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OPTIMIZATION OF PHYSICO-CHEMICAL PROPERTIES FOR PRODUCTION OF ALKALINE PROTEASE FROM *FUSARIUM GRAMINEARUM*

B. Lalitha Kumari[1*], P. Vijetha[2], P. Sudhakar[3]
[1]*BES Dept., NIET, Kantepudi, Sattenapalli, Guntur, A.P, India*
[2]*Pharmacy, SIPS, Kantepudi, Sattenapalli, Guntur, A.P., India*
[3]*Dept. of Biotechnology, ANU, Guntur, A.P. India*

Abstract
Alkaline proteases of microbial origin possess considerable industrial potential due to their biochemical diversity and wide applications in tannery and food industries, medicinal formulations, detergents and processes like waste treatment, silver recovery and resolution of amino acid mixtures. Discovering such species, producing proteases with novel characteristics will be of great value to the enzyme industry for different applications. Fusarium graminearum is potentially a good host for the production of proteins. The advantage of growing Fusarium graminearum in continuous flow culture is that theoretically, greater productivity can be achieved. Newly isolated Fusarium graminearum was grown with glucose as the sole carbon source. The Fungi isolates yielded maximum enzyme at pH 6.6 and 37°C temperature. Sucrose was found as the best source of carbon as the enzyme yield was found highest and the biomass turn over was also good. Of all the inorganic nitrogen sources ammonium nitrate was found to be best and of all the organic nitrogen sources Yeast extract was found to be best source of nitrogen for the mold to synthesize the enzyme. The inoculum level of 4.0% (v/v) was found optimum for production. Fe (NH3)2 SO3 is found essential for the enzyme synthesis. The objectives of this investigation, therefore is to isolate strains of filamentous fungi, which would produce significant quantities of extra cellular alkaline proteases and to determinate the values of their proteolytic activities.
Keywords: Alkaline protease, filamentous fungi, Fusarium sps., Optimization, Industrial enzyme.

Introduction

Filamentous fungi are used in many industrial processes for the production of enzymes and metabolites (Adrio et al., 2003). Proteases represent an important group of enzymes produced industrially and account for 60% of the worldwide sales value of the total industrial enzymes (Godfrey, 1996). Alkaline proteases of microbial origin possess considerable industrial potential due to their biochemical diversity and wide applications in tannery and food industries, medicinal formulations, detergents and processes like waste treatment, silver recovery and resolution of amino acid mixtures (Rao et al., 1998; Agarwal et al., 2004) The alkaline proteases find their largest use in house hold laundry with a worldwide annual production of detergents of approximately 13 billion tons (Nehra et al., 2002). Alkaline proteases were in fact the first enzyme produced in bulk. For the production of enzymes for industrial use, isolation and characterization of new promising strain is a continuous process (Kumar et al., 2002). They are generally produced by using submerged fermentation due to its apparent advantages in down stream in spite of the cost intensiveness for medium components.

The major uses of proteases are in the biotechnological production of detergents (Bailey et al., 1977), in dairy industries as milk-clotting agents (calf rennet composed mainly of amylases, protease, chymosine and pepsin) (Fox, 1982) and as an agent for meat tenderization (Bernholdt, 1975). Proteases have also clinical and medical application (reduction of tissue inflammation) (Bailey et al., 1977; Nout et al., 1990).

In general, most of the alkaline proteases applied for industrial purposes face some limitations, including low activity and stability towards anionic surfactants like SDS and oxidants; and their high total cost (Joo et al., 2003). Discovering such species, producing proteases with novel characteristics will be of great value to the enzyme industry for different applications.

The objectives of this investigation, therefore is to isolate strains of filamentous fungi, which would produce significant quantities of alkaline proteases and to determinate the values of their proteolytic activities.

Alkaline proteases are the enzymes, which catalyze the hydrolysis of peptide bonds of the proteins. The amino acid composition of proteins is very diverse so the proteases responsible for their hydrolysis are also diverse. Further the proteases are also classified into alkaline, acid and neutral proteases based on their

* Corresponding Author, *Email*: *lalithayadlapalli@rediffmail.com*

pH optima of activity. On the basis of the functional group present at the catalytic site these proteases are classified as serine proteases, cysteine proteases, aspartic proteases, threonine proteases, glutamic acid proteases and metalloproteases.

Methodology

Isolation of fungal strain

The selected *Fusarium graminearum* strain is highly branched (colonial), isolated; collected and preserved carefully in the refrigerator and were tested for proteolytic microbes within few days using enrichment technique. After isolation, the isolated organisms were purified through repeated plating method in PDA media. For the identification of selected isolates different morphological and cultural characteristics like size, shape, arrangement, colour, and growth on agar plate, agar slants, in liquid or in deep agar media were observed. Finally the characteristics were compared with the standard description of 'A manual of soil fungi 'by Gilman (Gilman 1957).

