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Isolation and screening of extracellular proteases produced by new Isolated *Bacillus* sp

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ABSTRACT

Bacterial are well known for their ability to excrete enzymes into the environment. *Bacillus* sp. being industrially important organisms produces a wide variety of extra-cellular enzymes including proteases. *Bacillus* sp. isolated from local marine samples collected from Saudi Arabia for produced protease enzyme. The aim of this work was to evaluate protease production by different bacterial isolated from marine. Six bacterial isolates were screened for protease production. Two protease producing isolates, No.2 and No.3 were selected on the basis of gelatin hydrolyses. When the extracellular protease were examined using fermentation production medium, the date recorded the highest extracellular protease enzyme reached to (243 U/ml) by isolate No. 2. Finally the selected isolate was identify according to the morphological characteristics and named *Bacillus* sp No.2 EHN.

INTRODUCTION

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.

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Neutral proteases are mainly of plant origin. While proteases having optimum activity at pH range of 8 and above are classified as alkaline proteases produced from microorganisms. Proteases produced from microorganisms play important role in several industries example detergent, tanning, photographic industries, pharmaceutical and waste treatment etc. (Gupta *et al.*,2002). Proteases are widespread in nature, microbes serve as a preferred source of these enzymes because of their rapid growth, the limited space required for their cultivation and the ease with which they can be genetically manipulated to generate new enzymes with altered properties that are desirable for their various applications (Kocher and Mishra, 2009) *Bacillus* produces a wide variety of extracellular enzymes, including proteases. Several Bacillus species involved in protease production are e.g. *B. cereus*, *B. sterothermophilus*, *B. mojavensis*, *B. megaterium* and *B. Subtilis* (Shumi *et al*., 2004). The genus "Bacillus" is an important source of industrial alkaline proteases and are probably the only genera

being commercialized for alkaline protease production (Ferrari *et al.*,1993). They are widely distributed in soil and water, and certain strains tolerate extreme environmental conditions including highly alkaline conditions. Screening of proteases producing *Bacillus* sp. from different ecological environments can result in isolation of new alkaline proteases with unique physiochemical characteristics (Singh *et al.*,1999). One of the most important characteristics that determine the industrial suitability of proteases is their requirement of high pH for optimum enzyme activity. In this paper we aimed to isolate newer source of extracellular protease from the local marine sample in Saudi Arabia to potential application of the proteases for industrial applications.

MATERIALS AND METHODS

Source of samples

Samples of sea water and sediment (30-m depth) were collected in sterile containers along the Red Sea coast from Jeddah, Saudi Arabia.

Isolation of bacteria

Bacteria were isolated for protease enzyme using a serial dilution method described by Sjodahl *et al.,* (2002). Samples were inoculated on skim milk agar plates containing peptone (0.1%), NaCl (0.5%) and skim milk (10%) medium prepared using sea water, then incubated at 28 ± 2°C for three days (Uyar *et al.*,2011). Bacterial isolates were primarily purified on nutrient agar medium and routinely maintained at 4^0C on culture purity was determined from colony morphology.

Screening for best strain produced protease

The isolates were Screening for best strain produced protease by plate assay using protease specific medium containing (g/l) K_2HPO_4 2.0, glucouse1.0, peptone 5.0, gelatin 15.0, and agars 15. The clear zone diameters were measured after 24h of incubation at 28°C by flooded the plates with mercuric chloride solution, this method was referred as gelatin clear zone method (Abdel Galil 1992).

Quantities estimation of protease

The isolates strains were inoculated in 50 ml of protease specific medium broth containing (g/L) glucose, 5.0; peptone, 7.5; ($MgSO_4.7H_2O$, 5.0; KH_2PO_4, 5.0; and $FeSO_4.7H_2O$, 0. 1, pH-7.0 and were culture in a rotary shaker (180 rpm) at 28°C for 3 days. After the completion of fermentation, the whole fermentation broth was centrifuged at 10,000 rpm at 4°C, and the clear supernatant was recovered. The crude enzyme supernatant was subjected to further studies (Josephine *et al.*,2012).

Measurement of enzymatic activity

Protease activity in the culture supernatant was determined according to the method of Tsuchida *et al.,* (1986) by using casein as a substrate. A mixture of 500 µl of 1% (w/v) of casein in 50 mM phosphate buffer, pH 7 and 200 µl crude enzyme extract were incubated in a water bath at 40°C for 20 minutes. After 20 minutes, the enzyme reaction was terminated by the addition of 1 ml of 10% (w/v) trichloroacetic acid (TCA) and was kept at room temperature for 15 minutes. Then, the reaction mixture was centrifuged to separate the unreacted casein at 10,000 rpm for 5 minutes. The supernatant mixed with 2.5 ml of 0.4M Na2CO3. 1 ml of 3-fold diluted Follin Ciocalteus phenol reagent, was adding. The resulting solution was incubated at room temperature in the dark for 30 minutes and absorbance of the blue color developed was measured at 660 nm against a reagent blank using a tyrosine standard (Lowry *et al.*,1951). One unit of protease is defined as the amount of enzyme that releases 1 µg of tyrosine per ml per minute under the standard conditions of supernatant solution.

