Production of 2,3-dihydroxyisovalerate by *Enterobacter cloacae*

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Abstract

2,3-Dihydroxyisovalerate is an intermediate of the valine synthesis pathway. However, neither natural microorganisms nor valine producing engineered strains have been reported yet to produce this chemical. Based on the 2,3-butanediol synthesis pathway, a biological route of 2,3-dihydroxyisovalerate production was developed using a *budA* and *ilvD* disrupted *Klebsiella pneumoniae* strain in our previous research. We hypothesised, that other 2,3-butanediol producing bacteria could be used for 2,3-dihydroxyisovalerate production. Here a *budA* disrupted *Enterobacter cloacae* was constructed, and this strain exhibited a high 2,3-dihydroxyisovalerate producing ability. Disruption of *ilvD* in *E. cloacae* Δ*budA* further increased 2,3-dihydroxyisovalerate level. The disruption of *budA,* encoding an acetolactate decarboxylase, resulted in the acetolactate synthesized in the 2,3-butanediol synthesis pathway to flow into the valine synthesis pathway. The additional disruption of *ilvD,* encoding a dihydroxy acid dehydratase, prevented the 2,3-dihydroxyisovalerate to be further metabolized in the valine synthesis pathway. Thus, the disruption of both *budA* and *ilvD* in 2,3-butanediol producing strains might be an universal strategy for 2,3-dihydroxyisovalerate accumulation. After optimization of the medium components and culture parameters 31.2 g/L of 2,3-dihydroxyisovalerate was obtained with a productivity of 0.41 g/L h and a substrate conversion ratio of 0.56 mol/mol glucose in a fed-batch fermentation. This approach provides an economic way for 2,3-dihydroxyisovalerate production.

Key words: *Enterobacter cloacae*; 2,3-dihydroxyisovalerate; 2,3-butanediol; branched-chain amino acid

1. Introduction

2,3-Dihydroxyisovalerate is an intermediate of the valine biosynthesis pathway. Valine and leucine biosynthesis start from pyruvate. Two molecules of pyruvate condense to form one molecule of acetolactate. Acetolactate is then converted to 2,3-dihydroisovalerate, and this reaction is catalyzed by an acetohydroxy acid isomeroreductase. 2,3-Dihydroisovalerate is then converted to 2-ketoisovalerate with the catalysis of a dihydroxy acid dehydratase. 2-Ketoisovalerate is further used for valine and leucine synthesis [1]. In *Escherichia coli*, acetohydroxy acid synthase has three isoenzymes and encoded by *ilvBN*, *ilvGM*, and *ilvIH*, respectively [2]. Acetohydroxy acid isomeroreductase and dihydroxy acid dehydratase are encoded by *ilvC* and *ilvD*, respectively [3]. The branched-chain amino acid synthesis pathway is highly regulated and the expression of these genes is subject to different multivalent repression by branched-chain amino acids [4]. Metabolic engineering methods have been employed to disrupt the regulation for valine production by bacteria [5, 6]. However, there have been no reports of natural microorganisms or valine producing engineered bacteria accumulating 2,3-dihydroisovalerate.

2,3-Butanediol is a metabolic product of many different bacteria [7]. The 2,3-Butanediol synthesis pathway shares the step of acetolactate synthesis from pyruvate, and *budB* or *alsA* genes encoding acetolactate synthase catalyses this reaction. Acetolactate is converted to acetoin by the catalysis of acetolactate decarboxylase, and this enzyme is encoded by *budA*. Acetoin is further converted to 2,3-butanediol with the catalysis of *budC* encoding a dehydrogenase [8] (Fig. 1).

In our previous work, a 2,3-dihydroxyisovalerate producing *Klebsiella pneumoniae* was constructed based on manipulation of the 2,3-butanediol synthesis pathway and valine synthesis pathway. A *budA* disrupted *K. pneumoniae* mutant lost the ability to synthesize 2,3-butanediol and a high level of 2-ketoisovalerate was accumulated in the fermentation broth of this strain [9]. Further work found low levels of 2,3-dihydroxyisovalerate was accumulated in the culture broth at the early phase of culture and then reused by the cell. In order to prevent reuse of this intermediate we constructed a *budA* and *ilvD* double gene knock out *K. pneumoniae* mutant that accumulated a high level of 2,3-dihydroxyisovalerate [10].

As 2,3-dihydroxyisovalerate accumulation in *K. pneumoniae* was caused by the disruption of 2,3-butanediol synthesis pathway, we hypothesised that other 2,3-butanediol producing bacteria might be used for 2,3-dihydroxyisovalerate production employing the same approach. Here 2,3-dihydroxyisovalerate production by an engineered *E. cloacae* strain is reported in detail. *Enterobacter cloacae* is a Gram negative bacterium and in the family of *Enterobacter*, which is the same family as *K.*

pneumoniae [11]. *E. cloacae* S1 is a strain that was isolated from the soil, and this strain produces high levels of ethylene glycol and glycolic acid when cultured with xylonic acid as a carbon source. This strain is a high natural 2,3-butanediol producer and the gene replacement method suitable for this bacterium has been established [12]. The genome of *E. cloacae* S1 has been sent to GenBank with the accession number of VSZU00000000. The *bud* operon of *E. cloacae* S1 contains a transcriptional regulator *BudR*, acetolactate decarboxylase encoding gene *budA*, acetolactate synthase encoding gene *budB* and butanediol dehydrogenase encoding gene *budC*. *budA*, *budB* and *budC* are in the same direction transcribed. While *budR* is transcribed in the opposite direction. (Fig. 1). The structure of bud operon is the same as that of *K. pneumoniae*.

2. Material and methods

2.1 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.

2.2 Construction of mutants of *E. cloacae*

Red recombinase system was used for generation of *E. cloacae* gene knock outs. *E. cloacae* 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).

E. cloacae Δ*budA* construction. The flanking sequences of *budA* gene in *E. cloacae* were amplified by PCR using the primer pairs budA-left-s/ bud -left-a and budA -right-s/ budA -right-a. 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 773-s/773-a. The PCR products and a linearized vector were ligated together to form pMD-ΔbudA-773 according to the protocol of a One Step Cloning Kit (vazyme ®). pMD-ΔbudA-773 was hosted in *E. coli*.

pMD-ΔbudA-773 was used as the template for PCR of a linear DNA containing the apramycin resistance gene *aac(3)IV* with 600 bp of budA homologous regions on both sides. The primer pair used was budA-left-s/ budA-right-a. The linear DNA was transformed into *E. cloacae* S1, which already hosted the plasmid pSARI-red. Homologous recombination between the linear DNA and the chromosome was facilitated by Red recombinase and led to budA deletion in the genome of *E. cloacae* to obtain *E. cloacae* ΔbudA.

