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TRANSPORT-LIMITED SUCROSE UTILIZATION AND NEOKESTOSE PRODUCTION BY *PHAFFIA RHODOZYMA*

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Summary

Growth of an astaxanthin hyper-producing strain of *Phaffia rhodozyma* on sucrose is accompanied by the accumulation of glucose and fructose in the medium due to the limited capacity of the corresponding monosaccharide transport system or systems. This is accompanied by the production of the trisaccharide neokestose by transglycosylation reactions.

Introduction

Sugar-cane molasses, containing sucrose as the major constituent sugar, is a low-cost substrate which has been investigated for the cultivation of the astaxanthin-producing yeast *Phaffia rhodozyma* (Haard, 1988). Utilization of sucrose by this yeast, however, results in the temporary accumulation of glucose and fructose in the medium, indicating that glucose and fructose transport limits the sucrose utilization rate (Meyer and du Preez, 1994). This prompted us to investigate the utilization of sucrose by *P. rhodozyma* in relation to the rates of transport of its monomers. In addition, an unknown product that accumulated in the medium during growth on sucrose was isolated and identified. The results indicate that transport of glucose and fructose limits the utilization of sucrose by *Phaffia rhodozyma* and that the trisaccharide neokestose is produced by transfructosylation reactions during growth on sucrose.

Materials and Methods

Micro-organism and cultivation

An astaxanthin-overproducing strain of *Phaffia rhodozyma* (Meyer et al, 1993) was maintained on YM agar containing (per litre) 10 g glucose, 5 g peptone, 3 g yeast extract and 17 g agar. Batch cultivations were done at 21°C in a 2 l Multigen F-200 fermentor with a working volume of 1.5 l. The stirring rate was 500 rpm and the fermentor was aerated at 1 l/min. The pH was maintained at 5 by the automatic addition of 2 M NaOH. The cultivation medium contained (per litre): 10 g sucrose, 1 g yeast extract, 0.5 g citrate, 1.8 g NH_4Cl, 2 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 0.1 g $CaCl_2.2H_2O$, 35 mg $FeSO_4.7H_2O$, 7 mg $MnSO_4.7H_2O$, 11 mg $ZnSO_4.7H_2O$, 1 mg $CuSO_4.5H_2O$, 2 mg $CoCl_2.5H_2O$, 1.3 mg

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$Na_2MoO_4.2H_2O$, 2 mg H_3BO_4, 0.35 mg KI, 0.5 mg $Al_2(SO_4)_3$ and 0.5 ml antifoam A (Sigma, St. Louis). A 3% inoculum was used which was prepared by inoculating shake flasks containing YM medium from a fresh slant followed by incubation for 30 to 36 h at 21°C.

Analytical methods

Growth was monitored by measuring culture turbidity with a Klett-Summerson colorimeter at 640 nm. Dry biomass was determined gravimetrically. Sugars were analyzed by HPLC using either a Waters Dextro-Pak column or a Biorad Aminex HPX-42C column. Degassed water served as eluent in both cases. In some instances fractions of the HPLC eluate were collected for further analysis. Total carbohydrate was determined by the anthrone method (Herbert et al. 1971). Thin-layer chromatographic (TLC) separations of sugars were run on Merck F_{254} plates using butanol-ethanol (100:40) as mobile phase and sugars were detected as previously described (Schäffler and Morel du Boil, 1972). ^{13}C proton noise decoupled NMR spectra of the purified unknown compound were obtained at 75 Mhz on a Brucker 300 spectrometer. These samples were dissolved in D_2O and dioxane was used as internal reference.

Transport assays

Cells were collected and washed twice by centrifugation followed by resuspension in ice-cold water at approximately 70 mg dry cells/ml. The uptake of 0.02 M [U-^{14}C] labeled glucose or fructose was measured during 20 sec as previously described (Lucas and van Uden 1986).

