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Research Article

Protease from *Cohnella thermotolerans:* A laundry additive

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ABSTRACT

LONAR Lake, an impact crater, highly saline, is located in the Buldhana district of Maharashtra State, India, with a considerable amount of sodium carbonate and chloride. The present study deals with the production and optimization of wash performance analysis of Alkaline Protease produced from *Cohnella thermotolerans* isolated and identified by 16s rRNA ribotyping from the Alkaline Lonar Lake. The *Cohnella thermotolerans* produced protease at maximum rate after 72 h of incubation at 37^{0}C with agitation speed of 120 rpm and 5% of starter culture. It was found that proteases produced by *Cohnella thermotolerans* showed higher compatibility with Tide, Ariel, Nirma, Surf excel, Rin, Sargam plus and Active wheel at zero time. But after Enzyme solutions were incubated with the deactivated detergents in a final concentration of 7mg/ml at 50^{0}C for 30 minutes, it was found that proteases produced by *Cohnella thermotolerans* decreased compatibility with Tide, Ariel, Nirma, Surf excel, Rin, Sargam plus but remain constant in Active wheel. It was found that among the different washing condition, the mixture of alkaline protease produced by *Cohnella thermotolerans* exhibited better action with faintness of the chocolate spots, sauce spots and turmeric powder on the cloth. The *Cohnella thermotolerans* showed efficient removal of dirt / faintness from cloth among all other protease producing bacilli which has wide industrial applications.

Keywords: Alkaline Protease, *Cohnella thermotolerans*, wash performance test, compatibility test.

INTRODUCTION

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-alkalophilic proteases. 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 sediment of alkaline Lonar Lake [1]. The detergent industry is the largest single market for protease 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 [2]. It is also thermostable organism growing in naturally alkaline habitats may have proteases with special characteristics [3]. Therefore, a 16S rRNA gene sequence analysis was made to isolate new species of bacilli which can produce good quality of proteases useful in the detergent and leather industry.

Alkaline proteases produced by *Cohnella thermotolerans* are of great importance in detergent and leather industry due to their high thermo and pH stability which accounting for about 60% of total enzyme market [4,5]. Very less study has been done on protease from *Bacilli* of Lonar Lake which can withstand high temperature as well as high pH. Therefore, as there is large demand of proteases, isolation and production of protease enzyme is most important to fulfill this demand [6]. The present study deals with the compatibility and wash performance analysis of alkaline protease produced from *Cohnella thermotolerans* isolated from the alkaline Lonar Lake.

MATERIALS AND METHODS

Screening and Identification of the proteolytic isolates: Total four sediment and eight water samples were collected from alkaline Lonar Lake

for isolation and identification of bacteria followed by their screening for proteolytic activity. Isolated bacilli 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) and isolates with prominent zones of clearance on medium were processed for identifications based on Bergey's Manual of Determinative Bacteriology and Diagnostic Microbiology. The identified strains were maintained on nutrient agar slants having pH 10 at 4.0^0C as well as isolated strains were then analyzed by 16S rRNA at NCCS, Pune and BLAST identification was made.

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 *Bacillus pseudofirmus* 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.

Determination of proteolytic activity and Partial characterization of protease: Proteases activity was determined by a slightly modified method of Yang *et al* [7]. 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 was carried out as per Joo *et al*, [8].

Effect of pH, temperature, substrate and enzyme concentration 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 was 0.2 M: phosphate (pH 6-7), tris–HCl (pH 8-9) and Glycine-NaOH (pH 10-12). 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^oC to 90^oC. The effect of substrate concentration on alkaline protease activity was 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. 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 [9].

Compatibility with various commercial detergents: For commercial exploitation of enzyme, the isolated protease was analyzed for its compatibility with commercial detergent by incubating with locally available detergents at 40^0C for 30 minute and the residual activity was examined by assay method [10]. The detergent Tide, Ariel, Nirma, Surf excel, Rin, Sargam plus and Active wheel were diluted in distilled water to a final concentration of 7 mg/ml to stimulate washing conditions. The indigenous enzyme in the detergents was deactivated by heating at 100^0C for 10 min. Enzyme solutions were incubated with deactivated detergents at 50^0C for 30 minutes. The residual activities were determined under assay conditions and compared with control samples incubated at 50^0C for 30 minutes without any detergent. The enzyme activity of control was taken as 100%.

