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Research Article

Allitol bioproduction by recombinant *Escherichia coli* with NADH regeneration system co-expressing ribitol dehydrogenase (RDH) and formate dehydrogenase (FDH) in individual or in fusion

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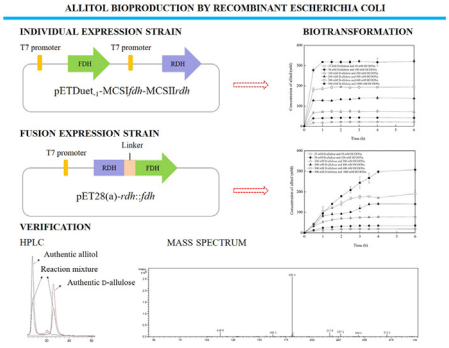
GRAPHICAL ABSTRACT



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ABSTRACT

Background: As a kind of rare sugar alcohol, allitol has important application values in food and medication. In addition, it can be used as a key substrate to produce other D/L-rare sugars. Allitol can be effectively produced by the resting-cell biotransformation method.
Results: Two recombinant *Escherichia coli* strains, one simultaneously expressing ribitol dehydrogenase (RDH) and formate dehydrogenase (FDH) in fusion (fusion expression strain for short) and the other expressing the above two enzymes individually (individual expression strain for short), were respectively constructed and used for allitol bioproduction. The produced allitol was confirmed by HPLC, mass spectrometry, and polarimetry. The individual expression strain had higher activity, which produced 58.5 g/L allitol from 90 g/L D-allulose (also named D-psicose) in 1 h with an allitol productivity of 58.5 g/L/h under optimized conditions.
Conclusions: The constructed individual expression strain had the highest allitol productivity among the reports. The production process developed in this study was simple, highly efficient, and had the potential for mass production of allitol.
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1. Introduction

Allitol is a six-carbon rare sugar alcohol that can be used as a sweetener and a bulking agent in the food industry [1]. Allitol also has important physiological functions. It can increase the water content in the small intestine and promote small intestinal transport so as to have a laxative effect and is used in the preparation of monosaccharide laxatives for the treatment of constipation [2]. Allitol is an important intermediate in the synthesis of azasugars, which can be used in the preparation of drugs against diabetes, cancer, and viral infections including AIDS [1]. Furthermore, allitol can be used as a substrate to produce other D/L-type rare sugars, such as D/L-allulose (also named D/L-psicose), according to Izumoring strategy [3,4,5].

Allitol is rare in nature and difficult to obtain from natural resources. It can be chemically synthesized, but has a low yield and toxic byproducts [1,6]. In contrast, the biotransformation method is more suitable as it has many advantages, such as mild reaction conditions, no side products, low pollution to the environment, low cost, and high yield, etc. Allitol can be biotransformed from D-allulose byribitol dehydrogenase (RDH) consuming NADH. The expensive coenzyme, NADH, plays an important role in oxidoreductase reactions, and can be regenerated by using enzyme or microbial technology [7,8,9,10]. In this research, formate dehydrogenase (FDH) is used for NADH regeneration, utilizing formate in the production of allitol (Fig. 1). As a coenzyme regeneration system, formate dehydrogenase has the following advantages: first, the reduction reaction catalyzed by formate dehydrogenase is irreversible; second, the required substrate formate is cheap and has little negative effect on the enzyme activity; and third, the by-product of carbon dioxide (CO_2) produced has little negative effect on the enzyme activity, and is easily spilled from the reaction system, which simplifies the product separation and purification [11].

Allitol bioproduction from D-allulose by using enzymatic catalysis and resting-cell biotransformation methods has been reported [12,13,14,15]. But the production was too low for real applications. In natural cells, enzymes are arranged in order rather than distributed randomly in the cell space. This ordered arrangement makes the enzyme molecules of the same metabolic pathway close to each other to overcome the diffusion limit of intermediate metabolites and the crosstalk of intermediate products of different metabolic pathways. Co-expressing RDH and FDH in an individual is a normal way, but in which expression mode the expressed RDH and FDH enzyme molecules could be far from each other inside the host cell and the intermediate metabolite of one enzyme needs to diffuse to the other enzyme to finish one reaction cycle, as shown in Fig. 1. The diffusion of the intermediate metabolite may also interfere with the metabolism of the host cells. Fusion expression is a simple method of enabling two recombinant proteins close to each other. Thus, fusion expression of RDH and FDH was investigated in this research and compared with the individual expression strain.

