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Construction of lactose-consuming *Saccharomyces cerevisiae* for lactose fermentation into ethanol fuel

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Abstract Two lactose-consuming diploid *Saccharomyces cerevisiae* strains, AY-51024A and AY-51024M, were constructed by expressing the *LAC4* and *LAC12* genes of *Kluyveromyces marxianus* in the host strain AY-5. In AY-51024A, both genes were targeted to the *ATH1* and *NTH1* gene-encoding regions to abolish the activity of acid/neutral trehalase. In AY-51024M, both genes were respectively integrated into the *MIG1* and *NTH1* gene-encoding regions to relieve glucose repression. Physiologic studies of the two transformants under anaerobic cultivations in glucose and galactose media indicated that the expression of both *LAC* genes did not physiologically burden the cells, except for AY-51024A in glucose medium. Galactose consumption was initiated at higher glucose concentrations in the *MIG1* deletion strain AY-51024M than in the corresponding wild-type strain and AY-51024A, wherein galactose was consumed until glucose was completely depleted in the mixture. In lactose medium, the Sp. growth rates of AY-51024A and AY-51024M under anaerobic shake-flasks were 0.025 and 0.067 h^{-1}, respectively. The specific lactose uptake rate and ethanol production of AY-51024M were 2.50 g lactose g CDW^{-1} h^{-1} and 23.4 g l^{-1}, respectively, whereas those of AY-51024A were 0.98 g lactose g CDW^{-1} h^{-1} and 24.3 g lactose g CDW^{-1} h^{-1}, respectively. In concentrated cheese whey powder solutions, AY-51024M produced 63.3 g l^{-1} ethanol from approximately 150 g l^{-1} initial lactose in 120 h, conversely, AY-51024A consumed 63.7 % of the initial lactose and produced 35.9 g l^{-1} ethanol. Therefore, relieving glucose repression is an effective strategy for constructing lactose-consuming *S. cerevisiae*.

Keywords Lactose · *S. cerevisiae* · *LAC* gene · *MIG1* gene · Glucose repression

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Introduction

Cheese whey, a byproduct of the dairy industry, represents an important environmental problem because of the large volumes produced and its high organic matter content. Whey production reaches over 160 million tons annually with a 1–2 % annual growth rate [37]. Lactose, which is largely found in cheese whey, has a high biochemical oxygen demand (BOD) of 30–50 g l^{-1} and a chemical oxygen demand (COD) of 60–80 g l^{-1} [5]. Numerous studies have investigated the use of whey in bioethanol production because of its high carbohydrate content and availability [6, 19]. However, the common distiller's yeast *Saccharomyces cerevisiae* cannot ferment lactose because it lacks both lactose permease and β-galactosidase, which are responsible for transporting the disaccharide into the cytoplasm for hydrolysis into monosaccharides. Over the last 20 years, the engineering of *S. cerevisiae* for lactose utilization has been addressed through different strategies [30]. However, most of the strains displayed undesirable characteristics, such as genetic instability or problems derived from the use of glucose–galactose mixtures, or were ineffective for ethanol production. The same situation was observed in other *S. cerevisiae* strains that express both *LAC4* and *LAC12 Kluyveromyces lactis* genes [31, 32].

In general, using a lactose-consuming strain to directly ferment whey to produce ethanol is not economically feasible because low lactose contents yield low ethanol titer (2–3 % v/v). Fermentation should begin with concentrated whey to obtain high ethanol concentrations at the end of the process. Therefore, a high ethanol and osmotic pressure tolerance would be useful for ethanol production from concentrated whey. In *S. cerevisiae*, the accumulation of trehalose is widely believed to be a critical determinant in improving the stress tolerance of this yeast [34]. Two enzymes can hydrolyze trehalose: a neutral cytosolic trehalase (encoded by *NTH1*) and an acidic vacuolar trehalase (encoded by *ATH1*) [16]. Shima et al. [35] reported that the degradation of intracellular trehalose is inhibited in Δ*nth1* and Δ*ath1* mutants. These mutants also exhibit higher levels of tolerance to dry conditions than their parent strains. Kim et al. [10] reported that abolishing acid trehalase activity by disrupting the *ATH1* gene induces the accumulation of high levels of cellular trehalose and increases ethanol tolerance.

The lactose that enters cells is first hydrolyzed into glucose and galactose moieties. However, in *S. cerevisiae*, the presence of glucose inhibits the uptake and metabolism of galactose through a process known as catabolite repression [12, 29]. *S. cerevisiae* utilizes galactose through the Leloir pathway using enzymes encoded by the structural gene *GAL* [28]. *GAL* transcription is controlled by the presence of glucose through repression of the transcription of Gal4, a transcriptional activator protein of *GAL*. This repression is mediated by a protein complex that consists of Ssn6, Tup1, and Mig1, the latter of which directs the complex to a specific consensus motif on *GAL4* gene promoters, thereby repressing *GAL4* gene expression [8, 39]. Deleting the *MIG1* gene reportedly abolishes the glucose control of *GAL* gene transcription [13, 26]. If glucose control is abolished, then glucose and galactose may be simultaneously consumed, and lactose may be consumed more quickly.

In the present study, we expressed the permease and β-galactosidase genes of the *Kluyveromyces marxianus* strain TY-3 in *S. cerevisiae* AY-5 to construct lactose-consuming strains. The loci of the *ATH1* and *NTH1* genes were used as integrative regions expressing the *LAC4* and *LAC12* genes, respectively. This process, in which the *LAC* genes were controlled by the *PGK1* promoter, created AY-51024A. To relieve glucose repression, the lactose-consuming strain AY-51024M, wherein the *MIG1* and *NTH1* loci were used as integrative regions, was constructed. The ability of the two lactose-consuming strains to utilize lactose was examined using anaerobic shake-flasks. In addition, the degree of glucose control was studied under anaerobic batch cultivations in glucose–galactose mixture medium.

