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ORIGINAL PAPER

Enhanced butanol production from cassava with *Clostridium acetobutylicum* by genome shuffling

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Abstract To obtain strains exhibiting high levels of solvent tolerance and butanol production, wild type strains of *Clostridium acetobutylicum* butanol-producing strain GX01 and *Lactobacillus mucosae* butanol-tolerant strain M26 were subjected to mutagenesis combining *N*-methyl-*N*-nitro-*N*-nitrosoguanidine induction with genome shuffling. After four successive rounds of genome shuffling, the *C. acetobutylicum* shuffled strain GS4-3 showing greater levels of fermentation performances (such as secreting a higher level of amylase, improving the thermal stability, and possessing greater environmental robustness) compared to the wild type strains was isolated. As a result, after optimization of culture conditions, mutant GS4-3 produced 32.6 g/L of total solvent, 20.1 g/L of butanol production, and 0.35 g/L/h of butanol productivity, which were, respectively, increased by 23.5, 23.3, and 40.0 % than the wild-type strain GX01, in a 10 L bioreactor. The enhanced production of butanol and tolerance of solvent of mutant associated with GS4-3 make it promising for acetone/butanol/ethanol fermentation from cassava (*Manihot esculenta*).

Graphical Abstract Compared to the parental strain (blue), the shuffled strain exhibited the more excellent performances for butanol production from different carbon sources.

Shu-Bo Li and Yi Qian contributed equally to this work.

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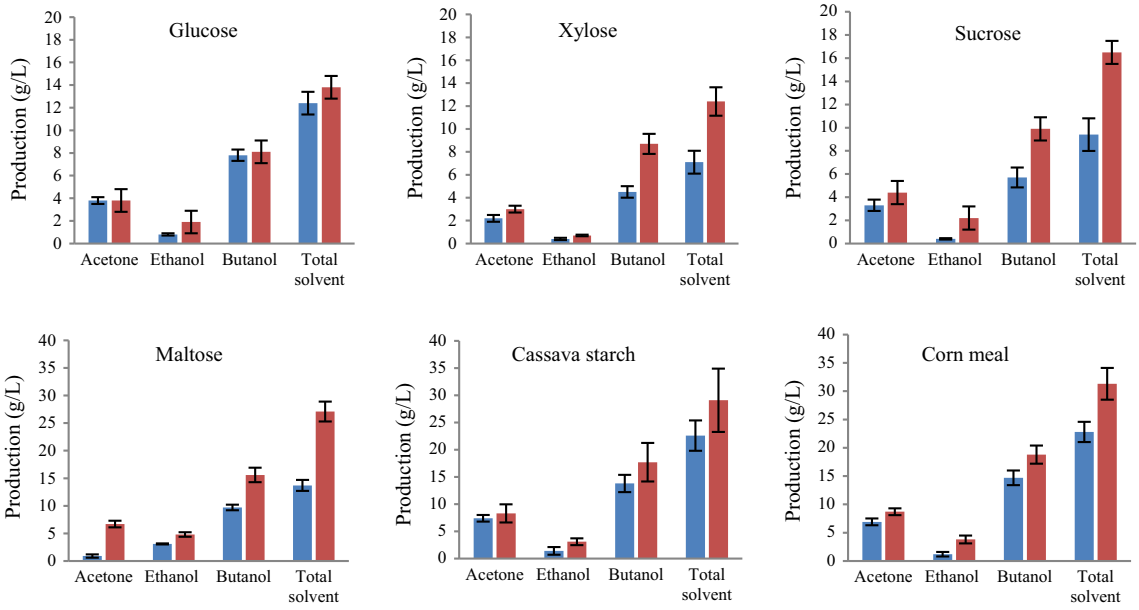

Keywords Butanol · Cassava · *Clostridium acetobutylicum* · *N*-methyl-*N*-nitro-*N*-nitrosoguanidine mutation · Genome shuffling

Introduction

In addition to a multi-purpose chemical feedstock, butanol can be regarded as one of the next-generation biofuels with remarkable advantages compared to bioethanol; butanol is less explosive and is associated with lower levels of corrosion and evaporation (Lee et al. 2008). The global depletion of fossil energy sources has prompted new interest in butanol; production has been established, opening new opportunities and presenting challenges for the biofuel industry. Recently, butanol production by acetone/butanol/ethanol (ABE) fermentation using clostridial species (including *Clostridium acetobutylicum* and *Clostridium beijerinckii*) has gained much improvement (Li et al. 2013). At the same time, however, this approach has suffered from an impact of butanol toxicity and feedstock cost, resulting in low yield and productivity, and high recovery and production cost (Knoshaug and Zhang 2009). Thus, obtaining higher butanol-yielding and butanol-tolerant strains that can use a less expensive substrate is vital for realizing the commercialization of ABE fermentation.

At present, various degrees of success have been obtained for improving butanol tolerance in microorganisms by classical improvement methods and modern molecular biotechnology (Mann et al. 2012). Owing to limited insight into the physiology and genetics of organisms available for butanol biosynthesis (Olano et al. 2008; Qureshi et al. 2007), serious impediments (including expensive and time-consuming procedures and the need for characterized genes) have been encountered for realizing desired phenotypes (Gong et al. 2009). Fortunately, genome shuffling, integrating classic and modern strain improvement approaches, was developed to improve the metabolic pathway and other complex phenotypes for uncharacterized bacteria (Patnaik et al. 2002). Similar to the effective whole-cell engineering approach, genome shuffling has been applied successfully for rapid improvement of microbial phenotypes and enhancing the production of target substances, including hydroxycitric acid (Hida et al. 2007), ethanol (Gong et al. 2008) and D-lactic acid (Zheng et al. 2010). However, there is a paucity of published work describing efforts to improve the ABE fermentation properties of *C. acetobutylicum* by genome shuffling (Gao et al. 2012).

