ARTICLE

CHARACTERIZATION OF AN EXTRACELLULAR CHITOSANASE FROM THE SOIL BACTERIUM *BACILLUS CEREUS* CH12

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ABSTRACT

An extracellular chitosanase producing bacterium was isolated from soil samples in Mysuru, Karnataka, India and identified as Bacillus cereus CH12 based on morphological, biochemical and 16S rRNA gene sequence. Presence of the chitosanase gene was detected by partial gene sequencing. The chitosanase showed an activity of 11.16 U/ml after three days of incubation with 90% deacetylated chitosan. The enzyme was purified by gel filtration and its molecular weight was 30 kDa. Optimum temperature of the enzyme was 40 ^{o}C and optimum pH was 6.0. The enzyme was stable at a pH range of 5.0-8.0 and up to a temperature of 50 ^{o}C. The enzyme showed high specificity towards carboxymethyl cellulose (CM-cellulose). Analysis of the hydrolytic product of the enzyme showed the presence of chitooligosaccharides of a degree of polymerization of 3-5. Bacillus cereus CH12 chitosanase can be useful in the degradation of cellulose and chitosan containing biomass and in the production of chitooligosaccharides.

KEY WORDS
Bacillus cereus; Chitosan; Chitosanase; Extracellular; Chitooligosaccharides

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INTRODUCTION

Chitosan is a D-glucosamine polymer made of N-acetyl-D-glucosamine linked by β-1,4-glycosidic bonds [1, 2]. Chitosan is a deacetylated derivative of chitin [1, 3]. Chitosan is manufactured in industries by chemical deacetylation of chitin and biologically by the deacetylation of chitin through a chitin deacetylase enzyme. Fungi like *Absidia, Mucor* and *Rhizopus* are known to have chitosan in the cell walls of their mycelia and sporangiophores. It is also present in the exoskeletons of the insects and predominantly in crustacean shells [1, 4]. Chitosan and the derivatives of chitosan have various applications - they are being used as permeability control agents, adhesives and as flocculating and chelating agents. They have also emerged as potential biomaterial for food, pharmaceutical, textile industry and in wastewater treatment [1, 5]. Chitin and chitosan have been receiving lot of importance in the recent times due to their derivatives - the chitooligosaccharides. Chitooligosaccharides have several beneficial properties [4, 5]. Chitooligosaccharides have low viscosity, they have shorter chains and most importantly, they are water soluble [6]. Chitosan can be converted to chitooligosaccharides either by chemical or enzymatic methods. However, chemical methods of chitosanase production have several shortcomings - production of large amounts of glucosamine, which is the monomer of chitin, low yields of chitooligosaccharides, cost effectiveness and problems of environmental pollution. Therefore, biological methods of chitooligosaccharide production have attracted more attention and are highly advantageous [7]. Chitosanases (EC 3.2.1.132) are enzymes that hydrolyze chitosan (1, 3, 8). Chitosanases have been classified under family 8, 46, 75 and 80 respectively based on their amino acid sequence similarities. Among these families, the glycoside hydrolase family 8 (GH-8) has the highest number of bifunctional enzymes with cellulolytic and chitosanolytic activities. The GH-8 family also contains cellulase, xylanase, lichenase and others [9, 10]. Chitosanases are used in the production of biologically functional chitooligosaccharides and in the control of fungal pathogens [11, 12]. Chitooligosaccharides produced enzymatically are a better alternative to chemical methods of degradation, which use acids for hydrolysis resulting in lower yields [13]. Use of chitosanases for the enzymatic degradation of the vast biomass of chitosan available in nature has become advantageous over chemical methods due to its environmental compatibility, reproducibility and low cost [14]. A chitosanase producing bacterium *Bacillus cereus* CH12 was isolated, the enzyme chitosanase was purified and its biochemical characteristics were studied. This may serve as a potential enzyme for the degradation of biomass and in the production of useful chitooligosaccharides.

MATERIALS AND METHODS

Materials

Chitosan of 75% degree of deacetylation, Glucosamine hydrochloride and chitin (Himedia laboratories, Mumbai, India), 90% degree of deacetylation chitosan, chitosan lactate (Everest Biotech, Bengaluru, Karnataka, India) and Sephadex G-75 (Sigma) were used. Other chemicals were of analytical grade (SRL, Mumbai India and Himedia Laboratories, Mumbai India). Pre-stained protein marker (Fermentas) was used to determine the molecular weight of the protein. The soil for screening was obtained from garden soil samples at Mysuru, Karnataka, India.

