

## Production of fructose from inulin using mixed inulinases from *Aspergillus niger* and *Candida guilliermondii*

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**Abstract** Two new effective microbial producers of inulinases were isolated from Jerusalem artichoke tubers grown in Thailand and identified as *Aspergillus niger* TISTR 3570 and *Candida guilliermondii* TISTR 5844. The inulinases produced by both these microorganisms were appropriate for hydrolysing inulin to fructose as the principal product. An initial inulin concentration of  $\sim 100 \text{ g l}^{-1}$  and the enzyme concentration of  $0.2 \text{ U g}^{-1}$  of substrate, yielded  $37.5 \text{ g l}^{-1}$  of fructose in 20 h at  $40^\circ\text{C}$  when *A. niger* TISTR 3570 inulinase was the biocatalyst. The yield of fructose on inulin was  $0.39 \text{ g g}^{-1}$ . Under identical conditions, the yeast inulinase afforded  $35.3 \text{ g l}^{-1}$  of fructose in 25 h. The fructose yield was  $0.35 \text{ g g}^{-1}$  of substrate. The fructose productivities were  $1.9 \text{ g l}^{-1} \text{ h}^{-1}$  and  $1.4 \text{ g l}^{-1} \text{ h}^{-1}$  for the mold and yeast enzymes, respectively. After 20 h of reaction, the mold enzyme hydrolysate contained 53% fructose and more than 41% of initial inulin had been hydrolysed. Using the yeast enzymes, the hydrolysate contained nearly 38% fructose at 25 h and nearly 36% of initial inulin had been hydrolysed. The *A. niger* TISTR 3570 inulinases exhibited both endo-

inulinase and exo-inulinase activities. In contrast, the yeast inulinases displayed mainly exo-inulinase activity. The mold and yeast crude inulinases mixed in the activity ratio of 5:1 proved superior to individual crude inulinases in hydrolysing inulin to fructose. The enzyme mixture provided a better combination of endo- and exo-inulinase activities than did the crude extracts of either the mold or the yeast individually.

**Keywords** Fructose · Inulin · Inulinases · *Aspergillus niger* · *Candida guilliermondii* · Jerusalem artichoke

### Introduction

Inulin is a natural storage polymer found widely in plants such as chicory, artichoke, and banana. Inulin is a polydisperse fructan that ranges in its degree of polymerization (DP) from 2 to 60, or higher. The fructosyl units in inulin are linked by  $\beta$  (2  $\rightarrow$  1) linkages with the polymer chains terminating in glucose residues. Inulin is commercially recovered from chicory roots and Jerusalem artichoke tubers (Hoebregs 1997; Frank and Leenheer 2002; Rhee et al. 2002; Stolzenburg 2005). It is used as a mostly indigestible soluble dietary fibre and thickener in foods. This work reports on enzymatic hydrolysis of inulin to produce fructose using single and mixed crude enzyme preparations obtained from new isolates of the mold *Aspergillus niger* and the yeast *Candida guilliermondii*.

Inulin is a potential source of fructose, or fruit sugar. Fructose is significantly sweeter than the table sugar sucrose and glucose. Consequently, fructose is an important sweetener in the food industry, especially as

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high-fructose-corn-syrup (HFCS), produced by isomerization of corn starch hydrolysates. HFCS is produced through a two-step process that involves enzymatic isomerization of corn starch hydrolysate to a mixture of 42% fructose and 50% glucose. Subsequently, a second step is used to purify the mixture to up to 95% fructose with the glucose being recycled to the isomerization step. An alternative to this two-step process is to produce fructose by a single-step direct enzymatic hydrolysis of fructan polysaccharides such as inulin (Kierstan 1978; Zittan 1981; Dahlmans et al. 1983; Guiraud and Galzy, 1990; Fontana et al. 1993; Nakamura et al. 1995).

Inulin is hydrolysed by enzymes known as inulinases. Inulinases are classified into endo- and exo-inulinases, depending on their mode of action. Endo-inulinases (2,1- $\beta$ -D-fructan fructanohydrolase; EC 3.2.1.7) are specific for inulin and hydrolyse it by breaking bonds between fructose units that are located away from the ends of the polymer network, to produce oligosaccharides. Exo-inulinases ( $\beta$ -D-fructohydrolase; EC 3.2.1.80), split terminal fructose units in sucrose, raffinose and inulin to liberate fructose (Onodera and Shiomi 1988; Uchiyama 1993; Ohta et al. 2002).

Inulinases are encountered in plants and many microorganisms. Among fungi, some well-known sources of these enzymes include *A. niger*, *Aspergillus ficuum*, *Chrysosporium pannorum* and *Penicillium purpurogenum*. Among yeasts, the best-known producers are *Kluyveromyces marxianus*, *Candida kefyr*, *Debaryomyces cantarellii* and *Pichia polymorpha*. These yeasts appear to produce only exo-inulinases whereas most inulin-hydrolysing molds produce both endo- and exo-inulinases (Guiraud and Galzy 1990; Frank and Leenheer 2002; Barta 1993; Hensing et al. 1993).

