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RESEARCH PAPER

Acetolactate synthase (AlsS) in *Bacillus licheniformis* WX-02: enzymatic properties and efficient functions for acetoin/butanediol and L-valine biosynthesis

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Abstract Acetolactate synthase catalyzes two molecules of pyruvates to form α-acetolactate, which is further converted to acetoin and 2,3-butanediol. In this study, by heterologous expression in *Escherichia coli*, the enzymatic properties of acetolactate synthase (AlsS) from *Bacillus licheniformis* WX-02 were characterized. Its K_m and k_{cat} for pyruvate were 3.96 mM and 514/s, respectively. It has the optimal activity at pH 6.5, 37 °C and was feedback inhibited by L-valine, L-leucine and L-isoleucine. Furthermore, the *alsS*-deficient strain could not produce acetoin, 2,3-butanediol, and L-valine, while the complementary strain was able to restore these capacities. The *alsS* overexpressing strain produced higher amounts of acetoin/2,3-butanediol (57.06 g/L) and L-valine (2.68 mM), which were 10.90 and 92.80% higher than those of the control strain, respectively. This is the first report regarding the in-depth understanding of AlsS enzymatic properties and its functions in *B. licheniformis*, and overexpression of AlsS can effectively improve acetoin/2,3-butanediol and L-valine production in *B. licheniformis*. We envision that this AlsS can also be applied in the improvement of acetoin/2,3-butanediol and L-valine production in other microbes.

Keywords Acetolactate synthase · *Baicllus licheniformis* · Acetoin/2,3-butanediol · L-valine · Enzymatic properties

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Introduction

Acetoin and 2,3-butanediol (2,3-BD) are important platform compounds, and their derivative compounds are also promising valuable chemicals because of their extensive applications [1–5]. Branched-chain amino acids (L-valine, L-leucine, and L-isoleucine) are used in dietary products, pharmaceuticals, cosmetics, animal feed additives, or as precursors of antibiotics or herbicides [6–8]. Acetolactate is the direct substrate for acetoin/2,3-BD biosynthesis, and the precursor of L-valine and L-leucine, while α-acetohydroxybutyrate is the precursor of L-isoleucine [9]. There are two kinds of acetolactate synthase for synthesizing acetolactate: anabolic acetohydroxyacid synthase (AHAS) and catabolic acetolactate synthase (cALS) [9]. Both enzymes can convert two molecules of pyruvates to one acetolactate, and AHAS can also catalyze the condensation of pyruvate and α-ketobutyrate to form α-acetohydroxybutyrate. Thus, the anabolic AHAS is responsible for biosynthesis of the branched-chain amino acids, while cALS is responsible for production of acetoin/2,3-BD [10, 11].

Generally, both anabolic AHAS and cALS require thiamine diphosphate (ThDP) and a divalent metal ion (Mg^{2+}) for catalytic activity [11]. The AHAS contains flavin adenine dinucleotide (FAD), and is inhibited by the branched-chain amino acids [12–15]. Compared to the anabolic AHAS, the cALS with a lower optimal pH at about 6.0 is FAD independent and lacks a regulatory subunit [16–20]. Considering that cALS exhibits higher affinity to pyruvate [1], cALS has attracted great attention and is served as a potential target of genetic engineering for bioproduct production, such as acetoin/2,3-BD and other high-value products. Recently, several recombinant strains have been developed via altering the expression levels of *alsS*. For example, the AlsS from *Bacillus subtilis* was overexpressed to improve the 2,3-BD production in *Saccharomyces cerevisiae* [21], and the ethanol titer was significantly improved by the koncking-out of *alsS* in *Pyrococcus furiosus* [22].

Bacillus licheniformis is a generally regarded as safe (GRAS) strain, which is capable of producing 2,3-BD at high yield [23], and its recombinant strain shows high capacity to produce L-valine [7]. However, the acetolactate synthase (AlsS) of *B. licheniformis* has not been characterized and whether it is involved in L-valine formation was unclear. The objective of this work was to investigate the enzymatic properties of acetolactate synthase AlsS in *B. licheniformis* WX-02 as well as its functions in the biosynthesis of acetoin/2,3-BD and L-valine. An in-depth understanding of AlsS characteristics and functions in *B. licheniformis* will help the re-direction of carbon flux distribution to improve the production of acetoin/2,3-BD and L-valine by enhancing the biosynthetic capacity of intercellular acetolactate.

Materials and methods

Bacterial strains, plasmids, and culture conditions

The strains and plasmids used in this study are listed in Table 1. *Escherichia coli* DH5α and BL21 (DE3) were used for the cloning procedures and protein purification, respectively. The wild-type WX-02 (CCTCC M208065) was used as the host strain [24]. The plasmid pET-28a (+) was used for the induced expression, the plasmid pT49 was applied for construction of expression vector in *B. licheniformis*, and the T2 (2)-ori vector was used for gene knock-out.