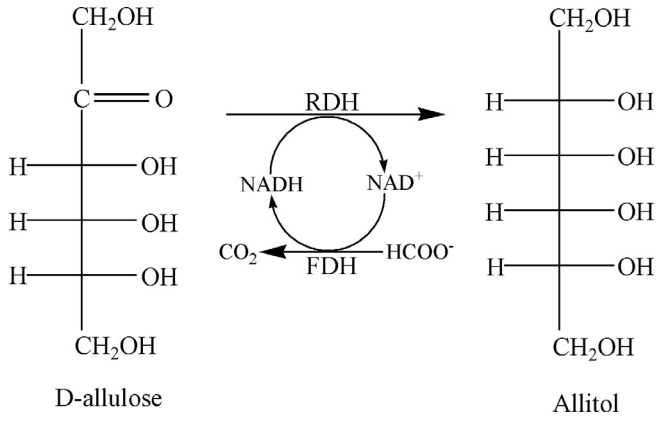

Fig. 1. Conversion diagram of allitol from D-allulose by using RDH and FDH with NADH regeneration. RDH, ribitol dehydrogenase; FDH, formate dehydrogenase.

Even if the substrate of D-allulose is a rare sugar, it can be produced from the cheap substrate D-fructose or D-glucose by some labs, including ours [16,17,18,19,20,21,22,23,24]. Therefore, D-allulose was used in the bioproduction of allitol in this research. According to the reports, the RDH from *Providencia alcalifaciens* RIMD 1656011 had high specificity and almost no by-products in catalyzing D-allulose into allitol [1,13], and was therefore used in this research. The consumed NADH in allitol production can be regenerated by FDH [25,26]. The FDH from *Pseudomonas* sp. 101 was reported to be stable [27] and was used in this research. Two gene expression strategies were used in constructing the recombinant strains co-expressing RDH and FDH in individual or fusion and the conditions for biocatalyst preparation and allitol biotransformation were optimized. Finally, 58.5 g/L of allitol was produced from 90 g/L of D-allulose, consuming 68 g/L of sodium formate for NADH regeneration by using the individual expression strains as biocatalysts. Allitol production was the highest among the reports, and the production process was simple and practical for large-scale production of allitol.

2. Materials and methods

2.1. Reagents, strains, plasmids, and primers

Allitol was purchased from TCI Development (Shanghai, China). Sodium formate was purchased from Macklin (Shanghai, China). D-Allulose was produced by our lab [17].The bacterial strains, plasmids, and primers used in this study are listed in Table 1.

2.2. Construction of recombinant E. coli strains expressing RDH or RDH and FDH

The only RDH-expressing recombinant strain named *E. coli* BL21 (DE3)-pETDuet$_{-1}$-MCSII*rdh* was obtained by inserting the pETDuet$_{-1}$-MCSII*rdh* into the competent cell *E. coli* BL21 (DE3). The recombinant strain named *E. coli* BL21 (DE3)-pET28(a)-*rdh, which* only expresses RDH, was constructed as follows: the *rdh* fragment was cloned by using pETDuet$_{-1}$-MCSII*rdh* as the template and *rdh*-pET28(a)-*Nco*I-U and *rdh*-pET28(a)-*Xho*I-D as the primers; and the cloned *rdh* gene fragment was then inserted into pET28(a) using *Nco*I and *Xho*I restriction sites; and finally, the obtained plasmid named pET28(a)-*rdh* was transformed into *E. coli* BL21 (DE3) to construct *E. coli* BL21 (DE3)-pET28(a)-*rdh*. The primers used in this study are listed in Table 1.

In constructing the recombinant strain that expresses the fused RDH and FDH named *E. coli* BL21 (DE3)-pET28(a)-*rdh::fdh* (fusion expression strain for short), the *rdh* fragment without TAA at C-terminus was cloned by using pETDuet$_{-1}$-MCSII*rdh* as the template and r*rdh*-pET28(a)-*Nco*I-U and r*rdh*-pET28(a)-D as the primers. Likewise, the *fdh* fragment was cloned by using pETDuet$_{-1}$-MCSII*fdh* as the template and r*fdh*-pET28(a)-U (containing linker: GGATCTGGC) and r*fdh*-pET28(a)-*Xho*I-D as the primers. Then, the cloned *rdh* and *fdh* fragments were used as the common templates to clone the fusion gene fragment *rdh*::*fdh* by using r*rdh*-pET28(a)-*Nco*I-U and r*fdh*-pET28(a)-*Xho*I-D as the primers. Finally, the *rdh*::*fdh* fusion gene fragment was inserted into pET28(a) by using *Nco*Iand *Xho*Irestriction sites. The obtained plasmid was named pET28(a)-*rdh*::*fdh* and was transformed into *E. coli* BL21 (DE3) to construct *E. coli* BL21 (DE3)-pET28(a)-*rdh*::*fdh*.

Table 1
Strains, plasmids, and primers used in this study.

