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Enhancement of cell growth and glycolic acid production by overexpression of membrane-bound alcohol dehydrogenase in *Gluconobacter oxydans* DSM 2003

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ABSTRACT

Membrane-bound alcohol dehydrogenase (mADH) was overexpressed in *Gluconobacter oxydans* DSM 2003, and the effects on cell growth and glycolic acid production were investigated. The transcription levels of two terminal ubiquinol oxidases (bo_3 and *bd*) in the respiratory chain of the engineered strain *G. oxydans-adhABS* were up-regulated by 13.4- and 3.8-fold, respectively, which effectively enhanced the oxygen uptake rate, resulting in higher resistance to acid. The cell biomass of *G. oxydans-adhABS* could increase by 26%–33% when cultivated in a 7L bioreactor. The activities of other major membrane-bound dehydrogenases were also increased to some extent, particularly membrane-bound aldehyde dehydrogenase (mALDH), which is involved in the catalytic oxidation of aldehydes to the corresponding acids and was 1.26-fold higher. Relying on the advantages of the above, *G. oxydans-adhABS* could produce 73.3 g l^{-1} glycolic acid after 45 h of bioconversion with resting cells, with a molar yield 93.5% and a space-time yield of 1.63 g l^{-1} h^{-1}. Glycolic acid production could be further improved by fed-batch fermentation. After 45 h of culture, 113.8 g l^{-1} glycolic acid was accumulated, with a molar yield of 92.9% and a space-time yield of 2.53 g l^{-1} h^{-1}, which is the highest reported glycolic acid yield to date.

1. Introduction

Gluconobacter oxydans, a gram-negative and obligate aerobic bacterium belonging to acetic acid bacteria, can rapidly and completely oxidize a wide range of sugar and sugar alcohols with low biomass, accumulating the corresponding oxidation products in large amounts (De Ley et al., 1984; Prust et al., 2005). The oxidation reactions are catalyzed by numerous membrane-bound dehydrogenases which are located on the periplasmic side of the cytoplasmic membrane and linked to the respiratory chain (Prust et al., 2005). The high oxidation rate usually causes a high demand for oxygen, and the oxidation products accumulate in the culture medium, often resulting in its acidification (Hanke et al., 2012).

The PQQ-containing membrane-bound alcohol dehydrogenase (mADH) is one of these key dehydrogenases in *G. oxydans* and catalyzes the oxidation of primary alcohols and diols to the corresponding acids or hydroxy acids (Wei et al., 2013). When oxidizing a substrate, electrons are transferred through the cytochrome *c* site of subunit I (heme I) to the subunit II through heme II_1, then to heme II_2 and finally to ubiquinone (Matsushita et al., 1996). The respiratory chain of *G. oxydans* contains two ubiquinol oxidases, bo_3 and *bd* (Prust et al., 2005). The bo_3 ubiquinol oxidase has a significantly higher oxygen affinity than the *bd* ubiquinol oxidase and plays a major role for the build-up of proton motive force and biomass formation, probably functioning as a rate-limiting factor of the respiratory (Miura et al., 2013; Richhardt et al., 2013). The *bd* oxidase route, a non-energy generating and cyanide-resistant bypass oxidase pathway, could be used to keep oxidation of substrates at a high speed in acidic environments (Matsushita et al., 1989; Miura et al., 2013; Tkac et al., 2009). Based on earlier studies, a mutant of *G. oxydans* lacking the subunit II of mADH shows relatively less cyanide resistance in the respiratory chain (Matsushita et al., 1991), yet under acidic conditions the amounts of subunit II are elevated parallel with the increase in the cyanide-resistant respiratory (Matsushita et al., 1989). These indicate that mADH

Abbreviations: mADH, membrane-bound alcohol dehydrogenase; mALDH, membrane-bound aldehyde dehydrogenase; mGLDH, membrane-bound sorbitol dehydrogenase; mGDH, membrane-bound glucose dehydrogenase; GA-2-DH, membrane-bound 2-keto-D-gluconate dehydrogenase; DCW, dry cell weight; OUR, oxygen uptake rate; DO, dissolved oxygen; qRT-PCR, quantitative real-time polymerase chain reaction; HPLC, high-performance liquid chromatography.

