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CONSTRUCTION OF THE INDUSTRIAL ETHANOL-PRODUCING STRAIN OF *Saccharomyces cerevisiae* ABLE TO FERMENT CELLOBIOSE AND MELIBIOSE

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The gene *mel1*, encoding α-galactosidase in *Schizosaccharomyces pombe*, and the gene *bgl2*, encoding and β-glucosidase in *Trichoderma reesei*, were isolated and co-expressed in the industrial ethanol-producing strain of *Saccharomyces cerevisiae*. The resulting strains were able to grow on cellobiose and melibiose through simultaneous production of sufficient extracellular α-galactosidase and β-glucosidase activity. Under aerobic conditions, the growth rate of the recombinant strain GC1 co-expressing 2 genes could achieve 0.29 OD_{600} h^{-1} and a biomass yield up to 7.8 g l^{-1} dry cell weight on medium containing 10.0 g l^{-1} cellobiose and 10.0 g l^{-1} melibiose as sole carbohydrate source. Meanwhile, the new strain of *S. cerevisiae* CG1 demonstrated the ability to directly produce ethanol from microcrystalline cellulose during simultaneous saccharification and fermentation process. Approximately 36.5 g l^{-1} ethanol was produced from 100 g of cellulose supplied with 5 g l^{-1} melibose within 60 h. The yield (g of ethanol produced/g of carbohydrate consumed) was 0.44 g/g, which corresponds to 88.0% of the theoretical yield.

Tremendous researches have been devoted to producing fuel ethanol from cellulosic raw materials, and cellulases are key factors in solving this problem. The two-step conversion of biomass to ethanol involves the enzymatic hydrolysis of cellulosic biomass to produce reducing sugars, and the conversion of the resulting sugars to ethanol. However, this is a very costly process due to the recalcitrance of cellulose, and therefore the low yield and high cost of the enzymatic hydrolysis process [1]. β-Glucosidases working synergistically with endoglucanases (EC 3.2.1.4) and exoglucanases (EC 3.2.1.91) on the degradation of cellulose [2] not only catalyze the final step in the degradation of cellulose, but also stimulate the extent of cellulose hydrolysis by relieving the cellobiose-mediated inhibition of exoglucanase and endoglucanase [3, 4].

Development of a yeast strain capable of producing ethanol by fermenting cellulosic substrates has received a great deal of interest over recent years. The advantages using this microorganism include: (i) high ethanol productivity and tolerance, (ii) large cells size, which simplify their separation from the culture broth and (iii) resistance to viral infection [5]. Although *Saccharomyces cerevisiae* is one of the most suitable microorganisms for practical purposes, it cannot degrade polysaccharides such as cellobiose. Since cellobiose (and longer chain cello-oligosaccharides) is the major soluble by-product of cellulose hydrolysis, its efficient utilization is of primary importance to cellulose bio-degradation process development. Enzymatic hydrolysis of cellobiose requires the action of β-glucosidases. This heterogeneous group of enzymes displays broad substrate specificity towards cellobiose, cello-oligosaccharides and different aryl- and alkyl-β-D-glucosides. β-Glucosidases were found widely in animals, plants, fungi and bacteria [6]. Though many efforts have been done to express heterogenous gene of β-glucosides in yeast and bacteria to improve the ethanol productivity, the strains they used mostly are haploid auxotrophic strains and primarily for laboratory research [7–11]. In addition, melibiose, a disaccharide containing glucose and galactose linked through α-1,4 glycosidic bond, is one of the main non-reducing saccharides not effectively utilized during *S. cerevisiae*–mediated very high gravity ethanol fermentation from starchy materials such as wheat, corn, and cassava. Thus, it is necessary to enhance the ability of the yeast to ferment melibiose to improve the utilization rate of these materials and ethanol yield.

In this study, the gene *mel1*, encoding α-galactosidase in *Schizosaccharomyces pombe*, and the gene *bgl2*, encoding β-glucosidase in *Trichoderma reesei*, were isolated and separately expressed or co-expressed in the industrial ethanol-producing strain of *Saccharomyces cerevisiae*. The resulting strains were studied under anaerobic conditions and ethanol production from cellobiose and melibiose was achieved by expressing these genes.

