Master's Thesis of Science in Agriculture

Flavor enhancement by developing CRISPR-Cas9 technology in *Saccharomyces cerevisiae*

CRIPSR-Cas9 기술을 이용한 *Saccharomyces cerevisiae*의 향미 증진 연구

August 2022

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for the Degree of Master of Science in Agriculture

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Abstract

Saccharomyces cerevisiae is used worldwide to produce ethanol and various by-products. Aroma compounds are the desired group of secondary metabolites produced by *S. cerevisiae.* In a previous study, an interesting result was accidentally discovered, which has the improvement of tropical and rosy flavors in mutants after the CRISPR-Cas9 system without other side effects. Previous strains, applied CRISPR system, indicated that both deletion and disruption of *CAR1* gene result in improved specific flavor production.. Here, the hypothesis is tested by inactivating the *CAR1* gene of another *S. cerevisiae* species using CRISPR-Cas9 genome editing. Additionally, in this research, the CRISPR-Cas9 system was built to establish sgRNA synthesis *in vitro* without foreign plasmid. And it could make non-sense mutation and complete mutation of the target gene, the *CAR1* in SNUws strain. All of the SNUws mutants create equally improved production of specific flavors than wild type including other strain types such as SNUit strain and KCCM 51299 strain using by GC-MS analysis. As expected, results explained an increase in the relative abundance of aroma compounds such as isoamyl alcohol and phenethyl alcohol in modified the microbes with development of the CRISPR-Cas9 system. The connection from the inactivation of the *CAR1* gene to both isoamyl alcohol like tropical fruit flavor and phenethyl alcohol like rose flavor is unknown. This study shows that although it is not clear that the arginase concentration of the gene affects the flavor pathways until now, there is a specific link. Therefore, more research is needed in the future. And yeast's unique volatile compounds will provide important information to making natural additives in modern the fragrance industries.

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Research background

1. Yeast

In microbiology, yeast is classified into the fungus kingdom and unicellular eukaryotic cells. The yeast has now been identified as a model representing useful eukaryotes whose genome can be manipulated easily. Although yeast is a eukaryotic cell, it has a simpler genome than other eukaryotic organisms and applies to many technical advantages that permit rapid progress in the molecular genetics of prokaryotes and their viruses (Sherman, 2002). Among yeasts, *Saccharomyces cerevisiae* is a common representative model of baker's yeast and brewer's yeast. *S. cerevisiae* has been associated with human society, leading to the idea that its use in fermentation leads to its differentiation of fermented products. Properties of yeast that make it suitable for biological research contain rapid growth, dispersed cells, the ease of replica plating, and mutation isolation. Especially, a Well-known genetic system is essential for applying a highly versatile DNA transformation system. Yeast sources are available commercially and can be provided cheaply to research (Sherman, 2002).

1.1 Yeast genome

Through genome evolution, high throughput sequencing technology can analyze the population structure of *S. cerevisiae* (Liti et al., 2009). Yeast genome is similar to eukaryotes that include mammal to human. So, analyzing simple yeast genome is helped to understand gene location, structures and expression level. Also it is cost effective to identify gene function as yeast model than other higher organisms (Levy, 1994). Whole genome sequence of *S. cerevisiae* is completed by international collaboration of 600 scientists and published article in 1996. It is first to put entire eukaryotic genome puzzle that comprise large size. A number of public datas that have the mapping nucleotide and protein sequence information come from 16 yeast chromosomes. Genetic map is defined the haploid set of 16 chromosomes and 6000 genes. The complete genome sequence has open reading frames (ORFs) that encode protein specially. The yeast genome is compact than other eukaryotes due to placing at each chromosome. In the Table 1., DNA sequencing information has many different genomic elements that cover length, unit, the number of gene, open reading frames (ORFs), untranslated region (UTR), intron, and protein (Table 1.1.) (Goffeau et al., 1996).

Table 1.1. Genome sequence elements of *S. cerevisiae*.

Element	Chromosome number																
	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	XV	XVI	Total
Sequenced length (kb)	230	813	315	1,532	577	270	1,091	563	440	745	667	1,078	924	784	1,091	948	12,068
Name of unit				*ENA2* and Y	tel	*CUP1*				rDNA and Y'							
Length of unit (kb)				4 and 7	<1	2				9 and 7							
Number of units				2 and 2	1	13				±140 and 2							
Length of repeats (kb)				8 and 14	<1	26				1,260 and 14					1,321		
Total length (kb)	230	813	315	1,554	577	271	1,091	589	440	745	667	2,352	924	784	1,091	948	13,389
ORFs (n)	110	422	172	812	291	135	572	288	231	387	334	547	487	421	569	497	6,275
Questionable proteins (n)	3	30	12	65	13	5	57	12	11	29	20	41	30	23	3	36	390
Hypothetical proteins (n)	107	392	160	747	278	130	515	276	220	358	314	506	457	398	566	461	5,885
Introns in ORFs (n)	4	18	4	30	13	5	15	15	8	13	11	17	19	15	15	18	220
Introns in UTR (n)	0	2	0	1	2	0	5	0	0	0	0	3	0	2	0	0	15
tRNA genes (n)	2	13	10	27	20	10	36	11	10	24	16	22	21	16	20	17	275
snRNA genes (n)	1	1	2	1	2	0	3	1	1	4	1	3	8	3	7	2	40

1.2 Yeast Engineering

Yeast has played a key role in fermentation since ancient times. Yeast is used for baking and alcoholic fermentation. Traditional yeast engineering is composed of time-consuming methods like hybridization and evolutionary engineering. These methods are also not only need lots of costs but lack stability of change. Another example of the previous methods in brewing has been developed by switching fermentation conditions and adding substrates from other collaborations, which can change through producing a variety of compounds (Table 1.2.) (G. G. Stewart, 2017b). In 1996, due to the complete genome sequence of *S. cerevisiae*. yeast engineering is changed to focus genetic modification (Fig. 1.1.).

Table 1.2. Correlation between various fermentation conditions and possible results. Possible results contained diversity of aroma compound flux.

Fermentation condition Variable	Possible results
Higher temperature	Esters ↑ alcohols ↑
Trub present in the fermenter	Esters ↓ Alcohols ↑
Fedding nutrients of yest added to wort	Esters ↑ Alcohols ↑
Increased wort oxygen at pitching	Esters ↓
Pressure fermentation	Esters ↓ Alcohols ↓
Higher wort gravity	Esters ↑ Alcohols ↑
Increased wort gravity and maltose	Esters↓
Increased wort lipids	Esters↓

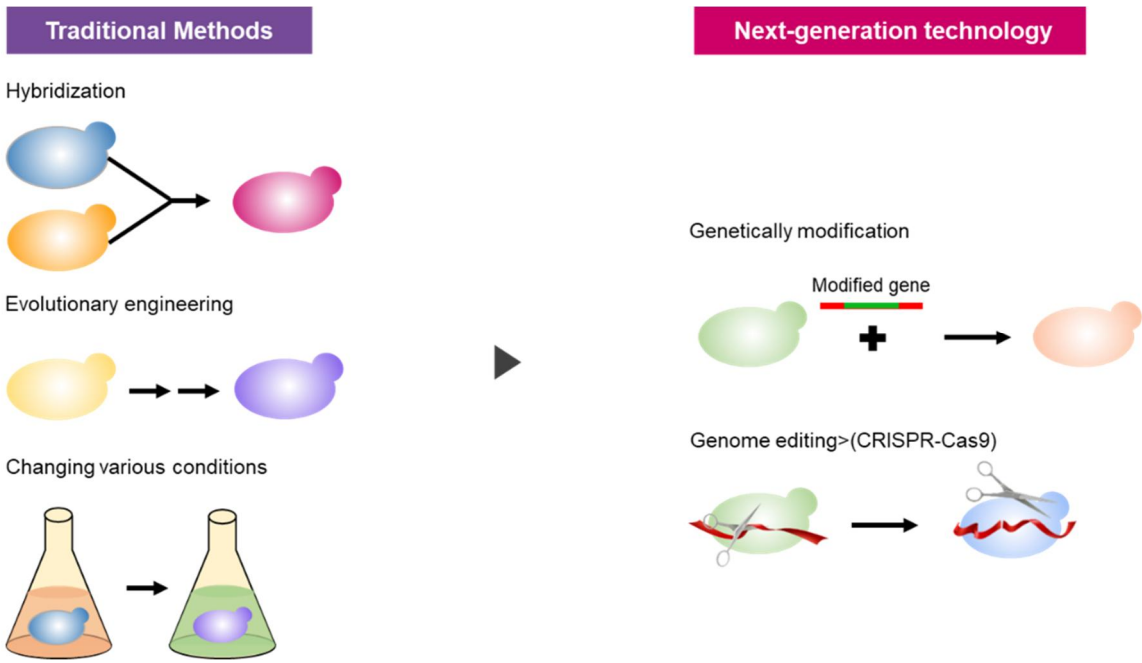

Fig. 1.1. Comparison of traditional yeast technology with next generation technology. Both traditional methods and next-generation technology also made changes of the flavors phenotype of yeast strain. However there was a difference in time and target efficiency.

2. Secondary metabolites

Yeast has a complex chemical flux that is the result of numerous metabolic reactions in yeast cells. Various metabolic pathways in living cells are connected to other pathways continuously. Secondary metabolites, in addition to major metabolites, produce a wide range of different types. In secondary metabolites, some substances are beneficial to humans while others are classified as harmful. Beneficial metabolites for humans are typically ethanol and flavor agents, especially in the food industry and the alcoholic industry. In the fermentation of beer, wine, and liquor, fermented sugars are metabolized into ethanol, by-products, and CO_2 (Fig. 1.2.). These metabolites influence the properties of alcoholic beverages but in flavor properties they have little impact. Volatile compounds such as esters and higher alcohols are an important part of the aroma of alcoholic beverages, determined by secondary metabolites of the yeast (G. Stewart, 2017).

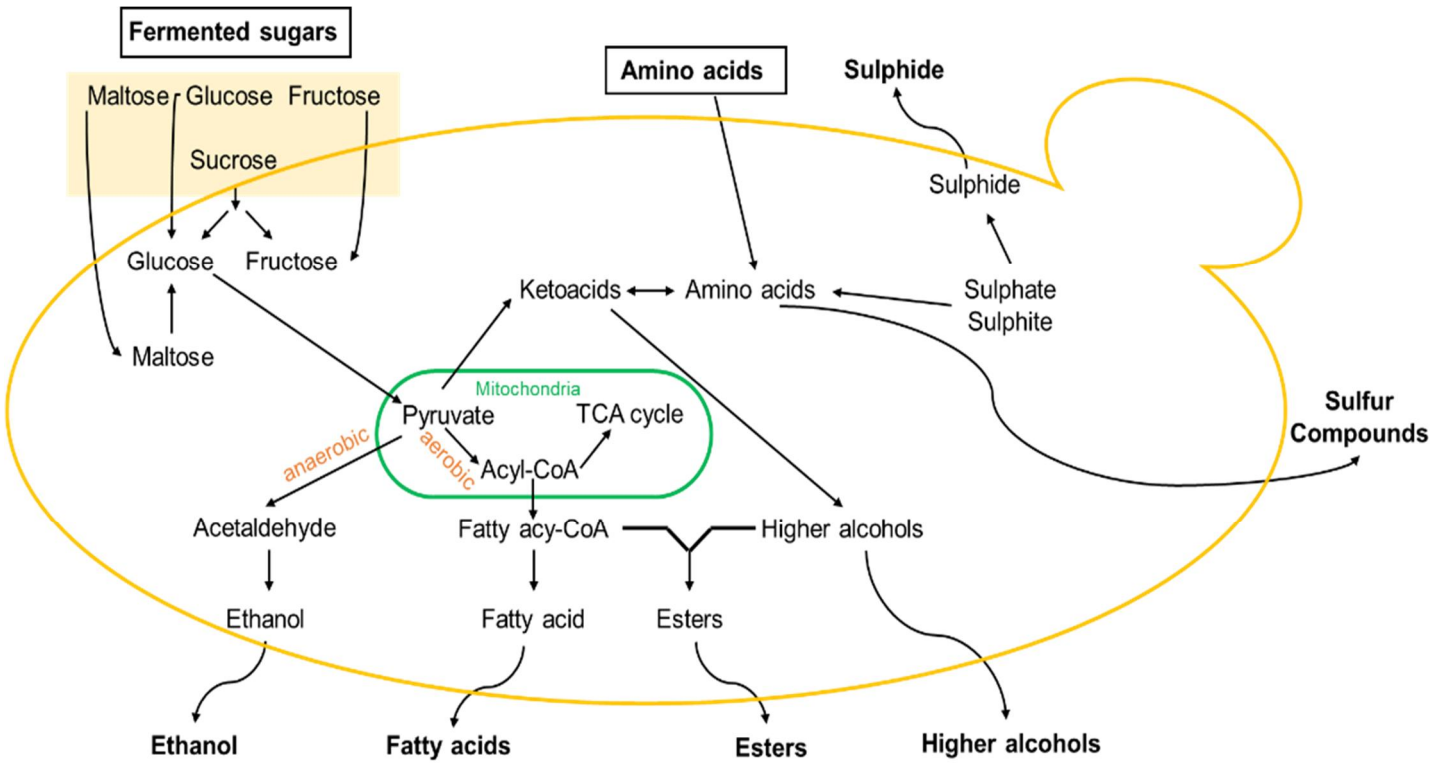

Fig. 1.2. Schematic metabolic pathways of yeast in fermentation.