Identification of isolated bacteria

The isolated bacteria with strong productivity for protease were purified according to the procedure described by Peciulytė (2007). Finally, the isolated and purified bacterial strain producer was identified by means of morphological examination, cultural studies and biochemical characterization according to the methods of Buchanon and Gibbons (1974).

RESULTS AND DISSECTION

Six bacterial strains were isolated from marine samples. For all the 6 isolates, both qualitative (zone of inhibition) and quantitative (U/ml) protease assays were done. The best isolate produced a maximum amount of extracellular protease was identified.

Screening and isolation of proteolytic bacteria

The proteolytic activities of all strains were assayed using skim milk agar and gelatin agar, and exhibited as diameter of clear zone. Gelatine agar was the best than skim milk agar for qualitative test of protease. As showed in Fig (1) among, the six isolates the isolates No. 2 and 3 showed high proteolytic activity.

Fig. 1: The zone of inhibition by isolates No.2 and 3 on gelatin agar plates.

Gupta *et al.,* (2005) performed isolation of bacterial strains from environmental samples and screened their capability of protease

production using skim agar and reported that the *Streptomycin sp*. CD3 was the maximum producer of protease among the isolated strains. On the hand seventy fungal isolates were screened by Chekireb *et al.,* (2009) for their abilities to produce extracellular protease by means of formation of clearing zones around the fungal growth in gelatin ager plates.

Quantitative analysis of extracellular protease

The objective of present investigation was to select the bacterial strains with high level of protease producing ability. In order to achieve the aims, we have selected during the initial screening, a total of 6 different bacterial strains were isolated on gelatin agar medium. The six isolates were checked for quantitative test of extracellular protease in liquid medium. Table (1) clear that, all the bacterial under study secreted protease enzyme at varied levels. The maximum protease activity (243 U/ml) was attained after 72 h by isolate No 2. It was found that maximum production occurred at end of exponential phase. Other three strains showed high production of extracellular protease, isolates No 4, 5, and 6 are (155,175 and 149 U/ml respectively). The lowest extracellular enzyme activity was observed by isolates No 1 and 3 with enzyme activity (55 and 75U/ml) respectively.

Table. 1: Protease activity from different isolate in production medium .

Isolate No	Optical density	Enzyme activity (U/ml)
Isolate 1	0.266	55
Isolate 2	1.17	243
Isolate 3	0.362	75
Isolate 4	0.747	155
Isolate 5	0.825	171
Isolate 6	0.716	149

Several investigations have been done for screening new isolates for proteases production. Other investors, reported that both *Bacillus anthraces*, S-44 and *Bacillus cereus* S-98 exhibited their maximum ability to biosynthesize proteases within 60 h incubation period since the productivity reached up to 126.09 units/ml-1 for *Bacillus anthracis*, S-44 corresponding to 240.45 units/ml-1 for *Bacillus cereus*, S-98 respectively (Johnvesly *et al.*,2012). On another hand , the maximum protease production was noticed among 14 isolated *Endhatia parasitica and Muco. Miehei*, which ranged between 5.1 to 369 U/ml.(Brown *et al.*,1991).

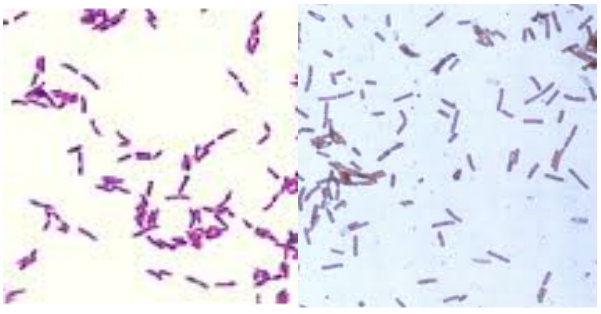

Fig. 2: Gram-positive Rod Shaped Bacillus strain.

Identification protease producing bacterium

Bacterial strain producing protease enzymes were isolated from marine. Among them, isolate No.2 showed the highest protiolatic enzymes productivity. The potent *bacteria was* identified based on morphological characterizations. The morphological characteristics are presented in Fig (2). The results showed that the strain No. 2 is a motile, Grame-positive, rod-shaped, spore-forming bacterium it identified to be a strain of *Bacillus* (Kim *et al.,* 1998) and named it *Bacillus* sp. 2 EHN.

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