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Effects of carbon sources, oxygenation and ethanol on the production of inulinase by *Kluyveromyces marxianus* YX01

ABSTRACT

Inulinase is one of the most important factors in consolidated bioprocessing, which combines enzyme production, inulin saccharification, and ethanol fermentation into a single process. In our study, inulinase production and cell growth of *Kluyveromyces marxianus* YX01 under different conditions were studied. Carbon source was shown to be significant on the production of inulinase, because the activity of inulinase was higher using inulin as a carbon source compared with glucose or fructose. The concentration of the carbon source had a repressive effect on the activity of inulinase. When the concentration was increased to 60 g/L, inulinase activity was only 50% compared with carbon source concentration of 20 g/L. Enzyme activity was also strongly influenced by aeration rate. It has been shown that the activity of inulinase and cell growth under anaerobic conditions were maintained at low levels, but aeration at 1.0 vvm (air volume/broth volume minute) led to higher activity. Inulinase activity per unit biomass was not significantly different under different aeration rates. Ethanol had a repressive effect on the cell growth. Cells ceased growing when the level of ethanol was greater than 9% (v/v), but ethanol did not affect the activity of secreted inulinase and the enzyme was stable at ethanol concentration up to 15%.

Key words: inulinase, *Kluyveromyces marxianus*, carbon source, aeration rate, ethanol

Introduction

Future shortages in petroleum could be a grave threat to the sustainable development of society, which may be exacerbated in China because of the fast rate of its economic development. Consequently, the production of fuel ethanol from cheap non-grain materials may be the key for this problem. Jerusalem artichoke is a rich source of carbohydrates (inulin), a linear biopolymer made up of fructose residues linked by β-2,1 bond (Silva-Santisteban et al., 2009). Inulin can be hydrolyzed into fructooligosaccharide or single fructose, which can be utilized by microbes (Yu et al., 2010). Therefore, Jerusalem artichoke, or inulin could be considered to be an ideal material for the production of fuel ethanol. For now though, consolidated bioprocessing (CBP) would be the most competitive technology in the use of Jerusalem artichoke tubers to produce ethanol. CBP would combine inulinase production and inulin hydrolysis with ethanol production (Yuan et al., 2008a).

Inulinase, as a kind of hydrolases, can be divided into endoinulinase and exoinulinase (Gong et al., 2008). The endoinulinases, without invertase activity, can only cut the internal linkages in inulin to yield inulooligosaccharides, while the exoinulinases remove the terminal fructose residues from the non-reducing end of the inulin to yield fructose or glucose (Chi et al., 2009; Yuan et al., 2012).

Inulinase production has largely been explored using various microbiological methodologies. However, most studies have focused on the optimization of culture media and operating parameters, such as pH, temperature, agitation, and aeration (Gill et al., 2003; Yuan et al., 2008b; Sheng et al., 2009; Ghasemi et al., 2012). As a consequence, there are contrasting opinions about the effect of carbon types and concentrations on the synthesis and secretion of inulinase. It has been observed that glucose was responsible for catabolic

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repression, whereas sucrose and fructose acted as weak inducers compared with inulin, in *Kluyveromyces fragilis* (Gupta et al., 1994). Other strains did not exhibit any induction mechanisms (Cruz-Guerrero et al., 1995; Schwan et al., 1997).

Aeration and dissolved oxygen also have an effect on inulinase activity (Silva-Santisteban & Filho, 2005). Oxygen supply is crucial for both inulinase activity and biomass concentration, and agitation speed strongly influences inulinase production, but the effect of aeration rates was less significant. Previous research indicates that the mechanisms of regulation for inulinase production are complicated, and are often strain-dependent (Silva-Santisteban et al., 2009). To date, to the best of our knowledge, no systematic studies have been conducted that clearly outline the factors that affect inulinase production. The mechanisms of its regulation, especially under conditions required for ethanol fermentation, have yet to be reported.