2.1 Flavor agents .

Many flavor agents come not from supplied sources but rather from primary or secondary compounds formed during yeast metabolism reactions related to alcoholic fermentation (Styger et al., 2011). During fermentation, flavor compounds are metabolized via various metabolic pathways through microbial community activity (Meng et al., 2015). The flavor diversity and production level also depend on the yeast strain-specific properties (Lambrechts & Pretorius, 2019). Flavoring agents arise from a connected process that begins with fermented sugars and amino acids. In yeast fermentation, the major volatile compound is ethanol, glycerol, and CO_2. Although these are weak flavoring agents, they have the fundamental basic flavor of yeast fermentation. Key compounds of flavor specifically determine flavor characteristics. They could become both amino acids and sugars. Esters, Sulphur compounds, higher alcohols, organic acids and carbonyl compounds are example of flavor compounds via yeast metabolisms (He et al., 2014). Yeast strain can communicate with culture medium adding various substrates and fermentation condition, which used to produce combination of attractive flavor. Also yeast can have many different flavor type depending on their genetic factors and environment (Fig. 1.3.)

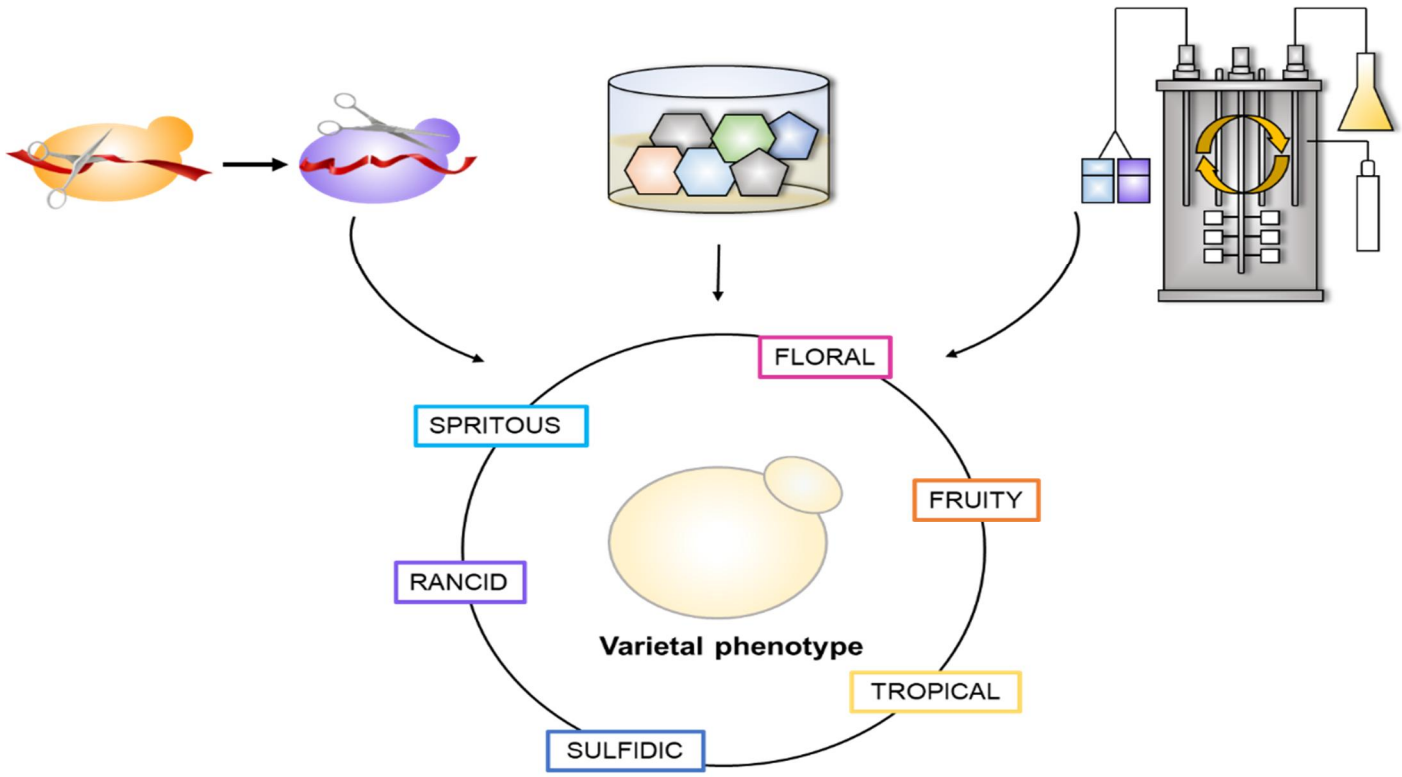

Fig. 1.3. Varietal phenotype of yeast that originated from different flavor compounds.

2.1.1 Esters

Ester biosynthesis pathways are mainly broaden way by enzyme catalyzed condensation reaction which catalyzed an organic acid with higher alcohol by acyl-coenzyme A (acyl-CoA) (Fig. 1.2.) (G. G. Stewart, 2017a). The acetate esters are formed from acetic acid with ethanol or high alcohol. Alcohol acetyl transferases (AATase) encoded *ATF1* and *ATF2* genes are usually used for the formation of acetate esters. Also, acyl-CoA: ethanol O-acyltransferases (AEATases) encoded *EHT1* and *EEB1* genes are employed in both synthesis of ethyl esters and the hydrolysis of esters. The hydrolyzing esterase in the *IAH1* gene plays a role in the rate of ester accumulation (He et al., 2014). Volatile esters that offer flavor to the final product can divide into 6 components: ethyl acetate (solvent-like flavor), isoamyl acetate (banana flavor), isobutyl acetate (fruity flavor), phenylethyl acetate (roses and honey flavor), ethyl hexanoate (sweet apple flavor), and ethyl octanoate (sour apple flavor) (Table 1.4.). Esters often create a synergy effect with other agents at concentrations below their individual threshold values. That effect can create various flavors for humans (G. G. Stewart, 2017a).

2.1.2 Higher alcohol

Higher alcohols are produced by side products of amino acid metabolism or through pyruvate produced from fermented sugar metabolism (Fig. 1.2.). In *S. cerevisiae,* the anabolic pathway of pyruvate can manufacture higher alcohols directly. In this pathway, branched-chain amino acids derived from glucose are synthesized to form higher alcohols by the catalytic reaction of transaminases. Also amino acid catabolism by the Ehrlich pathway at 1907 is tacked into *S. cerevisiae* cell slowly during fermentation. The catabolic pathway involves firstly the degradation of amino acids to their corresponding alcohols, α-keto acids, and called transamination step. Next, α -keto acid is converted into aldehyde or acids according to each compound type, which called decarboxylation step. In the last step using by dehydrogenases, aldehyde was reduced to higher alcohols (Hazelwood et al., 2008; Olaniran et al., 2017; Parapouli et al., 2019; Querol et al., 2018) (Fig.1.4.) (Table 1.3.). Common volatile higher alcohols bringing flavor to product include isoamyl alcohol, *n*-propanol, isobutyl alcohol, and phenyl ethanol (Table 1.4.). Large part of these higher alcohols (>300 ppm) in fermented products can give them sharp and acidic smell, while optimal level of higher alcohols offers desirable flavor. Isoamyl alcohol is the most important elements of higher alcohols quantitatively as well as qualitatively. It has positive impacts of drinkability due to higher isoamyl alcohol concentration. On the other hand, isobutyl alcohol has a negative impact on the beer quality if its concentration is greater than the total amount of n-propanol, isobutyl alcohol and isoamyl alcohol (Olaniran et al., 2017). Nevertheless, isoamyl alcohol and phenethyl alcohol represent a more fruity flavors than other flavor compounds. Isoamyl alcohol has more of a banana flavor whereas phenethyl alcohol displays a gummy bear and rosy flavors (Michel et al., 2016).

Table 1.3. Flavorous higher alcohols producing amino acid metabolism through Ehrlich pathway (Styger et al., 2011).

Amino acid	α-keto acid	Aldehydes	Higher alcohols
Leucine	α-ketoisocaproate	Isovaleraldehyde	Isoamyl alcohol
Isoluecine	α-keto-β-methylvaleate	2- Methylbutyraldehyde	Amyl alcohol
Valine	α-ketoisovalerate	Isobutyraldehyde	Isobutanol
Phenylalanine	Phenylpyruvate	Phenylacetaldehyde	Phenylethanol
Tyrosine	*p*-OH-phenylpyruvate	*p*-OH-phenylacetaldehyde	*p*-OH-phenylethanol
Tryptopham	Indole pyruvae	Indole-3-acetaldehyde	Tryptophol
Methinonine	α-ketobutyrate	3-Methylthiopropanal	3-Methylthiopropanol

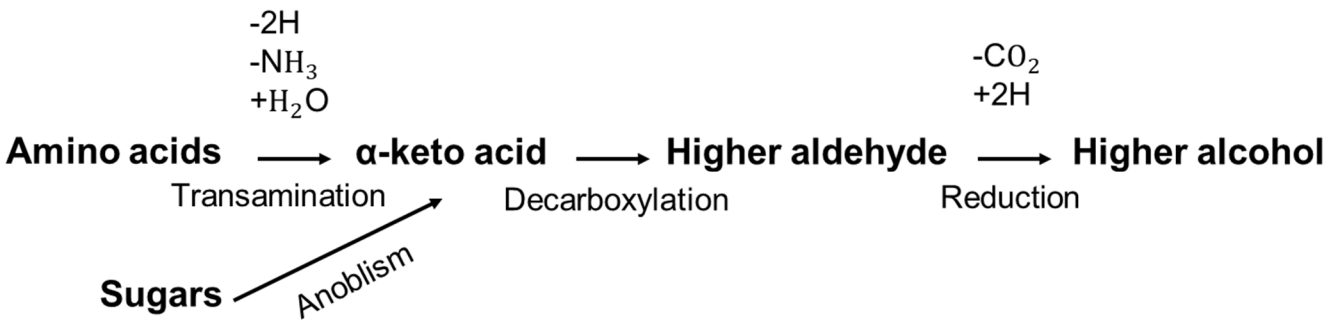

Fig. 1.4. Ehrlich pathway for higher alcohol production.

Table 1.4. Odor value of volatile compounds such as esters and higher alcohols (Niu et al., 2020; Kobayashi et al., 2006; Olaniran et al., 2017). Each ester and higher alcohol brought a different flavor to humans. The organoleptic threshold was the minimum level of the detection range for human sensory organs.

	Flavor description	Organoleptic threshold (mg/L)
Esters		
Ethyl acetate	Solvent, fruity, sweetish	30
Isoamyl acetate	Banana, apple, solvent, estery	1.2
Isobutyl acetate	Pineapple, fruity	0.9
Phenylethyl acetate	Roses, honey, apple, sweetish	0.4
Ethyl hexanoate	Sweet apple	0.05
Octanoate (Ethyl capylate)	Sour apple	0.9
Higher alcohols		
Isoamyl alcohol	Banana, alcohol	65
n-Propanol	Alcohol	200
Isobutanol	Alcohol	70
Phenetyl alcohol	Roses, sweetish, perfumed	125

2.2 Ethyl carbamate

In yeast metabolisms, a representative toxic by-product is ethyl carbamate (EC). EC is formed into an ethyl ester of carbamic acid through the reaction from ethanol and carbamyl compounds like urea. Especially arginine, the main nitrogen source of yeasts, is usually decomposed into ornithine and urea by arginase encoding the *CAR1* gene in yeast cells. Then urea combined with ethanol results in forming the EC (Fig. 1.5.) (Ryu et al., 2015). EC is classified as a genotoxic compound and as a group 2A carcinogen by the World Health Organizations International Agency for Research on Cancer (IARC) in 2010. Nevertheless, it can be found easily in the fermentation process or in fermented foods like alcoholic beverages, cheese, yoghurt or these product's storage process (Gowd et al., 2018a). The Joint FAO/WHO Expert Committee on Food Additives (JECFA) explain that the Benchmark Dose Lower Limit (BMDL) of EC is 0.3 mg/kg per day, and the average daily dietary intake (ADI) of EC derived food is about 15 ng/kg per day. In particularly alcoholic beverages are main courses to intake EC by yeast metabolic activity. Many countries regulate the level of EC concentration (Li et al., 2017). Intercellular metabolism of EC is connected to oxidative stress and DNA damage through producing DNA adducts that form covalent bonds between EC compounds and DNA, RNA, and protein. These could cause a risk to human health (Fig. 1.5.) (Gowd et al., 2018b).

