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Continuous Production of Fructose-Syrups from Inulin by Immobilized Inulinase from Recombinant *Saccharomyces cerevisiae*

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Recombinant exoinulinase was partially purified from the culture supernatant of *S. cerevisiae* by $(NH_4)_2SO_4$ precipitation and PEG treatment. The purified inulinase was immobilized onto Amino-cellulofine with glutaraldehyde as a cross-linking agent. Immobilization yield based on the enzyme activity was about 15%. Optimal pH and temperature of immobilized enzyme were found to be 5.0 and 60°C, respectively. The enzyme activity was stably maintained in the pH ranges of 4.5 to 6.0 at 60°C. 100% of enzyme activity was observed even after incubation for 24 hr at 60°C. In the operation of a packed-bed reactor containing 412 U inulinase, dahalia inulin of 7.5%(w/v) concentration was completely hydrolyzed at flow rate of 2.0 mL/min at 60°C, resulting in a volumetric productivity of 693 g-reducing sugars/L/h. Under the reaction conditions of 1.0 mL/min flow rate with 2.5% inulin at 60°C, the reactor was successfully operated over 30 days without loss of inulinase activity.

Key words: Immobilization, recombinant inulinase, fructose-syrup, packed-bed reactor

INTRODUCTION

Inulin is a polyfructan found as a carbohydrate reserve in the roots and tubers of plants like Jerusalem artichoke, chicory, and dahlia. It consists of linear chains of fructose residues linked by β-2,1 bonds and terminated by a glucose residue. Degree of polymerization varies widely from 3 to about 35 fructose units. Inulin has been received a cosiderable attention as a potential substrate for the production of ethanol, and sweeterners such as fructose, inulooligosaccharide and difructose anhydride.

The yeast *Kluyveromyces marxianus* has been known to produce extracellular inulinase (2,1-β-D-fructan fructanohydrolase, EC 3.2.1.7) that liberates fructose molecules from inulin successively [1]. Enzymatic hydrolysis of inulin is more efficient process for the production of fructose than the chemical hydrolysis and other enzymatic treatments of starch materials in which three enzymes are necessary, and yields up to 75% of fructose [2]. Until now, various immobilization methods employing inulinase-producing microbial cells or inulinase enzymes have been reported to produce fructose from inulin [3]. However, productivity or operational stability was unsatisfactory to produce large quantity of fructose for a prolonged period. Recently, the structural gene (*INU1*) of the *K. marxianus* inulinase was cloned and expressed in *Saccharomyces cerevisiae* [4]. The recombinant inulinase expressed in *S. cerevisiae* showed enhanced thermostability, due to hyperglycosylation in the recombinant enzyme. In this paper, we partially purified the recombinant inulinase from *S. cerevisiae*, and investigated optimal conditions for enzyme immobilization on Amino-cellulofine by cross-linking agent. Also, we evaluated the performance of packed-bed enzyme reactor for the continuous production of high-fructose syrups from inulin.

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MATERIALS AND METHODS

Materials

Amino-cellulofine (Seikagaku Kogyo, Japan), chitin and amberlite (Sigma, USA) were used as support materials for immobilization of recombinant inulinase. 1-ethyl-3-(3-dimethyl- aminopropyl)-carbodiimide hydrochloride (EDC), 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluene sulfonate (CMC) (Wako, Japan) or glutaraldehyde (Sigma, USA) was used as cross-linking agents. Pure inulin from dahlia tubers (Sigma, USA) and raw inulin prepared from Korean Jerusalem artichoke by autoclaving were used as substrates [3].

Purification of Inulinase

Recombinant *Saccharomyces cerevisiae* SEY2102 harboring plasmid pYI10 [5] was cultivated on YPDG medium (1% yeast extract, 1% Bactopeptone, 1% glucose, 1% galactose) in a 5 L jar fermentor at 30°C for 2 days. The culture supernatant was concentrated by PEG20,000 treatment and then precipitated with 70% ammonium sulfate. The resulting supernatant was dialyzed against DW and reconcentrated by PEG20,000 treatment. After freeze-drying the concentrated enzyme solution, a portion of dried enzyme was appropriately diluted with 0.1 M acetate buffer (pH5.0) and used for immobilization. Protein concentration was determined by Bicinchoninic acid method [6], using bovine serum albumin as a standard.

