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Partial characterization and optimization of protease production from newly isolated *Cohnella thermotolerans* from Lonar Lake

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ABSTRACT:

Lonar Lake, an impact crater located in the Buldhana district of Maharashtra State, India is occupied by saline water and harbors various unidentified, unique haloalkaliphilic bacterial bacillus species which produces thermo-halo-alkaliphilic proteases. The present study deals with the isolation, production dynamics, purification, characterization and optimization of a protease from *Cohnella thermotolerans* isolated and identified by 16s RNA ribotyping from the Alkaline Lonar Lake. The *C. thermotolerans* produced protease at maximum rate after 72 h of incubation at 37^0C with the agitation speed of 120 rpm and 5% of starter culture. The best carbon sources for this *C. thermotolerans* were fructose and maltose and best nitrogen source was yeast extract, while the most effective inorganic nitrogen source was ammonium carbonate. Supplementation of the culture medium with amino acid L -glutamic acid and metal ion Mg^{2+} improved the protease production substantially. Under these conditions, newly isolated *Cohnella thermotolerans* strain was found to produce alkaline protease at a maximum rate of optimum pH 10 and temperature at 75^0C.

Keywords:
Alkaline Protease, *Cohnella thermotolerans*, Environmental factors, Nutritional conditions.

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INTRODUCTION

LONAR Lake, an impact crater located in the Buldhana district of Maharashtra State, India is a circular lake occupied by saline water which was formed by meteoritic impact on basaltic rock and it has been well known as an inland saline lake with a considerable amount of sodium carbonate and chloride (Kanekar *et al,* 2002). Extracellular enzymes like amylase, lipase, protease and cellulases producing *Bacillus cereus, Bacillus firmus, Enterococcus caseliflavus, Bacillus fusiformis, Bacillus cohnii, Bacillus horikoshii* were isolated from water and sediments of alkaline Lonar Lake (Joshi *et al,* 2008). Proteases are industrially important enzymes used in the detergent, food, pharmaceutical and leather industries and also have application in silver recovery from photographic plates and in peptide synthesis which account for about 60% of total industrial enzyme sales (Horikoshii, 1999). The detergent industry is the largest single market for this enzyme. The enzyme has better resistance to alkali and some other denaturing chemicals in the reaction mixture and has a higher affinity towards proteinaceous substrates. It is also a thermostable organism growing in naturally alkaline habitats may have proteases with special characteristics (Norazizah *et al,* 2005). Therefore, attempt was made to isolate new species of bacillus which can produce good quality of proteases useful in the detergent and leather industry. A 16S rRNA gene sequence analysis had been made for identification of the isolated species.

Alkaline proteases produced by *Cohnella thermotolerans* are of great importance in detergent industry due to their high thermo-stability and pH stability and most important industrial enzymes accounting for about 60% of total enzyme market (Kanekar *et al,* 2002; Borsosi *et al,* 2005). Very less study had been done on protease from *Bacilli* of Lonar Lake which can withstand high temperature as well as high pH and has wide applications in different industries. As there is large demand of proteases, isolation and production of protease enzyme is most important to fulfill this demand (Srinivasan *et al*, 2009). The present study aims to deal with the isolation, purification, and characterization and production optimization of a protease from *Cohnella thermotolerans* isolated from the alkaline Lonar Lake.

MATERIALS AND METHODS

Collection of Lonar lake water and sediment sample: Totally four sediments and eight water samples were collected in the year 2009 from alkaline Lonar Lake. Water samples collected in sterilized plastic cans and sediments in sterilized plastic bags were transferred to laboratory for isolation and identification of bacteria followed by their screening for proteolytic activity. During sample collection the date, time and places were noted.

Isolation of Alkaliphiles: About 1.0 g of soil sample was transferred to 99.0 ml sterilized normal saline in 250 ml conical flask and agitated (100 rpm) at 37^0C for 15 minutes in water bath shaker. The sample was then heated at 80^0C for 15 minutes to destroy all the vegetative microbial cells. The suspension was then diluted to 10^{-7} dilutions. One ml of each diluted sample was lawn into petri plates containing nutrient agar medium (pH 10) and inoculated at 37^0C for 24 hours.

