

Energetics and Kinetics of Maltose Transport in *Saccharomyces cerevisiae*: a Continuous Culture Study

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In *Saccharomyces cerevisiae*, maltose is transported by a proton symport mechanism, whereas glucose transport occurs via facilitated diffusion. The energy requirement for maltose transport was evaluated with a metabolic model based on an experimental value of Y_{ATP} for growth on glucose and an ATP requirement for maltose transport of $1 \text{ mol} \cdot \text{mol}^{-1}$. The predictions of the model were verified experimentally with anaerobic, sugar-limited chemostat cultures growing on a range of maltose-glucose mixtures at a fixed dilution rate of 0.1 h^{-1} . The biomass yield (grams of cells \cdot gram of sugar $^{-1}$) decreased linearly with increasing amounts of maltose in the mixture. The yield was 25% lower during growth on maltose than during that on glucose, in agreement with the model predictions. During sugar-limited growth, the residual concentrations of maltose and glucose in the culture increased in proportion to their relative concentrations in the medium feed. From the residual maltose concentration, the in situ rates of maltose consumption by cultures, and the K_m of the maltose carrier for maltose, it was calculated that the amount of this carrier was proportional to the in situ maltose consumption rate. This was also found for the amount of intracellular maltase. These two maltose-specific enzymes therefore exert high control over the maltose flux in *S. cerevisiae* in anaerobic, sugar-limited, steady-state cultures.

Energy required for transport processes is derived from the gradient of the solute over the membrane (passive transport) or is delivered by metabolic processes at the expense of metabolic energy (active transport). The energy costs of transport are especially important in cellular metabolism when the actively transported solute serves as the source of carbon and energy. This affects growth in two ways: the ATP requirement for biomass formation is higher, and the energy yield of dissimilation is lower. The energy requirement for transport processes may take a large portion of the total energy budget of the cell when the energy yield of the substrate is low. An example is the growth of *Pseudomonas oxalaticus* on oxalate. In this case, half the energy obtained in respiration of the growth substrate is required for transport of the dicarboxylic acid (4, 5). However, also during the growth of yeasts on sugars in mineral media, the energy requirement for sugar transport can be substantial. Verduyn (23) calculated that the theoretical energy cost of sugar transport in the yeast *Candida utilis* growing on glucose is $8.2 \text{ mmol of ATP} \cdot \text{g of biomass}^{-1}$ or 20% of the total ATP requirement.

Maltose is an important sugar in the production of beer and in the leavening of certain doughs (1). So far, however, the specific effects of maltose on yeast physiology, such as the energetics of growth, have received little attention. Most investigations have been aimed at glucose metabolism. However, maltose is transported by proton symport in *Saccharomyces cerevisiae* (20, 22), whereas glucose is taken up by facilitated diffusion. In this study, an attempt is made to quantify the ATP requirements of maltose transport in *S. cerevisiae* via a comparison of growth on glucose and growth on maltose.

MATERIALS AND METHODS

Organism and cultivation conditions. *S. cerevisiae* CBS 8066 was obtained from the Centraalbureau voor Schimmelcultures (Delft, The Netherlands) and maintained on malt agar slopes at 4°C . Chemostat cultivation was performed with ADI 2-liter bioreactors (Applikon Dependable Instruments) at a dilution rate of 0.10 h^{-1} and a working volume of 1.00 liter. Cultures were grown under carbon and energy limitation on a mineral medium described below. The removal of effluent by the standard procedure, continuous, upwardly directed suction from the surface of the culture, gave rise to differences in cell density between the culture and the effluent of up to 20%. Under these conditions, the continuous culture theory is not valid (see also reference 13). Removing effluent from the middle of the culture when the culture surface made contact with an electrical sensor did not give rise to such a difference, and this method was used throughout this study.

The temperature was 30°C , and the stirrer speed was 750 rpm. The pH was kept constant at 5.0 by an ADI 1020 biocontroller by the automatic addition of 2 M KOH. To assure anaerobic conditions, the reactor and the reservoir vessel were flushed with nitrogen gas at a flow rate of $0.5 \text{ liter} \cdot \text{min}^{-1}$. The flow rate was kept constant by a Brooks 5876 gas flow controller. The whole experimental setup (reactor, reservoir, and waste vessel) was equipped with Norprene tubing (Cole-Palmer Corp.). The dissolved-oxygen tension of the culture was continuously monitored with an oxygen electrode (Ingold, no. 34 100 3002) and was below 0.1% air saturation.

The mineral medium contained (each per liter): 5.0 g of $(\text{NH}_4)_2\text{SO}_4$, 3.0 g of KH_2PO_4 , 0.5 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 15.0 mg of EDTA, 4.5 mg of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.3 mg of $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 1.0 mg of $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.3 mg of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 4.5 mg of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 3.0 mg of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.4 mg of $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 1.0 mg of

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H₃BO₃, 0.1 mg of KI, and 0.05 ml of silicone antifoam (BDH). After heat sterilization at 120°C and cooling, a filter-sterilized vitamin solution was added to final concentrations per liter of 0.05 mg of biotin, 1.0 mg of calcium pantothenate, 1.0 mg of nicotinic acid, 25.0 mg of inositol, 1.0 mg of thiamine HCl, and 0.2 mg of *para*-aminobenzoic acid. Ergosterol and Tween 80 were dissolved in pure ethanol and steamed at 100°C for 10 min before being added to the medium to final concentrations of 10 and 420 mg · liter⁻¹, respectively. Because of this addition, the medium feed always contained 10 to 12 mM ethanol, which was taken into account in the calculation of the ethanol yield and fluxes. Maltose monohydrate and glucose were sterilized separately (17) and added at the ratios indicated to a final sugar concentration of approximately 25 g · liter⁻¹.

