Introduction

Glucose isomerase (GI, also known as xylose isomerase, EC 5.3.1.5) is one of the three highest tonnage value enzymes, Amylase and protease being the other two (Bhosale et al., 1996). GI is industrially applied to the production of high-fructose corn syrup because of its ability to catalyze the reversible isomerization of D-glucose to D-fructose. Recent research on GI has largely focused on searching for an enzyme with broad optimum pH range (Xu et al., 2009), high glucose affinity (Siprapundh et al., 2000), improved reaction rate toward L-ribose (Santa et al., 2005), and lactulose synthesis (Hua et al., 2010).

Lactulose (4-O-β-D-galactopyranosyl-α-D-fructose) is a synthesized disaccharide formed by one molecule of fructose and one molecule of galactose via a β-(1,4)-glycosidic bond. As a prebiotic, it has gained increased interest (Agustín and Nieves, 2009). The chemical route involves cost-consuming purification steps and presents safety problems. Alternatively, the enzymatic conversion has been explored to overcome these limits. Known processes of enzymatic transformation consist of two reaction steps (Lee et al., 2004): firstly, lactose is converted into glucose and galactose catalyzed by the hydrolytic activity of β-galactosidase; subsequently, the formed galactose is transferred to fructose to produce lactulose via transgalactosylation by β-galactosidase. During the search for an enzymatic approach to the synthesis of lactulose, it has been observed that glucose may act as a competitive product inhibitor for the transgalactosylation reactions catalyzed by β-galactosidase. Therefore there is a high current interest in reducing the product inhibitory effect by addition of GI. If exogenous GI is added to the reaction mixture, the formed fructose can compete with glucose for the transgalactosylation reaction and react only with the galactosyl-enzyme intermediate as a galactose acceptor to generate lactulose (Kim et al., 2004). Hua et al. (2010) first reported the enzymatic synthesis of lactulose under the ca-
talyzation of an immobilized β-galactosidase and GI dual-enzyme system in organic-aqueous media. Under optimum reaction conditions, the maximum lactulose yield reached 151 g/L. The use of immobilized enzyme could overcome some problems, such as low stability and non-reusability of the enzymes, but there were still some drawbacks. To more efficiently produce lactulose, the development of co-immobilization of β-galactosidase and GIs are of particular interest. In this context, a thermostable β-galactosidase from *Bacillus stearothermophilus* was provided by the Research Centre of Food Biotechnology, Jiangnan University, Wuxi, China, and the recombinant β-galactosidase expressed in *B. subtilis* WB600 had an optimum temperature and pH value of 70 °C and 7.0, respectively. On the other hand, *A. missouriensis* showed an optimum temperature and pH value of 60 °C and 7.0, respectively. On the other hand, since most commercially available GI produced by various microorganisms, including *Streptomyces*, *Actinoplanes*, *Flavobacterium*, and *Bacillus* species (Rastall, 2007; Liao et al., 1995; Lama et al., 2001), is generally available only in the immobilized form, a bacterial strain of *Actinoplanes missouriensis* CICIM B0118(A) was used to produce the free form of GI.

An expression study with the cloned *A. missouriensis* GI in *Saccharomyces cerevisiae* showed that this gene is not expressed as a fully active protein (Amore et al., 1989). Compared to the use of *S. cerevisiae* as an expression system, the cloning and expression of *A. missouriensis* DSM 43046 GI gene in *E. coli* have been successfully conducted (Amore and Hollenberg, 1989; Rey et al., 1988; Jenkins et al., 1992; Karimäki et al., 2004). However, the previous reports mainly focused on molecular characterization, catalytic mechanism, and substrate specificity. Little information on optimization of the medium components for the production of recombinant GI (rGI) from *E. coli* BL21(DE3) is available in the literature. Hence, the enhanced production of rGI is certainly of importance due to the potential use in industrial production of lactulose.

