Blocking the 2,3-butanediol synthesis pathway of *Klebsiella pneumoniae* resulted in L-valine production

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Abstract:

Klebsiella pneumoniae is a 2,3-butanediol producing bacterium. Nevertheless, a design and construction of L-valine production strain was studied in this paper. The first step of 2,3-butanediol synthesis and branched-chain amino acid synthesis pathways share the same step of α-acetolactate synthesis from pyruvate. However, the two pathways are existing in parallel and do not interfere with each other in the wild-type strain. A knockout of *budA* blocked the 2,3-butanediol synthesis pathway and resulted in the L-valine production. The *budA* coded an α-acetolactate decarboxylase and catalyzed the acetoin formation from α-acetolactate. Furthermore, blocking the lactic acid synthesis by knocking out of *ldhA*, which is encoding a lactate dehydrogenase, improved the L-valine synthesis. 2-Ketoisovalerate is the precursor of L-valine, it is also an intermediate of the isobutanol synthesis pathway, while indole-3-pyruvate decarboxylase (*ipdC*) is responsible for isobutyraldehyde formation from 2-ketoisovalerate. Production of L-valine has been improved by knocking out of *ipdC*. On the other side, the *ilvE*, encoding a transaminase B, reversibly transfers one amino group from glutamate to α-ketoisovalerate. Overexpression of *ilvE* exhibited a distinct improvement of L-valine production. The *brnQ* encodes a branched-chain amino acid transporter, and L-valine production was further improved by disrupting *brnQ*. It is also revealed that weak acidic and aerobic conditions favor L-valine production. Based on these findings, L-valine production by metabolically engineered *K. pneumonia* was examined. In fed-batch fermentation, 22.4 g/L of L-valine was produced by the engineered *K. pneumoniae* Δ*budA*-Δ*ldhA*-Δ*ipdC*-Δ*brnQ*-*ilvE* after 55 hours of cultivation, with a substrate conversion ratio of 0.27 mol/mol glucose.

Key words: *brnQ* · 2,3-butanediol · *ilvE* · *ipdC* · *Klebsiella pneumoniae* · L-valine

Introduction

L-valine is a branched-chain amino acid. It is a nutrient-for animals and has been widely used in animal feed additives, food additives, drugs, cosmetics, and other fields (Park and Lee 2010). L-valine was first obtained in the nineteenth century from proteins. The historical method to obtain L-valine is its extraction from natural protein sources (Oldiges et al. 2014). Besides, L-valine can be produced by chemical synthesis and by microbial fermentation (Leuchtenberger et al. 2005). The world L-valine market is continuously increasing in recent years and there is also an increasing interest in the development of efficient L-valine-producing strains. Metabolic engineering has proved its superior potential for L-valine production. The microorganisms employed are limited to recombinant *Escherichia coli* (Park et al. 2011a, b), *Corynebacterium glutamicum* (Buchholz et al. 2013; Radmacher et al. 2002) and *Brevibacterium flavum* strains (Hou et al. 2012a, b). There are no reports of L-valine production by other bacteria.

Klebsiella pneumoniae is a gram-negative bacterium belonging to the family of *Enterobacteriaceae*. *K. pneumoniae* is also an important industrial microorganism. This bacterium has the advantage of growing quickly and can use plenty kinds of chemicals as carbon sources, including the hydrolysis of biomass. Wild-type strains have been used as producers for 2,3-butanediol (Chen et al. 2014) and 1,3-propanediol (Hao et al. 2008) production. Engineering strains were constructed for 2-ketogluconic acid (Wei et al. 2013), gluconic acid (Wang et al. 2016), acetoin (Wang et al. 2015), 2,3-dihydroxyisovalerate (Wang et al. 2020), 2-ketoisovalerate and isobutanol production (Gu et al. 2016).

When cultured with glucose or other chemicals as carbon sources, 2,3-butanediol is the main metabolite of wild-type *K. pneumoniae* (Chen et al. 2014). The synthesis pathway of 2,3-butanediol

starts from the condensation of two pyruvate molecules to yield α-acetolactate. The α-acetolactate is converted to acetoin with the catalysis of α-acetolactate decarboxylase (*budA*). α-Acetolactate is further converted to acetoin by a *budA* encoded α-acetolactate decarboxylase. Finally, acetoin is reduced to 2,3-butanediol by butanediol dehydrogenase (*budC*). In the branched-chain amino acid synthesis pathway, 2,3-dihydroxyisovalerate is synthesized from α-acetolactate and this reaction is catalyzed by the acetohydroxy acid isomeroreductase (*ilvC*). Then, 2,3-dihydroxyisovalerate is converted to 2-ketoisovalerate by dihydroxy acid dehydratase (*ilvD*) (Freundlich et al. 1962). Subsequently, L-valine is obtained from 2-ketoisovalerate, and this reaction is catalyzed by a transaminase (*ilvE*) (Fig. 1).

In a previous report of our group, a *budA* knocked out strain accumulated 2-ketoisovalerate and isobutanol in the culture broth. Indole-3-pyruvate decarboxylase (*ipdC*) was identified to catalyze the conversion of 2-ketoisovalerate to isobutyraldehyde, and the latter was further converted to isobutanol, *ipdC* knocked out strain increased the accumulation level of 2-ketoisovalerate (Gu et al. 2016). 2-Ketoisovalerate is a precursor of valine, thus *K. pneumoniae* Δ*budA* and derived strains have the potential to be used for valine production. Here, the metabolic engineering studies and manipulations were performed to explore and enable efficient valine production by *K. pneumoniae*.

Material and methods

Strains, plasmids, and primers

Strains, plasmids, and primers used were listed in Tables 1, 2, and 3.

Construction of gene knockout *K. pneumoniae* strains

Gene knockout strains were performed following the method of Wei et al (Wei et al. 2012). *K.*

pneumoniae Δ*brnQ* construction was described in detail.

The upstream and downstream flanking sequences of *brnQ* in the genome of the *K. pneumoniae* were amplified by PCR using a primer pair *brnQ*-up-s/*brnQ*-down-s. Streptomycin resistance gene (*aadA*) was amplified from pIJ778 with a primer pair *brnQ*-FRT-s/*brnQ*-FRT-a. The upstream and downstream flanking sequences of *brnQ* and *aadA* were ligated together using a ClonExpress Ultra One Step Cloning Kit ®. The obtained linear DNA contained the *aadA* and *brnQ* homologous regions on both sides. The linear DNA was transformed into *K. pneumoniae* strain containing pDK6-red. Red recombinase promotes homologous recombination between linear DNA and the strain's chromosomal DNA, resulting in *brnQ* deletion in *K. pneumoniae* genome and obtaining the *K. pneumoniae* Δ*brnQ*.

For the screening of engineered strains, the strains were grown in Luria-Bertani medium at 37 ºC. Antibiotics used in the selective medium were ampicillin (50 μg/mL), apramycin (50 μg/mL), kanamycin (50 μg/mL) and streptomycin (25 μg/mL). Then, the positively engineered strains were verified by PCR. The construction of other knockout strains followed the same method, except that the relevant primers and the antibiotic resistance were used (shown in Tables 2 and 3).

Construction of gene overexpression strains

The *ilvE* was amplified from the genomic DNA of *K. pneumoniae* using the primer pair *ilvE*-s/ *ilvE*-a. The PCR product was ligated into a pMD18-T simple vector to produce a pMD18-T-*ilvE*. pMD18-T-*ilvE* and pDK6 were digested with *EcoR I* and *Hind III,* and *ilvE* fragment was ligated with linear pDK6 vector to get pDK6-*ilvE*. pDK6-*ilvE* was transformed into *K. pneumoniae* to get *ilvE* overexpression strain. *budB* overexpression strains were constructed followed the same way.

Medium and culture conditions

The fermentation medium contains: 30 g/L glucose, 20 g/L $(NH_4)_2SO_4$, 2 g/L KH_2PO_4, 0.25 g/L

KH_2PO_4, 0.4 g/L $MgSO_4 \cdot 7H_2O$ and 1 mL of trace element solution.