Plasmids, strains and primers	Characteristics, sources and sequences	
Plasmids	**Relevant characteristics**	**Sources**
pET28(a)	Km^r, T7 promoter	Lab
pETDuet$_{-1}$	Amp^r, two multiple cloning sites	Lab
pETDuet$_{-1}$-MCSII*rdh*	*rdh*, Amp^r	Boshang (Jinan, China)
pETDuet$_{-1}$-MCSII*fdh*	*fdh*, Amp^r	Boshang (Jinan, China)
pET28(a)-*rdh*	*rdh*, Km^r	This study
pET28(a)-*rdh*::*fdh*	*rdh*::*fdh*, Km^r	This study
pETDuet$_{-1}$-MCSI*fdh*-MCSII*rdh*	*fdh*, *rdh*, Amp^r	This study
Strains	**Relevant characteristics**	**Sources**
E. coli DH5α	For gene cloning	TransGen Biotech (Beijing, China)
E. coli BL21 (DE3)	For gene expression	TransGen Biotech (Beijing, China)
E. coli BL21 (DE3)-pET28(a)	Containing pET28(a)	This study
E. coli BL21 (DE3)-pETDuet$_{-1}$	Containing pETDuet$_{-1}$	This study
E. coli BL21 (DE3)-pETDuet$_{-1}$-MCSII*rdh*	RDH	This study
E. coli BL21 (DE3)-pET28(a)-*rdh*	RDH	This study
E. coli BL21 (DE3)-pET28(a)-*rdh*::*fdh*	Fusion expression strain; RDH::FDH	This study
E. coli BL21 (DE3)-pETDuet$_{-1}$-MCSI*fdh*-MCSII*rdh*	Individual expression strain; RDH; FDH	This study
Primers	**Sequences (5′-3′)**	
rdh-pET28(a)-*Nco* I-U	CATG*CCATGG*CCATTAGCCTGGAAAATAAGGTGG	
rdh-pET28(a)-*Xho* I-D	CCG*CTCGAG*TTTAATGGTGATGATGATGATGCAGATCAACA CTATTCGGCAG	
r*rdh*-pET28(a)-*Nco* I-U	CATG*CCATGG*CCATTAGCCTGGAAAATAAGGTGG	
r*rdh*-pET28(a)-D	CAGATCAACACTATTCGGCAGAATAAC	
r*fdh*-pET28(a)-U	GTTATTCTGCCGAATAGTGTTGATCTGGGATCTGGCATGG CAAAAGTGCTGTGCGTGCTGTATG	
r*fdh*-pET28(a)-*Xho* I-D	CCG*CTCGAG*TTAATGGTGATGATGATGATGCACGGCTTTTT TGAATTTTGCTGCTTC	
fdh-pETDuet$_{-1}$MCSI-*Nco* I-U	CATG*CCATGG*CAAAAGTGCTGTGCGTGCTGT	
fdh-pETDuet$_{-1}$MCSI-*Not* I-D	AAGGAAAAAAG*CGGCCGC*CACGGCTTTTTTGAATTTTGCT GCTTCTTCGC	

Primers: restriction sites were indicated with italic and linker was indicated with underline.

In constructing the recombinant strain expressing RDH and FDH in an individual named *E. coli* BL21 (DE3)-pETDuet$_{-1}$-MCSI*fdh*-MCSII*rdh* (individual expression strain for short), the *fdh* fragment was cloned by using pETDuet$_{-1}$-MCSII*fdh* as the template, and *fdh*-pETDuet$_{-1}$MCSI-*Nco* I-U and *fdh*-pETDuet$_{-1}$MCSI-*Not* I-D as the primers. Then the *fdh* gene fragment was inserted into pETDuet$_{-1}$-MCSII*rdh* by using *Nco*I and *Not*I restriction sites to form the plasmid pETDuet$_{-1}$-MCSI*fdh*-MCSII*rdh*. Finally, the plasmid was transformed into *E. coli* BL21 (DE3) to construct *E. coli* BL21 (DE3)-pETDuet$_{-1}$-MCSI*fdh*-MCSII*rdh*.

2.3. Cultivation media and conditions

Seed culture was performed using 50 mL flasks containing 10 mLLB medium (1% tryptone, 0.5% yeast extract, and 1% NaCl) cultured at 37°C and 200 rpm for 12 h. Cell biocatalysts cultivation was performed using 500 mL flasks containing 100 mL of LBG medium (LB medium supplied with 5 g/L glucose) with 1 mL inoculation, started at 37°C and 200 rpm. After 3 h of cultivation, IPTG was added to the final concentration of 1 mM and the conditions changed to 28°C, 140 rpm, and continued the cultivation for a further 12 h. Kanamycin or ampicillin was added to the final concentration of 100 μg/mL in the initial medium.

2.4. Resting-cell biotransformation

The recombinant *E. coli* cells were harvested by centrifugation at 12,000 × g for 5 min and washed twice by using double distilled water. The washed cells were resuspended in a reaction solution containing 50 mM tris-HCl buffer, 50 mM D-allulose, and 100 mM sodium formate. The reaction was operated at 35°C (before temperature optimization) or the optimized value and pH 7.0 (before pH optimization), or the optimized value. All trials were performed in triplicates.