Determination of dry weight and elemental analysis. The dry weight of the cultures was determined with a microwave oven and 0.45- μ m-pore-size filters according to the method of Postma et al. (16). The carbon, hydrogen, and nitrogen composition of the biomass was determined with an Elemental Analyzer 240B (Perkin-Elmer) (24).

Sugar analysis. The sugar concentrations in the reservoir vessels were determined with a glucose analyzer (YSI 2000; Yellow Springs Instruments). Maltose was first hydrolyzed to glucose by α -glucosidase (Boehringer, no. 105 414) (19). Maltose was found to contain glucose and maltotriose as impurities (both approximately 3% [wt/wt] after heat sterilization). Maltotriose is also hydrolyzed to glucose by α -glucosidase and is not metabolized by *S. cerevisiae* CBS 8066 (see also references 9 and 26). The maltose concentrations in reservoir media and culture supernatants were therefore corrected for the amounts of maltotriose and glucose present.

For the determination of residual substrate concentrations, a culture sample was taken from the culture, frozen in liquid nitrogen within 2 s, and stored at -40°C. Prior to the determination of the concentrations, the sample was thawed and centrifuged at 0°C. The residual concentrations of maltose and glucose were determined with Boehringer test kit 676543 before and after treatment of the supernatant with α -glucosidase. Although rapid sampling was performed throughout this study, it can be calculated that this is not a prerequisite for obtaining accurate data on residual sugar concentrations. For example, the highest maltose consumption rate was 3.5 mmol · g⁻¹ · h⁻¹ at a residual maltose concentration of 0.6 mM. Thus, with the amount of cells present (1.6 g · liter⁻¹) in 2 s maximally 0.003 [1.6 × 3.5/(60 × 30)] mmol of maltose or 0.5% (0.003/0.6) disappeared during the sampling.

Metabolite analysis. Ethanol, glycerol, maltotriose, and organic acids (2-oxoglutaric acid, pyruvic acid, succinic acid, and fumaric acid) were determined simultaneously by high-pressure liquid chromatography (HPLC) analysis with an HPX-87H Aminex ion exclusion column (300 by 7.8 mm; Bio-Rad) at 30°C. The column was eluted with 5 mM sulfuric acid at a flow rate of 0.6 ml · min⁻¹. Organic acids were detected with a Waters 441 UV meter at 214 nm coupled with a Waters 741 data module. Ethanol, glycerol, and maltotriose were detected by an ERMA ERC-7515A refractive index detector coupled with a Hewlett-Packard 3390A integrator. The amount of ethanol produced was corrected for the amount of ethanol in the reservoir (approximately 10 mM ethanol, originating from the addition of ergosterol). Acetic acid could not be determined by this HPLC method, since it had the same retention time as one of the medium components. It was therefore determined with Boehringer test kit 148261.

Metabolite fluxes were calculated as $q = c \cdot D/X$, in which q stands for flux (mmoles · gram of biomass⁻¹ · h⁻¹), c stands for the amount of substrate or product consumed or produced (mmoles · liter⁻¹), D stands for the dilution rate (per hour), and X stands for the biomass concentration in the culture (grams [dry weight] · liter⁻¹).

Gas analysis. The gas flowing out of the reactor was cooled in a condenser (2°C) and dried with a Perma Pure Dryer (PD-625-12P). CO₂ was determined with a Beckman model 864 infrared detector. The effluent gas flow rate was measured with a device which we constructed. It consisted of an inverted glass cylinder filled with water. Under this cylinder but without touching the glass cylinder, a water reservoir was placed to prevent outflow of the water. The reservoir rested on an electronic balance. When a gas flow was directed into the cylinder, water flowed out into the reservoir. The amount of water, assessed by the electronic balance per unit of time, was a measure for the gas flow after corrections for pressure falls, temperature, and water tension. The accuracy was 3%, and the repeatability was 0.3%.

The CO₂ flux was calculated as $q = c \cdot V/X$, in which V stands for the gas flow rate (liters · min⁻¹). Other designations are as described above. The amount of CO₂ leaving the culture with the effluent medium was negligible.

Enzyme analysis. Enzyme assays were performed with a Hitachi model 100-60 spectrophotometer at 30°C. Reaction rates were linearly proportional to the amount of enzyme added. The preparation of cell extracts and assays of pyruvate decarboxylase (EC 4.1.1.1) and glucose-6-phosphate dehydrogenase (EC 1.1.1.49) were done according to Postma et al. (18). Citrate synthase (EC 4.1.3.7) activity was determined according to the method of Srere (21), and that of hexokinase (EC 2.7.1.1) was determined according to the method of Postma et al. (15).

Maltase (EC 3.2.1.20) activity was measured by a discontinuous assay. The reaction mixture contained 100 mM acetate buffer (pH 6.6) and 60 mM maltose. The reaction was carried out in 1.0 ml of buffer at 30°C and was started by the addition of cell extract. The reaction was stopped at different time intervals by the addition of 10 μ l of 75% (wt/vol) trichloroacetic acid. Before the amount of glucose in the reaction mixture was determined with the Boehringer test kit described above, the pH of the sample was neutralized by the addition of 4.5 μ l of 10 M NaOH. All enzyme activities are expressed as micromoles of substrate converted per minute · milligram of protein⁻¹.

Maltose transport assay. Maltose uptake rates were determined as described by van Leeuwen et al. (22) by measuring the alkalization of weakly buffered cell suspensions after the addition of maltose.

Protein determination. The protein content of whole cells was determined by a modified biuret method (24). The amount of protein in cell extracts was determined by the Lowry method.

