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Production of Alkaline Protease by *Bacillus pumilus* (MTCC7420) using Submerged Fermentation and Optimization of Process Parameters

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ABSTRACT

In the present study Bacillus pumilus (MTCC 7420) was employed for production of protease enzyme using submerged fermentation. The culture conditions were optimised for maximum enzyme production. The enzyme was stable between the pH 9.2 to 10.4 but the optimal pH was found 10.2. The enzyme was also stable between temperature ranges 40^{o}C to 70^{o}C but best temperature for enzyme activity was found to be 60^{o}C. Alkaline protease activity was influenced by various carbon and nitrogen sources. The carbon and nitrogen sources were found best for fructose and gelatin respectively.

Keywords: Alkaline protease, *Bacillus pumilus*, Process parameters, Submerged Fermentation.

INTRODUCTION

The genus *Bacillus* constitutes a diverse group of rod shaped, gram-positive bacteria characterized by their ability to produce robust endospores as a survival mechanism in response to adverse environmental conditions [Gibson, T. and Gordon, R.E., 1974]. Proteases are the most important group of industrial enzymes which account for about 60% of total enzymes in the market. This biocatalyst hydrolyzes peptide bonds in proteins. All proteases are heat resistant [Mehler, H.A., 1957]. On the basis of their acid-base behaviour, proteases are classified into three categories i.e. acid, neutral and alkaline proteases. The acid proteases are those which have pH optimum in the range of 2.0-5.0 and these are mainly fungal in origin. Proteases have optimum pH in the range of 7.0 or around are neutral and they are mainly originated from plants however some bacteria and fungi are also able to produce neutral proteases. While those which work in the pH range of 8.0-11.0 are alkaline proteases. Some of the important alkaline proteases are Solanain, Hurain and Proteolytic enzymes of *Bacillus sp., Streptomyces sp.* [Hameed, A. *et al*.,1996]. The alkaline protease is an extracellular enzyme. The bacterial alkaline proteases are produced in large amount for various applications in leather processing, Medical diagnostics, and recovery of silver from X-ray films. The single largest application for bacterial alkaline protease

is the recent incorporation of enzyme in laundry detergent by all the major detergent manufactures and the market share of such enzyme is between 30%-60% in most industrialized countries. Alkaline proteases are also widely used in protein recovery or solubilisation, meat tenderization, in the biscuit and cracker industries. Both solid state fermentation and submerged fermentation can be used for alkaline protease production.

MATERIALS AND METHODS

Chemicals and reagent used:

Chemicals which had been used during the study were: Ammonium chloride, Ammonium nitrate, Beef extract, Calcium carbonate ($CaCO_3$), Calcium chloride ($CaCl_2$), Casein, Dilute iodine, Dipotassium monohydrogen phosphate (K_2HPO_4), DNS reagent, Follins reagent, Fructose, Gelatin, Glucose, Hydrated magnesium sulphate ($MgSO_4.7H_2O$), Maltose, Magnesium sulphate ($MgSO_4$), Peptone Beef Extract Agar Medium (PBE), Peptone, Potassium dihydrogen phosphate (KH_2PO_4), Sodium hydroxide (NaOH) solution, Starch, Sodium carbonate (Na_2CO_3), Sucrose, Trichloro acetic acid (TCA), Tris HCl, Urea, Yeast extract.

Bacterial strain:

Bacillus pumilus (MTCC7420) were used for the production of alkaline protease. The culture were maintained in a refrigerator after periodic sub-culturing and growing at 50^{o}C for 48 hours on peptone beef extract(PBE) agar medium (nutrient agar medium).

Inoculum:

A loopful from the bacterial slant was transferred into culture tube having 2ml sterile PBE medium and incubated at 50^{o}C for 48 hours. The content was then transferred into 25ml test tube containing 10ml of growth medium and incubated at 50^{o}C for 48 hours and thus prepared 10ml culture was used for seeding 100ml medium under similar condition and used as inoculum at the rate of 5 percent during studies on enzyme production.

