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BIOENERGY AND BIOFUELS

Metabolic engineering of *Clostridium tyrobutyricum* for *n*-butanol production from maltose and soluble starch by overexpressing α-glucosidase

Le Yu[1] · **Mengmeng Xu**[1] · **I-Ching Tang**[2] · **Shang-Tian Yang**[1]

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Abstract *Clostridium tyrobutyricum* does not have the enzymes needed for using maltose or starch. Two extracellular α-glucosidases encoded by *agluI* and *agluII* from *Clostridium acetobutylicum* ATCC 824 catalyzing the hydrolysis of α-1,4-glycosidic bonds in maltose and starch from the non-reducing end were cloned and expressed in *C. tyrobutyricum* (Δ*ack*, *adhE2*), and their effects on *n*-butanol production from maltose and soluble starch in batch fermentations were studied. Compared to the parental strain grown on glucose, mutants expressing *agluI* showed robust activity in breaking down maltose and produced more butanol (17.2 vs. 9.5 g/L) with a higher butanol yield (0.20 vs. 0.10 g/g) and productivity (0.29 vs. 0.16 g/L h). The mutant was also able to use soluble starch as substrate, although at a slower rate compared to maltose. Compared to *C. acetobutylicum* ATCC 824, the mutant produced more butanol from maltose (17.2 vs. 11.2 g/L) and soluble starch (16.2 vs. 8.8 g/L) in batch fermentations. The mutant was stable in batch fermentation without adding antibiotics, achieving a high butanol productivity of 0.40 g/L h. This mutant strain thus can be used in industrial production of *n*-butanol from maltose and soluble starch.

Keywords α-Glucosidase · Butanol · *Clostridium tyrobutyricum* · Maltose · Metabolic engineering · Soluble starch

Introduction

n-Butanol is a promising gasoline substitute that can be produced by solventogenic *Clostridium* such as *C. acetobutylicum* and *Clostridium beijerinckii* in acetone-butanol-ethanol (ABE) fermentation (Wang et al. 2014; Xue et al. 2013; Zhao et al. 2013). Because of the high crude oil prices and concerns of the depletion of fossil energy, many countries including USA, China, and Brazil have planned or started to apply ABE fermentation for *n*-butanol production (Ni and Sun 2009; Mariano et al. 2013; Qureshi and Blaschek 2001). However, conventional ABE fermentation is limited by low productivity and butanol yield because of strong butanol cytotoxicity and the coproduction of acetone (Lee et al. 2008; Lütke-Eversloh 2014; Nicolaou et al. 2010; Papoutsakis 2008; Zheng et al. 2009). In addition, the feedstock usually accounts for more than 50 % of the product cost in ABE fermentation (Green 2011; Gu et al. 2011). Starchy materials from corn, cassavas, and potatoes are cheaper than glucose and have thus been widely used in ABE fermentation with *C. acetobutylicum* and *C. beijerinckii*, which have amylases and can directly use starch and its hydrolytic products including maltose, maltotriose, and maltodextrin as substrates. However, glucose, as the end product in starch hydrolysis, hinders the amylolytic activity through catabolite repression (Annous and Blaschek 1990; 1991; Chojecki and Blaschek 1986). Also, pH variation caused by the metabolic shift in the biphasic fermentation (acidogenesis and solventogenesis) by solventogenic clostridia can negatively affect the activity and stability of starch-hydrolyzing enzymes, especially glucoamylase and the rate-

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✉ Shang-Tian Yang
yang.15@osu.edu

[1] William G. Lowrie Department of Chemical and Biomolecular Engineering, The Ohio State University, 151 West Woodruff Ave, Columbus, OH 43210, USA

[2] Bioprocessing Innovative Company, 4734 Bridle Path Ct, Dublin, OH 43017, USA

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limiting α-amylase (Paquet et al. 1991; Soni et al. 1992; Madihah et al. 2001), causing process difficulties.

Recently, we have engineered *Clostridium tyrobutyricum*, an acidogen-producing butyrate as the main product with a high titer and yield (Liu et al. 2005), for *n*-butanol production by overexpressing *adhE2* encoding a bifunctional aldehyde/alcohol dehydrogenase (Yu et al. 2011, 2012). The engineered *C. tyrobutyricum* was able to produce *n*-butanol as the main product (without acetone) from glucose with a high titer and yield when additional NADH was available (Du et al. 2015). However, *C. tyrobutyricum* can only use a very restricted range of carbon sources, including a few monosaccharides (glucose, xylose, and fructose), mannitol, and lactate (Dwidar et al. 2012). It is unable to use disaccharides, such as maltose, and polysaccharides, such as starch, because of lacking some important transport and catabolic systems.

In this study, two α-glucosidase genes (CAC2252, CAC2891) were cloned from *C. acetobutylicum* ATCC 824 (Cac 824) and separately expressed in *C. tyrobutyricum* together with *adhE2* in a clostridia shuttle vector (Heap et al. 2009; Yu et al. 2012). The mutants were evaluated for their abilities to use maltose and soluble starch as substrates in batch fermentations at different pHs between 5.0 and 6.0, which affected the activity of α-glucosidase and butanol biosynthesis. The results showed that the mutant expressing *agluI* and *adhE2* had superior capability of utilizing maltose and starch and produced more butanol as compared to Cac 824. This is the first study realizing the direct utilization of maltose and starch in *C. tyrobutyricum*, which should have advantages for industrial production of biobutanol from low-cost feedstocks such as food wastes or processing by-products rich in maltose or starch. Although *Clostridium thermocellum* cellulases have been expressed as mini-cellulosomes on *C. acetobutylicum* (Kovács et al. 2013), this study is the first one successfully transferring α-glucosidase, an extracellular enzyme, between two different *Clostridium* species, indicating a similar recognition and export system functioning in both *C. acetobutylicum* and *C. tyrobutyricum*.

Materials and methods

Bacterial strains, plasmids, and culture media

Table 1 summarizes the bacterial strains and plasmids used in this study. All recombinant plasmids constructed in this work were transformed into the mutant strain of *C. tyrobutyricum* ATCC 25755 with *ack* knockout developed in a previous study (Liu et al. 2006). Clostridial growth medium (CGM) was used to culture *C. tyrobutyricum* at 37 °C under anaerobic conditions. The CGM contained (g/L): 4 tryptone, 2 yeast extract, 1.0 $K_2HPO_4{\cdot}3H_2O$, 0.5 KH_2PO_4, 2 $(NH_4)_2SO_4$, 0.1 $MgSO_4{\cdot}7H_2O$, and trace minerals (Zhu and Yang 2003). Carbon sources including glucose, maltose, and soluble starch (dextrose equivalent (DE) value of 25, Cargill, Eddyville, IA) were added as needed. *E. coli* strains used in the cloning were cultivated at 37 °C aerobically in liquid Luria-Bertani (LB) medium and on LB agar plates. After autoclaving at 121 °C for 30 min, media were supplemented with 25 μg/mL chloramphenicol, 30–45 μg/mL thiamphenicol, or 250 μg/mL cycloserine as needed.

