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PRODUCTION OF HEMICELLULASES BY THREE FUNGI PATHOGENIC TO SUGAR CANE*

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ABSTRACT

Three fungal pathogens, *Ceratocystis paradoxa* (*CP*), *Cephalosporium sacchari* (*CS*), and *Marasmius sacchari* (*MS*) were screened for the production of hemicellulose-degrading enzymes (hemicellulases) by induction on bagasse hemicellulose B, and on a commercial preparation of hemicellulose (crude xylan). All three pathogen initially grew poorly on hemicellulose B and "crude xylan" as carbon source. Profuse growth was induced, however, by using mixtures of hemicellulose B and sucrose in the culture media for *CP* and *CS* until the organisms were capable of growing on media containing only hemicellulose B. These isolates were classified as CS_1 and CP_1. Profuse growth occurred when *CS* and *CP* were grown on carboxymethylcellulose (CMC) and also when these cultures were transferred to media containing only hemicellulose B. These isolates were classified as CS_2 and CP_2. When the above four isolates were grown on hemicellulose B as carbon source in submerged liquid culture, only CP_1 did not produce any extra-cellular hemicellulase(s), and CP_2 produced the highest yield of enzyme. CS_2 and CP_2 also produced extra-cellular CM-cellulase(s). The CP_2-culture isolate was selected for the study of conditions for the optimal production of extra-cellular hemicellulase(s). A preliminary study of the action of enzymes from *CS* and *CP* isolated on hemicellulose is reported.

INTRODUCTION

Plant pathogens are known to produce enzymes which cleave virtually every kind of glycosidic linkage present in the plant cell-wall polysaccharides[1]. The synthesis of these enzymes (polysaccharases) appears to be controlled by a combination of induction and repression[2]. The carbohydrates in the environment of the pathogen regulate the production of polysaccharases, and the *in-vitro* production of these enzymes can often be induced by growth of the pathogen on cell-wall preparations of the plant, and on isolated polysaccharides as carbon source in culture media. The

*Studies of Hemicelluloses: Part I.

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production of polysaccharases, *e.g.*, hemicellulases and cellulases, is common among fungi and fungal pathogens[2–10], but several different bacteria[11–13], soil micro-organisms[14], rumen bacteria[15–18], rumen protozoa[19], and ruminant caecal bacteria[20] are also known to produce these enzymes in their native environments.

Ceratocystis paradoxa (pineapple disease, *CP*), *Cephalosporium sacchari* (*CS*), and *Marasmius sacchari* (*MS*) are fungal pathogens which attack the cell-wall components of sugar cane causing stem rot. These pathogens are of potential economic importance and can occur throughout the sugar-cane growing areas of tropical Queensland.

Related studies in these laboratories on ruminant digestion of polysaccharide constituents of pasture plants have shown that hemicelluloses are incompletely digested[21,22], probably due to the encrustation of the cell-wall polysaccharides by lignin which acts as a physical barrier to the rumen micro-organisms and their enzymes[23]. It was of interest, therefore, to study the degradation of hemicellulose(s) by pure enzymes, and fungal pathogens appeared to be a likely source of extracellular enzymes of this type.

This paper reports a preliminary investigation of conditions of growth of three fungi with a view to selecting the most suitable system for hemicellulase production for further detailed studies.

EXPERIMENTAL

Cultures of the fungal plant pathogens, *Ceratocystis paradoxa*, *Cephalosporium sacchari*, and *Marasmius sacchari* were kindly provided by Dr. C. G. Hughes of the Bureau of Sugar Experimental Stations, Brisbane, Queensland. The cultures were considered to be pure, and were maintained on potato-dextrose agar slants.

