Physiological Studies in Aerobic Batch Cultivations of *Saccharomyces cerevisiae* Strains Harboring the *MEL1* Gene

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Abstract: Physiological studies of *Saccharomyces cerevisiae* strains harboring the *MEL1* gene were carried out in aerobic batch cultivations on glucose–galactose mixtures and on the disaccharide melibiose, which is hydrolyzed by the enzyme melibiase (Mel1, EC 3.2.1.22) into a glucose and a galactose moiety. The strains examined (T200, T256, M24, and TH1) were all derived from the bakers' and distillers' strain of *S. cerevisiae*, DGI 342. All the strains showed a significant higher ethanol yield when growing on glucose, and half the biomass yield, compared with growth on galactose. The maximum specific uptake rates were 2.5–3.3-fold higher on glucose than on galactose for all the strains examined, and hence, ethanol production was pronounced on glucose due to respiro-fermentative metabolism. The T256 strain and the T200 strain having the *MEL1* gene inserted in the *HXK2* locus and the *LEU2* locus, respectively, hydrolyzed melibiose with low specific hydrolysis rates of 0.03 C-mol/g/h and 0.04 C-mol/g/h, respectively. This resulted in high biomass yields on melibiose in the order of 10 g/C-mol compared with 3.7 g/C-mol for M24 and 1.6 g/C-mol for TH1. The M24 strain, constructed by classical breeding, and the *mig1*/*gal80* disrupted and melibiase-producing strain TH1, were superior in their ability to hydrolyze melibiose into glucose and galactose showing specific melibiose hydrolysis rates of 0.17 C-mol/g/h and 0.24 C-mol/g/h, respectively. Hence, high ethanol yields on melibiose were obtained with these two strains. Growth on the glucose–galactose mixtures showed a reduction of glucose control successfully obtained in the M24 strain and the TH1 strain. © 2000 John Wiley & Sons, Inc. *Biotechnol Bioeng* **68:** 252–259, 2000.
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INTRODUCTION

Molasses is often used as a carbon source in the production of ethanol and bakers' yeast, which are both low-value-added products with a high production volume. In industrial production the yield of these products with respect to the substrate is one of the most important design parameters to obtain a profitable process. To achieve a high overall yield with molasses as substrate, it is important to utilize all the different sugars present in the molasses. Molasses mainly consist of glucose, fructose, and sucrose, but also the trisaccharide raffinose may be present in up to 2% dry weight (Rosen, 1987). The enzyme invertase (EC 3.2.1.26) encoded by the *SUC2* gene, is able to decompose raffinose into fructose and the disaccharide melibiose. In *Saccharomyces cerevisiae* both raffinose and melibiose are cleaved extracellularly and no specific uptake system for these sugars exist. Only a few strains of *S. cerevisiae* contain the gene(s) encoding the extracellular enzyme melibiase (EC 3.2.1.22) (Naumov et al., 1996) that cleaves the α-1,6-linkage of melibiose, whereby glucose and galactose are released to the medium.

Melibiase is encoded by a family of movable genes *MEL1-MEL11* of which the *MEL1* gene is the most well-characterized (Naumov, 1989; Naumov, 1996; Sumner-Smith et al., 1985). The *MEL1* gene is regulated at the transcriptional level similar to the structural *GAL* genes that encode the enzymes responsible for utilization of galactose, i.e., the Leloir pathway. These genes are tightly regulated, being induced by galactose and repressed by glucose. Transcription of these genes is mediated by the transcriptional activator protein Gal4 encoded by the *GAL4* gene (Johnston, 1987; Post-Beittenmiller et al., 1984). The Gal4 protein activates the transcription of these genes by binding to specific sequences upstream of the coding region, and the *MEL1* gene contains a single upstream-activator sequence (Johnston, 1987). The most well-described mechanism that down-regulates the expression of the *MEL1* gene and the *GAL* genes, involves the protein Gal80, which binds to the C-terminal end of Gal4, and hence, transcriptional activation of the *MEL1*/*GAL* genes does not occur (Johnston and Carlson, 1992; Melcher, 1997).

