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Enhancement of Butanol Tolerance and Butanol Yield in *Clostridium acetobutylicum* Mutant NT642 Obtained by Nitrogen Ion Beam Implantation[§]

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As a promising alternative biofuel, biobutanol can be produced through acetone/butanol/ethanol (ABE) fermentation. Currently, ABE fermentation is still a small-scale industry due to its low production and high input cost. Moreover, butanol toxicity to the *Clostridium* fermentation host limits the accumulation of butanol in the fermentation broth. The wild-type *Clostridium acetobutylicum* D64 can only produce about 13 g butanol/L and tolerates less than 2% (v/v) butanol. To improve the tolerance of *C. acetobutylicum* D64 for enhancing the production of butanol, nitrogen ion beam implantation was employed and finally five mutants with enhanced butanol tolerance were obtained. Among these, the most butanol tolerant mutant *C. acetobutylicum* NT642 can tolerate above 3% (v/v) butanol while the wide-type strain can only withstand 2% (v/v). In batch fermentation, the production of butanol and ABE yield of *C. acetobutylicum* NT642 was 15.4 g/L and 22.3 g/L, respectively, which were both higher than those of its parental strain and the other mutants using corn or cassava as substrate. Enhancing butanol tolerance is a great precondition for obtaining a hyper-yield producer. Nitrogen ion beam implantation could be a promising biotechnology to improve butanol tolerance and production of the host strain *C. acetobutylicum*.

***Keywords*:** butanol tolerance, butanol yield, *Clostridium acetobutylicum*, nitrogen ion beam implantation, fermentation

Introduction

Butanol, which is an important industrial chemical, can be widely used not only as a solvent but also as a chemical feedstock (Formanek *et al.*, 1997). With diminishing natural oil and gas resources, butanol has been considered as a promising advanced biofuel for its higher energy density and lower vapor pressure, for being less corrosive and having less water solubility compared to ethanol (Connor and Liao, 2009; Kataoka *et al.*, 2011). However, major bottlenecks in the current butanol fermentation process are low yield, low productivity and, especially, low titer due to the toxicity of butanol to its producing strains (Liu *et al.*, 2012).

Butanol toxicity is one of the major roadblocks that acetone/butanol/ethanol (ABE) fermentation currently faces. Even the native producer, *Clostridium acetobutylicum* only tolerates up to 1–2% (v/v) of this solvent (Winkler *et al.*, 2010). Butanol tolerance is a complex phenotype involving multiple loci (Makarova *et al.*, 2006; Papoutsakis, 2008; Winkler *et al.*, 2010), making the engineering of strains with enhanced tolerance to this solvent difficult. So far, efforts for enhancing butanol tolerance have mainly focused on the enrichment of mutants by serial transfers (Lin and Blaschek, 1983; Soucaille *et al.*, 1987). More recently, protoplast fusion between *E. coli* and *Lactobacillus brevis* generated a hybrid with enhanced butanol tolerance from 1% to 2% (v/v) butanol (Winkler *et al.*, 2010). These reports provided various valuable methods to enhance butanol tolerance; however, they share the problem that the chosen host can not produce butanol yet. Additional complex, high-risk work has to be done to get a solventogenic gene to express in these hosts.

As *C. acetobutylicum* has a complex genetic background and grows in rigorous anaerobic circumstances, it is difficult to enhance butanol tolerance via gene engineering. Although mutagenesis is random, it still has several advantages, such as being technologically easier and having a lower workload compared to other methods. Using chemical mutagenesis, Annous and Blaschek obtained a hyper-producing mutant *Clostridium beijerinckii* BA101 which produced 32.6 g total solvent/L and 18.6 g butanol/L with 60 mM sodium acetate and 8% glucose (Annous and Blaschek, 1991; Chen and Blaschek, 1999). Guo *et al.* (2011) reported that the mutant *C. beijerinckii* IB4 with high inhibitor tolerance was produced using low-energy ion implantation. Nitrogen ion beam implantation (NIBI) has been increasingly used in various fields, especially in industrial microbial mutagenesis and mutation breeding (Yu *et al.*, 1991). This is due to the low and controllable damage rate, higher mutation rate, and wider spectrum of mutations obtained by NIBI compared to traditional mutation methods (Feng *et al.*, 2006). However, there are as yet few reports on improving the butanol tolerance and butanol production of *C. acetobutylicum* by nitrogen ion beam implantation. The present study has employed NIBI to produce such a *C. acetobutylicum* mutant.