The high cost of feedstock is another important factor for limiting the commercial use of ABE fermentation. To lower the cost, cheap substrates [including cassava (Thang et al. 2010), molasses (Ni et al. 2012), wheat bran (Liu et al. 2010) and lignocellulosic materials (Baral and Shah 2014)] have been studied for ABE fermentation. Cassava (*Manihot esculenta* Crantz), the world's fourth most important agricultural crop, is grown on arid and semiarid land in many countries (including in Africa, Asia and Latin America) and contains the maximum theoretical concentration of starch (dry weight basis) among food crops (Dai et al. 2006). As a comparatively cheap raw material and nonfood crop in China, Thailand and Vietnam, cassava has been used for the production of several important chemicals, including ethanol (Choi et al. 2010) and L-lactic acid (Wang et al. 2010). Owing to its high carbohydrate content and low price, cassava can be used as an excellent substrate for ABE fermentation with

regard to both economic and geographical considerations. There are some problems, however, in using ABE fermentation of cassava starch. For example, owing to the lack of nitrogen source, the concentration of butanol and total solvent produced from cassava were only 13.0 and 19.4 g/L, respectively, which is far below the requirements of industrial production (Gu et al. 2009).

In this study, the aim was to breed high-yield producers of butanol by *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (NTG) mutagenesis and genome shuffling. The protocols for isolation, regeneration of protoplasts and successive rounds of protoplast fusion between *C. acetobutylicum* and *Lactobacillus mucosae* are described. A high producer of butanol, shuffled strain GS4-3, was obtained and its butanol productivity was determined and confirmed.

Materials and methods

Materials

Cassava (*M. esculentis* Crantz), potato (*Solanum tuberosum* L.), corn (*Zea mays*) and other carbon sources were obtained from Yulin County in Guangxi Province, China. Cassava starch (raw starch content 65–70 %, w/w) and other starch-based substrates were liquefied by incubation with 3.5 U/g of α-amylase (Termamyl SC, Novozymes Corp., Denmark) at 90 °C for 30 min before using. Lysozyme and phosphoenolpyruvate (PEP) were purchased from Sigma (USA); polyethylene glycol (PEG 6000) was purchased from Sinopharm Chemical Reagent Co. Ltd (China). All other chemicals used in this work were of analytical grade.

Media and culture conditions

Medium (A) for enriching a butanol-tolerant strain (A) contained cassava starch (90 g/L), peptone (5 g/L) and butanol (20 g/L). Medium B for further isolation contained: yeast extract (3 g/L), peptone (5 g/L), glucose (20 g/L), KH_2PO_4 (1 g/L), $MgSO_4$ (0.5 g/L), agar (12 g/L) and butanol (15 g/L). Medium C consisted of: medium B with the addition of butanol (15, 18, 20 and 22 g/L) and lithium chloride (1.0 g/L) for mutant isolation. Medium D contained: $MgSO_4$ (0.2 g/L), K_2HPO_4 (0.5 g/L), yeast extract (2 g/L), peptone (5 g/L), glucose (10 g/L), sucrose (10 g/L) and $FeSO_4$ (0.01 g/L). Medium for regeneration (RM) contained: NaCl (41 g/L), $CaC1_2$ (4.0 g/L), $MgCl_2$ (5.0 g/L), $FeSO_4$ (0.01 g/L), yeast extract (2 g/L), peptone (2 g/L), glucose (10 g/L), sucrose (10 g/L), agar (6 g/L) and L-cysteine (1 g/L). Seed medium contained cassava starch (60 g/L) and peptone (5 g/L). Fermentation medium contained cassava starch (90 g/L) and soybean meal (5 g/L). All media were adjusted to pH 7.0.

The seed culture was prepared as follows: 10 % (v/v) of the stock of spore suspension was placed at 100 °C for 2 min for heat shock, and then incubated at 37 °C for 24 h without shaking. Butanol fermentation took place in a 10 L fermentor (New Brunswick Scientific, Enfield, CT, USA), using a working volume of 7.2 L. The inoculation size was 10 % (v/v), which was followed by passing O_2-free N_2 gas for 15 min to keep the bioreactor contents anaerobic. The pH was controlled at 7.0 and batch fermentation was maintained at 39 °C for 68 h.

Isolation and identification of the butanol-tolerant strain

Samples ($n = 30$) were collected from soil and from waste residues associated with agriculture and biogas digesters in Guangxi Province, China. Portions (2 g) were placed into tubes containing 20 mL of medium A and mixed thoroughly. After incubation at 37 °C for 48 h, cells were collected and suspended by centrifugation (10,000×*g* for 20 s) in sterile water and then diluted serially to an optical density at 600 nm (OD_{660}) of 1×10^0, 1×10^{-1}, 1×10^{-2} and 1×10^{-3}; 10 μL of each dilution was spread onto plates containing agar, medium B and different concentrations of butanol. The plates were sealed with adhesive vinyl plastic tape to prevent evaporation of butanol and incubated at 37 °C. Colonies that grew on medium B were selected to determine their butanol-tolerant capability; high butanol-tolerant strains were dissolved in 20 % (v/v) glycerol at −80 °C for storage. A highly butanol-tolerant strain was identified by 16S ribosomal DNA (rDNA) sequence analysis (using primers 5′-AGAGTTTGATCCTGGCTCAG-3′ and 5′-GGTTACCTTGTTACGACTT-3′). The results were compared with sequences in the GenBank databases using the Basic Local Alignment Search Tool (BLAST) software.

