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ORIGINAL ARTICLE

Improving the acidic stability of *Staphylococcus aureus* α-acetolactate decarboxylase in *Bacillus subtilis* by changing basic residues to acidic residues

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Abstract The α-acetolactate decarboxylase (ALDC) can reduce diacetyl fleetly to promote mature beer. A safe strain *Bacillus subtilis* WB600 for high-yield production of ALDC was constructed with the ALDC gene *saald* from *Staphylococcus aureus* L3-15. SDS-PAGE analysis revealed that *S. aureus* α-acetolactate decarboxylase (SaALDC) was successfully expressed in recombinant *B. siutilis* strain. The enzyme SaALDC was purified using Ni-affinity chromatography and showed a maximum activity at 45 °C and pH 6.0. The values of K_m and V_{max} were 17.7 μM and 2.06 mM min^{-1}, respectively. Due to the unstable property of SaALDC at low pH conditions that needed in brewing process, site-directed mutagenesis was proposed for improving the acidic stability of SaALDC. Homology comparative modeling analysis showed that the mutation (K52D) gave rise to the negative-electrostatic potential on the surface of protein while the numbers of hydrogen bonds between the mutation site (N43D) and the around residues increased. Taken together the effect of mutation N43D-K52D, recombinant $SaALDC_{N43D-K52D}$ showed dramatically improved acidic stability with prolonged half-life of 3.5 h (compared to the WT of 1.5 h) at pH 4.0. In a 5-L fermenter, the recombinant *B. subtilis* strain that could over-express $SaALDC_{N43D-K52D}$ exhibited a high yield of 135.8 U mL^{-1} of SaALDC activity, about 320 times higher comparing to 0.42 U mL^{-1} of *S. aureus* L3-15. This work proposed a strategy for improving the acidic stability of SaALDC in the *B. subtilis* host.

Keywords α-Acetolactate decarboxylase · Diacetyl · Acidic stability · Site-directed mutagenesis · *Bacillus subtilis*

Introduction

Diacetyl, an important substance in the beer, can cause undesirable buttery off-flavor when its content is over flavor threshold (0.02–0.1 mg L^{-1}) (Dulieu et al. 2000). It is necessary to reduce the content of diacetyl during the traditional process of beer production. Diacetyl formation happens under the oxygenolysis of acetolactate, which is produced by the yeast to be an intermediate of amino acid leucine and valine synthesis. Diacetyl is then transformed to acetoin by an enzymatic reaction. Acetoin has a much higher flavor threshold and does not cause any off-flavor in the beer. However, the route of diacetyl formation during traditional beer fermentation is unavoidable as well as a rate-limiting step (Dulieu et al. 2000).

It was reported that α-acetolactate decarboxylase (ALDC, EC.4.1.1.5) could catalyze α-acetolactate to acetoin in one step without the formation of diacetyl (Loken and Stormer 1970; Suihko et al. 1990), and it was a good food additive for promoting mature beer fleetly. So ALDC application in the beer fermentation will bring great economic benefit. Besides involving in the catabolic degradation of acetolactate to acetoin in the 2,3-butanediol pathway, ALDC is also found to be as a key regulator of valine and leucine biosynthesis by controlling the acetolactate flux

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(Curic et al. 1999; GoupilFeuillerat et al. 1997; Monnet et al. 2000; Monnet and Corrieu 2007; Repizo et al. 2011). In the cell, synthesis of ALDC at high levels can have a negative effect on the valine and leucine synthesis. The cell can often adjust its amount of ALDC in response to different environmental conditions. It is assumed that ALDC may be required at high levels during carbon limitation or aerobiosis conditions that induce a shift from lactic homofermentation to mixed acid fermentation, and concomitant with high fluxes to acetoin (Goupil-Feuillerat et al. 2000). The utilization of ALDC had also been expanded for the synthesis of enantiomerically pure diols by Yan et al. (2009).

Recently, the enzyme ALDC has been reported to be found in several bacteria, such as *Lactococcus lactis* (Goupil-Feuillerat et al. 2000), *Enterobacter aerogenes* (Sone et al. 1988), *Streptococcus lactis* (Goelling and Stahl 1988), *Bacillus brevis* (Diderichsen et al. 1990), *Bacillus licheniformis* (Qin et al. 2000), *Bacillus subtilis* (Guo et al. 2001), *Acetobacter aceti* (Yamano et al. 1994) and *Leuconostoc oenos* (Garmyn et al. 1996). ALDC from different bacteria showed different physical and chemical properties. The optimum pH and K_m value of ALDC from *Lactococcus lactis* were found to be pH 6.0 and 20 mM, respectively. And this enzyme could be inhibited by Mg^{2+}, Mn^{2+}, Ba^{2+}, Ca^{2+}, Fe^{2+}, Zn^{2+} and Sn^{2+} inordinately (Kisrieva et al. 2000). Researches from O'Sulivan revealed that the purified ALDC from *Leuconostoc lactis* had an optimum pH of 6.0, pI of 4.2 and K_m value of 1.3 mM (O'Sullivan et al. 2001), while in Rasmussen's research, the optimum pH and temperature of the ALDC form *Lactobacillus casei* were pH 4.5 and 40 °C, respectively. The enzyme, with the molecular weight of 48 kDa and K_m value of 4.8 mM, could be inhibited by 1,10-phenanthroline (Phalip et al. 1994).

In this work, we isolated the ALDC from *Staphylococcus aureus* L3-15. The SaALDC (ALDC from *S. aureus* L3-15) showed a relatively high enzyme activity compared with other ALDC. To satisfy its application in the beer fermentation, the SaALDC gene *saald* was cloned and expressed in a safe host strain *Bacillus subtilis* WB600. The recombinant SaALDC was purified and characterized. For further use of this enzyme in beer maturation, its acidic stability was improved by site-directed mutagenesis. The recombinant enzyme activity was increased significantly by scale-up fermentation in the 5-L fermenter, and this study is of great importance to industrial application of ALDC.

