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Research article

Improving Engineered *Escherichia coli* strains for High-level Biosynthesis of Isobutyrate

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Abstract: Isobutyrate is an important platform chemical with various industrial applications. Previously, a synthetic metabolic pathway was constructed in *E. coli* to produce isobutyrate from glucose. However, isobutanol was found to be a major byproduct. Herein, gene knockouts and enzyme overexpressions were performed to optimize further the engineered *E. coli* strain. Besides *yqhD*, the knockouts of three genes *eutG*, *yiaY* and *ygjB* increased isobutyrate production in shake flasks. Furthermore, the introduction of an additional *padA* on a medium copy number plasmid under the constitutive promoter significantly reduced isobutanol formation. The IBA15-2C strain (BW25113, Δ*yqhD,* Δ*ygjB*; carrying two copies of *padA*) produced 39.2% more isobutyrate (0.39 g/glucose yield, 80% of the theoretical maximum yield) than IBA1-1C strain (BW25113, Δ*yqhD*; carrying one copy of *padA*). A scale-up process was also investigated for IBA15-2C strain to optimize the conditions for the production of isobutyrate in the fermentor. With $Ca(OH)_2$ as the base for pH control and 10% dissolved oxygen level, IBA15-2C strain produced 90 g/L isobutyrate after 144 h. This study has engineered *E. coli* to achieve biosynthesis of a nonnative compound with the highest titer and opened up the possibility of the industrial production of isobutyrate.

Keywords: synthetic biology; isobutyrate; isobutanol; fermentor; *E.coli*

1. Introduction

The extensive use of fossil fuels has led to the aggravation of environmental problems and the rapid depletion of natural reserves. To sustain the future development of human society, green and renewable processes to produce fuels and chemicals have to be explored. The U.S. Department of Energy has set goals to provide 30% of transportation fuels with biofuels, and to produce 25% of industrial chemicals from biomass by 2025 [1]. Though there are some successes in these aspects [2–10], most of chemicals are still not accessible from carbohydrates directly. To expand the scope of renewable chemicals, it is necessary to develop and optimize novel biosynthetic pathways to enlarge the metabolite repertoire of living systems.

Although there is no isobutyrate overproducer found in nature, it is possible to introduce an artificial synthetic metabolic pathway in *E. coli* to enable the production in light of our previous works [11]. Isobutyrate is an important platform chemical that is currently used to high volume industrial chemicals (more than 100 million pounds annually) such as emulsifier sucrose acetate isobutyrate, plasticizer 2,2,4-trimethyl-1,3-pentanediol monoisobutyrate (Texanol) or diisobutyrate (TXIB) [12,13]. Moreover, isobutyrate can be converted to 3-hydroxyisobutyrate by some microorganisms [14]. Since methacrylic acid is chemically obtained by either catalytic oxidative dehydrogenation of isobutyrate [15] or dehydration of 3-hydroxyisobutyric acid [16], biosynthesis of isobutyrate or 3-hydroxyisobutyrate will enable the sustainable production of the blockbuster molecule methyl methacrylate (MMA), an ester of methacrylic acid. MMA is produced in the quantity of 6 billion pounds annually for the synthesis of plastic materials such as polymethyl methacrylate (PMMA) [17].

The first generation *E. coli* strain we developed produced 11 g/isobutyrate from 40 g/L glucose in shake flask. The yield is 0.29 g/g glucose, only 59% of the theoretical maximum. Thus two concerns have to be immediately addressed for this process to be industrially relevant. Firstly, it is necessary to increase the carbon conversion from glucose to reduce the cost of raw material. Secondly, whether or not this process can be scaled up by a bioreactor determines its viability for the industrial production. In the present study, these challenges were solved by improving the engineered strains and optimizing the fermentation conditions.

A major byproduct of fermentation is isobutanol, with a level of 0.8 g/L representing a loss of 8.1% of the target isobutyrate product. As shown in Figure 1, the synthetic pathway starts from biosynthesis of ketovaline, which is converted to isobutyraldehyde by KIVD decarboxylase. Isobutyraldehyde is then oxidized into the target product isobutyrate by a promiscuous phenylacetaldehyde dehydrogenase

(PadA). However, endogenous alcohol dehydrogenases can compete with PadA and hence reduce the oxidation of isobutyraldehyde to isobutanol. There are six predicted alcohol dehydrogenases (AdhE, AdhP, EutG, YiaY, YqhD, and YjgB) in *E. coli* [18]. Except for YqhD, the other five dehydrogenases have not been characterized regarding their capabilities to produce isobutyrate. Our previous work demonstrated that *yqhD* knockout significantly reduced isobutanol accumulation and increased isobutyrate production [11]. Herein, two strategies were employed to further improve the yield of isobutyrate: inactivating one or more of the remaining five dehydrogenases and enhancing the cellular activity of aldehyde dehydrogenase PadA.

