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Extracellular Secretion of β-glucosidase in Ethanologenic *E. coli* Enhances Ethanol Fermentation of Cellobiose

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Abstract Consolidated bioprocessing of lignocellulose for ethanol production is realized by expressing cellulase enzymes on ethanologenic strain. In this study, an ethanologenic *Escherichia coli* ZY81 was constructed by integrating pyruvate decarboxylase gene *pdc* and alcohol dehydrogenase gene *adhB* from *Zymomonas mobilis* into the genome of *E. coli* JM109 to obtain the capability of ethanol production. Then, the β-glucosidase gene *bglB* from *Bacillus polymyxa* was cloned and secretively expressed in *E. coli* ZY81. The recombinant strain *E. coli* ZY81/*bglB* showed an obvious activity of β-glucosidase in extracellular location with more than half in periplasmic space. EDTA was found to promote the release of the periplasmic proteins by approximately tenfold. *E. coli* ZY81/*bglB* utilized cellobiose as sole carbon source for ethanol production with 33.99 % of theoretical yield.

Keywords *E. coli* · Ethanol fermentation · β-glucosidase · Secretive expression · Surfactant

Introduction

Cellulase enzyme has been considered as the most expensive factor for cellulosic ethanol production [1]. To reduce the cost of cellulase enzyme [2, 3], consolidated bioprocessing (CBP) concept is proposed by integrating cellulase production, cellulose hydrolysis, and ethanol fermentation into one single cell [4–6]. For CBP process, the strain construction with dual functions of cellulase production and ethanol fermentation is the most important step [7–9].

Cellulase responsible for degradation of lignocellulose includes endoglucanase, exoglucanase, and β-glucosidase [10, 11]. Commercial cellulase enzymes are cocktails of endoglucanase, exoglucanase, and β-glucosidase in different ratio produced and by different strains. For CBP strains, cellulases are required not only to express sufficient cellulases for cellulose hydrolysis, but also to secrete extracellularly in order to touch the substrates [12–14]. Unlike endoglucanase and exoglucanase act on solid cellulose substrate, β-glucosidase catalyzes the hydrolysis of soluble cellobiose [15] which easily passes through the cell wall [16]. Therefore, either extracellular expression or transmembrane expression in periplasmic space of β-glucosidase retained within the cell wall may work well for hydrolyzing cellobiose into

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glucose. For endoglucanase or exoglucanase, the proteins have to be expressed completely across the membrane and cell wall into the hydrolysate to take into effect, because cellulose fragments are not able to across the cell wall to reach the membrane [14]. Therefore, extracellular expression of β-glucosidase on an ethanologenic strain should be tried as the first step for construction of a practical CBP strain to reduce the use of β-glucosidase in the cellulase components.

In this study, an *Escherichia coli* ZY81 strain was constructed by integrating pyruvate decarboxylase gene (*pdc*) and alcohol dehydrogenase gene (*adhB*) from *Zymomonas mobilis* into the genome of *E. coli* JM109 to obtain the capability of ethanol production. Then the β-glucosidase gene *bglB* from *Bacillus polymyxa* was heterologously expressed in the *E. coli* ZY81. β-glucosidase was designed to be secreted extracellular through the functional signal peptide *NprB* [17], and the results indicated that *bglB* gene was correctly expressed in the host strain and the extracellular β-glucosidase activity was observed. To increase the secretion of β-glucosidase and promote the ethanol fermentation performance of the recombinant *E. coli* ZY81/*bglB*, 0.5 % EDTA was used as the surfactant.

Materials and Methods

Strains and Plasmids

The strains and plasmids used in this study are shown in Table 1. *E. coli* JM109 was used to construct the ethanologenic *E. coli* ZY81. pKD46 was used in the RED recombinant of the genome. Plasmid pCP20 was used to eliminate the antibiotic resistance gene.

Table 1 Strains and plasmids used in this study

	Genotype and/or salient characteristics	Sources
Strains		
E. coli JM109		Novagen
E. coli DH5α		
Z. mobilis ZM4	Wild-type strain, ATCC 31821	ATCC
B. polymyxa	Wild-type strain, CGMCC 1.794	CGMCC
E. coli ZY81	JM109 *pfl*::P_{tac}-*pdc-adhB*	In this study
Plasmids		
pUC19	Vector	
pGEX-4 T-1	Template plasmid, carrying P_{tac} promoter gene	Pharmacia
pMD19-T	Cloning T-vector	Takara
pKD4	Template plasmid, carrying kanamycin-resistance gene (Kan^R) flanked by the recognition sites (FRT sites) of the yeast FLP recombinase in direct repeats	Datsenko et al. (2000)
pKD46	Helper plasmid, carrying bacteriophage λ-Red recombinase genes under the control of arabinose-inducible ParaBAD promoter (*araBp-gam-bet-exo*)	Datsenko et al. (2000)
pCP20	Helper plasmid, carrying FLP recombinase gene under the control of an temperature sensitive promoter	Peter et al. (1995)

