Metabolic Engineering 41 (2017) 102–114

Contents lists available at ScienceDirect

Metabolic Engineering

journal homepage: www.elsevier.com/locate/meteng

Coordination of metabolic pathways: Enhanced carbon conservation in 1,3-propanediol production by coupling with optically pure lactate biosynthesis

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ARTICLE INFO

Keywords:
Co-production
1,3-Propanediol
Optically pure lactate
Metabolic engineering
Glycerol conversion
Metabolic flux coordination

ABSTRACT

Metabolic engineering has emerged as a powerful tool for bioproduction of both fine and bulk chemicals. The natural coordination among different metabolic pathways contributes to the complexity of metabolic modification, which hampers the development of biorefineries. Herein, the coordination between the oxidative and reductive branches of glycerol metabolism was rearranged in *Klebsiella oxytoca* to improve the 1,3-propanediol production. After deliberating on the product value, carbon conservation, redox balance, biological compatibility and downstream processing, the lactate-producing pathway was chosen for coupling with the 1,3-propanediol-producing pathway. Then, the other pathways of 2,3-butanediol, ethanol, acetate, and succinate were blocked in sequence, leading to improved D-lactate biosynthesis, which as return drove the 1,3-propanediol production. Meanwhile, efficient co-production of 1,3-propanediol and L-lactate was also achieved by replacing *ldhD* with *ldhL* from *Bacillus coagulans*. The engineered strains PDL-5 and PLL co-produced over 70 g/L 1,3-propanediol and over 100 g/L optically pure D-lactate and L-lactate, respectively, with high conversion yields of over 0.95 mol/mol from glycerol.

1. Introduction

Metabolism is pivotal to microbial life, as it fuels cells with energy and reducing power. In responding to environment changes or artificial manipulations, bacterial cells can integrate the altered conditions into intracellular signals via metabolism for better survival. Accordingly, a cascade of reactions is set off to rebalance flux magnitudes, rectify imbalances, and redistribute resources quickly and stably (Chubukov et al., 2014). Such rebalance, rectification, and redistribution could be used in metabolic engineering to manipulate cellular metabolism and channel carbon flux to the desired direction (Zhang et al., 2014). Through the process of artificial manipulation, balanced flux could be achieved to bypass cell growth retardation and metabolic imbalance due to accumulation of intermediates (Lee et al., 2012).

1,3-Propanediol (PD) is a bulk chemical that is widely used in the production of polymers, cosmetics, lubricants and drugs (Celinska, 2010). Of particular interest is its use for producing polytrimethylene terephthalate, which has superior stretching and stretch recovery characteristics. The global demand for PD is growing rapidly and is expected to increase from 60.2 kt in 2012 to approximately 150 kt by 2019 (Xin et al., 2016). Glycerol, the main by-product of the biodiesel industry, has been shown to be an excellent fermentation substrate for the PD production (Zeng and Biebl, 2002). Microbial production of PD from glycerol depends on the glycerol dismutation process, which involves two parallel branches, the oxidative and reductive branches. Through the oxidative branch, glycerol is metabolized and channeled into glycolysis, producing energy and reducing power for cell growth and resulting in formation of a series of by-products, such as CO_2, H_2, acetate, butyrate, lactate, ethanol, butanol, and/or 2,3-butanediol (BD). On the other hand, the reductive branch leads to PD biosynthesis through glycerol dehydration and 3-hydroxypropionaldehyde (3-HPA) reduction, with the consumption of NADH produced from the glycerol oxidative branch. This process is biologically important for maintaining intracellular redox balance (Celinska, 2010). Consequently, glycerol oxidation is indispensable for PD biosynthesis, and there is always considerable by-product formation in the PD production from glycerol (Sauer et al., 2008).

