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A Thesis for the Degree of Master of Science

CRISPR-Cas9 based Metabolic Engineering of Industrial *Saccharomyces cerevisiae* for Acrylamide Reduction

아크릴아마이드 저감화를 위한
산업용 ***Saccharomyces cerevisiae*** 의
CRISPR-Cas9 기반 대사공학

By

Byung Yeon Kim

Department of Agricultural Biotechnology
Seoul National University
February 2018

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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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指導敎授 徐鎭浩

이論文을 農學碩士學位論文으로 提出함
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2018年 **2**月

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ABSTRACT

Acrylamide, known as a toxic substance and potential carcinogen, is present in large amounts in foods including potato chips and breads. Acrylamide is produced from asparagine in raw food materials through the Maillard reaction during the cooking process. Therefore, reduction of asparagine is the key of the acrylamide reduction.

The objective of this thesis is to reduce acrylamide in foods by degrading asparagine by metabolically engineered *S. cerevisiae*. A recently developed CRISPR-Cas9 system was employed to engineer industrial *S. cerevisiae* since the CRISPR-Cas9 genome editing technology would ensure safety for food applications.

First, to isolate industrial *S. cerevisiae* to be used for acrylamide reduction, 7 stains of *S. cerevisiae* were isolated from nuruk which is a Korean traditional microbial resource. Among 7 strains of *S. cerevisiae*, *S. cerevisiae* N1 was selected as the host strain for further studies because *S. cerevisiae* N1 showed the best glucose fermentation ability. Also *S. cerevisiae* N1 was expected to be used efficiently under acidic pH conditions or osmotic stress conditions as compared with the other isolated *S. cerevisiae* strains.

Next, metabolic engineering was performed for reducing acrylamide using the CRISPR-Cas9 gene editing technology. To prove the rationality of metabolic engineering strategies for acrylamide reduction, a laboratory strain *S. cerevisiae* D452-2 was engineered prior to industrial *S. cerevisiae* N1. *S. cerevisiae* D452-2 was engineered by overexpression of the general amino acid permease (*GAP1*), overexpression of asparaginases (*ASP1*, *ASP3*), regulation of nitrogen catabolite repression (NCR). Engineered strains were grown in flasks containing asparagine rich YPD media to test the ability of asparagine degradation. For the laboratory *S. cerevisiae* D_P_{ASP3} strain overexpressing *ASP3* (cell-wall asparaginase) through a promoter replacement, the asparagine consumption rate was enhanced by 9.9 times relative to the D452-2 WT strain. Therefore, the *ASP3* overexpression strategy was applied to the industrial N1 strain. Since *APS3* did not exist in N1, an *ASP3* expression cassette was constructed and inserted into either the *URE2* site or the *GZF3* site of the N1 chromosome to construct N1_ΔURE2::ASP3 and N1_ΔGZF3::ASP3. To determine the asparagine consumption ability of the two strains, engineered strains were grown in flasks containing asparagine rich YPD media. For the *S. cerevisiae* N1_ΔURE2::ASP3, the asparagine

consumption rate increased by 20.6 times, compared with the N1 WT strain. For the *S. cerevisiae* N1_ΔGZF3::ASP3, the asparagine consumption rate increased by 11.4 times, compared with the N1 WT strain. When they were applied to food doughs, asparagine was consumed fast to result in 95% less acrylamide than the control strain N1 WT.

In this study, metabolically engineered industrial *S. cerevisiae* strains were constructed to reduce acrylamide in foods by degrading asparagine. The engineered strains reduced acrylamide in foods by 95%, and it is believe that these engineered strains could be used effectively for acrylamide reduction in food industry.

Keywords: Industrial *Saccharomyces cerevisiae*, food industry, metabolic engineering, CRISPR-Cas9, flask fermentation, asparagine, acrylamide reduction, cell-wall asparaginase (*ASP3*)

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I. INTRODUCTION

1. Metabolic engineering of industrial *Saccharomyces cerevisiae* based on CRISPR-Cas9

1.1. Necessity and difficulty of metabolic engineering of industrial *S. cerevisiae*

Saccharomyces cerevisiae is the core microorganism in fermentation of foods. During the fermentation with *S. cerevisiae*, raw materials of the foods were converted into new substances according to the metabolism of *S. cerevisiae* (Walker G., Stewart, 2016). This means the characteristics of fermented foods such as food quality, nutrition value, taste and flavor, strongly depend on metabolites of *S. cerevisiae.*

By the *S. cerevisiae*, varieties of organic acids, vitamins, minerals, antioxidants that were known as beneficial metabolites to human are produced (Adams, 1990; Steinkraus, 1998). But the problem is that harmful substances, such as biogenic amine, acetaldehyde, ethyl carbamate and formaldehyde, are also produced as metabolites. (AnnaHalasz. et al. 1994; Weber, Sharypov, 2009; Jayakody LN. et al., 2016).

If making *S. cerevisiae* to enhance the production of good substances

and to inhibit the production of harmful substances by using metabolic engineering, fermented foods can be easily improved with good flavor, high nutritional values and health functionalities (Guo, X et al., 2015, Jayakody LN. et al., 2016).

In spite of the necessity of metabolic engineering of industrial *S. cerevisiae*, however, application of metabolic engineered strains for food fermentation is limited and remained elementary level because of next two reasons.

The first is the absence of adequate metabolic engineering techniques while ensuring food safety. In case of plasmid-based metabolic engineering, the addition of toxic antibiotics or maintenance of auxotroph marker deficient condition is essential for the stability of the transformed plasmid (Rinji akada, 2002). But, the use of antibiotics is limited in the food industry and certain nutrients are not deficient in the food material. Therefore, integration-based metabolic engineering is essential for food industry application. However, when using the conventional genome engineering methods, there is a problem in terms of safety. This is because undesired DNA fragments such as multi-cloning sites, the origin of replication and scar are left. In the food industry, the use of antibiotic markers or presence of undesired DNA

fragments might be labeled *S. cerevisiae* as a genetically modified organism (GMO). And this will make them be avoided from producers and consumers (Jayakody LN. et al., 2016).

The second is difficulty of genome engineering due to increased ploidy (Ikushima S. et al., 2009). Most gene editing tools for *S. cerevisiae* have been developed based on nutritional requirement mutations and selectable marker systems. These systems are highly effective only for haploid *S. cerevisiae* strains, not for polyploid *S. cerevisiae* strains (Rinji A., 2002). Unfortunately, most of *S. cerevisiae* existed as polyploid in nature and industry. Polyploid strains are preferred for industrial applications because they have beneficial traits like rapid sugar fermentation and higher tolerance facing stressful conditions such as nutrient depletion, heat, osmotic stress, and oxidative stress (Anli G. et al., 2016).

1.2. CRISPR-Cas9 system

CRISPR (Clustered regularly interspaced short palindromic repeats)-Cas9 (CRISPR associated protein 9) was first discovered as an adaptive immune defense system against foreign nucleic acid infections in bacteria and archaea (Marraffini et al., 2010). Since then, the possibility

of CRISPR-Cas9 system has been raised as a tool for genome engineering, and has been made remarkable progress. Recently, CRISPR-Cas9 system has emerged as a powerful genome engineering tool and anticipated to solve the two difficulties described above in 1.2 (Cong et al., 2013; DiCarlo et al., 2013; Mali et al., 2013).

The use of CRISPR-Cas9 can induce deletion, introduction and overexpression of a desired gene without introduction of any undesired DNA fragments. If the plasmid is cured out appropriately after being used for the desired change, CRISPR–Cas9-based genome engineering can make scar-less and marker-free *S. cerevisiae* (Zhang GC. et al., 2014).

2. Acrylamide in foods

As a main target of the food industry in which an engineered strain could be effectively used, acrylamide reduction was chosen.

2.1. What is acrylamide?

Acrylamide is an odorless white crystal, monomeric chemical used in the manufacture of poly-acrylamide (Figure 1). Acrylamide was defined

as a probably carcinogenic to humans (Group 2A) by the International Agency for Research on Cancer (IARC) (IARC, 1994). Since the first report of the discovery of acrylamide in foods in 2002, a study for acrylamide reduction has been underway (Rosen J., Hellenas KE., 2002; Tareke E. et al., 2002).

Acrylamide is formed naturally when plant materials with high carbohydrate content and low protein content, such as potato or cereal, is heated at a 160°C or higher temperature. Therefore, acrylamide is contained in a large amount in french fries, potato chips, potato snacks, cereals, breads and biscuits (Table 1) (Lineback et al., 2012; Petersen, Tran, 2005).

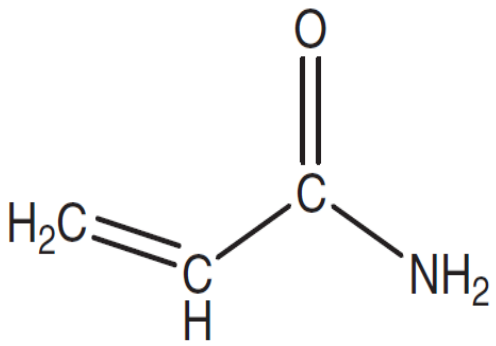


Figure 1. Structure of acrylamide.

It is generally known that asparagine and reducing sugars, which are naturally present in food raw materials, form acrylamide by the chemical reaction known as maillard reaction at a high temperature (Visvanathan R., Krishnakumar T., 2014; Javad K. et al, 2011). The key to reducing acrylamide is the reduction of asparagine (Xu F. et al., 2016; Morales F. et al., 2008; Pedreschi F. et al, 2011).

Table 1. Acrylamide contents in each food group (Mills *et al.,* 2009 & Petersen, Tran, 2005)

Product/ product group	Acrylamide range (ug/kg)	Product/ product group	Acrylamide range (ug/kg)
Potatoes (raw)	<10 – <50	Chocolate products	<2 – 826
Potato chips/ crisps	117 – 4,215	Roasted coffee	45 – 935
French fries/ chips	59 – 5,200	Coffee substitute	80 – 5,399
Bakery products/ biscuits	18 – 3,324	Coffee extract/ powder	87 – 1,188
Breads	<10 – 3,200	Meats	<10 – 116
Bread (toast)	25 – 1,430	Dairy products	<10 – 100
Breakfast cereals	<10 – 1,649	Baby food & infant formula	<10 – 130
Other fruit & vegetable	<10 – 70		

2.2. Toxicity of acrylamide and related regulations

The daily intake of acrylamide in human diets was estimated to 0.6 to 3.4 μg per kg body weight per day. Children have twice as much acrylamide intake as adults because of their low body weight (Lineback et al., 2012; EFSA, 2015; Visvanathan R., Krishnakumar T., 2014). Upon oral intake, acrylamide is absorbed in the gastrointestinal tract and distributed throughout all organs. Acrylamide in the body acts as a neurotoxin, genotoxin, reproductive toxin, and potential carcinogen (Lineback et al., 2012).

Experiments on the toxicity of acrylamide have been carried out in animal models. The panel on contaminants in the food chain (CONTAM Panel) selected $BMDL_{10}$ values of 0.43 mg/kg body weight per day for peripheral neuropathy in rats and of 0.17 mg/kg body weight per day for neoplastic effects in mice (EFSA, 2015).

Because of the toxicity of acrylamide, the use of mandatory measures to reduce content of acrylamide in foods and the validity of its effectiveness were discussed. Recently, the European Union issued regulation on acrylamide reduction measures and benchmark standards (Table 2) (EU, 2017). This regulation shall apply from 11 April 2018. Comparing the regulation with the amount of acrylamide present in

foods, an attempt to reduce acrylamide will be needed in many food groups (Table 1 & Table 2).

In Korea, it is still recommended that the food company autonomously keeps acrylamide level below 1 mg/kg (MFDS, 2015). However, as benchmark standards are set in Europe, specific acrylamide regulations are expected to be generated in Korea in the near future.

Table 2. Benchmark levels for the presence of acrylamide in foods (EU, 2017)

Product/ product group	Benchmark level (µg/kg)	Product/ product group	Benchmark level (µg/kg)
French fries	500	(c) Ginger bread	800
Potato crisps from fresh potatoes and from potato dough/ Potato-based crackers	750	Roast coffee	400
Soft bread			
(a) Wheat based bread	50	Instant (soluble) coffee	850
(b) Other than Wheat based bread	100		
Breakfast cereals		Coffee substitutes	
(a) Bran products and whole grain cereals, gun puffed grain	300	(a) coffee substitutes exclusively from cereals	500
(b) Wheat and rye based products		(b) coffee substitutes from a mixture of cereals and chicory	500
(c) Maize, oat, spelt, barley and rice based products	300 150	(c) coffee substitutes exclusively from chicory	4000
Biscuits and wafers		Baby foods	
(a) Crackers with the exception of potato based crackers	400	(a) Processed cereal based foods for infants and young children excluding biscuits and rusk	40
(b) Crispbread	350	(b) Biscuits and rusks for infants and young	150

2.3. Acrylamide reducing methods

As mentioned earlier, the key to reducing acrylamide is the reduction of asparagine. In order to reduce asparagine, it is possible to use asparaginase or a microorganism treatment method (Xu F. et al., 2016; Morales F. et al., 2008; Pedreschi F. et al., 2011; Renaissance bioscience corp., 2016).

In the case of Orion, a Korea's representative food company, the content of acrylamide in their products is controlled by asparaginase treatment process. In 2016, 13.5 tons of the enzyme (about 1.7 billion won) was imported and used. However, enzyme-treatment process is disadvantageous in that enzyme is expensive, not reuseable, instable. If the enzymatic treatment process is converted to a microorganism fermentation process, it can be expected to overcome the limitations of the enzyme and bring significant cost savings (Zhou W. et al., 2015).

3. Pre-treatment of *S. cerevisiae* to reduce acrylamide

3.1. Characteristic of *S. cerevisiae*

For the microbial fermentation process, *S. cerevisiae* was chosen. *S. cerevisiae* originally has an asparagine metabolic pathway, and it has advantages over other microorganisms in safety since it is generally recognized as safe (GRAS). Also, since *S. cerevisiae* has been used already in baking and brewing, it can be applied to foods without consumers' rejection.

