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Full Length Research Paper

Production of alkaline protease from a haloalkaliphilic soil thermoactinomycete and its application in feather fibril disintegration

Amit Verma[1]*, Hukum Singh[2], Mohammad Shahbaz Anwar[3], Mohammad W. Ansari[4] and Sanjeev Agrawal[1]

[1]Department of Biochemistry, GBPUA&T, Pantnagar, Uttarakhand 263145, India.
[2]Department of Plant Physiology, GBPUA&T, Pantnagar, Uttarakhand 263145, India.
[3]Department of Microbiology, GBPUA&T, Pantnagar, Uttarakhand 263145, India.
[4]Plant Molecular Biology Group, International Centre for Genetic Engineering and Biotechnology, Aruna Asaf Ali Marg, New Delhi 110 067, India.

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Proteases have found a wide application in several industrial processes, such as laundry detergents, protein recovery or solubilization, prion degradation, meat tenderizations, and in bating of hides and skins in leather industries. In the present study, a bacterial isolate, *Thermoactinomyces* sp. RS1, isolated from soil sample was taken for enzyme production by submerged fermentation technology. The classical "one-variable-at-a-time approach" was employed. Optimum incubation time, pH and temperature were found to be 24 h; pH 9.0 and 55°C, respectively. The enzyme production was highest at salt concentration of 5%; inoculum of 4% and agitation rate of 150 rpm. RS1 could grow in the presence of all carbon sources employed and was highest with glucose. In the case of organic nitrogen sources, enzyme production was highest with peptone and in the case of inorganic nitrogen sources, enzyme production was highest in urea. Overall, 1.5 folds of production was achieved after optimization of all conditions of previously used culture media. Protease in the present study shows good feather degradation within short incubation time, presenting its utilization for poultry feed production. The study holds significance as only few reports are available on the alkaline proteases having keratinolytic property from haloalkaliphilic bacteria.

Key words: Protease, feather disintegration, haloalkaliphilic isolate, thermoactinomyces, submerged fermentation.

INTRODUCTION

Alkaline proteases are the key industrial enzymes utilized in myriad of industrial works viz. laundry detergents, protein recovery or solubilization, prion degradation, meat tenderizations, and in bating of hides and skins in leather

*Corresponding author. E-mail: amibiochem@rediffmail.com. Tel: 09415468516.

industries. Extensive studies had been done on production and downstream processing to obtain purified enzyme both at laboratory and industrial scale. Various types of alkaline proteases have been characterized and their potential industrial applications have been explored (Gupta et al., 2002). Alkaline proteases are reported from various groups of bacteria but Actinomyces are not paid much attention. These groups of prokaryotes have ability to produce many bioactive compounds (Mehta et al., 2006; Gupta et al., 2002) but the production of commercial enzymes from them was totally unsighted.

Proteases of extracellular nature, having different other industrial potential are wide spread among the halo-alkaliphiles. In order to obtain commercially viable yields, it is essential to optimize fermentation media for the growth and protease production. Moreover, most of the studies on haloalkaliphilic bacteria in the past have largely focused on hyper saline environments and the exploration of relatively moderate saline and alkaline environments is only the beginning (Joshi et al., 2008)

Production of proteases was effected by various environmental factors and also the strategy of fermentation, that is, submerged or solid state. Usually, production of these enzymes was reported by submerged process due to its apparent advantages in consistent enzyme production characteristics with defined medium and process conditions and advantages in downstream inspite of the cost-intensiveness for medium components.

In the present study, *Thermoactinomyces* sp. RS1, isolated from soil sample was taken for enzyme production by submerged fermentation technology and crude protease from studied isolate shows good feather fibril disintegration within short incubation time, presenting its utilization for poultry feed production.

MATERIALS AND METHODS

Microorganism and culture conditions

Alkaline protease producing thermoalkaliphilic *Thermoactinomyces* sp. RS1 used in this investigation was isolated from soil sample of Rajasthan, India and maintained on a nutrient agar medium pH 9.0, stored at 4°C and sub-cultured at monthly intervals (Verma et al., 2011).

Culture media

Protease screening medium contained (g/L) peptone 1.0, NaCl 5.0, skimmed milk 20.0, and Agar 20.0 at pH 8.0.

