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Influence of L-Sorbose on Growth and Enzyme Synthesis of *Trichoderma reesei* C-5

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The effects of L-sorbose on the growth and biosynthesis of cellulases and other polysaccharide-degrading enzymes of *Trichoderma reesei* C-5 were studied. The specific growth rate and yield of this strain in batch culture were reduced by 23% and 46% respectively on addition of 1% (w/v) sorbose to Vogel's medium containing 1% (w/v) glucose. The specific consumption rate of both sorbose and glucose decreased in the presence of the other sugar at 1% (w/v) concentration. The addition of sorbose (1–5%) to cultures grown in 1% glucose resulted in enhanced activities of all cellulase enzymes, and particularly endoglucanase activity, which increased sevenfold in the presence of 5% sorbose. There was no significant effect on the activities of β-glucosidase, acid phosphatase and amylase. While the increased enzyme activities seemed to be correlated with a decreased rate of glucose consumption, a direct effect on some extracellular enzymes could not be ruled out.

INTRODUCTION

A high total surface to volume ratio and especially an increase in the number of growing hyphal tips are conducive to exoenzyme secretion in fungi (Stavy *et al.*, 1970). L-Sorbose, a ketohexose, has been shown to cause an aberration of 1,3-β-glucan synthesis in fungi, leading to distinct changes in morphology and growth pattern, a phenomenon known as 'paramorphogenesis', first described by Tatum *et al.* (1949) in *Neurospora crassa*. Bisaria *et al.* (1986) showed that the presence of sorbose in the growth medium of *Trichoderma reesei* QM9414 containing cellobiose resulted in the formation of tight mycelial pellets. A change in the composition of the cell wall, and increased branching and septation, were noticed. The uptake of cellobiose was also affected by sorbose. These factors have been shown to be involved in increased activities of extracellular cellulases in *T. reesei* QM9414.

The cellulases from *T. reesei* have been extensively studied for their potential applications in the hydrolysis of renewable lignocellulosic residues (Bisaria & Ghose, 1981; Gilbert & Tsao, 1983). The present work was undertaken to study the effect of sorbose on morphological changes in *T. reesei* C-5, a partially constitutive mutant derived from QM9414, and its relationship with the distribution of cellulase enzymes in various subcellular fractions of the fungus. The effects of sorbose on specific growth rate, yield and specific sugar consumption rate of the fungus were also studied for their possible involvement in increased enzyme synthesis.

METHODS

Organism and growth medium. *Trichoderma reesei* C-5, a partially constitutive cellulase-producing mutant derived from *T. reesei* QM9414 (Mishra *et al.*, 1982), was used. The culture was maintained on Vogel's minimal medium (Vogel, 1956) supplemented with 0·1% (w/v) peptone and 1% (w/v) sucrose. It was stored at 4 °C and subcultured every four weeks.

† *Abbreviation*: FPA, Filter paper activity.

The organism was grown in Vogel's minimal medium containing (w/v) 0·1% peptone, 0·2% Tween 80 and 1% glucose, 1% L-sorbose or 1% glucose plus 0·5–5·0% L-sorbose as appropriate for each experiment.

Batch cultures. These were grown with shaking at 28 °C in 500 ml flasks containing 100 ml medium. The cultures were inoculated and grown as described previously (Mishra & Gopalkrishnan, 1984). Samples were removed every 4 h (for 1% glucose and 1% glucose plus 1% sorbose) or 6 h (for 1% sorbose) during the exponential growth phase, and centrifuged at 687·5 *g* for 20 min. The supernatant was assayed for sugars, soluble protein and enzyme activities.

