1,2-Propanediol production from glycerol via a endogenous pathway of *Klebsiella pneumoniae*

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Abstract

Klebsiella pneumoniae is an important microorganism and is used as a cell factory for many chemicals production. When glycerol was used as the carbon source, 1,3-propanediol was the main catabolite of this bacterium. *K. pneumoniae* Δ*tpiA* lost the activity of triosephosphate isomerase, and prevented glycerol catabolism through the glycolysis pathway. But this strain still utilize glycerol and 1,2-propanediol became the main catabolite. Key enzymes of 1,2-propanediol synthesis from glycerol were investigated in detail. *dhaD* and *gldA* encoded glycerol dehydrogenases were both responsible for the conversion of glycerol to dihydroxyacetone, but overexpression of the two enzymes result in a decrease of 1,2-propanediol production. There are two dihydroxyacetone kinases (I and II), but the dihydroxyacetone kinase I had no contribution to dihydroxyacetone phosphate formation. Dihydroxyacetone phosphate was converted to methylglyoxal, and methylglyoxal was then reduced to lactaldehyde or hydroxyacetone and further reduced to form 1,2-propanediol. Individual overexpression of *mgsA*, *yqhD*, and *fucO* all resulted in increased production of 1,2-propanediol, but only the combined expression of *mgsA* and *yqhD* showed a positive effect on 1,2-propanediol production. The process parameters for 1,2-propanediol production by *Kp* Δ*tpiA-mgsA-yqhD* were optimized, with pH 7.0 and agitation rate of 350 rpm were found to be optimal. In the fed-batch fermentation, 9.3 g/L of 1,2-propanediol was produced after 144 hours of cultivation and the substrate conversion ratio was 0.2 g/g. This study provides an efficient way of 1,2-propanediol production from glycerol via an endogenous pathway of *K. pneumoniae*.

Key points

1,2-Propanediol was synthesis from glycerol by a *tpiA* knocked out *K. pneumoniae*

Overexpression of *mgsA*, *yqhD*, or *fucO* promote 1,2-propanediol production

9.3 g/L of 1,2-propanediol was produced in fed-batch fermentation

Key words: 1,2-propanediol; glycerol; *tpiA*; *mgsA*; *Klebsiella pneumoniae*

Introduction

1,2-Propanediol is a bulk chemical. It finds applications in the production of antifreeze, deicer, pharmaceuticals, polyester resin, detergents, cosmetics, and so on. Racemic 1,2-propanediol has been produced on a large scale by the petrochemical industry, using propylene oxide as a feedstock (Bennett and San 2001).

Many bacteria have a native 1,2-propanediol synthesis pathway that uses deoxy sugars as the feedstock. It was first identified in *Salmonella typhimurium* and *Klebsiella pneumoniae*. L-rhamnose is converted to L-rhamnulose-1-phosphate, and the phosphorylated sugar is cleaved by an aldolase to give dihydroxyacetone phosphate and lactaldehyde. Lactaldehyde is reduced to form 1,2-propanediol (Badía et al. 1985). Another natural 1,2-propanediol synthesis pathway uses glucose and other sugars as the feedstock and via the methylglyoxal pathway. Methylglyoxal is formed from dihydroxyacetone phosphate, an intermediate of glycolysis. Methylglyoxal is reduced to dihydroxyacetone or lactaldehyde, and further reduced to 1,2-propanediol. However, this pathway is restricted in some strains of *Clostridium thermosaccharolyticum* (Cameron and Cooney 1986). 1,2-Propanediol can be obtained from lactic acid by resting-cell of *Lactobacillus buchneri*, and lactaldehyde was proposed to be the intermediate of this pathway (Oude Elferink et al. 2001).

Wild-type *Escherichia coli* is not known to produce 1,2-propanediol from common sugars. An engineered strain that overexpressing NADH-linked glycerol dehydrogenase genes (*gldA* or *dhaD*) and a methylglyoxal synthase gene (*mgsA*) produced 1,2-propanediol from glucose (Altaras and Cameron 1999). After eliminated lactic acid synthesis and optimization of the fermentation process, 4.5 g/L of 1,2-propanediol was produced, and the substrate conversion ratio was 0.19 g/g (Altaras and Cameron 2000). Further metabolic engineering works that inactivation of triose phosphate isomerase (*tpiA*) and employ optimal enzymes were done in *E. coli*, and a final titer of 5.13 g/L of 1,2-propanediol was obtained with a yield of 0.48 g/g glucose (Jain et al. 2015). A 1,2-propanediol producing *Corynebacterium glutamicum* was constructed following a similar strategy (Siebert and Wendisch 2015). Besides glucose, glycerol was also used for 1,2-propanediol production. An engineered *E. coli* with expression of *gldA* and *mgsA*, and replacement of the native PEP-dependent dihydroxyacetone kinase with an ATP-dependent dihydroxyacetone kinase from *Citrobacter freundii,* produces 1,2-propanediol from glycerol (Clomburg and Gonzalez 2011). *Saccharomyces cerevisiae* was also used as the host cell for artificial 1,2-propanediol synthesis pathway construction, and 2.19 g/L of 1,2-propanediol was produced from a medium contains 10 g/L of glycerol (Joon-Young et al. 2011).

K. pneumoniae is an important industrial microorganism. When glycerol was used as the sole carbon source, the main metabolites of this strain were 1,3-propanediol and 2,3-butanediol (Hao et al. 2008). The glycerol catabolism pathway is a dismutation process. In the reductive pathway, glycerol is converted to 3-hydroxypropionaldehyde and further converted to 1,3-propanediol. In a coupled oxidative branch, glycerol is oxidized to dihydroxyacetone, and the latter is then phosphorylated to dihydroxyacetone phosphate and channeled into glycolysis (Wei et al. 2014). Dihydroxyacetone phosphate is an intermediate of the glycerol catabolism pathway and it is also an intermediate of all these artificial 1,2-propanediol synthesis pathways. Wild type *K. pneumoniae* contains *mgsA* and other genes used in the artificial 1,2-propanediol synthesis pathway construction. Thus an endogenous 1,2-propanediol synthesis pathway exists in this bacterium. However, no strains of *K. pneumoniae* strains are known to produce 1,2-propanediol from glycerol or glucose. In this work, this endogenous 1,2-propanediol synthesis pathway was awakened by blocking the dihydroxyacetone phosphate catabolism through glycolysis and a high level of 1,2-propanediol was produced with glycerol as a carbon source (Fig. 1).

Material and Methods

Strains, plasmids, and primers

Bacterial strains and plasmids used in this study are listed in Table 1. Primers used for PCR are listed in Table S1.

Knocking out genes in the genome of *K. pneumoniae*

For genes knock out, *K. pneumoniae* and *E. coli* were grown in Luria–Bertani (LB) medium at 37 °C. The antibiotics used in the selective medium were ampicillin (50 μg/mL), kanamycin (50 μg/mL), apramycin (50 μg/mL), and streptomycin (25 μg/mL).

Kp Δ*tpiA* Δ*gldA* construction

Kp Δ*tpiA* Δ*gldA* construction was generated using the Red recombinase associated gene replacement method as described previously (Wang et al. 2020; Wei et al. 2012). The up and drown flanking sequences of *gldA* gene in the genome of *K. pneumoniae* were amplified by PCR using the primer pair gldA-up-s/a and gldA-down-s/a. Apramycin resistance gene *aac(3)IV* was amplified with plasmid pIJ773 as the template using the primer pair gldA -FRT-s/a. The up and drown flanking sequences of *gldA* and *aac(3)IV* were ligated together with a ClonExpress Ultra One Step Cloning Kit® to generate a linear DNA containing the apramycin resistance gene *aac(3)IV* with 600 bp of *gldA* homologous regions on both sides. The linear DNA was transformed into *Kp* Δ*tpiA*, which already hosted the plasmid pDK6-red. Homologous recombination between the linear DNA and the chromosome was facilitated by Red

recombinase and led to *gldA* deletion in the genome of *Kp* Δ*tpiA* to obtain *Kp* Δ*tpiA* Δ*gldA*.

