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RESEARCH ARTICLE

Production of L-rhamnulose, a rare sugar, from L-rhamnose using commercial immobilized glucose isomerase

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ABSTRACT

A commercial immobilized D-glucose isomerase from *Streptomyces murines* (Sweetzyme) was used to produce L-rhamnulose from L-rhamnose in a packed-bed reactor. The optimal conditions for L-rhamnulose production from L-rhamnose were determined as pH 8.0, 60 °C, 300 g L^{-1} L-rhamnose as a substrate, and 0.6 h^{-1} dilution rate. The half-life of the immobilized enzyme at 60 °C was 809 h. Under the optimal conditions, the immobilized enzyme produced an average of 135 g L^{-1} L-rhamnulose from 300 g L^{-1} L-rhamnose after 16 days at pH 8.0, 60 °C, and 0.6 h^{-1} dilution rate, with a productivity of 81 g/L/h and a conversion yield of 45% in a packed-bed reactor.

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l-rhamnulose; rare sugar; glucose isomerase; isomerization

Introduction

Rare sugars are defined by the International Society of Rare Sugars (ISRS) as monosaccharides that rarely exist in nature (Izumori 2002) and have recently attracted much attention in the foods, pharmaceutical, and nutritional industries due to their many uses such as low-calorie sweeteners, bulking agents, antioxidants, glycosidase inhibitors, nucleoside analogues, and immunosuppressants (Doong et al. 1991; Levin et al. 1995; Muniruzzaman et al. 1996; Hossain et al. 2000; Levin 2002; Matsuo et al. 2002). Over 50 kinds of rare sugars exist in nature, including tetrose, pentose, hexose, and D- and L-forms of sugars (Izumori 2002). D-Tagatose and D-allulose have been formally approved by the US Food and Drug Administration (FDA) as Generally Recognized As Safe (GRAS) and are allowed for use in food industries (Levin 2002; Kim 2004; Mu et al. 2012).

One of the rare sugars, L-rhamnulose (6-deoxy-L-sorbose), is a precursor of furaneol, which has been used in the flavour industry and it plays important roles in sugar metabolism (Hecquet et al. 1996; Menavun et al. 2006). L-Rhamnulose has been produced using L-rhamnose isomerase, which has been isolated and characterized from various microorganisms including *Escherichia coli* (Badia et al. 1991), *Pseudomonas stutzeri* (Bhuiyan et al. 1999; Leang, Takada, Fukai, et al. 2004; Menavuvu et al. 2006), *Bacillus pallidus* (Poonperm et al. 2007), *Bacillus halodurans* (Prabhu et al. 2011), *Thermotoga mritima* (Park et al. 2010), *Mesorhizobium loti* (Takata et al. 2011), and *Dictyoglomus turgidum* (Kim et al. 2013). These L-rhamnose isomerases have been applied in the production of rare sugars such as L-lyxose, L-mannose, L-talose, D-glulose, D-allose, and L-rhamnulose.

However, using a commercial immobilized enzyme in the production of rare sugars provides enhanced stability and better process control. Therefore, here we used D-glucose isomerase (GI) from *Streptomyces murines* (xylose isomerase, Sweetzyme™), a commercially available GI obtained from Novozyme, Denmark, and characterized and optimized it for L-rhamnulose production. Under the optimized conditions, continuous L-rhamnulose production from L-rhamnose was performed in a packed-bed reactor.

Materials and methods

Materials

Immobilized GI from *Streptomyces murines* (Sweetzyme) was purchased from Novozyme (Kalundborg, Denmark) under the trade name Sweetzyme IT extra. Authentic standard sugars and all

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other regents were purchased from Sigma (St. Louis, MO, USA).

Enzyme assay

The reaction was performed in 50 mM EPPS buffer (pH 8.0) containing 10 mM L-rhamnose and 1.5 U·mL^{-1} enzyme at 60 °C for 10 min. One unit (U) of enzyme activity was defined as the amount of enzyme required to produce 1 μmol L-rhamnulose from L-rhamnose per minute at 60 °C and pH 8.0.

