

Optimization of Enniatin Production by Solid-Phase Fermentation of *Fusarium tricinctum*

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Enniatins are cyclic depsipeptides produced by fungi of the genus *Fusarium* that are of interest due to their pronounced biological activities; especially enniatins A, A₁, B, and B₁ possess anticarcinogenic and anti-HIV properties. In the present study, *F. tricinctum* was grown on seven solid media and in one liquid growth medium with or without addition of peptone or of amino acid precursors in order to identify favourable media with simple cultivation conditions for maximum enniatin production. Additionally, the optimal duration of growth was investigated for the highest yields of enniatins. From the different media analysed, white beans (*Phaseolus vulgaris*, solid medium) induced the highest accumulation of enniatins A, A₁, B, and B₁, that reached a maximum of 1,365 mg total enniatins in 1 L growth medium after 18 days of fermentation. Fermentation of *F. tricinctum* on white beans gave the highest yield of enniatins compared to all other media analysed in this study.

Key words: Enniatin, *Fusarium tricinctum*, Production Optimization

Introduction

Enniatins are cyclic hexadepsipeptides and consist of three D-2-hydroxyisovaleric acid (D-Hiv) residues linked alternatively to *N*-methyl-L-amino acid residues (*N*-methyl-L-valine, *N*-methyl-L-isoleucine, and *N*-methyl-L-leucine) (Strongman *et al.*, 1988). They are fungal metabolites first isolated by Gäumann *et al.* (1947) from *Fusarium orthoceras* var. *enniatinum* but have since then been reported from other *Fusarium* species such as *F. tricinctum* that was isolated as an endophyte from the host plant *Aristolochia paucinervis* (Debbab, 2007).

Due to ionophoric properties, enniatins have been shown to have insecticidal activity as well as antimycobacterial activity, one of the most potent being enniatin B (Ovchinnikov *et al.*, 1974; Lifson *et al.*, 1984; Visconti *et al.*, 1992; Doebler, 2000; Firáková *et al.*, 2007). They inhibit various enzymes, e.g. acyl-CoA-cholesterol-acyl transferase and cyclic nucleotide phosphodiesterase (Tomoda *et al.*, 1992). Additionally, Kamyar *et al.* (2004) showed that enniatins are easily in-

corporated into cellular membranes where they may form cation-selective pores. Furthermore, enniatins, especially enniatins A, A₁, B, and B₁, possess anticarcinogenic properties by induction of apoptosis and disruption of ERK signaling (Dornetshuber *et al.*, 2007; Lee *et al.*, 2008; Hyun *et al.*, 2009; Wätjen *et al.*, 2009). Interestingly, it has been shown that enniatins possess anti-HIV activity, whereby a mixture of enniatins B, B₁, and A exhibit the highest activity compared to all other enniatins isolated so far (Firáková *et al.*, 2007; Shin *et al.*, 2009).

Hence, enniatins are of considerable interest both as possible lead structures for the development of new anticancer or anti-HIV drugs and as molecular probes for the investigation of intracellular signal transduction pathways. An improved production of enniatins through a simple fermentation method of *Fusarium* sp. is therefore desirable. In this study, we investigated seven solid media and one liquid medium with or without the addition of peptone or of amino acid precursors for the optimization of the production of enniatins by *F. tricinctum*.

Results and Discussion

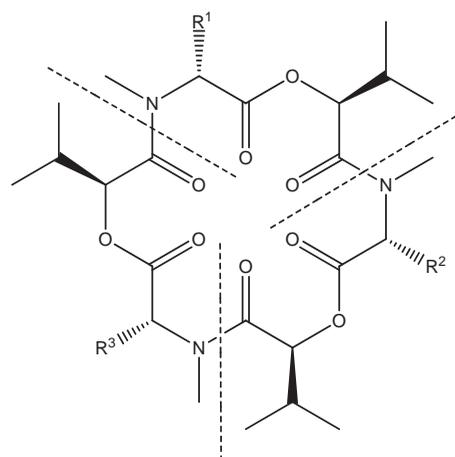
Effects of different media on enniatin production by *F. tricinctum*