Screening of chitosanase producing bacteria from soil

Soil samples were serially diluted and plated on to a chitosanase screening medium as described by Johnsen et al., 2010 [15]. The medium (1 litre) was prepared by adding 1.8 g of 90% deacetylated chitosan to 200 ml of distilled water and sterilizing it by autoclaving. At room temperature, 18 ml of sterile 1 molar HCl was added in order to bring the chitosan to solution. The chitosan solution was then dissolved by stirring for two hours. The dissolved chitosan was poured into 700 ml of an autoclaved, warm medium at a slow rate, while stirring vigorously so that the small colloidal particles were well maintained. This medium had the following composition; KH_2PO_4 – 9 g, K_2HPO_4 – 6 g, Tryptone – 8 g, Yeast extract – 4 g and Agar – 15 g. Vogel's trace elements (2 ml) were added to the medium. The composition of Vogel's trace elements is as follows – Citric acid H_2O – 5 g, $ZnSO_47H_2O$ – 5 g, $Fe(NH_4)_2(SO_4)_26H_2O$ – 1 g, $CuSO_45H_2O$ – 0.25 g, $MnSO_4H_2O$ – 0.05 g, H_3BO_4 – 0.05 g, $NaMoO_42H_2O$ – 0.05 g per 100 ml. Finally, 2 ml of sterilized $MgSO_46H_2O$ (203 g/l) was added. The final volume of the medium was adjusted to 1 litre. The bacterial isolates were incubated on the chitosanase detection medium for a period of 3-4 days. An isolate named CH12, showing significant chitosanase activity and a clear prominent halo around the bacterial growth was selected for further study. The isolate CH12 was identified taxonomically according to the Bergey's Manual of Systematic Bacteriology [16]. The identity of the organism was confirmed by Polymerase chain reaction (PCR) and the partial amplification of the 16S rRNA gene. The following primers were used for amplification: 27F- 5'- GAGTTTGATCCTGGCTCA-3', 1492R- 5'-TACGGCTACCTTGTTACGACTT-3' [17]. The sequence was amplified by Sanger Dideoxy method and the amplified sequence was compared with published sequences through an NCBI BLAST search. Chitosanase gene was identified by partial amplification of the gene by PCR. The primers were designed on the basis of the chitosanase gene of the bacterium *Bacillus cereus* ATCC 14579 (Accession number: NC_004722) obtained from a search in the NCBI database. The following PCR conditions were used – 95 ^{0}C for 15 minutes, 30 cycles at 95 ^{0}C for 30 seconds, 48 ^{0}C for 30 seconds, 72 ^{0}C for 1 minute and a final extension at 72 ^{0}C for 7 minutes. The primers had the following sequences: Forward primer: 5'-CTTCAGAAGGTCAAGGGTATGG-3', reverse primer: 5'-CCAATCAGATGGTCTCGTATCAA-3'.

Assay for chitosanase activity

Chitosanase was assayed by modifications of methods described by El – Sherbiny, 2011 [18] and Sinha et al., 2012 [12]. Chitosanase was assayed by measuring the reducing sugars produced using 0.18% of 90% deacetylated chitosan as a substrate. The reaction mixture consisted of 200 µl of enzyme solution, 500 µl of the substrate and 800 µl of sodium acetate buffer (pH 6.0). The mixture was incubated at 37 ^{0}C for 1 hour. The reaction was terminated by heating the mixture in a boiling water bath (100 ^{0}C) for 2 minutes. The supernatant was separated by centrifugation and the reducing sugars in the supernatant were measured by a method described by Imoto and Yagishita 1971 [19] using glucosamine hydrochloride as a calibration standard. One unit of chitosanase was defined as the amount of enzyme that liberated 1µmol of D-glucosamine per minute under the above mentioned conditions.

Purification of chitosanase

The isolate CH12 was cultured on the chitosanase detection medium (1 litre) except for agar. The culture was incubated for a period of 3-4 days. After incubation, the cell supernatant was separated by centrifugation at 8,000 rpm at 4 ^{0}C for 15 minutes. The resulting supernatant (950 ml) was subjected to ammonium sulphate precipitation. Ammonium sulphate (662 g/l) was added to the supernatant (950 ml) in order to achieve 90% saturation. This mixture was stored overnight at 4 ^{0}C and the resulting precipitate was centrifuged at 12,000 rpm at 4 ^{0}C for 15 minutes. The precipitate was pooled, and an appropriate amount of sodium acetate buffer (pH 6.0) was added in order to dissolve the precipitate. This resulting crude enzyme solution was concentrated by dialysis against the same buffer (Sodium acetate, pH 6.0) at 4 ^{0}C for a period of 48-72 hours. The dialyzed crude enzyme was then loaded on to a Sephadex G-75 gel filtration column (60x2 cm). 4 ml fractions were collected at a flow rate of 22 ml/hour. Protein profile was examined at 280 nm. Protein concentration of the enzyme was determined by Lowry's method [20] using Bovine Serum Albumin as a standard.

