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Construction of allitol synthesis pathway by multi-enzyme coexpression in *Escherichia coli* and its application in allitol production

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Abstract An engineered strain for the conversion of D-fructose to allitol was developed by constructing a multi-enzyme coupling pathway and cofactor recycling system in *Escherichia coli*. D-Psicose-3-epimerase from *Ruminococcus* sp. and ribitol dehydrogenase from *Klebsiella oxytoca* were coexpressed to form the multi-enzyme coupling pathway for allitol production. The cofactor recycling system was constructed using the formate dehydrogenase gene from *Candida methylica* for continuous NADH supply. The recombinant strain produced 10.62 g/l allitol from 100 mM D-fructose. To increase the intracellular concentration of the substrate, the glucose/fructose facilitator gene from *Zymomonas mobilis* was incorporated into the engineered strain. The results showed that the allitol yield was enhanced significantly to 16.53 g/l with a conversion rate of 92 %. Through optimizing conversion conditions, allitol was produced effectively on a large scale by the whole-cell biotransformation system; the yield reached 48.62 g/l when 500 mM D-fructose was used as the substrate.

Keywords Allitol · Multi-enzyme coupling pathway · Cofactor recycling system · Whole-cell biotransformation

Introduction

Allitol is a rare polyol found in nature that is useful not only as a sweetener or raw material for production of chemical compounds, but also as a research reagent [18]. Allitol may be an important intermediate for the preparation of azasugars, which represent potential medicines against diabetes, cancer, and viral infections, including AIDS [3]. Moreover, allitol is located at the center of hexoses in the Izumoring strategy. Thus, allitol should be useful as a substrate for the production of L-tagatose and L-psicose, from which several L-aldoses can be obtained by keto-aldol isomerases [7].

Since allitol has unique properties and is difficult to chemically synthesize, it is clearly important to discover an economic and high-yield method for allitol production. According to the location in the Izumoring strategy and the chemical formula of allitol, the production of allitol using biotransformation should be possible by D/L-psicose or D/L-tagatose reduction. Izumori et al. reported a method for converting allitol from D-psicose using *Enterobacter agglomerans* strain 221e [15]. However, D/L-Psicose and D/L-tagatose are expensive rare sugars, thereby making the cost of production of allitol from these sugars prohibitively expensive.

Allitol can also be produced from D-fructose via the intermediate D-psicose by coupling the enzymes D-tagatose-3-epimerase (DTE), ribitol dehydrogenase (RDH), and formate dehydrogenase (FDH) [18]. In this reaction system,

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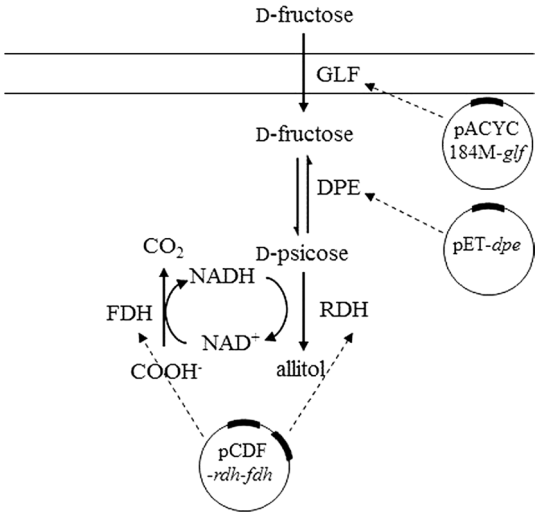

Fig. 1 The strategy for allitol production using whole-cell biotransformation. *GLF* glucose/fructose facilitator, *DPE* D-psicose 3-epimerase, *RDH* ribitol dehydrogenase, *FDH* formate dehydrogenase

D-fructose was transformed into D-psicose by DTE and then D-psicose was hydrogenated to form allitol by RDH. Nicotinamide adenine dinucleotide (NADH) was regenerated using FDH, thereby facilitating the conversion of D-psicose to allitol. The construction of a coenzyme recycling system effectively overcomes the problem on cofactor deficiency. However, the reaction was performed extracellularly and the yield of allitol was relatively low (10 g/l).

Compared with the extracellular enzyme reaction, whole-cell biotransformation has the advantages of maintaining cofactor recycling in vivo and circumvents the requirement to extract and purify every enzyme. Moreover, the separation and purification of products are more convenient because products are separated from enzymes and cofactors by the cell membrane. Sometimes the product can be crystallized from the fermentation liquid directly. Various whole-cell biotransformation systems using wild-type microorganisms, as well as recombinant strains, have been developed [22, 26, 27], such as the production of D-mannitol from D-fructose using resting cells of recombinant *E. coli* and *C. glutamicum* ATCC13032 [1, 9], and the synthesis of C4, C5, C6, and C7 rare ketoses using *C. glutamicum* engineered strain harboring an artificial pathway [24].

Compared with extracellular enzymatic reactions, it is important for whole-cell biotransformation to accumulate the substrate intracellularly in order to improve the conversion efficiency. The glucose/fructose facilitator (GLF) gene has been reported to increase significantly the D-fructose uptake rate during biotransformation [16, 21]. The *glf* gene from Gram-negative bacterium *Z. mobilis* was also reported to be coexpressed on a plasmid to accelerate the uptake of D-fructose, and thus a higher product yield was achieved by increasing the intracellular concentration of D-fructose [16, 21].