Trace element solution (1 L) contains: 200 mg $CoCl_2 \cdot 6H_2O$, 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% HCl.

K. pneumoniae strains were inoculated in a 250 mL flask containing 50 mL of medium and cultured on a rotating shaker at 37 ^oC and 200 rpm for 1 day.

Fermentation parameters optimization such as culture pH value and stirring speed were carried out in bioreactors. For seed culture, a 250 mL flask containing 50 mL LB medium was cultured overnight in a rotating shaker at 37 ^oC and 200 rpm. The seed culture was inoculated into a 5 L tank bioreactor (BIOSTAT-A plus, Sartorius) with a working volume of 2 L, and the airflow rate was 2 L/min. The pH value of the culture was automatically controlled by adding ammonia. For fed-batch cultivation, glucose was fed when the concentration of glucose in the fermentation broth decreased to 5 g/L.

All experiments were made in triplicate, and the data were expressed as mean ± standard error.

Analytical methods

A spectrophotometer was used to measure the biomass yield at set time intervals through optical density (OD600).

For quantification of L-valine, the culture was centrifuged (16,904×g for 10 min) and filtered through a 0.22 μm syringe filter to prepare cell-free supernatant. Then, the supernatant was precolumn derived as described in previous research (Gu et al. 2014). The samples obtained were HPLC analyzed using a Shimadzu Separations module connected to a Shimadzu SPD-M20A detector set to 254 nm. The samples were separated on an Agilent Zorbax SB-Aq column (250 mm × 4.6 mm, 5 μm) with 0.05 mol/L sodium acetate (pH 6.50 ± 0.05) (mobile phase A) and methanol/acetonitrile/water (20:60:20, v/v/v) (mobile phase B). The following gradient was used at a flow rate of 1.0 mL/min for 36 min:

from 0 min to 25 min, 95% solvent A + 5% solvent B; at 25 min, the ratio of solvent B increased to 32%; at 25.01 min, 0% solvent A + 100% solvent B; from 30 to 30.01 min, the ratio of solvent B decreased to 5%; and from 30.01 min to 36 min, 95% solvent A + 5% solvent B.

Glucose and other compounds in the fermentation broth were quantified by a Shimadzu 20AVP high-performance liquid chromatography (HPLC) equipped with RID-20A refractive index detector. 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 5 mmol/L H_2SO_4 solution, and the flow rate was 0.8 mL/min.

Results

L-valine production by *K. pneumoniae* Δ*budA*

K. pneumoniae Δ*budA* was a strain used for 2-ketoisovalerate and isobutanol production. *K. pneumoniae* Δ*ldhA* and *K. pneumoniae* Δ*budA*-Δ*ldhA* were constructed to eliminate lactic acid production (Gu et al. 2017). Here the L-valine production ability of these strains was determined. These strains and the wild-type strain were cultured in flasks in the fermentation medium and the results are presented in Fig. 2.

The amount of 30 g/L of glucose from the media was exhausted by the wild-type strain after 9 hours of cultivation. Cell growth coincided with glucose consumption and 9.6 OD units of cell density were achieved. The main metabolite of the process was 2,3-butanediol, and its final titer was 6.3 g/L. Low levels of lactic acid were also produced, while L-valine was not detected in the broth. The cell growth and glucose consumption rate of *K. pneumoniae* Δ*budA* were slower than that of the wild-type strain, and the cell density of 5.3 OD units was attained after 20 hours of cultivation. *K. pneumoniae* Δ*budA* lost the ability to synthesis 2,3-butanediol. However, 2.9 g/L of L-valine was produced by this strain

after 30 hours. During the cultivation, 2.2 g/L of 2-ketoisovalerate was accumulated after 12 hours and then decreased gradually with the formation of L-valine. In addition, 1.8 g/L of lactic acid was produced after 9 hours, and it was also reused by the cell in the following cultivation time. Furthermore, 2.3 g/L acetic acid was produced by the strain, which was not detected in the wild-type strain.

Cell growth of *K. pneumoniae* Δ*ldhA* was similar to that of the wild-type strain, and the final cell density of 8.8 OD units was achieved. The amount of 30 g/L of glucose was exhausted after 9 h of cultivation, and 6.5 g/L of 2,3-butanediol was produced. No lactic acid, acetic acid, 2-ketoisovalerate, or L-valine was detected in the broth.

Cell growth and metabolite production of *K. pneumoniae* Δ*budA*-Δ*ldhA* were similar to those of *K. pneumoniae* Δ*budA*. It took 20 hours to deplete the glucose, and a cell density of 5.9 OD units was achieved after 30 hours of cultivation. The main metabolites of this process were L-valine and acetic acid, and their final titers were 2.7 and 2.2 g/L, respectively. The highest titer of 2-ketoisovalerate was 2.2 g/L after 12 hours cultivation, and it was further consumed with the extension of fermentation time. No 2,3-butanediol or lactic acid was detected in the broth.

These results indicated that the *budA* knockout resulted in the production of L-valine by the *K. pneumoniae*, and L-valine titers were further improved by blocking the lactate synthesis pathway.

L-valine production by *ipdC* knocked out strain

Indole-3-pyruvate decarboxylase, encoded by *ipdC*, catalyzed the reaction of isobutyraldehyde formation from 2-ketoisovalerate. To erase isobutanol production, *ipdC* was knocked out to obtain *K. pneumoniae* Δ*budA*-Δ*ldhA*-Δ*ipdC*. This strain was cultured in the flasks, and the results are shown in Fig. 3.

The glucose consumption rate of *K. pneumoniae* Δ*budA*-Δ*ldhA*-Δ*ipdC* was slower than that of *K.*

pneumoniae Δ*budA*-Δ*ldhA*. It took 25 hours to consume the 30 g/L of glucose. The amount of only 0.05 g/L of isobutanol was produced by *K. pneumoniae* Δ*budA*-Δ*ldhA*-Δ*ipdC*, while the amount of 0.12 g/L has been produced by *K. pneumoniae* Δ*budA*-Δ*ldhA*. The peak value of 2-ketoisovalerate was 2.5 g/L and it was later reused by the cells, as that of *K. pneumoniae* Δ*budA*-Δ*ldhA*. L-valine produced by *K. pneumoniae* Δ*budA*-Δ*ldhA*-Δ*ipdC* was 3.2 g/L, which is 0.5 g/L higher than the obtained by *K. pneumoniae* Δ*budA*-Δ*ldhA*. The amount of 2.3 g/L of acetic acid was produced by *K. pneumoniae* Δ*budA*-Δ*ldhA*-Δ*ipdC* and it was similar to that of *K. pneumoniae* Δ*budA*-Δ*ldhA*. These results showed that knocked out of *ipdC* reduced isobutanol production and favored L-valine synthesis.

L-valine production by *ilvE* overexpression strain

The *ilvE* encodes a branched-chain amino acid transaminase, which catalyzes the transamination of 2-ketoisovalerate to produce L-valine. The *ilvE* was overexpressed in *K. pneumoniae* Δ*budA*-Δ*ldhA*-Δ*ipdC* to obtain *K. pneumoniae* Δ*budA*-Δ*ldhA*-Δ*ipdC*-*ilvE* (*K. pneumoniae* -*ilvE*). This strain was cultured in flasks, and the results are shown in Fig. 3.

The cell growth and glucose consumption rate of *K. pneumoniae* -*ilvE* was slower than that of *K. pneumoniae* Δ*budA*-Δ*ldhA*-Δ*ipdC*. The amount of 30 g/L of glucose was exhausted after 30 hours of cultivation. The L-valine yield produced by this strain was distinctly improved, a final L-valine titer of 5.7 g/L was obtained. The 2-ketoisovalerate reached its peak level after 12 hours with a titer of 1.1 g/L. It was interesting that 0.37 g/L of isobutanol was produced by this strain. The isobutanol titer was higher than that of *K. pneumoniae* Δ*budA*-Δ*ldhA*-Δ*ipdC*, and even of *K. pneumoniae* Δ*budA*-Δ*ldhA*. It is obvious that the overexpression of *ilvE* influenced a distinct increase of the L-valine titers and reduced the 2-ketoisovalerate accumulation. However, the increase of isobutanol titers required further study.

