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Extracellular Alkaline Protease by a Newly Isolated Halophilic *Bacillus* sp

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Abstract: A moderately halophilic bacterium, *Bacillus* sp. HS-4 was isolated from soil samples collected from Khewra salt range (Pakistan). Experiments were set to observe the effects of different pH, temperature, incubation time, inoculum's size, salt concentration (NaCl), agitation rate, carbon and nitrogen sources and metal ions on the growth of HS-4 and its protease activity. Optimum biomass and proteolytic activity was achieved after 24 hrs of incubation, at 37°C, pH 8 with 8 % salinity and 150 rpm agitation (295 U/ml). Among the various nitrogen sources investigated, different low cost agricultural byproducts in the form of defatted soybean meal was found to be the best inducer of alkaline protease, while inorganic nitrogen sources in the form of ammonium salts [NH_4Cl and $(NH_4)_2SO_4$]showed reduced bacterial growth and enzyme activity. Among the carbon sources tested, rice bran maximized the enzyme production. An increase in protease activity was observed in the presence of Ca^{2+} and Mg^{2+} ions while protease activity was significantly reduced by Cu^{2+} and Zn^{2+} ions. Thus *Bacillus* sp. HS-4 was proved to be an addition to the existing pool of extremophilic bacteria of industrial importance.

Key words: Alkaline protease · Halophiles · Detergents · Protease production · Rice bran · *Bacillus* sp.

INTRODUCTION

Extremophiles are organisms evolved to live in a variety of extreme environments like deep sea hydrothermal vents, hot springs and hyper saline environments. They can be classified as thermophiles, psychrophiles, acidophiles, alkalophiles, halophiles and others [1]. Halophiles have gained attention due to their extensive mechanism of adaptation to extreme hypersaline environments and differentiated based on salinity into non-halophiles (<1.2% NaCl), slight halophiles (1.2-3%), moderate halophiles (3-15%) and extreme halophiles (>15% NaCl) [2].

The halophiles exoenzymes exhibit unique structural and biochemical characteristics. The intriguing stabilities of these enzymes under extreme high saline conditions are still unknown [3]. It is speculated that this could be due to the presence of a relatively large number of negatively charged amino acid residues on their surface to prevent precipitation [4]. However, hydrolytic enzymes from halophiles are not only interesting from the basic scientific viewpoint but, they may also be of potential interest in many industrial and biotechnological applications, owing to their stability and activity at low water levels [5, 6].

A wide variety of biotechnological under harsh conditions, such as bacteriorhodopsins, halorhodopsins, biopolymers, biosurfactants, exopolysaccharides, polyhydroxyalkonates, flavoring agents, anti tumor drugs and enzymes are produced by halophilic bacteria [7]. 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 [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].

According to the market research report on world enzymes published in 2007, the world market for enzymes is expected to grow 7.6% per year to $6 billion in 2011 [10]. Microbial proteases account for approximately 60% of the total enzyme sales in the world [11]. Most of the Gram-positive or Gram-variable, endospore forming rods with halotolerantproperties, have been assigned to the genus *Bacillus* [12]. *Bacillus* sp. grows in a pH range of 7.0-11.0 and produces extracellular protease. Currently, a large proportion of commercially available alkaline proteases are

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derived from different strains of *Bacillus* [13]. Microbial Protease production is highly influenced by media and metal ions [14, 15]. Besides this, several other factors, such as aeration/agitation, pH, temperature and incubation time etc also affect the amount of protease produced [16, 17]. The aim of the present study was to optimize the medium and cultivation conditions for alkaline protease production by extremely halophilic *Bacillus* sp, HS-4 isolated from Khewra salt range, Pakistan.

MATERIALS AND METHODS

Bacterial Strain and Culture Conditions: The bacterial strain used in this study was isolated from the soil samples collected from Khewra salt range (Pakistan). Isolated colonies showing clear zones of casein hydrolysis on the casein agar plate containing 5M NaCl were selected. PH was adjusted to 8.0.