In the present study, we report the construction of recombinant plasmids and engineered strains to co-express the genes for converting D-fructose to allitol. The construction of the engineered strain for allitol production is shown in Fig. 1. The conditions for allitol production using whole-cell biotransformation were optimized, and allitol was inexpensively and efficiently produced using D-fructose as the substrate. This is the first report on allitol production using the whole-cell biotransformation of an engineered strain.

Materials and methods

Enzymes and chemicals

All restriction enzymes, T4 DNA ligase, the Taq DNA polymerase and protein molecular weight markers were obtained from Fermentas (Ontario, Canada). The DNA marker was obtained from Generay (Shanghai, China). The bacterial genomic DNA extraction kit, plasmid extraction kit, DNA gel extraction kit, and DNA purification kit were obtained from Tiangen (Tianjin, China). The Ni Sepharose resin and His SpinTrap kit for protein purification were obtained from GE (Uppsala, Sweden). Allitol, D-fructose and D-psicose were purchased from Sigma-Aldrich (St. Louis, MO, United States). Antibiotics, such as ampicillin (100 μg/ml), chloramphenicol (10 μg/ml), and streptomycin (100 μg/ml), were obtained from Solarbio (Beijing, China).

Plasmids and strains

The bacterial strains and plasmids used in this study are listed in Table 1. We used *E. coli* DH5α as the host strain for gene cloning and *E. coli* BL21 Star (DE3) for gene expression. The plasmid pCDFDuet™-1 was used as the co-expression vector. *Klebsiella oxytoca* CGMCC7662, from which the RDH gene (*rdh*) was found, was isolated from soil samples by our laboratory and deposited with the China General Microbiological Culture Collection Centre under the accession number of 7662 [4]. The D-psicose 3-epimerase (DPE) gene (*dpe*) from *Ruminococcus* sp. 5_1_39BFAA was synthesized by GeneWize (Beijing, China). The FDH gene (*fdh*) and GLF gene (*glf*) were from *Candida methylica* X81129 and *Zymomonas mobilis* subsp. *mobilis* ZM4, respectively.

Media and culture conditions

All strains were grown in Luria–Bertani medium and cultivated aerobically at 37 °C by rotating at 200 rpm.

Table 1 Plasmids and strains used in this study

Plasmids or strains	Relevant characteristics	Sources
pET-21a(+)	*amp*; expression vector with *T7* promoter	Novagen
pCDFDuet™-1	*sm*; Double T7 promoters, CloDF13ori	Novagen
pTrc99A	*bla*; expression vector with *trc* promoter	Novagen
pACYC184	*cat, tet*; low copy vector	Novagen
pACYC184 M	*cat*; replace *tet* with *lacI* and Ptrc of pTrc99A	This study
pET-*dpe*	*amp*; pET-21a(+) carrying gene for D-psicose 3-epimerase from *Ruminococcus sp.*	[12]
pCDF-*rdh-fdh*	*sm*; pCDFDuet™-1 carrying genes for FDH from *C. methylica* and RDH from *K. Oxytoca*	This study
pACYC184 M-*glf*	*cat*; pACYC184 M carrying the *glf* from *Z. mobilis*	This study
E. coli DH5	*endA1, supE44, recA1, gyrA96, relA1, deoR U169, Φ80dlacZΔM15, mcrA Δ(mrr–hsdRMS–mcrBC)*	Invitrogen
E. coli BL21 Star (DE3)	For gene expression	Invitrogen
Strain I	BL21 Star (DE3) pCDF-*rdh-fdh* pET-*dpe*	This study
Strain II	BL21 Star (DE3) pCDF-*rdh-fdh* pET-*dpe* pACYC184 M-*glf*	This study

Table 2 Primers used in this study (restriction sites are underlined)

Primers	Sequences
dpe-F	GGAATTCCATATGAAATATGGTATTTATTACG
dpe-R	CGGGATCCTTAGACTTCAAATACATG
rdh-F	CGGAATTCGATGAATACTTCCCTTAGCGGTAA
rdh-R	CCCAAGCTTTCAGAGATCCACGCTGTTCGG
fdh-F	GAAGATCTCATGAAGATCGTTTTAGTCT
fdh-R	GGGGTACCTTATTTCTTATCGTGTTTACCGT
glf-F	CGGAATTCAGTTCTGAAAGTAGTCAGGG
glf-R	AACTGCAGCTACTTCTGGGAGCG

Protein expression was conducted aerobically at 20 °C by rotating at 100 rpm with 1.0 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) present in the medium.

Construction of expression plasmids and strains

The *dpe* gene was synthesized into the vector pET-21a(+) at the *Nde* I and *Xho* I restriction sites by GeneWize to form pET-dpe. The *rdh* gene was amplified from the genomic DNA of *K. oxytoca* and ligated into the first multiple cloning site (MCS) of pCDFDuet™-1 at the *Eco*R I and *Hind* III site to create the recombinant plasmid, named pCDF-*rdh*. A 6×His-tag sequence was present in the vector to aid protein purification. The *fdh* gene was amplified from *C. methylica* genomic DNA and ligated into the second MCS of pCDF-*rdh* at the *Bgl* II and *Kpn* I site to create the recombinant plasmid pCDF-*rdh-fdh.* The *glf* gene was amplified from *Z. mobilis* genomic DNA. The amplified *glf* gene with the restriction sites *Eco*R I and *Pst* I was ligated into the vector pACYC184M, which was modified by pACYC184 and pTrc99A, resulting in vector pACYC184M-*glf*. The primers used in this work were synthesized by GeneWize and listed in Table 2. The recombinant plasmids pET-*dpe* and pCDF-*rdh-fdh* were transformed into *E. coli* BL21 Star (DE3) simultaneously to form Strain I. The recombinant plasmid pACYC184M-*glf* was transformed into strain I to form Strain II.

