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Molecular cloning and expression of *Enterobacter aerogenes* α-acetolactate decarboxylase in pyruvate decarboxylase-deficient *Saccharomyces cerevisiae* for efficient 2,3-butanediol production

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ABSTRACT

α-Acetolactate decarboxylase (ALDC) catalyzes the conversion of α-acetolactate into acetoin, a precursor of 2,3-butanediol (2,3-BD). In this study, we overexpressed the genes coding for various ALDCs from natural 2,3-BD producing bacteria in recombinant *Saccharomyces cerevisiae* SB strains with two essential enzymes for 2,3-BD production (α-acetolactate synthase and 2,3-BD dehydrogenase) and without pyruvate decarboxylase (PDC) activity. Expression of ALDCs from Bacillus subtilis and *Enterobacter aerogenes* gave 1.3–1.5 times higher 2,3-BD productivities than those from *Klebsiella pneumoniae* and *Klebsiella oxytoca oxytoca*. Kinetic analysis of purified ALDCs revealed that *E. aerogenes* ALDC exhibited an 1.7 fold higher k_{cat}/K_m ($22.9 \pm 0.2\ mM^{-1}\ s^{-1}$) than *B. subtilis* ALDC ($13.3 \pm 4.1\ mM^{-1}\ s^{-1}$). In fed-batch fermentations by intermittent addition of a concentrated glucose solution, the SB-Ea strain overexpressing *E. aerogenes* ALDC produced 132.4 g/L of 2,3-BD with a yield of 0.34 g 2,3-BD/g glucose and 0.41 g/L h productivity, which were 30% and 25% higher than those of the BD4 strain expressing *B. subtilis* ALDC. It was concluded that *E. aerogenes* ALDC was the most effective enzyme among four bacterial ALDCs for improving 2,3-BD production in PDC-deficient *S. cerevisiae*.

1. Introduction

2,3-Butanediol (2,3-BD) is a promising chemical for synthesis of useful products such as cosmetics, pharmaceuticals and industrial solvents [1–4]. Especially, 2,3-BD can be converted to 1,3-butadiene and methyl ethyl ketone (or 2-butanone) through dehydration processes which can be used as synthetic rubber prcursor and liquid fuel additive, respectively. In microbial production of 2,3-BD, bacterial fermentations of *Klebsiella pneumonia*, *Klebsiella oxytoca*, *Paenibacillus polymyxa* and *Enterobacter aerogenes* have been developed for more than 100 years [5–7]. Although most bacteria could produce 2,3-BD with up to yield of 0.5 g 2,3-BD/g glucose and 5.4 g/L h productivity, they were classified as potent pathogen, which is a hurdle for commerciallization of bacteria-based fermentation processes [8]. Meanwhile, *Saccharomyces cerevisiae* is a GRAS (Generally Recognized As Safe) microorganism and has been easily engineered for industrial production of biofuels and biochemicals [9,10]. As it can produce little amount of 2,3-BD naturally, many research groups introduced the foreign 2,3-BD pathway into *S. cerevisiae* by overexpressing three essential enzymes of α-acetolactate synthase (ALS), α-acetolactate decarboxylase (ALDC) and 2,3-BD butanediol dehydrogenase (BDH) [11,12]. To produce 2,3-BD with high yield, recombinant *S. cerevisiae* strains were developed by overexpressing the 2,3-BD biosynthetic enzymes and by eliminating the ethanol-producing pathway. Among them, the *S. cerevisiae* BD4 strain produced almost 100 g/L of 2,3-BD with more than yield of 0.3 g 2,3-BD/g glucose, which was more than 100 times improvement of 2,3-BD yield in comparison to that for a wild type of *S. cerevisiae* D452-2 [11]. Additionally, introduction of the xylose or cellobiose assimilating pathway into pyruvate decarboxylase (PDC)-deficient *S. cerevisiae* (BD4X and SOS2-CB) overexpressing the 2,3-BD biosynthetic enzymes resulted in yields of 0.27 g 2,3-BD/g xylose and 0.29 g 2,3-BD/g cellobiose [12,13].