Materials and methods

Strains, media, and growth conditions

The yeast strains used in this study are listed in Table 1. The *Escherichia coli* strain DH5α was grown in Luria–Bertani broth with ampicillin for plasmid maintenance. *S. cerevisiae* and *K. marxianus* strains were incubated in YPD medium at 30 °C. The recombination strains were screened in YPD medium supplemented with 1,000 μg ml^{-1} G418 and 250 μg ml^{-1} zeocin. Lactose fermentation medium (containing 5 g l^{-1} $(NH_4)_2SO_4$, 1 g l^{-1} KH_2PO_4, 0.5 g l^{-1} $MgSO_4{\cdot}7H_2O$, 10 ml l^{-1} trace element solution, 1 ml l^{-1} vitamin solution, and 60 g l^{-1} lactose) was prepared according to the method by Verduyn et al. [40] with slight

Table 1 Yeast strains used in this study

Yeast strain	Genotype	Source
TY-3		Guo et al. [7]
AY-5		Guo et al. [7]
AY-51024A	*nth1*(1215,1664)*::PGK-LAC4/ nth1*(1215,1664)*::PGK-LAC4/ ath1*(1678,2355)*::PGK-LAC12/ ath1*(1678,2355)*::PGK-LAC12*	This study
AY-51024M	*nth1*(1215,1664)*::PGK-LAC4/ nth1*(1215,1664)*::PGK-LAC4/ mig1*(499,1123)::*PGK-LAC12/ mig1*(499,1123)::*PGK-LAC12*	This study

Table 2 Primers used in this work

Primer name	Primer sequences
NTH1-up	5′-tatggatcctgatcccgaaacaggcttatccagg-3′
NTH1-down	5′-ttaggatccaaaaaccccgactcgtcatcccaca-3′
ATH1-up	5′-ataggtacccccctggacatctggtaag-3′
ATH1-down	5′-actggatccagcccgtaagtatggaag-3′
MIG1-up	5′-tatggtaccggcgaaagtggtgggaat-3′
MIG1-down	5′-attggatccttgtcgtgggcgtgga-3′
Kanmx-up	5′-gcagtcgaccagctgaagcttcgtacgc-3′
Kanmx-down	5′-gaggtcgacgcataggccactagtggatctg-3′
LAC4-up	5′-gacagatctatgtcttgccttattcctgagaa-3′
LAC4-down	5′-ggcagatctttattcaaaagcgagatcaaactca-3′
LAC12-up	5′-cccctcgagcttgagctcaaaatggcagatcatt-3′
LAC12-down	5′-cccctcgagcggtctagaatggctttaaacaga-3′
Zeo-up	5′-attgtcgaccccacacaccatagcttc-3′
Zeo-down	5′- acagtcgacagcttgcaaattaaagcc-3′

Relevant restriction endonuclease sites are *underlined*

modifications. The galactose and glucose media were prepared similar to lactose fermentation medium, replacing lactose with the corresponding sugar. The sugar mixture media (containing 30 g l^{-1} glucose, 30 g l^{-1} galactose, 5 g l^{-1} $(NH_4)_2SO_4$, 1 g l^{-1} KH_2PO_4, 0.5 g l^{-1} $MgSO_4{\cdot}7H_2O$, 10 ml l^{-1} trace element solution, and 1 ml l^{-1} vitamin solution) was used to analyze the glucose repression exerted on galactose metabolism. *S. cerevisiae* AY-5 was deposited in the China General Microbiological Culture Collection Center (CGMCC) under the number CGMCC 2.1364. *K. marxianus* was deposited in the China Center of Industrial Culture Collection (CICC) under the number CICC 1953.

Plasmid and strain construction

The primers used in this study are listed in Table 2. The genomic DNA used as a template for DNA amplification was isolated from AY-5 and TY-3.

The integrative plasmid pPNLZ was constructed (Fig. 1a) to express the *LAC4* gene of *K. marxianus*. First, a *Hin*dIII–*Hin*dIII fragment from the vector pHVXII containing the promoter (PGK_P) and terminator (PGK_T) sequences of the yeast phosphoglycerate kinase I gene (*PGK1*) was inserted into the unique *Hin*dIII site of the plasmid pUC19 to generate the vector pUC-PGK. A PCR-generated 3,078-bp *LAC4* fragment from the *K. marxianus* strain TY-3 was digested with *Bgl*II and subcloned into pUC-PGK. For the integration region, an *NTH1* sequence was amplified from AY-5 genomic DNA and inserted into the *Bam*HI site of PUC-PGK. A sequence containing the *sh ble* gene and its promoter *TEF1* was amplified from the plasmid pGAPZA. The *sh ble* gene sequence was subcloned into the *Sal*I site of pUC-PGK to produce the integrative plasmid pPNLZ.

The plasmids pPALK and pPMLK both expressed the *LAC12* gene (Fig. 1b, c). A 1,764-bp *LAC12* fragment amplified from TY-3 genomic DNA was subcloned into the *Xho*I site of pUC-PGK. The *KanMX* gene (amplified from the plasmid pUG6), used in the two plasmids as a resistance mark towards G418, was inserted into the *Kpn*I and *Sal*I sites of pUC-PGK. The distinctive components of these two plasmids are the integrative regions, including an *ATH1* sequence in pPALK and a *MIG1* sequence in pPMLK (Fig. 1b, c).

Yeast cell transformation was carried out via the lithium acetate method. The plasmids pPNLZ and pPALK were linearized by cleavage within the *NTH1* and *ATH1* sequences using the restriction endonucleases *Nsi*I and *Msc*I, respectively. The plasmids were transformed into AY-5 a/α, selecting for zeocin$^+$ and G418$^+$ mutant strains on the resistance plates. Finally, the expected phenotypes of AY-5 a/α were selected and mated to obtain the hybrid AY-51024A. The linearized pPNLZ and pPMLK were transformed into AY-5 a/α, which recovered two Lac$^+$ haploids, and were mated to obtain the diploid Lac$^+$ strain AY-51024M. The recombination of the three plasmids was verified by polymerase chain reaction (PCR) [21].

