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Purification and molecular characterization of chitinases from soil actinomycetes

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Microbial extracellular chitinases are used in agriculture as effective biocontrol agents and in waste degradation, pharmaceutical and food industry. Actinomycetes are widely tapped group for production of extracellular chitinases. In the present study, approximately 260 actinomycetes were isolated from various ecological habitats was subjected to primary analyses and screened for production of chitinase by plate assay method. Diameter of zones of hydrolysis ranged from 8 to 16 mm. Based on the results, isolates 130, 194, 184, NRRLB 24916 (*Streptomyces mexicanus*) and NRRLB 16746 (*Streptomyces albidoflavus*, positive control) were selected for secondary screening and purification. Enzyme activity was estimated in crude cell free extract and partially purified samples. Activity ranged from 7.16 to 14.12 IU/ml (in crude extracts) and 12.1 to 23.10 IU/ml (in partially purified samples). In case of highest chitinase producing isolate 130, effect of various fermentation conditions (pH, temperature and substrate concentration) was studied in crude extract. Furthermore, complete purification of isolate 130 was done by column chromatography and the activity in purified fraction was found to be 32.12 IU/ml. The K_m and V_{max} values of the purified fractions for isolate 130 were 2.11 µg/ml/min and 53.11mg/ml respectively. This shows that the enzyme has high affinity for the substrate. SDS gel electrophoresis of the purified fraction showed presence of single band of approximately 65 to 70 kDa. Analyses of purified chitinase were done using MS/MS technique. N-terminal sequence corresponded to chitinase, the gene encodes a protein of 453 amino acid residues. Comparison of deduced amino acid sequence to other chitinases in the database indicated that enzyme showed 70% similarity with chitinase from *Streptomyces plicatus* and belongs to glycoside hydrolase family 18. Homology modeling showed that the enzyme was folded into a domain of $(\alpha/\beta)_8$ barrel structure. Identification of secondary structure was done by CD spectroscopy. Isolate 130 was capable of degrading biodegradable wastes such as crustacean shells.

Key words: Actinomycetes, extracellular chitinase, primary screening, secondary screening, purification, MS/MS analyses, homology modelling, protein structure, biodegradation.

INTRODUCTION

Chitinases are enzymes that hydrolyse the β-1,4 linkage of N-acetyl glucosamine present in chitin chains. Due to vast availability, low cost, high stability and productivity, microbial chitinase is attaining prominence for waste management, pest control in agriculture, and human health care (Das et al., 2015, 2016; Rathore and Gupta, 2015). Improving the yield of the enzyme and consequent cost reduction depends on the selection of strains, optimization of fermentation conditions, genetic improvement of strains and kinetic studies of enzyme (Andualem,

2014, Fentahun and Kumari, 2017).

Classical approaches in extracellular chitinase characterization include isolation and screening of bacterial groups for their ability to produce chitinase enzyme. Wide range of bacteria in the environment is efficient producers of extracellular chitinases. Actinomycetes are well known producers of chitinases (Kumar and Singh, 2013; Mohanta, 2014). Researchers are exploring diverse untapped habitats in an attempt to discover new actinomycete strains for producing novel chitinase enzyme, having applications in various industries (Gurung et al., 2013; Anbu et al., 2015). The next step is fine-tuning of fermentation processes, aimed specifically at the production of purified, well characterized enzymes from selected strains on a large scale (Bui, 2014; Yassien et al., 2014; Kumar et al., 2016).

Developments in biotechnology, such as protein engineering and directed evolution, revolutionized the probability of producing novel enzymes by introducing or modifying the capability of specific genes (Sandgren et al., 2013; Chen et al., 2014; Walia et al., 2015; Enkhbaatar et al., 2016; Castillo et al., 2016). Advances in biotechnology is providing a plethora of enzymes displaying new activities and having adaptability to a range of conditions leading to their increased adoption for industrial purposes (Diaz et al., 2004; Brzezinska et al., 2013; Sriyapai et al., 2013; Munar et al., 2013).

With the advancement in industrialization and urbanization, dumping of enormous amount of materials as wastes has become a nuisance (Akhtar, 2014). Conventional techniques for management of biodegradable wastes are becoming increasingly expensive and energy inefficient. Secondly, the chemical treatment methods are hazardous to both environment and humans (Wilts et al., 2016). As a result, search for more sustainable approaches becomes important for conversion of wastes into by-products that can be directly used for commercial purposes (Benhabiles et al., 2013). One such approach is bioremediation, which makes use of the enzymatic potential of micro-organisms present in the environment for effectual degradation of biodegradable wastes (Karigar and Rao, 2011). Waste contains substances like cellulose, starch and lignin, which are susceptible to microbial degradation. It is an eco-friendly process which decomposes the wastes into useful raw materials (Ilangumaran et al., 2017).

The purpose of the current investigation was to screen actinomycete isolates for production of chitinase enzyme followed by optimization of fermentation parameters (pH, temperature and substrate concentration) for improving the yield of the enzyme. Next step was the use of molecular techniques to characterize the enzyme for identification of the type of protein and active site residues. This in turn will form the basis for protein engineering of enzymes and at a later stage will allow specific manipulation of the associated amino acids for desired enzymatic properties. From the application point view, role of the selected isolate in biodegradation of chitin present in crustacean/shrimp waste samples was also studied.

MATERIALS AND METHODS

Collection of soil samples and isolation of actinomycetes

Soil samples were collected from various ecological habitats (Table 1) and actinomycete isolates were isolated by plate dilution method. Single isolates were purified by restreaking on yeast extract-malt extract agar plates and stored as glycerol stocks at -20ºC/-80°C deep freezers (Vestfrost/Sanyo, Model- MDF-U55V) (Khanna et al., 2011; Solanki et al., 2011).

Primary screening of isolates for production of chitinase

Colloidal chitin was prepared by adding 5 gof chitin powder (HiMedia) in 60 ml conc. HCl. The mixture was then kept at room temperature overnight with vigorous stirring. It was then filtered through Whatman no.1 filter paper and the residue remaining on filter paper was added to 200 ml of 95% ethanol and stirred vigorously overnight. The mixture was centrifuged at 5,000 rpm for 20 min at room temperature. Precipitate was transferred to a glass funnel containing Whatman no. 1 filter paper and washed with sterile distilled water until the pH of the sample became neutral. The chitin that was retained on filter paper was removed and stored in dark at 4°C (Priya et al., 2011). Chitin agar medium supplemented with 1% colloidal chitin (pH 8.0) was prepared and autoclaved. The cultures were spot inoculated on the medium plates and incubated at 28°C for 14 to 21 days until the zone of chitin hydrolysis was observed around the isolates. The clear zone diameter was measured by subtracting the inoculum size from the total zone diameter used as an indicator for chitinase activity (Gadelhak et al., 2005; Priya et al., 2011).

Secondary screening or quantitative analyses of chitinase activity

Strains showing maximum zones of clearance during primary screening were selected for subsequent secondary screening (Das et al., 2015, 2016).

Standard inoculum preparation under submerged fermentation process

Isolates showing maximum zone of clearance were inoculated in 25 ml of 148G medium (composition (g/L^{-1}) Glucose 22, Beef extract 4, Bacto peptone 5, Yeast extract 0.5, Tryptone 3, NaCl 1.5, (pH 7.5) (Schupp and Divers, 1987) respectively. The flasks were incubated at 28°C on a rotary shaker (New Brunswick Scientific, Excella E24R) at 200 rpm for 5 days. After incubation,

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Table 1. Number of isolates from different ecological habitats.

S/N	Habitat
1	Agricultural soils Agricultural soil, Dhanaura, Uttar Pradesh Agricultural soil, Yamuna Bank, Delhi Agricultural soil, Nainital, Uttarakhand Agricultural soil, Kashipur, Uttarakhand
2	Industrial soils Sugar Plant, Dhanaura, Uttar Pradesh Chemical Plant, Faridabad
3	Landfill soils Dumping site, Sarai Kale Khan, Delhi
4	River/lake soils Yamuna Bank, Delhi Lake soil, Purana Quila, Delhi
5	Diversity park soils Great Himalayan National Park, Teerthan Valley, Himachal Pradesh

absorbance was measured at 600 nm in each case. Culture broths were serially diluted as follows in 148G medium going up to a dilution of 10^{-7}.

