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Production and immobilization of cellobiase from *Aspergillus niger* A20

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Abstract

The production of cellobiase was investigated using a submerged culture of *Aspergillus niger* A20. The maximum production occurred when the pH was controlled and maintained at 4.0 during the fermentation process. Lactose (0.2%, w/v) and cellulose (1.5%, w/v) were the most favourable carbon sources. Yeast extract (0.05%, w/v) peptone (0.025%, w/v), urea (0.025%, w/v) and $(NH_4)_2SO_4$ (0.07%, w/v) were the most favourable nitrogen sources. KH_2PO_4 used at a concentration of 0.2% (w/v) produced the highest enzyme yield. It was possible to increase the cellobiase yield to 27.5 U ml^{-1} in the modified medium. The enzymes immobilized on chitosan with 0.3% (v/v) glutaraldehyde and entrapped in 10% polyacrylamide with a 5% (w/v) crosslinking level had the highest activities. Maximal loading of the enzyme activity on chitosan was 1570 U gram $carrier^{-1}$. The K_m (Michaelis constant) value of the immobilized enzyme was higher than those of the native enzyme. Thermal stability was improved by the immobilization process.

Keywords: Cellobiase; Production; Immobilization

1. Introduction

Cellobiase (β-glucosidase, β-D-glucosidase glucohydrolase, EC 3.2.1.21) is an enzyme that hydrolyses variously β-linked diglucosides and aryl β-glucosides and has been studied using several microbial sources [1]. Interest in this enzyme centres on its role in the enzymatic hydrolysis of cellulose. The presence of cellobiase in cellulase preparation has been reported to stimulate the rate and extent of cellulose hydrolysis [2]. This effect has been explained by the concept that it reduces the inhibition of cellulase activity by cellulose-derived cellobiose. Cellobiase can thus be used to supplement cellulase preparation in order to enhance saccharification [3].

Immobilization of the enzyme on water insoluble supports has become a subject of interest for many applications. The advantages of this technique include the possibility of enzyme reutilization, enhanced stability, reduced product inhibition and facilitated automation. As far as cellobiases are concerned, however, there are only a few reports regarding their immobilization [4–7].

The objective of this project was to investigate some of the factors affecting the production of cellobiase from *Aspergillus niger* A20. This work included studies of the immobilization of *A. niger* A20 cellobiase on chitosan and polyacrylamide. The properties of the free and immobilized enzymes were also compared.

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2. Materials and methods

2.1. Microorganism

The fungal culture used in this project was obtained from the collection at the centre of culture at the National Research Centre, Cairo, Egypt.

2.2. Culture medium

The basal growth medium for enzyme production had the following composition (g l^{-1}): cellulose, 15; lactose, 2.0; wheat bran, 10; KH_2PO_4, 2.0; $CaCl_2.2H_2O$, 0.3; $MgSO_4.7H_2O$, 0.3; urea, 1.5; $(NH_4)_2SO_4$, 0.7; yeast extract, 0.5; peptone, 0.25; $FeSO_4.7H_2O$, 0.005; $ZnSO_4.7H_2O$, 0.0014; $MnSO_4.5H_2O$, 0.0022; $CoCl_2$, 0.002. Tween-80 was also included at a concentration of 1.0 ml l^{-1}. The pH of the medium was adjusted to 5.0.

2.3. Cultivation

For preparation of the inoculum, 1.0 ml of spore suspension (8×10^7 spore ml^{-1}) was transferred to 50 ml of the basal medium in a 250 ml Erlenmeyer flask and incubated in a rotary shaker (180 rev min^{-1}) at 30 °C for 5 days. Cultivation was also done in 250 ml Erlenmeyer flasks, each containing 50 ml of sterile medium. One millilitre of the inoculum (2%,

v/v) was transferred to the growth medium. The flasks were incubated at 30 °C in the rotary shaker at 180 rev min^{-1} for different incubation periods (i.e. 7, 10, and 14 days). The culture broth from each flask was filtered off (by using a sintered glass funnel, G5) and the clear culture filtrate was taken for enzyme assays.