However, the problem is that *S. cerevisiae* does not consume asparagine as a top priority for nitrogen source (Ljungdahl PO., Daignan-Fornier, 2012; Godard P. et al., 2007). Because there are various nitrogen sources which are more preferred than asparagine, asparagine is not reduced sufficiently in fermentation time. In other words, in order to use *S. cerevisiae* for acrylamide reduction, *S. cerevisiae* should be engineered metabolically to consume asparagine selectively and rapidly.

3.2. Asparagine metabolic pathway in *S. cerevisiae*

Various genes are involved in asparagine metabolism of *S. cerevisiae* (Figure 2) (Ljungdahl PO., Daignan-Fornier, 2012; Godard P. et al., 2007; Functional Technologies corp., 2012). Asparagine existing outside of the cell enters the cell as it is or decomposed form (aspartate & ammonia (NH_4^+)). After entering the cell, this is converted to glutamate or glutamine, the building block of cell growth via the metabolic pathway.

The genes involved in the asparagine metabolic pathway can be divided into three groups (Table 3) (Ljungdahl PO., Daignan-Fornier, 2012; Godard P. et al., 2007, Functional Technologies corp., 2012). The first group is related to asparagine permease, which transports asparagine into the cell. There is no permease that transports asparagine selectively. Asparagine is transported with other amino acids by high-affinity glutamine permease (Agp1p), glutamine permease (Gnp1p), general amino acid permease (Gap1p), dicarboxylic amino acid permease (Dip5p), and so on (Ljungdahl PO., Daignan-Fornier, 2012; Debailleul F. et al., 2013; Schreve JL. et al., 1998, Functional Technologies corp., 2012).

The second group is genes coding asparaginase, which directly degrades asparagine. *ASP1* coding cytosol asparaginase and *ASP3* coding cell-wall asparaginase belong to the second group (Jones GE, 1977; Lee SJ. et al., 1995, Functional Technologies corp., 2012). Interestingly, *ASP3* has a variable copy number. S288c, which is whole genome sequence identified, has four identical genes (*ASP3-1, ASP3-2, ASP3-3, ASP3-4*). But *ASP3* is completely absent in some other laboratory or industrial strains (League GP. et al., 2012)

The third group is a regulatory gene that controls the expression levels of the genes in the above two groups. Especially, the genes involved in the overall process of asparagine metabolism are regulated by Nitrogen Catabolite Repression (NCR). NCR is regulated by Ure2p, Gln3p, Gat1p, Gzf3p, and Dal80p (Georis I. et al., 2009; Magasanik B., Kaiser CA., 2002; Oliveira EM. et al., 2003, Functional Technologies corp., 2012). There is a specific sequence called the GATA box (5'-GATAAG-3' consensus sequence) at the promoter site of the NCR-sensitive genes. Gln3p, Gat1p, Gzf3p, and Dal80p compete to occupy this site. Gln3p and Gat1p act as transcriptional activators of the NCR-sensitive genes. On the other hand, Gzf3p and Dal80p act as transcriptional repressors

of the NCR-sensitive genes (Georis I. et al., 2009; Magasanik B., Kaiser CA., 2002; Oliveira EM. et al., 2003).

When amino acids are abundant or preferred, Gln3p and Gat1p are phosphorylated by TOR kinase (Figure 3A) (Cooper TG, 2002). Ure2p then complexes with phosphorylated Gln3p or Gat1p in the cytosol, interfering and consequently inhibiting the transcription of the NCR-sensitive genes. On the other hand, when amino acids are lacking or not preferred, Gln3p and Gat1p are dephosphorylated, entering the nucleus and consequently promoting the transcription of the NCR-sensitive genes (Figure 3B) (Cooper TG, 2002).

During the food fermentation, NCR works and nitrogen metabolism slows down because there is a large amount of the preferred amino acid in the foods. Therefore, in order to consume asparagine rapidly, NCR should be alleviated (Georis I. et al., 2009, Oliveira EM. et al., 2003). To alleviate NCR, *URE2, GZF3* and *DAL80*, which inhibit the expression of the NCR-sensitive genes, should be deleted. Or *GLN3* and *GAT1*, which promote the expression of the NCR-sensitive genes, should be overexpressed (Figure 3C).

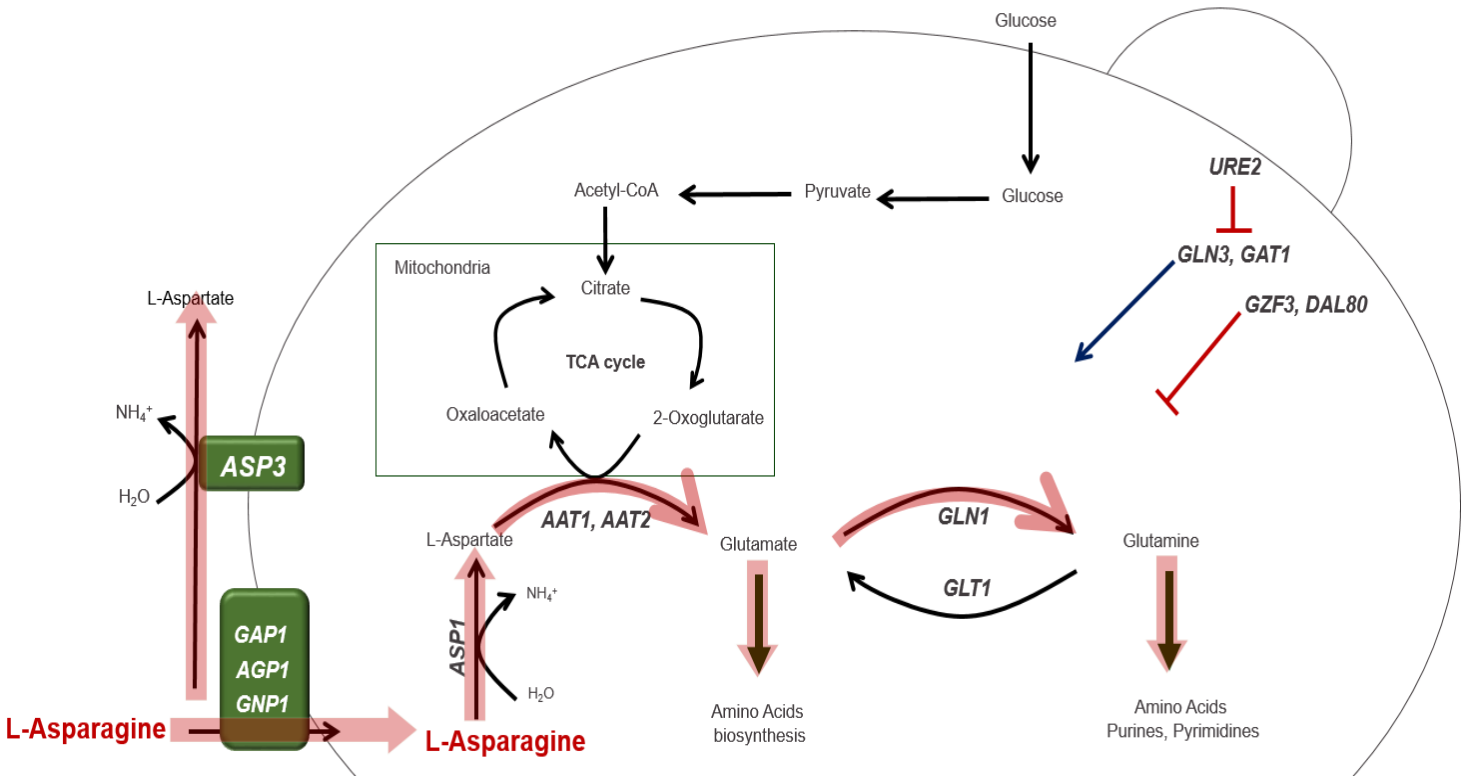


Figure 2. Asparagine metabolic pathway in *S. cerevisiae.*

Table 3. List of genes involved in asparagine metabolic pathway *GAAC : general amino acid control

Genes	Functional description (substrate specificity)	Regulation	Reference
Asparagine permease			
AGP1	Broad-Substrate range, medium capacity permease (Val, Ile, Phe, Met, Ser, Leu, Thr, Cys, **Asn**, Tyr, Ala, Gly, Gln)	NCR, GAAC*	Schreve *et al.* (1998)
GNP1	High-affinity glutamine permease (Thr, Gln, Ser, Cys, Leu, Met, **Asn**)	SPS-sensor	Zhu *et al.* (1996)
GAP1	General, high-capacity amino acid permease (All amino acid, GABA)	NCR, GAAC	Andre *et al*. (1993)
DIP5	Dicarboxylic amino acid permease (Ser, Ala, **Asn**, Asp, Gln, Gly)	NCR	Regenberg *et al.* (1998)
Asparaginase			
ASP1	Cytosol asparaginase	GAAC	Natarajan *et al*. (2001)
ASP3	Cell-wall asparaginase	NCR	Godard *et al.* (2007)
NCR regulatory gene			
URE2	Inhibitor of Gln3p and Gat1p		
GLN3	Positive transcription factor of NCR-sensitive genes	GAAC	Natarajan *et al*.(2001)
GAT1	Positive transcription factor of NCR-sensitive genes	NCR, GAAC	Godard *et al.* (2007)
DAL80	Negative transcription factor of NCR-sensitive genes	NCR	Godard *et al.* (2007)
GZF3	Negative transcription factor of NCR-sensitive genes	NCR	Godard *et al.* (2007)

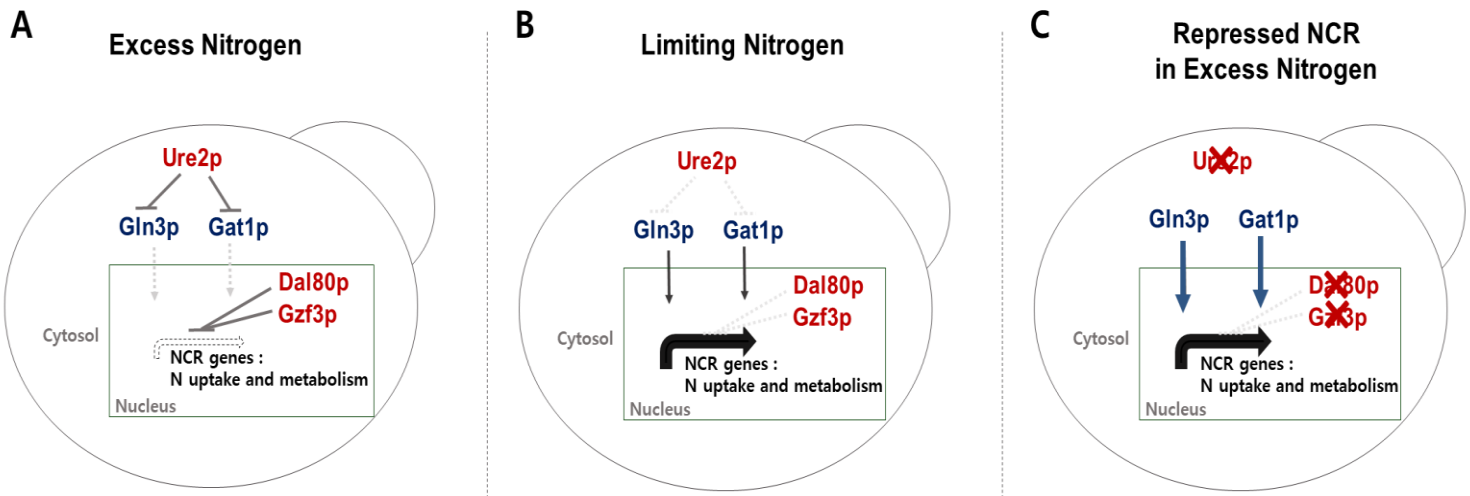


Figure 3. Nitrogen catabolite repression in *S. cerevisiae.*

Excess nitrogen condition(A), limiting nitrogen condition(B), and strategies for NCR alleviation (C).

4. Research objectives

For application to food industry, the research needs for industrial *S. cerevisiae* strains which are used in the industry fields, not laboratory *S. cerevisiae* strains which are developed for convenience of study.

The goal of this study is to propose a series of processes from the separation of the excellent industrial *S. cerevisiae* present in nuruk to the application of food industry for the acrylamide reduction by metabolic engineering.

The specific objectives of this research were described as follows:

(1) Isolation of industrial *S. cerevisiae* with excellent glucose fermentation ability from nuruk, and choosing a host strain among isolated industrial *S. cerevisiae* strains for further studies

(2) Construction of engineered industrial *S. cerevisiae* for acrylamide reduction. First: metabolic engineering of laboratory *S. cerevisiae* D452-2 to prove the rationality of metabolic engineering strategies for acrylamide reduction ; Second: metabolic engineering of industrial *S. cerevisiae* strain which was isolated form nuruk for using in acrylamide reduction ; Third: application of engineered industrial *S. cerevisiae* to foods for acrylamide reduction.

II. Materials and Methods

1. Reagents

All chemicals were reagent grade. Glucose, yeast nitrogen base and asparagine were purchased from Sigma-Aldrich (St. Louis, MO, USA); bacto-peptone, bacto-trypton, yeast extract and bacto-agar were obtained from Difco (Detroit, MI., USA); SC mix was purchased Sunrise Science product (San Diego, CA, USA); ampicillin from Fisher Scientific (Hampton, NH, USA); hygromycin B from Duchefa (Haarlem, Netherlands); aureobasidin A from Clontech Laboratories (Mountain view, CA, USA); ethidium bromide from Bioneer (Daejeon, Korea); agarose from Dongin Genomic (Seoul, Korea); NaOH, HCl, NaCl and H_2SO_4 from Duksan (Ansan, Korea).

2. Strains and Plasmids

2.1. Strains

Strains used in this study are described in Table 4. *Escherichia coli* TOP10 (Invitrogen, Carlsbad, CA, U.S.A) was used for gene cloning and manipulation. The N series of *S. cerevisiae* were industrial strains which were isolated from nuruk. The others industrial *S. cerevisiae*, La

Parisienne (LP) , Premier Cuvee (PC), were used as references for evaluation of ability of glucose fermentation. In the metabolic engineering for acrylamide reduction, metabolic engineering was also performed in the laboratory *S. cerevisiae* D452-2 [*Matα, leu2, his3, ura3, can1*], together with industrial *S. cerevisiae* to prove the rationality of metabolic engineering strategies. The engineered strains were stored on YPD or YNBD medium in a deep freezer at -80°C suspended in 15% glycerol.