L-valine production by *brnQ* over-expressed and knocked out strain

A gene *brnQ* encodes a branched-chain amino acid transporter and is responsible for the uptake of extracellular L-valine. *brnQ* was overexpressed or knocked out to obtain *K. pneumoniae* Δ*budA*-Δ*ldhA*-Δ*ipdC*-*brnQ* (*K. pneumoniae* -*brnQ*) and *K. pneumoniae* Δ*budA*-Δ*ldhA*-Δ*ipdC*-Δ*brnQ*. (*K. pneumoniae* -Δ*brnQ*). These strains were cultured in flasks, and the results are shown in Fig. 4.

A 2.3 g/L of L-valine was produced by *K. pneumoniae* -*brnQ*, which was lower than that of *K. pneumoniae* Δ*budA*-Δ*ldhA*-Δ*ipdC*, while L-valine level was increased to 3.9 g/L by *K. pneumoniae* -Δ*brnQ*. The cell growth of *K. pneumoniae* -Δ*brnQ* was weak compared with *K. pneumoniae* Δ*budA*-Δ*ldhA*-Δ*ipdC*. But glucose consumption rate was faster than that of *K. pneumoniae* Δ*budA*-Δ*ldhA*-Δ*ipdC*. A 2-ketoisovalerate peak level of *K. pneumoniae* -Δ*brnQ* was 2.1 g/L after 12 hours of cultivation. Acetic acid was a by-product with a final titer of 2.0 g/L. No isobutanol was detected in the broth of *K. pneumoniae* -*brnQ*, *K. pneumoniae* -Δ*brnQ*, or *K. pneumoniae* -Δ*brnQ*-*ilvE*. (data not shown). It can be concluded that the inhibition of the uptake of extracellular L-valine by knocking out of *brnQ* improved the L-valine production.

As the over-expression of *ilvE* improved L-valine production in *K. pneumoniae* Δ*budA*-Δ*ldhA*-Δ*ipdC*, the *ilvE* was further overexpressed in *K. pneumoniae* -Δ*brnQ* to obtain *K. pneumoniae* -Δ*brnQ*-*ilvE*. This strain was cultured in flasks and results are also shown in Fig. 4.

Like *K. pneumoniae* -*ilvE*, L-valine production by *K. pneumoniae* -Δ*brnQ*-*ilvE* had a remarkable improvement compared with that of *K. pneumoniae* -Δ*brnQ*. The final titer of L-valine reached 6.7 g/L, which was 1 g/L higher than that of *K. pneumoniae* -*ilvE*. The 2-ketoisovalerate peak value was 2.7 g/L, it was achieved after 20 hours of cultivation.

Further improving the *ilvE* expression levels by constructing a duplicate *ilvE* expression vector and

adjusting the IPTG levels were done. However, no further improvement of L-valine production was obtained, and therefore data is not shown.

Optimization of fermentation parameters

Culture pH optimization

Culture pH and oxygen supplementation are two key parameters of bioreactor processes. Here, they were optimized in experiments performed in bioreactors. *K. pneumoniae -ilvE* was cultured in 5 L bioreactor with fermentation medium. The stirring rate was kept at 600 rpm, and the airflow rate was set at 2 L/min, while the culture pH was varied and examined at 5.5, 6.0, 6.5, and 7.0, respectively. Fermentation results are shown in Fig. 5.

The cell growth and glucose utilization in bioreactors were significantly faster than that in shaking cultures. There was a positive correlation between cell growth and pH value in the studied experimental range. Culture pH at 5.5 and 6.5 had the lowest and highest cell densities, respectively, with the values of 11.3 and 16.6 OD units. Glucose consumption rates were similar in the range of pH 5.5-6.5, in both cases, it took 16 hours to consume the 30 g/L glucose. The fastest glucose consumption rate was at pH 7.0, and the glucose was exhausted after 12 hours of cultivation at pH 7.0. The results have shown that the weak acidic conditions favored L-valine production since 10.1 and 7.8 g/L of L-valine were produced at the culture pH 6.0 and 5.5, respectively. The production was decreased to 3.9 g/L at a culture pH of 6.5. The lowest titer of L-valine of 0.8 g/L was detected at pH 7.0. Low levels of 2-ketoisovalerate were detected at culture pH of 5.5 and 6.0 after 15 or 23 hours of cultivation, while this was not detected at other culture pH conditions. Levels of 2-ketoisovalerate accumulated at the weak acidic conditions coincided with that of optimal L-valine production. Acetic acid production was positively correlated with the culture pH. The amounts of 1.1 and 5.8 g/L acetic acids were produced at

culture pH 5.5 and 7.0, respectively. Therefore, pH 6.0 was selected as the optimal culture pH.

Oxygen supplementation

Oxygen supplementation is generally affected by the stirring rate and air-flow rate. Here, different stirring rates were used to get different oxygen supplementation. *K. pneumoniae -ilvE* was cultured in 5 L bioreactor with glucose as a carbon source. The culture pH and air-flow rate were set at 6.0 and 2 L/min, respectively. The stirring rate was examined at 300, 400, 500, 600, 700, and 800 rpm. Fermentation results are shown in Fig. 6.

Cell growth and glucose consumption had a positive relationship with the stirring rate. By employing the higher stirring rates, the higher cell densities and glucose consumption rates were achieved. The lowest and highest cell density reached 8.6 and 14.8 OD units at 300 rpm and 700 rpm, respectively. A cell density of 12.4 OD units was obtained at 800 rpm after 10 hours of cultivation. However, the cell growth was impeded by the depletion of glucose. At this time point, the cell density was significantly reduced. High L-valine titers were detected at the increased stirring rates between 300 rpm and 600 rpm. Further increasing the stirring rate reduced the L-valine titer. The highest level of 10.1 g/L of L-valine was obtained at a stirring rate of 600 rpm. The highest and lowest titers of 2-ketoisovalerate were obtained at 300 rpm and 800 rpm, respectively. Acetic acid was produced within the whole experimental range, and its titers were not very regular. According to the results, medium aerobic conditions were favorable for L-valine production and thus the stirring rate of 600 rpm was selected as the optimal.

Fed-batch fermentation

After the culture parameters were optimized, fed-batch fermentations were performed to increase the final L-valine titers. Previously determined as the best candidates for L-valine production, *K.*

pneumoniae -ilvE and K. pneumoniae -ΔbrnQ-ilvE were cultured under the optimal conditions. The initial glucose concentration was 30 g/L, 50 g glucose was fed to the bioreactor after the glucose levels decreased below 5 g/L. Fermentation results are shown in Fig. 7.

During the fed-batch fermentation of *K. pneumoniae -ilvE*, the highest cell density of 13.6 OD units was achieved after 27 h of cultivation, after that the cell density began to stabilize. 24 g/L of glucose was consumed by the cells in the first 12 h of cultivation. A pulse of highly concentrated glucose solution was added to the medium at 12, 14, 30, and 36 hours of cultivation. Coinciding with cell growth, the glucose consumption rate was gradually decreased after 30 h. L-valine titers increased to 15.2 g/L after 27 hours. Its concentration was not increased till 60 hours of cultivation. Acetic acid was mainly synthesized during 14-27 hours of cultivation and stretched to a final titer of 5.8 g/L. After 14 hours of cultivation, 2-ketoisovalerate was synthesized, and a peak value of 4.5 g/L was reached after 36 hours. 4.2 g/L of 2-ketoisovalerate was still in the broth at the end of the process. This was different from a batch fermentation, where all 2-ketoisovalerate was reused by the cells.

