Enzyme and Microbial Technology 49 (2011) 105–112

Contents lists available at ScienceDirect

Enzyme and Microbial Technology

journal homepage: www.elsevier.com/locate/emt

Development of an industrial ethanol-producing yeast strain for efficient utilization of cellobiose

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ARTICLE INFO

Article history:
Received 15 January 2011
Received in revised form 13 February 2011
Accepted 16 February 2011

Keywords:
Industrial strain
Cellobiose
β-glucosidase
Surface-engineered *S. cerevisiae*

ABSTRACT

The BGL1 gene, encoding β-glucosidase in *Saccharomycopsis fibuligera*, was intracellular, secreted or cell-wall associated expressed in an industrial strain of *Saccharomyces cerevisiae*. The obtained recombinant strains were studied under aerobic and anaerobic conditions. The results indicated that both the wild type and recombinant strain expressing intracellular β-glucosidase cannot grow in medium using cellobiose as sole carbon source. As for the recombinant EB1 expressing secreted enzyme and WB1 expressing cell-wall associated enzyme, the maximum specific growth rates (μ_{max}) could reach 0.03 and 0.05 h^{-1} under anaerobic conditions, respectively. Meanwhile, the surface-engineered *S. cerevisiae* utilized 5.2 g cellobiose L^{-1} and produced 2.3 g ethanol L^{-1} in 48 h, while *S. cerevisiae* secreting β-glucosidase into culture broth used 3.6 g cellobiose L^{-1} and produced 1.5 g ethanol L^{-1} over the same period, but no-full depletion of cellobiose were observed for both the used recombinant strains. The results suggest that *S. cerevisiae* used in industrial ethanol production is deficient in cellobiose transporter. However, when β-glucoside permease and β-glucosidase were co-expressed in this strain, it could uptake cellobiose and showed higher growth rate (0.11 h^{-1}) on cellobiose.

1. Introduction

Fuel alcohol is one of the most promising alternative liquid fuels among the primary renewable sources and energy. Ethanol production from cheap, abundant, and renewable material such as plant material or lignocellulose, is of great commercial and social interest. The two-step conversion of biomass to ethanol involves the enzymatic hydrolization of cellulosic biomass by cellulase, basically comprised of endoglucanases (EC 3.2.1.4), exoglucanases (EC 3.2.1.91), and β-glucosidase (EC 3.2.1.21), to produce reducing sugars, and the conversion of the resulting sugars to ethanol. However, cellobiose is usually accumulated because of the weak β-glucosidase (cellobiase) activity of most cellulolytic microorganisms making cellobiose hydrolyzation the rate-limiting step during this enzymatic hydrolysis process of the cellulose [1–3]. Although the yeast *Saccharomyces cerevisiae* is used widely for industrial ethanol production because of its ability to produce high concentrations of ethanol from hexoses and its high tolerance to ethanol and other inhibitory compounds, the species are unable to utilize cellobiose. Despite great efforts have been made to genetically engineer *S. cerevisiae* to grow on cellobiose by expressing heterologous genes encoding β-glucosidase, there are still no recombinant *S. cerevisiae* can achieve high ethanol yields [4–6]. Until now, few studies are concerning the transport system of cellobiose in *S. cerevisiae* and there is no clear evidence to support that a permease or a transport system of β-glycoside exists in this microorganism with the exception of strain C [7], nor does this organism could produce an extracellular cellobiase [8,9]. Engineering the yeast *S. cerevisiae* to utilize cellobiose by co-expressing cellobiose transporter and β-glucosidase is a novel strategy only reported recently but not well described in their work [10]. Our studies differ from their results in that we aim to determine whether the *S. cerevisiae* used in industrial ethanol production processes a transport system for cellobiose. Based on these findings and comparing different strategies to develop the most promising way in engineering the yeast with the ability to use cellobiose. The studies have both basic and applied research significance. After all, this is a step forward in developing industrial yeasts able to convert cellulosic materials into useful products.

In the present study, the gene *BGL1*, encoding β-glucosidase in *Saccharomycopsis fibuligera* which was already denoted as a suitable and efficient gene product can be heterologously expressed in *S. cerevisiae* [6], was intracellularly, secreted and cell-wall associated expressed in industrial ethanol-producing yeast. The enzymatic properties of the recombinant β-glucosidase were characterized. The obtained recombinant strains were studied under aerobic and anaerobic conditions in media supplemented with cellobiose. This work is dedicated to investigating the cellobiose transport ability

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doi:10.1016/j.enzmictec.2011.02.008

Table 1
Microbial strains and plasmids used in the present study.

Strains	Relevant genotype	Source of reference
S. cerevisiae	Polyploid, wild type	CICIMY0086, JU
S. cerevisiae IB1	P_{PGK}-*BGL1*	This investigation
S. cerevisiae EB1	P_{PGK}-*αfBGL1*	This investigation
S. cerevisiae WB1	P_{PGK}-*αfBGL1-AG1*	This investigation
S. cerevisiae BP	P_{PGK}-*bglP*	This investigation
S. cerevisiae IBP1	P_{PGK}-*bglP, BGL1*	This investigation
S. fibuligera	Wild type	CICIM Y0087(T)
B. subtilis	Wild type	CICIM B0629, JU
E. coli JM109	*recA*1 *supE*44 *endA*1 *hsdR*17 *gyrA*96 *relA*1 *thi* (*Lac-proAB*) F'[*traD*36 *proAB*$^+$ *lacI*^{q} *lacZ*M15	Strategene
Plasmids	Relevant genotype	Source of reference
pMD18-T simple	*bla*[a]	TaKaRa, Japan
pMG	*bla kan* $PGK1_{PT}$	This investigation
pMGKR	*bla kan* $PGK1_{PT}$-rDNA	This investigation
pMGKR-*BGL1*	*bla kan* $PGK1_{PT}$-*BGL1*	This investigation
pMGKR-*αfBGL1*	*bla kan* $PGK1_{PT}$-*αfBGL1*	This investigation
pMGKR-*αfBGL1-AG1*	*bla kan* $PGK1_{PT}$-*αfBGL1-AG1*	This investigation
pMGKR-*bglP*	*bla kan* $PGK1_{PT}$-*bglP*	This investigation

[a] β-Lactamase gene, confers resistance to Penicillin in *E.coli*.

of industrial ethanol-producing *S. cerevisiae* and engineering the strain to convert cellobiose to ethanol efficiently and effectively.