In experiments involving biomass estimation, cultures were grown (shaken) under identical conditions in 250 ml flasks containing 50 ml medium. The entire contents of the flasks were harvested at the end of every 6 h during the exponential growth phase and centrifuged. The mycelium was washed twice with distilled water, collected on Whatman no. 42 filter paper, and dried overnight at 85 °C to a constant weight. The biomass yield (Y) was calculated according to Trinci & Collinge (1973) using the following equation:

$$Y = \text{Maximum biomass (mg dry wt ml}^{-1}\text{)/Sugar consumed (mg ml}^{-1}\text{)}$$
$$= \Delta X/\Delta S$$

When a mixture of glucose and sorbose was used the yield (Y) was calculated as:

$$Y = \Delta X/[(\Delta S)_{\text{glucose}} + (\Delta S)_{\text{sorbose}}]$$

Estimation of sugars. Glucose was estimated in the growth medium by the Worthington Glucostat Reagent (Bergmeyer & Brent, 1974) and sorbose by the cysteine.HCl method (Dische & Devi, 1960).

The specific sugar consumption rate was defined as follows:

$$\text{Specific consumption rate} = \frac{\text{Sugar consumed (mg ml}^{-1}\text{ h}^{-1}\text{)}}{\text{Amount of biomass at time of assay (mg dry wt ml}^{-1}\text{)}}$$
$$= (dS/dt)/X$$

In the case of mixed sugars

$$\text{Specific consumption of total sugar} = [(dS/dt)_{\text{glucose}} + (dS/dt)_{\text{sorbose}}]/X$$

Subcellular fractionation. Cultures grown on different sugars (2% glucose, 2% sorbose, 1% glucose + 1% sorbose) were withdrawn in duplicate at specific time intervals and centrifuged at 687·5 *g* for 20 min. The supernatant was used to assay for extracellular enzymes. The mycelia were washed twice with distilled water and lyophilized.

A known quantity of lyophilized mycelia was suspended in a definite volume of 0·05 M-citrate buffer, pH 4·8, and sonicated (20 kHz) in an MSE sonicator (20 ml vessel, titanium probe, amplitude 6 μm) at 4 °C for 20 min with continuous cooling. The sonicated suspension was centrifuged at 11 000 *g* at 4 °C for 30 min. The supernatant was used as the source of intracellular enzymes while the residual pellet (washed three times with 0·05 M-citrate buffer, pH 4·8) suspended in a known volume of the same buffer, served as source of cell-wall-bound enzymes.

Enzyme assays. The samples were precipitated with acetone (9 vols) at 4 °C. The resulting precipitate was suspended in 0·05 M-citrate buffer, pH 4·8, in a volume equal to the original sample volume. These preparations were used for determination of enzyme activities. The assays were done in triplicate; the values for replicate assays in all cases differed by less than 10% from the mean. All enzyme activities except amylase are reported as IU ml^{-1}. One IU ml^{-1} represents the formation of 1 μmol product min^{-1} (ml enzyme solution)$^{-1}$ under the conditions of assay.

Filter paper activity (FPA) and endoglucanase activity were measured according to the method of Mandels *et al.* (1976), with 50 mg Whatman no. 1 paper (6 × 1 cm) and 1% (w/v) carboxymethylcellulose as substrate respectively. The reducing sugars released were measured by the dinitrosalicylic acid method (Sumner & Somers, 1949). β-Glucosidase was assayed according to Berghem & Pettersson (1974), with *p*-nitrophenyl β-D-glucopyranoside as the substrate.

Amylase and acid phosphatase activities were measured according to Nisizawa *et al.* (1972), with 0·2% amylose and 0·23 M-*p*-nitrophenyl phosphate as substrates. One amylase unit was defined as the amount of enzyme producing a 1·0% reduction of blue value.

Protein estimation. This was done by the Lowry method, with bovine serum albumin as the standard.

Chemicals. L-Sorbose was obtained from Fluka, and carboxymethylcellulose, cysteine hydrochloride, *p*-nitrophenyl β-D-glucopyranoside, *p*-nitrophenyl phosphate, amylose and bovine serum albumin from Sigma.

