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Maltotriose fermentation by *Saccharomyces cerevisiae*[1]

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Maltotriose, the second most abundant sugar of brewer's wort, is not fermented but is respired by several industrial yeast strains. We have isolated a strain capable of growing on a medium containing maltotriose and the respiratory inhibitor, antimycin A. This strain produced equivalent amounts of ethanol from 20 g l^{-1} glucose, maltose, or maltotriose. We performed a detailed analysis of the rates of active transport and intracellular hydrolysis of maltotriose by this strain, and by a strain that does not ferment this sugar. The kinetics of sugar hydrolysis by both strains was similar, and our results also indicated that yeast cells do not synthesize a maltotriose-specific α-glucosidase. However, when considering active sugar transport, a different pattern was observed. The maltotriose-fermenting strain showed the same rate of active maltose or maltotriose transport, while the strain that could not ferment maltotriose showed a lower rate of maltotriose transport when compared with the rates of active maltose transport. Thus, our results revealed that transport across the plasma membrane, and not intracellular hydrolysis, is the rate-limiting step for the fermentation of maltotriose by these *Saccharomyces cerevisiae* cells. *Journal of Industrial Microbiology & Biotechnology* (2001) **27,** 34–38.

Keywords: brewing; fermentation; maltose; maltotriose; *Saccharomyces*; transport

Introduction

The most abundant fermentable sugars in brewer's wort are maltose (50–60%), maltotriose (15–20%), and glucose (10–15%). Although maltotriose is the second most abundant sugar of brewer's wort, it has the lowest priority for uptake by yeast cells. In general, only when half of the wort glucose has been taken up by the yeast will the uptake of maltose and maltotriose commence, with a slower uptake rate for maltotriose than for maltose [20,29]. This slower, and sometimes incomplete, uptake of maltotriose leads to one of the problems experienced by some breweries, namely a high content of fermentable sugars in the finished beer, and atypical beer flavor profiles. The rate of uptake and metabolism of maltotriose during wort fermentation is, therefore, one of the major determinants of fermentation efficiency and product quality. However, maltotriose metabolism has received little attention compared with the mechanisms of maltose and glucose utilization by industrial yeast cells [3].

Maltose utilization requires the presence of at least one of five highly homologous and unlinked *MAL* loci: *MAL1* through *MAL4* and/or *MAL6* [17]. Each locus contains at least one copy of three separated genes encoding for maltose permease (*MALx1*, where *x* stands for one of the five loci), maltase (*MALx2*), and a positive regulatory protein (*MALx3*) that induces transcription of the two previous genes in the presence of maltose. Maltose permease transports maltose across the cell membrane, and subsequently, the cytoplasmic maltase (α-glucosidase) hydrolyzes maltose into two units of glucose, which are then chanelled through the glycolytic pathway.

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However, glucose is the preferred carbon source for *Saccharomyces cerevisiae*, and complex regulatory control circuits have evolved to ensure that the expression of alternative sugar utilization enzymes (including maltose permease and maltase) is repressed when glucose is present in the growth medium [17]. This situation is also observed during fermentation of industrial media, and it is aggravated by the use of sugar adjuncts, like glucose, fructose, or even sucrose [2,3]. The detailed molecular analysis of glucose repression of *MAL* transcription has allowed the development of strains with enhanced maltose fermentation by using either classical yeast strain selection or genetic techniques [18,21], altering the physiological condition of the cell [4,8], or by the use of recombinant DNA techniques [10,11,19].