Jerusalem artichokes (*Helianthus tuberosus* L.) are an important potential source of inulin (Stolzenburg 2005). Optimal growth conditions and the time of harvest of Jerusalem artichokes in Thailand were identified by Pinpong (1997). The structure of inulin in plants is known to depend on factors such as the specific plant source, the climatic conditions of growth, and the state of maturity at harvest. The influence of age at harvest and storage temperature of tubers, on inulin composition of Jerusalem artichoke grown in Thailand have been documented by Saengthongpinit and Sajjaanantakul (2005).

This study is focused on isolating two effective microbial producers of inulinases and the use of their crude enzyme extracts for hydrolysing Jerusalem artichoke inulin to fructose, as a potential industrial process. Earlier published processes for inulin hydro-

lysis made use of immobilized yeast cells (Workman and Day 1984; Abelyan et al. 1998), immobilized enzymes of *A. niger* (Nakamura et al. 1995), soluble enzymes from single microbial sources (Kim et al. 1997), and suspended single microbial species. A strong synergistic effect of exo- and endo-inulinases has been reported on inulin hydrolysis when these enzymes are used in combination (Barratti and Ettalibi 1993). Use of mixtures of bacterial and yeast inulinases has been reported (Cho et al. 2001). Construction of a flocculating yeast for inulin hydrolysis has been reported (Remize et al. 1998). This work reports on inulin hydrolytic capabilities of single and mixed enzymes sourced from new isolates of a mold and a yeast.

## Materials and methods

### Culture medium and Jerusalem artichoke tubers

The medium used in isolation and screening of producer microorganisms and production of inulinases, contained (per liter of 0.1 M McIlvaine buffer at pH 5.0): inulin (Frutafit<sup>®</sup> TEX, The Netherlands), 10 g; yeast extract, 12 g; MgSO<sub>4</sub>·7H<sub>2</sub>O, 2 g; and agar, 15 g (for agar medium only). All media were sterilized by autoclaving at 121°C for 15 min.

Jerusalem artichoke (*Helianthus tuberosus* L.) tubers were kindly provided by Kanchanaburi Research Station, Research and Development Institute for Agricultural Systems under Adverse Conditions (IA-SAC), Kasetsart University, Thailand.

### Isolation and screening of inulinase producers

#### Isolation

Rotten Jerusalem artichoke tubers were cut in small pieces that were placed on agar plates. The plates were incubated at 30°C for 7 days. The colonies produced were isolated and used to generate pure cultures.

#### Primary screening

All fungal colonies were point inoculated on agar plates. The plates were incubated at 30°C for 5 days. Colonies that displayed rapid growth, i.e. attained large colony diameter per unit growth time, were selected for further experiments and transferred to fresh plates.

#### Secondary screening

Selected fungi and other isolates were inoculated into separate 250-ml shake flasks that contained 50 ml of

liquid medium. Flasks were incubated at 30°C, 200 rpm agitation speed, for 24 h. Inocula (10% v/v) were transferred to 250-ml shake flasks containing 100 ml of liquid medium at an initial pH of 5.0. Flasks were incubated at 30°C, 200 rpm, for 5 days. Samples were withdrawn periodically and analysed for activities of inulinases and invertase.

#### Specific growth rate measurements

Biomass concentration in fungal broths was measured periodically by filtering a sample (3 ml) through a preweighed membrane filter (0.45 µm nominal pore size, cellulose acetate filter, No. 11106, Sartorius AG, Germany), washing with 2 × 5 ml of deionized water, drying (105°C, 24 h), cooling in a desiccator and weighing. For broths of yeast and bacteria, a 5 ml sample was placed in a preweighed test tube, centrifuged (1,700g, 10 min), washed with deionized water (2 × 5 ml) and dried as earlier. In all cases, specific growth rate was calculated as the slope of the linear portion of the semilog plot of biomass concentration versus time (Chisti and Moo-Young 1999).

#### Strain identification

Two potential isolates were selected and systematically identified at the National Institute of Health, Department of Medical Science (DMSC), Thailand, and Microbiological Resources Center, Thailand Institute of Scientific and Technological Research (TISTR), for identifying molds and yeasts, respectively.

#### Inulinase production

Isolates identified as the mold *A. niger* TISTR 3570 and the yeast *C. guilliermondii* TISTR 5844 were used for producing inulinases, as follows.

##### *Aspergillus niger* TISTR 3570

The inoculum was prepared by transferring a loopful of stock culture to 250-ml Erlenmeyer flasks containing 50 ml liquid medium, as described previously. A 10% v/v inoculum was transferred to two 500-ml Erlenmeyer flasks each containing 150 ml of liquid medium. The flasks were held at 30°C, 200 rev min<sup>-1</sup>, for 24 h. These cultures (300 ml) were inoculated into a 10-l stirred-tank fermentor (BEM; B. E. Marubishi, Tokyo, Japan) that contained 3 l of fresh medium. The fermentor was controlled at 30°C, 600 rpm impeller agitation speed, and 1 v/v min aeration rate, for 48 h. The

broth was harvested, cooled to 4°C, and filtered (Whatman No. 1 filter paper; Whatman PLC, UK) to remove the biomass. The filtrate was held at -20°C, as the crude preparation of the mold enzymes.