2. Materials and methods

2.1. Strains and media

The genotypes of the microbial strains and plasmids used in the present study are summarized in Table 1. All strains used in this study were provided by Culture & Information Center of Industrial Microorganism of China Universities, JU. *Escherichia coli* JM109 was used for plasmid construction. Industrial yeast *S. cerevisiae* CICIMY0086 (wild type) and *S. fibuligera* were routinely cultivated in a medium composed of 1% yeast extract, 2% Bacto peptone, and 2% glucose (YPD), solid media contained 2% agar. For selection of yeast transformants, G418 was added with the final concentration 500 μg ml^{-1}. The indicator plate for β-glucosidase activity was SD minimal medium supplemented with 1 mM *p*-nitrophenyl-β-D-glucoside (pNPG) [11]. To testify the ability of the recombinant β-glucosidase to hydrolyze cellobiose, yeast was aerobically cultivated at 30 °C in synthetic medium containing 10 g L^{-1} cellobiose and 6.7 g L^{-1} yeast nitrogen base without amino acids (YNBC). Aerobic cultivations were performed in defined medium containing 10 g cellobiose L^{-1}, 10 g yeast extract L^{-1}, and 20 g Bacto-peptone L^{-1}. The medium for fermentation contains 10 g L^{-1} cellobiose and supplemented with 7.5 g $(NH_4)_2SO_4$, 3.5 g KH_2PO_4, 0.75 g $MgSO_4{\cdot}7H_2O$, 0.5 g yeast extracts. The medium was supplemented with 420 mg Tween 80 and 10 mg ergosterol L^{-1}, which are necessary for anaerobic growth of *S. cerevisiae* [12] and with amino acids, in order to enhance heterologous protein production (aspartic acid [257 mg L^{-1}], glutamic acid [64 mg L^{-1}], glycine [33 mg L^{-1}] and serine [108 mg L^{-1}]) [13].

2.2. Construction of the plasmids

All primers used in this study are listed in Table 2. The plasmids used for yeast intracellular, secreted and cell-wall associated expressing β-glucosidase were constructed as follows: The plasmid pMGKR [14] containing *S. cerevisiae PGK1* promoter and terminator, G418 resistance gene and partial of 18S ribosomal RNA coding sequence (an rDAN fragment) was used for recombinant plasmids construction. The *BGL1* gene, encoding β-glucosidase in *S. fibuligera*, was cloned by PCR amplification using primers BF1 and BR2. A 2580-bp fragment including the entire coding region without signal sequence was obtained. After digested by *Eco*RI, this fragment was inserted into pMGKR and pPIC9K at the same site respectively, resulting in pMGKR-*BGL1* for expressing intracellular *BGL1* and pPIC9K-*BGL1*. Then, the fragment containing signal sequence of the yeast α-factor fusing with *BGL1* was obtained by PCR using pPIC9K-*BGL1* as template with primers ABF1 and ABR2 flanking with *Bam*HI and *Sal*I site on each 5′ end. This *Bam*HI/*Sal*I digested fragment was inserted into pMGKR at the same site to create pMGKR-*αfBGL1* for expressing secreted β-glucosidase. An anchor sequence of C-terminal half of the yeast α-agglutinin gene (*AGα1*) [15], designated *AG*1, was prepared by PCR using primers AGF1 and AGR2. This *Sal*I digested fragment was inserted into pMGKR-*αfBGL1*, resulting in pMGKR-*BLG1-AG*1 for expressing cell-wall associated β-glucosidase (Fig. 1).

The plasmid used for yeast expressing *bglP* was constructed as follows: the *bglP* gene, encoding β-glucoside permease, was obtained from *B. subtilis* genomic DNA by PCR amplification using primers bPF and bPR. The obtained 1830-bp fragment

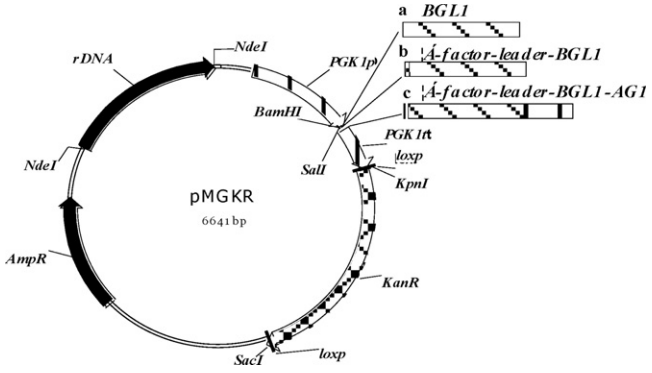

Fig. 1. Schematic summary of the construction of the different expression cassettes used for intracellular (a), secreted (b) and cell associated (c) expression of β-glucosidase in this study. (*PGK*1 promoter, *S. cerevisiae* glyceraldehyde 3-phosphate dehydrogenase gene promoter; α-factor leader, 269-bp fragment encoding the α-factor signal sequence for secretion in *S. cerevisiae; BGL1*, *S. fibuligera* β-glucosidase gene; *AG1*, half of the yeast α-agglutinin gene; *bglP*, *B. subtilis* β-glucoside permease gene; KanR, Kanamycin resistance gene from Tn*903* which confers resistance to Geneticin in *S. cerevisiae* and kanamycin resistance in *E. coli*).

was digested by *Bam*HI and *Sal*I and then inserted into the corresponding sites of pMGKR, resulting in pMGKR-*bglP*.

The plasmid used for expressing *BGL1* was linearized by *Sac*II and the plasmid for expressing *bglA* was digested by *Sac*I. After purified, the resultant linear DNA fragments were introduced into *S. cerevisiae* by the lithium acetate method [16]. For initial selection of yeast transformants, G418 was added with the final concentration 0.25 mg ml^{-1}. Transformants with the G418$^+$ phenotype were isolated. To delete the G418 resistance gene, the *cre*-recombinase expression vector pSH47 [17] was transformed into the relevant recombinant strains.

