

# A Shortcut to the Production of High Ethanol Concentration from Jerusalem Artichoke Tubers

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## Summary

*Aspergillus niger* SL-09, a newly isolated exoinulinase-hyperproducing strain, and *Saccharomyces cerevisiae* Z-06, with high ethanol tolerance, were used in a fed-batch process for simultaneous saccharification and fermentation of Jerusalem artichoke tuber mash and flour. *S. cerevisiae* Z-06 utilized 98 % of the total sugar and produced 19.6 % of ethanol in 48 h. In this process the conversion efficiency of the fermentation of Jerusalem artichoke and the production of ethanol were 90 % of the theoretical ethanol yield and the cost of the production of flour was cut nearly into half.

*Key words:* *Aspergillus niger*, ethanol, inulinase, Jerusalem artichoke, *Saccharomyces cerevisiae*

## Introduction

Jerusalem artichoke is a plant native to temperate regions of North America. This plant is very resistant to frost, plant diseases, desert conditions and can grow on poor land. It has been proposed for many years as a possible substrate for the production of ethanol. Today, the need for petroleum and the environmental damage make it essential to enlarge the planting area of Jerusalem artichoke and also to enhance the productivity of ethanol fermentation based on this biomass source.

Jerusalem artichoke contains nearly 20 % of carbohydrates, 70–90 % of which is inulin. Inulin consists of linear chains of  $\beta$  (2 $\rightarrow$ 1) linked D-fructose units. Each chain is terminated by a D-glucose residue linked to fructose by an  $\alpha$  (1 $\rightarrow$ 2) bond (1). Jerusalem artichoke has good potential for alcohol production when fermented by suitable microorganisms (2,3). After Hayashida *et al.* (4) had reported the production of 20.1 % of ethanol from raw ground corn by simultaneous saccha-

rification and fermentation processes, Ohta *et al.* and Nakamura *et al.* (5,6) recommended this method to produce ethanol from Jerusalem artichoke at the concentration of 21 %. However, both fermentation processes took up to 5 days. The aim of the present study was the production of high concentration of ethanol in 48 h by stepwise simultaneous saccharification and fermentation of mashed tuber and flour of Jerusalem artichoke using *A. niger* SL-09 and *S. cerevisiae* Z-06, and the evaluation and development of an optimized process of the production of ethanol.

## Materials and Methods

### Microorganisms and media

*A. niger* SL-09, a novel producer of inulinase, was isolated from the soil and subjected to mutagenesis with UV, <sup>60</sup>Co, and N-methyl-N'-nitro-N-nitrosoguanidine (NTG) as reported previously (7). The identification of this strain was performed according to the descriptions

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of Dai (8). It was maintained on slants of potato agar (9) and subcultured every month. The culture medium used for the production of inulinase contained (g/L): sucrose 30, sucrose fatty acid ester 5,  $\text{NH}_4\text{H}_2\text{PO}_4$  12, KCl 0.7,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.5, and  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  0.001, adjusted to pH=6.0 with 0.1 M of HCl or NaOH.

*S. cerevisiae* Z-06 was chosen for ethanol fermentation because of its high ethanol tolerance and high rate of fermentation activity. This strain was originally isolated from a fermented mash for Huating (a Chinese alcoholic drink) manufactured in Xuzhou, P. R. China, and was maintained on the slants of malt agar (9). For the preparation of inoculum, *S. cerevisiae* Z-06 cells from a slant culture were suspended in 100 mL of a liquid medium containing (g/L): yeast extract 10, peptone 20, and glucose 20 in a 500-mL shake flask, at 30 °C with shaking (140 rpm) for 30 h.

Tubers of Jerusalem artichoke grown in GanSu Province, P. R. China were harvested in November. Dry-matter content of the fresh tubers was 26.4 %. The whole tubers were washed with water, mashed directly and sterilized at 121 °C for 15 min. To prepare Jerusalem artichoke flour, the washed tubers were peeled, sliced, and dried in a forced air-flow oven at 40 °C for 48 h. The dried material was finely pulverized by ball milling. The resulting flour contained 2 % of moisture and 83 % of total sugar.

