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SCREENING AND PRODUCTION OF ALKALINE PROTEASE FROM HALOPHILIC BACTERIA ISOLATED FROM SOLAR SALTERN

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ABSTRACT

Halophiles are predominant organisms which are present in the hypersaline environment saltern ponds. They produce a variety of industrially important bioactive compounds including protease enzymes. Soil samples were collected from solar saltern of Vedaranyam, Tamilnadu. A total of 12 halophilic bacteria HPB1 –HPB12 isolated were screened for proteolytic activity by plate assay showed that seven isolates exhibited proteolytic activity by zone formation around the colonies. Isolate HPB2 produced high amount protease enzyme 35.4U/ml than the other seven isolates were identified as Bacillus megaterium. The effect of incubation time, medium pH, temperature, NaCl concentration and carbon source source for the protease production was investigated. Protease enzyme production by Bacillus sp was maximum at 40 hrs of incubation period 86.5 U/ml, pH 8 (107.2Uml^{-1}), temperature 40°C (98.3 Uml^{-1}), Nacl 2M (102.8 Uml^{-1}). and glucose (124.5 Uml^{-1}) as carbon source.

KEY WORDS

Halophiles, solar saltern , protease, Bacillus, plate assay

1. INTRODUCTION

Solar salterns are hyper saline water bodies located along the sea-coast and are the main source of generating salt through the vaporation of seawater [1]. They are generally of shallow ponds with salinities ranging from of seawater to supersaturated brines. In Tamilnadu, they are located in the coastal areas of Tuticorin, Nagapatinam, Ramanathapuram, Cuddalore, Thanjayur, Thiruvarur, Kancheepuram, villupuram, Pudukottai and Kanyakumari districts. These environments represent a unique group of halophilic and halotolerant microorganisms that survive at high salinities, high temperatures and tolerate severe solar radiations [2, 3]. Halophiles include Archaea, Bacteria and Eucarya and contain representatives of many different physiological types adapted to a wide range of salt concentrations as high as salt saturation [4] .

Halophiles, inhabiting saline environments, are considered as good source of useful salt stable enzymes [5]. Their enzymes possess unique structural features to catalyze the reactions under high salt conditions. Microbial proteases account for approximately 60% of the total enzyme sales in the world [6,7]. Proteases are one of the most important groups of industrial enzymes with broad applications including meat tenderization, detergents, cheese-making, de-hairing, baking, waste management and silver recovery [8,9].

Microbial proteases, especially from Bacillus species have traditionally held the predominant share of the industrial enzyme market of the worldwide enzyme sales with major application in detergents formulations [10]. Therefore, the aim of this study is to isolate and identify the halophilic bacteria from saltern that could produce protease enzyme and optimize fermentation conditions.

2. MATERIALS AND METHODS

2.1 Sample collection

Soil samples were collected from solar saltern of Vedaranyam situated at lattitude 10°22'31"N and longitude 79°51'1"E, Tamilnadu, India in a sterile container. These samples were brought to the laboratory aseptically and stored at 4°C under refrigerator until use.

2.2 Isolation of Halophilic Bacteria

Ten gram of soil sample was added to 90 ml of 5% NaCl in a conical flask and kept in a shaker for approximately 15 minutes at 100 rpm. It was diluted in upto 10 -4 in 5% Nacl . An aliquot of 10^{-4} dilution was transferred to halophilic agar medium (Himedia) by spread plate technique and incubated at 37°C for seven days. After incubation morphologically, different colonies were selected and investigated for further studies.

2.3 Screening of proteolytic activity by plate assay

To screen the halophilic bacteria for proteolytic activity each isolates were spot inoculated on Skim milk agar containing beef extract (3 g/L), peptone (10 g/L), NaCl (10 g/L), skimmed milk powder (10 g/L), and 18 g/L agar with pH adjusted to 8.5 [11] and incubated at 37°C for 48 hrs at 150rpm. The plates were examined for zone of hydrolysis for proteolytic activity.

2.4 Production of protease enzyme

Protease enzyme production medium contained (g l^{-1}) the following: NaCl 25, KCl 2, $MgSO_4$ 20, tri-sodium citrate 3, Skim milk 10, pH 7.2 [12]. 1% inoculum was added to the enzyme production medium and incubated at 37°C for 48 hrs at 150rpm. at 10,000 rpm for 15 min at 4°C. The cell free supernatant was used as source of protease enzyme.