Mutagenesis by NTG induction and rational screening

Portions of NTG solutions (0.3 mL of 1.0 mg/mL solutions in 0.2 mol/L phosphate buffer, pH 6.0) were added to 0.7 mL of the cell suspension (after incubation for 24 h) of the parental strain GX01 preserved in our laboratory (producing 14.0 g/L of butanol; Nanning, China). The mixtures were incubated at 37 °C for 30 and 45 min before centrifugation at 8000×*g* for 10 min. The pellet was given three washes with 3 mL of 0.85 % (w/v) NaCl to terminate the reaction, suspended and incubated in 1 mL of the seed medium at 37 °C for 12 h before the cells were cultivated repetitively in selective medium C. The adaptive strains were diluted with sterile physiological saline and 0.2 mL of the cell suspension was spread onto and incubated on solid selection medium at 37 °C for 2–3 days. Subsequently,

growing mutant colonies were selected to determine their butanol-production ability.

Preparation of protoplast

Some cells of each mutant were cultured in medium D at 37 °C for 12 h, harvested by centrifugation, washed and suspended in protoplast formation buffer (PFB; 4.1 % (w/v) NaCl, 0.5 % (w/v) $MgCl_2$, 1.0 % (w/v) peptone, 0.4 % (w/v) $CaCl_2$, 0.1 % (w/v) L-cysteine, pH 7.0). The cell suspension was diluted to OD_{660} 0.2. Lysozyme (0.5 mL of 10 mg/mL) was added and the mixture was incubated at 37 °C for 30 min. Protoplasts were collected by centrifugation at 2500×*g* for 10 min and suspended in PFB (Minton and Morris 1983).

Protoplast inoculation and genome shuffling

According to the results of earlier studies (Chalopagorn et al. 2014; Wang et al. 2013), equal numbers of protoplasts from different populations were mixed and divided into two equal parts. One part was inactivated with ultraviolet (UV, 30 W) at a distance of 15 cm for 40 min and the other was heat-treated at 80 °C for 20 min. The inactivated protoplast preparations were mixed 1:1, centrifuged, suspended in 100 µL of PFB and 50 µL of 30 % (w/v) PEG6000 was added. After incubation at 37 °C for 15 min, 5 mL of PFB was added. The fused protoplasts were centrifuged and suspended in 1 ml of PFB. A sample (1 mL) was grown and regenerated on RM fluid medium at 37 °C for 12 h, then incubated in fluid RM medium with the addition of butanol at various concentrations (15, 18, 20, 25 and 30 g/L) at 37 °C for 7 days. After incubation, fusant was transferred and grown on RM plates for further isolation. The colonies with high butanol tolerance were selected for the measurement of butanol production. Subsequent rounds of genome shuffling were achieved by repeating the protoplast fusion as described.

Verification of mutant heredity stability

According to an earlier study (Yu et al. 2014), the positive mutants from each mutation experiment were transferred from agar slant medium to the next for nine generations, and each generation was cultured under the same conditions to determine the butanol production ability.

Analytical methods

A sample (10 mL) of broth was centrifuged at 10,000×*g* for 10 min, cells were harvested, washed three times with deionized water and suspended to the original volume with deionized water to determine cell concentrations, which are expressed as OD_{660}. According to the earlier study (Guo et al. 2012a; Li et al. 2015), the reducing sugar content was measured using the 3,5-dinitrosalicyclic acid method, and the concentrations of products (acetone, butanol and ethanol) were measured by a gas chromatograph (GC-12A, Shimadzu Corporation, Tokyo, Japan) equipped with a flame ionization detector and a PEG-20 M capillary column (30 m × 0.32 mm × 0.4 µm) using high-purity nitrogen (99.99 %) as the carrier gas.

During the ABE fermentation, 2 mL of broth was centrifuged (10,000×*g* for 2 min) and the supernatant fluid was used for measuring the α-amylase activity with an α-Amylase Assay Kit (Kikkoman Corp., Chiba, Japan). One unit (U) of α-amylase activity was defined as the release of 1 µmol of 2-chloro-4-nitrophenol within 1 min. All measurements were made in triplicate independent experiments, and the results are expressed as mean ± SE.

Results

Selection of starting strains for genome shuffling

Isolation and identification of high butanol-tolerant strain

In this study, 30 enriched samples were diluted and spread onto medium B under anaerobic conditions. Colonies that grew on the medium B plates were inoculated into medium C for testing butanol-tolerant capability. It was found that eight strains could resist more than 15 g/L of butanol stress, and the strain that achieved the highest stress, including 25 g/L of butanol but produced only about 10.0 g/L of butanol, was named M26 (Table 1). Additionally, according to the 16S rDNA sequence, the taxonomic experiment showed the 16 rDNA sequence of strain M26 shared 99 % identity with those of *Lactobacillus mucosae*, demonstrating strain M26 belonged to *L. mucosae*, which was named *L. mucosae* M26.