Fermentation media:

Different production media were used for the production of alkaline protease. The pH of each of the following media was adjusted at 9 with 1N, HCl and 1N, NaOH solution and the media containing 100ml each, were sterilized at 15 psi for 20 minutes**.**

Batch fermentation:

100ml of each of the prepared media were taken in 250ml Erlenmeyer flask separately and sterilized at 15 psi for 20 minutes, the flasks were inoculated with 1ml of *Bacillus pumilus* (MTCC 7420) culture suspension into production media and incubated at 60^{o}C for 72 hours on a shaking incubator at 25 to 30 rpm then the fermented broth was centrifuged to separate the biomass and the cell extract was used for estimation of alkaline protease.

Alkaline protease estimation:

For estimation of alkaline protease casein solution was used as a substrate. Casein solution and buffer solution (carbonate-bicarbonate buffer) were taken in test tube and further enzyme solution (cell extract) was also added in test tube and the reaction mixture thus prepared, was incubated. The reaction was stopped by adding trichloroacetic acid solution. After filtration of this solution, filtrate was used for further experiment. Then after, small amount of aliquot and Na_2CO_3 was taken and C-reagent was added in the test tube. After few minutes follin reagent (double diluted) was added in the test tube. After 30 minutes the reading of O.D. was estimated.

Characterization of the crude alkaline protease enzyme:
The crude alkaline protease obtained from the *Bacillus* pumilus (MTCC 7420), which showed the highest potential for proteolytic activity, was further subjected to preliminary characterization study. Therefore, the effects of pH, temperature, carbon and nitrogen sources on activity were studied.

Optimization of pH for alkaline protease production:
The initial pH of the medium was shown to effect protease production. The effect of initial pH on alkaline protease production was studied. The production medium was adjusted at various levels of pH by NaOH solution (9.2, 9.4, 9.6, 9.8, 10, 10.2, and 10.4).

Determination of optimum temperatures for alkaline protease production:
Incubation temperature was shown to effect protease production. To study the effect of incubation temperature for maximum protease production, solution was incubated at various temperatures such as 40, 50, 60, and 70^{o}C.

Analysis of carbon sources on alkaline protease production:
Carbon sources chosen for the study were glucose, sucrose, fructose, and maltose. These carbon sources were used to replace the carbon source available in media.

Analysis of nitrogen sources on alkaline protease production:
Sources of nitrogen include gelatin, urea, ammonium chloride and ammonium nitrate. These nitrogen sources were used to replace the carbon source available in media.

RESULTS AND DISCUSSION

Alkaline protease estimation:
Batch fermentation was carried out with *Bacillus pumilus* (MTCC7420) in eight different media (M1 to M8) separately containing 100ml medium into 250ml conical flask. The results are shown in Figure 1. It is observed from figure 1 that medium M5 was found best among these media for alkaline protease production.

Effect of pH on alkaline protease production:
To study the effect of pH on enzyme production, fermentation was carried out by *Bacillus pumilus* (MTCC7420) in mediums M1 to M8 separately at pH 9.2 to 10.4. It is observed from respective (Figure 2), that the protease enzyme production took place at pH 9.2 and 9.6 the maximum yield was obtained in medium M1 and minimum was obtained in medium M5, at pH 9.4, 9.8, 10 and 10.2 the maximum yield was obtained in medium M4 while minimum was obtained in mediums M8, M7, M7 and M6 respectively and at pH 10.4 the maximum yield was obtained in medium M2 and minimum was obtained in medium M8. The combine effect of pH for all mediums shows that the maximum yield was obtained medium M4 at pH 10.2 and minimum was obtained in medium M^{7} at pH 9.8. Similar results have been reported by [Durham, D.R.,1987], [Hameed, A. *et al.*,1996], [Rao, M.B. *et al.*,1998], and [Tang, X.M. *et al.*, 2004].

