The Anaerobic Metabolism of Glucose and Fructose by *Saccharomyces bailii*

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In contrast to most yeasts, which ferment glucose more rapidly than fructose, *Saccharomyces bailii* ferments fructose first, then glucose. Thus, in a medium containing fructose and glucose, diauxic growth results. Cells of *S. bailii* that were grown on fructose were unable to ferment glucose when suspended in a glucose-containing buffer solution. Fructose-grown cells were cryptic for glucose fermentation but contained the enzymes for glucose metabolism. When suspended for 2 h in a growth medium containing glucose, fructose-grown cells acquired the ability to ferment glucose, due to the synthesis of a carrier protein. This induction was prevented by cycloheximide. In *S. bailii*, fructose was transported into the cells by a constitutive carrier system that was insensitive to uranyl ions. The inducible glucose carrier system was completely inhibited by 10^{-4} M-uranyl ions. If subsequent metabolism of hexoses was inhibited by iodoacetic acid, the uptake of hexoses could be measured by the increase in their intracellular concentrations. Fructose-grown cells took up only fructose whereas glucose-grown cells possessed an inducible glucose carrier and uptake of both glucose and fructose was observed. A model is proposed to explain the sequential fermentation of fructose and glucose in *S. bailii*.

INTRODUCTION

Dubrunfaut (1847) and Bourquelot (1886) observed that the hexoses glucose and fructose were metabolized at different rates during alcoholic fermentation. Most yeasts, including the typical wine yeasts, ferment glucose more rapidly than fructose (glucophilic yeasts) whereas a few yeasts belonging to the species *Saccharomyces rouxii* and *Saccharomyces bailii* ferment fructose preferentially (fructophilic yeasts) (Peynaud & Domercq, 1955). The glucophilic characteristic of yeasts was explained by the nature of their system of hexose transport. The metabolism of both sugars was similar, except for glucose 6-phosphate which is an intermediate of glucose metabolism only. Gottschalk (1946) regarded the preference of glucose as being due to the permeability of the cell membrane. Sols (1956) assumed that both sugars are transported by the same carrier but that fructophilic yeasts have a higher affinity for fructose.

The differential fermentation of glucose and fructose is of some interest for the wine industry, because the glucose:fructose ratio in the unfermented residual sugar can be used to detect an addition of saccharose or grape must to wine after fermentation. Furthermore, fructose has a much sweeter taste than glucose, which can be of advantage for some wines. In this paper, the fermentation of glucose and fructose by *Saccharomyces cerevisiae* and *S. bailii*, in growing and in resting cells is described. By determining the activity of the primary enzymes of the metabolism of glucose and fructose, it should be possible to determine whether the fermentation of these hexoses is regulated at the level of such enzymes. The inducible nature of the glucose fermentation was also investigated.

METHODS

Organisms and culture conditions. *Saccharomyces bailii*, strain N2120 (obtained from Dr F. E. M. J. Sand, Bussum, Netherlands) and *Saccharomyces cerevisiae* strain 43 from the collection of this institute were used. The

yeasts were cultivated on the synthetic B-medium (Heerde & Radler, 1978), and the carbon source varied as required. Cultures grown in 5 ml B-medium at 28 °C for 24 h were used to inoculate 50 ml of the same medium. This procedure was repeated until final cultures of 0.2–2 l were obtained. Experimental cultures were grown for 36 h at 28 °C in shaken Erlenmeyer flasks fitted with fermentation traps.

Preparation of glucose-grown cells, fructose-grown cells, glycerol-grown cells and cell extracts of S. bailii. Cultures were grown anaerobically as described in B-medium containing 10% (w/v) glucose or 10% (w/v) fructose respectively. Glycerol-grown cells were cultured on 5 g glycerol (l B-medium)⁻¹ with aeration. After 24 h at 28 °C, the cells were collected by centrifugation and washed twice in 0.1 M-KCl/HCl buffer, pH 3.1. Cell extracts were prepared by maceration with glass beads as described by Kuczynski & Radler (1982).

Measurement of hexose uptake. Cells of *S. bailii* were suspended in the above buffer [10 ml (g wet weight)⁻¹] and incubated at 30 °C in 200 ml Erlenmeyer flasks gassed with N₂ for 10 min on a water-bath shaker. Samples (2 ml) of this suspension were centrifuged for 1 min at 3000 g. To the sediment was added 5 ml ice-cold 10% (w/v) hexose solution in KCl/HCl buffer containing 1 mM-iodoacetic acid. After intensive shaking for 5 s, the suspension was transferred to screw-cap bottles and gassed with N₂ for 3 min. The bottles were incubated in a water-bath shaker at 30 °C and the reaction was stopped by immersing the bottles in an ice-bath. The cells were rapidly recovered by filtration on membrane filters (0.45 µm pore size) and rinsed three times with 5 ml ice-cold KCl/HCl buffer. After weighing, the cells were extracted in 1 ml absolute ethanol for 1 h. Water (2 ml) was added, before the cells were removed by centrifuging and the hexoses determined in the supernatant. The intracellular concentration of glucose and fructose was calculated by assuming that the composition of yeast cells is 67% water and 33% dry matter.

