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Secretive expression of heterologous β-glucosidase in *Zymomonas mobilis*

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Abstract

Background: *Zymomonas mobilis* is an efficient ethanol fermentation strain, but its narrow substrate range limits its fermentation in lignocellulose hydrolysate. As a potential consolidated bioprocessing (CBP) stain for bioethanol production, the ability of cellulose utilization was necessary. In this study, extracellular expression of β-glucosidase on *Z. mobilis* was studied as the first step for construction of a practical CBP strain to reduce the use of β-glucosidase in the cellulase components.

Results: The heterologous β-glucosidase from *Bacillus polymyxa* was expressed in the ethanologenic strain *Z. mobilis* (ZM4) and secreted extracellularly by an endogenous signal peptide and a fusion protein. The signal peptide SP1086 of the endoglucanase gene ZMO1086 from *Z. mobilis* was identified and facilitated 12 % of the endoglucanase encoded by ZMO1086 from *Z. mobilis* ZM4 and 16 % of the β-glucosidase encoded by *bglB* gene secreted out of the membrane of *Z. mobilis* ZM4. Another method for enhancement of the β-glucosidase secretion is to fuse the β-glucosidase encoded by *bglB* with the levansucrase encoded by *sacB* from *Z. mobilis* ZM4 to achieve the secretive expression. Its expression level was enhanced two times but only showed a 2 % secretion ratio in this situation.

Conclusions: The SP1086 signal peptide showed an obviously secreting capacity of the β-glucosidase protein. The fusion protein with SacB also showed the secretion effect, but it was less efficient.

Keywords: *Zymomonas mobilis*; β-glucosidase; Secretion; Signal peptide; Fusion protein

Background

Consolidated bioprocessing (CBP) integrates cellulase production, cellulose hydrolysis, and ethanol fermentation into one single unit; thus, the expensive cellulase enzyme production step is removed or the usage is reduced [1]. When cellulase enzyme is produced by CBP cells, the cellulase should be in effective contact with a solid cellulosic substrate for the hydrolysis to occur. To achieve this goal, not only the cellulase enzymes are sufficiently expressed but also the enzymes should be secreted extracellularly onto the periplasm space of the cell or directly to the extracellular medium. The practice of CBP concept was carried out on *Saccharomyces cerevisiae* strains by displaying cellulase enzymes on the surface [2].

Zymomonas mobilis is an efficient ethanol fermentation strain with low cell biomass generation, high-ethanol yield, and the tolerance to the toxicity of the final product, which makes it into a suitable candidate for CBP strain [3–5]. For the CBP process, unlike endoglucanase and exoglucanase acting on a solid cellulose substrate, β-glucosidase catalyzes the hydrolysis of soluble cellobiose, which easily passes through the glucan cell wall and contacts the enzymes on the periplasmic space of the cell [6, 7]. Therefore, either the extracellular expression into the fermentation broth or the trans-membrane expression in periplasmic space of β-glucosidase could work well for hydrolyzing cellobiose into glucose. In this work, the extracellular expression of β-glucosidase on *Z. mobilis* was tested as the first step for construction of a practical CBP strain to reduce the use of β-glucosidase in the cellulase components.

It has been tried to express cellulase genes in *Z. mobilis* [8–13], but their secretion was difficult; thus, a cellulase-expressing *Z. mobilis* without secretion is not able to function as a CBP cell in lignocellulose biorefining processes. Selecting native signal peptides or secretory pathways is a useful vehicle for enhancing the cellulase secretion on *Z. mobilis*. The signal peptide of the ZMO0130 gene encoding alkaline phosphatase and the ZMO0131 gene encoding hypothetical protein from *Z. mobilis* had been

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used to secret endocellulase and carboxymethyl cellulase from *Acidothermus cellulolyticus*, and the secretion ratio was more than 40 % of the total proteins [9]. To our knowledge, the secretion of heterologous β-glucosidase had not been successfully tested in *Z. mobilis.*