K. pneumoniae Kp Δ*tpiA* Δ*dhaD* Δ*gldA* was constructed following the same way of *Kp* Δ*tpiA* Δ*gldA* construction, with *Kp* Δ*tpiA* Δ*dhaD* replacing *Kp* Δ*tpiA* as the target strain.

The ORF of *mgsA* in *K. pneumoniae* was amplified using the primer pair mgsA-s and mgsA-a. The PCR product was digested with restriction enzyme *EcoR I* and *Hind III*. The DNA fragment was ligated into the pDK6 vector to generate pDK6-mgsA. pDK6-mgsA was transformed into *Kp* Δ*tpiA* to generate *Kp* Δ*tpiA-mgsA*. Other strains with the expression of *gldA*, *fucO*, *yqhD*, or *dhaD* were constructed in the same way as *Kp* Δ*tpiA-mgsA*.

Medium and culture conditions

The fermentation medium contained 30 g/L glycerol, 1.5 g/L yeast extract, 4 g/L $(NH_4)_2SO_4$, 0.69 g/L $K_2HPO_4 \cdot 3H_2O$, 0.25 g/L KH_2PO_4, 0.2 g/L $MgSO_4$, 0.05 g/L $FeSO_4 \cdot 7H_2O$ and 1 mL of trace element solution. One liter of trace element solution contained 200mg $CoCl_2 \cdot 6H_2O$, 100 mg $MnSO_4 \cdot 4H_2O$, 70 mg $ZnCl_2$, 60 mg H_3BO_3, 35 mg $Na_2MoO_4 \cdot 2H_2O$, 29.28 mg $CuSO_4 \cdot 5H_2O$, 25 mg $NiCl_2 \cdot 6H_2O$ and 0.9 mL 37% (w/w) HCl.

K. pneumoniae strains were inoculated into 250 mL flasks containing 50 mL medium and incubated for 120 hours at 37 °C and 120 rpm. 1g of $CaCO_3$ was added to each flask to adjust the pH stable at 5.8-6.5 , and all flask culture experiments were conducted in triplicate.

Experiment to optimize process parameters, such as pH and oxygen concentration were performed in bioreactors in batch conditions. For seed culture, 250 mL flasks containing 50 mL of LB medium were incubated overnight in a rotary shaker at 37 °C and 200 rpm. The seed culture was inoculated into 5-L tank bioreactor (BIOSTAT-A plus, Sartorius) with a working volume of 3 L and the air flow rate was 2 L/min. The pH was controlled automatically by adding 10 mol/L NaOH.

Analytical methods

Biomass yield at fixed time intervals was determined by optical density (OD600) with a spectrophotometer. Chemical compounds in the broth were quantified by a Shimadzu 20AVP high performance liquid chromatography system (HPLC) equipped with a RID-20A refractive index detector. An Aminex HPX-87H column (300×7.8 mm) (Bio-Rad, USA) was used and the column temperature was set at 65 ºC. The mobile phase was 0.005 mol/L H_2SO_4 solution with a flow rate of 0.8 ml/min.

Since the retention times of glycerol and dihydroxyacetone for were the same (10.3 min), and the retention times of 1,2-propanediol and 1,3-propanediol were the same (13.3 min) using HPLC method, Gas chromatography (GC) was used combined with HPLC to analyses these compounds. A gas

chromatography system (Shimadzu GC 2010) equipped with a flame ionization detector and a HP-FFAP column (30 m × 0.25 mm), with nitrogen as the carrier gas was used. The injector and detector were maintained at 250 °C and 280 °C, respectively. The column temperature initially started at 120 °C and was maintained for 1 min, then it was increased at a rate of 20 °C /min to 230 °C and then maintained at this temperature for 1.3 min.

Results

1,2-propanediol production from glycerol by *Kp* Δ*tpiA*

tpiA encodes triosephosphate isomerase, which catalysis the interconversion of dihydroxyacetone phosphate and glyceraldehyde 3-phosphate. *Kp* Δ*tpiA* was already used for dihydroxyacetone and glycerol production from glucose as described previously (Shaoqi et al. 2021). This strain and the wild-type strain were cultured in flasks with glycerol as the carbon source, and the results are shown in Fig. 2.

K. pneumoniae is a 1,3-propanediol producer. Glycerol (30 g/L) was completely consumed by the wild type strain after 15 h of cultivation, and 11.4 g/L of 1,3-propanediol was produced. Cells were grown rapidly and a cell density of 2.8 OD units was obtained after glycerol was exhausted. No 1,2-propanediol was produced with the wild type strain. Unlike the wild type strain, 1,3-propanediol was not synthesized by *Kp* Δ*tpiA*, and this strain grew very slowly. Glycerol was exhausted after 120 h, and a cell density of 2.2 OD units was obtained. 3.4 g/L of 1,2-propanediol was produced by this strain. 1.0 g/L acetic acid was synthesized by this strain after 96 h, but it was reused by the cells later.

Acetic acid is the main by-product of the process. Acetate kinase and phosphate acetyltransferase are key enzymes of this pathway, and their encoding genes were knocked out based on *Kp* Δ*tpiA* to obtain *Kp* Δ*tpiA*-Δ*pta acK*. This strain was cultured in shake flasks and results were shown in Fig 2.

The cell growth of *Kp* Δ*tpiA*-Δ*pta acK* was faster than that of *Kp* Δ*tpiA* (t test, $P < 0.05$), and a final cell density of 2.7 OD units was obtained. The highest concentration of acetic acid that was synthesized by *Kp* Δ*tpiA*-Δ*pta acK* was 0.7 g/L, which was lower than that of *Kp* Δ*tpiA*, but the difference was not significant in t test. Further, the 1,2-propanediol titer of *Kp* Δ*tpiA*-Δ*pta acK* was 3.1 g/L, which was lower than *Kp* Δ*tpiA* (t test, $P < 0.05$). Thus, *Kp* Δ*tpiA*-Δ*pta acK* was not used for further investigation.

Role of isoenzymes of glycerol dehydrogenase on 1,2-propanediol production

The conversion of glycerol to dihydroxyacetone is the first step of the 1,2-propanediol synthesis pathway. *dhaD* and *gldA* encoding two glycerol dehydrogenases in *K. pneumoniae*, individually. *dhaD* and *gldA*

were knocked out individually and combined to identify their roles on 1,2-propanediol synthesis. *Kp* Δ*tpiA*-Δ*dhaD*, *Kp* Δ*tpiA*-Δ*gldA*, and *Kp* Δ*tpiA*-Δ*dhaD*-Δ*gldA* were cultured in shake flasks and the results are shown in Fig 3.

