain was used for the large scale production of enniatins on solid white bean medium using flash chromatography and recrystallization. During our study a short and efficient HPLC-DAD method was developed and validated for the detection of enniatins in the fungal extract. The results are comparable to mainly those of Wang *et al*. (2013) who optimized enniatin production using a *F. tricinctum* strain with the main focus on the type of medium used. According to these authors and the conditions applied during their study, the best harvesting times for enniatin production was between days 14 and day 18 when the production of enniatins A, A1, B and B1 peaked 1365 mg/mL. During our study, the best harvest time for enniatin production was between day 24 and day 30. The enniatin B, B1 and B peaked at day 24 with 1228 mg/L. Moreover, it was demonstrated, that *F. tricinctum* O32 produced about two times more enniatins than the strain DSM 23357. The slight observed differences could be due to the fermentation conditions that were different in both studies, but also to toxin variability and loss of toxin production by a *Fusarium* strain caused by mutations of the strain cultures stored for long period in fungal collections, as these may result in such differences (Moretti *et al*., 2007). After finding the best enniatin producer strain among the two tested ones, our aim was to purify a mixture of enniatins A, A1, B1 and B from the ethyl acetate crude extract using a combination of flash chromatography and recrystallization. Enniatins were confirmed using HPLC-MS and the purity was calculated after comparison with pure standards. The purified enniatin mixture contained about 77% of enniatin B, 17% of enniatin B1, 6% of enniatin A1 and 0.3% of enniatin A. The quantity of enniatins (A, A1, B, B1, B4, J1) purified from a solid corn medium fermented with *F. tricinctum* ITEM 9496 using a double purification with semipreparative LPLC and LC column process, produced quantities ranging from 30 to 300

mg (Cuomo *et al*., 2013), whereas we obtained 3 g of enniatin mixture containing enniatin B, B1, A1 and A from 1 kg of white bean medium following flash chromatography and recrystallization. The combination of flash chromatography and recrystallization resulted in a higher yield of a mixture of enniatins A, A1, B1 and A compared to the data available in the literature using different fungal strains, extraction procedures, and purification techniques. No attempt was made to separate enniatin variants from each other, because during search for detoxifying microorganisms we wanted to have a scenario close to the natural conditions.

During last decades, much research has been conducted with the goal of decontaminating mycotoxin-contaminated food commodities and so as to reduce the health risk associated to high consumption of mycotoxins. Biodegradation is one of the techniques used for mycotoxin decontamination of feed or food; mycotoxins are removed under mild conditions, without harmful chemicals and significant losses in nutritive value and palatability of the decontaminated food and feed (Bata and Lásztity, 1999). Some examples of biological detoxification are reported in the literature. Enzymes isolated from the bacterium *Sphingopyxis* sp. MTA144 were demonstrated to detoxify fumonisin B1 (Heinl *et al*., 2010). The degradation of aflatoxin B1 by *F. aurantiacum* was studied by Line and Brackett (1995), it was found that aflatoxin degradation was independent of nutrients in the culture medium, suggesting a possibility of using this bacterium for aflatoxin detoxification in fermentation processes. Young *et al*., (2007) investigated the degradation of 12 trichothecenes by microorganisms isolated from chicken gut. Acethylation and deepoxydation were the two principal degradation pathways that were evidenced. The percentage of reduction detected ranged from 40-95%.

The second part of this study involved the search of microorganisms able to degrade enniatins into less toxic compounds, we first isolated 114 bacteria and 34 fungi that were able to grow on media containing enniatins as the only carbon source. Then these strains were screened for enniatin degradation using HPLC. The results showed that two classes of microorganisms were isolated, one category which probably adsorb the mycotoxins and another category able to degrade the mycotoxin leading to the formation of new compounds. Microorganisms able to grow on medium containing the mycotoxin DON were proven to adsorb the mycotoxin instead of degrading the mycotoxin DON (Völkl *et al*., 2004). 1285 microbial cultures were tested and a mixed culture D107 was the only culture able to transform DON into new products mainly 3-keto-4-deoxynivalenol. No pure culture of the mixed culture D107 was

able to consistently transform DON. In the present study, three pure cultures of two fungi and one bacterium were active regarding enniatin degradation. The culture supernatant of those microorganisms was analyzed using HPLC-MS. During our study, four enniatin degradation products were identified. Enniatin degradation products had protonated ions with m/z 658, 672, 686 and 700 for enniatin B, B1, A1 and A respectively, we assumed that these degradation products resulted from the hydrolysis of an ester (more likely) or an amide bond in the cyclic ring of enniatins. The degradation products obtained during the present study are different from those reported by Meca *et al*., (2014) who described two enniatins degradation products after the action of some *bacillus* strains on enniatins with loss of a hydroxyisovaleric acid residue in the structures of enniatin B and B1. Since the toxicity of enniatins is assumed to be derived from their ionophoric properties and from their action as uncouplers of oxidative phosphorylation (Shemyakin *et al.*, 1969), enniatin degradation products demonstrated during the present study may be less toxic. Nevertheless, the degradation products must be purified and more studies must be made to test the toxicity of these compounds.

