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Acetone, butanol, and ethanol production from gelatinized cassava flour by a new isolates with high butanol tolerance

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HIGHLIGHTS

- Direct fermentation of cassava flour to produce ABE was successfully achieved by SE36.
- Phase shift was smoothly triggered due to corn steep liquor and $MnSO_4$ supplementation.
- A high butanol tolerant strain with increased butanol production was obtained.
- The correlation between produced butanol and the butanol tolerance was investigated.

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ABSTRACT

To obtain native strains resistant to butanol toxicity, a new isolating method and serial enrichment was used in this study. With this effort, mutant strain SE36 was obtained, which could withstand 35 g/L (compared to 20 g/L of the wild-type strain) butanol challenge. Based on 16s rDNA comparison, the mutant strain was identified as *Clostridium acetobutylicum*. Under the optimized condition, the phase shift was smoothly triggered and fermentation performances were consequently enhanced. The maximum total solvent and butanol concentration were 23.6% and 24.3%, respectively higher than that of the wild-type strain. Furthermore, the correlation between butanol produced and the butanol tolerance was investigated, suggesting that enhancing butanol tolerance could improve butanol production. These results indicate that the simple but effective isolation method and acclimatization process are a promising technique for isolation and improvement of butanol tolerance and production.

1. Introduction

Due to the rising concerns over the environment and limited fossil fuel reserves, an increasing amount of attention has been recently devoted to developing the environmentally and cost-effective benign alternative energy resources (Ezeji et al., 2013; Li et al., 2014). Among the various fuels produced through biological route, butanol has many advantages over other solvents, such as higher octane number, less corrosive, higher energy content and lower solubility in water (Gottumukkala et al., 2013). Therefore, butanol is considered as one of the suitable candidates for biofuels. Moreover, commercial acetone, butanol and ethanol (ABE) fermentation process have also been investigated by some companies, such as Dupont and BP (Cheng et al., 2012). However, 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, productivity and conversion efficiency (Li et al., 2013). Therefore, this presents a key scientific challenge in ABE fermentation process. Several methods such as metabolic engineering, genetic engineering techniques, selecting solvent tolerant microorganisms, advanced fermentation techniques and downstream processing have been proposed to overcome the above challenge (Ezeji et al., 2010; Fatehi, 2013).

Among these methods, the economically desirable way is to enhance the tolerance of the producing microorganisms. Tomas et al. (2003) reported that the engineered strain 824 (pGROE) obtained by overexpressing *groESL* operon genes in *Clostridium acetobutylicum* ATCC 824, was found to grow better by a butanol challenge than that of the control strain. The final solvent titre of the strain 824 (pGROE) was 33% higher than the plasmid control strains. Borden and Papoutsakis (2007) reported that the strains

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824 (pCAC003) and 824 (pCaAC1869) were isolated by over-expressing two solvent tolerance genes identified by genomic-library enrichment in *C. acetobutylicum* ATCC 824. The butanol tolerance of strains 824 (pCAC003) and 824 (pCaAC1869) increased by 13% and 81%, respectively, than that of the plasmid control strain. Furthermore, the strain 824 (pCAC003) consistently exhibited higher cell densities in normal and challenged cultures. *C. beijerinckii* MUT3, a hyper-solvent tolerant mutant derived from *C. beijerinckii* L175 was produced using combined low-energy ion beam implantation and *N*-methyl-*N*-nitro-*N*-nitrosoguanidine induction. When cultured in batch fermentations in P2 medium, the production of butanol was 15.8 ± 0.7 g/L, a 46% higher than the wild-type strain (Li et al., 2013). Soucaille et al. (1987) reported that a butanol tolerant strain G1 isolated from *C. acetobutylicum* ATCC 824 by a serial enrichment procedure showed 40% increase in butanol tolerance and 17% butanol production higher than that of the wild-type strain.

According to Linggang et al. (2013), substrate cost is another important factor that influences the commercial production of butanol by ABE fermentation. Various renewable agricultural or raw materials, such as sago starch (Al-Shorgani et al., 2012) and corn (Qureshi and Blaschek, 2001), have been used as feedstock for ABE fermentation. However, these present a serious challenge to food security. Howbeit, in countries such as Vietnam, Thailand and China, cassava is not the staple food. Thus, concerns of food insecurity will be minimized (Thang et al., 2010a). Hence, cassava is a promising feedstock for ABE fermentation from renewable resources due to its high starch content (the maximum theoretical concentration of starch, dry weight basis among food crops) (Li et al., 2014). Recently, several research has been carried out on the use of cassava to produce butanol (Lu et al., 2012; Tran et al., 2010).

In this study, in order to obtain native strains resistant to butanol toxicity, butanol producing and tolerant microorganisms were isolated from environmental samples. The isolates were further acclimatized by serial subcultures into media containing increasingly higher sodium chloride and butanol concentrations. The effect of corn steep liquor and $MnSO_4$ on butanol production from cassava flour using the isolate and butanol tolerant mutant was investigated. Furthermore, the correlation between butanol produced and the butanol tolerance was also discussed.