Bacillus licheniformis seeds were incubated at 37 °C, 180 rpm for 8 h in 250-mL flasks containing 50 mL LB medium. The medium for acetoin and 2,3-BD production was described previously [25], consisting of (per liter): 120.00 g glucose, 33.00 g corn steep liquor, 9.00 g $(NH_4)_2SO_4$, 1.50 g $MgSO_4{\cdot}7H_2O$, 1.00 g $K_2HPO_4{\cdot}3H_2O$, 0.50 g NaCl, 0.12 g $ZnCl_2$, 1.00 mg $MnSO_4{\cdot}H_2O$, 1.00 mg $FeCl_3{\cdot}6H_2O$, pH 7.0. The seed culture was inoculated 1% (v/v) into 250-mL flasks containing 30 mL acetoin/2,3-BD production medium, and then cultivated at 37 °C, 180 rpm for 48 h. The L-valine production medium was prepared according to the previous research [7], consisting of (per liter): 100.00 g glucose, 2.00 g corn steep liquor, 15.00 g $(NH_4)_2SO_4$, 1.00 g $KH_2PO_4{\cdot}3H_2O$, 0.50 g $MgSO_4{\bullet}7H_2O$, 3.00 g $CaCO_3$, pH 7.0. The seed culture 2% (v/v) was inoculated into a 250-mL flask containing 30 mL L-valine production medium, and then cultivated at 37 °C, 180 rpm for 84 h.

Expression and purification of AlsS protein

The *alsS* encoding acetolactate synthase is located in the butanediol operon, and the gene was amplified from *B. licheniformis* WX-02 genomic DNA using the primers *alsS*-F/R (Table 2). Then, the DNA fragment was subcloned into pET-28a (+), confirmed by PCR verification and Sanger sequencing. The recombinant plasmid was further transferred into *E. coli* BL21 (DE3) to construct the recombinant strain, named as *E. coli* BL21 (DE3)/pET*alsS*.

The *E. coli* BL21 (DE3)/pET*alsS* was cultivated in the 50 mL LB medium containing 20 μg/mL kanamycin at 37 °C. Cells were induced by 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) until the OD_{600nm} value reached 1.0, and further cultivated for 6 h. Then, the culture was centrifuged at 8000 rpm, 4 °C for 10 min to collect the cell pellets. After washed twice with lysis buffer (50 mM Tris, 500 mM NaCl, 10% glycerol, 50 mM sodium dihydrogen phosphate, 20 mM imidazole, 5 mM β-Mercaptoethanol, pH 8.0), cell pellets were resuspended with 50 mL lysis buffer, and disrupted by high pressure. After centrifugation at 12,000 rpm for 30 min, the supernatant was collected for purification of AlsS using the Ni–NTA column [26]. The eluted AlsS was dialyzed in 500 mL buffer containing 20 mM Tris–HCl (pH 8.0) containing 10% glycerol, 1 mM EDTA (pH 8.0), 0.1 mM DTT and 0.002% Tritonx-100 at 4 °C for 24 h. The purified AlsS was analyzed by SDS-PAGE and quantified by Bradford protein kit according to the manual [27].

Determination of enzymatic properties of AlsS

The enzymatic activity of AlsS was determined according to the previously reported method [28]. Briefly, mixtures of 20 μL distilled water, 60 μL reaction buffer (75 mM potassium phosphate buffer containing 15 mM $MgCl_2$ and 5 mM ThDP, pH 7.5) and 5 μL AlsS were added into 96-well microplates, followed by incubation at 37 °C for 10 min. After the addition of 5 μL 0.6286 M sodium

Table 1 Bacterial strains and plasmids used in this study

Strains and plasmids	Characteristics[a]	Source or reference
Escherichia coli		
DH5α	F^- Φ80d/*lac*ZΔM15, Δ(*lacZYA-argF*) U169, *recA*1, *endA*1, *hsdR*17 (r_K^-, m_K^+), *phoA*, *supE*44, λ^-, *thi*-1, *gyrA*96, *relA*1	Laboratory stock
BL21(DE3)	F^-, *omp*T, $hsdS_B(r_B^-m_B^-)$, *gal*(λ*cI*857, *ind*1, *Sam*7, *nin*5, *lac*UV5-T7 *gene*1), *dcm*(DE3)	Laboratory stock
BL21(DE3)/pET-28a(+)	BL21(DE3) harboring pET-28a(+), Kan^r	This study
BL21(DE3)/pET*alsS*	BL21(DE3) harboring pET*alsS*, Kan^r	This study
Bacillus licheniformis		
WX-02	CCTCC M208065, Wild-type	Laboratory stock
WX-02/pT49	WX-02 harboring pT49, Kan^r	This study
WX-02/pT49*alsRS*	WX-02 harboringpT49*alsRS*, Kan^r	This study
WX-02Δ*alsS*	WX-02 with a 1269-bp deletion in *alsS* introducing a frameshift mutation	This study
WX-02Δ*alsS*/pT49	WX-02Δ*alsS* harboring pT49, Kan^r	This study
WX-02Δ*alsS*/pT49*alsRS*	WX-02Δ*alsS* harboring pT49*alsRS*, Kan^r	This study
Plasmids		
pET-28a(+)	*E. coli* expression vector, Kan^r	Laboratory stock
pET*alsS*	pET-28a(+) derivative harboring *alsS*, Kan^r	This study
pT49	*E. coli-Bacillus* shuttle vector, harboring P43 promoter and TamyL (amyl terminator), Kan^r	Laboratory stock
pT49*alsRS*	pT49 derivative harboring *alsRS*, Kan^r	This study
T2(2)-ori	*E. coli-Bacillus* shuttle vector, Ori_{pUC}/Ori_{ts}, Kan^r	Laboratory stock
T2Δ*alsS*	T2(2)-ori derivative containing homologous arms for *alsS*	This study

[a]Kan^r, Kanamycin resistance; CCTCC, China Center for Type Culture Collection

pyruvate, the mixture was incubated at 37 °C for 30 min. The enzymatic reaction was terminated by adding 10 μL 3 M H_2SO_4 and incubated at 60 °C for 20 min for the decarboxylation of acetolactate. Next, the volume of 100 μL 5% (w/v) α-napthtol solution and 100 μL 0.5% creatine (w/v) was added, respectively, followed by incubating at 37 °C for 20 min. The resulting sample was measured at 525 nm using a microplate reader (Bio-Rad, USA). One unit of AlsS activity is defined as producing 1 μmol acetolactate in 1 min.