#### *Inulinase and invertase production by A. niger SL-09*

The strain *A. niger* SL-09 was incubated in 500-mL conical flasks, each containing 100 mL of the culture medium. The media were inoculated with 0.5 mL of spore suspension (with the concentration of  $10^6$  per mL) and cultured at 30 °C for 4 days on a rotary shaker (140 rpm). To maintain the desired pH, calcium carbonate was added to give a concentration of 10 g/L after 24 h. During the process, mycelium was periodically withdrawn and centrifuged at 5000 rpm and 4 °C for 5 min. Supernatant was used for the analysis of inulinase activity, invertase activity, total sugar, pH and biomass.

#### *Fermentation tests of S. cerevisiae Z-06*

Fermentation tests for determining the maximum concentration of ethanol obtained by *S. cerevisiae* Z-06 were carried out in fed-batch culture with sequential feeding with sucrose. Either whole liquid culture or mycelium-free culture supernatant of *A. niger* SL-09 (150 mL) was supplemented with 30 g of sucrose, 0.45 g of  $(\text{NH}_4)_2\text{SO}_4$ , and 0.15 g of  $\text{KH}_2\text{PO}_4$ , and the two were compared as a fermentation medium. The initial pH was 5.0, and the pH was not controlled during the fermentation. All tests were conducted statically in 250-mL culture bottles, at 30 °C as described previously (5).

To determine the optimum concentration of Jerusalem artichoke flour for the conversion efficiency and productivity of fermentation using *S. cerevisiae* Z-06, experiments were carried out with Jerusalem artichoke flour as the medium (150 mL) at the concentration of 50–300 g/L in 500-mL culture bottles. The fermentations were run statically at 30 °C after the same quantity of *S. cerevisiae* Z-06 cells and 60 mL of whole liquid culture of *A. niger* SL-09 were added to each of the culture bottles.

In order to study the effect of enzyme activities on ethanol yield and productivity, fermentations were carried out using mashed tuber (200 g) as medium with 20–100 mL of liquid culture of *A. niger* SL-09, and the medium without liquid culture was used as control. These cultures were run at 30 °C in stationary flasks after the addition of the same quantity of *S. cerevisiae* Z-06 cells.

#### *Simultaneous saccharification and fermentation using A. niger SL-09 and S. cerevisiae Z-06*

In this process 200 g of mashed tubers were inoculated with *S. cerevisiae* Z-06 in 500-mL culture bottles, and fermentations were conducted at 30 °C for 6 h. The cells of *S. cerevisiae* Z-06 were collected from the inoculum culture and resuspended in the medium as described above. Then 60 mL of whole liquid culture of *A. niger* SL-09 were supplemented. The mass of 40 and 30 g of the flour was added after 10 and 25 h of fermentation, respectively. Due to high viscous nature of fermentation mash, it was necessary to shake the bottles to submerge the solid matter in the liquid, after which the cultures were incubated without shaking, and the viscosity decreased rapidly probably because of the inulin solubilization by endoinulinase. During the first 6 h, fermentations were very intense, so antifoam was added to the culture at a mass fraction of 0.1 ppm to secure complete fermentation.

