Tropical Journal of Pharmaceutical Research December 2016; 15 (12): 2701-2708
ISSN: 1596-5996 (print); 1596-9827 (electronic)

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v15i12.23

Original Research Article

Synthesis of allitol from D-psicose using ribitol dehydrogenase and formate dehydrogenase

Hinawi AM Hassanin, Rebaone Letsididi, Marwa YF Koko, Wanmeng Mu, Ammar Elferga and Bo Jiang*

State Key Laboratory of Food Science and Technology, School of Food Science and Technology, Jiangnan University. 1800 Lihu Avenue, Wuxi 214122, China

For correspondence:* ***Email:* *bjiang@jiangnan.edu.cn;* ***Tel:*** *+86-510-85329055;* ***Fax:*** *+86-510-85919625*

Received: 21 July 2016 *Revised accepted: 10 November 2016*

Abstract

Purpose: *To synthesize allitol from D-psicose by a combination of novel ribitol dehydrogenase (RDH) and formate dehydrogenase (FDH) under optimised production conditions.*
Methods: *RDH and FDH genes were cloned and introduced into pET-22b(+) vectors for expression in Escherichia coli to produce the corresponding enzymes. The effects of temperature, pH, shaking velocity (75, 100, 125, and 150 rpm), and shaking type (horizontal and vortex) were optimised to maximise the production yield of allitol. The final product was purified and subjected to nuclear magnetic resonance (NMR) spectroscopy, infrared (IR) spectrometry, and liquid chromatography-mass spectrometry (LC-MS) to confirm its structure.*
Results: *The optimal pH and temperature for the reaction were 7.5 and 40 °C, respectively. The results revealed that allitol yield significantly increased with increase in reaction shaking velocity and reached a maximum yield of 95.60 ± 0.54 % at 150 rpm shaking velocity after 6 h of reaction. When the reaction was run under horizontal shaking, allitol yield increased from 100.00 ± 6.05 (without shaking) to 124.20 ± 9.70 %. Twenty milligrams of D-psicose were successfully reduced to allitol under optimum conditions with a high production yield of 16.7 ± 0.62 mg after 6 h. No by-products were formed during or after the reaction. The produced allitol had a purity of 95 %, and its structure was confirmed by HPLC, IR, LC-MS, and NMR spectral analyses.*
Conclusion: *Using D-psicose as a substrate, allitol with 95 % purity was successfully produced by the combination of novel RDH and FDH.*

Keywords: *Allitol, Ribitol dehydrogenase, Formate dehydrogenase, D-Psicose, Providencia alcalifaciens*

Tropical Journal of Pharmaceutical Research is indexed by Science Citation Index (SciSearch), Scopus, International Pharmaceutical Abstract, Chemical Abstracts, Embase, Index Copernicus, EBSCO, African Index Medicus, JournalSeek, Journal Citation Reports/Science Edition, Directory of Open Access Journals (DOAJ), African Journal Online, Bioline International, Open-J-Gate and Pharmacy Abstracts

INTRODUCTION

Rare sugars are monosaccharides that are less abundant in nature than common sugars such as D-fructose and D-glucose. Their rarity limits their large-scale extraction and separation from natural sources. These compounds, which are classified as ‘sugars’ and ‘sugar alcohols,’ have interesting characteristics, and are used in both food production and pharmaceuticals [1-6]. The use of inexpensive raw substrates for the production of rare sugars is now attracting considerable attention. Previous studies confirmed the validity of this approach; for example, L-xylulose, D-tagatose, D-sorbose, D-psicose, D-talitol, and allitol were successfully produced using microorganisms [7-11]. Among these, allitol is a rare hexitol that is synthesised

by the reduction of D-psicose [12]. In the natural world, allitol is present in plants of the *Itea* genus (*Itea virginica* L.) and certain fungi [13,14]. Izumori [10] reported a useful strategy for processing most hexoses, called the Izumoring map, in which allitol and galactitol are placed at symmetric points of D and L in the production zone of hexose sugars.