The optimum temperature and pH of the AlsS were determined by measuring the enzyme activity as described above. The reaction was carried out at temperature from 4 to 80 °C. The optimum pH was measured using reaction buffer with different pH from 2.0 to 10.0.

The kinetic parameters of AlsS were determined by measuring the enzyme activity under the condition of different substrate concentrations. The values of K_m and V_{max} for the substrate were acquired according to the Michaelis–Menten equation.

The effect of branched-chain amino acids on the AlsS activity was also analyzed by the standard assay method with different concentrations of L-valine, L-leucine, and L-isoleucine at 37 °C. The inhibition constants of three amino acids were calculated, respectively.

Construction of *alsS*-deficient strain in *B. licheniformis*

The method of markerless gene deletion used in this study has been previously described by Qiu et al. [29]. Briefly, the fragments for deleting *alsS* were amplified from the WX-02 genomic DNA using the primers *alsS*-AF/AR and *alsS*-BF/BR, respectively (Table 2). The two fragments were fused by splice overlap extension PCR (SOE-PCR) using the primers *alsS*-AF/BR, and the fused fragment was inserted into T2 (2)-ori at *Bam*HI and *Sac*I restriction sites, generating the plasmid T2Δ*alsS*. The plasmid T2Δ*alsS* was transformed into *B. licheniformis* WX-02 via electroporation [30]. The positive clone was incubated in LB medium containing 20 μg/mL kanamycin 45 °C for 8 h, and then streaked on LB agar plates with kanamycin for another 8 h to obtain the single-crossover recombinants. The single-cross clones were grown in LB medium at 37 °C with serial subcultures to promote homologous recombination, The kanamycin sensitive colonies meaning a double

Table 2 The primers used in this study

Primer name	Sequence 5′ → 3′[a]
Primers for *alsS* cloning	
alsS-F	CATG**CCATGG**GCTTGAATAATGTAGCCGCT
alsS-R	CCG**CTCGAG**AGATTGCTTAGAGGCTTC
Primers for *alsS* deletion	
alsS-AF	CG**GGATCC**GAAGATCGATCTGCACGG
alsS-AR	AGCAGTCCATGCGGACGGTCAAAGCCCTCACTCCTCCA
alsS-BF	TGGAGGAGTGAGGGCTTTGACCGTCCGCATGGACTGCT
alsS-BR	GC**GAGCTC**CACTCCCTTGATTTTGTTC
Δ*alsS*-F	CCGTGTATGACTTCAGTTC
Δ*alsS*-R	CGCTGCTTGATTTCGTGATA
T2-F	ATGTGATAACTCGGCGTA
T2-R	GCAAGCAGCAGATTACGC
Primers for *alsS* complementation	
alsS-OF	CCG**CTCGAG**GGCAGCAATCGGCATTTAAC
alsS-OR	ATAAGAAT**GCGGCCGC**TCAAGATTGCTTAGAGGCTTC
pT49-YF	CCGTGATTTCGTGTATCATTGG
pT49-YR	CCTATGCGATTCAAACTGTCAGC

[a]Restriction sites were highlighted in bold. Underline stands for the overlap region for splicing by splice overlap extension PCR (SOE-PCR)

crossover event were verified by PCR using the corresponding primers (Table 2) and the gene-deleted clones were further verified by DNA- sequencing. The mutant strain was designated as WX-02Δ*alsS*.

Construction of *alsS* overexpressing strain and complementary strain in *B. licheniformis*

The *alsRS* fragment containing *alsS*, its promoter, and the positive regulatory gene of *alsR* [31] was amplified from WX-02 genome using primers *alsS*-OF/OR (Table 2). Then, the fragment was incorporated into pT49 vector at *Xho*I and *Not*I restriction sites. The recombinant plasmid was further verified by DNA sequence and designated as pT49*alsRS*.

The plasmid pT49*alsRS* was transformed into *B. licheniformis* WX-02 and WX-02Δ*alsS* by electroporation, respectively, and the transformants were selected by kanamycin (20 μg/mL), followed by PCR verification with the primers pT49-YF/YR (Table 2). In this study, the *alsS* overexpressing and complementary strains were designated as WX-02/pT49*alsRS* and WX-02Δ*alsS*/pT49*alsRS*, respectively. The recombinant strains WX-02/pT49 and WX-02Δ*alsS*/pT49 were served as the control strains in this research.

Analytical procedures

Cells were harvested from the fermentation broth via centrifugation at 12,000 rpm for 5 min. The separated cells were washed twice with deionized water and resuspended for determination of the cell density at 600 nm (OD_{600nm}) with a spectrophotometer (Bio-Rad, USA). Residual glucose was measured enzymatically by a Bio-analyzer (SBA 40C, Shandong Academy of Sciences, China). The concentrations of acetoin and 2,3-BD in the supernatant were analyzed by gas chromatography [23]. L-valine was determined by gas chromatography after derivatization process with ethyl chlorocarbonate [7].

Statistics analysis

All experiments were performed in triplicate, and the data were presented as the mean ± the standard deviation for each sample point. All data were conducted to analyze the variance at $P < 0.05$, and an approximate Duncan's multiple range test was applied to compare the mean values.

