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ORIGINAL RESEARCH PAPER

Deletion of *ldh*A and *ald*H genes in *Klebsiella pneumoniae* to enhance 1,3-propanediol production

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Abstract
Objectives To improve 1,3-propanediol (1,3-PD) production and reduce byproduct concentration during the fermentation of *Klebsiella pneumonia.*
Results *Klebsiella. pneumonia* 2-1Δ*ldh*A, *K. pneumonia* 2-1Δ*ald*H and *K. pneumonia* 2-1Δ*ldh*AΔ*ald*H mutant strains were obtained through deletion of the *ldh*A gene encoding lactate dehydrogenase required for lactate synthesis and the *ald*H gene encoding acetaldehyde dehydrogenase involved in the synthesis of ethanol. After fed-batch fermentation, the production of 1,3-PD from glycerol was enhanced and the concentrations of byproducts were reduced compared with the original strain *K. pneumonia* 2-1. The maximum yields of 1,3-PD were 85.7, 82.5 and 87.5 g/l in the respective mutant strains.
Conclusion Deletion of either *ald*H or *ldh*A promoted 1,3-PD production in *K. pneumonia*.

Keywords Acetaldehyde dehydrogenase · Gene deletion · *Klebsiella pneumonia* · Lactate dehydrogenase · 1,3-Propanediol · λRed recombination

Introduction

1,3-Propanediol (1,3-PD) has applications in polymer synthesis, cosmetics, foods, lubricants, and medicines. Its industrial production is of economic importance particularly as an key monomer in the synthesis of a new type of polyester, polytrimethylene terephthalate (Zeng and Biebl 2002). 1,3-PD is currently produced by chemical processes such as hydroformylation of ethylene oxide and hydration of acrolein. The microbial production of 1,3-PD, a socially beneficial route to obtain chemicals from renewable resources, has been widely researched and is considered as a competitor to the traditional petrochemical synthesis routes (Da Silva et al. 2009). Among the microorganisms that can convert glycerol to 1,3-PD, *Klebsiella pneumoniae*, *Clostridium butyricum* and *Citrobacter freundii* have been the major foci of attention because of their appreciable substrate tolerance, yield, and productivity (Celinska 2010). Biological manipulation including optimization of the natural glycerol utilizing fermentation process and metabolic engineering can contribute to increasing the yield of 1,3-PD (Mu et al. 2006).

Glycerol metabolism in the model organism *K. pneumoniae* involves two pathways—the oxidative

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branch and the reductive branch (Oh et al. 2013a) (Fig. 1). In the oxidative branch, glycerol is transformed to dihydroxyacetone by glycerol dehydrogenase, which then undergoes normal glycolysis to form pyruvate, and finally can be converted into various byproducts such as acids and alcohols. In the reductive pathway, glycerol is converted to 3-hydroxypropionaldehyde (3-HPA) by a coenzyme B_{12}-dependent glycerol dehydratase (GDHt), which is then reduced to 1,3-PD by a NADH-dependent 1,3-PD oxidoreductase (DhaT) (Ji et al. 2010; Jung et al. 2012). Activity of GDHt is the key rate limiting step for this biological process. The second metabolic pathway, which maintains the redox balance of the cell, is essential for the conversion of glycerol to 1,3-PD. However, during the biological production of 1,3-propanediol, a series of byproducts (such as lactate and ethanol) inhibit the pathway and increase the purification cost and time (Oh et al. 2013b). During glycerol metabolism, considerable amounts of metabolites are produced via the oxidative branch. Reduction or elimination of these metabolites is an important strategy to enhance 1,3-PD production during microbial fermentation. In this study, we investigated lactate and ethanol during 1,3-PD production in *K. pneumoniae* 2-1. We investigated whether direct inactivation of metabolic pathways leading to production of these major byproducts of the oxidative branch would enhance 1,3-PD production. Additionally, fermentation parameters were optimized to achieve this goal (Seo et al. 2009).