2.5 Protease Enzyme assay

Extracellular proteolytic activity was determined according to the modified method as described in [13] using casein as the substrate. The reaction mixture contained 1 ml of 1.5 (w/v) casein in 0.1 M citrate phosphate buffer (pH 6.0) and 1 ml of culture supernatant. The mixture was incubated at 40°C for 30 min. The enzyme reaction was terminated by addition of 6 ml of 5% (w/v) trichloroacetic acid (TCA). The mixture was allowed to stand for 10 min and filtered through Whatman No. 1 filter paper. To 1 ml of filtrate, 3 ml of 0.5 M Sodium carbonate solution and 1 ml of 3-fold diluted Folin-Ciocalteau reagent were added and mixed thoroughly. It was incubated at room temperature in dark for 30mins for the development of blue colour. A blank was prepared as described previously except that the TCA solution was added before the enzyme. The absorbance was read at 660 nm against reagent blank. One unit of protease activity was defined as the amount of enzyme required to liberate 1 µg of tyrosine per minute under standard assay conditions. All assays were carried out in triplicate.

2.6 Optimization of protease enzyme production

The various factors influencing the protease production were investigated, examining one factor at a time, keeping all other variables constant except one. They include incubation time (8, 16, 24, 32, 40,48, 56, 64 and 72hrs), initial pH (5, 6, 7, 8, 9, 10 and11), incubation temperature (25, 30, 35, 40, 45, 50 and, 55°C) and sodium chloride concentration (0.5M to 3.0 M NaCl) and carbon source 1%w/v (glucose, lactose, maltose, starch and sucrose).

3. RESULTS AND DISCUSSION

Solar saltern are extreme hypersaline habits which contain thriving microbial populations. Halophiles have been perceived as a potential source for the production of industrial important.

Table 1 Proteolytic activity of bacterial isolates in Skim milk agar with NaCl plate assay

Halophilic Bacteria	Zone of hydrolysis	Halophilic Bacteria	Zone of hydrolysis	Halophilic Bacteria	Zone of hydrolysis	Halophilic Bacteria	Zone of hydrolysis
HPB1	+	HPB4	++	HPB7	+	HPB10	-
HPB2	+++	HPB5	-	HPB8	-	HPB11	++
HPB3	-	HPB6	-	HPB9	++	HPB12	+

High activity (> 10 mm); ++, moderate activity (5 to 10 mm) +. Low activity (< 5 mm); -, no activity

Table 2. Identification of protease producing halophilic bacterial isolate

Isolate	a	b	c	d	e	f	g	h	i
HPB2	G+ve	Rods	motile	+	-	+	-	+	+

a. gram staining, b. Shape, motility, d. Spore staining, e.Indole, f.MR, g.VP, h.Citrate, I .Catalase

hydrolytic enzymes, exopolysaccharides, carotenoids, pigments with exceptional properties. The present study was intended to isolate halophilic bacteria from Solar saltern of Vedaranyam, Tamilnadu, India.

A total of twelve morphologically different bacteria were isolated from saltern soil in halophilic agar medium were screened for proteolytic activity by plate assay. Seven isolates exhibited proteolytic activity by zone of hydrolysis around the colonies in skim milk agar with 10g/l NaCl (Table1). Among the seven isolates the isolate HPB2 showed highest amount of protease enzyme 20.4 Uml^{-1} (figure1) compared to other was selected for further optimization studies.

3.1 Identification of the Isolate

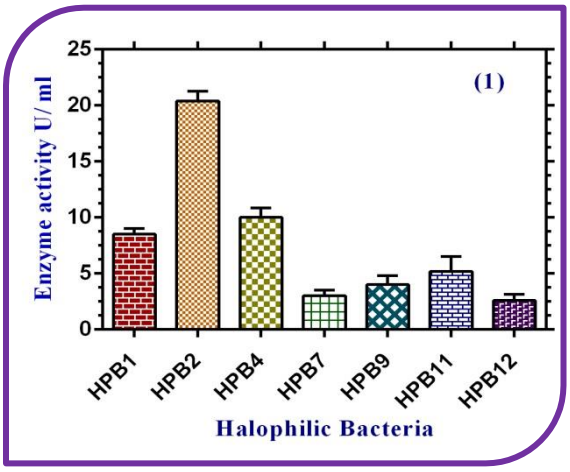
The halophilic bacteria which produces maximum protease enzyme production by submerged fermentation was selected as a novel protease producer and identified based on morphological, biochemical and physiological characteristics [14]. The halophilic bacterial isolate was confirmed to be *Bacillus megaterium* (Table 2).

