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Characterization of the sugar alcohol-producing yeast *Pichia anomala*

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Abstract Sugar alcohols have been widely applied in the field of food and medicine for their unique properties. Compared to chemical production, microbial production of sugar alcohol has become attractive for its environmental and sustainable pattern. In this study, a potential yeast isolated from soil of Beijing suburbs was identified as *Pichia anomala* TIB-x229, and its key enzyme of D-arabitol dehydrogenase for microbial production of sugar alcohols was functionally characterized. This yeast could simultaneously produce D-arabitol, xylitol, and/or ribitol from a different ratio of sugar substrates at a high efficiency by bioconversion, and no glucose repression happened when mixed sugars of xylose and glucose were used as the substrates during the bioconversion. This yeast could also efficiently convert complicated feedstock such as xylose mother liquor to D-arabitol, xylitol, and ribitol with 55 % yields. To elucidate the conversion relationship of the sugar alcohols, especially D-arabitol and xylitol, the key D-arabitol dehydrogenase gene from *P. anomala* was cloned, expressed and purified for further in vitro characterization. The results showed that this D-arabitol dehydrogenase could catalyze arabitol to xylulose further, which is significant for xylitol production from glucose. Our study laid the foundation for improving the production of sugar alcohols by metabolic and fermentation engineering strategies.

Keywords *Pichia anomala* · Sugar alcohol · Bioconversion · D-Arabitol dehydrogenase

Abbreviations

A_{600}	Absorbance at 600 nm
A_{340}	Absorbance at 340 nm
ArDH	D-Arabitol dehydrogenase
CGMCC	China General Microorganism Culture Center
HPLC	High-performance liquid chromatography
IPTG	Isopropyl β-D-1-thiogalactopyranoside
KDa	Kilodalton
NAD^+	Nicotinamide adenine dinucleotide
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
QPS	Qualified presumption of safety

Introduction

Sugar alcohols, also known as polyols, are ingredients used as sweeteners and bulking agents [1]. As a sugar substitute, they provide fewer calories than regular sugar and require little or no insulin to be metabolized. This makes them popular among individuals with diabetes [23]. As important five-carbon polyols, D-arabitol and xylitol have attracted much attention for their wide application as food sweeteners, dental caries reducer, and a sugar substitute for diabetics [7]. In addition, D-arabitol and xylitol have also been used as raw materials for the chemical synthesis of enantiopure compounds, immunosuppressive glycolipids,

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herbicides, as well as anti-pathogenic disease medicines [29]. Hence, D-arabitol and xylitol had been selected as one of the top 12 types of value-added building-block chemicals from biorefineries [31].

At present, sugar alcohols are still primarily produced by chemical reduction from monosaccharide. However, this method has many drawbacks such as the requirements of highly purified substrates, high pressure and temperature, and expensive chemical catalyst of Raney-Nickel (a fine-grained solid composed mostly of nickel derived from a nickel-aluminium alloy, and often used for the reduction of sugar to produce sugar alcohol) in the process. At the same time, extensive separation steps have to be followed in order to remove the by-products, which are mainly derived from the chemical reaction.

However, sugar alcohol production using microorganisms is eco-friendly and more cost-effective, as the process does not require pure sugar substrate and different biomass materials can be used as substrates. In an attempt to increase product yields, bioconversion processes based on whole-cell enzyme biocatalysts had been investigated [1, 9, 10, 13, 33]. Bioconversion, known as biotransformation, refers to the utilization of live microbial cells to carry out some multiple-enzyme-catalyzed reactions that are more costly or not feasible chemically. Compared to the traditional production by fermentation, it is not necessary to keep sterile strictly and add the nitrogen resource for the bioconversion process, and this will reduce the cost for industrial applications. In addition, the products from bioconversion are usually much simpler for down-stream separation [34]. Hence, the production of bio-based chemicals via bioconversion has attracted much attention for its practical application and become a promising technology [4].

