Bioresource Technology 137 (2013) 254–260

Contents lists available at SciVerse ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

# Acetone, butanol, and ethanol production from cane molasses using *Clostridium beijerinckii* mutant obtained by combined low-energy ion beam implantation and *N*-methyl-*N*-nitro-*N*-nitrosoguanidine induction

Han-guang Li [a,b], Wei Luo [a], Qiu-ya Gu [a], Qiang Wang [a], Wen-jun Hu [a], Xiao-bin Yu [a,*]

[a] The Key Laboratory of Industrial Biotechnology, Ministry of Education, School of Biotechnology, Jiangnan University, Wuxi 214122, China
[b] College of Bioscience and Engineering, Jiangxi Agricultural University, Nanchang 330045, China

## HIGHLIGHTS

- Mutagenesis technique with $N^+$ ion implantation and NTG mutation was developed for this study.
- We have established an effective and convenient method to select mutant strains.
- MUT3 could grow in agar medium supplemented 3% (g/g) butanol.
- Fatty acid (stearic acid) greatly influenced butanol production.
- MUT3 showed a superior ability to produce ABE from cane molasses.

## ARTICLE INFO

*Article history:*
Received 6 January 2013
Received in revised form 10 March 2013
Accepted 12 March 2013
Available online 21 March 2013

*Keywords:*
Butanol
*Clostridium beijerinckii*
Low-energy ion implantation
*N*-methyl-*N*-nitro-*N*-nitrosoguanidine
mutation
Solvent tolerance

## ABSTRACT

In order to obtain mutant strains showing higher solvent tolerance and butanol production than those of wild-type strains, the butanol-producing strain Clostridium beijerinckii L175 was subjected to mutagenesis using a combined method of low-energy ion beam implantation and *N*-methyl-*N*-nitro-*N*-nitrosoguanidine induction. With this effort, mutant strain MUT3 was isolated. When it was used for butanol fermentation in P2 medium, the production of butanol was 15.8 ± 0.7 g/L 46% higher than the wild-type strain. Furthermore, after optimization of butanol production from cane molasses with MUT3, the maximum butanol production of 14.9 ± 0.5 g/L were obtained in crew-capped bottles. When ABE production by MUT3 was carried out in a bioreactor, the production of butanol and total solvent were 15.1 ± 0.8 g/L and 22.1 ± 0.9 g/L, respectively. The remarkable butanol production and solvent tolerance of MUT3 make it promising for butanol production from cane molasses.



## 1. Introduction

In addition to as a multipurpose chemical feedstock, butanol can be also utilized as an alternative biofuel candidate to ethanol. Butanol presents many better properties than ethanol when used as fuel, such as higher octane number, higher energy content, less corrosive and lower solubility in water (Lee et al., 2008). Moreover, butanol can be directly consumed in the current automobile engines and transported in the transportation pipeline, thus it is an ideal candidate to replace gasoline (Jin et al., 2011; Lu et al., 2012). Butanol production through acetone–butanol–ethanol (ABE) fermentation, usually using some clostridial species, obtained much improvement during the early years of last century. However, this production style has then suffered from an impact of the burgeoning petrochemical industry, which was characterized by the closedown of hundreds of biobutanol plants (Khaliq et al., 2009). Producing butanol by Chemical synthesis can greatly reduce the production cost, which is ascribed to the availability of abundant and inexpensive crude oil resource, hence renders its commercial application in many fields. With the quick depletion of fossil fuels, rapidly increasing oil prices and alarming effects of environmental changes, ABE fermentation by clostridial species has regained much attention in both industrial and academic worlds recently (Lehmann and Lütke-Eversloh, 2011). In spite of that, butanol toxicity to the current producing microorganisms limits its accumulation in the fermentation broth, and for this reason the biobutanol production processes is of low yield, low titer, low productivity and high recovery cost (Knoshaug and Zhang, 2009). Thus, this problem should be taken into consideration in order to obtain high-yield butanol via ABE fermentation. Many solutions have been proposed, such as selecting solvent tolerant microorganisms, or eliminating/

* Corresponding author. Address: The Key Laboratory of Industrial Biotechnology, Ministry of Education, School of Biotechnology, Jiangnan University, No 1800 Lihu Avenue, Wuxi 214122, China. Tel./fax: +86 510 85918167.
*E-mail address:* xbyu@jiangnan.edu.cn (X.-b. Yu).

0960-8524/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.biortech.2013.03.084

reducing butanol toxicity to the culture by the method of in situ butanol recovery (Mariano et al., 2011).

Diverse degree of success has been made on improving the butanol tolerance level in different microorganisms by the modern breeding techniques (Mann et al., 2012). However, serious impediments have been frequently encountered during the application of those modern techniques due to limited insight into the biochemistry, physiology, and genetics of organisms with regarding to butanol biosynthesis (Olano et al., 2008). Therefore, traditional mutation, including physical or chemical methods, combining rational screening technologies, is still applied to obtaining strains with high yield of target products (Qureshi et al., 2007). Among the agents used in traditional mutation, *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (NTG) is an alkylating agent that induces point mutagenesis acting as G–C → A–T and is regarded as a super one with high mutagenicity. Additionally, the low-energy ion beam implantation ($N^+$ ion beam implantation) has remarkable advantages, including a wider spectrum of mutation, a low damage rate, and a higher mutation rate compared to conventional mutation methods. As a physical mutation method, $N^+$ ion beam implantation mutagenesis technique, has made significant progress in mutation breeding and transgenic transfer, and has created many good social and economic benefits (Feng et al., 2006; Kumar et al., 2009; Yu et al., 1991).

