

RESEARCH ARTICLE

Production, Optimization and Characterization of Chitin Deacetylase from Marine bacteria *Bacillus cereus* TK19

M. Ravikumar and K. Perinbam*

PG and Research Department of Botany, Government Arts College for Men (Autonomous), Nandanam, Chennai-600035, TN, India
drperinbam73@gmail.com*; rvkmr1011@gmail.com; +91-9566963344*, 9940867295

Abstract

About 15 marine derived chitinolytic bacteria were screened for their chitin deacetylase producing ability on the basis of chitin deacetylation. Based on enzyme production ability, one potent isolate TK19 was selected and identified as *Bacillus cereus* based on 16S rRNA sequence analysis. The process parameters were optimized for the better production of chitin deacetylase. The maximum production of chitin deacetylase was observed at 35°C and pH 8.0 after 48 h of incubation by *Bacillus cereus* TK19. The effect of pH and stability on the activity and stability of purified chitin deacetylase was assessed further. The marine derived *Bacillus cereus* TK19 serves as a potential candidate which can be further augmented for enhanced production of chitin deacetylase.

Keywords: Chitin deacetylase, *Bacillus cereus*, 16S rRNA sequence, process parameters, optimization.

Introduction

Chitin is the structural component of most of the fungi, crustaceans, protozoans, algae, etc. In case of crustaceans like shrimps and crabs, chitin accounts for the 30-60% of their body weight and were discarded as waste upon processing (Hao *et al.*, 2012). The seafood processing industries produces large amount of such chitinous waste every year which imposes serious environmental concern (Matroodi *et al.*, 2013). The derivative of chitin such as chitosan holds a stable position in pharmaceutical industry for their unique structural and functional properties. The microorganisms having the ability to produce chitin-degrading enzymes (chitinase) or deacetylating enzymes (chitin deacetylase) can be useful in their possible bioremediation and their effective management (Dahiya *et al.*, 2006; Waghmare and Ghosh, 2010). Chitosan is the biopolymer derived from the N-deacetylation of marine derived chitin. Chitosan is a water-soluble polymer possessing excellent biocompatibility and antimicrobial potential. In medical field, chitosan is used in the cancer therapy, cholesterol control, wound dressings and controlled drug release membranes (Linden *et al.*, 2000). Chitosan also acts as biopesticide for boosting the plant defense mechanism against fungal pathogens. Currently, NaOH pyrolysis method is used for the production of chitosan from chitin which requires high energy and results in chitosan of poor quality. As an alternative, employing chitin deacetylase from microbial source for N-deacetylation of chitin could overcome such drawbacks (Zhao *et al.*, 2010). Chitin deacetylase are the enzyme which hydrolyzes the acetamido group in the N-acetylglucosamine units of chitin to convert them into chitosan.

The enzyme was first isolated from the fungi, *Mucor rouxii* (Araki *et al.*, 1975) and further several fungal and bacterial chitin deacetylases have been characterized (Gao *et al.*, 1995; Li *et al.*, 2006; Gauthier *et al.*, 2008). Chitin deacetylase from bacterial sources are preferred more since, the fungal mediated production of enzyme requires high energy for enzyme extraction. Against these points, the present study was aimed to isolate the chitin deacetylase bacteria from marine origin and to optimize the fermentation conditions for maximum enzyme production.

Materials and methods

Preparation of colloidal chitin: For the isolation and screening studies, colloidal chitin was prepared from the chitin using the method of Wiwat *et al.* (1999). About 40 g of chitin was added with concentrated hydrochloric acid (600 mL) and vigorously stirred for 60 min at room temperature. Colloidal suspension of chitin was prepared by the addition of 2 L distilled water to the above mixture at 10°C. The suspension was filtered, washed and dissolved in 5 L distilled water. The process of washing was repeated till the pH attained 3.5. The colloidal chitin thus obtained was used for further studies.

Isolation of chitinolytic bacteria: Marine water samples of 1 m depth were aseptically collected from 5 different regions of Tuticorin, TN, India. The chitinolytic bacteria were isolated from the collected samples by plating them in agar medium amended with colloidal chitin. The serially diluted water samples were spread plated on medium consists (g/L): Na₂HPO₄, 6; KH₂PO₄, 3; NH₄Cl, 1; NaCl, 0.5; yeast extract, 0.05; agar, 15 and colloidal chitin, 10 (Saima *et al.*, 2013).

The plates were incubated at 37°C for 24 h and observed for chitinolytic bacteria. The formation of clear zones around the bacterial colony indicated their chitinolytic activity and was purified for further investigations.