Purification and characterization of RDH

Escherichia coli cells of Strain I were cultivated at 37 °C and rotated at 200 rpm in LB medium containing 100 μg/ml streptomycin and 100 μg/ml ampicillin. Protein expression was induced by the addition of IPTG when the OD_{600} reached 0.8, and the culture was grown at 20 °C and rotated at 100 rpm for a further 20 h. The cells were harvested by centrifugation and disrupted by sonication. The supernatant was obtained by centrifugation at 14,000×*g* for 20 min at 4 °C. The recombinant RDH was purified using the His SpinTrap kit and a standard method, and the purified RDH was desalted and concentrated using Amicon Ultra Centrifugal Filters (Millipore) against 25 mM Tris–HCl buffer (pH 8.0). The resulting solution was used as a purified enzyme.

The activity of RDH was determined spectrophotometrically by monitoring the change in A_{340} upon oxidation of NADH. The RDH assay mixture consisted of 2 mM NADH, 50 mM D-psicose, and 40 μg/ml enzyme [14]. The reaction was started by the addition of substrate. One unit of enzyme activity was defined as the amount of enzyme required to consume 1 μmol NADH per min. The optimum pH of RDH was determined at various pH values between 4.0 and 11.0 using four buffer systems. The optimum temperature of RDH was measured by assaying the enzyme samples over the range of 20–70 °C in 25 mM Tris–HCl buffer (pH 8.0). The thermal stability of the enzyme was investigated by incubating the enzyme in 25 mM Tris–HCl buffer (pH 8.0) at various temperatures. The activity was then

assessed under a standard condition. All trials were performed in triplicate.

Detection and determination of heterologous proteins

Strains I and II were cultivated aerobically at 37 °C by rotating at 200 rpm until the optical density (OD_{600}) reached 0.8 to 1.0, and then cultivated at 20 °C and rotated at 100 rpm with 1.0 mM IPTG for a further 20 h to induce the expression of heterologous proteins. The cells were harvested and disrupted by sonication. The supernatants were obtained by centrifugation at 14,000×*g* for 20 min and used as cell-free extracts for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis and enzymatic activity assays. For the detection of membrane proteins, the cell lysate of Strains I and II was centrifuged at 200,000×*g* for 90 min. The precipitate was suspended by 1 % DDM (*n*-Dodecyl-β-D-maltoside) and extracted overnight at 4 °C. The suspension was used for SDS-PAGE analysis.

The DPE activity was analyzed by determining the amount of D-psicose obtained from D-fructose [28]. The activity of RDH and FDH was determined spectrophotometrically by monitoring the change in A_{340} upon oxidation or reduction of NADH at room temperature [20]. Protein concentrations were determined by the Lowry method.

Whole-cell biotransformation

The engineered *E. coli* strains were harvested and washed twice with 50 mM Tris–HCl buffer (pH 7.0). The washed cells were suspended in 50 mM Tris–HCl buffer (pH 7.0) containing 100, 200, 300, and 500 mM D-fructose, and 200, 400, 600, and 1,000 mM sodium formate, respectively. The cell concentrations were 7, 14, 21, and 28 g/l (corresponding to OD_{600} = 20, 40, 60, and 80, respectively). The conversion experiment was performed at 40 °C by rotating at 200 rpm. Samples were obtained at different times to determine the allitol yield until the reaction time reached 18 h. The biomass, temperature, and pH were investigated as factors that may affect allitol yield. All trials were performed in triplicate.

Quantification of D-fructose and allitol

To determine the concentrations of D-fructose and allitol, the reaction products of the biotransformation experiments were subjected to high-performance liquid chromatography with a reflective index detector. The samples were filtered through 0.22 μm membrane filters and then injected onto two tandem gel pack GL-C611 columns (Hitachi Chemical, Japan) at 60 °C, using 0.1 mM NaOH as the mobile phase. The flow rate was 0.6 ml/min.

Results and discussion

Two key genes for allitol production

In the multi-enzyme coupling pathway for allitol production, the enzyme converting D-fructose to D-psicose is the most important. As reported, D-tagatose 3-epimerase or D-psicose 3-epimerase (DTE/DPE) catalyze the epimerization reaction between D-fructose and D-psicose [2, 5, 25]. In our previous work, a metal-independent DPE from *Ruminococcus* sp. was cloned and overexpressed in *E. coli*. It was shown that the DPE from *Ruminococcus* sp. was the most thermostable among all reported DTE/DPEs. Because of the high stability and activity, this DPE was used in large-scale production of D-psicose, which was produced at 125 from 500 g/l D-fructose using a small amount of DPE (1.2 U/ml) in a short period (3 h) [28].

The other key enzyme is ribitol dehydrogenase, which converts D-psicose to allitol. In our previous study, *Klebsiella oxytoca* CGMCC7662 was isolated from soil samples and used for allitol production from D-psicose [4]. The *rdh* gene of this strain was cloned and overexpressed by IPTG induction. SDS-PAGE analyses on the extract of *E. coli* harboring the *rdh* gene showed the presence of a protein around 26 kDa, which is in agreement with the predicted molecular mass for the RDH. The recombinant RDH was purified by affinity chromatography.