In our previous studies, it was revealed that *K. marxianus* YX01 can utilize raw Jerusalem artichoke to produce relatively high concentrations of ethanol *via* CBP technology without contamination by unwanted microorganisms (Yuan et al., 2012). But the low activity of inulinase produced is one of the major problems associated with using Jerusalem artichokes to produce ethanol by CBP technology.

Consequently, to provide more information on the release of extracellular inulinase, which may help us to know clearly about the mechanism of the inulinase production and to achieve the well controlled enzyme expression in the future studies, we have performed batch cultivation of *K. marxianus* YX01 with variations in the type and concentration of carbon sources, aeration rate as well as the addition of ethanol.

Materials and Methods

Microorganisms and culture medium

Strain *K. marxianus* YX01, domesticated from *K. marxianus* ATCC8554 was deposited at China Center for Type Culture Collection (CCTCC). The strain was streaked onto the YPD-agar slant composed of (g/L): glucose 20, yeast extract 10, peptone 20 and agar 15, and incubated at 30°C for 48 h. Then, the slant culture was collected and maintained at 4°C and used to inoculate the flask culture. Cultures were maintained on an agar slant at 4°C for routine use. For long-term preservation, cultures were stored at -80°C in 20% glycerol. The culture medium used for maintenance, seeds and enzyme production contained 4% (w/v) inulin, 2% (w/v) peptone, 1% (w/v) yeast extract, and 2% (w/v) agar (for solid medium only) and had a natural pH. The inulin was purchased from Elion CO., LTD (China).

Effect of the carbon source on the inulinase production

Batch fermentation was started with a 10% (v/v) inoculum in culture medium containing 4% (w/v) glucose, fructose or inulin. Fermentation was carried out in 500 mL flasks with 150 mL of culture medium at 30°C and 150 rpm. Samples were withdrawn every 24 h throughout the fermentation period to determine the levels of inulinase and biomass. The total period of fermentation was 120 h.

Effect of carbon concentrations on the inulinase production

Batch fermentation was started with 10% (v/v) inoculum in the culture medium containing 2% (w/v), 4% (w/v) and 6% (w/v) inulin, respectively. Fermentation was carried out in 500 mL flasks with 150 mL of culture medium at 30°C and 150 rpm. Samples were withdrawn every 24 h throughout the fermentation period to determine the levels of inulinase and biomass. The total period of fermentation was 120 h.

Effect of the aeration rate on the inulinase production

Batch fermentation was carried out using an initial 10% (v/v) inoculum at 30°C and 150 rpm. Aeration was applied at 0, 0.5, 1.0, 2.0, or 3.0 liters of air per minute per liter of medium (vvm) in a 5-L fermenter (BIOTECH-2000, Shanghai, China), with a 2.0 L working volume. The culture medium contained 4% (w/v) inulin. Samples were withdrawn every 12 h throughout the fermentation period to determine the levels of inulinase, biomass, ethanol and other major metabolites.

Effect of ethanol addition on the inulinase production

Batch fermentation was commenced with a 10% (v/v) inoculum in culture medium containing 4% (w/v) inulin. Ethanol was added to a final concentration of 0, 3, 6, 9, 12, or 15% (v/v). Fermentation was carried out in 500 mL flasks with 150 mL of culture medium at 30°C and 150 rpm. Samples were withdrawn every 12 h throughout the fermentation period to determine the levels of inulinase and biomass. The effect of ethanol addition on secreted inulinase activity was determined by centrifuging culture supernatant (120 h post-inoculation) at 6000g for 10 min. Ethanol was then added to the culture supernatant at a final concentration of 0, 3, 6, 9, 12, or 15% (v/v). After incubation at room temperature for 1 h, the inulinase in the supernatant was assayed.

