Utilization of water hyacinth cellulose for production of cellobiase-rich preparation by *Aspergillus niger* 1

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Abstract

Production of a cellobiase-rich preparation by *Aspergillus niger* 1 was achieved using water hyacinth cellulose as the sole carbon source in the culture medium. Production of cellobiase, carboxymethylcellulase (CMC-ase) and filter paper (FP)-cellulase was favoured by controlling the pH of the culture medium during fermentation at 5.0. Sodium citrate (0.5%), sodium phytate (0.1%), Tween-80 (0.2%, v/v) and asparagine (0.07%) had stimulating effects on the productivity of cellobiase, CMC-ase and FP-cellulase. Potassium dihydrogen phosphate doubled the yield of CMC-ase but had a slight effect on FP-cellulase and cellobiase. Wheat bran had a pronounced stimulating effect on the production of cellobiase and CMC-ase. The combined effects of these stimulators resulted in an enzyme preparation rich in cellobiase and contained 18.5, 0.29 and 2.21 U/ml of cellobiase, FP-cellulase and CMC-ase, respectively. A high cellobiase/FP-cellulase ratio of 63.8:1 was thus obtained with the fungal enzyme preparation. The cellobiase activity was maximal at pH 5.0 and showed good thermostability.

Introduction

Cellobiase (β-glucosidase, β-D-glucosidase glucohydrolase; EC 3.2.1.21) is an enzyme which catalyses the hydrolysis of various compounds with β-D-glucosidic linkages. It has enzymic properties depending on the source and conditions under which the enzyme is produced. Cellobiase plays a crucial role in large-scale saccharification by removing cellobiose (Berghem *et al.*, 1975; Wood, 1968). Cellobiase preparations can thus be supplemented to cellulase preparations which are poor in cellobiase to enhance cellulosic saccharification.

In Egypt, the water hyacinth (*Eichhornia crassipes*), may even prevent navigation in some areas of the River Nile. It contains a high proportion of cellulose, and a possible solution to the problems the water hyacinth creates is in its utilization as a carbon source for the fungal production of useful enzyme preparations. The present work is concerned with the use of hyacinth cellulose as a carbon source for the production of cellobiase-rich enzyme preparations by the local fungal strain *Aspergillus niger* 1.

Materials and methods

Micro-organism

Aspergillus niger 1, a local isolate, was used in the present work. It was obtained from the Centre of Cultures of the National Research Centre, Dokki, Cairo, Egypt.

Water hyacinth cellulose

Eichhornia crassipes, the water hyacinth, was collected, dried and milled. Cellulose was isolated from the plant according to the method of Whistler *et al.* (1948).

Corn steep

The concentrated corn steep liquor (48%) was supplied by the Egyptian Corporation for the Production of Starch and Glucose, Torra, Cairo, Egypt.

Maintenance of stock cultures

The original cultures were kept on potato dextrose agar slants with 5.0% cellulose at 28°C.

Culture medium

Unless otherwise stated, the culture medium (Mandels and Weber, 1969) consisted of (g/l): water hyacinth cellulose, 30.0; lactose, 1.0; $(NH_4)_2SO_4$, 1.4; urea, 0.3; proteose peptone, 1.0; KH_2PO_4, 2.0; $CaCl_2$, 0.3; $MgSO_4.7H_2O$, 0.3; together with (mg/l) $FeSO_4.7H_2O$, 5.0; $MnSO_4.H_2O$, 1.6; $ZnSO_4.7H_2O$, 1.4; $CoCl_2$, 2.0; and Tween-80, 1 ml.

Cultivation

A spore suspension of 7-day-old slants was transferred (1.0 ml) to 50 ml of the basal medium. The inoculum was incubated at 28°C on a rotary shaker at 200 rpm for 5 days. Cultivation was made in 250 ml Erlenmeyer flasks, each containing 50 ml of sterile medium. The inoculum (1.0 ml) was transferred to the culture medium and the flasks were incubated at 30°C on a rotary shaker at 200 rpm for the desired incubation period. The culture broth was then filtered off to obtain the culture filtrate.

Analyses

Protein was determined by the method of Lowry *et al.* (1951). Carboxymethylcellulase (CMC-ase) activity and cotton activity were determined using the method of Mandels and Weber (1969), while filter paper (FP) activity was measured following the technique of Mandels and Sternberg (1976), and phosphoric acid swollen cellulose activity was assessed according to Ogawa *et al.* (1982). The reducing sugars released were determined by the method reported by Neish (1952). Cellobiase activity was assessed by the method of Berghem and Pettersson (1974) and the released glucose was determined using a glucose oxidase/peroxidase reagent. One unit of enzyme activity is defined as μmoles of reducing sugars (or glucose) produced per min under the assay conditions.

