MYCOTOXINS AND ENZYMES OF ENTOMOPATHOGENIC FUNGUS *Lecanicillium saksenae* (Kushwaha) Kurihara and Sukarno AND THEIR BIOEFFICACY ON CROP PESTS

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(2016-21-011)

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KERALA, INDIA

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Thesis submitted in partial fulfillment of the requirement for the degree of

DOCTOR OF PHILOSOPHY IN AGRICULTURE

Faculty of Agriculture

Kerala Agricultural University

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DECLARATION

I hereby declare that this thesis entitled **"Mycotoxins and enzymes of entomopathogenic fungus *Lecanicillium saksenae* (Kushwaha) Kurihara and Sukarno and their bioefficacy on crop pests**" is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award of any degree, diploma, fellowship or other similar title, of any other University or Society.

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Date :18-08-2020 **(2016-21-011)**

CERTIFICATE

Certified that this thesis entitled **"Mycotoxins and enzymes of entomopathogenic fungus *Lecanicillium saksenae* (Kushwaha) Kurihara and Sukarno and their bioefficacy on crop pests"** is a record of bonafide research work done independently by Mrs. Sreeja. P. (2016-21-011) under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

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Sreeja. P.

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LIST OF ABBREVIATIONS AND SYMBOLS USED

@	At the rate of
%	Per cent
µg	Micro gram
µL	Microlitre
et al.	And other co workers
Fig.	Figure
h	Hour
kDa	Kilo Dalton
L^{-1}	Per litre
nm	Nanometer
min	Minutes
mL	Millilitre
pH	Negative logarithm of hydrogen ions
ppm	Parts per million
Rt	Retention time
rpm	Revolution per minute
s	seconds
U	Unit
viz.	Namely

INTRODUCTION

1. INTRODUCTION

Concerns over the deleterious effects of chemical insecticides on environmental and human safety lead to a strong impetus for the development of microbial control agents for integrated pest management. Of the diverse assemblage of microorganisms including viruses, bacteria and fungi, considerable effort has been focused on the development and utilization of entomopathogenic fungi. Entomopathogenic fungi have a tectonic role among microbials, due to their contact mode of action which render them pathogenic to sucking pests as well. *Beauveria bassiana* (Balsam) Vullemin, *Metarhizium anisopliae* (Metschn) Sorokin, *Lecanicillium lecanii* (Zimmermann) Zare and Gams, *Hirsutella thompsonii* (Fisher), *Nomuraea rileyi* (Farlow) Samson and *Isaria fumosorosea* (Wize) Brown and Smith are the fungal bioagents that are gaining importance in microbial pest management.

The genus *Lecanicillium* is known for its pathogenicity to insects, mites as well as nematodes infesting various crop plants of which some of them have been developed as commercial biopesticides (Hall and Papierok, 1982). Certain species of this genus are even antagonistic to plant pathogenic fungi (Kim *et al*., 2007). Regardless of their effectiveness in managing sucking pests, they are reported to be slow in action and sensitive to the environment. Identification of indigenous and geographically distinct isolates that are highly pathogenic and environmentally stable, would greatly reduce such drawbacks.

Indigenous isolates perform better than the exotic strains in terms of pathogenicity and virulence. *Lecanicillium saksenae* (Kushwaha) Kurihara and Sukarno (ITCC- LsVs1 -7714), an indigenous virulent isolate from soils of Vellayani isolated and characterised by Rani *et al*. (2015), was found infective to sucking pests and more adaptable under the normal temperature regime of Kerala. Apart from its infectivity and adaptability, it was found to be more effectual in terms of speed of kill. Preliminary studies carried out in the Dept. of Agrl. Entomology, College of Agriculture, Vellayani, Thiruvanthapuram, Kerala,

India, have proved its safety to crop plants and natural enemies. With an attempt to uplift this indigenous isolate as an effective and safe bioagent, basic information on its host range, pathogenicity, cross infectivity to crop plants and safety to non target organisms were gathered in the preliminary investigations. Amenability to mass production and field efficacy in managing sucking pests of rice had been already investigated by Sankar and Rani (2018). Its entomopathogenicity was further established by detecting the presence of major cuticle degrading enzymes. Quick insecticidal action reported by previous researchers was a strong impetus to profile its metabolites.

Entomopathogenic fungi are treasure of secondary metabolites with diverse chemistry and unique mode of action. Metabolomics provide valuable insight into the host pathogen interaction. They mediate infection process in insects and cause mortality through inhibition of different enzymes involved in the physiological functions. Furthermore, metabolite profiling of an entomopathogen would pave way for the development of safer biocide molecules that could be commercialised directly for pest management or would serve as templates to develop insecticidal molecules with novel mode of action. Identification of new and safer insecticidal molecule is thus a fascinating area of research for drug designing.

Recent developments in spectrometric analysis have made spectacular advancement in the study of metabolomics of entomopathogenic fungi. Accessibility to inbuilt spectral libraries and recent upgradation of such databases enable the structural and functional identification of metabolites easier.

A precise understanding of the underlying mechanisms, particularly the role of primary metabolites such as cuticle degrading enzymes that breach the insect cuticle and the secondary metabolites, the toxins that deprive the immune system of host insect is an essential stride to move forward to enhance its virulence through genetic modifications.

The present investigation on *L. saksenae* was therefore focussed to gather information on the following aspects

- Qualitative and quantitative detection of cuticle degrading enzymes
- Bioefficacy of major cuticle degrading enzymes on insect pest and plant parasitic nematode
- Purification and characterisation of the major cuticle degrading enzyme
- Detection of mycotoxins produced by the fungus
- Purification and characterisation of the major mycotoxin
- Bioefficacy of toxin on insect pest
- Effect of toxin on predators
- Mode of action of the major metabolites

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

Entomopathogenic fungi are successfully evolved organisms to overcome the formidable defense system in insects. Unlike bacteria and viruses, they have a unique mode of entry through the host cuticle, rendering them as a potent candidate in microbial control of insects, irrespective of their feeding habits. More than 750 species of fungi are reported to be entomopathogenic, of which *Metarhizium anisopliae* (Metschn) Sorokin, *Beauveria bassiana* (Balsam) Vullemin, *Lecanicillium lecanii* (Zimmermann) Zare and Gams, *Isaria fumosorosea* (Wize) Brown and Smith, *Hirsutella thompsonii* (Fisher) and *B. brongniartii* (Sacc) are the most popular and widely adopted species. *B. bassiana and M. anisopliae* have a broad host spectrum, while others have a narrow spectrum of host insects.

Under the genus *Lecanicllium*, *L. lecanii* is a popularly used bioagent for the management of sucking pests. Its efficacy in managing aphids, scales, mealybugs and whiteflies were reported from various parts of the world (Ekbom, 1979; Kanagaratnam *et al.,* 1982; Cuthbertson *et al.*, 2005; Faria and Wraight, 2007). The species*, L. saksenae* was not known for its entomopathogenicity until Rani *et al*. (2015) reported it to be infective to aphids *Aphis craccivora* (Koch), *A. gossypii* (Glover), whitefly *Bemisia tabaci* (Gennadius), jassid *Amrasca biguttula biguttula* (Ishida) and mealybug *Coccidohystrix insolita* (Green). Its infectivity to common sucking pests of vegetables was further demonstrated by Jasmy (2016) and the field efficacy in managing sucking pests of rice was proved by Sankar and Rani (2018). They reported paralysis and convulsions in rice bugs treated with the spore suspension at 10^7 spores mL^{-1} within 48 h of treatment. It was also reported to be safe to beneficial insects and predators of the rice ecosystem (Sankar and Rani, 2018).

Pathogenicity of an entomopathogenic fungus is a combination of mechanical and biochemical processes as well as its inherent trait. The most important step in pathogenesis is the germination of conidia followed by its

penetration through the cuticle facilitated by hydrolytic enzymes and mechanical pressure.

Latge *et al.* (1987) reported that successful germination of conidia resulted from the assimilation of the nutrients and their ability to overcome toxic compounds present in the cuticle. Hydrolytic enzymes played pivotal role in fungal pathogenesis which included a complex of biochemical, physiological and genetic processes (Petrisor and Stoian, 2017).

2.1 CUTICLE DEGRADING ENZYMES OF ENTOMOPATHOGENIC FUNGI

The exoskeleton or the cuticle is one of the physiological barriers that protect insect from infections. It is a multi layered structure composed of chitin, proteins, lipids, lipoproteins, wax, polyphenols, etc. Production of extracellular cuticle degrading ezymes (CDE) by entomopathogenic fungi under submerged fermentation was first reported by St. Leger *et al*. (1986). Gupta *et al.* (1994) supported the notion that CDE determine both specific virulence parameters and host specificity. They suggested that, establishing a direct relationship with CDE and virulence would help to undertake strain improvement programme. CDE produced sequentially by the fungus play a key role in the break down of cuticular layers, facilitating the penetration process.

Chitin constitute the major component of insect cuticle. It is a biopolymer of β (1,4)-linked N-acetyl-glucosamine, and is one of the most copious and renewable natural biopolymers, second to cellulose (Tharanathan and Kittur, 2003). The important chitin degrading enzymes of entomopathogenic fungi are chitinases, chitin deacetylase and chitosanase.

2.1.1 Detection and Activity of CDE

2.1.1.1 Chitinase

Chitinase is a glycoside hydrolases that hydrolyze the β-1, 4-glycosidic bonds between the N-acetyl-D-glucosamine residues of chitin. They are prevalent in microorganisms such as bacteria (Singh *et al.,* 2008), entomopathogenic, nematophagous and antagonistic fungi (Pino *et al*., 2011). Attempts have been made by many researchers to evaluate chitinase activity of entomopathogenic fungi both qualitatively and quantitatively.

Agrawal and Kotasthane (2012) reported a simple and sensitive method to detect chitinase activity of microorganisms. The composition of detection plate was standardised with colloidal chitin and pH indicator, bromo cresol purple and found to be an easy, reproducible and economic option to determine chitinase activity. Jenin *et al.* (2016) identified chitinase activity of *Aspergillus niger* (Tieghem) and *A. fumigatus* (Fresenius) using chitin-agar medium, wherein, spot inoculation on chitin agar plates produced a halo of clear zone around the colony which was confirmed using congo red dye solution.

Nahar (2004) investigated the time course for the production of chitinase in different isolates of *M. anisopliae.* The chitinase activity appeared after 72 h which ranged between 0.01 and 0.0398 U mL^{-1} among isolates of *M. anisopliae.*

Chitinase activity of different species and isolates of *Lecanicillium viz. L. lecanii*, *L. attenuatum*, *L. longisporum* and *L. muscarium* was evaluated by Ramanujam *et al*. (2011). Among the 15 isolates of *L. lecanii* tested, three isolates *viz*., Vl-7, Vl-24 and Vl-25 had high activity ranging between 100 and 126 µg mL^{-1}. The activity of Vl-22 of *L. attenuatum*, Vl-24 of *L. longisporum* and Vl-8 of *L. muscarium* were 90, 110 and 117µg mL^{-1} respectively. Yu *et al*. (2015) detected increase in chitinase activity of *L. lecanii* after 24 h in the

medium supplemented with chitosan which increased after 48 h, with a gradual decline thereafter (change in OD value from 0.02 to 0.45).

Chitinase activity of different isolates of *B. bassiana* and *M. anisopliae* were investigated by Chai *et al. (*2012). The activity ranged from 4.39 to 181.05 mU mL^{-1}, with peak activity on the 11^{th} day. Enzyme activity expressed a positive correlation with infection in the larvae of diamondback moth *Plutella xylostella* (Linn.). Mishra *et al.* (2013) detected the enzyme activity of native *B. bassiana* (HQ917687) from northern part of Uttar Pradesh and found that it increased gradually from 1^{st} to 6^{th} day of incubation, with peak activity of 5.24 UmL^{-1} on the 5^{th} day. An extracellular antifungal chitinase of *L. lecanii* strain 43H was assayed by Nguyen *et al.* (2015) who quantified it as 0.528 U mL^{-1} (66 Umg^{-1} protein) after 144 h at 40°C and pH 6.0. Elawati *et al*. (2018) noted that the release of extracellular chitinase into the culture medium was associated with the log phase of growth of *B. bassiana.* The activity (0.585 U mL^{-1}) was at its peak on the 4^{th} day.

2.1.1.2 Lipase

Lipase acts on the outer most wax layer of insect cuticle and hydrolyzes the ester bond of lipoproteins, lipids and waxes to facilitate the entry of fungus into insect integument (Ali *et al*., 2009a). Hasan *et al*. (2013) detected the lipolytic activity of *L. lecanii* using sorbitan monolaurate (Tween 20). Its activity was observed as a visible precipitate around the colony. The activity was highest at pH 7.0.

Silva *et al*. (2005) analysed lipase activity of a Brazilian strain of *M. anisopliae* and observed that its activity was high in olive oil substrate (108.38 U mL^{-1}). Ali *et al*. (2009b) also found that two per cent olive oil stimulated the production of lipase (97.44±1.96) in *M. anisopliae*. Enhanced lipase activity due to the supplementation of olive oil (125.33±2.96 U mL^{-1}) and surfactant SDS (110.66±3.52 U mL^{-1}) was observed in *I. fumosorosea*. In *B. bassiana* specific

activity of lipase increased from 2nd to 6th day of incubation (0.318 ± 0.004 to 0.686 ± 0.003 m OD min^{-1}) and then declined from 7th day onwards, with its peak on the 6th day at pH 7.1 (Zibaee *et al*., 2011).

2.1.1.3 Protease

Proteins comprise about 70 per cent of cuticle and hence proteolytic enzymes secreted during the early stages of penetration are considered as the most important pathogenicity factor (St. Leger *et al*., 1988)**.** Subtilisin like protease Pr1 and trypsin like protease Pr2 were the important proteolytic enzymes mostly studied by researchers.

Pinto *et al.* (2002) established the role of different substrates in the expression of the proteases, chymoelastase (Pr1) and trypsin (Pr2) of *M. flavoviride.* Pr1 and Pr2 activities were high in the medium containing cuticle of South-American grasshopper, *Rhammatocerus schistocercoides* (Rehn) compared to mineral medium with nitrate or casein. Dias *et al*. (2008) suggested an efficient mechanism for the secretion of Pr1 and Pr2. The induction and expression of Pr1 and Pr2 by strain CG425 of *B. bassiana* was influenced by factors such as composition of medium, pH and incubation time. Un buffered minimal medium (MM) supported highest production of PrI (0.88±0.17) compared to MM medium with cuticle (0.65±0.09). In the case of buffered medium, activity of Pr1 (1.17±0.04) was more in MM + cuticle medium compared to MM medium (0.70±0.0).

Chymoelastase serine protease Pr1 was the key enzyme involved in the infection process as evidenced by its high accumulation at the site of penetration peg and believed to be upregulated during appresorium formation and conidiogenesis (St. Leger *et al.* 1987, 1996a; Small and Bidochka, 2005). Extracellular serine protease (Ver112) from the nematophagous fungus *L. psalliotae* was studied by Yang *et al*. (2005)**.** Highest protease activity was observed on the 6th day of incubation (5.7 U mL^{-1}).

Kaur and Padmaja (2009) explained that fungi produce great quantities of Pr1 that degrades the proteinaceous insect cuticle while Pr2 helps Pr1 in the infection process. The solubilised proteins were acted upon by aminopeptidases and exopeptidases to release aminoacids which served as nutrient source for the penetrating fungus. The activity of Pr1 was higher than Pr2 and highest production was observed in the medium containing cuticle, after 24 h of incubation.

Lakshmi *et al.* (2010) noticed that the level of Pr1 and Pr2 of *B. bassiana* (UB9) and *M. anisopliae* (UM4) was high in media supplemented with casein compared to MM with colloidal chitin and MM alone. In *B. bassiana,* Pr1 activity was higher than Pr2 and the values at 72 h after incubation were 28.5±0.01U mL^{-1} and 4.26±0.02 U mL^{-1} respectively. In MM supplemented with colloidal chitin the values were 3.65±0.03 U mL^{-1} and 4.02±0.07 U mL^{-1} respectively.

Perinotto *et al.* (2014) suggested that the Pr1 activity could be used as a biochemical tool for screening the virulence of fungi there by opening up new avenues for genetic modification. Extracellular protease Pr1 and Pr 2 produced by *M. anisopliae* isolate IF 28.2 was evaluated by Ali *et al.* (2011). Pr1 and Pr 2 was more in media supplemented with one per cent cuticle of diamond back moth and glucose. The enzyme activity was 18.83±1.25 and 12.44±1.36 U mL^{-1} h^{-1} respectively. Cito *et al*. (2016) evaluated the key virulent factors of six strains of *B. bassiana* and reported a wide variation in total proteases, Pr1 and Pr 2 activity among the fungal strains. Total protease activity varied from 0.64 to 1.86 mU $mL^{-1,}$ Pr1 from 51.45 to 120.30 mU mL^{-1}, and that of Pr2 from 6.93 to 61.36 mU mL^{-1} after 72 h of incubation.

2.1.1.3.1 Keratinase

Keratinases are a specific class of proteolytic enzymes which catalyse the hydrolysis of keratin, the fibrous protein with a high degree of cross linkages by

disulfide and hydrogen bonds (Gupta *et al*., 2012). It is the structural protein of skin, hair and nails and also the body wall of nematodes. Keratinase is important in the biodegradation of keratinous waste material and the key enzyme in fungal invasion of keratinous cuticle.

Friedrich *et al.* (1999) screened the activity of around 300 fungal species by plate assay and spectrophotometric analysis. About 54 per cent of them expressed keratinase activity on agar plates with soluble keratin as the substrate. Among the fungi *Fusarium*, *Acremonium* and *Geotrichum* were the most active in a medium with porcine nail as the sole source of carbon and nitrogen. Of these *Aspergillus flavus* (Link) (22 mm, 78 mU mL^{-1}), *Alternaria radicina* (Meier) (23mm, 374 U mL^{-1}), *Trichurus spiralis* (Hasselbr) (25 mm, 278 mU mL^{-1}) and *Stachybotrys atra* (Corda) (21 mm, 225 mU mL^{-1}) had high levels of keratinase activity.

Kumar and Kushwaha (2014) evaluated 234 fungal strains for their keratinase producing ability on solid milk agar plates. Highest clearing zone (7 mm) was expressed by *Chrysosporium indicum* (Randhawa and Sandhu). Enzyme activity was measured to be high in *Acremonium strictum* (Gams) in minimal medium with chicken feather as substrate on the 8^{th} and 12^{th} day of incubation respectively (74.40 U mL^{-1} and 124.72 U mL^{-1}). *Lecanicillium* sp. (GPCK 3634) showed moderate activity of 12.15 U mL^{-1} and 35.23 U mL^{-1} on the 8^{th} and 12^{th} day respectively.

2.1.1.4 Chitosanase

Chitosanase is a hydrolytic enzyme catalysing the cleavage of b-1, 4-linked glycosidic linkage of chitosan. Chitosanases exist widly in micro-organisms such as viruses, fungi and bacteria (Alfonos *et al.* 1992; Kim *et al.* 2004; Sun *et al.* 2006). Zhou *et al.* (2008) optimized the production of chitosanase in *Gongronella* sp. JG. Its activity was 800 µmol $min^{-1}L^{-1}$, after 72 h, under optimum conditions. The major chitosanase, csn-1 was purified through

three chromatographic steps: CM (carboxymethyl)–Sepharose fast flow (FF), Sephacryl S200, SP (sulfopropyl) - Sepharose FF. The molecular weight, specific activity and the pH value of csn1 were 90 000 Da, 82 μ mol min^{-1} mg^{-1} and 5.8 respectively.

Singh and Sagar (2017) isolated, purified, and optimized chitosanase production of *A. fumigatus* by optimising physical parameters like pH, temperature, inoculum size, agitation speed and nutritional parameters like carbon and nitrogen sources. Highest chitosanase activity (1.3924 IU) was exhibited by the isolate SK-4.

2.1.1.5 Chitin deacetylase

Chitin deacetylase (CDA) converts chitin into its deacetylated form chitosan, a glucosamine polymer, which is amorphous and easily degraded by chitosanase. Chitin deacetylase hydrolyses the *N*-acetamido bonds in chitin to produce chitosan. It belongs to the family of hydrolases and act on C-N bonds other than peptide bonds in linear amide. CDA activity is reported in fungi (Kauss *et al*. 1983) and in insects (Arachami *et al.* 1986).

Nahar *et al.* (2004) opined that CDA is one of the key enzymes of *M. anisopliae* which is involved in cuticular degradation of gram pod borer *Helicoverpa armigera* (Hübner). It converted cuticular chitin to chitosan and softened the cuticle to facilitate the entry of the pathogen. They assayed the constitutive production of CDA in Yeast extract Peptone Glucose medium (YPG) in the absence of an inducer. CDA was detected 24 h after incubation and the peak activity was observed at 72 h (0.26 U mL^{-1}), which was later reduced to 0. 13 U mL^{-1} by 120 h. In chitin supplemented medium highest production was 0.07± 0.01 U mL^{-1} after 96 h.

The role of CDA in self defence mechanism of fungus was established by (Nahar *et al*., 2004). They assayed the CDA activity of different invading

structures of *M. anisopliae* M 161063. The activity was more in germinating conidia (12 h, 0.02 U mg^{-1}), followed by those in appressoria (24 h, 0.04 U mg^{-1}) and mycelia (24 h, 0.08 U mg^{-1}). It was low in conidia obtained from PDA slant (7 h, 0.005 U mg^{-1}) and lowest in blastospores (24 h, 0.01 U mg^{-1}). The authors concluded that CDA play a role in modifying fungal chitin to chitosan so as to protect itself from the action of insect chitinase.

A native isolate of *A. flavus* from south Kerala was evaluated for CDA activity by Narayanan *et al*. (2016). It was detected by colour reaction of 4-nitroacetanilide in selective agar plates which was further assayed using High-Performance Liquid Chromatography (HPLC). The study revealed an activity to the tune of 9.64±2.04 U in an unoptimized medium whereas under optimal conditions there was 5.98 fold increase.

2.1.1.6 Amylase

Many researchers have demonstrated amylase production in entomopathogenic fungi using plate assay method with starch as the substrate. Fernandes *et al.* (2012) evaluated amylase activity of *B. bassiana, M. anisopliae* and *Purpureocillium* sp. by enzyme indexing. *Purpureocillium* sp. GC 301 and *B. bassiana* CG 470 showed higher H/C index (2) for amylase compared to that of *M. anisopliae* CG 312 with H/C index 1. Sujeeta *et al.* (2017) screened various fungi from different sources for amylolytic activity by starch hydrolysis test using starch agar plate. Amylase hydrolyzing isolates were identified by the clear zones developed by hydrolysis of starch, followed by treatment with iodine solution.

High level of amylase had been correlated with decreased virulence of entomopathogenic fungi. Jackson *et al.* (1985) reported that amylase activity is inversely proportional to the virulence of entomopathogenic fungus. Small quantities of amylase were detected in the most virulent strains of *L. lecanii.* Conversely, Hasan *et al.* (2013) observed high amylolytic production in *L. lecanii* on the seventh day of incubation at pH 3, which was found to decline thereafter.

Barra *et al.* (2015) identified low quantities of amylase in *P. lilacinum* and reported that the activity was high after alkaline and neutral pH at three days of incubation compared to 15 days.

2.2 BIOEFFICACY OF CUTICLE DEGRADING ENZYMES

2.2.1 In Insect Pests

The urge for ecofriendly, sustainable and economically acceptable strategies for pest management encouraged the scientific community to explore the potential of primary and secondary metabolites in entomopathogens.

2.2.1.1 Chitinase

Fungal chitinases were studied for their potential effects and as a powerful biocontrol tool against different pests. Utilization of chitinase for homopteran pest control was reported much earlier by Fawcetts *et al*. (1969). Experiments conducted thereafter, emphasized the potential use of this enzyme in pest management. Saguez *et al.* (2005) evaluated the positive effect of chitinase on green peach aphid *Myzus persicae* (Sulzer) when delivered *via* transgenic potato plants. It was reported to be effective in degrading the cuticle of whitefly *B. tabaci* (Mubarik *et al.,* 2010) and *A. gossypii* (Nicho *et al.,* 2010).

Binod *et al.* (2007) evaluated the importance of fungal chitinase from *Trichoderma harzianum* (Rifai) against *H. armigera* as a tool in enzyme based biopesticide. Lyophilized filtrate had adverse effect on the growth and metamorphosis of the insect. Studies on anti feedent property revealed a clear dose dependent effect on its feeding, pupation and mortality. Moussa *et al*. (2014) studied the potency of chitinase of *B. bassiana* on the aphids *A. craccivora* and *Rhopalosiphum padi* (Linn.) by leaf dip method. The LC_{50} values reported were 24.94 ± 5.21 and 16.17 ± 8.48 ppm, respectively.

2.2.1.2 Lipase

The role of lipase of *M. anisopliae* on the cattle tick *Rhipicephalus microplus* (Canest.) infection was studied by Silva *et al.* (2010). They found that its activity profile enhanced from 0.03 ± 0.00 U to 0.312 ± 0.068 U during the infection process. Addition of purified lipase @ 2.75 U to conidia @ 10^6 spores mL^{-1} enhanced the germination of conidia of *N. rileyi* **(**Sam.) to 98 per cent, whereas it was 86.5 per cent in the treatment without lipase. Topical application of purified lipase along with conidial suspension enhanced action of *N. rileyi* towards *Spodoptera litura* (Fab.) larvae, bringing about 63.3 per cent mortality, 4–10 days post-exposure which was 2.7 times higher than the treatment with conidial suspension alone (Supakdamrongkul *et al*., 2010).

Khan *et al*. (2012) correlated the mortality of green peach aphid *M. persicae* with enzyme activity of *B*. *bassiana* and *L. lecanii.* Among the three isolates screened, culture filtrate of the isolate *L. lecanii* (L3) showed highest mortality of 88.36 per cent compared to the treatment with conidial suspension, where the mortality was 80.70 per cent. Enzyme assay revealed that lipase was predominant than protease and chitinase in total virulence or pathogenicity. The effect of extracellular lipase of *I. fumosorosea* on nymphs of gray pineapple mealybug *Dysmicoccus neobrevipes* (Cock.) was reported by Ali *et al*. (2014). Purified lipase @ 100 U combined with conidial suspension of $1x10^7$conidia mL^{-1} reduced medial survival time ST_{50} to 2.91 ± 0.12 whereas it was 6.37 ± 0.19 days in the treatment with conidial suspension alone.

2.2.1.3 Protease

St. Leger *et al*. (1996 b**)** established the enhanced virulence of genetically modified *M. anisopliae* with additional copies of Pr1 gene, in tobacco horn worm *Manduca sexta* (Linn.). It was reported that Pr1 constitutively over produced the enzyme in the haemolymph of *M. sexta* by activating the prophenoloxidase system. The modified fungus was found to reduce the time taken for mortality, by

25 per cent and food consumption by 40 per cent, compared to those observed in its wild-type fungus.

Kim *et al.* (2010) reported the effect of Pr1 and Pr2 of *B. bassiana* on *A. gossypii*. They attributed the mortality (94.1%) and degradation of the insect cuticle to the presence of cuticle degrading enzymes chitinase, Pr1 and Pr2 which were present in the protein pellet. The structural changes on cuticle were confirmed by transmission electron microscopy.

Keppanan *et al.* (2017) evaluated the mycotoxic effect of partially purified protease of *M. anisopliae* against fourth instar larva of *Galleria mellonella* (L). They reported 90 per cent mortality at 120 h after treatment with two per cent protease.

2.2.2 In Nematodes

Extracellular cuticle degrading enzymes of nematode parasitic fungi play key role in the infection process of nematode egg.

Miller and Sands (1977) studied the key role of hydrolytic enzymes on plant parasitic nematodes. Commercial preparations of chitinase, protease, lipase, papain and collagenase were tested either alone or in combination in stunt nematode *Tylenchorhynchus dubius* (Buet.) Filipjev, and the root lesion nematode *Pratylenchus penetrans* (Cobb) Filipjev and Stekhoven. Chitinase, papain and lipase @ 0.8 mg mL^{-1} water resulted in 100 per cent mortality within 48 h, while papain and chitinase caused around 80 per cent mortality. Collagenase immobilized all the treated *T. dubius* within 24 h.

The activity of egg shell degrading enzymes of *Pochonia chlamydosporia* (Zare and Gams) on *Globodera pallida* (Stone) Behrens, was evidenced by the areas of low electron density around the point of apressoria and penetration hyphae on the infected egg (Tickhonov *et al*., 2002 **)**

2.2.2.1. Chitinase

Chitinase of nematophagous fungi *L. antillanum* (Castañeda and Arnold) Zare and Gams, hydrolyzed the outer egg shell of *Meloidogyne incognita* (Kofoid and White) and degraded the egg shell resulting in transparent and vacuolated eggs (Nguyen *et al.*, 2007). Abbasi *et al.* (2017) reported enzyme activity of 32 fungal isolates infecting eggs of golden cyst nematode *Globodera rostochiensis* (Woll). Among the isolates, highest chitinase activity was exhibited by *T. atroviridae* (0.56 U mg^{-1}) and *Fusarium oxysporum* (Schlec.) (1.02 U mg^{-1}).

2.2.2.2 Protease

Llorca and Robertson (1992) observed that major serine protease produced by *P. rubescens*, was immunolocalized in appressoria of the fungus and facilitated infection process on eggs of *Heterodera schachtii* (Schm.). Segers *et al*. (1996) reported that pre-treatment with purified serine protease of *P. chlamydosporia* were susceptible to the eggs of *M. incognita* and *G. pallida.*. Llorca *et al.* (2002) studied the proteolytic activity of the extracts from conidia and germlings of fungal parasites *such as P. rubescens*, *P*. *chlamydosporia* and *L. lecanii* on *M. javanica*. They observed that the proteolytic activity of *P. chlamydosporia* was nearly six times higher than *P*. *rubescens* and that *L. lecanii* had the lowest activity. Addition of serine protease inhibitors to the fungal extracts was found to reduce the activity.

The nematicidal effect of purified and crude protease of *L. psalliotae* was investigated on vinegar nematode *Panagrellus redivius* (Linn.) Goodey. It was found that the enzyme immobilized and degraded the nematode cuticle within 12 h and caused 81-100 per cent mortality (Yang *et al*., 2005).

2.3 PURIFICATION AND CHARACTERIZATION OF MAJOR CUTICLE DEGRADING ENZYMES

Enzyme purification is generally a multi-step process exploiting a wide range of biochemical and biophysical characteristics of the target enzyme, such as its source, relative concentration, solubility, charge, hydrophobicity, etc. (Ali *et al*., 2010). The common purification steps are ammonium sulphate precipitation, affinity chromatography, and ion exchange chromatography.

2.3.1 Chitinase

Yu *et al*. (2015) purified chitinase from *L. lecanii* through successive column chromatography on phenyl sepharose, DEAE sepharose and CM sepharose. Characterization of the enzyme revealed that chitinases belongs to the family 18 of glycosyl hydrolases with a molecular weight of 42 kDa. The enzyme was purified 18.76 fold with a specific activity of 29.26 U mg^{-1}.

The extracellular chitinase from *L. lecanii* was purified by ammonium sulphate precipitation and DEAE Sephadex A-50 ion exchange chtromatography. The antifungal chitinase showed a molecular mass of 33 kDa with a specific activity of 167.5 U mg^{-1} protein. The optimum temperature and pH for enzyme activity was 40^0C and 6.0 respectively (Nguyen *et al.,* 2015).

2.3.2 Lipase

Microbial lipases of *B. bassiana* was purified and characterized with a molecular weight of 25 kDa (Zibaee *et al*., 2011). The enzyme was 9.91 fold purified with specific activity of 20816.8 ± 762.7 U mg protein $^{-1}$ and recovery of 25 per cent. The purified enzyme was stable for 36 to 72 h. The optimal pH and temperature for highest activity were 7 and 35^0C respectively.

Supakdamrongkul *et al*. (2010) characterised and purified lipase from *N. rileyi*. They could purify the enzyme 23.9 fold with 1.69 per cent yield using

ammonium sulfate precipitation followed by sephacryl S-100 HR column chromatography. The molecular weight of the homogenous lipase was identified as 81 kDa by SDS polyacrylamide gel electrophoresis. The optimum pH and temperature for enzyme activity were 8.0 and 35^0 C, at which it was stable for 1h.

Ali *et al.* (2014) characterized lipase of *I. fumosorosea* through three purifcation steps *viz*. dialysis, DEAE-cellulose chromatography and Sephadex G-100 chromatography. The specific activity of lipase was 72.25 U mg^{-1} proteins with DEAE-cellulose chromatography which increased to 180 U mg^{-1} with Sephadex G-100 chromatography. SDS–PAGE analysis identified lipase with molecular weight of 31 kDa.

2.3.3 Protease

Bidochka and Khachatourians (1987) purified and characterized protease in *B. bassiana* GK 2016 with a molecular weight of 35 kDa which had an optimum activity at pH 5.8 and temperature 35^{o}C. Yang *et al.* (2005) isolated and characterized serine protease (ver 112), from *L. psalliotae* by SDS PAGE which yielded a single band and a molecular weight 32 kDa. Lakshmi *et al.* (2010) charactarised protease of *B. bassiana* (UB9) and *M. anisopliae* (UM4) with molecular weight of 19 kDa and 21 kDa respectively.

Protease isolated from *B. bassiana* AM-118 was purified and characterized by Firouzbakht *et al.* (2015) with molecular weight of 105 kDa for Pr1, and 103 kDa for Pr2.

2.4 MYCOTOXINS OF ENTOMOPATHOGENIC FUNGI

Fungal metabolites exhibit a remarkable spectrum of biological activities such as insecticidal and antimicrobial which offer ample scope to develop them either directly as insecticide molecules or as templates for synthesis of compounds with noval mode of action.

2.4.1 Metabolites of Hypocrealian Entomopathogens

Bioactive compounds of hypocreales fall into the chemical groups such as polyketides, nonribosomal peptides, polyketides- peptide hybrid metabolites, lysine derive alkaloids, terpenoids etc (Table 1)

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Table.1. Review on bioactivity of entomofungal metabolites

Sl.no	Toxins	Fungi	Biological activity	Reference
Polyketides				
1	Oosporein	*Beauveria bassiana,* *B. brongniartii* *Lecanicillium lecanii*	Antiviral, antifungal, immunoregulator	Vining *et al*. (1962) Seger *et al.*(2005)
2	Phomalactone	*Hirsutella thompsonii var.synnematosa*	Antimicrobial, phytotoxic, cytotoxic, insecticidal	Krasnoff and Gupta. (1994)
3	Viridoxin	*M. flavoviride*	Insecticidal	Gupta *et al.(*1993)
4	Norlichexanthone	*P. cinnamomeus*	Cytotoxic	Isaka *et al*. (2007a)
5	Aurovertins D	*M. anisopliae*	Enzyme inhibitor	Azumi *et al*. (2008)
6	PyrenocineA and B	*L. hemipterigenum*	Cytotoxic	Nilanonta *et al*. (2003a)
Non ribosomal peptides				
7	Hirsutellic acid	*Hirsutella* sp	Antimalerial	Thongtan *et al*. (2006)
8	Hirsutide	*Hirsutella* sp	Cytotoxic	Lang *et al*. (2005a)
9	Bassiatin	*B. bassiana*	Cytotoxic	Kagamizono *et al*. (1995)
10	Vertihemiptellides	*L. hemipterigenum*	Antimycobacterial	Isaka *et al*. (2005a)
11	Leucinostatin (Paecilotoxins)	*P. lilacinum*	Cytotoxic	Arai *et al*. (1973)
12	Diketopiperazines I and II	*L. hemipterigenum*	Antimycobacterial	Isaka *et al*. (2005a)
13	Beauverolides	*B. bassiana,* *P. fumosoroseus*	Immulnosuppressive	Elsworth *et al*. (1980)

14	Hirsutatin A and B	*H. nivea*	Antiplasmodial	Isaka *et al* (2005b)
15	Paecilodepsipeptide A	*P. cinnamomeus*	Antiplasmodial	Isaka *et al.*(2007b)
16	Hirsutellide A	*H. kobayasil*	Antimycobacterial	Vongvanich *et al*. (2002)
17	Beauvericin	*B. bassiana, P. tenuipes*	Insecticidal, antifungal, antibacterial	Hamill *et al.* (1969)
Ribosomal peptides				
18	Enniatins	*L. hemiptererigenum*	Antibiotic, anti fungal, antihelmenthic	Nilanonta *et al*. (2003b)
19	Bassianolide	*Beauveria bassiana* *Lecanicillium lecanii*	Insecticidal	Xu *et al*. (2009) Keppanen *et al*.(2018)
20	Paecilosetin	*Paecilomyces farinosus*	Antimicrobial	Lang *et al*. (2005)
21	Bassianin	*B. bassiana*	Cytotoxic	Wat *et al.* (1977)
22	Tenellin	*B. bassiana*	Cytotoxic	Wat *et al.* (1977)
23	Destruxin	*M. anisopliae*	Insecticidal, cytotoxic, antibiotic	Roberts (1969)
24	Efrapeptins G	*Tolypocladium niveum*	Insecticidal, cytotoxic, antifungal, antibacterial	Krasnoff *et al*. (1991)
25	Verticilide	*Lecanicillium* sp	Insecticidal	Shiomi *et al.* (2010)
26	Hirsutellone	*Hirsutella* sp	Insecticidal	Isaka *et al.* (2005)
27	Bassiacridin	*B. bassiana*	Insecticidal	Moraga and Vey (2004)
Terpenoids				
28	Aphidicolin	*Lecanicillium , Cephalosporium aphidicola*	Cytotoxic	Claydon and Grove (1982)

29	Metarhizin A and B	*M. flavoviride*	Cytotoxic	Azumi *et al. (*2008)
Organic acids				
30	Fusaric acid	*Fusarium solani*	Insecticidal	Claydon *et al.* (1977)
31	Oxalic acid	*B. bassiana, P. farinosus*	Insecticidal	Claydon and Grove (1982)
32	Vertilecanin	*L. lecanii*	Insecticidal	Soman *et al*. (2001)
33	Dipicolinic acid	*P. fumosoroseus* *L. lecanii*	Insecticidal	Asaff *et al*.(2005) Claydon and Grove (1982)

2.4.2 Toxins of *Lecanicillium*

The genus *Lecanicillium* had been exemplified as a good source of novel compounds, the important ones and their role in entomopathogenicity are briefly reviewed below.

2.4.2.1 Bassianolide

Bassianolide is a cyclotetradepsipeptide. Kanoka *et al*. (1978) isolated the toxin bassianolide from the mycelia of *L. lecanii* and *B. bassiana* which was reported to cause muscular atony in silk worm larvae by inhibiting acetyl choline mediated muscle contraction. The entomotoxicity of bassionolide was attributed to its ionophoric nature which were exteriorly hydrophobic and interiorly hydrophilic. Xu *et al.* (2009) reported bassianolide as a highly significant virulence factor in *B. bassiana* and *L. lecani.*

2.4.2.2 Dipicolinic acid

Dipicolinic acid (DPA) or pyridine-2, 6-dicarboxylic acid, is a lysine derived metabolite produced by entomopathogenic fungi under different genera. DPA was reported as the principal insecticidal metabolite in *L. lecanii* by Claydon and Grove (1982). Its presence was also reported in *B. bassiana, P. farinosus* and *P. fumosoroseus, Penicillium* sp., (Asaff *et al*., 2005*)* and *Cordyceps militaris* (Linn.) (Watanabe *et al*., 2006). In bacteria it was reported to impart thermal resistance to the spores (Fichtel *et al*., 2007). Paterson (2008) reported that it interferes with the innate immune system of insects by inhibiting the prophenoloxidase system during melanin biosynthesis.

2.4.2.3 Oosporein

Oosporein is a red-coloured pigment of dihydroxybenzoquinone under the group of non reduced polyketides. Oosporein was first reported as a dye from endophytic fungus *Oospora colorans* (Beyma) by Kogl and Wessem in 1944. It was then described as a mycotoxin from different entomopathogenic fungi *viz. B. bassiana* (Vining *et al.,* 1962), *Chaetomium trilaterale* (Ames) (Cole *et al*., 1974) and *L. psalliotae* (Nagaoka *et al*., 2004). It acted as a virulent factor in *B. bassiana* by inhibiting host immunity and facilitating propagation in insects.

Oosporein was reported to be an inhibitor of Ca dependant ATPase of erythrocyte membrane at relatively high concentrations of 200 µg mL^{-1}. Immune interference studies by Feng *et al*. (2015) revealed that injection of oosporein inhibited prophenoloxidase (PPO) activity and down-regulated the antifungal peptide gallerimycin gene in insects following activation by fungal spores. Fan *et al.* (2017) reported that oosporein protected the host cadaver from bacterial infection and helped the fungus to utilize the host nutrients and complete its life cycle. It also exhibited antimicrobial and growth inhibitory effect against phyto pathogenic fungus *Phytophthora infestans* (Mont.) de Bary.

2.4.2.4 Vertilecanin A

It is reported as a phenopicolinic acid analogue from *L. lecanii* with a molecular mass of 229.07 and formula $C_{13}H_{11}O_3N$ (Soman *et al.*, 2001). The other secondary metabolites identified from *L. lecanii* were vertilecanin A methyl ester, vertilecanin B, vertilecanin B methyl ester, and vertilecanin C, which were determined by and NMR analysis. Among the different analogues, vertilecanin was the potent insecticidal compound.

2.4.2.5 Verticilide

It is a cyclic depsipeptide of cyclo [(2R)-2-hydroxyheptanoyl-N-methyl- L-alanyl] isolated from *Lecanicillium* strain FKI-1033 (Shiomi *et al*., 2010). The unique structure of small side-chain amino acids and very long, straight side-chain hydroxy acids, contributed to the interesting biological activity of this compound. The compound inhibited ryanodine binding to ryanodine receptors in cockroach at an IC_{50} value of 4.2 µM whereas, inhibition against mouse ryanodine receptors was weak (IC_{50}=53.9 µM). Biopotency of the molecule was studied on brine shrimp *Artemia salina* (Linn.), and the free living nematode *Caenorhabditis elegans* (Maupas) by microplate assay. The minimum growth inhibitory concentration against *C. elegans* and *A. salina* were 20 µg mL^{-1} each.

2.4.2.6 Aphidicolin

It is an important tetracyclic diterpenoid isolated from *L. lecanii* by Claydon and Grove (1982) and from *Cephalosporium aphidicola* (Petch) by Hanson *et al.* (1992). They reported that it exhibited potent cytotoxicity by inhibiting eucaryotic DNA polymerase.

2.4.2.7 Beauveriolide

Beauveriolide belong to the family of cyclic tetradepsipeptides with 3 hydroxy-4- methyl alkanoic acid units. It exhibited moderate insecticidal property against leaf caterpillar *S. litura* and pulse beetle *Callosobruchus chinensis* (Linn.), (Mochizuki *et al.,* 1993).

2.4.2.8 Verlamelin

Ishidoh *et al.* (2014) elucidated the structure of two antifungal compounds *viz*. Verlamelin A and B from *L. lecanii* for the first time. It is a cyclic

lipodepsipeptide [cyclo(5S-hydroxytetradecanoicacid-D-alloThr/Ser-D-Ala-L-Pro-L-Gln-D-Tyr-LVal)]. The molecular formula of verlamelin A was identified as $C_{45}H_{71}N_7O_{11}$ with m/z 886.52 and verlamelin B as $C_{44}H_{69}N_7O_{11}$ with m/z 872.5140. Bio activity of both the compounds revealed equal potency against the plant pathogens *Cochliobolus miyabeanus* (Ito and Kuribayashi) and *Alternalia solani* (Ell and Mart.). However, verlamelin B was less active against *Fusarium oxysporum* (Schlecht), *Cladosporium cucumerinum* (Ellis and Arthur) and *Ustilago maydis* (DC) Corda.

2. 4. 3 Isolation of Toxins from *Lecanicillium*

During the infection process, entomopathogenic fungi produce toxins in host insects which hamper their immune system and act as insecticidal molecules. They also produce secondary metabolites, when cultured in specific nutrient rich medium which can be retrieved from the culture filtrate and mycelia.

Kanaoka *et al*. (1978) extracted bassianolide, an insecticidal cyclodepsipeptide for the first time from *L. lecanii* cultured in Czapek Dox medium supplemented with two per cent yeast. The toxin was extracted from mycelia using methanol and purified through different chromatographic techniques.

Clydon and Grove (1982) evaluated the secondary metabolite production in seven strains of *L. lecanii* and found that all the strains except one produced dipicolinic acid (DPA) the most active principle responsible for insecticidal acivity of the fungal extract. They extracted mycotoxins such as aphidicolin and DPA from fungal culture grown in Czapek Dox medium and were identified through NMR analysis. Each strain was found to vary in terms of quantity and duration of production of DPA.

Many workers have attempted to isolate oosporein, a highly reactive compound produced by entomopathogenic fungi. Strasser *et al.* (2000) extracted oosporein from *B. brongniartii* (Sacc.) Petch multiplied in barley kernals. Nagaoka *et al*. (2004) isolated oosporein from *L. psalliotae* cultured in potato dextrose broth.

P. fumosoroseus cultured in nutritive medium suggested by Fargues *et al.* (1992) produced DPA and was the most abundant metabolite (0.041 g L^{-1}) (Asaff *et al*., 2005). DPA was extracted from culture filtrate using solvents of varying polarity and was detected through HPLC and confirmed by NMR analysis.

The phenopicolinic acid analogue, vertilecanin was isolated from *L. lecanii* grown under solid state fermentation by Soman *et al.* (2001). Extraction of the fermented rice substrate with ethyl acetate followed by fractionation through sephadex LH-20 column chromatography and semipreparative reversed phase HPLC revealed five acid phenopicolinic derivatives such as vertilecanin A (**1**), vertilecanin A methyl ester (2), vertilecanin B (3), vertilecanin B methyl ester (4), and vertilecanin C(5). Among these compounds, vertilecanin A (1) was the most effective and potent compound.

L. lecanii grown in Czapek dox medium supplemented with 0.5% bactopeptone yielded bassianolide (Keppanan *et al.,* 2018). The active toxin was extracted from the mycelia through series of purification steps including column chromatography, and preparative HPLC. The molecular weight and purity of the compound was asertained by FT-IR and NMR.

Recent advances in analytical techniques facilitated easier separation, identification and structural determination of biomolecules. GC-MS is a powerful tool for the identification and quantification of volatile molecules, based on their retention indices and mass spectral fragmentation patterns.

Many researchers reported secondary metabolites of entomopathogenic fungi with different bioactivity (Moraga and Vey, 2004; Asaff *et al.,* 2005; Shiomi *et al.,* 2010). GC-MS analysis of ethyl acetate fraction of mycelia of *B. bassiana* identified n-hexadecanoic acid, 9, 12, octadecadienoic acid, squalene, and octadecanoic acid (Ragavendran *et al*., 2017). Vivekanandhan *et al*. (2018) reported hexadecanoic acid from mycelia as the major compound of pathogenicity of *B. bassiana* 28. Ragavendran *et al*. (2019) identified mosquitocidal compounds such as 1-octadecene, 1-nonadecene, 9-octadecenoic acid and cyclobutane through GC-MS analysis of *Penicillium* sp. GC-MS analysis of the methanolic extract from *Cladosporium cladosporioides* (Fresen) yielded 26 compounds and while that from *P. lilacinum* yielded 19 compounds. The most bioactive compounds were linoleic acid and palmitic acid (Elbanhawy *et al*., 2019).

2.4.4 Bioefficacy of Mycotoxins

2.4.4.1 In Insects

Kanaoka *et al*. (1978) studied the toxicity of bassianolide from *L. lecanii* by oral administration and injection to silk worm larvae. Oral administration of bassianolide through diet @ 4 ppm developed atony symptom and a dose above 8 ppm was lethal. Injection of the active compound in water at a dose of 2µg resulted in atonic symptom and was lethal at a dose above 5µg/larva.

Gindin *et al*. (1994) investigated the toxicity of methanolic extract of *L. lecanii* against*, B. tabaci.* Crude mycelial toxin exhibited different levels of mortality to all stages of *B. tabaci*. The mortality rate observed at 0.5 % concentration was 90 per cent and with 0.1 % it was only 33.6 per cent.

Investigations of Rani (2000) revealed the bioefficacy of crude toxin extracted from entomopathogenic *F. pallidoroseum* (Cook) Sacc. The studies

revealed that the toxin at 500 and 1000 ppm caused 46 and 100 per cent mortality respectively to *A. craccivora* at the end of 72 HAT.

