Isolation and characterization of protease producing *Bacillus sp* from soil

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Abstract

Isolated *Bacillus* strains BP1 and BP2 were a good source of protease as they also showed high proteolytic activity. They showed a clear zone on casein hydrolysed medium plate of 3.5 & 2.2mm in diameter respectively. The strain BP1 and BP2 produce amount of Proteolytic enzyme 1.01μ/ml and 0.73μ/ml after the incubation of 42 hours and 72 hours respectivly at 37±2^{0}C. As the time of incubation increase the proteolytic activity decrease. Both the isolated *Bacillus* strains were producing protease was novel and makes it potential for industrial application

Key words: Bacillus, Protease, Casein hydrolysed

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. 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.

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. Some of them can detach the terminal amino acids from the protein chain (exopeptidases, such as amino peptidases, carboxypeptidase A); the other attack internal peptide bonds of a protein (endopeptidases, such as trypsin, chymotrypsin, pepsin, papain, and elastase). Proteases are also a type of exotoxin, which is virulence factor in bacteria pathogenesis. Bacteria exotoxic protease destroys extracellular structures. Protease enzymes used extensively in the bread industry as a bread improver. Most of the work is focused on alkaline protease producing thermophiles[1,2,3] . Acid proteases secreted into the stomach as pepsin and serine proteases present in duodenum as trypsin and chymotrypsin enables to digest the protein in food; proteases present in blood serum as thrombin, plasmin, Hageman factor, etc. play important role in bloodclotting, as well as in the lysis of the clots, and the correct action of the immune system. Proteases determine the lifetime of other proteins playing important physiological role like hormones, antibodies or other enzymes, so this is one of the fastest "switching on" and "switching off" regulating mechanisms in the physiology of an organism Microorganisms- Protease production is an inherent capacity of all microorganisms. In bioprocesses selecting the proper microorganism is important in order to obtain the desired product. The microorganism that is to be used should give adequate yields, be able to secrete large amounts of proteases and should not produce toxins or any other undesired products. Potential hosts should be suitable for industrial fermentations and produce large cell mass per volume quickly on cheap media. Extremophiles are considered an important source of enzymes and their specific properties are expected to Industrial uses of protease are vast and can be categorized broadly in to detergents, dairy industry, tanning, baking, and brewing[4]. Hence keeping this in mind the present investigation was carried out as follows.

Material and Methods

Sampling

The test soil samples were collected from the different sites of Kanya Gurukul Campus with aid of sterile spatula from 4-5 cm depth in to sterile plastic bags. Soil samples were air dried at room temperature.

Isolation and purification

Isolation of bacteria from soil carried out by serial dilution method[5] and isolated bacterial colonies were purified by sub culturing and stored as slants at 4±2^{0}C.

Characterization of bacterial culture

Various biochemical tests were performed for the identification and characterization of isolated bacteria viz- Gram staining, Catalase test, Starch hydrolysis, Casein hydrolysis, Fermentation test, IMViC test, **U**rease test,

Nitrate test. Morphological, cultural, physiological and biochemical properties of the isolated strains were studied according to the methods given in Bergey's manual of systemic bacteriology[6].

Growth curve study

For the growth curve 150 ml of nutrient broth was prepared and divided into 3 flasks, all containing 50 ml of media. All flasks were autoclaved and cooled at room temperature. Two of them were inoculated with bacterial culture and incubated at 37 ± 2^0C/200rpm. One flask containing 50ml media was kept as blank. After every 2 hours absorbance of inoculated flask was taken at 570nm against the blank.

Enzymatic study

a. Qualitative screening of bacteria (for protease)

The bacteria were streaked on casein hydrolysed medium and plates were incubated at 35 ± 2^0C for 24 hours. A clear zone around the growth indicates proteolytic activity of the strains and visible difference in the extent of zone of clearing was recorded for proteolytic activity

b. standard curve

Prepared different concentration of glucose (10^1- 10^{10}) in different test tubes by taking 0.1ml of protein and 0.9ml of distilled water and so on. Added 1mlof protein and 1 ml of 1N NaOH in test tube and kept in water bath at 100^oC for 10 min. Tubes were cooled at room temperature and after 10 min add 1ml Folin's reagent and leave it for 30 min and absorbance was measured at 750 nm.

c. quantitative determination (Proteolytic activity)