Reagents and Chemicals

Restriction enzymes were purchased from Fermentas (Vilnius, Lithuania). T4 DNA ligase and PrimerSTAR HS DNA polymerase used in PCR were purchased from Takara (Dalian, China). Tryptone and yeast extract were from Oxoid (Cambridge, UK). Bacterial DNA kit was from Omega Bio-Tek (Norcross, GA, USA). PCR purification kit, plasmid mini kit, and gel extraction kit were from Sangong Biotech (Shanghai, China). All other chemicals used in this study were purchased from Sinopharm Chemical Reagent (Shanghai, China).

Gene Cloning

All the primers used were shown in the Table 2. The *bglB* gene fragment was amplified from the genome of B. *polymyxa* CGMCC 1.794. The PCR product was cleaved, gel-purified, and ligated into pUC19-P_{43}-*NprB* to give the plasmid pUC19-*bglB* (Fig. 1b). The *bglB* gene fragment was located at the downstream of the promoter P_{43} and the signal peptide *NprB*. The *pdc* and *adhB* gene fragments were amplified from Z. *mobilis* genomic DNA. P_{tac} was amplified from pGEX-4 T-1. The kanamycin resistance gene was amplified from pKD4. The plasmid pUC19-*bglB* was introduced into the ethanologenic *E. coli* ZY81 to give the recombinant *E. coli* ZY81/*bglB*.

RED Recombination of Ethanologenic *E. coli* Strain

RED recombinant method [18] was used for the construction of *E. coli* ZY81 (Fig. 1a). The plasmids and primers used in RED recombinant are shown in Tables 1 and 2, respectively. The primers Ol1 and Ol2 were used to get P_{tac} fragment and Ol3 and Ol4 to get *pdc-adhB* fragment

Table 2 Primers used in this study

Fragments	Primers	Sequence (5′–3′)
pdc	Pdc1	GGAATTCCATATGAGTTATACTGTCGGTACCTA (*Nde* I)
	Pdc2	ACGCGTCGACTAGAGGAGCTTGTTAACAGG (*Sal* I)
adhB	Adh1	ACGCGTCGACGAGGTTATAGCTATGGCTTCTTCAACTTT (*Sal* I)
	Adh2	TCCGCTCGAGTTTTCCTGTTTTGAAATTAG (*Xho* I)
kan	Kan1	CGCAGTAAATAAAAAATCCACTTAAGAAGGTAGGTGTTACGT CTTGAGCGATTGTGTAGG
	Kan2	ACTCAATAAAGTTGCCGCTTTACGGGGAAATTAGAACATT CTCGAGTTAAGGTTTAACGG (*Xho* I)
P_{tac}-*pdc-adhB*	Ol1	GGGAATTCCATATGGGAGCTGTTGACAATTA (*Nde* I)
	Ol2	GTACCGACAGTATAACTCATGAATACTGTTTCCTGTGTGA
	Ol3	TCACACAGGAAACAGTATTCATGAGTTATACTGTCGGTAC
	Ol4	CCGCTCGAGGTTTTCCTGTTTTGAAATTAGA (*Xho* I)
kan-P_{tac}-*pdc-adhB*	RED1	CGCAGTAAATAAAAAATCCACTTAAGAAGGTAGGTGTT ACGTCTTGAGCGATGTGTAGG
	RED2	ACTCAATAAAGTTGCCGCTTTACGGGGAAATTAGAACATT CTCGGTTAAGGTTTAACGG
bglB	bgl1	CGGGATCCATGCGCAACTTGACCAAGAC (*Bam*H I)
	bgl2	CCCAAGCTTTTAAAACCCGTTCTTCGCC (*Hin*d III)

The underlined sections mean the restriction enzyme sequences indicated by the parentheses