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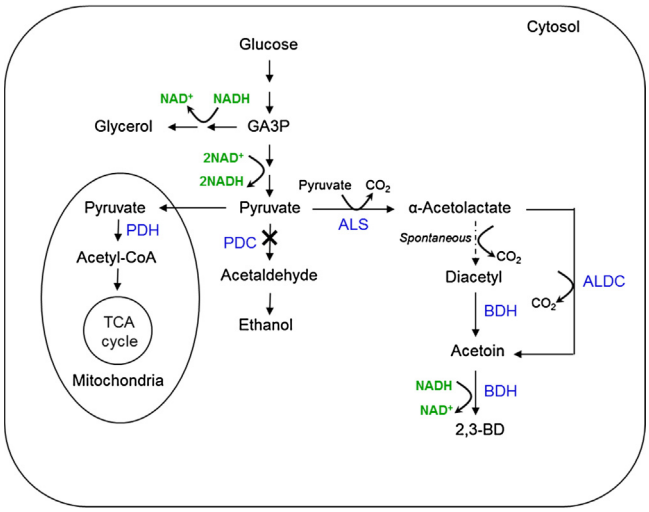

Fig. 1. 2,3-Butanediol biosynthetic pathway in recombinant *Saccharomyces cerevisiae*. The enzymes involved in the 2,3-butanediol production and metabolites are abbreviated as follow: ALS, α-acetolactate synthase; ALDC, α-acetolactate decarboxylase; BDH, 2,3-butanediol dehydrogenase; PDC, pyruvate decarboxylase; PDH, pyruvate dehydrogenase; GA3P, glyceraldehyde-3-phosphate; 2,3-BD, 2,3-butanediol.

In bacterial 2,3-BD metabolism α-acetolactate, an intermediate of 2,3-BD, faces two fates according to oxygen content in media. α-Acetolactate is converted into acetoin by ALDC in anaerobic condition, while it is spontaneously and slowly decarboxylated to diacetyl which is converted into acetoin by diacetyl reductase (DAR) in aerobic condition. Finally, acetoin is converted into 2,3-BD by butanediol dehydrogenase (BDH), also known as acetoin reductase (AR) [1]. In 2,3-BD metabolism in a wild type of *S. cerevisiae* (Fig. 1), α-acetolactate can be solely converted into diacetyl by spontaneous and slow decarboxylation because of the absence of ALDC, which is a rate-limiting step in 2,3-BD biosynthesis [14]. Also, *S. cerevisiae* BDH was more tightly bound to acetoin by three times, relative to diacetyl [15]. To minimize the unfavorable decarboxylation of α-acetolactate and increase the carbon flux to acetoin, direct conversion of α-acetolactate to acetoin without diacetyl accumulation has been deviced by expression of foreign ALDC in *S. cerevisiae* [16]. Over expression of *E. aerogenes* ALDC led to the improvement of wine and beer quality without formation of diacetyl, a source of unpleasant flavors [17,18]. In most cases of 2,3-BD production, ALDC from *Bacillus subtilis* and *E. aerogenes* was overexpressed in various recombinant *S. cerevisiae* strains even though other bacterial strains also produced high amounts of 2,3-BD with high fermentative performances [11–13,19]. In this study, we expressed various genes encoding ALDCs derived from *Klebsiella pneumoniae*, *K. oxytoca*, *E. aerogenes* and *B. subtilis* known to be natural 2,3-BD producers with high yield and titer in PDC-deficient *S. cerevisiae* transformed with the *B. subilis alsS* and endogeneous *BDH1* genes. Purified ALDCs from *B. subtilis* and *E. aerogenes* were subjected to enzymatic characterization in order to compare their catalytic efficiencies. Subsequently, fed-batch fermentations of the engineered *S. cerevisiae* strains were performed to evaluate the effects of ALDC overexpression on 2,3-BD production.