Enzyme assay was determined by the modified method of Hayashi et al., 1967 as followed by Meyers and Ahearn,1977. Four production Broth such as peptone –yeast extract-dextrose broth, tryptone-yeast extract-dextrose broth, gelatine-yeast extract-dextrose both and casein-yeast extract-dextrose broth were used for the enzymatic activity.

d Protease Assay

The Protease was assayed by 3ml of bacterial supernatant (centrifuged 24hr old culture at 5000 rpm for 20 min) take in sterile test tube add 3ml phosphate buffer and 3ml 1% casein solution and place it on water bath at 35^oC. now add 5ml 20% TCA (trichloroacetic acid) added in reaction. Content was immediately mix after adding Folin Ciocalteu Reagent in the mixture after 30 min 6 ml distilled water was added. Now absorbance was measured at 650 nm in every 24 hr till 72 hrs.

e. Paper chromatography for the detection of amino acids-

Test sample extract was loaded on a line 2.5 cm from one end of the Whatman paper strip by capillary. Keep the paper strip in solvent system containing Butanol:Acetic Acid:Distilled Water (4:1:5). Paper was air dried and dip in Ninhydrin Reagent. And dried in a oven for a minute.

Results

In the present study both the isolates BP1 and BP2 growth curve were plotted between optical density vs incubation time up to 48 hours and sigmoid curve were obtained. It involves lag, log, stationary and decline phase. The strain BP1 showed 3.5 mm zone of clearing around the colony whereas BP2 showed 2.2 mm zone of clearing around the colony as qualitative proteolytic activity. The strain BP1 and BP2 produce amount of proteolytic enzyme 1.01μ/ml and 0.73μ/ml after the incubation after 48 and 72 hours at 37 ± 2^0C respectively as quantitative. As the incubation time increases the proteolytic activity decrease. Different spots of solute were appeared on the Paper chromatographic assembly. In strains BP1 the amino acid were identified as Tyrosine, Leucine, Phenylalanine and BP2 identified as Asparagine, tyrosine and Histidine respectively when campared with the standared Rf values. Bacterial cultures were identified as Bacillus strains BA1 and BA2 by Gram staining and various biochemical tests.

Table I Culture morphology and Biochemical Characterization of *Bacillus* sp. BP1 & BP2

Characteristics	**BP1**	**BP2**
Culture characteristic		
Size	Big	Small
Shape	Irregular	Irregular
Margin	Uneven	Even
Pigmentation	Nill	Nill
Morphological characteristic		
Shape	Rod	Rod
Arrangement	Chain	Chain
Gram's reaction	Positive	Positive
Biochemical Tests		
Lactose Fermentation	-	±
Mannitol Fermentation	+	+
Sucrose Fermentation	AG	AG
Dextrose Fermentation	A	AG
Catalase Test·	+	+
Starch Hydrolysis	+	+
Casein Hydrolysis	+	+
Indole test	-	-
Methyl Red	+	+
Voges proskauer test	-	-
Citrate Utilization Test	+	+
Urease test	-	-
Nitrate Reduction	+	+

± Variation Reaction, + Positive, - Negative, AG Acid and Gas production, A- Acid production

Table II Quantitative screening of *Bacillus* sp, BPI & BP2 for proteolytic activity

Isolates	**Proteolytic activity(p/ml)Mean**					
	After 24 hrs.		**After 48 hrs.**		**After 72 hrs.**	
	Absorbance (At 540 nm)	Conc. (u/ml)	Absorbance (At 540 nm)	Cone, (p/ml)	Absorbance (At 540 nm)	Cone, (p/ml)
BP1	1.624	1.01	1.937	1.4	1.875	1.28
BP2	1.369	0.73	1.785	1.19	2.000	1.49

Table III Standard table for Rf value of amino acids

Amino acid	**Rf (Ratio of fraction)**
Alanine	0.38
Arginine	0.20
Asparagine	0.5
Aspartic acid	0.24
Cysteine	0.4
Glutamine	0.13
Glutamic Acid	0.30
Glycine	0.26
Histidine	0.11
Isoleucine	0.72
Leucine	0.73
Lysine	0.14
Methionine	0.55
phenylalanine	0.68
Proline	0.43
Serine	0.27
Threonine	0.35
Tryptophane	0.66
Tyrosine	0.45
valine	0.61