The performance of strain plays an important role in the bioconversion process. Screening or engineering the suitable strains will greatly contribute to the success of bioconversion for the production of bio-based chemicals. When exposed to osmotic stress, some microorganisms accumulate compatible solutes such as D-arabitol, glycerol, xylitol, and mannitol to balance the osmotic pressure across the cell membrane [12]. It is well known that many osmophilic yeast species, such as *Zygosaccharomyces* [26], *Debaryomyces* [18], *Metschnikowia* [19], *Candida* [2], and *Hansenula* [30] can produce different sugar alcohols. Of these species, *P. anomala*, belonging to the non-*Saccharomyces* yeasts, exhibits wide metabolic and physiological diversity for its capacity to grow under the environment of low pH, high osmotic pressure, and low oxygen tension [22]. In fact, *P. anomala* is one of several yeast species that have been given QPS status, and it had been employed for biocontrol purposes in food and feed [27]. Therefore, it has widespread biotechnological significance ranging from therapeutic protein production, biocontrol agents, and biofuels production.

In this study, we reported that a recently isolated *P. anomala* can produce important sugar alcohols as ribitol, D-arabitol, and/or xylitol from different substrates. In order to characterize the metabolic pathway of the production of sugar alcohols, D-arabitol dehydrogenase (ArDH) gene in *P. anomala* was cloned and expressed in *Escherichia coli* firstly. Its enzymatic activity and in vitro reactions have also been investigated with the purified recombinant protein to confirm its properties.

Materials and methods

Chemicals

Yeast extract and tryptone were procured from OXOID (Hampshire, UK). Agar powder was purchased from Solarbio (Beijing, China). Glucose, xylose, D-arabitol, xylitol, D-xylulose and enzyme cofactors such as NADH and NAD^+ were purchased from Sigma (St. Louis, MO, USA). The other chemicals were purchased from Sinopharm (Beijing, China).

Organisms and growth conditions

The *P. anomala* TIB-x229 (CGMCC No. 5482) and *Escherichia coli* strains BL21 (DE3) used in this study were non-pathogenic strains. The plasmid pET30a(+) (Novagen, San Diego, CA, USA) was used for expressing protein in *Escherichia coli* BL21(DE3).

The yeast strain was maintained on YPD agar plates with yeast extract 10 g/l, tryptone 20 g/l, agar 15 g/l, and glucose 20 g/l, incubated at 30 °C. The inoculums were prepared in YPD medium and the composition was 10 g/l yeast extract, 20 g/l tryptone, and 20 g/l glucose. *E. coli* strain was cultivated in LB medium with yeast extract 5 g/l, tryptone 10 g/l, and NaCl 10 g/l.

Identification and phylogenetic analysis of isolates

The taxonomical studies of isolate was conducted by 18S rDNA homologous analysis. The genomic DNA of *P. anomala* was extracted by Biomega Yeast gDNA Kit (Beijing, China) following the manufacturer's instruction. The 18S rDNA was amplified with universal primers 18s-FP and 18s-RP (Table 1). The cloned 18S rDNA was aligned in the GenBank database.

Bioconversion of different sugar substrates by *Pichia anomala* TIB-x229

A single colony of *P. anomala* TIB-x229 was inoculated into 20 ml of YPD medium in a 250-ml shake flask and cultured at 30 °C/200 rpm. After incubation for 12 h, the

Table 1 Oligonucleotide primers in this study

Primers	Sequences (5′–3′)
18s-FP	CCAACCTGGTTGATCCTGCCAGTA
18s-RP	CCTTGTTACGACTTCACCTTCCTCT
ardh-1	aattcCATATGATGGAWTCCGCATAC
ardh-2	aattCTCGAGCCARCAYTCGTAACCYCC

whole cells were harvested by centrifugation at 3,000×*g* for 3 min. After washing twice with distilled water, whole cells were suspended in isometric substrate solution for following bioconversion. The condition of bioconversion was 30 °C/250 rpm in a 250-ml shake flask.