Soman *et al.* (2001) evaluated the insecticidal activity of vertilecanin isolated from *L. lecanii* and recorded a reduction of 49 per cent weight gain in *H. zeae* at a concentration of 1500 ppm. Gurulingappa *et al*. (2011) revealed the insecticidal potential of metabolites in the mycelial and ethyl acetate extract of *L. lecanii* and *B. bassiana* against *A. gossypii*. Mortality ranging from 24 to 82 per cent and 45 to 97.5 per cent were observed with the methanolic fractions of mycelia of *L. lecanii* and *B. bassiana* respectively at 0.25 to 2.0 % concentrations. Shiomi *et al*. (2010) reported verticilide a new ryanodine binding inhibitor from *Lecaniicillium* sp. FKI-1033. They reported that verticilide inhibited ryanodine binding to ryanodine receptors in cockroach at an IC_{50} value of 4.2 µM whereas in mouse the binding was weak with IC_{50} of 53.9 µM.

The role of insecticidal toxins from two strains of *L. lecanii* strain V3450 and Vp28 were evaluated by Wang *et al.* (2007). They found that all the stages of *B. tabaci* were susceptible to both the toxins, with varying level of toxicity. LC_{50} for ovicidal activity was 447 and 629 mg L^{-1} for V3450 and Vp28 respectively. The nymphal stage was the most susceptible with LC_{50} values 111 mg L^{-1} and 216 mg L^{-1} for toxins V3450 and Vp28, respectively.

Keppanan *et al.* (2018) evaluated the toxicity of bassianolide from *L. lecanii* against third instar larvae of *P. xylostella*. Highest concentration of 0.5 mg mL^{-1} caused mortality of 88.8 ± 9.6 per cent at 120 h whereas, the mortality was 72.16 ±9.6 per cent was observed with 0.1 mg mL^{-1} of toxin.

Elbanhawy *et al*. (2019) investigated the toxicological effects of secondary metabolites from entomopathogenic fungi. The crude toxin from mycelia and spores

of *C. cladosporioides* and *P. lilacinum* exhibited highest insecticidal effect on adults of *A. gossypii* with a LC_{50} value of 57.60 and 94.18 ppm respectively at 24 h.

Sivakumar *et al*. (2011) reported the larvicidal and repellent activity of pure tetradecanoic against *Aedes aegypti* (Linn.) and *Culex quinquefasciatus* (Say). The LC_{50} values were 14.08 and 25.10 mg mL^{-1}. N-hexadecanoic acid from *B. bassiana* showed insecticidal activity against first, second, third and fourth instar larvae of *Anopheles stephensi* (Liston), *C. quinquefasciatus* and *A. aegypti* (Ragavendran *et al.,* 2017). Secondary metabolites of *B. bassiana* 28 showed strong mosquitocidal activity against larval and pupal stage of *C. quinquefasciatus.* They opined that the insecticidal property was due to the major secondary metabolites. N-hexadecanoic acid, Z, Z-9,12 dectadecadienoic acid 9, eicosone, heptacosane and tetrateracontane - -7- hexyleicosane (Vivekanandhan *et al*., 2018).

2.4.4.2 In Nematodes

Nematophagous fungi are well known for their toxic metabolites which are antagonistic to plant-parasitic nematodes. Toxin-producing fungi release nematicidal metabolites to attack and immobilise nematodes (Dong *et al*., 2006).

Many compounds were reported to have nematostatic property. Acetic acid produced by *P. lilacinum* immobilizes juvenile parasitic nematodes (Djian *et al.* 1991). Li *et al*. (2007) described 179 nematicidal compounds from fungi under deuteromycetes, ascomycetes and basidiomycetes. Latest in this category include, monocerin and deoxyphomalone from *Pseudobambusicola thailandica* (Rupcic) against nematodes (Rupcic *et al.,* 2018). Among nematophagous fungi, *Fusarium* and *Purpureocillium* were important genera from which toxins were isolated. However, literature on secondary metabolites from entomopathogenic fungi with nematicidal activity is meagre.

Ciancio (1995) evaluated toxins produced by *Fusarium* such as T2-toxin, moniliforrnin, verrucarin A and cytochalasin B at concentrations of 0.02, 0.2, 2, 20, and 200 ppm in 4 % methanol against *M. javanica.* All the toxins reduced the viability of juveniles at 2 ppm or higher concentrations after 72 h of exposure. T2-toxin exhibited nematicidal effect on *M. hapla* and *P. neglectus* at 0.2 and 2 ppm, respectively.

Liu *et al.* (2009) isolated and characterized a novel nematicidal compound 4-(4-carboxy-2-ethyl-hydroxypentyl)-5,6,-dihydro-6-ethylcyclo buta [b]-pyridine-3,6-dicarboxylic acid from *Paecilomyces* sp. LD_{50} at 24 h was 50.86, 47.1 and 167.7 mg L^{-1} against *P. redivivus, M. incognita* and *B. xylophilus* respectively. Bogner *et al*. (2016) isolated and demonstrated the potential of nematicidal compounds of *F. oxysporum.* Three most potent nematode-antagonistic compounds were 4-hydroxybenzoic acid, indole-3-acetic acid (IAA) and gibepyrone D and LC_{50} values of these compounds were 104, 117 and 134 gm L^{-1}, respectively, after 72 h. Shimada *et al.* (2010) observed nematicidal activity for beauvericin extracted from *Fusarium bulbicola* (Nirenberg & O'Donnell) against pine wood nematode *B. xylophilus* (46%) at a concentration of 1 mM, and weak nematicidal activity against free living nematode *Caenorhabditis elegans* (Maupas) at concentrations of 0.1 mM and 1 mM.

2.4.4.3 Adverse Effect of Mycotoxin to Predators

Effect of secondary metabolites produced by various entomopathogenic fungi on coccinellid predators was reported by many researchers. Toxicity of *L. lecanii* on whitefly predator, *Axinoscymnus cardilobus* (Pang and Ren) was studied by Xu *et al*. (2009) under laboratory conditions. They reported that there was only five per cent reduction in number of eggs (126.2 ± 34.85) in beetles treated with 1×10^{6} conidia mL^{-1} as against 133.2±32.18 in control. No significant difference was

observed in the per cent survival of any of the immature stages, mean generation time, intrinsic rate, finite rate of increase and longevity of the predator. The study concluded that *L. lecanii* was compatible with the predator for biological control of whitefly.

Wang *et al.* (2005) opined that application of *L. lecanii* or its toxins should be avoided when the whitefly predator *Delphastus catalinae* (Horne) is present in the field. Crude toxins of two strains of *L. lecanii*, V 3450 and Vp 28 were evaluated for their sublethal toxicity and their effect on consumption and functional response of the predator. The crude toxins expressed low toxicity to larval stage and LC_{50} was about 10 and 12-fold of the field rate (200 ppm) whereas for adult beetle the values were approximately 20 and 22 fold. The consumption and foraging capacity were significantly impaired in the second-instar larvae exposed for a longer time, but fecundity and longevity, were unaffected.

Wu *et al*. (2014) tested various isolates of *B.bassiana* for their virulence against *Frankliniella occidentalis* (Pergande) and its predator *Neoseiulus (Amblyseius) barkeri* Hughes. The potent strain SZ-26 showed high toxicity against the pest but no pathogenicity to the predatory mite.

2.4.5 Mode of Action of Metabolites

Entomopathogenic fungi are potential resource of biologically active metabolites. *In vitro* screening of these metabolites to identify a novel insecticidal molecule is a cumbersome process. Hence structure based virtual sreening is the most widely used strategy to identify the most promising compound for biological assay.

In silico molecular docking analysis is a powerful tool for the study of structure-activity relationship between two molecules with prediction of their

interaction (Hughes *et al*., 2011). The binding affinity and the 3D structure of the complex obtained designate the degree of interaction between them.

Keppanan *et al.* (2017) carried out *in silico* analysis to study the interaction between protease of *M. anisopliae* and its toxic effect on host protein, moricin like peptide C4. The energy and thermal stability of protease was more upon interaction, revealing the fact that protease get bound with the host protein naturally and subsequently degrading it. The same authors in 2018 carried out docking studies between defense protein of diamondback moth as the receptor and bassianolide as the ligand molecule. They found that bassianolide interacted with the target receptor protein and this interaction enhanced the binding energy and thermal stability of the compound. They could therefore confirm the interaction of bassianolide with the receptor protein of insect.

Such docking studies are more with plant metabolites rather than those of fungal metabolites. However it is an essential component in modern agrochemical industry to identify a potent molecule with specific mode of action.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

The research work entitled "Mycotoxins and enzymes of entomopathogenic fungus *Lecanicillium saksenae* (Kushwaha) Kurihara and Sukarno and their bioefficacy on crop pests" was carried out at the Biocontrol Laboratory for Crop Pest Management, Department of Agricultural Entomology, College of Agriculture, Vellayani and AINP on Vertebrate Pest Management Laboratory, Department of Agricultural Entomology, College of Horticulture, Vellanikkara during 2016-2019.

3.1 DETECTION AND ACTIVITY OF CUTICLE DEGRADING ENZYMES OF *L. SAKSENAE*

3.1.1 Source and Maintenance of Fungal Culture

L. saksenae (ITCC Ls.Vs.1 -7714) isolated from cultivated soils of Vellayani Thiruvanthapuram, Kerala in 2015 and maintained in Biocontrol Laboratory for Crop Pest Management, Department of Agricultural Entomology, College of Agriculture, Vellayani was utilized for the study. The fungus was sub cultured periodically on Sabouraud Dextrose Agar (SDA) slants at 27^0C and maintained at 4^0C. The culture was revived by periodical passage through the susceptible host, brinjal mealybug *Coccidohystrix insolita* (G).

3.1.2 Preparation of Spore Suspension

Fungal plug of 5 mm diameter of *L. saksenae* was inoculated using a cork borer at the centre of SDA plates and incubated at 27^0C. Fourteen day old cultures were used for the preparation of conidial suspension. The Petri plates were flooded with three mL sterile distilled water containing Tween 80 (0.03%) and the conidial suspension was collected. It was vortexed for one min and filtered through sterile

cheese cloth. Conidial concentration was determined as 10^7 conidia mL^{-1} under a compound microscope using Neubauer Haemocytometer.

3.1.3 Qualitative Assay

The extracellular cuticle degrading enzymes of *L. saksenae* were detected qualitatively by plate assay method. Enzyme activity of chitinase, lipase, protease, chitosanase, and amylase were detected on the basis of development of halo around fungal colony whereas, chitin deacetylase was detected by colour reaction using pH indicator medium. In addition to halo, chitinase was also detected by colour reaction of the medium.

Enzyme activity of *L. saksenae* was calculated based on the total diameter of colony with clear zone and diameter of colony.

$$\text{Enzyme activity index} = \frac{\text{Total diameter of colony + clear zone}}{\text{Diameter of colony}}$$

A value greater than one denotes the extracellular activity of the fungus (St. Leger *et al*., 1997)

3.1.3.1 Chitinase

Chitinase was detected by plate assay method using colloidal chitin as substrate. It was also detected by colour reaction using pH indicator medium of pH 4.7 with bromocresole purple as dye and colloidal chitin as substrat (Appendix I.1) . On the basis of colour rating chitinase activity was rated (Agrawal and Kotasthane, 2012).

3.1.3.1.1 Preparation of Colloidal Chitin

Colloidal chitin was prepared as per the procedure of Roberts and Selitrennikoff (1988). About 250 mL of ice cold concentrated HCl was added slowly into 40 g of chitin (HiMedia) at 4ºC with vigorous stirring for 1 h. The mixture was filtered through glass wool and the filtrate was collected into two litre ice cold water under stirring using a magnetic stirrer. The gelatinous white material in the filtrate was separated using Whatman No.1 filter paper. Colloidal chitin thus obtained by filtration was washed repeatedly in tap water until the pH turned neutral. It was stored at 4^0C for future use.

Fungal plug of 5 mm diameter from seven day old culture of *L. saksenae* was inoculated at the centre of each of the two types of chitinase detection plates and incubated at 27^{o}C for nine days. Three replications were maintained and the medium without colloidal chitin served as control.

In the first set of plates the diameter of the fungal colony and clear zone or halo around the colony was measured for a period of nine days in two dimensions at 90° to each other and the average was calculated. The second set of plates were observed for purple zone formation around the inoculated culture. Based on the relative increase in diameter as well as intensity of the colour the enzyme activity was classified into different groups low, medium, high and highest.

3.1.3.2 Lipase

Lipase was detected by the procedure of Hankin and Anagnostakis (1975) using minimal medium of pH 7 amended with Tween-20 as substrate (Appendix I.2). Inoculated plates were incubated at 28 $\pm$1^o C for nine days. Diameter of the colony and the zone of precipitation were measured for a period of nine days. Three replications were maintained and the plates without Tween 20 served as control.

3.1.3.3 Protease

Protease was detected by plate assay method suggested by Aneja (2007) using casein agar medium (Appendix I.3). Assay plates were inoculated with seven day old fungal plug and incubated for nine days at 25^0C. Diameter of the fungal colony and the halo around the colony was measured for a period of nine days as in 3.1.3.1. Three replications were maintained for observation and the plates without casein served as control.

3.1.3.4 Chitosanase

Chitosanase was detected by plate assay method suggested by Zhou *et al*. (2008) using minimal medium with colloidal chitosan as substrate (Appendix I.4).

3.1.3.4.1 Preparation of Colloidal Chitosan

Colloidal chitosan was prepared as per the procedure suggested by Fenton and Eveleigh (1981). Ten grams of powdered chitosan (HiMedia) was slowly added into 0.2 N HCl with continuous stirring. The pH of the resultant mixture was adjusted to 5.5 using 0.2 N NaOH. Colloidal chitosan was formed as a white gelatinous material and was separated by filtration through Whatman No.1 filter paper and stored at 4^0C for future use. Chitosanase detection plates were inoculated and incubated at 37^0C for nine days. The diameter of the fungal colony and halo around the colony was measured as in 3.1.3.1. Three replications were maintained and the plates without colloidal chitosan served as control.

3.1.3.5 Chitin deacetylase

Chitin deacetylase activity (CDA) was determined by visualization of colour change of medium amended with colloidal chitin (Naryanan *et al.,* 2016). Detection plates were prepared using CDA selective agar medium with 4-nitroacetanilide and

1.0 per cent colloidal chitin as the sole carbon source at pH 7.2 (Appendix I.5). Seven day old culture was inoculated on CDA detection plates and incubated at 30°C for nine days. Chitin deacetylase production was assessed by the colour reaction of 4-nitroacetanilide. Three replications were maintained and plates without colloidal chitin and 4-nitroacetanilide served as control.

3.1.3.6 Amylase

Amylase plate assay was conducted in minimal medium with starch as the carbohydrate source with pH 6 (Kaur and Padmaja, 2009) (Appendix I.6) The plates were inoculated with, seven day old fungal plug of 5 mm diameter and incubated at 28^{o}C for nine days. Colony diameter was measured on the 8th day and plates were flooded with iodine reagent (65 mg iodine crystals and 130 mg KI in 100 mL sterile distilled water) for 30 s. Then the reagent was drained and the clearing zone developed immediately around the colony was measured. Three replications were maintained and plates without starch served as control.

3.1.4 Quantitative Assay

The cuticle degrading enzymes detected in plate assay studies were quantified as detailed in the procedure below, except for chitin deacetylase due to the non availability of substrates for the enzyme assay.

3.1.4.1 Chitinase Assay

Chitinase activity was assessed based on the estimation of reducing sugars released during the hydrolysis of colloidal chitin (Chai *et al.* 2012).

L. saksenae was cultured in 250 mL Erlenmayer flasks with 50 mL of two different media *viz*. Enzyme Producing Medium (EPM) (Appendix I.7) with colloidal chitin as substrate and Sabouraud Dextrose Broth (SDB). The media were

sterilized at 120^0C for 20 min and inoculated with a spore suspension of $1x10^7$ mL^{-1} and incubated in a shaker at 200 rpm and 28°C for 14 days. Samples for enzyme analysis were drawn from second day onwards at two day intervals till 14^{th} day. Aliquots of five mL were collected aseptically and were centrifuged at 10000 rpm for 10 min at 4^0C. It was then filtered through 0.45 µm-pore-size filter (Millipore Corp) fitted on a 10 mL glass syringe. The supernatant served as the enzyme source.

Reaction mixture consisted of 125 µl each of the supernatant, 5 % (w/v) colloidal chitin and 0.1M acetate buffer of pH 5.0. Enzyme control for the assay consisted of the reaction mixture devoid of colloidal chitin whereas reaction mixture without enzyme source served as substrate control. The mixtures were incubated at 37°C for 20 min. The amount of reducing sugars liberated was estimated by the procedure suggested by Miller (1959) using dinitrosalysilic acid (DNS). The reaction was terminated by the addition of 250 µL of 1% (w/v) DNS reagent. The mixture was then kept in a water bath at 100^0 C for 10 min. After cooling five mL of distilled water was added to the reaction mixture. Three replications were maintained for the assay. The absorbance was measured at a wavelength of 540 nm in UV-VIS spectrophotometer.

Standard graph was plotted with different concentration of N-acetyl glucosamine (NAGA) (Sigma-Aldrich) under same conditions as that of samples (Fig. 1). One unit (IU) corresponds to the amount of enzyme that hydrolyzes 1 µmole of NAGA per min at 37°C. The chitinase activity was calculated by the formula

$$\text{Units mL}^{-1}\text{ enzyme} = \frac{(\mu\text{ mole NAGA released})\ (\text{Total volume of assay})}{V_1 x\ t\ x\ V_2}$$

Where,

V_1 - Volume of enzyme used in assay
V_2 - Volume used in colour development
T - Time of analysis

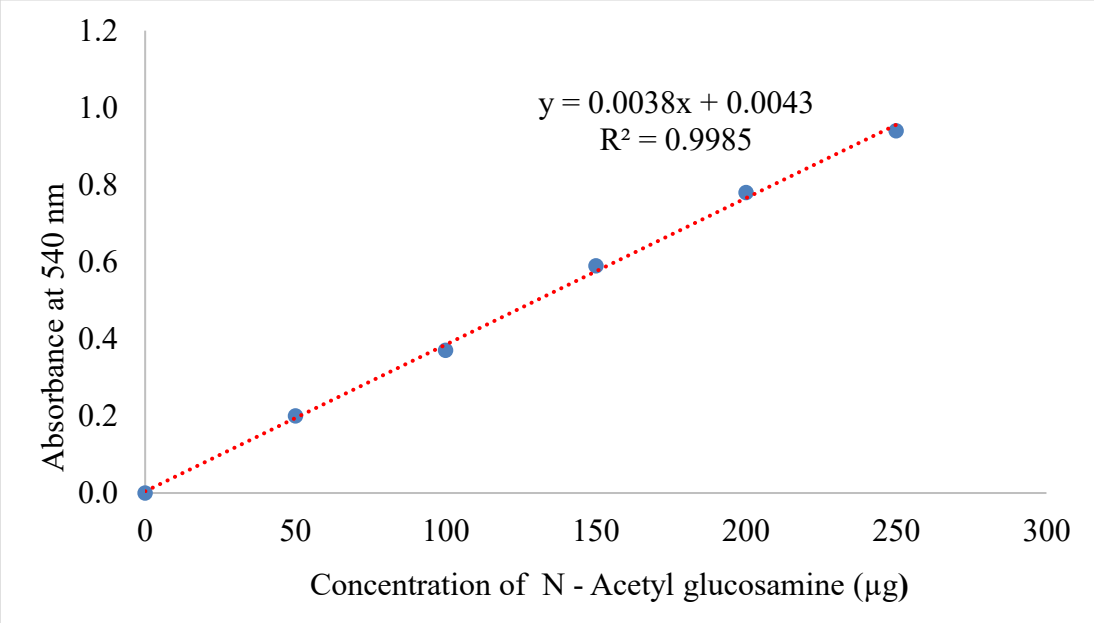


Fig. 1 Standard curve of N - Acetyl glucosamine

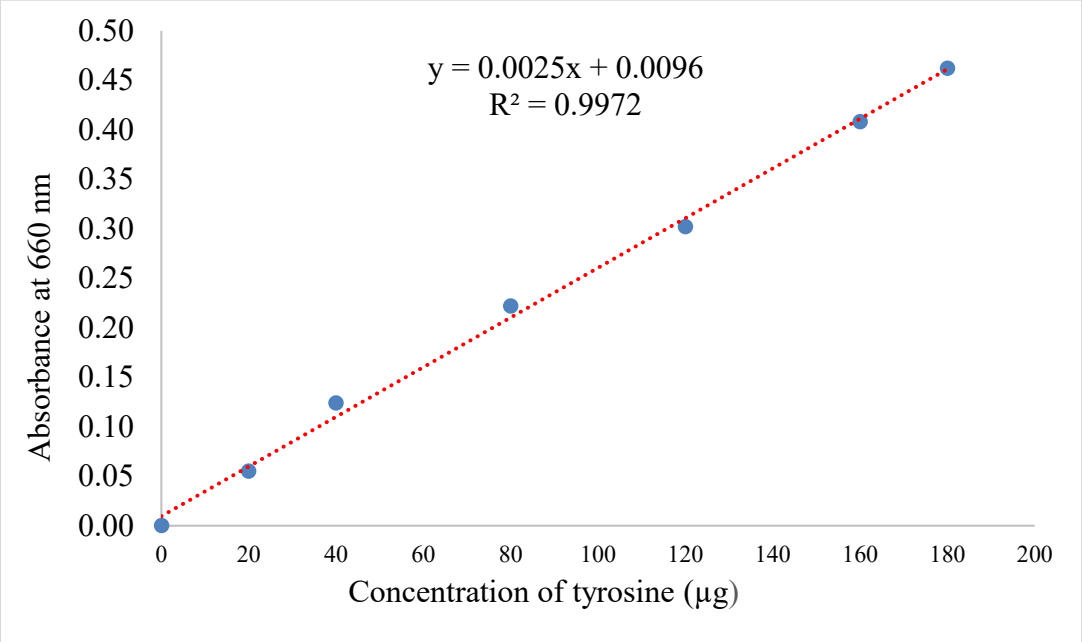


Fig. 2 Standard curve of tyrosine

3.1.4.2 Lipase Assay

The fungus was cultured in EPM (Appendix I.7) and samples for enzyme assay were drawn as described in para 3.1.4.1. Lipase activity was measured by titrimetric assay with 0.05N NaOH using emulsified olive oil as substrate (Kamimura *et al.,* 1999).

Reaction mixture consisted of one mL of enzyme, five mL emulsion containing 25 per cent (v/v) olive oil and 75 per cent (v/v) gum arabic and two mL of 10 mM phosphate buffer at pH 7. The reaction mixture was incubated at 37°C for 30 min. The reaction was stopped by the addition of 15 mL acetone–ethanol (1:1v/v). The amount of fatty acids liberated was titrated against 0.05 % NaOH using phenolphthalein as indicator. End point of the reaction was determined by the change in colour of the titrate from light yellow to pink at pH 9, which was ascertained using a pH paper. Each flask served as a replicate and three replications were maintained per treatment. One unit of lipase is defined as the amount of enzyme that released 1µmole of fatty acids per min under these conditions. Lipase activity was calculated by the formula

$$U\ mL^{-1}\ min^{-1} = \frac{Tv\ of\ NaOH\ (sample) - Tv\ of\ blank \times Normality\ of\ NaOH\ (N) \times 1000}{Time\ of\ incubation\ (min) \times Volume\ of\ enzyme\ solution\ used\ (mL)}$$

Where Tv- Titer value

3.1.4.3 Total Protease Assay

The fungus was cultured and samples were drawn as described in para 3.1.4.1. Total proteolytic activity was estimated as per the modified procedure of Hossain *et al.* (2006). The reaction mixture contained one mL of culture filtrate, three mL of 1% (w/v) casein and three mL 0.1M citrate-phosphate buffer, pH 7.0. One mL of crude enzyme mixed with three mL of 0.1M citrate-phosphate buffer at

pH 7.0 served as enzyme control whereas three mL of 1% (w/v) casein mixed with three mL of 0.1M citrate-phosphate buffer, pH 7 acted as substrate control. The reaction mixtures were incubated at 40 ± 1°C for 1 h. The reaction was stopped with 2% (w/v) trichloro acetic acid (TCA) followed by centrifugation at 12000 rpm for 10 min. Supernatant was collected and aliquots of 1.4 mL each were transferred to test tubes containing two mL of 0.4 M $NaCO_3$. To this mixture, 200 µl of 0.5 N Folin Ciocalteu's phenol reagent was added and incubated in dark at 37°C for 30 min. The absorbance was measured at a wavelength of 660 nm. Three replications were maintained for the assay.

A standard graph was plotted at different concentrations of tyrosine under same conditions as that of samples (Fig. 2). One unit (IU) of total protease was equated to the amount of enzyme that hydrolyzed 1 µ mole of tyrosine per min at 37°C. Protease activity was calculated by the formula

$$\text{Protease activity (Unit mL}^{-1}\text{min}^{-1}) = \frac{\mu \text{ mol of tyrosine released x } V_1}{V_2 x\ t\ x\ V_3}$$

Where,

V_1 - Total volume of assay (mL)
t - Time of analysis in min
V_2 - Volume of enzyme used (mL)
V_3 - Volume for calorimetric determination (mL)

3.1.4.3.1 Pr1 and Pr2 Assay

The fungus was cultured in minimal medium (Appendix I.8) with casein as protein source. Spore suspension of $1x10^7$ mL^{-1} was inoculated into 50 mL of the medium in 250 mL Erlen-Mayer conical flasks. The cultures were incubated for 96 h in an orbital shaker at 180 rpm and 28^0C. The cultures were centrifuged at 10,000 rpm for 10 min and the supernatant was used as the enzyme source for quantitative estimation of Pr1and Pr2.

Subtilisin (Pr1) and trypsin like (Pr2) enzymatic activity was assayed by modified method described by St. Leger *et al.* (1987). N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide (Sigma-Aldrich) was used as the specific synthetic substrate for Pr 1, while N-α-Benzoyl-DL-Arginine p-nitroanilide (BAPNA) (Sigma-Aldrich) was used as the specific substrate for the assay of Pr 2. Each of the substrates @ 0.05 mL was mixed with 0.85 mL of Tris buffer 0.05M (pH 8.0) and 0.025 mL of culture supernatant, separately. The reaction mixture was incubated for 45 min at 30 °C and the reaction was terminated using 0.25 mL of 30 per cent acetic acid. Samples were centrifuged at 4000 rpm for 10 min and placed in ice for 15 min. Absorbance of para-nitro aniline was measured at a wavelength of 410 nm.

A standard graph was plotted at different concentrations of para-nitro aniline (Sigma- Aldrich) under same conditions as that of samples (Fig. 3). One unit of Pr 1 and Pr 2 enzymatic activity was defined as the amount of enzyme that released one μ mol of para-nitro aniline per minute under the above conditions. Protease activity was calculated by the formula mentioned under 3.1.4.3.

3.1.4.3.1.1 Estimation of Total Protein

The total protein of the supernatant was estimated by Lowry's method (Lowry *et al*., 1951) at different days of incubation using Bovine Serum Albumin (BSA fraction no:5) as standard. A calibration curve was plotted with different concentrations of BSA (HiMedia) (Fig. 4). Enzyme activity was calculated using the formula mentioned in para 3.1.4.3. Specific activity of Pr1 and Pr2 was worked out on the basis of enzyme activity and total protein content.

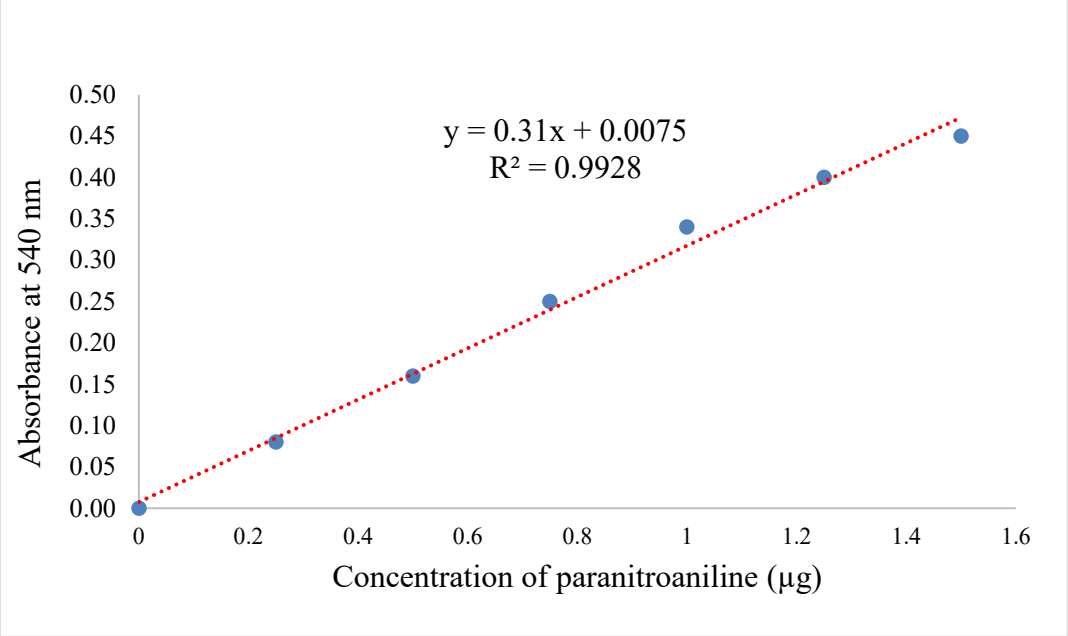


Fig. 3 Standard curve of paranitroaniline

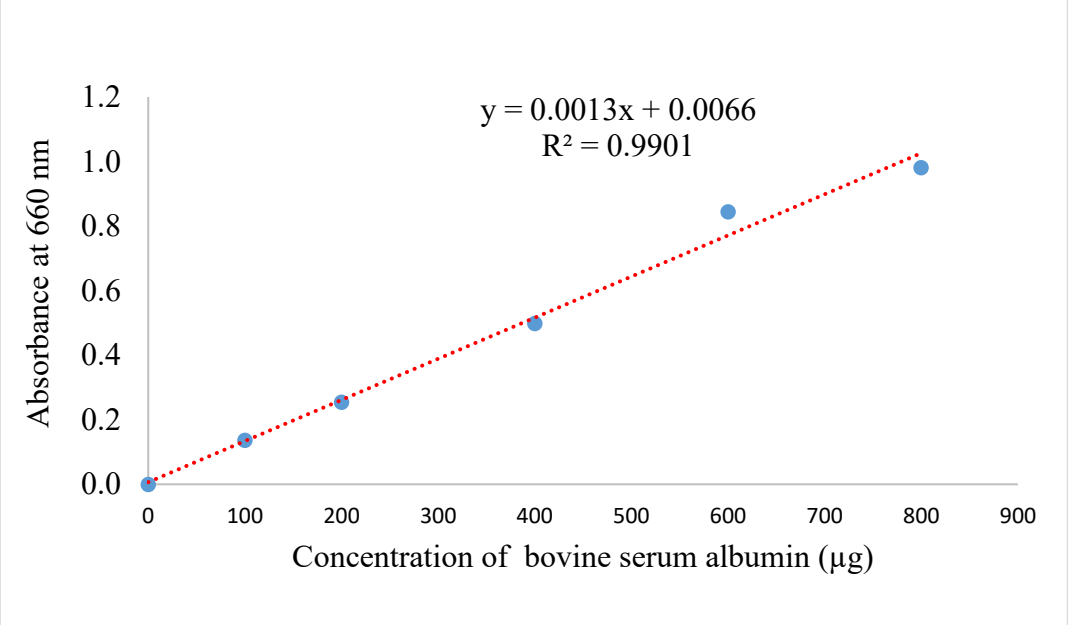


Fig. 4 Standard curve of bovine serum albumin

3.1.4.3.2 Keratinase Assay

3.1.4.3.2.1 Preparation of Keratin Substrate

Quantitative assay of keratinase was carried out using poultry feather as substrate, which was procured from poultry farm, Kerala Veterinary and Animal Science University, Mannuthy. It was processed as per the procedure suggested by Mini *et al.* (2012). It was cleaned thoroughly using 1:1 mixture of chloroform and methanol followed by distilled water and then dried in sunlight. Dried feathers were cut into pieces of one cm length and subjected to tyndalization for five successive days by exposing it to 120^0C for 30 min with intermediate incubation at 28^0C, so as to facilitate germination of contaminant spores if any. The sterilized feather was blended in a mixer grinder and passed through a 20 mesh sieve (840 µ). The coarse powder thus obtained was used as the substrate for keratinase assay.

3.1.4.3.2.2 Culturing of L. saksenae for Keratinase Assay

Fungal culture was prepared as described in para 3.1.4.1 and kept static in an incubator at 27^0C for 50 days. Mineral salt medium (Appendix I.9) supplemented with pre processed chicken feather was inoculated with seven day old culture to induce keratinase as suggested by Kumar and Kushwaha (2014). Medium without fungal culture served as control. Samples were drawn initially on 20^{th} day of incubation and subsequently at 10 days interval till 50^{th} day. The pH of medium was recorded on each day by using a pH meter. Total protein of the culture filtrate on each day of assay was quantified as described in para 3.1.4.3.1.1. At the end of the incubation period, the fungal mat and feather powder were separated from culture medium by filtering through Whatman No.1 filter paper. The final weight of the feather was recorded and percentage reduction in biomass was calculated.

3.1.4.3.2.3 Enzyme Assay

Keratinase activity of *L. saksenae* was assayed by Folin Ciocalteau method suggested by Cheng *et al*. (1995). An aliquot of 10 mL of culture medium from each flask was collected aseptically and filtered through glass wool followed by Whatman No 1 filter. The reaction mixture was prepared by adding one mL of the enzyme extract, 50 mg of keratin substrate and six mL of 10 Mm phosphate buffer (pH 8) which was then incubated for 30 min at room temperature. After incubation the reaction was arrested by adding two mL of 10 per cent TCA and then centrifuged at 3000 rpm for 10 min. One mL of the supernatant was added with 2.5 mL 0.5 M NaOH and 0.5 N Folin reagent and kept under incubation at room temperature. Colour development was measured at 660 nm against enzyme and substrate control. Amount of tyrosine released was quantified by plotting a standard graph of tyrosine. One mL of keratinase activity was defined as the amount of enzyme that released one microgram of tyrosine per min under standard assay condition. Keratinase activity was calculated using the formula

$$\text{Keratinase activity} = \frac{\mu g \text{ of tyrosine released} \times \text{dilution factor} \times \text{total volume}}{\text{Reaction time}}$$

3.1.4.4 Chitosanase Assay

Quantitative assay of chitosanase was carried out by culturing the fungus in a chitosanase inducing medium supplemented with 0.5 % colloidal chitosan at pH 5 (Composition of medium is given in Appendix I.4). Culturing and sample collection were carried out as described in para 3.1.4.1.

Chitosanase activity was assayed using colloidal chitosan as substrate suggested by Zhou *et al*. (2008). The reaction mixture constituted 1.8 mL one per cent colloidal chitosan in 50 mm sodium acetate buffer (pH 5.5) and 0.2 mL of crude enzyme. The mixture was incubated at 55^{o}C for 30 min and the reaction was

terminated with two mL of DNSA reagent (1%) and kept in a boiling water bath for 10 min. Insoluble chitosan was removed by centrifugation and the reducing sugars released were measured at 540 nm. Standard graph was plotted at different concentration of D-glucosamine (Sigma-Aldrich) under same conditions as that of samples (Fig. 5). One unit of chitosanase activity was defined as the amount of enzyme required to release one µmol of reducing sugars per min. Chitosanase activity was calculated by the formula

$$\text{Unit mL}^{-1}\text{min}^{-1} = \frac{\mu\text{mol of D-glucosamine x df x}V_1}{V_2 \text{ x } 10 \text{ x } 0.4}$$

Where,

V_1 - Total volume (mL)
df - Dilution factor
V_2 - Volume of enzyme used in assay
V_3 - Volume used in colour development

3.1.4.5 Amylase Assay

Amylase activity was assayed using minimal medium (Appendix1.10) supplemented with one per cent starch as per 3, 5-dinitrosalicylic acid method of Bernfeld (1955). Samples were drawn at two day intervals and the crude enzyme for the assay was prepared as described in para 3.1.4.1. The substrate for enzyme assay was one per cent soluble starch prepared in 100 mL 0.02 M sodium phosphate buffer at pH 6.9 and 0.06 M NaCl. Crude enzyme (0.5 mL) was incubated at 25^0C for three min followed by the addition of 0.5 mL of one per cent starch solution. The reaction mixture was then incubated at 30ºC for five min. The reaction was stopped by the addition of 1M one per cent DNS reagent and kept in water bath at 100ºC for five min. After cooling, eight mL water was added and absorbance was measured at 540 nm.

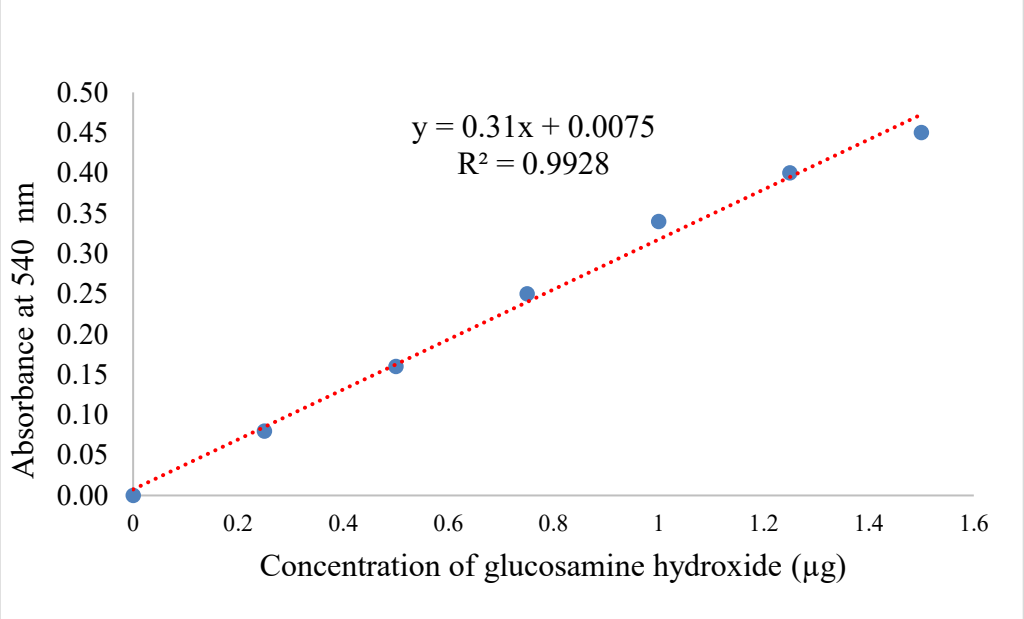


Fig. 5 Standard curve of D - glucosamine hydroxide

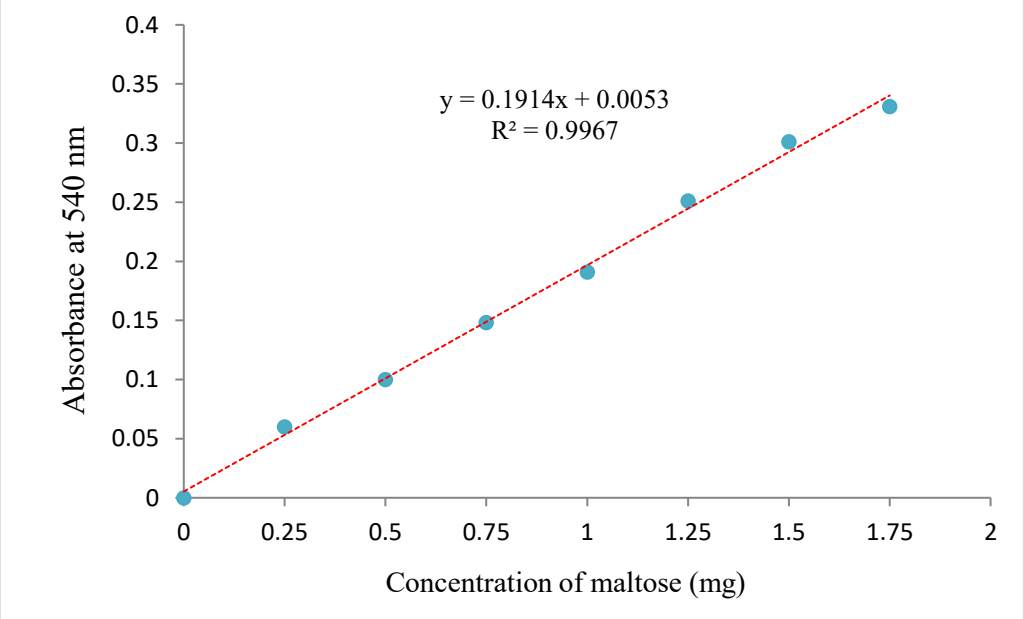


Fig. 6 Standard curve of maltose

Standard graph was plotted at different concentration of maltose (HiMedia) under the same conditions as that of samples (Fig.6). One unit (IU) of amylase corresponds to the amount of enzyme that hydrolyzes 1 µ mole of maltose per minute at 30°C. Amylase activity was calculated using the formula

$$\text{Units mL}^{-1}\ \text{min}^{-1} = \frac{\text{mg of maltose released x df}}{\text{mL of enzyme x time of incubation}}$$

Where,

df - dilution factor

3.1.5 Statistical Analysis

The data was subjected to analysis of variance (ANOVA) using WASP2 software.

3.2. BIOEFFICACY OF ENZYMES ON INSECT PESTS AND NEMATODES

Bioefficacy of partially purified enzymes was tested in one of the potential insect hosts of *L. saksenae viz*. brinjal mealybug *C. insolita* and the root knot nematode host *M. incognita.*

3.2.1 Purification of Major Cuticle Degrading Enzymes (CDE)

The major CDE *viz.* chitinase, lipase and protease were purified using the standard procedures.

3.2.1.1 Chitinase

Culture filtrate for purification was prepared from six day old culture of *L. saksenae* in EPM as in para 3.1.4.1. Partially purified chitinase was obtained through steps such as precipitation and dialysis. Total protein of the partially purified

chitinase was estimated. Different steps involved in the purification process are described below, sequentially.

3.2.1.1.1 Precipitation

The optimum concentration of ammonium sulphate required to precipitate target protein was standardized by its addition, at 30-90 per cent saturation to the culture filtrate. The quantity of crystal ammonium sulphate required for each per cent saturation was calculated by the formula (for 1 L of culture).

$$G = \frac{533\ (X-X_0)}{100 - 0.3X}$$

Where,

G is the quantity in gram of ammonium sulphate required
X- The required saturation
X_0-The initial saturation

Salting out was carried out with 50 mL of culture filtrate each at 4^0C at varying level of saturation using ammonium sulphate at 30, 40, 60, 70, 80 and 90 per cent separately. Different quantities of ammonium sulphate were added slowly under stirring and the mixture was kept overnight. The mixture was centrifuged at 10000 rpm for 15 min at 4^0C and the precipitated protein fraction was collected for further purification.

3.2.1.1.2 Dialysis

The precipitated proteins at different level of saturation were re suspended separately in two mL each of 0.01M Tris HCl buffer with pH 8 and transferred to dialysis tubes of size 8 x 2 cm^2. It was dialyzed against 0.01 M solution of the same buffer for 24 h at 4^0C with six changes of buffer and then assayed for total protein.

3.2.1.1.3 Estimation of Total Protein

The dialysate was subjected to total protein estimation as per para 3.1.4.1.1. Optimum quantity of ammonium sulphate [$(NH_4)_2SO_4$] for protein precipitation was standardized on the basis of highest recovery of protein.

3.2.1.1.4 Purification of Chitinase

L. saksenae was cultured in chitin amended medium (1L), precipitated with 80 per cent ammonium sulphate, partially purified and total protein was estimated as described in para 3.2.1.1.1 to 3.2.1.1.3.

3.2.1.2 Lipase

Basal medium supplemented with olive oil 2% (v/v) with an initial pH of 5.8 was used for optimum production of lipase. Composition of medium is given in Appendix I.11. Culture medium (50 mL) were taken in 250 mL Erlemeyer flasks and sterilized at 121^0C for 20 min. After cooling, olive oil (sterilized in hot air oven at 180^oC for 60 min) was added into the medium. It was inoculated with I mL spore suspension @$1x10^6$ along with a surfactant 0.25% (v/v) Tween 80. The flasks were incubated at 28^oC in an incubating shaker at 150 rpm for five days.

Protein precipitation, dialysis, estimation of total protein and partial purification were done as described vide para 3.2.1.1.1, 3.2.1.1.2, 3.2.1.1.3 and *3.2.1.1.4* respectively.

3.2.1.3 Protease

L. saksenae was grown as described in para 3.1.4.3.1 in medium supplemented with one per cent casein for five days. It was subjected to partial purification and protein estimation as described in para. 3.2.1.

3.2.2 Bio efficacy of Enzymes on *C. insolita*

3.2.2.1 Rearing of C. insolita

C. insolita was reared in sprouted potato tubers under laboratory conditions. Good quality potato tubers with robin eyed sprouts were procured from the local market for rearing mealybugs. The tubers were washed with sterile water and disinfected with 1% carbendazim. Incisions of 1 cm depth was made on the tubers with a sharp sterile blade and treated with 100 ppm giberillic acid solution for three hours**.** They were then air dried under shade and placed on sterile moist sand spread in a plastic tray of size 40 cm x 30 cm @10 tubers per tray. The trays were kept at 27^{o}C under darkness for one week to facilitate sprouting. After the initiation of sprouts they were shifted to ambient room conditions. The trays were regularly sprayed with sterile water to maintain moisture.

Pre adults of *C. insolita* collected from brinjal plants were released on to the sprouts to facilitate oviposition and subsequent buildup of population. Adequate food supply was ensured by replacing the exhausted potatoes with freshly sprouted one. Different life stages of the mealybugs were collected from the sprouts as and when required for carrying out the bioassay.

3.2.2.2 Bioefficacy of Chitinase

Bioefficacy of partially purified chitinase was tested against nymphs and adults of *C. insolita***.** Three different concentrations *viz*. 25 µg L^{-1}, 50 µg L^{-1} and 100 µg L^{-1} of protein were prepared with 0.1M Tris buffer (pH 8) and each test concentration was replicated five times with Tris buffer and sterile distilled water as negative and absolute control, respectively.

In vitro bioefficacy studies were conducted by leaf dip method suggested by Kim *et al*. (2010). Thirty numbers each of nymphs and adults were transferred separately using a fine camel hair brush from the stock culture on to brinjal leaf disc of 30 mm diameter. The discs were dipped in each of the test solution with gentle agitation for 10 s. The treated leaves were dried at room temperature. Each disc was placed on a brinjal leaf kept in a Petri plate lined with moistened tissue paper. Mortality of the test insects was recorded at 24 h interval for a period of four days.

3.2.2.3 Bioefficacy of Protease

Bioefficacy studies were conducted with different test concentrations of proteins at 25, 50 and 100 µg mL^{-1} on adult and nymphal stages of *C. insolita* as described in para 3.2.2.2.

3.2.2.4 Bioefficacy of Lipase

Three different test concentrations of lipase were tested for their efficacy on nymphs and adults of *C. insolita* as described in para 3.2.2.2.

3.2.2.5 Combined Effect of Protease, Chitinase and Lipase on C. insolita

Bioefficacy of all the three major enzymes in combination was tested at concentrations of 25, 50 and 100 µg mL^{-1} on nymphs and adults of *C. insolita* as described in 3.2.2.2.

The mortality in the control group was corrected using Abott's formula. The data were statistically analysed by one way ANOVA using WASP2 software.

3.2.3 Bioefficacy on *M. incognita*

3.2.3.1 Culturing of M. incognita

Pure culture of *M. incognita* obtained from Dept. of Nematology, College of Agriculture, Vellayani was utilized to maintain an axenic culture of the species. Tomato seedlings var. Anagha were grown in earthen pots containing sterilized potting mixture and were maintained in polyhouse. The plants were inoculated with juveniles (J2) of root knot nematode @1 per gram of soil, one week after planting. The plants were maintained under regular cultivation operations. Sequential planting was done to maintain continuous culture throughout the period of experiment.

Fibrous roots with tiny galls were collected and washed under tap water to remove the adhering soil particles. Protruding egg masses were picked up from the roots with a fine needle under a zoom stereo microscope.

