Trends in Applied Sciences Research

ISSN 1819-3579

Academic
Journals Inc.

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Trends in Applied Sciences Research 6 (10): 1206-1213, 2011
ISSN 1819-3579 / DOI: 10.3923/tasr.2011.1206.1213

Production and Characterization of Alkaline Protease from *Bacillus licheniformis* sp. Isolated from Iranian Northern Soils with Ram Horn Hydrolysate

[1]Masumeh Anvari and [2]Gholam Khayati

[1]Department of Microbiology, Faculty of Sciences, Islamic Azad University, Rasht Branch, P.O. Box 41335-3516, Rasht, Iran

[2]Department of Chemical Engineering, Technical Faculty, Guilan University, Rasht, 419961-3769, Iran

Corresponding Author: Masumeh Anvari, Department of Microbiology, Faculty of Sciences, Islamic Azad University, Rasht Branch, P.O. Box 41335-3516, Rasht, Iran Tel: +98 9112323482 Fax: +98 131 4223621

ABSTRACT

The aim of this study was the use of ram horn hydrolysate as a substrate for alkaline protease production using *Bacillus* spp. in submerged fermentation method. Sixty-three bacillus strains were isolated from Iranian alkali soils. Thirty were identified as *Bacillus licheniformis*. The enzyme solution from these *B. licheniformis* strains were investigated for their proteolytic activity. When tested on skim milk agar, the enzyme solution of *B. licheniformis* strain 5 exhibited the highest proteolytic activity (a clear zone with an average area of 480 mm^2). The results show that with the addition of RHH to the fermentation medium with a final concentration of 4% (optimal concentration), alkaline protease value reached a maximum value (93 U mL^{-1}) which is 69% higher than that of the control medium. Optimal pH and temperature activity of the alkaline proteases from *B. licheniformis* strain 5 were found to be at 9 and 50°C, respectively. The protease was unstable and rapidly decreased in activity when heated at 60°C. It was activated by Mn^{+2}, Ca^{+2}. The enzyme activity was strongly inhibited by PMSF, suggesting that it belong to the family of serine proteases.

Key words: Alkaline protease, alkali soils, *Bacillus licheniformis*, ram horn hydrolysate

INTRODUCTION

Proteolytic enzymes account for nearly 60% of the industrial enzyme market and are widely used in food industry for cheese ripening, meat tendering, the production of protein hydrolysate and bread making (Poldermans, 1990). Alkaline proteases are produced by a wide range of microorganisms including bacteria, moulds, yeast and also mammalian tissues. Currently, a large proportion of commercially available alkaline proteases are derived from *Bacillus* strains (Singh *et al*., 2004; Uyar and Baysal, 2004). *B. licheniformis* was the specie which has been used in industry for alkaline protease production (Calik *et al*., 2002). Use of waste biomaterials for biotechnologic products especially enzyme has been noticed in the recent years (Mahanta *et al*., 2008; Prakasham *et al*., 2006). Ram horns, known as fibrous protein, are widely produced in the world. Increasing concern about pollution that occurs from agricultural and industrial wastes has stimulated interest in converting waste materials into commercially valuable products. Furthermore, other fibrous proteins from, for example, feather, nail and hair are available as

waste. Ram horns consist of a-keratin which is relatively rich in cysteine (up to 22%). In addition, they contain most of the other common amino acids (Dalev, 1990). Ram Horn Hydrolysate (RHH) has been investigated only to a minor extent and its use in industrial processes is still low.

Alkaline protease production by *B. licheniformis* isolated from Iranian soils has previously reported (Eftekhar *et al.*, 2003). The objective of this study was to use RHH in a fermentation medium for the production of alkaline protease and the characteristics if the enzyme produced by using a local isolate of *B. licheniformis* and to compare alkaline protease production under laboratory conditions in batch fermentation with Control Medium (CM).

MATERIALS AND METHODS

Isolation method: Microorganisms isolated from alkali soils collected at different locations in northern Iran. Bacterial colonies were isolated and characterized by their morphological and physiological properties (Norris *et al.*, 1981; Sneath, 1986). These included cell shape, Gram staining, presence of spore and growth conditions (aerobic or anaerobic). Further classification was performed using biochemical tests as described by Norris *et al.* (1981).

