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SHORT COMMUNICATION

Production of D-arabitol by a newly isolated *Zygosaccharomyces rouxii*

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Abstract A newly isolated *Zygosaccharomyces rouxii* NRRL 27,624 produced D-arabitol as the main metabolic product from glucose. In addition, it also produced ethanol and glycerol. The optimal conditions were temperature 30°C, pH 5.0, 350 rpm, and 5% inoculum. The yeast produced 83.4 ± 1.1 g D-arabitol from 175 ± 1.1 g glucose per liter at pH 5.0, 30°C, and 350 rpm in 240 h with a yield of 0.48 g/g glucose. It also produced D-arabitol from fructose, galactose, and mannose. The yeast produced D-arabitol and xylitol from xylose and also from a mixture of xylose and xylulose. Resting yeast cells produced 63.6 ± 1.9 g D-arabitol from 175 ± 1.8 g glucose per liter in 210 h at pH 5.0, 30°C and 350 rpm with a yield of 0.36 g/g glucose. The yeast has potential to be used for production of xylitol from glucose via D-arabitol route.

Keywords *Zygosaccharomyces rouxii* · D-Arabitol production · Xylitol from glucose · Resting yeast cells · Osmophilic yeast

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Introduction

Xylitol, a five-carbon sugar alcohol, has attracted much attention because of its potential use as a natural food sweetener, a dental caries reducer, and a sugar substitute for diabetics [4]. It is currently produced by chemical reduction under alkaline conditions of xylose derived mainly from wood hydrolyzate [8]. Drawbacks of the chemical process are the requirements of high pressure (up to 50 atm) and temperature (80–140°C), use of an expensive catalyst (Raney-Nickel), and use of extensive separation and purification steps to remove the by-products that are mainly derived from the hemicellulose hydrolyzate.

The production of xylitol by fermentation is becoming more attractive because of the above-mentioned problems associated with its chemical production process. Many yeasts and mycelial fungi possess NADPH-dependent xylose reductase (EC 1.1.1.21) which catalyzes the reduction of xylose to xylitol as a first step in xylose metabolism [2]. Xylitol can be subsequently oxidized to xylulose by the action of xylitol dehydrogenase (EC 1.1.1.14), which preferentially uses NAD as an acceptor. In xylose fermenting yeasts, the initial reactions of xylose metabolism appear to be rate limiting. This results in accumulation of xylitol in the culture medium. We have studied xylitol production from xylose by three yeasts: *Candida entomaea* NRRL Y-7785, *Pichia guilliermondii* NRRL Y-2075, and *C. peltata* NRRL Y-6888 [12, 13]. However, these yeasts convert glucose to ethanol and L-arabinose to L-arabitol. There is a growing interest in the production of xylitol from a readily available and much cheaper substrate such as glucose.

In 1969, Onishi and Suzuki [10] described a three-step fermentation process for conversion of glucose to xylitol. First, glucose was converted to D-arabitol by an osmophilic yeast strain *Debaryomyces hansenii*. D-Arabitol was then

converted to D-xylulose by *Acetobacter suboxydans* and finally *C. guilliermondii* converted D-xylulose to xylitol. The yield of xylitol from glucose was about 11.6%. Recently, Suzuki et al. [14] reported a novel enzymatic method for conversion of D-arabitol to xylitol with *Gluconobacter oxydans*. In this method, D-arabitol was first oxidized to D-xylulose by a membrane-bound D-arabitol dehydrogenase (EC 1.1.1.11) and then to xylitol by reduction catalyzed by a NAD-dependent xylitol dehydrogenase. Therefore, the development of an efficient method for production of D-arabitol is essential for a xylitol production process from glucose.

As a first step to produce xylitol from glucose via the D-arabitol route, we have isolated a yeast, *Zygosaccharomyces rouxii* NRRL B-27,624, from honeybee hives. The yeast was able to utilize high concentrations of glucose and produce D-arabitol as the major metabolic product in a very good yield. In this report, the factors affecting the D-arabitol production by this yeast are presented.

Materials and methods

Screening, isolation, and identification of the yeast strain

The yeast strain was isolated by screening 125 honeybee hives collected from the Mackinaw, IL, area. The YMP medium used for screening contained 3 g yeast extract, 3 g malt extract, and 3 g peptone per liter (pH 5.0). Glucose (50 g/l) was used as the carbon and energy source. The samples of collected beehives (~0.25 g) were placed in test tubes (1.5 × 15 cm, 10 ml YMP medium) and incubated at 30°C and 200 rpm for 4 days. After five transfers (0.5 ml) in liquid culture (10 ml), samples of culture broth from D-arabitol producing cultures were serially diluted and grown on agar (20 g/l) plates containing the screening medium for 2–3 days. Isolates were purified via subsequent transfer on agar plates and after a few transfers in agar plates, single isolated colonies were transferred to test tubes containing 10 ml of the screening medium. The isolated D-arabitol producing strain was maintained throughout this study at 4°C on agar (20 g/l) slants containing YMP medium with glucose (20 g/l). It was identified as a strain of *Z. rouxii* (100% similarity by rRNA sequencing) by MIDI Labs, Inc., Newark, DE, and was deposited in the ARS Culture Collection, Peoria, IL (designated as NRRL Y-27,624).

