3.1 Screening and selection of strains for chitinase production by submerged fermentation.

3.1.1 Sample collection

Soil samples were collected from the premises of prawn peeling units in the coastal areas of Kochi in Kerala. The samples were taken from 2 to 3 cm depth using sterile spatula and put in clean plastic bags. The mouth of the bags were tied properly and brought to laboratory for further processing.

3.1.2 Screening for chitin degradation (Primary screening)

Five grams of soil was added to 250 mL flask containing 100 mL 1% suspension of dried and milled prawn shell waste in water. After incubation for five days at 37\(^{o}\) C the suspension was plated onto a screening medium (pH 7.5) containing (\%, w/v): prawn shell powder, 1g and agar, 2g. The plates were incubated at 37\(^{o}\) C for 48 h. Chitinase producing bacteria were selected for further studies based on the zone of clearance around the colonies. The bacteria were subcultured and maintained in nutrient agar slants at 4\(^{o}\) C. The bacteria which showed zone
of clearance of 1.5 cm or above around the colonies were selected for testing the yield of chitinase. Two chitinolytic MTCC strains, MTCC 1688 (*Pseudomonas aeruginosa*) and MTCC 2387 (*Bacillus subtilis*) were included for comparison.

### 3.1.3 Selection of high yielding strains under submerged fermentation (Secondary screening).

For the preparation of inoculum, the bacterium was first grown on the nutrient agar slants (pH 8.0) for 24 h. A loopful of the growth was then transferred to nutrient broth (pH 8.0) and was allowed to grow at room temperature for 24 h, with agitation at 150 rpm. This culture with a cell concentration adjusted to get an OD$_{600}$ corresponding to 2 mg dry cell weight per mL was used as the inoculum.

The overnight grown culture of each bacterium was inoculated into 100 mL of the 1% suspension of prawn shell powder in water (pH 6.5) and incubated at 37° C for 48 h in an incubator shaker agitated at 100 rpm. The culture was centrifuged at 8000 rpm for 20 min and the supernatant was collected to estimate the chitinase activity.

### 3.1.4 Assay of Chitinase

Chitinase production was measured in terms of chitinase activity exhibited by the culture supernatant in the enzyme assay. Chitinase activity was assayed by DNSA method (Miller, 1959). Chitinase activity was assayed in a reaction mixture containing 1 mL of suitably diluted enzyme extract and 1 mL of 1% of colloidal chitin (appendix) dissolved in 0.05 M phosphate buffer (pH 7) and incubated at 40° C for 1 h. The reaction mixture was then centrifuged at 8000 rpm for 20 min. 1.0 mL of supernatant was transferred to a clean test tube to which 1.0 mL
of DNS reagent (appendix) and two drops of NaOH (1%) were added, vortexed and boiled for 5 min. The solution was then cooled to room temperature and 5 mL of distilled water was added. Absorbance was read at 540 nm. Concentration of N-acetyl glucosamine was quantified using a standard curve already prepared. One unit of chitinase was defined as the amount of enzyme that liberated one micro mole of N-acetyl glucosamine per mL per minute under the assay conditions.

### 3.1.5 Identification of bacterium

The cultural, morphological, physiological and biochemical properties of the highest chitinase yielding bacterium (Mb126) were studied as part of the identification. Identification was done according to the guidelines in *Bergey’s Manual of Systematic Bacteriology* (Holt *et al.*, 1986). The results were also confirmed by 16S r RNA gene sequence based molecular identification. For this isolation of genomic DNA and PCR were conducted as per methods described by Sambrook *et al* (1986).

### 3.1.6 Genomic DNA Isolation

For the genomic DNA isolation, Mb126 was inoculated in to 10 mL of LB broth (appendix) and was incubated at 37° C for 24 h. From this, 1.5 mL was transferred to a micro centrifuge tube and was centrifuged for 2 min at 6000 rpm. The bacterial pellet was further resuspended in 467 µL TE buffer (appendix) followed by the addition of 30 µL of 10% SDS and 3 µL of 20 mg/ml proteinase K. This was then incubated for 1 hr at 37° C. Then equal volume of phenol/chloroform was added to the tube, mixed well and was centrifuged to collect the supernatant. The supernatant was further treated with chloroform /isoamyl alcohol, the aqueous phase was then transferred into a new tube. To this tube, 1/10 volume of sodium
acetate was added followed by the addition of 0.6 volumes of isopropanol. This was then mixed gently until the DNA precipitates. The DNA was collected by centrifugation further washed with 70 % ethanol. The DNA pellet was then air-dried and resuspended in 100 µL TE buffer. The quality of the DNA was checked by agarose gel electrophoresis and was quantified using Shimadzu UV-VIS spectrophotometer.