Screening of bacterial alkaliphiles: Individual bacterial colonies were screened for proteolytic activities on Skim milk agar medium (skim milk 1%, Peptone 1%, sodium chloride 0.5%, Agar-Agar 2%, pH 10). The pH of the medium was adjusted to 10 with 1N NaOH before sterilization at 121^oC for 15 minutes. The inoculated plates were incubated at 37^oC for 72 hrs and observed for zones of clearance, indicating proteolytic activities.

Identification of the proteolytic isolates: The bacterial isolates with prominent zones of clearance on casein agar medium were processed for identifications based on morphology, Gram characteristics, motility, citrate utilization, oxidase, urease, gelatin liquification, catalase, Vogous-proskaur, indol tests and acid production from glucose, arabinose, lactose, mannitol, galactose and maltose. The isolates were also tested for their growth at different temperatures and pH. These isolates were identified in accordance with the methods recommended in Bergey's Manual of Determinative Bacteriology and Diagnostic Microbiology. The identified strains were maintained on nutrient agar slants having pH 10 at 4.0 ^{o}C.

Phylogenetic analysis: 16S rRNA sequencing was performed at NCCS, Pune. Nearly-full-length 16S rRNA gene sequences were submitted to CHECK-CHIMERA, available on the Ribosomal Database Project release 10.26, in order to identify chimeras. Phylogenetic analyses were performed using the ARB software package. The 16S rRNA gene

phylogenetic analyses were performed by the maximum-likelihood method, using 1,292 nucleotide positions. The functional genes were translated into amino acid sequences, and these were included in phylogenetic analyses using the neighbor-joining method (Dayhoff PAM model).

Preparation of crude enzyme extracts: The 100 ml Yeast extract casein medium (Glucose 1%, Casein 0.5%, yeast extract 0.5%, KH_2PO_4 0.2%, K_2HPO_4 0.2%, $MgSO_4$ 0.1%, pH.10.5) was dispensed (50 ml each) into two 250 ml capacity conical flasks, after adjusting the pH to 10.5 and sterilized in autoclave. After cooling, the broth was inoculated with *Cohnella thermotolerans* cultures and incubated for 72 h at 37^0C in shaking incubator. After 72h incubation, centrifuged the broth at 5000-8000 rpm for 15 min. The supernatant served as crude enzyme source.

Optimization of crude enzyme protease: The standard graph of tyrosine was prepared by adding different concentration of standard tyrosine (1 mg/ml) into a series of test tubes and made the final volume in each test tube to 1 ml with distilled water. Estimation of proteases was carried out with 1 ml of casein in a test tube; 1 ml of enzyme source was added and incubated for 10min at room temperature. After incubation 2 ml of TCA was added to stop the reaction and centrifuged the reaction mixture at 5000-8000 rpm for 15 min. Supernatant was separated and 1ml of Folin-Ciacalteau reagent and 2 ml of Na_2CO_3 were added in 1 ml of supernatant. The reaction mixture was boiled for 1 min in a boiling water bath and 6 ml of distilled water was added to make a final solution to 10 ml. In control tube, the reaction was terminated the reaction at zero time and the absorbance was read at 650 nm (Lalitha *et al,* 2010).

Determination of proteolytic activity: Proteases activity was determined by a slightly modified method of Yang *et al*, 2001. The reaction mixture containing 1 ml of 1.0 % casein solution in 0.2 M Glycine-NaOH buffer having pH 10.5 and 1 ml of a given enzyme solution was incubated at 40°C for 10 minutes and the reaction was then stopped with 2 ml of 10 % tri-chloroacetic acid (TCA). The amount of tyrosine liberated was determined as per tyrosine assay procedure at 650 nm. The proteolytic unit was defined as the amount of the enzyme that released 1ug of tyrosine per minute under the assay conditions.

Partial characterization of protease: Partial characterization of protease was carried out using methodology. (Joo *et al*, 2002).

Effect of pH on alkaline protease activity: The effect of pH on alkaline protease from *Bacillus* spp. was determined by assaying the enzyme activity at different pH values ranging from 7.0 to 10.5 using the following buffer systems with concentration of each buffer at 0.2 M: phosphate (pH 6-7) tris–HCl (pH 8-9) and Glycine-NaOH (pH 10-12).

Effect of temperature on alkaline protease activity: The effect of temperature on alkaline protease activity was determined by incubating the reaction mixture (pH 10.5) for 20 minutes at different temperature ranging from 55°C to 90°C.