Table 4. List of the strains used in this study

Strains	Description	Reference
Escherichia coli		
TOP10	F– *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara leu*) 7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G	Invitrogen, Carlsbad, CA, USA
S. cerevisiae		
BJ3505	Used as positive control of a cell & negative control of α cell	
D452-2	*Matα, leu2, his3, ura3, can1* Used as positive control of α cell & negative control of a cell	Hosaka *et al*., 1992
La Parisienne	Wine fermentation industrial strain	France
Premier Cuvee	Wine fermentation industrial strain	USA
N1	Isolated from nuruk	In this study
N2	Isolated from nuruk	In this study
N3	Isolated from nuruk	In this study
N4	Isolated from nuruk	In this study
N5	Isolated from nuruk	In this study
N6	Isolated from nuruk	In this study
N7	Isolated from nuruk	In this study

Strains	Description	Reference
S. cerevisiae		
D_ΔURE2	D452-2 *ΔURE2*	In this study
D_ΔGZF3	D452-2 *ΔGZF3*	In this study
D_P_{GAP1}	D452-2 P_{GAP1}::P_{GPD}	In this study
D_P_{ASP1}	D452-2 P_{ASP1}::P_{GPD}	In this study
D_P_{GAP1}_P_{ASP1}	D452-2 P_{GAP1}::P_{GPD} P_{ASP1}::P_{GPD}	In this study
D_P_{GAT1}	D452-2 P_{GAT1}::P_{GPD}	In this study
D_P_{ASP3}	D452-2 P_{ASP3}::P_{GPD}	In this study
D_ ΔURE2_P_{ASP3}	D452-2 *ΔURE2* P_{ASP3}::P_{GPD}	In this study
D_ ΔGZF3_P_{ASP3}	D452-2 *ΔGZF3* P_{ASP3}::P_{GPD}	In this study
N1_ ΔURA3	N1 *ΔURA3*	In this study
N1_ΔURE2::ASP3	N1 *ΔURE2::ASP3* cassette	In this study
N1_ ΔGZF3::ASP3	N1 *ΔGZF3::ASP3* cassette	In this study

2.2. Plasmids

Construction of Cas9 expression plasmid, p414_Cas9-AUR, was performed by JW Kim (Kim thesis, 2015). Construction of the guide RNA (gRNA) expressing plasmid, p42H_gRNA-HYG, was performed according to the gRNA synthesis protocol of Addgene Inc. and zhang GC. with modifications (Zhang GC. et al., 2014). Protospacers of the target genes were selected using CRISPRdirect (Naito Y. et al., 2015). The gRNA expression cassettes under the *SNR52* promoter and *SUP4* terminator were synthesized by overlap extension PCR with F1_Sac1_gRNA, R2_KpnI_gRNA, F2_gRNA_gene and R1_gRNA_gene primer combinations (Table 6). Sequentially, the synthesized gRNA DNA fragments were digested with SacI and KpnI, and then ligated into plasmid p42H (EUROSCARF), which was digested with the same enzymes to construct plasmids p42H_gRNA-HYG.

Table 5. List of the plasmids used in this study

Plasmids	Description	Reference
p426GPD	*LEU2*, *TDH3* promoter, *CYC1* terminator, 2 μ origin, Amp^R	Mumberg *et al*., 1995
p414_Cas9-AUR	*AUR1-C, CEN6, ARS4,* Amp^R, P_{TEF1}_*Cas9*_T_{CYC1}	Constructed by JW Kim
p42H_gURA3-HYG	Hyg^R, 2 μ origin, Amp^R, P_{SNR52}_*gURA3*_T_{SUP4}	Constructed by JW Kim
p42H_gURE2-HYG	Hyg^R, 2 μ origin, Amp^R, P_{SNR52}_*gURE2*_T_{SUP4}	In this study
p42H_gGZF3-HYG	Hyg^R, 2 μ origin, Amp^R, P_{SNR52}_*gGZF3*_T_{SUP4}	In this study
p42H_gASP1-HYG	Hyg^R, 2 μ origin, Amp^R, P_{SNR52}_*gASP1*_T_{SUP4}	In this study
p42H_gGAP1-HYG	Hyg^R, 2 μ origin, Amp^R, P_{SNR52}_*gGAP1*_T_{SUP4}	In this study
p42H_gGAT1-HYG	Hyg^R, 2 μ origin, Amp^R, P_{SNR52}_*gGAT1*_T_{SUP4}	In this study
p42H_gASP3-HYG	Hyg^R, 2 μ origin, Amp^R, P_{SNR52}_*gASP3*_T_{SUP4}	In this study

Table 6. List of primers used in this study

Primers	Primer sequence (5' → 3')	Reference
F-SnaBI-AUR1	AGCTTGTCACCTTACGTAAAAGTGCCCATCAGTGTTC	Kim, 2016.
R-MfeI-AUR1	ATAACCGGGTCAATTGCAGAGGAAAGAATAACGCAA	Kim, 2016
F1_SacI_gRNA	CCGCGAGCTCTCTTTGAAAAGATAATGTATG	This study
R2_KpnI_gRNA	TATAGAGCGGCCGCGGTACCAGACATAAAAAACAAAAAAAG	This study
SCD_F	AGGAGTGCGGTTCTTTGTAAAGTG	Chang, 2007.
SCD_R	TACTTACCGAGGCAAGCTACATTCC	Chang, 2007.
delta 2	GTGGATTTTTATTCCAAC	Xufre, 2011
delta 12	TCAACAATGGAATCCCAAC	Xufre, 2011
delta 21	CATCTTAACACCGTATATGA	Xufre, 2011
HMLα-F	GAAATATTTAAACTCATTTATGGCTTTTAGAG	Huxley, 1990
HMRa-F	GTAATTTGACTAAAGTAGAGCAACATAC	Huxley, 1990
MAT_{loci}-R	CAAAAGTCACATCAAGATCGTTTATGG	Huxley, 1990
F_Cas9 check	GCCACCCAATCTTTGGCAATATCGTG	This study
R_Cas9 check	GGTATCCGAAATGTGAGGATTTTCTCAATC	This study
R1_gRNA_URA3	CCAAGTACAATTTTTTACTCGATCATTTATCTTTCACTGCG	Zhang, 2014
F2_gRNA_URA3	GAGTAAAAAATTGTACTTGGGTTTTAGAGCTAGAAATAGCAAGT	Zhang, 2014
R1_gRNA_URE2	ATGTTTACTTGACGGAGCGCGATCATTTATCTTTCACTGCG	This study
F2_gRNA_URE2	GCGCTCCGTCAAGTAAACATGTTTTAGAGCTAGAAATAGCAAGT	This study
R1_gRNA_GZF3	TCTTCTTCGCTTTGATTTAAGATCATTTATCTTTCACTGCG	This study
F2_gRNA_GZF3	TTAAATCAAAGCGAAGAAGAGTTTTAGAGCTAGAAATAGCAAGT	This study
R1_gRNA_GAP1	GAGCGAATTCCGGAAGAAAGGATCATTTATCTTTCACTGCG	This study
F2_gRNA_GAP1	CTTTCTTCCGGAATTCGCTCGTTTTAGAGCTAGAAATAGCAAGT	This study
R1_gRNA_ASP1	AAAGTGCGATGAGAGTGCTGGATCATTTATCTTTCACTGCG	This study
F2_gRNA_ASP1	CAGCACTCTCATCGCACTTTGTTTTAGAGCTAGAAATAGCAAGT	This study
R1_gRNA_GAT1	GGAGTGGCACACACCTATATGATCATTTATCTTTCACTGCG	This study

Primers	Primer sequence (5' → 3')	Reference
F2_gRNA_GAT1	ATATAGGTGTGTGCCACTCCGTTTTAGAGCTAGAAATAG CAAGT	This study
R1_gRNA_ ASP3	CAAAAGGATATCATGAGGTGGATCATTTATCTTTCACTG CG	This study
F2_gRNA_ASP3	CACCTCATGATATCCTTTTGGTTTTAGAGCTAGAAATAG CAAGT	This study
F_repair_URA3	TCCATGGAGGGCACAGTTAAGCCGCTAAAGGCATTATA AGCCAAGTACAATTTTTTACTC	Zhang, 2014
R_repair_URA3	ACCAATGTCAGCAAATTTTCTGTCTTCGAAGAGTAAAA AATTGTACTTGGCTTATAATGC	Zhang, 2014
F_repair_URE2	AAGTGTCGAATCTCTCCAATGCGCTCCGTCAAGTAAAC ATATAAAACAGGAACAGTAATA	This study
R_repair_URE2	ATTTATATTACTTTGATCGGTGGTTGTATTACTGTTCCTG TTTTATATGTTTACTTGACGGAGC	This study
F_repair_GZF3	AAGTGAAAACCTCAACAGCTTAAATCAAAGCGAAGAA GAATAGCATATTGGGAGATGGCC	This study
R_repair_GZF3	TACTGCTTCATAACCTAAAGGTGGCCATCTCCCAATATG CTATTCTTCTTCGCTTTGATTTA	This study
F_repair_GAP1	CGTGCCGCTATCAGGCAGCCTCACTAATCTACCCATTGA CCTCATGCAGCTCATTATCAATACTCGCCAT	This study
R_repair_GAP1	TGTTTCAGATTATCTGGATTATTCTTCTCGTACGAAGAA GTATTACTCATTCGAAACTAAGTTCTGGTGT	This study
F_repair_ASP1	AAAAAGATGATGACGATGACTTTGTTGAAGAAATGTGT TTGAAGACAGTGTCATTATCAATACTCGCCAT	This study
R_repair_ASP1	TTTTCAACATCTGGGCAGATGGTAGTGATTTCAACTGA ATCGCTTTTCATTCGAAACTAAGTTCTGGTGT	This study
F_repair_GAT1	GGTGTCCTCACACAACCCTGTCTCTGCACAACGTAATA CCTCCTTTTCCCGTTCATTATCAATACTCGCCAT	This study
R_repair_GAT1	GCGATGAACAGAACAGGGGAAGGGCGGAAAAGCAAA GGAAAGAAAACGTGCATTCGAAACTAAGTTCTGGTGT	This study
F_repair_ASP3	TATAATTTCGTTGCGCAAATTCAACTAAACCACCAATAT CCCCCCTACAATCATTATCAATACTCGCCAT	This study
R_repair_ASP3	CTGGACATTGCGACAAAGAGAGAAAGTAAAAGGGTAT TTAAAGATCTCATTCGAAACTAAGTTCTGGTG	This study
F_repair dURE2 :: ASP3 cassette	TTGAAAAGAATAGCAAAAATCTTTCCTTTTCAAACAGC TCATTTGGAATTTCATTATCAATACTCGCCAT	This study
R_repair URE2 :: ASP3 cassette	GACCAGAGTAATGGATTACCAGTCTCTTTGTAATATTTAT TTACCAAATGGCAAATTAAAGCCTTCGAGC	This study
R_repair dGZF3 :: ASP3 cassette	TCATCTTTCTCGTCGTTCCTAGTGATTAACGTTACTAAA ATTACTGATCCTCATTATCAATACTCGCCAT	This study
R_repair dGZF3 :: ASP3 cassette	TAGAGGTTAAACAGTTCTTACAAACAGGAACATTAGCA TCATTGGATACGGCAAATTAAAGCCTTCGAGC	This study
F_URA3 check	GAGCACAGACTTAGATTGGT	Zhang, 2014
R_URA3 check	ATAGAAATCATTACGACCGA	Zhang, 2014
F_URE2 check	CTGCCACTATTGAATTTTTG	This study
R_URE2 check	GTGAATGGAAGTATCTGAAA	This study
F_GZF3 check	GGGGAACCTTTTGATAGGTA	This study

Primers	Primer sequence (5’ → 3’)	Reference
R_GZF3 check	TTCAAACTAATTGGCCTGGG	This study
F_GAP1 check	TCAGCCGATCGCTTATCTGC	This study
R_GAP1 check	TGCCATTTGGACCCTGAACC	This study
F_ASP1 check	TGTTCATGAGGGTTCTCCATG	This study
R_ASP1 check	GATTTTGATTCTTGGTAAGCTACG	This study
F_GAT1 check	CCTACCCTATTTCATTATAGATGC	This study
R_GAT1 check	TTAAATTCAAGTCCGGGTCGAG	This study
F_ASP3 check	CTAAATTTTGAAATCGGCCGCA	This study
R_ASP3 check	TTGTTGCACTTGTCGAACCC	This study
F_RTq_ACT1	GAGCCCCAGAAGCTTTGTTCCATCC	This study
R_RTq_ACT1	CATGGTGGTACCACCGGACATAACG	This study
F_RTq_ASP3	ACATTTCCAACCTCACAGACCCTTCG	This study
R_RTq_ASP3	CTACCCGTAGCAGTCCAGGAACC	This study

3. DNA manipulation and transformation

3.1. Preparation of DNA

All enzyme kits were used according to the manufacturer's instruction. Preparation of plasmid DNA was carried out by using the Plasmid Miniprep Kit from Takara (Otsu, Japan). Preparation of chromosomal DNA for a PCR template was carried out by using the DNeasy Blood & Tissue Kit from QIAGEN (Düsseldorf, Germany). PCR amplified or enzyme treated DNA was purified by using the Hiyield™Gel/PCR DNA Extraction Kit from Real Biotech Corporation (Taipei, Taiwan). DNA sequencing was performed by SolGent (Daejeon, Korea).

3.2. Polymerase Chain Reaction (PCR)

PCR to amplify the gene for cloning was performed with the PrimeSTAR® HS PCR PreMix (Takara, Shiga, Japan) in GeneAmp PCR System 2720 (Applied Biosystems, CA, USA). PCR solution was composed of 10 pmol of forward and reverse primers, and 10 ng of DNA as a template. After the initial pre-denaturation step at 95°C for 10 min, 40 cycles of PCR amplification were performed with the

following conditions: 95°C for 30 sec, 50°C for 30 sec, 72°C for 1kb/min, followed by final extension at 72°C for 10 min. The amplified DNA was confirmed by gel electrophoresis.

