



PRODUCTION OF PROTEASE BY ALKALOPHILIC BACILLUSSUBTILIS IN BIOREACTOR AND ITS CHARACTERIZATION

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ABSTRACT: The current studies were aimed at to investigate role of pH, dissolved oxygen for production protease in bioreactor by alkalophilic bacterium and application of saw dust for its purification. The production of proteolytic enzyme by Bacillus subtilis IC-5 started as pH of medium falls to 9 and reached to maximum at pH 7 i.e., 4400 Uml⁻¹. Likewise dissolved oxygen decreased in the medium as the protease production progresses. Saw dust was successively utilized for partial purification of protease. The partial purification of protease increased the specific activity to 5.3 fold. The optimum pH and temperature for purified activity was 11 and 70°C, respectively. The purified enzyme was stable up to pH 12 and 80°C.

Key words: Protease, production, bioreactor, purification, tea leaves, pH, temperature.

I. INTRODUCTION

Proteases represent the class of enzymes, which occupy a key position in respect to their physiological roles as well as their commercial applications [1]. The current estimated value of the worldwide sales of industrial enzymes is US\$1 billion; among them 75% are hydrolytic [2,3]. The bacterial proteases have found wide scale industrial application because of their extensive use in many industrial applications such as food, detergent, pharmaceutical and leather [4-6] and as high as two-third of share in detergent industry alone [7]. Proteases can be produced from microbes, bacteria, fungi and yeast by solid-substrate or submerged fermentation [4-8]. Although protease production is an inherent property of all organisms, only those microbes that produce a substantial amount of extracellular protease have been exploited commercially [9].



Microbial proteases are now becoming popular and gaining more importance than conventional chemicals because of the cheaper production cost and use of renewable resources. Bacteria of the genus *Bacillus* are active producers of extracellular alkaline proteases. Currently, the alkaline proteases derived from *Bacillus* strains [4, 8, 10] has already occupied the major portion of the market. It is well known fact that extracellular protease production by microorganisms in bioreactors is greatly influenced by media components, physical factors such as, aeration, agitation, temperature, inoculum density, dissolved oxygen, pH and incubation time [10,11]. The aim of present work was to study how the pH, dissolved oxygen controlled the alkaline protease production in a lab scale bioreactor. Partial purification and characterization of the produced enzyme also carried out.

II. MATERIALS AND METHODS

Microorganism

The bacterial strain *Bacillus subtilis* IC-5 of Gul *et al.*[12] was used in the present studies. The culture was maintained on nutrient agar slants.

Media

The optimized media of Gul *et al.* [13] was used in the present studies. The composition of the medium was (g/l) soybean meal 18.0, glucose 21.1, sodium nitrate 2.9, KH_2PO_4 1.0 and Na_2CO_3 10.0 (pH 10.0).

Inoculum Preparation

The same inoculum preparation procedure was adopted as by Gul *et al.* [12].

Production of Protease

Bacillus subtilis IC-5 was grown in optimized nutritional medium [13] in a bioreactor, BioG-Micom, Biotron Inc. Korea, working volume 5 liters, used for protease production. The bioreactor was equipped with pH, foam, temperature, stirring rate and dissolved oxygen controllers and having exhaust condenser. The vessel of bioreactor was fitted with turbine blades for stirring. Butanol was used as antifoaming agent. The fermentation temperature was maintained at $37 \pm 1^\circ\text{C}$, throughout the studies. The variation in pH, dissolved oxygen during the production, were recorded.

Isolation of crude alkaline protease

The culture containing proteases obtained by cultivation of *Bacillus subtilis* IC-5 in Bioreactor was centrifuged at 10,000 rpm for 20 minutes at 4°C to obtain crude alkaline protease. The crude alkaline protease was preserved at 4°C for further studies [14].

