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Isolation of *Bacillus* producing Chitinase from Soil: Production and Purification of Chito-oligosaccharides from Chitin Extracted from Fresh Water Crustaceans and Antimicrobial Activity of Chitinase

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Abstract

In the present investigation *Bacillus* sp. strain was isolated and screened from the red soil collected from Doiwala region of Dehradun (U.K), India. Serial dilution technique was adopted to isolate the organism and was screened for its chitinolytic activity. The biochemical tests were performed to prove its validity. The microorganism was also screened by inoculating a loop full of the isolated strain in basic cresol red dye and incubated for about 18- 24 h. The conversion of colour of the red dye into purple (pH, 6.5- 8.8) was taken as an indication for the presence of *Bacillus* sp. Amylase production by the organism was also screened by introduction of iodine in the broth/agar culture having starch. The broth/agar medium having starch but no bacterial strain was used as the control. The disappearance of color confirmed the presence of *Bacillus* strain producing amylase which degrades the starch. The chitinous wastes were collected from fresh water crustaceans viz. fresh water crab (*Potamon* sp.) and fresh water prawn (*Palaemon* sp.) and the chitin extracted was used as the substrate for chitinase. The yield of chitin extracted from fresh water prawn (*Palaemon* sp.) was found to be comparatively higher than that of chitin extracted from fresh water crab (*Potamon* sp.). Standard colloidal chitin was used as the reference control. The enzyme activity of chitinase for degradation of chitin extracted from crab and prawn was compared. The results confirmed that chitinase activity for degradation of crab chitin was comparatively higher than that of degradation of prawn chitin. The enzyme activities were found to be 0.11 µg/ml/minute and 0.09 µg/ml/minute for degradation of crab and prawn chitin respectively. The antimicrobial activity of chitinase extracted was determined against the bacterial and fungal cultures. Potent antibacterial activity of chitinase was observed against the bacterial cultures but no antifungal activity was observed. The chitinase produced by the species was able to degrade the chitin and chito-oligosaccharides produced was separated by TLC and purified by HPLC.

Key Words: *Bacillus*, Amylase producing strain, Chitin, Chito-oligosaccharides, Chitinase, Fresh water crustaceans, TLC, HPLC

Introduction

Chitin, a polysaccharide of animal origin, is obtained from waste material of seafood industries. It occurs in the skeletal material of crustaceans such as crabs, lobsters, shrimps, prawns and crayfish. Chitin is also the important component of exoskeleton of Arthropods Chitin is also forming the important composition of fungus. Chitin hold great economic value due to their versatile biological activities and chemical applications, mainly in medical [1, 2] and pharmaceutical areas [3, 4]. Chito-oligosaccharides and their N-acetylated analogues are useful for applications in various fields because they have specific biological activities such as antimicrobial activity, antitumor activity, immune-enhancing effects [5]. Some chito-oligosaccharides such as (GlcNAc) and (GlcNAc) have been reported to possess antitumor activity [6, 7]. Chitinolytic enzymes have been widely used in various processes including the agricultural, biological and environmental fields

[8]. Several chitinolytic enzymes have been identified in various *Streptomyces* sp., including, *Streptomyces plicatus* [9], *S. lividans* [10], *S. virdificans* [11] and *S. halstedii* [12]. The chitinases were purified and characterized from marine bacterium [13]. The potent chitinolytic activity of marine actinomycetes species and enzymatic production of chito-oligosaccharides was investigated [14].

Materials and Methods

All the materials, reagents and media used for the study were procured from Ranchem, CDH and Hi-Media, India.

Collection of chitinous wastes

The chitinous wastes of fresh water crustaceans viz. fresh water crab (*Potamon* sp.) and prawn (*Palaemon* sp.) were collected from the fresh water areas of Dehradun and Rishikesh (U.K), India and were washed properly in order to remove the sand debris present on the surfaces. The chitinous wastes were then after air dried and powdered material obtained was used as chitin.

Demineralization of chitinous wastes

The demineralization of chitinous wastes was performed [15]. The chitinous wastes were treated with 1.75 N acetic acid at room temperature for about 12-15 hours. The ratio of waste to solvent were maintained (1:15 w/v). The demineralized material obtained were recovered by filteration and rinsing with de-ionized water and will be dried in forced hot air oven at 65^0C.