Plasmid construction

All plasmids were constructed from pMTL82151-*adhE2* (Yu et al. 2012). The *agluI* (CAC2891) and *agluII* (CAC2252) were extracted and amplified from *C. acetobutylicum* ATCC 824 genomic DNA by PCR with the primers shown in Table 1. The original ribosome binding sites were replaced with the consensus sequence "AGGAGG" to optimize their expression. The thiolase (*thl*) promoter from *C. tyrobutyricum* was used to drive gene expression. The plasmids pGluI and pGluII were constructed from pMTL82151-*adhE2* by inserting *agluI* and *agluII*, respectively, after *adhE2* at the *Sac*II site (see Supplemental Fig. S1) using In-fusion HD cloning kit from Clontech (Mountain View, CA).

Introduction of genes into *C. tyrobutyricum*

The plasmids were transformed into the host cells by conjugation (Yu et al. 2012). The donor cells, *E. coli* CA434, used to carry the plasmids to be transformed were cultivated in LB medium containing 25 μg/mL chloramphenicol at 37 °C overnight to reach optical density at 600 nm (OD_{600}) of 1.5–2.0. After transformation by electroporation, *E. coli* cells were collected by centrifugation at 4000×*g* for 2 min and washed once using 1 mL sterile phosphate-buffered saline (PBS). The donor cells were then mixed with 200 μL *C. tyrobutyricum* cells precultured at 37 °C overnight, and the mixture was pipetted onto CGM agar plates in an anaerobic chamber and incubated at 37 °C for 8–12 h. Then, cells were recovered and resuspended in 1 mL of PBS and spread onto CGM plates containing 30 μg/mL thiamphenicol and 250 μg/mL cycloserine for 2–3 days to select for positive transconjugants. A relatively high transfer frequency of $(3.32 \pm 1.3) \times 10^{-6}$ colony/donor cell was achieved using the plasmid pMTL82151 (Yu et al. 2012). Positive transconjugants were first identified by colony PCR and then confirmed by restriction enzyme digestion of the plasmids extracted from the transconjugants. Two mutant strains each containing one of the two recombinant plasmids were obtained and stored at −80 °C.

Enzyme activity assay

The activity of α-glucosidase was assayed under anaerobic conditions using the *p*-nitrophenyl α-D-glucoside (PNPG)

Table 1 Bacterial strains and plasmids used in this study

Strain/Plasmid	Characteristics	Reference/Source
Strains		
E. coli DH5α	Host cells for plasmids amplification	Invitrogen
E. coli CA434	Donor cells in conjugation	Williams et al. (1990)
Cac 824	*C. acetobutylicum* ATCC 824	ATCC
Ct(Δ*ack*)	*C. tyrobutyricum* ATCC 25755 with *ack* knockout	Liu et al. (2006)
Ct(Δ*ack*)-pM2	*adhE2* overexpression in Ct(Δack)	Yu et al. (2012)
Ct(Δ*ack*)-pGluI	*adhE2* and *agluI* overexpression in Ct(Δack)	This study
Ct(Δ*ack*)-pGluII	*adhE2* and *agluII* overexpression in Ct(Δack)	This study
Plasmids		
pMTL82151	ColE1 ori; Cm^r; pBP1 ori, *TraJ*	Heap et al. (2009)
pM2	pMTL82151; P-*thl adhE2*	Yu et al. (2012)
pGluI	pMTL82151; P-*thl adhE2 agluI*	This study
pGluII	pMTL82151; P-*thl adhE2 agluII*	This study
Primers	Sequence (5′–3′)	
AgluI-for	TTTGCTTCATTATCC AGGAGG GTAAAATATAATGTATAATAGTTCAAAAACACG	
AgluI-rev	GAAACAGCTATGACC TAAATAATTCTATAAAACCACAAGAAATC	
AgluII-for	TTTGCTTCATTATCC AGGAGG ACTACCACATAGGTTTGAGAAGTTTA	
AgluII-rev	GAAACAGCTATGACC ATTAACATATTTTGGGTGTATGTTTGA	

method (Albasheri and Mitchell 1995; McMahon et al. 1999). To test the presence of intracellular, cell-bound, and secretory α-glucosidase, cell crude extract, whole cells, and cell culture supernatant were assayed separately. All mutants were cultured overnight to reach a similar optical cell density. For the preparation of cell crude extract, cells in 50 mL of overnight culture broth were collected by centrifugation and washed twice in a potassium phosphate buffer (50 mM, pH 5.5). After centrifugation, the cell pellet was resuspended in the same buffer. Cells were then disrupted by using Mini-Beadbeater-16 (Biospec, Bartlesville, OK), and the cell debris was removed by centrifugation at 13,000 rpm for 10 min at 4 °C. For whole-cell preparation, 2 mL cells were harvested by centrifugation at 4000 rpm for 5 min and the cell pellet was then washed and suspended in 400 μL potassium phosphate buffer. The supernatant was prepared by filtration to remove all the remaining cells. The assay mixture (1 mL) containing 5 mM PNPG, 50 mM potassium phosphate (at pH 5.5 or as indicated otherwise) and an appropriate amount of crude cell extract, whole cell, or supernatant was incubated at 37 °C for 1 h. The reaction was stopped by adding 1-mL 0.5 M sodium carbonate solution. The assay mixture without cell extract was used as the blank. The activity of α-glucosidase was measured by monitoring the release of PNP in the mixture with a UV/vis spectrophotometer (UV-1601, Shimadzu) at 400 nm. The protein concentration was measured by the Bradford dye-binding assay (Bio-Rad Laboratories, Hercules, CA) with bovine serum albumin as standard. The specific enzyme activity present in crude cell extract, whole cell, or supernatant is reported as the amount of released PNP per minute per milliliter of the cell culture broth with OD normalized to 1.