Metabolite analysis

Aliquots of culture filtrate collected at different time intervals were centrifuged at 10,000×*g* for 10 min. Concentrations of glucose, xylose, xylitol, ribitol, and D-arabitol were measured by HPLC (Agilent, Santa Clara, CA, USA) equipped with an Hi-Plex Ca column (7.0 × 300 mm, Agilent, Santa Clara, CA, USA). The column was eluted with H_2O at a constant rate of 0.6 ml/min at 78 °C. The Agilent 1260 refractive index detector (Agilent, Santa Clara, CA, USA) was used. The resulting chromatograms were compared to the chromatograms of the known standards and calibration curves for identification and quantification of the sugar alcohols present.

Cloning, expression, and purification of *P. anomala* ArDH

To clone the ArDH gene from *P. anomala*, degenerate primers were designed based on sequence data of relevant sequenced *ardh* homologs in GenBank [14]. The degenerate primers were *ardh*-1 and *ardh*-2 (Table 1). The sense and anti-sense primers carry *Nde*I and *Xho*I restriction sites (underlined), respectively.

The purified PCR product was digested with *Nde*I and *Xho*I and was cloned into expression vector pET30a(+) digested with *Nde*I and *Xho*I. The resulting expression vector pET-ArDH was transformed into *E. coli* strain BL21(DE3). The recombinant cells were cultured at 37 °C/220 rpm in LB medium supplemented with 50 μg/ml kanamycin, and induced by the addition of 0.1 mM IPTG when the OD_{600} of the growing culture reached 0.4–0.6. Induced cells were cultured at 28 °C and 180 rpm for 6 h.

Cells were collected by centrifugation at 5,000×*g* for 5 min, washed twice with binding buffer, and lysed by sonication. The lysate was centrifuged for 20 min at 12,000×*g* at 4 °C to obtain the clear supernatant, and applied to a 5-ml nickel-charged Hi-Trap column (Pharmacia, New York, NY, USA) pre-equilibrated with binding buffer. The column was washed with 15 ml of binding buffer and then washed with washing buffer until no more protein was eluted. The column was then eluted with 20 ml of eluting buffer. Fractions were combined and passed through an ultra-filtration tube MWCO 3kD (Millipore, Billerica, MA, USA) for desalting.

Enzyme assay and in vitro reaction

The activity of recombinant enzyme ArDH was determined spectrophotometrically by monitoring the change of A_{340} reflecting oxidation or reduction of NAD(H) or NADP(H) [28]. The oxidative reaction mixture for ArDH consisted of 50 mM Tris–HCl (pH 7.5), 2 mM NAD^+ or $NADP^+$, 50 mM D-arabitol/xylitol, and purified enzyme. For the reductive reaction, the mixture contained 50 mM Tris–HCl (pH 7.5), 2 mM NADH or NADPH, 50 mM D-xylulose, and purified enzyme. All reactions were started by the addition of enzyme to a final volume of 0.2 ml at 30 °C. One unit of enzyme activity refers to 1 μmol of NADH produced per min. For determining the specific activity, the concentration of ArDH was determined by standard BCA protein assay kit (Themo, Rockford, IL, USA).

In vitro reaction mixture mainly included sugar alcohol/xylulose, coenzyme (NAD^+ or NADH) and purified ArDH enzyme. After reacting for 30 min at 30 °C, the mixture of products was centrifuged at 12,000 × *g* for 20 min and the supernatant was analyzed by HPLC with Hi-Plex Ca column at 50 °C with H_2O as the mobile phase at a flow rate of 0.6 ml/min. The oxidative reaction mixture (1.5 ml 20 mM with pH 7.5) for ArDH consisted of 2 mM NAD^+, 50 mM D-arabitol/xylitol, and purified enzyme. For the reductive reaction, 1.5 ml of 20 mM Tris/HCl (pH 7.5) buffer containing 5 mM NADH, 5 mM D-xylulose, and purified enzyme was used.

Nucleotide sequence accession numbers

The nucleotide sequences of 18S rDNA gene and *ardh* gene from *P. anomala* TIB-x229 have been deposited in the GenBank database under accession numbers KC800814 and KC800813, respectively.