2.4. Control of pH

This was achieved manually every 12 h using sterile 0.1 M HCl or 0.1 M NaOH.

2.5. Ultrafiltration

This was carried out by using a millipore pellicon cassette system polysulfone membrane (cut-off molecular weight, 10 000). The filtration rate was maintained at 100 ml min^{-1}.

2.6. Ethanol fractionation

Ethanol fractionation was carried out as follows: a proportion of the ultrafiltrate from the culture filtrate was kept in a cold water bath at 5.0 °C. An equal amount of ice cold absolute ethanol was added slowly and the mixture was stirred for 30 min. The resultant precipitate was separated using a refrigerated centrifuge at 4000 rev min^{-1} for 15 min. This fraction was dried over anhydrous calcium chloride under reduced pressure at 5 °C. This partially purified enzyme (specific activity 50 U mg $protein^{-1}$) was used for enzyme immobilization.

2.7. Enzyme immobilization on chitosan

Immobilization on chitosan was carried out as follows: 1 g chitosan (Sigma) was dissolved in 100 ml 0.5% acetic acid. To the chitosan solution was added 2 ml of the enzyme solution (containing 800–4000 U cellobiase). The mixture was stirred for 30 min at 25 °C. Glutaraldehyde (GA) was then added (2.5%, v/v, GA in 0.05 M citrate buffer at pH 4.5) to yield final concentrations of 0.1–0.4% (v/v). The reaction mixture was then stirred for 2 h at 25 °C. The mixture was then poured into six volumes of ice cold acetone (at 4.0 °C) with gentle stirring. The precipitates were separated by filtration (using a sintered glass funnel, G1) and washed with 0.05 M citrate buffer (pH 4.5) to remove any unbound enzymes. The washing procedure was repeated until no activity or soluble protein was detected. The immobilized enzyme was kept moist in a buffer for further use [7].

2.8. Enzyme immobilization by entrapment

The entrapment of the enzyme was carried out using different concentrations of acrylamide (2.5–12.5%, w/v) with different crosslinker (*N,N* methylene bisacrylamide) concentrations (2.5–10%, w/w), as described by Roy et al. [6].

2.9. Thermal stability

The thermal stabilities of both free and immobilized cellobiase were tested by incubating the enzymes in 0.1 M citrate buffer, pH 4.8, at a designated temperature for 15 to 75 min before activity assay.

2.10. Assay for cellobiase activity

The cellobiase activity was determined according to the method reported by Berghem and Petterson [8] as follows: to a 1.0 ml sample of 0.4% cellobiose, dissolved in 0.05 M citrate phosphate buffer (pH 4.8), 0.5 ml diluted culture filtrate or a weighed sample of immobilized enzyme was added. The reaction mixture was incubated in a water bath for 30 min at 50 °C. The reaction was stopped by heating the reaction mixture in a boiling water bath for 5 min. The glucose released was determined by using a glucose oxidase/peroxidase reagent. One enzyme activity unit (U) is defined as the amount of enzyme that release 1 μmole glucose from cellobiose per min.

2.11. Protein estimation

The protein was estimated using the method developed by Lowry et al. [9]. The protein content of the immobilized enzyme was calculated by subtracting the amount of unbound proteins from the originally added protein.

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

3. Results and discussion

Among the 12 fungal strains investigated for the production of extracellular cellobiase after different incubation periods (i.e. 7, 10 and 14 days) [10] *Aspergillus niger* A20 after 10 days' growth was found to be the most suitable for enzyme production (13.8 U ml^{-1}).