Protease production medium contained (g/L) peptone 7.5, glucose 5.0, salt solution 50 ml containing $MgSO_4$ 0.5%, KH_2PO_4 0.5% and $FeSO_4$ 0.01%, at pH 9.0.

Submerged fermentation

The classical “one-variable-at-a-time approach” was employed to evaluate the effect of growth conditions, including incubation time, temperature and initial pH of the medium, on cell growth and protease production. Experiments were conducted in triplicate and statistical analysis was performed.

Effect of incubation time on the production of protease

The effect of incubation time on protease production was determined by incubating the culture medium at different time intervals (24 TO 96 h) with an interval of 24 h.

Effect of initial pH on the production of protease

The effect of pH of culture media was examined by maintaining the pH 6.0 to 12.0 with increase of 1 and then inoculating the media and by incubating it at 60°C for 24 h.

Effect of temperature on the production of protease

The effect of incubation temperature on the growth and enzyme production was examined by incubating the flasks containing culture for 24 h at different temperatures ranging from 40 to 70°C with increase of 5°C.

Effect of salt concentration on the production of protease

The effect of salt concentration on the growth and enzyme production was examined by supplementing the culture media with NaCl 0 to 20 % with increase of 5%.

Effect of inoculation on the production of protease

The effect of inoculation on the growth and enzyme production was examined by varying inoculation volumes 2 to 10% (v/v) with increase of 2% inoculated into culture media.

Effect of carbon sources on the production of protease

Different carbon sources (glucose (as control), fructose, maltose, sucrose, starch and lactose) were supplemented at 1% (w/v) to culture media to investigate their effect on the growth and alkaline protease production. Samples were withdrawn after 48 h for the measurement of growth and protease estimation.

Effect of organic nitrogen source on protease production

Different nitrogen source (peptone (as control), yeast extract, tryptose, tryptone, malt extract and beef extract) were taken to study their effect on protease production. These nitrogen source were added into production media at same concentration, enzyme were harvested and assayed for activity.

Effect of inorganic nitrogen source on protease production

Different inorganic nitrogen sources, that is, $(NH_4)_2SO_4$, NH_4Cl, NH_4NO_3, urea and KNO_3 were taken to study their effect on protease production. These nitrogen source were added into production media at same concentration, enzyme were harvested and assayed for activity.

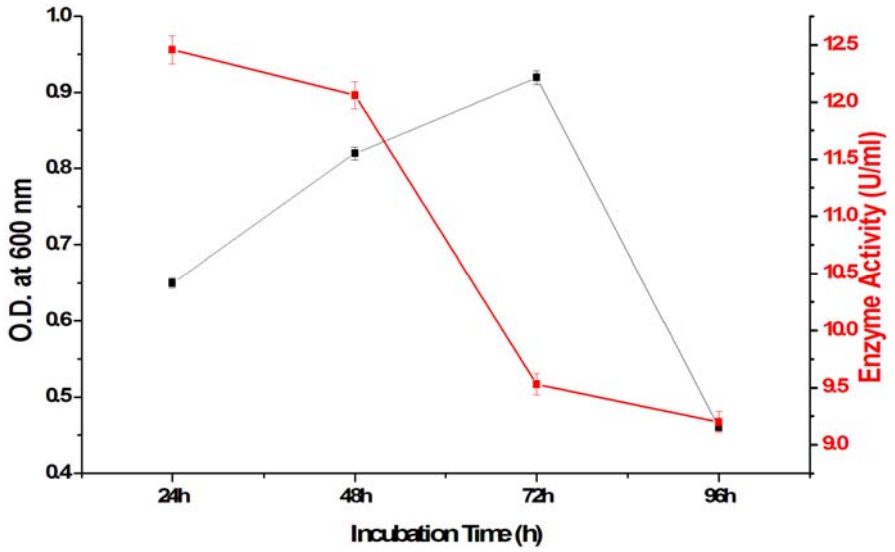

Figure 1. Effect of incubation time on growth and alkaline protease production.

Effect of agitation rate on the production of protease

The effect of agitation rate on the growth and enzyme production was examined by incubating the flasks containing culture for 24 h at different shaking speeds ranging from 0 to 250 rpm with increment of 50 rpm.