The majority of studies on maltotriose utilization by yeast cells have dealt with the analysis of environmental factors that may influence the uptake of this carbon source during brewing fermentations [2,31,34]. Early studies on sugar utilization by yeast cells revealed that maltose and maltotriose are transported across the plasma membrane by different transport systems [22,29]. Although maltotriose transport activity in yeast cells has been studied in a number of industrial strains [7,16,33], the molecular identity of this permease remained unknown until some years ago when a new permease gene (*AGT1*) showing wider substrate specificity was characterized [6]. The *AGT1* permease is an active H^+ symporter, which can transport a series of α-glucosides, including maltose, trehalose, melezitose, sucrose, α-methylglucoside, and maltotriose [25–28]. In accordance with the importance of maltotriose transport for the industrial applications of yeast, practically all brewing strains harbor the *AGT1* permease [9]. This transporter has a high affinity ($K_m \sim 8$ mM) for trehalose and sucrose, and a lower affinity ($K_m \sim 20$ mM) for maltose and maltotriose [25,28].

We have recently shown that maltotriose is not fermented by any of several industrial strains, but instead, this sugar is respired by the cells [32]. Similar results have been observed with another α-glucoside transported by the *AGT1* permease, the disaccharide

trehalose, which is also respired by yeasts [14]. In both cases, sugar influx into glycolysis seemed to be the rate-limiting step for the fermentation of the α-glucoside [14,32]. In order to gain insights into the molecular basis of maltotriose utilization by yeast cells, we have isolated a yeast strain able to ferment maltotriose, and performed a detailed analysis of several biochemical parameters that could be involved in maltotriose fermentation. Our results indicate that active maltotriose transport across the plasma membrane, and not intracellular hydrolysis, is the rate-limiting step for fermentation of this sugar.

Materials and methods

Materials

Media components were purchased from Difco (Detroit, MI, USA). Glucose, maltose, maltotriose (minimum 95% pure), *p*-nitrophenyl-α-D-glycopyranoside, phenylmethylsulfonyl fluoride, glass beads, and antimycin A were obtained from Sigma (St. Louis, MO, USA). Ampholine (pH 5.0–7.0 and pH 3.5–10.0) and pI protein markers (pI 3.5–9.3) were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden), and 5-bromo-4-chloro-3-indoyl-α-D-glucopyranoside (X-α-glucoside) was from Calbiochem (San Diego, CA, USA). All other chemicals were of analytical grade.

Strains and culture conditions

Two *S. cerevisiae* strains were used in this study. Strain *70* (kindly provided by Dr. Anita D. Panek, Universidade Federal do Rio de Janeiro, Brazil) was obtained originally from Labatt Brewing Co. Ltd. (London, Ontario, Canada) and has been described elsewhere [32]. Strain *B01* was isolated from the slurry of a local ale brewing company (see below). Both strains were grown in batch culture (28°C and 160 rpm) on YEP medium (pH 5.0) containing, per liter: Bacto peptone 20 g, yeast extract 10 g, and 20 g of the indicated carbon source (glucose, maltose, or maltotriose). Solid YPD medium also contained, per liter, 20 g of Bacto agar and, when indicated, 3 mg of antimycin A. Growth was followed by turbidity measurements at 570 nm and the amount of yeast was expressed as dry weight (determined with cells filtered, washed with distilled water, and dried at 80°C for 48 h). Cells were harvested (2500×*g*, 3 min) at the exponential phase of growth and washed twice with ice-cold distilled water before use. Alternatively, culture samples were centrifuged (5000×*g*, 3 min) and the supernatant was used for determination of ethanol and glycerol.

Glucose, ethanol, and glycerol determinations

Glucose was measured by the glucose oxidase and peroxidase method using a commercial kit from Biobras (Montes Claros, Brazil). Ethanol was determined in culture supernatants by gas chromatography (CG Instrumentos Científicos, São Paulo, Brazil) with a Poropak Q-80-100 column, a flame ionization detector, and a computing integrator system. Glycerol was determined with a commercial enzymatic assay based on glycerol kinase, glycerol-3-phosphate oxidase, and peroxidase (Biobras, Montes Claros, Brazil).

