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ORIGINAL PAPER

Identification of a Novel Alditol Oxidase from *Thermopolyspora flexuosa* with Potential Application in D-Glyceric Acid Production

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Abstract

Glycerol is a potential sustainable feedstock, and biorefining processes to convert glycerol into value-added chemicals have been developed over the past decade. Alditol oxidase (AldO) is capable of selectively oxidizing the primary hydroxyl groups of alditols such as glycerol. In this study, a new FAD-binding protein from *Thermopolyspora flexuosa* was expressed and identified as a novel alditol oxidase ($AldO_{T.\ fle}$). $AldO_{T.\ fle}$ displayed the optimal activity at pH 8.0 and 25 °C. $AldO_{T.\ fle}$ was not metal-dependent, but the activity was completely inhibited by Fe^{3+}. $AldO_{T.\ fle}$ had a wide substrate specificity and high catalytic efficiency for glycerol. Furthermore, the recombinant $AldO_{T.\ fle}$ could produce D-glyceric acid from glycerol with a conversion rate ranging from 86.6% (5 mM glycerol) to 20.5% (500 mM glycerol). The recombinant *E. coli* with $AldO_{T.\ fle}$ could also produce 23.8 mM D-glyceric acid from 100 mM glycerol. The recombinant $AldO_{T.\ fle}$ had the potential to produce other aldehyde products by selectively oxidizing the hydroxyl groups of alditols and many other commodity chemicals by redesigning glycerol metabolism.

Keywords Glycerol · Alditol oxidase · D-Glyceric acid

Introduction

Glycerol is one of the major biomass resources and generated in large amounts during the production of biodiesel [1]. However, the pressure to deal with these byproducts is becoming more pronounced. Therefore, glycerol transformations, including oxidation, hydrogenolysis, esterification, and reformation, have become increasingly significant in terms of the economy and environmental protection [2].

Biorefining by integrating systems biology, synthetic biology, and metabolic engineering has become an emerging approach that is mainly used for the development of biocatalysts platforms [3]. To date, microbial fermentation of glycerol to produce value-added products has been extensively studied [4–7]. Selective oxidation of glycerol produces many chemical intermediates, including glyceraldehyde, glyceric acid, dihydroxyacetone, glycolic acid, and oxalic acid [8, 9]. Although many efforts have been made regarding the catalytic oxidation of glycerol, how to control the selectivity of specific products remains lacking and challenging.

Several oxidizing enzymes can be utilized in processes requiring the selective oxidation of glycerol, such as glycerol dehydrogenase (GDH), which catalyzes the oxidation of glycerol into glyceraldehyde and ketone with the concomitant reduction of the electron acceptor $NAD(P)^+$. However, most GDHs convert glycerol to dihydroxyacetone [10]. The obvious drawback of these enzymes is the need for stoichiometric amounts of an expensive cofactor. To avoid the above deficiencies, alcohol oxidases have aroused wide interest [11]. These oxidases use molecular oxygen as a cheap and clean electron acceptor with hydrogen peroxide as the only byproduct. Alditol oxidase (AldO) from *Streptomyces coelicolor* A3 has been successfully expressed, and capable of selectively oxidizing the primary hydroxyl groups of alditols [12]. Meanwhile, it exhibits good biocatalytic potential towards glycerol for the production of D-glyceraldehyde and D-glyceric acid [13–15]. Soon afterwards, another HotAldO was identified from the proteome of *Acidothermus cellulolyticus* 11B, which shared 48% protein sequence identity with AldO. HotAldO is highly thermostable and has a half-life

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activity of 112 min at 75 °C [16]. Both of them have extensive substrate specificity; in particular, AldO has also been applied in the synthesis of D-glyceric acid [15, 17].

D-Glyceric acid is present in plants and humans as a metabolite [18]. It has been reported to be a building block for many agents, such as D-glycerate hemicalcium salt monohydrate [19], dialkylglycerate [20], monoalkylglycerate [21], and glycerate-phospholipid analogs [22]. Conversion of glycerol to D-glyceric acid is desirable for the industrial applications. There are several reports on the potential biological activity of D-glyceric acid and its derivatives, including accelerated ethanol and acetaldehyde oxidation, functional surfactants, and skin care [19, 21, 23]. D-glyceric acid, a byproduct during dihydroxyacetone production, was obtained by acetic acid bacteria *Gluconobacter* sp. [24], *Gluconacetobacter* sp. [25], and *Acetobacter* sp. [25, 26]. To date, only a few reports have examined D-glyceric acid synthetic pathway or the genes and enzymes involved in the reactions [17, 26, 27].

In this study, a putative flavin adenine dinucleotide (FAD)-binding protein from *Thermopolyspora flexuosa* was cloned and identified as a novel AldO. This oxidase was heterologously expressed and purified, and its biochemical properties, including the enzymatic properties and substrate specificity, are investigated in detail. To the best of our knowledge, this is the first report about $AldO_{T.\ fle}$, which has a wide substrate specificity and the highest catalytic efficiency for glycerol. It also showed the highest activity towards glycerol for D-glyceric acid production. $AldO_{T.\ fle}$ could be a new potential candidate for the biosynthesis of various functional aldehydes/carboxylic acids.

Materials and Methods

Chemicals, Materials, and Strains

D-Glyceraldehyde (D-GLA), D-glyceric acid sodium salt, and catalase were all purchased from Sigma-Aldrich (St. Louis, USA). Imidazole, isopropyl-β-D-1-thiogalactopyranoside (IPTG) and kanamycin were all purchased from Sangon (Shanghai, China). PCR primers and infusion DNA polymerase were obtained from Tian Lin Co., Ltd. (Wuxi, China). Restriction endonucleases and Ligation Mix were purchased from Takara (Japan). All other chemicals were of analytical grade and commercially available.