Protease assay

Proteolytic activity was assayed by modified folin method; using 0.5% casein as substrate dissolved in 50 mM Glycine NaOH buffer pH 10.0. The reaction mixture was incubated at 80°C for 30 min, and reaction was stopped using 10% TCA. Tyrosine released was estimated using Folin Ciocalteau's Reagent and absorbance taken at 670 nm. One unit of protease (1 PU) was defined as amount of enzyme required to release 1 µg of tyrosine under the assay condition when reaction was incubated for 1 min.

Protein determination

The protein content in crude enzyme was determined by Lowry method (Verma et al., 2011).

Feather disintegration studies

Feather fibril detachment and hydrolysis was studied using 70% ethanol washed chicken feathers (Bockle et. al., 1995) using crude enzyme treatment along with additive βME in different combinations (feathers + buffer (control); feathers + buffer + enzyme; feathers + buffer + filtered enzyme; feathers + buffer + filtered enzyme + βME (0.1%)). Patterns of detachment were recorded by periodic observation of the treated feathers using compound light microscope at 10x magnification.

Statistical analysis

For statistical analysis, a standard deviation for each experimental result was calculated using the Excel Spreadsheets available in the Microsoft Excel. Statistical validation was performed with STPR software and graphs were prepared using ORIGIN software Version 6.

RESULTS AND DISCUSSION

Submerged fermentation

Effect of incubation time on the production of protease

The effect of incubation time on protease production was determined by incubating the culture medium at different time intervals (24 to 96 h) with an interval of 24 h. The optimum incubation time was found to be at 24 h after which there was fall in enzyme production (Figure 1). The possible reason could be denaturation and degradation by other proteases secreted along with alkaline protease, autolysis or repression of the enzyme synthesis. Earlier, similar features have been reported by Joshi et al. (2008).

Effect of initial pH on the production of protease

The effect of pH of culture media was examined by maintaining the pH 6.0 to pH 12.0 with increment of 1 and then inoculating the media and by incubating it at 60°C for 24 h. The enzyme production was highest at pH 9.0 with enzyme activity of 16.22 ± 0.02 U/ml (Figure 2). Requirement of alkaline pH for optimum growth and protease production, clearly suggested the alkaliphilic nature of the organism and enzyme.

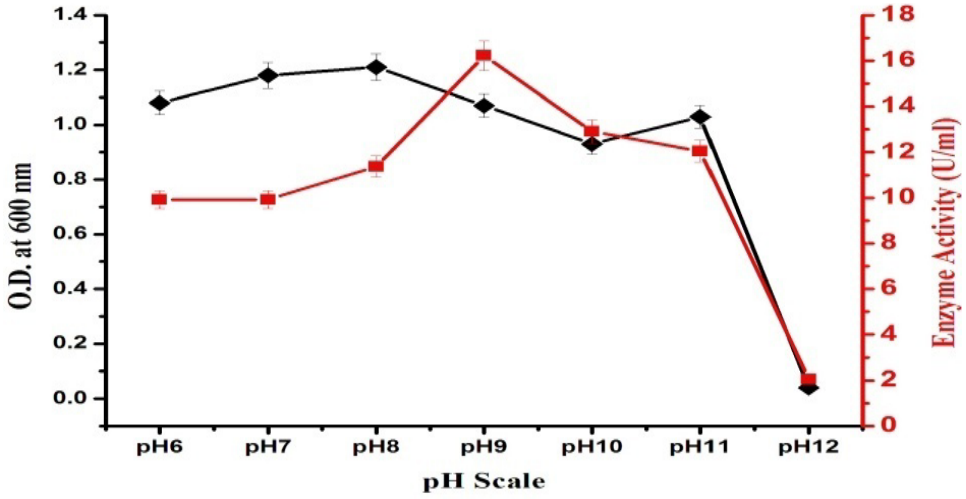

Figure 2. Effect of pH on growth and alkaline protease production.