Isolated bacteria were cultivated in 40 mL broth medium containing 1% glucose, 0.1% casein, 0.1% yeast extract, 0.1% K_2HPO_4, 0.05% $MgSO_4$ $7H_2O$ with shaking (150 rpm) at 34°C for 24 h. The culture was then centrifuged at 8000x g for 20 min at 4°C. The supernatant was collected and used as the enzyme solution.

Initially, protease activity was tested using skim milk agar (1% skim milk, 0.02% sodium azide and 2.0% agar). For this, 5 μL of the enzyme solution produced from isolated bacteria were spotted on skim milk agar plates. The plates were then incubated at 34°C for 10 h. Proteolytic activity of the enzyme solution was detected by observing the presence of clear zones. According to Cooper (1963) activity of biological substances (i.e., antibiotics and enzymes) can be expressed in terms of the square of the diameter of the clear zone.

After initial screening for proteolytic activity, *B. licheniformis* strain 5 exhibited highest activity of proteases. The strain was maintained on nutrient agar and used throughout the study.

Hydrolysis of ram horn: Hydrolyses were prepared by the method of Kurbanoglu (2003).

Culture conditions and fermentation process: The experimental medium contained 3% glucose, 0.1% casein, 0.1% yeast extract, 0.1% K_2HPO_4, 0.05% $MgSO_4$ $7H_2O$. In order to determine the effects of RHH on the alkaline protease production, 0 (control medium, CM), 1-6% v/v RHH (RHHM medium) were added to the production medium, respectively.

The experiments were conducted at different RHH concentrations (0-6% v/v). Batch fermentations were carried out in a 2-L fermentor with a working volume of 1 L. The airflow rate was adjusted at 0.7 v/v/m. The culture temperature and agitation rate was automatically maintained at 34°C and 200 rpm, respectively.

The biomass was determined following centrifugation at 8000x g for 20 min in 4°C, drying the cell mass at 80°C overnight and weighting the resulting dry cell biomass. The supernatant was used for the determination of the residual sugars and protease activity.

Analytical methods: The microbial growth, residual sugar and protease activity were determined. Bacterial growth was assessed measuring the optical density with a spectral photometer (Biosience, Ultrospec 2100 pro (UV/Vis) photometer) has been previously described (Anvari and Khayati, 2009).

The dinitrosallicylic acid method of Miller (1959) was employed for residual sugar assessment.

Protease activity was determined as released tyrosine from the supernatants according to a modified Lowry method (Meyers and Ahearn, 1977). One unit of enzyme activity was defined as the amount of the enzyme resulting in the release of 1 mg of tyrosine per min at 34°C under the reaction conditions.

All experiments were replicated twice and averaged values are presented in this study.

Effect of pH and temperature on the enzyme activity and stability: The pH stability of the alkaline protease was determined for 1 h at 34°C in the following buffer systems: 0.1 M sodium acetate (pH 4.0-5.5); 0.1 M sodium phosphate (pH 6.0-7.5); 0.1 M Tris-HCl (pH 8.0-9.0); 0.1 M glycine-NaOH (pH 9.5-11); and 0.1 M sodium carbonate (pH 11.5-12.0), respectively. To examine the thermal stability of the alkaline protease, the protease solution was in 50 mM Tris-HCl buffer (pH 8.0) was allowed to stand for 1 h at different temperatures in the range of 30-70°C, the reaction stopped in ice-cold water and then the residual activity was measured as described above.

Influence of various chemical reagents on protease activity: The effects of metal ions and chemical reagents on the enzyme were examine by incubating 500 μL of enzyme solutions with each of the metal ions and chemicals reagents at a concentration of 5 mM under optimum reaction conditions for 10 min. The remaining activity of the enzyme was measured under standard condition described above.

All experiments were repeated at least three times in order to acquire high accuracy. This procedure gave consistent and reproducible results.