Analytical methods

The monosaccharide concentrations were determined using a Bio-LC system (Dionex ICS-3000, Sunnyvale, CA, USA) with an electrochemical detector and a CarboPac PAI column. The column was eluted at 30 °C with 0.1 M NaOH (0–5 min), followed by a linear gradient (5–35 min) of sodium acetate (0–0.2 M) at 1 mL·min^{-1}.

Results and discussion

Effects of pH and temperature on L-rhamnulose production

The isomerization activity for L-rhamnulose production using immobilized GI was examined in 50 mM PIPES buffer (pH 6.5–7.5) and EPPS buffer (pH 7.5–9.0) containing 10 mM L-rhamnose at 60 °C for 10 min (Figure 1(a)). Maximum activity was observed at pH 8.0. The activity was >80% of the maximum at pH 7.0 and 9.0. The optimum pH values for L-rhamnulose production from L-rhamnose using L-rhamnose isomerase from *Bacillus pallidus* Y25 (Poonperm et al. 2007), *Pseudomonas stutzeri* (Leang, Takada, Fukai, et al. 2004, Takada, Ishimura, et al. 2004), and *Dictyoglomus turgidum* (Kim et al. 2013) were 7.0, 9.0, and 8.0, respectively.

Next, the isomerization activity for L-rhamnulose production using immobilized GI was examined in a temperature range from 40 °C to 95 °C at pH 8.0. Maximum activity was observed at 90 °C (Figure 1(b)). The thermostability of the immobilized GI during L-rhamnulose production from L-rhamnose was determined by measuring enzyme activity as a function of time and temperature. Thermal inactivation of the enzyme followed first-order kinetics. The half-lives of the enzyme at 50 °C, 60 °C, 70 °C, 80 °C, and 90 °C were 918, 809, 265, 24, and 13 h, respectively (Figure 2). The immobilized GI showed maximum activity at 90 °C, but the thermostability at 90 °C was too low for L-rhamnulose production. The optimum temperatures for L-rhamnulose production from L-rhamnose using L-rhamnose isomerase from *B. pallidus* Y25 (Poonperm et al. 2007), *P. stutzeri* (Menavuvu et al. 2006), and *D. turgidum* (Kim et al. 2013) are 65 °C, 60 °C, and 70 °C, respectively.