(a) 100 µl of culture broth + 900 µl of 148G medium (10^{-1} dilution)
(b) 100 µl of 10^{-1} dilution + 900 µl of 148G medium (10^{-2} dilution)
(c) 100 µl of 10^{-2} dilution + 900 µl of 148G medium (10^{-3} dilution)
(d) 100 µl of 10^{-3} dilution + 900 µl of 148G medium (10^{-4} dilution)
(e) 100 µl of 10^{-4} dilution + 900 µl of 148G medium (10^{-5} dilution)
(f) 100 µl of 10^{-5} dilution + 900 µl of 148G medium (10^{-6} dilution)
(g) 100 µl of 10^{-6} dilution + 900 µl of 148G medium (10^{-7} dilution)

The respective dilutions were plated on Yeast Extract Malt Extract medium (YM) and incubated at 28°C till the appearance of colonies. CFU's/ml were then calculated (El-Sersy et al., 2010; Shanmugapriya et al., 2012; Shaikh et al., 2013). The inoculum having an average viable count of 10^5 to 10^7 CFU's/ml was transferred to production broth (Chitin broth) (Tweddell et al., 1994). The respective media flasks were incubated at 28°C for 5 to 6 days on rotary shaker at 200 rpm.

Estimation of chitinase activity in culture broths (crude enzyme)

After incubation, culture broths were centrifuged at 10,000 rpm for 10 min at 4°C and the cell free supernatants were used as a source of crude enzyme. Chitinase activity was measured in each case, using p-dimethylaminobenzaldehyde (p-DMAB) method (Tweddell et al., 1994). Culture filtrate (0.5 ml) was added to 1.5 ml of colloidal chitin (10 mg/ml) prepared in 50 mM sodium acetate buffer (pH 6.8) in a test tube. To this, 0.1 ml potassium tetraborate was added and incubated at 100°C for 3 min. Tubes were cooled under tap water and 3 ml p-DMAB reagent was added and incubated at 36 to 38°C for 20 min. Tubes were cooled and OD was recorded at 585 nm using spectrophotometer (Elico, Model-SL-160). By using a calibration curve for N-acetylglucosamine, enzyme activity (U = 1 unit of chitinase) is defined as the amount of enzyme that releases 1 µmol of NAGA/ml (Reissig et al., 1995; Gadelhak et al., 2005). Enzyme activity was calculated by using the formula:

$$\text{Enzyme activity (IU/ml/min)} = \frac{\text{Concentration of NAGA} \times \text{dilution factor}}{\text{Time of incubation (min)} \times \text{volume of enzyme}}$$

Where,

$$\text{NAGA concentration} = \frac{\text{Actual absorbance (OD)}}{\text{Slope from graph}}$$

and actual OD = Test OD- (Enzyme blank OD + Substrate blank OD).

Estimation of protein content in cell free culture extract

Protein concentration in crude enzyme was determined by Lowry's method with BSA (Bovine serum albumin) as a standard (Lowry et al., 1951; Das et al., 2016).

Optimization of fermentation parameters (pH, temperature and substrate concentration) for enzyme production in crude extract

Various culture conditions like pH of medium (6 to 9), incubation temperature (30 to 55°C) and substrate concentration (0. 25 to 2.5%; w/v) were optimized for enhanced production of enzyme in submerged fermentation process (Kuddus and Ahmad, 2013; Karthik et al., 2015).

Statistical analyses of enzymatic activity using SPSS software

Data obtained after optimization of fermentation conditions (pH, temperature and substrate concentration) for isolate 130 was statistically analysed using one way ANOVA and multiple comparison test (Post-Hoc test) at significance level of $p < 0.05$. Both the tests were performed by using IBM SPSS Statistics 19 software (Gangwar et al., 2016).

Purification of enzymes and enzyme assay of purified products

Ammonium sulphate saturation and dialysis for chitinase enzyme

Crude extracts from the highest enzyme producers were subjected to partial purification. Crude enzyme solutions (250 ml) of the cultures were saturated by sequential addition of ammonium sulphate followed by dialysis and concentration. Enzyme activity and protein content were estimated in ammonium sulphate saturated, dialyzed and concentrated samples of isolates. The concentrated sample of isolate 130 was purified further by ion exchange chromatography using DEAE Bio-Gel A (Sigma) column (BioRad, 1.3×16 cm). Both unbound and bound fractions were tested for chitinase activity. Active fraction was used as purified enzyme solution. Enzyme activity as well as protein content was estimated in purified fractions (Karthik et al., 2015; Gangwar et al., 2016). The fractions were loaded on SDS-PAGE gel.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

For observing the protein profile of enzyme samples and determining the molecular weight of purified fractions, denaturing SDS- PAGE was used. A broad range prestained standard marker (BioRad, 15-210 kDa) was used in this case (Sowmya et al., 2012; Castillo et al., 2016).

Analysis of kinetic parameters

The effect of colloidal chitin on the chitinase activity of isolate 130 was evaluated by ranging the colloidal chitin concentration from 0.5 to 10 mg/ml. A Lineweaver-Burk plot was obtained by plotting 1/v against 1/s. Kinetic parameters (K_m and Vmax) were estimated by linear regression from Lineweaver-Burk plot (Nagpure and Gupta, 2013; Rahman et al., 2014).

Characterization of enzyme by molecular approach

Protein identification by mass spectrometry analyses

Slices of interest containing the protein bands from the semi-denaturing PAGE were cut and subjected to trypsin digestion. The final samples were submitted for MALDI-MS (ABI SCiex 5800 TOF/TOF system) and LC-ESI-MS/MS (Waters SYNAPT G2 with 2D nano ACQUITY system) analyses respectively. Raw data from both the analyses were transformed in mz data format and used to query non-redundant protein databases with a licensed version of MASCOT 2.1 (Matrix Science, Boston, USA) (Rashad et al., 2017).

Analysis of structure and catalytic sites

The peptide sequence of isolate 130 obtained after MS/MS analysis was used to investigate the structure and catalytic sites. The sequence was matched against the NCBI database and the FASTA protein sequence database. pBLAST was performed to study the homology with chitinases from related *Streptomyces* species.

For constructing the structure for the enzymes, N-terminal sequence of isolate 130 was submitted in SWISS-MODEL. The software searches the database for similarity with the query structure. The best suited structure was chosen by the SWISS-MODEL and based on the sequence of that model, a tentative three dimensional structure was made (Bienert et al., 2017).

Homology modeling approach was adopted for structural and functional study of catalytic site of isolate 130. The pBLAST analyses of amino acid sequence revealed the three dimensional structure of chitinase (Altschul et al., 1990). The most suitable high-resolution protein structure was selected as the template protein. Multiple sequence alignment of target and template protein sequences was performed by ClustalW. Modeling was performed with the help of MODELLER version 9.11 (Schwede et al., 2003; Arnold et al., 2007; Bienert et al., 2017). After aligning, the target and template sequences were used as input files in MODELLER, the software automatically calculates and gives a model containing all non-hydrogen atoms (Ubhayasekera and Karlsson, 2012; Hamid et al., 2013).

The final 3D model of chitinase was verified by the Structural Analysis and Verification Server (SAVES) which used PROCHECK software. Ramachandran Plot was constructed and analysed to check the percentage of residues present in most favored, allowed, generously allowed and disallowed regions respectively (http://nihserver.mbi.ucla.edu/SAVES). Compatibility of the 3D model with its own amino acid sequence (1D) was done by VERIFY 3D program. Identification of active sites that are responsible for substrate binding was done by using Catalytic Site Atlas (CSA) database of European Bioinformatics Institute (http://www.ebi.ac.uk/thorntonsrv/databases/CSA/) (Hoell et al., 2006).

Circular dichroism for protein secondary structure analysis

Circular dichroism measurements were performed on a Chirascan spectropolarimeter (Applied Photophysics). The CD spectra were recorded from 190 to 260 nm. The results were analyzed by Graphpad Prism processing software (Gangwar et al., 2016; Berini et al., 2017).

Role of extracellular enzymes in bioremediation of wastes

Collection of waste samples from various sites

Biodegradable waste samples such as fishery wastes (crustacean (prawns, shrimp and crab) shells) were collected (Table 2). Furthermore, raw wastes were pretreated to convert it into powdered substrates (Table 2) (Kumar and Sharma, 2017).