Analytical determinations. D-Glucose and D-fructose were determined enzymically with hexokinase, glucose-6-phosphate dehydrogenase (Bergmeyer *et al.*, 1974) and additional phosphoglucose isomerase for fructose (Bernt & Bergmeyer, 1974). Protein was determined using the Biuret method, or the spectrophotometric method of Warburg & Christian (1941). The cell mass was determined either by weighing on the membrane filter or photometrically (Zeiss PM 6) by measuring the turbidity at 610 nm and comparing with a calibration curve of a known cell suspension. The production of CO₂ was measured manometrically with a Warburg apparatus.

Enzyme determinations. Hexokinase was determined spectrophotometrically at 340 nm, with the substrates glucose or fructose according to Bergmeyer *et al.* (1974). For the determination of glucosephosphate isomerase, fructose-1,6-bisphosphate aldolase and 6-phosphofructokinase the procedures described by Conrad & Schlegel (1977) were used. One unit (U) is the enzyme activity that converts 1 µmol substrate min⁻¹. Specific activities are U (mg protein)⁻¹.

Chemicals. All enzymes and co-enzymes were purchased from Boehringer-Mannheim. Cycloheximide was obtained from Serva, and the other chemicals were from Merck.

RESULTS

A preliminary experiment confirmed that *S. cerevisiae* used glucose and fructose simultaneously when grown on a mixture of these sugars (Fig. 1a). Glucose was used more rapidly than fructose, so that the ratio of glucose to fructose changed during fermentation in favour of fructose. When *S. bailii* was grown under identical conditions, only fructose was metabolized and the concentration of glucose was not significantly changed during the growth (Fig. 1b). This striking difference in the utilization of sugars could be caused either by differences in the transport systems or by the subsequent intracellular metabolism of the sugars. At a total sugar concentration of less than 10%, curves showing diauxic growth were observed with *S. bailii*. During the first growth phase, fructose was consumed, then after a short 'lag' period, glucose was fermented during a second phase of growth.

To determine whether the presence of fructose prevented the metabolism of glucose, *S. bailii* was grown in a medium containing glucose or fructose as the only fermentable sugar. Such cells are termed glucose-cells or fructose-cells, respectively. Washed, resting glucose-cells (50 mg) suspended in KCl/HCl buffer (2.9 ml) fermented glucose, fructose and mannose producing 9.5, 38.4 and 21.6 µmol CO₂ h⁻¹, respectively. The concentration of each sugar was 20 mM. Fructose-cells fermented fructose at the same rate as glucose-cells (37.3 µmol CO₂ h⁻¹), but glucose was not fermented at all. Similar observations were made with cells grown aerobically on glycerol (glycerol-cells). Such cells behaved like fructose-cells: fructose was fermented, glucose was not. Thus it was shown that glucose-cells and fructose-cells were different and that the presence of fructose was not essential to prevent the metabolism of glucose.

If fructose-cells and glucose-cells of *S. bailii* that were originally grown from the same

Fig. 1. Fermentation of glucose (●) and fructose (○) by growing cultures of (a) *S. cerevisiae* and (b) *S. bailii*. Growth (□) was in B-medium, pH 3.2, containing 100 g glucose and 100 g fructose l⁻¹ at 27 °C in shaken fermentation vessels.

inoculum showed such different abilities to ferment sugars, the most likely basis would be the phenomenon of induction. In fact, glycerol-cells, initially unable to ferment glucose, acquired this ability within 2 h by incubation in a complete medium that contained glucose (Fig. 2). This induction was dependent on protein synthesis, since glycerol-cells incubated in glucose-medium plus cycloheximide did not acquire the capacity to ferment glucose. This new protein(s) could be either an enzyme involved in hexose metabolism or a protein that catalyses the transport of glucose. To investigate the first possibility, the activities of the enzymes involved in the metabolism of glucose or fructose were determined in fructose-cells and in glucose-cells.

