J Ind Microbiol Biotechnol (2015) 42:807–812
DOI 10.1007/s10295-015-1603-z

SHORT COMMUNICATION

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# Enhanced D-arabitol production by *Zygosaccharomyces rouxii* JM-C46: isolation of strains and process of repeated-batch fermentation

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Received: 9 February 2015 / Accepted: 14 February 2015 / Published online: 25 February 2015


**Abstract** A new strain producing high yield of D-arabitol was isolated from hyperosmotic environments and the ITS rDNA sequencing analysis revealed it as *Zygosaccharomyces rouxii*. In addition, using a pH control and repeated-batch fermentation strategy in a 5-L reactor, the maximum yield and the highest volumetric productivity of D-arabitol were 93.48 ± 2.79 g/L and 1.143 g/L h, respectively. Volumetric productivity was successfully improved from 0.86 to 1.143 g/L h, which was increased by 32.9 % after 72 h of fermentation. *Z. rouxii* JM-C46 has potential to be used for D-arabitol and xylitol production from glucose via D-arabitol route.

**Keywords** D-Arabitol · *Zygosaccharomyces rouxii* · Screening and identification · Repeated-batch fermentation

## Introduction

D-Arabitol, a five-carbon polyhydric alcohol, is a functional polyol with notable properties. D-Arabitol is a stereoisomer to xylitol. As enantiomers, D-arabitol and xylitol are likely to have some similar properties. Xylitol has many known applications in the food and pharmaceutical industry, particularly as a sugar substitute for diabetics, an anticariogenic agent and a natural food sweetener [11]. Similar to xylitol, as the catabolism of D-arabitol by *Escherichia coli* involves the formation of D-arabitol phosphate which induces the synthesis of compounds that inhibit oral bacterial metabolism [13], D-arabitol has the potential to be applied in oral health care and pharmaceutical industry [1, 7]. Furthermore, D-arabitol has much lower nutritional calorie compared to xylitol and sucrose, which makes it a natural, low-calorie sugar substitute for the diabetic patients [4].

Presently, the main preparation methods of D-arabitol are chemical synthesis and biosynthesis. In the former case, D-arabitol is currently produced by chemical and catalytic reduction under alkaline conditions of D-lyxose using Raney nickel as the catalyst. Drawbacks of the process of chemical synthesis are the requirements of high temperature, high pressure and use of an expensive catalyst. Instead, because of mild conditions, low energy consumption and little environmental pollution, biosynthesis method is the research hot point in the production of D-arabitol, in which microbial enzymes in natural yeasts could convert glucose into D-arabitol. D-Arabitol can also be effectively converted to xylitol by *Gluconobacter oxydans* which was capable of oxidizing D-arabitol to D-xylulose using the membrane-bound D-arabitol dehydrogenase and then converting D-xylulose to D-xylitol using the membrane-bound D-xylitol dehydrogenase [8]. This D-arabitol-to-xylitol route is an alternative to the rather expensive xylitol production processes based on the method of chemical reduction. Therefore, the screening of efficient strain has great realistic significance for the green production of D-arabitol and xylitol.

The production of D-arabitol from D-glucose using osmophilic yeasts belonging to the genera *Saccharomycopsis* [19], *Candida* [14], *Debaryomyces* [9], *Pichia* [3], *Hansenula* [16], *Metschnikowia* [12], and *Zygosaccharomyces* [5] has been reported. Osmotolerant yeast species can convert D-glucose to D-ribulose-5-$PO_4$/D-xylulose-5-$PO_4$ via the pentose pathway and then reduce D-ribulose/D-xylulose to D-arabitol by

