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Production of Alkaline Protease by *Bacillus subtilis* (MTCC7312) using Submerged Fermentation and Optimization of Process Parameters

O P Verma*, Prashansa Kumari, Shruti Shukla and Abha Singh

JSBB, Sam Higginbottom Institute of Biotechnology & Bioengineering, Allahabad

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ABSTRACT

Alkaline proteases 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. In leather industries alkaline proteases are exhibiting a prominent role in unhairing and bating processes. In the present study Bacillus subtilis MTCC7312 was used for the production of alkaline protease enzyme using submerged fermentation technique. The main purpose of the project was to study the different parameters of protease productivity and its yields. The maximum alkaline protease activity was 6.376U/ml in medium M6 using casein as substrate. A temp. of 60^{o}C was found to be optimum for enzyme production in medium M3. Similarly maximum protease activity was found at pH 10 in medium M4. Among the different sources glucose was found to be best carbon source for production of alkaline protease and gelatin was found to be the optimum nitrogen source for protease enzyme production by Bacillus subtils.

Key Words: Alkaline Protease, Bacillus subtilis, Submerged Fermentation, Process Optimization.

INTRODUCTION

Protease catalysis of peptide bonds (proteolysis) [Genckal, H. and Tari, C., 2004]. Proteases (peptidyl-peptide hydrolases) are a group of enzymes (also known as peptidases, proteinases or proteolytic enzymes) that hydrolyse (break down) a variety of proteins via the addition of water across peptide bonds (i.e., bonds that join two adjacent amino acids to form a polypeptide) and catalyse peptide synthesis in organic solvents and in solvents with low water content [Beg, Q.K. *et al.*, 2003; Sookkhe, B. *et al.*, 2000]. Proteases are one of the most important classes of enzymes, occupying a major share of 60% of total enzyme market. This biocatalyst hydrolyzes peptide bonds in proteins. All proteases are heat resistant [Mehler, H.A., 1957]. Proteases play a crucial role in many physiological and pathophysiological processes. Microbial proteases

account for approximately 40% of the total worldwide enzyme sales. Microbial proteases are preferred to the enzymes from plant and animal sources, since they possess almost all the characteristics desired for biotechnological applications. 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 [Mukhtar, H. and Haq, I. 2007]. Acidic proteases have application in meat tenderization, in the production of fermented foods and also in acidic cleaning compositions [Rao, M.B. *et al*., 2007]. 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 and Streptomyces species* **[**Hameed, A. *et al*., 1996, Lee, J.K. *et al*., 2002]. Neutral and alkaline proteases hold great potential for application in the detergent and leather tanning industries due to the increasing trend in developing environment friendly technologies.

MATERIALS AND METHODS

Bacterial strain

The bacterial strain of *Bacillus subtilis* has the characteristic nature of producing alkaline protease.

Inoculums

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

Fermentation media

Different production media were used for the production of alkaline protease.

The composition media for 100ml
M1: (Starch- 2g, Peptone-1g, $CaCO_3$-0.3g).
M2: (Starch-2g, Peptone-1g, Beef extract-1g, $CaCO_3$- 0.3g).
M3: (Starch-2g, Peptone-1g, Yeast extract-1g, $CaCO_3$- 0.3g).
M4: (Starch-2g, Peptone-0.5g, Beefextract-0.5g, $CaCO_3$- 0.3g).
M5: (Nutrient-Gelatin-1g, Glucose-1g, KH_2PO_4-0.05g, K_2HPO_4-0.05g, $CaCl_2$-0.05g).
M6: (Glucose-1g, Peptone-0.5g, yeast extracts-0.5g, KH_2PO_4-0.1g, $MgSO_4.7H_20$-0.02g, Na_2CO_3-0.5g**).**
M7: (Maltose-1g, Casein-0.5g, Yeast extract-0.5g, K_2HPO_4-0.1g, $MgSO_4$-0.2g, Na_2CO_3-0.5g).
M8: (Sucrose-1g, Casein-0.5g, Yeast extract-0.5g, K_2HPO_4-0.1g, $MgSO_4$-0.2g, Na_2CO_3-0.5g).

