Global Journal of Biotechnology & Biochemistry 5 (1): 44-49, 2010
ISSN 2078-466X

Production and Optimization of Haloalkaliphilic Protease by an Extremophile-*Halobacterium* Sp. Js1, Isolated from Thalassohaline Environment

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Abstract: An Extremely halophilic archaeon-*Halobacterium sp* JS1, isolated from solar salterns (Thalassohaline environment) was screened for the secretion of protease on casein plates containing 20% NaCl. Media and cultivation conditions were investigated to optimize bacterial growth and protease production which includes different Carbon and nitrogen sources, metal ions, in addition to different factors such as the incubation time, pH, temperature, NaCl concentrations and agitation levels. Optimum biomass and proteolytic activity was achieved after 96 hrs of incubation, at 40°C, pH-7.0 with 20% salinity and 200 rpm agitation. Among the various nitrogen sources investigated, defatted soybean meal was found to be the best inducer of alkaline protease, while inorganic nitrogen sources in the form of ammonium salts repressed bacterial growth and enzyme activity. Among the carbon sources tested, rice bran maximized the enzyme production. Combination of metal ions such as Ca, Mg and Mn enhanced proteolytic activity, where as Zn, Fe and CO strongly inhibited enzyme production.

Key words: Casaminoacids · Detergents · *Halobacterium* Sp · Extremozymes · Thalassohaline environment

INTRODUCTION

Hypersaline environments originate by the evaporation of sea water and are also called Thalassohaline environments [1]. As water evaporates sodium chloride (NaCl) precipitates and salinity increases above 300 PSU [2]. Despite the prevailing extreme environment, a great diversity of extremophiles, especially Haloarchaea has been observed in hypersaline bodies [3]. Extremophiles, the microbes dwelling in unusual habitats can potentially serve in a variety of industrial applications. As a result of adaptation to extreme environments, extremophiles have evolved unique properties, which can be of biotechnological and commercial significance [4]. Extreme halophiles have been proven to be a rich source of biological information. A wide variety of biotechnological products such as bacteriorhodopsins, halorhodopsins, biopolymers, biosurfactants, exopolysaccharides, polyhydroxyalkonates, flavoring agents, anti tumor drugs and enzymes are produced by halophilic bacteria [5]. Extremozymes, the enzymes isolated from extremophiles are now replacing chemical catalysts in many industries, including manufacturing of chemicals, textiles, Pharmaceuticals, detergents, food, paper and agricultural chemicals [6].

Out of the vast pool of Extremozymes, halophilic proteases are the most widely exploited enzymes in the processing of food, leather and detergents [7]. Proteases are hydrolytic enzymes, which can degrade different protein sources, so find potential application for waste treatment, bioremediation, wool quality improvement, meat tenderization, in food, leather, pharmaceutical and detergent industries. The enzyme to be used as detergent additives should be stable and active in the presence of surfactants, bleaching agents, fabric softeners and various other formulations [8]. Since, halophilic proteases are adapted to extreme environments, they are unusually stable and therefore they could serve as a suitable candidate for industrial processes that are performed under harsh conditions, such as high temperature, high ionic strength and in the organic solvents [9]. Microbial Protease production is highly influenced by media components and metal ions [10, 11]. Besides this, several other factors, such as aeration/agitation, pH, temperature, salinity and incubation time also affect the amount of protease produced [12, 13]. The aim of the present study was to optimize the medium and cultivation

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conditions for alkaline protease production by an extremely halophilic archaeon-*Halobacterium sp*, isolated from solar salterns.

MATERIALS AND METHODS

Bacterial Strain and Culture Conditions: The bacteria used in this study was isolated from solar evaporated ponds (Thalassohaline environment), Tuticorin, Tamilnadu, India. Isolated colonies showing clear zones of casein hydrolysis on the casein agar plate containing 4M NaCl were selected and were aerobically cultured at 40°C in a basal salt medium containing (g l^{-1}): NaCl:233.6; $MgCl_2$:10; KCl:5; Trisodium citrate:3 casein:10; casaminoacid:7.5 and peptone:10, pH was adjusted to 7.0(1).