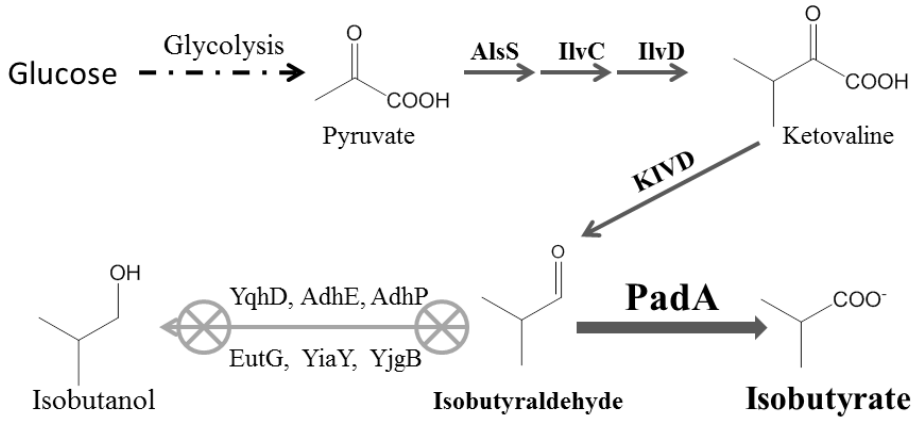

Figure 1. Artificial biosynthetic pathway for the production of isobutyrate in *E. coli*. Abbreviations: AlsS, acetolactate synthase from *Bacillus subtilis* (EC 2.2.1.6); IlvC, 2,3-dihydroxy-isovalerate: NADP+ oxidoreductase from *E. coli* (EC 1.1.1.86); IlvD, 2,3-dihydroxy-isovalerate dehydratase from *E. coli* (EC 4.2.1.9); KIVD, α-ketoisovalerate decarboxylase from *Lactococcus lactis* (EC 4.1.1.1); PadA, phenylacetaldehyde dehydrogenase from *E. coli* (EC 1.2.1.39).

2. Materials and Methods

2.1. Bacterial strains and plasmids

All primers were from Eurofins MWG Operon and were listed in Table 1. The *E. coli* strains used in this study were listed in Table 1, and derived from the wild-type *E. coli* K-12 strain BW25113 with *yqhD* deletion. All cloning procedures were carried out in the *E. coli* strain XL10-gold (Stratagene). Plasmids pIBA1 and pIBA7 used to produce isobutyrate were from previous works [11]. To construct the pIBA11 plasmid carrying two copies of *padA*, the *padA* gene was amplified by PCR with oligos padA_SacIfwd and padA_SacIrev, digested with *Sac*I and then ligated into pIBA7. In pIBA11, another

copy of *padA* was in the same operon with ampicillin resistance gene *bla*, under the regulation of a constitutive promoter. IBA1 strain with *yqhD* deletion was used as the start strain. Strains with their respective *adhE*, *adhP*, *eutG*, *yiaY* or *yjgB* deletion were obtained from the Keio collection [19]. The P1 phages were used to transfect the above each deletion into the IBA1 strain to construct double knockout strains according to the method provided by manufacturer. All knockout strains were then transformed with pCP20 plasmid to remove the kanamycin marker. The correct knockouts were verified by PCRs using detection primers listed in Table 1. To produce isobutyrate, each of knockout strains was transformed with plasmids pIBA1 plus pIBA7, or pIBA1 plus pIBA11.

Table 1. Strains, plasmids and primers used in this study.