Batch fermentation

100ml of each of the prepared media was taken in 250ml Erlenmeyer flask separately and sterilized at 15 psi for 20 mins, the flasks were inoculated with 1ml of *Bacillus subtilis* culture suspension into production media and incubated at 60^{o}C for 72 hrs 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, appendix 1.1) 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 5% trichloroacetic acid solution. After filtration of this solution, filtrate was used for further experiment. Then after, small amount of aliquot and Na_2CO_3 will be taken and C-reagent (10% trichloroacetic acid) 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 taken at 660nm.

Optimization of process parameters

Effect of pH on alkaline protease production

The production medium was adjusted at various levels of pH such as pH 9.2, 9.4, 9.6, 9.8, 10, 10.2 and 10.4. The effect of pH on alkaline protease production was studied.

Effect of carbon sources

Glucose, sucrose, fructose and maltose were taken for study. These were used to replace the carbon source available in media.

Effect of nitrogen sources

Sources of nitrogen chosen for the study were urea, ammonium chloride, ammonium nitrate and gelatin. These nitrogen sources were used to replace the nitrogen sources available in media.

Effect of temperature

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.

RESULTS AND DISCUSSION

Alkaline Protease Estimation

The maximum enzyme production was found 6.376 U/ml in medium M6.

Effect of pH on alkaline protease production

To study the effect of various pH on alkaline protease production different pH ranges (9.2, 9.4, 9.6, 9.8, 10, 10.2, and 10.4) were used separately for all fermentation media i.e. media M1 to M8. The results showed that the maximum protease production was 5.941 and 7.842 U/ml at pH 9.2 and 9.6 respectively in medium M1. And 13.466, 25.428, 26.537, 23.210 and 24.556 Units/ml at different pH 9.4, 9.8, 10, 10.2 and 10.4 respectively in medium M4. Thus from above results we found that the maximum yield was obtained in medium M4 even at different pH. The maximum alkaline protease production (26.537U/ml) was observed at pH 10 in medium M4.

Effect of Carbon sources on alkaline protease production

Different carbon sources had different impact on the production of alkaline protease by *Bacillus subtilis* MTCC7312. Among the various carbon sources tested, soluble glucose was found to be the best support protease production with 9.030 U/ml in medium M1. In addition, good protease activity was also observed in the media supplemented with sucrose, fructose and maltose in media M2, M3 and M4 respectively.

