World J Microbiol Biotechnol (2012) 28:2963–2971
DOI 10.1007/s11274-012-1107-1

ORIGINAL PAPER

# Screening and characteristics of a butanol-tolerant strain and butanol production from enzymatic hydrolysate of NaOH-pretreated corn stover

**Kai Gao · Yun Li · Shen Tian · Xiushan Yang**

Received: 19 April 2012 / Accepted: 11 June 2012 / Published online: 26 June 2012


**Abstract** As a gasoline substitute, butanol has advantages over traditional fuel ethanol in terms of energy density and hydroscopicity. However, solvent production appeared limited by butanol toxicity. The strain of *Clostridium acetobutylicum* was subjected to mutation by mutagen of *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine for 0.5 h. Screening of mutants was done according to the individual resistance to butanol. A selected butanol-resistant mutant, strain 206, produced 50 % higher solvent concentrations than the wild-type strain when 60 g glucose/l was employed as substrate. The strain was also able to produce solvents of 23.47 g/l in 80 g/l glucose P2 medium after 70 h fermentation, including 5.41 g acetone/l, 15.05 g butanol/l and 3.02 g ethanol/l, resulting in an ABE yield and productivity of 0.32 g/g and 0.34 g/(l h). Subsequently, Acetone-butanol-ethanol (ABE) production from enzymatic hydrolysate of NaOH-pretreated corn stover was investigated in this study. An ABE yield of 0.41 and a productivity of 0.21 g/(l h) was obtained, compared to the yield of 0.33 and the productivity of 0.20 g/(l h) in the control medium containing 52.47 mixed sugars. However, it is important to note that although strain 206 was able to utilize all the glucose rapidly in the hydrolysate, only 32.9 % xylose in the hydrolysate was used after fermentation stopped compared to 91.4 % xylose in the control medium. Strain 206 was shown to be a robust strain for ABE production from lignocellulosic materials and has a great potential for industrial application.

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**Keywords** *Clostridium acetobutylicum* · NTG mutation · Butanol resistance · ABE fermentation · Corn stover · Undetoxifed hydrolysate

## Introduction

Owing to a limited supply of petroleum oil, increased environmental concerns, and an awareness of global warming, bioproduction of automotive fuels from renewable feedstocks has become a global priority (Liu and Qureshi 2009). Compared to ethanol, butanol contains two more methylenes, thus is more hydrophobic than ethanol, with lower volatility, is miscible with gasoline and has higher energy density. Therefore, the fermentative production of butanol has received renewed attention in recent years (Jones and Woods 1986; Atsumi et al. 2008).

One of the major obstacles to commercial acceptance of the acetone/butanol fermentation is the high recovery cost of butanol caused by the rather low final concentration of butanol in the fermentation broth (approx. 1 %). Clostridial cellular metabolism ceases in the presence of 20 g/l or more solvents (Lee et al. 2008). This limits the concentration of carbon substrate that can be used for fermentation, thus resulting in low final solvent concentration and productivity.

Butanol has numerous harmful effects on *Clostridium acetobutylicum*. At a concentration high enough to inhibit growth, butanol tends to partition into the cytoplasmic membranes and changes the membrane structures interfering with normal functions and destroying the ability of the cell to maintain internal pH, lowers the intracellular level of ATP, and inhibits glucose uptake (Bowles and Ellefson 1985).

The modern techniques of gene engineering and metabolic engineering are considered to be effective methods in microorganism breeding. However, application of these modern techniques frequently encounters serious impediments due to limited insight into the genetics, physiology, and biochemistry of organisms (Olano et al. 2008). Thus, traditional mutation is still an effective approach to obtain the improved strains for overproduction of both primary and secondary metabolites (Khaliq et al. 2009). Rational screening on the basis of understanding the metabolism and pathway regulation, allows for a significant improvement in the efficiency of the selection process, by contrast with the time-consuming random selection method. Moreover, precursor-resistant mutants and product-resistant mutants are widely used for screening the high producing strains (Wu et al. 2008). Chemical mutation by *N*-methyl-*N*-nitro-*N*-nitrosoguandine (NTG) has been reported as a successful method for mutation and screening of high-yield strains (Kumar et al. 2009).

The high cost of conventional substrates (corn, molasees) is also a major factor affecting the economic viability of acetone-butanol-ethanol (ABE) fermentation (Jones and Woods 1986). Lignocellulosic biomass is therefore recognized as an effective potential substrate for ABE production because of its abundance, renewability, and cost-effective characteristics (Wang and Chen 2011). Corn stover is one of the most abundant agricultural residues in China. However, it is required to be pretreated and enzymatically hydrolyzed for producing fermentable sugars. A wide range of compounds, some of which are toxic to butanol-producing microorganisms, is generated during lignocellulose degradation. Several methods have been investigated, ranging from overliming (Qureshi et al. 2010a, b), alkaline peroxide pretreatment and activated charcoal pretreatment (Wang and Chen 2011), and ion exchange resins (Qureshi et al. 2008), for the removal of toxic compounds from lignocellulosic hydrolysate. However, detoxification is a very costly process, and may lead to loss of fermentable sugars.