Cell-free extracts

Washed cells were suspended at 15 mg dry yeast ml^{-1} in cold buffer A (100 mM MOPS–NaOH pH 6.8) containing 1 mM phenylmethylsulphonyl fluoride, 20% (by volume) glycerol, and 1 mM EDTA. Cells were disrupted by vigorous shaking on a vortex mixer in the presence of 1.5 g ml^{-1} glass beads (0.5 mm diameter) for five 1-min periods at room temperature, each followed by a 1-min interval where the tubes were kept on ice. The extracts were centrifuged (10,000×*g*, 5 min) and the supernatant fluids were used for total α-glucosidase determination and isoelectric focusing analysis.

α-Glucosidase assays

Total α-glucosidase activity present in cell extracts was determined by following the hydrolysis of 1 mM *p*-nitrophenyl-α-D-glucopyranoside in buffer A at 30°C. The *p*-nitrophenol released was determined at 400 nm (ε=7.28 mM^{-1} cm^{-1}), and one unit of α-glucosidase activity is defined as 1 μmol *p*-nitrophenyl-α-D-glucopyranoside hydrolysed per minute. The α-glucosidase activity toward maltose or maltotriose (at 0.5–100 mM) was determined *in situ* with permeabilized yeast cells as previously described [24]. All assays were done at least in duplicate, and controls using previously boiled permeabilized yeast cells or cell extracts were used. Specific activity is expressed as nmol glucose released from each substrate min^{-1} [mg dry yeast]$^{-1}$.

Analytical scale isoelectric focusing

Isoelectric focusing gels were prepared and processed as described elsewhere [23]. Electrophoresis was carried out in a vertical slab

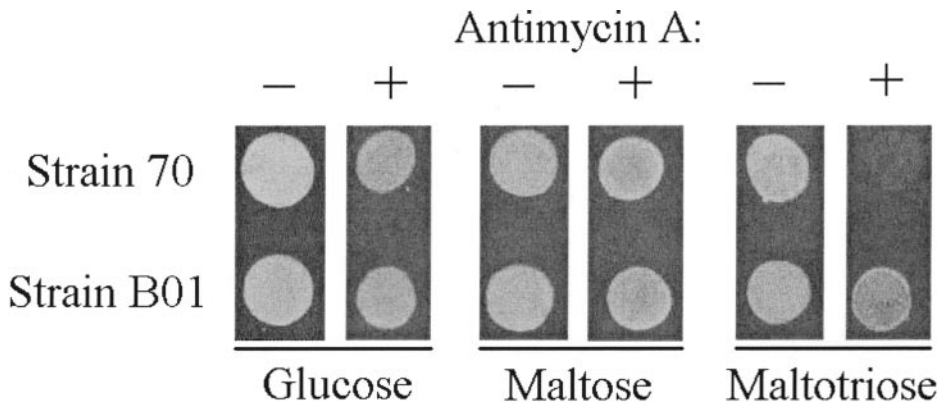

Figure 1 Growth on maltotriose is inhibited by antimycin A. Cells from strain *70* or *B01* were inoculated onto YEP agar plates containing 2% of the indicated carbon sources in the absence (–) or presence (+) of antimycin A, and incubated aerobically at 28°C for 3 days.

gel apparatus at 4°C using 150 V for 1 h followed by 400 V for 12–14 h at a constant current of 10 mA. After isoelectric focusing, the proteins present in the gels were stained with Coomassie Blue R250 or, alternatively, the different α-glucosidase isoforms present in the gel were stained with X-α-glucoside, which upon hydrolysis by α-glucosidases produces an easily detectable blue dye [12]. The activity toward maltose or maltotriose was detected in the gel by a modification of a previously described method [5]. Briefly, the gel was soaked in buffer A for 5 min and placed on top of two layers of Whatman paper no. 1 soaked with the glucose oxidase and peroxidase reagent used for glucose determination. A solution of 200 mM maltose or maltotriose in buffer A was spread over the gel surface and incubated for 20–30 min at room temperature to allow the enzymatic reagent to react with the glucose molecules liberated during hydrolysis of the substrates.

