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Production of α-ketoisovalerate with whey powder by systemic metabolic engineering of *Klebsiella oxytoca*

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Abstract

Background Whey, which has high biochemical oxygen demand and chemical oxygen demand, is mass-produced as a major by-product of the dairying industry. Microbial fermentation using whey as the carbon source may convert this potential pollutant into value-added products. This study investigated the potential of using whey powder to produce α-ketoisovalerate, an important platform chemical.

Results *Klebsiella oxytoca* VKO-9, an efficient L-valine producing strain belonging to Risk Group 1 organism, was selected for the production of α-ketoisovalerate. The leucine dehydrogenase and branched-chain α-keto acid dehydrogenase, which catalyzed the reductive amination and oxidative decarboxylation of α-ketoisovalerate, respectively, were inactivated to enhance the accumulation of α-ketoisovalerate. The production of α-ketoisovalerate was also improved through overexpressing α-acetolactate synthase responsible for pyruvate polymerization and mutant acetohydroxyacid isomeroreductase related to α-acetolactate reduction. The obtained strain *K. oxytoca* KIV-7 produced 37.3 g/L of α-ketoisovalerate from lactose, the major utilizable carbohydrate in whey. In addition, *K. oxytoca* KIV-7 also produced α-ketoisovalerate from whey powder with a concentration of 40.7 g/L and a yield of 0.418 g/g.

Conclusion The process introduced in this study enabled efficient α-ketoisovalerate production from low-cost substrate whey powder. Since the key genes for α-ketoisovalerate generation were integrated in genome of *K. oxytoca* KIV-7 and constitutively expressed, this strain is promising in stable α-ketoisovalerate fermentation and can be used as a chassis strain for α-ketoisovalerate derivatives production.

Keywords α-Ketoisovalerate, *Klebsiella oxytoca*, Whey powder, Metabolic engineering, L-Valine

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Introduction

α-Ketoisovalerate is a branched-chain α-keto acid with diversified applications. For example, it can be used in treatment of hyperphosphatemia in uremic patients [1, 2]. In addition, α-ketoisovalerate can also be used in synthesis of functional substances such as L-valine [3], L-leucine [4] and vitamin B5 [5]. Currently, α-ketoisovalerate is primarily produced from potassium cyanide and formaldehyde via Heine's method [6]. However, this process involves cumbersome steps and use of toxic raw materials. The green production of α-ketoisovalerate from renewable resources by microbial fermentation is thus highly desirable. α-Ketoisovalerate serves as the precursor for L-valine biosynthesis and it cannot be accumulated in most of the natural microorganisms. Various microorganisms including *Escherichia coli* [7, 8], *Corynebacterium glutamicum* [9, 10], *Pseudomonas putida* [11], *Klebsiella pneumoniae* [12], and *Streptomyces cinnamonensis* [13] have been metabolic engineered for fermentative production of α-ketoisovalerate.

K. oxytoca is a Risk Group 1 organism exhibiting high growth rate and wide substrate spectrum [14–16]. It has been used in the fermentative production of many platform chemicals including 1,3-propanediol [17], 2,3-butanediol [18], D-lactate [17, 19], succinate [20], and L-valine [21]. For example, Cao et al. recently redirected the metabolic flux of *K. oxytoca* from 2,3-butanediol production to L-valine synthesis [21]. Briefly, the key enzymes responsible for 2,3-butanediol generation from α-acetolactate were deleted while the enzymes related to L-valine production from α-acetolactate including acetohydroxyacid isomeroreductase (AHAIR), dihydroxyacid dehydratase (DHAD), and leucine dehydrogenase (LeuDH) were overexpressed. The obtained strain *K. oxytoca* VKO-9 was able to generate 122 g/L of L-valine with a yield of 0.587 g/g [21].

Whey is a by-product of cheese and yogurt production. Its disposal without appropriate treatment may lead to serious pollution problems due to high biochemical oxygen demand (BOD) and high chemical oxygen demand (COD) [22]. Whey powder typically contains approximately 77% lactose [23], which is a utilizable carbon source of many microorganisms. Extensive research has explored the use of whey powder in fermentative production of pyruvate [14], citrate [24], fatty acids [25], and isobutanol [26]. Recently, Darwin et al. constructed a recombinant *E. coli* strain W1262 to produce α-ketoisovalerate from whey powder but the final concentration of α-ketoisovalerate was only 3.22 g/L [8].

In this study, the L-valine producing strain *K. oxytoca* VKO-9 (referred to as KIV-0) was metabolic engineered to produce α-ketoisovalerate from whey powder. The leucine dehydrogenase, which catalyzes the amination of α-ketoisovalerate into L-valine, was inactivated in *K. oxytoca* KIV-0 to accumulate α-ketoisovalerate. Then, branched-chain α-keto acid dehydrogenase catalyzing the oxidative decarboxylation of α-ketoisovalerate was inactivated to prevent α-ketoisovalerate degradation. Mutant AHAIR ($AHAIR^M$) catalyzing α-acetolactate reduction and α-acetolactate synthase (ALS) for α-acetolactate synthesis were also overexpressed to further enhance the α-ketoisovalerate production (Fig. 1). Efficient production of α-ketoisovalerate with a concentration of 40.7 g/L and a yield of 0.418 g/g from whey powder was realized with the obtained strain *K. oxytoca* KIV-7.

Methods

Chemicals

α-Ketoisovalerate was purchased from Sigma-Aldrich (Louis, Missouri, USA). Whey powder containing 77.0% lactose, 11.2% protein, 1.1% fat, 1.9% moisture and 8.2% ash was purchased from Kuoquan Biotech (Jinan, China). Crude whey containing 4.0% lactose, 0.66% protein, 0.01% fat, 94% moisture and 0.6% ash was purchased from BASHIKE MILK SHOP (Tianjin, China). Restriction enzymes were acquired from Thermo Fisher (USA). Polymerase chain reaction (PCR) primers were supplied by Beijing Tsingke Biotech Co., Ltd (Qingdao, China). T4 DNA ligase and DNA polymerase were procured from Thermo Fisher (USA) and Vazyme Biotech Co., Ltd (Nanjing, China), respectively. All other chemicals were of analytical grade and commercially available.

Bacterial strains, plasmids and medium

The strains and plasmids used in this study are listed in Table 1. *E. coli* and *K. oxytoca* were generally cultured at 37 °C in LB medium. Plasmids pEcgRNA and $pEcCas_{Cm}$ were employed for genes knock-in, knock-out, and promoter replacement in *K. oxytoca*. Chloramphenicol and spectinomycin were added at a concentration of 40 and 50 μg/mL, respectively. The α-ketoisovalerate production capabilities of different *K. oxytoca* strains were compared in medium containing 5 g/L yeast extract, 2 g/L KH_2PO_4, 3.3 g/L $(NH_4)_2SO_4$, 10 g/L $K_2HPO_4{\cdot}3H_2O$, 0.1 g/L $MgSO_4{\cdot}7H_2O$, and 1 mL/L multicomponent metal ion solution [14]. Glucose, lactose, or whey powder were added in the medium with appropriate amounts to achieve the desired concentrations. Crude whey supplemented with 5 g/L yeast extract, 2 g/L KH_2PO_4, 3.3 g/L $(NH_4)_2SO_4$, 10 g/L $K_2HPO_4{\cdot}3H_2O$, 0.1 g/L $MgSO_4{\cdot}7H_2O$, and 1 mL/L multicomponent metal ion solution [14] was also used as a culture medium for α-ketoisovalerate production.

