微生物学报 *Acta Microbiologica Sinica*
51(3):332 – 339; 4 March 2011
ISSN 0001 – 6209; CN 11 – 1995/Q
http://journals.im.ac.cn/actamicrocn

Isolation and identification of a novel *Candida sp.* H2 producing D-arabitol and optimization of D-arabitol production

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Abstract: [**Objective**] To isolate a new osmophilic yeast for producing D-arabitol and research its optimal fermentation conditions for highest yield of D-arabitol from glucose. [**Methods**] The isolated strain was characterized by electron microscopy, Biolog (GN) test, G + C content measurement and 26S rDNA D1/D2 domain sequences analysis. The purified fermentation product was identified by IR, ^{1}H-NMR, ^{13}C-NMR, MS and optical rotation analysis. Then the fermentation conditions for D-arabitol production were optimized. [**Results**] A new osmophilic yeast was isolated and identified as *Candida sp.* H2. Through the single factor experiment, the optimum conditions of 250 g/L glucose, 10 g/L yeast extract, initial pH 6.0, 35℃ of culture temperature, 200 r/min of agitation, 200 mL medium in a 1000 mL flask of broth content, 1% (v/v) of inoculum size, 96 h of fermentation time were achieved. Based on the conditions above, weight yield of 35% (86.55 g D-arabitol from 250 g glucose) was obtained and 10% higher than the conditions not optimized. [**Conclusions**] *Candida sp.* H2 was a novel strain for producing D-arabitol and valuable for further study.

Keywords: Polyalcohol, D-arabitol, Xylitol, Osmophilic yeast, Candida

CLC number: Q935 **Document code**: A **Article ID**: 0001-6209 (2011) 03-0332-08

D-arabitol, a naturally occurring five-carbon polyalcohol presenting in lichens and mushrooms[1], is used as sweetener and pharmaceutical intermediate[2-4], as the product of osmophilic yeasts corresponding to osmotic pressure[5], and most specially, as a precursor for biosynthesizing xylitol[6], widely used as anticariogenic sweetener and low-calories sugar substitute[7].

Suzuki et al. isolated a *Gluconobacter oxydans* strain for converting D-arabitol to xylitol with a weight yield of xylitol from D-arabitol of about 98% after 27 h fermentation[8] Obviously, in the production of xylitol from glucose, the process of glucose to D-arabitol becomes a rate-limiting step, and thus, to isolate a high-weight yield D-arabitol-producing strain from glucose is essential and valuable.

Many osmophilic strains were reported capable of producing D-arabitol from glucose, including genus *Aspergillus*, *Candida*, *Debaryomyces*, *Hansenula*, *Metscbnikowia*, *Moniliella*, *Pichia and Zygosaccharomyces*[5,9-13], but drawbacks such as long fermentation

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Received: 11 October 2010/Revised: 24 November 2010

time, low weight yield and various by-products formation hindered the industrial application. Two biosynthetic pathways of D-aratitol including D-ribulose forming pathway[14,15] and D-xylulose forming pathway[16,17] were proposed. Currently, D-arabitol is synthesized from the chemical reduction of D-arabinose or D-lyxose.

In this study, an osmophilic yeast for producing D-arabitol was isolated and identified as *Candida* sp.. Its fermentation feature was investigated and fermentation conditions were optimized.

1 Materials and methods

1.1 Screening of strains

The screening medium consisted of 400 g/L glucose and 10 g/L yeast extract (pH 6.0). Both the slant and the plate medium were composed of 100 g/L glucose, 10 g/L yeast extract and 20 g/L agar (pH 6.0). The fermentation medium contained 200 g/L glucose and 10 g/L yeast extract (pH 6.0). Osmophilic yeasts were isolated from various samples including fresh fruits, spoiled fruits, pollens, soil, honeycombs and fresh honey. The samples were added into a 100-mL flask containing 20 mL screening medium and incubated at 30℃, 200 r/min for 72 h. After cultivation, the microorganisms were streaked on plate medium at 30℃ for 72 h. Individual colonies were picked and inoculated into a 100-mL flask with 20 mL fermentation medium and incubated at 30℃, 200 r/min for 144 h. D-arabitol-producing strains were then selected by qualitative assay of D-arabitol in the fermentation broth with thin layer chromatography (TLC) and further quantitative determination by HPLC.