MATERIALS AND METHODS

Yeast strains and media. *E. coli* JM109 {*recA*1 *supE*44 *endA*1 *hsdR*17 (r_K^-, m_K^+) *gyrA*96 *relA*1 *thi*-1 (*lac-proAB*) [F', *traD*36 *proAB*$^+$ *lacI*^{q} *lacZ*M15]} (Stratagene, USA) was used for plasmid transformation and propagation. *T. reesei* was grown in a medium containing (g/l): bean cake powder – 45.0, wheat bran – 10.0, corn meal – 20.0, KH_2PO_4 – 5.0, $CaCl_2$ – 3.0, NH_4Cl – 5.0.

The industrial yeast, *S. cerevisiae* CICIMY0086 (http://cicim-cu.sytu.edu.cn/, ethanol producing yeast used in industrial plants) was used for genetic manipulation. The yeasts including *S. pombe* were routinely grown in a medium composed of 1% yeast extract, 2% bactopeptone, and 2% glucose (YEPD), solid media contained 2% agar. For selection of yeast transformants, geneticin (G418) was added with the final concentration 300 μg/ml. Incubation conditions were standardized on the rotary shaker with 150 rpm at 30°C.

Construction of the strains. The plasmid for expressing β-glucosidase was constructed. The total RNA of *T. reesei* was extracted using with a guanidine thiocyanate-phenyl-chloroform method [12]. Poly A^+ mRNA was isolated from the obtained total RNA of *T. reesei* using Oligoex Kit (Qiagen, Germany) according to the manufacturer's instructions. The gene *bgl2*, encoding β-glucosidase in *T. reesei*, was obtained by PCR amplification using Qiagen One Step RT-PCR kit from the poly A^+ mRNA with primers P1(5'-CCG-*GAATTC*ATGTTGCCCAAGGACTTTCAGTGGG-3') and P2(5'-CCC*TTCGAA*ATTTCCCCTTTGA AGA-AGCATCAGG-3') [13, 14] containing *Eco*RI and *Hin*dIII sit, respectively. A 1538-bp PCR fragment including the entire coding region for β-glucosidase was obtained. This *Eco*R I/*Hin*dIII digested fragment was inserted into the vector pYX212 (Ingenenius MBV-028-10) at the same sit, resulting plasmid pYX-*BGL*. Kanamycin resistance gene which confers resistance to geneticin in *S. cerevisiae* was isolated from the vector pPIC9K and was inserted into the downstream of the target gene in pYX-*BGL* resulting in the plasmid pYX-*BGL-Km*. The β-glucosidase expressing cassette including *TPI* (triosephosphate isomerase) promoter, and geneticin resistance gene was isolated from the plasmid pYX-*BGL-Km* with primers P3, 5'-AAC-TTAACTTCCGGCCACTTGAATGCTGGTAGAA-AGAGAAGTTCCTCTTCTGTTAACGGGAGCG-TAATGGTGATGGAA-3' and P4, 5'-TAATTCTTCA-ATCATGTCCGGCAGGTTCTTCATTGGGTAGT TGTTGTAAACGATGAGATATCATGCGTAGTCA-GGCAC-3'. A 54-bp gene fragment (underlined) of the *S. cerevisiae* glycerol phosphate dehydrogenase gene (*GDP*1) was added to each primer used as homologous integration site. After purification, this *gpd1*'-P_{TPI}-*BGLII*-Km-*gpd1*' fragment was introduced into the industrial alcoholic yeast by the lithium acetate method [15]. The recombinants were screened on the YEPD plate containing 300 μg/ml G418. Correct insertion of the gene into the target locus was verified by PCR.

The gene *mel1* was amplified by PCR form the genomic DNA of *S. pombe* using primers P5(5'-CCG*G-GATCC*TTGCCACATTCGCCTCCGTA-3'), and P6 (5'-CCC*GGATCC*ATCATGTGCTAGGTCGATTCTG-GT-3') containing *Bam*H I sit on both ends. This *Bam*H I digested fragment was inserted into the same sit of vector pYX212, resulting in the plasmid pYX-*MEL*. After that, G418 resistance gene was inserted into the downstream of the gene *mel1* and the resulted plasmid was designated pYX-*MEL-Km*. The α-galactosidase expressing cassette including *TPI* promoter, and G418 resistance gene was isolated from the plasmid pYX-*MEL-Km* with primers P7 (5'-ATGTAATAAGCAAA-CAAGCACGAATGGGGAAAGCCTATGTGCAA-TCACCAAGGTAACGGGAGCGTAATGGTGAT-GGAA-3') and P8 (5'-TCGTGAACTTCTCTGCA-TGTGATTATCCCTTGGGCGGATTGACCGTTAA-GCAATGAGATATCATGCGTAGTCAGGCAC-3'). A 54-bp gene fragment (underlined) of the *S. cerevisiae* glycerol phosphate dehydrogenase (*gdp2*) gene was added to each primer used as homologous integration site. After purification, this *gpd2*'-P_{TPI}-*Mel*-Km-*gpd2*' fragment was introduced into the industrial alcoholic yeast. The recombinants were screened on the YEPD plate containing 300 μg/ml G418. Correct insertion of the gene into the target locus was verified by PCR.

