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SHORT COMMUNICATION

Inhibition of acetate accumulation leads to enhanced production of (*R,R*)-2,3-butanediol from glycerol in *Escherichia coli*

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Abstract This work describes the production of (*R,R*)-2,3-butanediol in *Escherichia coli* using glycerol by metabolic engineering approaches. The introduction of a synthetic pathway converting pyruvate to (*R,R*)-2,3-butanediol into wild-type *E. coli* strain BW25113 led to the production of (*R,R*)-2,3-butanediol at a titer of 3.54 g/l and a yield of 0.131 g product/g glycerol (26.7 % of theoretical maximum) with acetate (around 3.00 g/l) as the dominant by-product. We therefore evaluated the impacts of deleting the genes *ackA* or/and *poxB* that are responsible for the major by-product, acetate. This increased production of (*R,R*)-2,3-butanediol to 9.54 g/l with a yield of 0.333 g product/g glycerol (68.0 % of theoretical maximum) in shake flask studies. The utilization of low-priced crude glycerol to produce value-added chemicals is of great significance to the economic viability of the biodiesel industry.

Keywords 2,3-Butanediol · Glycerol · *Escherichia coli*

Introduction

In recent years, the tremendous growth in biodiesel production has created a huge surplus of glycerol which has turned it into an inexpensive and abundant carbon source [9, 17]. The availability of crude glycerol in large quantities provides an opportunity for bio-process industries to manufacture value-added chemicals at a relatively low raw material expense. On the other hand, developing processes to convert low-priced crude glycerol into higher-value chemicals has been regarded as an approach for the economic viability of the biodiesel industry [33]. In the past several years, glycerol has been used as a platform carbon source to produce a wide spectrum of products including ethanol, lactate, succinate, 1,2-propanediol, 1,3-propanediol, and 3-hydroxypropionic acid [3, 5, 13, 20, 23, 25]. In this study, we demonstrated efficient production of (*R,R*)-2,3-butanediol by metabolically engineered *Escherichia coli* using glycerol as the carbon source.

As a typical bulk chemical, 2,3-butanediol has broad applications. It can be converted to 1,3-butadiene which is an important monomer in the rubber industry [27]. It also has a high octane number and can be used as an "octane booster" for petrol [4, 16, 34]. The product of 2,3-butanediol dehydrogenation, diacetyl, has significant applications in the food industry serving as a flavoring agent and a bacteriostatic food additive [4, 16, 34]. The dehydration product of 2,3-butanediol, 2-butanone, is considered as an effective fuel additive and can also be utilized as a solvent for lacquers and resins [4, 16, 34]. In general, the derivative products of 2,3-butanediol possess an annual market of about 32 million tons, which is about US$43 billion in sales [18]. In addition, optically active 2,3-butanediol, such as (*R,R*)-2,3-butanediol, can be used as an antifreeze agent and has applications in the cosmetic industry [4, 16, 34].

2,3-Butanediol can be naturally produced by several species of the genera *Klebsiella, Lactococcus, Clostridium,* and *Bacillus* as a fermentative product [7, 12, 18, 22]. But

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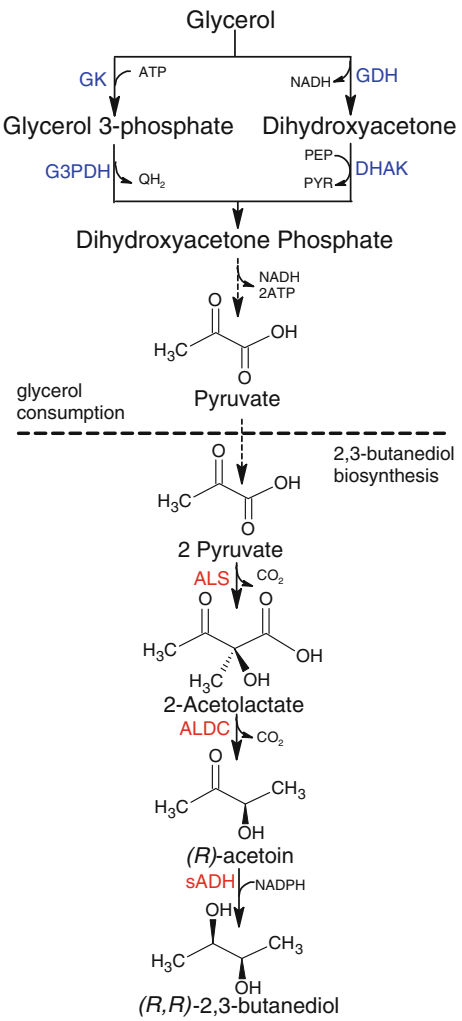