The use of microorganisms to produce rare sugars has various advantages, such as the use of inexpensive catalysts that do not require purification and in the avoidance of cofactor recycling *in vitro*, as enzymatic conversion is more controllable and scalable. The use of both bacterial reduction (using *Enterobacter* strain 221) and enzymatic reaction (using ribitol dehydrogenase: RDH) to produce allitol from D-psicose has been reported [12,15,16]. In recent studies, a new RDH from *Providencia alcalifaciens* RIMD 1656011 and formate dehydrogenase (FDH) from the methanol-assimilating yeast *Ogataea parapolymorpha* DL-1 were cloned and characterised [17,18]. The new RDH was found to possess high specificity towards ribitol and allitol and low specificity towards other polyols (D-sorbitol, L-arabitol, galactitol, D-mannitol, inositol, and glycerol) regarding catalytic targeting [17].

In the current paper, we report for the first time the use of new RDH from *P. alcalifaciens* RIMD 1656011 and FDH from the methanol-assimilating yeast *O. parapolymorpha* DL-1 for the production of allitol. Allitol was completely separated, and its structure was characterised using infrared (IR), liquid chromatography-mass spectrometry (LC-MS), and nuclear magnetic resonance (NMR) spectral data. We also investigated the effects of shaking velocity and shaking type to standardise the reaction time for maximising allitol yield.

EXPERIMENTAL

Chemicals and plasmids

An allitol standard was purchased from Sigma-Aldrich (Shanghai, China). D-psicose was previously prepared in our laboratory from D-fructose [19]. Two reconstructed plasmids, containing FDH from the methanol-assimilating yeast O. parapolymorpha DL-1 and RDH from *P. alcalifaciens* RIMD 1656011 were synthesised by Sangon Biological Engineering Technology and Services (Shanghai, China), in accordance with the protocol reported by Hassanin *et al* [17].

Expression and separation of crude RDH and FDH from *E. coli* BL21

A gene (gene locus_tag: HPODL_3660) with the protein ID EFW95288.1 and a target gene (gene locus_tag: HMPREF1565_2774) with the protein ID number EUD03977.1 have been identified as hypothetical sequences encoding FDH and RDH, respectively. The genes RDH and FDH were cloned and introduced into the pET-22b(+) vector using NdeI and XhoI sites and an in-frame fusion His6-tag sequence at the C-terminus in the reconstructed plasmids. The plasmids, pET-Pral-RDH and pET-Op-FDH, were transformed into *E. coli* BL21 for RDH and FDH overexpression. *E. coli* BL21 cells harbouring the plasmids were grown separately in 500-mL flasks containing 250 mL of Luria–Bertani medium supplemented with ampicillin (100 µg/mL) and incubated at 37 °C. When the culture had attained an optical density of 0.6 at 600 nm, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the culture to a final concentration of 1 mM, and then the expression of RDH and FDH was induced at 30 °C for 5 h.

Subsequently, the cells were collected by centrifugation (8,000 rpm; 10 min), rinsed with lysis buffer (50 mM Tris buffer, 100 mM NaCl, pH 7.5), resuspended in lysis buffer, and disrupted by ultra-sonication at 4 °C using a Vibra-Cell™ 72405 Sonicator (BioBlock Scientific, lllkirch, France). The supernatants obtained after centrifugation (10,000 rpm; 30 min) was used as the crude enzymes. The activities of RDH and FDH were 4.0 and 3.4 units/mL, respectively.

RDH and FDH assays

The protein concentration was measured following the standard Bradford method. Enzyme activities were assayed by measuring NADH absorbance at 340 nm. Furthermore, for the RDH assay, a reaction mixture consisting of 1 mL each of the following was used: 50 mM glycine–NaOH buffer (pH 10.0), 0.6 mM NAD^+, 50 mM ribitol, and enzyme solution; absorbance was measured at 340 nm.