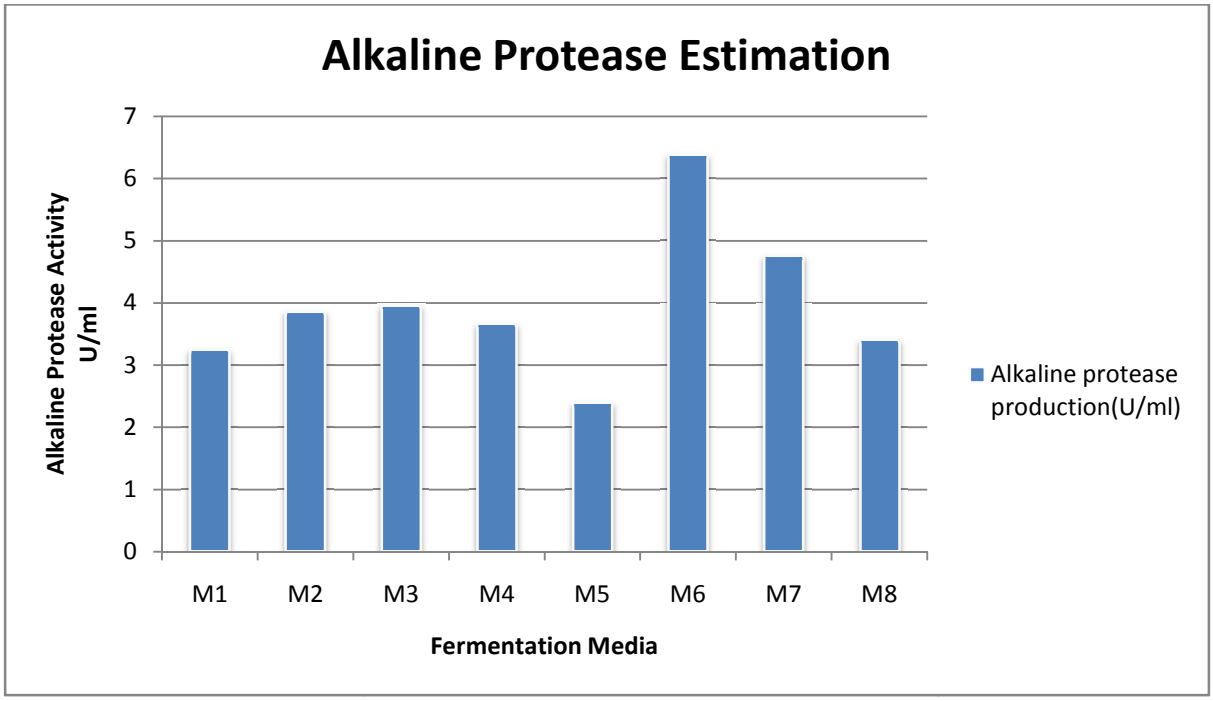

Figure 1: Estimation of alkaline protease

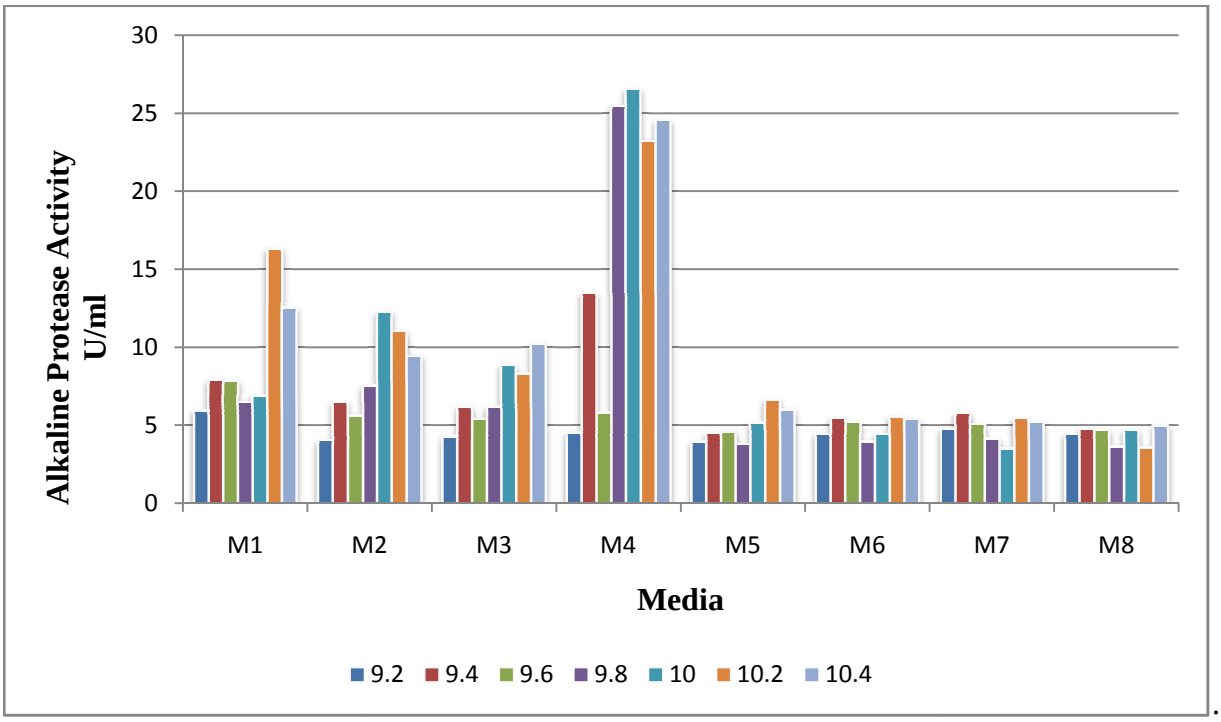

Figure 2: Effect of different pH on alkaline protease production by *Bacillus subtilis*

Effect of Nitrogen sources on alkaline protease production

Among the various nitrogen sources tested, gelatin was found to be the best support protease production with 13.07 U/ml in medium M4. In addition, good protease activity was also observed in the media supplemented with ammonium nitrate, urea and ammonium chloride in media M1, M2 and M3 respectively.