K. pneumoniae -ΔbrnQ-ilvE grew rapidly during the first 32 hours of cultivation and the highest cell density of 18.1 OD units was reached. The amount of 30 g/L of glucose was consumed by the cells after 12 hours. A pulse of highly concentrated glucose solution was added to the medium at 12, 20, 29, 36, and 47 hours of cultivation. The concentration of L-valine in the broth was continuously increasing, and 22.4 g/L of L-valine was achieved after 55 hours. 7.7 g/L of 2-ketoisovalerate was produced at the end of the process. Similar to fed-batch cultivation of *K. pneumoniae -ilvE*, 2-ketoisovalerate was not reused by the cells. The concentration of acetic acid produced in the process was 7.7 g/L. For the whole process, the conversion rate of glucose to L-valine was 0.27 mol/mol.

Discussion

Knock out of *budA* resulted in L-valine production

The main metabolite of wild-type *K. pneumoniae* was 2,3-butanediol, and no L-valine was detected in the broth of this bacterium. This is common for most microorganisms, and the concentration of amino acids synthesized by the cell would not exceed its usage (Kinoshita 1959). The first step of the 2,3-butanediol synthesis pathway shares the same step with the branched-chain amino acid synthesis. A gene *budB* is encoding an α-acetolactate synthase which catalyzes α-acetolactate formation in the 2,3-butanediol synthesis pathway. Acetohydroxy acid synthase I, II, and III encoded by *ilvBN*, *ilvGM*, and *ilvIH* are enzymes of the branched-chain amino acid synthesis pathway and catalyze α-acetolactate formation. Acetohydroxy acid synthase and α-acetolactate synthase are isozymes. Among them, α-acetolactate synthase is mainly responsible for the α-acetolactate formation in *K. pneumoniae* (Gu et al. 2017). It can be seen that the 2,3-butanediol and branched-chain amino acid synthesis are crossed with the same intermediate-α-acetolactate. However, the two pathways are existing in parallel and do not interfere with each other in the wild-type *K. pneumoniae*.

The activity of acetohydroxy acid synthase I and III inhibited L-valine formation in engineered *E. coli*, thus the production of L-valine did not exceed the cell consumption (Lawther et al. 1981). Overexpression of the key enzymes of the synthetic pathway, such as *ilvBNCED*, was a common strategy to improve the efficiency of the process during glucose-based fed-batch production using engineered *E. coli* (Park et al. 2011a, b) and *C. glutamicum* (Buchholz et al. 2013). In our current study, in engineered *K. pneumoniae ΔbudA* the 2,3-butanediol synthesis pathway was blocked. However, the activity of *budB* encoding α-acetolactate synthase in the cell was not affected. All α-acetolactate synthesized in the cell flowed into the branched-chain amino acid synthesis pathway thus enabling that

the L-valine production exceeded the cell usage and resulted in the L-valine accumulation (Fig. 1). This mechanism is the same as in our previous research dealing with 2,3-dihydroxyisovalerate and 2-ketoisovalerate production by *K. pneumoniae* (Gu et al. 2017). Many microorganisms hold 2,3-butanediol synthesis pathway, and some strains of *K. pneumoniae* (Ma et al. 2009), *Bacillus subtilis* (Fu et al. 2016), *Enterobacter cloacae* (Yang et al. 2020) have very high 2,3-butanediol producing ability. These strains might be very good chassis cells for the construction of L-valine-producing strains according to the same strategy.

Disruption of *ldhA* blocks the lactic acid synthesis pathway and improves L-valine synthesis

Lactic acid is synthesized from pyruvate under the control of *ldhA* gene which encodes lactate dehydrogenase in most bacteria (Yang et al. 2020). Pyruvate is the substrate used for α-acetolactate synthesis. The *ldhA* deletion significantly decreased lactate production, and in that case the L-valine synthesis was improved. Pyruvate is a central metabolite of the cell; lactic acid, formic acid, and acetic acid are all synthesized from pyruvate. Removal of these organic acids increased the metabolic flow of 2,3-butanediol in *K. pneumoniae* (Guo et al. 2014; Rathnasingh et al. 2016; Jung et al. 2014). As L-valine and 2,3-butanediol synthesis pathways share the same first step, an improvement of L-valine production could be expected in the strains with *ldhA* deletion.

The experimental results have shown that lactic acid produced by wild-type *K. pneumoniae* was not particularly high, the knock-out of *ldhA* had little influence on 2,3-butanediol production and did not lead to L-valine accumulation, this agreed with the previous reports (Gu et al. 2017). However, lactic acid concentrations were increased to a high level in the strain with the blocked 2,3-butanediol synthesis pathway. Thus, it can be concluded that the release of lactic acid synthesis was important for L-valine production. This validated our previous research dealing with 2,3-dihydroxyisovalerate,

2-ketoisovalerate, and isobutanol production by *K. pneumoniae* (Wang et al. 2020; Gu et al. 2017).

Block isobutanol synthesis pathway improve L-valine production

Besides as the precursor of L-valine, 2-ketoisovalerate can be converted to isobutyraldehyde by indole-3-pyruvate decarboxylase controlled by *ipdC* gene, and further converted to isobutanol (Gu et al. 2017; Atsumi et al. 2008). In a previous work of isobutanol production, the *ipdC* was over-expressed to improve the isobutanol titer (Gu et al. 2017). Here, the isobutanol synthesis pathway was blocked to enable higher 2-ketoisovalerate flow into L-valine synthesis. Though a low level of isobutanol was still synthesized by *K. pneumoniae* Δ*budA*-Δ*ldhA*-Δ*ipdC*, the level of accumulation of 2-ketoisovalerate and L-valine concentration of this strain were improved.

Increase L-valine production through overexpression of *ilvE*

IlvE is encoding a transaminase B in bacteria (Radmacher et al. 2002). The transaminase B reversibly transfers one α-amino group from aliphatic amino acids such as glutamate to α-ketoisovalerate, leading to the formation of L-valine and α-ketoglutarate. Overexpression of *ilvE* in a pyruvate-dehydrogenase negative *C. glutamicum* strains avoids intracellular and extracellular accumulation of ketoisovalerate and leads to enhancement of L-valine synthesis (Bartek et al. 2010). In contrast, deletion of *ilvE* in a *C. glutamicum* strain improved isobutanol production (Hasegawa et al. 2020). Similarly, a 2-ketoisovalerate producing *C. glutamicum* strain was constructed by deletion of *ilvE* (Krause et al. 2010). Here, it was shown that the L-valine level was significantly improved by overexpression of *ilvE*. Accordingly, the 2-ketoisovalerate peak level was decreased in the broth of *K. pneumoniae -ilvE*. In fed-batch fermentation, a high level of 2-ketoisovalerate was accumulated. It indicated that the transammonia reaction was a bottleneck of the L-valine production process. Further attempt for improving the expression level of *ilvE* was done (data not shown) but did not get positive results. Thus,

further research is needed to solve this problem.

Blocking the branched-chain amino acid transporter improves L-valine production

Knockout or weakening of branched-chain amino acid transporters gene can increase the production of L-valine (Park and Lee 2010). *BrnQ* is a low-affinity branched-chain amino acid transporter gene (Anderson and Oxender 1978). A gene *brnQ* is encoding a branched-chain amino acid transporter, a hydrophobic protein composed of 426 amino acid residues. There are 12 hydrophobic fragments, which can form transmembrane α-spiral. Leucine, isoleucine, and valine are transported by this carrier and that transport is driven by the proton motive force (Stucky et al. 1995). The *brnQ* plays an important role in isoleucine uptake in *C. glutamicum*. Disruption of *brnQ* significantly reduced the isoleucine uptake rate (and improved L-valine production), while the overexpression of *brnQ* increased the isoleucine uptake rate (Tauch et al. 1998). The *brnQ* was found in the genome of *K. pneumoniae*. Here, *brnQ* was knocked out or overexpression *K. pneumoniae* strains were constructed, and they have shown a similar behavior like that in *C. glutamicum*.

