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RESEARCH ARTICLE

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Biosynthesis of 1,4-butanediol from erythritol using whole-cell catalysis

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ABSTRACT

1,4-Butanediol (BDO) biosynthesis from renewable resources is of increasing interest because of global energy and environmental problems. We have previously demonstrated the production of BDO from erythritol by whole-cell catalysis. Here, the effects of several variables on BDO production were investigated, including cell density, temperature, substrate concentration and pH. It was found that the maximum BDO production was obtained at cell density (OD_{600}) of 30. Low temperature and weak alkaline environment were beneficial for the biotransformation. Regarding substrate concentration, 80 g/L of erythritol was found to be optimum for the bioconversion. Under the optimal conditions, the highest concentration of BDO reached 34.5 mg/L, resulting in 5.8-fold increment after optimization. These results will provide useful guidance for enhancing the bioconversion of erythritol to BDO.

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1,4-Butanediol; erythritol; whole-cell biocatalysis

1. Introduction

1,4-Butanediol (BDO) is an important commodity chemical which is widely used in the production of plastics, polyesters and spandex fibers (Zeng and Sabra 2011; Forte et al. 2016). Its annual demand is approximate one million metric tons (Liu and Lu 2015). However, BDO cannot be produced naturally in any known microorganism. It is only produced from petroleum-based feedstocks such as acetylene, propylene, and butadiene (Yang et al. 2015). Given the growing concerns over the environment and volatile fossil energy costs, it becomes an urgent need to develop a more sustainable process for BDO production from renewable feedstocks. In 2011, biosynthesis of BDO via engineered *Escherichia coli* was first achieved by Yim et al. (2011). This pathway requires six steps to convert TCA-cycle intermediate succinate into BDO, and involves the overexpression of six enzymes. Recently, other pathways for BDO biosynthesis have been reported (Liu and Lu 2015; Tai et al. 2016; Wang et al. 2017). These pathways convert lignocellulose-derived sugar into BDO via six steps and require five enzymes.

Erythritol, a four-carbon polyol, is a naturally occurring substance which is widely distributed in nature (Moon et al. 2010). Its chemical synthesis is inefficient and industrial production of this polyol is therefore based on fermentation, mostly using osmophilic fungi (van der Woude et al. 2016). The highest yield of erythritol from glucose is 61%, and a 56% yield can be obtained from glycerol (Amada et al. 2012). Considering the structures of erythritol and BDO are highly similar, it is attractive to explore an enzyme capable of reducing the number of hydroxyl groups, so as to convert erythritol to BDO.

Glycerol dehydratase (GDHt, EC 4.2.1.30) catalyzes the coenzyme B_{12}-dependent conversion of glycerol to 3-hydroxypropionaldehyde, which is further reduced to 1,3-propanediol by 1,3-propanediol dehydrogenase (Daniel et al. 1995). In our previous study, a mutant of GDHt has been obtained by site-directed mutagenesis. Coupling with alcohol dehydrogenase, the mutant can individually catalyze reduction of 1,2-butanediol, 1,2,4-butanetriol (BT), erythritol, 1,2-pentanediol, 1,2,5-pentanetriol and 1,2,6-hexanetriol to produce 1-butanol, 1,4-butanediol, 1,4-butanediol, 1-pentanol, 1,5-pentanediol and 1,6-hexanediol. These products are important chemicals with extensive applications in modern industry. For example, the 1-alkanols have been regarded as substitutes for diesel fuel and gasoline; the 1,n-alkanediols have been widely used for the production of polymers, polyesters and polyurethane resins. As most of these alcohols are non-natural compounds, the promiscuous functions of the GDHt

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mutant can be applied to extend natural cell metabolism for renewable production of these chemicals (Dai et al. 2017). We have previously demonstrated the recombinant strain with overexpression of GDHt can catalyze conversion of erythritol to BDO using whole-cell catalysis. This is a novel pathway which contains only four steps and requires only one heterologous gene. Comparing to previous reports, the corresponding gene regulation and genetic manipulation are simpler. Besides, as engineering a long heterologous pathway will bring significant metabolic burdens on cells, the BDO pathway from erythritol could commendably avoid this. Based on these advantages, the novel route is promising and viable. However, the titer of BDO is still unsatisfactory, leading us to investigate the effects of different factors on BDO production from erythritol.

