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BIOENERGY AND BIOFUELS

Thermotolerant *Kluyveromyces marxianus* and *Saccharomyces cerevisiae* strains representing potentials for bioethanol production from Jerusalem artichoke by consolidated bioprocessing

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Abstract Thermotolerant inulin-utilizing yeast strains are desirable for ethanol production from Jerusalem artichoke tubers by consolidated bioprocessing (CBP). To obtain such strains, 21 naturally occurring yeast strains isolated by using an enrichment method and 65 previously isolated *Saccharomyces cerevisiae* strains were investigated in inulin utilization, extracellular inulinase activity, and ethanol fermentation from inulin and Jerusalem artichoke tuber flour at 40 °C. The strains *Kluyveromyces marxianus* PT-1 (CGMCC AS2.4515) and *S. cerevisiae* JZ1C (CGMCC AS2.3878) presented the highest extracellular inulinase activity and ethanol yield in this study. The highest ethanol concentration in Jerusalem artichoke tuber flour fermentation (200 gL^{-1}) at 40 °C achieved by *K. marxianus* PT-1 and *S. cerevisiae* JZ1C was 73.6 and 65.2 gL^{-1}, which corresponded to the theoretical ethanol yield of 90.0 and 79.7 %, respectively. In the range of 30 to 40 °C, temperature did not have a significant effect on ethanol production for both strains. This study displayed the distinctive superiority of *K. marxianus* PT-1 and *S. cerevisiae* JZ1C in the thermotolerance and utilization of inulin-type oligosaccharides reserved in Jerusalem artichoke tubers. It is proposed that both *K. marxianus* and *S. cerevisiae* have considerable potential in ethanol production from Jerusalem artichoke tubers by a high temperature CBP.

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Introduction

Bioethanol serving as a form of renewable energy has been regarded as an alternative to fossil fuels. At present, bioethanol is essentially produced from crops such as corn and sugar cane (Rojey and Monot 2010). However, concerns have been raised about its production related to food shortage because such crops must be planted on farmland (Li and Chan-Halbrendt 2009). Thus, feedstocks that do not depend on fertile farmland have recently received much attention. Jerusalem artichoke can grow well on poor land without competition for good quality fertile land and possesses high tolerance to frost and plant diseases which normally attack traditional crops (Chubey and Dorrell 1974; Dorrell and Chubey 1977). The tuber yield of Jerusalem artichoke can reach 90 t/ha with a carbohydrate yield of 5~14 t/ha (Swanton et al. 1992). The tuber of the plant contains inulin, a dominant reserve carbohydrate (70~90 %) consisting of linear chains of β-2,1-linked D-fructofuranose molecules terminated by a glucose residue (Pandey et al. 1999). Inulin or its hydrolysates can be converted into ethanol by microorganisms. Consequently, Jerusalem artichoke is treated as a sustainable feedstock for bioethanol production.

In the process of ethanol fermentation from Jerusalem artichoke tubers, the feedstock is generally hydrolyzed into fructose and glucose before fermentation by acid or enzymatic hydrolysis to achieve high fermentation efficiency (Torandiaz et al. 1985; Kim and Hamdy 1986; Kim and Rhee 1990). However, acid hydrolysis produces fermentation inhibitors and enzymatic hydrolysis increases production cost (Torandiaz et al. 1985). The yeast *Kluyveromyces*

marxianus can efficiently utilize inulin and has been used in ethanol production from Jerusalem artichoke tubers without hydrolysis pretreatment (Margaritis and Bajpai 1982a, b; Yuan et al. 2008). Recently, consolidated bioprocessing (CBP) was used in ethanol fermentation from Jerusalem artichoke tubers by *K. marxianus* in a publication, where CBP means integrating inulinase production, inulin hydrolysis, and fermentation in one step (Yuan et al. 2011). The yeasts that can directly utilize inulin and produce ethanol are potential CBP strains. In CBP, it is desirable that the temperatures of saccharification and fermentation are consistent. However, yeast inulinases responsible for inulin digestion function efficiently at a temperature between 40 and 50 °C, while the optimum fermentation temperature of the yeasts is between 30 and 35 °C (Kushi et al. 2000; Chi et al. 2009; Zhang et al. 2009). Thus, thermotolerant inulin-utilizing yeast strains are economically important and preferred for CBP.

To obtain thermotolerant inulin-utilizing yeast strains for ethanol fermentation from Jerusalem artichoke by CBP, we isolated 21 naturally occurring yeast strains by an enrichment method and evaluated 65 previously isolated *Saccharomyces cerevisiae* strains by a high throughput assay. These yeast strains were further investigated by determination of the extracellular inulinase activity and by ethanol fermentation assay from inulin and Jerusalem artichoke tuber flour. This study suggested that not only *K. marxianus* PT-1 but also *S. cerevisiae* strain JZ1C was thermotolerant and both of them represent potential candidate strains for ethanol production from Jerusalem artichoke by an elevated temperature CBP.

Materials and methods

Isolation of thermotolerant inulin-utilizing yeast strains

To isolate yeast strains, pieces of samples were directly placed into sterile plastic tubes containing 3 mL liquid selective medium at the sampling locations. The cultures were incubated overnight at 40 °C with shaking at 200 rpm. The samples included the rhizosphere soil of Jerusalem artichoke, tubers of Jerusalem artichoke, and fruits from two markets in Qingdao, China. The selective medium was 1/2 YPI containing 10 gL^{-1} of inulin, 10 gL^{-1} of peptone, and 5 gL^{-1} of yeast extract (pH 5.0). The inulin (chicory) was purchased from Bio Basic Inc (BBI), Canada. The diluted culture (100 μL) was spread on YPI plate (20 gL^{-1} of inulin, 20 gL^{-1} of peptone, 10 gL^{-1} of yeast extract, and 20 gL^{-1} of agar) supplemented with 200 mg L^{-1} chloramphenicol and 50 gL^{-1} ethanol. After 5 days of incubation at 40 °C, colonies were transferred into YPI slants and then stored in glycerol at −80 °C for further investigation (Table 1).

Table 1 Strains isolated in this study and sources

Species	Strain	Source
Candida tropicalis	FQ-1	Tomato
	HMG-6	Hami melon
	PT-2	Grape
	XJ-5	Banana
Clavispora lusitaniae	LZ-5	Plum
Kluyveromyces marxianus	PT-1	Grape
Meyerozyma guilliermondii	LL-1	Durian
	SG-3	Apple
	SG-7	Apple
	SL-6	Pomegranate
	SL-4	Pomegranate
	LZ-1	Plum
	YZ-23	Pomelo
	YZ-2	Pomelo
	YZ-16	Pomelo
M. caribbica	LZ-2	Plum
	LZ-12	Plum
Torulaspora delbrueckii	PT-13	Grape
	XG-1	Watermelon
Saccharomyces cerevisiae	S22	Grape
	S30	Banana

Identification of yeast strains

The yeast strains were identified by sequence analysis of the 26 S rDNA D1/D2 domain. Nuclear DNA was extracted by using the method of Makimura et al. (1994). The sequences of the rDNA D1/D2 domain were amplified and sequenced as described by Lu et al. (2004). The phylogenetic tree was generated by the neighbor-joining method. Reference sequences were retrieved from GenBank under the accession numbers indicated.