Results and discussion

Identification of sugar alcohol-producing strain

A sugar alcohol-producing yeast strain, designated as TIB-x229, was screened from natural environment. It is capable of utilizing glucose and xylose to produce valuable sugar alcohols. In order to further characterize the yeast and to confirm its species, homology analyses of sugar alcohol-producing strain by 18S rDNA were performed. Based on the alignments of the 18S rDNA sequencing data at the National Center for Biotechnology Information (NCBI),

the result showed that this strain belonged to the genera of *Pichia*. The 18S rDNA sequence of this strain had 99 % similarity with the partial sequence of *Pichia anomala* KCTC7104. From these results, strain TIB-x229 was identified as a strain of *P. anomala*, and preserved at China General Microbiological Culture Collection Center with the collection number of CGMCC No. 5482.

Pichia anomala is an interesting species, known as *Wickerhamomyces anomalus* and *Hansenula anomala*, and it can combat the undesirable mold *Aspergillus flavus*, which contaminates food sources and produces aflatoxins. The details of sugar alcohol production by this yeast strain belonging to the genus *Pichia* are still unclear, although it has been mentioned that D-arabitol was produced by *P. anomala* [6].

Production of sugar alcohols via bioconversion from glucose/xylose substrates

In order to evaluate the performance of *P. anomala* TIB-x229 for the production of sugar alcohols, the bioconversion