DNA manipulation in *K. oxytoca*

The primers are listed in Table S1. Knock-in, knock-out, and promoter replacement of genes in *K. oxytoca* were conducted through CRISPR/Cas9 gene editing [27].

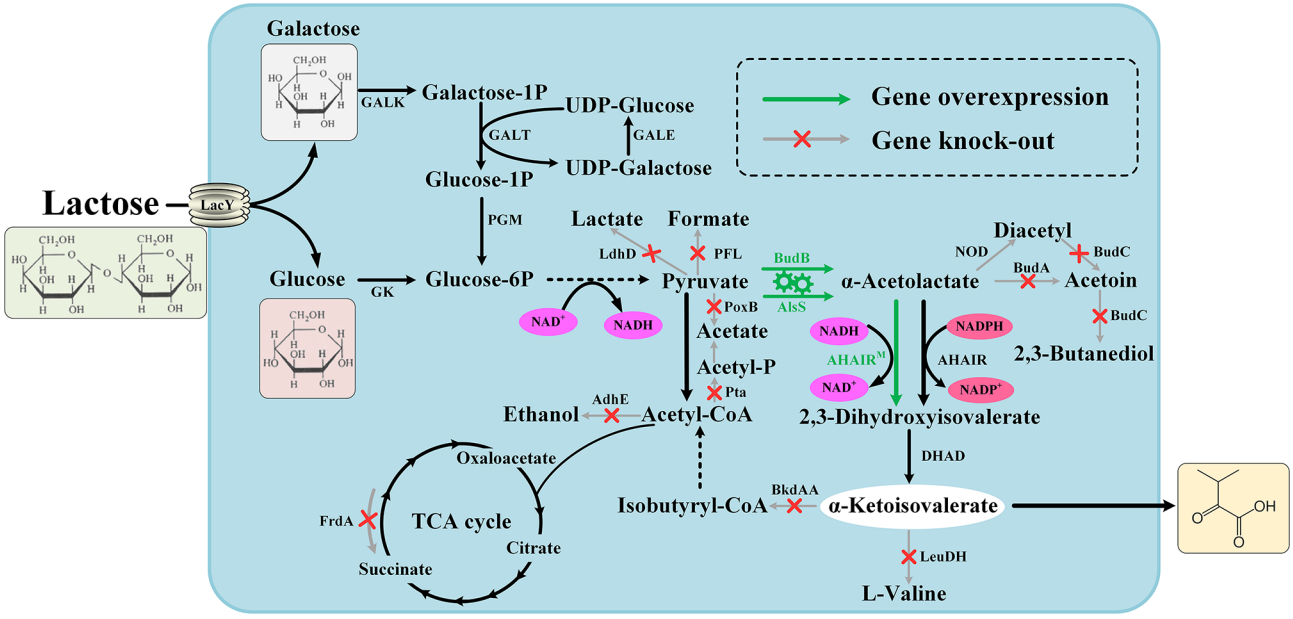

Fig. 1 Metabolic engineering strategies for α-ketoisovalerate production from lactose by recombinant *K. oxytoca*. NOD, non-enzymatic oxidative decarboxylation; GALT, galactose-1-phosphate uridylyltransferase; LacY, lactose permease; GALK, galactokinase; GK, glucose kinase; GALE, UDP-galactose-4-epimerase; PGM, phosphoglucomutase; Pta, phosphate acetyltransferase; PFL, pyruvate formate-lyase; LdhD, D-lactate dehydrogenase; PoxB, pyruvate oxidase; AdhE, ethanol dehydrogenase; FrdA, α-subunit of fumarate reductase. BudB, α-acetolactate synthase of *K. oxytoca*; AlsS, α-acetolactate synthase of *B. subtilis* 168; DHAD, dihydroxyacid dehydratase from *E. coli* W3110; AHAIR, acetohydroxyacid isomeroreductase of *E. coli* W3110; AHAIR^{M}, mutant acetohydroxyacid isomeroreductase of *E. coli* W3110; BudA, α-acetolactate decarboxylase; BudC, *meso*-2,3-butanediol dehydrogenase; BkdAA, α-subunit of branched-chain α-keto acid dehydrogenase complex; LeuDH, leucine dehydrogenase of *B. subtilis* 168

Herein, the replacement of *pox* encoding pyruvate oxidase with *budB* encoding α-acetolactate synthase under the *trc* promoter was taken as an example. The primer pair (gRNA-*pox*-1 and gRNA-*pox*-2) was annealed to form dsDNA targeting *pox*. Then, the dsDNA was ligated into linearized pEcgRNA using the T4 DNA ligase, resulting in plasmid pEcgRNA-Δ*pox*. The donor DNA, comprising homologous arms of *pox* and P_{trc}-*budB*, was obtained with the primer pairs Δ*pox*::P_{trc}-*budB*-1/Δ*pox*::P_{trc}-*budB*-2, Δ*pox*::P_{trc}-*budB*-3/Δ*pox*::P_{trc}-*budB*-4, and Δ*pox*::P_{trc}-*budB*-5/Δ*pox*::P_{trc}-*budB*-6 by overlap PCR. The donor DNA and the plasmid pEcgRNA-Δ*pox* were co-transformed into *K. oxytoca* harboring the plasmid pEcCas$_{Cm}$ via electrotransformation. Positive colonies were selected on LB agar plates with spectinomycin and chloramphenicol and identified by colony PCR. The verified positive colonies were then inoculated in LB medium containing chloramphenicol and rhamnose to cure the plasmid pEcgRNA-Δ*pox*. The appropriately diluted bacterial solution was then coated on LB agar plates containing 100 g/L sucrose and incubated at 37 °C overnight to eliminate the plasmid pEcCas$_{Cm}$. Antibiotic marker-free strains were identified as those growing only on LB agar plates. Other mutants of *K. oxytoca* were constructed by using the same procedure.

Batch fermentation and fed-batch fermentation

Batch fermentation with glucose as the carbon source and fed-batch fermentation with lactose or whey powder as the carbon source were both conducted under aerobic condition in a 1-L bioreactor (Infors AG, Bottmingen, Switzerland) with 0.8 L medium. The cultivation was conducted at 37 °C, with a stirring speed of 500 rpm and an aeration rate of 1.6 vvm. The pH was controlled at 6.8 by 10 M NaOH via a program-controlled peristaltic pump. In batch fermentation, the initial glucose concentration was controlled at 60.0 g/L. In fed-batch fermentation, the initial lactose concentration was about 45 g/L. When its concentration dropped below 5.0 g/L, lactose or whey powder was added to maintain the concentration at around 20 g/L.

Analytical methods

The concentrations of α-ketoisovalerate, pyruvate, acetate, and lactose were detected through high performance liquid chromatography (HPLC) [14, 21]. Glucose concentration was detected by an SBA-40D bioanalyzer (Shandong Academy of Sciences, China). The concentrations of acetoin and 2,3-butanediol were determined by gas chromatography (GC) (Shimadzu, GC2014c) with a capillary GC column [21].

