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Identification of Chitin Degrading Bacterial Strains Isolated from Bulk and Rhizospheric Soil

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The subsequent application of insecticides and pesticides in agriculture results in several health and environment issues. To overcome, such devastating effect of synthetic chemicals, an eco friendly measure is required. Chitinolytic bacteria and their enzymes can be adopted as potent substitutes to chemicals required controlling the agriculture loss. The aim of this research was to isolate bacterial strains with significant chitinase activity. Isolates were screened based on method *viz.* zone inhibition, colorimetric, biochemical and were identified based on 16S rDNA sequences. Observed chitinase activity was in range 0.181Uml^{-1} to 1.594Uml^{-1} with zone inhibition in the range of 6mm to 29mm. Among the recovered strains only two, MSCP10 and MSCW8 showed good response when tested against insect and showed 80% and 95% mortality respectively after 72 hours of treatment. Based on 16S rDNA sequencing, MSCP10 and MSCW8 exhibited similarity with *Serratia marcescens* strain S308 and *Staphylococcaceae bacterium* HDMd_5 respectively. Through insect bioassay it was concluded that these bacterial strains were effective against Lepidopteran insect *P. xylostella*.

Keywords: 16S rDNA sequencing, Chitinolytic bacteria, Chitinase, *Plutella xylostella*.

The use of naturally occurring chitinolytic bacteria, actinomycetes and fungi as potential supplements for insecticides pesticides have been reported in many studies[1]. Chitin, the second most abundantly found and widely distributed natural renewable resource next to cellulose in nature. It is a homopolymer of ß-1, 4-linked N-acetyl-D-glucosamine. Chitin is the main structural component of shells of crustaceans, exoskeleton of insects, fungal cell wall and protozoa. The annual worldwide turnover of chitin is around 100 billion tons[2]. Based on amino acid sequences present chitinases are the enzymes that catalyze chitin degradation and divided into Family 18 and 19 of glycosyl hydrolase[1]. Several bacteria produces chitinases to degrade chitin and utilize it as an energy source and thereby helpful in recycling these resources in soil ecosystem[2].

A large number of chitinolytic soil bacteria have been isolated from soil[3], shellfish waste[4], shrimp shell-enriched soil[5] and vermicompost[6]. Phytospheres, such as rhizosphere and phylloplane, are important habitats for chitinolytic bacteria[7,8]. There is a considerable interest in chitinolytic bacteria for efficient bioconversion of chitinaceous waste based on the exploitation of chitinases. Soil bacteria are excellent sources of chitinases and could be used for catabolic conversion

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of chitinaceous waste into useful molecules for application in agriculture, biotechnology and medicine[9,10]. Bacteria from genera like *Bacillus*, *Serratia*, *Pseudomonas*, *Streptomyces* and *Aeromonas* frequently occur in soil and are potentially suitable sources of enzymes. Recycling of chitinous waste using chemical treatments is a costly process[11]. The continous use of chemicals in agriculture leads to numerous environment and public health problems. Hence, there is a need to look forward for an environmentally sound and cost effective approach. The use of chitinolytic bacteria and chitinases (exochitinase and endochitinase) can be adopted as an alternative to both, degradation of chitinous waste and as biocontrol agent.

In the present study, bulk soil, rich in chitinous waste and rhizospheric soil rich in microbes' were collected randomly for isolation, screening of chitinolytic bacteria and performed insect mortality bioassay with selected strains.

MATERIALS AND METHODS

Local market (fish and chicken) of Delhi NCR and rhizospheric regions of Ludhiana and Meerut were randomly selected for soil collection (Table 1). Soil sampling was done using the quadrat method of sampling and was processed.

Preparation of colloidal chitin

Colloidal chitin was prepared by method described by Roberts and Seltrennikoff with modification[12]. To 2gm of chitin powder 35ml of concentrated HCl was added, incubated overnight at 4°C and next day ice cold 200ml ethanol was added to the mixture, stand overnight at room temperature and centrifuge it at 10,000(g) for 30min. The mixture was filtered through fine muslin cloth with continuous washings of distilled water. Recovered colloidal chitin was stored at 4°C until use.