Results and discussion

Sucrose utilization by *P. rhodozyma* was accompanied by the accumulation of fructose and glucose in the medium (Fig. 1), confirming previous results (Meyer and du Preez, 1994). In addition, however, HPLC analysis indicated the accumulation of an unknown compound in the medium (Fig. 1). Both the monomers and the unknown were utilized following the depletion of sucrose, with glucose disappearing first (Fig. 1). To determine whether glucose and fructose utilization was limited by their rates of transport, the specific rates of glucose and fructose transport were determined at substrate concentrations similar to the maxima observed during cultivation. The specific rates of utilization of glucose and fructose were also calculated from the rates of sucrose utilization and of glucose and fructose accumulation in the medium, assuming that all the sucrose utilized was first hydrolyzed into glucose and fructose and that no uptake of the intact disaccharide occurred. These calculated rates of utilization were initially substantially higher than the measured rates of transport of each sugar, but declined to values similar to those of transport following sucrose depletion (Fig. 2). These results indicated that sucrose utilization was limited by the capacity of the transport system or systems for its monomeric constituents. In addition, it seemed probable that sucrose hydrolysis was accompanied by the extracellular formation of a product from sucrose or its monomers or both. The unknown compound accumulating in the medium was, therefore, investigated further. Fractions were collected from HPLC analysis of the fermentation broth after 12 h cultivation using a Biorad HPX-42C column. Anthrone analysis of these fractions indicated that the unknown compound was probably a carbohydrate (results not shown). This compound was purified by collecting fractions following HPLC separation on a Waters Dextro-Pak column of samples withdrawn at various intervals during cultivation. The purified compound was hydrolyzed with 1 N HCl for 1 h in a boiling water-bath and the hydrolysate analyzed by HPLC using a Biorad Aminex HPX-42C column. The results showed that the unknown compound consisted of glucose and fructose in a 1:2 ratio, which indicated that it was a

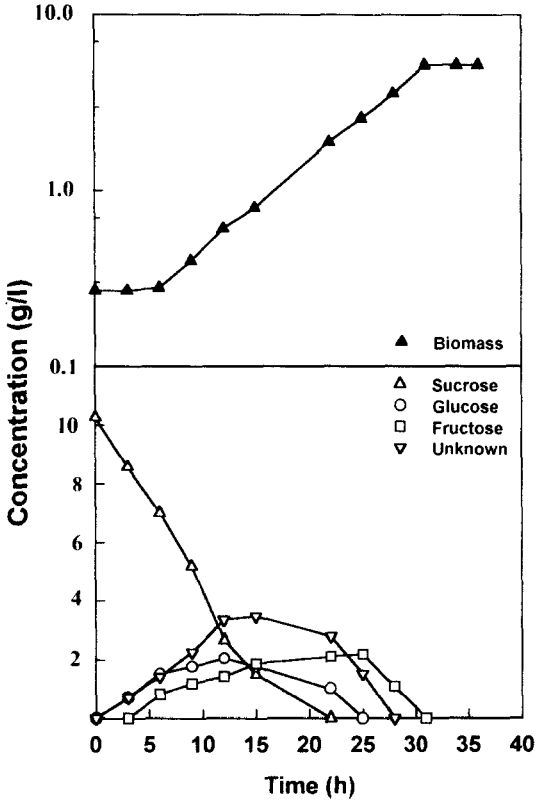

Fig. 1. Growth, substrate utilization and product formation by *Phaffia rhodozyma* at pH 5 and 21°C in a fermentor stirred at 500 rpm and aerated at 1 l/min. The approximate concentration of the unknown compound was calculated using sucrose as standard.

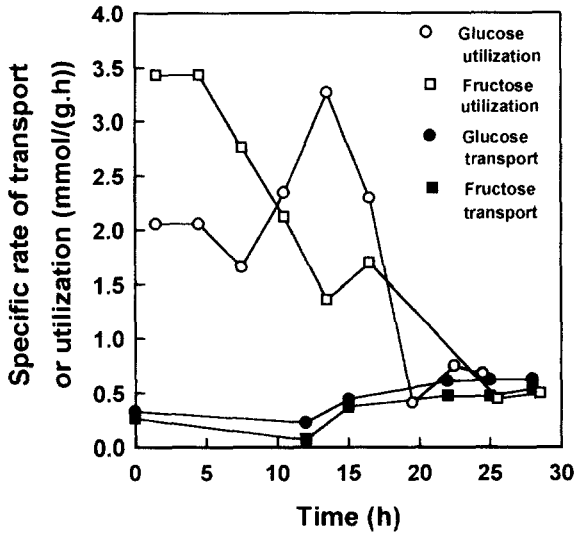

Fig. 2. Specific rates of uptake and utilization of glucose and fructose during the cultivation depicted in Fig. 1. The specific rates of sugar utilization were calculated from the rates of sucrose utilization and the changes in glucose and fructose concentrations in the medium.

trisaccharide. Three trisaccharides consisting of one glucose and two fructose molecules are frequently produced during enzymatic sucrose hydrolysis by a transglycosylase or the transfructosylation activity of invertase itself. This involves the transfer of a fructosyl unit to sucrose (Myrbäck 1959; Jung et al. 1987; Hayashi et al. 1989; Gupta and Bhatia 1980) to produce 1-kestose (1^F -ß- fructosylsucrose), 6-kestose (6^F - ß - fructosylsucrose) or neokestose (6^G - ß - fructosylsucrose) (Straathof et al. 1986).