Effect of temperature on alkaline protease production:
In order to study the effect of temperature on alkaline protease production, fermentation was carried out at five different temperatures (40^{o}C, 50^{o}C, 60^{o}C, 70^{o}C) in mediums M1 to M8. It was observed from figure 3 that at temperature 40^{o}C, 50^{o}C, 60^{o}C and 70^{o}C the yield of enzyme was maximum in mediums M2, M7, M4 and M1 and minimum yield was obtained in mediums M8, M4, M3 and M5 respectively. The combine effect of temperature for all mediums shows that the

maximum yield was obtained in medium M4 at temperature 60°C and minimum was obtained in medium M8 at temperature 40°C. Similar results have been reported by [Durham, D.R.,1987], [Hameed, A. *et al.*,1996], [Rao, M.B. *et al.*,1998], and [Tang, X.M. *et al.*, 2004].

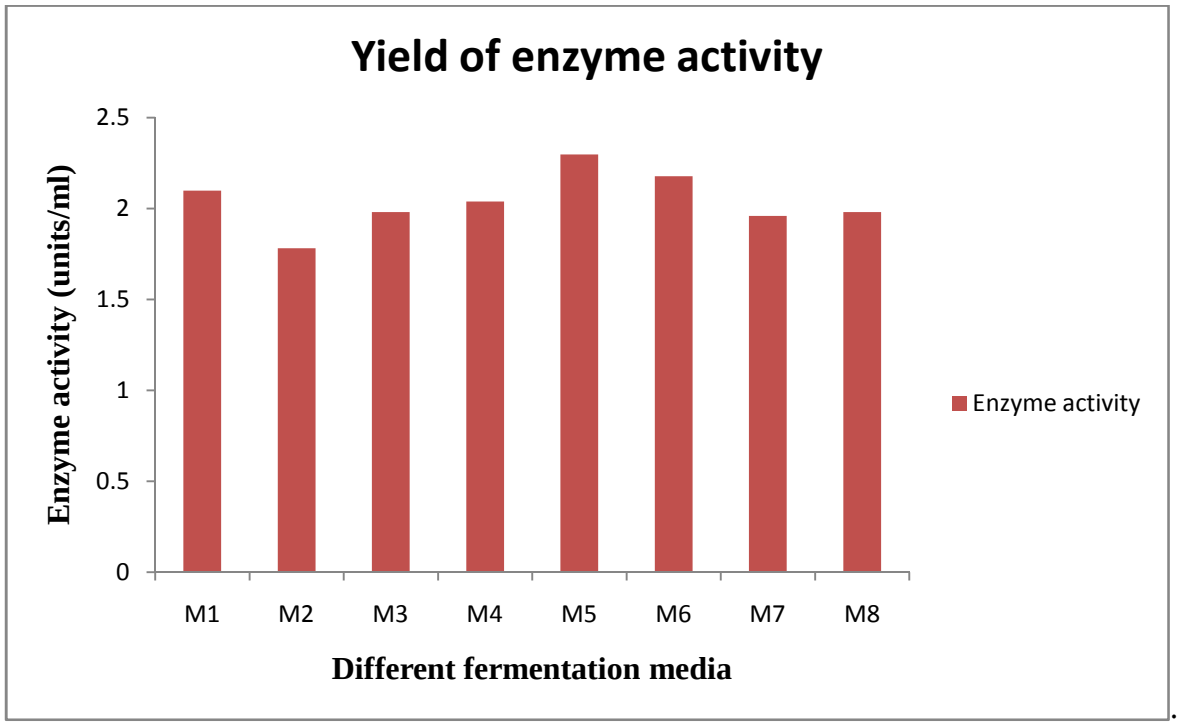

Figure 1: Best media for alkaline protease production

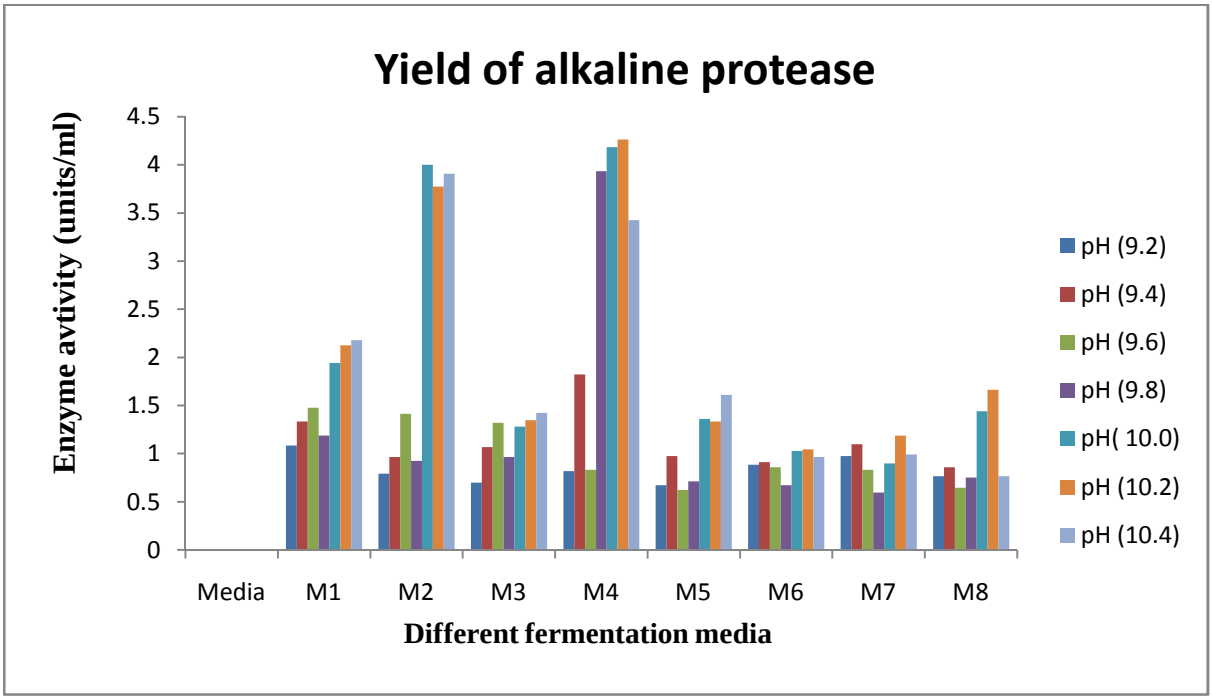

Figure 2: Effect of different pH on alkaline protease production in different fermentation media (M1 to m8)

