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Thermo stable alkaline protease production from *Bacillus thuringiensis* MTCC 1953: Optimisation and kinetic studies.

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Abstract: The aim is to optimize alkaline protease production from *Bacillus thuringiensis* MTCC 1953. Growth affecting component, carbon source was optimized for various sources namely glucose, sucrose and starch, and growth was observed. The best source found was glucose for *Bacillus thuringiensis*. Effect of glucose concentration and initial pH on cell and alkaline production was studied. Based on the optimum condition alkaline protease production was investigated in submerged batch fermentation process. The crude enzyme obtained from fermentation was subjected to acetone precipitation. Then partially purified enzyme was collected. Effect of temperature, pH, and substrate concentration on alkaline protease activity was studied. The enzyme showed maximum activity at 50ºC and at pH 10. Thermal stability of enzyme was studied and half life period of partially purified enzyme was found to be 18.73 min.
Key words: Alkaline protease, *Bacillus thuringiensis,* thermostable, kinetic study.

INTRODUCTION

Bacillus thuringiensis is a gram-positive, spore-forming and insecticidal crystal protein-producing bacteria. The crystal consists of protein named δ-endotoxin, which exhibit larvicidal toxicity upon ingestion by susceptible insect larvae, dissolution and activation by larval gut-juice proteases[1]. Bacterial protease has one of the high potential industrial enzyme, accounting to 60% of the total enzyme market in the world[2]. It is widely used in detergent, baking, tanning, pharmaceutical and food industry[3]. Protease is not a single enzyme, but consists of proteinases, peptidases and amidases[4]. Protease obtained from Bacillus spp., has more potential compared with fugal species and animal cells[5]**.** A protease producing bacteria *Bacillus thuringiensis* cc7 was isolated from soil collected near detergent industry and identified as *Bacillus species*[6]. Alkaline protease obtained from thermophilic and alkaliphilic Bacillus sp., able to withstand high temperature, pH, chemicals denaturing agents, and non-aqueous environment. Thermostable alkaline protease production was achieved by alkaliphilic and neutrophylic *Bacillus spp.*in a complex medium[7,8,9]. The protease production efficiencies of these organisms were measured with different carbon sources, incubation time, pH and temperature. Enzyme production was better in *Bacillus licheniformis* than in *Bacillus coagulans*[10]**.** Kinetics and thermodynamic properties of alkaline protease from newly isolated *Bacillus spp.,* was studied[11]**.**

Due to great commercial application of protease, it is necessary to study thermostable and kinetic charecterisation of this enzyme in order to improve design of enzymetic reactors. In this work, thermos table alkaline protease from *Bacillus thuringiensis* MTCC1953 production was achieved in submerged fermentation. The kinetic description of alkaline protease and the effect of parameters such as substrate concentration, pH, temperature and thermostability on enzyme activity were studied.

MATERIALS AND METHODS

Microbial Culture

Bacterial strain *Bacillus thuringiensis* MTCC1953 was purchased from MTCC, Chandigarh, India. It was transferred to growth medium (Beef extract: 1g/l, yeast extract: 2g/l, peptone: 5g/l, NaCl: 5g/l and agar: 15g/l). *B. thuringiensis* was allowed to grow for 24 hours in the above medium at a temperature of 28 ˚C. 10% V/V of this inoculum was introduced into 150 ml sterilized production medium (glucose: 6g/l, ammonium sulphate: 10 g/l, potassium dihydrogen phosphate: 4g/l, magnesium sulphate: 0.5 g/l, calcium chloride: 0.02 g/l) in 250 ml Erlenmeyer flask which was then kept in a rotary shaker at 150 rpm and 30 ˚C.

Various carbon sources such as starch, sucrose and glucose were tried as carbon source in the production medium to screen the best carbon source. Maximum dry cell weight concentration and soluble protein concentration were observed when glucose was used as a carbon source and same was used as a carbon source for further analysis.

Batch fermenter studies

Batch fermenter (Bioflow110, New Brunswick Scientific, USA) study was carried out with a working volume of 1.5L at room temperature. Fermentation was carried out under optimum condition. The aeration and agitation rates were maintained at 1.5 vvm and 150 rpm respectively.