#### *Analytical methods*

Exoinulinase ( $\beta$ -D-fructan fructohydrolase, E.C. 3.2.1.80) was distinguishable from endoinulinase (2,1- $\beta$ -D-fructan fructohydrolase, E.C. 3.2.1.7) by their ability to hydrolyze sucrose. The extracellular inulinase activity (I) and invertase activity (S) in culture supernatants were assayed by measuring the reducing sugar released from inulin and sucrose, respectively, as described by Pessoni *et al.* (10). One unit of inulinase activity was defined as the amount of enzyme in 1 mL that releases 1  $\mu\text{mol}$  of fructose from inulin per min. One unit of invertase activity was defined as the amount of enzyme in 1 mL that catalyzes the hydrolysis of 1  $\mu\text{mol}$  of sucrose per min. Reducing sugars were estimated with 3,5-dinitrosalicylic acid (DNS) (11), using fructose as standard. Total reducing sugar was assayed by the same method after acid hydrolysis (adjusted to pH=1.0 with sulfuric acid and heated to 100 °C for 30 min). Yeast cell populations were determined by direct microscopic count in a counting chamber. The pH was measured by pH-meter. Ethanol was assayed by gas liquid chromatography (12). Biomass concentration was determined by harvesting the mycelia pellets by filtration and freeze-drying them to a constant mass, the dry mass was expressed as gram per liter of the fungal culture.

## Results and Discussion

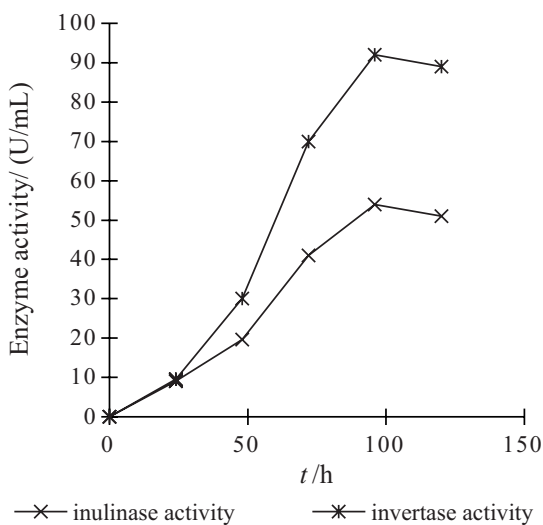
#### *Morphological and physiological characteristics of S. cerevisiae Z-06*

Vegetable cells of this strain were of globular to ellipsoidal shape, 4.0 to 4.8 by 5.2 to 8.5  $\mu\text{m}$  in size, and

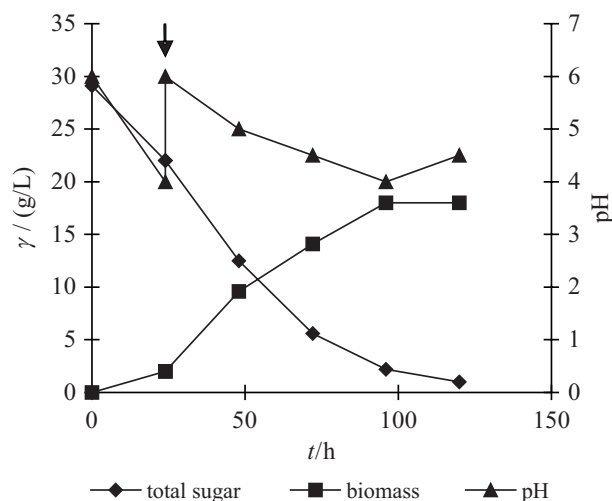
reproduced by multilateral budding. White and smooth colonies were formed on potato-glucose agar but pseudomycelium was not found. Sporulation was observed on the acetate agar, and two or three round ascospores were produced per ascus. Strain Z-06 was unable to utilize citrate, methanol, nitrate or nitrite, and was sensitive to 1 % of acetic acid. It was able to ferment glucose, galactose, sucrose and maltose. On the basis of the morphological and physiological properties, the strain was identified as *S. cerevisiae* (13).