F. tricinctum was cultivated on seven solid media and in a liquid modified Wickerham medium (static vs. shaking cultures). Cultures on solid media were harvested and extracted after 10 days, when the fungus had completely overgrown the various media. Cultures in liquid media were harvested after 24 days (static culture), due to slow growth, or after 7 days when cultures were shaken. Total enniatin concentrations (expressed in mg/L medium) and profiles of individual compounds (Fig. 1) were assessed by high-performance liquid chromatography (HPLC). Total enniatin concentrations varied considerably among the different growth media analysed (Fig. 2) and were highest (1,113 mg/L) when the fungus was grown on white beans (*Phaseolus vulgaris*). Soybeans proved to be the second best medium compared to white beans and resulted in a total enniatin concentration of 541 mg/L. All other media (solid or liquid) were clearly inferior and yielded total enniatin concentrations less than 100 mg/L. Shaking of the cultures of *F. tricinctum* in liquid modified Wickerham growth medium vs. static cultures

reduced the total enniatin concentrations from 63 to 9.1 mg/L (Fig. 2).

As a result of this comparative investigation, legume crops such as white beans or soybeans that are rich in proteins give rise to the highest enniatin concentrations and are hence preferable as growth media compared to other media rich in carbohydrates such as grains or potatoes. Liquid Wickerham medium (static or shaking cultures) gave likewise low yields of enniatins compared to white beans or soybeans and, therefore, offers no feasible alternative for production of the investigated compounds.

Enniatin patterns on the other hand proved to be remarkably homogenous between the various treatments analysed (data not shown). These findings are in accordance with the study of Pieper *et al.* (1992) who isolated enniatin synthetases from several *Fusarium* species and found that each synthetase preferably accepts certain nutrients resulting in a distinct production pattern of enniatins. In our study, enniatin B was consistently found to be the major constituent for all growth media analysed followed by enniatins B₁, A₁, and A. For cultures growing on white beans the average percentages of compounds were 43% for enniatin B, 38% for enniatin B₁, 12% for enniatin A₁, and 7% for enniatin A (Fig. 3).



| | R ¹ | R ² | R ³ |
|----------------|--|--|--|
| A | -CH(CH ₃)CH ₂ CH ₃ | -CH(CH ₃)CH ₂ CH ₃ | -CH(CH ₃)CH ₂ CH ₃ |
| A ₁ | -CH(CH ₃)CH ₂ CH ₃ | -CH(CH ₃)CH ₂ CH ₃ | -CH(CH ₃) ₂ |
| B | -CH(CH ₃) ₂ | -CH(CH ₃) ₂ | -CH(CH ₃) ₂ |
| B ₁ | -CH(CH ₃) ₂ | -CH(CH ₃) ₂ | -CH(CH ₃)CH ₂ CH ₃ |

Fig. 1. Chemical structures of enniatins A, A₁, B, and B₁.

Influence of peptone and of amino acid precursors on enniatin production on solid and in liquid media

Peptone and selected amino acid precursors (L-valine, L-leucine, and L-isoleucine) that constitute biogenetic building blocks of the investigated enniatins were studied for their effects on enniatin production by *F. tricinctum* on solid (white beans) as well as in liquid media (Fig. 4). Neither addition of peptone nor of amino acids had a significant effect on enniatin production on white beans when compared to controls. There was a slight increase of the yields of total enniatins in the presence of 2.5 g peptone/L and of 0.5 g of each of the three amino acids analysed/L. This was, however, not statistically significant (Fig. 4A). Apparently, white beans supply all necessary nutrients for the growth of *F. tricinctum* and precursors for the production of enniatins in sufficient quantities. Hence, supplementation by addition of peptone or by selected precursors does not provide a further stimulus for the production of these compounds.

