2,3-Dihydroxyisovalerate production by *Klebsiella pneumoniae*

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Abstract

2,3-Dihydroxyisovalerate is an intermediate of valine and leucine biosynthesis pathway, however, no natural microorganism has been found yet that can accumulate this compound. *Klebsiella pneumoniae* is a useful bacterium that can be used as a workhorse for the production of a range of industrially desirable chemicals. Dihydroxy acid dehydratase, encoded by the *ilvD* gene, catalyses the reaction of 2-ketoisovalerate formation from 2,3-dihydroxyisovalerate. In this study, an *ilvD* disrupted strain was constructed which resulted in the inability to synthesize 2-ketoisovalerate, yet accumulate 2,3-dihydroxyisovalerate in its culture broth. 2,3-Butanediol is the main metabolite of *K. pneumoniae* and its synthesis pathway and the branched-chain amino acid synthesis pathway share the same step of the α-acetolactate synthesis. By knocking out the *budA* gene, carbon flow into the branched-chain amino acid synthesis pathway was upregulated which resulted in a distinct increase in 2,3-dihydroxyisovalerate levels. Lactic acid was identified as a by-product of the process and by blocking the lactic acid synthesis pathway a further increase in 2,3-dihydroxyisovalerate levels was obtained. The culture parameters of 2,3-dihydroxyisovalerate fermentation were optimized, which include acidic pH and medium level oxygen supplementation to favor 2,3-dihydroxyisovalerate synthesis. At optimal conditions (pH 6.5, 400 rpm), 36.5 g/L of 2,3-dihydroxyisovalerate was produced in fed-batch fermentation over 45 hours, with a conversion ratio of 0.49 mol/mol glucose. Thus a biological route of 2,3-dihydroxyisovalerate production with high conversion ratio and final titer was developed; providing a basis for an industrial process.

Key words: 2,3-Dihydroxyisovalerate; 2,3-Butanediol; 2-Ketoisovalerate; *Klebsiella pneumoniae*; *ilvD*

Introduction

Klebsiella pneumoniae is a Gram-negative bacterium that is ubiquitous in the natural environment and on mucosal surfaces in mammals. It is also a bacterium of industrial importance as it is used for the production of numerous industrial chemicals. When this bacterium is cultured with glycerol as the main carbon source, 1,3-propanediol is the main metabolite and this process has a high conversion ratio and a high productivity (Hao et al. 2008). 1,3-Propanediol can be used to synthesize polytrimethylene terephthalate (PTT) and other industrial polyester fibers. This has resulted in the commercialization of 1,3-propanediol production in China using *K. pneumoniae.* When *K. pneumoniae* is grown on other carbon sources such as glucose, the main metabolite is 2,3-butanediol (Chen et al. 2014). Acetoin is the metabolic precursor of 2,3-butanediol and high levels of acetoin have been produced in a strain (*K. pneumoniae* Δ*budC)* in which the butanediol dehydrogenase gene has been inactivated (Wang et al. 2015). Acetoin is synthesized from α-acetolactate, and this reaction is catalysed by α-acetolactate decarboxylase. In another study, *K. pneumoniae* Δ*budA*, the α-acetolactate decarboxylase inactivated strain, produced 2-ketoisovalerate and isobutanol at neutral pH conditions (Gu et al. 2017). The 2-ketoisovalerate and isobutanol synthesis pathway, the 2,3-butanediol synthesis pathway and the branched-chain amino acid synthesis pathway share some common steps in which pyruvate, a principal metabolite of the cell, is their main precursor compound. Two molecules of pyruvate condense to synthesize one molecule of acetolactate and this reaction is catalysed by isoenzymes of acetohydroxy acid synthase and α-acetolactate synthase. 2,3-Dihydroxyisovalerate is synthesized from α-acetolactate and this reaction is catalysed by the acetohydroxy acid isomeroreductase. 2,3-Dihydroxyisovalerate dehydrates to form 2-ketoisovalerate and the latter is further converted to valine and leucine (Fig. 1). α-Acetolactate synthase and α-acetolactate decarboxylase are encoded by the *budB* and *budA* genes, and they are located in the *bud* operon in *K. pneumoniae* (Blomqvist et al. 1993). *ilvBN*, *ilvGM*, *ilvIH* encode acetohydroxy acid synthase I, II and III. *ilvC, ilvD* and *ilvE* encoding acetohydroxy acid isomeroreductase, dihydroxy acid dehydratase and transaminase. *ilvGMED* and threonine dehydratase encoding gene *ilvA* forms a single operon, while *ilvBN*, *ilvIH*, and *ilvC* are independent operons in *Escherichia coli* and *K. pneumoniae* (Lawther et al. 1987, Gu et al. 2017).

2,3-Dihydroxyisovalerate is an intermediate of the valine and leucine synthesis pathway, however, there are no reports which confirm that this chemical can be produced naturally by microorganisms. An engineered *Saccharomyces cerevisiae* strain which was used for isobutanol production secreted 0.3 g/L of 2,3-dihydroxyisovalerate under microaerobic fermentation conditions (Generoso et al. 2017). Racemic

sodium salt of 2,3-dihydroxyisovalerate can be synthesized via a chemical approach (Cioffi et al. 1980). This was a laboratory method for 2,3-dihydroxyisovalerate preparation and the product was used to detect the activity of dihydroxyisovalerate dehydratase. It has been reported to be in the R-form in The Human Metabolome Database, and its main biological role being a nutrient, energy storage, energy source, and membrane stabilizer (http://www.hmdb.ca/metabolites/HMDB0012141). The specific chiral structure of (R)-2,3-dihydroxyisovalerate is highly attractive for modification which can lead to the formation of compounds of interest in the pharmaceutical industry. In particular, (R)-2,3-dihydroxyisovalerate is an intermediate of azinomycins synthesis which is known to exhibit potent antitumor activities (Bryant et al. 1998). Like lactic acid, 2,3-dihydroxyisovalerate has hydroxyl groups and a carboxyl group, which might allow possible applications in polymers synthesis. Currently, 2,3-dihydroxyisovalerate is not available in large quantity, especially the optical isomer, which limits its application. The biological route of 2,3-dihydroxyisovalerate production might provide an efficient way for the large scale production of this chemical, and enables its application in industry.

Here, we show for the first time 2,3-dihydroxyisovalerate synthesis by mutants of *K. pneumoniae* and partial optimization of the production process with regard to culture pH and agitation using a triple deletion mutant.

Materials 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 2.

Construction of mutants of *K. pneumoniae*

For mutant constructions, *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).

K. pneumoniae Δ*ilvD* construction

Mutants were generated by following the methodology outlined by Wei et al (2012). The *ilvD* gene in *K. pneumoniae* and flanking sequences were amplified by PCR using the primer pair ilvD-s and ilvD-a. The PCR product was ligated into the pMD18-T vector to generate pMD18-T-ilvD. A linear DNA with 39 and 40 nt homologous extensions flanking apramycin resistance gene *aac(3)IV* was amplified with plasmid pIJ773 as the template using the primer pair ilvD-FRT-s/ilvD-FRT-a. pMD18-T-ΔilvD was

constructed by replacing *ilvD* in the plasmid pMD18-T-ilvD with the *aac(3)IV* cassette using the Red recombination system in *E. coli*.

pMD18-T-ΔilvD was used as the template for PCR of a linear DNA containing the apramycin resistance gene *aac(3)IV* with 500 bp of *ilvD* homologous regions on both sides. The primer pair used was ilvD-a/ilvD-s. The linear DNA was transformed into *K. pneumoniae* CGMCC 1.6366, which already hosted the plasmid pDK6-red. Homologous recombination between the linear DNA and the chromosome was facilitated by Red recombinase and led to ilvD deletion in the genome of *K. pneumoniae* to obtain *K. pneumoniae* Δ*ilvD*.