#### Time for inulinase and invertase production by *A. niger* SL-09

The time for inulinase and invertase activities, total sugar, pH, and biomass were determined in shake flask culture of *A. niger* SL-09 at 30 °C (Figs. 1 and 2). Nearly



**Fig. 1.** The time course of enzyme production in shake flask culture of *A. niger* SL-09. Fermentation was carried out using 200 g of mashed tubers with 20–100 mL of liquid culture of *A. niger* SL-09. Cultures were run at 30 °C after the addition of *S. cerevisiae* Z-06



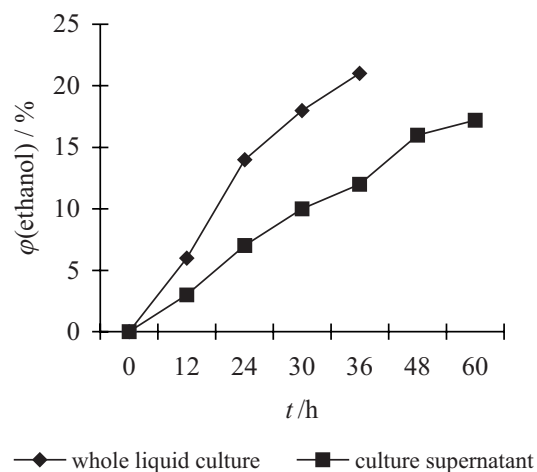
**Fig. 2.** Growth course of *A. niger* SL-09 in shake culture. The media were inoculated with 0.5 mL of spore suspension ( $10^6$ /mL) and cultured at 30 °C for 4 days on a rotary shaker. The arrow indicates the addition of calcium carbonate at the concentration of 10 g/L

all of the sugar was exhausted in 5 days. Invertase activity reached a maximum of 92 U/mL after 4 days, while inulinase activity was 54 U/mL (I/S ratio, 0.57). In a previous work the enzyme activities in the liquid culture of *A. niger* 817 were 64.8 U/mL for inulinase and 8.8 U/mL for invertase (I/S ratio, 7.8) (6). The comparison of these results shows that *A. niger* SL-09 is a more favourable strain for the production of enzyme with high invertase activity. The pH and I/S values remained near the constant through the culture period and the biomass maximum reached 18.5 g/L, which is in agreement with most reports (14).

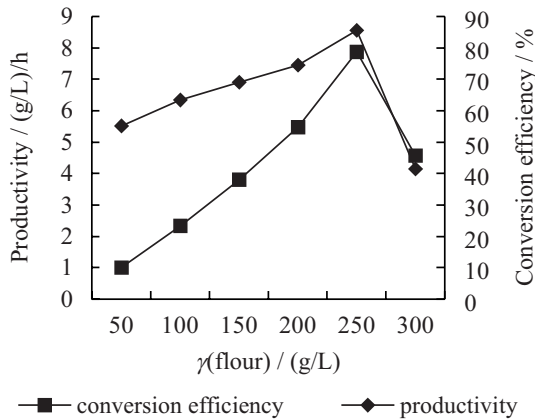
#### Ethanol tolerance of *S. cerevisiae* Z-06 and the optimum flour concentration for conversion efficiency and productivity

A total of 70 g of sucrose was added sequentially to the mycelium-containing culture during ethanol fermentation, which stopped in 36 h and the final ethanol concentration reached 21 %. In the mycelium-free culture, however, a lower ethanol concentration of 17.2 % was produced from a total of 60 g of sucrose in 60 h (Fig. 3). The comparison showed that the fungal mycelia were responsible for the enhanced growth and fermentation activities as well as for the ethanol tolerance of *S. cerevisiae* Z-06 (5,15). However, it took *S. cerevisiae* 1200 as long as 5 days to produce the same concentration of ethanol, therefore, *S. cerevisiae* Z-06 is more profitable for ethanol fermentation.

Maximum conversion efficiency of the fermentation and production of ethanol from Jerusalem artichoke were determined by calculating ethanol concentration in the culture. As shown in Fig. 4, maximum conversion efficiency and productivity were 86 % and 8.5 (g/L)/h, respectively, when flour concentration was 250 g/L. However, both conversion efficiency and productivity dropped dramatically when flour concentration was over 250 g/L. Therefore, it is very important to add no more than 250 g/L of Jerusalem artichoke flour to the culture to obtain the optimum conditions for fermentation.