The specific growth rates of *Kp* Δ*tpiA*-Δ*dhaD* and *Kp* Δ*tpiA* were 15.5 h^{-1} and 21.5 h^{-1}, respectively. The glycerol consumption rates of *Kp* Δ*tpiA*-Δ*dhaD* and *Kp* Δ*tpiA* were 0.34 g/Lh and 0.30 g/Lh, respectively (*Kp* Δ*tpiA* shown in Fig 2). However, the concentration of 1,2-propanediol produced by *Kp* Δ*tpiA*-Δ*dhaD* was only 0.8 g/L for 72 h, and after that, the 1,2-propanediol was reused by the cells. After 120 h, 1.6 g/L of 1,3-propanediol was produced. This differs from that of *Kp* Δ*tpiA*, which loses the ability to synthesise 1,3-propanediol. The specific growth rate and the glycerol consumption rate of *Kp* Δ*tpiA*-Δ*gldA* were 39 h^{-1} and 0.22 g/Lh, which were both slower than that of *Kp* Δ*tpiA*. 16.2 g/L of glycerol was still not consumed by the cell after 120 h of cultivation. Accordingly, the 1,2-propanediol productivity was lower than that of *Kp* Δ*tpiA*, and a final titer of 2.0 g/L of 1,2-propanediol was produced. No 1,3-propanediol was synthesized by *Kp* Δ*tpiA*-Δ*gldA*. Glycerol consumption by *Kp* Δ*tpiA*-Δ*dhaD*-Δ*gldA* was almost stopped, and the cell growth of this strain was very slow. Neither 1,2-propanediol nor 1,3-propanediol was produced by this strain. 1.1 g/L of acetic acid was produced by *Kp* Δ*tpiA*-Δ*dhaD* after 72 h of cultivation, but the acetic acid titer was decreased to 0.4 after 120 h of cultivation. Acetic acid produced by *Kp* Δ*tpiA*-Δ*gldA* and *Kp* Δ*tpiA*-Δ*dhaD*-Δ*gldA* were all 0.1 g/L.

These results indicated that either *dhaD* or *gldA* encoded glycerol dehydrogenases were both key enzymes responsible for the conversion of glycerol to dihydroxyacetone. To further investigate these two enzymes on 1,2-propanediol synthesis. *dhaD* and *gldA* were individually overexpressed in *Kp* Δ*tpiA* to obtain *Kp* Δ*tpiA*-*dhaD* and *Kp* Δ*tpiA*-*gldA*. They were both cultured in shake flasks and results are shown in Fig 3.

The specific growth rate and the glycerol consumption rate of *Kp* Δ*tpiA*-*dhaD* were 15 h^{-1}, and 0.31 g/Lh, respectively. While the specific growth rate and the glycerol consumption rate were both in low rates for *Kp* Δ*tpiA*-*gldA*. 2.1 and 1.3 g/L of 1,2-propanediol were produced by *Kp* Δ*tpiA*-*dhaD* and *Kp* Δ*tpiA*-gldA, respectively, which were lower compared to those of *Kp* Δ*tpiA*. The concentration of acetic acid produced by *Kp* Δ*tpiA*-*dhaD* and *Kp* Δ*tpiA*-gldA was higher than that of *Kp* Δ*tpiA*, with the titer of 2.7 and 2.6 g/L, respectively. The distinct characteristic of *Kp* Δ*tpiA*-*dhaD* and *Kp* Δ*tpiA*-gldA was 1.1 and 1.4 g/L of dihydroxyacetone were synthesized by the two strains, respectively. No dihydroxyacetone was accumulated in *Kp* Δ*tpiA*.

Role of isoenzymes of dihydroxyacetone kinase on 1,2-propanediol production

Dihydroxyacetone phosphate formation was the second step of the pathway of 1,2-propanediol synthesis from glycerol. There are two kinases in *K. pneumoniae* that catalysis dihydroxyacetone phosphate formation from dihydroxyacetone. Dihydroxyacetone kinase I is encoded by *dhaK*, and it uses ATP as the phosphate donor. Dihydroxyacetone kinase II contains three subunits and they are encoded by *dhaK1, dhaK 2*, and *dhaK3*, respectively. Dihydroxyacetone kinase II uses phosphoenolpyruvic acid (PEP) as the phosphate donor. It has been revealed in our previous research that dihydroxyacetone kinase II was the main functionally enzyme responsible for dihydroxyacetone phosphate formation in glycerol catabolism (Wei et al. 2014). In this paper, the role of two dihydroxyacetone kinases on 1,2-propanediol synthesis was investigated in detail. The encoding genes of the two dihydroxyacetone kinases were knocked individually and combined in *Kp* Δ*tpiA* to obtain *Kp* Δ*tpiA*-Δ*dhaK*, *Kp* Δ*tpiA*-Δ*dhaK1*, *Kp* Δ*tpiA*-Δ*dhaK2*, *Kp* Δ*tpiA*-Δ*dhaK3*, and *Kp* Δ*tpiA*-Δ*DHAK*. *Kp* Δ*tpiA*-Δ*DHAK* was a strain in which all of these encoding genes were knocked out. These strains were cultured in shake flasks and the results are shown in Fig 4.

Similar to *Kp* Δ*tpiA*, *Kp* Δ*tpiA*-Δ*dhaK* and *Kp* Δ*tpiA*-Δ*dhaK2* produced 3.0 and 2.8 g/L of 1,2-propanediol, respectively, after 96 h of cultivation. The acetic acid produced by the two strains was 2.2 and 2.3 g/L, respectively. The glycerol consumption and the cell growth of *Kp* Δ*tpiA*-Δ*dhaK* was faster than that of *Kp* Δ*tpiA*, whereas glycerol was completely consumed after 96 h of cultivation. The physical properties of *Kp* Δ*tpiA*-Δ*dhaK1*, *Kp* Δ*tpiA*-Δ*dhaK3*, and *Kp* Δ*tpiA*-Δ*DHAK* were significantly different from those of *Kp* Δ*tpiA*. The 1,2-propanediol produced by these strains was decreased to 0.3, 0.6, and 0.4 g/L, respectively. While 0.2 and 0.1 g/L of 1,3-propanediol was produced by *Kp* Δ*tpiA*-Δ*dhaK1* and *Kp* Δ*tpiA*-Δ*DHAK*. 0.7, 0.7, and 0.8 g/L of dihydroxyacetone was produced by *Kp* Δ*tpiA*-Δ*dhaK1*, *Kp* Δ*tpiA*-Δ*dhaK3*, and *Kp* Δ*tpiA*-Δ*DHAK*, respectively. The acetic acid produced by these strains was 0.6, 1.2, and 1.4 g/L.

Enhanced production of 1,2-propanediol by overexpression of key enzymes

Methylglyoxal synthase gene (*mgsA*) was responsible for methylglyoxal formation from dihydroxyacetone phosphate. There are two pathways for the formation of 1,2-propanediol from methylglyoxal, via lactaldehyde or hydroxyacetone as the intermediate. It was noted in the KEGG that the conversion of methylglyoxal to lactaldehyde and hydroxyacetone to 1,2-propanediol is catalysed by GldA or DhaD. The conversion of methylglyoxal to hydroxyacetone was catalysed by YqhD. FucO catalysed the formation of 1,2-propanediol from lactaldehyde (Fig. 1). The influence of GldA and DhaD

on 1,2-propanediol production was investigated (Fig. 3). Here, the *mgsA*, *yqhD*, and *fucO* were overexpressed individually in *Kp* Δ*tpiA* to obtain *Kp* Δ*tpiA-mgsA*, *Kp* Δ*tpiA-yqhD*, and *Kp* Δ*tpiA-fucO*. These strains were cultured in shake flasks and the results are shown in Fig 5.

Individual overexpression of *mgsA*, *yqhD*, or *fucO* resulted in an increase in 1,2-propanediol, with final titers of 4.1, 4.6 and 4.1 g/L respectively. Glycerol was depleted by *Kp* Δ*tpiA-mgsA* and *Kp* Δ*tpiA-yqhD* after 120 h of cultivation. By contrast, 6.8 g/L of glycerol was still present in the broth of *Kp* Δ*tpiA-fucO*. Acetic acid produced by *Kp* Δ*tpiA-mgsA*, *Kp* Δ*tpiA-yqhD*, and *Kp* Δ*tpiA-fucO* was 0.7, 0.4 and 2.0 g/L respectively.