Name	Relevant genotype	Reference
Strains		
BW25113	*rrnB*$_{T14}$ ΔlacZ$_{WJ16}$ *hsdR514* Δ*araBAD*$_{AH33}$ Δ*rhaBAD*$_{LD78}$	[20]
IBA1	BW25113 Δ*yqhD*	[11]
IBA11	BW25113 Δ*yqhD* Δ*adhE*	This work
IBA12	BW25113 Δ*yqhD* Δ*adhP*	This work
IBA13	BW25113 Δ*yqhD* Δ*eutG*	This work
IBA14	BW25113 Δ*yqhD* Δ*yiaY*	This work
IBA15	BW25113 Δ*yqhD* Δ*ygjB*	This work
IBA1-1C	BW25113 Δ*yqhD* + pIBA1 and pIBA7	This work
IBA11-1C	BW25113 Δ*yqhD* Δ*adhE* + pIBA1 and pIBA7	This work
IBA12-1C	BW25113 Δ*yqhD* Δ*adhP* + pIBA1 and pIBA7	This work
IBA13-1C	BW25113 Δ*yqhD* Δ*eutG* + pIBA1 and pIBA7	This work
IBA14-1C	BW25113 Δ*yqhD* Δ*yiaY* + pIBA1 and pIBA7	This work
IBA15-1C	BW25113 Δ*yqhD* Δ*ygjB* + pIBA1 and pIBA7	This work
IBA1-2C	BW25113 Δ*yqhD* + pIBA1 and pIBA11	This work
IBA13-2C	BW25113 Δ*yqhD* Δ*eutG* + pIBA1 and pIBA11	This work
IBA14-2C	BW25113 Δ*yqhD* Δ*yiaY* + pIBA1 and pIBA11	This work
IBA15-2C	BW25113 Δ*yqhD* Δ*ygjB* + pIBA1 and pIBA11	This work
plasmids		
pIBA1	p15A ori, Kan^R, P$_L$lacO$_1$::*alsS ilvD*	[11]
pIBA7	ColE1 ori, Amp^R, P$_L$lacO$_1$::*kivD padA*	[11]
pIBA11	ColE1 ori, Amp^R, P$_L$lacO$_1$::*kivD padA padA*	This work
Primers		
adhEKOC-F	TTGCTTACGCCACCTGGAAGT	

adhEKOC-R	GAACGGTCGCATGAGCAGAAAG
adhPKOC-F	TGACGATAATTTCTGGCAAGC
adhPKOC-R	GCAGGCTGACATTAAGTTCGT
eutGKOC-F	AGATTTGGCCTGCGGTGAAA
eutGKOC-R	CTGTTAGTTGTTATTTATTGGCGG
yiaYKOC-F	CATTTATTGCGCGACGCATTAT
yiaYKOC-R	ATAGCGGGCTTTTAACTTGAGG
yjgBKOC-F	CACTGAAGAGGTATGCGGAAAA
yjgBKOC-R	CTGGGCATTTTATGCCGGTAG
padA_SacIfwd	CTAGTAGAGCTCAAGGAGATATACCATGACAGAGCCGCATGTAGC
padA_SacIrev	GACTATGAGCTCTTAATACCGTACACACACCGACTTAGTT

2.2. Cell cultivation and shake flask fermentation

Unless otherwise stated, cells were grown in test tubes at 37 °C in 2×YT rich medium (16 g/L Bacto-tryptone, 10 g/L yeast extract and 5 g/L NaCl) supplemented with 100 mg/L ampicillin and 50 mg/L kanamycin. 200 μL of overnight cultures were transferred into 5 ml M9 minimal media supplemented with 5 g/L yeast extract, 40 g/L glucose, 100 mg/L ampicillin and 50 mg/L kanamycin in 125 ml conical flasks. Isopropyl-β-D-thiogalactoside (IPTG) was added at a final concentration of 0.1 mM to induce the overexpression of introduced genes for the production of isobutyrate. The fermentation broth was buffered by the presence of 0.5 g $CaCO_3$. Fermentation was carried out in a shake incubator with a vigorous agitation at 250 rpm, 30 °C.

2.3. Culture media in the fermentor

The seeding medium for *E. coli* culture, in grams per liter, is as follows: glucose, 10; $(NH_4)_2SO_4$, 1.8; K_2HPO_4, 8.76; KH_2PO_4, 2.4; sodium citrate, 1.32; yeast extract, 15; ampicillin, 0.1; kanamycin, 0.05. Fermentation medium for fermentor contains the following composition, in grams per liter: glucose, 30; $(NH_4)_2SO_4$, 3; K_2HPO_4, 14.6; KH_2PO_4, 4; sodium citrate, 2.2; yeast extract, 25; $MgSO_4.7H_2O$, 1.25; $CaCl_2.2H_2O$, 0.015, calcium pantothenate, 0.001; Thiamine, 0.01; ampicillin, 0.1; kanamycin, 0.05; and 1 ml/L of trace metal solutions. Trace metal solutions are (in grams per liter): NaCl, 5; $ZnSO_4.7H_2O$, 1; $MnCl_2.4H_2O$, 4; $CuSO_4.5H_2O$, 0.4; H_3BO_3, 0.575; $NaMoO_4.2H_2O$, 0.5; $FeCl_3.6H_2O$, 4.75; 6N H_2SO_4, 12.5 ml. The feeding solution contains, (in grams per liter): glucose, 600;