2.3. Enzyme assay

For first insolating the recombinant yeast colonies secreting or expressing cell-wall associated β-glucosidase, a solution of 0.25 M Na_2CO_3 was poured onto colonies grown on the indicator plate. The colonies turned into yellow were picked out and cultured for 3 days in liquid YEPD medium. The culture supernatant was recovered by centrifugation of the culture broth at 8000 × *g* for 5 min to determinate the extracellular β-glucosidase activity. The cells were collected, washed with buffer (50 mM citrate buffer, pH 5.0) and disrupted by sonication (VC750, Sonics, USA). Then, the cell wall fraction was recovered by centrifugation of the homogenate at 8000 × *g* for 5 min and washed with the same buffer. The cell extract was used to determine the intracellular enzyme activity. Activities of β-glucosidase were determined by measuring pNP (*p*-nitrophenol) concentration derived from pNPG. The reaction mixtures consisting of 450 μl reaction buffer (50 mM citrate buffer, pH 5.0), 50 μl of 10 mM pNPG, and 100 μl enzyme-contained solution (supernatant of the medium, appropriately diluted suspension of the yeast cells, cell extracts and cell-wall fractions, respectively), were incubated for 20 min at 30 °C. One unit of pNPGase activity was defined as the amount of enzyme required for releasing total reducing sugar equivalent to 1 mol pNP min^{-1}. pNP was quantified at 420 nm.

2.4. Measurement of intracellular accumulation of cellobiose

The wild-type strain and the recombinant *S. cerevisiae* expressing β-glucoside permease were cultivated aerobically in YEPD medium for 48 h at 30 °C. The cells were recovered by centrifugation of the culture broth at 6000 × *g* for 5 min at room temperature, washed three times with distilled water. To initiate the cellobiose uptake reaction, the cells were then inoculated into 100-ml YEPD medium supplemented with 20 g L^{-1} cellobiose to yield an OD_{600nm} of 0.5, incubated at 30 °C with shaking at 200 rpm. Samples (10 ml) were taken periodically from 0 to 5 h. The yeast cells of each sample were recovered by centrifugation of the homogeneous culture at 6000 × *g* for 5 min at 4 °C, washed twice and resuspended with the ice-cold water. 10 ml of cell suspension was vortexed with 10 g glass beads (0.5 mm diameter) for 15 min at 0 °C. The supernatant was recovered by centrifugation of the homogenate at 8000 × *g* for 5 min to determine the intracellular content of cellobiose. The water used for washing the cells was used to detect the released cellobiose from the yeast cells. The amount of cellobiose was determined by high performance liquid chromatography (HPLC) as described in following section.

2.5. Characterization of the recombinant β-glucosidase and comparison of the stability

The recombinant strains expressing secreted or cell-wall anchored β-glucosidase were cultivated in YEPD medium for 3 days at 30 °C. The cells were collected by centrifugation of the culture broth at 6000 × *g* for 5 min at 4 °C and

Table 2
The sequences of the oligonucleotide primers used in this study.

Primer names	Sequence (5′–3′) restriction sits are italic/underlined	Restriction sites
PF1	ATTTTAGATTCCTGACTTCAACTC	
PR2	GCG*GAATTC*[a]*GGATCC*[b]TGTTTTATATTTGTTGTAAAAAG TAG	*Eco*RI[a], *Bam*HI[b]
TF1	CCG*GAATTC*[a]*GTCGAC*[b]TTCTTTGGAATTATTGGAAGGTA	*Eco*RI[a], *Sal*I[b]
TR2	GCG*GAGCTC*[a]*GGTACC*[b]GAACGCAGAATTTTCGAGTTAT	*Sac*I[a], *Kpn*I[b]
KF1	AG*GGTACC*[a]*ATAACTTCGTATAATGTATGCTATACGAAGTTAT*[b]GCCCAGTAGTAGGTTGAGG	*Kpn*I[a], *loxp*[b]
KR2	AG*GAGCTC*[a]*ATAACTTCGTATAATGTATGCTATACGAAGTTAT*[b]TTGAAGTCGGACAGTGAGT	*Sac*I[a], *loxp*[b]
RF1	CCG*CATATG*[a]CTCTATCCCCAGCACGA	*Nde*I[a]
RR2	CCC*CATATG*[a]GAGAAACGGCTACCACATC	*Nde*I[a]
BF1	CCG*GAATTC*[a]ATGGTCCCAATTCAAAACTATAC	*Eco*RI[a]
BR2	CGC*GAATTC*[a]TCAAATAGTAAACAGGACAGA	*Eco*RI[a]
ABF1	GGC*GGATCC*[a]ATGAGATTTCCTTCAATTTTTA	*Bam*HI[a]
ABR2	CGC*GTCGAC*[a]TCAAATAGTAAACAGGACAGA	*Sal*I[a]
AGF1	CCG*GTCGAC*[a]AGCGCCAAAAGCTCTTTTATC	*Sal*I[a]
AGR2	CCG*GTCGAC*[a]TTTGATTATGTTCTTTCTAT	*Sal*I[a]
bPF	GCG*GGATCC*[a]ATGGATTATGATAAATTATCGAA	*Bam*HI[a]
bPR	CCG*GTCGAC*[a]TCAAGATAAAGCAAGCAGC	*Sal*I[a]

([a, b]) Restriction sit with corresponding restriction enzyme.

then washed twice with distilled water. The supernatant containing secreted β-glucosidase and the suspension of cell pellets displaying β-glucosidase were used to study the characterizations of the expressed recombinant β-glucosidase. The same activity of the secreted β-glucosidase (4.73 U ml^{-1} supernatant) and displayed β-glucosidase on cell surface (4.73 U ml^{-1} suspension) were used in all experiments. The optimal pH was assayed by measuring β-glucosidase activity with 1 mM pNPG as the substrate. Buffers used for the determination of the pH profiles were 50 mM glycine–HCl (pH 2.0), 50 mM citrate/acetate (pH 3.0–7.2), potassium phosphate (pH 7.0–8.2), 50 mM Tris/HCl (pH 7.0–8.2). The pH values of buffers were adjusted at the temperatures of use. The stability of recombinant β-glucosidase at various pH values was determined by pre-incubating the β-glucosidase at various pH, ranging from 3.0 to 7.0, at 30 °C for 4 h and followed by analyzing the remaining activity at the same pH of the incubation using pNPG as the substrate at 30 °C. The optimal temperatures for the secreted and displayed β-glucosidase were determined using pNPG as substrate at pH 5.0 over the temperature range of 30–70 °C. The thermostability of the enzyme in a 50 mM citrate buffer (pH 5.0) was determined by incubating the enzyme at various temperatures, ranging from 30 to 70 °C, for 4 h incubation period and detecting the remaining activity using pNPG as the substrate.

