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ALKALINE PROTEASE PRODUCTION BY THERMOPHILIC AND ALKALOPHILIC HALOTOLERANT *BACILLUS* SP. STRAIN TD : A PROMISING ENZYME PRODUCER FOR BIOTECHNOLOGICAL APPLICATION

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ABSTRACT
Alkaline protease producing halotolerant bacteria were isolated from marine water, Gulf of Cambay, Gujarat. Isolates were screened for alkaline protease and potential enzyme producer was selected for further studies. Isolate was identified by biochemical test and identified as *Bacillus* sp. strain TD. Environmental conditions were optimized for higher enzyme production suggests that isolate was able to produce enzyme at 45 ºC and at pH 11. Effect of NaCl indicates that organism was able to tolerate 0.5% NaCl. Effect of organic and iorganic nitrogen source suggests that cells produce higher enzyme in response to organic compounds than inorganic compounds. Enzyme was purified using Sephadex G-200 column chromatography. Molecular weight determined by gel electrophoresis showed it to be greater than 66KD.

KEY WORDS: *Bacillus*, alkaline protease, halotolerant, zymogram.

INTRODUCTION

In recent years, chemical catalysts are substituted by the microbial enzymes in manufacturing chemicals, leather goods, food, paper, pharmaceuticals and textiles (Oberoi et al. 2001). Lesser generation time of microorganisms compared to plant and animals has also attracted the most industries to mitigate the current demand of industrial important enzymes. Furthermore, microbial enzymes produced by fermentation methods can easily be scaled up. The majority of microbial enzymes produced on a commercial scale are extracellular, which exhibit stability to chemical and physical changes in the medium and are potentially employed in industries. Proteases constitute one of the most important groups of industrial enzymes, accounting 40 – 60 % of total enzyme sales with two-thirds of the proteases produced commercially are from microbial origin (Joshi et al. 2007). Of late, greater emphasis has been on protease production and hence, several microbes have been investigated for their ability to secrete these enzymes. However, little information is available for marine halotolerant bacteria for alkaline protease production. Hence, present study aims to examine alkaline protease production by marine halotolerant bacteria and its characterization.

MATERIALS AND METHODS

Isolation: Halotolerant organisms were isolated from the marine water collected from Gulf of Khambhat on nutrient agar (pH 8) containing 0.5 % NaCl. Isolates were further purify and stored on nutrient agar slant at 4 °C until the use. ***Screening and identification:*** Halotolerant bacteria were screened for the alkaline protease production by casein agar and milk agar plates contain 0.5 % NaCl. Isolates showed cleared zone near colony were selected for further screening by quantitative enzyme assay. Potential organisms was identified by morphological and biochemical tests. ***Enzyme assay:*** Enzyme activity of alkaline protease in the culture supernatant was measured as method described by Hagihara (1958) using casein as the substrate. One unit of the alkaline protease activity (U) was defined as the amount of enzyme liberating 1 µg of tyrosine per min per ml under the standard assay conditions. The estimations were based on a tyrosine calibration curve.

Identification of bacterial isolate: The colony characteristics and cellular morphology, staining reactions, physiolocgical and biochemical characteristic were examined by standard methods given in Bergey's Manual of Systematic Bacteriology.

Optimization of environmental conditions : pH and temperature were optimized for the maximum production of alkaline protease by isolates. Enzyme production was estimated by growing cells at 25, 37 and 45 °C temperature and at pH ranges from 7 - 12. Cells were removed and enzyme production was estimated as mentioned above.

Repression studies with nitrogen and NaCl : To investigate the catabolite repression of enzyme synthesis in the presence of different nitrogen (skim milk, peptone, casein, yeast extract, KNO_3 and $NaNO_3$) and at different NaCl concentration (0.5-3% w/v), enzyme production was monitored at their different concentrations. The enzyme activity was monitored after 48h as mentioned above.