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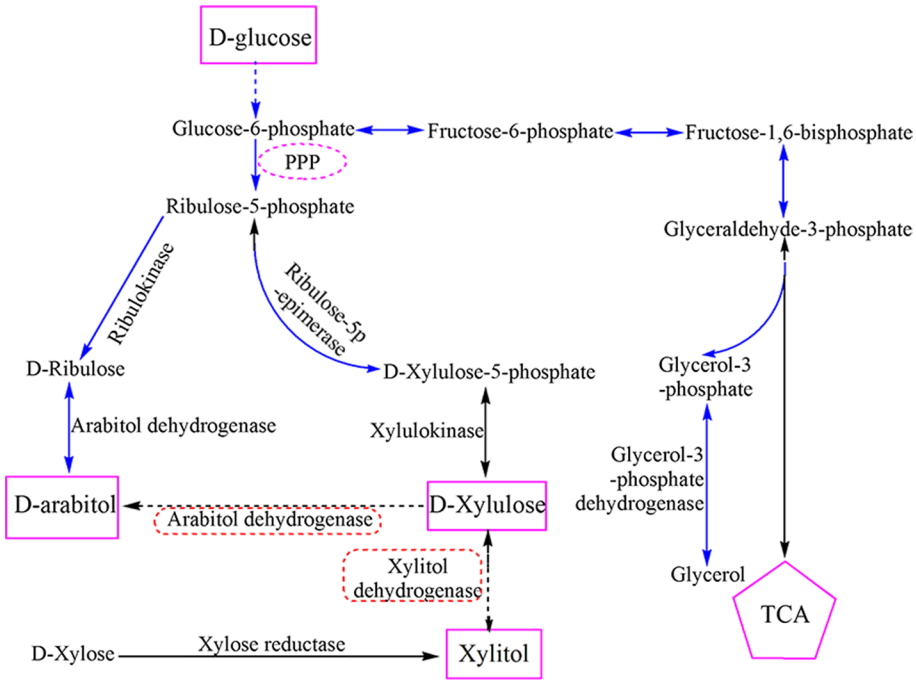


**Fig. 1** Possible pathways for the conversion of various substrates to arabitol by *Zygosaccharomyces rouxii* JM-C46 (*PPP* pentose phosphate pathway, *TCA* tricarboxylic acid cycle)

an NADH/NAD-dependent pentitol dehydrogenase [17, 18]. The two possible routes are summarized in Fig. 1. It has been shown that accumulation of D-arabitol or other polyols in yeasts may be bound up with water activity and osmophilic stress in the medium, because D-arabitol and other undesirable by-products acted as compatible solutes can balance the osmotic pressure across the cell membrane [8]. Nozaki et al. [12] described a process for the production of 81.4 g/L D-arabitol by *Metschnikowia reukaufii* in 116 h with 0.701 g/L h productivity, which is the highest research level in the field of D-arabitol production initial from D-glucose. However, the long cultivation time and the low productivity of D-arabitol were still two of the major problems in D-arabitol production. In accordance with the problems, the screening of osmophilic yeast strains with high-D-arabitol yield and optimizing the culture conditions is extremely urgent for D-arabitol industrialization.

In present study, we isolated an efficient D-arabitol-producing strain and optimized the productivity of D-arabitol from D-glucose in flask-shaking experiments. In addition, expanding the scale of the fermentation in a 5-L bioreactor was investigated using a pH control and repeated-batch fermentation strategy.

## Materials and methods

### Materials

Pollen samples of sweet-scented osmanthus and honey of various flowers were collected from different bee houses of Zhenjiang, China.

### Screening and isolation

The osmophilic microorganisms were isolated from natural osmophilic sources as follows. The Medium S used for screening contained 400 g D-glucose, 10 g yeast extract, 10 g tryptone per liter. One gram of each collected sample was added into 50-mL Erlenmeyer flask, containing 10 mL of medium, and then incubated at 30 °C and 200 rpm for 3–4 days. After six transfers (1 mL) in liquid culture (9 mL), the samples of D-arabitol-producing culture broth were serially diluted and then transferred to Medium S plates containing 400 g/L D-glucose and 20 g/L agar for 2–3 days. Isolates were purified on agar plates via subsequent transfer, and these plates with single colonies were stored in freezer with 4 °C. Then individual isolated colonies were picked and inoculated into liquid Medium S and incubated at 30 °C for 1 day.