Many microorganisms, such as *Klebsiella* sp., *Clostridia* sp., and *Citrobacter* sp., can produce PD from glycerol. Among them, the facultative anaerobe *Klebsiella* sp. is considered a good producer and

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http://dx.doi.org/10.1016/j.ymben.2017.03.009
Received 16 September 2016; Received in revised form 9 January 2017; Accepted 31 March 2017
Available online 07 April 2017

has received much attention because of its appreciable substrate tolerance, yield, productivity, and genetic tractability (Saxena et al., 2009). Therefore, several strategies have been proposed to eliminate by-product formation and enhance PD production in *Klebsiella* sp., including disrupting the genes involved in by-product formation and overexpressing the genes of the PD biosynthetic pathway (Chen et al., 2009; Kim et al., 2013; Lin et al., 2016; Seo et al., 2009; Xu et al., 2009; Zhang et al., 2006). However, reducing the formation of a by-product usually resulted in increased formation of other by-products (Jantama et al., 2015; Sangproo et al., 2012). Furthermore, total inactivation of all genes involved in by-product formation is unreasonable because of the tight control of flux in the central carbon metabolism. In fact, the variety of by-products reflects the delicate redox balance of the cell (Bro et al., 2006; Celinska, 2010). Therefore, readjusting by-product formation while maintaining the overall redox balance is crucial to improve the efficiency of glycerol utilization and PD production. To overcome this problem and achieve optimal substrate utilization, the carbon flux toward the glycerol oxidative branch may be redirected to a useful product, which may realize co-production of PD from the reductive branch and another valuable product from the oxidative branch. This strategy can not only guarantee the generation of ATP and reducing power for cell growth and PD biosynthesis, but can also increase the efficiency of glycerol utilization considerably. To date, few reports have focused on developing a rebalance between glycerol reduction and oxidation by co-producing PD and another end product from the glycerol oxidative branch to achieve maximum conversion of substrate.

In the present study, we used *K. oxytoca* as a host strain and aimed to establish a co-production strategy involving PD and another product from the glycerol oxidative branch. After careful evaluation, lactate was selected as the co-product. Systematic metabolic engineering approaches were then applied to develop *K. oxytoca* into an efficient cell factory for co-production of PD and lactate from glycerol (Fig. 1a).

2. Materials and methods

2.1. Bacterial strains, plasmids and culture conditions

Strains used in this study were listed in Table S1. *K. oxytoca* strain PDL-0(WT), which is able to produce PD and lactate as its main products, was screened from a soil sample collected from Shanghai, China. Soil samples were collected from various areas of Shanghai. Approximately 2g of each sample was enriched in 50 mL of fermentation medium with glycerol as the sole carbon resource at 37 °C, 220 rpm for 24 h. Then, the cultures were diluted by 10, 100, 1000 and 10,000 times with 0.85% NaCl solution, and were plated on fermentation medium with glycerol, agar and $CaCO_3$. After 24 h of static incubation at 37 °C, colonies surrounded with bigger acid production zone were selected and incubated in fermentation medium with glycerol at 37 °C, 220 rpm for 24 h. The PD and lactate titers of each culture were detected. The strain showing the best ability of co-production of PD and lactate was selected for further study. Determination of the optical purity of lactate suggested that all the lactate produced by this strain was D-lactate. Thus, the strain was defined as PDL-0. The 16 s rDNA of strain PDL-0 was cloned and sequenced. The result showed that this strain was a *K. oxytoca* strain.

Escherichia coli DH5α (Novagen) was used for plasmid replication. *E. coli* S17-1 λ*pir* was used for conjugation with *K. oxytoca* for its capability of hosting pKR6K and its derivatives. pKR6K was derived from pK18*mobsacB* by substituting the replicon for the ori_{R6K} replicon. Because *K. oxytoca* PDL-0 is kanamycin-resistant, chloramphenicol resistance gene was cloned by PCR from pDK7, a plasmid owing a tac promoter used for gene expression in *Klebsiella* sp., using primers Cm-F (BglII) and Cm-R(NcoI) (Table S2). Then the original kanamycin resistance gene was replaced with chloramphenicol resistance gene through *Bgl*II and *Nco*I sites. The new plasmid $pKR6K_{Cm}$ was used for gene knock-out by homologous recombination in *K. oxytoca*. *K. oxytoca* and *E. coli* were cultured in lysogeny broth (LB) medium at 37 °C, supplemented with chloramphenicol (20 μg/mL) as required. The fermentation medium described previously was used for cultivation of *K. oxytoca* PDL-0 and its mutants for PD production (Huang et al., 2002).