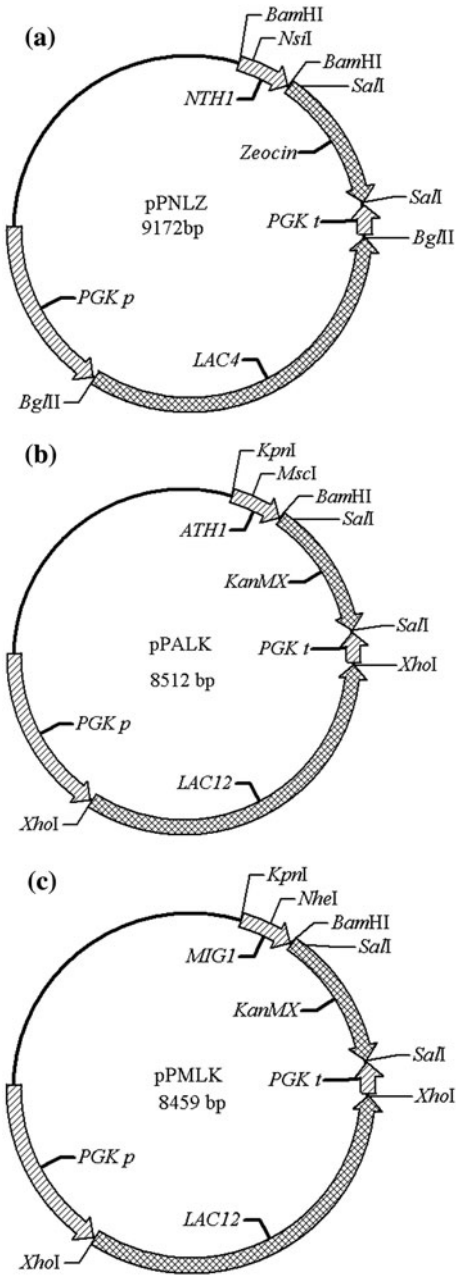

Fig. 1 Maps of plasmids **a** pPNLZ, **b** pPALK, and **c** pPMLK. The three plasmids all used pUC19 as vector, and the target gene was controlled by the *PGK1* promoter. The pPNLZ expressed the *LAC4* gene towards zeocin resistance and used a *NTH1* fragment as integrative region. The pPALK expressed the *LAC12* gene towards G418 resistance and used an *ATH1* fragment as integrative region. The only difference between pPALK and pPMLK was the integrative region, which was a *MIG1* fragment in pPMLK

Standard solutions for the medium used

The trace-element solution and vitamin solution had the following compositions:

Trace element solution: 3 g l^{-1} EDTA, 0.09 g l^{-1} $CaCl_2 \cdot 2H_2O$, 0.90 g l^{-1} $ZnSO_4 \cdot 7H_2O$, 0.60 g l^{-1} $FeSO_4 \cdot 7H_2O$, 200 mg l^{-1} H_3BO_3, 156 mg l^{-1} $MgCl_2 \cdot 2H_2O$, 80 mg l^{-1} $Na_2MoO_4 \cdot 2H_2O$, 60 mg l^{-1} $CoCl_2 \cdot 2H_2O$, 60 mg l^{-1} $CuSO_4 \cdot 5H_2O$, and 20 mg l^{-1} KI. The pH of the trace element solution was adjusted to 4.00 with NaOH, and the solution was autoclaved afterwards.

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

Calculation of Sp. growth rate, sugar uptake, and ethanol yield

The cells were cultivated in different sugars. In the exponential phase, the cell dry weight was separately measured. The change in the cell dry weight logarithm versus the time is the Sp. growth rate. In the exponential phase, the change in sugar concentration versus the change in the cell dry weight multiplied by the Sp. growth rate is the sugar uptake. The ethanol yield on sugar was calculated based on the amount of ethanol produced during fermentation versus the actual consumed sugar.

Determination of β-galactosidase activity

Crude extracts were prepared using the Mahoney et al. [17] and Ribeiro et al. [25] methods to determine enzyme activities. Galactase activity was determined following the Ribeiro et al. [25] method, which made use of using *O*-nitrophenyl-β-D-galactopyranoside (ONPG) instead of *p*-nitrophenyl-β-D-galactopyranoside. The protein concentration in the extracts was measured according to Bradford's method [3] using the Bio-Rad protein reagent and ovalbumin as the standard. One ONPG unit of enzyme activity is defined as the amount of enzyme that liberates 1 μM *O*-nitrophenol per minute under the conditions described. Specific enzyme activities were expressed as units per milligram of protein or per gram of yeast. Statistical analysis was carried out using a completely randomized design.

Colony survival after ethanol/high osmotic pressure shock

Ethanol shock: The AY-51024A, AY-51024M, and wild-type AY-5 strains were cultured in YEPD medium for 36 h at 30 °C. Culture samples of 20 OD600 units of cells were washed twice in H_2O at room temperature. The cell pellets were resuspended in glucose fermentation medium containing 20 % ethanol at a cell concentration of 5 OD600 units of cells per milliliter and incubated at 30 °C. The samples were removed every hour for 5 h, and serial dilutions of the ethanol-treated samples were spread on YEPD plates in triplicate. Viable colonies were counted after 48 h at 30 °C.

High osmotic pressure shock: The three strains were prepared in the same way as the ethanol shock experiment. NaCl (20 %) was added in YEPD medium to simulate high osmotic pressure. The cells were resuspended in YEPD medium containing 20 % NaCl. The samples were removed every hour for 5 h, and serial dilutions of the shock samples were spread on YEPD plates. The number of viable colonies was counted after 48 h at 30 °C.