3.2.3.2 Pathogenicity of L. saksenae on M. incognita Eggs

In order to derive a preliminary assessment regarding the pathogenicity of *L. saksenae* to *M. incognita*, pathogenicity test was carried out in the egg stage before the conduct of bioefficacy studies.

Pathogenicity studies were conducted using agar plates (1%) inoculated with five day old fungal plug (5 mm). Four replications were maintained and the plates were then incubated at 27^0C for one week in darkness. Egg masses of *M. incognita* collected from laboratory culture were surface sterilized with sodium hypochlorite solution (0.05 %) followed by washing with sterile water. The egg masses were placed on the growing edges of the fungal colony @ three egg masses per plate. After an incubation period of one week, each egg mass was observed under a zoom stereo microscope (Leica^{TM}) for parasitism or fungal growth.

3.2.3.3 Effect of Enzymes on Egg Hatching

3.2.3.3.1 Preparation of Eggs

M. incognita egg masses were carefully hand-picked from root galls under a zoom stereo microscope. They were surface sterilized with 0.05 % sodium hypochlorite solution and vortexed for two min to break up the gelatinous matrix and to disburse the individual eggs. Debris was allowed to settle for 30 s. The supernatant with eggs were collected and allowed to settle for five min. The eggs were then washed with three changes of sterile distilled water to remove the traces of sodium hypochlorite from the egg suspension. Finally the pellet was resuspended in 250 µL sterile distilled water and number of eggs per µL were counted and standardized using a counting dish.

3.2.3.3.2 Effect of Partially Purified Enzymes on Eggs

Chitinase and protease prepared as in para 3.2.1.1.4 and 3.2.1.3 were used for the study. The suspension (10 µL) containing 100 eggs were transferred to each well of the micro plates. One mL each of partially purified protease, chitinase and the combination of protease plus chitinase in 0.1M Tris buffer (pH 8) at three different concentrations of 25, 50 and 100 μgmL^{-1} were added to the egg suspension under aseptic conditions. The plates were incubated at 27^{o} C for five days. Egg suspension in Tris buffer and sterile distilled water served as negative and absolute control respectively. After incubation, number of eggs hatched were counted under a zoom stereo microscope. Unhatched eggs were subjected to Scanning Electron Microscopy to observe any structural changes on the outer egg shell.

3.2.3.3.3 Scanning Electron Microscopy (SEM)

Nematode eggs for SEM were prepared as per the procedure of Bozzola and Russell (1999) with some modifications. Chemicals used for preparations of eggs are enlisted in Appendix 11.

Different steps involved in sample preparation for SEM are represented in the flow chart below

Enzyme treated eggs
↓
Washed with double distilled water
↓
Fixed with 2.5% glutaraldehyde (24 h)
↓
Washed with 0.05M phosphate buffer (3 changes, 30 min)
↓
OsO_4 fixation (2 h)
↓
Washed with 0.05 M phosphate buffer (pH 7.2) (3 changes, 30 min)
↓
Dehydrated using 30%, 50 %, 70 %, 90% and 100% ethyl alcohol (30 min each)

The dehydrated eggs were then mounted on aluminum stubs using a micropipette. Electric current was passed at 10 mA for 20 s in vacuum and coated with gold to a thickness of 20 nm in an ion sputter coater.

3.2.3.3.3 SEM Imaging

The specimens were viewed and photographed by Scanning Electron Microscope (TESCAN VEGA3) at Central Instrumentation Facility, KVASU, Mannuthy, Thrissur, Kerala.

3.3. PURIFICATION AND CHARACTERIZATION OF ENZYMES

Based on the bioefficacy studies, the most effective CDE was subjected to further purification and characterization.

3.3.1 Ammonium Sulphate Precipitation

L. saksenae was cultured as described in para 3.1.4.3.1 for the production of protease. Extraction of the enzyme was carried out by ammonium sulphate precipitation method at 80 per cent saturation as described in para 3.2.1.1.1. The precipitate was resuspended in 100 mM Phosphate Buffered Saline (PBS) (pH 7).

3.3.2 Dialysis

The ammonium sulphate precipitate was desalted by dialysis. Pre-treatment of dialysis bag (HiMedia, Molecular Weight Cut off (MWCO) of 12 kDa was done with distilled water at 65º C for 10 min and then soaked in 10 mM disodium ethylene diamine tetraacetic acid.It was then treated with 10mM sodium bicarbonate solution and finally washed with distilled water. The samples were then dialyzed against PBS of pH 7.0 at 4^0C by changing buffer in every four hours. The samples were carefully drawn to determine the protease activity and protein content.

3.3.3 Ion Exchange Chromatography

The dialysed protein was resuspended in PBS and applied to a Sephacryl G-100 column (Sigma-Aldrich) pre-equilibrated with 100 mM PBS (pH 7.0). The protein elution (20 mLh^{-1}) was done with the same buffer, and the bound enzyme was eluted with 0.1-1.0 M NaCl gradient. Eluted proteins were pooled, collected and resuspended in PBS.

3.3.4 DEAE-Cellulose Fast Flow Column

Protease was further purified by affinity column chromatography. DEAE-Cellulose Fast Flow column (Sigma-Aldrich) was packed and equilibrated with 100 mM PBS (pH 7.0). The protein was eluted (25 fractions) with 0.1 to 0.5 M NaCl gradient with the flow rate of 1mL min^{-1}. All the steps were carried out at 4°C to prevent protein denaturation.

3.3.5 SDS-PAGE Analysis of Purified Protease

SDS Polyacrylamide Gel Electrophoresis (PAGE) was done with resolving gel 12% and stacking gel, 4% with the the buffer system described by Laemmli (1970). A constant voltage of 60 V was maintained till the samples concentrated on the stacking gel, and was then increased to 100 V for the remaining separation time. Electrophoretic separations were kept for 120 min. After running the gel casting system, the gel was taken out and was subjected to Commassie brilliant blue staining and was followed by de staining. The molecular weight of protease was determined by comparison with unstained protein molecular weight marker (Thermo Fisher Scientific). The image of gel was captured using gel documentation system (BioRad Gel DOC™-EZ imaging system). As the individual proteins could not be purified, aminoacid sequencing was not attempted.

3.4 ISOLATION AND CHARACTERISATION OF MYCOTOXINS

3.4.1 Isolation

3.4.1.1 Preparation of Fungal Culture

L. saksenae was sub cultured on SDA plates and incubated for 14 days at 27^0C. Conidial suspension was prepared as described in 3.1.2.

3.4.1.2 Standardization of Media for Mycotoxin/Pigment Production

Different growth media *viz*. Potato Dextrose Broth (PDB), Czapek Dox medium (CDM), Fargues medium (FM) and rice media (RM) were evaluated for the production of red coloured mycotoxin/pigment from *L. saksenae* (Appendix IV). Peptone was used as the as nitrogen source in CDM, glucose and yeast extract were used as carbon and nitrogen source respectively in FM.

The flasks were inoculated with spore suspension @$1x10^7$ mL^{-1} prepared as per 3.1.2 and were kept static and incubated in darkness for 14 days at 25^0C. The optimal medium was standardized visually on the basis of intensity of pigmentation.

3.4.1.3 Isolation and Purification of Metabolite

The culture of *L. saksenae* in PDB (1000 mL) was centrifuged at 10000 rpm at 4ºC and pH of the culture filtrate was adjusted to 2.0 with 2 M HCl. Culture filtrate was divided into aliquots of 100 mL each and added with equal quantity of ethyl acetate in the ratio of 1:1(v/v) and were shaken overnight in a rotary shaker at 200 rpm. The ethyl acetate layer was separated and concentrated under reduced pressure at vacuum 168^{mbar} and 45^0C in vacuum evaporator (Heidolph^{TM}). The dried extract was dissolved in 5 mL of methyl cyanide (MeCN) (HPLC Grade). Purification of crude toxin was carried out by collecting the insoluble filtrate, which was further purified by series of washing with hexane. Sufficient quantities were generated for further analysis.

3.4.1.4 Characterization of Purified Compound

The compound was subjected to different spectrometric analysis for the structural characterisation and identification of the toxin.

3.4.1.4.1 UV-VIS Spectroscopy

UV-VIS spectrum of the compound was recorded in dimethyl sulfoxide (DMSO), scanned at the wavelength range 100-1000 nm using UV-VIS double beam spectrophotometer (Systronics-AU-2701) to determine the wavelength at which maximum absorbance occur.

3.4.1.4.2 Fourier Transform Infrared Spectroscopy (FTIR) Analysis

FTIR analysis was conducted to identify functional groups and the chemical nature of the purified compound. Sample weighing 1.0 mg was mixed with 100 mg of KBr (binding agent) using a clean mortar and a pestle to make it into a powder. The powder was then made to pellets using a hydraulic press. The pellets were subjected to FTIR analysis on a BRUKER T FTIR spectrometer. The scanning range was from 4000 to 500 cm^{-1} with a resolution of 4 cm^{-1}. FTIR analysis was carried out at Sophisticated Analytical Instrumentation Facility, Cochin University of Science and Technology, Kochi, Kerala.

3.4.1.4.3 High Resolution Liquid Chromatography Mass Spectroscopy (HR-LCMS) Analysis

Mass spectra of the compound was recorded on Finnigan Tripple Stage Quadrupol Spectrometer (TSQ-70) with Electron Spray Ionization (ESI) method. Thermo X caliber Qual computer software was used in analysis of the mass chromatogram. The HR-LCMS analysis was done at Indian Institute of Technology (IIT), Mumbai, Maharashtra.

3.4.1.4.4 Nuclear Magnetic Resonance (NMR) Spectroscopy

The ^{1}H, ^{13}C, NMR spectra were recorded on the Bruker Avance 400 MHz NMR spectrometer at Central Laboratory for Instrumentation and Facilitation

(CLIF), University of Kerala, Thiruvanthapuram, Kerala. The measurements were done in Deuterated DMSO and chemical shifts assigned by comparison with the residue proton and carbon resonance of the solvent. Tetramethyl silane (TMS) was used as an internal standard and chemical shifts were given as δ (ppm).

3.4.2 Identification of Known Entomofungal Metabolites

Crude toxin of *L. saksenae* was screened for the presence of metabolites such as dipicolinic acid, beauverolide, beauvericin etc. that are commonly produced by entomopathogenic fungi.

3.4.2.1 Dipicolinic Acid (DPA)

3.4.2.1.1 Culturing of the Fungus

Minimal medium with glucose and yeast extract as carbon and nitrogen sources respectively were used for culturing *L. saksenae* **(**Fargues *et al.,* 1992). The pH of the medium was adjusted to 5.6 with 0.1N HCl. The sterilized medium was inoculated with spore suspension @ $1x10^7$ mL^{-1} and cultures were incubated in an incubator shaker at 27°C under 150 rpm for six days.

3.4.2.1.2 ***LC-ESI-MS Analysis***

Crude toxin extracted from culture filtrate of *L. saksenae* in FM was subjected to LC-ESI-MS analysis. LC-ESI-MS analysis was conducted on Agilent 6520 accurate mass Q-TOF LC-MS coupled with Agilent LC 1200 equipped with Extend-C18 column of 1.8 µm, 2.1 mm x 50 mm. Gradient elution was performed with water 0.05% formic acid (solvent A) and acetonitrile (solvent B) at a constant flow rate of 0.8 mL min^{-1}. The MS analysis was performed using ESI in the negative mode. The conditions for mass spectrometry were : drying gas (nitrogen) flow 5L min^{-1}; nebulizer pressure 40 psig; drying gas temperature 325ºC; capillary voltage-

3000 V; fragmentor volt 125V and Oct RF Vpp 750 V. LC-MS analysis was conducted at Advanced Instrumentation Facility (AIF), Arya Vaidya Sala, Kottakkal, Malappuram, Kerala.

The presence of DPA in the crude toxin was detected on the basis of retention time using commercial standard of DPA (Sigma-Aldrich) (Rf=0.92).

3.4.2.2 Beauverolide

3.4.2.2.1 Preparation of Mycelial Toxin of L. saksenae

L. saksenae was cultured in CDM and the biomass was separated through Whatman No.1. The fungal mat was washed with sterile water to remove the adherent filtrate, and then blotted between folds of sterile filter paper. Fungal mats from each flask were weighed separately under aseptic conditions. They were then soaked in 100 mL methanol for a week at 27^0C for cold extraction. The methanol fractions were separated from mycelia by filtration through Whatman No. 1filter paper and finally concentrated by vacuum evaporation at 45^0C. It was then reconstituted in methanol (HPLC grade).

3.4.2.2.2 Purification of Insecticidal Compound

3.4.2.2.2.1 Thin Layer Chromatography (TLC)

TLC plates were prepared using glass slides of dimension 5cm x 2 cm. Silica G (E-Merck) powder was mixed well with distilled water in the ratio 1:2 to form a slurry. It was uniformly applied on the plate using TLC applicator to a thickness of 0.2 mm and was kept for air drying. The plates were activated by placing in a hot air oven at110^oC for 30 min.

TLC was performed to find out the suitable solvent systems for eluting different compounds through column chromatography. Different combinations of solvent systems ranging from nonpolar to high polar were tested for their effectiveness to separate compounds from methanol fraction of crude extract. The combinations tried were chloroform : ethyl acetate (1:1), hexane: chloroform (1:1), hexane : ethyl acetate (1:1), ethyl acetate : methanol (1:1), hexane : methanol (1:1) , chloroform : methanol (1:1).

The solvent combinations were taken separately in 100 mL beaker and covered with a lid to fill the chamber with the solvent vapor. The activated TLC plates were then spotted with 10µl of crude toxin at a point of about 2 cm from the bottom of the plate using a capillary tube. The plates were then kept in the beakers containing solvents with approximately 1cm level solvent at the bottom till the solvent front reached the top of the plate. They were then removed from the tank and kept under open air at room temperature to evaporate solvent. Based on the movement of crude extract spotted on TLC plates, different combinations of solvents were selected to perform column chromatography.

3.4.2.2.2.2 Column Chromatography

Methanol fraction of crude toxin of *L. saksenae* was partitioned into different fractions using column chromatography. The purification of the fraction was done using a column of 22 cm x 1.6 cm dimension packed with 20 g silica gel having a particle size from 0.063 to 0.2 mm. The column was eluted with varying proportions of ethyl acetate and methanol at the rate of 2.6 mL min^{-1}. Twelve fractions were collected @10 mL per test tubes. Fractions with similar TLC pattern were grouped together to obtain five pooled fractions serially numbered from F_1 to F_5.

The fractions were then air dried and reconstituted in sterile water with Tween 80 (0.03%) and tested for its bioefficacy on third instar nymphs of *C. insolita.*

Uniform stages of the test insects were collected from the culture maintained in the sprouted potato tubers in the laboratory.

3.4.2.2.2.3 Identification by LC-MS

The active fractions with insecticidal activity were pooled together, reconstituted in methanol and subjected to chemical characterization using LC-MS at Central Instrumentation Facility, Kerala University, Thiruvananthapuram.

3.4.2.3. Beauvericin

3.4.2.3.1. Sample Preparation for HPLC

L. saksenae was cultured in PDB at 27^0C incubated at 120 rpm for eight days. Sufficient quantities were generated for further analysis. Mycelia were separated from the culture filtrate and washed to free the media particles attached to the fungus and dried in folds of sterile filter paper. Air dried mycelia were soaked in 100 mL methanol (HPLC Grade) for one week. The solvent was filtered through Whatman filter paper No: 1 and evaporated to dryness under vacuum evaporator at 40°C and re dissolved in methanol.

Culture filtrate was centrifuged at 10000 rpm 4^0C and the filtrate was passed through a 0.2 µm sterile filter equipped with a sterile glass syringe. The filtrate (250 mL) was freeze dried in an freeze drier and then lyophilised. The freeze drying and lyophilisation was carried out at National Coir Research and Management Institute, Thiruvanthapuram, Kerala. The lyophilised sample was reconstituted in methanol and subjected to HPLC analysis along with the standard beauvericin (Sigma-Aldrich) at Central Instrumentation Facility, Kerala Forest Research Institute, Peechi, Thrissur, Kerala.

3.4.3 Metabolite Detection by OSMAC Technology

One strain many compounds (OSMAC) technology suggested by Bode *et al*. (2002) is a method to produce diverse metabolite under different growth media. Therefore *L. saksenae* was cultured under different growth conditions and substrate composition in four different media.

3.4.3.1 Culturing of the Fungus

3.4.3.1.1 Czapek Dox Medium (pH 7)

The fungus was grown in 250 mL Erlen-mayer flasks, each containing 100 mL CDM fortified with 2% yeast extract. The flasks were inoculated with I mL of conidial suspension @10^7 conidia mL^{-1} and incubated statically at 27ºC for 12-15 days. Sufficient number of cultures were maintained to meet the analysis requirement.

3.4.3.1.2 Fargues Medium (pH 5.6)

FM supplemented with glucose and yeast extract as carbon and nitrogen sources respectively was used for culturing the fungus. The pH of the medium was adjusted to 5.6 with 0.1N HCl . The sterilized medium was inoculated and incubated under 150 rpm for six days.

3.4.3.1.3 Potato Dextrose Broth (pH 6)

Potato dextrose medium (HiMedia) 100 mL each, 250 mL Erlenmayer conical flasks were inoculated and incubated under 27ºC, at 120 rpm for eight days. The whole experiment was repeated three times to get sufficient quantity of sample for analysis**.**

3.4.3.1.4 Rice Medium (pH 7)

About 50 g of rice medium was taken in 250 mL Erlenmayer flasks. The spore suspension of $1x10^7 mL^{-1}$ prepared as per para 3.1.2. was used as inoculum. The flasks were kept static at 27 ^{0}C for 13 days.

3.4.3.2 Sample Preparation for Analysis

After the incubation period, culture filtrate from each of the medium was centrifuged at 10,000 rpm, 4°C for 10 min to remove mycelia and spores.

3.4.3.3 Extraction of Secondary Metabolites

The secondary metabolites present in the culture filtrate as well as the mycelia were recovered by liquid-liquid extraction using different organic solvents.

3.4.3.3.1 From Culture Filtrate

Three different solvents of varying polarity *viz*. methanol (high polar), ethyl acetate (medium polar), and hexane (non polar) were evaluated for their efficiency to recover the metabolites in the culture filtrate. Culture filtrate prepared as in 3.4.3.2 from different growth media were divided into aliquots of 100 mL each. Equal quantity of solvent [1:1(v/v)] was added to the filtrate and the flasks were shaken overnight in a rotary shaker at 200 rpm. Extraction procedure was repeated thrice. After the extraction process, organic layer containing metabolites were separated from the aqueous phase with the help of separating funnels. The aliquots of same solvents were pooled together and concentrated in vacuum at 45°C using a rotary flash vacuum evaporator. The residue from each solvent was reconstituted separately in 4 mL methanol.

3.4.3.3.2 From Mycelia

The fungal biomass from CDM was separated and processed as described para 3.4.2.2.1.

3.4.3.3.3 From Rice Grain

After incubation period each flask was added with 100 mL of ethyl acetate (HPLC grade) and kept overnight. The extract in each flask was pooled together and filtered. The organic layer was then subjected to vacuum evaporation at 40^0C.

3.4.4 Characterization of Mycotoxins

The crude toxin extracted through various processes as detailed in para 3.4.3.3 and mycelial toxin from CDM were subjected to analytical techniques such as FTIR to identify the functional groups and chemical nature. The ethyl acetate extracts obtained from culture filtrates of different media were subjected to analytical procedures such as GC-MS and HR-LCMS to identify the mycotoxins.

3.4.4.1 FTIR Analysis

The analysis was done as detailed in para 3.4.1.4.2

3.4.4.2 Gas Chromatography-Mass Spectrophotometry (GC-MS)

Culture filtrates from CDM and PDB were subjected to GC-MS analysis to detect the volatile metabolites. It was carried out on a Perkin Elmer (clarus 680) series GC-MS (Marathon, USA) system equipped with clarus 600 (EI) auto-sampler coupled with an Elite-5 MS capillary column (30 mx 0.25 mm i.d, and 0.250 µm) (PerkinElmer, Inc, made in USA). Helium was used as the carrier gas at a flow rate of 1 mL min^{-1}; split ratio of 10:1; mass scan 50–600 Da; ionization energy, 70 eV;

ion source temperature, 240^0 C; injector temperature, 250^0C. The oven temperature was programmed with the initial temp at 60^0 C for 2 min, rising at 10^0 C min 1^{-1} to 300 ^{0}C and then held isothermally at 300^0 C (6 min) with a total run time of 32 min. The composition of the crude extract constituents was expressed as percentage of the peak area. The chemical compounds were identified and characterized based on their retention time (RT).The mass spectral data retrieved was matched with those of standards available in the NIST data base.

The analysis was carried out at Dept of Microbiology, Tamil Nadu Agricultural University, Coimbatore.

3.4.4.3 HR-LCMS analysis

The crude toxin prepared in methanol from CDM, FM, PDB and RM was subjected to HR-LCMS (G6550A system Agilent technologies, USA) analysis to identify the secondary metabolites produced by *L. saksenae*. The sample was run isocratically for 30 min using acetonitrile (95%) as solvent. The MS analysis was carried out by ESI positive and negative ionization modes. MS source conditions were capillary voltage 3500 V, Gas temperature 250^0C, drying gas flow 13 $Lmin^{-1}$, sheath Gas temp 300^0C, sheath Gas Flow 11, nebulizing gas pressure 35 (psig), fragmentor 175 V, Skimmer 65 V, Octopole RF Peak 750 V, and mass range m/z 50–1000. The resolution was 40,000 FWHM.

The structure of metabolites was ascertained on the basis of high resolution mass spectrometry and confirmed using Metlin database. The analysis was carried out at Sophisticated Analytical Instrument Facility (SAIF), IIT Mumbai.

3.5 BIOEFFICACY OF MYCOTOXIN

The purified mycotoxin was tested for its bio efficacy in nymphs and adults of the test insect *C. insolita* according to the method suggested by Ragavendran *et al*. (2017) with slight modification. Its efficacy was compared with that of the crude toxin extract, based on mortality data. Effect of these toxins on natural enemies was also tested in the coccinellid predator *Cheilomenes sexmaculata* (Fab.).

3.5.1 Preparation of Samples for Bioassay

3.5.1.1 Purified Toxin

Test concentrations were prepared by dissolving 10 mg of the purified toxin in one mL of DMSO which was then and diluted using sterile distilled water to six different concentrations *viz.* 10 ppm, 50 ppm, 100 ppm, 250 ppm, 500 ppm and 1000 ppm. DMSO dissolved in distilled water served as treatment control and distilled water only served negative control.

3.5.1.2 Crude Toxin

Test concentrations 10 ppm, 50 ppm, 100 ppm, 250 ppm, 500 ppm and 1000 ppm were prepared by dissolving 100 mg dry weight of the toxin in one mL of sterile water as in para 3.5.1.1. Distilled water treatment served as absolute control**.** Ethyl acetate extract of uninoculated medium was also tested to rule out the effect of culture medium, if any on test insects.

3.5.2 Bioassay

3.5.2.1 C. insolita

Adults and third instar nymphs collected from laboratory culture (Para 3.2.2.1) were used for the study. Separate set of test insects were used for assaying purified and crude toxin. Thirty numbers of each were transferred carefully with a fine camel brush on to brinjal leaf kept inside a Petri dish lined with a moist tissue paper. The insects were sprayed uniformly with different test concentrations using an atomizer and were kept for air drying. Air dried plates were then sealed with perforated parafilm and kept at 27°C.

The experiment was laid out in CRD with eight treatments and four replications. Mortality was recorded at 24 h interval for a period of 4 days. The mortality in the treatments and control was corrected using Abbott's formula. The LC_{50} and LC_{90} values for crude toxin were calculated using probit analysis with IBM SPSS software.

3.5.2.2 M. incognita

The effect of crude toxin on J_2 of *M. incognita* was carried out with different test concentrations *viz*.1, 10, 50, 100, 250, 500 ppm. Distilled water and fraction from un inoculated Czapek Dox medium served as absolute and negative control.

Second stage juveniles of *M. incognita* were obtained from the egg masses by Petri plate method. Nematode suspension was concentrated with 500 J_2 mL^{-1}. An aliquot of 2 mL of each of the test solutions was pipetted into 24 welled tissue culture plates. Nematode suspension (200µL) was added to each well containing test solutions. Juvenile mortality was recorded at 24, 48, 72 and 96 h after treatment. Mortality was confirmed by mechanical prodding of J_2 with a fine needle.

3.5.2.3 C. sexmaculata

The effect of crude toxin on different stages of *C. sexmaculata* was carried out using the extremely concentrated crude toxin from 1000 to 10000 ppm (Approximately 5X, 10X, 20X, 25X, 50X of lethal dose calculated for *C. insolita*) as per the procedure suggested by Pandi *et al*. (2013).

C. sexmaculata was reared in plastic jars of 22.5 cm x 15 cm under ambient laboratory conditions. Rearing was initiated with the adult beetles collected from cowpea field of Model Organic Farm, College of Agriculture, Vellayani. They were sexed and transferred to plastic jars in pairs along with aphid infested leaves and twigs. Aphid infested twigs were supplied afresh as and when needed. The jars were covered with muslin cloth and fastened with a rubber band. Multi folded tissue papers were placed in the jar as substratum to facilitate egg laying by the mated females. Egg masses were collected from jars and kept inside plastic boxes of 7 cm X 2.5 cm at ambient conditions for hatching. On emergence, the first instar grubs were separated and transferred to different plastic jars along with corrugated papers to avoid cannibalism and fed with cowpea aphids till they attain full growth. Final instars were then transferred into jars provided with cowpea leaves to facilitate pupation. Upon emergence, the beetles were again kept for oviposition to continue the rearing process, so as to get sufficient number of test insects. Cowpea aphids *A. craccivora* were multiplied on cowpea var. Bhagyalakshmi in polyhouse to assure the continuous availability of aphids as food source to coccinellids.

3.5.2.2.1 Egg

Toxicity to eggs was assessed by leaf dip method. Freshly laid egg masses on cowpea leaves were dipped in different concentrations of toxin for 30 s and dried under air for 10 min. These were placed in Perti plates lined with tissue paper, for observations. Number of eggs per egg mass, and number of un hatched eggs were

recorded, for a period of 6 days. The unhatched eggs were assumed to be dead and the per cent mortality was calculated.

3.5.2.2.2 Larva

Diet contamination method was followed using newly hatched third instar grubs. Cowpea twigs with uniform sized aphids were treated with different concentrations of crude toxins using an atomizer. These were air dried and transferred to Petri plates. Twenty grubs each of *C. sexmaculata* were transferred to the Petri plate containing treated aphids and was allowed to feed on it for 24 h. Thereafter, the grubs were fed with untreated aphids for a period of seven days. Control group was fed with untreated aphids. Mortality of grubs was recorded at 24 h interval for a period of five days.

3.5.2.2.3 Pupa

Assay was carried out using leaf dip method. Cowpea leaves with one day old pupae were dipped in different concentrations of toxin for 30 s and air dried for 10 min. They were then placed @ 10 per plate, lined with moist tissue paper. Observations were recorded on emergence, at 24h interval for a period of five days.

3.5.2.2.4 Adults

Topical application method was followed for adults. The beetles of uniform size and age were treated with different concentrations of crude toxins using an atomizer. Treated beetles were placed in Petri plates lined with moistened tissue paper @ 10 per plate and were fed with aphids. Mortality of beetles was recorded at 24 h interval, over period of five days.

Distilled water treatment served as absolute control**.** The whole experiment was laid out in CRD with six treatments and four replications. The data was subjected to statistical analysis using probit analysis with IBM SPSS software.

3.6. *In silico* MOLECULAR DOCKING STUDIES OF METABOLITES OF *L. SAKSENAE*

In silico studies were conducted to elucidate the mode of action of insecticidal metabolites of *L. saksenae.*

3.6.1. Software

The software used for molecular docking was Discovery Studio 4.0 developed and distributed by Accelryl, USA.

3.6.2 Ligands and Protein Targets

Different ligands were selected from the list of secondary metabolites in the HR-LCMS data of *L. saksenae* from IIT, Mumbai. Compounds that showed >80 per cent best match in mzCloud results were selected for *in silico* analysis. The three dimensional structures of metabolites were downloaded from NCBI Pubchem compound database.

The X-ray crystallographic structure of target protein of actylcholinesterase of *Drosophila melanogaster* (*Dm* AchE, PDB Code IDX4) was retrieved from Protein Data Bank (PDB).

The ligands were filtered using Veber and Lipinski's rule and active sites of the target receptor were identified by inhibitory property of the amino acid residues present in the binding sites. Each ligand was docked with the target protein and the interacting ligands were scored using CDOCKER programme. Ligand-protein

interaction was thereafter interpreted on the basis of CDOCKER energy, CDOCKER interaction energy, hydrogen bonds and binding energy etc. *In silico* analysis was carried out at Distributed Information centre, Centre for Plant Biotechnology and Molecular Research (CPBMB), College of Horticulture, Vellanikkara, Thrissur. Kerala.

RESULTS

4. RESULTS

The results of the study on 'Mycotoxins and enzymes of entomopathogenic fungus *Lecanicillium saksenae* (Kushwaha) Kurihara and Sukarno and their bioefficacy on crop pests was carried out at the Biocontrol Laboratory for Crop Pest Management, Department of Agricultural Entomology, College of Agriculture, Vellayani and Department of Agricultural Entomology, College of Horticulture, Vellanikkara during 2016-2019 are presented here under.

4.1. CUTICLE DEGRADING ENZYMES IN *L. SAKSENAE*

4.1.1. Enzymes Detected

Qualitative analysis revealed the presence of extracellular hydrolytic enzymes such as chitinase, lipase, protease, chitosanase, chitin deacetylase and amylase.

4.1.1.1 Chitinase

Chitinase activity of *L. saksenae* was detected by two methods as in para 3.1.3.1. In the screening plate method, with colloidal chitin a clear halo was developed on the 5th day of incubation around the fungal colony. Diameter of halo was found to increase over incubation time. The enzyme index ranged from 1.03 to 1.08 from 5th to 8th day (Table 2). The index decreased to 1.05 on the 9th day of incubation. The fungal colony attained a diameter of 3.8 cm in treatment plate and 4.6 cm in control plate on the 9th day.

In the pH indicator medium, a purple colouration was found to develop around the inoculated fungal plug indicating the release of chitinase and utilization of chitin from the medium on the 5th day. Diameter and intensity of coloured zone increased with the incubation period and the medium turned completely purple on

Table 2. Chitinase index of *Lecanicillium saksenae* in minimal medium with colloidal chitin

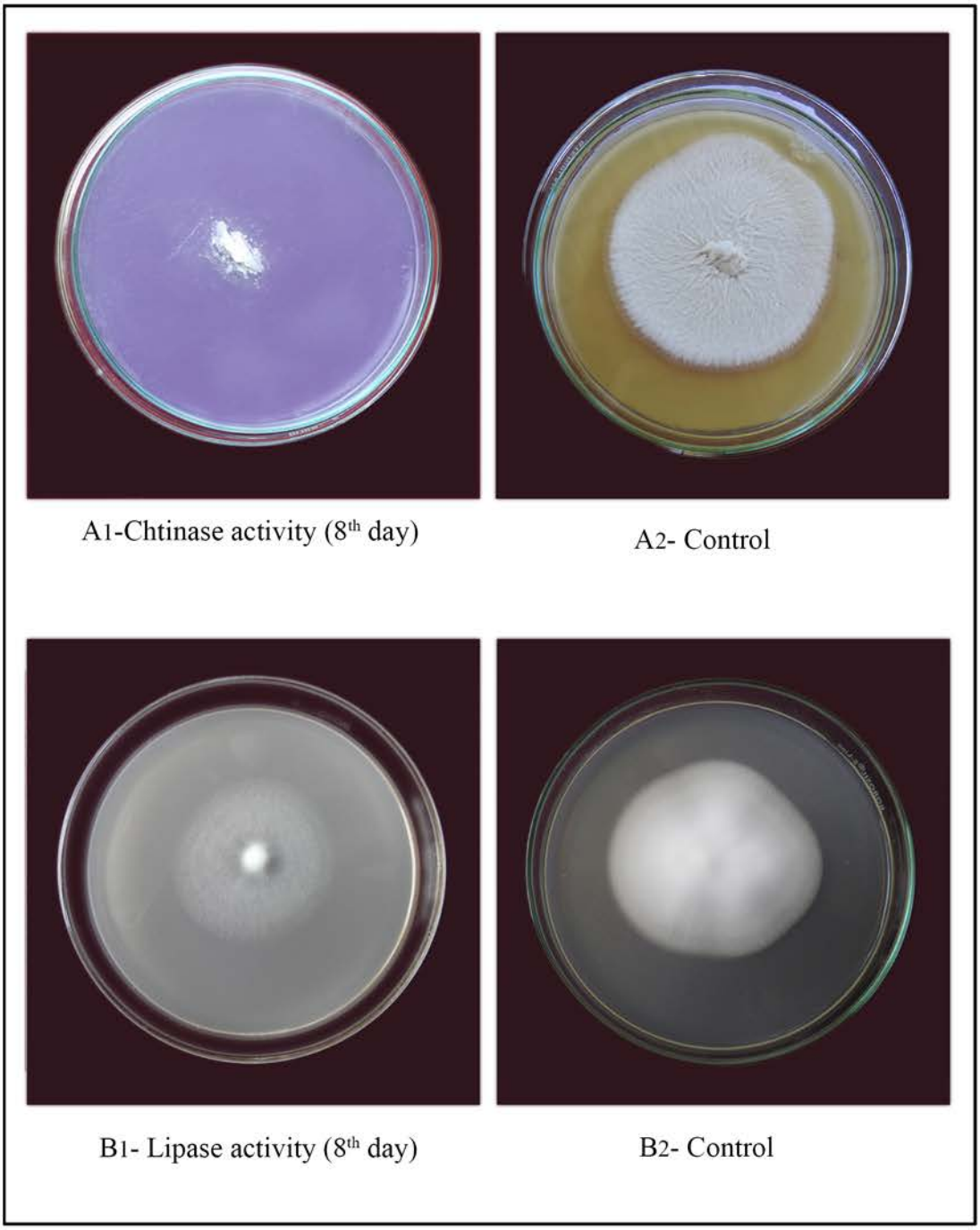
Incubation period (days)	Colony diameter without halo (cm)	Colony diameter with halo (cm)	Colony diameter in control (cm)	Enzyme Index
5	1.55	1.60	1.90	1.03
6	2.30	2.45	2.70	1.04
7	2.80	3.00	3.20	1.07
8	3.40	3.70	4.10	1.08
9	3.60	3.80	4.60	1.05

Mean of 3 observations

Table 3. Lipase index of *Lecanicillium saksenae* in minimal medium with Tween 20

Incubation period (days)	Colony diameter without halo (cm)	Colony diameter with halo (cm)	Colony diameter in control (cm)	Enzyme Index
5	0.50	1.50	2.30	3.00
6	0.80	2.40	2.80	3.00
7	0.80	2.50	3.20	3.12
8	0.80	3.40	4.20	4.25
9	1.00	4.20	4.50	4.20

Mean of 3 observations



A1-Chtinase activity (8th day)

A2- Control

B1- Lipase activity (8th day)

B2- Control

Plate 1. Chitinase and lipase activity of *Lecanicillium saksenae*

the 8th day (Plate 1A1). On the basis of colour rating given by Agrawal and Kotasthane (2012) chitinase activity was rated as high.

4.1.1.2 Lipase

Presence of lipase was detected by the zone of precipitation around the fungal colony (Plate 1B1). It was observed on the 5th day of incubation. Enzyme index worked out was 3.00 on the 5th day which gradually increased to 4.25, attained the highest on the 8th day (Table 3). The fungus attained a colony diameter of 1 cm in treatment plate on the 9th day while it was 4.5 cm in the control plate.

4.1.1.3 Protease

General protease was detected on screening plate containing minimal medium amended with casein. A clear halo was observed on the 5th day of incubation around the fungal colony which indicated the zone of activity of protease. Colony and halo diameter were found to increase with the increase in incubation period. Enzyme index worked out ranged from1.05 to 1.11 over a period of nine days (Table 4). The lowest index of 1.05 was recorded on the 5th day of incubation and the highest index was noted on the 7th day (1.11). The activity remained same on the 8th day and thereafter it decreased gradually to 1.10. In control plates, no halo was observed. The diameter of the fungal colony was 3.8 cm with the corresponding growth of 5 cm in the control plate.

4.1.1.4 Chitosanase

Presence of chitosanase was visualized in the screening plate prepared with colloidal chitosan as sole carbon source. Chitosanase was detected on the 5th day of incubation as a clear halo around the fungal colony (Plate 2 A1), diameter of which was found to increase with incubation time. The index ranged from 1.18 to 1.36

Table 4. Protease index of *Lecanicillium saksenae* in casein agar medium

Incubation period (days)	Colony diameter without halo (cm)	Colony diameter with halo (cm)	Colony diameter in control (cm)	Enzyme Index
5	1.70	1.80	2.10	1.05
6	2.30	2.50	2.80	1.09
7	2.60	2.90	3.20	1.11
8	3.40	3.80	4.10	1.11
9	3.80	4.20	5.00	1.10

Mean of 3 observations

Table 5. Chitosanase index of *Lecanicillium saksenae* in colloidal chitosan agar medium

Incubation period (days)	Colony diameter without halo (cm)	Colony diameter with halo (cm)	Colony diameter in control (cm)	Enzyme Index
5	1.60	1.90	2.10	1.18
6	2.60	3.20	3.40	1.23
7	3.20	4.30	4.60	1.34
8	3.80	5.20	5.70	1.36
9	4.20	5.40	6.20	1.28

Mean of 3 observations

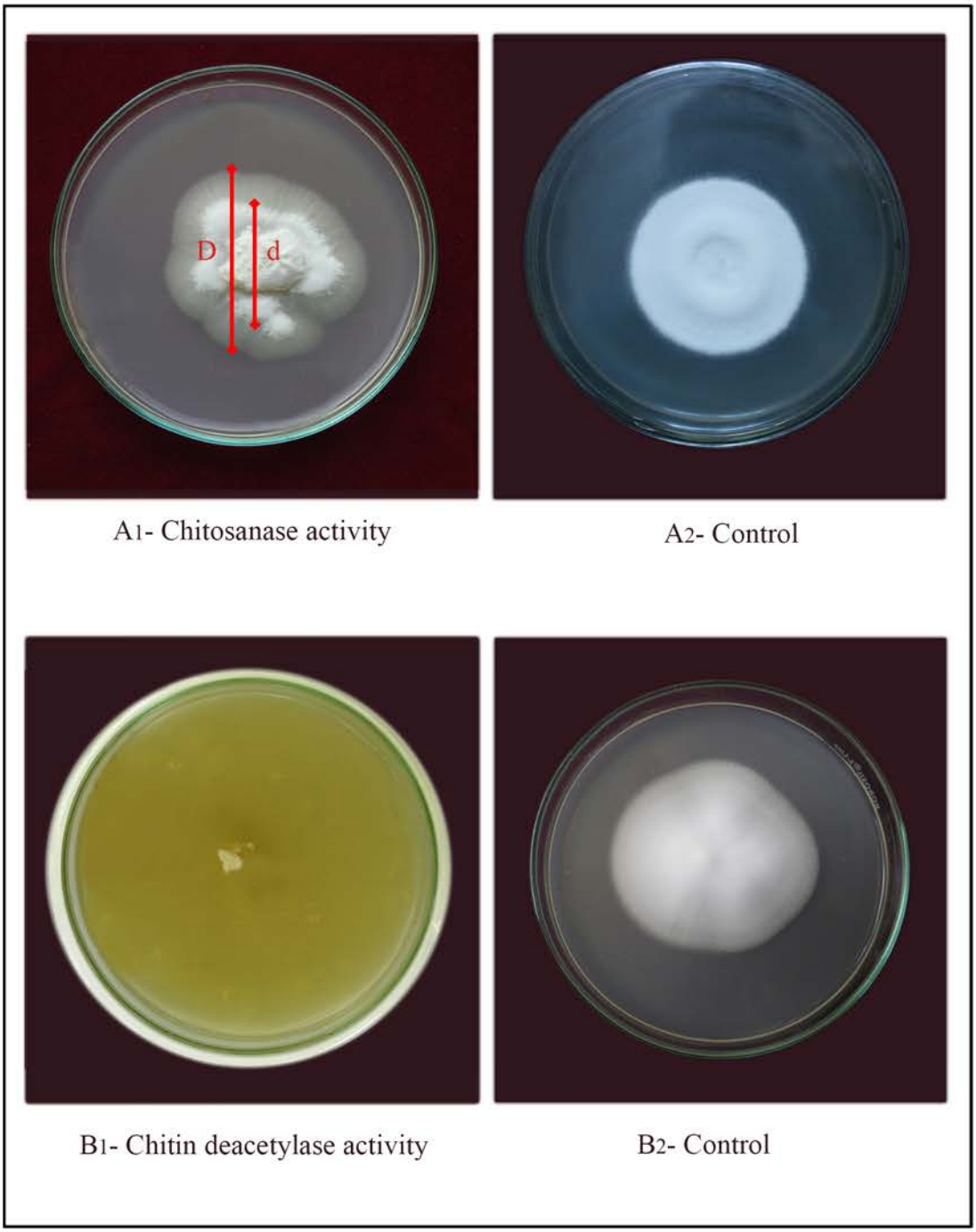


Plate 2. Chitosanase and chitin deacetylase activity of *Lecanicillium saksenae*
D- Total diameter (Colony diameter + halo) d- Colony diameter

from 5th to 8th day (Table 5). It decreased to 1.35 on the 9th day of incubation. The fungus attained a colony diameter of 4.2 cm on the 9th day while it was 6.2 cm in the control plates.

4.1.1.5 Chitin deacetylase

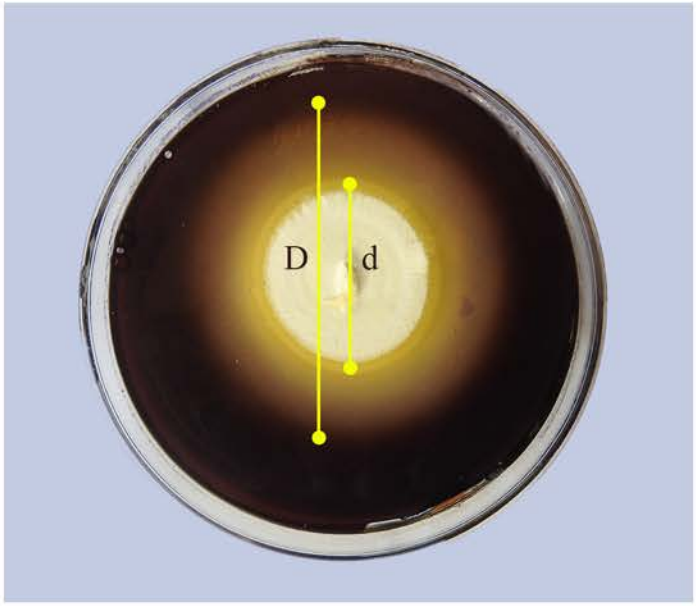
Chitin deacetylase (CDA) was detected by the colour reaction with pH indicator medium using the substrate, N-nitroacetanilide. The detection plate was creamy white initially at a pH of 7.2. Five days after inoculation, the colour of CDA plates turned yellowish. The intensity of colour increased over time and finally a bright yellow colour was observed on the 8th day (Plate $2B_1$) which indicated the production of chitin deacetylase. No fungal growth was observed around the fungal plug though there was colour change in the detection plate. The fungus was found to grow normally in the control plates without colloidal chitin and N-nitroacetanilide.

4.1.1.6 Amylase

Amylase was detected using starch agar plate. The zone of hydrolysis around fungal colony was observed as a halo on the 5th day after incubation and the diameter of halo was found to increase with incubation period. Treatment of plates with iodine reagent on the 8th day of incubation produced a clear yellow zone (zone of hydrolysis) around the fungal colony which indicated amylase activity (Plate $3A_1$). Diameter of the halo developed was 1.6 cm and the index was calculated as 1.57 on the 8th day of incubation.

4.1.2 Enzyme Activity

Activity of the enzymes detected in *L. saksenae* was quantified in different culture media which differed in nutrient composition.



A_1- Zone of hydrolysis

D- Total diameter (Colony diameter + halo) d- Colony diameter

A_2- Control

Plate 3. Amylase activity of *Lecanicillium saksenae*

4.1.2.1 Chitinase

Results revealed that the induced and constitutive production of chitinase varied significantly with media and incubation period (Table 6).

Chitinase activity was assessed as a measure of the breakdown product of colloidal chitin *viz.* N-acetyl glucosamine in enzyme producing medium (EPM) and sabouraud dextrose broth (SDB). Chitinase activity was 0.07 U mL^{-1} min^{-1}on the 2^{nd} day of incubation which increased significantly to 0.21 U mL^{-1} min^{-1} on the 4^{th} day. Significantly higher activity (1.35 U mL^{-1} min^{-1}) was noted on the 6^{th} day, which was the peak. A significant decline was observed thereafter, with the lowest value (0.28 U mL^{-1} min^{-1}) on the 14^{th} day.

A similar trend was observed in SDB also. The activity was found to increase from 0.01 on the 2^{nd} day to 0.03 U mL^{-1} min^{-1} on the 4^{th} day and 0 .08 U mL^{-1} min^{-1} on the 6^{th} day which was statistically different from each other. Highest activity (0.09 U mL^{-1} min^{-1}) was noted on the 8^{th} day of incubation. A significant decline was noticed thereafter, which reached a minimum of 0.02 U mL^{-1} min^{-1} on the 14^{th} day.

Though chitinase activity was detected in SDB, the enzyme level was very low compared to that in EPM. Highest chitinase activity was observed on the 6^{th} day in EPM, whereas in SDB it was on the 8^{th} day. Hence, the optimum time for highest production of chitinase was found to be 6^{th} day in EPM amended with colloidal chitin at a pH of 5.5 kept at a temperature of 27^0C.

4.1.2.2 Lipase

As observed with chitinase, notable difference was observed in the activity of lipase in EPM and SDB (Table 6). In EPM it ranged from 0.20 to 0.7 U mL^{-1} min^{-1}

Table. 6. Activity of major cuticle degrading enzymes of *Lecanicillium saksenae*

Enzyme	Medium	*Enzyme activity (U mL^{-1} min^{-1}) Incubation period (days)							CD (0.05)
		2	4	6	8	10	12	14	
Chitinase	EPM	0.07^{g}	0.21^{f}	1.35^{a}	1.09^{b}	0.78^{c}	0.52^{d}	0.28^{e}	0.029
	SDB	0.01^{g}	0.03^{e}	0.08^{b}	0.09^{a}	0.05^{c}	0.04^{d}	0.02^{f}	0.008
Lipase	EPM	0.66^{b}	0.70^{a}	0.63^{c}	0.54^{d}	0.33^{e}	0.28^{f}	0.20^{g}	0.011
	SDB	0.62^{b}	0.67^{a}	0.62^{b}	0.53^{c}	0.32^{d}	0.26^{e}	0.18^{f}	0.024
Protease	EPM	0.78^{c}	1.07^{c}	1.53^{b}	1.99^{a}	1.07^{c}	0.43^{d}	0.21^{d}	0.336
	SDB	0.24^{f}	2.26^{d}	4.89^{a}	4.39^{b}	3.59^{c}	2.05^{d}	0.78^{e}	0.319

*Means of three replications EPM-Enzyme producing medium
SDB-Sabouraud dextrose broth
Values sharing same alphabets in superscript are statistically on par based on LSD

Table 7. Pr1 and Pr2 activity of *Lecanicillium saksenae* in casein medium

Incubation period (days)	Pr1			Pr2		
	Activity (UmL^{-1} min^{-1})	Total protein (µg)	Specific activity (U mg^{-1})	Activity ($UmL^{-1}min^{-1}$)	Total protein (µg)	Specific activity (U mg^{-1})
2	0.18	463.10	0.39	0.36	463.10	0.78
3	0.18	504.18	0.35	0.20	504.18	0.40
4	0.19	553.02	0.34	0.20	553.02	0.36
5	0.22	761.69	0.28	0.18	761.69	0.24
6	0.14	612.54	0.22	0.13	612.54	0.21
7	0.09	504.85	0.17	0.09	504.85	0.18
8	0.08	418.52	0.19	0.08	418.52	0.19
9	0.04	397.79	0.10	0.03	397.79	0.08

Values are means of three replications

over a period of 14 days of incubation whereas, the corresponding activity in SDB was 0.18 to 0.67 U mL^{-1} min^{-1}.