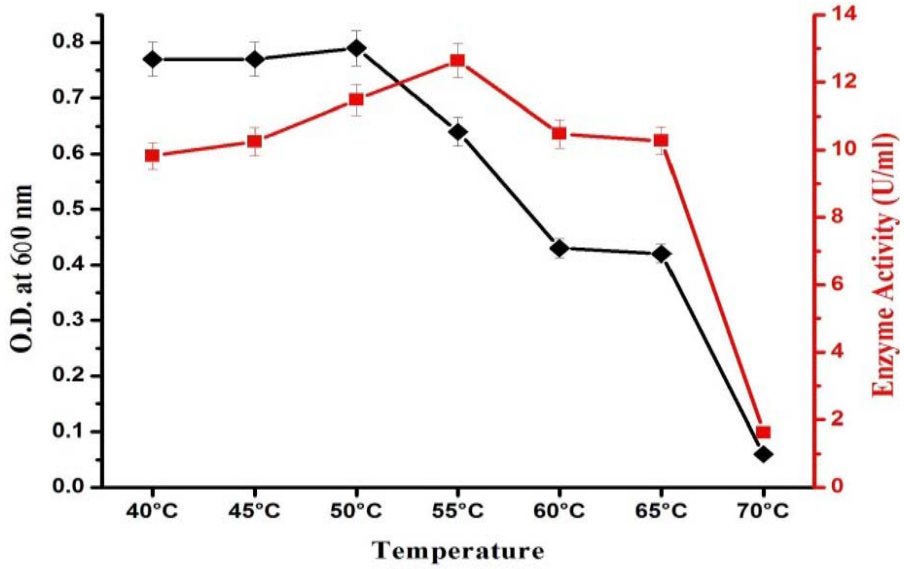

Figure 3. Effect of temperature on growth and alkaline protease production.

Effect of temperature on the production of protease

The effect of incubation temperature on the growth and enzyme production was examined by incubating the flasks containing culture for 24 h at different temperatures ranging from 40 to 70°C with increase of 5°C. The enzyme production was highest at 55°C with enzyme activity of 12.63 ± 0.02 U/ml (Figure 3). Temperature is a critical parameter that has to be controlled and varied from organism to organism. The mechanism of temperature control of enzyme production is not well understood (Chaloupka, 1985). However, studies by Frankena et al. (1986) showed that a link existed between enzyme synthesis and energy metabolism in *Bacilli*, which was controlled by temperature.

Effect of salt concentration on the production of protease

The effect of salt concentration on the growth and enzyme production was examined by supplementing the culture media with NaCl 0 to 20 % with increase of 5%. The enzyme production was highest at salt concentration

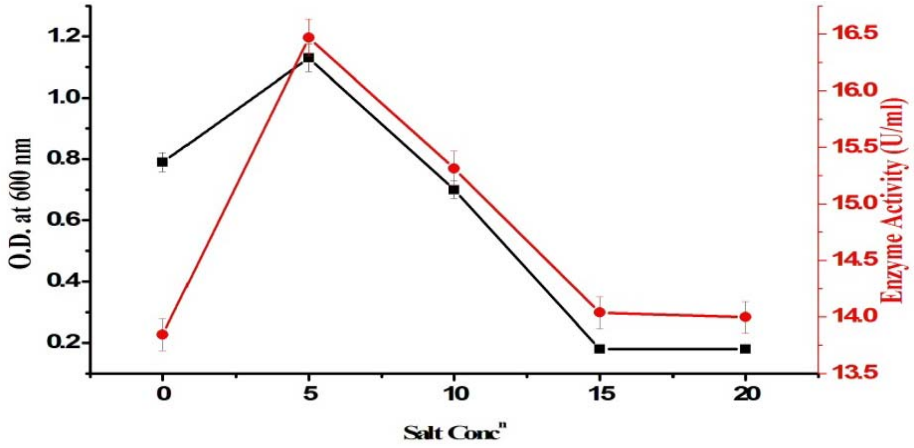

Figure 4. Effect of salt concentration on growth and alkaline protease production.