Materials and methods

Strains, plasmids and chemicals

The strains and plasmids used in this study are shown in Table 1. *Staphylococcus aureus* was screened and stored in our laboratory. *Escherichia coli* JM109, *B. subtilis* WB600 and the shuttle expression plasmid pMA5 were preserved in our laboratory. The cloning vector pMD18-T and restriction enzymes were purchased from TaKaRa Co., Ltd. (Dalian, China). T_4 DNA ligase and 4-morpholineethanesulfonic acid (MES) monohydrate were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). Ethyl-2-acetoxy-2-methylacetocetate was purchased from Sigma Aldrich Co. LLC (Shanghai, China). The *Fast* Mutagenesis System was purchased from TransGen Biotech Co., Ltd. (Beijing, China). All other chemicals of high grade were obtained from commercial source.

Strain cultivation

Staphylococcus aureus and *B. subtilis* were grown at 37 °C in Luria–Bertani (LB) medium. Kanamycin with the concentration of 50 mg L^{-1} was added to the culture medium

Table 1 Characteristics, source or reference of the strains and plasmids using in this study

Strains/plasmids	Characteristics	Source or reference
Strainis		
Staphylococcus aureus	The source of *saald*	Our lab
E. coli JM109	Host for gene cloning	TaKaRa
B. subtilis WB600	Host for gene expression	Our lab
B. subtilis WB600/pMA5	*B. subtilis* WB600 harboring plasmid pMA5	This work
B. Subtilis WB600/pMA5-*saald*	*B. subtilis* WB600 harboring plasmid pMA5-*saald*	This work
Plasmids		
pMD18-T	Cloning vector, 2,692 bp, Amp^R, lacZ	TaKaRa
pMA5	*B. subtilis* expression vector, Amp^R in *E. coli*, Km^R in *B. subtilis*, His-Tag coding sequence, *Hpa*II promoter	Our lab
pMA5-*saald*	A derivative of pMA5, Km^R, harboring the *saald* gene	This work

Amp^R ampicillin-resistant phenotype, *Km^R* kanamycin-resistant phenotype

Table 2 Primes used for site-directed mutagenesis

Primes	Primer sequences 5′–3′
P_{N43D} F	TACACTAACAGGTTCA**G**ACGGTGAGGTAAT
P_{N43D} R	**C**TGAACCTGTTAGTGTAGCGATACCTAAGT
P_{N43E} F	TACACTAACAGGTTCAGA**A**GGTGAGGTAAT
P_{N43E} R	**T**TCTGAACCTGTTAGTGTAGCGATACCTAA
P_{K52E} F	AATCTTTTTAGATGGA**G**AAGCTTACCATGC
P_{K52E} R	**C**TCCATCTAAAAAGATTACCTCACCGTTTG
P_{K52D} F	AATCTTTTTAGATGGAGA**T**GCTTACCATGC
P_{K52D} R	**A**TCTCCATCTAAAAAGATTACCTCACCGTT
P_{R124D} F	TTAAAAAAATGCATGTA**GA**TATGATGCCGG
P_{R124D} R	**TC**TACATGCATTTTTTTAAATAAGCCTGAA
P_{R124E} F	TTAAAAAAATGCATGTAGA**A**ATGATGCCGG
P_{R124E} R	**T**TCTACATGCATTTTTTTAAATAAGCCTGA

Mutation sites are shown in bold and underlined parts

if necessary. For growing on solid medium, 20 g L^{-1} agar was added. *Bacillus subtilis* was grown at 37 °C in LB in the phase of engineering bacteria construction and then grown at 37 °C in fermentation medium (g L^{-1}): soybean tryptone, 10; glucose, 50; corn steep liquor, 15; urea, 3; $K_2HPO_4{\cdot}3H_2O$, 1.7; KH_2PO_4, 2.3; $MgSO_4{\cdot}7H_2O$, 0.75; NaCl, 50; pH 6.8–7.0.

Cloning of the gene and site-directed mutagenesis

The gene *saald* was obtained from *S. aureus* chromosome and amplified by PCR using the forward (5′-ATCGGATCCATGACGAATGTCTTGTATC) and reverse (5′-TGACTGCGGGGGCCTTCAGCTTCTCTAATTT) primers (underlined parts were the restriction sites). The *BamH* I/*Not* I site of *saald* fragment was cloned onto the plasmid pMA5. Transformation to the *B. subtilis* WB600 cell was carried out by the procedure described by Spizizen (Spizizen 1958). The recombinant strain *B. subtilis* WB600/pMA5-*saald* was selected by resistance to kanamycin. *B. subtilis* WB600 harboring the empty plasmid pMA5 was used as control. Site-directed mutagenesis on the *saald* was carried out by using the overlapping PCR-based method (Sambrook and Russell 2001). All mutants with single substitutions were constructed with plasmid encoding wild-type *saald* as the template. The overlap extension PCR for the generation of mutants was performed using the primers listed in Table 2 and followed the instructions of *Fast* Mutagenesis System. The successful introductions of the desired mutations were confirmed by sequencing.

Expression of SaALDC in *B. subtilis* WB600

The recombinant strain *B. subtilis* WB600/pMA5-*saald* was grown in LB medium containing 50 μg mL^{-1} kanamycin at 37 °C. At the end of the exponential growth phase, cells were harvested by centrifugation of culture for 15 min at 10,000×*g* and 4 °C. The cells were washed twice using MES I buffer (50 mM 4-morpholineethanesulfonic acid, 0.5 g L^{-1} Brij35, 0.6 M NaCl, pH 6.0), then resuspended in the same buffer. The harvested cells were sonicated and centrifuged at 15,000×*g* for 30 min at 4 °C to remove the cell debris. The supernatant was used for SaALDC enzyme activity assays. SDS-PAGE analysis (12 % separation gel) was basically performed according to the method described by Laemmli (1970). The protein concentration was determined by Bradford method using BSA as standard protein (Bradford 1976).

Measurement of α-acetolactate decarboxylase activity

The substrate for SaALDC enzyme assay, α-acetolactate, was generated by hydrolyzing ethyl-2-acetoxy-2-methylacetocetate according to the reported method (Garmyn et al. 1996; Loken and Stormer 1970) with minor modification. 100 mg ethyl-2-acetoxy-2-methylacetocetate was hydrolyzed with 6.0 mL 0.5 M NaOH at 20 °C for 30 min. The pH was adjusted to 6.0 with 0.5 mM HCl. The resulting solution was diluted to 50 mL with MES II (50 mM 4-morpholineethanesulfonic acid, pH 6.0), defined as substrate solution and used for α-acetolactate decarboxylase activity assay immediately.