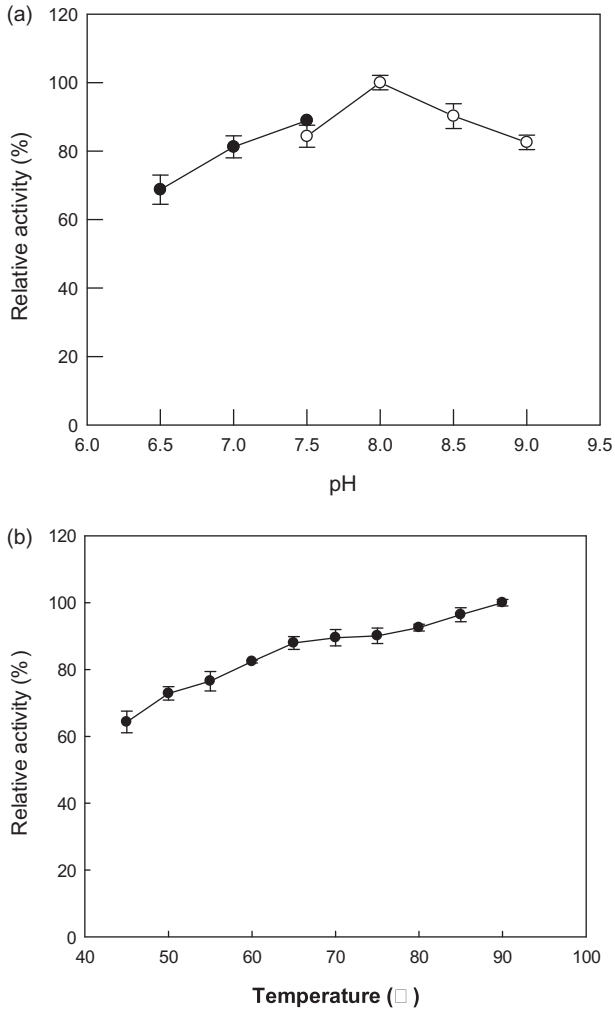

Figure 1. Effect of pH (a) and temperature (b) the immobilized glucose isomerase (GI) activity. (a) The reactions were performed in 50 mM PIPES buffer (pH 6.5–7.5) and EPPS buffer (pH 7.5–9.0) containing 10 mM L-rhamnose at 60 °C for 10 min. (b) The reactions were performed in 50 mM EPPS buffer (pH 8.0) containing 10 mM L-rhamnose at 45–90 °C for 10 min. Data represent the means of three experiments and error bars represent standard deviation.

These results suggested that the optimum pH and temperature for L-rhamnulose production is pH 8.0 and 60 °C, respectively, and the conversion yield of L-rhamnose to L-rhamnulose was approximately 45%. The conversion yield of L-rhamnulose from L-rhamnose using L-rhamnose isomerase from *B. pallidus* Y25 (Poonperm et al. 2007), *P. stutzeri* (Shompoosang et al. 2014), and *D. turgidum* (Kim et al. 2013) were similar at 40–45%. The optimum pH and temperature for

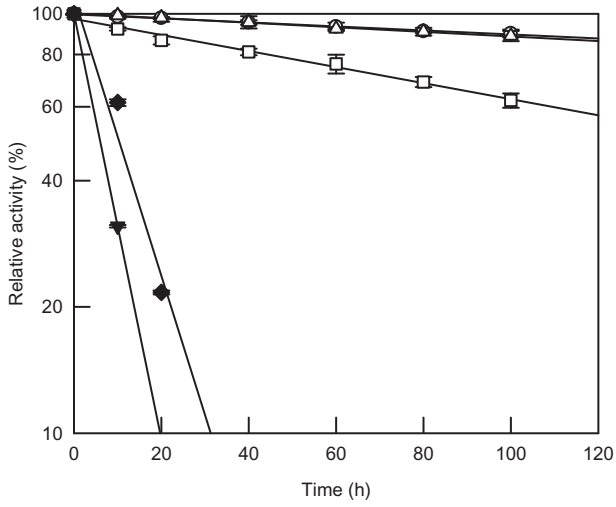

Figure 2. Thermal inactivation of immobilized GI at 50 °C (*open circle*), 60 °C (*open triangle*), 70 °C (*open square*), 80 °C (*closed diamond*), and 90 °C (*closed inverted triangle*). The enzymes were incubated at temperatures ranging from 50 °C to 90 °C for varying periods of time. A sample was withdrawn at each time interval and assayed for the remaining enzyme activity in 50 mM EPPS buffer (pH 8.0) containing 10 mM L-rhamnose at 60 °C for 10 min. The experimental data for thermal deactivation of the enzyme were fitted to a first-order curve and the half-lives of the enzyme were calculated using Sigma plot 10.0 software (Systat software, San Jose, CA, USA). Data represent the means of three experiments and error bars represent standard deviation.

D-fructose production from D-glucose using GI were pH 8.0–8.5 and 60–70 °C, respectively.

Optimization of L-rhamnulose production using a packed-bed reactor

Next, the substrate concentration of L-rhamnose required to produce L-rhamnulose using GI was investigated. Immobilized GIs were packed into a bioreactor (XK 16; Amersham Pharmacia Biotech, Uppsala, Sweden) with a working volume of 40 mL. A solution of 50 mM EPPS buffer (pH 8.0) containing 50–500 g L^{-1} L-rhamnose in the feeding reservoir was fed continuously into the reactor, and the effluent was allowed to flow out of the reactor to the outside reservoir using a peristaltic pump (Watson-Marlow 101 U/R, Cornwall, UK). The temperature was maintained at 60 °C using a water circulator (VTRC-620; Jeio Tech, Daejon, Korea). The optimum concentration of L-rhamnose for L-rhamnulose production using immobilized GI was determined as 300 g L^{-1} (Figure 3). As the concentration of L-rhamnose varied from 50 to 500 g L^{-1}, maximum L-rhamnulose production was observed using 400 g L^{-1} L-rhamnose after 2 h. L-Rhamnulose production reached a plateau at about 400 g L^{-1} L-rhamnose. The conversion yield of L-rhamnulose from L-rhamnose