RESULTS

Growth and cellulase synthesis on glucose and sorbose. T. reesei strain C-5 was grown on 1% glucose, 1% sorbose and a mixture of 1% glucose and 1% sorbose, and growth, biomass yield and

enzyme production were measured. The exponential phase of growth lasted for 24 h, 48 h and 28 h on the three respective media. Growth occurred in the form of tight pellets in the sorbose-grown cultures, compared to the long sparse mycelial growth in the cultures with glucose alone. The maximum biomass produced on 1% glucose was 6·0 mg ml^{-1}, attained after 24 h, while on 1% sorbose only 4·7 mg ml^{-1} accumulated after 52 h (Table 1).

The rate of consumption of the sugars differed. About 98% of the glucose was consumed in 24 h and the remainder after 30 h. Sorbose was taken up relatively slowly: 20% was consumed in 24 h and the remaining 80% after 96 h. The cultures grown on 1% sorbose gave more FPA (1·4 times), protein (1·1 times), endoglucanase (3·0 times) and β-glucosidase (1·5 times) on day 6 than the glucose-grown cultures (Table 1). The drop in the culture pH was greater (pH = 3·2) in glucose cultures than in sorbose cultures (pH = 5·0) (Table 1).

Table 1 also gives the values of maximum biomass, soluble protein and enzyme activities obtained on glucose plus sorbose. No significant increase in biomass was observed over the glucose-grown cultures, the maximum of 6·35 mg ml^{-1} being attained after 24 h. The culture exhibited increased enzyme activity values for both the FPA and the endoglucanase components of cellulase. Specific endoglucanase activity was 2·4-fold higher than on glucose but slightly lower than in sorbose cultures.

Effect of sorbose on specific growth rate and yield. Addition of sorbose resulted in lowered specific growth rate of strain C-5 on 1% glucose: a sorbose concentration of 1% gave a decrease of 23%, while 5% sorbose gave a decrease of 64% (Table 2). Similarly the yield of strain C-5 decreased by 46% and 62% when 1% and 5% sorbose respectively were added to 1% glucose medium (Table 2).

Specific consumption rate of sugar. Sorbose at a concentration of 1% decreased the specific consumption rate of glucose by 15% in a culture containing 1% glucose (Table 3). This effect was more pronounced (decrease of 71·5%) when 5% sorbose was added to 1% glucose medium. The

Table 1. *Synthesis of cellulase by T. reesei C-5 on glucose and sorbose medium after 6 d*

All parameters were measured on three independent cultures. Values for replicate assays in all cases differed by less than 7% from the mean.

Carbon source	Soluble protein (mg ml^{-1})	Maximum biomass* (mg ml^{-1})	Minimum pH†	FPA‡ (IU ml^{-1})	Endoglucanase‡ (IU ml^{-1})	β-Glucosidase‡ (IU ml^{-1})
Glucose (1%)	0·78	6·0 (24 h)	3·2	0·63 (0·8)	4·8 (6·15)	0·4 (0·51)
Sorbose (1%)	0·88	4·7 (52 h)	5·0	0·85 (0·96)	14·2 (16·13)	0·61 (0·69)
Glucose (1%) + sorbose (1%)	1·38	6·35 (24 h)	3·2	1·3 (0·94)	20·0 (14·50)	0·53 (0·38)

* Maximum biomass attained after the time indicated in parenthesis.
† Minimum pH attained after 24 h of culture.
‡ The numbers in parentheses indicate the specific activity [IU (mg protein)$^{-1}$].

Table 2. *Effect of sorbose on growth parameters of T. reesei C-5 in submerged culture on 1% glucose*

Each specific growth rate (= ln2/doubling time) and yield is the mean of three experiments. The values for replicate assays in all cases differed by less than 15% from the mean.