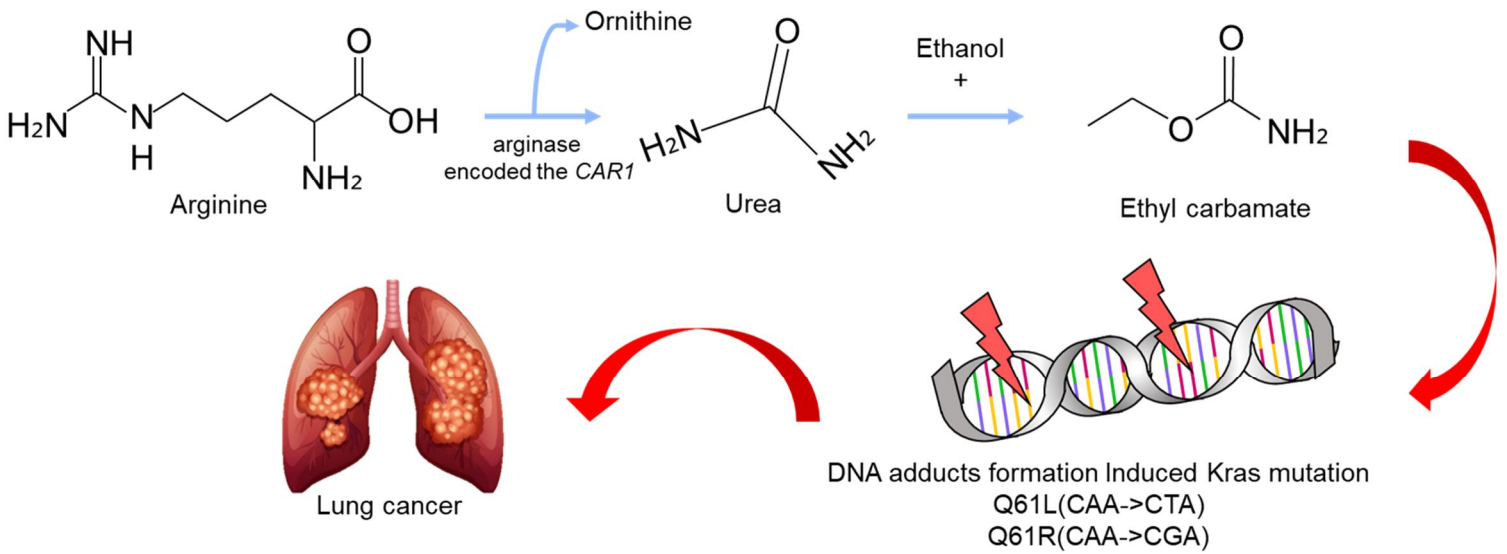

Fig. 1.5. The formation process of ethyl carbamate in yeast cell that pose a risk of human health. Arginase could produce the ethyl carbamate that effects a carcinogen compound to humans because of existing the urea production pathway.

3. Genome editing

Sequencing platforms is established by accessibility of molecular biology and micro mechanics. It provides humans with an advantage in manipulating unique genetic code on broad levels (Khan, 2019). Over the last decades, active advances in genome editing skills have revolutionized the study of different editing technologies, which has allowed researchers to better understand eukaryotic gene modification in utilizing many fields such as food, the environment and disease (Li et al., 2020). Cutting edge technique also has ability offering rapidity of sequencing, economically analysis and accurate genome modification in target sequence of any organisms (Khan, 2019). The history of genome editing is in order nuclease, zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR-associated protein (Fig. 1.6.). Since TALENs and CRISPR-Cas9 systems can recognize precise genomic sequences, they have driven animated scientific research as well as genome editing (Gaj et al., 2016).

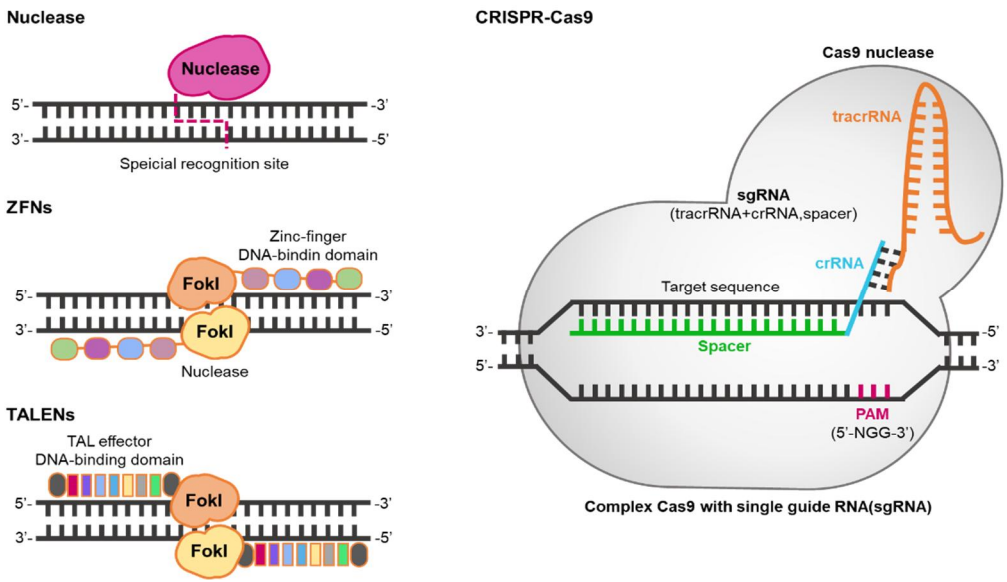

Fig. 1.6. The history of schematic genome editing technology. Nuclease was the first genome editing tool. Since then several technologies have developed such as Zinc-finger nucleases (ZFN) and transcription activator-like effector complex (TALENs). And CRISPR-Cas9 technology was state-of-the-art and can accurately design and verify the target sequence.

3.1 CRISPR-Cas9 system

High-throughput genome editing technology is clustered regularly interspersed short palindromic repeats (CRISPR) with CRISPR -associated protein and also calls CRISPR-Cas9 system (Zhang & Showalter, 2020). It has been modified to bacteria adaptive immune system that protects DNA from invading viruses. In bacteria, the type-II-CRISPR system functions by cleaving a foreign genome that guided RNA to complement the target sequence. Also in 2005, scientists discovered that short repeat sequences have characteristics that correspond short segments of foreign DNA due to the process of integration within the CRISPR locus. This segment goes through transcription to CRISPR RNA (crRNA), which is connected with *trans*-activating crRNA (tracrRNA). Single guide RNA (sgRNA) containing crRNA and tracrRNA collaborates with Cas9 protein which functions nuclease (Fig. 1.6.)(Gaj et al., 2016). Mechanistically, a complex of sgRNA and Cas9 can function as a genome editing system. The sgRNA usually contains spacer which has a specific 20 base pair and spacer sketches target DNA sequence complementary. The design of guide RNA spacer must be located upstream of three DNA sequences that are also called protospacer adjacent motif (PAM) encoding "NGG" (Fig. 1.6.). When this condition is met, the CRISPR-Cas9 complex can be attached to the target gene and also work by cutting a desirable gene. Generating double strand breaking (DSB) of a gene targeted is a first stage of this system application. Then the DSB repair system of the cell operates to cure the damaged DNA. Targeted DNA is relocated from efficient repair mechanisms specially in cells. Alteration results in integration or disruption of DNA a precise DNA site (Li et al., 2020). Control of the CRISPR-Cas9 system has more simple components than other genome editing

techniques. One component is Cas9 nuclease, which applies identically in all situations. Another is sgRNA that changes only the target site. Consequently, this engineering is a convenient tool when a new site is targeted. The Advantages of CRISPR-Cas9 are that it is cheaper, easier to design, highly targeted, and more efficient than previous tool boxes (Ran et al., 2013).

3.2 DNA repair pathway

All organisms have several DNA repair mechanisms for re-ligation. Once DNA double-strand breaking is induced in organisms, urgent events can be repaired by one of two major repair pathways for each organism: homology-directed repair (HDR) and non homologous end-joining (NHEJ) (Fig. 1.7.) (Li et al., 2020). Also, double-strand breaking can be induced intentionally by precise genome cleavage at the target sequences, which allows genome editing to start to deliver the editing machines *in vitro* or i*n vivo*. The DNA repair system is common to recover double strand breaking from recombination machinery system with adding, removing, and substituting nucleotides. Specially DNA repair systems can result in sophisticated modification of genes when similar regions exist aside from the target gene (Li et al., 2020).

3.2.1 Non-homologous end joining (NHEJ)

NHEJ pathway is usually used to fulfill gene knockout through random indels, while resulting in base insertions or base deletions (Gaj et al., 2016) (Fig. 1.7.). NHEJ makes indels, which leads to error-prone during the repair process. This outcome explains the possibility of genetic disruption and gene knockout (Sansbury et al., 2019).

3.2.2 Homology-directed repair (HDR)

Alternatively, during DSB, if organisms have a donor template with a homology region to the targeted chromosome, HDR can occur, which achieves gene integration into the chromosomal gene or base correction being an intentional gene combination (Gaj et al., 2016) (Fig. 1.7.). Donor DNA can take any form including plasmid, single stranded oligonucleotide, and double stranded nucleotide as long as it has homologous region to the target chromosome. Early in the repair process, a D-loop molecular structure is formed by attaching one broken strand to the donor DNA template. And broken strand is synthesized and ligated using its own repair systems based on complementary sequence (Kim & Mirkin, 2018; Storici et al., 2006). Most DNA pairing systems have been studied by research on homologous recombination in fission yeast, budding yeast, and fungus.

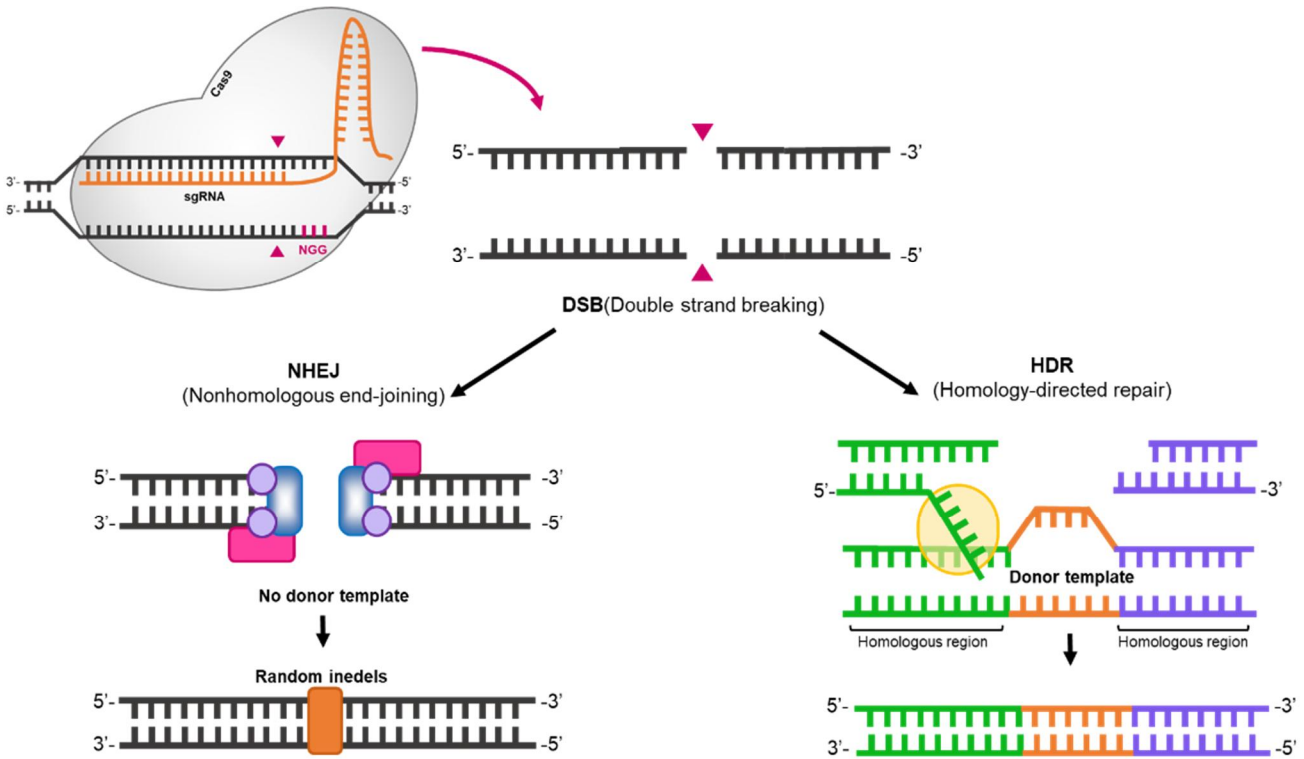

Fig. 1.7. Major repair DNA mechanisms to solve the urgent events during double strand breaking.