Effect of substrate on alkaline protease activity: The effect of ***substrate concentration on*** alkaline protease activity is determined by incubating the reaction mixture (pH 10.5) for 20 minutes with different substrate concentration, ranging from 5 mg/ml to 40 mg/ml.

Fig. 1: Km of isolated protease enzyme

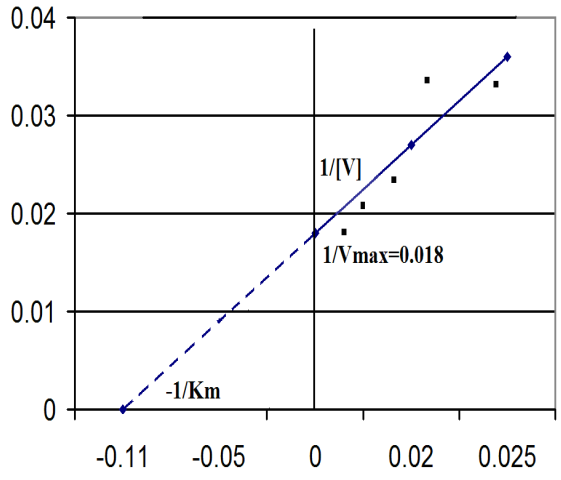


Fig 2: Effect of enzyme concentration on protease activity

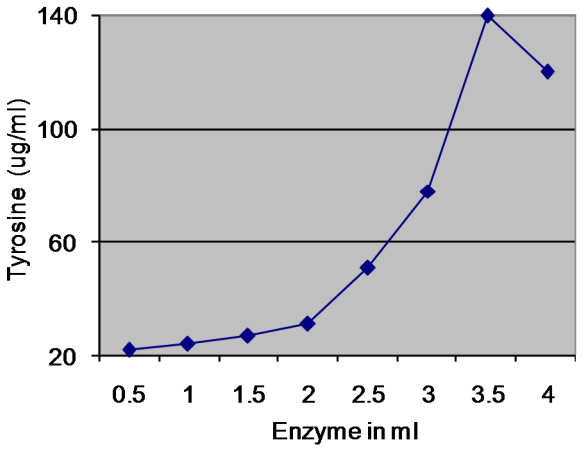


Fig. 3: Effect of pH on proteases activity

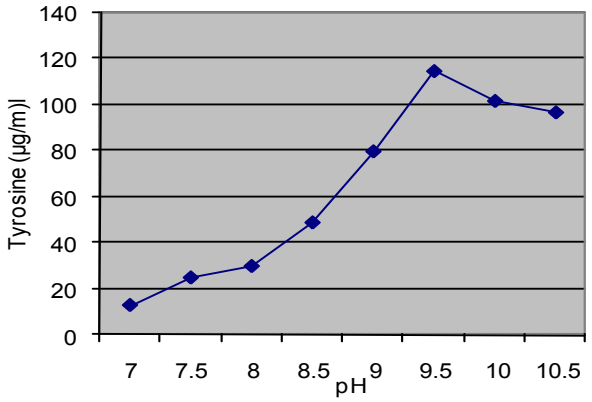
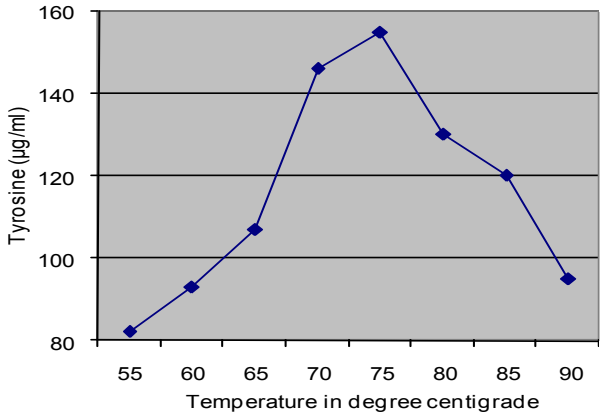


Fig. 4: Effect of Temperature on enzyme activity



Effect of enzyme on alkaline protease activity: The effect of enzyme concentration on alkaline protease activity was determined by incubating the reaction mixture (pH 10.5) for 20 minutes at different enzyme concentration ranging from 0.5ml to 4ml. The activity of the protease was then measured as per assay procedure.