The *MEL1* gene and the structural *GAL* genes are all subject to glucose control as is the case for a number of genes (Klein et al., 1998). "Glucose control" covers the

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different mechanisms responsible for the decrease or termination of the consumption of disaccharides (e.g., sucrose, maltose) or slower fermentable carbon sources (e.g., galactose), when rapid-fermentable carbon sources (e.g., glucose and fructose) are present in the medium. Glucose control exerted on the *MEL1* gene and the *GAL* genes mainly occurs by Mig1-mediated glucose repression on the transcriptional level. This repression is mediated by a protein complex consisting of Ssn6, Tup1, and Mig1 of which the latter directs the complex to a specific consensus motif on the promoters of the target genes, whereby the expression of these genes is repressed (Keleher et al., 1992; Treitel and Carlson, 1995). Mig1 affects the expression of the *MEL1* gene indirectly by binding to the promoter of the *GAL4* gene, but it has also been suggested that Mig1 represses the *MEL1* gene directly by binding upstream of the *MEL1* gene, as verified by DNase footprinting (Lundin et al., 1994).

From an industrial point of view it is desirable to obtain a strain that can utilize melibiose which can be achieved by introducing one of the *MEL* genes into the host strain. Melibiose utilization may result in an increased biomass yield and a waste stream with reduced biological oxygen demand (BOD). In a recent study by Vincent et al. (1999), bakers' yeast strains able to hydrolyze melibiose were produced by genetic engineering and classical breeding. We used the same two approaches for construction of melibiose hydrolyzing strains, but we examined these strains with respect to their ability to hydrolyze melibiose in addition to the extent of glucose control exerted on the galactose metabolism of the constructed strains. The M24 strain was produced by classical breeding, and T200 and T256 were constructed by genetic engineering (Nielsen, 1991). Because melibiase hydrolyzes melibiose into glucose and galactose, and galactose metabolism is subject to glucose control, melibiase-producing strains were constructed, in which glucose control of the galactose metabolism was partly relieved, resulting in TH1 (constructed in the work of Rønnow et al., 1999). The ability to hydrolyze melibiose of all the strains containing one or more copies of the *MEL1* gene, was examined in aerobic batch cultivations, and hence, the maximum specific melibiose hydrolysis rates of the individual strains were determined. Further, the degree of glucose control of the individual strains, was studied in aerobic batch cultivations on glucose–galactose mixtures.

MATERIALS AND METHODS

Yeast Strains

All *S. cerevisiae* strains used in this study (Table I) were generated from the bakers' and distillers' yeast strain DGI 342 (Danisco Distillers, Copenhagen, Denmark).

Yeast-Strain Development

NRRL Y-12533 (Mel^+ Suc^-) is a homothallic distillers' strain, meaning that **a** or α spores originating from such a strain will rapidly give rise to **a**/α diploids without having to encounter cells of different clonal origin and mating type. One method of hybridizing homothallic prototrophic yeasts is crossing of individual germinating spores, and monitoring the mating visually. Sporeclones of the Mel^+ Suc^- bakers' and distillers' strain DGI 342 (e.g., D850) with defined mating type were mated with homothallic sporeclones from the *S. cerevisiae* strain NRRL Y-12533. The selection for hybrids; melibiase synthesis, giving green colonies on 5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside, and growth on sucrose medium, confirming the presence of invertase, resulted in several hybrids, of which M24 was chosen for further analysis (Nielsen, 1991).

T200, a Mel^+ transformant of DGI 342, was constructed by transforming DGI 342 with an integrative plasmid, harboring an expression cassette containing *MEL1*, controlled by the constitutive phosphoglycerate kinase (*PGK*) promoter and a selectable marker conferring resistance towards G418. The expression cassette was located within *LEU2*, directing integration to this locus, as described by Nielsen (1991).

T256, a Mel^+ transformant of DGI 342, was constructed as described by Nielsen (1991) by transforming DGI 342 concomitantly with a plasmid containing a selectable marker, conferring resistance towards G418 and a linear DNA fragment containing *MEL1*, controlled by *PGK*, within *HXK2*, using these sequences as target for integration. After transformation, the transformants were cured for the autonomous-replicating plasmid, carrying the selectable marker.

In TH1, all *MIG1* and *GAL80* genes were disrupted by combining classical breeding and plasmid technology.

Table I. Yeast strains used in this study.

