Simultaneous Saccharification of Inulin and Ethanol Fermentation by Recombinant *Saccharomyces cerevisiae* Secreting Inulinase

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Various *Saccharomyces cerevisiae* strains were transformed with a 2μ-based multicopy expression plasmid, pYIGP, carrying *Kluyveromyces marxianus* inulinase gene under the control of *GAPDH* promoter. Among them two strains, SEY2102 and 2805, showed high levels of cell growth and inulinase expression, and were selected to study their fermentation properties on inulin. Jerusalem artichoke inulin was more effective for cell growth ($10 \sim 11$ g-dry wt./L at 48 hr) and inulinase expression (1.0 units/mL with SEY2102/pYIGP and 2.5 units/mL with 2805/pYIGP) than other inulin sources such as dahlia and chicory. It was also found that maximal ethanol production of 9 g/L was obtained from Jerusalem artichoke inulin at the early stationary phase (around 30 hr), indicating that recombinant *S. cerevisiae* cells secreting exoinulinase could be used for the simultaneous saccharification of inulin and ethanol fermentation.

Key Words : inulinase, recombinant yeast, constitutive expression, ethanol

INTRODUCTION

Inulin, a polyfructan found as an energy reserve in the roots and tubers of plants (e.g., Jerusalem artichoke, chicory, and dahlia), consists of linear chains of fructose residues linked by β-2,1 bonds and terminated by a glucose residue. The degree of polymerization varies widely 3 to 40 fructose units. Inulin has been recieved cosiderable attention as a potential substrate for the production of ethanol, and food sweeteners such as high-fructose syrup, inulooligosaccharide and difructose anhydride [1-3]. A variety of microorganisms can ferment inulin to ethanol and biomass, but traditional brewing yeasts, *Saccharomyces cerevisiae* with high ethanol and sugar tolerance cannot utilize inulin because they lack inulinase (exo-or endo-inulinase).

The yeast strains of *Kluyveromyces* have been known to produce extracellular exoinulinase (2,1-β-D-fructan fructanohydrolase, EC 3.2.1.7) that liberates fructose molecules from inulin successively [4]. Recently, two exoinulinase genes (*INU1* and *INU1A*) from *K. marxianus* were cloned and expressed in *S. cerevisiae* under the control of *GAL*s and *GAPDH* promoters [5-7]. Yeast transformants carrying these 2μ-based multicopy plasmids secreted the cloned inulinase by its own signal sequence, and therefore are expected to be able to grow on inulin as a sole carbon source.

In this article, we examine various yeast host cells for the constitutive expression of inulinase gene under the control of *GAPDH* promoter, and describe the fermentation properties of selected *S. cerevisiae* transformants carrying the 2μ-based multi-copy plasmid. In addition, the effects of three inulin sources derived from Jerusalem artichoke, dahlia, and chicory on cell growth, inulinase expression, and ethanol production are analyzed and compared.

MATERIALS AND METHODS

Yeast Host Strains and Plasmid

All yeast host strains used in this study are haploid and uracil auxotrophs [8]. The construction and restriction map of plasmid pYIGP were previously described [7]. The inulinase gene (*INU1A*) in pYIGP is constitutively expressed by the *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) promoter. Transformation of yeast cells was carried out by the LiCl method [9].

Media and Culture Conditions

For selection and maintenance of yeast transformants, a minimal SD medium (0.67% Bacto-yeast nitrogen base without amino acids, 2% dextrose, 0.05% casamino acids) was used. Fermentations were performed in YP media (1% Bacto-yeast extract, 2% Bacto-peptone) plus 2% carbon

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source (dextrose, sucrose, Jerusalem artichoke inulin, dahlia inulin, or chicory inulin) at 30℃ and 170 rpm. Yeast transformants were precultured on SD medium for 24 hr, and then inoculated with 5% (v/v) into 500 mL baffled-flask containing 50 mL YP medium.

Analytical Methods

Cell growth was measured by optical density at 600 nm (OD_{600}), and converted to the dry cell weight (DCW) by a conversion factor (0.32 g-DCW/L), where necessary.

Residual reducing sugars were determined by the dinitrosalicylic acid (DNS) method [10]. Total sugars were estimated as reducing sugars after acid hydrolysis (0.1 N HCl, 30 min, 100℃).