1.2 Identification of strain

The strain was identified based on its morphological, biochemical properties and 26S rDNA D1/D2 domain sequence. The method to observe the morphology of isolate, Biolog GN test to determine the ability of the strain to oxidize various carbon sources and G + C content measurement were performed according to Pan[18]. The 26 S rDNA D1/D2 domain sequence, cloned and sequenced by TaKaRa Biotechnology (Dalian) Co., Ltd, was compared with the sequences in the GenBank databases using BLAST program. CLUSTALX 1.8.1 with default settings and MEGA 4.1 using neighbor-joining method with bootstrapping 1000 times were used to construct phylogenetic tree.

1.3 Purification and analysis of the fermentation product

Purification of D-arabitol was performed according to the method by Lavin[19]. The fermentation product was identified by IR, ^{1}H-NMR, ^{13}C-NMR, MC and optical rotation analysis.

1.4 Fermentation

A loopful of slant cultures was inoculated into a 100-mL flask with 20 mL seed medium containing 20 g/L glucose and 10 g/L yeast extract (pH6.0), and incubated at 30℃, 200 rpm till OD_{660} reached 20. A 0.2 mL portion of the above seed cultures was inoculated into a 100-mL flask with 20 mL fermentation medium containing 250 g/L glucose and 10 g/L yeast extract (pH 6.0) and cultured at 35℃, 200 r/min for 144 h.

1.5 Analytical methods

Thin layer chromatography (TLC) for qualitative detection of D-arabitol was performed using a solvent system of ethylmethylketone/acetone/water (10∶1∶1, v/v) on a silica-gel plate (Qingdao Haiyang Chemical Co., Ltd, China) which was pretreated in advance at 105℃ for 20 min and placed in the preservation tank until used. After about 2 h of migration, the plates were dried in a fume hood and sprayed with the color-developing agent containing sodium periodate and benzidine. D-arabitol and glucose were quantitatively assayed by HPLC (Agilent Co., Ltd, USA) equipped with a 2000ES evaportive light scattering detector (ELSD, Alltech Co., Ltd, USA). The determination was performed at 30℃ of column temperature and 1 mL/min of flow rate using a ZORBAX Carbohydrate Analysis Column (4.6 × 250 mm, 5 μm, Agilent Co., Ltd, USA). The mobile phase consisted of acetonitrile∶water (80∶20). The elution was monitored by an

ELSD with the conditions optimized in advance, i. e. 86℃ of drift tube temperature, 2. 2 L/min of the carrier gas flow rate and 1 of the gain. The biomass production of fermentation broth was measured as optical density at 660 nm using UV spectrophotometer (UNICO Instruments Co., Ltd, China).

2 Results

2.1 Isolation and identification of strains

420 osmophilic strains were isolated from samples of fresh fruits, spoiled fruits, pollens, soil, honeycombs and fresh honey. Among them, 105 isolates were found capable of producing D-arabitol detected by TLC and 10 were able to produce at least 40 g D-arabitol from 250 g glucose. A yeast designated H2, producing 50 g D-arabitol from 250 g glucose, thus obtained with the highest weight yield of D-arabitol and selected for further study.

On the screening plate, strain H2 formed a circular, smooth, shiny, creamy and white colony with approximately 3 mm after two days incubation at 30℃.

