

***Penicillium verruculosum* Strain BS3 Produces Aurantioclavine and Rugulosuvine B Alkaloids**

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Research Article

Abstract

The present study describes rapid screening of *Penicillium verruculosum* strain BS3 (novel strain previously reported from this laboratory) for the production of secondary metabolites in potato-dextrose agar and broth media. Metabolites were extracted from both mycelia (intracellular) and supernatant (extracellular). For intracellular secondary metabolites, the 5 days old mycelia were extracted in the solvent system containing methanol, dichloromethane and ethyl acetate (1:2:3 ratio, respectively) with 1% volume of formic acid; while the extracellular metabolites in the supernatant were extracted in ethyl acetate. The concentrated intra- and extra-cellular extracts were pooled in methanol for analysis using liquid chromatograph/quadrupole-time of flight/mass spectrometry (LC/Q-TOF/MS). Alkaloids such as aurantioclavine and rugulosuvine B were identified from the extract of *P. verruculosum* BS3, upon the database search of 465 secondary metabolites produced by various fungi. It indicates that *P. verruculosum* BS3 is a potent fungus capable of producing valuable leads for drugs such as aurantioclavine, an intermediate for the synthesis of fungal communisin and rugulosuvine B, an antitumor and antimicrobial agent.

Keywords: *Penicillium verruculosum* BS3; Aurantioclavine; Rugulosuvine B; LC/Q-TOF/MS.

1. Introduction

Microbial metabolites are biologically active compounds produced to fulfill the metabolism and growth activities of microorganisms. They are of two types, i.e., primary and secondary metabolites. Among microorganisms, fungi are the rich source of thousands of secondary metabolites, comprising many groups of low molecular weight compounds that are usually regarded as not essential for their life. Most of the secondary metabolites are produced towards the end of the growth phase of the organism. The secondary metabolites isolated from fungi exhibits some antimicrobial (antibacterial, antifungal and antiprotozoal), antitumor or antiviral activities [1].

Fungal secondary metabolites can be divided in to four main chemical classes; polyketides, terpenoids, shikimic acid derived compounds and non-ribosomal peptides [2]. Of diverse bioactive metabolites synthesized by the fungus *Penicillium verruculosum*; ergot and quinoline alkaloids and diketopiperazines got special consideration. Recently, profile of secondary metabolites offers an important component of fungal identification and classification. Selective extraction procedures required for obtaining a full-fledged profile of secondary metabolites. Secondary metabolites have been extracted and separated on the basis of chemical polarity using diverse array of chromatographic techniques such as Thin Layer Chromatography (TLC), High Pressure Liquid Chromatography (HPLC), Liquid Chromatography - Mass Spectrometry (LC-MS) and Liquid Chromatograph/Quadrupole-Time of Flight/Mass Spectrometry (LC-Q-TOF/MS), etc. [3,4].

Many members of the genus, *Penicillium* are known to synthesize a variety of secondary metabolites of alkaloid in nature, i.e., ergots, diketopiperazines, quinolines, quinazolines, benzodiazepines and polyketide [5]. Out of them, aurantioclavine and rugulosuvine received much attention [6]. Aurantioclavine, the ergot alkaloid is believed to be a biological precursor to the communes in (a class of heptacyclic indole alkaloids with cytotoxic and insecticidal properties) family [7,8]. The bioactive rugulosuvines are diketopiperazine alkaloids [9]. Thus, the focus of this study is to screen *Penicillium verruculosum* strain BS3 for the production of bioactive secondary metabolites, with the following objectives: (a) cultivation and screening of *P. verruculosum* for the secondary metabolites; (b) extraction of potent secondary metabolites in suitable solvents; and (c) biophysical characterization of the active compounds extracted.

2. Materials and Methods

2.1 Materials

Analytical grade chemicals from HiMedia (India) and Nice (India) were used for the preparation of culture media and reagents for the extraction of metabolites.

