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ISOLATION AND CHARACTERIZATION OF A MODERATELY HALOPHILIC PROTEASE PRODUCING *PSEUDOMONAS* SP. FROM BRINE SAMPLES OF A COASTAL AREA SOLAR SALTERN

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ABSTRACT
Screening of the brine samples of a solar saltern has yielded one moderately halophilic protease producing bacteria. The bacterial isolate has been identified as a *Pseudomonas* sp. and it produced an extracellular protease and an amylase. Maximal protease production was detected at the early stationary phase. The enzyme showed the highest activity at 40^{0}C and pH 8.0 in 15% NaCl. The enzyme was active over a range of salt (0-25%), pH (6.5-10.0) and temperature (20^{0}C-55^{0}C). These broad ranges of tolerance of the enzyme indicate potential applications of this enzyme in fish sauce fermentation and detergent formulations.

INTRODUCTION

Moderately halophilic bacteria constitute a heterogeneous physiological group of microorganisms that grow optimally in media containing 3-15% NaCl (Ventosa *et al*., 1998). Several halophilic biotopes have been identified, including saline lakes, evaporite lagoon sediments and coastal salterns. Saline soils and the salt-excreting surfaces of animals are less well-explored habitats, but almost all hypersaline biotopes are thought to harbour significant populations of microorganisms (Grant *et al*., 1998). Halophilic microorganisms possess haloadaptation mechanisms such as accumulation of high cytoplasmic concentrations of compatible solutes to grow and survive in saline habitats (Galinski, 1993).

Halophilic microorganisms, able to live in saline environments, offer a multitude of actual applications in various fields of biotechnology (Rosa Margesin and Franz Schinner, 2001). The technical applications of bacteriorhodopsin comprise holography, spatial light modulators, optical computing, and optical memories. Compatible solutes are useful as stabilizers of biomolecules and whole cells, salt antagonists or stress-protective agents. Biopolymers, such as biosurfactants and exopolysaccharides, are of interest for microbially enhanced oil recovery (Ventosa & Nieto, 1995; Ventosa *et al*., 1998).

Other useful biosubstances are enzymes, moderately halophilic bacteria are a source of hydrolytic enzymes such as amylases, DNases, lipases, proteases and pullulanases (Sánchez-Porro *et al*., 2003). Proteases from these organisms are one interesting group of hydrolytic enzymes with wide range of commercial applications that have not been extensively studied. These enzymes play an essential role in food biotechnology for the production of fermented food and food supplements. Some of the previous reports of proteases from moderately halophilic bacteria have been from *Pseudomonas* sp. A-14 (Duong Van Qua *et al.,* 1981), *Pseudomonas* sp. CP76 (Sanchez-Porro *et al.,* 2003), *Salinivibrio costicola* 18AG (Lama *et al.,* 2005), *Filobacillus* sp. RF2-5 (Kazumi Hiraga *et al.,* 2005), *Halobacillus* sp. SR5-3 (Sirilak Namwong *et al.,* 2006) and *Salinivibrio* sp. strain AF-2004 (Karbalaei-Heidari *et al.,* 2006)

Solar salterns consist of small ponds fed with sea water. They act as crystallizers where salt precipitates. These salterns become an abode for a wide variety of halophilic microorganisms as the salinity of these ponds increases gradually with the evaporation of sea water. The present study is focused on isolation of moderately halophilic bacteria that are capable of protease production from the brine samples (saline water samples) from the solar salterns of Kakinada, Andhra Pradesh, India.

MATERIALS AND METHODS

Isolation of halophilic bacteria from the brine samples

Brine samples collected from the sea water salterns of Kakinada, Andhra Pradesh, India have been serially diluted and plated on to a halophile medium (1% [w/v] Yeast

extract, 0.2% [w/v] Peptone, 10% [w/v] NaCl, 0.2% [w/v] $MgSO_4$, 0.1% [w/v] Tri sodium citrate and 2% [w/v] agar pH 7.5) by spread plate method and incubated for five days at 37^{0}C. The pure cultures of the isolates obtained through repeated plating method were transferred to the halophile agar slants and preserved as stock cultures.

Screening for proteolytic activity

Proteolytic activity of the isolates was assayed by performing casein hydrolysis test. Sterile halophile media plates supplemented with 1% (w/v) casein were spotted with loop full of the 24 h old culture of the isolates and incubated at 37^{0}C. At the end of the incubation period the casein plates were flooded with 10%Trichloro acetic acid to observe the zone of hydrolysis.