The strains and plasmids are listed in Table S1. DH5α was used for plasmid construction. *Escherichia coli* BL21 (DE3) was used for protein expression and D-glyceric acid production in this study. The *E. coli* strains were grown at 37 °C with shaking at 220 rpm in an LB medium (5 g/L yeast extract, 10 g/L tryptone, and 10 g/L NaCl) supplemented with kanamycin (50 μg/mL) as required. A TB medium (24 g/L yeast extract, 12 g/L tryptone, 16.4 g/L $K_2HPO_4{\cdot}3H_2O$, and 2.31 g/L KH_2PO_4) was used for protein expression and fermentation supplemented with streptomycin (50 μg/mL) and glycerol as required.

Cloning, Expression, and Purification of $AldO_{T.\ fle}$

The sequence of AldO from *S. coelicolor* A3 (UniProt: Q9ZBU1) was applied as a template to explore new potential alditol oxidases using the BLAST tool of the National Center for Biotechnology Information (NCBI). A putative FAD-binding protein with the NCBI reference sequence of WP_142259226.1 was selected as a candidate. The codon-optimized (based on *E. coli* preferences) gene of *aldO* from *T. flexuosa* encoding alditol oxidase ($AldO_{T.\ fle}$ gene sequence in Table S2) was synthesized by Tian Lin Co., Ltd. (Wuxi, China). The $aldO_{T.\ fle}$ gene and peroxidase genes (*prx02* and *katE*) from *E. coli* MG1655 were amplified by PCR. The primers are listed in supplementary Table S3. The PCR product of $aldO_{T.\ fle}$ was inserted into the pET28a vector and multiple cloning sites (MCS1) of the plasmid pCDFDuet-1 with *Bam*H I and *Hin*d III to generate plasmids pET28-*aldO and* pCDFDuet-*aldO*, respectively. The PCR product of $prx02_{E.\ coli}$ and $katE_{E.\ coli}$ was inserted into the multiple cloning sites (MCS2) of the plasmid pCDFDuet-*aldO* with *Nde* I and *Xho* I to form plasmids pCDFDuet-*aldO-prx02* and pCDFDuet-*aldO-katE*, respectively. The recombinant plasmid pET28-*aldO* was transformed into *E. coli* BL21 (DE3) for expression. The positive transformant was confirmed by colony PCR, which was grown in LB medium for plasmid extraction and sequencing. The recombinant plasmids pCDFDuet-*aldO*, pCDFDuet-*aldO-prx02*, and pCDFDuet-*aldO-katE* were transformed into *E. coli* BL21 (DE3) for D-glyceric acid production by fermentation.

To facilitate the purification of $AldO_{T.\ fle}$, *E. coli* BL-01 carrying the plasmid pET28a-*aldO* was grown at 37 °C in a TB medium with 50 μg/mL kanamycin. When the OD_{600} reached 0.6–0.8, 0.1 mM IPTG was added to induce the expression of $AldO_{T.\ fle}$. Then, the cells were further cultured at 16 °C for another 20–24 h. To purify the $AldO_{T.\ fle}$, the recombinant *E. coli* cells were harvested and washed with 0.9% (wt/vol) sodium chloride solution by centrifugation at 5, 000 rpm for 10 min. Subsequently, the cell pellet was suspended in a binding buffer (pH 8.0; 50 mM Tris–HCl, 20 mM imidazole, and 150 mM NaCl) and disrupted by sonication in an ice bath. The supernatant containing $AldO_{T.\ fle}$ was collected by centrifugation at 15,000 rpm for 30 min at 4 °C and purified through a HisTrap HP column (Ni–NTA agarose, Sigma). Then, the purification steps were as follows: 1) unbound proteins were removed by elution buffer A (pH 8.0; 50 mM Tris, 20 mM imidazole, and 150 mM NaCl) and buffer B (pH 8.0; 50 mM Tris, 60 mM imidazole, and 150 mM NaCl); 2) recombinant $AldO_{T.\ fle}$ with a histidine

tag was washed with elution buffer C (pH 8.0; 50 mM Tris, 500 mM imidazole, and 150 mM NaCl). The fraction containing $AldO_{T.\ fle}$ was dialyzed with Tris–HCl buffer (pH 7.5, 50 mM Tris, 10 mM EDTA) to remove metal ions and then dialyzed with Tris–HCl buffer (pH 7.5, 50 mM Tris) to remove EDTA.

Enzyme Activity and Molecular Mass Determination

The assay of $AldO_{T.\ fle}$ activity with 10 mM corresponding substrate was performed at 25 °C and in a 50 mM Tris–HCl buffer (pH 7.5) for 30 min. The oxidase activity of $AldO_{T.\ fle}$ was measured by coupling H_2O_2 production [12]. The H_2O_2 production was determined by ABTS kit (Sangon, Shanghai, China). One unit of $AldO_{T.\ fle}$ activity was defined as the amount of enzyme necessary for 1 μmol of H_2O_2 per minute.

The molecular mass of $AldO_{T.\ fle}$ subunit was detected by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) using 12% separating gel and 5% stacking gel. The voltage for SDS-PAGE was 80 V in stacking gel for 30 min and 120 V in separating gel for another 70 min. Then the gel was stained using Coomassie Blue G-250 for 30 min. The native molecular mass of $AldO_{T.\ fle}$ was detected by a high-performance liquid chromatography (HPLC) system (Waters 2695, USA) equipped with an ultraviolet detector and TSK-GEL G3000SW column (TSK, Japan). The mobile phase containing PBS buffer (pH 7.4) flowed at 0.5 mL/min, and the injection volume was 20 μL.