The culture broth of yeast transformants was centrifuged to separate the cells from the media. The cells were lysed by treatment of Zymolyase 100T (Seikagaku Kogyo, Japan) and vortexed with glass beads [6]. After centrifugation at $3000 \times g$ for 10 min, the supernatant was designated as the cellular fraction and used for the measurement of cellular inulinase activity. The medium and cellular fractions were incubated with 1.5% inulin (Jerusalem artichoke, Sigma) in 0.1 M Na · acetate buffer (pH5.0) at 50℃ for 10 min. The amount of reducing sugar released was determined by the DNS method. One unit of inulinase was defined as the amount of enzyme liberating 1 μ mole of fructose equivalent from inulin per minute at 50℃. The secretion efficiency was defined as [(inulinase activity secreted)/(total inulinase activity expressed)] $\times 100$

Plasmid stability was determined by spreading the diluted broth onto YPD agar plates and incubating them at 30℃ for 2 days. About 200 colonies were then toothpicked onto SD agar plates. The ratio of the number of colonies on SD to those transferred was used to calculate the percentage of plasmid-containing cells or plasmid stability.

Ethanol was estimated by gas chromatography (Hewlett Packard, Model 5890, USA) equipped with a dual-flame ionization detector and a Porapak Q (80~100 mesh) column (Waters Associates, Inc.). Injector, column, and detector temperatures were 200℃, 150℃, and 250℃, respectively. Equal volume of 5% (v/v) isopropanol was used as an internal standard.

RESULTS AND DISCUSSION

Selection of Optimal Host Strains

Since fast-growing cells are suitable for the constitutive expression of inulinase by the *GAPDH* promoter, ten uracil auxotrophic strains of *S. cerevisiae* with high genetic diversity were transformed with pYIGP plasmid, and then the recombinant yeasts obtained were cultivated in test tubes (10 mL) to examine their growth properties on YP+glucose (YPD) medium. Among them, six strains showing relatively fast growth were chosen and grown in shake flasks containing the YPD medium.

Table 1 shows their final biomass, plasmid stability, and distribution of inulinase expressed after 48 hr cultivation. Cell growth was strongly dependent on the host strains, and reached as low of 12 OD_{600} (for SC3 and YNN281) and as high of 23~25 OD_{600} (for YNN27, 2805, and SEY2102). Except for YNN27 cell, transformants with higher cell growth gave a greater expression of inulinase. The growth-associated production or expression of inulinase was clearly demonstrated by the two transformants of 2805 and SEY2102. The distribution or secretion efficiency of inulinase activity in each strain was also very different. With low producers such as SC3 and X1266-1C cells, about 50% of inulinase was retained in the cellular fraction. Most of inulinase, however, expressed in other transformants was secreted into the culture medium. Irrespective of host strains, a low plasmid stability of less than 40% was observed. By comparing the plasmid stability and total expression level, it appeared that high plasmid stability alone did not guarantee a high production yield.

Transformants of 2805 and SEY2102 displaying growth-associated expression of inulinase were selected as optimal recombinant cells for the simultaneous saccharification of inulin and ethanol fermentation.

Effect of Inulin Sources on Cell Growth and Inulinase Expression

From the observation that yeast transformants secreted efficiently inulinase into the culture medium, it is likely that inulin can be hydrolyzed to fructose by extracellular inulinase and then used as a good carbon source for recombinant

Table 1. Expression and distribution of inulinase in different yeast strains transformed with the plasmid pYIGP (*GAPDH* promoter). The recombinant yeast cells were grown on YPD medium for 48 hr in baffled-flasks.

S. cerevisiae Host Strains	Cell Growth (OD_{600})	Plasmid Stability (%)	Inulinase Activity (units/mL)		Secretion Efficiency (%)
			Medium	Cell	
YNN27	25.1	37	0.51	0.18	74
SC3	12.1	32	0.12	0.13	48
X1266-1C	17.5	26	0.13	0.09	59
YNN281	12.0	40	0.54	0.12	82
2805	24.7	42	1.20	0.41	75
SEY2102	23.4	17	0.75	0.15	83

yeast. It is well known that the complete hydrolysis of inulin is strongly dependent on the inulin sources as well as the degree of polymerization [11, 12]. To assess the effects of inulin sources on cell growth and inulinase expression, recombinant *S. cerevisiae* SEY2102 and 2805 cells were cultivated on three inulin sources, and the results were compared with the cases grown on glucose and sucrose.

When grown on sucrose, SEY2102 transformant showed slower growth and lower expression of inulinase than 2805 cell on sucrose (Fig. 1 and Fig. 2). This is presumedly due to the defectiveness of the major invertase gene (*SUC2*) in SEY 2102 host cell (*Δsuc2-9*) [13], resulting in a decreased assimilation rate of sucrose. The maximum

specific growth rates of two transformants grown on glucose were estimated to be 0.34 hr⁻¹, and final biomass on glucose and sucrose reached to $22 \sim 30$ OD_{600} $(7.0 \sim 9.6$ g-DCW/L). The expression of inulinase was clearly observed after 12 hr cultivation, and continued for the following growth phase(ethanol-consuming phase) (Fig. 2). However, a decreased cell growth of SEY2102 transformant in sucrose medium resulted in a low expression of inulinase, even at the end of cultivation.