On the other hand, feedstock cost is another important factor limiting the commercial production of butanol via ABE fermentation (Jang et al., 2012). Although various raw materials or renewable agricultural crops such as corn (Qureshi and Blaschek, 2001) and sago starch (Al-Shorgani et al., 2012) can be used as substrates for ABE fermentation, they possess a shortcoming by resulting in food shortage. Cane molasses is a byproduct of the sugar industry (Qureshi et al., 2001), which consists of water, approximately 50% (w/w) total sugars (sucrose, glucose and frucose), heavy metals, suspended colloids, vitamins and nitrogenous compounds, etc. (Najafpour and Poi Shan, 2003). Additionally, it is a comparatively cheap raw material, and has already been used for the production of numerous important industrial chemicals, such as butanol (Jones and Woods, 1986), polysaccharide (Roukas, 1998), ethanol (Ergun and Ferda, 2000), lactic acid (Kotzamanidis et al., 2002), sorbitol (Cazetta et al., 2005), gluconic acid (Sharma et al., 2008). In china about 3,000,000 tons of molasses are produced annually. However, to the best of our knowledge, cane molasses has rarely used in the fermentative production of butanol by *Clostridium beijerinckii* in china.

In this study, mutagenesis was performed by using a combination of $N^+$ ion implantation and NTG mutation, resulting in a solvent-tolerant mutant strain, namely, MUT3 which produces butanol with a higher yield compared with original strains. Furthermore, Sugars were released from cane molasses by the pretreatment with dilute sulfuric acid. The production of butanol by MUT3 from pretreated cane molasses were studied, and the effects of complex nitrogen sources, initial sugar concentration and fatty acid concentration on butanol production from molasses were also investigated.

## 2. Methods

### 2.1. Microorganism and medium

*C. beijerinckii* L175 was selected from the waste water of Daqing Oilfield Company in the north China, A stock culture of L175 was routinely maintained as a cell suspension in 20% (v/v) sterile glycerol at −20 °C in screw-capped bottles.

L175 cell suspension was heat shocked for 2 min at 80 °C followed by cooling to room temperature on ice. Then, the heat shocked cell suspension was cultivated at 37 °C for 12–18 h in anoxic presterilized tryptone-yeast extract-acetate medium (TYA medium) containing 0.4% (w/v) glucose, as described previously (Baba et al., 2012). Following growth, 5–7 mL of the culture was inoculated into 50 mL of inoculum development P2 medium, prepared in 125 mL screw capped bottle.

TYA development medium was used for the pre-culture, and its consisted of the following: 40 g/L glucose, 2 g/L yeast extract, 6 g/L tryptone, 3 g/L $CH_3COONH_4$, 0.2 g/L $MgSO_4{\cdot}7H_2O$, 0.5 g/L $K_2HPO_4$, 0.5 g/L $KH_2PO_4$, and 10 mg/L $FeSO_4{\cdot}7H_2O$ (Zheng et al., 2013). The medium was sterilized at 121 °C for 15 min.

The development P2 medium consisted of the following: 60 g/L glucose, 3 g/L yeast extract. 1 mL each of filter-sterilized stock solutions (buffer: 50 g/L $K_2HPO_4$, 50 g/L $KH_2PO_4$, 220 g/L ammonium acetate, mineral: 1 g/L $MnSO_4{\cdot}H_2O$, 20 g/L $MgSO_4{\cdot}7H_2O$, 1 g/L NaCl, 1 g/L $FeSO_4{\cdot}7H_2O$; and vitamin: thiamin 0.1 g/L, para-aminobenzoic acid 0.1 g/L, biotin 0.001 g/L) was added to 1 L of P2 medium (Qureshi and Blaschek, 1999).

The experiment to study the effect of different types of carbon sources at a concentration of 60 g/L and the effect of initial total sugar concentration of pretreated molasses (ranging from 40 to 100 g/L) was carried out using 3.0 g/L yeast extract as nitrogen source. The experiment to study the effect of different types of nitrogen sources at a concentration of 3 g/L and the effect of yeast extract (ranging from 0 to 8 g/L) was carried out using 60 g/L total sugar concentration of pretreated molasses as carbon sources. The study on the effect of different fatty acids were carried out using 60 g/L total sugar concentration of pretreated molasses as carbon sources and 3.0 g/L yeast extract as nitrogen source.

### 2.2. Pretreatment of cane molasses

Cane molasses, obtained from Jiangmen sugar-refinery (Guangdong, PRC), contained 35% (w/w) sucrose, 10% (w/w) converted sugars (glucose and fructose), 2.5% (w/w) other carbohydrates, 4.3% (w/w) crude protein, 0.06%(w/w) crude fat, 9.6% (w/w) ash, 8.9% (w/w) metal ions (potassium, calcium, iron, sodium, copper, magnesium, etc.), 4.6% (w/w) salt, and 25% (w/w) water. After the crude molasses was diluted with distilled water, 40–100 g/L of total sugar was obtained. For sulfuric acid treatment, the crude molasses solution was firstly adjusted to pH 3.5 with 5 M $H_2SO_4$, and heated at 60 °C for 2 h. The supernatant was collected by centrifugation at 8000 r/min for 15 min, and was adjusted to pH 6.5 with 10 M NaOH (Jiang et al., 2009).

