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Downstream Processing of Inulinase

Comparison of Different Techniques

ADALBERTO PESSOA, JR.,*[,1] AND MICHELE VITOLO[2]

[1]*Departamento de Biotecnologia/FAENQUIL-CP116, CEP. 12.600-000-Lorena/SP-Brazil; and* [2]*Biochemical and Pharmaceutical Technology Departmento/FCF/USP-P.O. Box 66083- CEP 05315-970-São Paulo/SP-Brazil*

ABSTRACT

Candida kefyr DSM 70106 was cultivated in a medium containing inulin as a carbon source. About 92% of the inulinase was recovered directly from the medium. Different concentration (C_f) and enrichment (E_f) factors were obtained, using the following methods: Cross-flow filtration (microfiltration and cell diafiltration were carried out using a rotary filter; enzyme ultrafiltration and diafiltration were performed using a cassette module): C_f = 7.5 and E_f = 2.2; liquid–liquid extraction of *N*-Benzyl-*N*-Dodecyl-*N*-*bis*[2-hydroxyethyl] ammonium chloride (BDBAC) reversed micelles: C_f = 2.5 and E_f = 2.7; and expanded-bed adsorption: C_f = 2.8 and E_f = 4.3.

Index Entries: Inulinase; downstream; cross-flow filtration; expanded-bed adsorption; reversed micelles; *Candida kefyr*.

Nomenclature: E_f; Enrichment Factor; C_f; Concentration Factor.

INTRODUCTION

Fructose has drawn much attention because of its organoleptic and sweetening properties *(1)*. This sugar is generally obtained in the form of syrup, by isomerization of glucose coming from maize hydrolysis, which normally involves the activities of several enzymes *(2)*. An alternative to this process is the enzymatic hydrolysis of inulin, a polyfructosan composed of a linear chain of D-fructofuranosides.

Inulinase (2,1 β-D-fructanfructanohydrolyse EC 3.2.1.7) is an enzyme that hydrolyzes inulin (β-D-fructofuranoside bearing a single α-D-glucopyranosyl unit) *(3)*. It is mainly produced by yeasts *(4)* and plants *(5)*.

* Author to whom all correspondence and reprint requests should be addressed.

Candida kefyr has high potential for producing this enzyme, since it reaches enzymatic activity levels comparable to those of the best strains of other species tested *(6,7)*. The industrial application of inulinase will only be viable if this enzyme is available on the market in large quantities at competitive prices *(8)*. Thus, to enhance inulinase production appropriate and feasible recovery techniques would be needed.

The costs of the extracellular enzyme recovery directly from the medium are significant. Among several separation techniques described in the current literature, with potential for scaling-up the process, cross-flow filtration, liquid–liquid extraction by reversed micelles, and expanded-bed adsorption are promising *(7,9)*.

In the case of cross-flow filtration, some fluid-dynamic characteristics have to be considered, as well as the kind of membrane, the filter geometry, and the filtration modules (rotary and cassette). The liquid–liquid extraction by reversed micelles is highly selective, specific, and, in general, compatible with proteins *(10)*. This method is able to provide desirable levels of protein concentration and purification *(11)*. Expanded-bed adsorption of proteins using Streamline™ is a recent method developed by Pharmacia (Uppsala, Sweden). It permits the recuperation of proteins without previous cell removal from the cultivated media, and efficiently replaces unitary operations such as centrifugation, extraction, and filtration *(12)*.

This work compares the yields and the purification and concentration factors of inulinase recovered directly from the medium.

MATERIALS AND METHODS

Inulinase Production

For inulinase production, *C. kefyr* DSM 70106 was grown in a 300 L fermenter containing 200 L of medium: inulin (10.0 g/L); yeast extract (2.8 g/L); peptone (6.5 g/L); $MgSO_4 \cdot 7H_2O$ (0.05 g/L); urea (2.25 g/L); KH_2PO_4 (0.30 g/L); and $CaCO_3$ (0.01 g/L). The culture was carried out batchwise, for 72 h under the following conditions: 30°C, pH 5.0, and K_La/43 h. The inulinase content in the supernatant was in the range of 30–40 U/mL, and the total protein concentration was 4–5 mg/mL.

Inulinase Activity Measurement

Assays were made to measure the inulinase (2,1 β-D-fructan-fructanohydrolase, EC 3.2.1.7) in the fermentation broth, as follows: 0.20 mL of enzyme solution was mixed in a test tube with 0.80 mL of 4.0%(w/v) buffered inulin solution (0.10 *M* acetic acid/acetate buffer, pH 5.0), and the mixture was maintained at 50°C for 10 min. The reaction was stopped by immersing the test tube in boiling water. The amount of fructose was determined by the Boehringer enzymatic assay (Boehringer Mannheim,

Mannheim, FRG). One inulinase unit was defined as the amount of enzyme that catalyzes the formation of one μmol of fructose per minute under the test conditions.