Determination of trehalose and protein contents

Culture samples of 5 OD 600 units of cells were washed twice in cold YEPD, resuspended in 200 μl of cold H_2O, and then divided into two 100-μl aliquots in 1.7-ml microcentrifuge tubes. One aliquot was lysed with glass beads. The acid-washed glass beads (0.45–0.55 mm in diameter) were added at 80 % of the resuspended cell volume. The suspension was mixed by vortexing at a maximum speed for 1 min. In the control experiments, the maximal protein extraction measured using the Lowry

Table 3 β-Galactosidase activity of AY-51024A and AY-51024M in different sugar media

Carbon source	β-Galactosidase activities			
	AY-51024A		AY-51024M	
	Toluene (IU g^{-1} yeast)	Vortex (IU mg^{-1} protein)	Toluene (IU g^{-1} yeast)	Vortex (IU mg^{-1} protein)
Glucose	155.32 ± 8.42	762.23 ± 56.14	267.74 ± 4.54	793.56 ± 38.25
Galactose	295.41 ± 7.33	798.25 ± 48.54	304.12 ± 10.59	822.42 ± 43.24
Lactose	300.52 ± 7.78	803.22 ± 68.21	320.00 ± 7.62	828.45 ± 38.67
Glycerol	251.46 ± 11.67	781.13 ± 51.54	282.27 ± 9.89	812.24 ± 36.12

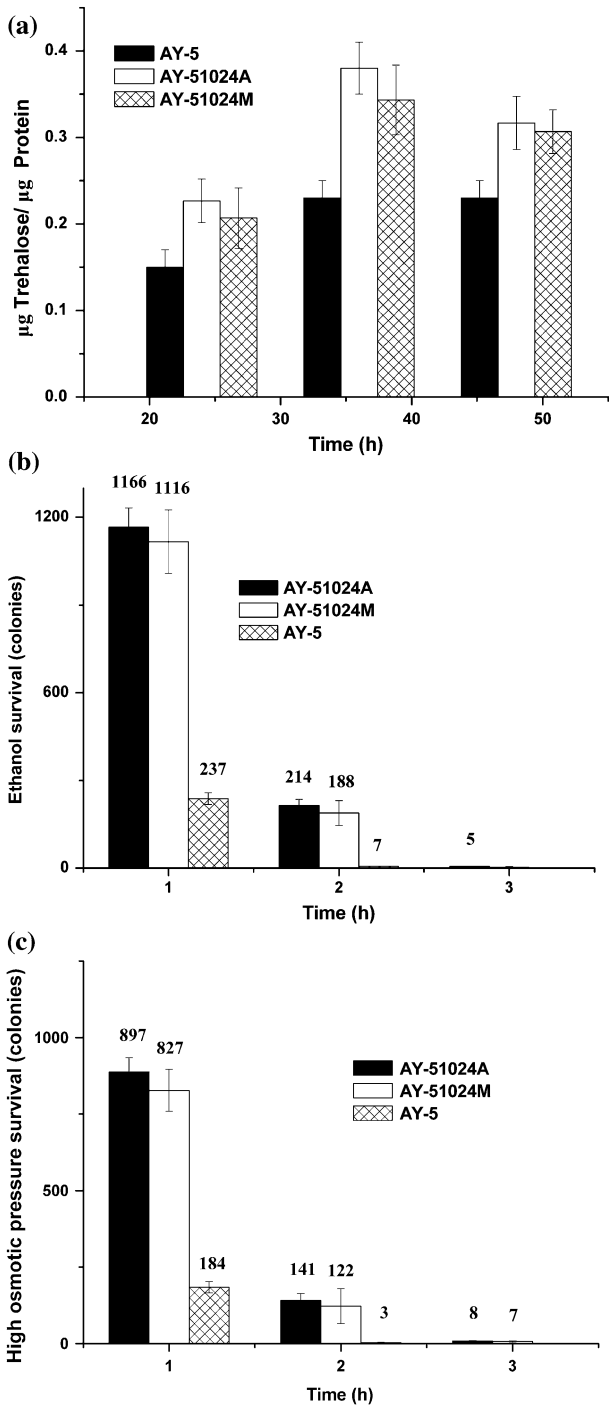

Fig. 2 Colony survival of Δ*ath1*Δ*nth1* AY-51024A, wild-type AY-5, and Δ*nth1* AY-51024M after 20 % exogenous ethanol shock and high osmotic pressure shock. **a** The cells were removed at the indicated time points and assayed for total cellular trehalose and protein as described in the "Materials and methods" section. The data shown are an average number from triplicate experiments. **b** Surviving colonies after ethanol shock and the numbers is indicated above each *graph bar*. **c** Surviving colonies after high osmotic pressure shock and the *numbers* is indicated above each *graph bar*

Table 4 Data from anaerobic cultivations on glucose and galactose

Strain	Sp. growth rate on glucose (h^{-1})	Sp. glucose uptake[a] (g glu g CDW^{-1} h^{-1})	Biomass yield on glucose[b] (g CDW g glu^{-1})	Sp. ethanol prod. on glucose[c] (g Eth g CDW^{-1} h^{-1})	Ethanol yield on glucose[d] (g Eth g glu^{-1})	Sp. growth rate on galactose (h^{-1})	Sp. galactose uptake[a] (g gal g CDW^{-1} h^{-1})	Biomass yield on galactose[b] (g CDW/g gal)	Sp. ethanol prod. on galactose[c] (g Eth/g CDW/h)	Ethanol yield on galactose[d] (g Eth/g gal)
AY-5	0.097 ± 0.0081	1.11 ± 0.095	0.035 ± 0.0025	0.334 ± 0.015	0.43 ± 0.022	0.074 ± 0.0047	0.593 ± 0.031	0.047 ± 0.0021	0.2 ± 0.012	0.44 ± 0.023
AY-51024A	0.090 ± 0.0079	0.98 ± 0.098	0.037 ± 0.0023	0.327 ± 0.022	0.43 ± 0.021	0.081 ± 0.0067	0.723 ± 0.035	0.048 ± 0.0019	0.313 ± 0.028	0.43 ± 0.022
AY-51024M	0.135 ± 0.0089	1.28 ± 0.11	0.042 ± 0.0027	0.746 ± 0.024	0.42 ± 0.024	0.104 ± 0.0089	1.067 ± 0.042	0.053 ± 0.0023	0.416 ± 0.031	0.42 ± 0.023