**Fig. 3.** Ethanol production by *S. cerevisiae* Z-06 in fed-batch fermentation with sequential addition of sucrose. Either whole liquid culture or culture supernatant of *A. niger* SL-09 were used as medium. Tests were conducted statically in 250-mL culture bottles at 30 °C



**Fig. 4.** Productivity and conversion efficiency of the fermentation with 50–300 g/L of flour concentration. Experimental conditions: 200 g of mashed tubers were inoculated with *S. cerevisiae* Z-06. The cells were collected and resuspended in the medium to which 60 mL of whole liquid culture of *A. niger* SL-09 were supplemented and total of 70 g of flour was added step by step

#### Effect of the enzyme activities on ethanol yield and productivity

Ethanol concentration, productivity and conversion efficiency obtained during the fermentations of six different media are given in Table 1. The results show that in the first 6 h of fermentations the maximum productivity was obtained in the basal medium. High content of reducing sugar hydrolyzed from inulin by liquid culture of *A. niger* SL-09 slowed down the initial fermentations. However, in 12 h the total sugar of the media with the addition of the liquid culture, especially the medium with 60 mL of the liquid culture, was consumed more completely than that of the basal medium.

The juice of Jerusalem artichoke tubers (containing amino acids, proteins, vitamins, and metal ions, besides carbohydrates) had been proved to be an appropriate medium for the production of ethanol (16). Therefore, to obtain the maximum productivity in ethanol fermentation, mashed tubers should be used as substrate. How-

ever, to produce high ethanol concentration with maximum conversion efficiency in short period of time, 60 mL of the liquid culture of *A. niger* SL-09 should be added to the medium.

#### Simultaneous saccharification and fermentation of Jerusalem artichoke mashed tubers and flour

On the basis of 70 g of sucrose fed into the mycelium-containing culture (Fig. 3), optimum flour concentration was 250 g/L (Fig. 4), a total of 70 g of flour was added to the 200 g of mashed tubers step by step. At first, the viscosity of the culture was high, while it decreased rapidly during the fermentation. To avoid the inhibition caused by high concentration of sugar, during first 6 h of the fermentation, liquid culture of *A. niger* SL-09 was not added to the culture. Due to these conditions in first 6 h, the maximum volumetric productivity of ethanol was obtained. When the flour was added, there was a short slow-moving period for *S. cerevisiae* Z-06. Fermentation was completed in 48 h, final ethanol concentration was 19.6 %, and conversion efficiency of Jerusalem artichoke to ethanol was 90 % of the theoretical ethanol yield (Fig. 5).

Because the level of total sugar in the mashed tubers was mostly 20 %, the final ethanol concentration was lower than 11 %, but the fermentation was rapid and complete. High concentration of reducing sugar in the concentrated juice inhibited the final ethanol concentration of juice below 15 %, and the fermentable sugar was not consumed completely (17). To obtain a high concentration of ethanol, inulin or flour was used as medium, but the fermentation courses were up to 5 days long, although mycelia were used (5,6), and the step of the production of flour might increase the cost of process significantly.

In this process, to economically produce high concentration of ethanol in short period of time, the yeast *S. cerevisiae* Z-06 with high ethanol tolerance and fermentation activity was used, and the fermentation cycle needed by *S. cerevisiae* 1200 was cut into half (6). Moreover, mashed tubers were used first as medium, after which the flour was added below the concentration of 250 g/L,