Acid pretreatment of a wide range of cellulosic materials (Qureshi et al. 2008, 2010a, b) has been investigated and butanol production from dilute sulfuric acid-pretreated wheat straw without detoxification has proven to be efficient and successful (Qureshi et al. 2007). However, fermentation of undetoxified corn stover using the same pretreatment method showed no growth and ABE production (Qureshi et al. 2010b). Wang and Chen (2011) investigated ABE fermentation from undetoxified steam-exploded corn stover and obtained a rather low ABE production of 3.71 g/l. On the contrary, in a similar study, alkali-pretreated enzymatic hydrolysate of wheat straw was directly converted to butanol and acetone by *C. acetobutylicum*, with a solvent concentration of 17.3 g/l (Marchal et al. 1984). Pretreatment of corn stover with 2 % NaOH was reported recently to substantially increase the lignin removal and enhance the accessibility and digestibility of cellulose (Chen et al. 2009). Compared with acid or oxidative reagents, alkali treatment appears to be the most effective method in breaking the ester bonds between lignin, hemicellulose and cellulose, and avoiding fragmentation of the hemicellulose polymers (Gaspar et al. 2007).

The objectives of this study are twofold. One is to develop a butanol-resistant mutant from *C. acetobutylicum* ATCC 824 and investigate the characteristics of the mutant to produce butanol using glucose as the substrate, the other is to evaluate the strain's ability to ferment enzymatic hydrolysate of NaOH-pretreated corn stover as the feedstock to produce butanol in order to decrease the cost further.

## Materials and methods

### General microbiological conditions

All microbiological work was performed under aseptic and strict anaerobic conditions. Anaerobic incubation of the cultures was performed in an anaerobic suction system or in sealed serum vials or anaerobic tubes. Cultures were continuously vented to prevent the build up of gas pressure.

### Strains and maintenance

The wild-type strain of *C. acetobutylicum* ATCC 824 was purchased from China General Microbiological Culture Collection Center (CGMCC).

Cultures of this strain and of the mutants derived from it were routinely maintained as suspensions in seed medium containing (per liter) 3 g of yeast extract, 5 g of peptone, 5 g of soluble starch, 5 g of glucose, 2 g of ammonium acetate, 2 g of NaCl, 3 g of $MgSO_4$, 1 g of $KH_2PO_4$, 1 g of $K_2HPO_4$, 0.1 g of $FeSO_4{\cdot}7H_2O$, pH 6.0 at 4 °C. Spores in the seed medium were heat shocked for 2 min at 90 °C and transferred to fresh seed medium. Three milliliters of actively growing cells were inoculated into 50 ml of inoculum development P2 medium, prepared in a 100 ml screw-capped bottle. The inoculum development P2 medium contained 30 g/l glucose, 1 g/l yeast extract, and stock solutions (minerals, buffer, and vitamins) (Qureshi et al. 2008). The solution containing glucose and yeast extract was sterilized at 115 °C for 20 min and 0.5 ml of each of the filter-sterilized stock solutions were added to 50 ml glucose-yeast extract solution. Then the bottles were placed in an anaerobic jar for 24 h. The culture (inoculum) was

allowed to grow for approximately 18 h at 37 °C when it was ready to be inoculated into the ABE production medium.

### Selection of optimal mutagenesis conditions

The mutagenesis process was performed in anaerobic tubes containing 10 ml seed medium. The parent strain was cultivated for 16–18 h. Then different amounts of 10 mg/ml NTG solution were inoculated into the cultures by syringe through rubber stoppers resulting in a final concentration of NTG ranging from 0.2 to 1.4 mg/ml. The mutagenesis lasted for 30 min. After each run, 1 ml of NaOH solution (1 mol/l) was added into the cultures to stop the mutagenesis process. The cell suspension was diluted properly and spread over the agar seed medium in plates. The individual colonies on each screening plate and control plate were separately counted. The lethal rate was calculated by the equation below:

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

### Mutagenesis and rational screening

It has been reported that more positive mutants can be obtained when lethal rate was about 70 % (Xu et al. 2011). In this study, 0.8 mg/ml NTG was employed to yield more butanol-resistant mutants because lethal rate under this condition was 72.1 %. Cells were collected by centrifugation, washed three times and diluted properly with sterilized saline, then plated on screening agar plates (supplemented with 8, 10, 12, 14 g/l butanol). Plates were incubated for 1–2 days at 37 °C in an anaerobic jar. All the individual colonies on each screening plate were inoculated to 50-ml flask containing 20 ml of sterilized seed medium and were incubated anaerobically at 37 °C for 16–18 h, before they were evaluated for their solvent production.

