A Thesis for the Degree of Master of Science

Ethanol production from inulin by recombinant *Saccharomyces cerevisiae* with inulinase gene from *Kluyveromyces marxianus*

이눌린 분해효능 유전자를 가진 재조합 효모에서 이눌린으로부터 바이오에탄올 생산에 관한 연구

By

Soo-Jeong Hong

School of Agricultural Biotechnology
Seoul National University
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Advisor : Professor Jin-Ho Seo

Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science

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農 學 碩 士 學 位 論 文

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이눌린 分解性能을 가진 再組合 酵母에서 이눌린으로부터 바이오에탄올 生産에 관한 硏究

指導敎授 徐 鎭 浩

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委 員 長 장 판 식 (인)
副委員長 서 진 호 (인)
委 員 강 동 현 (인)

ABSTRACT

In recent years, efforts to produce biofuels from renewable biomass have been done for overcoming the problems of uncertain fuel supply and carbon dioxide emissions. One of the most representative biofuels is bioethanol because it has greater octane booster properties, is not toxic and does not contaminate water sources. Inulin consisting of linear β-2,1-linked polyfructose chains terminated by a glucose residue ($C_{6n}H_{10n+2}O_{5n+1}$) is a polyfructan which is present in plants such as Jerusalem artichoke, chicory and dahlia. It can be converted to fructose by inulinase, then fructose can be utilized easily by microorganisms such as *Saccharomyces cerevisiae*. However, *S. cerevisiae* cannot consume inulin itself because it does not have inulin-degrading enzymes.

This thesis was carried out to produce ethanol from inulin by recombinant *S. cerevisiae* with the inulinase gene from *Kluyveromyces marxianus* by metabolic engineering approach. Three types of promoters (*GPD*, *PGK1*, truncated *HXT7*) and signal sequences (*KmINU*, *MFα1*, *SUC2*) of an expression cassette were compared to select the optimized expression system when the inulinase gene from *Kluyveromyces marxianus* (*KmINU*) was introduced into *S. cerevisiae* D452-2 used as a host. Nine plasmids having different combinations of promoter and signal sequence were constructed and introduced into *S. cerevisiae* D452-2. The recombinant *S. cerevisiae* carrying the *PGK1* promoter and *MFα1* signal sequence (*S. cerevisiae* D452-2/p426PM) not only had the highest

KmINU activity per dry cell weight, but also exhibited the most outstanding fermentation properties among the nine recombinant *S. cerevisiae* strains. It was observed that *KmINU* accumulated in the medium and the specific activity (U/mg dry cell weight) increased with cultivation time. The fermentation performances of a wild type strain grown in an acid-hydrolyzed inulin solution were compared with those of the recombinant *S. cerevisiae* grown in the same amount of inulin. Ethanol production based on inulin needs three steps including acid hydrolysis, neutralization and fermentation. In aspects of the cost and efficiency, the use of the *S. cerevisiae* with the inulinase gene is competitive because the recombinant yeast strain allows inulin hydrolysis and ethanol production simultaneously. Finally, a batch fermentation of *S. cerevisiae* D452-2/p426PM in a bioreactor with 200 g/L inulin was performed and resulted in 0.47 g ethanol/g inulin of ethanol yield and 1.02 g/L·h of ethanol productivity.

Keywords : ethanol, inulin, Jerusalem artichoke, inulinase, *Saccharomyces cerevisiae*, metabolic engineering

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I. Introduction

1. Bioethanol

In recent years, largely in response to uncertain fuel supply and efforts to reduce carbon dioxide emissions, bioethanol has become one of the most promising biofuels today because it has greater octane booster properties, is not toxic, and does not contaminate water sources (Zhang, Chi, Zhao, *et al.* 2010). Therefore, it is considered as alternative to fossil transport fuels in the world. Bioethanol is a form of renewable energy that can be produced from agricultural feedstocks. These fuel crops include maize, corn and wheat crops, waste straw, cord grasses, Jerusalem artichoke, sorghum plants and so on.

However, production of bioethanol from food crops has led to increase the food prices. This has become an international issue because of the global increase in food prices. Therefore, the feedstocks such as Jerusalem artichoke grown in non-arable lands are regarded as a sustainable source for production of bioethanol.

Meanwhile, *Saccharomyces cerevisiae* is the current industrial strain for bioethanol production as it has numerous characteristics such as high concentration of ethanol producer, high ethanol tolerance, the existence of well-developed tools for manipulation of yeast DNA and so it has a long history of commercial scale fermentation.

2. Jerusalem artichoke

Jerusalem artichoke (*Helianthus tuberosus L.*) is drawing attention as an alternative sugar crop. Jerusalem artichoke is a perennial herbaceous plant belonging to the sunflower family. It has a lot of advantages as biomass for bioethanol. It is well adapted to a wide variety of climates, does not require soil fertility and develops underground stolons forming shaped tubers, which are similar to potatoes (Yuan *et al.* 2012). Unlike most starch-based plants, it contains inulin polysaccharide and does not need to be replanted each year because having 3 ~ 4 life cycle. Also, if the Jerusalem artichoke crop is harvested when the sugar content in the stalk reaches a maximum for avoiding the harvesting of the tubers, the harvesting equipment and procedures are essentially the same as for harvesting sweet sorghum or corn for ensilage, reducing operation costs (Negro *et al.* 2006). Above all, lignocellulosic biomass is needed to be pretreated such as elimination of lignin. However, Jerusalem artichoke can be used directly as a fermentable carbon source after a simple pretreatment process. This means that the cost for a pretreatment process can be reduced dramatically by using Jerusalem artichoke compared to lignocellulosic biomass.

The dry tubers of Jerusalem artichoke contain about 60 ~ 80% (w/w) inulin (Pandey *et al.* 1999). Therefore these tubers are an important source for inulin.

3. Inulin

Inulin, a non-digestible carbohydrate, is not only found in many plants as a storage carbohydrate, but has also been part of human's daily diet for several centuries. It is a polyfructan consisting of linear β-2,1-linked polyfructose chains terminated by a glucose residue and exists as a reserve carbohydrate in the roots and tubers of plants such as Jerusalem artichoke, chicory, dahlia and yacon (Liu *et al.* 2010). Fructose formation from complete hydrolysis of inulin is a single-step inulinase reaction and yields up to 95% of fructose, therefore, microorganisms can produce ethanol from hydrolyzates of product of inulin (Park *et al.* 2001). Inulin is regarded as a renewable carbohydrate source for production of fructose syrup, ethanol and inulooligosaccharide. Furthermore, inulin is easy to be dissolved in water and the solution with high concentration of inulin has low viscosity (Zhang, Chi, Zhao*, et al.* 2010).

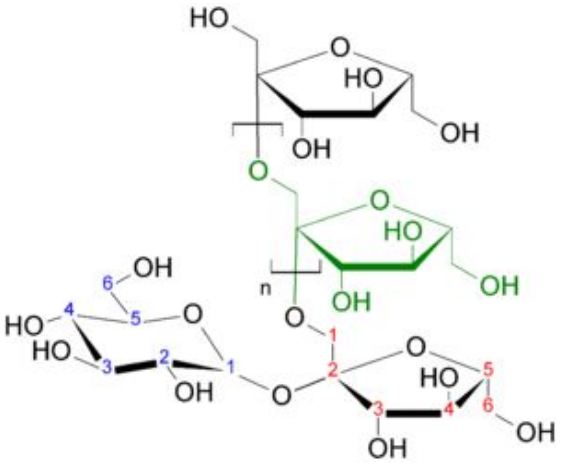

Figure 1. Structure of inulin

3.1 Hydrolysis of inulin

Inulin can be hydrolyzed by a chemical method such as acid hydrolysis. However, the chemical method has many drawbacks. For example, fructose is easily degraded at low pH and the process gives rise to coloring of the inulin hydrolysate and by-product formation in the form of difructose anhydrides (Barthomeuf *et al.* 1991). Also fructose is liable under acidic conditions to form 5-hydroxymethylfurfural, a known inhibitor of microbial cell growth (Zhang *et al.* 2012).

Microbial inulinases (exo-inulinase) are usually inducible and exo-acting enzymes, which catalyze the hydrolysis of inulin by splitting off terminal fructosyl units (D-fructose) by cleaving the glycosidic linkages in polymer moiety (Pandey *et al.* 1999). After this enzymatic hydrolysis of inulin, 95% pure fructose can be obtained and separation of fructose from the fructose syrup process is not required. However, hydrolysis of inulin using microbial inulinases increases production cost (Torandiaz *et al.* 1985).

So, the best procedure of ethanol production from inulin is to use microorganisms that can produce ethanol with a high yield and have inulinase activity. In other words, simultaneous saccharification and fermentation (SSF) can be used in ethanol fermentation from inulin. This means integration of inulinase production, inulin hydrolysis, and fermentation in one step. The microorganisms that can directly utilize inulin and produce ethanol are potential SSF strains. By using this method, a step of hydrolysis pretreatment of inulin can be eliminated (Hu *et al.*

2012). The SSF strategy is more economically competitive and effective for ethanol production from inulin than chemical hydrolysis. Because no sugar and toxic materials accumulation occurs during ethanol fermentation, contamination can be prevented effectively (Yuan *et al.* 2012) and above all, the neutralization process is not needed any more. So the SSF strategy using microorganisms for ethanol production from inulin has a lot of advantages than chemical or enzymatic hydrolysis of inulin.

3.2 Inulinase

Inulin can be hydrolyzed to fructose and glucose by inulinases. There are two-types of inulinase, exo-inulinases and endo-inulinases (Moriyama *et al.* 2003). The exo-inulinases (β-D-fructan fructohydrolase, EC 3.2.1.80) are distinguishable from endo-inulinases (2,1-β-D-fructanfructohydrolase, EC 3.2.1.7) by their ability to hydrolyze sucrose and catalyze the hydrolysis of inulin by splitting the terminal fructosyl units (D-fructose) due to the cleavage in the glycosidic linkages at the polymer moiety (Vandamme *et al.* 1983). The exo-inulinases catalyze removal of the terminal fructose residues from the non-reducing end of the inulin molecule in one step. As a result, fructose can be produced as a main product and glucose as a minor product.

Although inulinases could be obtained from vegetable and animal sources, microorganisms are the best sources for commercial production of inulinases mainly due to their easy cultivation (Astolfi *et al.* 2011). Many microbial species such as *Kluyveromyces marxianus*, *Penicillium sp*,

Bacillus polymyxa, *Pichia guilliermondii* and *Aspergillus niger* excrete inulinase and can use inulin as a growth substrate. However, it has been known that yeast strains generally have higher inulinase production than fungal and bacterial strains (Chi *et al.* 2009).

Above all, inulinase from *K. marxianus* (KmINU) has shown good thermostability and activity (Rouwenhorst *et al.* 1988). Its size is about ~ 60 kDa and signal sequence information of the gene is known. Therefore, the *KmINU* gene was selected to use for this study and introduced into host *S. cerevisiae* strain.

4. Gene expression cassettes

The exploitation of microorganisms for the overproduction of useful heterologous proteins is a field of increasing economic importance (Chung *et al.* 1996). In contrast to the operons that can be employed in prokaryotic cells, a series of independently transcribed genes must be introduced in yeast. Both the level and the timing of enzyme synthesis can be essential for the successful introduction of new pathways. In addition to copy number and transcription level/timing, translational and post-translational control can be important for modulating protein levels (Da Silva *et al.* 2012).

Expression of heterologous gene in *S. cerevisiae* to introduce new metabolic pathway or increase the yield of target products has been researched by various approaches. For example, engineered *S. cerevisiae*

was used to conferment mixtures of xylose and cellobiose. This recombinant strain hydrolyze the cellobiose through the action of an intracellular β–glucosidase following import by a high-affinity cellodextrin transporter (S. J. Ha *et al.* 2011). And engineered *S. cerevisiae* which was overexpressed NADH-preferable xylose reductase mutant, NAD+-dependent xylitol dehydrogenase and endogenous xylulokinase produced reduced xylitol, also more modulation including disruption of the chromosomal *ALD6* gene encoding aldehyde dehydrogenase improved the performance of ethanol production from xylose (S. H. Lee *et al.* 2012).

Protein expression is very complicated phenomenon and affected by many factors which are promoter strength, type of signal sequence, copy number and so on.

4.1 Promoter

The efficient expression of heterologous proteins in yeast considerably relies on yeast promoters. Therefore, different promoters have been used to successfully direct expression of heterologous genes in yeast and well-characterized promoters are essential for pathway engineering and synthetic biology efforts in *S. cerevisiae* (Blazeck *et al.* 2012).

Constitutive promoters offer fairly constant gene expression levels at the single-cell level without the need for specific inducers or media formulations (Da Silva *et al.* 2012). The most widely used constitutive promoters have often been from the yeast glycolytic pathway. They are

highly-expressed and used successfully to express a number of proteins. These glucose-dependent promoters include those for glyceraldehyde-3-phosphate dehydrogenase (*GPD*) promoter (Holland *et al.* 1980), phosphoglycerate kinase (*PGK1*) promoter (Ogden *et al.* 1986), pyruvate decarboxylase (*PDC1*) promoter (Kellermann *et al.* 1986), triosephosphate isomerase (*TPI1*) promoter (Russell 1985), alcohol dehydrogenase I (*ADH1*) promoter (Hitzeman *et al.* 1981) and pyruvate kinase (*PYK1*) promoter (Nishizawa *et al.* 1989). Other commonly used native promoters include iso-1-cytochrome c (*CYC1*) promoter (Guarente *et al.* 1984), actin 1 (*ACT1*) promoter (Gallwitz *et al.* 1980), mating factor alpha-1 (*MFα1*) promoter (Brake *et al.* 1984) and those for hexose transport, for example *HXT7* promoter (Reifenberger *et al.* 1995). Under de-repressed conditions *HXT7* is by far the most strongly expressed *HXT* gene, but it is strongly repressed at high glucose concentrations (Reifenberger *et al.* 1997). However, 5' deletion of the *HXT7* promoter region, leaving only the 390 bp upstream of the ATG start codon leads to strong constitutive transcription of the gene on glucose media (Hauf *et al.* 2000). Therefore, a truncated *HXT7* promoter is used widely in glucose-based studies.