Screening of thermotolerant inulin-utilizing *S. cerevisiae* strains

A panel of *S. cerevisiae* strains isolated before from diverse ecological sources and geographic locations in China was used in this study (Table 2). These strains were isolated using an enrichment method described in Wang and Bai (2008). To evaluate the inulin assimilation of the isolates, their growth dynamics using inulin as the sole carbon source was determined in Bioscreen C MBR reader (Oy Growth Curves Ab Ltd., Helsinki, Finland) at 30 and 40 °C, respectively. The strains were inoculated into 3 mL YPD medium (20 gL^{-1} glucose, 20 gL^{-1} peptone, and 10 gL^{-1} yeast extract) and incubated at 30 °C with shaking at 200 rpm. The cultures were centrifuged, washed twice with sterile water, and then resuspended into YPI medium to a final cell

Table 2 *Saccharomyces cerevisiae* strains examined by using a Bioscreen C MBR reader

Strain	Location	Source	Date
SH17-1	Xinjiang, China	Vineyard grape	2006
SH26-1	Xinjiang, China	Vineyard grape	2006
SH28-1	Xinjiang, China	Vineyard grape	2006
XL4	Xinjiang, China	Pear in orchard	2006
XL10-1	Xinjiang, China	Pear in orchard	2006
XL11-2	Xinjiang, China	Pear in orchard	2006
XL14-1	Xinjiang, China	Pear in orchard	2006
P7-1	Xinjiang, China	Apple in orchard	2006
P8-1	Xinjiang, China	Apple in orchard	2006
P11	Xinjiang, China	Apple in orchard	2006
P16	Xinjiang, China	Apple in orchard	2006
JZ1C	Yunnan, China	Orange in orchard	2006
BL6	Yunnan, China	Pineapple in plantation	2006
BL8	Yunnan, China	Pineapple in plantation	2006
BL11C	Yunnan, China	Pineapple in plantation	2006
BJPTE	Beijing area, China	Vineyard grape	2008
WA3-4	Beijing area, China	Vineyard grape	2008
BJPG4	Beijing area, China	Apple in orchard	2008
BJPG8	Beijing area, China	Apple in orchard	2008
BJL3	Beijing area, China	Pear in orchard	2008
BJL7	Beijing area, China	Pear in orchard	2008
BJL10	Beijing area, China	Pear in orchard	2008
AQPT45	Shandong, China	Vineyard grape	2008
AQT2	Shandong, China	Grapevine	2008
QFPT32	Shandong, China	Vineyard grape	2008
YC77	Jiling, China	Plum	2008
YC81	Jiling, China	Peach	2008
A2	Shaanxi, China	Oak bark	2006
A9	Shaanxi, China	Oak bark	2006
A11	Shaanxi, China	Oak bark	2006
A7	Shaanxi, China	Wild tomato	2006
HLB2	Shaanxi, China	Oak bark	2006
HTJ1	Shaanxi, China	Oak bark	2006
HHT2	Shaanxi, China	Oak bark	2006
ZX4	Yunnan, China	Oak bark	2006
YDX4	Yunnan, China	Soil	2008
YNT3	Yunnan, China	Soil	2008
SHM1	Hainan, China	Oak bark	2006
SHM4	Hainan, China	Oak bark	2006
BW4	Hainan, China	Rotten wood	2008
DL3	Hainan, China	Rotten wood	2008
JF1	Hainan, China	Rotten wood	2008
HNG26	Hainan, China	Soil	2008
BJ3	Beijing area, China	A peach flower	2006
LS23	Beijing area, China	Oak bark	2008
LS26	Beijing area, China	Oak bark	2008
LS542	Beijing area, China	Oak bark	2008
LSYS62	Beijing area, China	Oak bark	2008
LSLL69	Beijing area, China	Oak bark	2008
AQSZ8	Shandong, China	Oak bark	2008
TABL2	Shandong, China	Oak bark	2008
TAHT7	Shandong, China	Oak bark	2008
TS7	Shandong, China	Oak bark	2008
BYC1	Jiling, China	Oak bark	2008
AS2.3	England	Beer	Pre-1952
AS2.4	England	Beer	Pre-1952
AS2.24	England	Brewary	Pre-1952
AS2.381	Russia	Paper mill	Pre-1960
AS2.395	Russia	Paper mill	Pre-1960
AS2.521	Unknown	Ratafee	Pre-1960
AS2.666	Guizhou, China	Distilled spirit	Pre-1960
AS2.1422	Japan	Distilled spirit	1955
AS2.1427	Japan	Distilled spirit	Pre-1943

density with an OD_{600} reading of 0.02. Three hundred microliters of the YPI resuspension was pipetted into 100-well Honeycomb plate and incubated in Bioscreen C MBR reader without shaking. The optical density at 600 nm was automatically read every 30 min. Three duplications were performed for each strain.

Determination of extracellular inulinase activity

The yeast strains were inoculated into a 250-mL flask containing 50 mL of YPI medium and incubated at 40 °C with shaking at 200 rpm. The culture of 1 mL sampled at various time points was centrifuged at 4 °C, and the supernatant was employed for inulinase activity determination. Enzyme activities were assayed using inulin or sucrose as substrates by determining the concentration of reducing sugars released. The reaction mixture containing 0.1 mL of diluted crude extract and 0.9 mL of 20 gL^{-1} inulin (dissolved in 0.1 mol L^{-1} acetate buffer, pH 5.0) was incubated at 50 °C for 30 min. The reaction was terminated at 100 °C for 10 min, and the concentration of the reducing sugar in the mixture was determined (Spiro 1966). Absorbance was read at a wavelength of 540 nm. One inulinase (U) unit was defined as the amount of enzyme which yields 1 μmol fructose min^{-1} under the assay conditions used in this study.

Effect of pH and temperature on crude enzyme stability

The crude enzyme solutions were collected from the YPI cultures after growing for 36 h. The effect of pH and temperature on the stability of the crude enzyme solutions was investigated according to a previously described method (Yuan et al. 2012). In brief, pH stability was evaluated by

pre-incubating the solutions for 120 min at 4 °C in the pH range of 3.0 to 8.0. The temperature stability of the solutions was tested by pre-incubating them at temperatures from 30 to 70 °C for 120 min in 0.1 M sodium acetate buffer (pH 5.0).

Effect of pH and temperature on growth

The effect of pH and temperature on growth was evaluated in the strains *K. marxianus* PT-1 and *S. cerevisiae* JZ1C. The optimum growth temperature was investigated in YPD medium by using 250 mL flasks. The effect of pH on the strains cultured in the YPD medium at the optimum temperature was detected by using Bioscreen C MBR reader. The maximum growth temperature was tested on YPD slants at various temperatures.

