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Microbiological Purification of L-Arabitol from Xylitol Mother Liquor

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As a rare sugar alcohol, L-arabitol can be used in food and can prevent extra fat deposits in the intestinal tract. Commercially, L-arabitol is prepared from pure L-arabinose by hydrogenation, which needs a high temperature and high pressure, leading to a high production cost for L-arabitol. Therefore, this study describes a novel L-arabitol production method based on biological purification from the xylitol mother liquor, a cheap and readily available raw material that contains a high concentration of L-arabitol. First, a novel *Bacillus megaterium* strain was screened that can utilize xylitol, sorbitol, and mannitol, yet not L-arabitol. The isolated strain was inoculated into a medium containing the xylitol mother liquor under formulated culture conditions, where a high L-arabitol yield (95%) and high purity (80%) were obtained when the medium was supplemented with 50 g/l of xylitol mother liquor. Upon further purification of the fermentation broth by ion exchange and decolorization, L-arabitol was crystallized with a purity of 98.5%.

Keywords: *Bacillus megaterium*, xylitol mother liquor, L-arabitol

L-Arabitol, a rare sugar alcohol with a molecular weight of 152, belongs to the pentitol family, which also includes xylitol, ribitol, and D-arabitol. Various studies have shown that rare sugars and sugar alcohols play important roles in biological processes. Thus, many members of this family are already used in the food industry and human therapeutics, including D-ribose, L-ribose, D-arabinose, L-arabinose, tagatose, D-allulose, euonymus alcohol (galactitol), xylitol, erythritol, ribitol, and D-arabitol [5, 7, 8]. Rare sugar alcohols possess a similar sweetness to sucrose, yet have low or zero dietary calories because of their slow or lack of absorption by the human digestive tract. Furthermore, as the vast majority of oral bacteria cannot metabolize rare sugar alcohols, their consumption does not lead to dental cavities. Therefore, they have become increasingly popular as alternative sweeteners, playing important roles in energy-controlled diets [9, 10], plus the vast majority of chewing gums now contain a certain amount of rare sugar alcohols. Studies have also shown that L-arabitol, which has similar physiological effects to xylitol, can significantly reduce the adipose tissue in the body and prevent the deposition of fat in the digestive tract [14]. Moreover, its efficacy is similar to soluble dietary fibers. Nonetheless, although applications of rare sugar alcohols in the medical field and as pharmaceutical intermediates are increasing, such applications are significantly limited by the current high cost of production.

L-Arabitol can be synthesized either by chemical reduction or microbiological fermentation from L-arabinose [1, 13]. Yet, there is no reported procedure for the production of L-arabitol *via* the fermentation of glucose. As L-arabinose itself is expensive, L-arabitol derived from L-arabinose is even more costly, thereby greatly limiting the use of L-arabitol in foods and medicine. Consequently, the development of a low-cost method for the preparation of L-arabitol is critical in promoting its applications.

The xylitol mother liquor is the product of the chemical hydrogenation of the xylose mother liquor, which is the by-product of xylose prepared from corncob. The xylitol mother liquor contains L-arabitol (28–33%), xylitol (40–45%), sorbitol (8–10%), D-mannitol (5–8%), and small amounts of other unidentified polyols (Fig. 2a). However, the complex composition of the mother liquor makes it very difficult and expensive to extract L-arabitol using routine methods, such as simulated moving bed chromatography (SMBC). However, isolating L-arabitol using microorganisms may be efficient and cost-effective.

Accordingly, this study identified a microorganism, *Bacillus megaterium* BM314, which cannot utilize L-arabitol, yet can metabolize other sugar alcohols, such as

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xylitol, sorbitol, and mannitol. Thus, technically, it should be possible to produce L-arabitol from the xylitol mother liquor economically by culturing this microorganism in a medium that contains inexpensive raw materials. Under such conditions, *B. megaterium* BM314 was able to enrich the L-arabitol in the xylitol mother liquor by metabolizing the other sugar alcohols.