Results

Cloning, expression, and purification of AlsS

The 1719 bp *alsS* fragment was cloned into pET-28a (+) vector to form the recombinant plasmid pET*alsS*, which was then transformed into *E. coli* BL21 (DE3). The induced protein showed the size at 63 kDa on SDS-PAGE gel (lane 3) (Fig. 1a), which was consistent with the molecular weight of the AlsS of *B. licheniformis* WX-02, indicating that the AlsS was expressed successfully. After

purification by Ni-NAT, the total intracellular proteins showed a single band of purified AlsS on the SDS-PAGE gel (Fig. 1b), and the concentration of the purified AlsS was 30.71 mg/L.

AlsS enzymatic properties and feedback inhibitory effects of branched-chain amino acids

To obtain the optimum temperature and pH for the AlsS, the enzyme activity was measured under different temperatures (4–80 °C) and pH (2.0–10.0). Based on our results, the AlsS exhibited considerable activity in the range of pH 6.0–7.5, with a maximum activity at pH 6.5 under different pH buffer conditions measured at 37 °C (Fig. 2a). And the apparent optimum temperature for AlsS activity was 37 °C measured at pH 6.5, while the activity above 50 °C was less than 50% and then completely decreased at higher temperatures (Fig. 2b).

A discontinuous colorimetric analysis was used to determine the kinetic parameters of the AlsS. The kinetic parameters of the AlsS were determined at different concentrations of pyruvate under the optimal pH and temperature. As shown in Fig. 3, the K_m and V_{max} values for pyruvate were calculated at 3.96 mM and 0.75 mM/min by Lineweaver–Burk plot, respectively.

The feedback inhibitory effects of branched-chain amino acids (L-valine, L-leucine, and L-isoleucine) on the AlsS activity were further measured. The inhibition constants (K_i) of L-valine, L-leucine, and L-isoleucine were 2.73×10^{-3}, 1.19×10^{-2}, and 0.19 mM, respectively. These results implied that the AlsS, participating in production of acetoin and 2,3-BD, was also feedback inhibited by the three branched-chain amino acids, and L-valine displayed the strongest inhibitory effect on the AlsS activity.

Effects of *alsS* deletion on acetoin/2,3-BD and L-valine biosynthesis

To further investigate the role of the AlsS on biosynthesis of acetoin/2,3-BD and L-valine, the *alsS*-deficient strain WX-02Δ*alsS* was constructed. The wild-type strain WX-02 produced 51.61 g/L acetoin/2,3-BD and 1.53 mM L-valine in the corresponding medium, respectively, while WX-02Δ*alsS* could not produce any of those products under same conditions (Fig. 4a, c). Meanwhile, the complementary strain WX-02Δ*alsS*/pT49*alsRS* successfully restored the biosynthesis capacity, which could produce 9.54 g/L acetoin/2,3-BD and 0.93 mM L-valine, respectively (Fig. 4a, c). Also, the deletion of *alsS* resulted in a poor cell growth, which could be partially recovered by the complementation of *alsS* (Fig. 4b, d). Previous research has reported that acetoin and 2,3-BD biosynthesis pathways can prevent acidification through conversion of excess

