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Direct production of ethanol from raw sweet potato starch using genetically engineered *Zymomonas mobilis*

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The direct production of ethanol from sweet potato starch by *Zymomonas mobilis* required the construction of four fused glucoamylase genes from *Aspergillus awamori* using recombinant polymerase chain reaction (PCR) and inserted into the broad -host-range vector pBBR1MCS-2. After electro transformation of the four recombinant plasmids into *Z. mobilis,* it was discovered that only the plasmid pGA0 offered the transformants the phenotype of growth on rich medium (RM) plates containing 1.5% sweet potato starch. One transformant had the highest glucoamylase activity of 157 U/mL and about 80% of enzyme activities were detected in extra cellular fraction, indicating that the glucoamylase- coding sequence of *Aspergillus awamori* is expressed as an active enzyme. All transformants of pGA0 could directly ferment sweet potato starch to ethanol. One transformant displayed higher efficiency of ethanol production with 14.73-fold of control strain and 92.69% of the theoretical yield of ethanol. Kinetics of this transformant was also investigated, including growth curve, total sugar consumption, and ethanol production. Our results provide a basis for further constructing a genetically engineered *Z. mobilis* strain directly fermenting sweet potato starch with higher efficiency.

Key words: Ethanol, sweet potato starch, *Zymomonas mobilis*, *Aspergillus awamori,* glucoamylase.

INTRODUCTION

The advantages that *Zymomonas mobilis* holds over traditional yeast processes has led to more economical methods of producing ethanol (Swings and Deley et al., 1977; Jeffries et al., 2005; Panesar et al., 2006; Rogers et al., 2007). However, its narrow spectrum of fermentable carbohydrates has limited its use, especially for fuel ethanol production from varied sources, such as starchy materials, available in abundance as carbon sources for production of ethanol. To overcome this limitation, a number of strategies have been adopted for the construc-tion of starch- utilizing systems, which include the addition of amylolytic enzymes in culture broth, mixed-culture (Dostálek and Häggström et al., 1983; Reddy and Basappa et al., 1996; Abate et al., 1999; Kleerebezem and Loosdrecht et al., 2007) and introduction of heterologous genes into *Z. mobilis* for secretive production of the enzymes to ferment starch for ethanol production. For example, -amylase gene from *Bacillus licheniformis* was transferred into *Z. mobilis*, and the enzyme was released by secretion to extracellular fraction (Brestic-Goachet et al., 1990). Cloning of gluco-amylase gene from *Aspergillus niger* was also attempted but stable transconjugants were not obtained in *Z. mobilis* (Skotnicki et al., 1983). Transfer of the -glucosidase gene of *Xanthomonas albilineans* to *Z. mobilis* was also achieved and the transformants could produce ethanol from cellobiose (Su et al., 1989). However, expression level of the recombinant enzymes was low in *Z. mobilis*. On the other hand, the stability of plasmids in Z. *mobilis* in the absence of selective pressure is another problem.

In China, sweet potato has not only been shown as the least costly for ethanol production by comparing the feedstock cost of bioethanol among sweet potato, corn and cassava (Jikun Huang et al., 2004; Jikun Huang et al., 2002), but also, has a huge production per year. In this report, we described the construction of a genetically engineered strain of *Z. mobilis* by introduction of a foreign

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Table 1. Strains and plasmids used in this study.

Strain or plasmid	Description	Reference or source
Z. mobilis ATCC29191	Wild-type strain	China Center of Industrial Culture collection
Aspergillus awamori SG1	*Aspergillus niger* variety	This laboratory
Plasmids		
pBBR1MCS-2	Broad-host-range vector, Km^R	(Kovach et al. 1995)
pGA0	pBBR1MCS-2::glucoamylase	This study
pGFOR-F1	pBBR1MCS-2::GFOR (Tat)::glucoamylase	This study
pGFOR-F2	pBBR1MCS-2::GFOR::glucoamylase	This study
pSacC-F3	pBBR1MCS-2::SacC::glucoamylase	This study

Table 2. Primers sequence used for construction of recombinant plasmids.