Screening for chitin deacetylase production: About 15 chitinolytic bacteria were isolated and further screened for the production of chitin deacetylase. The pure cultures of the bacteria were streaked on the chitin deacetylase screening medium consists (g/L): NaNO₃, 2; K₂HPO₄, 1; KH₂PO₄, 1; MgSO₄, 0.5; Colloidal chitin, 10; agar, 15 (Zhou *et al.*, 2010). The plates were incubated at 37°C for 48 h and observed for yellow coloration due to the action of chitin deacetylase. The characteristic chitin deacetylase producing bacterial strain TK19 was selected and sub-cultured for further studies.

Molecular identification of bacteria: Chitin deacetylase producing bacterial strain TK19 was identified by 16S rRNA sequence analysis. For the same, the genomic DNA of the bacteria was extracted by phenol-chloroform method (Ruzante *et al.*, 1996). From the isolated genomic DNA, 16S rRNA region was amplified using PCR with universal primers, 27F (5'GAGAGTTTGATCCTGGCTCAG3') and 1492R (5'CTACGGCTACCTGTTACGA3') (Setia and Suharjono, 2015). The PCR amplified region of partial 16S rRNA gene was sequenced using Automated Sequencer in Chromous Biotech, Chennai. The sequence similarity search with nucleotide database in Gene Bank was then performed using BLASTN (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Production of chitin deacetylase: The production of chitin deacetylase was carried out by inoculating the overnight grown culture of *Bacillus cereus* TK19 into 100 mL of chitin deacetylase production medium consists (g/L): (NH₄)₂SO₄, 7; K₂HPO₄, 0.1, NaCl, 1; MgSO₄·7H₂O, 0.1, Peptone, 1, Yeast extract, 1 and Colloidal chitin, 10 (Ischaider *et al.*, 2014). The flask was incubated on shaker (120 rpm) at 37°C for 60 h. An aliquot of production medium was withdrawn every 12 h, centrifuged (12000 rpm for 1 min) and the supernatant was assayed for chitin deacetylase and total protein content.

Chitin deacetylase assay: Chitin deacetylase activity was measured using glycol chitin as substrate (Tokuyasu *et al.*, 1996). The reaction mixture consists of 20 mM sodium tetraborate/HCl buffer with glycol chitin (final concentration 0.15%) and 40 µL of culture filtrate to 160 µL of reaction mixture was incubated for 20 min at 30°C. The reaction was terminated by the addition of 200 µL of 33% (v/v) acetic acid. To 0.5 mL of the reaction mixture, 0.5 mL of a 5% sodium nitrite and 0.5 mL of a 33% acetic acid, shaken and left standing for 10 min. The excess nitrous acid was removed by the addition of 0.5 mL of 12.5% ammonium sulfamate, and shaking for 30 min.

To 2 mL of the above solution, 2 mL of 5% HCl and 0.2 mL of a 1% indole in alcohol are added and kept in boiling water bath for 5 min. About 2 mL of alcohol was added to remove the turbidity and the absorbance of the orange colour developed was read at 492 nm. The control was prepared without adding the glycol chitin. One unit of enzyme activity was defined as the amount of enzyme producing 1 µmol of glucosamine residues per min when incubated with glycol chitin as the substrate. The total protein content was measured using Lowry's method (Lowry *et al.*, 1951).

Optimization of chitin deacetylase production: The effect of the initial pH on chitin deacetylase production was studied by altering the pH of the production medium from 4 to 11 and the production was carried out for optimized incubation time. Next to pH, the effect of temperature on the production of chitin deacetylase was determined by incubating the production medium at different temperatures (2, 30, 35, 40, 45, 50 and 55°C) for optimized period of time and at optimized pH. The chitin deacetylase assay was performed as described above.

Partial purification of chitin deacetylase: For the purification of chitin deacetylase, two-step purification method was followed. The protein present in the culture filtrate was precipitated using Ammonium sulphate precipitation and the impurities were removed by dialyzing the precipitated protein overnight using phosphate buffer (Kaur *et al.*, 2012).

Effect of pH and temperature on enzyme activity and stability: The effect of pH on the activity and stability of the partially purified chitin deacetylase was assayed by determining the enzyme activity after incubation of the enzymes at different pH (6-11) for 1 h. Similarly, the effect of temperature on the activity and stability of chitin deacetylase was determined by determining the enzyme activity after incubation of the enzymes at different temperatures ranging 30-90°C for 1 h in a constant temperature water bath. The residual chitin deacetylase activity and stability was estimated quantitatively using spectrophotometer.