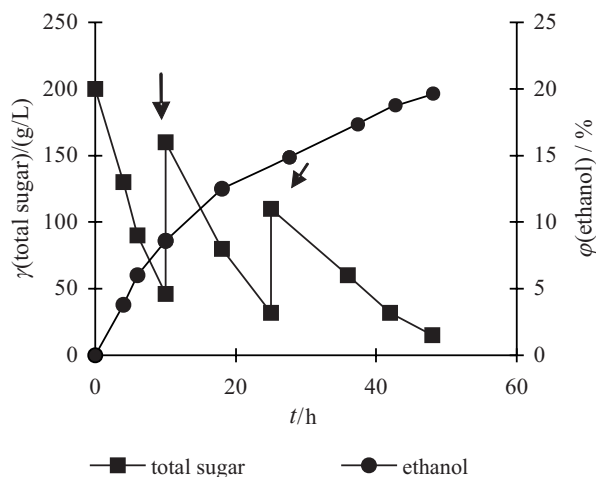
**Table 1.** Ethanol production parameters on medium containing various volumes of liquid culture of *A. niger* SL-09 after 6 and 12 h of fermentation by *S. cerevisiae* Z-06

V(liquid culture) mL	Inulinase activity U	Invertase activity U	t(fermentation)/h				
			6		12		Conversion efficiency % <sup>b</sup>
			$\phi$ (ethanol) %	Productivity (g/L)/h <sup>a</sup>	$\phi$ (ethanol) %	Productivity (g/L)/h <sup>a</sup>	
0	0	0	6.8	11.3	7.6	3.17	54.1
20	1080	1839	6.2	10.3	8.1	3.38	63.0
40	2160	3672	5.4	9.0	9.8	4.08	76.3
60	3240	5510	4.9	8.1	12.0	5.00	93.4
80	4320	7344	4.3	7.1	11.5	4.80	89.5
100	5400	9180	3.8	6.3	10.0	4.17	77.8

Sucrose consumption was calculated assuming 90 % of the theoretical conversion of sucrose to ethanol plus CO<sub>2</sub>, and the mass of sucrose fermented (in g) equal to 2.16 times the mass of CO<sub>2</sub> evolved (in g)

<sup>a</sup>The average volumetric productivity of ethanol (ethanol concentration in g/L was divided by the fermentation time)

<sup>b</sup>Conversion efficiency as percentage of the theoretical maximum on the basis of total sugar concentration



**Fig. 5.** Production of ethanol from Jerusalem artichoke. Experimental conditions: 200 g of mashed tubers were inoculated with *S. cerevisiae* Z-06, then 60 mL of liquid culture of *A. niger* SL-09 were supplemented. The arrows indicate the addition of 40 and 30 g of flour after 10 and 25 h of fermentation, respectively

and therefore high conversion efficiency of 90 % was obtained. These observations lead to the conclusion that the higher the concentration of total initial sugar, the

slower the fermentation of the media, which is in agreement with the results of Atiyeh *et al.* (18).

*A. niger* SL-09 was selected among many strains because of its high inulinase activity and enhanced fermentation activity, which is due to high content of proteolipids (PL) in its mycelia (15,19). The liquid culture of *A. niger* SL-09 is characterized by predominant exoinulinases, which catalyze the hydrolysis of inulin by splitting off terminal D-fructose. Because fructose is consumed more efficiently than high-polymerized polyfructosans of inulin, liquid culture of *A. niger* SL-09 was used to hydrolyze inulin and permitted better sugar assimilation of *S. cerevisiae* Z-06. In order to avoid the inhibition caused by high sugar concentration, especially by the reducing sugars hydrolyzed from inulin, no more than 60 mL of the liquid culture from *A. niger* SL-09 should be added to the medium.

Table 2 summarizes and compares the results of ethanol fermentation of three types of inulin sources obtained from Jerusalem artichoke tubers (5,6). Disregarding the effect of other factors, such as yeasts, the composition of fermentation medium, *etc.*, the data in Table 2 were analyzed using SAS Microsoft, and the standardized regression coefficients of the fermentation parameters were given in Table 3. The results confirm