### 2.3. Mutagenesis by $N^+$ ion beam implantation and NTG induction

The mutation by $N^+$ ion beam implantation was implemented at Hefei Institute of Physics, Chinese Academy of Sciences. The freshly culture, grown in TYA medium and harvested at the exponential phase, was diluted by 0.9% sterile physiologic saline solution to an optical density at 540 nm ($OD_{540}$) of 0.1. Thereafter, 0.1 mL of the cell suspension was spread on a Petri dish (60 mm diameter) and desiccated by sterile air so as to create a dry membrane of cells. The dishes were placed on the sample holder and implanted with 15 keV energy of $N^+$ ion beam. The dose for implantation ranged from $0.1 \times 2.6 \times 10^{15}$ to $0.9 \times 2.6 \times 10^{15}$ ions $cm^{-2}$ and the vacuum of the implantation room is $10^{-3}$ Pa. The interval of implantation is 55 s and ion impulse for 5 s. Afterwards the dry membrane of cells were eluted with 0.9% sterile physiologic saline solution and diluted properly before grown on the selection agar medium in Petri dishes.

For NTG mutation, the mutant obtained from low-energy ion beam implantation was grown in TYA solid medium at 37 °C for 37 h and washed with 0.9% sterile physiologic saline solution. One aliquot of $1 \times 10^8$ cells was transferred to a 15 mL tube, and

centrifuged at 4000 r/min for 5 min. The pellets were resuspended in 2.0 mL sodium phosphate buffer (0.1 M and pH 6.5), which was mixed with NTG solution to give a final concentration of NTG ranging from 0.3 to 1.5 mg/mL for 30 min. After each run, 1 mL of 1 mol/L NaOH was added into the reaction mixtures to stop the mutagenic process. The cell suspension was properly diluted and plated on the selection agar medium in Petri dishes. The individual colonies on control petri dishes and each selection petri dishes were counted, respectively. The lethality or survival rate was determined as follow,

$$\text{Lethal rate}(\%) = (\text{Total colonies under control condition} - \text{Survival colonies})/\text{Total colonies under} \times \text{control conditions} \times 100$$

Survival rate (%) = Survival colonies/Total colonies under control condition × 100

### 2.4. Rational screening

The selection agar TYA medium was supplemented with 0.02 g/L resazurin (commonly used as a redox indicator) (Guo et al., 2013), and various concentrations (10, 15, 20, 25, 30 and 35 g/L) of butanol (Hermann et al., 1985). Furthermore, 1.0 g/L lithium chloride was added to the selection agar medium for NTG mutagenesis. The cell suspension after mutagenesis was incubated in selective agar petri dishes at 37 °C for 1–2 days to achieve the mutants with high solvent tolerance and high production of butanol. All the individual colonies showing large haloes on each selection Petri dishes, which indicated high solvent tolerance and high production of butanol, were transferred to 100 mL flask with 50 mL of sterilized development P2 medium and were incubated at 37 °C for 18–20 h for vegetative culturing.

### 2.5. Batch fermentation

Screw-capped bottles (500 mL) containing 250 mL of P2 medium were used throughout these studies. The culture medium was autoclaved at $10^5$ Pa (115 °C) for 15 min and cooled to room temperature. The initial pH of the broth was adjusted to 6.0 in all cultures; however, pH was not controlled during the course of fermentation. Anaerobic conditions were created by keeping this screw-capped bottles containing medium in anaerobic jars for 36 h before inoculation. The batch fermentation was initiated by adding 10% (v/v) inoculum, which was grown for 18–20 h. Additionally, subculturing experiment was also carried out in 500 mL screw-capped bottles containing 250 mL of P2 medium. The method for the subculturing experiment is the same as batch fermentation. All fermentation was performed at 37 °C until cells ceased ABE production. By the end of the culture, samples were taken for sugar, acetic acid, butyric acid and ABE analysis. All experiments were carried out as biological duplicates.

Batch fermentation was carried out in a LiFlus GX bioreactor (7 L, Biotron) with an initial working volume of 4 L containing the pretreated molasses, sterilized in an autoclave (121 °C for 15 min), then filter-sterilize P2 stock solutions were added. The culture medium was purged with nitrogen gas to remove oxygen before and after inoculation; the culture temperature was maintained at 37 °C for 84 h. During the course of fermentation, there was no agitation or pH control; 10 mL samples were regularly collected for analysis of ABE, total sugar, and organic acids (acetic and butyric).

### 2.6. Analytical methods

The concentrations of ABE and organic acids (acetic and butyric) were quantified by internal standard method using gas chromatograph (GC) system (GC-2010, Shimadazu Scientific Instruments, Japan) equipped with a flame ionization detector. ABE fermentation products were separated in a PEG-20 M capillary column (30 m × 0.32 mm × 0.4 μm) from Agilent Technologies. The injector temperature was maintained at 280 °C, and detector temperature at 240 °C, and the column temperature at 210 °C.

The concentrations of total sugar were measured according to the method described previously (Jiang et al., 2009).

## 3. Results and discussion

### 3.1. $N^+$ ion beam implantation for survival rate

As shown in Fig. 1, $N^+$ ion beam implantation induced a dose-dependent survival rate in *C. beijerinckii* L175, which was characterized by a saddle-shaped curve. The survival rate had a rapid decrease from 0 to $0.3 \times 2.6 \times 10^{15}$ ions/cm$^2$ and a temporary increase within a short range ($0.3 \times 2.6 \times 10^{15}$–$0.7 \times 2.6 \times 10^{15}$ ions/cm$^2$), and then decreased as the dose continued to increase. The falling, rising, and then falling survival pattern is called a "saddle-shaped curve", as earlier report (Xu et al., 2010). The dose range between $0.1 \times 2.6 \times 10^{15}$ and $0.9 \times 2.6 \times 10^{15}$ ions/cm$^2$ was selected so as to obtain a high mutagenesis rate.