Partial purification of alkaline protease

The partial purification of supernatant crude alkaline protease was carried out as follows: The pH of the crude protease was adjusted to 7.50 by using 0.1 N NaOH/HCl. The chilled crude enzyme was precipitated by adding ammonium sulphate (up to 80 percent saturation) as per standard chart [15]. The precipitates formed were collected by centrifugation at 10,000 rpm at 4°C for 15 minutes, dissolved in minimum amount of 0.01 M calcium acetate. The isolated enzyme was partially purified by using saw dust as an adsorbent of enzyme protein. The sawdust was boiled in distilled water until free from coloring material and then packed in the column (2.60 cm dia). The precipitated enzyme was dialyzed against 0.001 M calcium acetate and passed through saw dust column which was previously washed with distilled water, 1 M ammonium sulphate and distilled water. After passing the enzyme solution, the column was washed with distilled water to remove non adsorbed substances. The bound enzyme was eluted with 1 M NaCl solution and analyzed.

Characterization of purified alkaline protease

The effect of pH was investigated in the range of 7-13 by using buffers, 0.02 M Tris-HCl (pH 7-9), 0.02 M Carbonate-Bi-carbonate (pH 9-10), 0.02 M Na_2HPO_4 -NaOH (pH 10-11), 0.02 M KCl-NaOH (pH 12-13) buffers. The pH stability of enzyme was investigated by incubating enzyme in different pH buffers for 30 minutes and the residual activity was determined after incubation period. The residual activity is the percentage of enzyme activity as compared to control (untreated sample). The effects of temperature on the activity of enzyme was studied by carrying out enzyme reaction at 30,40,50, 60,70 & 80°C . Temperature stability of enzyme was determined by incubating enzyme at 30,40,50, 60,70 & 80°C temperatures for 30 minutes before assaying the residual activity. The percent residual activity was calculated, keeping the activity of enzyme 100% at particular temperature without treatment.

Analytical Techniques - Enzyme Assay

The activity of proteolytic enzymes was assayed by the method of Horikoshi [16]. One unit of proteolytic activity was defined as the amount of enzyme that produced TCA soluble material equivalent to 1.0 μg of tyrosine from casein per minute at pH 11.5 for alkaline protease at 37°C .

Protein estimation

The protein in the culture filtrate and purified enzymes was estimated by using Lowry method[17].



III. RESULTS AND DISCUSSION

Production of alkaline protease in bioreactor

The pH of the culture medium strongly affects many enzymatic processes and transport of compounds across the cell membrane [18] during fermentation. Effect of initial pH on production of alkaline protease production and solubility of oxygen in the medium were investigated. First batch was performed under uncontrolled pH. The production of alkaline protease, variation in pH and solubility of oxygen in the medium were monitored. The average rate of decrease in pH was 0.05 per hr in initial 10 hrs of fermentation. Later on it become 0.14 per hr till 24th hr. The dissolved oxygen of the medium started to slow down as the fermentation progresses and was maximum between 16th to 18th hrs i.e., 10.42% (Fig. 1). The synthesis of alkaline protease and protein was started from 6th hr and reached to maximum in 18 hrs i.e., 4400 Uml⁻¹. The production of protein and alkaline protease was significant (p=0.01) between 10 to 18 hours. Maximum production/specific activity of alkaline protease recorded was 4400 Uml⁻¹/ 866.86 U mg⁻¹ proteins at 18th hr of fermentation. The pH and dissolved oxygen stabilized as the enzyme and protease production reached at peak. The pH and dissolved oxygen at the end of the fermentation period was 7.50 and 13 percent, respectively. The second batch was performed under controlled pH (10.0) of the medium throughout the fermentation period. The dissolved oxygen decreased as the fermentation progresses. The synthesis of proteins and enzyme was initiated with the decrease in dissolved oxygen of the medium. The dissolved oxygen of the medium was 43% at the end of batch i.e., at 24th hr. It is as high as 70% at the same time in uncontrolled pH fermentation. The maximum alkaline protease production achieved was 465 Uml⁻¹ with specific activity 130.99 Umg⁻¹ (Fig. 2). The production of protease remained far low under controlled than uncontrolled pH of the medium during fermentation i.e., only 11.3%. This revealed that the pH for growth and protease production was different for the bacterium used in the present studies i.e., *Bacillus subtilis* IC-5. The parameter pH and DO concentration of the medium could be utilized for controlling the fermentation process.