Assay of Inulinase Activity

The inulinase activity was assayed by measuring the

concentration of reducing sugar released from inulin [5]. One unit of inulinase activity was defined as the amount of enzyme that can liberate 1.0 μmole of fructose equivalent from inulin per minute at 55°C.

Immobilization of Recombinant Inulinase

The immobilization method was as follows [7]. Support resins were washed with DW. 500 mg wet wt. of each support and condensing reagent (20 mg EDC or CDC, or 2% glutaraldehyde) were added to 1.0 mL of inulinase solution (pH6.0), and then stirred gently at 4°C for 24 hr. Immobilized enzyme was collected by filtration, and washed with DW until no prtein was detected in the washings. The immobilized enzyme was suspended in 0.1M acetate buffer (pH5.0) and stored at 4°C until used. The immobilization yield (%) was defined as [inulinase activity of the immobilized enzyme/ (inulinase activity loaded - inulinase activity in the filtrate and washings)] × 100.

Operational Conditions for Packed-bed Reactor

Two packed-bed column reactors were designed with glass columns (1×9 cm or 1×18 cm) surrounded by water jacket and were packed with immobilized inulinase to bed height of 9 or 18 cm. The packed column reactor was operated at different temperatures of 30°C to 60°C. Inulin solution of 2.5% to 10% in 0.1 M acetate buffer (pH5.0) was continuously fed into the reactor with flow rates of 1.0-6.0 mL/min. The volumetric productvity of the reactor, Q_p (grams of product/L/h), was calculated from $Q_p=C_pF/V$ where V is reactor volume (liter), F is the flow rate (L/h), and Cp (g/L) is the concentration of reducing sugars produced.

Analysis of Reaction Products by HPLC

Hydrolysed inulin in the effluent from the reactor was analysed by HPLC (Waters LC-1, Aminex HPX-42C column). The concentration of total sugar was also determined by HPLC after acid hydrolysis of inulin in 0.1 N HCl at 100°C for 1.0 hr. Extent of inulin hydrolysis (%) was calculated as (amounts of reducing sugars/amount of tatal sugars in loaded inulun) × 100.

RESULTS AND DISCUSSION

Purification of Recombinant Inulinase

From the batch culture of recombinant *S. cerevisiae* strain containing pYI10 plasmid, 5.9 U/mL inulinase was produced in the extracellular medium. Preliminary experiments indicated that the recombinant inulinase could not be precipitated successfully by ammonium sulfate, acetone or ethanol. However, it was found that about 70% of the inulinase activity was retained in the supernatant after 70% ammonium sulfate precipitation. Therefore, we initially reduced the volume of the culture supernatant by PEG treatment and then removed most of other proteins by ammonium sulfate precipitation. The resulting supernatant was concentrated to 37.2 mL by PEG treatment. Overall purification steps are summarized in Table 1. As shown in Fig. 1, a broad band of inulinase about 90 to 150 kDa was detected on SDS-PAGE, indicating that the recombinant inulinase was hyperglycosylated as that of *K. marxianus* [8]. The specific activity of partially purified inulinase was evaluated to be 18.3 U/mg-protein toward inulin and 88.7 U/mg-protein toward sucrose, resulting in the S/I ratio of 5.8.

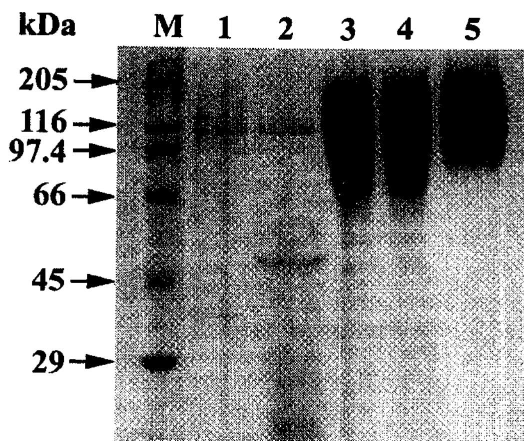

Fig. 1. SDS-PAGE of crude and purified recombinant inulinase. Lanes M, protein marker (Sigma, MW-SDS-200); lane 1, culture supernatant; lane 2, precipitates after 70% ammonium sulfate precipitation; lane 3, precipitates after acetone precipitation; lane 4, precipitates after ethanol precipitation; lane 5, purified recombinant inulinase.