Hemicellulose B, an arabino-4-*O*-methylglucuronoxylan[24], was extracted from delignified, sugar-cane bagasse with 10% sodium hydroxide[25]. A commercial sample of hemicellulose, *viz.*, "crude xylan", obtained from Calbiochem (La Jolla, California, U.S.A.) was also used. After commencing this work, we found that this "crude xylan" contains major amounts of mannan (probably a glucomannan), in addition to the expected 4-*O*-methylglucuronoxylan. Total acid hydrolysis of Calbiochem "crude xylan" was carried out by the method of Saeman *et al.*[26], and the absolute glycose composition, determined by g.l.c. of the derived alditol acetates as described previously[21], indicated the presence of 1.0% of arabinose, 31% of xylose, 42% of mannose, and 17% of glucose residues. In addition, 4-*O*-methylglucuronic acid was identified by paper chromatography.

The culture media employed for the growth of the fungal organisms, and for the production of hemicellulases was that of Hultin and Nordstrom[27], using 1% (w/v) of hemicellulose. The hemicelluloses (1 g) used in the culture medium, and as substrates for enzyme assays, were solubilized by trituration in M NaOH (10 ml), neutralised with M acetic acid (10 ml), and diluted to 100 ml with water.

Small amounts of each fungus were transferred from the potato-dextrose agar

slant to agar plate media containing the different polysaccharides and varying amounts of sucrose (Table I) and incubated at 32° under illumination. The resultant isolates, which continued to show vigorous growth when further subcultured on hemicellulose in agar, we designate CS_1 and CS_2 for *CS* induced *via* sucrose and CMC, respectively, and similarly for CP_1 and CP_2. The isolate CP_2 grew profusely on "crude xylan" also, and this substrate was utilised for the large-scale production of hemicellulases.

TABLE I

GROWTH RESPONSE OF *C. sacchari*, *C. paradoxa*, AND *M. sacchari* TO VARIOUS CARBON SOURCES IN AGAR MEDIUM

Carbon source	*Growth response*[a]		
	C. sacchari	C. paradoxa	M. sacchari
Hemicellulose B	+	+	−
"Crude xylan"	++	++	−
Sucrose + Hemicellulose B (5:5)[b]	++++	++++	+++
Sucrose + Hemicellulose B (2:8)	++++	+++	++
Sucrose + Hemicellulose B (1:9)	++++	++	N.d.
Hemicellulose B	+++	+	N.d.
Carboxymethylcellulose[b]	++++	++++	++++
Hemicellulose B	++++	++++	N.d.

[a]Growth after 2 days at 32° (*CS* and *CP*) or 25° (*MS*) under illumination: ++++, very profuse growth; +++, moderate growth; ++, slight growth; +, poor growth; −, no growth; N.d., not determined. [b]These experiments represent successive sub-cultures on the substrates shown.

The four isolates were grown in submerged liquid culture on media containing hemicellulose B to examine the comparative yields of extra-cellular hemicellulase(s). After inoculation, the culture media (50 ml) were incubated at 32° with shaking (80 strokes/min). After 97 h, a portion of the culture fluid (10 ml) was removed, and centrifuged at 20,000 *g* for 0.5 h at 5° to remove mycelia and spores before being used in enzyme assays.

The activity of total hemicellulase and CM-cellulase was determined by hydrolysis of hemicellulose B, and CMC, respectively. The enzymic digests contained 2 ml of a 0.5% polysaccharide solution in 0.1M sodium acetate (pH 5.5) and 0.1 ml of the fungal extra-cellular fluid, and were incubated at 37° for 1 h. Reducing sugars were estimated as xylose or glucose by the method of Nelson[28]. Enzymic activity was expressed as μg equiv. of D-glucose produced per min per ml of extra-cellular fluid for CM-cellulase, and as μg equiv. of D-xylose per min per ml of extra-cellular fluid for hemicellulase. The results are shown in Table II. 0.1M Acetate ion had no significant effect on the colour produced by xylose by the reducing method of Nelson.

Since CP_2 produced most hemicellulase (Table II), it was selected for further investigation of growth conditions. CP_2 cultures were grown at 25°, 32°, and 37°,

TABLE II

COMPARATIVE HEMICELLULASE AND CARBOXYMETHYL-CELLULASE ACTIVITIES OF CULTURE ISOLATES GROWN IN SUBMERGED LIQUID CULTURE ON HEMICELLULOSE B FOR 97 h AT 32°

Culture isolate[a]	*Hemicellulase activity*	*CM-cellulase activity*
CS_1	0.207	0
CS_2	0.230	0.003
CP_1	0	0
CP_2	1.083	0.042

[a]See text for classification.