Isolation of chitinase producers

Chitinolytic bacteria from collected soil samples were isolated by serial dilution and spread plate method. On each plate 0.1ml of dilution was plated in triplicates on minimal salt media (MSM) containing $Na_2HPO_4.2H_2O$ (3.5g), KH_2PO_4 (1.0g), $(NH_4)_2SO_4$ (0.5g), $MgCl_2.6H_2O$ (0.1g), Ca $(NO_3)_2.4H_2O$ (0.05g) and chitin (5.0g) as carbon source and incubated at 30°C for 3days. The total 28 chitinase producers were selected based on the morphology, color and area of clearance around the colonies.

Screening of chitinase producing bacteria

Quadrant streak of all the isolates were carried out on MSM plate amended with 0.5% chitin to isolate the potential bacteria based on the chitinase produced.

Further, isolates were screened using different concentrations of chitin (0.5, 1.0, 1.5, 2.0 and 2.5%) in MSM plates and incubated at 30°C for three to five days. Colonies with larger clear zone size (>=10mm) were selected. The pure isolates were preserved in chitin containing nutrient broth glycerol stock at -80°C to maintain viability.

Characterization of Bacterial isolates

Identification of chitinolytic bacterium

The isolates were identified through their morphological and physiological properties according to Bergey's manual of systematic bacteriology[13] (Table 2).

Chitinase assay

Colorimetric method described by Setia and Suharjona was used to determine the chitinase activity with three replications[14]. The reaction mixture consists of 1ml crude enzyme, 1.5ml of 1% colloidal chitin substrate in 200mM (pH6) potassium phosphate buffer. The reaction mix was incubated at 30°C for 2 h and boiled for 10 min to stop the reaction. Then centrifuge at 8000rpm for 20 min. collected the supernatant and added 1ml of Dinitrosalicylic acid (DNS) in 1ml of supernatant boiled for 5 min and left at room temperature to cool down. Absorbance was measured at 540nm against the standard curve of N-acetylglucosamine (GlcNAc) plotted between GlcNAc concentrations and GlcNAc absorbance values. One unit of chitinase activity was defined as the amount of enzyme required to liberate 1.0mg of GlcNAc per h.

PCR amplification of genomic DNA for 16S rDNA sequencing

The total genomic DNA was isolated from the samples. Approximately (1µl DNA) 1.3/1.5Kb, 16S rDNA fragment was amplified using high fidelity PCR polymerase. The PCR products were sequenced bi-directionally using 16S forward and reverse primer 5'-AGHGTBTGHTCMTGNCTCAS-3'and 5'-TRCGGYTMCCTTGTWHCGACTH-3' respectively using gradient polymerase chain

reaction (ABI 3500 Genetic Analyzer). The PCR amplification was performed with initial denaturation (96°C; 5min), denaturation (96°C; 30s), hybridization (50°C; 30s) and elongation (60°C; 1.30min). The PCR amplified product was analysed on 1% agarose gel and with 500bp ladder. The amplified sequence was analysed using Data analysis software (seq Scape- v 5.2).

Phylogeny tree construction

For the construction of phylogeny, 16S rDNA sequences was matched with reference strains sequences (Table 3) in *Genebank* database (http://www.ncbi.nlm.nih.gov) and was aligned using *Clustal W Multiple Alignment tool* in *MEGAV.*7 *program.*

Statistical analysis

The standard statistical software Graph Pad Prism was used to carry out the data analysis. The mean and standard deviation were used to summarize the collection of data for each measurement. Two-way analysis of variance was used to evaluate the influence of independent bacterial strains. *Bonferroni multiple comparision* procedure was used to determine whether the data show evidence of difference between the various classes of chitinolytic bacteria.

Table 1. Location of collected soil sample

Type of sample	Location of sample
Rhizospheric soil	Sugarcane crop, Ludhiana
Rhizospheric soil	Wheat crop, Ludhiana
Non-rhizospheric soil	Industrial area of Meerut
Non-rhizospheric soil	Fish and poultry market Delhi-NCR

RESULTS

A total of 28 chitinase producing bacterial strains were isolated from the soil samples collected from different sites (Fig. 1). Out of 28 strains only 12 showed clear zone (>=10mm) when incubated in different concentrations (0.5, 1.0, 1.5, 2.0 and 2.5%) containing chitin media plates were selected and labeled as (MCPB1, MCPB2, MCPB3, MCPB4, MCPB5, MCPB6, MCPB7, MSCW8, MCPB9, MSCP10, MCPB11 and MCPB12) (Fig. 2). The formation of clear zone around the colonies indicates the presence of chitinase activity, to utilize chitin as a source of carbon and nitrogen. These finally selected 12 pure isolates were subjected to identification through biochemical tests. Observed results of biochemical tests presented in tabulated form