### Identification and phylogenetic analysis of screening strain

The method of strain identification was based on YEAST characteristics and identification, taxonomically [10]. Preliminary identification was carried out through the observation of morphology of colonies and cells and the determination of biochemical properties. The ITS sequence of the strain was amplified by PCR with universal primers of yeast. The forward primer of ITS1 is 5′-TCCGTAGG TGAACCTGCGG-3′ and the reverse primer of ITS4 is 5′-TCCTCCGCTTATTGATATGC-3′. PCR procedures were performed with denaturation at 95 °C for 5 min, followed by 35 cycles at 94 °C for 2 min, 55 °C for 30 s, and

extension at 72 °C for 45 s, with final extension for 10 min at 72 °C. The molecular size of the PCR products was confirmed by conducting electrophoresis (Bio-rad, USA) in 1 % Biowest Agarose gel. The ITS sequencing was completed by Jinsite Biotechnology (Nanjing, China), and was analyzed by BLAST program in the database of National Center for Biotechnology Information (NCBI). Close relatives were selected as the reference, and the construction method of unrooted neighbor joining phylogenetic tree of the strain was to utilize MEGA 5.03 with bootstrapping 1000 times by aligning the chosen references.

### Preparation of seed culture

The medium for seed cultures contained 50 g glucose, 10 g yeast extract, and 10 g tryptone per liter (pH 5.0, after sterilization). A loopful of cells transferred from a stock slant was inoculated into a 250-mL Erlenmeyer flask containing 50 mL of the seed medium. The culture was grown on a rotary shaker with 220 rpm at 30 °C for 1 day to inoculate flasks for fermentation experiments. 5 % inoculum of the final broth volume was used in the subsequent experiments of culture conditions.

### Batch fermentation and fed-batch fermentation experiments in 5-L fermenter

Medium F contained 200 g glucose, 10 g yeast extract, and 10 g tryptone per liter (pH 5.0) was used in Erlenmeyer flask for the optimization of fermentation conditions. Then, the scale up of biotransformation was carried out in a 5-L fermenter to validate control parameters of D-glucose fermentation in industrial production of D-arabitol. Fermentation Medium P used in this process contained 200 g glucose, 10.66 g yeast extract, 7.2 g tryptone and 2.44 g $(NH_4)_2SO_4$ per liter, pH 5.0. A 5-L fermenter containing 2 L Medium P was inoculated with 400 mL of the seed culture and cultivated at 30 °C at 300 rpm. During fermentation, the flow rate of sterile air was 4 L/min and the value of pH was maintained approximately at 5.0. For the fed-batch cultivation, the culture from the first generation was used as the seed inoculum for the second batch fermentation. When the D-glucose of the culture was exhausted (<5 g/L), 1600 mL of the fermentation broth was withdrawn and replenished with 1600 mL fresh Medium P.

### Analytical methods

The cell growth during fermentation was monitored by optical density at 660 nm. The production of D-arabitol and by-products were detected by TLC (Thin layer chromatography), with a developing solvent system of ethyl acetate/pyridine/glacial acetic acid/water (16:10:2:3) on microcrystalline cellulose plate. The quantitative analysis of residual glucose and D-arabitol-product yield in the culture was performed by HPLC(high-performance liquid chromatography) equipped with a reflective index detector using a Shodex SP0810 column (Showa Denko Co., Ltd., Tokyo, Japan). The ratio of acetonitrile and water in mobile phase was selected as 80:20 of acetonitrile: water (v/v). The flow rate was 0.8 mL/min and 20 μL of sample solution was injected into the HPLC–RID (refractive index detector) system. Column temperature was selected as 35 °C. Chromatographic grade chemicals of D-glucose, D-arabitol and glycerol were loaded as mix standards. Peaks were detected by refractive index and identified and quantified by comparison to retention times of authentic standards.

## Results

### Screening and isolation of D-arabitol-producing strains from natural sources

260 osmophilic strains from six different raw honey samples were obtained and isolated on agar plates containing 400 g/L glucose, among which 92 strains could produce D-arabitol in flask culture. Quantitative analysis of D-arabitol production by HPLC suggested that 13 strains were able to produce more than 60 g/L of D-arabitol from 200 g/L glucose. A strain from chaste honey could produce over 70 g/L D-arabitol from the Medium F containing 200 g/L glucose. This strain was named as JM-C46 and then was selected as the best producer of D-arabitol for further investigation.