Culturing and identification of the isolate (Ydc)

An isolate with profound proteolytic activity was selected for further studies and is designated as Ydc. The isolate Ydc was inoculated into the halophile broth medium and incubated at 37^{0}C for 72 h on a rotary shaker at 120 rpm. The isolate was characterized morphologically and biochemically by performing the standard phenotypic tests as described previously (Ventosa *et al.*, 1982; Quesada *et al.*, 1984; Garcia *et al.*, 1987).

Comparison of the effect of different salts on the growth of Ydc

A comparative study of the effect of different salts on the growth of Ydc has been carried out by replacing NaCl from the halophile media with different salts. A standard 2M concentration of the selected salts (KCl, NaCl and $MgCl_2$) was added to the halophile broth media. Growth of the culture was followed turbidimetrically by reading the optical density at 600 nm against an uninoculated blank.

Effect of growth on protease production

Halophile broth medium supplemented with 0.2% (w/v) of casein and 0.1% (w/v) soluble starch, pH 7.5 was used for the production of protease. Production medium was seeded with 5% inoculum of the 24 h old culture of the isolate. The isolate was cultured at 37^{0}C in an orbital shaker at 120 rpm. The culture broth was harvested at 8 h intervals by centrifugation at 10,000 rpm for 30 min and the supernatant was assayed for protease activity.

Protease assay

Protease activity was assayed by Folin – Ciocalteau method (K. Hiraga *et al.* 2005). The reaction mixture consisted of 0.35ml of 1.0% (w/v) casein in 25mM Tris-HCl buffer (pH-7.5) containing 10% (w/v) NaCl. The reaction was started by adding 50μl of enzyme solution. After incubation for 2h at 37^{0}C, the reaction was stopped by adding 0.44M trichloroacetic acid followed by centrifugation at 12,000 rpm for 10min. To 0.5ml of the supernatant, 2.5ml of the 0.44M sodium carbonate and 0.5ml of Folin – Ciocalteau reagent was added and incubated for 2h at 37^{0}C for 20min.The optical density of the supernatant was measured at 660nm. One unit of activity is defined as the amount of enzyme that liberates 1μg of tyrosine per ml of the reaction mixture per minute.

Determination of the optimal pH for the protease activity

Effect of pH on the protease activity was studied by carrying out the standard assay at different pH values within the range of 6.0-11.0. The buffers used for maintaining the necessary pH include: phosphate buffer (pH 6.0-8.0), borate buffer (pH 8.0-10.0) and carbonate buffer (pH 10.0-11.0).

Determination of the optimal temperature for the protease activity

Optimal temperature for protease activity was determined by incubating the reaction mixture at different temperatures within the range of 20^{0}C-60^{0}C at a pH of 7.5 under the standard assay conditions.

Determination of the optimal NaCl concentration for the protease activity

Effect of varying concentrations of NaCl on the protease activity was studied to determine the optimal NaCl concentrations for enzyme activity. Under standard assay conditions mentioned earlier, protease assay was performed at different NaCl concentrations (0, 5, 10, 15, 20 and 25% [w/v]) using casein as substrate.

Amylase activity

Amylolytic activity of the isolate Ydc was determined by Cowan (1991) method using sterile halophile agar media (pH 7.5) plates supplemented with 1% (w/v) soluble starch. A loop full of the 24 h old culture of the isolate Ydc was spotted on to the plates and incubated at 37^{0}C. After an incubation period of 24 h, the plates were flooded with 0.3% I_2 / 0.6% KI solution: a clear zone around the growth indicated hydrolysis of starch.

Amylase assay

Dextrinogenic amylase activity was assayed by using soluble starch as a substrate by the modified method of Fuwa (1954): 2 ml of 0.5% soluble starch in 25 mM tris(hydroxymethyl)aminomethane(Tris)-hydrochloride buffer (pH 7.0) containing 2 M NaCl was mixed with 1 ml of enzyme solution. After incubation at 37^{0}C for 10 min, a 0.2 ml portion of the reaction mixture was added to 5 ml of 0.167 mM I_2-KI solution. The optical density at 700 nm was measured in a spectrophotometer. Hydrolysis of 0.1 mg of soluble starch in 1 min was defined as 1 U of enzyme activity.

Study of amylase production with respect to growth

Growth versus amylase production was studied by inoculating 5% inoculum of the 24 h old of Ydc into sterile halophile broth medium (pH 7.5) supplemented with 1.0% [w/v] soluble starch. Culturing was carried out at a temperature of 37^{0}C in an orbital shaker at 120 rpm. At 8 h intervals the culture was harvested by centrifugation at 10,000 rpm for 30 min and assayed for amylase activity.

