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Original Article

Fructose production from Jerusalem artichoke using mixed inulinases

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ABSTRACT

Mixed inulinases of the fungus *Aspergillus niger* TISTR 3570 and the yeast *Candida guilliermondii* TISTR 5844 were used in producing fructose from freshly prepared extracts of Jerusalem artichoke (*Helianthus tuberosus*). Fructose production was optimized using the Taguchi method involving five factors at four levels. The optimal conditions for maximizing the concentration and yield of fructose were: a mixed inulinase concentration of 4 U/g substrate; a fungus and yeast inulinase activity ratio of 25:1; pH 4.5; 40 °C; and a 24 hr reaction time. Under these conditions, the peak concentration of fructose was 46.9 g/L, the yield on the substrate was 0.60 g/g and the productivity was 1.84 g/L hr. The most important factor was the reaction time for maximizing the final concentration and yield of fructose, whereas the factor significantly affecting fructose productivity was enzyme concentration. All the experimental factors had a significant effect on the production of fructose. The identified optimal conditions for a batch operation were used in the fed-batch production of fructose which had a fructose concentration of 117.6 g/L at a productivity of 1.03 g/L hr. Glucose was the sole by-product with a relatively low concentration of 30.2 g/L.

Introduction

Inulin is a storage carbohydrate that occurs mainly in the roots and tubers of plants such as Jerusalem artichoke, chicory, dahlia and yacon and dried tubers may contain up to 50% inulin by weight (Zhao et al., 2010). Parker et al. (2010) described the procedure for producing fructose involving inulin which is a fructan, consisting of β-2,1-linked linear chains of polyfructose with a terminal glucose unit, and is a potential source of fructose and inulo-oligosaccharides for use in food and pharmaceuticals. Generally, fructose as fructose corn syrup (HFCS) is commercially produced from cornstarch. HFCS is enzymatically produced cornstarch, initiated as fructose and glucose. Thus, glucose is isomerized with glucose isomerase to finally produce fructose at the highest yield of up to 95%. However, a single process has been reported that produced fructose and/or inulo-oligosaccharides by direct conversion of fructans polysaccharide or inulin (Sirisansaneeyakul et al., 2007a; Kango and Chand Jain, 2011). Fructose is the sweetest of the natural sugars and an alternative sweetener to sucrose; fructose has a low glycemic index compared to sucrose and is often recommended for diabetics (Singh et al., 2007). Inulo-oligosaccharides are functional sweeteners in a low-calorie diet and a source of dietary fiber in food preparations (Yun et al., 1999; Chen et al., 2009; Mutanda et al., 2014; Singh et al., 2016). Both fructose and inulo-oligosaccharides can be produced from inulin by the enzymatic action of an exo-inulinase (β-D-fructan fructohydrolase, EC 3.2.1.80) acting either alone or in synergy with an endo-inulinase (2,1-β-D-fructan fructanohydrolase, EC 3.2.1.7) (Zhengyu et al., 2005; Ricca et al., 2007; Sirisansaneeyakul et al., 2007a; Chen et al., 2009; Lima et al., 2011; Mutanda et al., 2014). Chemical hydrolysis of inulin to fructose is possible, but produces unwanted by-products and coloration, so the use of inexpensively produced microbial inulinases is preferred for producing fructose and inulo-oligosaccharides from inulin as enzymatic hydrolysis of inulin may yield up to 95% pure fructose (Jing et al., 2003; Ricca et al., 2007).

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Inulinases are divided into exo- and endo-inulinases depending on their modes of action on inulin, where exo-inulinases act by successively splitting off terminal fructose units in sucrose, raffinose, and inulin to liberate fructose (Sirisansaneeyakul et al., 2007a). In contrast, endo-inulinases act randomly on the internal links in inulin to release inulotriose, inulotetraose and inulopentaose as the major products (Xu et al., 2016). Inulinases are produced by microorganisms such as *Kluyveromyces marxianus*, *Aspergillus* sp., *Staphylococcus* sp., *Xanthomonas* sp. and *Pseudomonas* (Chi et al., 2009; Vijayaraghavan et al., 2009; Risso et al., 2012).

Jerusalem artichoke (*Helianthus tuberosus*) tubers are a major source of inulin (Chi et al., 2011; Khuenpet et al., 2016). The degree of polymerization of inulin of Jerusalem artichoke tubers is between 2 and 60, or higher (Sirisansaneeyakul et al., 2007a; Bekers et al., 2008). This study focused on optimizing the production of fructose from inulin of Jerusalem artichoke by using mixed crude inulinases derived from a fungus and a yeast. A mixture of crude inulinases from two microbial sources was used because previous work had shown the crude enzymes from different microbial sources had different ratios of exo- and endo-inulinase activities (Sirisansaneeyakul et al., 2007a). Therefore, mixing enzymes from the two sources allows for optimizing the ratio of the two types of activities in the reaction mixture to improve the rate of hydrolysis of inulin and to obtain a more complete conversion of the polymer to fructose.

The fungus *A. niger* TISTR 3570 and the yeast *C. guilliermondii* TISTR 5844 used in producing the crude enzymes had been isolated from Jerusalem artichoke tubers (Sirisansaneeyakul et al., 2007a). Furthermore, the inulinase produced by these microorganisms has been found to have excellent potential for hydrolyzing inulin (Sirisansaneeyakul et al., 2007a). The Taguchi method was used to optimize the mixed enzyme treatment of inulin to produce fructose in a batch reaction. The optimized reaction conditions were then implemented in a fed-batch operation to produce fructose.

