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DEVELOPMENT OF BIOINSECTICIDES AGAINST *SPODOPTERA FRUGIPERDA* USING CHITINOLYTIC BACTERIA

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ABSTRACT

Actinomycetes play an important role in the biological control of insects by producing insecticidal active compounds against various insects. *They* are predominantly found in *soil*, in the silt of water bodies, in the air and in plant remains. Chitinase is increasingly finding applications in various fields such as biomedicine, in ultra-structural studies, in *the* preparation of chitooligosaccharides, in single-cell protein production, biocontrol agents, agriculture, etc. Chitinase is originally an enzyme used by insects to degrade the structural polysaccharide of insects. Chitinase brings about hydrolysis of chitin which is widely distributed in cell wall of fungi, plants and insects. An attempt has been made to use chitinase producing bacteria for the development of bioinsecticides against *Spodoptera frugiperda,* which feeds on over more than 60 species of plants and it is periodic. The Spodoptera frugiperda armyworm (Lepidoptera) is a serious pest of cabbage and other crop. It is also major pest of maize, rice, sorghum, turf grasses, cotton, and peanuts, etc. In this attempt enrichment and isolation of chitinase producing bacteria from soil was carried out. Chitinolytic activities of the three isolates were tested against larvae of *Spodoptera frugiperda.* Three isolates were individually applied against *Spodoptera frugiperda.* The isolates named Ai, A2 and A3 were found to be more effective against *Spodoptera frugiperda*. The insecticidal activity of isolate A1was tested and control within 5 hours. Isolate A2 was treated with larvae *Spodoptera frugiperda* killed after 8 hours whereas, isolate A3 controlled after 24 hours. The evaluated insecticides provided a significant reduction in the infestation level, for variable periods.

KEYWORDS: ***Biological Control, Bioinsecticides, Chitinase, Spodoptera frugiperda***

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INTRODUCTION

Insecticides of synthetic origin have been used to manage insect pests during 1940 s[1]. Due to adverse effects of insecticides on the environment, their rational use is being controlled. Actinomycetes are the important group of microorganisms, not only as degraders of organic matter in the natural environment but also as producers of antibiotics and other useful compounds of commercial interest [2,3,4]. Actinomycetes play an important role in the biological control of insects through the production of insecticidal active compounds against the house fly *Musca domestica*[5]. Commercial synthetic sexual pheromones provided a significant reduction in the infestation level, for variable periods, depending on the chemical suggesting that the pheromone trap can be a useful tool in integrated pest management program in maize crop in Brazil[6]. Chitin is the major polysaccharide present in insects and many other invertebrates as well as in several microbes, including fungi. Structurally, it is the simplest of the glycosaminoglycans, being a β (1, 4) linked linear homopolymer of N-acetylglucosamine (GlcNAc, $[C_8H_{13}O_5N]n$, where n>>1). It serves as the skeletal polysaccharide of several animal phyla, such as the Arthropoda, Annelida, Mollusca and Coelenterata. In insects, it is found in the body wall or cuticle, gut lining or peritrophic matrix (PM), salivary gland, trachea, egg shells, and muscle attachment points. All of these structures are primarily composed of chitin fibers and proteins with varying degrees of hydration and trace materials distributed along the structures. The insolubility and structural complexity of the cuticle have limited its study. However, sclerotized cuticle can be modeled as an interpenetrating network of chitin fibers with embedded cross-linked protein and pigments. Both synthesis and degradation of chitin take place at multiple developmental stages in the cuticle and the PM. It is usually synthesized as portions of the old endocuticle and PM and tracheas are resorbed, and the digested materials are recycled. Although primarily composed of poly-GlcNAc, chitin also can contain a small percentage of unsubstituted (or N-deacetylated) glucosamine (GlcNAc) residues, making it a GlcNAc-GlcNheteropolymer[7,8]. Degradation of chitin is carried out by two enzymes chitinase (CHIs,) and *β-N*-acetylglucosaminidases (NAGs,). These enzymes are produced by various bacteria and fungi. CHIs catalyze the endohydrolysis of chitin at random internal positions within the chitin polymer. Exochitinases, which cleave the exohydrolysis of the chitin polymer from the non-reducing end, is divided into two subcategories: chitobiosidases and NAGs[9]. Chitobiosidases catalyze the progressive release of diacetylchitobiose, while NAGs produce monomers of GlcNAc[10]. *Spodoptera frugiperda* is a major army worm insect pest of maize (*Zea mays* L.) in theAmerica[11,12]. In Brazil, this pest causes serious economic damage, estimated at more than 400 million dollars annually. The importance of the pest has increased due to selection of insect populations resistant to chemical insecticides [13]. Hence an attempt has been made to develop an environment friendly bioinsecticides for biocontrol of this insect pest.