Since individual overexpression of *mgsA*, *yqhD*, and *fucO* all had positive effects on 1,2-propanediol production, they were overexpressed combined to obtain *Kp* Δ*tpiA-mgsA-yqhD*, *Kp* Δ*tpiA-mgsA-fucO*, *Kp* Δ*tpiA-yqhD-fucO*, and *Kp* Δ*tpiA-mgsA-yqhD-fucO*. They were cultured in shake flasks and the results are shown in Fig 5.

The results obtained with *Kp* Δ*tpiA-yqhD-fucO* were different from other strains, 2.2 g/L of glycerol was still unused by this strain after 120 h of cultivation, whereas glycerol was exhausted by other strains. The final concentration of 1,2-propanediol produced by this strain was 4.2 g/L, which was lower than other strains. *Kp* Δ*tpiA-mgsA-yqhD* produced the highest concentration of 1,2-propanediol, with a titer of 5.3 g/L, followed by *Kp* Δ*tpiA-mgsA-yqhD-fucO* and *Kp* Δ*tpiA-mgsA-fucO*, with the titer of 5.0 and 5.0 g/L.

These results indicated that the expression level of *mgsA* was a bottleneck of the pathway. Overexpression of *yqhD* or *fucO* should be combined with overexpression of *mgsA* to get the high 1,2-propanediol level. However, combined overexpression of *yqhD* and *fucO* was poor than overexpression of *yqhD* alone. *Kp* Δ*tpiA-mgsA-yqhD* was selected for further research.

Fermentation parameters optimization

Culture pH optimization.

Kp Δ*tpiA-mgsA-yqhD* was cultured in 5 L bioreactors, where the culture pH was stabilized at 6.0, 6.5, 7.0, and 7.5. Fermentation results are presented in Fig. 6.

It is interesting to observe that the cell growth was better in bioreactors than in flasks. The final cell densities were 7.6, 7.9,7 .6, and 3.3 OD units in experiments performed at pH of 6.0, 6.5, 7.0, and 7.5, respectively. In the experiment performed at pH 7.5, after 96 h, the cell density reached the peak value of 4.4 OD units and it decreased after 120 h of cultivation. The glycerol consumption rate was increased with the pH increase, and it was high at pH 7.0 and 7.5. 1,2-Propanediol titers were increased with culture

pH increase in the range of 6.5-7.0, and 8.2 g/L of 1,2-propanediol was obtained at culture pH 7.0. Further increase of pH resulted with the low 1,2-propanediol titer. Acetic acid production was inversely related to the pH. No acetic acid was produced at pH 6.0. 4.3 g/L of acetic acid was produced in the culture pH of 7.5. Thus pH 7.0 was selected as the optimal pH.

Agitation rate

Oxygen concentration is an important parameter for cell growth and products synthesis. The air flow rate was set at 2 L/min and the stirring rate of the bioreactor was set at 150, 250, 250, and 450 rpm to obtain different aerobic conditions. The pH was kept constant at pH 7.0 and the fermentation results are presented in Figure 7.

The cell growth of *Kp* Δ*tpiA-mgsA-yqhD* showed a positive correlation with agitation rate with cells grown at 450 rpm having the highest cell densities (14.5OD units), while those grown at 150 rpm had the lowest cell density (3.3OD units). The glycerol consumption rate was similar to that of the cell growth, with cells grown at 450 rpm having the fastest glycerol consumption rate, and those grown at 150 rpm had the lowest rate. 1,2-Propanediol production was positively correlated during agitation rates from 150 to 350 rpm. However, the 1,2-propanediol titer was decreased in the condition of 450 rpm. Thus, the medium aerobic condition seems to favor 1,2-propanediol production, so 450 rpm was selected as the optimal agitation rate.

Fed-batch fermentation

Once the optimal pH and the agitation rate were identified, fed-batch fermentations were carried out at pH 7.0 and 350 rpm to achieve high final product content. Fermentation results are presented in Fig. 8.

In fed-batch experiments, the glycerol concentration in the fermentation broth decreased to 12.0 g/L after 96 h of cultivation. After the addition of glycerol, the glycerol concentration increased to 20.8 g/L. After 120 h, another glycerol feed had to be performed. The cell growth reached a cell density of 8.0 after 120 h of cultivation, after which cell density decreased. The highest 1,2-propanediol titer of 9.3 g/L was achieved after 144 h of cultivation, and the titer decreased after 168 h of cultivation. The conversion ratio of glycerol to 1,2-propanediol was 0.20 g/g after 144 h of cultivation. 4.0 g/L of acetic acid was produced in the fed-batch fermentation. The production of 1,3-propanediol started after 96 h, reaching a final titer of 1.5 g/L. 1-Propanol was detected at the same time as 1,3-propanediol. The highest 1-propanol level was achieved after 102 h, and its concentration decreased afterwards.

Discussion

1,2-Propanediol production by *K. pneumoniae*

The interconversion of dihydroxyacetone phosphate and glyceraldehyde 3-phosphate was blocked in the cell *Kp* Δ*tpiA*. When this strain is cultivated using glucose as a carbon source, the dihydroxyacetone phosphate cannot be further catabolized through the TCA cycle and dephosphated to form dihydroxyacetone by dephosphorylase (Shaoqi et al. 2021). Since further catabolism of dihydroxyacetone phosphate was blocked, it was assumed that this strain cannot grow on glycerol as a carbon source. Though the growth of *Kp* Δ*tpiA* was slower than the wild-type strain, this strain still catabolizes glycerol, as shown in Fig 2. This is because the endogenous 1,2-propanediol synthesis pathway was awakened. In which, dihydroxyacetone phosphate is converted to methylglyoxal, and the latter is further catabolized to 1,2-propanediol and propanoyl-CoA (Fig. 1). Methylglyoxal can be converted to pyruvate with the catalysis of aldehyde dehydrogenase A (encoded by aldA). Pyruvate and propanoyl-CoA were used as carbon and energy sources for cell growth (Fig. 1).

1,2-Propanediol formation from dihydroxyacetone phosphate consumes two molecules of NADH, while only one molecule of NADH was formed in the process of dihydroxyacetone phosphate formation from glycerol. The additional NADH was more likely produced in the process of pyruvate formation from methylglyoxal and further catabolisms like TCA cycle. The cofactor balance limited the entire conversion ratio of glycerol to 1,2-propanediol. This is similar to that of 1,3-propanediol production from glycerol, part of glycerol was oxidized to provide NADH and building blocks of cell growing (Zeng et al. 1996).

Acetic acid was the main by-product of 1,2-propanediol production by *Kp* Δ*tpiA*. Acetic acid can be formed from pyruvate via several pathways. *pta* and *ackA* responsible for acetyl-P formation from pyruvate and acetic acid formation from acetyl-P. Acetic acid production was decreased by blocking this pathway in 1,3-propanediol production with glucose as the carbon source (Lama et al. 2020). Here, *pta* and *ackA* were knocked out to generate *Kp* Δ*tpiA*-Δ*pta acK*. Though the acetic acid level was decreased, it had no positive effects on 1,2-propanediol production. Some acetic acid produced in the process was reused by the cells. It is the same in researches of 2,3-dihydroxyisovalerate and dihydroxyacetone production by *K. pneumoniae*, that the acetic acid produced can be reused by the cells (Wang et al. 2020) (Shaoqi et al. 2021). The mechanism of acetic acid reuse by the cell was not investigated in detail. It has been noted in the KEGG, that the reaction of pyruvate converted to acetic acid with the catalysis of pyruvate dehydrogenase (encoded by *poxB*) was a reversible reaction. Thus, acetic acid can be converted to pyruvate and used by the cells.