When the two transformants were cultured on inulin media, the rapid-growing phase proceeded for 24 hr or 30 hr, and then the slow growth or stationary phase followed (Fig. 3A and Fig. 4A). The start of the stationary phase was concomitant with the depletion of inulin or total sugars (Fig. 3B and Fig. 4B). During the fast-growing phase, all of three inulins gave growth retardation, compared with the cases grown on glucose or sucrose. This retardation was more predominant with SEY 2102 than 2805 transformant. The maximum specific growth rate was about $0.21 \sim 0.22$ hr⁻¹, and final biomass of $27 \sim 33$ OD_{600} $(8.6 \sim 10.6$ g-DCW/ L) was obtained. This final biomass is comparable
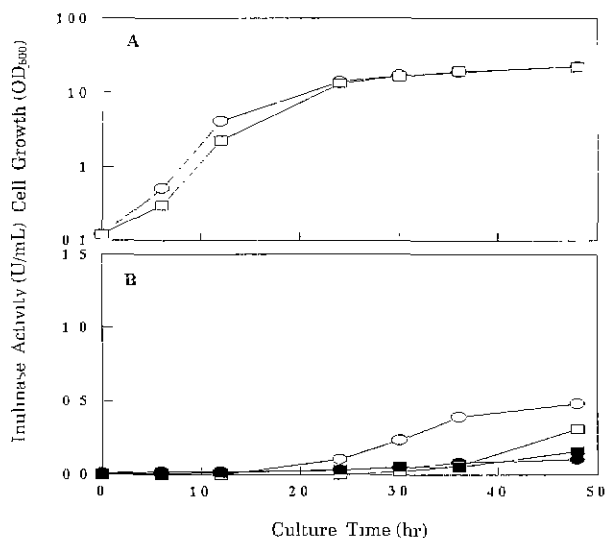

Fig. 1. Cell growth (A) and inulinase expression (B) in *S. cerevisiae* SEY2102 containing pYIGP plasmid grown on YP plus dextrose or sucrose medium.
Symbols in (A) and (B) : (○, ●), dextrose; (□, ■), sucrose (B) : (open symbols), extracellular inulinase; (closed symbols), intracellular inulinase.
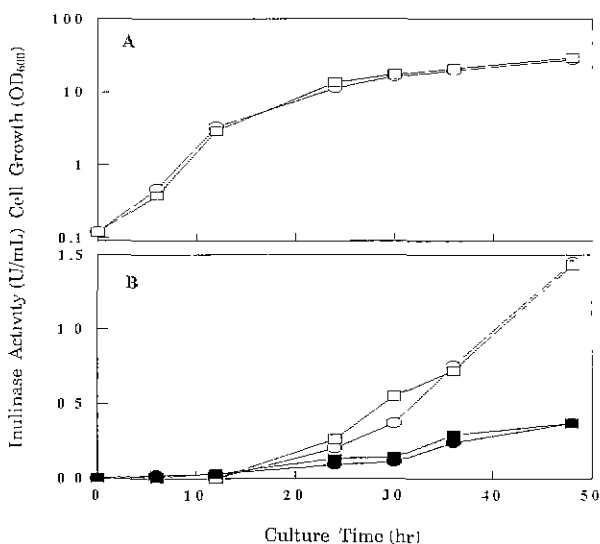