Effect of pH, Temperature, and Metal Ions on the Activity of Recombinant $AldO_{T.\ fle}$

To determine the optimal pH of recombinant $AldO_{T.\ fle}$, 50 mM Tris–HCl buffer (pH 7.0–9.0) was employed to evaluate its effect on the activity of recombinant $AldO_{T.\ fle}$ in 1 mL at 25 °C for 12 h. To investigate the optimal temperature of recombinant $AldO_{T.\ fle}$, temperatures ranging from 20 to 40 °C were adopted to determine their influence on the activity of recombinant $AldO_{T.\ fle}$ in 50 mM Tris–HCl buffer (pH 8.0) for 12 h. To explore the pH stability, $AldO_{T.\ fle}$ was added to a Tris–HCl buffer with different pH values (7.0–9.0) for 2 h at 4 °C, and the residual oxidase activity was measured. The thermostability was monitored as follows: $AldO_{T.\ fle}$ was incubated in a Tris–HCl buffer (pH 8.0) at various temperatures (40–55 °C) for 2 h. The residual oxidase activity was determined as usual. The initial activity without any treatment was set as 100%. To investigate the influence of metal ions on the activity of recombinant $AldO_{T.\ fle}$, eight different types of metal ions, namely Zn^{2+}, Ni^{2+}, Ca^{2+}, Mg^{2+}, Fe^{3+}, Cu^{2+}, Co^{2+}, and Mn^{2+}, were used. The final concentration (1 mM) of the above metal ions was determined in a Tris–HCl buffer (pH 8.0) at 25 °C. The enzyme activity was defined as the same as the above.

Kinetic Parameter Determination and Substrate Specificity

In order to study the kinetic parameters of recombinant $AldO_{T.\ fle}$ to glycerol and xylitol, various concentrations of glycerol (5–800 mM) and xylitol (0.1–10 mM) were used in a Tris–HCl buffer (50 mM, pH 8.0) for 30 min. Kinetic parameters including K_m (mM), k_{cat}/K_m ($M^{-1}\ s^{-1}$), and k_{cat} (s^{-1}) were determined from the Lineweaver–Burk plot after nonlinear regression fitting. 10 mM of various substrates, including methanol, ethanol, ethanediol, *n*-propanol, isopropanol, 3-aminopropanol, S-aminopropanediol, R-aminopropanediol, *n*-butanol, mannitol, and xylitol, were used to explore the substrate specificity of recombinant $AldO_{T.\ fle}$. The above experiments were performed in a Tris–HCl buffer (pH 8.0) at 25 °C for 12 h. The enzyme activity was defined as the same as the above.

D-Glyceric Acid Production Using Recombinant $AldO_{T.\ fle}$

The production of D-glyceric acid from glycerol using recombinant purified $AldO_{T.\ fle}$ was performed at 25 °C in a Tris–HCl buffer (50 mM, pH 8.0) containing 0.5 mg/mL $AldO_{T.\ fle}$ (250 U/mL), various concentrations of glycerol (5, 10, 50, 100, and 500 mM) substrate, and supplemented with catalase (250 U/mL) and FAD cofactor (1 mM) as required. The amount of D-glyceric acid was examined at fixed time intervals by HPLC as stated below until 24 h.

D-Glyceric Acid Production Using Fermentation

To produce D-glyceric acid with *E. coli* strains in flasks, *E. coli* strains stored as glycerol stocks at − 80 °C were streaked on LB plates with appropriate antibiotics and incubated at 37 °C overnight. Single colonies were picked from LB plates to inoculate 5 mL of LB medium with appropriate antibiotics in a 25 mL tube. The cultures were shaken at 37 °C and 220 rpm in a rotary shaker and used as seed cultures to inoculate 200 mL of TB medium at 1% (v/v) with appropriate antibiotics in 1 L conical flasks. This culture was shaken at 37 °C and 220 rpm until an optical density of 0.8 OD_{600} was reached. Then, 100 mM glycerol and 0.1 mM IPTG were added to the medium, and the cells were shaken at 25 °C and 220 rpm for 60 h.

Analytical Methods

The oxidization of glycerol to D-glyceric acid was measured by an HPLC system (Waters 2695, USA) equipped with a refractive index detector and Bio-Rad column (Aminex

HPX-87H, 300 × 7.8 mm). The mobile phase containing 5 mM H_2SO_4 flowed at 0.5 mL/min (60 °C), and the injection volume was 20 µL [13].

Results and Discussion

Expression, Purification, and Molecular Mass of $AldO_{T. fle}$

The protein sequence of AldO from *S. coelicolor* was adopted to perform BLAST searches to identify putative oxidases. Several sequences were identified, and a FAD-binding protein from *T. flexuosa* was selected. The purified $AldO_{T. fle}$ was used for SDS-PAGE analysis, and then a single band with a molecular mass of approximately 50 kDa was observed (Fig. 1A), which was consistent with the theoretical value of 50.6 kDa with a single 471 amino acid polypeptide chain plus a 6×His tag. The native protein was analyzed by HPLC with a TSK-GEL G3000SW column. The total molecular mass of $AldO_{T. fle}$ was approximately 50 kDa based on a standard protein curve (Fig. 1B). According to the above results, $AldO_{T. fle}$ should be a monomeric protein similar to $AldO_{S. coe}$ [28].