Continuous production of alkaline protease

These studies were carried out under uncontrolled pH of the medium for three succeeding fermentation. 50 percent of fermented broth was harvested after one hour of maximum production of alkaline protease and replaced by fresh sterilized medium. For monitoring and analysis, the broth was harvested after regular interval of 2 hours since the start of batch. The results of the complete fermentation are presented in Fig. 3. The pattern of production of protease, changes in pH and DO was more or less same in all three fermentation. The pH in 1st, 2nd & 3rd fermentation at final harvest was 7.6, 7.72 & 7.75, respectively. Data showed that the time for significant decrease in pH was shortened. In 1st, 2nd & 3rd fermentation. Maximum decrease in pH of the medium was significant (p=0.01) from 11th, 4th & 3rd hours, respectively, from the start of the fermentation to the harvest time. The dissolved oxygen started decreasing from the beginning of the all three succeeding fermentations and remained significant till the conclusion time of the fermentations. The dissolved oxygen at 1st, 2nd & 3rd harvest was 13.53, 13.21 & 14.02%. LSD analysis showed that the dissolved oxygen at the time of all the three harvests was significantly the same. The increase in protein synthesis was significant at p=0.01/0.10 at the end of all three fermentations. Maximum production of protein recorded in 1st, 2nd & 3rd fermentation was 5.41, 5.19 & 5.23 mg mL⁻¹ respectively. The production/specific activity of alkaline protease at 1st, 2nd & 3rd harvest was 4215/801.33, 3832/672.28 & 3515/599.83, respectively. These figures show that the production of alkaline protease decreased, significantly at 2nd & 3rd fermentation, whereas a significant difference in specific activity was observed in between 1st & 2nd and 2nd & 3rd harvest. The time for reaching maximum production of 2nd & 3rd fermentation was shortened may be because of enough population of applied bacterium for initiation synthesis of extracellular protease.

Protease purification

The cell free culture supernatant of *Bacillus subtilis* IC-5 having initial protease activity of 4535.0 Uml⁻¹ was used as a source of crude enzyme. Alkaline protease was concentrated by ammonium sulfate precipitation (80% (w/v) saturation), followed by dialysis and saw dust column filtration. As shown in table 1 ammonium sulfate precipitation resulted in increase in specific activity from 867.11 to 2556.12 Umg⁻¹ with a purification of 2.94 fold. Nassar *et al.* [14] reported an increase in specific activity from 1504 to 5143 Umg⁻¹ i.e., 2.6 purification fold by using 70% w/v ammonium sulphate saturation. The protease was subjected to dialysis against 0.001 M calcium acetate raised the specific activity to 3469.09 Umg⁻¹ protein and purification of 4 fold w.r.t. cell free filtrate. Finally the dialyzed enzyme was passed through washed saw dust column which further raise the specific activity to 4596.8 Umg⁻¹ protein with a 5.30 fold purification w.r.t. crude enzyme.

Characterization of partially purified enzyme Effect of pH on Activity and stability of protease activity

The pH activity of partially purified protease was studied by using different pH buffers. The pH had significant influence on activity of protease (Fig. 3a). The enzyme was active in pH range of 7 to 12 with an optimum pH of 10 where the activity was 4425 Uml⁻¹. The activity of purified enzyme was appreciably deteriorated above pH 11. Proteases that have pH optima in the range of 8.0–11.0 are grouped under the category of alkaline proteases [19–21]. There was no significant difference in activity at pH 7 and 8. Minimal activity of enzyme was observed at pH 13. These results show that the partially purified enzyme of *Bacillus subtilis* IC-5 to be categorized as true alkaline protease. Ibrahim et al. [22] also reported an alkaline protease which had maximum activity at pH 10.0. Different researcher had also reported optimum activity of proteases at different pH values such as pH 7.5 [23], 8 [24], 9.8–10.2 [25], 10.5 [23] and 12–13 [26]. The pH stability was determined by diluting enzyme with 0.02 M buffers of various pH and incubating at 37°C for 2 hours before assay. The enzyme retained more than 80% activity between pH 7–9 however, 100 % retention in activity was observed at pH 10 to 11. As the pH increased from 12 there was a sharp loss in activity was observed. The minimum relative protease activity was observed at pH 13.0 i.e., 67.31 % (Fig. 3b). In similar study Jelloluli *et al.* [27] revealed that optimum pH for protease in their studies was 10 and it was stable at alkaline pH ranges.