Primer	Sequence (5'-3') (Underline represent restriction site)	Gene to be amplified (Accession No.)
GFOR-F1	TGCTCTAGACAGAAATAATTATCTGACA	Promoter of *Z. mobilis* glucose-fructose oxidoreductase (gfor) gene (M97379)
GFOR-R1	GCTCAACCACGAATCCAAGGTCGCTGCCTGAAGACCACTGGCTAAG	
GFOR-R2	AGAAGAGATCGGAACGACATAATCCTTGTTTCTTTCTTAACTAACC	
SacC-F	TGCTCTAGATCTTCTAATTCTGCGGC	Promoter of *Z. mobilis* extracellular sucrase (sacC) gene (L33403)
SacC-R3	AGAGATCGGAACGACATTTAAAGTAATATACACT	
GA-F0	TGCTCTAGAATGTCGTTCC GATCTCTTC	Coding region of glucoamylase gene (AB083161)
GA-F1	CTTAGCCAGTGGTCTTCAGGCAGCGACCTTGGATTCGTGGTTGAGC	Mat peptide of glucoamylase gene
GA-F2	GGTTAGTTAAGAAAGAAACAAGGATTATGTCGTTCCGATCTCTTCT	Coding region of glucoamylase gene
GA-F3	AGTGTATATTACTTTAAATGTCGTTCCGATCTCT	Coding region of glucoamylase gene
GA-R	CGAGCTCCTACCGCCAGGTGTCAGTCA	Coding region of glucoamylase gene

gene encoding glucoamylase from *Aspergillus awamori* SG1 and it was used to convert raw sweet potato starch directly to ethanol. This will make the commercial ethanol production processes from starchy materials more economical.

MATERIALS AND METHODS

Bacterial strains, plasmids, and cultural conditions

All bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* JM109 was used as the host strain for recombinant DNA manipulations. *Z. mobilis* ATCC29191 was used as the breeding host strain for fermentation of sweet potato starch, and *Aspergillus awamori* SG1 was used as the source of the glucoamylase gene. *E. coli* was cultured in Luria-Bertani medium or on Luria- Bertani agar plates at 37°C, while *Z. mobilis* was cultivated statically in Rich media (RM) (Goodman et al., 1982) at 30°C. Appropriate antibiotics (45 µg/mL of kanamycin for *E. coli* and 200 µg/mL for *Z. mobilis*, respectively) were added in bacterial culture if needed. Broad-host-range vector pBBR1MCS-2 was used for construction of fusion expression unit and gene transformation (Kovach et al., 1995).

Construction of fused glucoamylase gene

All DNA manipulation, including plasmid preparation from *E. coli*, restriction enzyme digestion, ligation, *E. coli* transformation and agarose gel electrophoresis were performed according to standard protocols (Sambrook et al., 1989). All PCRs were carried out using Ex-Taq polymerase (TaKaRa, Dalian, China), and the oligonucleotides (Invitrogen, Shanghai, China) used to construct the expression plasmids are listed in Table 2.

The cDNA encoding glucoamylase were amplified by reverse transcriptase polymerase chain reaction(RT-PCR) from total RNA of *A. awamori* using each primer pair of GA-F0, GA-F1, GA-F2, GA-F3 or GA- R, and resulting in the corresponding DNA fragments named as F0, F1, F2 and F3, respectively. Fragment F0, including 1920 bp of glucoamylase gene, was digested with *Xba* /*Sac* and directly inserted into the broad-host-range vector pBBR1MCS-2, yielding pGA0 (Figure 1). The other fragments (F1, F2, F3) containing full glucoamylase gene with a set of nucleotides at 5' were used to fuse various signal sequences and promoters by the recombinant PCR (Kanagasundaram and Scopes, 1992). Three DNA fragments encoding the 53-amino acid of secretion signal peptide of glucose–fructose oxidoreductase (GFOR), the promoter of GFOR and extracellular sucrase (SacC) were amplified from *Z. mobilis* genomic DNA using the primer pair of GFOR-F1/GFOR -R1, GFOR-F1/GFOR-R2, SacC-F/SacC-R3, respectively. Each of the three DNA fragments was then mixed with fragment F1 (1848 bp), F2(1920 bp), F3 (1920 bp), respectively, heated at 94°C for 20 min, and incubated at 37°C for 45 min to form a heteroduplex from which the fused glucoamylase gene was amplified by using primers GFOR-F1, GFOR-F2, SacC-F and GA-R, respectively, resulting in three corresponding fusion DNA fragments of GFOR-F1 (2256 bp), GFOR-F2 (2172 bp) and SacC-F3 (2117 bp). The fused glucoamylase genes were then digested with *Xba* /*Sac* and inserted into