CDW cells dry weight

[a] Specific glucose/galactose uptake rate was calculated from the biomass yield and specific growth rate

[b] Calculated based on the biomass formed during growths on glucose/galactose

[c] Specific ethanol production rate was calculated from the ethanol yield and the specific growth rate

[d] Ethanol yield on glucose/galactose was calculated based on the amount of ethanol produced during glucose/galactose depletion in relation to the amount of glucose/galactose consumed

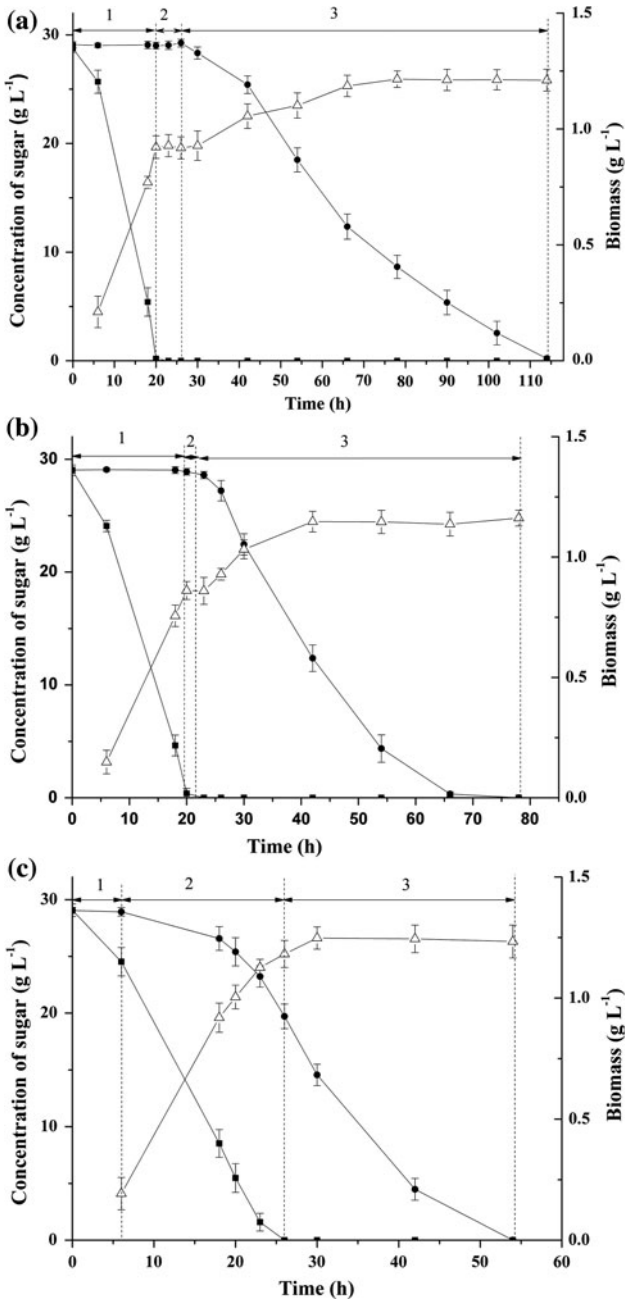

Fig. 3 Concentrations of glucose (*filled squares*) and galactose (*filled circles*) and growth curves (*open triangle*) during the anaerobic cultivation of strains **a** AY-5, **b** AY-51024A, and **c** AY-51024M on a glucose–galactose mixture. The *numbers 1*, *2*, and *3* above each panel refer to the different growth phases as described in the *text*

method was achieved after approximately 30 s of vortexing. After glass bead lysis, the protein concentration of the crude lysate, at an appropriate dilution, was measured via the Lowry method using the commercial Pierce bicinchoninic acid protein assay kit with bovine serum albumin fraction V as the standard. Using 0.5 M trichloroacetic acid on ice, cellular trehalose was extracted from the second aliquot for 30 min, as previously described [15]. Trehalose content was determined using the anthrone method [9]. The trehalose extracted from approximately 1.5 OD600 units of cells gave a signal within the linear range of the anthrone assay. Trehalose concentration was normalized and expressed as micrograms of trehalose per microgram of the total protein.

Analysis of extracellular metabolites

The extracellular metabolites were determined by immediately filtering 2 ml of the sample from the flask through a 0.45-μm pore cellulose acetate filter. The filtrate was frozen and kept at −20 °C until analysis. Ethanol, galactose, lactose, and glucose contents were analyzed by high-performance liquid chromatography (HPLC) using an Aminex HPX-87H column at 65 °C with 5 mM H_2SO_4 as the mobile phase at a flow rate of 0.6 ml min^{-1}. The sugars were detected using a refractive index detector (Waters 410).

GenBank accession numbers of *LAC4* and *LAC12*

The *LAC4* and *LAC12* genes of *K. lactis* was published in GenBank in 1993 and 2006 under the accession numbers of M84410.1 and X06997.1, respectively. The primers of *LAC4* and *LAC12* in this paper were designed according to the upper two gene sequences published in GenBank.

Results

Determination of β-galactosidase activity

The β-galactosidase activity of the AY-51024A and AY-51024M strains cultured in different carbon sources was determined to confirm *LAC4* gene expression. Toluene and vortex methods were used to autolyze the yeast cells to measure the activities. In the two mutants, β-galactosidase was synthesized. However, the activities, in which toluene was used to autolyze cells, were significantly higher in other carbon sources than in glucose (Table 3). Although the *LAC4* and *LAC12* genes are induced specifically by lactose or galactose in *K. lactis* [14, 41], the *LAC4* gene may not be induced in the two mutants because it was promoted by the constitutive *PGK1* promoter. This phenomenon might result from the different autolysis efficiencies in toluene. Therefore, the vortex method was used to autolyze the cells. The activity of β-galactosidase, which was obtained by vortexing, in galactose was close to that in lactose and still higher than that in glucose. The activity of β-galactosidase in glycerol was between galactose/lactose and glucose (Table 3).