$(NH_4)_2SO_4$, 5; $MgSO_4.7H_2O$, 1.25; yeast extract, 5; $CaCl_2.2H_2O$, 0.015; calcium pantothenate, 0.001; Thiamine, 0.01; ampicillin, 0.1; kanamycin, 0.05, 0.2 mM of IPTG; and 1 ml/L of trace elements.

2.4. Fermentor culture conditions

Culture of *E. coli* was performed in a 1.3 liter Bioflo 115 fermentor (NBS, Edison, NJ USA) using a working volume of 0.6 liter. The fermentor was inoculated with 10% of overnight seeding cultures and then cells were grown at 37 °C, 10% dissolved oxygen (DO) level, and pH 7.0. After OD_{600} reached 8.0, 0.2 mM IPTG was added and the temperature was shifted to 30 °C to initiate isobutyrate production. The pH was controlled at 7.0 by automatic addition of 10 M sodium hydroxide solution, 50% v/v ammonia hydroxide, or 200 g/L calcium hydroxide suspension, respectively. Air flow rate was maintained at 1 vvm in each fermentation. DO was maintained at 10% with the saturated air by adjusting agitation speed from 300 to 800 rpm. The glucose level in the fermentor was maintained around 10 g/L by adding feeding medium automatically. When DO was over 40% and the isobutyrate yield did not increase, the fermentation was stopped. Samples at different time points were taken to determine the optical density and the concentrations of metabolites.

2.5. Metabolite analysis and dry cell weight determination

Fermentation products were analyzed using an Agilent 1260 Infinity HPLC equipped with an Aminex HPX 87H column (Bio-Rad, USA) and a refractive-index detector. The mobile phase is 5 mM H_2SO_4 with a flow rate 0.6 ml/min. The column temperature and detection temperature are 35 °C and 50 °C, respectively. Dry cell weight was determined by filtering 5ml samples through a 0.45 μm glass fiber filter (Michigan, USA). After removal of the culture medium, the filter was washed with 15 ml of MilliQ water, dried in an oven and then weighed. Dry cell weight was determined in triplicate.

2.6. Preparation of cell extracts and measurement of enzyme activity

The recombinant *E. coli* strains were cultivated in 2×YT medium to mid-logarithm phase at 37 °C, and then 0.1 mM IPTG was added to induce the protein expression. After induction for 4 h, the cells were harvested by centrifugation at 13,000×g for 5 min at 4 °C and washed with the sterilized water twice. The cells were then resuspended in a lysis buffer (50 μM Tris. HCl buffer, pH 7.0, 1 mM $MgSO_4$, 1mM DTT), and then the protein was extracted using a vortex with 200 μL glass beads. The resultant lysates were centrifuged at 14,000×g for 10 min at 4 °C, and the supernatants obtained were then analyzed for enzymatic activities towards isobutyraldehyde in a reaction buffer described in [21]. Enzyme concentrations in the supernatants were determined with a Bio-Rad protein analyzer. One unit

of enzyme activity was defined as the amount of enzyme that reduced or oxidized 1 μmol $NAD(P)^+$ or NAD(P)H per minute. These enzyme activities were determined with a Cary 50 MPR microplate reader coupled with a Cary 50 Bio UV-visible spectrophotometer (Varian, Inc.).