Optimization of Immobilization

For the immobilization of recombinant inulinase, three kinds of support materials such as Amino-cellulofine, amberite, and chitin were examined. On the basis of activity yields, Amino-cellulofine showed better recovery yield of 15% than those of chitin (10%) and amberite (4%) when 2% glutaraldehyde was used as a cross-linking agent. The immobilization capacity of cross-linking agents such as EDC, CMC and glutaraldehde was compared at their various concentrations. The 500 mg (wet wt.) of the Amino-cellulofine and 1.0 mL of enzyme solution (153 U/mL) were gently mixed and then cross-linked at desirable concentrations. Among them, glutaraldehyde showed the highest immobilization yield of 15% at 2% glutaraldehyde. Various inulinase concentrations ranging from 1.0 to 27 mg/mL were examined for the determination of optimal enzyme concentration. The activity of the immobilized in-

Table 1. Summary of purification of extracellular inulinase from recombinant *S. cerevisiae*

Procedure	Volume (mL)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purified fold	Yield (%)
Supernatant	1800	9551.8	10656.0	1.1	1.0	100.0
PEG	565	6252.5	10079.7	1.6	1.5	94.6
70% $(NH_4)_2SO_4$ Supernatant	1360	1677.3	7126.4	4.2	3.8	66.9
PEG	37.2	427.8	6553.8	15.3	13.9	61.5

Table 2. Effect of reaction pH on immobilization of inulinase from *S. cerevisiae* SEY2102/pYI10 onto Amino-cellulofine

pH	Activity loaded (U)	Activity in supernatant (U)		Activity in immobilized inulinase (U)		Activity yield (%)	
		CMC	Glutaraldehyde	CMC	Glutaraldehyde	CMC	Glutaraldehyde
4.0	153.2	76.1	54.5	13.8	25.6	8.98	13.00
5.0	153.2	67.1	54.2	11.8	22.4	6.89	11.29
6.0	153.2	64.2	54.1	16.0	29.4	8.94	14.84

ulinase increased up to 10 mg/mL, and reached 29.4 U/g-support. Over the range of 10 to 27 mg/mL, total activity of the immobilized enzyme was nearly constant, while the immobilization yields were gradually decreased from 15% to 12%. As a consequence, 10 mg/mL of enzyme solution (about 306.4 U/g-support) was used in the subsequent experiments. When diluted inulinase solutions (153.2 U/mL) with different pH values were used for the immobilization, maximum immobilization yield of 15% was observed at pH 6.0 (Table 2). These optimized immobilization conditions for the recombinant inulinase are summarized in Table 3.

Properties of Immobilized Inulinase

Optimal pH and temperature of the immobilized inulinase were found at pH 4.5-5.0 and 60°C, respectively, while those of free recombinant enzyme were pH5.0 and 55°C. The activity of the immobilized inulinase was stably maintained at 60°C. About 75% of the immobilized inulinase activity was remained after incubation for 1 hr at 65°C, which was superior when compared to 40% activity of the free enzyme at 60°C after 1.0 hr incubation. In addition, pH stability of the immobilized enzyme was greatly improved, since loss of enzyme activity was not observed after 24 hr incubation in both the pH ranges of 4.0 to 6.0 at 40°C and 5.0 to 6.0 at 60°C.

Continuous Production of Fructose-Syrups from Inulin

To determine operating conditions for complete hydrolysis of inulin, two packed-bed reactors (1× 9 cm and 1× 18 cm) containing immobilized inulinase (total activities, 206 U and 412 U) were operated with various inulin concentrations (2.5-10%), flow rates (1.0-6.0 mL/min), and temperatures (30-60°C). As shown in Fig. 2, extent of inulin hydrolysis with 1× 9 cm reactor gradually decreased according to increase in the inulin concentration or the flow rate. When the reactor volume was doubled (1× 18 cm reactor), inulin hydrolysis of more than 65% was observed even at 60°C, 10% inulin, and 6 mL/min flow rate. At 60°C, the complete hydrolysis of inulin was obtained with 7.5% inulin and 2 mL/min flow rate. Under these conditions, the volumetric productivity was evaluated to be 693 g-reducing sugars/L/h (equivalent to 614 g-fructose/L/h). This productivity was superior to that of immobilized inulinase from *Aspergillus niger*, in which the productivity of 410 g-reducing sugars/L/h was obtained with 5% inulin [7].