Purification of enzymes: Purification of enzyme will be made by chilled acetone, isopropyl alcohol and Ammonium sulphate precipitation method.

Optimization of environmental and nutritional conditions for the production of alkaline protease: Optimization of proteases production was studied with the help of fermentor by the optimization of medium composition (variation in carbon, nitrogen sources and metal ions) and environmental conditions such pH, temperature, incubation period etc (Kumar *et al,* 2002). The present investigation was aimed at optimization of medium components which have been predicted to play a significant role in enhancing the production of alkaline proteases. Carbon sources chosen for the study were glucose, sucrose, starch, fructose, maltose and lactose. These carbons occur which led to cell lysis and increased cell sources were used to replace the carbon source available permeability due to abrasion by shear forces (Gupta *et al*, 2002). Sources of nitrogen include organic nitrogen, inorganic nitrogen and amino acid in which sources throughout the study were soy tone, soya bean cake, beef extract, yeast extract, peptone, ammonium nitrate, ammonium carbonate, urea, lysine, L-aspartic acid, glutamic acid and glycine. Metal cations tested to replace metal ion source in the Media were Ca^{2+}, Cu^{2+}, Mg^{2+} and Mn^{2+} (Adinarayana *et al,* 2003).

RESULTS AND DISCUSSION

In the present study, a total of 104 bacterial isolates were isolated from water and sediment sample of Lonar Lake, maintained on slant of nutrient agar (pH 10.5) and various tests were performed for identification of bacteria. Out of them 67 were from water and 37 from sediments. Then these cultures were inoculated on alkaline skim milk agar at pH 10.5 for studying their proteolytic activity and using morphological and biochemical characteristics. Out of 104 cultures, 37 isolates were identified as *Bacillus*. Out of 37 isolates of sediments, only 15 isolates were efficient in protease production and most efficient bacillus species were used to study different enzyme parameter. Among them, a bacterial culture identified as *Cohnella thermotolerans* by 16S rRNA analysis were used for detail study of protease production and optimization (**Table 1**). Alkaline protease production was maximum at pH 9 -10.5. Maximum protease production was recorded after 72 h of incubation at 37°C. In the effect of substrate concentration on enzyme activity of protease, the Michalies Menten constant (K_M) and Maximum velocity (V_{Max}) was found to be 9.09 mg/ml and 0.018 mg/ml by Line weaver-Burk plot. The optimum enzyme concentration required for maximum activity of protease is 3.5 ml.

Effects of carbon sources on protease production: It has been reported that pure sugars affected protease production considerably. Utilization of pure sugars as carbon and energy sources was also shown to result in good growth with increased protease production. This observation was in agreement with previous studies which suggested that larger amount of enzyme was synthesized when carbon sources used were poorly utilized for growth purposes (Ismail and Fattah,

Fig. 5: Effect of pH on protease production

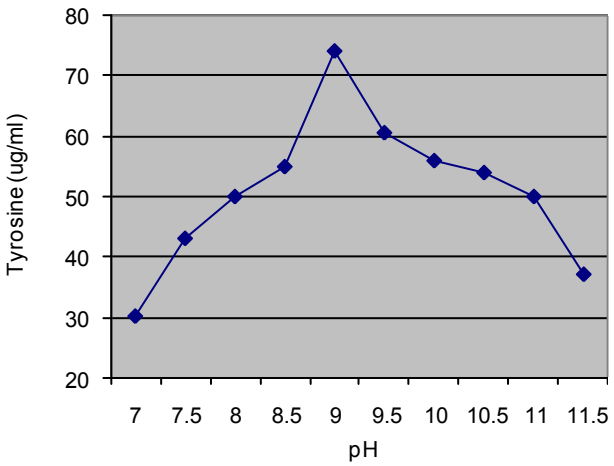
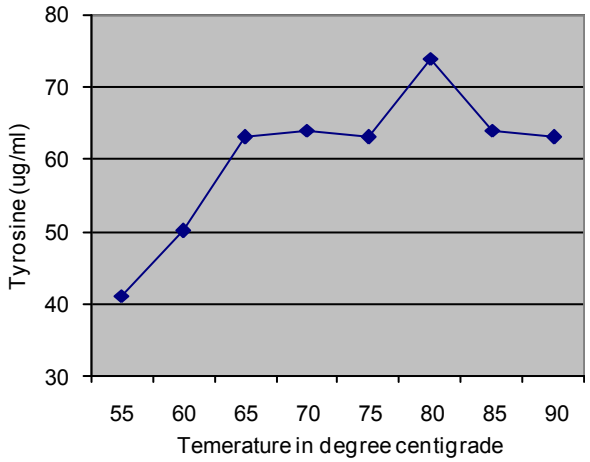