2.2 Source of organism

Pure fungal culture, *Penicillium verruculosum* strain BS3 (GenBank accession No. HQ876770), reported previously from this Laboratory, was used in the present study [10].

2.3 Culture medium

Stock cultures were maintained on basal salt medium (BSM)-agar slants supplemented with carboxy methyl cellulose (CMC) at 28°C. The fungus was cultured on/in potato dextrose-agar (PDA) or potato dextrose (PD) broth. The PDA contained the following ingredients (g/l): 200 potato infusion; 20 dextrose; 15 agar in 1 L distilled water, and PD broth contained: 200 potato infusion; 20 dextrose in 1 L distilled water.

2.4. Extraction of secondary metabolites

2.4.1.Extraction of intracellular metabolites

Secondary metabolites were extracted from the mycelia of 5 days old *P. verruculosum* strain BS3 culture in solvent mixture (methanol, dichloromethane and ethyl acetate in ratio 1:2:3; containing 1% by volume formic acid), by crushing the mycelia using a mortar and pestle, and subsequent centrifugation at $12800 \times g$ for 15 min. The fungal extracts were stored at 4°C for further analysis.

2.4.2. Extraction of extracellular metabolites

Extracellular secondary metabolites were extracted from the mycelia of 5 days old culture of *P. verruculosum* from potato-dextrose broth in ethyl acetate. Using 100 ml separating funnel, the extract was concentrated in rotary evaporator at 77°C. Finally, intra- and extra-cellular extracts in methanol were pooled for further analysis.

2.5 Preliminary screening by TLC

The crude sample was loaded on silica gel coated plate. Solvent mixtures were toluene, ethyl acetate and formic acid in ratio 3:2:1 and 4:3:2, respectively. The chromatograms on the TLC plates were visualized by iodine reagent.

2.6. LC/Q-TOF/MS analysis

The crude extract was used for LC/Q-TOF/MS analysis using the facility available at the Inter-University Instrumentation Center, MG University in Kottayam. The Instrument used was Acquity H class (Waters) Ultra Performance Liquid Chromatography and Xevo G2 (Waters) Quadrupole-Time-of-Flight (Q-TOF). BEH C18 column (50 mm $\times$ 2.1 mm $\times$ 1.7 μ m) was used at a flow rate of 0.3 ml/min. The total run time was 8 min. The source type was Electro-Spray Ionization (ESI) with the capillary temperature of 135°C. Capillary voltage of positive mode of ESI was 3.50 KV and for negative mode, it was 2.50 KV. The mobilization gas flow was nitrogen at 0.3 ml/min, approximately.

3. Results and Discussion

3.1. Effects of culture media

P. verruculosum strain BS3 grown in BSM supplemented with CMC in PDA and PD broth media showed significant growth within 5 days of incubation at 25°C in both solid (on PDA) and liquid culture states. The fungus at its mycelial stage was whitish, and then upon sporulation (after 4 days of incubation), its color notably changed to light green. Well-developed fruiting bodies with spores appeared after 4 days of incubation at 25°C (Figures 1 A and 1B). Brick-red colored pigment was released in 5 days of growth, which contained the secondary metabolites; thus 5 days old culture was used for the extraction of secondary metabolites. The brick-red colored pigment production was prominent on PDA medium. In PD broth, the mycelia were appeared as a viscous mat.

The components of culture media would affect production of secondary metabolite in fungi. The carbon or nitrogen ratio can affect the type and yield of the synthesis of secondary metabolites. Glucose, phosphate or ammonium at higher concentrations is generally regarded as the repressors of secondary metabolism. Several reports convincingly demonstrate that the profiles of fungal secondary metabolites varied under different culture conditions [11]. Growth of *P. verruculosum* strain BS3 on BSM-CMC, PDA media and PD broth showed significant growth in 4 days. The carbon and nitrogen sources in PDA and PD broth media influenced the growth of *P. verruculosum* strain BS3.