Strain	Genotype	Origin	Source/reference
DGI 342	*mel SUC*	Bakers' and Distillers' strain	Danisco Distillers
D850	*mel SUC*	Derivative of DGI 342	Nielsen, 1991
NRRL Y-12533	*MEL suc*	Distillers' strain	Olivieri et al., 1984
M24	*MEL SUC*	D850 × derivate of NRRL Y-12533	Nielsen, 1991
T200	*leu2::PGK-MEL1*	DGI 342	Nielsen, 1991
T256	*hxk2::PGK-MEL1*	DGI 342	Nielsen, 1991
TH1	*mig1::MEL1/mig1::MEL1/ mig1::MEL1/mig1::MEL1 gal80::ILV2-SMR/gal80::ILV2-SMR/ gal80::PGK-MEL1/gal80::PGK-MEL1*	DGI 342	Rønnow et al., 1999

MIG1 was disrupted with *MEL1* in DGI 342, a few resulting transformants were sporulated, diploid maters were isolated, and homozygous Δ*mig1*/Δ*mig1* spontaneous recombinants were selected. After integrative inactivation of both *GAL80* genes in these recombinants, strains having all *MIG1* and *GAL80* genes inactivated, were reconstituted as described by Klein et al. (1996) and Rønnow et al. (1999).

Inoculum

The strains were stored at −80°C in Eppendorf tubes containing YPD medium (Rose et al., 1990) with 20% (v/v) glycerol. The medium was thawed and streaked out onto YPD agar plates where a single colony was picked to inoculate a shake flask.

Standard Solutions for the Media Used

The media used for precultures and for the batch cultivations was prepared according to Verduyn et al. (1990) with slight modifications. The trace-element solution and vitamin solution contained the following compositions:

Trace element solution: 3 g/L EDTA; 0.90 g/L $CaCl_2 \cdot 2H_2O$; 0.90 g/L $ZnSO_4 \cdot 7H_2O$; 0.60 g/L $FeSO_4 \cdot 7H_2O$; 200 mg/L H_3BO_3; 156 mg/L $MgCl_2 \cdot 2H_2O$; 80 mg/L $Na_2MoO_4 \cdot 2H_2O$; 60 mg/L $CoCl_2 \cdot 2H_2O$; 60 mg/L $CuSO_4 \cdot 5H_2O$; and 20 mg/L KI. The pH of the trace element solution was adjusted to 4.00 with NaOH, and autoclaved.

Vitamin solution: 50 mg/L d-biotin; 200 mg/L *para*-amino-benzoic acid; 1 g/L nicotinic acid; 1 g/L Ca-pantothenate; 1 g/L pyridoxine · HCl; 1 g/L thiamine · HCl; and 25g/L *m*-inositol. The pH was adjusted to 6.5 and stored at 4°C after sterile filtration.

Medium for Precultures Grown in Shake Flasks

Baffled, cotton-stopped, 500-mL Erlenmeyer flasks were used to obtain precultures for inoculation of the bioreactors. These flasks contained a volume of 100-mL medium with the following composition: 7.5 g/L $(NH_4)_2SO_4$; 14 g/L KH_2PO_4; 0.74 g/L $MgSO_4 \cdot 7H_2O$; 10 mL/L trace-element solution; 1 mL/L vitamin solution; 50 μL/L synperonic antifoam (Sigma® A-5551); and 0.33 C-mol/L glucose. The pH of the mineral medium was adjusted to 6.5 with NaOH and autoclaved separately from the glucose solution, which was added after autoclaving. The vitamin solution was added to the medium by filter sterilization. The shake flasks were grown at 30°C at 120 rpm for approximately 24 h until the cell mass concentration reached 1–1.5 g/L (dry weight). Subsequently, the bioreactors were inoculated to give 1 mg/L of cell mass (dry weight).

Media for the Batch Cultivations

Media for the batch cultivations had the following composition: 7.5 g/L $(NH_4)_2SO_4$; 3.5 g/L KH_2PO_4; 0.74 g/L $MgSO_4 \cdot 7H_2O$; 10 mL/L trace element solution; 1 mL/L vitamin solution; 50 μL/L synperonic antifoam (Sigma® A-5551); and 0.5 ± 0.05 C-mol/L (either as melibiose only, or as a mixture of glucose and galactose). The sugar solution was autoclaved separately from the mineral medium, and subsequently added to the bioreactor, as was the case for the vitamin solution that was added by filter sterilization.

Cultivation Conditions

The cells were grown as batch cultivations with the carbon source being the growth-limiting component. The cultivations were carried out aerobically in well-controlled four-baffled 5-L in-house-manufactured bioreactors with working volumes of 4 L. The bioreactors were equipped with two disk-turbine impellers rotating at 600 rpm, and the pH was kept constant at 5.0 by automatic addition of 2*M* H_2SO_4 or 4*M* NaOH. The air flow was 4 L/min (1 vvm), and the off gas passed through a condenser to avoid evaporation of ethanol from the bioreactor.