Determination of molecular weight of chitosanase

The molecular weight of the purified chitosanase enzyme was determined using 12% Sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE) [21] and stained with Coomassie Brilliant Blue R-250. Pre-stained protein marker was used for identification of the molecular weight of the protein.

Effect of pH, temperature, metal ions and substrates on enzyme activity

Enzyme activity for the purified enzyme was tested for its behavior with respect to different parameters like temperature, pH, effect of metal ions and specificity to substrates [3, 13, 22]. To determine the optimum pH, the enzyme and substrate were incubated in buffers of different pH values. The buffers used were 100 mM solutions of McIlvaine buffer (pH 3.0, pH 4.0), sodium acetate buffer (pH 5.0), potassium phosphate buffer (pH 6.0, 7.0, 8.0) and bicarbonate-carbonate buffer (pH 9-10). For the determination of pH stability, the enzyme solution was pre-incubated at 4 ^{0}C for 1 hour in the buffers as described above and the residual activity was measured. For the determination of optimum temperature, a mixture of enzyme,

substrate and buffer was incubated at various temperature ranges from 10-80 ^{0}C at pH 6.0. Temperature stability was determined by checking the residual activity after pre-incubation of the enzyme for 1 hour at pH 6.0 and various temperatures as described above without the substrate. Metal ion effect was determined by pre-incubating the enzyme with 10 mM metal ions like Mn^{2+}, Ca^{2+}, Mg^{2+}, Mo^{2+}, Ba^{2+}, Zn^{2+}, Cu^{2+}, Hg^{2+} and Fe^{2+}. Substrate specificity was tested by selecting substrates of chitosan and cellulosic nature. Chitosan of different degrees of deacetylation (75%, 90%), chitosan lactate, colloidal chitin, CM-cellulose, insoluble cellulose and xylan were used to analyze the substrate specificity.

Analysis of the hydrolytic products of chitosanase

An enzymatic hydrolysate was prepared from the enzyme solution as described by Wang et al., 2012 [14]. For the production of chitooligosaccharides, 90% deacetylated chitosan was used as a substrate (0.18% w/v, 50 mM sodium acetate buffer pH 6.0). 1ml of enzyme was incubated with 1ml of substrate at 40 ^{0}C for 2 hours. The enzymatic hydrolysate was collected after incubation and this solution was concentrated to 1/5 of its original volume in a vacuum concentrator. This was followed by the addition of methanol solution (90% concentration, v/v) until yellow agglomerates were formed in solution. The resulting solution was concentrated in a vacuum concentrator and was collected after drying completely. This sample was subjected to Matrix - assisted laser desorption/ ionization time of flight (MALDI-TOF) mass spectral analysis (UltrafleXtreme MALDI TOF/TOF (Bruker Daltonics)).

RESULTS

Screening of chitosanase producing bacteria from soil

The bacterial isolates were screened for chitosanase production and one isolate CH12 was selected [Fig. 1]. The strain CH12 was identified by its biochemical and molecular characteristics. The organism was Gram positive, aerobic, rod shaped and motile. The bacterium was positive for Malonate, Voges-Proskaeur, Nitrate, β- haemolysis and catalase tests. It was negative for Citrate, O-Nitrophenyl-β-D-galactopyranoside (ONPG) and arginine. It was positive for the hydrolysis of sucrose, glucose and trehalose and negative for mannitol and arabinose. The organism could grow up to a temperature of 45 ^{0}C, could not grow in acidic pH but tolerated an alkaline pH up to 10.0. According to sequencing of the 16S rRNA gene, the organism showed close similarity (99%) to *Bacillus cereus*. Therefore, based on the morphological, biochemical characters and the results of the 16S rRNA gene sequencing, CH12 was identified as *Bacillus cereus* and named as *Bacillus cereus* CH12. The partial 16S rRNA gene sequence was deposited in GenBank with the accession number KR818841. The partial chitosanase gene was sequenced and deposited to Genbank with the accession number KX375814. The sequence showed high homology (99%) to *Bacillus* chitosanases.