### Growth inhibition by butanol

The seed medium was also used to investigate the effects of added butanol on the growth of both mutant and wild-type strain. The cultures were carried out in 100 ml screw-capped anaerobic bottles containing 50 ml medium. Addition of butanol was performed by injection with a syringe through the rubber stoppers of the sterile bottles, and then the bottles were inoculated with 1 ml 16-h-old culture on the same medium and incubated at 37 °C. Cell growth was estimated by measurement of $A_{600}$ with a 722 spectrophotometer.

### Raw material

Corn stover was collected from rural area of Beijing, China. After harvest, the corn stover was air dried and stored at room temperature. Before any pretreatment, the stover was ground to 40 mesh and the sample was stored in a plastic bag at room temperature for use.

### Pretreatment

Corn stover sample (20 g) was soaked in 160 ml of 2 % NaOH aqueous solution and kept at 120 °C for 30 min. Solid residues were thoroughly washed with water until neural pH, and then filtered by nylon cloth (200 mesh). The pretreated sample was dried at 65 °C in an oven to constant weight and stored at room temperature.

### Hyrolysate preparation

Enzymatic hydrolysis experiments were conducted with solid substrate concentration of 10 % (w/v) in distilled water and an enzyme loading of 20 FPU/g WIS using Celluclast 1.5 l complemented with 20 IU/g WIS of Novozyme 188. The pH was adjusted to 4.8 ± 0.2. The hydrolysis reaction was conducted at 50 °C, 150 rpm for 72 h. This was followed by centrifugation at 2,000×*g* for 5 min to remove sediments. The hydrolysate was stored in a presterilzed bottle at 4 °C. The sugar composition of the hydrolysate was glucose 36.04 g/l, xylose 21.04 g/l. The hydrolysate was used for fermentation studies.

### Fermentation

All Fermentation studies were conducted in 100 ml screw-capped bottles containing 50 ml of fermentation medium without any agitation or mixing. The preparation of the fermentation medium was the same as that of the inoculum development P2 medium. The only difference was that the fermentation medium contained 6, 8 or 10 % glucose or 60 g/l mixed sugars (glucose and xylose in the ratio of 2:1) as carbon source.

For the hydrolysate fermentation, the pH was adjusted to 6.5 using 5 M NaOH solution. To the bottle, 0.5 ml of sterile 100 g/l yeast extract solution and 0.5 ml of each stock solution were added to reach the same nutrient concentration level as in P2 medium. Following this the bottles were kept in an anaerobic jar for 24 h for anaerobiosis. Then the bottles were inoculated with 5 ml of actively growing culture followed by incubation at 37 °C. Samples were taken intermittently and centrifuged at 12,000 rpm. Clear liquid was stored at −18 °C for ABE and sugar analysis.

### Analytical methods

An Agilent 7890A gas chromatograph equipped with flame ionization detector was used for solvent assays. The samples were injected by Agilent 7694E Headspace sampler. Determination conditions for solvents were as follows: the chromatographic column was HJ-PEG-20 M (60 m × 0.32 mm × 0.5 μm), column temperature was 120 °C, injection temperature was 120 °C, detector temperature was 200 °C, $N_2$ was used as the carrier gas, and the flow rate was 4 ml/min.

Determination of sugar content was performed using a high performance liquid chromatograph (Waters 2690). NUCLEOSIL 100-5 NH2 column (250 × 4.6 mm) and Waters 410 differential detector were used, the column temperature was 40 °C, acetonitrile in mobile phase: water = 75:25, flow rate was 1 ml/min, and sample size was 20 μl.

Productivity was calculated as the maximum ABE concentration achieved (g/l) divided by the fermentation time and is expressed as g/(l h). Yield was calculated as the total amount of solvents produced divided by the amount of fermentable sugar utilized and is expressed as g/g.

The chemical composition of raw and pretreated corn stover was determined according to the previous methods (Liu 2004).

At least three parallel samples were used in all analytical determinations, and data are presented as the mean of three replicates.

## Results and discussion

### NTG mutagenesis

NTG concentration was investigated to get optimal NTG mutagenesis conditions. Exposure time was fixed at 30 min. The NTG concentration ranged from 0.2 to 1.4 mg/ml. The lethal rate was shown in Fig. 1. The lethal rate increased with the increase of NTG concentration. The highest value approached 98.7 % at the condition of 1.4 mg/ml NTG. And the lowest level was 18.7 % when 0.2 mg/ml NTG was used. It was speculated that the NTG concentration in these ranges was either too weak to induce mutation or so strong it damaged the cells. On the other hand, more positive mutants may be obtained when the lethal rate was between 60 and 80 %, therefore 0.8 mg/ml NTG (lethal rate at this condition was 72.1 %) was used in this study to obtain butanol-resistant mutants.