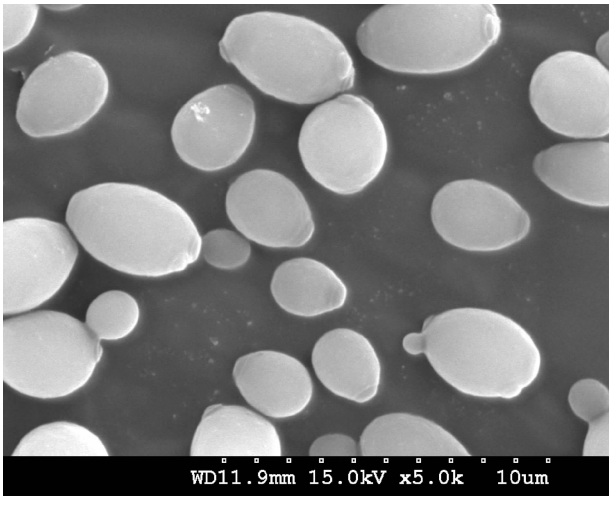

Fig. 1 Scanning electron micrograph of *Candida sp.* H2.

Furthermore, it was roundish or elliptical (size 3 – 5 μm) and procreated by budding reproduction (Fig. 1). The result of Biolog GN test showed the strain H2 was high similar to the species of *Candida*. The content of (G + C) from strain H2 was 38. 7%. A 26S rDNA D1/D2 domain fragment of strain H2 with 511 bp in length was cloned and deposited in GenBank database under accession number HQ269807. Multiple