RESULTS AND DISCUSSION

Identification of microorganisms: In this study, several samples were collected from different locations with alkali soils in northern Iran. To destroy all vegetative cells and thus selectively isolate bacteria in the genus *Bacillus*, each sample was resuspended in distilled water, incubated at 80°C for 20 min and spread on plates. Sixty three bacterial isolates were obtained and further identified using biochemical assays as suggested by Norris *et al.* (1981). Key identification of *Bacillus* species included catalase assay, Voges Proskaur (VP) test, growth under anaerobic condition and starch hydrolysis. Of all isolated bacteria, 30 (47.6%) isolates were classified as *B. licheniformis*. The *B. licheniformis* isolates were then characterized for protease production. Using skim milk agar, the proteolytic activity could be detected by the presence of a clear zone. It was found that *B. licheniformis* strain 5 yielded the highest protease activity with a clear zone of approximately 480 mm^2 (Fig. 1).

Effects of culture conditions: A series of experiments was carried out to study the effect of different initial RHH concentration (0-6%) on the alkaline protease production. These results demonstrated that up to 4% RHH addition could result in promoted *B. licheniformis* strain 5 growth and increased alkaline protease production (Table 1). As seen in Table 1, the highest biomass (6.7 g L^{-1}) and alkaline protease (87 U mL^{-1}) were obtained from 4% RHH. It was found that applications higher than 4% had an inhibitory effect. This inhibitory effect is probably due to the high concentrations of salt and resulted in high osmotic pressure in the medium that may be cause critical problems during fermentation as they inhibit the growth of *B. licheniformis* strain 5.

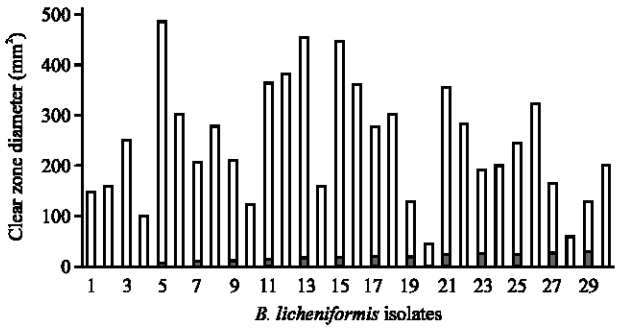

Fig. 1: Comparative results of proteolytic activity of *B.licheniformis* strains isolated. The proteolytic activity was assayed using skim milk agar and expressed as a square of the clear zone diameter

Table 1: Biomass and alkaline protease yields of *B.licheniformis* strain 5 after 20 h at different RHH concentration

Media	Biomass yield (g L^{-1})	Alkaline protease yield (U mL^{-1})
1% RHH+CM	4.73	56
2% RHH+CM	5.48	66
3% RHH+CM	6.11	78
4% RHH+CM	6.70	87
5% RHH+CM	3.17	34
6% RHH+CM	1.32	18
CM	3.48	36

Kurbanoglu and Kurbanoglu (2007) also reported the same results in xanthan gum production. These results suggested that the increase in RHH had a negative effect on the cell growth rate and the increase in alkaline protease production rate was due to the increase in the cell growth rate.

The effects of incubation time on the alkaline protease, biomass yield and residual sugar of the cultures were shown in Fig. 2. Notably, the use of RHH in the fermentation medium has a significantly effect on the alkaline protease yield and sugar consumption. The results of RHHM (4% RHH+CM) (Fig. 2a) are higher than that of CM (Fig. 2b). The highest alkaline protease yield in the RHHM was observed at 26 h. This value was 93 U mL^{-1} and the rate of sugar consumption was approximately 100%. However, the alkaline protease yield in the CM for the same incubation time was measured as 48 U mL^{-1} and the rate of sugar consumption for this medium was about 84%.The highest alkaline protease yield in the CM was observed at 30 h. This value was 55 U mL^{-1}; the rate of sugar consumption was approximately 100%. Nevertheless, this value is lower than that of RHHM. According to the 30 h incubation time, the content of alkaline protease in the RHHM is 69% higher than that of CM.

Although the CM gave the maximum enzyme production after a long time (30 h), the RHHM gave it after a short time (26 h). Therefore, the RHHM on protease production may be more economic. It resulted in an improvement in both a short time and fermentation rate.