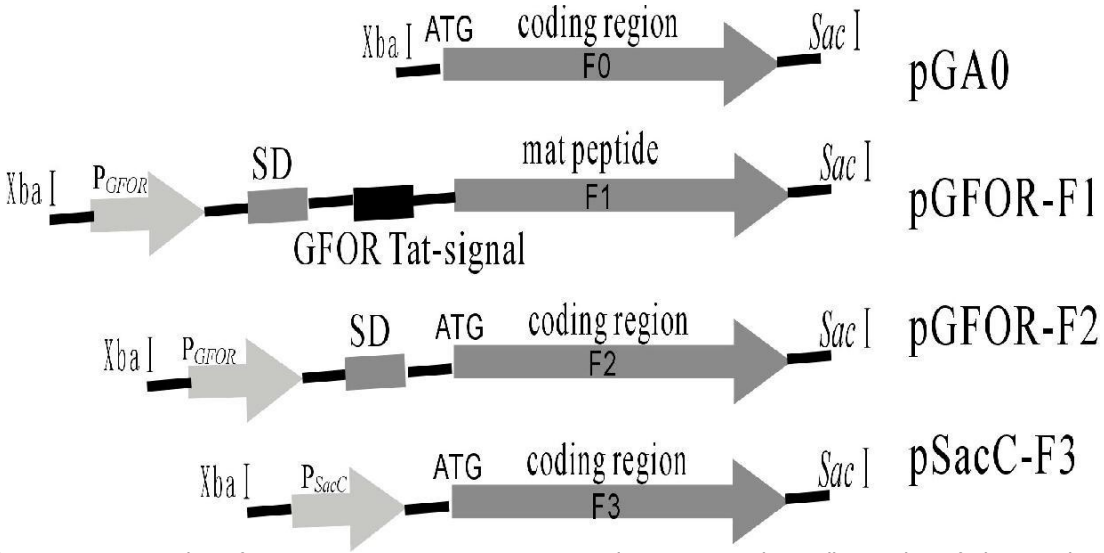

Figure 1. Construction of pGA0, pGFOR- F1, pGFOR-F2 and pSacC-F3. The coding region of glucoamylase gene was amplified by RT-PCR from total RNA of *A. awamori* and then inserted into the *Xba* and *Sac* site of pBBR1MCS-2, yielding pGA0. The recombinant PCR was used to construct different fused glucoamylase genes and the resulted genes were inserted into the *Xba* and *Sac* site of pBBR1MCS-2, yielding pGFOR-F1 (mature peptide-coding region of glucoamylase gene fused with the Tat secretion signal peptide and the promoter of GFOR gene), pGFOR-F2 (coding region fused with promoter of GFOR gene) and pSacC-F3 (coding region fused with promoter of extracellular sucrase gene).

vector pBBR1MCS- 2, yielding pGFOR-F1, pGFOR-F2 and pSacC-F3 (Figure 1).

Expression of glucoamylase in *Z. mobilis*

These plasmids (1-3 g for each) were transformed into *Z. mobilis* by electro transformation method described previously (Zhang et al., 1995). Two hundred microliters of transformed cells were directly dispensed on RM plate containing 1.5% soluble starch and 1 mg/mL Trypanblau and were incubated for 3 days at 30°C. Screening of transformants was performed by halo formation on the above agar plates.

Glucoamylase activity was assayed by the method described previously (Goto et al., 1994). One milliliter of transformant cells was harvested by centrifugation for 5 min at 5,000 × *g* and 4°C and the supernatant was collected as extracellular fraction sample. The pellet was washed twice with distilled water and then resuspended in 1 ml distilled water as intracellular fraction sample. Five milliliters of 2.0% soluble starch solution mixed with 1 ml 0.1 M HAc buffer (pH 4.8) was preheated for incubation at 50°C for 10 min. One hundred microliters of the enzyme sample were added to the mixture and incubated at the same temperature for 30 min. The reaction was stopped by boiling the mixture for 10 min and the concentration of glucose was determined by the 3,5- dinitrosalicylic acid method (Miller, 1959). One unit of glucoamylse was defined as the amount of enzyme required to release 1 mg of glucose per hour from soluble starch. Stability of expression plasmid was investigated as previously described (Brestic -Goachet et al., 1987) in a medium without kanamycin, and then one hundred colonies was randomly selected to test their ability to resist kanamycin.