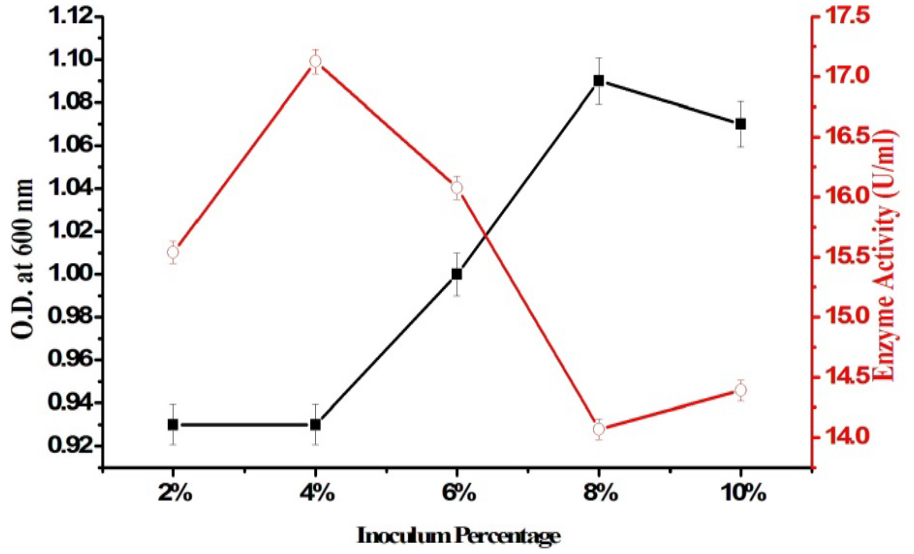

Figure 5. Effect of inoculum percentage on growth and alkaline protease production.

of 5% with enzyme activity of 16.47 ± 0.03 U/ml (Figure 4). The NaCl required for optimum growth and protease production was fairly lower as compared to other halo-alkaliphilic protease producers isolated from other saline habitats (Kostrikina et al., 1991). The optimum protease production at 15~16% NaCl for some halophilic bacteria has been reported which indicates that our protease producer was mild halophilic in nature (Horikoshi, 1971; Sen and Satyanarayana, 1993).

Effect of inoculation on the production of protease

The effect of inoculation on the growth and enzyme production was examined by varying inoculation volumes 2 to 10% (v/v) with increase of 2% was inoculated into culture media. The enzyme production was highest at inoculum percentage of 4% with enzyme activity of 17.12 ± 0.58 U/ml (Figure 5). The growth of bacterium increases with inoculum percentage; however, it had pronounced effect on protease production. The repression of protease at higher inoculation volume (>4%, v/v) was quite comparable with some of the earlier reports, where protease production was enhanced at lower inoculation volume (Mao et al., 1992).

Effect *of carbon sources on the production of protease*

Different carbon sources (glucose, fructose, maltose, sucrose, starch and lactose) were supplemented at 1%

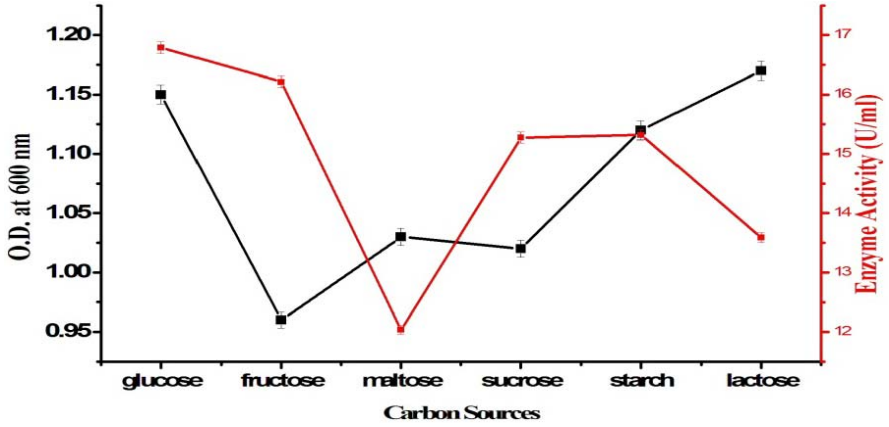

Figure 6. Effect of different carbon sources on growth and alkaline protease production.

