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Statistical optimization of culture conditions for the production of enniatins H, I, and MK1688 by *Fusarium oxysporum* KFCC 11363P

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The aim of this study was to optimize the culture conditions for the production of biological cyclic hexadepsipeptides (enniatins H, I and MK1688) from *Fusarium oxysporum* KFCC 11363P. Tests of 10 complete or chemically defined liquid culture media revealed that *Fusarium* defined medium was the best for the production of enniatins (produced amounts: enniatin H, 185.4 mg/L; enniatin I, 349.1 mg/L; enniatin MK1688, 541.1 mg/L; and total enniatins, 1075.6 mg/L). On the eighth day after inoculation, the maximal production of enniatins was observed at 25°C in *Fusarium* defined medium. The optimal carbon and nitrogen sources for producing biological cyclic hexadepsipeptides (enniatins H, I, and MK1688) were sucrose and $NaNO_3$, respectively, and their optimal concentrations were determined by the principle of response surface methodology. It was confirmed that using the optimized growth medium compositions increased the amounts of enniatins H, I, and MK1688, and total enniatins produced to 695.2, 882.4, 824.8, and 2398.5 mg/L, respectively. These findings will assist in formulating microbiological media useful for enniatin research.

[**Key words:** Cyclic depsipeptide; Enniatins; Response surface methodology; Culture conditions optimization; *Fusarium oxysporum*]

A recent review of secondary metabolites from fungi has revealed that strains belonging to the common terrestrial genera *Aspergillus* and *Penicillus* produce most of the reported metabolites (1). However, *Fusarium* strains are also capable of producing various secondary metabolites including mycotoxins, such as trichothecenes, fumonisins, fusaric acid, moniliformin, and fusaproliferin (2,3). *Fusarium* strains are also able to produce several biologically active cyclic depsipeptides that are formed in infected plants in the field. These cyclic depsipeptides have numerous pharmaceutical benefits including acting as enzyme inhibitors, antimicrobial agents, immunomodulating substances, and anticancer drugs (3–8). Various *Fusarium* strains have been found to produce enniatin analogues with a cyclic hexadepsipeptide structure. Cyclic hexadepsipeptides consist of alternating residues of D-2-hydroxyisovaleric acid and a branched-chain *N*-methyl-L-amino acid linked by peptide and ester bonds; the ionophoric properties that arise from their molecular structure are related to their biological activities (9). Enniatins act as ionophores by forming dimeric sandwich structures that transport monovalent ions across membranes (particularly mitochondrial membranes) and uncouple oxidative phosphorylation (10). These basic mechanisms underlie diverse biological activities, such as antibiotic effects against several Gram-positive bacteria (11), insecticidal, herbicidal (12) and cholesterol acyltransferase-inhibiting (3) properties. Recently, it was reported that enniatins inhibit one of the major multidrug efflux pumps (Pdr5p in *Saccharomyces cerevisiae* cells) at nontoxic concentrations, via a mechanism that clearly differs from their function as ionophores (13). This property of enniatins may have important clinical applications in combination with chemotherapeutic drugs.

Recently, several *Fusarium* strains producing cytotoxic cyclic hexadepsipeptides were isolated from potato in Korea (6,8,14–16). Some *Fusarium* isolates produced recently defined cyclic hexadepsipeptides, such as enniatins H, I, and MK1688 as well as beauvericin (14) (Fig. 1). Enniatins H, I and MK1688 exhibit antiplasmodial, antimycobacterial (17), cytotoxic activities (6,8) and potent inhibitory activity against human immunodeficiency virus type-1 integrase (18). Due to the various biological activities of these cyclic hexadepsipeptides, the production of beauvericin was optimized in liquid culture media in our previous report (19). In the present study we further investigated the production of enniatins H, I, and MK1688 by response surface methodology (RSM), in which the compositions of culture medium were optimized with respect to the types of carbon and nitrogen sources and their concentrations.

MATERIALS AND METHODS

Chemicals The enniatins H, I and MK1688 produced in the submerged culture of *F. oxysporum* KFCC 11363P were identified and purified in our previous study (16). The purified enniatins were lyophilized and used as standards in the current study. Water, acetonitrile and methanol for high-performance liquid chromatography (HPLC) were obtained from J.T. Baker (Phillipsburg, NJ, USA). All other chemicals were of analytical grades.