The purified RDH showed reduction activity toward D-psicose, and exhibited an optimum activity at 40 °C and pH 8.0 (Fig. 2a,b). The thermal stability was investigated by exposing the enzyme in Tris–HCl buffer (pH 8.0) to temperatures of 40, 45, and 50 °C for different time intervals. RDH was found to be stable at 40 °C, but the stability decreased rapidly at higher temperatures (Fig. 2c). The half-life of RDH at 40, 45. and 50 °C was 601.8, 88.5, and 15.7 min, respectively.

Construction of the allitol production pathway in *E. coli*

To construct the allitol production pathway in *E. coli*, the *dpe* and *rdh* gene, which are responsible for epimerization between D-fructose and D-psicose and reduction from D-psicose to allitol, were transformed into the *E. coli* BL21 Star strain. The *fdh* gene, the product of which maintained a continuous NADH supply, was transformed into the strain simultaneously and formed the cofactor recycling system with the *rdh* gene. Strain I containing the recombinant plasmids of pET-*dpe* and pCDF-*rdh-fdh* was obtained and induced the expression of heterologous proteins . SDS-PAGE analysis showed that the cell lysate of Strain I gave three distinct protein bands, corresponding to DPE, RDH, and FDH proteins (Fig. 3a, lane 1, 2).

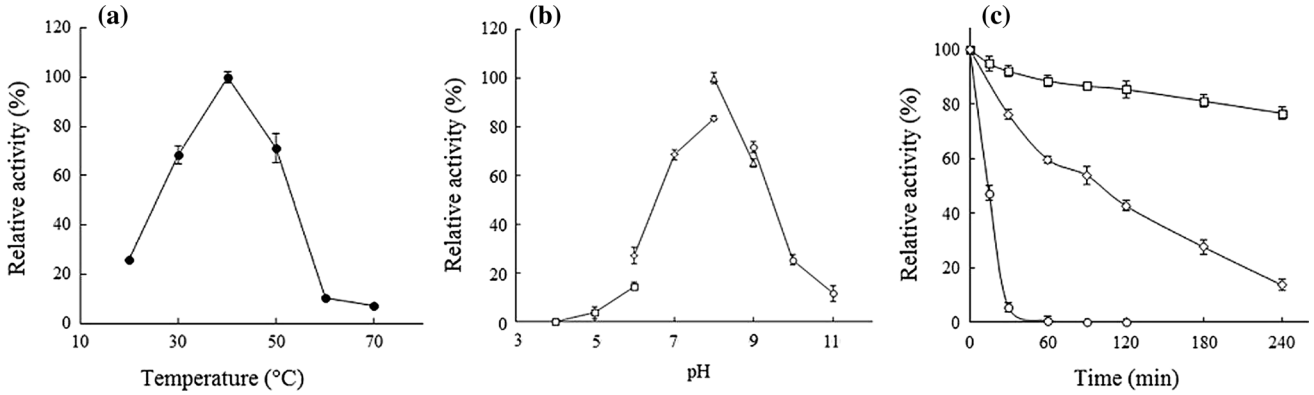

Fig. 2 Effect of temperature (**a**) and pH (**b**) on RDH activity and the thermal stability of the enzyme (**c**). Mean and standard deviation (*error bars*) were calculated based on triplicate experiments. Symbols **b** *square*, sodium acetate buffer; *diamond* sodium phosphate buffer; *triangle* Tris–HCl buffer; *circle* glycine-NaOH buffer. Symbols **c** *square* 40 °C; *diamond* 45 °C; *circle* 50 °C

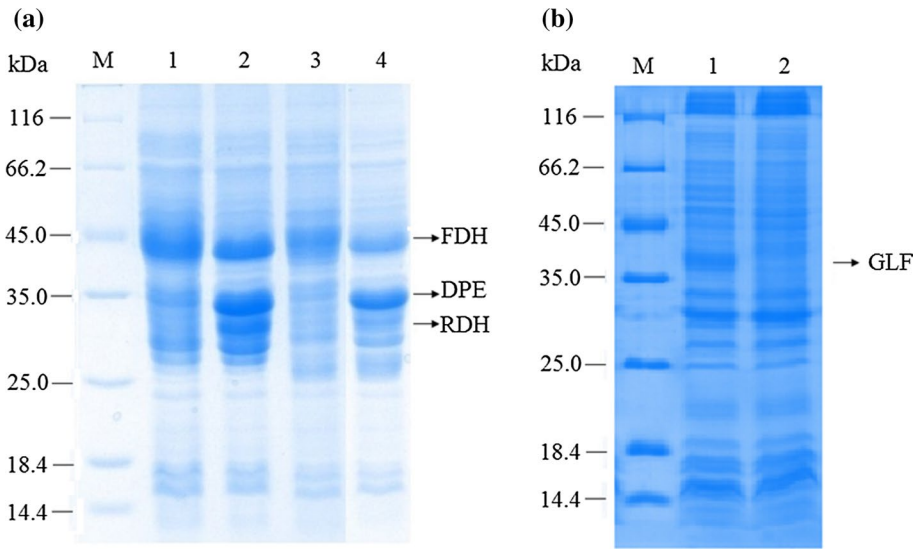

Fig. 3 SDS-PAGE analysis of the co-expression recombinant proteins. **a** The soluble recombinant proteins. M, protein marker; *lane 1* soluble proteins in the supernatant of strain I without IPTG; *lane 2* soluble proteins in the supernatant of Strain I with IPTG; *lane 3* soluble proteins in the supernatant of Strain II without IPTG; lane 4, soluble proteins in the supernatant of Strain II with IPTG. **b** The membrane proteins. M, protein marker; *lane 1* membrane proteins in the precipitate of Strain II; lane 2, membrane proteins in the precipitate of Strain I

Cell-free extracts of the recombinant Strain I were used for the enzymatic activity analysis, as described in the Materials and methods. The activities of DPE, RDH, and FDH were 6.0, 75, and 56 U/mg, respectively. As previously reported, when the epimerization reaction catalyzed by DTE/DPEs reaches its equilibrium, the relative content of D-psicose is no more than 30 % [13]. In the multi-enzyme coupling pathway, RDH and DPE were coexpressed, so that D-psicose was reduced to allitol under the role of RDH. Consequently, the reaction equilibrium was disrupted and more fructose was converted to D-psicose, which increased the utilization efficiency of the substrate.