Diacetyl could be produced from acetolactate decarboxylase by oxidation of acetoin under extreme alkaline conditions. The diacetyl formed in turn reacted with the guanine group of creatine to produce a strong red color in the presence of naphthol (Garmyn et al. 1996). The reaction mixture contained 200 μL substrate solution and 200 μL enzyme solution. The whole reaction was carried out for 30 min at 20 °C and pH 6.0, The mixture was incubated at 30 °C for 10 min and then 4.6 mL creatine-naphthol reagent (α-naphthol, 5 g L^{-1}; creatine monohydrate, 0.5 g L^{-1}; NaOH, 10 g L^{-1}) was added. The absorbance of the resulting reaction mixture was measured at 520 nm. A control reaction was run without enzyme. One unit of SaALDC activity was defined as 1 μmol acetoin produced per minute.

SaALDC purification and characterization

The supernatant of cell extraction from *B. subtilis* strains were loaded onto a Ni-NTA column. After allowing binding to proceed for 30 min, the resin was washed with 20 column volumes of buffer McAc-0 (20 mM Tris pH 8.0, 500 mM NaCl, 100 g L^{-1} glycerol) and eluted with 10 mL buffer McAc-100 (McAc-0 added with 100 mM imidazole). The eluted protein was monitored by SDS-PAGE.

To determine the optimal temperature for SaALDC, the purified enzyme was assayed from 20 to 80 °C in buffer

MES I (pH 6.0). The following buffer systems were used to investigate the pH dependence of SaALDC: 50 mM sodium acetate buffer (pH 4.0–6.0), 50 mM phosphate buffered saline buffer (PBS buffer, pH 6.0–8.0) and 50 mM glycine-NaOH buffer (pH 8.0–11.0). The enzyme stability was investigated using the standard ALDC assay after it had been incubated for certain time under different conditions.

The kinetic parameters of SaALDC

The reaction was performed in MES I (pH 6.0) at 30 °C for the determination of kinetic parameters. Assays were performed with active enzyme and α-acetolactate with different concentrations (10–500 μM). The Eadie-Hofstee plots were used to calculate kinetic parameters K_m and V_{max} according to the reactions.

Fermentation of the recombinant *B. subtilis*

The recombinant *B. subtilis* strain was cultured in LB medium supplemented with 50 μg mL^{-1} kanamycin at 37 °C for 16 h; then it was inoculated into fermentation medium for 48 h at 37 °C. Furthermore, the fermentation was scaled up in a 5-L fermenter with a working volume of 2.5 L for 60 h at 37 °C with optimized fermentation medium (g L^{-1}): glucose, 50; soybean tryptone, 12; corn steep liquor, 20; urea, 3; $K_2HPO_4{\cdot}3H_2O$, 3.6; KH_2PO_4, 4.9; $MgSO_4{\cdot}7H_2O$, 0.75; $NiSO_4{\cdot}6H_2O$, 0.3; NaCl, 5; pH 6.8–7.0. The fermentation was carried out with the stirring speed of 300 rpm and the ventilation volume of 1 vvm. Glucose was supplemented when its concentration was under 10 g L^{-1}. The cell density and SaALDC activity were measured over the fermentation period.

Measurement of diacetyl in beer fermentation

Beer fermentation with or without 100 μL L^{-1} SaALDC enzyme was conducted in the 5 L fermenter, following by the description of Lei et al. (Lei et al. 2013). Fermentation duration was 20 days and the temperature kept at 10 °C. Determination of diacetyl was conducted during the fermentation period. Diacetyl was evaporated by the distillation of beer sample. Then it was reacted with *o*-phenylenediamine to produce 2,3-dimethylchinaxolin which was absorbed at 335 nm.

Results and discussion

Cloning of ALDC from *S. aureus*

More than 20 bacteria that could produce ALDC were previously isolated from nature samples (data were not shown). Among these bacteria, *S. aureus* had relatively high-yield production of ALDC. However, *S. aureus* was pathogenic bacterium from which the enzyme product could not be used for beer fermentation. Because of that, on the GRAS (generally regarded as safe) list of Food and Drug Administration (Schallmey et al. 2004), *B. subtilis* was selected as a safe host bacterium for the production of many new and improved products (Kunst et al. 1997). The ALDC encoding gene *saald* obtained from *S. aureus* genome was cloned to *B. subtilis* WB600 with vector pMA5, and the recombinant strain *B. subtilis* WB600/pMA5-*saald* was constructed. The *B. subtilis* WB600 transformed with vector pMA5 (*B. subtilis* WB600/pMA5) was used as the control. The gene *saald* had an open reading frame of 705 bp that encodes a protein of 234 amino acids. Comparing with those reported proteins in NCBI database (http://www.ncbi.nlm.nih.gov/protein/312830942), ALDC of this work shared a 100 % similarity with that from *S. aureus* subsp. *aureus* ECT-R 2 (accession No.FR714927) in amino acid sequence.

Functional expression of SaALDC in *B. subtilis*

SDS-PAGE analysis (Fig. 1) showed that one obvious band of about 28 kDa was observed in the cell-free extracts of *B. subtilis* WB600/pMA5-*saald*. So, the enzyme SaALDC was expressed as a soluble protein. The recombinants were grown in 250 mL shake flasks containing 50 mL LB medium at 37 °C for 16 h. The SaALDC activity in *B. subtilis* WB600/pMA5-*saald* was 36.0 U mL^{-1}, improved by about 90 times compared to 0.42 U mL^{-1} of *S. aureus* and 120 times compared to 0.3 U mL^{-1} of the control strain *B.*

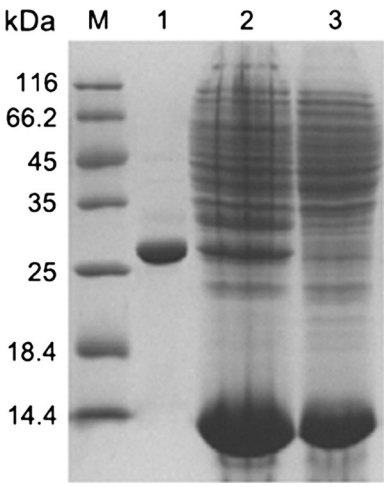


Fig. 1 SDS-PAGE analysis of SaALDC expression and purification in *B. subtilis* WB600. *Lane M* molecular weight markers. *Lane 1* The purified recombinant SaALDC; *Lane 2 B. subtilis*/pMA5-*saald*; *Lane 3 B. subtilis*/pMA5

subtilis WB600/pMA5. These results suggested that the enzyme was functionally expressed in *B. subtilis* W600. Before we cloned the SaALDC gene to *B. Subtilis*, we also expressed the SaALDC in *E. coli*. But the specific activity of SaALDC in *E. coli* was only 12.5 U mL^{-1}. By comparison of *E. coli*, *B. Subtilis* demonstrated itself a good host bacteria for SaALDC.