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Materials and Methods

Microorganisms and media

Clostridium acetobutylicum D64 was cultivated in a yeast extract/peptone/starch (YPS) medium (Jin *et al.*, 2008). The YPS medium contained (per L) 3.0 g yeast extract, 5.0 g peptone, 10.0 g soluble starch, 2.0 g ammonium acetate, 2.0 g NaCl, 3.0 g $MgSO_4{\cdot}7H_2O$, 1.0 g KH_2PO_4, 1.0 g K_2HPO_4, 0.1 g $FeSO_4{\cdot}7H_2O$. pH was adjusted to 6.0 with 1 M HCl. The selection agar plate was supplemented with 0.02 g resazurin/L and 2% 1-butanol (v/v) in YPS medium. Colonies that grew well and exhibited large transparent zones on the selection agar plate indicated that they could produce high amylase activity and reducing power, characteristics of a hyper-producing strain (Jin *et al.*, 2008).

Nitrogen ion beam implantation

The NIBI machine (Supplementary data Fig. S1) was designed by the Chinese Southwestern Institute of Physics. It is driven by an ion implantation machine with accelerator, high energy ion beams and is operated at a vacuum and low gas temperature. It is called the LZD-900 instrument (Guo *et al.*, 2011). *C. acetobutylicum* D64 cells grown in YPS medium were harvested in the exponential phase. The fresh cells were diluted in sterile, physiological saline solution to an optical density at 600 nm (OD600) of 0.15, and then 0.5 ml of the suspension was spread on an empty sterile Petri dish (9 cm diameter). These dishes were desiccated at 37°C in the electric vacuum drying oven until the suspension formed a dry membrane of cells. The dishes were put into the target chamber and implanted with 15 keV energy by a beam of N^+ ions under vacuum conditions. The dose for implantation ranged from 0.2×10^{16} to 1.2×10^{16} ions/cm^2.

Mutant screening

After treatment, the cells were washed off into a test tube using sterile saline. The suspension was spread on the selection agar plates and cultured in a sealed box adding deoxidant at 37°C for 3 days. Colonies that grew well and exhibited large halos were selected and inoculated in YPS medium (20 ml) containing 2% (v/v) butanol in 25 ml screw-capped test tubes to establish their butanol tolerance. This experiment was carried out in triplicate.

Measurements of growth kinetics and sporulation rates

Cultures were grown overnight in YPS medium at 37°C. Growth kinetic measurements for each strain at given concentrations of 1-butanol were taken in 25 ml screw-capped test tubes. The total volume of the culture was 20 ml with an initial OD_{600nm} of 0.01. Tubes were sealed to prevent evaporation of 1-butanol during the incubation. OD_{600nm} measurements were taken every hour. Measurements of sporulation rate were taken in Petriff-Hausse counter for each strain during different phases. The resulting growth kinetic data were plotted and the exponential growth regions were identified. The growth kinetic parameters of the growth rate, maximum cell density and sporulation ratios were calculated for each strain at given conditions. All experiments regarding growth monitoring or spore counting were carried out in triplicate.

Fermentation

Screw-capped test tubes (25 ml) containing 20 ml medium were used. In all experiments, medium was inoculated with a 10% (v/v) active cell suspension and purged with nitrogen gas to remove dissolved oxygen. The culture was incubated at 37°C with no agitation or pH control for 72 h. Fermentation medium containing 70 g/L of either corn or cassava flour liquefied for 30 min at 100°C by thermostable α-amylase was sterilized at 121°C for 15 min. After fermentation for 72 h, 5 ml was taken for ABE analysis.

Analytical methods

The OD_{600nm} of cells was measured by an UV-Vis spectrophotometer. Fermentation products were analyzed by gas chromatography using a KB-FFAP column (0.25 mm×30 m, Kromat Corporation, USA). Total ABE was the sum of acetone, butanol and ethanol. The ABE yield was expressed in g/L.

Results and Discussion

Survival rate curve and paramerers

The energy of the ion beam can be controlled depending on the specific purpose. In this study, the energy was set to 15 keV. Figure 1 shows the effects of different ion implantation doses on the survival rate of *C. acetobutylicum* D64 cells. According to earlier reports (Feng *et al.*, 2006; Xu *et al.*, 2008), the "saddle" region of the survival dose curve is considered to be the appropriate dose. Thus, a dosage of 0.6×10^{16} ions/cm^2 was chosen as optimal for further mutation treatments.