Fermentation kinetics

Batch fermentation was studied in a 1-L stirred-tank bioreactor containing 600 mL of CGM with glucose, maltose, or soluble starch as the substrate and 30 μg/mL thiamphenicol, with pH controlled at the desired level (5.0, 5.5, or 5.8) by adding 40 % ammonium hydroxide, unless otherwise noted. The bioreactor was inoculated with an overnight culture at a volume ratio of 5 %, and liquid samples were collected twice a day at regular intervals to monitor cell density, sugar consumption, and product formation. Each fermentation condition was repeated at least once.

Analytical methods

Cell growth was monitored by measuring the OD_{600} with a spectrophotometer (UV-16-1, Shimadzu, Columbia, MD). Glucose, maltose, and soluble starch in samples were measured using a high-performance liquid chromatograph (HPLC) equipped with an organic acid analysis column (HPX-87H, Bio-Rad) and a refractive index detector (Shimadzu RID-10A) at 45 °C (Yu et al. 2011). A series of various concentrations of starch solutions were used as standards for calibration. Acetone, butanol, ethanol, acetate, and butyrate were analyzed with a gas chromatograph (GC, Shimadzu GC-2014) equipped with a flame ionization

detector and a 30-m fused silica column (0.25-m film thickness and 0.25-mm ID, Stabilwax-DA). Detailed description of the GC method has been given elsewhere (Jiang et al. 2014).

Segregational stability

Segregational stability of the plasmid pGluI was evaluated in a series of subcultures in CGM containing glucose or maltose without antibiotics following the method previously described (Yu et al. 2012). Briefly, a single colony of the mutant was picked and cultured in 1 mL CGM medium with 20 g/L glucose and 30 μg/mL thiamphenicol for 24 h at 37 °C. Cells were collected and washed with PBS and then subcultured in antibiotic-free CGM containing 20 g/L glucose or maltose at 1 % inoculum every 24 h for 96 h. At 96 h, cells were diluted by 10^4 times with PBS and plated on CGM agar plates (containing 20 g/L glucose) with or without antibiotics at 37 °C for 5 days to determine colony-forming units (CFU). The segregational stability P can be calculated from the following equation: $R=P^n$ or $P=\sqrt[n]{R}$, where R is the fraction of cells carrying the plasmid and n is the number of generations in 96 h, which was −28 based on the observed specific growth rate of −0.20/h (generation time=3.465 h).

Statistical analysis

Unless otherwise noted, batch fermentation for each condition was studied in duplicate, and the means with standard errors for product yields and productivities are reported. Student's t test analysis was performed using JMP software with the significance level $\alpha=0.05$.

Results

α-Glucosidase activity

Firstly, the mutants expressing Aglu I or Aglu II were examined for their α-glucosidase activities at pH 5.5. As shown in Table 2, high enzyme activity was found with the whole cell, while only negligible or trivial activity was detected in the supernatant and cell crude extract. On the other hand, no enzyme activity was found with Ct(Δ*ack*)-pM2 as the negative control. The results suggested that both Aglu I and Aglu II were exported and associated with cell membrane, instead of being kept intracellularly or secreted into the medium. The trivial activity detected in the supernatant was probably caused by cell lysis. Also, the mutant Ct(Δ*ack*)-pGluI had about 60 % higher enzyme activity compared to Ct(Δ*ack*)-pGluII. It was found that the Aglu activity was sensitive to the pH, decreasing from 48.0 nmol PNP/mL/min at pH 5.0 to 7.4 nmol PNP/mL/min at pH 6.5 for the assay with Ct(Δ*ack*)-pGluI. To study if the Aglu expression was affected by the substrate used in the culture, the enzyme activity was also investigated with glucose, maltose, and glucose/maltose (1:1, total 20 g/L) pregrown cultures of Ct(Δ*ack*)-pGluI and Cac 824 (Table 3). As expected, regardless of the substrate used in culturing the cells, comparable Aglu activities were detected for Ct(Δ*ack*)-pGluI, confirming the constitutive expression of *aglu* in the mutant. In contrast, for Cac 824, the Aglu activity was significantly lower for cells grown on glucose and glucose/maltose, suggesting that the expression of *aglu* was both induced by maltose and repressed by glucose in Cac 824. The glucose-mediated catabolite repression on the expression of α-amylase was also observed previously in Cac 824 (Annous and Blaschek 1990). It should be noted that Cac 824 also showed significant intracellular α-glucosidase activity that might be attributed to another α-glucosidase (CAC1085) present in its genome.

Aglu I and Aglu II, encoded by *agluI* (CAC2891) and *agluII* (CAC2252), respectively, on the chromosome of Cac 824, were both annotated as α-glucosidase with conserved family 31 of glycosyl hydrolase domain located at 55–775 and 27–779 amino acids, respectively (see Fig. S2 in Supplemental Materials). Alignment of these two proteins shows a high level of similarity with 75 % identities. SignalP analysis of the sequences gave a signal peptide score of 0.839 for Aglu I and 0.449 for Aglu II, suggesting the presence of signal peptides in these proteins for their export

Table 2 α-Glucosidase activities in cell extract (intracellularly), associated with cells (exported to cell membrane), and in supernatant (secreted extracellularly) at different pHs

Strain	pH	Cell extract (nmol PNP/mL/min)	Whole cell (nmol PNP/mL/min)	Supernatant (nmol PNP/mL/min)
Ct(Δ*ack*)-pGluI	5.0	NA	48.0±1.1	NA
	5.5	0.023±0.003	32.7±2.1	0.22±0.01
	6.0	NA	14.1±0.9	NA
	6.5	NA	7.4±1.0	NA
Ct(Δ*ack*)-pGluII	5.5	0.013±0.007	20.5±2.0	0.10±0.04
Ct(Δ*ack*)-pM2	5.5	0.0010±0.0003	0.06±0.02	0.002±0.001

Data shown are mean±SD (n=3)

NA data not available

Table 3 α-Glucosidase activities of *C. tyrobutyricum* Ct(Δ*ack*)-pGluI and *C. acetobutylicum* ATCC 824 pregrown on glucose, maltose, and glucose/maltose at pH 5.5

Strain	Pregrown carbon source	Cell extract (nmol PNP/mL/min)	Whole cell (nmol PNP/mL/min)	Supernatant (nmol PNP/mL/min)
Ct(Δ*ack*)-pGluI	Glucose	0.023±0.003	32.7±2.1	0.22±0.01
	Glucose/Maltose	NA	28.9±0.9	NA
	Maltose	NA	34.8±0.5	NA
Cac 824	Glucose	0.017±0.001	3.2±0.4	ND
	Glucose/Maltose	2.3±0.3	18.4±1.3	0.003±0.000
	Maltose	6.1±0.5	29.3±1.1	0.28±0.09

Data shown are mean±SD (n=3)

NA data not available, *ND* activity not detected

or secretion (Bendtsen et al. 2004; Servinsky et al. 2010). Variations in the protein sequence, especially the signal peptides, might have caused differences in export efficiency and thus influenced the enzyme activity, as observed in this study with the two mutants expressing Aglu I and Aglu II, respectively.