Several strategies for secretively expressing β-glucosidase in *Z. mobilis* ZM4 were investigated. First, a putative homologous endoglucanase gene *ZMO1086* [14] in *Z. mobilis* ZM4 was overexpressed to ensure the secretive function of its signal peptide SP1086 by using a strong enolase promoter P*eno*. Then, the signal peptide SP1086 of *ZMO1086* was used for secretively expressing a heterologous β-glucosidase gene *bglB* from *Bacillus polymyxa* in *Z. mobilis* ZM4. Finally, a fusion protein of β-glucosidase with levansucrase (SacB) from *Z. mobilis* ZM4 was also constructed to utilize its original secretion mechanism of levansucrase. The results showed that these methods significantly improved the secretion of β-glucosidase in *Z. mobilis* and provided a way for further practical *Z. mobilis*-based CBP strain.

Methods

Strains and plasmids

The strains used in this study are shown in Table 1. *Escherichia coli* DH5α was used in gene clone and plasmids construction. *Z. mobilis* ZM4 (ATCC 31821) was the host strain for the recombinant strain. Plasmid pHW20a is the shuttle vector with different gene expression [15].

Reagents and chemicals

The restriction enzymes were purchased from Fermentas (Vilnius, Lithuania). The T4 DNA ligase and PrimerSTAR HS DNA polymerase used in PCR were purchased from Takara (Dalian, China). The Tryptone and yeast extract were from Oxoid (Cambridge, UK). The Bacterial DNA kit was from Omega Bio-Tek (Norcross, GA, USA). The 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

The primers used in this study are shown in Table 2. The construction of recombinant plasmids is shown in Fig. 1. The *bglB* gene fragment was amplified from the genome of *B. polymyxa* CGMCC 1.794. The *ZMO1086* gene fragment was amplified from the genome of *Z. mobilis* ATCC 31821. *NprB* signal peptide fragments were amplified from the genome of *Clostridium thermocellum* DSM 1237.

The enolase promoter (P*eno*) PCR product was cleaved, gel purified, and ligated into pHW20a by the restriction enzyme site *EcoRI* and *BamHI* and then constructed the plasmid pHW20a-P*eno*. The *bglB*- and *ZMO1086*-gene-amplified fragment was located at the downstream of the promoter P*eno* by the same method and got the plasmid p*bglB* and p*ZM1086* (Fig. 1). Fragment P*eno*-SP1086 was amplified from p*ZM1086*, and the PCR product was cleaved, gel purified, and ligated into pHW20a by the restriction enzyme site *EcoRI* and *BamHI* to get the plasmid pHW20a-P*eno*-SP1086. Then, the *bglB* fragment was inserted and located at the downstream of the promoter P*eno* and signal peptide by the same method and got the plasmid pSP1086-*bglB* (Fig. 1). The P*eno-NprB* fragment was amplified by PCR and ligated into pHW20a by the restriction enzyme site *EcoRI* and *BamHI* and then

Table 1 Strains and plasmids used in this study

Strains	Genotype and/or salient characteristics	Sources
E. coli DH5α	F-, φ80dlacZΔM15, Δ(*lac*ZYA-*arg*F) U169, deoR, *rec*A1, *end*A1, *hsd*R17(*rk*-,*mk*+), *pho*A, *sup*E44, λ-, *thi*-1, *gyr*A96, *rel*A1	Invitrogen
Z. mobilis ZM4	Wild type strain, ATCC 31821	ATCC
B. polymyxa	Wild type strain, CGMCC 1.794	CGMCC
C. thermocellum	Wild type strain, DSMZ 1237	DSMZ