3.2. Extraction of secondary metabolite

Fungal secondary metabolites may be cell bound (intracellular) or secretory (extracellular); therefore, both mycelia and supernatant were subjected to extraction.

3.2.1. Extraction of intracellular metabolites

The intracellular metabolites were extracted using 6 ml solvent mixture containing methanol, dichloromethane and ethyl acetate (1:2:3) in 1% of formic acid (v/v). The mycelia were disrupted using

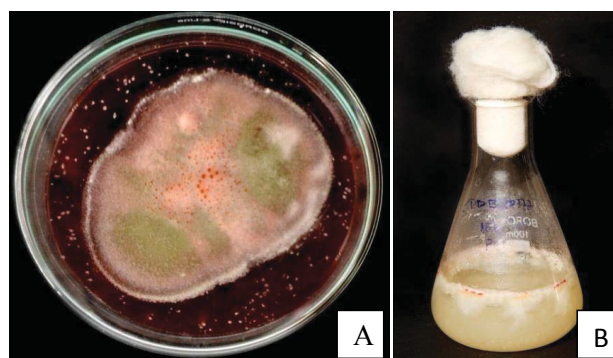


Figure 1. Growth of *P. verruculosum* strain BS3 on fifth day of incubation: (A) PDA and (B) PD broth.

mortar and pestle or by sonication, with an output voltage of 15 db in 15 min. The extract was soluble in methanol, giving a pale yellowish color; which was stored at 4°C for further analysis.

3.2.2. Extraction of extracellular metabolites

The extracellular metabolites were extracted in ethyl acetate. The upper layer containing the dissolved metabolites was collected and the lower layer was discarded (Figure 2). The extract was concentrated by evaporating the ethyl acetate using a rotary evaporator (77°C at 120 rpm).

3.3. Screening by TLC

Preliminary screening of fungal secondary metabolites was carried out by TLC method. The solvent mixture contained toluene, ethyl acetate and formic acid in 3:2:1 and 4:3:2 ratios, respectively. A clear light orange-red spot was observed on both the TLC plate, run in two different solvent systems (Figure 3). The R_f of 3:2:1 solvent system was 0.557 cm, while that of 4:3:2 solvent system was 0.371 cm.

3.4. LC/Q-TOF/MS analysis

The analysis provided data based on mass to charge (m/z) ratio, chemical formula and molecular mass of the analytes from the crude sample. Using the information provided by LC/Q-TOF/MS analysis, the set of known secondary metabolites were identified from the database of fungal secondary metabolites [12]. The data from the LC/Q-TOF/MS obtained in both positive and negative modes. Many peaks



Figure 2. Separation of metabolites using separating funnel: upper layer contains metabolites in ethyl acetate.

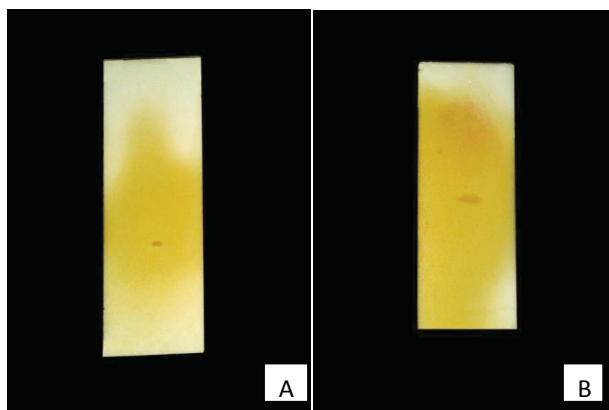


Figure 3. TLC profile of single secondary metabolite. (A) TLC profile of crude fungal extract in toluene:ethyl acetate:formic acid (4:3:2); and (B) TLC profile of crude fungal extract in toluene: ethyl acetate: formic acid (3:2:1).

with corresponding mass, m/z ratio and chemical structure are utilized for the identification of present study.