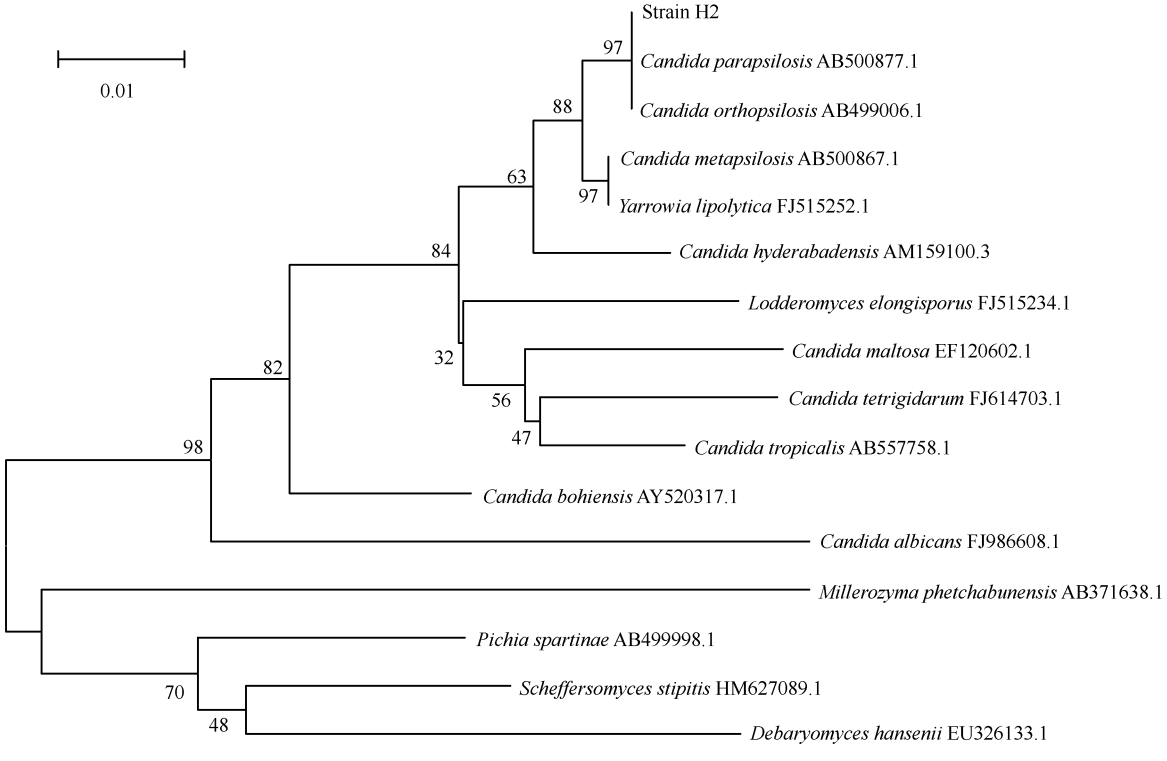

Fig. 2 Phylogenetic tree of strain H2 and related strains based on 26 S rDNA D1/D2 domain sequence. The tree was constructed by the neighbor-joining approach. Bootstrap values (%) were indicated at the nodes. Different accession numbers were following the species names.

alignments indicated its 26S rDNA D1/D2 domain sequence was 100% identity to *Candida parapsilosis* AB499006.1 and *Candida orthopsilosis* AB500877.1. To determine the phylogeny of strain H2, strains from different genera were used to construct the phylogenetic tree based on 26S rDNA D1/D2 domain sequence. The result shown in Fig. 2 indicated the strain H2 clustered closely with *Candida parapsilosis* and *Candida orthopsilosis*.

Based on the results of morphological, biochemical properties and 26S rDNA D1/D2 domain sequence, the strain H2 typically belonged to genus *Candida* and was denominated as *Candida* sp. H2.

2.2 Identification of the fermentation product

The purified product from fermentation broth was identified by IR, ^{1}H-NMR, ^{13}C-NMR, MS and optical rotation analysis. The result of IR of the fermentation product showed that the signal at 3332.39 represented OH. Moreover, ^{1}H-NMR spectrum displayed three signals at δ 4.690 (－OH), δ 3.478－3.531 (－CH) and δ 3.783－3.400 (－CH_2). Thereinto, the signal of CH consisted of multiple peaks. In the spectrum of ^{13}C-NMR, signals with －CH_2OH (δ 62.914 and δ 63.006), －CHOH－ (δ 70.219 and δ 70.408) and －CHOH－ (δ 70.886) were detected. In addition, MS spectrum indicated that the molecular weight of the product was 152.9 corresponding to the standard value of D-arabitol (152.12). Then, the polarimeter was used to detect its optical rotation and the value of specific rotation +11.42 ($[\alpha]_D^{20}$) in 8% borax solution with 5% fermentation product was obtained identical to that of the standard D-arabitol. All of