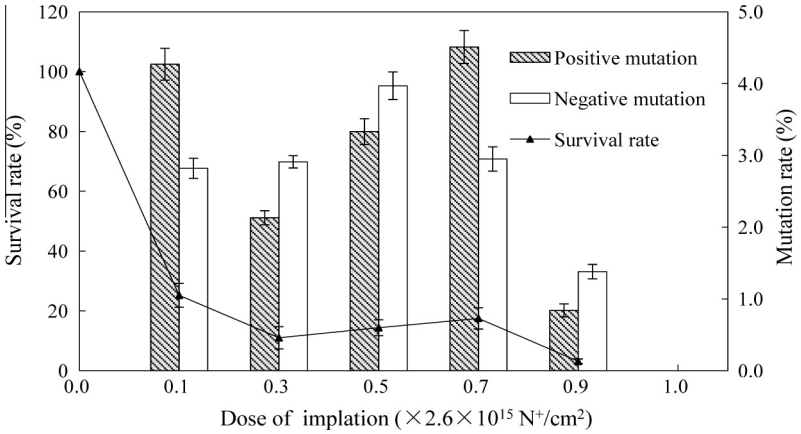


**Fig. 1.** Survival rate and mutation rate of L175 by $N^+$ ion implantation. All experiments were carried out in duplicates.

In this study, the positive mutation is defined as 5% enhancement of the butanol yield of mutant compared to that of the start strain, and negative mutation means that the butanol yield reduced by 5%. As shown in Fig. 1, with the increase in the implantation dose, the positive mutation rate increased firstly and then decreased. The highest positive mutation rate was achieved at the dose of $0.7 \times 2.6 \times 10^{15}$ ions/cm$^2$. Furthermore, the survival rate was all higher others at the dose of $0.1 \times 2.6 \times 10^{15}$ ions/cm$^2$ and $0.7 \times 2.6 \times 10^{15}$ ions/cm$^2$, respectively. Out of these doses, the negative mutation rate was slightly higher than the positive mutation rate. So the dose of $0.7 \times 2.6 \times 10^{15}$ ions/cm$^2$ was chosen in the subsequent study.

Strains producing high productions of butanol and ABE can be selected with resazurin for strong deoxidization activity (Jin et al., 2008). Forty colonies, which showed comparatively large transparent zones, were singled out in the selection agar medium containing resazurin and butanol, and then cultured in the fermentation medium. The concentrations of butanol and total solvent of those mutants were higher than the wild-type strain (10.8 ± 0.6 g/L butanol and 14.4 ± 0.8 g/L total solvent). The maximum production of butanol and total solvent were 12.5 ± 0.6 g/L and 16.9 ± 0.3 g/L, respectively, in the mutant IB26, which were about 16% and 17%, respectively, higher than that of the wild-type strain. Therefore, the mutant IB26 was selected as a preferable butanol-producer for further studies.

### 3.2. Mutagenesis with NTG induction and isolation of mutant with high butanol yield

In order to obtain a new strain for the hyper-production of butanol, IB26 was treated with NTG and then plated on the selection agar medium containing resazurin, butanol and lithium chloride (see Section 2).

NTG concentration was investigated to obtain optimal NTG mutagenesis conditions. The results shown that the lethal rate also increased with the increase of NTG concentration. The highest value approached 99% when 1.5 mg/mL NTG was performed, but this is not the optimal concentration for the achievement of butanol hyper-producing mutants. This is ascribed to the result that more positive mutants would be emerged when the lethal rate was between 60% and 75%, therefore the final concentration of 1.0 mg/mL was used in this study.

Many single colonies could form in the selection agar medium plate containing 10–30 g/L butanol, but they were arrested in the plates supplemented with high than 35 g/L butanol. Therefore, 20 g/L and 30 g/L butanol was added and five colonies were isolated. They are cultured in the fermentation medium. It could be found that strain MUT3 shown the highest butanol yield (15.8 ± 0.7 g/L butanol) among these five strains, which was about 26% higher than that of the strain IB26. Butanol production of the MUT4 strain (12.8 ± 0.7 g/L) was slightly higher than that of IB26 strain, while the production of other mutant strains was lower than that of IB 26 strain. Butanol-producing stability of MUT3 strain was determined by continuous subculture experiment at 37 °C in a shaking flask. During five generations of MUT3, the average production of butanol and total solvent were 15.7 ± 0.6 g/L and 19.2 ± 0.8 g/L, respectively. The results indicated that the butanol production remained relatively stable in subsequent generations.

### 3.3. Medium optimization

#### 3.3.1. Sugar utilized spectrum of MUT3

To investigate the feasibility of using molasses as substrate in ABE fermentation, anaerobic cultivation of MUT3 using various carbon sources were studied in P2 medium at pH 6.0. As shown in Table 1, MUT3 could utilize glucose, fructose, lactose, xylose, cellobiose and sucrose to produce butanol. When cells were grown in P2 medium supplemented with glucose as carbon sources, butanol production (15.7 ± 0.6 g/L) and butanol yield (0.26 g/g) were the

**Table 1**
Comparison of various carbon sources for ABE fermentation with MUT3 in P2 medium at pH 6.0.[a]

| Substrate | Acetone (g/L) | Ethanol (g/L) | Butanol (g/L) | Total solvent (g/L) | Butanol yield (g/g)[b] |
|---|---|---|---|---|---|
| Glucose | 2.2 ± 0.1[c] | 1.8 ± 0.1 | 15.7 ± 0.6 | 19.7 ± 0.8 | 0.26 |
| Fructose | 3.8 ± 0.2 | 2.1 ± 0.1 | 14.0 ± 0.4 | 19.9 ± 0.4 | 0.23 |
| Lactose | 3.3 ± 0.2 | 2.1 ± 0.1 | 14.1 ± 0.3 | 19.5 ± 0.3 | 0.24 |
| Xylose | 1.8 ± 0.0 | 0.4 ± 0.0 | 3.4 ± 0.1 | 5.6 ± 0.1 | 0.06 |
| Cellobiose | 3.6 ± 0.1 | 2.8 ± 0.0 | 13.4 ± 0.2 | 19.8 ± 0.3 | 0.22 |
| Sucrose | 2.3 ± 0.0 | 2.9 ± 0.1 | 13.0 ± 0.2 | 18.2 ± 0.3 | 0.22 |
| Sugar mixture[d] | 3.7 ± 0.1 | 1.2 ± 0.1 | 13.0 ± 0.4 | 17.9 ± 0.4 | 0.22 |