Protease Production in Shake Flasks: The medium used for the protease production was the basal salt medium mentioned above.1 ml of a week old culture of *Halobacterium sp.* JS1 was inoculated into a sterilized 250 ml Erlenmeyer flask containing 50 ml of the above growth medium and incubated at 40°C in an incubator shaker at 200 rpm for 4 days. The flasks were removed at regular intervals, the contents were centrifuged at 10,000 g for 10 min at 40°C to remove cells and insoluble materials (sediment) and the cell free supernatant was filtered through a 45µm pore size membrane filter and was used as the source of the crude enzyme [14].

Protease Assay by Gelatin Cup Method: The medium containing 2% (w/v) agar, 1% (w/v) gelatin in 50mM glycine, NaOH buffer of pH-10 with 20% NaCl was sterilized at 121°C for 15min. After sterilization, about 15ml of the medium was poured in a petriplate under sterile conditions. Using sterilized cork borer, two 6mm diameter cups were made in the agar plate. The petriplates were then incubated at 37°C for 48 hours. After incubation, the plates were developed with 15% (w/v) mercuric chloride in 20% HCl. After 10 minutes, a clear transparent zone indicated hydrolysis of gelatin by extracellular protease; where as the rest of the plate became opaque because of the coagulation of gelatin by $HgCl_2$. The diameter of the clear zone was used as a measure of protease activity.

Protease Assay: Protease activity was assayed by the modified method of Anson., [15]. The reaction mixture contained 1 ml of 1% casein and 0.5 ml of enzyme in the presence of 50 mM glycine NaOH buffer of pH 10.0 containing 20% NaCl and incubated at 37°C for 20 min and the reaction was stopped by adding 3 ml of 10% TCA. After standing for 1 hr the solution was filtered through Whatman filter paper. To 0.5 ml supernatant, 2.5 ml of Folin-ciocalteau reagent was added, the reaction mixture was incubated at room temperature for 30 min and then the absorbance was measured at 660 nm.

One unit (U) of enzyme is defined as the amount of enzyme that liberates 1µg tyrosine per min from casein under assay conditions.

Biomass Yield: Bacterial biomass was determined by measuring the absorbance at 600 nm [16].

Optimization of Culture Conditions for Bacterial Growth and Protease Production: The effect of temperature, pH and NaCl Concentrations on the growth and protease production was studied. This were carried out by cultivating the isolate at different temperatures (25°C-55°C), different pH values (pH4.0-11.0), different concentrations of NaCl (1.0-5.0M) and different agitation speeds(0-250). Bacterial growth and Protease activity were measured at optimum growth (96hrs).

Effect of Various Carbon Sources: The Bacterial strain was grown in the basal salt medium containing different soluble carbon sources (1% W/V) including glucose, fructose, glycerol, sucrose, starch, maltose and different low cost-agro industrial residues such as wheat bran, wheat flour and Rice bran to study their effect on bacterial growth and protease production.

Effect of Various Nitrogen Sources: The growth medium was supplemented with different nitrogen sources (1%W/V) including organic nitrogen sources such as peptone, beef extract, casein, gelatin, yeast extract, corn steep liquor and skim milk powder, inorganic nitrogen sources such as NH_4Cl_2, $NaNO_3$, $(NH_4)_2SO_4$ and urea, different low cost agricultural byproducts in the form of defatted meals such as soybean meal, cotton seed meal, corn seed meal and chick Pea flour to investigate their effect on bacterial biomass and protease production.

Effect of Various Metal Ions: Effect of metal ions such as $MnSO_4$, $ZnSO_4$, $CaCl_2$, $FeSO_4$, $COCl_2$ and $MgSO_4$ on the protease production and bacterial growth were studied.