FDH was added to the multi-enzyme coupling pathway to form the cofactor recycling system. As previously shown, non-growing *E. coli* cells may lose cofactors due to cell permeabilization caused by toxic agents, such as organic solvents or excess carbon dioxide [19]. Supplementation with a cofactor to the biotransformation reaction system could increase the specific productivity [8, 11, 23]. However, most cofactors are very expensive, resulting in increased production costs. Hence, the cofactor recycling system was constructed for cofactor NADH regeneration to ensure the presence of sufficient cofactor for ketohexose reduction. In this case, sodium formate was added into the reaction mixture together with the substrate, and thus, the cost for allitol production was significantly decreased.

Effect of *glf* overexpression on the conversion

The *glf* gene that encodes the glucose facilitator protein was overexpressed in the study to increase the D-fructose concentration in the cell. The plasmid pACYC184M-*glf* was constructed and transformed into Strain I. After induction by IPTG, three distinct protein bands of DPE, RDH, and FDH were observed in the SDS-PAGE analysis (Fig. 3a, lane 3, 4); however, the protein band of GLF, which is a

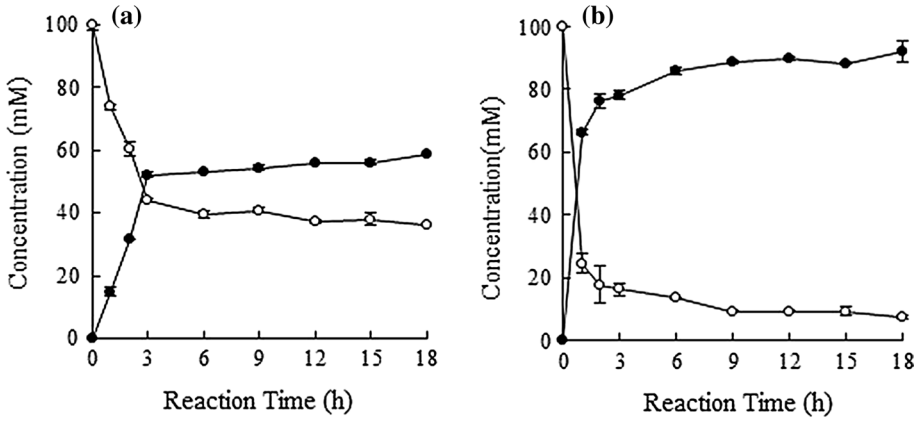

Fig. 4 Comparison of allitol production between Strain I (**a**) and Strain II (**b**). Mean and standard deviation (*error bars*) were calculated based on triplicate experiments. Symbols *circle* D-fructose; *filled circle* allitol. The initial concentration of D-fructose was 100 mM

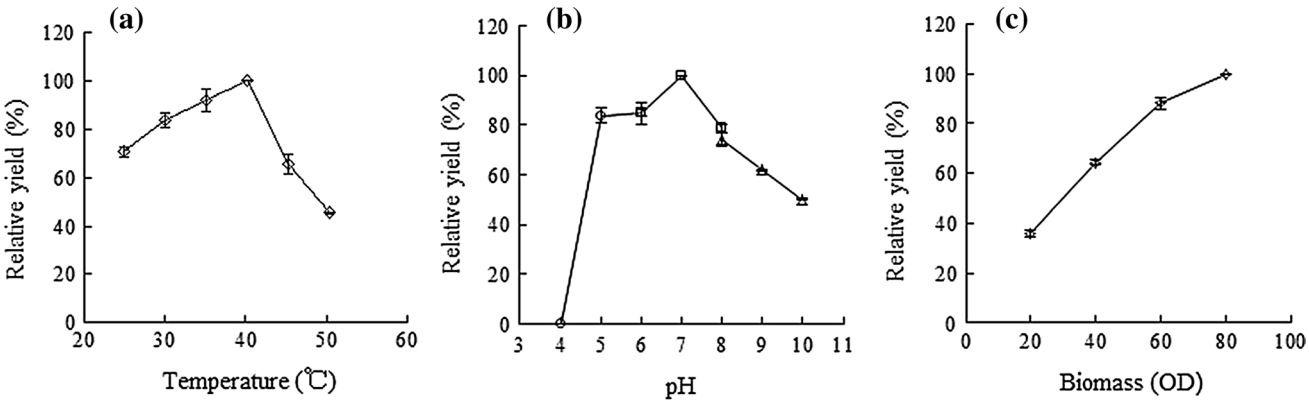

Fig. 5 Effect of temperature (**a**), pH (**b**), and cell biomass (**c**) on the allitol yield from Strain II. Mean and standard deviation (*error bars*) were calculated based on triplicate experiments. Symbols **b** *circle* sodium acetate buffer; *square* Tris–HCl buffer; *triangle* glycine−NaOH buffer

membrane protein, was not observed. Hence, the membrane proteins of Strain I and II were extracted and detected by SDS-PAGE. Different from Strain I, there was a unique protein band among the membrane proteins of Strain II (Fig. 3b). The molecular mass of GLF is ~52.1 kDa, but the protein band observed in the SDS-PAGE analysis was ~40 kDa. As reported, the gel shift is common for membrane proteins and originates from altered detergent binding [17].