[a] Cells were grown in screw-capped bottles at pH 6.0 for 60 h with an initial total sugar concentration of 60 g/L, and the nitrogen source was yeast extract with a concentration of 3 g/L.
[b] The butanol yield was defined as follow, butanol yield (g/g) = Butanol value (g)/Total sugar (g).
[c] All experiments were carried out as biological duplicates.
[d] Sugar mixture was 8 g/L fructose, 10 g/L glucose, and 42 g/L sucrose.

**Table 2**
Effects of different nitrogen sources on ABE fermentation from pretreated molasses with MUT3.[a]

| Nitrogen sources | Acetone (g/L) | Ethanol (g/L) | Butanol (g/L) | Total solvent (g/L) | Butanol yield (g/g)[b] |
|---|---|---|---|---|---|
| Yeast extract | 3.4 ± 0.2[c] | 1.7 ± 0.1 | 14.0 ± 0.6 | 19.1 ± 0.6 | 0.23 |
| Urea | 2.4 ± 0.2 | 0.7 ± 0.0 | 6.6 ± 0.2 | 9.7 ± 0.3 | 0.11 |
| Ammonium sulfate | 2.2 ± 0.1 | 1.0 ± 0.1 | 7.7 ± 0.3 | 10.9 ± 0.4 | 0.13 |
| Tryptone | 3.7 ± 0.2 | 1.5 ± 0.1 | 12.7 ± 0.4 | 17.9 ± 0.7 | 0.21 |
| Peptone | 3.5 ± 0.2 | 1.5 ± 0.1 | 13.1 ± 0.4 | 18.1 ± 0.7 | 0.22 |
| Beef extract | 2.8 ± 0.2 | 2.0 ± 0.1 | 12.5 ± 0.4 | 17.3 ± 0.6 | 0.21 |
| Corn steep liquor | 3.5 ± 0.2 | 1.9 ± 0.1 | 13.1 ± 0.5 | 18.5 ± 0.7 | 0.22 |

[a] The concentrations of nitrogen sources were also 3 g/L. Cells were grown in crew-capped bottles at pH 6.0 for 60 h with different nitrogen sources and 60 g/L sugar.
[b] The butanol yield was defined as follow, butanol yield (g/g) = Butanol value (g)/Total sugar (g).
[c] All experiments were carried out as biological duplicates.

**Table 3**
Effects of yeast extract concentration on the ABE fermentation with MUT3.[a]

| Parameter/ performance | Yeast extract concentration (g/L) | | | | |
|---|---|---|---|---|---|
| | 0 | 1 | 3 | 5 | 8 |
| Acetone (g/L) | —[b] | 1.1 ± 0.1[c] | 3.9 ± 0.2 | 3.4 ± 0.1 | 2.0 ± 0.1 |
| Ethanol (g/L) | — | 2.0 ± 0.1 | 1.1 ± 0.1 | 0.8 ± 0.0 | 1.6 ± 0.2 |
| Butanol (g/L) | — | 5.4 ± 0.1 | 13.7 ± 0.4 | 10.7 ± 0.4 | 9.3 ± 0.3 |
| Total solvent (g/L) | — | 8.5 ± 0.3 | 18.7 ± 0.4 | 14.9 ± 0.4 | 12.9 ± 0.4 |
| Butanol yield (g/g)[d] | — | 0.09 | 0.23 | 0.18 | 0.16 |

[a] Cells were grown in screw-capped bottles at pH 6.0 for 60 h with an initial total sugar concentration of 60 g/L.
[b] ND means not detectable.
[c] All experiments were carried out as biological duplicates.
[d] The butanol yield was as followed: Butanol yield(g/g) = Butanol value (g)/Total sugar (g).

highest among the tested. Considering the constituent of cane molasses, a mixture of fructose, glucose and sucrose which was prepared to approach the ratio in molasses was used as carbon sources in the fermentation. The results showed that the sugar mixture could be efficiently fermented by MUT3 to produce 13. 0 ± 0.4 g/L butanol (butanol yield 0.22 g/g), suggesting that cane molasses could be used as a great potential material for the economical production of butanol.

### 3.3.2. Effect of different nitrogen sources

To obtain a better nitrogen source for butanol fermentation, the effect of different organic and inorganic nitrogen sources on butanol production were compared with yeast extract in screw-capped bottles, including urea, ammonium sulfate, tryptone, peptone, beef extract and corn steep liquor. As can be seen from Table 2, all the inorganic nitrogen sources tested gave poor production of butanol and total solvent, and yeast extract was the best nitrogen source for butanol production by MUT3.

The optimum yeast extract concentration for ABE fermentation from cane molasses was also studied in screw-capped bottles supplemented with 0–8 g/L of yeast extract. As can be seen from Table 3, cells could slightly grow due to pretreated molasses fermentation without yeast extract. When the yeast extract concentration was 3 g/L, the maximum production of butanol and total solvent was obtained. When the yeast extract concentration was beyond 3 g/L, the butanol concentration decreased with the increase of yeast extract concentration. Therefore, the optimum yeast extract concentration of 3 g/L was selected for ABE fermentation with pretreated molasses.