For the biological route of 1,2-propanediol production, most studies followed the work of Altaras who set up the 1,2-propanediol synthesis pathway from methylglyoxal (Altaras and Cameron 1999). The 1,2-propanediol produced through this pathway was R-form. Another artificial 1,2-propanediol synthesis pathway was through lactic acid as the intermediate, but obtained 1,2-propanediol was a mixture of R and S- form (Niu et al. 2018). The synthesis pathway of 1,2-propanediol produced by *Kp* Δ*tpiA* was similar to that of Altaras' research, but all enzymes of this pathway were endogenous enzymes. Thus,1,2-propanediol produced by Kp Δ*tpiA* was supported to be R-form.

A *tpiA* knocked out *E. coli* strain that heterogeneous overexpression of *mgsA*, *gldA* and *fucO* produced 1,2-propanediol with glucose as the carbon source (Jain et al. 2015). Glucose was also used as a carbon source to culture *Kp* Δ*tpiA*. However, the 1,2-propanediol production rate was very low (data not shown). Besides glucose, glycerol was also used for 1,2-propanediol production. 1,2-Propanediol was produced by an *E. coli* strain that co- overexpression of *mgsA*, *gldA*, and *yqhD* (Clomburg and Gonzalez 2011). But, the *tpiA* gene was not knocked out in that research.

Medium aerobic conditions favored 1,2-propanediol production by *Kp* Δ*tpiA-mgsA-yqhD*, too much air supply inhibited 1,2-propanediol synthesis. This is different from the research of 1,2-propanediol production by *E. coli* or *C. glutamicum* (Altaras and Cameron 2000; Lange et al. 2017; Niu et al. 2018), which were both cultured under anaerobic or microaerobic conditions. 9.3 g/L of 1,2-propanediol produced by *Kp* Δ*tpiA-mgsA-yqhD* with the conversion ratio of 0.20 g/g. The 1,2-propanediol titer obtained here was higher than all those researches of engineered strains that the pathway use methylglyoxal as an intermediate. But the engineered *E. coli* which uses lactic acid as the intermediate of the 1,2-propanediol synthesis pathway had a higher 1,2-propanediol titer of 17.3 g/L (Niu et al. 2018).

1,3-Propanediol synthesis was inhibited in *K. pneumoniae* Δ*tpiA*

Genes responsible for glycerol catabolism and 1,3-propanediol synthesis form the *dha* regulon. Glycerol dehydrase and 1,3-propanediol dehydrogenase catalysis reactions of glycerol conversion to 3-hydroxypropionaldehyde and further to 1,3-propanediol. The two enzymes were encoded by *dhaB123* and *dhaT*, respectively. *tpiA* is not in the *dha* regulon, but no 1,3-propanediol was synthesized by *tpiA* disrupted strain (Fig.2). It was suspected that the expression of *dha* regulon was inhibited in *Kp* Δ*tpiA*. It had been reported that the expression of the *dha* regulon was induced by dihydroxyacetone (Forage and Lin 1982). However, a high level of dihydroxyacetone was produced by Kp Δ*tpiA-hdpA* with glucose as the carbon source, but the expression of *dha* regulon was inhibited (Shaoqi et al. 2021).

dhaK encoded dihydroxyacetone kinase I contributed little to the dihydroxyacetone phosphate formation from dihydroxyacetone in the process of 1,2-propanediol production (Fig. 4). This is consistent with the results obtained in the process of 1,3-propanediol production. Dihydroxyacetone kinase II is not only an enzyme that catalysis the dihydroxyacetone phosphate formation, and it is also involved in the regulation of the expression of the *Dha* regulon (Wei et al. 2014). The mechanism of dihydroxyacetone kinase II in the regulation of *dha* regulon expression had been revealed in detail in *E. coli*. DhaK1 contains the dihydroxyacetone binding site, DhaK2 contains ADP as a cofactor for the double displacement of phosphate from DhaK3 to dihydroxyacetone, and DhaK3 provides a phospho-histidine relay between the PEP sugar phosphotransferase system (PTS) and DhaK2: ADP. DhaR is a transcription activator of the *dha* regulon. In the presence of dihydroxyacetone, DhaK2 is dephosphorylated and DhaK2: ADP displaces DhaK1 and stimulates DhaR activity. In the absence of dihydroxyacetone, DhaK2: ADP is converted by the PTS to DhaK2: ATP, which does not bind to DhaR (Bächler et al. 2005). Dihydroxyacetone kinase II has the same physical function in *K. pneumoniae* (Wei et al. 2014). A low level of 1,3-propanediol was produced by *Kp* Δ*tpiA*-Δ*dhaK1* and *Kp* Δ*tpiA*-Δ*DHAK* (Fig. 4) indicated the expression of *dha* regulon was slightly recovered by deletion of *dhaK1*. The synthesis of 1,2-propanediol by these strains was blocked, but dihydroxyacetone was accumulated in the broth (Fig. 4). This suggests that the dihydroxyacetone phosphate levels in the cell were decreased compared to that of *Kp* Δ*tpiA*. Thus, dihydroxyacetone phosphate might be a signaling molecule that inhibited the expression of *dha* regulon in *Kp* Δ*tpiA*. The detailed mechanism of this inhibition needs further investigation.

Key enzymes responsible for the 1,2-propanediol synthesis

Glycerol dehydrogenases encoded by *dhaD* or *gldA* are both key enzymes responsible for the conversion of glycerol to dihydroxyacetone. However, the two enzymes' roles in 1,2-propanediol synthesis were different. The activity of GldA was important for cell growth and glycerol consumption, and the activity of DhaD was important for 1,2-propanediol synthesis. Besides DhaD and GldA, no other isoenzymes were responsible for the conversion of glycerol to dihydroxyacetone. The native expression levels of the two enzymes were enough for 1,2-propanediol synthesis. Excessive expression of the two glycerol dehydrogenase resulted in dihydroxyacetone accumulation, which had a negative effect on 1,2-propanediol production. In the first report on the biological route of 1,2-propanediol production, *E. coli gldA* or *K. pneumoniae dhaD* was used in the pathway constructed (Altaras and Cameron 1999).

Individual overexpression of *mgsA*, *yqhD*, and *fucO* all had positive effects on 1,2-propanediol production, but only the combined expression of *mgsA* and *yqhD* showed additional positive effects.

YqhD was an NADPH-dependent aldehyde reductase, and the substrates of this enzyme were broad. This enzyme catalyzes the conversion of glycolaldehyde to ethylene glycol in *E. coli* and *Enterobacter cloacae* (Liu et al. 2013) (Zhang et al. 2020). This enzyme catalyzes 1,3-propanediol formation from 3-hydroxypropionaldehyde (Nakamura and Whited 2003). FucO also catalyzes the ethylene glycol formation from glycolaldehyde in *E. coli*, but this enzyme uses NADH as a cofactor (Wang et al. 2018). The substrates of YqhD and FucO were overlapped and the cofactors used by the two enzymes were different. This could be the reason why the combined overexpression of the two enzymes led to poor results.

1,2-Propanediol catabolism in *K. pneumoniae*

1,2-Propanediol is neither a building block nor an energy storage chemical of cells. In nature, 1,2-propanediol is expected to be present in the intestinal environment, and the degradation of 1,2-propanediol is important for enteric pathogenesis. The 1,2-propanediol catabolism pathway was investigated in detail in *Salmonella enterica*. This pathway initiates with the conversion of 1,2-propanediol to propionaldehyde by an AdoCbl-dependent diol dehydratase. Subsequently, propionaldehyde is converted to propionyl-CoA or 1-propanol by aldehyde dehydrogenase or alcohol dehydrogenase, respectively. The genes required for 1,2-propanediol degradation cluster at the *pdu* locus on the chromosome of *S. enterica* (Jeter and R. 1990). The expression of *pdu* operon was induced by the presence of 1,2-propanediol, but not other carbon sources (Bobik et al. 1999). *pdu* operon was also found in the genome of *K. pneumoniae*. 1,2-Propanediol produced in fed-batch fermentation reached 9.3 g/L after 144 h of cultivation, further increase culture time had no help to increase its level. At the same time, 1-propanol was produced. This indicated the activity of 1,2-propanediol catabolism exceeds its synthesis, and more 1,2-propanediol was catabolized to form 1-propanol or propionyl-CoA. *E. coli* didn't contain the 1,2-propanediol catabolism pathway. Thus 1,2-propanediol produced by engineered *E. coli* will not use by the cell. If introducing the 1,2-propanediol catabolism pathway to the 1,2-propanediol producer of *E. coli*, 1-propanol was produced (Jain et al. 2015). Similarly, introducing the 1,2-propanediol catabolism pathway of *Klebsiella oxytoca* to *C. glutamicum*, 1-propanol was produced by that 1,2-propanediol producer (Siebert and Wendisch 2015).