2.6. Cultivation conditions

Yeast cells were pre-cultured in 500-ml Erlenmeyer flasks at 30 °C in YPD medium for 24 h. Then, this stationary phase culture was used to inoculate the cellobiose-growth medium to yield an OD_{600} of 0.45 and the yeast strains were grown at 30 °C, 150 rpm. Ethanol fermentation was carried out under oxygen limited conditions in a 1 L shake flask contains 500 ml fermentation medium. In order to maintain anaerobic conditions in batch fermentations, the shake-flasks were plugged with a rubber in which a vent-pipe obdurated by sterile water was drilled through. Then, the pre-cultured yeast cells were used to inoculate the fermentation medium at a rate of 1.68×10^7 CFU ml^{-1}. During the fermentation process, the flasks were kept at 30 °C in a thermostatic chamber with no air supplied and magnetic force stirred. Fermentation experiments were performed in triplicate.

2.7. Analysis of product formation and determination of dry weight

Cellobiose concentration was determined by HPLC using a NH_2 type-column (Econosphere-NH_2, Alltech Corp., Deerfield, IL) with 60% acetonitrile as mobile phase at a flow rate of 1.0 ml min^{-1} and subsequently detected with a refractive index detector (Agilent, USA); The concentration of glucose, ethanol, glycerol, pyruvic acid and acetic acid in filtered samples withdrawn from the batch cultivations were separated by a SH1011 column (Agilent, USA), at a column temperature of 50 °C with 0.01 M H_2SO_4 as mobile phase at a flow rate of 0.8 ml min^{-1} and subsequently detected with a refractive index detector (Agilent, USA); The biomass concentration in the medium was measured gravimetrically as described earlier [18]. Two samples (10 ml each) were centrifuged at 6000 × *g* for 15 min, washed twice with water and subsequently dried at 105 °C for 24 h and weighed. The other two samples were resuspended in 3 ml NaOH and total protein was determined by a modified biuret method [19] using bovine serum albumin as a standard. The product yield was calculated as the ratio of the amount of product obtained divided by the amount of substrate consumed.

3. Results

3.1. Expression of the β-glucosidase and Co-expression of bglP and BGL1 in strain S. cerevisiae CICIMY0086

The plasmids pMGKR-*BGL1*, pMGKR-*αfBGL1* and pMGKR-*BLG1-AG1* used for expressing β-glucosidase and the plasmid pMGKR-*bglP* used for expression of *B. subtilis* β-glucoside permease, were constructed successfully and subsequently electrotransformed into *S. cerevisiae* CICIMY0086 (wild type). Transformants with the G418$^+$ phenotype were isolated. Correct integration of the heterologous genes into the chromosome of *S. cerevisiae* CICIMY0086 was verified by PCR. Successful expression of β-glucoside permease in *S. cerevisiae* was confirmed by Northern blotting (data not shown) and the recombinant strain was designated SP1. Transformants capable of producing secreted or cell-wall associated β-glucosidase activity were then selected by their ability to form yellow halos around colonies on the indicator plates containing pNPG. The transformants essentially contained two copies of the *BGL*1 gene integrated into their genome were identified by southern blot analysis (data not shown) and then subcultured to determine their β-glucosidase activity. Meanwhile, the growth of the transformants on YNBC plate containing cellobiose as sole carbon source was analyzed. Except for the recombinant strain expressing intracellular β-glucosidase, both the recombinant strains expressing secreted and cell-wall anchored enzyme can grow on YNBC plate. Three recombinant strains designated IB1, EB1 and WB1 expressing intracellular, secreted and cell associated β-glucosidase, respectively,

Table 3
Distribution of β-glucosidase activity in wild-type and recombinant strains.

Parameter	Enzyme activity (U mg^{-1})			
	Crude extract[a]	Cell pellet[a]	Cell wall[a]	Culture supernatant[a]
IB1	0.139 ± 0.021	ND[b]	ND	ND
EB1	0.099 ± 0.013	0.021 ± 0.009	ND	0.126 ± 0.015
WB1	0.077 ± 0.011	0.235 ± 0.014	0.178 ± 0.022	ND

(±) Standard deviation.
[a] Triplicate experiments.
[b] Not detectable.

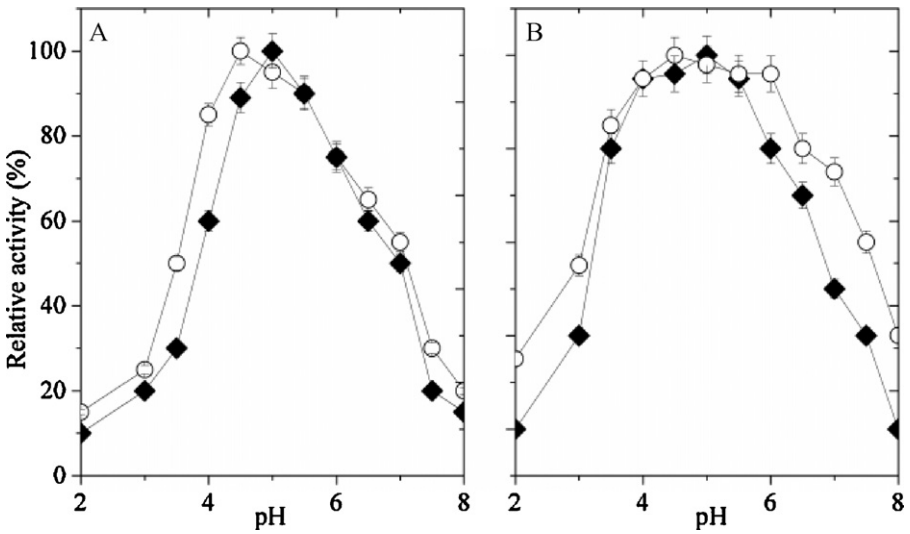

Fig. 2. (A) Effect of pH on secreted β-glucosidase (diamond) and displayed β-glucosidase (circle) activity at 30 °C. (B) Effect of pH on secreted β-glucosidase (diamond) and displayed β-glucosidase (circle) stability at 30 °C. Each data point represents the mean of three independent experiments and the error bar indicates the standard deviation.

showed apparently high β-glucosidase activities as compared to the wild-type strain were isolated and used in the subsequent experiments (Table 3).

