Mycopath (2013) 11(1)**:** 51-54 **Brief Article**

Isolation and screening of protease producing bacterial species

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Abstract

Bacteria are well known for their ability to excrete enzymes into the environment. This research aimed to isolate and to screen the potential bacterial isolates for protease production. Twenty three bacterial strains were procured from different sources and screened for protease production. Two protease producing isolates, *Streptococcus* sp. 1 and 2 were selected on the basis of casein hydrolyses by showing clear zones of 4.53 mm and 4.90 mm, respectively. When the proteases were examined using fermentation production medium, the data recorded the highest protease enzyme reached to 155 U mL^{-1} by *Steptococcus* sp. 2. While *Streptococcus* sp. 5 and 6 showed minimum protease production by showing clear zone of 0.5 and 0.8 mm and enzyme activity 2.0 and 1.0 U mL^{-1}, respectively.

Keywords: Bacterial species, protease, submerged fermentation, *Streptococcus* sp.

Introduction

Proteases are a group of enzymes whose catalytic function is to hydrolyze peptide bonds of proteins and to break them down into polypeptides or free amino acids. They constitute 59% of the global market of industrial enzymes, which was expected to exceed $ 2.9 Billion by 2012 (Deng *et al*., 2010). They got wide range of commercial usage in detergents, leather, food and pharmaceutical industries (Bhaskar *et al*., 2007; Jellouli *et al*., 2009). Numerous proteases have been reported to be produced by microorganisms depending upon the species of the producers or the strains even belonging to the same species. Proteases are ubiquitous in nature and found in a wide diversity of sources such as plants, animals and microorganisms (Rao *et al*., 1998). Proteases are important enzymes that carry out various biological processes. Protease is one of the most important industrial enzymes occupying nearly 60% of the enzyme sales (Beg *et al*., 2003; Adinarayan *et al*., 2003). Of different types of proteases, alkaline proteases is one of the most widely studied groups of enzymes because of their wide use in many industrial applications in food, leather, pharmaceutical and detergent industries (Kumar and Takagi, 1999; Dayanandan *et al.*, 2003). Moreover, they are also used for cleaning of membranes used in protein ultrafiltration (Kumar and Tiwari, 1999). Of these different applications, the main use of alkaline protease is mainly focused on detergent industries. .

The cost of the enzyme is a major issue in enzyme production and their applications in various industrial processes. Therefore, industrial waste was used in the present study to make the protease production economical. The effects of various fermentation process parameters were optimized, such as pH, temperature, age and size of inoculum and growth medium on the yield of protease production (Bhaskar *et al*., 2007). Proteases are found in wide diversity of sources such as plant, animals and microorganisms. Microbial cells are the usual sources of proteases for industrial use except for traditionally used plant and animal enzymes. Microbial proteases account for approximately 40% of the total worldwide enzyme sale (Godfrey and West, 1996). Numerous proteases have been reported to be produced by microorganisms depending upon the species of the producers or the strains even belonging to the same species. In this paper we aimed to isolate newer source of protease from the local sample in Pakistan to potential application of the proteases for industrial applications.

Materials and Methods

Selection of microorganism

In the present study, some bacterial species namely *Bacillus* sp., *Bacillus theorengensis, Escherichia coli* and *Xanthomonas maltifolia* were obtained from First Fungal Culture Bank of Pakistan (FCBP), Institute of Agricultural Sciences, University of the Punjab Lahore,

Pakistan. The *Bacillus lichiniformis, B. subtilus* and eight strains of *B. subtilus* were collected from Institute of Industrial Biotechnology (IIB), GC University Lahore. In addition to these strains, some other strains were also isolated from the soil.

Isolation of microorganism

Some bacterial species used in the present study were isolated from the garden soil of Nasir Bagh, Lahore through serial dilution method. One gram of soil was suspended in 100 mL of sterilized water in 250 mL Erlenmeyer flask, and was vigorously shaken. From this stock solution, serial dilution was prepared up to the 10^{-6}. A 0.5 mL suspension from each dilution was pippetted out and spread on the solidified surface of nutrient casein agar medium plates. The Petri plates were gently rotated clockwise and anti-clockwise to facilitate a uniform spreading of soil suspension. The Petri plates were incubated at 37 °C for 24 h, after which the independent colonies forming larger haloes of casein hydrolysis on the plates were picked up. The isolates were transferred and maintained on nutrient agar slants and were stored in cooled cabinet at 4 °C for further use.

Microorganism maintenance

Slants were prepared using a medium consisting of 1.8% agar and 0.8% nutrient broth. Five milliliters of the medium was poured in each test tube and the test tubes were plugged with cotton. After sterilization at 121 °C for 15 minutes and kept in slanting position at 30° angle. The micro-organisms were maintained in slants for sub-culturing.

Screening of microorganism

Screening of micro-organism was done by casein hydrolysis on the plates which were prepared by dissolving 1.8% agar, 1% yeast extract, 1% peptone, 1% casein and 0.5% NaCl. It was sterilized in an autoclave at 121 °C for 15 minutes. Then this was poured into sterilized Petri plates.