Selection of high butanol-producing strain by NTG mutagenesis

To further increase genetic diversity, the parental strain GX01 was treated with 1.0 mg/mL of NTG as described (Li et al. 2013). After mutagenesis in response to NTG, butanol-tolerant mutants were incubated on medium C. Only eight colonies were isolated following the addition of 15 g/L butanol and no colony was present following the addition of 20 g/L butanol. During ABE fermentation, mutants NTG-1 (14.6 g/L), NTG-2 (15.3 g/L), NTG-3 (14.8 g/L), NTG-4 (14.5 g/L) and NTG-5 (15.1 g/L) showed an increase in the production of butanol, from 14.1–15.3 g/L, compared to strain GX01 (Fig. 1). Additionally, butanol-producing stability was assayed by

Table 1 Isolation of strains selected for solvent tolerance and production of butanol

Strains	Butanol stress[a] (g/L)	Butanol production[b] (g/L)	Strains	Butanol stress (g/L)	Butanol production (g/L)
M11	20.0	7.8 ± 0.2	M22	18.0	7.7 ± 0.4
M12	18.0	6.5 ± 0.5	M23	18.0	8.2 ± 0.3
M13	20.0	9.5 ± 0.3	M26	25.0	10.1 ± 0.5
M21	15.0	8.3 ± 0.2	M27	20.0	8.8 ± 0.2

[a] Cells were grown in shake flasks with medium C at pH 7.0 for 3 days

[b] Cells were grown in shake flasks with fermentation medium for butanol production at pH 7.0 for 68 h. All experiments were done independently in triplicate and the results were expressed as mean ± SE

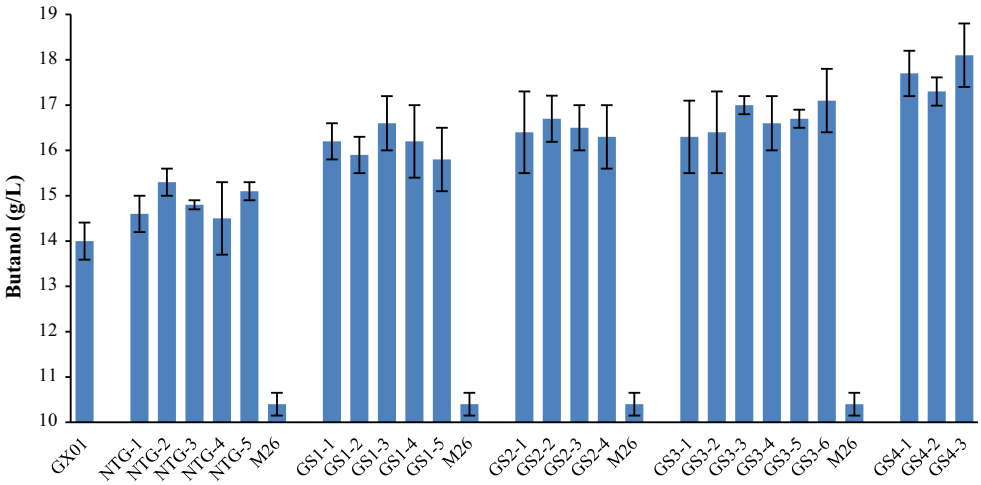

Fig. 1 Improvement of butanol production using the combination of NTG mutagenesis with genome shuffling. All experiments were done independently in triplicate and the results were expressed as mean ± SE

continuous subculture experiment and we found each strain could keep butanol-producing ability constant for 10 generations. Based upon the results above, five mutants and strain M26 were selected as the starting population for genome shuffling.

Improving the butanol-producing ability by genome shuffling

Four successive rounds of genome shuffling were carried out with the six strains (NTG-1–NTG-5 and M26) in an attempt to enhance production of the butanol mutants. As shown in Fig. 1, the production of butanol was increased steadily with each round of genome shuffling. After the first round, 15 colonies exhibiting butanol resistance of 18 g/L were screened for butanol production. Among these colonies, five mutants with higher butanol yield were named GS1-1 (16.2 g/L), GS1-2 (15.9 g/L), GS1-3 (16.6 g/L), GS1-4 (16.2 g/L) and GS1-5 (15.8 g/L) and used for the second fusion. During the second and third fusion, the production of butanol was increased slightly, reaching 16.7 and 17.1 g/L, respectively. Fortunately, the shuffled strain GS4-3, with 20 g/L butanol resistance and the highest level of butanol production (reaching 18.1 g/L, with an increase of 29.3 % compared to the parental strain GX01), was selected as a potential industrial production strain after the fourth round of genome shuffling. Meanwhile, the heredity stability of GS4-3 was assayed; its butanol production was stable and it exhibited excellent heredity stability (Fig. 2).

Investigation of physiological characteristics for mutant GS4-3

Superior physiological characteristics were obtained in mutant GS4-3 (Fig. 3). (1) This mutant could secrete a higher level of amylase, in which its highest level of amylase activity was 34.3 U/mL, with an increase of 1.4-fold compared to the parental strain GX01, decreasing the amount of α-amylase to be added. (2) The thermal stability of the mutant was improved slightly compared to the control (37 °C). The maximum relative growth ratio of mutant GS4-3 was 104.8 % at 39 °C and that of strain GX01 was 101.9 % at 35 °C. (3) Although the optimum pH of strains GX01 and GS4-3 was 7.0, mutant GS4-3 could grow better under acidic conditions (pH 5.0–6.0), which was favorable for butanol synthesis (Jiang et al. 2014). Mutant GS4-3 possessed greater environmental robustness and better performance compared to the parental strain GX01.