To co-express the β-glucoside permease and β-glucosidase in *S. cerevisiae*, the G418 resistance gene of the recombinant strain IB1 was excised by expressing *Cre*-recombinase induced by 2% D-galactose. The excision of the *kan*^{r} gene was verified by PCR. After that, the plasmid pMGKR-*bglP* used for expressing *B. subtilis* β-glucoside permease was electrotransformed into recombinant strain IB1. Correct integrating of the gene *bglP* into the chromosome of recombinant strain IB1 was verified by PCR. Transformants capable of growth on YNBC plate using cellobiose as sole carbon source were then isolated. The recombinant strain named IBP1 showed comparably fast growth rate on selecting plate was used in the subsequent experiments.

3.2. Characterization and comparison of the recombinant β-glucosidase

The recombinant β-glucosidase was characterized by evaluating the effects of pH (2.0–8.0) and temperature (30–70 °C) on secreted and displayed β-glucosidase activity on substrate (pNPG). As shown in Fig. 2A and B, the optimal pH for activity of secreted β-glucosidase was 5.0 and this recombinant enzyme was stable at pH ranging from 4.0 to 6.0, whereas the optimal pH for displayed β-glucosidase was 4.5 and this form of the enzyme was stable at pH 3.0–6.5. Besides, the relative activity of displayed β-glucosidase was higher than that of secreted enzyme at both acid and alkaline pH conditions. On the other hand, measurement of the enzymatic activities of secreted and displayed β-glucosidases at various temperatures at pH 5.0 showed that the secreted enzyme exhibited the maximal activity at 50 °C (Fig. 3A) and the enzyme was stable at 30–40 °C after a 4-h incubation (Fig. 3B). Despite the optimal temperature for β-glucosidase activity of displayed β-glucosidase also was 50 °C (Fig. 3A), this kind enzyme had a moderate wider stable range of temperature between 30 and 50 °C (Fig. 3B).

3.3. Cellobiose uptake in the recombinant strains of S. cerevisiae

The recombinant strain SP1 (expressing *B. subtilis* β-glucoside permease) and the wild-type strain were used to test their abilities for uptake and accumulation of cellobiose. Since both the

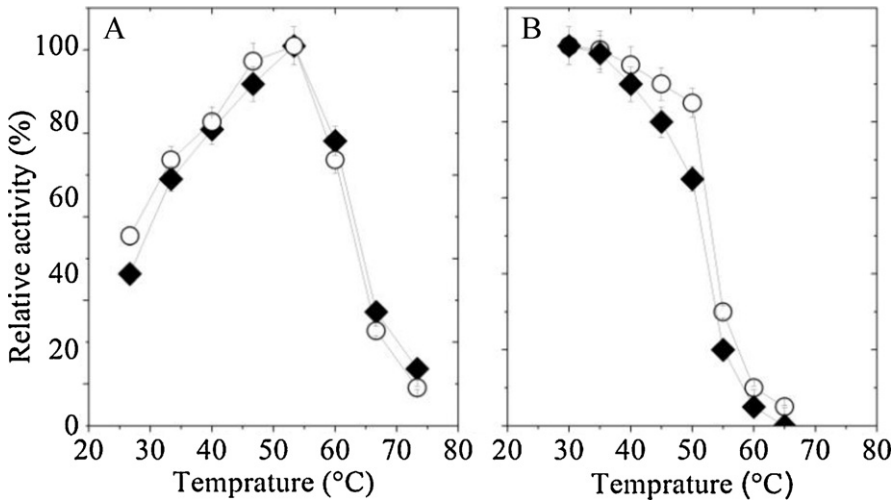

Fig. 3. (A) Effect of temperature on secreted β-glucosidase (diamond) and displayed β-glucosidase (circle) activity in a 50 mM citrate buffer (pH 5.0). (B) Effect of temperature on secreted β-glucosidase (diamond) and displayed β-glucosidase (circle) stability in a 50 mM citrate buffer (pH 5.0). Each data point represents the mean of three independent experiments and the error bar indicates the standard deviation.

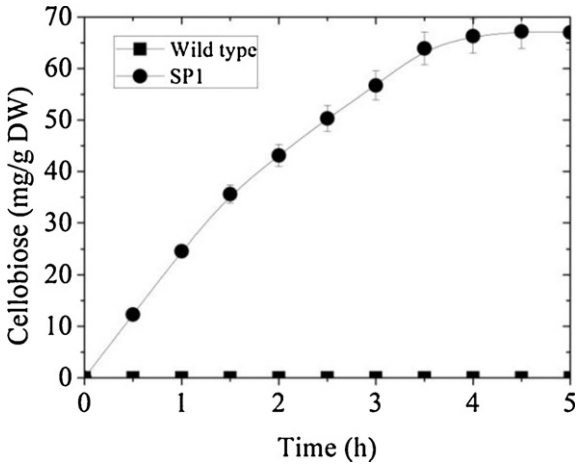

Fig. 4. Time course of intracellular cellobiose accumulation by recombinant strain SP1 (circles) and wild-type strain (square). Each data point represents the mean of three independent experiments and the error bar indicates the standard deviation.

strains cannot utilize the cellobiose, cellobiose should be accumulated inside the cells if only the strains could transport the cellobiose into cells. As a result, no cellobiose was detected in washing buffer for both the strains, as well as in cell extract of the wild type, but cellobiose was found in cell extract of the recombinant strain SP1. Measuring intracellular concentration of cellobiose illustrated this substrate was accumulated during the incubation period of the yeast cells of SP1 and achieved the maximal amount of $67.3 \pm 0.35\,mg\,g^{-1}$ DW by 4 h (Fig. 4). The above results, together with the fact that recombinant strain expressing intracellular β-glucosidase cannot utilize cellobiose suggest that the wild type *S. cerevisiae* used in industrial ethanol production is likely deficient in cellobiose transporter and expressing β-glucoside permease in this strain can enable the yeast to uptake cellobiose. Although this method was rough estimation of the transport ability of cellobiose for recombinant strain expressing β-glucoside permease and therefore more definitive studies are needed, our results, however, confirmed the validity of β-glucoside permease to transport cellobiose in *S. cerevisiae*.