TABLE III

HEMICELLULASE PRODUCTION BY CP_2 IN LIQUID CULTURE ON MEDIA CONTAINING HEMICELLULOSE B, UNDER DIFFERENT CONDITIONS OF GROWTH

Growth condition	*Time of growth (h)*	*Hemicellulase activity*
(*a*) 25°		
Stationary	23	0.07
	47	0.17
	63	0.20
Shake	23	0.08
	47	0.20
	70	0.60
	92	1.27
	119	1.41
	143	1.26
	183	1.06
(*b*) 32°		
Stationary	20	0.04
	67	0.29
	98	0.79
Shake	4	0.48
	28	1.22
	44	1.77
	75	1.75
	97	1.83
(*c*) 37°		
Stationary	23	0.38
	47	0.68
	70	0.70
	92	0.69
Shake	23	0.38
	47	0.68
	70	0.70
	92	0.68

and by stationary and shake culturing to determine the optimal conditions for production of enzyme. The results are shown in Table III.

Enzymic reactions were terminated by heating at 100° for 10 min. Solutions were concentrated, where necessary, under diminished pressure at 40°. Enzyme digests and acid hydrolysates (neutralised with $BaCO_3$) were deionized with mixed ion-exchange resins [Amberlite IRC-50(H^+) and IR-45(OH^-)] prior to paper chromatography.

Products resulting from the enzymic hydrolysis of hemicellulose B and "crude xylan" by the extra-cellular fluid of CS_1, CS_2, and CP_2 were chromatographed on Whatman No. 1 paper with ethyl acetate–pyridine–water (10:4:3) for neutral sugars, and ethyl acetate–acetic acid–formic acid–water (18:3:1:4) for uronic acids, using alkaline silver nitrate[29] and *p*-anisidine hydrochloride[30] spray reagents on duplicate papers.

RESULTS AND DISCUSSION

Table I shows that the fungi initially grew poorly on agar media containing hemicellulose B and "crude xylan". However, rapid growth (within 1.5 to 2 days) of all three species followed the inoculation of agar plates containing hemicellulose B in the presence of sucrose. Table I also shows that when these cultures were successively sub-cultured on media containing decreasing amounts of sucrose and increasing amounts of hemicellulose B, then CS_1 retained its induced capacity to grow vigorously, whereas CP_1 lost most of this ability. It was also observed that *MS* would not grow at 32°, but grew slowly at 25°, taking at least 10–14 days to attain a profuse growth. When *CS* and *CP* were pre-cultured once on agar media containing CMC, they developed the ability to grow readily on agar media containing hemicellulose B, and these isolates were called CS_2 and CP_2.

CS_1, CS_2, CP_1, and CP_2 isolates were subsequently grown in submerged liquid culture on media containing hemicellulose B and, with the exception of CP_1, grew at a rate (1.5 to 2 days, under constant shaking conditions) similar to that of cultures on agar plates. Examination of the extra-cellular fluid from the liquid cultures showed hemicellulase activity as in Table II. CP_2 produced most hemicellulase activity and was selected for the production of enzyme on a large-scale basis. The same extra-cellular fluid showed that the fungi induced on CMC, and grown on media containing hemicellulose B in liquid culture, (CS_2 and CP_2) produced small amounts of extra-cellular enzyme(s) capable of degrading CMC, (CM-cellulase), see Table II and Fig. 1. *MS* grew well on media containing CMC in liquid culture, but no extra-cellular CM-cellulase(s) were detected in the extra-cellular fluid even after 14 days of growth. This suggests that CMC is degraded by intra-cellular cellulases (*cf.* Ref. 31).