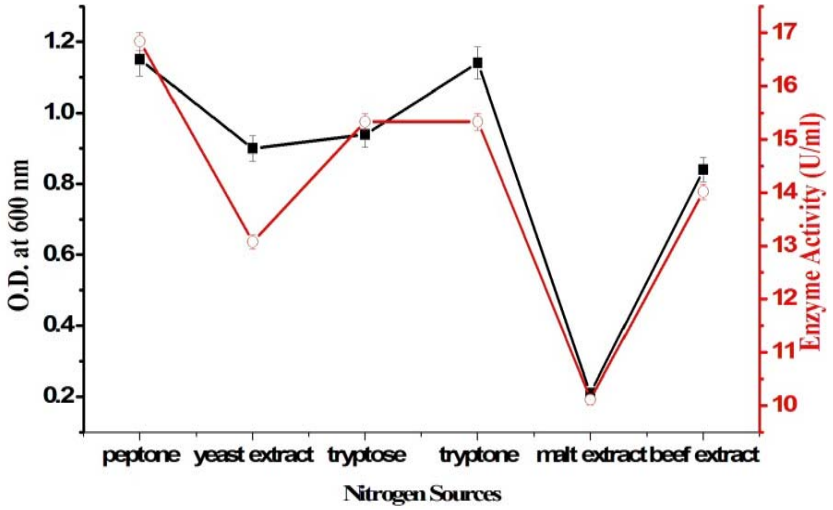

Figure 7. Effect of different organic nitrogen sources on growth and alkaline protease production.

(w/v) to culture media to investigate their effect on the growth and alkaline protease production. The enzyme production was highest with glucose (16.79 ± 0.01 U/ml) followed by fructose (16.21 ± 0.19 U/ml); starch (15.32 ± 0.57 U/ml); sucrose (15.27 ± 0.01 U/ml); lactose (13.58 ± 0.56 U/ml); maltose (12.03 ± 5.74 U/ml) (Figure 6). RS1 could grow in the presence of all carbon sources employed; the protease production was not suppressed by any of the carbon sources. But in some cases e.g. production of extracellular protease CP1 from moderately halophilic *Pseudoalteromonas sp.* strain CP76 was significantly inhibited in the presence of maltose, glucose, and lactose (Sanchez-Porro et al., 2003). Earlier, a catabolic repression mechanism for extracellular protease production has been also reported (Matsubara and Feder, 1971; Fujiwara and Yamamoto, 1987).

Effect of organic nitrogen source on protease production

Different nitrogen source (peptone, yeast extract, tryptose, tryptone, malt extract and beef extract) were taken to study their effect on protease production. These nitrogen source were added into production media at same concentration, enzyme were harvested and assayed for activity. The enzyme production was highest with peptone (16.84 ± 0.01 U/ml) followed by tryptose (15.33 ± 0.02 U/ml); tryptone (15.33 ± 0.01 U/ml); beef extract (14.01 ± 0.01 U/ml); yeast extract (13.07 ± 0.02 U/ml); malt extract (10.11 ± 0.10 U/ml) (Figure 7). Complex nitrogen sources are usually used for alkaline protease production and the requirement for a specific nitrogen supplement differs from organism to organism.

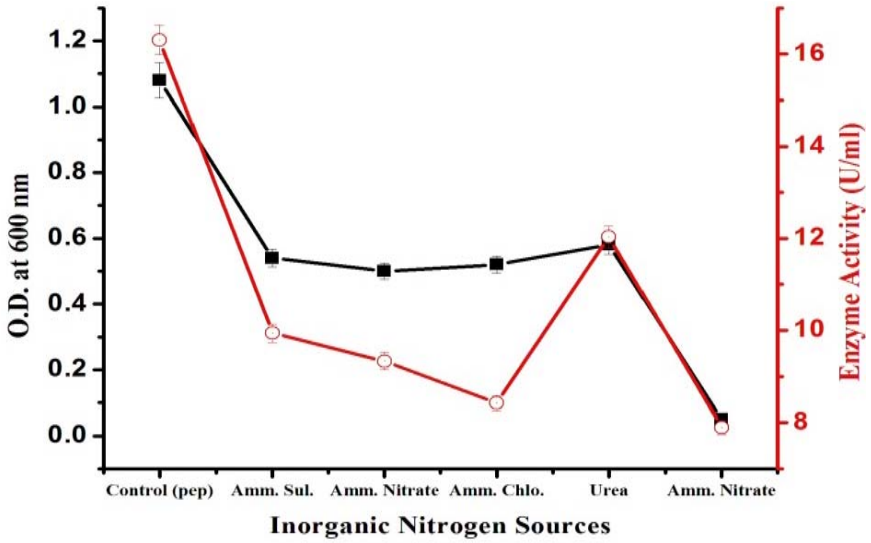

Figure 8. Effect of different inorganic nitrogen source on growth and alkaline protease production.