Cell-Mass Determination

The cell-mass concentration on a dry weight basis was determined by the use of nitrocellulose filters with a pore size of 0.45 μm (Gelman Sciences, Ann Arbor, MI). Initially, the filters were predried in a microwave oven at 150 W for 10 min, and weighed. A known volume of cell culture was filtered, and the residue was washed with distilled water. Finally, the filter was dried in the microwave at 150 W for 15 min, and weighed.

Analysis of Extracellular Metabolites

For determination of the extracellular metabolites, 2 mL of sample was taken out of the bioreactor and immediately filtered through a 0.45 μm pore-size cellulose acetate filter (Sartorius AG, Göttingen, Germany). The filtrate was frozen and kept at −20°C until analysis.

Glucose, galactose, melibiose, ethanol, and acetate were separated on an Aminex HPX-87H column (Biorad, Hercules, CA) at 65°C, using 5 m*M* H_2SO_4 as the mobile phase at a flow rate of 0.6 mL/min. Glucose, galactose, melibiose, and ethanol were detected refractometrically (Waters 410 Differential Refractometer Detector; Millipore Corp., Milford, MA). Acetate was measured spectrophotometrically with a Waters 486 Tunable Absorbance Detector set at 210 nm.

Off-Gas Analysis

A Brüel and Kjær 1308 acoustic gas analyser (Christensen et al., 1995) (Brüel & Kjær, Nærum, Denmark) was applied for determination of the carbon dioxide concentration in the off gas.

RESULTS AND DISCUSSION

Batch Cultivations on Glucose and Galactose

Growth characteristics of the different strains grown in aerobic batch cultivations on glucose–galactose mixtures were examined because melibiose is hydrolyzed into a glucose moiety and a galactose moiety. There was a distinct difference of the growth characteristics obtained on glucose and galactose for all the strains examined (Table II). The 1.8–2.7-fold higher biomass yield obtained during the growth phase on galactose, compared with the biomass yield obtained during the growth phase on glucose, was a common feature for all the strains. This is presumably due to the low consumption rate of galactose (0.03–0.04 C-mol/g/h) that causes a lower glycolytic flux, and thus, nearly all the pyruvate formed can be oxidized through the tricarboxylic acid cycle. As a result, more ATP is formed from growth on galactose compared with growth on glucose. The higher ATP formation will support biosynthesis of precursors for biomass formation, and consequently the biomass yield on galactose exceeds the biomass yield on glucose. Additionally, a significant lower ethanol yield and ethanol-production rate was observed on galactose in comparison with glucose and all the strains had a lower maximum specific growth rate on galactose than on glucose. The 2.5–3.3-fold higher maximum specific uptake rate of glucose compared with galactose resulted in respiro-fermentative metabolism due to the Crabtree effect (for further details see Alexander and Jeffries, 1990), and hence, a higher ethanol productivity and ethanol yield were obtained during growth on glucose (see Table II). The higher specific growth rates on glucose and the higher specific glucose-uptake rates may be results of an evolutionary adaptation of yeast that preferentially consumes glucose, as this sugar is present excessively in nature, compared with galactose. Various genes encode proteins that are designated glucose transporters (Bisson et al., 1993) and only the galactose permease encoded by the *GAL2* gene is able to transport galactose with an affinity comparable with the glucose transporters (Reifenberger et al., 1997). This clearly indicates that the glucose-uptake system has evolved far beyond the galactose-uptake system.

The maximum specific glucose uptake rates of 0.13 C-mol/g/h and 0.12 C-mol/g/h for T200 and T256, respectively, were significantly higher than the maximum specific glucose uptake rate of 0.10 C-mol/g/h for DGI 342, M24, and TH1. Also the maximum specific growth rates on glucose for T200 and T256 (0.44 h^{-1} and 0.42 h^{-1}, respectively) were higher than the maximum specific growth rate on glucose of 0.37 h^{-1} for DGI 342. No obvious reason can explain the higher maximum specific glucose uptake rates and the higher maximum specific growth rates on glucose between T200 and T256 on the one hand, and DGI 342 on the other hand, because the two recombinant strains only differ from DGI 342 by the *MEL1* gene expressed from a *PGK* promoter (Table I). Nevertheless, the higher maximum specific ethanol productivity of T200 and T256 is a result of the higher maximum specific glucose-uptake rate of these two strains compared with DGI 342 (respiro-fermentative metabolism). Additionally, the maximum specific galactose-uptake rate was slightly higher for T200 and T256 in addition to TH1, in comparison with DGI 342. T256 and TH1 exhibited the highest maximum specific growth rates on galactose (0.32 h^{-1} and 0.30 h^{-1}, respectively) among all the strains, while the maximum specific growth rates on galactose for T200 (0.25 h^{-1}) was the same as for DGI 342. The physiological studies on glucose–galactose mixtures showed that M24 was almost similar to DGI 342 with respect to the growth characteristics determined (Table II). The values in Table II obtained from the different growth phases of the glucose–galactose mixtures, corresponded well with the maximum specific growth rates, the biomass yields, and the ethanol-production rates determined for the strains when grown on glucose or galactose as the sole carbon source (data not shown).