Effect of Temperature on alkaline protease production

To study the effect of various temperatures, different temperature ranges (40^{o}C, 50^{o}C, 60^{o}C and 70^{o}C) were used. The results indicated that at temperature 40^{o}C the maximum alkaline protease production (22.391U/ml) was observed in medium M5 and the minimum yield was observed in medium M8 (13.816U/ml). Similarly, the maximum alkaline protease production at temperatures 50^{o}C, 60^{o}C and 70^{o}C was observed in media M4 (31.443U/ml), M3 (56.693U/ml), and M3 (40.495U/ml) respectively.

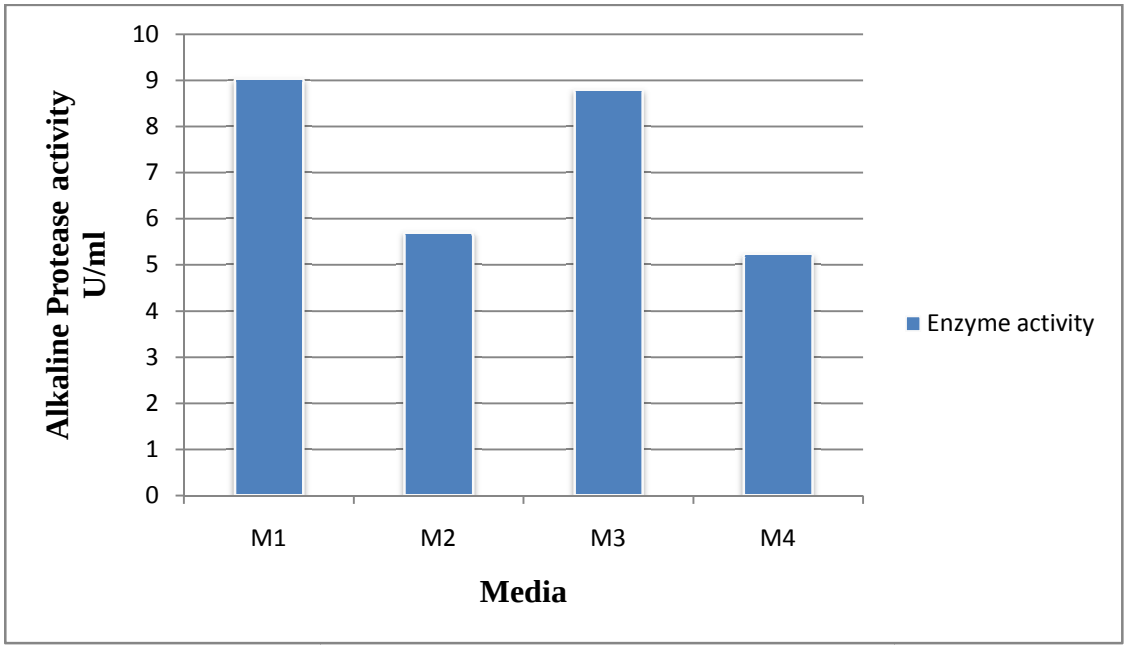

Figure 3: Effect of Carbon sources on alkaline protease production by *Bacillus subtilis*