Table IV Different amino acids present in enzymatic crude of *Bacillus* sp. BP1 & BP2

S.No.	Amino acid	BP1	BP2
1.	Tyrosine	+	-
2.	Leucine	+	-
3	Phenylalanine	+	-
4	Asparagines	-	+
5	Valine	-	+
6	Tyrosine	-	+
7	Histidine	-	+

(+) Positive (-) Negative

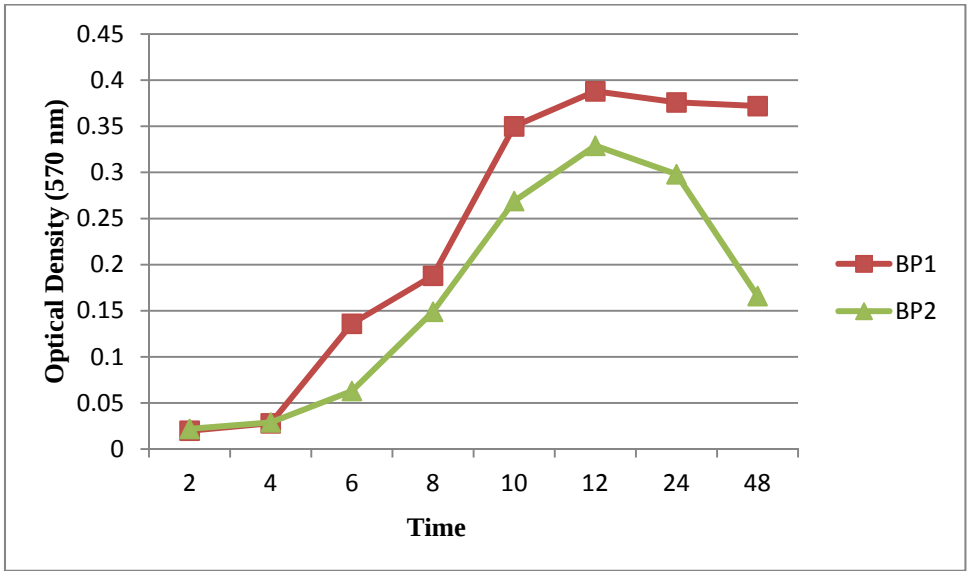

Figure I Growth curve of *Bacillus* sp. BP1 and BP2

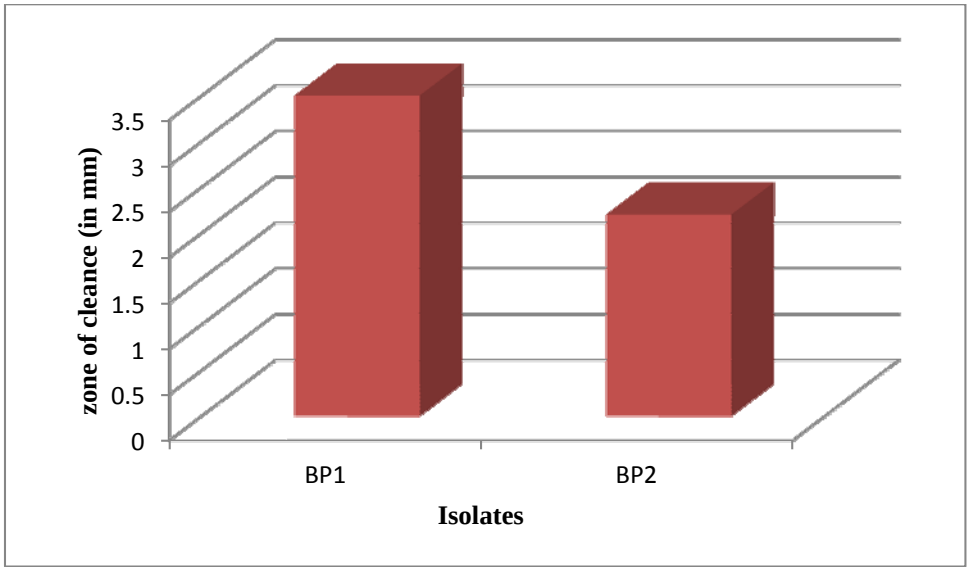

Figure II: Qualitative screening of *Bacillus* sp for proteolytic activity

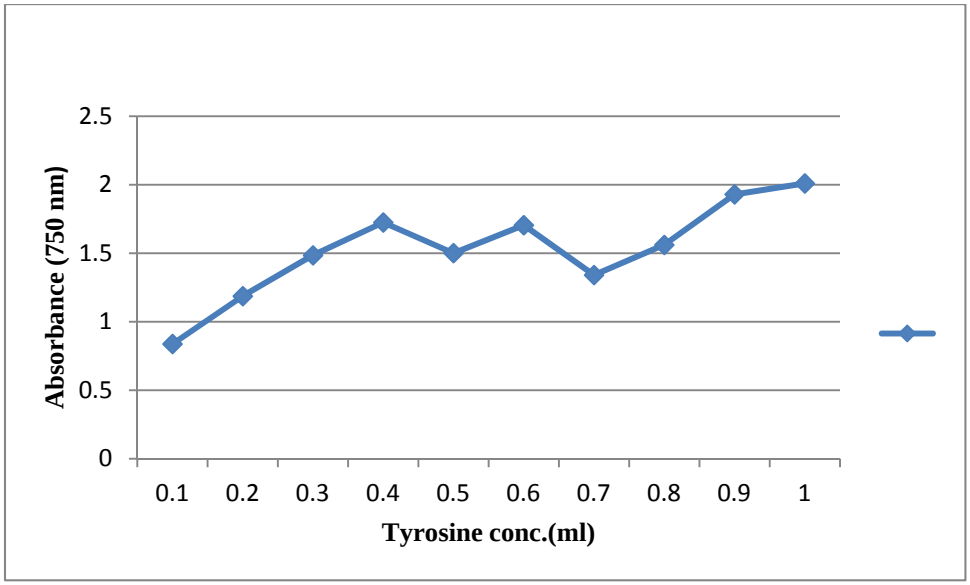

Figure III: Standard curve of Tyrosine

Discussion

Protease producing organisms are generally isolated from soil and most of the work is focused on alkaline protease. Information is lacking in the area of acid and neutral protease. Therefore, the present study deals with the isolation of protease producing bacteria from soil. Members of the genus *Bacillus* produce a large variety of extracellular enzymes of which protease are of particularly significant industrial importance. Isolation of protease producing bacteria was performed by the serial dilution spread plate technique. Similar method has been used by Clark et al. 1958. Identification of selected *Bacillus* strain was identified on the basis of standard morphological and biochemical tests according to the method described in Bergey's Manual of Determinative Bacteriology[10].Out of many bacteria only, 2 *Bacillus* bacterial strains produced zone of clearance on casein, hydrolysate media. That was designated as BP I & BP2. Maximum zone of diameter was observed in BPI i.e. 3.5mm in comparison to BP2 i.e. 2.2. Weiss and Ollis, 1980 also report that *B. licheniformis* produced very narrow zone of hydrolysis on casein agar despite giving very