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ORIGINAL PAPER

Inulinase production by a marine yeast *Pichia guilliermondii* and inulin hydrolysis by the crude inulinase

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Abstract Marine yeast strain 1, isolated from the surface of a marine alga, was found to secrete a large amount of inulinase into the medium. This marine yeast was identified as a strain of *Pichia guilliermondii* according to the results of routine yeast identification and molecular methods. The crude inulinase produced by this marine yeast worked optimally at pH 6.0 and 60°C. The optimal medium for inulinase production was seawater containing 4.0% (w/v) inulin and 0.5% (w/v) yeast extract, while the optimal cultivation conditions for inulinase production were pH 8.0, 28°C and 170 rpm. Under the optimal conditions, over 60 U ml^{-1} of inulinase activity was produced within 48 h of fermentation in shake flasks. A large amount of monosaccharides and a trace amount of oligosaccharides were detected after the hydrolysis, indicating that the crude inulinase had a high exoinulinase activity.

Keywords Inulinase · Marine yeasts · Marine environment · *Pichia guilliermondii* · 18S rDNA · ITS

Introduction

Inulin is a linear β-(2, 1)-linked fructose polymer that occurs as a reserve carbohydrate in Jerusalem artichoke, dahlia tubers and chicory root [13, 15]. This polymer is a recognized source for the production of either ultra-high fructose syrups, with D-fructose content over 95%, or for production of oligofructose syrups [15]. Fructose is a GRAS sweetener, sweeter than sucrose (up to 1.5 times), with lower cost, and has functional properties that enhance flavor, color, and product stability, and is thus widely used in many foods and beverages instead of sucrose. Furthermore, fructose metabolism bypasses the known metabolic pathway of glucose and therefore does not require insulin [9]. Oligofructose is a prebiotic, and its positive effect on human health has been widely known [9]. Inulin can be hydrolyzed by a chemical approach. However, the chemical approach has many drawbacks [5, 13]. Fructose can also be produced from starch by enzymatic methods involving α-amylase, amyloglucosidase and glucose isomerase, resulting in the production of a mixture consisting of oligosaccharides (8%), fructose (45%) and glucose (50%) [5]. However, separation of fructose from this fructose syrup is costly and thus makes this method uneconomical [5]. The best procedure involves the use of microbial inulinase, which after one step enzymatic hydrolysis of inulin, yields 95% pure fructose. Inulinase is produced by many microorganisms, such as *Kluyveromyces*, *Aspergillus*, *Staphylococcus*, *Xanthomonas* and *Pseudomonas*. Yeasts such as *Kluyveromyces fragilis*, *Kluyveromyces marxianus*, *Candida kefyr*, *Debaryomyces cantarelli* and fungi, *Penicillium* and *Aspergillus* species are common inulinase producers [8, 13]. Among the yeasts which can produce inulinases, such as strains of *Candida* sp., *Sporotrichum* sp., *Pichia* sp., and *Kluyveromyces* sp, two species of *K. fragilis* and *K. marxianus* have high potential for producing commercially acceptable yields of the enzyme [13]. The genes encoding inulinase have been cloned and sequenced in *Kluyveromyces cicerisporus* CBS4857 and *K. marxianus* ATCC12424

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[12, 24]. In our recent studies, we found that diversity of marine yeasts is very rich and several marine yeast strains can produce protease, amylase, lipase and killer toxin [4]. However, there has been no report about inulinase and inulinase production from marine yeasts.

After we screened over 400 marine yeast strains from different marine environments, we found that some marine yeast strains could secrete a large amount of inulinase into medium prepared with seawater. The main purpose of the present study was to optimize the conditions for inulinase production by the marine yeast *Pichia guilliermondii*. We also identified the hydrolysis products of inulin by the crude inulinase produced by this marine yeast strain. To our knowledge, this is the first report of inulinase produced by a marine yeast.

Materials and methods

Yeast strain

The yeast strain, *P. guilliermondii*, was isolated from the surface of a marine alga collected at 100 m depth of seawater at Changdao island in Penglai, China. This yeast strain was maintained in YPD medium containing 2.0 g glucose, 2.0 g yeast extract, 1.0 g polypeptone and 100 ml of seawater at 4°C.