### Characterization of the D-arabitol producing strain JM-C46

After 48 h of incubation on YEPD agar plates at 30 °C, strain JM-C46 formed a smooth, circular, white and creamy colony with roughly 2.5–3.5 mm. In addition, the shape of vegetative cells was spherical, with approximately 2–4 μm in diameter, and multiplied by budding reproduction. Strain JM-C46 could assimilate many kinds of monosaccharides and produce D-arabitol, such as D-glucose, D-xylose and D-galactose. However, JM-C46 could not grow on medium with sucrose, L-arabinose, lactose or starch as the sole carbon source.

The ITS sequencing analysis was then performed to identify the strain JM-C46 (GenBank accession number: KM249341). Based on the ITS rDNA domain sequence and sequence alignments in the database of NCBI, strains from related genera were joined in constructing the phylogenetic tree of strain JM-C46. The Homology analyses results with ITS rDNA domain sequencing showed that the strain JM-C46 had 99 % similarity with the partial

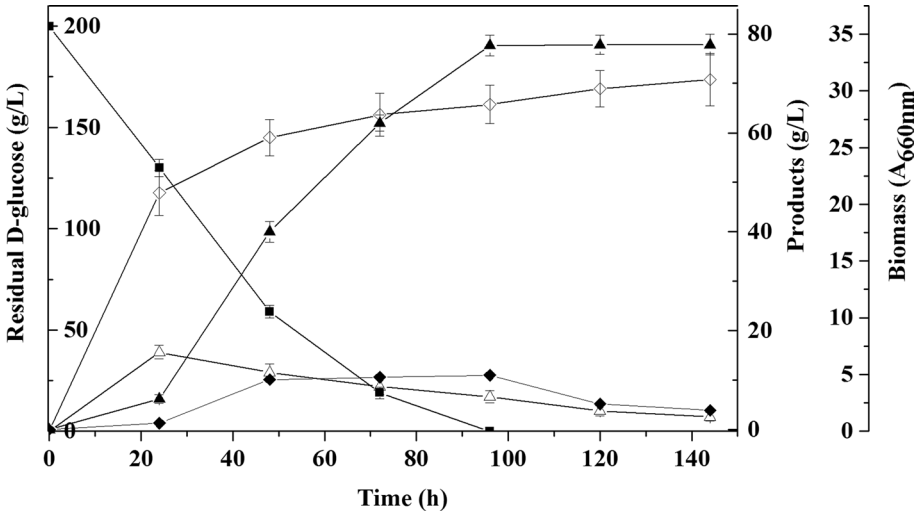


**Fig. 2** Time courses of biomass, residual D-glucose, D-arabitol production, glycerol concentration and ethanol concentration in shake flasks (500 mL) by the strain JM-C46. The cultivations were performed in the 100 mL Medium P containing 200 g D-glucose, 10.66 g yeast extract, 7.20 g peptone, 2.44 g $(NH_4)_2SO_4$ per liter with 5 % inoculum size, at pH 5.0, 220 rpm for 96 h. Biomass (*open diamond*); residual D-glucose (*filled square*); the yield of D-arabitol (*filled triangle*); glycerol concentration (*filled diamond*); ethanol concentration (*open triangle*)