Enzyme purification : After optimization, cells were grown in media with optimized conditions for enzyme purification. The organism was grown for 48 hours as described previously. The cells were separated by centrifugation, and the supernatant was fractionated by precipitation with ammonium sulfate between 50% and 70% of saturation. All subsequent steps were carried out at 4°C. The protein was resuspended in 0.1M Tris-HCl buffer, pH 7.8, and dialyzed against the same buffer. Protein concentration was measured spectrophotometrically by Lowry's method (Lowry et al. 1951) with bovine serum albumin as a standard.

Gel Filtration Chromatography: Dialyzed samples were loaded onto a column of Sephadex G-200 (1.5 × 24 cm) (Sigma-Aldrich, St Louis, MO) equilibrated with Tris-HCl buffer, pH 7.8. The column was eluted at a flow rate of 60 mL/h with a 1:1 volume gradient from 0.1M to 1M NaCl in the same buffer. Fractions with high protease activities were pooled and used for further studies.

Gel electrophoresis and zymography: Electrophoresis was carried out according to the method by Laemmli (1971) using 12% crosslinked polyacrylamide gel on an electrophoresis unit (Bio-Rad, USA) to find molecular weight of enzyme. Silver staining was used to visualize protein bands on the gel. Alkaline protease activity was analyzed using zymography by copolymerizing 0.1% casein as substrate (Oliver *et al.*, 1999). Gel was stained by coomassie brilliant blue in a single step based the method given by Leber and Balkwill (1997). The protease activity band was visualized as a clear colorless band against blue background.

RESULTS AND CONCLUSION

The present study is an attempt to find halotolerant alkaline protease producers from marine site. A sample of marine water was collected from Gulf of Khambhat. Organisms grown on nutrient agar containing NaCl were purified on the basis of their colony morphologies for further studies. Total 13 bacteria were isolated. Results of primary screening by plate assay revealed that six isolates out of thirteen produced clear zone on both casein and milk agar plates (Figure 1). This bacteria were designates as M1 to M6. Morphological studies of the isolates which shown clear zone indicates all are Gram positive rod shape microbes.

Further screening of the bacteria for alkaline protease production revealed that isolate M4 (375 U/ml) showed higher production among the rest of isolates (Figure 2.). Hence, M4 was selected for further studies and identified by biochemical test. On the basis of morphological characteristics by Gram's reaction and spore staining and various biochemical reactions, M4 was identified as *Bacillus* sp. strain TD (Table 1).

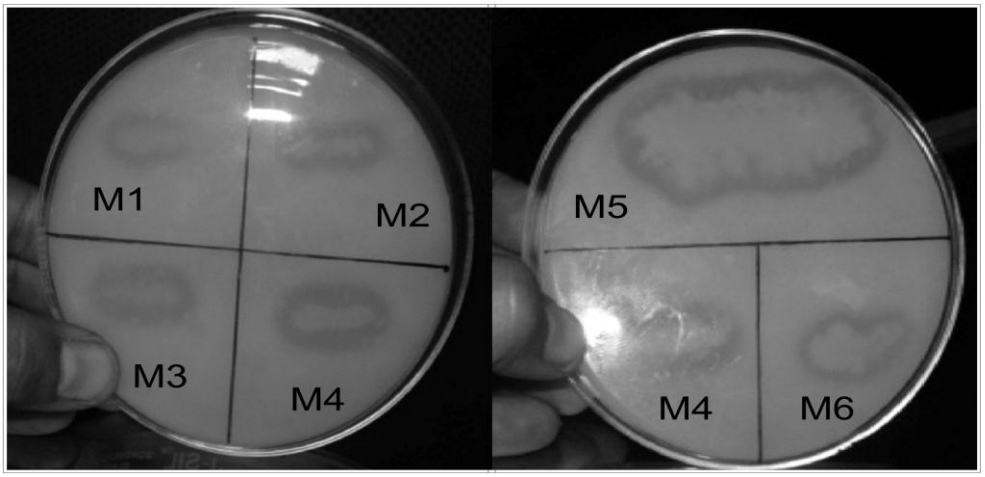

Figure 1. Alkaline protease production on milk agar plate by marine isolates

Effect of temperature on enzyme production showed that isolate produced higher enzyme (442 U/ml) at 45 ºC. (Fig III). Further increases in temperature reduce enzyme production by the isolates. Effect of pH on enzyme production indicated that highest enzyme activity was obtained at pH 11 by isolate (Figure 4). Hence, further experiments were carried out at optimized temperature and pH.