### 3.3.3. Effect of initial sugar concentration

Urbance et al., (Urbance et al., 2004) reported that cell growth and metabolites production could be influenced by the initial sugar concentration. Therefore, the butanol production with pretreated molasses in different dilution rates were studies. In the present study (Table 4), MUT3 could grow in the medium with the initial sugar concentrations from 40 g/L to 100 g/L, and butanol was produced as a major product. The production of butanol significantly increased with the increase of initial sugar concentrations from 40 g/L to 60 g/L. The maximum value of butanol concentration (14.0 ± 0.6 g/L) was obtained when the initial total concentration was 60 g/L. However, when the initial total sugar concentration was over 60 g/L, the concentration and yield of butanol would decrease.

Previous study indicated that the sugar utilization decreased with high sugar concentration due to the osmotic effects (Kotzamanidis et al., 2002). In this study, high sugar concentration in cane molasses may inhibit the cell growth and butanol accumulation, which was probably due to the inhibitory effects of excessive substrate, a common problem in batch fermentation.

### 3.4. Effect of different concentrations of stearic acid and palmitic acid on the production of butanol and total solvent by MUT3

The ratio of saturated fatty acids in the membrane would increase when saturated fatty acids were added to the medium (Rogers, 1986), which could enhance the butanol tolerance, ATPase activity and cell growth (Jones and Woods, 1986). Therefore, different concentrations of stearic acid and palmitic acid were added into the culture in order to enhance the butanol production. The results shown that the optimal concentration of stearic acid for ABE fermentation was 0.75 g/L, where the concentration of butanol and total solvent were 14.9 ± 0.5 g/L and 21.3 ± 0.6 g/L, respectively; which were about 12% and 13% higher than that of the control group (13.3 ± 0.5 g/L butanol and total solvent 18.8 ± 0.8 g/L). Meanwhile, it is interesting to note that all the production of butanol in the groups added with straric acid were higher than that of the control group. However, the production of butanol was not necessarily higher than that of the control group when palmitic acid was added into the medium. Therefore, results from this study also indicated that fatty acid (stearic acid) greatly influenced solvent production, but the underlying mechanisms of this phenomenon are unclear. Studies of the mechanism of fatty acid on solvent production are underway in our laboratory.

### 3.5. Production of ABE in batch bioreactor

In order to verify the above experimental results in a larger scale, ABE production by MUT3 and the wild type strain was carried out using pretreated molasses as the carbon source in a bioreactor (7 L). The concentration of initial total sugar and yeast extract were 60 g/L and 3 g/L, respectively, and stearic acid (0.75 g/L) was also added into the medium. As shown in Fig. 2(a). about 95% of the total sugar was consumed during the 84 h fermentation when MUT3 was used, resulting in a total ABE production of 22.1 ± 0.9 g/L (4.8 ± 0.3 g/L acetone, 2.2 ± 0.1 g/L ethanol, 15.1 ± 0.8 g/L butanol). The total acid concentration was 2.31 ± 0.07 g/L (1.10 ± 0.03 g/L acetic acid and 1.21 ± 0.04 g/L butyric acid). The ABE productivity

**Table 4**
Effects of initial total sugar concentration on the production of ABE from pretreated molasses using MUT3.[a]

| Parameter/performance | Initial total sugar concentration (g/L) | | | | |
|---|---|---|---|---|---|
| | 40 | 50 | 60 | 70 | 100 |
| Acetone (g/L) | 3.3 ± 0.2[b] | 3.0 ± 0.2 | 3.7 ± 0.2 | 2.9 ± 0.1 | 2.2 ± 0.1 |
| Ethanol (g/L) | 1.0 ± 0.1 | 1.4 ± 0.0 | 2.1 ± 0.1 | 1.7 ± 0.1 | 1.0 ± 0.1 |
| Butanol (g/L) | 8.2 ± 0.2 | 12.6 ± 0.4 | 14.0 ± 0.6 | 12.4 ± 0.4 | 9.5 ± 0.3 |
| Total solvent (g/L) | 12.5 ± 0.4 | 17.0 ± 0.6 | 19.8 ± 0.8 | 17.0 ± 0.6 | 12.7 ± 0.5 |
| Butanol yield (g/g)[c] | 0.21 | 0.25 | 0.23 | 0.18 | 0.1 |

[a] Cells were grown in crew-capped bottles at pH 6.0 for 60 h with different initial total sugar concentrations from 40 g/L to 100 g/L, and were supplied with yeast extract concentration of 3 g/L.
[b] All experiments were carried out as biological duplicates.
[c] The butanol yield was as followed: Butanol yield(g/g) = Butanol value (g)/Total sugar (g).