In this study, the effects of cell density, temperature, substrate concentration, and pH on BDO production were investigated. It was found that the maximum concentration of BDO was obtained at cell density (OD_{600}) of 30, 16 °C and pH 8.0 by using 80 g/L erythritol as substrate. These results offer useful guidance for enhancing the biotransformation of erythritol to BDO.

2. Materials and methods

2.1. Bacterial strains, chemicals and culture conditions

E. coli BL21 (DE3) with overexpression of the GDHt mutant was constructed in the previous study, which was designated as strain EGDHt. Erythritol (99%), BT (98%) and BDO (99%) were purchased from Aladdin (Shanghai, China). Isopropyl β-D-thiogalactoside (IPTG) and kanamycin were obtained from Sangon Biotech (Shanghai, China). Other chemicals were of analytical grade.

2.2. Preparation of resting cells for bioconversion of erythritol to BDO

For whole-cell catalysis, resting cells were prepared with strain EGDHt. The recombinant *E. coli* was inoculated in Luria-Bertani (LB) medium and incubated at 37 °C with 200 rpm agitation. When OD_{600} reached 0.6, 1 mM IPTG was added and the culture was induced at 20 °C for 12 h. Subsequently, cells were harvested by centrifugation (5000 rpm, 15 min) and washed twice with PBS buffer (pH 7.4). The precipitates were stored at 4 °C and used for the following biotransformation.

2.3. Effects of cell density, temperature, substrate and pH on BDO production

Experiments were performed with 20 mL of reaction mixture in a 100-mL Erlenmeyer flask. To investigate the effect of cell density on BDO production, cell precipitates prepared before were suspended in 50 mM potassium phosphate buffer (pH 8.0). The final optical density (OD_{600}) of resting cells was set as 10, 20, 30 and 40. With addition of 60 g/L erythritol and 15 μM coenzyme B_{12}, the suspensions were incubated at 30 °C and 200 rpm for 20 h. Optimal bioconversion temperature was determined over a range of temperatures under the optimal cell density. Cells were suspended in 50 mM potassium phosphate buffer (pH 8.0), followed by the supplement of 60 g/L erythritol and 15 μM coenzyme B_{12}. The mixtures were individually incubated at 16, 25, 30, 37 and 45 °C for 20 h. To optimize the substrate concentration, the reactions were conducted based on the optimal cell density and temperature. Erythritol concentrations from 40 to 100 g/L were evaluated. The effect of initial pH on BDO production was studied under the optimal cell density, temperature and erythritol concentration; 50 mM potassium phosphate buffer with different pH (5.0, 6.0, 7.0, 8.0 and 9.0) was used as the reaction buffer. For each experiment, 1 mL of reaction mixture was collected for GC-MS/MS analysis.

2.4. Analytical methods

BDO and BT were derivatized by N,O-Bis(trimethylsilyl) trifluoroacetamide (BSTFA) and quantified by GC-MS/MS. Samples were centrifuged at 12,000 rpm for 5 min; 800 μL of the supernatant was mixed with supersaturated NaCl followed by the addition of 800 μL acetonitrile. The organic phase was collected and dried completely by using a rotary evaporator. Then, 20 μL of 1 mM cyclohexanol (dissolved in dimethylformamide) and 50 μL BSTFA were subsequently added, and the mixture was incubated at 70 °C for 30 min. The derivatized samples were injected into triple quadrupole GC-MS/MS system (Thermo & TSQ 8000) equipped with a HP-5 MS column (0.25 μm, 0.25 mm × 30 m), and quantified in SIM mode. The oven program was as follows: 80 °C for 1.5 min, raised to 140 °C at 10 °C/min, held for 3 min, increased to 300 °C at 30 °C/min, held for 5 min. Selected ion monitoring was performed by monitoring m/z 75, 129 and 157 for cyclohexanol, m/z 101, 116 and 177 for BDO, m/z 103, 129 and 219 for BT.