Fig. 1 Pathways involved in glycerol assimilation in *E. coli* under oxygen-limited conditions and for (*R,R*)-2,3-butanediol biosynthesis. *GK* glycerol kinase, *G3PDH* glycerol 3-phosphate dehydrogenase, *GDH* glycerol dehydrogenase, *DHAK* dihydroxyacetone kinase, *ALS* acetolactate synthase, *ALDC* acetolactate decarboxylase, *sADH* secondary alcohol dehydrogenase, QH_2 ubiquinol, *PEP* phosphoenolpyruvate, *PYR* pyruvate

industrial applications of such microorganisms are expected to be limited due to grave issues of pathogenicity, strict growth conditions, and few genetic and physiological manipulation approaches [21]. However, *E. coli*, as a well-characterized model microorganism, has a set of readily available tools for genetic manipulation and physiological regulation, and therefore, has been shown to be a suitable host for the production of many value-added chemicals [1, 14, 15, 29, 30, 32]. In our previous work, we engineered *E. coli*, an amendable workhorse in the biotechnology industry, to produce enantiopure (*R,R*)-2,3-butanediol from glucose by introducing a synthetic pathway consisting of an acetolactate synthase (ALS), an acetolactate decarboxylase (ALDC), and a stereospecific secondary alcohol dehydrogenase (sADH) (Fig. 1) [31]. Here, we present the use of this synthetic pathway to produce (*R,R*)-2,3-butanediol from glycerol. In addition, we also evaluated the impacts of competing pathways on (*R,R*)-2,3-butanediol production through gene knockout approaches, which led to the identification of an optimal overproducing strain. A substantial increase in both titer and yield was achieved.

Materials and methods

Chemicals, strains, and plasmids

(*R,R*)-2,3-Butanediol and *meso*-2,3-butanediol were purchased from Sigma Aldrich (St. Louis, MO, USA). *E. coli* strain XL1-Blue (Stratagene, La Jolla, CA, USA) was used for plasmid propagation. Wild-type *E. coli* strain BW25113 (CGSC, The Coli Genetic Stock Center, New Haven, CT, USA) was used for shake flask experiments. In addition, two single-knockout strains BW25113/*ΔackA* and BW25113/*ΔpoxB* derived from Keio collection and one double-knockout strain BW25113/*ΔackA,ΔpoxB* created by P1 transduction were also employed for shake flask experiments [2, 6, 28]. Plasmid pZE12-luc was employed for gene expression in *E. coli* [19]. For the expression of the synthetic pathway converting pyruvate to (*R,R*)-2,3-butanediol, we utilized the previously constructed plasmid pZE12-*alsS-alsD*-CBADH [31]. The features of the used strains and plasmids are listed in Table 1.

Media and cultivation conditions

For the purposes of plasmid propagation and inoculum preparation, *E. coli* cells were grown in Luria–Bertani (LB) medium at 37 °C with rigorous shaking. The modified M9 minimal medium was used for shake flask experiments containing (per liter): glycerol (30 g), yeast extract (5 g), NH_4Cl (1 g), Na_2HPO_4 (6 g), KH_2PO_4 (3 g), NaCl (0.5 g), $MgSO_4$ (0.24 g), $CaCl_2$ (0.011 g), vitamin B_1 (0.002 g). For the strains carrying plasmids, 100 μg/ml ampicillin was added. For all shake flask experiments, the strains were inoculated into 3 ml of LB medium and grown overnight at 37 °C in a shaker. After overnight incubation, the culture was transferred (2 % v/v) into 10 ml of the modified M9 minimal medium in 125-ml conical flasks with screw caps and tightened in order to provide oxygen-limited conditions. After the culture had grown at 37 °C for 7 h, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.2 mM to induce protein expression. Following this, production was conducted at 30 °C with shaking (250 rpm). The samples for HPLC analysis were collected after 48 h and then analyzed by HPLC-RID.