Primary and secondary screening of isolates for degradation of wastes

For screening of isolate 130 for its ability to degrade wastes, the culture was spot inoculated on chitin agar medium supplemented with 0.4% crustacean (prawns, shrimp and crab) shells powdered substrate. The clear zone diameter was measured by subtracting the inoculum size from the total zone diameter in order to observe the potential of isolate for degradation of chitin (Subramaniam et al., 2012; Setia and Suharjono, 2015). For quantitative screening, enzyme activity was estimated in crude extracts under solid state fermentation. Isolate was inoculated in 25 ml of 148G medium. The culture flask was incubated at 28°C on a rotary shaker at 200 rpm for 5 days. After incubation, absorbance was measured at 600 nm in each case. Culture broth was serially diluted in 148G medium going up to a dilution of 10^{-5}. The respective dilutions were plated on Yeast Extract Malt Extract medium (YM) and incubated at 28°C till the appearance of colonies. CFU's/ml were then calculated. Inoculum having an average viable count of 10^4 to 10^5 CFU's/ml was transferred in mineral salt broth supplemented with specific substrate and incubated at 28°C for 5 to six days on rotary shaker at 200 rpm.

Table 2. Details regarding collected biodegradable waste sample.

Waste material used as substrate	Collection site	Collected by	Pre-treatment procedure followed
Crustacean (prawns, shrimp and crab) shells	Ghazipur fish market, New Delhi	Undergraduate student, working under Delhi University Innovation Project)	Crustacean shells were treated using chemical method.

Table 3. Clear zone produced by isolates due to production of chitinase.

Strain	Clear zone diameter (mm)
Isolate 130 (Sugar plant, Dhanaura, U.P.)	16
NRRLB 16746 *Streptomyces albidoflavus* (chitinase control)	13
Isolate 194 (Dumping site, Sarai Kale Khan, Delhi)	12
Isolate 184 (Chemical plant, Faridabad, Delhi)	10
NRRLB 24916 *Streptomyces mexicanus (*xylanase control)	8

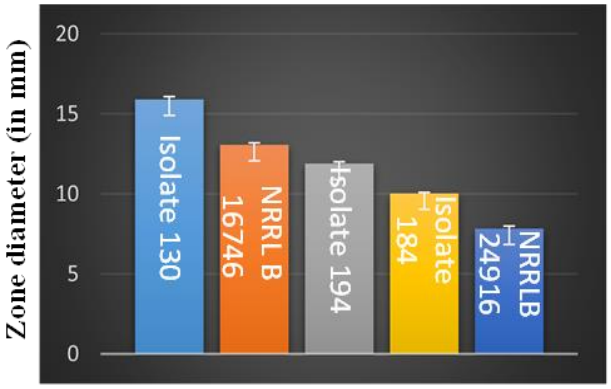


Figure 1. Comparison of activity of chitinase producing isolates (colloidal chitin was used as substrate). *Error bars presented mean values of ± standard deviation of triplicates of three independent experiments; SD determined was in the range of 0.05 to 0.1.

Enzyme activity was estimated in crude cell free extract by p-dimethylaminobenzaldehyde (p-DMAB) method. Culture filtrate (0.5 ml) was added to 1.5 ml of colloidal chitin (10 mg/ml) prepared in 50 mM sodium acetate buffer (pH 6.8) in a test tube. To this, 0.1 ml potassium tetraborate was added and incubated at 100°C for 3 min. Tubes were cooled under tap water and 3 ml p-DMAB reagent was added and incubated at 36 to 38°C for 20 min. Tubes were cooled and OD was recorded at 585 nm using spectrophotometer. By using a calibration curve for N-acetylglucosamine, enzyme activity (U = 1 unit of chitinase) is defined as the amount of enzyme that releases 1 µmol of NAGA/ml (Reissig et al., 1995; Gadelhak et al., 2005). Enzyme activity was calculated by using the formula as mentioned earlier (Hoang et al., 2011; Brzezinska et al., 2014).

RESULTS AND DISCUSSION

Collection of soil samples and isolation of actinomycetes

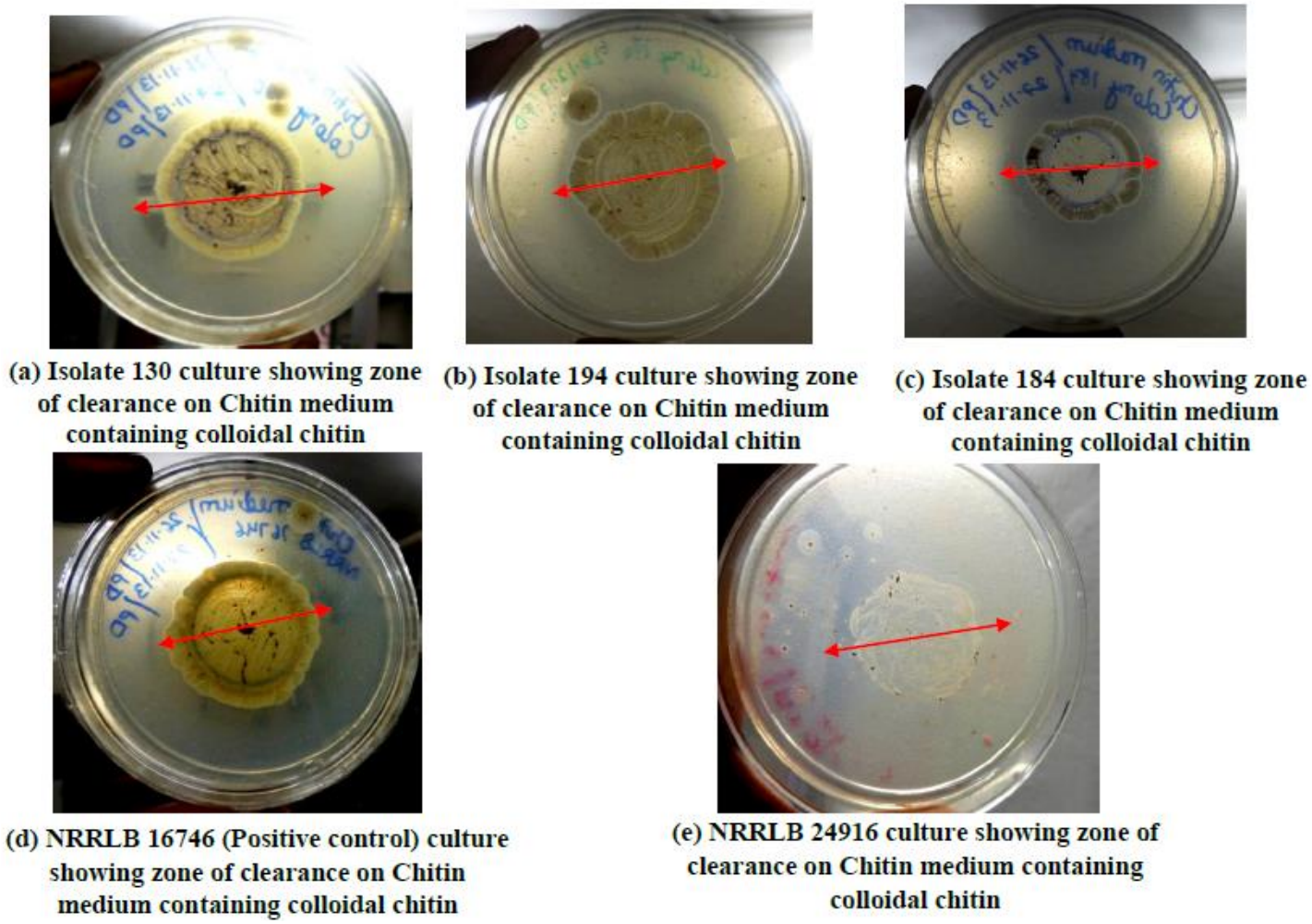
In the course of our study, a total of 260 actinomycete bacterial isolates were isolated. Isolates from diverse ecological habitats were subjected to primary screening to select actinomycetes capable of producing comercially important extracellular chitinase. Isolation of actinomycetes from varied ecological environments has also been reported by researchers for identifying producers of extracellular enzymes (Lekshmi et al., 2014; Mohanta, 2014).

Primary screening of isolates for production of chitinase

Among the 105 strains tested, 72% were found to be chitinase producers. Based on the results of primary screening, isolate no 130, 194, 184, *Streptomyces albidoflavus* (NRRLB 16746) and *Streptomyces mexicanus* (NRRLB 24916) that showed high chitinase activity and representing different ecological habitats were selected for further analyses. Comparison of chitinase activity of isolates is shown in Table 3 and Figure 1. The zone of clearance produced due to hydrolysis of chitin is shown in Figure 2.