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Analytical methods

Inulinase activity was measured as described by Parekh & Margaritis (1986). Culture broth samples were centrifuged at 6000g for 10 min. The supernatant was then collected and diluted appropriately with distilled water, and subjected to inulinase activity assay. Briefly, 0.5 mL culture supernatant was incubated with 2% (w/v) inulin prepared in 0.02 M sodium acetate buffer (pH 4.6) at 55°C for 10 min, and the reducing sugar was analyzed by the dinitrosalicylic acid method (Miller, 1959). One enzyme unit was defined as the amount of fructose (μmol) hydrolyzed per min under the above conditions (Parekh & Margaritis, 1986). Fructose was used as the standard substance to plot a standard curve. Cell mass concentration was determined using optical density at 620 nm. Ethanol concentration was determined as previously described (Xu et al., 2005). Briefly, the ethanol was analyzed by gas chromatography (Agilent 6890A, USA; solid phase: cross-linked polyethylene glycol; carrier gas: nitrogen; 90°C isothermol capillary column; injection temperature 160°C; flame ionization detector temperature 230°C; Agilent ChemStation Data Analysis System) and isopropanol was used as an internal standard. Duplicate analysis was applied to ethanol, biomass and residual sugars, and the mean values were given in the results.

Results

Effect of the carbon source on the inulinase production

Batch cultivations of *K. marxianus* YX01 were performed in culture medium containing glucose, fructose and inulin. Inulinase activity and biomass concentration were both increased and reached maximum levels of 10 g/L at 120 h. The three carbon sources did not have a significant effect on the cell growth (Figure 1A). Maximum inulinase activity was 6.6 U/mg biomass (Figure 1B), which was higher in the medium using inulin as carbon source compared with the other two sources.

Effect of the carbon concentration on the inulinase production

Carbon concentration had a strong effect on the inulinase activity, with a high carbon concentration reducing inulinase activity (Figure 2). When the concentration of glucose was 20, 40 and 60 g/L, inulinase activity was 58.59, 48.48 and 37.65 U/mL, respectively at 96 h. Similar results were observed for the other two carbon sources (Table 1).

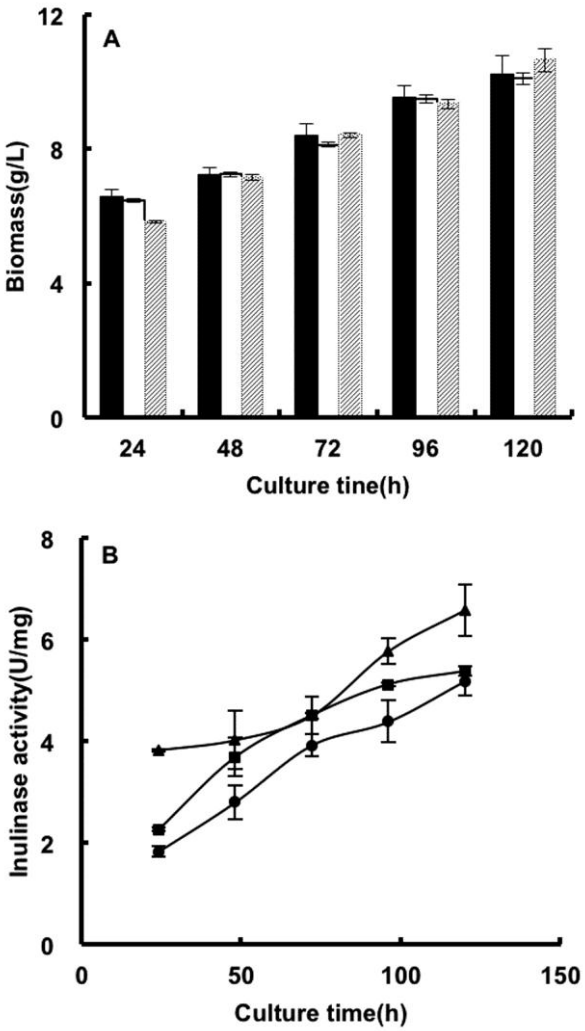

Figure 1. *Effect of carbon sources on the cell growth and inulinase production. Seed culture (15 mL) was inoculated into a 500 mL flask containing 150 mL of growth medium. Cultures were incubated at 30℃ and 150 rpm under aerobic conditions. Data are given as means ± SD, n = 3.*
(**A**) *Biomass. Glucose* (■), *fructose* (□) *and inulin* (▨).
(**B**) *Inulinase activity per unit biomass. Glucose (filled circle), fructose (filled square) and inulin (filled triangle).*

Biomass concentration was expressed as dry cell weight. As the carbon concentration increased, biomass concentration increased correspondingly. Maximum biomass concentration was between 10–11 g/L at 120 h. Comparing inulinase activity with biomass concentration at 96 h, we observed that as carbon concentration increased, inulinase activity per unit biomass decreased (Table 1).