Cultivation of *Fusarium* isolate The *Fusarium* strain was isolated from potato cultivated in Korea as we described previously (15), and this strain was deposited in the KCCM (Korean Culture Center of Microorganisms) as number 11363P. *F. oxysporum* KFCC 11363P was cultivated on potato dextrose agar (PDA) (Difco, Detroit, MI, USA), which

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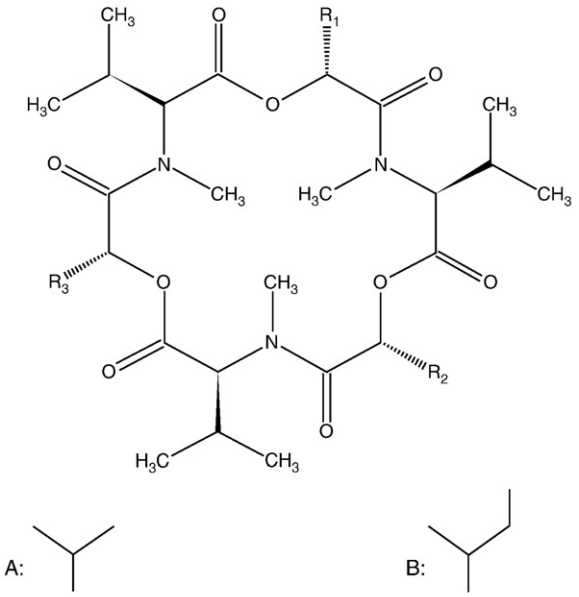

Compound	R_1	R_2	R_3	R_4	R_5	R_6	$[M+H]^+$
Enniatin H	B	A	A	A	A	A	654
Enniatin I	B	A	A	A	B	A	668
Enniatin MK1688	B	A	B	A	B	A	682

FIG. 1. Chemical structure of enniatins H, I, and MK1688.

consists of potato extracts (4.0 g/L), dextrose (20.0 g/L), and agar (15.0 g/L). The spore suspensions were prepared for submerged cultures grown on PDA for 7 days at 25°C and aseptically filtered through sterile filter paper (Whatman International, Maidstone, United Kingdom) to remove mycelial debris. Approximately, 10^5 spores were inoculated in 100 mL of culture medium in a 250 mL Erlenmeyer flask, and incubated at 25°C with shaking at 125 rpm for 7 days.

Analysis of cyclic hexadepsipeptides The cyclic hexadepsipeptides were analyzed using the high-performance liquid chromatography (HPLC) method described by Song et al. (14). The liquid culture of *F. oxysporum* KFCC 11363P including mycelia was extracted twice with a double volume of chloroform (14). The bottom layer was evaporated to dryness, and the residue was resuspended in methanol. The extract in methanol was filtered through an MF3 filter (pore size, 0.5 μm; Advantec MFS, Pleasanton, CA, USA) and then injected into an HPLC system equipped with a C18 column (0.46 × 25 cm; Shiseido, Tokyo, Japan) and an ultra-violet detector at 210 nm. HPLC was performed for 40 min at a constant flow rate of 1 mL/min with acetonitrile: water (70:30, v/v) as the eluent. With the HPLC operation conditions above, the retention times of enniatins were as follows: enniatin H (16.5 min); enniatin I (21.5 min) and enniatin MK1688 (28.5 min). The concentrations of enniatins in the submerged culture of *F. oxysporum* KFCC 11363P were calculated by comparing the peak areas of the samples to a calibration curve of peak areas obtained with enniatins standards. The limit of detection of enniatins was 0.15 mg/L and the limit of quantification was 0.50 mg/L. All samples were analyzed in triplicate.

Selection of culture medium To optimize the production of enniatins H, I, and MK1688, *F. oxysporum* KFCC 11363P was cultivated in the following 10 types of complete or chemically defined culture media at 25°C and 125 rpm (amounts are listed per liter): 1, Potato dextrose broth (PDB): infused potato, 200 g; and dextrose, 20 g; 2, Malt extract broth (MB): malt extract base, 6 g; maltose, 1.8 g; dextrose, 6 g; and yeast extract, 1.2 g; 3, Yeast and malt extract broth (YMB): malt extract base, 3 g; yeast extract, 3 g; peptone,

TABLE 1. Central composite design arrangement (real and coded values) and response for production amounts of enniatin analogues by *Fusarium oxysporum* KFCC 11363P.

Exp. no.	Concentration (mM)		Production amount (mg/L)			
	Carbon source (X_1)	Nitrogen source (X_2)	Enniatin H (Y_1)	Enniatin I (Y_2)	Enniatin MK1688 (Y_3)	Total enniatins (Y_4)
1	50 (−1)	100 (−1)	106.9	106.4	148.8	362.1
2	50 (−1)	200 (+1)	220.2	247.5	273.8	741.5
3	150 (+1)	100 (−1)	455.3	491.7	368.6	1315.6
4	150 (+1)	100 (−1)	502.3	649.4	527.9	1679.6
5	29.3 (−1.414)	150 (0)	205.8	172.1	146.8	524.7
6	100 (0)	79.3 (−1.414)	279.8	372.6	592.9	1145.3
7	100 (0)	220.7 (+1.414)	462.6	635.9	526	1624.5
8	170.7 (+1.414)	150 (0)	416.9	678.5	466.9	1562.3
9	100 (0)	150 (0)	626.2	774.8	798.8	2199.8
10	100 (0)	150 (0)	670.2	721.1	710.9	2102.2
11	100 (0)	150 (0)	626.5	722.5	775.2	2124.2
12	100 (0)	150 (0)	759.5	988.4	840	2587.9
13	100 (0)	150 (0)	638.8	892.5	850.3	2381.6