RESULTS AND DISCUSSION

Isolation and Identification of the protease producing moderately halophilic bacteria

Among an array of isolates that have been obtained from the brine samples of solar salterns of Kakinada, India; one isolate with profound proteolytic activity has been chosen for further studies and was designated as Ydc. According to Kushner and Kamekura (1988) the isolate, Ydc has been identified as a moderately halophilic bacterium. The results of the study of morphological and biochemical characteristics of the isolate (Ydc) (data not shown) on comparison with those standard characteristics described in Bergey's Manual of Determinative Bacteriology (8th Edn.) have indicated that the moderately halophilic bacterial isolate belongs to the genus *Pseudomonas*.

Cultural characteristics of Ydc

Ydc produced light yellow coloured glossy colonies with undulate margin on halophile media with 10% NaCl. The increase in salt concentration above 20% (w/v) reduced the pigment production. In the halophile broth media with 10% NaCl, Ydc has shown an exponential growth till the 64th hour. The culture Ydc has been found to produce an extra-cellular protease and amylase with profound proteolytic and amylolytic activity.

The effect of different salts on the growth of Ydc was studied by replacing NaCl of the halophile medium with other salts ($MgCl_2$, KCl and NaCl) of 2M concentration (Fig. 1). Highest growth was observed in media containing 2M KCl followed by the one in NaCl and $MgCl_2$.

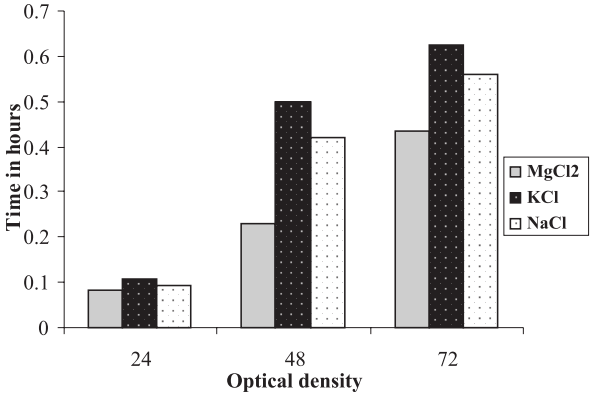

Figure 1: Effect of different salts ($MgCl_2$, KCl and NaCl on the growth of Ydc

Growth of the culture Ydc was studied at a standard concentration of 2M in NaCl, KCl and $MgCl_2$. Highest growth was observed in KCl followed by NaCl and $MgCl_2$.

Characterization of Protease

Protease production was found to reach its maximum at the 72nd hour of growth i.e., at the early stationary phase (Fig. 2). the effect of pH (Fig. 3), temperature (Fig. 4) and sodium chloride (Fig. 5) on the activity of protease produced by Ydc has been investigated. Optimum protease activity was recorded at a pH of 8.0, temperature of 40^{0}C and 15% concentration of Sodium chloride. The enzyme had retained its activity at the pH range of 6.5-9.5 and at temperatures within the range of 25^{0}C -55^{0}C. Activity of the enzyme was detected in the range of 0-25% NaCl concentration (Fig. 6).

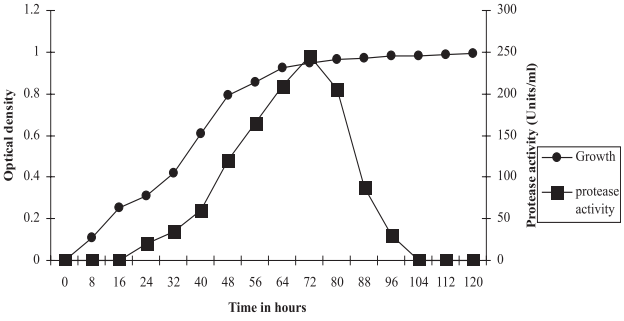

Figure 2: Growth Vs Protease activity

Growth versus protease activity has been studied for the culture Ydc at 8h intervals. Fig.1 shows the growth pattern of the isolate and Ydc is found to enter the stationary phase by 64th hour of growth. Fig. 2 corresponds to the protease production with respect to growth and highest protease production can be seen at 72nd hour of growth i.e., during the early stationary phase

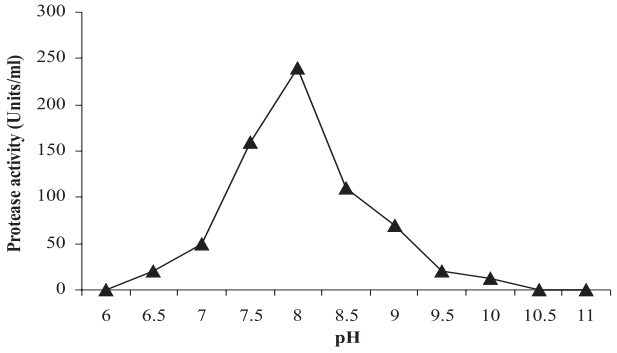

Figure 3: Effect of pH on protease activity

Assay of the protease activity at different pH values within the range of 6-11 has indicated 8.0 as the optimum pH.