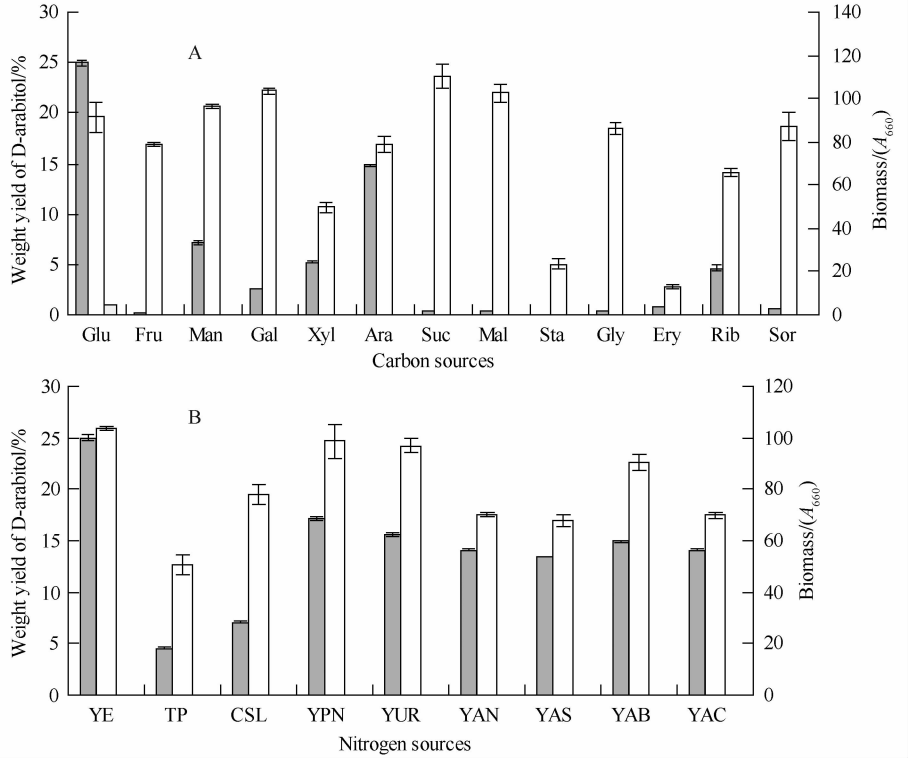

Fig. 3 Effect of carbon sources (A) and nitrogen sources (B) on weight yield of D-arabitol (hatched bar) and biomass (white bar). Cultivation was carried out at 30℃, pH 7.0 for 144 h. A: Fermentation medium contained 200 g/L carbon source and 10 g/L yeast extract. B: Fermentation medium contained 200 g/L glucose and 10 g/L organic nitrogen source or 10 g/L yeast extract and different inorganic nitrogen (total inorganic nitrogen was 45 mmol/L). Abbreviations: Glu, glucose; Fru, fructose; Man, mannose; Gal, galactose; Xyl, xylose; Ara, D-arabinose; Suc, sucrose; Mal, maltose; Sta, starch; Gly, glycerol; Ery, *meso*-erythritol; Rib, ribitol; Sor, sorbitol; YE, yeast extract; TP, tryptone; CSL, corn steep liquor; YPN, yeast extract and potassium nitrate; YUR, yeast extract and urea; YAN, yeast extract and ammonium nitrate; YAS, yeast extract and ammonium sulfate; YAB, yeast extract and ammonium bicarbonate; YAC, yeast extract and ammonium chloride. All tests were performed in triplicate and the average values were documented.

results above indicated the fermentation product was D-arabitol.

2.3 Optimazation of flask fermentation

2.3.1 Effect of carbon sources and nitrogen sources: The effect of various carbon sources on D-arabitol production of *Candida sp.* H2 was investigated in this study. Results shown in Fig. 3-A indicated that most monosaccharides and polyalcohols could be assimilated and transformed to D-arabitol by *Candida sp.* H2 with glucose as the most favorite carbon source for producing

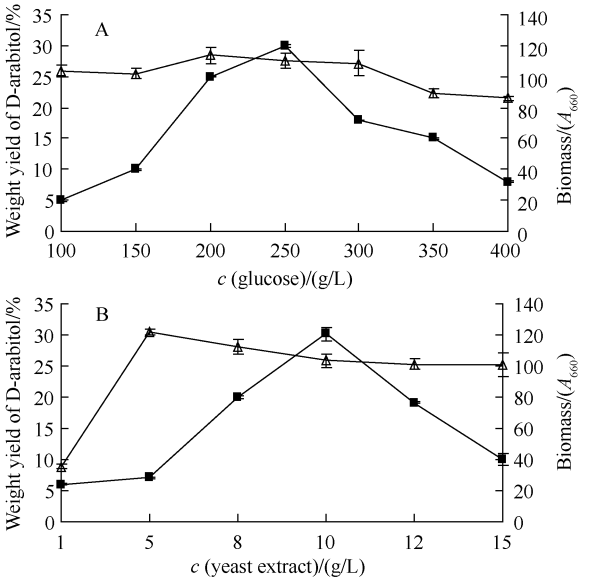

Fig. 4 Effects of the concentration of glucose (A) and yeast extract (B) on weight yield of D-arabitol (■) and biomass (Δ). Cultivation was carried out at 30℃, pH 7.0 for 6 to 144 h. A: Fermentation medium containing 10 g/L yeast extract and 100 g/L-400 g/L glucose. B: Fermentation medium containing 250 g/L glucose and 1 g/L-15 g/L yeast extract. All tests were performed in triplicate and the average values were documented.

