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Production and Characterization of Partially Purified Chitosanase Enzyme of *Bacillus cereus* A4/B4 Isolated from Biowaste Soil Samples: Bioactive Chitooligosaccharide

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Abstract

Microbial chitosanase has received the attention of producing chitooligosaccharides. In this study, Chitooligosaccharide was developed by chitosanase enzyme hydrolysis and evaluated for its bioactivity. Chitosan degrading *Bacillus cereus* was isolated from biowaste sample and its extracellular enzyme chitosanase was characterized. The chitosanase enzyme production was induced by chitosan substrate and the enzyme production reached the maximum in 72 hours. The optimum pH and temperature for the production was 6.5 and 30°C respectively. The culture medium with 1.0 to 1.5% of substrate level with xylose served as significantly for enzyme production. The molecular weight of the enzyme 43KDa was determined by SDS-PAGE. The chitooligosaccharide produced by chitosanase hydrolysis inhibited the growth of the pathogens and showed MIC_{50} as 0.2 µg/ml against all the test pathogens and has proved to be a potential antimicrobial agent against skin and other infections.

Keywords: Bacillus Species, Bioproduction, Chitosanase, Chitooligosaccharides, Microbial Enzymes

1. Introduction

Chitooligosaccharides from chitosan and chitin have various versatile functional properties including water solubility, antimicrobial activities and antitumor activity. Chitooligosaccharides obtained by the chemical conversion of chitosan lead to low yield, short chain oligosaccharides, high cost of separation and environmental pollution. The enzymatic conversion of the chitosan is the advantageous method due to the environmental compatibility, low cost, reproducibility, mild condition and controllable conditions [1].

Many chitosanases have been isolated from microorganisms like bacteria, fungi, actinomycetes. Chitosanases belong to glycosyl hydrolase family acting on β1, 4 glycosidic linkage of chitosan to depolymerize into chitosanoligosaccharides. According to the similarity of amino acid sequence chitosanase are grouped into five glyco-

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syl hydrolase (GH) families such as GH5, GH 8, GH46, GH75,and GH 80.Mostly bacterial chitosanses belong GH 45 family and fungal chitosanase belong GH 75 [2].

Chitosanolytic enzymes are recently getting attraction as it depolymerise chitosan to chitosanoligomers which has varied applications in the field of medicine, biotechnology and agriculture. The utilization of chitosanoligosaccharide obtained by chitosanase hydrolysis is limited due to the higher cost of production and purification, unavailability of the enzymes in bulk quantity [3, 4] and so for the extensive application of the chitooligosaccharide enzyme hydrolysate could be used [5].

Hence the present work was focused on the preparation of chitooligosaccharides from chitosanase enzyme of the strain isolated from the bio waste soil sources.

2. Materials and Method

Chitosan (high M.W> 75% deacetylated), D-Glucosamine, 3,5 Dinitrosalicylic acid and CM Cellulose are purchased from Sigma Aldrich (Mumbai).The multi drug resistant pathogens were procured from KMCH Hospitals, Coimbatore, and CMC Vellore, Tamilnadu.

2.1 Screening of Chitosanase Producing Organisms

The bio waste dumped soil samples Chicken feather dumped soil, Agriculture land soil, Garden soil, Horn decomposed soil, Market waste decomposed soil, Egg shell decomposed soil, Fish market waste soil, Cockroach feathers dumped soil, Poultry waste dumped soil, Domestic waste dumped soil were collected from various places in a sterile condition were collected from different areas of Kerala and Tamil Nadu. These samples were taken from the depth of 10-15 cm and were brought to the laboratory for further processing.

One gram of collected soil sample was weighed and mixed with 5ml of sterile distilled water. From the suspension 0.1ml was spread plated on to the CDA [6] plates having basal medium M9 along with (0.5% ,1% ,2%) substrate chitosan or colloidal chitosan of 0.5% and 1% [7-9], Minimal Salt Chitosan (MS) medium [10, 11], Minimal Synthetic (MSM) Medium [12] and all the plates were incubated at 30° C for 3-5 days.