In this study, the *xylA* gene was cloned and overexpressed in *E. coli* BL21(DE3) as a fusion protein containing a six-His tag at its N-terminus. The present work also reports the enhancement of the expression of wild-type rGI in *E. coli* BL21(DE3) through optimization of the medium components. Furthermore, rGI purified by a one-step affinity chromatography procedure was characterized biochemically in detail.

### Material and Methods

#### Bacterial strains, plasmid, and cultivation

*A. missouriensis* CICC11008 and CICIM B0118(A) were obtained from the China Center of Industrial Culture Collection and the Culture and Information Center of Industrial Microorganisms of China Universities, respectively. *E. coli* BL21(DE3) and pET-28a(+) were used as host cell and expression vector, respectively. The recombinant *E. coli* harbouring GI was routinely grown in a 250-mL flask containing 50 mL of Luria-Bertani (LB) medium supplemented with 50 μg/mL of kanamycin at 37 °C and agitation at 200 rpm. Induction was initiated by adding isopropyl-β-D-thiogalactopyranoside (IPTG) at a final concentration of 0.6 mM at an optical density of 0.8 at 600 nm (OD600); the culture was incubated with shaking at 200 rpm at 30 °C for further 10 h.

#### Gene cloning and expression

For cloning the GI gene (*xylA*, 1185 bp) from *A. missouriensis* CICC11008 and CICIM B0118(A), two specific oligonucleotides, which introduced an *NdeI* site at the 5’-end and an *HindIII* site at the 3’-end, respectively, were designed to amplify the *xylA* gene. The primers used were as follows: 5'-TTTCATATGTTGAATAAAC-3' (*NdeI*) and 5'-TTCTCGACACACTATCG-3' (*HindIII*). The amplified 1.2-kb *xylA* gene was cleaved by *NdeI* and *HindIII* restriction enzymes and inserted into the pET-28a expression vector (Novagen, Madison, WI, USA) to obtain pET-28a-*xylA*008 and pET-28a-*xylA*118, respectively. The clones containing the desired *NdeI*-*HindIII* fragment were selected and confirmed by sequencing. The positive recombinant plasmids were used to transform chemically competent *E. coli* BL21(DE3) cells. A single colony of *E. coli* BL21(DE3) cells harbouring the plasmid pET-28a-*xylA*118 was inoculated into 10 mL LB medium containing 50 μg/mL kanamycin and grown at 37 °C overnight. The culture was then diluted to 1:100 with five typical media (Whittaker and Whittaker, 2009; Li et al., 2010), including LB, terrific broth (TB), super broth (SB), super optimal broth (SOB), and super optimal broth with catabolite repression (SOC), and then incubated at 37 °C on a rotatory shaker at 200 rpm.
Media optimization and culture conditions

The LB medium was used as basal medium with some minor modifications. The factors, such as initial pH value of medium, mineral salts, and various sources of carbon and nitrogen that influence the production of GI, were optimized by varying one parameter at a time while other components were kept constant. The effect of pH value in the range 6–9 on rGI production was first studied in LB medium. Following this, the effect of supplementation with various nitrogen and carbon sources at their respective equivalent nitrogen and carbon concentrations on rGI production was compared. The influence of mineral salts such as MgSO$_4$, MnSO$_4$, and CoCl$_2$ on rGI production was also investigated. All experiments were carried out at 30 °C in 250-mL Erlenmeyer flasks containing 50 mL of the medium on a shaker platform at 200 rpm.