3.2 Optimization of protease enzyme production

The incubation time for enzyme production is governed by the characteristics of the culture. The protease enzyme production increases gradually from 8 hrs of incubation period 34 $Uml^{-1}$and reaches its maximum at 40 hrs 86.5 Uml^{-1} and decreases above 40 hrs (figure 2). Similarly, for *B. subtilis* PE-11 [15] and *B. licheniformis* LBBL-11 [16] maximum growth and protease enzyme production was observed at 48hr of incubation period.

The initial pH of the culture medium was found to be one of the most important critical environmental parameters which affects the enzyme production by *Bacillus megaterium*. Maximum protease enzyme production (107.2 Uml^{-1}) was obtained at an initial medium pH of 8.0 (Figure 3). Protease production decreased significantly at pH of 8.0 and above. Results suggest that there is a stimulation of enzyme production at alkaline pH. The obtained results coincide with Kumar et al. [17] who has reported that protease production was maximum at pH 7 and 9 for *Bacillus* sp. strain S4 and *Pseudomonas* sp. strains S22 respectively. Medium with slightly alkaline (pH 8.0–8.5) has been reported to be optimum for protease production by *B. licheniformis* IKBC-17, *B. subtilis* IKBS 10, *Bacillus macerans* IKBM-11 [18] and Bacillus *amovivorus* [19]. Most of the *Bacillus* sp. reported have optimum pH from 7.0 to 11.0 for the production of protease [20,21].

The Influence of temperature on protease production of *Bacillus megaterium* was investigated by different temperatures ranges from 25˚C to 55˚C at an interval of 5˚C .The protease enzyme production was found to be maximum (98.3 Uml^{-1}) at 40˚C (Figure 4).and the enzyme production was affected and decreased after increase of temperature above 40°C. A similar temperature of 40ºC has been reported to be best for production of protease by *Bacillus* sp. 2–5 [22], *B. licheniformis* GUS1 [23] , *V. pantothenticus* [24].



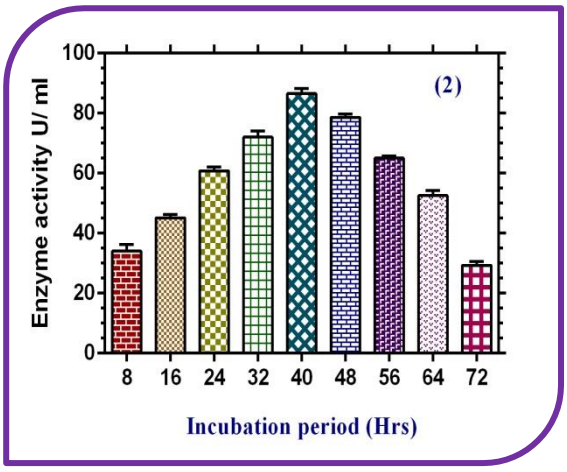
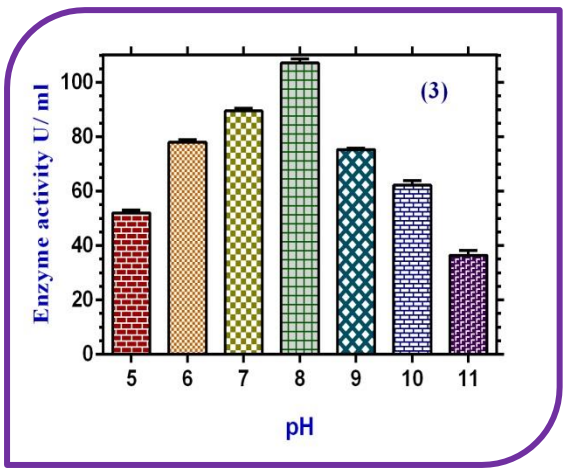


Figure 1. Production of protease by halophilic bacterial isolate, Figure 2. Effect of incubation time on protease production.