The results obtained during primary screening were correlated to data reported in literature. Priya et al., 2011 isolated 36 *Streptomyces* strains and screened them for production of chitinase. Out of these, only 10 isolates showed clear zones of hydrolysis on colloidal chitin agar medium.

The strain VMCH2 showed the maximum zone of size 13 mm and in others, the zone size varied from 5 to 8 mm. A total of 58 actinomycetes were isolated from various habitats of Lucknow, India by Kuddus and Ahmad (2013). Isolates were then screened for their ability to produce chitinase enzyme. Based on the results, six isolates showing zones of clearance above 0.2 cm were selected for further studies. Similar work has been reported by Thirumurugan et al. (2015) and Wang et al.



(a) Isolate 130 culture showing zone of clearance on Chitin medium containing colloidal chitin

(b) Isolate 194 culture showing zone of clearance on Chitin medium containing colloidal chitin

(c) Isolate 184 culture showing zone of clearance on Chitin medium containing colloidal chitin

(d) NRRLB 16746 (Positive control) culture showing zone of clearance on Chitin medium containing colloidal chitin

(e) NRRLB 24916 culture showing zone of clearance on Chitin medium containing colloidal chitin

Figure 2. Plates showing zone of clearance of isolates due to production of chitinase enzyme.

Table 4. Chitinase enzyme activity and protein content in crude culture extract.

Culture	Absorbance at 585 nm	Concentration of NAGA (mg)	Protein content (mg/ml)	Protein content after equalization (mg/ml)	Enzyme activity (IU/ml)
Isolate 130	0.702	0.812	0.71	0.39	14.12
NRRLB 16746 (Positive control)	0.680	0.718	0.70	0.38	12.81
Isolate 194	0.542	0.598	0.55	0.35	11.6
Isolate 184	0.482	0.401	0.52	0.34	10.45
NRRLB 24916	0.29	0.212	0.33	0.33	7.16

(2015).

Secondary screening for chitinase activity

For quantitative analyses of chitinase activity in crude extracts, isolate 130, 184, 194 and *Streptomyces mexicanus* (NRRLB 24916) were selected. *Streptomyces albidoflavus* (NRRLB 16746) was taken as positive control for chitinase activity. The results are shown in Table 4.

The results obtained during secondary screening were compared to studies reported in literature. Chitinase activity in *Streptomyces* sp. strain A was estimated by

Table 5. Optimization of fermentation conditions (pH, temperature and substrate concentration) for highest chitinase producer (in crude), isolate 130 utilizing colloidal chitin as a substrate.

Cultures/ collection site	pH	Enzyme activity (*IU/ml)	Temperature (°C)	Enzyme activity (*IU/ml)	Substrate concentration (%)	Enzyme activity (*IU/ml)
Isolate 130 (Chemical Plant, Faridabad)	6.5	2.00	2	1.20	0.25	3.12
	7.0	5..89	25	16.14	0.5	14.22
	7.5	7.56	3	17.56	1	17.68
	8.0	16.28	35	17.05	1.5	17.12
	8.5	17.12	4	2.12	2	10.22
	9.0	0.9	45	0.16	2.5	4.01

Deepthi et al. (2012). The culture was inoculated in 50 ml of colloidal chitin broth, incubated for seven days at 30°C and centrifuged to obtain cell free extract. Strain A showed 1.356 µmol/ml/min of enzyme activity, 0.225 mg/ml protein content in crude. Kumaran et al. (2012) estimated enzyme activity in *Streptomyces* sp. CDB20. The colloidal chitin medium was inoculated with culture spores and incubated for 10 days at 28°C. Crude supernatant was obtained after centrifugation and specific activity was measured by using N-acetyl glucosamine as standard and protein content was measured by Bradford method. Activity shown by CDB20 was found to be 1.22 IU/ml.

Optimization of fermentation parameters (pH, temperature and substrate concentration) for enzyme production in crude extract

Based on the results of primary screening, isolate 130 was selected for secondary or quantitative analyses by submerged fermentation process at a range of temperature, pH and substrate concentration conditions to determine maximum enzyme activity. Isolate 130 showed maximum chitinase activity, 17.12 IU/ml at pH range of 8.0 to 8.5, 17.56 IU/ml activity at temperature range of 25 to 35°C and 17.68 IU/ml at substrate concentration range of 1 to 1.5% (Table 5 and Figure 3A to C).

As reported previously, Santhi (2016) performed optimization of fermentation conditions in crude extract for improving chitinase activity of *S. albus* FS2. The result showed the following: Maximum activity was observed on 5^{th} day of incubation (80 IU/ml), at temperature 37°C (82 IU/ml) and pH 8 (90 IU/ml). Similar results have been reported in *Streptomyces* sp. by Singh et al. (2008) and Subramaniam et al. (2012). The values for enzyme activity and protein content mentioned in aforementioned references was first multiplied by the total volume of the sample and then subsequently used for calculating specific activity. However, in the present study, activity and protein content values “per millimeter” have been reported.

Statistical analyses of enzymatic activity using SPSS software

Statistical analyses of fermentation conditions (pH, temperature and substrate concentration) using one way ANOVA showed that there is a significant effect of pH, temperature and substrate concentration on the enzyme activity shown by isolate 130. The values obtained were $F(5,12) = 66581.475$, $p = 0.000$ for pH, $F(5,12) = 137621.778$, $p = 0.000$ for temperature and $F(5,12) = 111638.172$, $p = 0.000$ for substrate concentration. This was also proved by Post Hoc test (Turkey HSD) analyses which demonstrated that there lies a statistically significant difference in the activity observed at different pH and temperature values. This means that with the increasing pH, temperature and substrate concentration, activity initially increases, attains a maximum level then gradually decreases to produce a bell shaped curve.

In a similar study, Gherbawy et al. (2012) analyzed the data recorded for chitinase activity in case of 7 actinomycete isolates by two-way ANOVA by using ‘Proc Mixed”. The level of statistical significance was checked with $P<0.05/P<0.01$. However, the results showed statistically no significant differences ($P<0.05$ or $P<0.01$) in the data.

Apart from using ANOVA/Post Hoc test (Turkey HSD) analyses, other statistical methods such as Plackett-Burman and response surface methodology can also be used to optimize the medium components and improve chitinase production from strains (Meriem and Mahmoud, 2017).

Purification of enzyme and enzyme assay in purified products

Purification by ion exchange chromatography

Chitinase enzyme activity and protein content were determined in the partially purified protein samples using NAGA and BSA standard curves, respectively as already mentioned in the analyses of crude extracts. It was found that protein content also increased after purification. For chitinase enzyme activity comparison,