Table 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Origin
Strain		
Bacillus subtilis 168	Wild-type	Lab stock
E. coli W3110	Wild-type	Lab stock
Klebsiella oxytoca PDL-0	Wild-type	Lab stock
K. oxytoca VKO-9 (*K. oxytoca* KIV-0)	*K. oxytoca* PDL-0 Δ*pox*Δ*pta*Δ*frdA*Δ*ldhD*::*ilvD*Δ*pflB*::*brnFE*Δ*adhE*::*leuDH*Δ*budA*::*ilvC*^{M}Δ*budC*::*alsS*Δ*gldA*::*ilvC*	Lab stock
K. oxytoca KIV-1	*K. oxytoca* KIV-0 Δ*leuDH*	This study
K. oxytoca KIV-2	*K. oxytoca* KIV-1 Δ*pox*::P_{trc}-*budB*	This study
K. oxytoca KIV-3	*K. oxytoca* KIV-2 Δ*bkdAA*	This study
K. oxytoca KIV-4	*K. oxytoca* KIV-3 ΔP_{bud}::P_{trc}-*ilvC*^{M}	This study
K. oxytoca KIV-5	*K. oxytoca* KIV-4 Δ*frdA*::P_{trc}-*budB*	This study
K. oxytoca KIV-6	*K. oxytoca* KIV-4 Δ*frdA*::P_{trc}-*ilvBN*	This study
K. oxytoca KIV-7	*K. oxytoca* KIV-4 Δ*frdA*::P_{trc}-*alsS*	This study
K. oxytoca KIV-8	*K. oxytoca* KIV-4 Δ*frdA*::P_{trc}-*alsS*^{M}	This study
Plasmid		
pEcCas	Kan^{r}, vector for constitutive expression of Cas9 and inducible expression of λ-Red recombinase, *sacB*, P_{rhaB}-sgRNA-pMB1, pSC101	Addgene
$pEcCas_{Cm}$	Cm^{r}, vector for constitutive expression of Cas9 and inducible expression of λ-Red recombinase, *sacB*, P_{rhaB}-sgRNA-pMB1, pSC101, derived from pEcCas	Lab stock
pEcgRNA	Spe^{r}, gRNA expression vector	Addgene
pEcgRNA-Δ*leuDH*	Spe^{r}, pEcgRNA derivative for transcribing sgRNA to knock out *leuDH*	This study
pEcgRNA-Δ*pox*	Spe^{r}, pEcgRNA derivative for transcribing sgRNA to replace *pox* with P_{trc}-*budB*	This study
pEcgRNA-Δ*bkdAA*	Spe^{r}, pEcgRNA derivative for transcribing sgRNA to knock out *bkdAA*	This study
pEcgRNA-ΔP_{bud}::P_{trc}	Spe^{r}, pEcgRNA derivative for transcribing sgRNA to replace the *bud* promoter with *trc* promoter	This study
pEcgRNA-Δ*frdA*	Spe^{r}, pEcgRNA derivative for transcribing sgRNA to replace *frdA* with P_{trc}-*budB*, P_{trc}-*ilvBN*, P_{trc}-*alsS*, and P_{trc}-*alsS*^{M}, respectively	This study

[a] Cm^{r}, chloramphenicol resistant; Kan^{r}, kanamycin resistant; Spe^{r}, spectinomycin resistant

Enzymatic assays

The α-acetolactate synthase activities in the crude extracts of different recombinant *K. oxytoca* strains were detected followed the procedure of Gao et al. with some modifications [28]. The reaction solution contained 40 mM pyruvate, 0.1 mM TPP, 0.1 mM FAD, 10 mM $MgCl_2$, and 50 μL appropriately diluted crude extract. The reaction was conducted at 37 °C and 120 rpm for 15 min and then pyruvate consumption was measured using HPLC. One unit of activity is defined as the amount of enzyme required to catalyze the condensation of 1 μmol of pyruvate per minute.

Results and discussion

Blocking L-valine synthesis to accumulate α-ketoisovalerate

K. oxytoca KIV-0 is a recombinant strain genetically engineered to produce L-valine from glucose [21]. α-Ketoisovalerate is the direct precursor of L-valine. Thus, *K. oxytoca* KIV-0 was chosen as the starting strain for α-ketoisovalerate production. The *leuDH* gene encoding an NADH-dependent leucine dehydrogenase (LeuDH) in *B. subtilis* 168 was previously integrated into genome of *K. oxytoca* KIV-0 to transform α-ketoisovalerate into L-valine [21]. This gene was knocked out to prevent the consumption of α-ketoisovalerate, resulting in strain KIV-1.

As expected, the inactivation of *leuDH* gene in strain KIV-0 resulted in accumulation of α-ketoisovalerate. *K. oxytoca* KIV-1 produced 8.09 g/L of α-ketoisovalerate from 60.0 g/L of glucose within 24 h, with a yield of 0.135 g/g (Fig. 2a). As shown in Fig. 2b, the main by-product generated during α-ketoisovalerate production by *K. oxytoca* KIV-1 was pyruvate (24.9 g/L). Actually, 42.5% of the carbon ratio was diverted to pyruvate production while only 20.9% of the carbon ratio was directed to α-ketoisovalerate accumulation (Fig. 2c). Other by-products, including acetate (0.686 g/L), acetoin (1.06 g/L), and 2,3-butanediol (0.266 g/L), were accumulated at rather low concentrations (Fig. 2b and c).

Increasing the expression of α-acetolactate synthase BudB to enhance α-ketoisovalerate generation

The initial step in α-ketoisovalerate biosynthesis is the α-acetolactate synthase catalyzed α-acetolactate generation from pyruvate. The endogenous α-acetolactate synthase BudB encoded by *budB* in *K. oxytoca* KIV-0 is responsible for polymerization of pyruvate in this L-valine producing strain [21]. Accumulation of pyruvate by *K. oxytoca* KIV-1 suggested that the activity of the endogenous BudB may not be sufficient to convert pyruvate into α-acetolactate. The *pox* gene in *K. oxytoca* encodes pyruvate oxidase, which catalyzes the oxidization of pyruvate with oxygen to produce acetate. Thus, the second copy of *budB* with constitutive *trc* promoter was introduced in *K. oxytoca* KIV-1 at the location of *pox* to simultaneously strengthen pyruvate condensation and decrease acetate generation.

