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RESEARCH ARTICLE

ISOLATION AND BIOCHEMICAL CHARACTERIZATION OF PROTEASE ISOLATED FROM *BACILLUS SP* FROM AL-RAZZAZA LAKE, KARBALA, IRAQ

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ABSTRACT

Proteases the most important group of enzymes produced commercially are used in detergent, protein, brewing, and meat, photographic, leather and dairy industries. Al-Razzaza, is the second largest lake located,10 kilometers west of Karbala province, Iraq. It is a unique ecosystem and harbors various haloalkaliphilic bacterial species which produces biotechnologically important thermo-haloalkaliphilic enzymes. The present study deals with the production and partial characterizations of enzyme protease isolated from Al-Razzaza Lake, Karbala. An effective proteolytic enzyme producing microbial strain has been isolated and evaluated its extracellular protease production properties with respect to different fermentative physiological parameters. The strain has been identified based on biochemical tests according to Bergey's Manual of Systematic Bacteriology as *Bacillus* sp. A total fifteen bacterial strains were isolated from the Al-Razzaza Lake water, sediment and matt samples. Out of these, one bacterium showing prominent proteolytic activity was characterized on the basis of morphological, cultural and biochemical parameter and identified as *Bacillus* sp. The alkaline protease produced by *Bacillus sp*showed optimum activities at pH 8, at temperature 70^0C. It proves that the enzyme alkaline protease produced by this bacillus has potential applications in food, pharmaceutical and detergent industries.

INTRODUCTION

Enzymes are a specialized protein produced in an organism which is capable in catalyzing a specific chemical reaction. Protease is an important group of enzyme which conducts proteolysis by hydrolysis of the peptide bonds that link amino acid together in the polypeptide chain. Protease production is an inherent capacity of all microorganisms (Kumar, 1999). Proteases are a group of enzymes, whose catalytic function is to hydrolyze peptide bonds of proteins and break them down into polypeptides or free amino acids. They constitute 59%of the global market of industrial enzymes, which is expected to exceed $ 2.9 Billion by 2012 (Deng *et al.,* 2010). They have got wide range of commercial usage in detergents, leather, food and pharmaceutical industries (Bhaskar *et al.,* 2007 and Jellouli *et al.,* 2009). Sources of proteases include all forms of life, that is, plants, animals and microorganisms. Based on their acid-base behavior, proteases are classified in to three groups, that is, acid, neutral and alkaline proteases. Acid proteases performed best at pH range of 2.0-5.0 and are mostly produced by fungi. Proteases having pH optima in the range of 7.0 or around are called neutral proteases. Bacteria are the predominant group of alkaline protease producers the genus Bacillus being the most common source. Uses of protease are vast and can be categorized broadly into detergents, dairy industry, tanning, baking and brewing industries.

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Bacterium secretes proteases to hydrolyse the peptide bonds. Proteases are involved in digesting long protein chains into short fragments, splitting the peptide bonds that link amino acid residues. Al-Razzaza was created in 1969 when a Spanish contractor built a drainage canal to divert the annual floodwaters of the Euphrates river in to the desert to prevent flooding across southern Iraq. The lake, with a surface area of 1810sqkm, is 40meters above sea level and can hold some 26billion cubic meters of water with length 70kmform north to the south and 40km width from the east to the west. Al-Razzaza Lake is located10 km north west of Karbala. It is part of a wide valley that includes al-Thar thar, al-Habbaniya,al-Razzaza and Bahr Najaf (Najaf Sea). The lakeis supplied by eight sources, including the River Euphrates; Lake Habaniya, east of Ramadi; Rashidiya, north of Karbala; ground waters prings in Ayn al-Tamr, 80km west of Karbala; rainwater and seasonal flows. Located to the west of the Iraqi holy city of Karbala with Coordinates 32°45'35"N 43°39'22"E, as shown in Figure (1). Iraq features various ecosystems including extreme environments such as high salinity soil and water in which microbial diversity has been poorly studied (Kornijów *et al.,* 2001; Zdanowski, 2001; Rahi and T. Halihan, 2010; Mohammad, 2014). Lake Razazah, is also known as Bahr Al-Milh (literally Sea of Salt). No study had been done on protease from *Bacilli* of Al-Razzaza Lake, which can withstand high temperature and high pH and has wide applications in different industries. In present study, aim was to deal with the isolation, characterization, production and optimization of aprotease from bacterial strain isolated from

the Al-Razzaza Lake and which could be useful in the food, pharmaceutical, detergent and leather industry.