Enzymatic properties of recombinant SaALDC

The purification of SaALDC from the recombinant strain *B. subtilis* WB600/pMA5-*saald* was performed by a Ni-NTA column (Fig. 1). The effect of pH on SaALDC activity was measured at different pH ranging from 4.0 to 11.0. The recombinant SaALDC exhibited the maximum activity at pH 6.0 (Fig. 2a). SaALDC could catalyze α-acetolactate to acetoin in acidic pH conditions. However, when pH was lower than 5.0 or higher than 7.0, the SaALDC activity declined rapidly and was completely inactivated at pH 2.0–4.0, and the recombinant SaALDC in acidic pH conditions was not as stable as at pH 7.0–9.0 (Fig. 2b). Comparing to more than 75 % retained enzyme activity at pH 7.0–9.0 after pre-incubated for 10 h, the enzyme activity at pH 6.0 retained only 41 % after 10 h and decreased much more rapidly at pH 5.0 after 2.5 h. The optimal temperature for the recombinant SaALDC activity was between 40 and 50 °C with a maximum temperature at 45 °C at pH 6.0 (Fig. 3a). Studies on temperature stability were conducted with the temperature ranging from −20 to 65 °C. The SaALDC activity retained nearly 90 % at −20 °C and about 80 % from 0 to 35 °C after 10 h, while the rapid inactivation was observed over 45 °C (Fig. 3b).

The kinetics of the recombinant SaALDC was analyzed using α-acetolactate as substrate with different concentrations. The K_m and V_{max} values were determined by nonlinear fit analysis based on Eadie-Hofstee plots. The recombinant SaALDC catalyzed α-acetolactate decarboxylation with Michaelis constant (K_m) of 17.7 μM and maximum reaction rate (V_{max}) of 2.06 mM min^{-1} (Fig. 4).

Modeling and site-directed mutagenesis of SaALDC

The amino acid sequences of SaALDC were subjected to the SWISS-MODEL Workspace (http://swissmodel.expasy.org/workspace/) (Arnold et al. 2006; Biasini et al. 2014; Bordoli et al. 2009) and the I-TASSER server (http://zhanglab.ccmb.med.umich.edu/I-TASSER/) (Roy et al. 2010, 2012; Zhang 2008) to obtain a relative accurate tertiary-structure model. After online prediction and simulation, the crystal structure of the unknown function protein (PDB ID: 1xv2) with a resolution of 2.00 Å

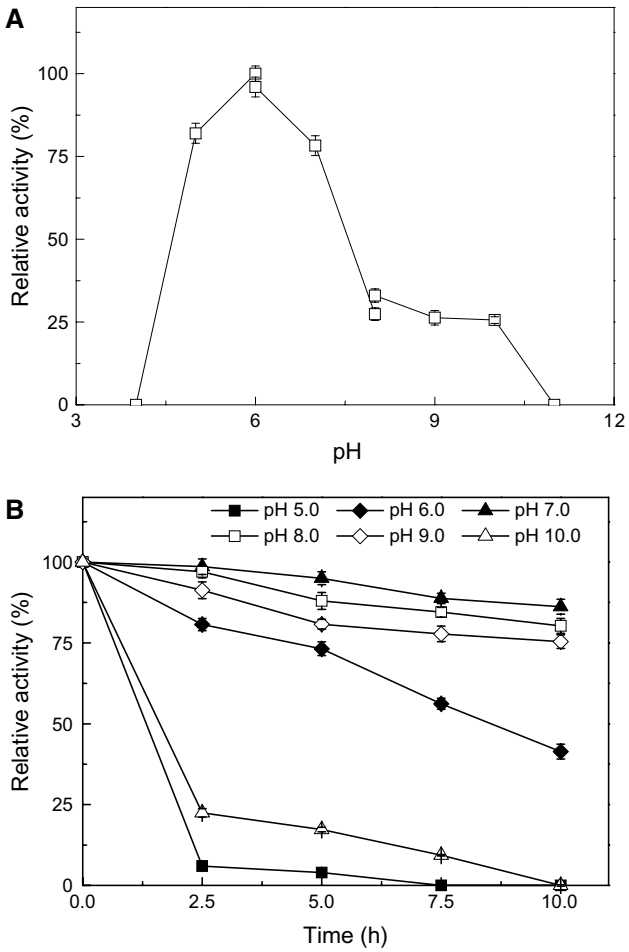


Fig. 2 Effect of pH on recombinant SaALDC activity. **a** Optimum pH was decided by assaying the recombinant SaALDC activity at 30 °C with pH ranging from 4.0 to 11.0. **b** Stability of SaALDC under different pH conditions. After incubating the enzyme in different pH values (5.0–10.0) at 4 °C, the residue activities were determined at pH 6.0 and 30 °C. (All assays were performed by triplicate cultures; standard deviations of the biological replicates are represented by *error bars*)

that was similar to α-acetolactate decarboxylase was identified as having the highest similarity to SaALDC. The estimated accuracy model by I-TASSER has the TM-score of 0.99 ± 0.04 and RMSD (root-mean-square deviation) of 1.6 ± 1.4 Å (TM-score and RMSD are known standards for measuring structural similarity between two structures) and has the *C*-score (confidence score of the model) = 2. In general, the models with *C*-score > −1.5 have a correct fold, and TM-score >0.5 indicates the correct topology of the predicted models. With low $C\text{-score}^{EC}$ (confidence score of the enzyme commission number) and no consensus of the EC numbers among the identified analogs in the function prediction by I-TASSER, we were advised to consult the GO (gene ontology) term predictions, wherein the analogs were associated with the