Extraction of enzyme:

Sample from the fermentation process was centrifuged at 12000 × g for 20 min at 4 ˚C and supernatant was collected and added with equal volume of 0.5 M acetone for precipitation. The mixture was further centrifuged and the pellet was collected. Then pellet was dissolved in minimum amount of 0.02 M phosphate buffer, pH 6.0 and total soluble protein concentration was estimated by Lowry's method with bovine serum albumin as standard[12] **.**

Enzyme Assay

Protease activity was measured with UV Spectrophotometer at 660 nm using casein as a substrate [13]**.** One unit of protease activity is defined as the amount of enzyme required to produce 1micromoles of tyrosine from casein per min under specified condition.

Enzyme Kinetics and optimisation:

Effect of pH (4, 6, 7, 8 and 10), substrate concentration (0-6 %wt) and temperature (30˚C to 60˚C) on enzyme activity were studied. The thermal stability of the enzyme was studied by exposing the enzyme to 60 ˚C for varying time durations prior to enzymatic reaction.

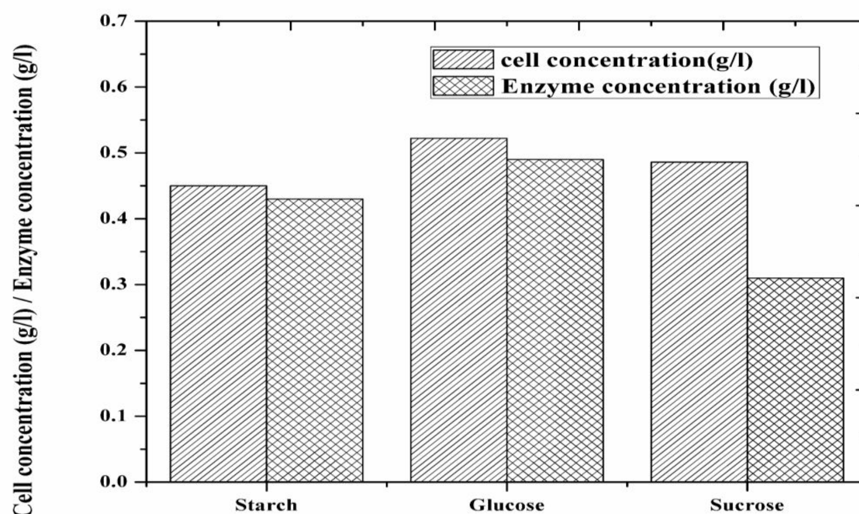


Figure 1: screening of carbon source for cell and alkaline protease production.