All of them, *GPD* promoter, *PGK1* promoter and truncated *HXT7* promoter are known as powerful promoters in expression of heterologous proteins relatively.

In this study, to increase the specific activity of recombinant inulinase, the promoter of an expression cassette was changed and the promoter strength was compared. Therefore, in this study, three promoters

(*GPD* promoter, *PGK1* promoter and truncated *HXT7* promoter) were used and compared.

4.2 Signal sequence

Protein secretion in *S. cerevisiae* involves a complex series of events (Marten *et al.* 1989). First, proteins pass through the endoplasmic reticulum where core glycosylation takes place and then into the Golgi apparatus where outer chain oligosaccharides are added (Esmon *et al.* 1981). Then, the glycosylated protein is packaged in secretory vesicles and release the processed protein (Tkacz *et al.* 1973). Some proteins are retained in the periplasmic space, and the others pass through the cell wall into the medium.

Proteins secreted from yeast usually possess a hydrophobic amino-terminal extension, called a signal peptide (Chung *et al.* 1996) or signal sequence. This signal sequence is usually composed of about 20 amino acids and is cleaved from the protein during passage through the secretion pathway. For secreting heterologous proteins from yeast effectively, the strategies using homologous signal sequence, heterologous signal sequence from other genes or artificially designed signal sequence are mainly used.

K. marxianus inulinase (KmINU) has already its homologous signal sequence which is leading the inulinase gene. The *KmINU* gene from *K. marxianus* encoding inulinases have several properties which make it attractive for the development of a yeast secretion vector system

(Kalil *et al.* 2001). KmINUs have putative signal sequences consisting of 23 amino acids, which share a common feature with other characterized signal sequences (Chung *et al.* 1996).

The most extensively used signal sequence for heterologous protein secretion from *S. cerevisiae* is the prepro region from α-factor (*MFα1*). *MFα1* encodes a 165 amino acid protein, prepro-α-factor, which comprises a signal sequence of 19 amino acids (the pre region) and a pro region, followed by four tandem repeats of the mature 13 amino acid α-factor sequence (Romanos *et al.* 1992).

Smith *et al*. fused the *SUC2* signal sequence to prochymosin protein instead of the native signal sequence and they obtained activate prochymosin. However, less than 5 percent of the prochymosin produced is secreted (Smith *et al.* 1985).

In this study, as data for the promoter, the signal sequence of expression cassettes was changed and compared their effects to recombinant inulinase activity. Therefore, *KmINU*, *MFα1* and *SUC2* signal sequences have been used.

5. Manufacturing technologies for bioethanol production using inulin

Ethanol has been produced from hydrolyzed Jerusalem artichoke by acid using various microorganisms such as *Kluyveromyces fragilis*, *S. cerevisiae*, *S. diastaticus*, *S. rasei* and *Zygosaccharomyces fermentati* (Schorrgalindo *et al.* 1995), (Remize *et al.* 1998).

Nakamura *et al*. used *Aspergillus niger* mutant produced exo-inulinases and endo-inulinases in combination with an ethanol tolerant strain *S. cerevisiae* 1200 in the simultaneous saccharification and fermentation of pure inulin (Nakamura *et al.* 1996). The inulinase produced by the marine-derived *Pichia guilliermondii* strain 1 has been purified, characterized and overexpressed (Gong *et al.* 2008). This *INU1* gene encoding exo-inulinase from *P. guilliermondii* was transformed into the uracil mutant of *Saccharomyces sp*. W0 and the positive transformant Inu-66 producing 34.2 U/ml of extracellular inulinase was obtained (Zhang, Chi, Chi*, et al.* 2010). Following this research, this gene ligated into the 18S rDNA integration vector, transformed into the uracil mutant of *Saccharomyces sp.* W0 and integrated into its chromosomes. Then, ethanol was produced from Jerusalem artichoke and inulin using this recombinant strain (Wang *et al.* 2011).

The efforts to increase an expression level of inulinase have been done. The high-level secretory production of highly active inulinase from *K. marxianus* in *Pichia pastoris* was researched. For this, the own signal sequence of inulinase from *K. marxianus* was replaced to mating alpha

factor signal sequence (Zhang *et al.* 2012).

S. cerevisiae has been used the most in ethanol production from various substrates. They can produce high concentration of ethanol and his high ethanol tolerance. Also, it is a genetically tractable, well-characterized organism, the current industrial strain alcohol producer, generally recognized as safe and it has been previously manipulated to produce other heterologous metabolites. So, in this study, *S. cerevisiae* was used as a host strain to produce ethanol from inulin. Many researches of ethanol production from inulin or Jerusalem artichoke using *S. cerevisiae* have been performed, and the results of these researches were summarized in Table 1.

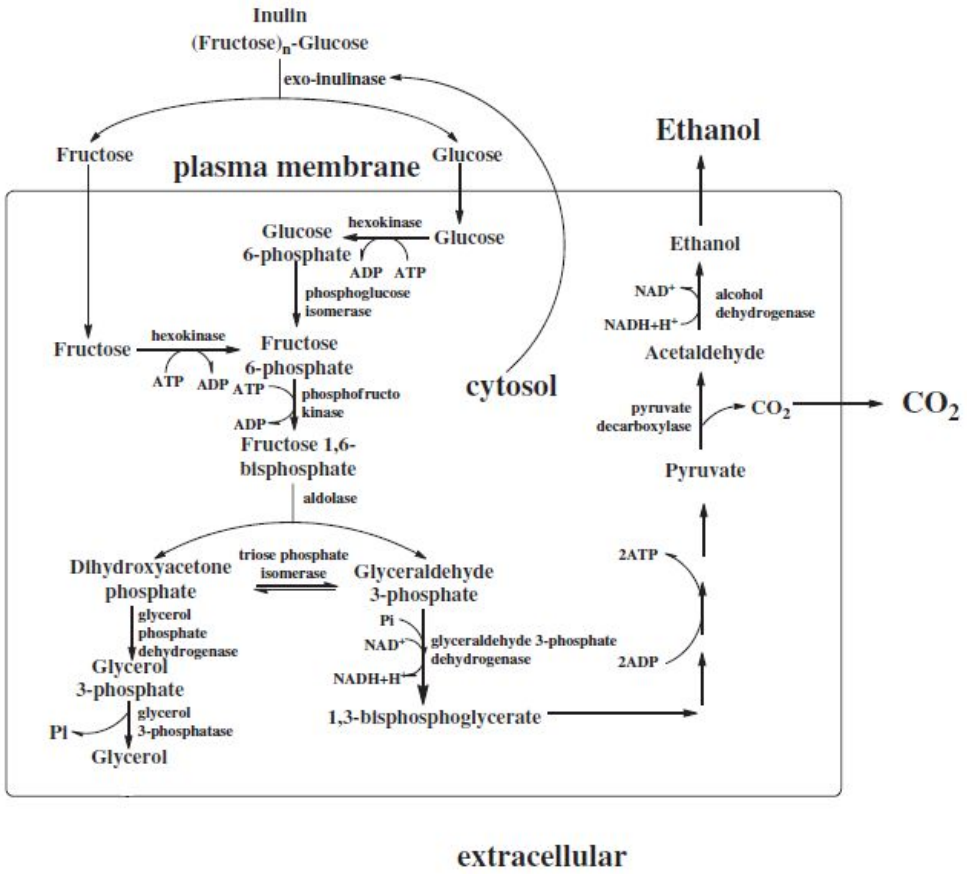

Figure 2. Metabolic pathway of ethanol from inulin in *S. cerevisiae*

Table 1. Researches of ethanol production from inulin-containing substrate

Reference	Strain	Maximum biomass (g D.C.W./L)	Ethanol yield (g ethanol/ g inulin)	Ethanol productivity (g/L·h)
Y. H. Kim *et al.* 1998. Biotechnol. Bioprocess Engineering	*S. cerevisiae*	10.2	0.46	0.43
Zhang, T., Z. Chi, Z. Chi *et al*. 2010. Microbial Biotechnology		3.5	0.24	1.22
Wang, J. M., T. Zhang *et al*. 2011. Biomass and Bioenergy		3.4	0.39	0.97
S. H. Lim *et al*. 2011. Bioresource Technology		-	0.20	1.06
Zhang, T., Z. Chi, C. H. Zhao *et al*. 2010. Bioresource Technology		3.7	0.32	0.66

6. Research objectives

For production of ethanol from inulin in engineered *S. cerevisiae*, several factors should be considered including heterologous inulinase source, expression system and conditions of fermentation.

This study was focused on introduction of the heterologous inulinase gene from *K. marxianus* in *S. cerevisiae* and production of ethanol directly from inulin by the recombinant *S. cerevisiae*.

The specific objectives of this study are as follows.

(1) To optimize expression of the inulinase gene from *K. marxianus* in *S. cerevisiae*.
(2) To characterize and optimize simultaneous hydrolysis of inulin and ethanol production by recombinant *S. cerevisiae*.

II. Materials and methods

1. Reagents

All chemicals used were of reagent grade. Glucose, agarose, ampicillin, ethidium bromide, yeast synthetic drop-out supplement, yeast nitrogen base (YNB, w/o amino acid), bovine serum albumin and EDTA were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, U.S.A). Bacto-peptone, bacto-trypton, yeast extract and bacto-agar were obtained from Difco (Detroit, MI, U.S.A); glycine from Junsei (Tokyo, Japan); ethanol from Merck (Darmstadt, Germany); HPLC-grade water from J.T. Baker (Phillipsburg, NJ, U.S.A); 1 N NaOH and 1 N HCl, NaCl, H_2SO_4 and potassium phosphate from Duksan (Ansan, Korea).

2. Strains and plasmids

2.1 Strains

Escherichia coli TOP10 (Invitrogen, Carlsbad, CA, U.S.A) was used for the propagation and preparation of plasmid DNA. *S. cerevisiae* D452-2 [*Matα, leu2 his3 ura3 can1*] was used as host strains for the expression of inulinase. *S. cerevisiae* D452-2 strain was kindly donated by Prof. Makino at Kyoto University. The wild type and recombinant strains were stored on YEPD and YNB selective medium respectively in a deep freezer at - 80℃ suspended in 15% glycerol.

Table 2. List of strains used this study

Strains	Genotype	Reference
E. coli TOP10	F^- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *deo*R *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L(Str^R) *end*A1 *nup*G	-
S. cerevisiae D452-2	*Matα*, *leu2 his3 ura3 can1*	Hosaka, Nikawa *et al*. 1992
D452-2/p426GI	D452-2, ura3::URA3, P_{GPD}-KmINU signal sequence-KmINU-T_{CYC1}	This study
D452-2/p426PI	D452-2, ura3::URA3, P_{PGK1}-KmINU signal sequence-KmINU-T_{CYC1}	This study
D452-2/p426HI	D452-2, ura3::URA3, $P_{HXT7(truncated)}$-KmINU signal sequence-KmINU-T_{CYC1}	This study
D452-2/p426GM	D452-2, ura3::URA3, P_{GPD}-MFα1 signal sequence-KmINU-T_{CYC1}	This study
D452-2/p426PM	D452-2, ura3::URA3, P_{PGK1}-MFα1 signal sequence-KmINU-T_{CYC1}	This study
D452-2/p426HM	D452-2, ura3::URA3, $P_{HXT7(truncated)}$-MFα1 signal sequence-KmINU-T_{CYC1}	This study
D452-2/p426GS	D452-2, ura3::URA3, P_{GPD}-SUC2 signal sequence-KmINU-T_{CYC1}	This study
D452-2/p426PS	D452-2, ura3::URA3, P_{PGK1}-SUC2 signal sequence-KmINU-T_{CYC1}	This study
D452-2/p426HS	D452-2, ura3::URA3, $P_{HXT7(truncated)}$-SUC2 signal sequence-KmINU-T_{CYC1}	This study

2.2 Plasmids

Plasmid p426GPD, p426PGK1 and p426HXT7 were used as mother vectors (Figure 3) which has the *GPD* promoter, *PGK1* promoter and truncated *HXT7* promoter respectively and cloning vectors for expression of *KmINU* (Inulinase gene from *K. marxianus*) gene. The expression cassettes were comprised of the one signal sequence among *KmINU* signal sequence, *MFα1* signal sequence and *SUC2* signal sequence, *KmINU* gene, *myc* tag and *CYC1* terminator (from *S. cerevisiae*). *KmINU* signal sequence and *KmINU* gene were isolated from chromosomal DNA of *K. marxianus* by the polymerase chain reaction (PCR) using primers based on the *KmINU* sequence from *K. marxianus* reported in NCBI. *MFα1* and *SUC2* signal sequence were isolated from chromosomal DNA of *S. cerevisiae* by PCR using primers listed in Table 2. To fuse *myc* tag to C-terminal of *KmINU* gene, the reverse primers for amplifying the C-terminal part of *KmINU* contained *myc* tag sequence. Signal sequences were fused to N-terminal of *KmINU* attached the *myc* tag by overlap PCR using primers listed in Table 2. The amplified expression cassettes were cloned into p426GPD, p426PGK1 and p426HXT7 respectively.