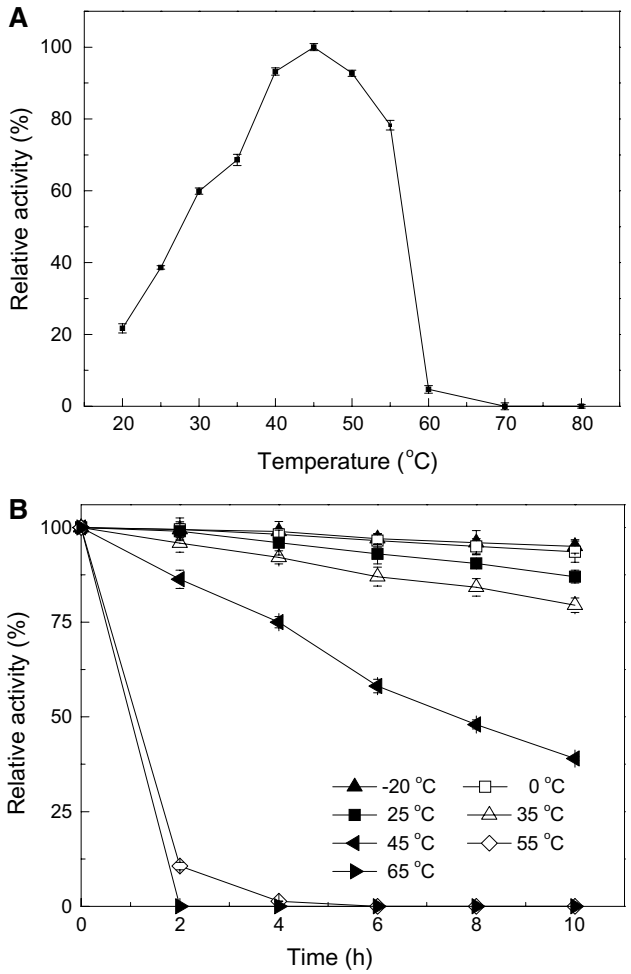


Fig. 3 Effect of temperature on recombinant SaALDC activity. **a** Optimum temperature was examined by assaying the recombinant SaALDC activity at various temperatures ranging from 20 °C to 80 °C. **b** Thermal stability of the purified enzymes was determined by pre-incubation at different temperatures from −20 to 65 °C, then assaying the residue activity under standard assay conditions. (All assays were performed by triplicate cultures; standard deviations of the biological replicates are represented by *error bars*)

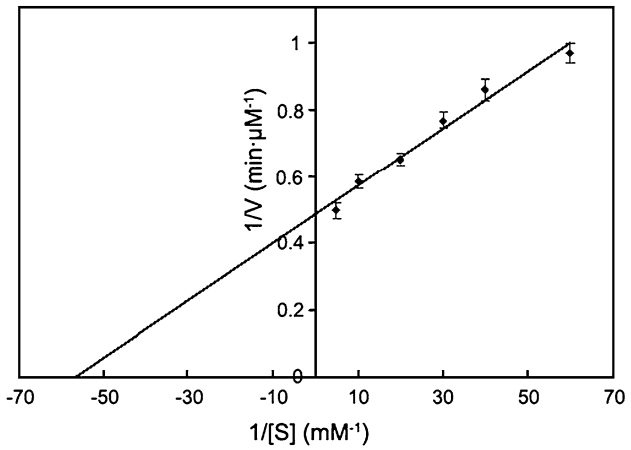


Fig. 4 Lineweaver-Burk plot for kinetics determinations of purified SaALDC using α-acetolactate as the substrate

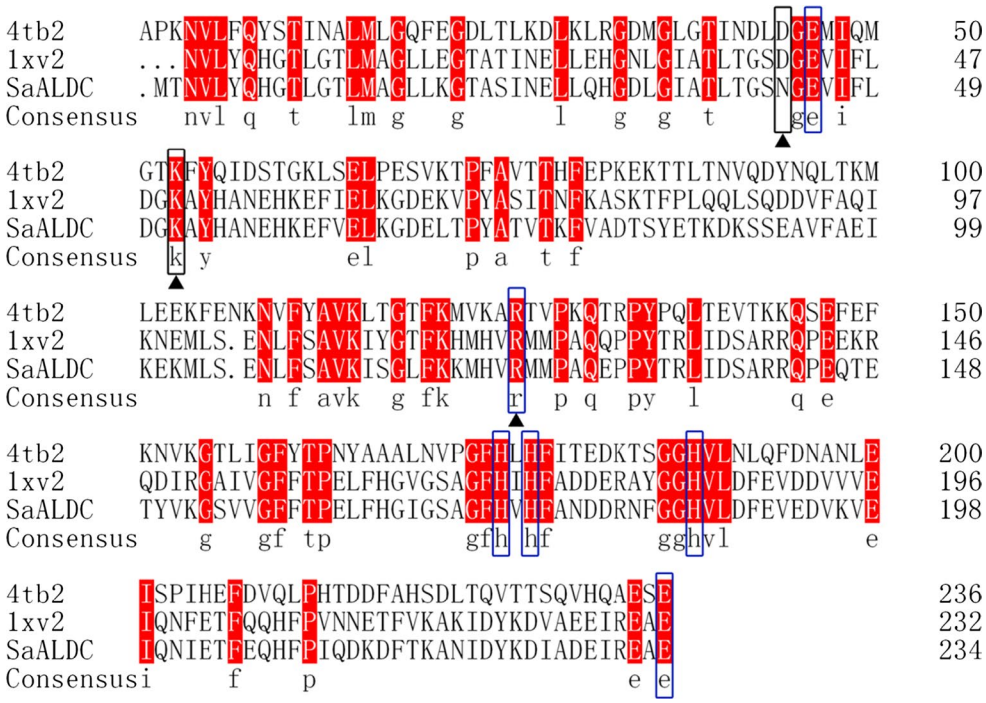
highest level of molecular function, biological process and cellular location in the GO hierarchy. The model with the highest *C*-score^{GO} (confidence score of GO term) of 0.80 was also associated with the PDB hit of 1xv2 and was assigned to the acetolactate decarboxylase activity (GO: 0047605) and polyol metabolic process (GO: 0019751). The predicted binding site residues were E45, H173, H175 and H186, which should be avoided while the enzyme was restructured by site-directed mutagenesis.