Table 1. Morphological and biochemical characterization of isolate

No	Morphological and Biochemical Characters	Result
1	Cultural character	White growth
2	Gram staining	Positive
3	Motility	Motile
4	Spore staining	Positive
5	Shape	Rod
6	Indole production	-
7	Methyl red test	+
8	Voges-proskauer test	±
9	Citrate utilization test	-
10	Catalase test	-
11	Oxidase test	+
12	Urease test	
13	Carbohydrate fermentation	
	Glucose	+
	Sucrose	+
	Fructose	-
	Lactose	-
	Galactose	+

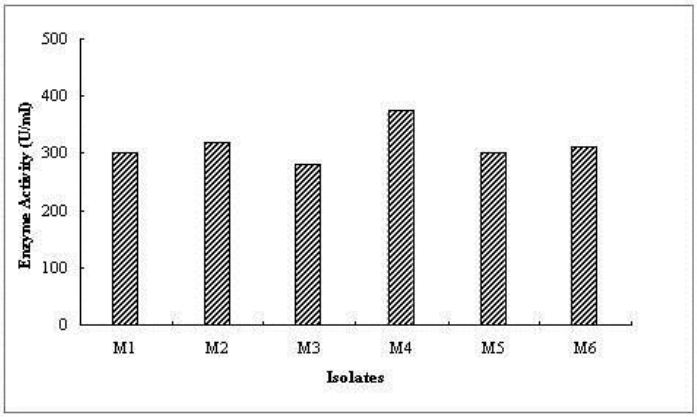

Figure 2. Screening of marine isolates for alkaline protease production

Effect of NaCl on enzyme production was shown in Fig V indicates higher enzyme production was found at 0.5 % NaCl concentration. As concentration increase the production decreased indicates organism is not able to tolerate the higher salt concentration.

Nitrogen source play a key role in protease production. Among different organic and inorganic nitrogen sources used, casein was found to have significant effect on the enzyme production. Isolates showed higher enzyme production (675 U/ml) when casein is used as nitrogen source compared to other while least with KNO_3 and $NaNO_3$ (Figure 6). There is meager difference for skim milk and yeast extracts the isolates. Inorganic nitrogen sources caused significant reduction in the protease yields.