Figure 1. The Area of Study (Al-Razzaza lake, Karbala, Iraq)

MATERIALS AND METHODS

Screening of proteolytic bacteria

A total of 25 samples (sediment, matt and water) were collected from Lake was screened for proteolytic enzymes. About 1g of sediment and matt sample was transferred to 100mL sterilized distilled water in 250 mL conical flask and agitated (100 rpm) at 37^0C for 15 min on rotary shaker. The sample was then heated at 800C for 15 min to destroy all the vegetative microbial cells. The suspension was then diluted to 10^7 dilutions. One ml of each diluted sample was lawn into Petri plates containing Horikoshi medium (A, B, C and D) and incubated at 37^0C for 72h and four time repeated sub culturing was made in the same medium. After enrichment, it was inoculated on Nutrient agar (pH 10) and incubated at 37^0C for 24h for isolation pure bacterial culture.

Biochemical and phenotypic characterization

Selective isolate was identified through its biochemical and physiological properties according to Bergey's Manual of Systematic Bacteriology.

Preparation and Partial characterization of crude enzyme protease

The 100 mL Yeast extract casein medium was inoculated with isolated culture and incubated at 37^0C in shaking incubator. After 72h incubation, the broth was centrifuged at 5000-8000 rpm for 15 min. The supernatant served as crude enzyme source. The standard graph with tyrosine was prepared. Estimation of proteases was carried out with casein and the absorbance was read at 650 nm 2, 6. The proteolytic activity was defined as the amount of the enzyme that released 1µg of tyrosine per minute under the assay conditions.

Partial characterization of protease was determined by assaying the enzyme activity at different parameters such as pH (7.0 to 10.5), temperature (55^0C to 80^0C), substrate concentration (5 mg/mL to 40 mg/mL) and enzyme concentration (Gupta *et al*., 2005).

Protease assay

Protease activity was assayed according to Anson method and was slightly modified. The reaction mixture contained 2.5 ml of 0.65% Hammerstein casein and 0.5 ml of appropriately diluted enzyme in the presence of 50mMGlycineNaOH buffer pH 9.0. The reactants were incubated at 37°C for 10 min and the reaction was stopped by adding 2.5 ml of 110mM trichloroacetic acid (TCA). A suitable blank was run simultaneously, in which TCA was added to the enzyme solution, followed by substrate addition. After incubating at room temperature for 30 min, both test and blank solutions were centrifuged at 10,000 rpm for 10min. To the 0.4ml supernatant, 1.0 ml 50mM Na_2CO_3 and 0.2 ml *Folin-ciocalteau* reagent was added, the reaction mixture was incubated at room temperature for 30 min and the absorbance was measured at 660nm. One unit (U) of proteolytic enzyme activity was defined as the amount of enzyme that liberated 1µg tyrosine per ml per minute from casein under specified assay conditions (Lowry *et al.,* 1951).

Optimization of culture conditions for the bacterial growth and the protease production by isolated strain

To select the optimum pH, temperature, aeration and agitation, enzyme production was investigated at different pH environments (pH 5.0-12.0), at different temperatures (25^oC-45^oC), aeration conditions with respect to volume of media in 250ml conical flasks (25ml-150ml) and speed of agitation from static to 150 rpm, respectively in separate flasks. The samples were collected every 24 h for 72h to measure the enzyme activity (Mabrouk, 1999).

RESULTS AND DISCUSSION

In the present study, a total of 15 different bacterial species were isolated from water, sediment and matt samples from Lake. Out of 15, three isolates were showed maximum casein hydrolysis activity on skim milk agar at pH 8. Out of them one isolate AHK 9(**A**bdul Hussain Kadhim) was selected for further study since it showed prominent proteolytic zone of 25mm. These isolate was characterized by cultural, morphological and biochemically by commercially available Hi-media Rapid detection kit. The isolate AHK 9 was Gram positive, rod shape and motile. Growth was detected at different pH (7 to12) and salt concentration of NaCl (1 to 8%). Proteolytic enzymes are ubiquitous in occurrence, being found in all living organisms, and are essential for cell growth and differentiation.