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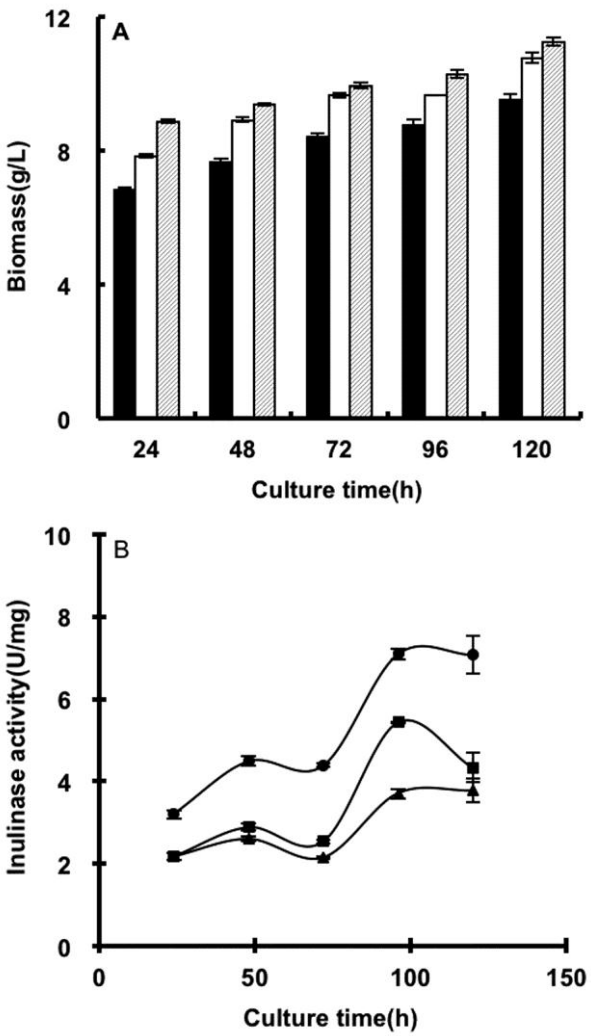

Figure 2. *Effect of the carbon concentration on the inulinase production and cell growth. Seed culture (15 mL) was inoculated into a 500 mL flask containing 150 mL of growth medium. Cultures were incubated at 30 ℃ and 150 rpm under aerobic conditions. Data are given as means ± SD, n = 3.*
(**A**) *Biomass. 20 g/L* (■), *40 g/L* (□) *and 60 g/L* (▨).
(**B**) *Inulinase activity per unit biomass. 20 g/L (filled triangle), 40 g/L (filled square) and 60 g/L (filled circle).*

Effect of the aeration rate on the inulinase production

As shown in Figure 3, inulinase production required an oxygen supply. Under anaerobic conditions, inulinase activity and the biomass concentration were both kept at low levels (10 U/mL and 6 g/L, respectively) (Figure 3A, 3B). As the aeration rate was increased to 0.5 vvm, inulinase production and biomass concentration was obviously improved. When the aeration rate was increased further to 1.0 vvm, inulinase production and cell growth were optimal, enzyme activity was maximal (>40 U/mL), and biomass concentration was greater than 10 g/L. When the aeration rate was 2.0 or 3.0 vvm, the activity of inulinase was not obviously improved, and was at a level similar to that observed when aeration was at 1.0 vvm. Inulinase activity per unit biomass was not significantly different when aeration was 0.5–3.0 vvm (Figure 3C). The highest concentration of ethanol produced was around 12 g/L under anaerobic conditions, with the increased aeration rate, the ethanol was decreased, 10 g/L at 0.5 vvm, compared with 5 g/L at 3.0 vvm.