Transport assays

The rates of active maltose- or maltotriose-H^+ symport were assayed as previously described [25–27,32] using sugars at 0.2–150 mM. All assays were carried out at least in duplicate, and the maximum deviation of the pair of assays was less than 10%. Transport activity is expressed as nmol glucose equivalents transported min^{-1} [mg dry yeast]$^{-1}$.

Results and discussion

Selection of a maltotriose-fermenting yeast strain

We have reported [32] that the respiratory inhibitor, antimycin A, completely inhibited the growth of several wild-type and industrial yeast strains on maltotriose, while growth on glucose or maltose was not affected (see strain *70* in Figure 1). We used the inhibition of growth by antimycin A to isolate under aerobic incubation a yeast strain capable of fermenting maltotriose. Yeast cells from a brewing slurry were streaked into 2% maltotriose YEP agar plates containing antimycin A, and after 3 days, selected colonies which had grown on the plates were further purified. A colony showing vigorous growth on maltotriose plus antimycin A (see strain *B01* in Figure 1) was further characterized.

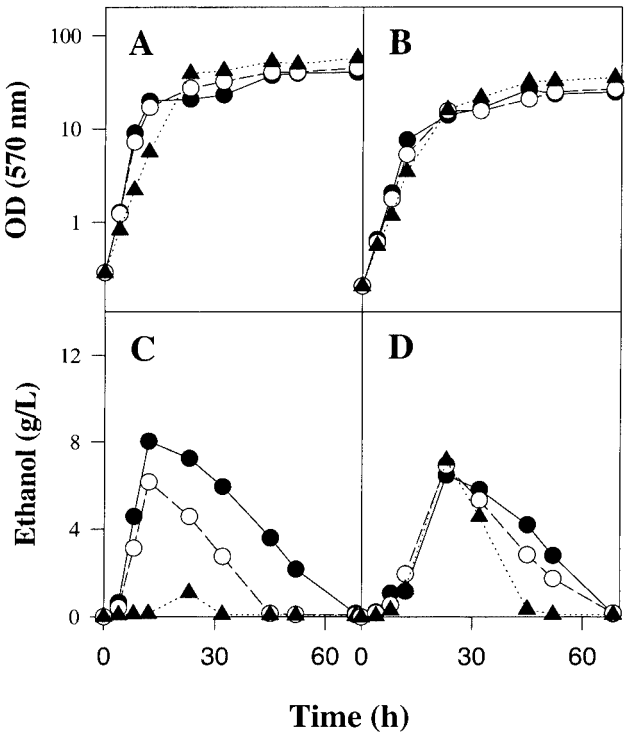

Figure 2 Batch growth and ethanol production by strain *70* (A and C) or strain *B01* (B and D). Cells from each strain were inoculated on YEP medium containing 2% of (●) glucose, (○) maltose, or (▲) maltotriose as carbon source.