3. Results

3.1. Effect of alcohol dehydrogenases knockouts on isobutyrate production in shake flasks

Isobutyraldehyde is the immediate *precursor* of isobutyrate. It can be reduced to isobutanol by-product by endogenous alcohol dehydrogenases such as AdhE, AdhP, EutG, YiaY, YjgB and YqhD in *E. coli* [22,23]. YqhD has already been confirmed to play an important role in isobutanol formation since its deletion improved 50% of isobutyrate production [11]. However, even *yqhD* was knockout, isobutanol was still present as a major fermentation byproduct with a concentration of 0.8 g/L. Therefore, it is important to investigate if knockouts of the other five genes in addition to *yqhD* can decrease isobutanol formation and then increase isobutyrate production. According to the fermentation results, the isobutanol production in all the knockout strains was in the range of 0.7–0.9 g/L (Figure 2B), suggesting that the double knockout strains did not decrease the isobutanol production significantly. The enzymatic assay of alcohol dehydrogenase was not significantly different among all the studied strains, which indicated that none of the other alcohol dehydrogenases played a key role to reduce isobutanol formation like YqhD in the recombinant *E. coli* (Table 2). Deletion of *adhE* or *adhP* did not affected the isobutyrate production (Figure 2A, ii and iii). The knockout strains of *eutG*, *yiaY* and *ygjB* produced 12.2, 12.4 and 12.9 g/L isobutyrate, which was 10.7%, 12.3% and 17.5% more than the control strain (Figure 2A, iv–vi). The ratio between isobutyrate and isobutanol was significantly increased in *eutG*, *yiaY* and *ygjB* knockout strains, which was 16.1, 15.2 and 15.6 vs 13.4 in control strain.

Recently, one report used the same knockout strategy to investigate their effect on isobutylaldehyde production, and it was found that the knockouts of *eutG*, *yiaY* and *ygjB* significantly increased the isobutylaldehyde but not decrease the isobutanol significantly [24]. Because isobutylaldehyde is the direct precursor of isobutyrate, suggesting that the deletion of *eutG*, *yiaY* and *ygjB* did increase the carbon flux towards isobutyrate via isobutyraldehyde even with unknown details.

Table 2. Enzymatic assay results in different cell extracts.

Strain	Isobutanol dehydrogenase
IBA1(ΔyqhD)	0.12 ± 0.023
IBA11(Δ*yqhD* Δ*adhE*)	0.11 ± 0.023
IBA12(Δ*yqhD* Δ*adhP*)	0.11 ± 0.010
IBA13(Δ*yqhD* Δ*eutG*)	0.13 ± 0.013
IBA14(Δ*yqhD* Δ*yiaY*)	0.13 ± 0.026
IBA15(Δ*yqhD* Δ*ygjB*)	0.11 ± 0.024
Strain	**Isobutyraldehyde dehydrogenase**
IBA1-1C (single padA)	0.32 ± 0.03
IBA1-2C (double padA)	1.13 ± 0.14

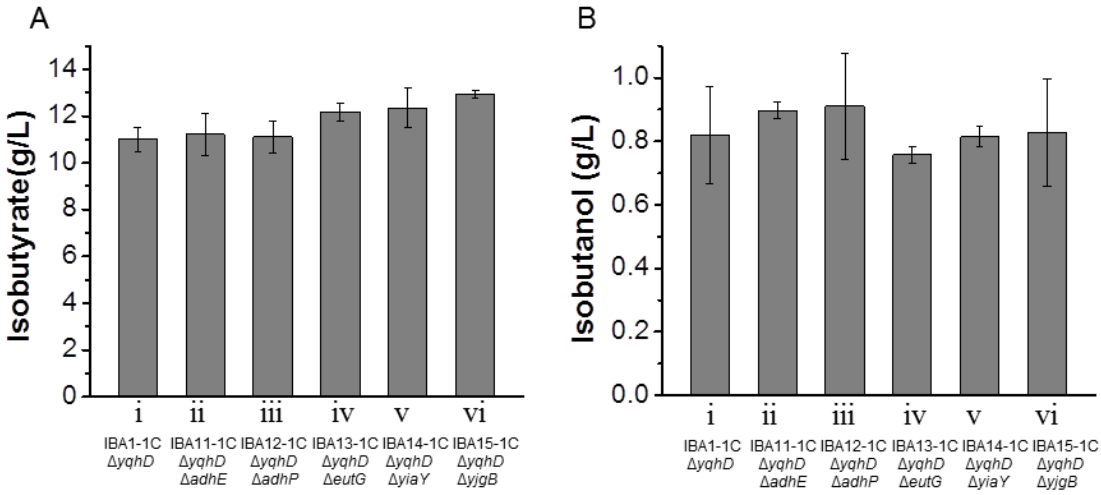

Figure 2. Effect of alcohol dehydrogenase knockouts on the isobutyrate production in shake flasks. (A) Isobutyrate production in different knockout strains. (B) Isobutanol formation in corresponding knockout strains.