RESULT AND DISCUSSION

An extremophile producing haloalkaliphilic extracellular protease was isolated from the solar evaporated salt pond of Tuticorin, Tamilnadu, India. Based on the morphological, biochemical characters, lipid analysis and 16s rDNA sequence the isolate was identified as *Halobacterium sp.*JS1.

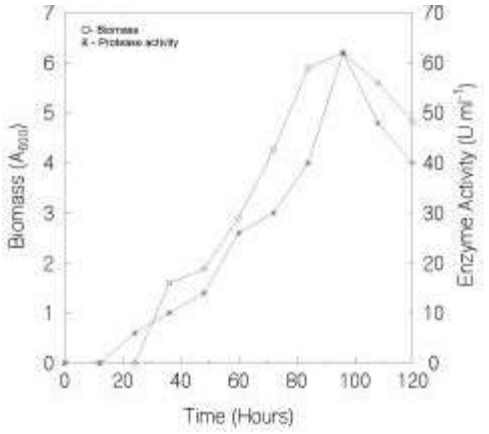

Fig. 1: Effect of Incubation Time on Growth and Production of Protease

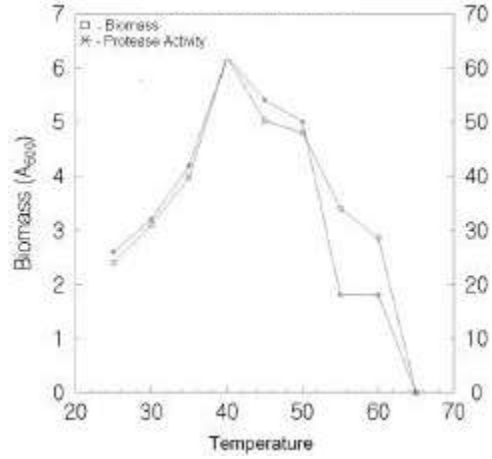

Fig. 2: Effect of Temperature on Growth and Protease Production

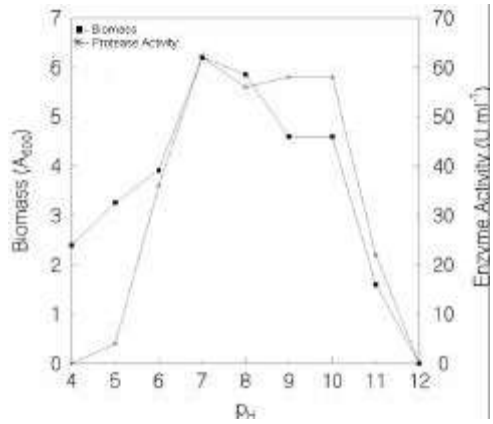

Fig. 3: Effect of pH on Growth and Protease Production

Effect of Incubation Time: Incubation time plays a substantial role in maximizing bacterial growth and protease production. Protease production by

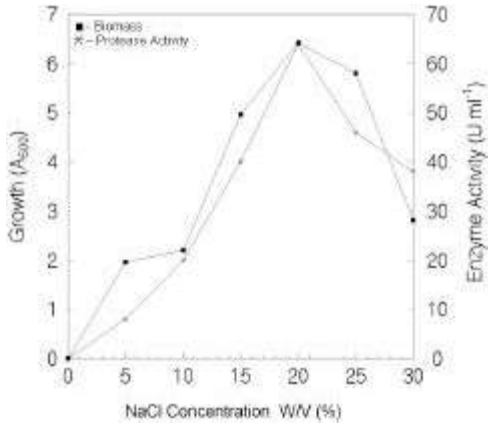

Fig. 4: Effect of Salinity on Growth and Protease Production

Halobacterium sp was found to be growth dependent, as maximum enzyme production was achieved during the stationary phase 96 hrs of growth [Fig.1]. In this respect, the isolate resembled *Halogeometricum sp.* [17] and *Halobacterium salinarum* [18].