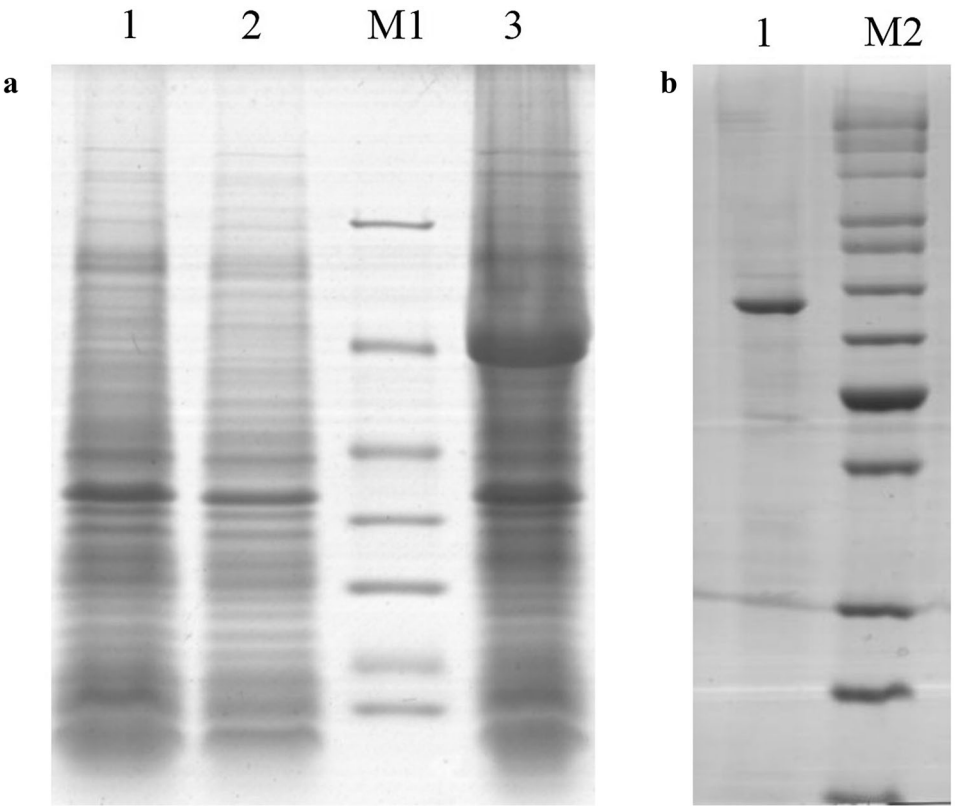

Fig. 1 SDS-PAGE analysis of induced expression (**a**) and purification (**b**) of the AlsS in *E. coli*. **a** Induced expression of AlsS in *E. coli*. Lane M1: Protein Marker (116, 66.2, 45, 35, 25, 18.4 and 14.4 KDa); Lane 1: The total intracellular proteins of *E. coli* BL21 (DE3)/pET-28a(+) induced by IPTG; Lane 2: The total intracellular proteins of *E. coli* BL21 (DE3)/pET*alsS* non-induced by IPTG; Lane 3: The total intracellular proteins of *E. coli* BL21 (DE3)/pET*alsS* induced by IPTG. **b** SDS-PAGE analysis of the purified AlsS. Lane M2: Protein markers (200, 150, 120, 100, 85, 70, 60, 50, 40, 30, 25, 20, 15 and 10 kDa); Lane 1: Purified AlsS

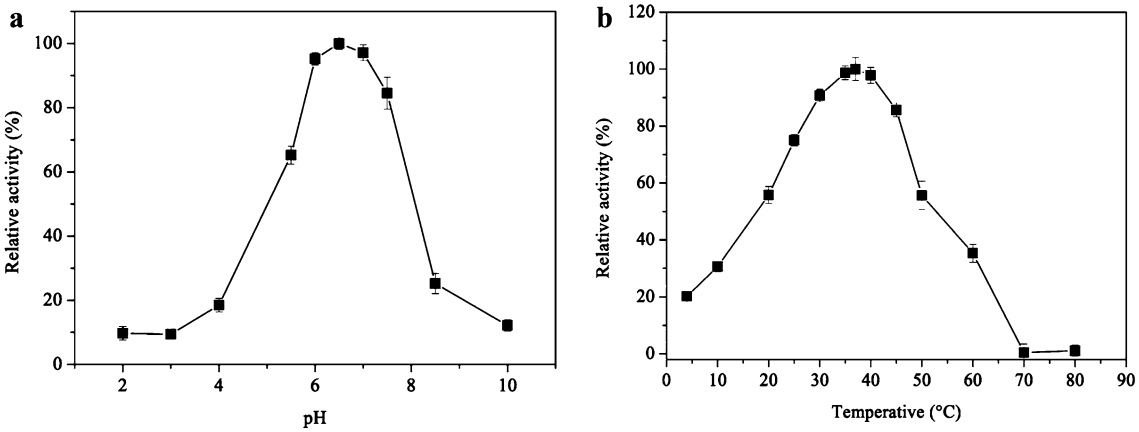

Fig. 2 Effects of different temperatures and pH on the catalytic activity of theAlsS. **a** pH. **b** Temperature. The optimum pH value was measured at 37 °C using the reaction buffer with different pH. The optimum temperature was determined at different temperatures under the condition of pH 6.5. Data were expressed as mean ± standard errors of three replicates

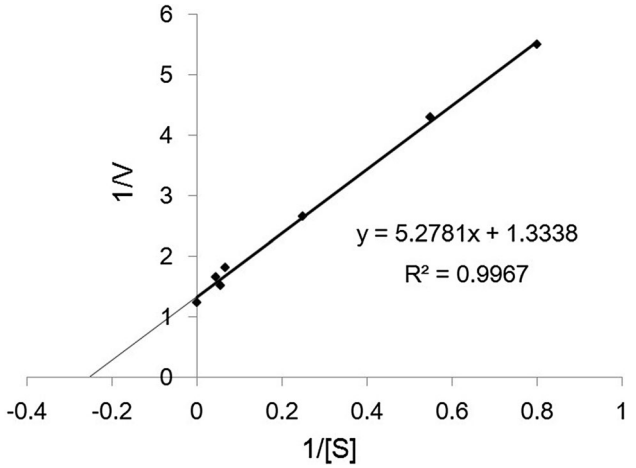

Fig. 3 Lineweaver–Burk plot of the AlsS. The enzymatic reaction was carried out under the optimum conditions (temperature 37 °C, pH 6.5) to measure the reaction rate of pyruvate at the different concentrations. The K_m and V_{max} value for pyruvate were calculated according to the formula: $1/v = (K_m/V_{max})(1/[S]) + 1/V_{max}$. Data represented the average of more than three independent measurements

pyruvate into neutral compounds (acetoin and 2,3-BD) [32]. Our results indicated that the deletion of *alsS* could eliminate the biosynthesis of acetoin/2,3-BD and L-valine.

Effects of *alsS* overexpression on acetoin/2,3-BD and L-valine production

Since the AlsS was proven to be essential in the biosynthesis of acetoin/2,3-BD and L-valine, the recombinant strain WX-02/pT49*alsRS* was constructed to enhance the acetolactate biosynthesis pathway, which might further lead to increase of acetoin/2,3-BD and L-valine yields.

The metabolite profiles were determined to evaluate the performance of WX-02/pT49*alsRS* on the production of acetoin/2,3-BD and L-valine. The output of acetoin has no significant difference between WX-02/pT49*alsRS* and the control strain at the first 16 h of the fermentation, but the acetoin titer of WX-02/pT49*alsRS* was higher after 16 h (Fig. 5a). The titer of 2,3-BD increased rapidly until 32 h, then decreased gradually in both WX-02/pT49*alsRS* and WX-02/pT49, and the output of 2,3-BD produced by WX-02/pT49*alsRS* was higher during the whole fermentation (Fig. 5a). The combined production of acetoin/2,3-BD produced by WX-02/pT49*alsRS* reached the highest titer at 57.06 g/L, which was 10.9% higher than that of WX-02/pT49 (Fig. 5a). Also, as shown in Fig. 5c, WX-02/pT49*alsRS* produced more L-valine during the fermentation process between 24 and 72 h, and the maximum titer of L-valine produced by WX-02/pT49*alsRS* reached 2.68 mM, increased by 92.8% compared with 1.39 mM L-valine produced by WX-02/pT49. However, the cell growth of WX-02/pT49*alsRS* was slightly impaired during the fermentation process of acetoin/2,3-BD and L-valine production (Fig. 5b, d). These results implied that overexpression of the AlsS could enhance the titers of acetoin/2,3-BD and L-valine in *B. licheniformis*.