With the development of synthetic biology, many artificial pathways were constructed in *E. coli* and other microorganisms for 1,2-propanediol and other chemicals production. However, silent endogenous 1,2-propanediol synthesis pathways exist in some bacteria. This silent pathway was awakened in *K. pneumoniae* in this study. Other microorganisms that contain these genes of artificial 1,2-propanediol

synthesis pathways might also be suitable as hosts for 1,2-propanediol producing strains construction followed this strategy.

Authors' contributions

JH designed this study. SS, LS, XL, and QH conducted the research. SS, MT, CZ, JS, FB, GL, and JH analyzed the data. SS and JH wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and material: The authors declare that the data supporting the findings of this study are available within the article and its supplementary information files.

Compliance with Ethical Standards: This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of Interest

Authors declare that they have no conflict of interest.

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Figure captions

Fig. 1 Pathways of 1,2-propanediol synthesis from glycerol in *K. pneumoniae*.

DHA: Dihydroxyacetone; DHAP: Dihydroxyacetone phosphate

Fig. 2. The cell growth, glycerol depletion and products formation during *K. pneumoniae* strains cultivation in shake flasks with glycerol as a carbon source.

Fig. 3. The cell growth, glycerol depletion and products formation during *K. pneumoniae* strains knocked out or overexpression of encoding genes of glycerol dehydrogenase.

Fig. 4. The cell growth and products formation of *K. pneumoniae* strains that knocked out encoding genes of dihydroxyacetone kinases.

Fig. 5. The cell growth and product formation of *K. pneumoniae* strains that overexpression of *mgsA*, *yqhD*, and *fucO*.

Fig. 6. The cell growth and metabolites production of *Kp* Δ*tpiA-mgsA-yqhD* in the batch culture at different pH values in 5L bioreactors. The air flow rate was set at 2 L/min and the stirring rate was operated at 350 rpm.

Fig. 7. The cell growth and metabolites production of *Kp* Δ*tpiA-mgsA-yqhD* in the batch culture at different agitation rates in 5L bioreactors operated at pH 7.0.

Figure 8. The cell growth and metabolites production of *Kp* Δ*tpiA-mgsA-yqhD* in the fed-batch culture at pH 7.0 values in a 5L bioreactor operated at 350 rpm. A: cell density; B: Glycerol; C: 1,2-propanediol; D: acetic acid; E: 1,3-propanediol; F: n-propanol.

Tables

Table 1 Strains and plasmids

Strains or plasmids	Relevant genotype and description	Reference
E.coli DH5α	Host of plasmid	Lab stock
K.pneumoniae CGMCC 1.6366	TUAC01 Wild type, Amp^r	(Hao et al. 2008)
Δ*tpiA*	Δ*tpiA*, Apr^r	This work
Δ*tpiA*-Δ*dhaK*	Δ*tpiA*, Δ*dhak*, Apr^r	This work
Δ*tpiA*-Δ*dhaK1*	Δ*tpiA*, Δ*dhak1*, Apr^r	This work
Δ*tpiA*-Δ*dhaK2*	Δ*tpiA*, Δ*dhak2*,Apr^r	This work
Δ*tpiA*-Δ*dhaK3*	Δ*tpiA*, Δ*dhak3*, Apr^r	This work
Δ*tpiA*-Δ*DHAK*	Δ*tpiA*, Δ*dhak*, Δ*dhak123*, Apr^r	This work
Δ*tpiA*-Δ*pta acK*	Δ*tpiA*, Δ*pta*, Δ*ackA*, Apr^r, Str^r	This work
Δ*tpiA*-Δ*dhaD*	Δ*tpiA*, Δ*dhaD*, Apr^r	This work
Δ*tpiA*-Δ*gldA*	Δ*tpiA*,Δ*dhaD*, Apr^r	This work
Δ*tpiA*-Δ*gldA*-Δ*dhaD*	Δ*tpiA*,Δ*gldA*, Δ*dhaD*, Apr^r	This work
Δ*tpiA*-gldA	Δ*tpiA*, Kan^r, carries the *gldA* gene	This work
Δ*tpiA*-dhaD	Δ*tpiA*, Kan^r, carries the *dhaD* gene	This work
Δ*tpiA*-mgsA	Δ*tpiA*, Kan^r, carries the *mgsA* gene	This work
Δ*tpiA*-yqhD	Δ*tpiA*, Kan^r, carries the *yqhD* gene	This work
Δ*tpiA*-fucO	Δ*tpiA*, Kan^r, carries the *fucO* gene	This work
Δ*tpiA*-mgsA-yqhD	Δ*tpiA*, Kan^r, carries the *mgsA* and *yqhD* gene	This work
Δ*tpiA*-mgsA-fucO	Δ*tpiA*, Kan^r, carries the *mgsA* and *fucO* gene	This work
Δ*tpiA*-yqhD-fucO	Δ*tpiA*, Kan^r, carries the *yqhD* and *fucO* gene	This work
Δ*tpiA*-mgsA-yqhD-fucO	Δ*tpiA*, Kan^r, carries the *mgsA*, *yqhD* and *fucO* gene	This work
Plasmids		
pIJ773	Apr^r, *aac(3)IV* with FRT sites, 4334 bp	(Gust et al. 2003)
pIJ778	Str^r, *aadA* with FRT sites, 4337 bp	(Gust et al. 2003)
pIJ790	Cm^r, encodes λ-Red genes (gam, bet, exo), 6084 bp	(Gust et al. 2003)
pDK6	Kan^r, $lacI^Q$, tac, 5118bp	(Kleiner et al. 1988)
pDK6-red	Kan^r, carries λ-Red genes (gam, bet, exo), 7.1 kb	(Wei et al. 2012)
pDK6-flp	Kan^r, carries the yeast FLP recombinase gene, 6.3 kb	(Wei et al. 2012)
pDK6-mgsA	Kan^r, carries the *mgsA* gene, 5532 bp	This work
pDK6-yqhD	Kan^r, carries the *yqhD* gene, 6237 bp	This work
pDK6-fucO	Kan^r, carries the *fucO* gene, 6222 bp	This work
pDK6-gldA	Kan^r, carries the *gldA* gene, 6177 bp	This work
pDK6-dhaD	Kan^r, carries the *dhaD* gene, 6171bp	This work

pDK6-mgsA-yqhD	Kan^r, carries the *mgsA* and *yqhD* gene, 6798 bp	This work
pDK6-mgsA-fucO	Kan^r, carries the *mgsA* and *yqhD* gene, 6783 bp	This work
pDK6-yqhD-fucO	Kan^r, carries the *mgsA* and *yqhD* gene, 7488 bp	This work
pDK6-mgsA-yqhD-fucO	Kan^r, carries the *mgsA* and *yqhD* gene, 8049 bp	This work
pMD18-T-simple	Amp^r, TA cloning vector, 2692 bp	Takara
pMD18-T-Δ*tpiA*	Amp^r, Apr^r, carries part of *tpiA*, 5.3kb	This work
pMD18-T-Δ*ubiC*	Amp^r, Str^r, carries part of *ubiC*,5.3 kb	This work
pMD18-T-Δ*pta-ack*	Amp^r, Str^r, carries part of *pta* and *ackA*, 5.3 kb	This work
pMD18-T-Δ*gldA*	Amp^r, Apr^r, carries part of *gldA*, 5.3 kb	This work
pMD18-T-Δ*dhaD*	Amp^r, Str^r, carries part of *dhaD*, 5.3 kb	This work