Wash performance test/ Destaining efficiency of enzymes: The dirty cloth piece were washed with commercial detergent, isolated alkaline proteases at various temperatures and examined for the removal dirt / faintness from cloth [10]. Pieces of white cotton clothes (5 cm x 5 cm) were stained with chocolate spots, turmeric powder and tomato sauce spots separately and taken in separate flasks as described below.

1. Distilled water (100 ml) + stained cloth
2. Distilled water (100 ml) + stained cloth+ 1ml detergent (7mg/ml).
3. Distilled water (100 ml) + stained cloth + 2ml enzyme solution.
4. Distilled water (100 ml) + stained cloth + 1ml detergent (7mg/ml) + 2ml enzyme solution.

All sets of flasks were kept for 15 minutes. After incubation, cloth pieces were taken out and dried. Visual examination of the cloth pieces exhibited the effect of enzymes in the removal of stains. Untreated cloth pieces stained with spots were taken as control [11].

RESULTS AND DISCUSSIONS

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 using morphological and biochemical characteristics. Out of 104 cultures, 37 isolates were identified as *Bacillus*. Out of 37 isolates of sediments, only 8 isolates were efficient in protease

production and most efficient bacillus species were used to for detail study. Among them, a bacterial culture identified as *Cohnella thermotolerans* by 16S rRNA analysis at NCCS, Pune and BLAST identification were used for detail study of protease production and optimization. On the basis of 16S rRNA gene sequence analysis, it was confirmed "*Bacilli*" and order *Bacillales* as *Cohnella thermotolerans* with <98.5% sequence similarity.

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 3.5 ml. The optimum pH and temperature required for maximum activity of protease was 9.5 (Fig.3) and 75^{0}C respectively (Fig.4). Effect of pH and temperature on protease production of *Cohnella thermotolerans* strain produced maximum alkaline protease at pH 9 (Fig.5) and temperature at 80^{0}C (Fig.6).