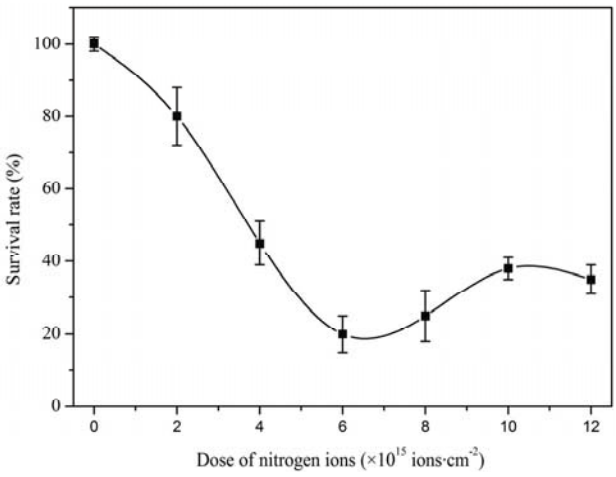

Fig. 1. Effects of ion implantation doses on the survival rate of irradiated *C. acetobutylicum* D64. The survival rate was calculated using the number of colonies scored on control plates without ion implantation as 100% (~600 colonies).

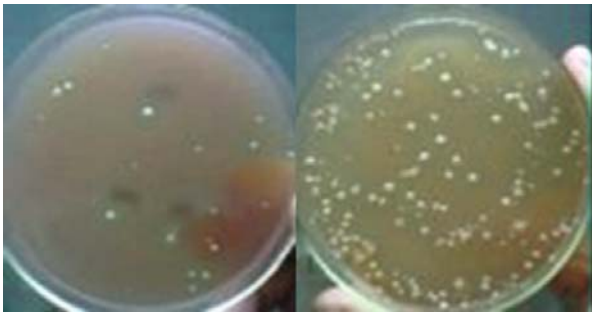

Fig. 2. Growth of the mutans and the parent strain on selection agar plates. (A) Growth of the parent strain. (B) Growth of the mutants.

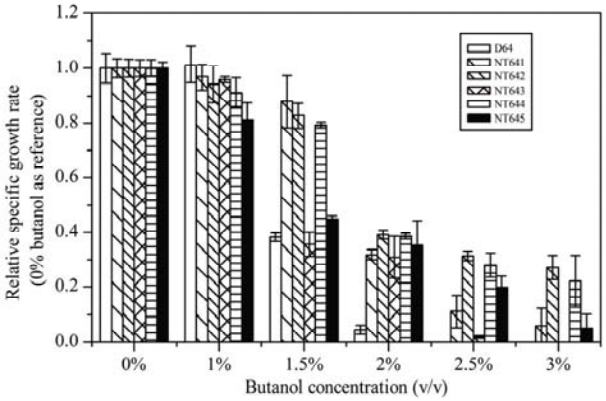

Fig. 3. Inhibition of growth by butanol in YPS medium at 37°C. Values and error bars are from three replicate measurements for each strain at each condition. Reference specific growth rates at 37°C are: 0.48 h^{-1} (D64), 0.42 h^{-1} (NT641), 0.45 h^{-1} (NT642), 0.47 h^{-1} (NT643), 0.48 h^{-1} (NT644), 0.50 h^{-1} (NT645). The butanol concentration of 0.5% (v/v) was not done in this experiment.

Screening using selection agar plates

Thirty colonies that grew well and exhibited large transparent zones were selected by selection agar plates containing resazurin and 1-butanol (Fig. 2), and then inoculated into YPS medium containing 2% (v/v) butanol to evaluate the stability of butanol tolerance. Five mutants of the thirty were comparatively stable at 2% (v/v) butanol. Table 1 shows the colonial morphology of the wild-type and five mutants on YPS medium. Morphologies of the NT642, NT644, and NT645 strains were most similar to each other, while only the NT643 strain was similar to that of the wild type.