3.1.7 Agarose gel electrophoresis

Agarose gel electrophoresis was carried out for the visualization of isolated DNA. For this, 0.8% (appendix) agarose in 1X TAE buffer (appendix) was melted in a microwave oven. The molten agarose was poured into a gel casting tray and allowed to solidify at room temperature. From the isolated DNA sample, 5µL was loaded onto a gel along with 6X gel loading dye and the electrophoresis was carried out at 80V for 1h. Double digest of Lambda DNA with EcoR1/Hind111 (Genei,Bangalore) was used as marker. After the gel run, the gel was stained in freshly prepared 0.5 mg/mL ethidium bromide solution for 10 min and was viewed on an UV transilluminator.

3.1.8 PCR amplification

Primers used for the amplification of 16S rRNA gene from Mb126 were got synthesized from Sigma. The sequence of forward primer (10-30 F) was 5’-GAG TTT GAT CCT GGC TCA G-3’ and that of reverse primer (530 R) was 5’-G(A/T)A TTA CCG CGG CGG CTG-3’. PCR was performed in 50µL reaction volume containing 50 ng of isolated genomic DNA, 10 pmoles of each primer, 200 µM of each dNTP, 1X PCR buffer, and 1.25U of Taq DNA polymerase. The conditions used for PCR were: initial denaturation at 95º C for 5 min, then 30
cycles of denaturation at 94º C for 30 seconds, annealing at 58º C for 30 seconds and extension at 72 ºC for 45 seconds. This was followed by the final extension at 58º C for 30 seconds for 29 times. The PCR product was analysed by agarose gel electrophoresis.

3.1.9 Analysis of PCR Products

For this, 1.5% agarose (appendix) gel electrophoresis was used. From the final PCR product, 5 µL was used along with 6X gel loading dye for the gel analysis. The DNA ladder used was low range DNA ladder from Genei, Bangalore and the gel run was carried out at 80 V for 3 h. After the gel run, the gel was stained with ethidium bromide solution and the PCR product was viewed on an UV transilluminator. The PCR product was further gel purified and was used as template for DNA sequencing.

3.1.10 DNA Sequencing

The DNA sequencing was performed at the DNA Sequencing Facility of Sci Genome, Cochin. Both the forward and reverse primers were used in separate sequencing reactions to obtain the complete sequence of the PCR product. The DNA sequence thus obtained was used for detailed comparative sequence and phylogenetic analysis. A similarity search for the nucleotide sequence of 16S rRNA gene of the test isolate was carried out using a BLAST search at NCBI (Altschul et al, 1990).

3.1.11 Stability of the selected isolate

Ability to maintain high yielding nature was studied by subculturing and testing the chitinase yield at monthly intervals till the end of this work.
3.2 Optimization of culture conditions for the production of chitinase by *Kurthia gibsonii* Mb126 under the submerged fermentation method.

The factors were studied in a sequential manner. One factor was optimized at a time. The optimal level of this factor was incorporated in the next step. The preparation of inoculum, inoculation and incubation were same as described under section 3.1.3 unless otherwise specified.

3.2.1 Effect of growth phase & incubation period.

The culture was inoculated into 100 mL of the fermentation medium containing 1% prawn shell powder (pH 6.5) and incubated at 37°C in an incubator shaker agitated at 100 rpm for different periods of time and the enzyme production was assayed.

3.2.2 Agitation

The production of chitinase was monitored varying the agitation rate (50-250 rpm) of the incubator shaker.

3.2.3 Substrate Concentration

Different concentrations of prawn shell powder were incorporated into the fermentation medium. Fermentation was performed for 3 days at 37°C and the enzyme was assayed.

3.2.4 Temperature

The optimum temperature for enzyme production were selected by varying temperature of incubation (20-60°C).
3.2.5 Salts

To evaluate the effect of salts the bacterium was grown in the presence of various salts (5mM) such as MnCl$_2$, CaCl$_2$, HgCl$_2$, CuSO$_4$, ZnCl$_2$ and MgCl$_2$.