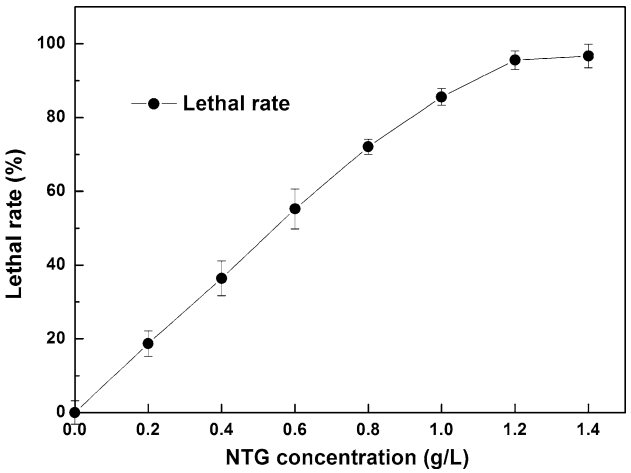


**Fig. 1** Lethal rate curve of *C. acetobutylicum* by 30 min of NTG mutagenesis; (*filled circle*) lethal rate

### Isolation of butanol-resistant mutants

Butanol-resistant mutants of strain *C. acetobutylicum* were obtained as described in materials and methods in an effort to select better solvent-producing strains. After 48 h of incubation, slight growth inhibition was observed on plates containing 8 and 10 mg/ml butanol. A clear background with no growth was observed on plates containing 14 mg/ml butanol. Butanol-resistant colonies were obtained on plates containing 12 mg/ml butanol and no spontaneous mutant was obtained on control plates containing the same concentration of butanol after 48 h incubation. Several of these mutants were inoculated into 50 ml flask containing 25 ml sterile seed medium and incubated at 37 °C in an anaerobic jar for 16 h under static condition before they were used for fermentation experiments.

### Solvent production by butanol-resistant mutants

Screening for better solvent producers among butanol-resistant mutants was first carried out in anaerobic tubes with 6 % glucose as carbon source. Many mutants were found to produce higher concentrations of solvent than those produced by the wild-type strain (data not shown), and the most promising strain 206 was used for further study.

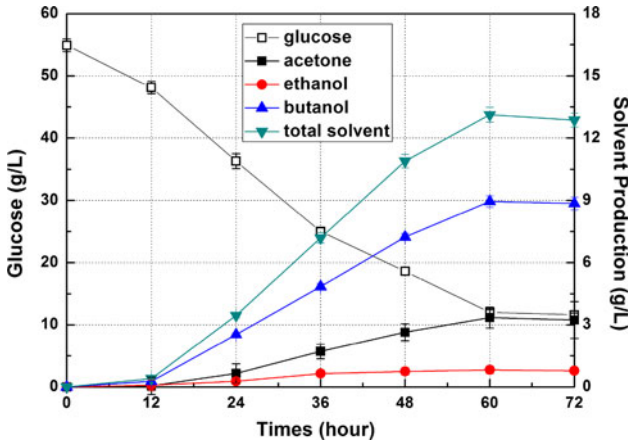


**Fig. 2** Production of solvents by wild-type strain of *C. acetobutylicum*; (*open square*) concentration of glucose; (*filled square*) concentration of acetone; (*filled circle*) concentration of ethanol; (*filled triangle*) concentration of butanol; (*filled inverted triangle*) total solvents

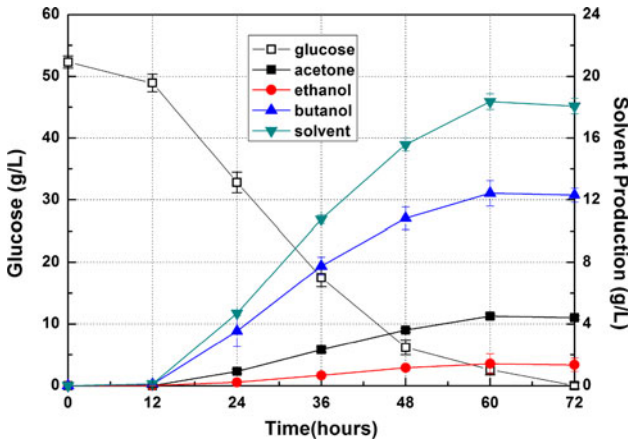


**Fig. 3** Production of solvents by the 206 butanol-resistant strain of *C. acetobutylicum*; (*open square*) concentration of glucose; (*filled square*) concentration of acetone; (*filled circle*) concentration of ethanol; (*filled triangle*) concentration of butanol; (*filled inverted triangle*) total solvents

More detailed comparative experiments were performed in 100 ml screw-capped bottles containing 50 ml of P2 medium with 6 % glucose as carbon source between wild-type strain and a selected butanol-resistant mutant (strain 206). Similar fermentation patterns were obtained in these conditions and are illustrated in Figs. 2 and 3. Both strains reached their maximum solvents production within 60 h.

