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Research Article

Partial characterization of protease from *Bacillus flexus*

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Abstract

Halo-alkaliphiles grow high pH and high salt concentration can be a source of novel enzymes. The enzymes produced by these bacteria have great importance in industry due to its high thermo and pH stability. The alkaline protease enzymes have great importance in industries due to its thermo and pH stability. The alkaline protease producing bacteria are generally found in sea water or alkaline lakes such as Lonar Lake. Present study deals with the isolation, characterization, production dynamics and optimization of protease from bacteria. A total of 6 bacterial cultures were isolated from alkaline Lonar lake, one strain DHT13 showed prominent proteolytic activity which was studied further for its phenotypic and biochemical characters. The bacterium DHT13 was screened for production and partial characterizations of protease, and 16SrRNA sequencings identified as *Bacillus flexus*. Protease from *Bacillus flexus* is active at high temperature of 80^0C and pH 10 and finds potential applications in food, pharmaceutical, leather and detergent industries.

INTRODUCTION

Proteases are enzymes occurring everywhere in nature being inside or on the surface of living organisms such as plants, animals and microbes. These enzymes carry out proteolysis by hydrolysis of the peptide bond that exists between two amino acids of a polypeptide chain (Singhal *et al.,* 2012). The proteases available today in the market are mostly derived from microbial sources. Many bacteria like *Bacillus cerus*, *Bacillus fermus*, *Bacillus pseudofirmus* , *Enterococcus caseliflavus* are well known for the production of thermostable and alkali stable protease enzyme (Tambekar *et al.,* 2011). Proteases are industrially important enzymes used in the detergent, food, pharmaceutical, 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 (Horikoshi *et al.,* 1999). The detergent industry is the largest single market for this enzyme. The alkaline enzyme has better resistance to alkali and some other denaturing chemicals in the reaction mixture and has a higher affinity towards proteinaceous substrates. The microorganism growing in alkaline and thermostable habitats produces the alkaline-thermostable protease may have with special characteristics (Shafee *et al.,* 2005). Very less study had been done on protease from *Bacilli* of Lonar Lake, which can withstand high temperature and high pH and has wide applications in different industries (Srinivasan *et al.,* 2009). In present study, aimed to deal with the isolation, purification, characterization, production and optimization of a protease from bacterial strain isolated from the alkaline Lonar Lake and which is useful in the food, pharmaceutical, detergent and leather industry.

MATERIALS AND METHODS

Collection, Enrichment, Isolation and Identification of protease producing bacteria: Total 12 samples (sediment and matt) were collected in august 2014 from alkaline Lonar Lake. Sample is diluted to 1/100 in sterilized normal saline and heated at 80^0C for 15 min to destroy all the vegetative microbial cells. The suspension was further diluted to 10-7 dilutions one ml each was subcultured and screened for proteolytic activities on skim milk agar medium at 37^0C for 72 h and observed for zones of clearance, indicating proteolytic activities. The bacterial isolates with prominent zones of clearance on skim milk agar medium were processed for identifications based on cultural, morphological and biochemical was done by commercially available Hi-media Rapid detection kit KB003 and KB009. The 16S rRNA gene sequence was perform at Agharkar Research Institute, Pune

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 10.5 and sterilization, the bacterial culture nd was inoculated with culture and incubated for 72 h at 37^0C in rotary shaking incubator. After 72h incubation, centrifuged the broth at 5000- 8000 rpm for 15 min. The supernatant served as crude enzyme source.

Preparation of standard graph: 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).

Assay and kinetics alkaline Lipase: 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 40^0C for 10 min 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 1µg of tyrosine per min under the assay conditions. The effect of pH on alkaline lipase from *Bacillus flexus* spp. was determined by assaying the enzyme activity at different pH ranging from 7.0 to 10.5 and effect of temperature at different temperature ranging from 40^0C to 100^0C. The effect of substrate and enzyme concentration on alkaline lipase activity was determined by incubating the reaction mixture (pH 10.5) for 30 min with variable substrate and enzyme concentrations (Adinarayan *et al.*, 2003).

RESULTS AND DISCUSSION

In the present study, a total of 17 different bacterial species were isolated from sediment and matt samples on Lonar Lake. Out of 17, six isolates were showed maximum casein hydrolysis activity on skim milk agar at pH 10.5. Out of them one isolate DHT13 was selected for further study since it showed maximum proteolytic activity is 30mm (Fig. 1). These isolate was characterized by cultural, morphological and biochemically by commercially available Hi-media Rapid detection kit KB003 and KB009. The isolate DHT13 was gram positive, rod shape and motile. Growth was detected with different pH 7 to 11 and NaCl concentration from 1-5% NaCl. The growth of isolate DHT13 was found to be at 40^0C to 55^0C temperature (Table 1). Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain DHT13 was identified as a *Bacillus flexus*. The optimum enzyme concentration required for maximum activity of protease 151 µg/mL (Fig.4) and substrate concentration on enzyme activity of protease, the Michalies Menten constant (*K*m) and Maximum velocity (*V*max) was found to be 6.66 mg/mL and 0.01µg/mL by Line weaver-Burk plot. From the data it showed that, the maximum activity was observed on pH 10 and in case of temperature; maximum temperature was showed at 80^0C by *Bacillus flexus* (Fig.2 and Fig.3). In the similar study (Nasser *et al.*, 2007) reported that the optimum temperature for proteolytic activity of protease producing bacteria was 37^0C-50^0C. Thus, the result was also related with Abdel (Nasser *et al.*, 2007) result.

