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Studies on process and nutritional parameters for production of alkaline protease by *Thermoactinomyces thalpophilus* PEE 14

P Ellaiah*, G Divakar, P Vasu, M Sunitha and P Udaya Shankar

Pharmaceutical Biotechnology Division, Department of Pharmaceutical Sciences, Andhra University
Visakhapatnam 530 003, India

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Studies on process and nutritional parameters for the production of extracellular alkaline protease employing *Thermoactinomyces thalpophilus* PEE 14 was carried out. Different initial *p*H, incubation temperatures and time and inoculum levels and their age were studied. Effects of different nutritional parameters, trace elements and metabolic inhibitors on protease production were also studied. The maximum enzyme activity was obtained with incubation temperature of 55ºC, initial *p*H of 10, incubation time of 24 h, level of inoculum 10 per cent and age of the inoculum 72 h. Results of nutritional and trace element parameters showed that pyridoxin, L-cysteine and sodium molybdate showed best vitamin, amino acid and trace elements, respectively. Silver nitrate, antibiotic framycitine and surfactant cetrimide showed strong inhibitory effect on protease production.

Keywords: alkaline protease, inhibitors, nutritional factors, submerged fermentation, *Thermoactinomyces thalpophilus* PEE 14

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Introduction

Proteases are one of the most important industrial enzymes accounting for nearly 60 per cent of the total worldwide enzyme sales[1-3]. Of these, alkaline proteases are physiologically and commercially important group of enzymes used primarily as detergent additives[4]. They play a specific catalytic role in the hydrolysis of proteins and are also used in industries like leather, food, photography and pharmaceuticals. The use of enzyme detergents is expected to increase, since the addition of enzymes compensates for the decreased efficiency of detergents at lower temperatures. Another industrial process, which has received attention, is the enzyme-assisted dehairing of animal hides and skin in the leather industry. Traditionally the process is carried out by treating the animal hides with a saturated solution of lime and sodium sulphide, which is expensive and unpleasant to carry out. Alkaline proteases also find potential application in the bioprocessing of used X-ray films, which contain approximately 1.5 to 2.0 per cent (by weight) silver in their gelatin layers. In recent years, the use of thermostable alkaline enzymes has increased in a wide range of other biotechnological applications such as silver recovery[5,6] in feeds[7,8] and peptide synthesis[9]. Hence, there is a considerable interest in the production of alkaline protease. However, no defined medium has been established for the better production of alkaline proteases from different microbial sources. Each organism or strain has its own special conditions for maximum enzyme production. With a view to develop an economically feasible technology to improve yield of alkaline protease, the authors have studied a number of factors which include, optimization of environmental and fermentation parameters such as *p*H, temperature, incubation period, size and age of inoculum and effect of addition of some nutrients, trace elements and inhibitors on protease production. A mutant strain of *T. thalpophilus* PEE 14, isolated in our laboratory, was used in the present study[10].

Materials and Methods

Inoculum Preparation

Cell suspension from a 24 h old slant of *T. thalpophilus* PEE 14 was transferred at 10% level, into 250 mL Erlenmeyer flasks containing 45 mL of sterile inoculum medium containing (g/L): Soluble starch, 10; casein, 3; KNO_3, 2; NaCl, 2; K_2HPO_4, 2;

*Author for correspondence:
Tel: 91-891-2701852 / 3105186; Fax: 91-891-2755547
E-mail: ellaiah2@rediffmail.com

$MgSO_4.7H_2O$, 0.05; $CaCO_3$, 0.02 and $FeSO_4.7H_2O$, 0.01. The flasks were kept on a rotary shaker (70 rpm) at 55ºC for 24 h. The contents of the flasks were centrifuged at 3000 rpm for 10 min and the supernatant was decanted. The cell pellets were washed thoroughly with sterile distilled water. Finally the cell mass was adjusted with sterile distilled water to give a final concentration of 6.24×10^7 spores/mL.