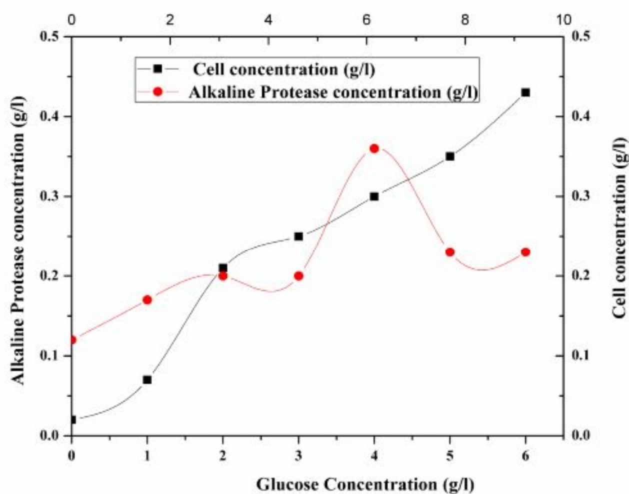


Figure 2: Effect of glucose concentration on cell and alkaline protease

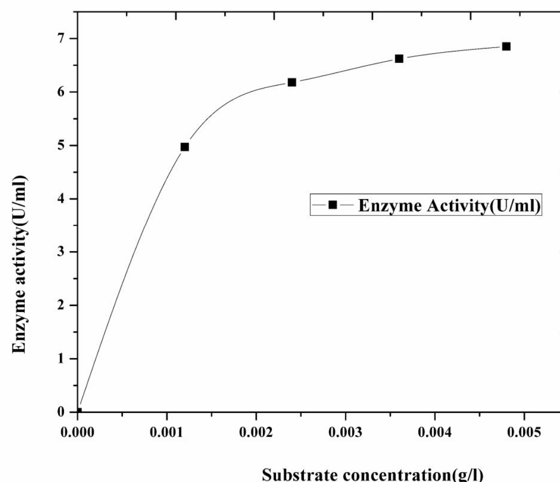


Figure 5: Effect of substrate concentration on enzyme activity.

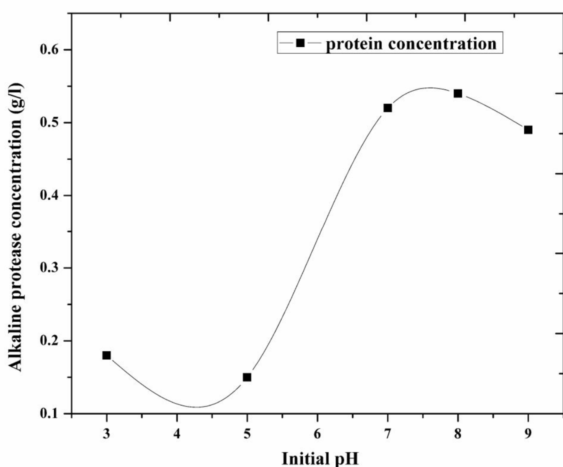
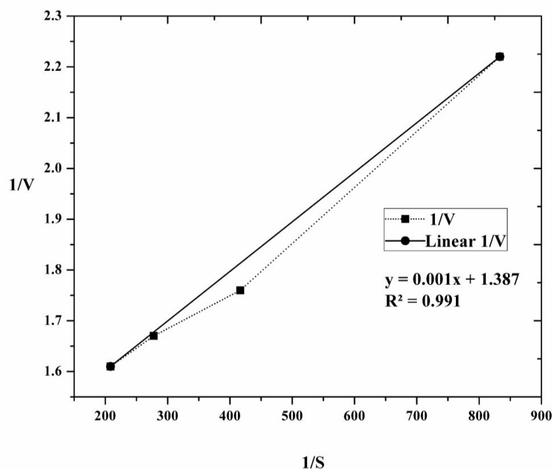


Figure 3: Effect of initial pH on alkaline protease concentration.



Figue 6: Enzyme kinetic parameters Estimation using Line-weaver plot.

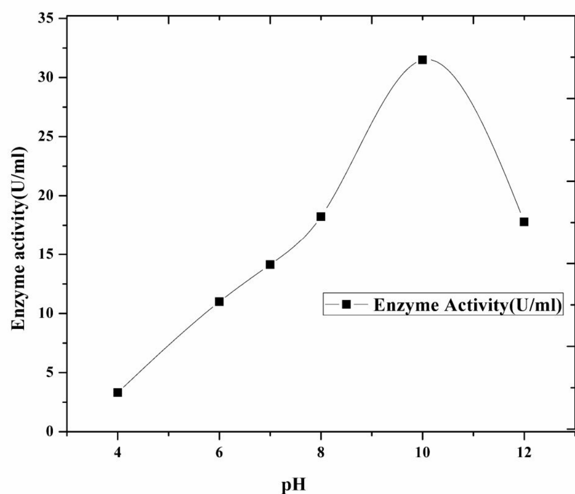


Figure 4: Effect of pH on enzyme activity.

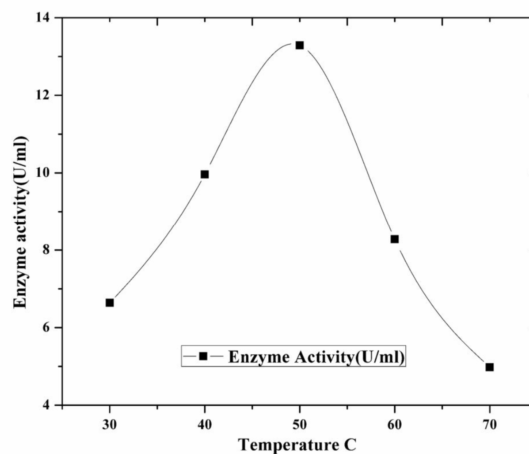


Figure 7: Temperature study on Enzyme activity.