Seed culture preparation

The YMP medium, containing glucose (20 g/l) was used to prepare the seed cultures for production experiments. The pH was adjusted to 5.0 after sterilization. A 250-ml Erlenmeyer flask containing 50 ml of the medium was inoculated with a loopful of cells taken from a stock slant and incubated at 30°C on a rotary shaker (200 rpm) for 24 h to inoculate flasks for fermentation experiments.

Fermentation experiments

The experimental medium contained 175 g glucose and 15 g yeast extract per liter. The pH was adjusted to 5.0 after sterilization. Erlenmeyer flask (250 ml) containing 50 ml medium were inoculated with 2.5 ml of the seed culture and cultivated at 30°C on a rotary shaker (350 rpm). Samples were withdrawn periodically to determine cell growth, sugar utilization, and product yield.

Resting cells experiment

The *Z. rouxii* NRRL Y-27,624 cells grown in 50 ml of the YMP medium with glucose (20 g/l) at pH 5.0, 30°C, and 200 rpm for 24 h were harvested by centrifugation at 8,000×*g* for 15 min at 4°C and then washed twice with 10 mM potassium phosphate buffer, pH 5.0. The resulting cells were suspended in a 250 ml Erlenmeyer flask containing 50 ml medium (175 g glucose and 1 g $MgSO_4 \cdot 7H_2O$ in 50 mM phosphate buffer, pH 5.0 per liter) and incubated with shaking at 30°C at 450 rpm.

Preparation of xylose–xylulose mixture

A reaction mixture containing 400 g xylose, 5 mM $MgSO_4 \cdot 7H_2O$, and 3 ml enzyme preparation (glucose isomerase, EC 5.3.1.5, from *Streptyomyces murianus*, a product of Novozyme purchased from Sigma Chem. Co., St. Louis, MO, USA) in 50 mM phosphate buffer, pH 7.0 per liter was incubated at 60°C for 24 h. The reaction mixture was then boiled for 10 min to inactivate the enzyme. The product profile was monitored by using high performance liquid chromatography (HPLC). The xylose and xylulose ratio in the final mixture was 3:1.

Analytical methods

Cell growth was monitored by measuring optical density of the appropriately diluted culture broth at 660 nm. Sugar utilization and product (D-arabitol, ethanol, glycerol) analyses were performed by HPLC (Spectra-Physics, San Jose, CA, USA) using an Aminex HPX-87P column (300 × 7.8 mm, Bio-Rad Laboratories, Hercules, CA, USA) with a Carbo-P (30 × 4.6 mm) micro-guard column. The column was maintained at 85°C, and the sugars and products were eluted with deionized water (Milli-Q water, Millipore Corp., Bedford, MA, USA) at a flow rate of 0.6 ml/min. Peaks were detected by refractive index and identified and quantified by comparison to retention times of authentic standards.

Results and discussion

Attempts were made to optimize the D-arabitol production from glucose (175 g/l) by the newly isolated *Z. rouxii* NRRL Y-27,624 in shake flasks. The effect of temperature (25–40°C) on D-arabitol production from glucose at pH 5.0 is presented in Table 1. Cell density was highest at 25°C, and the yeast could not grow at 40°C. D-Arabitol production was optimal at 30°C. The effect of pH on D-arabitol production from glucose by the yeast at 30°C was also studied. The optimum pH for D-arabitol production was at pH 5.0 even though there is not much difference in D-arabitol production between pH 4.0 to 6.0 (data not shown). The inoculum size influenced the D-arabitol production. The D-arabitol production increased with the increase of inoculum size, and these values were 70.5 ± 0.5, 72.2 ± 0.7, 73.7 ± 0.3, and 73.9 ± 0.3 g from 175 ± 0.3 g glucose per liter at pH 5.0, 30°C, and 350 rpm after 96 h using 1.0, 2.5, 5.0, or 10.0% inoculum size, respectively. Agitation was essential for D-arabitol production and maximal D-arabitol production in shake flasks occurred at 300–450 rpm as no difference in D-arabitol production was observed at agitation speed of 300, 350, 400 and 450 rpm. The time course of D-arabitol production from glucose in shake flasks by the *Z. rouxii* strain is shown in Fig. 1. The yeast produced glycerol and ethanol in addition to D-arabitol as the main product. However, the ethanol slowly disappeared from the fermentation broth during the fermentation. The maximum D-arabitol produced was 83.4 ± 1.1 g from 175.0 ± 1.1 g glucose per liter with a yield of 0.48 g/g glucose. The yeast could completely utilize 250 g glucose per liter (data not shown). Even though it had the capacity to grow well (OD at 660 nm, 20.6 ± 0.6) in media containing 550 g glucose per liter, it could utilize only 25% of the available substrate. In order to improve culture aeration, the fermentation was also run in 2-l fermenters with 1-l working volumes at 30°C, pH 5.0, and 800 rpm. However, the D-arabitol yield was not improved in both batch and fed-batch fermentations (data not shown).