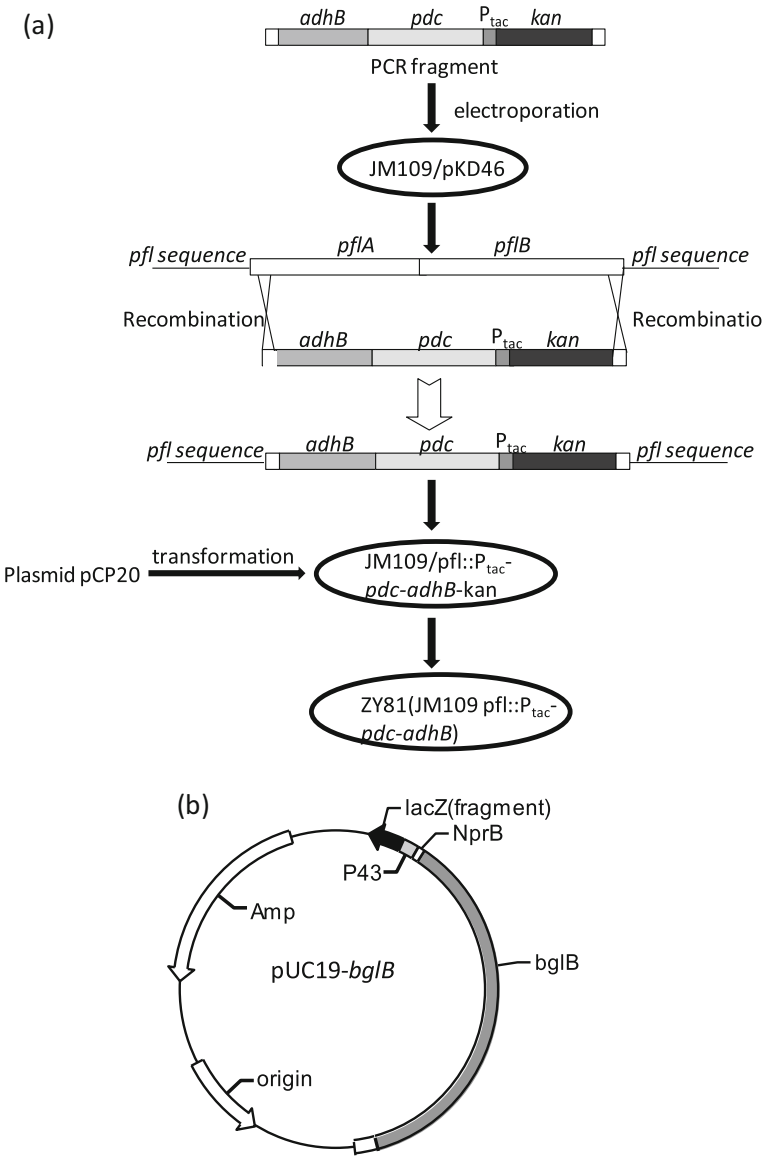


Fig. 1 Construction of the recombinant strain *E. coli* ZY81/*bglB*

for overlap PCR. Then Ol1 and Ol4 were used to get the production P_{tac}-*pdc-adhB* fragment by overlap PCR. This fragment was located at the downstream of the kanamycin resistance gene by restriction enzyme site. Primers RED1 and RED2 which own the homologous pyruvate formate lyase (*pfl*) gene sequence of *E. coli* were used to get the insertion fragment kan-P_{tac}-*pdc-adhB* by PCR.

pKD46 was introduced into *E. coli* JM109 by calcium chloride transformation method to get the strain *E. coli* JM109/pKD46. Then the insertion fragment kan-P_{tac}-*pdc-adhB* was introduced into the *E. coli* JM109/pKD46 through electroporation method. The positive clone

can be selected from the LB plate with 100 μg/mL kanamycin at 30 °C, and the pKD46 can be eliminated by incubating the recombinant strain at 37 °C. The kanamycin resistance gene can be eliminated by introducing the pCP20 into the recombinant strain by the recognition of FLP sequence to give the ethanologenic *E. coli* ZY81.

Enzyme Activity Assay

The recombinant strain was 1 % inoculated and cultivated in 20 mL LB medium with 100 μg/mL ampicillin at 37 °C, 220 rpm for 24 h. Then the cells were centrifuged at 10,000 rpm for 5 min at 4 °C, and the supernatant was collected. The proteins in the supernatant were precipitated by using ammonium sulfate at 60 % (*w*/*v*) saturation and redissolved in 1 mL 50 mM citric acid buffer (pH 6.0) to give the crude enzyme solution. The enzyme extract in periplasm and cytoplasm was got by osmotic shock method [19]. The cell was suspended in STE buffer (20 % sucrose, 30 mM Tris-HCl (pH 8.0), 1 mM EDTA) and incubated on ice for 10 min and then centrifuged at 8,000 rpm for 10 min. Discard the supernatant and resuspended the cell by water, incubated on ice for 10 min and centrifuged at 8,000 rpm for 10 min to obtain the periplasmic fraction in supernatant. The cell pellet was crushed to make the cytoplasmic fraction.