Effect of ethanol addition on the inulinase production

Under high gravity condition, the concentration of ethanol was high. Whether the activity of inulinase has been affected by ethanol? According our results, the cell growth was strongly influenced by initial ethanol concentration in the culture medium. When concentration of the ethanol was over 6% (v/v), the cells stopped to grow. Cell growth was seriously restricted when the ethanol concentration was 6% (v/v). However, the biomass concentration was only half of the control. Inulinase activity per unit biomass was not affected by the addition of ethanol (Figure 4). The effect of ethanol addition on the secreted inulinase activity was determined after 120 h of culture. Ethanol did not repress the activity of secreted inulinase. Inulinase secreted by *K. marxianus* YX01 was highly stable when the ethanol concentration was also high (15% v/v) (Table 2).

Table 1. *Inulinase activity and biomass concentration for the three different carbon sources at three different concentrations after 96 h of culture.* A_m, *inulinase activity*; X_m, *biomass concentration*; A, *inulinase activity/biomass.*

	Glucose (g/L)			Fructose (g/L)			Inulin (g/L)		
	20	**40**	**60**	**20**	**40**	**60**	**20**	**40**	**60**
A_m (U/mL)	58.59 ± 2.28	48.48 ± 2.65	37.65 ± 1.86	62.20 ± 1.92	52.45 ± 1.86	38.01 ± 1.78	67.97 ± 3.03	66.17 ± 3.65	52.09 ± 4.08
X_m (g/L)	8.32 ± 0.14	9.55 ± 0.12	10.52 ± 0.09	8.78 ± 0.14	9.65 ± 0.10	10.27 ± 0.13	9.08 ± 0.44	10.09 ± 0.15	10.18 ± 0.25
A (U/mg)	7.04 ± 0.16	5.08 ± 0.16	3.58 ± 0.08	7.09 ± 0.13	5.44 ± 0.13	3.70 ± 0.11	7.49 ± 0.67	6.56 ± 0.27	5.12 ± 0.51