The effect of various carbon and energy sources on the production of D-arabitol by *Z. rouxii* NRRL Y-27,624 was investigated (Table 2). The yeast could assimilate a variety of monosaccharides and produce D-arabitol. It also utilized xylose and produced D-arabitol and xylitol. From a mixture of xylose and xylulose, the yeast produced both xylitol and D-arabitol. Surprisingly, it could not utilize L-arabinose. Also, the yeast did not grow on lactose, sucrose or starch.

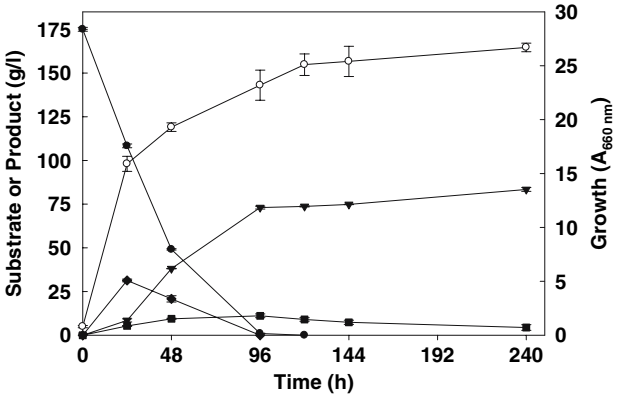

Fig. 1 Time course of D-arabitol production from glucose by *Zygosaccharomyces rouxii* NRRL Y-27,624. The experiments were performed in shake-flasks (250 ml) containing 50 ml of the medium (175 g glucose and 15 g yeast extract per liter) at pH 5.0, 30°C, and 350 rpm. The data presented are averages of two replicate experiments. *Filled circle* glucose; *filled inverted triangle* D-arabitol; *filled diamond* ethanol; *filled square* glycerol; *open circle* cell density (A_{660nm})

The time course of production of D-arabitol from glucose (175 g/l) by the resting cell suspension (OD at 660 nm, 23.2 ± 2.0) of *Z. rouxii* was studied. The glucose utilization and D-arabitol production increased proportionately with time. Glycerol was produced initially and then decreased very slowly throughout the time course study. No ethanol was detected during any time period. In 210 h, the cells utilized all the glucose provided and produced 63.6 ± 1.9 g D-arabitol from 175.0 ± 1.8 glucose with a yield of 0.36 ± 0.01 g/g glucose. Initially, 13.0 ± 1.0 g glycerol per liter was produced which then decreased to 9.5 ± 0.9 g in 210 h. Thus, the resting cells can also be used for production of D-arabitol.

To our knowledge, this is the highest yield of D-arabitol production from glucose by *Z. rouxii* reported in literature. Groleau et al. [6] investigated the production of polyols and ethanol by *Z. rouxii*. They obtained 30 g ethanol, 8.5 g

Table 1 Effect of temperature on the production of D-arabitol from glucose by *Zygosaccharomyces rouxii* NRRL Y-27,624

Temperature (°C)	Cell density (A 660)	Unutilized glucose (g/l)	Products (g/l)		
			D-Arabitol (g/l)	Ethanol (g/l)	Glycerol (g/l)
25	27.0 ± 0.9	1.3 ± 0.3	61.0 ± 1.2	3.4 ± 0.4	18.1 ± 1.8
30	25.1 ± 1.0	1.0 ± 0.2	73.7 ± 0.3	0.0 ± 0.0	11.1 ± 1.1
35	17.6 ± 0.6	2.7 ± 1.8	47.6 ± 1.1	39.4 ± 0.8	10.8 ± 0.1
40	0.0 ± 0.0	–	–	–	–

