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ORIGINAL PAPER

High-level extracellular production of D-Psicose-3-epimerase with recombinant *Escherichia coli* by a two-stage glycerol feeding approach

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Abstract The aim of this study is to achieve high-level extracellular production of D-Psicose-3-epimerase (DPE) with recombinant *Escherichia coli*. High-level production of DPE is one of the key factors in D-Psicose production. In the present study, the gene AAL45544.1 from *Agrobacterium tumefaciens* str. C58 was modified by artificial synthesis for overexpression in *E. coli*. The total DPE activity reached 3.96 U mL^{-1} after optimization of the media composition, induction temperature, and concentration of inducer. Furthermore, it was found that addition of glycine had a positive effect on the extracellular production of DPE, which reached 3.5 U mL^{-1}. Finally, a two-stage glycerol feeding strategy based on both the specific growth rate before induction and the amount of glycerol residues after induction was applied in a 3-L fermenter. After a series of optimal strategies in the 3-L fermenter, the total and extracellular DPE activity were 5.08- and 3.11-fold higher than that noted in the shake flask. The extracellular and intracellular DPE activity reached 10.9 and 13.2 U mL^{-1}, achieving 25.5 and 31.1 % conversion of D-fructose to D-psicose, respectively. The systemic strategies presented in this study provide valuable novel information for the industrial application of DPE.

Keywords D-Psicose-3-epimerase · *Agrobacterium tumefaciens* · *Escherichia coli* · Extracellular expression · Two-stage glycerol feeding · Bioconversion

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Introduction

The International Society of Rare Sugars defines rare sugars as monosaccharides and their derivatives which rarely exist in nature [1–7]. According to this definition, D-Psicose is a rare sugar. Rare sugars have recently attracted much attention due to their various applications, including their use as low-calorie sweeteners and bulking agents, as immunosuppressants in allogenic orthotopic liver transplantation in rats, as potential inhibitors of various glycosidases, in ischemia reperfusion injury of rat liver, and in segmented neutrophil production without other detrimental clinical effects. Nowadays, rare sugars can be produced through microbial and enzymatic reactions with ketose epimerase, aldose isomerase, aldose reductase, and oxidoreductase. The bioproduction strategies for all rare sugars have been illustrated with ring-form structures [8].

D-Psicose, a carbon-3 epimer of D-fructose, is present in small quantities in commercial carbohydrate or agricultural

products. This rare sugar is poorly absorbed in the digestive tract [9] and hardly supplies energy for growth. D-Psicose is used as a sweetener aiding in weight reduction, and is found to repress the activity of hepatic lipogenic enzyme without producing any toxicity [1, 5, 10, 11]. Due to its outstanding advantage in health care, the conversion of D-fructose to D-Psicose using the D-tagatose-3-epimerase (DTE) family enzymes has been investigated for the commercial production of D-Psicose.

Currently, although the genes coding the DTE family enzymes occur in various microorganisms, only few studies have investigated the ability of DTE to convert D-fructose into D-Psicose. DTE from *Pseudomonas cichorii* was the first to be purified and characterized, and has been used for D-Psicose production [12]. Recently, Zhang et al. and Mu et al. [13, 14] characterized another two DTE family enzymes from *Rhodobacter sphaeroides* SK011 and *Clostridium cellulolyticum* H10. Furthermore, when compared with the above-mentioned DTE family enzymes, the one from *Agrobacterium tumefaciens* str. C58 has been found to specifically catalyze the interconversion of D-Fructose and D-Psicose, with a conversion yield of 32.9 %, and has been named D-Psicose-3-epimerase (DPE) on account of its high substrate specificity for D-Psicose [15].

Escherichia coli is a common host microorganism for heterologous protein expression. Although DPE has been successfully produced in the cytoplasm of *E. coli* [15], targeting secretion of the protein into the culture medium has several advantages such as facilitating downstream processing and achieving higher level expression. In the present study, extracellular production of DPE from *A. tumefaciens* str. C58 with highest substrate specificity [15, 16] in recombinant *E. coli* BL21 (DE3) was accomplished. A strategy was developed to increase the secretion of DPE by *E. coli* into the liquid medium in shake flasks, and the DPE activity in the medium was found to reach 3.5 U mL^{-1}. Furthermore, the DPE activity was investigated using synthetic medium. Different cultivation conditions, such as feeding strategies and induction methods, were examined in a 3-L fermenter to further promote DPE secretion into the culture medium. The results showed that a two-stage feeding strategy with complex nitrogen sources, such as peptone and yeast extract, could achieve high cell density culture overproducing DPE. When the culture with intermediate cell density of 30 g L^{-1} was induced by isopropylthio-β-galactoside (IPTG) at 25 °C, the extracellular DPE yield and extracellular DPE concentration were significantly enhanced (10.9 U mL^{-1} and 1,240 mg L^{-1}), achieving a 25.5 % conversion of D-fructose to D-Psicose after incubation of 700 g L^{-1} D-fructose for 100 min. Thus, it can be concluded that the strategies presented in this study are suitable for high cell density culture as well as DPE and D-Psicose production under ideal industrial conditions.

Materials and methods

Bacterial strain and plasmid

The DPE coding gene AAL45544.1 from *A. tumefaciens* str. C58 was modified by artificial synthesis for overexpression. *E. coli* JM109 (Invitrogen) was used as the host for gene cloning. *E. coli* BL21 (DE3) (Novagen) was used as the recombinant DPE production host. Plasmid pET22b (+) with a *pelB* leader sequence was employed for the construction of pET22b (+)/*pelb-DPE* and pET22b (+)/*DPE*.

Culture media

For shake flask cultivation, LB media supplemented with 100 μg mL^{-1} ampicillin were used. LB media consisted of (g L^{-1}): peptone 10, yeast extract 5, and NaCl 10. The modified TB medium in shake flask contained (g L^{-1}): glycerol 10, yeast extract 24, peptone 12, KH_2PO_4 17 mM, K_2HPO_4 72 mM, and ampicillin 1 mM. The modified TB medium in fermenter cultures was supplied 150 mM glycine at 24 h in the middle of the logarithmic phase. The feeding solutions contained (g L^{-1}): glycerol 500, yeast extract 50, and peptone 50. The SOB medium consisted of (g L^{-1}): peptone 20, yeast extract 5, NaCl 0.5, KCl 2.5 mM, and $MgCl_2$ 10 mM. SOC medium contained (g L^{-1}): peptone 20, yeast extract 5, NaCl 0.5, KCl 2.5 mM, MgCl2 10 mM, and glucose 20 mM. SB medium contained (g L^{-1}): peptone 30, yeast extract 20, and glucose 20.

