Expression, Enzymatic Characterization, and High-Level Production of Glucose Isomerase from *Actinoplanes missouriensis* CICIM B0118(A) in *Escherichia coli*

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High-level production of recombinant glucose isomerase (rGI) is desirable for lactulose synthesis. In this study, the *xylA* gene encoding glucose isomerase from *Actinoplanes missouriensis* CICIM B0118(A) was cloned and expressed in *E. coli* BL21(DE3), and high-level production was performed by optimization of the medium composition. rGI was purified from a recombinant *E. coli* BL21(DE3) and characterized. The optimum pH value of the purified enzyme was 8.0 and it was relatively stable within the pH range of 7.0–9.0. Its optimum temperature was around 85 °C, and it exhibited good thermostability when the temperature was lower than 90 °C. The maximum enzyme activity required the presence of both Co^{2+} and Mg^{2+}, at the concentrations of 200 μM and 8 mM, respectively. With high-level expression and the simple one-step chromatographic purification of the His-tagged recombinant enzyme, this GI could be used in industrial production of lactulose as a potential economic tool.

Key words: D-Glucose Isomerase, Medium Optimization, Enzymatic Characterization

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). Current industrial production of lactulose is mainly based on the chemical isomerization reaction of lactose in basic media using different types of catalysts (Aider and Halleux, 2007). 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-

talysis 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, 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 (OD_{600}); 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 *Nde*I site at the 5'-end and an *Hin*dIII site at the 3'-end, respectively, were designed to amplify the *xylA* gene. The primers used were as follows: 5-TTTCATATGGTGAATAAAGCACACTATCG-3′ (*Nde*I) and 5′-TTCTCGAGTTCGACCCCTTCTA-3′ (*Hin*dIII). The amplified 1.2-kb *xylA* gene was cleaved by *Nde*I and *Hin*dIII 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 *Nde*I-*Hin*dIII 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 diafiltered at 4 °C against 20 mM sodium phosphate buffer (pH 7.0). Finally, the purity of the obtained enzyme was examined by SDS-PAGE. The protein concentration in the solution was estimated by the Bradford method using bovine serum albumin as a standard.

Enzyme assay

Under the standard assay conditions, 1.25 mL of the reaction mixture contained 250 μL of 8 mM $MgCl_2$, 250 μL of 200 μM $CoCl_2$, and 0.75 mL of a suitable amount of purified enzym. [The endogenous GI of *E. coli* was not active above 50 °C, hence the performance of the rGI enzyme assay ruled out the possibility that *E. coli* GI could have disturbed the enzyme assays performed with rGI (Karimäki *et al.*, 2004).] The reaction was started by addition of 1.25 mL of D-glucose to a final concentration of 1.5 M. After 60 min of incubation at 70 °C, the reaction was stopped by adding 2.5 mL of 500 mM perchloric acid. The amount of fructose produced was measured by the cysteine-carbazole-sulfuric acid method (Dische and Borenfreund, 1951). One unit of GI was defined as the amount of enzyme required to produce 1 μmol of fructose from D-glucose per min at 70 °C in this assay system.

Enzymatic characterization of rGI

The effect of the pH value on the activity and stability of rGI was investigated using the following buffers: 20 mM sodium acetate at pH 4.0 to 6.0, 20 mM sodium phosphate at pH 6.0 to 8.5, and 20 mM Tris-HCl at pH 8.0 to 9.0. To determine the pH stability, samples were pre-incubated in the above-mentioned buffers for 30 min at 70 °C. The optimum temperature of rGI activity was determined at temperatures ranging from 50 to 95 °C. Thermostability of rGI was monitored by incubating the enzyme solution at different temperatures (70, 80, and 90 °C) in 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-*xylA*118 after 10 h of induction at 30 °C using 0.6 mM IPTG, while a lower enzyme activity was achieved with pET-28a-*xylA*008. The enzyme activity of *xylA*118 was about seven times higher than that of *xylA*008. Thus the recombinant *E. coli* BL21(DE3) containing pET-28a-*xylA*118 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 SOB 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.

Table I. Effects of different culture media on expression of rGI. *E. coli* cells were cultured in each medium with 0.6 mM IPTG at 30 °C for 10 h.

Culture medium	GI activity [U/mL]
TB	3.29 ± 0.24
SB	3.77 ± 0.37
SOC	4.00 ± 0.15
SOB	5.64 ± 0.27
LB	5.77 ± 0.24

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 $MgCl_2$, 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).