Inulinase production

One loopful of the cells of the yeast strain was transferred into 50 ml of YPD medium prepared with seawater in a 250 ml flask and aerobically cultivated for 24 h. The cell culture (5.0 ml, $OD_{600\ nm} = 20.0$) was transferred to 45 ml of the production medium (prepared with seawater), which contained 4.0% inulin, 0.5% yeast extract and initial pH 8 and grown by shaking at 170 rpm and 28.0°C for 2 days. In order to determine the optimal medium and cultivation conditions for inulinase production by the yeast strain, different carbon and nitrogen sources as well as NaCl with different concentrations were added to the medium prepared with distilled water and the yeast strain was grown at different values of pH and temperature.

Determination of inulinase activity

The fermented broth was centrifuged at 5,000 rpm and 4°C for 5 min and the supernatant was taken as a crude enzyme. The reaction mixture containing 0.1 ml of the crude enzyme and 0.9 ml of phosphate buffer (0.1 M, pH 6.0) containing 2.0% inulin was incubated at 60°C for 10 min. The reaction was inactivated immediately by keeping the reaction mixture at 100°C for 10 min. The amount of reducing sugar in the reaction mixture was assayed by the method of Nelson–Somogyi [20]. One inulinase unit (U) was defined as the amount of enzyme that produces one micromole of reducing sugar per minute under the assay conditions used in this study.

DNA extraction and PCR

The total genomic DNA of the yeast strain was isolated and purified by using the methods as described by Sambrook et al. [16]. The common primers for amplification of 18S rDNA in yeast were used, the forward primer P1: 5′-ATCTGGTTGATCCTGCCAGT-3′ and the reverse primer P2: 5′-GATCCTTCCGC AGGTTCACC-3′ [21] and the common primers for amplification of ITS in yeasts were used, the forward primer P11: 5′-TCCGTAGGTGAACCTGCGG-3′ and the reverse primer P21: 5′-TCCTCCGCTTATTG ATATGC-3′ [7]. The reaction system (25 µl) was composed of 10 × buffer 2.5 µl, dNTP 0.8 µmol/l, $MgCl_2$ 1.5 mmol/l, P1 or P11 0.5 µmol/l, P2 or P21 0.5 µmol/l, Taq DNA polymerase 1.25 U, template DNA 1.0 µl and H_2O 16.6 µl. The conditions for the PCR amplification were as follows: initial denaturation at 94°C for 10 min, denaturation at 94°C for 1 min, annealing temperature at 53°C for 1.0 min, extension at 72°C for 2.0 min, final extension at 72°C for 10 min. PCR was run for 32 cycles and PCR cycler was GeneAmp PCR System 2400 made by Perkin–Elmer. PCR products were separated by agarose gel electrophoresis and recovered by using UNIQ-column DNA gel recovery kits (BIOASIA, Shanghai). The recovered PCR products were ligated into pGEM-T easy vector and transformed into the competent cells of *Escherichia coli* JM109. The transformants were selected on plates with ampicilin. The plasmids in the transformant cells were extracted by using the methods as described by Sambrook et al. [16]. In order to confirm that the PCR products had been ligated into the vector, the purified plasmids were used as templates for amplification of 18S rDNA and ITS in the yeast strain, respectively. The reaction system and the conditions for PCR amplification were the same as described above. The 18S rDNA fragment and ITS fragment inserted on the vector were sequenced by Shanghai Sangon Company.

Phylogenetic analysis and identification of the yeast

The sequences obtained above were aligned by using BLAST analysis (http://www.ncbi.nlm.nih.gov/BLAST). For comparison with currently available sequences, 20 sequences were retrieved with over 98% similarity

belonging to 20 different genera from NCBI (http://www.ncbi.nlm.nih.gov) and performed multiple alignment by using Bioedit 7.0. The routine identification of the yeasts was performed by using the methods as described by Kurtzman and Fell [10].

Effects of pH and temperature on inulinase activity

The effects of pH on the enzyme activity were determined by incubating the culture supernatant at different pH values between 5.0 and 8.0 using the standard assay conditions described above. The buffers used were 0.1 M acetate butter (pH 5.0) and 0.1 M phosphate buffer (pH 6.0–8.0). The optimal temperature for activity of the enzyme was determined at 40, 50, 60, 65 and 70°C in the same buffer as described above.

