

## In Vitro and In Vivo Induction, and Characterization of Toxins Isolated from *Beauveria bassiana*

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### ABSTRACT

Entomopathogenic fungi produce secondary metabolites which may bio actively help fungus in its virulence toward insect hosts. *Beauveria bassiana* produces several toxic low molecular compounds in vitro as well as in vivo, the most important of them is Beauvericin. The *Beauveria bassiana* fungus isolated was selected for Toxin assay. Various Toxins was obtained from surface and submerged cultures of the fungus in PDYB and PDA, culture filtrates, and in vivo conidia harvested from insect cadavers. Results indicated that in vivo fungal conidia contained the most Beauvericin, causing a higher mortality compared with in vitro fungal products in their different concentrations. Beauvericin chromatogram revealed that Beauvericin was in its greatest quantity in comparison with the other secondary metabolites of *Beauveria bassiana* isolate. The impact of Beauvericin on mean and on paralysis time was in agreement with bioassay data in treated with metabolites of the insect-derived conidia.

**Keywords:** *Beauveria bassiana*, HPLC, MALDI-TOF, Toxin Identification.

### INTRODUCTION

In general, entomopathogenic fungi involve an infective spore stage, in which it germinates on the host cuticle, forming a germ tube that penetrates the cuticle and invades the haemocoel of the insect host<sup>11</sup>. The fungus then multiplies within the insect body and kills it. Death occurs due to toxin production by the fungus and/or multiplication to inhabit the entire insect body<sup>7</sup>. Such circumstantial evidences as reduced activity, reduced microbiota on the host cadaver, paralysis and some other phenomena are consistent with the involvement of fungus derived biocides in pathogenesis<sup>4</sup>.

Entomopathogenic fungi are prolific producers of bioactive secondary metabolites<sup>13,14,20</sup>, which are predicted to play key roles as virulence factors for fungi, infecting arthropods<sup>25</sup>. Metabolites produced by entomopathogenic fungi would serve one or more of the following functions: (1) toxic to the host and help to cause death; (2) to aid the fungus overcome host defence; (3) to suppress competition from other pathogens and saprophytes on the insect cadaver; (4) to provide a defence outside the host against mycophagous organisms<sup>4</sup>.

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Nonribosomal peptides, alkaloids, terpenes, and polyketides are the main classes of fungal secondary metabolites the expression and secretion of which appear to be controlled by various genetic and cellular regulatory mechanisms<sup>3,12</sup>. These are not needed for growth or development of the producing organism under laboratory conditions, but are thought to aid the fungus in successfully competing with other organisms in its natural habitat. Accordingly, many secondary metabolites tend to be compounds that bear toxic or inhibitory effects on other organisms<sup>29</sup>. Only a few studies to date have unequivocally demonstrated the significance of entomopathogenic secondary metabolites, including toxins, in the disease process<sup>25</sup>.

The entomopathogenic fungus, *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin (1912) (Ascomycota: Cordycipitaceae) is a ubiquitous fungus which has been found and isolated from a wide variety of insects of different orders<sup>19, 16, 18, 8</sup> and is the most widely used fungal species available commercially<sup>7</sup>. It is generally found on infected insects both in temperate and tropical areas throughout the world. Some molecular studies have recently confirmed a teleomorph, *Cordyceps bassiana* for *B. bassiana*<sup>10</sup>. The biosynthesis of secondary metabolites is an outstanding hallmark of the fungal organisms. They may operate as either defensive or offensive chemical weapon against animals<sup>25</sup>. *Beauveria bassiana* produces several toxic compounds in vitro and in vivo<sup>30, 32</sup>. A majority of these insecticidal molecules are low molecular weight secondary metabolites. Beauvericin, bassianin, bassianolide, beauverolides, beauveriolides, tenellin, oosporein<sup>30, 32</sup>, oxalic acid<sup>24</sup> bassiacridin<sup>5,23</sup> are some of the important metabolites of *B. bassiana*. Among them, Beauvericin is the most important compound which was reported first from *B. bassiana*. Beauvericin is a toxic cyclic hexadepsipeptide and comprising a cyclic repeating sequence of three molecules of N-methyl phenylalanine that alternate with three

molecules of 2-hydroxyisovaleric acid. Not all isolates of *B. bassiana* produce beauvericin in vitro<sup>5,21</sup>. Beauvericin carries moderate insecticidal properties<sup>31, 15,2, 22, 9, 2</sup>. Nevertheless, there are some reports of no toxicity against certain insects<sup>2</sup>.