3.2.6 pH

The optimum pH for enzyme production were selected by varying the pH of the medium (3-8).

3.2.7 Surfactants

To evaluate the effect of surfactants the bacterium was grown in the presence of surfactants (1%v/v) such as Tween 20, Triton X–100 and Tween -80.

3.3 Screening and selection of strains for chitinase production by solid substrate fermentation

3.3.1 Bacterial strains

The same chitinolytic bacterial strains isolated from marine environments of Kochi, mentioned under section 3.1.2 were subjected to SSF studies. Two chitinolytic MTCC strains, MTCC 1688 (*Pseudomonas aeruginosa*) and MTCC 2387 (*Bacillus subtilis*) were included for comparison.

3.3.2 Preparation of substrate

Prawn shell waste was collected from shellfish processing units in Kochi, Kerala. The waste was then sun dried, milled into small pieces of uniform size (0.4 mm).

Selection of high yielding strains was done by SSF in two steps.
3.3.3 Step 1 Preliminary studies

The SSF experiments were carried out in 250ml Erlenmeyer flasks using 10 g of prawn shell waste moistened with 20 mL mineral salt solution (pH 7) containing (g/l): KH$_2$PO$_4$-2, NH$_4$NO$_3$-10, NaCl-1, and MgSO$_4$.7H$_2$O-1. Cotton-plugged flasks were autoclaved at 121° C under 151bs pressure for 15min, cooled to room temperature and uniformly inoculated with 1ml inoculum (3X10$^7$ CFU / ml) of 24 hr culture and incubated at 37° C for 72 h. After incubation the fermented substrate was mixed with 50 mL of distilled water on a rotary shaker for 1 h. The suspension was then centrifuged at 8000 rpm at 4° C for 10 min and the supernatant was assayed for enzyme activity. Chitinase activity was assayed as mentioned under section 3.1.4 and expressed in U/g dry substrate (U/gds). Dry weight of the substrate was determined by drying it in a hot air oven set at 80° C till a constant weight was obtained.

Percent moisture content of the solid substrate was calculated as follows,

Percent of moisture content = (wt. of the substrate - dry wt.) X 100.
                             dry wt.

3.3.4 Step 2 - Detailed solid substrate fermentation studies (Selection of high yielding strain).

Mb 14, Mb126 and Ch135, the top three strains found to be high yielding strains by preliminary studies were tested further with different moisture levels (Substrate: moistening solution ratio 1:1, 1:1.5, 1:2, 1:2.5) and incubated for different periods (48, 72, 96 and 120 h). Enzyme activity was assayed as in the preliminary studies.
3.3.5 Monitoring the stability of yield potential

Stability of the highest yielding strain Mb126 was studied by subculturing and testing the yields at monthly intervals till the end of this work.

3.4 Optimisation of process parameters on chitinase production by SSF

Medium preparation, inoculum and incubation were as described in section 3.3.3 except for the modifications mentioned under each experiment.

Parameters

3.4.1 Incubation period.

Growth media containing 10 g of prawn shell powder were incubated at 37°C (pH 7) for varying time periods (12, 24, 36, 48, 60, 72, 84, 96 h) and the enzyme produced was assayed.

3.4.2 Effect of initial moisture content

Fermentation was carried out with prawn shell powder medium moistened with different volumes of moistening solution. Enzyme production was assayed after 60 h of incubation.
3.4.3 Inoculum size

Effect of inoculum size were carried out by inoculating the medium with inoculum containing varying concentrations of bacterial cells as 1, 2, 3, 4, 5 and 6 x 10^7 CFU/mL.

3.4.4 pH of the medium.

The effect of pH on chitinase production was studied by performing SSF using moistening solution adjusted to different pH (3-10).

3.4.5 Incubation temperature.

The solid substrate medium was inoculated and the yield of chitinase was determined after incubation at different temperature from 20 to 50°C.

3.4.6 Effect of various sugars

Effect of additional sugars were tested by adding each of starch, glucose, lactose, maltose, fructose, colloidal chitin and N-acetyl glucosamine to the moistening solution so that the final concentration was (1%, w/w).

3.4.7 Effect of nitrogen source

Effect of additional nitrogen source were tested by adding each of yeast extract NaNO₃, (NH)₄SO₄, NH₄Cl, NH₄NO₃ and KNO₃ to the moistening solution at a final concentration of 1% (w/w).