D-arabitol and sucrose for biomass' growth. The optimal concentration of glucose in fermentation medium was found 250 g/L in a test range of 100 g/L-400 g/L with 30% of weight yield of D-arabitol (Fig. 4-A). Significant decline in weight yield of D-arabitol and little difference of biomass were observed whether the glucose concentration was higher or lower than 250 g/L.

Yeast extract was found the optimal sole nitrogen source as compared with tryptone and corn steep liquor (Fig. 3-B). However, both of D-arabitol production and biomass both decreased due to the addition of inorganic nitrogen source, including potassium nitrate, urea, ammonium nitrate, ammonium sulfate, ammonium bicarbonate or ammonium chloride, in yeast extract. Whether yeast extract concentration was higher or lower than 10 g/L in a test range of 1 g/L-15 g/L, the weight yield of D-arabitol decreased rapidly and the peak value was obtained at 10 g/L of yeast extract concentration in fermentation medium, as described in Fig. 4-B. There are no significant differences of biomass in the range of 5 g/L-15 g/L of yeast extract concentration in fermentation medium. However, 1 g/L of yeast extract resulted in low biomass yields perhaps because low concentration of nitrogen was far insufficient for vigorous growth.

2.3.2 Effect of initial pH, cultural temperature, inoculum size and broth content: For D-arabitol production, initial pH, fermentation temperature, inoculum size, and broth content were also potential impact factors. pH 6.0 was found the favorite initial pH in the range of pH 4.0-pH 10.0 (Fig. 5-A). The test results performed between 25℃ to 40℃, shown in Fig. 5-B, indicated that the cell density reached peak at 25℃, declined with increase of the culture temperature and D-arabitol production is maximum at 35℃. Fermentation was carried out in 10 mL-60 mL medium of 100 mL flask and 20 mL medium of 100 mL flask was selected for further study (Fig. 5-C). Inoculum size of 0.5%-10% (v/v) were experimented, and 1% was optimal for yield of D-arabitol (Fig. 5-D).

2.3.3 Time course of biomass concentration and D-arabitol production and batch fermentation: Based on the results above, the optimal conditions for D-arabitol production were: fermentation medium containing 250 g/L glucose and 10 g/L yeast extract (pH 6.0), 35℃ of culture temperature, 200 mL in a 1000-mL flask of broth content and 1% (v/v) of inoculum size. Under the optimized conditions, the time courses of biomass, glucose concentration and D-arabitol content were plotted in Fig. 6. From the figure, the production of D-arabitol was rising along with biomass increasing and glucose consumed till 96th h when glucose was