3.4. Characterization of the strain expressing β-glucosidase in aerobic cellobiose fermentation

The characteristics of the yeast strains expressing intracellular, secreted and cell-wall associated β-glucosidase, as well as the recombinant strain co-expressing *BGL1* and *bglP* were investigated under aerobic growth conditions with cellobiose as sole carbon source. The wild type, as a control strain, was also tested. Fig. 5 shows that both the wild type and recombinant yeast IB1 expressing intracellular β-glucosidase cannot grow on cellobiose medium. By contrast, the recombinant strains EB1 and WB1, expressing secreted and displayed enzyme, consumed 3.3 and $5.0\,g\,L^{-1}$ cellobiose over 48 h, respectively, according to the growth phase of the yeast strains (Fig. 5). EB1 sustained growth aerobically at $0.04\,h^{-1}$ and WB1 exhibited higher growth rate at $0.06\,h^{-1}$ (versus $0.29\,h^{-1}$ on glucose, data not shown) on cellobiose. It should be noticed that although high residual sugar remained in medium, no longer consumption of this substrate was observed for both the strains after 48 h (Table 4). Hence, anchoring the β-glucosidase on the yeast cell wall did not result in significant improved utilization ability of cellobiose. On the other hand, the *BGL1* and *bglP* co-expressing yeast strain consumed all the $10.0\,g\,L^{-1}$ cellobiose over 72 h and exhibited fast cellobiose consumption rate. Besides, this strain showed

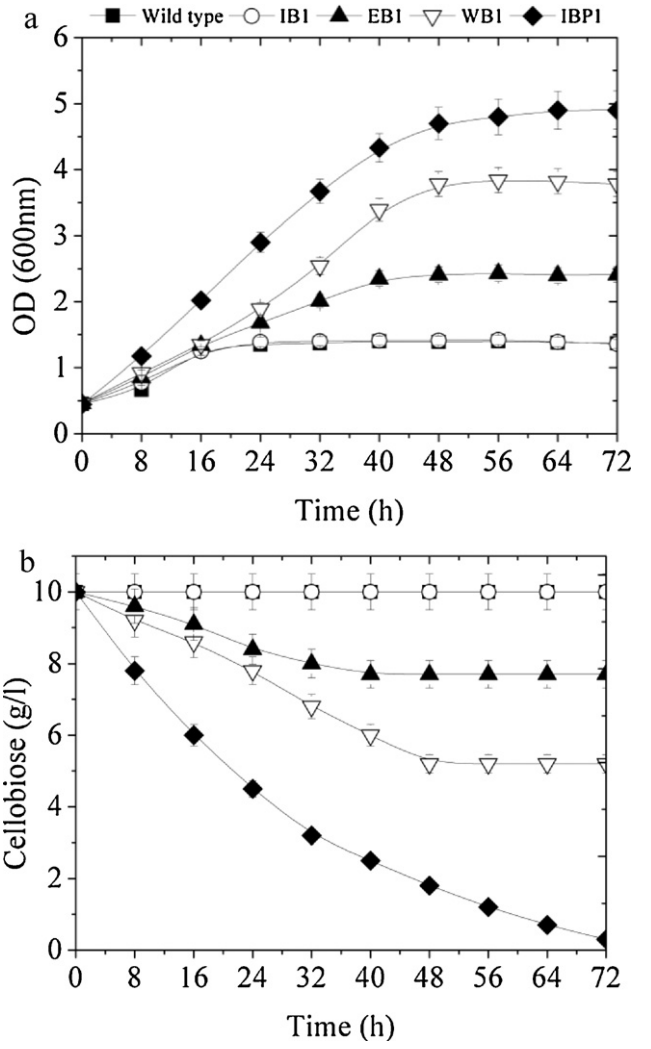

Fig. 5. Changes in measured parameters during aerobic batch cultures of *S. cerevisiae* CICIMY0086 wild type, IB1 (P_{PGK}-*BGL1*), EB1 (P_{PGK}-*αfBGL1*), WB1 (P_{PGK}-*αfBGL1-AG1*) and IBP1 (P_{PGK}-*BGL1-bglP*), respectively, during aerobic batch growth on $10\,g\,L^{-1}$ cellobiose as carbon and energy source. Shown are (A) OD_{600nm}, optical density at 600 nm, (B) cellobiose concentration versus time.

higher growth rate ($0.11\,h^{-1}$) and biomass yield than other strains during aerobic growth on cellobiose.

3.5. Characterization of the strain expressing β-glucosidase in anaerobic cellobiose fermentation

We next carried out cellobiose fermentation using wild type and all the recombinant strains. Similar to what was found in aerobic growth experiments, both the wild type and recombinant yeast expressing intracellular β-glucosidase cannot grow on cellobiose (Fig. 6a). The recombinant strain expression secreted and displayed enzyme consumed 3.6 and $5.2\,g\,L^{-1}$ cellobiose and produced 1.5 and $2.3\,g\,L^{-1}$ ethanol over 48 h, corresponding to 81.7% and 86.7% of theoretical maximum yield, respectively (Fig. 6b and c). However, the maximum specific growth rate (μ_{max}) of EB1 and WB1 on cellobiose was slightly lower than that of the strains under aerobic conditions (Table 5). The strain IBP1 co-expressing *BGL1* and *bglP*, however, consumed all the $10.0\,g\,L^{-1}$ cellobiose and produced $4.4\,g\,L^{-1}$ ethanol over 96 h. For this strain, the specific cellobiose consumption rate was 0.289-cellobiose g^{-1}-DW h^{-1} and the specific ethanol production rate was 0.076-ethanol g^{-1}-DW h^{-1} (Table 5). Both the specific cel-

Table 4
Comparison of growth and compound yields of *S.cerevisiae* CICIMY0086 wild type, IB1 (P_{PGK}-*BGL1*), EB1 (P_{PGK}-*αfBGL1*), WB1 (P_{PGK}-*αfBGL1-AG1*) and IBP1 (P_{PGK}-*BGL1-bglP*), respectively, during aerobic batch growth on 10 g L^{-1} cellobiose.