4. Overall objectives

Traditionally, the fermentation section has put a lot of effort into eliminating toxic agents like urea, a precursor to the carcinogenic ethyl carbamate (Weber & Sharypov, 2009; Wu et al., 2012; Wu et al., 2017). Various methods for reducing arginase in the pathway of urea production have been continuously performed. Especially as the *CAR1* gene is key of urea formation, it has been inactivated for genomic methods (Chin et al., 2016a; Chin et al., 2021; Guo et al., 2016).

In Chin's study, flavor alteration tendency was stumbled on additional results via mutants analysis after main study (Chin et al., 2021). So, our approach was used to characterize the specific flavor flux allowed by arginine decomposition constraints. Predicting intracellular secondary metabolites was planned to be examined through consistent experiments of genome editing on an equal gene in different yeast species. It could be explained by having completely different genotypes, which makes two types with the exact genomic modification tool, the CRISPR system. One type, also called the △*CAR1* type, was not only unlike the genome of the wild type but also had 1000bp short nucleotide of the *CAR1* gene. Another type, also called Gln26stop type, resembled wild type due to the same genome except for only 4bp of difference on *CAR1*. Thus, the deletion strain had nothing in common with the distruption strain genetically, but had the inactivation genomic part about the target gene, *CAR1*. And to eliminate the possibility of strain specificity we performed the same with 3 kinds of yeast strains. As a result, a general theory would be made either strain characteristic specifically only the KCCM 51299 strain that used Chin's study at 2021 or characteristic of metabolic connection rings with target gene and flavor compounds.

Chapter 2.

CRISPR-Cas9 system in *Saccharomyces cerevisiae*

1. Introduction

Cell factories that create primary and secondary metabolites used in the food, medical, and biotech industries have benefited humans. The first generation cell factories are composed of *Escherichia coli* in bacteria species and *S. cerevisiae* in yeast species (Lee et al., 2012). In particular, the performance of *S. cerevisiae* has been improved progressively from traditional engineering with untargeted mutagenesis to next-generation engineering, integrated metabolic engineering with systems biology and synthetic biology (Borodina & Nielsen, 2014; Crook et al., 2016; Hong & Nielsen, 2012; Lee et al., 2005; Lee et al., 2012; Nielsen, 2019; Tsai et al., 2015). Genetic modification is applied to enhance cell factories through next-generation engineering, which supports CRISPR-Cas9 technology that utilizes specially guided RNA and nuclease-activity protein. (Ishino et al., 2018).

Recently CRISPR technology has become an important tool in all aspects of effective and powerful genetic manipulation in microbial cell factories In 2020, two scientists who creatively used CRISPR technology were awarded the Noble Prize in Chemistry. The CRISPR system is used in yeast specifically for editing target genes, achieving products with flexible donor DNA. And the using homology

recombination pathway in yeast, different genes can be manipulated by expression of various guide RNA designed. Guide RNA is basically composed of a promoter and a terminator. And a combination of individual guide RNAs can use multi-cite gene modification of yeast. These modifications include not only expression levels but Customizable exchange (Meng et al., 2020).

Homology-direct repair system occurs voluntarily at lethal double strand breaking in the strain, but it is not happen at all strains. According to donor template sequences, repair system causes gene disruption, gene deletion, gene integration, or multiple repairs simultaneously, while the cell carefully assembles its DNA (Fig. 2.1.) (Lian et al., 2018).

The target gene, *CAR1* encodes a degrading enzyme of arginine. It might be inferred that flavor alteration is related to amino acid metabolism. In yeast amino acid biosynthesis was a major component of the metabolic flux. Amino acids serve as precursors for protein synthesis, intermediates of nucleotides, cofactors of various reactions and members of redox buffer. From these points of view, specific genome approaches of metabolism enable functional effects According to Mulleder's reports, metabolite quantities were measured by the deletion of orthotropic genes which share functional or biosynthetic properties on a genome scale (Mulleder et al., 2016). Until recently, more than half of the budding yeast gene and their effects were poorly characterized. Genetic modification approaches were available to show different yeast metabolism. For instance, in research boosting glycerol yield, it was found that yeast cells accumulated extremely flavor compounds such as acetaldehyde, acetate, and acetoin through overexpressing target gene for glycerol production (Kutyna et al., 2010; Zhao et al., 2015). As correlated metabolites originate from similar precursors and share chemical functions such as donors and receptors, it may also occur flavor accumulation in

metabolism flux by genetic modification for CRISPR-Cas9. It has not been clearly established that circuit accounts for between amino acids such as arginine, ornithine, glutamate, leucine, and phenylalanine and metabolites, especially volatile compounds. Pre-test results showed experiments of flavor performance using accomplished mutants and it had a meaningful difference. It may cause to exist connecting link in yeast metabolism (Fig 2.2.).

In chapter 2, gene disruption and gene deletion among various results of the CRISPR-Cas9 system had inactivate a specific target site, the *CAR1* gene, that is connected with production of the toxic agent in *S. cerevisiae* (Fig. 2.1.). The point is that study uses the CRISPR-Cas9 systems to one gene of various yeast strains under the same condition. Expectably, flavor enhancement should be found to connect either strain or target gene specificity. An important thing was that a target gene revealed not to have any relations with aromatic compounds. So after making a strain line, all line of various yeast was analyzed flavor prints using by GC-MS. If specific flavor compounds increase in the same manner as the result of a prior study, unknown pathways definitely exist.

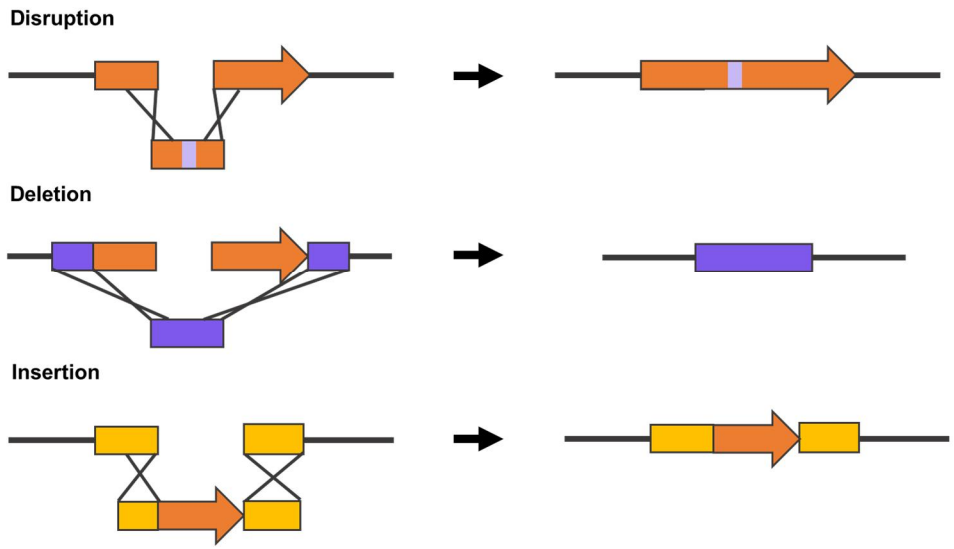

Fig. 2.1. Results of various type to homologous recombination depending on design of donor. Donors with homologous regions could be made tailored sequences by various DNA synthetic methods. So mutation of the target gene was made as point alteration form using stop codon of donor template, and complete deletion using front and back sequences of the target gene. Additionally, it could insertion of specific sequences between homologous sequences.

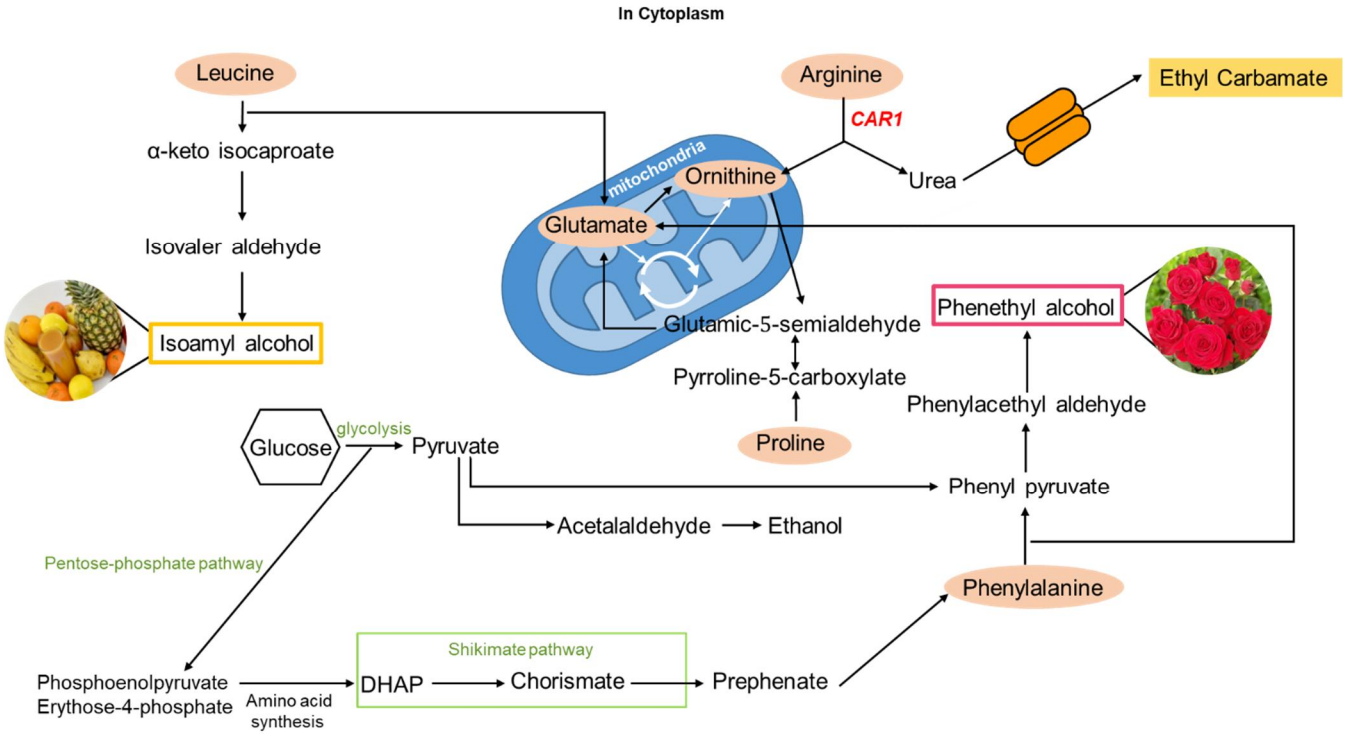

Fig. 2.2. An overview of schematic metabolic pathway in yeast (Holt et al., 2019; Styger et al., 2011).

2. Materials and Methods

2.1. Strains, plasmid, primer

The *Escherichia coli* TOP10 strain (Invitrogen Co, Carlsbad, CA, USA) was supplied as the host of the cloning system. Plasmids in *E.coli* were propagated in Luria-Betani (LB) agar plate with 50 μg/ml ampicillin at 37 ℃. The target strain SNUws was isolated from Korean traditional fermented food. A list of strains used in this chapter study is available in Table 2.1.

2.2. Culture condition

Yeast strains were cultured at 220 rpm in shacking incubator, 30 ℃ on YPD (10 g/L yeast extract (BD Difco™, Franklin lakes, USA), 20 g/L peptone (BD Difco™, Franklin lakes, USA), and 20 g/L glucose (Junsei chemical, Japan) medium. In order to regulate the fermentation condition, sugar was added according to the various glucose concentrations in the YPD medium. Competent cells using a preparation of transformation initial step were grown in YPD 2% medium to 0.8 observances, O.D_{600}. Then, competent cells could start the transformation step using Frozen-EZ-Yeast Transformation Kit (Zymo Research, Irvine, CA, USA). After plasmid transformation, the transformant is cultured in YPD added 100 μg/ml g418 (Sigma-Aldrich, St. Louis, MO, USA). And the guide RNA was synthesized *in vitro* transcription and transformed in same culture medium.