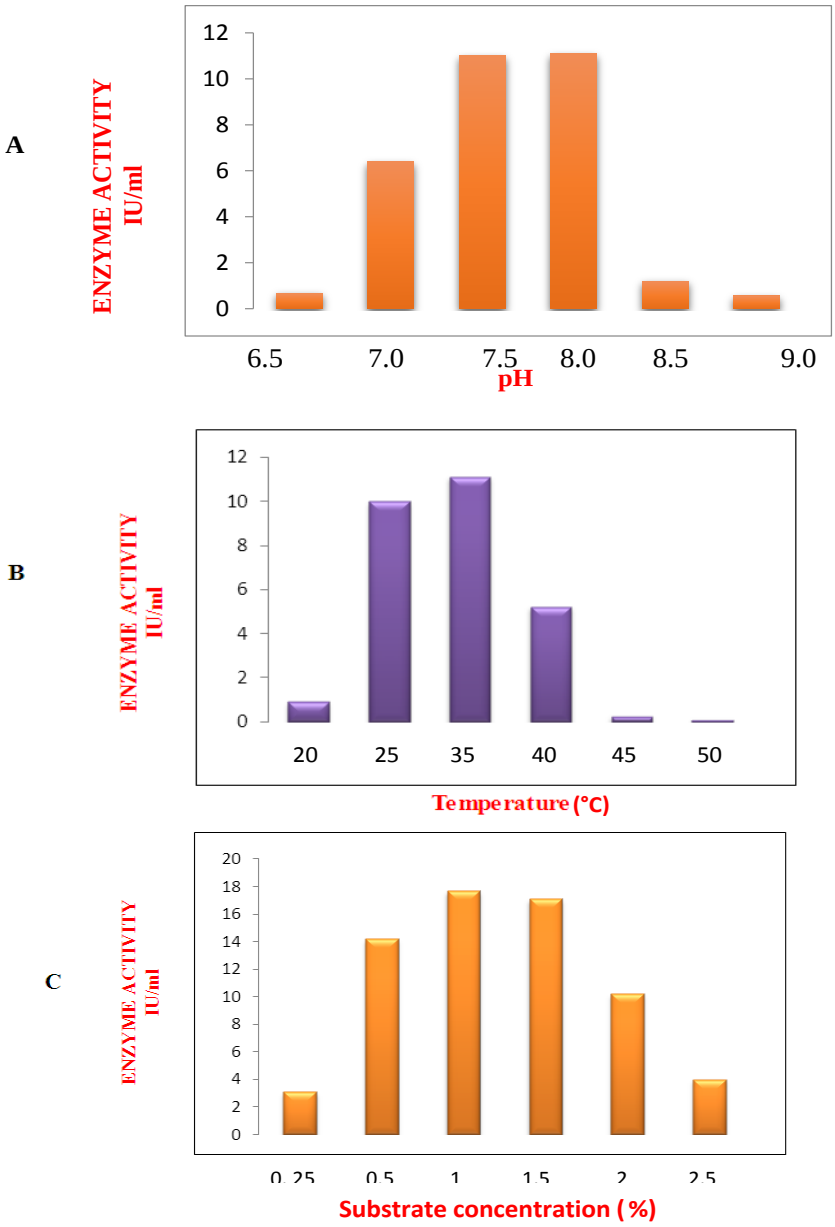


Figure 3. Comparison of fermentation conditions for highest chitinase producer (in crude at different pH (A), temperature (B) and substrate concentration (C).

protein content in all samples was equalized. Chitinase activity increased in culture extracts after purification as indicated by the higher values of released NAGA (Table 6 and Figure 4). Enzyme activity was found maximum in isolate 130, followed by NRRLB 16746, isolate 194, 184 and NRRLB 24916. Therefore, it can be concluded that isolate 130 is an efficient producer of extracellular chitinase enzyme in comparison to even the known chitinase producer like NRRLB 16746. The highest chitinase producing isolate 130 was selected for further purification by ion exchange column chromatography. Enzyme activity in purified fraction was found to be 32.12 IU/ml/min.

The results obtained after purification were compared with data reported in literature. Narayana and Vijayalakshmi (2009) performed a single-step purification of chitinase from *Streptomyces* sp. ANU 6277. The crude culture supernatant was 80% saturated with ammonium sulphate, dialyzed and then concentrated. The sample was loaded on Sephadex G-100 column. Activity and protein content in purified fraction was examined. Molecular size of purified fraction was estimated by SDS-PAGE. Total activity (U), total protein content (mg) and specific activity (U/mg) recorded in ammonium sulphate saturated sample was 3120 U, 118 mg and 26.4 U/mg, whereas in case of Sephadex G-100 purified sample it

Table 6. Protein content and chitinase enzyme activity in partially purified partially purified samples.

Culture	Protein content (mg/ml)	Protein content after equalization (mg/ml)	Enzyme activity (IU/ml) in partially purified	Enzyme activity as previously observed in crude culture extracts (IU/ml)	Enzyme activity in extract purified by ion exchange chromatography (IU/ml)
Isolate 130	1.071	0.59	23.10	14.12	32.12
NRRLB 16746 (control)	0.930	0.59	19.08	12.81	-
Isolate 194	0.813	0.569	13.19	11.6	-
Isolate 184	0.740	0.560	12.0	10.45	-
NRRLB 24916	0.572	0.553	12.1	7.16	-

*The highest chitinase producing isolate 130 was selected for further purification by ion exchange column chromatography.

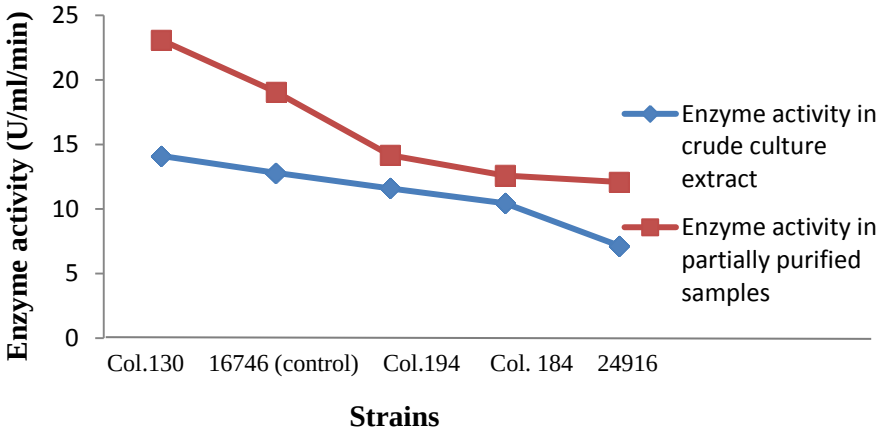


Figure 4. Comparison of chitinase enzyme activity of crude culture extracts and partially purified samples.

was found to be 1649 U, 27.5 mg and 59.9 U/mg. A single protein band of size approximately 45 kDa was obtained.

Mander et al. (2016) purified chitinase from *Streptomyces anulatus* CS242. The crude sample was precipitated by ammonium sulfate followed by dialysis and concentration. The resultant sample was purified using gel permeation chromatography with Sepharose CL-6B column. Protein content and chitinase activity was assayed in each fraction by Bradford and DNS method respectively. Total activity (U), total protein content (mg) and specific activity (U/mg) recorded in ammonium sulphate saturated sample was 5300659 U, 51.24 mg and 10356 U/mg whereas in case of Sepharose CL-6B purified sample it was found to be 270102 U, 10 mg and 27010 U/mg.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Partially purified samples (crude cell extract, dialyzed and concentrated) of isolate 130 were run on denaturing SDS-PAGE for observing the protein profile. Multiple bands of different sizes were observed in all crude extracts. However, number of bands started decreasing when the samples were dialyzed and concentrated. This showed the extent of purification in each case (Figure 5a). Purified fraction was also run on gel and showed presence of one band of approximately 65 to 70 kDa corresponding to purified chitinase enzyme (Figure 5b). The purified band from the gel was eluted and processed for further studies.

Molecular sizes of bacterial chitinases reported in literature were 28, 35 and 45 kDa from *Streptomyces* sp. NK 1057, 43 and 45 kDa from *S. albovinaceus* S-22(5), 49 kDa from *S. griseus* HUT 6037, 20 to 70 kDa, 38 kDa from *S. anulatus* CS242 (El-Sayed et al., 2000; Nawani and Kapadnis, 2004; Bhattacharya et al., 2007; Narayana and Vijayalakshmi, 2009; Mander et al., 2016).

Analysis of kinetic parameters

The K_m and V_{max} values of the purified fractions for isolate 130 were found to be 2.11 µm/ml and 53.11 mg/ml respectively (Figure 6). This shows that the enzyme have high affinity for the substrate and moderate turnover

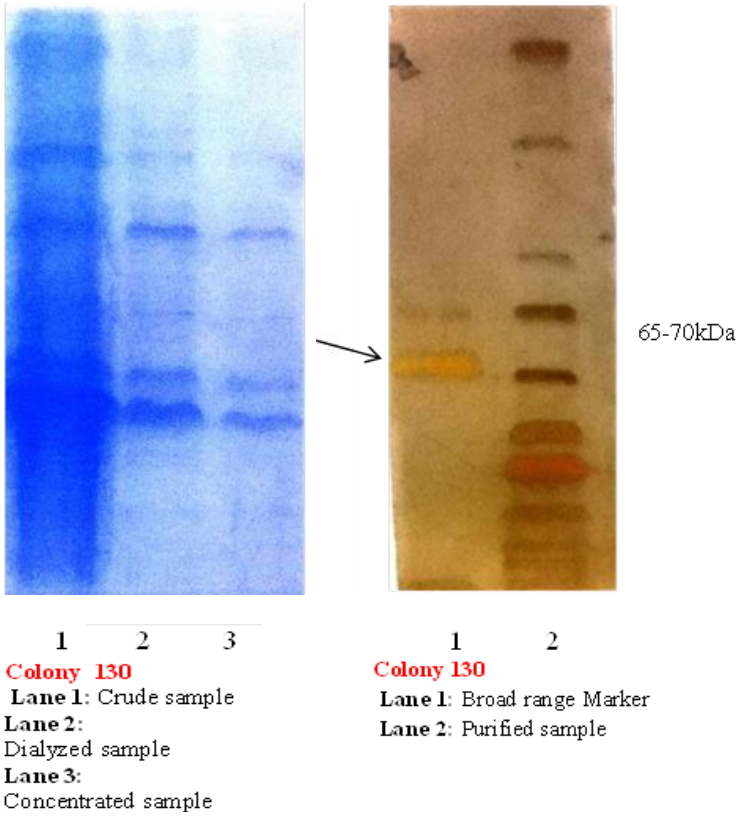


Figure 5. (a) SDS-PAGE profile of partially purified samples of isolate 130; (b) SDS-PAGE profile of isolate 130 purified fractions.