Ethanol fermentation

Ethanol fermentation from inulin and Jerusalem artichoke tuber flour was performed as follows: The seed medium consisted of 50 gL^{-1} of inulin, 10 gL^{-1} of peptone, 6 g L^{-1} of yeast extract, 2 gL^{-1} of ammonium sulfate, 1 gL^{-1} of potassium dihydrogen phosphate, 1.5 gL^{-1} of magnesium sulfate heptahydrate, and 0.55 gL^{-1} of calcium chloride. Yeast cells were inoculated into 50 mL of fermentation seed medium and incubated at 30 or 40 °C with shaking at 200 rpm for 16 h. The seed culture incubated at 40 °C was transformed into 250 mL flasks containing 100 mL of fermentation medium at an inoculum size of 10 % (*v*/*v*) for ethanol fermentation at 40 °C without shaking. The seed culture incubated at 30 °C was used in the fermentation of Jerusalem artichoke tuber flour at 30, 35, and 40 °C with shaking at 120 rpm. The inulin fermentation medium contained 100 gL^{-1} of inulin and equal concentrations of other components as in the seed medium. For fermentation of Jerusalem artichoke, 200 gL^{-1} of tuber flour without any other nutrient supplements was adjusted to pH 4.0 and used in the process. The Jerusalem artichoke tubers were purchased from Hengshui, Hebei Province, China. Dry matter content of the fresh tubers was 23.6 %. To prepare the flour, tubers were washed, peeled, sliced, and dried in a forced air flow oven at 60 °C. The dried material was finely pulverized by ball milling. The total sugar content in the flour (80.2 %) was estimated after acid hydrolysis.

Three duplications were performed for all the tests. The ethanol concentration was determined by Biosensor SBA 40C (Biology Institute of Shandong Academy of Sciences, Jinan, China). Reducing sugar and total sugar were determined by phenol–sulfuric acid method (Masuko et al. 2005).

High-performance anion-exchange chromatography

The inulin-type oligosaccharides before and after YPI fermentation by *K. marxianus* PT-1 and *S. cerevisiae* JZ1C were detected using high-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD). Analyses were performed on a Dionex ICS-3000 chromatography system (Dionex Corporation). The standard sample contained 0.01 gL^{-1} of fructose and sucrose. The initial YPI medium was diluted tenfold and the fermentation supernatant was diluted fivefold before analyses. A volume of 25 μL of samples was injected on a Dionex PA200 column at a flow rate of 0.5 mL min^{-1} and the oligosaccharides were separated by elution with 100 mM NaOH.

Sequence submission

The nucleotide sequences of the 26 S rDNA D1/D2 domain were deposited in the GenBank database under the accession numbers JQ686899 to JQ686919.

Results

Isolation and identification of thermotolerant inulin-utilizing yeast strains

To obtain thermotolerant and inulin-utilizing yeast strains, we employed an enrichment method. Inulin was used as the sole carbon source, and ethanol (50 gL^{-1}) and high temperature (40 °C) served as selection pressures. Under the enrichment condition, 21 fast growing yeast strains were isolated. These strains were identified as belonging to seven yeast species and the six genera of *Kluyveromyces*, *Meyerozyme*, *Saccharomyces*, *Torulaspora*, *Candida*, and *Clavispora* (Table 1). The phylogenetic relationships of the strains were inferred in the neighbor-joining tree based on the sequence data of 26 S rDNA D1/D2 domain (Fig. 1). *K. marxianus* and *Meyerozyme guilliermondii* were previously documented with a high efficiency in inulin utilization and identified in this study (Gong et al. 2007; 2008; Margaritis and Bajpai 1982a, b; Yuan et al. 2008; Zhang et al. 2010). Interestingly, *S. cerevisiae* and *Meyerozyme caribbica* were also observed capable of utilizing inulin, which were not well known in inulin metabolism. In particular, the *S. cerevisiae* strains identified here can grow well at 40 °C.

Screening of thermotolerant inulin-utilizing *S. cerevisiae* strains

The identification of two *S. cerevisiae* strains growing well on the enrichment medium indicated that *S. cerevisiae* can utilize inulin, but it is unknown how efficiently does the yeast convert the substrate. We examined the growth dynamics of 65 *S. cerevisiae* isolates on the sole carbon source of inulin by Bioscreen C MBR reader at 40 and 30 °C,

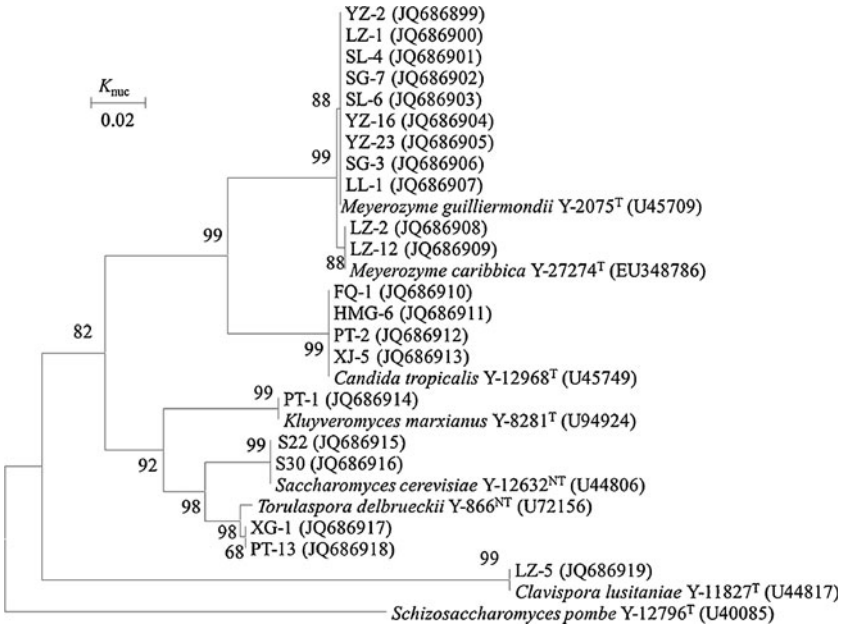

Fig. 1 Phylogenetic tree drawn from neighbor-joining analysis of the 26 S rDNA D1/D2 domain, depicting the relationships of the isolates. Bootstrap percentages over 50 % from 1,000 bootstrap replicates are shown. Reference sequences were retrieved from GenBank under the accession numbers indicated

respectively. Most of them were previously isolated from diverse sources and geographic locations in China (Table 2). The strains of JZ1C, BJL3, and BL11C represented advantageous growth dynamics at 40 °C (Fig. 2a). In order to investigate whether the advantage is due to thermotolerance or efficient inulin utilization, we selected 15 strains and examined their growth dynamics at 30 °C. Advantageous growth was achieved by JZ1C, BJL3, BJL7, and TABL2 (Fig. 2b). These observations indicated that JZ1C and BJL3 were thermotolerant inulin-utilizing strains, BL11C was a thermotolerant strain, while BJL7 and TABL2 were inulin-utilizing strains. The strain JZ1C exhibited a slightly higher growth rate than BJL3 at 40 °C and was applied in the next assays (Fig. 2a).