The Chitosanase activity was indicated by the clear zone formed on the CDA plates. The strains with big clear zone was selected and named as B1, B2 , B3…….

The selected strains were cultivated on CDA broth at 30° C for 5 days. The culture broth was centrifuged for 15 min at 6000 rpm; the supernatant crude enzyme was used for the enzyme analysis. The strains shown the highest chitosanase activity was selected for further study.

2.2 Characterization and Identification of the Isolates

The selected strains were identified by the morphological, physiological and biochemical characteristics. PCR amplification of 16S rDNA coding region was performed for the molecular characterization. The nucleotide sequence data was deposited in Gene Bank and obtain an accession number was obtained.

2.3 Optimization of the Chitosanase Production

For the commercial production of the chitosanse enzyme the fermentation conditions and medium were optimized.

2.4 Effect of Incubation Time and Substrate Concentration on Chitosanase Production

To infer the optimum incubation period for chitosanase production, the flasks were incubated at 30° C temperature for about eight days. Every 24 hours the enzyme activity was checked. The production medium prepared with different substrate concentration of 0.5%, 0.6%, 0.8%, 1%, 1.5%, 2% and then all the flasks were incubated with inoculating the strains. The enzyme activity was checked every day

2.5 Effect of pH and Temperature on Chitosanase Production

The CDA broth was taken at various pH (4, 5, 6, 7, 8) and the selected strains was inoculated and were incubated at room temperature for 8 days. Every 24 hours, the enzyme activity was measured. For temperature standardization, the culture was inoculated and incubated at various temperatures (27° C, 30° C, 45° C, and 55° C) for eight days. Every 24 hours the enzyme activity was measured.

2.6 Effect of Carbon Source and Nitrogen on Chitosanase Production

After selecting the suitable pH and temperature, various carbon sources(glucose, sucrose, fructose, xylose,

D-glucosamine, Glycerol, starch) and various nitrogen sources (Peptone Yeast Extract, Ammonium Chloride (NH_4Cl), Ammonium nitrate (NH_4NO_3)) were tested for the maximum activity Every 24 hours the enzyme activity was measured by DNS method for 8 days.

2.7 Enzyme Analysis (DNS- Miller Method)

Chitosanase activity was determined by quantitative estimation of reducing sugar during the hydrolysis of chitosan with 3, 5 Dinitrosalisalic acid. The crude enzyme solution of 0.5ml was mixed with 0.5ml of chitosan (pH 6.0) as substrate and the mixture was incubated in water bath at 55° C for 30 minutes. After incubation, 3ml of 3, 5-Dinitrosalisalic acid was added and to stop the reaction the mixture was immersed in boiling water for 3 minutes and was centrifuged to remove the insoluble chitosan. The reducing sugar was measured in UV Spectrophotometer at 540 nm. One unit of chitosanase was defined as the amount of enzyme that could liberate 1 µmol of reducing sugar per minute, with D-Glucosamine as the standard.

2.8 Estimation of Protein Content

The protein content of the crude extract was estimated by Lowry *et al*. [13] method with bovine serum albumin as standard calibration.

2.9 Partial Purification of Enzyme: Ammonium Sulfate Fractionation and Dialysis

Ammonium sulphate was added in increments to a concentration of 100% of saturation while gently stirring and allowed to dissolve and equilibrate between additions. The precipitated proteins were regimented by centrifugation for 15min. The resulted pellet was dissolved in 5ml of phosphate buffer at (pH 7.0). The supernatant was mixed again with ammonium sulfate to achieve 100% (w/v) saturation and was dialysed and used for SDS-PAGE analysis.

2.10 Determination of Molecular Mass by SDS-PAGE

Ammonium sulfate precipitated fractions molecular weight was further characterized by SDS-PAGE was performed in 10% polyacrylamide gel with 0.1% SDS according to Laemmli [14] protocol.