The enzymatic activity of *bglB* was determined by using *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG) as the substrate. The *p*NPG-hydrolyzing activity was estimated by incubating 1 mL enzyme extract with 1 mL of 8 mM *p*-NPG in 50 mM citric acid buffer (pH 6.0) at 30 °C for 10 min. 1 mL 0.5 M Na_2CO_3 was added to stop reaction. The optical density at 405 nm ($OD_{405\ nm}$) of the solution was determined to obtain the release of *p*-nitrophenol (*p*-NP). The enzyme activity was expressed in unit per gram DCW, and one unit (U) was defined as the 1 μmol of *p*-nitrophenol released per minute.

Fermentation and Medium

E. coli ZY81/*bglB* and other control strains were 1 % inoculated and cultivated in 20 mL LB medium with 100 μg/mL ampicillin at 37 °C, 220 rpm in the flask. When the strain grew to the exponential phase (OD_{600}=0.4–0.6), 0.1 mM IPTG was added. The surfactant was added at 12 h. The component of the fermentation broth was assayed by HPLC (LC-20 AD, refractive index detector RID-10A, Shimadzu, Kyoto, Japan) fitted with a Bio-Rad Aminex HPX-87H column at 65 °C. The mobile phase was 5 mM H_2SO_4 at 0.6 mL/min.

LB medium contained 10 g/L tryptone, 10 g/L NaCl, and 5 g/L yeast extract. M9 medium contained 6 g/L Na_2HPO_4, 3 g/L K_3PO_4, 0.5 g/L NaCl, 1 g/L NH_4Cl, 2 mM $MgSO_4$, and 0.1 mM $CaCl_2$.

Results and Discussion

Secretive Expression of β-glucosidase by the Recombinant *E. coli* ZY81/*bglB*

The β-glucosidase activity of the recombinant strain *E. coli* ZY81/*bglB* was assayed by using *p*NPG as the substrate. Two *E. coli* strains, *E. coli* JM109/pUC19 and *E. coli* ZY81/pUC19, were used as the controls. Figure 2 shows that the high whole cell β-glucosidase activity at 4.64 U/g DCW was detected in the recombinant *E. coli* ZY81/*bglB*, while the two control strains were approximately 0.02 U/g DCW. This result indicates that *bglB* gene was correctly expressed in *E. coli* ZY81.

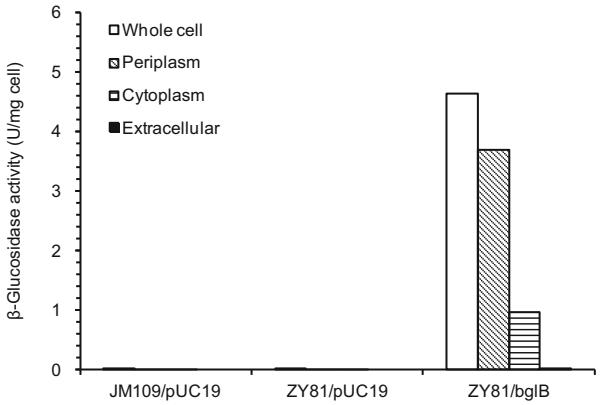


Fig. 2 The enzyme activity of *E. coli* ZY81/*bglB*. The glucosidase activities were determined using *p*NPG substrate at 37 °C for 10 min and strains *E. coli* JM109/pUC19 and *E. coli* ZY81/pUC19 were used as the control

In *E. coli* ZY81/*bglB*, 79.02 % of the expressed β-glucosidase was located in the periplasmic place and its activity reached 3.69 U/g DCW, while the β-glucosidase activity in the cytoplasm was 0.95 U/g cell and counted for 20.47 % of the total whole cell activity. The extracellular β-glucosidase activity was 0.03 U/g DCW, approximately 0.64 % of total protein. The results indicated that the signal peptide *NprB* used for secretion of the *bglB* helped the most of the heterologous protein transport onto periplasmic space. The β-glucosidase in the periplasmic space is still able to contact cellobiose and hydrolyze it to glucose. So we measured the cellobiose utilization ability of *E. coli* ZY81/*bglB*.