Peptone is one of common nitrogen source for protease production but in some cases tryptone (2%) and casein (1-2%) also serve as excellent nitrogen sources (Ong and Gaucher, 1976; Phadatare et al., 1993).

Effect of inorganic nitrogen source on protease production

Different inorganic nitrogen sources, that is, $(NH_4)_2SO_4$, NH_4Cl, NH_4NO_3, Urea and KNO_3 were taken to study their effect on protease production. These nitrogen source were added into production media at same concentration, enzyme were harvested and assayed for activity. The enzyme production was highest with peptone (16.30 ± 0.15 U/ml) used as control followed by urea (12.03 ± 0.54 U/ml); ammonium sulphate (9.94 ± 0.01 U/ml); ammonium nitrate (9.33 ± 0.07 U/ml); ammonium chloride (8.42 ± 0.03 U/ml); potassium nitrate (7.89 ± 0.03 U/ml) (Figure 8). Usually low levels of alkaline protease production were reported with the use of inorganic nitrogen sources in the production medium (Sen and Satyanarayana, 1993; Chaphalkar and Dey, 1994). Enzyme synthesis was found to be repressed by rapidly metabolizable nitrogen sources such as amino acids or ammonium ion concentrations in the medium (Cruegar and Cruegar, 1984), although inorganic nitrogen sources are preferred in industrial process for their low cost.

Effect of agitation rate on protease production

The effect of agitation rate on the growth and enzyme production was examined by incubating the flasks containing culture for 24 h at different shaking speeds ranging from 0 to 250 rpm with increment of 50 rpm. The enzyme production was highest at agitation rate of 150 rpm with enzyme activity of 17.77 ± 0.01 U/ml (Figure 9). The variation in the agitation speed influences the extent of mixing in the shake flasks or the bioreactor and will also affect the nutrient availability. Protease production optimizes in the range of 150 to 200 rpm e.g. optimum yields of alkaline protease are produced at 200 rpm for *Bacillus subtilis* ATCC 14416 (Chu, 1992) and *Bacillus licheniformis* (Sen and Satyanarayana, 1993). In one study, *Bacillus* sp. B21-2 produced increased enzyme production when agitated at 600 rpm (Fujiwara and Yamamoto, 1987).

Feather disintegration studies

Gradual changes in the feather upon enzymatic treatment with 0.1% β-ME could be visibly observed, and clean shaft was obtained after 18 h. Untreated control feather shows the hollow supporting shaft (rachis) of feather and its side branches (barbs) to which are attached a set of fine barbules which could be clearly observed in microscopic images. Disintegration of barbules apparently started after 2 h of enzymatic treatment along with βME and after 12 h, barbs started disintegrating and within 18 h, degradation of the barbs, leaving behind the thick basal portion of the rachis was seen (Figure 10). Efficiency of degradation in the absence of reductant was lower and took nearly 48 h to show clean shaft. The base of the shaft remained undigested even after prolonged incubation.