Fig. 1: Culture plate of *Bacillus cereus* CH12 showing clear zone of chitosan hydrolysis around the bacterial growth after 3 days of incubation.

Assay for chitosanase activity

Bacillus cereus CH12 was grown on chitosanase detection media of various chitosan concentrations. A medium with 0.18% of 90% deacetylated colloidal chitosan [15] was found to be most suitable for chitosanase production in this study. Maximum chitosanase activity was seen when cultures were grown at 37 ^{0}C for three days. At the end of the incubation period, a maximum activity of 11.16 U/ml of chitosanase was seen. According to previous reports of chitosanase activities among *Bacillus* species, chitosanase from *Bacillus circulans* WL-12 showed an activity of 1.2 U/ml after two days of cultivation [8], wild strain of *Bacillus* sp. TS showed an activity of 5 U/ml [7]. *Bacillus megaterium* P1 and *Bacillus cereus* D11 and showed activities of 1 U/ml and 4.85 U/ml respectively [2, 22]. Activity of chitosanase of *Bacillus cereus* CH12 (11.16 U/ml) was higher than many chitosanases produced by wild strains of *Bacillus* species so far when grown in a chitosan medium.

Purification of chitosanase

Culture supernatant (950ml) of *Bacillus cereus* CH12 was purified by precipitation with 90% ammonium sulphate, dialysis and Sephadex G-75 gel filtration chromatography. The gel filtration yielded 60 fractions of 4ml each. The fractions were assayed for chitosanase activity and the protein profile was monitored by checking the absorbance at 280 nm. The active fractions were pooled and concentrated by a vacuum concentrator. After the final purification step, 16.2 mg of protein and 5.9 fold purity was obtained. Percentage yield was 18.1.

Determination of molecular weight of chitosanase

Purity of the protein was analysed by SDS-PAGE and a single band of 30 kDa was obtained [Fig. 2].

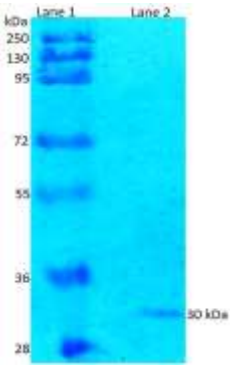

Fig. 2: SDS-PAGE of the purified protein. Lane 1: Molecular marker, Lane 2: Purified enzyme

Effect of pH, temperature, metal ions and substrates on enzyme activity

Optimum pH of the enzyme was 6.0. The activity was low in the pH below 5.0 and above 7.0. The enzyme was stable at pH 5.0-8.0 when incubated in various buffers at 4 ^{0}C as described earlier. The pH stability of chitosanases from *Bacillus* species ranged from 4-10 [Fig. 3A]. The enzyme had an optimal temperature of 40 ^{0}C, was stable up to a temperature of 50 ^{0}C and all its activity was intact at this temperature. When heated to higher temperatures for one hour, the enzyme retained 66% of its activity at 60 ^{0}C. There was a gradual decline of activity at higher temperatures and almost no activity was seen above 60 ^{0}C [Fig. 3B]. In order to determine the effect of various metal ions, CH12 chitosanase was incubated with different metal ions [table 1] at 10 mM concentration. The enzyme was completely inhibited by Hg^{2+} and Mn^{2+}. The activity was reduced by 67% when incubated with Zn^{2+} and Fe^{2+} ions when compared to the control. However, the enzyme was completely inhibited by Mn^{2+} ions. This result was different when compared to other chitosanases. Chitosanase inhibition by Mn^{2+} ions is an unusual characteristic among chitosanases and most microbial chitosanases are not inhibited by Mn^{2+} ions [5]. A similar inhibition by Mn^{2+} was reported in chitosanase of *Serratia marcescens* TKU011 [23]. Chitosanase from *Bacillus* sp. Strain KCTC 0377BP showed 69% inhibition in enzyme activity in the presence of Mn^{2+} ions [5]. Analysis of the substrate specificity of the purified enzyme was done with different substrates [Table 2] ranging from colloidal chitosan of different degrees of deacetylation, chitin to cellulosic substrates and chitosan lactate. The activity was highest in 90% deacetylated chitosan. The enzyme also retained 91% of activity on CM-cellulose. The enzyme showed little activity on xylan, insoluble cellulose and colloidal chitin.