Graphical representation of data. All metabolic parameters are plotted as a function of the composition of the sugar mixture that is utilized in terms of hexose units on a molar basis according to (maltose in feed - residual maltose) × 2 / [(maltose in feed - residual maltose) × 2 + glucose in feed - residual glucose].

RESULTS

The disaccharide maltose is a good model substrate to evaluate the energy requirements of active sugar transport in *S. cerevisiae*. Since maltose hydrolysis does not require

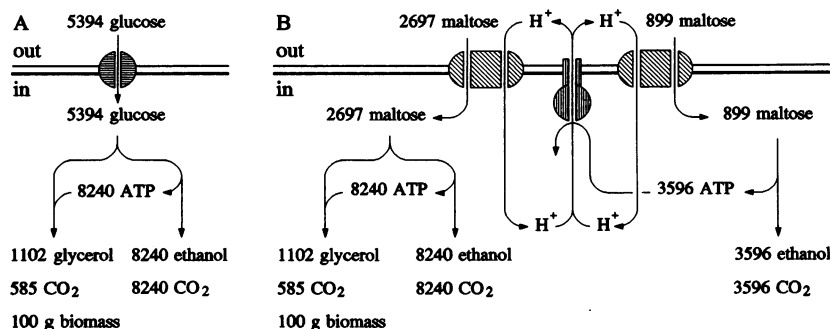
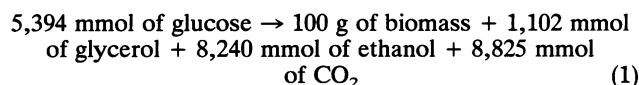


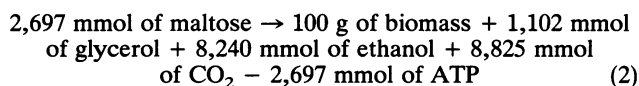
FIG. 1. Metabolic model for production of 100 g of cells of *S. cerevisiae* CBS 8066 under anaerobic conditions with glucose as a carbon source (24) (A) and transformation of the model to the utilization of maltose, assuming that maltose transport requires 1 mol of ATP per mol of maltose (B). One quarter of the maltose that is utilized [899/(2,697 + 899) mmol] is required to generate the ATP required for transport of the disaccharide.

ATP, the only difference between the energetics of maltose and glucose metabolism resides in the transport step: transport of maltose is active, whereas transport of glucose is passive. It is therefore appropriate to use established data on the energetics of growth on glucose for predicting the growth efficiency on maltose.

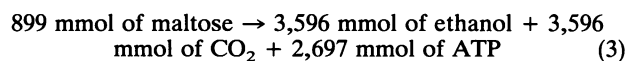
Metabolic model. A metabolic model for the anaerobic growth of *S. cerevisiae* CBS 8066 with glucose as a carbon and energy source (24) is summarized in Fig. 1A. The overall equation for biomass formation is



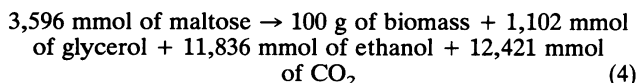
where maltose is regarded as two glucose units (in fact, maltose consists of two glucose units minus one water molecule). It was therefore convenient to use maltose monohydrate as the carbon source. Assuming that the ATP requirement for maltose transport is 1 mol of ATP, caused by a proton-maltose stoichiometry of 1 of the maltose transporter (20, 22) and a proton-ATP stoichiometry of 1 of the plasma membrane ATPase (11, 12, 14), the net ATP production from 1 mol of maltose is 3 mol of ATP (Fig. 1B). Therefore, compared with growth on glucose, a deficit of 2,697 (5,394/2) mmol of ATP will occur in the formation of 100 g of biomass from an equivalent amount of maltose:



This can be replenished by the additional dissimilation of 899 (2,697/3) mmol of maltose:



Therefore, the overall equation for the formation of 100 g of biomass from maltose under anaerobic conditions is



This model can be used to predict the production of dry weight, ethanol, CO₂, and glycerol during growth of *S. cerevisiae* with glucose and maltose as carbon sources in chemostat cultures at a dilution rate of 0.1 h⁻¹. Thus, for example, whereas the biomass yield during growth on glucose is 0.103 [100/(5.394 × 180)] g · g⁻¹ (equation 1), the

theoretical biomass yield on maltose is 0.077 [100/(3.596 × 360)] g · g⁻¹ (equation 4). The ethanol production during growth on glucose is 82.4 (8,240/100) mmol · g of biomass⁻¹ (equation 1). With a dilution rate of 0.1 h⁻¹, this is produced in 10 h or 8.24 mmol · g of biomass⁻¹ · h⁻¹. Similarly, from equation 4 it follows that the theoretical specific production rate on maltose is 11.84 (11,836/100 × 0.1) mmol · g of biomass⁻¹ · h⁻¹ (Table 1).

Methodology of chemostat cultivation on sugar mixtures.

For a comparison of anaerobic glucose and maltose metabolism it is important to keep the growth conditions, e.g., growth rate, temperature, and pH, etc., the same. The only suitable cultivation apparatus that can meet these requirements is the chemostat. Moreover, since carbon-limited chemostat cultivation gives rise to low residual substrate concentrations, it is possible to circumvent repression effects and to cultivate organisms on two carbon sources simultaneously (8). This gives the opportunity to cultivate *S. cerevisiae* on various mixtures of glucose and maltose in the feed (7). The metabolic model can thus be tested not only for growth on glucose and maltose as sole carbon sources, but also for growth on various mixtures. The theoretical yields, production, and consumption rates with mixtures of glucose and maltose can be calculated as the summation of the parameters for growth on the separate sugars. Thus, since a