Gene cloning and protein expression

To amplify the modified DPE coding gene AAL45544.1, the sense primer (5′-CCATGGATATGAAACACGGTAT CTACTATTCTT-3′) and the antisense primer (5′-CC GGAATTCTCAGCCACCCAGAACGA-3′) were used for the construction of pET22b (+)/*pelb-DPE*, while the sense primer (5′-CATATGATATGAAACACGGTATCTACTA TTCTT-3′) and the antisense primer (5′-CCGGAATTCT CAGCCACCCAGAACGA-3′) were used for the construction of pET22b (+)/*DPE*. PCR was conducted with PrimeSTAR® HS DNA Polymerase (Takara Biotechnology, Dalian, China). The amplified *pelb-DPE* DNA fragment obtained by PCR was purified and digested with *Nco*I and *Eco*RI endonucleases (Takara, Dalian, China). The digested *pelb-DPE* DNA fragment was cloned into the *Nco*I–*Eco*RI sites of pET22b (+) to generate pET22b (+)/*pelb-DPE*. The *DPE* DNA fragment was digested with *Nde*I and *Eco*RI. The digested *DPE* DNA fragment was cloned into the *Nde*I–*Eco*RI sites of pET22b (+) to generate pET22b (+)/*DPE*. Then the two plasmids were transformed into BL21 (DE3) and plated on LB agar containing 50 μg mL^{-1} ampicillin.

The *E. coli* BL21 (DE3) harboring pET22b (+)/*pelb-DPE* and pET22b (+)/*DPE* were cultured in LB medium containing (g L^{-1}): 5 yeast extract, 10 peptone, 5 NaCl and 0.1 ampicillin. After an overnight culture, the culture was inoculated with an inoculum size of 3 % (v/v) into TB. When the OD_{600} reached 0.6, different amounts of isopropyl 1-thio-β-D-galactopyranoside (IPTG) and lactose were added to induce the expression of DPE at different temperatures for 90 h. To confirm the expression of DPE in *E. coli*, both cultures of the recombinant strain were grown with and without IPTG and lactose induction. Expression of the gene encoding DPE was determined by both SDS-PAGE and enzyme activity assay.

Bioreactor culture conditions

Bioreactor cultivation was performed in a 3-L fermenter (BioFlo 110, New Brunswick Scientific Co.). For fed-batch cultivation, 5 % (v/v) of seed culture was inoculated into the modified TB medium (containing 100 μg mL^{-1} of ampicillin). The whole culture process comprised three phases. The first batch phase commenced with an initial glycerol concentration of 10 g L^{-1} and a temperature of 37 °C. The end of glycerol consumption was detected by a sudden increase in both DO and pH. In the second exponential feeding (pre-induction) phase of fed-batch cultivation, exponential feeding occurred according to the following equation:

$$F(t) = \frac{\mu_{\text{set}} X_0 V_0 \exp(\mu_{\text{set}} t)}{S_{\text{f}} Y_{X/S}}$$

where $F(t)$ is the continuous feeding rate (L h^{-1}), X_0 is the cell concentration (g_{cell} L^{-1}), V_0 is the culture volume (L) at the beginning of fed-batch phase, S_f is the glycerol concentration in feeding solution (g L^{-1}), μ_{set} is the predetermined specific growth rate (h^{-1}), $Y_{X/S}$ is the growth yield obtained on glycerol (g_{cell} g_{glycerol}^{-1}). In the present study, the feeding rate F was adjusted every hour to simulate exponential feeding process.

During the second phase, when a certain dry cell weight (DCW) (15, 30, and 50 g L^{-1}) was reached, 1.5 mM IPTG was added and the temperature was decreased to 25 °C for DPE production. Finally, in the third phase, constant feeding (post-induction) occurred. During the whole process, the pH was maintained at 7.0 by automatic addition of ammonia solution (25 %, v/v). Antifoam was added manually when necessary. To maintain the DO level at around 30 % of air saturation, the agitation speed was varied from 300 to 900 rpm. The air flow rate was 1.8 L min^{-1}.

Dry cell weight (DCW) analysis

Cell growth was monitored by measuring the optical density of culture at 600 nm using a spectrophotometer (BioPhotometer plus, Eppendorf Co., Hamburg, Germany). Samples were diluted with 0.9 % (w/v) NaCl when the OD_{600} exceeds 0.8. To determine the DCW, 5 mL of culture broth was centrifuged (10,000×g; 4 °C; 10 min) and the pellet was washed with 0.9 % (w/v) NaCl, centrifuged, and dried to constant weight at 105 °C.

Cell fractionation

Before cell fractionation, the OD600 of each culture was measured to guarantee the same cell density (5 of OD600) could be obtained. Culture supernatants were obtained by centrifugation at 10,000×g and 4 °C for 10 min, and the supernatant was used as an extracellular fraction. The residual cells were resuspended in 500 μL of PBS buffer and sonicated (Xinchen Bio CO., Nanjing, China) to release soluble intracellular proteins. Cell lysates were centrifuged (7,000×g; 4 °C; 10 min) to recover the soluble intracellular contents.

Analytical methods

Protein concentration was determined according to the method of Bradford using bovine serum albumin as a standard. SDS-PAGE gel electrophoresis was performed using 5 % stacking gel and 12 % separating gel (Bio-Rad Laboratories, Hercules, CA, USA).

Determination of glycerol and acetate

Fermentation supernatant was used for glycerol and acetate analysis using an Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA, USA) with a differential refractive index detector. Five microliters of sample was injected into the Shodex SUGAR SH1011 column (8.0 mm ID × 300 mm, Showa Denko, Japan) with a constant temperature of 50 °C. The mobile phase consisted of 5 mM H_2SO_4 at a flow rate of 0.8 mL min^{-1}. The detector temperature was maintained at 30 °C during the analysis process.

Assay of DPE

The enzyme activity was determined by measuring D-Psicose formed using D-fructose as a substrate. Unless otherwise stated, the reactions were carried out in a 50 mM glycine/NaOH buffer (pH 8.0) containing 50 mM D-fructose and 0.5 μM DPE. The concentrations of D-Psicose were analyzed by HPLC equipped with a refractive index detector and Ca^{2+}-carbohydrate column (Waters Sugar-Pak 1, Waters Corp., Milford, MA, USA), which was eluted with water at 80 °C and 0.4 mL min^{-1}. One unit of

enzyme activity was defined as the amount of enzyme producing 1 μmol D-Psicose per min at 50 °C and pH 8.0.

Bioconversion between D-Psicose and D-fructose

The equilibrium ratio between D-Psicose and D-fructose was 32:68 at 50 °C and pH 8.0 with 0.5 μM enzyme when DPsicose and D-fructose were at a total concentration of 0.1 % (wt/vol). For high levels of production of D-Psicose from D-fructose, extracellular DPE in media culture and cell lysates containing intracellular DPE were used as catalyst, when reaction buffer containing 700 g L^{-1} D-fructose was incubated at 50 °C and pH 8.0 for 100 min.

Assay of outer and inner membrane permeability

Outer membrane permeability was determined by using *N*-phenyl-1-naphthylamine (NPN) access assay. Briefly, *E. coli* cells were collected and centrifuged at 3,000×*g* and suspended in 10 mM sodium phosphate buffer (pH 7.4) to an OD_{600} of 0.5. NPN was added with a concentration of 10 μM into quartz cuvettes containing 2 mL of cell suspension. The sample was mixed by inverting the cuvette immediately prior to fluorescence monitoring. Fluorescence was measured using a 650-60 spectrofluorometer (model 650-60; Hitachi, Tokyo, Japan) with slit widths set to 5 nm and excitation and emission wavelengths set to 350 and 428 nm, respectively.