Production of Protease in Shake Flask Fermentation: The casein gelatin medium was used for the inoculum's development with the following composition (gLl^{-1}): Gelatin 1.5; Casein hydrolysates 0.72; glycerol 1.8; and NaCl 7.5. After sterilization, 1.8 ml (20%) glycerol solution sterilized in dry oven at 100°C for 30 min. was poured into the medium aseptically in each flask. After that 15 ml of the inoculum (10%) was added in the different flasks. The fermentation conditions were maintained at 37°C, 120 rpm agitation for 3 days. Samples were collected after every 12 hrs. The culture broth was harvested and centrifuged at10,000 rpm for 20 min at room temperature to remove cells and insoluble materials (sediments) and the cell free supernatant was filtered through Whatmann filter paper 1 and was used as the source of crude enzyme [18].

Protease Assay: Alkaline protease activity was estimated by the modified method of Kunitz [19] and this method is based on the determination of split products of casein soluble in 5 % trichloroacetic acid (TCA). To 1ml of 1% casein solution (pH 8.0), 1 ml of crude enzyme solution was added (pH 8.0). The mixture was incubated for 60 minutes at 37° C. After incubation, 1 ml of 5% TCA was added and tubes were placed in ice for 30 minutes. The precipitates were removed by using Whatmann filter paper No.1. The optical density of the supernatant was measured at 600 nm. Blanks were prepared in the same way except that 1ml of 5 % TCA was added before incubation. All assays were carried out in triplicate and the average of the three was taken to evaluate the activity units. One unit of enzyme activity is defined as the amount of enzyme which releases 1ì mole of tyrosine under standard conditions.

Biomass Yield: Bacterial biomass was determined by measuring the growrhat 600 nm [20].

Optimization of Culture Conditions for Bacterial Growth and Protease Production: The effect of temperature, pH, NaCl concentrations, inoculum's size and agitation speed on the bacterial growth and protease production was studied by cultivating the isolate at different temperatures (30, 37 and 50°C), different pH values (pH 6.0-11.0), different concentrations of NaCl (3-14%), different inoculum's size (1%, 5%, 10% and 15%) and different agitation speeds (120, 150 and 180 rpm). Bacterial growth and Protease activity were measured at optimum growth (24 hrs).

Effect of Various Carbon Sources: The Bacterial strain was grown in the casein-gelatinmedium containing different carbon sources (1% w/v) including glucose, fructose, glycerol, lactose, 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: Growth of the bacterial isolate and the protease production were observed in casein-gelatin mediumprovided with various organic nitrogen sources such as casein, gelatin, peptone, meat extract, yeast extract and inorganic nitrogen sources such as urea, NH_4Cl, $(NH_4)_2SO_4$ and different low cost agricultural byproducts in the form of defatted meals such as soybean meal and corn seed meal.

Effect of Various Metal Ions: Effects of metal ions such as $ZnCl_2$, $CaCl_2$, $CuSO_4$ and $MgSO_4$ on the protease production and bacterial growth were studied.

RESULTS AND DISCUSSION

An extremophile producing haloalkaliphilic organism was isolated from soil samples collected from Khewra salt range (Pakistan). Based on the morphological and biochemical characters, the isolate was identified as *Bacillus* sp [21].