Effects of pH

The optimal pH for *C. tyrobutyricum* grown on glucose is ~6.3 (Liu et al. 2005; Yu et al. 2011; 2012), whereas the optimal pH range for Aglu activity is expected to be between 4.5 and 5.5, the pH usually used for growing *C. acetobutylicum* ATCC 824 (Huang et al. 1986). To find the optimal pH for butanol production from maltose by the mutants Ct(Δ*ack*)-pGluI and Ct(Δ*ack*)-pGluI, the effects of pH on their growth, maltose consumption, and acid and alcohol production in batch fermentation of maltose were evaluated with pH controlled at 5.0, 5.5, 5.8, and 6.0, respectively. As can be seen in Fig. 1, increasing the pH from 5.0 to 5.8 decreased cell growth and maltose consumption slightly (Fig. 1a, b). At pH 5.0 and 5.5, all maltose, ~120 g/L, was consumed or hydrolyzed in ~70 h, whereas ~7 g/L of maltose remained at the end of the fermentation at pH 5.8. Meanwhile, a large amount of glucose was accumulated (up to ~30 g/L) in the fermentation at pH 5.0, 5.5, and 5.8, indicating that Aglu I was actively breaking down maltose into glucose at a faster rate than glucose uptake by cells in the fermentation. Maltose hydrolysis (consumption) slowed down significantly after cells had entered the stationary phase at ~35 h. The final (maximum) cell density was lower at pH 5.8 than at pH 5.0 and 5.5. Also, compared to the lower pHs, much less acids (acetate and butyrate) but more alcohols (butanol and ethanol) were produced at pH 5.8. The final butanol concentration reached ~6.5 g/L at pH 5.0, ~12.0 g/L at pH 5.5, and ~17.2 g/L at pH 5.8, with corresponding butanol yields of 0.20 g/g at pH 5.8 and ~0.10 g/g at pH 5.0 and 5.5 (see Table 4). The higher butanol yield at pH 5.8 can be attributed to the lower acid production. At pH 5.0 and 5.5, 15.7–18.2 g/L of butyrate and 4.8–5.7 g/L of acetate were produced, while acid production decreased to ~8.3 g/L butyrate and ~3.3 g/L acetate at pH 5.8. The butanol productivity was also much higher at pH 5.8, 0.29 vs. 0.19 g/L h at pH 5.5 and 0.11 g/L h at pH 5.0. Clearly, pH 5.8 was more favorable for alcohol production, probably because it was closer to the optimal pH of ~6.0 for the bifunctional aldehyde/alcohol dehydrogenase, which catalyzes the biosynthesis of butanol and ethanol from butyryl-CoA and acetyl-CoA, respectively (Fontaine et al. 2002).

However, Ct(Δ*ack*)-pGluI was unable to consume maltose to support its growth at pH 6.0. As shown in Table 2, Aglu activity was compromised at pH above 6.0. Since Aglu was exported to the cell membrane, its activity and stability would be sensitive to the medium pH. Similar phenomena have also been reported for other extracellular amylolytic enzymes found in *C. acetobutylicum*, which also suffered from low activity and poor stability under inappropriate pH conditions (Paquet et al. 1991; Soni et al. 1992; Madihah et al. 2001). The optimal pH for Ct(Δ*ack*)-pGluI to produce butanol from maltose was thus determined to be 5.8, compromising between butanol biosynthesis and Aglu I activity. Similar pH effects were also observed with Ct(Δ*ack*)-pGluII (data not shown), which also had the best butanol production when the pH was controlled at 5.8.

Fermentation kinetics using maltose as substrate

Figure 2 shows batch fermentation kinetics of maltose at pH 5.8 by Ct(Δ*ack*)-pGluI, Ct(Δ*ack*)-pGluII, and Cac 824. As expected, all strains were capable of growing on maltose to produce butanol, butyrate, acetate, and ethanol. For Cac 824, acetone was also produced as a main product. However, the fermentation with Ct(Δ*ack*)-pGluI was faster than Ct(Δ*ack*)-pGluII and Cac 824, indicating a more robust α-glucosidase encoded by *agluI* in the hydrolysis of maltose, which was