Materials and methods

Microorganisms

A. niger TISTR 3570 and *C. guilliermondii* TISTR 5844 (Sirisansaneeyakul et al., 2007a) were obtained from the Microbiological Resources Center, Thailand Institute of Scientific and Technological Research (TISTR). The medium used in maintaining stock cultures contained the following (per liter of 0.1 M McIlvaine buffer, pH 5.0): 10 g inulin (Frutafit®, the Hague, the Netherlands), 12 g yeast extract, 2 g $MgSO_4 \cdot 7H_2O$ and 15 g agar.

Preparation of the crude inulinase

The inoculum for *A. niger* TISTR 3570 was prepared by aseptically transferring a loopful of spores to 30 mL of a liquid medium (1×10^5 spores/mL) that contained the following (per liter of 0.1 M McIlvaine buffer, pH 5.0): inulin 10 g, yeast extract 12 g and $MgSO_4 \cdot 7H_2O$ 2 g. The Erlenmeyer flasks were incubated at 30 °C on a rotary shaker at 200 rpm for 24 hr. An amount of 10% volume per volume (v/v) inoculum (30 mL) was transferred to 500 mL flasks each containing 270 mL of the above-specified liquid medium. The flasks were incubated as specified above. These cultures were inoculated into a 5 L stirred-tank fermenter (Biostat B; B. Braun Biotech International; Melsungen, Germany) containing 2.7 L of the above-specified fresh medium. The fermenter was operated for 48 hr at 30 °C at an agitation speed of 600 rpm and an aeration rate of 1 vessel volume per minute. The pH and the dissolved oxygen level were not controlled. The broth was then harvested and filtered (Whatman No.1 filter paper; Whatman PLC; Maidstone, UK) to remove the biomass. The filtrate was used directly as the crude fungus inulinase.

The yeast inoculum of *C. guilliermondii* TISTR 5844 was prepared by one loopful inoculation of yeast grown on agar slant to 30 mL of liquid medium initially with 0.1 optical density at 600 nm. The yeast was cultivated as described above for *A. niger*. The supernatant of the fermentation broth was recovered using centrifugation (10,000 × *g*, 20 min) at 4 °C. The supernatant was used directly as the crude yeast inulinase.

Preparation of Jerusalem artichoke extract

Jerusalem artichoke tubers were obtained from the Petchaboon Research Station, Faculty of Agriculture, Kasetsart University, Thailand. The tubers were washed with tap water, peeled and steamed at 100 °C for 15 min. Inulin was extracted by crushing 1 kg of cooked tubers into a fine pulp in 1 L of hot water. The resulting slurry was filtered through a double-layered muslin cloth. The extract contained approximately 100 g/L total carbohydrate and 2 g/L reducing sugars. The filtered extract was centrifuged (10,000 × *g*, 30 min) at 4 °C. The recovered supernatant was concentrated in a rotary vacuum evaporator at 45 °C under a vacuum of 72 ± 3 mm Hg until the total carbohydrate content was 370 g/L. The concentrate was pasteurized by holding at 62 ± 2 °C for 30 min. This concentrate was used as the substrate for the production of fructose.

Experimental design

The experimental factors were: mixed enzyme activity in the reaction mixture (A); the ratio of the fungus and the yeast inulinase activities in the mixed enzyme (B); the initial pH of the reaction mixture (C); the incubation temperature (D); and the reaction time (E). The Taguchi L_{16} orthogonal array table for the experimental design, consisting of five factors at four levels, is shown in Table 1 (Roy, 2001). The experimental data were processed using the Qualitek-4 software (Nutek, Inc.; Bloomfield Hills, MI, USA) with 'bigger is better' quality criterion selected for determining the optimum conditions for producing fructose from inulin. The relevant statistical treatments have been previously discussed (Sirisansaneeyakul et al., 2007b, 2011; Songpim et al., 2011; Vaithanomsat et al., 2011; Dejsungkranont et al., 2017).

Batch production of fructose

Sixteen batch experiments for the production of fructose were carried out in triplicate in 250 mL shake flasks at the factor levels shown in Table 1. Each shake flask contained 50 mL of Jerusalem artichoke extract and 0.1% (g/100 mL) benzoic acid. The flasks were incubated at various temperatures (Table 1) at a shaker speed of 200 rpm. The flasks were sampled after an appropriate incubation period (Table 1). The sample was quenched by boiling for 10 min, centrifuged (10,000 × *g*, 10 min) and the supernatant was analyzed for fructose and fructo-/inulo-oligosaccharides using high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD). All experiments were carried out in triplicate.

Fed-batch production of fructose

The optimal conditions identified using the batch production data guided the production of fructose in the fed-batch experiments. The loss in the enzyme activity in the reaction mixture as a consequence of feeding the inulin extract and prolonged incubation was compensated for by intermittently feeding mixed inulinases

Table 1
Experimental design factor levels for the optimization (layout of the L_{16} (4^5) orthogonal arrays) and the results of the final concentration (C_F), yield of fructose ($Y_{F/CHO}$), and productivity of fructose (Q_F).