Fermentation of raw sweet potato starch by recombinant *Z. mobilis*

Fermentation experiment was initially performed in test tubes with 5 ml of RMS20 medium (same as Rich media, except for replacing glucose by sweet potato starch to a final concentration of 20 g/L). The tubes were then inoculated with 1% (v/v) 48 h seed cultures, and cultivated anaerobically at 30°C. Ethanol was assayed using GC103 with a glass column (0.26×200 cm) filled with Porapak type QS (80–100 mesh, Waters, Milford, MA) at 150°C and a FID detector at 80°C. Molecular Nitrogen (N_2) was the carrier gas (30 ml/min).

A larger-scale fermentation experiment was performed as follows: 500 ml of RMS50 medium (same as Rich media, except for replacing glucose by sweet potato starch to a final concentration of 50 g/L) in a 1000 mL flask was inoculated with 1% (v/v) 48 h seed culture of the recombinant showing the highest ethanol production in previous experiments and the fermentation was carried out as described above. Cell growth was determined by optical density measurements at 600 nm. Total sugar used for indicating the undigested starch was determined by the dinitrosalicylic acid (DNS) method (Miller 1959), after concentrated HCl hydrolysis (30 min at 100°C, pH adjusted to 7.0 with NaOH) . Ethanol product was assayed using GC103 as described above.

RESULTS AND DISCUSSION

Construction of fused glucoamylase genes

To achieve expression of glucoamylase gene of *A. awamori* in *Z. mobilis*, two promoters were selected to construct the fused genes, including promoters of the

periplasmic enzyme glucose–fructose oxidoreductase and extracellular sucrase gene from *Z. mobilis*. To secrete glucoamylase of *A. awamori* into the periplasm of *Z. mobilis*, the enzyme was tagged with a typical 53-aa secretion signal peptide of GFOR, which secretes the target protein via Tat- dependent pathway (Blaudeck et al., 724 Afr. J. Microbiol. Res.
2001). Previous study showed that a fused -glucosidase gene tagged with the 53-aa Tat signal enabled 61% of the enzyme activity to be transported through the cytoplasmic membrane of *Z. mobilis* and produced 0.49 g ethanol/g cellobiose (Yanase et al., 2005) . The *sacC* gene of *Z. mobilis* encodes the extracellular sucrase (Preziosi et al., 1990; Kannan et al., 1995). Therefore, four expression plasmids containing the different recombinant genes (Figure 1), pGA0, pGFOR-F1, pGFOR -F2 and pSacC-F3 were constructed and transformed into *Z. mobilis* ATCC29191, respectively.

Expression of glucoamylase in *Z. mobilis*

Plate assay was first performed to determine whether the transformants gained amylolytic activity. Unexpectedly, six transformants harboring plasmid pGA0 without any tag of Tat signal peptide or extracellular sucrase promoter showed haloes on the starch plate, indicating that they could hydrolyze starch. Expression of the glucoamylase gene may be driven by upstream promoter before the Kanamycin unit. However, no halo formation was observed around the cells harboring other plasmids pGFOR-F1, pGFOR-F2 and pSac-F3, and the reason is still unknown. We randomly selected 2 of 6 transformants for further identification on starch plate. As shown in Figure 2, both of them could grow on the plate. However, the transformant carrying pBBR1MCS-2 was unable to grow on the same plate. Further activity assay illustrated that the enzyme activities of the six transformants was between 117-157 U/mL. About 80% of the total activity was detected in the extracellular fraction (Table 3), indicating that the enzyme was secreted into the culture medium. Similar result was also observed when
-amylase gene from *Bacillus licheniformis* was transferred into *Z. mobilis* (Brestic-Goachet et al., 1990). The extracellular localization of -amylase or glucoamy-lase activity may be due to the result of an unknown secretory mechanism in *Z. mobilis.*

Stability of plasmid pGA0 in *Z. mobilis*

Previous study showed that the broad host range vector pBBR1MCS-2 was stably maintained for at least 50 generations and evaluated as an expression vector for *Z. mobilis* (Jeon et al., 2005). Stability of expression plasmid pGA0 was also investigated as previously described (Brestic-Goachet et al., 1987) in a medium without kanamycin. One hundred colonies were tested and all of them were found to be resistant to kanamycin, indicating that the plasmid pGA0 in *Z. mobilis* ATCC29191 in the absence of selective pressure was stably maintained. Furthermore, the plasmid pGA0 was also stably maintained in *E. coli* JM109 strain without kanamycin (data not shown).