The effect of GLF on the conversion reaction was analyzed by comparing Strain I and Strain II at the same substrate concentration (Fig. 4). When 100 mM D-fructose and 200 mM sodium formate were used as the substrate, the yield of allitol produced by Strain I and II after 18 h reaction was 10.62 and 16.53 g/l, respectively. This result indicated that the yield of allitol was increased due to the overexpression of GLF, which could transport more substrate into the cells.

As reported, D-fructose could be phosphorylated and then taken up by the phosphoenolpyruvate-dependent phosphotransferase (PTS) system in *E. coli* cells [12]. However, phosphorylated fructose cannot be converted to D-psicose by DPE [10]. Consequently, the phosphorylated fructose would be consumed by the cells. Hence, in the case of Strain I, the conversion rate of D-fructose was only 59 %, and part of the substrate was lost through metabolic consumption. The heterogenous expression of GLF from *Z. mobilis* accelerated the rate of uptake of D-fructose in the non-phosphorylated form. Thus, more fructose was converted to D-psicose, rather than being consumed by the strain. The result indicated that the conversion rate by Strain II was increased to 92 % thereby the yield of allitol was 1.6-fold higher than that observed using Strain I.

Optimization of biotransformation conditions

In order to maximize the allitol yield, different biotransformation conditions, such as temperature, pH value, and cell biomass were optimized using Strain II. To determine the effect of temperature on allitol yield, the reaction was carried out at various temperatures (25, 30, 35, 40, 45, and 50 °C), and the relative yields of allitol were calculated. As the temperature increased, the yield of allitol increased until the temperature reached 40 °C (Fig. 5a). The optimum

temperatures for DPE [28] and RDH (Fig. 2a) were 60 and 40 °C, respectively. Moreover, RDH was stable at 40 °C, but the stability dramatically decreased with increasing temperature. Hence, the stability of RDH was the key factor of the whole multi-enzyme coupling system. The optimal temperature for allitol production using whole-cell biotransformation was 40 °C, which is the same as the optimum temperature for RDH.

To determine the effect of pH on allitol yield, the reaction was carried out at various pH values (4.0, 5.0, 6.0, 7.0, 8.0, 9.0, and 10.0). The results indicated that the highest yield of allitol occurred at pH 7.0 in 50 mM Tris–HCl buffer (Fig. 5b). This pH value is lower than the optimum pH (pH 8.0) for DPE [28] and RDH (Fig. 2b), which may arise from the steady increase in pH due to the level of formic acid in the reaction mixture gradually reducing during transformation [9].

For optimization of cell biomass, the resting cells at different biomasses (OD_{600} = 20, 40, 60, and 80) were prepared and used for allitol production. As shown in Fig. 5c, the allitol yield increased as the cell biomass increased from OD_{600} = 20 to OD_{600} = 80, and the allitol yield at OD_{600} = 80 was 2.5-fold that measured at an OD_{600} = 20. However, the rate of increase of the allitol yield did not increase linearly. When the cell biomass reached OD_{600} = 60, the rate of increase dropped. Taking into consideration cost factors, we selected a cell biomass of OD_{600} = 60 as the most suitable biomass for allitol production.

Allitol production at different substrate concentrations

To determine the effect of substrate concentration on the yield of allitol, different initial substrate concentrations were used for the biotransformation under the optimal conditions found. The allitol yields at different initial substrate concentrations were 16.53, 32.33, 42.04, and 48.63 g/l when the concentrations of D-fructose and sodium formate were increased from 100–500 to 200–1,000 mM, respectively (Fig. 6). The biotransformation reaction reached equilibrium after 9 h at low concentrations of D-fructose (100 and 200 mM). However, it took 12 and 15 h to reach equilibrium when the concentration of the D-fructose was 300 and 500 mM, respectively.

Moreover, at low initial substrate concentrations (100 and 200 mM), almost all D-fructose was converted into allitol without any intermediate accumulation, and only a small amount of the D-fructose was consumed by the *E. coli* cells. However, at high substrate concentrations (500 mM), only half of the D-fructose was converted into allitol and the accumulation of D-psicose was observed (Fig. S1). This was caused by the stability of RDH, which has a half-life of ~10 h at 40 °C. The majority of the RDH enzyme had lost its activity after a 15 h reaction, thus suspending the conversion of D-psicose to allitol. Since the epimerization reaction between D-fructose and D-psicose had already reached its equilibrium, D-fructose was no longer converted.