For co-expressing 2 genes, the recombinant strain expressing β-glucosidase was re-transformed by the α-galactosidase expressing cassette and higher concentration of G418 and nearly 800 μg/ml was used which was determined by the resistance experiment of the initial recombinant. Correct insertion of the gene into the target locus was verified by PCR.

Measurement of enzyme activity. The recombinant strains were cultivated at 30°C for 48 h in YEPD medium and the resulting fermentation fluid was used as enzyme solution. Activities of β-glucosidase and α-galactosidase were determined by measuring pNP (*p*-nitrophenol) concentration derived from pNPG. To assay the β-glucosidase activity, the reaction mixture (final volume, 4.0 ml) containing 0.2 ml of enzyme solution, 1.8 ml of 0.2 M Na_2HPO_4 and 2.0 ml of 5.0 mM 4-nitrophenyl-β-D-glucopyranoside (Sigma, USA) in 0.1 M citric acid buffer (pH 4.5), was mixed and incubated at 30°C for 10 min. Reaction was stopped by adding 2.0 ml of 1.0 M Na_2CO_3. The enzyme reaction was monitored by spectrophotometry (400 nm) at room temperature for 5 min [16]. For measuring the activity of α-galactosidase, 4-nitrophenyl-β-D-glucopyranoside was substituted by 4-nitrophenyl-α-D-galactopyranoside used as chromogenic substrate [16]. One unit of pNPGase activity was defined as the amount of enzyme required for releasing total reducing sugar equivalent to 1 μmol pNP min^{-1}.

OD_{600} of the host *S. cerevisiae* 0086 and recombinants during growth in cellobiose/melibiose-containing medium

Time, h	OD_{600}				
	S. cerevisiae 0086	Recombinant strains			
		1	9	15	24
24	0.18 ± 0.01	0.28 ± 0.02	0.21 ± 0.01	0.16 ± 0.02	0.19 ± 0.02
48	0.21 ± 0.01	0.81 ± 0.02	0.54 ± 0.03	0.20 ± 0.02	0.26 ± 0.01
Max. specific growth rate, h^{-1}	0.17 ± 0.01	0.29 ± 0.02	0.24 ± 0.02	0.16 ± 0.02	0.20 ± 0.01
DCW, g l^{-1}	0.19 ± 0.02	7.33 ± 0.02	7.61 ± 0.01	7.82 ± 0.01	7.25 ± 0.02

Note: ± – the standard deviation.

Aerobic growth of recombinants in cellobiose and melibiose mixed medium. Cultivations were carried out under aerobic conditions in a flask with a working volume of 100 ml. The transformed yeast or the parental yeast were tested for the ability to grow in a medium containing 10.0 g l^{-1} of cellobiose and 10.0 g l^{-1} of melibiose as carbon source supplemented with 7.5 g of $(NH_4)_2SO_4$, 3.5 g of KH_2PO_4, 0.75 g of $MgSO_4 \cdot 7H_2O$ and 0.5 g of yeast extract. The colonies from YPD slant were inoculated in this medium. During the cultivation process, the flasks were kept at 100 rpm at 30°C in a thermostatic chamber. The experiments were performed in triplicate. The optical density at 600 nm (OD_{600}) of the broth was monitored using the fresh medium as control.