3.3. Digestion and ligation of DNA

All enzymes were used according to the manufacturer's instruction. Restriction enzymes SacI, KpnI and calf intestinal alkaline phosphatase (CIP) were purchased from New England Biolabs (Beverly, MA, USA). Plasmid p42H_gRNA-HYG and amplified DNA fragments were digested with SacI and KpnI. T4 Ligation Mix (Mighty Mix) obtained from Takara (Shiga, Japan) was used for ligation of PCR products and plasmids.

3.4. Transformation of *E. coli*

Transformation of *E. coli* was carried out as described by sambrook (Sambrook J., Russell DW., 1989). *E. coli* Top10 was cultured in 5 mL LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl) for 12 hr. 0.5 mL of the culture was transferred to fresh 50 mL LB medium and cultured until $O.D._{600}$ reached 0.5. Cells harvested by

centrifugation at 6000 rpm for 5 min at 4°C were resuspended in 5 mL of 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°C for 45 sec and 1 mL of LB medium was added to the tubes and incubated at 37°C for 40 min to allow the bacteria to express the antibiotic resistance. Transformed cells was spread on LB agar plates with an ampicillin selection marker.

3.5. Transformation of *S. cerevisiae*

S. cerevisiae transformation was performed by a standard lithium acetate transformation method (Gietz RD., Schiestl RH., 2007). A single colony of the *S. cerevisiae* was inoculated and incubated overnight in 5 mL YPD medium at 30°C. After 12 hour of growth, 2.5×10^8 cells of the cultures transferred to fresh 50 mL 2×YPD medium and cultured until $O.D._{600}$ reached 2. Cells harvested by centrifugation at 6000 rpm for 5 min at 4°C were washed twice with double-distilled water (DDW). Washed cells were mixed with 50% (w/v) PEG 3350, 1.0 M lithium acetate, carrier DNA, repair DNA and plasmid. They were subjected to heat-shock at 42°C for 30 min and incubated with 1 ml of YPD at 30°C

for 1.5 hours. Transformants were selected on YP medium containing 20 g/L glucose. Amino acids, nucleotides and antibiotics were added as necessary.

3.6. CRISPR-Cas9 genome engineering procedure

In this study, every genome engineering method of *S. cerevisiae* were performed based on CRISPR-Cas9. A detailed CRISPR-Cas9 system procedure was described below (Figure 4) (Zhang, 2014).

First, plasmid p414_Cas9-AUR was transformed into *S. cerevisiae*.

Second, double-stranded repair DNA was amplified by PCR with F_repairDNA and R_repairDNA. F_repairDNA and R_repairDNA were designed to contain about 50-bp homology with the target gene. Gene disruption to block undesired protein was performed by placing a stop codon in the middle of the Open Reading Frame (ORF). For gene overexpression, a *GPD* promoter, known as a constitutive strong promoter, was inserted into the native promoter (Blazeck J. et al., 2012). Repair DNA were designed to confusing the protospacer adjacent motif (PAM) sequence or protospacer. In case of disruption, for example, the stop codon replaced the PAM sequence to disrupt the target gene, simultaneously to prevent repetitive Cas9 cleavage of the target site by

removing PAM.

Third, p42H_gRNA-HYG plasmid was transformed together with the repair DNA into *S. cerevisiae* which plasmid p414_Cas9-AUR are transformed already (Zhang, 2014). The inserted sequences were confirmed by DNA sequencing.

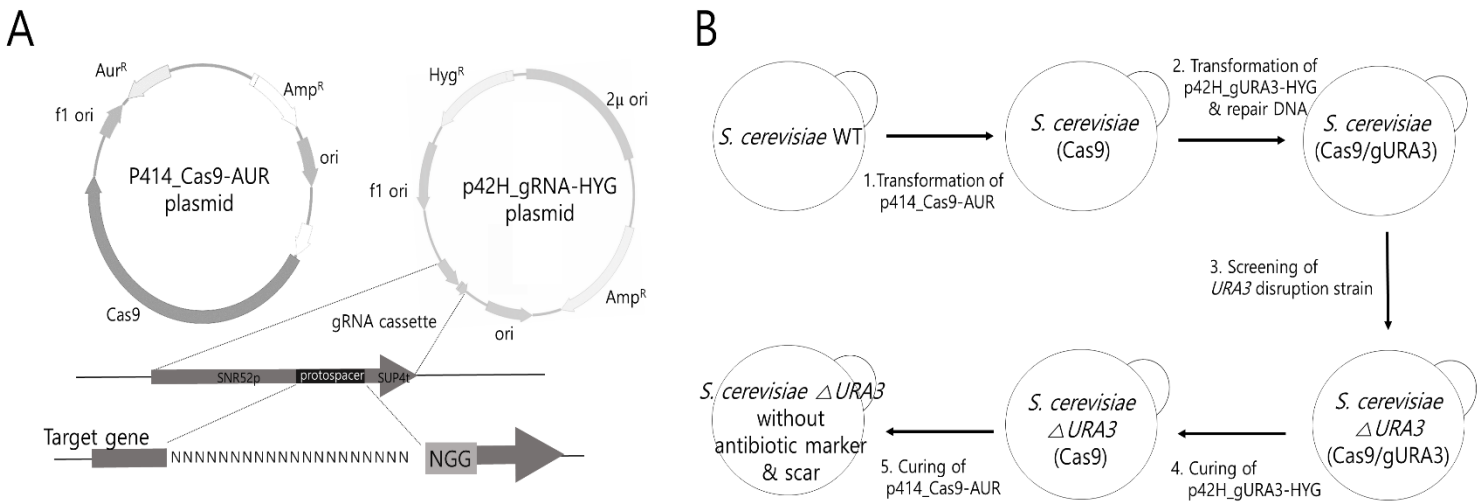


Figure 4. Schematic illustration of plasmids and diagram of CRISPR-Cas9 based genome engineering procedure. P414_Cas9-AUR & p42H_gRNA-HYG (A), diagram of CRISPR-Cas9 genome engineering procedure (in case of *URA3* deletion) (B).

4. Media and Culture conditions

4.1. Media

Luria-Bertani (LB) medium (5 g/L yeast extract, 10 g/L tryptone, 10 g/L NaCl) containing 75 μg/mL of ampicillin was used for recombinant *E. coli* cultivation. Yeast extract-peptone (YPD) medium (10 g/L yeast extract, 20 g/L peptone) containing 20 g/L glucose and appropriate antibiotics (0.1 μg/mL of aureobasidin A or 375 μg/mL of hygromycin B) was used for incubation of engineered *S. cerevisiae*. YPD and SCD medium (SC mix 2 g/L, YNB 6.7 g/L) containing 200 g/L glucose was used for assessing fermentation ability of isolated *S. cerevisiae*. An asparagine rich YPD medium (YP and contain 80 g/L glucose and 5 g/L asparagine) was used to assess asparagine consumption rate of engineered *S. cerevisiae.* The concentration of asparagine in the medium was set equal to the average concentration of asparagine in the potatoes (Granby K. et al., 2008)

4.2. Culture conditions

To prepare the inoculums, engineered *S. cerevisiae* stock was cultivated in a 5 mL test tube with YPD medium containing 20 g/L

glucose at 30°C, 250 rpm for 12 h. The grown cells were transferred to a 500 mL baffled-flask with 100 mL YPD medium containing 20 g/L glucose at 30°C, 250 rpm until the mid-exponential phase ($O.D._{600}$ 5-6). The grown cells were harvested by centrifugation at 7,000 rpm for 1 min and washed twice with DDW. The harvested cells were inoculated into the main culture at the initial $O.D._{600}$ 1.0.

To compare glucose fermentation ability between isolated industrial *S. cerevisiae* and commercially used industrial *S. cerevisiae*, the main flask cultures were performed in both YPD medium and SCD medium containing 200 g/L glucose. Main cultures were carried out in 250 mL flasks with a working volume of 50 mL at 80 rpm for oxygen-limited conditions.

To assess asparagine consumption rate of engineered *S. cerevisiae*, an asparagine rich YPD medium was used. Main cultures were carried out in 250 mL flasks with a working volume of 50 mL at 80 rpm for oxygen-limited conditions.

5. Analysis

5.1. Isolation of industrial *S. cerevisiae* from nuruk

10 kinds of Korean nuruk were selected. 10 g of each nuruk sample was diluted using 0.85% NaCl solution and homogenized by a stomacher (AES, France). The mixed samples were serially diluted in 0.85% NaCl solution and spread on Potato Dextrose Agar (PDA) with sodium propionate 3.5 g/L, kanamycin 10 mg/mL, ampicillin 50 mg/mL and adjusted to pH 5.03 with HCl.

5.1.1. *S. cerevisiae* specific PCR screening

Colonies were picked randomly and colony PCR was conducted with SCD_F, SCD_R primers for *S. cerevisiae*-specific isolation (Chang HW. et al., 2007). Through a species-specific PCR assay, the presumptive *S. cerevisiae* has a band of 320-bp, while the non-*S. cerevisiae* does not have the band.

5.1.2. VITEK2 Compact analysis

VITEK2 Compact (bioMerieux, France) is an automated system able to identify microorganisms by testing 47 biochemical properties (Pincus

DH., 2005). VITEK2 Compact was used to reconfirm isolated *S. cerevisiae* strains by analyzing and comparing the phenotypes. The isolated strains were individually grown on a PDA plate. Colonies were picked and mixed in 0.45% NaCl solution until the McFarland standard measured 2.0 ± 0.2 on the VITEK2 DensiCheck instrument (bioMerieux). Yeast colorimetric identification cards (bioMerieux) containing the yeasts were assembled in a cassette and assayed using the VITEK2 Compact system. Data was analyzed by the VITEK 2 software version VT2-R03.1.

5.2. Characterization of isolated industrial *S. cerevisiae*

5.2.1. Inter-delta RAPD PCR typing

To differentiate *S. cerevisiae* at a strain level, inter-delta RAPD (Randomly Amplified Polymorphic DNA) PCR typing was conducted (Xufre A. et al., 2011; Franco-Duarte R. et al., 2011). Primers for delta12 + delta2 and delta12 + delta21 combinations were used in the RAPD PCR reaction (Table 6). After the initial pre-denaturation step at 95°C for 10 min, 40 cycles of PCR amplification were performed with the following conditions: 95°C for 30 sec, 50°C for 30 sec, 72°C for 90 sec, followed by a final extension at 72°C for 10 min. The

amplification products were separated by electrophoresis on 2% (w/v) agarose gels submitted to 135 V (constant voltage) for 60 min in 0.7X TAE buffer. After staining with ethidium bromide (0.5 µg/mL), DNA bands were visualized using an UV transilluminator and photographed. GelJ version 2 software (BMC Bioinformatics) was used to analyze the band patterns. Two RAPD PCR profiles were compared.

5.2.2. Assessment of *S. cerevisiae* mating type

S. cerevisiae can live as either a haploid (a or α) or a diploid (a/α). The haploid cell can mate with other haploid cell of the opposite mating type. An a cell can only mate with an α cell, and vice versa (Ohnishi G. et al., 2004). Mating type is determined by two different alleles of the mating-type (MAT) locus, MATα or MATa on chromosome III. MATα and MATa alleles, distinguished by their Yα (750-bp) or Ya (650-bp) regions, share X and Z_1 regions for homology with Hidden MAT Right (HMR), while the W and Z_2 regions for homology with Hidden MAT Left (HML) (Figure 5) (James E. Haber, 2012).

To assess the mating type of the isolated *S. cerevisiae* strains, HMLα-F, HMRa-F and MATloci-R primer were designed with sequence modifications (Huxley C. et al., 1990). After the initial pre-denaturation

step at 95°C for 10 min, 40 cycles of PCR amplification were performed with the following conditions: 95°C for 30 sec, 52°C for 30 sec, 72°C for 50 sec, followed by a final extension at 72°C for 10 min. The amplification products were separated and visualized by the methods described in 5.2.1.

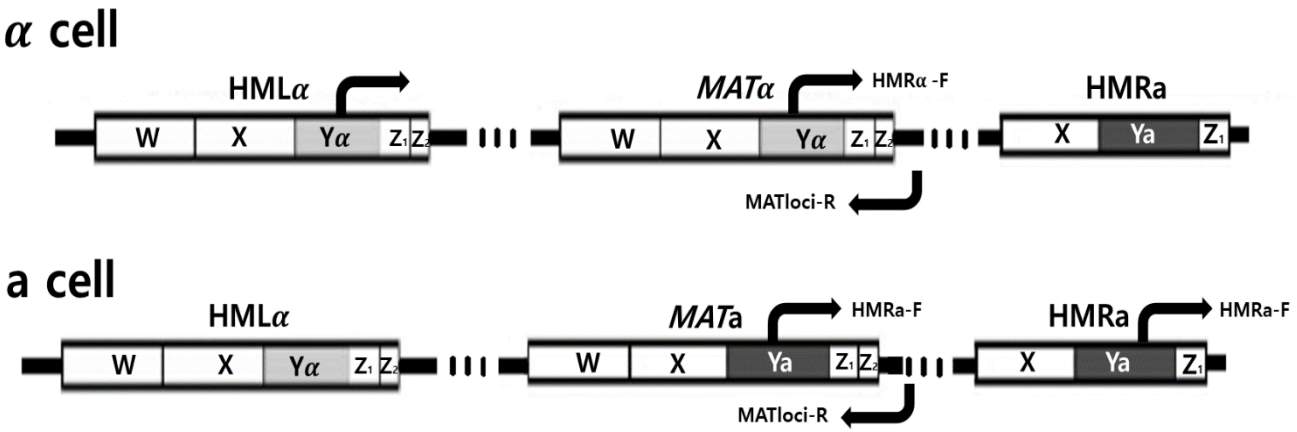


Figure 5. Structure of MATa and MATα alleles, distinguished by Ya or Yα.