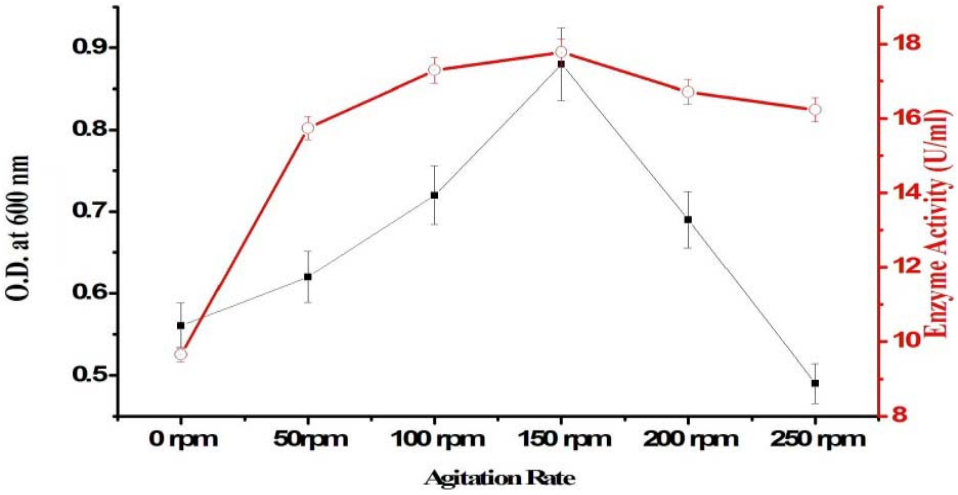

Figure 9. Effect of agitation rate on growth and alkaline protease production.

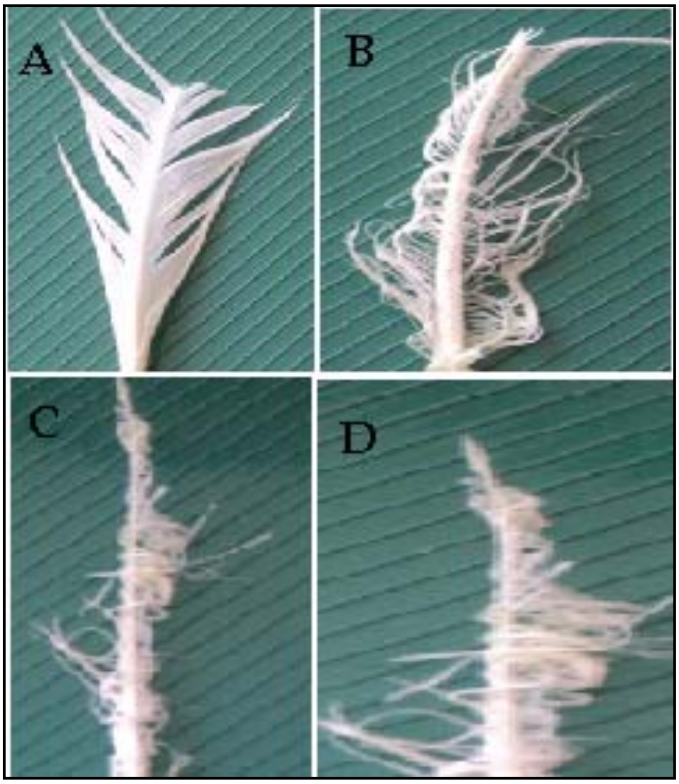

Figure 10. Feather disintegration studies: (1) Chicken feather disintegration using protease preparation from *Thermoactinomyces* sp. RS1. A) Control; B) filtered crude enzyme (20 U/ml) + buffer; C) crude enzyme (20U/ml) + buffer; D) filtered crude enzyme (20U/ml) + buffer + β ME.

Conclusions

Alkaline proteases are generally produced by submerged fermentation. In commercial practice, the optimization of medium composition is aimed to maintain a balance between the various medium components, thus minimizing

the amount of unutilized components at the end of fermentation. Research efforts have been directed mainly toward the evaluation of the effect of various carbon and nitrogenous nutrients as cost-effective substrates on the yield of enzymes; requirement of divalent metal ions in the fermentation medium; and optimization of environmental and fermentation parameters such as pH, temperature, aeration and agitation. In addition, no defined medium has been established for the best production of alkaline proteases from different microbial sources. Each organism or strain has its own special conditions for maximum enzyme production (Kumar and Takagi, 1999). So, in the present study, the classical "one-variable-at-a-time approach" was employed to evaluate the effect of growth conditions, including incubation time, temperature and initial pH of the medium, on cell growth and protease production. Overall, 1.5-fold production was achieved after optimization of all conditions of previously used culture media. Apart from the above, protease from the above culture was found to be keratinolytic in nature and solubilizes the keratin of chicken feathers which is beneficial for poultry feed production.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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