Figures

Figure 1

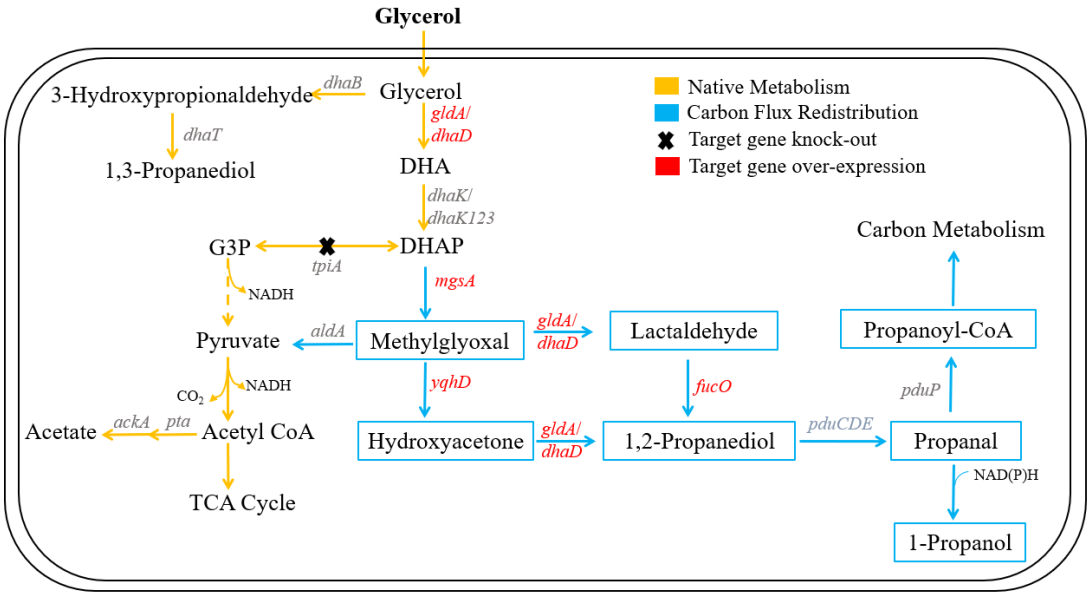

Figure 2

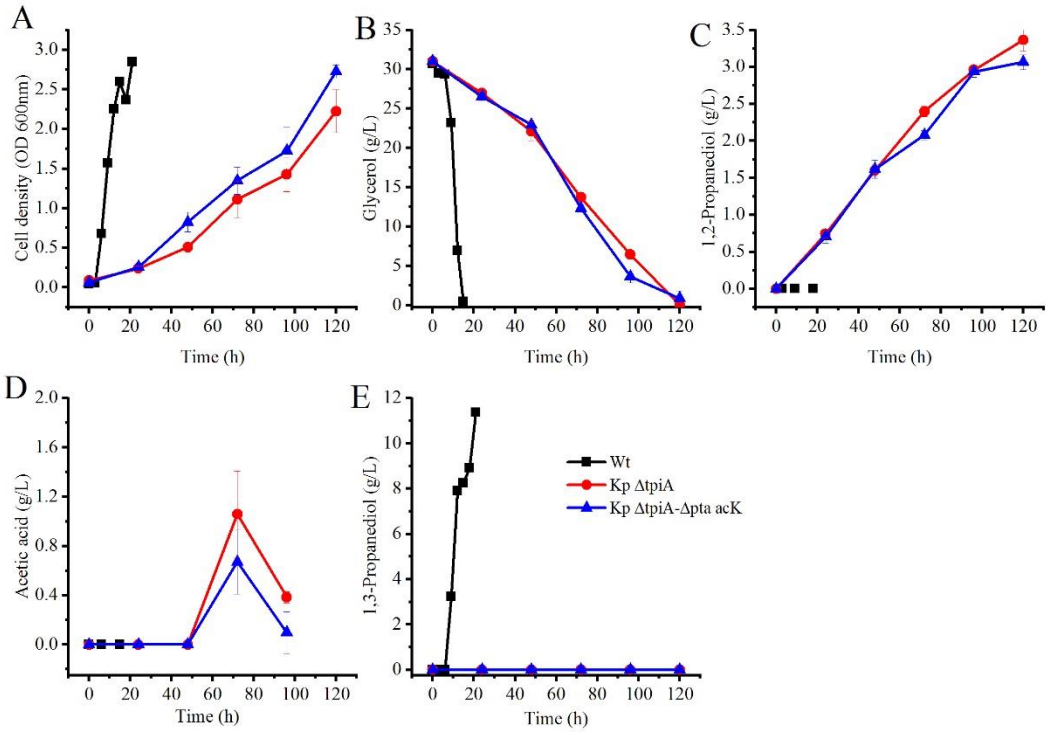

Figure 3

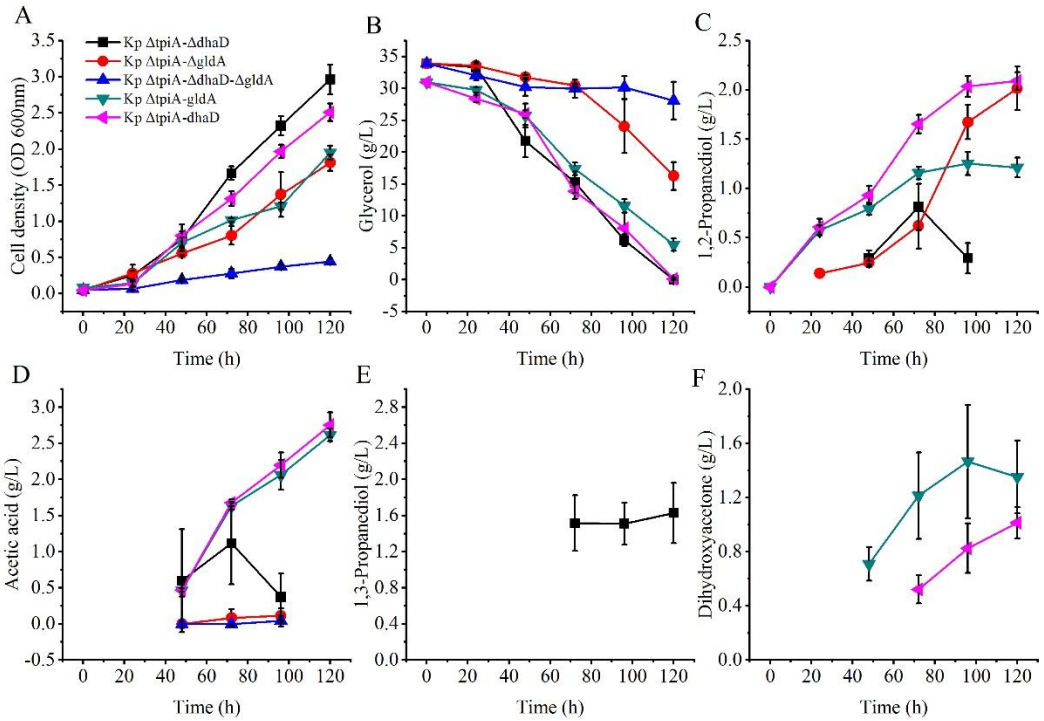

Figure 4