2.11 Preparation of Chitooligosaccharide

Chitooligosaccharides are prepared by the hydrolysis of chitosan by partially purified chitosanase enzyme.1ml of the enzyme solution was dissolved in 1ml 0f 0.05M acetate buffer (pH 5.0) and added to 1ml of 1% chitosan. Then mixture was incubated for 30 minutes at 55°C. The reaction was stopped by heating at 100°C for 5minutes and after cooling the mixture was mixed with 0.25M of sodium hydroxide and was centrifuged for 20 minutes at 1,000 X g. The supernatant chitooligosaccharides was collected [15]. A portion of the mixture was mixed with concentrated alkali and precipitate formation was observed [16]. Then the aliquots were lyophilized.

2.12 Assessment of Solubility

Chitosan oligosaccharide solubility was evaluated at concentration of 5mg/ml in different solvents including water, ethanol, diethyl ether, ethyl acetate and glacial acetic acid at room Temperature [17].

2.13 Antimicrobial Activity of Chitooligosaccharides

The multi drug resistant pathogens *Escherichia coli*, *Pseudomonas* sp, *Staphylococcus aureus* were procured from KMCH Laboratories, Coimbatore and CMC, Vellore, Tamilnadu. Overnight broth cultures of *Escherichia coli*, *Pseudomonas* sp, *Staphylococcus aureus* was used to check the antimicrobial activity. The pathogens were swabbed on the muller hinton agar plates and 3mm well was marked. Then the enzyme solution, Chitosan and Chitooligosaccharides at various concentrations (10-100µl) was added into the well and incubated at 37°C for 24 hours.

2.14 Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The determination of the MIC and MBC values for each strain was evaluated using broth dilution method. The chitooligosaccharide samples were diluted to give the final concentrations of 0.25, 0.5, 1, 2, 4, 5, 10, 15, 20, mg/ml. About 100 µl of 10^5 CFU/ml of the test culture was inoculated in tubes with equal volume of nutrient broth and chitooligosaccharide samples. The tubes were incubated aerobically at 37° C for 24 hours. Two control

tubes were maintained for each strain (media control and organism control). The dilutions that showed no turbidity were incubated further for 24h at 37°C. The lowest concentration that produced no visible turbidity after a total incubation period of 48h was regarded as final MIC. MBC value was determined by sub culturing the test dilution which showed no visible turbidity on to freshly prepared nutrient agar media. The plates were incubated further for 24-48 hours at 37° C. The highest dilution that yielded no single bacterial colony on the nutrient agar plates was taken as MBC.

2.15 Statistical Analysis

The analysis was performed in triplicates and average values are reported. All data were expressed as mean ± standard deviation and significance were analysed using SPSS software package.

3. Result and Discussion

3.1 Screening of Chitosanse Enzyme Producing Organism

The Bio waste soil samples from various places were collected and aseptically transferred to the laboratory. 1ml serially diluted soil sample was taken and spread plated on to CDA(M9 medium with 0.5% and 1% chitosan), M9 medium with 1% colloidal chitosan, Minimal Salt chitosan medium(MS) with 0.5% chitosan, Minimal Synthetic Medium (MSM) . Growth was observed only in CDA plates of chitosan 0.5% and in 1% plates after 3 days.

0.5% soluble chitosan was used (Shaida *et al.*,2012) for the isolation of chitosan degrading organism and the microbial colonies with large halos was screened on M9 chitosan agar (CDA) [17, 18]. Crispinus *et al.* [19] isolated the chitosanase producing organism with 2% chitosan. but no growth was observed in the present study (Figure 1). The chitosanase producing organism was isolated from soil sample forming a clear zone of halos on 1% colloidal chitosan medium [8, 9] and 2% colloidal chitosan [7] whereas no growth was observed in M9 medium with 0.5% and 1%colloidal chitosan medium, Minimal salt (MS) medium along with 0.5% chitosan, Minimal Synthetic medium and Enriched medium.