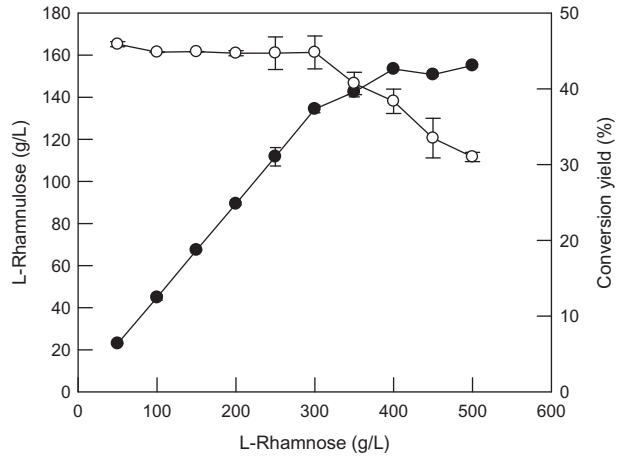

Figure 3. Effect of substrate concentration on L-rhamnulose production (closed *circle*) from L-rhamnose, and conversion yield of L-rhamnulose from L-rhamnose (open *circle*) in a packed-bed reactor. The reactions were performed in 50 mM EPPS buffer (pH 8.0) containing 50–500 g L^{-1} L-rhamnose at 60 °C with a dilution rate of 0.6 h^{-1}. Data represent the means of three experiments and error bars represent standard deviation.

was constant for 300 g L^{-1} L-rhamnose, but decreased with increasing L-rhamnose concentration above 300 g L^{-1}. The decrease of the conversion yield with increasing the concentration of L-rhamnose may be due to substrate inhibition at high concentrations.

Next, we monitored L-rhamnulose production at different dilution rates ranging from 0.075 to 2.25 h^{-1} using 300 g L^{-1} L-rhamnose in a packed-bed reactor (Figure 4). Until a dilution rate of 0.6 h^{-1}, the productivity increased with constant conversion yield. However, beyond 0.6 h^{-1}, the conversion yield began to decrease. At higher dilution rate reduced L-rhamnose retention time would lead to decrease productivity. Thus, the optimal dilution rate for continuous L-rhamnulose production was determined as 0.6 h^{-1}. Consistent with this result, Kim et al. (2013) previously reported the optimum conditions for L-rhamnulose production from L-rhamnose using immobilized L-rhamnose isomerase from *D. turgidum* as 300 g L^{-1} L-rhamnose as a substrate with a dilution rate of 0.6 h^{-1}.

Continuous L-rhamnulose production in a packed-bed reactor

The optimal conditions for L-rhamnulose production from L-rhamnose were determined as pH 8.0, 60 °C, and a dilution rate of 0.6 h^{-1}. We then used these conditions to evaluate the long-term operation of the immobilized GI for continuous L-rhamnulose production in a packed-bed reactor for 30 days.

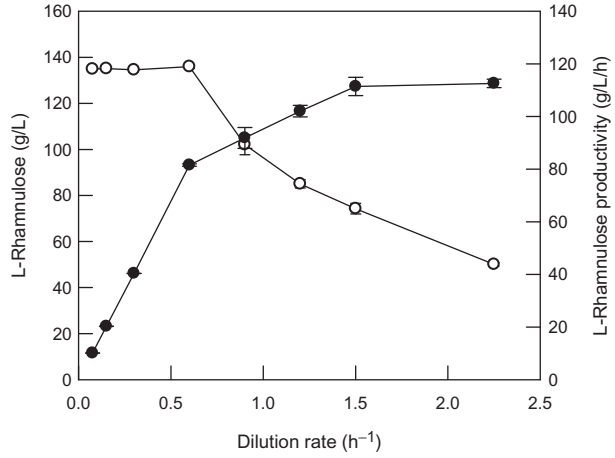

Figure 4. Effect of the dilution rate on L-rhamnulose production (*closed circle*) and productivity (*open circle*) from L-rhamnose in a packed-bed reactor. The working volume of the reactor was 40 mL. A solution of 50 mM EPPS buffer (pH 8.0) containing 300 g L^{-1} L-rhamnose in the feeding reservoir was fed continuously into the reactor, and the effluent was allowed to flow out of the reactor to the outside reservoir using a peristaltic pump. The temperature was maintained at 60 °C using a water circulator. Data represent the means of three experiments and error bars represent standard deviation.