Materials and Methods

Bacterial Strains and Media

The strains used in this study are listed in Table 1. *B. megaterium* BM314 was isolated earlier from soil samples in the authors' laboratory. Briefly, aliquots of soil samples were vigorously suspended overnight in sterilized water. The suspended cells were directly seeded into an enriched medium [M9 medium plus 1% (w/v) xylitol] and cultivated at 37°C for 24 h at 200 rpm, and then spread onto solid agar plates (M9 medium with 1% xylitol and 2% agar). After incubation at 37°C for 72 h, the morphologically distinct colonies present on the plates were transferred to a solid M9 medium with 1% sorbitol or 1% mannitol and cultivated again at 37°C. Only those strains that were able to utilize xylitol, sorbitol, and mannitol were selected for further evaluation as regards enriching L-arabitol from the xylitol mother liquor. After successive screening procedures, a superior bacterium strain was isolated that could utilize xylitol, sorbitol, and mannitol well, yet not L-arabitol. This strain was initially identified as a *B. megaterium* species according to *Bergey's Manual of Systematic Bacteriology* (8th Ed.). The strain was then further analyzed based on the 16S rRNA sequence. A set of bacterial 16S rRNA universal primers (forward: 5'-AGAGTTTGATCATG GCTCAG-3'; and reverse: 5'-GGTACCTTGTTACGACTT-3') was used to amplify the 1.5 kb 16S rRNA gene *via* the following isolation procedure: an initial denaturation step at 94°C for 5 min, followed by 30 cycles at 94°C for 30 s, annealing at 55°C for 40 s, and an extension at 72°C for 90 s, followed by a final extension step at 72°C for 5 min. The amplified 1.5 kb DNA fragment was then subcloned into a TA cloning vector, pMD18-simple (Takara, Dalian, China), for sequencing.

All the strains listed in Table 1 were inoculated into a liquid medium (1.5% yeast extract and 1% tryptone) supplemented separately with 1% xylitol, 1% sorbitol, 1% L-arabitol, or 1% mannitol, and cultivated at 37°C for bacteria (*Bacillus*, *Brevibacillus*, and *Geobacillus*) or 32°C for yeasts (*Candida* and *Saccharomyces*) at 200 rpm. Samples were regularly withdrawn and the contents of the four sugar alcohols measured by HPLC.

Table 1. Bacterial strains used in this study.

Strains	Source
Bacillus subtilis 168	BGSC
Bacillus licheniformis DSMZ13	DSMZ
Bacillus megaterium DSMZ319	DSMZ
Bacillus megaterium BM314	This study
Bacillus megaterium QMB1551	BGSC
Bacillus amyloliquefaciens CGMCC1.1099	CGMCC
Bacillus coagulans CGMCC1.2009	CGMCC
Brevibacillus brevis DSMZ30	DSMZ
Geobacillus stearothermophilus DSMZ22	DSMZ
Candida tropicalis CGMCC2.1975	CGMCC
Saccharomyces cerevisiae ATCC18824	ATCC

BGSC: *Bacillus* Genetic Stock Center.
DSMZ: Deutsche Sammlung von Mikroorganismen und Zellkulturen Gmbh.
CGMCC: China General Microbiological Culture Collection.
ATCC: American Type Culture Collection.

Optimization of Xylitol Mother Liquor Concentration for Fermentation Assays

The strain selected for its ability to utilize all sugar alcohols, except L-arabitol, was cultured in media with varying xylitol mother liquor concentrations from 5% to 20% (v/v) to determine the optimum level. In addition to the components detailed in Table 2, the medium contained 0.2% (w/v) $MgSO_4 \cdot 7H_2O$ and 1% (w/v) KH_2PO_4. The cultivation was carried out in a 500-ml shake-flask with a 100-ml working volume and 10% (v/v) inoculum size at 37°C and 200 rpm for an 80-h cultivation period. Samples were taken at intervals to determine the content and relative purity of L-arabitol.