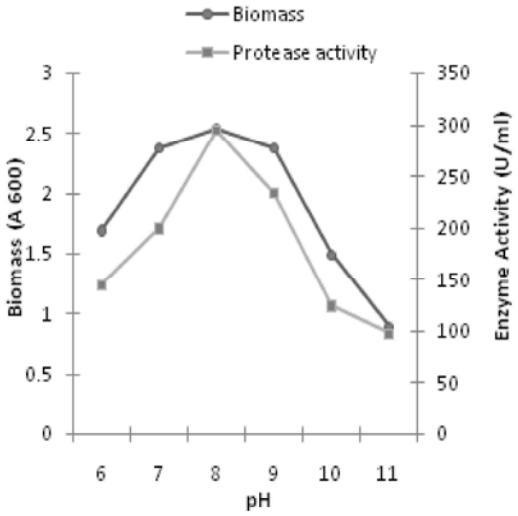

Fig. 1: Effect of pH on Bacterial Growth and Production of Protease

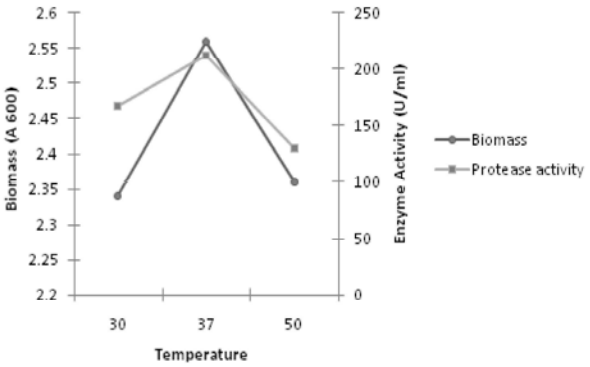

Fig. 2: Effect of temperature on Bacterial Growth and Production of Protease

Effect of pH: *Bacillus* sp. HS-4 grew and produced protease optimally at pH 8, with slightly suppressed growth at pH 7 and significantly reduced growth at pH 11. Bacterial growth and protease production were not observed below pH 5 and above pH 11 (Fig. 1). The bacterial biomass(2.54) and proteolytic activity (295 U ml^{-1}) were optimized atpH-8.0, resembling *Bacillus* sp. strain SMIA-2 [22]. Alkaline protease from *Bacillus subtilis*NCIM 2713 [23] was maximally active at pH 8 and from *Halomonascampisalis*at pH 8-11 [24].

Effect of Temperature: Temperature is one of the most critical parameters that have to be controlled in bioprocessing [25]. Both bacterial growth and enzymatic activity of *Bacillus* sp. were optimum at 37°C (Fig.2). Similarly an extracellular protease purified from a psychrophilic *Pseudomonas*sp., displayed optimal activity at 40°C [26]. In contrast, chi *et al*. [25] reported that

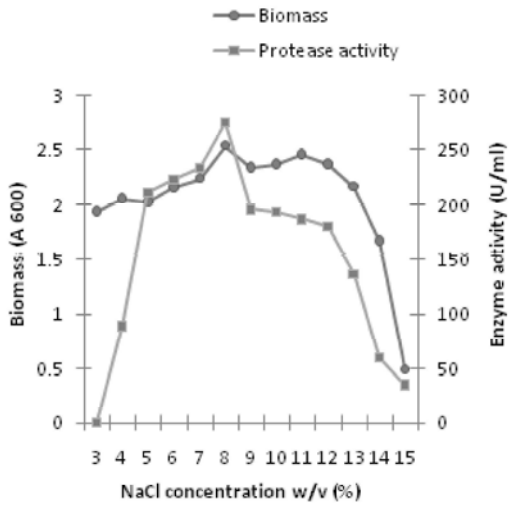

Fig. 3: Effect of Salinity on Bacterial Growth and Production of Protease

optimum temperature for alkaline protease production by *Aureobasidiumpullalans*was much lower than theoptimum growth temperature. Optimum temperature for protease production by *Bacillus* sp. MIG was 60°C [27].

Effect of Salinity: The isolate under study could grow in the range of 3-15 % (w/v) NaCl, the optimum being at 8 % (w/v) with protease activity at 276 U ml^{-1} (Fig. 3). These results indicated the halophilic nature of the strain HS-4. Similar trends were also evident in *Salinicoccus alkaliphilus* sp.nov., a moderately halophilic and alkalophiliccoccus isolated form Baer Soda Lake in Mongolia, which could grow over a wide range of NaCl, 0-25 % (w/v) with an optimum at 10 % (w/v) [26]. The growth of HS-4 was reduced extensively in the absence of salt with no protease production and reduction in protease production at 15 % (w/v) NaCl was also evident. The results clearly indicated the halophilic nature of the protease. Similar results have also been reflected by the haloalkalophilicarchaeon and *Natronococcus occultus* in which protease secretion was optimum at 1-2 M NaCl [28]. However, in the case of the archaebacterium *Halobacteriummediterranei*, a much higher salt requirement (25 % w/v) for serine protease secretion was reported [29]. The salt requirement for optimum enzyme secretion varied significantly among the isolates. The salt dependency was; however, relatively lower when compared to the extreme haloalkalophilicarchaean isolated from Soda Lake [30].