MATERIALS AND METHODS

This study was carried out during the period of June 2017 to January 2018, at department of Botany, Annasaheb Magar Mahavidyalaya, Pune, India.

Preparation of Colloidal Chitin[14]

Colloidal chitin was prepared from chitin (Hi-Media) by the modified method of Hsu and Lockwood 1975. 40 g Chitin powder was slowly added to 600 ml of concentrated HCl and kept for 60 min at 30^0C with vigorous stirring. Chitin was precipitated as a colloidal suspension by adding it slowly to 2 litre of distilled water at 4–10^0C. The suspension was collected by filtration with suction on a coarse filter paper and washed by suspending it in about 5 litre of distilled water. Washing was repeated 3 times until the pH of the suspension was 3.5. After the above treatment, the loose colloidal chitin was obtained and used as substrate.

Screening and Isolation of Chitinolytic Bacteria[14]

Garden soil sample was collected in sterile petri-dish; 1g of this soil sample was added into 10 ml sterile distilled water and mixed on cyclo mixer. A loopful of soil suspension was streaked on chitin agar. The medium consists of colloidal g/lit K_2HPO_4-0.7; KH_2PO_4- 0.3; $MgSO_4$-0.5; $FeSO_4.7H_2O$- 0.01; $MnCl_2.4H_2O$- 0.01; $ZnSO_4.7H_2O$- 0.001; chitin 1% W/V, pH- 8.0 +/- 0.2 at 25^0C. Plates were incubated at room temperature for 4 days. The colonies showing clearance zones on a creamish background were selected as chitinase-producing bacteria.

Production and Purification of Chitinase

Bacterial isolates were selected on the basis of a larger hydrolysis zone after 4 days of incubation

and further screened for maximum enzyme production. Isolates were inoculated each in 100 ml in liquid M9 minimal medium (12.8 g, $Na_2HPO_4 \cdot 7H_2O$; 3g, KH_2PO_4; 0.5 g, NaCl; 1 g, NH_4Cl and 4 g glucose per litre of distilled water) containing.5% (v/w) insoluble chitin and incubated for 5 days at 28^0C. After 5 days the cultures were centrifuged at 10000 rpm for 15 min. and supernatants were stored at 4^0 C till further use as crude chitinase enzyme.

Assays of Chitinase Activity[15]

Reaction mixture contained 1 ml crude enzyme, 1.5 ml of 1% colloidal chitin substrate in 200 mM Potassium phosphate buffer (pH 6.0). The mixture was incubated at 30˚C for 2 hrs, boiled for 10 min to stop the reaction, and centrifuged at 8000 rpm for 20 min. Then 1 ml of test supernatant was added to 1 ml of DNSA (Dinitrosalicylic Acid), boiled for 5 min, cooled down to room temperature. Released by NAG (N-acetylglucosamine) was measured at 540 nM. Standard curve of NAG was plotted between NAG concentration and NAG absorbance. One unit of chitinase activity was described as the amount of enzyme which liberates 1.0 mg NAG per hour from chitin substrate under reaction condition. Data of chitinase activity were analyzed for the enzyme from each isolate.

Characterization of Bacterial Isolates

Bacterial isolates were characterized on the basis of colony characters and Gram's staining (Bergey's manual of determinative bacteriology).

Insecticidal Activity of Bacterial Isolates against Spodoptera frugiperda

Healthy larvae of *Spodoptera frugiperda* were collected from infected cabbage and kept in a beaker with some cabbage leaves; care was taken to keep adequate aeration and humidity to keep larvae alive. Larvae were immediately brought to the laboratory and used for checking the insecticidal activity of bacterial isolates as follows. A suspension of the active culture of each bacterial isolate was prepared in sterile saline to get 10^7 cells/ml as compared with McFarland tube no 5[16, 17] and few drops of this suspension were applied onto healthy larvae in a big petri-dish. Control was prepared by applying sterile saline onto healthy larvae.