K. pneumoniae Δ*budA*-Δ*ilvD* construction

K. pneumoniae Δ*budA*-Δ*ilvD* was constructed following the same way of *K. pneumoniae* Δ*ilvD* construction, with *K. pneumoniae* Δ*budA* replacing wild-type *K. pneumoniae* as the target strain.

2,3-Dihydroxyisovalerate purification and structure confirmation

2,3-Dihydroxyisovalerate produced by *K. pneumoniae* Δ*budA*-Δ*ldhA*-Δ*ilvD* was purified from the fermentation broth and its structure was confirmed by nuclear magnetic resonance (NMR) spectroscopic analysis. The cell-free broth of *K. pneumoniae* Δ*budA*-Δ*ldhA*-Δ*ilvD* was discolored by activated carbon. The discolored broth was subsequently passed through an anion exchange (D311) column, which had been treated with 1 mol/L NaOH. The column was rinsed with water to neutral pH and gradient eluted with NH_3 solution (0-1 mol/L). Eluate fractions that contained the target compound were concentrated using a rotary evaporator. Crystals obtained were used for ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopic analysis. A Bruker spectrometer was used and chemical shift values are reported in ppm (δ).

Medium and culture conditions

The fermentation medium contained 30-50 g/L glucose, 5 g/L yeast extract, 4 g/L corn steep liquor, 5 g/L $(NH_4)_2SO_4$, 3 g/L sodium acetate, 0.4 g/L KCl, 0.1 g/L $MgSO_4$, 0.02 g/L $FeSO_4$, and 0.01 g/L $MnSO_4$. Corn steep liquor free fermentation medium is a modified fermentation medium without corn steep liquor and the concentration of yeast extract is 1.5 g/L.

A single colony grown on plate was selected to inoculate into 50 ml of LB medium and grown for 12 hours at a rotary shaker at 37°C and 200 rpm overnight. Then 1 ml of seed culture was inoculated into 250 mL flasks containing 50 mL of fermentation medium for flask culture.

M9 minimal medium with different carbon sources (4 g/L) were used for carbon sources utilization experiments.

Fermentation parameters focusing on culture pH and agitation rate optimization experiments were performed in stirred tank bioreactors operated in batch mode. 50 ml of seed culture was inoculated into 5-L bioreactor (BIOSTAT-A plus Sartorius) with a working volume of 3 L and the air flow rate of 2 L/min. The culture pH was automatically controlled by 2M NaOH solution.

Fed-batch cultures were performed at optimized conditions, with culture pH 6.5, culture temperature 37°C and stirring rate of 400 rpm. When the glucose level in the broth decreased to 10 g/L, 200 mL of 600 g/L glucose solution was added batch wise.

Analytical methods

The biomass titer at set time intervals was determined by optical density (OD600) with a spectrophotometer. Cell dry weight (CDW) per liter of fermentation broth was calculated from OD values using the following formula: CDW (g/l)=0.387×OD+0.334.

Chemical compounds in the broth were quantified by a Shimadzu 20AVP high performance liquid chromatograph system (HPLC) equipped with a RID-20A refractive index detector and a SPD-M20A photodiode array 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 0.025 mol/L H_2SO_4 solution (pH 1.6) using a flow rate of 0.8 ml/min.

Carbon balance

For carbon balance calculations, the elemental composition of *K. pneumoniae* was assumed to be $CH_{1.73}O_{0.43}N_{0.24}$ (Esener et al. 1982). Carbon recovery was calculated as percentage of total product carbon divided by total substrate carbon at the time point where the highest 2,3-dihydroxyisovalerate titer was reached.

Results

2,3-Dihydroxyisovalerate purification and structure validation

The *ilvD* gene which encodes dihydroxy acid dehydratase catalysed the reaction of 2-ketoisovalerate formation from 2,3-dihydroxyisovalerate. *K. pneumoniae* Δ*budA*-Δ*ldhA*-Δ*ilvD* was constructed in our previous research of 2-ketoisovalerate and isobutanol production, and 2-ketoisovalerate and isobutanol synthesis was blocked in this strain (Gu et al. 2017). We suspected 2,3-dihydroxyisovalerate might have accumulated in the broth of this strain during fermentation and the potential fraction was purified from the broth. The fermentation broth of *K. pneumoniae* Δ*budA*-Δ*ldhA*-Δ*ilvD* contained a range of organic acids such as succinic acid and acetic acid. The potential fraction in the fermentation broth was purified

by ion-exchange chromatography. ^{1}H and ^{13}C NMR spectral data of the sample are given in Table 3.

The ^{1}H NMR data correlate with the report of the chemical synthesized racemic sodium salt of 2,3-dihydroxyisovalerate (Cioffi et al. 1980) and reports from urine samples from patients (Holmes et al. 1997). ^{13}C NMR data of 2,3-dihydroxyisovalerate were not found in other reports. The predicted ^{13}C NMR shift by ChemDraw was 173.2, 86.3, 70.9, and 23.1 for C1, C2, C3, and C4, C5. Thus, the structure of 2,3-dihydroxyisovalerate produced by *K. pneumoniae* Δ*budA*-Δ*ldhA*-Δ*ilvD* was confirmed.

2,3-Dihydroxyisovalerate production by mutants of *K. pneumoniae*

To investigate the mechanism of 2,3-dihydroxyisovalerate accumulation, *K. pneumoniae* Δ*ilvD* and *K. pneumoniae* Δ*budA*-Δ*ilvD* were constructed. These two strains and the wild-type *K. pneumoniae, K. pneumoniae* Δ*budA* and *K. pneumoniae* Δ*budA*-Δ*ldhA*-Δ*ilvD* were cultured in shake flasks with the corn steep liquor free fermentation medium and the results are shown in Fig. 2.

The cell growth of wild-type strain, *K. pneumoniae* Δ*budA* and *K. pneumoniae* Δ*ilvD* were very similar during the first 6 hours of cultivation. After that time, the wild-type strain continued to grow at a fast rate until all the glucose was consumed reaching a final cell density of 8.6±0.9 OD units after 10 hours. By contrast, growth of the *K. pneumoniae* Δ*ilvD* mutant ended after about 8 hours reaching a final cell density of 3.6±0.2 OD units. After that point, the cell density decreased slightly though the cells still consumed glucose until the end of the cultivation. The *K. pneumoniae* Δ*budA* mutant reached a cell density of 5.8±0.3 OD units after 12 hours when most of the glucose was consumed. From that point the cell density decreased until it reached a final value of about 4.1±0.01 OD units after 20 hours. In contrast to the single mutants, growth of the *K. pneumoniae* Δ*budA*-Δ*ilvD* and *K. pneumoniae* Δ*budA*-Δ*ldhA*-Δ*ilvD* mutants was very weak, and the final cell densities were only 1.5±0.2 OD and 0.5±0.1 OD units, respectively. Despite, the slow growth both mutants consumed most of the glucose by end of the cultivation. The glucose consumption of all strains coincided with cell growth and was similar for the first 6 hours (Fig. 2A). After the initial growth period clear differences in glucose utilization could be seen with the wild-type strain exhibiting the highest rate of consumption followed by *K. pneumoniae* Δ*budA*, *K. pneumoniae* Δ*ilvD*, *K. pneumoniae* Δ*budA*-Δ*ilvD,* and *K. pneumoniae* Δ*budA*-Δ*ldhA*-Δ*ilvD* that had the lowest glucose consumption rate. 2,3-Butanediol was the main metabolite of the wild-type strain yielding 5.1 g/L. Yet although the 2,3-butanediol production was lower for the *K. pneumoniae* Δ*ilvD* mutant with a titer of 1.3 g/L, it was still the main metabolite of this strain. Acetic acid was produced by all strains and *K. pneumoniae* Δ*budA* had the highest titer of 2.5 g/L after 15 hours. The others mutants reached similar levels of about 2 g/L at the end of the cultivation. Lactic acid was the