On the 2nd day of incubation, activity was to the tune of 0.66 and 0.62 U mL^{-1} min^{-1}in EPM and SDB respectively. Highest activity was observed on the 4th day of incubation in both SDB and EPM which was statistically different from enzyme activity on all other days of incubation. Subsequently, the activity declined expressing the lowest level of 0.20 in EPM and 0.18 U mL^{-1} min^{-1} in SDB on the 14th day. The optimum time for highest production of lipase was found to be fourth day of incubation in both SDB and EPM at pH 5.5 and temperature 27^0C.

4.1.2.3. Total Protease

Total protease activity of *L. saksenae* in culture filtrate was quantified at different incubation period (Table 6). Notable differences were observed between the protease activities in two different media. Activity was more in SDB compared to EPM, except for the second day.

In EPM, activity varied between 0.21 U mL^{-1} min^{-1} and 1.99 U mL^{-1} min^{-1} during the incubation period. The activity on the 2nd and the 4th day of incubation was on par with each other and significantly increased to 1.53 U mL^{-1} min^{-1} on the 6th day. Peak of activity was recorded on the 8th day (1.99 U $mL^{-1}min^{-1}$) which statistically declined thereafter to 0.21 U mL^{-1} min^{-1} on the 14th day.

In SDB, marginal activity of protease was observed on the 2nd day (0.24 U mL^{-1} min^{-1}) onwards, with an increasing trend till 6th day (4.89 U mL^{-1} min^{-1}). Thereafter, the activity was found to decline significantly. The enzyme activity noted on the 12th day was statistically on par with that on the 4th day. Lowest activity noted was 0.78 U mL^{-1} min^{-1} on the 14th day.

The optimum time for maximum production of protease was found to be six days after inoculation in SDB medium at a pH of 5.5 kept at a temperature of 27^0C.

4.1.2.3.1 *Pr1 and Pr2*

Subtilisin like Pr1 and trypsin like Pr2, the isoenzymes of protease were assayed separately. They were detected in culture medium supplemented with one per cent casein as sole nitrogen source. The activity was noted from the 2nd day onwards, with significant increase on subsequent days (Table 7).

Pr1 activity was found to increase significantly from 0.18 U mL^{-1} min^{-1} on the 2nd day to 0.22 U mL^{-1} min^{-1} on the 5th day. A sudden drop was observed from 6th day of incubation and the lowest activity of 0.04 U mL^{-1} min^{-1} was observed on the 9th day of incubation. The optimum period for Pr1 production was found to be five days of incubation in casein enriched medium.

Highest activity of Pr2 (0.32 U mL^{-1} min^{-1}) was observed on the 2nd day which declined statistically day by day, reaching a minimum of 0.03 U mL^{-1} min^{-1} on the 9th day.

Highest specific activity of 0.39 U mg^{-1} was observed on the 2nd day which decreased to 0.01 on the 9th day. In the case of Pr2, highest specific activity noted was 0.78 U mg^{-1} on 2nd day of incubation which decreased to 0.08 on the 9th day of incubation.

Total protein showed a similar trend as that of Pr1 where the highest total protein was recorded on the 5th day of incubation. The protein content on the 2nd day of incubation was 463.10 µg which gradually increased to 761.69 µg over a period of five days of incubation and thereafter declined to 397.79 µg with increase in incubation period.

4.1.2.3.2. *Keratinase*

Keratinolytic activity was assessed by measuring the turn over product of tyrosine, change in pH and total protein in the minimal medium with poultry feather as substrate (Table 8). The keratinase activity varied from 15.93 to 73.61 U mL^{-1} min^{-1} during an incubation period of 50 days. The lowest activity was observed on the 20^{th} day with a gradual increase to 40.91 on the 30^{th} day. The highest activity of 73.61 U mL^{-1} min^{-1} was observed on the 40^{th} day after incubation which showed a decreasing trend from 50^{th} day onwards.

The pH of the culture medium recorded on each day revealed a shift in pH from neutral to alkalinity as against slight changes in control. The pH of the medium gradually increased from 7.0 in control to 7.2 on the 20^{th} day to 7.6 on 30^{th} day and 8.1 on 40^{th} and 50^{th} day.

Total protein revealed an increasing trend with the period of incubation and varied from 214.23 to 750.46 µg mL^{-1}over a period of five days of incubation. The peak activity was observed on the 40^{th} day and thereafter it declined to 520.42 µg mL^{-1}.

Keratinase activity was further ascertained by observing the degradation of feathers in culture medium. Feather degradation was visually observed from 30 days of incubation by the turbidity of the medium (Plate $4A_1$) as against clear medium with visible feather pieces in the control (Plate $4A_2$). A reduction in feather weight from 2 g to 0.38 g (80.20 %) was observed after a period of 50 days of incubation.

4.1.2.4 Chitosanase

Chitosanase activity was recorded as a measure of substrate utilization and turnover of D-glucosamine at pH 5.5. Significant difference was observed in chitosonase production with increase in incubation period (Table 9). It ranged from 0.003 to 0.260 U mL^{-1} min^{-1} from 2^{nd} to 14^{th} day, with highest activity of 0.260 on the 8^{th} day which was statistically different from the activity on all other days. The activity was statistically reduced from the 10^{th} day onwards reaching lowest level of 0.054 U $mL^{-1}min^{-1}$ on the 14^{th} day. The optimum period of incubation for highest production of chitosanase was found to be eight days.

4.1.2.5 Amylase

Starch used in the minimal medium (pH 6) induced amylase activity in *L. saksenae.* On the 2^{nd} day of incubation minimum activity of 1.36 U mL^{-1} min^{-1} was observed which was statistically on par with the activity on the 4^{th} day (2.04 U mL^{-1} min^{-1}). The activity increased significantly on the 6^{th} day (13.83 U mL^{-1} min^{-1}) and the 8^{th} day (19.14 U mL^{-1} min^{-1}). The peak activity was on the 10^{th} day (23.42 U mL^{-1} min^{-1}) (Table 10). Consequently there was a significant decrease to 14.86 U mL^{-1} min^{-1} on the 14^{th} day, which was statically on par with that observed on the 6^{th} day. The optimum time for highest production of amylase was therefore found to be 10^{th} day.

Table 8. Keratinase activity of *Lecanicillium saksenae* on chicken feather substrate

Incubation period (days)	Keratinase activity ($UmL^{-1}min^{-1}$)	Total protein (µg)	pH		Biomass of substrate (g)	
			Control	Culture medium	Before incubation	After incubation
20	15.93	214.23	7.00	7.20	2.00	0.38
30	40.91	485.65	7.00	7.60		
40	73.61	755.46	7.10	8.10		
50	52.69	520.42	7.10	8.10		

Table 9**.** Chitosanase activity of *Lecanicillium saksenae* in minimal medium with colloidal chitosan

Activity ($U\ mL^{-1}\ min^{-1}$)* at 2 day interval during the incubation period							CD (0.05)
2	4	6	8	10	12	14	
0.003^{g}	0.011^{f}	0.116^{c}	0.260^{a}	0.200^{b}	0.070^{d}	0.054^{e}	0.004

*Values are means of three replications
Values sharing same alphabets are statistically on par based on LSD

Table 10. Amylase activity of *Lecanicillium saksenae* in minimal medium with soluble starch

Activity ($U\ mL^{-1}\ min^{-1}$)* at 2 day interval during the incubation period							CD (0.05)
2	4	6	8	10	12	14	
1.35^{d}	2.04^{d}	13.83^{c}	19.14^{b}	23.42^{a}	19.86^{b}	14.86^{c}	1.279

*Values are means of three replications
Values sharing same alphabets are statistically on par based on LSD

A1- Degradation of feather (50th day)

A2- Control

Plate 4. Keratinase activity of *Lecanicillium saksenae* on chicken feather substrate

4.2. BIOEFFICACY OF ENZYMES ON INSECT PEST AND NEMATODE

Bio efficacy of partially purified chitinase, lipase and protease as well as their combination on the test insect, brinjal mealybug *C. insolita* (G) and root knot nematode *M. incognita* were studied.

4.2.1 Bioefficacy on *Coccidohystrix insolita*

4.2.1.1 On C. insolita Nymphs

4.2.1.1.1 Efficacy of Chitinase

On the first day after treatment, mortality noted in nymphs treated with lower concentration of 25µg mL^{-1} was on par with negative control. It was 12.74 and 5.00 per cent respectively. Whereas, at 48 h after treatment (HAT), higher concentrations of 50 and 100 µg mL^{-1}, resulted in significantly higher mortality of 30.87 and 46.79 per cent, respectively (Table 11).

On the third day, cumulative mortality significantly increased to 23.32, 36.27 and 57.10 per cent with 25, 50 and 100 µg mL^{-1}, respectively. The corresponding mortality observed on the 4^{th} day was 25.96, 45.12 and 58.10 per cent, respectively and the mortality at the lowest concentration diferred statistically from that of negative control.

4.2.1.1.2 Efficacy of Lipase

Application of partially purified lipase @ 25µg mL^{-1} caused 12.13 per cent mortality after 24h, which differed significantly from that of control. A significantly higher mortality of 20.47 and 38.08 per cent was recorded with 50 µg mL^{-1} and 100 µg mL^{-1}, respectively (Table 12).

Table 11. Bioefficacy of chitinase (partially purified) on *Coccidohystrix insolita* nymphs

Concentration of protein (µg mL^{-1})	Cumulative corrected mortality* of nymphs at 24 h interval (%)			
	24	48	72	96
25	12.74^{c}	17.96^{c}	23.32 ^{c}	25.96^{c}
50	23.15^{b}	30.87^{b}	36.27^{b}	45.12^{b}
100	34.49^{a}	46.79^{a}	57.10^{a}	58.10^{a}
Tris buffer	5.00^{c}	11.65^{c}	14.94^{d}	15.91^{d}
SEM	2.83	2.60	1.56	2.17
CD (0.05)	8.447	7.809	4.668	6.501

*Mean of five replications,
Values sharing same alphabets in superscript are statistically on par based on LSD

Table 12. Bioefficacy of lipase (partially purified) on *Coccidohystrix insolita* nymphs

Concentration of protein (µg mL^{-1})	*Cumulative corrected mortalityof nymphs at 24 h interval (%)			
	24	48	72	96
25	12.13^{c}	16.77^{c}	21.61c	23.99^{c}
50	20.47^{b}	30.93^{b}	35.60^{b}	36.86^{b}
100	38.08^{a}	51.76^{a}	53.61^{a}	54.23^{a}
Tris buffer	5.00^{d}	11.65^{c}	14.94^{c}	15.91^{c}
SEM	2.54	2.78	2.64	2.84
CD (0.05)	7.613	8.349	7.927	8.540

*Mean of five replications
Values sharing same alphabets in superscript are statistically on par based on LSD

Table 13. Bioefficacy of protease (partially purified) on *Coccidohystrix insolita* nymphs

Concentration of protein (µg mL^{-1})	*Cumulative corrected mortality of nymphs at 24 h interval (%)			
	24	48	72	96
25	16.71^{c}	18.90^{c}	20.85^{c}	26.28^{d}
50	24.34^{b}	32.28^{b}	38.56^{b}	43.56^{b}
100	43.30^{a}	65.46^{a}	70.17^{a}	71.79^{a}
Tris buffer	5.00^{c}	11.65^{d}	14.94^{d}	15.91^{c}
SEM	2.53	2.12	2.67	1.94
CD (0.05)	13.388	6.380	5.806	8.007

*Mean of five replications
Values sharing same alphabets in superscript are statistically on par based on LSD

After 48 HAT the death rate at lowest concentration and negative control were on par statistically (16.77 % and 11.65 % respectively). Whereas, the higher concentrations 50 and 100 µg mL^{-1}, mortality observed was 30.93 and 51.76 per cent which varied significantly among them.

The trend observed was same even after 72 and 96 HAT. In both the cases the mortality at lowest concentration 25 µg mL^{-1} was on par with that of negative control. Significantly higher mortality of 35.60 and 36.86 per cent was observed with 50 µg mL^{-1} at 72 and 96 h, respectively. Corresponding death rate noted with 100 µg mL^{-1} was 53.61 and 54.23 per cent respectively which were statistically different.

4.2.1.1.3 Efficacy of Protease

At its lowest concentration of 25 µg mL^{-1}, protease caused 16.71 per cent mortality in the nymphs, 24 HAT which was on par with that in negative control (Table 13). As the concentration increased to 50 and 100 µg mL^{-1}, mortality increased significantly to 24.34 and 43.3 per cent respectively.

After 48 h, the mortality recorded was 18.9, 32.28 and 65.46 per cent with 25, 50 and 100 µg mL^{-1} respectively.

The trend observed after 72 h and 96 h was exactly the same. In the nymphs there was 20.85 and 38.56 per cent mortality with 25 and 50 µg mL^{-1} respectively, whereas the death rate was significantly high (70.17%) when treated with 100 µg mL^{-1}. The corresponding mortality after 96 h of treatment was 26.28, 43.56 and 71.79 per cent, respectively.

4.2.1.1.4. Effect of Combined Application of Enzymes

Treatment with the combination of all the three enzymes resulted in a higher mortality of nymphs compared to individual application. After 24h, concentration

dependant mortality was observed as with the case of individual application (Table 14). Significantly high mortality of 43.30 per cent was observed with 100 µg mL^{-1}, followed by 26.58 and 18.39 per cent with 50 and 25 µg mL^{-1}, respectively and all differed statistically from that of control.

At 48 HAT, there was 20.05 per cent mortality in insects treated with 25 µg mL^{-1} of enzyme combination. Mortality significantly increased to 34.08 and 68.36 per cent when higher concentrations of 50 and 100 µg mL^{-1} were tested.

Similar trend was observed at 72 and 96 HAT. At all the concentrations mortality differed significantly among each other. Highest cumulative mortality of 74.00 per cent was recorded with 100 µg mL^{-1} after 72h, while it was 21.99 and 40.39 per cent with the lower concentrations of 25 µg mL^{-1} and 50 µg mL^{-1}. After 96 h, the death rate noted was 78.79 per cent with 100 µg mL^{-1} and 46.28 per cent with 50 µg mL^{-1} and 29.28 per cent with 25µg mL^{-1}.

4.2.1.2 On C. insolita Adults

Individual application of partially purified chitinase, lipase, and protease at highest concentration of 100 µg mL^{-1} caused very low mortality in adults *viz.* protease (35.64%), chitinase (30.25%) and lipase (26.75%) at 96 HAT. Hence the results of combined application only are presented in Table 15.

At 24 HAT the mortality with two lower concentrations such as 25µg mL^{-1} and 50 µg mL^{-1} were statistically on par with that of negative control. A significant increase in mortality (29.99%) was observed with 100 µg mL^{-1}. At 48 and 72 HAT exactly similar trend was observed where the lower concentrations were on par with negative control, whereas, the higher concentrations of 100 µg mL^{-1}, resulted in significantly higher mortality of 33.43 and 36.15 per cent respectively at 48 and 72

Table 14. Combined effect of Chitinase, protease and lipase on *Coccidohystrix insolita* nymphs

Total concentration of protein (µg mL^{-1})	*Cumulative corrected mortality of nymphs at 24 h interval (%)			
	24	48	72	96
25	18.39^{d}	20.05^{d}	21.99^{c}	29.28^{d}
50	26.58^{b}	34.08^{b}	40.39^{b}	$46.28.^{b}$
100	43.30^{a}	68.36^{a}	74.00^{a}	78.79^{a}
Tris buffer	5.00^{c}	11.65^{c}	14.94^{d}	15.91^{c}
SEM	2.95	3.10	2.30	1.87
CD (0.05)	13.388	6.380	5.806	8.007

*Mean of five replications

Values sharing same alphabets are statistically on par based on LSD

Table 15. Combined effect of Chitinase, protease and lipase on *Coccidohystrix insolita* adults

Concentration of protein (µg mL^{-1})	*Cumulative corrected mortality at 24 h interval (%)			
	24	48	72	96
25	17.38^{b}	17.92^{c}	18.25^{c}	23.68^{c}
50	19.54^{b}	22.64^{b}	25.09^{b}	35.29^{b}
100	29.99^{a}	33.43^{a}	36.15^{a}	40.88^{a}
Tris buffer	13.47^{b}	14.10^{c}	14.92^{c}	16.84^{d}
SEM	2.33	2.53	2.03	1.30
CD (0.05)	6.282	3.921	5.478	3.526

*Mean of five replications

Values sharing same alphabets are statistically on par based on LSD

HAT. After 96 h mortality was 40.88 35.29 and 23.68 per cent with 100, 50 and 25 µg mL^{-1}. The corresponding mortality in negative control was 16.84 per cent.

4.2.2 On *M. incognita*

4.2.2.1 *Pathogenicity of L. saksenae* on *M. incognita*

Pathogenicity of *L. saksenae* on root knot nematode eggs was ascertained prior to bioefficacy studies on *M. incognita.*

Sequence of events in the pathogenicity of *L. saksenae* on eggs of *M. incognita* is presented in Plate 5. Growth of hyphae towards the egg mass was observed on the 3rd day of incubation. The hyphae were found to come in contact with the gelatinous matrix and then started growing on the surface of the egg mass on the 5th day. Prolific growth of mycelia was observed on the surface of the egg mass on the 7th day and was completely covered on the 8th day. Observation of the infected egg mass on the 10th day under zoom stereo microscope revealed complete disintegration of egg mass leading to total inhibition on hatching (Plate 5 F).

4.2.2.2 Efficacy of Chitinase

The effect of partially purified chitinase on *M. incognita* eggs is given in Table 16. Hatching was found to be significantly suppressed compared to absolute control and negative control. The number of juveniles emerged was inversely proportional to protein concentration of chitinase.

After 24 h of incubation, the hatching observed was 3.80 per cent in the eggs treated with 25 µg mL^{-1}. When treated with 50 and 100 µg mL^{-1}, the hatching was reduced to 2.2 and 0.8 per cent. In eggs treated with Tris buffer, the negative control it was 4.0 per cent and in absolute control it was 5.8 per cent.

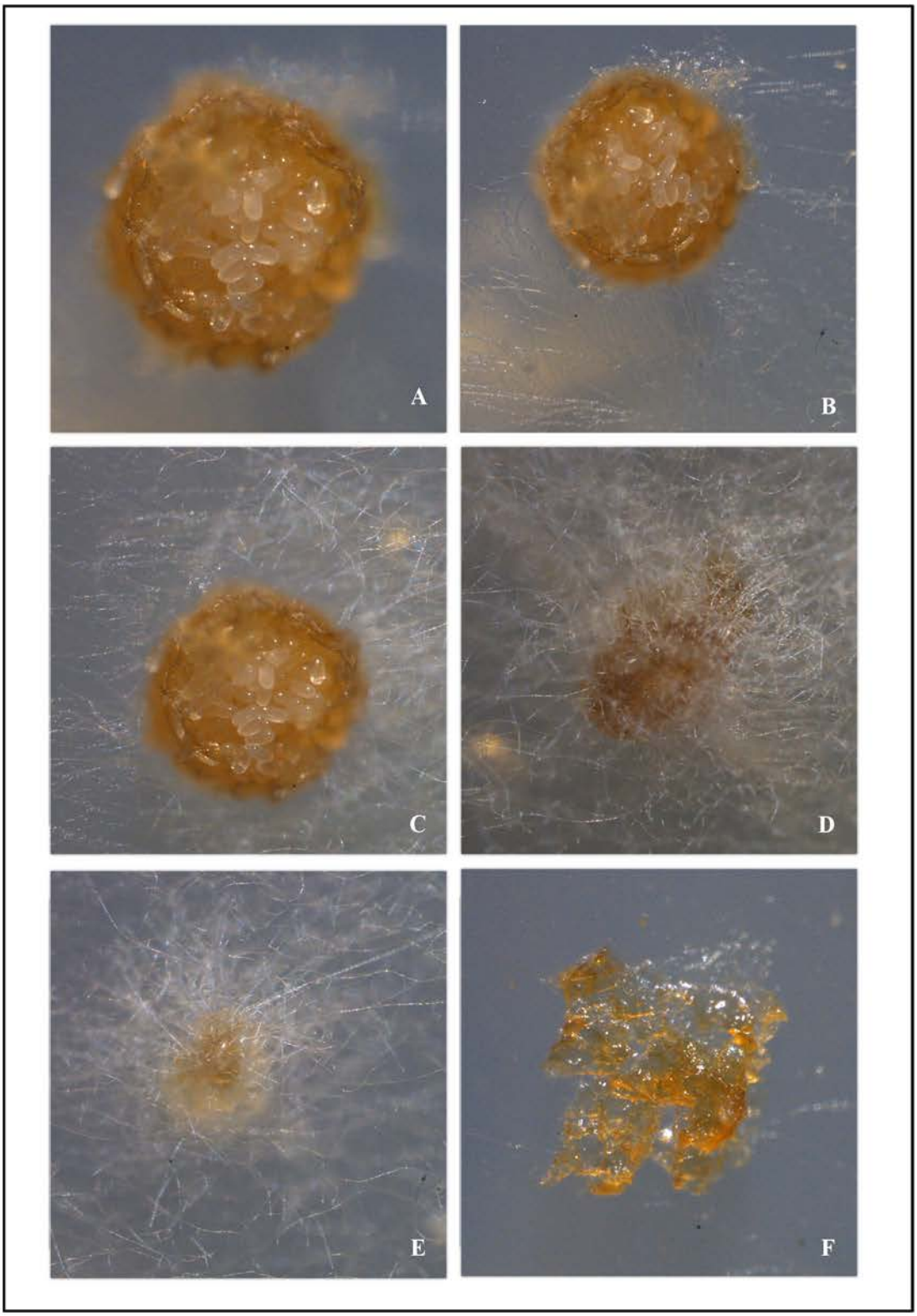


Plate 5. Sequential events in parasitism by *Lecanicillium saksenae* on *Meloidogyne incognita* egg mass (Magnification 80X).

A. Egg mass of *M. incognita* B. Growing of hyphae towards egg mass.
C. Hyphal attachment to egg mass. D. Proliferation of mycelia E.Proliferation of mycelia F. Disintegration of egg mass

Table 16. Effect of partially purified chitinase on *Meloidogyne incognita* eggs

Concentration of protein (µg mL^{-1})	* Hatching at 24 h interval (%)				
	24	48	72	96	120
25	3.80^{b} (11.04)	7.00^{b} (15.29)	11.60^{c} (18.61)	16.00^{c} (23.55)	29.02^{c} (32.68)
50	2.20^{c} (8.39)	6.20^{bc} (14.36)	10.60^{c} (19.50)	15.00^{c} (20.93)	25.80^{d} (25.67)
100	0.80^{c} (6.70)	5.40^{c} (12.64)	10.00^{bc} (20.24)	12.60^{c} (22.73)	18.80^{c} (30.45)
Tris buffer	4.00^{ab} (11.47)	7.00^{b} (15.27)	13.40^{b} (21.44)	33.60^{b} (36.49)	55.4^{b} (48.23)
Control	5.80^{a} (13.83)	12.6^{a} (20.73)	16.20^{a} (23.39)	41.40^{a} (42.34)	70.8^{a} (58.75)
SEM	0.82	0.69	0.62	1.06	1.15
CD (0.05)	2.429	2.035	1.816	3.125	3.412

*Mean of five replications. Figures in parentheses are arc sin transformed values. Values sharing same alphabets are statistically on par based on LSD

Table 17. Effect of partially purified protease on *Meloidogyne incognita* eggs

Concentration of protein (µg mL^{-1})	* Hatching at 24 h interval (%)				
	24	48	72	96	120
25	2.60 ^{bc} (8.98)	7.40^{b} (15.61)	9.80^{b} (17.50)	16.60^{c} (23.96)	17.60^{a} (24.74)
50	3.60^{b} (10.73)	6.60^{b} (14.84)	8.20^{bc} (16.57)	11.00^{d} (19.29)	11.80^{d} (20.03)
100	1.60^{c} (7.54)	5.00^{c} (11.35)	6.00^{c} (13.99)	9.00^{d} (17.38)	10.40^{d} (18.76)
Tris buffer	4.00^{ab} (11.47)	7.00^{b} (15.27)	13.40^{a} (21.44)	33.60^{b} (36.49)	55.4^{b} (48.23)
Control	5.80^{a} (13.83)	12.60^{a} (20.73)	16.20^{a} (23.39)	41.10^{a} (42.34)	70.80^{a} (58.75)
SEM	0.96	0.94	0.94	1.11	1.07
CD (0.05)	2.844	2.783	2.782	3.284	3.166

*Mean of five replications. Figures in parentheses are arc sin transformed values Values sharing same alphabets are statistically on par based on LSD

After 48 h, the hatching was significantly lower when treated with 50 and 100 µg mL^{-1} (6.2 and 5.4%) compared to the treatment with lower concentration 25 µg mL^{-1} which was on par with negative control (7.00%) but differed significantly from absolute control (12.6%).

After 72 h, the hatching noted was statistically on par in all the concentrations but significantly lower than those in control. It was 11.60, 10.60 and 10.00 per cent with 25 µg mL^{-1}, 50 µg mL^{-1} and 100 µg mL^{-1} respectively.

After 96 h, hatching noted was 16.00, 15.00, and 12.60 per cent, which were statistically on par each other but differed significantly from negative and absolute control (33.60 and 41.40 % respectively).

At the end of the experimental period (120 h) the trend observed was same. The hatching was significantly lower in all the test concentrations compared to control. Highest inhibition was noted with 100 µg mL^{-1} where the hatching was only 18.8 per cent, followed by 25.80 per cent (50 µg mL^{-1}) and 29.02 per cent (25 µg mL^{-1}). The corresponding values observed in absolute control was 70.8 per cent and that in negative control was 55.4 per cent.

4.2.2.3 Efficacy of Protease

Protease inhibited hatching significantly at varying levels. After 24 h, there was significant reduction in hatching compared to that in control (Table 17). Hatching was 3.60 per cent with 25 µg mL^{-1} which reduced to 2.60 and 1.6 per cent with 50 µg mL^{-1} and 100 µg mL^{-1} respectively. Hatching was 4.00 and 5.80 per cent with negative and absolute control.

Similarly, after 48 h, hatching was lower in all the treatments and differed significantly from control. Hatching noted with 25 and 50 µg mL^{-1} was 7.40 and

6.60 per cent which were on par while it was significantly reduced to 5.00 per cent with the highest concentration of 100 µg mL^{-1}. The corresponding hatching in negative control and absolute control was 7.0 and 12.6 per cent, respectively and both of them deferred significantly among each other.

The trend observed was more or less same after 72 h. Significant differences were observed with different concentrations. Lowest hatching (6.00 %) was observed with 100 µg mL^{-1} followed by 8.20 and 9.80 per cent with 50 and 25 µg $mL^{-.1}$ respectively. In the absolute control, the egg hatching was 16.2 per cent, while in negative control the hatching was 13.4 per cent.

Inhibition in hatching was more evident after 96 h of treatment, where there was only 9.00 per cent hatching in 100 µg mL^{-1} which was on par with that of 50 µgmL^{-1}. The corresponding value in absolute control was 41.1 per cent and that in negative control was 33.6.

Similar trend was observed at 120 h, the hatching was 17.6 per cent with 25 µg mL^{-1}, which significantly reduced to 11.8 and 10.4 at 50 and 25µg mL^{-1} which were statistically on par. Highest hatching was observed with absolute control (70.8 %) while it was 55.40 per cent with negative control.

4.2.2.4 Combined Effect of Chitinase and Protease

4.2.2.4.1 On Egg Hatching

Potease and chitinase when applied in combination, strongly inhibited hatching in *M. incognita* (Table 18). Hatching inhibition was inversely proportional to the concentration of protein. After 24 h, all the treatments significantly reduced hatching. It was only 1.00 per cent with 100 µg mL^{-1} and 1.20 and 2.4 per cent with 50 and 25 µg mL^{-1}, respectively. The corresponding values in control were 5.8 per cent (absolute control) and 4.00 per cent (negative control).

Table18. Combined effect of chitinase and protease on *Meloidogyne incognita* eggs

Concentration of protein (µg mL^{-1})	* Hatching at 24 h interval (%)				
	24	48	72	96	120
25	2.40^{c} (8.33)	6.40^{b} (14.61)	8.00^{c} (16.37)	10.60^{c} (18.95)	11.40^{c} (19.71)
50	1.20^{cd} (6.69)	6.00^{b} (13.99)	7.40^{b} (15.59)	9.80^{c} (17.50)	10.40^{c} (17.76)
100	1.00^{d} (5.739)	3.40^{c} (10.51)	5.60^{d} (13.60)	7.20^{d} (15.46)	7.80^{d} (16.14)
Tris buffer	4.00^{ab} (11.47)	7.00^{b} (15.27)	13.40^{a} (21.44)	33.60^{b} (36.49	55.40^{b} (48.23)
Control	5.80^{a} (13.83)	12.60^{a} (20.73)	16.20^{a} (23.39)	41.10^{a} (42.34)	70.80^{a} (58.75)
SEM	0.69	0.75	0.70	1.08	0.97
CD (0.05)	2.057	2.220	2.086	3.204	2.875

*Mean of five replications. Figures in parentheses are arc sin transformed values
Values sharing same alphabets are statistically on par based on LSD

Table 19. Activity and yield of purified protease of *Lecanicillium saksenae*

Purification Steps	Total Enzyme Activity (U)	Total Protein (mg)	Specific Activity (U mg^{-1} of protein)	Purification fold	Yield (%)
Crude extract	908	61.91	14.67	1.0	100
Ammonium sulphate, post dialysis	693	5.73	120.82	8.23	76.30
Sephacryl G-100 column	560	0.84	665.90	45.38	61.69
DEAE- Cellulose	133	0.10	1306.62	89.04	14.68

After 48 h, hatching observed with 25 and 50 µg mL^{-1} were on par with that of negative control, the values being 6.4, 6.0 and 7.0 per cent respectively. The hatching was 8.00, 7.40 and 5.60 per cent at different concentrations of 25, 50, 100 µg mL^{-1} respectively, while in negative control it was 13.40 per cent and in absolute control it was 16.20 per cent.

Similar results were observed after 96 h also. Lowest hatching was observed with 100 µg mL^{-1}, where only 7.2 per cent of the eggs hatched which differed significantly from that in control. Hatching observed with 25 and 50 µg mL^{-1} was 10.6 and 9.8 per cent respectively which were on par with each other.

After 120 h, the same trend was observed where the lowest hatching observed was 7.80 per cent in eggs treated with 100 µg mL^{-1}. At 25 and 50 µg mL^{-1} the hatching observed was 11.4 and 10.4 per cent respectively which were on par with each other. In absolute control 70.8 per cent of eggs hatched while it was 55.4 per cent in negative control.

In all the treatments significant differences were observed between the hatching of eggs in Tris buffer and water, except at 72 h.

4.2.2.4.2 Morphological Changes in Egg Shell

Combined application of chitinase and protease exhibited aberrant changes in the egg shell. Microscopic examination of treated eggs revealed characteristic and visible deformities. The eggs were found to be vacuolated and transparent, indicating damage to the developing embryo and egg content, whereas the water treated eggs developed normally and hatched readily in water.

Scanning Electron Microscopy (SEM) analysis of the enzyme treated eggs revealed morphological changes in the outer vitelline layer of the egg shell (Plate

A1- Egg treated with partially purified protease + chitinase

A2- Control

Plate 6. SEM image of *Meloidogyne incognita* egg

6A). The structural damages included uneven and deformed egg shell surface along with shrinkage and loss of egg shell integrity. Lines of breakage were observed at different points on the outer vitelline layer. Aberrant changes in shape and reduction in size were also observed. Eggs treated with sterile distilled water (control), were of normal size and shape and the outer protein layer was intact and smooth (Plate 6B). They developed normally and hatched to second stage juveniles (J_2).

4.3. MAJOR ENZYME PURIFIED AND CHARACTERISED

4.3.1. Purification of Protease

Protease was the most bioactive enzyme identied in the bioefficacy studies compared to chitinase and lipase. Hence, protease was purified through three step process (Table 19). It was observed that with each level of purification the total enzyme activity and total protein got reduced. When the total enzyme activity of crude extract was 908 U L^{-1}, the purification fold was 1 per cent and at 8.23 fold purification total enzyme activity and total protein were 693 U L^{-1} and 5.73 mg L^{-1} respectively. As the purification fold increased to 45.38 and 89.04 per cent, total enzyme activity was found to decrease from 560 to 133 UL^{-1} and total protein from 0.84 to 0.10 mg L^{-1} respectively.

The specific activity of purified enzyme was found to increase logarithmically with each step of purification. When the crude enzyme recorded 14.67 U mg^{-1}, 89.04 per cent purified enzyme exhibited 1306.62 U mg^{-1}. The yield of protease was found to reduce at each step and on completion of purification the yield was 14.68 per cent.

The elution profile of the enzyme preparations in Sephacryl G-100 chromatography and DEAE-Cellulose Fast Flow column are depicted in Fig. 7 and Fig. 8 respectively. In Sephacryl G-100 chromatography, fractions 8, 9 and

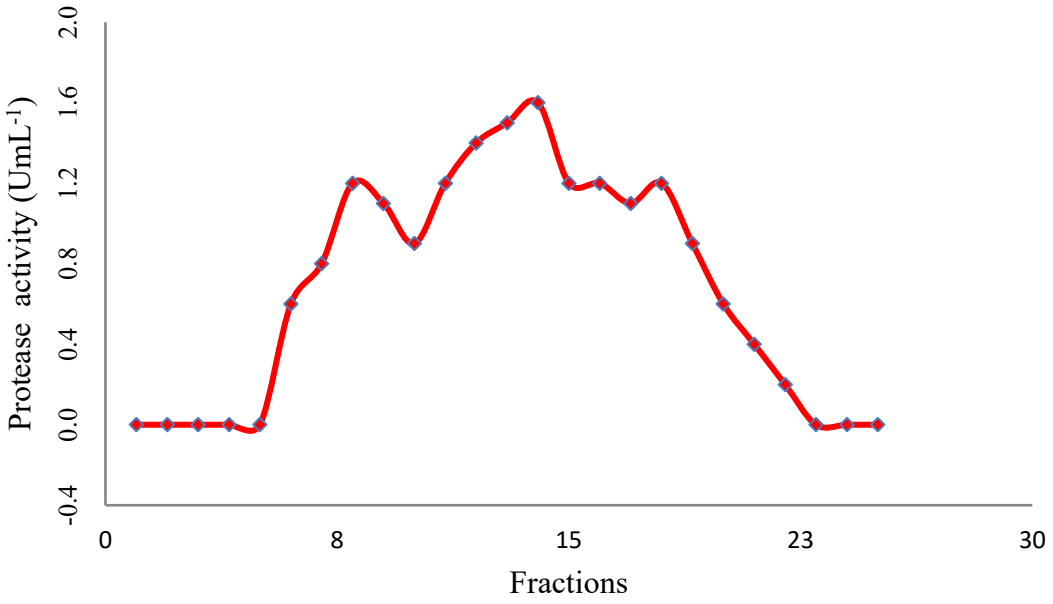


Fig.7 Elution profile of protease on Sephacryl G-100 column

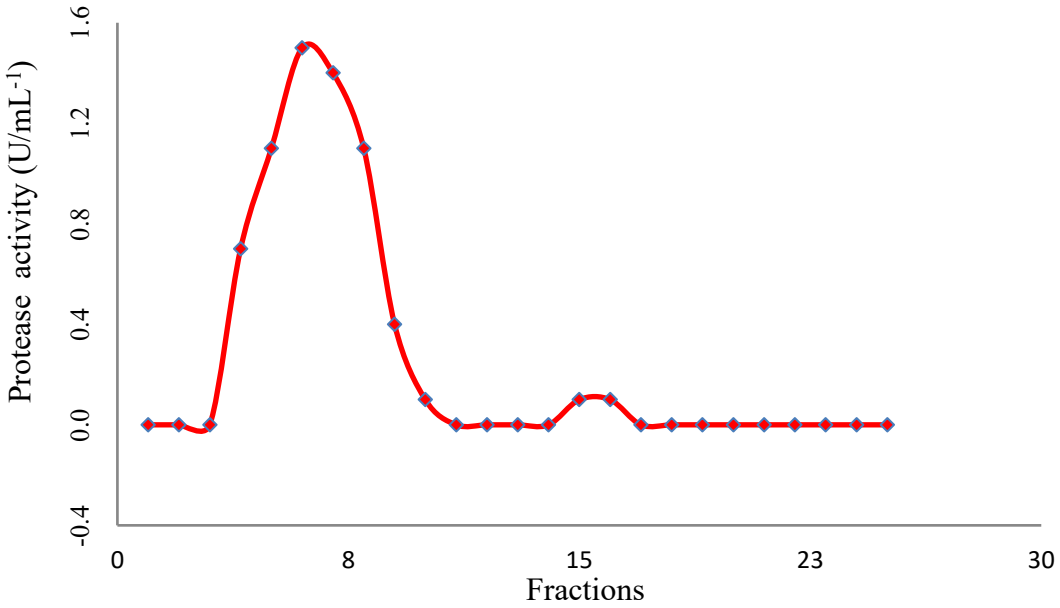


Fig. 8 Elution profile of protease on DEAE Cellulose column

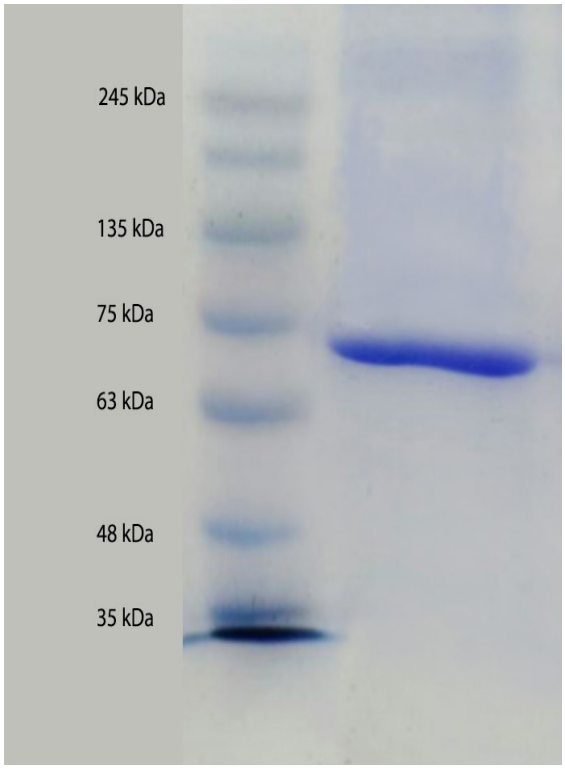


Fig. 9 SDS -PAGE Pattern of protease of *Lecanicillium saksenae*

11-18 showed the highest proteolytic activities (Fig. 7). In DEAE-Cellulose Fast Flow column, fractions 5-8 showed highest protease activities (Fig. 8).

4.3.2. Determination of the Molecular Weight of Purified Protease

The purified protease on analysis with SDS - PAGE showed a single band on staining with Coomassie brilliant blue G 250 indicating the homogeneity of the preparation. A single band was visualised on SDS - PAGE using gel documentation system and molecular weight of the purified protease was identified as 71 kDa (Fig. 9).

4.4 MYCOTOXINS PRODUCED BY *L. SAKSENAE*

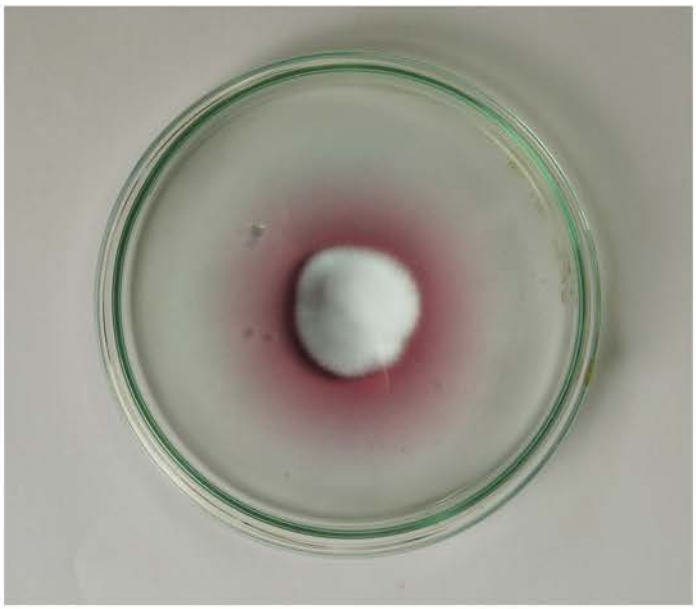
The major toxins of *L. saksenae* identified from ethyl acetate fractions of culture filtrate of different growth media are described below.

4.4.1 Major Toxins Detected and Characterised

4.4.1.1 Oosporein

L. saksenae cultured in PDA was found to support highest production of pigment/ toxin compared to other media such as Czapek Dox medium and Fargues medium (Plate 7). Its production could be detected in the medium from the 3rd day onwards by the presence of pink pigmentation which later turned wine red by the 7th day (Plate 8).

Ethyl acetate extract fraction of 1L of culture filtrate under vacuum evaporation yielded 600 mg of reddish brown crude toxin (Plate 9 A). Crystallisation of the crude toxin in acetonitrile yielded an insoluble compound, which upon further purification by washing with hexane yielded 100 mgL^{-1} pure toxin. Finally orange red crystals (100 mg) were obtained as the purified compound (Plate 9B).



A. 6th day after inoculation

B. 12th day after inoculation

Plate 7. Pigment/ Mycotoxin (oosporein) in *Lecanicillium saksenae*

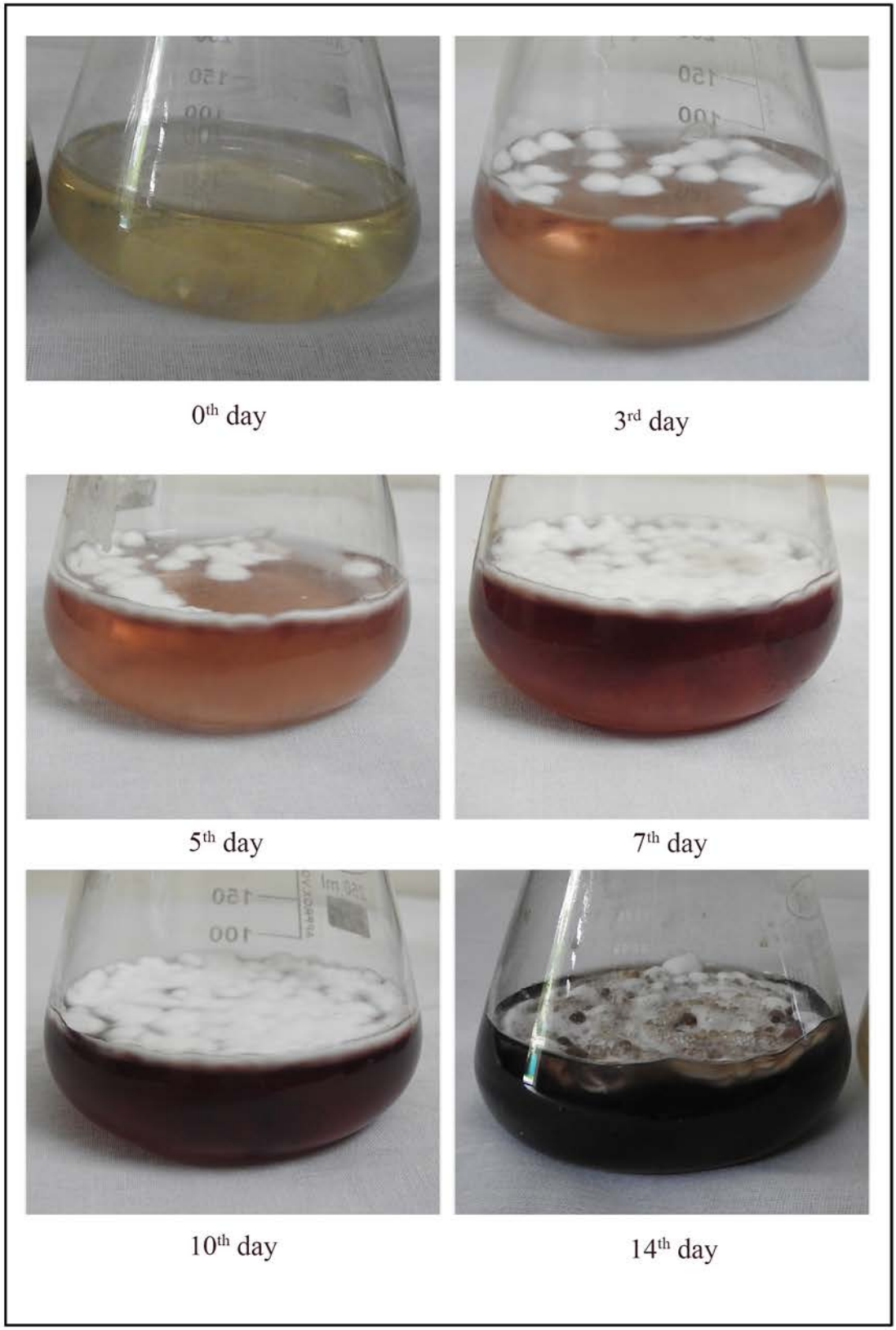


Plate 8. Pigmentation of *Lecanicillium saksenae* in PDB

A. Crude toxin

B. Purified mycotoxin (Oosporein)

Plate 9. Mycotoxin in *Lecanicillium saksenae*

4.4.1.1.1 Structural Characters

Structural characterisation of the purified compound carried out using a combination of spectral analysis tools *viz.* UV spectrometry, FTIR, HR-LCMS, ^{1}H NMR and ^{13}C NMR analysis are described here under.

The uv max wave length (λ max) of the compound detected under UV spectrometry was 281 nm (Fig. 10), which revealed one of the chemical identities of oosporein. The molecular weight (m/z) of the purified compound detected through HR-LCMS analysis was 306.22 (Fig. 11), with an empirical formula $C_{14}H_{10}O_{8}$.

The functional groups present in the molecule, studied through FTIR spectrum analysis of the compound showed the presence of different absorption peaks that confer various functional groups of the molecule. The prominent peak observed at 3308.37 cm^{-1}denoted the chemical bonds due to stretching of O-H groups (Fig. 12). The absorption peak at 1648.40 cm^{-1} is attributed to C=O stretching of ketone group, while the strong band at 1625.01cm^{-1} is assigned to C=C stretching. Presence of the functional groups O-H, C=O and C=C suggests a dibenzoquinone molecule. The other peaks detected in the compound and their corresponding wave numbers are depicted in Table 20.

Analysis of the ^{1}H NMR data revealed resonances for two methyl and four hydroxyl groups. A broad peak at δ=11.093 ppm confirmed four hydroxyl protons and single peak at δ=1.822 ppm confirmed the presence of two methyl protons (Fig. 13). The ^{13}C NMR spectrum did not reveal all the carbon signals though three carbon resonances including one methyl carbon at δ_C 8.06 and two olefinic carbons at δ_C 113.27 and δ_C 107.78 (Fig. 14), were expressed. NMR analysis thus clearly indicated the number of C, H, OH and CH_3 groups which revealed the characteristic of oosoprein.