Based on the LC/Q-TOF/MS profile and of the database, aurantioclavine and rugulosuvine B alkaloids were identified as the significant compounds (Figures 4A-4C). As seen in Figure 4A, about 19 components were detected from the total ion chromatogram obtained from the extract of *P. verruculosum* strain BS3. The peak with m/z 227.1548 at 3.3 of RT was identified as aurantioclavine. Auranitioclavine is an ergot alkaloid, whose molecular structure is characterized by a tetracyclic ergoline system with a seven carbon ring and a ring D [7,8]. A resourceful producer of this alkaloid is *P. nalgiovense* Lax (1932) VKM F-229, formerly known as *P. aurantiovirens* VKM F-229. Optimal media for the synthesis of aurantioclavine and other ergot alkaloids by *Penicillium* spp. fungi contain succinate and manitol as carbon source and ammonium salt as nitrogen source [13]. It serves as an intermediate in the biosynthesis of fungal communisin. The dynamics of aurantioclavine in the liquid culture of the fungus also displayed a biphasic pattern. The extracellular concentration of the alkaloids increased concurrently with the biomass during the lag phase of active growth in succinate, and it will decrease in log phase. With manitol, increased active growth was seen, whereas at the beginning of the stationary growth phase, the concentration of alkaloid reached the maximum [14]. Within the retention time 7.7 of mass 443.3543 is identified as rugulosuvine B. Kozlovsky isolated the diketopiperazine of tryptophan and phenylalanine, an alkaloid metabolite from the culture of *P. rugulosum* VKM F-182 and named it after the producer (rugulosuvine) [15]. Absolute structure of rugulosuvine B was demonstrated as identical to that of rugulosuvine A. Rugulosuvine B showed antibiotic and antitumor activities [6,15]. *Penicillium verruculosum* (IMI 3521 19) was found to produce three closely related macro cyclic poly lactones: BK223-A, BK223-B and BK223-C. BK223-A

