Culture conditions for enhanced cellulase production by a native strain of *Penicillium purpurogenum*

J. Steiner*, C. Socha and J. Eyzaguirre

A cellulolytic wild-type strain of *Penicillium purpurogenum* was isolated from a soil sample in southern Chile. It grew best at 28°C from an inoculum of 4 × 10^7 spores/100 ml medium. Highest endoglucanase activity was with Sigmacell as carbon source and corn steep liquor as nitrogen source. Wheat bran enhanced the production of endoglucanase and β-glucosidase. The enzymes in the crude supernatants were stable up to 50°C and between pH 4.4 and 5.6 for 48 h.

Key words: Cellulase, enzyme production, β-glucosidase, *Penicillium purpurogenum*, xylanases.

Enzymatic hydrolysis of cellulose on an industrial scale is hindered by the high cost of the enzymes involved. There has therefore been much research directed towards finding new microbial sources of the enzymes, particularly amongst fungi of the genus *Trichoderma* (Wood 1968; Mandels 1982). However, although *Trichoderma* spp. produce high activities of cellulases, they secrete low amounts of β-glucosidase, an enzyme necessary for the conversion of cellobiose to glucose (Mandels & Andreotti 1978). Other fungi, such as members of the genus *Penicillium*, have received less attention, although they may secrete similar amounts of cellulases and relatively high amounts of β-glucosidase (Sasaki *et al.* 1983; Brown *et al.* 1987b; Tischler *et al.* 1989).

We have isolated a strain of *Penicillium purpurogenum*, from a soil sample (Musalem *et al.* 1984), that grows well on wheat straw and produces high cellulase activity. The properties of the β-glucosidase from this strain have also been studied (Hidalgo *et al.* 1992). In the present study, some of the culture parameters that influence the production of cellulases by *P. purpurogenum*, and the effects of temperature and pH on the stability of these enzymes in culture supernatants, have been studied.

J. Steiner and C. Socha are with the Facultad de Ciencias Quimicas y Farmaceuticas, Universidad de Chile, Casilla 233, Santiago 1, Chile; fax: (562) 222 7900. J. Eyzaguirre is with the Laboratorio de Bioquimica, Pontificia Universidad Católica de Chile, Casilla 114-D, Santiago, Chile. *Corresponding author.

Materials and Methods

Fungal Strain and Growth

The *P. purpurogenum* strain used, which can use cellulose as the only carbon source, has been described previously (Musalem *et al.* 1984). Liquid media for enzyme production were inoculated either with 4 × 10^7 spores/100 ml or with mycelium from pre-cultures containing carbon sources as indicated, inoculated with 4 × 10^6 spores/100 ml, and incubated in an orbital shaker (200 rev/min) at 28°C for varying periods of time (the whole content was used as inoculum).

Production of Cellulases in Shake Flasks

Cultures were grown in 500-ml Erlenmeyer flasks containing 100 ml of Mandels' medium (Mandels & Weber 1969) plus a carbon source (see below). The pH, 5.5 after sterilization, was adjusted when necessary with sterile acid or base. Flasks were shaken at 28°C at 200 rev/min and samples were taken aseptically, centrifuged and the supernatants kept at −20°C until analysed for enzymatic activities.

Carbon Sources

The carbon sources used, alone or combined, were cellulose (Sigmacell type 50; Sigma), wheat straw (average particle size 0.6 mm) and wheat bran.

Nitrogen Sources

Mandels' medium contains (g/l) three nitrogen sources: $(NH_4)_2SO_4$, 1.4; urea, 0.3; and peptone (Difco), 0.75. In some experiments the sulphate and urea were replaced, as indicated; peptone was always present.

Enzyme Assays

Endoglucanase activity was assayed using 4 g carboxymethylcellulose (Sigma)/l in 0.1 M acetate buffer, pH 4.8. Reducing sugars were estimated with alkaline ferricyanide after incubating for 20 min at 50°C (Anon. 1978). Filter paper cellulolytic activity (FPA) was estimated using 0.5 ml culture supernatant with 50 mg filter paper (Whatman No. 1) in 0.5 ml 0.04 M acetate buffer (pH 5.0) at 50°C for 60 min (Mandels *et al.* 1974). Xylanase activity was determined by incubating enzyme for 10 min at 50°C in 0.05 M acetate buffer (pH 4.8) using 1% oat spelt xylan (Sigma) as substrate (Poutanen *et al.* 1987). For FPA and xylanase activities, the reducing sugars liberated were quantified by the Somogyi-Nelson method (Nelson 1944). One unit of enzyme activity was defined as the amount of enzyme required to release 1 μmol reducing sugar per min. β-Glucosidase activity was determined by the release of *p*-nitrophenol from *p*-nitrophenyl-β-D-glucopyranoside at 50°C for 30 min (Hoffman & Wood 1985); 1 unit of activity was defined as the amount of enzyme which releases 1 μmol *p*-nitrophenol per min.