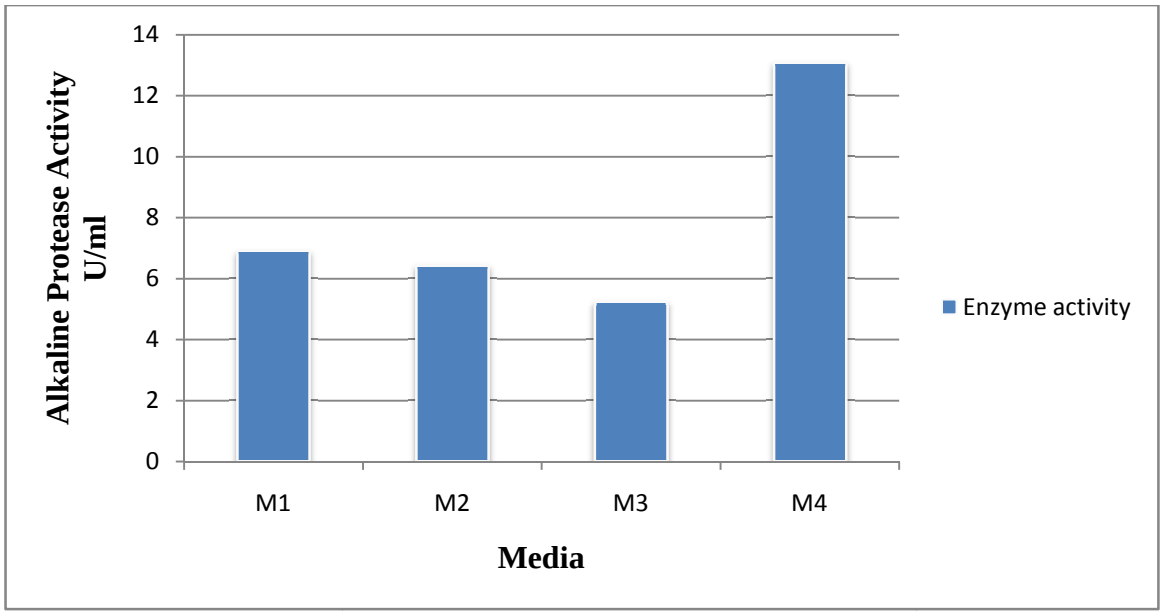

Figure4 : Effect of Nitrogen sources on alkaline protease production by *Bacillus subtilis*

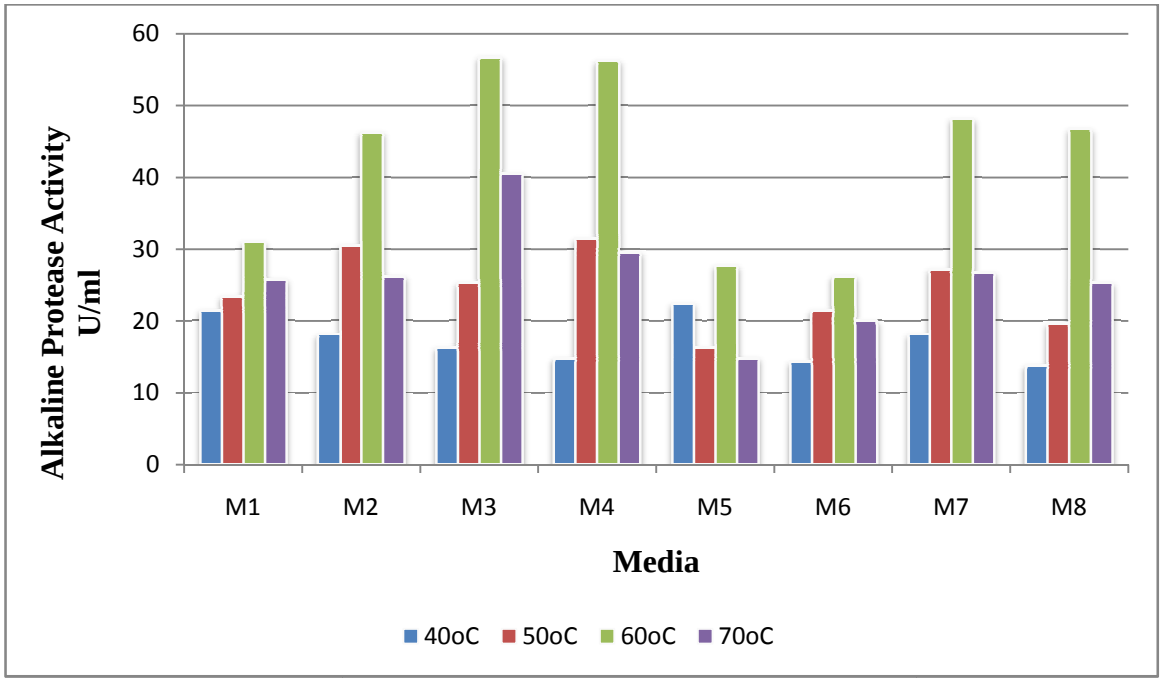

Figure5 : Effect of various temperatures on alkaline protease production by *Bacillus subtilis*

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