Among the forty samples taken for studies the chicken feather and domestic waste dumped soil have shown to be the prominent chitosanase producers. The highest zone producing four bacterial isolates were selected as the chitosanase producers and was named as B4, B5, B16, and B23. The strain B4 was selected as it showed the highest chitosanase activity. Based on the taxonomic identification, the morphological and biochemical characteristics, the strain resembled *Bacillus* sp. The strain was then designated as A4/B4 and the nucleotide sequence of 16SrRNA studies was submitted to Gene bank with the accession number KR063701. The isolate A4/B4 was designated as *Bacillus cereus* A4/B4 .

3.2 Crude Enzyme Assay (DNS- Miller Method., 1959)

The enzyme activity of the isolates was carried as per Miller 1959 [20] with D-glucosamine as standard [21]. After 5 days of incubation, the activity of enzyme was at maximum of 1.8 mg/ml of the chitosanase activity was detected which was lower when compared to 4.8U/ml of *Bacillus cereus* [22] and 2-3.8U/ml^2 [23].

3.3 Effect of Time and Substrate for Chitosanase Production

The strain B4/A4 showed the high activity on the 3rd day and the activity was seen higher if the concentration of the substrate is increased (0.5%, 0.6%, 0.8%, 1%, 1.5%, 2%). The strain showed the higher activity of 2.198 mg/ml with 1.5% on the 5th day. No growth was seen at 2.0% chitosan due to high viscosity of the medium with limited oxygen availability and also high concentration of chitosan inhibited the growth of the organisms [24, 25].

Figure 1. Chitosanase production of the isolate indicating the clear zone formation on the CDA media.

3.4 Effect of pH and Temperature for Chitosanase Production

The organism showed the higher enzyme activity in pH 6.5. B4/A4 enzyme was sensitive to pH below 5 and above 7 which might be due to the instability of the proteins (Figure 2)The crude enzyme showed the higher activity when the organisms were subjected at 30°C and 37°C [25]. But the enzyme activity was decreased at 45°C, 55°C (Figure 3). The specific growth maximized at pH 6.5 and 30°C yielding a higher production of the enzyme.

3.5 Effect of Carbon and Nitrogen for Chitosanase Production

The chitosanase activity was higher in glucose [22] and starch [10] containing medium. The bacterial colonies utilized 1% glucose, 1% fructose and 1% xylose as a major carbon source and showed higher enzyme activity (Figure 4). The activity increased at 2% concentration of Xylose and fructose to 13.2 mg/ml. Among the nitrogen sources, the present study revealed that the strain utilized peptone (0.5%) and ammonium chloride (0.5%) for the higher activity. The yeast extract (1%) showed higher chitosanolytic activity [22]. The activity was repressed when yeast extract of 0.5% employed in the production medium. The activity ceased in the presence of urea [26].

The strain B4/A4 showed higher activity in the Peptone 10% with all carbon sources of 20%, but among the carbon 20% and nitrogen20% the higher activity was seen only in Ammonium Chloride 20% and Sucrose10%. The activity was also seen higher in the proportion of peptone 20% and fructose 10%. From the results its clear that the proportion of peptone with carbon sources induced the organism to produce the enzyme. The chitosanase activity reached maximum by the optimization of carbon, nitrogen sources and of culture conditions.

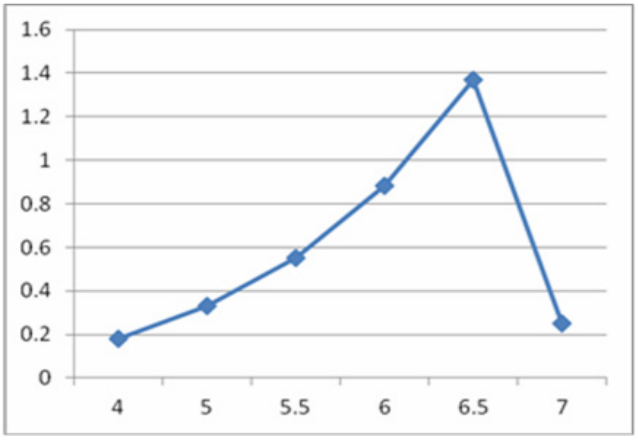

Figure 2. Effect of pH on Chitosanase activity.