5.3. Dry cell weight

Cell growth was estimated by measuring optical density (O.D.) at 600 nm using a spectrophotometer (OPTIZEN POP, Mecasys, Daejeon, Korea). Dry cell weight (DCW) was calculated by the pre-estimated conversion equation.

$$\text{Dry cell weight (g/L)} = 0.3 \times \text{O.D.}_{600}$$

5.4. Metabolite detection

Concentrations of glucose, glycerol, acetate and ethanol were measured by a High Performance Liquid Chromatography (HPLC) (Agilent Technologies 1260 Series, Santa Clara, CA, USA) with a Rezex ROA-organic acid column (Phenomenex, CA, USA). The column maintained at 60°C was eluted with 5 mM sulfuric acid at flow rate of 0.6 ml/min. Detection was made with a reflective index (RI) detector at 35°C.

5.5. Real-time quantitative PCR

Real-time quantitative PCR (RT-qPCR) was performed to compare transcriptional levels by replacing the chromosomal native promoter

with the *GPD* promoter. Degradation of RNA was protected by treating with the RNA protect reagent (Qiagen, Valencia, CA) before cell lysis. The total RNA was isolated using RNeasy mini kit (Qiagen, Valencia, CA). The cDNA was amplified by using Maxima H Minus First Strand cDNA Synthesis Kit (Thermo SCIENTIFIC). The qPCR experiment was carried out on a MiniOpticon Real-Time PCR system (Bio-Rad) using the SYBR premix Ex Taq (Takara). The *ACT1* gene, a highly invariant gene, was used as a reference gene for normalization of qPCR values. The primers used in qPCR experiments were summarized in Table 6.

5.6. Quantification of asparagine and acrylamide

Quantitative analysis of asparagine in the medium or in the foods was measured using the K-ASNAM enzyme kit (Megazyme, USA) according to the manufacturer's instruction.

Quantitative analysis of acrylamide present in the foods after heated was measured by LC-MS/MS system at Nongshim Food Safety Research Center.

5.7. Application of engineered industrial *S. cerevisiae* to foods

Because a large amount of acrylamide was detected in potato chips and breads, wheat-based dough and potato-based dough were selected for food applications. Wheat-based dough and potato-based dough were kneaded with 10 g of cells per 200 g of dough (Table 7) (Renaissance bioscience corp., 2016). Each kneaded dough was fermented at 30℃ for 3 hours and samples were collected every 30 min to quantify the amount of asparagine in the dough. Each sample was treated immediately with liquid nitrogen in order to halt asparaginase, and was stored at -80℃ (Renaissance bioscience corp., 2016).

After three hours, the wheat-based dough was heated in an oven at 200℃ for 20 min and the potato-based dough was fried in oil at 170°C for 5 min. And then, the each food samples taken from the both cooked doughs were extracted and purified to quantify the amount of acrylamide by LC-MS/MS at Nongshim Food Safety Research Center.

To measure asparagine levels using the K-ASNAM kit, frozen samples were pretreated with 80℃ DDW, and incubated in 80℃ water bath for 20 min to inactivate enzyme. After that, the supernatant

was separated by centrifugation at 13,000 rpm for 10 min. The supernatant was diluted appropriately and the amount of asparagine was measured by the K-ASNAM kit.

Table 7. Composition of doughs for food applications

	Control	Strain treatment
Wheat-based dough (Bread)		
Flour	25 g (+5 g_{Asn}/kg_{dough})	25 g (+5 g_{Asn}/kg_{dough})
Salt	0.5 g	0.5 g
Sucrose	2 g	2 g
DDW	16.25 g	16.25 g
S. cerevisiae	(Baking powder) 0.65 g	0.84×10^{10} cell
Potato-based dough (Stackable potato chip)		
Mashed potato	25 g	25 g
Corn starch	1.5 g	1.5 g
Salt	1.5 g	1.5 g
DDW	35 g	35 g
S. cerevisiae	-	0.84×10^{10} cell

III. RESULTS AND DISCUSSIONS

1. Isolation of industrial *S. cerevisiae* from nuruk

From 10 kinds of nuruk, many colonies were obtained on PDA plates. From among these, 316 colonies that had different color, shape, size were picked and evaluated with PCR using the SCD_F and SCD_F primer sets.

Of the 316 colonies, 15 colonies had a detectable band at 320-bp and were predicted as *S. cerevisiae* (Data not shown). To confirm the identity of the 15 colonies, isolated strains were investigated with the VITEK2 Compact system (Pincus DH, 2005). As shown in Table 8, finally 7 *S. cerevisiae* were isolated from 15 colonies.

After that, Inter-delta RAPD-PCR typing was performed to differentiate *S. cerevisiae* at a strain level (Figure 6). Two sets of Inter-delta RAPD-PCR typing (delta2 + delta12 and delta12 + delta 21) were performed (Figure 6A & 6B). When two RAPD PCR results were compared, all 7 *S. cerevisiae* showed different RAPD PCR patterns. This means that all of the isolated 7 *S. cerevisiae* are different at a strain level.

Table 8. VITEK2 Compact results

Strains	Vitek2 Compact	Identity
4-5 (N1)	*S. cerevisiae*	98%
4-9	*Candida pelliculosa*	98%
5-1	*C. pelliculosa*	99%
5-2	*C. pelliculosa*	99%
5-4	*C. pelliculosa*	98%
5-8	*C. pelliculosa*	99%
5-9	*C. pelliculosa*	97%
8-1 (N2)	*S. cerevisiae*	98%
8-3 (N3)	*S. cerevisiae*	98%
8-4 (N4)	*S. cerevisiae*	98%
8-5 (N5)	*S. cerevisiae*	98%
8-10	*Candida colliculosa*	98%
8-26 (N6)	*S. cerevisiae*	98%
8-27 (N7)	*S. cerevisiae*	98%
9-26	*C. pelliculosa*	99%

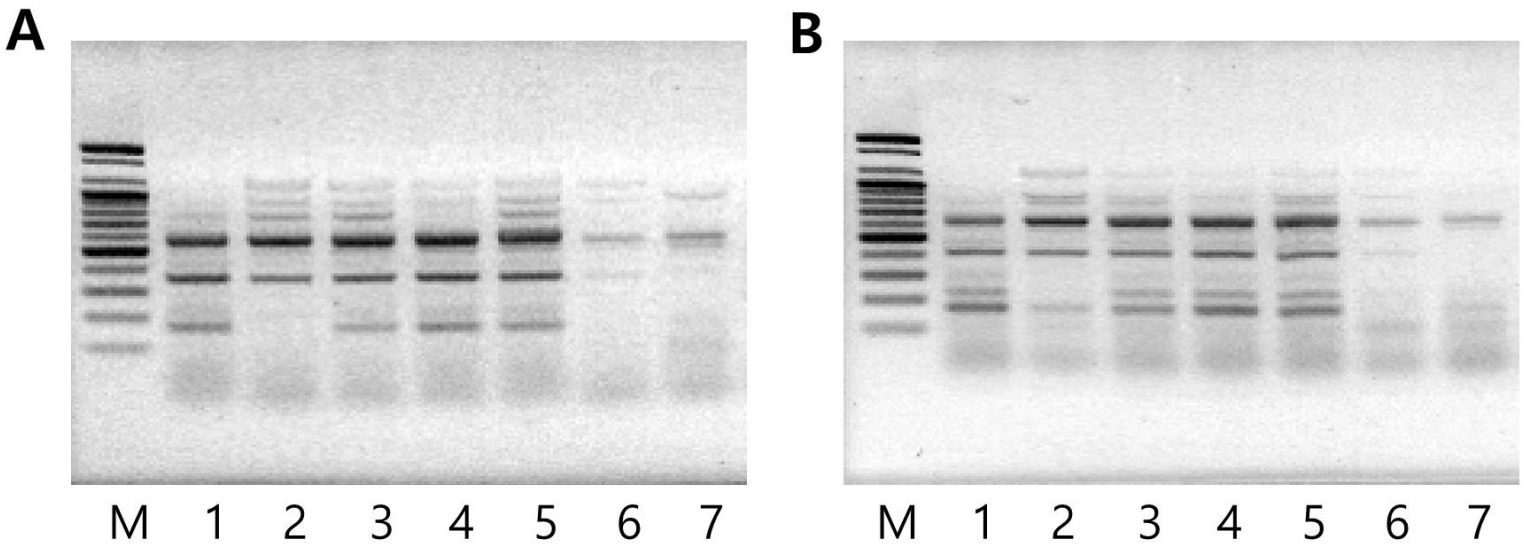


Figure 6. Inter-delta RAPD-PCR typing results with delta2 + delta12 primers (B) and with delta12 + delta 21primers (C). Lane M : DNA ladder (1 kb), lane 1 : *S. cerevisiae* 8-1 (N2), lane 2 : *S. cerevisiae* 8-3 (N3), lane 3 : *S. cerevisiae* 8-4 (N4), lane 4 : *S. cerevisiae* 8-5 (N5), lane 5 : *S. cerevisiae* 8-26 (N6), lane 6 : *S. cerevisiae* 8-27 (N7), lane 7 : *S. cerevisiae* 4-5 (N1).

2. Characterization of isolated industrial *S. cerevisiae*

To confirm the mating type of isolated *S. cerevisiae*, the PCR reaction was performed respectively with HMLα-F + MATloci-R or HMRa-F + MATloci-R primer sets. If isolated *S. cerevisiae* is haploid, one band is observed only once in two PCRs with either HMLα-F + MATloci-R or HMRa-F + MATloci-R primer sets. If isolated *S. cerevisiae* is diploid, one band is observed twice in two PCRs. Positive & negative controls were used *S. cerevisiae* BJ3505 as α cell type and *S. cerevisiae* D452-2 as a cell type.

Amplification with the HML α -F and MATloci-R primer sets generated 757-bp band product in all *S. cerevisiae* strains isolated from nuruk except for the *S. cerevisiae* D452-2 (Figure 7A). An amplification with the HMRa-F and MATloci-R primer sets generated 525-bp band product in all *S. cerevisiae* strains isolated from nuruk except for the *S. cerevisiae* BJ3505 (Figure 7B).This results suggested all *S. cerevisiae* strains isolated from nuruk are diploid or more.

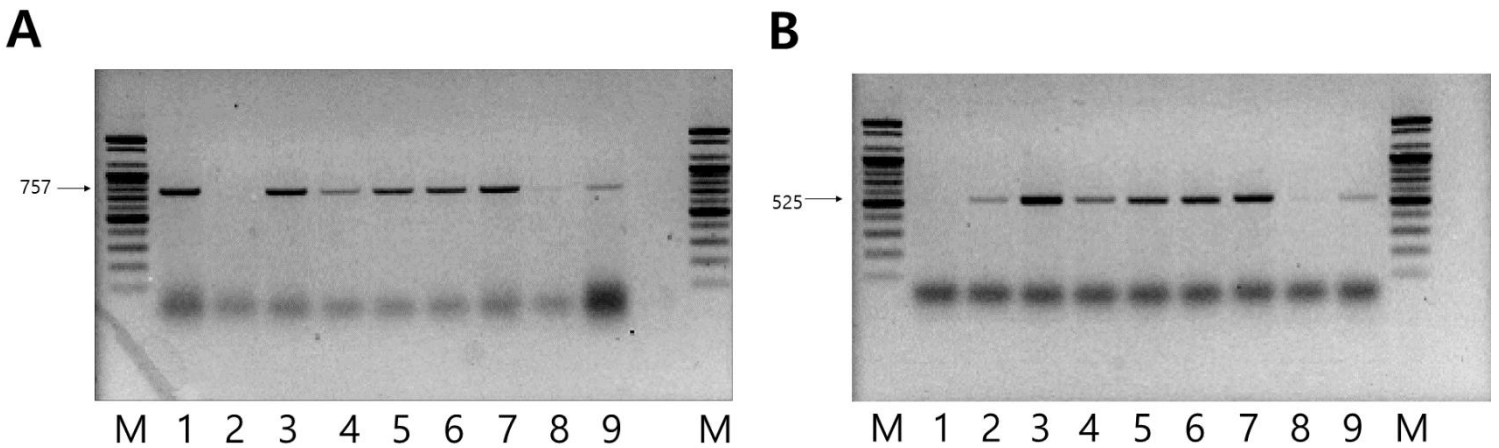


Figure 7. The results of PCR with HMLα-F and MATloci-R primer (A) and with HMLa-F and MATloci-R (B). Lane M : DNA ladder (1 kb), lane 1 : *S. cerevisiae* BJ3505, lane 2 : *S. cerevisiae* D452-2, lane 3 : *S. cerevisiae* 8-1 (N2), lane 4 : *S. cerevisiae* 8-3 (N3), lane 5 : *S. cerevisiae* 8-4 (N4), lane 6 : *S. cerevisiae* 8-5 (N5), lane 7 : *S. cerevisiae* 8-26 (N6), lane 8 : *S. cerevisiae* 8-27 (N7), lane 9 : *S. cerevisiae* 4-5 (N1).

3. Assessment of glucose fermentation ability of isolated *S. cerevisiae* from nuruk

Fermentation ability of 7 *S. cerevisiae* strains isolated from nuruk was compared with the others industrial *S. cerevisiae* (La Parisienne and Premier Cuvee). Growth rate, glucose consumption rate, ethanol productivity and glycerol accumulation were used as a representative factor of fermentation characteristics. The end of fermentation was determined by glucose depletion.

Among 7 *S. cerevisiae* strains, 4 strains (N1, N2, N3, N4) were reselected based on good fermentation factors as mentioned above. Their fermentation profiles were compared with the other industrial strains' profiles (Figure 8). In YPD medium, every strain depleted glucose within 18 h, and pH was changed from 6.8 to 4.0-4.5 during the fermentation. A similar pattern of fermentation profiles was observed for isolated *S. cerevisiae* and the others industrial strains (Figure 8A). On the other hand, in SC medium known as more acidic condition than YP, only PC and N1 can consume glucose within 60 hr, and pH was changed from 4.0 to 2.2-2.5 during the fermentation. While LP, N2, N3, N4 left glucose (Figure 8B). These results suggest that *S. cerevisiae* N1

show more acidic tolerance than the other isolated *S. cerevisiae*, although N1 has slower glucose consumption rate than PC in acidic medium.

Also, among isolated *S. cerevisiae* strains, *S. cerevisiae* N1 showed the highest glycerol producing ability in both YPD and SCD medium. Commonly, accumulation of glycerol plays a major role in growth under osmotic condition in *S. cerevisiae* (Kofli NT. et al., 2006).