Inulin hydrolysis

Inulin hydrolysis was carried out by incubating the reaction mixture containing 2.0% inulin in 0.1 M phosphate buffer (pH 6.0) and 20 µl of 120 U ml^{-1} of the crude enzyme at 60°C for 12 h. The end products of inulin hydrolysis after 12 h of incubation at 60°C were withdrawn and identified to ascertain the extent of hydrolysis by ascending thin layer chromatography (Silica gel 60, MERCK, Germany) with the solvent system of *n*-butanol–pyridine–water (6:4:3) and a detection reagent comprising 2.0% diphenylamine in acetone—2.0% aniline in acetone—85% phosphoric acid (5: 5: 1 by volume).

Results and discussion

Screening and identification of the inulinase-producing marine yeast

Many terrestrial yeasts have been confirmed to have the capacity to produce a large amount of inulinase [5, 13]. Therefore, we wanted to know whether such yeasts exist in marine environments. After over 400 yeast strains from seawater, sediments, guts of the marine fish and marine algae were screened, we found that strain 1, which was isolated from the surface of the marine algae collected at 100 m depth of seawater, could secrete large amount of inulinase into the production medium (data not shown). The colony of the yeast strain 1 was white to cream and butyrous. The vegetative cells of this strain reproduced by budding. There was a simple to elaborate pseudohyphae. The ascospores formed were hat-shaped. The yeast strain could ferment glucose, sucrose and raffinose, but could not ferment galactose, maltose, lactose and melibiose. However, it could assimilate glucose, galactose, sucrose, maltose, cellobiose, trehalose, raffinose, L-arabose and D-xylose, but could not assimilate D-arabose and soluble starch. It did not require vitamins for growth, but it could grow on the medium containing 50 and 60% (w/v) glucose and could not utilize nitrate. The results show that this strain is closely related to *P. guilliermondii* [10].

18S rRNA gene and ITS sequences of yeast strain 1 were deposited in NCBI server and the accession numbers are DQ821711 and DQ681368, respectively. Phylogenetic analysis of 20 18S rRNA gene sequences and ITS sequences with over 97% similarity belonging to 20 different genera from NCBI server shows that the similarities between 18S rRNA gene sequences of yeast strain 1 and *P. guilliermondii* and between ITS sequences of yeast strain 1 and *P. guilliermondii* were 99%, respectively. Therefore, the yeast strain 1 was finally identified as a strain of *P. guilliermondii* [10]. It has been reported that extracellular inulinase was produced by *Pichia* sp [14].

Effects of different temperature and pH on activity of the crude inulinase

The results in Figs. 1 and 2 show that the optimal pH and temperature for the crude inulinase were 6.0 and

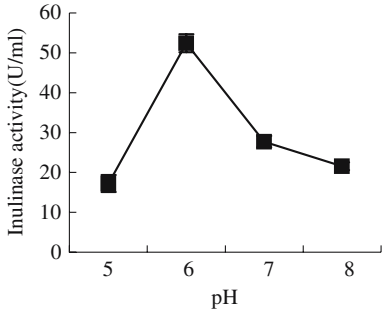

Fig. 1 Effects of different pH on activity of the crude inulinase. Data are given as means ± SD, $n = 3$

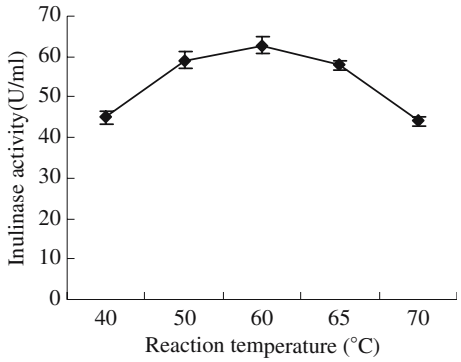

Fig. 2 Effects of different temperatures on activity of the crude inulinase. Data are given as means ± SD, $n = 3$