E. cloacae ΔilvD was constructed in the same way as *E. cloacae* ΔbudA construction. Streptomycin resistance gene *aadA* that cloned from pIJ773 was replaced apramycin resistance gene *aac(3)IV*. *E.*

cloacae ΔbudA-ΔilvD was constructed in the same way as *E. cloacae* ΔilvD construction, with *E. cloacae* ΔbudA replacing wild type *E. cloacae* as the target strain.

2.3 Medium and flask culture conditions

The primary fermentation medium used for 2,3-dihydroxyisovalerate production contained 20 or 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 trihydrate, 0.4 g/L KCl, 0.1 g/L $MgSO_4$, 0.02 g/L $FeSO_4$, and 0.01 g/L $MnSO_4$. Xylose or glycerol fermentation media were the same as the fermentation medium, except glucose was replaced by xylose or glycerol.

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

2.4 Culture parameters optimization

Culture pH was optimized in stirred tank bioreactors by single-factor experiment. Other culture parameters and fermentation medium were optimized in shake flasks. Plackett-Burman design was used to identify key parameters, and Response Surface Methodology was used for key parameters optimization.

For bioreactor culture, the seed culture was inoculated into a 5-L stirred tank bioreactor (BIOSTAT-A plus, Sartorius) with a working volume of 3 L, air flow rate of 2 L/min, stirring rate was 400 rpm and culture temperature 34.5 °C.

2.5 Fed-batch culture condition

Fed-batch cultures were performed at optimized conditions, with culture pH 6.5, culture temperature 34.5 °C, air flow rate of 2 L/min and stirring rate of 400 rpm. When glucose in the broth was reduced to 15 g/L, 100 ml of 500 g/L glucose solution was added batch wise. All experiments were done in triplicate, and data were expressed as the mean ± standard error.

2.6 Analytical methods

The biomass titer at set 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 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 with a flow rate of 0.8 ml/min.

2.7 Experimental design for culture parameters and fermentation medium optimization

The Plackett-Burman design (PBD) was used to identify critical variables for the 2,3-dihydroxyisovalerate production. Culture temperature, the volume of medium and the medium content were selected as variables. Effects of each variable and its significance were calculated using the Minitab statistical software. The response surface methodology (RSM) was used to further optimize the three most notable factors identified by the PBD. Analysis of variance (ANOVA) was conducted to determine the main, interactive and quadratic effects using the Design-Expert software.

3. Results

3.1 Deletion of *budA* in *E. cloacae* redirects metabolite production from 2,3-Butanediol to 2,3-Dihydroxyisovalerate

E. cloacae S1 is a strain used for ethylene glycol and glycolic acid production [12]. Here the 2,3-butanediol production ability of this strain was tested. *E. cloacae* S1 was cultured in flasks using fermentation medium and the results are presented in Fig. 2.

After 3 hours of lag phase, wild type cells grew into the exponential phase, and a final cell density of OD 10.3 was obtained. 20 g/L of glucose was exhausted after 9 hours of cultivation, and 7.0 g/L of 2,3-butanediol was produced. The acetic acid in the medium was consumed by the cells and its concentration was decreased in the process. Succinic acid was a by-product of the process, and 1.3 g/L was produced by the end of the process. No lactic acid was detected in the broth.

E. cloacae ΔbudA was constructed by replacing part of *budA* in the chromosome of *E. cloacae* S1 by an apramycin resistant gene *aac(3)IV*. It was described in detail in the material and method section. *E. cloacae* ΔbudA was cultured in flasks with fermentation medium and the results are presented in Fig. 2.

Glucose utilization of *E. cloacae* ΔbudA was slower than that of the wild type strain, and 22 g/L of glucose was exhausted after 17.5 hours of cultivation. Cell growth rate of *E. cloacae* ΔbudA was also slower than that of the wild type strain, and it reached only OD 4.4 after 9 hours of cultivation. However, a higher final cell density of OD 12.1 after 14 h was obtained. In contrast to the wild type, the 2,3-butanediol level reached only 1.1 g/L. Acetic acid consumption and succinic acid production were also slower compared to the wild type. The final titer of acetic acid and succinic acid were 0.8 g/L and 1.4 g/L, respectively. The significant characteristic of this strain was the production of 7.0 g/L of 2,3-dihydroxyisovalerate at the end of the batch process.

3.2 2,3-Dihydroxyisovalerate production by *E. cloacae* ΔbudA-ΔilvD

In the branched-chain amino acid synthesis pathway, *ilvGMEDA* forms a single operon and *ilvC* is located nearby and separated by a regulator gene *ilvY*. (Fig. 1). To determine the effect of the dihydroxy-

acid dehydratase activity on 2,3-dihydroxyisovalerate production, an *ilvD* knock-out strain and an *ilvD* and *budA* double genes knock-out strain were constructed. The two strains were cultured with fermentation medium in flasks and the results are given in Fig. 2.

Fermentation results of *E. cloacae* ΔilvD were nearly the same as the wild type strain. 19 g/L of glucose was consumed after 9 hours of cultivation, and a final cell density of OD 10 was reached. 6.9 g/L of 2.3-butanediol was produced in the broth, and it was the main metabolite of the cell. The final acetic acid concentration was 0.5 g/L and the succinic acid level was 1.2 g/L. No 2,3-dihydroxyisovalerate was accumulated in the broth. Fermentation results of *E. cloacae* ΔbudA-ΔilvD were comparable to that of *E. cloacae* ΔbudA. Cells grew to a density of OD 12.6 after 15 hours of cultivation and 19 g/L of glucose was consumed. 1.4 g/L of 2,3-butanediol and 1.0 g/L of succinic acid were produced. The main metabolite of *E. cloacae* ΔbudA-ΔilvD was 2,3-dihydroxyisovalerate, and its level was improved to 8.6 g/L.

3.3 Xylose or glycerol are suitable carbon sources for 2,3-dihydroxyisovalerate production

Utilization of xylose and glycerol as carbon sources for chemicals production are increasingly used in biorefinery processes. Here, xylose or glycerol fermentation medium was used to culture *E. cloacae* ΔbudA-ΔilvD for 2,3-dihydroxyisovalerate production.