Extracellular inulinase activity of the yeast strains

Inulin metabolism in microorganisms is essentially due to the secretion of extracellular inulinase. To further evaluate inulin utilization of the yeast isolates, we measured the extracellular inulinase activity of the 21 isolates and the strain *S. cerevisiae* JZ1C grown in YPI medium at 40 °C. High inulinase activities were found in the five strains of *K. marxianus* PT-1, *M. guilliermondii* SL-6, *M. guilliermondii* YZ-16, *M. caribbica* LZ-2, and *S. cerevisiae* JZ1C (Fig. 3). As depicted in Fig. 3, the strain *K. marxianus* PT-1 showed an obviously advantageous enzyme activity dynamics and the highest enzyme activity of 30.4 U mL^{-1}. The strain *S. cerevisiae* JZ1C showed the highest enzyme activity of 5.8 U mL^{-1}, which was slightly higher than those of the other three strains. The sucrose/inulin (S/I) hydrolysis ratio of the crude enzyme was 3.19±0.55 for *K. marxianus* PT-1 and 13.33±2.34 for *S. cerevisiae* JZ1C.

The effect of pH and temperature on the stability of the extracellular crude enzyme solutions was investigated in *K. marxianus* PT-1 and *S. cerevisiae* JZ1C. The crude enzyme solution from *K. marxianus* PT-1 showed pH stability in the range of 4.0 to 5.5 and temperature stability below 55 °C. At

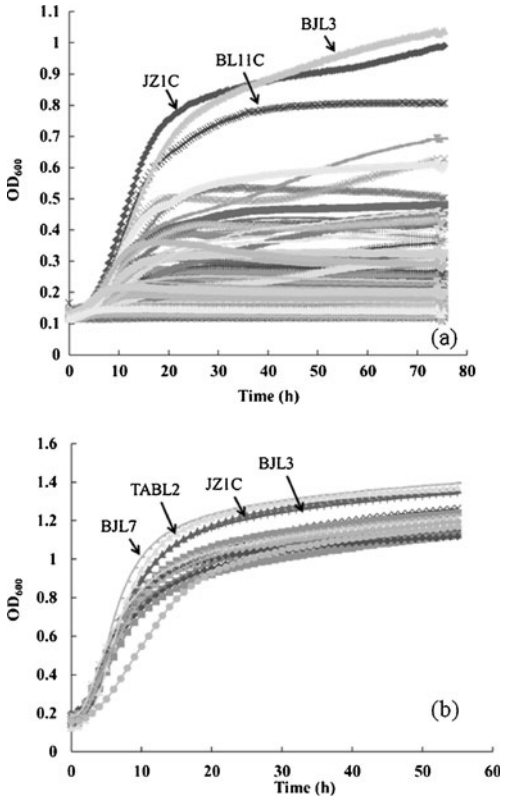

Fig. 2 The growth dynamics of *S. cerevisiae* strains using inulin as the sole carbon source determined by Bioscreen C MBR reader. **a** 40 °C, 65 strains; **b** 30 °C, 15 strains

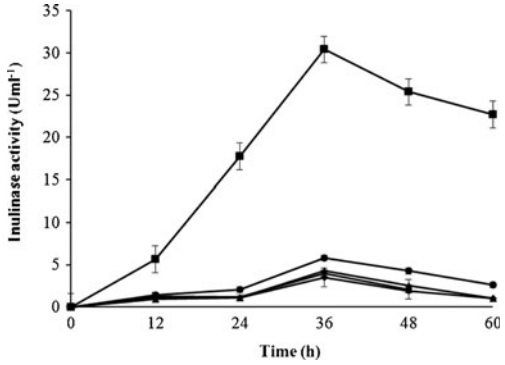

Fig. 3 Extracellular inulinase activity dynamics of the five strains *K. marxianus* PT-1 (*square*), *M. guilliermondii* SL-6 (*diamond*), *M. guilliermondii* YZ-16 (*triangle*), *M. caribbica* LZ-2 (*times symbol*), and *S. cerevisiae* JZ1C (*circle*)

a temperature higher than 55 °C, the inulinase activity decreased substantially and only 26 % of activity remained after 2 h of treatment at 60 °C. The crude enzyme solution from *S. cerevisiae* JZ1C displayed pH stability in the range of 4.0 to 7.5 and temperature stability below 45 °C. After 2 h of treatment at 55 °C, the solution completely lost the inulinase activity.

Ethanol fermentation at 40 °C

Ethanol fermentation from inulin was performed at 40 °C by using the five strains of *K. marxianus* PT-1, *M. guilliermondii* SL-6, *M. guilliermondii* YZ-16, *M. caribbica* LZ-2, and *S. cerevisiae* JZ1C. The highest ethanol production and specific inulin hydrolysis rates were achieved by *K. marxianus* PT-1 (Fig. 4). This strain produced 43 gL^{-1} of ethanol within 48 h from 100 gL^{-1} of inulin and that was 78 % of the theoretical ethanol yield. *S. cerevisiae* JZ1C showed the fastest growth and the largest biomass concentration (Fig. 4). The final ethanol production of JZ1C was 28 gL^{-1} (51 % of the theoretical ethanol yield), which was higher than those of *M. guilliermondii* and *M. caribbica* strains (Fig. 4a).

Due to the advantages of ethanol fermentation from inulin, the two strains *K. marxianus* PT-1 and *S. cerevisiae* JZ1C were further tested in ethanol fermentation from 200 gL^{-1} of Jerusalem artichoke tuber flour at 40 °C without shaking (Fig. 5). The total sugar content in the tuber flour accounted for 80.2 %. The highest ethanol concentration achieved by *K. marxianus* PT-1 and *S. cerevisiae* JZ1C was 69.6 and 65.1 gL^{-1}, which corresponded to the theoretical ethanol yield of 84.9 and 79.5 %, respectively. The ethanol productivity was 0.9 gL^{-1} h^{-1} for *S. cerevisiae* JZ1C versus 0.83 gL^{-1} h^{-1} for *K. marxianus* PT-1. These data displayed the distinctive superiority of the two strains in thermotolerance and ethanol fermentation of Jerusalem artichoke tuber flour. They have been deposited in the China

