

Construction of engineered *Saccharomyces cerevisiae* strain to improve that whole-cell biocatalytic production of melibiose from raffinose

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Abstract There are excessive by-products in the biocatalysis process of this whole-cell biocatalytic production of melibiose from raffinose with current *Saccharomyces cerevisiae* strains. To solve this problem, we constructed engineered strains based on a liquor yeast (*S. cerevisiae*) via gene deletion (*mell* gene), heterologous integration (*fsy1* or/and *ffzi1* gene from *Candida magnoliae*), and gene over-expression (*gcr1* gene). Functional verification showed that deletion of the *mell* gene led to elimination of the reactions catalyzed by α -galactosidase, as well as elimination of the degradation of melibiose and the formation of galactose by-product. Insertion of the *fsy1* or/and *ffzi1* gene and over-expression of the *gcr1* gene could contribute to fructose

transport for enhancing the biopurification rate of the fructose by-product. Compared with the wild-type strain, the optimal engineered strain of MP8 (Δ *mell*::*fsy1*_{cm}::*ffzi1*_{cm}::*gcr1*_{sc}) had improved about 30% on yield, 31% on productivity, and 36% on purity of the melibiose product.

Keywords Melibiose · Raffinose · *Saccharomyces cerevisiae* · Whole-cell biocatalysis · Engineered strain · Biopurification

Introduction

Melibiose (α -D-Gal-(1 \rightarrow 6)- α -D-Glc), an important natural oligosaccharides with moderate sweetness, is widely used as an additive in manufactured foods, beverages, and cosmetics [13]. In particular, increasing evidence indicates that melibiose can be used as a main ingredient in human functional foods, animal-feed supplements, and pharmaceutical formulations. For instance, because of its prebiotic properties, melibiose can promote proliferation of the intestinal probiotic group such as *Bifidobacteria* and *Lactobacillus* [29]. Melibiose can adjust and maintain the balance of the human immune system to relieve allergic dermatitis symptoms [26, 27]. Additionally, it has been shown that melibiose is a potential therapeutic drug for polyQ-mediated neurodegenerative diseases such as Alzheimer's disease [21]. Therefore, melibiose possesses potential applications for disease treatment, assisted food therapy, and animal disease prevention and treatment, which should expand the market for melibiose greatly over traditional sweeteners.

Melibiose was first identified in honey and later in many plants. Despite the higher content of melibiose in cottonseed, sugar beet, and mallow than in other plant sources, it is arduous to extract melibiose from these plants on a

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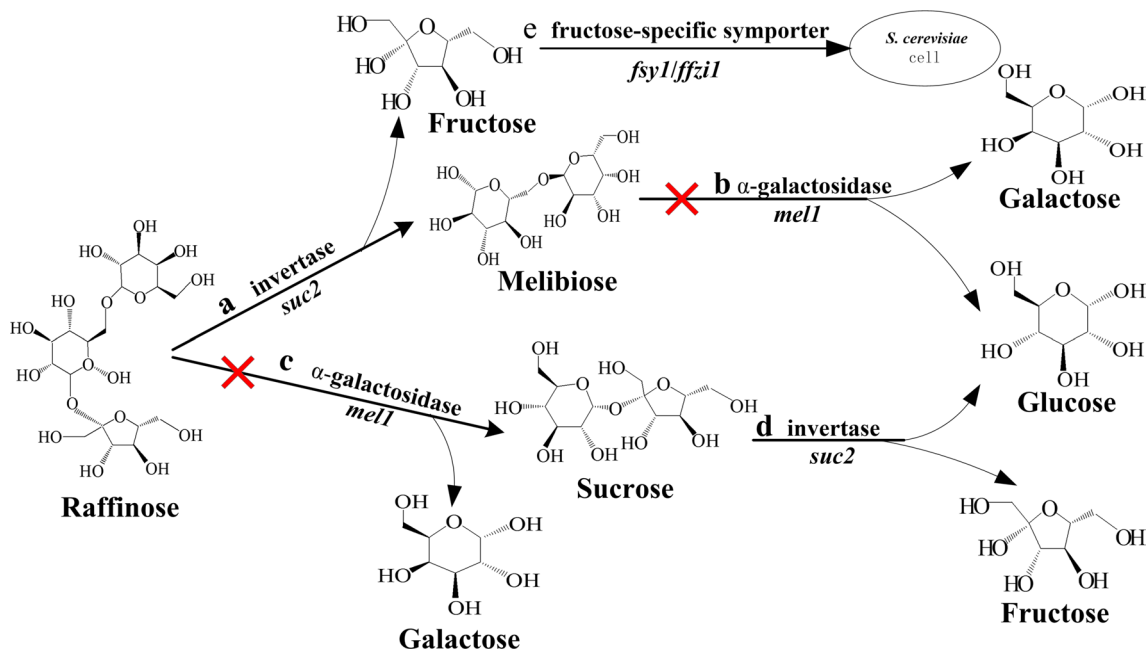


Fig. 1 Raffinose is whole-cell biocatalyzed by *Saccharomyces cerevisiae* and the strategies are presented for reducing by-products in this whole-cell biocatalysis. **a** Raffinose catalyzed by invertase (encoded by the *suc2* gene) secreted from *S. cerevisiae* cell to product melibiose (target product) and fructose (by-product). **b** Further, melibiose is catalyzed by another secreted enzyme, α -galactosidase (encoded by the *mell* gene), to product galactose (by-product) and glucose (by-product). **c** Raffinose also can be hydrolyzed by α -galactosidase to product galactose and sucrose. **d** The sucrose is then hydrolyzed by the invertase to product glucose and fructose. In order to reduce

the formation of by-products, such as sucrose, galactose, glucose, and fructose, it is essential for the hydrolytic reaction induced by α -galactosidase to be inhibited (labeled with red cross). Consequently, only the target product (melibiose) and one by-product (fructose) should be produced in this reaction system. **e** Finally, the fructose-specific symporter(s) (such as encoded by the *fsy1* or/and *ffz1* gene(s)) was/were heterologous expressed in this *S. cerevisiae* strain for improvement of the fructose's trans-membrane transport to reduce the by-product fructose (color figure online)

commercial scale because of the high cost of the purification process [8, 14, 25]. However, in some by-products of the agricultural industry, such as cottonseed and bean pulp, the content of raffinose (α -D-Gal-(1 \rightarrow 6)- α -D-Glc-(1 \rightarrow 2)- β -D-Fru), a trisaccharide composed of one melibiose and one fructose, is high enough for economical extraction [11, 25]. Hence, melibiose can be produced from raffinose by enzyme hydrolysis with invertase or by whole-cell biocatalysis with *Saccharomyces cerevisiae* [16, 24, 30]. Compared with enzyme hydrolysis, whole-cell biocatalysis has several advantages such as more flexible reaction conditions and convenient cell-cycle utilization for production cost control without needing the expensive enzyme. However, our previous study shown that whole-cell biocatalytic production of melibiose by *S. cerevisiae* strains with raffinose as the reaction substrate has the drawback of producing excessive by-products in this reaction system, such as sucrose, galactose, glucose, and fructose [30].

To solve this problem, it is essential to inhibit unwanted reactions induced by the α -galactosidase (labeled with red \times in Fig. 1). Furthermore, since the α -galactosidase is encoded by the *mell* gene [22], the screening of wild-type *S. cerevisiae* strains that lack the *mell* gene and the deletion

of the *mell* gene in a *S. cerevisiae* strain are effective strategies to prevent such reactions. Consequently, only the target product (melibiose) and one by-product (fructose) should be produced in this reaction system after implementation of this strategy.