Cellobiose Utilization and Ethanol Fermentation by *E. coli* ZY81/*bglB*

E. coli ZY81/*bglB* was fermented in M9 medium containing 10 g/L cellobiose to test whether the secretive expression of *bglB* could help the recombinant strain utilized the cellobiose. The result showed that *E. coli* ZY81/*bglB* behaved the ability of cellobiose utilizing and 2.5 g/L cellobiose was consumed in 48 h (Fig. 3) and grew to $OD_{600\ nm}$=1.94, but no ethanol was produced. The two control strains showed slightly cell growth ($OD_{600\ nm}$ <0.2) with no cellobiose utilization and ethanol production were observed. This indicated that the recombinant *E. coli* ZY81/*bglB* could utilize cellobiose through the expression of *bglB*.

M9 minimal medium only contained simple inorganic salts (Na_2HPO_4, K_3PO_4, NaCl, NH_4Cl, $MgSO_4$, and $CaCl_2$) and usually used for *E. coli* culture with nutrients supplementation such as yeast extract, peptone, thiamine, etc. [20–32]. These rich nutrient components are important to support the anabolic and catabolic pathways of *E. coli*. The previous results show that *E. coli* recombinants able to produce ethanol occasionally are cultured in M9 medium, but the above nutrients are supplemented [24, 32]. In this study, the M9 medium was used in its simple inorganic ingredients without addition of complicated nutrients such as yeast extract and peptone for the purpose of accurate measurement of cellobiose decomposition to glucose. Under this restricted nutrient condition, the metabolic flux of pyruvate, the precursor of ethanol, tended to anabolism only for cell growth, instead of the secondary metabolite generation such as ethanol [33]. On the other hand, the acetaldehyde dehydrogenase enzyme (ADHB) in the ethanol formation pathway is NADH dependent when it converts acetaldehyde to ethanol. The lack of carbon source and nutrients could also limit the NADH generation and then affect the activity of ADHB [34]. In this circumstance, both *E. coli* ZY81/*bglB* and the

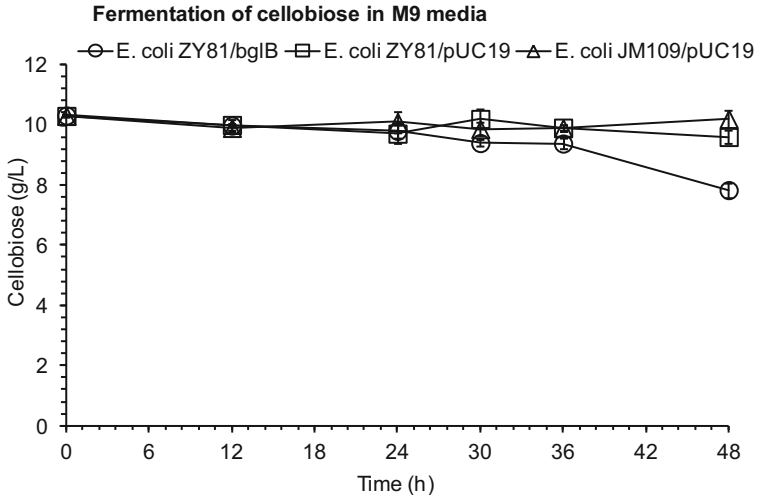


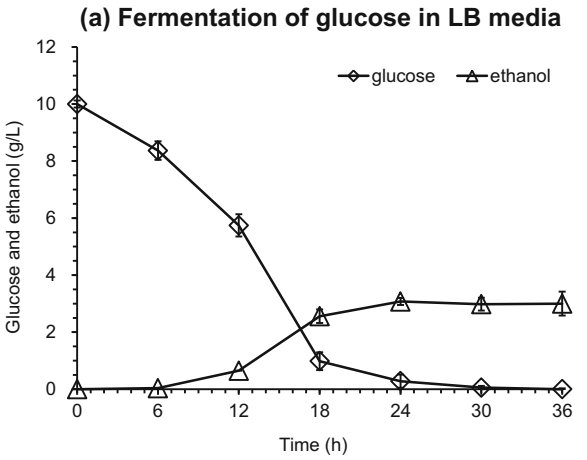
Fig. 3 The fermentation by *E. coli* ZY81/*bglB* in M9 media with 10 g/L cellobiose. *E. coli* JM109/pUC19 and *E. coli* ZY81/pUC19 were used as the control strains and cultivated in 20 mL LB medium with 100 μg/mL ampicillin at 37 °C, 220 rpm in flask. (lt for *E. coli* ZY81/*bglB*, (lB for *E. coli* ZY81/pUC19, and (*white triangle*) for *E. coli* JM109/pUC19. None of these strains produced ethanol