Protein Determination

The amount of total protein in the filtration and adsorption tests was measured according to the Comassie blue method described by Bradford *(13)*; Lowry's method *(14)* was employed in the reversed-micelle liquid–liquid extraction. Bovine serum albumin was used as a protein concentration standard.

Cell Mass Determination

The cell concentration was obtained by means of a calibration curve to correlate optical density (OD) with dry wt (g/L).

Cross-Flow Microfiltration (Concentration and Diafiltration)

Cell harvesting by microfiltration was carried out batchwise using the rotary filter, Biodruckfilter (BDF), a prototype developed by Sulzer AG, Winterthur, Switzerland. The filter unit consists of two coaxial cylinders made of stainless steel, of which the inner is rotary and the outer cylinder is fixed. The inner cylinder is driven by an electrical motor. From a reservoir, the suspension is fed to the inlet at the lower end by a metering pump, and the concentrate is discharged by a second metering pump from the outlet at the top. The filtrate leaves the machine through a central filtrate channel at the bottom of the rotor. The outer housing and the filtrate line are cooled during processing. The suspension is pumped through the module. The retentate was continuously concentrated by repeated returning to the reservoir. The BDF technical specifications and operational conditions used in the experiments were: total membrane (PTFE 0.2 μm) area, 0.04 m^2; annular distance,: 4 mm; length of rotary cylinder, 200 mm; radius of rotary cylinder, 33 mm; radius of cylinder mantle, 37 mm; rotation speed, 3300 rpm (peripheral velocity of 11.6 m/s); pressure regime (transmembrane), ~0.5 bar; and feed rate of 30 L/h. The operation pressure was controlled by valves used for adjusting the transmembrane pressure.

Cross-Flow Ultrafiltration and Diafiltration

Plate and frame cassette devices from Filtron (Karlstein, Germany) were employed, respectively, for enzyme concentration by ultrafiltration and removal of low molecular compounds by diafiltration. Cassettes are multiple layers of ultrafiltration membranes placed between polymeric retentate separators. This type of filter consists of plates and frames arranged alternately and supported by a pair of rails. The plate has a ribbed surface. The feed channel is formed by a hole that admits feed into the

frame, and at the bottom there is an outlet for the filtrate. The surface area of the PTFE membrane was 0.07 m^2, the "cutoff" 100 kDa, the transmembrane pressure about 1.0 bar, and the feed flow 225 L/h. The temperature was maintained constant at 25°C during the filtration process. Retentate and filtrate samples were withdrawn and analyzed at intervals. At the end of the process, the membranes were rinsed with 0.10 *M* sodium hydroxide, and with detergent solutions (including proteases).

Liquid–Liquid Extraction by Reversed Micelles

The enzyme was extracted from the whole clarified fermentation broth by *N*-Benzyl-*N*-Dodecyl-*N*-*bis*[2-hydroxyethyl] ammonium chloride (BDBAC) reversed micelles in iso-octane by a two-step procedure. The first step (forward extraction) was carried out by mixing 5.0 mL of the aqueous inulinase solution with an equal volume of micellar microemulsion (BDBAC in iso-octane/hexanol), the aqueous phase solution being adjusted to pH 6.0 by adding phosphate buffer. The phase equilibrium was obtained by intense agitation on a vortex (type Bender and Hobein AG, Zürich) for 1 min. Then the phases were separated by centrifugation at 2800*g* for 5 min. After that, 4.0 mL of inulinase-BDBAC-micellar phase was mixed with 4.0 mL of fresh aqueous phase (0.10 *M* acetate/acetic acid buffer containing 0.5 *M* NaCl, pH 4.0), in order to transfer the enzyme from the micelles to this aqueous phase (backward extraction), which was finally collected by centrifugation (2800*g*; 5 min) after intense agitation. Both aqueous phases were assayed as for enzyme activity. The extraction results are reported in terms of total recovered-activity (%) in the strip phase, using the inulinase content in the initial aqueous phase as a reference.