Further, to obtain higher purity melibiose, it is necessary to remove the by-product fructose by a low cost and highly efficient method. One strategy for removing fructose is to promote efficient trans-membrane transport of fructose at the same time as the whole-cell biocatalysis, which depends on a high-affinity fructose-specific symporter operating efficiently in the chosen *S. cerevisiae* strain. Previously, high-affinity fructose-specific symporters were identified in some fructophilic yeasts, such as in *Saccharomyces carlsbergensis* [10], *Kluyveromyces lactis* [7], *Saccharomyces bayanus* [6], *Zygosaccharomyces bailii* [23], wine yeast *S. cerevisiae* [9], *Zygosaccharomyces rouxii* [19], and *Candida magnoliae* [20]. Hence, the biological purification for the fructose (by-product) could be feasible through expression of the heterologous fructose-specific symporters (encoded by the *fsy1* or/and *ffz1* gene) in this *S. cerevisiae* strain for improvement of fructose's trans-membrane transport (Fig. 1e).

In addition, the uptake and consumption of hexose (such as glucose or fructose) is a complex physiological process involving not only the trans-membrane transport of hexose but also the glycolysis. In *S. cerevisiae*, the efficient transcription of glycolytic genes and ribosomal protein (RP) requires the RAP1p/GCR1p regulatory complex [5]. Especially, over-expression of *gcr1* gene (GCR1p) solely in *S. cerevisiae* can improve the uptake and consumption of glucose, via up-regulation of the expression levels of some RP genes (glucose-responsive target genes such as *rpl11a*, *rps18b* and *rpl30*) and some glycolytic genes (such as *hxt1* and *adh1*) [18]. Since the glycolysis of fructose is similar as glucose, over-expression of *gcr1* gene in the *S. cerevisiae* strain should contribute to the fructose transport and consumption for enhancing the biopurification rate of the fructose by-product in this whole-cell biocatalysis.

Based on the above strategy, in this study we constructed a series of engineered *S. cerevisiae* strains based on the chosen liquor yeast [30], involving deletion of the *mell* gene, insertion of the *fsy1* or/and *ffzi1* gene(s) from *C. magnoliae*, and overexpression of the *gcr1* gene, both individually and combined. We implemented functional verification of the engineered *S. cerevisiae* strains and determined the optimal strain. Furthermore, we systematically evaluated an improvement strategy for construction of engineered *S. cerevisiae* strain to produce melibiose via whole-cell biocatalysis of raffinose. This study could contribute to improve the technology of melibiose production and provide a model for the development of such similar whole-cell biocatalysis to obtain product with higher yield, productivity and purity.

Materials and methods

Chemicals and materials

Raffinose and fructose were obtained from China National Sugar & Alcohol Group Co., Ltd (Beijing, China). The standards of raffinose, sucrose, melibiose, galactose, glucose, and fructose for HPLC analysis were purchased from China National Pharmaceutical Group Co., Ltd (Beijing, China).

Yeast genomic extract kit and PCR polymerase Q5 were purchased from OMEGA Co., Ltd (Manchester, United Kingdom) and New England Biolabs (Beijing, China), respectively. ClonExpress® Entry One Step Cloning Kit and ClonExpress® MultiS One Step Cloning Kit were purchased from Vazyme Biotech Co., Ltd (Nanjing, China).

Strains, plasmids, and media

The strains and the plasmids used in this study are described in Table 1. *E. coli* DH5 α cells were used as hosts

in the construction of plasmids (pMP2 to pMP8). *C. magnoliae* (CGMCC 2.1919) was obtained from China General Microbiological Culture Collection Center (CGMCC; Beijing, China), and its genome was used as the PCR template for cloning of *fsy1* (GenBank: KC147727.1) and *ffzi1* (GenBank: KC147728.1) genes. A series of engineered *S. cerevisiae* strains (MP1 to MP8) were derived from liquor yeast (*S. cerevisiae*: CGMCC 2.773), a typical wild-type Chinese liquor brewing industry strain obtained from CGMCC (Beijing, China). The genome of the liquor yeast strain (*S. cerevisiae*: CGMCC 2.773) was used as a PCR template for cloning of *gcr1* gene (NCBI Reference Sequence: NM_001183889.1).

Plasmid pUG6 was used as a PCR template to amplify the loxP-Kan^RMX-loxP fragment. Plasmid pUC19 was used as a vector to construct a series of plasmids: pMP2 to pMP8. Plasmid pPICZ α A::Cre contains the expression cassette of Cre recombinase and the selective marker gene for zeocin resistance, which was used for the removal of the selective marker Kan^R in the construction of the engineered *S. cerevisiae* strains.

LB medium (5 g/L yeast extract, 10 g/L tryptone, 10 g/L NaCl) was used for the culture of *E. coli* DH5 α cells and the construction of the plasmids (pMP2–pMP8). YPD medium (10 g/L yeast extract, 20 g/L tryptone, 20 g/L glucose) was used for the cultures of *C. magnoliae* and *S. cerevisiae*. Resistance media (LB medium with 100 μ g/mL of ampicillin, or/and 50 μ g/mL of kanamycin, or/and 20 μ g/mL of zeocin; YPD medium with 400 μ g/mL of G418, or/and 20 μ g/mL of zeocin) were used for the selection of positive clones when necessary.

DNA manipulation techniques

The manipulation of recombinant DNA was performed using standard techniques of in vitro fusion PCR [4, 12] and multi-fragments in vitro recombination [2]. A DNA fragment (UP_{*mell-5'*}-loxP-Kan^RMX-loxP-DW_{*mell-3'*}) was constructed for the deletion of the *mell* gene in the liquor yeast *S. cerevisiae*, to develop the engineered *S. cerevisiae* strain MP1 by using standard fusion PCR. The construction strategy is shown in Figure S1, and the primers used are listed in Table S1. Similarly, the recombinant expression cassette of the *fsy1* gene from *C. magnoliae* (P_{*tdh3*}-*fsy1*_{*cm*}-T_{*cyc1*}; Figure S2; Table S2), the recombinant expression cassette of the *ffzi1* gene from *C. magnoliae* (P_{*tdh3*}-*ffzi1*_{*cm*}-T_{*cyc1*}; Figure S3; Table S3), and the recombinant expression cassette of the *gcr1* gene from *S. cerevisiae* (P_{*tdh3*}-*gcr1*_{*sc*}-T_{*cyc1*}; Figure S4; Table S4) were constructed using standard fusion PCR.

The recombinant DNA fragments for the development of engineered *S. cerevisiae* strains MP2 to MP8 were PCR amplified from the constructed corresponding plasmid. The