Level	Factor												
	A: Enzyme concentration (U/g substrate)					B: Inulinases activity ratio (fungus: yeast)		C: Initial pH			D: Temperature (°C)		E: Reaction time (hr)
1	2					10:1		4			40		6
2	3					15:1		4.5			45		12
3	4					20:1		5			50		18
4	5					25:1		5.5			55		24
Experiment no.	Factor[a] ($L_{16}(4^5)$)										Production of fructose		
	A	B	C	D	E	A	B	C	D	E	C_F (g/L)	$Y_{F/CHO}$ (g/g)	Q_F (g/L hr)
1	1	1	1	1	1	2	10:1	4	40	6	7.55 ± 0.15	0.07 ± 0.01	1.03 ± 0.03
2	1	2	2	2	2	2	15:1	4.5	45	12	7.47 ± 0.40	0.08 ± 0.01	0.55 ± 0.04
3	1	3	3	3	3	2	20:1	5	50	18	8.17 ± 0.70	0.08 ± 0.01	0.39 ± 0.03
4	1	4	4	4	4	2	25:1	5.5	55	24	17.73 ± 1.02	0.2 ± 0.01	0.69 ± 0.04
5	2	1	2	3	4	3	10:1	4.5	50	24	25.71 ± 0.83	0.33 ± 0.01	1.02 ± 0.04
6	2	2	1	4	3	3	15:1	4	55	18	22.52 ± 1.49	0.29 ± 0.03	1.17 ± 0.09
7	2	3	4	1	2	3	20:1	5.5	40	12	16.89 ± 0.61	0.21 ± 0.01	1.28 ± 0.05
8	2	4	3	2	1	3	25:1	5	45	6	4.17 ± 0.16	0.04 ± 0.00	0.51 ± 0.03
9	3	1	3	4	2	4	10:1	5	55	12	24.63 ± 0.38	0.31 ± 0.01	1.82 ± 0.05
10	3	2	4	3	1	4	15:1	5.5	50	6	8.81 ± 0.44	0.09 ± 0.01	1.01 ± 0.06
11	3	3	1	2	4	4	20:1	4	45	24	28.07 ± 3.09	0.37 ± 0.04	1.08 ± 0.13
12	3	4	2	1	3	4	25:1	4.5	40	18	39.76 ± 3.15	0.55 ± 0.06	2.15 ± 0.17
13	4	1	4	2	3	5	10:1	5.5	45	18	19.36 ± 2.76	0.29 ± 0.04	1.01 ± 0.15
14	4	2	3	1	4	5	15:1	5	40	24	39.12 ± 3.32	0.58 ± 0.07	1.57 ± 0.14
15	4	3	2	4	1	5	20:1	4.5	55	6	10.42 ± 0.43	0.13 ± 0.01	1.43 ± 0.08
16	4	4	1	3	2	5	25:1	4	50	12	21.34 ± 2.04	0.31 ± 0.03	1.63 ± 0.18

[a] Each of the 16 experiments was carried out in triplicate.

every 2–6 hr. The Jerusalem artichoke concentrate was fed at 20 hr, 40 hr, 60 hr and 84 hr to achieve final total carbohydrate concentrations of 150 g/L, 220 g/L, 340 g/L and 320 g/L, respectively. The sugars and fructo-/inulo-oligosaccharides in the quenched reaction samples were analyzed using HPAEC-PAD.

Enzyme assays

Inulinase activity

The crude enzyme extract was diluted to the required level with 0.05 M McIlvaine buffer at pH 5.0. The diluted extract (0.5 mL) was mixed with a 1 mL solution of 0.5% weight per volume (w/v; g/100 mL) inulin (chicory root inulin, Sigma I-2255; www.sigmaaldrich.com) made in the aforementioned buffer. The mixture was incubated at 40 °C for 30 min. The reducing sugar produced was measured using the 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959) and a fructose standard curve. One unit of inulinase activity was defined as the quantity liberating 1 μmol of fructose per minute from a 0.5% w/v standard solution of inulin in 0.5 M McIlvaine buffer at pH 5.0 and 40 °C.

Invertase activity

The crude enzyme extract was diluted to the required level with 0.05 M McIlvaine buffer at pH 5.0. The diluted extract (0.5 mL) was mixed with a 1 mL solution of 0.5% w/v sucrose (analytical grade; Ajax Finechem 500; Auburn, NSW, Australia) in the above-mentioned buffer. The mixture was incubated at 40 °C for 30 min and analyzed for reducing sugars, as described earlier for the inulinase assay. One unit of invertase activity was defined as the quantity liberating 1 μmol of fructose (or glucose) per minute from sucrose under the above-mentioned conditions.

Analytical methods

The initial total carbohydrate in the Jerusalem artichoke extract (prior to any hydrolysis) was measured using the phenol-sulfuric acid method (Dobois et al., 1956) with fructose as a standard. The inulin concentration was calculated as 91% of the fructose produced by hydrolysis of inulin (degrees of polymerization; DP > 2), as described by Raessler et al. (2008).

The products of the enzymatic reaction were analyzed using HPAEC-PAD. A Dionex BioLC chromatograph (Sunnyvale, CA, USA) was used. The chromatograph was equipped with an ED 50-pulsed electrochemical detector with a gold working electrode and a silver chloride reference electrode (Sirisansaneeyakul et al., 2007a). Glucose (Ajax Finechem A783; Auburn, NSW, Australia), fructose (Ajax Finechem A775; Auburn, NSW Australia), sucrose (Ajax Finechem 500; Auburn, NSW, Australia), 1-kestose (Wako 295-64111; Osaka, Japan), nystose (Wako 292-64121; Osaka, Japan) and 1^F-fructofuranosyl nystose (Wako 299-64131; Osaka, Japan) were used as standards for identifying products of hydrolysis with DP values of 1–5, respectively.