2.5. Analytical methods

Allitol was identified by using high performance liquid chromatography (HPLC), mass chromatography, and optical rotation. The qualitative and quantitative analyses of allitol and D-allulose were measured by using HPLC at a column temperature of 78°C and eluted with double distilled water at a flow rate of 0.5 mL/min using a Carbomix Pb-NP column (7.8 × 300 mm, 10 μm, Sepax Technologies). A mass spectrometry (BRUKER, Germany) was performed in negative ion detection mode with an ESI ion source. Around 5 μL allitol of10 μg/mL (dissolved in double distilled water) was injected and eluted at a flow rate of 0.4 mL/min. The optical rotation of allitol (5% in double distilled water) was determined by using a polarimeter (INESA, China) at 25°C.

3. Results

3.1. Expressionof proteins RDH, FDH, and RDH::FDH

For analyses of the expression of the recombinant proteins, the above two recombinant *E. coli* strains, *E. coli* BL21 (DE3)-pET28(a)-*rdh*::*fdh* or *E. coli* BL21 (DE3)-pETDuet$_{-1}$-MCSI*fdh*-MCSII*rdh,* and the two control *E. coli* strains containing empty plasmid pET28(a) or pETDuet$_{-1}$ were, respectively, cultivated in LB medium induced by IPTG for 5 h. The cells were harvested by centrifugation at 12,000 × g for 5 min, and the intracellular proteins were extracted for analyses. SDS-PAGE analyses of the total proteins from the *rdh*::*fdh* fusion expression strain showed a protein band around 71 kDa (Fig. 2A), and that of the *fdh* and *rdh* individual expression strain showed two protein bands around 45 kDa and 26 kDa, respectively (Fig. 2B). The RDH molecular weight was consistent with the report [13].

In order to verify that the expressed FDH had real function in the biotransformation, we compared the allitol yield produced at

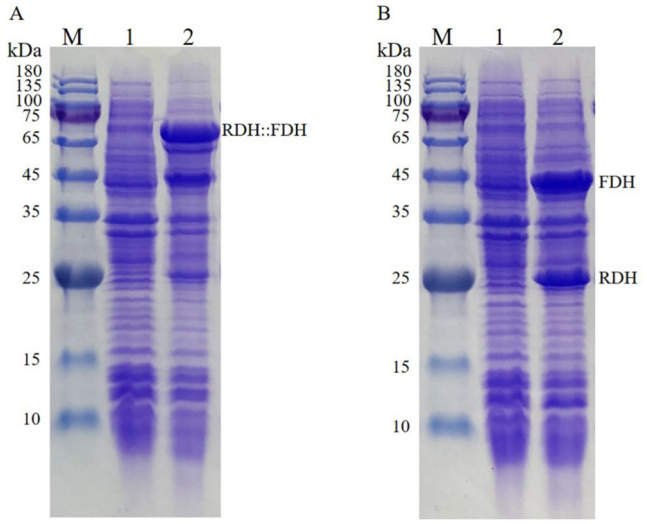

Fig. 2. SDS-PAGE analyses of fusion protein RDH::FDH (A) and individual expression proteins of FDH and RDH (B), respectively, expressed by *E. coli* BL21 (DE3). A: Lane M, protein marker; lane 1, the total proteins of *E. coli* BL21 (DE3)-pET28(a); lane 2, the total proteins of *E. coli* BL21 (DE3)-pET28(a)-*rdh*::*fdh*; B: Lane M, protein marker; lane 1, the total proteins of *E. coli* BL21 (DE3)-pETDuet$_{-1}$; lane 2, the total proteins of *E. coli* BL21 (DE3)-pETDuet$_{-1}$-MCSI*fdh*-MCSII*rdh*.

OD_{600} 40 by the co-expression strains to the control strains expressing only RDH. The results showed that allitol production increased by about 8.5 or 8.3 times by using the individual expression strain or fusion expression strain, respectively, compared with the corresponding controls (Fig. 3).

3.2. Identification of allitol

The allitol produced was purified by cooling crystallization and recrystallization before identification. The result of HPLC analysis shown in Fig. 4A showed that the retention time of allitol and D-allulose in the reaction mixture was the same as that of the authentic allitol and D-allulose. The result of the mass chromatography showed that the mass of the produced allitol was 182.1 from the mass spectrum (Fig. 4B, which was equal to the molecular weight of allitol and the report [14]. The result of the polarimetric analysis showed that the specific optical rotation of the produced allitol was 0.0°, as expected. As a result, the produced product was confirmed to be allitol.

