

PRODUCTION AND IMMOBILIZATION OF ALKALINE PROTEASE FROM *BACILLUS MYCOIDES*

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Abstract

The production of alkaline protease was investigated in submerged culture of *Bacillus mycoides*. The optimal conditions for enzyme production were pH 9.0, 3% (w/v) dextrin as carbon source, $(\text{NH}_4)_2\text{SO}_4$ (0.1%, w/v) and peptone (0.1%, w/v) as nitrogen sources and KH_2PO_4 at 1.0 g/l. The enzyme was immobilized on various carriers by different methods of immobilization including physical adsorption, ionic binding, covalent binding, and entrapment. The enzymes prepared by physical adsorption on chitosan, ionic binding on Amberlite IR-120, covalent binding on chitin, and entrapment in 2% crosslinked polyacrylamide had the highest activities. The optimal reaction temperature of the immobilized enzymes was shifted from 50 to 55°C. The thermal and storage stabilities of the free enzyme were significantly improved by the immobilization process. © 1998 Elsevier Science Ltd. All rights reserved

Key words: Alkaline protease, production, immobilization.

INTRODUCTION

Applications of alkaline protease are concentrated in laundry detergents, leather processing, brewing, and food and pharmaceutical industries (Lee *et al.*, 1991; Kembhavi *et al.*, 1993). The proteolytic enzymes of industrial value are preferably obtained from microbial sources, but few microbial strains are used for industrial applications. Moreover, the increasing demand for protease as a detergent supplement justifies a study aiming at a search for proteolytic enzymes from various microbial sources.

For industrial applications, the immobilization of protease on a solid support can offer several advantages, including repeated usage of enzyme, ease of product separation, improvement of enzyme stability and continuous operation in packed-bed reactors.

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The activities of the immobilized proteases so far reported are rather low when compared to those reported for other enzymes (Kise & Hayakawa, 1991; Gauthier *et al.*, 1991; Hyndman *et al.*, 1992).

This work was undertaken to investigate some factors affecting the production of alkaline protease from *Bacillus mycoides*. The results also discussed in this paper include the immobilization of *B. mycoides* alkaline protease onto various carriers using physical adsorption, ionic binding, covalent binding and entrapment. The properties of the free and immobilized enzymes were compared.

METHODS

Organisms

The bacterial cultures used were from the culture collection of The National Research Centre (NRC), Dokki, Cairo, Egypt.

Carriers for enzyme immobilization

Chitin, chitosan, Sepharose Cl-4B, and hydroxyapatite were from Sigma Chemical Co., USA. Acrylamide, bis-acrylamide, alumina were from BDH Chemicals Ltd, England. Amberlite IR-120, Dowex 50W, Dowex 1 × 4 (Cl) were from Fluka, Switzerland. DEAE-cellulose DE-52 was obtained from Whatman Lit, England. DEAE-Sephadex A25 was obtained from Pharmacia Fine Chemicals Inc., Sweden. All other chemicals were of analytical grade.

Medium composition and cultivation

The basal medium for liquid cultures consisted of (g/l): glycerol, 20; $(\text{NH}_4)_2\text{SO}_4$, 1.0; KH_2PO_4 , 0.5; MgSO_4 , 0.3; CaCO_3 , 1.0; NaCl, 1.0; peptone, 1.0. After autoclaving, 10 ml of 0.05% ZnSO_4 was added. The pH was adjusted to 7.0. The same medium was also used for inoculum preparation for all the fermentation experiments. Cultivation was in 250 ml Erlenmeyer flasks containing 50 ml of sterile medium. The flasks were inoculated with 1 ml of a

24 h old culture (optical density at 650 nm of about 1.0). The flasks were incubated at 30°C with shaking at 180 rev/min. The cells were harvested by centrifugation at 8000 rev/min. The clear culture filtrates were assayed for enzyme activity. The whole culture filtrate from the optimized medium, after dialysis and lyophilization, was used for enzyme immobilization. The specific activity of this crude enzyme was 29.3 U/mg protein.

Immobilization methods

Physical adsorption

Tannin-chitosan and tannin-sepharose were prepared by the method of Watanabe *et al.* (1978). One hundred milligrams of the carriers (tannin-chitosan, tannin-sepharose, alumina, chitin, ceramics and chitosan) were incubated with 100 U *B. mycooides* protease dissolved in 1 ml of 0.1 M Tris-HCl buffer (pH 9.0) at 4°C overnight (in the case of chitosan 200 U of *B. mycooides* protease was used). The unbound enzymes were removed by washing with Tris-HCl buffer (0.1 M, pH 9.0).