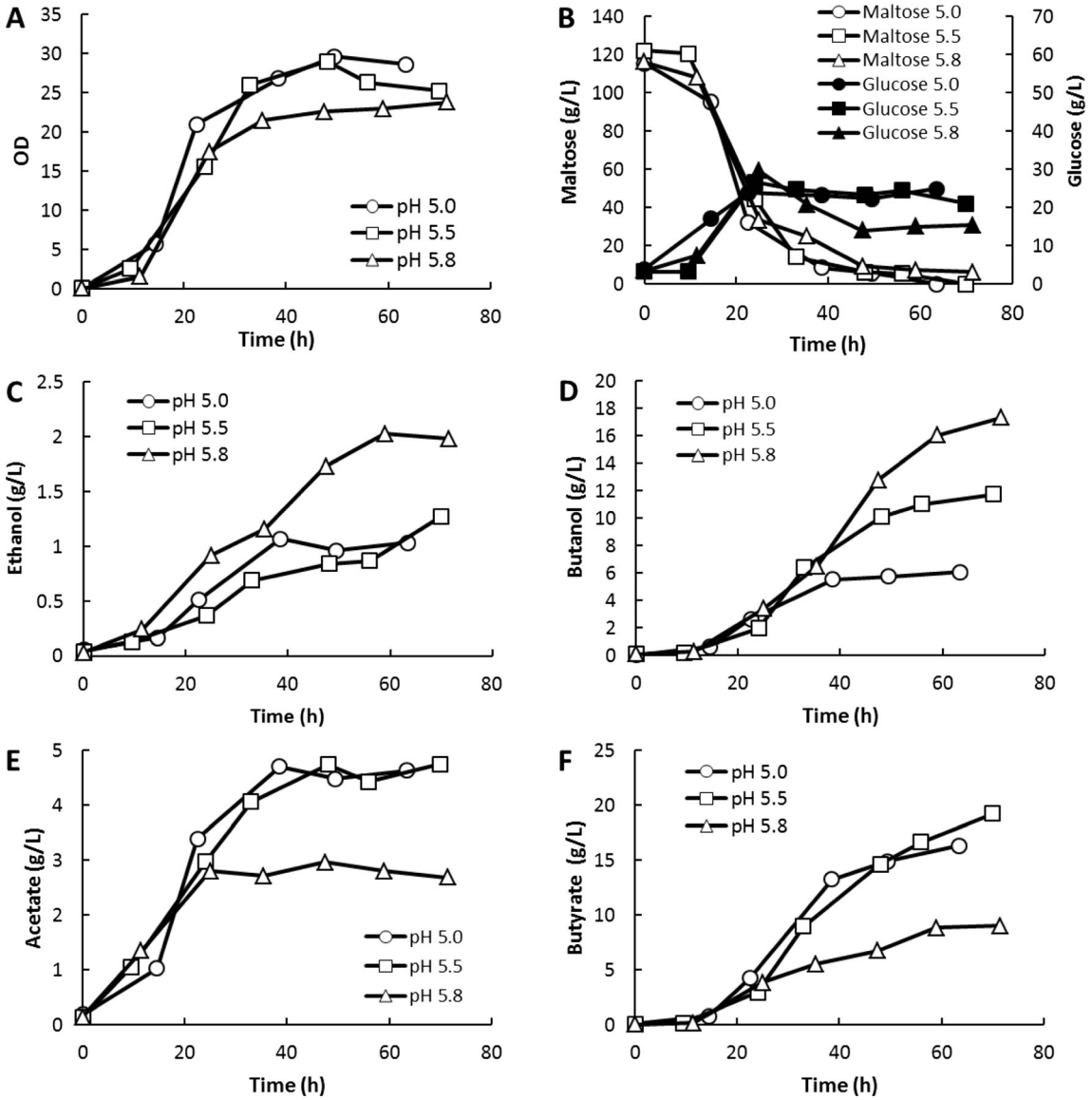

Fig. 1 Effects of pH on cell growth (OD); maltose consumption; and butyrate, acetate, ethanol, and butanol production in batch fermentations by *C. tyrobutyricum* Ct(Δ*ack*)-pGluI with pH controlled at 5.0 (*circle*), 5.5 (*square*), and 5.8 (*triangle*). The fermentation kinetics was studied with the medium containing 30 μg/mL thiamphenicol

consistent with the higher enzyme activity for Ct(Δ*ack*)-pGluI. Ct(Δ*ack*)-pGluI also produced more butanol (17.2 g/L) than Ct(Δ*ack*)-pGluII (11.2 g/L) and Cac 824 (11.2 g/L) did, giving a higher butanol productivity of 0.29 g/L h (vs. 0.17 g/L h for Ct(Δ*ack*)-pGluII and 0.10 g/L h for Cac 824) (see Table 4). The butanol yield from maltose by Ct(Δ*ack*)-pGluI was 0.20 g/g, which was comparable to that by Cac 824 and ~82 % higher than that of Ct(Δ*ack*)-pGluII (0.11 g/g). Ct(Δ*ack*)-pGluI also produced significantly more butyrate (8.3 vs. 5.6 g/L for Ct(Δ*ack*)-pGluII and 2.5 g/L for Cac 824), while similar levels of ethanol and acetate were produced by all three strains. The lower butyrate production (accumulation) by Cac 824 was attributed to its CoA-transferases, which reassimilated butyrate and acetate, leading to the production of acetone to ~8.8 g/L. Overall, Ct(Δ*ack*)-pGluI has good maltose catabolism and butanol synthesis capabilities and would be a better choice for industrial butanol production from maltose.

It should be noted that growth on maltose by Ct(Δ*ack*)-pGluI was much slower at 0.14/h compared to the control strain Ct(Δ*ack*)-pM2 (0.24/h) on glucose (see Table 4). The slower growth could be attributed to the additional metabolic burden in expressing Aglu I for maltose hydrolysis. The total duration of the maltose fermentation was thus longer (~70 vs. ~35 h for Ct(Δ*ack*)-pM2). However, more butanol was produced from maltose fermentation compared to glucose by Ct(Δ*ack*)-pM2, which produced ~9.5 g/L of butanol, at a

Table 4 Fermentation kinetics of *C. tyrobutyricum* and *C. acetobutylicum* grown on maltose and starch

Strain	Sugar	pH	Specific growth rate (/h)	Butanol			Butyrate (g/L)	Acetate (g/L)	Ethanol (g/L)	Acetone (g/L)
				Titer (g/L)	Yield (g/g)	Productivity (g/L h)				
Ct(Δ*ack*)-pM2	Glucose	6.0	0.24±0.02	9.5±0.4	0.10±0.01	0.16±0.01	22.5±0.8	9.0±0.4	2.1±0.4	–
Cac 824	Maltose	5.0	0.17±0.00	11.2±0.5	0.20±0.02	0.10±0.02	2.5±0.3	3.0±0.3	1.7±0.7	8.8±0.5
	Starch	5.0	0.16±0.01	8.8±0.2	0.14±0.02	0.10±0.03	2.1±0.6	2.6±0.7	2.2±0.9	6.0±1.1
Ct(Δ*ack*)-pGluII	Maltose	5.8	0.12±0.01	11.2±0.3	0.11±0.01	0.17±0.01	5.6±0.3	2.7±0.2	2.0±0.2	–
	Starch	5.8	0.10±0.01	13.4±0.5	0.14±0.01	0.13±0.01	11.7±1.0	7.5±0.8	2.6±0.6	–
Ct(Δ*ack*)-pGluI	Maltose	5.8	0.14±0.03	17.2±0.2	0.20±0.02	0.29±0.01	8.3±0.9	3.3±0.8	2.5±0.7	–
		5.5	0.16±0.01	12.0±0.3	0.11±0.01	0.19±0.01	18.2±1.0	4.8±0.1	1.7±0.5	–
		5.0	0.20±0.01	6.5±0.3	0.07±0.01	0.11±0.01	15.7±0.9	5.7±1.0	1.3±0.3	–
	Starch	5.8	0.12±0.01	16.2±0.2	0.17±0.02	0.20±0.01	9.5±1.2	4.5±0.3	2.7±0.2	–
Ct(Δ*ack*)-pGluI no antibiotics	Maltose	5.8	0.24±0.01	17.3±0.3	0.17±0.01	0.40±0.01	13.1±0.1	3.0±0.3	2.6±0.8	–

Data shown are mean±SD (n=2)

much lower yield of 0.10 g/g (vs. 0.20 g/g by Ct(Δ*ack*)-pGluI). This was because faster cell growth led to higher butyrate production (accumulation) and thus limited butanol biosynthesis, which competed for the same substrate, butyryl-CoA with the native butyrate biosynthesis pathway (Yu et al. 2011). Therefore, it would be desirable to produce butanol from maltose, instead of glucose, not only for the lower substrate cost but also for the higher product yield.