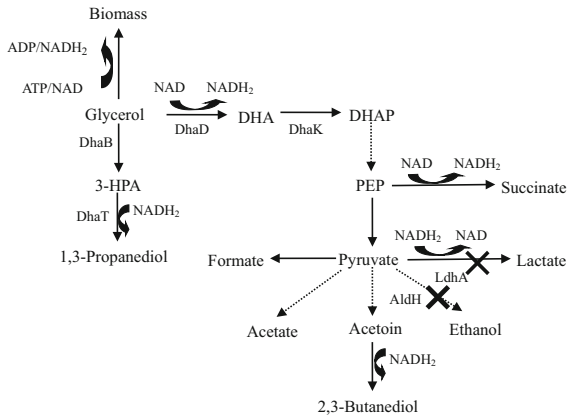

Fig.1 The glycerol metabolic pathway in *K. pneumoniae*

Materials and methods

Materials

Klebsiella pneumoniae 2-1 was previously described (Meng et al. 2011). *Escherichia coli* DH5α was used for DNA manipulation. λRed recombinase (Datsenko and Wanner 2000) and the FLP recombinase (Datsenko and Wanner 2000) were expressed by the helper plasmids pKD46 (Biosci Biotech, Hangzhou, China) and pCP20 (Biosci Biotech, Hangzhou, China), respectively; replication of these plasmids is temperature sensitive, and the plasmids can thus be easily eliminated. The plasmid pMD19-T Simple (TaKaRa Biotech, Dalian, China) was employed for cloning. The primers and restriction endonuclease are shown in Supplementary Table 1. The primers were synthesized by Shanghai Sangon. Restriction endonucleases were purchased in Shanghai Sangon.

The preculture medium contained (g/l)

glycerol, 20.0; KH_2PO_4, 1.3; K_2HPO_4, 3.4; $(NH_4)_2SO_4$, 2.0; $MgSO_4{\cdot}7H_2O$, 0.2; $FeSO_4{\cdot}7H_2O$,5 × 10^{-2}; $CaCl_2$,2 × 10^{-3}; yeast extract, 1; citric acid, 0.42; trace element solution A, 2 ml.

The fermentation medium contained (g/l)

glycerol, 50; $(NH_4)_2SO_4$, 6.6; NaH_2PO_4 1.38; Na_2SO_4, 0.28; KCl, 0.75; $MgCl_2{\cdot}6H_2O$, 0.26; $CaCl_2{\cdot}2H_2O$, 0.29; yeast extract, 1; citric acid, 0.42; trace element solution B, 5 ml.

Trace element solution A contained (g/l)

$CoCl_2{\cdot}6H_2O$, 0.2; $MnCl_2{\cdot}4H_2O$, 0.1; $ZnCl_2$ 0.07; H_3BO_3, 0.06; $Na_2MoO_4{\cdot}2H_2O$, 0.035; $CuCl_2{\cdot}2H_2O$, 0.02; $NiCl_2{\cdot}6H_2O$, 0.025.

Trace element solution B contained (g/l)

$CuCl_2{\cdot}2H_2O$, 0.17; $MnCl_2{\cdot}4H_2O$, 2; H_3BO_3 0.6; $ZnCl_2{\cdot}6H_2O$, 0.68; $Na_2MoO_4{\cdot}2H_2O$, 0.005; $CoCl_2{\cdot}6H_2O$, 0.47; $FeCl_3{\cdot}6H2O$.

Deletion of *ldh*A and *ald*H genes

To make an *ldhA* deletion mutant, the *ldh*A gene was cloned using primers A1/A2 (Supplementary Table 1) and the *K. pneumoniae* 2-1 genome as a template. The recombinant plasmid pMD19-T-*ldh*A was made from the *ldh*A gene and the vector pMD19-T. Secondly, in order to obtain linear fragments *ldh*A was amplified with a 300 bp homologous arm using A3/A4 as primers and pMD19-T-*ldh*A as template. The *Cm*^{r} gene was cloned by using L1/L2 as primers and FRT sites in pKD3 (Datsenko and Wanner 2000) as a template. Then the *ldh*A1-T-*ldh*A2 and *Cm*^{r} genes were digested and ligated with *Kpn*I and *Xho*I to obtain the plasmid pMD19-T-*ldh*A1-*Cm*^{r}-*ldh*A2 which was selected by ampicillin and chloramphenicol resistance. Finally, linear fragments *ldh*A1-*Cm*^{r}-*ldh*A2 with the 300 bp homologous arm were generated using A1/A2 as primers and pMD19-T-*ldh*A1-*Cm*^{r}-*ldh*A2 as a template. The linear fragments were introduced into *K. pneumoniae* 2-1 by electroporation to induce homologous recombination. Correct integration of the DNA fragment was confirmed by colony PCR with A1/A2 and L1/L2 as primers.