Discussion

Due to the important roles of acetolactate in microorganisms, acetolactate synthase (ALS), which condenses two molecules of pyruvate into acetolactate, is of great interests for researchers. Two different types of ALSs have been reported in microorganisms, anabolic acetohydroxyacid synthase (AHAS) and catabolic acetolactate synthase (cALS) [9, 10]. The AHAS is involved in the biosynthesis of branched-chain amino acids, and overexpression of AHAS could improve the yields of L-valine in *E. coli* and

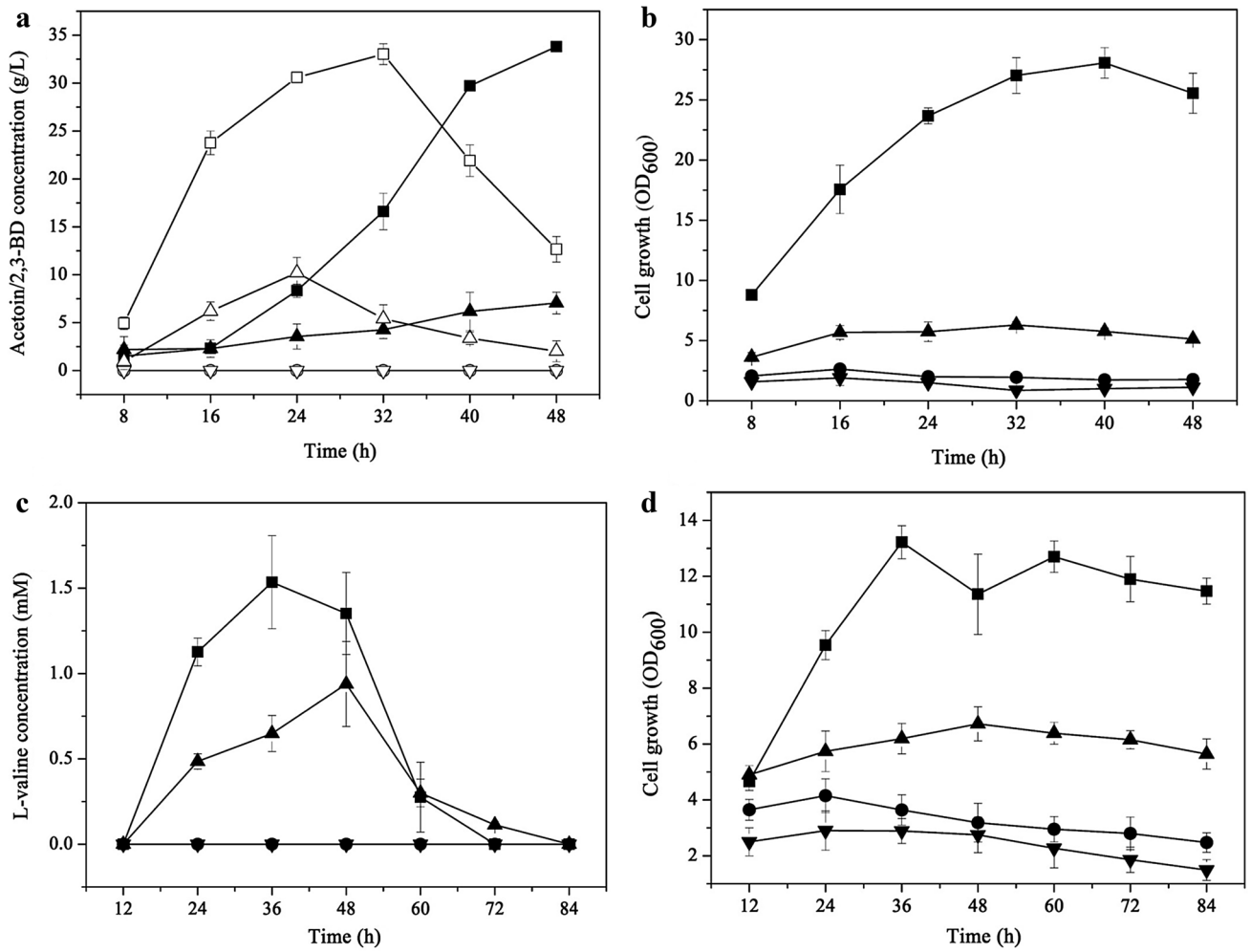

Fig. 4 Effects of the *alsS* deficient on the acetoin/2,3-BD and L-valine metabolism in *B. licheniformis*. **a** Time course of the acetoin (solid symbols) and 2,3-BD (open symbols) produced by the *alsS* deficient strain (WX-02Δ*alsS*) (circles), the complementary strain (WX-02Δ*alsS*/PT49*alsRS*) (triangles), WX-02Δ*alsS*/PT49 (inverted triangles), and wild-type strain (WX-02) (squares), circles overlaps with inverted triangles. **c** Time course of L-valine produced by the *alsS* deficient strain (WX-02Δ*alsS*) (black circles), the complementary strain (WX-02Δ*alsS*/PT49*alsRS*) (black triangles), WX-02Δ*alsS*/PT49 (black inverted triangles), and wild-type strain (WX-02) (black squares), black triangles overlaps with white circles. **b**, **d** Time course of the cell growth in the *alsS* deficient strain (WX-02Δ*alsS*) (black circles), the complementary strain (WX-02Δ*alsS*/PT49*alsRS*) (black triangles), WX-02Δ*alsS*/PT49 (black inverted triangles), and wild-type strain (WX-02) (black squares) in the acetoin/2,3-BD and L-valine production medium, respectively. Data were expressed as mean ± standard errors of three replicates