Determination of Optimal Aeration Rate for L-Arabitol Biological Purification

The experiments were carried out in a 3-l fermenter (Bioflo 110, New Brunswick Scientific Co. Inc, NJ, USA) with a 1.8-l working volume using a fermentation medium containing 5% (v/v) xylitol mother liquor, 1.5% yeast extract (Oxoid), 0.5% tryptone (Oxoid), 0.2% (w/v) $MgSO_4 \cdot 7H_2O$, and 1% (w/v) KH_2PO_4. The primary inoculum of *B. megaterium* BM314 was cultivated in a 50 ml test tube with 5 ml of an LB medium for 12 h at 37°C and 200 rpm, and then transferred to a 1,000-ml shake-flask containing 195 ml of the fermentation medium, cultivated at 37°C and 200 rpm for 12 h, and finally inoculated into the 3-l fermenter to culture at 37°C. The stirring was set at 300, 400, 500, or 600 rpm with an aeration rate of 1 vvm. Samples were taken at regular intervals to determine the OD_{600} and relative purity of the L-arabitol.

Isolation and Purification of L-Arabitol

After fermentation, the bacteria were removed by centrifugation to give 1.8-l of supernatant, which was further concentrated to 200 ml using a rotary evaporator at 70°C and then rotary decolorized for 90 min at 70°C by adding 8 g of powdered activated carbon. The colorless fermentation broth obtained after removing the activated carbon *via* filtration was then concentrated to 50 ml using a rotary evaporator at 70°C, and 300 ml of ice-cold anhydrous ethanol was

Table 2. Components of the fermentation medium for optimizing the xylitol mother liquor concentration[a].

Numbers	Yeast extract (w/v)	Peptone (w/v)	Xylitol mother liquor (v/v)
1	0.5%	0	5%
2	0.5%	0.5%	10%
3	1%	0.5%	15%
4	1.5%	0.5%	20%

[a]In order to keep the constant of C/N ratio, the content of nitrogen sources increased with the increasing xylitol mother concentration.

added. The mixture was allowed to crystallize for 24 h at 4°C and the crystals were then isolated by centrifugation, washed with ice-cold 95% ethanol, and centrifuged again. The purity was then analyzed by HPLC.

Analytical Method

The L-arabitol, xylitol, sorbitol, and other sugar alcohols were analyzed by HPLC (Shodex SKO 801 sugar column, distilled ultrapure water as the mobile phase, with a flow rate of 1.2 ml/min, column temperature 70°C, Shodex RI 101 refractive index detector).

RESULTS

Screening and Identification of Superior Xylitol-Utilizing Bacteria

To enrich the L-arabitol from the xylitol mother liquor with a short fermentation time, strains were selected for their ability to utilize xylitol, sorbitol, and mannitol efficiently during fermentation, and then successive screening steps of solid cultures were repeated to confirm each strain screened. Sixty-nine strains that could grow on a solid minimal salt medium with 1% xylitol were obtained. Among these strains, one strain, named BM314, had the highest growth rate in both solid and liquid minimal salt media (M9) containing sorbitol, mannitol, or xylitol, indicating that the strain could readily utilize those sugar alcohols (Fig. 1). BM314 was systematically identified based on the phenotypic and genotypic characters for strain classification. It was found to be positive for nitrate utilization and Gram-stain negative for catalase, oxidase, Voges–Proskauer, starch hydrolysis, and the production of acid from lactose and mannitol, and did not grow at 65°C. Comparisons of ribosomal RNA (rRNA) gene sequences offered a powerful method for estimating the phylogenetic relationships. The 16S rRNA gene sequence analysis revealed a quite high homology (>99%) with the species of *B. megaterium*. The physiological properties and level of 16S rRNA identity strongly suggest that strain BM314 is a strain of *B. megaterium*; therefore, it was named *B. megaterium* BM314 and deposited in the China General Microbiological Culture Collection (CGMCC) under No. CGMCC 3041.