Table 3. Optimized conditions for the immobilization of recombinant inulinase

Parameters	Conditions
Support	500 mg-wet wt. Amino-cellulofine
Cross-linking Agent	2% Glutaraldehyde
Temperature	4°C
Enzyme Concentration	10 mg/mL (153.2 U/mL)
pH	6.0

When the inulin hydrolysate in the effluent of reactor was analysed by HPLC, it consisted of 86% fructose, 11% glucose, and 3% inulo-oligosaccharides respectively (data not shown). Complete hydrolysis of raw Jerusalem artichoke inulin occurred at flow rate of 2 mL/min with 5% inulin and 1.0 mL/min with 7.5% inulin at 60°C (Fig. 2). Major products of raw inulin hydrolysate were fructose (68%), glucose (11%), and inulo-olgosaccharides (21%) (data not shown).

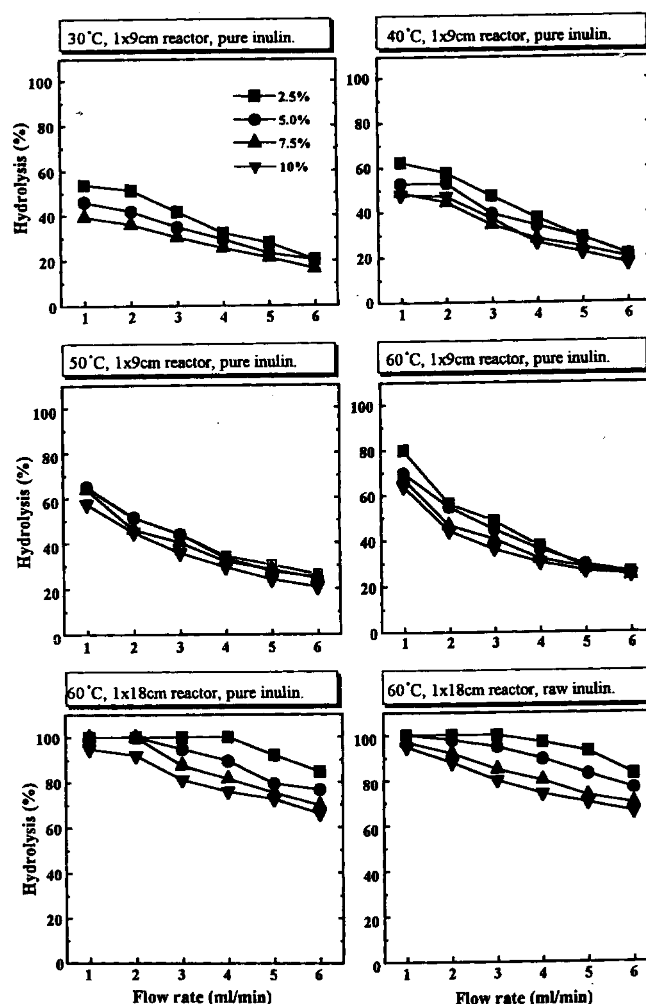

Fig. 2. Inulin hydrolysis as functions of inulin concentration and flow rate in packed-bed column reactors (1× 9 cm or 1× 18 cm) containing immobilized recombinant inulinase. Symbols: ■, 2.5% inulin; ●, 5% inulin; ▲, 7.5% inulin; ▼, 10% inulin.

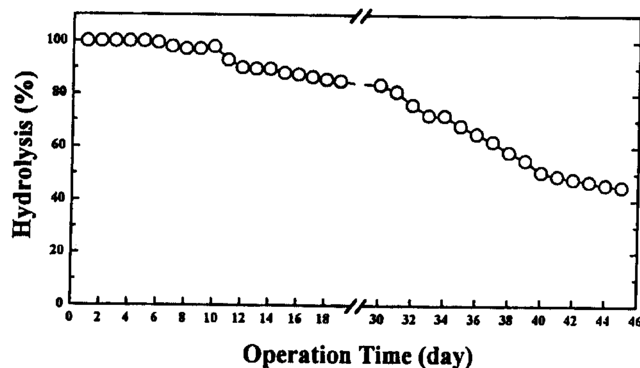

Fig. 3. Operational stability of packed-bed reactor containing immobilized recombinant inulinase. Packed-bed reactor was operated with inulin solution of 2.5% and flow rate of 1 mL/min at 60°C.

Operational Stability

Operational stability of the reactor was determined with 2% inulin and flow rate of 1 mL/min at 60°C (Fig. 3). Hydrolysis of inulin to reducing sugars proceeded continuously up to 30 days at 60°C. The half life of reactor at 60°C was about 40 days.

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