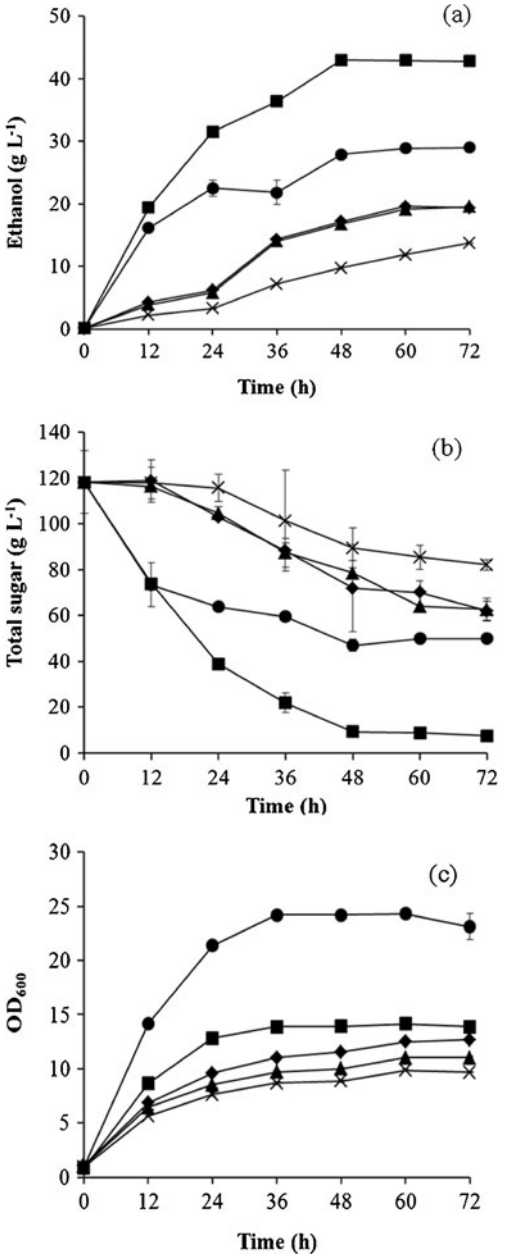

Fig. 4 Ethanol fermentation from inulin. **a** Ethanol production; **b** residual total sugar; **c** biomass. *K. marxianus* PT-1 (*square*), *M. guilliermondii* SL-6 (*diamond*), *M. guilliermondii* YZ-16 (*triangle*), *M. caribbica* LZ-2 (*times symbol*), and *S. cerevisiae* JZ1C (*circle*)

General Microbiological Culture Collection Center, Academia Sinica, Beijing, China, as AS2.3878 for *S. cerevisiae* JZ1C and AS2.4515 for *K. marxianus* PT-1.

Effect of temperature on ethanol fermentation from Jerusalem artichoke tuber flour

The effect of pH and temperature on growth was estimated before the comparison of ethanol fermentation in the strains *K. marxianus* PT-1 and *S. cerevisiae* JZ1C.

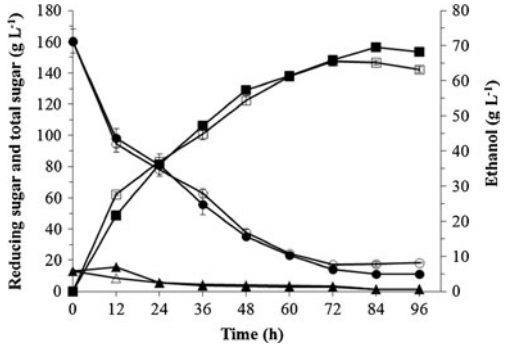

Fig. 5 Ethanol fermentation from Jerusalem artichoke tuber flour at 40 °C. Ethanol yield, *K. marxianus* PT-1 (*black square*), *S. cerevisiae* JZ1C (*white square*); total sugar, *K. marxianus* PT-1 (*black circle*), *S. cerevisiae* JZ1C (*white circle*); reducing sugar, *K. marxianus* PT-1 (*black triangle*), *S. cerevisiae* JZ1C (*white triangle*)

The favorable pH was found in the range of 4.0 to 7.0 for *K. marxianus* PT-1 and 4.0 to 8.0 for *S. cerevisiae* JZ1C. The optimum growth temperatures were observed at 42 °C for *K. marxianus* PT-1 and 37 °C for *S. cerevisiae* JZ1C. The maximum growth temperature was estimated at 50 °C for *K. marxianus* PT-1 and 45 °C for *S. cerevisiae* JZ1C.

In view of the fact that the optimum fermentation temperature was generally lower than the optimum growth temperature, ethanol fermentation from Jerusalem artichoke tuber flour by *K. marxianus* PT-1 and *S. cerevisiae* JZ1C was performed at 30, 35, and 40 °C with shaking at 120 rpm, respectively (Fig. 6 and Table 3). The result showed that the effect of temperature in the range from 30 to 40 °C on ethanol fermentation process was not significant (Fig. 6). The highest ethanol concentration achieved by *K. marxianus* PT-1 and *S. cerevisiae* JZ1C was 74.4 gL^{-1} at 35 °C and 66.9 gL^{-1} at 30 °C, respectively. At 40 °C, the highest ethanol concentrations of 73.6 gL^{-1} for *K. marxianus* PT-1 and 65.2 gL^{-1} for *S. cerevisiae* JZ1C were observed, which corresponded to the theoretical ethanol yield of 90.0 and 79.7 %, respectively. The ethanol productivity at 40 °C was 0.91 gL^{-1} h^{-1} for *S. cerevisiae* JZ1C and 1.53 gL^{-1} h^{-1} for *K. marxianus* PT-1.

Inulin-type oligosaccharide utilization by the strains PT-1 and JZ1C

The inulin-type oligosaccharide utilization was examined in *K. marxianus* PT-1 and *S. cerevisiae* JZ1C by HPAE-PAD analysis. The data indicated that *K. marxianus* PT-1 could almost completely utilize all the inulin-type oligosaccharides while *S. cerevisiae* JZ1C could not efficiently hydrolyze oligosaccharides with a high degree of polymerization (DP) (Fig. 7).

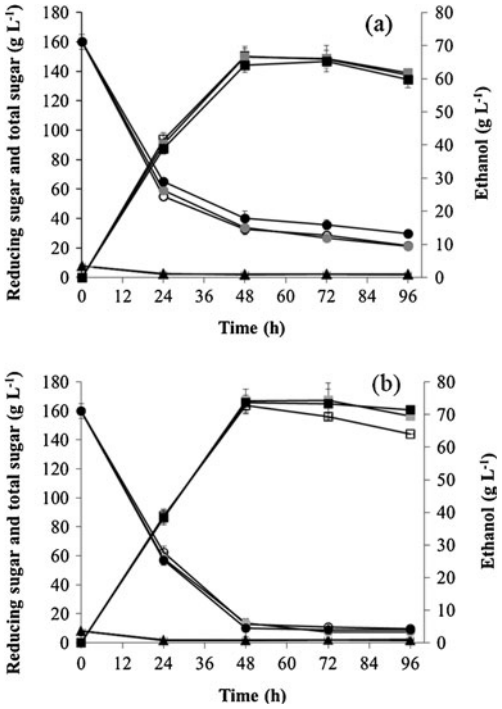

Fig. 6 Effect of temperature on ethanol fermentation from Jerusalem artichoke tuber flour. **a** *S. cerevisiae* JZ1C. Ethanol, 30 °C (*white square*), 35 °C (*gray square*), 40 °C (*black square*); total sugar, 30 °C (*white circle*), 35 °C (*gray circle*), 40 °C (*black circle*); reducing sugar, 30 °C (*white triangle*), 35 °C (*gray triangle*), 40 °C (*black triangle*). **b** *K. marxianus* PT-1. Ethanol, 30 °C (*white square*), 35 °C (*gray square*), 40 °C (*black square*); total sugar, 30 °C (*white circle*), 35 °C (*gray circle*), 40 °C (*black circle*); reducing sugar, 30 °C (*white triangle*), 35 °C (*gray triangle*), 40 °C (*black triangle*)