Permeability of the inner membrane was assessed by measuring the access of *o*-nitrophenyl-β-D-galactoside (ONPG) to the cytoplasm. Briefly, ONPG was added with a concentration of 100 μg ml^{-1} to the *E. coli* BL21 (DE3) suspension as described above. Substrate cleavage by β-galactosidase was determined by measuring OD_{420} using a spectrophotometer (UV-2450; Shimadzu Co., Kyoto, Japan).

Results

Gene cloning and expression

The DPE coding gene AAL45544.1 from *A. tumefaciens* str. C58 was modified by artificial synthesis and cloned. In order to achieve the expression and secretion of DPE in *E. coli*, the DPE gene was cloned into the downstream of the T7 promoter in pET-22b (+) to construct the expression vector pET22b (+)/*DPE* in which the pelB signal peptide was removed. In addition, the DPE gene was inserted into the downstream of the pelB signal peptide sequence in pET22b (+), resulting in the pET22b (+)/*pelb-DPE*. Both recombinant vectors were transformed into *E. coli* BL21 (DE3) and induced with 1.0 mM IPTG at 30 °C for 90 h. The recombinant strain harboring pET22b (+)/*pelb-DPE* produced 0.23 U mL^{-1} extracellular DPE. However, the strain carrying pET22b (+)/*DPE* obtained no DPE activity. SDS-PAGE analysis revealed the significant amount of a 33-kDa protein, which was similar to theoretic molecular weight of the DPE [15, 17].

Effect of medium composition on cell growth and DPE production

The composition of growth medium can significantly affect the recombinant protein expression. In the present study, *E. coli* BL21 (DE3) harboring the pET22b (+)/*pelb-DPE* were grown in five different complex media (LB, SOB, SOC, SB and TB supplemented with ampicillin) until the OD_{600} reached 0.6. The cells were induced with the addition of 1.0 mM IPTG at 30 °C for 90 h. Although both the complex and minimal media considerably supported the growth of cells, the highest DCW and total DPE activity were achieved in the complex media (Table 1). When cultured in TB and SB media, the biomass reached 8.57 and 8.36 g L^{-1}, respectively, while the total DPE yield reached 2.31 and 2.24 U mL^{-1}, respectively. As shown in Table 1, the maximum yield of extracellular activity and protein concentration of DPE obtained in the TB medium reached 0.64 U mL^{-1} and 75 mg L^{-1}, which were slightly higher than those observed in the SB medium (0.61 U mL^{-1} and 70 mg L^{-1}) and 2.78-fold higher than those noted in the LB medium (0.23 U mL^{-1} and 26 mg L^{-1}). The specific productivity (extracellular activity/DCW) of the recombinant DPE was almost the same in the SOC (70.2 U g_{cell}^{-1}), SB (73.0 U g_{cell}^{-1}), and TB (74.7 U g_{cell}^{-1}) media, but was lower in the LB (44.9 U g_{cell}^{-1}) and SOB (50.6 U g_{cell}^{-1}) media. Furthermore, the maximum

Table 1 Effects of media composition on cell growth and DPE production

Culture medium	DCW (g L^{-1})	Total activity (U mL^{-1})	Extracellular activity (U mL^{-1})	Specific productivity (U g_{cell}^{-1})	Secretion capability (%)
LB	5.12	1.02	0.23	44.9	22.4
SOB	6.32	1.19	0.32	50.6	27.0
SOC	6.98	1.96	0.49	70.2	24.9
SB	8.36	2.24	0.61	73.0	27.5
TB	8.57	2.31	0.64	74.7	27.7

secretion capability (extracellular activity/total activity) of the recombinant DPE reached a value of 27.7 % in the TB medium, and was slightly lower in the SOB and SB media.

These results indicate that nutrient-rich media support higher biomass and promote secretion of the recombinant protein into the culture medium. Complex media could provide abundant amino acids, vitamins, and trace elements to support growth and product expression in *E. coli*. Furthermore, a high content of yeast extract in the TB and SB media cannot be ignored, because it is known that yeast extract helps in the secretion of recombinant proteins into the periplasmic space [18]. Besides, the higher buffer capacity of the TB medium facilitates cell growth and recombinant enzyme stability [18]. Therefore, the TB medium was chosen for further studies to improve the secretion of recombinant DPE.

Effects of induction temperature and inducer concentrations on cell growth and DPE production

Induction temperature and inducer concentration are important parameters for recombinant protein production in *E. coli* [19, 20]. In the present study, the influence of different induction temperatures in the presence of 1.0 mM IPTG on DPE production was investigated. As shown in Fig. 1, with the increase in induction temperature, the DCW increased. At an induction temperature of 30 °C, the biomass reached 8.7 g L^{-1}, which was 1.32- and 1.12-fold higher than that observed at 20 and 25 °C, respectively. However, at 25 °C, the extracellular yield of DPE and protein concentration (1.42 U mL^{-1} and 169 mg L^{-1}) were much higher, and the recombinant strain exhibited the highest total yield of 3.53 U mL^{-1}, whose capability of extracellular secretion reached the highest level of 40.2 %. These results indicated that cell growth and DPE synthesis rate were not completely synchronous, and that the inhibitory effect on DPE production was obvious when the induction temperature was increased from 30 to 37 °C. Although high temperature can enhance the fluidity of the cell membrane and the rate of protein synthesis, the enzyme synthesis rate may affect the cell physiology and protein transport, leading to the accumulation of inactive protein aggregates [21]. Moreover, high temperature often makes the plasmid unstable, thus causing a negative effect on foreign protein expression. However, lower temperature could reach the equilibrium of protein accumulation in periplasm and membrane-associated cellular functions such as periplasmic transport. Thus, in the present study, induction at 25 °C resulted in a better coordination between DPE synthesis and translocation, producing the highest total DPE yield of 3.53 U mL^{-1} with the highest extracellular secretion level of 40.2 %.