Fig. 6: Effect of temperature on protease production



1999). Various sources of carbon such as glucose, fructose, sucrose, maltose, starch and lactose were used in enhancing the production of alkaline proteases. Results obtained showed that starch instigated highest protease production compared to other carbon sources at 72 h of incubation due to the prolonged incubation time perhaps led to auto digestion of proteases and proteolytic attack by other proteases. Fructose, maltose and lactose caused slightly low protease production.

Effect of nitrogen sources on protease production:

Effect of organic nitrogen sources on protease production: In this study, sources of organic nitrogen like soy ton, soya bean cake, beef extract, yeast extract and peptone were used. It was showed that soy ton resulted in the highest level of protease activity compared to other sources of organic nitrogen. Despite the luxurious bacterial growth the presence of beef extract, peptone and tryptone resulted in low protease production. This observation contradicted (Phadatare *et al,* 1993) which reported that protease production in *Conidiobolus coronatus* was enhanced by organic nitrogen sources like yeast extract, peptone and tryptone.

Effects of inorganic nitrogen sources on protease production: Inorganic nitrogen sources like ammonium carbonate, ammonium nitrate and urea were tested on the growth and protease production. Among them, ammonium carbonate led to high protease activity at 48 and 72 h. Urea did not enhance protease production at early stages of incubation but at later stages (48 and 72 h) protease production increased. Even though growth was stimulated, only moderate levels of enzyme