##### *Candida guilliermondii* TISTR 5844

A loopful of stock culture was inoculated in a 250-ml Erlenmeyer flask containing 50 ml liquid medium. After 24 h incubation at 30°C, 200 rev min<sup>-1</sup>, a 10% v/v inoculum was transferred to a 500-ml Erlenmeyer flask containing 150 ml liquid medium. Further incubation occurred at 30°C, 200 rev min<sup>-1</sup>, for 24 h. The resulting broth was used to inoculate a second stage of 200 ml liquid medium in a 500-ml Erlenmeyer flask. The flask was incubated as above, for 48 h. The broth was harvested, cooled to 4°C, and centrifuged (8,000g, 20 min) to remove the biomass. The supernatant was held at -20°C and used as the crude preparation of the yeast enzymes.

#### Enzyme assays

##### *Inulinase activity*

Crude enzyme extract (0.5 ml) was diluted to the required level with 0.05 M McIlvaine buffer at pH 5.0. Diluted extract (0.5 ml) was mixed with a 1 ml solution of 0.5% w/v inulin (chicory root inulin; Sigma I-2255) in the aforementioned buffer. The mixture was incubated at 40°C for 30 min. The reaction was terminated by boiling for 5 min (Benfeld 1955). The reducing sugars were subsequently analysed by the 3,5-dinitrosalicylic acid (DNS) method (Miller 1959). One unit of inulinase activity was defined as the quantity liberating 1 µmol of fructose in 1 min, from a 0.5% w/v solution of inulin standard in 0.5 M McIlvaine buffer at pH 5.0 and 40°C.

##### *Invertase*

Crude enzyme extract (0.5 ml) was diluted to the required level with 0.05 M McIlvaine buffer at pH 5.0. Diluted extract (0.5 ml) was mixed with a 1 ml solution of 0.5% w/v sucrose (analytical grade; Ajax Finechem 500, Australia) in the mentioned buffer. The mixture was incubated and analysed for reducing sugars, as described earlier for the inulinase assay. One unit of invertase activity was defined as that liberating 1 µmol of fructose (or glucose) in 1 min from a 0.5% w/v solution of sucrose in 0.5 M McIlvaine buffer at pH 5.0 and 40°C.

## Inulin hydrolysis

The capabilities of crude enzyme preparations described earlier for hydrolysing inulin were assessed in reaction mixtures that contained various concentrations of either single or mixed enzyme preparations, inulin substrate (chicory root inulin; Sigma, I-2255) and 0.1% w/v benzoic acid. The latter was used to inhibit microbial growth. Reactions were carried out in 250-ml Erlenmeyer flasks containing 70 ml of reaction mixture. The flasks were held on a 200 rev min<sup>-1</sup> shaker at a temperature of 40°C. Samples were withdrawn periodically, quenched by boiling for 10 min, centrifuged (10,000g, 10 min) and analysed for sugars, fructo-oligosaccharides and inulo-oligosaccharides as described in the next section.

When inulin was hydrolysed with mold or yeast inulinases individually, a substrate solution that initially had 50 g l<sup>-1</sup> (or 100 g l<sup>-1</sup> in separate experiments) was mixed with the enzyme solution to provide inulinases activity 0.1 U g<sup>-1</sup> inulin (or 0.2 U g<sup>-1</sup> inulin for the higher concentration of substrate) in the reaction mixture. All dilutions were with 0.1 M McIlvaine buffer at pH 4.5.

When mixed mold and yeast inulinases were used in hydrolysing inulin, a solution with the initial substrate concentration of 50 g l<sup>-1</sup> was mixed with the crude enzyme solution so that enzyme activity in the mixture was 0.2 U g<sup>-1</sup> inulin. In this case the enzyme activity was measured for the specific mixture of the enzymes. All dilutions were with McIlvaine buffer as specified earlier.

## Inulin analyses

The initial concentration of inulin prior to any hydrolysis was measured with the phenol-sulfuric acid method (Dobois et al. 1956) using chicory root inulin (Sigma, I-2255) as a standard. During hydrolysis, only the major oligosaccharides produced were analysed by high-performance anion exchange chromatography with pulsed amperometric detector (HPAEC-PAD), using a Dionex BioLC chromatograph (Sunnyvale, CA, USA) equipped with an ED 50-pulsed electrochemical detector with gold working electrode and silver chloride as a reference electrode. HPAEC-PAD was also used to obtain the degree of polymerization (DP) profile of inulin hydrolysates (Chatterton et al. 1993).