Table 1 List of strains and plasmids in this study

Strains	Genotype
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F' *proAB lacIqZ ΔM15 Tn10* (TetR)]
BW25113	F-, Δ(*araD-araB*), ΔlacZ (::*rrnB-3*), *λ*-, *rph-1*, Δ(*rhaD-rhaB*), *hsdR*
BW25113/*ΔackA*	BW25113, disruption of *ackA*
BW25113/*ΔpoxB*	BW25113, disruption of *poxB*
BW25113/ *ΔackA, ΔpoxB*	BW25133, disruption of both *ackA* and *poxB*
Plasmids	**Description**
pZE12-luc	ColE1 ori; Amp^R; $P_L$lacO-1; *luc*
pZE-*alsS-alsD-CBADH*	Derivative of pZE12-luc with *alsS-alsD-CBADH*

Analytical method

The samples were analyzed by HPLC-RID (Shimadzu) equipped with a Coregel-64H column (Transgenomic). All the samples (1.0 ml) were centrifuged at 15,000 rpm for 10 min and the supernatants were filtered and used for product analysis. H_2SO_4 (4 mN) was used as the mobile phase. The flow rate was 0.6 ml/min. The oven temperature was maintained at 60 °C [11].

Results and discussion

(*R,R*)-2,3-Butanediol production from glycerol in *E. coli*

In the oxygen-limited conditions defined in Sect. "Media and cultivation conditions", glycerol assimilation in *E. coli* can occur through two primary routes to dihydroxyacetone phosphate (DHAP), which further channels into glycolysis (Fig. 1). Glycerol dehydrogenase (GDH), encoded by *gldA*, and dihydroxyacetone kinase (DHAK), encoded by *dhaKLM*, form a fermentative route, which generates one molecule of NADH and requires one molecule of phosphoenolpyruvate (PEP) for dihydroxyacetone (DHA) phosphorylation. The enzymes glycerol kinase (GK) and aerobic glycerol-3-phosphate dehydrogenase (G3PDH) constitute a respiratory route. The enzymes were encoded by the genes *glpK* and *glpD*, respectively. This route consumes one molecule of ATP to perform substrate phosphorylation; however, it does not generate NADH as reducing power, but instead produces one molecule of reduced ubiquinone [10]. In addition, a membrane-associated protein, glycerol facilitator encoded by *glpF*, facilitates diffusion of glycerol into the cell for intracellular utilization [26]. In general, *E. coli* can use glycerol as the sole carbon source for growth; however, it lacks the capability to produce 2,3-butanediol. To overcome this disability, we introduced the previously constructed plasmid pZE12-*alsS-alsD-CBADH* into *E. coli*. The plasmid has been shown to selectively produce (*R,R*)-2,3-butanediol [31]. It carries a synthetic operon consisting of the genes *alsS*, *alsD* from *Bacillus subtilis* and *adh* from *Clostridium beijerinckii*. A synthetic ribosomal binding site (RBS) (AGGAGA) followed by 6–8 nucleotides for optimal translation is located in front of each gene. The operon is regulated by a $P_L$lacO-1 promoter. The plasmid also contains an ampicillin-resistant marker and a *ColE1* replicon. Transformation was performed by electroporation to introduce this plasmid into the wild-type *E. coli* strain BW25113. The obtained transformants were used for shake flask experiments as described in Sect. "Media and cultivation conditions". The wild-type *E. coli* strain BW25113 carrying the plasmid pZE12-*alsS-alsD-CBADH* was able to produce 3.54 g/l (*R,R*)-2,3-butanediol, while 27.04 g/l of glycerol was consumed, representing a yield of 0.131 g product/g glycerol. The theoretical maximum yield of (*R,R*)-2,3-butanediol from glycerol is 0.49 g product/g glycerol which can be calculated from the pathway in Fig. 1. Therefore, we only achieved 26.7 % of the theoretical maximum. Acetate was the dominant by-product with a concentration of 3.00 g/l. For other fermentative by-products, ethanol and succinate were only generated at small quantities (0.34 and 0.01 g/l, respectively), whereas lactate and formate were not detected. Intermediates pyruvate and acetoin were also only accumulated at small amounts (0.11 and 0.23 g/l, respectively) (Table 2).