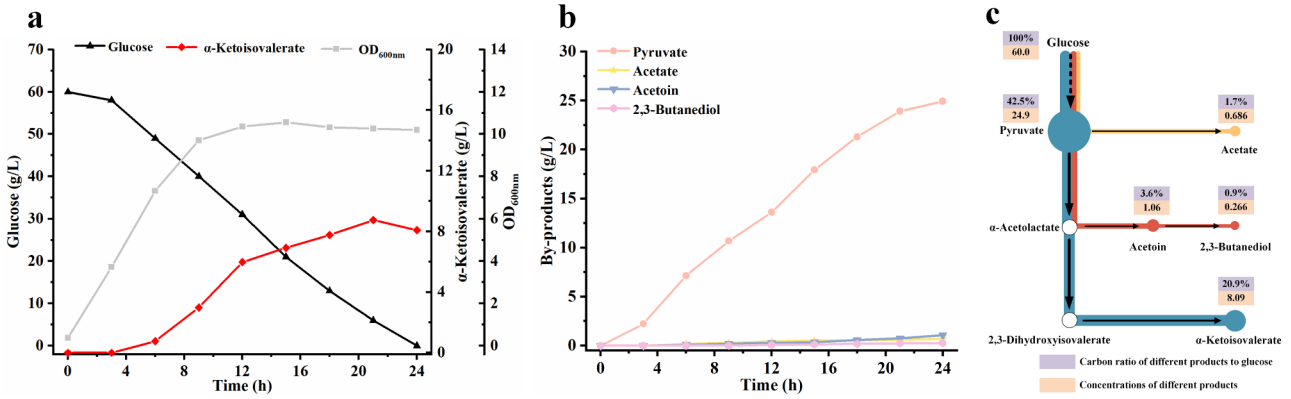

Fig. 2 Blocking L-valine synthesis to accumulate α-ketoisovalerate. (**a**) Biomass, substrate consumption, and α-ketoisovalerate generation of *K. oxytoca* KIV-1. (**b**) By-products generation by *K. oxytoca* KIV-1. (**c**) Metabolic flux of *K. oxytoca* KIV-1. Solid circles represent accumulated products; hollow circles indicate non-accumulated products; the area of the circles represents the carbon ratio of different products to glucose. The carbon ratio (%) was defined as the ratio of the theoretical amount of glucose required for production of different products to the total consumed glucose

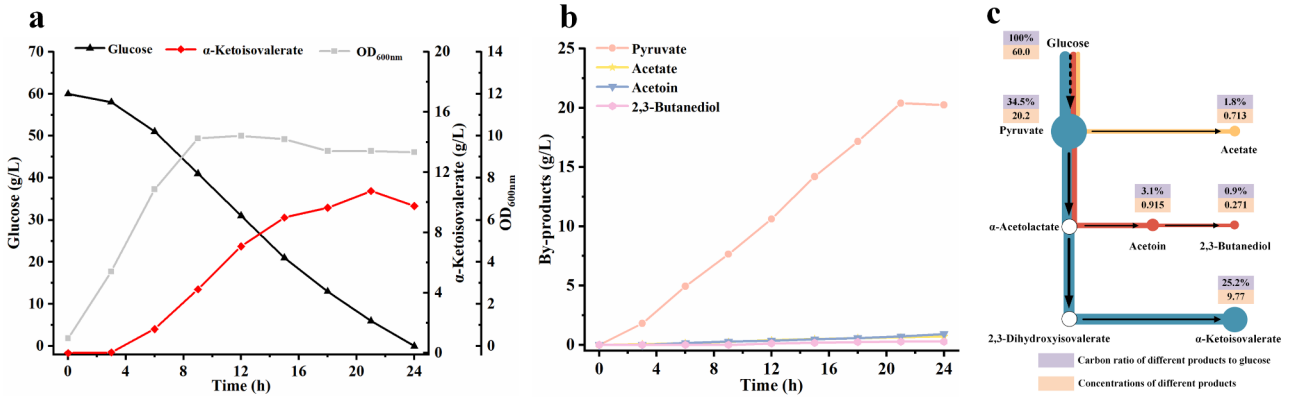

Fig. 3 Enhancing α-ketoisovalerate production through increasing α-acetolactate synthase expression. (**a**) Biomass, substrate consumption, and α-ketoisovalerate generation of *K. oxytoca* KIV-2. (**b**) By-products generation by *K. oxytoca* KIV-2. (**c**) Metabolic flux of *K. oxytoca* KIV-2. Solid circles represent accumulated products; hollow circles indicate non-accumulated products; the area of the circles represents the carbon ratio of different products to glucose. The carbon ratio (%) was defined as the ratio of the theoretical amount of glucose required for production of different products to the total consumed glucose

As shown in Fig. 3a, K. *oxytoca* KIV-2 produced 9.77 g/L of α-ketoisovalerate from 60.0 g/L of glucose, with a yield of 0.163 g/g within 24 h. The production of α-ketoisovalerate of *K. oxytoca* KIV-2 increased by 20.8%. Pyruvate was still the major by-product of *K. oxytoca* KIV-2, but its concentration decreased from 24.9 g/L to 20.2 g/L (Fig. 3b). The carbon ratio diverted into pyruvate decreased from 42.5 to 34.5%, while the carbon ratio diverted into α-ketoisovalerate increased from 20.9 to 25.2% (Fig. 3c).

Inactivating branched-chain α-keto acid dehydrogenase to enhance α-ketoisovalerate accumulation

The concentration of α-ketoisovalerate decreased at the end of fermentation by *K. oxytoca* KIV-1 and KIV-2, suggesting the α-ketoisovalerate degradation by these two strains (Figs. 2a and 3a). The branched-chain α-keto acid dehydrogenase (BCKDH) complex catalyzes oxidative decarboxylation of different branched-chain α-keto acids including α-ketoisovalerate, α-ketoisocaproate and α-ketomethylvalerate [29]. In previous reports, the *bkdAA* gene encoding the α-subunit of BCKDH complex was studied in *P. putida* [11], *P. aeruginosa* [29], *Listeria monocytogenes* [30], *B. subtilis* [31] and *Staphylococcus aureus* [32]. Comparative genomics revealed that homolog of BkdAA was also present in *K. oxytoca* KIV-0 (Additional file 1: Figure S1). To prevent the degradation α-ketoisovalerate through BCKDH complex, the *bkdAA* gene in *K. oxytoca* KIV-2 was deleted, resulting in strain KIV-3.

As shown in Fig. 4a, K. *oxytoca* KIV-3 exhibited a slight reduction in biomass generation and a significantly increase in α-ketoisovalerate production (12.7 g/L). The yield of α-ketoisovalerate of *K. oxytoca* KIV-3 increased to 0.212 g/g. Pyruvate, the structural analog of α-ketoisovalerate, may also serve as a substrate