TABLE 1. Predicted yields of biomass and ethanol and specific fluxes of substrates and products in anaerobic, carbon-limited chemostat cultures of *S. cerevisiae* CBS 8066 with maltose monohydrate as carbon sources

Yield or flux ^a	mM carbon source ^b		Maltose/glucose (%) ^c
	Glucose	Maltose	
<i>Y</i> _{biomass}	0.103	0.077	75
<i>Y</i> _{ethanol}	0.39	0.42	108
<i>q</i> _{ethanol}	8.24	11.84	143
<i>q</i> _{CO₂}	8.83	12.42	140
<i>q</i> _{glycerol}	1.10	1.10	100
<i>q</i> _{glucose}	5.29	0	
<i>q</i> _{maltose}	0	3.60	
<i>q</i> _{hexose}	5.29	7.20	133

^a Yields (*Y*) are in grams · gram⁻¹ and fluxes (*q*) are in mmol · gram⁻¹ · hour⁻¹. *D* = 0.1 h⁻¹.

^b Established data for growth on glucose (24) were used to predict growth parameters on maltose with the model outlined in Fig. 1.

^c Values of parameters for growth on maltose as percentages of the values for growth on glucose.

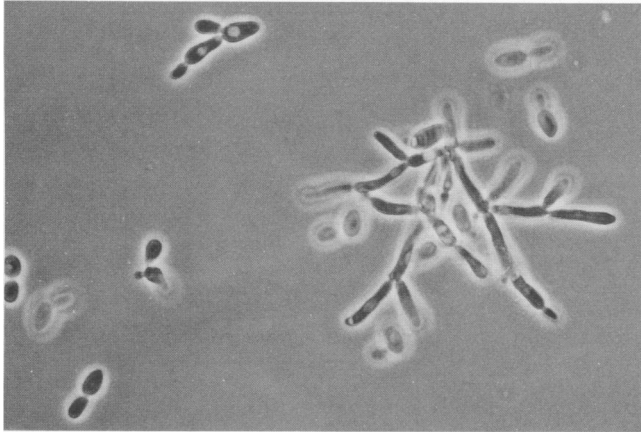


FIG. 2. Phase-contrast micrograph of *S. cerevisiae* CBS 8066 cultivated in carbon-limited chemostat culture at a D of 0.1 h^{-1} on a mixture of 75% maltose and 25% glucose. For an explanation, see the text.

25% decrease in cell yield is predicted according to the model (Table 1), growth on a 1:1 mixture of maltose and glucose (in terms of hexose units) should result in a 12.5% decrease. The advantage of this approach is obvious: the calculation of the energetics of maltose transport is not dependent anymore on one measurement, but the model can be checked by many measurements.

During the growth of *S. cerevisiae* CBS 8066 on mixtures of maltose and glucose, a peculiar problem was encountered: cells tended to change their morphology upon changes in cultivation conditions. Cultures grown on mixtures of these sugars gave rise to pseudohypha formation (Fig. 2). This was associated with a decrease in biomass densities in the culture after steady-state conditions should have been reached (i.e., after approximately five volume changes). This phenomenon occurred despite the precautions with respect to the effluent removal system (see Materials and Methods). Regular checks on the biomass densities in the culture and the culture effluent made it clear that the surface level sensing for effluent removal was appropriate: the biomass density in the culture was always within 1% of that of the culture effluent, suggesting that occurrence and selection of pseudohyphae could not be ascribed to inadequate effluent removal.

The formation of pseudohyphae occurred only during growth on glucose-maltose mixtures, not with glucose or maltose as the sole carbon source. It was not reproducible but seemed to be triggered by changes in the glucose-maltose composition of the medium. For example, when a culture was switched from a steady-state situation with maltose as the sole carbon source to growth on a 75% glucose-25% maltose feed mixture, no pseudohyphae were detected after 1 month. When this culture was subsequently grown on glucose as the sole carbon source until steady state was established and then switched to a 25% glucose-75% maltose feed mixture, pseudohypha formation started after 2 days (Fig. 2). Plating of this culture on malt agar gave rise to the development of two colony types, one consisting of normal cells and one consisting of elongated cells. To exclude the possibility that the elongated cells were an infection, both colony types were tested by the Centraalbureau voor Schimmelcultures by standard determination tests and were found to be genuine *S. cerevisiae* cells.

The volume/surface ratio of cells is known to influence the

cellular energetics (10). In order to circumvent a possible effects of cell morphology on the bioenergetics of growth, a standard procedure was adopted for steady-state cultivation on mixtures of maltose and glucose that avoided changes in morphology as follows: for each mixture, cultivation was started in a sterilized fermentor by batch cultivation. The medium pump was switched on immediately after inoculation, and cells were allowed to grow aerobically for 2 h and then switched to anaerobic conditions. To check for a steady-state situation, regular analyses of biomass and product concentrations were performed. By this procedure, steady states without pseudohyphae were obtained within six volume changes.

Verification of the metabolic model. *S. cerevisiae* CBS 8066 was grown anaerobically at a D of 0.1 h^{-1} on glucose-maltose mixtures ranging from 100% glucose to 100% maltose according to the procedure described above at a total reservoir sugar concentration of approximately $25 \text{ g} \cdot \text{liter}^{-1}$. With increasing maltose concentrations, the biomass decreased in a linear fashion from $2.42 \text{ g} \cdot \text{liter}^{-1}$ on glucose to $1.62 \text{ g} \cdot \text{liter}^{-1}$ on maltose. The glycerol concentration decreased in parallel to the dry weight. The ethanol concentration and the amount of CO_2 in the off-gas increased slightly (Table 2). Not all products were identified by HPLC, but the unidentified products were found under all conditions, and their concentrations did not change significantly. Furthermore, the carbon recovery of identified products in all steady states was between 98 and 104%.