Table 2. Morphological and Biochemical characterization of selected bacterial strains

Properties	MCPB1	MCPB2	MCPB3	MCPB4	MCPB5	MCPB6	MCPB7	MSCW8	MCPB9	MSCP10	MCPB11	MCPB12
Gram reaction	+	-	+	+	-	+	+	+	+	-	-	-
Cell shape	Thin rods	Rods	Thick rods	Rods	Rods	Thin rods	Cocci	Cocci	Cocci	Rods	Rods	Rods
Colony color	Cream	Cream	Dull white	Cream	White	Cream	Cream	Cream	Yellow	Red	Cream	Yellowish
Motility	Non motile	Non motile	Non motile	Non motile	Motile	Motile	Non motile	Non motile	Non motile	Motile	Motile	Motile
Catalase Test	-	-	+	+	+	+	+	+	+	+	+	+
Oxidase Test	-	-	-	-	-	-	-	-	-	-	-	+
Nitrate Reductase	+	+	+	+	+	+ light change	+	+	+	+	+	+
Nitrite reductase	-	-	-	-	-	-	-	-	-	-	-	-
Utilization of Glucose A/G	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/-	+/+	+/-
Lactose A/G	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	-/-	+/+	+/+
Sucrose A/G	No acid/gas production. Remains red with growth											
Starch hydrolysis	No clear halo around colonies, Media turns dark blue.											
+/+ = Yellow/Bubble + = bubble formation - - = no color change												

Table 3. Reference strains for construction of phylogeny based on 16S rDNA sequences

Sl. No.	Organism Name	Strain	Accession No.
1	*Serratia marcescens*	S308	KP718760.1
2	*Serratia marcescens*	E3	KX215147.1
3	*Serratia marcescens*	FZSF02	KU145144.1
4	*Serratia marcescens*	SYJ1-9	KR262852.1
5	*Serratia marcescens*	MUGA199	KJ672369.1
6	*Serratia marcescens*	KtPC3-9	KF017546.1
7	*Serratia marcescens subsp. sakuensis*	PSB23	HQ242736.1
8	*Serratia nematodiphila*	YS8	KY887776.1
9	*Serratia marcescens*	HX-3	KX461911.1
10	*Serratia marcescens*	JNDKHCo-24B	KT894728.1
11	*Staphylococcaceae bacterium*	HDMd_5	JN392843.1
12	*Staphylococcus warneri*	PSTJ-65	KY608110.1
13	*Staphylococcus warneri*	TJ41	KY575133.1
14	*Staphylococcus warneri*	TJ21	KY569426.1
15	*Staphylococcus warneri*	MT-Y-S2	KU671206.1
16	*Staphylococcus warneri*	RmL10	KY442753.1
17	*Staphylococcus pasteuri*	H75	KU922389.1
18	*Staphylococcus warneri*	L6	KU922371.1
19	*Staphylococcus pasteuri*	H83	KU922347.1
20	*Bacillus subtilis*	H67	KU922346.1

Table 4. Bacterial strains bioassay with *P.xylostella* larvae

Insect: *Plutella xylostella* (10d old) No. of Larvae: 10		
1% Bacterial stock (~cell count 1.5X10^8 CFU/ml)	Treating concentration	Mortality rate after 3days of treatment (in percent)
MCPB1	50µl	0
MCPB2	50µl	5
MCPB3	50µl	10
MCPB4	50µl	25
MCPB5	50µl	10
MCPB6	50µl	15
MCPB7	50µl	85
MSCW8	50µl	95
MCPB9	50µl	70
MSCP10	50µl	80
MCPB11	50µl	75
MCPB12	50µl	40
Control (Distilled Water)	50µl	0

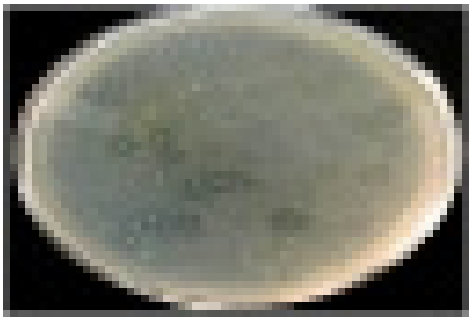

Fig. 1. Isolation of chitinolytic bacterial strains from soil

(Table 2) and also compared with each other (i) comparing zone diameter (in mm) using different concentrations of chitin amended MSM plates (Fig. 3) (ii) and measuring chitinase activity (Uml^{-1}) in chitin containing MSM broth (Fig. 4). On the basis of preliminary bioassay data two isolates (MSCW8 and MSCP10) were found to be more potent among 12 isolates and further selected for molecular identification (16S rDNA sequencing). The biochemical characteristics inferred that MCPB1 and MCPB2 shared similar characteristics

