SCREENING AND CHARACTERIZATION OF PROTEASE FROM *BACILLUS* SP

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ABSTRACT: Protease production by alkalophilic *Bacillus* sp were isolated from different regions of Bangalore and used to screen for the protease production by using casein and skim milk agar plate assay. The agro wastes are also used to screen and produce protease. The agro wastes are rice bran, paddy straw and pigeon pea waste, among these substrate rice bran showed maximum synthesis of protease. At 48 hr it showed maximum 0.13 IU protease production in rice bran medium was used for partial purification studies. Fermented medium were used for salt precipitation, dialysis. The dayalyste were used for studies on effect of Ph, temperature, inhibitors and metal ions. The optimum pH 10, temperature 65^0C were optimum and the inhibitors and metal ion studies, metalions like Mn^{2+} and Ca^{2+} were found to be potent enhancers. The complete loss of enzyme activity was found in presence of PMSF

Key words- casein-skim milk agar, plate assay, solid state fermentation, rice bran, al ions inhibitors and metal ions.

INTRODUCTION

Proteolytic enzymes play an important part in the metabolism of almost all organisms (plants, animals, fungi, bacteria, and viruses). Investigation of proteases is a central issue in enzymology due to both their immense physiological importance and wide application in research and economical activities. Proteases are one of the most important groups of industrial enzymes and are used in a variety of industrial applications as laundry detergents, pharmaceuticals, leather products, as meat tenderizers, protein hydrolyzates, food products, and even in the waste processing industry (Joo and Chang, 2005). This enzyme accounts for 30% of the total worldwide production of enzymes (Horikoshi, 1996).

The genus *Bacillus* contains a number of industrially important species and approximately half of the present commercial production of bulk enzymes derives from the strains of *Bacillus* sp. (Beg and Gupta, 2003; Priest 1977). These strains are specific producers of extracellular proteases (Singh,et al.,2001) and can be cultivated under extreme temperature and pH conditions to give rise to products that are, in turn, stable in a wide range of harsh environments (Han et al.,1997).

Bacillus sp. Mostly produces two groups of proteases, alkaline and neutral (Rao et al., 1998). Bacterial neutral proteases are active in narrow Ph range (pH 5.0-8.0) and have relatively low thermo tolerance (Barett, 1994).This property is advantageous for controlling their activity during the production of food hydrolysis. Bacterial neutral proteases generate less bitterness in hydrolyzed food proteins than animal proteases and, hence, are valuable for use in food industry (Rao et al., 1998).The aim of the present study was to isolate the *Bacillus* sp from the soil, screen the protease producing *bacillus* sp as well as substrate for the production of protease and characterize protease.

MATERIALS AND METHODS

Microorganism and culture condition

The bacterial strain used in this study was alkalophilic *Bacillus* sp. Isolated from a local soil samples from different regions of Bangalore.

Screening of protease production by plate assay

Bacillus sp. Was also screened for its proteolytic activity. This was done by inoculating the organisms on the agar plates containing casein (1% w/v) and milk powder (1% w/v), incubated for 48 h. The plates were flooded with 25% TCA (trichloro acetic acid) solution and incubated for 15 min at 45^0C. Plate. 1.

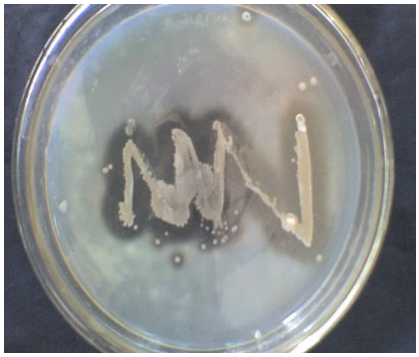

PLATE-1. CASEIN HYDROLYSED PLATE

Screening of substrate for extracellular protease production by Solid State Fermentation

Production of protease from *Bacillus* sp. Was carried out in Erlenmeyer's flask containing 20 g of rice bran, paddy straw and pigeon pea waste figure-1. The agro waste was moistened with 20 ml of mineral salt media containing K_2HPO_4, NaCl, $MgSO_4$, $CaCl_2$, Peptone, Yeast extract, Na_2CO_3. The Na_2CO_3 was autoclaved separately and added into flasks while inoculating. The flasks were inoculated with 1 ml of 18 h culture and incubated at 55^0C for 72 h.