After the cells were exposed to glucose, the period of induction of the *GAL* genes could be observed from the cultivations on the glucose–galactose mixtures. When glucose was depleted from the medium, biosynthesis of the structural proteins of the Leloir pathway took place, and

Table II. Growth characteristics for DGI 342 (wild-type), M24, T200, T256, and TH1 when grown on a mixture of glucose and galactose.

	Max. sp. growth rate on glucose (h^{-1})	Max. sp. glucose uptake (C-mol/g/h)	Biomass yield[a] on glucose (g/C-mol)	Max. sp. ethanol prod. on glucose (C-mol/g/h)	Ethanol yield[b] on glucose (C-mol/C-mol)	Max. sp. growth rate on galactose (h^{-1})	Max. sp. galactose uptake (C-mol/g/h)	Biomass yield[a] on galactose (g/C-mol)	Max. sp. ethanol prod. on galactose (C-mol/g/h)	Ethanol yield[b] on galactose (C-mol/C-mol)
DGI 342[c]	0.37	0.10	4.2	0.05	0.42	0.25	0.03	8.1	0.009	0.12
M24	0.37	0.10	4.9	0.05	0.50	0.24	0.03	8.6	0.009	0.18
T200	0.44	0.13	3.2	0.11	0.54	0.25	0.04	8.7	0	0
T256	0.42	0.12	4.0	0.09	0.55	0.32	0.04	9.6	0.007	0.13
TH1[c]	0.36	0.10	N.D.[d]	0.04	N.D.[d]	0.30	0.04	N.D.[d]	0.013	N.D.[d]

[a]Calculated on the basis of the biomass formed during the growth phase on glucose or galactose, respectively.
[b]Calculated on the basis of the ethanol formed during the growth phase on glucose or galactose, respectively.
[c]Data taken from Rønnow et al. (1999).
[d]Not determined, since no distinct diauxy was observed.

subsequently, growth on galactose occurred. From an industrial point of view, consumption of glucose and galactose should, ideally, occur simultaneously, if glucose control was completely abolished, to minimize the consumption time of both sugars. TH1 was constructed for this purpose and a partial simultaneous uptake of glucose and galactose was achieved. Figure 1 shows the galactose profiles of the five cultivations carried out on mixtures of glucose and galactose, where time zero is set to the time of glucose depletion from the media. All the cultivations on glucose–galactose mixtures with DGI 342, T200, T256, and M24 were carried out with initial sugar concentrations of 0.24–0.3 C-mol/L of both glucose and galactose. The time span between glucose depletion and galactose depletion is independent of the initial glucose and galactose concentrations, as long as they are the same, because a given increase of the initial sugar concentrations will increase the biomass concentration comparatively. This was confirmed as DGI 342 showed a time span of 6 h between depletion of glucose and galactose in this study, and the same time span was also reported by Rønnow et al. (1999) where the initial sugar concentrations were 0.5 C-mol/L. The cultivation of TH1 on a glucose–galactose mixture was carried out with initial sugar concentrations of 0.5 C-mol/L (data taken from Rønnow et al., 1999), and at the time of glucose depletion less than 0.4 C-mol/L galactose was present, as depicted in Figure 1. Hence, a partly simultaneous uptake of glucose and galactose was achieved with the TH1 strain, and all the galactose was consumed less than 3 h after the glucose was depleted which corresponded to 45% of the time span exhibited by DGI 342. Although M24 exhibited a time span between glucose and galactose depletion of 1.5 h longer than TH1, the time span was 30% shorter than for DGI 342. Contrary to this, no remarkable reduction in the time span between glucose and galactose depletion was observed for T200 and T256.