For the FDH assay, the reaction mixture consisted of 1 mL each of the following reactants: 50 mM phosphate buffer (pH 6.5), 1.6 mM NAD^+, 160 mM sodium formate, and enzyme solution; absorbance was again measured at 340 nm. The reaction was started by adding the substrates. One unit of enzyme activity was defined as the amount of enzyme able to catalyse the conversion of 1 µmol NAD^+ to NADH per minute at 30 °C.

Optimisation of temperature and pH for allitol production

A reaction mixture of 1 mL was used (10 mg of D-psicose, 10 mg of sodium formate, 2 mM NAD^+, and 0.5 units of RDH and FDH). Three buffer systems, i.e. phosphate buffer (50 mM, pH 5.0 – 7.0), Tris-HCl (50 mM, pH 7.5 – 9.0), and glycine – NaOH (50 mM, pH 9.5 – 11.0), were used to determine the optimal pH for the two enzymes in the reduction of D-psicose to allitol. The optimal temperature for RDH and FDH activities in the reaction was measured by assaying the coupled enzymes reaction over the temperature range of 25 – 60 °C at intervals of 5 °C.

Optimisation of shaking velocity and shaking type for allitol production

To investigate the effects of shaking velocity and shaking type on allitol production, the enzymatic reaction was carried out as follows. A reaction mixture containing 1 % D-psicose, 1 % HCOONa, 2 mM NAD^+, and 1.0 unit each of RDH and FDH in 2.0 mL of 50 mM Tris-HCl buffer (pH 7.5) was introduced into test tubes. This mixture was shaken using four different shaking velocities (75, 100, 125, and 150 rpm) and two shaking types (horizontal and vortex) in a water bath shaker.

Allitol production

The reduction of D-psicose to allitol by the coupled RDH and FDH reaction was carried out as follows using the above-mentioned conditions. The reaction mixture consisted of 1 % D-psicose, 1 % HCOONa, 2 mM NAD^+, and 1.0 unit each of RDH and FDH in 2.0 mL of 50 mM Tris-HCl buffer (pH 7.5). The mixture was shaken at 150 rpm (horizontal shaking) and 40 °C for 6, 24, 48, and 72 h. The rate of allitol production was measured by high-performance liquid chromatography (HPLC) (1200 Series; Agilent Technologies, Santa Clara, CA, USA).

HPLC

For the HPLC analysis, samples were heated at 95 °C for 5 min to inactivate enzymes, and then centrifuged at 10,000 rpm for 10 min at ambient temperature. The supernatants obtained after centrifugation were then passed through a 0.45 -µm filter and analysed by HPLC coupled with an IR detector (Shodex RI-101) and SUGAR-PAK column (6.5 × 300 mm) at a column temperature of 85 °C. Water was used as the mobile phase at a flow rate of 0.4 mL/min.

Allitol purification

Allitol purification was achieved by HPLC. It was separated on a SUGAR-PAK column (6.5 × 300 mm) with water as mobile phase at the flow rate of 0.4 mL/min at 85 °C. The collected eluate of pure allitol was placed in a freeze-dryer (Labconco Corporation, Kansas City, USA). To measure the purity of the purified allitol (freeze-dried), 2 mg of allitol powder was dissolved in 1 mL of distilled water and detected by HPLC.

NMR and IR spectroscopy

A total of 10 mg of freeze-dried allitol (purity 95 %) was dissolved in D2O (NMR solvent) at 25 °C. Then, the sample was loaded into a Bruker Avance III Digital NMR Spectrometer (Bruker, Karlsruhe, Germany) for 13C NMR spectroscopy. The spectra were processed with Bruker Topspin 2.1 software (Bruker). The IR spectra were processed using a Thermo/Nicolet Nexus 470 FT-infrared spectrometer.

LC-MS

LC-MS (Waters Acquity UPLC and Waters Maldi Synapt Q-T of MS) was performed in negative-ion detection mode. Ultra-pure synthetic air was used as the desolvation gas (flow rate, 700 L/h), and MS fragment ions were obtained with collision energy of 6 eV. The mobile phase consisted of 80 % acetonitrile and 0.1 % ammonia, and the run time was 15 min, with a flow rate of 0.3 ml/min and injection volume of 1 µL.