Table 20. Functional groups detected in the infrared spectrum of oosporein

Sl. No	Wave numbers (cm^{-1})	Peak assignment	Mode of vibration	Functional groups
1	3440.18	-OH Stretching	Broad	Alcohol or phenol
2	3308.37	-NH Stretching	Medium	Aliphatic amines
3	2931.26	-CH Stretching	Medium	Alkane
4	1648.40	-C=C Stretching	Strong	Alkene
5	1625.01	-C=C Stretching	Strong	Unsaturated Ketone

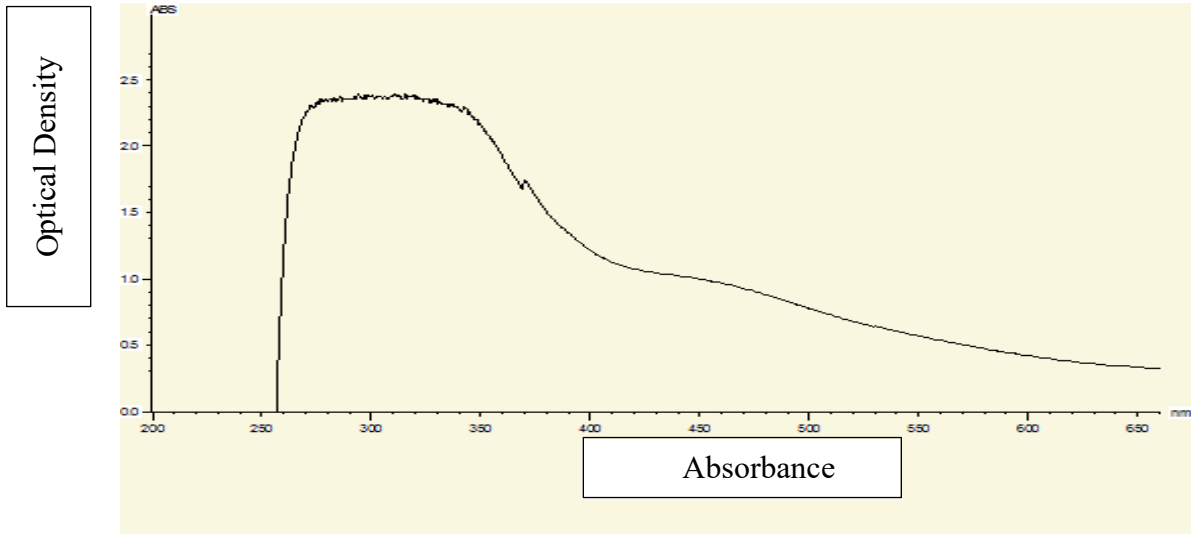


Fig. 10 UV Spectrum of oosporein

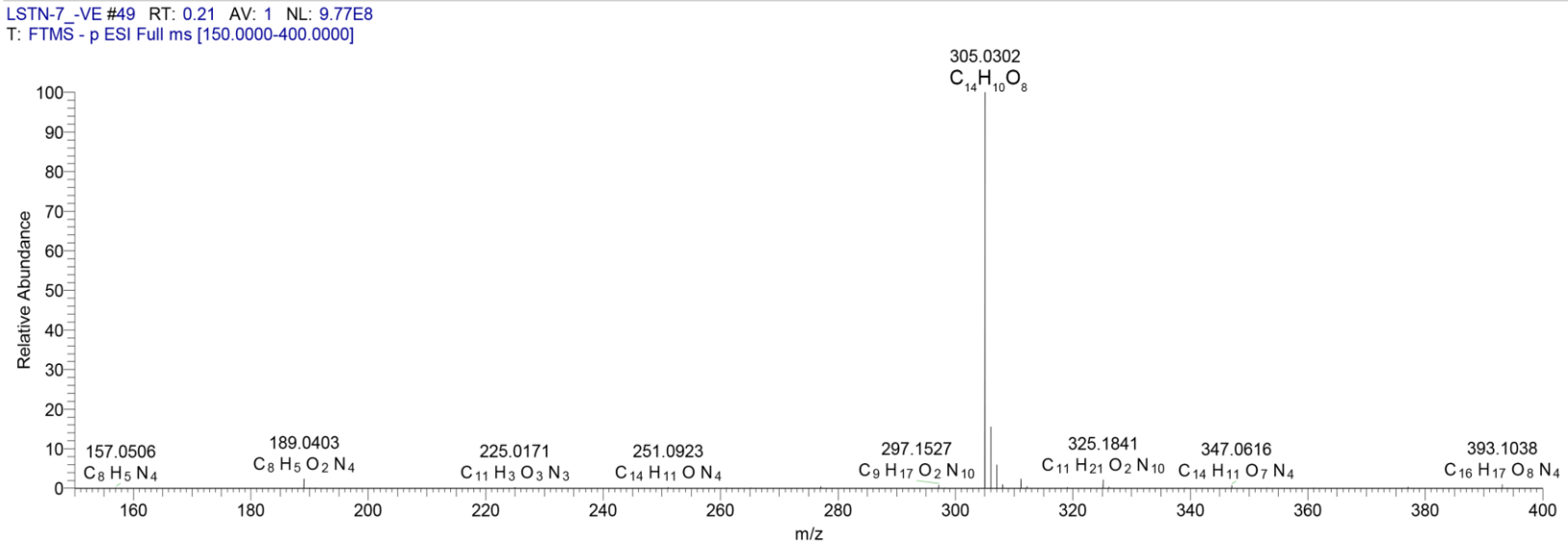


Fig. 11 HR-ESI-MS spectrum of oosporein

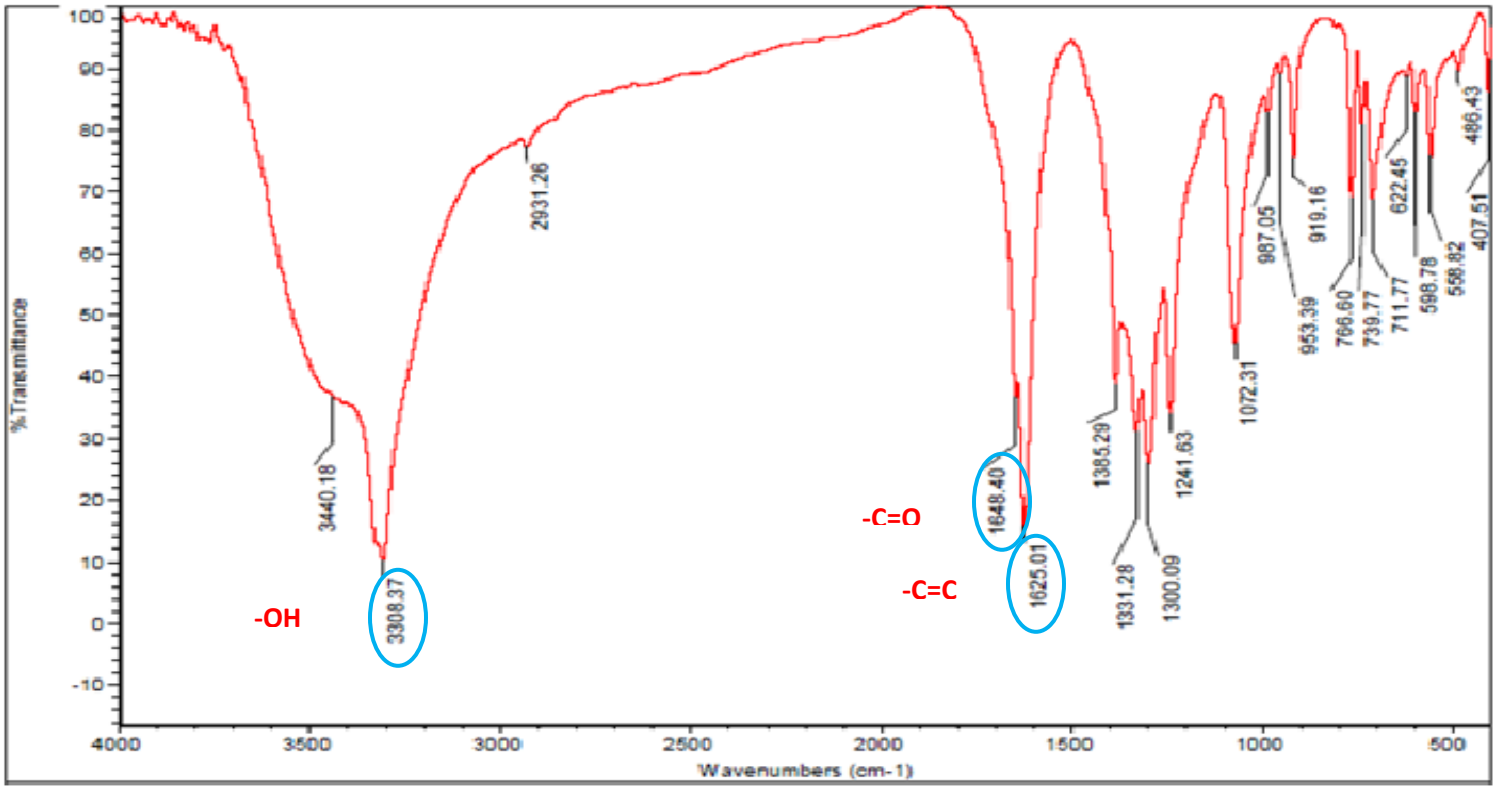


Fig. 12 FTIR spectrum of oosporein

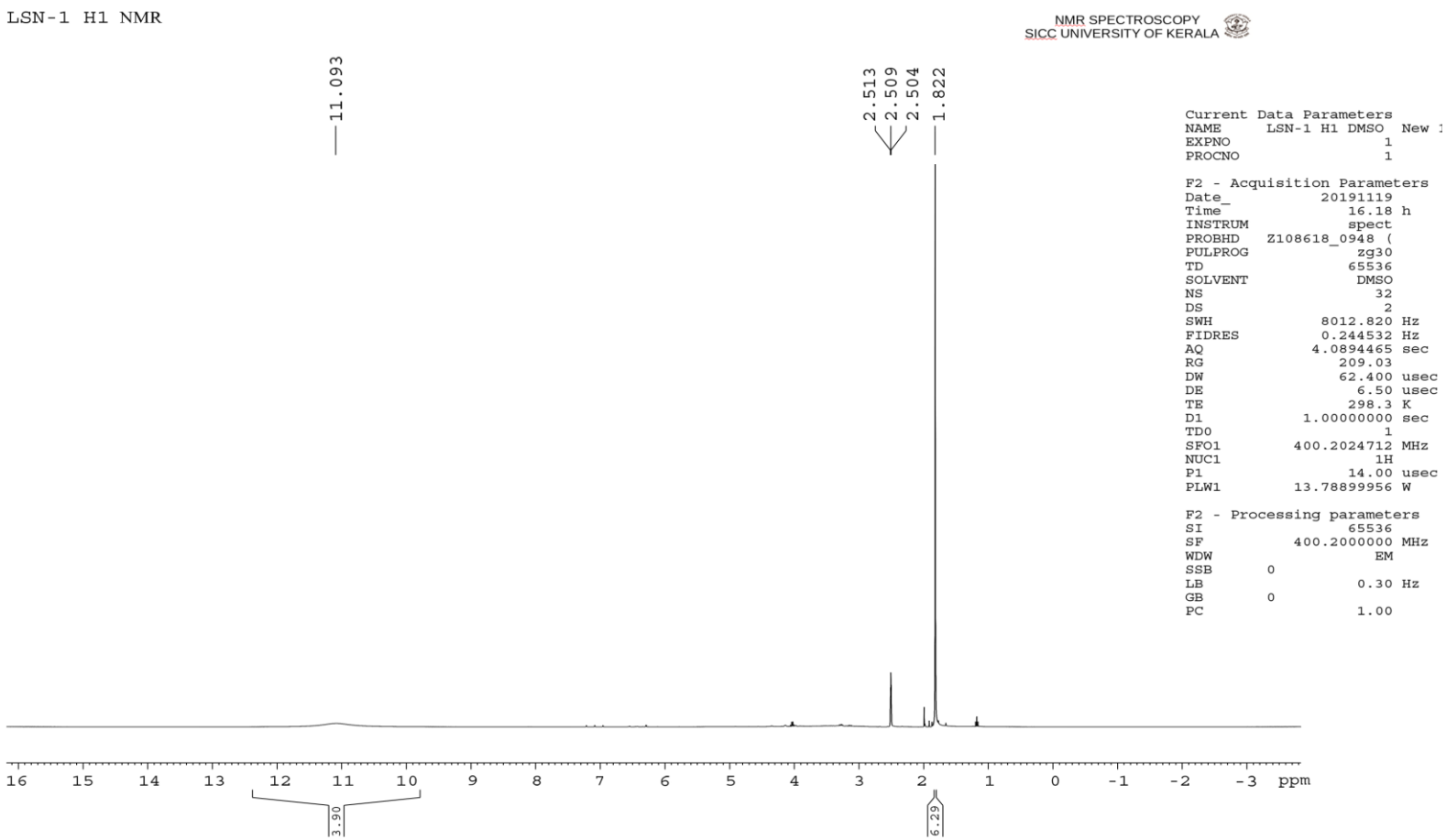


Fig. 13 ^{1}H NMR spectrum of oosporein

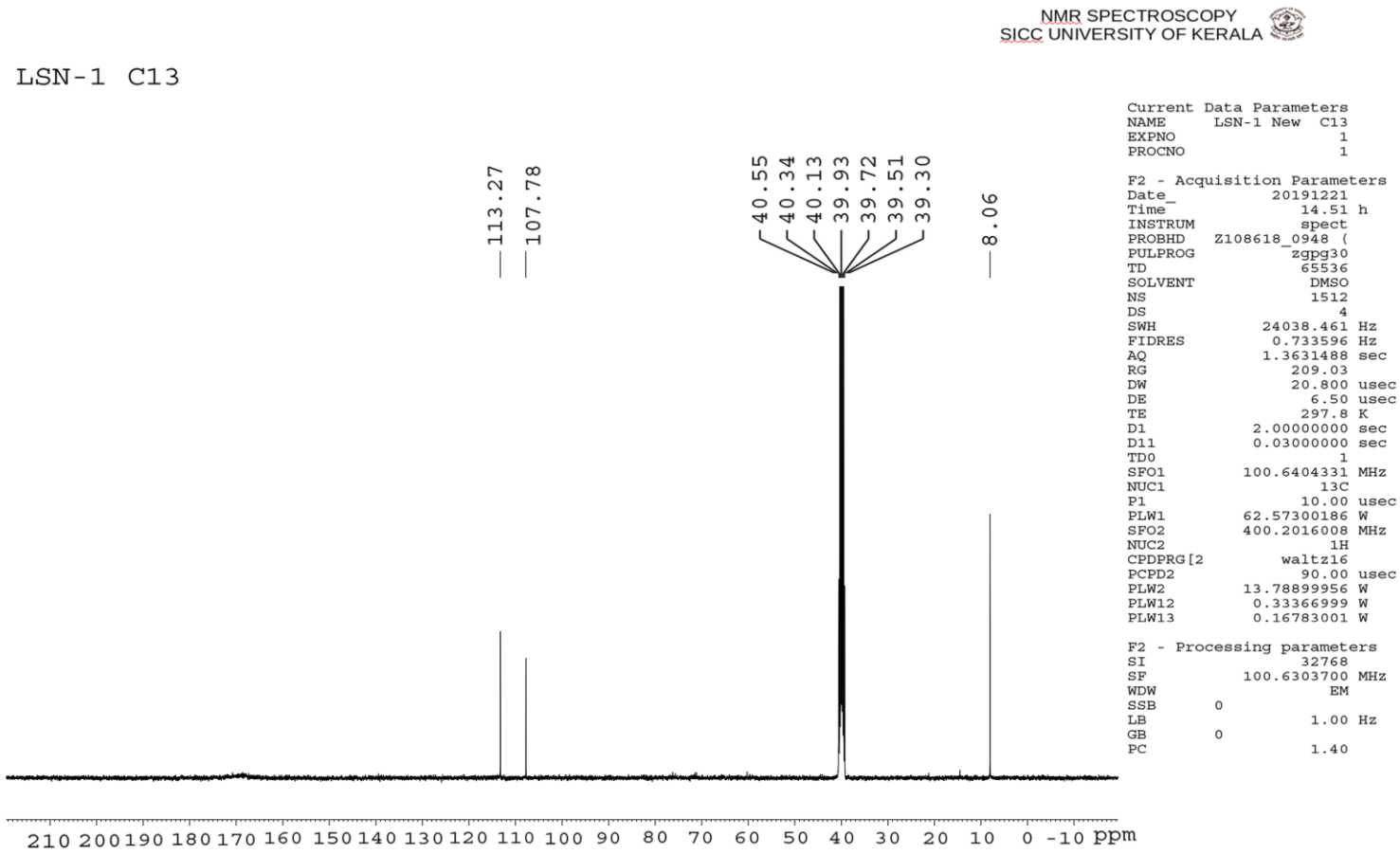
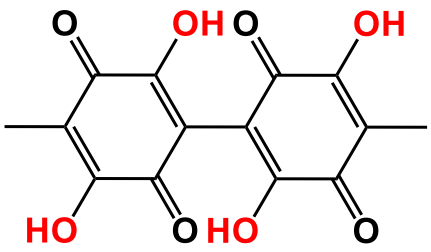


Fig. 14. ^{13}CNMR spectrum of oosporein

Spectral analysis thereby proved unambiguously the metabolite as 3,3',6,6'-tetrahydroxy-4,4'-dimethyl-1,1'-bi-*p*-benzoquinone, *i.e.* oosporein. The empirical formula of the purified compound was thus confirmed to be $C_{14}H_{10}O_8$.

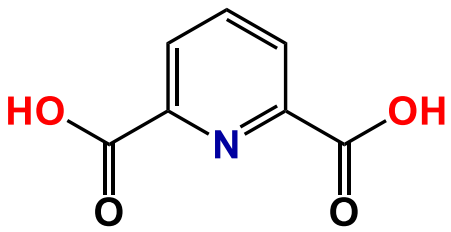
4.4.1.1.3 Structure Elucidation

The structural formula of the compound drawn using the software Chem draw professional, Cambridge Soft from Perkin Elmer using the NMR data is as below



4.4.1.2 Dipicolinic Acid (DPA)

L. saksenae grown in FM, subjected to LC-ESI- MS analysis revealed the presence of DPA which is a known insecticidal compound. LC-ESI-MS chromatogram of technical DPA standard (Fig.15 A), whose electron ionization (EI) MS spectrum data showed a single major peak, had a retention time (RT) of 2.012 min and mass (m/z) of 166.0317. The crude toxin of *L. saksenae* in methanol also exhibited a similar dominant peak (Fig. 15B) corresponding to that of the standard with RT, 2.146 min and m/z 166.0230, which revealed the presence of DPA.



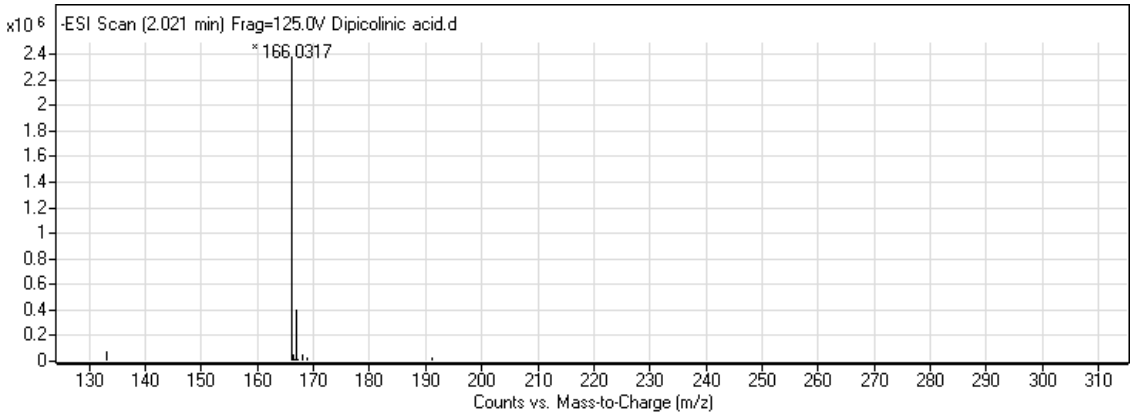


Fig. 15 A. LC-ESI- MS chromatogram of dipicolinic acid standard

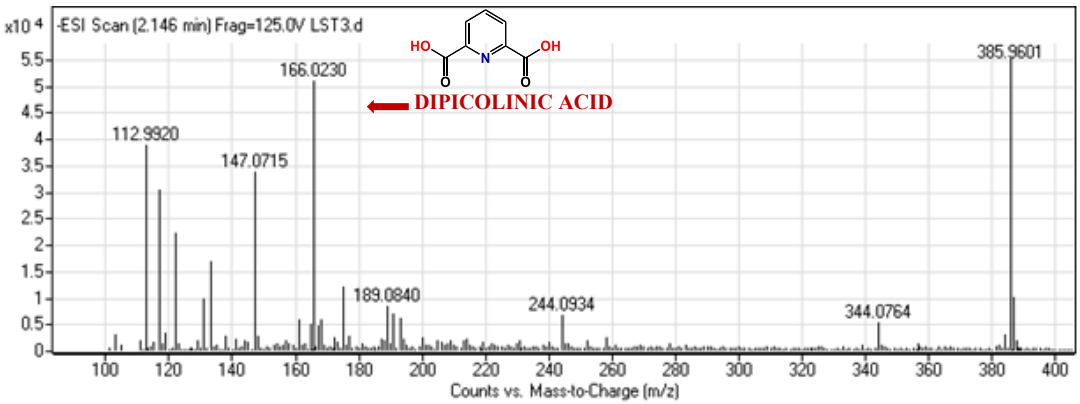


Fig.15 B LC ESI MS chromatogram of crude toxin

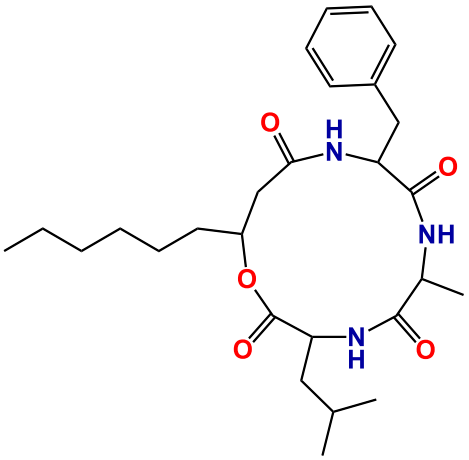
Identity of the other three dominant peaks seen in the chromatogram with different m/z of 112.9920, 147.0145 and 385.9601 could not be confirmed. Presence of dipicolinic acid was also detected in different culture media such as PDB, CDM and FM through GC-MS and HR-LCMS analysis

4.4.1.3 Beauverolides

The crude toxin of *L. saksenae* subjected to silica gel chromatography yielded nine fractions which were pooled together into five fractions (F1 to F5), based on TLC patterns.

Fractionation of crude toxin on *C. insolita* through column chromatography and the bioefficacy carried out with the fractions indicated that the fraction F4 was more bioactive causing 64. 23 per cent mortality 96 HAT (Fig. 16). All other fractions expressed weak activity ranging from 0 - 47.50 per cent.

LC-MS chromatogram of F4 in the negative mode illustrated only a single peak with a RT of 1.059 to 2.013 min. The spectral data of the peak showed strong signal for ion with m/z 485.6 (Fig. 17), which was the identity of beauverolide. The structure of beauverolide interpreted using chem draw is shown below.



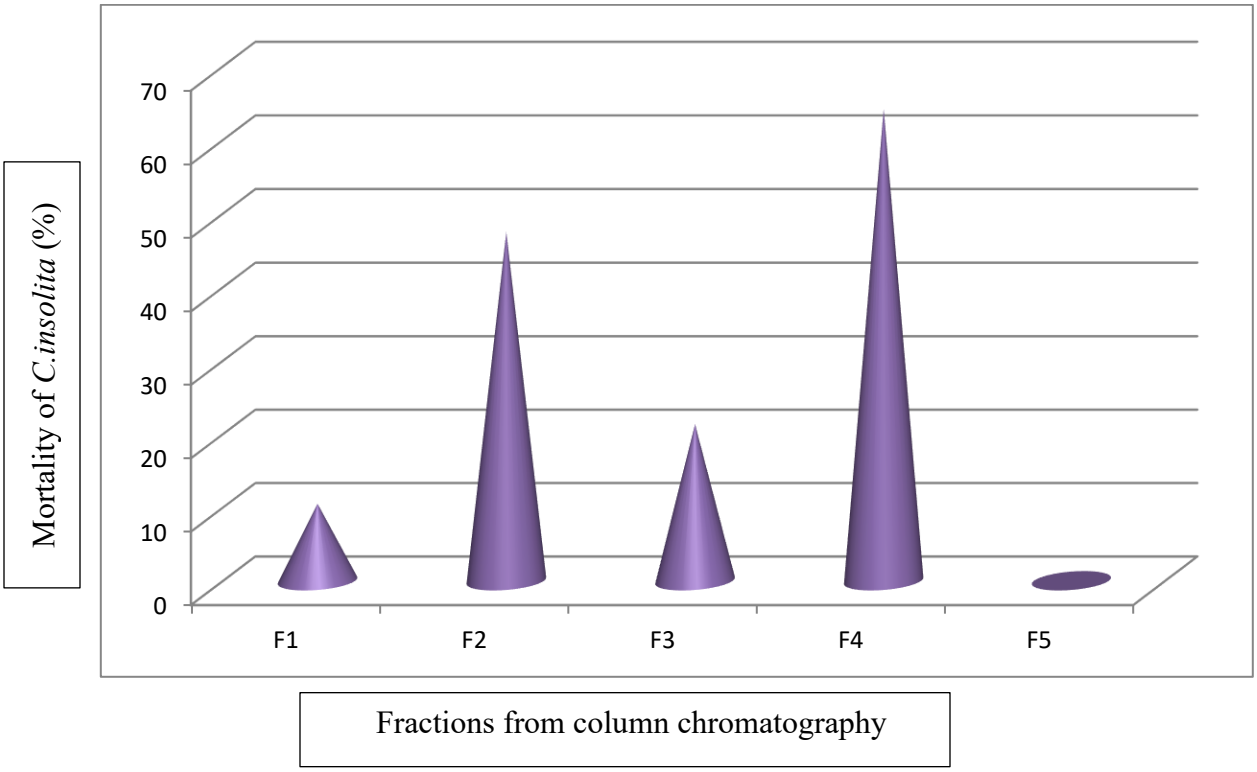


Fig. 16 Insecticidal activity of different fractions of *Lecanicillium saksenae* toxins

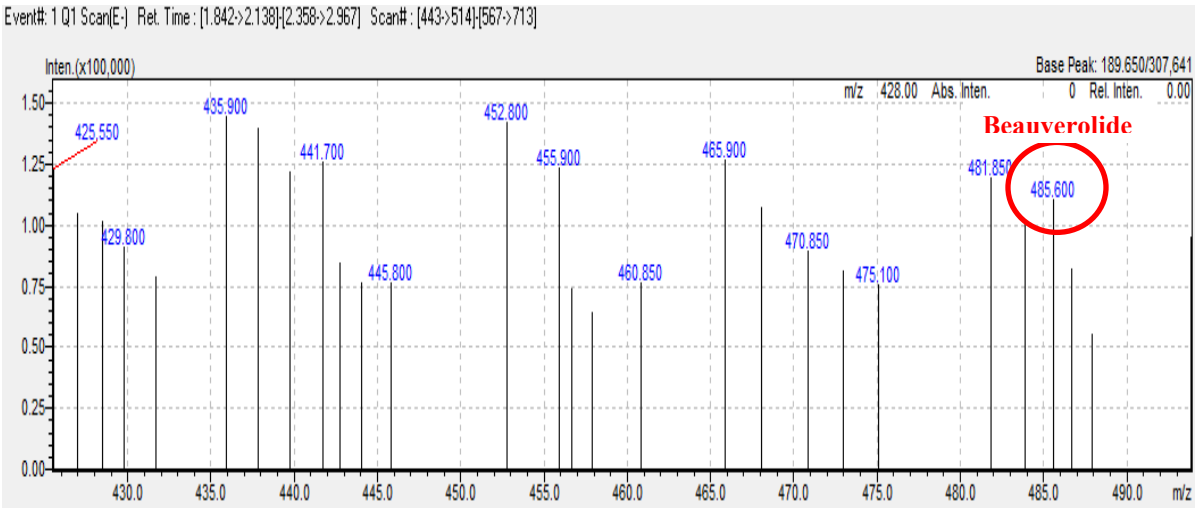


Fig. 17 Mass spectral analysis of the insecticidal fraction of metabolites

4.4.1.4 Absence of Beauvericin

The crude toxin of *L. saksenae* cultured in PDB was analysed by High Performance Liquid Chromatography (HPLC) using beavericin as standard. HPLC chromatogram of technical beauvericin is represented in Fig. 18 A. The single confirmatory peak of beauvericin (in addition to solvent peak) had a RT of 10.82 min occupying nearly 100 per cent area of the spectrum. The chromatogram of the samples from culture filtrate and mycelia subjected to analytical HPLC are depicted in Fig. 18 B and 18 C, respectively. The peak corresponding to beauvericin standard was not detected in either of the chromatogram, indicting its absence in the crude toxin of *L. saksenae.*

4.4.2 Other Secondary Metabolites of *L. saksenae*

4.4.2.1 Compounds Detected Through HR-LCMS/ GC-MS

The crude toxin from culture filtrate subjected to advanced spectroscopic methods such as HR-LCMS and GC-MS revealed the presence of innumerable number of other secondary metabolites whose functional groups were detected through FTIR. The compounds thus determined were those belonging to alcohols, phenols, alkanes, aromatic and aliphatic amines, carboxylic acids and ketones. These functional groups were determined on the basis of wave numbers in the functional group and finger print regions of the spectra as detailed below.

Fig. 19 indicates two major and four minor peaks obtained through FTIR spectrum of crude toxin from culture filtrate. The prominent peak at 3439.17 cm^{-1}ndicated O-H group of hydrogen bonded alcohols or phenols. The intense peak at 2959.05 cm^{-1} could be attributed to C-H stretching of alkanes. The peak at 1384 cm^{-1}denotes C-H bending in gem dimethyl groups present in the components (eg. isobutyl phthalate) due to strong aromatic amine group and the peak at 1307.86 cm^{-1}may be attributed to C-O stretching of carboxylic ester groups. The peak at 1076.83 cm^{-1} and the corresponding

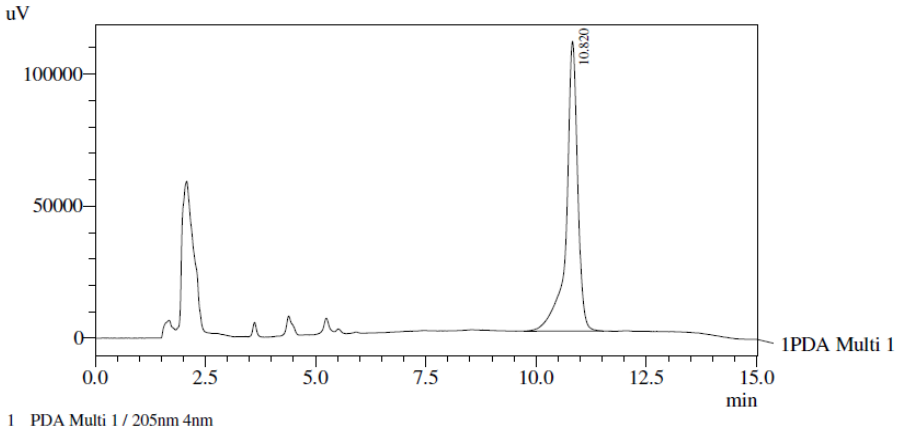


Fig.18 A. HPLC chromatogram of beauvericin standard

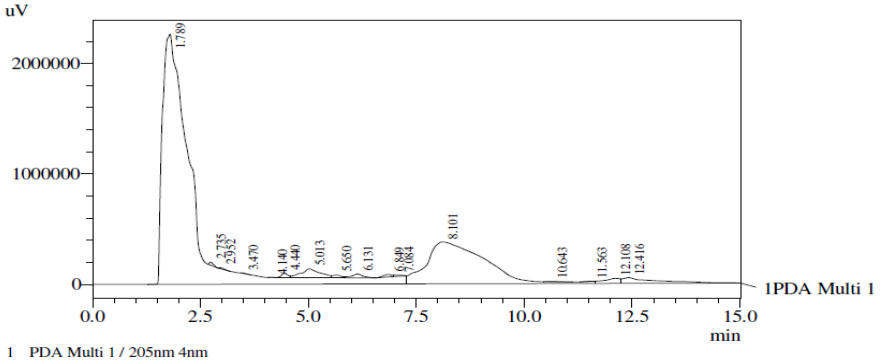


Fig.18 B. HPLC chromatogram of mycelial toxin of *Lecanicillium.saksenae*

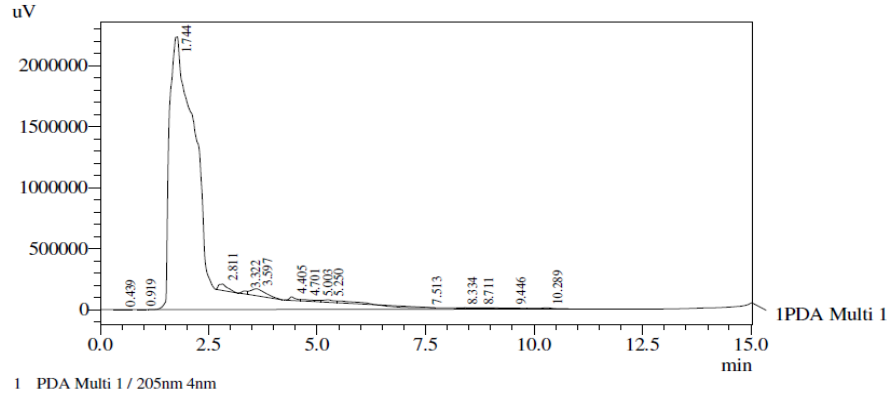


Fig. 18 C HPLC chromatogram of culture filtrate toxin of *Lecanicillium saksenae*

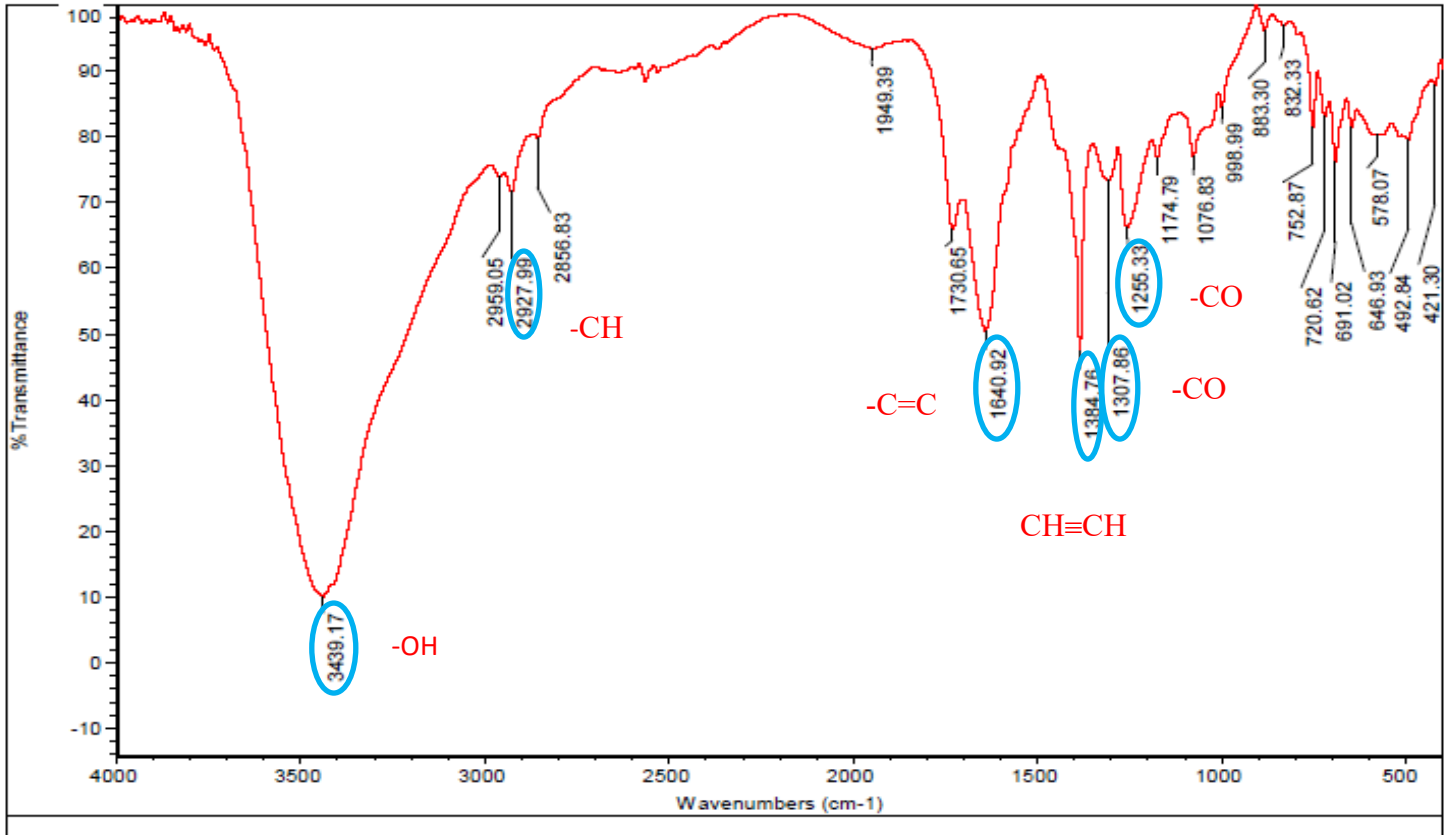


Fig. 19 FTIR spectrum of ethyl acetate fraction of *Lecanicillium saksenae* in Czapek Dox medium

S=O stretching support the presence of sulphate groups. The medium peak at 720.62 cm^{-1} corresponds to C-H bending in aromatic species present in the components. The other minor peaks visible in the spectrum with their representative functional group details are presented in Table 21.

Mycelial toxins from *L.* primary *saksenae* (Fig. 20) also revealed the presence of aromatic compounds such as amines, alkyls, aliphatic amines, carboxylic acids and alkyl halides which were detected as described afore. The prominent peak observed at 3433.8 cm^{-1} indicates stretching of O-H groups present in phenolic compounds. The intense peaks 1631.42 cm^{-1} corresponds to C=C stretching vibrations in alkenes and the band at 1399.87cm^{-1} represents S=O vibrations. The medium peak 1075.70 cm^{-1} corresponds to C-O stretching. The vibrational bands at 934.38 cm^{-1} and 898.42 cm^{-1} denotes H-C=C bending. The other minor peaks in the spectrum with their representative functional groups are presented in Table 22.

4.4.2.2 Metabolites Detected Through OSMAC Technology

Metabolites detected by HR-LCMS analysis of culture filtrate of *L. saksenae* grown in various media (OSMAC technology) were retrieved under both –ve and +ve mode of ESI. More than 100 secondary metabolites coming under important chemical classes were detected (Appendix IV). The predominant class detected was organic acids (43%) followed by phenolic acid (9%), alkaloid (8%), and fatty acid ester (3%) (Fig. 21). Important organic acid metabolites included DPA, picolinic acid, anthranilic acid, 4-dodecylbenzene sulfonic acid, vanillic acid, isophthalic acid, etc. The major phenolic compounds were aminophenol, 6-methoxy salicylic acid, etc. Alkaloids detected were nicotinic acid, 6-hydroxy nicotinic acid and trigonelline. Fatty acid esters detected were dibutyl phthalate, di isobutyl phthalate and the fatty acid amide compounds included oleamide and erucamide.

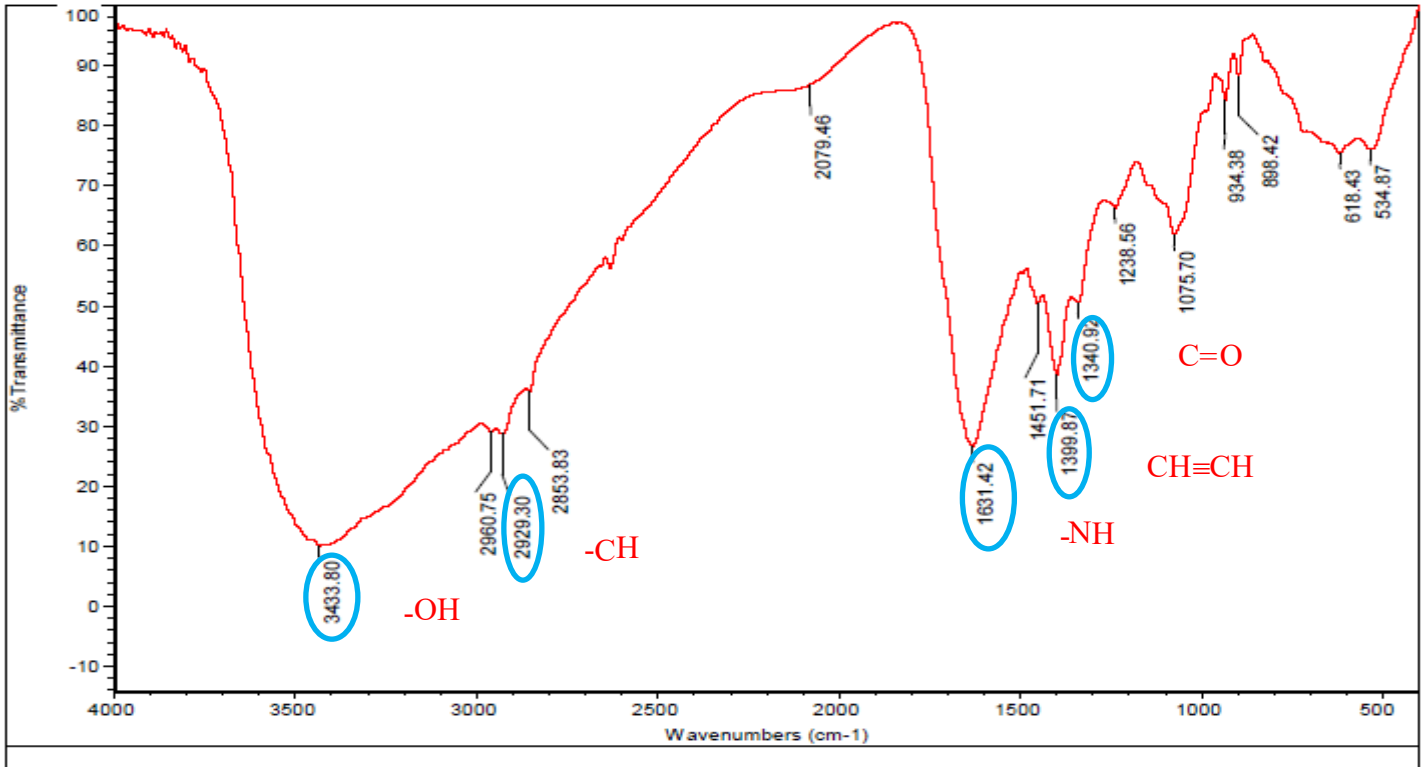


Fig. 20 FTIR spectrum of mycelial toxin of *Lecanicillium saksenae*

Table 21. Functional groups in the FTIR spectrum of culture filtrate of *Lecanicillium saksenae*

Sl. No	Wave numbers (cm^{-1})	Peak assignment	Mode of vibration	Functional groups
1	3439.17	-OH stretching	Strong, broad	Alcohol or phenol
2	2959.05	-CH Stretching	Medium	Alkene
3	2927.99	-CH Stretching	Medium	Alkane
4	2856.83	-CH Stretching	Medium	Alkane
5	1730.65	-C=O Stretching	Strong	Carbonyl
6	1640.92	-C=C Stretching	Strong	Alkene
7	1307.86	-CO Stretching	Strong	Acids
8	1255.33	-CO Stretching	Strong	Ether
9	1174.79	-CN Stretching	Weak	Amine
10	1076.83	-SO Stretching	Sharp	Sulfate
11	998.99	H-C=C Bending	Sharp	Aliphatic
12	883.30	H-C=C Bending	Weak	Alkene
13	832.33	H-C=C Bending	Sharp	Alkene
14	752.87	-NH Wagging	Medium	Amine
15	720.62	C-H Bending	Sharp	Aromatic
16	691.02	-C-H Bending	Medium	Aromatic

Table 22. Functional groups in the FTIR spectrum of mycelial toxins of *Lecanicillium saksenae*

S. No	Wave numbers (cm^{-1})	Peak assignment	Mode of vibration	Functional groups
1	3433.80	-OH Stretching	Strong, broad	Alcohol or phenol
2	2960.75	-CH Stretching	Medium	Alkene
3	2929.30	-CH Stretching	Medium	Alkane
4	2853.83	-CH Stretching	Medium	Alkane
6	1631.42	-NH Bending	Medium	Primary amine
7	1451.71	-CH Bending	Medium	Alkanes
8	1399.87	-S=O	Sharp	Sulphonic acid
9	1340.92	-C-N Stretching	Medium	Amines
10	1238.56	-CO Stretching	Strong	Ether
11	1075.70	-S=O Stretching	Sharp	Sulfate
12	934.38	-C=C-Bending	Sharp	Alkene
13	898.42	C=C-H Bending	Strong	Aromatic

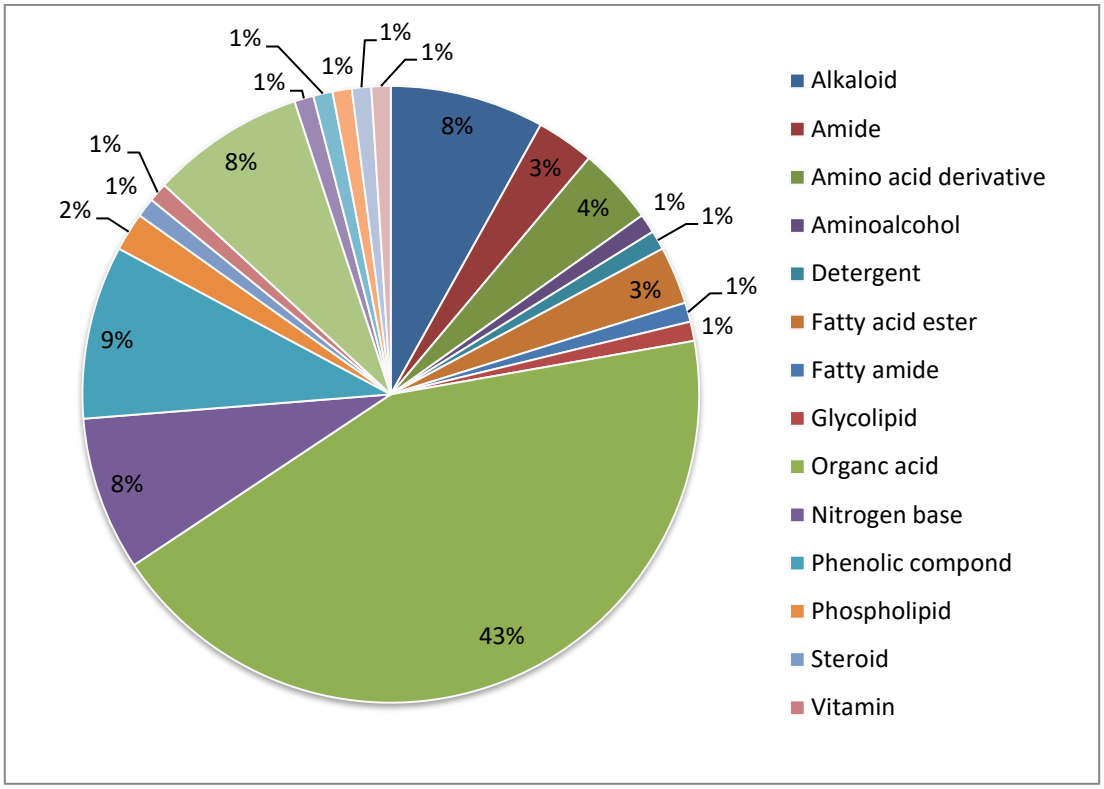


Fig. 21. Relative abundance of secondary metabolites in *Lecanicillium saksenae*

4.4.2.3 Bioactive Metabolites

HR-LCMS analysis of the crude toxin from Czapex Dox medium (CDM), Fargues medium (FM) potato dextrose broth (PDB) and rice medium (RM) revealed many secondary metabolites and many of them were common in all the media (Fig. 22 to 24). GC-MS chromatogram of crude toxin extracted from CDM and PDB cultures are depicted in Fig. 25 and 26 respectively.

Biological properties of metabolites detected through HR-LCMS and GC-MS analysis were ascertained from general chemical databases such as PubChem and Dr. Duke's phytochemical and ethanobotanical databases.Their insecticidal, nematicidal and antimicrobial activities revealed are detailed below.

4.4.2.3.1 Insecticidal Metabolites

The compounds such as oosporein, DPA, picolinic acid, cordycepin, dibutyl phalate, di isobutyl phthalate, dodecyl sulphate, 4-pyridine acetic acid, anthranilic acid, erucamide, octadecanoic acid, etc. detected through HR-LCMS of the crude toxin of *L. saksenae* were revealed as insecticidal, based on Pubchem and Dr. Duke's phytochemical and ethanobotanical databases. The molecular weight, molecular formula, best match and mz cloud ID of these compounds are depicted in Table 23 and 24.

Other insecticidal compounds such as DPA, 2-piperidinone, harmine, Dl-mevalonic acid lactone and n- hexadecanoic acid with their RT values (Table 24) were those detected in GC-MS chromatogram.

The biochemical properties, chemical and structural formulae of the major insecticidal compounds drawn using chem draw software are illustrated below.