Results and discussion

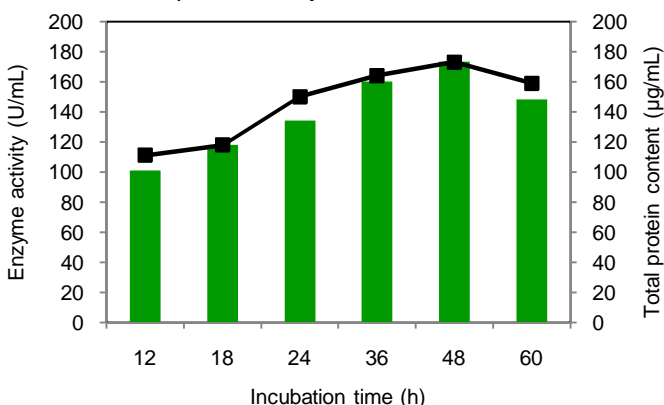
In the present investigation, a total of 36 bacteria were isolated from 5 marine water samples collected from different sites of Tuticorin, India. Among them, 20 chitinolytic bacteria were selected based on the hydrolysis of colloidal chitin via zone of clearance above 0.3 mm on screening medium. The strains were labeled as TK01 to TK20 and further screened for chitin deacetylase activity. In the chitin deacetylase screening, the isolate TK19 showed chitin deacetylase activity by the formation of yellow coloration in screening medium was selected for further studies (Fig. 1). Based on the basic biochemical characterization, the isolated bacterial strain TK19 was found to belong to *Bacillus* sp.

Fig. 1. Colony morphology of *Bacillus cereus* TK19.



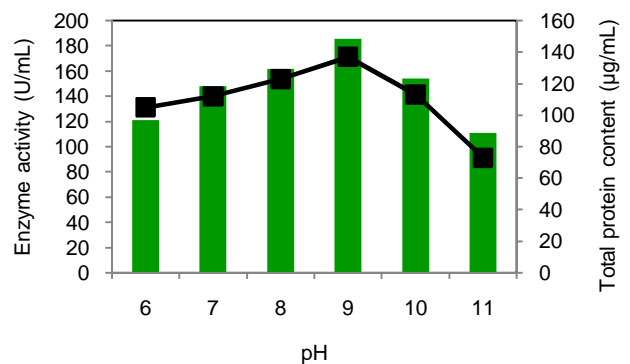
The isolate bacterial isolate TK19 was found to be aerobic, Gram positive, rod shaped and motile bacteria. The subsequent 16S rDNA sequence analysis confirmed the isolate TK19 as being *Bacillus cereus*. The 16S rRNA sequence of *Bacillus cereus* TK19 was submitted to GenBank nucleotide sequence database (Accession number: KU352779). Among the marine bacteria, various researchers have found that *Bacillus* sp. as the major producer of chitin deacetylase including *Bacillus* sp. (Natsir, 2002; Kuldeep Kaur *et al.*, 2012), *Bacillus thermoleovorans* (Toharisman and Suhartono, 2008), and *B. stearothersophilus* (Toharisman *et al.*, 2000). The effect of incubation time on chitin deacetylase production was evaluated and found *Bacillus cereus* TK19 produced maximum chitin deacetylase after 48 h of incubation (Fig. 2). There observed a decrease in enzyme production after 48 h which might be attributed by the depletion of the nutrients or production of toxic chemicals by the bacteria upon death phase (Nochure *et al.*, 1993). Wang and Hwang (2001) also reported similar observations with *B. cereus* and *B. sphaericus*.

Fig. 2. Effect of incubation time on chitin deacetylase production by *B. cereus* TK19.



The optimization of production parameters are important in the enzyme production to reduce the production cost and increased yield of the enzyme. Hence, the pH and temperature were optimized for the production of chitin deacetylase by *Bacillus cereus* TK19 strain. The bacteria was allowed to grow at different pHs (4-11) and among the tested pH, pH 8 was found to be best for the maximum production of chitin deacetylase (Fig. 3). The total protein content of the medium also found to be maximum at pH 8 which revealed that, the pH not only helps in the enzyme production but also plays a major role in the bacterial growth. Previous reports also suggested that alkaline conditions favoured the maximum yield of chitin deacetylase by the bacteria (kaur *et al.*, 2012; Zhou *et al.*, 2012).

Fig. 3. Effect of pH on chitin deacetylase production by *B. cereus* TK19.