Corynebacterium glutamicum [6, 13, 33–35]. The cALS is a catabolic enzyme required for 2,3-BD fermentation, which has been employed for high-level production of acetoin/2,3-BD or diacetyl in *Candida glabrata*, *B. subtilis,* and *S. cerevisiae* [4, 21, 36].

In this study, the amino acid sequence of the AlsS of *B. licheniformis* WX-02 was compared with the previously characterized cALSs (*Enterococcus faecalis*, *Lactococcus lactis*, *B. subtilis*, *Klebsiella pneumonia*, *Enterococcus aerogenes*, *Serratia marcescens*) and AHASs (*E. coli* I, *E. coli* II, *E. coli* III, *B. subtilis*, *Bacillus anthracis* and *S. cerevisiae*) of other species. The sequence alignment showed that the AlsS of *B. licheniformis* WX-02 has shared about 52–74% sequence identity with cALSs and about 27–30% with AHASs from other species. The results suggest that AlsS of *B. licheniformis* is a catabolic acetolactate synthase. The enzymatic properties of the AlsS of WX-02 were firstly characterized. The specific activity of the AlsS of WX-02 was 35.4 U/mg, which was higher than those from other microorganisms (Table 3), indicating that the AlsS of WX-02 is more active than that in other microorganisms. The K_m of AlsS was 3.96 mM with pyruvate, which apparently low, compared to 13.6 mM in *B. subtilis* [37], 70 mM in *L. lactis* (70 mM) [18] and 8 mM *in K. pneumonia* [20], except 1.37 mM in *E. faecalis*. Meanwhile, its k_{cat} for pyruvate was 514/s, which was higher than those strains mentioned above (Table 3). The K_m and k_{cat} showed the important properties among all