Multiple Sequence Alignment of AldO from Different Microorganisms

To date, only a few AldOs have been identified, including AldO from *S. coelicolor* [12] and HotAldO from *A. cellulolyticus 11B* [16]. The sequence alignment was performed using DNAMAN software. As shown in Fig. 2, $AldO_{T. fle}$ shared sequence identity with $AldO_{S. coe}$ (56.71% identity, NCBI Reference Sequence: WP_011030685.1), followed by Hot $AldO_{A. cel}$ (52.45% identity, NCBI Reference Sequence: WP_011719559.1).

The pyrophosphate group of FAD is involved in several hydrogen-bonding contacts with the main chain atoms of the loop described by residues ranging from Gly43 to Ser47. The residues ranging from Gly43 to Ser47 of $AldO_{T. fle}$, which were concerned with forming hydrogen-bonding contacts with the pyrophosphate group of FAD, were strictly conserved in all resolved AldOs [28]. The side chain of His46 approached the isoalloxazine ring from the *re* face of the flavin. Three residues, Ser106, Glu320, and Thr345, anchoring the O-2, O-3, O-4, and O-5 sites of xylitol were also seriously conserved, indicating that the AldOs had the same catalytic mechanism.

Effect of Temperature and pH on the Activity of $AldO_{T. fle}$

In general, the optimal pH and temperature are important for the catalytic properties of the enzyme. The optimal pH of $AldO_{T. fle}$ was investigated using a Tris–HCl buffer ranging from 7.0 to 9.0. As shown in Fig. 3A, $AldO_{T. fle}$ exhibited maximum activity in the Tris–HCl buffer system of pH 8.0 and showed nearly 80% of the maximum activity from pH 7.5 to 8.5. The optimal temperature was assessed in a Tris–HCl buffer (pH 8.0) at different temperatures (20 to 40 °C). As shown in Fig. 3B, $AldO_{T. fle}$ exhibited maximum activity at 25 °C, and the enzyme activity only decreased

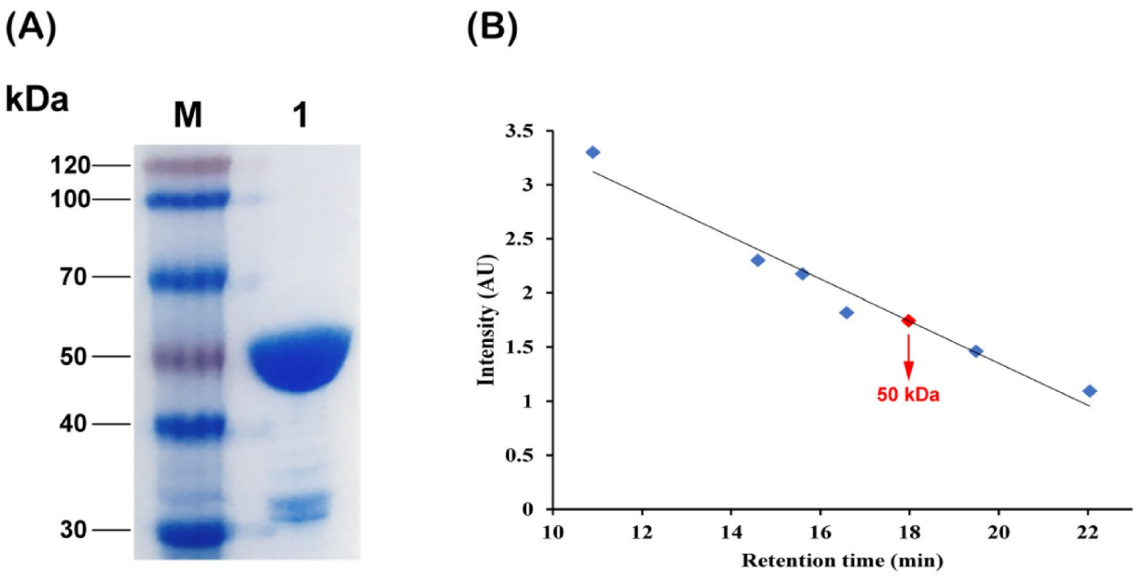

Fig. 1 Purification and molecular mass of $AldO_{T. fle}$. **A** SDS-PAGE analysis of $AldO_{T. fle}$. **B** HPLC analysis of $AldO_{T. fle}$ using gel filtration chromatography