Parameter	IB1[a]	EB1[a]	WB1[a]	IBP1[a]	Wild type[a]
μ_{max}[b] (h^{-1})	ND	0.04 ± 0.007	0.06 ± 0.005	0.11 ± 0.005	ND
v_{cello}[c] (g g^{-1} h^{-1})	ND	0.189 ± 0.010	0.210 ± 0.016	0.334 ± 0.012	ND
$Y_{X/S}$[d] (g g^{-1})	ND	0.036 ± 0.002	0.040 ± 0.001	0.044 ± 0.002	ND
Residual sugar (g L^{-1})	10.0 ± 0.3	6.7 ± 0.2	5.0 ± 0.1	ND	10.0 ± 0.4

(±) Standard deviation. ND: not detectable.
[a] Triplicate experiments.
[b] μ_{max}: maximum specific growth rate.
[c] v_{cello}: specific cellobiose consumption rate (g cellobiose g^{-1} dry cell weight h^{-1}).
[d] $Y_{X/S}$: biomass yield (g DW g^{-1} cellobiose).

Table 5
Comparison of growth and compound yields of *S.cerevisiae* CICIMY0086 wild type, IB1 (P_{PGK}-*BGL1*), EB1 (P_{PGK}-*αfBGL1*), WB1 (P_{PGK}-*αfBGL1-AG1*) and IBP1 (P_{PGK}-*BGL1-bglP*), respectively, during anaerobic batch growth on 10 g L^{-1} cellobiose.

Parameter	IB1[a]	EB1[a]	WB1[a]	IBP1[a]	Wild type[a]
μ_{max}[b] (h^{-1})	ND	0.03 ± 0.002	0.05 ± 0.005	0.09 ± 0.004	ND
v_{cello}[c] (g g^{-1} h^{-1})	ND	0.166 ± 0.001	0.197 ± 0.002	0.289 ± 0.001	ND
$Y_{X/S}$[d] (g g^{-1})	ND	0.029 ± 0.002	0.033 ± 0.001	0.041 ± 0.001	ND
$Y_{P/X}$[e] (g/100g)	ND	43.12 ± 2.1	44.36 ± 2.5	48.32 ± 1.8	ND
$Y_{Eth/S}$[f] (g g^{-1})	ND	0.433 ± 0.015	0.448 ± 0.017	0.488 ± 0.011	ND
$Y_{Ace/S}$[g] (mg g^{-1})	ND	3.05 ± 0.14	3.19 ± 0.21	3.19 ± 0.24	ND
$Y_{Gly/S}$[h] (g g^{-1})	ND	0.034 ± 0.002	0.037 ± 0.002	0.033 ± 0.001	ND
$Y_{Pyr/S}$[i] (mg g^{-1})	ND	0.354 ± 0.012	0.368 ± 0.022	0.348 ± 0.030	ND
Residual sugar (g L^{-1})	10.0 ± 0.5	6.4 ± 0.2	4.8 ± 0.2	ND	10.0 ± 0.5

(±) Standard deviation. ND: not detectable.
[a] Triplicate experiments.
[b] μ_{max}: maximum specific growth rate.
[c] v_{cello}: specific cellobiose consumption rate (g cellobiose g^{-1} dry cell weight h^{-1}).
[d] $Y_{X/S}$: biomass yield (g DW g^{-1} cellobiose).
[e] $Y_{P/S}$: protein (g 100 g^{-1} biomass).
[f] $Y_{Eth/S}$: ethanol yield (g ethanol g^{-1} cellobiose).
[g] $Y_{Ace/S}$: acetate yield (mg acetate g^{-1} cellobiose).
[h] $Y_{Gly/S}$: glycerol yield (g glycerol g^{-1} cellobiose).
[i] $Y_{Pyr/S}$: pyruvic acid (mg pyruvic acid g^{-1} cellobiose).

lobiose consumption rate and the specific ethanol production rate were higher in comparison to those of the recombinant strains EB1 (0.166-cellobiose g^{-1}-DW h^{-1} and 0.047-ethanol g^{-1}-DW h^{-1}, respectively) and WB1 (0.197-cellobiose g^{-1}-DW h^{-1} and 0.056-ethanol g^{-1}-DW h^{-1}, respectively). Besides, the strain IBP1 exhibited higher growth rate and biomass yield in cellobiose-fermentation experiments. The ethanol yield of IBP1 was 0.488 g-ethanol g^{-1}-consumed cellobiose, corresponding to 94.0% of theoretical maximum yield, whereas the ethanol yield of EB1 (0.433 g-ethanol g^{-1}-consumed cellobiose) was similar to that of WB1 (0.448 g-ethanol g^{-1}-consumed cellobiose). In addition, the yields of three main byproducts evaluated in our experiments were somewhat lower than those using glucose as substrate (compared to that 3.59 mg acetate g^{-1} glucose, 0.073 g glycerol g^{-1} glucose and 0.610 mg pyruvic acid g^{-1} glucose, respectively) (Table 5). The changes in synthesis of byproducts might be an example of metabolic adjustment by yeast to minimize the ATP-consumption when energy supply was limited.