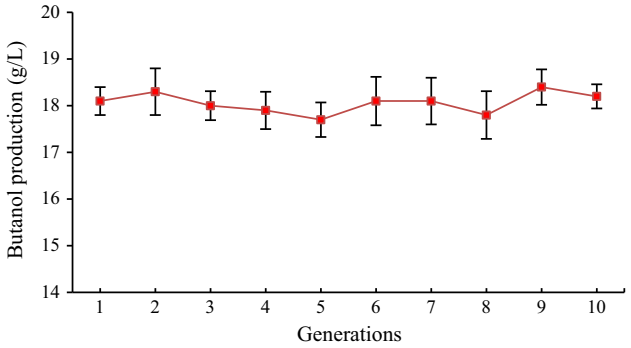

Fig. 2 Measuring the hereditary stability of strain GS 4-3 for butanol production through 10 generations. All experiments were done independently in triplicate, and the results were expressed as mean ± SE

Optimization of fermentation medium

Effect of various carbon sources on butanol production

The effects of different carbon sources on ABE fermentation were studied in order to investigate the feasibility of using cassava starch as substrate for the production of butanol. Mutant GS4-3 produced a higher level of butanol using various substrates, including glucose (8.1 g/L), xylose (8.7 g/L), sucrose (9.9 g/L), maltose (15.6 g/L), cassava starch (18.8 g/L) and corn meal (17.8 g/L), compared to strain GX01 (Fig. 4). Taking the cost of production into consideration, cassava starch was selected as a potential substrate for ABE fermentation; the production of acetone, ethanol, butanol and total solvent was 8.3, 3.1 and 17.8 g/L, which were increases of 12.2, 121.4 and 29.0 %, respectively, compared to strain GX01.

We investigated the effects of the initial concentration of cassava starch chosen for ABE fermentation. When the concentration of cassava starch was increased from 60 to 110 g/L, the butanol titer rose from 12.3 to 18.5 g/L but the butanol yield was decreased from 0.21 g/g to 0.17 g/g. Moreover, less butanol was produced when the initial concentration of cassava starch was increased by an extra 110 g/L (Table S1). Based upon these results, we chose 100 g/L as the initial concentration of cassava starch to be used as the substrate for ABE fermentation.

Effect of nitrogen source on butanol production

The effects of different organic and inorganic nitrogen sources on butanol production were investigated in order to

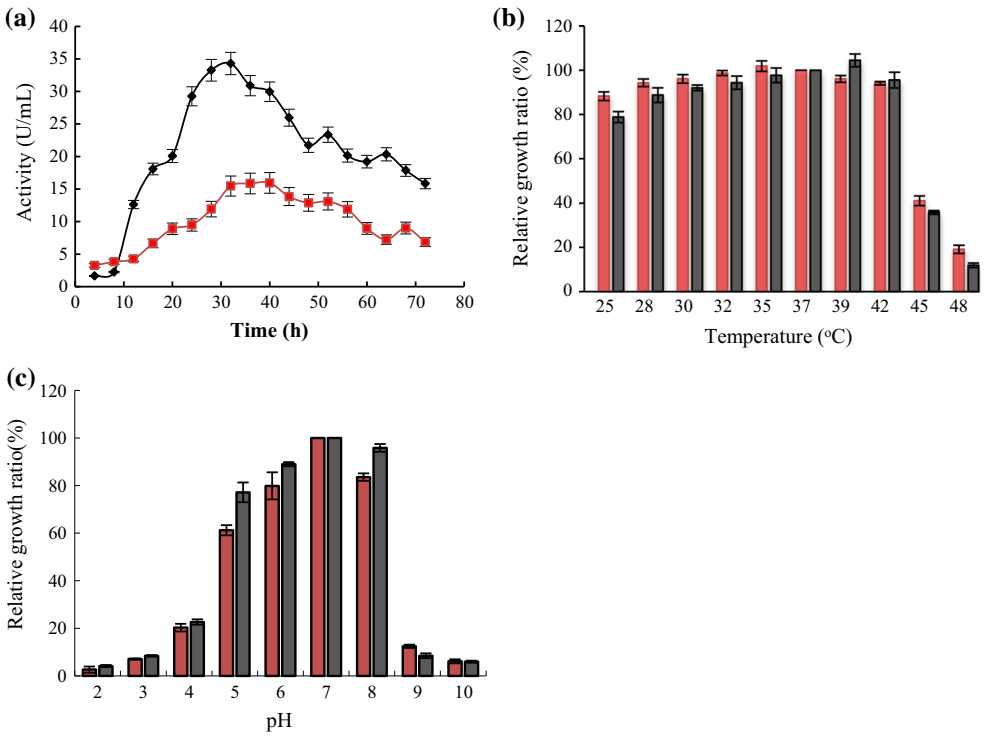

Fig. 3 The comparison of physiological characteristics between *C. acetobutylicum* GX01 (*red*) and mutant GS4-3 (*black*). Cells were grown in 200/250 mL of fermentation medium for 72 h and 2 mL samples were taken every 4 h for assay. **a** Amylase activity; cells were cultured for 48 h, measuring the effects on cell growth of **b** different temperatures and **c** different initial pH using 37 °C and pH 7.0 as the control, setting $OD_{660} = 2.11$ and $OD_{660} = 2.15$ as 100 % of relative growth ratio, respectively. All experiments were done independently in triplicate and the results were expressed as mean ± SE