Discussion

CBP has attracted great attention for the biological conversion of cellulosic biomass. The process integrating cellulase production, cellulose hydrolysis, and fermentation in one step has the potential to provide the lowest cost route for biomass conversion (Lynd et al. 2005; van Zyl et al. 2007; Anasontzis et al. 2011; Jin et al. 2011). This strategy was recently introduced into ethanol fermentation from Jerusalem artichoke, where CBP featured inulinase production, inulin hydrolysis, and fermentation in one step (Yuan et al. 2011). To realize the low cost through CBP in fermentation from Jerusalem artichoke, yeast strains that ferment inulin with high conversion rates must be used. The optimum fermentation temperature of yeast strains is between 30 and 35 °C, while presently identified yeast inulinases function efficiently at a temperature between 40 and 50 °C (Kushi et al. 2000; Chi et al. 2009; Zhang et al. 2009). Thus, strains tolerant to a high temperature are desirable in CBP for the consistency between the temperature of enzymatic hydrolysis and that of fermentation. Furthermore, high temperature fermentation has some other advantages, such as reducing the risk of contamination and decreasing the

Table 3 Comparison of kinetic parameters in fermentation of Jerusalem artichoke tubers by *K. marxianus* or *S. cerevisiae* strains

Kinetic parameters	*K. marxianus*			*S. cerevisiae*	
	PT-1	IGC2671	ATCC8554	JZ1C	KCCM50549
Substrate	Flour	Juice	Flour	Flour	Flour
Temperature (°C)	40	40	35	40	30
Maximum ethanol concentration (g L^{-1})	73.6	84	88	65.2	36.2
Ethanol productivity (g L^{-1} h^{-1})	1.53	0.35	1.05	0.91	1.06
Ethanol yield (g g^{-1})	0.46	0.4	0.467	0.41	0.35
Theoretical ethanol yield (%)	90	78	91.5	79.7	70
References	This study	Rosa et al. (1987)	Yuan et al. (2008)	This study	Lim et al. (2011)

costs of cooling and ethanol extraction (Abdel-Banat et al. 2010). It was demonstrated that a 5 °C increase in the fermentation temperature can greatly reduce fuel ethanol production costs (Abdel-Banat et al. 2010). In this study, we obtained thermotolerant inulin-utilizing strains *K. marxianus* PT-1 and *S. cerevisiae* JZ1C, which showed potential in bioethanol fermentation from Jerusalem artichoke at 40 °C by CBP.

K. marxianus is well known for thermotolerance and it could even grow at 52 °C, ferment glucose to produce ethanol at 50 °C, and generate the highest ethanol yield at 37 °C (Banat et al. 1992). *K. marxianus* can utilize various substrates, such as cellobiose, xylose, xylitol, arabinose, glycerol, lactose, and inulin (Banat and Marchant 1995; Nonklang et al. 2008; Lane et al. 2011; Rocha et al. 2011). This is an advantage for the conversion of feedstock containing mixed carbon sources. It was reported that fermentation of sugar cane juice containing 22 % total sugars by one *K. marxianus* strain produced 60.4 and 77.5 % of theoretical ethanol yield at 40 and 37 °C, respectively (Limtong et al. 2007). Recently, *K. marxianus* was employed in cellulosic ethanol production at high temperature, and it represented potential in fermentation at 45 and 48 °C, though the fermentation efficiency remained to be improved (Yanase et al. 2010; Kang et al. 2011; Pessani et al. 2011). Due to the advantages of thermotolerance and inulin utilization ability, *K. marxianus* has popularly been used in ethanol production from Jerusalem artichoke and the reported optimum ethanol fermentation temperature was 35 °C (Margaritis and Bajpai 1982a, b; Bajpai and Margaritis 1987; Yuan et al. 2008, 2011). The ethanol yield from Jerusalem artichoke tuber fermentation by *K. marxianus* strains was generally over 0.4 gg^{-1} of available sugars (Table 3), which was significantly higher than that from lactose with values less than 0.32 (Lane et al. 2011). The strain *K. marxianus* PT-1 obtained in this study could ferment 200 gL^{-1} of Jerusalem artichoke tuber flour without any other nutrient supplements to produce 90 % of the theoretical ethanol yield at 40 °C, representing its superiority in ethanol fermentation from Jerusalem artichoke by a high temperature CBP (Table 3). The strain PT-1 was isolated from a grape sample, while no microbial strains were isolated from the environments related to Jerusalem artichoke in this study. A recent study reported yeast strains isolated from Jerusalem artichoke and soil of Jerusalem artichoke tubers by using a non-selective wort medium; however, these yeasts were not taxonomically identified and fructose fermentation instead of inulin fermentation was tested by them (Bonciu et al. 2010). From the inulin sources of dahlia and chicory, bacteria and fungi that can secrete inulinases and belong to several different genera have been isolated, while only the yeast *Sporotrichum* sp. has been found in these sources (Pandey et al. 1999).

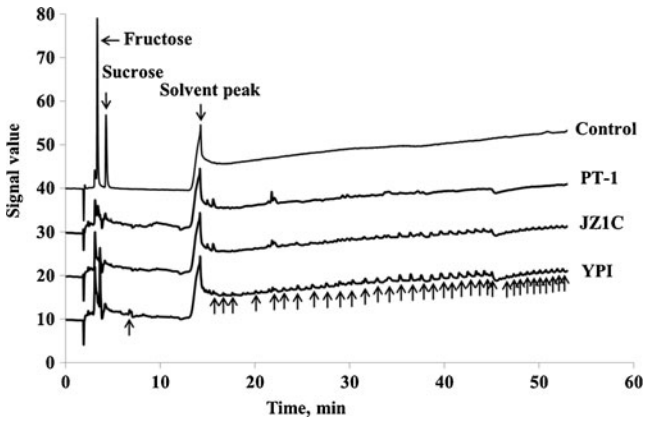

Fig. 7 Oligosaccharides analysis by HPAE-PAD. Control, fructose and sucrose; JZ1C, the YPI supernatant after fermentation by JZ1C; PT-1, the YPI supernatant after fermentation by PT-1. YPI, tenfold diluted YPI medium containing 2 gL^{-1} of inulin, 2 gL^{-1} of peptone, and 1 gL^{-1} of yeast extract. *Arrows* denote the typical oligosaccharides, but their specific DP values are unknown