Results and discussion

Optimization of the fructose production in shake flasks

Based on the fructose production from inulin using mixed inulinases previously reported (Sirisansaneeyakul et al., 2007a), the factors and levels influenced fructose production with mixed inulinases being optimized from the inulin used as a substrate. The final fructose concentration, the yield and productivity values for the 16 experiments are shown in Table 1. Experimental run 12 (Table 1) produced the highest values of both the fructose concentration (39.8 ± 3.2 g/L) and productivity (2.15 ± 0.17 g/L hr). Inulo-oligosaccharides production was approximately 2.22 ± 0.39 g/L. For this run, the factor settings were: a mixed enzyme concentration of 4 U/g substrate; an inulinase activity ratio of the fungus/yeast enzymes of 25:1; an initial pH of 4.5; 40 °C; and a reaction time of 18 hr. The highest yield of fructose (0.58 ± 0.07 g/g) occurred in run 14 (Table 1). For run 14, the factor settings were: a mixed enzyme concentration of 5 U/g substrate; an inulinase

activity ratio of the fungus/yeast enzymes of 15:1; an initial pH of 5.0; 40 °C; and a reaction time of 24 hr. In addition, a small amount of inulo-oligosaccharides was recorded (0.77 ± 0.14 g/L). The peak values of the fructose concentration, the yield and productivity were 6-fold–15-fold greater than the corresponding lowest values. The inulo-oligosaccharides (DP 3–5) from the runs were all in the range 0.77–19.53 g/L (data not shown).

The data in Table 1 were processed using the Qualitek-4 software with the bigger-the-better attribute selected for establishing the optimum conditions for fructose production. The main effects of the factors at the assigned levels on the production of fructose are summarized in Table 2, which also provides values for the percent main effect of the factors on fructose concentration, yield, and volumetric productivity. The main effect is a difference between the maximum and minimum values of a factor average at each factor level and the percent main effect of a factor is the percentage of its main effect divided by the sum of the main effects of all the factors (Roy, 2001; Dejsungkranont et al., 2017).

The percent main effects values (Table 2) showed that all the factors (A–E) significantly impacted the production of fructose based on it's the concentration, yield, and productivity. Of the five factors considered, the mixed enzyme concentration (factor A), the temperature (factor D) and the reaction time (factor E), were the most influential on fructose production. The reaction time and the mixed enzyme concentration were the most significant factors (62–63% main effect) with respect to fructose concentration and yield. The mixed enzyme concentration and the temperature had the greatest impact (approximately 66% main effect) on fructose productivity (Table 2). The factors of inulinases activity ratio (factor B) and pH (factor C) affected fructose production to a lesser level (approximately 8–12% main effect each) than the other three factors. However, as discussed later in this work, the inulinases activity ratio was an important factor in influencing the composition of inulin hydrolysates.

Table 2
Analysis of factors affecting fructose production.

Level	Factor[a]				
	A	B	C	D	E
(a) Fructose concentration (C_F, g/L)					
1	10.23	19.31	19.87	25.83	7.74
2	17.33	19.48	20.84	14.77	17.58
3	25.32	15.89	19.02	16.01	22.45
4	22.56	20.75	15.70	18.83	27.66
Minimum	10.23	15.89	15.70	14.77	7.74
Maximum	25.32	20.75	20.84	25.83	27.66
Main effect[b]	15.09	4.86	5.14	11.06	19.92
% Main effect	26.91	8.67	9.17	19.72	35.53
(b) Yield ($Y_{F/CHO}$, g/g)					
1	0.11	0.25	0.26	0.35	0.08
2	0.22	0.26	0.27	0.19	0.23
3	0.33	0.20	0.25	0.20	0.30
4	0.33	0.27	0.20	0.23	0.37
Minimum	0.11	0.20	0.20	0.19	0.08
Maximum	0.33	0.27	0.27	0.35	0.37
Main effect[b]	0.22	0.07	0.07	0.16	0.29
% Main effect	27.16	8.64	8.64	19.75	35.80
(c) Volumetric productivity (Q_F, g/L hr)					
1	0.67	1.22	1.23	1.51	1.00
2	1.00	1.08	1.29	0.79	1.32
3	1.52	1.05	1.07	1.02	1.18
4	1.41	1.24	1.00	1.28	1.09
Minimum	0.67	1.05	1.00	0.79	1.00
Maximum	1.52	1.24	1.29	1.51	1.32
Main effect[b]	0.85	0.19	0.29	0.72	0.32
% Main effect	35.86	8.02	12.24	30.38	13.50

[a] See Table 1 for an explanation of the factors A–E.
[b] The main effect was the difference between the maximum and minimum values of the factor averages at each factor level (Main effect = maximum − minimum), while the percent main effect of each factor was calculated as the percentage of its main effect divided by the sum of the main effects of all factors; thus, percent main effect = (main effect × 100)/∑ all main effects (Roy, 2001).