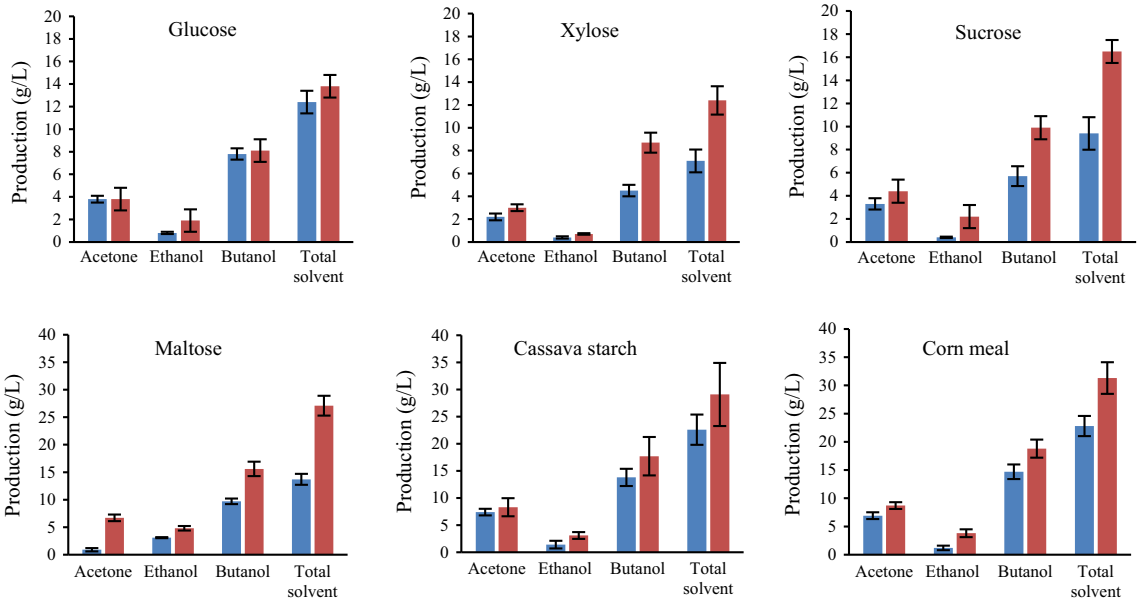

Fig. 4 Comparing the effects on ABE fermentation of different carbon sources between *C. acetobutylicum* GX01 (*blue*) and mutant GS4-3 (*red*). Cells were grown in 200/250 mL shake flasks for 68 h at pH 7.0. All experiments were done independently in triplicate, and the results were expressed as mean ± SE

Table 2 Effects of nitrogen sources on ABE fermentation with cassava flour

Nitrogen sources[a]	Acetone (g/L)[b]	Ethanol (g/L)	Butanol (g/L)	Total solvent (g/L)	Residual sugar (g/L)
Control	6.9 ± 0.2	3.3 ± 0.1	10.3 ± 0.8	20.5	26.7 ± 3.8
$(NH_4)_2SO_4$	5.8 ± 0.4	3.5 ± 0.2	15.6 ± 1.2	24.9	10.3 ± 1.8
$NH_4H_2PO_4$	6.8 ± 0.5	2.8 ± 0.2	15.8 ± 0.7	25.4	11.4 ± 3.1
NH_4NO_3	7.1 ± 0.1	4.8 ± 0.3	17.2 ± 1.0	29.1	9.7 ± 0.7
Urea	6.6 ± 0.4	4.7 ± 0.3	14.5 ± 0.8	25.8	10.1 ± 1.6
CH_3COONH_4	8.9 ± 0.5	3.6 ± 0.1	18.5 ± 0.9	30.6	8.3 ± 1.3
NH_4Cl	7.7 ± 0.3	4.5 ± 0.2	15.5 ± 1.1	27.7	13.2 ± 2.2
Yeast extract	8.1 ± 0.2	3.9 ± 0.3	14.9 ± 0.7	26.9	12.3 ± 4.1
Wheat bran	8.3 ± 0.5	3.4 ± 0.1	16.8 ± 1.2	28.5	10.7 ± 1.6
Peptone	8.3 ± 0.6	2.3 ± 0.1	14.7 ± 0.5	25.3	15.5 ± 2.1
Soybean meal	8.3 ± 0.2	2.9 ± 0.3	17.8 ± 1.4	29.0	7.3 ± 0.5
Peanut bran	7.1 ± 0.3	2.6 ± 0.3	15.5 ± 0.8	25.2	11.6 ± 1.9

Cells were grown in shake flakes at pH 7.0 for 60 h with 100 g/L of cassava flour

[a] The concentrations of inorganic and organic sources were 0.3 and 1 % (w/v), respectively

[b] All experiments were done independently in triplicate and the results were expressed as mean ± SE

identify the best nitrogen source for ABE fermentation. All the nitrogen sources we tested produced higher levels of butanol compared to the control and CH_3COONH_4 was the best nitrogen source for butanol production (18.5 g/L) by mutant GS4-3 (Table 2).