Table 23. Insecticidal metabolites of *Lecanicillium saksenae* detected by HR-LCMS

Sl No	Name of the compound	Molecular formula	Molecular weight	Best match	mz cloud ID
1	Anthranilic acid	C_6 H_7 N O	137.04768	86.1	113
2	Cordycepin	$C_{10}H_{13}N_5O_3$	251.10184	80.2	5866
3	Dibutyl phthalate	$C_{16}H_{22}O_4$	278.15181	85.6	32
4	Dodecyl benzene sulfonic acid	$C_{18}H_{30}O_3S$	326.19157	84.9	1484
5	Dodecyl sulphate	$C_{12}H_{26}O_4S$	266.15518	86.7	1348
6	3-Hydroxy-2-methylpyridine	C_6 H_7 NO	109.0527	80.0	1736
7	Isophthalic acid	$C_8H_6O_4$	166.02661	89.1	3201
8	Oleamide	$C_{18}H_{35}NO$	281.27186	85.2	530
9	Piperidone	C_5H_9NO	99.06841	89.2	8395
10	4-Pyridineacetic acid	C_7 H_7 NO_2	137.04768	87.7	244
11	Picolonic acid	C_6 H_5 NO_2	123.03203	88.9	547
12	Isobutylphthalate	$C_{16}H_{22}O_4$	278.15181	86.5	32
13	Erucamide	$C_{22}H_{43}NO$	337.33309	90.0	282
14	6, 2 Fluorinated telomere sulphonate	C_8 $H_5F_{13}O_3S$	427.97518	92.5	8374
15	Azelaic acid	$C_9H_{16}O_4$	188.10486	92.1	331
16	Benzoic acid	$C_7H_6O_2$	122.03678	91.8	332
17	4- Hydroxy benbenzyl alcohol	$C_7H_8O_2$	124.05243	83.9	1689

Table 24. Insecticidal metabolites of *Lecanicillium saksenae* detected by GC-MS

Sl No	Name of the compound	Molecular formula	Retention time (min)	Chemical Group
1	Dl- mevalonic acid lactone	$C_6H_{10}O_3$	8.140	Terpenoid
2	Harmine	$C_{13}H_{12}N_2O$	24.942	Alkaloid
3	N-hexadecanoic acid	$C_{16}H_{32}O_2$	20.591	Fatty acid
4	2, piperidinone	$C_9H_{19}Br$ NO	6.925	Alkaloid
5	2,6-pyridine dicarboxylic acid	$C_7H_5NO_4$	13.208	Organic acid

Table 25. Nematicidal metabolites of *Lecanicillium saksenae* detected by HR-LCMS

Sl No	Name of the compound	Molecular formula	Molecular weight	Best match	mz cloud ID
1	Dodecyl benzene sulfonic acid	$C_{18}H_{30}O_3S$	326.19157	91.7	1484
2	Harmine	$C_{13}H_{12}N_2O$	212.09496	88.4	2182
3	8-Hydroxyl quinone	C_9H_7NO	145.05276	84.7	2633
4	Nicotinic acid	$C_6H_5NO_2$	123.03203	94.0	519
5	2,6 - pyridine dicarboxylic acid	$C_7H_5NO_4$	167.02180	85.4	2578

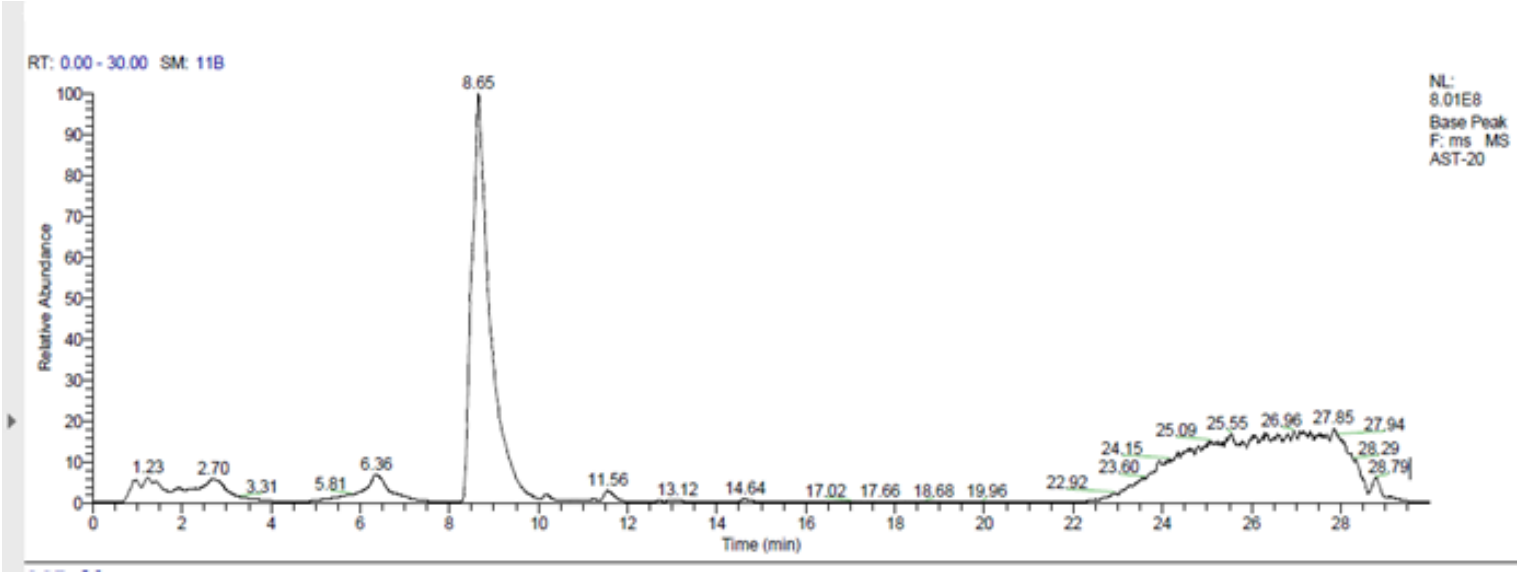


Fig. 22 HR-LCMS chromatograms of ethyl acetate fraction of *Lecanicillium saksenae* in Czapek Dox medium

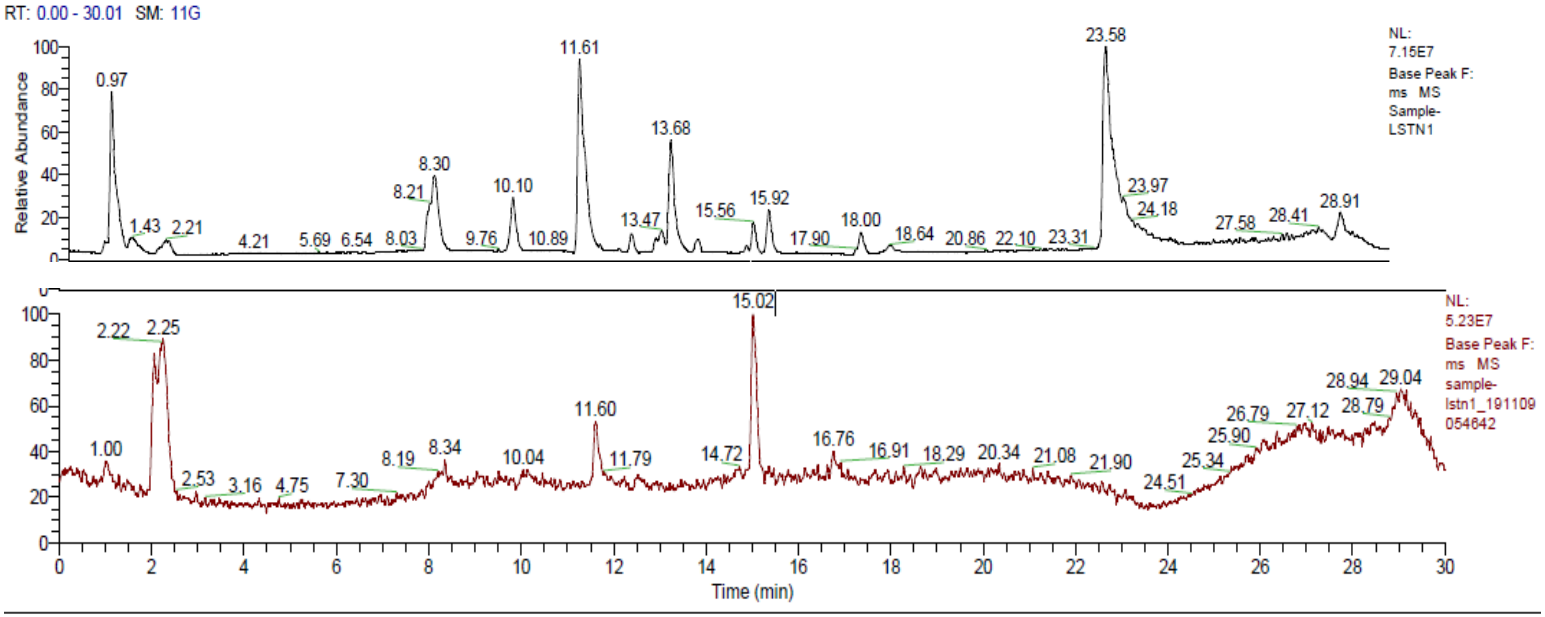


Fig. 23 HR-LCMS chromatograms of ethyl acetate fraction of *Lecanicillium saksenae* in Fargues medium

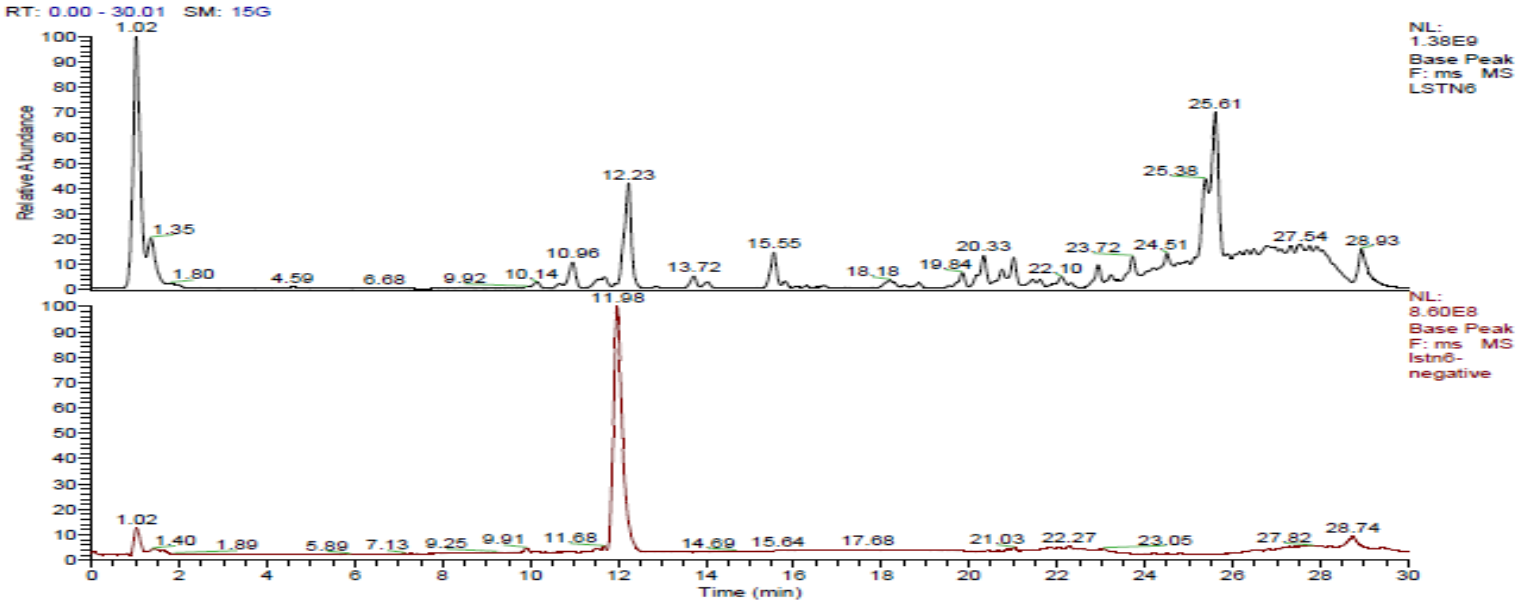


Fig. 24 HR LCMS chromatograms of ethyl acetate fraction of *Lecanicillium saksenae* in rice medium

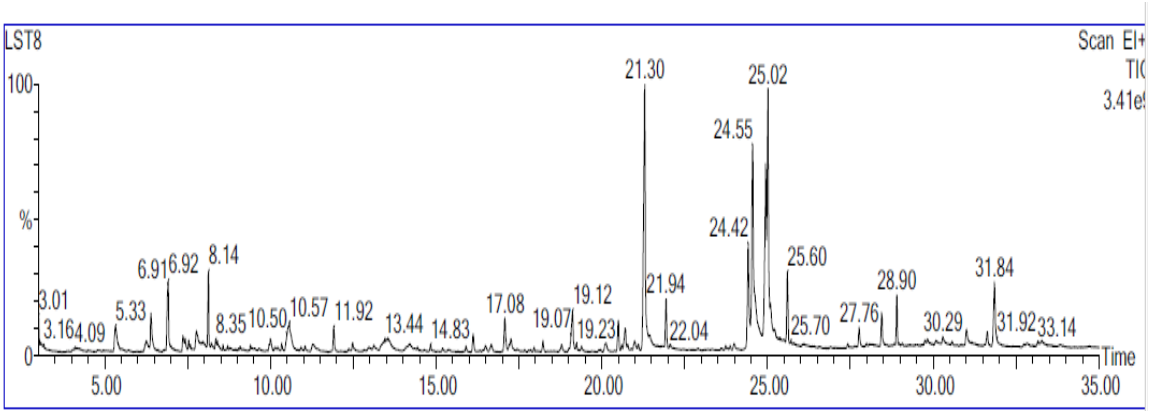


Fig. 25 GC-MS Chromatogram of ethyl acetate fraction of *Lecanicillium saksenae* in Czapek Dox medium

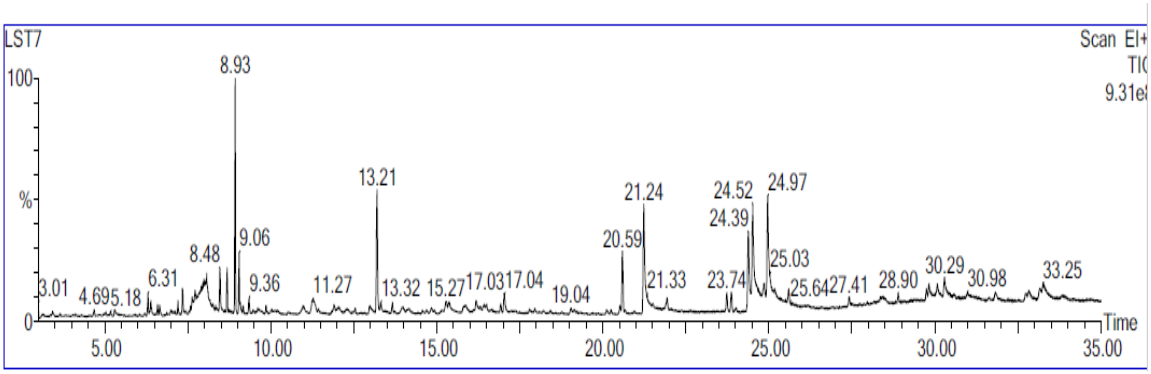
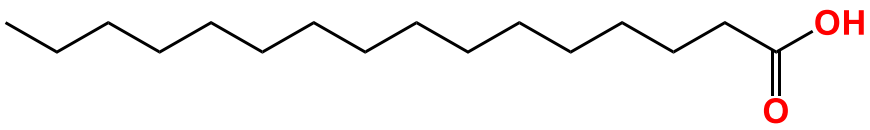


Fig. 26. GC-MS Chromatogram of ethyl acetate fraction of *Lecanicillium saksenae* in PDB medium

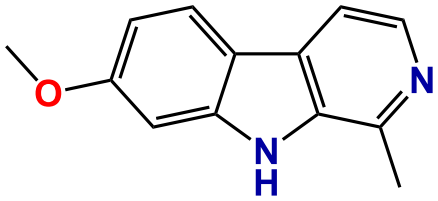
4.4.2.3.1.1 Hexadecanoic acid

It is a volatile fatty acid expressed at RT 21.30 min covering an area of 11.603 per cent. Its molecular formula is $C_{16}H_{32}O_2$.



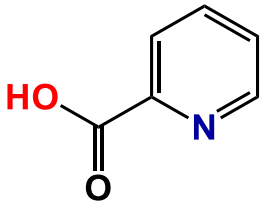
4.4.2.3.1.2 Harmine

It is a volatile alkaloid expressed at a RT of 24.942 and occupied an area of 6.216 per cent with a molecular formula $C_{13}H_{12}N_2O$.



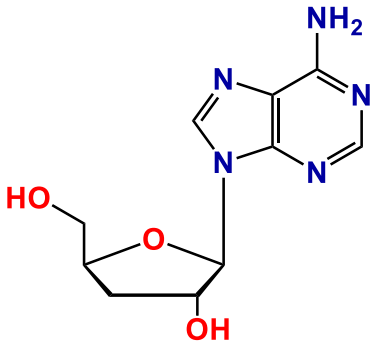
4.4.2.3.1.3 Picolinic acid

It is an organic acid with a molecular formula of $C_6H_5NO_2$ and a molecular weight of 123.03203.



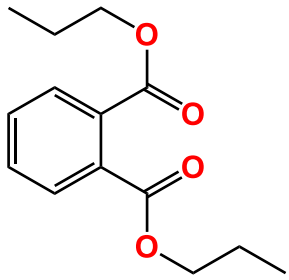
4.4.2.3.1.4 Cordycepin

It is a nucleoside analogue with a molecular formula $C_{1O}H_{13}N_5O_3$ and molecular weight of 251.10184.



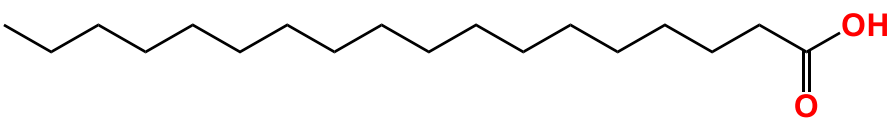
4.4.2.3.1.5 Dibutyl phthalate

A fatty acid ester compound with a molecular formula $C_{16}H_{22}O_4$ and a molecular weight of 278.15181.



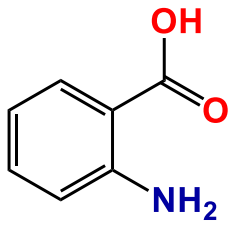
4.4.2.3.1.6 Octadecanoic acid

Volatile fatty acid, expressed at a retention time 25.017with an area of 10.234 per cent with a molecular formula of $C_{18}H_{36}O_2$.



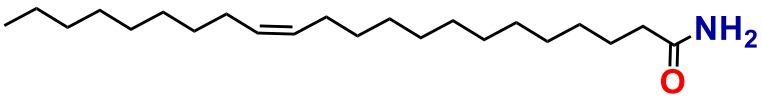
4.4.2.3.1.7 Anthranilic acid

An organic acid with a molecular formula of C_7H_7NO and a molecular weight of 137.0476



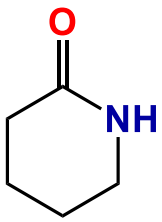
4.4.2.4.1.8 Erucamide

It is a fatty acid amide with a molecular formula of $C_{22}H_{43}NO$ and molecular weight 337.33447



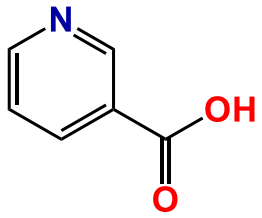
4.4.2.3.1.9 Piperidinone

A volatile alkaloid compound identified at a RT of 6.925 m and with an area of 2.664 per cent. Its molecular formula is $C_9H_{19}BrNO$.



4.4.2.3.1.10 Nicotinic acid

Alkaloid with a molecular formula of $C_6H_5NO_2$ and a mass of 123.03203.



4.4.2.3.2. Nematicidal Metabolites

HR-LCMS analysis of *L. saksenae* in different nutrient media CDB, PDB, FM and RM revealed the presence of various metabolites with nematicidal properties. All the four media indicated the presence of the alkaloid, nicotinic acid with known nematicidal property. Other nematicidal compounds detected were DPA, oleamide, hydroxyl quinone and many pyridine dicarboxylic acid derivatives and an organic acid dodecyl benzene sulfonic acid. The predominant nematicidal metabolites with their molecular formula, molecular weight and best match percentage are illustrated in Table 25.

GC-MS analysis of PDB also revealed the presence of DPA, n-hexadecanoic acid which are known to be nematicidal. The alkaloid harmine and the fatty acid, octadecanoic acid were detected in CDM. Retention time, molecular formula and peak area of these metabolites are depicted in Table 26.

4.4.2.3.2. Antimicrobial Metabolites

HR-LCMS analysis of crude toxin from various growth media identified many compounds with antimicrobial property. Those known to be antimicrobial are aminophenol, vanillic acid, dioctyl phthalate, 6 hydroxy nicotinic acid, isonicotinic 1.oxide salisylamide, dapsone, oxacilin, mandipropamid, netilmicin, oleamide and erucamide. List of metabolites with their molecular formula, molecular weight and best match are depicted in Table 27.

Table 26. Nematicidal metabolites of *Lecanicillium saksenae* detected by GC-MS

Sl.No	Name of the compound	Molecular formula	Retention time (min.)	Chemical Group
1	2,6 pyridine dicarboxylic acid	$C_7H_5NO_4$	13.208	Organic acid
2	N-hexadecanoic acid	$C_{16}H_{32}O_2$	20.591	Fatty acid
3	Harmine	$C_{13}H_{12}N_2O$	24.942	Alkaloid
4	Octadecanoic acid	$C_{18}H_{36}O_2$	25.017	Fatty acid

Table 27. Antimicrobial metabolites of *Lecanicillium saksenae* detected by HR-LCMS

Sl No	Name of the compound	Molecular formula	Molecular weight	Best match	mz cloud ID
1	Aminophenol	C_6H_7NO	109.05276	91.6	225
2	Anthranilic acid	$C_6 H_7N O_2$	137.04768	86.1	593
3	Dapsone	$C_{12} H_{12}N_2O_2S$	248.06195	79.7	1410
4	Dehydroacetic acid	$C_8H_8O_4$	168.04226	87.4	380
5	Dodecyl benzene sulfonic acid	$C_{18}H_{30}O_3S$	326.19157	91.7	1484
6	Erucamide	$C_{22}H_{43}NO$	337.33447	94.9	282
7	Mandipropamid	$C_{23}H_{22}ClNO_4$	411.12374	81.5	3324
8	Methyl succinic acid	$C_5H_8O_4$	132.04226	85.7	1337
9	Netilmicin	$C_{21}H_{41}N_5O_7$	475.30060	91.2	2443
10	Oleamide	$C_{18}H_{35}NO$	281.27186	85.2	530
11	Oxacilin	$C_{19}H_{19}N_3O_5S$	401.10454	92	2709
12	Picolinic acid	$C_6H_5NO_2$	123.03203	88.9	547
13	Salisylamide	$C_7H_7NO_2$	137.04768	78.0	113
14	Terephthalic acid	$C_8H_6O_4$	166.02661	88.3	1499

The GC-MS analysis showed the presence of metabolites coming under different chemical groups (Table 28). The predominant antimicrobial compounds such as acetamide (amide), 4-H pyran-4-one, 2, 3, dihydro 3, 5 dihydroxy l6 methyl (ketone), harmine (alkaloid) Dl-mevalonic acid lactone (terpenoid) were identified.

4.5. BIOEFFICACY OF MYCOTOXINS

Results of bioassay of toxins carried out in the insect pest, *C. insolita,* root knot nematode *M. incognita* and the coccinellid predator, *C. sexmaculata* are presented below.

4.5.1 *C. insolita*

Bioefficacy of the purified mycotoxin oosporein, in comparison with that of crude toxin, on nymphs and adults of *C. insolita* is presented in Tables 29 and 30.

4.5.1.1 On Nymphs

Analysis of data on corrected mortality revealed a concentration - dependant mortality with both the purified and crude toxins.

At 24 HAT, 1000 ppm oosporein recorded highest mortality (31.31%), while those with 500 and 250 ppm ranked second (18.56% and 16.31%) and were on par. A significantly lower rate of death was observed with 100 ppm (2%) and the lowest recorded was 1.51 per cent, with 10 ppm. The corresponding mortality noted with the crude toxin was much higher. It was 85.23 per cent with 1000 ppm and 78.28 per cent with 500 ppm which were statistically similar. Crude toxin at 250 ppm resulted in 60.82 per cent mortality, which was significantly higher than that caused by 50 and 10 ppm (25.44% and 2.63% respectively).

After 48 h, oosporein at 1000 ppm resulted in 42.81 per cent death of nymphs, while the percentage was significantly lower with 500 ppm (23.38%), followed by 20.88 per cent with 250 ppm. The mortality observed was negligible with 100, 50 and 10 ppm (18.38, 7.18 and 2.10% respectively). The corresponding death rate noted with crude toxin was much higher. It was 95.98 and 95.75 per cent with 1000 and 500 ppm, which were statistically on par.

Table 28. Antimicrobial metabolites of *Lecanicillium saksenae* detected by GC-MS

Sl. No.	Name of the compound	Mol. formula	Retention time (min.)	Chemical group
1	Acetamide, N-(2-phenylethyl)	$C_{10}H_{13}NO$	11.917	Amide
2	3- deoxy-d- mannoic lactone	$C_6H_{10}O_5$	13.462	Cyclic ester
3	4H Pyran -4-one, 2, 3, dihydro3, 5 dihydroxyl6 methyl	$C_6H_8O_4$	6.400	Ketone
4	Harmine	$C_{13}H_{12}N_2O$	24.942	Alkaloid
5	Dl-mevalonic acid lactone	$C_6H_{10}O_3$	8.140	Terpenoid
6	Pyrrolo (1,2) pyrazine 1,4-dione hexahydro	$C_7H_{10}N_2O_2$	17.07	Diketopiperazine

Table 29. Bioefficacy of oosporein and crude toxin on *Coccidohystrix insolita* nymphs

Concentration (ppm)	*Mean cumulative corrected mortality of nymphs at 24 h interval (%)							
	24		48		72		96	
	Oosporein	Crude toxin	Oosporein	Crude toxin	Oosporein	Crude toxin	Oosporein	Crude toxin
10	1.51^{e}	2.63^{e}	2.10^{e}	3.95^{e}	3.28^{f}	5.56^{e}	5.30^{f}	6.25^{d}
50	4.00^{d}	25.44^{d}	7.18^{d}	30.56^{d}	11.37^{e}	43.06^{d}	16.50^{e}	46.88^{c}
100	2.00^{c}	41.74^{c}	18.38^{c}	54.02^{c}	28.19^{d}	62.83^{c}	40.50^{d}	73.44^{b}
250	16.31^{b}	60.82^{b}	20.88^{bc}	72.26^{b}	31.81^{c}	84.40^{b}	45.68^{c}	95.31^{a}
500	18.56^{b}	78.28^{a}	23.38^{b}	95.75^{a}	39.43^{b}	$I00.00^{a}$	51.06^{b}	100.00^{a}
1000	31.31^{a}	85.23^{a}	42.81^{a}	95.98^{a}	51.18^{a}	100.00^{a}	60.25^{a}	100.00^{a}
SEM	0.72	3.31	0.84	5.18	0.71	3.11	0.94	3.66
CD (0.05)	2.402	11.009	2.791	17.206	2.346	10.345	3.125	12.15

*Mean of five replications
Values sharing same alphabets in superscript are statistically on par based on LSD

The mortality recorded with 250 ppm was significantly lower than those of the higher concentrations (72.26%), but significantly higher than those observed with the lower concentrations, 100, 50 and 10 ppm, where the death rate was only 54.02, 30.56 and 3.95 per cent.

Observations recorded after 72 h, revealed 51.18 per cent mortality with 1000 ppm oosporein, which was statistically higher than the death rate noted with 500 ppm (39.43%). The mortality observed with 250 ppm was 31.81 per cent, while it was 28.19, 11.37 and 3.28 with lesser concentrations of 100 ppm, 50 ppm and 10 ppm respectively. At this point of time, there was 100 per cent death in crude toxin treatment at higher concentrations such as 1000 and 500 ppm. When the concentrations were lowered to 250, 100, 50 and 10 ppm, the mortality recorded was 84.40, 62.83, 43.06 and 5.56 per cent respectively, each being significantly lower than the preceding.

Even at the end of the experimental period (96 HAT) highest mortality noted with 1000 ppm was only 60.25 per cent, while it was still lower (51.06), with 500 ppm. The death rate noted with 250 and 100 ppm was 45.68 and 40.50 per cent respectively, which were statistically lower than the former, but higher than those noted with 50 and 10 ppm (16.5% and 5.3%). At this point of time, crude toxin even at lower concentration of 250 ppm resulted in 95.31 per cent mortality which was on par with those of 500 and 1000 ppm (100%). The mortality at lower concentrations of 10, 50 and 100 ppm were 6.25, 46.88 and 73.44 per cent respectively.

4.5.1.2 On Adults

In adults, the death rate was comparatively less when compared to nymphs **(**Table 30). At 24 HAT, even at higher concentrations 1000 and 500 ppm oosporein resulted only 24.5 and 19.5 per cent mortality, while 250 and 100 ppm recorded still lower death rate of 10.75 and 7.5 per cent. It was 2.25 and 1.25 per cent with 10 and 50 ppm. The corresponding death rate noted with crude toxin was much higher, the values being 83.36, 71.84 and 67.89 per cent, where the

Table 30. Bioefficacy of oosporein and crude toxin on *Coccidohystrix insolita* adults

Concentration (ppm)	* Cumulative corrected mortality of adults at 24 h interval (%)							
	24		48		72		96	
	Oosporein	Crude toxin	Oosporein	Crude toxin	Oosporein	Crude toxin	Oosporein	Crude toxin
10	1.25^{e}	2.50^{d}	1.25^{e}	2.50^{d}	1.75^{f}	4.02^{d}	4.48^{e}	5.56^{d}
50	2.25^{e}	7.57^{d}	4.19^{e}	7.63^{d}	7.00^{e}	8.19^{d}	9.90^{d}	13.12^{d}
100	7.50^{d}	22.96^{c}	11.50^{d}	26.29^{c}	19.25^{d}	27.34^{c}	31.08^{c}	31.12^{c}
250	10.75^{c}	67.89^{b}	17.93^{c}	73.79^{b}	26.25^{c}	75.22^{b}	34.50^{c}	79.86^{b}
500	19.50^{b}	71.84^{b}	25.81^{b}	76.29^{b}	37.63^{b}	86.26^{a}	46.69^{b}	100.00^{a}
1000	24.50^{a}	83.36^{a}	35.69^{a}	85.51^{a}	46.69^{a}	89.04^{a}	51.00^{a}	100.00^{a}
SEM	0.74	2.52	1.03	2.3	1.28	2.38	1.42	2.61
CD (0.05)	2.457	8.379	3.450	7.642	4.254	7.809	4.704	8.692

*Mean of five replications .Values sharing same alphabets are statistically on par based on LSD

former two were statistically on par. The mortality noted with 100, 50 and 10 ppm was 22.96, 7.57 and 2.5 per cent respectively.

Similar trend was observed at 48 HAT. Highest mortality of 35.69 per cent was observed with 1000 ppm oosporein. Death rate noted with 500 ppm was significantly lower (25.81%). The mortality observed with 250, 100, 50 and 10 ppm was significantly lower among each other (17.93, 11.50, 4.19 and 1.25% respectively). With crude toxin 1000 ppm, the mortality noted was 85.51 per cent, which was significantly higher than those observed with 500 ppm (76.29%) and 250 ppm (73.79%). The mortality observed at lower concentrations 100, 50 and 10 ppm was 26.29, 7.63 and 2.50 per cent respectively.

At 72 HAT, mortality of adults varied significantly among each other. The highest mortality observed with 1000 ppm was 46.69 per cent. The mortality noted at different concentrations differed significantly. It was 37.63, 26.25, and 19.25 per cent with 500, 250 and 100 ppm concentrations. The corresponding death rate observed with 1000 ppm and 500 ppm of crude toxin was 89.04 and 86.26 per cent, respectively which did not differ significantly. At 250 ppm the mortality recorded was 75.22 per cent which was significantly higher than all other lower concentrations. Crude toxin at 100 ppm resulted in 27.34 per cent death, while the death rate was significantly less in lower concentrations 50 and 10 ppm (8.19% and 4.02%).

At 96 HAT, 1000 ppm oosporein resulted in 51 per cent mortality, while it was 46.69 per cent in 500 ppm and 34.50 per cent in 250 ppm, which differed significantly among them. The death rate was negligible with 10 and 50 ppm (4.48 and 9.90%). At this point of time, crude toxin at 1000 and 500 ppm caused 100 per cent mortality, while it was 79.86 and 31.12 per cent with 250 and 100 ppm which differed significantly. Death rate noted with 50 and 10 ppm was 13.12 and 5.56 per cent which were on par with each other.

Table 31. Toxicity of crude toxin of *Lecanicillium saksenae* on *Coccidohystrix insolita*

Stage of insect	Time interval (h)	LC_{50} (ppm)	(LCL-UCL)	LC_{90} (ppm)	(LCL-UCL)	$\chi 2$ (df =4)
NYMPH	24	155.81	128.91 - 187.67	1219.35	894.17 - 1819.11	1.85
	48	87.14	72.96 - 102.81	465.85	371.40 - 615.94	6.74
	72	63.94	45.87 - 85.00	261.88	185.28 - 435.88	6.99
	96	51.06	43.46 - 59.19	176.93	147.21 - 221.75	2.12
ADULT	24	221.71	136.22 - 366.25	1223.43	647.79 - 4534.68	15.56
	48	193.61	111.27 - 330.65	994.54	522.97 - 4011.99	19.39
	72	164.34	85.71 - 297.61	770.57	397.32 - 3583.74	25.57
	96	118.51	7.13 - 845.55	383.41	153.39 - 3211.41	94.82

Table 31 represents the LC_{50} and LC_{90} values calculated for nymphs and adults of *C. insolita.* In nymphs, LC_{50} values were 155.81, 87.14, 63.94 and 51.06 ppm at 24, 48, 72 and 96 h respectively. The corresponding LC_{9O} values were 1219.35, 465.85, 261.88, 176.93 ppm respectively. In adults, LC_{50} values were 221.71, 193.61, 164.34 and 118.51 ppm at 24, 48, 72, and 96 h, while LC_{90} values calculated were 1223.43, 994.54, 770.57 and 383.41 ppm.

4.5.2 *M. incognita*

As the crude toxin was found to be more toxic than the purified toxin oosporein, effect on the root knot nematode was carried out using the former only.

All the tested concentrations showed a dose dependant nematicidal effect on *M. incognita* and significant mortality was observed within a short period of 24 h after treatment (Table 32).

At 24 h exposure, highest mortality of 95.22 per cent was observed with 250 ppm followed by 91.68, 90.67, 65.25, 24.94 and 4.28 per cent respectively with 100, 50, 25, 10 and 1 ppm concentrations.

At 48 HAT, there was 100 per cent mortality in juveniles treated with 50 ppm and above. The mortality observed with 25 ppm was 70.91 per cent, whereas it was lesser in 10 and 1 ppm (27.54 and 5.61%). The mortality at 72 h was 82.06, 29.74 and 8.46 with 25, 10 and 1 ppm, respectively. At 96 h, 97.16 per cent mortality was observed with 25 ppm whereas it was 30.30 and 10.62 with 10 and 1 ppm, respectively.

Juveniles treated with ethyl extract of the culture medium (negative control) and those treated with water (absolute control) did not cause any mortality in the test organism.

Table 32. Nematicidal effect of crude toxin of *Lecanicillium saksenae* on *Meloidogyne incognita* juveniles

Concentration (ppm)	*Mean mortality at 24 h interval (%)			
	24	48	72	96
1	4.28±1.32	5.61±1.58	8.46±1.41	10.6±0.87
10	24.94±1.77	27.54±1.24	29.74±0.85	30.300±.81
25	65.25±1.90	70.91±1.54	82.06±2.25	97.162±.20
50	90.67±1.34	100.00±0.00	100.00±0.00	100.00±0.00
100	91.68±1.27	100.00±0.00	100.00±0.00	100.00±0.00
250	95.22±1.11	100.00±0.00	100.00±0.00	100.00±0.00
Control	0.00	0.00	0.00	0.00

*Mean of five replications

4.5.3 *Cheilomenes sexmaculata*

As the crude toxin was found to be more toxic than the purified toxin oosporein, effect on predatory coccinellid *C. sexmaculata* was carried out using crude toxin only. The effect of extremely concentrated crude toxin (1000 to 10000 ppm) which was five to 56 times higher than that determined for the pests (LC_{90} =176.93 ppm at 96 h), on egg, larvae, pupae and adults of the predator is enumerated below.

4.5.3.1 Egg

At 1000 ppm, the mortality (unhatched eggs) recorded was 30.00±2.04 per cent which was further increased to 36.25±3.15, 45±2.04 and 53.75±2.39 per cent at 2000, 4000, 5000 ppm respectively (Table 33A). Highest mortality noted with 10000 ppm was 78.75±5.15 per cent. At 10, 000 ppm the treated eggs appeared brownish and finally disintegrated. The LC_{50} value calculated was 3524.8 ppm which was 69 fold higher than that calculated for the pest (51.06 ppm at 96 h). The LC_{90} value was 38087.50 ppm which was 215 fold higher than that calculated for the pests (176.93 ppm at 96 h), invariably proving its non toxicity.

4.5.3.2 Larva

Larval mortality of *C. sexmaculata* exposed to varying doses at different time intervals was recorded (Table 33 B).

Grubs exposed to 1000 ppm of crude toxin did not die at 24 HAT. The death rate was meagre (7.5 ±2.50 %) after 48h and 22.50±2.5 and 35.00±2.89 per cent after 72 and 96 h respectively. At 2000 ppm the corresponding mortality was 5.00±2.89, 17.50±2.5, 27.5±2.5 and 40±4.08 per cent respectively. The death rate increased to 6.00 ±2.89, 20.50±4.79, 32.50±4.79 and 45.00±6.45 with 4000 ppm and to 7.5±2.5, 21.75 ±6.25, 35.00 ± 6.46 and 52.50±4.79 at 5000 ppm. Mortality of grubs exposed to 10000 ppm was 10.00± 4.08, 25.00±2.89, 47.50±6.29 and 65.00±2.89 respectively.

Table 33.Toxicity of crude toxin of *Lecanicillium saksenae* on coccinellid beetle *Cheilomenes sexmaculata*

A. Egg

	Conc.(ppm)	Mortality(%) ± SE	LC_{50} (ppm) (LCL-UCL)	LC_{90} (ppm) (LCL-UCL)	$\chi 2$ (df =3)
	1000	30.00±2.04	3524.80 (1772.18-7788.96)	38087.50 (12933.79-101946.55)	7.22
	2000	36.25±3.15			
	4000	45.00±2.04			
	5000	53.75±2.39			
	10000	78.75±5.15			
	0	0			

B. Larva

Duration (h)	Conc. (ppm)	Mortality(%) ± SE	LC_{50} (ppm) (LCL-UCL)	LC_{90} (ppm) (LCL-UCL)	$\chi 2$ (df =3)
24	1000	0.00±0.00	177237.13 (41186.14 - 106730296.51)	3666826.13 (267553.40 – 424717.1.37)	3.35
	2000	5.00±2.89			
	4000	5.00±2.89			
	5000	7.50±2.50			
	10000	10.00±4.08			
	0	0.00±0.00			
48	1000	7.50±2.50	123567.03 (29988.47 - 64263419.38)	14314885 (560929.35 - 33208221.91)	2.3
	2000	17.50±2.50			
	4000	20.50±4.79			
	5000	21.75±6.29			
	10000	25.00±2.89			
	0	0.00±0.00			
72	1000	22.50±2.50	15643.53 (8577.12 - 80590.81)	1279669.24 (171036.45 - 5796116.39)	0.76
	2000	27.50±2.50			
	4000	32.50±4.79			
	5000	35.00±6.46			
	10000	47.50±6.29			
	0	0.00±0.00			
96	1000	35.00±2.89	4040.74 (2854.53 - 6251.11)	204161.93 (56702.89 - 508440.48)	1.77
	2000	40.00±4.08			
	4000	45.00±6.45			
	5000	52.50±4.79			
	10000	65.00±2.89			
	0	5.00±2.89			

C. Pupa

Stage	Conc. (ppm)	Mortality (%) ± SE	LC_{50} (LCL-UCL)	LC_{90} (LCL-UCL)	χ^2 (df =3)
Pupa	0	0.00±0.00	9448.13 (6941.42-15780.17)	125330.69 (52666.56-64612.81)	0.63
	1000	15.00±2.89			
	2000	20.00±4.08			
	4000	32.50±2.50			
	5000	37.50±2.50			
	10000	52.50±4.79			

The LC_{50} computed at 24 h was 177237.13 ppm and at 48h was 123567.03 ppm. Whereas, with longer exposure period the value was 15643.53 ppm at 72 h and 4040.74 ppm at 96 h which was 306 and 79 fold higher than that calculated for the pests (51.06 ppm).

4.5.3.3 Pupa

Pupal mortality (non-emergence) observed was 15 ± 2.89 per cent in those exposed to 1000 ppm, while it increased to 20±4.08, 32.5±2.50 and 37.50±2.50 per cent and 52.50±4.79 per cent with 2000, 4000, 5000 and 10000 ppm. The LC_{50} value calculated was 9448.13 ppm which was 185 times higher than that computed for the pest.

4.5.2.4 Adult

Adult beetles exposed to the toxin did not exhibit any mortality till the end of 96 h.

4.6 MODE OF ACTION OF THE METABOLITES

In silico molecular docking with metabolites of *L. saksenae* revealed that, 18 of them interacted with AChE at specific sites (Table 34). The interacting sites were the esteratic site of the catalytic triad *viz.* serine, histidine and glutamate residues. The binding sites of AChE (*Dm* AchE, PDB Code IDX4) were SER 238, HIS 480, TRY 370 and GLU 237. These metabolites had effective interaction with AChE as exhibited by optimum CDOCKER energy (>20Kcal), CDOCKER interaction energy (>20 Kcal) and binding energy (41.5717 to -133.24).

Among the 18 metabolites, six metabolites showed highest interaction with acetyl cholinesterase with the binding energy varying from -111.212 to -133.24 kcal mol^{-1}. Of these, 3-hydroxy-2-methyl pyridine exhibited the highest binding energy (-133.24 kcal mol^{-1}) and therefore and highest interaction with

AchE followed by 2- hydroxy benzamide (-131.48 kcal mol^{-1}), 6- hydroxy picolinic acid (-121.21 kcal mol^{-1}), and picolinic acid N-oxide (-113.28 kcal mol^{-1}) (Fig. 27). Other metabolites with comparatively high binding energy were 2-amino benzoic acid (-111.212 kcal mol^{-1}) and 4-aminobenzoic acid (-107.18 kcal mol^{-1}).

Table 34 Docking scores of metabolites of *Lecanicillium saksenae* and the protein target acetyl cholinesterase for their inhibitory property

Ligand	(-) C docker energy (Kcal mol-1)	(-) C docker interaction energy (Kcal mol-1)	H bond amino acid residue	Hydrogen bond distance (A°)	Binding energy (Kcal)
Picolinic Acid	20.5057	27.3388	SER238	2.25627	-87.1397
			SER238	2.10394	
4-Aminophenol	23.9748	27.513	SER238	1.89879	-79.1248
			HIS480	1.75174	
3-Hydroxy-2-methylpyridine	25.8203	28.9507	SER238	1.85532	-133.24
			HIS480	2.02604	
3-Pyridine acetic acid	25.7084	26.6188	SER238	2.09434	-77.2754
			SER238	2.39698	
			HIS480	2.13704	
5-Hydroxy indol 3-acetic acid	31.6487	33.6039	SER238	2.04307	-62.7932
			HIS480	2.26632	
			TYR370	1.99321	
2-Amino benzoic acid	21.0629	25.8711	SER238	2.41947	-111.212
			SER238	2.11311	
			HIS480	2.33956	
6-Hydroxy picolinic acid	24.0854	25.4103	SER238	2.16393	-121.211
Dapsone 2955	24.578	32.493	HIS480	2.07457	-91.4038
Di butyl phathalate	42.2215	43.3687	HIS480	2.10697	-81.9556

4- Pyridine acetic acid	27.9961	29.829	SER238	2.25208	-74.0751
			SER238	2.00171	
			HIS480	2.41963	
2-Hydroxy benzamide	29.2461	30.0365	SER238	1.8434	-131.485
			HIS480	2.09465	
Di isobutyl phathalte	38.6794	40.2163	SER238	2.4003	-47.0943
Picolinic acid N-oxide	25.2795	27.6947	SER238	2.07954	-113.28
			SER238	1.95494	
			HIS480	2.47054	
Epicatechin	33.5368	43.0284	HIS480	2.0662	-94.3098
			GLU237	2.01465	
6-Hydroxy nicotinic acid	20.6616	24.7541	SER238	2.16764	-41.5717
2- pyridine acetic acid	24.7427	27.4075	SER238	2.37187	-69.2855
			SER238	2.07187	
			HIS480	2.17812	
Catechin	43.0324	47.3831	TYR370	2.47713	-52.7294
4-Amino benzoic acid	23.9896	25.6474	SER238	2.09453	-107.182
			HIS480	2.49788	
			GLY151	2.4589	

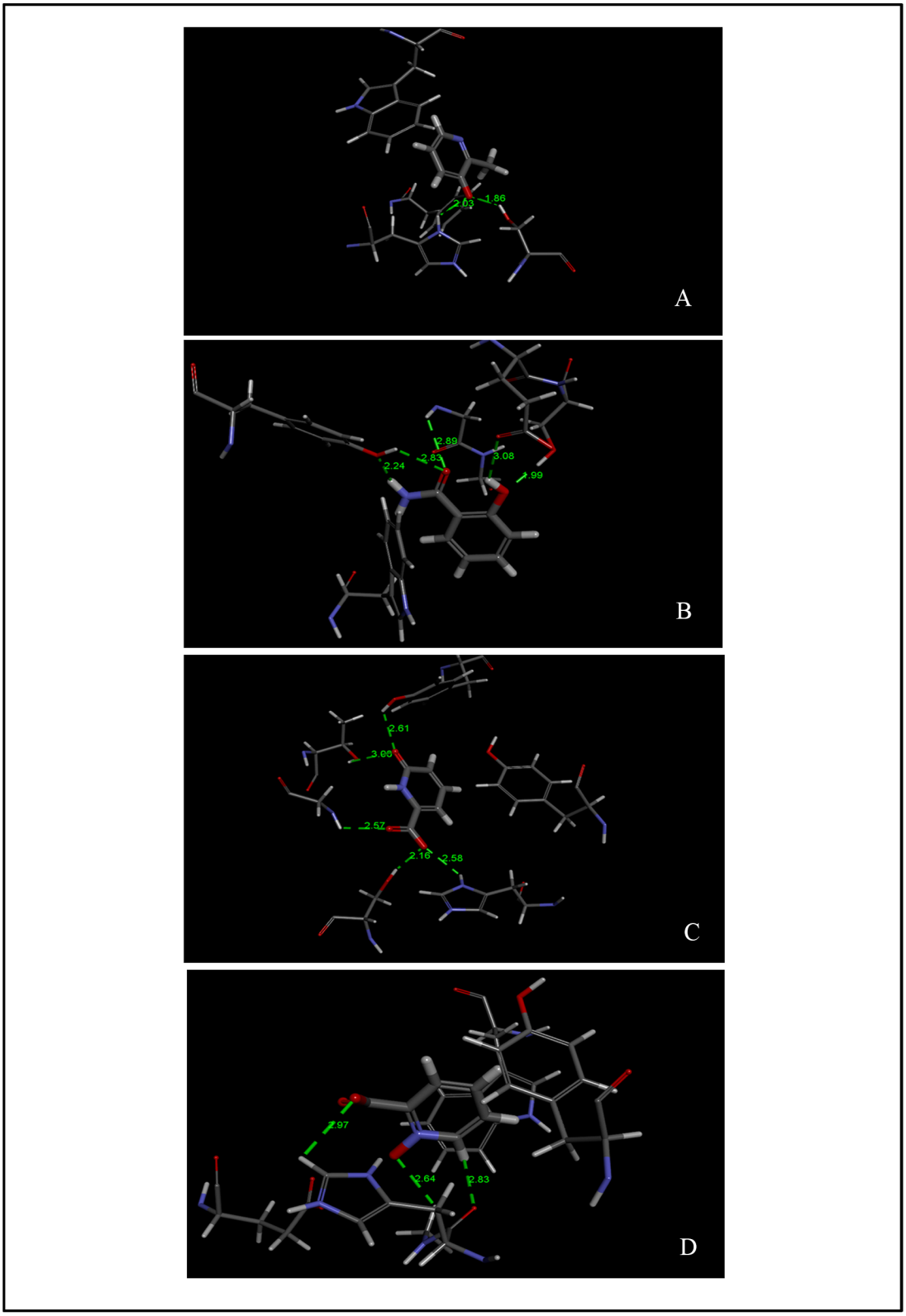


Fig. 27 Three dimensional representation of AChE-ligand complex.