3. Results and discussion

3.1. Effect of cell density on BDO production

The BDO pathway from erythritol by whole-cell catalysis has been validated in our previous study, which only involves four steps and overexpression of one heterogeneous gene. In this route, erythritol is first converted to 3,4-dihydroxy-butyraldehyde by the GDHt mutant; 3,4-dihydroxy-butyraldehyde is then converted to BT through alcohol dehydrogenase; followed by another dehydration reaction, BT is converted to 4-hydroxy-butyraldehyde by the GDHt mutant; and 4-hydroxy-butyraldehyde is finally reduced by alcohol dehydrogenase to produce BDO (Figure 1). However, the titer of BDO is not satisfactory, leading to the investigation of effects of several variables on BDO biosynthesis. As shown in Figure 2, BDO concentration was optimal at cell density (OD_{600}) of 30 and decreased when the optical density reached 40. The highest BDO titer obtained was about 6 mg/L. BT is the precursor of BDO. The conversion from erythritol to BDO requires two rounds of dehydration-hydrogenation, while BT is formed via one dehydration-hydrogenation of erythritol (Figure 1). In the reaction culture, significant amount of BT was accumulated and the concentration of BT increased with the rise in cell density.

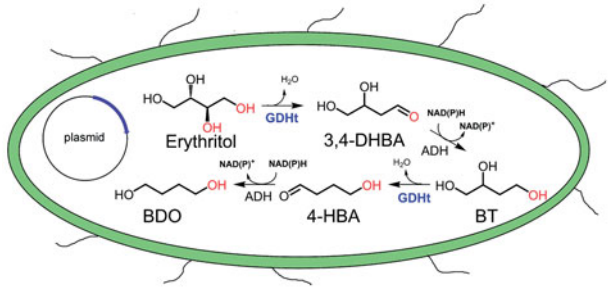

Figure 1. BDO production from erythritol by whole-cell catalysis. The ellipse indicates a cell of the engineered *E. coli* BL21 (DE3). GDHt: glycerol dehydratase; 3,4-DHBA: 3,4-dihydroxy-butyraldehyde; BT: butanetriol; 4-HBA: 4-hydroxy-butyraldehyde; BDO: 1,4-butanediol.

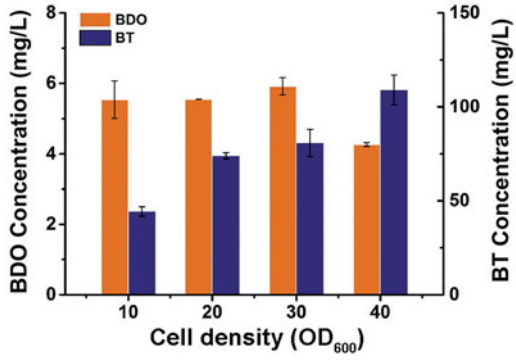

Figure 2. Effect of cell density on BDO and BT production by recombinant *E. coli*.

3.2. Effect of temperature on BDO production

To evaluate the optimum temperature for whole-cell biocatalysis, the cultures of EGDHt were incubated at 16–45 °C. It was found that BDO production was maximum at 16 °C with a titer of 19.9 mg/L, and decreased remarkably with increasing temperature (Figure 3). These results indicate that low temperature is more beneficial for BDO production. We speculate this may be attributed to the stability of NAD(P)H at low temperature (Rover et al. 1998). As shown in Figure 1, the transformation of erythritol to BDO is coupled with the NAD(P)H consumption. For every mole of BDO to be formed, two moles of NAD(P)H are required which are all derived from intracellular reducing equivalents. Thus, the stability of NAD(P)H is crucial for BDO production. It has been previously reported that low temperature is advantageous for the stability of NAD(P)H, which may thereby lead to the maximum production of BDO (Wu et al. 1986). Besides, significant amount of BT was accumulated in the reaction mixtures, and the highest concentration was obtained at 16 °C with a titer of 118.5 mg/L (Figure 3). It is notable that the relative content of BT is different at high and low temperature. We speculate this is probably because the temperature has

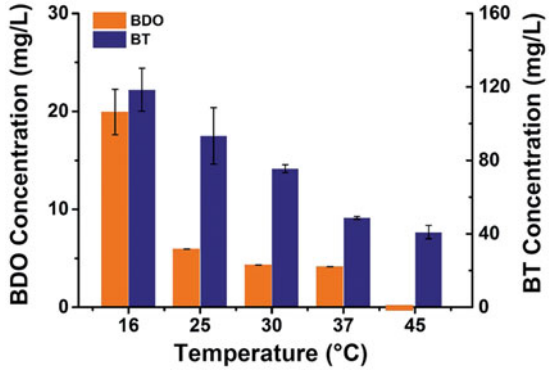

Figure 3. Effect of temperature on BDO and BT production by recombinant *E. coli*.

less effect on reaction rates of the first round of dehydration-hydrogenation and has a greater effect on that of the second round of dehydration-hydrogenation.