Table 1 Strains and plasmids used in this study

Strain or plasmid	Description	Source
<i>E. coli</i> DH5 α	F- ϕ 80 <i>dlacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>)U169 <i>endA1 recA1 hsdR17</i> (rK ⁻ mK ⁺) <i>deoR thi-1 phoA supE44</i> λ - <i>gyrA96 relA1</i>	Stratagene
<i>C. magnoliae</i>	Wild-type, genome was used as the PCR template for <i>fsyI</i> and <i>ffzI</i> genes	CGMCC2.1919
<i>S. cerevisiae</i>		
Liquor yeast	Wild-type, typical Chinese liquor brewing industry strain	CGMCC2.773
MP1	Liquor yeast derivative, Δ <i>mell</i>	This study
MP2	Liquor yeast derivative, Δ <i>mell</i> ::P _{<i>tdh3</i>} - <i>fsyI</i> _{<i>cm</i>} -T _{<i>cyc1</i>}	This study
MP3	Liquor yeast derivative, Δ <i>mell</i> ::P _{<i>tdh3</i>} - <i>ffzI</i> _{<i>cm</i>} -T _{<i>cyc1</i>}	This study
MP4	Liquor yeast derivative, Δ <i>mell</i> ::P _{<i>tdh3</i>} - <i>fsyI</i> _{<i>cm</i>} -T _{<i>cyc1</i>} ::P _{<i>tdh3</i>} - <i>ffzI</i> _{<i>cm</i>} -T _{<i>cyc1</i>}	This study
MP5	Liquor yeast derivative, Δ <i>mell</i> ::P _{<i>tdh3</i>} - <i>gcrI</i> _{<i>sc</i>} -T _{<i>cyc1</i>}	This study
MP6	Liquor yeast derivative, Δ <i>mell</i> ::P _{<i>tdh3</i>} - <i>fsyI</i> _{<i>cm</i>} -T _{<i>cyc1</i>} ::P _{<i>tdh3</i>} - <i>gcrI</i> _{<i>sc</i>} -T _{<i>cyc1</i>}	This study
MP7	Liquor yeast derivative, Δ <i>mell</i> ::P _{<i>tdh3</i>} - <i>ffzI</i> _{<i>cm</i>} -T _{<i>cyc1</i>} ::P _{<i>tdh3</i>} - <i>gcrI</i> _{<i>sc</i>} -T _{<i>cyc1</i>}	This study
MP8	Liquor yeast derivative, Δ <i>mell</i> ::P _{<i>tdh3</i>} - <i>fsyI</i> _{<i>cm</i>} -T _{<i>cyc1</i>} ::P _{<i>tdh3</i>} - <i>ffzI</i> _{<i>cm</i>} -T _{<i>cyc1</i>} ::P _{<i>tdh3</i>} - <i>gcrI</i> _{<i>sc</i>} -T _{<i>cyc1</i>}	This study
Plasmids		
pUG6	Template for PCR to amplify of the loxP-Kan ^R MX-loxP sequence	Novagen
pUC19	Kan ^R , as based vector to construct plasmid series	Novagen
pPICZ α A::Cre	Zeo ^R , Cre recombinase, for removal of the selective marker Kan ^R	Novagen
pMP2	UP _{<i>mell-5'</i>} -P _{<i>tdh3</i>} - <i>fsyI</i> _{<i>cm</i>} -T _{<i>cyc1</i>} -loxP-Kan ^R MX-loxP-DW _{<i>mell-3'</i>} ::pUC19	This study
pMP3	UP _{<i>mell-5'</i>} -P _{<i>tdh3</i>} - <i>ffzI</i> _{<i>cm</i>} -T _{<i>cyc1</i>} -loxP-Kan ^R MX-loxP-DW _{<i>mell-3'</i>} ::pUC19	This study
pMP4	UP _{<i>mell-5'</i>} -P _{<i>tdh3</i>} - <i>fsyI</i> _{<i>cm</i>} -T _{<i>cyc1</i>} -P _{<i>tdh3</i>} - <i>ffzI</i> _{<i>cm</i>} -T _{<i>cyc1</i>} -loxP-Kan ^R MX-loxP-DW _{<i>mell-3'</i>} ::pUC19	This study
pMP5	UP _{<i>mell-5'</i>} -P _{<i>tdh3</i>} - <i>gcrI</i> _{<i>sc</i>} -T _{<i>cyc1</i>} -loxP-Kan ^R MX-loxP-DW _{<i>mell-3'</i>} ::pUC19	This study
pMP6	UP _{<i>mell-5'</i>} -P _{<i>tdh3</i>} - <i>fsyI</i> _{<i>cm</i>} -T _{<i>cyc1</i>} -P _{<i>tdh3</i>} - <i>gcrI</i> _{<i>sc</i>} -T _{<i>cyc1</i>} -loxP-Kan ^R MX-loxP-DW _{<i>mell-3'</i>} ::pUC19	This study
pMP7	UP _{<i>mell-5'</i>} -P _{<i>tdh3</i>} - <i>ffzI</i> _{<i>cm</i>} -T _{<i>cyc1</i>} -P _{<i>tdh3</i>} - <i>gcrI</i> _{<i>sc</i>} -T _{<i>cyc1</i>} -loxP-Kan ^R MX-loxP-DW _{<i>mell-3'</i>} ::pUC19	This study
pMP8	UP _{<i>mell-5'</i>} -P _{<i>tdh3</i>} - <i>fsyI</i> _{<i>cm</i>} -T _{<i>cyc1</i>} -P _{<i>tdh3</i>} - <i>ffzI</i> _{<i>cm</i>} -T _{<i>cyc1</i>} -P _{<i>tdh3</i>} - <i>gcrI</i> _{<i>sc</i>} -T _{<i>cyc1</i>} -loxP-Kan ^R MX-loxP-DW _{<i>mell-3'</i>} ::pUC19	This study

plasmids pMP2 to pMP8 were constructed using ClonExpress[®] MultiS One Step Cloning Kit, for which the construction strategy and primers used are shown in the supplementary materials (pMP2: Figure S5, Table S5; pMP3: Figure S6, Table S6; pMP4: Figure S7, Table S7; pMP5: Figure S8, Table S8; pMP6: Figure S9, Table S9; pMP7: Figure S10, Table S10; and pMP8: Figure S11, Table S11).

Development of engineered strains

The DNA fragments for the development of engineered strains MP1 to MP8 were transformed into the liquor yeast *S. cerevisiae* strain using the AcLi induced method (standard), and positive clones were selected using resistance medium (YPD medium with 400 μ g/mL of G418). The genomes of candidate clones were extracted for PCR identification of the gene deletion and recombination after expanding cultured in YPD with 400 μ g/mL of G418. For removal of the selective marker Kan^R, plasmid pPICZ α A::Cre was transformed into the confirmed clones, and positive clones were selected using resistance medium (YPD medium with 20 μ g/mL of zeocin). After the candidate clones were subcultured for 3–5 generations in YPD medium to remove the plasmid pPICZ α A::Cre, PCR identification was implemented using extracted genome

from subculture of the candidate clones as templates. The confirmed clones were denoted as MP1 to MP8.

Whole-cell preparation of wild-type and engineered strains

A single colony of the liquor yeast (wild-type) or engineered strain (MP1–MP8) was placed into 5 mL of liquid YPD medium and cultivated at 30 °C and 200 rpm for 24 h. Subsequently, the cultures were transferred into 50 mL of liquid YPD and cultivated at 30 °C and 200 rpm for 14–18 h. Cells were collected by centrifugation at 4 °C and 7000 \times g for 20 min. The collected cells were washed twice with sterile water precooled on ice and centrifuged at 4 °C and 7000 \times g for 20 min.

Functional verification of the engineered strains in whole-cell biocatalysis

Functional verification of the engineered strain with the *mell* gene deletion

Samples (extracellular) from the whole-cell catalytic process of the MP1 (Δ *mell*) strain with the raffinose substrate

were analyzed by HPLC, and the chromatogram maps were compared with those of the wild-type strain (liquor yeast) at 0, 2, 4, and 6 h. Those whole-cell catalytic reactions were performed at that whole-cell concentration of each testing strain was OD_{600} of 1 in 50 mL of raffinose substrate (50 g/L) cultivated at 30 °C and 200 rpm for 6 h.

Functional verification of the engineered strains with that heterologous integration of the fructose transporter gene(s)

Concentrations of fructose (extracellular) at 6 h in those whole-cell biocatalytic processes of that MP2 ($\Delta mell::fsyI_{cm}$), MP3 ($\Delta mell::ffziI_{cm}$), and MP4 ($\Delta mell::fsyI_{cm}::ffziI_{cm}$) strains with fructose or raffinose substrates were analyzed by HPLC, and compared with the liquor yeast (wild-type) strain and/or MP1 ($\Delta mell$) strain. Those whole-cell catalytic reactions were performed at that whole-cell concentration of each testing strain was OD_{600} of 5 in 50 mL of fructose (or raffinose) substrate (50 g/L) cultivated at 30 °C and 200 rpm for 6 h.

*Functional verification of engineered strains with overexpression of the *gcr1* gene*

Concentrations of fructose (extracellular) at 6 h in those whole-cell biocatalytic processes of the MP5 ($\Delta mell::gcr1_{sc}$), MP6 ($\Delta mell::fsyI_{cm}::gcr1_{sc}$), MP7 ($\Delta mell::ffziI_{cm}::gcr1_{sc}$), and MP8 ($\Delta mell::fsyI_{cm}::ffziI_{cm}::gcr1_{sc}$) strains with fructose or raffinose substrates were analyzed by HPLC, and compared with the MP1 ($\Delta mell$), MP2 ($\Delta mell::fsyI_{cm}$), MP3 ($\Delta mell::ffziI_{cm}$), and MP4 ($\Delta mell::fsyI_{cm}::ffziI_{cm}$) strains correspondingly. Those whole-cell catalytic reactions were performed at that whole-cell concentration of each testing strain was OD_{600} of 5 in 50 mL of fructose (or raffinose) substrate (50 g/L) cultivated at 30 °C and 200 rpm for 6 h.