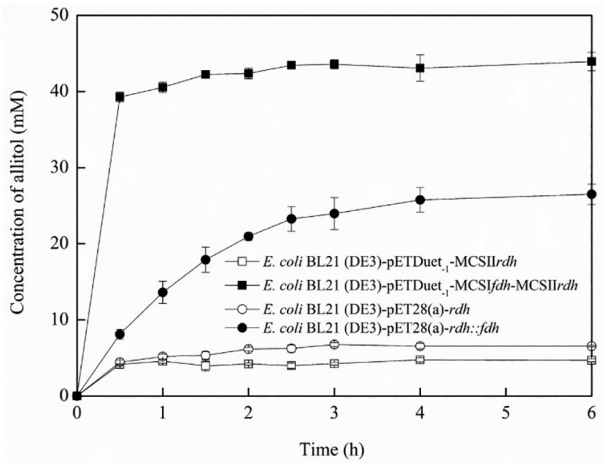

Fig. 3. The time courses of allitol production by using *E. coli* BL21(DE3)-pETDuet$_{-1}$-MCSI*fdh*-MCSII*rdh* or *E. coli* BL21(DE3)-pET28(a)-*rdh*::*fdh* compared with the controls *E. coli* BL21(DE3)-pETDuet$_{-1}$-MCSII*rdh* or *E. coli* BL21(DE3)-pET28(a)-*rdh*.

3.3. Optimization of medium carbon source for the cultivation of cell biocatalysts

The amounts of 1% (w/v) glucose, 1% (v/v) glycerol, and 1% glucose plus 1% glycerol were, respectively, added into the LB medium for cultivation of the catalytic cells, with the control without carbon source addition. After cultivation, cells were collected and allitol biotransformation was performed. The results indicated that the addition of carbon sources into the LB medium in the cultivation of the cells could increase allitol yield remarkably (Fig. 5A). The allitol yield reached its highest when 1% glucose or 1% glucose plus 1% glycerol were added into the cell cultivation medium. Taking into consideration the medium cost, only glucose was selected as the optimal carbon source. Then, glucose concentration was optimized using the individual expression strain, and the allitol yield was the highest when 5 g/L of glucose was added to the medium (Fig. 5B).

3.4. Optimization of pH, temperature, and cell mass for allitol biotransformation

The pH effect on allitol yield was investigated using four buffer systems of 50 mM, which were sodium acetate buffer (pH 4.0–6.0), sodium phosphate buffer (pH 6.0–8.0), tris-HCl buffer (pH 8.0–9.0), and glycine-NaOH buffer (pH 9.0–11.0), reacted at the optimal temperatures of 45°C for the fusion expression strain and 50°C for the individual expression strain, respectively. The results indicated that the highest allitol yield was obtained at pH 8.0 and pH 9.0 in 50 mM tris-HCl buffer for the fusion expression strain and individual expression strain, respectively (Fig. 6A).

The temperature effect on allitol production was investigated at various temperatures of 30, 35, 40, 45, 50, 55, and 60°C, respectively, using a reaction time of 0.5 h. The results showed that 45 and 50°C were optimal for the fusion expression strain and the individual expression strain, respectively (Fig. 6B).

The cell dosage effect on allitol yield was investigated at various cell concentrations with OD_{600} 20, 40, 60, 80, and 100, corresponding to dry cell weights of 11.7, 23.3, 34.9, 46.5, and 58.0 g/L for the fusion expression strain and 14.7, 29.4, 44.0, 58.7, and 73.4 g/L for the individual expression strain, respectively. As shown in Fig. 6C, the optimal cell concentrations were OD_{600} 40 and 80 for the individual expression strain and the fusion expression strain, respectively.

3.5. Optimization of D-allulose to sodium formate ratio for allitol biotransformation

The effect of D-allulose to sodium formate ratio (mM/mM) on allitol yield was investigated at ten values of 50:0, 50:1, 10:1, 5:1, 5:2, 5:4, 5:8, 1:2, 1:3, and 1:4, respectively, using the individual expression strain. The optimal D-allulose:sodium formate (mM/mM) ratio was 1:2 (Fig. 7A).

The effect of D-allulose concentration on allitol production of the two recombinant *E. coli* strains was investigated at six different D-allulose concentrations from 25 to 500 mM. For the fusion expression strain, the allitol concentration increased rapidly within the first 1 ∼ 4 h for all D-allulose concentrations (Fig. 7B), while the allitol yields decreased from 88.1 to 61.5% as the D-allulose concentration increased from 25 mM to 500 mM. For the individual expression strain, the allitol concentration increased rapidly within the first 0.5 ∼ 1 h, almost unchanged afterwards (Fig. 7C), and the

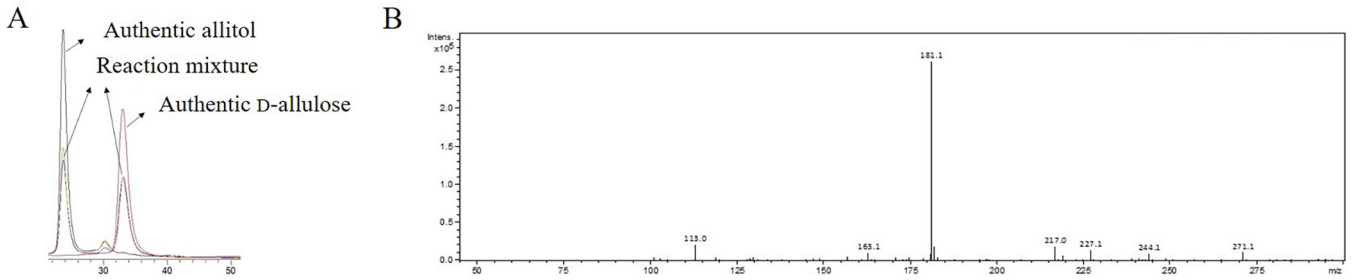

Fig. 4. HPLC analyses of authentic allitol, authentic D-allulose and the reaction mixture (A), and the mass spectrum of purified allitol product (B).