Ionic binding

0.2 g of the cation (Dowex-50W, Amberlite IR-120) or anion (DEAE-Sephadex A-25, Dowex 1 × 4 Cl, Ecteola cellulose, DEAE-cellulose D-E52) exchanger was equilibrated with acetate buffer (0.1 M, pH 6.0) or Tris-HCl buffer (0.1 M, pH 9.0) and incubated with 1 ml of protease solution containing 200 U protease in the same buffer at 4°C for 12 h. The unbound enzymes were removed by washing with the same buffer.

Covalent binding

0.4 g of chitosan was shaken in 5 ml of acetate buffer (0.1 M, pH 5.0) containing 2.5% (v/v) glutaraldehyde (GA) for 2 h at 30°C. The solubilized chitosan was precipitated by the addition of 1 ml of 0.1 M NaOH. The precipitate was collected by filtration and washed with distilled water to remove excess GA. The wet chitosan was mixed with 1 ml of enzyme solution (400 U). After being shaken for 1 h at 30°C, the unbound enzyme was removed by washing with distilled water.

Hydroxyapatite, chitin and alumina (0.2 g) were treated with 2 ml of 1% (v/v) GA for 2 h at 30°C. The carriers were washed with distilled water to remove the excess GA. The wet carriers were mixed with 0.5 ml of the enzyme solution (200 U) and incubated for 6 h at 30°C. The unbound enzyme was removed by washing with distilled water.

Cyanogen bromide-activated sepharose (CNBr-sepharose) was prepared by the method of March *et al.* (1974) and 0.3 g incubated at 4°C for 12 h with 0.3 ml of the enzyme solution (300 U dissolved in 0.1 M Tris-HCl buffer, pH 9.0). The unbound enzyme was removed by washing with distilled water.

Entrapment

The gel was prepared according to the method of Roy *et al.* (1984). The polymerization mixture contained 10 ml of enzyme solution (10000 U *B. mycooides* protease), 50 ml of 9.8% (w/v) acrylamide, and 40 ml of Tris-HCl buffer (0.1 M, pH 9.0). The amount of crosslinking compound (N,N-methylenebis acrylamide) added was at the levels of 1, 2, 4, 6, and 8% (w/v) of the total monomer content in separate experiments. The catalyst system consisted of the following: 0.1 ml N,N,N,N-tetramethylethylenediamine and 0.1 g of potassium persulphate dissolved in 0.2 ml of distilled water. It was finally added to the acrylamide mixture, and the stirring was continued for 5 min. The total volume of the mixture was brought to 100 ml with deionized water. Finally, the mixture was transferred to the gel-casting glass cassettes for polymerization at 24°C. After the polymerization, the gel was washed with saline, cut into 1 mm fragments and kept in Tris-HCl buffer (10 mM, pH 9.0) at 4°C for 24 h to remove the unbound enzyme.

Determination of protease activity

The proteolytic activity was measured by using a reaction mixture of 1.0 ml of 1.5% (w/v) water soluble casein (Sigma) in Tris-HCl buffer (0.1 M, pH 9.0), and 1 ml of enzyme solution or weighed sample of the immobilized enzyme. The reaction mixture was incubated for 30 min in a water bath at 37°C and stopped by adding 2 ml of 15% (w/v) trichloroacetic acid and centrifuged (4000 g). The solubilized proteins in the supernatant were assayed using the phenol method of Greenberg (1957). One unit of the enzyme activity (U) was defined as the amount of the enzyme that released 1 μmol tyrosine per min.

The protein content of the free and immobilized enzymes was determined by the method of Lowry *et al.* (1951).

All the results of the growth and assays are the means of at least three separate experiments.