The level of butanol tolerance in the mutants

To establish the butanol tolerance of the mutants generated in this study, growth kinetics were estimated in YPS medium in the presence of up to 3% (v/v) butanol for comparison with the tolerance level of parental *C. acetobutylicum* D64. These results are shown in Fig. 3. All mutants grew robustly in YPS media without butanol or with 1% (v/v) butanol, achieving specific growth rates from 87 to 104% and final OD values similar to that of *C. acetobutylicum* D64. A mutant (NT643) exhibited nearly the same level of butanol tolerance as *C. acetobutylicum* D64, while the other four (NT641, NT642, NT644, and NT645) exhibited increased tolerance to butanol. Though *C. acetobutylicum* D64, NT643, and NT645 growth was dramatically inhibited at 1.5% (v/v) butanol respectively, the growth of the other three mutants were not inhibited at 1.5% (v/v) butanol. The mutants grew in up to 2% (v/v) butanol and achieved a similar final OD of approx. 40% of that without butanol. After growth in YPS medium with 2.5% (v/v) butanol, the three most butanol tolerant mutants (NT642, NT644, and NT645) maintained their morphology (Circular, convex) and grew well on YPS medium (data not shown). However, only two mutants (NT642 and NT644) could grow at 3% (v/v) butanol with a similar relative growth rate of ~40%.

Interestingly, some samples in one out of three replicates exhibited growth at higher butanol concentrations (but not at a lower butanol concentration) and a growth lag also occurred concomitantly. A similar result was described previously (Winkler *et al.*, 2010). The result suggested that growth at higher butanol concentration was derived from adaptive mutations conferring enhanced butanol tolerance. It is unlikely that these instances resulted from contamination as no such growth emerged in the negative controls. Moreover, the other two replicates were from the same overnight inoculum. If there were same contaminants in the inoculum, growth should also be observed in the others. However, the growth only occurred in a single replicate in this case. A further investigation should be carried out to investigate these outlier growths.

Table 1. Morphology of mutant strains compared with the parent strain on YPS medium

Strain	Morphology
D64	Circular, flat
NT641	Irregular, flat
NT642	Circular, convex
NT643	Circular, flat
NT644	Circular, convex
NT645	Circular, convex

Effect of butanol tolerance on sporulation rate

C. acetobutylicum can produce spores under the control of sporulation genes to resist severe conditions such as poor nutrition, unfavorable pH challenge and solvent toxicity (Tomas *et al.*, 2004; Mao *et al.*, 2010). Butanol toxicity is the most severe among the fermentation products and sporulation will occur when butanol concentration reaches 10 g/L in the fermentation broth (Bowles and Ellefson, 1985). To identify the relationship between sporulation and butanol tolerance, five mutants with different levels of butanol tolerance were tested in YPS containing 2% (v/v) butanol (Fig. 4). Each mutant exhibited vigorous growth with sporulation rate of only ~30% in 12 h which was during the logarithmic growth phase. After overspreading, the sporulation rate increased quickly from ~60% in 18 h to above 90%. Remarkably, the two most butanol tolerant mutants (NT642 and NT644) had a sporulation rate of only 10% compared to that of 30% for the others (NT641, NT643, and NT645) with lower levels of butanol tolerance in 12 h. Furthermore, these changes in

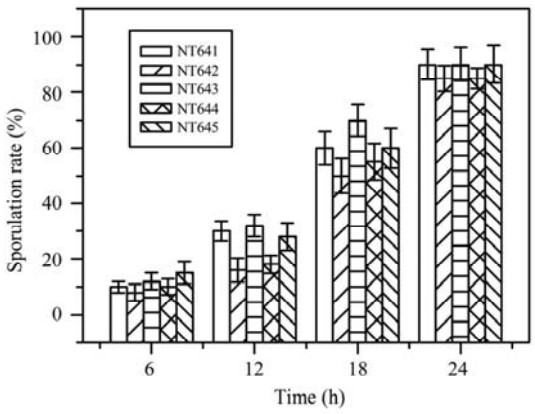

Fig. 4. Comparison of sporulation rate for each strain in YPS medium containing 2% (v/v) butanol during different phases. *C. acetobutylicum* D64 was not done in this experiment because of its dramatic inhibition under this butanol condition. The number of spores was scored using a Petriff-Hausse counter at a magnification of 100×. The time of observation began from the beginning of incubation at 37°C.

sporulation rates emerged simultaneously through all the experiments. It is unlikely that these changes were the result of general culture conditions, as each strain was incubated under the same conditions. Thus, we conclude that the level of butanol tolerance affects the sporulation rate in the presence of butanol. The mutants that tolerate a higher concentration of butanol undergo sporulation less readily than the wild type strain with low level of butanol tolerance in the fermentation broth of equal butanol cocentration. Consequently, the mutants with a higher butanol tolerance should produce a higher titer of butanol under normal conditions in ABE fermentation.