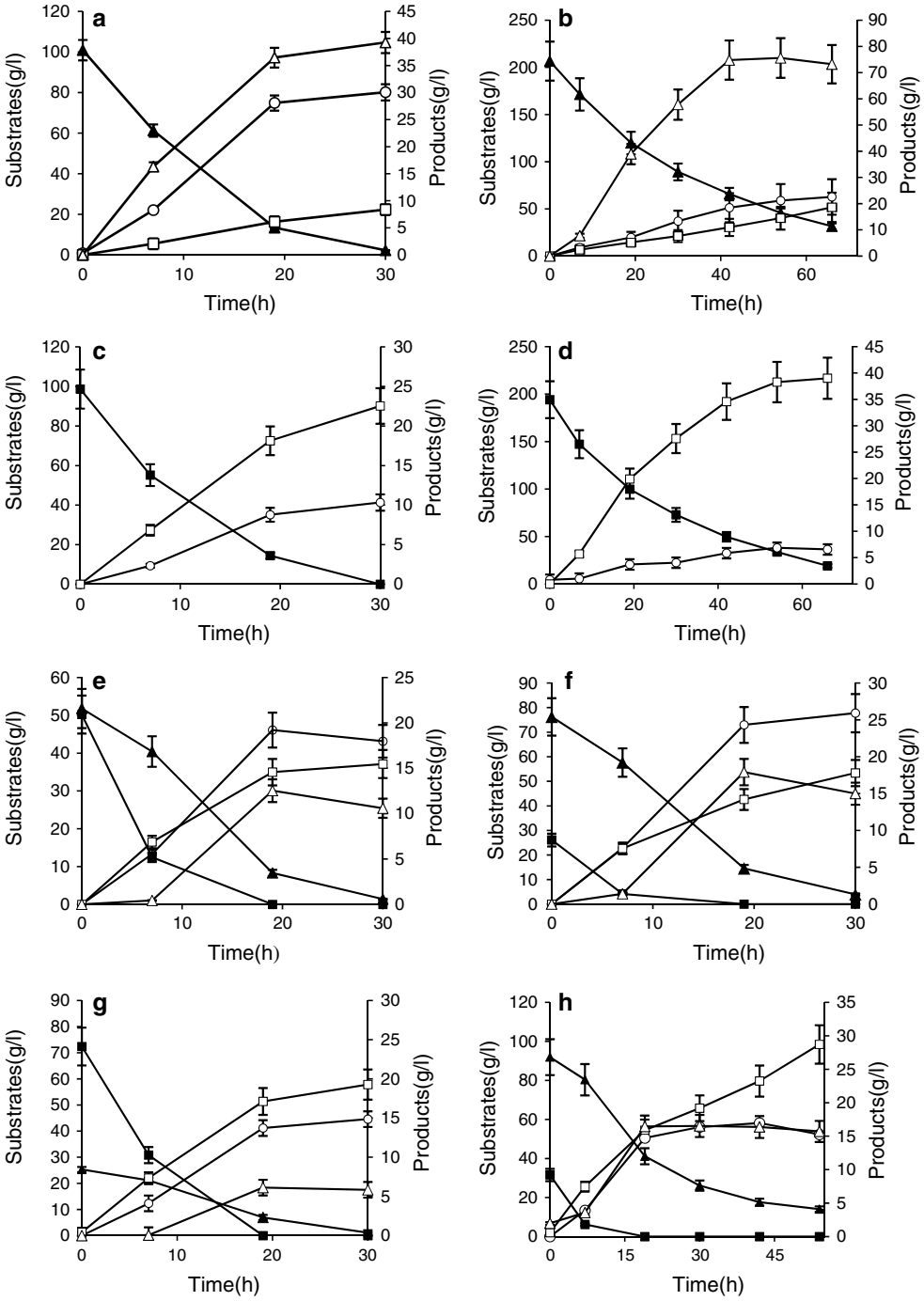

Fig. 1 Production of sugar alcohols from different substrates by bioconversion. The culture was performed in shaker-flasks at 30 °C and 250 rpm. **a** 10 % xylose; **b** 20 % xylose; **c** 10 % glucose; **d** 20 % glucose; **e** 5 % glucose + 5 % xylose; **f** 2.5 % glucose + 7.5 % xylose; **g** 7.5 % glucose + 2.5 xylose; **h** xylose mother liquor. Residual glucose (*filled squares*), D-arabitol (*open squares*), residual xylose (*filled triangles*), xylitol (*open triangles*), ribitol (*open circles*). Data represented the average values of three independent experiments with deviation varying between 5 and 10 % about the mean

of the resting yeast cell was conducted in single glucose/xylose substrate firstly. The results showed that the yeast could efficiently utilize 10 % xylose and produce xylitol (39 g/l), ribitol (30 g/l), and D-arabitol (8.4 g/l) as the major metabolic products with a yield of 0.77 g/g (Fig. 1a). The yield of sugar alcohol could also reach 0.57 g/g from 20 % xylose in 60 h (Fig. 1b). However, there were only two sugar alcohols (D-arabitol and ribitol) produced from glucose, and the productivity could achieve 33 % in glucose substrates (Fig. 1c, d). Moreover, the glycerol, ethanol, and other by-products were not nearly detected in any bioconversion process, which was significant for the downstream separation in practical application.

In addition, the effect of the proportion of glucose/xylose substrate was also investigated to test the performance of *P. anomala* for the utilization of mixed sugars. The results showed that *P. anomala* could synchronously convert 10 % total sugar into 2–3 kinds of sugar alcohols in 30 h with productivity 42.5, 57.5, and 40 % in different proportion of glucose/xylose substrate as 1:1, 1:3, and 3:1, respectively (Fig. 1e–g).

Since most microbes possess carbon catabolite repression (CCR), mixed sugars derived from the biomass are consumed sequentially, reducing the efficacy in the overall process [3]. To overcome this barrier, microbes exhibited the simultaneous consumption of mixed sugars have been isolated and developed. For example, specific strains of *Escherichia coli*, *Saccharomyces cerevisiae* [16], and *Zymomonas mobilis* [15] had been engineered for simultaneous sugars utilization via mutagenesis or introduction of a xylose metabolic pathway, but it is still not suitable for the efficient utilization of mixed sugar substrates with respect to yield and productivity.

In this study, *P. anomala* showed indeed excellent characteristics on utilization of single and mixed sugars. Especially glucose repression did not exist in the bioconversion of mixed sugars, and the mixed sugar substrate could be utilized simultaneously. However, glucose repression still existed in mixed sugar fermentation process for *P. anomala* (data not shown).