RESULTS AND DISCUSSION

Evaluation of the thermal stability of the proteolytic activity produced by the bacterial cultures

Among 14 bacterial strains investigated for extracellular alkaline protease, *Bacillus megaterium* (8.2 U/ml), *B. mycooides* (54.0 U/ml), *B. subtilis* (11.53 U/ml) and *Staphylococcus* sp. (23.9 U/ml) at the 48 h incubation period were the best for the enzyme production. The culture filtrates of these isolates were evaluated according to their thermal stabilities. The highest thermal stability was recorded for *B. mycooides* (Table 1). In terms of thermal stability, this enzyme is superior to the alkaline proteases from an alkilophilic *Bacillus* sp. (Durham *et al.*, 1987) and *Bacillus* sp. CW-1121 (Lee

Table 1. Thermal stability of alkaline protease activity of the bacterial cultures

Culture	Time of heating (h)	Residual activity (%)				
		0	1	2	3	4
<i>Bacillus megaterium</i> 10	Temperature of heating (°C)					
	50	100	48.0	19.2	14.9	9.5
	60		25.4	11.7	8.2	0.0
	70		3.3	0.0	0.0	0.0
<i>B. mycoides</i> c. 1088	80		0.0	0.0	0.0	0.0
	50	100	100	100	100	100
	60		100	100	100	100
	70		100	100	100	41.8
<i>B. subtilis</i> 3	80		7.1	4.0	0.0	0.0
	50	100	69.6	65.6	60.1	50.5
	60		63.6	60.2	53.6	43.6
	70		35.3	29.1	15.7	7.7
<i>Staphylococcus</i> sp. 9	80		2.8	0.0	0.0	0.0
	50	100	40.9	31.3	20.5	8.8
	60		25.7	17.6	4.9	5.3
	70		3.8	1.8	0.0	0.0
	80		0.0	0.0	0.0	0.0

Bacillus megaterium 100% = 8.3 U/ml.

B. mycoides c. 1088 100% = 54.0 U/ml.

B. subtilis 3 100% = 11.5 U/ml.

Staphylococcus sp. 100% = 24.0 U/ml.

et al., 1991). Therefore, *B. mycoides* was selected for further studies.

Culture conditions for maximization of the production of alkaline protease by *B. mycoides*

The effect of the initial pH of the culture medium on the production of alkaline protease was investigated over the pH range 6–11. The results indicated that maximal enzyme production was attained at an initial pH of 9.0. These results are similar to those reported by Novekova *et al.* (1986).

The production of alkaline protease was compared when *B. mycoides* was grown on the basal medium containing different carbon sources (glucose, galactose, arabinose, xylose, fructose, lactose, sucrose, maltose, dextrin and starch). The carbon level was kept equivalent to 2.0% (w/v) glycerol. Of these carbon source, dextrin was the most effective inducer for enzyme production (114.9 U/ml). These results are similar to those reported by Valdimirova & Komienko (1987). The effect of dextrin concentration was also investigated and the results indicated that maximal proteolytic activity (140 U/ml) was attained at 3.0% (w/v) dextrin concentration.

Glycerol in the basal medium was substituted by dextrin (3.0%, w/v) and on an equivalent nitrogen basis, the nitrogen source from the basal medium (0.1%, w/v, peptone, and 0.1%, w/v, (NH₄)₂SO₂) was substituted by different nitrogen sources. Casein, yeast extract, or meat extract produced only small amounts of alkaline protease (7–15 U/ml). Allison & Macfarlane (1990) reported on the fall in protease levels with the use of some organic and complex

nitrogen sources. They ascribed this to the feed-back inhibition mechanism resulting in the presence of certain amino acids repressing the enzyme biosynthesis. Inorganic nitrogen sources (NaNO₃, NH₄Cl, (NH₄)₂SO₄) seemed to be ineffective (14–17 U/ml). In our results the mixed nitrogen source in the basal medium (0.1% (w/v) peptone+0.1% (w/v) (NH₄)₂SO₄) was the most favourable (140 U/ml).

Using the same conditions of the previous experiment, the effect of phosphate level in the culture medium (0.02–0.25% (w/v) KH₂PO₄) was investigated. The highest activity (188 U/ml) was obtained at 0.1% (w/v) KH₂PO₄. On the other hand, Moon & Parulekar (1993) showed that protease production with *B. firmus* needed a phosphate level of 0.2 g/l.

By replacing water in the medium by Tris-HCl (0.05 M, pH 9.0), the enzyme yield was increased by 8% (203.0 U/ml).