Results and discussion

The effect of controlling the pH of the culture medium during fermentation indicated that for a culture of *Aspergillus niger* 1 which was 9 days old, the CMC-ase, FP-cellulase and cellobiase yields at pH 5.0 were 3.5-, 17- and 8-fold those at pH 3.0 (Table 1). This demonstrated the sensitivity of these enzymes to acidic pH. These results are in agreement with those reported

Table 1 Effect of pH control of the culture medium on the production of cellulases and cellobiase by *A. niger* 1

Incubation period (days)	pH	Enzyme activities (U/ml): CMC-ase	FP-cellulase	Cellobiase
1	UC (1.97)	0.02	0.0	0.20
	3	0.018	0.0	0.146
	4	0.018	0.0	0.16
	5	0.02	0.0	0.176
2	UC (2.53)	0.47	0.0	0.25
	3	0.05	0.0	0.20
	4	0.042	0.0	0.198
	5	0.051	0.0	0.23
3	UC (3.4)	0.115	0.005	0.78
	3	0.08	0.004	0.483
	4	0.094	0.006	0.70
	5	0.134	0.006	0.61
4	UC (4.18)	0.49	0.093	1.40
	3	0.142	0.087	0.97
	4	0.54	0.10	1.32
	5	0.66	0.11	1.40
5	UC (4.19)	0.61	0.093	1.60
	3	0.55	0.078	1.00
	4	0.89	0.10	1.72
	5	0.91	0.11	2.00
6	UC (5.18)	0.67	0.10	1.93
	3	0.63	0.056	0.80
	4	0.9	0.12	2.40
	5	1.19	0.142	1.80
7	UC (5.6)	0.8	0.11	2.30
	3	0.44	0.027	0.73
	4	0.88	0.138	2.60
	5	1.177	0.145	3.05
8	UC (6.3)	0.81	0.12	2.80
	3	0.40	0.014	0.61
	4	0.92	0.16	3.40
	5	1.18	0.158	3.90
9	UC (6.7)	0.93	0.13	3.40
	3	0.34	0.01	0.60
	4	0.90	0.17	3.66
	5	1.20	0.17	4.80
10	UC (6.83)	0.95	0.13	3.70
	3	0.31	0.01	0.55
	4	0.90	0.17	3.60
	5	1.21	0.17	4.80
11	UC (6.9)	0.87	0.12	3.65
	3	0.30	0.0	0.50
	4	0.89	0.165	3.54
	5	1.14	0.16	4.60

UC, uncontrolled.

Table 2 Effect of adding substances at various concentrations on the production of cellulases and cellobiase by *A. niger* 1

Substance added	Concn (g/l)	Enzyme activity: CMC-ase	FP-cellulase	Cellobiase
Basal medium		1.29	0.19	6.0
Sodium citrate	0.5	1.18	0.16	7.56
	1.0	1.0	0.16	5.7
	1.5	0.91	0.15	5.4
	2.0	0.74	0.14	5.16
Sodium phytate	0.5	0.97	0.15	7.26
	1.0	0.8	0.13	10.44
	1.5	0.58	0.13	10.14
	2.0	0.47	0.12	9.0
Asparagine	0.3	1.27	0.194	6.66
	0.5	1.84	0.20	7.06
	0.7	1.94	0.20	7.44
	0.9	1.87	0.20	6.5
KH_2PO_4*	2.0	1.29	0.19	6.0
	2.5	2.30	0.21	6.6
	3.0	2.44	0.24	6.7
	3.5	1.73	0.17	5.2
	4.0	1.71	0.16	4.48
Wheat bran	10.0	4.5	0.19	13.8
	15.0	4.9	0.22	14.4
	20.0	5.5	0.25	17.6
	25.0	5.1	0.22	16.4
Tween-80 (ml/l)	1.0	1.29	0.19	6.0
	1.5	1.79	0.17	6.42
	2.0	1.81	0.19	7.2
	2.5	1.2	0.17	6.0
	3.0	0.87	0.13	5.4

*With basal medium.

by other authors (Mandels and Sternberg, 1976; Mandels and Andreotti, 1978). On the other hand, the yield of cellobiase increased 1.25-fold by increasing the inoculum size from 2.0 to 5.0% (from 4.8 to 6.0 U/ml).

Addition of 0.5% sodium citrate to the culture medium effected a 1.25-fold increase of cellobiase yield but produced a slightly unfavourable effect on cellulase yield (Table 2). Although the addition of 0.1% sodium phytate to the culture medium effected a 1.47-fold increase in cellobiase yield (10.44 U/ml)

Table 3 Effect of various nitrogen sources on the production of cellulases and cellobiase by *A. niger* 1