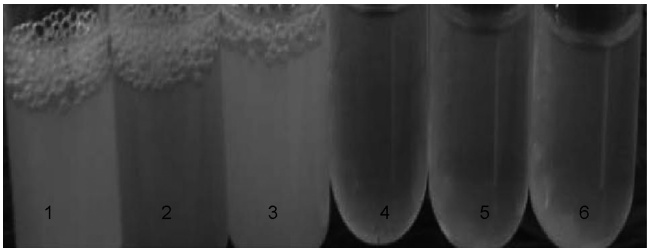

Fig. 1. Growth status of *B. megaterium* BM314 in M9 medium with different sugar alcohols [1, M9+mannitol; 2, M9+sorbitol; 3, M9+xylitol; 4, M9+L-arabitol; 5, M9 without sugar alcohol; 6, M9 +D-arabitol. The M9 medium was purchased from Ameresco (Framingham, MA, USA)].

Gas bubbles formed during its growth in the M9 medium containing xylitol, sorbitol, or mannitol, whereas no gas formed in the medium containing L-arabitol.

Characterization of Sugar Alcohol Utilization by Tested Strains

Because of their biological safety, *B. megaterium* BM314 and another eight types of bacteria (Table 1) from strains available in the authors' laboratory were further selected to culture in a medium supplemented with 1% xylitol, sorbitol, L-arabitol, or mannitol, as described in the Materials and Methods section. The purpose was to test which strain could metabolize xylitol, sorbitol, and mannitol at the highest rate, yet could not metabolize L-arabitol. *B. megaterium* BM314 could exhaust the xylitol, sorbitol, and mannitol, yet was unable to use L-arabitol during 30 h of cultivation. *B. megaterium* DSMZ319 had a similar performance but over 48 h with the same inoculum quantity and culture conditions. *B. megaterium* QMB1551 did not use L-arabitol, but utilized xylitol poorly. Meanwhile, the other six bacteria and two yeasts had little ability to utilize xylitol. Table 3 summarizes the sugar alcohol utilization by all the strains tested. Thus, since *B. megaterium* BM314 was able to efficiently assimilate xylitol, sorbitol, and mannitol, while leaving L-arabitol intact, it was selected to study the enrichment of L-arabitol from the fermentation of the xylitol mother liquor.

Effect of Xylitol Mother Liquor Concentration on Enrichment of L-Arabitol by *B. megaterium* BM 314

A higher xylitol mother liquor concentration in the fermentation medium might be expected to increase the L-arabitol content after *B. megaterium* BM314 has exhausted the xylitol, sorbitol, and mannitol in the fermentation medium. However, in this case, the fermentation time might also be extended and the xylitol, sorbitol, and mannitol may not be fully eliminated from the fermentation medium, which would result in a relatively low purity of L-arabitol compared with that arising from an optimal xylitol mother liquor concentration. Therefore, it is important to determine the optimum concentration of the xylitol mother liquor to maximize the purity of the L-arabitol, which is the most important property when subsequently crystallizing the L-arabitol from the fermentation medium.

Therefore, different concentrations of the xylitol mother liquor were tested, from 5% to 20%. The changes in the content of L-arabitol, xylitol, and sorbitol in the fermentation media containing 5% to 20% xylitol mother liquor during 72 h of fermentation by *B. megaterium* BM314 are shown in Fig. 2. The results showed that the selected strain in a medium containing 5% xylitol mother liquor completely metabolized the xylitol after 36 h, leaving only trace amounts

Table 3. Sugar alcohols utilization by strains tested in this study.

Strains	Sugar alcohols			
	L-Arabitol	Xylitol	D-Sorbitol	D-Mannitol
Bacillus subtilis 168	-	-	+	+
Bacillus licheniformis DSMZ13	-	-	+	+
Bacillus megaterium DSMZ319	-	+	+	+
Bacillus megaterium BM314	-	++	++	+
Bacillus megaterium QMB1551	-	weak	+	+
Bacillus amyloliquefaciens CGMCC1.1099	-	weak	+	+
Bacillus coagulans CGMCC1.2009	-	weak	+	+
Brevibacillus brevis DSMZ30	-	+	+	+
Geobacillus stearothermophilus DSMZ22	-	weak	+	+
Candida tropicalis CGMCC2.1975	-	very weak	+	weak
Saccharomyces cerevisiae ATCC18824	-	-	very weak	very weak

"-", no growth; "+", moderate growth; "++", good growth.

of the other sugar alcohols (Fig. 2B), and increased the purity of the L-arabitol from 36% to 75%, the minimal purity required to crystallize it from a solution. In addition to the desired product, the strain produced about 5% acetic acid (data not shown), yet this can easily be removed by ion exchange, without affecting the crystallization.