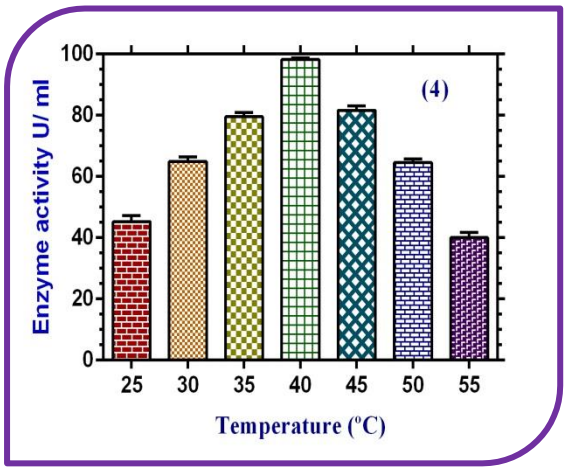
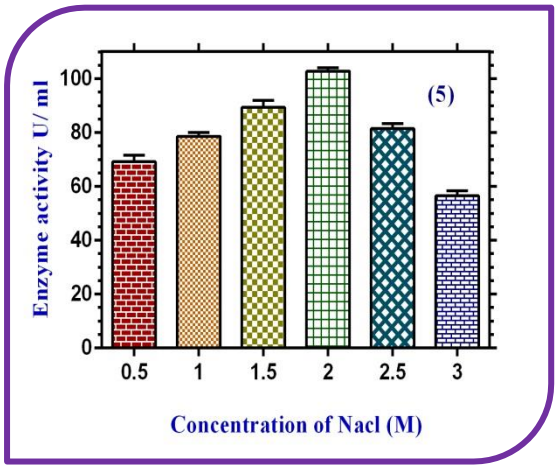


Figure 3. Effect of pH on protease production, Figure 4. Effect of temperature on protease production



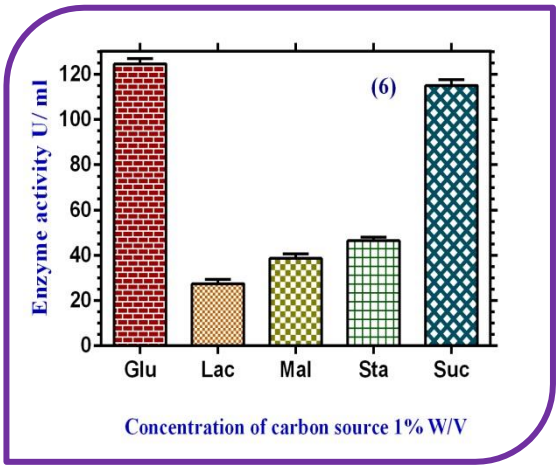


Figure 5. Effect of sodium chloride on protease production and Figure 6. Effect of carbon source on protease production by *Bacillus megaterium*

increase of temperature above 40°C. A similar temperature of 40ºC has been reported to be best for production of protease by *Bacillus* sp. 2–5 [22], *B. licheniformis* GUS1 [23], *V. pantothenticus* [24].

3.3 Effect of NaCl and carbon source on protease production

The effect of salt on protease production was shown in (Fig. 5). Maximum protease production was observed in the medium containing 2M NaCl (102.8 Uml^{-1}). The growth and production of protease was gradually reduced when salt concentration increases above 2M NaCl. An increased salt concentration creates change in the lipid composition of cell membrane. So, the growth rate decreases along with enzyme production. Mostly, gram positive moderate halophiles are often reported in the reduction of enzyme production at high salt concentration [25]. Concentration of 1M NaCl was found to be optimum for the production of protease from *Bacillus aquimaris* strain VITP4 [26] and Sinsuwan et al. [27] reported that 5 % NaCl was the optimum concentration for production of protease from *Virgibacillus* sp. SK33.