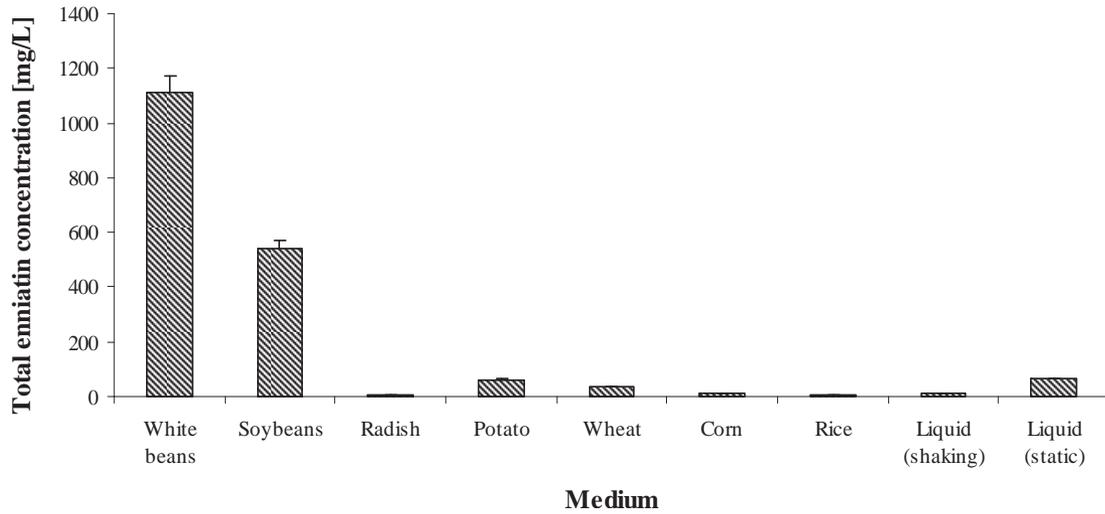


Fig. 2. Comparison of the enniatin production in the various tested media. Cultures were harvested after 10 days (solid media), 24 days (shaking liquid medium), and 7 days (static liquid medium).

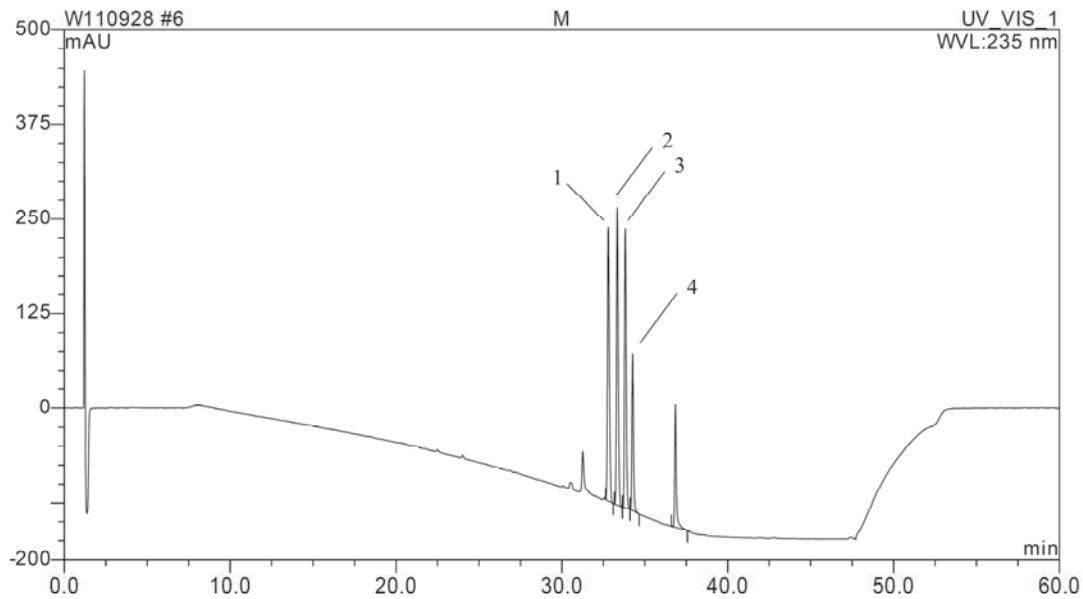


Fig. 3. HPLC chromatogram showing the composition of enniatins B with 43% (1), B₁ with 38% (2), A₁ with 12% (3), and A with 7% (4) in cultures of *F. tricin* grown on white beans extracted after 18 days. The retention times were detected at 235 nm: enniatin B with 32.797 min (1), B₁ with 33.337 min (2), A₁ with 33.823 min (3), and A with 34.267 min (4).