The taxonomy of the enniatins degrading microorganisms was performed and revealed their classification as *Bacillus licheniformis*, *Clonostachys rosea* and *Acremonium strictum*. This is the first report concerning the degradation of enniatins by the microbial cultures of *Bacillus licheniformis*, *Clonostachys rosea* and *Acremonium strictum*. Moreover this is the first report about enniatin degradation products generated from the hydrolysis of an ester bond (this is more likely because the hydrolysis of an ester bond requires less energy than that of an amide bond) as reported in the case of zearalenone. The enzyme zearalenone lactonohydrolase present in *C. rosea* cleaves the lactone rings from the backbone and transforms zearalenone to a far less potent compound (Kakeya *et al*., 2002). Although *C. rosea* does not degrade fumonisins, it was shown that it suppresses the synthesis of fumonisnins by *F. verticillioides* in co-cultures (Chatterjee *et al*., 2016). The ability of *C. rosea* to degrade enniatins as described in our study highlights the importance of *C. rosea* for mitigation of toxins in foodstuffs. We assumed that there is a hydrolase in the detoxifying microorganisms found in the present study capable of targeting the ester bond in the structure of enniatins.

B. licheniformis has also been demonstrated to degrade zearalenone *in vitro*, the idea of adsorption of the mycotoxin to the cell wall was excluded since an analysis of the bacterium cell wall was made after lysis (Yi *et al*., 2011). High capacity of secretion of the alkaline serine protease has made *B. licheniformis* one of the most important bacteria in industrial

enzyme production (Schallmey *et al*., 2004). According to the evidence of bio-transformation of enniatins A, A1 and B1 demonstrated in this study, its potential use as a probiotic demonstrated previously and after further investigations concerning enterotoxins nonproduction by *Bacillus licheniformis* (Yi *et al*., 2011), the possibility of applying bacterium K4 as a food and feed supplement for bio-detoxification of enniatins should be considered.

Acremonium strictum which is not known to produce mycotoxins in food (Gams, 1971) is recognized for the production of compounds with biological activities such as cellulosic enzymes (Goldbeckt *et al*., 2013), acremostatin which exhibits a weak antibacterial activity against *Micrococcus luteus, Salmonella typhimurium and Proteus vulgaris,* as well as a moderate antioxidant activity leading to protection against oxidative stress induced cell death (Julianti *et al*., 2011). These findings suggest the use of *A. strictum* for the production of new enzymes with potential biotechnological applications.

Beauvericin, zearalenone and fumonisin detoxifying microorganisms *Nocardia globulera* (ATCC 55847) (Duvick and Rood, 2000), *Rhodococcus erythropoli* (ATCC 55851) (Duvick and Rood, 1998) and *Exophiala spinifera* (ATCC 74269) (Duvick* and Rood, 1998) originated from mouldy wheat, mouldy corn and field-grown kernels respectively. *B. licheniformis* and *A. strictum* were isolated from a soil sample originating from a wheat field, while *C. rosea* was isolated from Hazelnut sample. Since high enniatins concentration have been associated with cereal products such as wheat and with some nuts also, the hypothesis according which microorganisms present in the same habitat with some mycotoxins may possess or acquire through mutations the genes encoding for enzymes responsible for those mycotoxins degradation may be true.

More studies focusing on the purification of degradation compounds, the degradation mechanisms, the gene and enzymes involved in the mechanism must be carried out. Moreover a study of toxicity of the degradation products must be performed.

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Summary

Mycotoxins are secondary metabolites produced by fungi. Enniatins are a group of cyclohexadepsipeptides made of three hydroxyisovaleric acid molecules and alternating amino acids mostly valine, leucine and isoleucine. Enniatins have been associated with mainly cereal and cereal-based products, but also with nuts and some dried fruits. There is no evidence that high concentrations of enniatins in food commodities are not harmful for human and animal health in the long term. Therefore, methods to reduce enniatin content in food by transforming them into less toxic compounds are of crucial importance. Decontamination strategies for mycotoxin contaminated food or feed involve physical and chemical treatments which are to some extent successful but also pose some disadvantages like limited efficacy and possible losses of the sensorial and nutritive value. Therefore, intensive work has been done in the field of biodegradation of mycotoxins in food. Within that framework, the main objective of this study was to find microorganisms (bacteria, yeast or fungi) able to degrade enniatins into less toxic compounds. The strategy used involved a random search of such microorganisms followed by their classification.