For HPAEC-PAD analyses, the quenched reaction mixtures were diluted to appropriate concentration with deionized water and filtered through a 0.45 µm Sartorius cellulose acetate membrane filter before injection. The samples were injected automatically

(AS50 autosampler) and the injection volume was 25 µl. A CarboPac PA1 column (2 mm × 250 mm) with a guard column was used. A gradient elution involving two eluents was used, as described by Saengthongpinit and Sajjaanantakul (2005). The eluents were 150 mM sodium hydroxide and 150 mM sodium hydroxide mixed with 500 mM sodium acetate. The eluent flow rate was 0.25 ml min<sup>-1</sup>. Glucose (Ajax Finechem A783, Australia), fructose (Ajax Finechem A775, Australia), sucrose (Ajax Finechem 500, Australia), 1-kestose (Wako 066-03423, Japan), nystose (Wako 064-03424, Japan) and 1<sup>F</sup>-fructofuranosyl nystose (Wako 068-03422, Japan) were used as standards for identifying inulin hydrolysates of DP 1–5, respectively. The HPAEC-PAD methodology in relation to analysis of inulin hydrolysates is further discussed by Chatterton et al. (1993).

## Results and discussion

### Inulinase-producing microorganisms

#### Isolation and screening

Initial isolation of microorganisms from rotten tubers of Jerusalem artichoke yielded ten molds, three bacteria, one actinomycete and a yeast that could grow on the agar medium that contained inulin as the sole carbon source. Four mold isolates were selected after primary screening at 30°C on agar plates as they displayed high colony growth rates of 1.50–2.25 cm d<sup>-1</sup> for 5 days, after point inoculation on the plates. These growth rate values were 1.1 to 1.7 times greater than the growth rate of *A. niger* ATCC 20611, that was used as a reference fungus (Sirisansaneeyakul et al. 2000a, b).

Secondary screening based on the measured production of inulinases, narrowed the total number of isolates to four, including a mold, a yeast, one actinomycete and one bacterium. These were designated as isolates KU-F1, KU-Y1, KU-A1, and KU-B3, respectively. Compared with the reference fungus (*A. niger* ATCC 20611), these isolates showed relatively good production of inulinases after 3- to 5-day incubation at 30°C in a liquid medium that had 1% inulin as the only carbon source (Table 1). The fungal isolate, KU-F1 exhibited the highest inulinase activity (Table 1). The actinomycete isolate KU-A1 also had a similarly high inulinase activity (Table 1). The highest invertase activity was detected in the culture broth of the yeast isolate KU-Y1 (Table 1). Interestingly, invertase activity was not found in the broth of the bacterial isolate KU-B3.

**Table 1** Growth and enzymes production by selected isolates

Isolate <sup>a</sup>	Specific growth rate (h <sup>-1</sup> )	Enzyme activity (U l <sup>-1</sup> )			Y <sub>I</sub> (U g <sup>-1</sup> )	Q <sub>I</sub> (U l <sup>-1</sup> h <sup>-1</sup> )
		Inulinases <sup>b</sup>	Invertase	I/S <sup>c</sup>		
KU-F1	0.38	120.2	634.8	0.19	8.7	0.75
KU-Y1	0.73	103.4	946.3	0.11	7.0	0.65
KU-A1	0.51	119.3	397.1	0.30	11.3	0.94
KU-B3	0.42	65.1	ND	ND	15.5	0.68
<i>A. niger</i> ATCC 20611	0.67	23.3	340.9	0.07	6.19	0.32

<sup>a</sup> F = fungus, Y = yeast, A = actinomycete, B = bacteria

<sup>b</sup> Culture times were: 5-d for KU-F1, KU-Y1, KU-A1; 4-d for KU-B3; 3-d for *A. niger* ATCC 20611

<sup>c</sup> I = inulinases, S = invertase, I/S = activities ratio

ND = not detected, Y<sub>I</sub> = inulinases yield on initial substrate (inulin), Q<sub>I</sub> = inulinases productivity

Hydrolysis of inulin (50 or 100 g l<sup>-1</sup>) by crude inulinases of the four promising isolates was measured as the reducing sugars produced with reaction time (Fig. 1). Enzymes of only the mold (KU-F1) and the yeast (KU-Y1) isolates attained high reducing sugar levels of 8.6 g l<sup>-1</sup>, or more, within 25 h of reaction (Fig. 1). In view of the high rates of hydrolysis with KU-F1 and KU-Y1, these microorganisms were selected for further study as competent producers of inulinases that also had relatively good ratios of activities of inulinases and invertase (I/S = 0.11–0.19; Table 1). The selected isolates had substantially higher yield and productivity of inulinases in comparison with the reference mold (Table 1). The new isolates KU-F1 and KU-Y1 were further systematically identified as explained in Identification.