Fermentation using different substrates

C. acetobutylicum could utilize various sugar and starch-containing substrates without any additional enzymatic hydrolysis (Mutschlechner *et al.*, 2000). Both corn and cassava contained above 60% (w/w) starch, which were also used as fermentation medium. Thus, we chose 7% (w/v) of corn or cassava flour as fermentation medium in this study. The productions of wild-type and five mutants are listed in Table 2. Using corn flour as medium, *C. acetobutylicum* NT642 and NT644, the two most butanol tolerant mutants, achieved the highest butanol (15.4 g/L and 14.0 g/L yield respectively) and solvent (22.3 g/L and 21.8 g/L yield respectively), compared to those of the wide-type and the other mutants. However, when fermented with cassava flour, each strain generated nearly equal yields of ~10.7 g butanol/L and 18.2 g ABE/L except for *C. acetobutylicum* NT642 with 12.2 g butatol/L and 20.4 g ABE/L. From Fig. 3, we can speculate that the butanol concentration of 10 g/L would hardly inhibit the growth of cells, and there must be some other reasons for the lower yields, such as poorer nutrition compared to corn. Although the other three mutants had higher butanol tolerance as compared to the wild-type, their production of butanol was not enhanced. *C. acetobutylicum* with high butanol tolerance, which is only one of the requirements of high-yield strains, and is not enough in itself for high production (Dunlop *et al.*, 2011). However, *C. acetobutylicum* NT642 (the most butanol tolerant mutant) produced the highest butanol and solvent yields in batch fermentation with both corn and cassava.

Maddox (1980) reported that different substrates affect the ABE ratio which agrees with the results of the present study. When corn was used in the medium, *C. acetobutylicum* NT642 produced ABE at 22.3 g/L with a ratio of 26:72:9, whereas, using cassava as substrate, it produced ABE at 20.4 g/L with a ratio of 27:56:13. Similar results were obtained with these two different feedstocks for wild-type and the other 4 mutants.

Conclusions

The present study shows that five *C. acetobutylicum* mutants with enhanced butanol tolerance and butanol production were obtained using nitrogen ion beam implantation. The production of butanol and ABE achieved by the most butanol tolerant mutant *C. acetobutylicum* NT642 were both higher

Table 2. ABE production from corn mash and cassava mash (70 g/L) in fermentation test by mutants and wild-type

Strains	Feedstock	Acetone (g/L)	Butanol (g/ L)	Ethanol (g/ L)	ABE yield (g/ L)
C. cetobutylicum D64	corn flour	5.3±0.2	12.8±0.3	1.3±0.1	19.4±0.2
NT641		4.5±0.1	13.5±0.2	1.6±0.2	19.6±0.2
NT642		5.2±0.1	15.4±0.6	1.8±0.2	22.3±0.7
NT643		4.9±0.4	13.7±0.5	1.5±0.5	20.1±0.6
NT644		6.6±0.6	14.0±0.2	1.2±0.1	21.8±0.8
NT645		4.5±0.2	13.5±0.3	1.7±0.4	19.7±0.5
C. cetobutylicum D64	cassava flour	5.0±0.1	10.8±0.2	2.1±0.4	17.8±0.4
NT641		4.5±0.7	10.2±0.3	2.9±0.5	18.0±0.8
NT642		5.5±0.3	12.2±0.7	2.6±0.3	20.4±1.1
NT643		4.8±0.2	10.6±0.8	2.8±0.3	18.2±1.1
NT644		5.0±0.4	10.9±0.4	2.4±0.4	18.3±0.4
NT645		4.8±0.3	10.6±0.3	3.5±0.4	18.9±1.2

Strains were grown in screw-capped test tube containing a fermentation medium with 70 g corn or cassava flour/L liquefied for 30 min at 100°C by thermostable α-amylase. The initial pH of culture was adjusted to 6. The temperature was maintained at 37°C. There was no agitation or pH control. Data are expressed as the mean±SD from three replications

than those of its parental strain and the other mutants regardless of whether corn or cassava was used as feedstock. Although the level of yield has been limited, the effect of nitrogen ion beam implantation on the enhancement of butanol tolerance and butanol yield in *C. acetobutylicum* was remarkable. Such increases in butanol tolerance and butanol yield using the mutant make the ABE fermentation process economically viable.

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