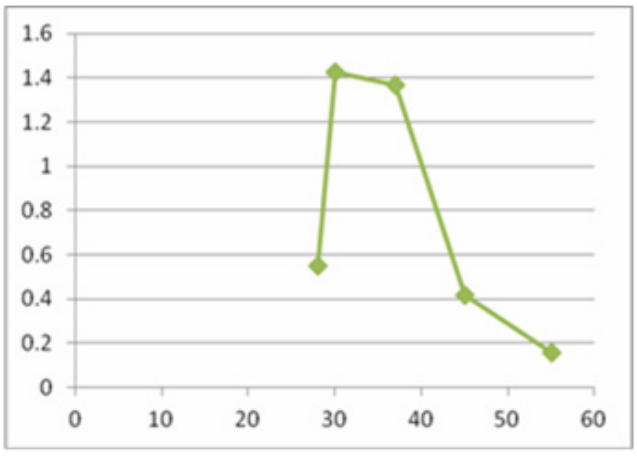

Figure 3. Effect of temperature on Chitosanase activity.

3.6 Molecular Mas-SDS-PAGE

SDS – PAGE pattern of Partially purified enzyme of A4/B4 was found 43 KDa (Figure 5) was similar to Chuan

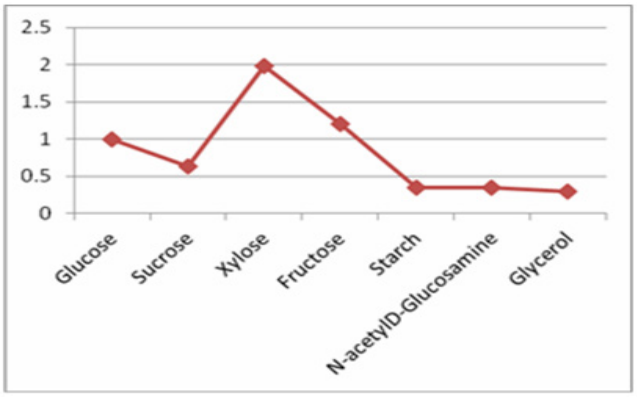

Figure 4. Effect of carbon sources on Chitosanase activity.

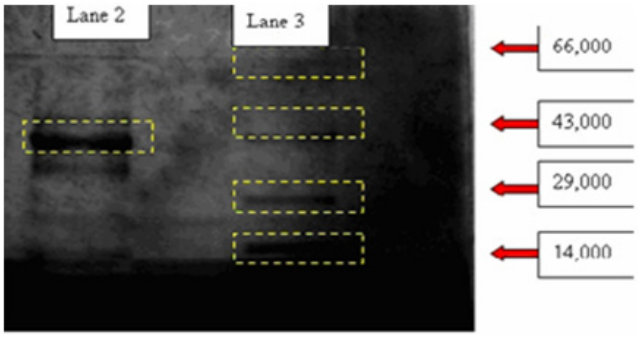

Figure 5. SDS PAGE of the chitosanase from the isolates. Lane2 :Partial purified enzyme extract of B4/A4 Lane 3: Marker molecule.

et al. The molecular weight ranged to be 41 KDa [22] to 45 KDa [10].

3.7 Preparation of Chitooligosaccharides and its Solubility

A portion of the mixture was mixed with concentrated alkali and no precipitate formation indicated that the chitosan has depolymerised into small oligomers [15] and the similar result was observed.

The solubility of chitooligosaccharides was investigated in various solvents such as water, ethanol, diethyl ether, ethyl acetate and glacial acetic acid at room temperature. Liu *et al*. [16] reported that the chitooligosaccharide dissolved in water, ethanol, ethyl acetate and glacial acetic and not in diethyl ether where as in the present investigation the chitooligosaccharide dissolved only in water.