According to the above results, it was speculated that glucose repression of *P. anomala* in bioconversion might be weak in comparison to that in the fermentation process. The plausible reason was that much more metabolites produced in fermentation process resulted in suppressing the transport or utilization of xylose. It was well known that yeast preferentially utilizes glucose in the presence of xylose and glucose mixtures, due to repression of xylose utilizing enzymes [8]. However, since there is no additional nitrogen resource during bioconversion process, only the substrate sugars were metabolized by the cell, and this would not contribute to producing excessive inhibitive metabolites. Kastner et al. [11] had also reported a similar study that induction of xylose metabolism before inoculation into a mixed-sugar environment could realize simultaneous utilization of mixed-sugars and alleviate the lag period in *Candida shehatae*. This may implicate the possibility for co-utilization of different sugars in complex biomass via bioconversion with this yeast.

The utilization of xylose mother liquor by *P. anomala*

Biomass resource is an attractive carbon source for bio-based fuel and chemical production; however, its compositional heterogeneity hinders its commercial use. Xylose mother liquor was waste hydrolysate of sugar-cane bagasse or corncob after xylose extraction. This biomass substrate containing xylose, glucose, and other monosaccharides is abundant and low in cost but difficult to use as the feedstock for biological production since it contains two major inhibitors for biological metabolism, furfural and 5-hydroxymethylfurfural (HMF) [21, 25]. In other words, efficient biological utilization of xylose mother liquor is a great challenge.

Due to no glucose repression and robustness of *P. anomala* [5], it is promising to study the performance on biomass utilization to widen its application field. In subsequent study, we utilized the *P. anomala* to convert xylose mother liquor containing 90 g/l xylose and 30 g/l glucose. The result showed that *P. anomala* could effectively convert liquor into 28.7 g/l arabitol, 15.3 g/l ribitol, and 15.7 g/l xylitol with 60 % productivity in 55 h (Fig. 1h). It was interesting that the whole process was not obviously affected by glucose and other inhibitors such as furfural and 5-hydroxymethylfurfural (HMF), which is still an

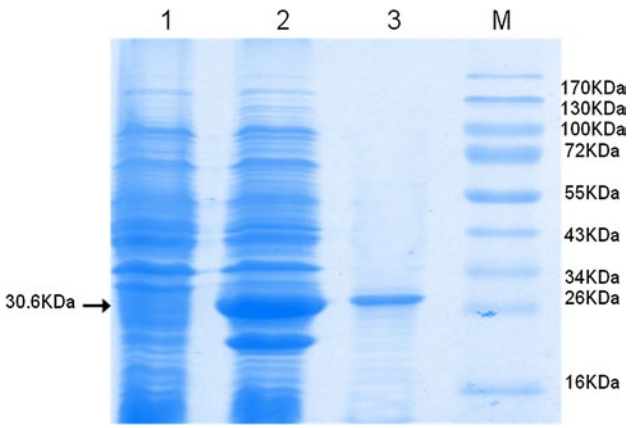

Fig. 2 The SDS-PAGE of ArDH expressed in *E. coli* BL21 (DE3). *Lane 1* the whole cell with *ardh* gene before induced; *lane 2* the whole cell with *ardh* gene after induced by IPTG; *lane 3* the purification of ArDH; *M* low molecular weight marker

important bottleneck for sustainable development of biomass resource utilization at present.

Cloning and characterization of arabitol dehydrogenase from *P. anomala*

In single-sugar bioconversion, the xylose could be converted into three kinds of sugar alcohols, including D-arabitol, xylitol, and ribitol. However, the single glucose was only converted into D-arabitol and ribitol. Hence, it is attractive to explore the difference of three sugar alcohols synthetic pathways from different substrates in *P. anomala*, especially the different roles of key sugar alcohols dehydrogenase.

It has been reported that D-arabitol produced from glucose by two alternative pathways [32]. Some organisms can convert glucose to D-ribulose-5-phosphate, dephosphorylate D-ribulose 5-phosphate, and then reduce D-ribulose to D-arabitol by NADP-dependent D-arabitol dehydrogenase (D-ribulose-forming pathway). Other strains can convert glucose to D-xylulose-5-phosphate, dephosphorylates D-xylulose-5-phosphate, and then reduce D-xylulose to D-arabitol by NAD-dependent D-arabitol dehydrogenase (D-xylulose-forming pathway).