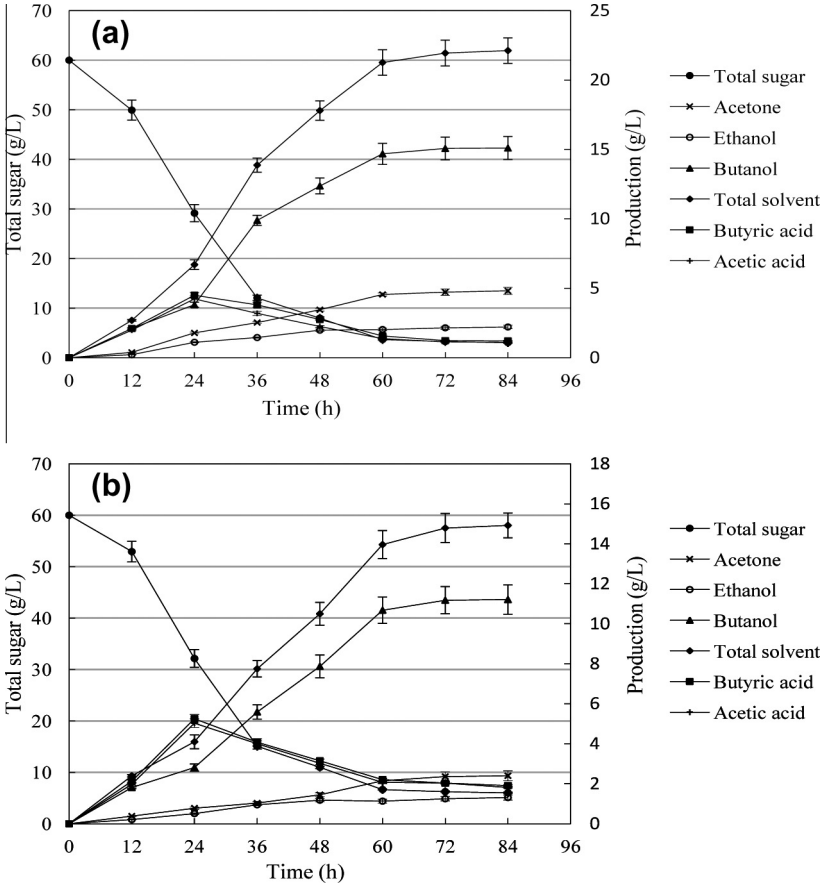


**Fig. 2.** Production of ABE from pretreated molasses in a batch reactor by MUT3 (a) and the wild type strain (b). The ABE productivity (g/(L·h)) = ABE concentration (g)/fermentation time (h). All experiments were carried out as biological duplicates.

was 0.26 g/(L·h) when pretreated molasses was used. However, when the wild type strain was performed on the tests under the same conditions, the production of butanol and total ABE were 11.2 ± 0.8 g/L and 14.9 ± 0.7 g/L, respectively (Fig. 2(b)). The ABE productivity was 0.18 g/(L·h) when the wild type strain was used. Therefore, the results indicated that mutant MUT3 was superior to the wild type strain in producing ABE from cane molasses.

In order to facilitate industrial-scale fermentation, improving the solvent tolerance of microorganisms is a promising method. The results presented here demonstrate that MUT3 is a highly solvent tolerance, butanol-production strain, which can utilize cane molasses to produce butanol.

## 4. Conclusions

In this study, a highly solvent tolerance, butanol-producing mutant strain MUT3 has been obtained. Additionally, butanol production from cane molasses by MUT3 was also studied. After the optimization of nitrogen sources, carbon sources and fatty acid concentration, the maximum butanol production of 14.9 ± 0.5 g/L and butanol yield (0.25 g/g) were obtained. When ABE production by MUT3 was carried out in a bioreactor, the production of butanol and total solvent were 15.1 ± 0.8 g/L and 22.1 ± 0.9 g/L, respectively. Therefore, MUT3 showed a superior ability to produce ABE from cane molasses, and thus reducing the production cost.

## Acknowledgements

This work was supported by the National Natural Science Foundation of China (Grant No. 21176105), the Fundamental Research Funds for the Central Universities (Grant No. JUSRP111A24), Program of the Key Laboratory of Industrial Biotechnology, Ministry of Education, China (Grant No. KLIB- KF201105), the 111 Project (No. 111-2-06) and a Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions.

## References

Al-Shorgani, N.K.N., Kalil, M.S., Yusoff, W.M.W., 2012. Fermentation of sago starch to biobutanol in a batch culture using Clostridium saccharoperbutylacetonicum N1–4 (ATCC 13564). Ann. Microbiol. 62 (3), 1059–1070.

Baba, S., Tashiro, Y., Shinto, H., Sonomoto, K., 2012. Development of high-speed and highly efficient butanol production systems from butyric acid with high density of living cells of *Clostridium saccharoperbutylacetonicum*. J. Biotechnol. 157 (4), 605–612.

Cazetta, M., Celligoi, M., Buzato, J., Scarmino, I., Da Silva, R., 2005. Optimization study for sorbitol production by *Zymomonas mobilis* in sugar cane molasses. Process Biochem. 40 (2), 747–751.

Ergun, M., Ferda Mutlu, S., 2000. Application of a statistical technique to the production of ethanol from sugar beet molasses by *Saccharomyces cerevisiae*. Bioresour. Technol. 73 (3), 251–255.

Feng, H., Yu, Z., Chu, P.K., 2006. Ion implantation of organisms. Mater. Sci. Eng. 54 (3), 49–120.

Guo, T., He, A.-y., Du, T.-f., Zhu, D.-w., Liang, D.-f., Jiang, M., Wei, P., Ouyang, P.-k., 2013. Butanol production from hemicellulosic hydrolysate of corn fiber by a Clostridium beijerinckii mutant with high inhibitor-tolerance. Bioresource Technol. 135, 379–385.

Hermann, M., Fayolle, F., Marchal, R., Podvin, L., Sebald, M., Vandecasteele, J., 1985. Isolation and characterization of butanol-resistant mutants of *Clostridium acetobutylicum*. Appl. Environ. Microbiol. 50 (5), 1238–1243.

Jang, Y.-S., Malaviya, A., Cho, C., Lee, J., Lee, S.Y., 2012. Butanol production from renewable biomass by *clostridia*. Bioresour. Technol. 123, 653–663.

Jiang, L., Wang, J., Liang, S., Wang, X., Cen, P., Xu, Z., 2009. Butyric acid fermentation in a fibrous bed bioreactor with immobilized *Clostridium tyrobutyricum* from cane molasses. Bioresour. Technol. 100 (13), 3403–3409.