RESULTS AND DISCUSSIONS

During this study, colloidal chitin was prepared; three isolates A1, A2 and A3 were found on chitin agar and identified from morphological and cultural characteristics. Isolate A1 appears to be a genus Actinomycetes where A2 and A3 were found to be *Bacillus sp*. During the screening maximum production of chitinase after 24 hours of incubation, three isolates from the cotton soil were selected for further study. Three isolates named A1, A2 and A3 were found to be most effective for chitinase production. Isolate A1 (*Actinomycetes spp.*) was found to be more effective followed by A2 then A3 (both *Bacillus spp.*).Insecticidal activity was found against larvae of *Spodoptera frugiperda.* Larvae were killed by isolate A1 within 5 hours and larvae treated with isolate A2 were killed after 8 hours and whereas larvae treated with isolate A3 was killed after 24 hours. Hence insecticidal activity of three isolates was found to be in the order of A1> A2> A3 as shown below. In our study, we found actinomycetes are the most effective organisms. *Actinomycetes* are large group of Gram positive filamentous bacteria produce variety of insecticidal compounds and play an important role in biocontrol of insects[18]. *Bacillus* species are also reported to be used as biocontrol agents [19].

Table1
Colony Characters of Bacterial Isolates on Chitin Agar Incubated at Room Temperature for Four Days

S.N.	Colony Character	Isolate A_1	Isolate A_2	Isolate A_3
1	Size	2mm	1.5mm	1.00 mm
2	Shape	Circular	Circular	Circular
3	Margin	Wavy	Wavy	Entire
4	Colour	White	White	off white
5	Elevation	Flat	Flat	Convex
6	Surface	Rough	Wrinkled	Smooth
7	Opacity	Opaque	Opaque	Translucent
8	Consistency	Powdery	Buterous	Buterous
9	Grams nature	Gram positive slender filaments	Gram positive sporulating rods	Gram positive sporulating rods
10	Motility	Nonmotile	Actively Motile	Motile

N.B.: Colonies of Isolate A_2 and A_3 appeared after 48 hours. Therefore colony characters and motility was recorded at the same time.

Table 2
Standard Graph of NAG

S.N.	Conc. of NAG µg/ml	Optical Density at 540 nm
1	100	0.042
2	200	0.061
3	300	0.08
4	400	0.158
5	500	0.191
6	600	0.229
7	700	0.262
8	800	0.325
9	900	0.334
10	100	0.375

N.B: Stock solution of N- acetyl D-glucosamine=1000 µg/ml in distilled water

Table 3
Chitinase Assay

S.N.	Enzyme	Optical Density at 540 nm
1.	A_1	0.2792
2.	A_2	0.2705
3.	A_3	0.194

NB: A_1 =Chitinase from isolate A_1
A_2 =Chitinase from isolate A_2 A_3 =Chitinase from isolate A_3

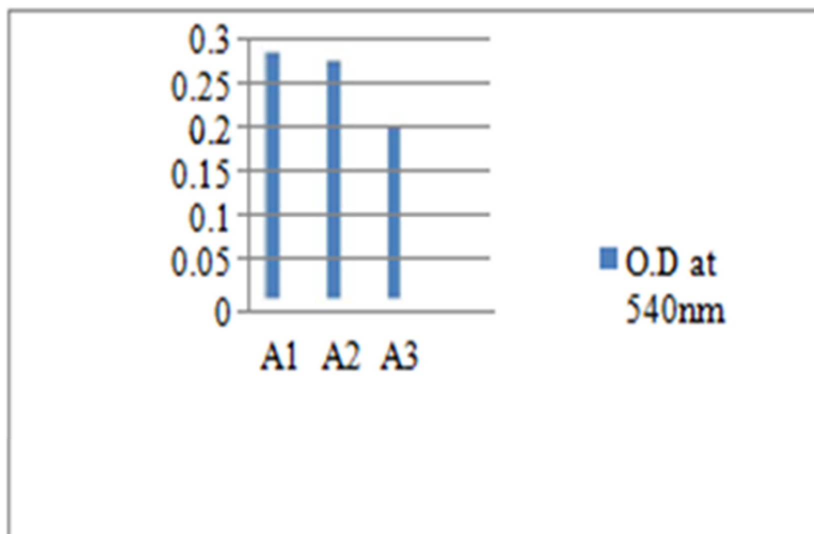
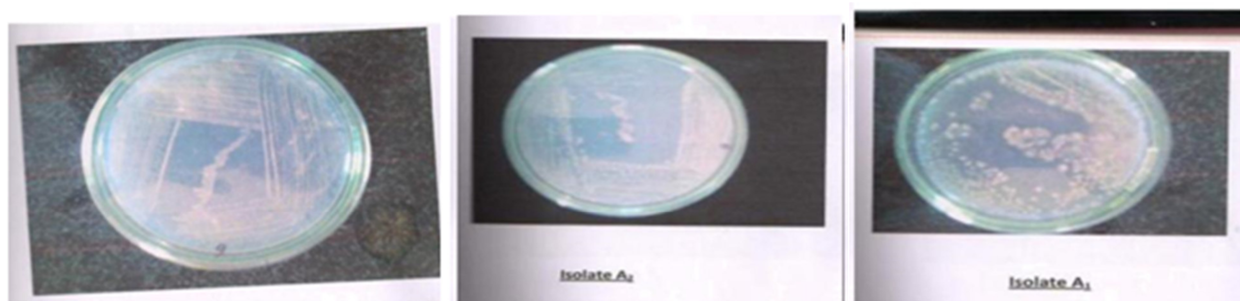