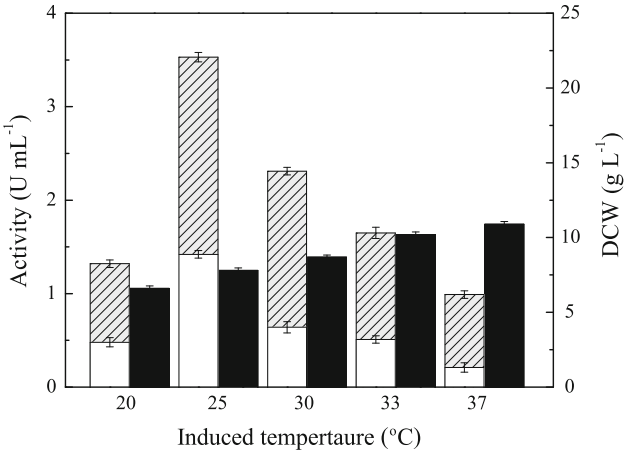

Fig. 1 Comparison of D-Psicose-3-epimerase activity and cell growth at different induced temperatures. Extracellular activity (*white*), intracellular activity (*slash*), and DCW (*black*)

The effects of IPTG and lactose concentration on cell growth and enzyme production were investigated at the optimal induction temperature of 25 °C. As shown in Table 2, the cell growth was inhibited at a high IPTG concentration, while extracellular activity was increased. The highest extracellular DPE activity and protein concentration were reached (1.70 U mL^{-1} and 211 mg L^{-1}) when the cells were induced with 1.5 mM IPTG. The levels of excreted DPE significantly varied with the dosage of IPTG [22]. On the other hand, when compared with

Table 2 Effects of inducer on cell growth and DPE production

Inducer (L^{-1})	DCW (g L^{-1})	Total activity (U mL^{-1})	Extracellular activity (U mL^{-1})	Specific productivity (U g_{cell}^{-1})	Secretion capability (%)
Control	8.6	2.64	0.83	96.5	31.4
0.5 mM IPTG	8	3.38	1.24	155.0	36.7
1.0 mM IPTG	7.8	3.53	1.42	182.1	40.2
1.5 mM IPTG	7.6	3.96	1.7	223.7	42.9
2.0 mM IPTG	7.7	3.34	1.36	176.6	40.7
2.5 g lactose	8.8	3.42	1.1	125.0	32.2
5.0 g lactose	8.9	3.84	1.42	159.6	37.0
7.5 g lactose	9.1	3.65	1.24	136.3	34.0
10.0 g lactose	9.4	3.51	1.22	129.8	34.76

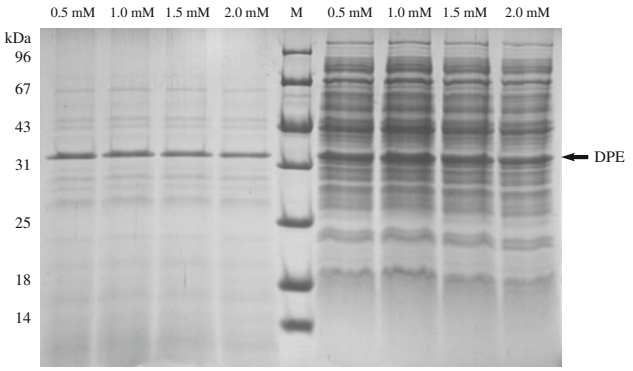

Fig. 2 SDS-PAGE analysis of distribution of DPE in culture supernatants fractions and soluble intracellular fractions

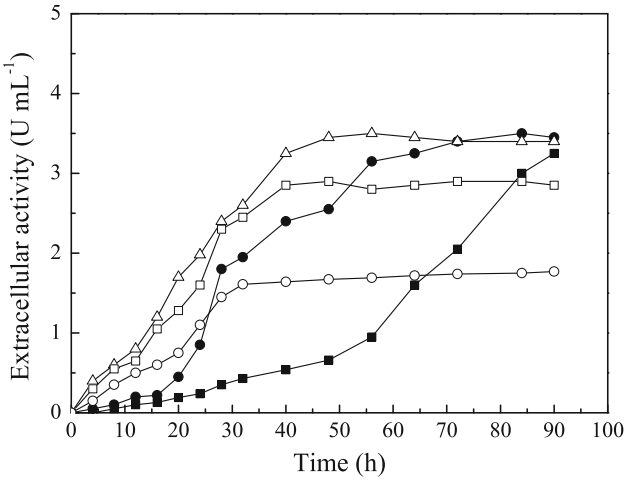

Fig. 3 Effect of glycine concentration on extracellular activity of D-Psicose-3-epimerase. *E. coli* cells were grown in optimal TB (*filled square*), optimal TB medium supplemented with 75 mM (*filled circle*), 150 mM (*open triangle*), 200 mM (*open square*), 300 mM (*open circle*) glycine. Each value represents the mean of three independent measurements, and the deviation from the mean is below 5 %

IPTG induction, the cell growth increased (8.9 g L^{-1}) at a high lactose concentration (5.0 g of lactose), while the extracellular activity and protein concentration reached values of 1.42 U mL^{-1} and 170 mg L^{-1}, which was 16.5 and 19.4 % lower than that observed with the addition of 1.5 mM IPTG, respectively. SDS-PAGE was used to determine the distribution of DPE in the fractions of culture supernatants and the soluble intracellular fractions, respectively (Fig. 2). It was found that with the increase in IPTG concentration from 0 to 2.0 mM, the quantity of DPE in the supernatants, along with the proportion of insoluble intracellular DPE in the total DPE, increased.

Effect of glycine addition on the extracellular production of DPE by *E. coli*

One of the disadvantages of extracellular secretion of recombinant proteins is the difficulty in protein translocation across the two membranes of the *E. coli* cells [6, 7]. Various attempts have been made to facilitate extracellular secretion of recombinant proteins in *E. coli*. To determine the advantages of glycine supplementation in extracellular secretion [23–25], the effect of glycine addition was investigated in the present study. As shown in Fig. 3, the extracellular secretion of the recombinant DPE was significantly affected by glycine. In previous studies, glycine supplementation at the beginning of culture was found to have a negative effect on cell growth, as demonstrated by the reduction in the total cell number and viability as well as the obvious increase in cell lysis. Therefore, in the present study, the TB medium was supplemented with 0–300 mM glycine at 14 h of incubation in the middle of the logarithmic phase [26]. As shown in Fig. 3, the highest extracellular activity (3.5 U mL^{-1}) and protein concentration (408 mg L^{-1}) were achieved in the presence of 150 mM glycine. At lower concentration of glycine, the enzyme activity in the culture medium was low and the extracellular production efficiency was lower than that obtained with the addition of 150 mM glycine. On the other hand, at a high glycine concentration (300 mM), the enhancing effect of glycine on extracellular activity was significantly reduced.

Furthermore, to elucidate the mechanism of increased extracellular production of DPE in recombinant cells with the addition of glycine, the permeability of the inner and outer membrane of the recombinant cells cultured under optimal and control conditions at 40 h of incubation was quantitatively determined. As shown in Fig. 4, when the *E. coli* cells were cultured in regular TB medium, both outer and inner cell membranes had very low permeability. On the other hand, when the *E. coli* cells were cultured in optimal medium with 150 mM glycine, the outer membrane had high permeability initially, which was twofold higher than that observed in the regular medium.

Effect of a two-stage glycerol feeding strategy in a 3-L fermenter

In the present study, a two-stage glycerol feeding strategy was proposed and applied. During the pre-induction phase, the glycerol feeding rate was found to increase exponentially, according to the exponential feeding method [27], and the cell growth was controlled at a specific growth rate (μ_{set}) of 0.20 h^{-1}. When the DCW reached a certain value (30 g L^{-1}), the post-induction phase commenced and the glycerol feeding rate shifted to 15 mL h^{-1}. In the fed-batch culture process, acetate and glucose concentrations were maintained below 1.0 and 0.7 g L^{-1}, respectively, and the DCW of the *E. coli* cells increased from 0.12 to 83.6 g L^{-1} (Fig. 5).