In the medium with 10% xylitol mother liquor, although the sorbitol was completely consumed after 60 h, some residual xylitol remained (Fig. 2C). Meanwhile, the media containing 15% and 20% xylitol mother liquor resulted in more residual sorbitol and xylitol after 60 h (Fig. 2D, 2E).

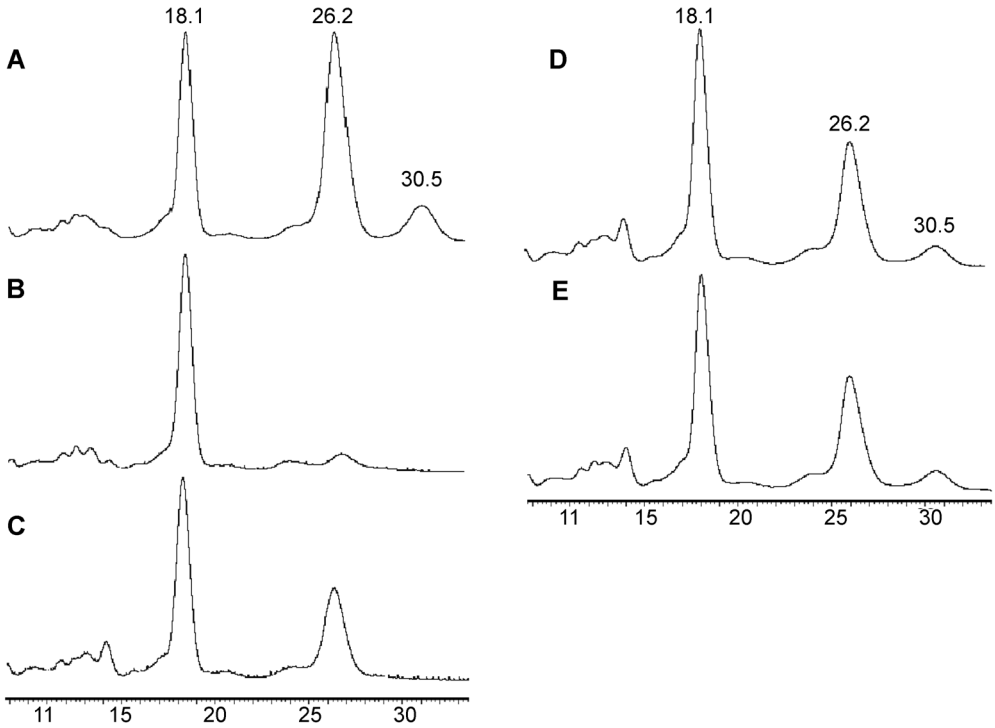

Fig. 2. HPLC analysis of *B. megaterium* BM314 fermentation medium.
(**A**) HPLC profile of 5% xylitol mother liquor. The fermentation medium of BM314 contained 5% (**B**), 10% (**C**), 15% (**D**), and 20% (**E**) xylitol mother liquor and some content of yeast extract and tryptone (see Table 2). The fermentation time was 36 h for (**B**) and 60 h for (**C–E**). The retention time of L-arabitol, xylitol, and sorbitol was 18.1 min, 26.2 min, and 30.5 min respectively. The peaks before 15 min represent unknown substances produced during the *B. megaterium* BM314 fermentation process.