Fig. 1: Km of isolated protease enzyme

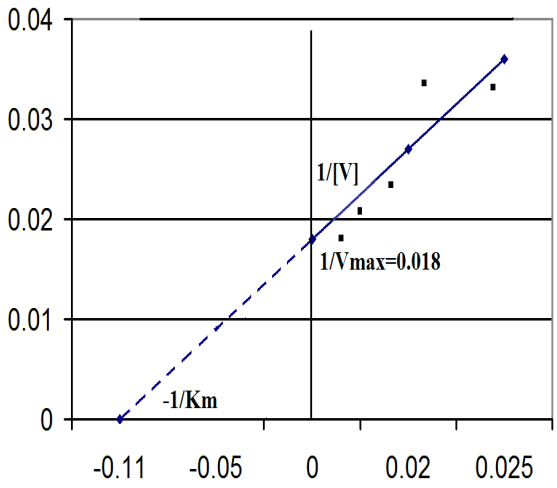


Fig 2: Effect of enzyme concentration on protease activity

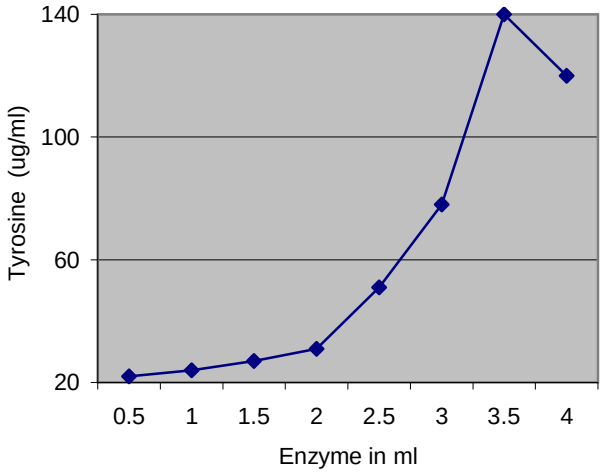


Fig. 3: Effect of pH on proteases activity

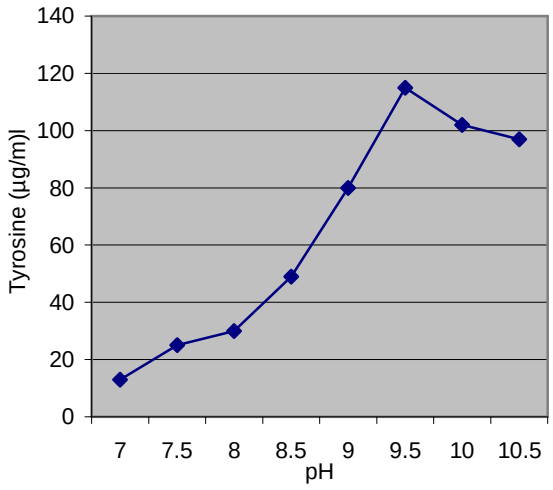
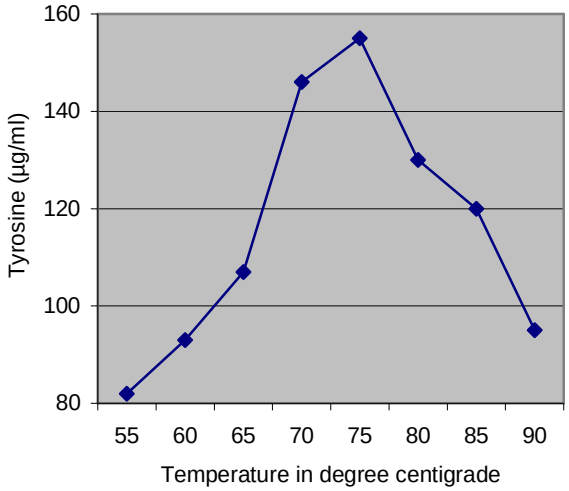
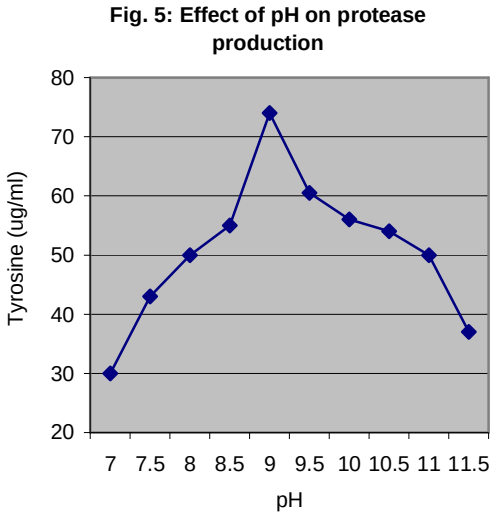
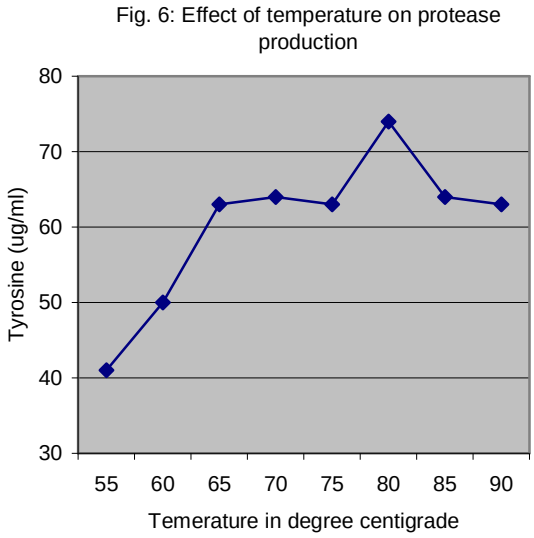