4. Discussion

In this study, we demonstrate that the used *S. cerevisiae* in industrial ethanol production does not possess an active transporter for cellobiose. Usually, the strain can be easily engineered by expressing heterologous β-glucosidase with the ability of utilizing cellobiose. However, the hydrolysis efficiency of secreted β-glucosidase on cellobiose is likely hampered particularly when high concentration of ethanol is present [20]. Thus, an adequate level of high active β-glucosidase is required for efficient breakdown of cellobiose. Expressing the enzyme on yeast cell surface is a good option as the enzymatic stability will be improved, but its effects are still limited as can seen from the lower utilizing rate of cellobiose in our experiments. Nonetheless, it should be noticed that although high amount of cellobiose still remained in the medium, the used yeast strains cannot use it as carbon source under both aerobic and anaerobic conditions. The finding that secreted or cell-wall associated BGL activity was insufficient for compete utilization of cellobiose is somewhat inconsistent with previous results [6,21]. McBride et al. [21] reported that the amount of copies of the *BGL* gene in the cell can make the difference between successful and unsuccessful utilization of cellobiose by these strains. Since genomic analysis by repeated PCR experiments showed that two copies of the *BGL*1 gene were remained in the recombinant strains and the relevant recombinant enzymatic activities can also be monitored at end of the fermentations (data not shown), it is likely that cellobiose, a non-native substrate, cannot be efficiently used by yeast as the carbon and energy source to support the cycle of metabolism due to the limited β-glucosidase activities. Low β-glucosidase activities would give a lower rate of hydrolyzation of cellobiose with slow glucose release rate cannot produce sufficient ATP for cell maintenance [22].

Since the industrial ethanol-producing yeast did not produce any extracellular enzyme, it is difficult to greatly improve the expressing level of heterologous genes in this strain as our experiences suggested. Furthermore, the yeast intracellular environment where most metabolic activities are undergoing would be a suitable place for cellobiase to effect on cellobiose. The limited cellular space will make it easier for the enzyme to combine its substrate and the

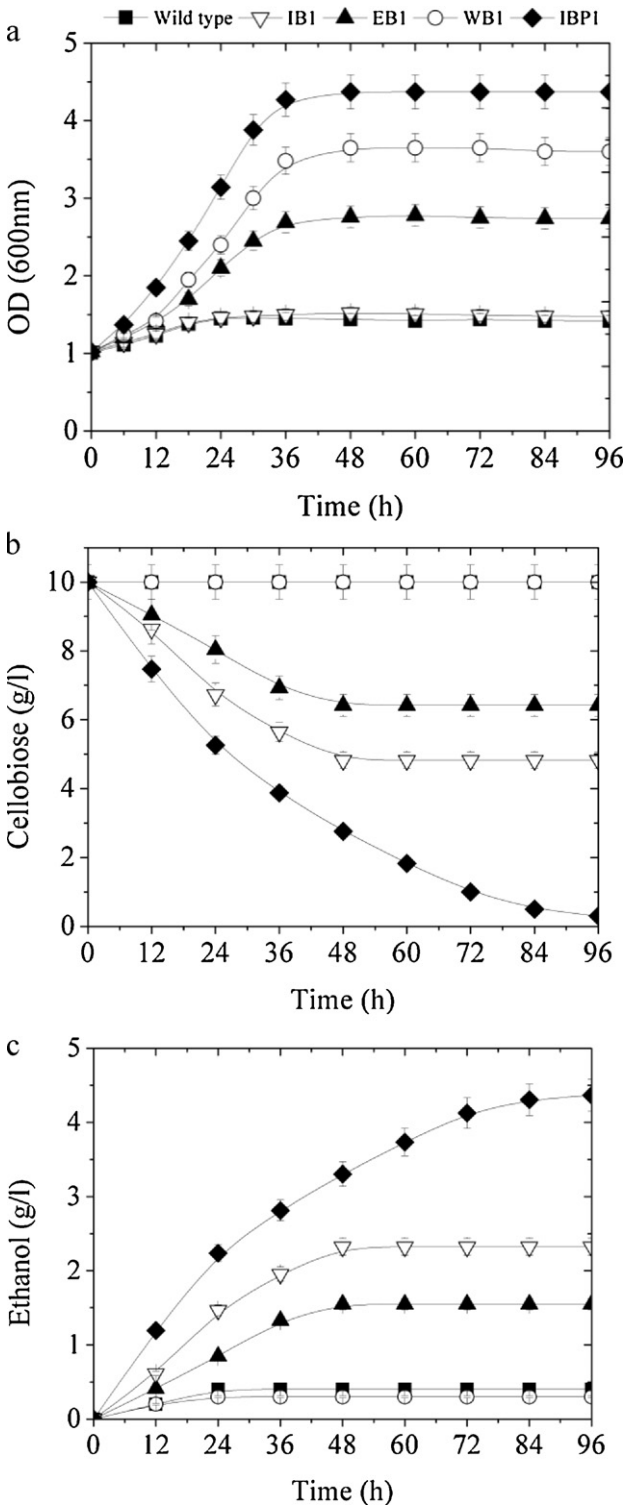

Fig. 6. Changes in measured parameters during aerobic batch cultures of *S. cerevisiae* CICIMY0086 wild type, IB1 (P_{PGK}-*BGL1*), EB1 (P_{PGK}-*αfBGL1*), WB1 (P_{PGK}-*αfBGL1-AG1*) and IBP1 (P_{PGK}-*BGL1-bglP*), respectively, during anaerobic batch growth on 10 g L^{-1} cellobiose as carbon and energy source. Shown is (A) OD_{600nm}, optical density at 600 nm, (B) cellobiose and (C) ethanol concentration versus time.

comparably stable environment will also facilitate the hydrolyzing reaction. In this study, the engineered yeast co-expressing β-glucoside permease and β-glucosidase showed extremely higher fermentation rate and ethanol yield as compared to the wild-type strain. To produce ethanol from cellobiose using this novel yeast is meaningful since the strain could be applied to industrial fermentation directly and this offers interesting perspectives for large-scale ethanol production from lignocellulosic hydrolysates. Nevertheless, the cellobiose consumption rate was still lower than that using glucose in ethanol fermentation. It was reported that seven gene clusters related to utilization of cellobiose might exist in yeast *Pichia stipitis* [23]. As for the bacteria, usually more than one ways exist to ensure the efficient assimilation of cellobiose. As the uptake rate of cellobiose was significantly lower in comparison to that of glucose, introducing a more efficient transport system for cellobiose companied with expressing high cellobiase activity in this strain will facilitate the utilization of this disaccharide.

Acknowledgements

This work was financially supported by the Natural Science Foundation of China (20706024), the "863" Program (2006AA020101, 2007AA10Z359), and the Program of Introducing Talents of Discipline to Universities (No. 111-2-06).

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