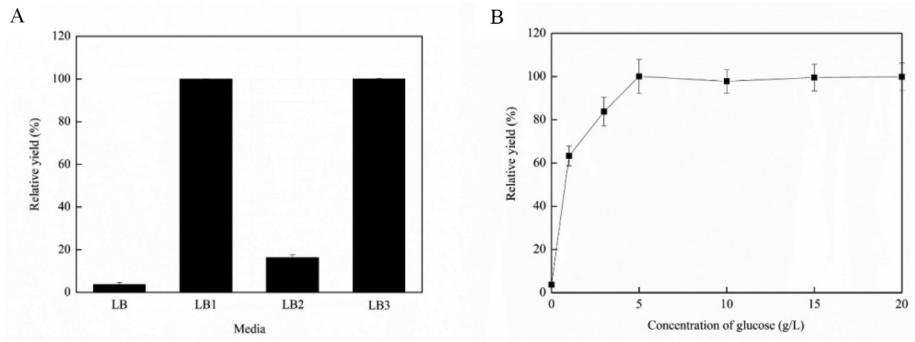

Fig. 5. The relative allitol yield obtained by the individual expression strain cultivated in LB medium supplied with various carbon sources (A) or at various glucose concentrations (B). LB1: LB medium containing 1% glucose; LB2: LB medium containing 1% glycerol; LB3: LB medium containing 1% glucose and 1% glycerol.

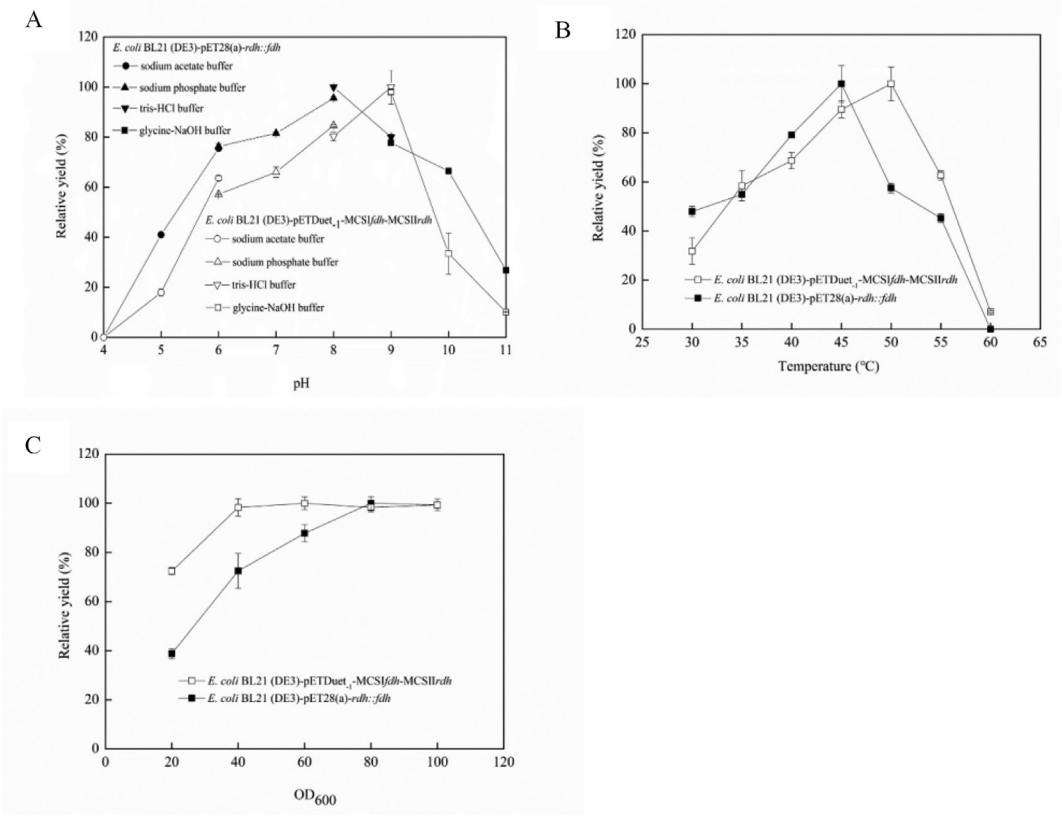

Fig. 6. Effects of pH (A), temperature (B) and cell concentration (C) on allitol relative yields for the fusion expression strain and individual expression strain.