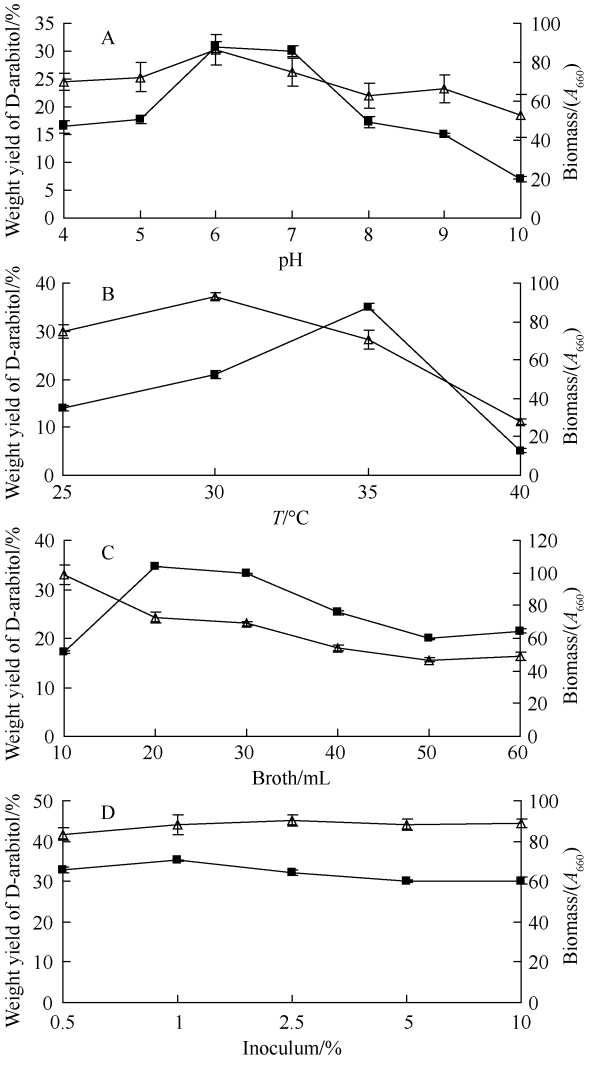

Fig. 5 Effects of pH (A), temperature (B), broth content (C) and inoculum (D) on weight yield of D-arabitol (■) and biomass (Δ). Fermentation medium contained 250 g/L glucose and 10 g/L yeast extract. A: Cultivation was carried out at 30℃, pH 4.0 – 10.0 for 144 h. B: Cultivation was carried out at 25℃ – 40℃, pH 6.0 for 6 144 h. C: Cultivation was carried out at 35℃, pH 6.0 for 6 144 h with 10 mL-60 mL medium in a 100-mL flask of broth content. D: Cultivation was carried out at 35℃, pH 6.0 for 144 h with 1%-10% of inoculum size. All tests were performed in triplicate and the average values were documented.

transformed totally and 86.55 g D-arabitol was obtained. From 96th h of fermentation, the growth entered a stationary stage and D-arabitol concentration remained a stable level. Therefore, 96 h of fermentation was optimal for D-arabitol production. Moreover, the results from batch fermentation of the strain *Candida sp.* H2 in 15 L jar fermentor with 8 L working volumes at 1.5 vvm, 450 r/min, 35℃ and pH 6.0 for 96 h and the results showed that the yield of D-arabitol was not improved (data not shown).

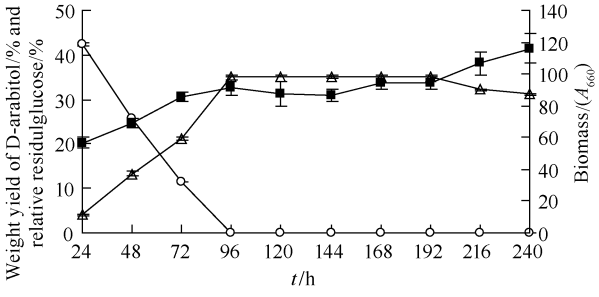

Fig. 6 Time course of D-arabitol (■), biomass (Δ) and residual glucose (○). Fermentation medium contained 250 g/L glucose and 10 g/L yeast extract. Cultivation was carried out at 35℃, pH 6.0. All tests were performed in triplicate and the average values were documented.

3 Discussion

To our knowledge, polyol especially D-arabitol was the main product of osmophilic strains. The mechanism for the biosynthesis of D-arabitol in osmophilic strains was studied in past and two hypothesizes were proposed. Schobert claimed that D-arabitol functioned as a compatible solute and formed hydrated biopolymers to protect the cells from the high pressure surrounding[20]. Rose suggested that the role of D-arabitol acted as a compatible solute for equilibrating the osmotic pressure between intracellular and extracellular circumstance[21]. Based on the mechanism, we designed screening method and isolated many D-arabitol producing strains in which *Candida sp.* H2 was the best one for producing D-arabitol. However, *Candida* species were considered as the pathogen in clinic and few for high-producing D-arabitol. So far, the biosynthesis pathway of D-arabitol in fungi had been elucidated in the past researches (Fig. 7)[14-17]. Therefor, it is possible that D-arabitol is converted from ribitol and xylose in *Candida sp.* H2 by two routes as described in Fig. 7.