The retention times of the unknown compound relative to those of sucrose were calculated from HPLC and TLC analysis and were compatible with those of neokestose reported in or calculated from literature but not with those of the other two trisaccharides (Table 1). In addition, the ^{13}C nuclear magnetic resonance (NMR) chemical shifts of the purified unknown were compatible with those previously reported for neokestose but not with those of the other two trisaccharides (Table 2).

We concluded, therefore, that the isolated compound was the fructo-oligosaccharide neokestose and that it was produced by transfructosylation associated with sucrose hydrolysis. During the growth of *P. rhodozyma* on sucrose, hydrolysis of the substrate at a rate that exceeds the capacity of the transport system or systems for glucose and fructose results in the accumulation of these sugars in the medium. This in turn results in the production of neokestose by transfructosylation. A carbon balance using the concentrations reported in Fig. 1 indicated that sucrose was nearly stoichiometrically converted into glucose, fructose and neokestose during the first nine hours of incubation, confirming the validity of the assumption that the disappearance of sucrose from the medium is not the result of uptake of intact sucrose but is mostly due to its conversion into extracellular products. Although the production of neokestose during the *in vitro* hydrolysis of sucrose by fungal invertases has previously been reported (Bacon 1954; Dickerson 1972), this is, to our knowledge, the first report of its production during yeast cultivation.

Table 1. Relative retention times ($R_{sucrose}$) of the unknown compound following HPLC and TLC separations.

	$R_{sucrose}$			
Separation	Unknown[a]	Neokestose	1-Kestose	6-Kestose
HPLC	1.92	2.10[b]	1.50[b]	0.70[b]
TLC	0.75	0.75[c]	0.51[c]	0.41[c]

[a]This work
[b]Smouter and Simpson (1991)
[c]Schäffler and Morel du Boil (1972)

Table 2. ^{13}C-NMR chemical shifts of the unknown, neokestose, 1-kestose and 6-kestose

Unknown		Neokestose[a]		1-Kestose[b]		6-Kestose[c]	
Chemical shift	C	Chemical shift	C	Chemical shift	C	Chemical shift	C
105.86	2''	105.86	2''	105.38	2''	105.86	2''
105.86	2'	105.84	2'	105.92	2'	105.86	2'
94.15	1	94.15	1	94.15	1	94.23	1
83.51	5''	83.49	5''	82.88	5'	83.28	5''
83.31	5'	83.29	5'	82.78	5''	82.37	5'
78.90	3''	78.93	3''	78.36	3''	78.71	3''
78.34	3'	78.38	3'	78.34	3'	78.32	3'
76.47	4''	76.49	4''	76.16	4''	76.86	4''
76.04	4'	76.08	4'	75.54	4'	76.52	4'
74.57	3	74.58	3	74.27	3	74.70	3
73.69	5	73.68	5	74.09	5	74.50	5
73.16	2	73.15	2	72.81	2	73.16	2
71.32	4	71.32	4	70.90	4	71.39	4
64.54	6'	64.57	6'	64.01	6''	65.09	6'
64.50	6''	64.50	6''	63.86	6'	64.71	6''
63.58	1'	63.60	1'	62.59	1'	63.39	1'
62.45	6	62.46	6	62.12	1''	62.45	6
62.31	1''	62.35	1''	61.82	6	61.99	1''

C = carbon nuclei. Unprimed carbons are glucose, primed carbons the fructose moiety of sucrose, double-primed carbons the fructose linked to sucrose.

[a]Shiomi (1993), [b]Calub and Waterhouse (1990), [c]Liu and Waterhouse (1991).

The anomeric carbon of the glucose moiety, readily distinguished by its well-isolated chemical shift (O'Reilly and Scott 1993), was used as reference to adjust literature values for instrument differences.

Fructo-oligosaccharides have applications in the food and feed industries. These sugars selectively stimulate the growth of health-promoting *Bifidobacterium* species in the gut of humans and other animals (Hidaka et al., 1986; Mitsuoka et al., 1987) and are currently commercially produced by enzymatic conversion of sucrose and used as feed supplements (Hidaka et al., 1986; van Balken *et al* 1991). In contrast to other oligosaccharides, the bifidogenic potential of neokestose has not been reported in literature. The possible application of *P. rhodozyma* for the production of neokestose and the effect of neokestose on the growth of *Bifidobacterium* sp. is under investigation with the aim of extending the range of products obtainable from this yeast.

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