2.3. Plasmid and sgRNA target design

Using the *E. coli* TOP10 strain as a cloning host, a plasmid is extended to produce targets. The plasmid p414-TEF1p-Cas9(KanMX) used in previous study (Chin., et al 2021) was formed from mother vetor, p414-TEF1p-Cas9-CYC1t (Addgene incorporation, #43802) and also had g418 antibiotics activity encoded *KanMX* gene. To construct guide RNA expression, 20 nucleotide sequences located upstream of the NGG sequence are designed as complementary sequences with the *CAR1* gene. The guide RNA was designed for successful homologous recombination with the donor DNA, including the homologous region (Fig. 2.3.).

Table 2.1. List of yeast strains and plasmids used this study (Fig. 2.4.).

Strain/plasmid	Relevant description	Reference
SNUws wild type	*S. cerevisiae* isolated from fermented food	
SNUws Gln26stop	*S. cerevisiae CAR1* (CAGGG→ TAAG)	This study
SNUws △*CAR1*	*S. cerevisiae*△*CAR1*	This study
KCCM 51299 Wild type	*S. cerevisiae* KCCM 51299 isolated from Nuruk, diploid	(Hwang et al., 2020)
KCCM 51299 Gln26stop	*S. cerevisiae* KCCM 51299 *CAR1* (CAGGG → TAAG-)	(Chin et al., 2021)
KCCM 51299 △*CAR1*	*S. cerevisiae* KCCM 51299D*CAR1*	(Chin et al., 2021)
SNUit Wild type	*S. cerevisiae*	This study
SNUit Gln26stop	*S. cerevisiae* (CAGGG → TAAG-)	This study
SNUit △*CAR1*	*S. cerevisiae* Δ*CAR1*	This study
Escherichia coli Top10	*E. coli*	Invitrogen Co.(Carlsad, CA, USA)
p414-TEF1p-Cas9(KanMX)	Replacement of auxotrophic marker (TRP1) of p414-TEF1p-Cas9-CYC1t with antibiotic marker ($G418^R$)	(Chin et al., 2021)

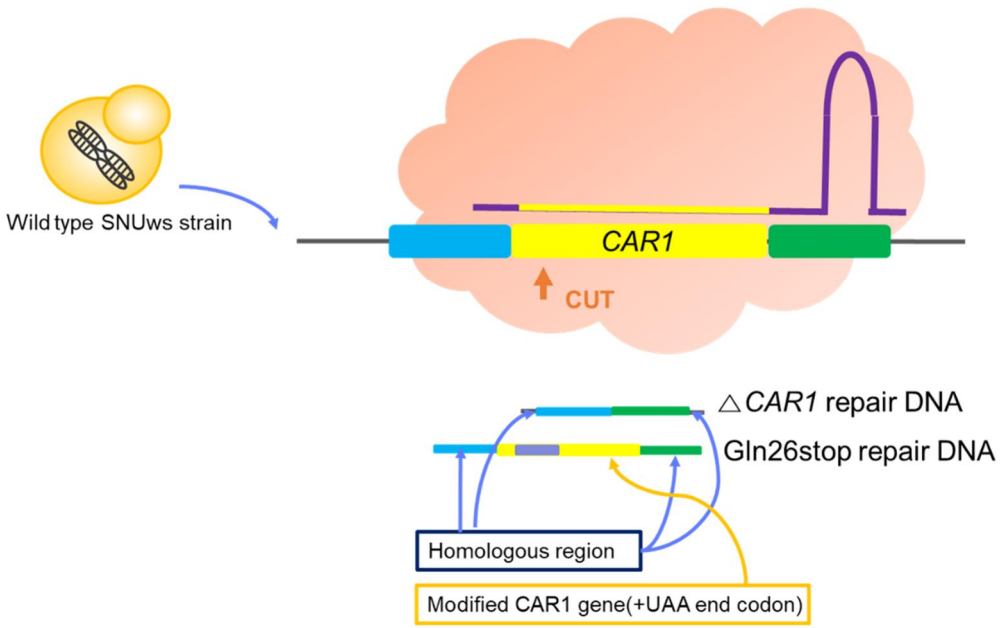

Fig. 2.3. Schematic mechanism of CRISPR-Cas9 system with the homologous direct repair. Overview described components of the CRISPR-Cas9 system such as Cas9 protein, sgRNA, and repair template. In particular, it needs to design sgRNA and template for each desired expression phenotype.

2.4. Preparation of *in vitro* transcriptions

To operate the CRISPR-Cas9 complex, a single guide RNA (sgRNA) is necessary. It was synthesized that single-strand RNA was architecturally made up of three sections: a promoter part, a spacer section with the target gene's complementary sequence, and a scaffold section *in vitro*. Forward and reverse primers construct to construct sgRNA frames, which are used to overlap PCR reaction making T7 promoter and gRNA scaffold (Fig. 2.4.). sgRNA is designed to cleavage the target gene through leading Cas9 complex. The target gene's 3' end should include the PAM sequence (5'-NGG-3'). To inactivate the target gene, a 20 nt spacer of sgRNA is designed upstream of the target DNA. DNA templates of sgRNA were assembled for PCR reaction. A DNA template is transcribed by using the MEGAscript T7 kit (Thermo Fisher Scientific, Walthan, MA, USA) according to the reference protocol carefully. Synthesized RNA is purified to improve the quality as described by the MEGA clear^{TM} (Thermo Fisher Scientific, Walthan, MA. USA). The purity and quality of sgRNA were measured using the Nano Vue Plus Spectrophotometer (GE Health Care Co., Nordrhein-Westfalen, Germany). The purified sgRNA (1200 ng/ul) was transformed into transformants revealed the Cas9 activity.

Fig. 2.4. Protocol of overlap PCR reaction. Overlap PCR could create long DNA templates with forward and reverse primers. These primers' sequences overlapped, therefore two short DNA strands were synthesized in order to employ the sgRNA's DNA backbone as a repair template.

2.5. Transformation

The CRISPR-Cas9 technique was used to inactivation the *CAR1* gene in the SNUws strain. Following with the Frozen-EZ-Yeast Transformation Kit (Zymo Research, Irvine, CA, USA), the wild type SNUws strains were converted into competent cells. For selection of strains transformed with plasmid, they were applied to YPDG 1L (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose, agar 20 g/L and g418 100 μg/ml) agar plate. To check Cas9 expression, living colonies at YPDG were employed as template for RT-PCR. RNA is extracted from the selected colonies according to the Universal RNA Extraction kit (Bioneer Co., Daejeon, Republic of Korea) protocol. Then the quantity and purity of extracted RNA were measured by the NanoVue Plus spectrophotometer (GE Health Care Co., Nordrhein-Westfalen, Germany). And cDNA (complementary DNA) that produced messenger RNA to coding DNA by reverse transcriptase was synthesized with AccuPower RT premix kit (Bioneer Co., Daejeon, Republic of Korea). For cDNA synthesis reactions of 20 ul volume, 1 ug of high purified RNA was reacted with oligo dT_18. RT-PCR was processed to check Cas9 expression in Cas9 (KanMX) plasmid with and special primer set, F_Cas_expression and R_Cas_expression. Thermal condition was in Table 2.3. Using primer sets, F_rDNA_non, R_rDNA-non, F_rDNA_el, R_rDNA_el, for overlap-PCR, repair template was synthesized including homologous region in each reaction (Fig. 2.3.). Using Frozen-EZ-Yeast Transformation Kit (Zymo Research, Irvine, CA, USA), the sgRNA and repair template were co-transformation into SNUws strain expressed Cas9 protein activity.

2.6. PCR analysis to search desired SNUws △ *CAR1* transformants

Colonies growing on YPDG agar plate were tested through colony PCR. Due to the occurrence of non-homologous end joining and homology direct recombination following CRISPR-Cas9, there was a chance that two genotypes might coexist. To secure desired colonies that got deletion of the *CAR1* gene by the HDR system, colonies were collected and lysed with a solution of 20 mM NaCl and centrifuged to collect the lytic supernatant. PCR amplification was performed with DNA template in supernatant using AccuPower *Taq* PCR mastermix (Bioneer Co., Daejeon, Republic of Korea) and the PCR condition is marked in Table 2.3. Primers (F_CAR1_2034bp, R_CAR1_2034bp) amplified a 2034bp fragment that is included in the genomes of wild type strains and a 1032bp fragment that is included in the genomes of the SNUws△*CAR1* candidates. Gene deletion was evaluated by gel electrophoresis as the size difference (Fig. 2.5.).

2.7. Positive selection to search SNUws Gln26stop transformants

The SNUws strain originated in a natural environment. Genetic engineering was considerd to create genetic modification, but the SNUws might not be foreseen due to strain factor. A positive selection to select only transformants of the *CAR1* gene disruption was developed from a specific medium method, which was followed at various concentrations according to our strain characteristics (Kitamoto et al., 1993). The principle of this selection medium is connected to the target gene, *CAR1*. If *CAR1* gene encoding arginase was completely inactivated, arginase deficient

mutants were made. Although mutants could grow in medium containing ornithine as the only nitrogen source, not in medium containing arginine alone as the nitrogen source. Without sequencing technology, it is practically impossible to isolate strains creating a tailored mutation in the *CAR1* gene with only 1 sequence size difference. Unlike complete mutant like SNUws△*CAR1* type mutant, there is no size difference between wild type and non-sense mutation like SNUws Gln26stop type strain. It could not be observe using electrophoresis because of the size difference. It was found the adaptive medium by following this method with a small modification. CAO medium (1.7 g/L yeast nitrogen base nitrogen free (BD Difco™, Franklin lakes, USA), 150 mg/L canavanine (Sigma-Aldrich, St. Louis, MO, USA), 5 mM ornithine (Sigma-Aldrich, St. Louis, MO, USA), 1 mM arginine (Sigma-Aldrich, St. Louis, MO, USA), 20g/L glucose (Junsei chemical, Japan)) was made for positive selection to grow only mutants excluding the rest of wild type strains and incomplete mutants (Kitamoto et al., 1993; Kuribayashi et al., 2013) (Fig. 2.7.). To examine the appropriate level of inhibition, SNUws△*CAR1* type mutants is cultured on various concentrations of canavanine. And it could endure the specific screening level, 150 mg/L canavanine, to apply to SNUws Gln26stop type candidates.

2.8. Enzyme reaction to search for SNUws Gln26stop transformants

Although SNUws△*CAR1* candidate could separate from wild types, Gln26stop type candidate that had the similar length of the *CAR1* gene size on gel electrophoresis (Fig. 2.5.). So through the specific enzyme reaction, the difference between amplified fragments is generated. The putative Gln26stop type colonies survived on CAO 150 mg/L agar plate and were used as DNA templates in colony PCR. For digestion of DNA templates, MseI is used with applied buffers from New England Biolabs (NEB). A DNA template of the desired Gln26stop type transformants is modified from original CAGG to TAA. The MseI recognition site exists only in Gln26stop type mutants, where it is the TTAA sequence cleaved by restriction enzyme.

Table 2.2. List of primer used this study.

Primers	Sequence (5' to 3)	Description
F_sgRNA	TAATACGACTCACTATAGGGGCTCCATTCAGCGGCGGTC**GTTTTAGAGCTAGAA**	*In vitro* transcription of sgRNA
R_sgRNA	AGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTAT**TTCTAG CTCTAAAAC**	
F_rDNA_non	CAACTACTACAAAAATCGCGAATTGTCCAT**CGTTCTGGCTCCATTCAGCGGCGGTTAAGT**	Repair template synthesis through overlap PCR
R_rDNA_non	TATTTAGGGCCCTTCTCGACACCAAGCTT**ACTTAACCGCCGCTGAATGGAGCCAGAACG**	
F_rDNA_el	GAAACAACAACAACAACTATATCAATAACAA**TAACTACTATCAAGTTTATATCATCATCC**	
R_rDNA_el	ATAAAAAGAGAATGCTTATTTTGATAAAAG**GGATGATGATATAAACTTGATAGTAGTTA**	
ttaa_115_F	ATGGAAACAGGACCTCAT	Gln26stop-type transformant analysis
ttaa_115_R	GCATGTATTTAGGGCCC	
Oligo dt 18	TTTTTTTTTTTTTTTTT	cDNA synthesis
F_Cas9 expression	AAAGCCAGCATTCCTGTCTG	Checking to Cas9 protein translation
R_Cas9 expression	ATCCACGTACATGTCCCTGC	
F_Car1_2034bp	GTCTCTAGCTCTTGCCCTTC	Amplification of *CAR1* gene
R_Car1_2034bp	CCGAATGGGAAGTCCAACAA	

Table 2.3. In case of various PCR, and thermal value.