Due to the high quantity of mycotoxins that are involved in such studies resulting in great expense, the first objective of this study was to purify enniatins from fungal extract. Therefore two strains of *F. tricinctum* (DSM 23357 and O32) were tested for enniatin production in solid white bean medium. Enniatins were extracted every 3 days. A short method for the detection and quantification of enniatins A, A1, B and B1 in the fungal crude extracts was established and validated using HPLC-DAD. For both studied strains, enniatin concentrations increased until 24 days with 614 g/kg and 479g/kg for *F. tricinctum* O32 and DSM 23357 respectively. Throughout the 30 cultures days *F. tricinctum* O32 produced about two times more enniatins than the strain DSM 23357. As a result *F. tricinctum* O32 was used for the large scale production of enniatins over 30 days on solid white bean medium. Enniatin in the fungal crude extract were separated using flash chromatography and recrystallization. 3.18 g enniatins mixture/kg of white bean medium with a purity of 96.25% and containing about 77% enniatin B, 17% enniatin B1, 6% enniatin A1 and 0.3% enniatin A was obtained.

The second part of this study involved the search for microorganisms having the ability to degrade enniatins. For this purpose, microorganisms were first isolated from several sources (grains, nuts, water, soil, cereal based-products, juice) based on their ability to grow on a minimal medium (MM) containing enniatin as the unique carbon source. Wash fluid from several materials were mixed with MM containing enniatins (final concentration 1 mg/ml) and incubated for 7 days at 20°C. The cultures were diluted 25-times in MM with enniatins (final concentration 1 mg/ml) and incubated for further 21 days at 20°C. In total 114 bacteria or yeast-like microorganisms and 34 fungi were isolated. The major sources of bacteria were water, grains and nuts from which 38, 35, 20 and 7 bacteria were isolated respectively. Whereas the majority of fungi originated from nuts (16), grains (10) and soil (3) samples. 20 bacteria and 10 fungi were not able to be cultivated and after a second selection using single culture in MM containing (final concentration 1 mg/ml), 6 bacteria and 1 fungus did not grow anymore. Then 88 bacteria (and yeast-like microorganisms) and 23 fungi were screened for their property to break down enniatins into new compounds using HPLC-DAD (as described previously) in MM and in a minimal medium containing a limited amount of glucose and tryptone (MMGP). The results showed that enniatin concentrations were reduced in all the tested samples (enniatin A was mostly reduced than enniatin B). The HPLC-UV chromatograms of a bacterium (K4) and two fungi (F18 and F20) produced new signals compared to those of the controls. K4 and F18 originated from wheat field soil sample, whereas F20 was isolated form hazelnuts sample. These samples were further analyzed using LC-MS, differential metabolic profiling (Noise reduction, chromatogram alignment, normalization) and the mass of enniatins products were deduced and confirmed using extracted ions chromatograms and the corresponding mass spectra. Four enniatin products were identified using this technique; they were probably the products of hydrolysis of either an ester or an amide bond in the ring structure of enniatins, leading to the opening of the ring and therefore probably producing less toxic compounds. The molecular masses of the protonated ions of enniatins degradation products were 658, 672, 686 and 700 for enniatin B, B1, A1 and A respectively.

Finally the identification of the pure cultures responsible for enniatin degradation was carried out using phenotypic (visual and microscopic), biochemical (for the bacterium only, gram test, catalase, sulfide-indole-motility, starch hydrolysis, citrate, urease tests…) and molecular characteristics of the microorganisms. The bacterium was preliminary identified as *Bacillus* sp. and the sequence of the 16S rRNA gene analysis enabled the species identification of the

bacterium K4 as *Bacillus licheniformis*. Based on morphological characteristics (visual and microscopic observations, spore size, conidia, conidiophores, presence or absence of septae…), fungi F18 and F20 were identified as *Acremonium* sp. and *Clonostachys* sp. respectively. The sequence of the ITS1, 5.8S RNA subunit and ITS2 of the DNA revealed that F18 and F20 were *Acremonium strictum* and *Clonostachys rosea*.

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Publications

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Declarations

1. I, hereby, declare that this Ph.D. dissertation has not been presented to any other examining body either in its present or a similar form.
Furthermore, I also affirm that I have not applied for a Ph.D. at any other higher school of education.

Gottingen,
....

2. I, hereby, solemnly declare that this dissertation was undertaken independently and without any unauthorized aid.

Gottingen,
....