major product of the *K. pneumoniae* Δ*budA* mutant with levels increasing to 2.2 g/L after 15 hours. However, only 0.3 g/L of lactic acid was produced by *K. pneumoniae* Δ*budA*-Δ*ilvD* and no lactic acid was detected in the broth of *K. pneumoniae* Δ*budA*-Δ*ldhA*-Δ*ilvD*. In this mutant, the lactic acid synthesis pathway was blocked by disruption of the *ldhA* gene, which encodes a lactate dehydrogenase. As expected no 2,3-Butanediol was synthesized by *K. pneumoniae* Δ*budA*-Δ*ilvD* because the 2,3-butanediol synthesis pathway was blocked by disruption of *budA*. 2,3-dihydroxyisovalerate produced by this double deletion mutant was 3.3 g/L and the titer was further increased to 3.7 g/L by *K. pneumoniae* Δ*budA*-Δ*ldhA*-Δ*ilvD* at the end of the cultivation. These titers were much greater than the 0.2 g/L produced by *K. pneumoniae* Δ*ilvD*. *K. pneumoniae* Δ*budA* produced 1.6 g/L of 2,3-dihydroxyisovalerate after 17 hours of cultivation, which was comparable to that produced by *K. pneumoniae* Δ*budA*-Δ*ilvD* at the same time. However, after the glucose was exhausted, the accumulated 2,3-dihydroxyisovalerate was reused by the *K. pneumoniae* Δ*budA* cells. Due to the high production of 2,3-dihydroxyisovalerate coupled with the lowest by-product formation only *K. pneumoniae* Δ*budA*-Δ*ldhA*-Δ*ilvD* was further characterized.

2,3-Dihydroxyisovalerate production using different carbon sources

K. pneumoniae is a bacterium that is capable of utilizing a range of different carbon sources such as glucose, xylose and glycerol for 2,3-butanediol and other chemicals production. Thus these were used as the sole carbon source in minimal medium to evaluate their utilization for the 2,3-dihydroxyisovalerate production by *K. pneumoniae* Δ*budA*-Δ*ldhA*-Δ*ilvD* (Fig. 3).

After 90 hours of cultivation, the cells consumed about 3 g/L of these three carbon sources considering the slightly different starting concentrations. Final 2,3-Dihydroxyisovalerate titers of 1.0, 1.0, and 0.7 g/L using glucose, xylose or glycerol as carbon sources, respectively were obtained. In addition, 0.025 g/L of 1,3-propanediol was produced with glycerol as the carbon source.

Cell growth in minimal medium with either glucose, xylose, or glycerol as the sole carbon source was very weak, with all cell densities obtained lower than 0.3 OD units (data not shown). The likely reason for the poor growth was the lack of branched-chain amino acids in the medium. To further investigate carbon source utilization, a complex nutrient-rich medium containing corn steep liquor was used. *K. pneumoniae* Δ*budA*-Δ*ldhA*-Δ*ilvD* was cultured in this fermentation medium with either glucose, xylose or glycerol as the main carbon source, and results are shown in Fig. 4.

The cell growth was similar for the first 10 hours of cultivation on all three carbon sources tested. Cells on xylose continued to grow at a fast rate reaching a final OD of 4.3 after 23 hours of cultivation, while consuming about 68% (23 g/L) of the initial xylose concentration. Cells on glucose grow slightly

slower reaching an OD of 2.7 after 23 hours at which point all the glucose supplied was consumed. Whereas the cells grown on glycerol only reached an OD of 1.6 after 17 hours of cultivation when the glycerol ran out, and 9.9 g/L of 1,3-propanediol was produced. The 2,3-dihydroxyisovalerate synthesis was at a similar rate using these carbon sources leading to 7.4 g/L, 7.0 g/L, and 4.6 g/L of 2,3-dihydroxyisovalerate being produced, with the conversion ratio of 0.25, 0.32, and 0.16 (g/g) using glucose, xylose and glycerol, respectively. Thus both glucose and xylose are suitable carbon sources for 2,3-dihydroxyisovalerate production. Considering the lower cost of glucose compared to xylose, glucose was selected as carbon source for further studies.

Fermentation parameters optimization

K. pneumoniae has been used as producer for many chemicals production requiring different process conditions with regard to oxygen levels and culture pH. Here culture pH and agitation rates for 2,3-dihydroxyisovalerate production were optimized separately.

Culture pH optimization.

Based on the shake flasks data *K. pneumoniae* Δ*budA*-Δ*ldhA*-Δ*ilvD* was cultured in 5 L bioreactors with fermentation medium using glucose as the main carbon source, the stirring rate was keep at 300 rpm and the air flow rate was set at 2L/min, where the culture pH was controlled at 6.0, 6.5, 7.0, 7.5, and 8.0. Fermentation results are presented in Fig. 5.

When cultured at pH 6.0, the cell growth and glucose consumption rates were the slowest amongst all the experimental pH ranges investigated. However, 2,3-dihydroxyisovalerate production was the highest at this condition, obtaining a titer of 15.9 g/L after 27 hours. The cell growth, glucose consumption rate, and 2,3-dihydroxyisovalerate production at the culture of pH 6.5 followed a similar pattern of the cells cultured at pH 6.0, with 14.7 g/L of 2,3-dihydroxyisovalerate being produced after 18 hours. Cell growth rates and glucose consumption rates at the culture pH 7.0-8.0 were similar. Based on these results it appears that 2,3-dihydroxyisovalerate production was inversely related to the culture pH. The 2,3-dihydroxyisovalerate level decreased to 8.2 g/L at the condition of culture pH 8.0.

The organic acid by-products generated by the cells, which included succinic acid, acetic acid, and formic acid, all appeared to have a positive correlation with the culture pH. The higher the pH culture, the more organic acids were produced. 3.5 g/L of succinic acid, 8.9 g/L of acetic acid, and 9.5 g/L of formic acid were produced at the culture pH of 8.0. By contrast, much less organic acids were produced by the cells grown at pH 6.0 i.e. 1.8 g/L, 0.4 g/L, and 0.5 g/L, respectively. Ethanol was another by-product of these cells, where a maximum ethanol titer of about 4.5 g/L was produced from the 6.5-7.5

pH cultures, whilst the lowest titers of about 2.5 g/L were obtained from cells grown at 6.0 and 8.0. Based on the production of metabolites and biomass, the carbon balance calculated for culture under pH 6.0, pH 6.5, pH 7.0, pH 7.5, and pH 8.0 are 73%, 75%, 81%, 81% and 78%, respectively. Thus, most of the carbon from glucose consumed is accounted for. The missing carbon is likely to be CO_2 generated from TCA cycle and decarboxylation reactions and additionally a small amount of other chemicals that were undetectable with the analysis method used here may have been formed.