RESULT AND DISCUSSION

Effect of carbon source on cell and alkaline protease production:

Screening of carbon source was investigated for *B.thuringiensis* MTCC 1953 using starch, glucose and sucrose as
a carbon source with production media (figure 1). It was found that the best suited carbon source for *B.thuringiensis* and alkaline protease production was glucose. Present finding coincides with earlier report of chudasama et al. chudasama et al explained that maximum concentration of *Bacillus thuringiensis* cc7 for alkaline protease production was obtained using glucose as a carbon source[6].

Effect of glucose concentration on cell concentration and alkaline concentration:

Cell concentration and alkaline protease concentration were studied by varying glucose concentration from 1g/l
to 7g/l shown in figure 2. Cell concentration was maximum at 6 g/l glucose concentration, where as alkaline protease concentration was high at 4g/l glucose concentration. After 4 g/l glucose concentration, alkaline protease concentration was decreased. It is similar to the finding of previous reports. Huang et al reported that optimum concentration of carbon source such as glucose (3.6 g/l) in the complex medium would enhance the production rate of thermostable protease from *Bacillus*. sp. HS08[14].

Effect of initial pH on alkaline protease production:

In this study, initial pH of the medium was varied from 2 to 10 at 4 g/l glucose concentration (**figure 3).** Alkaline protease concentration was high at initial pH 8 in the medium. It is consistent with earlier report of chudasama et al and Borris. It has been found that alkaline protease production rate was maximum at 8.5 from *B.thuringiensis* cc 7 [6]**.** Alkaline protease production was found to be maximum at pH 9-13[15].

Enzyme Kinetics and optimisation

Effect of pH on enzyme activity

In this study, optimum pH for enzyme activity was found to be 10 from *B.thuringinensis* (figure 4). Peek et al., Dhandapani and Vijayaragavan explained that optimum pH of 10.0–10.5 for protease obtained from *Bacillus sp.,Thermusaquaticus, Xanthomonas maltophila*, and *Vibrio metscnikovii* [16,17]. Hamid Mukhar and Ikram-Ul-Haq reported that optimum pH for alkaline protease activity from *B.subtilus* IH-72 was found to be 8.5[18]**.** Manavalan Arulmani et al observed that protease enzyme was relatively stable in the pH range 7.0–12.0 and optimum pH for maximum activity of the purified alkaline protease was found to be 9.0 [19]**.** These reports are similar to our finding.

Effect of substrate concentration

Figure 5 shows that effect of substrate concentration on enzyme activity. Kinetic parameters such as K_m and V_m
for alkaline protease from *B.thuringensis* were 0.72 mg/l and 7.952 U/ml (figure 6). Hamid mukhtar and Ikram-
ul-haq reported that K_m and V_m for alkaline protease from *Bacillus subtilus* IH-72 at 50ºC was 1 mg/ml and 15 U/mg/ml respectively[18]. Compare with previous report lower value of Km was obtained. Lower Km value denotes high affinity of enzyme with substrate[20]**.**

Effect of temperature and heat on enzyme activity

Temperature plays a vital role for activating and deactivating enzyme activity. Figure 7 shows that effect of temperature on enzyme activity. Enzyme activity was increased from 30ºC to 50ºC. Alkaline protease showed maximum activity at 50ºC reaction temperature. Kobayashi et al. observed that alkaline protease showed more stability till 50 º C from alkalophilic *Bacillus sp.* KSMK16[21]. It is consistent with our observation. Thermo stability of alkaline protease was checked by incubating enzyme solution at 60ºC. Half life period of alkaline protease was 18.73 min which was higher value than finding of Abu Sayem et al. Abu Sayem et al reported that halflife period of alkaline protease from novel Bacillus licheniformis MKZ03 was found to be 17 min[22].

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