The experimental data of Table 2 were used to calculate the actual biomass yields and specific fluxes of ethanol, glycerol, CO_2 , glucose, and maltose. These data, plotted in Fig. 3, fit well with the metabolic model. For example, during growth on a mixture of 36.7 mM maltose and 52.2 mM glucose in the medium feed (Table 2) it can be calculated that the biomass yield (defined as grams of biomass per gram of sugar) equals $0.086 \{1.92/[(36.7 - 0.45) \times 0.360 + (52.2 - 0.36) \times 0.180]\} \text{ g} \cdot \text{g of sugar}^{-1}$ [grams of biomass/(maltose in feed - maltose in culture + glucose in feed - glucose in culture)]. The metabolic model (Table 1) predicts a value of $0.088 \{[(36.7 - 0.45) \times 0.360 \times 0.077 + (52.2 - 0.36) \times 0.180 \times 0.103]/[(36.7 - 0.45) \times 0.360 + (52.2 - 0.36) \times 0.180]\} \text{ g of biomass} \cdot \text{g of sugar}^{-1}$ [(maltose in feed - maltose in culture) \times biomass yield on maltose + (glucose in feed - glucose in culture) \times biomass yield on glucose]/(maltose in feed - maltose in culture + glucose in feed - glucose in culture) and is within the accuracy of the biomass weight assay. Note that in this calculation the molecular mass of maltose is taken as 360 rather than $342 \text{ g} \cdot \text{mol}^{-1}$ to make the biomass yield on maltose comparable to that on glucose when expressed on a gram-per-gram basis. For the calculation of the ethanol production, take into account that the reservoir medium contained approximately 10 mM ethanol, which was used to dissolve ergosterol and Tween 80. The ethanol yield is defined as (ethanol in culture - ethanol in feed)/(maltose in feed - maltose in culture + glucose in feed - glucose in culture). On glucose, the ethanol yield equals $0.37 \{193 \times 0.046/[(132.1 - 0.48) \times 0.180]\} \text{ g of ethanol} \cdot \text{g of biomass}^{-1}$. On the sugar mixture with the highest amount of maltose, the ethanol yield is $0.43 \{197 \times 0.046/[(57.5 - 0.59) \times 0.360 + (3.33 - 0.08) \times 0.180]\} \text{ g of ethanol} \cdot \text{g of biomass}^{-1}$. The higher ethanol production during growth on maltose is close to the predictions listed in Table 1.

The model assumes that the cell composition is constant. Differences in cell composition may change the ATP requirements for biomass formation, thus leading to changes in the metabolite fluxes related to assimilation and dissimulation.

TABLE 2. Reservoir and residual sugar concentrations, metabolite and biomass concentrations, and standard deviations of single steady states in anaerobic chemostat cultures of *S. cerevisiae* CBS 8066 on different glucose-maltose mixtures^a

% Maltose in mixture ^b	Concn (mM) of:				Concn of metabolite in culture										Carbon recovery (%)
	Reservoir sugar		Residual sugar in culture		Dry wt (g · liter ⁻¹)	Ethanol (mM) ^c	Glycerol (mM)	Acetic acid (mM)	Succinic acid (mM)	Pyruvic acid (mM)	2-Oxoglutaric acid (mM)	Fumaric acid (μM)	CO ₂ (mmol · h ⁻¹ · liter ⁻¹)		
	Glucose	Maltose	Glucose	Maltose											
-0.1 ± 3.7	132.1 ± 1.2	0.0 ± 2.3	0.48 ± 0.01	0.06 ± 0.06	2.42 ± 0.02	193 ± 4	22.8 ± 0.3	0.35	1.4 ± 0.0	0.90 ± 0.01	0.28 ± 0.00	23 ± 0	22.0 ± 0.3	97.6 ± 6.9	
18.5 ± 2.9	107.5 ± 0.0	12.3 ± 2.2	0.54 ± 0.01	0.23 ± 0.06	2.25 ± 0.00	189 ± 2	20.5 ± 0.1	0.28	1.8 ± 0.1	0.76 ± 0.03	0.10 ± 0.01	11 ± 0	23.8 ± 0.3	97.5 ± 8.2	
37.3 ± 1.6	75.1 ± 1.4	22.4 ± 1.0	0.43 ± 0.02	0.34 ± 0.08	1.98 ± 0.01	193 ± 6	19.1 ± 1.0	0.19	1.2 ± 0.0	0.75 ± 0.06	0.15 ± 0.01	11 ± 0	22.3 ± 0.3	104.2 ± 8.6	
58.5 ± 1.1	52.2 ± 0.6	36.7 ± 1.2	0.36 ± 0.01	0.45 ± 0.06	1.92 ± 0.01	197 ± 1	18.6 ± 0.0	0.00	1.4 ± 0.1	0.57 ± 0.04	0.09 ± 0.01	7 ± 0	22.2 ± 0.4	99.9 ± 5.9	
77.9 ± 1.4	27.0 ± 0.8	47.2 ± 2.2	0.23 ± 0.01	0.45 ± 0.06	1.71 ± 0.01	198 ± 4	17.7 ± 0.2	0.44	1.2 ± 0.0	0.48 ± 0.03	0.07 ± 0.02	6 ± 1	21.1 ± 0.4	102.1 ± 8.8	
97.2 ± 0.1	3.33 ± 0.0	57.5 ± 2.2	0.08 ± 0.01	0.59 ± 0.06	1.62 ± 0.02	197 ± 3	16.0 ± 0.1	0.23	1.5 ± 0.1	0.51 ± 0.04	0.06 ± 0.01	5 ± 0	22.2 ± 0.4	103.1 ± 8.3	

^a D = 0.1 h⁻¹.