The effect of varying the pH of the culture medium (at 3, 4 and 5) during the fermentation process was investigated. A similar experiment without pH control was also conducted. The results (Fig. 1) indicate that the maximum yield of cellobiase was obtained when the pH of the culture was maintained at 4.0 and after 8 days' growth (22.8 U ml^{-1}). The maximum cellobiase activity obtained (at pH 4.0) was 2.12 times that of the uncontrolled pH conditions after the same incubation period (10.75 U ml^{-1}). In addition, the results also demonstrated the sensitivity of the enzyme to a lower pH value (i.e. 3.0). These results are similar to those reported for cellobiase production from *A. niger* 1 [11].

Mycelial inoculum (from a 5-day-old culture) was superior to spores produced from a 7-day-old slant (with a concentration of 8×10^7 spores ml^{-1}). The maximum cellobiase production was at a concentration of 5% (v/v) mycelial

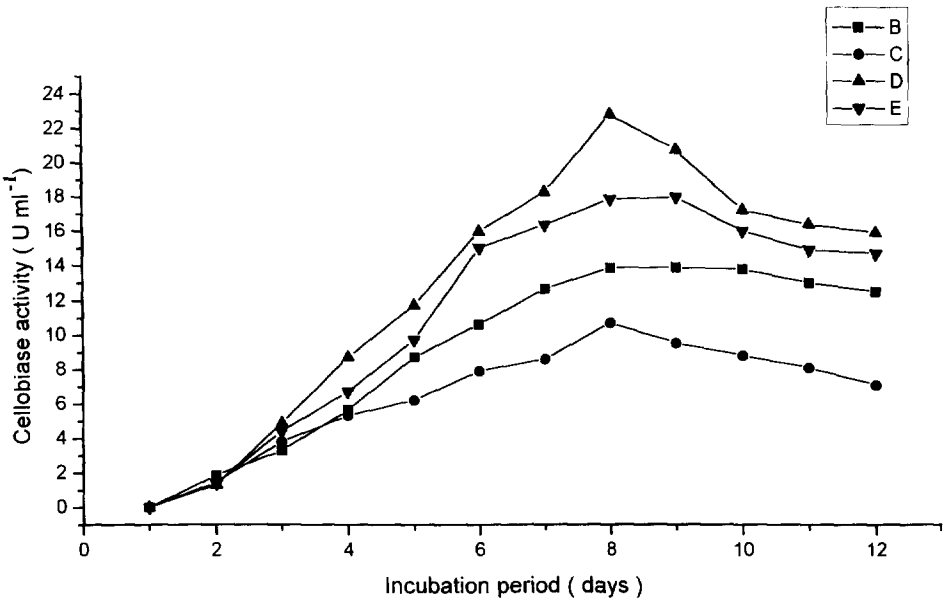

Fig. 1. Effect of pH control on the production of cellobiase by *A. niger* A20: uncontrolled pH (B), pH controlled at 3.0 (C), pH controlled at 4.0 (D), pH controlled at 5.0 (D).

inoculum (23.63 U ml^{-1}). These results are similar to those reported by Srivastava et al. [12].

On an equivalent carbon basis, the soluble carbon source (lactose) from the basal medium was substituted by glucose, sucrose, maltose, or cellobiose. The results (Table 1) indicate that none of the alternative sugars enhance productivity compared to lactose. These results are similar to those reported for the production of cellobiase from *A. niger* 1 [11]. The replacement of wheat bran in the basal medium at different concentrations (5 to 25 g l^{-1}) by rice bran produced a negative effect on the enzyme yield. The effect of wheat bran concentration was also investigated and the maximum activity was detected at 10 g l^{-1}.

On an equivalent nitrogen basis, the nitrogen sources in the basal medium (peptone, 0.025% (w/v); yeast extract, 0.05% (w/v); $(NH_4)_2SO_4$, 0.07% (w/v); urea, 0.15% (w/v)) were replaced by yeast extract, peptone, corn steep, casein, $(NH_4)_2SO_4$, urea (0.15%) plus $NaNO_3$ (0.97%) and urea (0.15%) plus $(NH_4)_2SO_4$ (0.75%). Of the nitrogen sources investigated (Table 2), the mixed nitrogen source in the basal medium was the most favourable for cellobiase production from *A. niger* A20 (23.63 U ml^{-1}). These results agree with those reported for β-glucosidase production using *Trichoderma viride* [13].