Expanded-Bed Adsorption Process

A 5.0-cm id chromatography column Streamline C50 (Pharmacia) was filled with 200 g Streamline DEAE. The bed was prepared by pumping the loading buffer (20 m*M* Tris-HCl, pH 6.5) upwards and allowing the bed height to stabilize at a twofold expansion. The cell suspension (viscosity: 2.2 mPas; density: 1.014 g/cm) was then introduced into the column from the bottom. Protein and enzyme activity in the effluent were subsequently measured. The OD (at 280 nm) of the stream leaving the bed was continuously monitored. After the adsorption step, the bed was washed with buffer flowing upwards. Then the adaptor was set down after settling of the gel bed to the initial volume of 200 mL, i.e., the expanded bed was transformed into a new fixed bed. Afterwards, the column was washed in reversed flow (downwards) with 1 bed-vol of buffer. Subsequently, elution was carried out using 20 m*M* Tris-HCl buffer, pH 6.5. After that, the bed was washed with 20 m*M* Tris-HCl containing 1.0 *M* NaCl, pH 6.5,

before cleaning in place and reequilibration with the loading buffer. The flow rates through the columns working in the expanded-bed modes were 4 L/h (= 200 cm/h) and 1.2 L/h (= 60 cm/h), respectively.

RESULTS AND DISCUSSION

Filtration Process

Cell concentration, using microfiltration, was performed in the BDF rotary filter, and the filtrate flux obtained was higher than 100 L/h m^{-2}. Thus, this filtration process can be considered economically feasible *(15)*. The concentrated cells were washed with acetate/acetic acid buffer (0.1 *M*, pH 5.0), and the inulinase recovery increased by about 6.5%. The enzymes present in the microfiltrate were ultrafiltered using a 100 kDa cutoff membrane, which provided a flux of 106.1 L/hm^{-2} and 99% of enzyme retention. Thereby, the concentration and enrichment factors for inulinase were 7.5 and 2.2, respectively (Table 1).

Liquid–Liquid Extraction by BDBAC-Reversed Micelles

Of the variables studied, those that promoted the highest extraction yields were: forward extraction: pH 6.5, 30 m*M* phosphate buffer, 37°C, 0.15 *M* BDBAC 7.5% hexanol, 92.5% isooctane, and ~5.0 mS/cm electrical conductivity; and backward extraction: pH 4.5, 100 m*M* acetate buffer, 25°C, and ~50 mS/cm electrical conductivity. After the forward and backward extractions, 91.0% of the inulinase was recovered from the original medium, without previous cell removal. The enrichment and concentration factors were 2.8 and 1.6, respectively (Table 1).

Expanded-Bed Adsorption

During inulinase adsorption, most of the contaminant proteins were eliminated from the column by flowing through 2 bed vol of acetate buffer. Approximately 2.8 expanded-bed volumes were used to completely remove the particulate materials from the column. Total protein concentration and optical density were used to monitor such variables. The passage of cells through the expanded bed neither blockaded nor destabilized the column and/or bed. The cells showed low affinity with the adsorbents and did not affect the enzyme adsorption. After that, the enzyme was completely eluted with 6.2 bed vol. After this chromatographic step, the inulinase enrichment and concentration factors were 4.3 and 2.8, respectively. Total recovered inulinase activity, compared to that injected into the column, was 93.1% (Table 1).

As can be seen in Table 1, the highest enrichment factor was obtained during the enzyme purification by expanded-bed adsorption, followed by the liquid–liquid extraction by reversed micelles and cross-flow filtration. The selectivity and specificity of the chromatographic technique were

Table 1
Enrichment Factor, Concentration Factor, and Enzyme Recovered by Different Processes of Enzyme Separation

Separation process	Enrichment factor (E_f)	Concentration factor (C_f)	Enzyme recovered (%)
Cross-flow filtration	2.2	7.5	92.7
BDBAD Reversed micelles liquid–liquid extraction	2.8	1.6	91.0
Expanded-bed adsorption	4.3	2.8	93.1

higher than those of the other techniques tested. However, compared to the cross-flow filtration, expanded-bed adsorption presented low capacity of enzyme concentration. Reversed-micelles liquid–liquid extraction showed neither high selectivity nor high concentration factor. To select the most appropriate technique to purify inulinase, directly from the fermented medium, both the potential industrial applications of this enzyme and their economic implications have to be considered. Through a rough analysis of the three techniques employed, it could be said that cross-flow filtration is the most adequate technique, since this technique provides a substantial increase in the concentration factor, and it exclusively depends on the initial volume employed.

CONCLUSIONS

To recover and purify inulinase, at least three techniques can be employed: cross-flow filtration, reversed-micelle liquid–liquid extraction, and expanded-bed adsorption. The recovery factor varies in the range of 91–93% for all of them. To choose the most appropriate technique for enzyme separation, two parameters were used: concentration and purification factors. Thereby, cross-flow microfiltration/ultrafiltration/diafiltration was considered the best method to produce inulinase for industrial application.

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