Statistical analysis

All experiments were performed in triplicate for each sample. The data are presented as mean ± standard deviation. The experimental data were analysed by one-way analysis of variance using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). Comparison of group means was accomplished using Duncan's multiple range tests. Unless noted otherwise, $p < 0.05$ was considered to indicate a significant difference.

RESULTS

Optimum pH and temperature

To determine the optimal pH and temperature, the reaction mixture was incubated for 6 h. Figure 1A shows that the optimal pH for allitol production was 7.5, whereas at pH 5.0, 5.5, 6.0, 6.5, 7.0, 8.0, 9.0, 10.0, and 11.0, the relative yield were 34.2 ± 3.9, 69 ± 4.3, 86.0 ± 4.5, 97.0 ±

5.2, 98.0 ± 4.1, 71.4 ± 5.5, 59.3 ± 5.7, 37.7 ± 6.5, and 29 ± 4.2 %, respectively.

As shown in Figure 1B, the reaction was also carried out within the temperature range of 25 – 60 °C. In the reaction mixture, maximum allitol production was achieved at a temperature of 40 °C. The production of allitol significantly increased from 68 ± 2.1 % at 25 °C and then decreased above 40 °C to 91.0 ± 3.2 % at 45 °C, reaching 0 % at 60 °C.

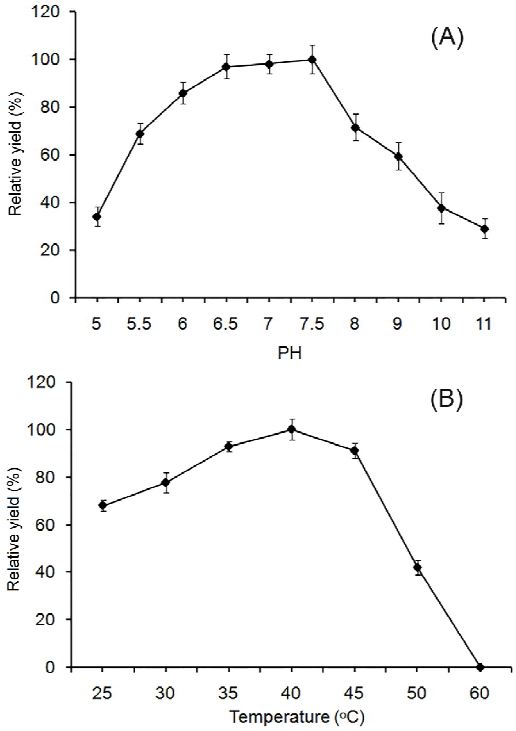

Figure 1: (A) Effect of pH on the reaction system. Values are the means of three replicates ± standard deviation. (B) Effect of temperature on the reaction system. Values are the means of three replicates ± standard deviation.

Effect of shaking velocity and shaking type

The effects of shaking velocity and shaking type on allitol production by the two enzymes (RDH and FDH) were also investigated in this study. Allitol production was investigated with two types of shaking (horizontal and vortex). The production yield of allitol increased by 24.2 ± 9 % and 13.7 ± 5 % for horizontal and vortex shaking, respectively, compared with that in the control (without shaking).

In addition, allitol production was tested with four different shaking velocities (75, 100, 125, and 150 rpm) for up to 24 h at 40 °C and pH 7.5. As shown in Figure 2, allitol production was significantly increased at 150 rpm, with the production yield reaching 94 ± 0.73 % after just 6 h. Shaking velocities of 0, 75, 100, and 125 rpm for 6 h resulted in production yields of 69.79 ± 8.6, 64.0 ± 3.5, 59.52 ±2.7 and 77.4 ± 2.4 %, respectively.