^b The mixtures are defined as the percentage of maltose metabolized (in hexose units) per total amount of sugar metabolized (in hexose units).

^c The ethanol concentration in the culture was corrected for the amount of ethanol in the reservoir.

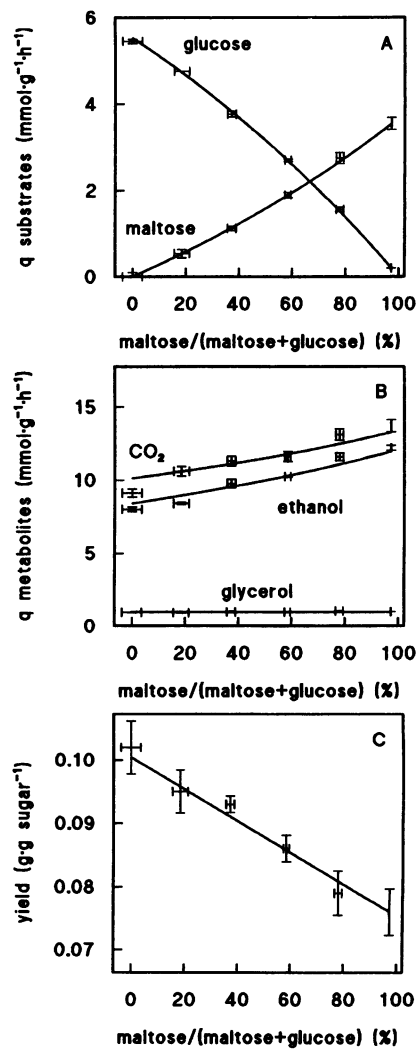


FIG. 3. Effect of amounts of maltose and glucose utilized on specific rates of glucose and maltose consumption (A), specific production rates of ethanol, CO₂, and glycerol (B), and biomass yields of anaerobic, carbon-limited chemostat cultures of *S. cerevisiae* CBS 8066 (C). The measured values are fitted with the metabolic model described in Results. The model graphs for sugar consumption, ethanol, and CO₂ production are curved because of the linear decrease in cell yield with the increasing amount of maltose in the medium feed. The values on the x axis are the percentages of maltose from the total amount of sugar utilized. Standard deviations are indicated by bars.

Over the entire range of sugar mixtures, the carbon, hydrogen, and sulfur contents of the cells did not change significantly (Fig. 4). The nitrogen content, however, increased from 7.5 to 8.0%. The protein content of the cells increased from 45.3 ± 0.5% on 100% glucose to 48.7 ± 0.9% (grams of protein · grams of biomass⁻¹) on 100% maltose. In terms of the theoretical ATP requirement for biomass formation, this increase in protein content would mean a 3% increase in energy expenditure. Therefore, this small change in biomass composition was neglected in the evaluation of the energetics of growth on maltose-glucose mixtures.

During anaerobic growth on glucose, *S. cerevisiae* produces organic acids that can be potent uncouplers. If the

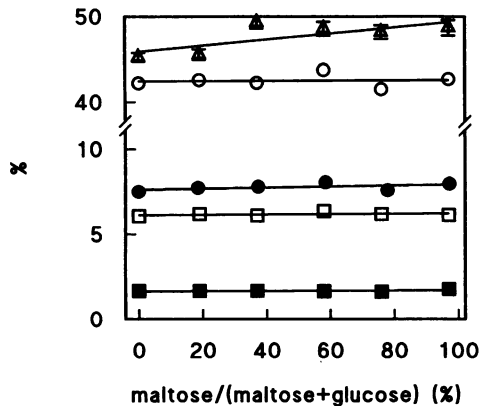


FIG. 4. Effect of amounts of maltose and glucose utilized on carbon (○), hydrogen (□), nitrogen (●), sulfur (■), and protein (△) contents of biomass in anaerobic, carbon-limited chemostat cultures of *S. cerevisiae* CBS 8066. The standard deviation for the nitrogen, carbon, hydrogen, and sulfur contents is 0.08, and those for protein are indicated. The values on the x axis are the percentages of maltose from the total amount of sugar utilized.

production rate of these acids were proportional to the rate of maltose metabolism, this would have severe consequences for the validity of the model, as this would cause a progressive decrease of the cell yield with increasing amounts of maltose in the reservoir feed. However, the amounts of acetic acid and pyruvic acid, as well as 2-oxoglutaric acid and succinic acid, were low and decreased with increasing concentrations of maltose in the feed (Table 2). The specific production rates of these acids were constant (data not shown). The decrease of fumaric acid was pronounced, but the absolute concentrations were very low. Uncoupling of metabolism by such low concentrations of acetic acid and pyruvic acid can be neglected (25).

Metabolic fluxes and enzyme activities. During growth of *S. cerevisiae* on glucose-maltose mixtures, the specific rate of sugar consumption (expressed as mmoles of hexose · gram of cells⁻¹ · hour⁻¹) slightly increased with increasing maltose concentrations in the medium feed (Table 1). This is due to the biomass yield on maltose being lower than that on glucose. This small increase in flux had no significant effect on key enzymes of the glycolytic pathway, such as hexokinase and pyruvate decarboxylase, which remained approximately constant over the whole range of sugar concentrations. The same was true for glucose-6-phosphate dehydrogenase and citrate synthase (Fig. 5) (which under anaerobic growth conditions fulfill only assimilatory functions). As expected, a different pattern was encountered for the maltose-specific enzymes, maltase and the maltose carrier. Maltase was present in glucose-limited cultures at an activity of approximately 1 U · mg of protein⁻¹. Its amount increased nearly sixfold with increasing maltose concentrations in the feed (Fig. 6A). The amount of maltose carrier also increased with increasing maltose concentrations in the medium feed. This may be envisaged as follows: the amount of maltose carrier can be calculated according to the equation $V = V_{\max} \cdot S / (K_m + s)$, in which V equals the specific maltose consumption rate in the culture (q_{maltose}), V_{\max} is equivalent to the amount of carrier, s is the residual maltose concentration in the culture (Fig. 7), and K_m is the Michaelis constant of the carrier for maltose. By using a value of 4 mM for the last parameter (22) it can be calculated that the