The effect of different carbon sources on protease production was investigated and the results showed that protease production was highest in the medium containing glucose (124.5 Uml^{-1}) followed by sucrose (115 Uml^{-1}) shown in (Fig. 6) and enzyme production was minimum in the medium containing lactose (27.5 Uml^{-1}). Similar result of glucose as carbon source in the medium increased the protease production of *B. thuringiensis* [28] and *B.cereus* [29] . Different carbon sources are reported as optimum carbon sources such as maltose [30], lactose [31] , starch [32], fructose [33] and sucrose [34].

CONCLUSION

In the present investigation halophilic bacteria *Bacillus megaterium* was isolated from solar saltern of Vedharanyam and evaluated for optimization of protease enzyme production. The optimum incubation time, pH, temperature, NaCl and carbon source were determined as 40hrs, 8pH, 40°C, 2M NaCl and 1% glucose. Further research on purification and characterization of enzyme will enable it to be used for different industrial purposes.

REFERENCES

[1] Mani K., Salgaonkar BB., Das D., Bragança JM. Community solar salt production in Goa, India. *Aquatic Biosystems*, 8(1): 30, (2012).

[2] Ma Y., Galinski EA., Grant WD., Oren A., Ventosa A. Halophiles 2010: Life in Saline Environments. Applied and Environmental Microbiology, 76 (21): 6971–6981, (2010).

[3] Jamadar SAG., Shaikh ZAS., Vinod PS., Sulochana MB. Molecular characterization and screening of halophiles for the production of biopolymers. European Journal of Biotechnology and Bioscience, 4(2): 32-36, (2016).

[4] Aljohny BO. Halophilic bacterium–a review of new studies. *Biosciences Biotechnology Research Asia*, 12(3): 2061-2069, (2015).

[5] Oren A. Microbial life at high salt concentrations: Phylogenetic and metabolic diversity. Saline Systems, 4(1): 2, (2008).

[6] Banik RM., Prakash M. Laundry detergent compatibility of the alkaline protease from *Bacillus cereus*. Microbiological Research, 159(2): 135-140, (2004).

[7] Sharma KM., Kumar R., Panwar S., Kumar A. Microbial alkaline proteases: optimization of production parameters and their properties. Journal of Genetic Engineering and Biotechnology, 15(1): 115-126, (2017).

[8] Gupta R., Beg Q., Lorenz P. Bacterial alkaline proteases: molecular approaches and industrial applications. Applied microbiology and biotechnology, 59(1): 15-32, (2002).

[9] Singh R., Mittal A., Kumar M., Mehta PK. Microbial proteases in commercial applications. J. Pharm. Chem. Biol. Sci, 4: 365-74, (2016).

[10] Gupta R. Purification and characterization of an oxidation stable thiol-dependent serine alkaline protease from *Bacillus mojavenesis*. Enzyme Microbial Technol, 32:294-304, (2003).

[11] Cui H., Wang L., Yu Y. Production and characterization of alkaline protease from a high yielding and moderately halophilic strain of SD11 marine bacteria. Journal of Chemistry, 798304, (2015).

[12] Sekar A., Packyam M., Kim K. Halophile isolation to produce halophilic protease, protease production and testing crude protease as a detergent ingredient. African Journal of Microbiology Research, 10(36):1540-1547, (2016).

[13] Berla ET., Suseela RG. Purification and characterization of alkaline protease from *Alcaligenes faecalis*. Biotechnol. Appl. Biochem, 35: 149-154, (2002).

[14] Holt JG, Krieg NR, Sneath PHA, Staley JT, Williams ST. Bergeys Manual of Determinative Bacteriology. 9th edn. Baltimore: Williams &Wilkins, (1994)

[15] Adinarayana K., Ellaiah P., Prasad DS. Purification and partial characterization of thermostable serine alkaline protease from a newly isolated *Bacillus subtilis* PE-11. Aaps Pharmscitech, 4(4): 440-448, (2003).

[16] Olajuyigbe FM., Ajele JO. Some properties of extracellular protease from *Bacillus licheniformis* LBBL-11 isolated from iru, a traditionally fermented African locust bean condiment. African Journal of Biochemistry Research, 2(10):206-210, (2008).

[17] Kumar A., Sachdev A., Balasubramanyam SD., Saxena AK., Lata A. Optimization of conditions for production of neutral and alkaline protease from species of *Bacillus* and *Pseudomonas.* Ind. J. Microbiol, 42: 233-236, (2002).