On the other hand, peptone and amino acids had a strong influence on enniatin production in cultures growing in liquid modified Wickerham medium. Addition of peptone (5.0 g/L) to stat-

ic cultures of *F. tricin* raised the total enniatin concentrations from 63 mg/L in controls to 510 mg/L (Fig. 4B). Addition of amino acids (0.5 g of each amino acid/L) also caused a strong

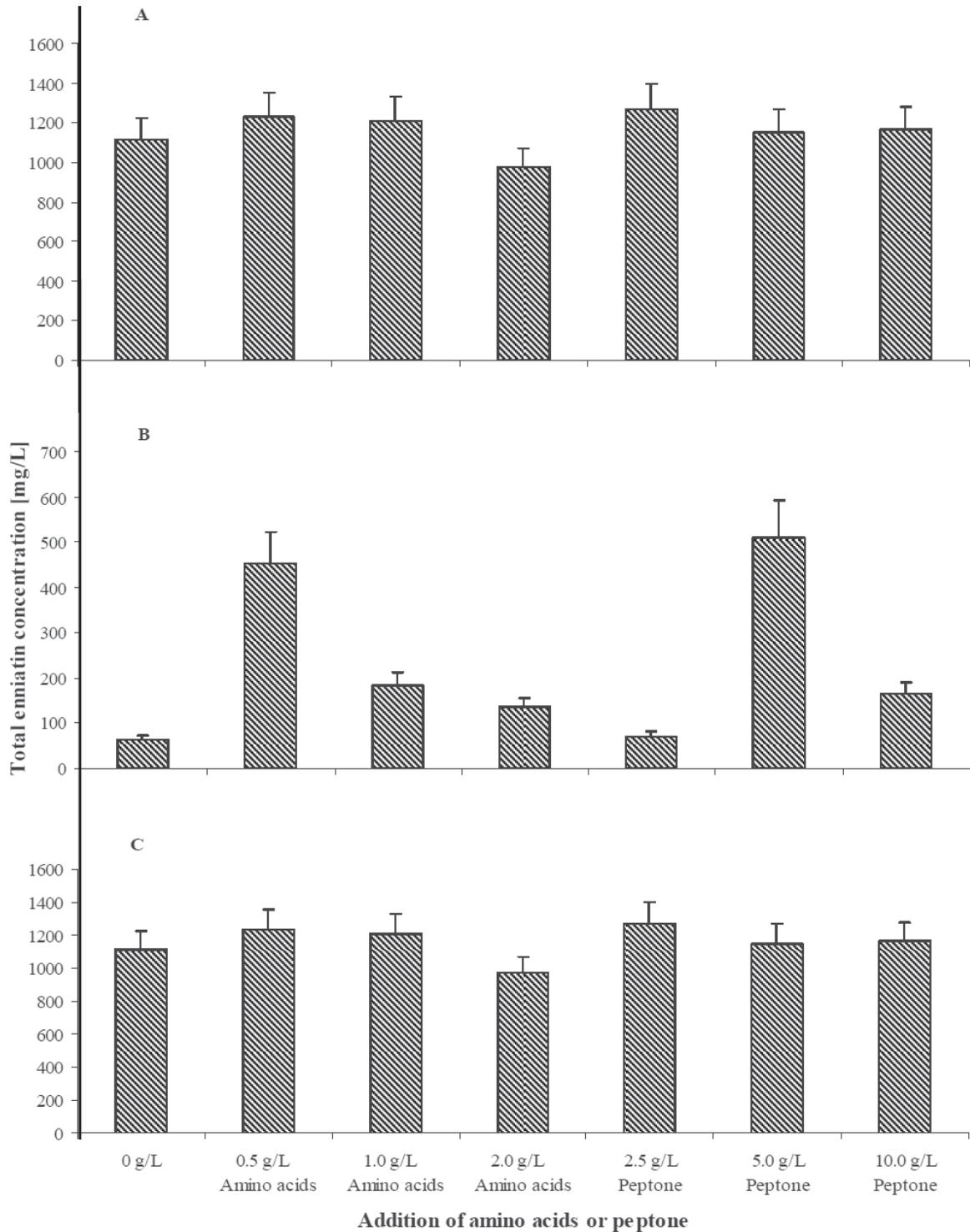


Fig. 4. Influence of peptone and amino acid mix (L-valine, L-leucine, and L-isoleucine) on total enniatin concentration in different media: (A) white beans; (B) liquid Wickerham medium (static culture); (C) liquid Wickerham medium (shaking at 150 rpm).