60°C, respectively. The optimum pH for inulinase secreted by *K. marxianus* was 4.4 and optimal temperatures for inulinase from *K. fragilis* and *K. marxianus* were 55°C [11, 13], respectively. It was also observed that the inulinase activity increased as the temperature increased, reaching the optimum at 60°C. The maximum inulinase activities for *K. marxianus* var. *bulgaricus* were observed at 50 and 60°C [3]. High temperatures were also observed by Wenling et al. [23] being 50°C for free inulinases and 55°C for immobilized inulinase. The optimal hydrolysis temperature for sucrose, raffinose and inulin was 55°C and the optimal pH for sucrose was 4.75 [14]. This means that the optimal pH and temperature for the crude inulinase produced by the marine yeast strain were in agreement with those for inulinases produced by terrestrial yeasts.

Effects of different carbon sources on inulinase production and cell growth

It has been reported that different carbon sources have significant influences on inulinase production by terrestrial yeasts [19]. Indeed, as shown in Table 1, inulinase production by the marine yeast strain was also influenced greatly by different carbon sources (including inulin, maltose, glucose, sucrose and Jerusalem artichoke extract) in the medium. It can be seen clearly from Table 1 that inulin was the best carbon source for inulinase production, and maltose was the second better source. However, the lowest inulinase was produced in the medium containing glucose. This may be due to both glucose repression on inulinase synthesis in the marine yeast and the inducible inulinase by inulin [6, 19] as inulinase activity was still low when the marine yeast was grown in the medium containing glycerol as nonfermentable carbon source (Table 1). However, the different carbon sources had no influences on cell growth of the marine yeast strain (Table 1). It was found that the optimal concentration of inulin for inulinase production was 4.0%. However, beyond this concentration, enzyme yield declined gradually (data not shown). Singh et al. [19] also found that the best source of carbohydrate for inulinase production by *K. marxianus* YS-1 is inulin, but the sucrose is the second better source [19]. An increase in inulinase production (15 U ml^{-1}) was observed in the media after 60 h with the increase in inulin concentration up to 3.5%. Factorial design and response surface analyses were used to optimize the production of inulinase by *K. marxianus* ATCC 16045, using sucrose as carbon source. The best production was 176 U ml^{-1} (sucrose was used as substrate of the enzyme) at 450 rpm and 1.0 vvm [2].

Table 1 Effects of different carbon sources on inulinase production and cell growth by the marine yeast strain

Carbon sources (w/v)	$OD_{600\ nm}$ of the cell culture	Inulinase activity (U ml^{-1})
Inulin (2.0%)	27.5 ± 0.3	60.1 ± 1.5
Maltose (2.0%)	28.1 ± 0.3	57.7 ± 1.8
Glucose (2.0%)	20.9 ± 1.4	10.8 ± 0.9
Sucrose (2.0%)	17.3 ± 1.4	27.9 ± 1.7
Glycerol (2.0%)	21.7 ± 0.8	42.6 ± 2.9
JAE (7.0%)	21.3 ± 1.0	33.1 ± 2.8

Data are given as means ± SD, $n = 3$

JAE Jerusalem artichoke extract

Effects of different nitrogen sources on inulinase production and cell growth

Different nitrogen sources were supplemented in the medium containing 4.0% inulin. Among all the nitrogen sources used, yeast extract (0.5% w/v) increased inulinase production (58.9 U ml^{-1}) and polypeptone was the second better nitrogen source, as shown in Table 2. In order to determine the optimum concentration of yeast extract for inulinase production, different concentrations (0.3–1.0%) of yeast extract were used in the production medium. A concentration of 0.5% (w/v) of yeast extract was found to be optimal for inulinase production (data not shown). Higher concentrations of yeast extract in the fermentation medium repressed inulinase activity. Ammonium sulphate, ammonium chloride and urea were found to be inhibitory for inulinase synthesis, presumably because of the release of ammonium ions [19]. However, *K. marxianus* YS-1 seems to be only efficient for inulinase production in the media containing proteic nitrogen. Inhibitory effect of ammonium salts on inulinase production by *Streptomyces* sp. and *K. fragilis* has also been reported earlier [19]. It was found that beef extract increased inulinase production (18.4 U ml^{-1}) and a concentration of 0.5% of beef extract has been found optimal for inulinase production [19]. The results in Table 2 also show that the yeast grew best in the medium containing 0.5% yeast extract.