The main effect plots in Fig. 1 reveals how the changes in a factor level affected the response of the fructose production process. The information in Fig. 1 along with Table 4 was used to identify the optimum conditions for the production of fructose. In Fig. 1, the adjacent main effect values for a factor are joined by straight lines. For the five factors (A–E), each at four levels (1–4), only one of the levels maximized the value of the main effect (Fig. 1). From Fig. 1, the conditions for the maximal concentration (Fig. 1A) and yield of fructose (Fig. 1B) were identified as: a mixed enzyme concentration 4 U/g substrate; a fungus/yeast inulinase activity ratio of 25:1; pH 4.5; 40 °C; and a reaction time of 24 hr. On the other hand, maximizing the productivity of fructose (Fig. 1C) required a shorter reaction time of 12 hr, but the other conditions were the same as the conditions for maximizing the yield and concentration.

Analysis of variance (ANOVA) (Table 3) indicated that all the factors significantly affected the fructose concentration, yield, and volumetric productivity at a confidence level of 99.9%. ANOVA confirmed that the mixed enzyme concentration (factor A) and reaction time (factor E) had the most profound effects on the concentration and yield of fructose, while the volumetric productivity was most influenced by the mixed enzyme concentration (factor A) and the reaction temperature (factor D).

The predicted values for the fructose concentration, yield, and productivity under optimal conditions are compared in Table 4 with the actually observed experimental values obtained under the same conditions. The expected or predicted values ($Y_{expected}$) were estimated using the equations given in Table 4. The predicted and observed values were in excellent agreement (Table 4). The measured data of the maximum concentration of fructose at 24 hr, the fructose yield at 24 hr and the fructose productivity at 12 hr were in generally good agreement with the predictions as shown in Table 4. The predicted/observed values of fructose concentration and yield were highest at a mixed enzyme concentration of 4 U/g substrate, a fungus/yeast inulinase activity ratio of 25:1, pH 4.5, 40 °C and a reaction time of 24 hr. This set of conditions was used in all subsequent experiments.

The compositions of the inulin hydrolysates of the 16 experimental trials are summarized in Table 5. The inulo-oligosaccharides (IOS) levels in the hydrolysate were the highest for the treatments 3 and 8 (Table 5). The conditions used in these treatments were rather different (Table 1) and not the same as the conditions that maximized the fructose content of the hydrolysate (Table 1, Table 5). Evidently, the conditions of hydrolysis can be tailored to predictably control the composition of the hydrolysate (Zhengyu et al., 2005; Chen et al., 2009) because the ratio of the exo- and endo-inulinase activities in the reaction mixture influences the product composition and the operational conditions of the hydrolysis influence these two activities differently. For example, use of only *A. niger* TISTR 3570 enzymes for the hydrolysis has previously been shown to produce a higher content of fructose in the hydrolysate compared to the case of using only *C. guilliermondii* TISTR 5844 enzymes (Sirisansaneeyakul et al., 2007a). Furthermore, the crude enzymes of *A. niger* TISTR 3570 have been found to be stable over a wider pH range of 4–10 (Sirisansaneeyakul et al., 2012) compared to the crude inulinases of *C. guilliermondii* TISTR 5844.

For the above-identified optimal conditions, the reaction profiles for the hydrolysis carried out in shake flasks are shown in Fig. 2. The concentration of inulin declined progressively as the hydrolysis progressed. The concentration of fructose and the co-product glucose rose progressively with time (Fig. 2A). The composition of the hydrolysates changed with time (Fig. 2B). By the end of the reaction, most (more than 90%) of the original inulin had been hydrolyzed to fructose and glucose.

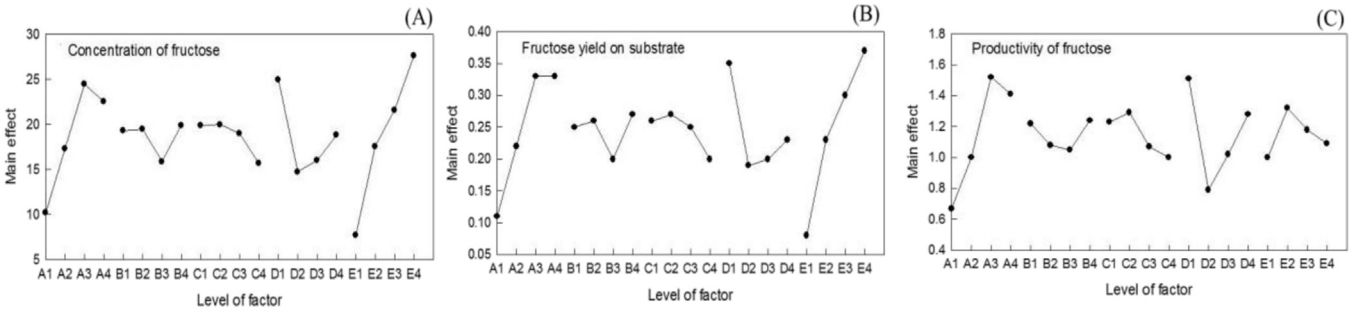

Fig. 1. Average values of main effects for combinations of various factors (A–E, Table 1) and level (1–4, Table 1): (A) concentration of fructose; (B) fructose yield on substrate; (C) productivity of fructose, where optimal conditions are indicated by the peak values of the main effect.

Table 3
Analysis of variance of factors affecting fructose production.