The *ald*H deletion method was performed in a similar way. First of all, the *ald*H gene was cloned by using Y1/Y2 as primers and the *K. pneumoniae* 2-1 genome as a template. Then the recombinant plasmid pMD19-T-*ald*H was obtained from the *ald*H gene and pMD19-T. Secondly, the linear fragments with the 300 bp homologous arm were generated by using Y3/Y4 as primers and pMD19-T-*ald*H as template for the reverse cloning. The *Cm*^{r} gene was cloned by using L1/L2 as primers and FRT sites with pKD3 as a template. The *ald*H1-T-*ald*H2 and *Cm*^{r} gene were then digested and ligated with *Kpn*I and *Xho*I to obtain a plasmid with pMD19-T-*ald*H1-*Cm*^{r}-*ald*H2 selected by ampicillin and chloramphenicol resistance. Finally, Y1/Y2 primers were used in order to obtain linear fragments *ald*H1-*Cm*^{r}-*ald*H2 with the 300 bp homologous arm using pMD19-T-*ald*H1-*Cm*^{r}-*ald*H2 as a template for cloning. Linear fragments were then introduced into *K. pneumoniae* 2-1 by electroporation to induce homologous recombination. Correct integration of the DNA fragment was confirmed by colony PCR with Y1/Y2 and L1/L2 as primers.

To make the Δ*ald*HΔ*ldh*A double mutant, the *ldh*A mutant linear fragment was PCR-amplified as described above and introduced into *K. pneumoniae* 2-1Δ*ald*H by electroporation to induce homologous recombination. Correct integration of the *ldh*A deletion linear fragment was confirmed as described above.

Fermentation of *K. pneumoniae* strains

Cells were seeded in a 250 ml shake-flask containing 50 ml medium at 37 °C for 14 h. Fermentations were carried out in a 5 l stirred-vessel system (Kobiotech. Co. Ltd) with 2 l medium. The culture was grown at 37 °C, pH 7, and agitation at 200 rpm. The pH was controlled at 7 by automatic addition of 2.5 M KOH. Anaerobic conditions in the bioreactors were maintained by N_2 at 0.4 vvm. In fed-batch fermentation, the limiting substrate, glycerol, was initially at 50 g/l. In the glycerol concentration feedback feeding strategy, after the residual glycerol dropped below 20 g/l, feeding was started to continuously pump concentrated glycerol solution into the bioreactor to maintain it between 15 and 25 g/l.

Analytical methods

Cell concentration was measured as the OD_{650} value (Oh et al. 2013a). The determination of 1,3-PD and ethanol levels was by GC using a Chromosorb 101 column 2 m × 0.5 mm with N_2 as a carrier gas at

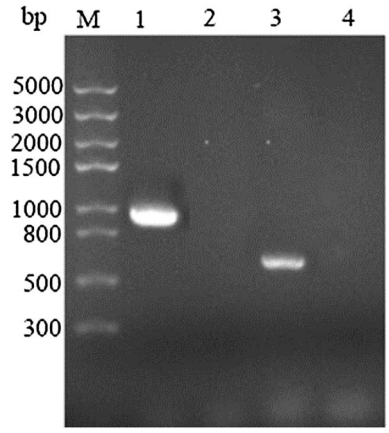

Fig.2 Confirmation of construction of the Δ*ldh*A of *K. pneumonia* 2-1 by colony PCR identification. *Lane M* 5000 bp DNA Ladder Marker; *Lane 1 ldh*A gene of *K. pneumonia* 2-1; *Lane 2 Cm*^{r} gene of *K. pneumonia* 2-1; *Lane 3* linear fragments *ldhA*1-*Cm*^{r}-*ldhA*2 of *K. pneumonia* 2-1Δ*ldh*A; Lane *4 Cm*^{r} gene of *K. pneumonia* 2-1Δ*ldh*A

40 ml/min. The detector was at 240 °C and column at 210 °C) (Wang et al. 2010). Glycerol was measured by a modified titration method (Meng et al. 2011). Lactate was determined by HPLC with an XDB-C_{18} column (250 mm × 4.6 mm, 5 μm; Agilent, USA) (Saxena et al. 2009). The mobile phase was of 2 % (v/v) H_3PO_4/acetonitrile (96.5:3.5, v/v), at 1 ml/min. Detection was at 214 nm. The column was held at 35 °C (Xiu and Zeng 2008). All samples tested were initially filtered through a membrane filter (0.45 μm pore size).