It can be concluded from Fig. 5, that acidic conditions favor 2,3-dihydroxyisovalerate synthesis, but extreme acidic conditions inhibited the cell growth and affected the productivity. Considering both the substrate conversion ratio and the productivity, pH 6.5 was selected as the optimal culture pH.

Optimization of agitation rate

K. pneumoniae Δ*budA*-Δ*ldhA*-Δ*ilvD* was cultured in 5L bioreactors with fermentation medium controlled at pH 7.0, and the air flow rate was set at 2L/min. The stirring rate of the bioreactor was set at 200, 300, 400, 600, and 800 rpm to obtain different aerobic conditions. Fermentation results are presented in Fig. 6.

Cell growth and glucose consumption appeared not to differ in these conditions. However, the amount of 2,3-dihydroxyisovalerate produced by these cultures were distinctly different. The highest 2,3-dihydroxyisovalerate titer of 18.7 g/L was produced at the stirring rate of 400 rpm, followed by stirring rate of 200 and 300 rpm generating 14.7 and 14.0 g/L of 2,3-dihydroxyisovalerate, respectively. Both growth and product formation were almost identical despite a 10% lower initial glucose concentration used in the cultivation operated at 300 rpm. Cultures grown with stirring rates of 600 and 800 rpm had the lowest 2,3-dihydroxyisovalerate production, with final titers of 10.5 and 9.3 g/L, respectively.

The amount of succinic acid, formic acid, and ethanol produced appears to have an inverse correlation with agitation rate. Using a stirring rate of 200 rpm, succinic acid, formic acid, and ethanol production was 2.4 g/L, 8.3 g/L, and 5.2 g/L, respectively. While using a stirring rate 600 and 800 rpm, these metabolites were not detected. However, 9.7 g/L and 8.7 g/L of acetic acid were produced at high agitation rates of 600 and 800 rpm, respectively. The carbon balance calculated for stirring rate of 200 rpm, 300 rpm. 400 rpm, 600 rpm and 800 rpm are 77%, 75%, 68%, 53% and 48%, respectively. The lower carbon recoveries at stirring rates of 600 and 800 rpm are likely due to increased TCA cycle activity under fully aerobic conditions generating more CO_2.

Looking at the data there is a very sharp change in metabolite production between low (200 - 400 rpm) vs. high (600-800 rpm) agitation conditions, especially for succinic acid, ethanol and formic acid

production. The increase of stirring rate from 400 rpm to 600 rpm can be considered as the change from medium to fully aerobic condition. The results suggests that the medium level of agitation (400 rpm) favors 2,3-dihydroxyisovalerate synthesis.

Fed batch fermentation

After the culture pH and agitation rate were optimized individually, fed batch fermentations were conducted to obtain a high final product level. Fermentation results are presented in Fig. 7.

In fed batch fermentation, cells exponentially grew for the first 10 hours, which resulted in a rapid depletion of glucose. Cells then reached a stationary phase and the glucose concentration in the broth dropped to 10 g/L after 12 hours. At this point a pulse of highly concentrated glucose solution was added and the glucose concentration increased to 45 g/L. After 24 hours, another glucose pulse feeding was performed. However at this stage, the cell density started to decrease, yet 2,3-dihydroxyisovalerate accumulation in the broth continued at a slow rate. Based on the production of 2,3-dihydroxyisovalerate (Fig,. 7C), the process can be divided into three phases. From 0 to 6 hours there was a slow initial production phase coinciding with onset of cell growth. Once the cells entered the exponential growth phase the productivity increased rapidly with an average value of 1.85 g/L h between 6-21 hours. After the cells entered stationary phase the productivity in the third phase (21 - 45 hours) decreased to about 0.28 g/L h. Overall, 36.5 g/L of 2,3-dihydroxyisovalerate was produced after 45 hours of cultivation. The maximum theoretical yield of 2,3-dihydroxyisovalerate synthesis from glucose is 1.0 mol/mol. The conversion ratio of 2,3-dihydroxyisovalerate from glucose was 0.58 mol/mol over the first 21 hours, and the conversion ratio of the whole process was 0.49 mol/mol. In agreement with the batch fermentation under the same conditions the main by-product was acetic acid, achieving a titer of 14.3 g/L; whereas the succinic acid formed was fully consumed by end of the process, and formic acid and ethanol were present at low concentrations, with 1.2 g/L and 0.3 g/L, respectively. The carbon recovery calculated was 76% for the whole process. This value is in agreement with the carbon recovery of the batch fermentation performed under these conditions.

Discussion

2,3-Dihydroxyisovalerate and branched-chain amino acid synthesis pathway

Branched-chain amino acids, including valine, leucine and isoleucine, are major building blocks of the cell. Their synthesis in the cell is strictly controlled by feedback inhibition and repression. The activity of IlvBN and IlvIH was inhibited by valine (Lawther et al. 1981). The *ilvBN* and *ilvGM* operons are repressed in cells grown in media containing excess branched-chain amino acids. The *ilvIH* operon is

repressed in cells growing in a medium containing leucine (Platko et al. 1990). Thus, synthesis of these amino acids will not exceed cell demand. In this study, the disruption of *ilvD* blocked the branched-chain amino acid pathway and induced 2,3-dihydroxyisovalerate accumulation in the broth (Fig. 2). The accumulation of intermediates by blocking metabolic pathways is common in bacteria. In our previous research acetoin accumulated in the broth of *K. pneumoniae*-ΔbudC, in which butanediol dehydrogenase was inactivated (Wang et al. 2015). Furthermore, gluconic acid accumulated in the broth of *K. pneumoniae*-Δ*gad*, in which gluconate dehydrogenase was inactivated (Wang et al. 2016). The 2,3-dihydroxyisovalerate accumulated by *K. pneumoniae*-Δ*ilvD* was at a low concentration which suggested that the metabolic flux of the branched-chain amino acid synthesis pathway was not strong in *K. pneumoniae*. The stereochemistry of valine synthesis pathway is known. (S)-acetolactate is the substrate of acetohydroxy acid isomeroreductase and (R)-2,3-dihydroxyisovalerate is the product (Hill and Yan. 1971; Chunduru et al. 1989; Dumas et al. 2001). Thus, 2,3-dihydroxyisovalerate obtained here was enantiomerically in R-form. The structure of this chemical was confirmed by NMR, and this optical isomer has important economic value.

