Isolation of chitinase yielding *Bacillus cereus* JF68 from soil employing an edible crab shell chitin

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Chitin degradation is one of the major steps in nutrients’ cycling, especially in marine environment. Chitinolytic bacteria play primary role in depolymerizing the complex polysaccharide in natural as well as sea food waste areas. Chitinolytic bacterium was isolated from a soil sample in medium containing edible crab shell waste as major carbon source. The strain showed clear zone of hydrolysis in colloidal chitin agar medium. Using morphological, biochemical and 16S rDNA analysis the isolate was identified as *Bacillus cereus* under accession No. KC849453. The G + C content of the genomic DNA were 53.58%. Maximum production of chitinase occurred after 5 days of cultivation at 30°C, pH 7.0 and with peptone as nitrogen source. Highest chitinolytic index of the *B. cereus* JF68 was 2831.75U/ml. The enzyme was partially purified by ammonium sulfate precipitation and dialysis. The precipitation gave a 3.72 fold increase of the specific activity with a yield increase of 72.26%. Extensive production of stable microbial chitinolytic enzymes has a promising potential for biological control of insect as well as fungal pests.

**Keywords:** Chitinolytic bacteria; *Bacillus cereus*, Colloidal chitin, Callinectes sapidus.

**Introduction**

Chitin is the second most abundant biopolymer in nature consisting of α 1,4-linked N- acetyl-D-glucosamine. It is insoluble in water and organic solvents. About 10% of the global landing of aquatic products consists of organisms rich in chitinous material. It constitutes the major structural component in shells and cuticles of crustaceans and insects and cell wall of fungi. However, the main commercial sources of chitin are shells of crustaceans such as shrimps, crabs, lobsters and krill that are supplied in large quantities by the shellfish processing units. Chitin has achieved biotechnological importance as the polysaccharide and its derivatives are of great economical values because of their diverse and important biological activities. Their industrial and biomedical applications include antitumor activity, cosmetics preparation, treatment of osteoarthritis, used for immune-enhancers and elicitors’ actions. Chitin can be transformed into saccharides under certain conditions. Chitin can also be used as a slowly degrading substrate in microbial fuel cells. Natural recycling of chitin wastes’ generated from the natural chitin depositing routes and seafood industries, by both terrestrial and marine bacteria is of valuable economic and environmental significance. Due to polymeric nature, chitin must undergo first partial hydrolysis before accumulation by microbial cells. This is accomplished by chitinolytic enzymes which cleave GlcNAc residue producing oligomeric or dimeric transportable across membrane units which can then be metabolized. Chitinases degrade chitin by hydrolyzing glycosidic bonds to low molecular weight products. Almost all of the reported chitinase producing strains use chitin or colloidal chitin as a carbon source. The diversity of chitinolytic bacteria is quite rich in soil and bacteria from *Bacillus, Pseudomonas, Streptomyces, Serratia* and *Aeromonas* genera are frequently found in soil. Soil bacteria are the major source of chitinases and could be used for bioconversion of chitinous waste into useful molecules for application in medicine, biotechnology and agriculture. *Bacillus* spp. produces variety of chitinases with different molecular masses. Chitinase producing *Bacillus cereus* has also been reported by many workers with its potentials in biological control. The present study was aimed at isolation and characterization of chitinase producing soil bacterium employing *Callinectes sapidus* as chitin source.

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Experimental Section

Crabs’ collection and chitin extraction

Edible crab (*Callinectes sapidus*) was purchased from local market was washed with tap water and then meat was separated from the shells. The solid parts were oven dried (105°C), crushed and milled to powder. Chitin extraction was carried out according to the method of Rhazi *et al.*. Accordingly, to remove minerals demineralization was carried out by two to three times washings; taking initial quantity of 4 g of crab shell powder with 45 ml of 0.55 M hydrochloric acid for 2 h at room temperature. Further to remove protein, deproteinization was performed by alkaline treatment with 0.3 M sodium hydroxide solutions at 80 to 85°C for 1 hr. This treatment was repeated three to four times. The powder was ultimately separated by filtration and washed with distilled water for two to three times. Finally thus processed chitin powder was dried and saved in dried capped bottles.