We used shake flasks supplemented with 0–9 g/L of ammonium acetate in an attempt to determine the optimum concentration of ammonium acetate for ABE fermentation from cassava starch by mutant GS4-3. When the addition of ammonium acetate was increased from 0 to 3 g/L, the production of butanol was increased from 10.3 to 18.4 g/L, but decreased along with any further increase of the concentration of ammonium acetate (Table S2). Therefore, 3 g/L ammonium acetate was selected as the initial concentration for ABE fermentation with cassava starch.

Effect of trace elements on butanol production

The effect of trace elements (including Fe^{2+}, Mg^{2+}, Cu^{2+}, Mn^{2+} and Ni^{2+}) was studied, in which the production of

butanol reached 18.1, 16.5, 15.2, 17.4 and 14.2 g/L, respectively (Fig. S1). Most of the trace elements we examined inhibited the accumulation of butanol slightly during ABE fermentation, which was not in accord with a report Mn^{2+} and Ni^{2+} were beneficial for ABE fermentation (Ni et al. 2012). It is possible the cassava starch we used contained sufficient trace elements for ABE fermentation.

Based upon the above results, the optimum fermentation medium and conditions for mutant GS 4-3 were determined as follows: 100 g/L cassava starch, 3 g/L ammonium acetate, pH 7.0 and temperature 39 °C.

Batch fermentation for butanol production

We used fed-batch fermentation under optimal conditions in a 10 L bioreactor to test the performance of mutant GS4-3 for ABE fermentation with parental strain GX01 as the control. Strain GX01 produced 26.4 g/L of total solvent, 16.3 g/L of butanol, 0.25 g/L of butanol in 1 h and 0.18 g/g of butanol during 66 h fermentation with glucose as the substrate (Fig. 5a). Under the same conditions, however, about 95 % of the glucose substrate was consumed by mutant GS4-3 during fermentation for 58 h, resulting in

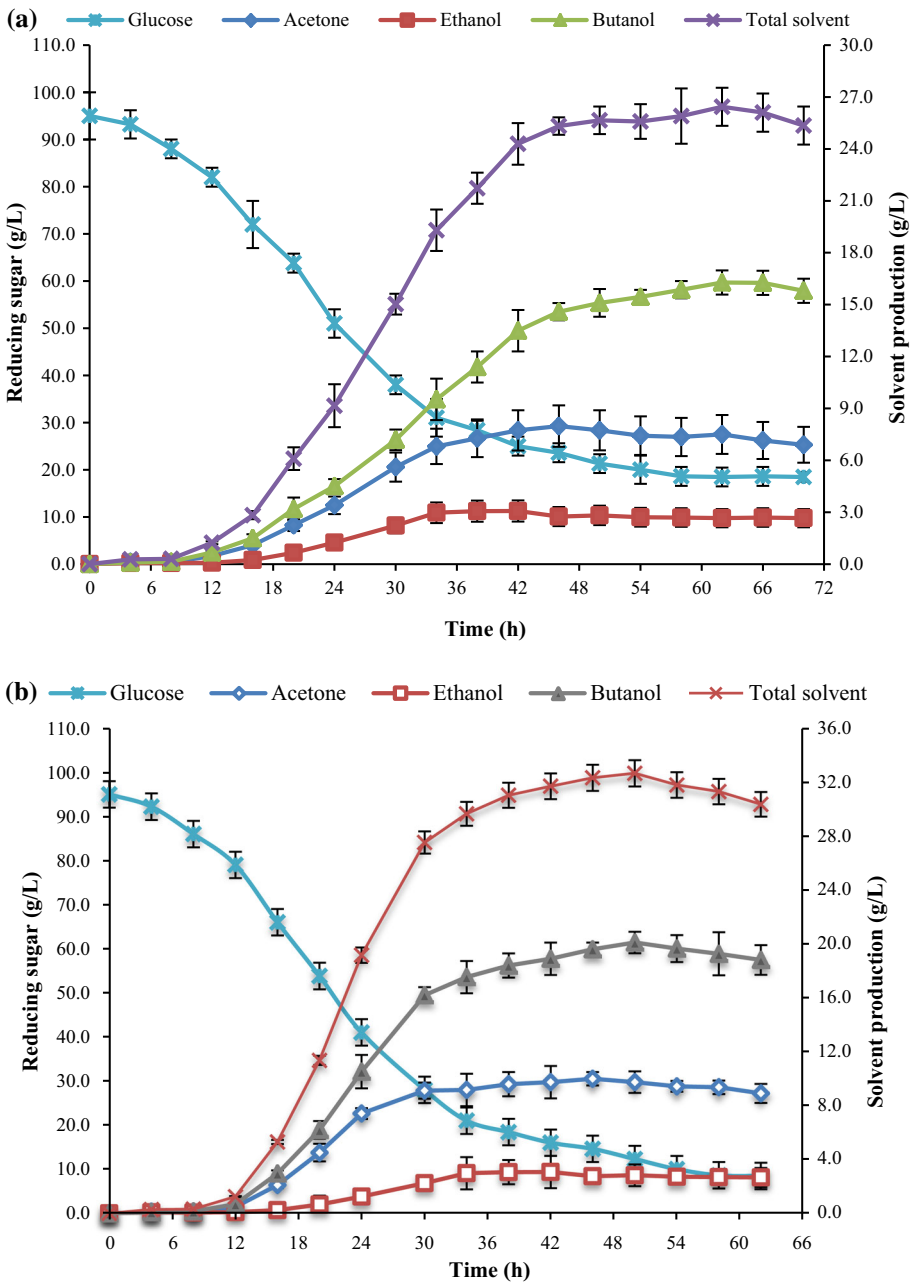

Fig. 5 Solvent production by batch fermentation of cassava starch in a 10 L fermenter: **a** Strain GX01 and **b** shuffled strain GS4-3. Cells were cultured in the working volume of 7.2 L at 39 °C for 68 h, the initial pH was adjusted to 7.0. All experiments were done independently in triplicate and the results were expressed as mean values ± SE