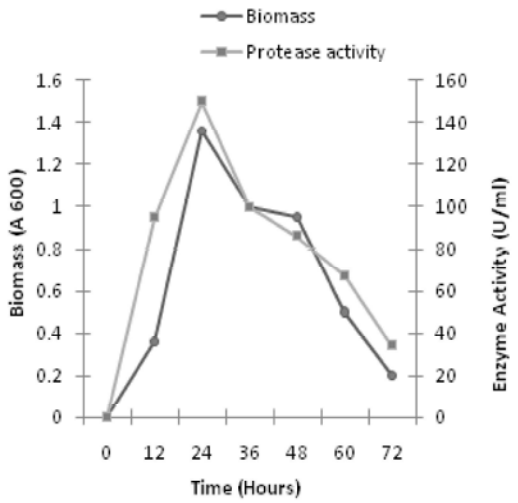

Fig. 4: Effect of Incubation Time on Bacterial Growth and Production of Protease

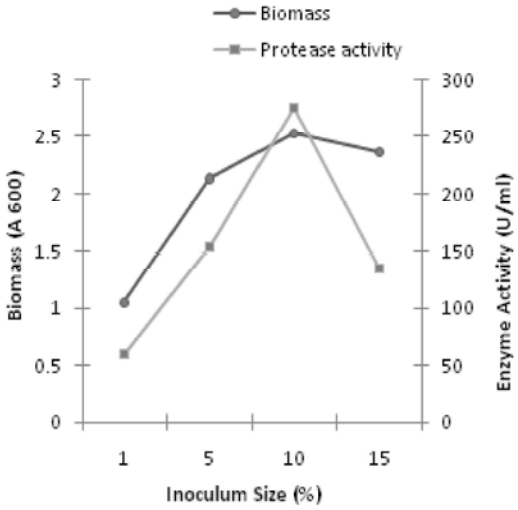

Fig. 5: Effect of Inoculum's Size on Bacterial Growth and Production of Protease

Effect of Incubation Time: Incubation time plays a substantial role in maximizing bacterial growth and protease production. Results showed that the maximum growth and protease production was observed with 24 hours incubation time (Fig. 4). Similar results showed that among the isolates, S5 was the most potent producer of alkaline protease. The level of protease production was maximum at the late exponential phase after 24 hours of growth [31].

Effect of Inoculum's Size: Results showed that the maximum growth and protease production was observed with 10 % inoculum's size after 12 hours of incubation (276 U ml^{-1}) (Fig. 5).

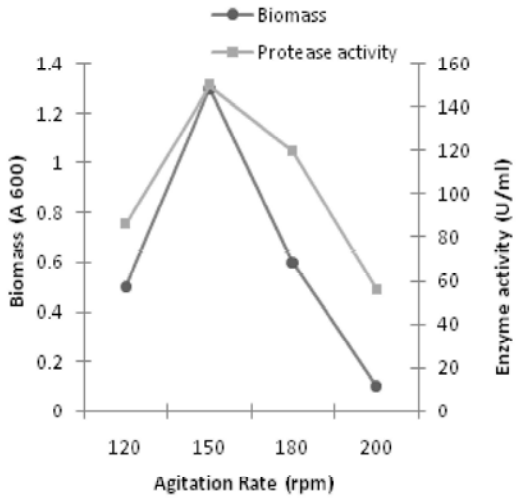

Fig. 6: Effect of Aeration/ Agitation on Bacterial Growth and Production of Protease

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 [22]. The result represented in figure 6 revealed that maximum protease activity observed with 150 rpm rate was 150 U ml^{-1}; while at 120 rev/min was 86 U ml^{-1}. But at 180 rpm rate, it was more than 120 rpm rate but less than 150 rpm rate i.e., 120 U ml^{-1} (Fig. 6). Similarly at 200 rpm rate, protease activity was greatly reduced. This was perhaps due to the denaturation of enzyme caused by high agitation speed.