Table 2 Primers used in this study

Target fragments	Primers	Sequences (5′–3′)
bglB	bgl1	CGGGATCCATGCGCAACTTGACCAAGAC (*BamHI*)
	bgl2	CCCAAGCTTTTAAAACCCGTTCTTCGCC (*HindIII*)
P*eno*	pe1	CGGAATTCTCGGCCATTGTCTATACTC (*EcoRI*)
	pe2	CGGGATCCATCGAAACCTTTCTTAAAATC (*BamHI*)
P*eno-NprB*	pn1	CGGAATTCTCGGCCATTGTCTATACTC (*EcoRI*)
	pn2	CGGGATCCTGCAGCTGAGGCATGTGTT (BamHI)
ZMO0186	zc1	CGGGATCCATGACCTATAGTCGTCGTTTTATC (*BamHI*)
	zc2	CCCAAGCTTAAAGATTTAGCGGTTATGCG (*HindIII*)
P*eno*-SP0186	pes1	CGGAATTCTCGGCCATTGTCTATACTCCAGTTAC (*EcoRI*)
	pes2	CGGGATCCGGCCGCTTTACCCTTCCG (*BamHI*)
sacB-bglB	p1	CGGGATCCATGTTGAATAAAGCAGGCATTG (*BamHI*)
	p2	ATAAAGGTATTCTCGCTCATACCACCACCACC ACCACCACCTTTATTCAATAAAGACAGGGCT
	p3	TGTCTTTATTGAATAAATAAGGTGGTGGTGGTGG TGGTGGTATGAGCGAGAATACCTTTATATTTC
	p4	CCAAGCTTTTAAAACCCGTTCTTCGCC (*HindIII*)

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

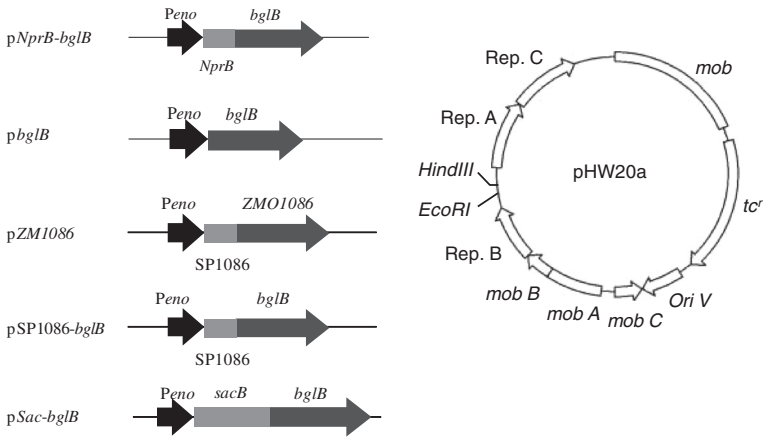

Fig. 1 Construction of plasmids. Construction of plasmids p*NprB-bglB* (express β-glucosidase gene *bglB* with promoter P*eno* and signal peptide *NprB*), p*bglB* (express β-glucosidase gene *bglB* with promoter P*eno*), p*ZM1086* (express *ZMO1086* gene with promoter P*eno*), pSP1086-*bglB* (express β-glucosidase gene *bglB* with promoter P*eno* and signal peptide SP1086), and p*SacB-bglB* (express fusion protein SacB-bglB with promoter P*eno*)

constructed the plasmid pHW20a-P*eno-NprB*. The *bglB* fragment was located at the downstream of the promoter P*eno* and signal peptide *NprB* and got the plasmid p*NprB-bglB* (Fig. 1).

The primers p1 and p2 were used to get the *sacB* fragment and p3 and p4 to get the *bglB* fragment for the overlap PCR. Then, p1 and p4 were used to get the production *sacB-bglB* fragment by the overlap PCR. Then, the overlap fragment was located at the downstream of the promoter P*eno* to get the plasmid p*Sac-bglB* (Fig. 1).

Enzyme activity assay

The recombinant strain was 1 % inoculated and cultivated in 20 mL RM medium with 20 μg/mL tetracycline and 30 μg/mL nalidixic acid, stationary culture at 30 °C for 24 h. The cells were centrifuged at 10,000×*g* for 5 min at 4 °C, and the supernatant was collected. The proteins in the supernatant were precipitated using ammonium sulfate at 60 % (*w*/*v*) saturation and redissolved in 1 mL 50 mM buffer (pH 6.0) to give the extracellular enzyme solution. The enzyme extract in periplasm and cytoplasm was obtained by osmotic shock method [16]. 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 8000 rpm for 10 min. The supernatant was discarded and the cell resuspended by water. And then, the cell was incubated on ice for 10 min again and centrifuged at 8000 rpm for 10 min to obtain the periplasmic fraction in supernatant. The cell pellet was crushed to make the cytoplasmic fraction.