Jin, C., Yao, M., Liu, H., Lee, C.F., Ji, J., 2011. Progress in the production and application of n-butanol as a biofuel. Renewable Sustainable Energy Rev. 15 (8), 4080–4106.

Jin, X., Zhou, H., Wu, X.-m., Zhang, G.-d., He, B., 2008. A rapid screening method of producing strain in acetone-butanol fermentation. Chin. J. Process Eng. 6, 1185–1189.

Jones, D.T., Woods, D.R., 1986. Acetone–butanol fermentation revisited. Microbiol. Rev. 50 (4), 484.

Khaliq, S., Akhtar, K., Afzal Ghauri, M., Iqbal, R., Mukhtar Khalid, A., Muddassar, M., 2009. Change in colony morphology and kinetics of tylosin production after UV and gamma irradiation mutagenesis of *Streptomyces fradiae* NRRL-2702. Microbiol. Res. 164 (4), 469–477.

Knoshaug, E.P., Zhang, M., 2009. Butanol tolerance in a selection of microorganisms. Appl. Biochem. Biotechnol. 153 (1), 13–20.

Kotzamanidis, C., Roukas, T., Skaracis, G., 2002. Optimization of lactic acid production from beet molasses by *Lactobacillus delbrueckii* NCIMB 8130. World J. Microbiol. Biotechnol. 18 (5), 441–448.

Kumar, K.S., Manimaran, A., Permaul, K., Singh, S., 2009. Production of β-xylanase by a *Thermomyces lanuginosus* MC 134 mutant on corn cobs and its application in biobleaching of bagasse pulp. J. Biosci. Bioeng. 107 (5), 494–498.

Lee, S.Y., Park, J.H., Jang, S.H., Nielsen, L.K., Kim, J., Jung, K.S., 2008. Fermentative butanol production by *clostridia*. Biotechnol. Bioeng. 101 (2), 209–228.

Lehmann, D., Lütke-Eversloh, T., 2011. Switching *Clostridium acetobutylicum* to an ethanol producer by disruption of the butyrate/butanol fermentative pathway. Metab. Eng. 13 (5), 464–473.

Lu, C., Zhao, J., Yang, S.T., Wei, D., 2012. Fed-batch fermentation for n-butanol production from cassava bagasse hydrolysate in a fibrous bed bioreactor with continuous gas stripping. Bioresour. Technol. 104, 380–387.

Mann, M.S., Dragovic, Z., Schirrmacher, G., Lütke-Eversloh, T., 2012. Over-expression of stress protein-encoding genes helps *Clostridium acetobutylicum* to rapidly adapt to butanol stress. Biotechnol. Lett. 34 (9), 1643–1649.

Mariano, A.P., Qureshi, N., Ezeji, T.C., 2011. Bioproduction of butanol in bioreactors: new insights from simultaneous in situ butanol recovery to eliminate product toxicity. Biotechnol. Bioeng. 108 (8), 1757–1765.

Najafpour, G.D., Poi Shan, C., 2003. Enzymatic hydrolysis of molasses. Bioresour. Technol. 86 (1), 91–94.

Olano, C., Lombó, F., Méndez, C., Salas, J.A., 2008. Improving production of bioactive secondary metabolites in actinomycetes by metabolic engineering. Metab. Eng. 10 (5), 281–292.

Qureshi, N., Blaschek, H., 1999. Butanol recovery from model solution/fermentation broth by pervaporation: evaluation of membrane performance. Biomass Bioenergy 17 (2), 175–184.

Qureshi, N., Blaschek, H., 2001. ABE production from corn: a recent economic evaluation. J. Ind. Microbiol. Biotechnol. 27 (5), 292–297.

Qureshi, N., Lolas, A., Blaschek, H., 2001. Soy molasses as fermentation substrate for production of butanol using *Clostridium beijerinckii* BA101. J. Ind. Microbiol. Biotechnol. 26 (5), 290–295.

Qureshi, N., Saha, B.C., Cotta, M.A., 2007. Butanol production from wheat straw hydrolysate using *Clostridium beijerinckii*. Bioprocess Biosyst. Eng. 30 (6), 419–427.

Rogers, P., 1986. Genetics and biochemistry of *Clostridium* relevant to development of fermentation processes. Adv. Appl. Microbiol. 31, 1–60.

Roukas, T., 1998. Pretreatment of beet molasses to increase pullulan production. Process Biochem. 33 (8), 805–810.

Sharma, A., Vivekanand, V., Singh, R.P., 2008. Solid-state fermentation for gluconic acid production from sugarcane molasses by *Aspergillus niger* ARNU-4 employing tea waste as the novel solid support. Bioresour. Technol. 99 (9), 3444–3450.

Urbance, S.E., Pometto, A.L., DiSpirito, A.A., Denli, Y., 2004. Evaluation of succinic acid continuous and repeat-batch biofilm fermentation by *Actinobacillus succinogenes* using plastic composite support bioreactors. Appl. Microbiol. Biotechnol. 65 (6), 664–670.

Xu, T.T., Bai, Z.Z., Wang, L.J., He, B.F., 2010. Breeding of D (−)-lactic acid high producing strain by low-energy ion implantation and preliminary analysis of related metabolism. Appl. Biochem. Biotechnol. 160 (2), 314–321.

Yu, Z., Deng, J., He, J., Huo, Y., Wu, Y., Wang, X., Lui, G., 1991. Mutation breeding by ion implantation. Nucl. Instrum. Methods Phys. Res. Sect. B 59, 705–708.

Zheng, J., Tashiro, Y., Yoshida, T., Gao, M., Wang, Q., Sonomoto, K., 2013. Continuous butanol fermentation from xylose with high cell density by cell recycling system. Bioresour. Technol. 129, 360–365.