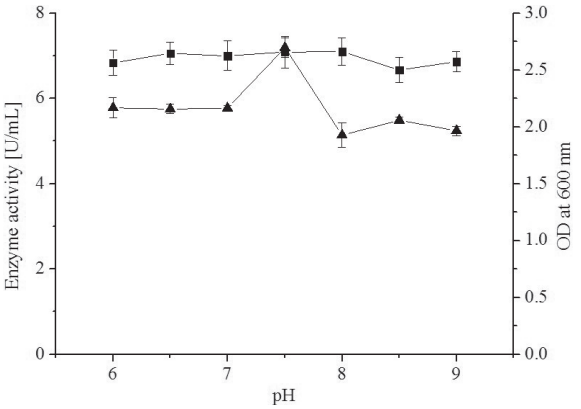

Fig. 1. Effect of initial medium pH value on rGI production of *E. coli* BL21(DE3)/pET-28a-*xylA*118. The bacterium was cultivated at 30 °C in LB medium and the results shown are the means of duplicate assays of duplicate shake cultures. Symbols: ■, cell density (optical density at 600 nm); ▲, enzyme activity.

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 (Prabhakar 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

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

Carbon source (1%, w/v)	GI activity [U/mL]
Xylose	10.21 ± 0.46
Glucose	8.48 ± 0.19
Sucrose	8.56 ± 0.24
Maltose	7.98 ± 0.31

not shown). 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, $MgSO_4$ and $CoCl_2$ at concentrations ranging from 0 to 1 mM were added to the basal medium. The enzymatic activity reached a maximum at 180 μM of Co^{2+}, while addition of Mg^{2+} 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 $CoCl_2 \cdot 6H_2O$ 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 Ni^{2+} 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.

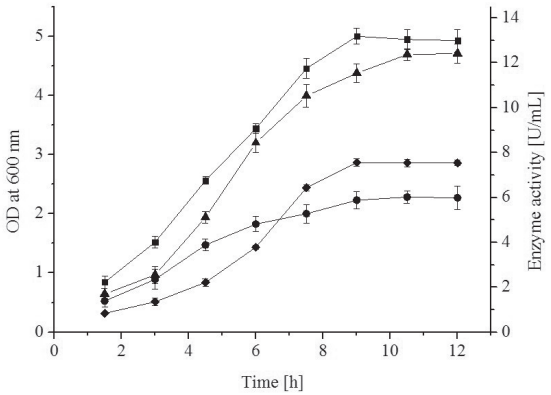

Fig. 2. Time-course profile of expression of *xylA* gene in *E. coli* and the growth curve of *E. coli* BL21(DE3)/pET-28a-*xylA*118 in optimized medium and LB medium. Symbols: ◆, growth curve in LB medium; ●, rGI production in LB medium; ▲, growth curve in optimized medium; ■, rGI production in optimized medium.

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*

Table III. Summary of rGI purification steps.

Purification step	Total protein [mg]	Total activity [U]	Specific activity [U/mg]	Yield (%)	Purification (fold)
Crude extract[a]	404.70	6589.31	16.28	100	1
Heat treatment (70 °C, 20 min)	206.96	6401.42	30.93	97	1.9
Ni^{2+} affinity chromatography	31.62	2240.37	70.85	34	4.4

[a] Crude extract from 1 L of *E. coli* culture.

al., 1980). 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).

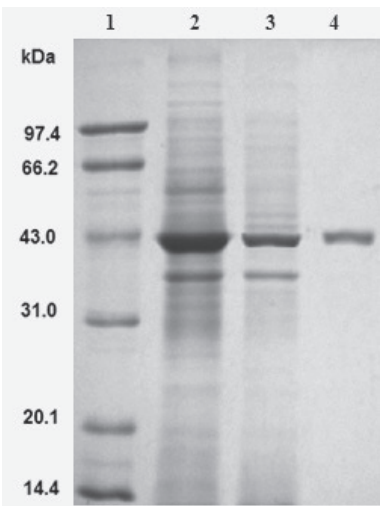

Fig. 3. Coomassie blue-stained SDS-PAGE analysis of purified rGI. Lane 1, molecular weight marker; lane 2, total crude extract; lane 3, sample from lane 2 after heat treatment; lane 4, purified GI after Ni^{2+} chelate affinity chromatography.

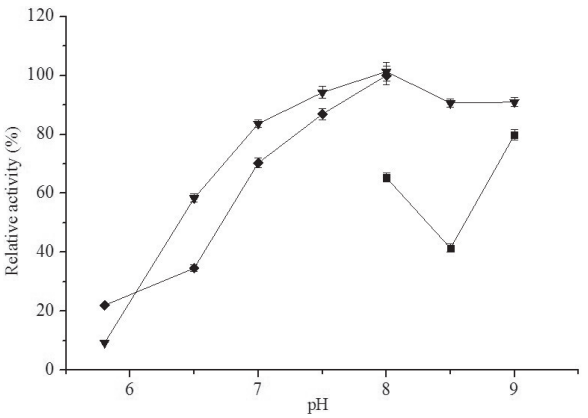

Fig. 4. Effect of pH value on rGI enzyme activity and pH stability. Enzyme activity was measured at 70 °C in buffers with different pH values. The activity at optimum pH was taken as 100%. Enzyme assays for stability were performed after 30-min exposure to different pH values. Error bars correspond to the standard deviation of three measurements. Symbols: ▼, pH stability; ◆, sodium phosphate buffer; ■, Tris-HCl.