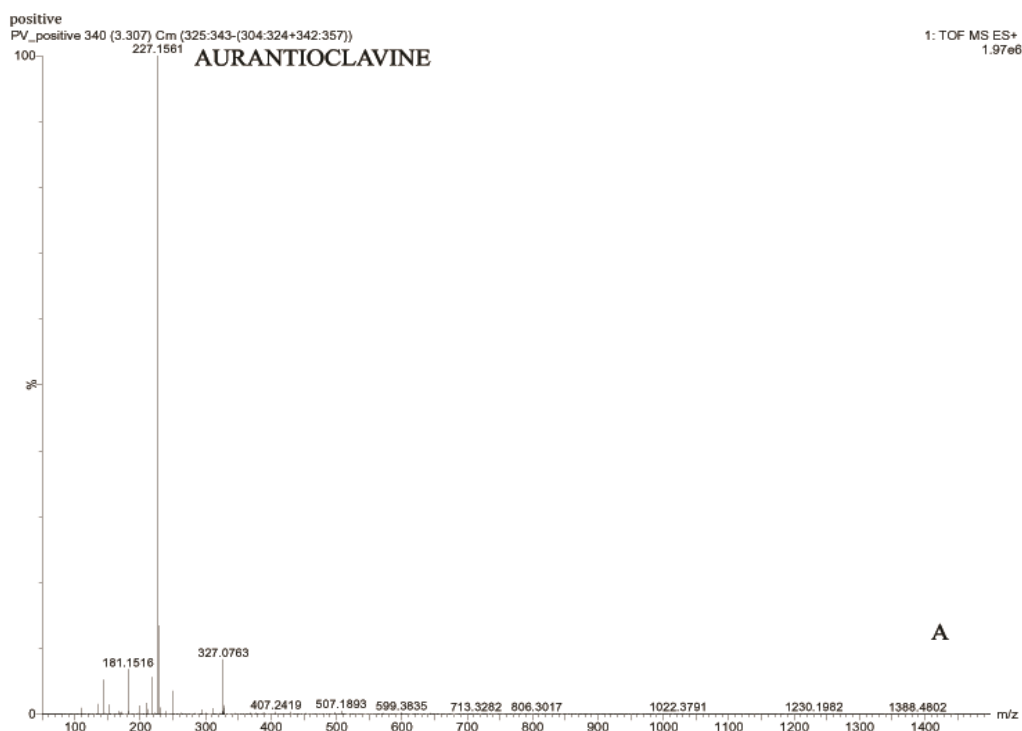


Figure 4A. Analysis of an extract from *P. verruculosum* strain BS3 by LC-Q-TOF MS: Total ion chromatogram with corresponding to aurantioclavine.

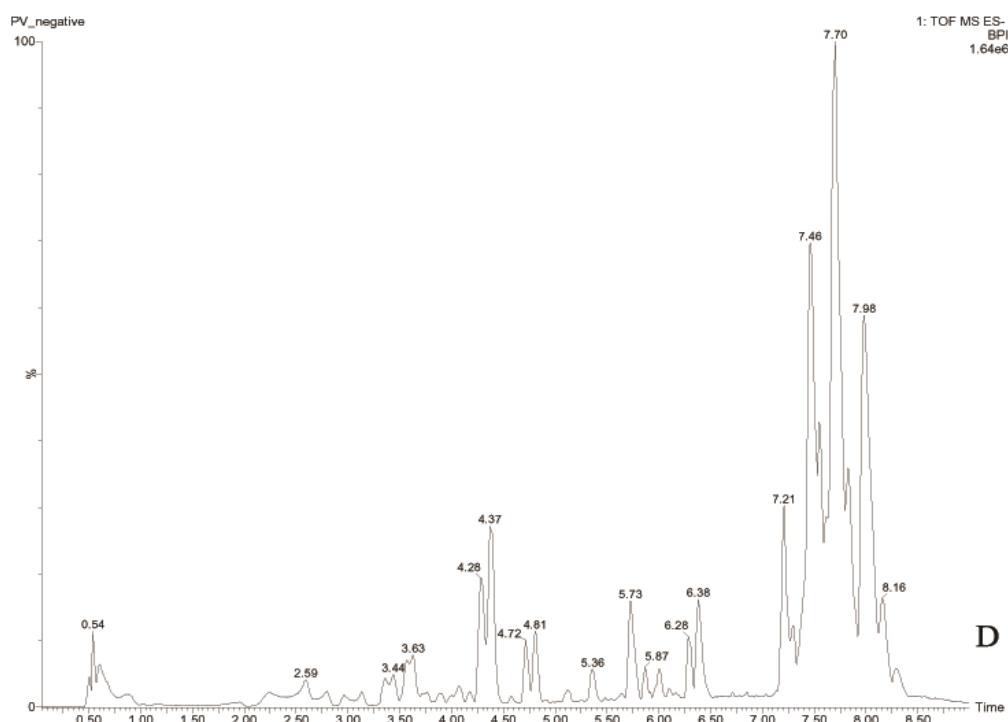


Figure 4B. Analysis of an extract from *P. verruculosum* strain BS3 by LC-Q-TOF MS: Total ion chromatogram with corresponding to rugulosuvine B.

was identified as NG-012, a nerve growth factor potentiator isolated from *P. verruculosum* F-45421; BK223-Band BK223-C, were found as novel. All these compounds exhibit growth inhibiting activity against plant pathogenic fungi including *Botrytis cinerea*, *Phoma lingam*, *Phoma betae*, *Pyrenophora*

teres, *Sclerotinia sclerotiorum*, *Monilinia fructigena*, *Ascochyta pisi* and *Alternaria alternata* [16].