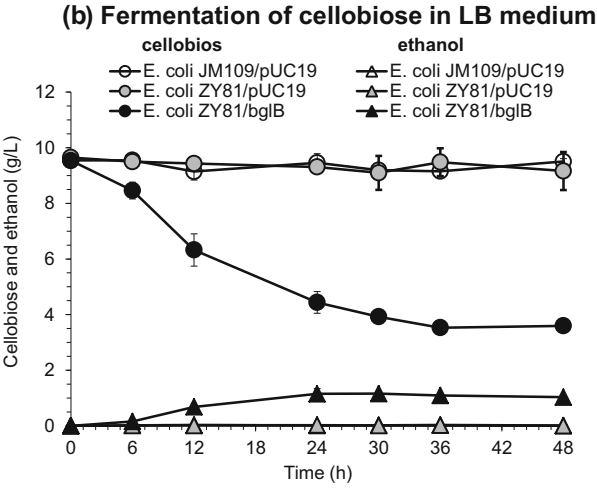
control *E. coli* ZY81/pUC19 strains were unable to produce ethanol in M9 medium with 10 g/L cellobiose.

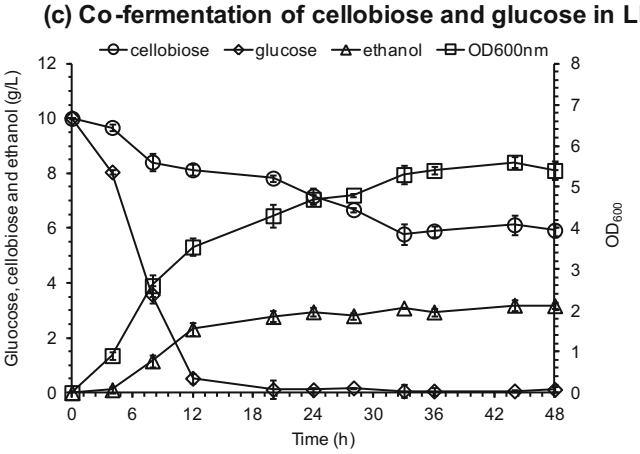
The CBP ability of the recombinant *E. coli* ZY81/*bglB* was tested to produce ethanol by using cellobiose as the carbon source in LB medium. Figure 4a shows the ethanol fermentation of *E. coli* ZY81/*bglB* by using 10 g/L glucose and 3.08 g/L ethanol was produced within 36 h, achieving 61.6 % of the theoretical yield. Figure 4b shows the ethanol fermentation of *E. coli* ZY81/*bglB* by using 10 g/L cellobiose in LB medium. Two *E. coli* strains, *E. coli* JM109/pUC19 (without ethanol fermentation capacity) and *E. coli* ZY81/pUC19 (without β-glucosidase activity), were used as the control strains. All the three strains grew well but only *E. coli* ZY81/*bglB* utilized 5.94 g/L cellobiose and produced 1.00 g/L ethanol, achieving 33.99 % of the theoretical yield. Figure 4c shows the ethanol fermentation of *E. coli* ZY81/*bglB* by using the mixed sugars, 10 g/L glucose and 10 g/L cellobiose, to test whether the presence of glucose affects the utilization of cellobiose. The results show that 1.79 g/L cellobiose was utilized within 12 h when glucose was present. Compared to the fermentation by using 10 g/L cellobiose only (3.21 g/L cellobiose within 12 h), the cellobiose consumption rate of the co-fermentation was decreased by approximately 44 %.

Effect of Surfactant Addition on Enzyme Secretion and Ethanol Fermentation

The recombinant strain *E. coli* ZY81/*bglB* demonstrated the secretive expression of β-glucosidase and utilization of cellobiose for ethanol fermentation, but the complete release of β-glucosidase to the culture is still limited. In order to enhance the release of proteins from periplasm space into the medium, the addition of surfactants was used to increase the permeability of cell membrane and promote the secretion of BglB [35]. Different surfactants including 2 % glycine, 0.5 % EDTA, and 0.1 % Tween-80 [36, 37] were added into the growth media of *E. coli* ZY81/*bglB* and then test the secretion capacity for β-glucosidase. The addition of the surfactants may affect the β-glucosidase activity, so a preliminary experiment


(a) Fermentation of glucose in LB media
glucose
ethanol
Glucose and ethanol (g/L)
Time (h)


(b) Fermentation of cellobiose in LB medium
cellobios
ethanol
E. coli JM109/pUC19
E. coli ZY81/pUC19
E. coli ZY81/bglB
Cellobiose and ethanol (g/L)
Time (h)


(c) Co-fermentation of cellobiose and glucose in LB medium
cellobiose
glucose
ethanol
OD600nm
Gluocose, cellobiose and ethanol (g/L)
OD600
Time (h)