Fig. 2. Cell growth (A) and inulinase expression (B) in *S. cerevisiae* 2805 containing pYIGP plasmid grown on YP plus dextrose or sucrose medium Symbols are the same as in Fig. 1.
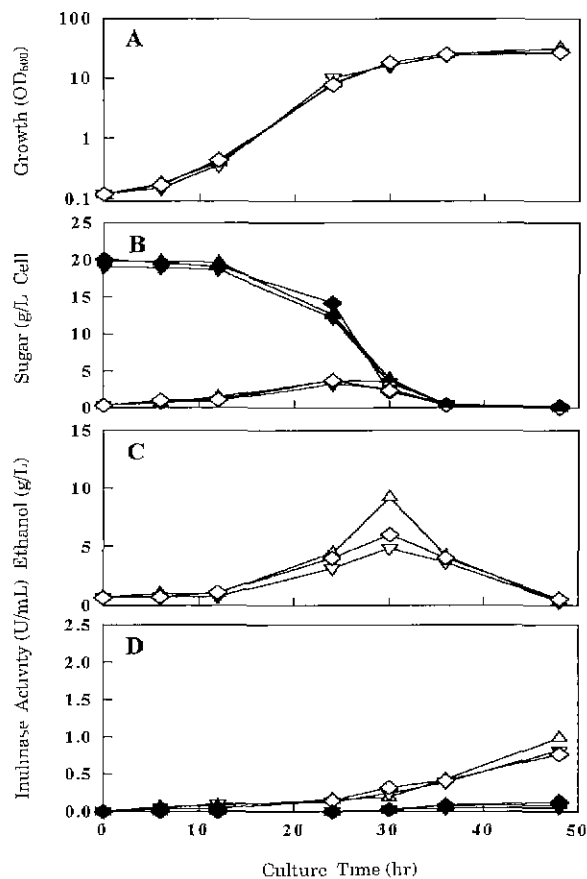

Fig. 3. Effect of inulin sources on the cell growth (A), sugar consumption (B), ethanol production (C), and inulinase expression (D) in *S. cerevisiae* SEY2102 containing pYIGP plasmid.
(A) : (△), Jerusalem artichokes inulin; (▽), dahlia inulin; (◇), chicory inulin
(B) : (open symbols), reducing sugar; (closed symbols), total sugar
(D) : (open symbols), extracellular inulinase; (closed symbols), intracellular inulinase
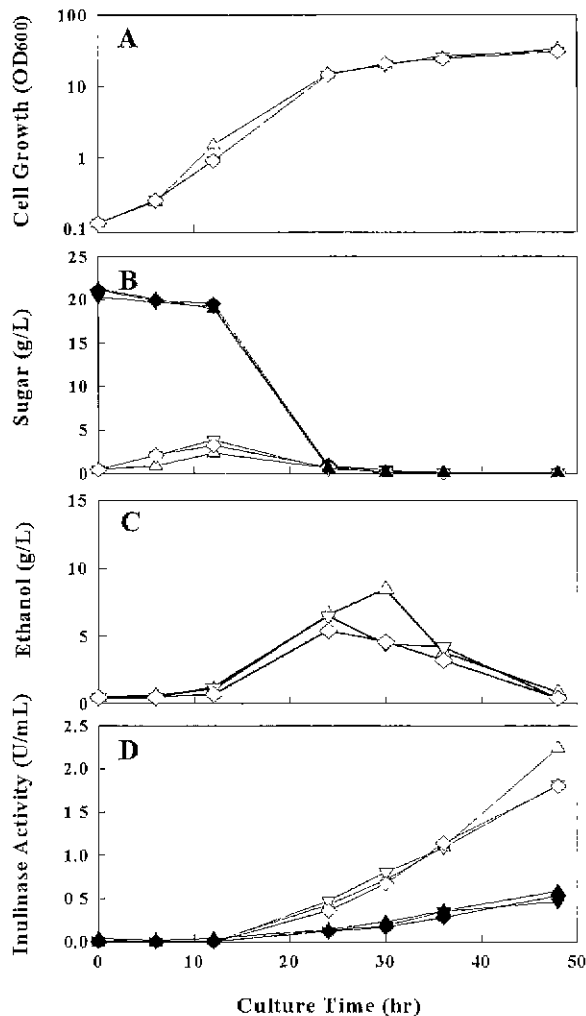
Fig. 4. Effect of inulin sources on the cell growth (A), sugar consumption (B), ethanol production (C), and inulinase expression (D) in *S. cerevisiae* 2805 containing pYIGP plasmid. Symbols are the same as in Fig. 3.

to that of the host cells grown on dahlia inulin, $15 \sim 18$ OD$_{600}$ (data not shown). The reducing sugar was accumulated at a maximal level of 3 g/L in $12 \sim 24$ hr, irrespective of inulin sources, and then exhausted in the following 12 hr. From the observation that the growth rate, final cell concentration, and reducing sugars level accumulated were similar, it is assumed that hydrolysis rate of inulin by the extracellular inulinase and assimilation rate of inulin hydrolysate have no significant differences between inulin sources and yeast transformants.

The expression of inulinase was kept at a very low level until 24 hr (late exponential phase or early stationary phase), and thereafter increased drastically to 48 hr, even after inulin and reducing sugar were depleted. The continuation of cloned-gene expression after exhaustion of primary carbon source (glucose) was also observed in the production of HBsAg and human lysozyme, which genes were under the control of *GAPDH* promoter [14, 15]. The persistence of expression capacity of the yeast cell seems to be due to the intracellular pool of metabolites or energy-generating inter-

mediates.