The amino acid residues involved in the decarboxylation and rearrangement steps of ALDC with the PDB deposition of 4bt2 (1.10 Å) from *Bacillus brevis* had been experimentally identified (Marlow et al. 2013). The catalytic mechanism of BbALDC on its molecular-level was also proposed. By amino acid sequences alignment of 4tb2, 1xv2 and SaALDC, they are dramatically conserved in the predicted active center sides (E62, R142 and E251 of BbALDC was involved in the catalytic mechanism, which were corresponded to E45, R124 and E234 of SaALDC; the highly conserved H194, H196 and H207 of BbALDC coordinate a Zn^{2+} ion, corresponded to H173, H175 and H186 of SaALDC). Thus, the tertiary structure of 4tb2 was used as the template for SaALDC molding and mutagenesis.

The conventional method for down-shifting the pH optimum was to change the basic residues to acidic residues (Huang et al. 2012). To select the candidate residues, the amino acid sequences alignment of several α-acetolactate decarboxylases was conducted (Fig. 5), in which the highly conserved sequences of K52 have the probability to be substituted with either aspartic acid or glutamic acid residues. The recombinant $SaALDC_{K52D}$ and $SaALDC_{K52E}$ were constructed. The second substitution of N43D was also constructed to obtain recombinant $SaALDC_{N43D}$, because the glutamic acid at this site was conserved in other two ALDCs. Due to the divergence of predicted active residues (R124 and E234) between 1xv2 and 4tb2, the basic residue of R124 was also substituted to construct $SaALDC_{R124D}$, $SaALDC_{R124E}$.

Enzymatic stability at low pH conditions of mutants

The pH sensitivities of the wild-type WT (SaALDC) and the mutants, with respect to their functional stability, were studied by incubating the enzymes for 2.5 h at various pH values ranging from pH 4.0 to 8.0 followed by measurement of ALDC activity at pH 6.0 (Fig. 6). The wild SaALDC showed very unstable property at low pH conditions; only 6.0 and 4.0 % activity left after it had been incubated for



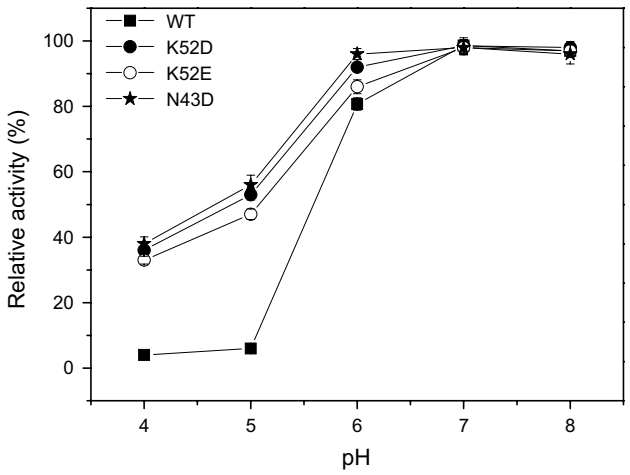


Fig. 6 Effect of pH (ranging from 4.0 to 8.0) on enzyme stability of mutants K52D, K52E and N43D. (All assays were performed by triplicate cultures; standard deviations of the biological replicates are represented by *error bars*)

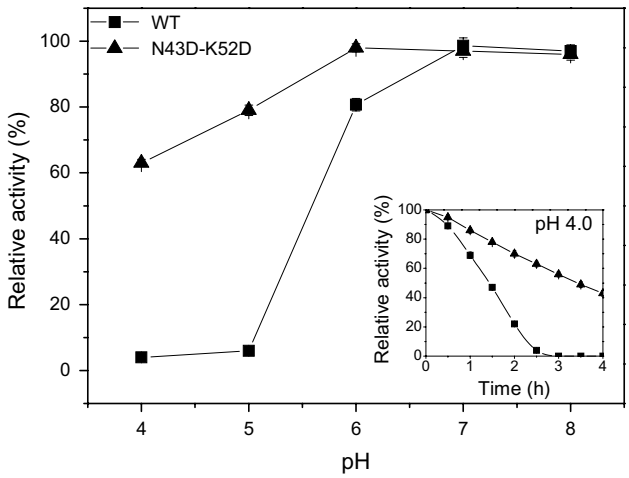


Fig. 7 Comparison of effect of pH (ranging from 4.0 to 8.0) on enzyme stability of mutant N43D-K52D and WT. The half-lives of N43D-K52D and WT at pH 4.0 were also studied. (All assays were performed by triplicate cultures; standard deviations of the biological replicates are represented by *error bars*)

2.5 h at pH 5.0 and 4.0, respectively. On the contrary, this enzyme was very stable at neuter and alkalescence conditions (pH 6.0–8.0). After site-directed mutagenesis, mutants of N43D, K52D and K52E have almost no variation on enzyme activity, but exhibited improved stability over pH 4.0–5.0 than the WT. After pre-incubation for 2.5 h at pH 5.0, N43D, K52D and K52E retained 56, 53 and 47 % of their enzyme activity, respectively, whereas their residue activity was 38, 36 and 33 % at pH 4.0, respectively. The results indicated that the acidic stability of the enzyme has been improved after rationally changing basic residues to acidic ones. As might have been expected, mutants of R124D and R124E retained only about 20 % of its activity at pH 6.0 and about 10 % at pH 4.0, indicating a basic residue is necessary for catalysis in this position (Marlow et al. 2013). Based on the significant improvement of the acidic stability of N43D and K52D, mutant of N43D-K52D was constructed, purified and identified by mass spectrometry compared to the WT (Fig. 7; Fig. S1).

The N43D-K52D mutant showed dramatically improved acidic stability at pH 4.0 (retained 63 % activity) and pH 5.0 (retained 79 % activity) after incubated for 2.5 h, and its half-life at pH 4.0 was prolonged from 1.5 to 3.5 h; meanwhile, the range of the enzyme stability at various pH conditions was broadened.