ΔackA and ΔpoxB are beneficial to (*R,R*)-2,3-butanediol production

Acetate is an undesirable by-product in many cases during bioconversion or fermentation. It is toxic to cells, inhibits enzymes, depletes carbon sources, and prevents the formation of target products [24]. Its accumulation can be avoided by reducing the NADH/NAD+ redox ratio in cells [35]. In our case, to maintain NADH at a low level that will not trigger acetate accumulation while still efficiently driving (*R,R*)-2,3-butanediol formation, which requires reducing power, is quite challenging. Alternatively, we attempted to eliminate acetate accumulation by blocking the corresponding pathways. In *E. coli*, acetate biosynthesis is controlled by the enzymes pyruvate oxidase and acetate kinase, encoded by the genes *poxB* and *ackA*, respectively. We tested their individual contribution to acetate accumulation by employing the *E. coli* single-knockout strains BW25113/*ΔackA* and BW25113/*ΔpoxB* which are deficient in one of the two known pathways for acetate generation. Once transformed with the plasmid pZE12-*alsS-alsD-CBADH*, both strains produced much less acetate. The titers

Table 2 (*R,R*)-2,3-Butanediol production in *E. coli* strains carrying pZE-*alsS-alsD-CBADH*

	OD_{600}	Glycerol consumed (g/l)	Ethanol (g/l)	Lactate (g/l)	Succinate (g/l)	Formate (g/l)	Acetate (g/l)	Pyruvate (g/l)	Acetoin (g/l)	(*R,R*)-2,3-butanediol (g/l)	Yield (g/g)
BW25113	3.7 ± 0.3	27.04 ± 0.08	0.34 ± 0.13	0	0.01 ± 0.01	0	3.00 ± 0.19	0.11 ± 0.01	0.23 ± 0.05	3.54 ± 0.22	0.131
BW25113/*ΔackA*	4.3 ± 0.5	28.64 ± 0.03	0.53 ± 0.40	0	0.10 ± 0.03	0	0.07 ± 0.03	0.04 ± 0.02	0	9.54 ± 0.81	0.333
BW25113/*ΔpoxB*	4.5 ± 0.2	27.00 ± 0.02	0.34 ± 0.11	0	0.06 ± 0.01	0	0.13 ± 0.02	0.07 ± 0.03	0	7.67 ± 0.24	0.284
BW25113/*ΔackA, ΔpoxB*	3.4 ± 0.1	28.75 ± 0.23	0.19 ± 0.11	0	0.13 ± 0.11	0	0.09 ± 0.05	0.18 ± 0.13	0	9.56 ± 0.44	0.332

of (*R,R*)-2,3-butanediol increased dramatically (Table 2). The *E. coli* strain with *poxB* deletion led to only 0.131 g/l of acetate accumulation, while production of (*R,R*)-2,3-butanediol reached 7.67 g/l. The yield (0.28 g product/g glycerol) was about 57.1 % of theoretical maximum. The *E. coli* strain with *ackA* deletion led to even less acetate accumulation (0.07 g/l). As a consequence, (*R,R*)-2,3-butanediol was produced at 9.54 g/l, which gave a yield of 0.333 g product/g glycerol, 68.0 % of theoretical maximum. We also proceeded to investigate the effect of *ackA and poxB* double knockout on (*R,R*)-2,3-butanediol production. The performance of BW25113/*ΔackA, ΔpoxB* is very similar to that of the single-knockout strain BW25113/*ΔackA*: 9.56 g/l (*R,R*)-2,3-butanediol was produced by consuming 28.75 g/l glycerol, representing a yield of 0.332 g product/g glycerol, 67.8 % of the theoretical maximum. However, the double knockout was not able to eliminate acetate accumulation, which is consistent with previous reports [3, 5, 8]. Thus, 0.09 g/l acetate could still be detected in the cultures. It should be noted that compared with wild-type *E. coli* strain BW25113, all knockout strains that we used avoided acetoin accumulation. Small amounts of ethanol, succinate, and pyruvate generation could still be observed, whereas no lactate and formate could be detected with the knockout strains. Overall, BW25113/*ΔackA* was identified as the optimal strain for (*R,R*)-2,3-butanediol production from glycerol in this study.

Conclusions

In this study, we achieved efficient production of (*R,R*)-2,3-butanediol from glycerol using metabolically engineered *E. coli.* The oxygen-limited conditions defined in Sect. "Media and cultivation conditions" were employed for production, which prevented the formation of fermentative by-products. No lactate and formate were detected in the cultures. Only small amounts of ethanol and succinate were co-produced. Acetate was the main by-product. Both *ΔackA* and *ΔpoxB* strains efficiently reduced acetate formation and led to enhanced (*R,R*)-2,3-butanediol production. In particular, *ackA* deletion can increase production of (*R,R*)-2,3-butanediol to a titer of 9.54 g/l by consuming 28.64 g/l glycerol with only a trace amount of acetate accumulation. This work forms the basis for a promising solution to glycerol waste and in the manufacture of a high-value chemical, (*R,R*)-2,3-butanediol.

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