Factor[a]	DOF	SS	Variance	F-ratio	Confidence (%)	*P*-value
(a) Fructose concentration (C_F, g/L)						
A	3	1586.30	528.80	176.72	100	<0.001
B	3	156.20	52.10	17.40	100	<0.001
C	3	179.80	59.90	20.03	100	<0.001
D	3	881.70	293.90	98.22	100	<0.001
E	3	2587.80	862.60	288.29	100	<0.001
Other/Error	32	95.80	3.00			
Total	47	5487.50				
(b) Yield of fructose ($Y_{F/CHO}$, g/g)						
A	3	0.39	0.13	144.85	100	<0.001
B	3	0.04	0.01	13.28	100	<0.001
C	3	0.04	0.01	15.10	100	<0.001
D	3	0.20	0.07	72.77	100	<0.001
E	3	0.54	0.18	199.66	100	<0.001
Other/Error	32	0.03	0.00			
Total	47	1.24				
(c) Productivity of fructose (Q_F, g/L hr)						
A	3	5.53	1.84	199.99	100	<0.001
B	3	0.36	0.12	12.99	100	<0.001
C	3	0.65	0.22	23.48	100	<0.001
D	3	3.50	1.17	126.71	100	<0.001
E	3	0.69	0.23	24.93	100	<0.001
Other/Error	32	0.29	0.01			
Total	47	11.03				

DOF = degree of freedom, SS = sum of squares.
[a] See Table 1 for an explanation of the factors A–E.

Production of fructose in reactors

Batch operation

For the optimal reaction conditions (a mixed enzyme concentration of 4 U/g substrate, a fungus/yeast inulinase activity ratio of 25:1, pH 4.5, 40 °C) identified in shake flasks, the reaction was repeated in a 2 L stirred tank batch reactor with an initial working volume of 1 L. The agitation rate was set at 200 rpm. The results are shown in Fig. 2C and D. In general, the results at the 1 L scale (Fig. 2C,D) were quite comparable to the shake flask data obtained at the 50 mL scale (Fig. 2C,D). By 24 hr, close to 99% of the inulin had been hydrolyzed. The final product contained mainly fructose (more than 67%; Fig. 2C,D) and lower levels of glucose. The fructose yield and productivity were 0.61 g/g and 2.2 g/L hr, respectively. Concomitantly, the inulo-oligosaccharides (DP3–5) were finalized at 0.75 g/L.

Fed-batch operation

The fed-batch hydrolysis of inulin to fructose was carried out in a 2 L stirred tank reactor under the optimized conditions (a mixed enzyme concentration of 4 U/g substrate, a fungus/yeast inulinase activity ratio of 25:1, pH 4.5, 40 °C, 200 rpm) of the batch operation.

Four feedings of the concentrated inulin substrate (feedings F_{1-4}) occurred at 20 hr, 40 hr, 60 hr, and 84 hr of operation. This increased the final volume in the reactor to around 1716 mL. As a consequence of the feeding-associated dilution and denaturation during prolonged operation at 40 °C, the enzyme activity in the reactor would have declined from the starting value of 4 U/g substrate. This was prevented by feeding the enzyme mixture intermittently at 2–6 hr intervals so that the activity remained nearly at 4 U/g substrate (see Fig. 3). The activity was calculated after the feeding (F_{1-4}) at 20 hr, 40 hr, 60 hr, and 84 hr (Fig. 3).

The profiles of fed-batch hydrolysis are shown in Fig. 3. The total quantities of the mixed enzyme activity, inulin, and the various products of hydrolysis in the reactor are shown. After 111 hr of operation, the amounts of fructose and glucose in the reactor were 204.1 g and 52.5 g, respectively. As the final volume of the hydrolysate was 1.716 L, the final concentration of fructose was 117.6 g/L,

Table 4
Comparison of predicted and experimental values of fructose concentration, yield, and productivity under optimal conditions.

Parameter	Factor level[a]					Value under optimal conditions	
	A	B	C	D	E	Predicted[c]	Experimental
Fructose concentration (g/L)	4	25:1	4.5	40	24	44.96 ± 1.9	46.85 ± 2.96[a]
Yield of fructose (g/g substrate)	4	25:1	4.5	40	24	0.62 ± 0.04	0.60 ± 0.04[a]
Productivity of fructose (g/L hr)	4	25:1	4.5	40	12	2.28 ± 0.11	2.48 ± 0.15[b]
$Y_{expected}$	Prediction equation for optimal conditions						
Fructose concentration (g/L)	$Y_{expected} = \overline{A}_3 + \overline{B}_4 + \overline{C}_2 + \overline{D}_1 + \overline{E}_4 - 4\overline{T}$						
Yield of fructose (g/g substrate)	$Y_{expected} = \overline{A}_3 + \overline{B}_4 + \overline{C}_2 + \overline{D}_1 + \overline{E}_4 - 4\overline{T}$						
Productivity of fructose (g/L hr)	$Y_{expected} = \overline{A}_3 + \overline{B}_4 + \overline{C}_2 + \overline{D}_1 + \overline{E}_2 - 4\overline{T}$						

[a] Optimal condition for fructose concentration and yield.
[b] Optimal condition of productivity of fructose.
[c] Confidence interval (CI) under optimal conditions at 99% confidence level.

Table 5
Composition of Jerusalem artichoke inulin hydrolysates.