Literature shows that aurantioclavine is produced both as intracellular and extracellular metabolite [13]; while, rugulosuvine B is produced chiefly

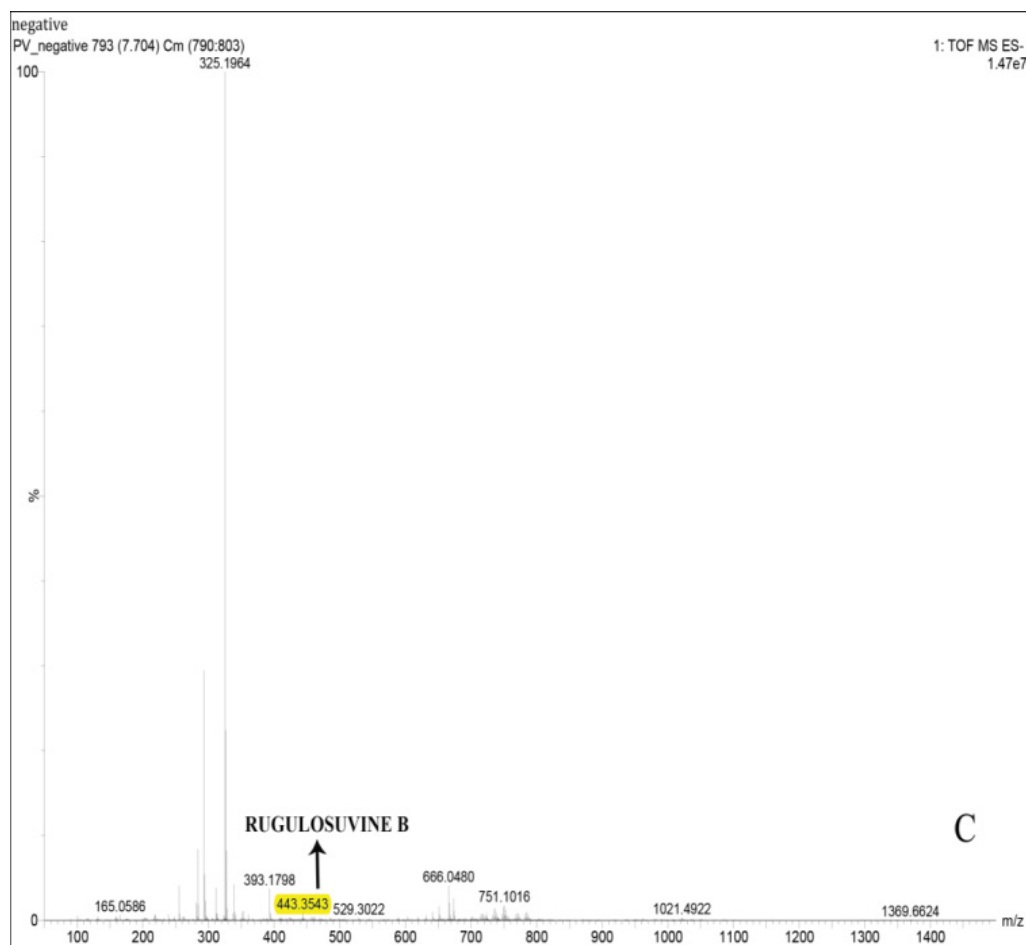


Figure 4C. Analysis of an extract from *P. verruculosum* strain BS3 by LC-Q-TOF MS: Total ion chromatogram in negative mode.

as an extracellular metabolite [6,17]. Thus, both extracellular and intracellular metabolites are pooled for the rapid screening of useful secondary metabolites by LC/Q-TOF/MS technique.

5. Conclusion

P. verruculosum strain BS3 sub-cultured in media such as basal salt medium supplemented with carboxy methyl cellulose, PDA and PD broth. Two weeks old culture was extracted in ethyl acetate, which was subjected to LC-QTOF-MS analysis. Aurantioclavine and rugulosuvine B were the major secondary metabolites identified from the analysis of data. Rugulosuvine B is known to have antibiotic and antitumor properties.

6. Acknowledgement

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