To understand the role of enzyme in the metabolic pathway of sugar alcohol production, the ArDH gene of *P. anomala* was cloned and aligned in GenBank. The result suggested that the sequence had nearly 88 % similarity to short-chain D-arabitol dehydrogenase of *Candida tropicalis* (GenBank accession No. U00675), but there was very little relative study about this kind of arabitol dehydrogenase [17].

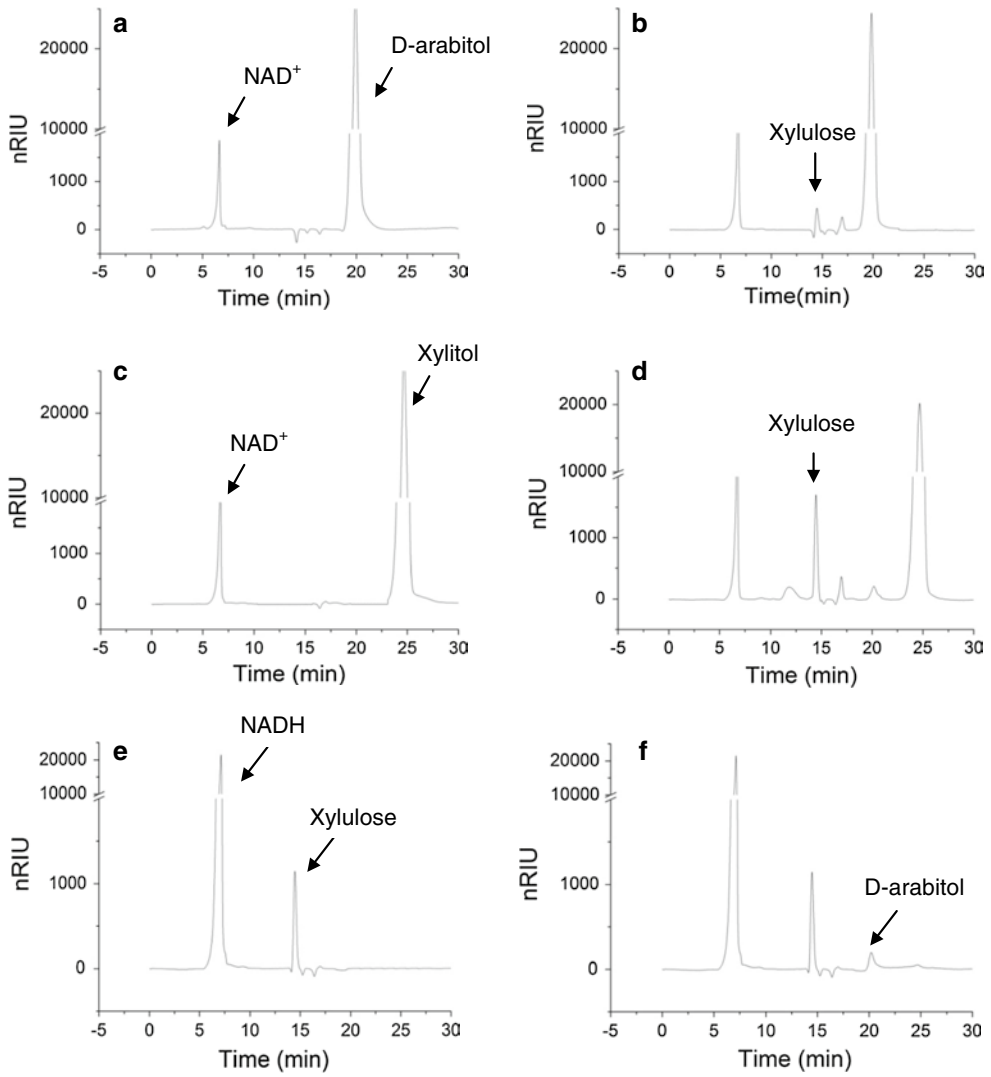

Fig. 3 HPLC analysis of the partial reaction products catalyzed by recombinant ArDH. **a** D-arabitol + NAD^+, **b** D-arabitol + NAD^++ArDH, **c** xylitol + NAD^+, **d** xylitol + NAD^++ArDH, **e** xylulose + NADH, **f** xylulose + NADH + ArDH