Weak acidic and aerobic conditions favor L-valine production

The optimal culture pH for *K. pneumoniae* cells growth is neutral (Sun et al. 2014). The conversion rate of 2,3-butanediol production was high in the pH range of 5.5 - 6.5 (Zeng et al. 1990; Priya et al. 2016). It is already known that L-valine and 2,3-butanediol synthesis pathways share the first step and that the *budB* gene which encoded α-acetolactate synthase was mainly responsible for α-acetolactate formation in *K. pneumoniae* (Gu et al. 2017). Thus, it is reasonable to expect that the optimal culture pH for L-valine production would be similar to that of 2,3-butanediol production. This was also experimentally determined in our current study. The L-valine production by *K. pneumoniae* is the aerobic process. However, this is different from that of 2,3-butanediol production, which is performed

under microaerobic conditions (Chen et al. 2014). The oxygen supplementation of a process is related to the redox state of the product. In our previous works using a similar pathway, isobutanol production was performed at microaerobic conditions (Gu et al. 2017), while 2,3-dihydroxyisovalerate production was performed at aerobic conditions (Wang et al. 2020), similar to the L-valine production performed in this study. To sum up, this study proved its superior potential for L-valine production by *K. pneumoniae* engineering.

In a fed-batch production process with glucose as a feedstock, an engineered *E. coli* produced 60.7 g/L of L-valine, with a productivity of 2.06 g/L·h (Park et al. 2011a). An engineered *C. glutamicum* produced 86.5 g/L of L-valine in fed-batch fermentation, with an overall yield of 0.36 mol/mol glucose and a productivity of 1.6 g/L·h (Buchholz et al. 2013). Though L-valine produced by *K. pneumoniae* was less than *E. coli* and *C. glutamate*. This is the first report of L-valine production by *K. pneumoniae*, and a novel strategy for design and construction of L-valine producers that use 2,3-butanediol producers as the chassis cells is provided.

Conclusion

L-valine production by *K. pneumoniae* was explored here and it expands the product range that can be produced by *K. pneumoniae*. L-valine was produced by *K. pneumoniae* Δ*budA*, which pointed to the relationship between the 2,3-butanediol synthesis pathway and branched-chain amino acid synthesis pathway. This provides a novel strategy for the design and construction of L-valine producers that use 2,3-butanediol producers as the basic cells.

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Statements & Declarations

Funding

This study was funded by National Key R&D Program of China (Grant No. 2019YFE0196900), Natural Science Foundation of Shanghai (Grant No. 19ZR1463600), Royal Society-Newton Advanced Fellowship (Grant No. NAF\R2\180721).

Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

JH designed this study. QHW, JJG, LS, and WYJ conducted the research. LM, ZKJ, JPS, GL, FB, WSX and JH analyzed the data. QHW and JH wrote the manuscript. All authors read and approved the final manuscript.

Data availability

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethical approval

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

Consent to participate

Not applicable

Consent to publish

Not applicable

Tables

Table 1. Strains

Strains	Basic characteristics	Sources
E. coli DH5α	Host of plasmid	Lab stock
K. pneumoniae CGMCC 1.6366	(TUAC01) Wild type, Amp^r	(Hao et al. 2008)
Δ*budA*	Δ*budA*, Str^r	(Wei et al. 2013)
Δ*ldhA*	Δ*ldhA*, Apr^r	(Gu et al. 2017)
Δ*budA*-Δ*ldhA*	Δ*budA*, Δ*ldhA*, *Str^r*, Apr^r	(Gu et al. 2017)
Δ*budA*-Δ*ldhA*-Δ*ipdC*	Δ*budA*,Δ*ldhA*, Δ*ipdC*, Apr^r	(Gu et al. 2017)
Δ*budA*-Δ*ldhA*-Δ*ipdC*-*ilvE*	Δ*budA*, Δ*ldhA*, Δ*ipdC*, carries pDK6-*ilvE*, Apr^r, Kan^r	This work
Δ*budA*-Δ*ldhA*-Δ*ipdC*-*brnQ*	Δ*budA*, Δ*ldhA*, Δ*ipd*C, carries pDK6-*brnQ*, Apr^r, Kanr	This work
Δ*budA*-Δ*ldhA*-Δ*ipdC*-Δ*brnQ*	Δ*budA*, Δ*ldhA*, Δ*ipdC*, Δ*brnQ*, Str^r, Apr^r	This work
Δ*budA*-Δ*ldhA*-Δ*ipdC*-Δ*brnQ*-*ilvE*	Δ*budA*, Δ*ldhA*, Δ*ipdC*, Δ*brnQ*, carries pDK6-*ilvE*, Str^r, Apr^r, Kan^r	This work

Table 2. Plasmids

Plasmids	Basic characteristics	Sources
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)
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)
pDK6-*ilvE*	Kan^r, carries *ilvE*, 6.0 kp	This work
pDK6-*brnQ*	Kan^r, carries *brnQ*, 6.4kp	This work
pMD18-T-simple	Amp^r, TA cloning vector, 2692 bp	Takara
pMD18-T-Δ*brnQ*	Amp^r, Str^r, carries *brnQ*	This work

Table 3. Primers

Primer name	Sequence (5′-3′)
brnQ-up-s	CGGGGATCCTCTAGAGATATCGAGCGCTTCTATCGGGTGG
brnQ-up-a	GCAGCTCCAGCCTACAAAATTCATTACTGCCTGTGGATGTG
brnQ-FRT-s	ATTTTGTAGGCTGGAGCTGCTTCG
brnQ-FRT-a	TTGTCCTCAACGCCGGATTCCGGGGATCCGTCGA
brnQ-down-s	GAATCCGGCGTTGAGGACAAGC
brnQ-down-a	TGCCTGCAGGTCGACGATATCTAATGATGCCGAACCCGGC
ilvE-s	TTCACACAGGAAACAGAATTCATGACGACGAAAAAAGCTGATTAC
ilvE-a	TCCGCCAAAACAGCCAAGCTTTTACTTACTTACCGGATCCAACCA
pDK6-*brnQ*-s	TTCACACAGGAAACAGAATTCATGACCCATCAGTTAAAATCTCGC
pDK6-*brnQ*-a	TCCGCCAAAACAGCCAAGCTTTTAGTGCGCGCTGGAGGTC
pDK6-s	CGCTACGGCGTTTCACTTC
pDK6-a	CCAATACGCAAACCGCCTC
pMD18-T-s	ATCGTCGACCTGCAGGCA
pMD18-T-a	ATCTCTAGAGGATCCCCGGGT

Figure captions

Fig. 1. L-valine synthesis–related metabolic pathways of *K. pneumoniae*.

Solid line: one step of reaction; dash line: contains several steps of the reaction. EMP pathway: glycolytic pathway; *ldhA*, lactate dehydrogenase; *budB*, α-acetolactate synthase; *ilvBN*, *ilvGM*, *ilvIH*, acetohydroxy acid synthase; *budA*, α-acetolactate decarboxylase; *budC*, butanediol dehydrogenase; *ilvC*, acetohydroxy acid isomeroreductase; *ilvD*, dihydroxy acid dehydratase; *ilvE*, transaminase; *ipdC*, indole-3-pyruvate decarboxylase; *leuA*, 2-isopropylmalate synthase; *brnQ*, branched-chain amino acid transporter; Green barrier, cytomembrane.

Fig. 2. Cell growth and product formation of *K. pneumoniae* strains in shake flasks.

WT: Wild-type; ΔldhA: *K. pneumoniae* Δ*ldhA*; ΔbudA: *K. pneumoniae* Δ*budA*; ΔbudA-ΔldhA: *K. pneumoniae* Δ*budA*-Δ*ldhA*. Data points are the average of n = 3; error bars represent standard error about the mean.

Fig. 3 Cell growth and products formation of *K. pneumoniae* strains.

ΔbudA-ΔldhA: *K. pneumoniae* Δ*budA*-Δ*ldhA*; ΔbudA-ΔldhA-ΔipdC: *K. pneumoniae* Δ*budA*-Δ*ldhA*-Δ*ipdC*; -ilvE: *K. pneumoniae* Δ*budA*-Δ*ldhA*-Δ*ipdC*-*ilvE*. Data points are the average of n = 3; error bars represent standard error about the mean.