Purification of rGI

All purification steps were performed at 4 °C unless otherwise specified. Using the designed medium, the induced cells were harvested by centrifugation at 8,000 × g for 5 min, resuspended in 20 mM sodium phosphate buffer (pH 7.0), and sonicated on ice using a Vibra Cell™ 72405 sonicator (Sonics and Material Inc., Newtown, CT, USA). The supernatant fraction was obtained by centrifugation at 15,000 × g for 20 min and subsequent heat treatment at 70 °C for 20 min to remove unwanted proteins, followed by a second centrifugation at 15,000 × g for 20 min resulting in the soluble fraction used for enzyme activity measurements and further protein purification. The partially purified recombinant protein was then loaded onto a Ni$^{2+}$ chelating Sepharose HP chromatography column (GE Healthcare Biosciences AB, Uppsala, Sweden) and eluted with a linear gradient from 10 to 250 mM imidazole at a flow rate of 1 mL/min. The active fraction was collected and dialyzed at 4 °C against 20 mM sodium phosphate buffer (pH 7.0). An aliquot of the enzyme solution was withdrawn at each time interval, and the residual activity was measured under standard assay conditions. Before studying the effects of Mg$^{2+}$ and Co$^{2+}$ on the rGI activity, the enzyme solution was dialyzed at 4 °C for 24 h against 20 mM sodium phosphate buffer (pH 7.0) containing 10 mM EDTA, followed by dialysis against the same buffer without EDTA. The requirement of the recombinant protein for Mg$^{2+}$ and Co$^{2+}$ was investigated at concentrations ranging from 1 to 10 mM and 0 to 1 mM, respectively.

Results and Discussion

Cloning and expression of xylA

Two xylA genes were cloned and sequenced from A. missouriensis CICC11008 and CICIM B0118(A). Their sequences were registered in the GenBank as FJ858194 for A. missouriensis CICC11008 and FJ858195 for A. missouriensis CICIM B0118(A). In order to increase
the expression level of recombinant enzyme, a set of induction experiments was carried out to determine the optimum conditions for inducing the expression of GI. Highest enzyme activity was achieved with pET-28a-xylA118 after 10 h of induction at 30 °C using 0.6 mM IPTG, while a lower enzyme activity was achieved with pET-28a-xylA008. The enzyme activity of xylA118 was about seven times higher than that of xylA008. Thus the recombinant E. coli BL21(DE3) containing pET-28a-xylA118 was employed in the following experiments.

In order to improve the production of the laboratory-scale microbial process for rGI, the culture conditions for the high-level production of rGI in E. coli BL21(DE3) were optimized with five typical media. As shown in Table I, the optimum culture medium was LB which was used as basal medium for further investigations. Recombinant E. coli cultivation in S0B medium was also suitable to produce enzyme. However, the use of a rich medium like TB medium resulted in a low enzyme yield. This observation was consistent with the results reported by Choosri et al. (2010).

**Effect of initial pH value of medium on enzyme production**

As described above, the effect of the initial pH value was investigated between pH 6.0 and 9.0. The pH of the media was adjusted by NaOH or HCl. E. coli BL21(DE3) could grow and produce the desired enzymes within a rather broad pH range of 6.0–9.0; however, the highest enzymatic activity was obtained at an initial pH value of 7.5 (Fig. 1). Besides higher enzyme activity, a relatively higher biomass production was achieved, as indicated by Fig. 1.

**Effect of carbon and nitrogen source on enzyme production**

Synthesis of enzymes depends on the type of nutrients available to the organism (Bertolin et al., 2003). Optimization of medium composition is necessary to produce the desired enzyme. The effect of various carbon and nitrogen sources on recombinant enzyme production was studied based on the basic medium in a one-factor design. Xylose was found to be the best source to enhance enzyme production, which was 17% higher than that seen with glucose (Table II). Several early studies have demonstrated that GI production is growth-associated, therefore an increase in the biomass would improve the yield of GI (Prabakar and Raju, 1993; Deshmukh et al., 1994; Givry and Duchiron, 2008; Pınar et al., 2009). Addition of glucose to the medium would be useful to increase the volumetric productivity of E. coli cultures to high cell concentration (Tsai et al., 1987). In order to determine the optimum concentration of carbon source for enzyme production, different concentrations of glucose and xylose were used in the media. It was found that optimal enzyme production was obtained when 1.8 g/L xylose was used in combination with 0.25 g/L glucose (data