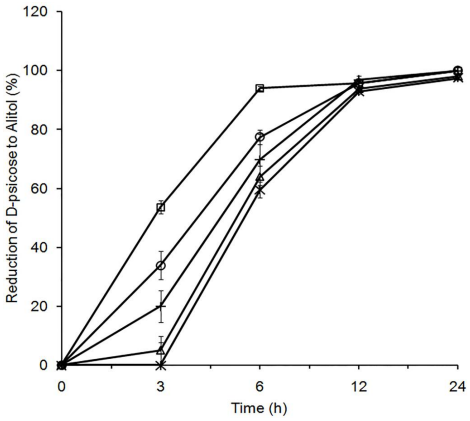

Figure 2: Effect of shaking velocity on allitol production. The shaking velocity was tested by measuring the reduction of D-psicose at (□) 150 rpm, (○) 125 rpm, (⁎) 100 rpm, (Δ) 75 rpm and (**+**) 0 rpm. Values are mean of three replicates ± standard deviation

Reduction of D-psicose to allitol under optimal conditions

The reduction of D-psicose to allitol was then conducted under optimal conditions (40 °C, Tris-HCl pH 7.5, 150 rpm, and horizontal shaking) using one unit of RDH and one unit of FDH. The reaction revealed a reduction of D-psicose to allitol with production yields of 16.75 ± 0.62, 17.86 ± 0.6, 18.3 ± 0.8, 18.85 ± 0.6, and 18.93 ± 0.82 mg mL^{-1} at 6, 12, 24, 48, and 72 h, respectively, without any by-product formation (Figure 3).

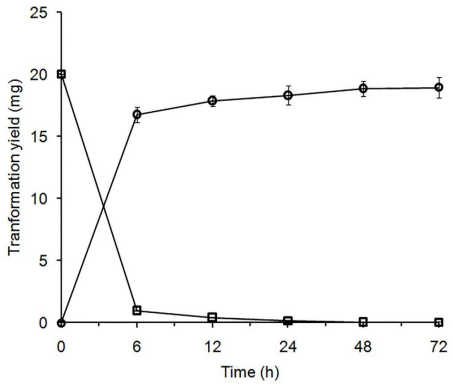

Figure 3: Reduction of D-psicose to allitol by ribitol dehydrogenase and formate dehydrogenase. (□) D-psicose; (○) allitol. The initial concentration of D-psicose was 1 %. The production of allitol and decrease in D-psicose are presented as the transformation yield (mg). Values are means of three replicates ± standard deviation

Product structure identification

The reaction mixture was analysed by HPLC; the retention times of the produced allitol and the authentic allitol standard were identical (Figure 4A). The purity of the purified allitol was approximately 95 %. The ^{13}C NMR (Peak no. 1: 63.20, Peak no. 2: 73.01 and Peak no. 3: 73.17) and IR data (Figure 4B) analyses confirmed that the product was allitol. The product was also analysed by LC-MS; as shown in Figure 5, the measured mass from the spectrum was 182.1, which is identical to the molar mass of allitol.

DISCUSSION

In this study, two novel enzymes, RDH and FDH from *Providencia alcalifaciens* RIMD 1656011 and the methanol-assimilating yeast *Ogataea parapolymorpha* DL-1, respectively, were used for the production of allitol from D-psicose. Only a few studies have reported the use of RDH for producing allitol from D-psicose, such as isolation of RDHs from *Klebsiella pneumonia* IFO 3321 [16].