The results presented in Fig. 3 suggest that xylose or glycerol can be used as the main carbon sources for growth and production of 2,3-butanediol by wild-type *E. cloacae* S1. 27 g/L of xylose were exhausted after 19 hours of cultivation, while it took 35 hours to consume the 20 g/L of glycerol supplied. 8.0 g/L and 3.0 g/L of 2,3-butanediol were produced by *E. cloacae* S1 with xylose or glycerol as the main carbon source, respectively. The 2,3-butanediol titer obtained with xylose as the main carbon source was comparable to that using glucose as the main carbon source (see Fig. 2). The glycerol consumption rate was slower than the glucose consumption rate and 2,3-butanediol titer obtained was lower than that using glucose as the main carbon source. However, a high cell density of OD 20.2 was obtained with glycerol as the main carbon source. 3.0 g/L of succinic acid were produced in the process, and the highest titer of acetic acid reach 2.8 g/L. Fig. 3 shows that *E. cloacae* ΔbudA-ΔilvD can catabolise xylose and glycerol for 2,3-dihydroxyisovalerate production. 2,3-Dihydroxyisovalerate titers when using xylose or glycerol as the main carbon source were 8.6 g/L and 6.0 g/L, respectively. Xylose and glycerol consumption rates of *E. cloacae* ΔbudA-ΔilvD were both slower than that of using glucose as the main carbon source (data shown in Fig. 2). 2.9 g/L of 2,3-butanediol was synthesized by *E. cloacae* ΔbudA-ΔilvD using xylose as the main carbon source. But no 2,3-butanediol was detected in the broth when using glycerol as the main

carbon source. The substrate conversion ratios were 0.46, 0.38 and 0.33 g/g, and the 2,3-dihydroxyisovalerate productivities were 0.57, 0.45 and 0.17 g/L h with glucose, xylose or glycerol as the carbon source, respectively. Thus, glucose was selected as the carbon source for further research.

3.4 Culture parameters and fermentation medium optimization

Culture conditions and fermentation medium used here were based on that previously used for 2,3-dihydroxyisovalerate production by *K. pneumoniae*. For the purpose of improving the 2,3-dihydroxyisovalerate production, culture parameters and the fermentation medium were optimized here.

The Plackett-Burman design was used to identify critical variables for the 2,3-dihydroxyisovalerate (Y) production. Variables including culture temperature (X_1), the volume of medium (X_2), the medium content of corn steep liquor (X_3), $(NH_4)_2SO_4$ (X_4), yeast extract (X_5), KCl (X_6), $MgSO_4$ (X_7), $FeSO_4$ (X_8) and $MnSO_4$ (X_9) were selected. Nine factors in 12 experiments were evaluated at high and low levels, and results are presented in supplementary Table 1. The effects of each variable and its significance were calculated and presented in supplementary Table 2.

The Plackett-Burman design results indicate that there was a large variation in the 2,3-dihydroxyisovalerate production in the twelve runs in a range from 0.6 g/L to 6.49 g/L. The P-value of culture temperature (X_1), and $(NH_4)_2SO_4$ (X_4) were less than 0.05 indicating they were significant factors. The P-value of corn steep liquor (X_3) was close to 0.05 suggesting that it might also be a significant factor. Culture temperature and corn steep liquor both showed negative effects on 2,3-dihydroxyisovalerate production, while $(NH_4)_2SO_4$ had a positive effect on 2,3-dihydroxyisovalerate production. Thus culture temperature (X_1), corn steep liquor (X_3) and $(NH_4)_2SO_4$ (X_4) were selected for further investigation.

To further optimize the culture temperature (X_1), corn steep liquor (X_3) and $(NH_4)_2SO_4$ (X_4), the face-centered central composite design (FCCD) was used. 20 experiments were carried out and the results are presented in supplementary Table 3.

Statistical analysis of the FCCD model was performed to evaluate the analysis of variance, and the results are presented in supplementary Table 4.

The *F*-value of the model was 20.76. It implies the model was significant. There was only a 0.0001 chance that the "Model *F*-value" could occur due to noise. The model showed a high coefficient of determination (R^2=0.95), indicating a 95% confidence that the results of the 2,3-dihydroxyisovalerate production obtained were significant. The adjusted R^2 was also high (0.90), indicating a good term fit for the model. The $P < 0.05$ showed the significant influence of the coefficients. X_3, X_4, X_3^2 and X_3 X_4 were

found to be significant model terms. The model equation was expressed as follows:

$Y=8.64 + 0.28\ X_1 - 0.49\ X_3 + 0.54X_4 - 0.53X_1^2 - 1.68X_3^2 - 0.061X_4^2 - 0.13X_1X_3 + 0.013\ X_1X_4 + 0.75\ X_3X_4$

Interaction between the variables on 2,3-dihydroxyisovalerate production is shown in the response surface plot (Fig. 4). The 2,3-dihydroxyisovalerate level was significantly affected by culture temperature, corn steep liquor and $(NH_4)_2SO_4$ in the medium. The optimum factors calculated were culture temperature 34.3 ºC, 3.14 g/L of corn steep liquor and 15 g/L of $(NH_4)_2SO_4$. In this condition, the 2,3-dihydroxyisovalerate level predicted was 9.17 g/L. Confirmation experiments were performed at the optimized conditions and 8.89 ± 0.05 g/L of 2,3-dihydroxyisovalerate was produced, which was close to the predicted value.

After the culture temperature and content of the fermentation medium were optimized, the culture pH was optimized in bioreactors individually. After preliminary experiments, culture pH of 6.25, 6.5 and 6.75 was selected in batch fermentations. After 16 hours of cultivation, glucose (20 g/L) was completely exhausted at these conditions (data not shown).

As presented in Fig. 5A 8.3, 9.3 and 8.8 g/L of 2,3-dihydroxyisovalerate was produced in culture pH of 6.25, 6.5 and 6.75, respectively. Thus, culture pH of 6.5 was selected as the optimal condition. The HPLC chromatograms of the broth sample from flask culture of confirmation experiment at optimal conditions and the broth sample from 16 hours of bioreactor culture at pH 6.5 are shown in Fig. 5 B and C respectively. The main product was 2,3 dihydroxyisovalerate and nearly no by-products were generated in the bioreactor culture, while many by-products were produced in flask culture. Peaks at the retention time of 9 min, 11.3 min, 14.8 min and 16.3 min were 2,3-dihydroxyisovalerate, acetic acid, 2,3-butanediol, and ethanol, respectively.

3.5 Fed-batch fermentation

After the culture parameters were optimized, fed-batch fermentations were performed in a bioreactor with the purpose of increasing the final 2,3-dihydroxyisovalerate titer, and the results are presented in Fig. 6.