The extracellular proteases are commercial value and find multiple applications in various industrial sectors. Although there are many microbial sources available for producing proteases, only a few are recognized as commercial producers (Gupta *et al.,* 2002). Of these, strains of Bacillus sp. dominate the industrial sector. In addition to that, several workers investigated the production of protease and alkaline protease from *Bacillus subtilis* (Lalitha, 2010). In the present study a total 15 different bacterial species were isolated from water, sample of Lake. Out of 15, 3 bacterial strains were found protease enzymes producers. Some extracellular enzymes are used in the food, dairy, pharmaceutical, and textile industries and are produced in large amounts by microbial synthesis (Singhal, 2012). Proteases are one of the most important group of industrial enzymes and account for nearly 60% of the total enzyme sale The major uses of free proteases occur in dry cleaning, detergents, meat processing, cheese making, silver recovery from photographic film, production of digestive and certain medical treatments of inflammation and virulent wounds (Cowan,1994).

Table 1. Morphological, Cultural and Biochemical characterization of lake *Bacillus* sp

Isolates	Gram character	Shape	Colour of colony	Motility	Catalase	Oxidase	Endospore	Enzyme	Biochemical test							
								Protease	Glucose	Fructose	Sucrose	Lactose	Mannose	Arabinose	Galactose	Xylose
AB01	+	rods	Cream	+	+	+	+	+	+	+	+	+	+	+	+	+
AHK02	+	rods	Cream	+	+	+	+	+	+	+	+	+	+	+	+	+
AHK03	+	rods	Cream	+	+	+	+	+	+	+	+	+	+	+	+	+
AHK04	+	rods	Cream	+	+	+	-	+	+	+	+	-	-	-	-	-
AHK05	+	rods	Cream	+	+	+	+	+	+	+	+	+	+	+	+	+
AHK06	+	rods	White	+	+	+	-	-	+	+	+	-	-	-	-	
AHK07	+	rods	White	+	+	+	+	+	+	+	+	+	+	+	+	+
AHK08	+	rods	Cream	+	+	-	-	-	+	-	+	+	+	+	+	+
AHK09	+	rods	Cream	+	+	+	+	+	+	+	+	+	+	+	+	+
AHK10	+	rods	Cream	+	+	+	+	+	+	+	+	+	+	+	+	+
AHK11	+	rods	Cream	-	+	+	-	-	+	+	+	+	+	+	+	+
AHK12	+	rods	Cream	+	+	-	-	-	+	+	+	+	+	+	+	+
AHK13	+	rods	Cream	+	+	+	+	+	+	+	+	+	+	+	+	+
AHK14	+	rods	Cream	+	+	+	+	-	+	+	+	+	+	+	+	+
AHK15	+	rods	Cream	+	+	+	+	+	+	+	+	+	+	+	+	+

Table 2. Screening for Protease activity on Skimmed milk agar

Isolates	Zone of solubilization (mm)		
	24hrs	48hrs	72hrs
AHK 03	15	30	35
AHK09	18	22	26
AHK10	9	14	18

Table 3. Physiological Characteristics of Bacillus sp isolated from

Temp	Results
25^0C	+
30^0C	+
37^0C	+
42^0C	+
45^0C	+
Growth at pH	
5	+
6	+
7	+
8	+
9	+
10	+
12	-
Growth on Nacl	
2.5	+
5	+
7.5	+
10	-

Currently, the enzyme industry is thriving worldwide, with the US market responsible for about one third of the total market. The estimated value of the worldwide sales of industrial enzymes in 2003 was $2.3 billion peryear. Approximate distribution between sectors is food enzymes (29%), feed enzymes (15%), and general technical enzymes (56%). Proteases account for about 60% of the market. The detergent industry represents the single most important industry, accounting for about 35% of total enzyme sales. Proteases find significant uses in many more industries, including the textile and waste water management industries. In addition to their dominant role in detergents and their uses in food processing, alkaline proteases are used for diverse applications, including silk processing, silver recovery, and in some nutritional applications. Recently, proteases have found novel applications in the silk industry wherein they find use in the process of degumming of silk.

Conclusion

In the present study, different bacterial species were isolated from water, sediment and matt sample of Lake. Out of them, one bacterial strain AHK 9 was found protease producer and screened for production and the partial characterizations of protease. The strain has been purified and characterized in terms of its biochemical and physiological growth properties. Based on biochemical tests, the isolate has been identified up to genus level and observed that this strain belongs to *Bacillus* sp. Extracellular enzyme production properties were studied and observed that this strain produces extracellular enzyme protease. This strains ability towards protease production and since the strain belongs to *Bacillus* sp. hence, the produced

protease is considered as serine type of protease which is known for its commercial importance.

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