allitol yields decreased from about 90.3 to 64.3% when the D-allulose concentration was increased from 25 mM to 500 mM. However, the cell concentration of the fusion expression strain used in the biotransformation was about twice that of the individual expression strain to achieve a similar allitol yield. Overall, the individual expression strain had higher allitol biotransformation

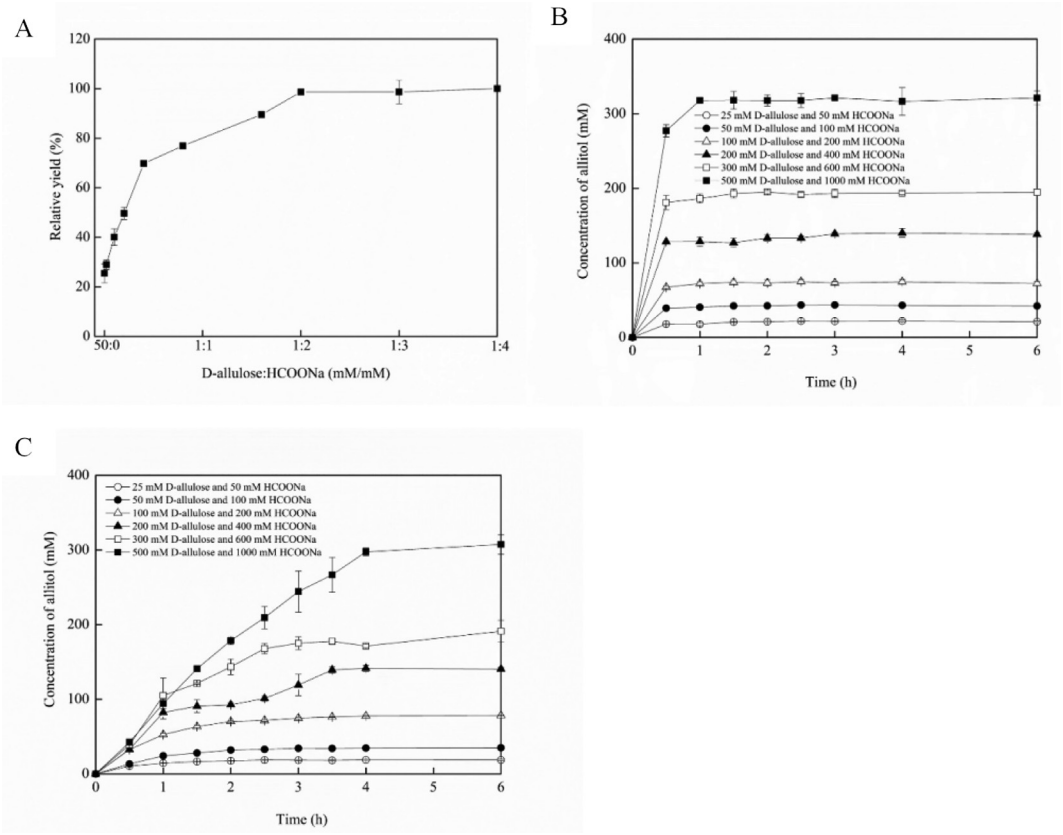

Fig. 7. Effects of D-allulose:HCOONa ratio (A) and the concentrations of D-allulose and HCOONa (B) on allitol relative yield of the individual expression strain at OD_{600} 40, and effects of the concentrations of D-allulose and HCOONa on allitol relative yield of the fusion expression strain at OD_{600} 80 (C).

activity. After 1 h of reaction, 58.5 g/L (321 mM) allitol and 6.5 g/L (36 mM) D-allulose remained in the reaction mixture with an allitol content of 90% (58.5/(58.5 + 6.5)) and a productivity of 58.5 g/L/h obtained at the conditions of OD_{600} 40, initial D-allulose and sodium formate concentrations of 90 and 68 g/L, respectively. Crystallization can be applied after concentration of the reaction mixture to obtain allitol crystals.

4. Discussion

In this study, two recombinant strains used for allitol production from D-allulose were constructed. The produced allitol was purified and successfully confirmed by HPLC, mass spectrometry, and polarimetry.

The carbon source in the medium for cultivation of individual expression strains was optimized and glucose was found more effective than glycerol to improve the biotransformation rate. The reason may be that the addition of a suitable carbon source in the cultivation of the biocatalysts can increase the intracellular NADH concentration and further increase the biotransformation rate, which was supported by the reports [28,29].

The fusion expression method has the advantage of channeling the intermetabolites inside the cells, in which case, the product of the first enzyme is the substrate of the second enzyme, and vice versa, so as to increase the local substrate concentrations and avoid diffusion inside the cells to overcome the crossover effects of the intermetabolites in interference with the host cell metabolism [30]. However, the fusion expression strain of RDH::FDH had lower activity than the individual expression strain. The reason could be that the linker peptide (-Gly-Ser-Gly-) used is not suitable for the fusion of ribitol dehydrogenase and formate dehydrogenase. This may lead to the independent deterioration of the two enzymes and may also lead to the misfolding and degradation of the fusion protein. In the future, other types of linker peptides, such as anti-protease degradation linker peptides that are rich in Pro-Thr, can be attempted [31,32,33,34]. In addition, the application of intracellular scaffold systems, including protein, DNA, and RNA scaffolds, which can realize co-regionalization of enzyme molecules, can also be tried to improve the fluidity of the protein, increase the local concentration of metabolites, and promote product synthesis [35,36,37,38].