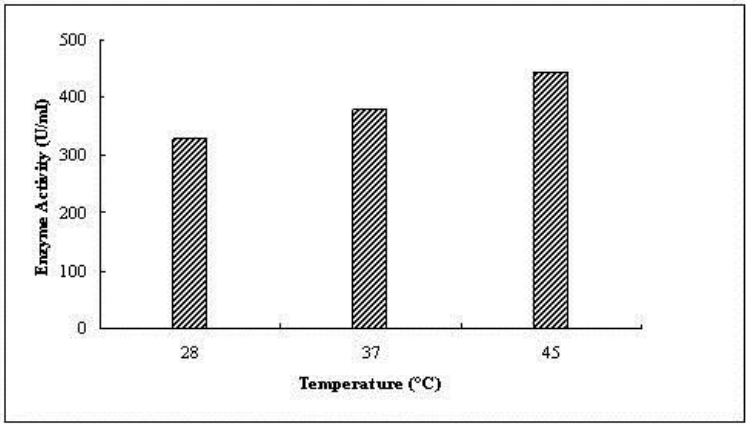

Figure 3. Effect of temperature on enzyme production by isolate

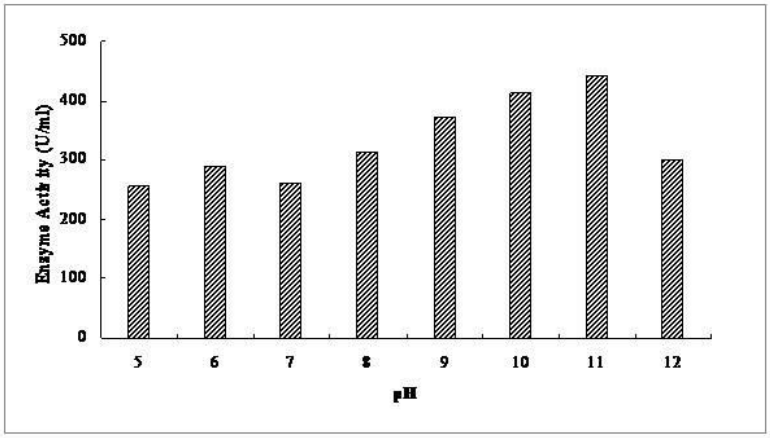

Figure 4. Effect of pH on enzyme production by isolate

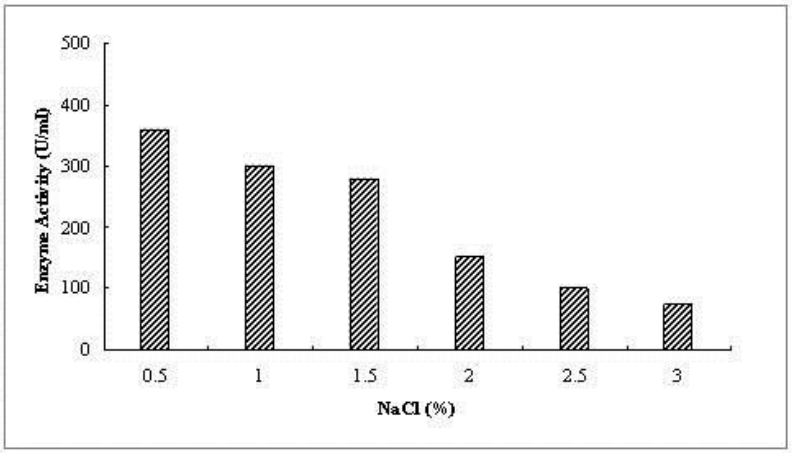

Figure 5. Effect of NaCl concentration (%) on enzyme production by *Bacillus* sp. strain TD

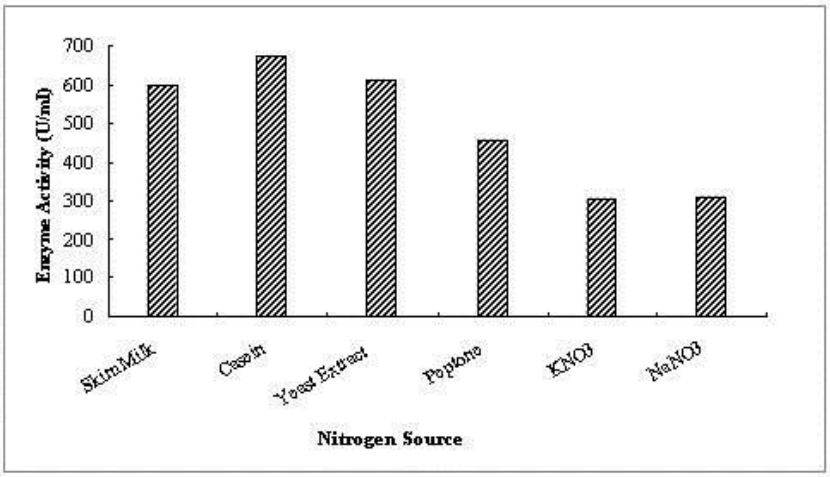

Figure 6. Effect of different nitrogen source on enzyme production by *Bacillus* sp. strain TD

Purified enzyme showed, 96.3 and 87.4 % yield after ammonium sulfate precipitation and gel filtration chromatography respectively by isolate. Molecular weight analysis by electrophoretic analysis showed that crude enzyme (Lane 6) and purified enzyme (lane 7) shows almost identical band above 66 KD with marker (Lane 1 and Lane 2) indicating molecular weight greater than 66 KD (Figure 7.). Activity stain by zymogram showed clear band indicates alkaline protease enzyme.