Among three types of inulin tested, one from Jerusalem artichoke displayed 10-30% higher cell growth than dahlia and chicory inulins, and showed an elevated expression level of inulinase after 36 hr (late stationary phase). This result indicates that Jerusalem inulin has higher degree of polymerization of fructose than that of other types of inulin. Considering the average degree of polymerization, 33.6 for chicory and 40.3 for dahlia inulin [12], a value of much greater than 40 seems to be estimated for Jerusalem artichoke inulin. The high degree of polymerization in Jerusalem inulin might result in an increased cell growth, leading to an elevated expression level of inulinase. It is interesting to note that the hydrolysis rate of inulins by the recombinant inulinase is independent on the degree of polymerization (Fig. 3B and Fig. 4B).

Most of inulinase activity was found in the culture medium, and SEY2102 cells revealed a slightly higher secretion level than 2805 cells (Fig. 3D and Fig. 4D). The inulinase activity in cell lysate was found in the periplasmic space (data not shown), as observed previously [6, 7]. The final expression level of inulinase in 2805 transformant was 2- or 3-fold higher than that of SEY 2102 cell, irrespective of inulin sources. The elevated expression in 2805 cell might be caused by the defectiveness of proteases (*pep4, prb1*) and/or strong secretory function of host itself. The expression of homologous or heterologous genes such as human lysozyme, human albumin, or carboxypeptidase Y was enhanced by using protease-deficient or supersecreting mutants [16-18].

Plasmid stabilities at the end of cultivation were about $10 \sim 20\%$ with both transformants (data not shown). The low plasmid stability was inevitable in the constitutive expression of heterologous genes in yeast, as reported with mouse α-amylase (*PGK* promoter) and *Clostridium* endoglucanase (*GAPDH* promoter) [19, 20].

Ethanol Production

Fig. 3C and Fig. 4C show ethanol production by SEY2102 and 2805 transformants when grown on various inulin sources. Until the stationary phase (24 to 30 hr), the transformants produced ethanol in a growth-associated manner, and then consumed it rapidly. Among the inulin sources examined, Jerusalem artichoke inulin gave the highest production level of ethanol of 9 g/L for both cells. These ethanol concentrations were about 2-fold higher than those of other inulins, and were similar levels with those attained with sucrose.

The fermentation results were summarized in Table 2. Even though no differences in growth rates between inulin sources were observed, values of other fermentation parameters (i.e., final biomass, growth yield, ethanol yield, ethanol production rate, and inulinase expression rate) in each transformant decreased in the following order: Jerusalem artichoke inulin, chicory inulin, and dahlia inulin. Besides the degree of polymerization,

Table 2. Comparison of fermentation parameters in shake flask cultures of recombinant *S. cerevisiae* SEY2102 and *S. cerevisiae* 2805 strains grown on different inulin sources

Fermentation parameter	SEY2102/pYIGP			2805/pYIGP		
	Jerusalem	Dahlia	Chicory	Jerusalem	Dahlia	Chicory
Specific growth rate (hr^{-1})[a]	0.21	0.21	0.21	0.22	0.22	0.22
Maximum biomass (g-DCW/L)	10.2	8 6	8.8	10.7	10.0	10 1
Growth yield (g-DCW/g-inulin)[b]	0.51	0.43	0.44	0.54	0.50	0.49
Maximum ethanol (g/L)	9.2	4.9	6.0	8.8	5.4	6.5
Ethanol yield (g/g-inulin)[b]	0.46	0.25	0.30	0.44	0.27	0.33
Ethanol production rate (g/L/hr)[c]	0.43	0.23	0.25	0 44	0.29	0.35
Inulinase expression rate (units/L/hr)[d]	48	35	36	106	71	82

[a] specific growth rate between 6 hr and 24 hr
[b] g-inulin means the amount of consumed inulin
[c] average ethanol production rate between 12 hr and 30 hr
[d] average inulinase expression rate between 30 hr and 48 hr

more understanding on the fructan structure and molecular weight of each inulin would be helpful for precise analysis of the fermentation parameters. Moreover, taking into account the low plasmid stability (10~20%) with the pYIGP episomal plasmid used in this work, the ethanol production rate would be enhanced if the plasmid stability could be kept at high level. Autoselective mutant [21] or integration of inulinase gene into the chromosome [22] can be a solution to overcome the plasmid instability.

As a consequence, the availability of recombinant *S. cerevisiae* secreting exoinulinase would allow development of a simultaneous saccharification and fermentation process of inulin to ethanol.

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