In summary, it is expected that *S. cerevisiae* N1 can be used efficiently under acidic pH conditions or osmotic stress conditions as compared with the other isolated *S. cerevisiae*. Therefore, *S. cerevisiae* N1 was chosen as the final strain to be used in future experiments.

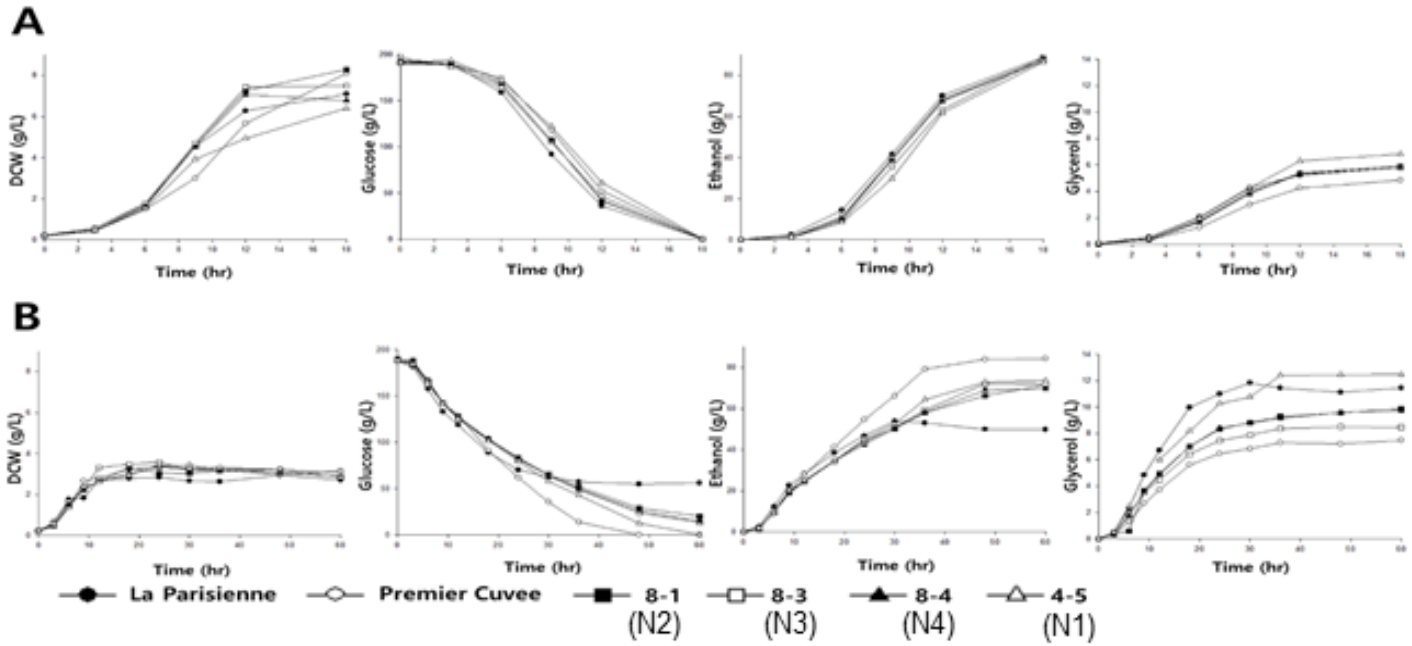


Figure 8. Assessment of glucose fermentation ability of isolated *S. cerevisiae* from nuruk. Cell growth rate, glucose consumption, ethanol production, glycerol accumulation of the *S. cerevisiae* isolates and the others industrial *S. cerevisiae*, La Parisienne & Premier Cuvee in YP medium (A) or SC medium (B) containing glucose 200 g/L.

4. Verification of genome engineering feasibility based on CRISPR-Cas9 in industrial *S. cerevisiae*

To demonstrate suitability and efficiency of CRISPR-Cas9 based genome engineering techniques, *S. cerevisiae* N1 was conducted Cas9-mediated *URA3* disruption (Figure 4). *URA3* encodes Orotidine 5'-phosphate decarboxylase (ODCase) that catalyzes the synthesis of pyrimidine ribonucleotides (Flynn PJ., Reece RJ., 1999). So, the *URA3* mutant can be easily recognized by replica plating on YNB ura- (ura deficient condition).

After carried out procedure of Cas9-mediated *URA3* disruption, colonies predicted as *URA3* disruption *S. cerevisiae* N1 were obtained. *URA3* disruption was confirmed by sequencing (data not shown). A point mutation was created in the middle of *URA3* region by homologous recombination with mutated repair DNA. The resulting amino acid sequence was changed from serine to TAA stop codon at the 81th codon of the Ura3 protein.

Additionally, both p414_Cas9-AUR and p42H_gURA3-HYG plasmids could be easily cured out from the engineered strain by culturing under nonselective culture conditions for 24 hours (data not

shown). This means that if the plasmid is cured after carry out the desired genome engineering, it did not leave any unnecessary DNA fragments and scars. This could be an effective strategy to solve the GMO issues caused by using engineered *S. cerevisiae* for food applications (Zhang GC. et al., 2014)

5. CRISPR-Cas9 based metabolic engineering of laboratory *S. cerevisiae* D452-2

5.1. Construction of engineered *S. cerevisiae* D452-2 for acrylamide reduction

To prove the rationality of metabolic engineering strategies for acrylamide reduction, the laboratory *S. cerevisiae* D452-2 was engineered prior to industrial *S. cerevisiae* N1. Strategies to promote asparagine consumption in *S. cerevisiae* are as follows. *S. cerevisiae* D452-2 was metabolic engineered according to the genome editing strategies.

First strategy: Overexpression of the asparagine permease gene (*GAP1*; general amino acid permease). If asparagine levels in the cell

are increased, asparagine can be consumed quickly. To overexpress *GAP1* without regulation by NCR, the *GAP1* promoter on the *S. cerevisiae* chromosome was replaced with the constitutive strong *GPD* promoter (Debailleul F. et al., 2013).

Second strategy: Overexpression of asparagine degradation genes (*ASP1*; cytosol asparaginase, *ASP3*; cell-wall asparaginase). Asparagine present in the cell or outside the cell can be rapidly degraded into aspartate and ammonia (NH_4^+) by asparaginase (Lee SJ. et al., 1995; Oliveira EM. et al., 2003). To overexpress *ASP1* or *ASP3* without NCR regulation, the *ASP1* or *ASP3* promoter on the *S. cerevisiae* chromosome was replaced with the *GPD* promoter.

Third strategy: Control of the asparagine metabolism regulatory genes to alleviate NCR (*URE2*; inhibitor of amino acid metabolism transcriptional activator, *GZF2*; negative transcription factor of NCR-sensitive genes, *GAT1*; positive transcription factor of NCR-sensitive genes). If the regulatory genes involved in overall amino acid metabolism were controlled, asparagine can be degraded rapidly under excess nitrogen conditions. The stop codon was inserted between the ORFs of *URE2* and *GZF3* to delete the corresponding gene. In addition,

the *GAT1* promoter was replaced with the *GPD* promoter so that it was not regulated by NCR (Cooper TG., 2002).

5.2. Flask fermentation of engineered *S. cerevisiae* D452-2 strains in asparagine rich YPD media

To assess an asparagine consumption ability of each engineered *S. cerevisiae* D452-2 strains, batch fermentations were performed in asparagine rich YPD media (Figure 9).

In order to compare the asparagine consumption rates of each strain, the residual asparagine levels were plotted (Figure 10). If the strain is not treated (black, X), asparagine is not spontaneously degraded in the medium, as the amount of asparagine in the medium remains unchanged for 12 hours. In the case of D452-2, the asparagine consumption rate was about 0.148 g_Asn/L/h, and the strategy to transport asparagine into the cell or to consume asparagine in the cell was not effective. On the other hand, in the case of D_Pasp3 overexpressing cell-wall asparaginase (*ASP3*) that degrades asparagine outside the cell, all asparagine in the medium was consumed in 3 hours. The asparagine consumption rate of D_Pasp3 was 1.463 g_Asn/L/h which is increased by 10 times, compared with the D452-2 WT strain.

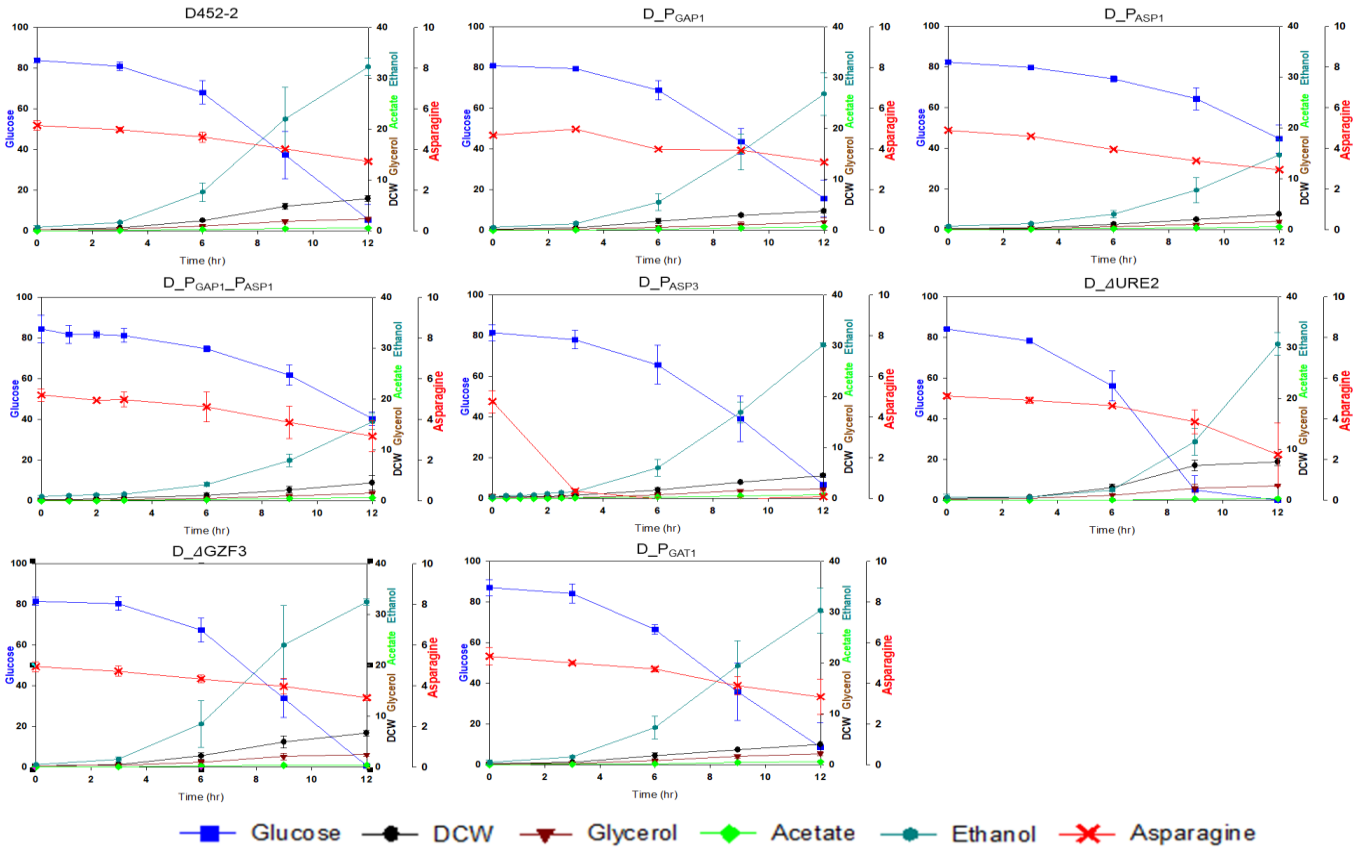


Figure 9. Flask fermentation of engineered *S. cerevisiae* D452-2 strains in asparagine rich YPD media.

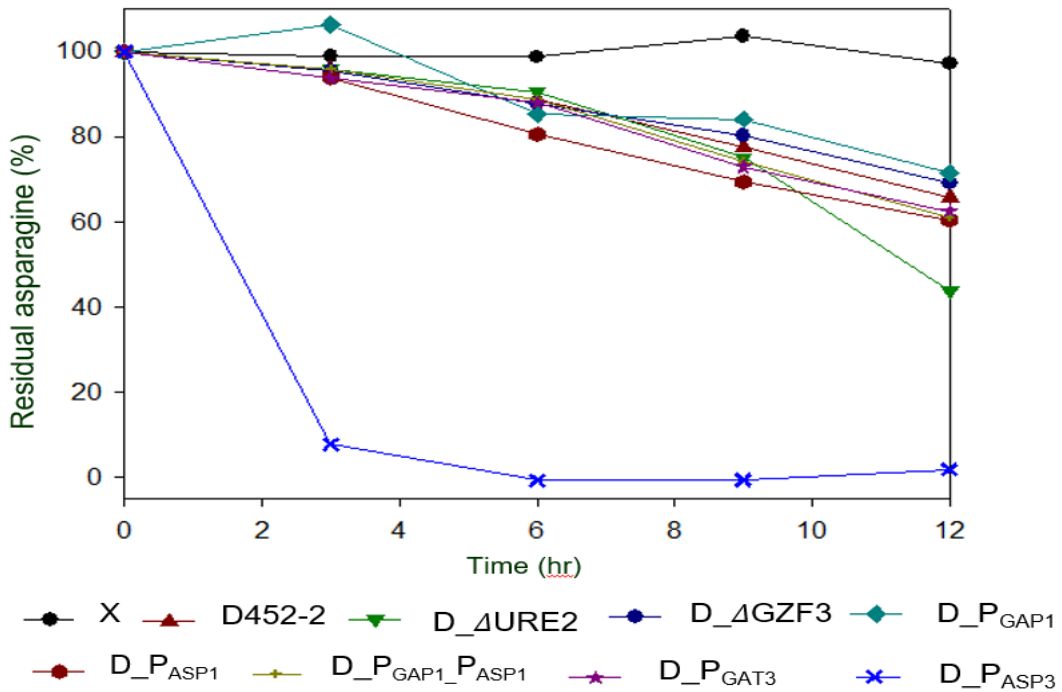
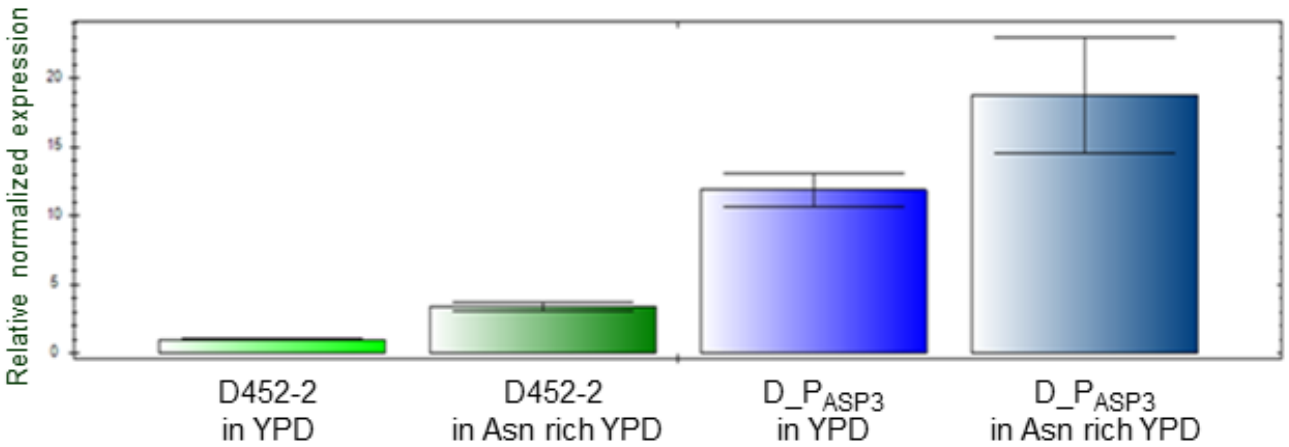


Figure 10. Residual asparagine levels in the asparagine rich YPD media.