A. AChE and 3- hydroxy-2-methyl pyridine B. AChE and 2-hydroxy benzamide
C. AChE and 6-hydroxy picolinic acid D. AChE and picolinic acid-N-oxide

DISCUSSION

5. DISCUSSION

Insect pathogenic microbes are globally researched for their exploitation in the biological control of insect pests. Conversely, insects have formidable range of defensive mechanisms against these microbes that are ubiquitous in the environment that they inhabit. Entomopathogenic fungi have a unique mode of infecting the host insects, by gaining their entry through the cuticle, unlike the other groups such as bacteria and virus. Infectivity, the innate trait of these fungi is a complex and multi-factorial phenomenon, which includes mechanical pressure, cuticle degrading enzymes and secondary metabolites *viz*., toxins, pigments, etc. Present investigation therefore, focused on profiling the mycotoxins and enzymes of the entomopathogenic fungus *Lecanicillium saksenae* (Kushwaha) Kurihara and Sukarno, a potent indigenous isolate (ITCC Ls Vs1-7714) from the cultivated soils of Vellayani, Kerala, India.

CUTICLE DEGRADING ENZYMES OF *L. SAKSENAE*

Cuticle degrading enzymes (CDE) play a pivotal role in the interaction between entomopathogenic fungi and host insects. Enzyme secretion is believed to be a key factor for its virulence.

Studies conducted in this line revealed the presence of a multitude of hydrolytic enzymes such as chitinase, protease, lipase, chitosanase, chitin deacetylase, keratinase and amylase. Plate assay, an easy and time saving but sensitive method was adopted to detect the secretion of extracellular enzymes produced by the fungus.

Chitinase was detected by colour change, in the detection plates containing colloidal chitin and the p^H indicator, bromocresol purple. Chitinase production by the fungus resulted in the breakdown of colloidal chitin into N-Acetyl glucosamine, the basic monomeric units of chitin that caused the colour change from yellow to purple. Agrawal and Kotasthane (2012), observed the chitinase activity in *Trichoderma viride* (Pers) using the same method.

L. saksenae exhibited strong lipase activity by the zone of precipitation caused by degradation of Tween 20, a commonly used substrate in the lipase detection plate. Kumar *et al*. (2012) attributed this action of Tween 20 to its constituent, oleic acid esters. A comparable zone of precipitation was reported by Hasan *et al*. (2013) while detecting lipase activity in *L. lecanii*. The zone formation is due to the release of fatty acids due to hydrolysis of Tween 20, by the action of lipase (Sanivada and Challa, 2014).

Presence of protease in *L. saksenae* was detected by the zone of clearance around the colony in casein agar plate, which is attributed to the proteolytic activity that utilized casein, the protein source from the medium. Use of casein in detecting protease production in *B. bassiana* was reported by Kaur and Padmaja (2009).

Chitosanase activity is a common feature in soil inhabiting fungi which utilize chitosan as nutritional source for their carbon requirement. In the plate assay of *L. saksenae*, carried out using colloidal chitosan, there was a positive response to chitosan. Its facultative mode of life might have necessitated the production of chitosanase to utilize the polysaccharide molecules from soil organic matter. Similar results on extracellular chitosanase activity in fungi were reported in other species too. Zone of clearance on chitosanase detection plate was reported by Nampally *et al*. (2015) in soil saprotrophs such as *Acremonium, Aspergillus, Fusarium* and *Pencillium*.

Activity of chitin deacetylase in *L. saksenae* was detected using p-nitroacetanilide, which has a unique property to cause colour change in the detection medium. Chitin deacetlylase hydrolyses N-acetamido bonds in chitin and produce chitosan by generating glucosamine units and acetic acid**,** which changes the pH from neutral to alkaline and colourless medium to deep yellow. Colour reaction of P- nitroacetanilide was used for detecting chitin deacetylase in the bacterium *Bacillus cereus* TK19 (Kaur *et al*., 2012); yeast *Saccharomyces*

cerevisiae (Meyen) (Kashyap and Garg, 2014) and the fungus *Aspergillus flavus* (Link) (Naryanan *et al*., 2016).

Extracellular amylase was detected by the development of a clear zone around the colony of *L. saksenae* in the minimal medium amended with starch when flooded with iodine solution. The halo denoted the area of utilization of complex starch molecules and its break down into monomers due to amylase activity. A comparable halo development and amylase activity in *L. lecanii* had been reported by Hasan *et al*. (2013).

Enzyme index is a semi quantitative measurement of the enzyme activity for initial screening of fungal isolates. It is likely to vary with the detection methodology, especially the inducing medium used in assay. Enzyme activity of *L. saksenae* reached its peak on the 8^{th} day of incubation with quantitative variation among them (Fig. 28). The indices of chitinase, lipase, protease, chitosanase, chitin deacetylase and amylase were 1.08, 4.25, 1.11, 1.36 and 1.57, respectively. Although *L. saksenae* recorded the lowest enzyme index for chitinase in the halo method, the colour reaction test proved high chitinase activity, demonstrating its high predominance. A strong lipolytic mechanism observed in the fungus is remarkable, as indicated by a high index of 4.25.

The same isolate, when evaluated by Jasmy (2016), for the effect of natural biopolymers in inducing enzymes showed varying indices in the amended and unamended media. When grown in chitin amended SDB, the values were higher for chitinase, lipase and protease (4.13, 3.07, 7.03) while it was 3.23, 1.77 and 5.57 respectively in SDB without chitin. Higher indices reported in the study might be attributed to the high nutrient content of the biopolymers, chitin and chitosan, in contrast to the minimal medium used in this study.

In a study conducted by St. Leger *et al*. (1997) in three different strains of *L. lecanii*, grown in the minimal medium supplemented with 0.1 per cent yeast extract, elastin (0.1%), mucin (0.3%) or protein azure (0.1%), the protease index

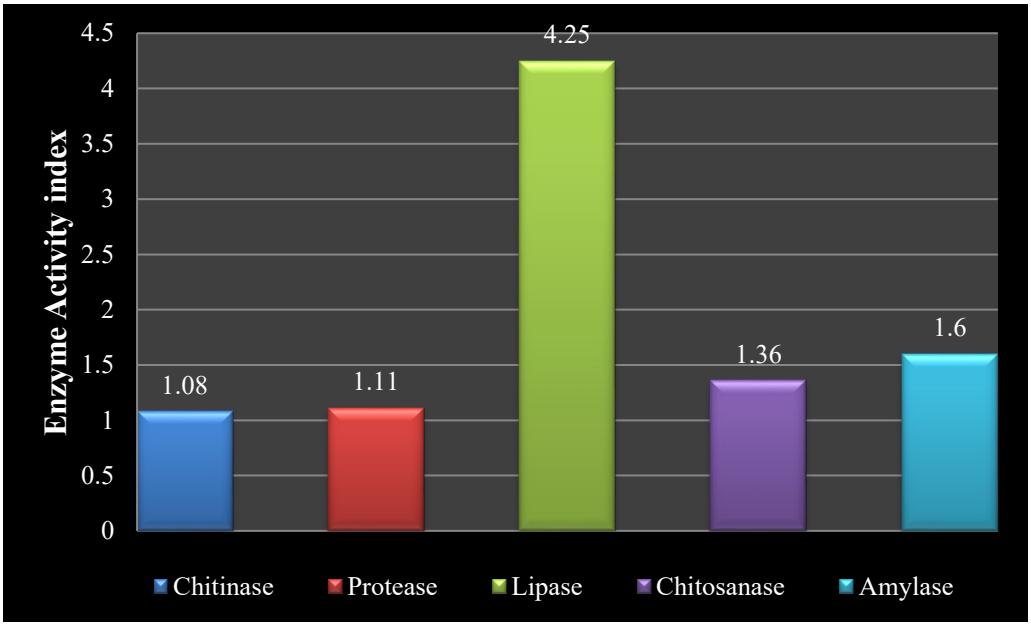


Fig. 28 Enzyme index of *L. saksenae* on 8th day of incubation

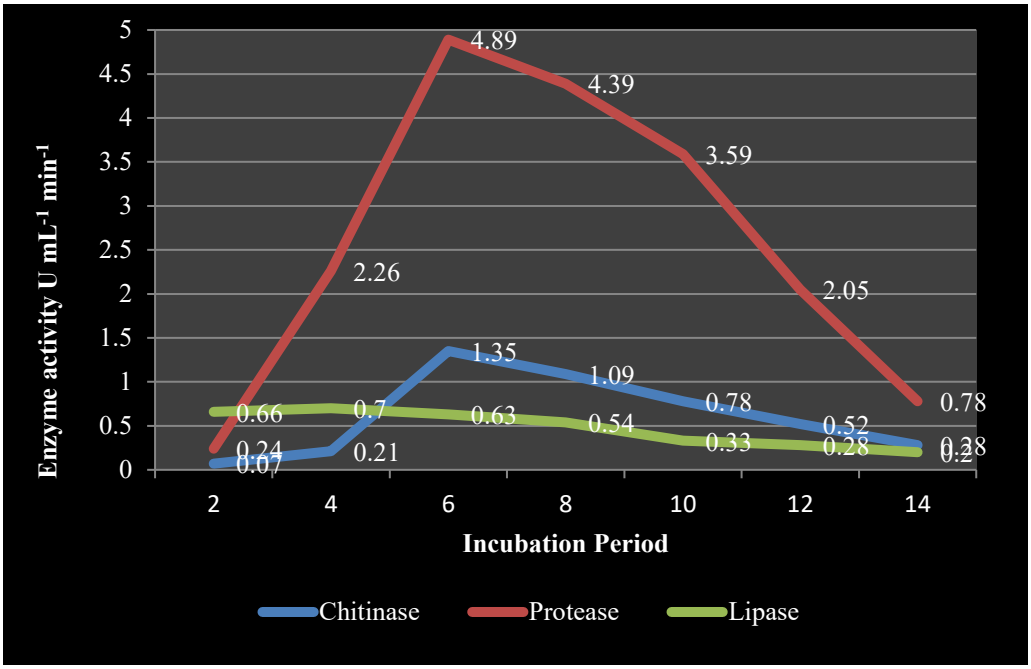


Fig. 29 Enzyme activity of major cuticle degrading enzymes on 8th day of incubation

varied from 1.0 to 1.6. Meanwhile, in the minimal medium supplemented with yeast extract (0.05%) and colloidal chitin (0.2%), the chitinase index was 1.1 in all the three strains. In another study conducted in the same species, Hasan *et al*. (2013) reported indices to the tune of 2.14 for lipase and 2.19 each for protease and amylase on the 7^{th} day of incubation.

The enzyme indices reported in other entomopathogenic fungi such as *B. bassiana* and *M. anisopliae* also indicated slight variations attributed to the assay medium. While studying the extracellular enzyme secretion pattern of *B. bassiana*, Kaur and Padmaja (2009) reported higher indices for lipase and amylase, compared to chitinase and protease. For most of the isolates, lipase and amylase indices were in the range of 3.06 to 3.80 and 3.03 to 4.25 respectively, while for protease it was 1.52 to 3.06 and chitinase 1.19 to 1.90. Similar results were reported by Bai *et al*. (2012) in different isolates of *M. anisopliae* where the chitinase index ranged from 1.5 to 2.2, lipase from 1.15 to 7.1 and protease from 1.2 to 3.3. Here the indices were calculated on different media with respective substrates *viz*. colloidal chitin (2%), skimmed milk (1%) and tributyrin (1%) amended PDB.

Enzyme production pattern of entomopathognic fungi are unique and distinctive for each isolate. Geographical and strain variations, host and substrate variability are some of the factors that accounted for varying enzyme activity.

Quantitative assay of enzymes disclosed that in *L. saksenae*, chitinase activity was more in enzyme producing medium (EPM) than in SDB. In EPM the peak activity was noted on the 6^{th} day (1.35 U mL^{-1} min^{-1}) while it was 0.09 U mL^{-1} min^{-1}) in SDB, on the 8^{th} day. Increase in activity of chitinase in EPM is accounted to the presence of colloidal chitin in the medium, which is in corroboration with the findings of Jasmy (2016). The study infers that chitinase production in *L. saksenae* is profoundly high compared to other genera of entomopathogenic fungi or even to the different species of the same genus.

Ramzi and Zibaee (2014) reported a low chitinase activity of 0.061 ± 0.009 U mg^{-1} protein at 12 days of incubation when *L. lecanii* was grown in the medium containing cuticle of *Chilo suppressalis* (Walker). So also, Nguyen *et al*. (2015) reported only 0.528 U mL^{-1} on the 6^{th} day in the case of *L. lecanii* strain 43 H.

Likewise, in other species such as *M. anisopliae* and *B. bassiana* also chitinase levels reported were much lesser. In *M. anisopliae* it ranged from 0.01 to0.039 U mL^{-1} (Nahar *et al*., 2004) and 0.093 ± 0.003 U mg^{-1} protein (Ramzi and Zibaee, 2014) and in *B. bassiana* it was 0.65 U mL^{-1} on the 6^{th} day (Dhawan and Joshi, 2017).

Quantitative assay of lipase carried out in *L. saksenae*, revealed its presence to the tune of 0.20 to 0.7 U mL^{-1} min^{-1} in EPM and 0.18 to 0.67 U mL^{-1} min^{-1} in SDB. However, a higher value was expected as its index calculated in the preliminary assay was higher than those of all other enzymes. It might be attributed to the lack of inducing agents or specific surfactants as mentioned in other methods. Surfactants added in the medium enhance lipase activity by increasing cell permeability, and facilitating export of several molecules across cell membrane. High lipase index of *L. saksenae* (4.25) observed is advantageous, as it would expedite the initiation of infection process. The lipophylic nature of the fungus could enable better adherence with the insect cuticle.

Silva *et al*. (2010) established the role of lipase as a prominent extracellular enzyme, in the infection process of *M. anisopliae* and opined that, high lipase content increases the lypophylic interactions of the conidia with host cuticle, facilitating better adhesion to insect cuticle.

Nahar (2004) reported a lower level (0.153 and 0.500 U m L^{-1}) of lipase in *M. anisopliae,* while a higher level (97.44 ± 1.96 U mL^{-1}), was reported by Ali *et al*. (2009b) when the medium was amended with olive oil (2%).

Zibaee *et al*. (2011) quantified lipase activity in *B. bassiana* and reported that the highest specific activity was on the 6th day (0.686 $\pm$ 0.003 m OD min^{-1}). These variations in enzyme levels noticed in different research work might be due to species variation of the fungi or due to variation in the substrates selected for the assay.

Quantitative assay on total protease of *L. saksenae* revealed that its activity was more when compared to those of lipase and chitinase (Fig. 29). It was 4.89 in SDB and 0.99 U mL^{-1} min^{-1} in EPB. Higher levels in SDB might be attributed to the presence of peptone in SDB, whereas in EPB it was colloidal chitin. This observation is in concurrence with that of Jasmy (2016) who reported that it was protease that dominated (3.45 U mL^{-1}) among the major cuticle degrading enzymes of *L. saksenae.* High quantity of protease speeds up the degradation of proteinaceous cuticle into its monomeric units of amino acids which serve as nutrient source to the fungi. It play a key role in cuticle hydrolysis and in turn accorded for the high virulence and pathogenicity reported by earlier researchers who worked with this indigenous isolate.

Dominance of protease in other entomopathogenic fungi was earlier reported by St. Leger *et al*. (1987) who reported a rapid production of proteases by *M. anisopliae.* Dominance of protease in the reports of Shinde *et al*. (2010) indicated that it served as major cuticle degrading enzyme in *L. lecanii*. In *B. bassiana* and *L. lecanii* 1037 it was 3.001 and 0.8946 U mL^{-1} respectively in casein amended medium, on 6thand 8th days of inoculation (Dhar and Kaur 2010 ; Quan and Hanh, 2015).

Among the fungal proteases, subtilisin-like serine protease (Pr1) and trypsin-like protease (Pr2) play a significant role in cuticle penetration. In *L. saksenae*, their activities were fairly high. Pr1 reached its peak on the 5th day (0.22 U mL^{-1} min^{-1}) while Pr2 exhibited a peak of 0.32 U mL^{-1} min^{-1} , on the 2nd day itself establishing the fact that it is Pr2 that trigger Pr1 as suggested in several studies conducted across the globe.

Gillespie *et al*. (1998) observed the presence of Pr2, 24 h before that of Pr1, in the culture medium of *Metarhizium* spp. with *Schistocerca gregaria* (Forsskal) cuticle, advocating the role of Pr2 in induction or activation of Pr1. Tiago *et al*. (2002) reported the dominance of Pr2 activity than Pr1 in *M. favoviridae* (Gams and Roszypal) and suggested a differential expression of Pr1 and Pr2 with minimal medium and medium with cuticle of *S. pallens*. Fang *et al*. (2009) suggested that Pr1 play a key role in cuticle degradation, whereas Pr2 regulate the activity of Pr1 without involving directly in cuticle degradation. They also suggested that Pr1 is secreted during the early stages of infection process *via* a signal transduction mechanism which activates the protein kinase A (PKA). Pr1and Pr2 had also been reported from supernatant of *N. rileyi* (Perez *et al*., 2014). In *L. lecanii* the activity reported by Ramzi and Zibaee (2014) was 0.003 ± 0.001 (Pr1) and 1.06 ± 0.06 (Pr2), while in *B. bassiana*, Pr1 peak observed on the 5th day was 4.66 U mL^{-1} and Pr2 on the 4th day was 5.48 U mL^{-1}.

Keratinase is an important class of protease enzyme which break down the insoluble protein, keratin. Different microorganism *viz.* fungi, bacteria and actinomycetes are sources of keratinase. It is an enzyme which is of great commercial and economic importance apart from its role in pathogenesis.

L. saksenae revealed fairly high keratinolytic activity to the tune of 15.93 U mL^{-1} min^{-1} to 73.61 U mL^{-1} min^{-1} , over a period of 50 days of incubation in chicken feather substrate (pH 7). During this period, a rapid increase in protein content of medium from 214.23 to 755.46 µg was noted with a hike in pH from 7 to 8.2. Change in total protein and hike in pH in keratin specific medium are characteristics of keratin degradation. Keratinolytic property of *L. saksenae* was earlier reported by Kushwaha (1980). Keratin degrades to sulphur containing amino acids and polypeptides which changes the pH of the medium to alkaline (Kaul and Sumbali, 1999). Kim (2003) studied keratinase activity of different species of *Aspergillu*s and observed a hike in total protein (40 to 59 µg mL^{-1}) and

pH (7.8 to 9.4). Singh (2014) and Kumar and Kushwaha (2014) reported it in the genus *Lecanicillium* though it is not in dermatophylic group.

In this study, it was also noted that keratinase activity of *L. saksenae* was more during the later stages (40 days of incubation), though its activity was detected even in the early days (20 days), which is in concurrence with the reports of Park and Son (2009), which suggested that keratinase activity takes place during the late exponential or stationary phase of growth though feather degradation starts during the early phase and continues. Potential of fungi *viz. Acremonium implicatum* (Gilman and Abbott) Gams, *T. harzianum* (Rifai), *Chrysosporium indicum* (Randhawa and Sandhu), *L. lecanii,* etc. in degradation of keratinous waste was also reported by Singh (2014).

Virulent strains of entomopathogens possess an innate mechanism to overcome the defense mechanisms such as melanisation developed by the insect. Chitin metabolizing enzymes such as chitin deacetylase and chitosanase help to overcome the resistance offered by melanised cuticle.

Quantitative assay of chitosanase secreted by *L. saksenae* expressed its activity ranging from 0.003 to 0.26 U mL^{-1} min^{-1} with its peak on the 8^{th} day. Nahar (2004) identified constitutive production of chitosanase in *M. anisopliae* with its highest activity of 0.31 ± 0.04 U mL^{-1} after 72 h. They suggested that chitin deacetylase secreted by the fungus converts chitin to amorphous chitosan which will be later acted upon by chitosanase.

Assay on quantitative estimation of amylase revealed its presence to the tune of 23.42 U mL^{-1} min^{-1}, with peak activity on the 10^{th} day (pH 6.9), which is high when compared to the units mentioned in other entomopathogenic fungi such as *M. anisopliae, B. bassiana, L. lecanii* and *P. lilacinum.* It is speculated that these fungi hydrolise starch and uses it as a source of energy for its growth and sporulation, during its facultative mode of life.

Murad *et al.* (2006) could not detect α-amylase in *B. bassiana* growing in *C. maculates*, cuticle and thus illustrated that amylase does not have role in infection process. Whereas, Hasan *et al*. (2013) reported the highest amylolytic production in strains of *L. lecanii* on the 7th day of incubation at pH 3. So also, Barra *et al*. (2015) detected low quantity of amylase in *P. lilacinum* against *Tribolium confusum* (J) on the 3rd day at a pH 9.

A comparatively high activity of major CDE such as chitinase, lipase and protease and other chitin hydrolyzing enzymes such as chitosanase and chitin deacetylase accounts for high virulence and speed of kill of *L. saksenae* reported by the earlier workers, Sankar and Rani (2018). Further, a remarkable level of keratinase activity noted in enzyme assay throws light to newer prospects of utilizing this fungus in keratinous waste degradation and leather industry as well.

As the genes encoding CDE are one of the targets for genetic improvement, information on the bioefficacy of individual enzyme is highly warranted. Although many of the researches focused on detection and quantification of enzymes produced by entomopathogenic fungi, efforts to test their bioefficacy in insects is meagre. A few studies conducted in this line were those using culture filtrate and spore suspensions.

In this study, partially purified enzyme of *L. saksenae* when tested for its bioefficacy in one of its susceptible host insect, the brinjal mealybug *C. insolita,* revealed that it was protease (100 µg mL^{-1}) that exhibited highest mortality compared to chitinase and lipase (Fig. 30). The cumulative mortality recorded was 71.79 per cent in nymphs, 96 h after treatment. The corresponding death rate observed with chitinase was 58.10 per cent and with lipase was 54.23 per cent. A marginal increase in mortality was observed when combination of all the three enzymes was applied. A mortality of 78.79 per cent was recorded with 100 µg mL^{-1} after 96 h, while it was 46.28 and 29.28 per cent with 50 and 25µgmL^{-1}. Cumulative mortality in adults were relatively low (46.88, 35.29 and 23.68 % at 100, 50, 25 µg mL^{-1}, respectively). Mortality of test insects in the

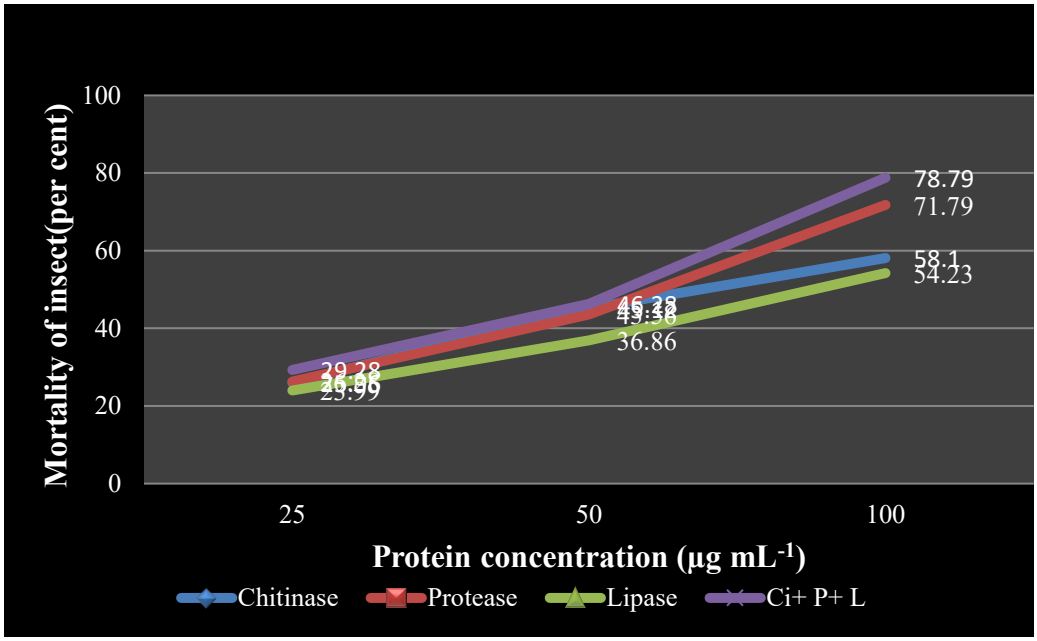


Fig. 30 Comparative bioefficacy of enzymes on *C. insolita* nymphs at 96 HAT

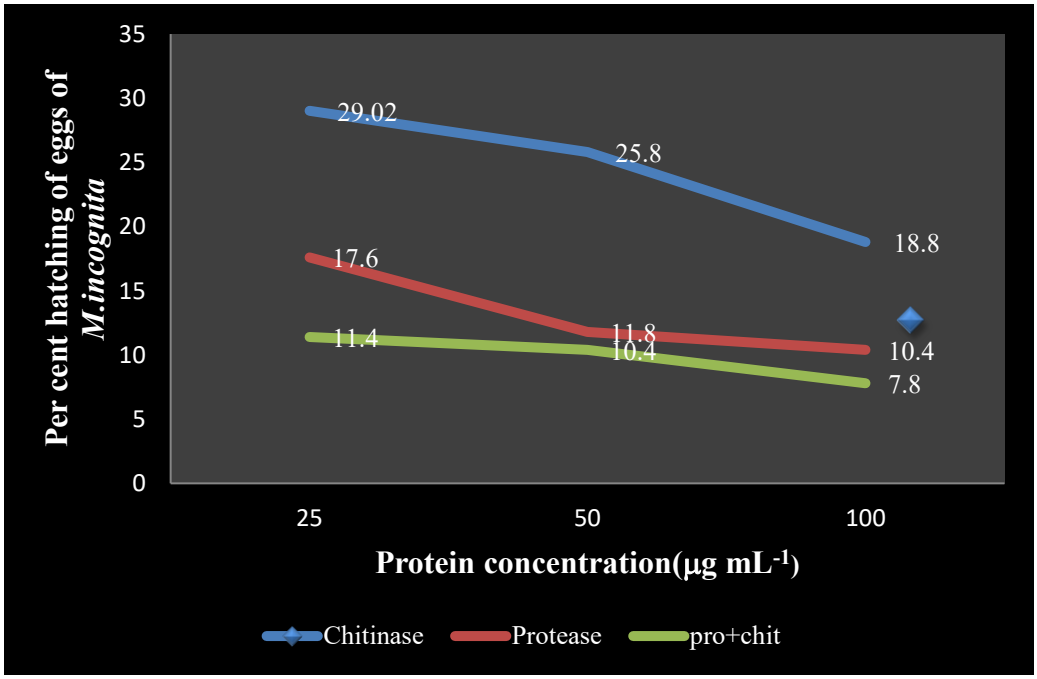


Fig. 31 Effect of partially purified enzymes on *M. incognita* eggs at 120 h after incubation

enzyme bioassay studies is a common phenomenon reported in various studies. Wu *et al*. (2010) reported larval and pupal mortality of diamondback moth, *P. xylostella* using chitinase present in the culture filtrate of *M. anisopliae.* They observed a larval mortality of 67.89 ± 3.11 per cent with the fraction having chitinase activity of 600 mU mL^{-1}. In a similar study, Kim *et al*. (2010) established the insecticidal activity of chitinase containing supernatant of culture filtrate of *B. bassiana*. They reported 77.5 per cent mortality of *A. craccivora* while the filtrate in which chitinase was inhibited caused only 7.4 per cent mortality. Moussa *et al*. (2014) confirmed the potential of chitinase over an insecticide primicarb at 15.63, 31.25, 62.50, 125.00, 250.00 ppm on *A. craccivora* though the effect of the former was not stable. They reported that chitinase exhibited less LC_{50} of 24.95 ppm as against 45.25 ppm for the standard chemical. Efficacy of chitinase enzyme had also been reported from soil fungi *Candida albicans* (Robin) Berkhout @ 3.42 ± 0.02 Um L^{-1} and *A. fumigates* @ 2.35 ± 0.03 U mL^{-1} on cattle tick *Boophilus microplus* (Canes.) exhibited a mortality of 90 and 80 per cent respectively, after seven and eight days of application (Hassan *et al*., 2015).

Mortality caused by CDE in *S. litura* was explained by Regev *et al*. (1996) as degradation of structural polysaccharides in the cuticle, which was further confirmed through transmission electron microscopy (TEM). TEM revealed the disintegration of cuticle of the treated insects with deformation of haemocoel. Cuticular laminations were not observed in enzyme treated insects while it was normal in control. Chai *et al*. (2012) suggested that the insecticidal activity of entomopathogenic fungi is directly linked with the CDE.

The role of lipase to speed up conidial germination and mortality of test insects were demonstrated by Supakdamrongkul *et al.* (2010). They reported that purified lipase of *N. rileyi* at a concentration of 2.75 U with the conidial suspensions of 10^6 spores mL^{-1} caused 63.3 per cent mortality in *S. litura* at 4–10 day post-exposure period, which was about 2.7 times more when compared to

that observed with conidial suspensions alone. The insecticidal effect of lipase was also vivid in the study carried out by Ali *et al*. (2014). They observed a low median survival time (ST_{50}) of 2.91 $\pm$ 0.12 days in the combined application of lipase 100 mU mL^{-1} and conidial suspension ($1x10^7$ mL^{-1}) of *I. fumosoroseus* while it was high when treated with conidial suspension alone (6.37 $\pm$ 0.19).

Though *L. saksenae* was found to have a prolific enzyme secretion mechanism, the mortality caused by either chitinase or lipase or protease resulted in less mortality (Highest 71.79%). This is due to the fact that morbidity and mortality of insects treated with entomopathogenic fungi is the combined effect of enzymatic degradation along with the mechanical pressure exerted by germ tube and insecticidal toxins released by the fungus. High mortality in protease treatment, compared to the other two enzymes is attributed to the fact that protein being the major constituent of insect cuticle, the most important lytic enzyme would be protease.

Examination of ectoparasitic nature of *L. saksenae* on egg mass of *M. incognita* revealed complete disintegration of egg mass within 10 days of treatment. Parasitism was initiated by the attachment of hyphae on the gelatinous matrix over the egg mass, with subsequent proliferation of the fungus over its surface. Nevertheless, there was no fungal structures or growth within the eggs when viewed under zoom stereo microscope (Leica^{TM}). Jasmy (2016) stated that *L. saksenae* could not establish as an endoparasite in the individual eggs of *M. incognita* and its ectoparasitic nature was also not observed in her study.

In a study carried out with a different isolate (CGMCC5329) of *L. saksenae* in China by Cao *et al*. (2012), its nematophagous nature was reported in *M. incognita.* Such intraspecific variations are not unusual among geographical isolates.

Endoparasitic nature of fungi such as *Pochonia chlamydosporia (*Zare and Gams) *P. lilacinum* and *Hirsutella* sp on *M. incognita* eggs was explained by many workers. Sharon and Spiegel (2007) stated the endoparasitic nature of *T. asperellum (*Samuels, Lieckf. and Nirenberg) and *T. atroviride.* They suggested that these fungi utilize fucose and N-acetyl glucosamine present in the gelatinous matrix of *Meliodogyne* eggs as its carbohydrate source. Normally the matrix acts as a defensive surface which could be invaded only by a virulent strain of the invading microorganism, whose proteolytic and chitinolytic enzyme secretion will be triggered upon parasitisation. In contradiction to the observations of the present study, Moosavi *et al*. (2011) detected the penetration peg of *P. chlamydosporia*, *P. lilacinum* and *Hirsutella* sp. in the parasitized eggs of *M. arenaria* (Neal) Chitwood and *M. javanica* (Treub) Chitwood.

Complete disintegration of egg shell noted in this study may be attributed to the presence of high level of extracellular cuticle degrading enzymes produced by *L. saksenae* which enabled the fungus to degrade the egg shell which is chitinacious and proteinacious in structure. Nematicidal metabolites produced by the fungus might also have contributed to the disintegration of eggs. This hypothesis was supported by the findings of Singh and Mathur (2010), who reported that nematophagous fungi either parasitize or produce toxic compounds harmful to plant parasitic nematodes. Similar observations were made by Hussain *et al*. (2018) who reported the parasitic potential of *L. muscarium* on different life stages of *M. incognita* and their disintegration. Parasitic nature of *L. saksenae* observed in this *in vitro* experiment, warrants *in vivo* evaluation to exploit its potential for the management of this pest.

M. incognita eggs treated with *L. saksenae* enzymes were found to be inhibited. In chitinase treated eggs, hatching was only 18.8 per cent while with protease, it was 10.8 per cent. Combined application of chitinase and protease drastically reduced hatching to 7.8 per cent as against 70.80 per cent in absolute control (Fig. 31). This ovicidal effect is attributed to the ability of the enzyme to

degrade the chitinaceous and protienaceous layers of egg shell. These finding are in concurrence with the reports of Khan *et al*. (2004) where, chitinase and protease obtained from *P. lilacinum* caused a drastic reduction in the hatching of *M. javanica* eggs to 17 and 5 per cent respectively, while in combined application it was 9 per cent. So also, these enzymes from *P. lilacinum* were reported to be inhibitory to *M. javanica* eggs by Huang *et al*. (2004). They also observed that combined application exhibited a synergistic effect. Similar effect was also reported Yang *et al.* (2005) where, inhibitory action of crude enzymes of a closely related species *L. psalliotae* in the eggs of vinegar nematode *Panagrellus redivivus* (Linn.) was observed. They also reported that crude enzyme was more inhibitive than the purified protease and suggested the role of other enzymes too, in the degradation process.

In this study, Scanning Electron Microscopic (SEM) analysis of the enzyme treated eggs revealed morphological changes in the outer vitelline layer of the egg shell, where it was found to be shrunken and uneven with loss of egg shell integrity. Furthermore, lines of breakage were observed at different points on the outer vitelline layer, indicating the protease activity on the outer vitelline layer which is composed of protein. Such effects of enzymes from *P. rubescens* was earlier reported by Tikhonov *et al*. (2002) wherein they reported structural deformity on the egg shell of the cyst nematode G*lobodera pallida* (Stone) Behrens. Khan *et al*. (2004) confirmed the disintegration of different egg shell layers of *M. incognita* by chitinase, protease and lipase of *P. lilacinum* by SEM. They also stated that combined application of protease and chitinase caused complete digestion of inner lipid and chitin layers and that the outer vitelline layer lost its integrity.

Extracellular protease play a key role in the hydrolysis of insect cuticle and facilitate fungal penetration and infection process. The purification and characterization of extracellular protease of *L. saksenae* revealed a single band of 71 kDa in SDS-PAGE. This protease shares similar characteristics in terms of

high molecular mass with that of Pr1 and Pr2 of *B. bassiana* reported by Firouzbakht *et al*. (2015) which were novel with high molecular weight of 105 and 103 kDa respectively.

Proteases have been isolated and characterized from different entomopathogenic fungi. Bidochka and Khachatourians (1987) identified a protease of *B. bassiana* with a molecular weight of 35 kDa, whereas in *M. anisopliae* it was of 27 kDa (Pei *et al.,* 2000). The purified extracellular protease (Ver112) of *L. psalliotae* showed apparent homogeneity giving a single band on SDS-PAGE with a molecular mass of 32 kDa (Yang *et al.*, 2005). It was further reported by Zibaee and Badani (2009) who observed a molecular weight of 47 kDa with protease purified from *B. bassiana* cultured in the presence of corn bug *Eurygaster integriceps* (Puton).

MYCOTOXINS OF *L. SAKSENAE*

Secondary metabolite production is a characteristic feature of hypocrealean entopathogenic fungi. Broad spectrum activity of these fungal metabolites include benefits to the producing organism such as development, intercellular communication, and interaction with other organisms in complex niches (Brakhage, 2013). These metabolites which include toxins, pigments etc. play a pivotal role in determining their virulence (Singh *et al*., 2016) and to overcome the defense mechanism of host insects, through diverse biological reactions.

The indigenous isolate of (ITCC LsVs1-7714) *L. saksenae,* was reported to be a potent entomopathogen with high speed of kill in hemipteran pests (Sankar and Rani, 2018). They suggested the role of some toxic metabolites in addition to the role of conidia in pathogenesis. This was the real instinct to profile its secondary metabolites.

The most important metabolite isolated and purified from *L. saksenae* grown in potato dextrose broth (PDB) was oosporein. It is a symmetrical, red pigmented 1, 4-bibenzoquinone molecule originally described as a dye from the endophytic fungus *Oospora colorans* (Beyma). The first report of oosporein dates back to 1944 by Kogl and Wessem. Since then, it was described as a mycotoxin from *B. bassiana* (Vining *et al.,* 1962)*, Chaetomium trilaterale* (Chiv.), (Cole *et al*., 1974) and *L. psalliotae* (Nagaoka *et al*., 2004). It exhibits antibiotic activity against gram +ve bacteria (Brewer *et al*., 1984), antiviral property (Terry *et al*., 1992) and antimicrobial activity against plant pathogenic oomycetes (Nagaoka *et al*., 2004). Its role in virulence of an entomopathogenic fungus to evade the host immune system and facilitate fungal development within the insect was first reported by Amin *et al*. (2010).

L. saksenae in PDB had a unique, wine red pigmentation on incubation, unlike that grown in SDB, suggesting that its production is influenced by the composition of culture medium, which is typical to certain entomopathogens. Its production was detected from third day onwards and the turnover was relatively high (100 mg L^{-1}) in PDB. *Beauveria* is another genus in which certain strains are known for their pigment production. Strasser *et al*. (2000) reported oosporein in different isolates of *B. brongniartii,* the content of which was found to vary with culture conditions. In submerged culture the production was 30-240 mg L^{-1}, in barley kernel it was 1.97-3.27 mg Kg^{-1} and in mycosed cockchafer larvae, it was 30 to 230 µg $larva^{-1}$. The genus *Lecanicillium* is also known for its pigment production. Nagaoka *et al*. (2004) reported higher quantity of oosporein (125 mg L^{-1}) in *L. psalliotae*.

Elevated levels of oosporein in *L. saksenae* detected during the earlier phase of incubation, indicates its prime role in pathogenicity and virulence of this indigenous isolate. This mycotoxin might interfere with its infection process in the initial days to tackle the host immune system, consequently paving way to the action of other insecticidal metabolites produced during the subsequent phases of

pathogenesis. Oosporein was reported to be a primary virulent factor in *B. bassiana* by Feng *et al.* (2015). They also stated that, oosporein in *B. bassiana* supresses the gene encoding the antifungal peptide, gallerimycin in insects and also inhibits the pro phenol oxidase (PPO) activity in haemocoel, thereby supressing the host defence mechanism. Fan *et al*. (2017) reported that it protects the host cadaver from bacterial infection and helps *B. bassiana* to utilize the host nutrients and complete its life cycle.

Many researchers have focussed on the safety aspects of myco insecticides as there is a concern on risk factors associated with mycotoxins. Oosporien comes under the polyketide (PK) group of mycotoxins. Mycotoxins produced by *B. bassaina, Cordyceps* spp. and *Isaria* spp. produces PK mycotoxins. Tenellins in *B. bassiana*, opaliferin in *Cordyceps* are some examples of PK mycotoxins. Oosporein exhibits a broad spectrum of antimicrobial, antioxidant and cytotoxic activities. The toxic effects of oosporein (20 to 200 μM) on kidney and spleen cells of Balb/C mouse were reported by Remesha *et al*. (2015). It was reported to increase the level of reactive oxygen species, mRNA expression in apoptosis or oxidative stress inducing gene and loss of mitochondrial membrane potential.

However, Strasser *et al*. (2000) suggested that amounts of oosporein released into the soil from the formulated product or mycosed insects will be negligible and either non-toxic to plants or is quickly detoxified by the plant. Hu *et al*. (2016) concluded that oosporein cannot be absorbed by organisms and therefore its entry into food chain is implausible. Owing to this fact, they also stated that it is unlikely to affect non target organisms including human beings.

Upon purification, oosporien had an orange red crystalline structure. Chemical characterization by FTIR, HR-LCMS and ^{1}HNMR and ^{13}CNMR analysis were consistent. Structural characters determined in this study is in concurrence with that of Alaruppa *et al.* (2014), who characterized and

confirmed the identity of oosporein from the endophytic fungus *Cochliobolus kusanoi* (Nisik).

Another important metabolite identified in *L. saksenae* was dipicolinic acid (pyridine-2, 6-dicarboxylic acid) or DPA, a pyridine derived compound with potent biological activities in insect - fungal interaction. It is ubiquitous among bacterial spores and reported to confer thermal resistance and spore dormancy (Fichtel *et al*., 2007).

In this study, DPA was detected through LC-ESI-MS analysis of chromatogram of ethyl acetate fraction of *L. saksenae* grown in Fargues medium. The peak expressed at 2.146 with a m/z of 166.0230 was similar to that of the standard, confirming its presence in the crude toxin. Other peaks noted in the chromatogram (Fig. 11) indicated the presence of other metabolites. Presence of DPA in *L. saksenae* was reported by Jasmy (2016) through HPTLC.

DPA is one of the important insecticidal metabolites reported in the genus *Lecanicillium* (Claydon and Grove, 1982). It was also reported from other clavicipitaceous fungi such as *B .bassiana* (Shima, 1955). It comes under the fungal aromatic acid group which are known to inhibit defensive enzymes explicating their role in insect pathogenesis. Gindin *et al.* (1994) studied the toxic effect of methanolic extract of *L. lecanii* in the nymphs of *B. tabaci*. The toxins exhibited 33.6 and 90 per cent mortality at 0.1 and 0.5 per cent concentrations respectively. They attributed the insecticidal activity of this toxin to phoshospholipid entity. Its bioactivity in insect pathogenesis and mortality had been documented by various authors. Asaff *et al.* (2005) reported DPA from *P. fumosoroseus* and the entomotoxicity of DPA or their calcium salts against *B. tabaci* and blowflies (*Calliphora erythrocephala,* Meigen) with varying degree of toxicity.

The structural character of DPA offers a strong ion chelating property to the compound. Hunt and Ginsburg (1981) suggested its role in inhibiting

enzymes by removal of essential ions from metalloenzymes, especially Zn. The chelating activity DPA suggests that it indirectly inhibits the PPO by interacting with Ca2+ ion which is an essential ion for the activation and activity of enzymes (Dowd, 1999). DPA inhibits PPO system during melanin synthesis in insects, thereby interfering with the innate immune system (Paterson, 2008).

Beauverolide, the third dominant metabolite in *L. saksenae* was identified through bioassay guided fractionation of crude toxin. Spectral data of LC-ESI-MS analysis of the fraction showed strong signal for the ion with m/z 487.64 which confirmed its identity. It belongs to the family of cyclic tetradepsipeptides consisting of 3-hydroxy-4-methylalkanoic acid unit.

Beauverolide has been reported from *B. bassiana, B. tenella* and *P. fumosoroseus* (Jegorov *et al.*, 1994). It is one among the prime metabolites that interferes with the insect immune system making the host insect conducive for fungal invasion and parasitism. It may interfere with any one of the humeral defense mechanisms involving phenol oxidases, lectins, antimicrobial peptides, reactive oxygen species or protease inhibitors (Vey *et al*., 2001)

FTIR spectrum of the culture filtrate and mycelial extract of *L. saksenae* reflected the presence of prominent functional groups identified on the basis of the wave numbers such as O-H, hydrogen bonded alcohols or phenols (3433.80), C-H alkanes (2927.99), -C=C-alkanes (1640.92), C-C aromatics (1451.71),C-O carboxylic acid or alcohols (1307.86), C=O ketones etc. Similar functional groups were revealed in *B. bassiana* by Ragavendran *et al.* (2017) who reported insecticidal activity of many of these and Vivekanandhan *et al*. (2018) who reported mosquitocidal activity of more or less similar functional groups in *B. bassiana*-28.

Under normal fermentation conditions, when a single nutrient source is used for expression of proteins or toxins, silencing of gene clusters may prevent the expression of all metabolites (Scherlach and Hertweck, 2009; Wasil *et al*.,

2013). Recent report by Pan *et al*. (2019) suggested that fungus when grown in different growth media may activate many silent biogenic gene clusters to produce diverse metabolites. Therefore, investigations using "One strain many compounds (OSMAC) technology" which utilizes the ability of fungus to produce different metabolites under different nutritional sources was attempted to reveal all possible metabolites in *L. saksenae*. The crude extracts from these media were subjected to spectral analysis, following the commonly adopted metabolomics tools such as chromatography for separation of metabolites and spectroscopy for their identification.

HR-LCMS and GC-MS analysis of ethyl acetate fraction of the crude toxin revealed wide array of metabolites under different chemical groups of fatty acid, fatty acid amides, alkaloid, organic acids, phenolic compounds, ketones etc. The bioactivities of the dominant metabolites were gathered using Dr. Duke's phytochemical and ethanobotanical database as well as PubChem data bases.

Of the metabolites detected 22 were insecticidal, seven were nematicidal and 20 were antimicrobial.

Insecticidal compounds that fetch special mention other than oosporein, DPA, and beauveroide that were earlier discussed in detail were hexadecanoic acid, octadecanoic acid, harmine, piperidinine, picolinic acid, anthranilic acid, cordycepin and dibutyl phthalate. Perusal of literature shows that most of these metabolites identified in *L. saksenae* have excellent biological activities against insect pests, plant parasitic nematodes and pathogenic microorganisms.

Hexadecanoic acid and octadecanoic acid identified from *L. saksenae* have been previously reported from several other species of entomopathogenic fungi. Rahuman *et al.* (2000) reported the bioactivity of hexadecanoic acid from acetone extract of *Feronia limonia* (Swingle) against fourth instar larvae of mosquitoes, *A. aegypti* and *C. quinquefasciatus*. Further, Vivekanandhan *et al.* (2018) reported its bioactivity on larvae of *C. quinquefasciatus* extracted from

mycelia of *B. bassiana* 28. Ragavendran *et al*. (2019) identified these metabolites in *B. bassiana* through GC-MS analysis, whose insecticidal activity was observed in the larval and pupal stages of *C. quinquefascitus* wherein, the LC_{50} values reported for pure hexdecanoic acid were 2.27 µg for nymphs and 0.69 µg for pupae after 24 h. Recently, Elbanhawy *et al.* (2019), detected these metabolites from the entomopathogenic species *P. lilacinum* and *Cladosporium cladosporiodes* (Fresen), through GC-MS analysis.

Alkaloids such as harmine and piperidinine, detected in this investigation were those which were formerly identified from different insecticidal plant species. Piperidinine was one of the major metabolite in the plant, *Peganum harmala* (Linn.) which has insecticidal activity in *S. littoralis* (Boisd) (Shonouda *et al*., 2008). Harmine is a β-carboline alkaloid which is known to have insecticidal, fungicidal and plant growth regulatory properties (Zeng *et al*., 2010). Bouayad *et al.* (2012) demonstrated the adverse effect of harmine on metamorphosis in Indian meal moth *Plodia interpunctella* (Hüb.) which reduced the larval weight and delayed pupation as well as adult emergence.

The metabolite picolinic acid identified in *L. saksenae* is a compound that interferes with DOPA oxidation by inhibiting phenoloxidase. Phenoloxidase is an enzyme known for its role in cuticular sclerotization and defensive functions in insects. Phenoloxidase inhibitors have profound applications as bioinsecticides in agriculture. Bacon *et al*. (1996) reported picolinic acid from *Fusarium* spp.