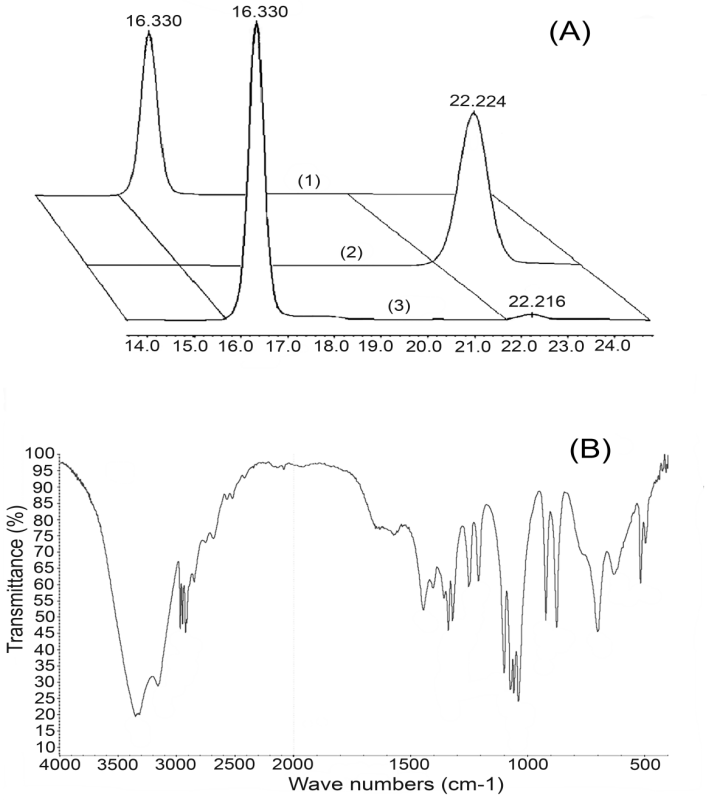

Figure 4: (A) High-performance liquid chromatography analysis of the reduction of D-psicose to allitol by ribitol dehydrogenase (RDH) and formate dehydrogenase (FDH). (1) Authentic allitol, (2) Authentic D-psicose, and (3) Reaction system. (B) Infrared spectrum of allitol produced by RDH from *Providencia alcalifaciens* RIMD 1656011

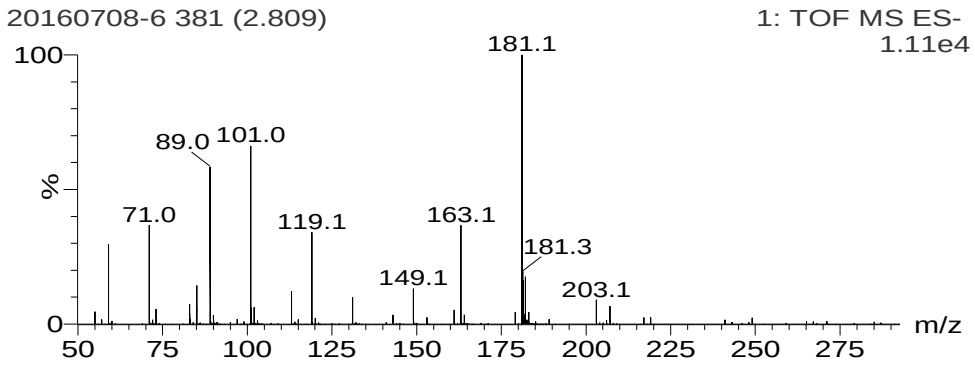

Figure 5: The mass spectrum of purified allitol produced from D-psicose

In addition, a recent study by Zhu *et al* [20] achieved the co-expression of multiple enzymes in *E. coli* using whole cells for allitol production. RDH enzymes have been isolated from many microorganisms, such as RDH from *E. agglomerans* strain 221e, *Rhodobacter sphaeroides*, *Gluconobacter oxydans* and *Zymomonas mobilis* [16,21-23]. However, only RDH from *K. pneumonia* IFO 3321 was coupled with FDH and optimised for allitol production [16]. Allitol can also be produced directly from D-fructose through three enzymes: D-tagatose-3-epimerase (DTEase), RDH, and FDH. The important consideration when producing allitol directly from D-fructose is that RDH should be active on allitol and not active on D-sorbitol or D-mannitol. If polyol dehydrogenase is active on these substrates, D-fructose could be reduced to D-sorbitol or D-mannitol, which would result in impurities in the final product [16]. However, the RDH from *P. alcalifaciens* that we investigated was not active on D-sorbitol and D-mannitol, which indicated that the enzyme can be used for the production of allitol directly from D-fructose [17]. To the best of our knowledge, there is also an RDH from *K. pneumonia* which is not active on D-sorbitol and D-mannitol [16].