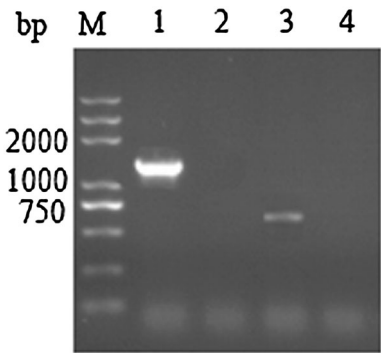

Fig.3 Confirmation of construction of the Δ*ald*H of *K. pneumonia* 2-1 by colony PCR identification. *Lane M* 5000 bp DNA Ladder Marker; *Lane 1 ald*H gene of *K. pneumonia* 2-1; *Lane 2 Cm*^{r} gene of *K. pneumonia* 2-1; *Lane 3* linear fragments *ald*H1-*Cm*^{r}-*ald*H2 of *K. pneumonia* 2-1Δ*aldH*; *Lane 4*, *Cm*^{r} gene of *K. pneumonia* 2-1Δ*ald*H

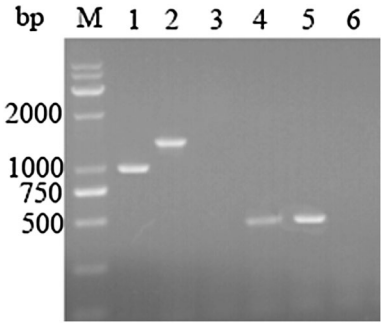

Fig.4 Confirmation of construction of the Δ*ldh*AΔ*ald*H of *K. pneumonia* 2-1 by colony PCR identification. *Lane M* 5000 bp DNA Ladder Marker; *Lane 1 ldh*A gene of *K. pneumonia* 2-1; *Lane 2 ald*H gene of *K. pneumonia* 2-1; *Lane 3 Cm*^{r} gene of *K. pneumonia* 2-1; *Lane 4* linear fragments *ldhA*1-*Cm*^{r}-*ldhA*2 of *K. pneumonia* 2-1Δ*ldh*AΔ*ald*H; *Lane 5* linear fragments *ald*H1-*Cm*^{r}-*ald*H2 of *K. pneumonia* 2-1Δ*ldh*AΔ*ald*H; *Lane 6 Cm*^{r} gene of *K. pneumonia* 2-1Δ*ldh*AΔ*ald*H

Results and discussion

Construction of Δ*ldh*A, Δ*ald*H and Δ*ald*H Δ*ldh*A mutant strains

To examine the effect of elimination of the major glycerol oxidative metabolic pathways involved in the synthesis of lactate or ethanol on 1,3-PD production, *K. pneumoniae* 2-1 was used as the starting strain for the construction of derivatives that were lactate dehydrogenase-deficient, acetaldehyde dehydrogenase-deficient, or deficient in both enzymes. *K. pneumoniae*

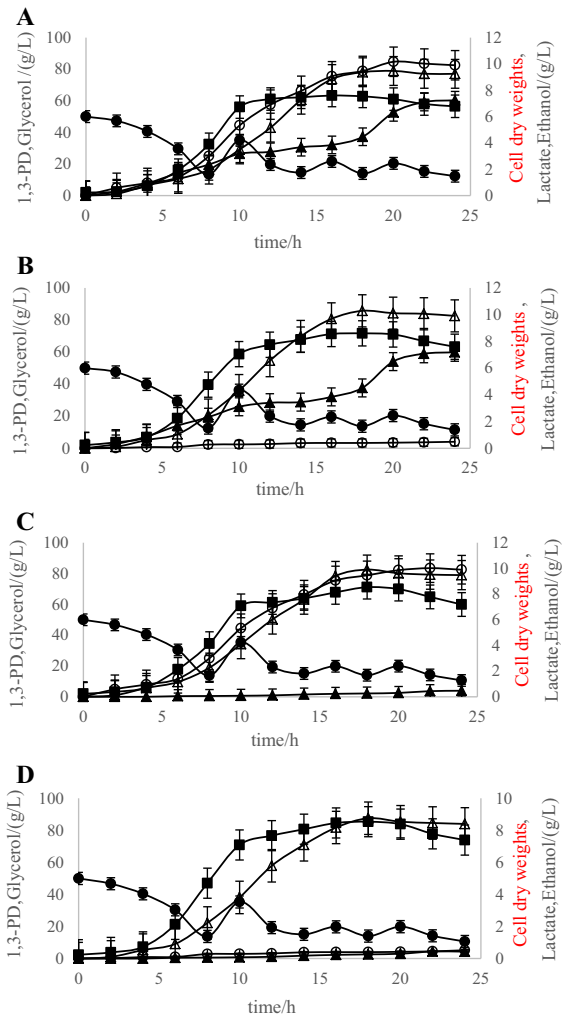

Fig. 5 Fed-batch fermentation of *K. pneumoniae* 2-1 (**a**), Δ*ldh*A (**b**), Δ*ald*H (**c**), and Δ*ldh*AΔ*ald*H strains (**d**). *Closed squares* cell growth, *closed circles* glycerol concentration, *open triangles* 1,3-PD levels, *open circles* lactate levels, *closed triangles* ethanol levels