Table 2 Effects of different nitrogen sources on inulinase production and cell growth

Nitrogen sources (w/v)	$OD_{600\ nm}$ of the culture	Inulinase activity (U ml^{-1})
Yeast extract (0.5%)	23.9 ± 1.6	58.6 ± 0.6
Polypeptone (0.2%)	16.1 ± 0.2	47.6 ± 0.9
Wheat bran (2.0%)	12.2 ± 0.4	4.7 ± 1.1
Urea (2.0%)	10.4 ± 0.7	36.8 ± 0.9
Ammonium sulfate (2.0%)	10.9 ± 0.4	29.3 ± 0.7
Ammonium chloride (2.0%)	10.9 ± 0.5	26.9 ± 0.8

Data are given as means ± SD, $n = 3$

Effects of different concentrations of NaCl and seawater on inulinase production and cell growth

Many studies have shown that many enzyme activities in halotolerant yeasts and moderately halotolerant bacteria were enhanced in the presence of NaCl [1, 22]. For example, the activity of NADP–glutamate dehydrogenase activity is fivefold higher when the halotolerant yeast *Debaryomyces hansenii* is grown in the presence of 1 M NaCl [1]. "*M. varians* subsp. *halophilus*" produced the highest amylase activity when grown in 2 M NaCl [22]. Because the yeast strain used in this study was isolated from a marine environment, it is very important to examine the effects of different concentrations of NaCl and seawater on inulinase production and cell growth by the marine yeast. Our results show that when NaCl concentrations of added NaCl were increased from 0 to 2.0% (w/v), inulinase activity was increased from 25 to 55 U ml^{-1}, whereas when the concentrations of NaCl was further increased from 2.0 to 9.0%, inulinase activity was decreased gradually (data not shown). These results indicate that 2.0% (w/v) of added NaCl was the most suitable for inulinase production by the marine yeast (dada not shown). Especially, when the marine yeast strain was grown in the medium prepared with seawater, the inulinase activity reached the highest (61.5 U ml^{-1}). However, it is still completely unknown why the inulinase production by the marine yeast was enhanced in the presence of 2.0% (w/v) NaCl or seawater. This may be related to the marine environment where the marine yeast strain was obtained. Sawabe et al. [17, 18] reported that the components of seawater may be concerned with folding or maintaining stability of the extracellular alginate lyase from the bacterium and the enzyme expression is enhanced by the components of seawater at transcriptional level. We are purifying and characterizing the extracellular inulinase and cloning the gene encoding the inulinase from the marine yeast. Then, the mechanisms of the enhanced inulinase production by 2.0% (w/v) or seawater will be understood.

The results also show that different concentrations of NaCl had no profound effects on cell growth. However, the yeast strain could grow better in the medium prepared with sea water (data not shown).

Effects of different initial pH and temperature on inulinase production and cell growth

Effects of different initial pH in the medium prepared with seawater on inulinase production by the marine yeast strain were tested. The results in Fig. 3 reveal that the optimal initial pH in the medium for inulinase production was 8.0, while the cells of the marine yeast strain grew best at initial pH 7.0. The results also show that when initial pH was higher or lower than 8.0, inulinase activity decreased sharply, indicating that inulinase production by the marine yeast strain was very sensitive to change in initial pH. However, it was reported that initial pH (4–8) of the fermentation medium has shown an increase in enzyme production (36.6 U ml^{-1}) up to pH 6.5 and thereafter there was a decline in this function [3]. There was very less enzyme production at pH 4.5 (2.5 U ml^{-1}) and pH 8 (7.2 U ml^{-1}). Optimum pH for the production of inulinase from *Kluyveromyces* has been reported between 6 and 7 [3]. This suggests that the optimal initial pH for inulinase production by the marine yeast was higher than that by terrestrial yeasts. This may be related to the marine environment where the marine yeast was isolated.