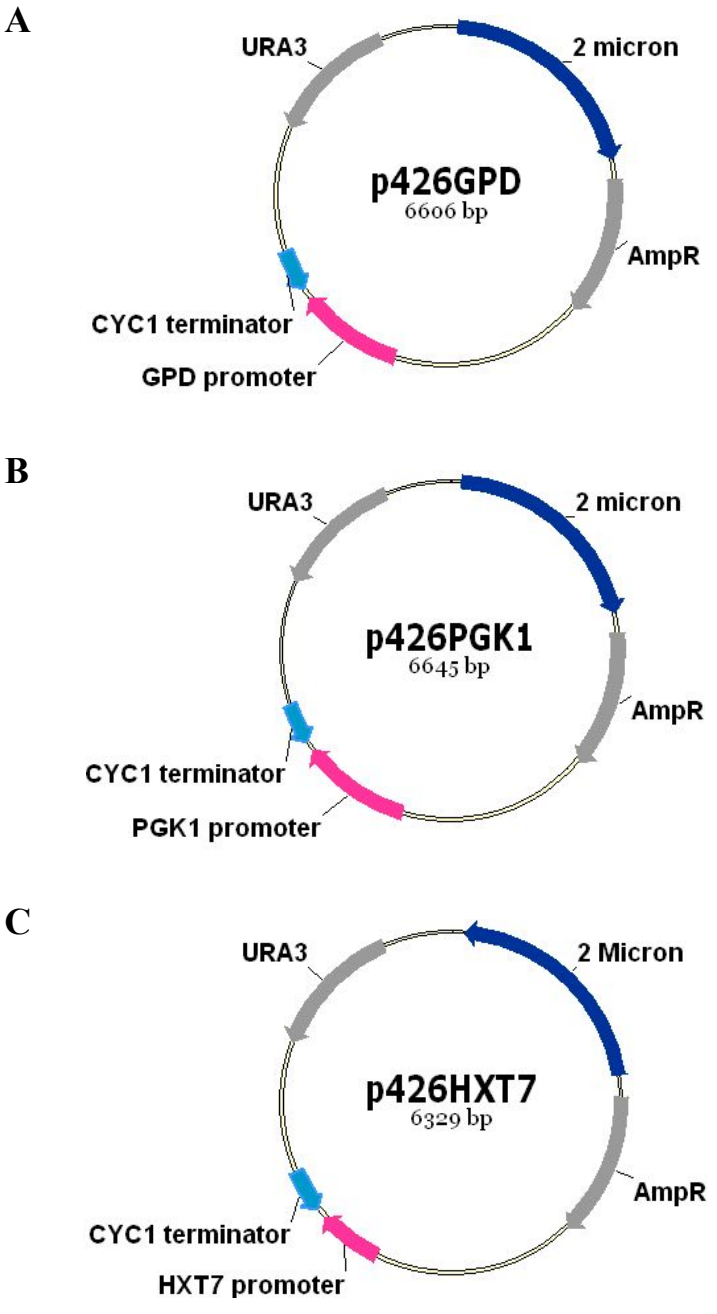

Figure 3. Mother vector used for this study (A) p426GPD, (B) p426PGK1, (C) p426HXT7

Table 3. List of primers used in this study

Name	Sequence	Relevant work
F-SpeI_KmINU	gactagtATGAAGTTCGCATACTCCCTCTTGCTT	Cloning KmINU cassette into p426GPD, p426PGK1
R-HindIII_KmINU_myc	cccaagcttTCACAGATCCTCTTCTGAGATGAGTTTTGTTCAACGTTAAATTGGGTAACGTTAAA	Cloning KmINU cassette into p426GPD
F-BamHI_KmINU	cgggatccATGAAGTTCGCATACTCCCTCTTGC	Cloning KmINU cassette into p426HXT7
R-XhoI_KmINU_myc	ccgctcgagTCACAGATCCTCTTCTGAGATGAGTTTTGTTCAACGTTAAATTGGGTAACGTTAAATGAGTTAATGGT	Cloning KmINU cassette into p426PGK1, p426HXT7
F-BamHI_ovMF	cgggatccATGAGATTTCCTTCAATTTTTACTGCTG	Overlapping KmINU cassette and *MFα1* signal sequence, cloning into p426GPD, p426PGK1, p426HXT7
R-ovMFKmINU	GGCCTTGCTGTCACCATCAGCTTCAGCCTCTCTTTTCTCG	Overlapping KmINU cassette and *MFα1* signal sequence

(Continued)

Name	Sequence	Relevant work
F-ovKmINU	GATGGTGACAGCAAGGCCATCA	Overlapping KmINU cassette and signal sequence
R-SalI_ovKmINU_myc	acgcgtcgacTCACAGATCCTCTTCTGAGATGAGTTTTTGTTCAACGTTAAATTGGGTAACGTTAAATGAGTTAATGG	Overlapping KmINU cassette and signal sequence, cloning into p426GPD, p426PGK1, p426HXT7
F-BamHI_ovSUC2	cgggatccATGCTTTTGCAAGCTTTCCTTTTCCTT	Overlapping KmINU cassette and *SUC2* signal sequence, cloning into p426GPD, p426PGK1, p426HXT7
R-ovSUC2KmINU	GGCCTTGCTGTCACCATCTGATGCAGATATTTTGGCTGCAAAAC	Overlapping KmINU cassette and *SUC2* signal sequence

The lowercase sequences correspond to restriction enzymes site.

3. DNA Manipulation and Transformation

3.1 Enzymes

Restriction enzymes, calf intestinal alkaline phosphatase (CIP) and Endoglycosidase H (Endo H, 10,000 unit) were purchased from New England Biolabs (Beverly, MA, U.S.A). T4 DNA ligation mix was obtained from Takara (Tokyo, Japan). Exo-inulinase for standard was purchased from Megazyme (Wicklow, Ireland).

3.2 Transformation of *E. coli*

Transformation of *E. coli* was carried out as described by Sambrook *et al*. (Sambrook). *E. coli* Top10 was cultured in 5 mL LB medium for 12 h. The half a milliliter of the culture was transferred to fresh 50 mL LB medium and cultured until OD_{600} reached 0.5. Cells harvested by centrifugation at 6,000 rpm for 5 min at 4℃ were resuspended in 5 mL of ice-cold 100 mM $CaCl_2$ solution containing 15% (v/v) glycerol. Resuspended cells were aliquoted to 100 μL, mixed with DNA, and kept on ice for 30 min. They were subjected to heat-shock at 42℃ for 45 sec, and 1 mL of LB medium was added to the test tubes and incubated at 37℃ for 40 min to allow the bacteria to express the antibiotic resistance. An appropriate volume of the transformed cells was spread on LB agar plates with an ampicillin selection marker.

3.3 Preparation of plasmid DNA and yeast genomic DNA

Mini-scale preparation of plasmid DNA was carried out using *AxyPrep*™ Plasmid Miniprep Kit from Axygen Biosciences Co. (San Francisco, U.S.A) according to the manufacturer's instruction.

Preparation of yeast genomic DNA to obtain a template for the gene was carried out using DNeasy Blood & Tissue Kit from QIAGEN (Düsseldorf, Germany) according to the manufacturer's instruction.

3.4 Isolation of DNA fragments and DNA sequencing

DNA was digested with restriction enzymes and separated on a 0.1% (w/v) agarose gel. After full separation of the desired DNA band from the gel, the gel containing the DNA fragment was solubilized and further purified by using Gel Extraction Kit from Real Genomics (Taipei, Taiwan). DNA sequencing was performed by Mbiotech (Hanam, Korea).

3.5 Polymerase chain reaction (PCR)

PCRs were performed with the AccupowerTM PCR PreMix (Bioneer Co., Daejon, Korea) in the GeneAmp PCR System 2400 (Applied Biosystems, Lincoln, CA, U.S.A). PCRs for cloning of genes were performed in 50 μL of dyemix solution from Enzynomics Co. (Korea) containing 10 pM each of forward and reverse primers, and 10 ng *S. cerevisiae* D452-2 genomic DNA or plasmid DNA as a template. After heating the reaction tubes for 5 min at 94℃, 30 cycles of PCR amplification were performed as follows: 30 sec at 94℃, 30 sec at 53℃, and 1 min 30 sec at 72℃ (time is dependent on product size, 1 min for 1 kb), followed by 7 min at 72℃ during the last cycle. In case of colony

PCR, 50 sec was set up for annealing time. The amplified gene was confirmed by gel electrophoresis.

3.6 Yeast transformation

Transformation of expression vectors was performed using the yeast EZ-Transformation kit (BIO 101, Vista, Calif). Transformants were selected on YNB medium containing 20 g/L glucose. Amino acids and nucleotides were added necessary.

4. Media and Culture conditions

4.1 Media

LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl) was used for *E. coli* cultivation. YEPD medium (1% yeast extract, 2% bacto-peptone, 2% glucose) and YNB medium which lacked appropriate amino acid were used for selection of yeast strains. YNB Synthetic Complete medium (0.67 g/L YNB, 2.0 g/L amino acids mixture) which contains amino acids mixture were used for cultivation of yeast strains.

4.2 Inoculation

Recombinant *S. cerevisiae* stock was transferred to a test-tube containing 5 mL of appropriate medium and incubated overnight at 30℃, 250 rpm in a shaking incubator (Vision, Korea). Pre-culture was carried out in a 500 mL baffled flask with 100 mL working volume at 30℃, 250 rpm for appropriate time in YNBD (2% glucose) selective medium. The inocula were prepared by growing cells overnight to an OD_{600} of 5 ~ 10. The cells

were transferred into 250 mL glass flask containing 50 mL YEPD medium. In flask culture, the cells were transferred into 250 mL glass flask containing 50 mL working volume and culture was carried out at 30℃ in shaking incubator (Vision, Korea) and shaking rate was maintained at 80 rpm. In bioreactor cultivation, 100 mL of the pre-culture grown in YNBD medium at 30℃ and shaking speed of 250 rpm for 60 h was transferred into 500 mL bioreactor.

4.3 Cultivations in bioreactor

Batch fermentation was performed using a bench-top fermentor (KoBioTech, Korea). Cultivation were performed in 500 mL YEP medium supplemented with 20% (w/v) inulin or fructose at 30℃ and pH 5.0 (adjustment by 1 N HCl and 1 N NaOH). For anaerobic conditions, and agitation speed of 200 rpm and aeration of 0 vvm (no-aeration) were maintained throughout the cultivation.

5. Immunoblot analysis

5.1 Preparation of protein

To detect inulinase secreted into medium from *S. cerevisiae* D452-2/p426PM on cultivation time, cell-free culture medium was taken at an interval of twenty four hours (0, 24, 48, 72 h). Fifty milliliters of the culture broth (YNB + 2% glucose) was taken from the flask, the culture was centrifuged at 5,000 x *g*, 4℃ for 10 min for separation cell precipitates and extracellular medium. The cell-free culture medium was concentrated to 50 μL through a Vivaspin 30,000 MWCO (GE Healthcare Life science, NJ, U.S.A). Fifty microliters of the concentrated medium was applied onto Sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and subjected to Western blot analysis.

To determine the glycosylation state of the KmINU, samples were treated by Endo H that cleaves the linkage between the innermost GlcNAc and asparagine residues of high mannose, hybrid and complex oligosaccharides from N-linked glycoproteins (Maley *et al.* 1989). Briefly, the KmINU sample was combined 1 μL of 10X Glycoprotein Denaturing Buffer and H_2O to make a 10 μL total reaction volume. Then the sample was denatured by heating reaction at 100℃ for 10 min. After heating, this solution was added 2 μL of 10X G5 Reaction Buffer, 1 ~ 5 μL Endo H and H_2O to make a total reaction volume of 20 μL. Finally, the reaction mixture was incubated at 37℃ for 1 h.

5.2 Western blotting

Concentrated medium samples were loaded on 12% SDS-PAGE. Following SDS-PAGE, the gel proteins were immune-blotted onto 0.45 μm polyvinylidene difluoride (PVDF) membranes (PALL, NY, U.S.A). Blots

were rinsed three times in 1X phosphate-buffered saline (PBS) buffer and blocked with 5% non-fat skim milk powder (Bio-Rad, CA, U.S.A) in PBS for 1.5 h at room temperature. Then, the blots were washed three times in PBS and probed with anti-*myc* mouse monoclonal antibody (IG Therapy Co., Kangwon, Korea) at a 1/1,000 dilution in PBS for 1 h at room temperature. The blots were further washed as above and the membranes reprobed with Anti-Mouse lgG (Fc specific) - Peroxidase, antibody produced in goat (A2554, Sigma, MO, U.S.A) at a 1/5,000 dilution in PBS for 40 min at room temperature. Washing was repeated further with an additional 2 x 5 min washes in PBS only. The blots were incubated in the detection reagents, WEST-ZOL (Intron biotechnology, Sungnam, Korea) for 2 min in dark room and then exposed to films in cassette for 30 sec ~ 10 min. The films were dipped in developer buffer for several minutes, stopped the reaction in stop buffer and dipped in fixer buffer sequentially.

6. Acid hydrolysis of inulin

Hydrolysis of inulin by acid has been deal with many researches. B. Pekic & B. Slavica (1985) carried out the hydrolysis of artichoke inulin at pH 2.0 and 0.5 h was needed for 95% hydrolysis of artichoke inulin at pH 2.0 (Pekic *et al.* 1985). Also, Pakvirun & Pornthap (2007) conducted the acid hydrolysis of inulin at pH 2.0 adjusted with sulfuric acid (H_2SO_4) and heated at 80℃ for 40 min. In this condition, they obtained highest reducing sugar content and ethanol yield (Thuesombat *et al.* 2007).

In this study, for acid hydrolysis, the pH of inulin solution was adjusted to 2.0 with H_2SO_4 and incubation was at 80℃ for 40 min ~ 1 h. For neutralization, the pH of inulin solution hydrolyzed by H_2SO_4 was adjusted to 5.0 by addition of sodium hydroxide (NaOH)

7. Analytical methods

7.1 Dry cell weight

Dry cell weight concentration was estimated by measuring absorbance at 600 nm by a spectrophotometer (UV-1601, Shimadzu, Kyoto, Japan). Optical density was converted into dry cell mass by using the following conversion equation.

$$\text{Dry cell weight (g/L)} = 0.30 \times OD_{600}$$

7.2 Sugar and ethanol concentration

Concentrations of inulin, fructose, glycerol, acetic acid and ethanol were measured by a high performance liquid chromatography (HPLC) (Agilent 1100LC, U.S.A) equipped with the Organic Acid column (Phenomenex, CA, U.S.A). The carbohydrate analysis ion exclusion column heated at 60℃ was applied to analyze the 20 μL of diluted culture broth. Detection was made with a reflective index detector at 35℃. HPLC operation conditions were set according to the instruction manual of the column supplier.