The activities of various enzymes were determined in cell extracts of glucose-cells, fructose-cells, and glycerol-cells of *S. bailii* (Table 1). As expected, there were quantitative differences in the enzyme activities. However, all cells contained hexokinase that phosphorylated glucose and fructose. This enzyme (hexokinase) of which at least three isoenzymes exist (Gancedo *et al.*, 1977), was found to be very similar in *S. bailii* and *S. cerevisiae* (W. Emmerich, unpublished results). Although a special glucokinase is known in yeast, this activity is associated with fructokinase and was found in glucose-cells and in fructose-cells of *S. bailii*. The yeast cells always contained hexokinase and not a separate and different glucokinase or fructokinase. Furthermore, the important enzyme glucose phosphate isomerase, which is involved in the metabolism of glucose only (Fig. 3), was present in fructose-cells and in glucose-cells. Thus there was no evidence that the metabolism of glucose and fructose in *S. bailii* was regulated at the level of the repression of catabolic enzymes. Therefore, it was assumed that the sugar metabolism of *S. bailii* is controlled at the level of membrane transport.

By measuring the production of CO₂ by glucose- and fructose-cells, the apparent substrate affinities for the fermentation of glucose and fructose were determined. Glucose-cells showed a low apparent affinity for glucose ($K_m = 80$ mM) and a much higher affinity for fructose ($K_m = 20$ mM). Fructose-cells showed a similar apparent substrate affinity for fructose ($K_m = 25$ mM) as did glucose-cells. Fructose-cells did not produce CO₂ from glucose.

The existence of two separate mechanisms for the uptake of glucose or fructose was further demonstrated by the effect of uranyl nitrate. This compound is known to inhibit hexose

Fig. 2. Induction of the glucose-metabolizing system in glycerol-cells of *S. bailii*. Glycerol-cells were suspended in B-medium with 10% glucose (open symbols) or in B-medium with 10% glucose and 10^{-3} M-cycloheximide (filled symbols) for 2 h. The cells were then washed and the formation of CO_2 from the sugars fructose (○, ●) and glucose (□, ■) was determined manometrically.

Table 1. Activities of enzymes of carbohydrate metabolism in cell extracts of *S. bailii* grown on different carbon sources

Enzyme	Specific activity (U mg^{-1}) with carbon source:		
	Glucose	Fructose	Glycerol
Phosphoglucose isomerase	0.31	0.12	1.09
Fructose-1,6-bisphosphate-aldolase	0.04	0.002	0.21
6-Phosphofructokinase	0.05	0.10	0.13
Hexokinase (glucose)	0.42	0.36	ND
Hexokinase (fructose)	0.65	0.48	ND

ND, Not determined.

transport in yeast (Kotyk & Michaljanicova, 1968). The metabolism of fructose was very insensitive to uranyl nitrate; even at 10^{-2} M the formation of CO_2 from fructose by fructose-cells or glucose-cells of *S. bailii* was only inhibited by about 30% (Table 2). Fructose-cells did not metabolize glucose. In glucose-cells, glucose metabolism was very sensitive to uranyl ions and 10^{-4} M-uranyl nitrate inhibited the formation of CO_2 from glucose completely. Thus two different mechanisms or carrier systems exist for the uptake of glucose and fructose in *S. bailii*, one was resistant, and the other very sensitive to uranyl ions.

This observation was confirmed in a further experiment, in which the production of CO_2 from glucose by glucose-cells of *S. bailii* was measured in the presence of uranyl nitrate (Fig. 4). If uranyl nitrate was present from the beginning, no CO_2 was produced. If the inhibitor was added to glucose-fermenting cells after 20 min, the formation of CO_2 stopped immediately. However, these cells were not inactivated or destroyed, for if fructose was added, the substrate was fermented. CO_2 is, of course, the final product of hexose metabolism. However, it is generally assumed that because of the high substrate and catabolite affinities of the glycolytic enzymes, membrane transport is the limiting reaction that controls the rate of formation of CO_2 (Becker & Betz, 1972; Barnett & Sims, 1976b; Höfer, 1977).

Because of the rapid metabolism of hexoses and other substrates, their intracellular concentration is generally very small in yeast cells. In order to measure the anaerobic uptake of hexoses

Fig. 3. Pathway of glucose and fructose metabolism in yeast.

Table 2. Inhibition by uranyl nitrate of hexose fermentation by *S. bailii*

Carbon source in growth medium	Substrate for fermentation	Inhibition of CO ₂ formation (%) at uranyl nitrate concns (M) of:					
		10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	5 × 10 ⁻⁵	0
Glucose	Glucose	100	100	100	96	46	0
	Fructose	57	32	26	2	0	0
Fructose	Glucose	—	—	—	—	—	—
	Fructose	ND	31	ND	ND	ND	0

ND, Not determined.