good protease production in submerged condition. For quantitative screening Casein yeast extract dextrose broth is used and inoculated with isolated Bacillus sp. which was incubated at 37°C for 24 hours. Abo-Aba et al., (2006) produced alkaline protease from *Bacillus circulance*, *B. Alvei*, *B sphaericus* and *B. Pumilus* using Luria-Bertani Broth, Luria-Bertani Agar milk and measured the maximum activity at 30°C after 40 hrs. All the isolates were grown in submerged condition and activity of protease are estimated in culture filtrate. Atalo et al., (1993) showed that yeast extract and peptone can induce the alkaline protease production in growth medium. On the place of peptone we prefer casein, which have high protein concentration. After incubation protease activity was measured. which was estimated as 1.0 1 µ /rnl in BPI and 0.73µ/ml in BP2 after 24 hrs. Although the maximum of alkaline protease production in other approach was observed 580.5 µ /ml in *B. polymixa* and 535.5 µ /ml in B.cereus after 24 hrs. Maximum production of protease with 48 & 72 hrs of incubation of bacteria was reported by Hoshino et al., (1994) and Shumi et al., (2004). The detection of amino acid in crude of enzyme was done by paper chromatography using Butanol: Acetic acid: Distilled water as a solvent. Gange and Simpson, 1993 also investigated the use of chromatographic technique for the detection of amino acid by using Ethanol: water (7:1000(v/v) as a solvent. By paper chromatography Phenylalanine, Asparagine, Tyrosine and Histidine were detected from BPI and Valine, Leucine and Tyrosine were detected from BP2. Hence due to maximum number of amino acid present in BP 1 was found more competent as compared to BP2