Anthraquinone dimers identified in *L. saksenae* was reported from other entompathogenic species *Aschersonia samoensis* (Henn) which revealed strong cytotoxic activity against the insect cell line Sf 9 of *S. frugiperda* with ID_{50} values 1.2 and 9.6 µg mL^{-1}. It is noteworthy that, this metabolite had only a weak activity in mammalian cells (Watts *et al*., 2003). The insect repellent property of dibutyl phthalate, another metabolite detected in this study was reported against *T. castaneum* (Herbst) by Wu *et al*. (2014). This compound once isolated from

tea leaves was reported to be insecticidal with LC_{50} value 357.72 ppm for 48 h against green peach aphid *M. persicae* (Khoshraftar *et al*., 2018).

Insecticidal activity of cordycepin which was also detected in *L. saksenae* was reported by Kim *et al*. (2002). They found that at 500 mg L^{-1} caused 78 and 100 per cent mortality at 2 days after treatment (DAT) and 4 DAT respectively in *P. xylostella* and suggested to be a stomach poison.

The major nematicidal compounds identified through spectrometric analysis were nicotinic acid, DPA, 8-hydroxyl quinone, hexadecanoic acid, octadecanoic acid and harmine, of which the latter three were discussed for their insecticidal properties. Presence of volatile compounds such as hexadecanoic acid, octadecanoic acid, harmine etc. in *L. saksenae* also reflects its nematicidal activity as these compounds were reported to exhibit mortality in *M. incognita* (Gu *et al.,* 2007; Rajeswari *et al*., 2012). Nicotinic acid the most abundant phenolic compound present in the fruit pulp of *Melia azedirachta* (*Linn.*) which is universally known for its insecticidal properties. It was reported to have nematicidal property as well and found to be effective against *M. incognita* (Aissani, 2013). Sharma *et al*. (2018) attributed nematicidal effect of the volatile compounds at concentrations above 100 ppm, against second stage juveniles of *M. incognita* which were isolated from the bacteria *Pseudomonas jessenii* (Verhille) strain R 62 and *P. synxantha* (Holland) strain R 80.

The compound DPA is also known to be nematicidal as per the findings of Liu *et al.* (2009), who isolated it from *Purpureocillium* strain YMF 1.0716. A pyridine carboxylic acid derivative, 4-(4′-carboxy-2′-ethylhydroxypentyl)-5, 6-dihydro-6-ethylcyclobuta [b] pyridine-3,6-dicarboxylic acid was found to be nematicidal with LD_{50} value 47.1 µg mL^{-1} to *M. incognita.*

L. saksenae has antimicrobial properties as evidenced by the presence of metabolites such as aminophenol, acetamide, N-(2-phenylethyl, 4H pyran-4-one, 2,3,dihydro 3, 5 dihydroxyl-6-methyl, dl-mevalonic acid lactone and pyrrolo

1,2 pyrazine 1,4-dionehexahydro etc. identified through GC-MS analysis and vanillic acid, anthranilic acid, salisylamide, erucamide, oleamide, terephthalic acid etc. detected by HR-LCMS analysis.

Mohana *et al.* (2011) suggested antifungal activity of aminophenol against *Fusarium oxysporum* and strong antibacterial activity against gram –ve and +ve bacteria. Use of aminophenol as ligand in synthesis of new metal complexes is being used nowadays in the development of new antibiotics against multi resistant strains of bacteria. Antimicrobial activity of vanillic acid was reported by Srivastava *et al*. (2013). It was reported to inhibit mycelial growth of the stem canker pathogen *Botryo sphaeriarbis* (Howd.) to the tune of 85 per cent in tree crops. Fatty acid amide compounds such as oleomide and erucamide detected in this study were reported from endophytic fungi such as *Botryodiplodia theobromae* (Pat) as a self defensive molecule (Zahar *et al.,* 2015). Tanvir *et al*. (2018) reported its antimicrobial, nematicidal and antifungal properties. Kiran *et al*. (2018), reported the antibiotic property of pyrrolo (1,2) pyrazine 1,4-dionehexahydro against a drug resistant strain of *Staphylococcus aureus,* after isolating it from the marine bacterium *Bacillus tequilensis* strain, MSI45 (Gatson).

Bioefficacy studies carried out with the purified oosporein revealed 60.25 per cent mortality in nymphs of the brinjal mealybug, *C. insolita*, while it was still lower (51.06%), with 500 ppm, 96 hours after treatment (HAT). The corresponding mortality in adults was 51.00 per cent with 1000 ppm and 46.69 per cent with 500 ppm. However, crude toxin exhibited much more efficacy causing 95.98 per cent mortality with 1000 ppm in nymphs and 98.75 per cent death with 500 ppm within 48 HAT. In adults the corresponding mortality was 85.51 and 76.29 per cent with 1000 and 500 ppm respectively.

Probit analysis revealed that crude toxins were more effective to nymphs with lower LC_{50} of 87.14 ppm compared to 193.61 ppm in adults after 48 h. LC_{9O} values were 465.85 and 994.54 ppm in nymphs and adults at 48 HAT. Higher

mortality observed with crude toxin suggests the insecticidal potential of secondary metabolites as a whole that is present in the crude toxin, rather than the individual effect of oosporein. Mozaina *et al.* (2008) reported that 1, 4-benzoquinones caused 100 per cent mortality on subterranean termite while in whiteflies the mortality was only 20 per cent as against 60 per cent observed with spore suspension. Amin *et al*. (2010) observed that the fungal spores along with the red pigment, resulted 92 per cent mortality, probably accounted with the synergistic effect of spores and toxins.

Bioefficacy studies carried out with crude toxin on *M. incognita* revealed an increase in the mortality rate of juveniles with the concentration of toxin and exposure period. The crude toxin revealed 100 per cent mortality at 50 ppm and above at 48 HAT, whereas the mortality was 70.91 per cent at 25 ppm. It was lesser in 10 and 1 ppm (27.54 and 5.61 %).

Nematicidal activity of the crude toxin is attributed to the presence of secondary metabolites with different functional groups *viz*. alkaloids, phenolics, fatty acids and organic acids in *L. saksenae*. Similar observations have been made by Akhtar (2000) who reported nematicidal activity of *A.indica* (A.juss) due to the presence of secondary metabolites. Fumigant action of nematicidal compound is of great importance due to the cryptic nature of nematodes in soil which can be utilized in nematode management. Gupta *et al.* (2006) reported the nematicidal activity of secondary metabolites of *P. lilacinum*. Gu *et al.* (2007) reported strong nematicidal and fumigant activity of fungal volatile compounds against *M. incognita*. Volatile compounds *viz*. hexadecanoic acid, dipicolinic acid, harmine etc. with nematicidal activity were identified in the GC-MS profile of *L. saksenae* (Sreeja and Rani, 2019).

Although earlier reports of Jasmy (2016) and Sankar (2017) has un doubtfully proved the safety of *L. saksenae* to the natural enemy complex in the rice ecosystem including coccinnellids, spiders as well as the egg parasitoid *Trichogramma* spp, effect of its toxin was not screened against these beneficial

organisms. The effect of crude toxin (1000 to 10000 ppm) which is 5 to 56 times higher than that determined for the pests (LC_{90} =176.93 ppm at 96 h) was tested on all stages of *C. sexmaculata*. Its topical application at 1000 ppm was found to have 30 per cent ovicidal activity. Larval and pupal stages were relatively safer with 7.5 and 15 per cent mortality after 48 h and the adults were totally unaffected. Mortality of grub exposed to 10000 ppm was 65± 2.89 at 96 HAT. This observation might arouse an ambiguity while developing commercial insecticides based on toxins. Nonetheless, the concentration of the molecule while developing such a formulation is not going to exceed 10 fold the lethal dose determined for the pest.

The results revealed that the toxicity varied with stages of the insects and exposure time. Egg was the most sensitive stage to the toxin followed by larval and pupal stages. Adults were relatively safer to the effect of toxin and mortality to adults were marginal. Wang *et al*. (2005) observed that crude toxin of *L. lecanii* significantly affected feeding capacity and reduced the fecundity and longevity of the larval stage of coccinellid predator *D. catalinae* of the whitefly *B. tabaci* at a concentration of 400 ppm. Khan *et al*. (2015) observed the toxicity of biorationals against *Menochilus sexmaculatus* (Fab.). Emamectin benzoate was least toxic against egg, larva, pupa and adult whereas neem oil was highly toxic and caused a mortality of 80 and 86.67 per cent to egg and pupa respectively.

The insensitivity of adults of *C. sexmaculata* to different concentrations of crude toxins of *L. saksenae* observed in the study might be due to the activation of resistance mechanism in the predator to detoxify the toxins. Similar observations have been made by Wang *et al.* (2005) who reported that crude toxins of *L. lecanii* was safe to adults of *Delphastus catalinae* than larval and pupal stages.

It is quite cumbersome to study each of the fungal metabolites to identify a novel insecticidal molecule. Structure based virtual screening is the most

widely used strategy to identify the most promising compound for biological assay (Loza-Mejia *et al*., 2018). An insight into the insecticidal metabolites produced by entomopathogenic fungi and their active site of interaction within the host cell would also help to identify hyper virulent strains. In order to deduce the mode of action of the metabolites present in *L. saksenae* molecular docking was carried and the results were promising.

In silico molecular docking of the compounds detected from *L. saksenae* revealed that 18 of them interacted with acetylcholinesterase (AChE) at esteratic site of the catalytic triad, serine, histidine and glutamate residues. Among these, 3-hydroxy-2-methylpyridine exhibited the highest binding energy (-133.24 kcal mol^{-1}) and therefore highest interaction with AChE, followed by 2-hydroxybenzamide (-131.48 kcal mol^{-1}), 6-hydroxy picolinic acid (-121.21 kcal mol^{-1}), and picolinic acid N-oxide (-113.28 kcal mol^{-1}). Inhibition of AChE activity leads to the accumulation of acetylcholine causing cholinergic stress, leading to paralysis, ataxia and general lack of coordination in the neuromuscular system and eventual death (Singh and Singh, 2000). This finding supports the increased mortality and paralysis observed within a short period of exposure by Sankar and Rani (2018) while studying the pathogenesis of *L. saksenae* in rice bug *Leptocorisa acuta* (Thunberg).

Raghavendran *et al.* (2019), reported the acetyl cholinesterase inhibitory activity of the crude toxin containing different secondary metabolites extracted from *Pencillium* sp on 4^{th} instar larvae of *A. aegypti*. The activity was attributed to the monoterpenoid fractions in crude extracts which were inhibitory to AChE. It was further reported that secondary metabolites of *Pencillium* such as 1-octadecene, 1-nonadecene, 9-octadecenoic acid also inhibited acetyl cholinesterase activity in 4^{th} instar larvae of *C. quinquefasciatus*.

Metabolic versatility of *L. saksenae* in different nutrient media suggests the presence of many silent biogenetic gene clusters in the genetic makeup of the

organism. It also highlights the presence of many other metabolites whose chemistry is unknown, as of now.

From the foregoing findings it is concluded that *L. saksenae* is a resourceful fungus rich in primary as well as secondary metabolites, that would be tapped not only for their pest management prospects but also in the leather and poultry industry. Biocidal secondary metabolites identified in this study such as oosporein, DPA and harmine has immense scope in the development of insecticidal, nematicidal and antibacterial formulations, while its potential enzyme activity especially keratinase, may be made use of in leather industry and keratinous waste management. Innumerable number of other metabolites revealed in spectral analysis of crude toxin, need to be characterised further.

SUMMARY

6. SUMMARY

The study on 'Mycotoxins and enzymes of entomopathogenic fungus *Lecanicillium saksenae* (Kushwaha) Kurihara and Sukarno and their bioefficacy on crop pests was carried out at the Biocontrol Laboratory for Crop Pest Management, Department of Agrl. Entomology, College of Agriculture, Vellayani and AINP on Vertebrate Pest Management Laboratory, Department of Agrl. Entomology, College of Horticulture, Vellanikkara during 2016-2019, the results of which are summarized below.

The extracellular cuticle degrading enzymes produced by *L. saksenae* were detected qualitatively by plate assay method using inducing substrates. The activity of chitinase, lipase, protease, chitosanase and amylase were detected on the basis of development of halo around fungal colony, whereas, chitin deacetylase was detected by colour reaction using pH indicator medium. In addition to halo method, chitinase was also detected by colour reaction of the medium. Colour reaction revealed high activity of chitinase and chitin deacetylase. Enzyme indices worked out for chitinase, lipase, protease, chitosanase and amylase were 1.08, 4.25, 1.1, 1.36 and 1.57 respectively.

L. saksenae cultured in two different media *viz.* Enzyme Producing Medium (EPM) with colloidal chitin and Sabouraud Dextrose Broth (SDB) were assayed for the major cuticle degrading enzymes, chitinase, lipase and protease at two days interval for 14 days. The assay revealed activity of chitinase and lipase was high in EPM whereas protease was high in SDB. In EPM chitinase activity reached its peak (1.35 U mL^{-1} min^{-1}) on the 6^{th} day, whereas highest lipase activity (0.7 U mL^{-1} min^{-1}) was on the 4^{th} day. Highest protease activity of 4.89 U mL^{-1} min^{-1}) recorded in SDB was on the 6^{th} day of incubation.

The other enzymes such as Pr1, Pr2, keratinase, chitosanase and amylase was detected in minimal media with specific amendments. The activity of Pr1 and Pr2, the key proteolytic enzymes detected in casein amended medium was to

the tune of 0.22 U mL^{-1} min^{-1} and 0.32 U mL^{-1} min^{-1} respectively. The highest activity of Pr1 was on the 5^{th} day while that of Pr2 was on the 2^{nd} day. Keratinolytic activity of *L. saksenae* was assessed in medium amended with poultry feather as substrate. It was highest on the 40^{th} day (73.61 U mL^{-1} min^{-1}) with a change in pH from 7.0 to 8.2 and total protein from 214.23 to 755.46 µg mL^{-1}. The biomass reduction of 80.2 per cent in poultry feather substrate due to keratinase activity is remarkable. Chitosanase activity in the medium amended with colloidal chitosan ranged from 0.003 to 0.26 U mL^{-1} min^{-1} with its peak noted on the 8^{th} day of incubation. The highest activity of amylase was 23.42 U mL^{-1} min^{-1} on the 10^{th} day of incubation in minimal medium with soluble starch as substrate.

The efficacy of major enzymes of *L. saksenae* was studied on the 3^{rd} instar nymphs and adults of *Coccidohystrix insolita* (G). Among the three major cuticle degrading enzymes, partially purified protease (100 µg mL^{-1}) exhibited highest cumulative mortality of 71.79 per cent in *C. insolita* nymphs, 96 h after treatment (HAT) followed by chitinase (58.10%) and lipase (54.23%). A marginal increase in mortality (78.79%) was observed when applied in combination. Mortality was very low in adults with the individual application of partially purified enzymes. It was 35.64 per cent with protease, 30.25 per cent with chitinase and 26.75 per cent with lipase. However their combined application resulted in a mortality of 40.88 per cent, 96 HAT.

L. saksenae was found to be an efficient pathogen on the egg mass of *M. incogita.* A total inhibition in hatching, followed by disintegration of egg mass within a period of 10 days, was observed. Partially purified enzymes also showed profound inhibition on hatching. Only 18.8 per cent of the eggs were found to hatch when treated with chitinase @ 100 µg mL^{-1} as against 70.8 per cent observed in absolute control. Protease was more effective than chitinase causing only 10.4 per cent hatching. Combined application of protease and chitinase further decreased the hatching to 7.8 per cent.

Scanning Electron Micrograph (SEM) of eggs treated with enzymes revealed aberrant changes in the egg shell with uneven and deformed surface as well as shrinkage and loss of shell integrity. Lines of breakage were observed on the outer vitelline layer.

As protease was found to be the predominant cuticle degrading enzymes it was purified through saphacryl 100 column chromatography and DAEA Cellulose column chromatography. Its molecular weight was determined as 71 kDa through SDS- PAGE analysis.

In order to identify the mycotoxins and other secondary metabolites, *L. saksenae* was cultured in potato dextrose broth and the acidified culture filtrate was subjected to solvent extraction. Vacuum evaporation of the extract under pressure yielded crude toxin @ 600 mg L^{-1}. Purification of crude toxin was carried out by washing with acetonitrile and collecting the insoluble filtrate, which was further purified by washing with hexane which yielded 100 mg L^{-1} of pure toxin.

Structural characterisation and identification of the metabolites present in the purified toxin was done through a series of spectrometric analysis. "Oosporein" was detected as the major toxin produced by *L. saksenae* through various spectral analytical procedures. UV spectrometric analysis expressed high absorbance at 281 nm, while Fourier Transform Infrared spectrometry (FTIR) revealed the presence of functional groups such as O-H, C=O and C=C. High Resolution Liquid Chromatography Mass spectrometry (HR-LCMS) revealed the molecular weight, m/z of the compound as 306.22. Nuclear Magnetic Resonance (NMR) analysis designated the number of OH and CH_3 protons and carbon in the molecule. Spectral analysis invariably confirmed the identity of oosporein which had a unique orange red coloration.

"Dipicolinic acid" (DPA) was another metabolite identified from *L. saksenae* grown in Fargues medium. It was identified through LC-ESI-MS of

the crude toxin extracted through solvent extraction, with a retention time (RT) of 2.146 min and m/z 166.0230, which coincided with those of the standard DPA (RT 2.012 min and m/z 166.0317).

Mycelial crude toxin subjected to column chromatography yielded five different pooled fractions based on TLC. Bioassay guided fractionation detected high insectidal property in fraction 4 (F4) causing 64.23 per cent mortality of *C. insolita* nymphs, 96 HAT. LC-MS analysis of the insecticidal fraction detected the presence of the toxin "beauverolide" based on the m/z 485.6. HPLC analysis of the crude toxin extracted from culture filtrate as well as mycelia could not detect "beauvericin" a common mycotoxin in entomopathogenic fungi.

The chemical nature of the metabolites was identified by their functional groups in the FTIR spectrum of toxin isolated from culture filtrate and mycelial toxin. The functional groups detected in the culture filtrate were alcohols, phenols, alkanes, aromatic and aliphatic amines, carboxylic acids and ketones. Mycelial toxins revealed the presence of primary amines, alkyls, aliphatic amines, carboxylic acids and alkyl halides.

One Strain Many Compounds (OSMAC) technology enabled the detection many metabolites as it uses various substrates to culture the fungus. Crude toxin extracted from culture filtrates of *L. saksenae* grown in various nutrient media such as PDB, Czapek Dox, Fargues and rice grain when subjected to HR-LCMS spectra generated under both –ve and +ve mode of ESI, could detect as many as 100 secondary metabolites. Majority of these were under organic acid group, including the dominant ones such as DPA, picolinic acid, anthranilic acid, 4-dodecylbenzene sulfonic acid, vanillic acid etc. The important alkaloids detected were nicotinic acid, 6-hydroxy nicotinic acid and harmine. Fatty acid amide compounds included oleamide and erucamide, whereas fatty acid esters detected were dibutyl phthalate, di isobutyl phthalate and the phenolic compounds included aminophenol and 6-methoxy salicylic acid.

GC-MS spectra revealed the presence of numerous volatile compounds in *L. saksenae*. The predominant ones were DPA, hexadecanoic acid and octadecanoic acid. Other volatile alkaoids detected were piperidinone and harmine and the volatile ketone was 4-H Pyran-4-one, 2, 3,dihydro 3, 5 dihydroxyl 6 methyl.

Screening of metabolites using PubChem and Dr. Dukes phytochemical and ethanobotanical databases unveiled the properties of the metabolites detected through various spectral analysis. Accordingly they could be classified as insecticidal, nematicidal and antimicrobial compounds. There were 22 insecticidal metabolites of which the dominant ones were picolinic acid, cordycepin, dibutylthalate, diisobutyl phthalate, hexadecanoic acid, ocadecanoic acid pyridine acetic acid, anthranilic acid and erucamide. A total of seven nematicidal compounds could be identified of which nicotinic acid, hydroxyl quinone, DPA, oleamide, and many pyridine dicarboxylic acid derivatives and dodecyl benzene sulfonic acid, N-hexadecanoic acid, harmine and octadecanoic acid need to be mentioned. Of the 20 antimicrobial compounds, 4- H pyran-4-one, 2, 3, dihydro 3, 5-dihydroxyl 6 methyl, harmine and Dl-mevalonic acid lactone aminophenol, vanillic acid, dioctyl phthalate, 6- hydroxy nicotinic acid, isonicotinic1.oxide salisylamide, dapsone, oxacilin, mandipropamid, netilmicin, oleamide and erucamide, acetamide were predominant. Of these compounds DPA, harmine, hexadecanoic acid, ocadecanoic acid, 4 H pyran-4-one, 2, 3, dihydro 3, 5 dihydroxyl 6- methyl, and and Dl-mevalonic acid lactone were volatile metabolites.

Bioefficacy studies carried out in *C. insolita* using the purified as well as the crude toxin, revealed higher insecticidal effect of the latter. In general, nymphs were found to be more susceptible than adults, when treated topically. Both the toxins, purified as well as crude, exhibited a dose - dependent mortality in nymphs. At its highest concentration (1000 ppm), oosporein resulted in 31.33 per cent mortality, while it was 85.23 per cent in the case of crude toxin, at 24

HAT. The corresponding mortality noted with the lower concentration (50 ppm) was 4.00 and 25.44 per cent. At the end of the experimental period (96 HAT), the resultant mortality was 60.25 and 100 per cent respectively with oosporien and crude toxin at 1000 ppm, while with 50 ppm it was 16.5 and 46.88 per cent, respectively.

The same trend was observed in case of adults also. At highest concentration of 1000 ppm, oosporein caused 24.5 per cent mortality at 24 HAT while it was 83.36 per cent with crude toxin. The corresponding mortality observed with the lower concentration of 50 ppm was 2.25 and 7.57 per cent. At 96 HAT, the cumulative mortality with 1000 ppm was 51.00 and 100.00 per cent respectively with oosporein and crude toxin, whereas with 50 ppm the mortality was 9.90 and 13.12 per cent respectively.

LC_{50} values calculated for crude toxin were 155.81, 87.14, 63.94 and 51.06 ppm at 24, 48, 72 and 96 h respectively in nymphs, while in adults the values were 221.71, 193.61, 164.34 and 118.51ppm at 24, 48, 72, and 96 h.

Bioefficacy studies with the crude toxin on J_2 of *M. incognita* revealed the nematicidal potential of secondary metabolites of *L. saksenae.* At highest concentration of 250 ppm, mortality of 95.22 per cent was observed at 24 HAT. At 48 HAT, the mortality observed was 100 per cent with 50 ppm and above. At the end of experimental period (96 h) 97.16 per cent mortality was observed with 25 ppm whereas it was 30.30 and 10.62 per cent with the lower concentration of 10 and 1 ppm, respectively.

As the crude toxin was more effective than the purified, further test on its effect on coccinellid predator *C. sexmaculata* was carried out with the former. As a rule of safety test, the toxins are to be tested at a concentration that is ten times higher to the lethal dose. However in this study the toxins were tested at 5-56 fold of lethal dose to the pest. It was observed that at 1000 ppm crude toxin was slightly toxic to the eggs and larvae as it caused 30 and 35 per cent mortality at

the end of the 96 HAT. The LC_{50} value for ovicidal activity (3524.8 ppm) was 69 fold higher than that of the pest (51.06 ppm at 96 h). LC_{50} for coccinellid grub was 4040.74 ppm at 96 h which was 79 times higher than that of pest. The mortality observed in pupae was negligible (15%) even at the highest concencentration with 1000 ppm. The adults were relatively safe to different doses of toxin.

In silico molecular docking of the metabolites detected from *L. saksenae* revealed that 18 of them were those interacting with AChE at esteratic site of the catalytic triad, serine, histidine and glutamate residues. Among these, 3-hydroxy-2-methyl pyridine exhibited the highest binding energy (-133.24 kcal mol^{-1}) and therefore and highest interaction with AchE, followed by 2- hydroxyl benzamide (-131.48 kcal mol^{-1}), 6-hydroxy picolinic acid (-121.21 kcal mol^{-1}) and picolinic acid N-oxide (-113.28 k cal).

The salient findings of the study were

- *L. saksenae* produced the cuticle degrading enzymes *viz.* chitinase, lipase, protease, chitosanase, chitin deacetylase, keratinase and amylase the activity of which was detected during 2 to 14 days of incubation
- Among the major enzymes, protease was the predominant and the key enzymes, Pr1 and Pr2 were also detected
- Partially purified protease caused 71.79 per cent mortality to nymphs of *C. insolita* at 100 µg mL^{-1}followed by chitinase (58.10%) and lipase (54.23%)
- *L. saksenae* was found to be an efficient pathogen on *M. incognita* and the enzymes protease and chitinase drastically reduced egg hatching and development
- Protease was purified and characterised with a molecular weight of 71 kDa
- The red pigmented mycotoxin of *L. saksenae* was isolated, purified and characterised as oosporein

- Twenty two insecticidal metabolites were detected of which dipicolinic acid, beauveroloide, picolinic acid, cordycepin, harmine etc. were predominant
- Seven nematicidal compounds were detected of which 2, 6, pyridine dicarboxylic acid, 8-hydroxyl quinone, nicotinic acid and hexadecanoic acid were dominant
- Twenty antimicrobial compounds were detected of which vanillic acid, aminophenol, salisylamide, erucamide etc. were predominant
- The major volatile compounds detected were DPA, hexadecanoic acid, octadecanoic acid, harmine, piperidinone, etc.
- Oosporein at 1000 ppm caused 60.25 per cent mortality in *C. insolita* nymphs while the crude toxin caused 100 per cent mortality 72 h after treatment
- LC_{50} of the crude toxin was 51.06 ppm in *C. insolita* nymphs which was 79 fold high in *C. sexmaculata* grubs at 96 h after exposure.
- Crude toxin at 50 ppm and above caused 100 per cent mortality in *M. incognita* at 48 hour after treatment
- LC_{50} of the crude toxin to coccinellid grub was 4040.74 ppm at 96 h which was 79 times higher than that of pest. The adults were relatively safe.
- *In silico* molecular docking revealed interaction of metabolites of *L. saksenae* with AChE and highest interaction was exhibited by 3-hydroxy-2-methylpyridine (-133.24 kcal mol^{-1}).

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APPENDICES

APPENDIX I

1. pH indicator medium (g L^{-1})

$MgSO_4.7H_2O$	0.3
$(NH4)_2SO_4$	3.0
KH_2PO_4	2.0
Citric acid monohydrate	1.0
Agar	15.0
Tween-80	200 µl
Colloidal chitin	4.5
Bromocresol purple	0.15
Distilled water	1000 mL

2. Minimal medium- Lipase (%)

NaCl	0.003
$MgSO_4$	0. 03
K_2HPO_4	0.015
Agar	2.00
Distilled water	100 mL

3. Casein agar medium (g L^{-1})

Casein	10.00
Dextrose	5.00
KNO_3	3.50
KH_2PO_4	1.50
$MgSO_4.7H_2O$	0.75
Agar	20.00
Distilled water	1000 mL

4. Minimal medium- Colloidal chitosan (g dL^{-1})

Colloidal chitosan	0.50
Peptone	0.10
KH_2PO_4	0.20
K_2HPO_4	0.01
Glucose	1.50
$MgSO_4$	0.02
$CaCl_2$	0.01
Agar	2.00
Distilled water	100.00 mL

5. CDA selective agar medium (g L^{-1})

$NaNO_3$	2.0
K_2HPO_4	1.0
KH_2PO_4	1.0
$MgCl_2.6H_2O$	0.5
4- nitroacetanilide	0.5
Agar	20.0
Colloidal chitin	1% (w/v)
Distilled water	100 mL

6. Minimal medium with starch (g mL^{-1})

Starch	20 .0
Peptone	20.00
Yeast extract	10. 00
Agar	20.00
Distilled Water	1000 mL

7. Enzyme producing medium (g L^{-1})

Colloidal Chitin	20.00
NaCl	0.250
KH_2PO_4	0.375
$MgSO_4.7\ H_2O$	0.125
$(NH_4)_2\ C_6H_6O_7$	0.625
$CaCO_3$	0.375
Glycerol 87%	6.500
Distilled water	1000 mL

8. Minimal medium with casein (g L^{-1})

KH_2PO_4	0.4
$MgSO_4$	0.2
NaCl	1.0
Casein	4.0
Distilled water	1000 mL

9. Mineral salt medium with poultry feather (g L^{-1})

Chicken feather	5.0
Glucose	2.0
Peptone	5.0
Yeast extract	5.0
K_2HPO_4	1.0
KH_2PO_4	3.0
Ca Cl_2	1.0
$MgSO_4$ $7H_2O$	1.0
Distilled water	1000 mL

10. Minimal media composition for broth culture- Amylase (g L^{-1})

Yeast extract	4
K_2HPO_4	6
$MgSO_4$	6
NaCl	6
Starch	10
Distilled water	1000 mL

11. Basal medium with olive oil (g L^{-1})

Glucose	2.0
Peptone	5.0
$MgSO_4$	0.1
K_2HPO_4	1.0
Olive oil	20 mL
Tween 80	2.5 mL
Distilled water	1000 mL

APPENDIX II

Chemicals for SEM

1. **2.5% Glutaraldehyde**

Glutaraldehyde 25%	1 mL
0.05 M sodium Phosphate buffer (pH 7.2)	5 mL
Double distilled water	4 mL
Total	10 mL
Mixed and stored at 4°C	

2. **0.05 M sodium phosphate buffer pH 7.2**

Di Sodium Hydrogen Phospahte 0.033M	0.472 g
Monosodium Hydrogen Phosphate .016 M	0.2605 g
Double distilled water	80 mL
pH adjusted to to7. 2 with 0.2 N HCl.	Total volume adjusted to 100 mL.

3. **1% Osmium tetraoxide**

Osmium tetroxide	0.1 g
Phosphate buffer	10 mL
stored at 4°C.	

APPENDIX III

Poly Acrylamide Gel Electrophoresis (SDS-PAGE)

Resolving gel 12%

Acrylamide / bis-Acrylamide -	4.0 mL
Lower Tris (pH- 8.8)	2.5 mL
Distilled water	3.35 mL
SDS (10%)	100 µL
Ammonium per sulphate	50 µL
TEMED	10 µL

A. 100 ML Acrylamide stock solution

a.30% acrylamide

b.0.8 % bis acrylamide

B. 1.5 M Tris buffer- pH 8.8

a. Tris base - 18.15g

b. ddH_2O - 80 mL

Adjust pH to 8.8 make upto 100 mL

C. 0.25M Tris buffer - pH 6.8

a Tris base .- 3 g

b. dd H2O - 80mL

c. Use 1N HCl to adjust the pH and make up to 100 mL

Electrophoresis buffer

a. Tris- 3.03 g
b. Glycine- 14.4 g
c. SDS- 1g
d. Distilled water – 1000 mL

APPENDIX IV

i) Czapek Dox medium (g L^{-1})

Sucrose	30.00
K_2HSO_4	1.00
$MgSO_4$	0.50
KCl	0.50
$NaNo_3$	3.00
$FeSO_4 7\ H_2O$	0.01
Distilled water	1000 mL

ii) Fargues medium (g L^{-1})

Glucose	30.00
Yeast extract	3.00
KH_2PO_4	0.39
$Na_2HPO_4 \cdot 12H_2O$	1.42
$MgSO_4 \cdot 7H_2O$	0.60
NH_4NO_3	0.70
KCl	1,00
Distilled water	1000 mL

iii) Rice medium (mg kg^{-1})

$FeSO_4\ 7\ H_2O$	6.0
$MnCl_2\ 4\ H_2O$	6.0
$ZnSO_4\ 7\ H_2O$	6.0
$CuSO_4\ 5\ H_2O$	6.0
$CoCl_2\ 6\ H_2O$	6.0
Basmathi rice	1000 g
Distilled water	600 mL

APPENDIX V

List of Secondary metabolites in *L. saksenae*

Class	Name	Formula	Molecular Weight	Mz Cloud Best Match
Alkaloid	6-Hydroxynicotinic acid	$C_6H_5NO_3$	139.02694	87.40
	Cyclo(phenylalanyl-prolyl)	$C_{14}H_{16}N_2O_2$	244.12073	86.40
	Isonicotinicacid1-oxide	$C_6H_5NO_3$	139.02694	86.90
	Nicotinic acid	$C_6H_5NO_2$	123.03185	91.20
	Difenoxurone	$C_{16}H_{18}N_2O_3$	286.13174	82.60
	DL-Stachydrene	$C_7H_{13}NO_2$	143.09463	82.80
	Nicotinicacid 1 oxide	$C_6H_5NO_3$	139.02694	86.20
	Trigonelline	$C_7H_7NO_2$	137.04768	90.40
Amide	2-hydroxy benzamide	$C_7H_7NO_2$	137.14000	82.10
	Palmotoyilethanolamide	$C_{18}H_{37}NO_2$	299.28243	82.60
	Salicylamide	$C_7H_7NO_2$	137.04768	86.10
	Mandipropamide	$C_{23}H_{22}ClNO_4$	411.12374	81.50
	Oxacilin	$C_{19}H_{19}N_3O_5S$	401.10454	92.00
Aminoacid derivative	L-threo-3-Phenylserine	$C_9H_{11}NO_3$	181.07366	82.20
	N-Isovalerylglycine	$C_7H_{13}NO_3$	159.08946	88.60
	Betaine	$C_5H_{11}NO_2$	117.07898	97.70
	Oxoproline	$C_5H_7NO_3$	129.04259	94.30
Aminoalcohol	2-Amino-1,3,4-octadecanetriol	$C_{18}H_{39}NO_3$	317.29192	81.30
Aminoglycoside	Netilmycin	$C_{21}H_{41}N_5O_7$	475.30060	91.20
Detergent	Dodecyl sulphate	$C_{12}H_{26}O_4S$	266.15516	86.50

Fatty amide	Oleamide	C_{18} H_{35} N O	281.27117	85.20
Fatty acid ester	Diisobutyl phthalate	C_{16} H_{22} O_4	278.15099	80.30
	Dibutyl phthalate	C16 H_{22} O_4	278.15099	85.60
	Dioctyl phthalate	$C_{24}H_{38}O_4$	390.27701	89.20
Glycolipid	N,N dimethyl sphingosine	$C_{20}H_{41}NO_2$	327.31373	80.30
Nitrogen base	2-deoxy adenosine	$C_{10}H_{13}N_5O_3$	251.10184	91.50
	4-Cyanoindole	C_9 H_6 N_2	142.0531	81.10
	4-Indolecarbaldehyde	C_9H_7NO	145.05276	85.20
	5-Hydroxyindole 3-acetic acid	$C_{10}H_9NO_3$	191.05824	85.10
	Adenine	C_5 H_5 N_5	135.05449	92.30
	Erucamide	C_{22} H_{43} N O	337.33309	90.00
	Triethanolamine	C_6 H_{15} N O_3	149.10498	97.10
	Adenosine	$C_{10}H13N_5O_4$	267.09675	91.50
Organic acid	(1,3-Phenylenedioxy)diacetic acid	C_{10} H_{10} O_6	226.04765	80.20
	2 (5 hydroxy)indol acetic acid	$C_{10}H_9NO_3$	191.05824	94.60
	2, methyl amino isobeutric acid	$C_5H_{11}NO_2$	117.07898	84.80
	2,6 Pyridine dicarboxylic acid	$C_7H_5NO_4$	167.0218	85.40
	3- Methoxy salicylic acid	$C_8H_8O_4$	168.04226	82.50
	3-Hydroxy manidelic acid	$C_8H_8O_4$	168.04226	84.60
	3-Hydroxypicolinic acid	$C_6H_5NO_3$	139.02694	92.30
	Gluconic acid	$C_6H_{12}O_7$	196.0583	91.70
	4-Acetaminidobenzoic acid	$C_9H_9NO_3$	179.05824	83.00
	Phenylglyoxylic acid	$C_8H_6O_3$	150.03169	88.00

	4- Hydroxy mandelic acid	$C_{14}H_{30}O_4S$	294.18648	86.20
	4-Acetamido beutric acid	$C_6H_{11}NO_3$	326.19157	91.70
	4-Aminobezoic acid	$C_7H_7NO_2$	137.0476	84.30
	2—Aminobenzoic acid	$C_7H_7NO_2$	137.0476	84.3
	Sorbic acid	$C_6H_8O_2$	112.05243	83.10
	4-Dodecylbenzenesulfonic acid	$C_{18}\ H_{30}\ O_3\ S$	326.19151	86.70
	4-Methoxysalicylic acid	$C_8\ H_8\ O_4$	168.04233	82.60
	6-Hydroxypicolinic acid	$C_6H_5NO_3$	139.02694	92.90
	6-Methoxysalicylic acid	$C_8\ H_8\ O_4$	168.04211	87.80
	9-Oxo-10(E),12(E)-octadecadienoic acid	$C_{18}\ H_{30}\ O_3$	294.21865	83.70
	Anthranilic acid	C_7H_7NO	137.04768	86.10
	Dehydro acetic acid	$C_8H_8O_4$	168.04226	87.40
	D-α-Hydroxyglutaric acid	$C_5\ H_8\ O_5$	148.03712	83.90
	Azelaic acid	$C_9H_{16}O_4$	188.10486	92.10
	Ethylmalonic acid	$C_5\ H_8\ O4$	132.04231	85.80
	Disulfiram	$C_{10}H_{20}N_2S_4$	296.05093	82.80
	Glutaric acid	$C_5H_8O_4$	132.04226	82.20
	Homovanillic acid	$C_9\ H_{10}\ O_4$	182.05778	85.20
	Isophthalic acid	$C_8\ H_6\ O_4$	166.02659	89.10
	Isovallinic acid	$C_8H_8O_4$	168.04226	85.70
	Kynurenic acid	$C_{10}\ H_7\ N\ O_3$	189.04231	88.00
	L Threonic acid 1 ,4, lactone	$C_4H_6O_4$	118.02661	83.70
	Methylmalanoic acid	$C_6H_6O_4$	118.02661	91.00
	Methylsuccinic acid	$C_5\ H_8\ O_4$	132.04219	87.50

	Mono (2 ethylhexyl) phthalate	$C_{16}H_{22}O_4$	278.15181	81.30
	N-Acetylanthranilic acid	C_9 H9 N O3	179.05833	86.40
	Perfluorobutanoic acid	C_4 H F_7 O_2	213.9866	83.20
	Picolinic acid	C_6 H_5 N O_2	123.0321	82.60
	Picolinic acid N. oxide	$C_6H_5NO_3$	139.02	83.50
	1-Amino cyclohexane carboxylic acid	$C_7H_{13}NO_2$	143.09463	81.80
	Pyrrole 2-carboxylic acid	$C_5H_5NO_2$	111.03203	91.80
	Succinic acid	C_4 H_6 O_4	118.02658	94.20
	Terethalic acid	$C_8H_6O_4$	166.02661	88.30
	Vallinic acid	$C_8H_8O_4$	168.04226	82.00
Phenolic compound	2- Amino phenol	C_6H_7NO	109.05276	90.00
	2,4,6 trihydroxy acetophenone	$C_8H_8O_4$	168.04226	83.00
	3-Hydroxybenzoic acid	$C_7H_6O_3$	138.03169	90.90
	4-Hydroxy benzoic acid	$C_7H_6O_3$	138.03168	90.70
	Cyanidin	$C_{15}H_{10}O_6$	286.04774	80.30
	Benzoic acid	$C_7H_6O_2$	122.03678	91.80
	2-Amino phenol	C_6H_7NO	109.05276	89.00
	3-Aminophenol	C_6H_7NO	109.05276	83.40
	4-Aminophenol	C_6 H_7 N O	109.05258	91.60
	(-)-Epicatechin	$C_{15}H_{14}0_6$	290.07904	83.6
	Catechin	$C_{15}H_{14}O_6$	290.07904	83.6
	4- Hydroxy benzylalcohol	C7H8O_2	124.05243	83.9
Phospholipid	Cordycepin	$C_{10}H_{13}N_5O_3$	251.10184	80.20
	Choline	$C_5H_{13}NO$	103.09971	89.00

Qnine derivative	8 Hydroxy quinoline	C_8H_7NO	145.05276	84.70
Steroid	3'-Hydroxystanozolol	$C_{21}\ H_{32}\ N_2\ O_2$	344.2452	80.00
Sulfate ester	Myristyl sulfate	$C_{14}\ H_{30}\ O_4\ S$	294.1866	86.20
Vitamin	Pantothenic acid	$C_9\ H_{17}\ N\ O_5$	219.11065	90.20
Vitamin derivative	2 -pyridine acetic acid	$C_7H_7NO_2$	137.04768	84.80
	3-Hydroxy 2-methyl pyridine	C_6H_7NO	109.0527	87.00
	3-Pyridineacetic acid	$C_7H_7NO_2$	137.04768	88.80
	4-pyridine acetic acid	$C_7H_7NO_2$	137.04768	87.70
	4-Pyridoxic acid	$C_8\ H_9\ N\ O_4$	183.05321	82.70
	L- Norleucine	$C_6H_{13}NO_2$	131.09463	93.60
	N- Acetyl DL-nor valine	$C_7H_{13}NO_3$	159.08954	86.20
	N- Acetyl valine	$C_7H_{13}NO_3$	159.08954	87.60
Sulfonamide	DAPSONE (Sulfonyl dianilline)	$C_{12}H_{12}N_2O_2S$	248.06195	80.70
	6-2-Fluorinated telomere sulphonate	$C_8H_5F_{13}O_3S$	427.97518	92.5
Pyrole	2,6,Dimethyl-Y pyrone	$C_7H_8O_2$	124.05243	81.40
Halogeneated compound	Bromhexine	$C_{14}H_{20}BR_2N_2$	373.99932	81.80

MYCOTOXINS AND ENZYMES OF ENTOMOPATHOGENIC FUNGUS *Lecanicillium saksenae* (Kushwaha) Kurihara and Sukarno AND THEIR BIOEFFICACY ON CROP PESTS

by

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ABSTRACT

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ABSTRACT

Lecanicillium saksenae an indigenous isolate from soils of Vellayani isolated by Rani *et al*. (2015) was reported to have high speed of kill in major hemipteran pests. Its quick insecticidal action prompted to profile its toxic metabolites. Therefore the study entitled 'Mycotoxins and enzymes of entomopathogenic fungus *Lecanicillium saksenae* (Kushwaha) Kurihara and Sukarno and their bioefficacy on crop pests" was carried out in the Department of Agrl. Entomology, College of Agriculture, Vellayani and Department of Agrl. Entomology, College of Horticulture, Vellanikkara during2016-2019. The objective of the study was to characterize the cuticle degrading enzymes and mycotoxins produced by *L. saksenae* and explore their potential for safer pest management.

Qualitative assay revealed the presence of different cuticle degrading enzymes *viz*. chitinase, lipase, protease, chitosanase and amylase, with the Enzyme Indices 1.08, 4.3, 1.1, 1.36, and 1.57 respectively. Qualitative assay using pH indicator medium revealed high activity of chitin deacetylase.

Quantitative assay carried out at two day interval for a period of 14 days revealed the peak activity of chitinase to the tune of 1.35 U mL^{-1} min^{-1}, lipase 0.7 U mL^{-1} min^{-1}, protease 4.89 U mL^{-1} min^{-1}, chitosanase 0.26 U mL^{-1} min^{-1}, amylase 23.42 U mL^{-1} min^{-1} and keratinase 73.61 U mL^{-1} min^{-1}. The activity of Pr1 and Pr2, the key proteolytic enzymes were also detected to the tune of 0.22 U mL^{-1} min^{-1} and 0.32 U mL^{-1} min^{-1}respectively. The peak activity period of these enzymes varied from 4^{th} to 10^{th} day of incubation, whereas the peak activity of Pr2 was on the 2^{nd} day and that of keratinase was on the 40^{th} day, in their respective minimal media with inducing substrates. The biomass reduction of 80.2 per cent in poultry feather substrate due to keratinase activity is notable.

Bioefficacy studies of partially purified major cuticle degrading enzymes revealed that protease @ 100 µg mL^{-1} exhibited 71.79 per cent mortality of

brinjal mealybug, C*occidohystrix insolita* nymphs, 96 hours after treatment (HAT). The corresponding mortality with chitinase and lipase was 58.10 and 54.23 per cent respectively. These enzymes when applied in combination resulted in 78.79 per cent mortality. However, the efficacy of individual enzymes on adults was meagre and the combined application caused only 40.88 per cent mortality.

Bioefficacy studies on the root knot nematode *Meloidogyne incognita* revealed its inhibitory action in the hatching of eggs. The hatching percentage was drastically reduced in chitinase (18.8%), protease (10.4%) and its combination (7.8 %), as against 70.80 per cent in absolute control. Scanning electron microscopy of the treated eggs exhibited uneven and shrunken surface with distinct lines of breakage on the outer vitelline layer, leading to disintegration of egg mass within five days of treatment.

The predominant enzyme, protease was purified through Sephacryl G-100 column and DEAE-cellulose and was characterised with molecular weight 71 kDa by SDS - PAGE analysis.

Spectral analysis of crude toxin extracted through standard procedures could detect an array of metabolites, of which the dominant one was purified and characterised. Ethyl acetate extraction of culture filtrate followed by vacuum evaporation and purification yielded 100 mg L^{-1} of the purified compound. Structural characterisation and identification of the metabolite through UV spectrometry, Fourier Transform Infrared spectrometry (FTIR), High Resolution Liquid Chromatography Mass spectrometry (HR-LCMS) and Nuclear Magnetic Resonance Spectrometry (NMR) unambiguously identified the metabolite as oosporein, the empirical formula of which was confirmed to be $C_{14}H_{10}O_8$ and molecular weight, m/z 306.22.

Dipicolinic acid (DPA), another volatile insecticidal metabolite was identified through LC-MS. Bioassay guided fractionation of crude toxin could

identify a fraction that caused 64. 23 per cent mortality of *C. insolita* nymphs at 96 HAT. Spectral data of LC-ESI-MS analysis of this insecticidal fraction showed a strong signal for the ion of m/z 485.6 which was identified as beauverolide.

One Strain Many Compounds (OSMAC) technology was carried out to detect multiple metabolites produced by *L. saksenae*. HR-LCMS and GC-MS analysis of the toxin extracts from various media, identified multiple metabolites whose bioactivity was determined using Dukes phytochemical and ethno botanical and PubChem databases. Of the bioactive metabolites, 22 were insecticidal, seven were nematicidal and 20 were antimicrobial. The dominant insecticidal metabolites other than oosporien, DPA and beauverolide were picolinic acid, cordycepin, anthranilic acid, dibutyl phthalate, hexadecanoic acid, harmine etc. while the major nematicidal metabolites were 2,6, pyridine dicarboxylic acid, 8-hydroxyl quinone, nicotinic acid, hexadecanoic acid, harmine etc. The predominant antimicrobial metabolites were vanillic acid, aminophenol, anthranilic acid, salisylamide, oleamide, terephthalic acid 4-H pyran-4-one, 2, 3, dihydro 3, 5 dihydroxyl 6- methyl, and and Dl-mevalonic acid lactone etc. The volatile compounds detected were hexadecanoic acid, octadecanoic acid, harmine. 2,6, pyridine dicarboxylic acid, 4H pyran-4-one, 2, 3, dihydro 3, 5 dihydroxyl 6- methyl, and Dl-mevalonic acid lactone.

Bioefficacy studies of the purified toxin revealed a dose dependent mortality in *C. insolita*. Toxin at 1000 ppm caused 60.25 per cent mortality in nymphs, 96 HAT, whereas the corresponding mortality in adults was 51.00 per cent. However, the crude toxin caused 100 per cent mortality with the same concentration, justifying the multifaceted properties of the fungus in causing insect diseases. Probit analysis revealed LC_{50} value of 87.14 ppm in nymphs at 48 h whereas it was higher (193.61 ppm) in adults.

Bioefficacy studies on J_2 of *M. incognita* revealed the nematicidal potential of secondary metabolites of *L. saksenae.* At highest concentration of

250 ppm, mortality of 95.22 per cent was observed at 24 HAT whereas at 48 HAT, mortality was 100 per cent with 50 ppm and above.

The study revealed the safety of crude toxin to different life stages of the coccinellid predator *Cheilomenes sexmaculata* . LC_{50} value for ovicidal activity was 69 fold higher than that of the pest (51.06 ppm at 96 h) while for grubs it was 79 times higher than that of pest.

In silico molecular docking of the metabolites detected from *L. saksenae* revealed that 18 of them were interacted with acetylcholinesterase (AChE). Among these, 3-Hydroxy-2-methylpyridine exhibited the highest binding energy (-133.24 kcal mol^{-1}) and therefore highest interaction with AChE.

Metabolite profiling of *L. saksenae* revealed its potential entomopathogenic, nematicidal and keratin degrading properties. The study paves way to the possibilities of tapping the potential of the bioactive metabolites for safer pest management. It could be made use in leather industry and keratinous waste management as well.