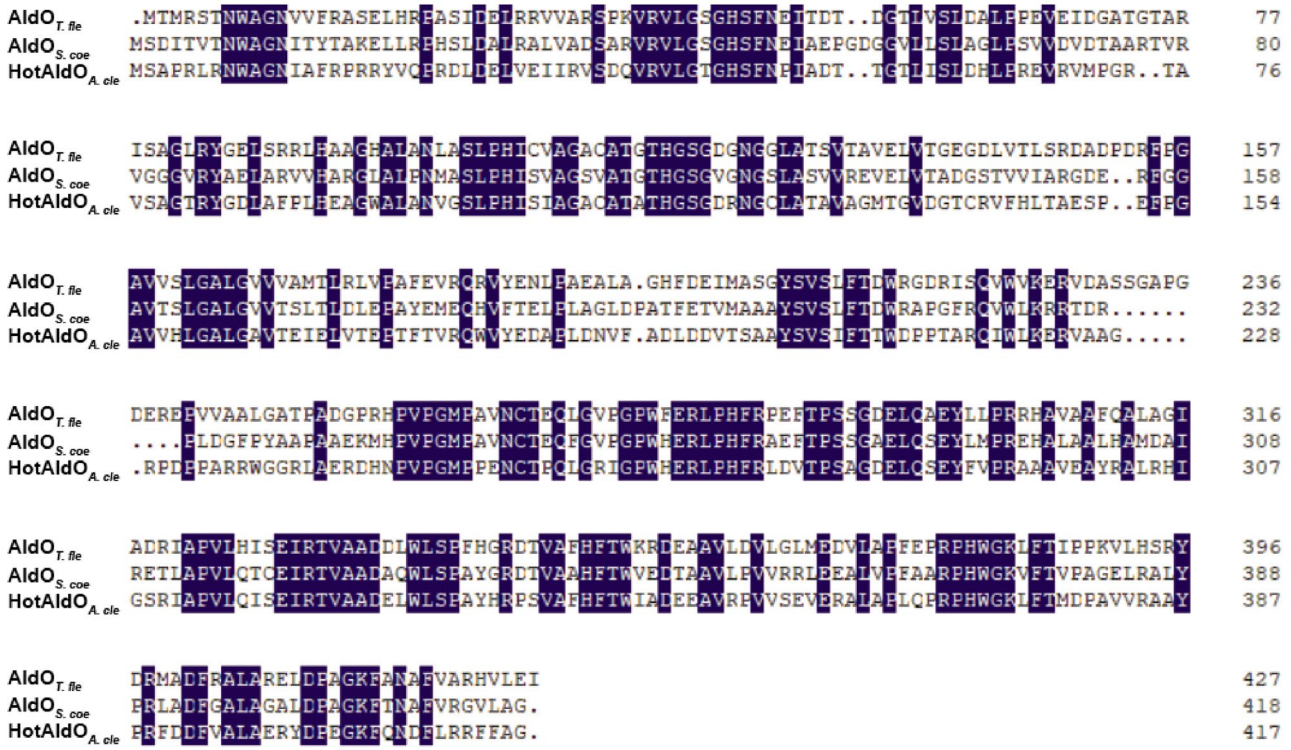

Fig. 2 Multiple sequence alignment of alditol oxidases from different microorganisms. The conserved residues are shown with a blue background

30% between 20 and 40 °C compared with the maximum activity. The effects of pH and temperature on recombinant $AldO_{T.\ fle}$ stability were also investigated. As shown in Fig. 3C and D, the residual enzyme activity was still more than 70% of the initial activity at various pH values and temperatures. These results indicate that $AldO_{T.\ fle}$ had relatively high pH stability and thermostability.

Effect of Metal Ions on the Activity of $AldO_{T.\ fle}$

To explore the effect of various metal ions on $AldO_{T.\ fle}$ activity, different metal ions with final concentrations of 1 mM were added into the metal-free enzyme system. The relative activity of $AldO_{T.\ fle}$ is shown in Fig. 4, metal-free $AldO_{T.\ fle}$ group exhibited the highest activity. However, most groups with metal ions had little effect on their activity, indicating that $AldO_{T.\ fle}$ was not metal-dependent. To our surprise, only 10% of the relative enzyme activity was retained in the presence of Cu^{2+}, and the activity of the enzyme was completely inhibited by Fe^{3+}. The related mechanisms need to be further studied.

Kinetic Parameters and Substrate Specificity

As shown in Table 1, $AldO_{T.\ fle}$ exhibited the highest k_{cat} value for glycerol, which was 3983 times for that of $AldO_{S.coe}$ and 4902 times for that of HotAldO, respectively. Additionally, the k_{cat} value for glycerol was 44.7 times for that of xylitol. To our delight, $AldO_{T.\ fle}$ showed the highest catalytic efficiency (k_{cat}/K_m) for glycerol, which was 3680 times for that of $AldO_{S.coe}$ and 3526 times for that of $HotAldO_{A.\ cel}$, respectively. Nevertheless, the catalytic efficiency for $AldO_{T.\ fle}$ toward xylitol was 2.2 times than that of glycerol, which was due to much higher K_m value for glycerol than that of xylitol.

Moreover, twelve different substrates including methanol, ethanol, ethanediol, *n*-propanol, isopropanol, 3-aminopropanol, S-aminopropanediol, R-aminopropanediol, *n*-butanol, mannitol, xylitol, and glycerol were applied to study the specific activities of $AldO_{T.\ fle}$. As shown in Fig. 5, all the above substrates could be converted by $AldO_{T.\ fle}$, which showed a broad substrate specificity. Meanwhile, it showed the highest activity towards glycerol. Subsequently, xylitol with five alcohol hydroxyls was selected to check the oxidation product. The product of xylose was identified from HPLC and compared with standard D-xylose (Fig. S4). These results manifested that the recombinant $AldO_{T.\ fle}$ had the potential to produce other aldehyde products by selectively oxidizing the primary hydroxyl group of alditols similar with $AldO_{S.\ coe}$ [12].