In the fed-batch fermentation, the highest cell density of OD 23 was achieved after 20 hours of cultivation, after that cell density began to decrease. 35 g/L of glucose was consumed by the cells in the first 20 hours of cultivation. A pulse of highly concentrated glucose solution was added to the medium at 20 and 56 hours of cultivation. Coinciding with cell growth, the glucose consumption rate was gradually decreased after 20 hours of cultivation. 2,3-dihydroxyisovalerate was synthesized by cells and its titer increased to 26 g/L after 49 hours of cultivation. After that, the product concentration increased

only slowly and the process was stopped after 76 hours of cultivation. A final titer of 31.2 g/L of 2,3-dihydroxyisovalerate was obtained, and the productivity was 0.41 g/L h. 2,3-Butanediol was nearly not produced in the first 40 hours of cultivation. After that 2,3-butanediol was synthesised and 5.2 g/L was produced by the end of the process. Acetic acid was a component of the fermentation medium and it was consumed by the cells during the first 12 hours of cultivation. After that acetic acid was synthesized by the cells and 8.3 g/L was produced at the end of the process. Over the whole process the yield of 2,3-dihydroxyisovalerate from glucose was 0.56 mol/mol.

4. Discussion

4.1 2,3-Butanediol production by *E. cloacae* S1

2,3-Butanediol is a metabolite produced by many different microorganisms. The Voges-Proskauer (VP) test used in classifying strains of bacteria is based on acetoin production [14], and acetoin is the precursor of 2,3-butanediol. Many bacteria, including *K. pneumoniae* [7], *Klebsiella oxytoca* [15], *Serratia marcescens* [16], *Paenibacillus polymyxa* [17], *Bacillus licheniformis* [18], *Enterobacter aerogenes* [19], and *E. cloacae* [20] have been reported of producing 2,3-butanediol in high levels. One molecule of 2,3-butanediol formation consumes two molecules of pyruvate. The theoretical maximum substrate conversion ratio of glucose to 2,3-butanediol is 1 mol/mol (0.5 g/g), and the real conversion ratios reported were around 0.3-0.4 g/g. Commonly, lactic acid is a by-product of 2,3-butanediol production by *Klebsiella spp* and *Enterobacter spp*, and reduction of lactic acid synthesis will increase the substrate conversion ratio [15, 19, 21]. Lactic acid was not synthesized in the process of 2,3-butanediol production by *E. cloacae* S1. This was an advantageous characteristic of this strain.

4.2 Disruption of *budA* caused 2,3-dihydroxyisovalerate accumulation

Disruption of *budA* blocks the 2,3-butanediol synthesis pathway. Acetolactate can be spontaneously converted to diacetyl by a nonenzymatic oxidation process. Diacetyl can be reduced to acetoin and the latter was further reduced to form 2,3-butanediol [22]. Thus, a low level of 2,3-butanediol was still generated by *E. cloacae* ΔbudA. In our previous work, *K. pneumoniae* ΔbudA also synthesised low level of 2,3-butanediol [7].

2,3-Dihydroxyisovalerate replaced 2,3-butanediol and became the main metabolite of *E. cloacae* ΔbudA in flask culture (Fig. 2). The control of *ilvC* and *ilvD* gene expression has been characterized in *E. coli* where the *ilvC* gene expression is positively controlled and induced in the presence of the acetolactate [3]. The expression of the *ilvGMEDA* operon is repressed when the three branched-chain amino acids are in ample supply but derepressed when the supply of any one of the three amino acids is

limited [23]. Structure of the *ilvCY* and *ilvGMEDA* operons of *E. cloacae* were similar to that of *E. coli*. Thus, the expression of *ilvC* and *ilvD* of *E. cloacae* might abbey a similar mechanism. Flow of acetolactate in the cell was likely divided to 2,3-butanediol synthesis pathway and valine synthesis pathway in wild-type *E. cloaca.* Disruption of *budA* might cause an increase of acetolactate level in the cell. Thus, *ilvC* expression was induced to a high degree, which could help to direct all acetolactate flowing to the valine synthesis pathway. As the expression of *ilvD* was not affected by the acetolactate, thus the enzyme that catalysises the 2,3-dihydroxyisovalerate formation and consumption was not balanced and this led to its accumulation.

2,3-Dihydroxyisovalerate accumulation in the broth indicated the activity of *ilvD* encoding dihydroxy acid dehydratase was a bottleneck step of the valine synthesis pathway in *E. cloacae* ΔbudA. This is different to *K. pneumoniae* ΔbudA where 2,3-dihydroxyisovalerate accumulated in the broth at a low level in the early phase of culture, and it was reused by the cell in the later stages of the culture. The main metabolite of *K. pneumoniae* ΔbudA was 2-ketoisovalerate [9]. While 2-ketoisovalerate was not detected in the broth of *E. cloacae* ΔbudA.

4.3 Disruption of *ilvD* enhanced 2,3-dihydroxyisovalerate accumulation

No 2,3-dihydroxyisovalerate was accumulated in the broth of *E. cloacae* ΔilvD (Fig. 2) indicating the carbon flux in the valine synthesis pathway was weak. The branched-chain amino acid synthesis pathway is carefully adjusted by the cell. The carbon flux of amino acid synthesis pathway is tightly controlled by the usage of amino acids by the cell. There have some reports using *ilvD* disrupted genotype of *E. coli* and *Salmonella enterica*, but no report mentioned the accumulation of 2,3-dihydroxyisovalerate [24] [25]. In our previous research of 2,3-dihydroxyisovalerate production by *K. pneumoniae*, a low level of 2,3-dihydroxyisovalerate (0.2 g/L) was detected in the broth of *K. pneumoniae* ΔilvD [10].

Part of 2,3-dihydroxyisovalerate formed in *E. cloacae* ΔbudA was converted to 2-ketoisovalerate with the catalysis of dihydroxy-acid dehydratase. This part of carbon flux was blocked by disruption of *ilvD*, thus the 2,3-dihydroxyisovalerate level was increased in the broth of *E. cloacae* ΔbudA-ΔilvD (Fig. 2). This agrees with the research of 2,3-dihydroxyisovalerate production by *K. pneumoniae*. Thus, disruption of *ilvD* and *budA* in a 2,3-butanediol producing strain might be an universal strategy for 2,3-dihydroxyisovalerate producing strain construction.