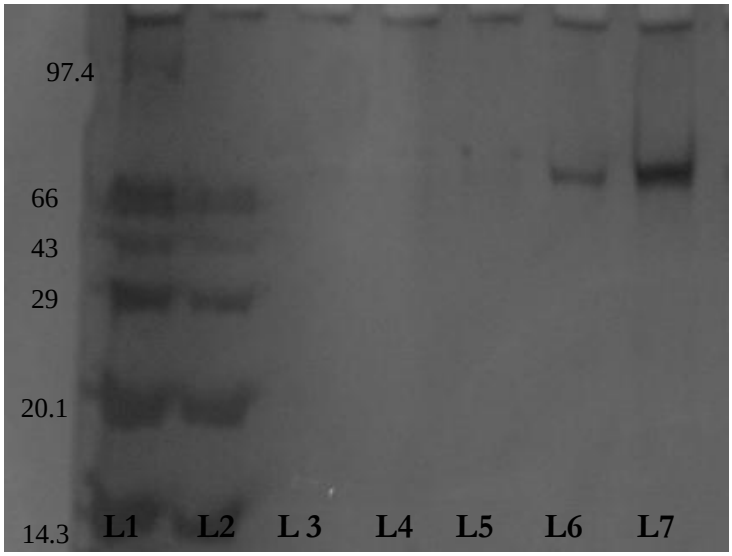

Figure 7. SDS-PAGE / silver staining of alkaline protease from *Bacillus* sp. strain TD (Lane 6, 7) with marker (Lane 1, 2)

Ecological niche play a key role in microbial metabolism. Here study aims to isolate halotolerant alkaline protease producer from marine sites. Marine ecosystem represents oligotrophic environment and hence isolates are more potential for enzyme production. Apart from halotolerance isolate was able to produce enzyme at thermophilic and alkalophilic condition. Isolate was identified as *Bacillus* sp. strain TD form marine sites. Bacillus species are well documented for alkaline protease production however; this isolate is novel as it is thermophilic, alkalophilic and halotolerant (Chauhan and Gupta 2004; Dodia et al. 2008; Akcan and Uyar 2011).

Enzyme production at higher temperature is in accordance with reported studies (Akcan and Uyar 2011; Horikoshi 1999; Gupta et al. 2002; Joo et al. 2002) but not by halotelerant isolate. Here optimum temperature for enzyme production was higher at 45 °C temperature. Isolate was able to produce enzyme at pH 11. The pH values above 11 could alter the three-dimensional structure of enzyme or ionization state of amino acids by disturbing the electrostatic interactions among the charged amino acids, resulting in loss of enzyme activity.

Different nitrogen source such as soybean meal, casamino acid and peptone were effective medium ingredients for the protease production by *Bacillus* sp. (Joo et al. 2002; Puri et al. 2002; Joo and Chang 2005; Patel et al. 2005; Chu 2007). Extreme halophilic archaeon *Halogeometricum* sp. TSS101 had maximum protease production in a medium containing 1% of skim milk powder (Vidyasagar et al. 2006). In present study isolate showed higher production when skim milk, casein, yeast extract and peptone were used as nitrogen source. However, production was decreased when KNO_3 and $NaNO_3$ were used as nitrogen source. In presence of inorganic nitrogen source, enzyme production was suppressed in *Bacillus.* sp. strain TD. Thus, isolate can be used under thermophilic and alakalophilic conditions for alkaline protease production. This triple advantage makes isolated an effective alternate for industrial application.