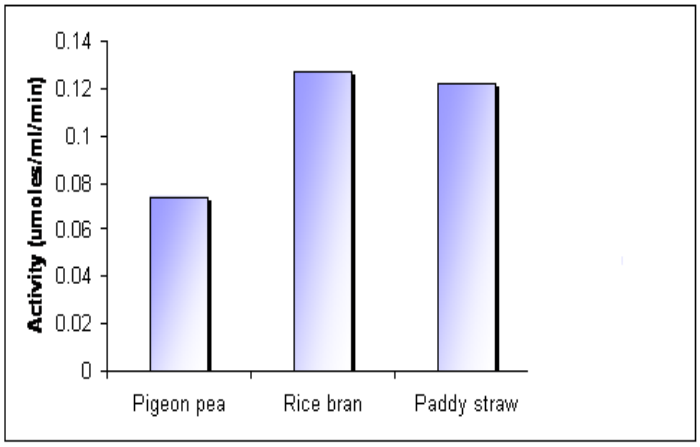

Figure.1. Screening of Substrate for Protease Production

After the completion of fermentation, the whole fermentation mix was flooded with 25 Mm glycine-NaOH buffer (pH 10.0) and filtered. The filtrate was further centrifuged at 10,000 rpm for 10 min and the clear supernatant was recovered. The crude enzyme supernatant was subjected for further studies.

Partial purification by Precipitation and Dialysis

The partial purification of enzyme has been carried out as per the method described by Sadashivam and Manikam (1998). The cell free supernatant was precipitated with different concentrations of ammonium sulphate i.e., from 10 – 90%. The precipitate was dissolved in small amount of 25 Mm glycine-NaOH buffer (pH 10) and dialyzed over night against the same buffer. The dialyzed enzyme was used for fallowing studies.

Characterization of protease

After fermentation process protease were separated out by using filtration, centrifugation, and ammonium sulfate salt precipitation for partially purification then protease enzyme sent for dialysis, dialysed enzyme were used for characterization studies.

Determination of optimum Ph

The optimum Ph of protease was determined with 1% casein (w/v) as substrate dissolved in different buffers (Acetate buffer pH 4.0, Citrate buffer, pH 5.0 and 6.0, phosphate buffer pH 7.0, Tris-HCl buffer, pH 8.0, glycine-NaOH buffer, pH 9, 10, 11 and 12) represented in figure-.2.

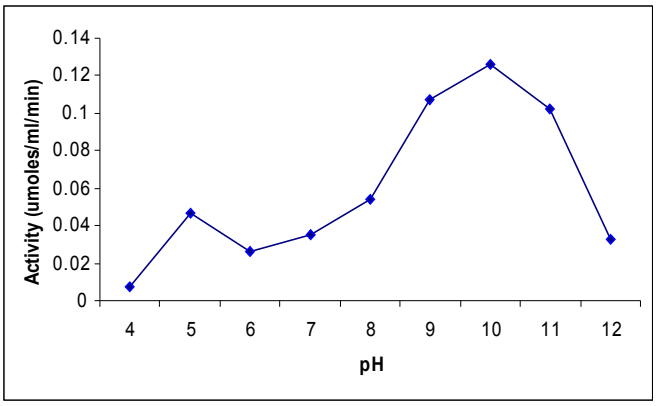

Figure.2. Effect of pH on Protease

Determination of optimum temperature

After the optimum pH was known, the optimum temperature of protease activity was determined by incubating the reaction mixture at different temperatures in a range from 37-95^{0}C in 25 Mm glycine-NaOH buffer (pH 10.0) for 10 min represented in figure-3.