H_2SO_4 (5 mM) solution was used as mobile phase at a flow rate of 0.6 mL/min.

7.3 Measurement of enzyme activities

Preparation of crude recombinant inulinase extract

The cultures were harvested and centrifuged at 5,000 x *g* and 4℃ for 10 min to separate the cells from the media. The supernatant was

designated as the extracellular fraction. The cells were lysed by treatment Y-PER (Thermo scientific, IL, U.S.A) and vortexed. After centrifugation at 5,000 x *g* for 10 min, the supernatant was designated as the intracellular fraction and used for the measurement of cellular inulinase activity.

To measure the recombinant inulinase used *SUC2* signal sequence, periplasmic fraction has to be prepared. After the culture were centrifuged at 5,000 x *g*, and 4℃ for 10 min to separate the cells from the media at first time, the cells were treated with Lyticase from *Arthrobacter luteus* (L4025, Sigma, St. Louis, MO, U.S.A). The supernatant was designated as the periplasmic fraction (Figure 4).

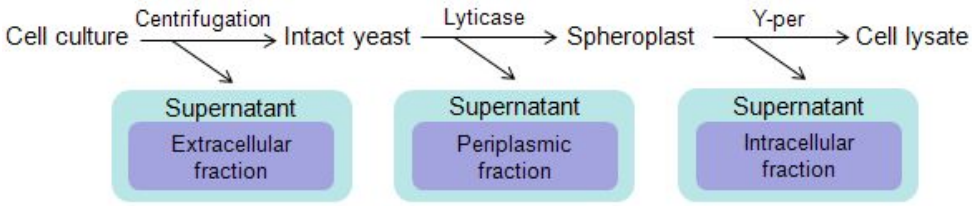

Figure 4. Process of preparation of crude inulinase extract

Determination of the recombinant inulinase activity

The extracellular, intracellular fractions and periplasmic fractions were incubated with 2% inulin in 0.1 M phosphate buffer (pH 6.0) at 60℃ for 10 min. The reaction was inactivated immediately by keeping the reaction mixture at 100℃ for 10 min. The amount of reducing sugar released was determined by the 3,5-dinitrosalicylic acid (DNS) method.

One unit of inulinase was defined as the amount of enzyme liberating 1 μmole of fructose equivalent from inulin per minute at 60℃

and specific activity means that sum of extracellular activity and intracellular activity per mg dry cell weight (U/mg dry cell weight). In case of recombinant *S. cerevisiae* containing the SUC2 signal sequence, specific activity means that sum of extracellular activity, intracellular activity and periplasmic activity per mg dry cell weight.

III. Results and discussions

1. Selection of promoter and signal sequence

1.1 Cloning of inulinase gene

Various microorganisms with inulinase activity have been known. Among them, the inulinase of *K. marxianus* has relatively high inulinase activity and it is the only microorganism whose information of signal sequences has been known. Therefore, the inulinase gene from *K. marxianus* (*KmINU*) was introduced into *S. cerevisiae* D452-2.

To increase the activity of KmINU, the expression system had to be optimized. To optimize the expression system, the optimal combination of promoter and signal sequence had to be investigated. Among the various promoters and signal sequences, *GPD, PGK1* and truncated *HXT7* promoter have been known as powerful promoters and *KmINU*, *MFα1* and *SUC2* signal sequence have been known as powerful signal sequences. Therefore, in this study, these promoters and signal sequences were used.

First, the *KmINU* gene encoding the inulinase was amplified from the genomic DNA of *K. marxianus* by PCR. Also, three types of signal sequence (*KmINU*, *MFα1* and *SUC2* signal sequence) were fused to the N-terminal of *KmINU* gene. *Myc* tag was fused to the C-terminal of *KmINU* gene because the expression of inulinase can be detected. These were called KmINU expression cassettes (various signal sequence - KmINU - *myc* tag) (Figure 5).

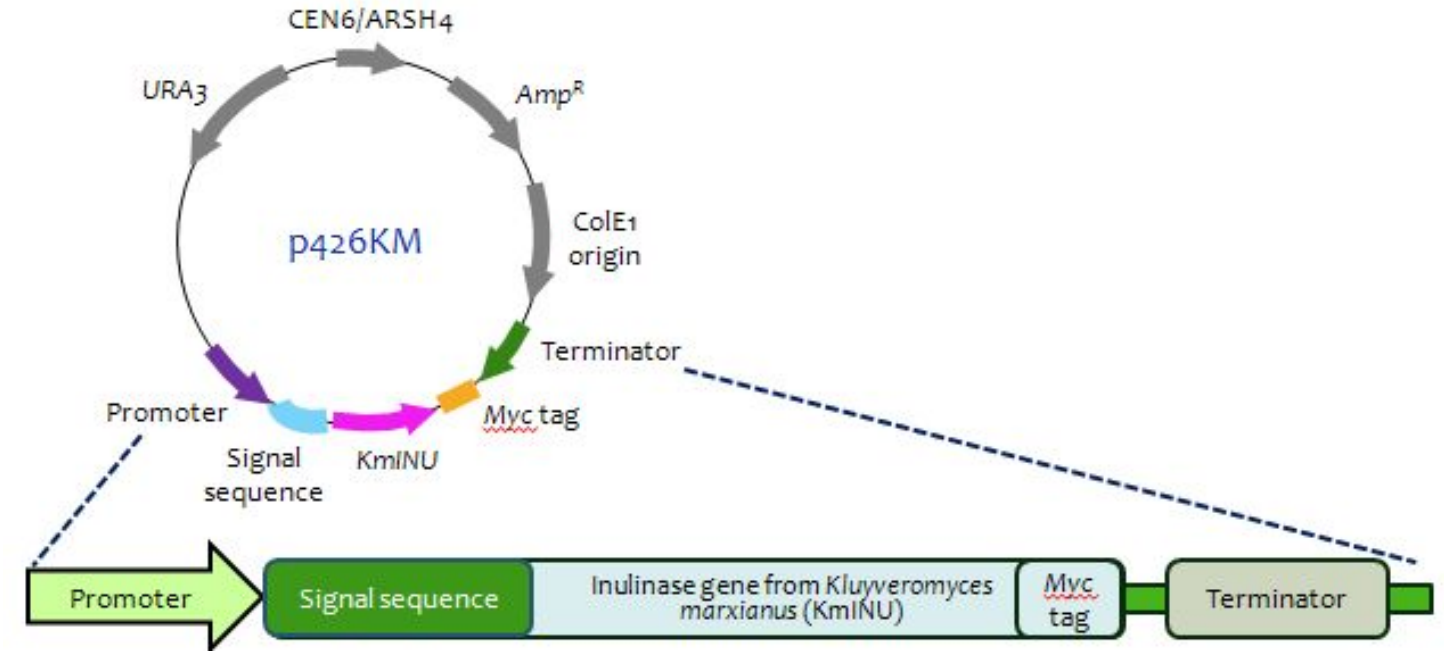

Figure 5. KmINU expression cassette

1.2 Construction of vectors carrying different promoter and signal sequence combinations

The amplified inulinase expression cassettes were inserted into the expression vectors p426GPD, p426PGK1, p426HXT7 containing the *GPD*, *PGK1* and truncated *HXT7* promoter respectively. The resulting plasmids were named p426GI (carrying the *GPD* promoter and the *KmINU* signal sequence), p426PI (carrying the *PGK1* promoter and the *KmINU* signal sequence), p426HI (carrying the truncated *HXT7* promoter and the *KmINU* signal sequence), p426GM (carrying the *GPD* promoter and the *MFα1* signal sequence), p426PM (carrying the *PGK1* promoter and the *MFα1* signal sequence), p426HM (carrying the truncated *HXT7* promoter and the *MFα1* signal sequence), p426GS (carrying the *GPD* promoter and the *SUC2* signal sequence), p426PS (carrying the *PGK1* promoter and the *SUC2* signal sequence), and p426HS (carrying the truncated *HXT7* promoter and the *SUC2* signal sequence) respectively (Figure 6).

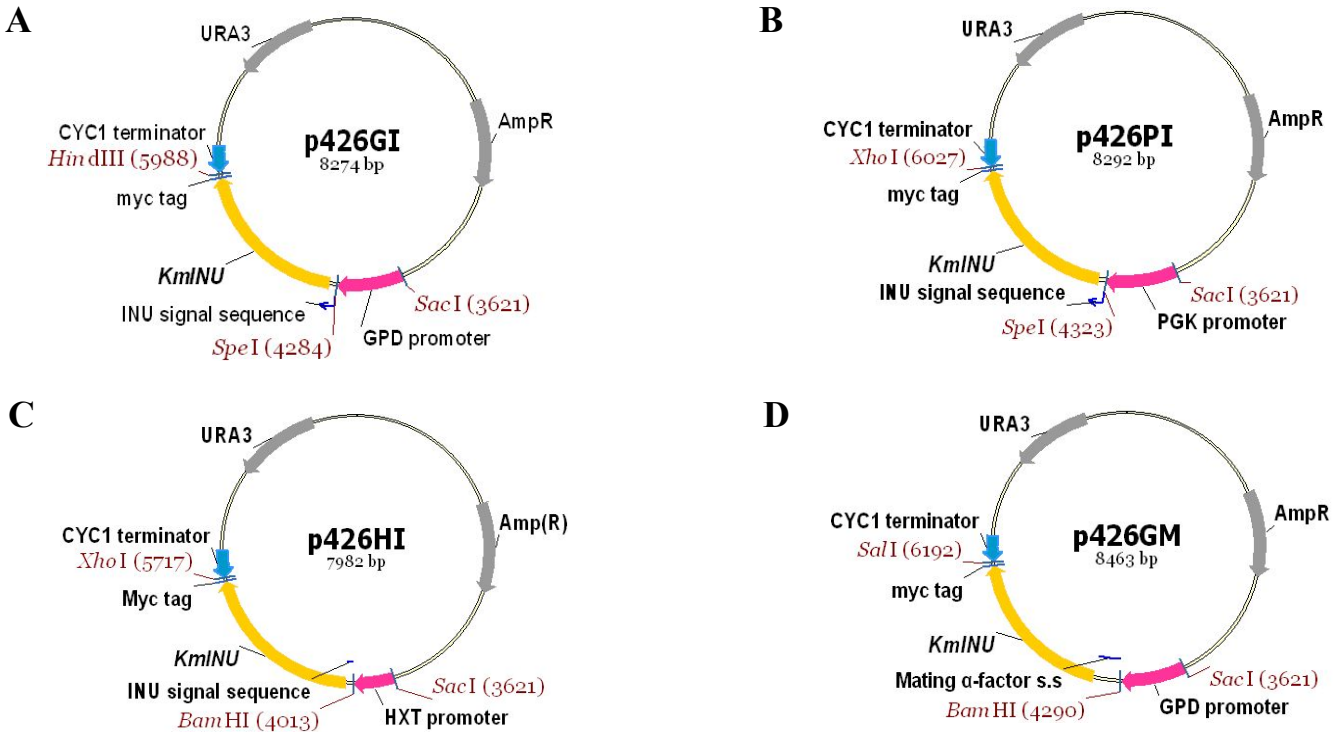

Figure 6. Genetic map of plasmids (A) p426GI, (B) p426PI, (C) p426HI, (D) p426GM, (E) p426PM, (F) p426HM, (G) p426GS, (H) p426PS and (I) p426HS (Continued)

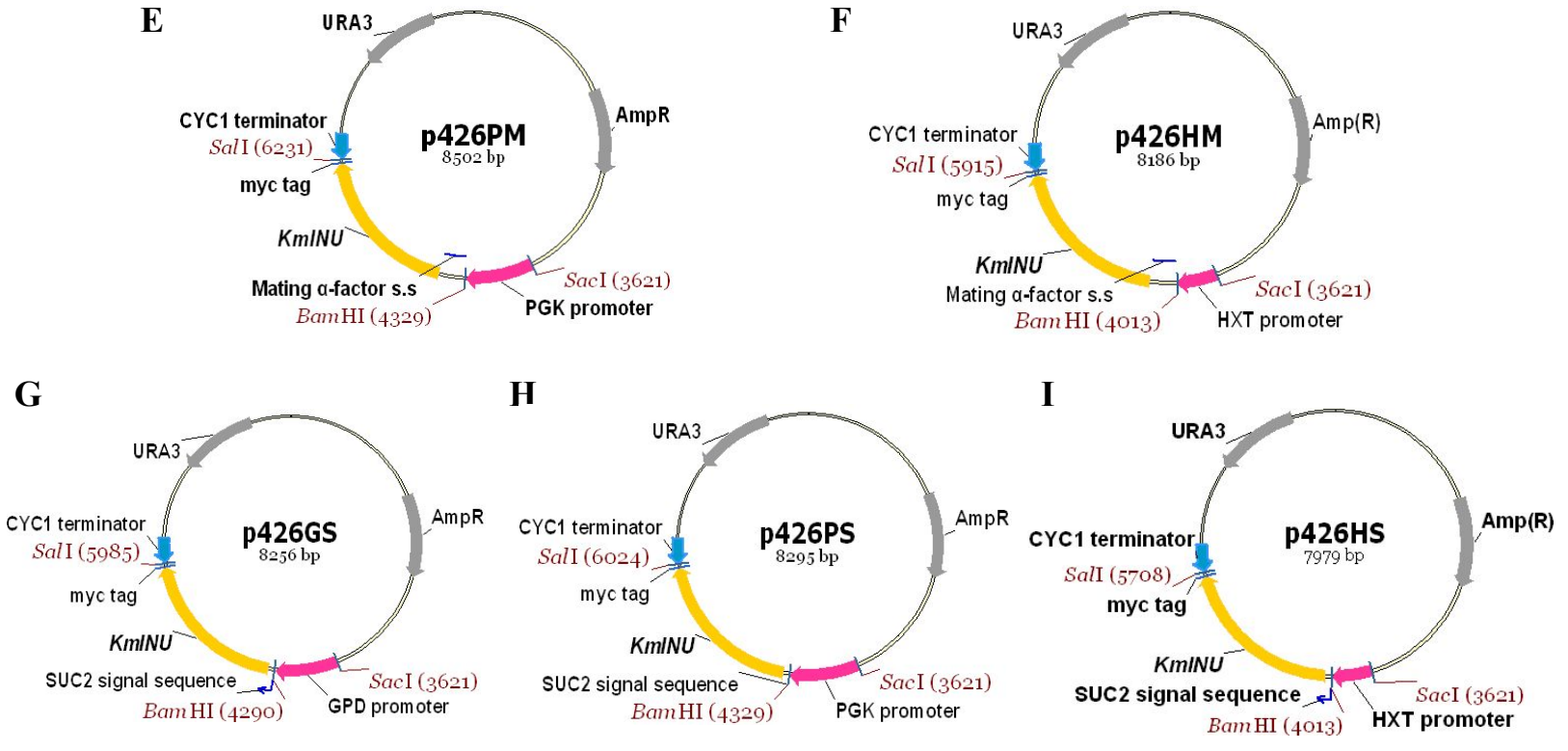

Figure 6. Genetic map of plasmids (A) p426GI, (B) p426PI, (C) p426HI, (D) p426GM, (E) p426PM, (F) p426HM, (G) p426GS, (H) p426PS and (I) p426HS

1.3 Comparison of inulinase activity

To test the activity of KmINU, plasmid p426GI, p426PI, p426HI, p426GM, p426PM, p426HM, p426GS, p426PS and p426HS was transformed into *S. cerevisiae* D452-2 respectively. Then, crude extract enzyme assays were performed.