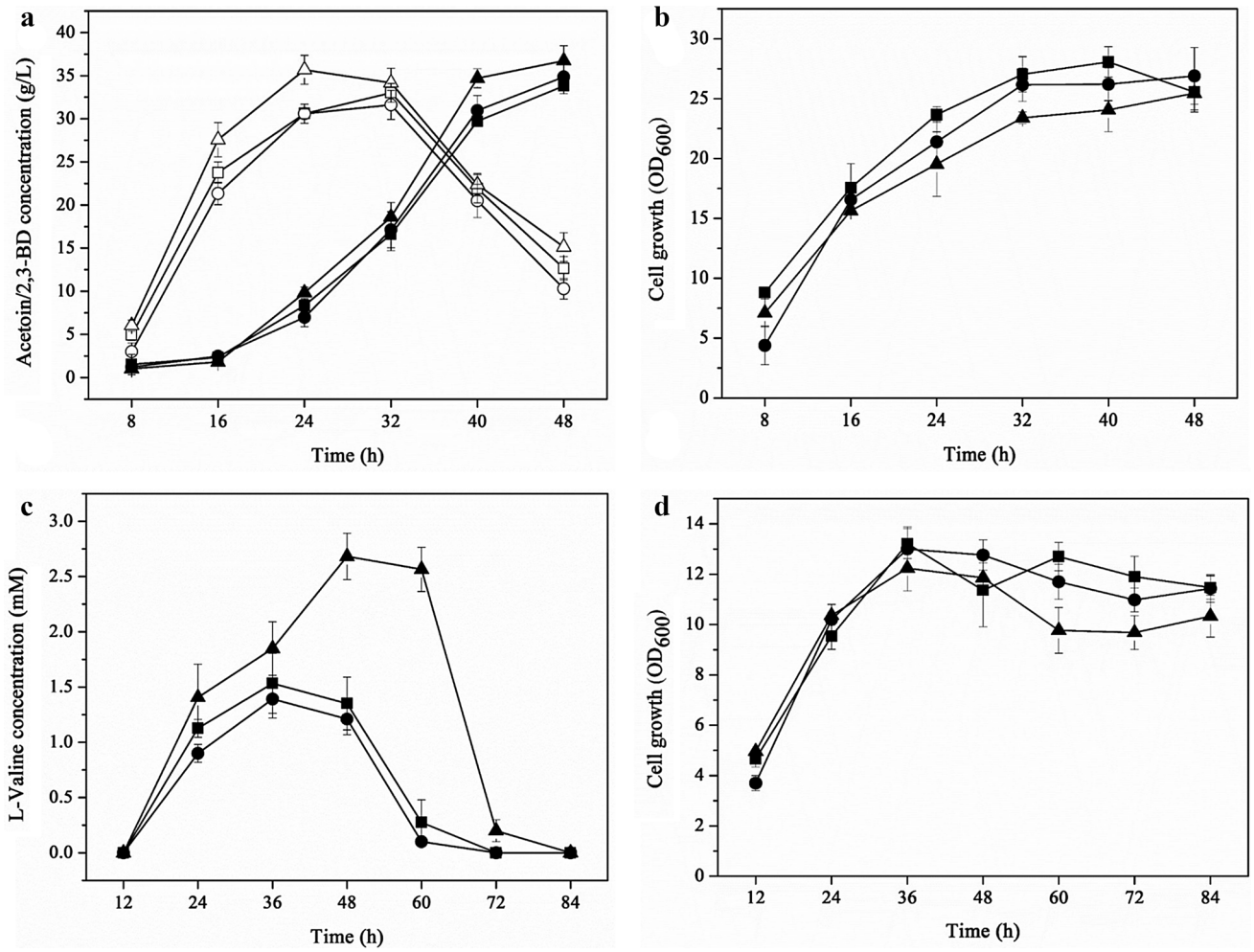

Fig. 5 Comparison of metabolite profiles among the different strains. **a** Acetoin yield (solid symbols)/2,3-BD (open symbols). **b** Cell growth in the acetoin/2,3-BD production medium. **c** L-valine. **d** Cell growth in L-valine production medium. Symbols:squares, WX-02; circles, WX-02/pT49; triangles, WX-02/pT49*alsRS*. Data were expressed as mean ± standard errors of three replicates

the enzyme kinetic parameters, and they were usually evaluated as parameters for donor gene selection for metabolic engineering [1]. The values of K_m and k_{cat} showed that the AlsS could be act as a candidate to improve the acetoin/2,3-BD production.

The previously reported catabolic ALSs were active only at pH 6.0 and anabolic ones at pH 8.0 [12, 14, 16, 19, 20]. The AlsS of *B. licheniformis* WX-02 was optimally active at pH 6.5, which could function well in a broad pH range (pH 6.0–7.5) (Fig. 2a). Generally, the

Table 3 Comparison of enzymatic properties of AlsSs from different strains

Strains	Specific activity (U/mg)	K_m for pyruvate (mM)	K_{cat} (1/s)	Optimum pH	References
*B. licheniformis*WX-02	35.4	3.96	514	6.5	This study
L. lactis	NR	70	NR	6.5–7.0	[18]
B. subtilis	8.28	13.6	121	7.0	[37]
L. mesenteroides	0.844	10	NR	5.3	[19]
E. cloacae	NR	20	NR	6.0–8.0	[38]
K. pneumoniae	NR	8.0	NR	5.8	[20]
E. faecalis	5.36	1.37	NR	6.8	[9]

NR not reported

anabolic AHAS was subjected to feedback control by branched-chain amino acids, while cALS was not [9]. In this study, L-valine, L-leucine, and L-isoleucine were found to be inhibitory to the AlsS of WX-02, and our results were consistent with the previous report in *E. cloacae* [38]. While, were different from other class reported that was not inhibited by the branched-chain amino acids [19]. These results indicated that the AlsS of WX-02 showed the properties of both the catabolic and anabolic enzymes. The regulatory mechanism of AlsS responsible for biosynthesis of branched-chain amino acids will be further investigated.

The functions of the AlsS on biosynthesis of the acetoin/2,3-BD and L-valine in *B. licheniformis* were further confirmed. Based on our results, the deficient strain WX-02Δ*alsS* could not produce acetoin/2,3-BD and L-valine, and the complementary strain WX-02Δ*alsS*/pT49*alsRS* partially restored its capability for the production of acetoin/2, 3-BD and L-valine. Although the complementary strain could express the *alsS*, the expression level or activity of *alss* in the complementary strain might be lower than those of wild-type strain, resulting in partially recovering the capacity. The insufficient AlsS also led to partially recovered the cell growth (Fig. 4b, d). We speculate that the activity of AlsS in cells can affect the acetoin/2,3-BD and L-valine production. Furthermore, the *alsS* overexpressing strain WX-02/pT49*alsRS* produced the highest titers of acetoin/2,3-BD and L-valine, which were 10.9 and 92.8% higher than those of the control strain WX-02/pT49, respectively. These results were consistent with previous reports in other strains such as *B. subtilis* and *S. cerevisiae* [21, 39]. The ability to accumulate acetolactate was observed in this study due to the increasing AlsS activity in the *alsS* overexpressing strain WX-02/pT49*alsRS*, and the accumulation of acetolactate led to the enhanced production of L-valine, acetoin, and 2,3-BD.

Generally, AHAS is involved in the biosynthesis of branched-chain amino acids, and it has rarely been reported for the impact of cALS on branched-chain amino acids biosynthesis. The activity of AlsS was inhibited by the branched-chain amino acids, especially L-valine, and the expression of AlsS might be positive correlation with the production of L-valine. These results indicate that AlsS is responsible for biosynthesis of L-valine. Taken together, we speculate that there is a synergy function between AHAS and cALS for branched-chain amino acids biosynthesis in *B. licheniformis*. In addition, this study provided a novel approach to enhance the L-valine production by enhancing the accumulation of intercellular acetolactate via AlsS overexpression.

Conclusions

In this study, the AlsS of *B. licheniformis* was proven to be an efficient acetolactate synthase with the properties of catabolic and anabolic acetolactate synthase, and the AlsS was proven to be involved in the biosynthesis of acetoin/2,3-BD and L-valine in vivo. Therefore, the AlsS could be used as a powerful genetic target for highly efficient production of value-added bioproducts in *B. licheniformis* WX-02. We have also proposed that overexpression of the AlsS could be an effective strategy to improve the production of acetoin/2,3-butanediol and L-valine in other microorganisms.

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Author contributions SC designed and supervised the study. YH and YZ performed the experiments. YH, YZ, QW, SL, SY, CN, CW and SC analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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