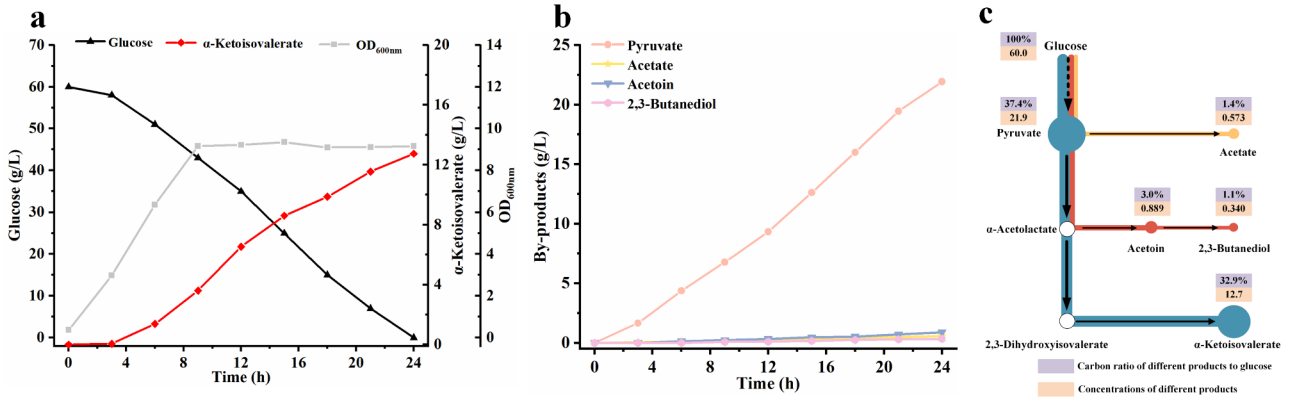

Fig. 4 Enhancing α-ketoisovalerate production through *bkdAA* deletion. (**a**) Biomass, substrate consumption, and α-ketoisovalerate generation of *K. oxytoca* KIV-3. (**b**) By-products generation by *K. oxytoca* KIV-3. (**c**) Metabolic flux of *K. oxytoca* KIV-3. Solid circles represent accumulated products; hollow circles indicate non-accumulated products; the area of the circles represents the carbon ratio of different products to glucose. The carbon ratio (%) was defined as the ratio of the theoretical amount of glucose required for production of different products to the total consumed glucose

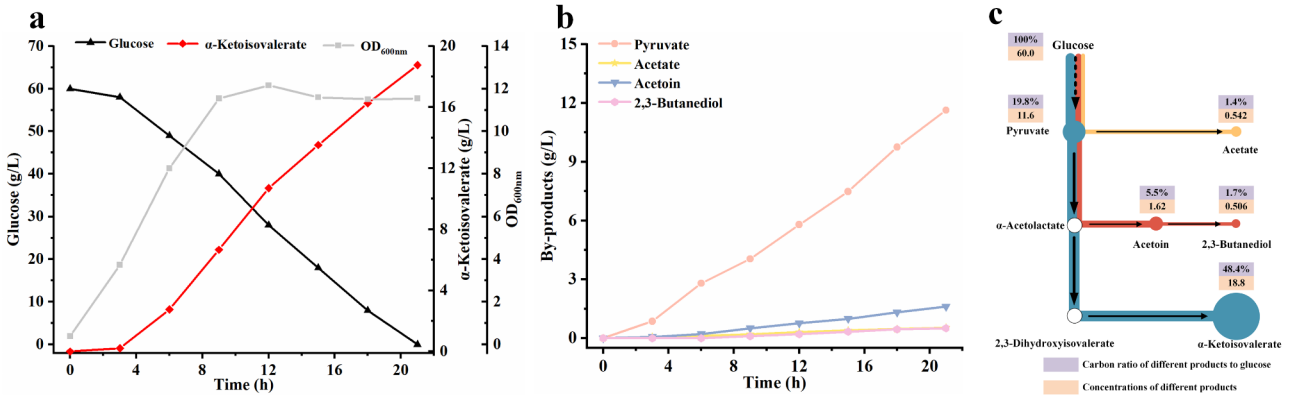

Fig. 5 Enhancement of α-ketoisovalerate production through increasing mutant acetohydroxyacid isomeroreductase expression. (**a**) Biomass, substrate consumption, and α-ketoisovalerate generation of *K. oxytoca* KIV-4. (**b**) By-products generation by *K. oxytoca* KIV-4. (**c**) Metabolic flux of *K. oxytoca* KIV-4. Solid circles represent accumulated products; hollow circles indicate non-accumulated products; the area of the circles represents the carbon ratio of different products to glucose. The carbon ratio (%) was defined as the ratio of the theoretical amount of glucose required for production of different products to the total consumed glucose

of BkdAA. Thus, the pyruvate production of *K. oxytoca* KIV-3 also slightly increased (Fig. 4b). The carbon ratio diverted to α-ketoisovalerate increased to 32.9% after *bkdAA* deletion (Fig. 4c).

Enhancing the expression of mutant acetohydroxyacid isomeroreductase to improve α-ketoisovalerate synthesis

The second step in α-ketoisovalerate biosynthesis is the acetohydroxyacid isomeroreductase (AHAIR) catalyzed α-acetolactate reduction. AHAIR of *E. coli* W3110 mainly utilizes NADPH as the cofactor, while the L67E, R68F, and K75E mutant of AHAIR ($AHAIR^M$) prefers NADH over NADPH as the cofactor [33, 34]. Previously, the *ilvC* encoding AHAIR and $ilvC^M$ encoding $AHAIR^M$ were integrated into the genome of *K. oxytoca* KIV-0 and under the control of promoters *gld* (the promoter of *gldA* gene) and *bud* (the promoter of *budA* gene), respectively [21]. Since *K. oxytoca* generally metabolizes glucose through glycolytic pathway and generates NADH, the expression of NADH utilizing $AHAIR^M$ was further enhanced through replacing the *bud* promoter of $ilvC^M$ with *trc* promoter.

Compared to *K. oxytoca* KIV-3, the obtained strain *K. oxytoca* KIV-4 exhibited significant increased substrate consumption and biomass generation (Figs. 4a and 5a). It produced 18.8 g/L of α-ketoisovalerate from 60.0 g/L of glucose in 21 h, with a yield of 0.313 g/g. The production of α-ketoisovalerate by *K. oxytoca* KIV-4 increased by 48% (Fig. 5a). In addition, the concentration of pyruvate accumulated by *K. oxytoca* KIV-4 decreased to 11.6 g/L (Fig. 5b). Enhancing the expression of $AHAIR^M$ increased the carbon ratio diverted to α-ketoisovalerate from 32.9 to 48.4% (Fig. 5c).

Introducing different α-acetolactate synthases to decrease pyruvate accumulation

Pyruvate was still the major by-product of *K. oxytoca* KIV-4 and its accumulation may potentially limit the production of α-ketoisovalerate. The *frdA* gene in *K. oxytoca* encodes α-subunit of fumarate reductase, which participates in reduction of fumarate to produce succinate. A third copy of *budB* with *trc* promoter was inserted into the *frdA* locus of stain KIV-4 to simultaneously enhance pyruvate condensation and decrease byproduct succinate generation, resulting in stain KIV-5. The α-acetohydroxyacid synthase IlvBN from *E. coli* W3110 and α-acetolactate synthase AlsS from *B. subtilis* 168 were reported to efficiently condense pyruvate into α-acetolactate [4, 7, 21]. The Q487S mutation of AlsS exhibited reduced decarboxylation activity toward α-ketoisovalerate, which may be beneficial for accumulation of α-ketoisovalerate [7]. The genes *ilvBN* (encoding IlvBN), *alsS* (encoding AlsS), and $alsS^{M}$ (encoding $AlsS^{M}$) were inserted into genome of strain KIV-4 under the control of *trc* promoter at the position of *frdA*, resulting in the strains KIV-6, KIV-7, and KIV-8, respectively.