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might be related to the cyanide-resistant bypass respiratory chain. Furthermore, mADH has another physiological role in the respiratory chain of *G. oxydans*. It mediates the electron transfer from another primary membrane-bound D-glucose dehydrogenase (mGDH) through the subunit II of mADH to ubiquinone and finally to oxygen (Shinagawa et al., 1990).

In our previous studies, three subunits (AdhA, AdhB and AdhS) of mADH were characterized, and overexpression of *adhABS* genes was found to effectively increase the mADH activity toward ethylene glycol to glycolic acid (Wei et al., 2010; Zhang et al., 2016). Glycolic acid is the smallest member of the α-hydroxy acid family, and widely used for the leather industry, the oil and gas industry, the laundry and textile industry and medical application, e.g., poly(lactic-co-glycolic acid)-based drug delivery systems (Fredenberg et al., 2011; Kataoka et al., 2001; Koivistoinen et al., 2013).

Glycolic acid is naturally produced by bioconversion of glycolonitrile using microbial nitrilases (He et al., 2010), but glycolonitrile and its degradation products formaldehyde and hydrogen cyanide are toxic. *Escherichia coli* was metabolically engineered for glycolic acid production from glucose via the glyoxylate shunt stably with the highest titer up to 56.44 g l^{-1} (Deng et al., 2015). Koivistoinen et al. (2013) engineered yeasts to produce glycolic acid at 15 g l^{-1} using D-xylose and ethanol as substrates. *Corynebacterium glutamicum* has been reported to be capable of glycolic acid production (Zahoor et al., 2014), and chemolithotrophic iron- and sulphur oxidizing bacteria are also used for producing glycolic acid (Ñancucheo and Johnson, 2010). Ethylene glycol is one of the cheap starting materials for glycolic acid production. Based on previous studies, *Pichia naganishii* AKU 4267 and *Rhodotorula* sp. 3Pr-126 could produce 105 and 110 g l^{-1} glycolic acid from ethylene glycol, with the space-time yield of 0.88 and 0.92 g l^{-1} h^{-1}, respectively (Kataoka et al., 2001). Gao et al. (2014) reported that *Burkholderia* sp. EG13 showed high potential application in the synthesis of glycolic acid from ethylene glycol, but only about 60 g l^{-1} glycolic acid could be obtained after 120 h of bioconversion. The activity toward ethylene glycol of these strains was not high enough and thus the titer of glycolic acid from the above methods was very low.

G. oxydans DSM 2003 was reported for the production of glycolic acid from ethylene glycol in an integrated bioprocess (Wei et al., 2009b). It found that 20 g l^{-1} ethylene glycol could inhibit the activity of resting cells. Accordingly, fed-batch bioconversion of ethylene glycol was carried out, and the highest concentration of glycolic acid was 74.5 g l^{-1}. To reduce the end-product inhibition, Wei et al. (2009b) developed an in situ product removal (ISPR) technique, which used anion exchange resin D315 as the adsorbent for selective removal of glycolic acid. This approach allowed the yield of glycolic acid to be increased to 93.2 g l^{-1}, with the space-time yield of 1.86 g l^{-1} h^{-1}. However, there were still some limitations in the ISPR system due to the extraction power of the resin and the loss of cell activity.

In this study, we investigated the effects of overexpression of mADH in *G. oxydans* DSM 2003 on biochemical and genetic features, leading to improved cell growth and accelerated respiratory rates. The biosynthesis of glycolic acid from ethylene glycol by the mADH overexpression strain was also performed.