Cellulose fermentation. 10 g of microcrystalline cellulose and 100 ml water were mixed in a 500 ml conical flask. The pH was adjusted to 4.8 with 6 M HCl and different amount of cellulase (Novozymes A/S, Denmark, 95 ± 4 filter paper Unit (FPU)/g) was added as follows: 6.0 FPU cellulase/g cellulose added at the beginning of fermentation process (process 1), or 4.0 FPU cellulase/g cellulose was added and the medium was incubated at 50° C for 1–1.5 h and then, another 2.0 FPU cellulase/g cellulose was added at the beginning of fermentation (process 2), or 4.0 FPU cellulase/g cellulose was added at the beginning and after incubated for 1.0–1.5 h at 50° C, another 2.0 FPU cellulase/g cellulose was added at the beginning and 2.0 FPU cellulase/g cellulose was supplemented at every 12 h thereafter during the fermentation process (process 3). For each experiment, 5 g l^{-1} of melibiose and a pre-culture of yeast (15 ml) were added, and the solution was incubated at 30°C with no air supplied. The cellobiose, glucose and melibiose concentrations were determined by HPLC using column HP1100 (Agilent, USA) eluted with 0.01 M H_2SO_4 at 50°C [17]; ethanol concentration was determined by GC [18].

RESULTS AND DISCUSSION

β-glucosidase and α-galactosidase activities of extracellular solution. Ethanol is a renewable energy source produced through the fermentation of the sugars and is widely used as a partial gasoline replacement in many countries. However, the high production cost of ethanol makes the cost of ethanol-based fuels comparably higher than that of fossil fuels. The potential mechanism to reduce the costs of ethanol production is the use of cellulosic raw materials or improvement of the ethanol yield from starchy materials. Since the most of the global ethanol is fermented from corn, it is necessary to improve the utilization rate of the starchy materials. Here, the gene *mel1*, encoding α-galactosidase in *S. pombe*, and the gene *bgl2*, encoding β-glucosidase in *T. reesei*, were co-expressed in the industrial ethanol-producing strain of *S. cerevisiae*. The engineered strain *S. cerevisiae* CG1 was used to incorporate cellulase for simultaneous saccharification and fermentation of cellulose and melibiose to ethanol. The enzymatic activities of β-glucosidase and α-galactosidase were measured from the enzyme solution of transformed yeast (Materials and Methods), and one unit of β-glucosidase activity was defined as the amount producing 1 μmol pNP in 1 ml of crude cell extracts per min. The results had shown that the highest activity of β-glucosidase of the transformants was 0.47 u/ml compared with the wild type which produced no detectable β-glucosidase activity. Meanwhile, the transformant showed much higher activity of α-galactosidase (1.58 u/ml) than the original strain (0.33 u/ml).

Characteristics of the recombinants under aerobic growth conditions. The ability of the industrial *S. cerevisiae* 0086 and its recombinant strains expressing β-glucosidase and α-galactosidase were tested during the growth in a medium with cellobiose and melibiose as carbon source. *S. cerevisiae* 0086 or positive recombinant colonies (1, 9, 15, and 24) were inoculated in cellobiose/melibiose-containing medium and the OD_{600} was measured after 24 and 48 h (Table).

Recombinants 1, 9, and 24 had OD_{600} at 24 and 48 h higher than those of the host yeast, indicating enhanced growth compared to wild-type. Recombinant 15 had a lower OD_{600} compared to wild-type at both time points. These results indicated that recombinants 1, 9, and 24 had enhanced growth in medium containing cellobiose and melibiose as carbon source com-

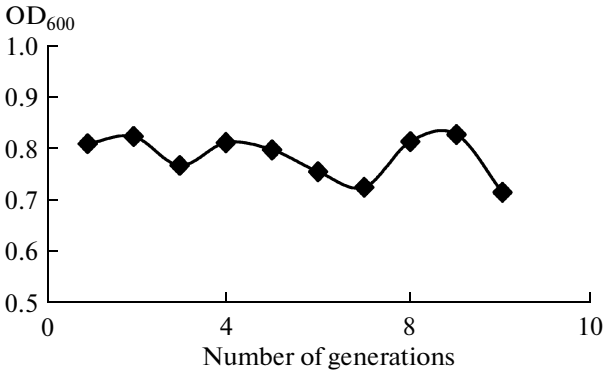

Fig. 1. OD_{600} of successive generation of *S. cerevisiae* CG1 using cellobiose and melibiose as carbon source in the medium of growth.

pared to host organisms. In contrast, recombinant 15 had reduced growth compared to wild-type. As recombinant 1 had the most marked increase in OD_{600} of the recombinants, it was used for further analysis and was designated *S. cerevisiae* CG1.

Inheritance capacity of *S. cerevisiae* CG1. We tested the inheritance capacity of the recombinant *S. cerevisiae* CG1. These cells were cultivated in a YEPD slant at 30°C and transferred to another slant every 24 h.