Description	Step	Temperature	Time(sec)	Cycles
In vitro sgRNA synthesis	Pre-denaturation	95℃	30	1
	Denaturation	95℃	30	35
	Annealing	55℃	30	
	Extension	72℃	30	
	Final Extension	72℃	300	1
cDNA synthesis	Denaturation of RNA secondary structure	70℃	300	1
		0℃	Place on ice	1
	cDNA synthesis	42℃	3600	1
	Rtase inactivation	94℃	300	1
PCR to search SNUws△*CAR1* transformants	Pre-denaturation	95℃	300	1
	Denaturation	95℃	20	
	Annealing	56℃	20	30
	Extension	72℃	60	
	Final Extension	72℃	180	1

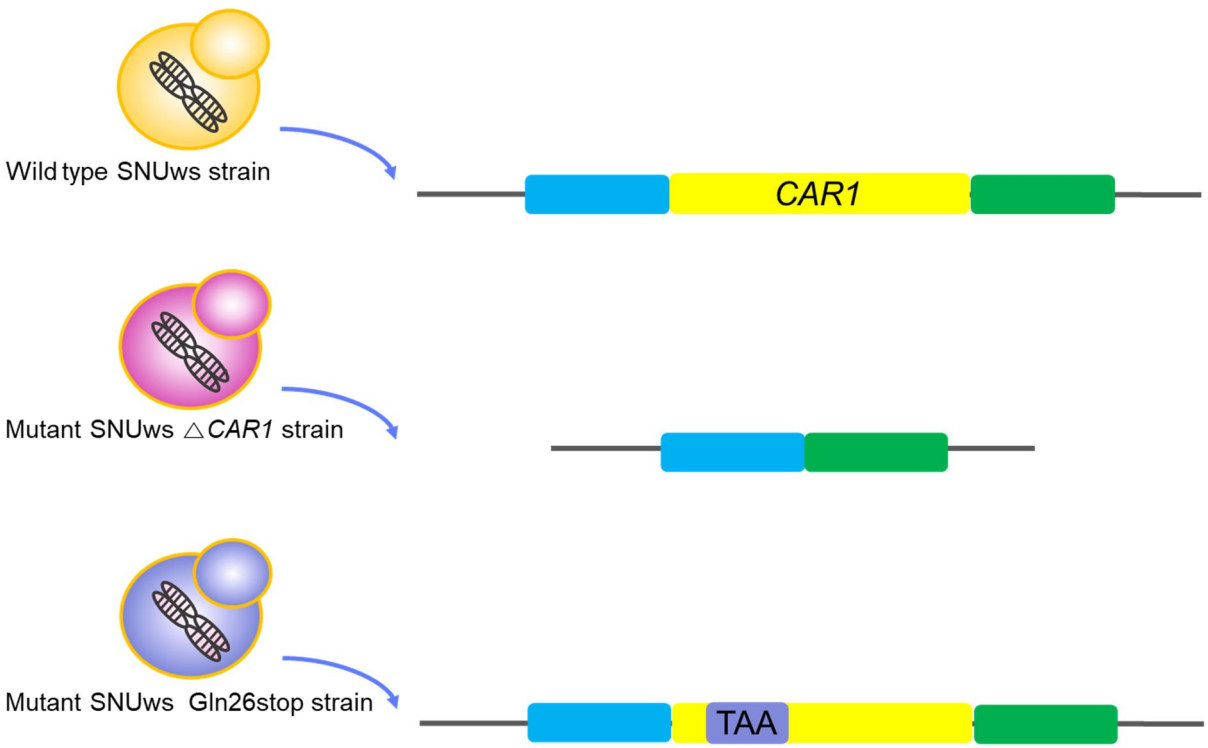

Fig. 2.5. Various types of SNUws strains. These genotypes were predicted by the designed sgRNA and each type of repair template.

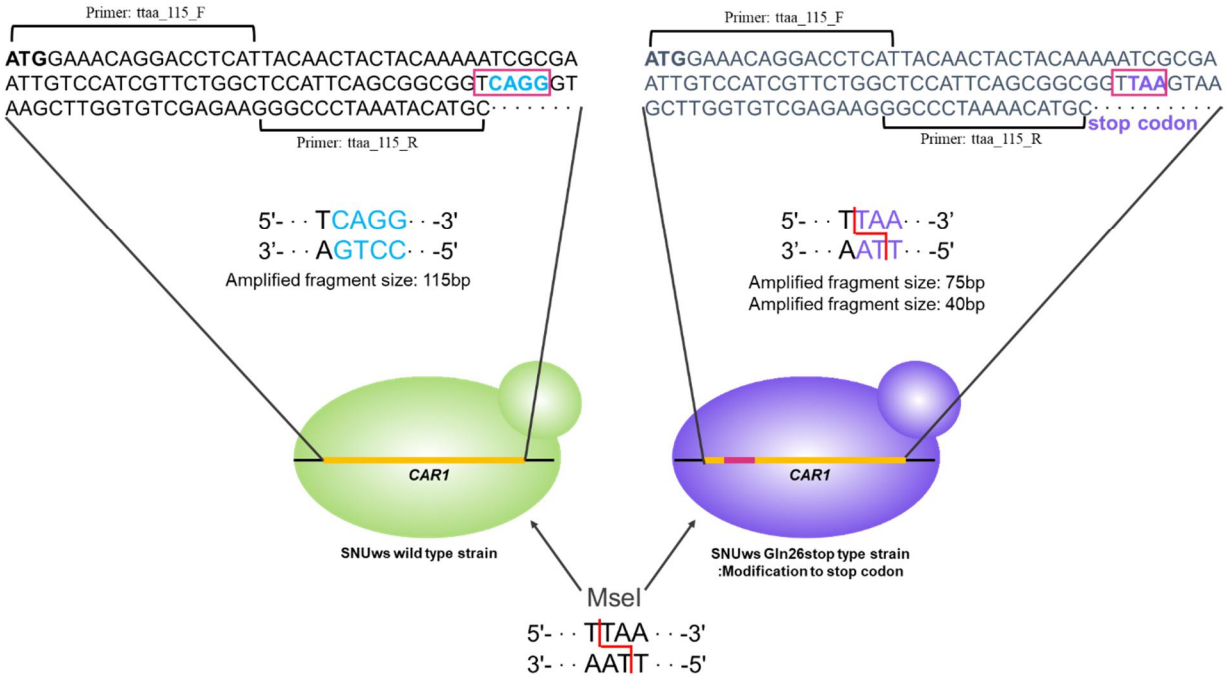

Fig. 2.6. Enzyme reaction methods for selection of Gln26stop type mutants. MseI had it's distinct restriction site (5'-T/TAA-3'). MseI restriction site was generated in SNUws Gln26stop type strain for point mutation at wild type sequence.

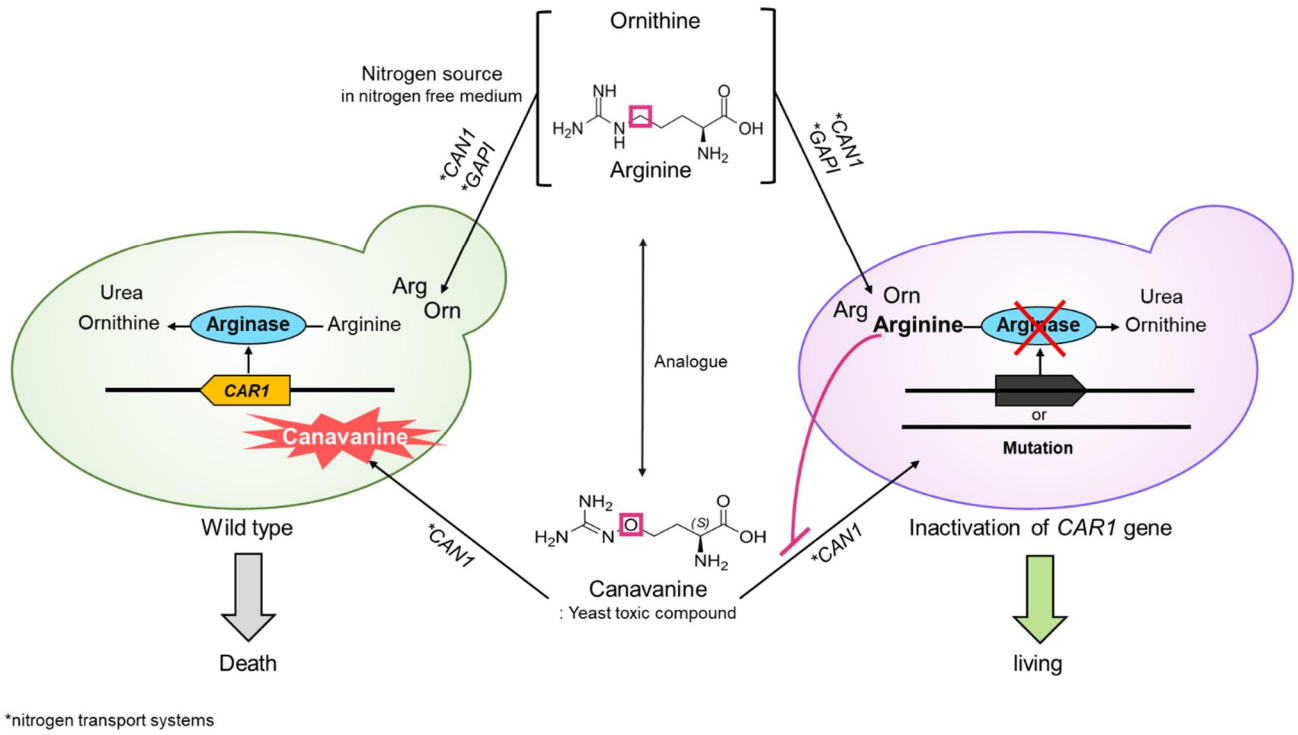

Fig. 2.7. Principle of CAO medium to search *CAR1* mutants.

2.9. Preparation for genomic DNA extraction and sequencing

For sequencing preparation, genomic DNA of wild type, SNUws△*CAR1*, and Gln26stop type of strains was extracted by the phenol chloroform isoamyl alcohol method according to the protocol of Dymond (Dymond, 2013). Purified genomic DNA was analyzed by Sanger sequencing system.

2.10. In comparison to wild type with mutants

To confirm the change between wild type SNUws and mutants, they were cultured in YPD (100 g/L of glucose) medium. And then fermented soup was collected in triflicate for growth measurement, HPLC and GC-MS sampling at 8-hour intervals. Using a UV-160 spectrophotometer (Shimadzu, Kyoto, Japan), cell growth was measured at 600 nm and converted to dry cell weight (DCW). And the production of glucose and ethanol was analyzed by using HPLC-RID, a high-performance liquid chromatography refractive index detector (Agilent, Santa Clara, CA, USA), with DB-WAX column (60 m × 0.25 mm I.D. × 0.25 μm film thickness) (Agilent, Santa Clara, CA, USA). As the mobile phase, sulfuric acid solution (0.005 N) was used at a flow rate of 0.6 ml/min. The sample injection volume was 20 μl and passed through the column at 60 ℃ for 25 min running time.

2.11. Identifying the flavor production using GC-MS

2.11.1 Sample preparation

For GC-MS analysis, various types of the SNUws, KCCM 51299, SNUit strains were cultured in YPD (10 % d-glucose) medium at 100 rpm, 30 ℃ consistently. And samples were collected in triplicates at 8-hour interval. At first preparation of GC-MS, samples were centrifuged at 10 min with maximal speed, and filtered with 0.2 μm pore size syringe filter, and stored at -70 ℃ prior to analysis of flavors. To establish standard, isoamyl alcohol and phenethyl alcohol were purchased from Sigma-Aldrich, St. Louis, MO, USA. They were diluted to 0.5 to 500 mg/L (ppm) using by 10 % ethanol to match the standard concentration.