Table 1 Glycerol consumed and metabolites produced by *K. pneumoniae* 2-1 and the Δ*ald*H, Δ*ldh*A, and Δ*ald*HΔ*ldh*A strains, after fed-batch fermentation

	2-1	Δ*ldh*A	Δ*ald*H	Δ*ldh*AΔ*ald*H
1,3-Propanediol (g/l)	78.8 ± 0.4	85.7 ± 0.3	82.5 ± 0.4	87.5 ± 0.4
Lactate (g/l)	10.1 ± 0.1	0.5 ± 0.1	10.0 ± 0.1	0.5 ± 0.1
Ethanol (g/l)	7.2 ± 0.1	7.1 ± 0.1	0.5 ± 0.1	0.4 ± 0.1
Formate (g/l)	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.1
Acetate (g/l)	16.4 ± 0.2	17.3 ± 0.2	17.3 ± 0.2	18.2 ± 0.2
Succinate (g/l)	8.5 ± 0.1	10.1 ± 0.2	9.9 ± 0.1	11.9 ± 0.2
1,3-PD conversion (g/g)	0.6	0.6	0.6	0.7
1,3-PD productivity (g/l h)	3. 3	3.5	3.4	3.6

2-1Δ*ldh*A, *K. pneumoniae* 2-1Δ*ald*H and *K. pneumoniae* 2-1Δ*ald*HΔ*ldh*A strains were constructed via homologous recombination with the λRed recombination system (see Materials and methods section). The mutants were identified by colony PCR identification as shown in Figs. 2, 3, and 4 respectively.

The glycerol metabolism properties of Δ*ldh*A, Δ*ald*H and Δ*ald*H Δ*ldh*A strains

A large amount of metabolites, ethanol and lactate, are co-produced with 1,3-PD from glycerol as byproducts via oxidative metabolism (Oh et al. 2012). Fed-batch fermentation of *K. pneumoniae* 2-1 resulted in the production of 1,3-PD to a maximum of 78.8 g/l (Fig. 5a). The concentration of lactate and ethanol were 10.2 and 7.2 g/l, respectively. In the *ldhA*-mutant strain, the synthesis of lactate was decreased, as expected, to 0.5 g/l. 1,3-PD increased to 85.7 g/l (Fig. 5b). The results indicate that the yield of 1,3-PD was increased by deleting the *ldh*A gene.

In the *ald*H-mutant strain, the synthesis of ethanol was also decreased, as expected, to 0.5 g/l. 1,3-PD increased to 82.5 g/l (Fig. 5c). Thus the yield of 1,3-PD was increased by deleting the *ald*H gene. In the *K. pneumonia* 2-1Δ*ldh*AΔ*ald*H, the synthesis of both lactate and ethanol were decreased, as expected. Lactate and ethanol were 0.5 and 0.4 g/l, respectively. 1,3-PD increased to 87.5 g/l (Fig. 5d). This shows that the yield of 1,3-PD was increased by co-deletion of the *ald*H and *ldh*A genes. The figures in Table 1 also demonstrate that the concentrations of formate, acetate and succinate are increased in the mutant strains compared with the parental strain. This shows that the metabolic flux of carbon and hydrogen reduction are transferred to other pathways owing to the deletion of the *ldh*A and *ald*H (Seo et al. 2009).

Conclusion

Abolishing the major glycerol oxidative metabolic pathway involved in the synthesis of lactate and ethanol, was studied. Deletion of the *ldh*A gene encoding lactate dehydrogenase (catalyzing the synthesis of lactate) and the *ald*H gene encoding acetaldehyde dehydrogenase (catalyzing the synthesis of ethanol) enhanced 1,3-PD production. The *K. pneumoniae* 2-1Δ*ldh*A, *K. pneumoniae* 2-1Δ*ald*H and *K. pneumoniae* 2-1Δ*ald*HΔ*ldh*A variants were constructed via homologous recombination with the λRed recombination system. After 24 h fermentation by *K. pneumoniae* 2-1Δ*ldh*A, *K. pneumoniae* 2-1Δ*ald*H and *K. pneumoniae* 2-1Δ*ald*HΔ*ldh*A, the maximum yields of 1,3-PD were increased to 85.7, 82.5, 87.5 g/l respectively, compared with 78.8 g/l in the parental strain.

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Supporting Information Supplementary Table 1—Primers used in this study.

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