Then, *K. oxytoca* KIV-5, KIV-6, KIV-7, and KIV-8 were cultured in a 1-L bioreactor with 60.0 g/L of glucose and the biomass, α-ketoisovalerate production and by-products generation were assayed (Additional file 1: Figure S2). Compared with *K. oxytoca* KIV-5, KIV-6, and KIV-8, the strain *K. oxytoca* KIV-7 overexpressing AlsS exhibited a significant increase in α-ketoisovalerate production (Fig. 6a-d). It consumed 60.0 g/L of glucose and produced 24.8 g/L of α-ketoisovalerate within 18 h. The pyruvate accumulation of *K. oxytoca* KIV-7 decreased to 2.97 g/L while the yield of α-ketoisovalerate increased to 0.413 g/g. The carbon ratio diverted to pyruvate and its oxidative decarboxylation product acetate in *K. oxytoca* KIV-7 decreased to 5.6% (Fig. 6e), while the carbon ratio diverted to downstream products of α-acetolactate, including α-ketoisovalerate, acetoin, and 2,3-butanediol, increased to 76.1% (Fig. 6f). These results indicated that the introduction of AlsS from *B. subtilis* 168 was more favorable for the pyruvate condensation and α-ketoisovalerate production in *K. oxytoca*. The reduction in pyruvate concentration significantly improved the biomass, glucose consumption, and α-ketoisovalerate production of *K. oxytoca* KIV-7 (Fig. 6c).

The activities of α-acetolactate synthase in *K. oxytoca* KIV-4, KIV-5, KIV-6, KIV-7, and KIV-8 were also measured (Additional file 1: Figure S3). The results indicated that the α-acetolactate synthase activity was highest in strain *K. oxytoca* KIV-7 expressing AlsS. α-Ketoisovalerate is a structural analogue of pyruvate and can competitively inhibit the activity of α-acetolactate

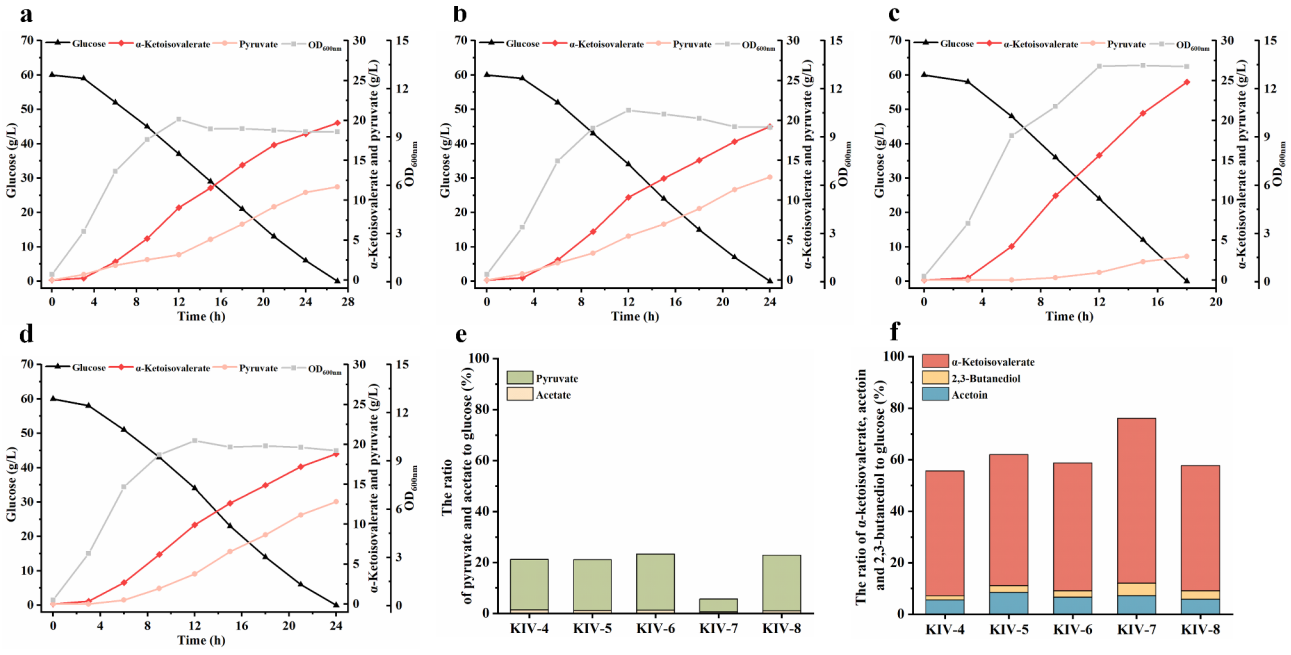

Fig. 6 Introducing different α-acetolactate synthases to increase α-ketoisovalerate production. (**a**) Biomass, substrate consumption, pyruvate and α-ketoisovalerate generation of *K. oxytoca* KIV-5. (**b**) Biomass, substrate consumption, pyruvate and α-ketoisovalerate generation of *K. oxytoca* KIV-6. (**c**) Biomass, substrate consumption, pyruvate and α-ketoisovalerate generation of *K. oxytoca* KIV-7. (**d**) Biomass, substrate consumption, pyruvate and α-ketoisovalerate generation of *K. oxytoca* KIV-8. (**e**) The carbon ratio of pyruvate and acetate to consumed carbon source during batch fermentation of *K. oxytoca* KIV-4, KIV-5, KIV-6, KIV-7, and KIV-8. (**f**) The carbon ratio of downstream products of α-acetolactate to consumed carbon source during batch fermentation of *K. oxytoca* KIV-4, KIV-5, KIV-6, KIV-7, and KIV-8. The carbon ratio (%) was defined as the ratio of the theoretical amount of glucose required for production of different products to the total consumed glucose

synthase [9]. As expected, the activities of α-acetolactate synthase in *K. oxytoca* KIV-4, KIV-5, KIV-6, KIV-7, and KIV-8 decreased in the presence of α-ketoisovalerate. The α-acetolactate synthase activity of KIV-7 was still the highest among these five strains. The lower pyruvate accumulation and higher α-ketoisovalerate production by *K. oxytoca* KIV-7 might be due to the high activity and the weak inhibition by α-ketoisovalerate of AlsS in the strain.

Utilization of lactose or whey for α-ketoisovalerate production

Whey is a by-product in dairying [35]. It contains lactose as the utilizable carbon source of many microorganisms. Batch fermentations using *K. oxytoca* KIV-7 with glucose, lactose, or crude whey as the carbon source were conducted in a 1-L bioreactor. α-Ketoisovalerate at a concentration of 14.5 g/L was produced from 38.0 g/L lactose in crude whey. The concentration and yield of α-ketoisovalerate with crude whey were slightly lower than those of with glucose or lactose as the substrate (Additional file 1: Figure S4). Fed-batch fermentation using *K. oxytoca* KIV-7 with lactose as the substrate was also performed in a 1-L bioreactor. As shown in Fig. 7a, K. *oxytoca* KIV-7 consumed 94.9 g/L of lactose and produced 37.3 g/L of α-ketoisovalerate in 33 h. The yield of α-ketoisovalerate produced by *K. oxytoca* KIV-7 with lactose was 0.393 g/g.