Disruption of *budA* resulted in 2,3-dihydroxyisovalerate accumulation

2,3-Butanediol is the main metabolite of *K. pneumoniae*, suggesting that the carbon flux through the 2,3-butanediol synthesis pathway was very high. 2,3-Butanediol is less toxic than organic acids and 2,3-butanediol synthesized from different carbon sources prevents lethal acidification which is typically caused by the production of organic acids (Van Houdt et al. 2007). The first step of the 2,3-butanediol synthesis pathway shares the same step of the branched-chain amino acid synthesis, although the enzymes catalysing this step are different. α-Acetolactate synthase encoded by *budB* is present in the 2,3-butanediol synthesis pathway, and acetohydroxy acid synthase I, II, and III encoded by *ilvBN*, *ilvGM*, *ilvIH* are used in the branched-chain amino acid synthesis pathway. Among these isoenzymes, α-acetolactate synthase is mainly responsible for α-acetolactate formation in *K. pneumoniae*. Branched-chain amino acid synthesis consumes a small percentage of α-acetolactate, and most α-acetolactate is used for 2,3-butanediol synthesis in *K. pneumoniae* (Gu et al. 2017). The 2,3-butanediol synthesis pathway and the branched-chain amino acid synthesis pathway are existing in parallel and do not interfere with each other in the wild-type *K. pneumoniae*. The 2,3-butanediol synthesis pathway was blocked by the disruption of *budA*, however, the activity of α-acetolactate synthase was not affected. Thus, α-acetolactate synthesized in the cell flowed into the valine and leucine synthesis pathway and resulted in 2,3-dihydroxyisovalerate accumulation (Fig 1). This is in agreement with previously published research

on 2-ketoisovalerate accumulation by *K. pneumoniae* Δ*budA* (Gu et al. 2017). The blocking of 2,3-butanediol synthesis pathway is critical for 2,3-dihydroxyisovalerate accumulation, as *K. pneumoniae* Δ*budA* accumulated 1.6 g/L of 2,3-dihydroxyisovalerate, which is 9 times higher than that accumulated by *K. pneumoniae* Δ*ilvD* (Fig. 2). 0.7 g/L of 2,3-butanediol was produced by *K. pneumoniae* Δ*budA* after 23 hours of cultivation (Fig. 2). The likely reason is that α-acetolactate is unstable and can be converted to diacetyl by nonenzymatic oxidation, and diacetyl formed can be converted to acetoin and further to 2,3-butanediol (Xiao and Xu. 2007). There are numerous reports about 2,3-butanediol production by *K. pneumoniae*, and some strains have a very high 2,3-butanediol producing ability (Ma et al. 2009). These strains might be better chassis cells for 2,3-dihydroxyisovalerate producing strain construction.

Disruption of *ilvD* prevented conversion of 2,3-dihydroxyisovalerate

2-Ketoisovalerate is an intermediate product of the valine and leucine synthesis pathway. Disruption of the *ilvD* gene will block 2-ketoisovalerate synthesis from 2,3-dihydroxyisovalerate and will result in amino acid auxotrophy. Beside branched-chain amino acids, pantothenic acid is also synthesized from 2-ketoisovalerate, and this strain was also auxotrophic for pantothenic acid. When cultured in the corn steep liquor free fermentation medium, the available nutrition limited the growth of *K. pneumoniae* Δ*ilvD* and affected the 2,3-butanediol synthesis (Fig 2). When cultured in fermentation medium, cell growth and 2,3-butanediol production were similar between *K. pneumoniae* Δ*ilvD* and the wild-type strain (data not shown). As 2,3-dihydroxyisovalerate can further be converted to 2-ketoisovalerate, the accumulated 2,3-dihydroxyisovalerate can be reused by the *K. pneumoniae* Δ*budA* cells. Combined disruption of ilvD and budA resulted in a high and stable 2,3-dihydroxyisovalerate production.

Blocking of lactic acid synthesis enhanced 2,3-dihydroxyisovalerate production

Lactic acid is synthesized from pyruvate and lactic acid production bypasses other pathways, which use pyruvate as the initial substrate. Conversion of pyruvate to lactic acid consumes one molecule of NADH, and the saved NADH might affect other chemicals synthesis. As such, the final level of 1,3-propanediol distinctly increased by *K. pneumoniae* Δ*ldhA* (Xu et al. 2009). When using glucose as the sole carbon source, the amount of lactic acid produced by wild-type *K. pneumoniae* was not particularly high, and the knock-out of *ldhA* had little influence on 2,3-butanediol production (Guo et al. 2014). However, when the 2,3-butanediol synthesis pathway was blocked, lactic acid synthesis increased to a high level (Fig.2). This validated our previous research on 2-ketoisovalerate and isobutanol production by *K. pneumoniae* (Gu et al. 2017). Beside lactic acid, removal of other by-products such as acetic acid and ethanol increased 2,3-butanediol synthesis by *K. pneumoniae* (Guo et al. 2014; Rathanasingh et al. 2016). These

metabolic engineering strategies might be adopted to improve 2,3-dihydroxyisovalerate synthesis.

Acidic and medium aerobic conditions favor 2,3-dihydroxyisovalerate synthesis

Culture parameters of 2,3-dihydroxyisovalerate production such as culture temperature, air flow rate and the content of the medium were based on those used for 2,3-butanediol and 2-ketoisovalerate production in our previous research (Gu et al. 2017). Thus only agitation rate and culture pH were optimized here. The optimal culture pH for 1,3-propanediol production by *K. pneumoniae* was in neutral pH conditions (Hao et al. 2008), which is in agreement with the optimal pH for *K. pneumoniae* cell growth (Sun et al. 2014). However, in the pH range from 5.5-6.5, 2,3-butanediol production by *K. pneumoniae* had the highest conversion ratio (Zeng et al. 1990), which was similar for acetoin production (Wang et al. 2015). Similarly, when *K. pneumoniae* was grown with glycerol as the carbon source it was shown that lower pH favor 2,3-butanediol formation and inhibit acetic acid synthesis (Biebl et al. 1998). The 2,3-butanediol production by an *Enterobacter cloacae* strain also showed an optimal culture pH of 6.5 (Priya et al. 2016). The transcription of the *bud* operon of *Klebsiella terrigena* is induced at oxygen-limited acidic conditions, 2,3-butanediol production prevents acetate overproduction and subsequent intracellular acidification (Blomqvist et al. 1993). 2,3-Dihydroxyisovalerate synthesis shares the first step of 2,3-butanediol synthesis pathway. Thus the optimal pH for 2,3-dihydroxyisovalerate production coincided with that of the 2,3-butanediol and acetoin production. In this study, a higher pH culture favored synthesis of succinic acid, acetic acid, and formic acid by-products. Formic acid was formed from pyruvate and catalysed by pyruvate formate-lyase. Higher formic acid levels suggested that more pyruvate was converted to acetyl-CoA (Zhou et al. 2017). Acetyl-CoA is a central metabolite and used for synthesis of many building blocks or catabolism through the TCA cycle. Acetyl-CoA can be converted to acetaldehyde and further converted to acetic acid. This resulted in higher acetic acid levels produced at higher culture pH values in this study. Succinic acid is an intermediate metabolite of the TCA cycle. Higher succinic acid production at higher culture pH might be the result of more acetyl-CoA flowing into the TCA cycle.