### Identification

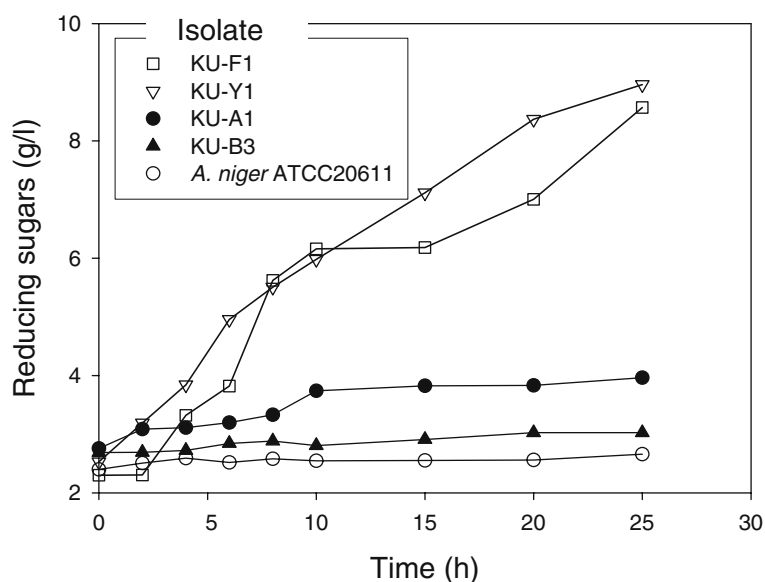
The mold isolate KU-F1 was identified as *A. niger*, based on morphological characteristics (Fig. 2), by the

National Institute of Health, Department of Medical Science (DMSC), Thailand. The yeast isolate KU-Y1 was identified as *C. guilliermondii*, by the Microbiological Resources Center, Thailand Institute of Scientific and Technological Research (TISTR), using the APILAB (bioMérieux, Inc., Durham, NC, USA) identification system. The two isolates were deposited at TISTR Culture Collection, Bangkok MIRCEN, as *A. niger* TISTR 3570 and *C. guilliermondii* TISTR 5844, respectively.

Inulinases from *A. niger* TISTR 3570 and *C. guilliermondii* TISTR 5844

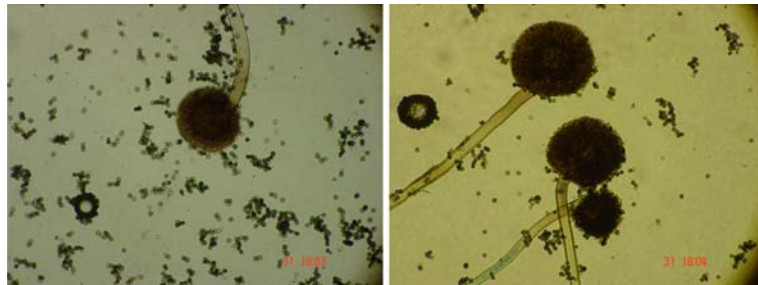
Inulin hydrolysis capabilities of separate crude preparations of the two microbial isolates were investigated using an initial substrate concentration of 100 g l<sup>-1</sup> and inulinases activity level of 0.2 U g<sup>-1</sup> inulin. The observed time profiles of concentrations of various sugars and fructo-/inulo-oligosaccharides during inulin hydrolysis are shown in Fig. 3 for the two isolates. The