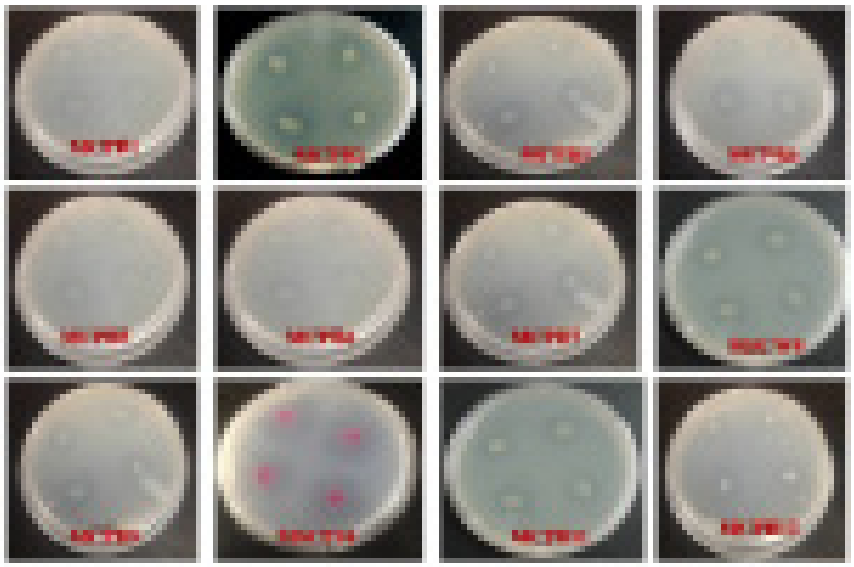

Fig. 2. Selection of different bacterial strains on the basis of zone size (>=10mm) after 72 h of incubation

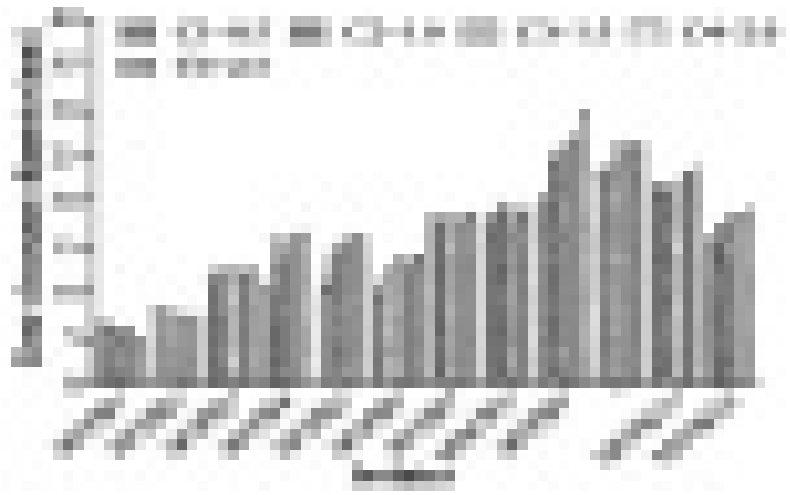

Fig. 3. Zone of inhibition at different concentration of chitin (C1=0.5, C2=1.0, C3=1.5, C4=2.0 and C5=2.5% respectively). Experiment was performed in triplicates, the error bars represents mean ± standard deviation at P value 0.0001<0.01<0.05

with *Lactobacillus*. MCPB3, MCPB4 and MCPB6 with *Bacillus*. MCPB7, MCPB9 and MSCW8 matches with *Staphylococcus* and *Micrococcus*. MCPB5 and MCPB11 were observed similar to *Escherichia*. MSCP10 and MCPB12 were found similar to *Serratia* and *Pseudomonas* respectively.