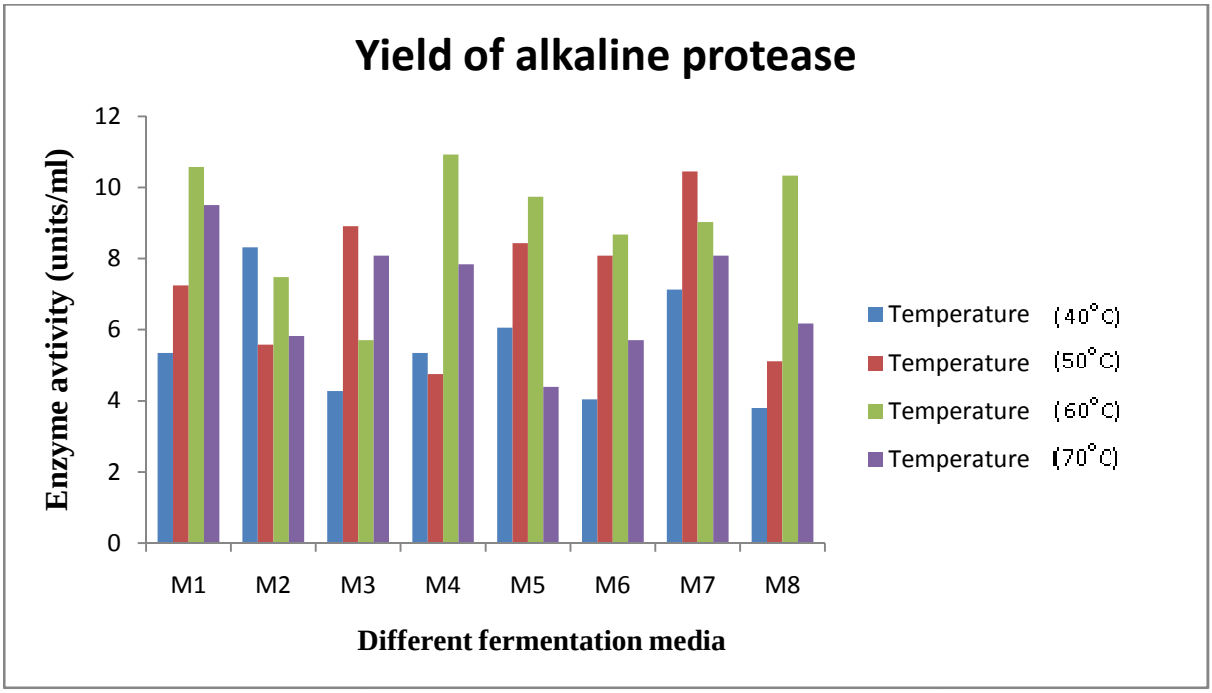

Figure 3: Effect of temperatures in different fermentation media (M1 to M8) for alkaline protease production

Effect of carbon sources on alkaline protease production:

In order to study the effect of carbon sources mediums M1 to M4 was used for production of alkaline protease by replacing only the starch with various other carbon sources like glucose, sucrose, fructose and maltose respectively as shown in figure 4. It is observed that alkaline protease production was appreciably good in all cases but maximum enzyme production was obtained using fructose and minimum production was obtained using sucrose as carbon sources as shown in the figure. Similar results have been reported by [Durham, D.R.,1987], [Hameed, A. *et al.*,1996], [Rao, M.B. *et al.*,1998], and [Tang, X.M. *et al.*, 2004].