Table III shows the hemicellulase activity when CP_2 was grown in liquid culture on hemicellulose B at different temperatures and under either stationary or shaking conditions. Maximal production of enzyme resulted after 98 h of growth

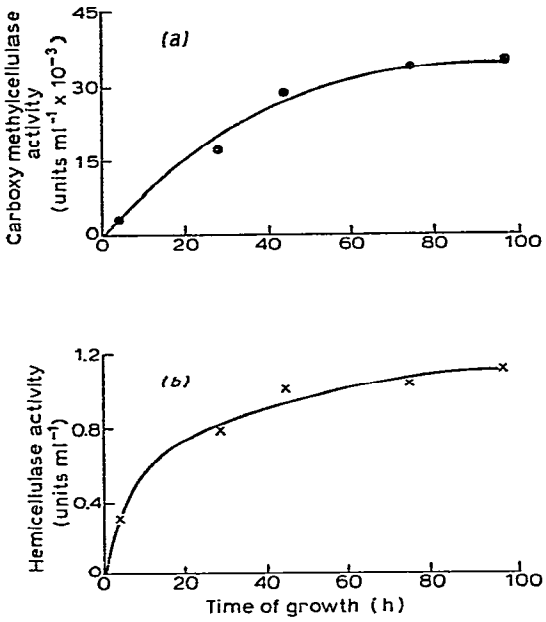

Fig. 1. Production of extra-cellular (*a*) carboxymethyl-cellulase and (*b*) hemicellulase by CP_2.

at 32°, under shaking conditions. CP_2 appeared to grow equally well on "crude xylan" and hemicellulose B.

The crude, extra-cellular hemicellulase(s) of CS_1, CS_2, and CP_2 were used to degrade hemicellulose B and "crude xylan", and the results are shown in Tables IV and V. The hemicellulases of CS_1 and CS_2 degraded hemicellulose B mainly to xylose (Table IV), which was present from the early stages of reaction and suggests the presence of exo-xylanase(s). The early presence of xylose disaccharide in the digests may also indicate the presence of endo-xylanase(s). The extra-cellular fluid from CS_2 (but not CS_1) also released arabinose as a major product from hemicellulose B. This suggests that this organism also produced an extra-cellular α-L-arabinofuranosidase which attacked the (1→3)-α-L-arabinofuranoside branch-points of hemicellulose B. Such enzymes have been isolated from fungal culture fluids[32–34], and from rumen bacterial[15,17,35] and protozoal[19,36] systems. In addition to the xylose oligosaccharides, glucose was found in the enzymic hydrolysates of hemicellulose B. This probably arose from the enzymic hydrolysis of a small glucose-containing fraction in the hemicellulose, possibly a degraded cellulose, or perhaps another glucan[37].

The production of xylose di- and tri-saccharides by the action of the hemicellulase system of CP_2 on hemicellulose B and "crude xylan" (Tables IV and V) suggests the presence of mainly endo-type hemicellulase(s). The time course of hydrolysis of "crude xylan" by the hemicellulases of CP_2 (which was grown on "crude xylan") (Fig. 2) shows that hydrolysis is still continuing after 72 h, with an apparent 32% conversion into xylose equivalents. Table V shows that, during the