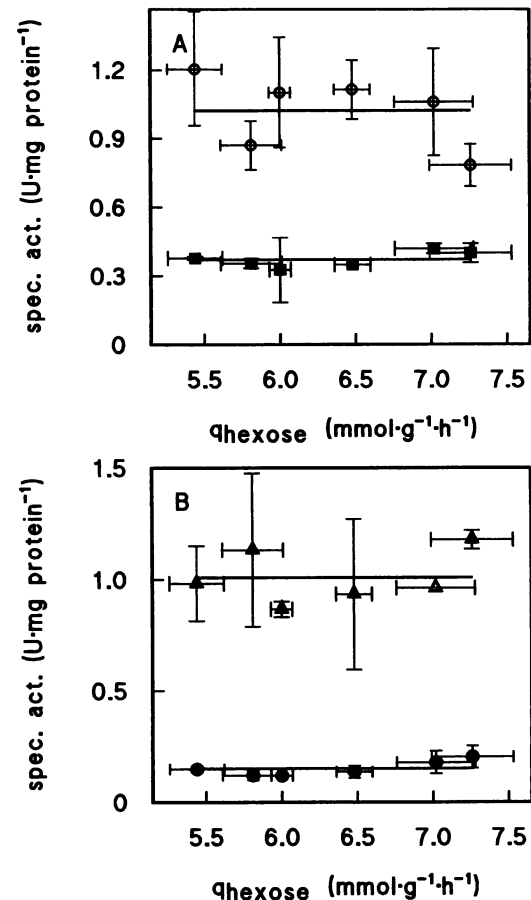


FIG. 5. Effect of glycolytic flux (in mmoles of hexose · gram of biomass⁻¹ · h⁻¹) on hexokinase (○) and glucose-6-phosphate dehydrogenase (■) (A) and pyruvate decarboxylase (△) and citrate synthase (●) (B) activities of *S. cerevisiae* CBS 8066.

amount of maltose carrier (expressed as V_{\max} in Fig. 6B) also increased with increasing amounts of maltose in the medium feed.

DISCUSSION

A quantitative analysis of anaerobic chemostat cultures of *S. cerevisiae* CBS 8066 growing on mixtures of maltose and glucose showed that growth and product formation fitted in a model based on an ATP requirement for maltose transport of 1 mol of ATP per mol of maltose (Fig. 1). The ATP requirement for maltose transport is due to the symport with protons which subsequently must be expelled by the plasma membrane ATPase at the expense of ATP. The model as presented in Fig. 1 predicts the various parameters for anaerobic growth as listed in Table 1. The experimental data (Fig. 3) show an excellent fit with these predictions. For example, the cell yield on maltose was 25% lower than that on glucose. As expected, this decrease in cell yield is proportional to the amount of maltose utilized.

Prerequisites for validation of the model. Relating cell yields to medium composition is often very difficult, since many factors, such as biomass composition and the formation of by-products, may affect cell yields. For this reason, it was decided to study cell yields not only on the individual

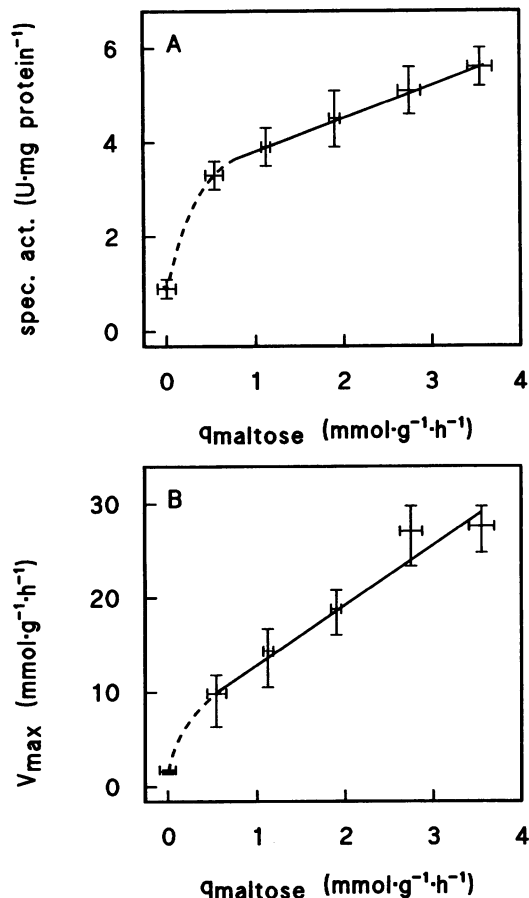


FIG. 6. Relationship between rate of maltose utilization (mmoles of maltose · gram of biomass⁻¹ · h⁻¹) and the amount of maltose (A) and maltose carrier (B) during growth of *S. cerevisiae* CBS 8066 on maltose-glucose mixtures in anaerobic, carbon-limited chemostats. The V_{\max} value for maltose uptake, with V_{\max} being the amount of maltose carrier, was calculated from the residual substrate concentration, the rate of maltose utilization, and a K_m of 4 mM. Standard deviations are indicated by bars.

sugars but also on mixtures. In this way, the model could be not only tested for growth on maltose as the sole carbon source but also verified under additional conditions. A slight increase in protein content was observed with increasing maltose concentrations in the medium feed. This, however, cannot explain the strong decrease in cell yield. Also, a maltose-associated increase in maintenance energy requirement due to uncoupling could be excluded (Table 2).