Fig. 4: Effect of Temperature on enzyme activity

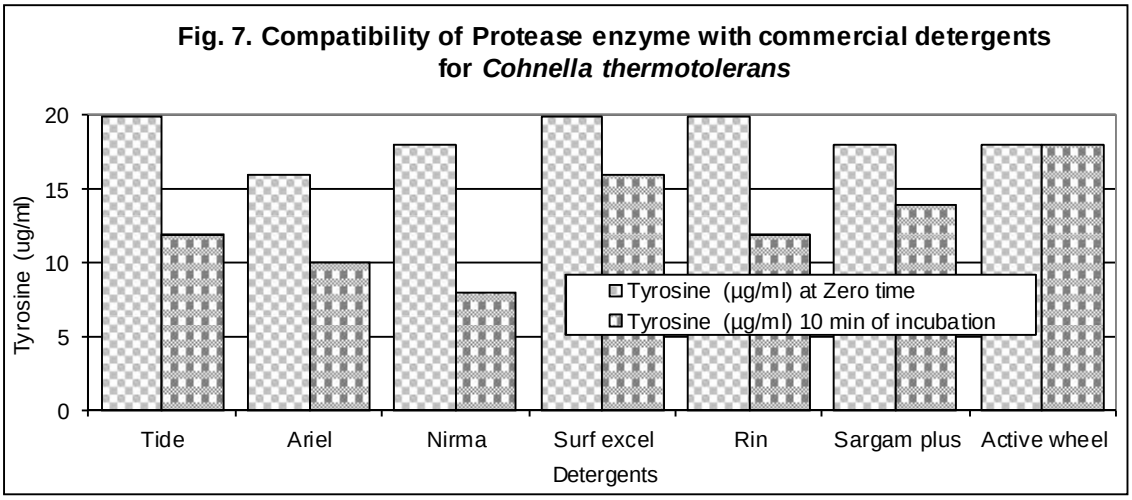






Compatibility with various commercial detergents: Besides pH and temperature stability, a good detergent enzyme should also be stable to various detergent ingredients such as surfactants, chelators and oxidants. Enzyme exhibiting activities in the high alkaline range are recognized as potential detergent additives and stain removing formulations. Taking this into consideration, alkaline protease are characterized for their stability in presence of bleaching agents, surfactants and optical brightener that are normally used as ingredients of the washing detergents. The enzyme activities were assayed and expressed in terms of residual activity (%) considering control as 100%.

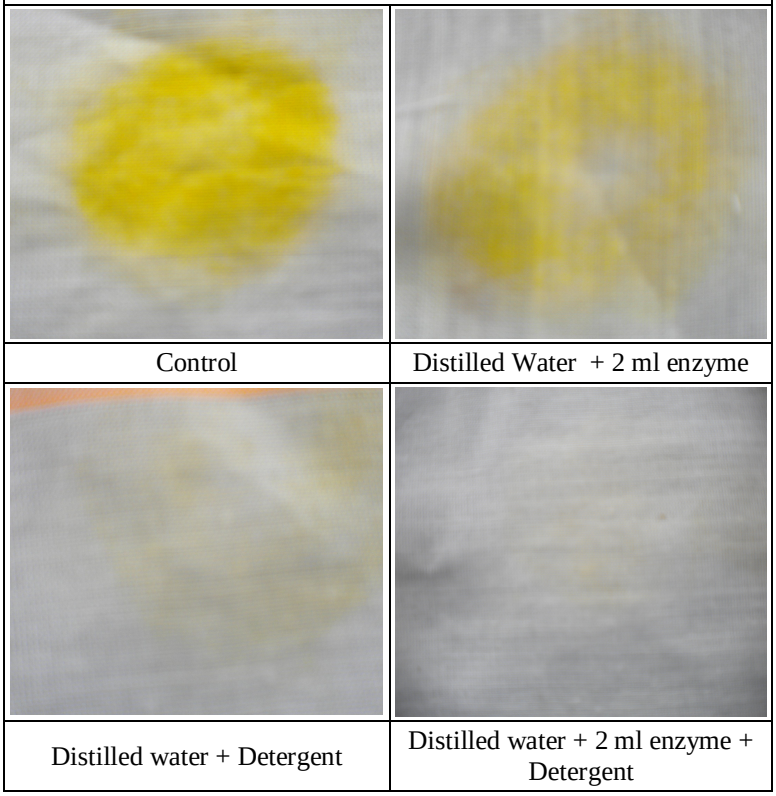
In the detergent industry, several chemical detergents were used to formulate industrial products and hence detergent stable enzymes were suitable for such industries [12]. A good detergent enzyme is expected to be stable in presence of commercial detergents. Enzyme solutions were incubated with the deactivated detergents in a final concentration of 7mg/ml and incubated at 50^0C for 30 minutes. It was found that proteases produced by *Cohnella thermotolerans* showed higher compatibility with Tide, Ariel, Nirma, Surf excel, Rin, Sargam plus and Active wheel at zero time. But after Enzyme solutions were incubated with the deactivated detergents in a final concentration of 7mg/ml and incubated at 50^0C for 30 minutes, it was found that proteases produced by *Cohnella thermotolerans* decreased compatibility with Tide, Ariel, Nirma, Surf excel, Rin, Sargam plus but remain constant in Active wheel (Fig.7).