2.2. Gene disruption in K. oxytoca

Gene disruption mutants of *K. oxytoca* PDL-0 were constructed using $pKR6K_{Cm}$. *E. coli* S17-1 λ*pir* was used as the host strain to realize the replication of $pKR6K_{Cm}$, and transfer the plasmid to the recipient strain. The left and right flanking sequences of *budB* gene were amplified from the genomic DNA of *K. oxytoca* PDL-0 using primer pairs *budB*-A-F(EcoRI) / *budB*-A-R(BamHI) and *budB*-B-F(BamHI) / *budB*-B-R(PstI), respectively. Then, the left fragment was digested by *Bam*HI and *Eco*RI, and the right fragment was digested by *Bam*HI and *Pst*I. The two fragments were ligated to $pKR6K_{Cm}$ digested with *Eco*RI and *Pst*I, producing $pKR6K_{Cm}$-Δ*budB*::BamHI. *E. coli* S17-1 ($pKR6K_{Cm}$-Δ*budB*::BamHI) was used as the donor in conjugation with *K. oxytoca* PDL-0. The *budB* mutant (Fig. 1b) was then constructed following protocols described previously (Wang et al., 2014b). Plasmids used for *budA*, *adhE*, *ackA-pta*, *pox*, *frdA* and *pflB* disruption were constructed using the procedure described above except that the ligation was performed via a recombination-ligation technique by *pEASY*-Uni Seamless Cloning and Assembly Kit (TransGen Biotech, China). FastPfu DNA polymerase was acquired from TransGen Biotech (China). Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs (Beverly, MA, USA). Genomic DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA).

2.3. Gene insertion or replacement in the chromosome of K. oxytoca

To realize the insertion of $ldhL_{Bc}$ gene in the chromosome of *K. oxytoca*, $pKR6K_{Cm}$-△*budAB*::$ldhL_{Bc}$ carrying a mutant allele of *budAB* gene and the complete coding region of $ldhL_{Bc}$ gene was constructed. The left and right fragments of *budAB* gene were cloned from the genomic DNA of *K. oxytoca* PDL-0 primer pairs, △*budAB*::$ldhL_{Bc}$-A-F /△*budAB*::$ldhL_{Bc}$-A-R and △*budAB*::$ldhL_{Bc}$-B-F /△*budAB*::$ldhL_{Bc}$-B-R, respectively. $ldhL_{Bc}$ was amplified from the genomic DNA of *Bacillus coagulans* 2–6 using primers △*budAB*::$ldhL_{Bc}$-F and △*budAB*::$ldhL_{Bc}$-R. Then, linearized $pKR6K_{Cm}$ plasmid digested by *Eco*RI and the three DNA fragments were ligated via DNA recombination by following the introduction of *pEASY*-Uni Seamless Cloning and Assembly Kit (TransGen, China). Gene insertion or replacement was performed using the same procedures as gene disruption.

2.4. Cloning and overexpression of ldhL gene in K. oxytoca

The genomic DNAs of *Lactobacillus casei* ATCC 334 (Zheng et al., 2012), *K. oxytoca* PDL-0 and *B. coagulans* 2–6 (Wang et al., 2014a) were used as templates for the amplification of *ldhL* genes by PCR with the primer pairs pDK7-$ldhL_{Lc}$-F(EcoRI) / pDK7-$ldhL_{Lc}$-R(EcoRI), pDK7-$ldhL_{Ko}$-F(EcoRI) / pDK7-$ldhL_{Ko}$-R(EcoRI) and pDK7-$ldhL_{Bc}$-F(EcoRI) / pDK7-$ldhL_{Bc}$-R(EcoRI), respectively. The genes $ldhL_{Lc}$, $ldhL_{Ko}$ and $ldhL_{Bc}$ were then ligated into pDK7 digested by *Eco*RI to produce pDK7-$ldhL_{Lc}$, pDK7-$ldhL_{Ko}$ and pDK7-$ldhL_{Bc}$, respectively. These resultant recombinant plasmids were then transformed into *K. oxytoca* via electrotransformation using a standard protocol described previously (Fournet-Fayard et al., 1995). The transformants were picked, cultured in LB medium containing chloramphenicol and screened through colony PCR. The correct transformant was cultured in 500-mL flasks filled with 100 mL of fermentation medium with initial glycerol concentration of about 20 g/L. Seed culture was inoculated (1%, v/v) into the medium and the flasks were cultured at 37 °C in a rotary shaker at 180 rpm. When OD_{620} of the culture reached 0.6–0.8, IPTG was added to a final concentration of 1 mM to induce expression of L-lactate dehydrogenase (L-LDH). Cells were cultured at 37 °C for further 10 h.