The CMC cellulolytic activity was determined by using carboxymethyl cellulose (CMC) as the substrate. The activity was measured by incubating 1 mL of enzyme extract with 1 mL of 10 g/L CMC in 50 mM Tris–HCl buffer (pH 6.0) at 30 °C for 1 h. The reducing sugars released were estimated by using the dinitrosalicylic acid (DNS) reagent. The enzyme activity was expressed in U/g dry cell weight (DCW), and one unit (U) was defined as the 1 g/L reducing sugars released per min.

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 of enzyme extract with 1 mL of 8 mM *p*-NPG in 50 mM citric acid buffer (pH 6.0) at 37 °C for 15 min. To stop the reaction, 1 mL 0.5 M Na_2CO_3 was added. The optical density at 405 nm (OD_{405nm}) of the solution was determined to obtain the release of *p*-nitrophenol (*p*-NP). The enzyme activity was expressed in U/g DCW, and one unit (U) was defined as the μmol of *p*-nitrophenol released per min.

Fermentation and media

The RM medium contained 10 g/L yeast extract, 2 g/L KH_2PO_4, and 20 g/L glucose. The glucose can be replaced to some other carbon source in fermentation.

Z. mobilis and recombinant strains were 1 % inoculated and cultivated in 20 mL RM medium with 30 μg/mL nalidixic acid (20 μg/mL tetracycline was used additionally for the recombinant strains), stationary culture at 30 °C in the flask. The component of the fermentation broth was assayed by HPLC (LC-20AD, refractive index detector RID-10A, Shimadzu, Kyoto, Japan) fitted with a Bio-Rad Aminex HPX-87H column (Hercules, CA, USA) at 65 °C. The mobile phase was 5 mM H_2SO_4 at 0.6 mL/min.

Results and discussion

Expression of the homogenous cellulase gene ZMO1086 in *Z. mobilis*

The *ZMO1086* gene is a putative cellulolytic gene with an endogenous signal peptide SP1086 according to the *Z.*

mobilis ZM4 genome annotation, providing the carboxymethyl cellulose (CMC) cellulolytic activity but with very low activity [9–11]. To test the secretive expression performance of SP1086, the ZMO1086 expression was enhanced by replacing its original promoter with P*eno*, a strong promoter in *Z. mobilis* ZM4. The recombinant plasmid p*ZM1086* was constructed to overexpress the *ZMO1086* gene in *Z. mobilis* ZM4 (Fig. 1). The *ZMO1086* gene fragment was located at the downstream of the promoter P*eno*, and the signal peptide SP1086 was included in the CDS of *ZMO1086* at the 5′ end. The recombinant plasmid was introduced into *Z. mobilis* by conjugation method to give a recombinant *Z. mobilis* ZM4/p*ZM1086*.

The CMC cellulolytic activity of the recombinant *Z. mobilis* ZM4/p*ZM1086* was measured by using CMC as the substrate. Figure 2 shows that the whole cell CMC activity of *Z. mobilis* ZM4/p*ZM1086* was at 6.50 U/g dry cell weight (DCW), approximately 25 folds greater than that of the control strain which was only 0.27 U/g DCW. Approximately 90 % of the CMC activity was located in the cytoplasm (5.36 U/g DCW), while the activity in the periplasmic space and extracellular medium only accounted for 6.31 % (0.41 U/g DCW) and 5.90 % (0.38 U/g DCW) of the whole cell activity, respectively. The results indicated that the secretive expression of *ZMO1086* using the signal peptide in *Z. mobilis* ZM4 was successful although the secretion ratio was not satisfactory. In the previous CMCase expression works, the absence of a signal peptide in *Z. mobilis* led to the absence of CMC activity in the extracellular space [11, 12].