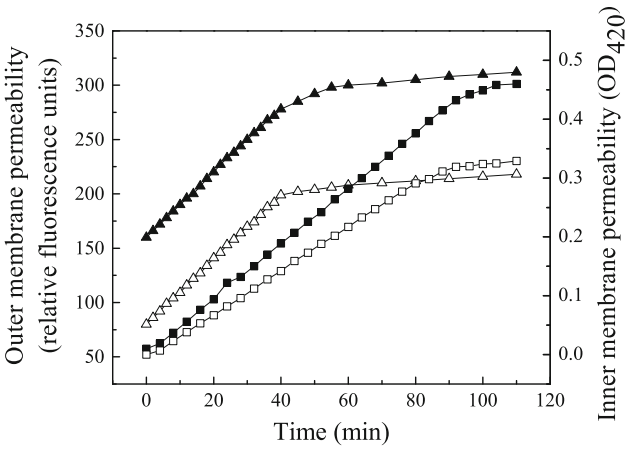

Fig. 4 Concurrent measurements of *E. coli* outer and inner cell membrane permeability. Outer cell membrane permeability: the culture without glycine addition (*open triangle*), the culture with glycine addition (*filled triangle*). Inner cell membrane permeability: the culture without glycine addition (*open square*), the culture with glycine addition (*filled square*)

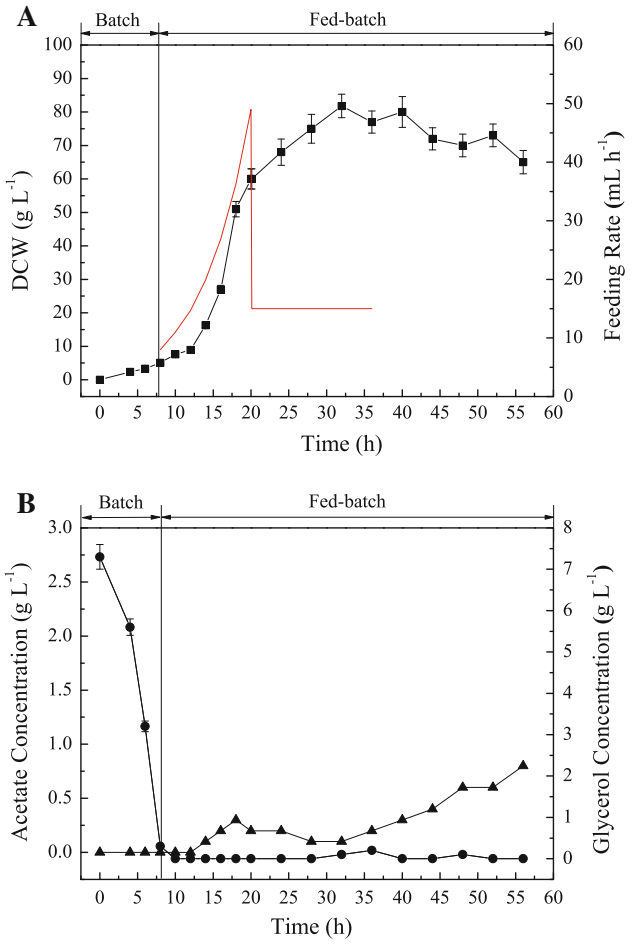

Fig. 5 Fed-batch culture course of *E. coli* BL21 (DE3) (pET-22b (+) DPE). **a** DCW (*filled square*) and glycerol feeding rate (*solid line*). **b** glycerol (*filled circle*) and acetate (*filled triangle*). *Arrows* indicated the starting point of induction

Although the fed-batch processes are primarily focused on increasing the biomass concentration, the induction conditions can also affect the specific yield of the target proteins. Recombinant protein expression generally imposes a certain metabolic burden on cell growth, and this may reduce the cell growth rate, cell yield, protein expression, and plasmid stability.

To reduce the metabolic burden imposed on the cells, the optimal point for DPE induction was investigated in the present study, and IPTG (1.5 mM) was added at three different cell densities (15, 30, and 50 g L^{-1}). Figure 6 shows the time profiles of biomass, extracellular activity and total DPE activity. The cell growth was severely inhibited when induced at an early log phase (15 g L^{-1}) when the maximum DCW was only 21.71 g L^{-1} (Fig. 6). As a consequence, reduced extracellular DPE activity (3.6 U mL^{-1}) (Fig. 6) and protein concentration (423 mg L^{-1}) were noted. Although no significant inhibition was observed when the cells were induced at high cell density (50 g L^{-1}), the extracellular DPE yield and protein concentration were low, reaching values of only 7.1 U mL^{-1} and 836 mg L^{-1}. On the other hand, when the cells were induced at an intermediate cell density (30 g L^{-1} of DCW), the total DPE yield increased to 24.1 U mL^{-1}, while the extracellular specific productivity (130.4 U g_{cell}^{-1}) was lower than that (165.8 U g_{cell}^{-1}) observed at an intermediate cell density (15 g L^{-1} of DCW). In this case, the biomass, extracellular DPE activity, extracellular DPE concentration and extracellular specific productivity were 83.6 g L^{-1}, 10.9 U mL^{-1}, 1,240 mg L^{-1}, and 130.4 U g_{cell}^{-1}, respectively. Therefore, intermediate cell density was concluded to be the optimal point for DPE induction.

Effect of composition of feeding solution on cell growth and DPE production

Nitrogen is a critical medium component for bacteria growth and production performance. With the aim to improve DPE production by recombinant *E. coli*, the effect of composition of feeding solution on cell growth and enzyme production was investigated. Two kinds of composition of feeding solution were investigated: inorganic nitrogen (glycerol 500 g L^{-1} and NH_4Cl 42 g L^{-1}) and complex nitrogen (glycerol 500 g L^{-1}, yeast extract 50 g L^{-1}, and peptone 50 g L^{-1}), which were designed in accordance with the principle of equal mass of nitrogen. For the control, no extra nitrogen was added.

The results showed that the cell concentration obviously increased when the feeding solution was supplemented with nitrogen sources. When cultured with feeding solution containing inorganic nitrogen and complex nitrogen, the highest DCW reached 76.4 and 83.6 g L^{-1}, respectively,