Our results were in agreements with several reports of alkaline protease cited in literature. In this regard, Venugopal and Saramma [13], reported that alkaline protease obtained from *Bacillus circulans* was considerably stable with commercial detergents like Ariel, Rin, Henko, Surf and Tide. After one hour incubation, the enzyme was reported to have retained 72% activity with Ariel and Rin, 74% with Henko and 70% with Surf and Tide.

Wash performance test/ Destaining efficiency of enzymes: To ascertain the application of alkaline protease as a wash detergent additives, the experiment was carried out which included the soaking of dirty white cloth pieces in different solutions for 15 min. It was found that among the different conditions of washing tested, the mixture of alkaline protease produced by *Cohnella thermotolerans* exhibited better action by showing faintness of the chocolate spots, sauce spots and turmeric powder on the cloth. The *Cohnella thermotolerans* showed efficient removal of dirt / faintness from cloth among all other protease producing bacilli which has wide industrial applications (Fig.8).

Fig. 8. Wash performance test: Efficacy of protease from *Cohnella thermotolerans*



Control	Distilled Water + 2 ml enzyme
Distilled water + Detergent	Distilled water + 2 ml enzyme + Detergent

These results were collaborated with previous findings of Jaswal and Kocher [10], who study the enzyme incubated with detergent solution (either in water or in buffer) revealed that when used in water, Fena and Rin showed maximum compatibility whereas the buffered detergent solution revealed maximum compatibility of alkaline protease with Tide. Enzymes from microorganisms that can survive under extreme pH could be particularly useful for commercial applications under highly alkaline reaction conditions, e.g. in the production of detergents. Alkaline proteases produced by *Bacillus* species were of great importance in detergent industries due to their high thermal and pH stability [14]. Nadeem *et al,* [15] studied protease production by alkalophilic *B. licheniformis* N-2 for removal of blood stains from cotton fabric also indicates its potential use in detergent formulations. Adinarayana *et al* [2] studied on purification and characterization of thermostable serine alkaline protease from a newly isolated *Bacillus subtilis.* The enzyme produce by *Cohnella thermotolerans* improved the cleansing power of various detergents. It removed bloodstains completely when used with detergents in the presence of 10 mM $CaCl_2$ and 1M glycine.

CONCLUSION

In Conclusion, isolated *Cohnella thermotolerans* species from Lonar Lake produce alkaline protease and maximum growth at pH 8.5-10.5. The isolated bacterial *Cohnella thermotolerans* strain produces the proteases enzyme which was theomorphic, alkaliphilic and has potential to produce good quality proteases which can use in the industry. It was found that proteases produced by *Cohnella thermotolerans* showed higher compatibility with Tide, Ariel, Nirma, Surf excel, Rin, Sargam plus and Active wheel at zero time. But after Enzyme solutions were incubated with the deactivated detergents in a final concentration of 7mg/ml and incubated at 50^0C for 30 minutes, it was found that proteases produced by *Cohnella thermotolerans* decreased compatibility with Tide, Ariel, Nirma, Surf excel, Rin, Sargam plus but remain constant in Active wheel. It was found that among the different conditions of washing tested, the mixture of alkaline protease produced by *Cohnella thermotolerans* exhibited better action by showing faintness of the chocolate spots, sauce spots and turmeric powder on the cloth. The isolates of bacilli *Cohnella thermotolerans* showed efficient removal of dirt / faintness among all other protease producing bacilli. The protease produced from this species was highly efficient at high temperature, high salt concentration and tolerate the other environmental conditions. Protease enzymes produced in the present investigation have found to be important in various industries like detergent and leather etc. The present investigation indicates the use of this enzyme in detergent formulation and leather industry.

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