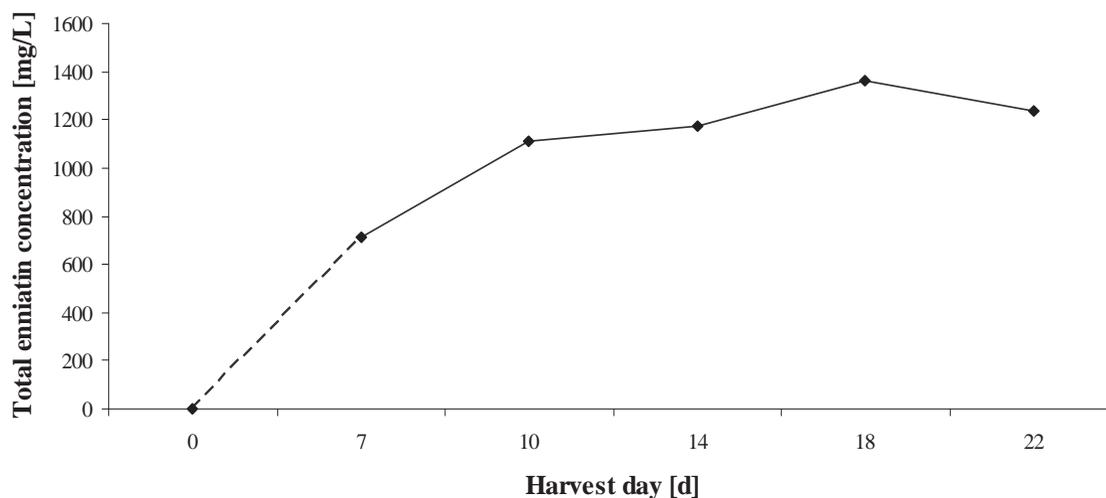


Fig. 5. Time course of enniatin production by *F. tricinctum* on white beans solid medium. The carry over is small and can be neglected. It is therefore shown only as a dashed line from day 0 until day 7.

increase in compound production and raised the total enniatin concentrations to 451 mg/L (Fig. 4B). Higher concentrations of amino acids resulted in a decrease in enniatin production which is probably due to toxicity of the amino acids. When peptone or the mix of amino acids were added to growing cultures of *F. tricinctum* maintained under shaking, an increase in enniatin production was likewise observed (Fig. 4C). Addition of 5.0 g peptone/L raised the total enniatin concentrations from 9 to 102 mg/L, whereas addition of 0.5 g of each of the three amino acids/L increased the total enniatin concentra-

tions to 135 mg/L. Total concentrations of enniatins that resulted from addition of peptone or amino acids to liquid Wickerham medium were, however, less than those observed for static cultures of *F. tricinctum* on white beans or on soybeans.

Time course and peak production of enniatins on white beans solid medium

Production of enniatins by *F. tricinctum* on white beans was monitored over a period of 22 days (Fig. 5). After 10 days of growth (harvest time for the comparative experiments shown in

Table I. Comparison of reported studies with this current study concerning *Fusarium* species, growth medium, supplements, duration of cultivation in days, yield of enniatins A, A₁, B, B₁ (in mg/L), and type of enniatins.

| Parameter | This study | Audhya and Russell (1973) | Audhya and Russell (1974) | Madry <i>et al.</i> (1983) | Visconti <i>et al.</i> (1992) | Meca <i>et al.</i> (2010) |
|-----------------------|---------------------------------------|--|--|---|---|---------------------------------------|
| <i>Fusarium</i> sp. | <i>F. tricinctum</i> | <i>F. sambucinum</i> Fuckel HLX316 | <i>F. sambucinum</i> Fuckel HLX316 | <i>F. oxysporum</i> ETH 1536/9- C9/5/82 | <i>F. acuminatum</i> MRC 3308 | <i>F. tricinctum</i> |
| Growth medium | White beans | Liquid | Liquid | Liquid FDM | Liquid | Corn-derived |
| Supplements | – | Lactose, tryptone | Lactose, tryptone | Amino acids | – | – |
| Days of cultivation | 18 | 28 | 34 | 4 | 7 | 30 |
| Enniatin yield (mg/L) | 1365 | 2000–3000 | 1724 | 5000 | 300 | 1320 |
| Enniatin type | A, A ₁ , B, B ₁ | A, B, C | A, B, C (calculated as A) | A, A ₁ , B, B ₁ (calculated as A) | A, A ₁ , B, B ₁ , B ₂ , B ₃ , B ₄ | A, A ₁ , B, B ₁ |