Results and Discussion

Influence of the Inoculum on Enzyme Production

Judging from the rate of colony growth on potato/dextrose/agar (PDA) plates, *P. purpurogenum* grew best at 28°C. Mycelia grown in 1% cellulose for 4 days and then used as inoculum gave slightly higher endoglucanase after 12 days in production flasks (12.2 U/ml) than a similar inoculum grown for 1 day (9 U/ml). No significant differences in FPA were observed: values ranged from 0.20 to 0.23 U/ml.

Pre-cultures grown in Mandels' medium containing 1% glucose or a mixture of 0.5% cellulose plus 0.5% glucose as carbon source gave dense fungal growth. However, when these cultures were transferred to a medium with 1% cellulose, only 5.5 U/ml endoglucanase and 0.18 U/ml FPA were obtained after 12 days' incubation.

When shake-flasks containing 100 ml of Mandels' medium, with 1% cellulose as carbon source, were inoculated with 4×10^4 to 4×10^8 spores/100 ml medium, best endoglucanase activity was obtained with 4×10^7 and 4×10^8 spores (10.0 and 11.0 U/ml). Sternberg (1976b) reported rapid production of enzymes after using a mycelial inoculum of *T. reesei*, because this shortened the lag period. However, a mycelial inoculum of *P. purpurogenum* did not decrease the lag period and all further experiments used a spore inoculum, of 4×10^7/100 ml medium, which gave the shortest total cultivation time.

To determine if the composition of the sporulation media influenced enzyme production, *P. purpurogenum* was grown for 7 days on different solid media and the spores were then inoculated with Mandels' medium with 1% cellulose. Similar endoglucanase activities (about 10 U/ml) were produced after 12 days whether the spores came from plates containing PDA, 1% (w/v) acid-swollen cellulose (Tansey 1971) or 1% Sigmacell. Lower activities were obtained from malt extract/agar (8.7 U/ml) and Sabouraud agar (8.0 U/ml). Spores from PDA plates were used in all subsequent experiments.

Influence of Nitrogen Source on Cellulase Production

Highest enzyme activities were obtained with corn steep liquor (CSL) as nitrogen source; this gave 3.5-fold higher endoglucanase and FPA activities, and more than 2-fold higher β-glucosidase and xylanase activities than those obtained in Mandels' medium (Table 1). When the nitrogen concentration was doubled (0.88 g N/l), or when mixed

Table 1. Effect of nitrogen source on enzyme production by *Penicillium purpurogenum*.

Nitrgoen source*	Enzymatic activity (U/ml)†			
	Endoglucanase	FPA	β-Glucosidase	Xylanase
0.44 g N/l				
Mandels'	8.2	0.21	0.39	2.1
Corn steep liquor (CSL)	29.2	0.75	1.04	5.5
$NaNO_3$	6.9	0.22	0.01	1.1
NH_4NO_3	5.2	0.13	0.01	0.6
Urea	3.8	0.23	0.08	1.1
$(NH_4)_2SO_4$	3.6	0.09	0.01	0.6
0.88 g N/l				
Mandels'	5.3	0.09	0.48	1.8
CSL	0.0	0.03	0.23	0.5
CSL + $NaNO_3$	0.0	0.03	0.14	0.3
CSL + NH_4NO_3	34.5	0.57	0.81	5.6
CSL + Urea	0.0	0.04	0.10	0.5

*Cultures were performed in Mandels' medium with the nitrogen sources indicated and 1% Sigmacell.

†Determined after 10 days of cuture.

FPA—Filter paper activity.

Table 2. Enzyme production by cultures of *P. purpurogenum* grown with different carbon and nitrogen sources.

Culture medium*	Enzymatic activity (U/ml)†			
	Endoglucanase	FPA	β-Glucosidase	Xylanase
S + M	10.0	0.33	0.46	2.31
S + L	29.2‡	0.75‡	1.51	5.40
S + M/L	18.8	0.29	0.38	3.90
S/B + M	12.5	0.21	1.60	3.00
S/B + L	43.1‡	0.12	2.25	6.25
S/B + M/L	30.3	0.38	2.70	8.37
W + M	5.9	0.16	1.05	10.40
W + L	0.3	0.01	1.04	2.92
W + M/L	3.1	0.05	1.04	6.60
W/B + M	5.5‡	0.19	4.80	8.29
W/B + L	0.3	0.02	2.56	6.78
W/B + M/L	1.6	0.03	2.08	5.65

*Each with carbon source (1% final concentration) [S—Sigmacell; S/B—Sigmacell + wheat bran (3 : 1); W—wheat straw; W/B—wheat straw + wheat bran (3 : 1)] and nitrogen source (0.05% N) [M—Mandels' medium; L—corn steep liquor; M/L—Mandels' medium + corn steep liquor (1 : 1)].
†Determined after 12 days unless stated otherwise.
‡Determined after 10 days.
FPA—Filter paper activity.

nitrogen sources were used, enzyme production did not increase, except for that of endoglucanase in the medium containing CSL plus NH_4NO_3. Enzyme production was very sensitive to CSL concentration; when this was doubled, very little enzyme activity was produced.