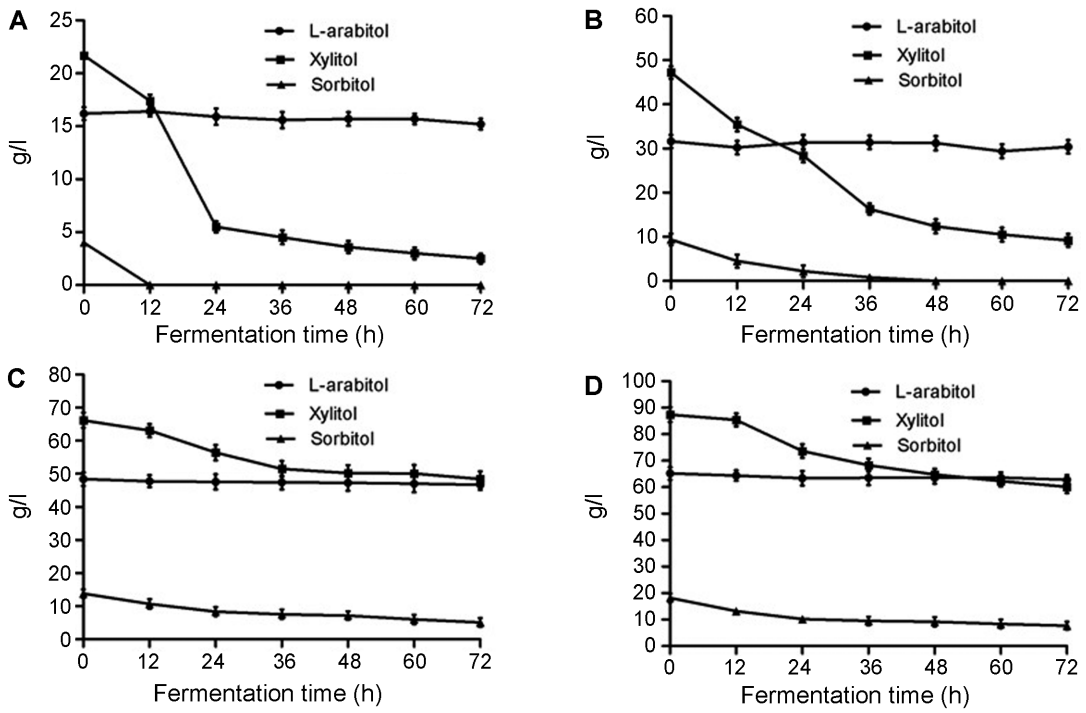

Fig. 3. Changes in L-arabitol, xylitol, and sorbitol concentrations during fermentation with *B. megaterium* BM314 in medium containing 5% (**A**), 10% (**B**), 15% (**C**), and 20% (**D**) xylitol mother liquor.
The values reported are the mean of duplicate experiments.

In the fermentation medium containing 5% xylitol mother liquor, the consumption rate of xylitol and sorbitol was the highest: the sorbitol was exhausted after 12 h and the xylitol decreased from 24 g/l to 3.1 g/l, while the L-arabitol concentration remained constant at 16.8 g/l (Fig. 3A). The highest purity of L-arabitol, up to 75%, was thus achieved and impurities were minimized, which facilitates L-arabitol purification from the fermentation medium.

In the medium with 10% xylitol mother liquor, the sorbitol metabolism was completed after 48 h, yet after 72 h there was still 12.5 g/l residual xylitol and the purity of the L-arabitol was 15% lower than when using 5% xylitol mother liquor (Fig. 3B). In the media containing 15% and 20% xylitol mother liquor, there were significant residual amounts of both sorbitol and xylitol after 72 h, giving L-arabitol concentrations of 45.6% and 47.2%, respectively (Fig. 3C and 3D), which would be difficult to crystallize. Although L-arabitol with a high purity would also be gathered with less than 5% xylitol mother liquor after 10 h, the quantity of L-arabitol would be reduced, which would increase the cost of separation. Therefore, the optimal xylitol mother liquor concentration for the purification of L-arabitol by *B. megaterium* BM314 on a shake-flask scale was 5%. At this concentration, *B. megaterium* BM314 metabolized the sorbitol and xylitol efficiently over a short time and achieved the highest purity of L-arabitol, which could then be easily crystallized.