Fig.4 Cell growth and products formation of *K. pneumoniae* strains that with knocked out or overexpressed *brnQ*: -brnQ; *K. pneumoniae* Δ*budA*-Δ*ldhA*-Δ*ipdC*-*brnQ*; -ΔbrnQ; *K. pneumoniae* Δ*budA*-Δ*ldhA*-Δ*ipdC*-Δ*brnQ*; -ΔbrnQ-ilvE; *K. pneumoniae* Δ*budA*-Δ*ldhA*-Δ*ipdC*-Δ*brnQ*-*ilvE*. Data

points are the average of n = 3; error bars represent standard error about the mean.

Fig. 5 Cell growth and products formation of *K. pneumoniae* Δ*budA*-Δ*ldhA*-Δ*ipdC-ilvE* in bioreactors at different culture pH. The strain was grown in stirred tank bioreactors operated at an agitation rate of 600 rpm and an aeration rate of 2 L/min. Data points are the average of n = 3; error bars represent standard error about the mean.

Fig. 6 Cell growth and products formation of *K. pneumoniae* Δ*budA*-Δ*ldhA*-Δ*ipdC-ilvE* in the bioreactor with different stirring rates. The strain was grown in stirred tank bioreactors and the air-flow rate was set at 2 L/min, culture pH 6.0. Data points are the average of n = 3; error bars represent standard error about the mean. (600 rpm data were the same shown in Fig. 5)

Fig. 7 Cell growth and products formation of *K. pneumoniae* Δ*budA*-Δ*ldhA*-Δ*ipdC-ilvE* and *K. pneumoniae* Δ*budA*-Δ*ldhA*-Δ*ipdC-ΔbrnQ-ilvE* in fed-batch fermentation. The strain was grown in stirred tank bioreactors operated at an agitation rate of 600 rpm, culture pH 6.0, and an aeration rate of 2 L/min. Data points are the average of n = 3; error bars represent standard error about the mean.

Figures

Fig. 1

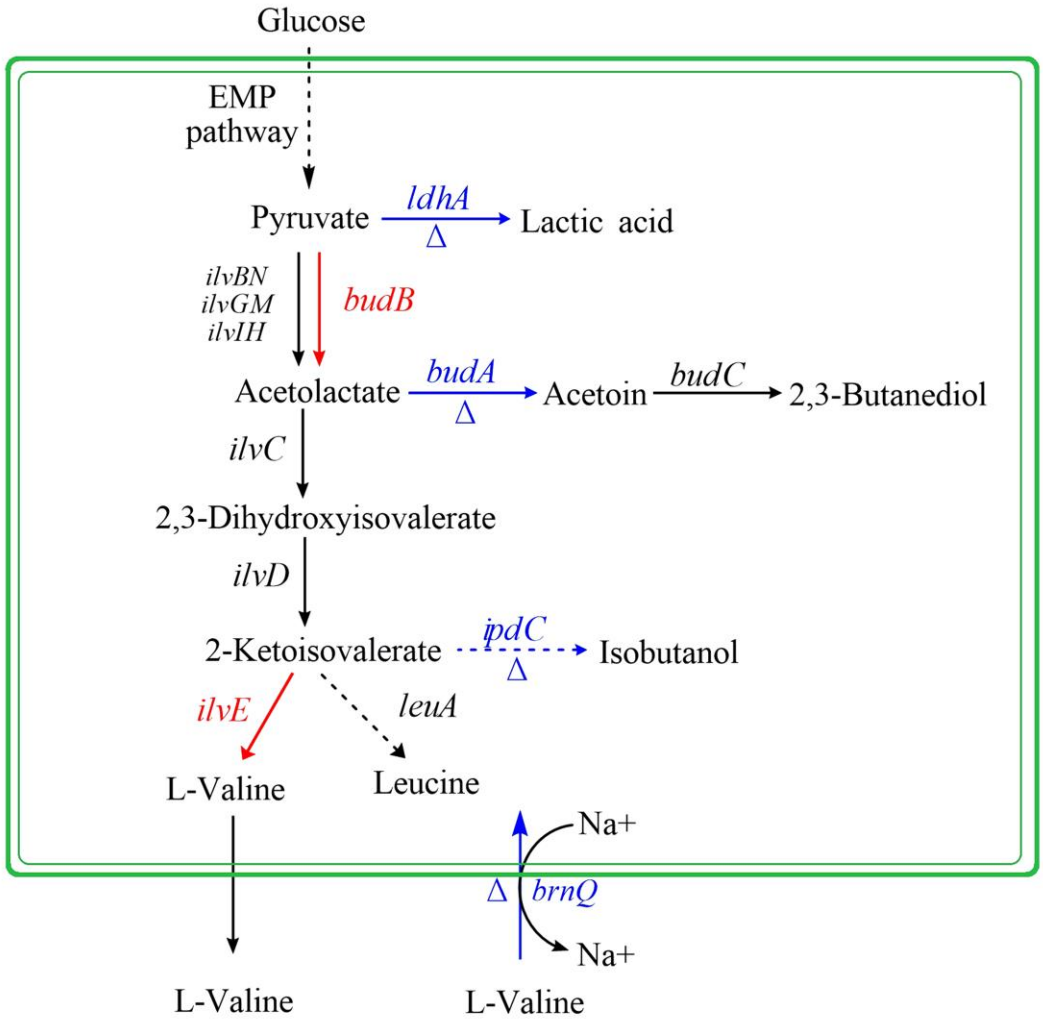


Fig. 2

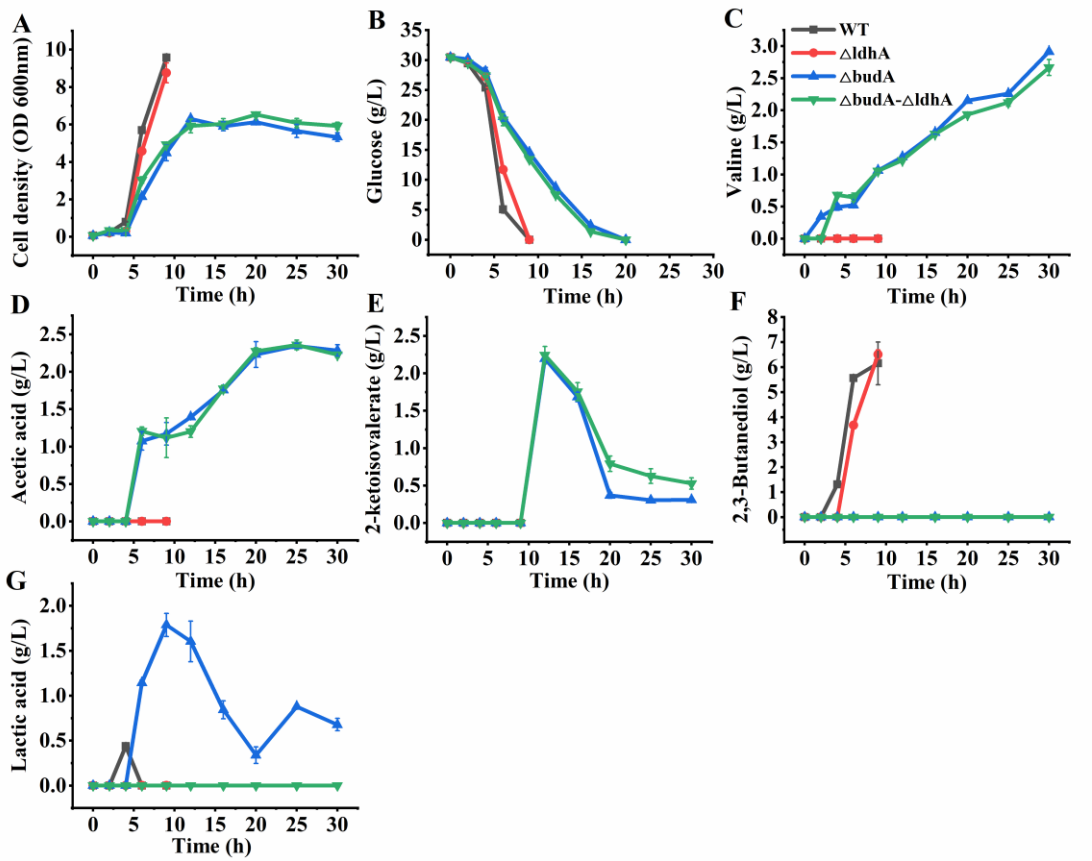

A
Cell density (OD 600nm)
Time (h)
B
Glucose (g/L)
Time (h)
C
WT
△ldhA
△budA
△budA-△ldhA
Valine (g/L)
Time (h)
D
Acetic acid (g/L)
Time (h)
E
2-ketoisovalerate (g/L)
Time (h)
F
2,3-Butanediol (g/L)
Time (h)
G
Lactic acid (g/L)
Time (h)