Structure analysis of wild SaALDC and mutants

The structure of SaALDC and $SaALDC_{N43D\text{-}K52D}$ was modeled using the crystal structure of BbALDC (PDB ID: 4tb2) from *Bacillus brevis* as template. The resulting models were used to explain the differences in acidic stability between the mutants and WT in terms of structure using the software of PyMOL. The electrostatic surfaces of the WT and mutant enzyme were modeled (shown in Fig. 8). The charge distribution of the WT enzyme showed a negative-positive–negative amino acid residue chain (GLU64-LYS52-ASP50-LYS113-GLU198-LYS196-GLU86) (Fig. 8a). The mutant enzyme gives rise to a negative potential on the structure surface around the mutant site (K52D) by disruption of the negative–positive–negative chain, which significantly changes the modeled electrostatic potential on the surface of the protein (Fig. 8b), so the resistance to the acidic condition of the proteins is increased.

Analysis of the hydrogen-bond interaction between the mutant site of N43D and K52D and their surrounding amino acid residues showed that N43D increased the hydrogen-bond numbers of the surface structure of the catalytic center from 11 to 14 while K52D showed no changed hydrogen-bond numbers (Fig. 8c, d). Since increasing the hydrogen-bond numbers can contribute to the enzyme stability (Joshi et al. 2000; Le Nours et al. 2003; Yang et al. 2013), the mutation also increased the rigidity of this structure, in which GLU45 (catalysis related amino acid) has two interaction hydrogen-bonds between N43 and D43, respectively. However, unexpected, the substitution of N43 by D43 decreased the interaction hydrogen-bonds of the catalytic center from 8 to 7 by losing the hydrogen-bond interaction between R124 and E234 (Fig. 8e, f), which may increase the flexibility for substrate docking and also improve the acidic stability of this enzyme (Liu et al. 2014; Marlow et al. 2013). Taken together, the results showed that the acidic stability of SaALDC had been improved after changing amino acids on the structure surface into the acidic ones.

Fermentation for the recombinant variant $SaALDC_{N43D\text{-}K52D}$ production in 5 L fermenter

The recombinant strain *B. subtilis* WB600/pMA5-$saald_{N43D\text{-}K52D}$ was cultivated for 48 h at 37 °C in 250 mL shaker flasks containing 50 mL fermentation medium at first. During the fermentation, the total $SaALDC_{N43D\text{-}K52D}$ activity reached the highest value of 66.5 U mL^{-1}. Then the fermentation was scaled up to a 5-L fermenter with a working volume of 2.5 L. In the growth phase, the maximum cell concentration in the fermenter was 25.3 (OD_{600}). The $SaALDC_{N43D\text{-}K52D}$ enzyme reached the maximum of 135.8 U mL^{-1} at 48 h (Fig. 9), which was 320-fold excess of *S. Aureus*. To our knowledge, the recombinant $SaALDC_{N43D\text{-}K52D}$ showed a very high enzyme activity. This could be attributed to not only the screening of bacterium for high-yield ALDC production but also the successful expression of *saald* gene to the right host bacterium *B. subtilis*.

Formation of diacetyl with $SaALDC_{N43D\text{-}K52D}$

The effect of $SaALDC_{N43D\text{-}K52D}$ enzyme on beer fermentation can be seen in Fig. 10. Addition of $SaALDC_{N43D\text{-}K52D}$ accelerated the transformation of α-acetolactate to acetoin. The maximum concentration of diacetyl in beer fermentation with $SaALDC_{N43D\text{-}K52D}$ was 0.35 mL L^{-1} and appeared at the fourth day of fermentation, which indicated that the enzymatic removal of α-acetolactate served to reduce the content of diacetyl indirectly. Sometimes the content of diacetyl rises during the storage stage of finished beer, attributed to the presence of a certain amount of α-acetolactate. This could also be improved by adding of ALDC enzyme.

As one approach to prevent the formation of diacetyl, adding the enzyme ALDC requires an ALDC high producing microorganism which is also safe for food industry. Then for the first time, the recombinant SaALDC with a high activity of 135.8 U mL^{-1} was expressed in *B. Subtillis*, a very safe strain for industrial application. It can be shown that the enzyme $SaALDC_{N43D\text{-}K52D}$ can be used in beer fermentation with improved acidic stability. The results demonstrate that highly active recombinant $SaALDC_{N43D\text{-}K52D}$ can be produced at sufficient levels and can thus be used as the biocatalyst for the reduction of diacetyl in a cost-effective way. Moreover, a further investigation with an aim of improving the enzyme SaALDC stability and activity in fermentation is in progress.

Conclusions

Heterologous over-expression of ALDC from *S. aureus* in *B. subtilis* WB600 was successfully achieved in this work. The recombinant enzyme SaALDC was expected to promote mature beer by reducing of diacetyl. However, SaALDC showed very low enzyme stability at low pH conditions (pH 4.0), which is needed during beer maturation process. Based on protein molecular structure modeling and site-directed mutagenesis, the mutant protein