3.8 Antimicrobial Activity of Chitooligosaccharides

The chitooligosaccharides showed the higher zone of inhibition from 60 µl for the all the organism. The crude enzyme showed no zone of inhibition where as the 100 µg of chitosan showed the inhibition zone lower to that of chitooligosaccharides (Table 1). The MIC & MBC of the chitooligosacharides was also determined from 0.2µg to100µg. The MIC & MBC activity was similar against *E.coli*, *Staphylococcus aureus* of 10mg/ml (Table 2, Figure 6). The chitooligosaccharides showed the higher zone of inhibition from 60 µg/ml for the all the tested organism.

The crude enzyme showed no zone of inhibition where as the 100 µl of chitosan showed the inhibition zone lower to that of chitooligosaccharides (Table 1). You-Jin *et al*. [27] reported that chitosan was effective against *E.coli* than chitoligosaccharide. From the study, chitooligosaccharides hydrolysate showed advantageous antibacterial activity compared to chitosan against both the organisms. The MIC & MBC of the chitooligosacharides was also determined from 0.2µl to100µl and the activity was similar against *E.coli*, *S. aureus* (Table 2) of 10mg/ml [28, 29]. The antimicrobial activity of chitooligoaccharides is similar to the chitosanase enzyme [30] but where as in the present investigation the chitosanase enzyme showed no inhibitory effect against the selected pathogens.

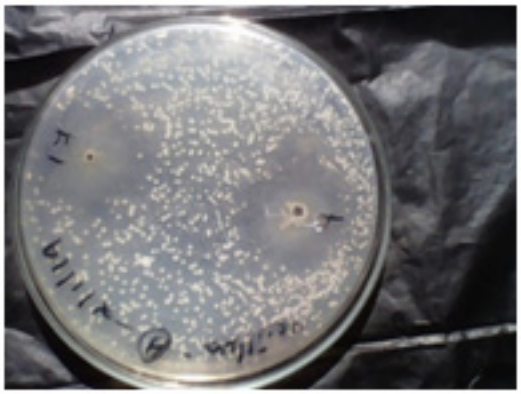

Figure 6. Antimicrobial activity of the chitooligosaccharide hydrolysate.

Table 1. Antibacterial screening activities of Chitooligosaccharides by using Well diffusion assay

Pathogens	Zone of inhibition in mm										Crude Enzyme 100µg / well	Chitosan -100µg/well Zone in mm
	Concentration of CHOS in µg											
	10	20	30	40	50	60	70	80	90	100		
E.coli	-	2	5	7	10	11	12	13	13	15	-	11
P.aeruginosa	3	9	12	14	16	16.7	17	18.5	19	20	-	14
S. aureus	4	6.2	7	10	11	11	12	13	14	15.1	-	9

Table 2. MIC and MBC values of Chitosan Oligosaccharide Samples

Organism	MIC and MBC values (mg/ml) of Oligochitosan Samples		
	Initial MIC [mg/ml]	Final MIC [mg/ml]	MBC [mg/ml]
Escherichia coli	0.1 ±0.01	0.2 ±0.01	0.1 ±0.01
Staphylococcus aureus	0.1 ±0.01	0.2±0.02	0.1 ±0.02
Pseudomonas aeruginosa	0.15 ±0.02	0.19±0.01	0.15±0.03

4. Conclusion

Chitosanase have been extensively applied in industry for the most important application is the preparation of the chitooligomers by the enzymatic hydrolysis of chitosan. For the large scale production of the enzymatic chitooligosaccharide production requires a cheap enzyme source. Hence the present study was focused on the selection of bacteria for the production of chitosanase for the good yield of bio functional chitooligomers. The chitooligosaccharide hydrolysate prepared from the partial purified enzyme has also exerted a better antimicrobial activity against resistant organisms than the chitosan.

Our results conclude that the bio waste dumped soil sample is a promising source for isolating a novel chitosanase producing organisms for the production of bio functional chitoligosaccharides.

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