Figure 7 show the specific activity of KmINUs obtained by overexpression in nine recombinant *S. cerevisiae*. *S. cerevisiae* D452-2/p426PM strain expressed the highest inulinase activity among nine recombinant *S. cerevisiae*. The results in Table 3 indicate that KmINUs activities were in the range of 0.09 ± 0.01 ~ 1.34 ± 0.06 U/mg dry cell weight and also show that KmINU activity from *S. cerevisiae* D452-2 harboring p426PM was the highest (1.34 ± 0.06 U/mg dry cell weight). This means that KmINU has the highest inulinase activity when KmINU is expressed by the *PGK1* promoter and secreted by the *MFα1* signal sequence. It can be observed some tendency that KmINU activities were generally high when using the *MFα1* signal sequence of expression cassettes, while these were generally low when using the *SUC2* signal sequence of expression cassettes generally. It might be more influenced by the type of signal sequence than the type of promoter.

It has been known that being cleaved of signal sequence is related to sequence of amino acids and whole structure of target protein. Although, the reason of the result that the specific activity of inulinase was the highest when KmINU was expressed by the *PGK1* promoter and secreted by the *MFα*1 signal sequence have not been identified, it seems that the *MFα1* signal sequence can be interacted effectively with the KmINU.

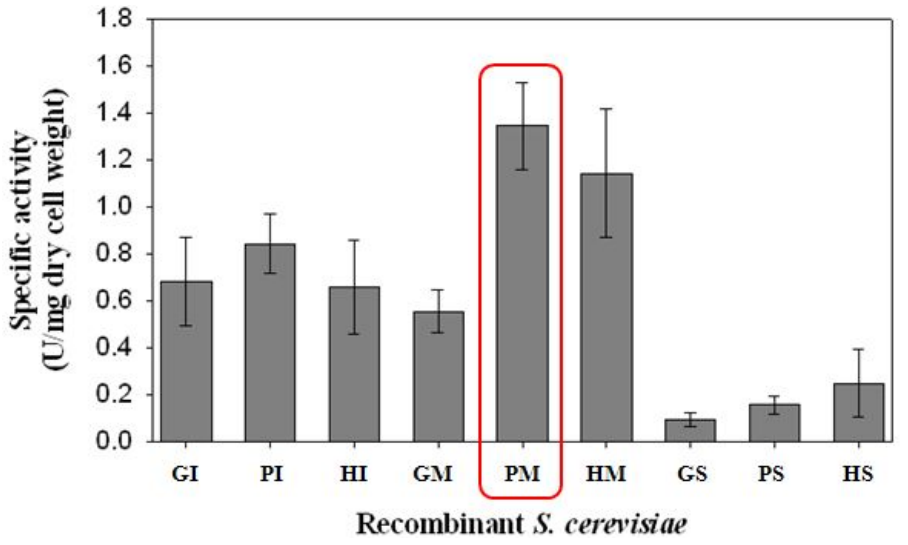

Figure 7. Specific activity of KmINUs obtained by overexpression in *S. cerevisiae* using different promoter and signal sequence combinations

Table 4. Specific activity of KmINUs from nine recombinant *S. cerevisiae*

Strain	Promoter	Signal sequence	Specific activity (U/mg dry cell weight)
S. cerevisiae D452-2/p426GI		*KmINU*	0.68 ± 0.07
S. cerevisiae D452-2/p426PI	*GPD*	*MFα1*	0.84 ± 0.04
S. cerevisiae D452-2/p426HI		*SUC2*	0.66 ± 0.07
S. cerevisiae D452-2/p426GM		*KmINU*	0.73 ± 0.18
S. cerevisiae D452-2/p426PM	*PGK1*	*MFα1*	1.34 ± 0.06
S. cerevisiae D452-2/p426HM		*SUC2*	1.14 ± 0.09
S. cerevisiae D452-2/p426GS		*KmINU*	0.09 ± 0.01
S. cerevisiae D452-2/p426PS	Truncated *HXT7*	*MFα1*	0.16 ± 0.02
S. cerevisiae D452-2/p426HS		*SUC2*	0.25 ± 0.06

1.4 Comparison of fermentation profile

To test the fermentation profile of constructed nine recombinant *S. cereivisae* with inulin, flask fermentation with 20 g/L inulin was performed. The results in Figure 8 and Table 4 show the fermentation properties of nine recombinant *S. cerevisiae*.

Fermentation properties of nine recombinant *S. cerevisiae* were similar. It was observed that inulin was degraded and fructose was created and accumulated in media at early time. This means that inulin was degraded to fructose by inulinases excreted from strains and fructose was accumulated in media because the inulin degradation rate of inulinases were faster than the fructose consumption rate of the strains. Finally, accumulated fructose was gradually consumed by the strains.

Also, the higher the specific activity of recombinant *S. cerevisiae* D452-2 was, the faster inulin consumption rate was and higher the value of ethanol yield and productivity also were. The inulin consumption rate was the fastest and maximum ethanol concentration, ethanol yield and productivity were the highest when *S. cerevisiae* D452-2 harboring p426PM which has the highest KmINU activity. Therefore, *S. cerevisiae* D452-2/p426PM not only has the highest KmINU activity, but also reveal the most outstanding fermentation properties.

Furthermore, *S. cerevisiae* D452-2/p426PM was used in the subsequent investigations.

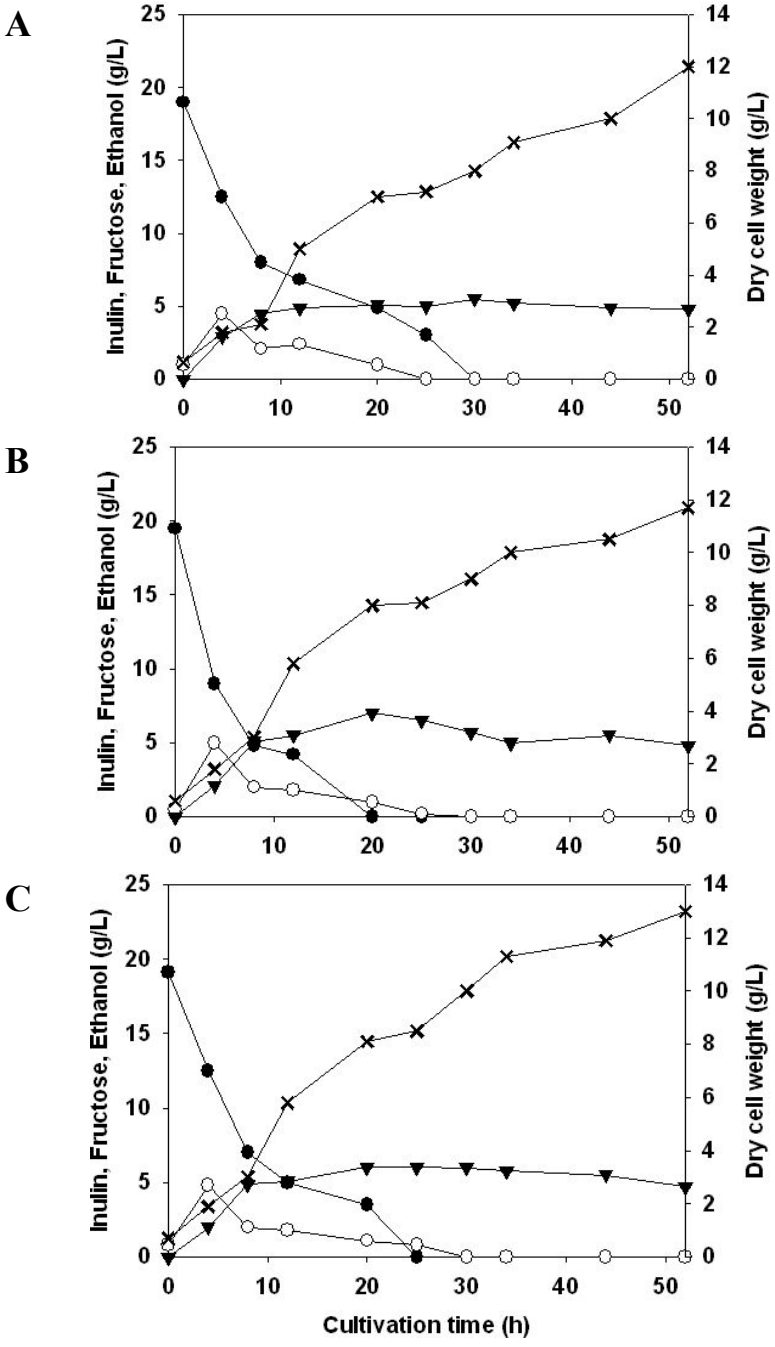

Figure 8. Flask fermentation profiles of (A) *S. cerevisiae* D452-2/p426GI, (B) D452-2/p426PI, (C) D452-2/p426HI, (D) D452-2/p426GM, (E) D452-2/p426PM, (F) D452-2/p426HM, (G) D452-2/p426GS, (H) D452-2/p426PS and (I) D452-2/p426HS (Continued)
(● : Inulin, ○ : Fructose, ▼ : Ethanol, × : Dry cell weight)

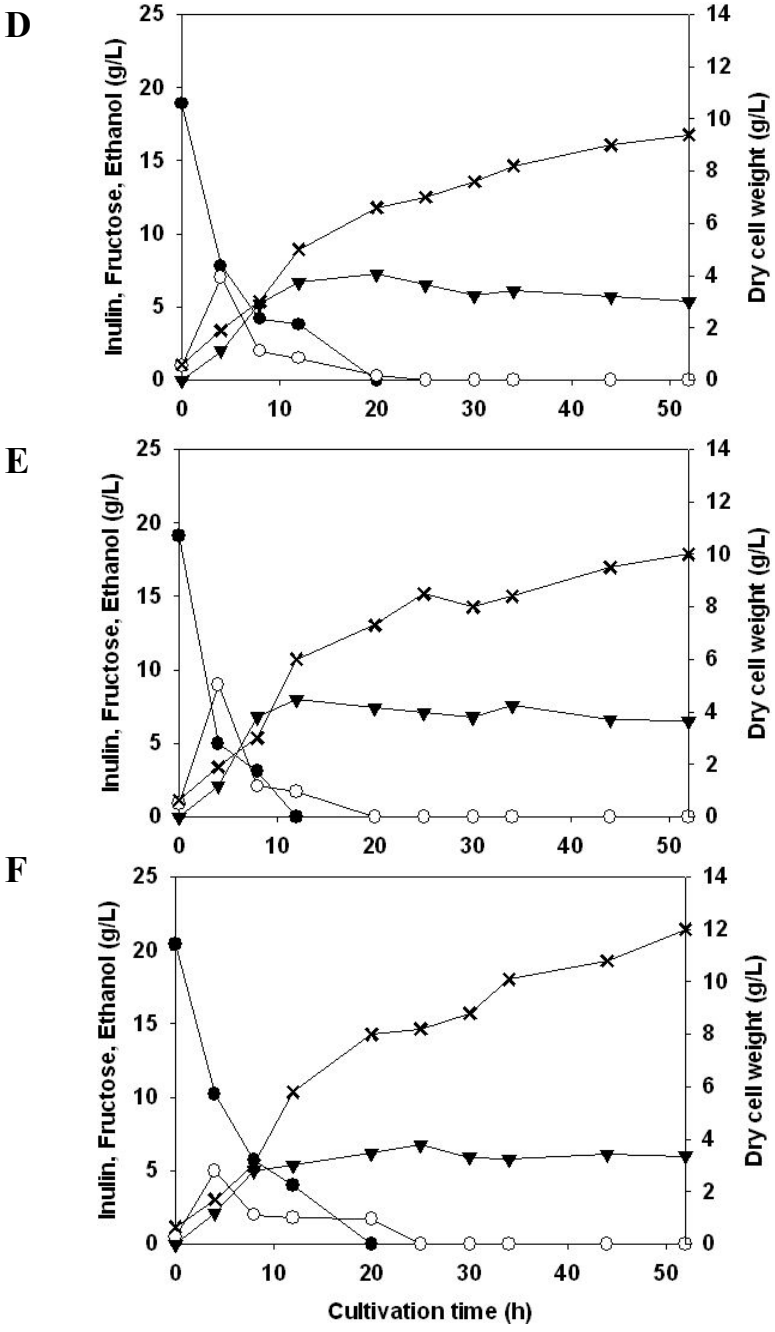

Figure 8. Flask fermentation profiles of (A) *S. cerevisiae* D452-2/p426GI, (B) D452-2/p426PI, (C) D452-2/p426HI, (D) D452-2/p426GM, (E) D452-2/p426PM, (F) D452-2/p426HM, (G) D452-2/p426GS, (H) D452-2/p426PS and (I) D452-2/p426HS (Continued)
(● : Inulin, ○ : Fructose, ▼ : Ethanol, × : Dry cell weight)