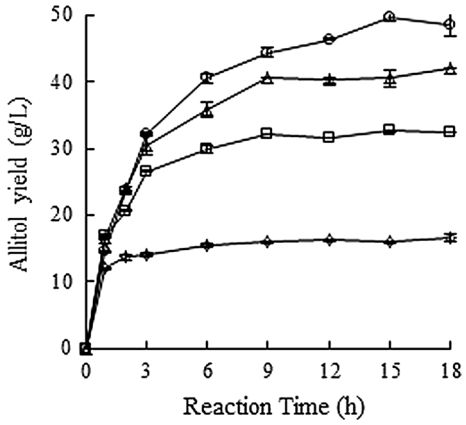

Fig. 6 Effect of the initial D-fructose concentration on the allitol yield from Strain II. Mean and standard deviation (*error bars*) were calculated based on triplicate experiments. Symbols *diamond* 100 mM D-fructose and 200 mM sodium formate; *square* 200 mM D-fructose and 400 mM sodium formate; *triangle* 300 mM D-fructose and 600 mM sodium formate; *circle* 500 mM D-fructose and 1,000 mM sodium formate

Whole-cell biotransformation has several advantages over an extracellular enzyme reaction system. (1) The reduction reaction from D-psicose to allitol catalyzed by RDH requires a cofactor, NADH, which is very expensive and restricts the use of the enzyme in industrial processes. In the case of allitol production via whole-cell biotransformation, NADH in *E. coli* cells was used as a cofactor and regenerated by FDH. Only the addition of sodium formate was required for the reaction system. However, a small amount of NADH was required when allitol was produced by an extracellular enzyme coupling transformation [18]. (2) The whole-cell biotransformation overcomes the problems that exist in the extracellular enzyme reaction system, such as complex steps of enzyme extraction and difficulty in product purification. After the reaction, the allitol was isolated from the reaction mixture by centrifugation or filtration and desalination with ion exchange resins. The step of removing proteins and cofactors may be omitted. (3) In order to further improve the yield of allitol, GLF was coexpressed and the presence of GLF in the reaction was significant. The maximum yield of allitol produced by whole-cell biotransformation reached up to 48.62 g/l, which is the highest among all reports describing allitol production. In contrast, the maximum yield of allitol produced by an extracellular enzyme coupling transformation was only 10 g/l [18].

The allitol produced through whole-cell biotransformation was purified with a column (2.0 × 100.0 cm) of

Dowex 50 W-X2 (Ca^{2+} form) [6]. The purified allitol was confirmed by HPLC, ^{13}C NMR analysis, and optical rotation measurements. The HPLC profile showed the retention time of the product was indistinguishable from authentic allitol. The ^{13}C NMR spectrum of the product was identical to that of authentic allitol (Fig. S2). The specific optical rotation of the product was +0.2 (20 °C, 1.0 % in H_2O), indicating a lack of optical activity of the product. Therefore, the product was identified as allitol.

Conclusions

In summary, we constructed a recombinant *E. coli* Strain II for the conversion of D-fructose to allitol. The enzymes DPE and RDH were coexpressed to form the multi-enzyme coupling pathway for allitol production, and FDH with RDH constituted the cofactor recycling system for NADH supply. To increase the intracellular concentration of D-fructose, the membrane protein GLF was introduced to the system, which enhanced the yield of allitol. Through optimization of the conditions, the maximum yield of allitol was 48.62 g/l using the whole-cell biotransformation system. Based on these results, Strain II shows enormous potential for application in the production of allitol.