Determination of the optimal engineered strain

To determine the optimal engineered strain, we compared the melibiose yield, productivity, and purity of the MP1–MP8 engineered strains at 6 h in those whole-cell biocatalytic processes. Those whole-cell catalytic reactions were performed at the whole-cell concentration of each testing strain was OD_{600} of 5 in 50 mL of raffinose substrate (50 g/L) cultivated at 30 °C and 200 rpm for 6 h. The substrate (raffinose), product (melibiose), and by-product (glucose) concentrations (extracellular) were determined by HPLC analysis. The melibiose yield (g/L) was determined by HPLC analysis directly; the melibiose productivity (%) was defined as that (determined melibiose yield/theoretical melibiose yield) \times 100%; the melibiose purity (%) was defined as that [determined melibiose yield/

(determined melibiose yield + determined fructose by-product yield) \times 100%. The melibiose yield, productivity, and purity of the optimal engineered strain were compared with the wild-type strain similarly.

HPLC analysis

All samples from the above whole-cell biocatalyses were centrifuged at $20,000\times g$ for 20 min to remove cell precipitation. Supernatant samples were incubated at 100 °C for 10 min, and centrifugation at $20,000\times g$ for 20 min was performed on those samples to obtain the supernatant. These supernatant samples were filtered with 0.22 μ m membranes for HPLC analysis to determine the substrate and product concentrations.

The HPLC system used was an Agilent 1200 series (Agilent Co., Ltd, CA, USA). Experiments were performed with a sugar-ParTM chromatographic column (6.5 \times 300 mm) and Refractive Index Detector (RID), with deionized water as the mobile phase at a flow rate of 0.4 mL/min and column temperature of 80 °C.

Statistical analysis

Experiments were performed in three replicates. Data were expressed as arithmetic means or average \pm standard deviation. Student's *t* test or Mann–Whitney rank sum test was used to analyze the statistical significance of the observed differences. A *p* value of less than 0.05 was considered statistically significant. All the analyses were performed with Sigmaplot for Windows Version 12.0 (Systat Software, Inc).

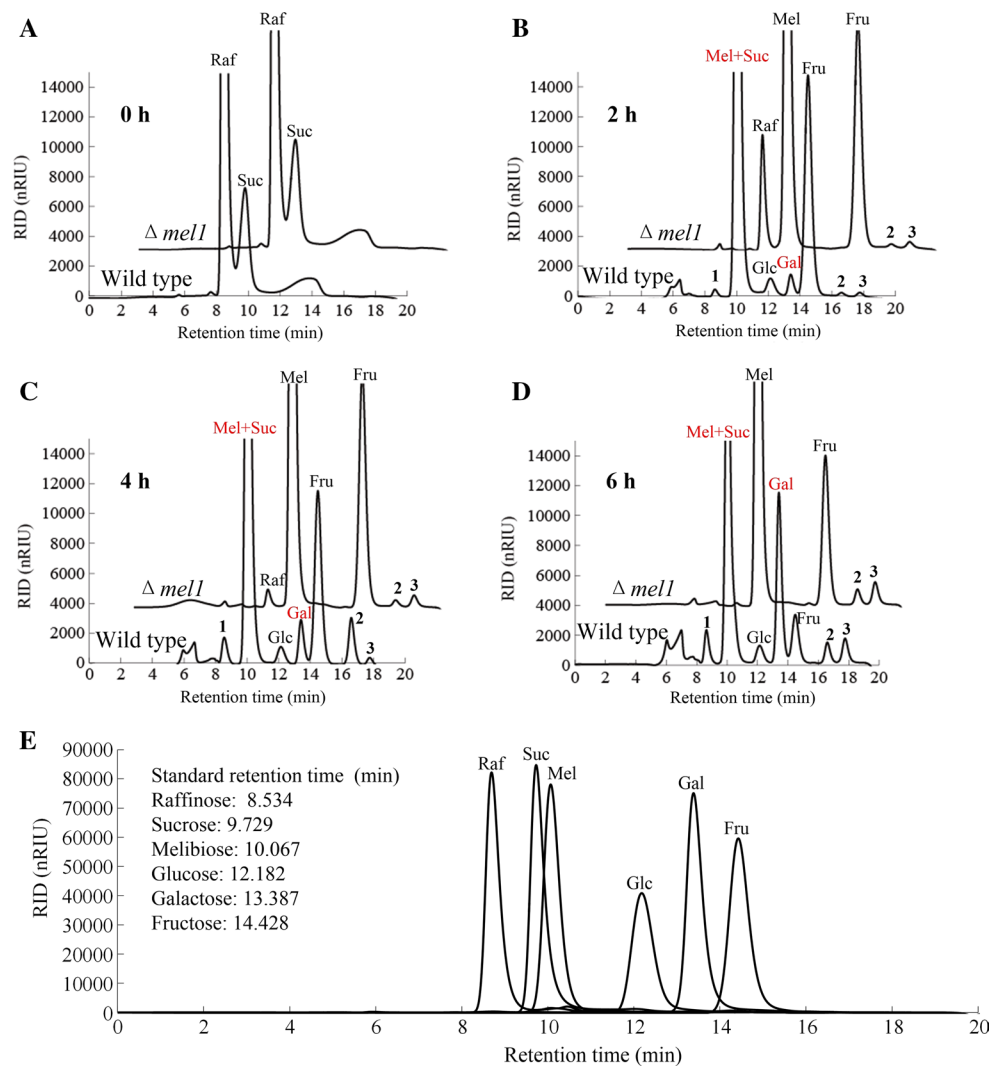
Results

Engineered strain with the *mell* gene deletion could eliminate side reactions

For functional verification of the engineered strain with the *mell* gene deletion ($\Delta mell$), samples from that whole-cell catalytic process of the MP1 ($\Delta mell$) strain with raffinose substrate were analyzed by HPLC, and the chromatogram maps were compared with of the wild-type strain (liquor yeast) at 0 (Fig. 2a), 2 (Fig. 2b), 4 (Fig. 2c), and 6 h (Fig. 2d).

At 0 h, the two chromatogram maps of the $\Delta mell$ and wild-type strains were identical as they are the signal from the raffinose substrate. However, there were some miscellaneous peaks in addition to the characteristic peaks of raffinose in the maps, which indicated that the raffinose substrate contained some impurities such as sucrose, galactose, glucose, and fructose (Fig. 2a).

Fig. 2 Functional verification of engineered strain with the *mell* gene deletion through comparison of that whole-cell catalytic process with raffinose substrate by the wild-type and $\Delta mell$ strains using HPLC analysis. Samples from the whole-cell catalytic process of the MP1 ($\Delta mell$) strain with raffinose substrate were analyzed by HPLC and were compared with the wild-type strain (liquor yeast) at 0 (a), 2 (b), 4 (c), and 6 h (d). Those whole-cell catalytic reactions were performed at that whole-cell concentration of each testing strain was OD_{600} of 1 in 50 mL of raffinose substrate (50 g/L) cultivated at 30 °C and 200 rpm for 6 h



When whole-cell catalytic reactions were performed for 2 h, the map of the wild-type strain showed that some by-products had been generated; especially galactose, which indicated that the raffinose substrate or melibiose product had been hydrolyzed by the α -galactosidase (Fig. 2b). However, the map of the $\Delta mell$ strain showed that no galactose was generated, which indicated that the unwanted reactions catalyzed by the α -galactosidase were eliminated. This confirms that the $\Delta mell$ strain had achieved the desired effect.