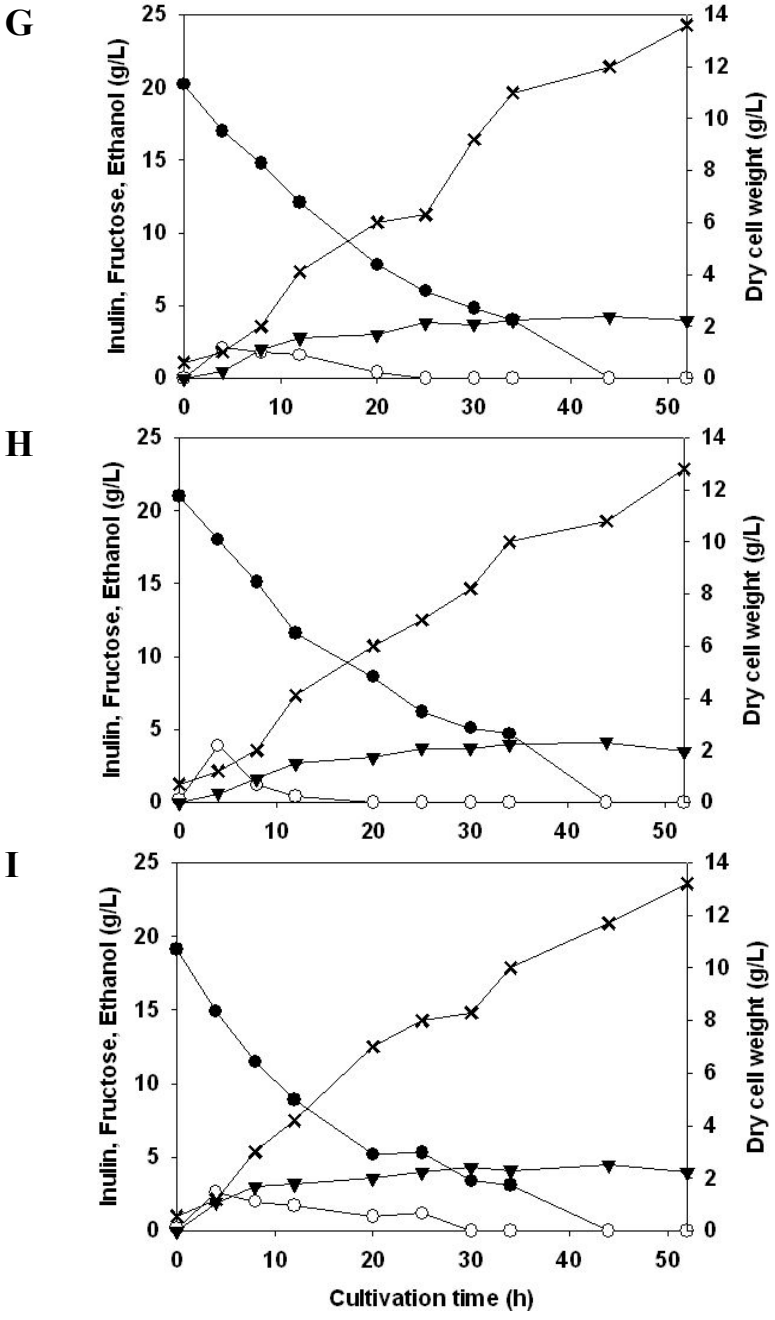

Figure 8. Flask fermentation profiles of (A) *S. cerevisiae* D452-2/p426GI, (B) D452-2/p426PI, (C) D452-2/p426HI, (D) D452-2/p426GM, (E) D452-2/p426PM, (F) D452-2/p426HM, (G) D452-2/p426GS, (H) D452-2/p426PS and (I) D452-2/p426HS

(●: Inulin, ○: Fructose, ▼: Ethanol, ×: Dry cell weight)

Table 5. Summary of flask fermentation of nine recombinant *S. cerevisiae*

Strain	Final Dry cell weight (g/L)	Specific activity (U/mg dry cell weight)	Inulin consumption rate (g/L·h)	Maximum ethanol concentration (g/L)	Ethanol yield (g/g inulin)	Ethanol productivity (g/L·h)
S. cerevisiae D452-2/p426GI	13.5	0.68 ± 0.07	0.67	5.86	0.29	0.29
S. cerevisiae D452-2/p426PI	14.6	0.84 ± 0.04	1.00	7.03	0.35	0.35
S. cerevisiae D452-2/p426HI	16.1	0.66 ± 0.07	0.80	6.02	0.32	0.18
S. cerevisiae D452-2/p426GM	11.3	0.73 ± 0.18	1.00	7.26	0.36	0.36
S. cerevisiae D452-2/p426PM	12.0	1.34 ± 0.06	1.67	8.00	0.40	0.67

(Continued)

Strain	Final Dry cell weight (g/L)	Specific activity (U/mg dry cell weight)	Inulin consumption rate (g/L·h)	Maximum ethanol concentration (g/L)	Ethanol yield (g/g inulin)	Ethanol productivity (g/L·h)
S. cerevisiae D452-2/p426HM	13.6	1.14 ± 0.09	1.00	6.76	0.34	0.27
S. cerevisiae D452-2/p426GS	16.2	0.09 ± 0.01	0.44	4.26	0.21	0.14
S. cerevisiae D452-2/p426PS	15.3	0.16 ± 0.02	0.44	4.12	0.21	0.14
S. cerevisiae D452-2/p426HS	16.8	0.25 ± 0.06	0.44	4.49	0.22	0.18

2. Expression of inulinase gene with cultivation time

2.1 Expression level of inulinase

To identify the expression of KmINU with cultivation time, inulinase in the supernatant produced by *S. cerevisiae* D452-2/p426PM was detected by immunoblot analysis. The cell-free culture medium was taken at an interval of twenty four hours and used for immunoblot analysis. After immunoblot analysis, the thickness of band was regarded as the amount of KmINU in medium.

The bands were thicker with cultivation time (Figure 9A). At 0 h, no band was detected and the bands were detected since 24 h. Then the band was the thickest at 72 h after cultivation. The result indicated that KmINU protein expressed from *S. cerevisiae* D452-2/p426PM accumulated with cultivation time, therefore, the protein concentration in the medium increased over time.

And immunoblot analysis of the culture supernatants revealed that there were protein bands around 90 kDa, which was about 30 kDa larger than the calculated molecular weight of the mature KmINU. When the sample was denatured and treated with Endo-H, the band which was about 30 kDa appeared (Figure 9B). These results suggested that the KmINU was likely glycosylated by the expression host and that the oligosaccharide chains could be removed.

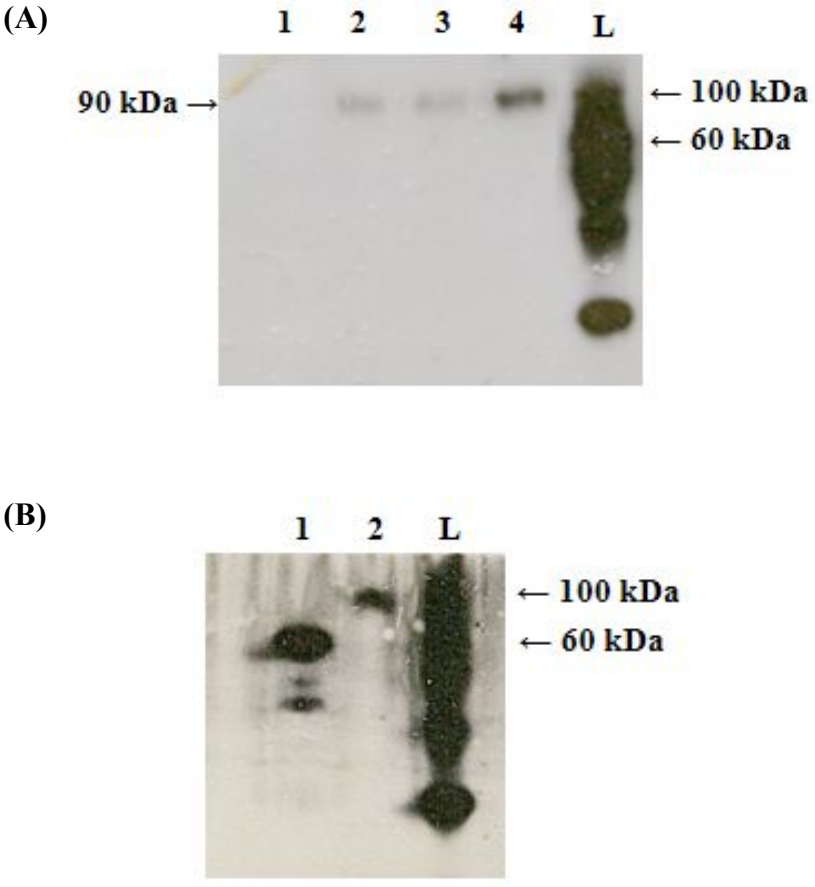

Figure 9. Immunoblot analysis of KmINU from *S. cerevisiae* D452-2/p426PM. (A) Expression of KmINU in the culture medium of *S. cerevisiae* D452-2/p426PM with cultivation time. Lanes 1, 2, 3, and 4 refer to samples after 0, 24, 48, 72 h, respectively. L refers to the protein marker. (B) Native and carbohydrate-depleted KmINU. Lane 1, Endo-H treated KmINU. The major band is the targeted protein. Lane 2, native KmINU. L refers to the protein marker.

2.2 Specific activity change of inulinase

During cell growth in the pre-cultured medium, KmINU from *S. cerevisiae* D452-2/p426PM specific activity in the extracellular and intracellular and cell mass were measured. Figure 10 showed that time profiles of specific activity of KmINU and cell mass of *S. cerevisiae* D452-2/p426PM. The specific activity of KmINU from *S. cerevisiae* D452-2/p426PM was increased with cultivation time and reached maximum value (1.34 U/mg dry cell weight) within 60 h after cell cultivation. Generally, in early time of cultivation, it can be observed that as *S. cerevisiae* D452-2/p426PM grew, the specific activity of KmINU increased. The specific activity of KmINU was constantly increased until 60 h after cultivation and since then, it decreased gradually.

To perform the high-capacity fermentation in bioreactor with inulin, pre-cultured medium which contain inulinase in medium excreted as cell grow is transferred to bioreactor. Therefore, to hydrolyze the inulin substrate in bioreactor rapidly, the inulinase activity in transferred pre-cultured medium has to be high.

The specific activity of KmINU from *S. cerevisiae* D452-2/p426PM was the highest at 60 h after cultivation. Therefore, it is effective to transfer the inocula after 60 h of the cell cultivation when the specific activity of KmINU is the highest.

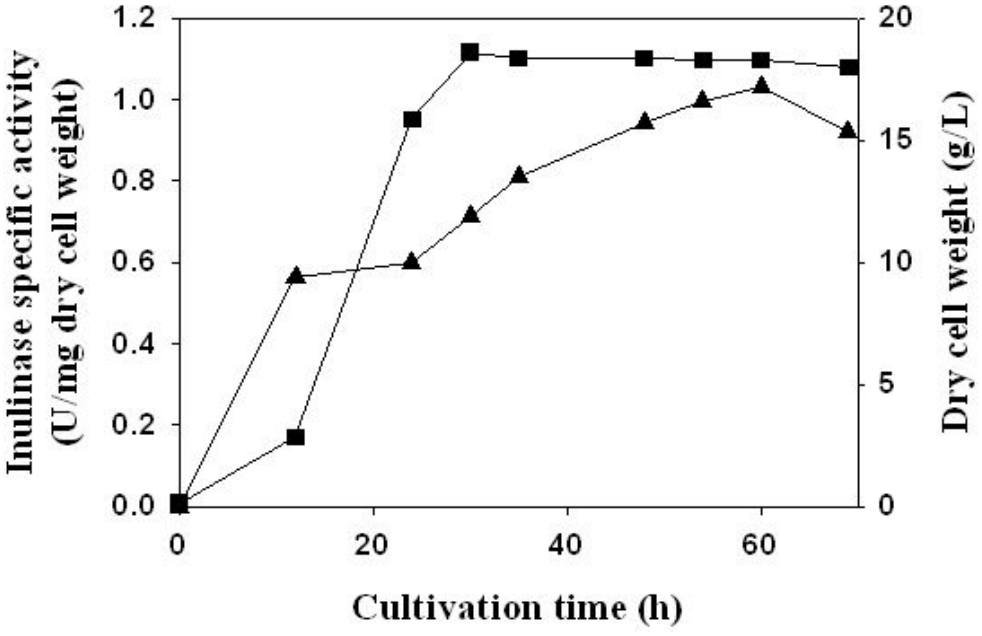

Figure 10. Specific activity change of KmINU from *S. cerevisiae* D452-2/p426PM with cultivation time

(▲ : Specific activity, ■ : Dry cell weight)

3. Comparison of hydrolysis type of inulin

As previously mentioned in part of introduction, hydrolysis of inulin using microorganism which has inulinase activity (SSF strategy) has many advantages over chemical hydrolysis of inulin. Therefore, to construct a recombinant strain expressed inulinase enzyme and to produce ethanol from inulin using the recombinant strain was research objective.

To compare the fermentation profiles according to hydrolysis type of inulin substrate, the experiment was performed under three conditions. 1) Fermentation using wild *S. cerevisiae* D452-2 with inulin substrate hydrolyzed by H_2SO_4, 2) Fermentation using wild *S. cerevisiae* D452-2 with inulin substrate neutralized by NaOH after hydrolyzed by H_2SO_4, 3) Fermentation using recombinant *S. cerevisiae* D452-2/p426PM with inulin substrate.