Effect of pH and temperature on the activity and stability of the enzyme: The effect of pH on the catalytic activity was studied by using casein as a substrate under the standard assay condition. The protease exhibited maximum activity at pH 9.0. The high optimum pH is a feature

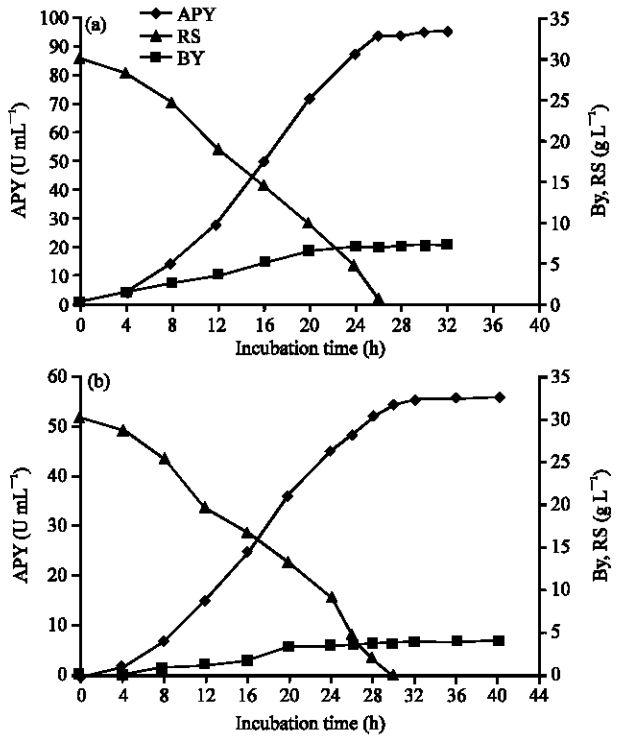

Fig. 2: Residual Sugar (RS), Biomass Yield (BY) and Alkaline Protease Yield (APY) on RHHM (a) and CM (b) at various incubation times

of alkaline proteases (Jasvir *et al*., 1999; Mehrotra *et al*., 1999). Similar results were described for several *Bacillus* spp. in literature, optimum pH range being between 7.0 and 11.0 (Prakasham *et al*., 2006).

The pH stability profile of the protease was determined by the measurement of the residual activity at pH 8.0 after incubation at various pH values at 34°C for 1 h. The protease was stable between pH 8.0 and 10.0 and retained more than 80% of its original activity (Fig. 3).

The optimum temperature for the protease activity was found to be 50°C. To examine the thermal stability of the protease, the protease solution in 50 mM Tris-HCl buffer (pH 8) was allowed to stand for 1 h at various temperatures and the residual activity was measured. The results showed that at temperatures higher than 50°C, the residual activity was decreased significantly, but at 35°C, the residual activity was maintained nearly 90%. At 45°C, the enzyme retained 60% of its activity (Fig. 4). The protease activity was completely inactivated at 60°C. In a similar work on alkaline protease activity of *B. licheniformis* isolated from Iranian soils on different pH value (7 to 12) and temperatures (30 to 55°C) the enzyme was considerably active within the pH range of 9-12 and a temperature range of 35-55°C (Eftekhar *et al*., 2003). Other reports concerning alkaline protease production by different *Bacillus* species have shown a pH range of activity within 9-12 and temperatures between 40 to 70°C (Johnvesly and Naik, 2001; Kumar, 2002). Also Fujiwara and Yamamoto, 1987) found temperature and pH optimum for *B. licheniformis* and *B.coagulans* protease was 60°C and 10, respectively. Hence, protease of these bacteria was quite suitable for detergent applications. Our results were quite reproducible and showed a similar range of activity for the alkaline protease from the strain.