**Fig. 1** Hydrolysis of inulin by crude inulinases of the four isolates and the reference fungus *A. niger* ATCC 20611



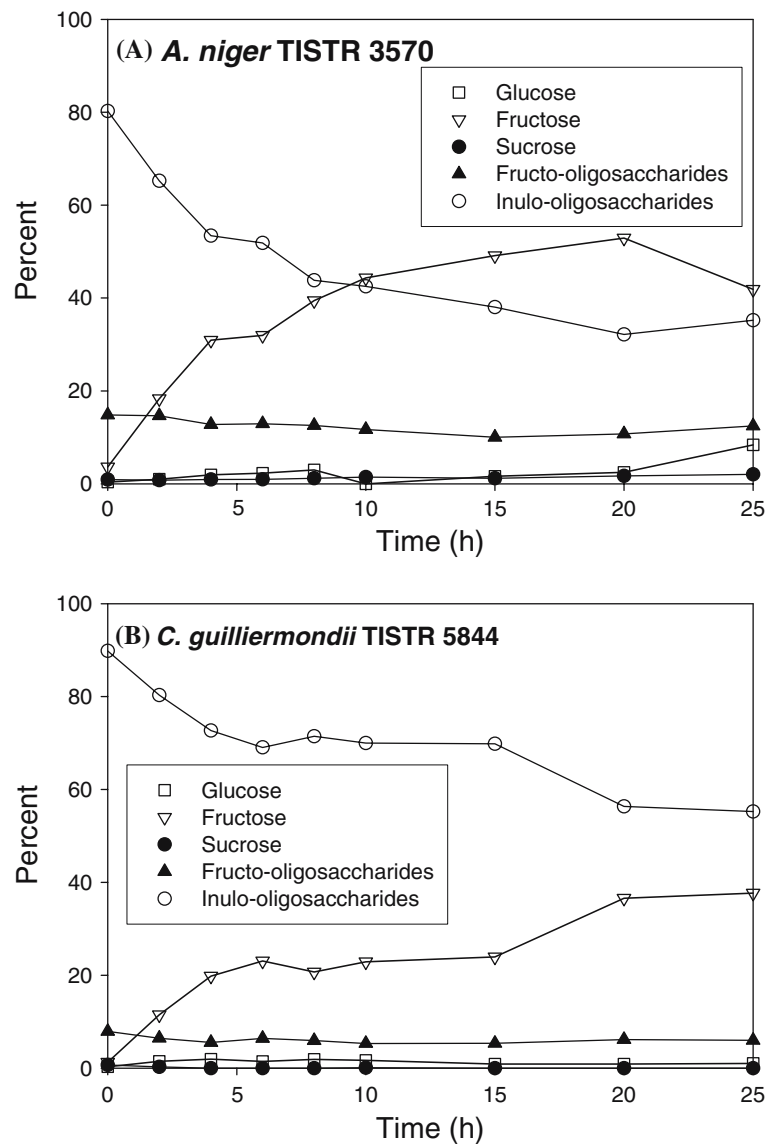


**Fig. 2** Morphology of *Aspergillus niger* TISTR 3570



initial level of the substrate in Fig. 3 is different for the two strains because the crude enzyme extracts of the strains had different levels of enzyme activities and, therefore, different volumes of crude enzyme solution had to be mixed with the substrate solution to achieve the specified enzyme activity level of  $0.2\text{U g}^{-1}$  of substrate in the reaction mixture.

**Fig. 3** Effect of inulinase source on changes in degree of polymerization (DP) during inulin hydrolysis: (a) *A. niger* TISTR 3570 and (b) *C. guilliermondii* TISTR 5844. Fructo-oligosaccharides and inulo-oligosaccharides refer to products with DP 3–10 and DP >10, respectively



The amount of inulo-oligosaccharides (Fig. 3) remaining at any time during the hydrolysis was estimated by subtracting the measured concentrations of the other carbohydrates (i.e. glucose, fructose, sucrose and fructo-oligosaccharides) from the initial concentration of inulo-oligosaccharide (i.e. inulin); thus,

$$[\text{Inulo} - \text{oligosaccharides}] = [\text{initial inulin}] - ((\text{DP1}) + [\text{DP2}] + [\text{DP3} - 10])$$

where

$$[\text{Initial inulin}] = [\text{DP1}] + [\text{DP2}] + [\text{DP3} - 10] + [\text{DP11} - 20] + [\text{DP21} - 30] + [\text{DP} > 30]$$

Both the mold and the yeast enzymes produced similar patterns of hydrolysis. In both cases, fructose was the main product. Levels of fructo-oligosaccharides were generally low. *A. niger* TISTR 3570 enzymes provided more rapid hydrolysis, compared to the yeast enzymes under the same conditions (Fig. 3).

As shown in Table 2, the fructose productivity with the mold enzymes was significantly greater than with yeast enzymes, at both enzyme activity levels tested. The mold enzymes hydrolysed a greater proportion of the initial inulin, in comparison with the yeast enzymes. Clearly, therefore, *A. niger* TISTR 3570 inulinases were superior to the yeast enzymes, for producing fructose from inulin. Other authors have generally observed similar differences in capabilities between inulinases of molds and yeasts (Guiraud and Galzy 1990; Frank and Leenheer 2002; Barta 1993; Hensing et al. 1993). Apparently, the synergistic effect of endo- and exo-inulinases in crude enzyme preparations of inulin hydrolysing molds contributes to the better hydrolytic performance by these enzymes.

The relative contents of monosaccharides (i.e. glucose, fructose), the disaccharide sucrose, and inulin oligosaccharides with various degrees of polymerization (DP), in the hydrolysates obtained from different treatments (i.e. enzyme activity, enzyme source, and initial substrate concentration), are shown in Table 3. Clearly, the hydrolysates generated using the yeast enzymes contained a larger fraction of oligosaccharides with DP values of 11–20 and 21–30, in comparison with the hydrolysates generated using the mold enzymes (Table 3). This suggests a poorer endo-inulinase activity in the yeast enzymes compared with the mold enzymes. The hydrolysates generated using the mold enzyme always had a higher fraction of fructose than did the hydrolysates generated using the yeast enzymes. In keeping with the enzyme activity data in Table 1, the residual sucrose in the hydrolysates generated with the mold enzymes (Table 3) confirmed that the invertase activity of the mold enzymes was not as good as that of the yeast enzymes.

In view of the lower amounts of sucrose and oligosaccharides with DP 3–10 present in the hydrolysates generated with the yeast enzymes (Table 3), the endo-inulinases of the yeast appeared to have a higher affinity for low DP fructans than did the mold enzymes.