Effect of Aeration on Biological Purification of L-Arabitol from Xylitol Mother Liquor by *B. megaterium* BM314

The metabolic rates of xylitol and D-sorbitol utilization were studied by culturing BM314 in media containing 5% xylitol mother liquor in four 3-l fermenters with a 10% (v/v) inoculum, an aeration rate of 1.0 vvm, and stirring rates of 300, 400, 500, and 600 rpm, respectively. At 600 rpm, the xylitol and sorbitol were consumed in about

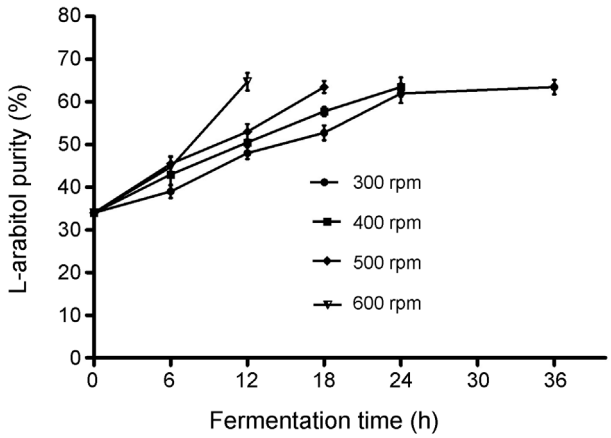

Fig. 4. Correlation between L-arabitol purity and aeration rate throughout the fermentation process.
The values reported are the means of duplicate experiments.

12 h, while 36, 24, and 18 h were required to achieve the same result at 300, 400, and 500 rpm, respectively. Fig. 4 shows the correlation between the L-arabitol purity and the aeration rate for different fermentation times. When increasing the aeration rate from 300 to 600 rpm, the biological purification time decreased from 36 to 12 h, reaching a maximum L-arabitol purity of 68.5%. The rate of consumption of the other sugar alcohols also increased when increasing the aeration rate, thereby significantly enhancing the purity of the L-arabitol in a shorter fermentation time. The sorbitol was consumed much faster than the xylitol (data not shown), probably because the content of sorbitol was present at one fourth the quantity of xylitol in the mother liquor.

Isolation and Purification of L-Arabitol from Fermentation Broth

White powdery crystals of L-arabitol were the final product obtained from the biological purification of the fermentation broth using *B. megaterium* BM314. The biological purification resulted in an L-arabitol purity greater than 65% in the fermentation broth, which is suitable for L-arabitol crystallization from a solution. The cells were removed by centrifugation, and then the supernatant was decolorized by shaking with activated carbon at 80°C and applied to an ion-exchange resin to remove the metal ions, reaching a conductivity of less than 20 μs/cm. After being concentrated to 70% (w/v) L-arabitol, the solution was slowly cooled from 70°C to 4°C. The precipitated white powdery crystals suspended in the solution were then centrifuged, washed with 95% ethanol, and dried. This purified white powder was dissolved in water and showed a purity of 98% based on an HPLC analysis (Fig. 5). There was about 5% acetic acid in the fermentation broth (data not shown), but this was removed during the purification by cation exchange.

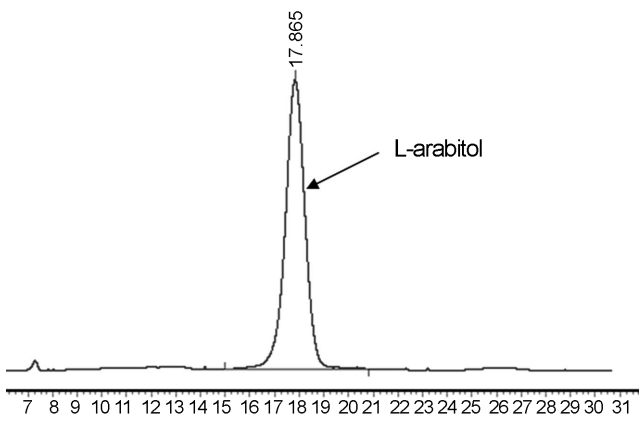

Fig. 5. HPLC profile of powdery crystallized L-arabitol biologically purified by *B. megaterium* BM314 fermentation.
The purity reached 98.5%. The analytical method is described in Materials and Methods.