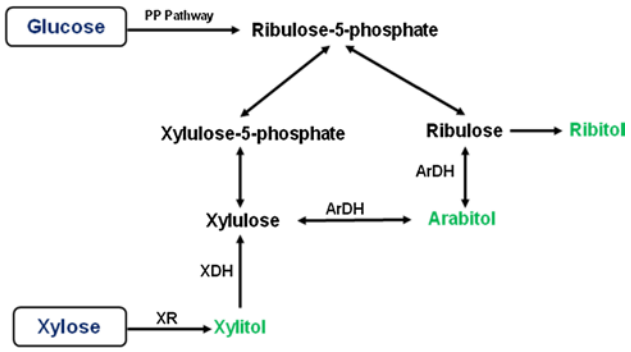

Fig. 4 The possible synthetic pathways for sugar alcohol production in *P. anomala*. The *blue* and *green* fonts represent substrates and products, respectively. *ArDH* arabitol dehydrogenase, *XDH* xylitol dehydrogenase, *XR* xylose reductase (color figure online)

In order to characterize its catalytic reaction further, ArDH of *P. anomala* was expressed in the *E. coli* BL21 and the activity of recombinant enzyme was measured. SDS-PAGE showed that soluble ArDH protein was expressed with calculated molecular weight of 30.6 kDa (Fig. 2). The purified recombinant ArDH gave a specific activity of 12.85 U/mg protein when 50 mM D-arabitol was used as substrate and 4.96 U/mg protein with xylitol as substrate. Like most other short-chain polyol dehydrogenases that possessed reductase activity [24], ArDH from *P. anomala* also had reductase activity; D-xylulose could be reduced to D-arabitol with 20.25 U/mg in the presence of NADH. Furthermore, ArDH was strictly specific to NAD^+/NADH, and no enzymatic activity was observed when $NADP^+$/NADPH was used as the coenzyme.

Comparison of these results clearly showed that the NAD^+/NADH-dependent ArDH gene in *P. anomala* has considerable practical importance, since the reaction product D-xylulose is considered as the intermediate compound for the production of xylitol.

Then, the in vitro catalytic reaction of recombinant ArDH was conducted to further figure out the difference between these substrates. The results suggested that D-xylulose production can be detected, which was catalyzed from D-arabitol and xylitol (Fig. 3a–d). In addition, a small amount of D-arabitol was detected from D-xylulose substrate (Fig. 3e, f), and it may suggest that ArDH of *P. anomala* could catalyze the reversible reaction between D-xylulose and arabitol, and the reaction from xylitol to D-xylulose, but not from D-xylulose to xylitol. Although the important xylitol dehydrogenase was still not characterized, this study implicates that the inter-conversion of D-arabitol and xylitol may be limited by the irreversible reaction from D-xylulose to xylitol in *P. anomala* (Fig. 4).

In nature, many osmophilic yeast strains can convert glucose to D-arabitol, but cannot further convert D-arabitol to D-xylulose since they lack a D-xylulose-forming D-arabitol dehydrogenase [19, 25, 34]. In our study, it was interesting that D-arabitol dehydrogenase from *P. anomala* could convert D-arabitol to D-xylulose, but could not further convert to xylitol. Meanwhile, in vitro reaction showed that D-xylulose could be produced from xylitol by recombinant D-arabitol dehydrogenase from *P. anomala* (Fig. 4). If introducing an enzyme which could catalyze the reaction from D-xylulose to xylitol, thus it will be possible to produce xylitol from glucose with relative cheaper cost than that from xylose.

A number of osmotolerant yeasts such as *Z. rouxii*, *D. hansenii*, *C. albicans*, *C. pelliculosa*, *C. famata*, and *P. miso* produce sugar alcohol from glucose [20]. However, low yield and substrate restriction are two of the major problems that may restrict the development of a commercial process for making sugar alcohol from biomass resource. This paper presents the first comprehensive study on the production of sugar alcohols by *P. anomala* bioconversion, which makes it a potentially useful microorganism for the production of value-added compounds from biomass resource.

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