The initial amount of sugar in the fermentation medium, which was 55 ± 1 g/l due to the loss of glucose during autoclaving, was reduced to 2.6 g/l by strain 206 and 12 g/l by wild-type strain after 60 h. This indicated that butanol-resistant culture utilized more sugar (95 %) for its metabolic activity in the production of solvents as compared with the wild-type strain (78.1 %). Total solvents produced by mutant strain 206 was 18.43 g/l (including 4.51 g/l acetone, 12.40 g/l butanol, and 1.42 g/l ethanol), 48.4 % higher than that of wild-type strain. Also, butanol production was 39.3 % higher than that of the wild-type strain. The ABE yields by mutant strain 206 and wild-type strain were 0.35 and 0.30 g/g, respectively, and ABE productivities by both strains were 0.31 and 0.22 g/(l h), respectively. Additionally, strain 206 produced a higher proportion of butanol than the wild-type strain, butanol to acetone ratio was 2.75:1 compared with 2:1 in a typical ABE fermentation (the typical products ratio of ABE fermentation is 3:6:1).

## Growth inhibition by butanol

The influence of various concentrations of butanol on the growth curves of wild-type strain and its butanol-resistant mutant 206 was compared. The growth curves obtained in the presence of butanol are shown in Figs. 4 and 5. Both strains can not grow in the medium containing 14 g/l butanol (data not shown). With both strains, inhibitory effects of added butanol became important only at concentrations approaching that of complete inhibition (12 g/l). In the case of lower concentrations of butanol (8 and 10 g/l butanol), inhibitory effects are more gradual. When the concentration of butanol increased to 12 g/l, the wild-type strain showed very restricted growth, while strain 206 showed a significant growth after incubation for 6 h.

## Effects of glucose concentration on solvent formation

The effect of various concentrations of glucose (6–10 %) on solvent formation by *C. acetobutylicum* and strain 206

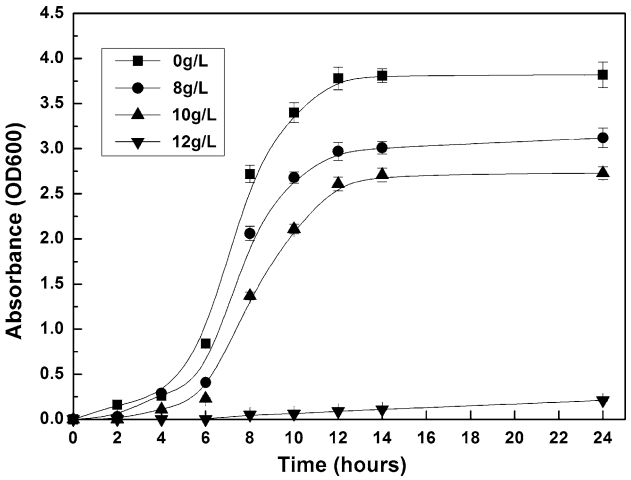


**Fig. 4** Effect of different concentrations of butanol on the growth of wild-type strain; (*filled square*) the OD value when no butanol was added into the culture; (*filled circle*) the OD value when 8 g/l butanol was added into the culture; (*filled triangle*) the OD value when 10 g/l butanol was added into the culture; (*filled inverted triangle*) the OD value when 12 g/l butanol was added into the culture

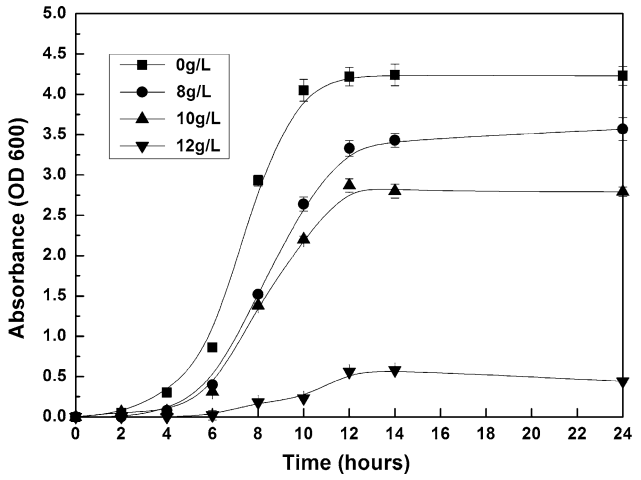


**Fig. 5** Effect of different concentrations of butanol on the growth of butanol-resistant strain 206; (*filled square*) the OD value when no butanol was added into the culture; (*filled circle*) the OD value when 8 g/l butanol was added into the culture; (*filled triangle*) the OD value when 10 g/l butanol was added into the culture; (*filled inverted triangle*) the OD value when 12 g/l butanol was added into the culture