4.4 2,3-Dihydroxyisovalerate production with different carbon sources

Xylose is the second most abundant sugar in nature after glucose. It is the main compound of the hemicellulose in biomass. Using non-food raw materials, especially the hydrolysate of biomass as a

carbon source for microorganism growth is a tendency in biotechnology. However, the catabolism of xylose by microorganisms is not as simple as that of glucose [26]. Here both glucose and xylose can be used as the main carbon source for *E. cloacae* growth and 2,3-butanediol or 2,3-dihydroxyisovalerate production. In addition, the productivity and final titer of 2,3-dihydroxyisovalerate were comparable by using glucose or xylose as the main carbon source. Glycerol is a by-product of biodiesel manufacture. With the development of the biodiesel industry, glycerol became abundant in recent years [27]. Using glycerol as a carbon source for microorganism growth or other chemical production became a hot topic. Conversion of glycerol to 2,3-dihydroxyisovalerate by *E. cloacae* ΔbudA-ΔilvD provides a novel way of glycerol utilization. 2,3-Butanediol and 2,3-dihydroxyisovalerate are both synthesised from pyruvate. Glucose, xylose, or glycerol transfer into cells through different transporters, and they were catabolized to form pyruvate through glycolysis pathway, pentose phosphate pathway, or glycerol oxidization pathway, respectively. Thus, the different pathways of pyruvate formation might affect the efficiencies of 2,3-butanediol and 2,3-dihydroxyisovalerate synthesis.

4.5 Optimal conditions of 2,3-dihydroxyisovalerate production by *E. cloacae* ΔbudA-ΔilvD

Culture temperature is an important factor for bacterial growth. Different strains of *E. cloacae* were cultured at the temperature range of 30-54 ºC to synthesize different products [28-30]. The optimal culture temperature of 34.3 ºC obtained here was a medium temperature. The optimal content of corn steep liquor and $(NH_4)_2SO_4$ in the medium were 3.14 g/L and 15 g/L, respectively. It indicated that an inorganic nitrogen source was favorable for 2,3-dihydroxyisovalerate production. The reason might be the activity of key enzymes in the branched-chain amino acid synthesis pathway was inhibited by the free branched-chain amino acids in the complex medium [31]. In the face-centered central composite design results, the optimal concentration of $(NH_4)_2SO_4$ was the upper boundary of the experimental range. We have further increased the concentration of $(NH_4)_2SO_4$ to 20 g/L or 30 g/L, however the 2,3-dihydroxyisovalerate level decreased (data not shown). Thus, 15 g/L of $(NH_4)_2SO_4$ seems to be the optimal concentration.

Succinic acid, lactic acid, acetic acid, and ethanol are common by-products of 2,3-butanediol production by *E. cloacae* in bioreactors [28]. It was similar for 2,3-dihydroxyisovalerate production by *E. cloacae* ΔbudA-ΔilvD in flask cultures. As shown in Fig. 1 low levels of 2,3-butanediol and succinic acid were produced in the process. In contrast, nearly no by-products were generated in the bioreactor batch culture (Fig. 5 C). The difference may be due to the different aeration and agitation conditions in shake flasks and bioreactors resulting in different oxygen levels that could affect the product formation

of the cells as has been shown for 2,3-dihydroxyisovalerate production by *K. pneumonia* [10]. Further investigation of the effect of aeration and agitation rate on product formation is needed to explain this result.

4.6 2,3-dihydroxyisovalerate production by *E. cloacae* ΔbudA-ΔilvD in fed-batch fermentation

A final high titer of 2,3-dihydroxyisovalerate was obtained in the fed-batch fermentation. However, relatively high levels of by-products such as 2,3-butanediol and acetic acid were produced. Thus, batch or fed-batch fermentation can be selected with different requirements for the final products. Fewer by-products in the fermentation broth is helpful for the purification process. Shorten the fed-batch process would reduce the formation of by-products but still achieving a higher titer compared to batch process.

Synthesis one molecule of 2,3-dihydroxyisovalerate consumes two molecules of pyruvate. Two molecules of pyruvate are formed from one molecule of glucose. Thus, the theoretically maximum conversion ratio of 2,3-dihydroxyisovalerate from glucose is 1 mol/ mol. A substrate conversion ratio of 0.56 mol/mol was obtained in the fed-batch fermentation. It was higher than the 2,3-dihydroxyisovalerate production by *K. pneumoniae*, which was 0.49 mol/mol. However, the titer and the productivity of 2,3-dihydroxyisovalerate production by *E. cloacae* ΔbudA-ΔilvD was lower than that of *K. pneumoniae*, which produced 36.5 g/L of 2,3-dihydroxyisovalerate after 45 hours of fed-batch fermentation.

Currently, commercial 2,3-dihydroxyisovalerate is not available in large quantities, and its price was about 500 USD per 50 mg, which limits its application. Here a new biological route of 2,3-dihydroxyisovalerate production was developed with a high substrate conversion ratio. This technology provides an economic way for 2,3-dihydroxyisovalerate production.

Conflict of Interest:

Authors declare that they have no conflict of interest.

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

Authors' contributions

JH and FB designed this study. YY, ZZ, XLu, JG, YW and YY conducted the research. XLiao, JP, GL, FB and JH analysed the data. YY, FB and JH wrote the manuscript. All authors read and approved the final manuscript.

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Tables

Table 1 Strains and plasmids

Strain or plasmid	Relevant genotype and description	Reference or source
Strains		
E. cloacae S1	Wild type	[12]
E. cloacae Δ*budA*	Δ*budA*, Apr^r	This work
E. cloacae Δ*ilvD*	Δ*yjhH*, Str^r	This work
E. cloacae Δ*budA-ilvD*	Δ*budA,* Apr^r, Δ*ilvD,* Str^r	This work
E. coli DH5α	Host of plasmid	Lab stock
Plasmids		
pIJ773	Apr^r, aac(3)IV with FRT sites, 4334 bp	[13]
pIJ778	Strr, aadA with FRT sites, 4337 bp	[13]
pSARI	Kan^r, 4,914 bp	Lab stock
pSARI-red	Kanr, carries λ-Red genes (gam, bet, exo), 6,799 bp	[12]
pMD 18T	Amp^r, cloning vector	ClonExpress ®
pMD-ΔbudA-773	Amp^r, Apr^r, carries part of budA	This work
pMD -ΔilvD-778	Amp^r, Str^r, carries part of ilvD	This work

Table 2 Primers

Primer	Sequence (5'-3')
budA-left-s	CCCGGGGATCCTCTAGAGATCTGGGTGCAGAAGCGCTG
budA-left-a	GCTCCAGCCTACAGCTTTATTTACTCCCGTCTGACTATTAA
budA-right-s	GAATTGATGGCCGAGTCACAACCC
budA-right-a	CATGCCTGCAGGTCGACGATTCAGCACCGCCACGCCTG
ilvD-left-s	CCCGGGGATCCTCTAGAGATCTGGGTGCAGAAGCGCTG
ilvD-left-a	GCTCCAGCCTACATGCTTTATTTACTCCCGTCTGACTA
ilvD-right-s	ACGGATCCCCGGAATATGATGGCCGAGTCACAACC
ilvD-right-a	CATGCCTGCAGGTCGACGATTCAGCACCGCCACGCCTG
773-s	TAAAGCATGTAGGCTGGAGCTGCTTCG
773-a	ATCATATTCCGGGGATCCGTCGA
778-s	TAAAGCATGTAGGCTGGAGCTGCTTCG
778-a	ATCATATTCCGGGGATCCGTCGA

Figure captions

Fig. 1. 2,3-Dihydroisovalerate synthesis related pathways.