2. Materials and methods

2.1. Bacterial strains and growth conditions

G. oxydans DSM 2003 belonged to the culture collection of our laboratory. Construction of the mADH overexpression strain *G. oxydans-adhABS* and the control strain *G. oxydans*-pBBR1MCS5 were described in our recent study (Zhang et al., 2016).

G. oxydans strains were cultivated at 30 °C in shake flasks or a 7L bioreactor in sorbitol medium, consisting of 80 g l^{-1} sorbitol, 20 g l^{-1} yeast extract, 1 g l^{-1} KH_2PO_4, 0.5 g l^{-1} $MgSO_4 \cdot 7H_2O$, and 0.1 g l^{-1} glutamine with initial pH 6.0. When cultivated in a bioreactor, agitation and aeration rates were regulated at either 400 rpm and 0.6 vvm for low aeration conditions or 600 rpm and 1.2 vvm for high aeration conditions, with pH value and temperature maintained at 6.0 and 30 °C automatically. The optical density at 600 nm (OD_{600}) of cultures was measured to monitor cell growth, which was converted into dry cell weight (DCW) using the relation of 1 OD_{600} = 0.463 g DCW l^{-1}.

2.2. Quantitative real-time (qRT)-PCR

Quantitative real-time polymerasechain reaction (qRT-PCR) analysis was performed to investigate the transcriptional abundance of the overexpression strain. To minimize the effect of the growth state on qRT-PCR analysis, bacterial cells that used for RNA isolation were cultured for approximately 18 h in sorbitol medium. Total RNA was extracted using RNAiso Plus (TaKaRa, Dalian, China) according to the manufacturer's instructions and treated with DNase I (TaKaRa) to eliminate any residual genomic DNA. The concentration of each RNA sample was quantified by NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). cDNA was synthesized using PrimerScript 1st strand cDNA synthesis kit (TaKaRa) by following manufacturer's instructions. All primers used for qRT-PCR in this study were designed with Primer 5.0 (Table 1). The quantitative expression analysis of target genes was performed using StepOnePlus™ Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA). The 16S rRNA gene, known as a housekeeping gene, was used as internal standard. Samples were run in triplicate with appropriate controls, and data were analyzed by the $2^{-\Delta\Delta Ct}$ method.

2.3. Preparation of the membrane fractions

The *G. oxydans* cells were harvested as described above, and washed twice with ice-cold 50 mM sodium phosphate buffer (pH 7.5) containing 1 mM dithiothreitol (DTT) and 1 mM $MgCl_2$. The washed cells were resuspended with the same buffer at a cell wet weight concentration of 0.2 g l^{-1} and passed twice through a French pressure cell press (Union-Biotech, Shanghai, China) at 14 000 psi. After centrifugation at 8000 × *g* for 15 min to remove the cell debris, the supernatants were centrifuged at 40 000 × *g* for 60 min. The precipitate was resuspended with 10 mM sodium phosphate buffer (pH 6.0) subsequently and used as the membrane fractions. Protein content was measured by the method of Bradford (1976), using bovine serum albumin as a standard.

2.4. Enzyme assay

The dehydrogenase activity in the cytoplasmic membrane was determined at 30 °C by measuring the initial reduction rate of 2,6-dichlorophenolindophenol (2,6-DCIP, Sigma, St. Louis, MO, United States) at 600 nm on a SpectraMax 190 instrument (Molecular Devices, California, United States). The basal reaction mixture consisted of 5 mM DCIP and 6.5 mM phenazine methosulfate (PMS, Sigma) in 50 mM potassium phosphate buffer (pH 6.0). The reaction mixtures (200 μl) for enzyme assay containing 80 μl of basal reaction mixture and 0.03 mg of protein in 50 mM potassium phosphate buffer (pH 6.0) was incubated at 30 °C for 5 min, and the reactions were started by adding 50 mM substrates (benzaldehyde, *meso*-erythritol, D-glucose, and D-gluconate). One unit of the dehydrogenase activity was defined as the amount of the enzyme that