Many works have been directed towards fungal penetration into host cuticle, but there is little knowledge as regards the role of toxins in fungal pathogenesis. In this study, in vitro and in vivo production of Beauvericin, as an important fungal toxin of *B. bassiana*, was investigated and its virulence assayed against the greater wax moth larvae.

## MATERIALS AND METHODS

### Fungal Isolate

In this study, an isolate of *B. Bassiana* was utilized. *Beauveria bassiana* had been isolated from the soil in open atmosphere by National Fungal Culture Collection of India (NFCCI), Pune. It was initially cultured on PDA (Potato Dextrose Agar) medium (Merck, Germany). Cultures were kept at 27°C in darkness for 15 days and then transferred to fridge (4°C) until further use.

### Toxin Extraction from Mycelia and Filtrates

Fungal mycelia were separated through filtering over a vacuum in Buchner funnel through a No. 1 Whatman® filter paper. Air-dried mycelia were incubated with 100 ml of methanol (Fisher Scientific) for one hour. The solvent was evaporated in distillation column at 55°C. Residuals were passed through a 0.2 µm sterile filter (Sartorius, Germany) equipped with a sterile syringe and re-dissolved in 5 ml of methanol for collection and dried again in cupboard into powder in a 30 ml glass container on a hot plate (45°C) and with nitrogen current involvement.

Crude filtrates (separated from mycelia) were filtered through a 0.2 µm sterile filter (Sartorius, Germany) equipped with a sterile syringe being freeze dried to dryness and used in HPLC analysis and in bioassays.

### **In vivo Bioassays with Toxins**

Each powdered toxin of mycelia and dried crude filtrates was re-dissolved in 1 ml double distilled sterile water with injection of solutions of fungal toxins performed through a Desaga micro-injector to greater wax moth. The fresh in vivo prepared serum, containing toxins were selected. The toxicity of all mentioned fungal materials was tested by injection 20 µl into HPLC instrument. Technical Beauvericin (Sigma) was diluted to 1 mM with double distilled water and directly injected to larval haemocoel. Three replicates of twenty larvae were treated with each fungal preparation. Control insects were injected with sterile broth, except mentioned otherwise. Larvae were placed in sterilized 500 ml glass containers and held at 25°C. Signs of toxic effects on insect host were observed and the mortality recorded for 12 days.

### **Toxin Production by Conidia**

Pure conidia from artificial medium (In vitro conidia) and conidia from sporulated cadavers (In vivo conidia) of *Beauveria bassiana* were extracted with 1:1 acetonitrile/water at 0.2 ml of solvent per mg of conidia<sup>17</sup>. Extracts were then passed through 0.2 µm sterile filter (Sartorius, Germany) and diluted 10,000-fold with double distilled water before 10 ml of the extract being directly injected into the HPLC-ESI/MS and analysed as described below. These conidial extracts were also used for bioassays.

### **High Performance Liquid Chromatography (HPLC)**

*B. bassiana* entomotoxin was analysed on a HPLC (Schimadzu LC/10AD, Japan) equipped with an injector (20 µl loop) and C-18 column (5µm particle size) (250 mm x 4.6 mm I.D) using CH<sub>3</sub>OH/H<sub>2</sub>O (1:1 v/v) at a flow rate of 0.8 ml min<sup>-1</sup> with detection gradient from 3% MeOH (Himedia, India) and increased up to 70% MeOH by UV absorption at 280 nm using a detector (SPD-10A/UV-Vis). Purification was executed by analytical HPLC (Schimadzu) using a LC/10ADODS, 250 mm X 4.6 mm column, MeOH/H<sub>2</sub>O linear elution gradient starting from 3% MeOH during 5 min and increased up to 70% MeOH in 30 min with a flow rate of 1.0

ml min<sup>-1</sup>. The analytical chromatogram showed one major compound UV maximum at 3000 nm. The procedure yielded 5 mL of a pure white column solution. The compound was subjected to MALDI-TOF for identification of *B. bassiana* entomotoxin as described below.