Screening of the isolates

Screening for proteases was performed in steps, primary and secondary. After primary selection, the isolates were selected for the protease activity in liquid medium by quantitative method.

Culture media

The medium of stock slants consisted of PDA medium according to Lin (2000) and used for our preliminary experiments.

Culture conditions

Stock slants were incubated at 26°C for 10 days before storing at 4°C. For seed cultivation, mycelia from fresh slants was inoculated into 100 ml of fermentation medium in 500 ml Erlenmeyer flasks, incubated on a rotary shaker incubator at 180 rpm and 27°C for 3 days. For original culture in shake flasks, 10% (v/v) inoculum was inoculated into 100 ml of fermentation medium in 500 ml Erlenmeyer flasks, incubated on a rotary shaker incubator for 4 or 5 days at 180 rpm at 27°C.

Production media

For the in vitro production of alkaline protease by the isolates, tryptone-dextrose-yeast extract broth medium (Matta et al., 1997), the flasks were inoculated with selected isolates (4 days old fungal culture) and incubated at 37°C for 5days. The fungal culture mat was filtered through Whatman no .1 filter paper. The filtrates were then centrifuged at 5000 rpm for 10min. at 4°C with few drops of toluene to avoid bacterial contamination. Enzyme activity was measured by the modified method of Hayashi (Hayashi et al., 1967) as followed by Meyers and Ahearn (Mayers and Ahearn, 1977). The amount of amino acids released was calculated and compared with a standard curve plotted from a known concentration of tyrosine.

Enzyme assay

3 ml of reaction mixture containing 0.5% casein in 2.95 ml of 0.1 M Tris-Hcl buffer, and 0.1 ml of enzyme was incubated at 50°C. After 10 min. 3ml of cold 10% TCA was added and after 1 hour, the culture filtrate was centrifuged at 8,000 rpm for 5 min to remove the precipitate and absorbance of the supernatant was read spectrophotometrically at 280nm. Enzyme activity was calculated by measuring mg of tyrosine equivalent released and compared with the standard. One unit (U) of enzyme activity represents the amount of enzyme required to liberate 1 µg of tyrosine under standard assay conditions.

Calculations

$$\text{Units of enzyme} = \frac{\mu\text{mole of tyrosine released X total volume}}{\text{Time of assay X volume of enzyme X volume used colorimetric determination}}$$

Optimization of cultural parameters

An attempt was also made to ascertain the optimum culture conditions such as pH, Temperature, Incubation period, carbon and nitrogen source requirements for their maximum growth and activities and yield of alkaline protease production of the selected isolate were recorded.

Results and Discussion

Filamentous fungi such as *Penicillium* spp., *Aspergillus* spp., *Fusarium* spp., *Alternaria* spp., *Curvularia* spp. and *Trichoderma* spp. are the most important group of micro-organisms used owing to their physiological and biochemical properties (Han and Anderson, 1975; Reese, 1977; Gervais *et al*., 1988; Oriol *et al*., 1988a, 1988b).

Fusarium graminearum

Fusarium graminearum is potentially a good host for the production of proteins. Recently systems have been developed for its transformation and for expression of heterologous proteins. The advantage of growing *Fusarium graminearum* in continuous flow culture is that, greater productivity can be achieved.

Production of alkaline proteases in submerged fermentation

Newly isolated *Fusarium graminearum* was grown with glucose as the sole carbon source. During early exponential phase and the fungus was growing predominantly on glucose. The pH profile of the extracellular proteases produced was similar, with proteolytic activity in the culture filtrate increasing with increasing concentrations of casein. For batch cultures grown on 5 g glucose and *5* g casein, an increase in proteolytic activity was detected at 48hrs and at every interval of 24hrs post-inoculation during the late exponential phase of growth.

Effect of pH:

To determine the optimum medium pH on alkaline protease production was carried out in corn broth at pH 5.8. The enzyme activity was monitored after 24 hrs. growth and production under shaking conditions. Similarly the growth and enzyme production were also monitored at pH in the range of 5.8 –7.00 (Table 1).

Table 1: Effect of pH on the synthesis of alkaline protease by *Fusarium graminearum*

Initial pH	24hrs. E.A units/ml	48hrs. E.A units/ml	72hrs. E.A units/ml	96hrs. E.A units/ml	120hrs. E.A units/ml
5.8	0.30	0.64	0.82	0.98	1.21
6.0	0.42	0.82	1.21	1.34	1.42
6.2	0.42	0.96	1.21	1.56	1.82
6.4	0.64	1.21	1.82	2.24	2.35
6.6	1.21	2.16	2.46	3.03	4.85
6.8	1.34	1.82	2.34	2.78	3.64
7.0	1.29	1.81	2.40	2.51	2.63

The secretion was adversely affected at lower pH compared to that at pH 6.60, it displayed optimum alkaline protease activity 4.85 units/ml.