No.	Composition (%)						
	Glucose	Fructose	Sucrose	IOS DP 3–10	Inulin DP 11–20	Inulin DP 21–30	Inulin DP > 30
1	10.98	29.37	13.07	32.81	10.87	2.26	0.63
2	7.89	27.53	12.36	37.75	11.21	2.33	0.93
3	13.22	25.22	5.01	42.31	11.81	2.01	0.41
4	9.14	51.19	7.66	21.25	8.53	1.94	0.29
5	21.01	59.99	4.94	8.21	4.50	1.22	0.14
6	21.85	57.89	6.95	8.99	3.51	0.73	0.08
7	18.45	47.55	8.70	17.10	6.55	1.34	0.31
8	8.24	22.89	12.97	39.39	12.08	3.42	1.02
9	17.67	50.74	5.40	17.25	6.98	1.65	0.32
10	14.92	33.27	4.37	31.93	10.56	3.73	1.23
11	20.81	58.57	5.48	9.17	4.40	1.25	0.33
12	28.19	61.73	2.23	4.14	2.74	0.88	0.10
13	16.78	42.20	6.94	20.88	8.65	3.03	1.54
14	26.43	65.09	0.46	1.96	2.36	3.07	0.64
15	15.34	43.41	11.34	19.08	7.06	2.69	1.08
16	22.78	57.18	6.83	8.50	3.89	0.79	0.04

No. = experimental number; IOS = inulo-oligosaccharide; DP = degrees of polymerization.

or nearly 2.5-fold the final concentration in the batch operation. However, the fed-batch operation had a substantially lower fructose productivity compared to the batch operation. This accumulated high fructose retarded the catalytic enzyme reaction (Mutanda et al., 2009). Finally, the inulo-oligosaccharides level from the fed-batch operation was 4.14 g/L at 111 hr of reaction time.

The optimal conditions for fructose production by hydrolysis of Jerusalem artichoke inulin using mixed inulinases of *A. niger* TISTR