When the reactions proceeded for 4 (Fig. 2c) and 6 h (Fig. 2d), it was more obvious that the desired function of the $\Delta mell$ strain had been achieved by eliminating reactions induced by the α -galactosidase. In the catalytic system of the wild-type strain, the by-products of glucose, galactose, and fructose were still detected. Importantly, the galactose by-product significantly increased from 4 to 6 h and was accompanied by other by-products with also increased significantly (the peaks of other by-products are

marked as 1, 2, and 3 in the maps of the wild-type strain in Fig. 2c, d). However, in the catalyzed system of the MP1 ($\Delta mell$) strain, no galactose by-product was generated, which was accompanied by the complete conversion of the raffinose substrate. The only significant peaks were of the target product melibiose and the fructose by-product, which could be observed at 4 or 6 h, although there were some other by-products detected (marked with 2 and 3 in the maps of the MP1 ($\Delta mell$) strain in Fig. 2c, d).

The sugar concentrations (extracellular) for the MP1 ($\Delta mell$) and wild-type strain at (only) 6 h whose were determined by HPLC were presented in Table 2, a description on data.

In summary, by comparison of the whole-cell catalytic processes of the MP1 ($\Delta mell$) strain and the wild-type strain with raffinose substrate, the side reactions mediated by α -galactosidase were eliminated, although there was a reduction in the whole-cell catalyzed speed of raffinose after *mell* gene deletion. The degradation of the

Table 2 Sugar concentrations for the MP1 ($\Delta mel1$) and wild-type strains at 6 h in the whole-cell biocatalytic processes (g/L)

Strain	Raffinose (substrate)	Melibiose (product)	Galactose (by-product)	Glucose (by-product)	Fructose (by-product)
Wild-type	0	25.25 ± 1.46	9.58 ± 0.46	2.16 ± 0.65	4.58 ± 0.56
MP1 ($\Delta mel1$)	0	32.86 ± 1.53	0	0	10.84 ± 0.86

Those whole-cell catalytic reactions were performed at that whole-cell concentration of each testing strain was OD₆₀₀ of 1 in 50 mL of raffinose substrate (50 g/L) cultivated at 30 °C and 200 rpm for 6 h. Sugar concentrations (extracellular) were determined by HPLC analysis. Mean values of the results of triplicate experiments are shown with error bars indicating the relative standard deviation

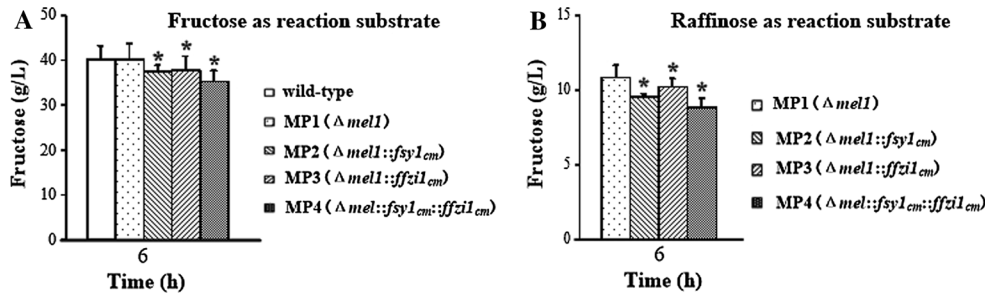


Fig. 3 Functional verification of engineered strains with that heterologous integration of fructose transporter genes. The fructose concentration (extracellular) of engineered strains were compared with those of the wild-type and/or $\Delta mel1$ strains, at 6 h of in the whole-cell catalytic process with fructose substrate (a), or with raffinose substrate (b). Those whole-cell catalytic reactions were performed at

that whole-cell concentration of each testing strain was OD₆₀₀ of 5 in 50 mL of fructose (or raffinose) substrate (50 g/L) cultivated at 30 °C and 200 rpm for 6 h. Fructose concentrations (extracellular) were determined by HPLC analysis. Mean values of the results of triplicate experiments are shown with error bars indicating the relative standard deviation. **p* < 0.05 (compared to the control strain)

target product melibiose and formation of the by-product galactose were eliminated in the MP1 ($\Delta mel1$) strain. This indicates that the function of the engineered strain MP1 ($\Delta mel1$) reached the expected goal.

Engineered strains with that heterologous integration of fructose transporter gene(s) could increase the fructose transporter

In the fructose substrate test (Fig. 3a), when compared with the liquor yeast (wild-type) strain and MP1 ($\Delta mel1$) strain, there were significant reductions in fructose concentrations (extracellular) in the whole-cell biocatalytic process of the MP2 ($\Delta mel1::fsy1_{cm}$), MP3 ($\Delta mel1::ffzi1_{cm}$), and MP4 ($\Delta mel1::fsy1_{cm}::ffzi1_{cm}$) strains at 6 h. Strikingly, there was a significant difference with the MP4 ($\Delta mel1::fsy1_{cm}::ffzi1_{cm}$) strain, which has simultaneous integration of *fsy1* and *ffzi1* genes, when comparing the fructose concentrations (extracellular) with the control strain. This indicates that the engineered strains with that heterologous integration of fructose transporter genes from *C. magnoliae* showed significant improvement in fructose transport during the whole-cell biocatalysis.

In the raffinose substrate test (Fig. 3b), fructose concentrations (extracellular) in the whole-cell biocatalytic process of the MP2 ($\Delta mel1::fsy1_{cm}$), MP3 ($\Delta mel1::ffzi1_{cm}$),

and MP4 ($\Delta mel1::fsy1_{cm}::ffzi1_{cm}$) strains at 6 h were all significant reductions compared with the MP1 ($\Delta mel1$) strain. Incidentally, engineered strains with that heterologous integration of fructose transporter genes from *C. magnoliae* also had significant improvement in fructose transport during the whole-cell biocatalysis with raffinose substrate. Integration of the *fsy1* gene in an engineered strain was more efficient in improving fructose transport than integration of the *ffzi1* gene, and the simultaneous integration of the *fsy1* and *ffzi1* genes exhibited some accumulated or synergistic effects on fructose transport.

With fructose and raffinose substrates, the engineered strains consistently had enhanced fructose transport capacity. Hence, the function of engineered strains with heterologous integration of fructose transporter genes from *C. magnoliae* was confirmed during the whole-cell biocatalytic processes.

Engineered strains with overexpression of the transcription factor (GCR1) could increase the fructose transport and consumption

For functional verification of engineered strains with overexpression of a transcription factor gene (*gcr1*), the concentrations of fructose (extracellular) at 6 h during the whole-cell biocatalytic processes of the MP5

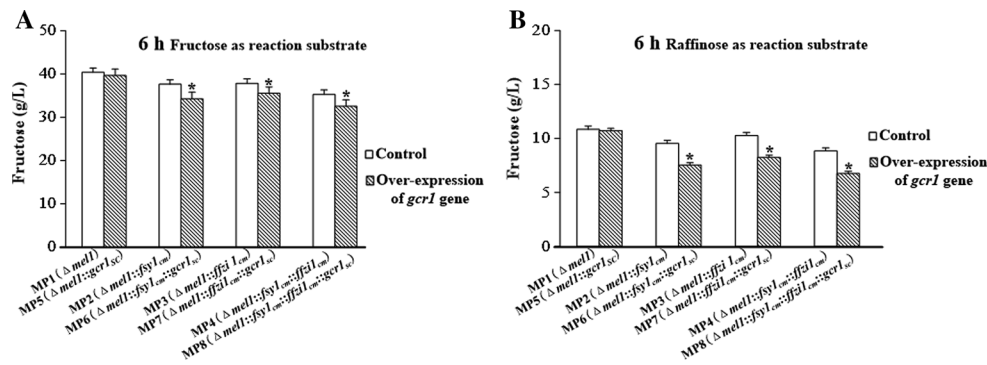


Fig. 4 Functional verification of engineered strains with overexpression of a transcription factor (*GCR1*). Concentrations of fructose (extracellular) at 6 h in the whole-cell biocatalytic process of the MP5 ($\Delta mel1::gcr1_{sc}$), MP6 ($\Delta mel1::fys1_{cm}::gcr1_{sc}$), MP7 ($\Delta mel1::ffz1_{cm}::gcr1_{sc}$), and MP8 ($\Delta mel1::fys1_{cm}::ffz1_{cm}::gcr1_{sc}$) strains in fructose or raffinose substrates were compared with that of the of respective MP1 ($\Delta mel1$), MP2 ($\Delta mel1::fys1_{cm}$), MP3 ($\Delta mel1::ffz1_{cm}$), and MP4 ($\Delta mel1::fys1_{cm}::ffz1_{cm}$) strains. Those

whole-cell catalytic reactions were performed at that whole-cell concentration of each testing strain was OD_{600} of 5 in 50 mL of fructose (or raffinose) substrate (50 g/L) cultivated at 30 °C and 200 rpm for 6 h. Fructose concentrations (extracellular) were determined by HPLC analysis. **a** 6 h with fructose substrate; **b** 6 h with raffinose substrate. Mean values of the results of triplicate experiments are shown with error bars indicating the relative standard deviation. * $p < 0.05$ (compared to the controlled strain)