Olive color: 2,3-Butanediol synthesis pathway; Blue color: valine synthesis pathway. *Bud* operon contains *bud*RABCD genes and responsible for 2,3-butanediol synthesis; *ilv*CY and *ilv*GMEDA are two nearby operons and responsible for valine synthesis. The red color arrows indicate the likely mechanism of 2,3-dihydroisovalerate accumulation. Disruption of *budA* resulted in the acetolactate synthesized in the 2,3-butanediol synthesis pathway flowing into the valine synthesis pathway. Disruption of *ilvD* prevents 2,3-dihydroxyisovalerate to be further metabolized in valine synthesis pathway.

Fig. 2. Growth and product formation of *E. cloacae* strains grown in batch culture in fermentation medium in shake flasks. EC: *E. cloacae* S1; ΔbudA: *E. cloacae* ΔbudA; ΔilvD: *E. cloacae* ΔilvD; ΔbudAΔilvD: *E. cloacae* ΔbudA-ΔilvD. Data points are the average of n = 3; error bars represent standard error about the mean. 2,3-Butanediol was the main metabolite of *E. cloacae* S1 and *E. cloacae* ΔilvD. 2,3-Dihydroxyisovalerate was produced by *E. cloacae* ΔbudA, and further improved by *E. cloacae* ΔbudA-ΔilvD.

Fig. 3 Growth and product formation of *E. cloacae* S1 and *E. cloacae* ΔbudA-ΔilvD with xylose or glycerol as the main carbon source of fermentation medium in shake flask culture. EC: *E. cloacae* S1; ΔbudAΔilvD: *E. cloacae* ΔbudA-ΔilvD. Data points are the average of n = 3; error bars represent standard error about the mean.

Fig. 4 Effect of culture temperature, corn steep liquor and $(NH_4)_2SO_4$ on 2,3-dihydroxyisovalerate production by *E. cloacae* ΔbudA-ΔilvD. A: effects of culture temperature and corn steep liquor; B: effects of culture temperature and $(NH_4)_2SO_4$; C: effects of corn steep liquor and $(NH_4)_2SO_4$.

Fig. 5 Effect of culture pH on 2,3-dihydroxyisovalerate production by *E. cloacae* ΔbudA-ΔilvD. A: 2,3-dihydroxyisovalerate levels; B: HPLC chromatograms of flask culture sample at optimal conditions. C: HPLC spectrum of 16-hour sample at culture pH of 6.5.

Fig. 6. 2,3-dihydroxyisovalerate production by *E. cloacae* ΔbudA-ΔilvD in fed-batch culture at pH 6.5 using a 5 L bioreactor operated at air flow rate of 2 L/min, 400 rpm and 34.3 ºC. A: cell density; B:

glucose; C: 2,3-dihydroxyisovalerate; D: 2,3-dihydroxyisovalerate; E: acetic acid. Data points are the average of n = 3; error bars represent standard error.