Discussion

The mechanism by which *Bacillus subtilis* utilizes sorbitol, mannitol, and xylitol has already been extensively studied [2, 3, 6, 11]. For sorbitol, before catalytic dehydrogenation to D-fructose with a small amount of L-sorbose by D-sorbitol dehydrogenase *gutB*, it is first transported intracellularly with the assistance of sorbitol permease *gutA*. It is then converted to fructose 6-phosphate in the presence of fructokinase *fruC*, transformed to fructose 1,6-diphosphate by phosphofructokinase, and finally enters the glycolytic pathway (EMP). For most *Bacillus* species [4, 15], mannitol is first converted to mannitol 1-phosphate and enters the cell through the phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS). It is then converted to fructose 6-phosphate by mannitol dehydrogenase, then transformed to fructose 1,6-diphosphate by fructose kinase, and finally enters the EMP. *B. subtilis* cannot use xylitol directly, but with the assistance of sorbitol-induced sorbitol dehydrogenase, xylitol can be oxidized to D-xylulose and enter the pentose phosphate pathway [4].

In this study, *B. megaterium* BM314 grew well on media with xylitol, sorbitol, or mannitol as the sole carbon source (Table 3). This indicates that a different mechanism exists in *B. subtilis* that enables the use of xylitol. The expression of the *gutB* gene in *B. subtilis* is induced by sorbitol, so xylitol cannot be used by *B. subtilis* until sorbitol is added to promote the sorbitol dehydrogenase gene expression. However, *B. megaterium* BM314 may utilize xylitol through the same mechanism as that of *Bacillus pallidus* Y25 by expressing the xylitol dehydrogenase gene [12]. In this way, xylitol could be oxidized to xylulose upon entering the cell, and then metabolized *via* the pentose phosphate pathway. However, the mechanism of xylitol and sorbitol metabolism in BM314 requires further investigation.

In the xylitol mother liquor, xylitol and sorbitol would be converted to D-xylulose and D-fructose, respectively, by dehydrogenases in the presence of NAD^+, and then further metabolized *via* the pentose phosphate and glycolytic pathways to provide energy for cell growth. Thus, the consumption of xylitol and sorbitol by *B. megaterium* BM314 in the xylitol mother liquor is a highly aerobic process. As *B. megaterium* BM314 is an aerobic bacterium, with high-volume aeration, rapid cell growth and metabolism facilitate the regeneration of NAD^+ from NADH and the subsequent oxidation of xylitol and sorbitol [6]. The first step in this process is the oxidation of xylitol and sorbitol, catalyzed by xylitol dehydrogenase or sorbitol dehydrogenase, to xylulose and fructose in the presence of the cofactor NAD^+ [4, 15]. The supply of NAD^+ must be adequate for the complete oxidation of xylitol and sorbitol. L-Arabitol can thus be enriched in the fermentation medium under these conditions.

The xylitol mother liquor is prepared by decolorization, followed by chemical hydrogenation, and contains large amounts of xylitol, L-arabitol, and sorbitol. Among these, L-arabitol is a rare sugar alcohol that can currently only be synthesized from expensive L-arabinose. Alternatively, chromatographic separation of L-arabitol from the xylitol mother liquor would involve very high equipment and operation costs.

Therefore, this study described a microbiological purification method for selectively consuming the low-value sugar alcohols, such as xylitol and sorbitol, from the mother liquor, thereby greatly improving the purity of the high-value L-arabitol. The enrichment of L-arabitol in the fermentation medium then makes routine crystallization possible. Therefore, this method can produce L-arabitol economically on a large scale from the very low-cost and readily available xylitol mother liquor raw material.

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