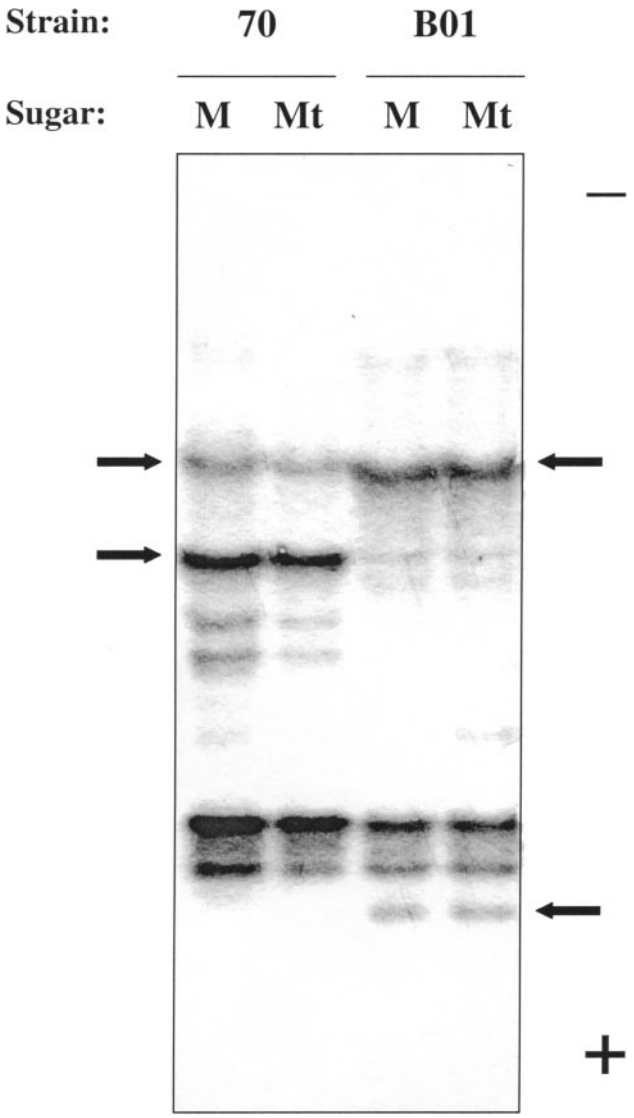

Figure 3 Characterization of yeast α-glucosidases by isoelectric focusing. Cell extracts, prepared from the indicated strains grown on maltose (M) or maltotriose (Mt) and containing the same amount of total α-glucosidase activity, were subjected to isoelectric focusing. The α-glucosidase isoforms present in the gel were stained with X-α-glucoside as described in *Materials and Methods*. The arrows indicate the isoforms that were able to hydrolyze both maltose and maltotriose.

Figure 2 shows that while strain *70* is able to produce ethanol from glucose or maltose, but not from maltotriose, strain *B01* produced equivalent amounts of ethanol from all three sugars. Similar results were obtained for glycerol production by both strains (data not shown). In order to gain insight into the molecular

basis of maltotriose fermentation by yeast cells, we analyzed the differences in the rates of transport and hydrolysis of both maltose and maltotriose by strain *70* and *B01*.

Analysis of maltose and maltotriose hydrolysis

Yeast cells harbor several α-glucosidase isoforms. While some isoforms are able to hydrolyze both maltose and maltotriose [13,15,30], other isoforms are specific for α-methylglucoside [15,23]. However, these α-glucosidase activities have always been purified or characterized from maltose-grown cells, and it is not known if maltotriose is able to induce the synthesis of an α-glucosidase specific for this sugar. We have performed analytical isoelectric focusing of yeast cell extracts obtained from both strains grown on either maltose or maltotriose (Figure 3). Although both strains had different patterns of α-glucosidase isoforms, the same isoforms were expressed in maltose- or maltotriose-grown cells, indicating that both sugars induced the synthesis of the same α-glucosidases. Moreover, the same isoforms that were able to hydrolyze maltose also hydrolyzed maltotriose (Figure 3). Thus, our results indicate that there is no maltotriose-specific α-glucosidase in these yeast strains.

Both strains showed a higher α-glucosidase activity towards maltose in maltose-grown cells than the hydrolysis of maltotriose by maltotriose-grown yeast cells (Figure 4), indicating that maltose is a stronger inducer of the enzymes when compared with maltotriose. However, since the kinetics of maltotriose (and maltose) hydrolysis by both strains was very similar (Figure 4), intracellular hydrolysis of maltotriose cannot be the rate-limiting step for the fermentation of this sugar.