Secretive expression using the signal peptide SP1086

From the expression of *ZMO1086*, we found that the cellulase can be secretively expressed in a restricted form. The signal peptide SP1086 demonstrated its ability of transporting the cellulase protein from cytoplasm onto the extracellular space of *Z. mobilis* ZM4. In the next step, SP1086 was used in the secretive expression of the heterologous β-glucosidase gene *bglB* from *B. polymyxa* CGMCC 1.794 in *Z. mobilis* ZM4. The *bglB* fragment was inserted at the downstream of promoter P*eno* and the signal peptide SP1086 (Fig. 1), then plasmid was introduced into *Z. mobilis* ZM4 by conjugation to give a recombinant *Z. mobilis* ZM4/pSP1086-*bglB*.

The β-glucosidase activity of the recombinant *Z. mobilis* ZM4/pSP1086-*bglB* was measured by using *p*NPG as the substrate. Figure 3 showed that the β-glucosidase activity of the whole cell was 2.48 U/g DCW, and the control strain was approximately 0.16 U/g DCW, indicating the correct expression of *bglB* in *Z. mobilis* ZM4. Approximately 16 % of the β-glucosidase enzyme was secreted. The β-glucosidase activity in the cytoplasm was 1.91 U/g DCW, accounting for more than 80 % of the total protein. The β-glucosidase activity in the periplasm was 0.35 and 0.05 U/g DCW in the extracellular medium and accounted for 14.11 and 2.02 % of total proteins, respectively. Compared to the secretive expression of *ZMO1086* using the same promoter and signal peptide, the proportion in the periplasmic space was more than doubled from 6.31 to 14.11 %, but the extracellular proportion of β-glucosidase was only half of ZMO1086.

Although the secretion ratio of β-glucosidase was still low, it was a significant improvement in *Z. mobilis* ZM4 compared to the *bglB* expression by the signal peptide *NprB* from *C. thermocellum*, which was almost no secretion (data not shown) although *NprB* was effective in other strains [17, 18]. The constructed plasmids p*NprB-bglB* (which attempted to express *bglB* with promoter P*eno* and signal peptide *NprB*) and p*bglB* (which attempted to express *bglB* with promoter P*eno*) (Fig. 1) were also not successful, except the present signal peptide SP1086. It is speculated that the expression and transportation of cellulase enzymes may affect the metabolism of the *Z. mobilis*

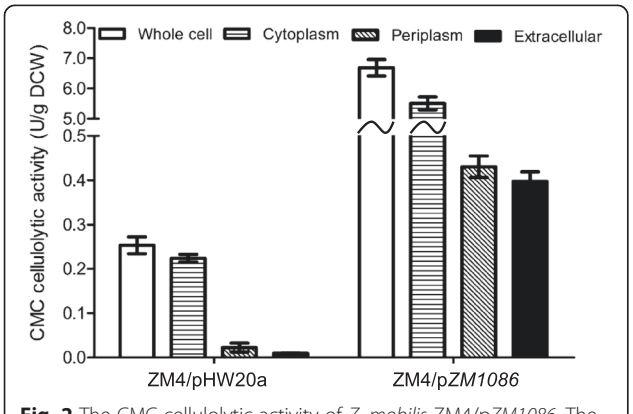

Fig. 2 The CMC cellulolytic activity of *Z. mobilis* ZM4/p*ZM1086*. The activity was determined by using CMC as the substrate at 30 °C for 1 h. The strain *Z. mobilis* ZM4/pHW20a was used as the controls