Effect of Temperature: Temperature is one of the most critical parameters that have to be controlled in Bioprocessing [19]. The temperature was found to influence extracellular enzyme secretion, possibly by changing the physical properties of the cell membrane [20]. Majority of the halophilic enzymes showed maximum activity in between 50-65°C [21]. Both Bacterial growth and enzymatic activity of *Halobacterium sp.* was maximized in between 35-45°C, with optimum at 40°C [Fig.2]. In contrast, chi *et al.* [19] reported that optimum temperature for alkaline protease production by *Aureobasidium pullalans* was much lower than the optimum growth temperature. Optimum temperature for protease production by *Bacillus sp.*MIG was 60°C [22].

Effect of pH: *Halobacterium sp.* grows in a broader pH (7-10.0) and its extracellular protease does not have a strict pH requirement for its activity. Bacterial biomass (6.20) and proteolytic activity (62 u ml^{-1}) was optimized at pH-7.0, resembling *Halogeometricum sp.* and *Bacillus Sp.*MIG [17, 22]. The above result is in contrast to *Bacillus sp*. strain SMIA-2 which showed optimum protease production at pH 8 [23]. Bacterial growth and protease production were not observed below pH 5 and above pH11 (Fig.3).

Effect of Salinity: Growth and proteolytic activity of *Halobacterium sp.* was observed in a broad range of NaCl concentrations (1-5 M). Since, the isolate has been

adapted to extreme halophilic environment, the maximum growth and proteolytic activity (64 Uml^{-1}) was achieved at high salt concentration (4M), resembling *Chromohalobacter sp.* strain TVSP 101 [24]. Whereas, a decrease in salt concentration (less than 1M) may lead to the reduction of growth and protease production [25]. In contrast, the extremophile was able to grow and produce protease in extreme halophilic condition of saturated NaCl concentration of 5.0 M (Fig.4).

Effect of Nitrogen Sources: The effect of various nitrogen sources including different organic, inorganic and cheap nitrogen sources [protein rich agricultural byproducts] were investigated after 96 hrs of incubation. *Halobacterium sp.* grew well in all media, but the proteolytic activity fluctuated considerably in various media. Among the organic nitrogen sources used, Casein, peptone and skim milk powder had significant effect on extracellular protease production (60 Uml^{-1}, 62 Uml^{-1} and 68 Uml^{-1} respectively).Interestingly maximum proteolytic activity (78 uml^{-1}) was achieved, when the cells were grown in a medium containing the combination of skim milk powder (1%) and peptone (1%), similar results were reported in *Chromohalobacter sp.* [24] and fungi [26]. Poor growth and enzyme secretion were observed in a medium containing gelatin, corn steep liquor, beef extract and yeast extract, similar to results reported in *Bacillus clausii* I-52 [27]. Simple inorganic nitrogen sources like urea, $(NH_4)_2SO_4$, NH_4Cl and $NaNO_3$ did not support the enzyme production.

As reported by John Vesly and Naik, [28] media containing protein rich agricultural by products like chick pea flour, soybean flour, cotton seed meal and corn seed meal showed high protease production [64 Uml^{-1}, 72 Uml^{-1}, 66 U ml^{-1} and 68 U ml^{-1} respectively] Table: 1. The most efficient natural N_2 source for protease production was soybean meal, which yielded 72U ml^{-1}. These results were in agreement with the findings of Elibola and Moreira [29].