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<table>
<thead>
<tr>
<th>Culture medium</th>
<th>GI activity [U/mL]</th>
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<tbody>
<tr>
<td>TB</td>
<td>3.29 ± 0.24</td>
</tr>
<tr>
<td>SB</td>
<td>3.77 ± 0.37</td>
</tr>
<tr>
<td>SOC</td>
<td>4.00 ± 0.15</td>
</tr>
<tr>
<td>SOB</td>
<td>5.64 ± 0.27</td>
</tr>
<tr>
<td>LB</td>
<td>5.77 ± 0.24</td>
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TB, 12 g/L tryptone, 24 g/L yeast extract, 2.31 g/L potassium phosphate monobasic, 12.54 g/L potassium phosphate dibasic, 4 mL glycerol; SB, 32 g/L peptone, 20 g/L yeast extract, 5 g/L NaCl; SOB, 0.5 g/L NaCl, 2.5 mM KCl, 10 mM MgCl₂, 5 g/L yeast extract, 20 g/L tryptone; SOC, SOB supplemented with 3.6 g/L glucose; LB, 10 g/L NaCl, 5 g/L yeast extract, 10 g/L tryptone. All culture media were supplemented with antibiotics as required for selection (kanamycin, 50 μg/mL).

Fig. 1. Effect of initial medium pH value on rGI production of E. coli BL21(DE3)/pET-28a-xylA118. The bacterium was cultivated at 30 °C in LB medium and the results shown are the means of duplicate assays of duplicate shake cultures. Symbols: ■, cell density (optical density at 600 nm); ▲, enzyme activity.
For nitrogen selection, tryptic soy, casein hydrolysate, and ammonium sulfate were used, however, a further increase in enzyme production was not observed (data not shown).

**Effect of metal ions on enzyme production**

To study the effect of mineral salts on rGI production, MgSO₄ and CoCl₂ at concentrations ranging from 0 to 1 mM were added to the basal medium. The enzymatic activity reached a maximum at 180 μM of Co²⁺, while addition of Mg²⁺ did not significantly increase the enzyme production (data not shown).

Based on the results obtained from the optimization of medium components, a culture medium consisting of 10.0 g/L NaCl, 5.0 g/L yeast extract, 10.0 g/L tryptone, 1.8 g/L xylose, 0.25 g/L glucose, and 180 μM CoCl₂·6H₂O was recommended for the production of rGI. The time course of rGI production and the growth curve of *E. coli* BL21(DE3) in the recommended medium and basal LB medium are shown in Fig. 2. As can be observed from the figure, *E. coli* BL21(DE3) grew quickly and reached the stationary phase after 9 h of cultivation on optimized medium, whereas on basal LB medium it was reached only after 10–11 h. Moreover, a maximum rGI production (13.18 U/mL) was obtained after induction by IPTG for 9 h in the optimized medium.

**Purification of rGI**

The purification scheme is shown in Table III. As described in Materials and Methods, the unwanted protein could be partially removed from the crude extracts by heat treatment. In this study, the pET-28a vector carries an N-terminal His tag and further purification of rGI could be accomplished in an one-step procedure by affinity chromatography. Consequently, partially purified rGI was purified further to apparent homogeneity by a single-step procedure based on Ni²⁺ affinity chromatography via His tag (Fig. 3). The protocol used for rGI purification from *E. coli* BL21(DE3) allowed a 4.4-fold purification and 34% yield from the crude extract (Table III). Earlier, purification of GI was achieved by multi-step purification procedures that were tedious and time-consuming (Mrabet, 1992; Gong et al., 1980; Santa et al., 2005). In contrast, the purification scheme employed here was relatively simple and highly reproducible.

**Optimum pH and pH stability of rGI**

Using glucose as substrate, the pH optimum of rGI was found to be around 8.0, while it was around 7.0 in the wild-type enzyme (Gong et al., 1980).