To make the inulin substrate using 2), 3) experiments, acid hydrolysis of inulin was performed. First, the conditions of acid hydrolysis had to be set. Using a lot of references about acid hydrolysis of inulin, the hydrolysis of inulin was carried out at pH 2.0 using H_2SO_4. Figure 11 show inulin peak in HPLC. At pH 2.0, 20 g/L inulin was mostly hydrolyzed to fructose and glucose within 40 min and 200 g/L inulin was mostly hydrolyzed to fructose and glucose within 1 h.

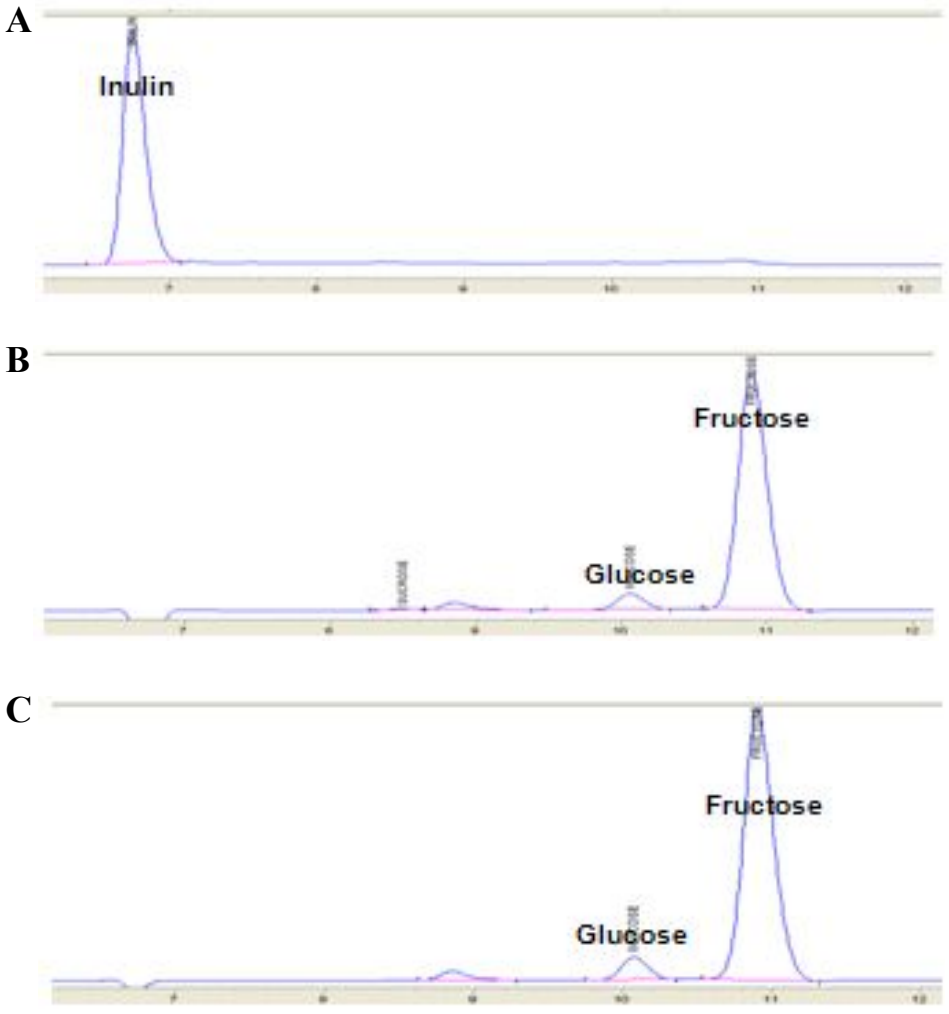

Figure 11. Inulin peak in HPLC. (A) 20 g/L inulin, (B) After acid hydrolysis of 20 g/L inulin at pH 2.0, 80℃ for 40 min, (C) After acid hydrolysis of 200 g/L inulin at pH 2.0, 80℃ for 1 h.

The experiments 1), 2) and 3) were performed under three conditions abovementioned using the prepared inulin substrate. The results in Figure 12 show that fermentation profiles according to hydrolysis type of inulin. Although the declined cell growth had been expected because of low pH in medium, the cell grew well when the inulin substrate hydrolyzed by H_2SO_4 was used to fermentation immediately. The reason for this may be neutralization of inulin substrate by medium because the entering volume of inulin substrate was 1/10 of the medium volume. However, as the occupying proportion of inulin substrate hydrolyzed by acid in medium is bigger, the neutralization step might be essential.

Table 5 shows comparison of the fermentation parameters such as final dry cell weight, maximum ethanol concentration, ethanol yield and ethanol productivity from inulin under three hydrolysis type. In case of using *S. cerevisiae* D452-2/p426PM, the final dry cell weight was the highest while the maximum ethanol concentration, ethanol yield and ethanol productivity were the lowest among three fermentation conditions. When using the inulin substrate hydrolyzed by H_2SO_4 and wild *S. cerevisiae* D452-2, the substrate consumption rate, maximum ethanol concentration, ethanol yield and ethanol productivity were higher than those of using *S. cerevisiae* D452-2/p426PM. The ethanol yield and productivity when using *S. cerevisiae* D452-2/p426PM was 82% and 86% of the ethanol yield and productivity when using inulin substrate hydrolyzed H_2SO_4 respectively.

Even though the ethanol yield and ethanol productivity may be increased by using acid hydrolysis of inulin, there are many disadvantages in acid hydrolysis of inulin. Above all things, three steps are needed (acid hydrolysis, neutralization, fermentation) until ethanol production in acid hydrolysis of inulin. However, *S. cerevisiae* D452-2/p426PM can hydrolyze

inulin and produce ethanol simultaneously (SSF strategy, only one step will be needed). In other words, the cost and complexity can be reduced by using this recombinant strain. Therefore, in aspects of the cost and efficiency, using the recombinant strain is more competitive in ethanol production industry.

A

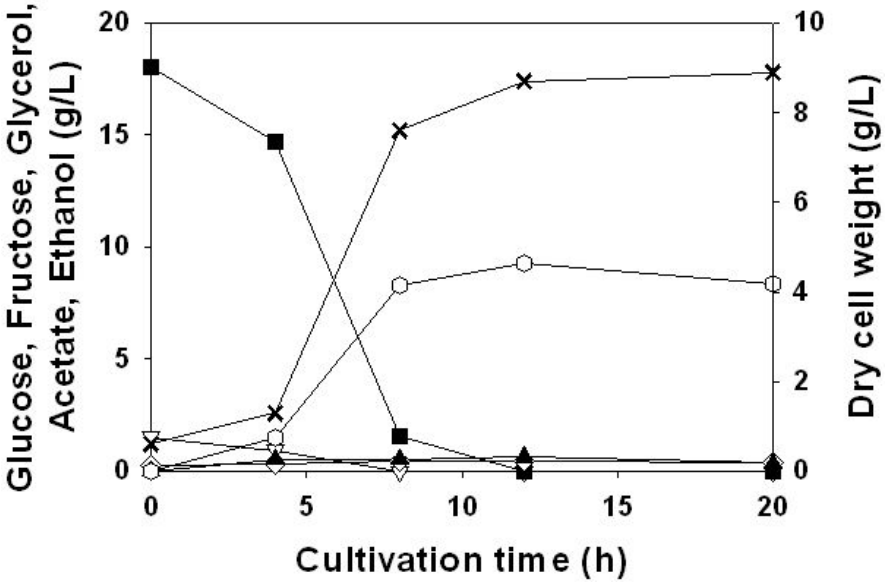

Figure 12. Fermentation profiles according to hydrolysis type of inulin (A) Fermentation using wild *S. cerevisiae* D452-2 with inulin substrate hydrolyzed by H_2SO_4, (B) Fermentation using wild *S. cerevisiae* D452-2 with inulin substrate neutralized by NaOH after hydrolyzed by H_2SO_4, (C) Fermentation using *S. cerevisiae* D452-2/p426PM with inulin substrate

(● : Inulin, ▽ : Glucose, ■ : Fructose, ◇ : Glycerol, ▲ : Acetate, ○ : Ethanol, ✕ : Dry cell weight) (Continued)

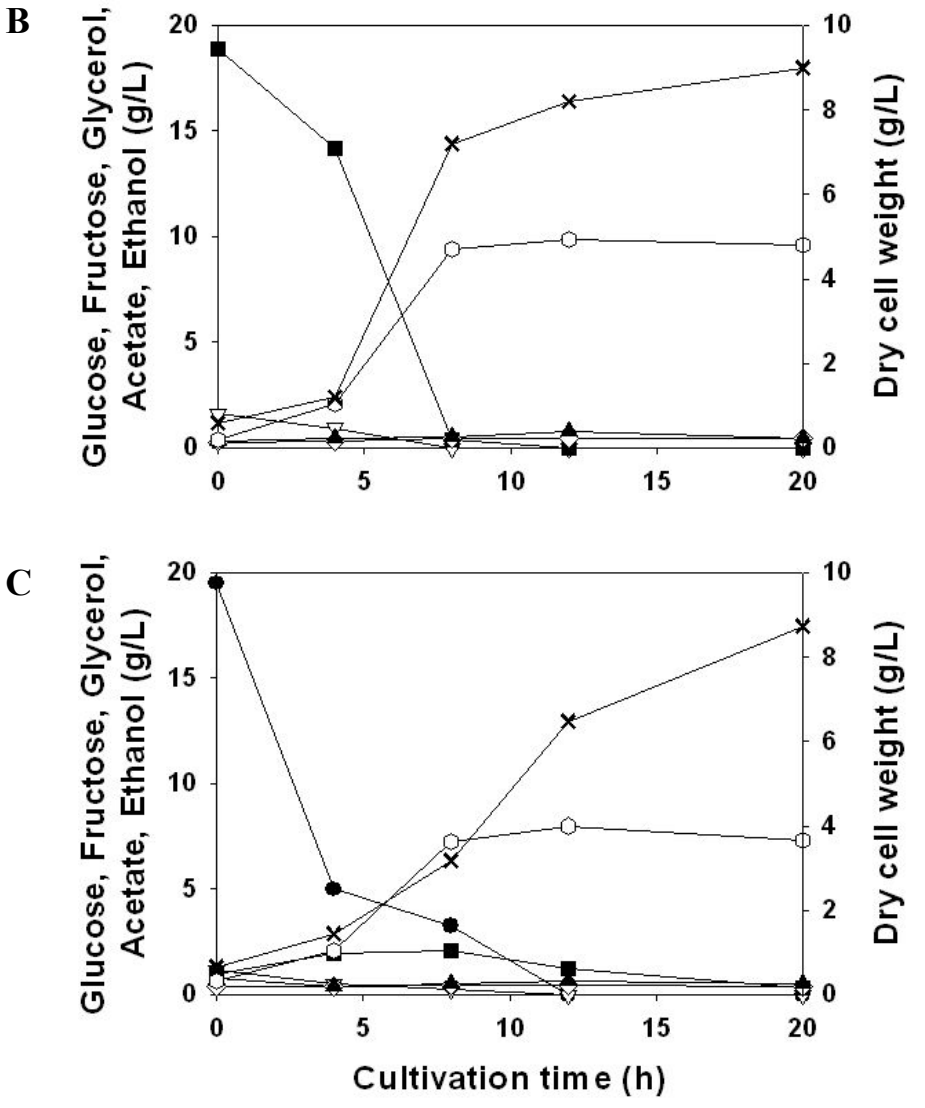

Figure 12. Fermentation profiles according to hydrolysis type of inulin (A) Fermentation using wild *S. cerevisiae* D452-2 with inulin substrate hydrolyzed by H_2SO_4, (B) Fermentation using wild *S. cerevisiae* D452-2 with inulin substrate neutralized by NaOH after hydrolyzed by H_2SO_4, (C) Fermentation using *S. cerevisiae* D452-2/p426PM with inulin substrate

(● : Inulin, ▽ : Glucose, ■ : Fructose, ◇ : Glycerol, ▲ : Acetate, ○ : Ethanol, ✕ : Dry cell weight)

Table 6. Summary of fermentation according to hydrolysis type of inulin

Condition	Final Dry cell weight (g/L)	Substrate consumption rate (g/L·h)	Maximum ethanol concentration (g/L)	Ethanol yield (g/g inulin)	Ethanol productivity (g/L·h)
Acid hydrolysis	8.90	1.50	9.30	0.47	0.78
Acid hydrolysis → Neutralization	9.00	1.57	9.89	0.48	0.82
Recombinant strain	8.73	0.97	8.00	0.41	0.67

4. Batch fermentation

In bioethanol production industry, high-capacity fermentation with high concentration of substrate is carried out. Therefore, ethanol production from inulin also has to be able to ferment with high concentration of substrate for applying industry. To demonstrate it, batch fermentation in bioreactor with 200 g/L substrate was performed using wild *S. cerevisiae* D452-2 and *S. cerevisiae* D452-2/p426PM. In bioreactor level, 200 g/L inulin and fructose was used as substrate.

Before the fermentation with 200 g/L substrate in bioreactor was performed, flask fermentation with 200 g/L inulin using *S. cerevisiae* D452-2/p426PM was performed (Figure 13). As a result, *S. cerevisiae* D452-2/p426PM produced 88.50 g/L ethanol in 72 h when the cell growth reached 16.04 g/L dry cell weight and ethanol yield was 0.46 g ethanol/g inulin and ethanol productivity was 1.48 g/L·h (Table 6).

The fermentation profiles in bioreactor are represented in Figure 14. A wild *S. cerevisiae* D452-2 consumed 200 g/L fructose within 60 h, on the other hand, *S. cerevisiae* D452-2/p426PM degraded 200 g/L inulin within 48 h, consumed fructose produced from inulin within 72 h. It can be observed that although inulin was degraded by inulinase from *S. cerevisiae* D452-2/p426PM within 48 h after cultivation, produced fructose from inulin was not consumed directly by *S. cerevisiae* D452-2/p426PM and accumulated in medium. So, *S. cerevisiae* D452-2/p426PM consumed accumulated fructose within 72 h and the ethanol concentration increased until 84 h. Thus, the ethanol productivity of fermentation using inulin and recombinant strain was lower than that of using fructose and wild strain.