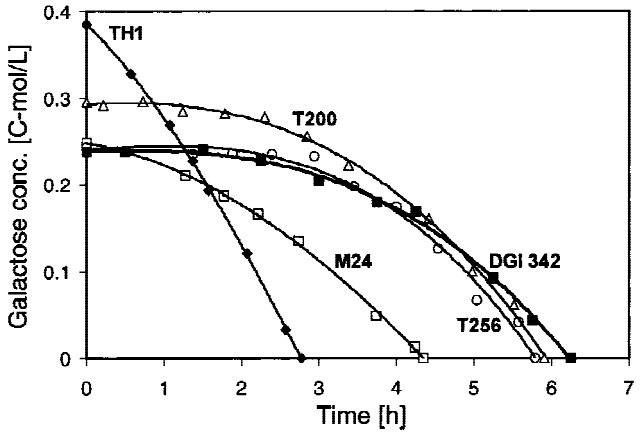

Figure 1. Profiles showing the galactose concentration for the five cultivations where the wild-type (DGI 342), M24, T200, T256, and TH1 were grown on mixtures of glucose and galactose. Time zero is set to be the time of glucose depletion from the media. Symbols: ■, DGI 342; □, M24; △, T200; ○, T256; ♦, TH1. The lines are depicted to facilitate the overview of the plot.

Batch Cultivations on Melibiose

Aerobic batch cultivations with melibiose as the sole carbon source were carried out to examine the capability of M24, T200, T256, and TH1 to hydrolyze melibiose. From these batch cultivations the differences in the maximum specific growth rates, the maximum specific rates of melibiose hydrolysis, and the product-formation patterns could be evaluated. The T200 strain and the M24 strain exhibited maximum specific growth rates on melibiose ($\mu = 0.28\ h^{-1}$ and $\mu = 0.32\ h^{-1}$, respectively) that were higher than with T256 and TH1 ($0.25\ h^{-1}$) (Table III). Figure 2 shows the sugar profiles in the four batch cultivations. Because the melibiose hydrolysis rate was higher than the consumption rate of glucose and galactose, these two sugars accumulated in the medium. The concentration of glucose and galactose in the bioreactor reached 0.03 C-mol/L and 0.04 C-mol/L, respectively, after the M24 strain had consumed the melibiose, and all the residual galactose was taken up 1 h after the glucose was depleted. Also T200 and T256 strain had time spans of 2 and 1.5 h, respectively, between the depletion of glucose and galactose, that was formed by melibiose hydrolysis, and in cultivations of these strains both glucose and galactose were built up in the medium to a concentration of 0.04 C-mol/L and 0.06 C-mol/L for T200 and T256, respectively. TH1 exhibited a somewhat different formation pattern of glucose and galactose compared with the three other strains. With this strain glucose was preferably taken up at the expense of galactose that accumulated in the medium to a concentration of 0.11 C-mol/L, whereupon galactose consumption was initiated at the time when glucose was depleted. Glucose repression cannot directly explain the extensive build-up of galactose in the medium, because the TH1 strain is the most successful construct obtained with respect to the reduction of glucose control exerted on the *GAL* genes, as illustrated in the previous section. The major reason for the increase in the galactose concentration was due to the high maximum specific rate of melibiose hydrolysis of 0.24 C-mol/g/h (Table III). Consequently, as glucose and galactose were liberated from melibiose, the glucose was taken up more preferentially than galactose. The high specific rate of melibiose hydrolysis resulted in the formation of 0.12 C-mol glucose/g/h and 0.12 C-mol galactose/g/h within the medium. These figures exceeded the maximum specific uptake rate of glucose of 0.10 C-mol glucose/g/h, and 0.04 C-mol galactose/g/h, determined when TH1 was grown on glucose or galactose as the sole carbon source, and hence, glucose but mostly galactose, accumulated in the medium.

As a result of the high specific melibiose hydrolysis rate of TH1, this strain could exhibit high specific glucose and galactose uptake rates when growing on melibiose, which caused a high ethanol yield of 0.32 C-mol ethanol/C-mol melibiose due to respiro-fermentative metabolism. M24 exhibited an ethanol yield on melibiose that was only half the yield of TH1 with an ethanol production rate one-third of TH1. This was a consequence of the lower melibiose hy-

Table III. Growth characteristics for M24, T200, T256, and TH1 when grown on melibiose.