TABLE IV

DEGRADATION OF HEMICELLULOSE B BY EXTRA-CELLULAR HEMICELLULASES

Culture isolate	*Time of hydrolysis (h)*	*Apparent hydrolysis[a] (%)*	*Products of hydrolysis[b,c]*	
			Major	*Minor*
CS_1	18	2.9	$\overline{\overline{\overline{Xyl}}}_1$, $\overline{Xyl}_2$	tr. (Ara, Glc)
	42	20.2	$\overline{\overline{\overline{Xyl}}}_1$, $\overline{Xyl}_2$	tr. (Ara, Glc)
	121	44.0	$\overline{\overline{\overline{Xyl}}}_1$	tr. (Ara, Glc, Xyl_2)
CS_2	18	2.6	$\overline{\overline{\overline{Xyl}}}_1$, $\overline{Xyl}_2$, $\overline{Ara}$	tr. (Glc)
	42	20.0	$\overline{\overline{\overline{Xyl}}}_1$, $\overline{\overline{Xyl}}_2$, $\overline{\overline{Ara}}$	tr. (Glc)
	121	44.7	$\overline{\overline{\overline{Xyl}}}_1$, $\overline{\overline{Ara}}$	tr. (Xyl_2, Glc)
CP_2	18	20.5	$\overline{\overline{Xyl}}_2$, $\overline{\overline{Xyl}}_3$	tr. (Xyl_1, Ara, Glc,
	42	28.6	$\overline{\overline{Xyl}}_2$, $\overline{\overline{Xyl}}_3$	Xyl_3, Xyl_4, >Xyl_4)
	121	40.7	$\overline{\overline{\overline{Xyl}}}_2$	$\overline{Xyl}$, $\overline{Ara}$, $\overline{Glc}$, tr. (Xyl_3, Xyl_4)

[a]Apparent conversion to xylose. [b]Xyl_1 refers to xylose, Xyl_n refers to (1→4)-linked xylose saccharides, Ara = arabinose, Glc = glucose, tr. refers to trace amounts. [c]Denotes relative amounts as judged visually by the intensity of staining of the sugars on the paper chromatogram.

TABLE V

DEGRADATION PRODUCTS FROM THE ENZYMIC HYDROLYSIS OF "CRUDE XYLAN" BY EXTRA-CELLULAR HEMICELLULASES OF CP_2 GROWN ON "CRUDE XYLAN"

Time of hydrolysis (h)	*Products of hydrolysis[a]*
1	Xyl_2, Xyl_3, Xyl_4
3	Xyl_2, Xyl_3, Xyl_4
6	Xyl_2, Xyl_3, Xyl_4, Glc, UD[b]
24	Xyl_1, Xyl_2, Xyl_3, Xyl_4, Glc, Man, UD
48	Xyl_1, Xyl_2, Xyl_3, Xyl_4, Glc, Man, UD
72	Xyl_1 (4), Xyl_2 (10), Xyl_3 (6), Xyl_4 (tr.), Glc (8) Man (6), UD (2)[c]

[a]See footnotes for Table IV; Man refers to mannose. [b]UD refers to unidentified higher oligosaccharides. [c]Numbers in brackets denote relative amounts.

early stages of hydrolysis, only xylose oligosaccharides were produced, and that, subsequently, glucose, mannose, and unidentified higher oligosaccharides appeared. The "crude xylan" used in these experiments yielded also mannose and glucose on acid hydrolysis, and probably contained a glucomannan. This type of component, therefore, has probably induced CP_2 to secrete extra-cellular mannanases as well as

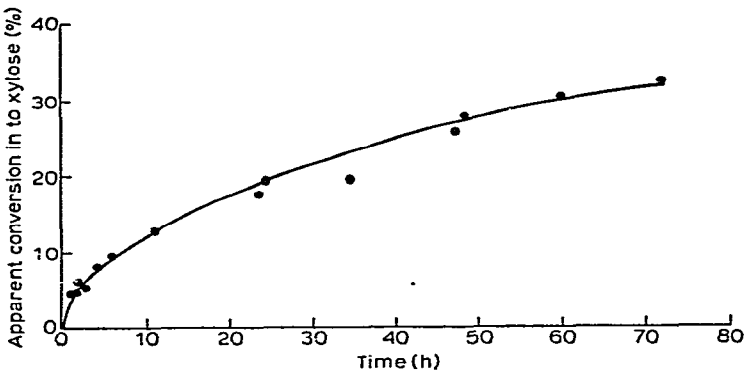

Fig. 2. Time course of hydrolysis of "crude xylan" by extra-cellular fluid hemicellulases of CP_2.

β-D-glucanases and xylanases when grown on "crude xylan". Extra-cellular mannanases from *Bacillus subtilis*[38] and *Aspergillus niger*[39] have been shown to hydrolyse both galacto- and gluco-mannans.

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