Deproteinization and removal of lipids

The new and advanced methodology for deproteinization of proteins from demineralized chitinous wastes was designed by using deproteinization agents. This process can be performed either by using proteolytic enzymes such as proteinase-K dissolved in buffer containing 0.05 M Tris-base (pH, 6.5-9.1) in a ratio 1:20 (w/v) in flasks at various temperatures in incubator-shaker for about 72 h and adding mixture of solvents (phenol: chloroform, 1:1 ratio) again and again to the residue obtained and centrifuging the mixture until the residue gives no test for the presence of protein content. After repeating the procedure for 3-4 times, finally the residue was treated with 2N sodium hydroxide (1:25 w/v) at 70^0C for 1 hour. The lipid content gets dissolved in phenol: chloroform mixture and was removed from the chitinous wastes. Greese spot test can be performed in order to determine qualitatively the presence of lipid content if any present in the residual material. The residual materials left were dried in hot air oven at 60^0 C and percent yield of chitin extracted from fresh water crab and prawn was calculated.

Preparation of colloidal chitin

The colloidal chitin was prepared by using 1g of standard chitin, fresh water crab chitin and fresh water prawn chitin separately in 1N HCl for 2 h at room temperature. The colloidal chitin prepared of each of the samples was washed several times with large volume of distilled water to adjust the pH to 7.0.

Isolation of microorganisms

The red soil was procured from Doiwala region of Dehradun (U.K), India by performing the serial dilution method and maintained on glycerol yeast medium plates at 37°C.

Screening and culture conditions

For the screening purpose, strain was inoculated in 100 ml of medium (3% w/v chitin, 0.1% KH_2PO_4, 0.05% $MgSO_4.7H_2O$, 50 mM Sodium Phosphate buffer, pH 6.0) in a 250 ml Erlenmeyer flask at 30°C. Further basic dye cresol red was added in a flask and kept for 18- 24 h. The conversion of colour of the red dye into purple (pH, 6.5- 8.8) was taken as an indication for the presence of *Bacillus* sp. Further biochemical tests such as amylase production/starch hydrolysis assay were performed in order to confirm the strain as *Bacillus* sp. Gram staining confirmed the strain as gram positive.

Chitinase Production

For the production of chitinase, strain was grown in 100 ml of fresh medium (3% w/v chitin; 0.1% KH_2PO_4; 0.05% $MgSO_4.7H_2O$; 50mM Sodium Phosphate buffer, pH 6.0) in a 250 ml Erlenmeyer flask at 30°C. For reflecting the growth of the culture in this medium OD at 660 nm was taken using blank as medium in which no inoculum was added. The supernatant (enzyme) was collected from 3 day old cultures by centrifuging the mixture at 12,000 g for 20 minutes. The enzyme was concentrated by condensing the solution in order to reduce its volume.

Measurement of enzyme activity

Chitinase activity was measured separately with colloidal chitin, fresh water crab chitin, fresh water prawn chitin as the substrate. Enzyme solution (0.5 ml) was added to 1.0 ml of substrate solution, which contained a 1.5 % suspension of each of the colloidal chitin prepared in a phosphate buffer (50 mM, pH 6.0) separately and the mixture were incubated at 37°C for 15 minutes. After centrifugation, the amount of reducing sugars produced in the supernatant was determined by the Dinitrosalicylic acid method for estimation of reducing sugars [16] using N-acetyl glucosamine as a reference compound [17]. One unit of chitinase activity was defined as the amount of the enzyme that produced 1 µmol of reducing sugar per minute.

Determination of antimicrobial activity of chitinase produced from strain

The antimicrobial activity of chitinase produced from *Bacillus* strain was screened for its antimicrobial activity against some microbial strains viz. *E.coli*, *S.pyogenes*, *Klebsiella pneumoniae, Lactococcus* sp., *A. niger*, *C. albicans* and *S. cerevisae* by well diffusion method [18]. The pure cultures of test microorganisms were procured from National Chemical Laboratory (NCL), Pune, Maharashtra, India. Nutrient agar medium/broth was used for the growth of bacterial cultures while Sabouraud's dextrose agar/broth was used for the fungal cultures. The wells were punctured in the agar plates with sterile borer and 10^5 cfu/ml of the bacterial and fungal cell suspension were introduced in the plates separately. The enzyme supernatant was introduced in the wells in each of the bacterial and fungal plates. The plates were left free for the thorough diffusion of the enzyme supernatant within the medium plates and were kept for 18-24 h and 72 h at 37^0 C for bacterial and fungal cultures respectively. The diameter of zone of inhibition observed was recorded.