2. Methods

2.1. Environmental samples and media

The soil and polluted water samples were obtained from Daqing Oilfield Company in North China. Each sample was divided into five aliquots ready for use, labeled and stored in sterile sealed bags.

The tryptone-yeast extract-acetate (TYA) development medium was used as seed medium and it comprised of the following: 35 g/L glucose, 6 g/L tryptone, 2 g/L yeast extract, 3 g/L CH_3COONH_4, 0.2 g/L $MgSO_4{\cdot}7H_2O$, 10 mg/L $FeSO_4{\cdot}7H_2O$, 0.5 g/L K_2HPO_4, and 0.5 g/L KH_2PO_4 (Zheng et al., 2013). The medium was autoclaved at 121 °C for 15 min.

The composition of the development P2 medium was as follows: 60 g/L glucose, 3 g/L yeast extract. One mL each of filter-sterilized stock solutions (buffer: 220 g/L CH_3COONH_4, 50 g/L KH_2PO_4, 50 g/L K_2HPO_4; mineral: 1 g/L $MnSO_4{\cdot}H_2O$, 20 g/L $MgSO_4{\cdot}7H_2O$, 1 g/L $FeSO_4{\cdot}7H_2O$, 1 g/L NaCl; and vitamin: biotin 0.001 g/L, thiamin 0.1 g/L, para-aminobenzoic acid 0.1 g/L) was added to 1 L of P2 medium. Buffer, carbon and nitrogen sources were sterilized separately at 121 °C for 15 min. Vitamins and minerals were filter-sterilized using sterile 0.2 μm membrane filters (Li et al., 2013).

Screening medium (TYA) supplemented with 20 g/L agar, 35 g/L starch soluble and 10 g/L butanol was used in the entire screening experiments.

2.2. Isolation of butanol producing strains

A "sandwich" isolation method was developed to obtain butanol producing strains. Sliced potato (about 0.5 cm^3) was placed in an 18 × 180 mL sterile tube, followed by the addition of soil and polluted water samples (3–5 g), and then sliced potato piece (about 0.5 cm^3) was again added until two-thirds of the volume. Finally, 15 g/L butanol (about 10 mL) was added to the tube, covered with a rubber plug and then heat-shocked at 100 °C for 30 s to eliminate the mixed bacteria which could not produce spores, and was cooled to room temperature afterwards. The heat-shocked mixture was cultivated at 37 °C for 16–20 h under anaerobic conditions. Following growth, the supernatant was inoculated into screening medium plate anaerobically for enrichment at 37 °C for 36–48 h. Single colonies were isolated from screening medium into TYA medium and then cultured at 37 °C for 16–18 h. Subsequently, 5–7 mL of exponential phase cultures were inoculated into 50 mL fermentation development P2 medium and or gelatinized cassava flour in 125 mL screw capped bottle.

2.3. Development of butanol tolerance and identification

Active phase samples cultures (one milliliter) of the parent strain were transferred into tubes (18 × 180 mL) containing 9 mL of TYA medium supplemented with various concentrations of sodium chloride (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 g/L) and butanol (10, 15, 20, 25, 30 and 35 g/L). The serial enrichment was started in the medium supplemented with sodium chloride and butanol with the initial concentrations of 0.5 g/L and 10 g/L, respectively. The concentration gradient of sodium chloride and butanol were 0.5 g/L and 10 g/L, respectively. The maximum supplemented concentration of sodium chloride and butanol were 3.0 g/L and 35 g/L, respectively. Growth was directly monitored by optical density measurements at 600 nm with spectrophotometer (Spectronic 200, Thermo scientific). When the optical density reached one, cultures were subsequently transferred to fresh TYA media containing increasing concentrations of sodium chloride and butanol (Lin and Blaschek, 1983). On the contrary, if the cells were inhibited, the enrichment procedure was restarted from the previous cycle again until the cells could tolerate this bottleneck size. At the same time, in order to prevent cells from being completely inhibited or going extinct before any resistant mutants occurred, a control experiment was performed which consisted of a low level concentration of sodium chloride and butanol of the subsequent experiment. Finally, the butanol tolerant mutant was identified by DNA sequencing the 16S rDNA.

The identity of the mutant strain SE36 was determined similar to that reported by Ting et al., (Ting et al., 2012). Genomic DNA of the mutant was extracted using kit from SK1201-UNIQ-10. The 16S rDNA was amplified using KOD-plus DNA polymerase (Invitrogen) and the universal primers set 7f (5′-CAGAGTTTGATCCTGGCT-3′) and 1540r (5′-AGGAGGTGATCCAGCCGCA-3′). PCR was performed using TECHNE TC-5000 (England) under the following conditions: an initial denaturation at 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 35 s and extension at 72 °C for 60 s. The final extension was performed at 72 °C for 8 min. The resulting PCR products were purified using agarose gel electrophoresis and sequenced by Sangon Biotech (Shanghai), China. Homology search of the mutant 16S rDNA was conducted using nBLAST program available from NCBI website (http://www.ncbi.nlm.nih.gov/BLAST/) and analyzed for species identification.