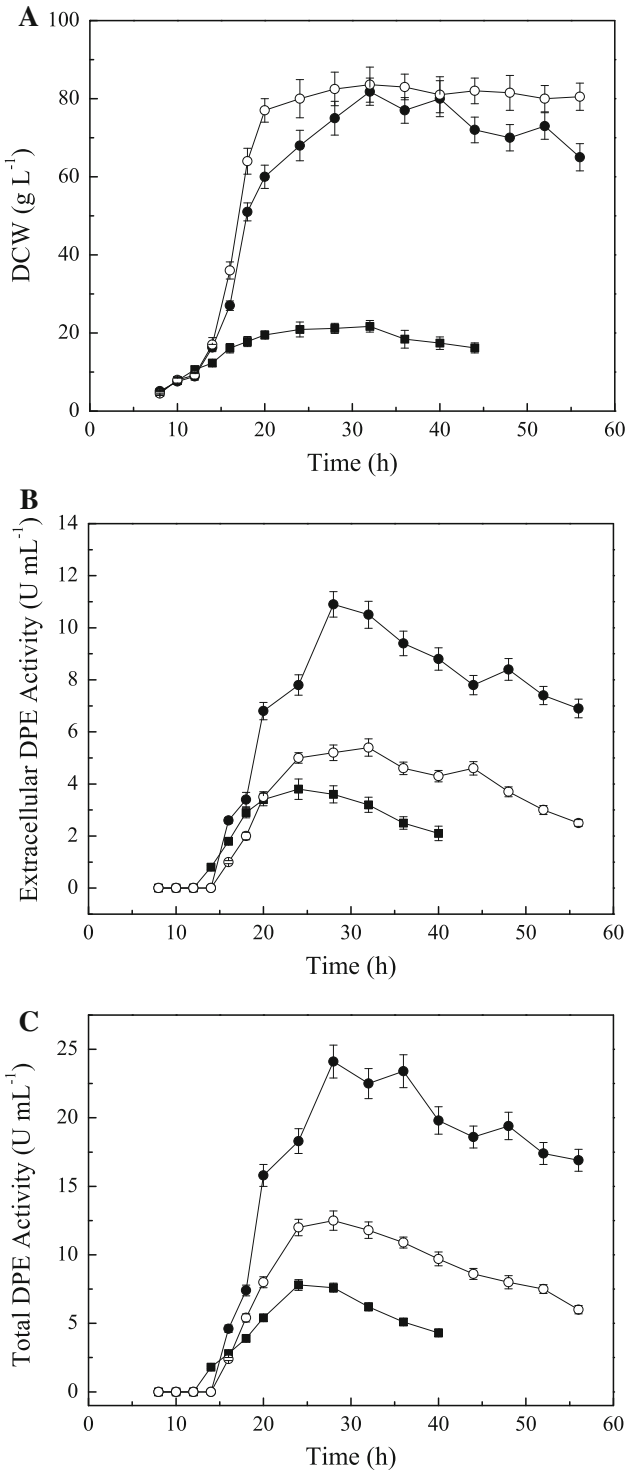

Fig. 6 Comparison of the time profiles for biomass (**a**), extracellular DPE activity (**b**), and total DPE activity (**c**) obtained from cultivations induced at low cell density (*filled square* 15 g L^{-1}), intermediate cell density (*filled circle* 30 g L^{-1}), and high cell density (*open circle* 50 g L^{-1})

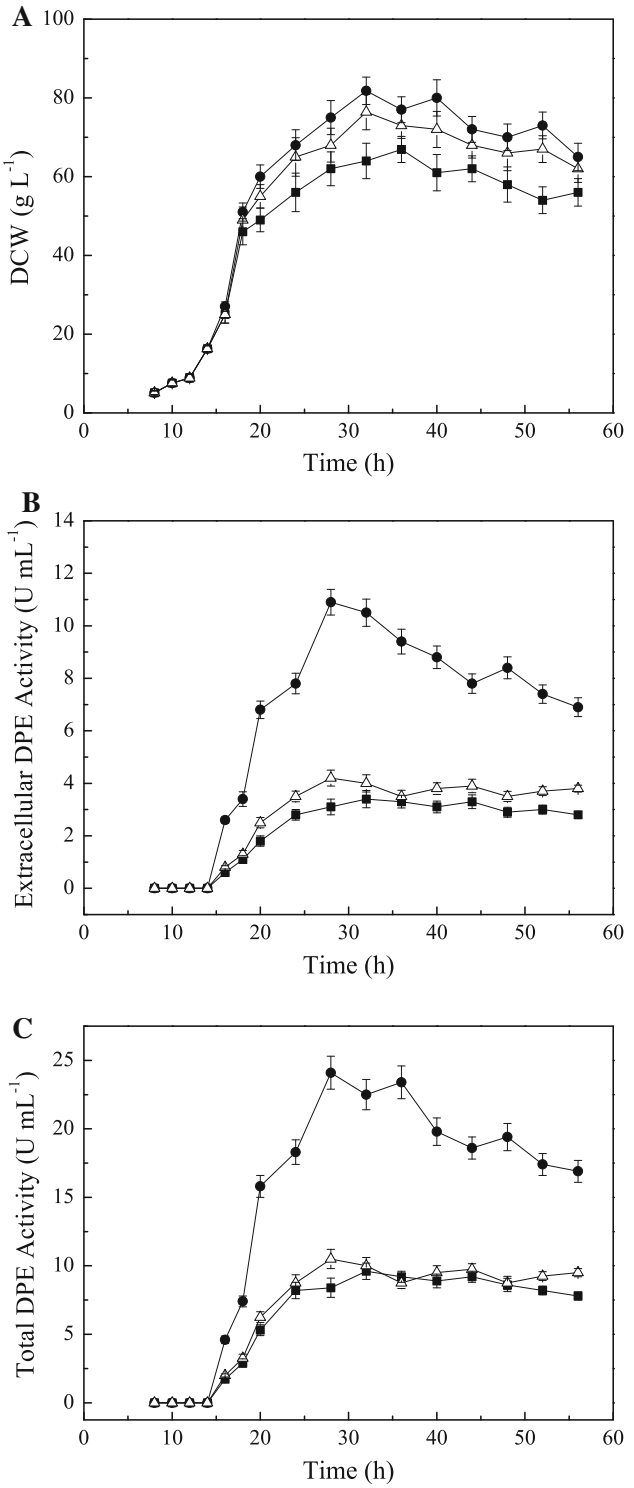

Fig. 7 Comparison of the time profiles for biomass (**a**), extracellular DPE activity (**b**), and total DPE activity (**c**) obtained from different composition of feeding solution. Control (*filled square*); inorganic nitrogen (*open triangle*); complex nitrogen (*filled circle*)

which were 1.1- and 1.3-fold that of control (66.9 g L^{-1}) (Fig. 7a). The extracellular activity and protein concentration obtained in feeding solution containing complex nitrogen reached 10.9 U mL^{-1} and 1,240 mg L^{-1} which was 3.4- and 3.3-fold that of control (3.4 U mL^{-1} and 374 mg L^{-1}) (Fig. 7b). The extracellular DPE specific

productivity obtained in complex nitrogen reached 130.4 U g_{cell}^{-1}, which was 2.2- and 2.6-fold that of inorganic nitrogen (54.9 U g_{cell}^{-1}) and control (50.8 U g_{cell}^{-1}).

In order to analyze the effect of composition of feeding solution, the time profiles of biomass, extracellular activity and total DPE activity were measured. As seen from Fig. 7c, the total activity obtained in feeding solution with complex nitrogen reached 23.1 U mL^{-1}, which was 2.4-fold that of control (9.6 U mL^{-1}). In the present study, it is known that complex nitrogen source may promote recombinant protein synthesis and translocation, which lead to an enhanced total activity of DPE.