2. Materials and methods

2.1. Strains and plasmids

Escherichia coli Top10 (Invitrogen, Carlsbad, CA, USA) was used for gene cloning. Recombinant *S. cerevisiae* SOS4 deficient in the *PDC1* and *PDC5* genes coding for pyruvate decarboxylase was used as a host for 2,3-BD production. All 2,3-BD producing *S. cerevisiae* strains expressed the *B. subtilis alsS* and endogeneous *BDH1* genes under the control of the constitutive *GPD* promoter. Strains and plasmids used in this study are listed in Tables 1 and 2, respectively.

2.2. Genetic manipulation

The genes encoding ALDC were amplified by the polymerase chain reaction (PCR) from the genomic DNAs of *B. subtilis* str. 168, *K. pneumoniae* KACC 14816, *K. oxytoca* ATCC 43863 and *E. aerogenes* KTCC 13732 using the corresponding PCR primers (Supplementary Table 1). After digesting the PCR products with restriction enzymes of *Bam*HI and *Xho*I, they were ligated with plasmid pRS426GPD, pRS423GPD, pRS426CYC1, pRS426ADH1 or pRS426tHXT, resulted in the series of ALDC expression vectors as described in Table 2. Plasmids pRS423_alsS and pRS425_BDH1 were transformed into recombinant *S. cerevisiae* SOS4 using the yeast EZ-Transformation kit (BIO 101, Vista, CA, USA) and the resulting strain was named recombinant *S. cerevisiae* SB. The transformants were selected on yeast synthetic complete (YSC) medium consisting of 6.7 g/L yeast nitrogen base without amino acids (Sigma–Aldrich, St. Louis, MO, USA), 20 g/L glucose and appropriate amino acid (histidine, leucine or uracil).

2.3. Culture conditions for batch and fed-batch fermentations

For batch fermentation, recombinant *S. cerevisiae* strains were pre-cultured in 5 mL of YSC medium at 30 °C and 250 rpm. The yeast cells collected at the mid-exponential phase was inoculated into main culture at an initial OD_{600} of about 1.0. Batch fermentation was performed in a 250 mL flask containing 50 mL of YP medium (10 g/L yeast extract and 20 g/L Bacto peptone) with 100 g/L glucose at 30 °C and 80 rpm of agitation. Fed-batch fermentation was carried out in a 1 L-bench-top bioreactor (Fermentec, Korea) containing 500 mL of YP medium with 120 g/L glucose at 30 °C. 1.0 vvm of aeration and 200 rpm of agitation were chosen for efficient production of 2,3-BD. Acidity was controlled at pH 5.5 by adding 2N HCl and 2N NaOH. Yeast cells precultured in YSC medium were harvested at the mid-exponential phase and inoculated into the bioreactor at an initial OD_{600} of about 10. During the fed-batch stage, a feeding solution composed of 800 g/L glucose was supplied four times.

2.4. Assay of α-acetolactate decarboxylase activity

The ALDC activity was determined as a previous study with some modifications [20]. For preparation of enzyme solution, optical density of culture broth was adjusted to be 10 by concentration or dilution. After the cell suspension was centrifuged, the cells were collected and disrupted by treating with the Y-PER solution (Thermo scientific, Rockford, IL, USA) for 20 min at room termperature. After centrifugation at 13,000 rpm and 4 °C for 10 min, the supernatant was collected and utilized as the crude enzyme solution. α-Acetolactate as a substrate was prepared by saponification of ethyl 2-acetoxy-2-methylacetoacetate [20]. For enzyme reactions, 200 μL of the crude enzyme solution or purifed ALDC was mixed with 2 mL of 15 mM α-acetolactate and then the reaction mixture was incubated at 10 °C for 20 min. The reaction was terminated by adding 2.2 mL of 2.5 M NaOH. Acetoin reacts with guanido groups of creatine, so that the reactant gradually turns pink. The color development was determined as absorbance at 525 nm [20]. In order to analyze acetoin produced by ALDC, 200 μL of the enzyme reactant was added into 2.5 mL of a color-reagent composed of 0.1% (w/v) creatine solution and 0.5% (w/v) α-naphtol dissolved in 2.5 M sodium hydroxide. After incubation at 20 °C for 40 min, an absorbance at 525 nm was measured using a

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