Figure 1
Chitinase Assay Standard Graph of N- acetyl D-glucosamine



Photoplate -1 Photoplate -2 Photoplate - 3

Figure 2
Screening and Isolation of Chitinolytic Bacteria from Soil

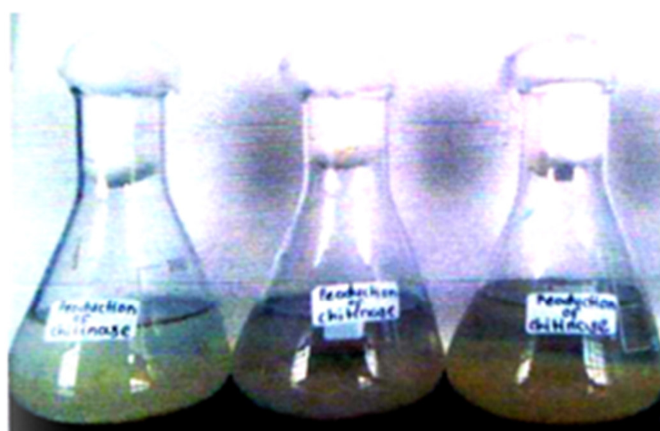


Figure 3
Production of Chitinase

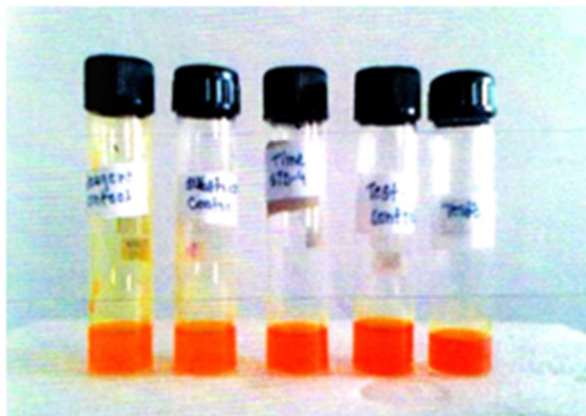


Figure 4
Chitinase Assay

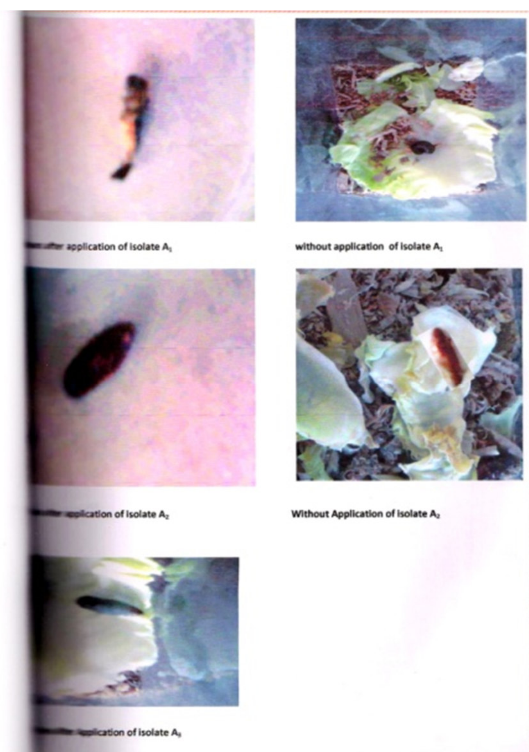


Figure 5
Spodoptera frugiperda

CONCLUSION

From the above results, it is concluded that all three isolates have potential insecticidal activity where isolate A1 which is an Actinomycetes species is the most effective and has great potential for the production of bioinsecticides against *Spodoptera frugiperda*. Other two isolates A2, A3 belong to genus *Bacillus* and also have considerable insecticidal activity. As these bacteria were isolated from garden soil and tested for their efficiency without any manipulation in their genetic makeup or growth conditions. Isolates could be mass produced easily in laboratories with limited resources and can be used as bioinsecticides in the fields without the threat to environment. Further studies for characterization of the isolates and other aspects are being undertaken.

CONFLICT OF INTEREST

Conflict of interest declared none.

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