Whey powder can be obtained by spray drying of crude whey. Batch fermentation using *K. oxytoca* KIV-7 with whey power as the carbon source was conducted in a 1-L bioreactor. α-Ketoisovalerate at a concentration of 16.0 g/L was produced from 38.8 g/L lactose in whey power. The yield of α-ketoisovalerate with whey power (0.412 g/g lactose) were slightly higher than that of with crude whey as the substrate (0.382 g/g lactose) (Additional file 1: Figure S4). Then, fed-batch fermentation was also conducted with *K. oxytoca* KIV-7 using whey powder as the substrate in a 1-L bioreactor. As shown in Fig. 7b, K. *oxytoca* KIV-7 produced 40.7 g/L of α-ketoisovalerate with a yield of 0.418 g/g lactose.

Many microorganisms have been used for the fermentative biosynthesis of α-ketoisovalerate (Table 2). For example, Zhou et al. constructed a recombinant strain *E. coli* 050TY/pCTSDTQ487S-RBS55 by overexpressing the key enzymes involved in α-ketoisovalerate biosynthesis, regulating NADPH supply, and dynamically regulating activity of pyruvate dehydrogenase complex. *E. coli* 050TY/pCTSDTQ487S-RBS55 produced 55.8 g/L of α-ketoisovalerate from glucose with a yield of 0.550 g/g [7]. In this study, *K. oxytoca* KIV-0, an efficient L-valine producing strain, was metabolic engineered to produce α-ketoisovalerate [21]. The oxidative decarboxylation of α-ketoisovalerate was blocked by inactivating BkdAA in the resultant strain *K. oxytoca* KIV-7. BkdAA is required for the production of branched-chain fatty acids [36], which plays a vital role in maintenance of appropriate membrane fluidity and stress tolerance of industrial microorganisms [37]. Thus, the directly inactivation of BkdAA may decrease the performance of *K. oxytoca* KIV-7 under industrial stress. Dynamic control of BkdAA expression may be beneficial for balancing stress tolerance and α-ketoisovalerate formation by the strain *K. oxytoca* KIV-7 [38]. The LeuDH encoding gene *leuDH* in *K. oxytoca* KIV-7 was deleted to block NADH-dependent reductive amination of α-ketoisovalerate. Then, the expression of NADH utilizing AHAIR^{M} was enhanced in *K. oxytoca* KIV-7 to consume excessive NADH and increase α-ketoisovalerate production. *K. oxytoca* metabolizes glucose into pyruvate mainly through glycolysis

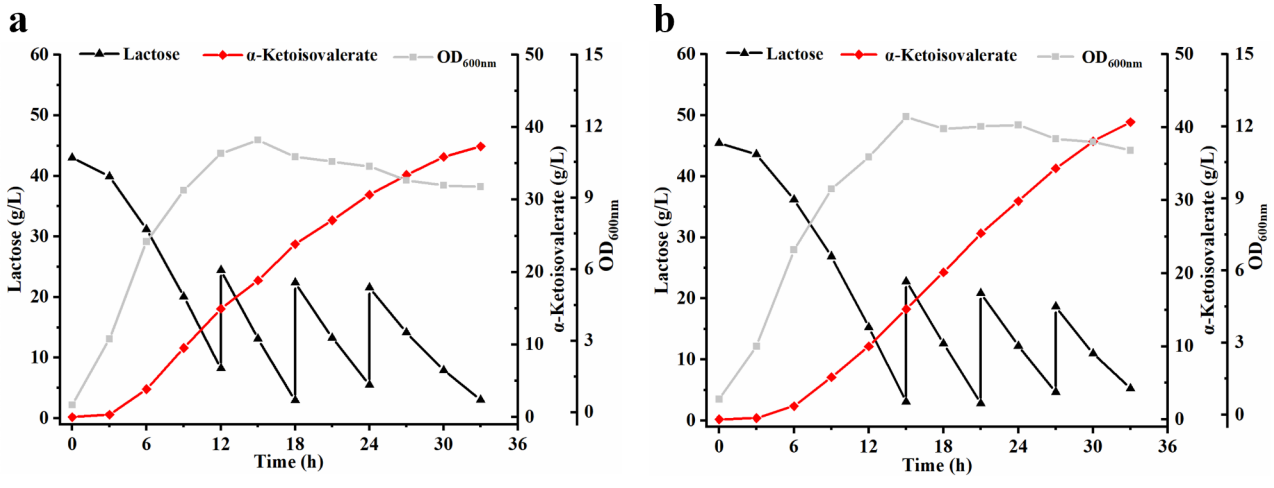

Fig. 7 α-Ketoisovalerate production through fed-batch fermentation with *K. oxytoca* KIV-7 with lactose or whey powder as carbon source. (**a**) Biomass, lactose consumption, and α-ketoisovalerate generation of *K. oxytoca* KIV-7 with lactose as the substrate. (**b**) Biomass, lactose consumption, and α-ketoisovalerate generation of *K. oxytoca* KIV-7 with whey powder as the substrate

Table 2 Comparison of α-ketoisovalerate production by different microorganisms

Strain	Relevant characteristics	Fermentation method	Substrate	Concentration (g/L)	Yield (g/g)	Productivity (g/L/h)	Reference
E. coli 050TY /pCTSDTQ487S-RBS55	*E. coli* B0016, Δ*ack-pta*Δ*pflB*Δ*adhE*Δ*frdA*Δ*ldhA*Δ*leuA* Δ*ilvE*::*T7RNAP*Δ*pntA*::P_{T7}, *aceF*-DAS + 4 tag, pETDuet plasmid harboring *alsS* Q487S, *ilvC* and *ilvD*	Fed-batch fermentation in 5-L bioreactor	Glucose	55.8	0.550	2.14	[7]
E. coli W1262	*E. coli* W, Δ*mdh*Δ*aceF*, pSEVA681 plasmid harboring *alsS*, *ilvC* and *ilvD*	Batch fermentation in 250-mL shake flasks	Glucose	2.18	0.21	0.0908	[8]
E. coli W1262	*E. coli* W, Δ*mdh*Δ*aceF*, pSEVA681 plasmid harboring *alsS*, *ilvC* and *ilvD*	Batch fermentation in 250-mL shake flasks	Whey powder	3.22	0.81	0.133	[8]
C. glutamicum Δ*aceE*Δ*pqo*Δ*ilvE* (pJC4ilvBNCD)	*C. glutamicum* ATCC 13,032, Δ*aceE*Δ*pqo*Δ*ilvE*, pJC4 plasmid harboring *ilvBN*, *ilvC* and *ilvD*	Fed-batch fermentation in 1-L bioreactor	Glucose and acetate	21.8	0.303	0.530	[9]
C. glutamicum aceE A16 Δ*pqo*Δ*ilvE* (pJC4ilvBNCD)	*C. glutamicum* ATCC 13,032, ΔP_{aceE}::P_{dapA16}Δ *pqo*Δ*ilvE*, pJC4 plasmid harboring *ilvBN*, *ilvC* and *ilvD*	Fed-batch fermentation in 1.5-L bioreactor	Glucose	33.7	ND	0.700	[10]
P. putida-2KIV	*P. putida* KT2440, Δ*aceEF*Δ*bkdAA*, pSEVAb84 plasmid harboring *alsS*, *ilvC* and *ilvD*, pSEVAb22 plasmid harboring *rhaR*, *rhaS*, *aceE* and *aceF*	Batch fermentation in 250-mL shake flasks	Glucose and acetate	1.28	0.400	ND	[11]
K. pneumoniae Δ*budA*Δ*ldhA-ilvIH*	*K. pneumoniae* CGMCC 1.6366, Δ*budA*Δ*ldhA*Δ*ilvIH*, pDK6 plasmid harboring *ilvIH*	Batch fermentation in 5-L bioreactor	Glucose	17.4	ND	ND	[12]
S. cinnamonensis BVR-13	*S. cinnamonensis* C-100-5, UV-irradiated mutant strain	Batch fermentation in shake flasks	Glucose	2.40	ND	ND	[13]
K. oxytoca KIV-7	*K. oxytoca* PDL-0, Δ*pta*Δ*adhE*Δ*bkdAA*Δ*ldhD*:: *ilvD*Δ*pflB*:: *brnFE*ΔP_{bud}-*budA*:: P_{trc}-*ilvC*^{M}Δ*budC*::*alsS*Δ*gldA*:: *ilvC*Δ*pox*::P_{trc}-*budB*Δ*frdA*::P_{trc}-*alsS*	Batch fermentation in 1-L bioreactor	Glucose	24.8	0.413	1.38	This study
K. oxytoca KIV-7	*K. oxytoca* PDL-0, Δ*pta*Δ*adhE*Δ*bkdAA*Δ*ldhD*:: *ilvD*Δ*pflB*:: *brnFE*ΔP_{bud}-*budA*:: P_{trc}-*ilvC*^{M}Δ*budC*::*alsS*Δ*gldA*:: *ilvC*Δ*pox*::P_{trc}-*budB*Δ*frdA*::P_{trc}-*alsS*	Fed-batch fermentation in 1-L bioreactor	Whey powder	40.7	0.418[a]	1.23	This study