The effect of substrate concentration on inulin hydrolysis was assessed using approximate initial inulin concentrations of 50 and 100 g l<sup>-1</sup> at the enzyme activity level of 0.2 U g<sup>-1</sup> inulin (Table 2). For both the mold and the yeast enzymes, the volumetric productivity of fructose and the fructose yield on inulin were

**Table 2** The production of fructose from inulin using crude inulinases

Parameter <sup>a</sup>	Fructose (g l <sup>-1</sup> )	Inulin hydrolysed (%) <sup>c</sup>	$Y_{F/S}$ (g g <sup>-1</sup> ) <sup>f</sup>	$Q_F$ (g l <sup>-1</sup> h <sup>-1</sup> ) <sup>g</sup>
Conditions of hydrolysis				
$[S] = 100 \text{ g l}^{-1}$ , $I = 0.2 \text{ U g}^{-1}$				
<i>A. niger</i> TISTR 3570 <sup>b,d</sup>	37.5	41.3	0.39	1.9
<i>C. guilliermondii</i> TISTR 5844 <sup>b,d</sup>	35.3	35.9	0.35	1.41
$[S] = 50 \text{ g l}^{-1}$ , $I = 0.2 \text{ U g}^{-1}$				
<i>A. niger</i> TISTR 3570 <sup>b,d</sup>	17.6	27.9	0.36	0.70
<i>C. guilliermondii</i> TISTR 5844 <sup>b,d</sup>	10.9	19.5	0.21	0.43
$[S] = 100 \text{ g l}^{-1}$ , $I = 0.1 \text{ U g}^{-1}$				
<i>A. niger</i> TISTR 3570 <sup>b,c</sup>	33.4	24.8	0.32	1.12
<i>C. guilliermondii</i> TISTR 5844 <sup>b,d</sup>	24.5	13.5	0.24	0.82

<sup>a</sup> At the instance of maximum fructose concentration

<sup>b</sup> Reaction time: <sup>c</sup> 20 h, <sup>d</sup> 25 h

$[S]$  = inulin concentration,  $I$  = inulinases,  $Y_{F/S}$  = fructose yield based on inulin,  $Q_F$  = volumetric productivity of fructose

<sup>c</sup> Percent inulin hydrolyzed =  $\frac{[S_0] - [S_t]}{[S_0]} \times 100$ , where  $[S_0]$  and  $[S_t]$  are concentrations of inulin at time 0 and  $t$ , respectively

<sup>f</sup>  $Y_{F/S} = \frac{[F_t] - [F_0]}{[S_0]}$ , where  $[F_t]$  and  $[F_0]$  are concentrations of fructose at time  $t$  and 0, respectively

<sup>g</sup>  $Q_F = \frac{[F_t] - [F_0]}{t - t_0}$

$S_0$  was always measured by the phenol–sulfuric acid method prior to hydrolysis. For above calculations,  $S_t$  was estimated as follows:

$$[S_t] = [S_0] - \sum [\text{DP1} - 5]$$

where the concentrations of the products with DP1–5 were estimated by HPAEC–PAD

**Table 3** Composition of inulin hydrolysates produced using crude inulinases

Microorganism	Conditions of hydrolysis	Composition (relative %) <sup>a</sup>							
		Monosaccharides	Glucose	Fructose	Sucrose	Inulin DP 3–10	Inulin DP 11–20	Inulin DP 21–30	Inulin DP >30
<i>A. niger</i> TISTR 3570 <sup>b,c</sup>	[S] = 100 g l <sup>-1</sup> , I = 0.2 U g <sup>-1</sup>	55.4	2.5	52.9	1.7	10.8	15.8	10.6	5.8
<i>C. guilliermondii</i> TISTR 5844 <sup>b,d</sup>		38.7	1.0	37.7	0.0	6.0	26.1	21.3	7.8
<i>A. niger</i> TISTR 3570 <sup>b,d</sup>	[S] = 50 g l <sup>-1</sup> , I = 0.2 U g <sup>-1</sup>	72.3	0.0	72.3	0.0	9.6	18.2	0.0	0.0
<i>C. guilliermondii</i> TISTR 5844 <sup>b,d</sup>		43.4	0.0	43.4	0.0	7.7	25.8	19.1	4.0
<i>A. niger</i> TISTR 3570 <sup>b,d</sup>	[S] = 100 g l <sup>-1</sup> , I = 0.1 U g <sup>-1</sup>	61.4	8.4	53.0	3.0	9.2	13.7	8.6	4.1
<i>C. guilliermondii</i> TISTR 5844 <sup>b,d</sup>		43.7	3.5	40.2	0.0	7.1	25.2	19.6	4.4

<sup>a</sup> At the instance of maximum fructose concentration

<sup>b</sup> Reaction time: <sup>c</sup> 20-h, <sup>d</sup> 25-h

[S] = inulin concentration, I = inulinases, DP = degree of polymerization, inulin DP = inulo-oligosaccharides DP

greater at the higher concentration of the substrate. No substrate inhibition was detected. An absence of substrate inhibition has previously been reported for inulin concentration as high as 150 g l<sup>-1</sup> during production of inulo-oligosaccharides using endo-inulinases of *Xanthomonas* sp. and *Pseudomonas* sp. (Cho et al. 2001).

At a constant initial substrate concentration of ~100 g l<sup>-1</sup>, changes in enzyme activity level from 0.1 to 0.2 U g<sup>-1</sup> inulin did increase the fructose productivity roughly in direct proportion to the enzyme activity (Table 2). As expected, the yield of fructose was not much affected by the changes in the enzyme dosage (Table 2), but the rate of fructose production was generally greater at the higher value of enzyme activity.