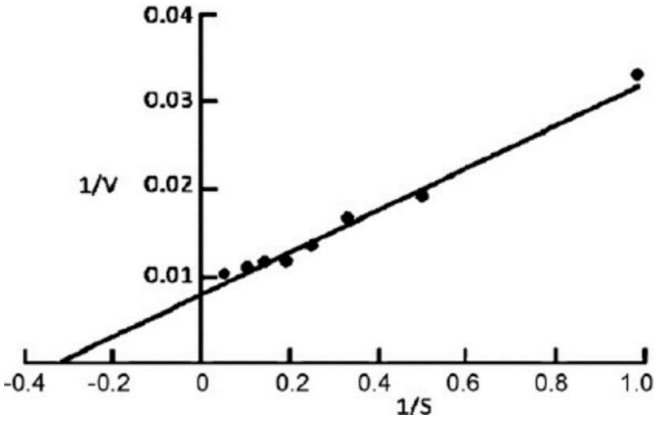


Figure 6. Lineweaer-Burk plot for isolate 130.

number.

The results obtained were compared with data reported in literature. Karthik et al. (2015) determined Km (6.74 mg/ml) and Vmax (61.3 U/mg) of purified chitinases produced by a *Streptomyces* sp. using colloidal chitin. Rabeeth et al. (2011) reported that the purified chitinase produced by *Streptomyces griseus* exhibited Km and Vmax values of 400 mg and 180 IU mL^{-1} respectively for colloidal chitin.

Characterization of enzymes by molecular approach

Protein identification by mass spectrometry

Sequences obtained after MS/MS for chitinase were assembled using MASCOT. ESI-MS spectra of isolate 130 are shown in Figure 7. Total assembled amino acid sequence obtained for isolate 130 had a length of 453 amino acids. The assembled sequences were then used for further analyses.

Analysis of structure and catalytic sites

For identifying the type of protein in isolate 130, the assembled amino acid query sequence was searched using pBLAST against Protein data base (PDB). The target sequence of isolate 130 showed high identity (70%) with chtA of family 18 from Streptomyces plicatus (PDB id: 1hp4). Multiple sequence alignment of N-terminal sequence of isolate 130 was done with sequences of known chitinase producing Streptomyces sp. using CLUSTALW software (Figure 8).

For constructing the structure for the enzymes, N-terminal sequences of isolate 130 were submitted in SWISS-MODEL. The software search the database for similarity with the query structure. The best suited

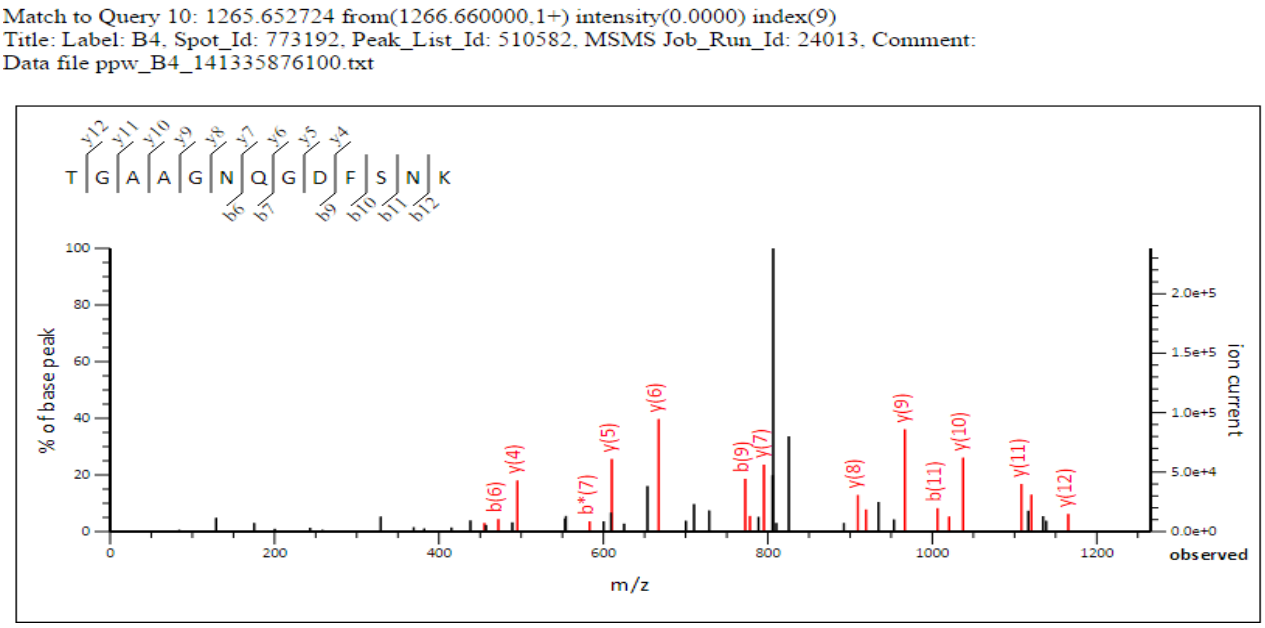


Figure 7. ESI-MS spectra for isolate 130.

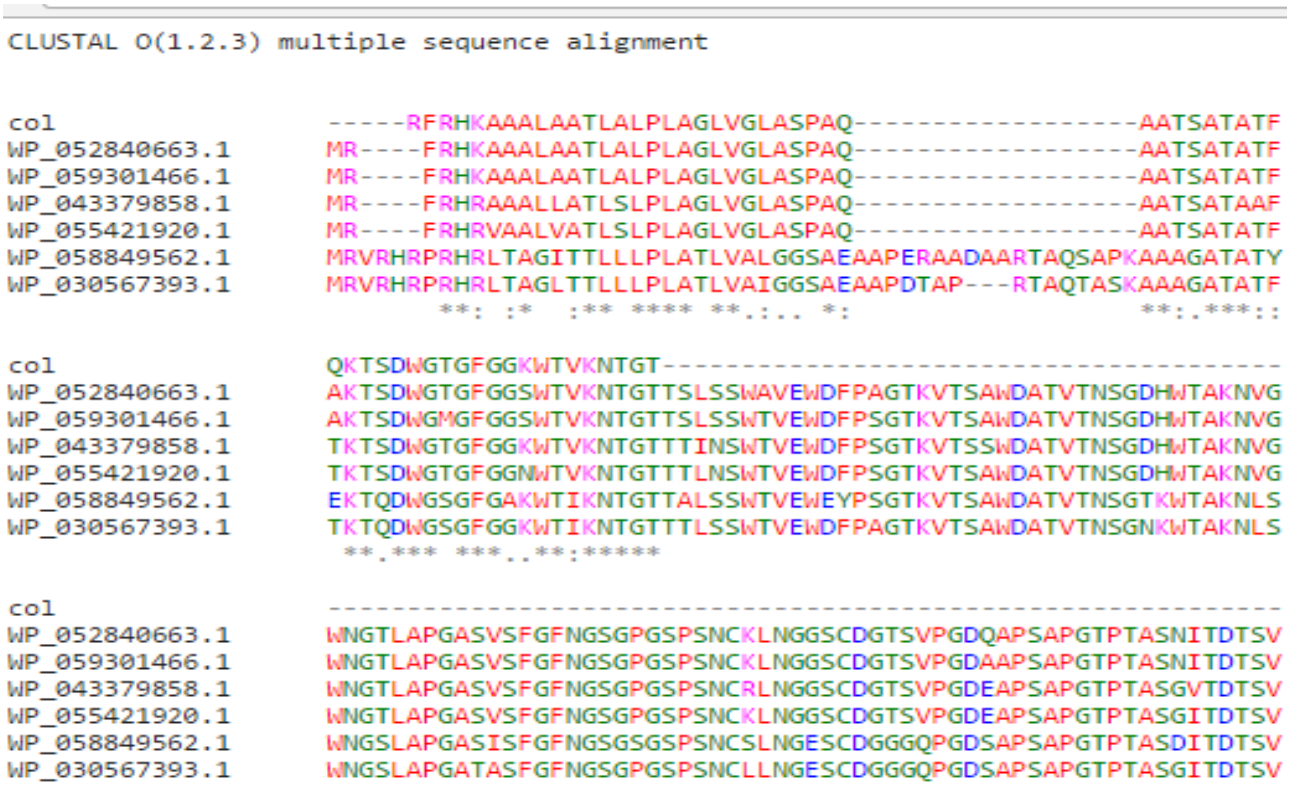


Figure 8. Multiple sequence alignment of target isolate 130 and template *Streptomyces plicatus (*PDB ID: 1hp4). The important residues from active site point of view are highlighted with red and hydrophobic residues with blue.