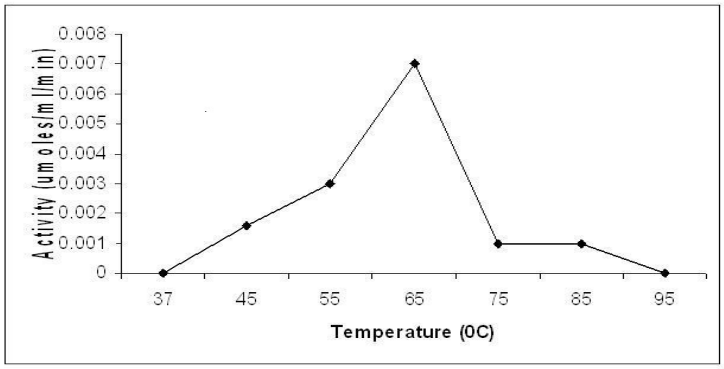

Figure.3. Effect of Temperature on Protease

Effect of Inhibitors and Metal ions and Inhibitors

Table.1, shows that the effect of various protease inhibitors such as Serine inhibitors (phenyl methyl sulphonyl fluoride[PMSF]), chelators of divalent cations (Ethylene diamine tetraaceticacid [EDTA] and 1,10-Phenanthroline) and the effects of Metalions (Ex: Hg^{2+}, Fe^{3+}, Cu^{2+}, Zn^{2+}, Na^{+} and Ca^{2+}) on the protease was assayed by studying the enzyme activity in their presence.

TABLE 1: EFFECT OF INHIBITORS AND METAL IONS ON PROTEASES

Inhibitors	**Activity**
Control	0.071
EDTA	0.032
PMSF	0.052
1,10-Phenantholine	0.082
Calcium chloride	0.057
$ZnSO_4$	0
$MgCl_2$	0.041
$HgCl_2$	0.066
$MnSO_4$	0.015
$CuSO_4$	0.022
$MgSO_4$	0.063
$FeCl_3$	0.005
$FeSO_4$	0
SDS	0.022
H_2O_2	0.054
NaCl	0.014

Assay of Protease

The protease activity was determined by the method proposed by Keay et al., (1970). 0.5 ml of suitably diluted enzyme was added to 1.0 ml of 1% casein and 0.5 ml of glycine-NaOH buffer (25 Mm, pH 10.0) whole mixture was incubated at 75^{0}C for 10 min. The reaction was terminated by the addition of 3 ml of 10% TCA solution. The solution was allowed to stand for 10 min in cool and was filtered. To the clear filtrate, 5 ml 0.4 M Na_2CO_3 and 0.5 ml of Folin Ciocalteau reagent (FCR) was added, mixed thoroughly and incubated at 37^{0}C for 30 min, in dark. The absorbance was measured at 660 nm.

International units (IU)

One protease unit was defined as the amount of enzyme that released 1 µg of tyrosine per ml per minute under the above assay conditions.

RESULT AND DISCUSSION

The isolated bacterial strain was identified as *bacillus* sp by staining and biochemical analysis. The isolated strain was used to screen for to produce protease by casein and milk powder plate assay by cleared zone around colony (Plate-1). Thus the culture isolated i.e. *Bacillus sp.* Has a proteolytic ability.

The hydrolysed strain was also used to screen the substrates for the production of protease through solid state fermentation. Among the agro wastes used rice bran showed best substrate for the maximum protease production. It showed 0.13 IU at 48 hr. The perusal of data indicated that the ammonium sulphate ppt. method showed 74.48 IU/mg protein specification and 87.18 IU/mg protein purification. This clearly indicated that there is 8.0 fold increases in the protein purification with 32.0% of recovery protein by employing ammonium sulphate method.