Enzyme reaction and thermal stability at different temperatures

The results depicted in Fig. 3c show that partially purified protease was active from 30 to 80°C and maximum activity was observed at 70°C. There was sharp decrease in activity above 80°C. As shown in Fig. 3c the optimum activity of the purified enzyme was 70°C, qualifying the rank of thermo-active proteases. This observation was comparable to the report of Nascimento *et al.* [28] that they worked on a thermophilic *Bacillus* sp. which produced a protease with maximum activity at 60°C. Maximum protease activity at high temperatures is an appropriate characteristic for industrial acceptability. Various workers had recorded different optimum temperatures for purified proteases, 30°C [29], 50°C [30] and 70°C [31]. The stability of the purified enzyme was experimented in the range of 30 to 90°C. The enzyme was given shock for 30 minutes at 30, 40, 50, 60, 70, 80, 90°C. The results showed that the purified protease was stable up to 80°C (93.69% relative activity) whereas above 80°C the stability decreased to 31.65 at 90°C (Fig. 3d). Yu et al. [32] also reported that enzyme from *B. licheni form is* MH31 was stable (75%) up to 60°C after 1 h of incubation, whereas Deng et al. [33] found that enzyme retained about 40% of its initial activity at 60°C after 1h of incubation in case of *Bacillus* sp. B001. The most commercially available subtilis in-type protease is also active at temperature ranging from 50 to 60°C [34, 35].

IV. CONCLUSIONS

From the present investigation it can be safely concluded that:

- The growth of alkalophilic bacterium and production of protease were pH and dissolved oxygen specific.
- Saw dust could be successively utilized for purification of protease.
- The isolated and purified enzyme was an alkaline and thermo stable protease.
- Enzymatic properties of the purified enzyme indicate possibility of its potential use for various industrial applications.

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Table 1 Purification of protease produced by *Bacillus subtilis* IC-5

Purification step	Protease activity, (Uml ⁻¹)	Protein contents (mg/ml)	Specific Activity (Units/mg protein)	Purification folds
Cell free filtrate	4535.00	5.23	867.11	1
Ammonium sulphate 80% fraction	7515.00	2.94	2556.12	2.94
Dialyzed against 0.001M calcium acetate	9540.00	2.75	3469.09	4.00
Passed through saw dust column	13065.00	2.50	4596.8	5.30

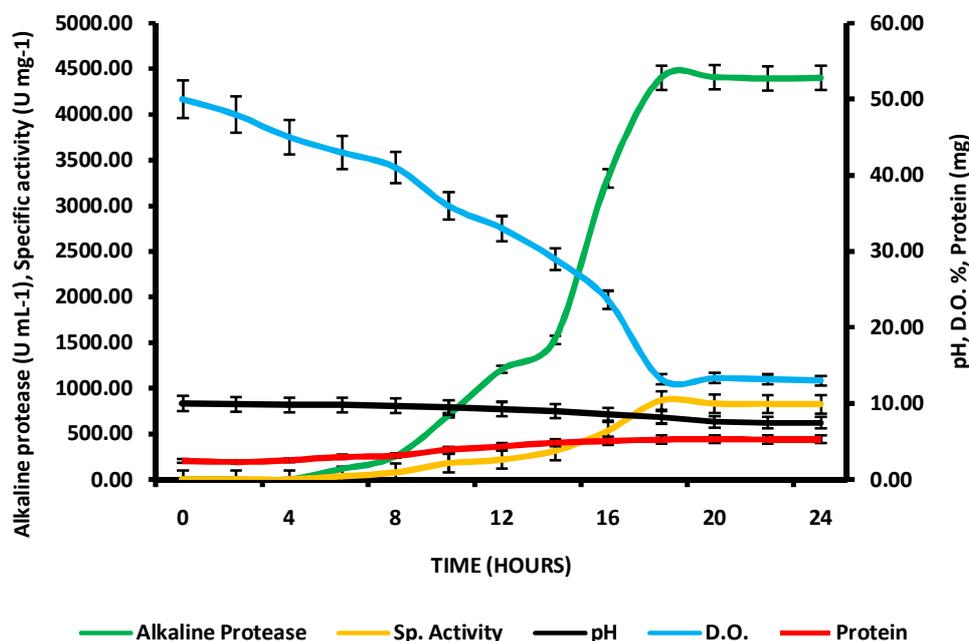


Fig. 1 Production of protease in uncontrolled medium pH by *Bacillus subtilis* IC-5 in bioreactor