Growth parameter	Sorbose concn in medium			Value on 1% sorbose expressed as percentage of 0% value
	0%	1%	5%	
Specific growth rate (μ, h^{-1})	0·215	0·165	0·078	76·7%
yield, (Y, g cell dry wt/g sugar)	0·52	0·28	0·20	53·8%

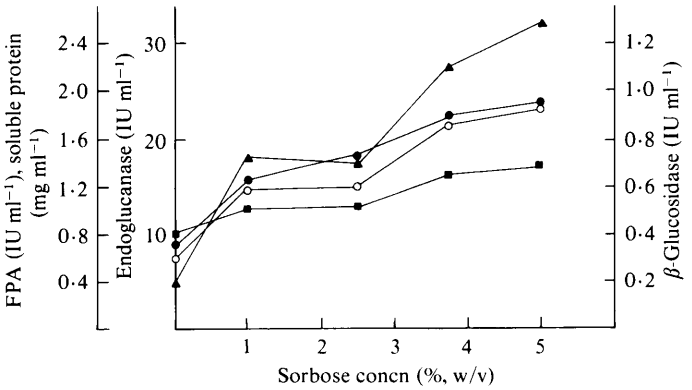

Fig. 1. Effect of increasing concentrations of sorbose on cellulase enzymes in *T. reesei* C-5 grown on 1% glucose. The extracellular supernatant harvested on day 6 was assayed for various enzymes as described in the text. The data points represent means of three experiments; values for replicate assays differed by less than 10% from the mean. Sorbose was added at the beginning of the experiment. ●, Soluble protein; ○, FPA; ▲, endoglucanase; ■, β-glucosidase.

Table 3. *Specific consumption rate of sugar(s) by T. reesei C-5 grown on glucose and/or sorbose*

All assays were done in duplicate on three independent cultures. The values for replicate assays in all cases differed by less than 7% from the mean.

Culture medium containing	Specific consumption rate (h^{-1}) of: Glucose	Sorbose	Total sugar (glucose + sorbose)
Glucose (1%)	0·2	–	0·2
Sorbose (1%)	–	0·03	0·03
Sorbose (5%)	–	0·10	0·1
Glucose (1%) + sorbose (1%)	0·17	0·025	0·195
Glucose (1%) + sorbose (5%)	0·057	0·06	0·117

specific consumption rate of sorbose was a function of the sorbose concentration in the medium; it decreased in the presence of 1% glucose.

Effect of sorbose concentration on cellulase synthesis. The presence of sorbose in the culture medium (either alone or in combination with glucose) led to increased enzyme synthesis (Table 1). The effect of increasing sorbose concentration (1–5%) in 1% glucose medium was investigated to determine if any further stimulation of enzymes could be achieved (Fig. 1). While all three enzyme activities measured were enhanced, the effect on endoglucanase was the most pronounced (sevenfold increase over the control 1% glucose culture).

Effect of sorbose on distribution of enzymes in subcellular fractions. Table 4 shows the distribution of FPA, endoglucanase, amylase, β-glucosidase and acid phosphatase in subcellular fractions of *T. reesei* C-5 grown in medium with glucose and/or sorbose. While sorbose increased the overall FPA and endoglucanase activity, the distribution of the enzymes in subcellular fractions remained unchanged when compared to glucose-grown cultures. For amylase, there was no increase in enzyme activity on addition of sorbose and the distribution also remained unchanged. In the case of β-glucosidase, sorbose markedly increased the proportion of extracellular enzyme, and both intracellular and cell-wall-associated activities were decreased when compared to glucose-grown cultures. The total β-glucosidase activity remained the same. For acid phosphatase, the total activity was increased by 38% in the mixed sugar culture compared with the glucose culture, and the extracellular fraction of the enzyme was increased by

Table 4. *Effect of* L-*sorbose on distribution of enzymes in subcellular fractions of T. reesei C-5*

Cultures were harvested at the end of day 4, and subcellular fractions were prepared and assayed for activities of various enzymes. All activities represent a mean of three separate experiments. The values in replicate assays in all cases differed by less than 10% from the mean value.