CONCLUSION

The main objective of our study was to characterize the two protease producing bacteria and to check their ability for enzyme production especially protease. Two *Bacillus* strains *BP1* & BP2 were isolated. They were good source of Protease as they showed high Proteolytic activity. Both isolated *Bacillus* strains which produce Protease was novel and make it potential for industrial applications. Enzymes are widely used in several industries, notably in detergent, food processing, brewing and pharmaceutical industries. They are also used for diagnostic, scientific and analytical purposes. The use of microorganisms to produce enzymes has a number of technical and economic advantages and in recent years has become the predominant mode of enzyme production. However, the cost of producing this enzyme is high and the cost of procurement by developing countries can be even higher, hence present potential *Bacillus* strain BP 1 could be alternative for industrial and commercial use.

References

[1] W. Sidler and H. Zuber. Isolation procedure for Thermostable neutral proteinases production by Bacillus stearothermophilus. EuropJ.appI. Microbiol., 1980, 10: 197 -209.

[2] H. Takami, T. Akiba and K. Horikoshi. Production of extremely Thermostable alkaline protease from Bacillus sp. No. AH-l 0 1. Applied Microbiol.Biotechnol., 1989, 30: 120-124.

[3] S. K. Purva, L. K. Gupta and J. K. Gupta. Thermostable alkaline protease from alkalophilic Bacillus sp. LS-3. Ind.J.Microbial.,1998, 38: 149-152.

[4] V. K. Joshi and A. Pandey. Biotechnology: Food Fermentation (Microbiology, Chemistry and Technology). Educational publishers & distributors., 1999, Vol-I: 1079-1101

[5] S.A. Waksman and E. B. Fred. A tentative outline of the plate method for determining the number of microorganisms in the soil. Soil Sci., 1922, 14: 27-28.

[6] G. John, Noel Holt, R. Krieg, et al. Bergey's manual of determinative bacteriology. 9th edition lippinocott . Willians. A Wolters kluwer campany., 1994, 559-562

[7] K. D. Hayashi, Fukushima. and K.Mogi. Alkaline proteinase of Aspergillus sojae. Physio-chemical properties, amino acid compositions and molecular conformation. Agric,Biol. Chern., 1967, 31 :642-43.

[8] S.P. Meyers, and D.G Ahearn. Extra cellular proteolysis by Candida lipolytica. Mycologia., 1977, 69:6464-651.

[9] H.E. Clark, G.E.F. Bordner, P.W. Kabler, and C. B. Huff. Applied microbiology. Appl Microbial. Biotechnol., 1958, 39:31-35.

[10] R. E. Buchana and N.E. Gibbons. Bergey's manual of determinative bacteriology. Baltimore: The Williams and Wilkins Co. 1974

[11] R.M. Weiss and F. Ollis. Extracellular microbial polysaccharides I. Substrate, biomass and product kinetic equation for batch xanthum gum fermentation. Biotechnol Bioeng.,1980, 22: 859-873.

[12] S.E.M. Abo-Aba, E.A.M. Soliman and A.A. Nivien. Enhanced production of extracellular alkaline protease in Bacillus ciculance through plasmid transfer. Res. J. Agic. BioI. Sci., 2006, 16: 526-530.

[13] K. Ataio and B. A. Gashe. Protease production by a Thermophilic Bacillus sp. (P-OOIA) which degrades various kinds of fibrous proteins. Biotechnol. Lett., 1993, 11: 1151-1156.

[14] T. Hoshino, K. Ishizaki, T. Sakamoto et al. Isolation of a Pseudomonas species from fish intestine that produces a protease active at low temperature. Applies Microbial., 1997, 25:70-72.

[15] W. Shumi, M.T. Hossain and M. N. Anwar. Proteolytic activity of a bacterial isolate Bacillus fastidiosus den Dooren de jong. J. BioI. Sci., 2004,4: 370- 374.

[16] N. Gange and B. K. Simpson. Use of proteolytic enzymes to facilitate recovery of chitin from shrimp wastes. Food Biotechnol., 1993, 7: 253-63.