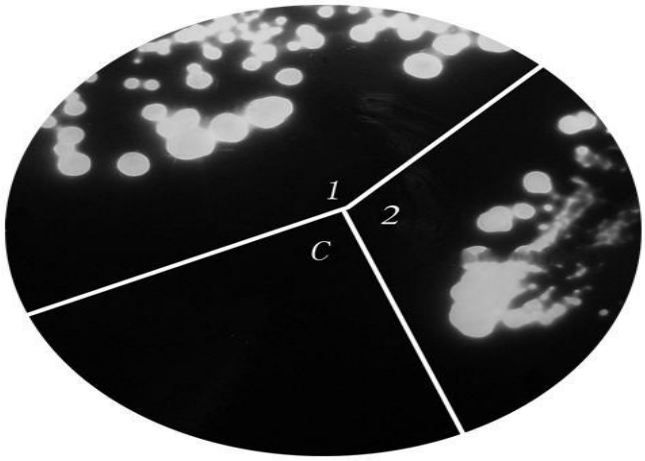

Figure 2. Growth test of genetically engineered *Z. mobilis* strains on sweet potato starch agar plate. 1-2, transformants of *Z. mobilis* ATCC29191/pGA0 c, *Z. mobilis* ATCC29191/pBBR1MCS-2 (as control).

Table 3. Distribution of glucoamylase in genetically engineered *Z. mobils* strains.

Transformant	Glucoamylase Activity (U/ml)		
	Total activity	Extracellular distribution (%)	Intracellular
1	157	126 (80)	31
2	120	96 (80)	24
3	125	100 (80)	25
4	127	102 (80)	25
5	147	123 (84)	24
6	117	94 (80)	23
c	ND	ND	ND

1-6 = transformants of *Z. mobilis* ATCC29191/pGA0; c = negative control, *Z. mobilis* ATCC29191/pBBR1MCS-2; ND = not detected

Ethanol production from sweet potato starch by genetically engineered *Z. mobilis*

All transformants harboring pGA0 could directly ferment sweet potato starch to ethanol. After 4 days cultivation of the transformant No. 4, the amount of ethanol accumu-lated reached 10.53 g/l, which is 92.69% of the theoretical yield of ethanol from 20.0 g starch/l, increased 14.73-fold compared with control strain (Figure 3). Kinetics of this transformant was also investigated in a larger-scale fermentation, including growth curve, total sugar, and ethanol production. In this fermentation experiment, a

higher concentration (50 g/l) of sweet potato starch was used. As shown in Figure 4, the strain transformant No. 4 carrying pGA0 was able to utilize sweet potato starch to produce ethanol, whereas *Z. mobilis* carrying pBBR1MCS-2 did not. After 6 days cultivation, the starch in the culture medium was partial exhausted, and the

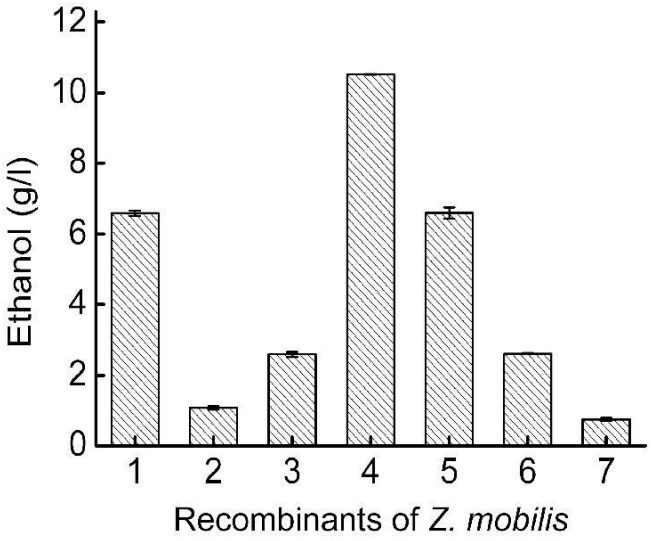

Figure 3. Ethanol production from sweet potato starch. 1-6, transformants of *Z. mobilis* ATCC29191/pGA07, *Z. mobilis* ATCC29191/pBBR1MCS-2 (as control). Data comes from two independent experiments.