Preparation of Chito-oligosaccharides

For the preparation of chito-oligosaccharides, 1% colloidal chitin prepared from standard chitin, fresh water crab and fresh water prawn were dissolved in 0.05M Phosphate buffer (pH, 5.5). About 10 ml of enzyme was added in 15 ml of 1% of each of the colloidal chitin in a 100 ml flask; further flask was kept at 30°C for 3 h. The reaction was terminated by immersing the tubes in boiling water for 5 minutes. After performing centrifugation at 3000 rpm, the insoluble materials were collected and condensed to obtain the chito-oligosaccharides.

Analysis of Chito-oligosaccharides by TLC

The TLC analysis of chito-oligosaccharides obtained from standard chitin, freshwater crab chitin and fresh water prawn chitin was performed by using the solvent system n- butanol: methanol: concentrated ammonia: water in the ratio of 5:4:2:1. The chromatogram was developed in the iodine chamber for the appearance of the spots and calculation of R_f values.

Analysis of Chito-oligosaccharides by HPLC

The purified compound obtained through gradient column chromatography and TLC was subjected to HPLC analysis. HPLC of the compound was performed in National Institute of Malaria Research, Hardwar (Uttarakhand), India using a Shimadzo LC- 2010 HPLC system (Kyoto, Japan), equipped with a Shimadzo LC 2010 UV-VIS detector with a thermostated flow cell. The detector signal was recorded on a Shimadzo LC2010 integrator. The column used was a C-18 block heating-type Shim-pack VP-ODS (4.6 mm interior diameter × 150 mm long) with a particle size of 5 µm. Then, a 20-µl of sample was chromatographed using linear gradients of $CH_3CN–H_2O$ from 70% to 55% in 30 minutes at a flow rate of 1 ml/minute. The oligosaccharides were monitored at 205 nm with a spectrophotometric detector.

Results and Discussion

Extraction of chitin from fresh water crustaceans

The chitin was extracted from fresh water crustaceans according to the methodology designed. The percent yield of chitin extracted from fresh water prawn (*Palaemon* sp.) was comparatively more in comparison to fresh water crab (*Potamon* sp.). The yield of chitin extracted from fresh water prawn and crab was found to be 80 and 70 % respectively. The results of percent yield of chitin extracted are reported in Table 1;Fig.1.

Table 1: Percent yield of Chitin extracted from fresh water crustaceans

S.No.	Sample	% Yield
1.	Fresh water prawn (*Palaemon* sp.)	80
2.	Fresh water crab (*Potamon* sp.)	70

Figure 1: Percent yield of Chitin extracted from fresh water crustaceans

Isolation and screening of microorganism from soil

The isolation of the strain was performed by serial dilution of the soil sample and maintaining the culture on glycerol-yeast medium plates. The microorganism was also screened by inoculating a loop full of the isolated pure single colony in basic cresol red dye and incubated for about 18- 24 h. The conversion of colour of the red dye into purple (pH, 6.5- 8.8) was taken as an indication for the presence of *Bacillus* sp. Further amylase production/starch hydrolysis assay was performed for its further confirmation. Amylase production by the organism was screened by introduction of iodine in the broth/agar culture having starch. The broth/agar medium having starch but no bacterial strain was used as the control. The disappearance of color confirmed the presence of *Bacillus* strain producing amylase which degrades the starch. Thus no purple coloured complex was formed on combination with iodine. The results for the screening of the isolated strain are represented in Fig. 2 (a) & (b).

Figure 2 (a): Screening of *Bacillus* sp. by using cresol red dye and bromophenol blue (the first photograph is of control medium with dyes separately in the test tubes with no inoculum while the second photograph is final having medium with dyes separately with the inoculum after 18- 24 h. The change in the colour of the plates is because of change in pH due to the presence of *Bacillus* strain)

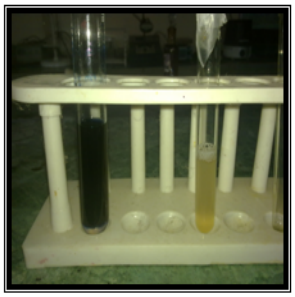

Figure 2 (b): Amylase production/ starch hydrolysis assay of *Bacillus* strain (first test tube is the control tube having no inoculum in the starch-broth after addition of few drops of iodine, the second tube showed the disappearance of colour due to the starch hydrolysis by amylase produced by the strain after 18-24 h incubation).