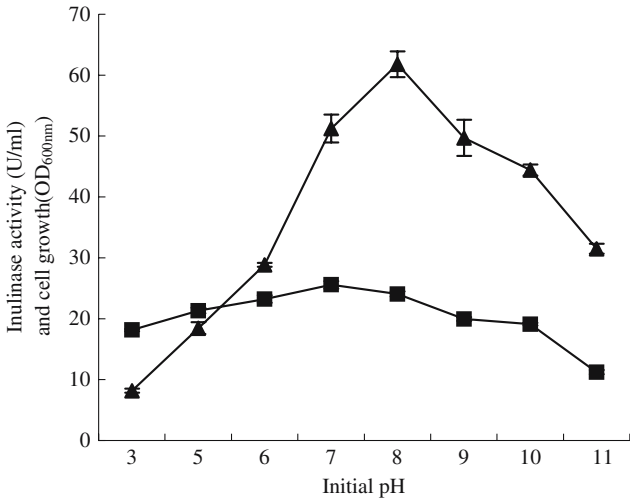

Fig. 3 Effects of different initial pH on inulinase production (*filled triangle*) and cell growth (*filled square*) by the marine yeast. Data are given as means ± SD, $n = 3$

The effect of temperature (24–37°C) on inulinase production was studied. Maximum inulinase production (60.0 U ml^{-1}) was observed at 28°C and thereafter a significant decrease in enzyme activity was seen at higher temperatures (Fig. 4). However, as the temperature was increased from 24 to 37°C, cell growth of the marine yeast decreased gradually (Fig. 4). Various researchers have reported 30°C as fermentation temperature for the production of inulinase by *K. marxianus* [3].

Time course of inulinase production and cell growth at shake flask level

All the data above showed that the optimal medium for inulinase production was seawater containing 4.0% inulin

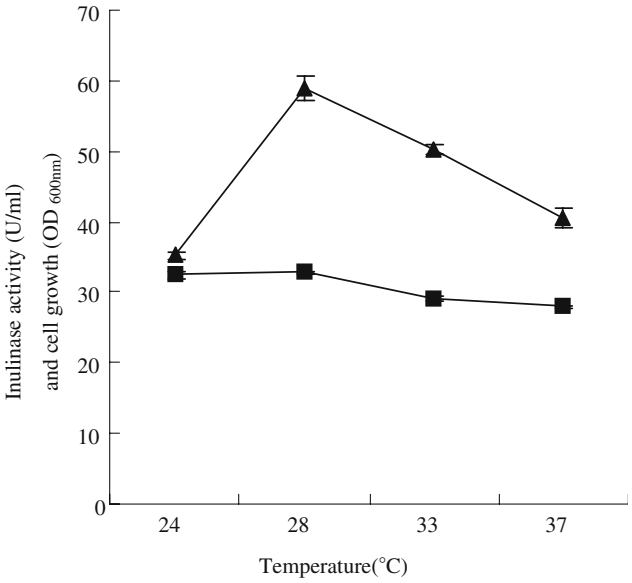

Fig. 4 Effects of different temperature on inulinase production (*filled triangle*) and cell growth (*filled square*) Data are given as means ± SD, $n = 3$

and 0.5% yeast extract, while the optimal cultivation conditions for inulinase production were pH 8.0, 28°C and 170 rpm. We also found that 10% (v/v) of 24 h old culture ($OD_{600\ nm} = 20.0$) was the best inoculation size to produce the maximum inulinase activity (data not shown). Therefore, the time course of inulinase production and cell growth during the fermentation was checked under the conditions. The results in Fig. 5 indicate that 61.6 U ml^{-1} of inulinase activity could be reached when cell growth was at the stationary phase. In inulin, the highest production of inulinase was 2.8 U ml^{-1} using *Candida pseudotropicalis*. When the yeasts grew in Jerusalem artichoke tubercles extract, around 90% of the enzymatic activity was accumulated