RDH from *P. alcalifaciens* achieved a high yield of allitol across a wide pH range, which is extremely interesting for industrial uses. The optimal pH for the reaction mixture with RDH from *K. pneumoniae* IF0 3321K was previously found to be 8.0 [16]. This shows that there is no significant difference between the optimal pH in this study and those in other reported studies on allitol production.

It was also revealed that temperature had a great effect on the use of RDH from *P. alcalifaciens* for allitol production. However, such variations in temperature were due to different optimal temperatures of RDH and FDH. Takeshita *et al* [16] reported that they produced allitol at a temperature of 30 °C. The previously reported optimal temperatures for RDH for producing allitol are in agreement with that for RDH from *P. alcalifaciens*, which exhibited high activity for allitol production within the range of 30 – 40 °C.

In addition, there was no significant difference in the level of allitol production between horizontal and vortex shaking. From the results of horizontal shaking, this was observed to be similarly effective for allitol production for both RDH and FDH, increasing the production yield of allitol.

There was no significant difference among the shaking velocities of 0, 75, and 100 rpm. However, shaking at 125 rpm resulted in a significantly higher yield ($p < 0.05$) compared with 75 and 100 rpm. Shaking at 150 rpm also resulted in a significantly higher yield ($p < 0.05$) compared with all other tested shaking velocities (0.0, 75, 100, and 125). This showed that the allitol production yield was increased by shaking velocities above 100 rpm. A further increase of shaking velocity to 150 rpm reduced the time until the end of the reaction was reached and accelerated the production of allitol from D-psicose. No previous studies examined the effects of shaking velocity and shaking type on allitol production.

In the next stage of the study, allitol was produced under the previously determined optimal conditions, and the reaction was characterised using HPLC after heating the reaction mixture at 95 °C for 5 min to inactivate the enzymes. The production yield reached approximately 17.86 ± 0.6 mg in just 12 h from 20 mg of D-psicose. Similarly, Takeshita *et al* [16] reported that almost all D-psicose was transformed into allitol after 48 h. Our enzyme system gave a high reduction yield of approximately 83 % within a short period of 6 h. The obtained product was then subjected by NMR, IR, and LC-MS analyses to confirm that it was allitol.

A comparison of the ^{13}C NMR results for allitol produced by RDH from *K. pneumoniae* IFO3321 (Peak no. 1: 63.20, Peak no. 2: 73.0 and Peak no. 3: 73.2), *Enterobacter agglomerans* strain 221e (Peak no. 1: 63.22, Peak no. 2: 73.01 and Peak no. 3: 73.18) and novel isolated strain of *Klebsiella oxytoca* G4A4 (Peak no. 1: 63.23, Peak no. 2: 73.02 and Peak no. 3: 73.18), revealing no differences among the ^{13}C NMR results [16,12.24]. IR spectrum of allitol produced by *P. alcalifaciens* is identical to the IR spectrum of allitol produced with the reaction system reported by Takeshita *et al* [16].

The mass spectrum obtained by LC-MS in negative-ion detection mode (182.1 g/mol) was identical in terms of the molar mass of allitol. This high allitol production yield using RDH from P. alcalifaciens can decrease the time required to produce allitol for commercial uses, and can thus reduce costs.

CONCLUSION

Allitol has been successfully produced by the combination of RDH/FDH and D-psicose as a substrate. Optimization of various reaction parameters results in high-yield allitol production under experimental conditions, such as shaking

velocity and shaking type, via the reduction of D-psicose by RDH and irreversible regeneration of NADH from NAD^+ by FDH. The allitol produced is pure, being successfully characterised and confirmed by HPLC, IR, and ^{13}C NMR analyses. Thus, low-cost production of allitol on a comparatively large scale using these novel enzymes can in turn extend the range of applications of allitol to pharmaceuticals and other types of food in future.

DECLARATIONS

Acknowledgement

This work was financially supported by National Natural Science Foundation of China (nos. 31371788 and 31230057) and National High Technology Research and Development Program of China (no. 2013AA102102).

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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