Preparation of colloidal chitin

Colloidal chitin was prepared according to the method of Skujins *et al.* with slight modification. Five grams processed crab shells powder were added very slowly in 60 ml concentrated HCl and kept in vigorous shaking for 1 h at room temperature. Then the material was filtered through glass wool and the filtrate was added to 200 ml of 50% ethanol with vigorous and continuous stirring during this process. The precipitate was filtered through Whatman filter paper No 1, and then washed with autoclaved distilled water for two to three times. Finally thus processed chitin powder was dried and saved in dried capped bottles.

Soil sampling and isolation of the chitinolytic bacterium

Soil sampling was done from Quaid-e-Azam campus area, University of the Punjab, Lahore, Pakistan. The samples were saved in tightly capped sterilized bottles and transported to laboratory for further processing. The soil was enriched in selective medium prepared after Furukawa *et al.*, following incubation of 72 h at 30°C when cultivated on selective agar medium was selected and characterized further through morphological and biochemical tests for catalase, oxidase, starch hydrolysis, methyl red, Voges Proskauer test and citrate utilization as described by Benson. The isolate was ultimately identified by 16S rDNA sequencing.

Chitinase assay

Chitinolytic activity of the isolate was determined by the estimation of released reducing sugars from the chitin as described by Sadafi *et al.* Accordingly 0.5 ml of cell free cultural fluid was mixed with 0.5 ml of 1% colloidal chitin suspension in the 0.1 M acetate buffer of pH 5.0. The mixture was incubated at 50°C for 30 min. The reaction was terminated by adding 1 ml of DNSA reagent. The reagent was prepared by adding 10 g NaOH, 10 g dinitrosalicylic acid, 2 g phenol, 0.5 g Na₂SO₃ and 200 g sodium potassium tartrate in total volume of 500 ml. Following the completion incubation at 3°C the reaction mixture was heated at 100°C for 10 mins in boiling water bath. After cooling to normal temperature, centrifugation of the mixture was carried out at 8,000 rpm for 10 mins and then absorption was measured at 540 nm. The standard curve was plotted with N-acetylglucosamine (NAG) in the range of 100 to 600 µg/ml with 100 class intervals. One unit of chitinolytic activity was described as 1 µmol of liberation of NAG per mg of protein per minute.

Protein test

The protein content was estimated by the method described by Bradford. Bovine serum albumin (BSA) prepared from 2 µg/ml to 10 µg /ml range with 2 class intervals was used as standard. Calibration curve was then plotted by performing regression analysis of A₅₉₅ absorbance versus corresponding concentrations of the standards.

Morphological and Biochemical analysis

The bacterium that yielded highest clearance zone (2.1 mm) following incubation of 72 h at 30°C when cultivated on selective agar medium was selected characterized further through morphological and biochemical tests for catalase, oxidase, starch hydrolysis, methyl red, Voges Proskauer test and citrate utilization as described by Benson. The isolate was ultimately identified by 16S rDNA sequencing.

PCR amplification of 16S rRNA gene and its sequencing

The bacterial isolate was tested for species identity using 16S rRNA gene sequencing. Freshly grow
bacterial colony from nutrient agar plate was suspended in 5 ml sterilized LB broth and grown for overnight. The culture was centrifuged at 10,000 for 10 min and pellet was saved for DNA extraction. Bacterial 16S rRNA gene was amplified by using the universal primers 27F(5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R(3'-TACGGYTACCTTGTTACG-5') (Oligo, USA). Five µl of template DNA was added to 50 µl PCR reaction solution containing. Amplification was performed using 35 cycles of 94 ºC for 45 sec, 53 °C for 45 sec and 72ºC for 1 min followed by a 7 min incubation at 72°C. The PCR products were electrophoresed on 1% w/v agarose gels and the target band was excised and purified with PCR purification kit (Fermentas). Sequencing was get done from Korea by using same primers. The sequence was matched with the nucleotide database available at Gene Bank, using BLAST tool in NCBI (http://www.ncbi.nlm.nih.gov) for recognition of the highest % similarity with the described species.