Determination of trehalose content and stress tolerance

The cellular trehalose levels in the AY-51024A, AY-51024M, and AY-5 strains were detected in the cells grown in glucose fermentation medium at different time intervals as described in the "Materials and methods" section. The cellular trehalose in the three strains peaked at 36 h, after which the trehalose levels correspondingly decreased. Higher trehalose levels accumulated in the *ATH1NTH1*-deletion strain AY-51024A and *MIG1NTH1*-deletion strain AY-51024M compared with AY-5 (Fig. 2a).

The three strains were grown in glucose fermentation medium to directly determine whether the AY-51024A and AY-51024M strains were resistant to higher ethanol levels and higher osmotic pressure compared with AY-5. At 36 h, the samples were removed and exposed to 20 % ethanol and 20 % NaCl, as described in the "Materials and methods" section. After 1 h of ethanol exposure, AY-51024A showed a fivefold increase in the number of surviving colonies, compared with AY-5 (Fig. 2b). After 2 h of incubation, the number of viable AY-51024A colonies decreased fivefold, whereas the viability of the AY-5 strain was nearly eliminated. No surviving colonies were detected after 4 h of incubation in 20 % ethanol medium. The cellular trehalose content of AY-51024M was increased relative to that of the wild-type strain AY-5 but slightly lower than that of AY-51024A (Fig. 2a). Compared with AY-5, the tolerance of AY-51024M to ethanol was improved. By the second hour of incubation, 118 AY-51024M colonies were detected and none after 3 h of ethanol exposure (Fig. 2b). In the high osmotic pressure test, the performance of AY-51024A was the best among the three strains (Fig. 2c), and the performance of AY-51024M was slightly inferior to that of AY-51024A. Therefore, both AY-51024A and AY-51024M exhibited higher levels of tolerance to toxic ethanol levels and high osmotic pressure compared with the wild-type strain AY-5.

Cultivation on glucose and galactose

In strains AY-51024A and AY-51024M, two key *K. marxianus* genes responsible for lactose hydrolyzation were expressed. However, the heterologous gene expression may result in morphologic modifications that negatively affect cell growth and alter cell size distribution, leading to cell lysis [1, 18, 21]. In addition, because lactose is hydrolyzed into glucose and galactose moieties, any change in glucose and/or galactose consumption would affect the lactose utilization. The physiologic characteristics examined under anaerobic conditions are listed in Table 4. A common feature of all the strains was a higher biomass yield obtained on galactose than on glucose. The higher specific growth rates on glucose and the higher specific glucose uptake rates for the three strains may result from an evolutionary adaptation. Various genes encode proteins that are able to transport glucose [2], and only the galactose permease encoded by *GAL2* gene is able to transport galactose in *S. cerevisiae* [29]. For the three strains, as the specific glucose uptake rates were higher than the specific galactose uptake rates, the higher the specific ethanol productivity obtained during growth on glucose. The specific glucose and galactose uptake rates of AY-51024M were significantly higher than those of AY-5 and AY-51024A, which might be due to the deletion of the *MIG1* gene (for further details see Klein et al. [12]). However, further study is needed to clarify the reason for the lower specific growth rate of AY-51024A on glucose compared with those of the other two strains. Considering the cultivation was performed under anaerobic conditions, the ethanol yield from glucose for each strain was similar to that on galactose. Thus, except for the AY-51024A in glucose, the heterologous gene expression did not impose physiologic burden on AY-51024A and AY-51024M, considering they exhibited higher specific growth rates than AY-5 on galactose and glucose.

Cultivation on mixed glucose–galactose

In *S. cerevisiae*, glucose was exhausted before galactose. AY-51024A, AY-51024M, and AY-5 were cultivated in a mixture of 30 g l^{-1} glucose and 30 g l^{-1} galactose to analyze the influence of glucose control on galactose metabolism under anaerobic shake-flask fermentations. The growth of AY-5 can be divided into three phases based on sugar consumption (Fig. 3a). The cells mainly grew on glucose in the first phase, during which 28.5 g l^{-1} glucose and 0.1 g l^{-1} galactose were consumed. The second phase was a lag phase, during which the enzymes necessary for galactose utilization were synthesized. The production of these enzymes was repressed during the first phase in the presence of glucose. By the third phase, the growth was based on galactose. The combined duration of the three phases lasted for approximately 114 h. The growth characteristics of AY-51024A, in which *ATH1* and *NTH1* were deleted, did not significantly differ from those of AY-5 in the first two phases (Fig. 3b). During the third phase, the galactose consumption rate of AY-51024A increased. The cultivation duration for the strain lasted for approximately 78 h, which was 32 % shorter than that of AY-5. The expression of the *LAC12* gene in AY-51024A, which transports lactose and galactose in *K. marxianus*, may be responsible for the change [33]. In the growth of AY-51024M, the lag phase was not observed (Fig. 3c). The cells mainly grew on glucose in the first phase, during which the proteins for the Leloir pathway were synthesized. During the second phase, galactose consumption

Table 5 Growth characteristics of AY-51024M and AY-51024A on lactose

Strains	Sp. growth rate on lactose (h^{-1})	Sp. lactose uptake rate[a] (g lac g CDW^{-1} h^{-1})	Biomass yield on lactose[b] (g CDW g lac^{-1})	Sp. ethanol prod. on lactose[c] (g Eth g CDW^{-1} h^{-1})	Ethanol yield on lactose[d] (g Eth g lac^{-1})
AY-51024A	0.025 ± 0.003	0.98 ± 0.15	0.029 ± 0.0021	0.32 ± 0.026	0.43 ± 0.028
AY-51024M	0.067 ± 0.002	2.50 ± 0.11	0.031 ± 0.0027	0.72 ± 0.024	0.47 ± 0.024