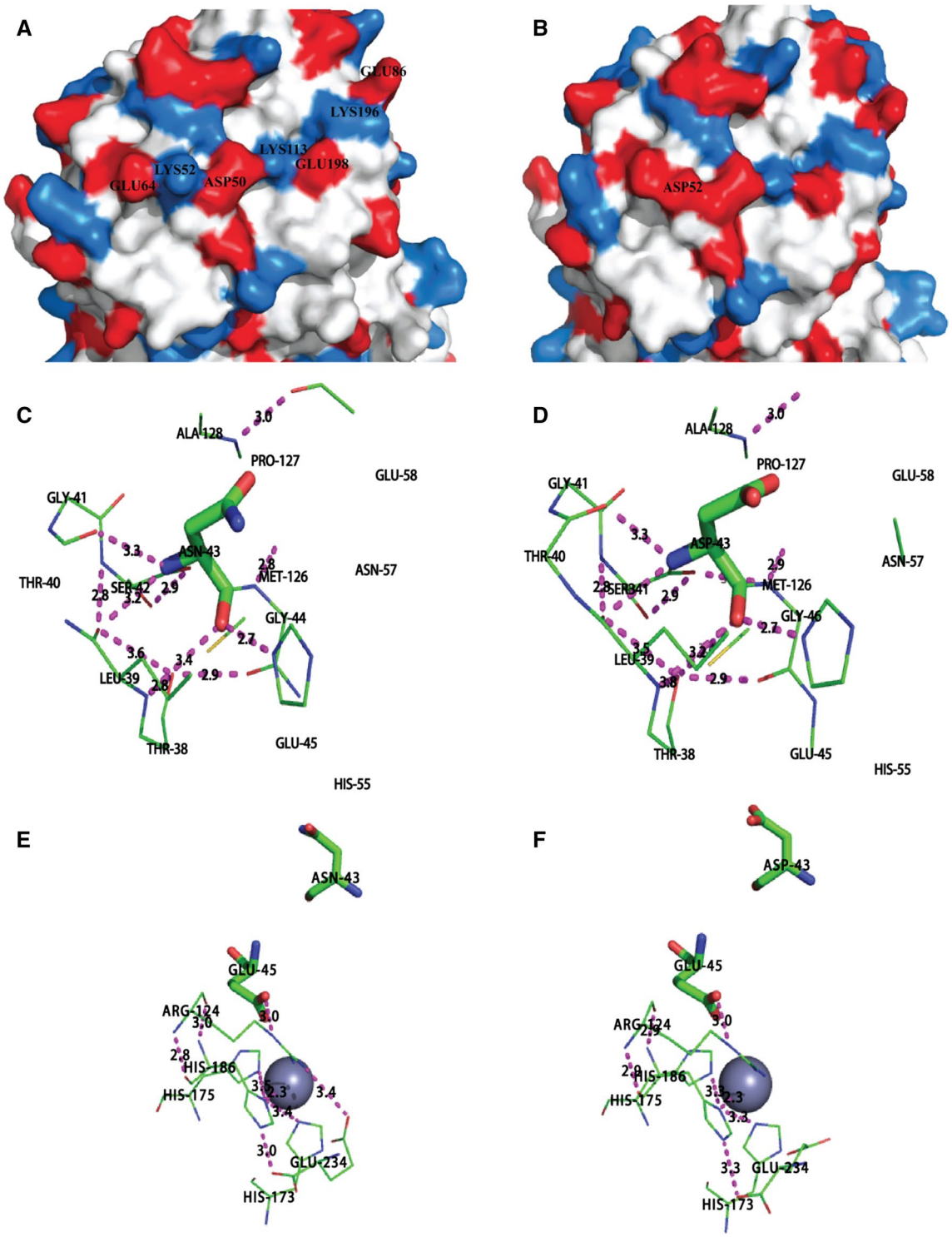


Fig. 8 Structure overview of SaALDC and $SaALDC_{N43D-K52D}$. **a** Charge distribution on the surface of SaALDC; **b** charge distribution on the surface of $SaALDC_{N43D-K52D}$, in which K52D showed significantly changed charge distribution; **c** interaction hydrogen bonds of N43 and its nearby residues; **c** interaction hydrogen bonds of D43 and its nearby residues; **e** interaction hydrogen bonds of catalytic-related residues, Zn^{2+} and N43; **f** interaction hydrogen bonds of catalytic-related residues, Zn^{2+} and D43, in which N43D resulted in the loss of hydrogen bond between E45 and E234. The hydrogen bonds are indicated by *broken pink lines*. This figure was generated using PyMOL software (color figure online)

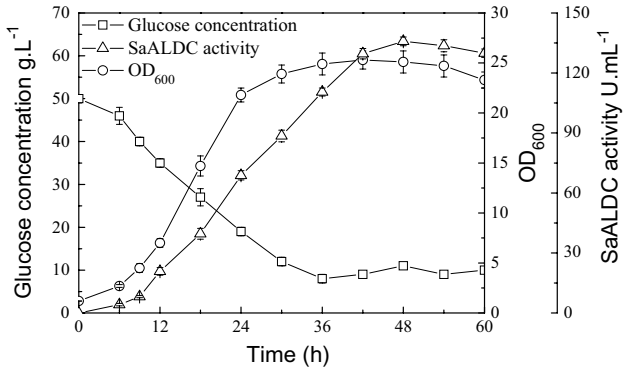


Fig. 9 Fermentation curves of *B. subtilis* WB600/pMA5-*saald*$_{N43D-K52D}$ in a 5-L fermenter. (All assays were performed by triplicate cultures; standard deviations of the biological replicates are represented by *error bars*)

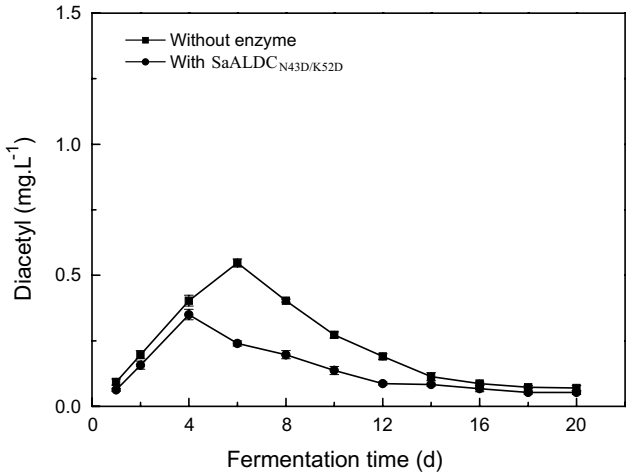


Fig. 10 Formation of diacetyl in beer without (*filled square*) and with 100 μL L^{-1} recombinant SaALDC enzyme (*filled circle*) (All assays were performed by triplicate cultures; standard deviations of the biological replicates are represented by *error bars*)

of SaALDC$_{N43DK52D}$ showed improved acidic stability and prolonged half-live at pH 4.0. Structure analysis of the mutant enzyme exhibited that mutation of K52D gave rise to the negative potential on the structure surface while mutation of N43D increased the hydrogen-bond interaction of the enzyme. This work provides reference for improving acidic stability of ALDC. The successful expression of *saald* gene in *B. subtilis* indicates it a suitable host for scale-up ALDC production.

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Conflict of interest The authors declare no conflict of interest.

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