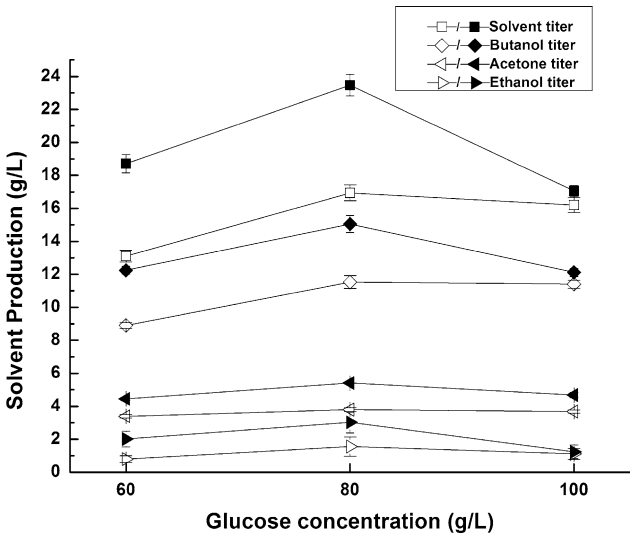


**Fig. 6** Effect of various concentrations of glucose on solvent formation by *C. acetobutylicum* ATCC 824 (*open symbols*) and butanol-resistant mutant 206 (*solid symbols*); (*filled square*) total solvent productions by strain 206 using different concentrations of glucose; (*open square*) total solvent productions by wild-type strain using different concentrations of glucose; (*filled diamond*) butanol concentrations by strain 206 using different concentrations of glucose; (*open diamond*) butanol concentrations by wild-type strain using different concentrations of glucose; (*filled left arrowhead*) Acetone concentrations by strain 206 using different concentrations of glucose; (*open left arrowhead*) Acetone concentrations by wild-type strain using different concentrations of glucose; (*filled right arrowhead*) Ethanol concentrations by strain 206 using different concentrations of glucose; (*open right arrowhead*) Ethanol concentrations by wild-type strain using different concentrations of glucose

can be seen in Fig. 6. The highest concentration of butanol was obtained when 8 % glucose was used as carbon source, with strain 206 producing 15.05 g/l of butanol, while wild-type strain under the same condition only produced 11.41 g/l butanol. Mutant 206 produced consistently higher levels of solvents than the wild-type strain between 6 and 10 % glucose.

It has been reported that if the carbohydrate in the medium is raised above 60 g/l, the concentration of solvent increases, although the fermentation may not be as efficient (Lin and Blaschek 1983). On contrary, the highest efficiency of conversion of glucose to total solvents was obtained by mutant strain 206 when higher glucose concentration was used as substrate (80 g/l). The initial glucose concentration was 74.2 g/l, which was reduced to 8.9 g/l after 70 h fermentation. The culture used 65.3 g/l glucose to produce 23.47 g/l ABE, corresponding to a solvent yield of 0.36 g/g and a solvent productivity of 0.34 g/(l h), both higher than the results achieved when 6 % glucose was used as substrate (0.35 g/g and 0.31 g/(l h)).

However, when glucose concentration was further increased to 100 g/l, both butanol and solvent titer decreased sharply, this was probably due to the inhibitory effects of excessive substrate.

## Chemical composition of NaOH-pretreated corn stover

To facilitate enzymatic hydrolysis of corn stover into fermentable sugars, pretreatment of raw material is essential. After NaOH pretreatment, the percent of cellulose and hemicellulose increased to 65.2 and 23.3 % from 38.7 and 21.7 % of the raw material, respectively. In contrast, the content of lignin decreased from 19.3 to 11.5 %.

## ABE fermentation from mixed sugars and enzymatic hydrolysate of corn stover by butanol-tolerant strain

Prior to carrying out cellulosic butanol fermentation, investigations were made to ascertain the ability of the butanol-tolerant strain to ferment the glucose and xylose concurrently. At the beginning of the fermentation, the initial total sugar level was 52.47 g/l, including 34.72 g/l glucose and 17.75 g/l xylose. As was shown in Fig. 7, the culture started to use glucose and xylose directly after the inoculation. Almost all the glucose was utilized by the culture at 36 h. In contrast, 91.0 % xylose was consumed at 84 h, leaving behind 1.6 g/l unused xylose in the medium. It was not surprising that the glucose utilization rate was far greater than the xylose as glucose is the preferred carbon source. In a closely related study, batch fermentation of the glucose/xylose mixture by *C. acetobutylicum* 824 on a complex medium showed that this bacterium metabolizes glucose first and rapidly before utilizing xylose for ABE production (El Kanouni et al. 1998). Results presented in

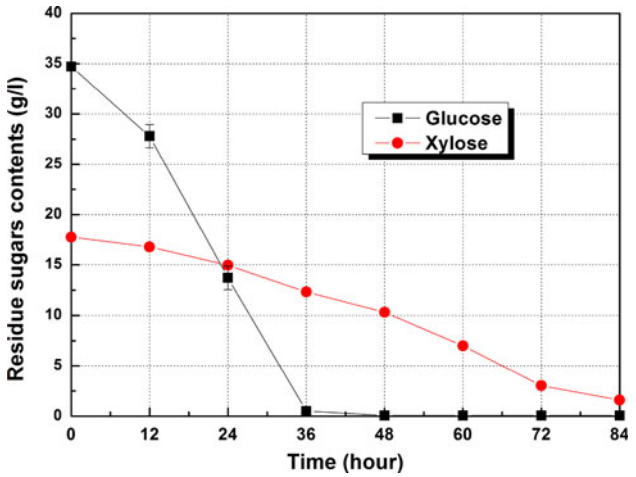


**Fig. 7** Sugar utilization in mixed sugar by the butanol-tolerant strain 206; (*filled square*) Residual glucose content in mixed sugar medium; (*filled circle*) Residual xylose content in mixed sugar medium