◀ **Fig. 4** The CBP fermentation by *E. coli* ZY81/*bglB*. **a** Fermentation with 10 g/L glucose in LB medium. *White diamond* indicate glucose concentration, and *white triangle* indicate ethanol concentration; **b** fermentation with 10 g/L cellobiose in LB medium, *white circle* for cellobiose concentration, and *triangle* for ethanol concentration. *White symbol* for JM109/pUC19, *gray symbol* for *E. coli* ZY81/pUC19, and *black symbol* for *E.coli* ZY81/*bglB*; **c** co-fermentation in LB medium with 10 g/L cellobiose and 10 g/L glucose. *White square* for cell growth, *white diamond* for glucose concentration, *white circle* for cellobiose concentration, and *white diamond* for ethanol concentration

was did to ensure the activity variation of BglB with the surfactant (data not shown). Approximately 10 % increase was observed when EDTA was added, while glycine and Tween-80 just affected the activity slightly.

Figure 5a and Table 3 show that when pNPG was used as the substrate, the addition of EDTA and Tween-80 significantly reduced the content of β-glucosidase in the periplasmic space from 79.02 to 51.80 % and 35.77 %, respectively, while glycine addition did not affect its content in the periplasmic space obviously. On the other hand, the secretion of β-glucosidase into the extracellular medium by the addition of EDTA, glycine, and Tween-80 was increased to 6.69, 4.01, and 2.21 % from 0.64 %, respectively. EDTA showed the most obvious effect for secreting about tenfold. Although EDTA could increase the activity of BglB

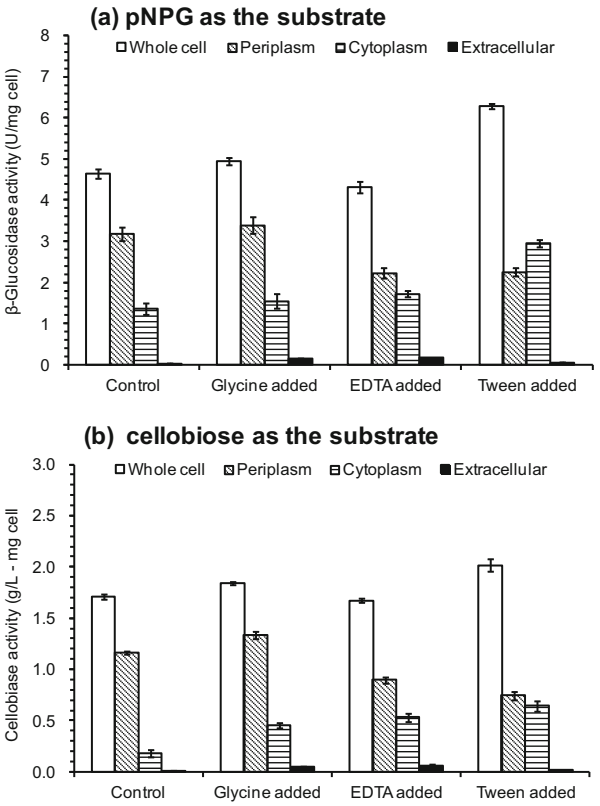


Fig. 5 The enzyme activity of *E.coli* ZY81/*bglB* when surfactant was added. 2 % glycine, 0.5 % EDTA, and 0.1 % Tween-80 was added separately as the surfactant. **a** The β-glucosidase activity of BglB; **b** the cellobiase activity of BglB. The surfactant was added at 12 h after the inoculation. The β-glucosidase activities were determined using *p*NPG substrate at 37 °C for 10 min; the cellobiase activities were determined using cellobiose substrate at 37 °C for 1 h. The recombinant strain incubated without addition of surfactant was used as the control

Table 3 Secretive proportion of β-glucosidase activities when surfactants were added

Secretive proportion	Control (%)	Glycine added (%)	EDTA added (%)	Tween added (%)
*p*NPG	0.64	4.01	6.69	2.21
Cellobiose	0.62	3.28	6.38	2.13

The surfactants were added at 12 h after the inoculation. The β-glucosidase activities were determined using *p*NPG substrate at 37 °C for 10 min; the cellobiase activities were determined using cellobiose substrate at 37 °C for 1 h. The recombinant strain incubated without addition of surfactant was used as the control

by 10 %, the promotion of extracellular activity of *E. coli* ZY81/*bglB* was much higher which could be ensured that it was increased by the addition of EDTA.

Similarly, when cellobiose was used as the substrate, the addition of EDTA reduced the periplasmic content of β-glucosidase from 68.14 to 53.57 %, and increased the extracellular activity from 0.62 to 6.38 % (Fig. 5b and Table 3).