Effect of Nitrogen Sources: Among the organic nitrogen sources used; casein-gelatin, peptone, yeast extract and beef extract had significant effect on extracellular protease production (197 U ml^{-1}, 100 U ml^{-1}, 96 U ml^{-1} and 87 U ml^{-1} respectively). Results showed that the maximum growth and protease production was observed with casein-gelatin after 24 hours of incubation (Table 1). Results of this study under discussion are also in consistence with the work that gelatin act as an organic source of protein synthesis and enhance the bacterial growth [32]. Actually extracellular protease are produced at exponential phase of bacterial growth, which is associated with the sporulation of *Bacillus subtilis* [33],whereas casein hydrolysate is a source of readymade amino acids and also encourages the foam formation to remove spores and cellular debris from the culture

Table 1: Effect of Different Nitrogen Sources on Biomass and Protease Production

N2 Sources (1% w/v)	Biomass (A 600)	Enzyme activity (U. ml)
Casein-Gelatin	3.95	197
Peptone	2.50	100
Beef extract	1.60	87
Yeast extract	1.75	96
Urea	1.25	57
NH_4Cl	0.10	13
$(NH_4)_2SO_4$	0.45	29
Soybean meal	4.56	195
Corn seed meal	4.25	180

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

Carbon Sources (1% w/v)	Biomass (A 600)	Enzyme activity (U. ml)
Glucose	2.85	130
Fructose	1.8	75
Glycerol	1.5	60
Lactose	2.1	93
Sucrose	3.1	230
Starch	3.8	268
Maltose	2.0	82
Wheat bran	4.1	268
wheat flour	4.56	270
Rice bran	5.01	295

Table 3: Effect of Different Metal Ions on Biomass and Protease production

Metal Ion (200 mM)	Biomass (A600)	Enzyme activity (U ml^{-1})
$CaCl_2$	3.6	80
$MgSO_4$	2.5	64
$CuSO_4$	0.8	14
$ZnCl_2$	0.3	5

medium. Simple inorganic nitrogen sources in the form of ammonium compounds showed reduced growth and protease production as compared to urea. As reported by Johnveslyand Naik, [34] media containing protein rich agricultural by products like soybean meal and corn seed meal showed high protease production [195 u ml^{-1} and 180 u ml^{-1} respectively] Table 1 shows that the most efficient natural N_2 source for protease production was soybean meal, which yielded 195 u ml^{-1}. These results are in agreement with the findings of Elibola and Moreira [35].

Effect of Carbon Sources: Results showed that the maximum growth and protease production was observed with starch after 24 hours of incubation (268 U ml^{-1}) (Table 2). This is similar to the previous reports which showed that starch caused high level of enzyme expression in *Bacillus* spand *Bacillus cereus* strain 146 respectively [36]. It seems that ‘’catabolic repression” phenomena is the best possible explanation for the reduction of protease production in the presence of glucose [37]. Therefore it is preferable to use starch as a carbon source. It was also reported that in the presence of glucose for growth of *Bacillus nesternkonia*sp. AL-20, the protease production was suppressed. [38].The most significant aspect of the present study isthe production of alkaline protease from *Bacillus* sp. by using cheaper and easily available substrates.Interestingly the use of cheap carbon sources like wheatbran, Rice bran and wheat flour instigated high biomass and proteolytic activity. Rice bran supported the maximumenzyme production of *Bacillus* sp [39].

Effect of Metal Ions: An increase in protease activity was observed in the presence of 200mM Ca^{2+} and Mg^{2+} with specific activities 80 and 64 U ml,$^{-1}$ respectively. These cations (Ca^{2+} and Mg^{2+}) have also been reported to increase activity of A21 from *B. mojavensis*A21 [40]. It is believed that these cations protect the enzyme against thermal denaturation and play a role in maintaining the active conformation of the enzyme at higher temperatures. In contrast, the protease activity was strongly inhibited by 200mM Cu^{2+} and Zn^{2+} with 14 and 5 U ml^{-1} respectively (Table 3).

It can be concluded that enzymes from halophiles are expected to show optimal activities in extreme conditions; thus, the possibility to have a wide variety of moderate halophiles producing extremozymes will be of invaluabletoolfor biotechnological applications.

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