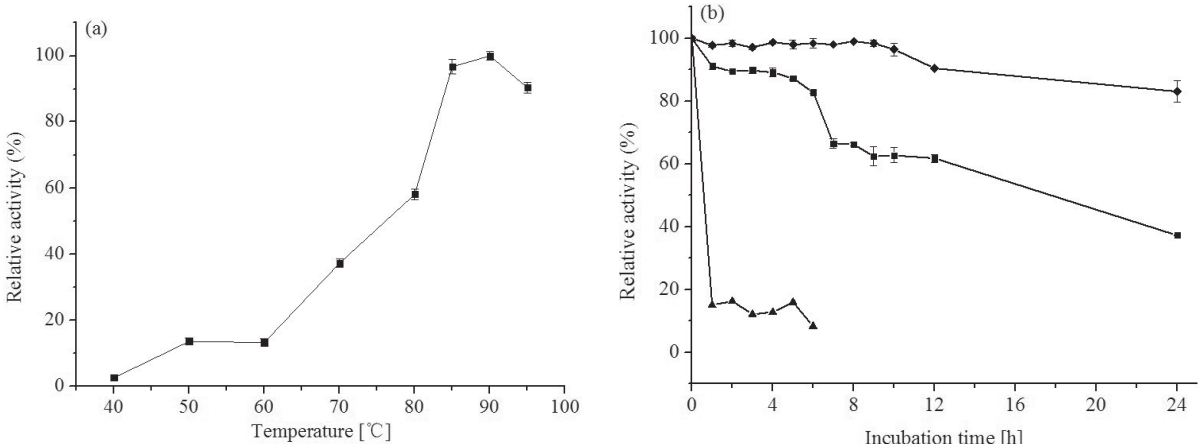

Fig. 5. Effect of temperature on rGI enzyme activity and thermal stability. (a) Temperature dependence of the enzyme activity of rGI determined at 40–95 °C. Maximum activity was taken as 100%. (b) Thermostability of the enzyme at 70 °C (◆), 80 °C (■), and 90 °C (▲). The activity of the enzyme without preincubation was taken as 100% in these experiments. Error bars correspond to the standard deviation of three measurements.

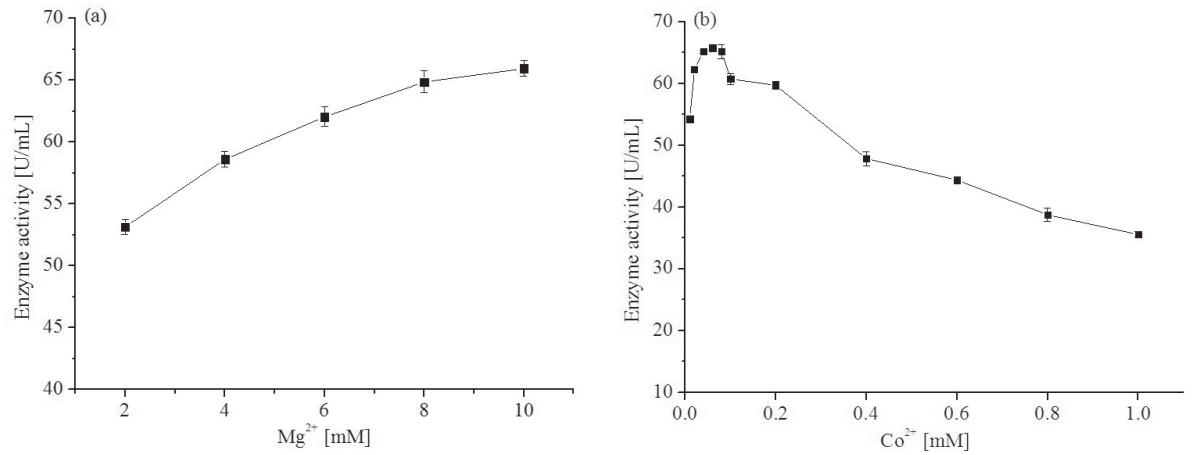

Fig. 6. Effect of bivalent metal ions on the enzyme activity of rGI. (a) Dependence of the enzyme activity on Mg^{2+} from 2 to 10 mM. (b) Dependence of the enzyme activity on Co^{2+} from 0.01 to 1 mM. Mg^{2+} or Co^{2+} was added to the standard assay at the final concentrations shown. Error bars correspond to the standard deviation of three measurements.

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.

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