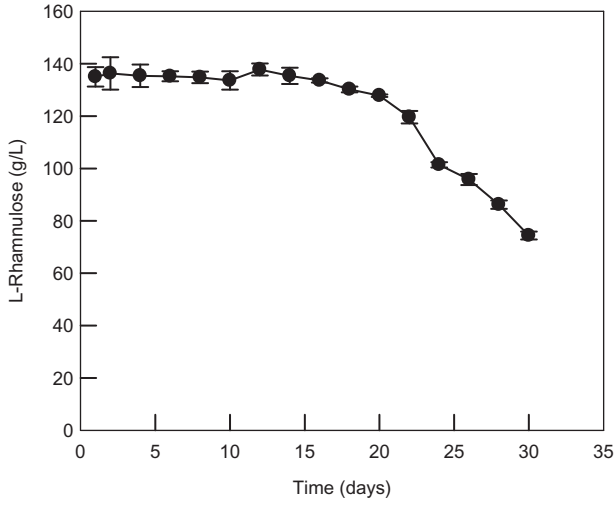

Figure 5. Continuous L-rhamnulose production from L-rhamnose using immobilized GI in a packed-bed reactor. A solution of 50 mM EPPS buffer (pH 8.0) containing 300 g L^{-1} L-rhamnose in the feeding reservoir was fed continuously into the reactor, and the effluent was allowed to flow out of the reactor, with a dilution rate of 0.6 h^{-1}. The reaction was performed at 60 °C for 30 days. Data represent the means of three experiments and error bars represent standard deviation.

The immobilized enzyme continuously produced an average of 135 g L^{-1} L-rhamnulose from 300 g L^{-1} L-rhamnose within 16 days, with a productivity of 81 g/L/h and a conversion yield of 45% (Figure 5). L-Rhamnulose concentration was reduced to approximately 55% at day 30. In a previous study, L-rhamnulose has been continuously produced using immobilized L-rhamnose isomerase from *D. turgidum* in a packed-bed bioreactor, and an average of 130 g L^{-1} L-rhamnulose was produced from 300 g L^{-1} L-rhamnose within 10 days, with a productivity of 78 g/L/h and a conversion yield of 43% (Kim et al. 2013). Thus, the two enzymes, immobilized GI and L-rhamnose isomerase from *D. turgidum*, for L-rhamnulose production show similar results with respect to L-rhamnulose production, productivity, and conversion, but the stability of the immobilized GI was three times higher than that of L-rhamnose isomerase from *D. turgidum* in a packed-bed reactor. The use of commercial immobilized GI also has the advantage of producing L-rhamnulose in an economical way without the need for microbial culture, enzyme purification, and immobilization.

Conclusions

We describe optimal operating conditions and long term stability for continuous L-rhamnulose production from L-rhamnose in a packed-bed reactor using commercial immobilized GI from *Streptomyces murines* (Sweetzyme). The packed-bed reactor system produced an average of 135 g L^{-1} L-rhamnulose from 300 g L^{-1} L-rhamnose within an operation time of 16 days at pH 8.0, 60 °C, and 0.6 h^{-1} dilution rate, with a productivity of 81 g/L/h and a conversion yield of 43%. This system provides enhanced L-rhamnulose productivity from L-rhamnose and offers long-term, continuous L-rhamnulose production. This is a new approach for the production of L-rhamnulose by commercial enzyme and to the best of our knowledge, this is the first report describing the enzymatic production of L-rhamnulose using immobilized GI. Although immobilized GI (Sweetzyme) has been mainly used for high fructose syrup production in the past, our findings indicate that it shows potential for use in the production of various rare sugars as well.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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