Inoculum preparation

For the preparation of inoculum, nutrient broth was prepared as: A 50 mL of nutrient broth was prepared in a 250 mL conical flask by using the peptone 0.4%, Yeast extract 0.4% in 100 mL distilled water. After sterilization, a loop full of bacteria from a fresh slant was transferred to the flask in laminar flow chamber. The flask was put in the rotary shaker at 200 rpm and 37 °C for 24 hrs for incubation.

Preparation of medium for submerged fermentation

Fermentation medium was prepared by using 2% soybean meal, 1% glucose, 1% peptone, 0.1% KH_2PO_4, 0.1% $(NH_4)_2SO_4$ and 0.5% Na_2CO_3. A 50 mL of water was taken in a flask and then added all the ingredients except soybean meal in a flask. Then measured 1 g soybean meal and added into each of the flask, shake the flask and then sterilized it in autoclave.

Fermentation

One milliliter of inoculum was taken with sterilized pipette and added into each of the flask containing 50 mL of the fermented medium as prepared above and incubated the flasks at 37 °C and 200 rpm in the rotary shaker for 48 h.

Protease assay

The activity of protease was assayed by the method of (McDonald and Chen, 1965). For quantitative test for proteases, the fermentation media was centrifuged at 6000 rpm for 15 min. Then 0.1 mL of supernatant and 0.9 mL of distilled water was added into it, 4 mL of 1% casein was also added to all of the test tubes with 1ml enzyme dilution. In controlled test tubes, 5 mL of TCA (5%) were added, incubated all the test tubes at 40 °C in water bath for 30 minutes. After 30 minutes 5 mL of 5% TCA was added to the experimental test tube, left it for 15 min then centrifuged it at 6000 rpm for about 15 minutes. Then 1 mL of supernatant was taken and added 1mL of 1N NaOH and 5mL of alkaline reagent along with 0.5 mL of folin reagent. Dark blue colour appeared which indicated that proteases had been produced. Leave it for 10 minutes. The absorbance was measured at 700 nm on U.V Spectrophotometer. One unit of protease is defined as the amount of enzyme required to produce an increase of 0.1 in optical density under optimal defined conditions.

Treatment mean and standard error were calculated for enzyme activity data obtained from various tests using software package COSTAT.

Results and Discussion

Twenty three bacterial strains were procured and isolated from samples. For all the twenty three isolates, both qualitative (zone of inhibition) and quantitative (U mL^{-1}) protease assays were done. The best isolate produced a maximum amount of protease was observed.

Screening and isolation of proteolytic bacteria

The proteolytic activities of all strains were assayed using Casein agar medium, and exhibited as diameter of clear zone. Casein agar was the best for qualitative test of protease. Among the twenty three isolates. the *Streptococcus* sp. isolates 1 and 2 showed high proteolytic activity. 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 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 23 different bacterial strains were isolated on Casein agar medium. The twenty three isolates were checked for quantitative test of protease in liquid medium. Table 1 clears that, all the bacterial under study secreted protease enzyme at varied levels. The maximum protease activity (155 U mL^{-1}) was attained after 24 h by *Streptococcus* sp. isolates 1. It was found that maximum production occurred at end of exponential phase. Other strains showed high production of protease, *Streptococcus* sp. isolates 1 is (145 U mL^{-1}). The lowest extracellular enzyme activity was observed by *Streptococcus* sp. isolates No. 6 and 8 with enzyme activity (1.0 U mL^{-1}). 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^{-1}. (Brown *et al.*, 1991).

Streptococcus sp. isolates 1 was found the best among different bacterial strains screened for the production of proteases in shake flasks . This strain might be suitable for many industrial applications. Further experiments to enhanced enzyme production for commercialized process is needed.

Table 1: Screening of bacterial strains for protease production in shake flasks.

Sr. No.	Isolates	Enzyme activity (UmL^{-1})	Size of clear zones (mm)
1	*Bacillus* sp. 1	7.0 ±0.5	2.00
2	*Bacillus* sp. 2	30.0 ±0.1	2.08
3	*Bacillus* sp. 3	4.0 ±01	1.00
4	*Bacillus* sp. 4	13.0 ±0.1	1.07
5	*Bacillus* sp. 5	5.0 ±0.1	1.00
6	*Bacillus* sp. 6	27.0 ±0.5	3.00
7	*Bacillus* sp. 7	63.0 ±01	2.55
8	*Bacillus* sp. 8	23.0 ±01	2.01
9	*Streptococcus* sp. 1	145.0 ±02	4.53
10	*Streptococcus* sp. 2	155.0 ±02	4.90
11	*Streptococcus* sp. 3	13.0 ±0.5	2.00
12	*Streptococcus* sp. 4	5.0 ±01	1.00
13	*Streptococcus* sp. 5	2.0 ±0.5	0.50
14	*Streptococcus* sp. 6	1.0 ±0.5	0.50
15	*Streptococcus* sp. 7	3.0 ±0.1	1.35
16	*Streptococcus* sp. 8	1.0 ±0.5	1.35
17	*Bacillus subtilus*	90.0 ±0.1	3.77
18	*B. lichieniformis*	50.0 ±0.5	3.00
19	*B. thurengensis*	1.0 ±0.1	1.02
20	*B. amyloliquefaciens*	10.0 ±0.1	1.45
21	*Escherichia coli* 1	3.0 ±0.5	1.34
22	*E. coli* 2	1.0 ±0.1	0.87
23	*Xanthomonas maltifolia*	25.0 ±0.5	3.02

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