Fermentation kinetics using soluble starch as substrate

Figure 3 shows batch fermentation kinetics at pH 5.8 with soluble starch as the substrate. The soluble starch used in this study had a dextrose equivalent (DE) value of 25 or 4–5 degrees of polymerization. Because of the longer α-1,4-glycosidic polymer chain, the hydrolysis and fermentation of soluble starch were slower than with maltose. Unlike in maltose fermentation, the glucose released from starch hydrolysis was maintained at a relatively low level of <5 g/L, with some maltose, maltotriose, and maltodextrin also present at a lower amount, for all three strains studied, indicating that starch hydrolysis could be limiting the fermentation. Similar to the results with maltose, Ct(Δ*ack*)-pGluI performed better with faster starch consumption and higher butanol production than Ct(Δ*ack*)-pGluII, again confirming that Aglu I was more robust than Aglu II in breaking down the α-1,4-glycosidic bonds in starch. Ct(Δ*ack*)-pGluI produced ~16.2 g/L butanol from ~97 g/L starch in ~80 h, while Ct(Δ*ack*)-pGluII produced ~13.4 g/L butanol from ~90 g/L starch in ~105 h. In contrast, Cac 824 produced ~8.8 g/L butanol and ~6.0 g/L acetone from ~71 g/L starch in ~80 h. Clearly, Ct(Δ*ack*)-pGluI gave higher butanol yield (0.17 vs. 0.14 g/g for Ct(Δ*ack*)-pGluII and Cac 824) and productivity (0.20 vs. 0.13 g/L h for Ct(Δ*ack*)-pGluII and 0.10 g/L h for Cac 824) and would be the better choice for industrial butanol production from starch. All fermentation kinetics data for these strains are summarized in Table 4 for direct comparison.

It is noted that only 89.5, 80.9, and 71.2 % of soluble starch in the medium was hydrolyzed and consumed in the fermentation by Ct(Δ*ack*)-pGluI, Ct(Δ*ack*)-pGluII, and Cac 824, respectively. This is because complete (efficient) starch hydrolysis usually requires multiple amylases, including α-amylase for starch liquefaction, β-amylase and glucoamylase or α-glucosidase for saccharification, and de-branching enzyme such as pullulanase to break down α-1,6-glycosidic linkage. The lack of pullulanase would not allow the complete utilization of soluble starch, which has a high percentage (>5 %) of maltodextrin with α-1,6-glycosidic bonds. Interestingly, Cac 824, a native starch-consuming strain, showed inferior ability in hydrolyzing and using starch in the fermentation at a rate of 1.01 g/h, which was much slower than that for Ct(Δ*ack*)-pGluI (1.52 g/h). Clearly, constitutively overexpressing *agluI* in Ct(Δ*ack*)-pGluI gave a more robust starch hydrolysis ability than the highly regulated starch hydrolysis mechanism used in Cac 824 involving multiple genes for a phosphotransferase system (PTS) for maltose transport, two α-amylases, a glucoamylase, a pullulanase, and two α-glucosidases, which may have to be induced with maltose, instead of starch itself (Servinsky et al. 2010), and may be repressed by glucose accumulated in the medium. In addition, the redundancy in expressing multiple enzymes and transport systems in Cac 824 could overload the cells with excessive ATP consumption and result in lower cell growth and metabolic activity.

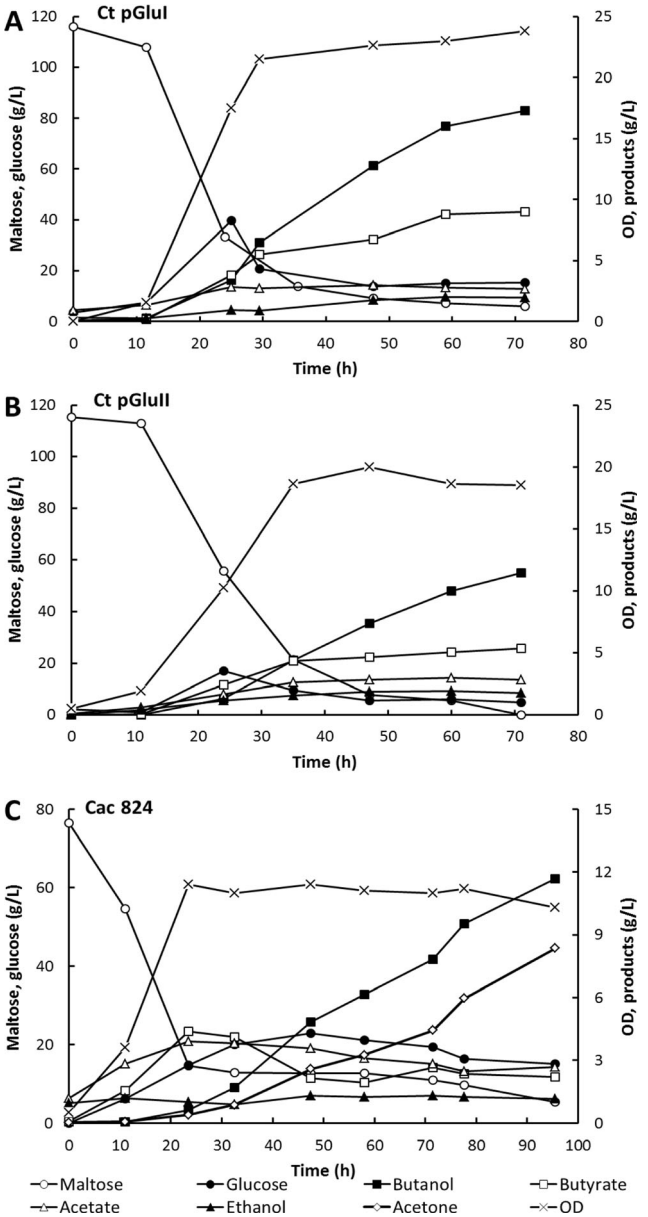

Fig. 2 Kinetics of batch fermentations of maltose by *C. tyrobutyricum* Ct(Δ*ack*)-pGluI (**a**) and Ct(Δ*ack*)-pGluII (**b**) at pH 5.8 and *C. acetobutylicum* ATCC 824 (**c**) at pH 5.0. For Ct(Δ*ack*)-pGluI and Ct(Δ*ack*)-pGluII, the fermentation was studied with the medium containing 30 μg/mL thiamphenicol