The colonies on every slant were inoculated in medium supplemented with cellobiose and melibiose in a rotary mixer (200 rpm) at 30°C every 48 h. OD_{600} of the broths was measured to determine the ability of the recombinants to use cellobiose and melibiose as the carbon source. OD_{600} was relatively stable after 10 generations (Fig. 1), demonstrating the stable inheritance capacity of *bgl2* in *S. cerevisiae* CG1.

Replication capacity of *S. cerevisiae* CG1. To test the replication capacity of the recombinant, *S. cerevisiae* CG1 and wild type were incubated in YEPD at 30°C with shaking (200 rpm) for 48 h, and then 20 h without shaking. After that the yeast concentration in the broth was counted. The results had shown that the amount of yeast in 1 ml of broth containing *S. cerevisiae* CG1 or wild type were 0.84×10^8 or 0.91×10^8, respectively. This indicated that the replication capacity of the recombinant did not significantly decrease compared to the host strain.

Yeast shape of *S. cerevisiae* CG1. Cell shapes of *S. cerevisiae* wild type and CG1 incubated in YEPD were determined by imaging using electron microscopy. The cells of *S. cerevisiae* CG1 were smaller and self-flocculated in comparison to wild type (Fig. 2). This suggests that the structure of the yeast cell had changed during construction of the cellobiose and melibiose metabolic pathway. However, this shape change had minimal effect on the yeasts' inheritance capacity or replication ability.

Cellulose fermentation by *S. cerevisiae* CG1. The ability of *S. cerevisiae* wild type and CG1 to mediate cellulose fermentation was investigated. In our experiments, three processes (1, 2 and 3) were tested, depending on the amount of cellulase added (see Materials and Methods). The broth was sampled every 12 h and cellubiose, melibiose, glucose, and residual sugar concentrations were measured. The alcohol concentration of the final fermentation broth was also determined. The results showed that alcohol concentration was increased and the cellobiose and melibiose accumulation markedly decreased in the broth fermented with *S. cerevisiae* CG1 compared to wild type, allowing to conclude that the feedback inhibition engendered by cellobiose accumulation could be eliminated by integration of *bgl2* into the chromosomal DNA of the parent yeast, as well as the residual melibiose. Furthermore, in the fermentation broth of *S. cerevisiae* CG1 of process 1, the glucose concentration maintained at a higher level and the cellobiose accumulated at lower level than in the parent yeast (Fig. 3). At the end of fermentation, recombinant yeast produced 32.2 ± 2.3 g l^{-1} ethanol, much higher than 13.1 ± 1.5 g l^{-1} ethanol of the reference strain. In process 2, 2.0 FPUs

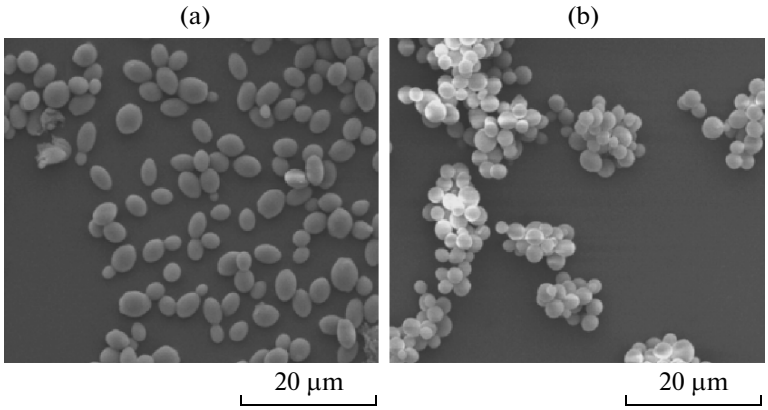

Fig. 2. Electron microscopy of *S. cerevisiae:* a – parental strain of *S. cerevisiae* 0086; b – recombinant strain of *S. cerevisiae* CG1. Images are taken at 2400× magnification. Scale bar – 20.0 μm.