($\Delta mel1::gcr1_{sc}$), MP6 ($\Delta mel1::fys1_{cm}::gcr1_{sc}$), MP7 ($\Delta mel1::ffz1_{cm}::gcr1_{sc}$), and MP8 ($\Delta mel1::fys1_{cm}::ffz1_{cm}::gcr1_{sc}$) strains with fructose or raffinose substrate were compared with those of the corresponding MP1 ($\Delta mel1$), MP2 ($\Delta mel1::fys1_{cm}$), MP3 ($\Delta mel1::ffz1_{cm}$), and MP4 ($\Delta mel1::fys1_{cm}::ffz1_{cm}$) strains.

With fructose substrate, at 6 h of the whole-cell biocatalytic process, the fructose concentrations of MP6 ($\Delta mel1::fys1_{cm}::gcr1_{sc}$), MP7 ($\Delta mel1::ffz1_{cm}::gcr1_{sc}$), and MP8 ($\Delta mel1::fys1_{cm}::ffz1_{cm}::gcr1_{sc}$) all showed significant differences compared with the respective control strains (Fig. 4a). However, overexpression of a transcription factor gene (*gcr1*) in the MP1 ($\Delta mel1$) strain did not shown a difference in the fructose concentrations (extracellular) in the whole-cell biocatalyzed process compared with the MP1 ($\Delta mel1$) strain (Fig. 4a). Taking these results together, we have found that overexpression of a transcription factor (*GCR1*) could contribute to improved fructose transport efficiency in engineered strains with the heterologous integration of fructose transporter gene(s), especially in which strains with the heterologous integration of the *fys1* gene and the simultaneous integration of the *fys1* and *ffz1* genes. Hence, the functions of engineered strains with overexpression of a transcription factor (*GCR1*) were confirmed in this fructose substrate testing.

To verify the fructose transport capacity of the engineered strains with overexpression of the transcription factor (*GCR1*) in the whole-cell biocatalyzed production of melibiose from raffinose, we implemented a reaction with raffinose substrate (Fig. 4b).

Compared with the control strain, there were some reductions in fructose concentrations in the whole-cell biocatalytic process of the MP6 ($\Delta mel1::fys1_{cm}::gcr1_{sc}$), MP7

($\Delta mel1::ffz1_{cm}::gcr1_{sc}$), and MP8 ($\Delta mel1::fys1_{cm}::ffz1_{cm}::gcr1_{sc}$) strains at 6 h (Fig. 4b). Notably, in the whole-cell biocatalytic process with raffinose substrate, fructose concentrations of MP6 ($\Delta mel1::fys1_{cm}::gcr1_{sc}$), MP7 ($\Delta mel1::ffz1_{cm}::gcr1_{sc}$), and MP8 ($\Delta mel1::fys1_{cm}::ffz1_{cm}::gcr1_{sc}$) showed significant differences compared with that of the respective control strains (Fig. 4b). Hence, the function of the engineered strains with overexpression of a transcription factor (*GCR1*) was confirmed in this raffinose substrate testing.

Summarizing the results from the above verification tests, we had concluded that overexpression of the transcription factor (*GCR1*) in the engineered strains with that heterologous integration of fructose transporter gene(s) could enhance the fructose transport capacity in this whole-cell biocatalyzed process using fructose or raffinose substrate.

Determination of the optimal engineering strain for the whole-cell biocatalytic production of melibiose from raffinose

We had compared yield, productivity, and purity of the target product (melibiose) in the whole-cell biocatalytic production of melibiose from raffinose using strains MP1–MP8 to determine the optimal engineered strain. Those whole-cell catalytic reactions were performed at that whole-cell concentration of each testing strain was OD_{600} of 5 in 50 mL of raffinose substrate (50 g/L) cultivated at 30 °C and 200 rpm for 6 h. The substrate (raffinose), product (melibiose), and by-product (glucose) concentrations (extracellular) were determined by HPLC analysis for calculation of yield, productivity, and purity of the target product (melibiose).

At 6 h in the whole-cell biocatalytic process with strains MP1–MP8 and the wild-type strain (liquor yeast), the raffinose substrates were all transformed completely. At this time, the wild-type strain (liquor yeast) had a melibiose yield of 25.25 ± 1.46 g/L (mixed with sugar by-product). However, all the engineered strains had higher melibiose yields compared with the wild-type strain (liquor yeast), achieving 32–33 g/L.

Comparing the yield, productivity, and purity of the target product (melibiose) at 6 h with strains MP1–MP8 in the whole-cell biocatalytic production of melibiose from raffinose, it was obvious that there were no significant differences in the yield and productivity. However, the purity of the melibiose from the MP4 ($\Delta mell::fys1_{cm}::ffzil_{cm}$), MP6 ($\Delta mell::fys1_{cm}::gcr1_{sc}$), MP7 ($\Delta mell::ffzil_{cm}::gcr1_{sc}$), and MP8 ($\Delta mell::fys1_{cm}::ffzil_{cm}::gcr1_{sc}$) strains was significantly different compared with the MP1 ($\Delta mell$) strain as a control (Fig. 5). Hence, the difference in performance of the engineered strains (MP1–MP8) was in the increase in the purity of the target product (melibiose) from the whole-cell biocatalytic production of melibiose from raffinose, which was meeting our expectation. Notably, the MP8 ($\Delta mell::fys1_{cm}::ffzil_{cm}::gcr1_{sc}$) strain had the most significant improvement in the purity of the target product (melibiose) among the engineered strains. Compared with the MP1 ($\Delta mell$) strain which purity of the melibiose product was $75.19 \pm 4.50\%$, the MP8 ($\Delta mell::fys1_{cm}::ffzil_{cm}::gcr1_{sc}$) strain had about 10% improvement in the purity of the melibiose product which was reach to $82.97 \pm 3.60\%$. Hence, we could determine that the MP8 ($\Delta mell::fys1_{cm}::ffzil_{cm}::gcr1_{sc}$) strain was the optimal engineered strain for the whole-cell biocatalytic production of melibiose from raffinose.

In addition, compared with the wild-type strain (melibiose yield: 25.25 ± 1.46 g/L, melibiose productivity: $75.76 \pm 4.38\%$, and melibiose purity: $60.74 \pm 3.52\%$), the optimal engineered strain MP8 ($\Delta mell::fys1_{cm}::ffzil_{cm}::gcr1_{sc}$) had significant improvements on the yield, productivity, and purity of the target product (melibiose) in this whole-cell biocatalytic production of melibiose from raffinose (Fig. 6), which produced a melibiose yield: 32.86 ± 1.43 g/L, melibiose productivity: $98.98 \pm 4.30\%$, and melibiose purity: $82.97 \pm 3.61\%$, and had improved about 30% on the yield, about 31% on the productivity, and about 36% on the purity of melibiose product compared with the wild-type strain.