S. cerevisiae strains are well known as ethanol-producing microorganisms; however, they have generally been considered unable to utilize inulin because no inulinase genes have been found in the genome of model strains. In this study, we observed that the *S. cerevisiae* strain JZ1C can ferment 200 gL^{-1} of Jerusalem artichoke tuber flour to produce 79.7 % of the theoretical ethanol yield at 40 °C and the ethanol productivity was 0.91 gL^{-1} h^{-1}. A recent study

reported that *S. cerevisiae* could ferment Jerusalem artichoke tuber flour to produce 70 % of the theoretical ethanol yield at 30 °C (Lim et al. 2011). These data indicated the superiority of *S. cerevisiae* JZ1C in ethanol fermentation from Jerusalem artichoke by a high temperature CBP (Table 3). The ethanol production of *S. cerevisiae* JZ1C in inulin fermentation was higher than those of *M. guilliermondii* and *M. caribbica* strains in this study. It was reported that *M. guilliermondii* secreted a large amount of active inulinase and the corresponding gene was cloned (Gong et al. 2007; 2008; Zhang et al. 2009). However, *S. cerevisiae* has no inulinase genes and the genes responsible for inulin utilization in *S. cerevisiae* JZ1C are obscure. *S. cerevisiae* possesses genes encoding invertases that are members of the superfamily β-fructofuranosidase as well as inulinases. The pH stability of the extracellular invertase in *S. cerevisiae* was reported in the range of 3.0 to 7.5, and its activity decreased substantially at temperatures above 50 °C (Gascón et al. 1968; Cavaille and Combes 1995). The crude enzyme solution from *S. cerevisiae* JZ1C was similar to the extracellular invertase in pH and temperature stability. In addition, the S/I hydrolysis ratio of the crude enzyme solution was over 10 for *S. cerevisiae* JZ1C supporting that the enzyme with inulinase activity was an invertase (Saber and El-Naggar 2009). Work is underway in our laboratory to validate the assumption.

In this study, the strain *S. cerevisiae* JZ1C represented a remarkably lower theoretical ethanol yield (51 %) than *K. marxianus* PT-1 (78 %) in inulin fermentation, while the theoretical ethanol yield significantly improved in Jerusalem artichoke flour fermentation for both strains and the difference between them minimized (79.7 % for JZ1C and 90 % for PT-1). The inulin used in this study was extracted from chicory and had an average DP of 12, while the average DP of oligosaccharides in Jerusalem artichoke tubers was reported to be 3.9 (Wack and Blaschek 2006; Slimestad et al. 2010). The strain *K. marxianus* PT-1 could efficiently utilize the inulin-type oligosaccharides with high DP, while *S. cerevisiae* JZ1C could not efficiently hydrolyze such oligosaccharides (Fig. 7). These findings demonstrated that the different metabolic abilities of the two strains to oligosaccharides with high DP were the cause of the discrepancy between Jerusalem artichoke tuber flour and inulin fermentation. The enzyme responsible for inulin utilization in *S. cerevisiae* JZ1C represented exoinulinase activity and could not hydrolyze inulin-type oligosaccharides with high DP. Endoinulinases can digest a long strand of inulin into oligosaccharides with low DP, which can be efficiently utilized by *S. cerevisiae* JZ1C. To improve Jerusalem artichoke tuber fermentation by CBP using the *S. cerevisiae* JZ1C, a heterologous gene expression is being performed in the strain by introducing an endoinulinase gene from *Aspergillus niger* in our laboratory.

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References

Abdel-Banat BMA, Hoshida H, Ano A, Nonklang S, Akada R (2010) High-temperature fermentation: how can processes for ethanol production at high temperatures become superior to the traditional process using mesophilic yeast? Appl Microbiol Biotechnol 85:861–867

Anasontzis GE, Zerva A, Stathopoulou PM, Haralampidis K, Diallinas G, Karagouni AD, Hatzinikolaou DG (2011) Homologous overexpression of xylanase in *Fusarium oxysporum* increases ethanol productivity during consolidated bioprocessing (CBP) of lignocellulosics. J Biotechnol 152:16–23

Bajpai P, Margaritis A (1987) The effect of temperature and pH on ethanol production by free and immobilized cells of *Kluyveromyces marxianus* grown on Jerusalem artichoke extract. Biotechnol Bioeng 30:306–313

Banat IM, Marchant R (1995) Characterization and potential industrial applications of five novel, thermotolerant, fementative, yeast strains. World J Microbiol Biotechnol 11:304–306

Banat IM, Nigam P, Marchat R (1992) Isolation of thermotolerant, fermentative yeasts growing at 52 °C and producing ethanol at 45 °C and 50 °C. World J Microbiol Biotechnol 8:259–263

Bonciu C, Tabacaru C, Bahrim G (2010) Yeasts isolation and selection for bioethanol production from inulin hydrolysates. Innov Rom Food Biotechnol 6:29–34

Cavaille D, Combes D (1995) Effect of temperature and pressure on yeast invertase stability: a kinetic and conformational study. J Biotechnol 43:221–228

Chi Z, Zhang T, Liu G, Yue L (2009) Inulinase-expressing microorganisms and applications of inulinases. Appl Microbiol Biotechnol 82:211–220

Chubey BB, Dorrell DG (1974) Jerusalem artichoke, a potential fructose crop for prairies. Can Inst Food Sci Technol J 7:98–106

Dorrell DG, Chubey BB (1977) Irrigation, fertilizer, harvest dates and storage effects on reducing sugar and fructose concentrations of Jerusalem artichoke tubers. Can J Plant Sci 57:591–596

Gascón S, Neumann NP, Lampen O (1968) Comparative study of the properties of the purified internal and external invertases from yeast. J Biol Chem 243:1573–1577

Gong F, Sheng J, Chi Z, Li J (2007) Inulinase production by a marine yeast *Pichia guilliermondii* and inulin hydrolysis by the crude inulinase. J Ind Microbiol Biotechnol 34:179–185

Gong F, Zhang T, Chi ZM, Sheng J, Li J, Wang XH (2008) Purification and characterization of extracellular inulinase from a marine yeast *Pichia guilliermondii* and inulin hydrolysis by the purified inulinase. Biotechnol Bioproc Eng 13:533–539

Jin MJ, Balan V, Gunawan C, Dale BE (2011) Consolidated bioprocessing (CBP) performance of *Clostridium phytofermentans* on AFEX-treated corn stover for ethanol production. Biotechnol Bioeng 108:1290–1297

Kang HW, Kim Y, Kim SW, Choi GW (2011) Cellulosic ethanol production on temperature-shift simultaneous saccharification and fermentation using the thermostable yeast *Kluyveromyces marxianus* CHY1612. Bioprocess Biosyst Eng. doi:10.1007/s00449-011-0621-0

Kim CH, Rhee SK (1990) Ethanol-production from Jerusalem artichoke by inulinase and *Zymomonas mobilis*. Appl Biochem Biotechnol 23:171–180

Kim K, Hamdy MK (1986) Acid-hydrolysis of Jerusalem-artichoke for ethanol fermentation. Biotechnol Bioeng 28:138–141

Kushi RT, Monti R, Contiero J (2000) Production, purification and characterization of an extracellular inulinase from *Kluyveromyces marxianus* var. *bulgaricus*. J Ind Microbiol Biotechnol 25:63–69