REFERENCES

Akcan N. and Uyar F. (2011). Production of extracellular alkaline protease from *Bacillus subtilis* RSKK96 with solid state fermentation. Eurasia J. Biosci. 5: 64-72.

Chauhan B. and Gupta R. (2004). Application of statistical experimental design for optimization of alkaline protease production from *Bacillus* sp. RGR-14. *Proc. Biochem.* 39: 2115-22.

Chu W.H. (2007). Optimization of extracellular alkaline protease production from species of *Bacillus*. *J. Ind. Microbiol. Biotechnol.* 34:241-245.

Dodia M.S., Rawal C.M., Bhimani H.G., Joshi R.H., Khare S.K. and Singh S.P. (2008). Purification and stability characteristics of an alkaline serine protease from a newly isolated haloalkaliphilic bacterium sp. AH-6. *J. Ind. Microbiol. Biotechnol.* 35: 121–31.

Gupta R., Beg Q.K. and Lorenz P. (2002). Bacterial alkaline proteases: molecular approaches and industrial applications. *Appl. Microbiol. Biotechnol.* 59: 15-32.

Hagihara B. (1958). In : The Enzymes (vol. 4). Academic press Inc., New York

Horikoshi K. (1999). Alkaliphiles: Some application of their products for biotechnology. *Microbial. Mol. Bio. Rev.* 63: 735-50.

Joo H.S. and Chang C.S. (2005). Production of protease from a new alkalophilic *Bacillus* sp. I-312 grown on soybean meal: optimization and some properties. *Proc. Biochem.* 40: 1263-1270.

Joo H.S., Kumar C.G, Park G.C., Kim K.M., Paik S.R. and Chang C.S. (2002). Otimization of the production of an extracellular alkaline protease from *Bacillus horikoshii*. *Proc. Biochem.* 38: 155 - 9.

Joshi G.K., Kumar S. and Sharma V. (2007). Production of moderately halotolerant, sds stable alkaline protease from *Bacillus cereus* MTCC 6840 isolated from lake Nainital, Uttaranchal state, India. *Braz J Microbiol 38: 773-9.*

Laemmli U.K. (1971). Cleavage of structural proteins during the as-sembly of the head of bacteria phage T4. *Nature.* 227: 680-85.

Leber T.M. and Balkwill F.R. (1997). Zymography: a single step staining method for quantitation of proteolytic activity on substrate gels. *Anal. Biochem.* 249: 24-28.

Lowry O.H., Rosebrough N.J., Farr A.L. and Randall R.J. (1951). Protein measurement with the Folin-Phenol reagent. *J. Biol. Chem.* 193: 265–75.

Oberoi R., Beg Q.K., Puri S., Saxena R.K., Gupta R. (2001). Characterization and wash performance analysis of an SDS-resistant alkaline protease from a Bacillus sp. *World. J. Microbiol. Biotechnol.* 17: 493-97.

Oliver G.W., Stetler-Stevenson W.G. and Kleiner D.E. (1999). *In: Proteolytic enzymes : tools and target, Ed. Sterchi E. and Stocker W. Springer-Verlag.* 63-76.

Patel R., Dodia M. and Singh S.P. (2005). Extracellular alkaline protease from a newly isolated haloalkaliphilic *Bacillus* sp.: Production and optimization. *Proc. Biochem.* 40: 3569-3575.

Puri S., Beg Q.K. and Gupta R. (2002). Optimization of alkaline protease production from *Bacillus* sp. using response surface methodology. *Curr. Microbiol.* 44: 286–90

Vidyasagar M., Prakash S.B. and Sreeramulu K. (2006). Optimization of culture conditions for the production of the production of haloalkaliphilic thermostable protease from an extremely halophilic archaeon *Halogeometricum sp.* TSS 101. *Lett. Appl. Microbiol.* 43: 385-391.