Medium agitation rates corresponding to medium aerobic conditions favor 2,3-dihydroxyisovalerate production, which is in agreement with 2-ketoisovalerate production by *K. pneumoniae* (Gu et al. 2017). In 2,3-butanediol production by *K. pneumonia*, high oxygen levels can completely hinder the butanediol formation (Silveira et al. 1993). It has been reported that under high aerobic conditions α-acetolactate synthase was rapidly and irreversibly inactivated in *K. pneumoniae* and prevented 2,3-butanediol synthesis (Kosaric et al. 1992). This is different from valine production by *Brevibacterium flavum* (Huang

et al. 2018) or *Corynebacterium glutamicum* (Blombach et al. 2007), which are all highly aerobic processes. The *bud* operon is induced by oxygen-limited conditions (Blomqvist et al. 1993). While, there has been no report about the effect of oxygen on *ilvC* expression, *ilvC* is induced by acetohydroxybutyrate or acetolactate in *E. coli* (Arfin et al. 1969). The medium aerobic condition favoring high 2,3-dihydroxyisovalerate production may be related to NADH regeneration to maintain the glycolytic flux. There are some reports about succinic acid and ethanol production by *K. pneumoniae*, and these studies were all performed under anaerobic or microaerobic conditions (Chandresh et al. 2006; Oh et al. 2013). This is agreement with our results of succinic acid and ethanol production that were inhibited under high aerobic conditions.

This is the first report to propose a biological route for the production of 2,3-dihydroxyisovalerate by the bacterium *K. pneumoniae*. A final titer of 36.5 g/L of 2,3-dihydroxyisovalerate were achieved in 45 hours of fed-batch fermentation equating to a conversion ratio of 0.49 mol/mol glucose. If the process would have been terminated after 21 hours, a conversion ratio of 0.58 mol/mol glucose and an overall productivity of about 1.4 g/L h, could have been obtained with a final titer of about 30 g/L. The conversion ratio and final titer are approaching that of valine production by *B. flavum* (0.6 mol/mol, 70 g/L) and *C. glutamicum* (0.6 mol/mol and 23 g/L) (Huang et al. 2018, Blombach et al. 2007). Thus this process has the potential to be industrialized for 2,3-dihydroxyisovalerate production assuming a similar product price to that of valine.

Conflict of Interest: Authors declare that they have no conflict of interest.

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Figure captions

Fig. 1. 2,3-Dihydroxyisovalerate synthesis related metabolic pathways. A: *K. pneumoniae* wild-type strain; B: 2,3-dihydroxyisovalerate producing strain *K. pneumoniae* Δ*budA*-Δ*ldhA*-Δ*ilvD.*

Solid line: one step of reaction; dash line: contains several steps of reaction; the line thickness represent the likely metabolic flux. *ldhA*: lactate dehydrogenase; *budB*: α-acetolactate synthase; *ilvBN*, *ilvGM*, *ilvIH*: acetohydroxy acid synthase; *budA*: α-acetolactate decarboxylase; *ilvC*: acetohydroxy acid isomeroreductase; *ilvD*: dihydroxy acid dehydratase; *ilvE*: transaminase; *leuA*: 2-isopropylmalate synthase. The blue color genes and reactions belong to 2,3-butanediol synthesis pathway; the red color genes and reactions belong to branched-chain amino acid synthesis pathway.

Fig. 2. Growth and product formation of *K. pneumonia* strains grown in batch culture on corn steep liquor free medium in shake flasks. A: Glucose; B: 2,3-dihydroxyisovalerate; C: Cell density; D: 2,3-butanediol; E: Lactic acid. F. Acetic acid. Data points are the average of n = 3; error bars represent standard error about the mean.

Fig. 3. Growth and product formation of *K. pneumoniae* Δ*budA*-Δ*ldhA*-Δ*ilvD* in minimal medium with different carbon sources. A: Carbon sources; B: 2,3-dihydroxyisovalerate; C: Cell density; D: 1,3-propanediol. Data points are the average of n = 3; error bars represent standard error about the mean.

Fig. 4. Growth and product formation of *K. pneumoniae* Δ*budA*-Δ*ldhA*-Δ*ilvD* in fermentation medium with different carbon sources. A: Carbon source utilization; B: 2,3-dihydroxyisovalerate; C: Cell density; D: 1,3-propanediol.

Fig. 5. Growth and product formation of *K. pneumoniae* Δ*budA*-Δ*ldhA*-Δ*ilvD* in fermentation medium with different culture pH. The strain was grown in stirred tank bioreactors operated at an agitation rate of 300 rpm and an aeration rate of 2 L/min. A: Cell growth; B: Glucose; C: 2,3-Dihydroxyisovlareate; D: succinic acid; E: Acetic acid; F: Formic acid; G: Ethanol. Data points for pH 7 cultivation are the average of n = 3; error bars represent standard error about the mean.

Fig. 6. Growth and product formation of *K. pneumoniae* Δ*budA*-Δ*ldhA*-Δ*ilvD* in fermentation medium (pH 7.0) with different agitation rates. The strain was grown in stirred tank bioreactors and the aeration rate set at 2 L/min. A: Cell growth; B: Glucose; C: 2,3-Dihydroxyisovlareate; D: succinic acid; E: Acetic acid; F: Formic acid; G: Ethanol. Data points for 300 rpm cultivation are the average of n = 3; error bars represent standard error about the mean.

Fig. 7. Growth and product formation of *K. pneumoniae* Δ*budA*-Δ*ldhA*-Δ*ilvD* in fed batch fermentation. Cells were grown in fermentation medium (pH 6.5) in a stirred tank bioreactor operated at 400 rpm and

565 and aeration rate of 2 L/min. A: Cell growth; B: Glucose; C: 2,3-Dihydroxyisovlareate; D: succinic acid;
566 E: Acetic acid; F: Formic acid; G: Ethanol. Data points are the average of n = 3; error bars represent
567 standard error about the mean.
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569 **Tables**

570

571 Table 1 Strains and plasmids

Strains or plasmids	Relevant genotype and description	Reference or source
K. pneumoniae strains		
CGMCC 1.6366	TUAC01 Wild type	(Hao et al. 2008)
Δ*ilvD*	Δi*lvD*, Apr^r	This work
Δ*budA*	Δ*budA*, Str^r	(Wei et al. 2013)
Δ*ldhA*	Δ*ldhA*, Apr^r	(Gu et al. 2017)
Δ*budA*-Δ*ilvD*	Δ*budA*, Str^r, Δ*ilvD*, Apr^r	This work
Δ*budA*-Δ*ldhA*	Δ*budA*, Str^r, Δ*ldhA*, Apr^r	(Gu et al. 2017)
Δ*budA*-Δ*ldhA*-Δ*ilvD*	Δ*budA*, Str^r, Δ*ldhA*, Δ*ilvD*, Apr^r	(Gu et al. 2017)
E. coli DH5α	Host of plasmid	Lab stock
plasmids		
pDK6-red	Kan^r, carries λ-Red genes (gam, bet, exo) 7.1 kp	(Wei et al. 2012)
pDK6-flp	Kan^r, carries the yeast FLP recombinase gene 6.3 kp	(Wei et al. 2012)
pIJ790	Cm^r, encodes λ-Red genes (gam, bet, exo), 6084 bp	(Gust et al. 2003)
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)
pMD18-T	Amp^r, TA cloning vector, 2,692 bp	Takara
pMD18-T-ilvD	Amp^r, carries *ilvD*	This work
pMD18-T-ilvD-773	Amp^r, Apr^r, carries part of *ilvD*	This work
pMD18-T-ilvD-778	Amp^r, Str^r, carries part of *ilvD*	This work

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579 Table 2 Primers

Primer name	Sequence (5'-3')
ilvD-s	GCACCGTCCCATTTAATAAAC
ilvD-a	ATGATTAGCCACCCAGTTTC
ilvD-FRT-s	CGCCGATCGTAAGGAGCTGTTCCTTAACGCCGGGAAACGATTCCGG GGATCCGTCGACC
ilvD-FRT-a	GGTGGTACGGATGCCCGCCGGGCCGGCGCGGAACATCTTTGTAGGC TGGAGCTGCTTC