Lane MM, Burke N, Karreman R, Wolfe KH, O'Byrne CP, Morrissey JP (2011) Physiological and metabolic diversity in the yeast *Kluyveromyces marxianus*. Antonie van Leeuwenhoek 100:507–519

Li SZ, Chan-Halbrendt C (2009) Ethanol production in (the) People's Republic of China: potential and technologies. Appl Energy 86: S162–S169

Lim SH, Ryu JM, Lee H, Jeon JH, Sok DE, Choi ES (2011) Ethanol fermentation from Jerusalem artichoke powder using *Saccharomyces cerevisiae* KCCM50549 without pretreatment for inulin hydrolysis. Bioresour Technol 102:2109–2111

Limtong S, Sringiew C, Yongmanitchai W (2007) Production of fuel ethanol at high temperature from sugar cane juice by a newly isolated *Kluyveromyces marxianus*. Bioresour Technol 98:3367–3374

Lu HZ, Ca YM, Wu ZW, Jia JH, Bai FY (2004) *Kazachstania aerobia* sp nov., an ascomycetous yeast species from aerobically deteriorating corn silage. Int J Syst Evol Microbiol 54:2431–2435

Lynd LR, van Zyl WH, McBride JE, Laser M (2005) Consolidated bioprocessing of cellulosic biomass: an update. Curr Opin Biotechnol 16:577–583

Makimura K, Murayama SY, Yamaguchi H (1994) Detection of a wide-range of medically important fungi by the polymerase chain-reaction. J Med Microbiol 40:358–364

Margaritis A, Bajpai P (1982a) Continuous ethanol-production from Jerusalem artichoke tubers. 1. Use of free-cells of *Kluyveromyces marxianus*. Biotechnol Bioeng 24:1473–1482

Margaritis A, Bajpai P (1982b) Continuous ethanol-production from Jerusalem artichoke tubers. 2. Use of immobilized cells of *Kluyveromyces marxianus*. Biotechnol Bioeng 24:1483–1493

Masuko T, Minami A, Iwasaki N, Majima T, Nishimura SI, Lee YC (2005) Carbohydrate analysis by a phenol–sulfuric acid method in microplate format. Anal Biochem 339:69–72

Nonklang S, Abdel-Banat BM, Cha-aim K, Moonjai N, Hoshida H, Limtong S, Yamada M, Akada R (2008) High-temperature ethanol fermentation and transformation with linear DNA in the thermotolerant yeast *Kluyveromyces marxianus* DMKU3-1042. Appl Environ Microbiol 74:7514–7521

Pandey A, Soccol CR, Selvakumar P, Soccol VT, Krieger N, Fontana JD (1999) Recent developments in microbial inulinases—its production, properties, and industrial applications. Appl Biochem Biotechnol 81:35–52

Pessani NK, Atiyeh HK, Wilkins MR, Bellmer DD, Banat IM (2011) Simultaneous saccharification and fermentation of Kanlow switchgrass by thermotolerant *Kluyveromyces marxianus* IMB3: the effect of enzyme loading, temperature and higher solid loadings. Bioresour Technol 102:10618–21064

Rojey A, Monot F (2010) Biofuels: production and applications. Wiley, Weinheim

Rocha SN, Abrahão-Neto J, Gombert AK (2011) Physiological diversity within the *Kluyveromyces marxianus* species. Antonie Van Leeuwenhoek 100:619–630

Rosa MF, Correia IS, Novais JM (1987) Production of ethanol at high temperature in the fermentation of Jerusalem artichoke juice and a simple medium by *Kluyveromyces marxianus*. Biotechnol Lett 9:441–444

Saber WIA, El-Naggar NE (2009) Optimization of fermentation conditions for the biosynthesis of inulinase by the new source; *Aspergillus tamarii* and hydrolysis of some inulin containing agrowastes. Biotechnology 8:425–433

Slimestad R, Seljaasen R, Meijer K, Skar SL (2010) Norwegian-grown Jerusalem artichoke (*Helianthus tuberosus* L.): morphology and content of sugars and fructo-oligosaccharides in stems and tubers. J Sci Food Agric 90:956–964

Spiro RG (1966) Analysis of sugars found in glycoproteins. Methods Enzymol 8:3–26

Swanton CJ, Cavers PB, Clements DR, Moore MJ (1992) The biology of Canadian weeds. 101. *Helianthus tuberosus* L. Can J Plant Sci 72:1367–1382

Torandiaz I, Jain VK, Allais JJ, Baratti J (1985) Effect of acid or enzymatic-hydrolysis on ethanol-production by *Zymomonas mobilis* growing on Jerusalem artichoke juice. Biotechnol Lett 7:527–530

van Zyl WH, Lynd LR, den Haan R, McBride JE (2007) Consolidated bioprocessing for bioethanol production using *Saccharomyces cerevisiae*. Adv Biochem Eng Biotechnol 108:205–235

Wack M, Blaschek W (2006) Determination of the structure and degree of polymerisation of fructans from *Echinacea purpurea* roots. Carbohydr Res 341:1147–1153

Wang SA, Bai FY (2008) *Saccharomyces arboricolus* sp. nov., a yeast species from tree bark. Int J Syst Evol Microbiol 58:510–514

Yanase S, Hasunuma T, Yamada R, Tanaka T, Ogino C, Fukuda H, Kondo A (2010) Direct ethanol production from cellulosic materials at high temperature using the thermotolerant yeast *Kluyveromyces marxianus* displaying cellulolytic enzymes. Appl Microbiol Biotechnol 88:381–388

Yuan B, Hu N, Sun J, Wang SA, Li FL (2012) Purification and characterization of a novel extracellular inulinase from a new yeast species *Candida kutaonensis* sp. nov. KRF1^{T}. Appl Microbiol Biotech. doi:10.1007/s00253-012-4108-y

Yuan WJ, Chang BL, Ren JG, Liu JP, Bai FW, Li YY (2011) Consolidated bioprocessing strategy for ethanol production from Jerusalem artichoke tubers by *Kluyveromyces marxianus* under high gravity conditions. J Appl Microbiol 112:38–44

Yuan WJ, Zhao XQ, Ge XM, Bai FW (2008) Ethanol fermentation with *Kluyveromyces marxianus* from Jerusalem artichoke grown in salina and irrigated with a mixture of seawater and freshwater. J Appl Microbiol 105:2076–2083

Zhang T, Chi Z, Parrou JL, Gong F (2010) Expression of the inulinase gene from the marine-derived *Pichia guilliermondii* in *Saccharomyces* sp. W0 and ethanol production from inulin. Microb Biotechnol 3:576–582

Zhang T, Gong F, Chi Z, Liu GL, Chi ZM, Sheng J, Li J, Wang XH (2009) Cloning and characterization of the inulinase gene from a marine yeast *Pichia guilliermondii* and its expression in *Pichia pastoris*. Antonie Van Leeuwenhoek 95:13–22