Fig. 3

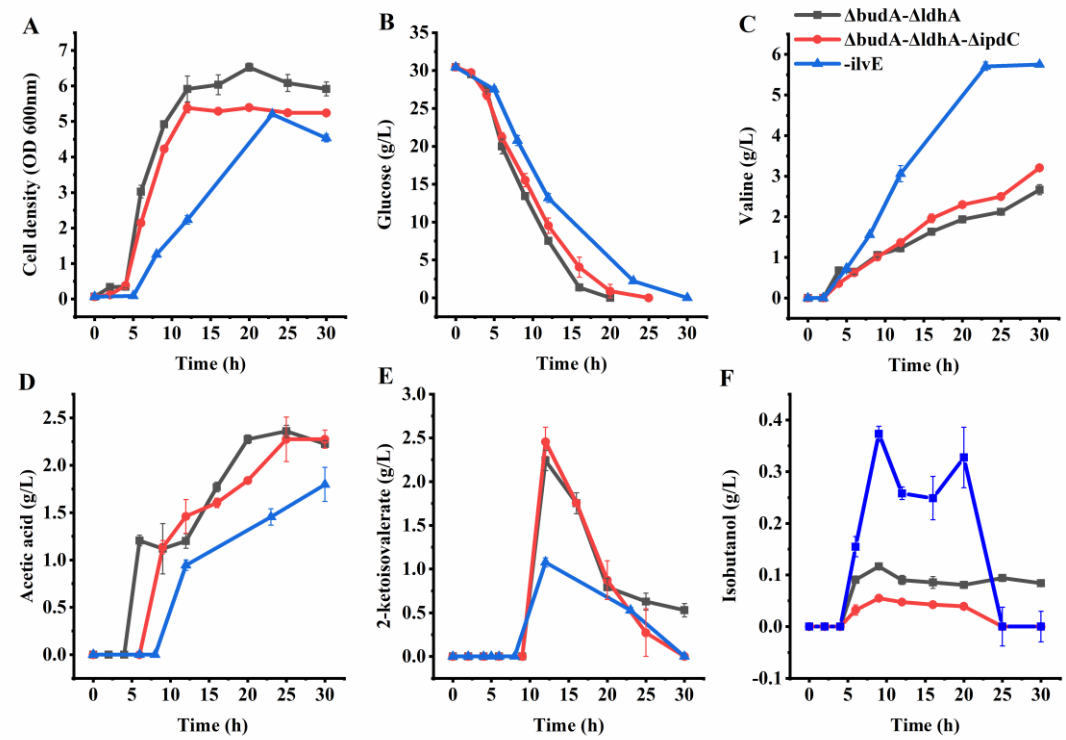

A
Cell density (OD 600nm)
Time (h)
B
Glucose (g/L)
Time (h)
C
ΔbudA-ΔldhA
ΔbudA-ΔldhA-ΔipdC
-ilvE
Valine (g/L)
Time (h)
D
Acetic acid (g/L)
Time (h)
E
2-ketoisovalerate (g/L)
Time (h)
F
Isobutanol (g/L)
Time (h)

Fig.4

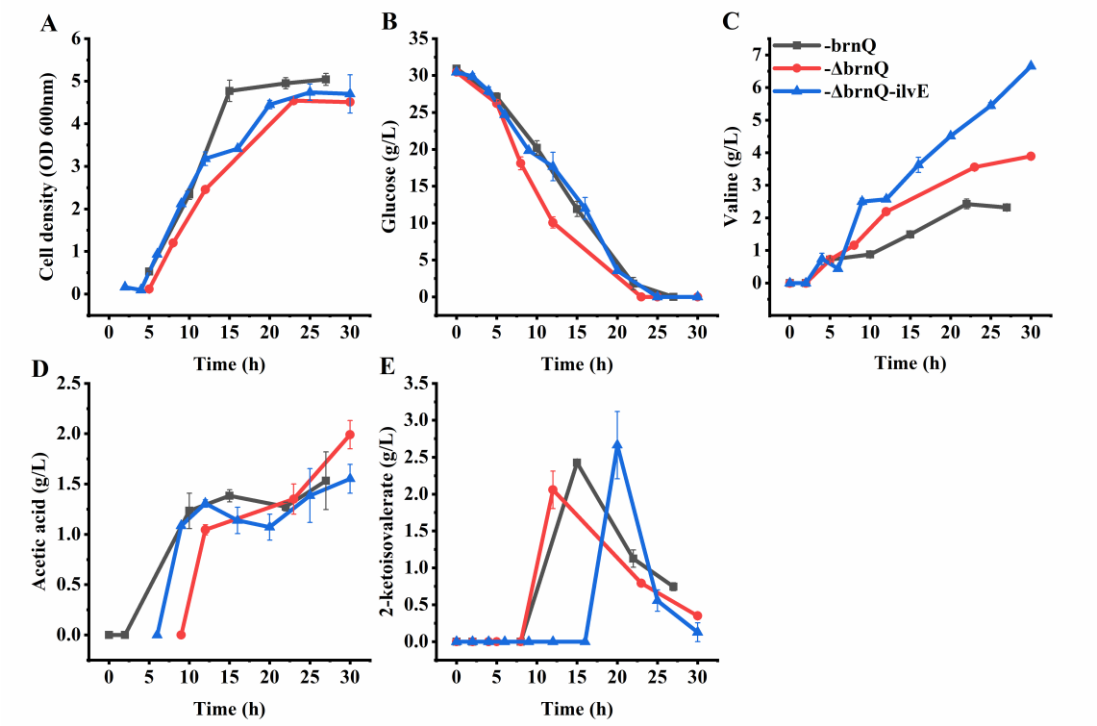

A
Cell density (OD 600nm)
Time (h)
B
Glucose (g/L)
Time (h)
C
-brnQ
-ΔbrnQ
-ΔbrnQ-ilvE
Valine (g/L)
Time (h)
D
Acetic acid (g/L)
Time (h)
E
2-ketoisovalerate (g/L)
Time (h)