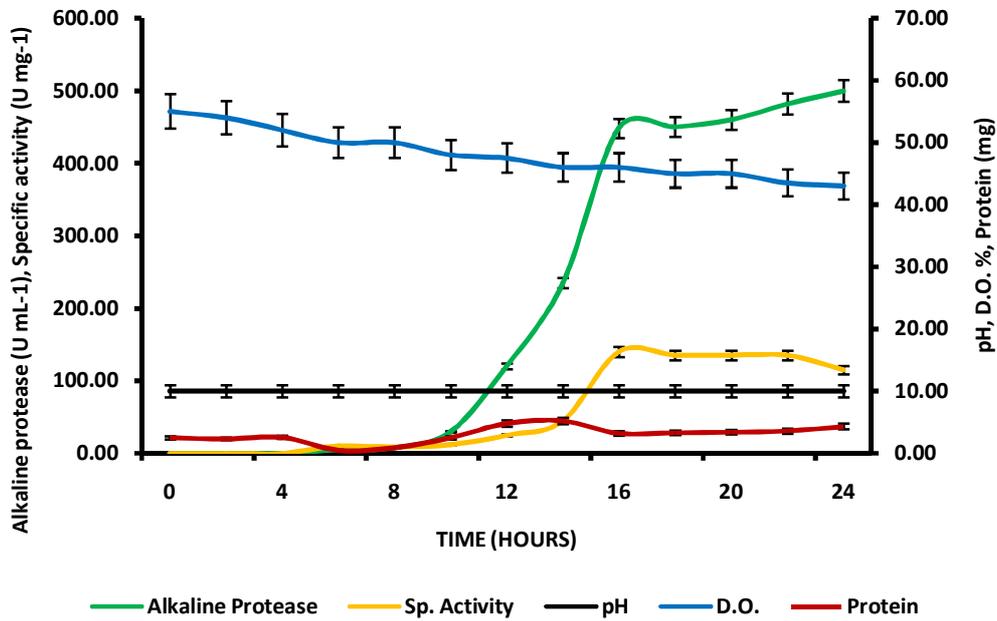


Fig. 2 Production of protease in controlled medium pH by *Bacillus subtilis* IC-5 in bioreactor