	Max. sp. melibiose hydrolysis (C-mol/g/h)	Max. sp. growth rate (h^{-1})	Biomass yield[a] (g/C-mol)	Max. sp. ethanol production (C-mol/g/h)	Ethanol yield[b] (C-mol/C-mol)	Max. sp. acetate production (C-mmol/g/h)	Acetate yield[c] (C-mmol/C-mol)
M24	0.17	0.32	3.7	0.01	0.16	0.9	4.9
T200	0.04	0.28	9.9	N.C.[d]	0.04	N.C.[d]	3.1
T256	0.03	0.25	9.8	0.00	0.00	N.C.[d]	2.5
TH1	0.24	0.25	1.6	0.04	0.33	0.6	6.5

[a]Calculated on the basis of the biomass formed during growth on melibiose.

[b]The ethanol yield on melibiose was calculated on the basis of the ethanol formed at the time of melibiose depletion in relation to the amount of melibiose consumed.

[c]Calculated on the basis of the highest acetate concentration obtained in relation to the melibiose consumed.

[d]Not calculated, due to insufficient data points.

drolysis rate of M24 compared with TH1, which resulted in a slower liberation of glucose and galactose to the medium. Hence, M24 had a lower specific sugar-uptake rate on melibiose and respiro-fermentative metabolism was less pronounced compared with TH1. In comparison with M24 and TH1, the T200 and the T256 strains exhibited far lower specific melibiose-hydrolysis rates, and the specific uptake rates of glucose and galactose were therefore also lower for these two strains when grown on melibiose. Consequently, there was much less ethanol production and thus, a high biomass yield on melibiose of almost 10 g/C-mol was observed for the T200 strain and the T256 strain.

In addition to cell mass and ethanol, acetate was formed during the cultivations on melibiose. The production of acetate continued to increase until all the galactose was depleted in the cultivations of M24 and TH1. T256 and T200 consumed the acetate formed simultaneously with the build up of glucose and galactose, which resulted in lower acetate yields compared with M24 and TH1. This difference could occur since M24 and TH1 have higher glycolytic fluxes on melibiose, according to the maximum specific uptake rates discussed above, and consequently, no acetate was taken up until all sugars were depleted. The higher specific acetate production rate of 0.9 C-mmol/g/h of M24 compared with