Fig. 5

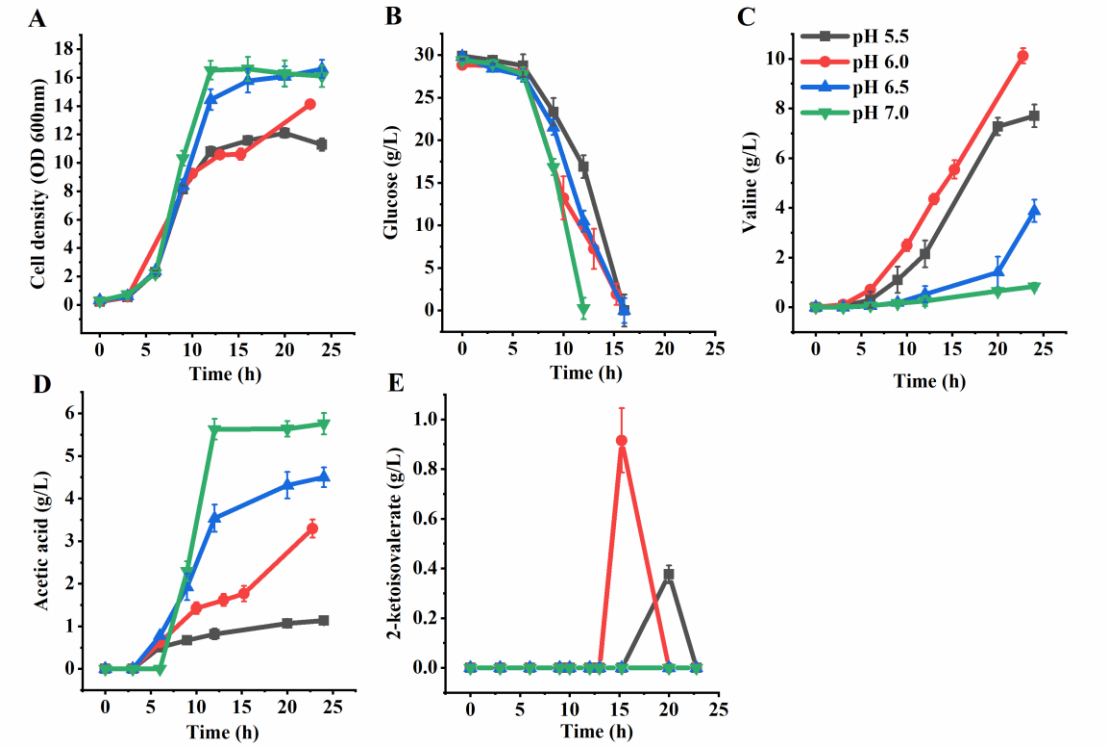

A
Cell density (OD 600nm)
Time (h)
B
Glucose (g/L)
Time (h)
C
pH 5.5
pH 6.0
pH 6.5
pH 7.0
Valine (g/L)
Time (h)
D
Acetic acid (g/L)
Time (h)
E
2-ketoisovalerate (g/L)
Time (h)

Fig. 6

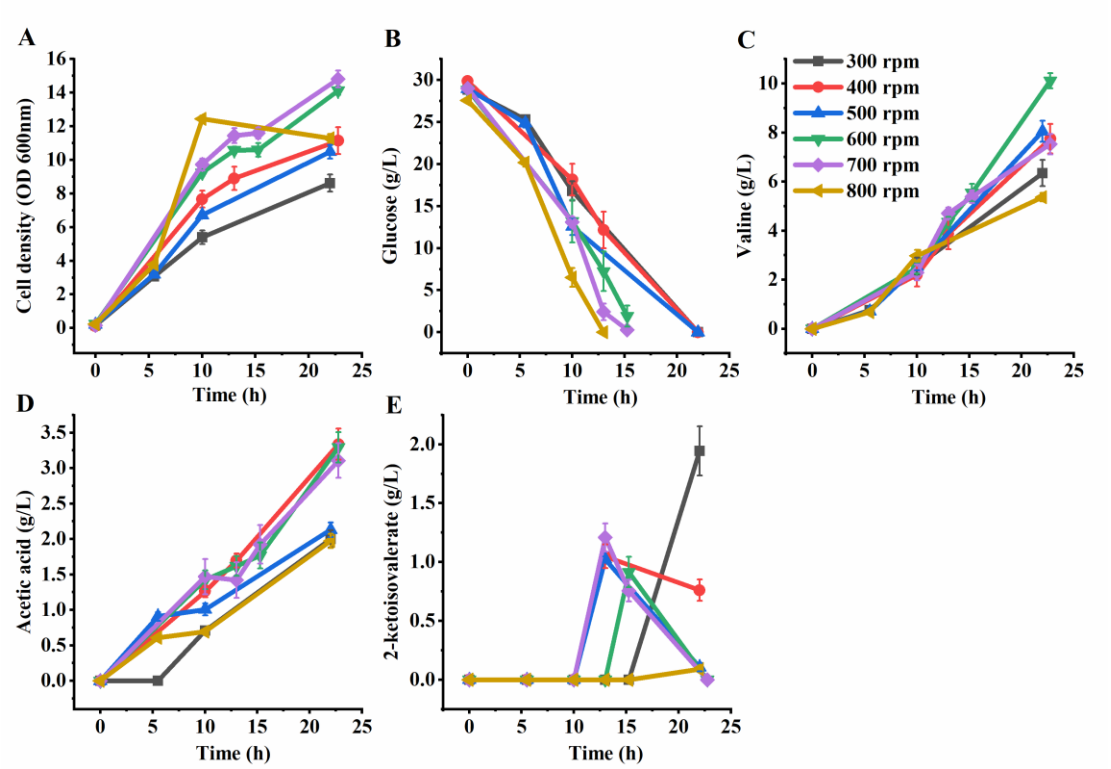
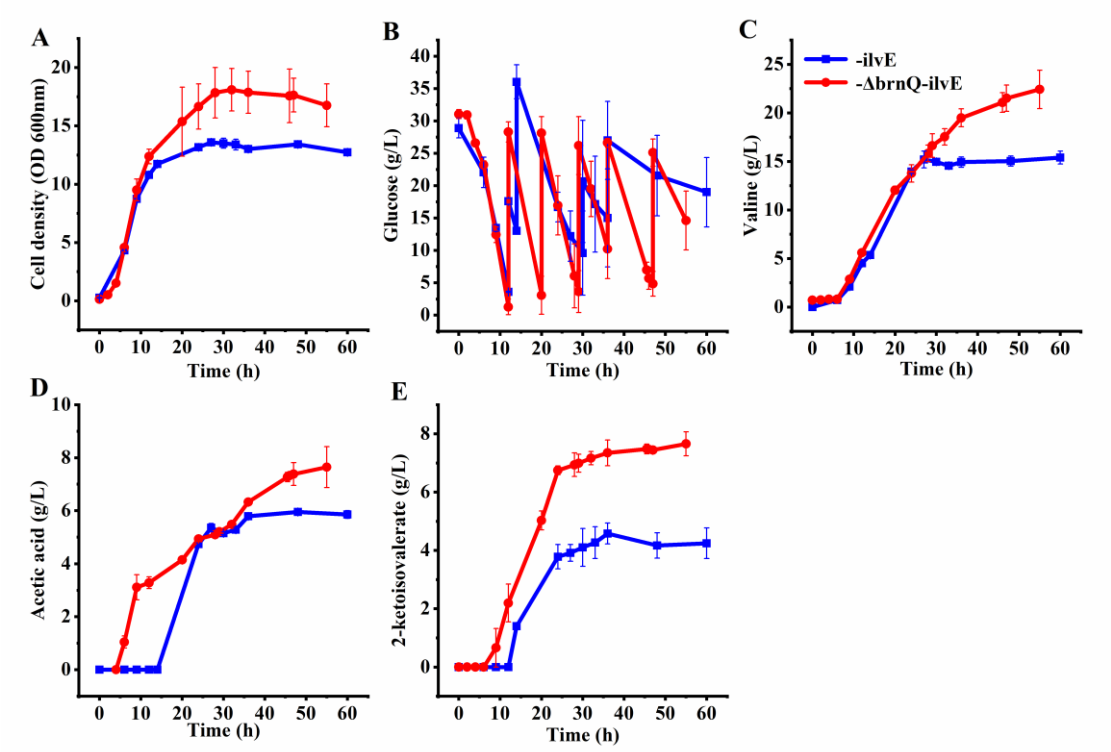

A
Cell density (OD 600nm)
Time (h)
B
Glucose (g/L)
Time (h)
C
300 rpm
400 rpm
500 rpm
600 rpm
700 rpm
800 rpm
Valine (g/L)
Time (h)
D
Acetic acid (g/L)
Time (h)
E
2-ketoisovalerate (g/L)
Time (h)

Fig. 7


A
Cell density (OD 600nm)
Time (h)
B
Glucose (g/L)
Time (h)
C
-ilvE
-ΔbrnQ-ilvE
Valine (g/L)
Time (h)
D
Acetic acid (g/L)
Time (h)
E
2-ketoisovalerate (g/L)
Time (h)