The effect of phosphate levels in the culture medium for the production of cellobiase was also investigated using different concentrations of KH_2PO_4 (0.1–3.0%, w/v). The maximum value for cellobiase production (25.95 U ml^{-1}) was observed at 0.25% (w/v) KH_2PO_4.

Trace metal ions (i.e. Fe^{2+}, Mn^{2+}, Zn^{2+}, and Co^{2+}) in the culture medium were found to support the production of cellulases and β-glucosidase [2]. However, the addition of these ions to double or triple the concentrations in the basal medium resulted in a 45.27% (i.e. 14.2 U ml^{-1}) or 57.6% (i.e. 11.05 U ml^{-1}) drop in the enzyme yield respectively. The inhibitory effect brought about by the high concentrations of trace metals may be partially due to their toxic effect on the fungal growth [14].

The effect of the presence of different surfactants on the production of cellobiase was also investigated (Table 3). The results indicate that the deletion of Tween-80 from the basal medium adversely affects enzyme production (from 25.95 to 14.2 U ml^{-1}). Lower yields were also obtained using 0.1% (v/v) Tween-20 (18.2 U ml^{-1}), 0.1% (v/v) Tween-40

Table 1
Effect of soluble carbon sources on the production of cellobiase by *Aspergillus niger* A20

Carbon source	Dry weight of mycelium (g 50 ml^{-1} culture medium)	Protein content of culture filtrate (mg ml^{-1})	Cellobiase activity (U ml^{-1})
None	1.16	1.47	13.7
Lactose (control)	2.23	1.93	23.63
Sucrose	0.97	1.66	12.01
Cellobiose	1.22	2.32	10.56
Maltose	1.65	1.77	10.84
Glucose	1.88	1.69	18.81
Galactose	1.79	1.59	17.2

Table 2
Effect of different nitrogen sources on the production of cellobiase by *Aspergillus niger* A20

Nitrogen source	Dry weight of mycelium (g 50 ml^{-1} culture medium)	Protein content of culture filtrate (mg ml^{-1})	Cellobiase activity (U ml^{-1})
Control [a]	2.23	1.93	23.63
Yeast extract	1.94	2.54	20.36
Peptone	2.1	2.53	14.8
Corn steep	1.55	2.11	18.72
Casein	1.63	2.5	21.31
$(NH_4)SO_4$	1.46	1.23	11.23
Urea + $NaNO_3$ [b]	1.55	0.94	14.86
Urea + $(NH_4)_2SO_4$ [c]	1.53	0.88	14.2

[a] The nitrogen source of the control in g l^{-1}: ammonium sulphate, 0.7; urea, 1.5; yeast extract, 0.5; peptone, 0.25.
[b] Urea, 1.5 g l^{-1}; $NaNO_3$, 9.7 g l^{-1}.
[c] Urea, 1.5 g l^{-1}; $(NH_4)_2SO_4$, 7.50 g l^{-1}.

Table 3
Effect of some surfactants on the production of cellobiase by *Aspergillus niger* A20

Surfactants	Concentration (%)	Dry weight of mycelium (g 50 ml^{-1} culture medium)	Protein content of culture filtrate (mg ml^{-1})	Cellobiase activity (U ml^{-1})
None		1.89	1.77	14.20
Tween-20	0.1 (v/v)	1.99	2.85	18.2
Tween-40	0.1 (v/v)	2.2	2.3	24.0
Tween-65	0.1 (v/v)	1.77	2.29	11.2
SDS	0.1 (w/v)	1.84	1.84	17.27
Tween-80	0.1 (v/v) [a]	2.4	2.29	25.95
	0.2 (v/v)	2.46	2.64	26.4
	0.3 (v/v)	2.1	2.73	21.73
	0.4 (v/v)	1.84	2.89	17.22

[a] Control.