[18] Olajuyigbe FM., Ajele JO. Production dynamics of extracellular protease from *Bacillus* species. African Journal of Biotechnology, 4(8): 776- 779, (2005).

[19] Sharmin S., Hossain MT., Anwar MN. Isolation and characterization of a protease producing bacteria *Bacillus amovivorus* and optimization of some factors of culture condition for protease production. J. Biol. Sci, 5(3): 358-362, (2005).

[20] Ravishankar K. Isolation of alkaline protease from *Bacillus subtilis* AKRS3. African J. Biotechnol, 11: 13415–13427, (2012).

[21] Devi KA. Screening, optimization of production and partial characterization of alkaline protease from haloalkaliphilic *Bacillus* sp. Int. J. Res. Eng. Technol,3: 435–443, (2014).

[22] Khosravi-Darani K., Falahatpishe HR., Jalali M. Alkaline protease production on date waste by an alkalophilic *Bacillus* sp. 2-5 isolated from soil. African Journal of Biotechnology, 7(10):1536 – 1542, (2008).

[23] Seifzadeh S., Hassan Sajedi R., Sariri R. Isolation and characterization of thermophilic alkaline proteases resistant to sodium dodecyl sulfate and ethylene diamine tetraacetic acid from *Bacillus* sp. GUS1. Iranian Journal of Biotechnology, 6(4): 214-221, (2008).

[24] Gupta A., Joseph B., Mani A., Thomas G. Biosynthesis and properties of an extracellular thermostable serine alkaline protease from *Virgibacillus pantothenticus.* World journal of Microbiology and Biotechnology, 24(2): 237-243, (2008).

[25] Rawway M., Taha TM., Eltokhey A., Abdul-Raouf UM. Optimization, partial purification and characterization of Halo-thermophilic alkaline protease from moderately halophilic bacterium AH10 isolated from Alexandria (Egypt). Int. J. Curr.Microbiol. App. Sci, 4(11): 304-317, (2015).

[26] Shivanand P., Jayaraman G. Production of extracellular protease from halotolerant bacterium, *Bacillus aquimaris* strain VITP4 isolated from Kumta coast. Process Biochem, 44: 1088 – 1094, (2009).

[27] Sinsuwan S., Rodtong S., Yongsawatdigul J. Production and characterization of NaCl-activated proteinases from *Virgibacillus* sp. SK33 isolated from fish sauce fermentation. ProcessBiochem, 43: 185 192, (2008).

[28] Chovatiya S., Dhola K., Patel P., Ingale S. Isolation, characterization and optimization of protease enzyme producing microorganism from gastrointestinal tract of *Labeo rohita*. Int. J. Pure App. Biosci, 2(3): 124-134, (2014).

[29] Santhi R. Microbial production of protease by *Bacillus cereus* using cassava waste water. Eur. J. Exp. Biol, 4(2): 19-24, (2014).

[30] Vijayaraghavan P., Lazarus S., Vincent, SGP. De-hairing protease production by an isolated *Bacillus cereus* strain AT under solid-state fermentation using cow dung:Biosynthesis and properties. J. Saudi Biol. Sci, 21: 27 34, (2014).

[31] Kumar RS., Ananthan G., Prabhu AS. Optimization of medium composition for alkaline protease production by *Marinobacter sp*. GACAS9 using response surface methodology - A statistical approach. Biocatalysis Agricult. Biotechnol, 3: 191 197, (2014).

[32] Padmapriya M., Williams CB. Purification and characterization of neutral protease enzyme from *Bacillus Subtilis.* J. Microbiol. Biotech. Res, 2(4): 612 618, (2012).

[33] Sevinc N., Demirkan E. Production of protease by *Bacillus* sp. N-40 isolated from soil and its enzymatic properties. J. Biol. Environ. Sci, 5(14): 95 103, (2011).

[34] Tsuchiya K., Ikeda I., Tsuchiya T., Kimura T. Cloning and expression of an intracellular alkaline protease gene from alkalophilic *Thermoactinomyces* sp. HS682. Biosci. Biotechnol. Biochem, 61: 298–303, (1997).

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