The experiments were performed in shake-flasks (250 ml) containing 50 ml of the medium (175 g glucose and 15 g yeast extract/l) at pH 5.0, 30°C and 350 rpm for 96 h. The data presented are averages of two replicate experiments

Table 2 Utilization of sugars by *Zygosaccharomyces rouxii* NRRL Y-27,624

Sugar	Products
Glucose	D-Arabitol, glycerol, ethanol
Xylose	D-Arabitol, xylitol
Xylose–xylulose mixture	D-Arabitol, xylitol
L-Arabinose	No growth
L-Arabitol	No growth
Fructose	D-Arabitol, glycerol, ethanol
Galactose	D-Arabitol
Mannose	D-Arabitol, glycerol, ethanol
Lactose	No growth
Sucrose	No growth
Starch	No growth

Two replicate experiments were performed for each sugar in shake-flasks (250 ml) containing 50 ml of the medium (50 g sugar and 15 g yeast extract/l) at pH 5.0, 30°C, and 350 rpm for 120 h. The products were identified by HPLC

glycerol, and 11.5 g polyol (D-arabitol–mannitol mixture) from 200 g glucose per liter in 108 h at 500 rpm. Escalante et al. [5] reported a yield of 0.14 g D-arabitol per gram glucose utilized in a fed batch culture of *Hansenula polymorpha* after 60 h at 45°C, pH 4.8, stirring rate 1,000 rpm, and using air at 1 vvm in a 2-l fermenter. Recently, Povelainen and Miasnikov [11] reported that expression of the arabitol phosphate dehydrogenase gene of *Enterococcus ovium* in the D-ribulose and D-xylulose-producing *Bacillus subtilis* strain resulted in a strain of capable of converting glucose to D-arabitol with a yield of 38%. Nozaki et al. [9] reported 42% yield of D-arabitol from glucose by flask fermentation with *Metschnikowia reukaufi*.

It has been reported that *Saccharomyces rouxii* can produce D-arabitol from glucose by two alternative pathways [1, 7]. It 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) [7]. Another strain of *S. rouxii* converts glucose to D-xylulose-5-phosphate, dephosphorylates D-xylulose-5-phosphate, and then reduces D-xylulose to D-arabitol by NAD-dependent D-arabitol dehydrogenase (D-xylulose forming pathway) [1]. The yeast *C. pelliculosa* produced D-arabitol from glucose but concomitantly produced ribose as a byproduct [3]. Certain osmophilic yeasts when grown in the presence of high concentration of glucose produce a variety of polyhydric alcohols (glycerol, erythritol, D-arabitol, and mannitol) [1, 10]. We could not detect erythritol, mannitol, ribose, or any intermediate product in the fermentation broth with glucose, fructose, mannose, or galactose as carbohydrate source. However, the yeast strain converted xylose as well as xylulose to xylitol and D-arabitol. Figure 2 represents a possible pathway for conversion of glucose to D-arabitol and xylose to xylitol and D-arabitol based on these results.

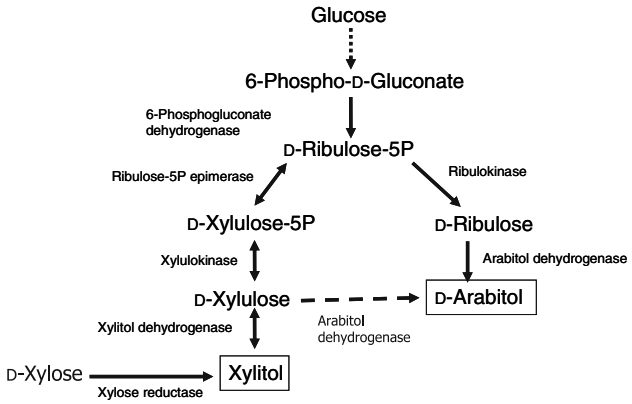

Fig. 2 Possible pathways for conversion of glucose to D-arabitol and xylose to D-arabitol and xylitol by *Zygosaccharomyces rouxii* NRRL Y-27,624

A number of osmophilic or osmotolerant yeasts such as *Z. rouxii*, *D. hansenii*, *C. albicans*, *C. peliculosa*, *C. famata*, and *P. miso* produce D-arabitol from glucose [10]. However, long cultivation times and low yields of arabitol are two of the major problems that may restrict the development of a commercial fermentation process for making D-arabitol. The newly isolated *Z. rouxii* NRRL Y-27624 can produce 83.4 ± 1.1 g D-arabitol from 175 g ± 1.1 glucose per liter with a yield of 48% (w/w). D-Arabitol can be converted to xylitol by biotransformation and enzymatic means. It may be possible to create a recombinant microorganism with the ability to produce xylitol from glucose.

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