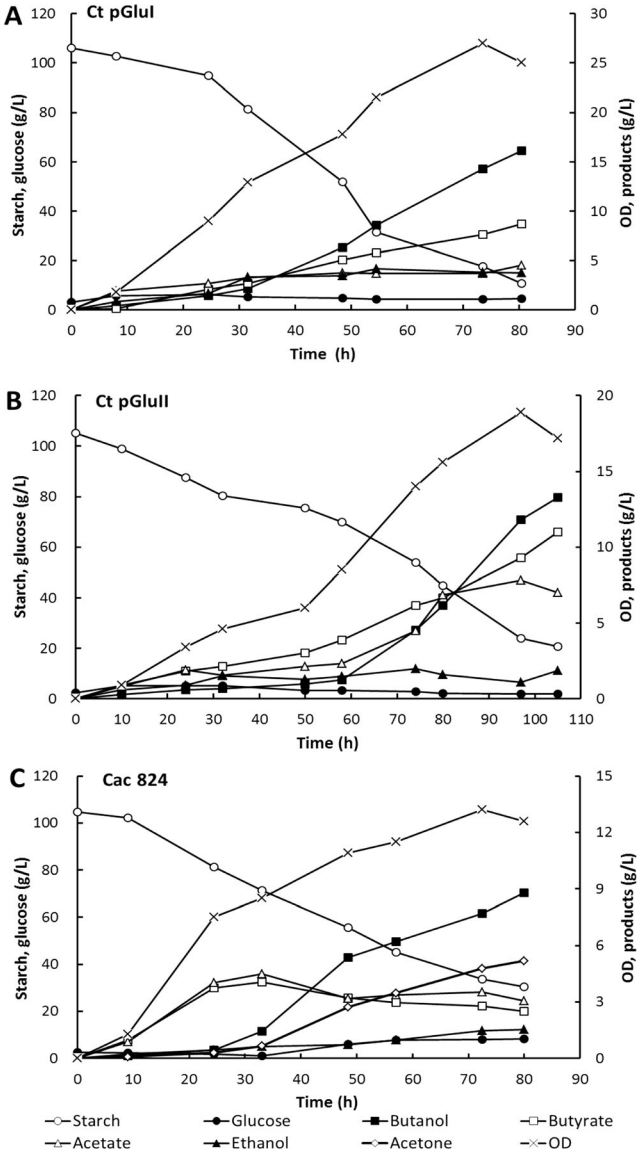

Fig. 3 Kinetics of batch fermentations of soluble starch by *C. tyrobutyricum* Ct(Δ*ack*)-pGluI (**a**) and Ct(Δ*ack*)-pGluII (**b**) at pH 5.8, and *C. acetobutylicum* ATCC 824 (**c**) at pH 5.0. For Ct(Δ*ack*)-pGluI and Ct(Δ*ack*)-pGluII, the fermentation was studied with the medium containing 30 μg/mL thiamphenicol

Fermentation kinetics without antibiotics

In the aforementioned fermentation kinetics studies, thiamphenicol was added in the fermentation medium to ensure that cells maintain the plasmids and express the heterologous genes. However, the addition of an antibiotic as the selection pressure is undesirable, as it would not only add cost but also impose additional metabolic burdens on cells in the fermentation. Since *C. tyrobutyricum* is a native maltose auxotroph, incapable of hydrolyzing or uptaking maltose, only the cells with cell-associated Aglu activity can survive in the medium with maltose as the sole carbon source. Therefore, theoretically maltose (and soluble starch) can be used as the selection pressure for the recombinant *C. tyrobutyricum* carrying the heterologous *aglu* (and *adhE2*) genes, allowing for stress-free cell-associated Aglu expression and fermentation to produce butanol in the absence of thiamphenicol. To prove this hypothesis, the fermentation without antibiotics addition was conducted with Ct(Δ*ack*)-pGluI and maltose as the carbon source, and the results are shown in Fig. 4. As expected, cells appeared to be more robust in the fermentation without antibiotics, growing at a faster rate of 0.24/h (vs. 0.14/h with

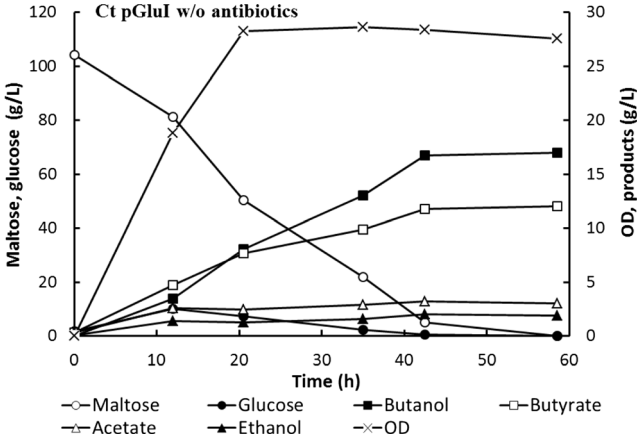

Fig. 4 Batch fermentation kinetics of *C. tyrobutyricum* Ct(Δ*ack*)-pGluI at pH 5.8 with maltose as carbon source in the absence of thiamphenicol

antibiotics) with almost no lag phase (vs. ~12 h with antibiotics) and reaching a higher cell density of OD 28 (vs. <24). Consequently, the fermentation was faster, consuming ~100 g/L of maltose and producing ~17.3 g/L of butanol in <45 h (vs. ~70 h with antibiotics). The amount of butanol produced in the absence of antibiotics was comparable to that with antibiotics, but at a much higher productivity of 0.40 g/L h (vs. 0.29 g/L h with antibiotics). It should be noted that the maltose hydrolysis (consumption) rate in the fermentation was comparable to that with antibiotics, indicating similar *agluI* expression level and Aglu I activity under both conditions (with or without antibiotics). However, the amount of glucose present in the fermentation medium was significantly lower at <10 g/L and there was no sharp accumulation of glucose, as observed in the fermentation with antibiotics (see Fig. 2a), which imposed additional metabolic burden and impeded cell growth and glucose consumption. Although butanol productivity was higher in the absence of antibiotics, butanol yield was slightly lower at 0.17 g/g (vs. 0.20 g/g) due to the higher cell growth and butyrate production (13.1 vs. 8.3 g/L). Butanol yield was usually higher when less butyrate was produced since both butyrate and butanol competed for the same precursor, butyryl-CoA, and a faster butyrate biosynthesis would result in lower butanol production (Yu et al. 2011).