32.6 g/L of total solvent, 20.1 g/L of butanol, 0.35 g/L of butanol in 1 h and 0. 23 g/g of butanol, which were increases of 23.5, 23.3, 40.0 and 27.8 %, respectively, compared to strain GX01 (Fig. 5b). Furthermore, a small quantity of by-products (acetone and ethanol) were produced by strains GX01 and GS4-3, to concentrations of about 8.0 and 3.0 g/L with strain GX01 and about 10.0 and 3.0 g/L, respectively, for strain GS4-3. The results given above indicated mutant GS4-3 was superior to GX01 for ABE fermentation from cassava starch, and could be exploited as a high solvent-tolerant and butanol-producing strain for ABE fermentation.

Discussion

At present, efforts to increase production of butanol by fermentation with industrial strains are focused on (1) overexpression of production pathway enzymes by metabolic engineering and (2) optimization of enzyme expression by codon bias. These approaches depend completely on characterized details of the biosynthetic pathway and regulatory mechanism of organisms. Unfortunately, as for *C. acetobutylicum*, the regulatory mechanisms and physiological complexity for the synthesis of butanol are not well understood. However, with the broad application of recombinant DNA technology, genome shuffling is designed to engineer whole genomes for uncharacterized organisms. In this study, we used *C. acetobutylicum* genome shuffling to increase butanol production. After four rounds of genome shuffling with butanol as the adaptive pressure, a *C. acetobutylicum* GS4-3 high-yield recombinant was obtained. Its butanol production increased to 20.1 g/L in a 10 L bioreactor, more than 23.3 % higher compared to the parental strain GX01. The butanol production and solvent tolerance properties of mutant GS4-3 make it promising for ABE fermentation from cassava starch.

As a powerful tool for breeding improved organisms, genome shuffling can combine the advantages of multiparental crossing allowed by DNA shuffling and the recombination of entire genomes associated with conventional breeding (Gong et al. 2009), and rapidly enhance complex phenotypes for microbes without knowledge of the detailed genomic information (Biot-Pelletier and Martin 2014). Therefore, multiple exchanges and multiple gene recombination can be created and/or removed rapidly and efficiently, resulting in much greater leaps in the desired phenotype compared to classical improvement methods (Zhang et al. 2002). Additionally, shuffled strains can be applied readily for many industries (including foodstuffs and pharmaceuticals) without the need for complex procedures to obtain approval to use these strains. At present, genome shuffling has been used to as an efficient method improve the production of many chemicals, including ethanol (Gong et al. 2008), D-lactic acid (Zheng et al. 2010) and succinic acid (Zheng et al. 2013).

During batch fermentation, the low concentration of cassava starch was favorable for higher productivity and yield of butanol (Table S1). A better sugar utilization, shorter fermentation time, and improved productivity could be achieved in semi-continuous compared to batch fermentation. Recently, continuous and semi-continuous fermentations have been used for many important fermentation processes (Bauer et al. 2005; Ezeji et al. 2007). For example, Ni et al. (2012) found two-stage continuous fermentation could obtain the higher butanol production of 15.27 g/L and butanol productivity of 0.61 g/L/h, compared to the batch ABE production. Additionally, as for *C. acetobutylicum*, butanol metabolism was associated with the ratio of NADH/NAD^+ and the activity of NADH-BDH, in which the highest levels of them were observed during solventogenesis at pH 5.5(Jiang et al. 2014). Therefore, pH has been regarded as a crucial factor for ABE fermentation and adjusting the extracellular pH might be an effective method to enhance butanol production (Guo et al. 2012b). Presently, the pH control strategy has been used to optimize the ABE fermentation process. For example, Guo adopted a two-stage controlled pH strategy, in which the pH was changed from 5.5 to 4.9 after achieving a dry cell weight of 0.5 g/L, and obtained a maximum ABE concentration and productivity of 20.3 g/L and 0.63 g/L/h, respectively (Guo et al. 2012a). Consequently, optimizing the fermentation process might contribute to increased cell growth and glucose consumption, and then enhance solvent production and productivity significantly.

In this study, genome shuffling was used to improve the butanol tolerance and butanol production for *C. acetobutylicum*, in which the shuffled strain GS4-3, exhibiting significantly higher ABE production, was obtained. However, owing to the dynamic interaction of genes and gene products distributed throughout a cell's genome, it is difficult to elucidate which genes were responsible for the enhancement of butanol production. Therefore, with the development of omics technologies, useful genes for butanol production will be elucidated further by systems biology, providing efficient strategies for increasing butanol production further and realizing commercialization of the ABE fermentation process.

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