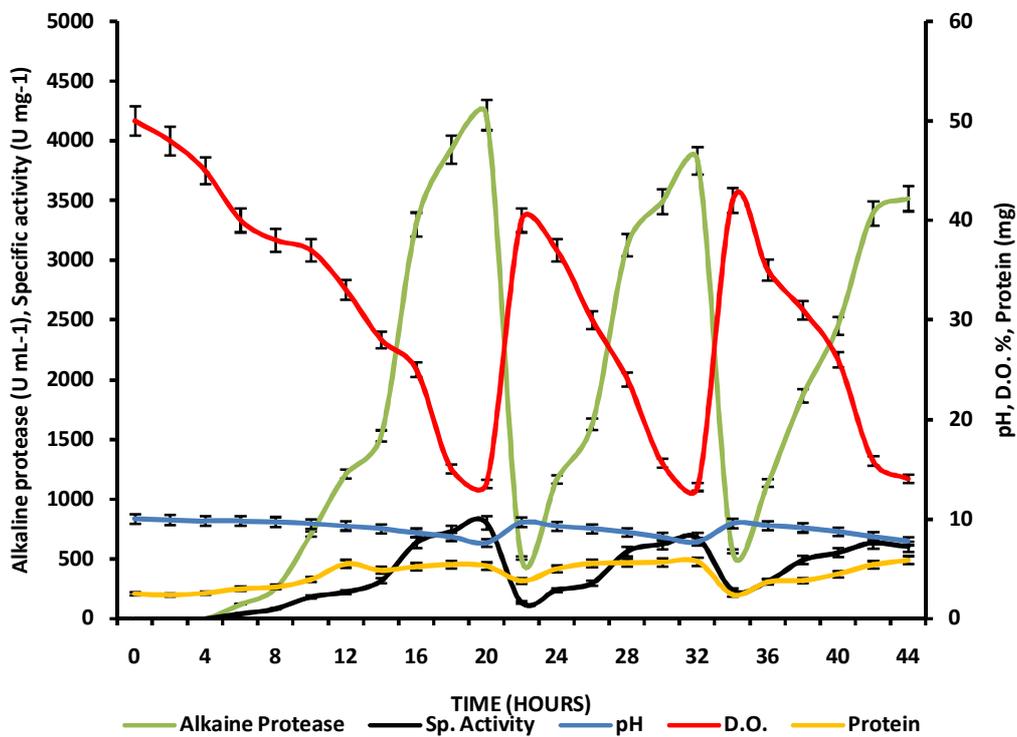


Fig. 3 Continuous production of protease in uncontrolled medium pH by *Bacillus subtilis* IC-5 in bioreactor

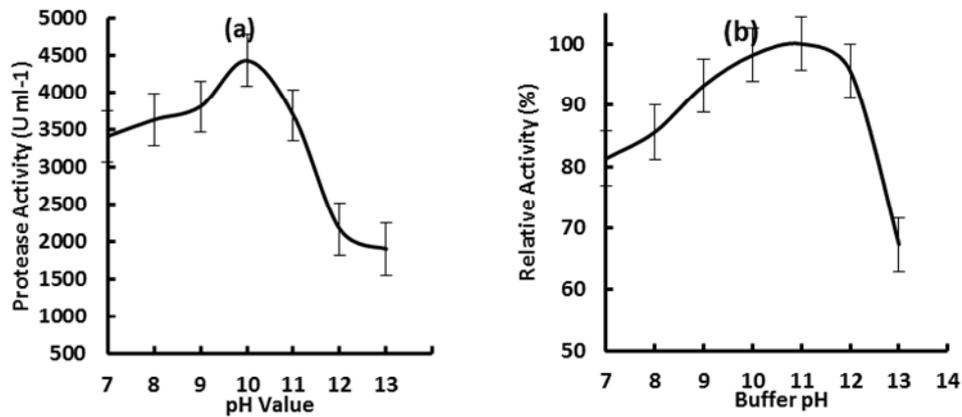


Fig. 4 Effect of pH on (a) activity and (b) stability of partially purified protease produced by *Bacillus subtilis* IC-5

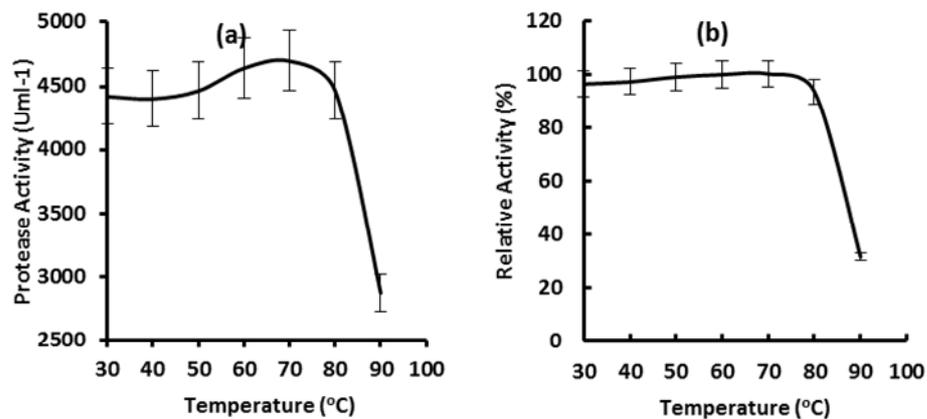


Fig. 5 Effect of temperature on (a) activity and (b) stability of partially purified protease produced by *Bacillus subtilis* IC-5