### Table II. Effect of different carbon sources on GI production by *E. coli* BL21(DE3).

<table>
<thead>
<tr>
<th>Carbon source (1%, w/v)</th>
<th>GI activity [U/mL]</th>
</tr>
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<tbody>
<tr>
<td>Xylose</td>
<td>10.21 ± 0.46</td>
</tr>
<tr>
<td>Glucose</td>
<td>8.48 ± 0.19</td>
</tr>
<tr>
<td>Sucrose</td>
<td>8.56 ± 0.24</td>
</tr>
<tr>
<td>Maltose</td>
<td>7.98 ± 0.31</td>
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</table>

### Table III. Summary of rGI purification steps.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein [mg]</th>
<th>Total activity [U]</th>
<th>Specific activity [U/mg]</th>
<th>Yield (%)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>404.70</td>
<td>6589.31</td>
<td>16.28</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Heat treatment (70 °C, 20 min)</td>
<td>206.96</td>
<td>6401.42</td>
<td>30.93</td>
<td>97</td>
<td>1.9</td>
</tr>
<tr>
<td>Ni²⁺ affinity chromatography</td>
<td>31.62</td>
<td>2240.37</td>
<td>70.85</td>
<td>34</td>
<td>4.4</td>
</tr>
</tbody>
</table>

* Crude extract from 1 L of *E. coli* culture.
The enzyme was active in a broad pH range (5.5–9.0). As shown in Fig. 4, rGI possessed good stability in the alkaline pH range of 7.5 to 9.0. At pH values lower than 5.8, rGI denatured quickly and almost no activity was measured. In Tris-HCl buffer (pH 8.0) the enzyme’s activity was 40% less than that in sodium phosphate buffer at the same pH. Most GIs reported show pH optima ranging from 7.0–8.5 (Lee and Zeikus, 1991; Chauthaiwale and Rao, 1994; Madhavan et al., 2009; Borgi et al., 2004) and are usually stable over a wide pH range.

**Optimum temperature and thermostability of rGI**

The enzymatic activity gradually increased in the range from 45 to 85 °C, and reached its maximal activity at 85 °C, followed by a slight decrease at temperatures above 90 °C (Fig. 5a). Bhosale et al. (1996) and Karimäki et al. (2004) reported that the wild-type enzyme has an optimum temperature of 75 °C. As shown in Fig. 5b, the purified enzyme retained more than 80% of its initial activity after incubation at 70 °C for 24 h, but was rapidly inactivated, retaining only 37% of residual activity at 80 °C. After a 30-min exposure to 90 °C, the purified enzyme showed only 6% of its initial activity.

**Effect of bivalent metal ions on enzyme activity of rGI**

For most GIs, divalent cations are necessary for substrate conversion. It has been reported earlier (Sanchez and Smiley, 1975) that the presence of Mg$^{2+}$ and Co$^{2+}$ was required for the optimum activity of GI. In the present work, treatment of the purified enzyme with EDTA resulted in the loss of 90% of its activity (data not shown). However, 85% and 75% of the original activity could be restored by the addition of 10 mM Mg$^{2+}$ and Co$^{2+}$, respectively. In addition, the influence of Mg$^{2+}$ on the enzyme activity of rGI was examined by incubating the metal-free enzyme in final concentrations ranging from 1–10 mM, while Co$^{2+}$ was set at a fixed concentration of 200 μM. To investigate the influence of Co$^{2+}$ on the enzyme activity of rGI, experiments were carried out in the same way as above except that the concentration of Mg$^{2+}$ was 8 mM. As depicted in Fig. 6a, the activity of the purified enzyme increased until the concentration of Mg$^{2+}$ was in the range of 8 mM. It was also found that a small amount of Co$^{2+}$, i.e. 200 μM, was necessary to reach maximal activity (Fig. 6b). No further increase was observed with increased concentration of Co$^{2+}$, contrasting with the report by Gong et al. (1980).
Conclusion

rGI activity in optimized medium was two-fold higher than in basal medium. High-yield rGI production in *E. coli* BL21(DE3) will provide the necessary free form of GI and promote its use in lactulose synthesis. Furthermore, the advantageous features of rGI and recombinant β-galactosidase, such as good temperature stability and similar pH optimum, suggest that they could be co-immobilized on a carrier to serve as an immobilized biocatalyst for lactulose production.

Acknowledgements

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