Using the optimum conditions reached from the previous experiments (3% dextrin as carbon source, KH₂PO₄ level at 0.1% w/v, and replacement of water in the basal medium by 0.05 M Tris-HCl, pH 9.0), the effect of addition of some metal ions, some vitamins and wheat bran was investigated. ZnCl₂ (0.05 mg%, w/v) enhanced the production of alkaline protease from *B. mycoides* by 14.3% (232 U/ml). Hiramatsu & Ouchi (1978) reported that zinc was essential for the stability of the proteinase from *Streptomyces naraensis*. On the other hand, CoCl₂ (1.0 mg%, w/v) seemed to exert an adverse effect on the enzyme activity. Macfarlane & Gibson (1993) reported on the inhibition of *Bacteroides splanchnicus* protease by cobalt ions. The

enzyme activity was greatly diminished by inclusion of Mn^{2+} and Fe^{2+} ions (1.0 mg%, w/v) in the culture medium. The addition of vitamins B₁, B₂ and B₁₂ (0.5 mg%, w/v) had no effect on the enzyme productivity. On the other hand, wheat bran (0.5%, w/v) showed about a 50% fall in the enzyme yield.

From the above results, the optimal medium consisted of (g/l): 30.0, dextrin: 1.0, $(NH_4)_2SO_4$: 1.0, peptone; 1.0, KH_2PO_4 : 1.0, $MgSO_4$. The constituents were dissolved in 0.05 M Tris-HCl buffer, pH 9.0. The optimum culture medium of *B. mycooides* yielded 232 U/ml alkaline protease activity which was about a 4.3-fold increase of proteolytic activity compared to the original basal medium.

Immobilization of *B. mycooides* alkaline protease

The whole culture-filtrate from the optimized medium, after dialysis and lyophilization, was used for enzyme immobilization. The specific activity of this crude enzyme was 29.3 U/mg protein.

Immobilization by physical adsorption

The results in Table 2 indicate that a low immobilization yield (1.74–3.11%) and bound enzyme (10.36–27 U/g carrier) was detected with alumina, tannin–chitosan, ceramic, and chitin. On the other hand, the highest bound enzyme (190.8 U/g carrier) and immobilization yield (18.8%) was found with chitosan. In the following experiments, the enzymes physically bound on chitosan were used as an example of this group.

Immobilization by ionic binding

A series of ion exchangers was used for the immobilization by ionic binding (Table 3). In

general, immobilization by ionic binding showed a low bound enzyme. Comparatively, Amberlite IR-120 was the most suitable ion exchanger for enzyme immobilization. Since this immobilized enzyme had the highest activity per gram carrier (28.09 U/g carrier) and the highest immobilization yield (15%), it was selected as a typical example of the immobilization by ionic binding.

Immobilization by covalent binding

Immobilization by covalent binding through a spacer group (glutaraldehyde) showed considerable bound enzyme activity (good loading efficiency) and immobilization yield (Table 4). This good loading efficiency for the immobilization by covalent binding might have been due to the formation of stable crosslinking between the carrier and the enzyme through a spacer group (glutaraldehyde). In addition, covalent binding through a spacer group probably increased the local surface area of the carrier and, consequently, reduced the steric hindrance around the active site of the enzyme molecule (Siso *et al.*, 1990). Moreover, Gauthier *et al.*, 1991 reported that increasing the length of the spacer group contributed to increase in the stability of immobilized proteases on agarose. The enzymes covalently bound to chitin showed the highest immobilization yield (79.29%) and the highest immobilized activity per gram carrier (227.59 U/g) and were taken as an example of the immobilization by covalent binding.

Immobilization by entrapment

Immobilization of *B. mycooides* alkaline protease by entrapment was achieved in 5% (w/v) acrylamide.

Table 2. Immobilization of *B. mycooides* alkaline protease by physical adsorption

Carrier	Added enzyme (A) (U/g carrier)*	Unbound enzyme (B) (U/g carrier)	Bound enzyme (I) (U/g carrier)	Immobilization yield = I/(A-B) %
Tannin–chitosan	1000	268.0	22.79	3.11
Alumina	1000	249.0	13.06	1.74
Chitosan	2000	985.0	190.89	18.8
Chitin	1000	311.1	27.08	3.93
Ceramics	1000	580.5	10.36	2.47
Tannin–sepharose	1000	354.5	92.65	14.35

*Unit per g dry weight of the carrier.

Table 3. Immobilization of *B. mycooides* alkaline protease by ionic binding

Carrier	Added enzyme (A) (U/g carrier)*	Unbound enzyme (B) (U/g carrier)	Bound enzyme (I) (U/g carrier)	Immobilization yield = I/(A-B) %
DEAE-Sephadex A 25	1000	819.5	13.25	7.34
Dowex-50W	1000	840.5	14.13	8.86
Amberlite IR-120	1000	813.0	28.09	15.02
Dowex 1 × 4 (CI)	1000	834.5	8.79	5.42
DEAE-Cellulose	1000	904.75	5.93	6.23
Eceola-Cellulose	100	829.5	8.87	5.2

*Unit per g dry weight of the carrier.