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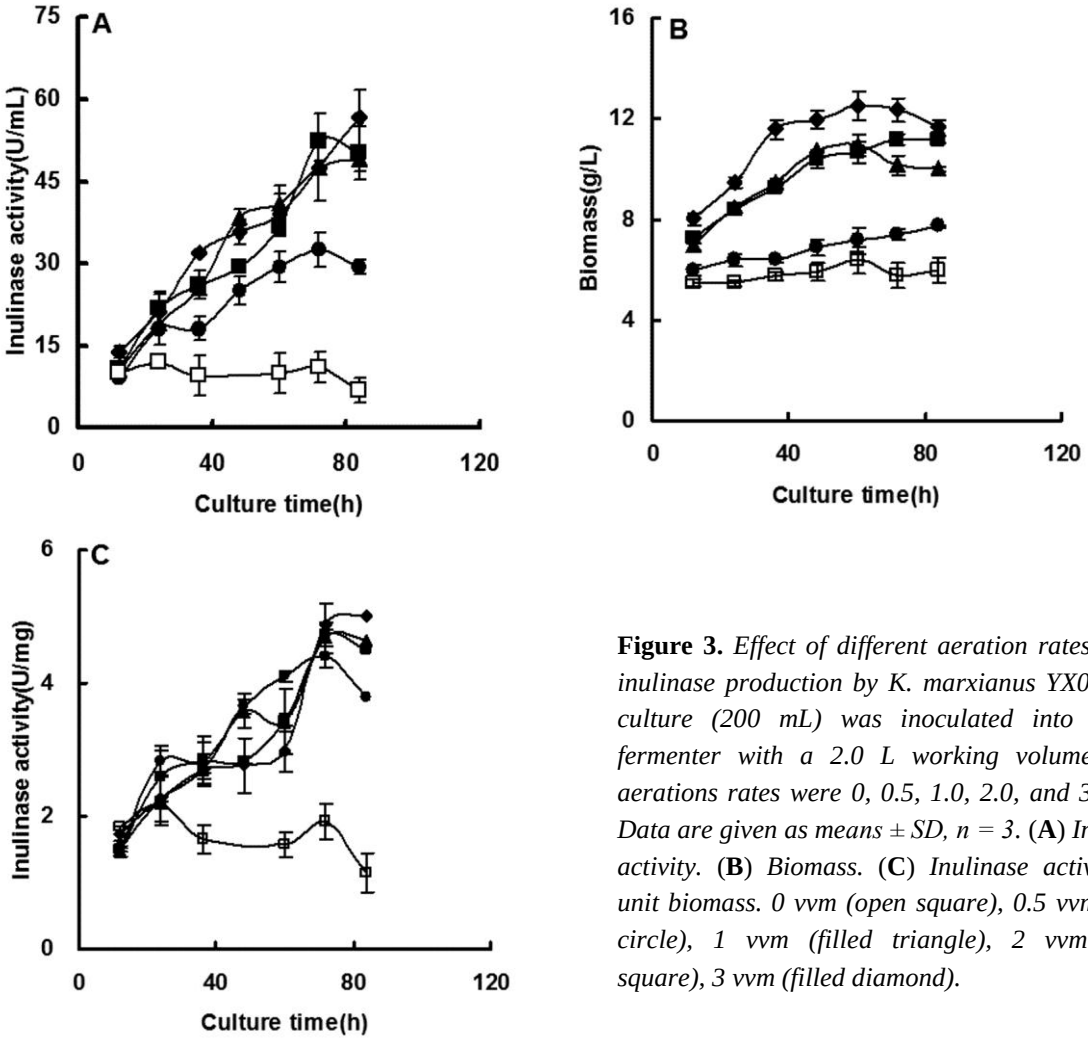

Figure 3. *Effect of different aeration rates on the inulinase production by K. marxianus YX01. Seed culture (200 mL) was inoculated into a 5 L fermenter with a 2.0 L working volume. Used aerations rates were 0, 0.5, 1.0, 2.0, and 3.0 vvm. Data are given as means ± SD, n = 3*. (**A**) *Inulinase activity.* (**B**) *Biomass.* (**C**) *Inulinase activity per unit biomass. 0 vvm (open square), 0.5 vvm (filled circle), 1 vvm (filled triangle), 2 vvm (filled square), 3 vvm (filled diamond).*

Discussion

The regulation of inulinase production is complicated and strain-dependent, and there are a number of differences observed under certain conditions by various strains. Inulinase secreted by *Streptomyces sp*. GNDU 1, regulated by a dual mechanism of substrate induction and product repression (Gill et al., 2003), appears to have reduced activity when its concentration is greater than 1% inulin. Glucose was responsible for catabolic repression, whereas sucrose and fructose acted as weak inducers compared with inulin, in *Kluyveromyces fragilis* (Gupta et al., 1994), but other strains did not exhibit any induction mechanisms (Cruz-Guerrero et al., 1995; Schwan et al., 1997). In our study, inulinase production showed obvious carbon-dependence, and inulin inducted inulinase production. Glucose had a repressive effect on inulinase production, a result that differed from those previously published (Yu et al., 2009). With respect to the induction of inulin, there were few differences compared with other studies (Gill et al. 2003; Silva-Santisteban & Filho, 2005; Silva-Santisteban et al., 2009; Yu et al. 2009). Our results also indicated that a high carbon concentration had obvious repressive effects on the enzyme activity, with the efficiency of inulinase production obviously lower in medium containing 60 g/L carbon (glucose, fructose, or inulin) compared with 20 g/L carbon. Inulinase production showed catabolic repression, which occurred at the transcriptional level (Yuan et al., 2006).