structure is chosen by the SWISS-MODEL and based on the sequence of that model a tentative three dimensional structure was made for the submitted sequence (Figures 9 and 10). The 453 amino acid residues of isolate 130

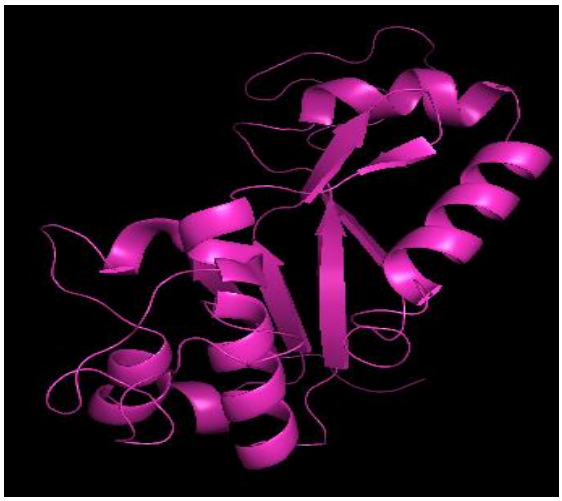


Figure 9. Modelled structure of isolate 130 using SWISS-MODEL.

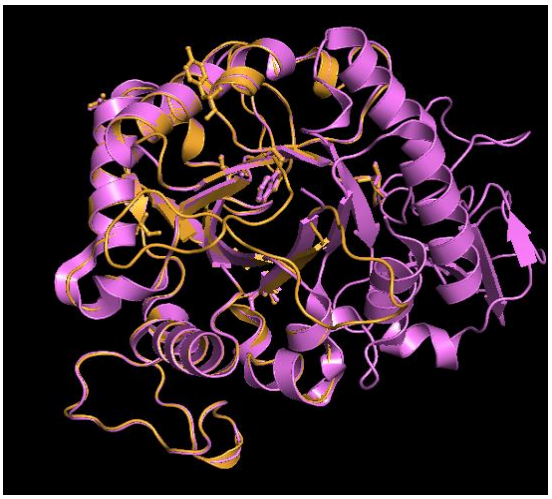


Figure 10. Superimposed modeled structure of isolate 130 by MODELLER (violet: 1hp4; orange: isolate 130).

were folded into a domain (β-jelly roll) structure comprising 3 antiparallel α-helices and five parallel β-sheets, like other enzymes belonging to the glycoside hydrolase family 18 (Watanabe et al., 1999, Hamid et al., 2013, Yan and Fong, 2015).

Ramachandran plot revealed that total residues in allowed region and additional allowed region were 89.8 and 9.5% respectively (Figure 11). The 453 amino acid residues of isolate 130 were folded into a domain of ((α/β)8 barrel) structure comprising of 3 antiparallel α-helices and five parallel β-sheets, similar to enzymes of the glycoside hydrolase family 18. Verify 3D comparison results for isolate 130 showed that in a 3D/1D profile, 88.75% of the residues had an average 3D-1D score >=0.2. The obtained data was also compared against protein database (PDB) using DaliLiteV3.1 server. In case of isolate 130, RMSD and Z score values for the top 5 matches were in the range of 0.7-0.9 and 24.9-28.3 respectively. This data further confirmed and validated the modeled structure obtained for the isolate.

Information about the active site was obtained through superimposing 3-D model structure of the target enzyme with that of template protein of chitinase from S. plicatus. This provided accuracy of homology between the structures, and also helped in positioning the conserved active site residues. Information related to active site of the template structure was obtained from Catalytic Site Atlas (CSA) data base of European Bioinformatics Institute. Overlapping of isolate 130 chitinase with the template placed the amino acid residues Aspartic acid (D) at position 477, Glutamic acid (E) at position 368, Tyrosine (Y) at positions 448, 475, 490, 524 and Arginine (R) at position 220. Combination of these amino acids constitutes the active site of the enzyme. Out of these, Glutamic acid (E) at position 368 and Tyrosine (Y) at position 524 were found to be shared by both isolate 130 and *S. plicatus* as shown in Figure 12.

Circular dichroism for protein secondary structure analysis

The far-UV CD spectrum of isolate 130 exhibited a pronounced maximum and minimum at 195 and 222 nm, respectively, which are characteristics of β-sheet and α-helix structures in aqueous solution, respectively (Figure 13). Thus isolate 130 chitinase is an autonomous structural protein that contains both α-helix and β-sheet secondary structures as predicted from homology modelling.

Results obtained in this study were compared with literature review. Mander et al. (2016) determined the N-terminal amino acid sequence of the purified enzyme of *S. anulatus* CS242 by the Edman degradation method. Sequence obtained was APGAPGTGAL. This was then searched against NCBI-BLAST database and it was found that chitinase from *S. anulatus* showed high degree of sequence similarity (80 %) with chitinase A1 from *Stigmatella aurantiaca* DW4/ 3-1, followed by enzymes from *Amycolatopsis mediterranei* S699 (70 %), *Streptomyces sp*. Mg1 (70%) and *Streptomyces* sp. AA4 (40%). Ubhayasekera and Karlsso (2012) performed homology modelling to determine the structure of chitinase producing *Streptomyces* sp. Mg1. Similar chitinase catalytic module structures were obtained from the Protein Data Bank (PDB), then superimposed and compared with the program O. Multiple sequence alignments were used to identify the best pair-wise alignment of the *Streptomyces sp.* Mg1 enzyme with that of *Lactococcus lactis* subsp. *lactis*. This pair-wise alignment was the basis of creating a homology model,