**Table 2.** Summary of simultaneous saccharification and fermentation of Jerusalem artichoke tubers

Experimental condition	Fermentation parameters									
	Type of substrate and enzyme	$V(\text{broth})$ mL	Total inulinase activity U	Total invertase activity U	$m(\text{total sugar})/V$ %	$\phi(\text{ethanol})$ %	$t(\text{fermentation})$ h	Volumetric productivity (g/L)/h <sup>a</sup>	$m(\text{final reducing sugar})/V$ %	Conversion efficiency % <sup>b</sup>
<b>A</b> Ground tubers and powdered enzyme		150	1200	280	17.8	10.4	15	5.5	0.3	92
<b>B</b> Juice concentrate and powdered enzyme		150	4800	1120	45.0	15.0	72	1.7	14.7	52
<b>C</b> Flour and fungal liquid culture		177	10300	1320	39.4	20.1	120	1.3	2.6	80
<b>D</b> Pure inulin and liquid culture		183	6000	1800	36.0	20.1–21.0	72	2.8	2.7	83–84
<b>E</b> Ground tubers, flour and liquid culture		265	3300	5520	38.6	19.6	48	4.1	1.5	90

The data of **A**, **B** and **C** are from reference 6, data of **D** is from reference 5, data of **E** is from this study

The sucrose consumption was calculated assuming 90 % of the theoretical conversion of sucrose to ethanol plus CO<sub>2</sub>, and the mass of sucrose fermented (in g) equal 2.16 times the mass of CO<sub>2</sub> evolved (in g)

<sup>a</sup>The average volumetric productivity of ethanol (ethanol concentration in g/L was divided by the fermentation time)

<sup>b</sup>Conversion efficiency as percentage of the theoretical maximum on the basis of total sugar concentration

**Table 3.** Standardized regression coefficients between variables and dependent variables in the ethanol fermentation of Jerusalem artichoke tubers

Variables	Dependent variables				
	$\phi(\text{ethanol})$ %	$t(\text{fermentation})$ h	Productivity (g/L)/h	$m(\text{sugar})/V$ %	Conversion efficiency %
Total inulinase activity/U	0.669	0.985	-0.862	0.092	-0.245
Total invertase activity/U	0.547	-0.06	0.135	-0.223	0.294
Total initial sugar concentration	0.664	0.702	-0.833	0.633	-0.670

that high invertase activity in the medium is responsible for shortened fermentation cycle, lowered residual sugar and enhanced productivity and conversion efficiency. To produce high ethanol concentration total inulinase activity and initial sugar concentration should also be taken into account.

It was found that if the flour of Jerusalem artichoke was added to the culture after 48 h, fermentation continued, and the final ethanol volume fraction reached 20 %. However, it was time consuming and the conversion efficiency decreased, so the fermentation should be stopped at 48 h.

## Conclusion

To shorten the fermentation cycle, to enhance the conversion efficiency and reduce the cost of the production of flour, *A. niger* SL-09, an exoinulinase-hyperproducing strain, and *S. cerevisiae* Z-06, with high ethanol tolerance, were used for rapid and complete saccharification and fermentation of the mash and flour of Jerusalem artichoke tubers. In this process the mashed tubers were used as substrate and flour was added to the culture under the inhibitory level, after which 19.6 % of ethanol were produced in 48 h, nearly half the cost of the production of flour was reduced, and the conversion efficiency of the fermentation and the production of ethanol from Jerusalem artichoke were 90 % of the theoretical ethanol yield.

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# Skraćeni postupak za proizvodnju velike koncentracije etanola od gomolja jeruzalemske artičoke

## Sadržaj

*Aspergillus niger* SL-09, novi izolirani soj hiperproducent egzoinulinaze, upotrijebljen je u šaržnom procesu za istodobnu saharifikaciju i fermentaciju kaše i brašna gomolja jeruzalemske artičoke. *S. cerevisiae* Z-06 koristio je 98 % ukupnog šećera i proizveo 19,6 volumnog udjela etanola u 48 h. U ovom postupku uspješnost konverzije jeruzalemske artičoke u etanol iznosila je 90 % teoretskog iskorištenja, a cijena proizvodnje brašna smanjena je približno na polovinu.