**Optimization of chitinase production**

Effects of temperature and pH on chitinase production were studied by growing the isolate in chitin containing broth at different temperature ranging from 20 to 60°C and different pH ranging from 4 to 11 for 5 days. Different nitrogen sources such as trypton, gelatin, peptone, yeast extract, urea, ammonium chloride, ammonium oxalate and ammonium dihydrogen phosphate were supplemented to the medium to study their influence on chitinase production.

**Enzyme characterization**

pH stability was checked by incubating the crude enzyme preparation at different pH values. The crude extract of enzymes with substrate was subjected to different pH levels from 4 to 11 by using different buffers acetate (4-5), phosphate (6-7), Tris HCl (8-9) and glycine NaOH buffers (10-11). The mixtures were incubated at 50°C for 30 min in shaking water bath. Thermal stability was investigated by incubating the enzyme preparation at different temperatures.

The crude extract of enzyme with substrate was incubated at temperatures ranging from 20 to 60°C for 30 min in shaking water bath. The crude enzyme was also incubated with different concentrations of chitin substrate at optimum temperature and pH for 30 min. The enzyme activity was measured, as described before.

**Partial purification**

Colloidal chitin broth (500ml) in 1 Litre capacity round bottle was inoculated with optimum inoculum and incubated at 30°C in shaking water bath for 5 days. The cultural fluid was centrifuged at 10,000 × g for 10 min and the supernatant was saturated with ammonium sulphate salt to 70% at 4°C and the precipitate was collected by centrifugation at 10,000 × g for 10 min. The precipitate was allowed to dissolve in acetate buffer (pH 5.0) and was then dialyzed against same buffer. The dialysate was considered partially purified chitinases.

**Results and Discussion**

**Processing of chitin containing waste**

Edible crab, *Callinectus sapidus*, was chosen as it represents enough source of waste product of food industries. The waste product utilized for the production of chitinases might serve a good source of chitinases production. Shells of the crab were processed for removal of minerals by demineralization and proteins by deproteinization. From 4 g of chitin wastes, 0.984 g (24.6%) chitin was obtained. This crude chitin was further processed for attaining colloidal chitin for the production of chitinases and from 5 g crude chitin 54 g (1080%) of colloidal chitin is was obtained.

**Isolation and screening of chitinase producing bacterium**

The chitinolytic bacterium JF68 has a zone to colony size ratio of 2.1mm. Wherein, the bacterium yielded chitinase enzyme upto 2831.75U/ml. Colonies of the isolate showed irregular margin, butyrous, convex, translucent and off white in color. Maximum size of well isolated colonies could measure upto 4.68 mm diameter and had lobate margins. The isolate was positive for gram, endospore catalase, oxidase, nitrate, citrate and Voges Proskauer tests but negative for sulfate reduction and methyl red tests. The isolate could express growth in fructose, maltose, sucrose, galactose, mannose, xylose and arabinose but did not express growth when cultivated in lactose, sacharose, sorbitol and mannitol sugars.

**Phylogenetic analysis of 16Sr RNA sequences**

This strain showed high homology with 99% similarly *Bacillus cereus* based on 16S rRNA gene sequences of the strain. Thus following the physiological and biochemical characteristics and finally relying comparison of its 16S rRNA gene sequence, the strain was identified as *Bacillus cereus*. 
The partial 16S rRNA gene sequence was deposited in the GenBank nucleotide sequence database under the accession number KC849453.