Analysis of maltose and maltotriose active transport

S. cerevisiae is a microorganism highly adapted for efficient utilization of sugars. As a consequence, it generally expresses several permeases, with different affinities for the substrate, which ensure that sugars are taken up from the medium efficiently. Maltose and maltotriose uptake by yeast cells is also mediated by high- and low-affinity transport systems [1,18,33]. We thus performed a detailed kinetic analysis of active maltose or maltotriose uptake by both yeast strains grown on the respective

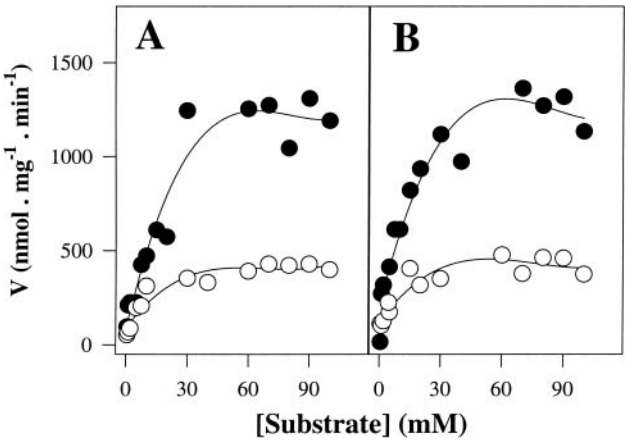

Figure 4 Kinetics of α-glucoside hydrolysis by strain *70* (A) or strain *B01* (B). Maltose (●) or maltotriose (○) hydrolysis was determined *in situ* with the indicated sugar concentrations in cells grown on maltose or maltotriose, respectively.

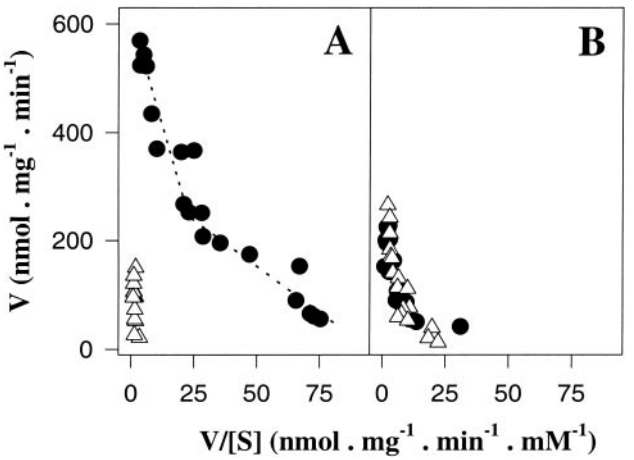

Figure 5 Kinetics of active α-glucoside transport by strain *70* (A) or strain *B01* (B). Maltose (●) or maltotriose (△) active transport was determine with 0.2–150 mM substrate concentrations in cells grown on maltose or maltotriose, respectively.

sugar substrate (Figure 5). Maltose uptake by strain *70* is mediated by two transport activities with high (K_m~4 mM) and a lower affinity (K_m~20 mM) for the substrate, as already described for several wild-type and industrial yeast strains [1,25]. In the case of maltotriose uptake, a single low-affinity (K_m~20 mM) transport system was observed (Figure 5), probably mediated by the *AGT1* permease.

Strain *B01* harbors only the lower-affinity (K_m~20 mM) transport systems for maltotriose and maltose (Figure 5). However, while strain *B01* had the same capacity (V_{max}~300 nmol min^{-1} mg^{-1}) for maltose or maltotriose transport, strain *70* had a significant lower capacity to transport maltotriose (V_{max}~180 nmol min^{-1} mg^{-1}), specially when compared with the V_{max} (~600 nmol min^{-1} mg^{-1}) for maltose uptake by this strain. Thus, the only metabolic parameter that could be correlated with the capacity of a given strain to ferment maltotriose is the active transport of the sugar across the plasma membrane.

Conclusions and further studies

The results reported indicate that maltotriose transport across the plasma membrane is the major limiting step for the fermentation of this sugar by these yeast cells. Attempts to improve maltose fermentation efficiency in brewing and baking yeasts are currently being undertaken and have revealed that the major limiting factor in the fermentation rate is also the expression of the maltose permease [10,11,19]. The maltotriose permease encoded by the *AGT1* gene is therefore expected to have great importance in the fermentation efficiency of industrial yeasts, and the genetic manipulation of strains containing this permease would be of major interest.

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