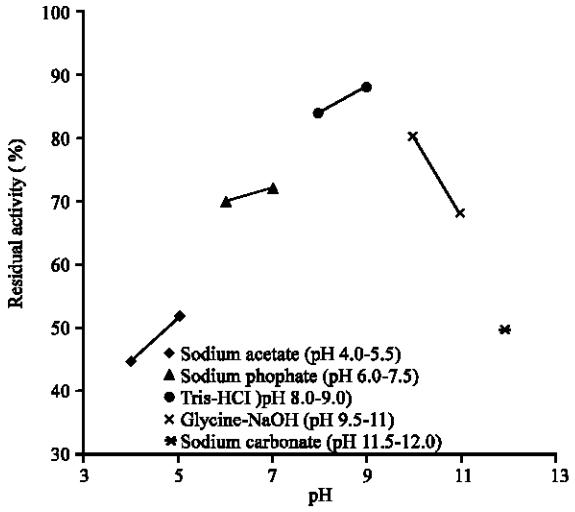

Fig. 3: Effect of pH on the stability of the protease produced by *B.licheniformis* strain 5

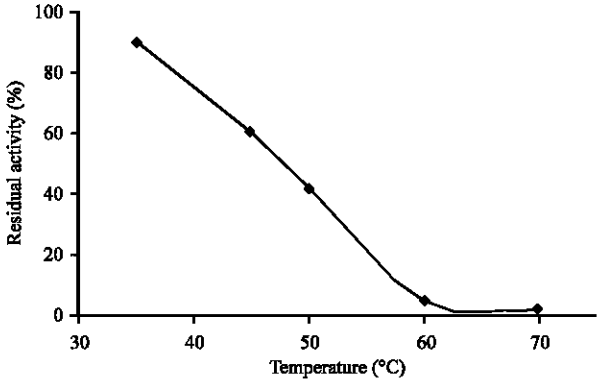

Fig. 4: Effect of temperature on the stability of the protease produced by *B.licheniformis* strain 5

Table 2: Effects of metal ions and various chemicals on *B.licheniformis* strain 5 protease activities

Reagents	Relative protease activity [a]
None	100
NaCl	97
$ZnSO_4$	89
$AlCl_3$	90
$MgSO_4$	116
$CaSO_4$	141
$MnSO_4$	125
$HgCl_2$	70
PMSF	4
EDTA	99

[a]The activities were assayed under the standard conditions and expressed as a percentage of the activity in the absence of the compound. EDTA: Ethylene diamine tetra acetic acid, PMSF: Phenyl methane sulphonyl fluoride

Influence of various reagents on enzyme activities: We investigated the effects of metal ions and other reagents on the activities of the alkaline protease and the results were shown in Table 2. The protease was strongly activated by Mn^{+2}, Ca^{+2}. The activities of the alkaline protease

in the presence of Mn^{+2}, Ca^{+2} were 25% and 41% higher than that observed in the control solution, respectively. They increased and stabilized the protease activity of the enzyme; this is possible because of the activation by the metal ions. These cations also have been reported to increase the thermal stability of other *Bacillus* alkaline proteases (Paliwal *et al*., 1994). Other cations such as Zn^{+2} and Al^{+3} slightly inhibited the protease activity. EDTA and Na^{+} did not show any appreciable effect on enzyme activity. In contrast, PMSF approximately led to complete inhibition of the protease. These results agree with an earlier report (Ates *et al*., 2007).

From the results that the examined alkaline protease was inhibited by PMSF, it may be concluded that this protease is a serine proteases (Gold and Fahrney, 1964).

CONCLUSION

The effect of different concentrations (1-6% v/v) of RHH on the production of alkaline protease was investigated and a concentration of 4% RHH was found to be optimal. The level of alkaline protease in the culture broth containing 4% RHH (93 U mL^{-1}) was 69% higher than that of the control culture broth (55 U mL^{-1}). From this result, RHH was demonstrated to be a suitable substrate for alkaline protease production and RHH may be a valuable supplement in biotechnology.

Characterization of the enzyme solution, without any purification, suggested the presence of a serine proteases produced by *B. licheniformis* strain 5 because it was inhibited in about 96% of its activity by PMSF. The optimum protease activity was observed at pH 9 and temperature of 50°C. The activity increased in the presence of metallic ions such as Ca^{2+} and Mn^{2+}.

ACKNOWLEDGMENT

This experiment was supported by the Islamic Azad University, Rasht Branch, Iran.

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