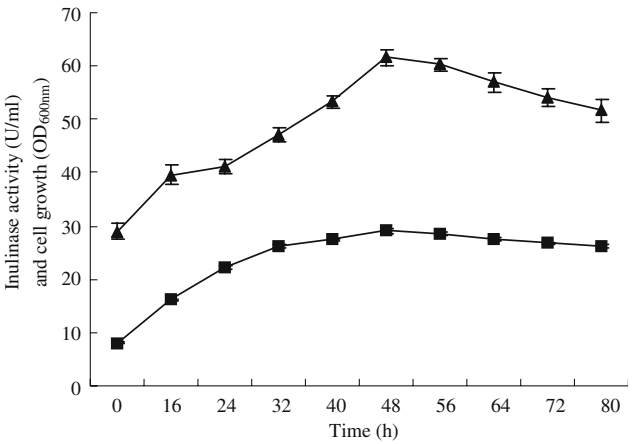

Fig. 5 Time course of cell growth (*filled square*) and inulinase production (*filled triangle*) by the marine yeast. Data are given as means ± SD, $n = 3$

in the medium and much higher levels of inulinase activity were obtained: 14.6 U ml^{-1} for *C. kefyr*, 18.7 U ml^{-1} for *C. pseudotropicalis*, 18.4 U ml^{-1} for *K. marxianus* var. *bulgaricus*, and 14.3 U ml^{-1} for *K. fragilis*. In dry Jerusalem artichoke extract, higher inulinase production was obtained after 7-day fermentation, using *K. marxianus* var. *bulgaricus* [3]. Inulinase yield of 40.5 U ml^{-1} of *K. marxianus* var. *bulgaricus* in an optimized medium containing inulin (3.5%), beef extract (0.5%), $MnSO_4$ (0.5 mM), $CoCl_2$ (0.05 mM), SDS (0.4 mM) and pH 6.5 at 30°C under agitation (150 rpm) for 60 h has been obtained at shake flask level [19]. Inulinase gene *inuA*1 from *Aspergillus niger* AF10 was overexpressed in *Pichia pastoris* and inulinase activity reached 50.6 U ml^{-1} in the fermentation liquid after 72 h [25]. The *INU1* gene encoding an exoinlinase from *K. marxianus* KW02 was expressed in *P. pastoris*. The enzyme activity of recombinant *P. pastoris* in the fermentation liquid was 52.0 U ml^{-1} [26]. This demonstrates that inulinase activity produced by the marine yeast reached very high levels within a short fermentation period (48 h). Therefore, inulinase produced by the marine yeast strain is potentially useful in industry. Purification and characterization of the inulinase and improved inulinase production by mutagenesis of the marine yeast strain are being undertaken in this laboratory.

Hydrolysis products

The hydrolysis products of inulin by the crude inulinase were analyzed by thin layer chromatography (TLC). A large amount of monosaccharides and a trace amount of oligosaccharides were detected after the hydrolysis (Fig. 6). This means that the crude inulinase

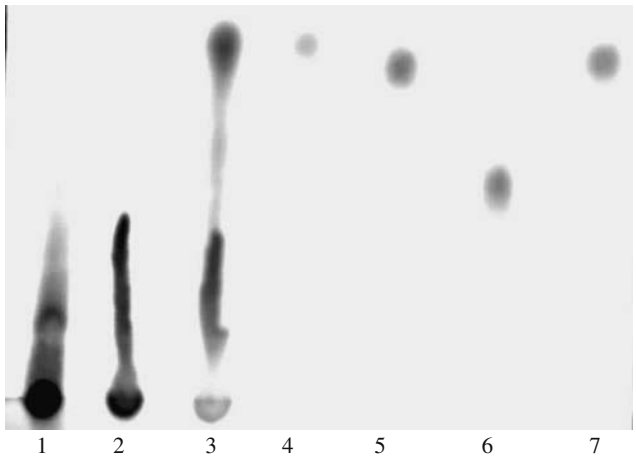

Fig. 6 Thin layer chromatography of hydrolysis products of inulin. *1* inulin, *2* control (2.0% inulin + 20 µl of 120 U ml^{-1} of the inactivated crude inulinase), *3* hydrolysis products, *4* fructose, *5* sucrose, *6* raffinose, *7* glucose

had a high exoinulinase activity. This characteristic may find potential applications in ultra-high fructose syrup production and high ethanol production. The monosaccharides and oligosaccharides were also detected after inulin hydrolysis for more than 2 h by the purified exoinulinase produced by *K. marxianus* var. *bulgaricus* [11].

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