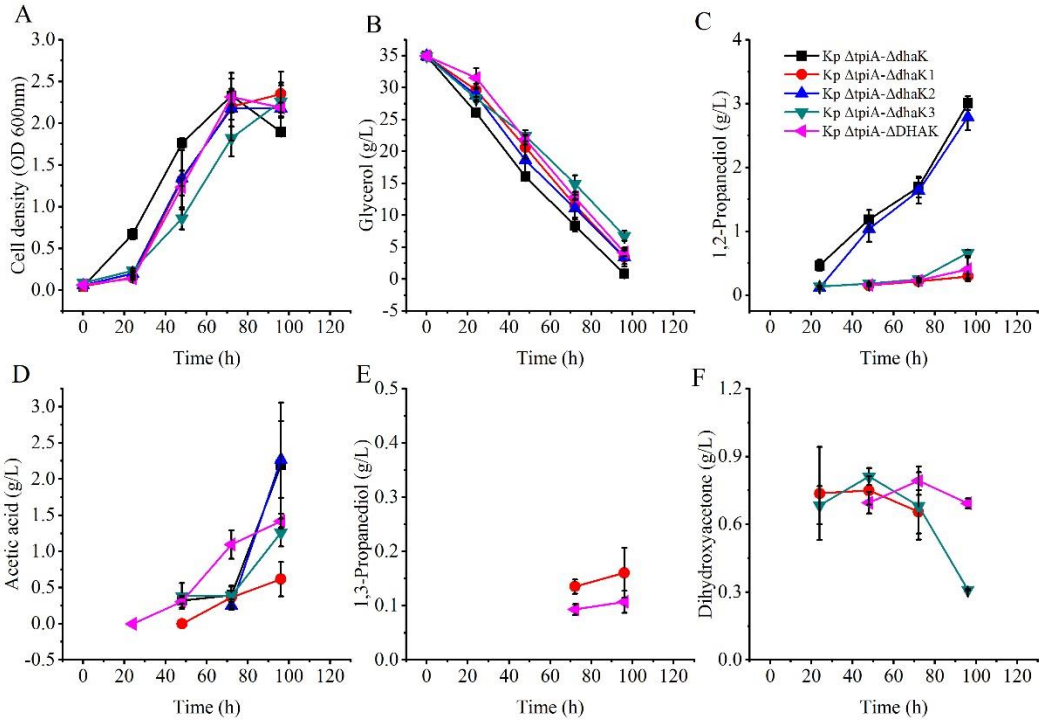

Figure 5

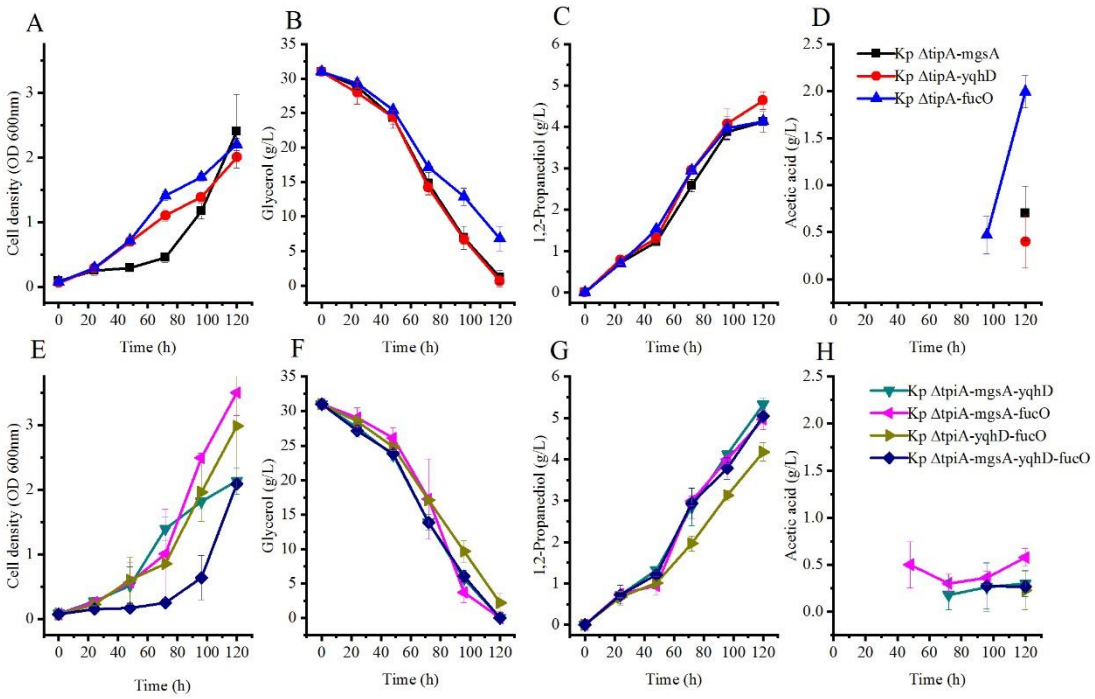

Figure 6

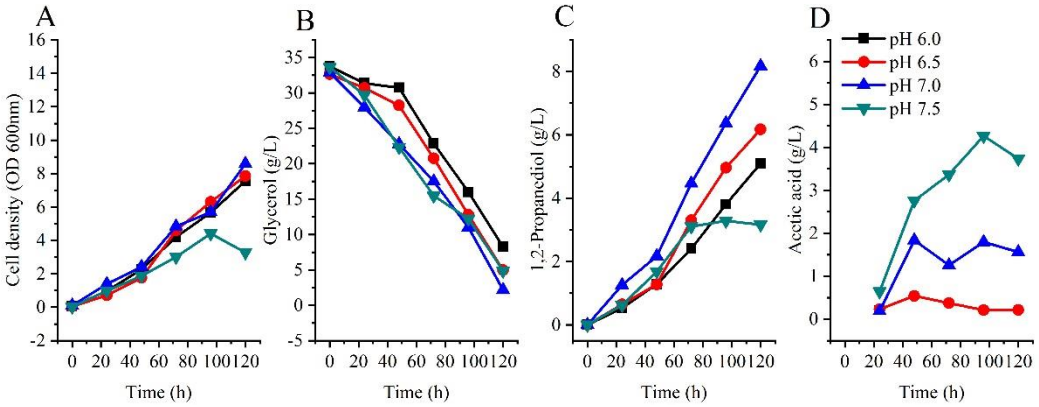

Figure 7

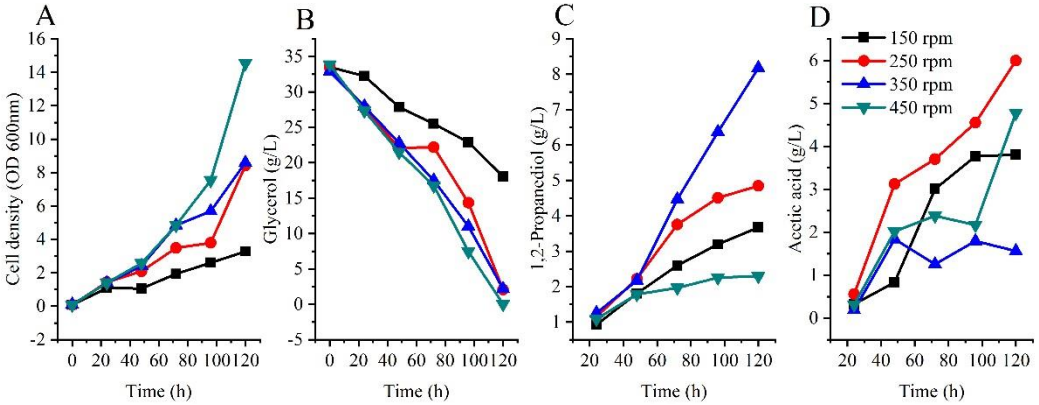

Figure 8