Effect of Carbon Source on Cellulase Production

Highest production of each of the four enzymes occurred with a different culture medium (see Table 2). The optimum medium for FPA contained Sigmacell and CSL (0.75 U/ml); for β-glucosidase, wheat straw plus wheat bran and Mandels' medium (4.8 U/ml); for endoglucanase, Sigmacell plus wheat bran and CSL (43.1 U/ml); and for xylanases, wheat straw and Mandels' medium (10.4 U/ml).

A stimulation in the production of several fungal hydrolases, especially β-glucosidase, has been observed when wheat bran is added to culture media (Brown *et al.* 1987b; Jain *et al.* 1990). However, when wheat bran was used as the only carbon source, enzyme activities were very low [Brown *et al.* 1987b; present study (data not shown)].

Penicillium purpurogenum compares favourably with other wild-type strains of *Penicillium* in enzyme production. Studying *P. funiculosum*, Joglekar & Karanth (1984) obtained 22.5 U endoglucanase, 2.0 U FPA and 7.0 U β-glucosidase per ml, and Hoffman & Wood (1985) reported 0.31 U FPA and 1.87 U xylanase per ml.

Effect of pH on Enzyme Production

When *P. purpurogenum* was grown in Mandels' medium, the pH values initially fell, then rose to 6 (Figure 1). In media with CSL, pH values fluctuated between 5.8 and 6.7. When ammonium salts were used as nitrogen source, pH fell to values between 3 and 4; such low pH values may have inactivated the enzymes or affected their production, thus explaining the low values reported in Table 1. With the remaining nitrogen sources examined, the pH rose to values over 7, probably producing a similar inhibitory effect. Similar observations were made by Stemberg (1976a) in studies on *T. viride* and by Joglekar & Karanth (1984) and Brown *et al.* (1987a) in studies on *P. pinophilum*.

Stability of the Enzymes

Enzyme activities in culture supernatants, when held at 50°C, were most stable at pH 4.8 to 5.2 (Table 3).

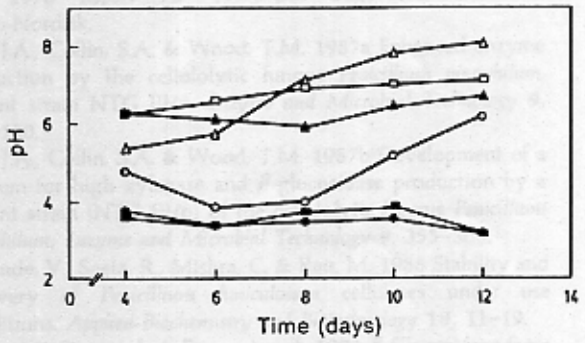

Figure 1. pH profiles of cultures of *P. purpurogenum* using Sigmacell (1% w/v) as carbon source and Mandels' medium (○), $(NH_4)_2SO_4$ (●), $NaNO_3$ (□), NH_4NO_3 (■), urea (△) or corn steep liquor (▲) as nitrogen source at 0.44 g N/l.

Table 3. Enzyme stability of crude supernatants at different pH values.*

pH	Residual activity (%)†							
	Endoglucanase		β-Glucosidase		FPA		Xylanase	
	4 h	48 h	4 h	48 h	3 h	48 h	4 h	48 h
3.6	66	38	34	3	20	0	ND	ND
4.0	82	57	81	2	50	10	ND	ND
4.4	97	83	91	37	68	11	49	23
4.8	81	78	100	59	83	37	59	19
5.2	100	82	85	43	88	56	58	20
5.6	90	77	73	53	80	54	49	17

*Incubations were performed at 50°C in 0.04 M acetate buffer in the absence of substrate.
†Note that 100% activity (7.0, 0.49, 0.27 and 7.5 U/ml for endoglucanase, β-glucosidase, filter paper activity (FPA) and xylanase, respectively) corresponds to the activity of the crude supernatant before incubation at the indicated pH value.
ND—Not determined.

Table 4. Enzyme stability of crude supernatants at different temperatures.*

Temperature (°C)	Residual activity (%)†			
	Endoglucanase	β-Glucosidase	FPA	Xylanase
4	100	72	75	95
28	100	70	66	100
40	98	76	59	67
50	78	59	37	19
60	16	10	<1	29

*Incubations, for 4 h (60°C) or 48 h (other temperatures), in 0.04 M acetate buffer at pH 4.8.
†100% activities of enzymes as given in Table 3.
FPA—Filter paper activity.

Endoglucanase was most stable at pH 4.8, with 98% activity retained after 48 h at 40°C (Table 4).

Our data are comparable with those obtained with the cellulases of *P. funiculosum*, which are most stable in the pH range of 4 to 5 (Desphande *et al.* 1988).

The results presented here indicate that this *P. purpurogenum* strain may be useful in studies on lignocellulose biodegradation. Work is in progress to characterize the enzymes participating in this process.

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