3.4.8 Effect of particle size

Effect of the size of substrate were tested by using different sized (0.1-1mm)
prawn shell powder for the preparation of the fermentation medium.

### 3.4.9 Effect of detergents

One percentage (w/w) of SDS, Tween-20, Tween-40, Tween-80 and Triton X-100 was included in moistening solution and the effect on the chitinase production was studied.

### 3.5 Purification of chitinase from *K. gibsonii* Mb126.

The enzyme was produced by solid substrate fermentation providing optimum conditions such as moisture content 75%, the pH of the moistening solution 8 and temperature of incubation 40°C and by using 0.6 mm sized prawn shell powder. The optimum inoculum size was 4-5 x 10⁹ CFU/mL.

The chitinase enzyme was purified by procedures including ammonium sulfate precipitation, affinity adsorption, ion exchange chromatography and gel filtration chromatography. All these purification steps were performed at 4°C. After each step chitinase activity and protein content were determined. The enzyme activity was determined as described under section 3.1.4. Protein concentration was determined spectrophotometrically at 280 nm.

#### 3.5.1 Ammonium sulfate precipitation

Ammonium sulfate was added into the culture supernatant and the precipitate obtained at 75% saturation was collected by centrifugation at 10000 rpm for 20 min. The precipitate was dissolved in 0.01 M potassium phosphate buffer of pH (7.5) and was dialyzed against the same buffer.
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3.5.2 Dialysis

3.5.2.1 Activation of dialysis tube

1 mM EDTA was prepared in 100 mL 2% Na₂CO₃ (solution ‘a’) and in 100 mL distilled water (solution ‘b’). The dialysis bag was immersed in the boiling solution of ‘a’ for 20 min. Then it was soaked in hot water to remove Na₂CO₃. Then the tubes were immersed in boiling solution of ‘b’ for 10 min. The activated dialysis tube was kept immersed in solution of ‘b’ in refrigerator.

3.5.2.2 Dialysis

The activated dialysis tube (cut off value 12 kDa) was tied at its one end and filled with ammonium sulfate fraction without trapping any air bubble. It was then tied at its upper end and suspended in a beaker containing 0.01 M phosphate buffer of pH (7.5) and kept overnight at 4°C. The buffer solution was changed regularly during dialysis. The concentrated dialysate was collected.

3.5.3 Affinity adsorption

Purified prawn shell powder purchased from Marine Chemicals, Cochin was dried and blended to fine powder. This was then suspended in 0.01 M phosphate buffer of pH (7.5) and was carefully packed in a column of size (0.5X10 cm). Sample solution was applied into the column. Elution was done with 2M NaCl. Active fractions were collected, pooled and kept for ion exchange chromatography.

3.5.4 Purification using DEAE cellulose column

Activated DEAE cellulose was carefully packed in a column of size 2.5 X 7 cm without trapping any air bubble. Sample was applied to the DEAE cellulose
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column pre equilibrated with 10 mM phosphate buffer (7.5). The enzyme elution was performed by gradient elution technique using NaCl gradient of 0 to 0.5 M in 20mM citrate - phosphate buffer (pH 5.5) at a flow rate of 24 mL/h. Fractions of 3 mL were collected and was assayed for enzyme activity. Protein concentration was measured at 280 nm mentioned under 3.5. The active fractions were pooled and dialyzed against 50 mM sodium acetate buffer (pH 4.5).

3.5.5 Gel filtration Chromatography

Sephadex G-100 was packed in a column of size 1.6X60 cm without any air bubble and it was pre equilibrated with 50 mM sodium acetate buffer (pH 4.5) before the dialysate was applied. Elution of the enzyme was carried out with 50 mM sodium acetate (pH 4.5) at a flow rate of 15 mL/h. 3 mL fractions were collected and each fraction of enzyme solution was assayed for enzyme activity and protein content was measured. The active fractions were pooled and dialyzed against 0.01 M phosphate buffer of pH (7.5).

3.5.6 Electrophoretic methods

Samples obtained at various stages of purification were run on 10 % SDS–PAGE gel(appendix) according to the method of Laemmli (1970). Gels were run at pH 8.8 at a constant current of 2mA per gel. Gel was stained with 1% Coomassie Brilliant Blue R-250 in 45% methanol-10% acetic acid solution for 4 h and destained in the same solvents for 4 h.
3.6 Properties of purified enzyme

The properties of purified enzyme from *K. gibsonii* Mb126 were studied. Experiments were done in triplicate.