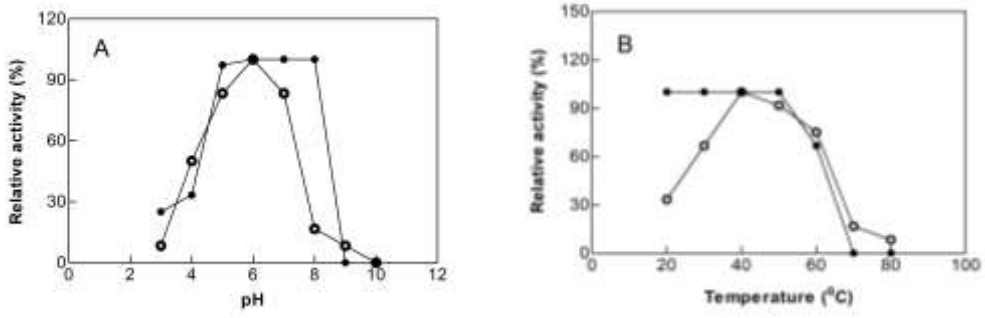

Fig. 3 [A]: Effect of pH (open circles) and pH stability (closed circles) of *Bacillus cereus* CH12 chitosanase. **B:** Effect of temperature (open circles), temperature stability (closed circles). 100% activity refers to the temperature and pH where the activity and stability are highest

Table-1: Effect of metal ions on chitosanase from *Bacillus cereus* CH12. 100% activity implies - incubation of the enzyme without metal ions.

Metal ion	Relative activity (%)
None	100
Mn^{2+}	0
Ca^{2+}	83
Mg^{2+}	83
Mo^{2+}	67
Ba^{2+}	75
Zn^{2+}	92
Cu^{2+}	33
Hg^{2+}	0
Fe^{2+}	33

Table-2: Substrate specificity of *Bacillus cereus* CH12 chitosanase. Activity values were relative to the maximum activity seen in 90% deacetylated chitosan.

Substrate	Relative activity (%)
Colloidal chitosan (90% deacetylated)	100
Colloidal chitosan (75% deacetylated)	83
Chitosan lactate	67
Colloidal chitin	0
CM-cellulose	91
Insoluble cellulose	47
Xylan (from oat spelts)	0

Analysis of hydrolytic products

The hydrolytic products of *Bacillus cereus* CH12 chitosanase were subjected to MALDI-TOF mass spectrometric analysis in the positive mode. The oligosaccharides present in the precipitate were obtained as sodium adducts [M+Na]. From the MALDI-TOF analysis, chitooligosaccharides of a degree of polymerization (DP) of 3 to 5 were identified. They had the *m/z* (mass to charge ratio) values of 524.3, 551.2, 647.7, 727.3, 749.3 and 888.7. The ionic compositions of the chitooligosaccharides were $(GlcN)_3$, $(GlcN)_4$, GlcNAc-$(GlcN)_3$, (GlcN)-$(GlcNAc)_3$, GlcNAc-$(GlcN)_4$. The results are similar to ionic compositions previously reported [24-27].

CONCLUSION

A bacterium with chitosan hydrolyzing property was isolated and identified as *Bacillus cereus* CH12. The presence of the chitosanase gene was identified in the bacterium by partial sequencing. When this sequence was translated into an amino acid sequence and subjected to a protein BLAST search, it showed homology to those sequences, which had the GH-8 signature motif - "ATDGDLDIAYSLLLAHKQW" [28]. GH-8 is a family with chitosanase, cellulase, lichenase, xylanase and other enzymes. The chitosanase enzyme also retained 91% of its initial activity on CM-cellulose. High activity on CM-cellulose is a characteristic of GH-8 enzymes [22]. An important feature of an enzyme that can be used in biodegradation is the production of oligosaccharides as a product of enzyme hydrolysis. Analysis of the hydrolytic product of *Bacillus cereus* CH12 chitosanase showed the presence of oligosaccharides of a degree of polymerization from 3-5. Absence of monosaccharides specifies that the enzyme may have an endo-splitting nature. However, this has to be confirmed with further studies. *Bacillus cereus* CH12 could be cultured up to a maximum temperature of 45 ^{0}C and the maximum chitosanase activity was at 40 ^{0}C. Activity of chitosanase of *Bacillus cereus* CH12 (11.16 U/ml) was higher than many chitosanases produced by wild strains of *Bacillus* species reported in literature so far when grown in a chitosan medium. In conclusion, chitosanase of *Bacillus cereus* CH12 had a high activity on chitosan, high relative activity on CM-cellulose and could produce chitooligosaccharides. This makes the organism very suitable for utilization in biodegradation and production of chitooligosaccharides.

CONFLICT OF INTEREST
There is no conflict of interest

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