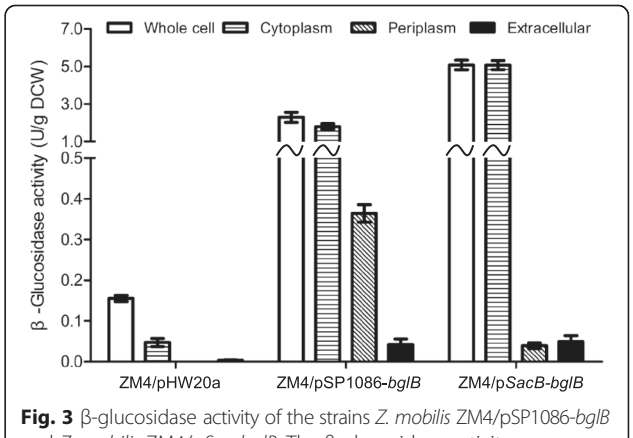

Fig. 3 β-glucosidase activity of the strains *Z. mobilis* ZM4/pSP1086-*bglB* and *Z. mobilis* ZM4/p*Sac-bglB*. The β-glucosidase activity was determined using *p*NPG as substrate at 37 °C for 15 min. The strain *Z. mobilis* ZM4/pHW20a was used as the control

cells due to their rigorous peptidoglycan cell wall under the strong influence of cellulase existence. The signal peptide from *Z. mobilis* ZM4 may mediate the transportation of cellulase enzyme out of the cytoplasm and distribute the cellulase in a unique way without disturbance or injury to the sensitive cell wall of *Z. mobilis* cells.

Secretive expression by fusion protein

Levansucrase gene *sacB* from *Z. mobilis* ZM4 is responsible for the sucrose hydrolysis into glucose and fructose in *Z. mobilis* ZM4 strain. Since sucrose is unable to transport through the cell membrane of *Z. mobilis* [19], the enzyme should be located in a position of the membrane and cell wall in contact with sucrose. From the published genome, no signal peptide for levansucrase was reported or annotated in the genome information of *Z. mobilis* ZM4. It is hypothesized that the protein structure of levansucrase encoded by *sacB* behaves with a trans-membrane property thus ensuring contact with the soluble sucrose for the hydrolysis. In this study, a fusion protein SacB-bglB linked by seven glycine groups was constructed by taking advantage of the secretion mechanism of SacB. The plasmid p*Sac-bglB* was constructed and introduced into *Z. mobilis* ZM4 by conjugation to give the recombinant *Z. mobilis* ZM4/p*Sac-bglB*.

The β-glucosidase activity of the recombinant ZM4/p*Sac-bglB* was measured by using *p*NPG as the substrate. Figure 3 showed that the whole expression of β-glucosidase was 5.26 U/g DCW, approximately two folds greater than that of *Z. mobilis* ZM4/pSP1086-*bglB*. However, the secretive proportion was not obviously promoted. The β-glucosidase activity was 0.05 and 0.06 U/g DCW in the periplasm space and in the extracellular medium, accounting for 1.0 and 1.1 % of the whole cell activity, respectively. The result indicated that the fusion protein strategy was not feasible for BglB secretion in *Z. mobilis* ZM4.

Conclusively, *Z. mobilis*-ZM4/p*Sac-bglB*-expressing fusion protein showed the highest β-glucosidase expression level in the cell, while *Z. mobilis* ZM4/pSP1086-*bglB* secreted most of the enzyme proteins into the periplasm up to 0.35 U/g DCW. The extracellular activities of two recombinants were similar, near to 0.05 U/g DCW. The use of the signal peptide SP1086 showed the better effect in secretive expression, and the fusion protein showed the highest expressive level of BglB but with less enzyme proteins secreted into periplasmic space.

The substrate-utilizing ability of the recombinants *Z. mobilis* ZM4/pSP1086-*bglB* and *Z. mobilis* ZM4/p*Sac-bglB* was tested by using 10 g/L cellobiose and 10 g/L glucose as the carbon source with *Z. mobilis* ZM4 harboring the empty pHW20a used as the control strain. Both the recombinants utilized glucose at a similar rate, and all the glucose was consumed in the fermentation, and the growth of *Z. mobilis* ZM4/pSP1086-*bglB* was faster with a slightly higher DCW at the end of fermentation (data not shown). Figure 4 showed that the control strain *Z. mobilis* ZM4/pHW20a did not use any cellobiose, but the recombinants *Z. mobilis* ZM4/pSP1086-*bglB* and *Z. mobilis* ZM4/p*Sac-bglB* also utilized only 0.4 and 0.2 g/L of cellobiose, respectively, while ethanol titer did not show obvious difference; the results indicated that, although the extracellular β-glucosidase activity was detected in the constructed recombinants, it was still not sufficient to give the effective CBP fermentation performance. Further enhancement of the β-glucosidase activity is required to give the practical CBP capacity.