Fig. 2), total enniatin concentrations amounted to 1,113 mg/L. Production of compounds peaked at day 18 with 1,365 mg/L followed by a gradual decline to 1,236 mg/L on day 22 (Fig. 5). The optimum harvest time for extraction of enniatins on white beans solid medium is thus between days 14 and 18 under the simple conditions chosen for our study. This compares favourably to reported yields of enniatins in other fermentation studies (Table I). For example, Audhya and Russell (1973, 1974) attained yields of 1,724–3,000 mg enniatins/L with liquid surface cultures of *F. sambucinum* Fuckel HLX316 which were supplemented with lactose and tryptone and grown for 28 and 34 days, respectively. Enniatins A, B, and C were determined as enniatin A with a photometric method rather than by HPLC detection of individual metabolites as done in our study. When considering the duration of cultivation (34 vs. 18 days) the yield in our study is superior. Another example is a yield of up to 5,000 mg enniatins/L liquid chemically defined production medium (*Fusarium*-defined medium, FDM) supplemented with amino acids and a submerged cultivation for 4 days of *F. oxysporum* ETH 1536/9-C9/5/82, a variant strain which was obtained by a duplicate treatment with nitrosoguanidine as described by Zocher *et al.* (1978, 1982) (Madry *et al.*, 1983). Whereas the yield of enniatins A, A₁, B, and B₁ reported in the latter study is superior to the yield obtained in our study, the quantification method employed lacks the selectivity of an HPLC-based method as employed in this study. Preparation methods of the media and of the strain are furthermore more complex and more time-consuming compared to our method. In a further study, Visconti *et al.* (1992) described a yield of an enniatin mixture (A, A₁, B, and B₁₋₄) of about 300 mg/L shaking liquid medium after 7 days of cultivation of *F. acuminatum* MRC 3308. Finally, Meca *et al.* (2010) obtained a yield of 1,320 mg enniatins (mixture of A, A₁, B, and B₁)/L corn-derived medium after cultivation of *F. tricinctum* for 30 days. Thus, the yield of enniatins produced on white beans as reported in our study compares well to other studies reported in the literature with regard to the simple cultivation methods and the short cultivation time needed until harvest of the cultures. This opens new opportunities for an easy and efficient production of enniatins A, A₁, B, and B₁. Nevertheless, it should be mentioned

that other enniatins, such as enniatins H, I, and MK1688, attained through submerged culture of *F. oxysporum* KFCC 11363P, gave optimum results employing an FDM (maximum production of total enniatins, 2,399 mg/L after 8 days) (Lee *et al.*, 2011).

Experimental

Microorganism

Fusarium tricinctum (GenBank accession number AB470859.1) was isolated from fresh healthy rhizomes of *Aristolochia paucinervis* Pomel (Aristolochiaceae) and identified as described previously (Debbab, 2007).

Reagents

All chemicals were of analytical grade and were purchased from Sigma (Deisenhofen, Germany). All fungi culture reagents were purchased from PAA (Coelbe, Germany). The reference substances of enniatin A, A₁, B, and B₁ (Fig. 1) were isolated and purified as described previously (Debbab, 2007). All spectroscopic data obtained were in accordance with published reference data (supplementary data containing ¹³C NMR spectra, HPLC chromatograms, and UV spectra can be obtained from the author for correspondence on demand).

Optimization strategies for enniatin production by *F. tricinctum*

Solid media for enniatin production by *F. tricinctum*

Erlenmeyer flasks (1 L each) containing 100 g of dry legumes [small white beans (Mueller's Muehle, Unna, Germany) or soybeans] or grains (wheat, corn or rice) and 100 mL of distilled water were autoclaved. Potatoes and radish were used fresh (140 g fresh weight after peeling) and sliced prior to autoclaving. Pieces of similar size from Petri dishes with the growing fungus were transferred under sterile conditions to the Erlenmeyer flasks containing the autoclaved medium. The fungus was grown on solid media at room temperature (25 °C) for 10 d followed by extraction. Four independent growth experiments were run separately, and all experiments were performed in triplicate.