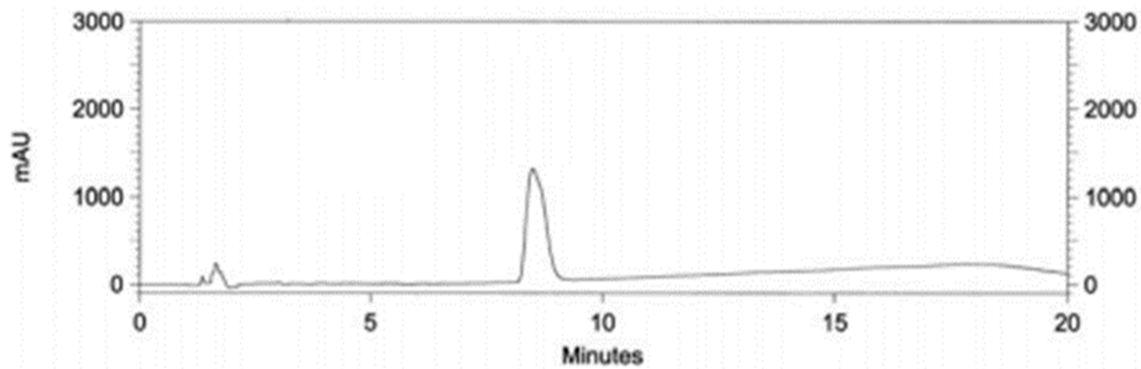
### **Matrix-assisted laser desorption/ionization-TOF (MALDI-TOF)**

Liquid *Beauveria bassiana* obtained from the preparative HPLC was allowed to dry and then subjected to MALDI-TOF MS analyses (Voyager-DETM PRO Bio spectrometry TM spectrometer (Applied Biosystems, Framingham, MA, USA) equipped with a model VSL-337ND Nitrogen Laser (Laser Science, USA). The accelerating voltage was 20 KV. The matrix was  $\alpha$ -Cyano-4 hydroxycinnamic acid (Sigma Aldrich, India), (10 mg/ml in 1:1 CH<sub>3</sub>CN/0.1% TFA). An equal amount of sample and matrix were dropped onto the MALDI sample plate and allowed to dry at room temperature. Time-to-mass conversion was achieved by external and/or internal calibration using standards of bovine pancreatic beta insulin (m/z 3496.9), bovine pancreatic insulin (m/z 5734.6), and apomyoglobin (m/z 16, 952.6) (Sigma Aldrich, India). Experiments were facilitated by the Voyager version 5 with Data Explorer TM software.