[a] Specific lactose uptake rate was calculated from the biomass yield and specific growth rate

[b] Calculated based on the biomass formed during growths on lactose

[c] Specific ethanol production rate was calculated from the ethanol yield and specific growth rate

[d] Ethanol yield on lactose was calculated based on the amount of ethanol produced during lactose depletion in relation to the amount of lactose consumed

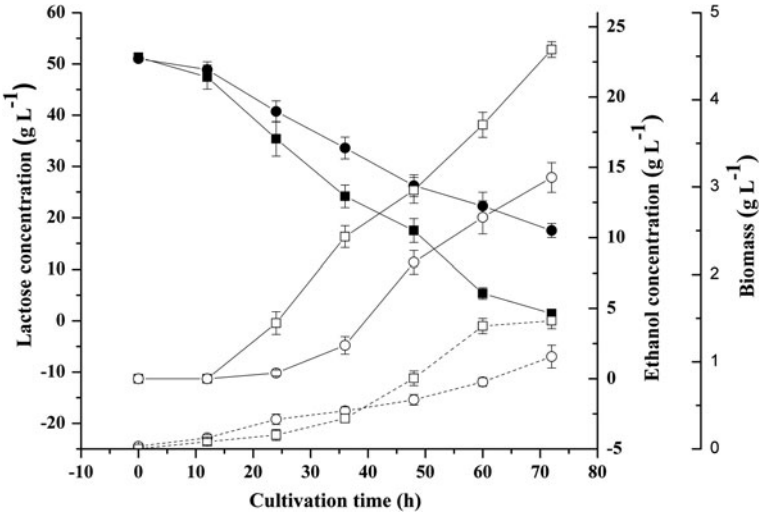

Fig. 4 Profiles of lactose consumption (*solid symbols*, *solid lines*) and ethanol production (*open symbols*, *solid lines*) and growth curves (*open symbols*, *dashed lines*) in AY-51024A (*solid* and *open circles*) and AY-51024M (*solid* and *open squares*). The duration of fermentation for the *MIG1* deletion strain was 72 h. The residual lactose was 1.5 g l^{-1}, and the final concentration of ethanol was 23.4 g l^{-1}. However, in this duration for AY-51024A, the residual lactose was 17.5 g l^{-1}, and the concentration of ethanol was 14.3 g l^{-1}

began, and the growth was partly based on glucose and galactose (24.5 g l^{-1} glucose and 9.2 g l^{-1} galactose were consumed). In the third phase, growth was mainly based on galactose. The whole fermentation process for AY-51024M lasted for 56 h, which is 51 % shorter than that for AY-5. Therefore, disrupting Mig1 activity relieves the glucose control of galactose metabolism in AY-5 and AY-51024A, which have functional Mig1.

Characterization of fermentation on lactose

AY-51024A and AY-51024M were cultivated in lactose medium under anaerobic shake-flask fermentations. AY-51024A showed growth in lactose medium, but the growth was slow. The growth characteristics of AY-51024A are listed in Table 5. The specific growth rate and lactose uptake rate of AY-51024A were 0.025 h^{-1} and 0.98 g lactose g CDW^{-1} h^{-1}, respectively. After 72 h of cultivation on 51 g l^{-1} lactose, the residual lactose was 17.5 g l^{-1}, and the concentration of ethanol was 14.3 g l^{-1}, which corresponds to 79.3 % of the theoretical conversion. The ethanol productivity of AY-51024A was 0.20 g l^{-1} h^{-1}, and the final biomass dry weight was 1.04 g l^{-1}. The performance of AY-51024M differed from that of AY-51024A. Lactose was depleted in 72 h. The growth characteristics of AY-51024M are listed in Table 5. The specific growth rate and specific lactose uptake rate of AY-51024M were 0.067 h^{-1} and 2.50 g lactose g CDW^{-1} h^{-1}, respectively. The final biomass concentration was 1.48 g l^{-1}, and the biomass yield on lactose was 0.031 g CDW g $lactose^{-1}$. The final ethanol concentration was 23.4 g l^{-1}, which corresponds to 87.1 % of the theoretical conversion yield (Fig. 4), and the ethanol productivity was 0.34 g l^{-1} h^{-1}. The different performances of AY-51024M and AY-51024A in utilizing lactose might be due to glucose repression. In AY-51024A, glucose and galactose were liberated from lactose, causing glucose to repress galactose consumption, thereby affecting lactose consumption. The deletion of the *MIG1* gene in AY-51024M removed the associated mechanism, accelerating the lactose consumption.

Characterization of fermentation on concentrated cheese whey powder solution

Two transformants were cultivated on CWPS, which corresponds to 150 g l^{-1} initial lactose under anaerobic shake-flask fermentations. During the fermentation of AY-51024M, the lactose was nearly depleted after approximately 120 h, resulting in the production of 63.3 g l^{-1} ethanol (Fig. 5), with a corresponding productivity of 0.53 g l^{-1} h^{-1}. The highest ethanol concentration was reached at the point of lactose exhaustion, which corresponds to 78.4 % of the theoretical conversion yield. The finial biomass dry weight was 2.46 g l^{-1}. AY-51024A utilized lactose to produce ethanol in CWPS. During the fermentation, only 63.7 % lactose was utilized in 120 h and 35.9 g l^{-1} ethanol was produced. The ethanol productivity was 0.21 g l^{-1} h^{-1}, with lactose-to-ethanol conversion yields 70.2 % of the theoretical yield, which is lower than that of AY-51024M. Therefore, AY-51024M utilized lactose in whey more efficiently to produce ethanol than AY-51024A.