Conclusion

An engineered strain was constructed to eliminate the by-products of the whole-cell biocatalytic production of melibiose from raffinose based on strategy involving the intracellular–extracellular interaction mechanism. Deletion of the *mell* gene could eliminate the side reactions mediated

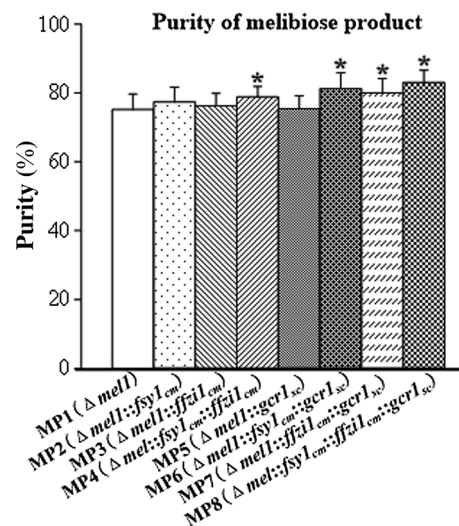


Fig. 5 Compared purity of the target product (melibiose) from the whole-cell biocatalytic production of melibiose from raffinose using the MP1–MP8 engineered strains at 6 h. Those whole-cell catalytic reactions were performed at that whole-cell concentration of each testing strain was OD_{600} of 5 in 50 mL of raffinose substrate (50 g/L) cultivated at 30 °C and 200 rpm for 6 h. The substrate (raffinose), product (melibiose), and by-product (glucose) concentrations (extracellular) were determined by HPLC analysis for calculation of yield, productivity, and purity of the target product (melibiose). The melibiose yield (g/L) was determined by HPLC analysis directly; the melibiose productivity (%) was defined as that (determined melibiose yield/theoretical melibiose yield) \times 100%; the melibiose purity (%) was defined as that [determined melibiose yield/(determined melibiose yield + determined fructose by-product yield) \times 100%. Mean values of the results of triplicate experiments are shown with error bars indicating the relative standard deviation. * $p < 0.05$ (compared to the control strain MP1($\Delta mell$))

by α -galactosidase and led to elimination of the degradation of melibiose in this whole-cell biocatalysis. Recombination of the *fys1* or/and *ffzil* gene(s), and over-expression of the *gcr1* gene could enhance the bio-purification rate of the fructose (by-product) in this whole-cell biocatalysis. The optimal engineered strain ($\Delta mell::fys1_{cm}::ffzil_{cm}::gcr1_{sc}$) had improved significantly on yield, productivity, and purity of the melibiose product compared with the wild-type strain in this whole-cell biocatalysis.

Discussion

Whole-cell biocatalysis is a kind of biological technology that uses living cells as biological catalysts to catalyze specific reactions for obtaining target product [3]. During the process of whole-cell biocatalysis, there may be some side reactions, such as the ineffective catalytic consumption of specific substrates and target products. We could analyze the principle underlying specific whole-cell biocatalysis, identify key genes that cause side reactions, and knock

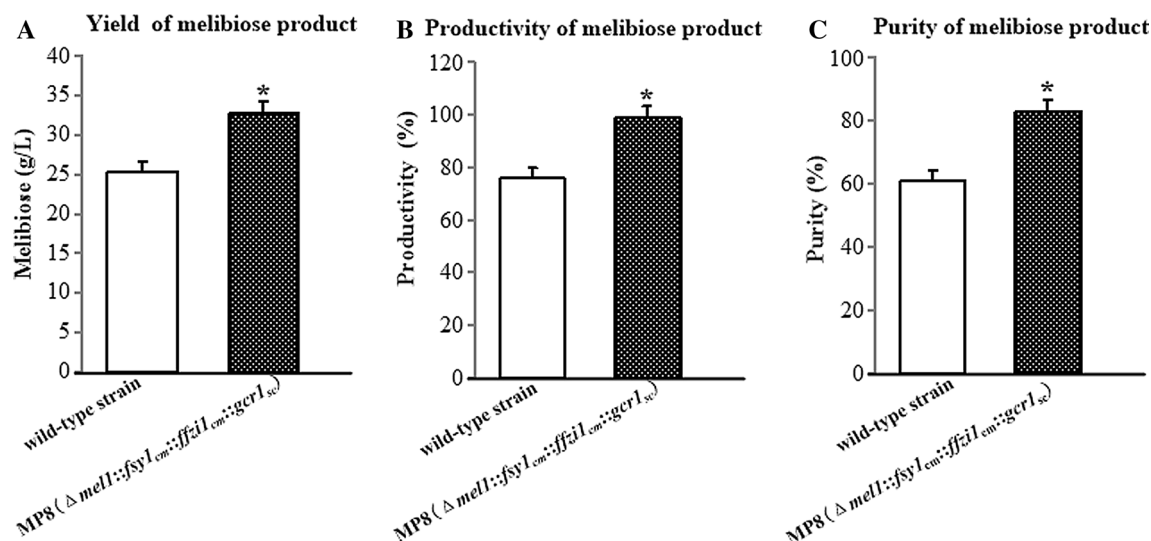


Fig. 6 The melibiose yield, productivity, and purity of the optimal engineered strain (MP8) were compared with the wild-type strain similarly. **a** Compared yield of melibiose product; **b** compared productivity of melibiose product; **c** Compared purity of melibiose prod-

uct. Mean values of the results of triplicate experiments are shown with *error bars* indicating the relative standard deviation. * $p < 0.05$ (compared to the wild-type strain)

them out to eliminate side reactions during the reaction process. For example, the *sucA* gene encoding α -ketoglutaric acid (α -KG) dehydrogenase was deleted to block α -KG degradation, further increasing the α -KG titer in a *Bacillus subtilis* whole-cell biocatalysis [15]. In this study, the *mell* gene encoding α -galactosidase was deleted to eliminate α -galactosidase expression (Fig. 7, ⑥ \rightarrow k), which blocked the ineffective degradation of raffinose (substrate) and melibiose (target product). This engineered strain with the *mell* gene deletion could increase the melibiose yield from 25.25 to 32.86 g/L compared with the wild-type strain in the whole-cell biocatalysis using raffinose as substrate.

Our hypothesis was that if the whole-cell biocatalysis was primarily extracellular, the by-product would be transported into the cytosol via an enhancement to reach a similar bio-purification. In this study, the raffinose substrate is hydrolyzed by invertase secreted from *S. cerevisiae* cell to produce melibiose and fructose (Fig. 7, I), which happens extracellularly. Fructose, as a major by-product, should be transmembrane-transported into the cytosol (Fig. 7, O \rightarrow P) in the whole-cell biocatalysis process to increase the extracellular melibiose purity. However, the fructose transport capacity of the wild-type strain is not strong enough for the whole-cell biocatalysis. Hence, we have carried out insertion of the *fSY1* or/and *ffzI* genes from *C. magnoliae*, which are coding genes for fructose transporters [20], to construct the engineered strains of MP2 ($\Delta mell::fSY1_{cm}$), MP3 ($\Delta mell::ffzI_{cm}$), and MP4 ($\Delta mell::fSY1_{cm}::ffzI_{cm}$) based on deletion of the *mell* gene.

We designed experiments with fructose as the sole substrate and simulated reaction conditions for whole-cell

biocatalysis to verify the function of the fructose transports in the engineered strains during whole-cell biocatalysis, which was different with verifying the fructose transport capacity in the growth process of the strain [20]. At the same time, we detected the transport capacity for the by-product fructose for the engineered strains during the process of whole-cell biocatalysis with raffinose substrate. Our results showed that integration of heterologous fructose transporter genes had enhanced the fructose transport capacity for the engineered strains with fructose or raffinose substrates. Hence, this confirmed our hypothesis that the by-product could be transported into the cytosol via an enhancement to reach a similar bio-purification during whole-cell biocatalysis.

Overexpression of suitable transcription factors could enhance transcription and expression of target genes for strengthening functions of the target gene. In this study, we hypothesized that overexpression of the *gcr1* gene in the *S. cerevisiae* strain could improve fructose consumption via enhanced transcription level of glycolytic genes [5]. We constructed engineered strains of MP6 ($\Delta mell::fSY1_{cm}::gcr1_{sc}$), MP7 ($\Delta mell::ffzI_{cm}::gcr1_{sc}$), and MP8 ($\Delta mell::fSY1_{cm}::ffzI_{cm}::gcr1_{sc}$) with overexpression of the *gcr1* gene (transcription factor) based on deletion of the *mell* gene and integration of the fructose transporters gene(s). In both fructose and raffinose substrates, we observe that the fructose concentrations (extracellular) were reduced significantly during the whole-cell biocatalysis using engineered strains compared with the control strains. In view of the whole-cell biocatalysis process, this provides a strategy in which overexpression of suitable transcription factors can improve the by-products bio-purification.