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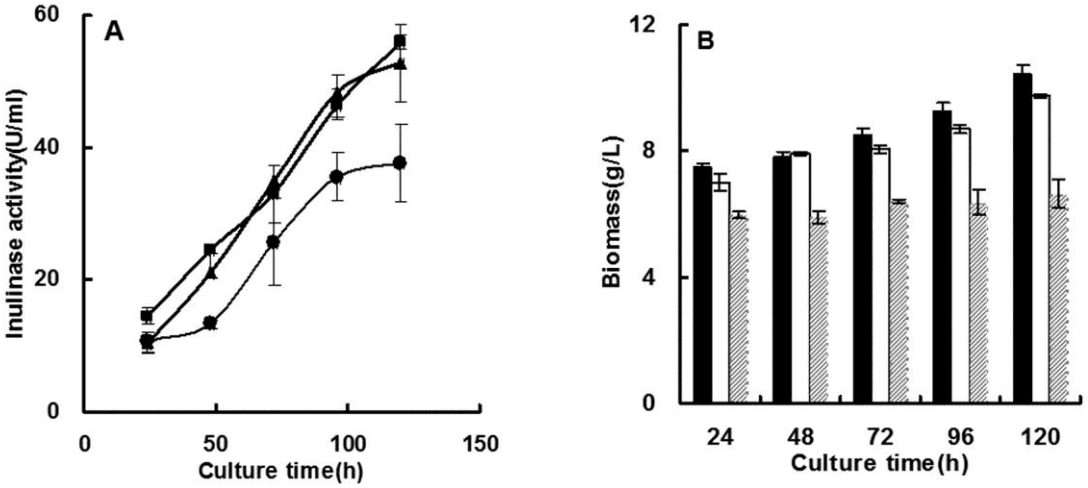

Figure 4. *Effect of different concentrations of ethanol on the inulinase production. Seed culture (15 mL) was inoculated into a 500 mL flask containing 150 mL of growth medium. The culture was incubated at 30°C and 150 rpm under aerobic conditions. Data are given as means ± SD, n = 3*. (**A**) *Inulinase activity. 0% ethanol (filled square), 3% ethanol (filled triangle), 6% ethanol (open triangle).* (**B**) *Biomass. 0% ethanol* (■), *3% ethanol* (□), *6% ethanol* (▨).

Table 2. *Effect of ethanol addition on the inulinase activity.*

Ethanol % (v/v)	**0**	**3**	**6**	**9**	**12**	**15**
Specific activity (%)	100 ± 2	93 ± 8	101 ± 1	95 ± 6	94 ± 4	97 ± 4

Moreover, the catabolic repression factor CreA and positive acting transcriptional factor InuR played an important role on the regulation of inulinase expression (Yuan et al., 2008). The removal of catabolic repression will be one of the key points in helping to increase enzyme activity under the ethanol fermentation conditions.

Inulinase production by *K. marxianus* YX01 was strongly influenced by aeration rate, similar with results previously published Silva-Santisteban & Filho, 2005; Silva-Santisteban et al., 2009). When compared with ethanol production using different aeration rates, we found that aeration repressed ethanol production, and that production was highest under anaerobic conditions. This finding was the opposite of the behavior demonstrated by *K. marxianus* var. *bulgaricus* (ATCC 16045) (Silva-Santisteban et al., 2009).

According to our research, the ethanol resistance of *K. marxianus* was obviously lower than that observed in *Saccharomyces cerevisiae*, with the growth of *K. marxianus* strongly influenced by high concentrations of ethanol. Inulinase activity was shown to be stable when the concentration of ethanol was high. Therefore improving the ethanol resistance of *K. marxianus* will be important when attempting to transform Jerusalem artichoke into ethanol by CBP.

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References

Chi ZM, Chi Z, Zhang T, Liu GL, Yue LX. 2009. Inulinase-expressing microorganisms and applications of inulinases. Appl. Microbiol. Biotechnol., 82(2): 211-220.

Cruz-Guerrero A, Garcia-Peña I, Barzana E, Garcia-Garibay M, Gomez-Ruiz L. 1995. *Kluyveromyces marxianus* CDBB-L-278: a wild inulinase hyperproducing strain. J. Ferment. Bioeng., 80(2): 159-163.