The production of fructose using mixed inulinases

Studies conducted with mixed inulinases (Table 4) used an initial substrate concentration of about 50 g l<sup>-1</sup> inulin. The mold and the yeast enzymes were mixed in the inulinases activities ratios of 1:1, 5:1 and 10:1, but the total activity of inulinases used in the reaction was always the same at 0.2 U g<sup>-1</sup>. As shown in Table 4, the 5:1 mixture of enzymes was distinctly better than the yeast enzymes alone, in terms of the fructose concentration obtained, the proportion of the initial inulin hydrolysed, the yield of fructose on inulin and the productivity of fructose. The 5:1 enzyme mixture was measurably better than the mold enzymes alone, but the difference was not very large. A 1:1 mixture was distinctly poorer than the mold enzymes alone, but still outperformed the yeast enzymes used alone.

The product profiles of the hydrolysates produced with the enzyme mixtures are shown in Table 5. The 5:1 mixture had a product profile that was comparable to the profile obtained using the mold enzymes alone. In view of the measurable enhancements in fructose yield and a satisfactory product profile in terms of the components present, an enzyme mixture of 5:1 was the most appropriate for hydrolysing inulin to fructose.

## Conclusions

Among the 15 microbial strains that were isolated from Jerusalem artichoke tubers grown in Thailand, *A. niger* TISTR 3570 and *C. guilliermondii* TISTR 5844 were

**Table 4** Comparison of fructose production from inulin using mixed-inulinases

Parameter <sup>a</sup>	Fructose (g l <sup>-1</sup> )	Inulin hydrolysed (%)	$Y_{F/S}$ (g g <sup>-1</sup> )	$Q_F$ (g l <sup>-1</sup> h <sup>-1</sup> )
Conditions				
<i>A. niger</i> TISTR 3570	17.6	27.9	0.36	0.70
<i>C. guilliermondii</i> TISTR 5844	10.9	19.5	0.21	0.43
Inulinases activity ratio (A:C)				
1:1	15.3	27.8	0.32	0.61
5:1	18.2	28.4	0.37	0.73
10:1	18.1	25.0	0.37	0.72

<sup>a</sup> At 25 h, the instance of maximum fructose concentration

A = *A. niger* TISTR 3570, C = *C. guilliermondii* TISTR 5844

$Y_{F/S}$  = fructose yield based on inulin;  $Q_F$  = volumetric productivity of fructose



**Table 5** Relative percentages of sugars and inulin in hydrolysates produced using mixed inulinases

Composition (relative %) <sup>a</sup>	Conditions			Inulinases activity ratios (A:C)		
	<i>A. niger</i> TISTR 3570	<i>C. guilliermondii</i> TISTR 5844		1:1	5:1	10:1
	Monosaccharides	72.3	43.4		66.0	78.2
Glucose	0.0	0.0		0.0	0.0	0.0
Fructose	72.3	43.4		66.0	78.2	77.7
Sucrose	0.0	0.0		0.0	0.0	0.0
Inulin DP 3–10	9.5	7.7		7.4	0.0	11.3
Inulin DP 11–20	18.2	25.8		17.0	10.2	11.0
Inulin DP 21–30	0.0	19.1		9.6	11.6	0.0
Inulin DP >30	0.0	4.0		0.0	0.0	0.0

<sup>a</sup> At 25 h, the instance of maximum fructose concentration  
*A* = *A. niger* TISTR 3570,  
*C* = *C. guilliermondii* TISTR 5844, DP = degree of polymerization, inulin DP = inulo-oligosaccharides DP

identified as novel isolates that produced significant quantities of inulinases. Crude inulinases of these microorganisms were assessed for their potential in producing fructose from inulin.

*A. niger* TISTR 3570 was a better overall producer of suitable inulinases. At 40°C, an initial inulin concentration of 100 g l<sup>-1</sup>, pH 4.5, and the enzyme activity level of 0.2 U g<sup>-1</sup>, inulinases of *A. niger* TISTR 3570 converted inulin to fructose with a yield of 0.39 g g<sup>-1</sup> at a productivity of 1.9 g l<sup>-1</sup> h<sup>-1</sup>. The final fructose concentration in the hydrolysate exceeded 37 g l<sup>-1</sup>. The hydrolysate contained 55.4% monosaccharides (i.e. glucose and fructose), 10.8% fructo-oligosaccharides (DP 3–10), and the remainder as disaccharide (i.e. sucrose; DP = 2) and inulo-oligosaccharides having a degree of polymerization of greater than 10.

An enzyme system consisting of the mold and yeast inulinases mixed in the activity ratio of 5:1, was better at hydrolysing inulin than were the yeast and mold enzymes alone. This mixed system produced a hydrolysate that contained 78.2% fructose. The enhanced capability of the mixed enzyme system relative to individual microbial enzymes, revealed the synergistic effect of a combined exo- and endo-inulinases system. The crude enzyme preparations were quite satisfactory in producing high levels of the desired inulin hydrolysis.

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