A pH range between 4.0 and 12.0 was used to study the effect of pH on protease activity (Fig. 2). Optimum Ph was found to be 10.0 and it shows 0.125 IU at Ph 4.0 only 0.01 IU of the enzyme activity was found. Sookkheo *et al*. (2000) reported to three proteases, S, N and B from thermophilic *Bacillus stearothermophilus* TLS33, optimum Ph values of 8.5, 7.5, and 7.0, respectively. The protease S was active over a very broad Ph range,

The protease activities were assayed at different temperatures ranging from 37ºC-95ºC at a constant pH of 10.0 (Figure. 3). Enzyme activity increased with temperature within the range of 37ºC to 65ºC. A reduction in enzyme activity was observed at values above 65ºC. The optimum temperature of this protease was 65ºC which was similar to that described for other *Bacillus* proteases by Banerjee, et al, (1999) and Horikoshi, (1990). At 65 ^{0}C the protease shows 0.007IU of enzyme activity.

The inhibition studies of the enzyme purified up to now gave a unique inhibitors and inducers list. The purified enzyme activity was induced up to 217.7% by Cu^{+2} ions, while 1,10-Phenanthraline gave 59.1% activity. Other metal ions like Hg^{2+}, Na^{+2}, EDTA; $MgCl_2$ gave a less activity of 193.9%, 100%, 13.8%, and 199.8%.Thus the inhibition studies show that the enzyme is sensitive to PMSF, which proves the presence of Serine in the active site of the enzyme. While Fe^{3+} and Cu^{2+} are the potent inhibitors and Mn^{2+} and Ca^{2+} are potent inducers of the protease studied in the present investigation.

In the inhibitors and metal ion studies, metalions like Mn^{2+} and Ca^{2+} were found to be potent enhancers. The complete loss of enzyme activity was found in presence of PMSF.

REFERENCES

1. U.C. Banerjee, R.K. Sani, W.Azmi, R.Soni (1999). Thermostable alkaline protease from *Bacillus brevis* and its characterization as a laundry detergent additive. Proc. Biochem: 35. 213-219
2. Q.K. Beg and R.Gupta (2003) Purification and characterization of an oxidationstable, thiol- dependent serine alkaline protease from *Bacillus mojavensis*. *Enzyme Microb. Technol.*, 32, 294-304
3. A.J. Baret (1994). Proteolytic enzyme: serine and cysteine peptidase. Methods Enzymol. 244. 1-15
4. X.Q. Han and S. Damodaran (1997). Isolation, identification and fermentation of *Bacillus* species producing a detergent-stable endopeptidase. J.Agric. Food Chem: 45. 4191-4195,
5. K. Horikoshi (1996). Alkalophiles – from an industrial point of view. *FEMS Microbiol. Rev*.18, 259-270,.
6. K. Horikoshi,(1990). Enzymes of alkalophilies. In: Microbial Enzyme and Biotechnology, 2nd, 275- 94,.
7. K. Horikoshi (1990). Enzymes of alkalophilies. In: Microbial Enzyme and Biotechnology: 2nd. 275-94.
8. H.S Joo, and C.S. Chang (2005). Production of protease from a new alkalophilic *Bacillus* sp. I-312 grown on soybean meal: optimization and some properties. *Proc. Biochem.*, 40, 1263- 1270.
9. L. Keay, P.W. Moser and B.S. Wildi (1970). Proteases of the genus *Bacillus I* alkaline proteases, Biotechnol. Bieng: 12. 213
10. F.G. Priest (1977). Extracellular enzyme synthesis in the genus *Bacillus*. Bacteriol.Rev: 41.711-753,
11. M.B.Rao, A.M.Tanksale, M.S, Ghatge and V.V.Deshpande (1998). Microbial and Biotechnol ogical aspects of microbial protease. Microbiol Mol Biol Rev: 62. 597-635
12. J.Singh, N.Batra, C.R.Sobti (2001). Serine alkaline protease from a newly isolated *Bacillus* sp. SSR1. *Proc. Biochem*., 36, 781-785.
13. B. Sookkheo, S. Sinchaikul, S.Phutrakul, S.T. Chen.(2000). Purification and characterization of the highly thermostable proteases from *Bacillus stearothermophilus* TLS 33. protl. Exp. Pur: 20. 142-151.
14. Sadashivam and Manicum (1998).Biochemical methods 2nd, Ed., 96.

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