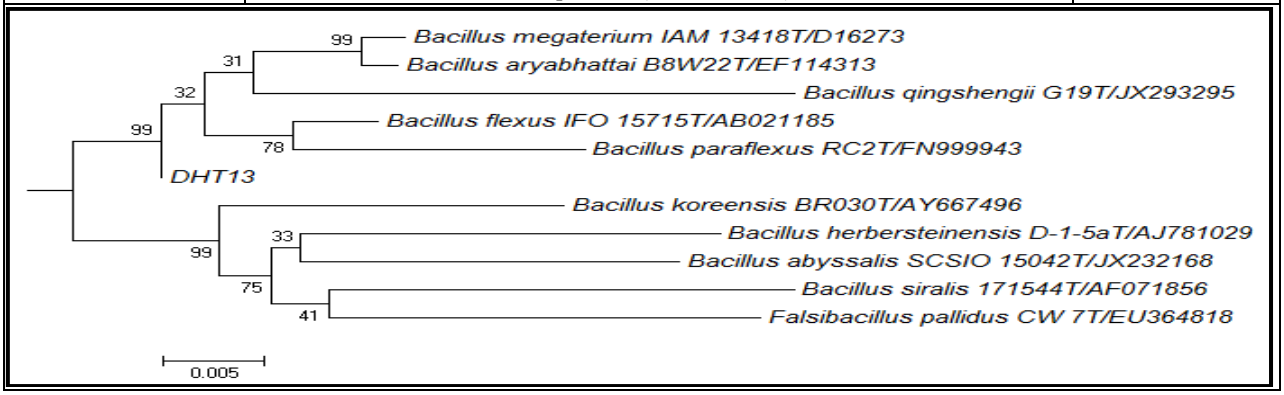
Tambekar *et al.*, 2011 reported that optimum pH for proteolytic activity of protease producing bacteria was observed between pH 9-10.5. Similar result was also observed in the present study. These indicate that for the growth of protease producing bacteria, alkaline environment is more suitable. This idea is also supported by Kuberan *et al.*, 2010. The phylogenetic position indicated the Lonar lake bacterial strains were related to phylum Firmicutes and belongs to genera Bacillus and are *Bacillus flexus*, *Bacillus pseudofirmus*, *Bacillus cereus*, *bacillus* sp. (Tambekar and Dhundale, 2013).

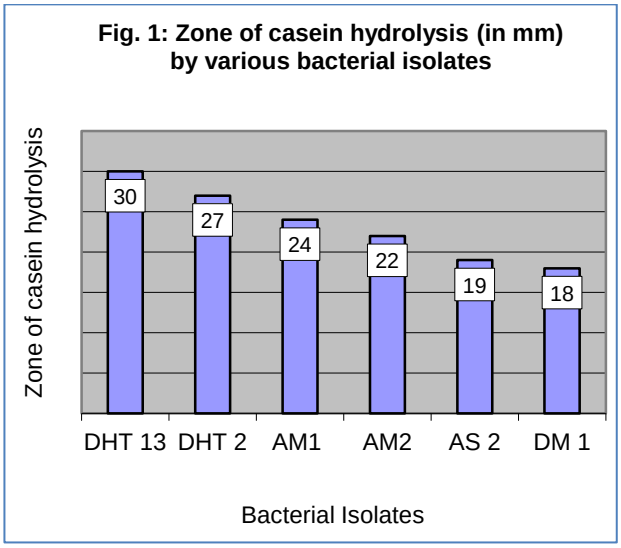
Table 1. Morphological and biochemical characteristics of bacteria isolated from Lonar Lake