Bioconversion between D-Psicose and D-fructose

For high levels of D-Psicose production, the reaction buffer containing 700 g L^{-1} of D-fructose along with the catalyst, extracellular DPE (DPE in the culture media) and intracellular DPE (recombinant *E. coli* whole cells harboring DPE), respectively, was incubated at 50 °C and pH 8.0 for 100 min. D-Psicose production using extracellular and intracellular DPE was 179 and 218 g L^{-1}, along with a 25.5 % turnover yield (1 mL of supernatant contained 10.9 U of extracellular DPE) and a 31.1 % turnover yield (1 mL of cell lysates contained of 13.2 U intracellular DPE), respectively (Fig. 8).

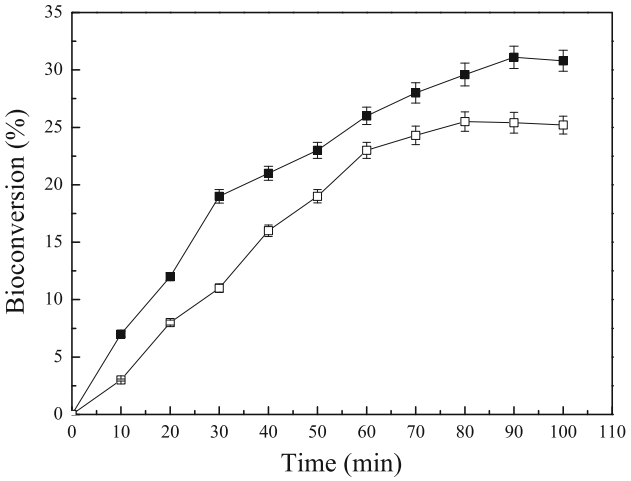

Fig. 8 Bioconversion of D-fructose into D-Psicose: turnover ratio in 1 L reaction system containing 700 g L^{-1} D-fructose at 50 °C and pH 8.0 for 100 min with supernatant containing extracellular DPE (*open square*), cell lysates containing intracellular DPE (*filled square*)

Discussion

In recent years, much effort has been taken to isolate and characterize DPE due to the increasing applications and demand for its converted product. For effective industrial production of D-Psicose, high concentrations of DPE are required in the immobilized biocatalyst reactor. Hence, high-level production of DPE is necessary. Although in previous studies, the DPE had been expressed in the cytoplasm of *E. coli* [15], targeting the secretion of DPE into the culture medium is considered significant for industrial-scale application because of its various advantages, such as facilitating downstream processing and achieving higher level expression. Thus, the specific aim of the present study was to enhance the extracellular expression of DPE in *E. coli* to obtain cost-effective production of D-Psicose for industrial applications.

An *E. coli* system was successfully constructed in this study for direct secretion of active DPE. To achieve extracellular expression of recombinant DPE, pelB secretion signal was applied. Application of the pelB signal peptide, which is generally recognized as an efficient secretory signal peptide in *E. coli*, produced extracellular DPE (0.23 U mL^{-1}). The secretory pathway mediated by pelB signal peptide is a two-step process, which is composed of translocation of cytoplasmic membrane and outer membrane. The pelB signal peptide may induce the accumulation of the DPE in periplasm, so increasing the extracellular DPE was studied. Subsequently, an extracellular DPE activity of 3.5 U mL^{-1} (Fig. 3) was achieved in shake flasks, indicating that the constructed *E. coli* system was suitable for extracellular production of DPE. Further investigation revealed that glycine could efficiently enhance the extracellular activity of the recombinant DPE. The potential mechanism for this increased activity was noted to be the glycine-induced modification of peptidoglycan structure in the cell wall [3]. Furthermore, the quantitative analysis (Fig. 4) carried out in the present study confirmed the role of glycine in increasing the cell membrane permeability, promoting translocation of DPE across the inner membrane.

Based on the above-mentioned results obtained in shake flasks, to enhance the productivity of recombinant DPE, a 3-L fermenter with increased biomass was employed. The yield of recombinant protein production was found to depend on both the biomass concentration and specific cellular productivity. To achieve a high biomass concentration in fed-batch cultivation, high cell density cultivation using appropriate feeding strategies was used [2].

As the feeding strategy is reported to affect the metabolic pathway fluxes, cell density, specific productivity of recombinant proteins, and formation of by-products such as acetic acid [28], it must be optimized for the high expression of recombinant proteins. Acetate is an extracellular by-product of aerobic fermentation, which inhibits cell growth and recombinant protein formation at a concentration higher than 2.0 g L^{-1} [29]. Usually, accumulation of acetate can be prevented by a limited supply of

carbon source, which can be achieved through exponential feeding. Accordingly, acetate formation can be minimized by controlling the specific growth rate below a certain value, such as 0.2 and 0.35 h^{-1}, in complex and minimal medium, respectively [30]. Thus, exponential feeding is a simple approach that allows cells to grow at a specified growth rate.

However, it has been reported that in exponential fed-batch cultures, the specific experiment growth rates (μ_{exp}) after induction were less than the those of μ_{set}, even when the acetate concentration was maintained at a lower level [31]. In addition, application of the same exponential feeding strategy during the post-induction phase has been found to usually lead to the accumulation of nutrients in the medium, which may be due to physiological and metabolic changes in the host cells after induction. In the present study, the results obtained in shake flasks demonstrated that, after induction and addition of glycine, the cell growth limited the production of recombinant DPE because of the negative effect of IPTG and glycine on cell growth. Therefore, a low constant feeding rate should be applied during the post-induction phase. Based on the above-mentioned analysis and specific expression systems employed, a two-stage glycerol feeding strategy was applied. When the DCW of the cell reached a certain value, the post-induction phase commenced and the feeding rate decreased (Fig. 5a). By applying the two-stage glycerol feeding strategy to the 3-L fermenter, an extracellular DPE activity of 10.9 U mL^{-1} was obtained, which was 3.11-fold higher than that achieved before optimization (3.5 U mL^{-1}). Thus, this is an effective tool to increase the extracellular production of DPE in *E. coli*. In previous studies, D-Psicose has been produced using purified intracellular DPE or cells containing DPE. However, due to the decreased activity resulting from the enzyme purification process, use of purified intracellular DPE to produce D-Psicose is less efficient. Similarly, utilization of cells which contain high levels of other cell materials may cause steric hindrance in the production of D-Psicose. The use of extracellular DPE for the production of D-Psicose can overcome these problems. In the present study, D-Psicose production from 700 g L^{-1} D-fructose reached 179 g L^{-1} when extracellular DPE was employed, with a turnover yield of 25.5 %. Considering the above-mentioned results, high-level extracellular production of DPE is vital for its use as a biocatalyst for D-Psicose production.

To our knowledge, this study is the first to report extracellular DPE production. A two-stage glycerol feeding strategy based on the approach of increasing the secretion of DPE into the liquid medium by recombinant *E. coli* BL21 (DE3) was developed in the present study. Under the optimum conditions, the extracellular DPE activity in the culture media reached 10.9 U mL^{-1} with a 25.5 % turnover yield, while the intracellular DPE activity reached 13.2 U mL^{-1} with a 31.1 % turnover yield. The results obtained reveal that systemic strategies can be applied for high-level extracellular production of DPE to produce D-Psicose, and provide valuable novel information for the industrial application of DPE.