5.3. Transcription level of the strains with *GPD* promoter replacement

To investigate the effect of the *GPD* promoter replacement at the mRNA transcription level, RT-qPCR was performed. *S. cerevisiae* D452-2 and D_P_{ASP3} were cultured in YPD and asparagine rich YPD medium. All the samples for transcription analysis were collected during the mid-exponential phase. The transcription level of *ASP3* was determined by using RT qPCR as described in 5.5.

In the case of YPD medium, the amount of *ASP3* expression was increased by 11.9 fold in *S. cerevisiae* D_P_{ASP3}, compared with *S. cerevisiae* D452-2 (Figure 11). In the case of asparagine rich YPD medium, the amount of *ASP3* expression was increased by 5.5 fold in *S. cerevisiae* D_P_{ASP3}, compared with *S. cerevisiae* D452-2.



Relative transcriptional level	YPD	Asparagine rich YPD
D452-2	1.0±0.09	3.39±0.31
D_P$_{ASP3}$	11.86±1.17	18.74±4.23

Figure 11. Relative transcription levels of *ASP3* in *S. cerevisiae* D452-2 and D_P$_{ASP3}$ in YPD and asparagine rich YPD media.

5.4. Target selection for application of industrial *S. cerevisiae*

Based on the results in laboratory *S. cerevisiae* D452-2, *ASP3* of the industrial strain *S. cerevisiae* N1 have to be overexpressed to consume asparagine quikly. However, there is a limit to apply the following experimental results directly to *S. cerevisiae* N1. This is because *ASP3* did not exist in *S. cerevisiae* N1 (data not shown). As mentioned earlier, *ASP3* has a variable copy number. *ASP3* is completely absent in some other laboratory or industrial strains (League GP. et al., 2012).

Thus, in order to overexpress *ASP3* in *S. cerevisiae* N1, the *ASP3* expression cassette have to be constructed and inserted into appropriate targets of the *S. cerevisiae* N1 chromosome. The criteria of the appropriate targets were selected based on the deletion target, *URE2* and *GZF3*.

To investigate the effect of deletion of *URE* or *GZF3* on the asparagine consumption rate when combined with *ASP3* over-expression, a combination strain was constructed using D452-2. To assess asparagine consumption ability of the combination strains, D_ *Δ*URE2_P_{ASP3} and D_*Δ*GZF3_P_{ASP3}, batch fermentations were performed in asparagine rich YPD media (Figure 12 & Table 9).

Only a single deletion of *URE2* showed a slight positive effect on asparagine consumption rate. However, when *URE2* deletion combined with *ASP3* overexpression, asparagine consumption rate was 10 times faster than that of WT. However, in the case of D_ΔURE2_PASP3, the glucose consumption rate and cell growth were slowed down, which was considered having influenced the overall fermentation ability.

As in the case of *URE2*, the deletion of *GZF3* alone showed a slight positive effect on asparagine consumption rate. However, when *ASP3* overexpression was combined with *GZF3* deletion, asparagine consumption rate was 9 times faster than WT. Compared to the combination of *URE2* and *ASP3*, the asparagine consumption rate is slightly lower, but the overall fermentation ability is similar to that of the WT strain.

In summary, the combination of *URE2* or *GZF3* deletion with *ASP3* overexpression had no synergistic effect. However, these deletions did not affect the overexpression of *ASP3*. Therefore, we selected *URE2* and *GZF3* as targets for N1 application.

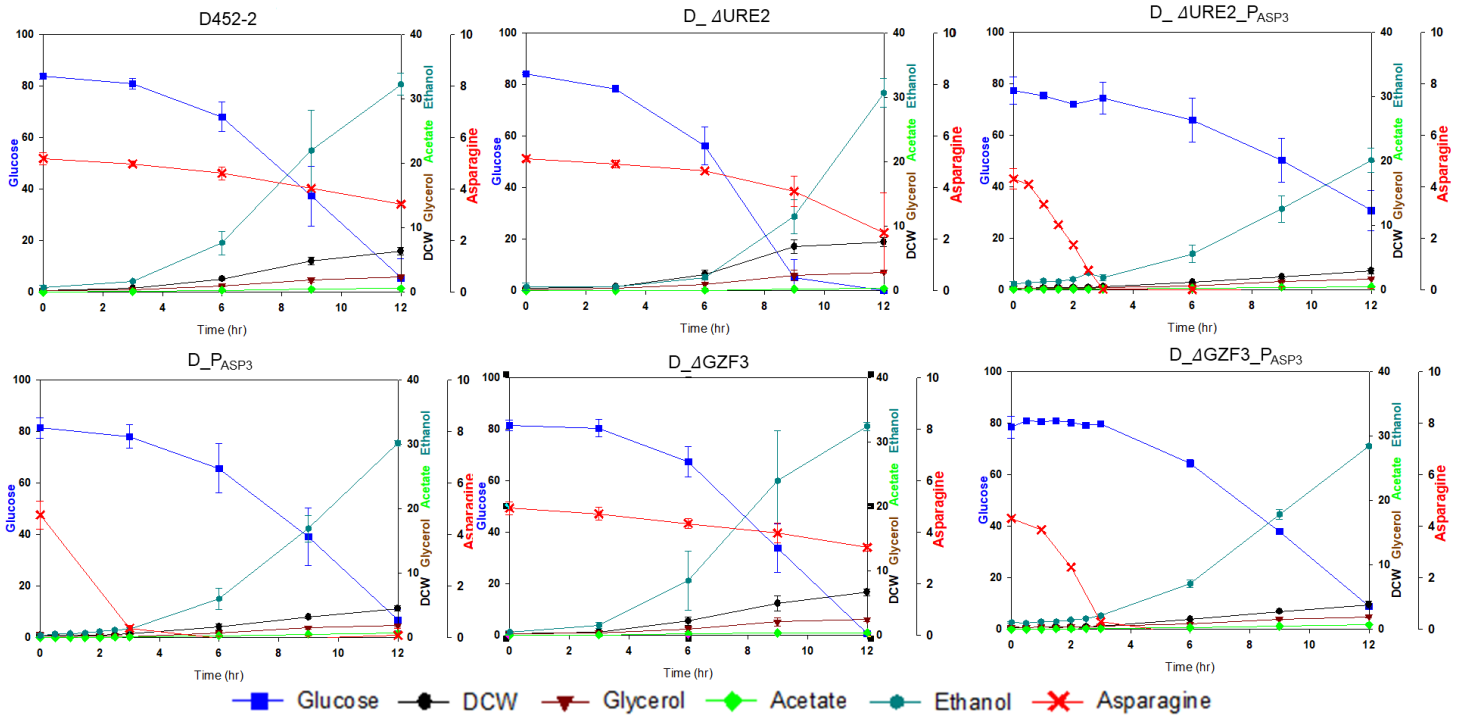


Figure 12. Flask fermentation of each combination strain in asparagine rich YPD media

Table 9. The results of flask fermentation of each combination strain in asparagine rich YPD media

Strains	Initial concentration of Asn (g/L)	Final concentration of Asn (g/L)	Consum-ption time (h)	Asn consumption rate (g/L/h)	Average DCW (g/L)	Specific Asn consumption rate (g/L/h/DCW)
D452-2	5.19±0.24	3.42±0.04	12	0.15	2.84	0.05
D_P_{ASP3}	4.76±0.55	0.37±0.11	3	1.46	0.46	3.18
D_ΔURE2	5.13±0.11	2.25±1.55	12	0.24	3.58	0.07
D_ΔURE2_P_{ASP3}	4.31±0.41	0.02±0.04	3	1.43	0.39	3.79
D_ΔGZF3	4.95±0.25	3.42±0.14	12	0.13	2.95	0.05
D_ΔGZF3_P_{ASP3}	4.31±0.00	0.29±0.00	3	1.34	0.41	3.31

6. CRISPR-Cas9 based metabolic engineering of industrial *S. cerevisiae* N1

6.1. Construction of engineered *S. cerevisiae* N1 for acrylamide reduction

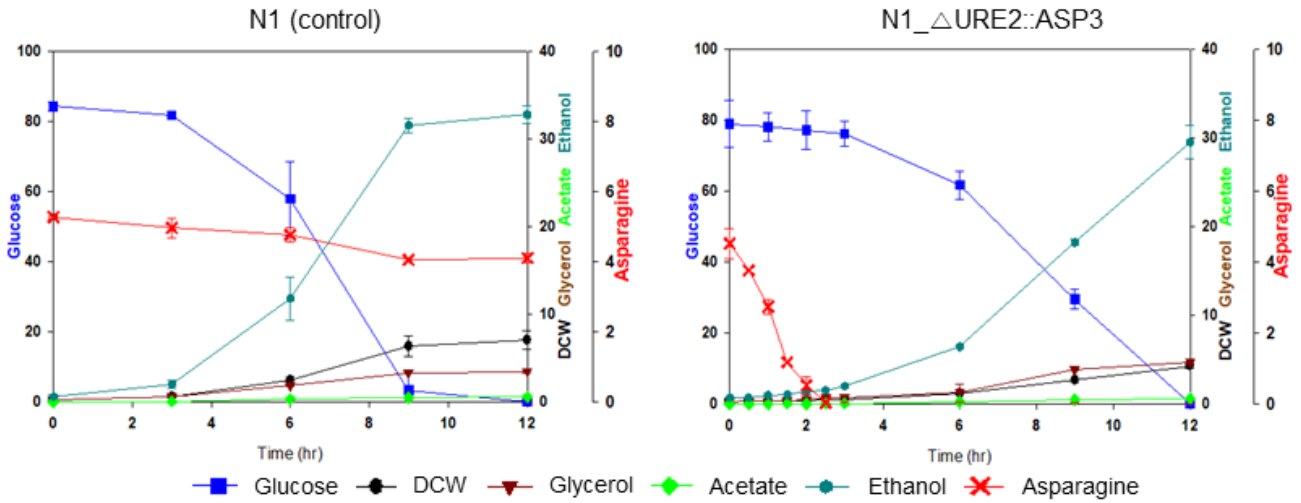
As mentioned above, there is no *ASP3* in *S. cerevisiae* N1. So, in order to express *ASP3,* the *ASP3* expression cassette has to be inserted into the appropriate target on the chromosome. According to the result of 5.4, it was confirmed that *ASP3* expression was not influenced by *URE2* or *GZF3* deletion. Therefore, the *ASP3* cassette was inserted in the *URE2* and *GZF3* regions on the chromosome of *S. cerevisiae* N1.

6.2. Flask fermentation of engineered *S. cerevisiae* N1 strains in asparagine rich YPD media

Figure 13 shows the results when the *ASP3* cassette was inserted into the *URE2* region on the N1 chromosome. The asparagine consumption rate of N1_ΔURE2::ASP3, 2.00 g_Asn/L/h, was increased by 20.6 times, compared with the N1 WT strain. However, similar to D_ ΔURE2_Pasp3 which *ASP3* was overexpressed with *URE2* deletion, the

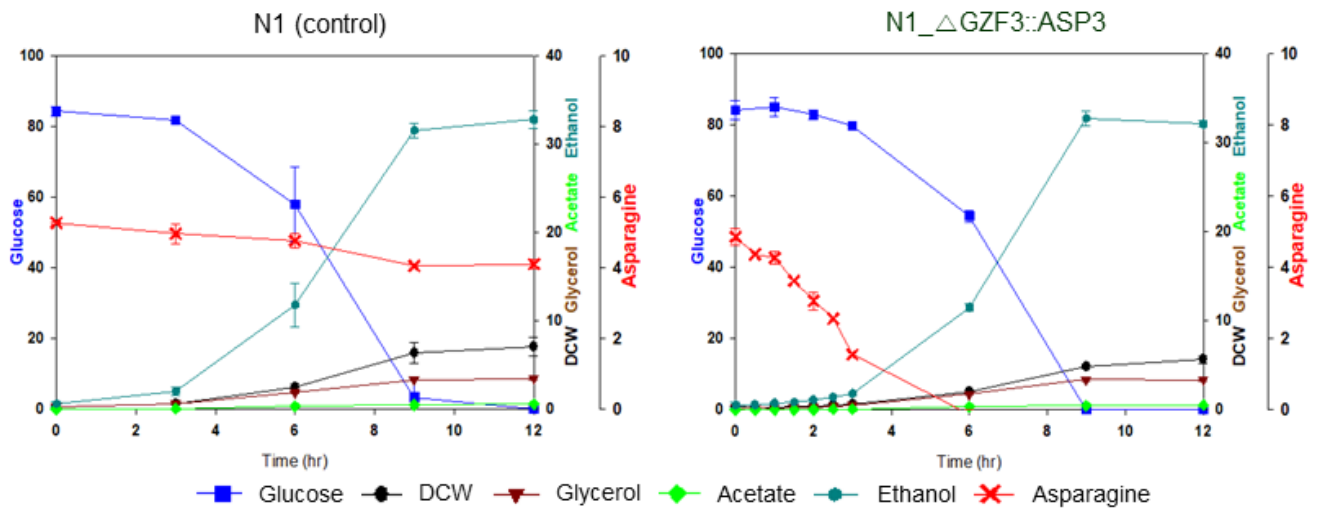
glucose consumption rate was reduced, resulting in a change in fermentation ability.