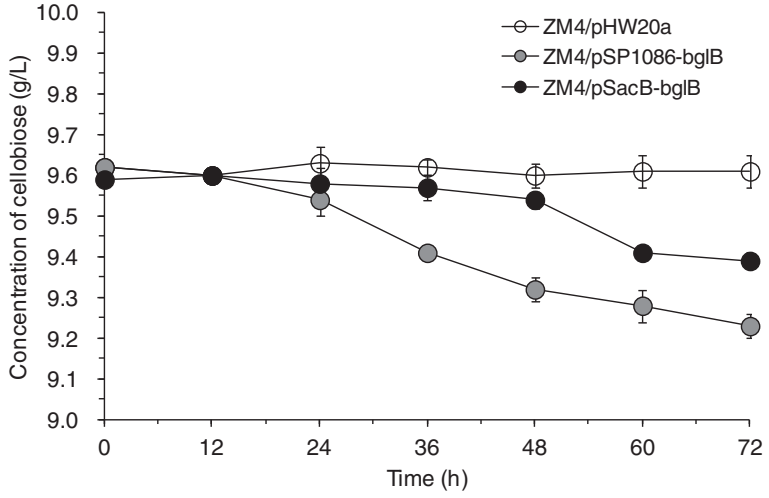

Fig. 4 Utilization of cellobiose by *Z. mobilis* ZM4/pSacB-bglB and *Z. mobilis* ZM4/pSP1086-bglB. *Z. mobilis* ZM4/pHW20a was used as the control strain. White circle for *Z. mobilis* ZM4/pHW20a, gray for *Z. mobilis* ZM4/pSP1086-bglB and black for *Z. mobilis* ZM4/ pSacB-bglB. 1 % of the strain culture were inoculated and cultivated in 20 mL RM medium with 1 with 30 μg/mL nalidixic acid and 20 μg/mL tetracycline, stationary culture at 30 °C in the flask

The exogenous signal peptide SP1086 realized the secretive expression of a heterologous β-glucosidase in *Z. mobilis* ZM4 and showed the more efficient secretive proportion than the fusion protein, which caused a higher total expression level with reducing secretion. As a potential CBP strain, both the recombinant strains *Z. mobilis* ZM4/pSP1086-*bglB* and *Z. mobilis* ZM4/p*Sac-bglB* did not utilize cellobiose well, and some further improvement should be done to meet the CBP fermentation.

Conclusions

In this study, we overexpressed *ZMO1086* in *Z. mobilis* and confirmed its signal peptide SP1086 could work for the secretion in *Z. mobilis*. On this basis, we realized the secretion of heterologous β-glucosidase gene *bglB* in *Z. mobilis* ZM4/pSP1086-*bglB* in which about 16 % of the total protein was secreted. Meanwhile, the recombinant *Z. mobilis* ZM4/p*Sac-bglB* which expressed a fusion-protein-combined β-glucosidase with levansucrase (SacB) also showed extracellular β-glucosidase activity with 2 % secretion ratio. In the fermentation by using RM media with 10 g/L glucose and 10 g/L cellobiose as the carbon source, *Z. mobilis* ZM4/pSP1086-*bglB* utilized 0.4 g/L cellobiose and *Z. mobilis* ZM4/p*Sac-bglB* utilized 0.2 g/L cellobiose during a 72-h fermentation. It was not an efficient result, and the secretion of β-glucosidase in *Z. mobilis* ZM4 needs improvement in further research.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

ZCL and JB designed the experiment, ZCL conducted the experiment, JB conceived the research, and ZCL and JB wrote this manuscript. Both authors read and approved the final manuscript.

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