Table 4
Effect of some amino acids and inducers on the production of cellobiase by *Aspergillus niger* A20

Substance added	Concentration (%)	Dry weight of mycelium (g 50 ml^{-1} culture medium)	Protein content of culture filtrate (mg ml^{-1})	Cellobiase activity (U ml^{-1})
None		2.46	2.64	26.4
L-phenylalanine	0.06	2.65	2.73	26.1
L-tryptophane	0.06	2.23	2.89	26.51
L-glutamine	0.06	2.1	2.63	26.0
Sodium phytate	0.03	2.09	1.69	20.33
L(-) sorbose	0.1	2.50	3.10	27.11
	0.5	2.54	3.44	27.53
	1.0	2.65	3.77	24.91

(24.0 U ml^{-1}), 0.1% (v/v) Tween-65 (11.2 U ml^{-1}) or 0.1% (w/v) SDS (sodium dodecyl sulphate) (17.27 U ml^{-1}). Due to the positive effect of Tween-80 on enzyme production, tests were conducted to find the optimum concentration. It was found that Tween-80 at 0.2% (v/v) produced the best results (26.4 U ml^{-1}).

The effect of some amino acids and some inducers on the production of the enzyme was also examined (Table 4). The addition of L-phenylalanine, L-tryptophane, L-glutamine (0.06%, w/v) showed no significant effect on enzyme production. These results are in contrast to those reported by Ryu and Mandels [2], who reported the positive effect of amino acids on cellulase and β-glucosidase production by *Trichoderma reesei*.

The addition of sodium phytate (0.03% w/v) resulted in a 23% inhibition (to 20.33 U ml^{-1}) of the cellobiase yield (Table 4). However, these results are in contrast to those reported by Abdel-Fattah et al. [15] and Ismail et al. [11], which showed the stimulating effect of sodium phytate on cellulase and cellobiase biosynthesis.

Cellobiase yield was improved (to 27.5 U ml^{-1}) by the addition of 0.5% (w/v) L(-) sorbose (Table 4). The most significant effect of L(-) sorbose addition was the increase in the protein content of the culture filtrate by about 32% to

Table 5
The effect of glutaraldehyde concentration on the immobilization of *A. niger* A20 cellobiase

Glutaraldehyde concentration (%)	Immobilized enzyme			Unbound enzyme		Immobilization yield (%), $\frac{I \times 100}{A-B}$
	Total activity (U g carrier^{-1}), I	Total protein (mg g carrier^{-1})	Specific activity (U mg protein^{-1})	Total activity (U g carrier^{-1}), B	Total protein (mg g carrier^{-1})	
0.1	792.0	21.36	37.07	696.0	10.64	87.60
0.2	912.0	23.52	38.70	592.2	8.48	90.47
0.3	1104.0	25.68	43.00	404.8	6.32	92.36
0.4	1064.0	31.04	34.27	88.0	0.96	74.71

Note: total activity of the enzyme added (A) = 1600 U g carrier^{-1}.

Table 6
Maximum loading of *Aspergillus niger* A20 cellobiase on chitosan

Enzyme added		Immobilized enzyme			Unbound enzyme		Immobilization yield (%), $\frac{I \times 100}{A-B}$
Total activity (U g carrier^{-1}), A	Total protein (mg g carrier^{-1})	Total activity (U g carrier^{-1}), I	Total protein (mg g carrier^{-1})	Specific activity (U mg protein^{-1})	Total activity (U g carrier^{-1}), B	Total protein (mg g carrier^{-1})	
800.0	16.0	560.0	13.36	41.90	96.00	2.64	79.54
1600.0	32.0	1104.0	25.68	43.00	404.80	6.32	92.36
2400.0	48.0	1570.4	38.88	40.39	720.00	9.12	93.47
3200.0	64.0	1728.0	44.80	38.57	1232.00	27.20	87.80
4000.0	80.0	1808.0	48.80	37.66	1656.00	32.00	77.10

Note: glutaraldehyde concentration used was 0.3%.