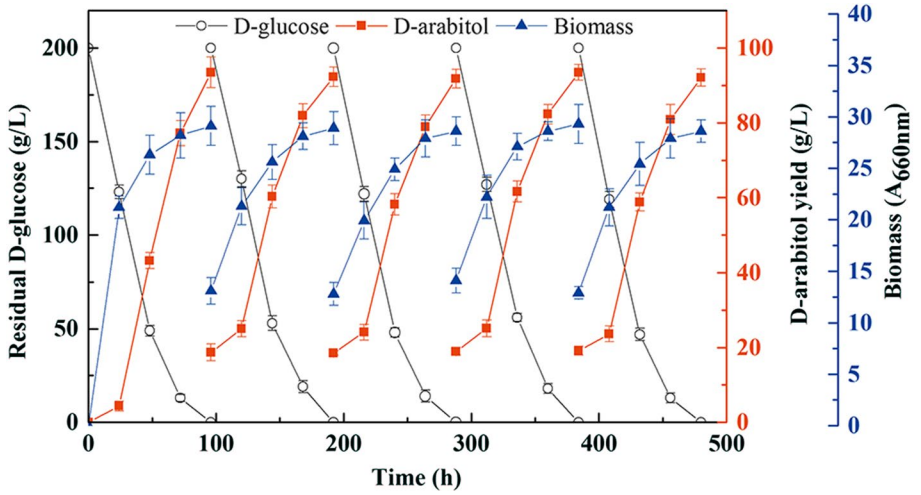


**Fig. 3** Fermentation profiles of *Z. rouxii* JM-C46 in 5-L bioreactor. Time courses of biomass, residual D-glucose and D-arabitol production in repeated-batch fermentation process. The cultivations were performed in Medium P with 10 % inoculum size, at 30 °C, 5.0 pH, 300 rpm for 480 h. The time interval of feeding was 96 h with 1600 mL fresh Medium P at the end of each cycle of fermentation. Biomass (*filled triangle*), residual D-glucose (*open circle*), the yield of D-arabitol (*filled square*)

sequence of *Zygosaccharomyces rouxii*. From these results, strain JM-C46 was identified as a strain of *Zygosaccharomyces rouxii*, and designated as JM-C46. *Z. rouxii* JM-C46 was deposited in the China Center for Type Culture Collection (CCTCC) and the preservation number is CCTCC M 2014205.

## Repeated-batch fermentation experiments by *Z. rouxii* JM-C46 in 5-L fermenter

At first, the optimization of fermentation conditions was performed in shake flasks to enhance the productivity of D-arabitol from glucose by *Z. rouxii* JM-C46, and the optimal condition for D-arabitol production by JM-C46 was 30 °C, pH 5.0, 220 rpm, 5 % inoculum in Medium F. 77.05 g/L of D-arabitol yield was obtained using response surface methodology (RSM) under the optimized culture medium P: D-glucose 200 g, yeast extract 10.66 g, peptone 7.20 g, $(NH_4)_2SO_4$ 2.44 g/L when the reaction parameters were set as: 30 °C, 220 rpm for 96 h. Under this optimized condition, Fig. 2 plotted the time courses of biomass, residual glucose and D-arabitol production in shake flask fermentation by strain JM-C46. The yeast cells produced 76.08 ± 0.49 g D-arabitol (maximum yield) from 200 g glucose per liter in 96 h with a yield of 0.38 g/g glucose.

To further enhance D-arabitol production, repeated-batch fermentation tests were carried out in 5-L fermenter with Medium P. On the basis of batch fermentation in shake flasks, the pH for D-arabitol production of repeated-batch fermentation experiment was kept at pH 5.0. Five cycles were performed over a time period of 480 h. Profiles of cell growth, D-arabitol production and D-glucose consumption during this repeated-batch fermentation are shown in Fig. 3. The repeated-batch fermentation process in 5-L fermenter had reached a state of dynamic equilibrium with regard to the biomass, substrate consumption rate, product formation rate and relevant parameters. The biomass reached a nearly constant value of 30 ($A_{660}$), approaching the value of batch

fermentation liquid. 400 mL of the residual culture broth was used as seed, and the initial biomass ($A_{660}$) of every cycle was about 13. The substrate consumption rate reached a nearly constant value of 2.16 g/L h. At the beginning of all fermentation cycles, almost no production of D-arabitol was observed. The D-arabitol yield at the end of each cycle reached 91.81 ± 1.83 to 93.48 ± 2.79 g/L by repeated-batch reaction with strain *Z. rouxii* JM-C46. The maximum yield of D-arabitol, 93.48 ± 3.09 g/L, was obtained at the end of the first cycle with a yield of 0.467 g/g D-glucose. D-Arabitol yield increased by 22.9 % compared with the maximum yield (76.08 ± 0.49 g/L) in batch fermentation. In addition, 82.31 ± 1.62 g/L D-arabitol was obtained after 72 h of each repeated feeding. In equal fermentation times (72 h), volumetric productivity increased from 0.86 to 1.143 g/L h compared to batch fermentation, which was increased by 32.9 % in a 5-L reactor using the method of repeated-batch fermentation.

