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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

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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.

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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.

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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/

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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 1×10^8 cells was transferred to a 15 mL tube, and

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