N_2	N_2 source (g/l)	Enzyme activities (U/ml): CMC-ase	FP-cellulase	Cellobiase
Control	Urea, 0.3; $(NH_4)_2SO_4$, 1.4; proteose peptone, 1.0	1.29	0.19	6.00
A	Urea, 0.3; $(NH_4)_2SO_4$, 1.4; yeast extract, 1.45; corn steep, N, 1.41 g	0.62	0.06	3.73
B	Urea, 0.3; $(NH_4)_2SO_4$, 1.4; yeast extract, 1.45; corn steep, N, 1.41 g; Na phytate, 2.0	1.45	0.193	4.15
C	Urea, 1.32; $(NH_4)_2SO_4$, 1.4; yeast extract, 1.45	1.60	0.17	0.50
D	Urea, 1.32; $(NH_4)_2SO_4$, 5.6; yeast extract, 1.45; wheat bran, 10	1.54	0.17	8.69
E	Casein, 15	0.61	0.10	0.60
F	Casein, 15; lactose, 20	1.84	0.14	1.90
G	Urea, 0.3; $(NH_4)_2SO_4$, 1.4	0.17	0.90	1.44
H	Urea, 0.3; $(NH_4)_2SO_4$, 1.4; yeast extract, 1.45	0.19	0.045	1.68

yet it resulted in a decrease in CMC-ase (~31%) and FP-cellulase (38%) yields (Table 2). In this case, the cellobiase/FP-cellulase ratio reached 80:1 compared with 31:1 without added sodium phytate.

The production of cellobiase by *A. niger* 1 was improved by up to 20%, when the culture medium was supplemented with 0.2% (v/v) Tween-80, but a higher concentration resulted in lower enzyme yields (Table 2). The addition of higher concentrations of Tween-80 had an adverse effect on the production of FP-cellulase. These results are in accord with those reported for cellulases and cellobiases from *Aspergillus terreus* (Ghosh and Kundu, 1980) and *Aspergillus wentii* (Srivastava *et al.*, 1987). On the other hand, the addition of asparagine to the culture medium had almost no effect on FP-cellulase activity, but the addition of 0.07% asparagine afforded a 60% increase of CMC-ase activity and a 24% increase of cellobiase yield (Table 2). Likewise, increasing the level of potassium dihydrogen phosphate in the culture medium from 0.2% to 0.3% doubled the yield of CMC-ase but had only a slight effect on FP-cellulase and cellobiase (Table 2).

Table 4 Effects of pH and temperature of the reaction on cellulase and cellobiase activities of *A. niger* 1 culture filtrate

		Enzyme activities (U/ml):				
pH	°C	CMC-ase	FP-cellulase	Cotton	H_3PO_4 swollen cellulose	Cellobiase
3.5*		2.29	0.20	0.0016	0.0020	13.83
4.0*		3.00	0.25	0.0017	0.0030	12.96
4.5*		4.10	0.261	0.0022	0.0034	16.63
5.0*		4.80	0.296	0.00185	0.0034	18.50
5.2*		4.26	0.30	0.0017	0.0031	18.50
5.5*		4.17	0.27	0.0017	0.0028	17.00
	35**	3.60	0.20	0.0015	0.0021	12.40
	40**	3.90	0.22	0.0016	0.0023	16.53
	45**	4.40	0.26	0.0017	0.0030	16.56
	50**	4.80	0.30	0.0019	0.0037	18.50
	55**	5.17	0.32	0.0017	0.0026	20.50
	60**	5.00	0.25	0.0016	0.0021	17.50

* Temperature of reaction was 50°C.
** pH for CMC-ase was 5.2; FP, cotton and H_3PO_4 swollen cellulose were 5.0; and cellobiase was 4.5.

Wheat bran had the most stimulating effect on the production of CMC-ase and cellobiase. Thus on adding 2.0% of wheat bran to the culture medium the yield of CMC-ase, FP-cellulase and cellobiase reached 4.6-, 1.3- and 2.93-fold those found without added wheat bran (Table 2).

The effect of nitrogen sources and their levels in the culture medium on the productivity of cellobiase and cellulases by *A. niger* 1 indicated the suitability of the nitrogen sources in the basal culture medium (Table 3). The relatively higher cellobiase yield on using mixture D of nitrogen sources (Table 3) may be due to the stimulating effect of wheat bran. The combined stimulating effects of the foregoing additives to the culture medium afforded an enzyme preparation from *A. niger* 1, which was 9 days old, comprising 18.5, 0.29 and 2.21 U/ml of cellobiase, FP-cellulase and CMC-ase, respectively. This enzyme preparation of *A. niger* 1 was thus characterized by a high proportion of cellobiase and feeble FP-cellulase activity. The cellobiase activity obtained was higher than those reported for *A. phoenicis* (Allen and Andreotti, 1982) and *A. wentii* (Ghose *et al.*, 1985).

The cellobiase component of *A. niger* 1 enzyme preparation showed maximal activity at pH 5.0 and 55°C, while the cellulase component showed maximal activity on CMC at pH 5.0 and 55°C, on FP at pH 5.2 and 55°C and on cotton and phosphoric acid swollen cellulose at pH 4.8 and 50°C (Table 4). In general, the cellobiase component of the *A. niger* 1 enzyme preparation showed good stability since it retained 54% of its activity after heating at pH 5.2 and 55°C for 48 h. These results are in accord with those reported for other fungal cellobiases (Deschamps and Huet, 1984; Cauchon and Le Duy, 1985).

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