3.2. Effect of PadA expression on the isobutyrate production

Since endogenous dehydrogenases AdhE, AdhP, EutG, YiaY, and YjgB cannot significantly decrease isobutanol formation, it may be a good selection to screen potential enzymes from other microorganisms to complete this process. However, it is time-consuming. In this study, an alternative approach was employed by increasing the expression level of PadA to be more competitive towards the substrate than other alcohol dehydrogenases. Therefore, another copy of *padA* was introduced into the plasmid pIBA7 under the control of the constitutive promoter, yielding the plasmid pIBA11. Strains IBA1-2C, IBA13-2C, IBA14-2C and IBA15-2C bearing the plasmid pIBA11 were chosen to investigate their fermentation performance.

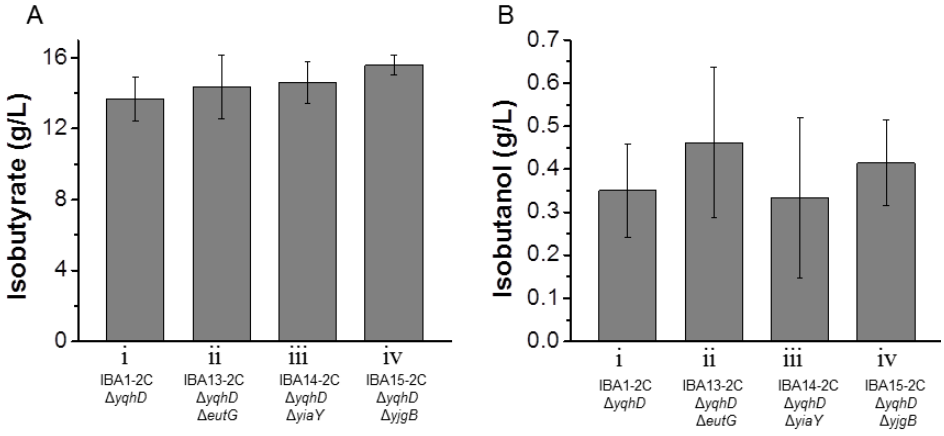

Figure 3. Effect of PadA expression on the isobutyrate production in shake flasks. (A) Isobutyrate titers in different knockout strains with two copies of PadA. (B) Corresponding isobutanol formation.

Results from shake flasks showed that all the strains carrying additional padA produced isobutanol in the range of 0.3–0.5 g/L, which decreased about 50% comparing to that in the strains with single copy padA (Figure 3B). However, the isobutyrate titer of IBA1-2C, IBA13-2C, IBA14-2C and IBA15-2C produced 13.7, 14.3, 14.6, and 15.6 g/L isobutyrate (Figure 3A), which was 24.4%, 17.7%, 18.3% and 20.6% higher than IBA1-1C, IBA13-1C, IBA14-1C and IBA15-1C, respectively. These results demonstrated that boosting the expression level of PadA did greatly decrease isobutanol accumulation and increase isobutyrate production. Compared to control strain IBA1-2C, strains IBA13-2C, IBA14-2C and IBA15-2C with *eutG*, *yiaY*, *ygjB* knockout produced 4.8%, 6.8% and 13.9% more isobutyrate. The production profiles of isobutanol and isobutyrate among these four strains were consistent with above results. Activities of PadA enzymes from recombinant strains are shown in Table 2. Obviously, the

activity of PadA from the IBA1-1C strain was much lower than those from the other strains. There was in good agreement with PadA activities and isobutyrate yields in these strains. Intriguingly, the decreased amount of isobutanol was less than the increased amount of isobutyrate. The possible reason may be that the production of isobutyrate is less stressful than that of isobutanol, so that cells produced some other byproducts and wasted less energy. For example, acetate was reduced from 0.6 g/L in IBA1-1C to 0.1 g/L in the IBA1-2C strain. The yield of isobutyrate from the best strain IBA15-2C was 0.39 g/g glucose, 80% of the theoretical maximum. However, there was still some isobutanol left in the fermentation medium. The future work can therefore be directed to further increase the expression level of PadA or identify unknown alcohol dehydrogenases in *E. coli*.