Measurement of enzyme (chitinase) activity

The specific activity of chitinase (µg/ml/minute) was determined by using colloidal chitin prepared from standard chitin, fresh water prawn chitin and fresh water crab chitin as the substrates separately. Standard linear plot was prepared by taking aliquots of N-acetyl glucosamine as standards and the concentration of N-acetyl glucosamine obtained as the product after degradation of colloidal chitin from each of the substrates was determined by DNSA method. Enzyme activity obtained after degradation of standard chitin was 0.087µg/ml/minute while in case for degradation of fresh water crab chitin was 0.11 µg/ml/minute and in case of degradation of fresh water prawn chitin was 0.09 µg/ml/minute. The results for specific activity of chitinase for degradation of chitin extracted from fresh water crab were found to be comparatively higher than that of the specific activity of chitinase for degradation of prawn chitin. The results of specific activity of chitinase are represented in Table 2 and Fig. 3.

Table 2: Specific activity (µg/ml/minute) of chitinase used for degradation of chitin extracted from fresh water crustaceans

S.No.	Sample	Specific activity (µg/ml/minute)
1.	Fresh water prawn (*Palaemon* sp.) chitin	0.09
2.	Fresh water crab (*Potamon* sp.) chitin	0.11
3.	Standard chitin	0.087

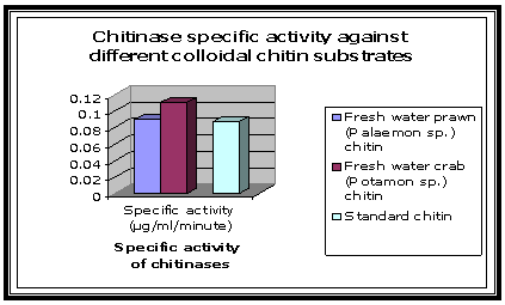

Figure 3: Specific activity (µg/ml/minute) of chitinase used for degradation of chitin extracted from fresh water crustaceans

Antimicrobial activity of chitinase produced from strain

The antimicrobial activity of chitinase produced from *Bacillus* sp. was determined *in vitro* by well diffusion method. The enzyme purified showed potent activity against the bacterial cultures but no activity was observed against the fungal test cultures. Amongst the test bacterial cultures the chitinase showed maximum inhibition against *S.pyogenes* (diameter of zone of inhibition: 26 mm) followed by *K.pneumoniae* (diameter of zone of inhibition: 25 mm); *Lactococcus* sp. (diameter of zone of inhibition: 23 mm) and *E.coli* (diameter of zone of inhibition: 21 mm). Our results strictly contradict the findings [19-21] which confirmed the antifungal activity of chitinase. Although the previous investigations on the antifungal activity of chitinase were against the phytopathogenic fungi. We have used the fungal test cultures such as *Aspergillus niger* and *Candida albicans* which are dreadful to animals and humans for determination of antifungal activity of chitinase. Thus from the present study it can be said that chitinase are acting as antifungal agents against phytopathogenic fungi and not against all clinical isolates. The results of antimicrobial activity of chitinase are reported in Table 3 and Fig. 4.

Table 3: Antimicrobial activity of chitinase produced from the strain

S.NO.	Diameter of zone of inhibition (mm)						
	ECO	KPN	SP	LAC	CA	AN	SC
Chitinase	21.0	25.0	26.0	23.0	NA	NA	NA

ECO, *E.coli*; KPN, *K.pneumoniae*; SP, *S.pyogenes*; LAC, *Lactococcus* sp;
CA, *C.albicans*; AN, *A.niger*; SC, *S.cerevisae*.
NA, No activity.

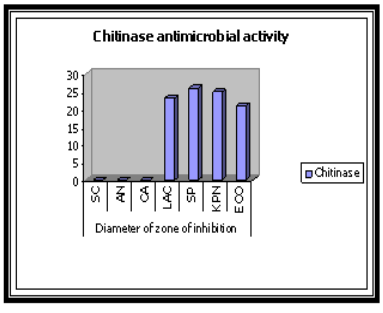

Figure 4: Antimicrobial activity of chitinase produced from the strain

Analysis of Chito-oligosaccharides by TLC

The chito-oligosaccharides obtained were analyzed by TLC coated by Silica gel G-250 and Rf values of standard, prawn and crab chito-oligosaccharides were determined by using Iodine chamber for the development of colour. Almost similar R_f values of all the extracted chito-oligosaccharides were observed. R_f values obtained in case of standard chitin (chito-oligosaccharide) was 0.59 while in case of fresh water crab chito-oligosaccharide and fresh water prawn chito-oligosaccharide, it was found to be 0.59 and 0.61 respectively. The results of TLC of chito-oligosaccharides are represented in Fig.5.