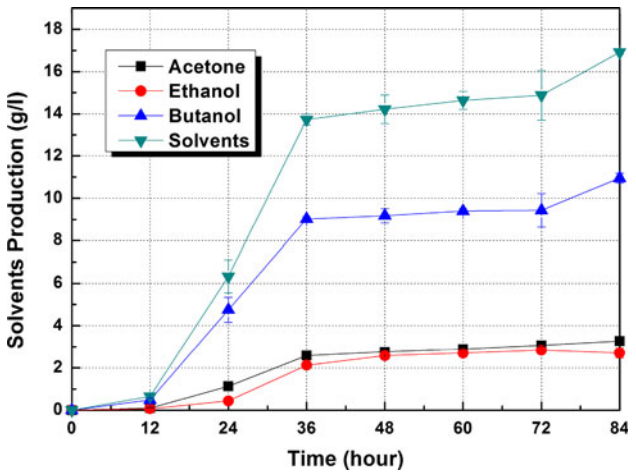


**Fig. 8** Profiles of solvents production from mixed sugar by the butanol-tolerant strain 206; (*filled square*) Acetone production from mix sugar medium by butanol-tolerant strain; (*filled circle*) Ethanol production from mix sugar medium by butanol-tolerant strain; (*filled triangle*) Butanol production from mix sugar medium by butanol-tolerant strain; (*filled inverted triangle*) Solvent production from mix sugar medium by butanol-tolerant strain

Fig. 7 suggested that the butanol-tolerant strain 206 was able to utilize glucose and xylose simultaneously (Fig. 8).

A solvent concentration of 16.92 g/l was achieved after 84 h fermentation with 3.27 g/l acetone, 2.71 g/l ethanol and 10.96 g/l butanol. Yield and productivity of the solvent were 0.33 g/g and 0.20 g/(l h). The results of solvent production and productivity was comparable to that of *C. beijerinckii* BA 101 (Ezeji et al. 2007), which was a hyper butanol production strain. In a similar study, *C. acetobutylicum* ATCC 824 was used for ABE fermentation from 50 g/l mixed sugars, consisting of 30 g/l glucose, 15 g/l xylose and 5 g/l cellobiose, resulting in an solvent concentration of 10.91 ± 0.06 g/l, corresponding to a yield of 0.23 and productivity of 0.15 g/l (Wang and Chen 2011), which was significantly lower than the results of the butanol-tolerant strain obtained in this study. It is speculated that the higher sugar utilization and solvent concentration by mutant strain 206 obtained in this study was probably due to elevated enzyme activity such as glucokinase resulted from mutagenesis.

Numerous studies have been conducted to investigate the possibility to use undetoxified enzymatic hydrolysate of lignocellulosic materials for successful butanol fermentations. One of the most successful cases is the use of *C. beijerinckii* P260 for butanol production from undetoxified enzymatic hydrolysate of dilute-acid pretreated wheat straw, in which 59.3 g/l sugar was utilized to produce 25.0 g/l ABE, with an ABE yield and productivity of 0.42 g/g and 0.60 g/(l h), respectively (Qureshi et al. 2007). In another case, alkali-pretreated and enzyme hydrolyzed wheat straw was used for butanol fermentation without detoxification, 17.7 g/l ABE was produced, with an ABE yield of 0.18 g/g and an ABE productivity of 0.47 g/(l h) (Marchal et al. 1984). However, fermentation result depends a lot on what type of substrate was used and whether a detoxification process was employed. Only 3.71 g/l solvent was produced when undetoxified enzymatic hydrolysate of steam-exploded corn stover was used as substrate (Wang and Chen 2011); fermentation of undetoxified acid-pretreated corn stover hydrolysate exhibited no growth and no ABE production (Qureshi et al. 2010b). In this study, undetoxified enzymatic hydrolysate of NaOH-pretreated corn stover was used for ABE fermentation by a butanol-tolerant strain from *C. acetobutylicum* ATCC 824. At 0 time, 49.5 g/l total sugars were present of which glucose and xylose were 31.5 and 18 g/l. After 60 h of fermentation, glucose was completely utilized. When the fermentation stopped at 72 h, only 32.9 % xylose was used, leaving behind 12.08 g/l xylose unused, compared to 1.6 g/l xylose when mixed sugar was used.

At the end of fermentation, the culture produced 15.42 g/l ABE, resulting in a productivity of 0.21 g/(l h). The individual levels of solvents were acetone 4.34 g/l, ethanol 1.24 g/l, and butanol 9.84 g/l. The culture used 37.42 g/l sugar to produce 15.42 g/l ABE, thus resulting in a yield of 0.41 g/g. The higher ABE yield and productivity obtained in hydrolysate fermentation than fermentation with glucose or mixed-sugar as carbon source may indicate that certain compounds present in the hydrolysate that the glucose or mixed-sugar medium does not contain may have stimulatory effect on ABE production. Even though, due to the inefficiency of xylose utilization by the culture, the final solvent production was lower than that of mixed sugar fermentation. At this stage, the interaction between degradation compounds and slow xylose utilization is still unknown (Figs. 9, 10).