3.44 mg ml^{-1}). L(-) sorbose has been reported to increase β-glucosidase activity in *T. reesei* [16,17].

From the results described above, the optimal medium consists of (g l^{-1}) cellulose, 15; lactose, 2.0; wheat bran, 10; KH_2PO_4, 2.5; $CaCl_2.2H_2O$, 0.3; $MgSO_4.7H_2O$, 0.3; urea, 1.5; $(NH_4)_2SO_4$, 0.7; yeast extract, 0.5; peptone, 0.25; L(-) sorbose, 0.5; $FeSO_4.7H_2O$, 0.005; $ZnSO_4.7H_2O$, 0.0014; $MnSO_4.5H_2O$, 0.0022; $CoCl_2$, 0.002; and Tween-80, 2 ml l^{-1}. Cultivation was carried out on a 5.0% mycelial inoculum and incubated for 8 days at 30 °C. The optimum culture of *A. niger* A20 yielded 27.53 U ml^{-1} of cellobiose. The volumetric activity of cellobiase obtained was higher than that reported by other investigators [11,12,15,18,19].

The culture filtrate was concentrated by ultrafiltration and fractionated using 50% ethanol. The partially purified enzyme produced, with a specific activity of 50 U mg protein^{-1}, was used for enzyme immobilization.

Immobilization on chitosan was achieved using glutaraldehyde (GA) as a bifunctional crosslinking reagent. The effect of GA concentration on the immobilized activity of *A. niger* A20 cellobiase was also investigated (Table 5). The maximum immobilization yield (i.e. 92.3%), with an immobilized enzyme activity of 1104 U g carrier^{-1}, was at a GA concentration of 0.3% (v/v). Above 0.3%, the values of both parameters (immobilized activity and immobilization yield) decreased. On the other hand, 0.2% (v/v) GA was used by Desai et al. [7]. The drop in the immobilized activity with higher GA concentration may be partially due to the increase in the crosslinking of the enzyme molecule to the support at different sites, which results in conformational changes to the enzyme molecule [20].

The results in Table 6 show that the loading efficiency of the cellobiase activity immobilized on chitosan can be improved by increasing the amount of the enzyme initially added to the carrier. The maximum immobilization yield (93.47%), with a loading efficiency of 1570 U g carrier^{-1}, was reached when the enzyme added was 2400 U g carrier^{-1}. This result is comparable to that reported by Bissett and Sternberg [4] (1590 U g carrier^{-1}).

Immobilization of *A. niger* A20 by entrapment was then carried out using polyacrylamide gel. The effect of polyacrylamide concentration on the entrapped *A. niger* A20 cellobiase activity was investigated (Table 7). In all cases, the amount of crosslinking monomer (i.e. N,N-methylene bisacrylamide) added was 5% (w/w) of the total monomer content. The results indicate that an increase in gel concentration leads to a corresponding increase in cellobiase activity until the former reaches 10% (w/w) concentration. At this point, the maximum immobilization yield is obtained (i.e. 40%), and the total activity of the immobilized enzyme is 272 U 100 ml gel^{-1}.

The effect of porosity (crosslinking level) of the gel was investigated using a 10% gel concentration with different crosslinking levels (2.5–10%, w/w, of the total monomer content). The results (Table 8) show that an increase in crosslinking level has an adverse effect on the specific activity but leads to a gradual increase in the immobilization yield. These results are similar to those reported by Roy et al. [6].