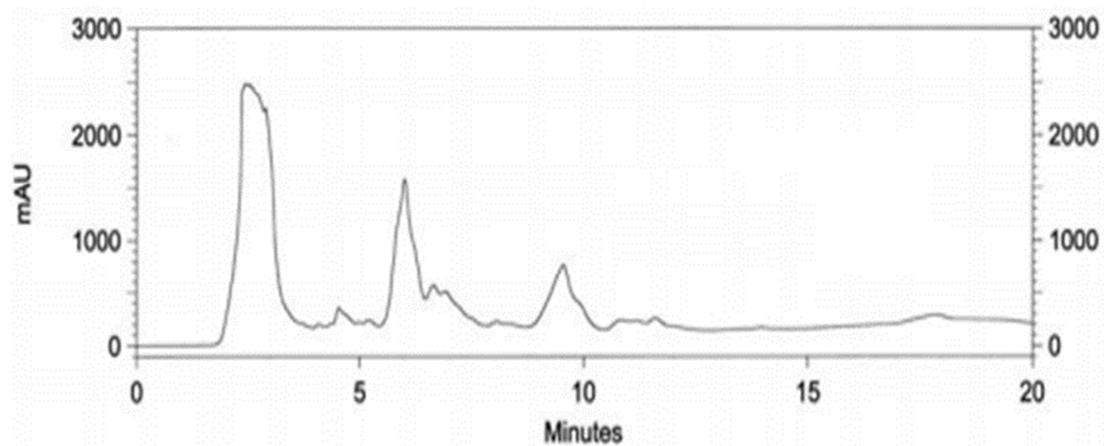
## **RESULTS**

### **HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)**

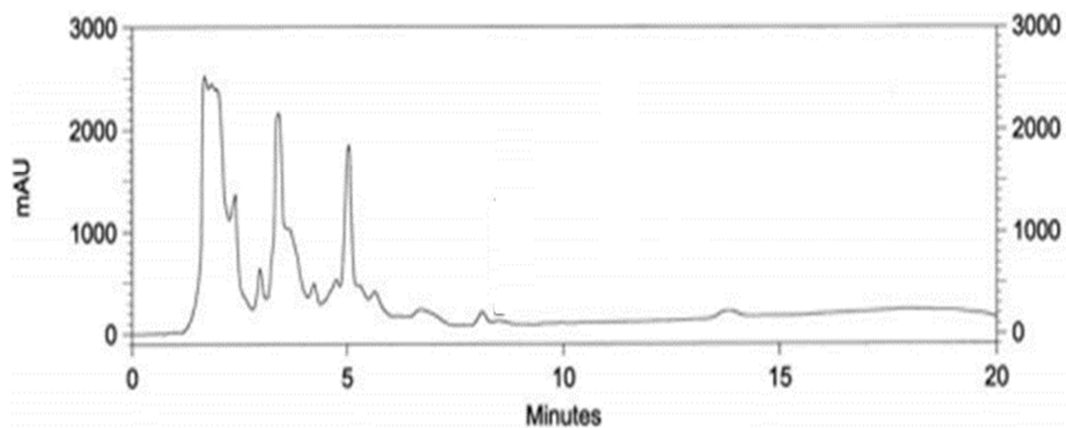
Since *Beauveria bassiana* has higher insecticidal activity, we are interested to analyse the same. *Beauveria bassiana* was analysed by HPLC for fractionation and separation of the active polypeptide. The samples were preliminarily subjected to Analytical HPLC. Two peaks were recorded with retention time of 4.262 min<sup>-1</sup> with the area of 95.69% and 5.915 min<sup>-1</sup> with the area of 4.1% (*Beauveria bassiana*). Then the fractionated HPLC sample *Beauveria bassiana* was subjected to preparative HPLC to record major polypeptide/peak. It has a retention time of 10.184 min<sup>-1</sup> (area 100%) with methanol as the solvent.



The Graph shows Signal generated at 205nm by injecting 20 $\mu$ l of 1000 $\mu$ M standard beauvericin. Signal Measured in mAU



The Graph shows at 250nm by injecting 20 $\mu$ l of GHA Methanol extraction obtained bassianolide