Effect of Carbon Source: In a preliminary study, various soluble carbon sources (1%) such as glucose, fructose, sucrose, maltose and starch were used to replace trisodium citrate which was the original carbon source in the growth medium. Results obtained (Table: 2) showed that the bacterium was able to utilize most of the carbon sources tested except glucose and starch. Where as, maltose and sucrose showed good growth but low protease production (30 Uml^{-1} and 34 Uml^{-1}protein respectively) after 96 hrs of incubation. This is in contrast to the previous reports which showed that starch and glucose caused high level of enzyme expression in

Table1: Effect of different Nitrogen sources on Biomass and Protease Production

N_2 Sources (1% w/v)	Biomass (A_{600})	Enzyme activity (U ml^{-1})
Gelatin	2.60	14
Casein	5.258	60
Peptone	5.80	62
Skim Milk Powder	6.0	68
Skim Milk Powder (1%) + Peptone (1%)	6.10	78
Beef Extract	1.0	00
Yeast extract	2.20	04
Urea	00	00
$(NH_4)_2SO_4$	00	00
NH_4Cl	00	00
$NaNo_3$	00	00
Soya bean flour	6.20	72
Cotton Seed meal	5.9	66
Chick Pea Flour	5.8	64
Corn seed meal	6.0	68

Table 2: Effect of different Carbon Sources on Biomass and Protease production

Carbon Sources	Biomass (A_{600})	Enzyme activity (U ml^{-1})
Glucose	00	00
Fructose	4.8	12
Sucrose	5.6	22
Maltose	5.2	20
Starch	00	00
Wheat Bran	5.9	52
Rice Bran	6.2	64
Wheat Flour	5.0	53

Table 3: Effect of different metal ions on Biomass and Protease production

Metal Ion (200 mM)	Biomass (A_{600})	Enzyme activity (U ml^{-1})
Control	6.20	62
$CaCl_2$	6.50	70
$MgSO_4$	4.84	56
$MnSO_4$	5.28	52
$FeSO_4$	00	00
$COCl_2$	00	00
$ZnSO_4$	00	00
$CaCl_2$+$MgSO_4$+$MnSO_4$	6.96	86

Table: 4 Effect of aeration rate on Biomass and Protease production

Agitation Rate	Biomass (A_{600})	Enzyme activity (U ml^{-1})
0	1.68	10
50	2.90	16
100	4.86	28
150	5.6	42
200	6.2	62
250	4.9	28

Bacillus sp and *Bacillus cereus* strain 146 respectively30, [31]. The most significant aspect of the present study is the production of alkaline Protease from *Halobacterium sp*. by using cheaper and easily available substrates. Interestingly the use of cheap carbon sources like wheat bran, Rice bran and wheat flour instigated high biomass and proteolytic activity. Rice bran supported the maximum enzyme production resembling *Bacillus sp* [32].

Effect of Metal Ions: Supplementation of culture media with metal cations improved substantially the protease production and Bacterial Biomass of *Halobacterium sp.* Among the different metals examined, Ca^{++}, Mg and Mn showed increased proteolytic activity at concentrations of 200mM with specific activities of 70 Uml^{-1}, 56 Uml^{-1} and 52 Uml^{-1} protein respectively [Table: 3] where as in corporation of Ca, Mn, Mg together (200mM) in the growth medium showed higher enzyme production than using them individually, with maximum production of 86 Uml^{-1} protein.A similar effect of metal ions was reported in *Bacillus halodurans* [14].

Effect of Aeration/agitation: Microorganisms vary in their oxygen requirement. In particular, O_2 acts as a terminal electron acceptor for oxidative reactions to provide energy for cellular activities. The variation in the agitation speed has been found to influence the extent of mixing in the shake flasks and also affect the nutrient availability [23]. Growth and protease production by *Halobacterium sp.* was investigated at different agitation rates. The result represented in Table: 4 revealed that growth and enzyme production increases with increasing agitation rates and the maximum protease production was observed at 200 rpm rate (62 Uml^{-1}). A similar profile was determined for the effect of agitation speed on alkaline protease production by *Teredinobacter turniae* [29] and *Bacillus halodurans* [14]. However, both growth and enzyme production was very poor under static conditions.

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