Table 7
Effect of acrylamide concentration on the immobilization yield of *Aspergillus niger* A20 cellobiase

Acrylamide concentration (%)	Immobilized enzyme			Unbound enzyme		Immobilization yield (%), $\frac{I \times 100}{A-B}$
	Total activity (U 100 ml gel^{-1}), I	Total protein (mg 100 ml gel^{-1})	Specific activity (U mg protein^{-1})	Total activity (U 100 ml gel^{-1}), B	Total protein (mg 100 ml gel^{-1})	
2.5	169.6	7.20	23.55	304.0	8.80	34.19
5.0	203.2	8.96	22.67	240.0	7.04	36.28
7.50	240.0	11.77	20.39	152.0	4.16	37.00
10.00	272.0	14.24	19.10	120.0	1.76	40.00
12.50	275.0	14.62	18.80	48.0	1.36	36.56

Note: total activity of the enzyme added (A) = 800 U 100 ml gel^{-1}; initial total protein added = 16 mg 100 ml gel^{-1}; crosslinking concentration = 5% (w/w) of the total monomer content.

Table 8
Effect of crosslinking level of a 10% polyacrylamide gel on *Aspergillus niger* A20 cellobiase

Crosslinking of polyacrylamide (%)	Immobilized enzyme			Unbound enzyme		Immobilization yield (%), $\frac{I \times 100}{A-B}$
	Total activity (U 100 ml gel^{-1}), I	Total protein (mg 100 ml gel^{-1})	Specific activity (U mg protein^{-1})	Total activity (U 100 ml gel^{-1}), B	Total protein (mg 100 ml gel^{-1})	
2.50	236.0	11.36	21.37	89.0	4.96	35.11
5.00	272.0	14.24	19.10	120.0	1.76	40.00
7.50	288.0	15.52	18.55	69.2	0.48	39.40
10.00	290.0	15.84	18.30	32.0	0.16	37.77

Note: total activity of enzyme added (A) = 800 U 100 ml gel^{-1}; initial total protein added = 16 mg 100 ml gel^{-1}.

Table 9
Maximum loading of *Aspergillus niger* A20 cellobiase in polyacrylamide gel

Added enzyme		Immobilized enzyme			Unbound enzyme		Immobilization yield (%), $\frac{I \times 100}{A-B}$
Total activity (U 100 ml gel^{-1}), A	Total protein (mg 100 ml gel^{-1})	Total activity (U 100 ml gel^{-1}), I	Total protein (mg 100 ml gel^{-1})	Specific activity (U mg protein^{-1})	Total activity (U 100 ml gel^{-1}), B	Total protein (mg 100 ml gel^{-1})	
400	8	140.0	7.50	18.66	24.0	0.54	37.23
800	16	272.0	14.24	19.10	120.0	1.76	40.00
1200	24	428.0	20.68	20.69	200.0	3.32	42.80
1600	32	612.0	27.50	22.23	273.0	4.48	46.12
2000	40	720.0	30.40	23.68	394.2	9.60	44.80

Note: acrylamide concentration was 10%; crosslinking concentration 5%.

Other experiments have been performed using a 6% crosslinking gel for β-xylosidase immobilization [21].

The loading of the enzyme within the gel was investigated by varying the activity (Table 9). The results indicate that the maximum immobilization yield (i.e. 46.12%), with an immobilized activity of 612 U 100 ml gel^{-1}, was reached when the added total enzyme activity was 1600 U 100 ml gel^{-1}. Since 100 ml gel contained 10 g acrylamide monomer, the maximum loading of the enzyme can be given as 61.2 U g carrier^{-1}. This value was low compared to those recorded for chitosan (1570 U g carrier^{-1}) (Table 6). Therefore, the enzyme immobilized on chitosan was used for the remainder of the experimental work.

The immobilized enzyme was most active at pH 4.5–5.0, similar to the free enzyme. This optimal pH range is similar to that of *Trichoderma reesei* cellulase, which was between 4.8 and 5.0 [2]. This may provide an additional advantage for both free and immobilized *A. niger* A20 cellobiase for practical applications.

The free *A. niger* A20 cellobiase had an optimal temperature of 55 °C, whereas the optimal temperature for the immobilized enzyme increased to 65 °C. The shift in the optimal temperature to a higher value indicates that the applied immobilization procedure contributes to a greater stability.