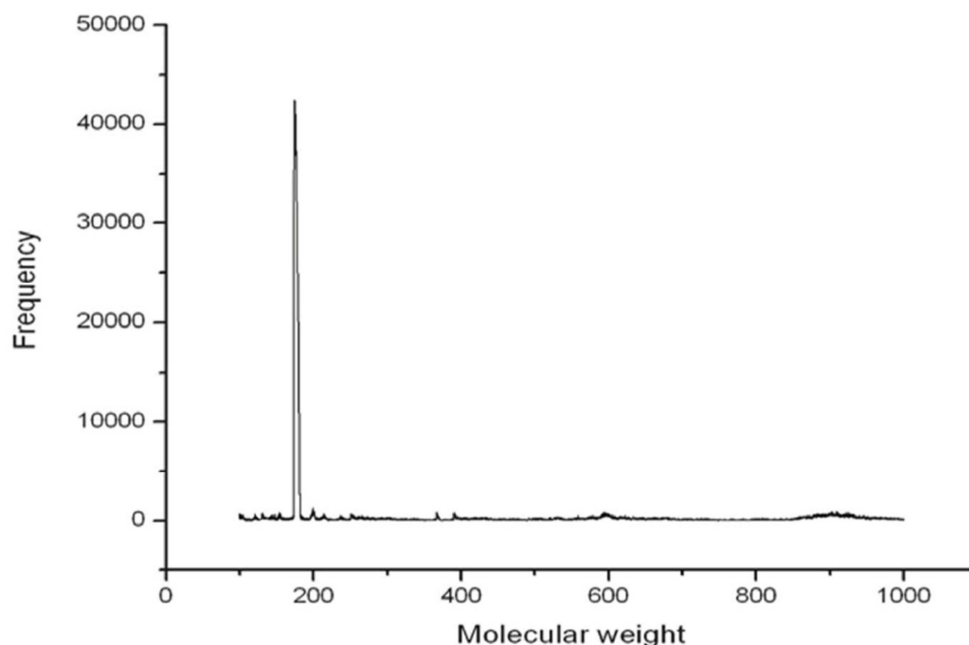


The Graph shows the obtained toxic components in Fungi *Beauveria bassiana* are Beauvericin (28kDa), Destruxins (22kDa), Bassianin (18kDa), Bassianolides (390kDa), Tenellin (60kDa), Oosporein (83kDa) and Bassiacridin (89kDa). With the HPLC the various components are identified with the Gradients Methanol 100% (CH<sub>3</sub>OH) and Gradient MeOH 70% (Wood Alcohol) by UV absorbance 280nm (SPD-10A/UV-Vis). (CH<sub>3</sub>OH AND MeOH are part of Methanol.)

### Matrix-assisted laser desorption/ionization- Time of Flight (MALDI-TOF)

The purified single peptide obtained by the preparative HPLC was analysed by MALDI-TOF. A peak was obtained at the molecular

weight of 174 Da and it was designated as *Beauveria bassiana*. Unfortunately no specific molecules were identified by searching PMF databases using the MASCOT search engine.



The Graph shows the MALDI-TOF Analysis of *Beauveria bassiana* entomotoxin protein Purification using HPLC

### DISCUSSION

Analytical HPLC spectrum of *B. bassiana* entomotoxin revealed two polypeptides present in *Beauveria bassiana* at the retention time of 4.2 min (95.6%) and 5.9 min (4.4%). Previously, reported tyrosine betaine, a novel entomotoxin secondary metabolite from the fungus *Metarhizium* sp. having the retention time of 4.1 min. In another study identified a neuropeptide from midgut of Colorado potato beetle by HPLC at the retention time of 4.4 min. These findings clearly showed that a toxic peptide was identified at the retention time of 4.1–4.4 min in HPLC spectrum. Hence, the result obtained from the present study confirms the toxic nature of *B. bassiana*. The major peak of *Beauveria bassiana* was collected by preparative HPLC at the retention time of 10.1 min and later identified with MALDI-TOF showed 172 Da molecular weight. No previous report is available about this fraction. Most fungal toxins are secondary metabolites and low molecular mass

compounds<sup>4</sup>. Well-characterized classes of toxins include polyketides (e.g. aflatoxins – 28 kDa), cyclic peptides, alkaloids and sesquiterpenoids (e.g. trichothecenes).

In most cases, application of purified toxin is sufficient to cause death in susceptible insects. This implies that by killing the insects with a secreted, soluble toxin, the fungi are able to circumvent the innate defences of insects. Most of the studies reported the insecticidal activity of different strains of *B. bassiana* spores at different concentrations. Few reports are available about the insecticidal activity of *B. bassiana* toxins such as beauvericin, destruxins, bassianin etc. against insect pests<sup>18,26</sup>. In the present study, the *B. bassiana* entomotoxin showed high mortality. Consumption of toxic substances makes complicated physiological changes in insects<sup>11</sup>. There are several reports available about the insecticidal activity and after effects of *B. bassiana* toxic metabolites against insect pests<sup>32,23,20,25</sup>.

Beauvericin (M.W. 28 kDa), destruxins (M.W. 22 kDa), bassianin (M.W. 18 kDa), bassianolide (M.W. 390 kDa), beauverolides (M.W. 72 kDa)<sup>32</sup>, tenellin (M.W. 60 kDa), oosporein (M.W. 83 kDa)<sup>9</sup> bassiacridin (M.W. 89 kDa)<sup>23</sup> are some of the important metabolites of *B. bassiana*.

Among them, Beauvericin is the most important virulent compound which was reported from *B. bassiana*<sup>23</sup>. The proteolytic enzymes from *B. bassiana* were intensively studied as the mortality factor. Protease is one of the most important and earliest enzymes involved in the invasion followed by chitinase after the eventual exposure of chitin in the host cuticle after the proteolytic degradation of cuticular proteins. Dose dependent mortality was recorded in the present study. It must be said that very few pieces of literature are available about the effect of toxins from *B. bassiana* against live insects; most studies were proven enzymatically under spectrometric conditions.

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