Figures

Fig.1

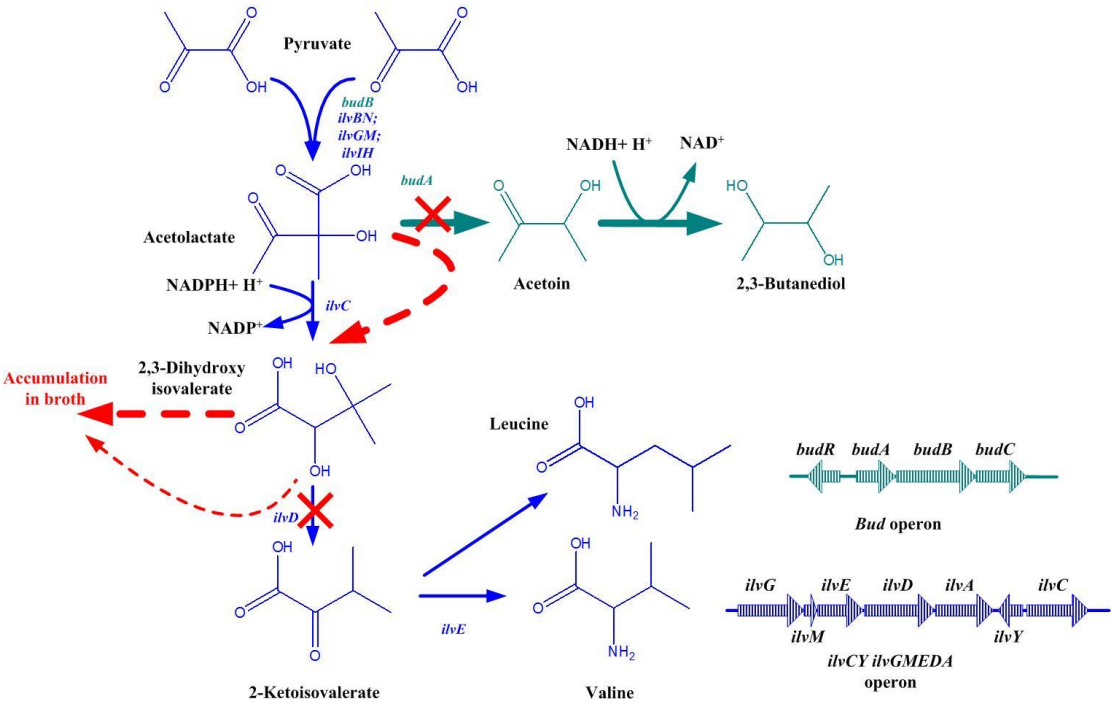


Fig. 2

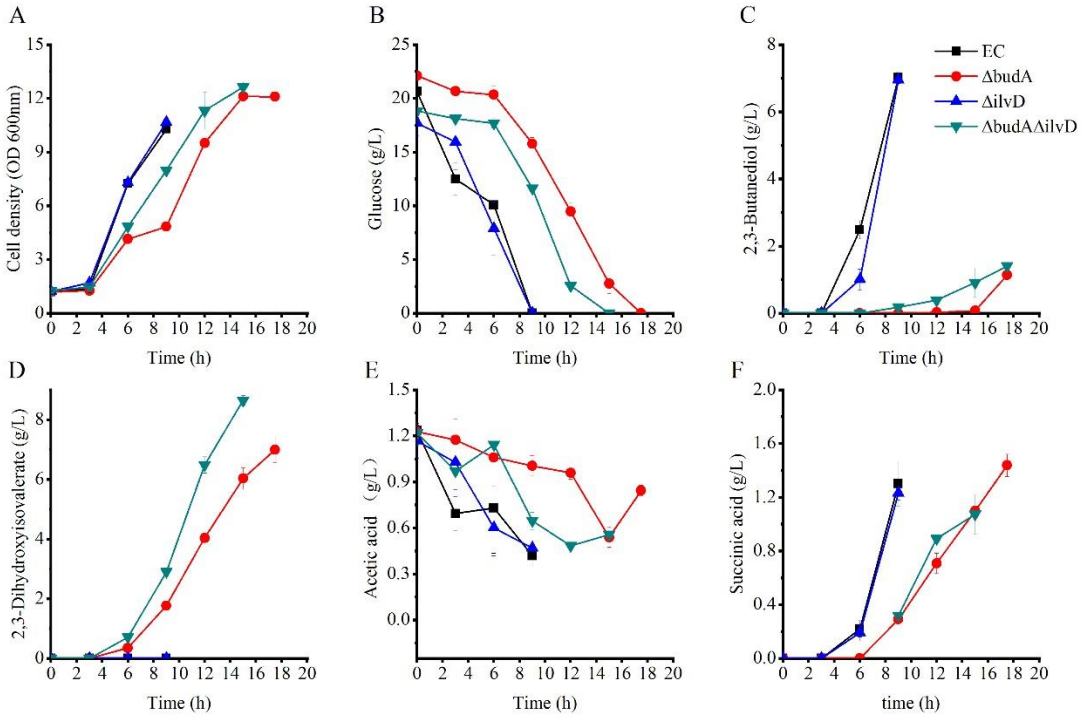


Fig.3

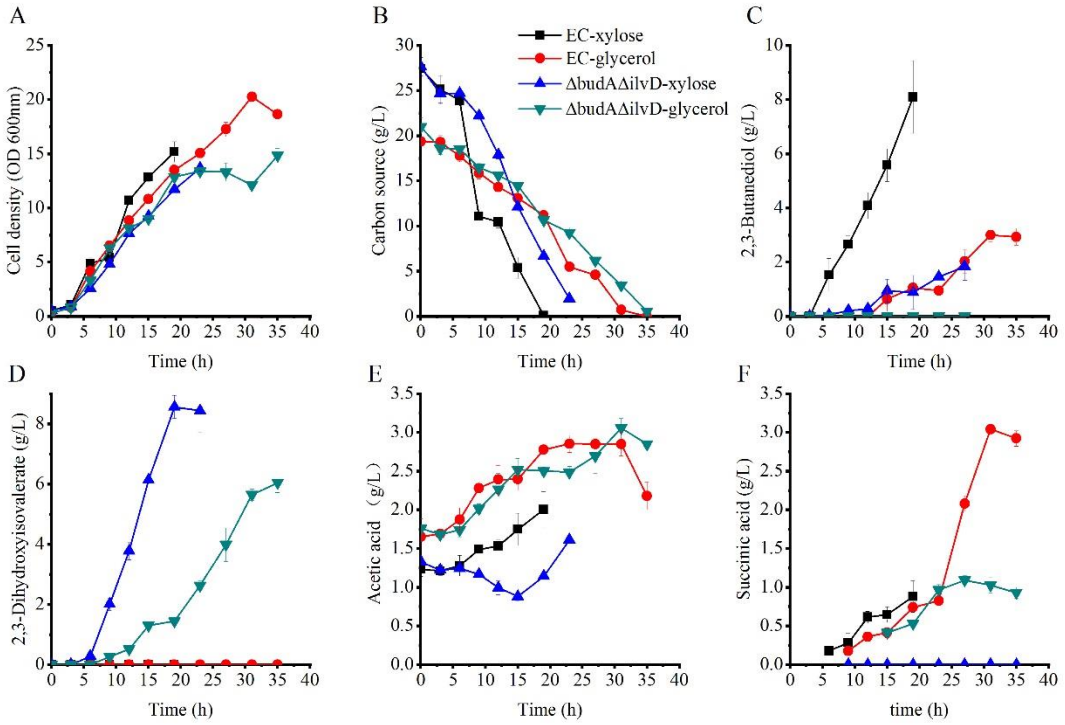

A
B
C
D
E
F
Cell density (OD 600nm)
Carbon source (g/L)
2,3-Butanediol (g/L)
2,3-Dihydroxyisovalerate (g/L)
Acetic acid (g/L)
Succinic acid (g/L)
Time (h)
time (h)
EC-xylose
EC-glycerol
ΔbudAΔilvD-xylose
ΔbudAΔilvD-glycerol