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References

1. Bäumchen C, Bringer-Meyer S (2007) Expression of *glf* $_{Z.m.}$ increases D-mannitol formation in whole cell biotransformation with resting cells of *Corynebacterium glutamicum*. Appl Microbiol Biotechnol 76:545–552. doi:10.1007/s00253-007-0987-8
2. Chan HC, Zhu Y, Hu Y, Ko TP, Huang CH, Ren F, Chen CC, Ma Y, Guo RT, Sun Y (2012) Crystal structures of D-psicose 3-epimerase from *Clostridium cellulolyticum* H10 and its complex with ketohexose sugars. Protein Cell 3:123–131. doi:10.1007/s13238-012-2026-5
3. Chandrasekhar B (2010) Synthesis of some monocyclic and bicyclic polyhydroxy nitrogen heterocyclic compounds (Azasugars). PhD Thesis, Indian Institute of Chemical Technology, Hyderabad
4. Han W, Zhu Y, Men Y, Yang J, Liu C, Sun Y (2014) Production of allitol from D-psicose by a novel isolated strain of *Klebsiella oxytoca* G4A4. J Basic Microbiol 53:1–7. doi:10.1002/jobm.201300647
5. Ishida Y, Kamiya T, Itoh H, Kimura Y, Izumori K (1997) Cloning and characterization of the D-tagatose 3-epimerase gene from *Pseudomonas cichorii* ST-24. J Ferment Bioeng 83:529–534. doi:10.1016/S0922-338X(97)81132-4
6. Itoh H, Sato T, Izumori K (1995) Preparation of D-psicose from D-fructose by immobilized D-tagatose 3-epimerase. J Ferment Bioeng 80:101–103. doi:10.1016/0922-338X(95)98186-O
7. Izumori K (2006) Izumoring: a strategy for bioproduction of all hexoses. J Biotechnol 124:717–722. doi:10.1016/j.jbiotec.2006.04.016
8. Kataoka M, Yamamoto K, Kawabata H, Wada M, Kita K, Yanase H, Shimizu S (1999) Stereoselective reduction of ethyl 4-chloro-3-oxobutanoate by *Escherichia coli* transformant cells coexpressing the aldehyde reductase and glucose dehydrogenase genes. Appl Microbiol Biotechnol 51:486–490. doi:10.1007/s002530051421
9. Kaup B, Bringer-Meyer S, Sahm H (2004) Metabolic engineering of *Escherichia coli*: construction of an efficient biocatalyst for D-mannitol formation in a whole-cell biotransformation. Appl Microbiol Biotechnol 64:333–339. doi:10.1007/s00253-003-1470-9
10. Kim HJ, Hyun EK, Kim YS, Lee YJ, Oh DK (2006) Characterization of an *Agrobacterium tumefaciens* D-psicose 3-epimerase that converts D-fructose to D-psicose. Appl Environ Microbiol 72:981–985. doi:10.1128/AEM.72.2.981-985.2006
11. Kizaki N, Yasohara Y, Hasegawa J, Wada M, Kataoka M, Shimizu S (2001) Synthesis of optically pure ethyl(*S*)-4-chloro-3-hydroxybutanoate by *Escherichia coli* transformant cells coexpressing the carbonyl reductase and glucose dehydrogenase genes. Appl Microbiol Biotechnol 55:590–595. doi:10.1007/s002530100599
12. Kornberg HL, Lambourne LTM, Sproul AA (2000) Facilitated diffusion of fructose via the phosphoenolpyruvate/glucose phosphotransferase system of *Escherichia coli*. Proc Natl Acad Sci USA 97:1808–1812. doi:10.1073/pnas.97.4.1808
13. Mu W, Zhang W, Feng Y, Jiang B, Zhou L (2012) Recent advances on applications and biotechnological production of D-psicose. Appl Microbiol Biotechnol 94:1461–1467. doi:10.1007/s00253-012-4093-1
14. Muniruzzaman S, Kunihisa Y, Ichiraku K, Izumori K (1995) Purification and characterization of a ribitol dehydrogenase from *Enterobacter agglomerans* strain 221e. J Ferment Bioeng 79:496–498. doi:10.1016/0922-338X(95)91269-B
15. Muniruzzaman S, Tokunaga H, Izumori K (1995) Conversion of D-psicose to allitol by *Enterobacter agglomerans* strain 221e. J Ferment Bioeng 79:323–327. doi:10.1016/0922-338X(95)93989-W
16. Parker C, Barnell WO, Snoep JL, Ingram LO, Conway T (1995) Characterization of the *Zymomonas mobilis* glucose gacilitator gene product (*glf*) in recombinant *Escherichia coli*: examination of transport mechanism, kinetics and the role of glucokinase in glucose transport. Mol Microbiol 15:759–802. doi:10.1111/j.1365-2958.1995.tb02350.x
17. Rath A, Glibowicka M, Nadeau VG, Chen G, Deber CM (2009) Detergent binding explains anomalous SDS-PAGE migration of membrane proteins. Proc Natl Acad Sci USA 106:1760–1765. doi:10.1073/pnas.0813167106
18. Takeshita K, Ishida Y, Takada G, Izumori K (2000) Direct production of allitol from D-fructose by a coupling reaction using D-tagatose 3-epimerase, ribitol dehydrogenase and formate dehydrogenase. J Biosci Bioeng 90:545–548. doi:10.1016/S1389-1723(01)80038-4
19. Walton AZ, Stewart JD (2004) Understanding and improving NADPH-dependent reactions by nongrowing *Escherichia coli* cells. Biotechnol Prog 20:403–411. doi:10.1021/bp030044m
20. Wang Q (2006) Metabolic engineering of *Escherichia coli* for high yield of succinic acid. Ph.D. Dissertation, Tianjin University, Tianjin, China
21. Weisser P, Krämer R, Sahm H, Sprenger GA (1995) Functional expression of the glucose transporter of *Zymomonas mobilis* leads to restoration of glucose and fructose uptake in *Escherichia coli* mutants and provides evidence for its facilitator action. J Bacteriol 177:3351–3354

22. Wu J, Du G, Zhou J, Chen J (2013) Metabolic engineering of *Escherichia coli* for (2*S*)-pinocembrin production from glucose by a modular metabolic strategy. Metab Eng 16:48–55. doi:10.1016/j.ymben.2012.11.009
23. Yamamoto H, Matsuyama A, Kobayashi Y (2003) Synthesis of ethyl (*S*)-4-chloro-3-hydroxybutanoate using fabG-homologues. Appl Microbiol Biotechnol 61:133–139. doi:10.1007/s00253-002-1188-0
24. Yang J, Zhu Y, Li J, Men Y, Sun Y, Ma Y (2014) Biosynthesis of rare ketoses through constructing a recombination pathway in an engineered *Corynebacterium glutamicum*. Biotechnol Bioeng. doi:10.1002/bit.25345
25. Zhang L, Mu W, Jiang B, Zhang T (2009) Characterization of D-tagatose-3-epimerase from *Rhodobacter sphaeroides* that converts D-fructose into D-psicose. Biotechnol Lett 31:857–862. doi:10.1007/s10529-009-9942-3
26. Zhao J, Li Q, Sun T, Zhu X, Xu H, Tang J, Zhang X, Ma Y (2013) Engineering central metabolic modules of *Escherichia coli* for improving β-carotene production. Metab Eng 17:42–50. doi:10.1016/j.ymben.2013.02.002
27. Zhou P, Li S, Xu H, Feng X, Ouyang P (2012) Construction and co-expression of plasmid encoding xylitol dehydrogenase and a cofactor regeneration enzyme for the production of xylitol from D-arabitol. Enzyme Microb Tech 51:119–124. doi:10.1016/j.enzmictec.2012.05.002
28. Zhu Y, Men Y, Bai W, Li X, Zhang L, Sun Y, Ma Y (2012) Overexpression of D-psicose 3-epimerase from *Ruminococcus* sp. in *Escherichia coli* and its potential application in D-psicose production. Biotechnol Lett 34:1901–1906. doi:10.1007/s10529-012-0986-4