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583 Table 3. ^{1}H and ^{13}C NMR chemical shift for 2,3-dihydroxyisovalerate

Hydrogen	CH	CH_3	Carbon	C1	C2	C3	C4, C5
Chemical shift ppm (δ)	4.03	1.22; 1.24		176.32	77.27	72.3	24.54; 24.67

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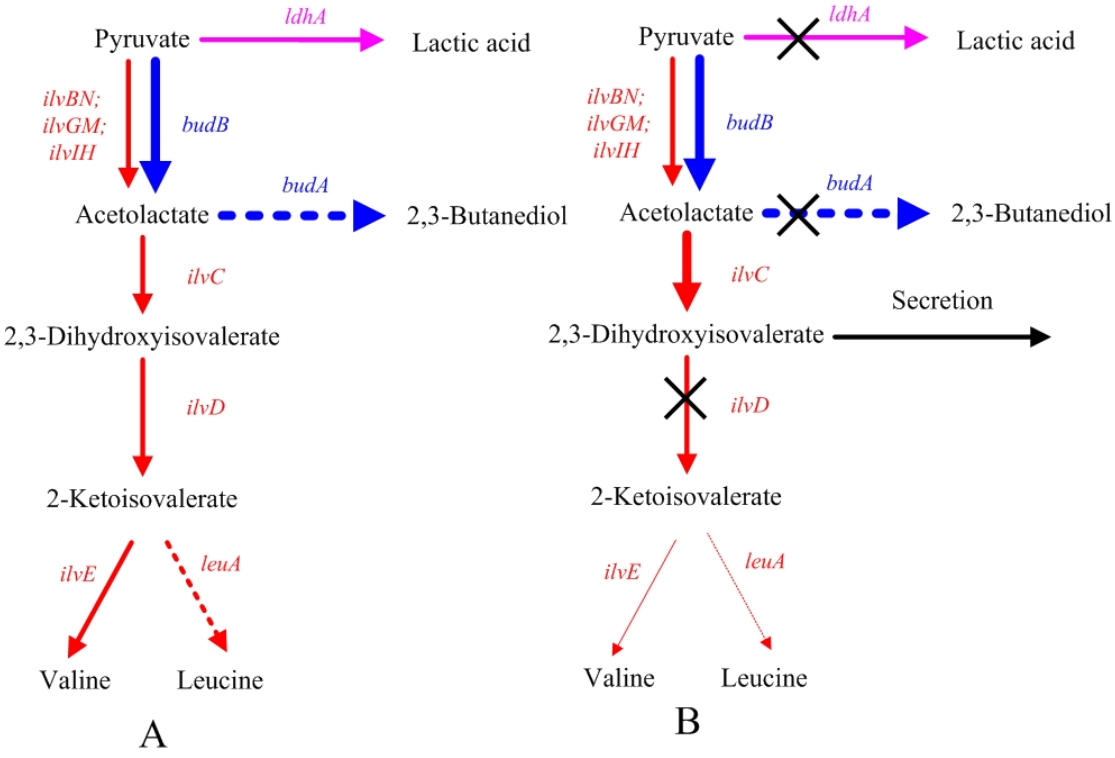
587 Figures