Liquid medium for enniatin production by *F. tricinctum*

Liquid cultures of the fungus were kept in Erlenmeyer flasks (1 L each) containing 200 mL liquid Wickerham medium (3 g yeast extract, 3 g malt extract, 5 g peptone, 10 g glucose, distilled water added up to 1000 mL, pH adjusted to 7.2–7.4) at room temperature under static conditions for 24 d or under shaking (150 rpm) for 7 d.

Addition of branched-chain amino acids or peptone to solid or liquid media

L-Valine, L-leucine, and L-isoleucine were dissolved in 10 mL sterile water and added to the autoclaved media by sterile filtration (pore size, 0.45 μm) resulting in final concentrations of 0.5, 1.0, 2.0 g of each amino acid/L, respectively. Peptone was dissolved in 10 mL sterile water and added to the autoclaved media by sterile filtration resulting in final concentrations of 2.5, 5.0, 10.0 g/L, respectively. Fungi were allowed to grow for 10 d at room temperature on solid media until extraction. Fungi in liquid modified Wickerham medium (static culture) were kept for 24 d until extraction, whereas fungi kept under shaking (150 rpm) were harvested after 7 d.

Extraction and quantification of enniatins

For cultures on solid media, 250 mL ethyl acetate (EtOAc) were added to the cultures at the time of harvest and left overnight. Culture media containing the mycelia were then cut into pieces inside the flasks to allow complete extraction and were kept on a shaker (150 rpm) for 3 d. After filtration, fresh EtOAc (250 mL) was added to the cultures, and extraction was repeated as described above. The combined EtOAc phases were then washed with distilled water, and the solvents were removed by rotary evaporation. The dry residue obtained from the EtOAc extract was dissolved in 2 mL methanol (MeOH), and 5 μL were injected into an analytical HPLC instrument to determine the content of enniatins.

For liquid cultures, 250 mL of EtOAc were added at the time of harvest, and the content was thoroughly mixed with an Ultraturrax at 4000 rpm for cell destruction for 10 min followed by filtration under vacuum using a Buchner funnel. The liquid phase was transferred to a separation funnel, and EtOAc and H₂O phases were separated after vigorous shaking. Extraction of

the aqueous phase was repeated twice by adding fresh EtOAc. The combined EtOAc phases were reduced to dryness by rotary evaporation, the residue dissolved in 2 mL MeOH, and 5 μL were subjected to HPLC analysis for quantification of the enniatins.

Individual enniatins were identified by HPLC in comparison to previously isolated reference compounds (Debbab, 2007) using a linear gradient starting with MeOH/nanopure H₂O (10:90), adjusted to pH 3.5 with phosphoric acid, and reaching 100% MeOH after 35 min. All peaks were detected by a UV-VIS photodiode array detector. The HPLC instrument consisted of a pump (Dionex P580A LPG; Morgan Hill, CA, USA), a detector (Dionex photodiode array detector UVD 340S), an injector, a separation column, and the reservoir of the mobile phase. The separation column (125 x 2 mm, ID) was pre-filled with Eurospher-100 C18 (5 μm), with an integrated pre-column (Knauer, Berlin, Germany).

The temperature of the column oven was set at 25 °C. The wavelength for detection of the enniatins was set at 235 nm. The flow rate was 1.0 mL/min. Calibration tests with previously isolated reference compounds (Debbab, 2007) were performed, and a suitable calibration curve was established. A correlation coefficient value higher than 0.9997 indicated linearity within the used concentration range.

For total enniatin quantification, clear separation of enniatins A, A₁, B₁, and B (given in the order of increasing retention times) was readily achieved with the procedure described above. Total enniatin contents were calculated based on the calibration curves.

Statistical analysis

Analysis of variance (one-way ANOVA) was performed to test the significance of differences between means obtained in each experiment at the 5% level of significance ($p < 0.05$).

Acknowledgements

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