2.11.2. Flavor Analysis

For GC-MS analysis, the extraction of the aromatic compounds in 500 μl of each sample were passed through the SPME Arrow (Solid Phase Micro Extraction) in a 20 ml headspace screw-top vial (Thermo Fisher Scientific, Rockford, USA). It was designed for good resolution of volatile compounds that had various boiling points with SPME Arrow 1.10 mm (DVB/PDMS (Divinylbenzene), Violet) prior to GC-MS analysis. Analytes were processed through the TriPlus RSH ™ autosampler by Thermo Scientific™ at Table 2.4. The volatile compounds were separated as their boiling points on the GC Column HP-5MS (60 m × 0.25 mm i.d. × 0.25 μm film thickness, Agilent J&W Scientific Inc., Folsom, CA, USA) using by Trace 1310 GC with a TSQ 9000 triple quadrupole MS (Thermo Fisher Scientific, Rockford, USA) in triplicate. In order to set samples of equilibrium, the SPME Arrow fiber was exposed at headspace at 30 ℃ for 30 min with 1,000 rpm. Samples were

injected to 5:1 spilt mode and desorbed at 210 ℃ for 10 min. The fiber should be post conditioned to eliminate any possible contamination at 230 ℃. GC oven temperature program was written on Table 2.5. They were inserted into the GC inlet at 210 ℃ and gradually increased to 220 ℃. Helium was used as a carrier gas at a 1 ml/min flow rate constantly. for 5 minutes before the next sample runs. The MS transfer line temperature was maintained at 230 ℃. The MS system was operated in EI mode. It has an ionization energy of 70 eV and an ion source temperature of 280 ℃. The MS data were collected between 35 and 550 m/z range in a full scan mode with a dwell time of 0.1 scan per second. For gathering data of interest volatile compounds, the SIM (Selected ion monitoring) was used to identify isoamyl alcohol and phenethyl alcohol with a dwell time of 0.05 scan per second.

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Process	SPME conditions		
	Temperature (°C)	Time (min)	Speed (rpm)
Equilibrium of samples	30	15 min	500
Extraction	30	30 min	1,000
Desorption	210	10 min	0
Post conditioning	230	5 min	0

Table 2.4. SPME parameter.

	Rate	Temperature (℃)	Hold time (min)
Initial		50	5
1 part	2	80	0
2 part	4	220	0

Table 2.5. GC oven parameter.

3. Results

3.1. Establishment of transformants with Cas9 sequence

The Cas9 activity must be assessed prior to the application of the CRISPR-Cas9 system. The p414-TEF1p-Cas9-CYC1t as a plasmid including Cas9 expression had antibiotic ability, g418. YPDG agar plate then assisted in selection of colony harboring an adaptive plasmid. To confirm Cas9 expression transcription level, living colonies on YPDG were collected to whole DNA. And RNA from each colony was extracted to construct cDNA with oligo dT primer. The next step, RT-PCR amplification was performed using F_Cas9 expression and R_Cas9 expression primers. Fig. 2.8 (a). represents antibiotic screening using YPD agar plate, adding g418 antibiotics. Visible colonies on YPDG were considered to have p414, which consists of Cas9 encoding sequence and g418 resistance. In order to check Cas9 protein expression in yeast cells described the gel image on electrophoresis about transcription level of Cas9. As shown in Fig. 2.8 (b)., Cas9 expression appeared as band intensity in the gel image.

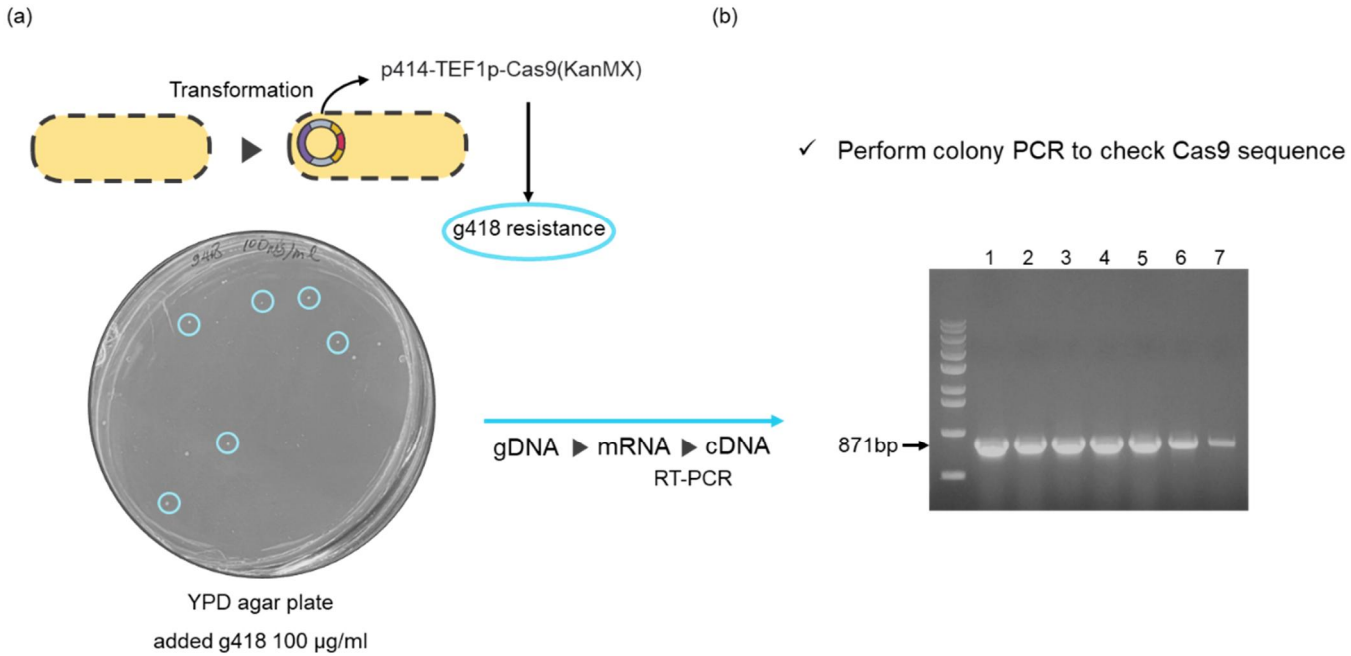

Fig. 2.8. Check for Cas9 plasmid into the organism via transformation. (a) Obtaining transformants on g418 (100 ug/ml) selection medium (b) analysis of the Cas9 transcription level intensity. lane 1, 2, 3, 4, 5, and 6: Cas9 transformed colonies: lane 7: p414-TEF1p-Cas9 (KanMX).

3.2. Design of the CRISPR-Cas9 system

For tailored genome editing, colonies induced Cas9 needed to introduce gRNA and each repair template to induce homologous direct recombination. Colonies in YPDG that introduced plasmids with Cas9 sequence were converted into competent cell to prepare secondary transformation. The designed gRNA was then synthesized *in vitro* and purified to confirm the high purity level of the transformation condition. Select colonies were co-transformed with gRNA and a specified repair template in accordance with the homologous region of each type using the Frozen-EZ-Yeast Transformation Kit (Zymo Research, Irvine, CA, USA). For inactivation of the target gene, the efficiency of gRNA should be confirmed for various controls. At Fig. 2.9, (a), also called "– control," was transformant of SNUws strain carrying the p414 plasmid with g418 antibiotics. (b) was, also referred to as "+ control," transformed only specific gRNA in (a). For desired genome editing using CRISPR-Cas9 system, each repair DNA in accordance with Fig. 2.2. and Fig. 2.3. was co-transformed with gRNA. (c), (d) were likely to live through homologous direct recombination with each type repair template. In Fig. 2.9 (c)., transformants of SNUws△*CAR1* were counted as about 32 colonies. In comparison with (b) plate that transformed the same concentration of gRNA, when Cas9 induced double strand breaking occurs in the transformants (c) and (d) were additional live due to the repair system, homologous recombination with repair DNA. The design of gRNA was a critical factor in Cas9 cleavage activity. Theoretically, if gRNA and Cas9 were co-transformed into cells without repair DNA, they should be nearly extinct. Although the few colonies could live through random mutation near the Cas9 binding site, due to the error-prone Non Homologous End Joining (NHEJ) of the repair system in yeast, only a few colonies chose it instead of HDR. Nevertheless, if the number of colonies was large on plate,

it was proven the less efficiency of gRNA caused low Cas9 activity in yeast cells. According to (Laughery et al., 2019), the experimental CRISPR-Cas9 under the existence of repair DNA should produce ten times the number of colonies than under the absence repair DNA. But based on (b) and (c) in Fig. 2.9., the growth of SNUws△*CAR1* strain appeared to only a little difference than "+ control" which had without repair template. This meant poor Cas9 cutting activity at the target gene. In that the repair template of Gln26stop type was compact sequence with the target gene than the DNA template of SNUws△*CAR1*, colonies survived more. Because the SNUws strain originated at the wild and not a lab, There was one more consideration to make. Therefore, in the experiment, not only strain specific but also gene locus heterogeneity should be considered. As a result, living colonies on the selection medium were made of unediting colonies, undesirable error-prone repaired colonies, and desired homologous directed repaired colonies.

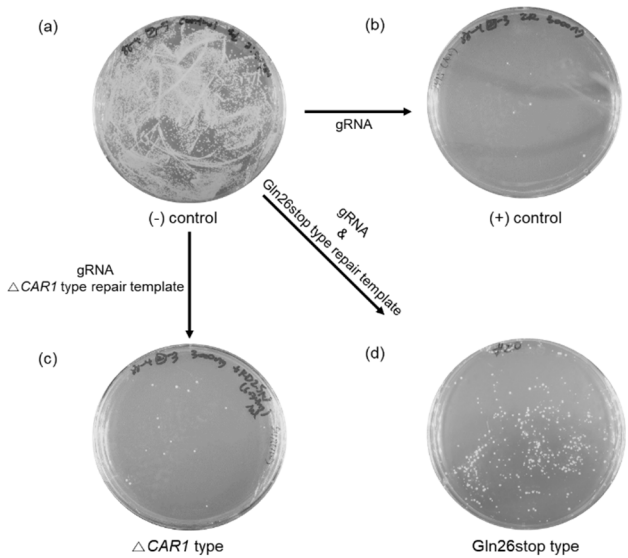

Fig. 2.9. Identification of transformants to build up the CRISPR-Cas9 system. (a) – control plate showed that SNUws wild type colonies in 50 µl volume were expressed only Cas9 activity. (b) + control showed that CRISPR-Cas9 system applied to SNUws wild type colonies adding Cas9 and sgRNA in same 50 µl volume. After double strand breaking by CRIPSR-Cas9 system some colonies survived using by each homologous template type through HR repair system.(c) SNUws△*CAR1* plate and (d) SNUws Gln26stop type plate appeared many candidates that survived various pathways.

3.3. PCR screening of the SNUws△*CAR1* strain

The putative transformants were determined by PCR amplification of the *CAR1* gene from colonies on YPDG plate using a primer set. Amplificated size was expected to be about 1 kb since the size of the sequence flanking both upstream and downstream was about 1 kb if the *CAR1* gene is not included. Therefore, the amplificated product containing the *CAR1* gene was about 2kb (Fig. 2.10.). As shown in Fig. 2.10., PCR products of 1kb size were aligned on gel image through electrophoresis. Also, some mutant's genotype showed two bands with 1 kb and 2 kb on the gel image. It should test to be assessed individually as strain specifically (Wen et al., 2021). It is meant that at the same time having antibiotic resistance ability, the CRISPR-Cas9 was not fully operating on the target gene. As a colony could be mixed with some of any type of mutant in colony levels, genomic DNA was extracted and amplified by PCR. Additionally, it was performed in sub-culture on YPD agar plate to eliminate the possibility another mixed type. Finally, it could be confirmed to have a desired genotype, deletion of the *CAR1* gene.

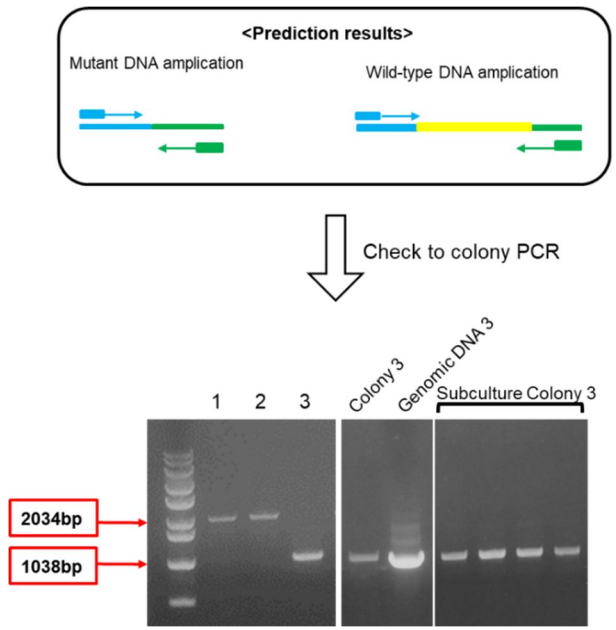

Fig. 2.10. PCR amplification of the near *CAR1* gene to confirm deletions by size comparison. At first gel image, lane 1: wild type; lane 2: candidates ; lane 3: putative SNUws△*CAR1* transformants. This was confirmed by extracting genomic DNA of colony 3 in second gel image. Finally it was performed through repetitive sub-culturing in YPD at third gel image.