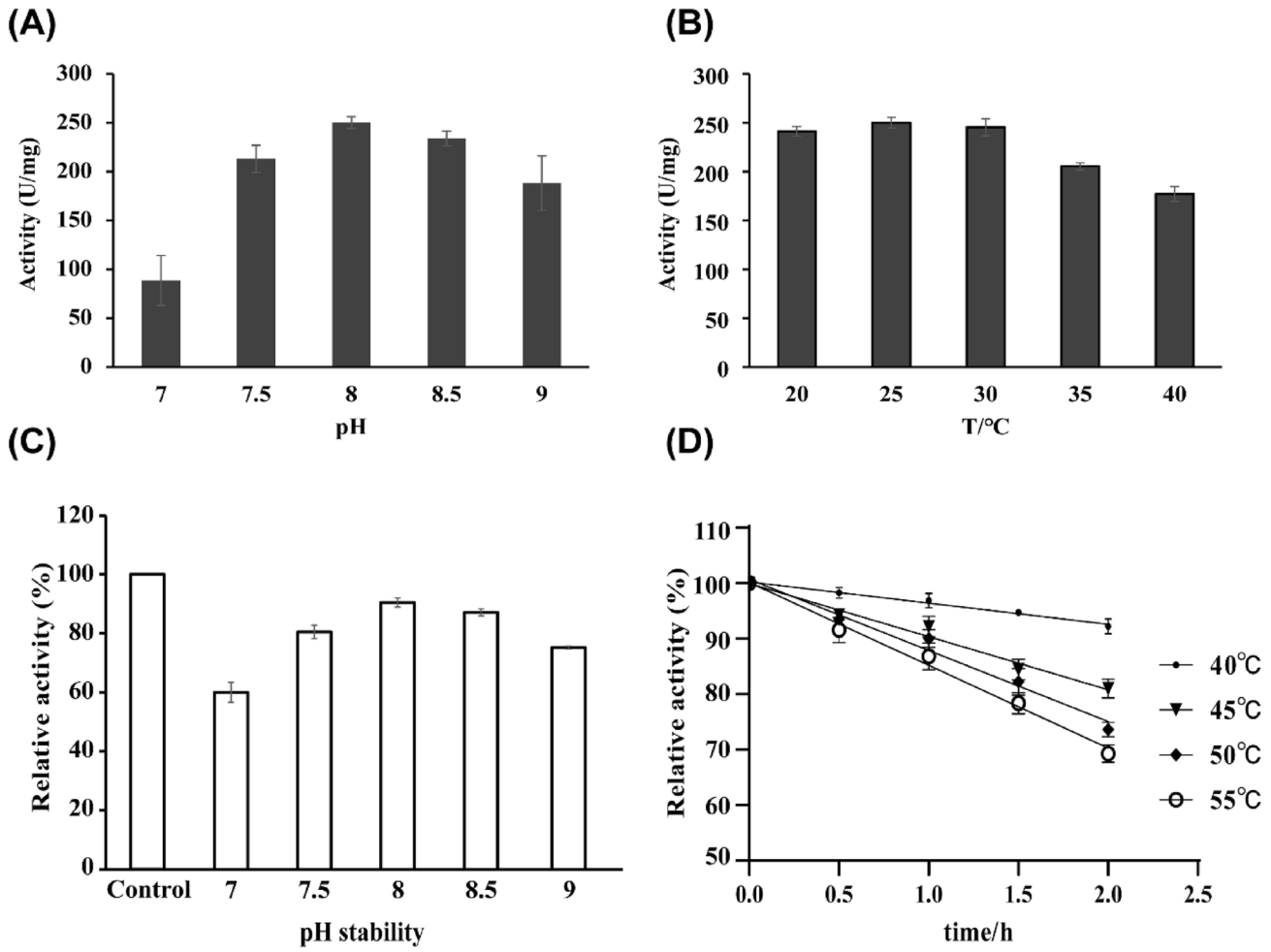

Fig. 3 Effect of pH and temperature on the activity of $AldO_{T.\ fle}$. **A** pH. **B** Temperature. **C** pH stability. **D** Thermostability. All experiments were carried out in triplicate

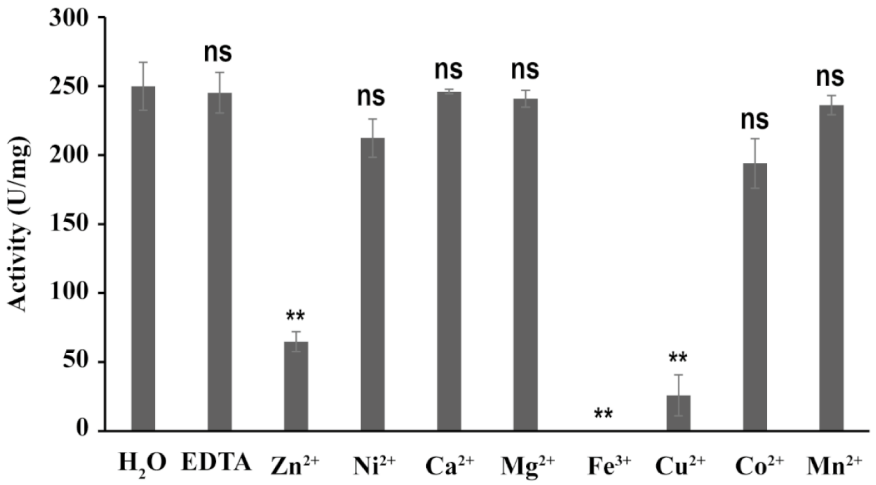

Fig. 4 Effect of metal ions on the activity of $AldO_{T.\ fle}$. $AldO_{T.\ fle}$ with ddH_2O or EDTA as the control. The group of ion-free $AldO_{T.\ fle}$ with ddH_2O was normalized to the maximum activity (100%)

ns means no significant differences. *means $p<0.05$, ** means $p<0.01$.