Effect of temperature

To determine the optimum temperature for enzyme production, the culture medium was incubated at different temperatures.

Optimum temperature for the production of alkaline protease was at 30°C for all the substrate at 1% (w/v). After which there is a decrease in the activity of the enzyme. The maximum activity of enzyme was 3.26 Units / ml with this production medium.

The best temperature identified was between 30°C to 40°C for maximum production and we maintained 37°C for further investigations.

Table 2: Effect of temperature

Temperature	24hrs. E.AUnits/ml	48hrs. E.AUnits/ml	72hrs. E.AUnits/ml	96hrs. E.AUnits/ml	120hrs. E.AUnits/ml
25º C	0.88	1.17	1.44	1.70	1.93
30º C	11.7	1.46	1.93	2.22	2.46
35º C	1.58	1.93	2.22	2.46	2.32
37º C	1.40	2.10	2.57	2.98	3.26
40º C	1.40	2.16	2.46	2.87	3.04

Effect of carbon source

The present investigation is carried out to determine the best source of carbon, which promotes growth, and synthesis of the enzyme is investigated incorporating substances like Sucrose, Glucose, Maltose, Potato starch, starch, at the same concentration as sugar in the broth medium. Fermentation was carried out as before. Of these Sucrose was found as the best source of carbon as the enzyme yield was found highest and the biomass turn over was also good (Table: 3). Microscopically

mycelium appeared healthy with no vacuoles. Potato starch was found the next best source of carbon. Maltose, Glucose, Starch are less preferred sources of carbon and stand in descending order of preference for the synthesis of the enzyme. Therefore, maize starch was selected as the best carbon source and used.

The culture media after inoculation are incubated at 30°C for 72hrs under shaking flask conditions followed by the estimation of alkaline protease activity for sucrose 3.39 units/ml was studied.

Table 3: Effect of carbon source

Carbon Source	24 hrs E.AUnits/ml	48 hrs E.AUnits/ml	72hrs E.AUnits/ml	96hrs E.AUnits/ml	120 hrs E.AUnits/ml
Sucrose	0.84	1.24	2.24	3.64	4.62
Glucose	0.64	1.17	1.53	2.62	3.64
Maltose	0.64	1.28	2.36	3.04	3.39
Potato Strach	0.52	0.98	1.26	2.10	2.36
Strach	0.48	1.10	1.52	2.12	2.36

Effect of nitrogen source

Generally in addition to an energy source and trace elements, a suitable nitrogen source in adequate quantities should be essential for the rapid growth of the mold. A variety of inorganic and complex organic nitrogen substances have been incorporated in the basal medium and their effect on the synthesis of the enzyme have been studied.

Of all the inorganic nitrogen sources ammonium nitrate (Table 4) was found to be best inorganic source of nitrogen for the mold to synthesize the enzyme. Sodium nitrate was found next best source of nitrogen but inferior to sodium nitrate.Urea, Yeast extract, Soya bean meal, Peptone and Beef extract were used as organic source of nitrogen

Table 4: Effect of inorganic & Organic nitrogen source

Nitogen Sourse	24 hrs E.AUnits/ml	48hrs E.AUnits/ml	72hrs E.AUnits/ml	96hrs E.AUnits/ml	120 hrs E.AUnits/ml
Ammonium Nitrate	1.17	1.64	2.24	2.88	3.46
Yeast Extract	1.17	1.82	2.62	3.36	4.26

Effect of different volumes of inoculum

The effect of different size of inoculum on the production of alkaline protease by *Fusarium graminearum* in shake flask was studied at the level of 1-8% for an enzyme. The inoculum level of 4.0% (v/v) was found optimum for production.

Table 5: Effect of different volumes of inoculum

Inoculum (ml/100ml broth)	1	2	3	4	5	6	7	8
Enzyme Activity (Units/ml)	1.17	1.82	3.04	3.46	2.24	1.86	1.32	1.22

Conclusion

Alkaline proteases (EC.3.4.21–24, 99) are defined as those proteases which are active in a neutral to alkaline pH range. They either have a serine center (serine protease) or are of metallo-type (metalloprotease) and the alkaline serine proteases are the most important group of enzymes exploited commercially. *Bacillus sp*. - the most widely exploited alkaline proteases producer, are often commercially used in bioremediation mixes, or as probiotic agent in aquaculture. Recently, the use of alkaline protease in the management of wastes from various food-processing industries and household activities opened up a new era in the use of proteases in waste management. Alkaline proteases play a crucial role in the bioprocessing of used X-ray or photographic films

for silver recovery. These waste films contain 1.5-2.0% silver by weight in their gelatin layer, which can be used as a good source of silver for a variety of purposes.

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