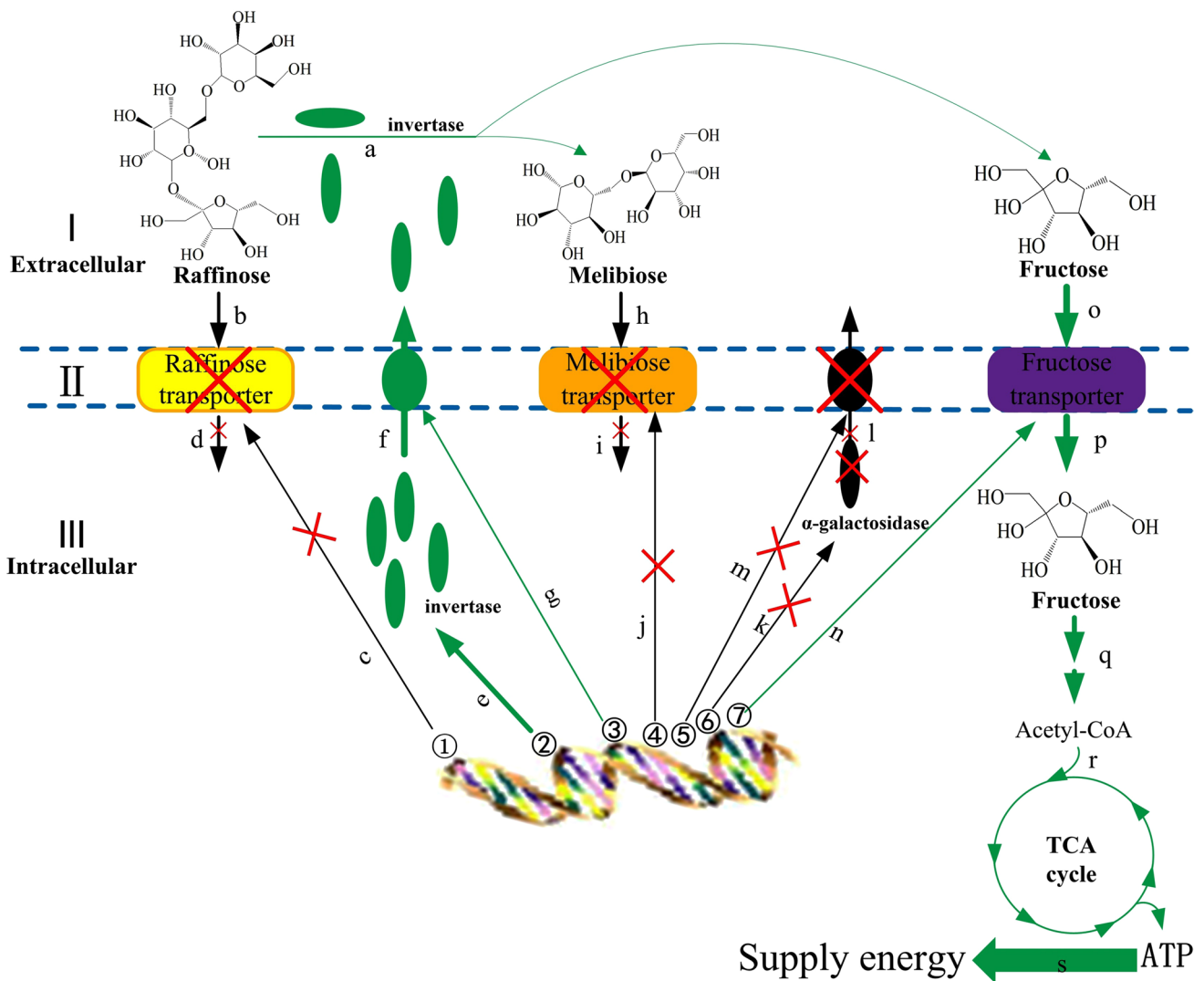


Fig. 7 Construction of engineered strains for that whole-cell biocatalytic production of melibiose from raffinose. *I* Extracellular system. Raffinose substrate is catalyzed by invertase secreted from *S. cerevisiae* cell to produce melibiose and fructose. *II* Cell wall and cell membrane. There are raffinose, melibiose, and fructose transporters, as well as secretory pathways for invertase and α -galactosidase. *III* Extracellular system. There are that gene expression and regulation of two key enzymes (invertase and α -galactosidase), gene expression and regulation of three sugar transporter proteins (raffinose, melibiose, and fructose transporter proteins), and the fructose metabolism. Fructose metabolism results in acetyl-CoA via a series of metabolic

conversion, which run into the TCA cycle to produce ATP as the energy supply maintaining the whole-cell biocatalysis. *Cross symbol* indicate that no α -galactosidase expression via the deletion of its coding gene, and no raffinose, and melibiose transporter expression via deletion of their coding genes. *Green arrow* indicate that enhancement of that whole-cell biocatalytic production of melibiose from raffinose, which are including invertase expression and its secretion, hydrolysis reaction of raffinose substrate, fructose transporter expression, fructose transport (from extracellular into intracellular), and intracellular metabolism of fructose to produce ATP

Compared with the wild-type strain, the engineered strains constructed in this study have significantly increased yield, productivity, and purity of the target product (melibiose) for whole-cell biocatalytic production of melibiose from raffinose. However, further studies are still needed, such as how to increase the rate of substrate catalysis, increase the yield of the target product (melibiose), enhance the bio-purification rate for the fructose by-product, and maintain the state of whole-cell biocatalysis for a

longer duration. Hence, insights into construction strategies for engineered strains based on this study are necessary, which should provide ideas and theoretical support for further study (Fig. 7).

In increasing the rate of substrate (raffinose) catalysis, invertase is the key enzyme in the whole-cell biocatalysis (Fig. 7a); hence, based on engineering enzymes to fit the manufacturing process [1], directed mutagenesis can be performed in the coding gene for invertase and its cofactor

genes for secretion to improve its expression and secretion (Fig. 7, ② → e → f, ③ → g) and to strengthen its stability and catalytic activity (Fig. 7a).

In order to increase the yield of the target product (melibiose), the loss of raffinose substrates and the melibiose product, which could be transported into the cytosol by the raffinose transporter (Fig. 7, b → d) and melibiose transporter (Fig. 7, h → i), should be considered. Based on membrane transporter engineering in industrial biotechnology and whole-cell biocatalysis [17], raffinose transporter genes should be deleted to yield no raffinose transporter expression (Fig. 7, ① → c). Similarly, melibiose transporter genes should be deleted to yield no melibiose transporter expression (Fig. 7, ④ → j).

In this study, we realized the heterologous integration of fructose transporter genes (Fig. 7, ⑦ → n) and overexpression of the transcription factor (*gcr1* gene) in engineered strains to enhance transmembrane-transport (Fig. 7, o → p) and consumption of the fructose by-product for increasing the extracellular melibiose purity.

In addition, after the extracellular fructose by-product is transported into the cytosol (Fig. 7, o → p), it will change into acetyl-CoA via a series of metabolic conversion, and run into the TCA cycle to produce ATP and supply energy maintaining the whole-cell biocatalysis (Fig. 7, q → r → s). Then, fructose metabolism should contribute to enhance the bio-purification rate of the fructose by-product and extend the duration of state maintenance for this whole-cell biocatalysis. Hence, transformation of the metabolic pathways of fructose, such as improving key enzymes expression involved in fructose metabolism to strengthen metabolic pathway flow, is worth considering in further studies [28].

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Compliance with ethical standards

Conflict of interest We declare that we have no financial or personal relationships with other people or organizations, which would inappropriately influence our work; there is no professional or other personal interest of any nature in any product, service, and/or company that could be construed as influencing the position presented in, or the review of, this manuscript.

Ethical approval This article does not include any studies involving human participants or animals.

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