Thermal stability investigations of the free and chitosan immobilized *A. niger* A20 cellobiase were carried out

Table 10
Thermal deactivation of free and chitosan immobilized *Aspergillus niger* A20 cellobiase

Enzyme	Incubation temperature (°C)	Rate constant of deactivation ($\times 10^{-2}$ min^{-1})	Activation energy of denaturation (kcal mol^{-1})
Free	55.0	2.57	29.0
	65.0	3.60	
	75.0	6.60	
Chitosan immobilized	55.0	1.33	33.0
	65.0	1.60	
	75.0	2.70	

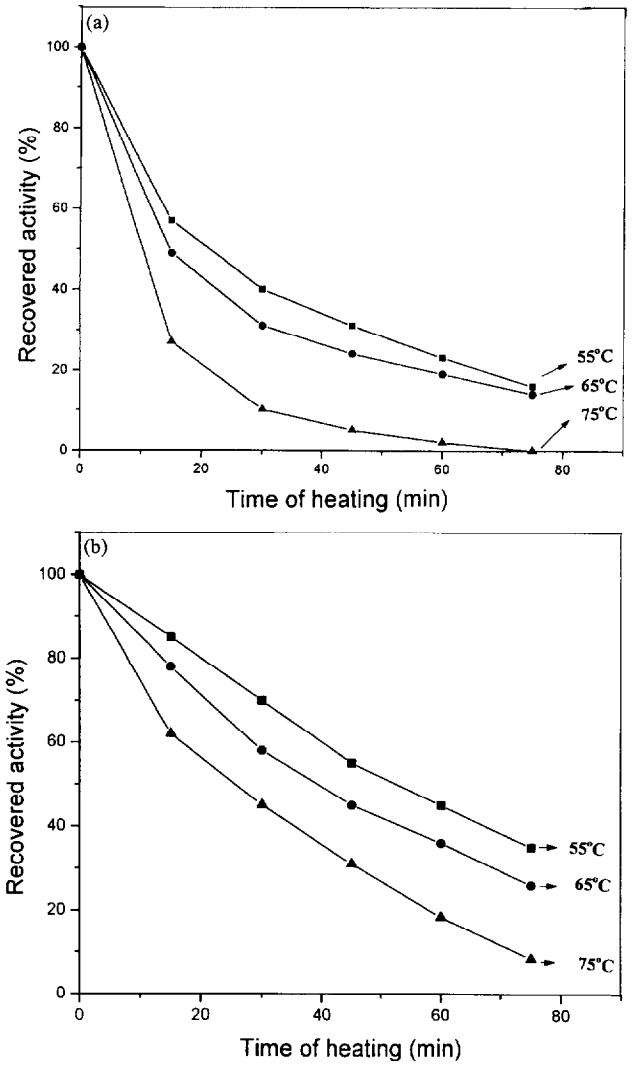

Fig. 2. Thermal stability of free and immobilized *A. niger* A20 cellobiase: (A) free enzyme, (B) chitosan immobilized enzyme.

(Fig. 2). In general, the immobilized enzyme was more stable than the free enzyme. For example, the deactivation rate constant at 65 °C for chitosan immobilized cellobiase was 1.6×10^{-2} min^{-1}, which is lower than that of the free enzyme (3.6×10^{-2} min^{-1}) (Table 10). Using the Arrhenius equation, the activation energy for thermal denaturation of the free enzyme is about 29 kcal mol^{-1}, which is 4 kcal mol^{-1} lower than that of the chitosan immobilized enzyme.

The immobilized enzyme exhibited a K_m value (1.24 mM) slightly higher than the free enzyme's (1.05 mM). On the other hand, the maximum reaction rate (V_{max}) of the immobilized enzyme was slightly lower than the free enzyme's (60 and 71 U mg protein^{-1} respectively). These results may suggest a diffusional limitation of the substrate. In addition, the fixation process of the enzyme on the carrier may impair or prevent the proper conformational changes required for catalysis [22]. The increase in K_m values and the decrease in V_{max} after the immobilization of β-glucosidases have already been reported [7].

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