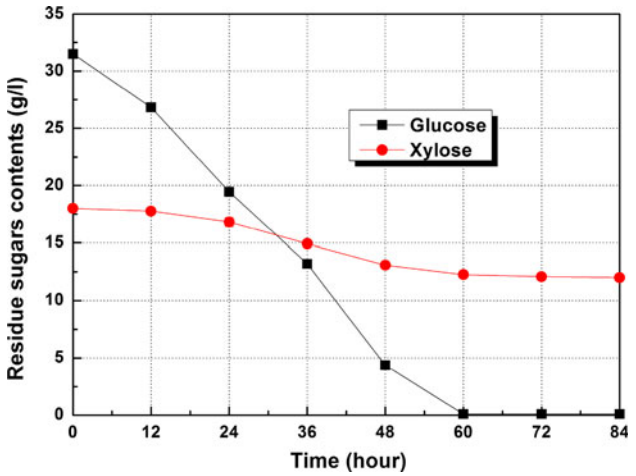


**Fig. 9** Sugars consumption of enzymatic hydrolysate of NaOH pretreated corn stover by the butanol-tolerant strain 206; (*filled square*) Residual glucose content in enzymatic hydrolysate of NaOH-pretreated corn stover; (*filled circle*) Residual xylose content in enzymatic hydrolysate of NaOH-pretreated corn stover

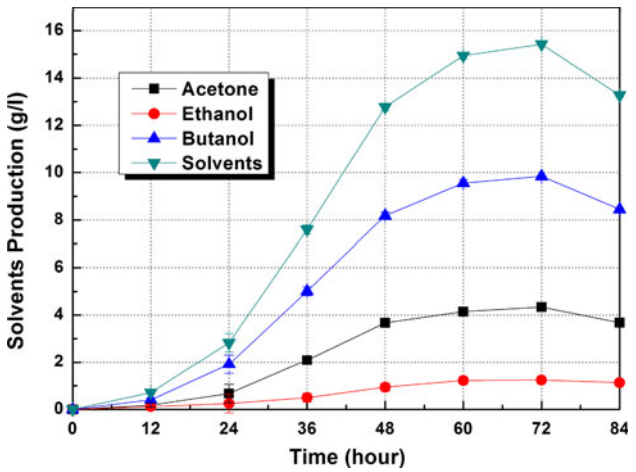


**Fig. 10** Solvents concentration from enzymatic hydrolysate of NaOH-pretreated corn stover by the butanol-tolerant strain 206; (*filled square*) Acetone production from enzymatic hydrolysate of NaOH-pretreated corn stover by butanol-tolerant strain; (*filled circle*) Ethanol production from enzymatic hydrolysate of NaOH-pretreated corn stover by butanol-tolerant strain; (*filled triangle*) Butanol production from enzymatic hydrolysate of NaOH-pretreated corn stover by butanol-tolerant strain; (*filled inverted triangle*) Solvent production from enzymatic hydrolysate of NaOH-pretreated corn stover by butanol-tolerant strain

## Conclusion

In this study, the wild-type strain of *C. acetobutylicum* ATCC 824 was mutated with 0.8 mg/ml NTG for 30 min, and the butanol-resistant mutants were screened on agar plates supplemented with 12 g/l butanol. The isolated mutant, strain 206 can tolerate up to 12 g/l butanol. Fermentation experiments verified that butanol and solvents titers produced by the strain were 39.3 % and 48.4 % higher than the wild-type strain when 6 % glucose P2 medium was used. Mutant 206 was able to produce up to 15 g/l butanol using 8 % glucose P2 medium, and the final solvent titer reached 23.5 g/l. However, further increase in substrate concentration exhibited severe inhibitory effects on solvent production.

Subsequently, the butanol-tolerant strain 206 was used to ferment mixed sugar and enzymatic hydrolysate of NaOH-pretreated corn stover, respectively. The results showed that a better solvent productivity (0.21 vs 0.20 g/(l h)) and yield (0.41 vs 0.33 g/g) than the mixed sugar fermentation was achieved when corn stover hydrolysate was used as carbon source, though the final solvent production was lower than the mixed sugar fermentation (15.42 vs 16.92 g/l), which was probably due to the toxic compounds present in the hydrolysate that inhibit xylose utilization of the culture.

The butanol-tolerant strain 206 showed a superior ability to produce ABE from enzymatic hydrolysate of NaOH-pretreated corn stover, which provides an opportunity for further simplifying the process for cellulosic butanol production, and thus reducing the production cost.

**Acknowledgments** We would like to express our deep appreciation to the National Key Technology R&D Program (2010AA101606, 2011BAD22B01) for its funding.

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