Fig 7: Effect of carbon sources on protease production

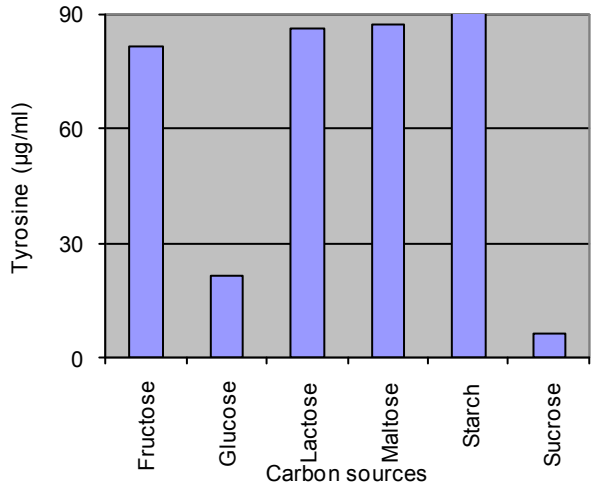


Fig 8: Effect of nitrogen sources on protease production

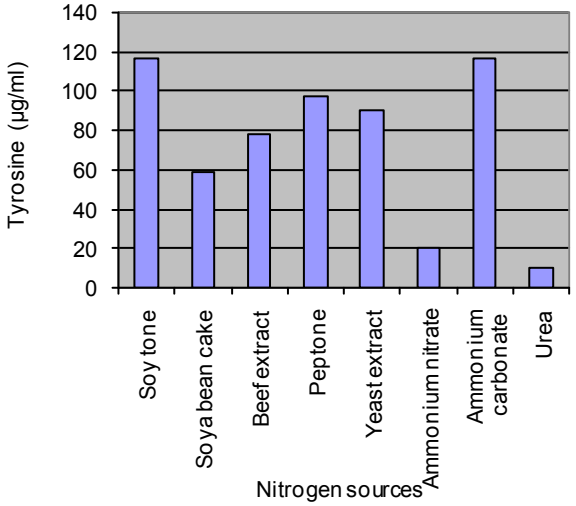


Fig 9: Effect of amino acid on protease production

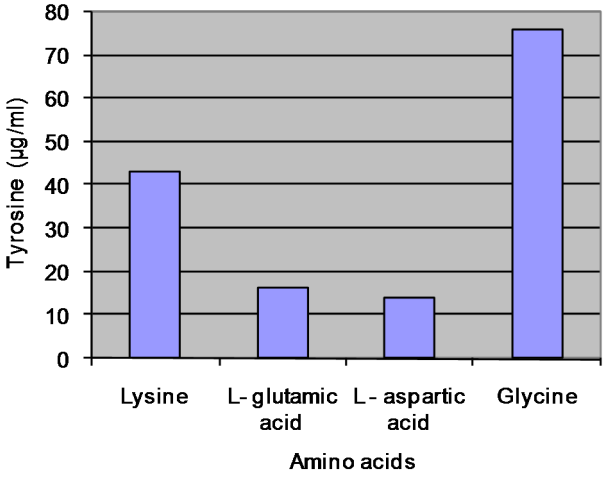
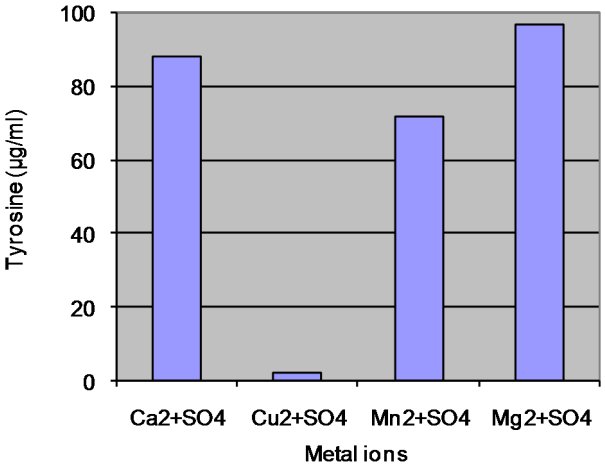


Fig 10: Effect of metal ion on protease production



activities were obtained when ammonium nitrate was used as a nitrogen source. This was perhaps due to the inability of bacteria to utilize ammonia in the media (Ellaiah *et al,* 2005).

Effects of amino acid on protease production: This observation was corroborated by L-aspartic acid; glutamic acid and glycine were also tested as sources of amino acids for protease production in *Cohnella* strain 146. In the presence of Glycine, protease production was observed to be high.

Effects of metal ions on protease production: The highest level of protease activity was observed in the presence of Mg^{2+} at 72 h incubation. Addition of Ca^{2+}, Cu^{2+} and Mn^{2+} resulted in high protease production only at 48 h incubation. It was suggested that these metal ions increased stability of proteases, even though effects of the different metal cations on protease production vary, their presence in the culture medium improved the growth of *Cohnella* strain. Supplementation of culture medium with metal cations improved substantially the protease production of *Cohnella thermotolerans*. This observation strongly suggested the requirement of some metal ions for protease production by this organism. These results were in agreement with the earlier findings which showed enhancement of protease activity in the presence of metal ions and it was suggested that these metal ions increased stability of proteases. (Banerjee *et al*, 1999).

Effect of pH and temperature on protease production: This observation was corroborated by different pH and Temperature range. *Cohnella thermotolerans* strain produced maximum alkaline protease at pH 9 and temperature at 80^0C.

In summary, isolated *Cohnella thermotolerans* species from Lonar Lake produce alkaline protease and maximum growth at pH 9-10.5. The isolated bacterial *C. thermotolerans* strain produces the proteases enzyme which was theomorphic, alkaliphilic and has potential to produce good quality proteases which can use in the industry. The *C. thermotolerans* species were most efficient for protease producing at pH 10.5 incubated at 37^0C for 72h. The protease produced from this species were highly efficient at high temperature, high salt concentration and tolerate the other environmental conditions. This bacterial species is ubiquitous and non-pathogenic, not causing any diseases to human beings and most efficient for protease production among all isolated protease-producing bacteria. Protease enzymes have importance in various industries. *Bacillus* species particularly *Cohnella thermotolerans*, were known for their ability to produce proteolytic enzymes with potential use in industries (Kampfer *et al*, 2006). In addition to the limited number of reports, protease production by this microorganism also was shown to be affected by various environmental and nutritional conditions. In the present investigation, we have determined the optimum parameters for maximum production of alkaline protease by the newly isolated thermophilic bacterium *Cohnella thermotolerans*. This information has enabled the ideal formulation of media composition for maximum protease production by this organism.

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