3.3. Optimization of fermentation conditions in a fed-batch bioreactor

To explore the possibility of the scale-up production of isobutyrate, fermentation experiments at the fermentor level with the strain IBA15-2C were performed. To avoid the over-accumulation of acetate in the fermentor, glucose feeding rate was adjusted to keep glucose at a level below 10 g/L [25]. Since two molecules of NADH were generated for each molecule of isobutyrate produced, dissolved oxygen (DO) level was maintained at 10% to burn excess NADH. Higher DO level was not used in order to prevent excessive oxidation of substrate into CO_2 through the TCA cycle.

During biosynthesis of isobutyrate, pH decreased sharply if the base was not added. The effect of three bases, NH_4OH, NaOH and $Ca(OH)_2$ which were used to maintain the pH at 7.0 on the production of isobutyrate was investigated. As shown in Figure 4A-C (closed square), for all bases used, the biomass increased exponentially at the first 20 h, and then decreased gradually. The maximum biomass obtained using both NH_4OH and NaOH was about 7.5 g/L, whereas it was about 10 g/L when $Ca(OH)_2$ was used. These results suggest that excessive ammonium or sodium ion may have a negative impact on the cell growth. Ammonia hydroxide was previously used to control pH as well as supply nitrogen source [26,27], but it is not apparently optimal for isobutyrate production. With NH_4OH, the final titer of isobutyrate was only 51.1 g/L after 140 h (Figure 4A, open circle). While the maximal titer was 65.4 g/L with NaOH (Figure 4B, open circle), and 90.3 g/L with $Ca(OH)_2$ (Figure 4C, open circle). The production of isobutyrate was inversely proportional to the acetate formation. Cultures adjusted by NH_4OH accumulated 12.6 g/L acetate, while only 7.1 g/L and 3.4 g/L of acetate accumulated in the cultures adjusted by NaOH and $Ca(OH)_2$, respectively (Figure 4A-C, closed up triangle). This was consistent with previous reports that acetate was a major inhibitor of *E. coli* fermentation [28,29]. In summary, using $Ca(OH)_2$ to adjust pH had many benefits: increasing cell density, improving isobutyrate titer and decreasing acetate byproduct.

As a control, the fermentation of a single gene *yqhD* knockout strain IBA1-2C in the fermentor was also investigated. This strain produced 57.6 g/L isobutyrate and 1.0 g/L acetate after 122 h (Figure 4D),

confirming that calcium hydroxide helps decrease acetate formation and increase isobutyrate production. However, IBA15-2C strain produced 57% more isobutyrate than IBA1-2C strain under the same condition, which suggested that *ygjB* knockout did significantly improve isobutyrate production in both shake flasks and the fermentor.