Shake Flask Fermentation

Five mL of inoculum (equivalent to 0.03 g dry cell weight)[11] was inoculated into 45 ml of basal production medium (g/L): glucose, 10; soya bean meal, 20; $CaCl_2$, 0.4 and $MgCl_2$, 2 (*p*H 10) contained in 250 mL Erlenmeyer flask and incubated at 55°C on incubator shaker maintained at 70 rpm for 48 h. At the end of fermentation, 5 mL broth was centrifuged at 3000 rpm for 10 min and assayed for enzyme activity.

Alkaline Protease Assay

Alkaline protease activity was determined by the modified procedure based on the method of Tsuchida *et al*[12]. Casein, 2 per cent (Hammerstan casein, Merck, Germany) in 0.2 *M* carbonate buffer (*p*H 10) was used as substrate. Casein solution, 0.5 mL, with an equal volume of suitably diluted enzyme solution was incubated at 55°C. After 10 min, the reaction was terminated by adding 1 mL of 10 per cent trichloroacetic acid. The mixture was centrifuged (3000 rpm) and 5 mL 0.44 *M* Na_2CO_3 and 1 mL two-fold diluted Folin Ciocalteau reagents were added to the supernatant. After 30 min the colour developed was red at 660 nm against a blank prepared in the same manner. Tyrosine served as the standard. One protease enzyme unit is defined as the amount of enzyme that releases 1 μg of tyrosine per ml per min under the above assay conditions. All the experiments were conducted in triplicate and the mean of the three is presented.

Optimization of Process Parameters

To investigate influence of initial *p*H, the production medium was adjusted at various levels of *p*H (6.0 to11.5). Fermentation was carried out and samples were assayed as described earlier. The optimal *p*H obtained was used in all the subsequent experiments. To study the effect of incubation, production medium was inoculated and incubated at various temperature ranges from 37 to 60°C for 48 h. The optimal temperature obtained at this level was fixed for further studies. Study of the optimal incubation period was carried out by withdrawing samples periodically at every 12 h up to 96 h and assayed for protease activity. The effect of different inoculum size was studied by varying it 4 to 15% for optimal alkaline protease production. The flasks with the basal production medium were inoculated as above and incubated at 55°C for 48 h. The optimal level of inoculum obtained was used in all the further experiments. The effect of inoculum age was studied from 12 to 72 h at 10 per cent level.

Optimization of Nutritional Parameters

Vitamin solutions were prepared by adding the specific amounts of vitamins to distilled water, sterilized by filtration and then added aseptically to the sterile basal medium at concentrations of 5 μg/mL. A control was run with water instead of vitamins. The following vitamins viz., B_1, B_2, C, biotin, pyridoxine, pyridoxine HCl, PABA and folic acid were used for study. After fermentation, protease activity was measured as described previously.

The solutions of individual amino acids viz., D-alanine, DL-alanine, L-alanine, L-arginine, L-asparagine, L-cysteine, L-cysteine HCl, glycine, L-glutamic acid, L-glutamic acid sodium salt, L-leucine, L-lysine, L-histidine, L-tryptophan and L-tyrosine were prepared in distilled water, sterilized by filtration and added aseptically to the sterile basal medium at a concentration of 0.5%. A control was run using water.

Solutions of all trace elements ($CaCO_3$, $CuSO_4$, $CoCl_2$, $CdCl_2$, $ZnSO_4$, $SrCl_2$, sodium molybdate, sodium tungstate and ferrous phosphate) were prepared in distilled water, sterilized and added to the medium in graded amounts to attain 10 μg/mL concentration. One set of control (without any trace element) was taken for comparing the effect.

Inhibitors [Silver nitrate ($AgNO_3$), Ethylene Diamine Tetra Acetate (EDTA), potassium iodate (KIO_3), Isoniazide (INH), 2,4-Dinitrophenol (2,4-DNP), Sodium fluoride (NaF), Mercuric chloride ($HgCl_2$), EDTA ferric chloride salt and Potassium permanganate ($KMnO_4$)] in solution form were separately sterilized and added individually at concentrations of 0.05% to the sterile basal medium and fermentations were conducted along with a control as described earlier. After fermentation, enzymatic activities were measured as described earlier.