Since the model is based on the assumption that the only difference between growth on glucose and that on maltose is the transport step, other factors such as morphological changes due to a different medium composition should be avoided, especially since the surface/volume ratio can affect the cellular energetics (10). The irregular formation of pseudohyphae by *S. cerevisiae* growing under sugar limitation on mixtures of glucose and maltose interfered with attempts to quantify the energetics of maltose transport (Fig. 2). Usually, pseudohypha formation by *S. cerevisiae* is associated with nutrient limitation (2). Since the cell elongation did not occur in sugar-limited cultures growing on glucose and maltose alone, nutrient limitation cannot be the

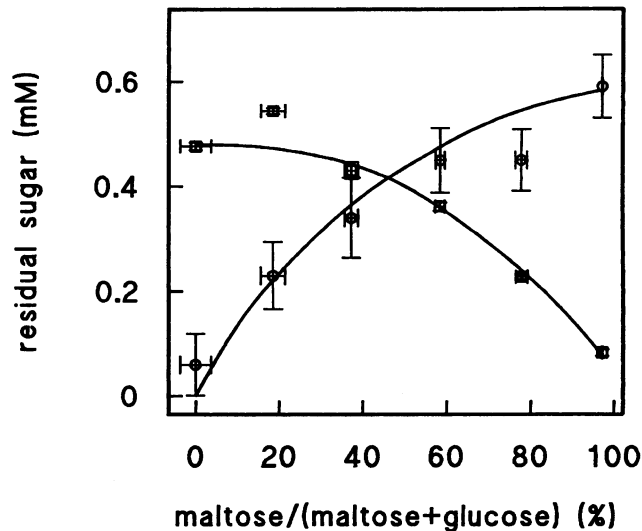


FIG. 7. Effect of amounts of maltose and glucose utilized by *S. cerevisiae* CBS 8066, cultivated in anaerobic, carbon-limited chemostats, on residual glucose (\square) and maltose (\circ) concentrations. The values on the x axis are the percentages of maltose from the total amount of utilized sugar. Standard deviations are indicated by bars.

only cause for the formation of pseudohyphae in this case. Fortunately, pseudohypha formation could be avoided by adopting a standard procedure that allowed steady-state growth with uniform yeast-like morphology.

Kinetics of mixed substrate utilization. Our results confirm and extend the original observations by Egli et al. obtained for *Escherichia coli* (6), that during growth on mixed substrates the residual concentrations of the individual substrates are lower than those during growth on the single substrate (Fig. 7). The residual sugar concentration depended on the composition of the medium feed. The residual sugar concentrations in carbon-limited chemostat cultures of *S. cerevisiae* were 2 orders of magnitude higher than those in carbon-limited *E. coli* cultures. This is a reflection of the affinity of the sugar uptake systems in *S. cerevisiae* being much lower than those in *E. coli*.

By the Michaelis-Menten equation, the residual substrate concentrations, and the K_m of the maltose carrier for its substrate, it was calculated that the amount of maltose carrier increased with increasing maltose consumption rates. In a separate experiment it was established that a small but significant amount of maltose carrier was still present in glucose-limited cultures. The same was also true for the amount of maltose. The most drastic adaptation in enzyme levels occurred at low maltose concentrations in the medium feed (i.e., at low maltose consumption rates). Above a maltose flux of 0.5 mmol · g⁻¹ · h⁻¹ the amount of these enzymes was linearly proportional to the consumption rate (Fig. 6). The clear correlation of the amounts of both enzymes with the in situ rate of maltose consumption by the cultures confirms that these enzymes exert a strong control over maltose flux in the cells.

Anaerobic cultivation for the evaluation of the bioenergetics of sugar transport. For the study of the ATP requirement of maltose-proton symport, anaerobic growth conditions were chosen, since particularly during anaerobic growth the energy requirements of sugar transport become apparent. This is due to the low ATP yield in catabolism compared with that

under aerobic growth conditions. Under anaerobic conditions, a 25% difference between cell yield on glucose and maltose can be expected (Table 1; Fig. 3). During aerobic growth, the relative effect of the energy requirement for sugar transport is much smaller. For example, with a P/O ratio (number of molecules of ATP formed per atom of oxygen used) of 1, aerobic dissimilation of 1 mol of maltose yields 32 mol of ATP. If maltose transport requires 1 mol of ATP, this would be only 1/32 of the ATP produced in catabolism. As a result, the cell yield (grams of cells · gram of maltose⁻¹) would be only 3% lower on maltose than on glucose, which is almost within the accuracy of yield determination.

Practical implications of active maltose transport. The results described above may have practical implications for ethanol production with yeasts. When starch (cereals) is used as a feedstock, it may be profitable to use hydrolysates with high maltose contents: in this way a higher yield of ethanol (grams of ethanol · gram of sugar⁻¹) can be expected (Table 1). With respect to the effects of sugar transport on cellular energetics, it would also be of interest to study various *Saccharomyces* strains that differ with respect to their modes of hexose uptake. It has been previously reported, for example, that certain strains of brewer's yeast have the ability to take up fructose by a sugar-proton mechanism (3). In such a case the energetics of growth on sucrose and maltose would be comparable. In contrast to maltose, sucrose is not taken up by yeasts but is hydrolyzed extracellularly by invertase or inulinase to glucose and fructose. If fructose transport would require 1 mol of ATP, the ATP yield of anaerobic catabolism of sucrose would be 3 mol/mol, identical to that of maltose.

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