The amplicon 16S rDNA sequences of isolate MSCW8 and MSCP10 on 1% agarose

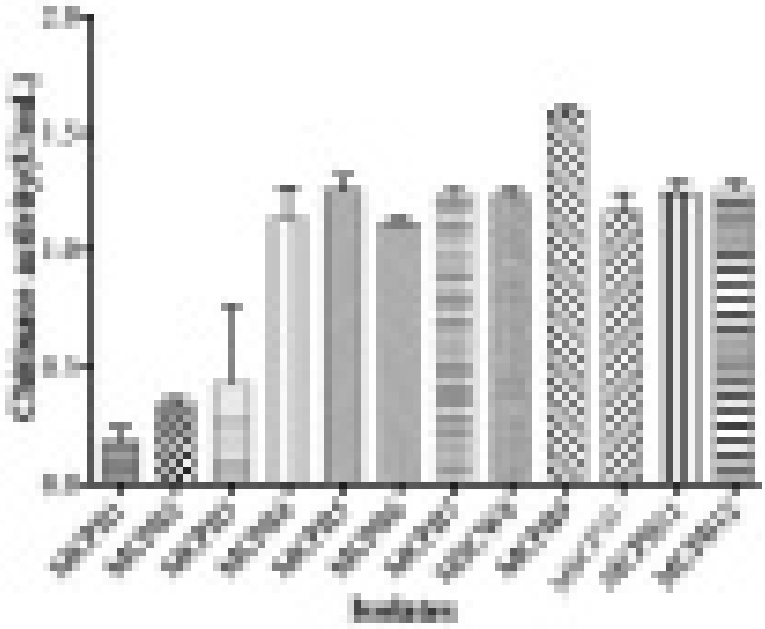

Fig. 4. Chitinase activity of selected bacterial strains. One unit of chitinase activity was defined as the amount of enzyme required to liberate 1.0mg of GlcNAc per h. Experiment was performed in triplicates, the error bars represents mean ± standard deviation at P value 0.001<0.01<0.05

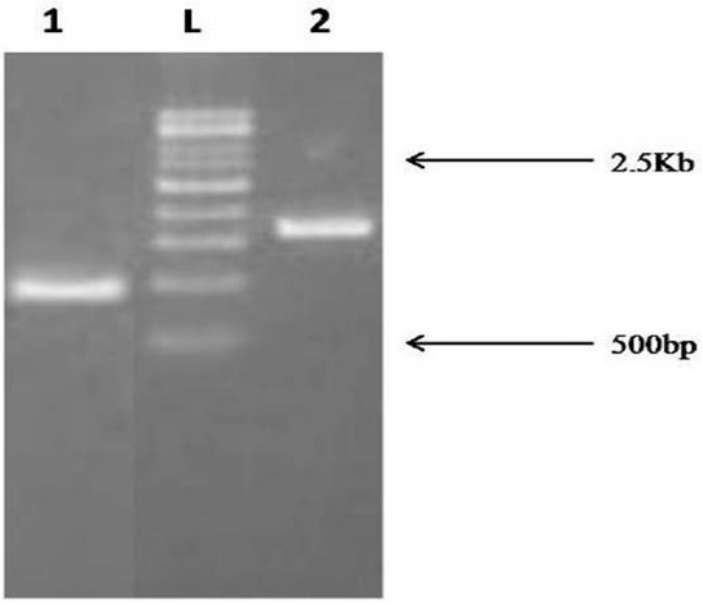

Fig. 5. PCR amplification of 16S rDNA fragment from Bacterial sample. The size of PCR amplified product is ~1.5Kb Lane description: L – 500bp ladder 1. MSCP10 2. MSCW8