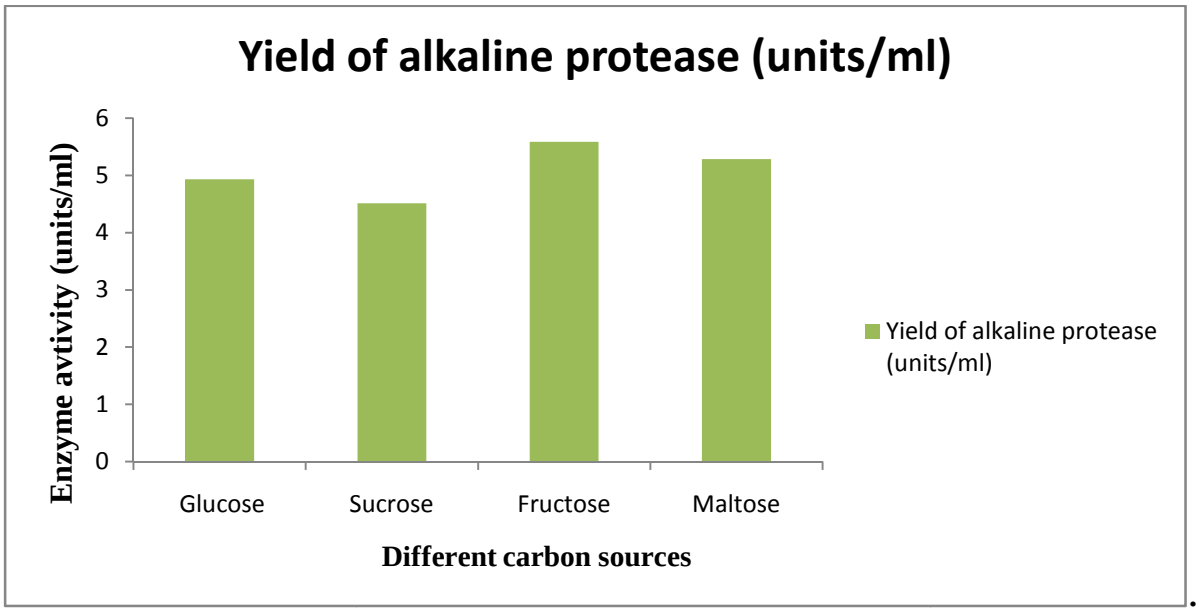

Figure 4: Effect of different carbon sources on alkaline protease production

Effect of nitrogen sources on alkaline protease production:

Alkaline protease production were carried out in mediums M1 to M4 separately along with various nitrogen sources by replacing only peptone with various other nitrogen sources like gelatin, urea, ammonium chloride, ammonium nitrate respectively as shown in figure 5. It is observed that alkaline protease production was appreciably good in all cases but maximum enzyme production was obtained using gelatine and minimum was obtained using urea as nitrogen source as shown in the figure. Similar results have been reported by [Durham, D.R.,1987], [Hameed, A. *et al.*,1996], [Rao, M.B. *et al.*,1998], and [Tang, X.M. *et al.*, 2004]..

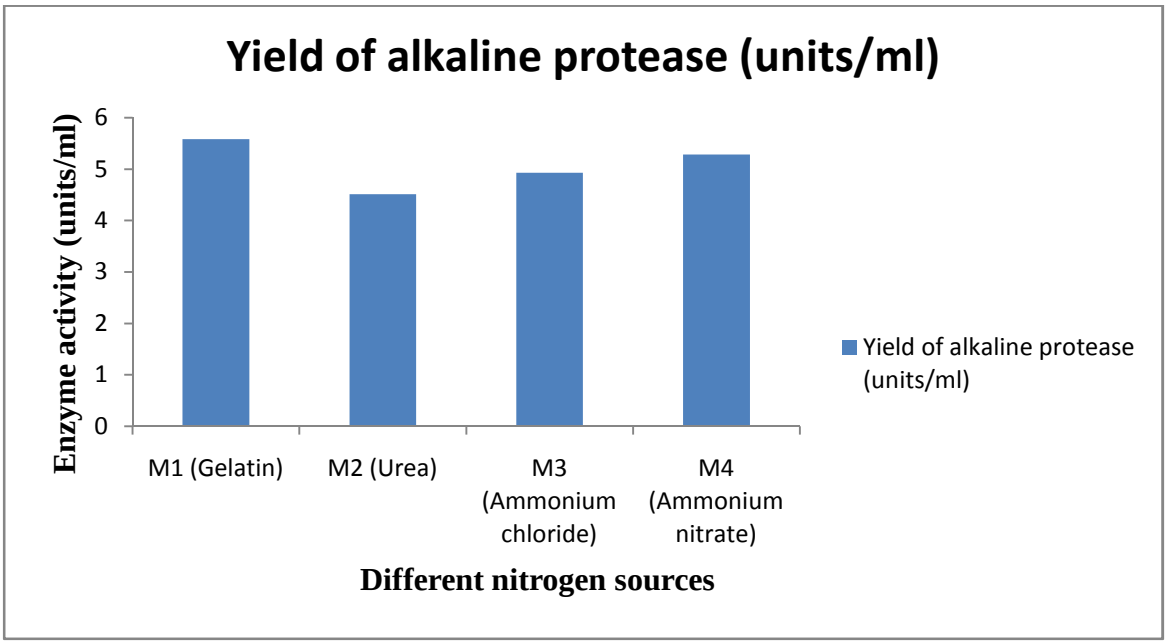

Figure 5: Effect of different nitrogen sources on alkaline protease production

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