**Time course of chitinase production**

*B. cereus* JF68 was grown aerobically in 100 ml of the selective medium in a 250 ml Erlenmeyer flask at 30°C. During the process of incubation chitinase activity was measured daily. The chitinase production increased progressively following the incubation and reached at maximum level after 5 days. In the absence of chitin nutrient broth chitinolytic activity was not expressed. Thus the chitinolytic enzymes of *B. cereus* JF68 require proper induction for their expression.

**Effects of pH, temperature and nitrogen source on chitinase production**

Culture conditions are critical parameters that affect cell growth and product yield. In this study, the effects of various variables, including incubation temperature, initial pH and nitrogen sources on the efficiency of chitinase production were examined. Maximum chitinase production (U/ml) by *B. cereus* JF68 was observed at initial pH of 7.0 (Fig. 1). However, significant amounts of the enzyme are also produced at pH 8.0 and 9.0 and could attain 78.30% and 54.75% of the level obtained at 7 pH, respectively. Chitinase production up to 558.42 U/ml was achieved at 30°C after 120 h of incubation (Fig. 2). Considerable decrease in chitinase production was observed at elevated temperatures so that at 50°C incubation temperature a reduction in the yield of 70.71% was recorded. Optimal chitinase production by *Bacillus thuringiensis* has also been reported to occur at pH 7.0 and 30°C. Karunya *et al.* has also reported optimum pH 7.0 for chitinase production by *B. subtilis*. Bacterial chitanases productions and their activity expression within relatively wide range of pH and temperature render them potential candidates for field applications. Among various organic and inorganic sources tested peptone was identified as the best nitrogen source supporting the enzyme yield (Fig 3). Supplementation of diammonium hydrogen phosphate in the media increased chitinase production by 1.54 folds. Addition of casein has been reported to increase chitinase activity in *B. thuringiensis* and *B. licheniformis*. Chitinase production by *B. cereus* reduced in the presence of ammonium chloride. The results of the present study indicated that both organic and
inorganic nitrogen sources are suitable for obtaining higher production of chitinase enzymes under the described culture conditions from B. cereus.

Characterization of enzyme

The extracellular chitinase from B. cereus JF68 showed best performance in natural pH but could remain active with buffers between 5 and 6 pH. Chitinases from different microbial sources have different ranges of physicochemical parameters for their activities. For instance B. ccirculans and Bacillus licheniformis are known to produce chitinase at wide range of pH from 4 to 11. Regarding thermostability the chitinase reported in the present study showed highest activity at 45°C to 50°C. Even following the exposure of 30 min to the 50°C, the activity of chitinase from B. cereus remained higher than at 40°C and the values obtained at other low temperatures. Following the exposure of 60°C for 30 min the enzyme could express but only 46% activity as compared to the corresponding values obtained at 45°C though the value is higher than temperature below 45°C. Nawani and Kapadnis also reported highest chitinolytic activity at 47°C for Serratia marcences. The chitinolytic activity appeared maximum, when the enzyme preparation was incubated for two hours thereafter the activity went down. In case of substrate concentration the bacterium showed optimum chitinase activity at 0.6% substrate concentration.

Partial purification

Following ammonium sulphate precipitation and dialysis, 59740.16U/mg specific activity of the chitinolytic enzyme was obtained. The partial purification gave a 3.717 fold increase of the specific activity and had a yield of 72.26%.

Conclusion

Microbial production of chitinolytic enzymes has got relevance to industrial sector and agriculture environment with their subsequent wide applications. The present study identified food industrial waste chitin as a reliable substrate for chitinolytic bacterial isolation as well as chitinase production. The Bacillus cereus JF68 isolated during the course of this study was found capable of producing chitinase enzyme upto 2831.75U/ml under the optimized growth conditions. Partial purification of the enzyme could enhance upto 3.72 fold higher specific chitinase activity. Thermostability of enzyme upto 50°C and pH range of 5 to 9 permit the bacterium to consider a potential biological control agent for chitin characterized pests.

References

17 Gomaa E Z, Chitinase production by Bacillus thuringiensis and Bacillus licheniformis: Their potential in antifungal biocontrol, J Microbiol, 50 (2012) 103-111.