It was reported that the optimum pH and the temperature stability of FDH from *Pseudomonas* sp.101 were 6.0–9.0 and 55°C, respectively [11,39]. The optimum pHs of the fusion expression strain (pH 8.0) and individual expression strain (pH 9.0) were all within the above pH range, while the optimum temperatures of the fusion expression strain (45°C) and individual expression strain (50°C) were lower than 55°C, which probably resulted from the RDH properties. Hassanin et al. reported that the optimum temperature and optimum pH of RDH from *Providencia alcalifaciens* RIMD 1656011 were 35°C and 10.0, respectively [13]. Taking into consideration the above facts, the optimum temperatures and pHs of the fusion expression strain (45°C, pH 8.0) and individual expression strain (50°C, pH 9.0) obtained in this study were reasonable. The imparity of the optimum temperature and optimum pH comes from the fusion expression strain and individual expression strain, which may be due to the different construction methods of the recombinant strains. In real applications, formic acid can be used in pH control in the production process to overcome the pH increase due to the consumption of formate and to provide the substrate for NADH regeneration as well [40]. Normally, sodium formate is chosen as the substrate for formate dehydrogenase (FDH) to realize the NADH regeneration. In order to boost the

Table 2
Allitol production from D-allulose by using enzymatic and whole cell biotransformation methods.

Biocatalyst	D-allulose (g/L)	NADH (NAD^+)	Temperature (°C)	pH	Time (h)	Productivity (g/L/h)	Reference
Enzymes							
RDH, FDH	10	+	30	8.0	48	0.2	[46]
RDH, FDH	10	+	40	7.5	6	1.4	[14]
Strains							
Enterobacter agglomerans Strain 221e	20	–	30	9.0	12	0.9	[29]
Klebsiella oxytoca G4A4	10	–	37	8.0	72	0.1	[12]
Engineered *E. coli*	20	–	30	7.0	12	0.7	[15]
Fusion expression strain	90	–	45	8.0	6	9.3	This study
Individual expression strain	90	–	50	9.0	1	58.5	This study

"+", NADH(NAD^+) was added into the reaction solution; "–", no NADH(NAD^+) was added into the reaction solution.

reaction with sufficient coenzyme NADH, the amount of sodium formate is usually excessive. Thus, the optimal D-allulose:sodium formate (mM/mM) ratio of 1:2 was achieved in this study.

Natural allitol mainly exists in Itea plants and *Tylopilus plumbeoviolaceus*. Only 14 g or 1.2 g of allitol can be extracted from 1 kg of fresh leaves of *Itea virginica* and 485 g fresh fruit of *T. plumbeoviolaceus,* respectively [41,42]. Allitol production by extraction from natural resources has the problems of consuming a large amount of raw materials, being harmful to the environment, and being expensive, which is not suitable for allitol's large-scale production. Allitol can also be chemically synthesized, and 11.3 g of allitol was obtained from the reaction mixture comprising 13 g of D-allose, 85 mL of water, and 4 g of sodium borohydride [1]. Although the chemical synthetic method has high efficiency, the substrate is also a rare sugar and is of high cost. Besides, the reaction process is complicated and produces by-products, making the product separation and purification difficult. Therefore, chemical synthesis is also not an ideal method. Table 2 shows the reported allitol productivities using the enzymatic catalysis and resting-cell biotransformation methods, respectively. As shown in Table 2, the allitol productivity of this study was up to 58.5 g/L/h without extra NADH/NAD^+ addition, which was much higher than the reports. Overall, allitol production by using biotransformation methods developed in this study is environment-friendly, high-yielding, and economical, which can overcome the disadvantages of extraction and chemical synthetic methods, and is suitable for commercial production of allitol. In addition, the allitol portion in the reaction mixture was as high as 90%, which was high enough to be used for crystallization without further purification so as to simplify the product purification process and decrease the production cost [43]. In the future, further optimization of the biotransformation process will be carried out according to the reaction kinetics of the catalytic process of allitol production [44,45].

5. Conclusions

In this research, the recombinant strain co-expressing RDH and FDH in individual or in fusion showed higher allitol productivity compared with the reports. Between them, the individual expression strain had higher activity and was the highest among the reports, which produced 58.5 g/L allitol from 90 g/L D-allulose in 1 h with an allitol productivity of 58.5 g/L/h under optimized conditions. The reaction mixture contained 90% allitol, which could be used in crystallization without further purification so as to simplify the production process and decrease the production cost. The method developed in this research is practical for large-scale allitol production.

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Conflict of interest

No conflict of interest associated with this work.

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