Discussion

The development of microorganisms capable of converting cheese whey into useful products is an important biotechnological objective [36]. In the present study, two lactose-consuming *S. cerevisiae* strains, AY-51024A and AY-51024M, were constructed through the expression of the *LAC4* and *LAC12* genes of *K. marxianus*. Through measuring the β-galactosidase activity, the *LAC4* genes were expressed in the two mutants. Although the *PGK1* promoter is generally regarded as constitutive, it can be regulated to some extent by carbon sources [4, 20]. *PGK1* mRNA levels are high in yeast cells grown in glucose medium but low in yeast cells grown in media containing carbon sources such as pyruvate and acetate. However, no references on galactose/lactose impairing the expression of *PGK1* were reported. The activities in glycerol were close to those in galactose/lactose. Therefore, the β-galactosidase activity was lower in glucose than in other carbon sources, such as galactose, lactose, and glycerol (Table 3). However, the reason behind this finding needs further study.

Galactose utilization was repressed in the glucose–galactose mixture of AY-5 and AY-51024A (Fig. 3). This repression is mediated by a protein complex consisting of Ssn6, Tup1, and Mig1, the latter of which directs the complex to a specific consensus motif on the *GAL4/LAC9* gene promoters [29]. Many examples of *MIG1* gene deletion approaches were used in relieving glucose repression on other sugars, such as maltose and galactose [11, 21, 22, 26]. Klein et al. [11] reported that in a *MIG1*-disrupted haploid strain, maltose metabolism is initiated at high glucose concentrations. However, *LAC* gene expression with *MIG1* gene deletion has not been previously reported in lactose-consuming *S. cerevisiae*. In this paper, the *Δmig1* lactose-consuming strain AY-51024M, in which the *MIG1* gene was deleted, was constructed to promote *LAC* expression (Table 3) and *GAL* transcription, thereby alleviating the glucose control of galactose metabolism. Galactose metabolism was initiated despite the high glucose

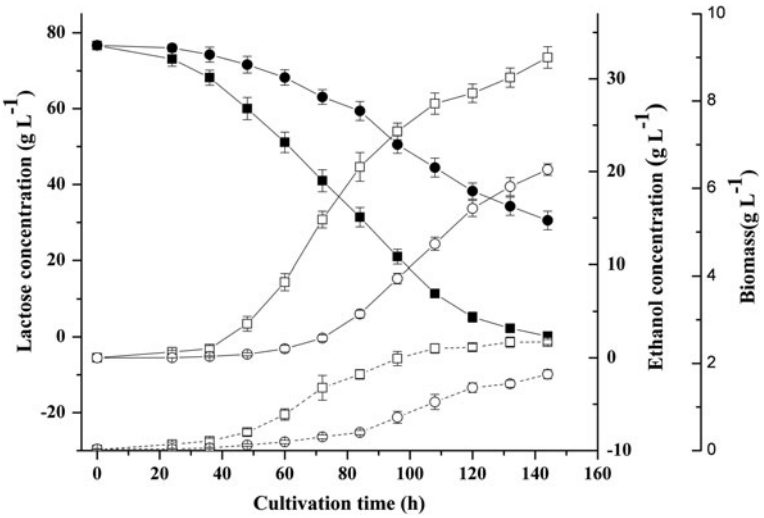

Fig. 5 Profiles of lactose consumption (*solid symbols*, *solid lines*) and ethanol production (*open symbols*, *solid lines*) and growth curves (*open symbols*, *dashed line*) in AY-51024A (*solid* and *open circles*) and AY-51024M (*solid* and *open squares*) during fermentation in CWPS. The duration of fermentation for the AY-51024M was 120 h. The residual lactose was 1.1 g l^{-1}, and the final concentration of ethanol was 63.3 g l^{-1}. However, in this duration for AY-51024A, the residual lactose was 54.1 g l^{-1} and the concentration of ethanol was 35.9 g l^{-1}

concentration, and the cultivation duration was reduced by 51 % compared with the wild-type AY-5 (Fig. 3). The results are similar to those reported by Klein et al. [12], in which the lag phase during the batch cultivation of a *Δmig1* strain grown on a glucose–galactose mixture was reduced by 50 %.

A difference in lactose utilization was observed between AY-51024A and AY-51024M. The AY-51024A strain slowly metabolized lactose. When the cells were cultivated for 72 h in lactose medium, only 65.7 % lactose was consumed and ethanol concentration was 14.3 g l^{-1}. However, AY-51024M completely consumed the lactose in the medium. For CWPS with high initial lactose concentration (150–200 g l^{-1}), the elevated osmotic pressure caused by the high concentration of lactose and other cheese whey powder components may impact yeast fermentation performance [23, 24, 27]. Given that the *ATH1* and/or *NTH1* was disrupted and the stress resistance of the cells was increased (Fig. 2), the two strains could utilize and ferment lactose in CWPS (Fig. 5). However, in the same conditions, the lactose consumption of AY-51024A was inferior to that of AY-51024M (Figs. 4, 5). We assumed that the suboptimal behavior of AY-51024A is caused by a low copy of both *LAC* genes or by glucose repression of lactose/galactose metabolism. However, Sreekrishna and Dickson [38] elucidated that the ability of lactose utilization did not seem to result from the increased gene expression because the Lac^+ and Lac^- transformants contained equal numbers of *LAC4* and *LAC12* transcripts. Therefore, the second possibility proved to be correct. The *MIG1* deletion strain AY-51024M may efficiently utilize lactose under anaerobic conditions. The cells fermented lactose (approximately 149 g l^{-1}) and produced 63.4 g l^{-1} ethanol, which corresponds to 79.7 % of the theoretical conversion yield (Fig. 5). This phenomenon shows that glucose was taken up more preferentially than galactose, although both glucose and galactose were liberated from lactose (Fig. 3b). Conversely, lactose consumption was affected with a fully functioning *MIG1* gene, such as in AY-51024A. The deletion of the *MIG1* gene eliminated its associated mechanism in AY-51024M, in which glucose and galactose were almost simultaneously utilized (Fig. 3c), subsequently increasing lactose consumption. Thus, eliminating glucose repression provides an effective strategy for constructing a lactose-consuming *S. cerevisiae* strain.

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