Table 1 Comparison of kinetic parameters among various alditol oxidases

Enzyme	Microorganisms	K_m (mM)		k_{cat} (s^{-1})		k_{cat}/K_m (M^{-1} s^{-1})		References
		Xylitol	Glycerol	Xylitol	Glycerol	Xylitol	Glycerol	
AldO	*T. flexuosa*	3.8	376.5	142.5	6373.1	37,500	16,927.2	This study
AldO	*S. coelicolor*	0.32	350	13	1.6	41,000	4.6	[12]
HotAldO	*A. cellulolyticus*	0.07	270	1.9	1.3	27,000	4.8	[16]

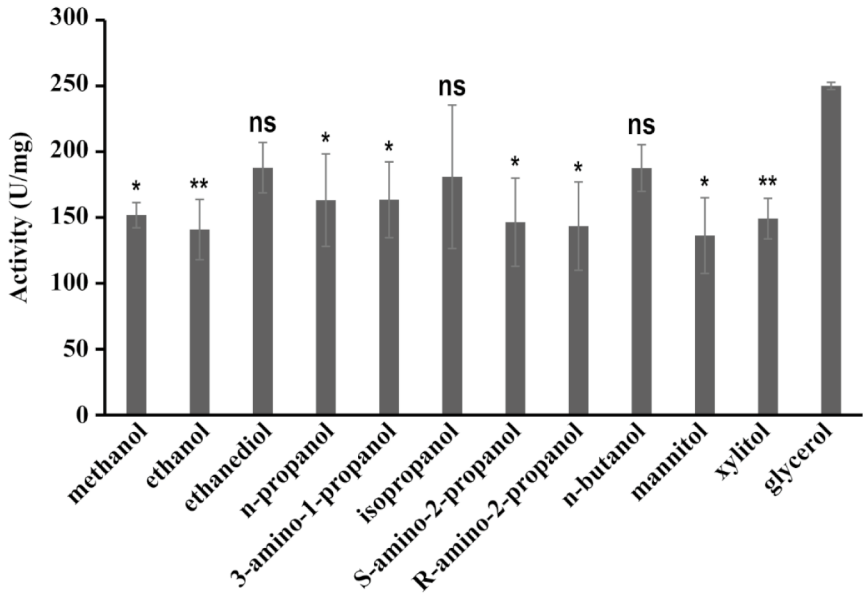

Fig. 5 Substrate specificity of recombinant $AldO_{T. fle}$

ns means no significant differences. *means $p<0.05$, ** means $p<0.01$.

Production of D-Glyceric Acid by Pure $AldO_{T. fle}$

First, D-glyceric acid production using different concentrations of glycerol (1, 5, 10, 50, 100 mM) was performed under optimized condition and detected by HPLC. As displayed in Fig. S1, only D-glyceric acid was detected at glycerol concentrations of 1 and 5 mM. Both D-glyceric acid and D-glyceraldehyde were detected at high glycerol concentrations, and higher concentrations of glycerol tended to synthesize D-glyceraldehyde. Interestingly, only D-glyceric acid was detected when catalase was added (Fig. S2). Perhaps more oxygen was needed for the end product of D-glyceric acid. Furthermore, the effect of FAD cofactor on $AldO_{T. fle}$ activity was also checked. From Fig. S3, without FAD, the relative activity of $AldO_{T. fle}$ was still maintained at 90%. As is well known, there is a set of genes (*ribA*, *ribD*, *ribB*, *ribH*, *ribC*, and *ribF*) involved in the formation of FAD in *E. coli* [29]. It also reveals that $AldO_{T. fle}$ contains covalently bound FAD.

The production of D-glyceric acid based on different concentrations of glycerol (5, 10, 50, 100, 500 mM) at 25 °C was measured under the condition of 50 mM Tris–HCl buffer (pH 8.0) containing 0.5 mg/mL $AldO_{T. fle}$ (250 U/mL) and catalase (250 U/mL). As the concentration of glycerol increased from 5 to 500 mM, the concentration of D-glyceric acid was increased and the conversion rate was decreased from 86.6% (5 mM) to 20.5% (500 mM) (Fig. 6A and B). The decreasing conversion rate with the increase in concentrations of glycerol may be attributed to substrate inhibition at high concentrations or lack of oxygen.

D-Glyceric Acid Production Using Fermentation

Furthermore, the production of D-glyceric acid based on different concentrations of glycerol was also detected using fermentation. $AldO_{T. fle}$ converted glycerol to D-glyceric acid with the formation of hydrogen peroxide byproducts. The intermediate metabolites of aldehydes and hydrogen peroxide are toxic to cell growth. A previous study reported that reactive oxygen species (ROS) were generated by molecular oxygen colliding with a variety of flavoenzymes, and excess intracellular ROS result in enzyme inhibition and cell damage [30, 31]. To eliminate the damage caused by hydrogen peroxide, two different peroxidases encoded by *prx02* and

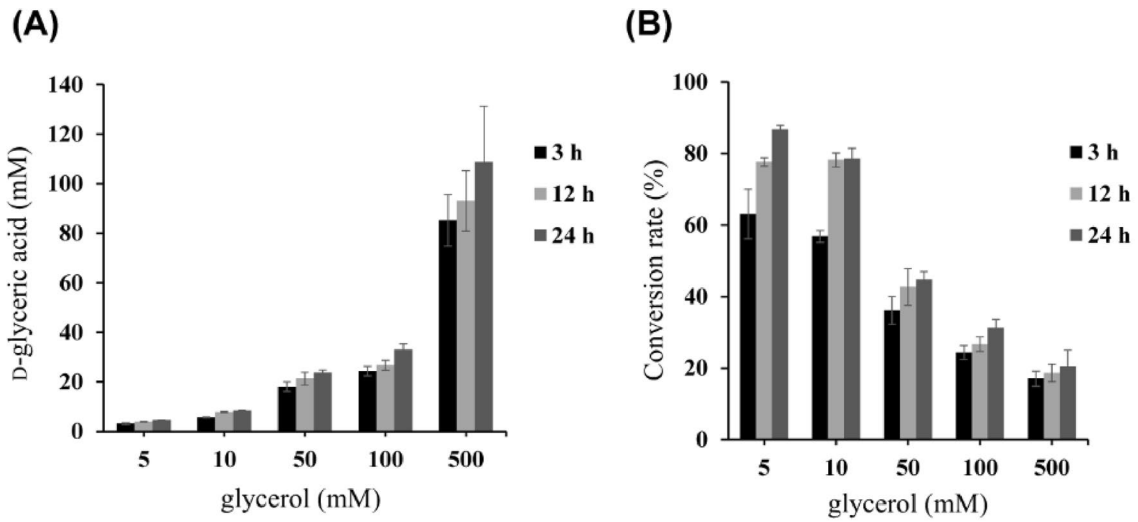

Fig. 6 Production of D-glyceric acid by pure $AldO_{T.\ fle}$. **A** The production of D-glyceric acid based on different concentrations of glycerol at 25 °C was measured in a Tris–HCl buffer (50 mM, pH 8.0). **B** The conversion rates of D-glyceric acid based on different concentrations of glycerol