Table 4. Immobilization of *B. mycoides* alkaline protease by covalent binding

Carrier	Added enzyme (A) (U/g carrier)*	Unbound enzyme (B) (U/g carrier)	Bound enzyme (I) (U/g carrier)	Immobilization yield = I/(A-B) %
Chitin	1000	713.0	227.59	79.29
Hydroxapatite	1000	714.0	186.68	65.45
CNBr-Sepharase	1000	704.8	187.19	63.91
Alumina	1000	709.19	176.50	60.69
Chitosan	1000	779.0	171.08	77.41

*Unit per g dry weight of the carrier.

The amount of crosslinker added was at the level of 1, 2, 4, 6, and 8% (w/w) of the total monomer content. The results (Table 5) indicated that a crosslinking concentration of 2% was sufficient for reaching maximal bound enzyme (entrapping efficiency) and immobilization yield. The decrease in yield with increase in crosslink might have been due to the decrease in the porosity of the gel matrix which caused diffusion limitation of the substrate. In the following experiments, protease entrapped in 5% acrylamide concentration (2% crosslinker) was used:

Properties of the immobilized enzymes

The immobilized enzymes retained 15–78% of the specific protease activity (Table 6). This drop of the specific activity suggests diffusional limitation on substrate or product flux due to the association of the enzyme within the pores of the carriers (Siso *et al.*, 1990). In addition to the limitations of substrate and product flux, the fixation process might impair or prevent the proper conformational changes required for catalysis (Hyndman *et al.*, 1992).

The optimum pH of the enzymes immobilized on chitosan and chitin were shifted to a more acidic

range (pH 8.7) from 9.0, which is the optimum for native alkaline protease. On the other hand, the enzymes ionically bound to Amberlite IR-120 or entrapped in polyacrylamide showed an optimum pH more alkaline (pH 10 and 9.7, respectively). These effects might be dependent on the ionic environment around the enzyme active site.

The optimum reaction temperature for the enzyme covalently bound to chitin was 50°C, which was the same as the free enzyme. However, the enzymes immobilized on chitosan, Amberlite IR-120, and entrapped in polyacrylamide showed optimal reaction temperatures at 55°C. The results indicated that the applied immobilization procedure contributed to improvement of the enzyme stability.

Heat treatment of the immobilized enzymes in the absence of the substrate at 70°C was investigated (Table 6). The results indicated that the immobilized enzymes retained 39–81.9% of the original activity after heating for 6 h. The free enzyme, however, retained only 21% of the original activity after the same treatment.

Storage stability at 5°C of the free and immobilized enzymes was also evaluated (Table 6). The free enzyme lost about 50% of its original activity after storage for 5–6 months, whereas the

Table 5. Immobilization *B. mycoides* alkaline protease by entrapment with polyacrylamide

Crosslinker concentration (%)	Enzyme added (A) (U/10 ml gel)	Unbound enzyme (B) (U/10 ml gel)	Immobilized enzyme (I) (U/10 ml gel)	Immobilization yield = I/(A-B) %
1	1000	553.25	268.9	60.19
2	1000	482.75	395.96	76.55
4	1000	454.15	251.7	46.11
6	1000	437.95	246.00	43.76
8	1000	415.8	209.4	35.84

Table 6. Properties of free and immobilized enzymes

Properties	Free enzyme	Carrier of immobilized enzyme			
		Chitosan	Amberlite IR-120	Chitin	Polyacrylamide*
Specific activity (U/mg protein)	29.3	5.5	4.35	22.9	21.7
Optimum pH	9.0	8.7	10.0	8.71	9.7
Optimum temperature (°C)	50	55	55	50	55
Thermal stability at 70°C, pH 9.0 for 6 h (%)	21.2	81.0	81.9	39.1	39.7
Stargae stability for 5–6 months at 5°C	50	90.1	88.4	100.0	87.9

*Total concentration = 5%, crosslinker concentration = 2%.

immobilized enzymes retained 88–100% of the original activity over the same period of time.

Both these sets of data show that immobilized *B. mycoides* alkaline protease was considerably more stable than the free enzyme, and could be stored for extended periods in both wet and dry forms before use. Various reports confirm that the thermal and storage stability of immobilized protease depend on the applied immobilization methods (Gauthier *et al.*, 1991; Kise & Hayakawa, 1991; Hyndman *et al.*, 1992).

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