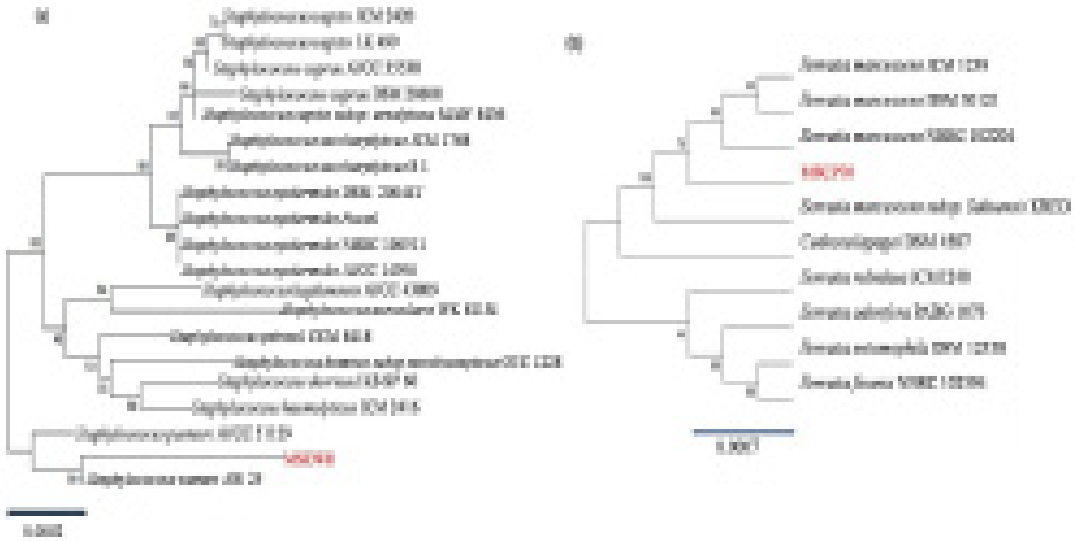

Fig. 6. Phylogeny tree of isolate (a) MSCW8; (b) MSCP10 compared with *Genebank* database (http://www.ncbi.nlm.nih.gov) and was aligned using *Clustal W Multiple Alignment tool* in *MEGAV.7 program*

gel was compared with 500bp ladder (Fig. 5). Amplified 16S rRNA sequences showed that isolate MSCW8 (Accession no. MG066581) and MSCP10 (Accession no. MG066582) were found to be most similar to *Staphylococcaceae bacterium* HDMd_5 and *Serratia marcescens* strain S308 respectively with 99% phylogenetic similarity (Fig. 6, a and b)

DISCUSSION

Total 28 isolates were recovered from chitinous rich bulk soil and agriculture land. These isolates were further screened on the basis of potential to utilize chitin. Out of 12, two efficient isolates (MSCW8 and MSCP10) were identified using 16S rDNA sequence. These isolates were found similar to *Staphylococcaceae bacterium* HDMd_5 *and Serratia marcescens* strain S308 respectively. In present study observed chitinase activity was in range 0.181Uml^{-1} to 1.594Uml^{-1}. The zone clearance diameter around colonies is in range 6mm to 29mm. This infers that these isolates produce exochitinases for the utilization of chitin. Suharjona and Satia reported highest chitinase activity at 30°C (pH7) after 4 days of incubation in *Streptomyces sp*. S242 (0.162Uml^{-1}), *Bacillus thuringiensis* (0.23Uml^{-1}), *Serratia marcescens* DSM3012 (0.556Uml^{-1})[14]. Other chitinase producing different species of *Bacillus* were previously mentioned such as *B. amyloliquefaciens*[15], *B. cereus*[16], *B. licheniformis*[17], *B. megaterium*[18], *B. circulans*[19], *B. subtilis*[20], *B. thuringiensis sub sp.aizawai*[21], *B. stereothermophilus*[22] and *B. thuringiensis sub sp. krustaki*[23]. Present research was employed with the aim of utilizing chitinase producers as an efficient biocontrol agent. Mubarik et al. reported chitinase from *Bacillus sp.* as biocontrol agent[24]. Isolate MSCP10 and MSCW8 shows effectiveness against *Plutella xylostella* larvae in preliminary bioassay as observed (**Table 4**). After 72 h of treatment larval mortality can be easily observed. Different concentrations of isolates were mixed with the diet and larvae were allowed to feed upon. As bacteria starts multiplying in the gut of larvae, it puncture the gut lining, larvae feels pain in stomach and feed less. As a result chitinase start degrading the gut lining of larvae made of chitin. From above conducted experiment we can interpret that chitinolytic bacteria as such can be used as biocontrol agent inspite of using simple spray of chitinase directly in field or in combination with delta toxin was found to be more effective[25,26] which is a long and tedious process. Application of chitin to a plant was an effective biocontrol agent for pest insects, as it attracts chitinolytic bacteria to produce chitinase[27].

CONCLUSION

Chitin degrading bacteria are promising and versatile agent in the field of agriculture, industry, medicine and other commercial uses. From current study data it can be concluded that isolated chitinolytic bacterial strains were effective against Lepidopteran insect *P. xylostella*. Further confirmation can be achieved by conducting insect bioassay with recovered strains in controlled laboratory conditions and consecutive field trials.

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