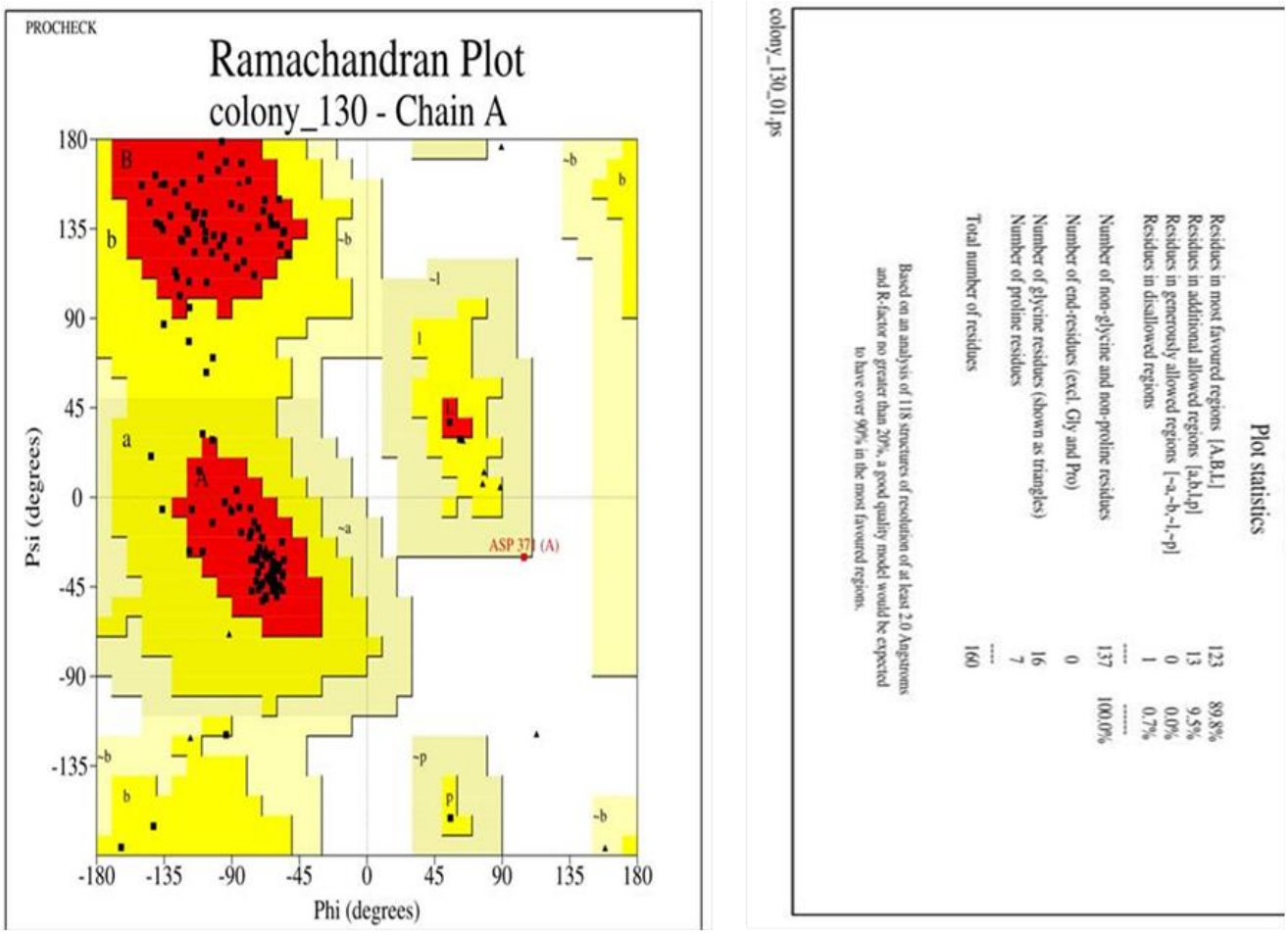


Figure 11. Ramachandran plot of modelled isolate 130 obtained by PROCHECK validation package.

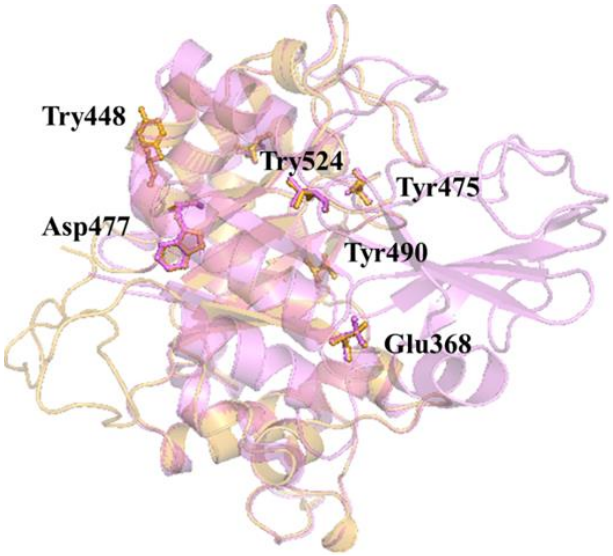


Figure 12. Superimposition of active site residues of modelled chitinase and template 1hp4. (Violet and orange color ribbons represents modelled and template proteins, respectively).

with PDB entry [PDB:3IAN] (*L. lactis* subsp. *lactis*) as the template in the program SOD. The model was adjusted in O, using rotamers that would improve packing in the interior of the protein. Homology modelling revealed that *Streptomyces* sp. Mg1 chitinase had a TIM barrel fold with six insertions and three deletions compared to the chitinase structure from *L. lactis* subsp. *Lacti.*

Role of extracellular enzymes in bioremediation of wastes

Primary and secondary screening of isolates for degradation of wastes

Results obtained during screening procedure showed that isolate 130 efficiently degraded crustacean (prawns, shrimp and crab) shells, with a zone of hydrolysis of 8 mm (Figure 14). The extent of degradation of commercial colloidal chitin by isolate 130 (zone of hydrolysis observed was 16 mm) versus its potential to degrade shell wastes has been shown in Figure 15.

For quantitative analyses of enzymatic activity, the

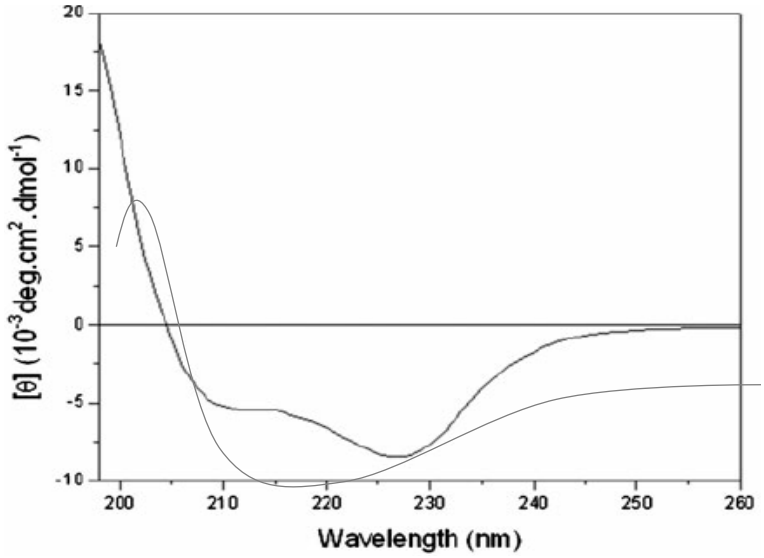
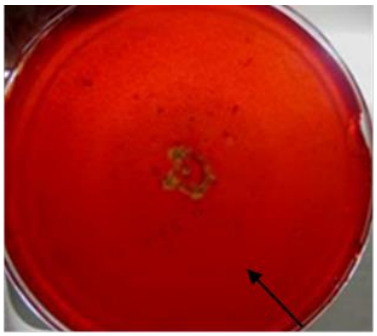


Figure 13. Far-UV CD spectra of isolate 130.



Isolate 130 (control) on CM + crustacean (prawns, shrimp and crab) shells (congo red staining)

Figure 14. Primary screening results for isolate 130.

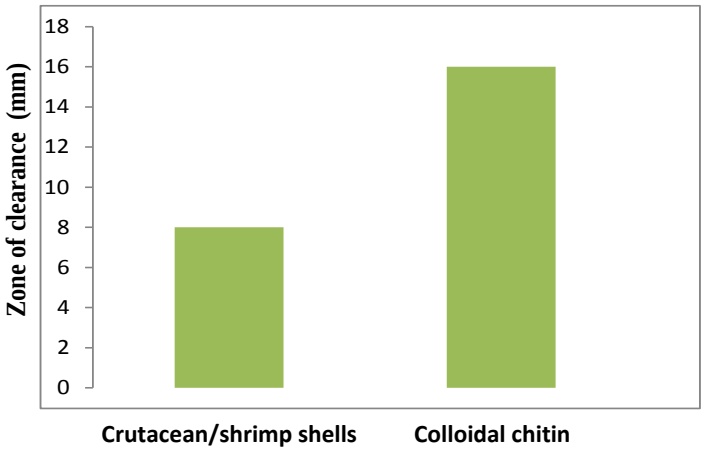


Figure 15. The extent of degradation of commercial colloidal chitin versus degradation of biodegradable waste.

production broth supplemented with 0.4% waste was inoculated with culture. Enzyme activity was measured under the solid state fermentation by using pDMAB method as mentioned in materials and methods. Activity observed was 9.66 IU/ml. As per literature, Brzezinska et al. (2014) reported efficient degradation (38.2%) of shrimp shells by *Streptomyces rimosus.* Similarly Hoang et al. (2011) reported the decomposition of shrimp shells by *Streptomyces* sp. TH-11 between 7 to 16 days. Similar results have been reported by Rabeeth et al. (2011).

Apart from chitin, there are many other derivatives which have large number of applications in industries. For example, chitosan, which is made by treating the chitin present in crustacean/shrimp shells with an alkaline substance. It can be used as biopesticide in agriculture, fining agent in winemaking, antibacterial agent in medicine and flocculent coagulant in removal of toxic metals etc. (Bouhenna et al., 2015, Ferhat et al., 2016).

For future studies, bacterial cultures with potential to degrade wastes can be converted into powdered form by lyophilization and can be packed in containers along with nutrient supplements. The sample can be dissolved in water to prepare a formulation and sprayed on to the waste materials for degradation. However, optimization of environmental parameters (pH, temperature and nutrients) is required to allow microbial growth and speed up the process of metabolism. Hence initially the *ex-situ* degradation of the waste samples can be done under controlled conditions.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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