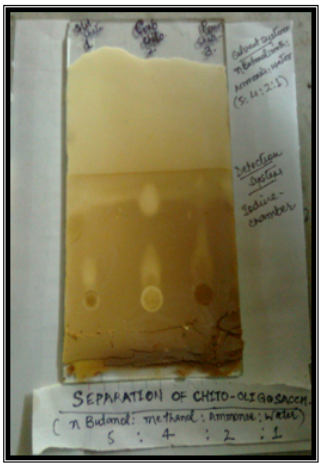

Figure 5: Separation and analysis of chito-oligosaccharides by TLC

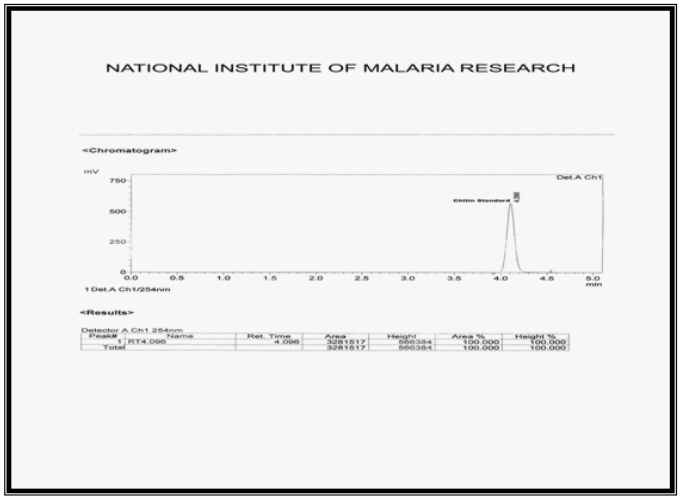

Figure 6 (a): Separation of standard chitin as revealed through HPLC chromatogram

Analysis of Chito-oligosaccharides by HPLC

The chito-oligosaccharides, chitin and chitosan (deacetylated form of chitin) were separated and purified by HPLC using the suitable solvent system. The value of retention time of extracted chitin was compared with that of standard. The results indicate that extracted chitin had almost similar value of retention time as that of standard chitin. The results of analysis and separation of chito-oligosaccharides are represented in Figure 6 (a) and (b).

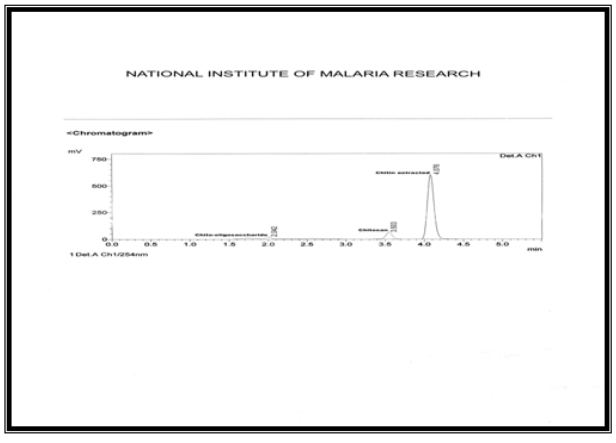

Figure 6 (b): Separation of extracted chitin, chitosan and chito-oligosaccharides as revealed through HPLC chromatogram

Conclusion

From the present investigation it is confirmed that strains isolated from soil such as *Bacillus* have chitinase activity and thus the enzyme extracted from these strains can be used as a catalyst for the degradation of chitin which is an abundant polysaccharide after starch. The enzyme chitinase can be immobilized to retain its activity for prolonged period and can be used commercially for the preparation of chito-oligosaccharides which have pharmacological activity [5-7]. Chitinase/chitinolytic enzymes have been widely used in various processes including the agricultural, biological and environmental fields [8]. Chitinase are acting as potent

antifungal agents [19-21]. From our studies it is revealed that chitinase has potent antibacterial activity. Thus there is a need to commercialize the production of chitinase/ chitinolytic enzyme from such microbial strains and production of chito-oligosaccharides which have beneficial aspects in pharmaceutical and food industries.

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