—, No CO₂ formation. Fig. 4. Inhibition of the anaerobic metabolism of glucose of resting cells of *S. bailii* by uranyl nitrate. Glucose-cells (50 mg) were suspended in 2.9 ml KCl/HCl buffer (0.1 M, pH 3.1) with 60 μmol glucose (○), and 1 mM-uranyl nitrate was added at the start (●) or when indicated by the arrow (■). Fructose (20 mM) was added when indicated by the arrow to cells previously inhibited by uranyl nitrate (▽).

Fig. 5. Transport of glucose (●) or fructose (○) by (a) glucose-cells or (b) fructose-cells of *S. bailii* in the presence of 1 mM-iodoacetic acid under N_2 .

by yeast cells, their metabolism has to be inhibited without interfering with the transport system. For this purpose several investigators (Holzer *et al.*, 1955; Cirillo, 1962; van Steveninck, 1969) have used iodoacetic acid successfully. Preliminary experiments with *S. bailii* have shown that hexose metabolism, as measured by production of CO_2 , was completely inhibited by 10^{-3} M-iodoacetic acid. Thus it was possible to measure the uptake of hexoses by determining their intracellular concentration in the yeast cells directly, provided that the transport itself was not inhibited by iodoacetic acid. Fructose-cells took up fructose only (Fig. 5); glucose was not found intracellularly. When glucose-cells were exposed to glucose or fructose, the intracellular concentration of both sugars increased. Similar experiments with cells of *S. cerevisiae* showed no significant difference in the uptake of glucose or fructose.

DISCUSSION

In contrast to *S. cerevisiae*, a yeast that ferments glucose and fructose simultaneously (although at different rates), the fermentation of glucose in *S. bailii* depends on the presence of an inducible system. However, although all strains of *S. bailii* tested fermented fructose preferentially, differences in the degree of fructose preference were observed.

Analysis of the activity of glycolytic enzymes failed to correlate the preferential fructose fermentation with differences in the enzyme spectrum. Neither a kinase specific for glucose (glucokinase) nor differences in the activity of glucose phosphate isomerase were observed in glucose-cells or fructose-cells of *S. bailii*. A special study of the hexokinase of *S. bailii* and *S. cerevisiae* (results not described in this paper) revealed that both organisms contained three hexokinases with a different ratio of phosphorylation of glucose and fructose. However, our results with *S. bailii* were very similar to the observations of Sols (1956) and of Gancedo *et al.* (1977) with *S. cerevisiae*.

Fructose-cells of *S. bailii* can be called cryptic with respect to their metabolism. Crypticity was regarded as being due to the absence of a transport system (Barnett, 1976). The transport of fermentable hexoses can be determined directly by measuring their intracellular concentration in the presence of a metabolic inhibitor that prevents the catabolism of hexoses (Kotyck & Janacek, 1975; Höfer, 1977; Sols, 1968). Since both hexoses were taken up by iodoacetic acid-

inhibited glucose-cells it was assumed that glucose and fructose were transported by facilitated diffusion independent of metabolic energy. This was originally shown for *S. cerevisiae* by Burger *et al.* (1959) and recently confirmed by Romano (1982). If the transport is rate-limiting (Becker & Betz, 1972; Barnett & Sims, 1976*a*) then the substrate affinity of the hexose carrier can be determined indirectly by measuring the products of hexose metabolism. Our experiments showed that cell extracts of *S. bailii* induced to catabolize glucose do not contain glucose or glucose 6-phosphate. This indicates that the metabolism was much more rapid than the influx of glucose.

The apparent K_m values for fructose fermentation in *S. bailii* ($K_m = 20$ mM) were very similar to those reported by Kotyk (1967) and Cirillo (1968) for fructose transport in *S. cerevisiae*. The apparent K_m values for glucose fermentation of glucose-cells of *S. bailii* were about 15–16 times higher than those found by Kotyk (1967) for glucose transport in *S. cerevisiae*.

Besides the transport system described in this paper a further regulatory mechanism has been observed that will be briefly mentioned. If glucose-cells of *S. bailii* were suspended in a buffer solution containing fructose, their ability to metabolize glucose was rapidly diminished and completely lost after 2 h. This inactivation was independent of the presence of cycloheximide. A similar observation has been described for the transport of galactose in *S. cerevisiae* (Alonso & Kotyk, 1978). This de-induction was explained by the activation of a protein-decomposing system by energy-providing substrates.

The results presented in this paper do not give an explanation for the biological significance of the preferential fructose fermentation of *S. bailii*. It may be that this characteristic is linked to the osmotolerance of these yeasts (Tilbury, 1980).

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