Fig 4

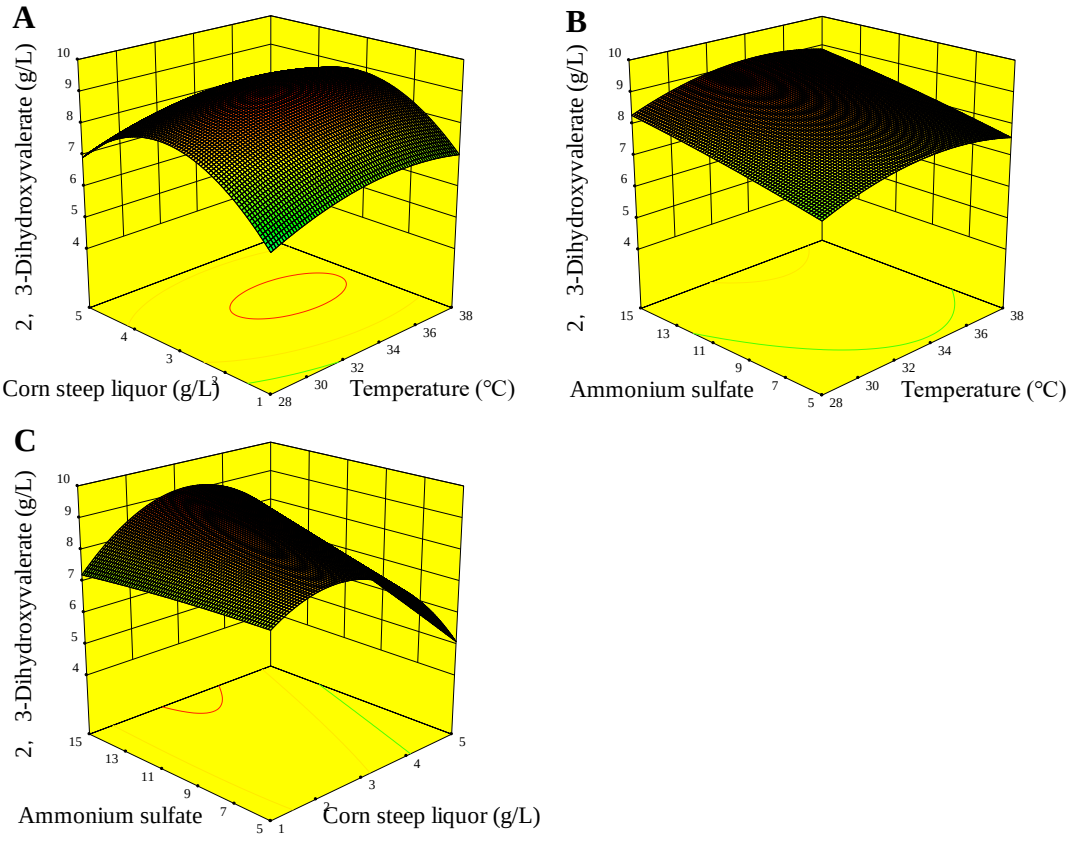

A
B
C
2，3-Dihydroxyvalerate (g/L)
Corn steep liquor (g/L)
Temperature (℃)
Ammonium sulfate
Temperature (℃)
Ammonium sulfate
Corn steep liquor (g/L)

Fig.5

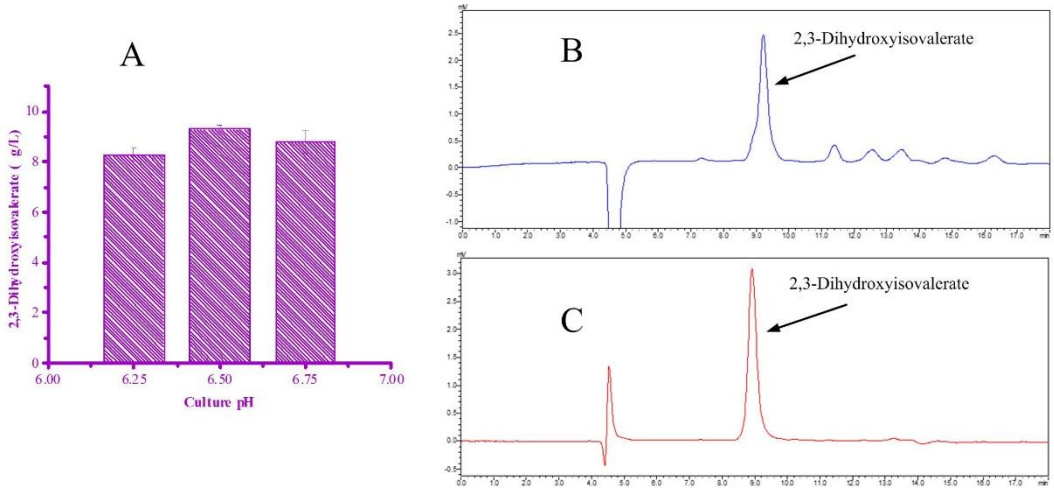
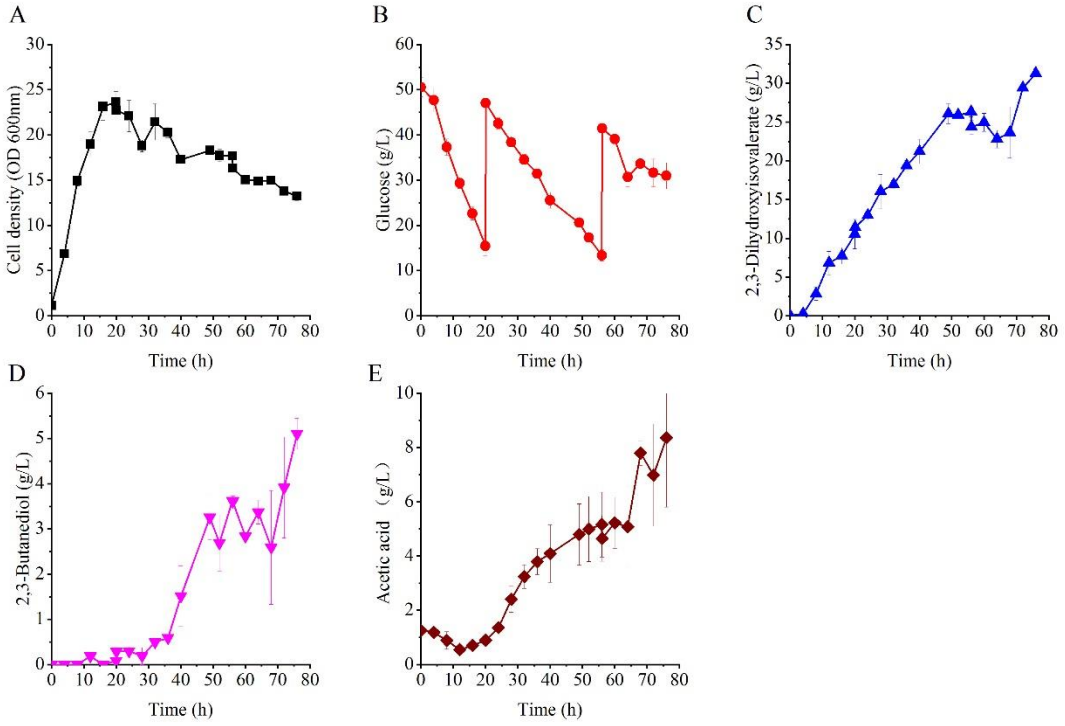

A
2,3-Dihydroxyisovalerate (g/L)
Culture pH
B
2,3-Dihydroxyisovalerate
C
2,3-Dihydroxyisovalerate

Fig. 6


A
Cell density (OD 600nm)
Time (h)
B
Glucose (g/L)
Time (h)
C
2,3-Dihydroxyisovalerate (g/L)
Time (h)
D
2,3-Butanediol (g/L)
Time (h)
E
Acetic acid (g/L)
Time (h)