## Discussion

A number of osmophilic or osmotolerant yeasts can produce D-arabitol from glucose. However, long cultivation times and low yields of D-arabitol are two of the major problems that may restrict the development of a commercial fermentation process for biosynthesis of D-arabitol [2, 15]. In recent years, considerable efforts have been made to shorten the cultivation time and increase volumetric productivity, including temperature-shifted methods and fed-batch fermentation [12]. To date, very few natural strains have both superiority of a short cultivation time and high productivity, even though novel D-arabitol-producing strains have been found constantly such as *Yarrowia lipolytica* [6]. In this study, a D-arabitol-producing strain *Z. rouxii* JM-C46 was isolated from osmotic environments and identified based on biochemical properties and ITS sequence. This strain can produce D-arabitol efficiently with a short cultivation time and high productivity.

The scale up of biotransformation was carried out in a 5-L reactor using a pH control and repeated-feeding strategies. This model in 5-L reactor would benefit the optimizing control of D-glucose fermentation in industrial production of D-arabitol. The maximum yield of D-arabitol by *Z. rouxii* JM-C46, 93.48 ± 2.79 g/L (measured in 96 h), was higher than reported yeasts producing D-arabitol, such as *K. ohmeri* NH-9 [19], *M. reukaufii* AJ14787 [12] and *Z. rouxii* NRRL 27624 [2]. Among them, the strain *Z. rouxii* NRRL Y-27624 can produce 83.4 ± 1.1 g D-arabitol from 175 g D-glucose per liter in 240 h with the volumetric productivity of 0.348 g/L h. Here, the highest volumetric productivity, 1.143 g/L h, was measured in 72 h by repeated-batch fermentation. As far as we know, this is the highest volumetric productivity of D-arabitol production from D-glucose by *Z. rouxii* reported in the literature. And strain *Z. rouxii* JM-C46 has potential to be used for xylitol production from glucose via D-arabitol route.

Certain osmophilic yeasts when grown in the presence of high concentration of D-glucose produce a variety of polyhydric alcohols (glycerol, D-arabitol, erythritol, and mannitol). The details of polyol production by yeast strains belonging to the genus *Z. rouxii* are still unknown, although it has been reported that D-arabitol was produced by *Z. rouxii* isolated from honeybee hives [2]. In the present study, ribose, mannitol, erythritol, or any intermediate product in the fermentation broth could not be detected when the single carbohydrate source was D-glucose, fructose, galactose, or mannose, respectively. However, the strain has capabilities to transform xylose to D-arabitol and xylitol (Fig. 1), and cannot transform sucrose, lactose and D-ribose into D-arabitol, which could be due to their deficiency of related enzyme or metabolic pathways.

Taken together, *Z. rouxii* JM-C46 is not only an efficient D-arabitol-producing strain, but it is able to convert xylose to D-arabitol or xylitol by biotransformation and enzyme action. Moreover, *Z. rouxii* JM-C46 has capability to transform D-glucose into D-arabitol but not xylitol due to its deficiency of D-arabitol dehydrogenase (ArDH). Therefore, it will be a new method to produce xylitol if the ArDH gene can be introduced into *Z. rouxii* JM-C46. Future research will focus on the identification and characterization of key enzymes in the D-arabitol biosynthetic pathway in *Z. rouxii* JM-C46. It may be possible to modify the key enzyme or create a recombinant microorganism with the ability to selectively produce desired fermentation products, thus accelerating the development of industrialized production of D-arabitol or xylitol.

**Acknowledgments** This work was supported by the National Natural Science Foundation of China under Grant No. 21006041, Science & Technology Development Plan of Nanning (No. 20121017), Research Fund of Jiangsu (08JDG009, 201410299024Z, 13A087), Jiangsu Government Scholarship for Overseas Studies and PAPD.

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