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 marcescents 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, Platella xylostella.

The use of naturally occurring chitinolytic bacteria, actinomycetes and fungi as potential supplements for insecticides pesticides have been reported in many studies¹. Chitin, the second most abundantly found and widely distributed natural renewable resource next to cellulose in nature. It is a homopolymer of β-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 tons². Based on amino acid sequences present chitinases are the enzymes that catalyze chitin degradation and divided into Family 18 and 19 of glycosyl hydrolase¹. Several bacteria produces chitinases to degrade chitin and utilize it as an energy source and thereby helpful in recycling these resources in soil ecosystem².

A large number of chitinolytic soil bacteria have been isolated from soil³, shellfish waste⁴, shrimp shell-enriched soil⁵ and vermicompost⁶. Phytospheres, such as rhizosphere and phylloplane, are important habitats for chitinolytic bacteria⁷. 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
of chitinaceous waste into useful molecules for application in agriculture, biotechnology and medicine. 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. The continuous 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 chitinaceous 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 Seltrennikhoff with modification. 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$_2$HPO$_4$.2H$_2$O (3.5g), KH$_2$PO$_4$ (1.0g), (NH$_4$)$_2$SO$_4$ (0.5g), MgCl$_2$.6H$_2$O (0.1g), Ca (NO$_3$)$_2$.4H$_2$O (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 (Table 2).

**Chitinase assay**

Colorimetric method described by Setia and Suharjona was used to determine the chitinase activity with three replications. 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‘-AGHGTBTGHTCTMGCTCAS-3’ and 5‘-TRCCGTYMCCCTGCTWHGACTH-3’ respectively using gradient polymerase chain reaction.
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 MEGAV7 program.

**Statistical analysis**

The standard statistical software Graph Pad Prism was used to carry out the data analysis.

| 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                |

**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 | +     | -     | +     | +     | -     | -     | +     | -     | -     | -      | -      | -     |
| Coll shape  | Thin rods | Rods | Thick rods | Rods | Rods | Thin rods | Ceci | Ceci | Ceci | Rods | Rods | Rods |
| Colony color | Cream | Cream | Pale white | Cream | White | Cream | Cream | Yellow | Red | Cream | Yellowish |
| Motility | Non motile | Non motile | Non motile | Non motile | Motile | Motile | Non motile | Non motile | Motile | Motile | Motile |
| Catalase Test | - | - | + | + | + | + | + | + | + | + | + | + |
| Oxidase Test | - | - | + | + | + | + | + | + | + | + | + | + |
| Nitrate Reductase | + | + | + | + | + | + | + | + | + | + | + | + |
| Nitrite reductase | - | - | - | - | - | - | - | - | - | - | - | - |
| Utilization of Glucose AG | +/− | +/− | +/− | +/− | +/− | +/− | +/− | +/− | +/− | +/− | +/− | +/− |
| Lactose AG | +/− | +/− | +/− | +/− | +/− | +/− | +/− | +/− | +/− | +/− | +/− | +/− |
| Sucrose AG | No acidic production, remains red with growth |
| Starch hydrolys | No clear halo around colonies, Media turns dark blue |

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.

**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.
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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            | MGJA199 | KJ672369.1   |
| 6      | Serratia marcescens            | KIPC3-9 | KF017546.1   |
| 7      | Serratia marcescens subsp. sakwensis | PSB23 | HQ242736.1 |
| 8      | Serratia nematodiphila         | YSR    | KY887776.1    |
| 9      | Serratia marcescens            | HX-3   | KX461911.1    |
| 10     | Serratia marcescens            | JNDK1Co-248 | K894728.1 |
| 11     | Staphylococcus pasteuri        | HMD0_5 | JN392843.1    |
| 12     | Staphylococcus warneri         | PSTJ-65 | KY083110.1   |
| 13     | Staphylococcus warneri         | TJ41   | KY575133.1    |
| 14     | Staphylococcus warneri         | TJ21   | KY569426.1    |
| 15     | Staphylococcus warneri         | MT-Y-82 | KU671206.1   |
| 16     | Staphylococcus warneri         | RnL10  | K442753.1     |
| 17     | Staphylococcus pasteuri        | H75    | KU922389.1    |
| 18     | Staphylococcus pasteuri        | L6     | KU922371.1    |
| 19     | Staphylococcus pasteuri        | H83    | KU922347.1    |
| 20     | Bacillus subtillis             | H67    | KU922346.1    |

Table 4. Bacterial strains bioassay with Plutella 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 3 days 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                        |
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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 (U/ml) 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. 1. Isolation of chitinolytic bacterial strains from soil

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

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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 gel.
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 MEGAV7 program.
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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