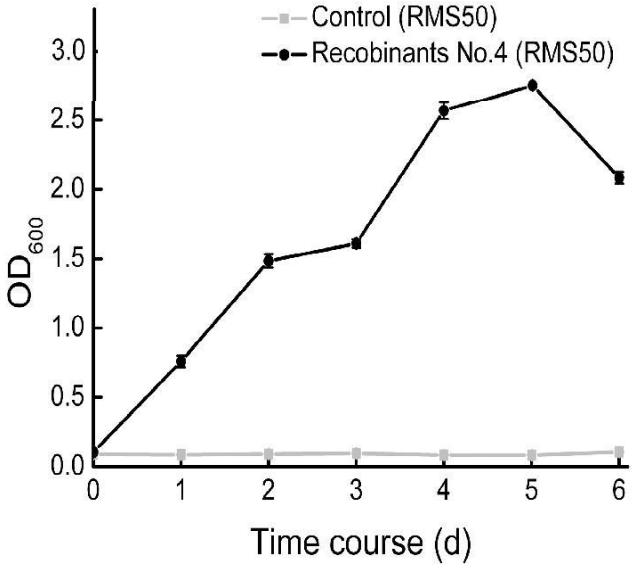

Figure 4a. Ethanol production from sweet potato starch by genetically engineered *Z. mobilis* (a) Cell growth of ATCC29191 and transformant in RMS50 medium.

starch/l. It indicated that the genetically engineered *Z. mobilis* strains yield lower ethanol from higher concentration of sweet potato starch, perhaps due to the low efficiency of expressed glucoamylase in the condition of higher sugar content. However, higher amount of total sugar remained in the culture of cells harboring the plas-

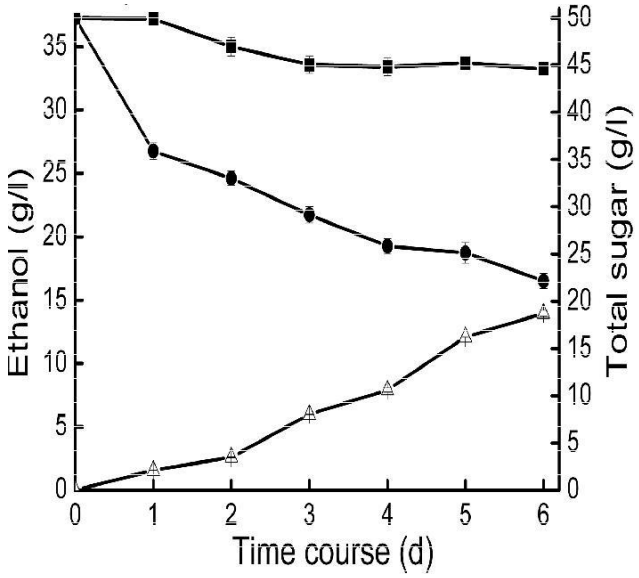

Figure 4b. Ethanol production from sweet potato starch by genetically engineered *Z. mobilis* (b) Total sugar and ethanol production by genetically engineered *Z. mobilis*. Open and filled symbols show ethanol and total sugar concentrations, respectively. *Z. mobilis* 29191/pGA0 (filled circles), ATCC29191/ pBBR1MCS- 2 (filled squares). Data comes from two independent experiments.

amount of ethanol accumulated reached 13.96 g/l, which is 49.14% of the theoretical yield of ethanol from 50.0 g

mids pBBR1MCS-2 due to low starch digestion efficiency during the 6 days cultivation. During the fermentation, almost no glucose (data not shown) was detected in the culture medium due to rapid utilization by the engineered *Z. mobilis* strains (Figure 4). So a new genetically engineered *Z. mobilis* strain was constructed, which could produce ethanol from sweet potato starch although at a low level. Interestingly, transformant No. 1 has shown the highest glucoamylase activity but lower ethanol yield than No. 4 (Table 3 and Figure 3). It may be indicated that enzyme activity and ethanol yield have no direct relationship in recombinant *Z. mobilis* or other unknown reasons. How- ever, our results provide a basis for further construction of an engineered *Z. mobilis* straindirectly fermenting raw sweet potato starch.

Still, a more efficient process will be essential for improving production of ethanol from sweet potato starch. In this regard, we have constructed a novel cell surface display system by using an outer membrane protein of *Z. mobilis* as anchor motif, which enables the expressed enzyme on the cell-surface of *E. coli* (He and Zhang et al., 2008), and studies are now under way to use this system for displaying glucoamylase on the cell surface of *Z. mobilis*.

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