Solutions of antibiotic substances (penicillin, streptomycin, neomycin, framycitin, kanamycin and cephalosporin) were prepared asceptically in sterilized distilled water and added asceptically to the sterilized basal production medium at 50 μg/mL and fermentations were conducted as described earlier. One control set (without any antibiotic supplementation) was used. After fermentation, enzymatic activities were measured as described earlier.

To the basal medium various surfactants (Tween-20, Tween-80, Sodium lauryl sulphate and Cetrimide) sterilized by filtration were added asceptically at 0.04% concentration. After fermentation, protease activities were assessed.

Results and Discussion

Effect of Process Parameters

Different initial *p*H values (6.0-11.5) were used to study their effect on the protease production. The highest enzyme activity was observed at *p*H 10 (226.2 U/mL), where it had maximum growth. So the optimum *p*H for protease production was found to be 10.0. The results of incubation temperature indicated that the organism grew over a wide range of temperatures (37-60°C). The maximum alkaline protease production (227 U/mL) was observed at 55°C. Increase in incubation temperature to 60°C decreased the yield to 186 U/mL. Hence, the optimum incubation temperature for protease production by this organism was 55°C. The results of incubation temperature indicated that the organism grew well in the medium and maximum protease production (274 U/mL) was achieved at 24 h. After that the protease production decreased gradually with increased incubation periods.

Initial microbial load to the medium does affect growth and in turn metabolite production. The results indicated that protease production was increased with increase in the level of inoculum up to 10 per cent level (280 U/mL) and further increase in inoculum level did not increase the protease production. The results of age of inoculum indicated that culture of 24 h age had maximum protease producing ability (282 U/mL).

Effect of Vitamins on Protease Production

Importance of vitamins for microbial nutrition was first introduced in the beginning of this century. Vitamins are considered as a distinct class of compounds, effective in minute quantities and essential for growth. From the results it can be observed that among the various vitamins tested only pyridoxine was able to stimulate the production of protease by 16%. Other vitamins could not stimulate the protease production.

Effect of Amino Acids on Protease Production

The results of effect of amino acids indicated that many of the amino acids (at 5μg/mL concentration) tested showed stimulating effect on protease production. Among them, L-cysteine showed maximum (198%) increase in protease production. The stimulating effect of amino acids were in the order of: L-cysteine > L-tyrosine, L-tryptophan (176% increase) > L-lysine HCl, L-cysteine HCl (48% increase) > L-alanine (25% increase) > DL-alanine (18% increase) > L-arginine, L-asparagine (16% increase). Other amino acids had an inhibitory effect on the protease production with L-glutamic acid having maximum inhibitory effect.

Effect of Trace Elements on Protease Production

The effect of various mineral salts on protease production has been studied. Among them, sodium molybdate, sodium tungstate, strontium chloride and ferrous phosphate exerted stimulatory effect while Zn^{2+}, Co^{2+}, Cd^{2+} and Cu^{2+} showed inhibitory effect at the concentration (0.04%) used in the test.

Effect of Inhibitors on Protease Production

The effect of various inhibitors on protease production showed that all the inhibitors used inhibit protease production at 0.05% level but only silver nitrate, mercuric chloride, 2,4-DNP, inhibited strongly. INH had almost no effect and other substances showed moderate inhibitory effect.

Effect of Antibiotics on Protease Production

Study of effect of different antibacterial antibiotics indicated that all antibiotics exerted inhibitory effect on protease production at the concentration used. Framycitin had highest inhibitory activity and penicillin showed the least inhibitory effect on enzyme production.

Effect of Surfactants on Protease Production

All the surfactants inhibited protease production at 0.04% concentration. Cetrimide exhibited the highest inhibitory activity followed by Tween-80, SLS and Tween-20.

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