ND, not determined; [a]The yield of α-ketoisovalerate produced from whey powder was calculated based on the lactose concentration in whey powder

and generates two NADH, while α-ketoisovalerate production from pyruvate with AHAIR^{M} only consumes one NADH [21, 33]. Thus, fine tuning the redox balance in *K. oxytoca* KIV-7 through control of the dissolved oxygen level or overexpression of NADH oxidase is still needed for further increasing α-ketoisovalerate generation.

Pyruvate is the major byproduct during α-ketoisovalerate fermentation by most of the derivative strains of *K. oxytoca* KIV-0. Thus, the α-acetolactate synthase BudB and AlsS were overexpressed to reduce pyruvate accumulation in *K. oxytoca* KIV-7. The significant improvements in final concentration and yield of α-ketoisovalerate were achieved, indicating the overexpression of BudB and AlsS is beneficial for the production of α-ketoisovalerate. Acetoin and 2,3-butanediol can be generated by non-enzymatic oxidative decarboxylation of α-acetolactate and subsequent diacetyl reduction (Fig. 1). Besides α-ketoisovalerate, these two chemicals are also the major downstream products of α-acetolactate. Decreased pyruvate accumulation and increased α-ketoisovalerate, acetoin and 2,3-butanediol production indicated that the carbon flux was successfully diverted to downstream products of α-acetolactate in *K. oxytoca* KIV-7 (Fig. 6f). In addition, the increased α-ketoisovalerate production

in *K. oxytoca* KIV-7 was accompanied by increased biomass and substrate consumption, implying the overall metabolic balance of strain was also improved (Fig. 6c). Importantly, all of the key genes for α-ketoisovalerate production were integrated into the genome of *K. oxytoca* KIV-7 and constitutively expressed under the control of the *trc* promoter. The plasmid and inducer free characteristic made *K. oxytoca* KIV-7 a promising strain for stable α-ketoisovalerate fermentation with low cost.

Approximately 180–190 million tonnes of whey is annually generated [39]. More than 50% of whey is directly disposed to natural water bodies, which results in a loss of resource and causes serious environmental pollution [40]. In this work, production of α-ketoisovalerate from crude whey was acquired using recombinant strain *K. oxytoca* KIV-7 (Additional file 1: Figure S4). Due to the low concentration of lactose in crude whey (38.0 g/L), the final concentration of α-ketoisovalerate was 14.5 g/L. Liquid crude whey can be transformed into solid whey powder by spray drying [41]. Whey powder has been widely used in the fermentative production of biochemicals such as pyruvate [14], citrate [24], isobutanol [26] and butyl acetate [42]. Darwin et al. recently inactivated the pyruvate dehydrogenase and malate dehydrogenase in *E. coli* W and introduced the α-ketoisovalerate-producing plasmid pKIV. The obtained strain *E. coli* W1262 produced 3.22 g/L of α-ketoisovalerate with whey powder [8]. In this work, *K. oxytoca* KIV-7 produced α-ketoisovalerate from whey powder with a concentration of 40.7 g/L and a yield of 0.418 g/g lactose, which was close to that of with glucose as the substrate (0.413 g/g glucose). The fermentative process is thus a promising alternative for both biotechnological α-ketoisovalerate production and resource utilization of whey. In addition, α-ketoisovalerate serves as the precursor for many important chemicals, such as L-leucine [4], vitamin B5 [5], and isobutyrate [43]. These chemicals might also be generated from whey powder by expressing different biosynthesis pathways into *K. oxytoca* KIV-7.

Conclusion

In summary, an efficient α-ketoisovalerate producing strain *K. oxytoca* KIV-7 was acquired through engineering of the L-valine producer *K. oxytoca* VKO-9. α-Ketoisovalerate at a concentration of 40.7 g/L and a yield of 0.418 g/g was generated by *K. oxytoca* KIV-7 with whey powder as the substrate. This work provides a promising process for both efficient α-ketoisovalerate production and industrially produced low-value whey utilization. The recombinant strain *K. oxytoca* KIV-7 could also be used as the chassis for producing other derivatives of α-ketoisovalerate.

Abbreviations

AHAIR	Acetohydroxyacid isomeroreductase
$AHAIR^M$	Mutant acetohydroxyacid isomeroreductase
DHAD	Dihydroxyacid dehydratase
LeuDH	Leucine dehydrogenase
ALS	α-Acetolactate synthase
BCKDH	Branched-chain α-keto acid dehydrogenase
BOD	Biochemical oxygen demand
COD	Chemical oxygen demand
HPLC	High performance liquid chromatography

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12934-024-02545-4.

Supplementary Material 1

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Author contributions

Weikang Sun: Writing original draft, Investigation, Data curation. Shuo Wang: Formal analysis, Software, Validation. Xiaoxu Tan: Software, Validation. Leilei Guo: Software, Validation. Wei Liu: Software, Validation. Wenjia Tian: Software, Validation. Hui Zhang: Software, Validation. Tianyi Jiang: Software, Funding acquisition. Wensi Meng: Funding acquisition. Yidong Liu: Software, Validation. Zhaoqi Kang: Software, Validation. Chuanjuan Lü: Software, Funding acquisition. Chao Gao: Conceptualization, Software, Funding acquisition. Ping Xu: Software, Supervision. Cuiqing Ma: Conceptualization, Supervision, Writing – review and editing, Funding acquisition. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Data availability

All data generated or analyzed during this study are included in this published article.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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