3.3. Effect of substrate concentration on BDO production

Varying concentrations of erythritol were used to elucidate the best concentration for maximum BDO production. As depicted in Figure 4, BDO concentration

increased first, and then decreased with the rising concentration of erythritol; 32.5 mg/L of BDO was achieved by feeding 80 g/L erythritol, which was the maximum BDO concentration. The titer of BT rose with the rising erythritol level during the low concentration range, and reduced during the high concentrate range. The highest concentration of BT was also obtained with 80 g/L erythritol as the substrate.

3.4. Effect of initial pH on BDO production

In order to evaluate the effect of initial pH on BDO production, the whole-cell biocatalysis was conducted at different pH (5.0–9.0). As shown in Figure 5, reaction with pH 8.0 was more productive in BDO production. Both an increase and decrease in pH caused the dramatic loss of BDO concentration and detection of BDO was not available when pH was lower than 7.0. The highest concentration of BDO obtained at pH 8.0 was 34.5 mg/L, which was 5.8-fold higher than that at cell density of 30. Optimum ranges of external pH are crucial for cell growth and survival (Beales 2004). During the whole-cell catalysis, acidic conditions may exhibit physical stress on cells, which thereby affects the state of organism. On the contrary, a weak alkaline environment may be favourable for the performance of cells, thus resulting in the higher production of BDO. Similarly, the maximum BT production was also obtained at pH 8.0. A significant loss of BT concentration was achieved with an increasing pH as well as a decreasing pH.

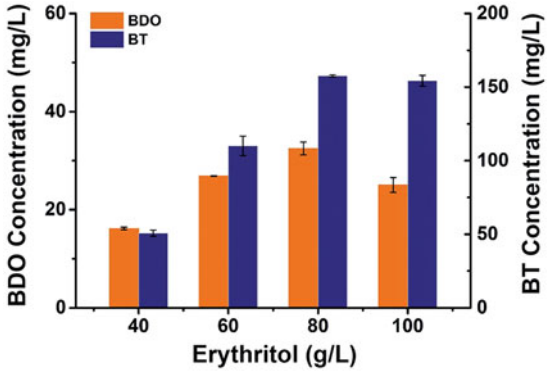

Figure 4. Effect of substrate concentration on BDO and BT production by recombinant *E. coli*.

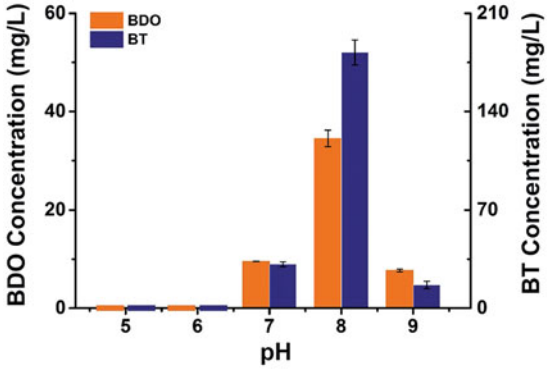

Figure 5. Effect of initial pH on BDO and BT production by recombinant *E. coli*.

4. Conclusions

This paper reported the effects of cell density, temperature, substrate concentration, and pH on BDO production. The highest concentration of BDO was obtained at cell density (OD_{600}) of 30. Other results included that high temperature and acidic pH were disadvantageous for BDO production. The maximum BDO production was achieved with 80 g/L erythritol as substrate. Under the optimal conditions, about 35 mg/L of BDO was produced, which was almost 6-fold higher than the unoptimized conditions. Besides, it was found that temperature and pH exerted significant impacts on the transformation, indicating that further optimization should give priority to these two factors. This will provide useful guidance for biotransformation of erythritol to BDO. During the bioconversion, significant amount of BT was accumulated in the reaction mixture, indicating the catalytic efficiencies of GDHt towards BT may be one of the limiting steps. Thus, further efforts include engineering the dehydratases by combination of structural biology techniques and computational approaches. Screening for more active homologues from other species might also be a promising strategy.

Disclosure statement

No potential conflict of interest was reported by the authors.

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