In conclusion, we isolated and identified a novel *Candida sp.* H2 for producing D-arabitol. However its yield was lower than those microorganisms producing

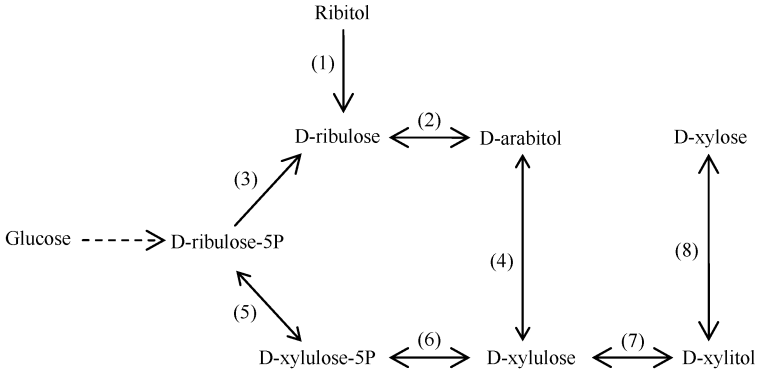

Fig. 7 Routes for synthesizing D-arabitol from glucose, ribitol and D-xylose. (1): Ribitol dehydrogenase; (2): 2-D-arabitol dehydrogenase; (3): Ribulokinase; (4): 4-D-arabitol dehydrogenase; (5): Ribulose 5P epimerase; (6): Xylulokinase; (7): Xylitol dehydrogenase; (8) Xylose reducase.

D-arabitol, such as *Metscbnikowia reukaufii*[12]. Therefore, the wild type *Candida sp.* H2 should be mutagenized to isolate higher D-arabitol-producing mutant. Furthermore, the genetic modification is an alternative method to obtain higher D-arabitol-producing strain.

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产 D-阿拉伯醇菌株的筛选、鉴定及其产 D-阿拉伯醇条件的优化

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摘要：【目的】产 D-阿拉伯醇的耐高渗酵母的筛选、鉴定和产 D-阿拉伯醇条件的优化。【方法】通过电镜、Biolog GN、(G + C)含量和 26S rDNA D1/D2 区序列分析法对所获得的菌株进行了描述。通过红外光谱、核磁共振氢谱和碳谱、质谱以及旋光度实验鉴定纯化产物的结构。通过单因素实验优化产 D-阿拉伯醇的发酵条件。【结果】本文筛选得到一株产 D-阿拉伯醇的新型菌株，经鉴定属于假丝酵母属并命名为 *Candida sp.* H2。200 mL 摇瓶发酵生产 D-阿拉伯醇的单因素优化实验表明，最适发酵条件为：葡萄糖 250 g/L、酵母抽提物 10 g/L、起始 pH 6.0、培养温度 35℃、摇床转速 200 rpm、装液量 200 mL/1000 mL 摇瓶、接种量 1%(v/v)、发酵时间 96 h，在该条件下，D-阿拉伯醇对葡萄糖的质量转化率达 35%，较优化前提高了 10%。【结论】*Candida sp.* H2 是一株有应用前景的新型产 D-阿拉伯醇菌株。

关键词： 多羟基醇，D-阿拉伯醇，木糖醇，耐高渗酵母，假丝酵母

中图分类号： Q935　　**文献标识码：**A　　**文章编号：**0001-6209 (2011) 03-0339-08

(本文责编：张晓丽)

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收稿日期：2010-10-11；**修回日期：**2010-11-24