Segregational stability

The segregational stability of pGluI in *C. tyrobutyricum* was investigated to confirm the plasmid stability in Ct(Δ*ack*)-pGluI in fermentation without applying the antibiotic selection pressure. In general, the plasmid pGluI was highly stable in *C. tyrobutyricum* even grown on glucose without a selection pressure (P=99.5±0.1 %), comparable to that found for pMTL82151 in a previous study (Yu et al. 2012). An even higher stability of 99.8±0.1 % was observed when cells were grown on maltose, confirming that maltose could also function as a selection pressure in the absence of the antibiotic. It is thus convincible that the expression of Aglu would be stable even when the hydrolytic product of maltose, glucose, was accumulated in the fermentation medium.

Discussion

ABE fermentation of corn (starch) with *C. acetobutylicum* has long been used in industrial production of *n*-butanol and acetone (Jones and Woods 1986). Starch and maltose are composed of glucose, and they share the same sugar metabolism in Cac 824. Based on the available genome data, Cac 824 has a putative maltose PTS (MalP), a maltose-6′-P glucosidase (MalH), two α-amylases, one glucoamylase, one pullulanase, and two α-glucosidases (Aglu I, Aglu II) (Tangney et al. 2001; Servinsky et al. 2010). Starch is first hydrolyzed extracellularly to maltose and glucose. There are two glucose PTS transporters: one is constitutively expressed, and another is induced by glucose (Servinsky et al. 2010). There are also two systems for maltose metabolism. One involves MalP, which phosphorylates and transports maltose into cell, and MalH, which hydrolyzes maltose-6-P to glucose and glucose-6-P (Thompson et al. 2004). The second system involves two putative α-glucosidase genes, *agluI* (CAC2252) and *agluII* (CAC2891), regulated by maltose regulator MalR (Novichkov et al. 2010). Both of these two systems are induced by maltose and starch and repressed in the presence of glucose through catabolite repression, which could limit starch and maltose utilization by Cac 824 in the fermentation as observed in this study.

In the present study, we introduced the α-glucosidase genes (*agluI*, *agluII*) from Cac 824 into *C. tyrobutyricum* and enabled the latter to use maltose and soluble starch for butanol production at a higher rate than Cac 824. Aglu I and Aglu II, consisting of 1157 and 1217 amino acids, respectively, have been annotated based on their genomic sequences but have not been well characterized (Servinsky et al. 2010). This study not only demonstrated the high activity of these two enzymes for starch and maltose hydrolysis, but also, for the first time, confirmed that these two enzymes are indeed translocated outside the cell as predicted by the presence of secretion (signal) peptide sequences on their N-terminal (Bendtsen et al. 2004). The improved fermentation can be attributed to the fact that α-glucosidase is constitutively expressed in *C. tyrobutyricum* without subjecting to gene regulation or glucose repression. The engineered mutant strain Ct(Δ*ack*)-pGluI is also genetically stable and can thus be used for industrial production of *n*-butanol from low-cost starch and maltose, which is a preferred substrate (than glucose) because of more balanced growth resulting in higher butanol (and lower butyrate) production. Soluble starch is produced after treating starch with α-amylase and is easier to use in fermentation than starch and cheaper

than glucose. Maltose is abundant in most germinating grains, which has been widely used in the brewing industry (Boulton and Quain 2006). The abundance and the economics of germinating grains could provide an economic feedstock for direct maltose fermentation in future industrial production of biobutanol.

Since only cells with Aglu activity can survive in a medium containing maltose or soluble starch as the sole carbon source, *aglu* can be used as a marker for selecting the transformants coexpressing a target gene with *aglu*. This could provide an antibiotic-free cloning vector for future strain construction. Antibiotic resistance genes are usually used for selection in cloning. However, the limitation in the available selective antibiotics has been an obstacle for cloning, especially for *Clostridium* (Al-Hinai et al. 2012; Heap et al. 2009). Using antibiotics during culture growth and fermentation to maintain the recombinant plasmids would increase costs, impose additional stress on cell growth and metabolism, which could negatively impact the fermentation performance, and raise concerns on the release or exposure of antibiotics to the environment, especially in large-scale industrial fermentation. Furthermore, many bacteria could mutate spontaneously to become antibiotic resistant without carrying the antibiotic resistance gene in the recombinant plasmid, rendering the antibiotic selection ineffective. Although stable mutants can be developed by gene knock-in or integrated into chromosome, without requiring the cell to carry the plasmids, the available techniques are limited by the relatively low efficiency in transformation, gene integration, and gene expression (Al-Hinai et al. 2012; Heap et al. 2007; Xu et al. 2015). Using the *algu* plasmid and a maltose auxotroph such as *C. tyrobutyricum* can provide a novel expression system with self-selection ability for stable clone maintenance and large-scale fermentation without antibiotics addition, as demonstrated in this study. It is thus desirable to further exploit the ability of using maltose for selectively culturing the transformant carrying the plasmid containing *aglu* gene as a useful tool for genetically engineering *C. tyrobutyricum* and other maltose auxotrophs incapable of hydrolyzing or uptaking maltose.

In conclusion, two α-glucosidases from *C. acetobutylicum* ATCC 824 were cloned and expressed in *C. tyrobutyricum* for butanol production from maltose and soluble starch. The mutant expressing *agluI* and *adhE2* showed multiple advantages in fermentation compared to *C. tyrobutyricum* expressing only *adhE2* (using glucose) and *C. acetobutylicum* ATCC 824 using maltose and soluble starch as substrate, including higher butanol titer, yield, and productivity. The mutant strain is stable without antibiotics and is thus a desirable candidate for use in future large-scale fermentation for *n*-butanol production from low-cost maltose and soluble starch.

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Compliance with ethical standards This research does not involve human participants or animals.

Conflict of interest The authors declare no conflict of interests.

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