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References

1. Matsuo T, Izumori K (2004) D-Psicose, a rare sugar that provides no energy and additionally beneficial effects for clinical nutrition. Asia Pac J Clin Nutr 13(Suppl):127
2. Hossain MA, Izuishi K, Tokuda M, Izumori K, Maeta H (2004) D-Allose has a strong suppressive effect against ischemia/reperfusion injury: a comparative study with allopurinol and superoxide dismutase. J Hepato Bill Pan Sci 11(3):181–189
3. Murata A, Sekiya K, Watanabe Y, Yamaguchi F, Hatano N, Izumori K, Tokuda M (2003) A novel inhibitory effect of D-Allose on production of reactive oxygen species from neutrophils. J Biosci Bioeng 96(1):89–91
4. Levin GV (2002) Tagatose, the new GRAS sweetener and health product. J Med Food 5(1):23–36
5. Matsuo T, Baba Y, Hashiguchi M, Takeshita K, Izumori K, Suzuki H (2001) Dietary D-Psicose, a C-3 epimer of D-Fructose, suppresses the activity of hepatic lipogenic enzymes in rats. Asia Pac J Clin Nutr 10(3):233–237
6. Hossain M (2000) Effect of the immunosuppressants FK506 and D-Allose on allogenic orthotopic liver transplantation in rats. Transpl Proc 32:2021–2023
7. Muniruzzaman S, Pan Y, Zeng Y, Atkins B, Izumori K, Elbein A (1996) Inhibition of glycoprotein processing by L-fructose and L-xylulose. Glycobiology 6(8):795–803
8. Granström TB, Takata G, Tokuda M, Izumori K (2004) Izumoring: a novel and complete strategy for bioproduction of rare sugars. J Biosci Bioeng 97(2):89–94
9. Matsuo T, Tanaka T, Hashiguchi M, Izumori K, Suzuki H (2003) Metabolic effects of D-Psicose in rats: studies on faecal and urinary excretion and caecal fermentation. Asia Pac J Clin Nutr 12(2):225–231
10. Matsuo T, Suzuki H, Hashiguchi M, Izumori K (2002) D-Psicose is a rare sugar that provides no energy to growing rats. J Nutr Sci Vitaminol 48(1):77
11. 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(6):1461–1467
12. Itoh H, Okaya H, Khan AR, Tajima S, Hayakawa S, Izumori K (1994) Purification and characterization of D-Tagatose 3-epimerase from *Pseudomonas* sp. ST-24. Biosci Biotechnol Biochem 58(12):2168–2171
13. 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(6):857–862. doi:10.1007/s10529-009-9942-3
14. Mu W, Chu F, Xing Q, Yu S, Zhou L, Jiang B (2011) Cloning, expression, and characterization of a D-Psicose 3-epimerase from

Clostridium cellulolyticum H10. J Agric Food Chem 59(14): 7785–7792
15. 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(2):981–985. doi:10.1128/aem.72.2.981-985.2006
16. Goodner B, Hinkle G, Gattung S, Miller N, Blanchard M, Qurollo B, Goldman BS, Cao Y, Askenazi M, Halling C (2001) Genome sequence of the plant pathogen and biotechnology agent *Agrobacterium tumefaciens* C58. Science 294(5550):2323–2328
17. Kim K, Kim H-J, Oh D-K, Cha S–S, Rhee S (2006) Crystal structure of D-Psicose 3-epimerase from *Agrobacterium tumefaciens* and its complex with true substrate D-Fructose: a pivotal role of metal in catalysis, an active site for the non-phosphorylated substrate, and its conformational changes. J Mol Biol 361(5):920–931. doi:10.1016/j.jmb.2006.06.069
18. Kelley R, Reddy CA (1986) Purification and characterization of glucose oxidase from ligninolytic cultures of *Phanerochaete chrysosporium*. J Bacteriol 166(1):269–274
19. Fu W, Lin J, Cen P (2010) Expression of a *hem*A gene from *Agrobacterium radiobacter* in a rare codon optimizing *Escherichia coli* for improving 5-aminolevulinate production. Appl Biochem Biotech 160(2):456–466
20. Pinsach J, de Mas C, López-Santín J (2008) Induction strategies in fed-batch cultures for recombinant protein production in *Escherichia coli*: application to rhamnulose 1-phosphate aldolase. Biochem Eng J 41(2):181–187
21. Martínez-Martínez I, Kaiser C, Rohde A, Ellert A, García-Carmona F, Sánchez-Ferrer Á, Luttmann R (2007) High-level production of *Bacillus subtilis* glycine oxidase by fed-batch cultivation of recombinant *Escherichia coli* Rosetta (DE3). Biotechnol Prog 23(3):645–651
22. Matsumoto T, Katsura D, Kondo A, Fukuda H (2002) Efficient secretory overexpression of *Bacillus subtilis* pectate lyase in *Escherichia coli* and single-step purification. Biochem Eng J 12(3):175–179
23. Tang J, Yang H, Song S, Zhu P, Ji A (2008) Effect of glycine and Triton X-100 on secretion and expression of ZZ-EGFP fusion protein. Food Chem 108(2):657–662
24. Rhee JI, Bode J, Diaz-Ricci JC, Poock D, Weigel B, Kretzmer G, Schügerl K (1997) Influence of the medium composition and plasmid combination on the growth of recombinant *Escherichia coli* JM109 and on the production of the fusion protein EcoRI: SPA. J Biotechnol 55(2):69–83
25. Kaderbhai N, Karim A, Hankey W, Jenkins G, Venning J, Kaderbhai MA (1997) Glycine-induced extracellular secretion of a recombinant cytochrome expressed in *Escherichia coli*. Biotechnol Appl Biochem 25(1):53–61
26. Macauley-Patrick S, Fazenda ML, McNeil B, Harvey LM (2005) Heterologous protein production using the *Pichia pastoris* expression system. Yeast 22(4):249–270
27. Pei X, Wang Q, Qiu X, Ying L, Tao J, Xie T (2010) The fed-batch production of a thermophilic 2-deoxyribose-5-phosphate aldolase (DERA) in *Escherichia coli* by exponential feeding strategy control. Appl Biochem Biotech 162(5):1423–1434
28. Norsyahida A, Rahmah N, Ahmad R (2009) Effects of feeding and induction strategy on the production of *BmR*1 antigen in recombinant *E. coli*. Lett Appl Microbiol 49(5):544–550
29. Shiloach J, Fass R (2005) Growing *E. coli* to high cell density—a historical perspective on method development. Biotechnol Adv 23(5):345–357
30. Eiteman MA, Altman E (2006) Overcoming acetate in *Escherichia coli* recombinant protein fermentations. Trends Biotechnol 24(11):530–536
31. Ramalingam S, Gautam P, Mukherjee KJ, Jayaraman G (2007) Effects of post-induction feed strategies on secretory production of recombinant streptokinase in *Escherichia coli*. Biochem Eng J 33(1):34–41