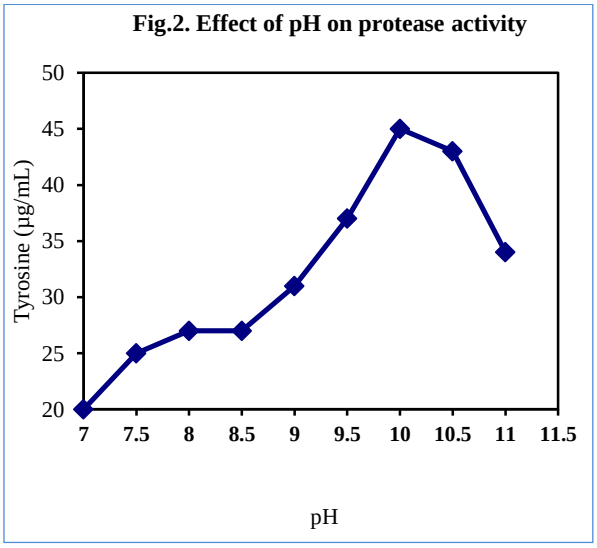
Test	Result	Test	Result	Test	Result
shape	Rod	Catalase	+	VP	-
Colour of colony	Milky White	Nitrate reduction	+	Arginine	+
Gram staining	+ve	Oxidase	+	Sucrose	+
Texture	Smooth	citrate	-	Maltose	+
Arrangement	single	Lactose	-	Fructose	+
Motility	Motile	Xylose	+	Dextrose	+
Growth at different temperature		Glucose	+	Melibiose	+
30^0c	+	Arabinose	-	Mannose	+
40^0c	+	Lysine Utilization		Sodium Gluconate	+
50^0c	+	Galactose	+	Glycerol	-
Growth at different pH		Raffinose	+	Salicin	-
pH 7	+	Trehalose	+	Dulcitol	-
pH 8	+	Mannitol	+	Inocitol	-
pH 9	+	Adonitol	-	Sorbitol	+
pH 10	+	Saccharose	-	Erythritol	-
pH 11	+	Ornithine	-	Melezitose	-
Growth at different salt concentration		Esculin hydrolysis	-	α- Methyl-D-Glucoside	-
1%	+	Rhamnose	-	Xylitol	-
2%	+	Cellibiose	-	Sorbose	-
3%	+	ONPG	-	L-Arabinose	-
4%	+	Esculin	-	Inulin	-
5%	+	Malonate	-	MR	-

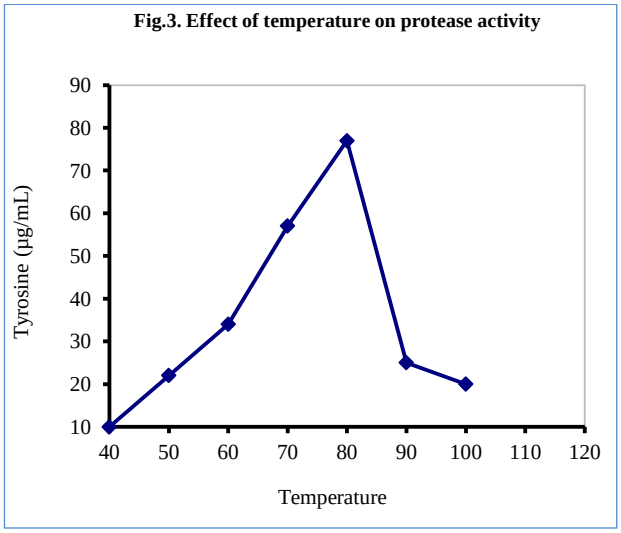
Table 2: The 16S rRNA gene sequencing Closest phylogenetic affiliation and pair similarity of isolated phenol degrading organism from Lonar lake

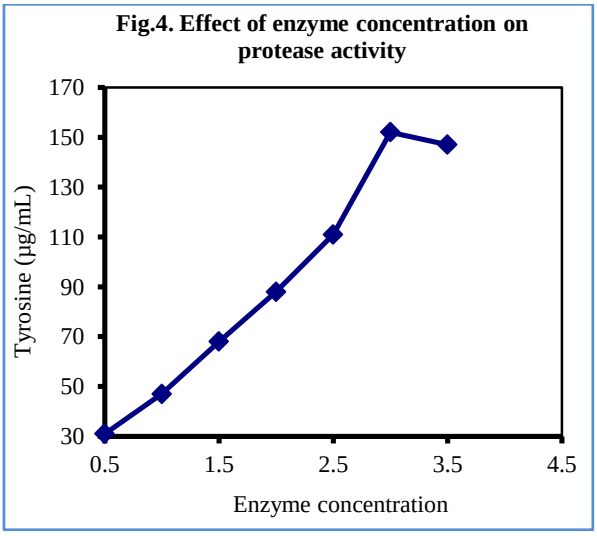
DHT 13	*Bacillus flexus* IFO 15715(T) 16S ribosomal RNA gene partial sequence (AB021185)	99.70%











Protease is one of the most important industrial enzymes known and is of great significance having approximately 25% of enzyme market and finds potential applications in food, pharmaceutical, leather and detergent industries. This study reports production of protease isolated from alkaline Lonar Lake. Protease from *Bacillus flexus* and *Bacillus sp.* is active at high
temperature, 80°C and pH 10 and finds potential applications in food, pharmaceutical and detergent industries

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