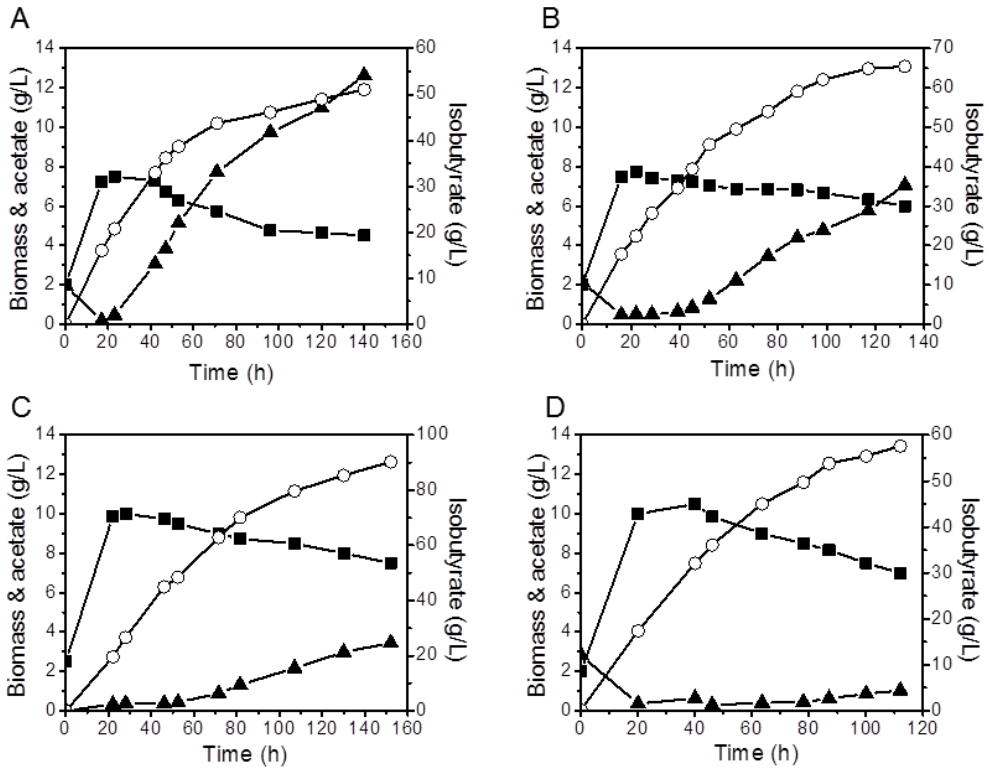

Figure 4. Scale-up fermentation of isobutyate by fed-batch culture in a bioreactor. (A) 50% NH4OH; IBA15-2C strain. (B) 10N NaOH; IBA15-2C strain. (C) 20% Ca(OH)2 suspension; IBA15-2C strain. (D) 20% Ca(OH)2 suspension, IBA1-2C strain. Symbols: closed square, biomass; closed up triangle, acetate; open circle, isobutyrate.

4. Discussion

Previously, our laboratory first reported the design of a synthetic metabolic pathway in *E. coli* to produce isobutyrate [11]. The purpose of this study was to investigate if this pathway could be further optimized to increase carbon conversion from glucose, and possible scale-up production in the fermentor. Among six knockout *E. coli* strains, one double knockout (Δ*yqhD*, Δ*yjgB*) produced 17% more isobutyrate than the single knockout strain (Δ*yqhD*). Furthermore, an additional copy of aldehyde dehydrogenase was introduced in a plasmid pIBA11 under the control of a constitutive promoter, which

significantly reduced isobutanol formation (50% less) and increased isobutyrate production (21% more). Thus, an engineered strain was successfully constructed with an isobutyrate yield of 0.39 g/g glucose, 80% of the theoretical maximum.

In addition, the fermentation process was also scaled-up from shake flask to 1 L fermentor. $Ca(OH)_2$ was found to be much better than NH_4OH and NaOH for pH adjustment during fermentation. $Ca(OH)_2$ increased cell density, decreased acetate formation and improved the final titer of isobutyrate to 90 g/L. Coincidently, a previous report showed that $Ca(OH)_2$ was superior to NaOH for lactate fermentation [30]. Since the specific mechanism for this phenomenon remains unknown, it is worth in the future to investigate the relevant biological details. The study will improve our fundamental understanding on how cells respond to specific cations as well as provide a guiding principle for fermentation processes which require a significant quantity of base addition.

The isobutyrate productivity decreased substantially during the fermentation process, with an initial volumetric productivity of 1 $g \cdot L^{-1} \cdot h^{-1}$, finally decreasing to about 0.22 $g \cdot L^{-1} \cdot h^{-1}$. There may be two reasons for this phenomenon. Firstly, increasing concentration of isobutyrate may inhibit enzymatic activities, glycolysis rate, cellular metabolism or membrane integrity. Secondly, aging cells may be less productive in the later fermentation stage. To make our process more economical, in the future we will need to boost the productivity by maximizing metabolic flux [31], improving *E. coli* tolerance to isobutyrate [32], increasing glycolysis rate [3] or enzymatic turnover rate.

5. Conclusion

This work demonstrated isobutyrate can be produced from engineered bacteria with a high titer of 90 g/liter. This is the highest titer among new biobased products reported for the past several years [2,5,33–38]. The production of isobutyrate is comparable to fermentation of lactate [39] or succinate [40], two of the most promising renewable chemicals currently in commercial production. In 2004, from a list of 300 candidates that can be produced from biomass, “top 12” chemical building blocks such as 3-hydroxypropionate and succinate were identified. So far, only a few of the “top 12” blocks that have achieved high-yield and high-titer biosynthesis are gaining momentum in commercial production. It is obvious that the “top 12” list should be a dynamic guide, and there are opportunities for new compounds to diversify the portfolio. Since isobutyrate described in this work is so amenable to microbial fermentation, it can serve as a new platform chemical. For example, besides existing applications, it can be reduced to isobutanol for biogasoline [2], or esterified to be green solvent [39].

Acknowledgements

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Conflict of Interest

All authors declare no conflicts of interest in this paper.

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