Figure 14 shows the results when the *ASP3* cassette was inserted into the *GZF3* region on the N1 chromosome. The asparagine consumption rate of N1_*Δ*GZF3::ASP3, 1.10 g_Asn/L/h, was increased by 11.4 times, compared with the N1 WT strain. This is lower than the *URE2* site but has the advantage that the overall fermentation pattern is similar to N1 WT.



Strains	Initial Concentration of Asn (g/L)	Final concentration of Asn (g/L)	Consumption time (h)	Asn consumption Rate (g/L/h)	Average DCW (g/L)	Specific Asn consumption rate (g/L/h/DCW)
N1	5.28±0.03	4.11±0.13	12	0.10	3.38	0.03
N1_△URE2::ASP3	4.52±0.42	0.52±0.25	2	2.00	0.36	5.50

Figure 13. Flask fermentation of N1_*Δ*URE2::ASP3 in asparagine rich YPD media.



Strains	Initial Concentration of Asn (g/L)	Final concentration of Asn (g/L)	Consumption time (h)	Asn consumption Rate (g/L/h)	Average DCW (g/L)	Specific Asn consumption rate (g/L/h/DCW)
N1	5.28±0.03	4.11±0.13	12	0.10	3.38	0.03
N1_△GZF3::ASP3	4.86±0.24	0.13±0.15	Less than 6	1.10	0.46	2.41

Figure 14. Flask fermentation of N1_ΔGZF3::ASP3 in asparagine rich YPD media.

6.3. Application of engineered *S. cerevisiae* N1 strains in food samples

Because a large amount of acrylamide was detected in potato chips and breads, wheat-based dough and potato-based dough were selected for food applications. The engineered industrial strains, N1_ΔURE2::ASP3 and N1_ΔGZF3::ASP3, were treated with wheat-based dough and potato-based dough. Each kneaded dough was fermented at 30℃ for 3 hours and samples were collected every 30 min to quantify the amount of asparagine in the doughs. After three hours, the wheat-based dough was heated in an oven at 200°C for 20 min and the potato-based dough was fried in oil at 170°C for 5 min.

As a result, it was confirmed that both N1_ΔURE2::ASP3 and N1_ΔGZF3::ASP3 rapidly decreased the amount of asparagine in the food doughs, compared with the N1 WT strain (Figure 15A & Table 10). After that, when doughs were heated as complete food, they contained about 95% less acrylamide than N1-treated foods (Figure 15B & Table 10).

Table 10. Acrylamide levels in food samples

(Unit: μg/kg)	T1	T2	T3	Average	Standard deviation
Wheat-based bread					
N1	6789	6576	6439	6601	176.4
N1_ΔURE2::ASP3	374	358	343	358	15.5
N1_ΔGZF3::ASP3	769	742	719	743	25.0
Potato-based chip					
N1	5598	5683	5401	5560	144.7
N1_ΔURE2::ASP3	72	74	69	71	2.5
N1_ΔGZF3::ASP3	140	143	134	139	4.6

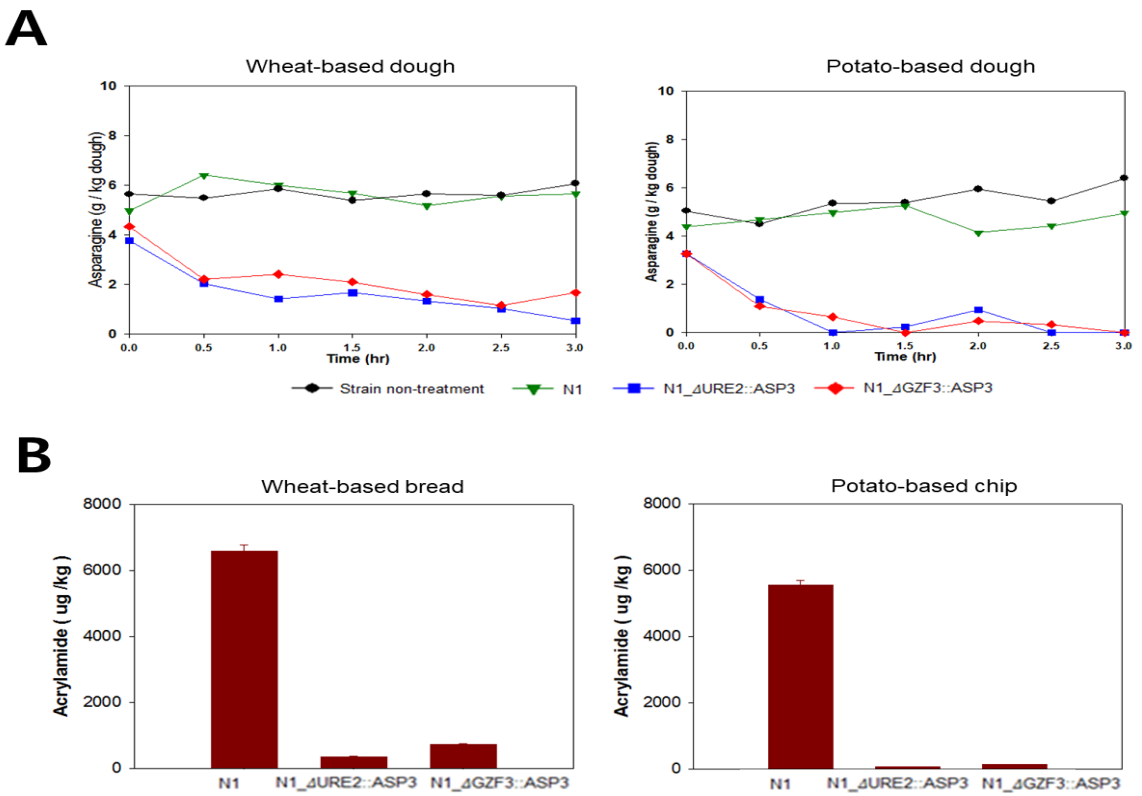


Figure 15. Asparagine (A) & Acrylamide (B) concentrations in food sample after N1_ΔURE2::ASP3 and N1 ΔGZF3::ASP3 were treated.

IV. CONCLUSIONS

This thesis can draw the following conclusions:

(1) 7 kinds of *S. cerevisiae* strains were isolated from nuruk and gene characteristics and fermentation ability were measured. Among the 7 *S. cerevisiae* strains, *S. cerevisiae* N1 showed the best glucose fermentation ability. Especially, *S. cerevisiae* N1 was expected to be used efficiently under acidic pH conditions or osmotic stress conditions as compared with the other isolated *S. cerevisiae* strains. So, *S. cerevisiae* N1 was chosen as the host strain for further studies. After that, to demonstrate suitability and efficiency of CRISPR-Cas9 based genome engineering, *S. cerevisiae* N1 was conducted Cas9-mediated *URA3* disruption. As a result, N1_ΔURA3, in which the stop codon was created in the middle of the *URA3* region, was obtained. After carry out the desired genome engineering, the plasmid was cured easily and it did not leave any unnecessary DNA fragments and scars. This mean that this system could be an effective strategy to solve the GMO issues caused by using engineered *S. cerevisiae* for food applications.

(2) As a main target of applying engineered industrial *S. cerevisiae* to food industry, isolated industrial *S. cerevisiae* N1 was applied to acrylamide reduction. To prove the rationality of metabolic engineering strategies for acrylamide reduction, the laboratory strain *S. cerevisiae* D452-2 was engineered prior to industrial *S. cerevisiae* N1. *S. cerevisiae* D452-2 was metabolic engineered by overexpression of the general amino acid permease (*GAP1*), overexpression of asparaginases (*ASP1*, *ASP3*), regulation of nitrogen catabolite repression (NCR). Engineered strains were grown in flasks containing asparagine rich YPD media to test the ability of asparagine degradation. For the laboratory *S. cerevisiae* D_P_{ASP3} strain overexpressing *ASP3* (cell-wall asparaginase) through a promoter replacement, the asparagine consumption rate was enhanced by 9.9 times relative to the D452-2 WT strain. Therefore, the *ASP3* overexpression strategy was applied to the industrial N1 strain. Since *APS3* did not exist in N1, an *ASP3* expression cassette was constructed and inserted into either the *URE2* site or the *GZF3* site of the N1 chromosome to construct N1_ΔURE2::ASP3 and N1_ΔGZF3::ASP3. To determine the asparagine consumption ability of the two strains, engineered

strains were grown in flasks containing asparagine rich YPD media. As a result, for the *S. cerevisiae* N1_ΔURE2::ASP3, the asparagine consumption rate increased by 20.6 times, compared with the N1 WT strain. For the *S. cerevisiae* N1_ΔGZF3::ASP3, the asparagine consumption rate increased by 11.4 times, compared with the N1 WT strain. When they were applied to food doughs, asparagine was consumed fast to result in 95% less acrylamide than the control strain N1 WT.

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국 문 초 록

잠재적 발암물질로 알려져 있는 아크릴아마이드는 감자칩이나 빵과 같은 식품에 다량 포함되어 있다. 이는 식품의 가공 중 식품 내 아스파라진이 마이야르 반응을 통해 아크릴아마이드로 전환되하기 때문이다. 아크릴아마이드의 저감화의 핵심은 아스파라진을 저감화하는 것이다.

본 연구의 목적은 CRISPR-Cas9 기반 대사공학을 통해 아스파라진을 선택적으로 소모하여 아크릴아마이드 저감화에 사용될 수 있는 산업용 *S. cerevisiae* 의 구축이다. 본 연구에서 산업용 *S. cerevisiae* 의 대사공학은 식품 적용의 안전성 확보를 위해서 CRISPR-Cas9 유전자 편집 기술은 이용하였다.

먼저, 아크릴아마이드 저감화에 사용될 산업용 *S. cerevisiae* 을 분리하기 위하여 누룩을 이용하였으며, 10 종의 누룩으로부터 7 종의 *S. cerevisiae* 을 분리하였다. 그 중 빠른 당 소모 속도와 더불어 산성 pH 조건 또는 삼투압 스트레스 조건에서 효과적으로 사용될 수 있는 가능성을 지닌 *S. cerevisiae* N1 을 모균주로 선정하였다. 분리된 N1 균주는 추후 진행될

대사공학의 적합성을 판단하기 위하여, CRISPR-Cas9 기반의 *URA3* 의 결손이 진행되었다. 그 결과, 효과적으로 *URA3* 부위에 종결 코돈이 삽입된 N1_ΔURA3 가 구축되었고, 이 후 플라스미드는 쉽게 제거되어 불필요한 DNA 시퀀스나 흉터는 남지않았다. 이는 CRISPR-Cas9 방법이 대사공학으로 구축된 산업용 *S. cerevisiae* 를 식품 산업에 사용할 때 발생할 수 있는 GMO 문제를 해결하는 효과적인 전략이 될 가능성이 있음을 의미한다.

다음으로 아크릴아마이드 저감화에 사용될 수 있도록 대사공학이 진행되었다. 앞서 분리된 산업용 균주 N1 의 대사공학에 앞서, 유전자 편집에 의한 효과를 확인하기 위하여 실험용 균주인 *S. cerevisiae* D452-2 에서 대사공학이 선행되었다. 아스파라진 소모를 촉진시킬 것이라 예상되는 general amino acid permease (*GAP1*)의 과발현, asparaginase (*ASP1, ASP3*)의 과발현, nitrogen catabolite repression 의 조절 전략에 따라 *S. cerevisiae* D452-2 에서 대사공학을 수행하였다. 5 g/L 의 아스파라진이 첨가된 YPD 배지에서 플라스크 발효를 통해

효과를 확인한 결과, 특이적으로 *ASP3* (cell-wall asparaginase)을 프로모터 교체를 통해 과발현 시킨 *S. cerevisiae* D_P_{ASP3} 에서 D452-2 WT 균주에 대비하여 9.9 배 빠른 아스파라진 소모 속도를 보였다.

위 결과에 따라 *ASP3* 과발현 전략을 산업용 균주인 N1 에 적용하였다. N1 에는 *APS3* 가 존재하지 않았기 때문에, *ASP3* 발현 cassette 을 제작하여 N1 의 *URE2* 와 *GZF3* 부위에 각각 삽입하였다. 그 결과 *S. cerevisiae* N1_ΔURE2::ASP3 와 *S. cerevisiae* N1_ΔGZF3::ASP3 를 구축하였다. 두 균주를 5 g/L 의 아스파라진이 추가로 첨가된 YPD 배지에서 플라스크 발효를 진행하였고, 그 결과 두 균주는 기존 N1 WT 균주 대비 각각 20.6 배, 11.4 배 빠르게 아스파라진을 소모하였다. 이후 두 균주를 밀가루 반죽과 감자반죽에 적용하였을 때에도 빠르게 반죽 내 아스파라진을 소모하는 것을 확인할 수 있었고, 반죽이 실제로 식품으로서 조리되었을 때는 기존 N1 WT 균주 대비 약 95% 이상 아크릴아마이드가 저감화되는 효과를 확인할 수 있었다.

주요어: 산업용 *Saccharomyces cerevisiae*, 식품 산업, 대사 공학, CRISPR-Cas9, 발효, 아스파라진, 아크릴아마이드 저감화, cell-wall 아스파라진분해효소

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