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Gill PK, Sharma AD, Harchand RK, Singh P. 2003. Effect of media supplements and culture conditions on inulinase production by an actinomycete strain. Bioresource Technol., 87(3): 359-362.

Ghasemi Y, Mohkam M, Ghasemian A, Rasoul-Amini S. 2012. Experimental design of medium optimization for invertase production by *Pichia sp*. J. Food Sci. Technol., doi:10.1007/s13197-011-0494-x

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. Bioprocess Eng., 13(5): 533-539.

Gupta AK, Singh DP, Kaur N, Singh R. 1994. Production, purificatioan and immobilization of inulinase from *Kluyveromyces fragilis*. J. Chem. Tech. Biotechnol., 59(4): 377–385.

Miller GL. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem., 31(3): 426-428.

Parekh S, Margaritis A. 1986. Production of inulinase (β-fructan fructanohydrolase) by *Kluyveromyces marxianus*. Agric. Biol. Chem., 50(4): 1085-1087.

Schwan RF, Cooper RM, Wheals AE. 1997. Endopolygalacturonase secretion by *Kluyveromyces marxianus* and other cocoa pulp-degrading yeasts. Enzyme Microb. Technol., 21(4): 234-244.

Sheng J, Chi ZM, Yan KR, Wang XH, Gong F, Li J. 2009. Use of response surface methodology for optimizing process parameters for high inulinase production by the marine yeast *Cryptococcus aureus* G7a in solid-state fermentation and hydrolysis of inulin. Bioprocess Biosyst. Eng., 32(3): 333-339.

Silva-Santisteban BO, Filho FM. 2005. Agitation, aeration and shear stress as key factors in inulinase production by *Kluyveromyces marxianus*. Enzyme Microb. Technol., 36(5-6): 717-724.

Silva-Santisteban BO, Converti A, Filho FM. 2009. Effects of carbon and nitrogen sources and oxygenation on the production of inulinase by *Kluyveromyces marxianus*. Appl. Biochem. Biotechnol., 152(2): 249-261.

Xu TJ, Zhao XQ, Bai FW. 2005. Continuous ethanol production using self-flocculating yeast in a cascade of fermentors. Enzyme Microb. Technol., 37(6): 634-640.

Yu J, Jiang JX, Zhang YQ, Lü H, Li YY, Liu JP. 2010. Simultaneous saccharification and fermentation of Jerusalem artichoke tubers to ethanol with an inulinase-hyperproducing yeast *Kluyveromyces cicerisporus*. Chin. J. Biotech., 26(7): 982-990.

Yuan XL, Goosen C, Kools H, van der Maarel MJEC, van den Hondel CAMJJ, Dijkhuizen L, Ram AFJ. 2006. Database mining and transcriptional analysis of genes encoding inulin-modifying enzymes of *Aspergillus niger*. Microbiology, 152(10): 3061-3073.

Yuan WJ, Ren JG, Zhao XQ, Bai FW. 2008a. One-step ethanol fermentation with *Kluyveromyces marxianus* YX01 from Jerusalem artichoke. Chin. J. Biotech., 24(11): 1931-1936.

Yuan WJ, Zhao XQ, Bai FW. 2008b. Optimization of culture conditions and characterization of inulinase of ethanol-producing *Kluyveromyces marxianus*Y1. Chin. J. Bioprocess Eng., 26(6): 25-29.

Yuan XL, Roubos J A, van den Hondel MJEC, Ram AFJ . 2008. Identification of InuR, a new zn(II)2cys6 transcriptional activator involved in the regulation of inulinolytic genes in *Aspergillus niger*. Mol. Genet. Genomics, 279(1): 11-26.

Yuan WJ, Chang BL, Ren JG, Liu JP, Bai FW, Li YY. 2012. Consolidated bioprocessing strategy for ethanol production from Jerusalem artichoke tubers by *Kluyveromyces marxianus* under high gravity conditions. J. Appl. Microbiol., 112(1): 38-44.