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589 Figure 1

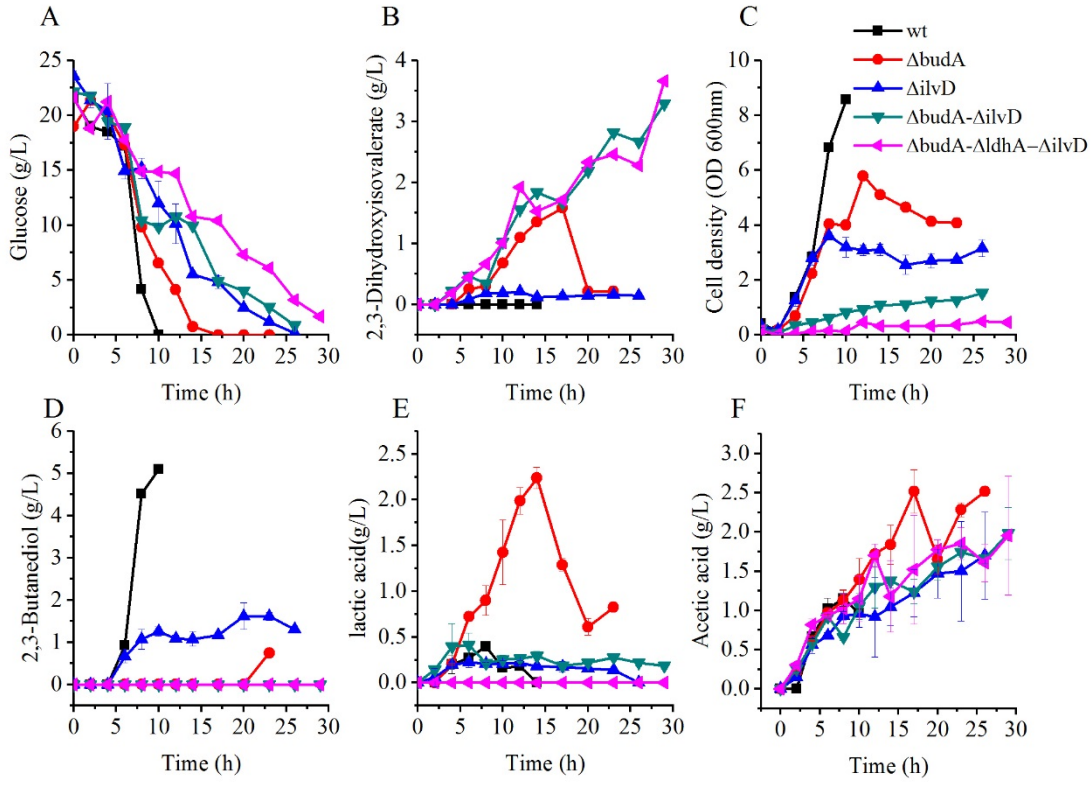

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Figure 2

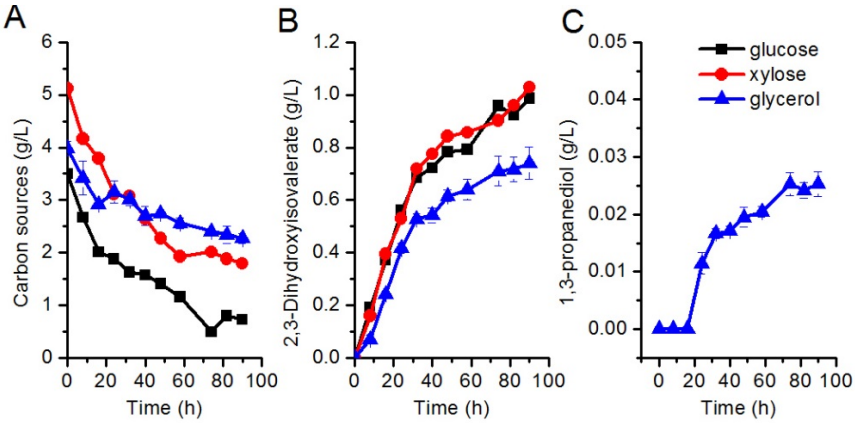

Figure 3

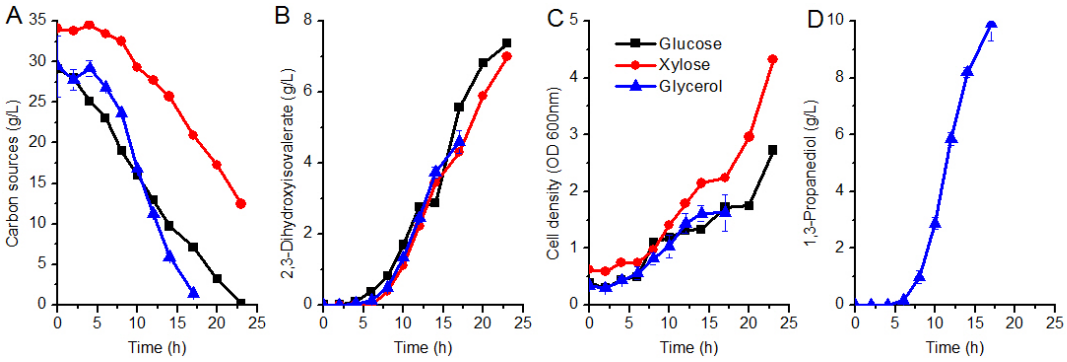

Figure 4

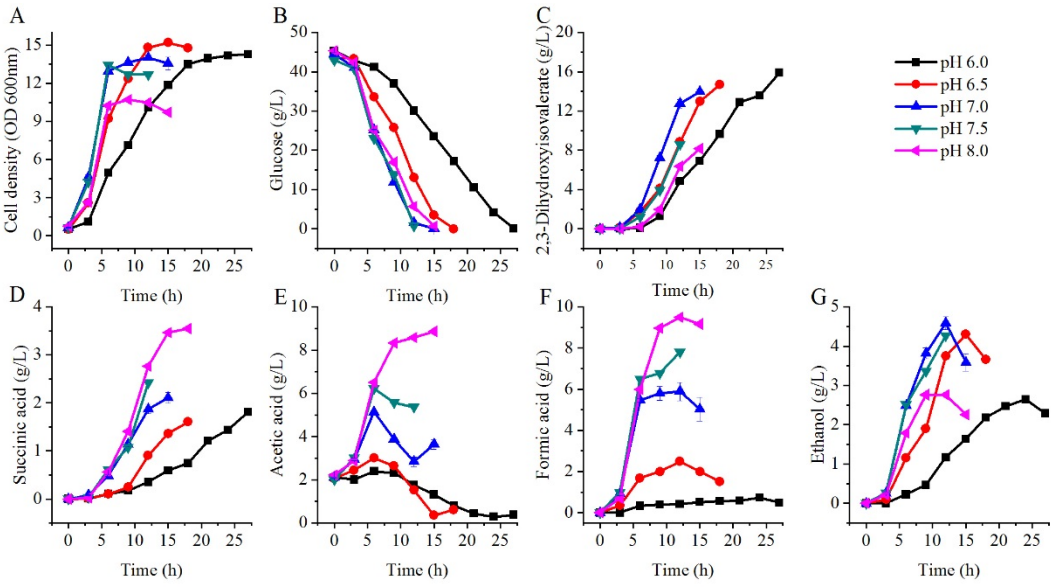

Figure 5

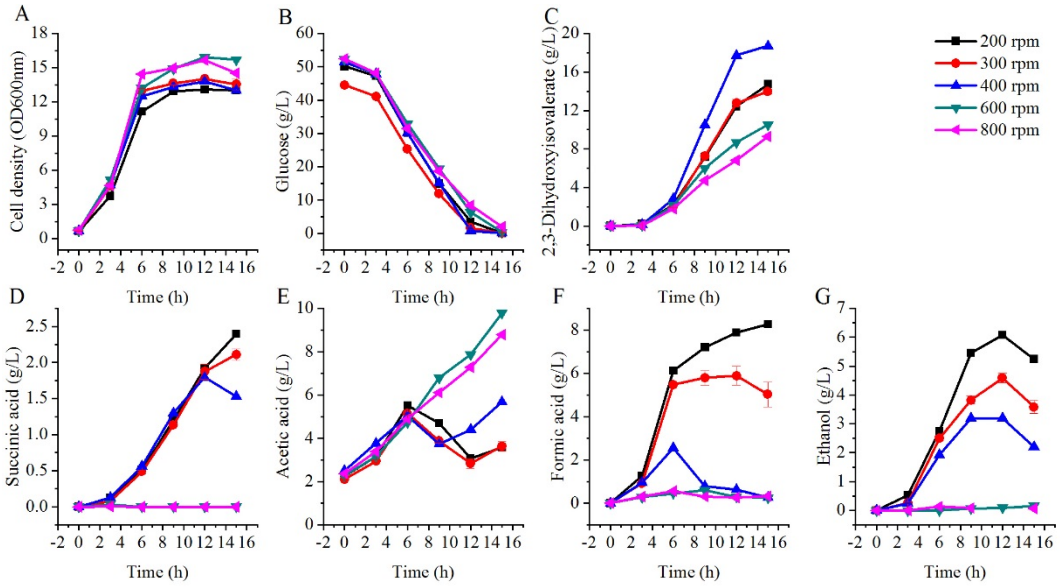

Figure 6

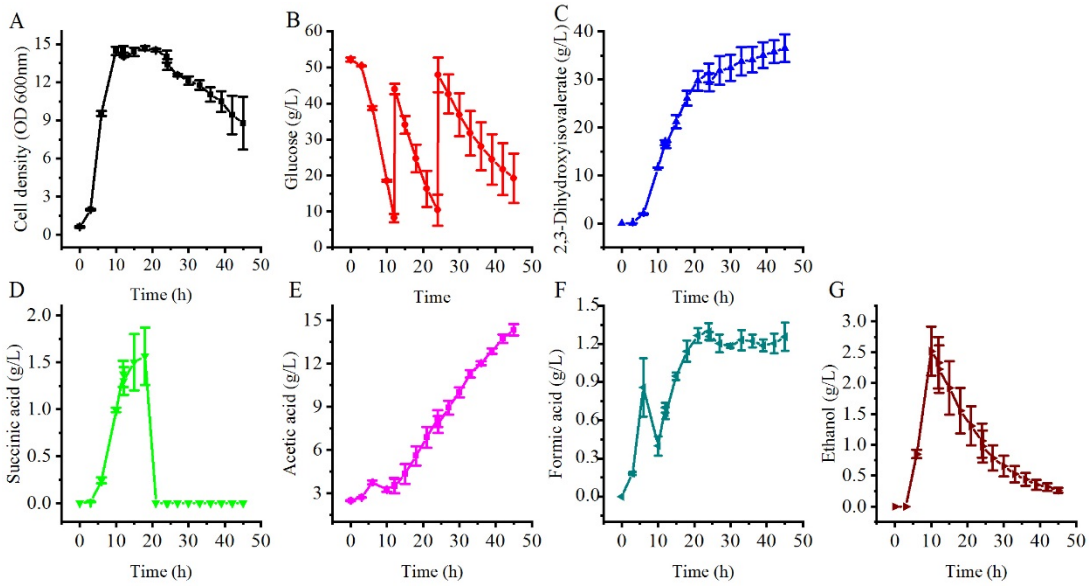

Figure 7