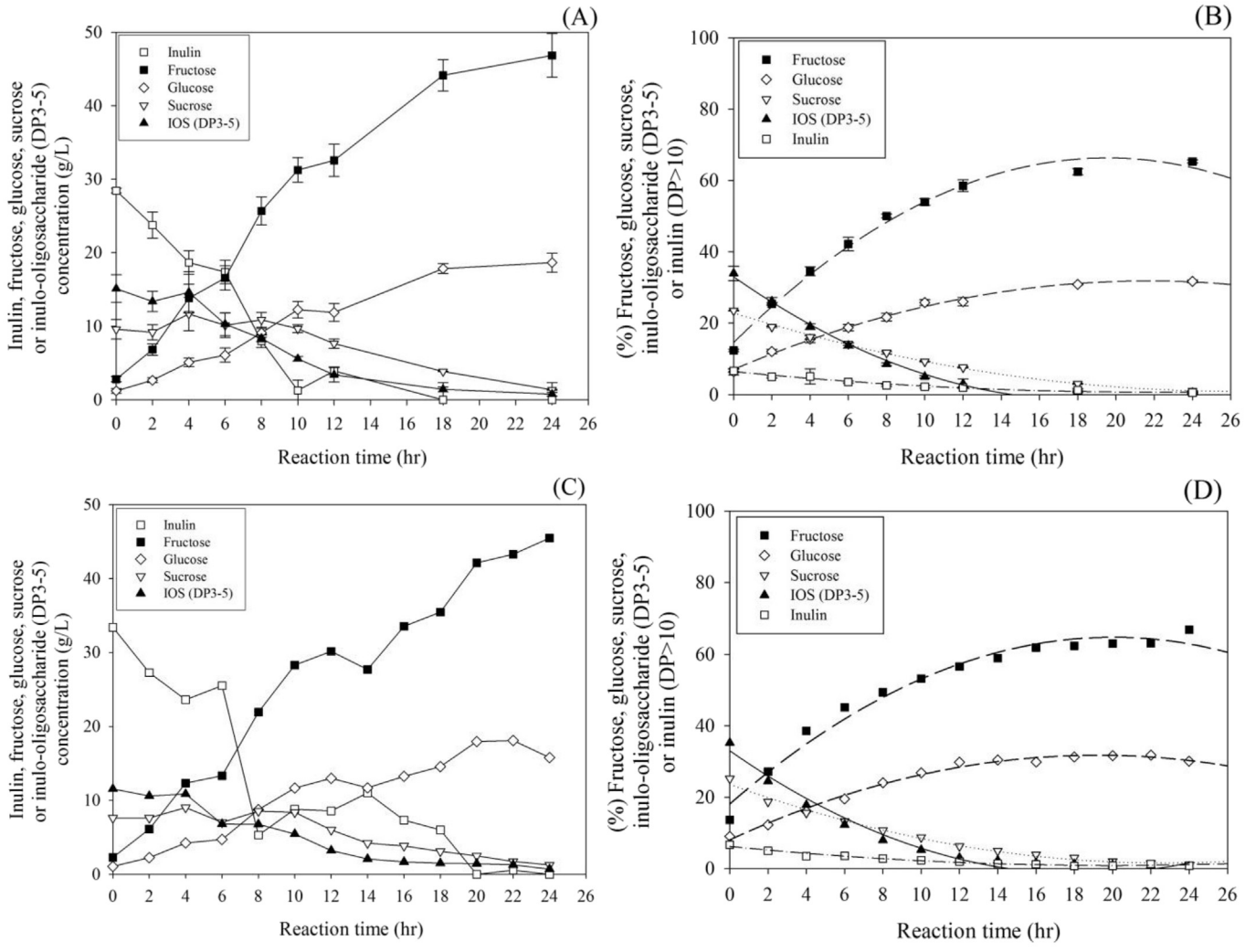

Fig. 2. Production of fructose from Jerusalem artichoke extract in shake flasks: (A) concentration profiles; and (B) percent composition profiles of the hydrolysate and 2 L stirred tank reactor: (C) concentration profiles; and (D) percent composition profiles of the hydrolysate under optimal conditions (enzyme concentration of 4 U/g substrate; fungus: yeast inulinases activity ratio of 25:1; pH 4.5; 40 °C), where IOS = inulo-oligosaccharide and DP = degrees of polymerization.

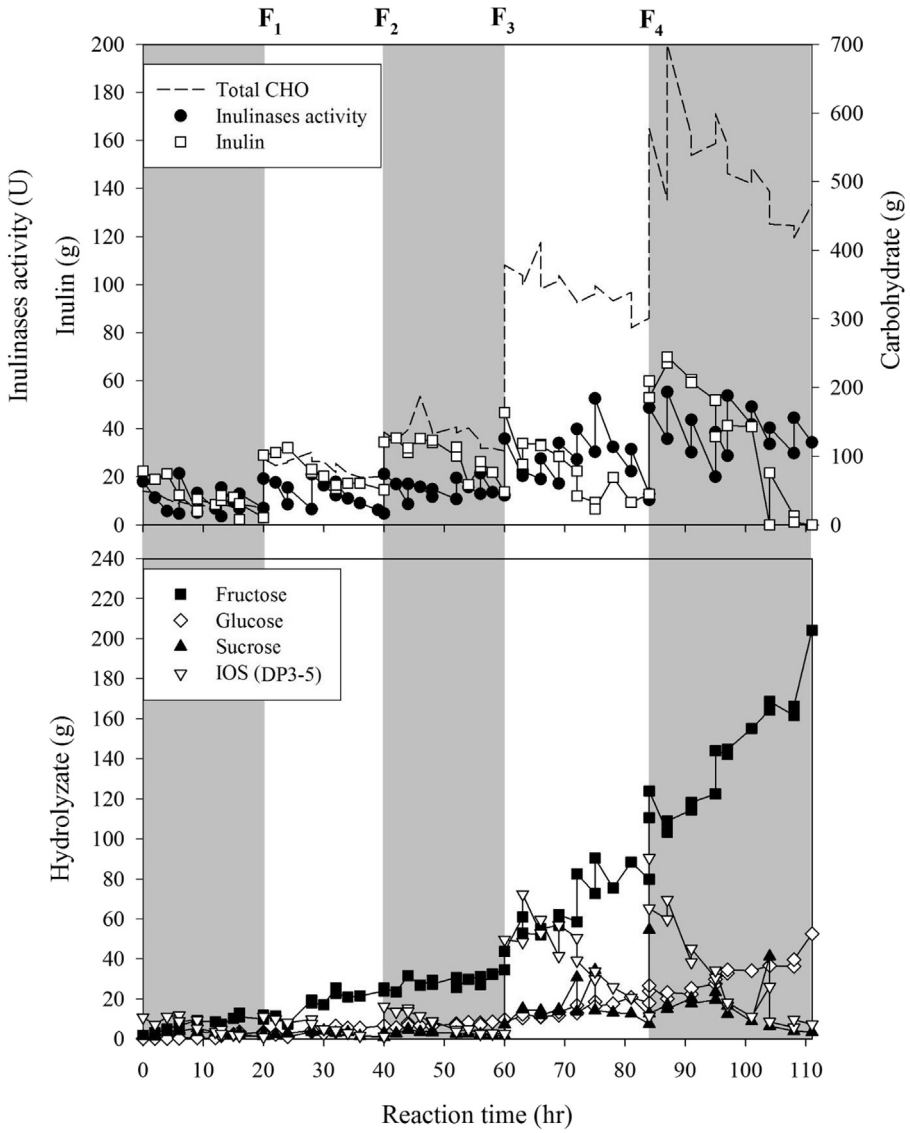

Fig. 3. Production of fructose from Jerusalem artichoke extract under optimal conditions (enzyme concentration of 4 U/g substrate; fungus: yeast inulinases activity ratio of 25:1; pH 4.5; 40 °C) in a fed-batch process, where IOS = inulo-oligosaccharide and DP = degrees of polymerization.

3570 and *C. guilliermondii* TISTR 5844 were: a mixed inulinase concentration of 4 U/g substrate; a fungus and yeast inulinase activity ratio of 25:1; pH 4.5; 40 °C; and a 24 hr reaction time. Under the specified optimal conditions, the observed concentration and yield of fructose were 46.9 g/L and 0.60 g/g, respectively. The productivity of fructose exceeded 1.8 g/L hr at 24 hr of reaction time. The mixed enzyme concentration played an important role in fructose production, in which a mixture of endo-/exo-inulinases synergistically led to a high fructose yield. Intermittent fed-batch operation under the optimal conditions provided a high fructose concentration of 117.6 g/L which was nearly 2.5-fold higher than for the batch operation.

Conflict of interest

The authors declare that there are no conflicts of interest.

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