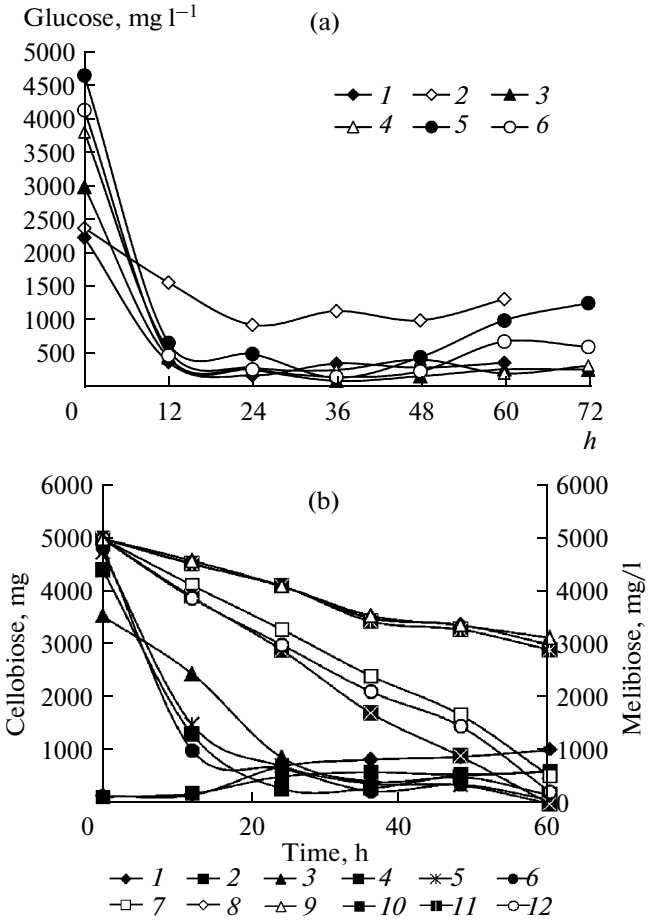

Fig. 3. Comparison of the time curves of fermentation. a – glucose concentration in the course of fermentation for *S. cerevisiae* 0086 wild type in process 1 (line *1*), 2 (line *3*), 3 (line *5*) and for *S. cerevisiae* CG1 in process 1 (line *2*), 2 (line *4*) and 3 (line *6*) respectively; b – cellobiose and melibiose concentrations in the course of fermentation for *S. cerevisiae* 0086 in process 1 (line 1, cellobiose; line 7, melibiose), 2 (line 3, cellobiose; line 9, melibiose), 3 (line *5*, cellobiose; line 11, melibiose) and *S. cerevisiae* CG1 in process 1 (line 2, cellobiose; line 8, melibiose), 2 (line 4, cellobiose; line 10, melibiose), 3 (line 6, cellobiose; line 12, melibiose) (see Materials and Methods).

of fresh cellulase/g of cellulose were supplied after the enzymatic reaction had proceeded for 1–1.5 h, so that the fermentation trends of *S. cerevisiae* CG1 and wild type were nearly the same at 50 h. However, thereafter the cellobiose accumulation of *S. cerevisiae* 0086 increased (Fig. 3b), indicating that some cellulase combined with cellulose was not reversible. As a result, 36.5 ± 1.0 g l^{-1} ethanol was produced by the recombinant strain from 100 g of cellulose supplied with 5 g l^{-1} melibose within 60 h. The yield (g of ethanol produced/g of carbohydrate consumed) was 0.44 g/g, which corresponds to 88.0% of the theoretical yield. In contrast, only 13.8 ± 0.8 g l^{-1} ethanol was produced by the parent strain during the same process. For process 3, the fermentation trend of *S. cerevisiae* CG1 was the same as that of wild type (Fig. 3). This was due to the continuous supplementation of the broth with cellulase, including small amounts of β-glucosidase, which resulted in cellobiose hydrolyzation. However, recombinant strain CG1 could produce 31.4 ± 1.6 g l^{-1} ethanol as compared to 18.5 ± 1.9 g l^{-1} of the parent strain. This demonstrates that addition of an adequate amount of β-glucosidase can be used to overcome the feedback inhibition of cellobiose. Although the recombinants could grow in the medium with cellobiose as the sole carbon source, the cellobiose was not well utilized to produce ethanol. Probably, the enzyme activity expressed in our system was not sufficient to hydrolyze cellulose. Future research will need to focus on designing yeast vectors with a high-level of *bgl* expression and a high mitotic stability under non-selective conditions. We demonstrated that cellobiose and melibiose accumulation decreased and alcohol productivity increased. It allows to conclude that the feedback inhibition engendered by cellobiose accumulation was partially relieved and the residual melibiose could be effectively utilized by the new strain. In future, more efforts should be made to enhance the usage of other kinds of unfermentable sugars for *S. cerevisiae* to improve the ethanol productivity.

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