Figure 6 shows the ethanol fermentation of *E. coli* ZY81/*bglB* using cellobiose with and without EDTA addition at 12 h of the fermentation. The results indicated that the cell growth of *E. coli* ZY81/*bglB* reached $OD_{600\ nm}$ at 4.62 in the fermentation with EDTA addition, while the $OD_{600\ nm}$ reached 5.83 in the fermentation without EDTA. Approximately 20 % decrease of cell growth was observed with the addition of EDTA, indicating that EDTA increased the permeability of cell membrane to release more proteins to extracellular medium, but might decrease the tolerance of the strain and inhibit the cell growth in a certain range. After 72 h fermentation, *E. coli* ZY81/*bglB* utilized 9.01 g/L cellobiose and produced 1.39 g/L ethanol with the yield of 31.03 % at 48 h when EDTA was added, approximately 1.85 g/L more cellobiose was consumed and 0.88 g/L more ethanol was produced. Although the growth of *E. coli* ZY81/*bglB* was inhibited by EDTA, the ability of ethanol production and cellobiose increased obviously. Cellobiose could not be consumed completely in the fermentation, but in the case of EDTA addition, the residue cellobiose was 1.0 g/L and it was much less than the common fermentation of cellobiose which was 3.6 g/L in Figs. 4b and 2.9 g/L in Fig. 6. The increasing extracellular β-glucosidase by the addition of EDTA might enhance the probability of the contact between the enzyme and substrate, and reduced the minimum reaction

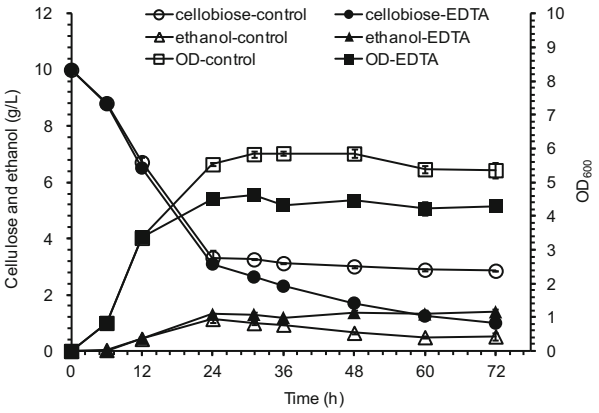


Fig. 6 The fermentation of *E.coli* ZY81/*bglB* when surfactant was added. The fermentation of recombinant without the addition of surfactant was used as the control. The *open symbols* indicate the control fermentation and the *closed* indicated the fermentation with the addition of EDTA. *Triangles* indicate the ethanol concentration, *square* the cell growth, and *round* the cellobiose concentration

concentration of cellobiose. The yield of ethanol did not change and kept the standard at 30 %, but the results suggest that EDTA helped the recombinant to utilize more cellobiose and produce more ethanol.

This study showed that *bglB* gene was successfully expressed and transported into the culture medium or cell location contacting cellobiose substrate in the ethanologenic *E. coli*. The results of cellobiose utilization enhancement by the *bglB* expression may reduce or even replace the enzyme usage of β-glucosidase, the major component of cellulase enzyme, in the hydrolysis and fermentation of lignocellulose materials for ethanol production. Therefore, *E. coli* ZY81/*bglB* could be considered as a preliminary consolidated bioprocessing strain (CBP) for its ethanol production and cellulose utilization, although the utilization is only effective for cellobiose in a limited conversion yield. This concept might be extended to *Saccharomyces cerevisiae*, *Z. mobilis*, or other ethanologenic strains with secretive expression of β-glucosidase or other cellulose enzymes, other than the surface display method.

Conclusion

In this study, ethanologenic *E. coli* ZY81/*bglB* which produced ethanol from cellobiose was constructed. About 0.64 % of the total β-glucosidase protein was secreted and the secretive proportion promotion could be enhanced tenfold by the addition of 0.5 % EDTA. ZY81/*bglB* could utilize glucose and cellobiose simultaneously, and when it fermented in LB medium with 10 g/L cellobiose, 5.94 g/L cellobiose was utilized with 1.00 g/L ethanol production achieving 33.99 % of theoretical yield. In the fermentation with the addition of 0.5 % EDTA at the same condition, the growth of the strain was inhibited about 20 % but cellobiose consumption and ethanol production increased obviously. This *E. coli* strain provided a preliminary CBP strain for converting cellobiose to bioethanol and a way for constructing a highly efficient and stable consolidated bioprocessing with secretive expression of cellulase enzymes.

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