Amylolytic activity

Ydc also has shown a profound amylolytic activity with maximal amylase production at 56th hour of growth, i.e., during the late exponential phase (Fig. 7).

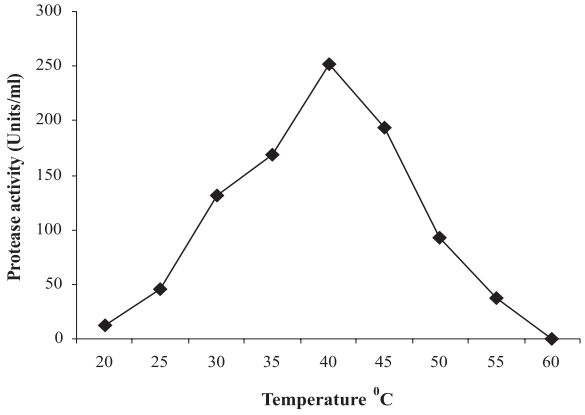

Figure 4: Effect of temperature on protease activity

Assay of the protease activity at different temperatures within the range of 20-60^{0}C has indicated 40^{0}C as the optimum temperature.

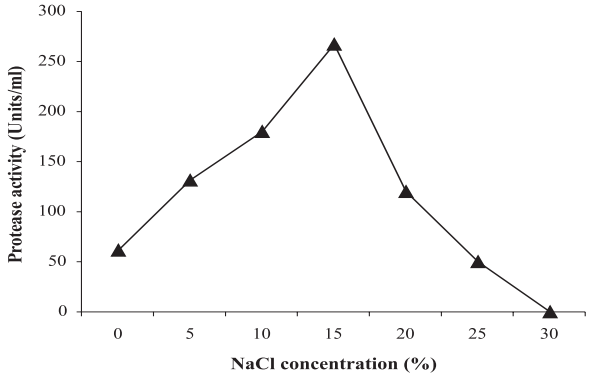

Figure 5: Protease activity with varying concentrations of NaCl

Protease activity assayed at varying concentrations of NaCl in the range of 0-25% has indicated 15% NaCl as an optimum concentration.

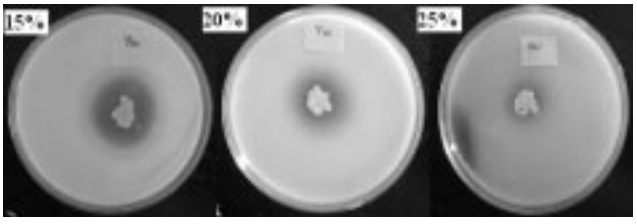

Figure 6: Zone of clearance as shown on casein plates with 15%, 20% and 25%NaCl by Ydc

CONCLUSION

The moderately halophilic *Pseudomonas* isolate has shown the production of an extracellular protease and amylase. The protease produced by the isolate has shown a broad range of salt (0-25%), pH (6.5-10.0) and temperature (20^{0}C -55^{0}C) tolerance. The wide range of tolerance shown by protease enzyme, towards the physiological conditions ensures its commercial viability and industrial applications.

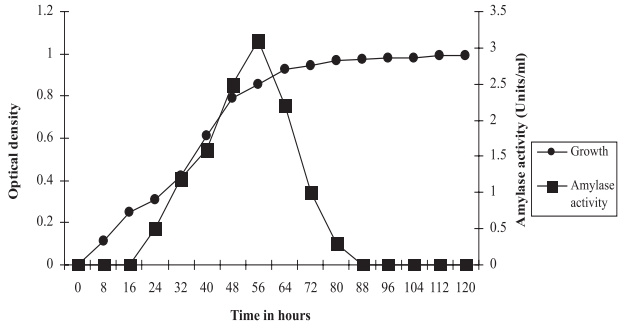

Figure 7: Growth Vs Amylase activity

Growth versus amylase activity has been studied for the culture Ydc at 8h intervals. Graph-1 shows the growth pattern of the isolate and Ydc is found to enter the stationary phase by 64th hour of growth. Graph-2 corresponds to the amylase production with respect to growth and highest amylase production can be seen at 56th hour of growth i.e., during the late exponential phase.

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