The fermentation parameters were summarized in Table 6. Wild *S. cerevisiae* D452-2 produced 84.22 g/L ethanol from 200 g/L fructose and resulted in 0.49 g ethanol/g inulin of ethanol yield, 1.24 g/L·h of ethanol

productivity and 3.29 g/L·h of substrate consumption rate. By comparison, *S. cerevisiae* D452-2/p426PM finally produced 85.41 g/L ethanol from 200 g/L inulin and resulted in 0.47 g ethanol/g inulin of ethanol yield, 1.02 g/L·h of ethanol productivity and 2.66 g/L·h of substrate consumption rate. It can be observed that ethanol yields in flask and bioreactor level were similar (0.46 g ethanol/g inulin in flask culture and 0.47 g ethanol/g inulin in bioreactor culture). However, the final dry cell weight of *S. cerevisiae* D452-2/p426PM in bioreactor was less than half that of flask culture. Therefore, *S. cerevisiae* D452-2/p426PM in bioreactor more produced the ethanol per gram cell (g ethanol/g cell) than that of *S. cerevisiae* D452-2/p426PM in flask. Also, the ethanol yield when using *S. cerevisiae* D452-2/p426PM was 96% of the ethanol yield when using fructose substrate and these yields were not significantly different. Thus, ethanol fermentation using high concentration inulin by recombinant *S. cerevisiae* D452-2 is competitive for applying to industry.

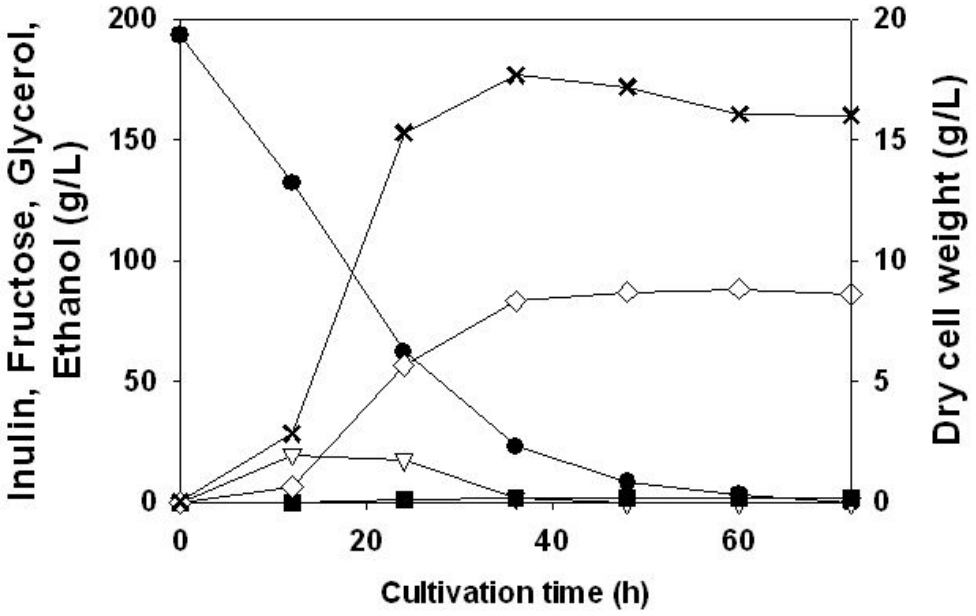

Figure 13. Flask fermentation profile of *S. cerevisiae* D452-2/p426PM with 200 g/L inulin

(● : Inulin, ▽ : Fructose, ■ : Glycerol, ◇ : Ethanol, ✕ : Dry cell weight)

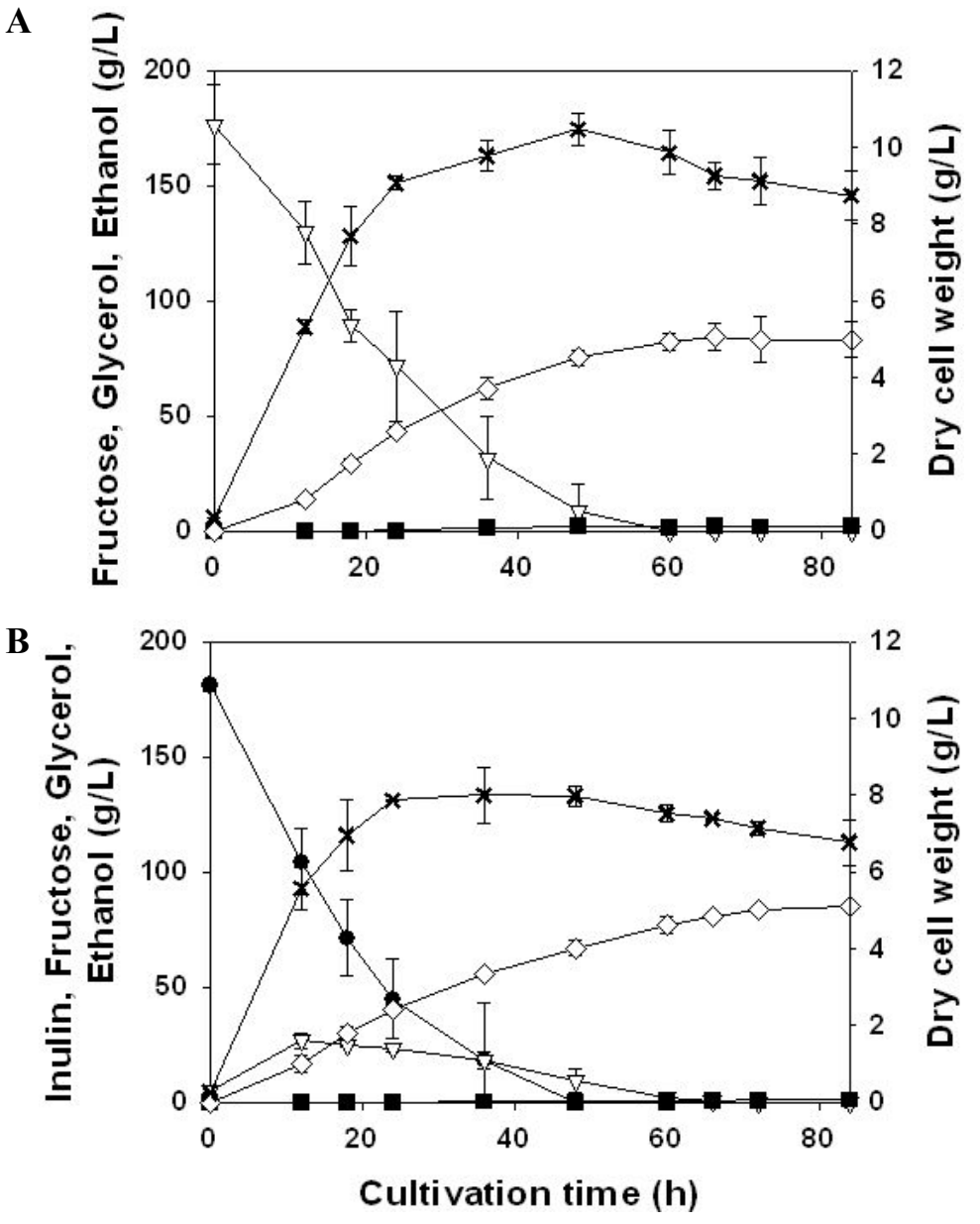

Figure 14. Bioreactor fermentation profiles of (A) wild *S. cerevisiae* D452-2 with 200 g/L fructose and (B) *S. cerevisiae* D452-2/p426PM with 200 g/L inulin

(● : Inulin, ▽ : Fructose, ■ : Glycerol, ◇ : Ethanol, ✕ : Dry cell weight

Table 7. Summary of batch fermentations

Condition	substrate	Final Dry cell weight (g/L)	Substrate consumption rate (g/L·h)	Maximum ethanol concentration (g/L)	Ethanol yield (g ethanol/ g substrate)	Ethanol productivity (g/L·h)
Flask culture	200 g/L Inulin	16.04 (72 h)	2.69	88.50	0.46	1.48
Bioreactor culture	200 g/L Fructose	9.26 (72 h)	3.29	84.22	0.49	1.24
	200 g/L Inulin	6.78 (84 h)	2.66	85.41	0.47	1.02

IV. Conclusions

This thesis can draw the following conclusions :

(1) The inulinase activity in *S. cerevisiae* carrying the *PGK1* promoter and the *MFα1* signal sequence was 1.34 ± 0.06 per dry cell weight.

(2) The simultaneous hydrolysis and fermentation (SSF) process using inulin could be achieved by the *S. cerevisiae* strain with the inulinase gene from *K. marxianus*.

(3) A batch fermentation of the recombinant *S. cerevisiae* in a bioreactor was performed with 200 g/L inulin at 30℃, pH 5.0, agitation speed of 200 rpm and aeration of 0 vvm (no-aeration) and resulted in 0.47 g ethanol/g inulin of ethanol yield and 1.02 g/L·h of ethanol productivity.

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국 문 초 록

최근 자원의 고갈과 환경 오염 문제를 극복하기 위하여 재생 가능한 바이오 매스로부터 바이오 연료를 생산하려는 노력이 행해지고 있다. 가장 대표적인 바이오 연료는 바이오 에탄올이며 이는 옥탄가가 높고 독성이 없으며, 수질을 오염시키지 않는 등 많은 장점을 지닌다. 이눌린은 Jerusalem artichoke, chicory와 같은 작물에 존재하는 고분자 물질 ($C_{6n}H_{10n+2}O_{5n+1}$)로, 과당 분자가 β-2,1 결합으로 연결되어 있고 말단에 포도당 한 분자가 결합되어 있는 형태이다. 따라서 이눌리네이즈라는 효소에 의해 분해되어 미생물이 쉽게 소모할 수 있는 여러 분자의 과당으로 전환되므로 과당시럽 생산이나 에탄올 발효를 위한 재생가능 자원으로 주목 받고 있다. 한편 *Saccharomyces cerevisiae*는 뛰어난 에탄올 생성능 및 내성 등으로 바이오 에탄올을 생성하는 데 가장 많이 쓰이는 산업용 미생물 중 하나이다. 하지만 이눌리네이즈를 거의 분비하지 않아 이눌린을 기질로 하여 에탄올을 생성할 수 없다.

본 연구에서는 대사공학 기법을 통해 외래의 이눌리네이즈 유전자를 *S. cerevisiae* 내에 도입시켜 이눌린 대사 경로가 생성된 재조합 균주를 구축하고 이를 이용하여 이눌린으로부터 에탄올을 생성하는 것을 목표로 하였다. 먼저 이눌리네이즈 역가가 비교적 높고 신호서열 정보가 알려져 있는 *Kluyveromyces marxianus* 유래의 이눌리네이즈 유전자를 도입하기로 하였고, 재조합 효소를 분비하는 최적의 발현 시스템을 선별하고자 발현 카세트 중 프로모터 3종 (*GPD*, *PGK1*, truncated *HXT7*)과 신호

서열 3종 (*KmINU*, *MFα1*, *SUC2*)을 교체하여 보았다. 각 프로모터, 신호서열 조합을 가지는 9개의 재조합 플라스미드를 구축하였고, 각 플라스미드로 형질전환된 9종의 재조합 *S. cerevisiae* 중 단일 세포 당 가장 큰 역가를 갖는 최적의 프로모터, 신호서열 조합을 선별하였다. 더블어 플라스크 발효를 통하여서 선별된 균주가 9개 균주 중 기질 소모 속도, 에탄올 수율, 생산성 등 발효 능이 가장 뛰어나다는 것을 확인하였다. 배양 시간이 지날수록 재조합 이눌리네이즈가 생성되어 배지에 축적되었고, 0시간부터 60시간까지 활성이 점차 증가한다는 것을 실험적으로 확인하였다. 또한 이눌린을 산으로 가수분해시킨 후 야생형 균주를 이용한 에탄올 발효와 재조합 균주를 이용한 에탄올 발효를 비교하여 보았다. 이눌린을 산으로 가수분해하여 에탄올을 생성하기까지 3단계 (산 가수분해, 중화, 발효)를 거쳐야 하는 화학적 처리의 비용 및 단계의 복잡성을 고려해볼 때, 재조합 균주를 이용하는 것이 충분히 산업적으로 경쟁성이 있다고 사료된다. 선별된 균주로 회분식 배양을 통하여 200 g/L의 고농도 이눌린으로부터 에탄올을 생성한 결과, 에탄올 수율은 0.47 g 에탄올/g 이눌린, 에탄올 생산성은 1.02 g/L·h 이었다.

주요어 : 에탄올, 이눌린, Jerusalem artichoke, 이눌리네이즈, 효모, 대사공학

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감사의 글

먼저 지금까지 지켜주신 주님께 감사합니다. 생물분자공학실에서 보낸 2년은 잊을 수 없는 정말 값진 경험이 되었고, 인생의 중요한 교훈들을 배울 수 있었던 시간이었습니다. 부족한 저를 제자로 받아 주셔서 연구뿐만 아니라 모든 부분에서 가르침을 주신 선생님께 진심으로 감사 드리고, 부끄럽지 않은 제자가 되도록 더욱 힘쓰겠습니다.

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럼 열심히 해서 승승장구 하자. 뿐만 아니라 식공과 동기들. 우리가 야식으로 먹은 치킨만 해도 양계장 하나 차리겠어. 정말 정말 즐거웠고 앞으로도 평생 연락하며 좋은 동료가 되자. 특히 SRC에서 항상 챙겨주고 큰 힘이 되어 준 혜림언니, 수진언니. 정말 고맙고, 얼마 안 됐는데 벌써 보고 싶네.

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