KatE from *E. coli* MG1655 were also coexpressed with $AldO_{T.\ fle}$ [31, 32]. The growth curve displayed in Fig. 7A shows that the strain coexpressing Prx02 or KatE significantly increased cell growth. However, overexpression of Prx02 or KatE only slightly improved the total production yield of D-glyceric acid, as shown in Fig. 7B. The highest yield of D-glyceric acid was approximately 23.8 mM from 100 mM glycerol. During the metabolism of glycerol in microorganisms, glycerol should first be applied to produce pyruvate, which is further transformed into high-value products. In fact, pyruvic acid can be produced with numerous additional enzymatic steps (glycerol dehydrogenase, ATP-dependent dihydroxyacetone kinase, glycerol kinase, NAD-dependent glycerol-3-phosphate dehydrogenase, triose phosphofructokinase, glyceraldehyde phosphate dehydrogenase, phosphoglycerate kinase, phosphoglyceromutase, enolase, and pyruvate kinase) and expensive cofactors (NAD, ADP, and ATP) in organisms, such as enolase and pyruvate kinase [17]. Here, glycerol was first converted into D-glyceric acid by $AldO_{T.\ fle}$. D-glyceric acid could be converted into pyruvate by DHAD from *S. solfataricus* with water as a byproduct [33, 34]. Glycerol could generate equal amounts of pyruvate without any other cofactors via two enzymatic steps. Furthermore, D-glyceric acid could also be converted into 3-hydroxypyruvate by 2-hydroxyglutarate-pyruvate transhydrogenase from *Saccharomyces cerevisiae* [35]. As a result, $AldO_{T.\ fle}$ had the potential to produce other commodity chemicals by redesigning the glycerol metabolism.

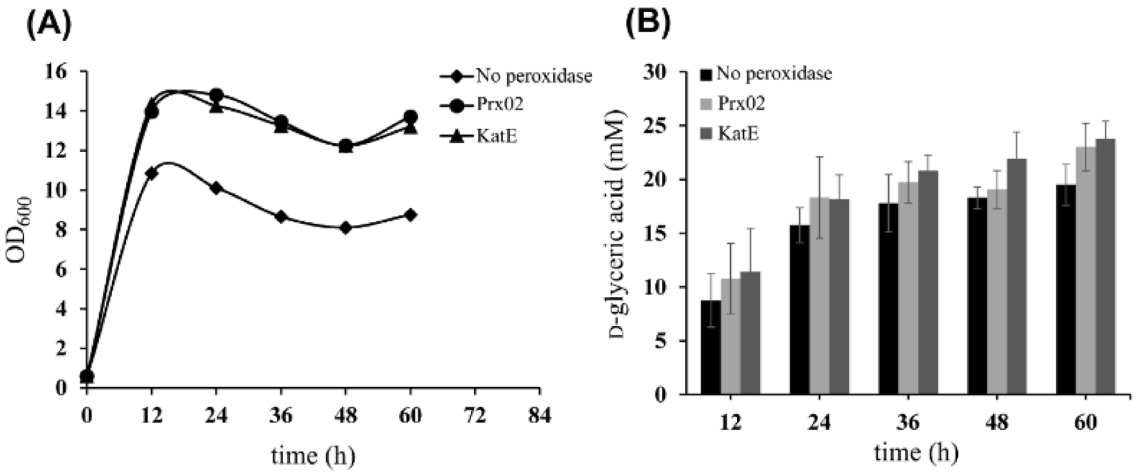

Fig. 7 Two different peroxidases, Prx02 and KatE, were coexpressed with $AldO_{T.\ fle}$, respectively. **A** The growth curve and the absorbance value were measured at 600 nm. **B** The production of D-glyceric acid using three different strains

Conclusion

In summary, a novel alditol oxidase from *T. flexuosa* was identified. $AldO_{T.\ fle}$ displayed the optimal activity at pH 8.0 and 25 °C. It showed a relatively high pH stability maintaining 70% of the initial activity at pH 7.5–8.5 and high thermostability at 50 and 55 °C. $AldO_{T.\ fle}$ was not metal-dependent, but its activity was completely inhibited by Fe^{3+}. $AldO_{T.\ fle}$ showed a broad substrate spectrum and a high catalytic capacity of glycerol (k_{cat}/K_m value was 16,927.2 M^{-1} s^{-1}). Thus, $AldO_{T.\ fle}$ could be applied to produce D-glyceric acid from glycerol with a conversion ratio ranging from 86.6% (5 mM) to 20.5% (500 mM). The recombinant *E. coli* expressing $AldO_{T.\ fle}$ also exhibited the capability to synthesize glyceric acid, and the yield of D-glyceric acid was 23.8 mM with a conversion rate of 23.8%. $AldO_{T.\ fle}$ had the potential to produce other aldehyde products by selective oxidation of alditols and produce other commodity chemicals by redesigning glycerol metabolism.

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Declarations

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

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