### 3.6.1 Effect of pH

The effect of pH on the activity of the purified enzyme was studied by measuring chitinolysis in buffers (50 mM) of different pH under standard assay conditions. Buffers used were glycine- HCl (pH 2-3), acetate (pH 3.5-5.5), phosphate (pH 6-8) and glycine -NaOH (pH 8.5-9).

### 3.6.2 Effect of temperature

Chitinase activity was tested at different temperatures ranging from 20-80°C in 50 mM phosphate buffer (pH6.5) using colloidal chitin as the substrate.

### 3.6.3 Effect of salts

The effect of various salts on the enzyme activity was studied. The enzyme was incubated with different concentrations of various salts (2-10 mM) in phosphate buffer (pH 6.5) at 40°C for 1 h and the enzyme activities were determined. The enzyme activity in buffer without any of these salts (control) was also determined.

### 3.6.4 Effect of various chemicals

Effect of various chemicals such as detergents (Triton X-100, Tween- 80, Tween -20 and SDS), β mercapto ethanol, dithiothreitol, EDTA and $\text{H}_2\text{O}_2$ were studied by adding different concentrations (2-10 mM) of each into the assay system. The enzyme activity in buffer without any of these chemical (control) was also determined.
3.6.5 Effect of pH on stability

pH stability of chitinase was studied. The enzyme was incubated in 50 mM acetate (pH 4 - 5.5) and phosphate (pH 6 - 7.5) buffers for 1 h at 25°C. Activities were determined before and after incubation.

3.6.6 Effect of temperature on stability

Thermal stability was examined by exposure of the enzyme solution in phosphate buffer (50 mM, pH 6.5) to different temperature for 2 h after which the enzyme solution was cooled rapidly. Enzyme activities were determined before and after heat treatments. The percentage of activities remaining after the heat treatments were calculated.

3.6.7 Kinetic properties

The apparent $K_m$ and $V_{max}$ were studied using colloidal chitin as the substrate. Varying concentrations of substrate were added to assay system containing 0.5 mL enzyme at its optimum pH (6.5) and temperature (40°C) and incubated for 1 h before the velocity of enzyme was measured. The amount of monomer released was extrapolated from the standard graph of GlcNac. The $K_m$ and $V_{max}$ values were determined.
3.7 Antifungal activity of *K. gibsonii* Mb126 chitinase against fungi associated with rice and its potential for the biocontrol of phytopathogenic fungi of rice

The effect of chitinase on phytopathogenic fungi was studied by assessing the inhibitory effect of the enzyme on the growth of mycelium. The experiments were done in triplicate.

3.7.1 Isolation of fungi from rice

Rice seed samples were collected from different locations. Seeds were surface sterilized with 0.1% mercuric chloride for 4 min followed by four washings with sterilized water. One gram of rice from each sample was placed on potato dextrose agar (PDA) medium and incubated at 28° C for 8 days. The plates were then examined for fungal growth.

3.7.2 Identification of fungi

The fungi were identified by studying the colony morphology and microscopic morphology. The microscopic morphology was studied by lactophenol cotton blue (appendix) staining of needle mount preparations. For identification, the guidelines of Hoog *et al* (2000) were followed. The percentage frequency of occurrence of various fungal species was calculated as follows;

Frequency of occurrence (%) =

\[
\frac{\text{Number of seeds on which a fungal species occurs}}{\text{Total number of seeds}} \times 100
\]
3.7.3 Effect of chitinase on phytopathogenic fungi

Loopfuls of test fungi were inoculated into 5 mL of potato dextrose broth and incubated at room temperature for 3 days. Then the mycelium from each was inoculated in the center of freshly prepared PDA plates with different concentration of chitinase (0.2, 0.4, 0.6, 0.8 and 1 U/ml) and in the control plates distilled water was used in place of the enzyme solution. The plates were incubated at 28° C for 12 day, and the radial growth was recorded. The percentage of inhibition was calculated by following the equation.

\[
\text{Percentage of Inhibition} = \left(\frac{C-T}{C}\right) \times 100.
\]

C - Colony diameter of the mycelium on the control plate (mm).

T – Colony diameter of the mycelium on the treatment plate (mm).

3.7.4 Effect of chitinase on the germination of rice

Preparation of spore suspension

The fungi were cultured on SDA for seven days at 25°C, fungal mycelia from pure culture were aseptically transferred to SDA and cultured for 10 days at 25°C. Spores were harvested and suspended them in distilled water containing 0.05% Tween 80. The concentration of spore suspension was adjusted to $10^4$-$10^5$/mL.