Temperature is an important factor which plays a major role in various biological processes including bacterial growth and enzyme production. The *Bacillus cereus* TK19 was allowed to grow in different temperatures ranging from 25 to 55°C and assayed for enzyme production. The results revealed that the maximum chitin deacetylase yield was obtained at 35°C beyond which there is a considerable decrease in the enzyme yield was observed (Fig. 4). Kaur *et al.* (2012) and Zhou *et al.* (2012) also reported maximum chitin deacetylase production around 35-40°C by *Bacillus* sp. The effect of pH on the activity and stability of partially purified chitin deacetylase was studied further. The chitin deacetylase was found to be active between the pH ranges of 6 to 9 with the maximum activity was observed at pH 9. A maximum of 100% relative enzyme activity was recorded at pH 9 beyond which the enzyme activity dropped (Fig. 5). Similar trend were observed by other researchers when characterizing *Cohnella* sp. A01 (Aliabadi *et al.*, 2016) and *Aeromonas hydrophila* HS4 (Saima *et al.*, 2013) producing chitinolytic enzymes. The effect of temperature was also considered while characterizing the partially purified chitin deacetylase enzyme. As expected, the maximum chitin deacetylase activity was recorded between 35 and 40°C and beyond which the activity decreased considerably (Fig. 6). The chitin deacetylase was found to be stable up to 40°C for 1 h incubation and lost its activity at 60°C (Fig. 7).

Fig. 4. Effect of temperature on chitin deacetylase production by *B. cereus* TK19.

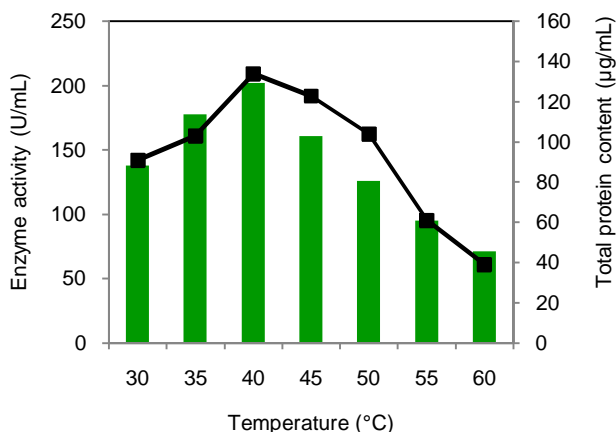


Fig. 5. Effect of pH on activity and stability of chitin deacetylase.

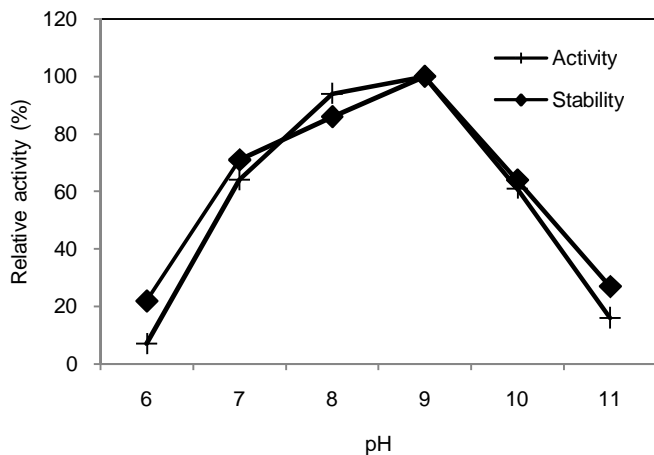
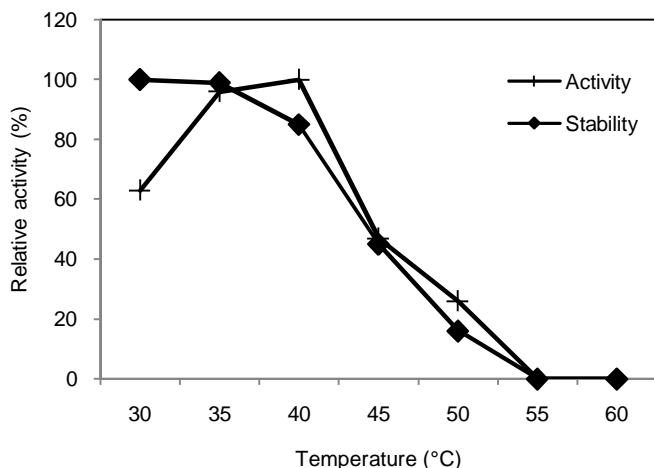


Fig. 6. Effect of temperature on activity and stability of chitin deacetylase.



Saima *et al.* (2013) and Aliabadi *et al.* (2016) also observed similar result while characterizing chitinolytic enzyme using *Cohnella* sp. A01 and *Aeromonas hydrophila* HS4 respectively.

Conclusion

Chitin deacetylase plays an important role in the management and utilization of chitinous as a renewable resource. The results of the present study concluded that the marine derived *Bacillus cereus* TK19 is a novel bacterial isolate having the ability to produce a promising amount of chitin deacetylase in a shorter time of 48 h under optimized conditions. Hence, chitin deacetylase produced by *Bacillus cereus* TK19 can be used in the management of chitinous waste in the sea food industries.

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