		Extracellular activity		Intracellular activity		Cell wall fraction		Total activity
Enzyme	Growth medium*	IU ml^{-1}	% of total activity	IU ml^{-1}	% of total activity	IU ml^{-1}†	% of total activity	IU ml^{-1}
FPA	G	0·38	71·6	0·15	28·3	<0·005		0·53
	S	0·63	73·0	0·23	27·0	<0·005		0·86
	G + S	0·85	75·2	0·28	24·8	<0·005		1·13
Endoglucanase	G	3·5	84·4	0·7	15·6	<0·01		4·2
	S	7·06	88·3	0·94	11·7	<0·01		8·0
	G + S	12·5	90·0	1·4	10·0	<0·01		13·9
Amylase‡	G	7·0	92·8	0·54	7·2	<0·05		7·54
	S	5·0	93·6	0·34	6·4	<0·05		5·34
	G + S	6·23	93·0	0·47	7·0	<0·05		6·7
β-Glucosidase	G	0·28	51·0	0·18	32·7	0·09	16·3	0·55
	S	0·48	81·3	0·07	11·8	0·04	6·9	0·59
	G + S	0·42	79·2	0·05	9·4	0·06	11·4	0·53
Acid phosphatase	G	0·33	56·9	0·25	43·1	<0·01		0·58
	S	0·34	82·9	0·068	16·5	<0·01		0·41
	G + S	0·65	81·2	0·15	18·7	<0·01		0·80

* G, 2% glucose; S, 2% sorbose; G + S, 1% glucose plus 1% sorbose.

† The values of 0·005 IU ml^{-1} (FPA), 0·01 IU ml^{-1} (endoglucanase, acid phosphatase) and 0·05 units ml^{-1} (amylase) correspond to the limit of detection for these assays.

‡ Amylase values are not in IU ml^{-1}. For details see Methods.

a similar proportion. Less enzyme (about 19%) remained associated intracellularly compared to 43% in the glucose-grown culture.

DISCUSSION

The effect of L-sorbose on growth parameters (specific growth rate and yield) and enzyme production in *T. reesei* C-5 has been investigated. The parent *T. reesei* QM9414 does not metabolize sorbose (Bisaria *et al.*, 1986). Both glucose and sorbose were taken up simultaneously by strain C-5 although the specific consumption rates were affected by the presence of the other sugar.

Addition of sorbose lowered the specific growth rate and yield of strain C-5 (Table 2). These results were different from those reported for *N. crassa* by Crocken & Tatum (1968) and Trinci & Collinge (1973), where addition of sorbose to glucose cultures caused no change in specific growth rate and yield.

Both FPA and endoglucanase activity were enhanced on addition of 1% sorbose to 1% glucose cultures. Such enhanced activities could not be correlated with increase in biomass content, as this remained comparable to that in glucose cultures. It seems likely that a decreased level of intracellular glucose in sorbose plus glucose cultures is responsible for this effect: both these enzymes are known to be regulated by glucose (Mandels & Weber, 1969). This explanation is supported by the observation that amylase, an extracellular constitutive enzyme in *T. reesei* (Nisizawa *et al.*, 1972) and known not to be affected by glucose, was synthesized to the same extent in glucose and in mixed sugar cultures (Table 4).

The enzyme β-glucosidase is reported to be predominantly associated with the cell wall in *T. reesei* QM9414 (Nanda *et al.*, 1982). It is also constitutive and not affected by the presence of various sugars in the medium (Mishra & Gopalkrishnan, 1984); hence its activity cannot be expected to be increased by sorbose. However, addition of sorbose is known to cause dissociation of cell-wall-associated enzymes. Thus in *T. reesei* C-5, although total β-glucosidase

activity remained the same for sorbose cultures as compared with glucose cultures, the percentage in extracellular fractions increased. Such increased release of enzymes by addition of sorbose has been attributed to increased branching and formation of an increased number of hyphal tips in this fungus (Bisaria *et al.*, 1986). The same pattern was also noticed for acid phosphatase, where more enzyme was released to the external medium in the presence of sorbose (Table 4).

Thus by carefully regulating the supply of glucose to *T. reesei* in continuous cultures it should be possible to increase the overall yield of cellulase. By supplementing the culture with paramorphogenic agents such as sorbose and certain colloids (Duff *et al.*, 1985) it should be possible to produce a mixture of enzymes, rich in all components of cellulase, ideal for efficient biomass conversion.

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