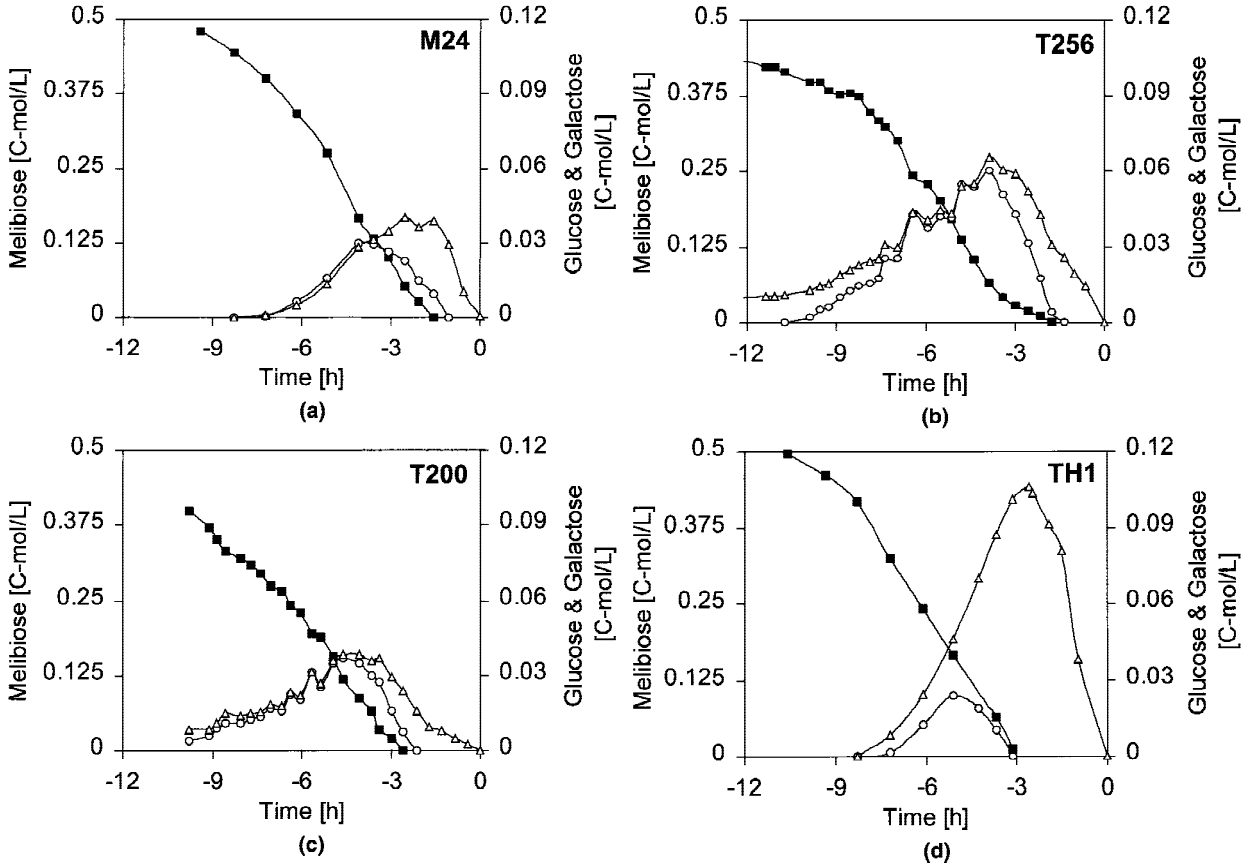

Figure 2. Concentration of melibiose, glucose, and galactose vs. time in aerobic batch cultivations of M24, T200, T256 and TH1 grown on melibiose. The time point of galactose depletion is set to zero. Symbols: ■, melibiose (C-mol/L); ○, glucose (C-mol/L); △, galactose (C-mol/L).

0.6 C-mmol/g/h of TH1, could be explained by the higher maximum specific growth rate on melibiose of M24 in comparison with TH1.

CONCLUSIONS

The *MEL1* gene was successfully introduced into a polyploid *S. cerevisiae* strain to enable growth on melibiose that may be present in industrial media such as beet molasses. The strains examined in this study were not isogenic, but they were all derived from the DGI 342 strain by classical breeding, genetic engineering, or a combination of the two.

This study illustrated that the disruptions of genes encoding proteins directly involved in the molecular events responsible for glucose repression at the transcriptional level, reduced the time span between glucose and galactose depletion, when grown on a mixture of the two. When genes such as *MIG1* and *GAL80* (TH1) were disrupted, the time span between depletion of glucose and galactose, was more than halved in comparison with the wild-type strain. Classical breeding given the M24 strain certainly affected the time span between glucose and galactose depletion due to unidentified differences in the genetic background of DGI 342 compared with M24, but the time span was not affected as extensively as was the case for the TH1 strain.

In the TH1 strain the presence of several copies of the *MEL1* gene in addition to constitutive expression of this gene from a *PGK* promoter, a far higher melibiose hydrolysis rate was observed for the TH1 strain compared with the other strains, which could be explained by the presence of several copies of the *MEL1* gene in addition to its constitution expression from a *PKG* promoter in the TH1 strain. The high glycolytic flux resulting from the melibiose hydrolysis rate of 0.24 C-mol/g/h of TH1, reveals a much higher ethanol yield due to respiro-fermentative metabolism, compared with T200 and T256. High hydrolysis rates of melibiose were not achieved by constitutive expression of the single-copy *MEL1* gene from a *PGK* promoter in T200 and T256, in comparison with M24 and TH1. Hence, the low melibiose hydrolysis rates of T200 and T256 cause low glycolytic fluxes that result in high biomass yields. Also the maximum specific growth rate on melibiose is an important design parameter for the production of bakers' yeast, and hence, T200 should be preferred over T256, because the maximum specific growth rate on melibiose of T200 exceeds the one of T256 with 0.03 h^{-1}. Obviously, fed-batch experiments will be necessary to carry out before appointing any of the strains presented here as being the best candidate(s) for large-scale bakers' yeast production, but this is beyond the scope of this study.

Introducing the *MEL1* gene into the polyploid Mel^- strain DGI 342, to enable the strains to hydrolyze melibiose, serves as another example of metabolic engineering that demonstrates the potential of targeting the cellular metabolism using a directed approach by means of genetic engineering. Also, the more classical approach of breeding and mating was applied to obtain strains able to hydrolyze melibiose. Partial alleviation of glucose control in industrial production strains may shorten the overall production time which makes an industrial process more profitable. The gain achieved from the performance of metabolic engineering on a system like the one described in this study, may seem small, because melibiose is present in molasses only to a limited extent. However, due to the tremendous production volume of both ethanol and bakers' yeast worldwide, minor improvements of the yield or productivity of these compounds may enhance the economic surplus involved with the production of these products significantly.

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