Seed Germination test

The germinability of seed was determined according to Nghiep and Gaur (2005). The surface sterilized seeds were put in spore suspension of each fungus.
for 12 h and then transferred to each treatment solution of chitinase containing 0.2, 0.4, 0.6, 0.8 and 1 U/ml, incubated for 12 h. The seeds were then placed on a moistened Whatman filter paper (No.1) which was carefully layered in petri plates. Ten seeds were placed per plate. Seeds were then covered with an another layer of moistened Whatman filter paper (No.1). Two types of controls were included. In control (A) seeds were soaked in distilled water for 24 h and were neither spore nor chitinase treated. In control (B) the seeds were treated with spore suspension only. The plates were then wrapped with a sheet of wax paper to reduce surface evaporation and placed in a germinator at 25ºC. Germinability and seedling vigour index were determined according to the recommended method by ISTA (1985). Each treatment was examined for seedlings. Germinated seeds were counted on 7\textsuperscript{th} day and the percentage of germination was calculated by the formula;

\[
\text{% germination} = \frac{\text{Total number of germinated seeds as on 7}^{\text{th}} \text{day}}{\text{Total number of seeds inoculated}} \times 100
\]

Seedling vigour index was calculated by the formula;

\[
\text{Percent Germination} \times \text{Seedling length} = \text{Seedling vigour index}
\]
3.8 PCR amplification and sequence characterization of Chitinase gene from *Kurthia gibsonii* Mb126

PCR amplification of chitinase gene from *K. gibsonii* Mb126 was conducted with degenerate primers using genomic DNA as the template.

3.8.1 Genomic DNA Isolation

Genomic DNA was isolated as per the methods explained under section 3.1.6 and the quality was analysed by electrophoresis using 0.8% agarose gel.

3.8.2 PCR amplification

The genomic DNA isolated was used as the template for PCR using the primers GA1F (5’-CGTCGACATCGACTGGGARTDBCC-3’) and GA1R (5’-ACGCCGGTCCAGCCNCKNCCRTA- 3’). The primer sequences which was described earlier by Williamson *et al* (2000) were got synthesized from Sigma. PCR was performed in 50 µL reaction volume containing 50 ng of isolated genomic DNA, 10 pmoles of each primer, 200 µM of each dNTP, 1X PCR buffer, and 1.25U of *Taq* DNA polymerase. The conditions used for PCR were: initial denaturation at 94º C for 3 min., then 30 cycles of cyclic denaturation at 94º C for 1 min, annealing at 50º C for 1 min and extension at 72º C for 1.5 min. This was followed by a final extension at 72º C for 7 min. The PCR product was analysed by agarose gel electrophoresis as per the methods explained earlier (3.1.9).

3.8.3 DNA Sequencing

The PCR product formed was further purified and sequenced at the DNA Sequencing Facility of Sci Genome, Cochin. Both the forward and reverse primers
were used in separate sequencing reactions to obtain the complete sequence of the PCR product. The DNA sequence thus obtained were translated to amino acid sequences and were further analysed by BLAST analysis (Altschul et al., 1997). The sequence was submitted to NCBI under the accession number of JQ739168. Multiple sequence alignment of the sequence was conducted along with other selected chitinases using BioEdit (Hall, 1999). Multiple sequence alignment of chitinases of *K. gibsonii* Mb126 (JQ739168) with other closely related bacterial chitinases from *Bacillus cereus* E33L(AAU16783), *Bacillus thuringiensis serovar konkukian* str.9727 (AAT60545), *Bacillus cereus biovar anthracis* st.CI (ADK 06287), *Bacillus mycoides* DSM 2048 (EEL 98244), *Clostridium botulinum* B Str. Eklund 17B (ACD23618) were performed. Molecular phylogeny were performed using MEGA 5 (Kumar et al., 1994). Crystal structure of chitinase from *K. gibsonii* Mb126 were predicted using SWISS-MODEL and the Swiss-PdbViewer, an automated protein homology-modeling server using the template 10.2210/ pdb3n11/pdb ie, crystal structure of wild-type chitinase from *Bacillus cereus* NCTU2 (Arnold et al., 2006; Schwede et al., 2003; Guex and Peitsch, 1997).