3.4. A counter selection to find desired mutants

Although it was obviously identified that genotype size of the SNUws△*CAR1* is amplified by PCR as a clear-cut difference, modified *CAR1* gene of Gln26stop type mutants have almost same size with the *CAR1* gene of the wild type. Because it was impossible to identify through PCR amplification by size, a positive selection method was established with the selected the SNUws△*CAR1*. If considering strain particularity, CAO medium is a suitable selection tool using inactivation of arginase completely. Prior to selecting of Gln26stop type, it must be validated that canavanine concentration has an effect on strain survival. Due to the *CAR1* gene deletion, strains of the *CAR1* mutants type did not metabolize arginine, resulting in a large amount of arginine. On the other hand, strains of wild type metabolized arginine to ornithine and urea, resulting in a lower amount of arginine (Fig. 2.11.). While canavanine was an arginine analogue, it was toxic to yeast cell via various arginine transport proteins (Shor et al., 2013). If mutants inactivated the *CAR1 gene* do spontaneous cell division in CAO medium, they will be less affected by the toxic agent, canavanine, than wild type. At a certain concentration, each strains of wild type were dead, and when the concentration increase. So it was chosen only tolerant mutants that have inactivated the *CAR1* gene entirely. By following the above theory, it was necessary to establish an appropriate canavanine concentration according to each strain.

Various strains, including wild type and mutants of each strain were needed to compare strain specificity. As assessed in Table 2.6., the SNUws strain did not grow well compared to the KCCM 51299 strain on toxic background. This result may be due to strain inherent features. While the SNUws strain metabolized toxic compounds using its moderate metabolism, the KCCM 51299 strain having better growth conditions had a high ability to metabolize toxic agents. When the

concentration of canavanine of toxic agents was gradually increased, the wild type could not metabolize if the concentration of canavanine is above specific quantities. They were cultured on triplicate medium adding any canavanine concentration. After testing at Table 2.6., the SNUws wild type strain could not grow at 140 mg/L canavanine in two plates, but it could not consistent because the wild type survival showed small of portion in one plate. It had to select the concentrations of canavanine where only complete SNUws△*CAR1* mutants survived in all repetitive medium for positive selection of SNUws strain. Therefore, looking at Fig. 2.11. finally, 150 mg/l of canavanine killed all types except for the perfect mutant type. To search for SNUws Gln26stop type, candidates of Gln26stop type were prepared to grow at YPD with g418 antibiotics. The living colony was diluted with 3 μl of distilled water and cultured at the CAO (150 mg/l of canavanine) medium. Some colonies did not appear on the CAO plate, others appear as a small circular part. Among colonies, it appears that a dropped section is changed to a white circle spot, representing full growth activity (Fig. 2.12.). it could be selected to putative Gln26stop type.

Table 2.6. Test to measure strain specific effects by triplicate. CAO medium with 1 mM arginine, 5 mM ornithine, and various canavanine concentration. It was performed by triplicate.

Strain/canavanine (mg/L)	90 mg	100 mg	110 mg	120 mg	130 mg	140 mg	150 mg
KCCM 51299 wild type	+++	+++	++	++	++	+	+
KCCM 51299△*CAR1*	+++	+++	+++	+++	+++	+++	++
SNUws wild type	+++	+++	++	+	+	+/-	-
SNUws△*CAR1*	+++	+++	+++	+++	+++	++	++

+++, very good; ++, good; +, moderate; +/-, intermediate results with both moderate and no growth condition; -, no growth.

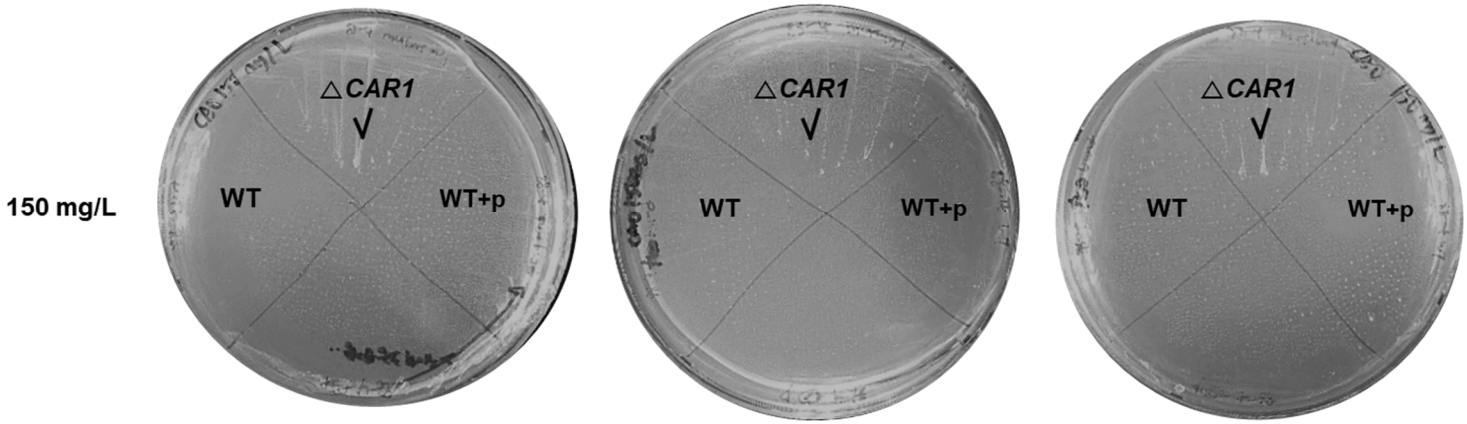

Fig. 2.11. Test to canavanine resistance of the SNUws strain depending on the phenotype by triplicate. △*CAR1*: complete SNUws△*CAR1* mutant that confirmed by PCR analysis; WT: SNUws wild type strain; WT+p: SNUws wild type harboring plasmid with Cas9 expression.

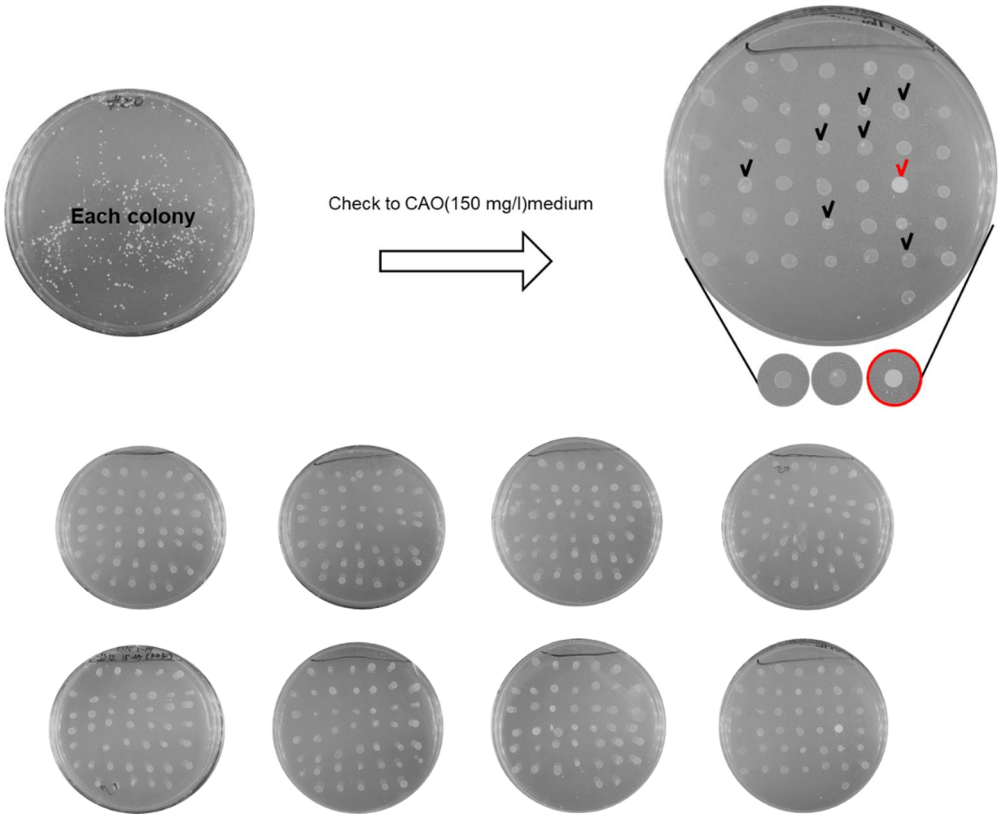

Fig. 2.12. SNUws Gln26stop type selection using 150 mg/l of canavanine. Each colony that transformed Cas9, gRNA, and Gln26stop type repair DNA was subcultured on canavanine selection medium (150 mg/L). Each circular pot represents one colony.

3.5. Screening of the Gln26stop type strain through enzyme reaction

The putative Gln26stop type could be checked that grow at CAO added 150 mg/l of canavanine, unlike the wild-type strain and mutants through non homologous end joining of SNUws (Fig. 2.12.). The *CAR1* gene's nucleotide sequence changed from "CAGG" to "TAA" in comparison to the wild type. Therefore, an additional method should be required to confirm the sequence. Since upstream of TAA was thiamine, a sequence that did not exist in the wild type, “TTAA,” was newly created in the expected mutants. So, using the ttaa_115_F and ttaa_115_R primers, a 115 bp specific sequence near the *CAR1* gene was generated for PCR reaction. Then, if restriction site of MseI enzyme was present, this enzyme treatment with a 155 bp DNA fragment resulted in the generation of two shorter-sized fragments. Thus, shown in Fig. 2.13., while amplification results of the wild type displayed one band of a 115 bp size that did not react to the enzyme. Due to not having a restriction enzyme site, mutants displayed two bands, which represented a 155 bp fragment and a 75 bp size fragment cut by MseI enzyme. The 40 bp fragment is probably not apparent on gel images because it is considerably shorter to identify, even on a 20 bp ladder. In addition to the using CAO medium, the enzyme reaction was used together to confirm more accurately, which could save on sequencing time and also economic costs.

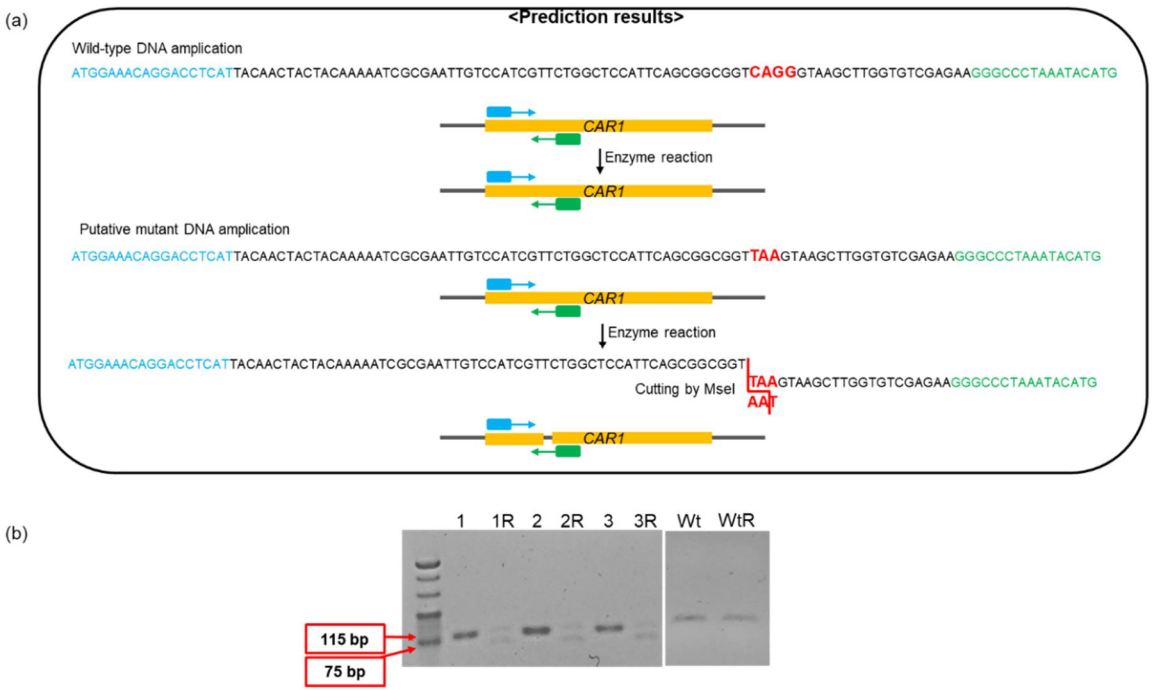

Fig. 2.13. Imaging results after restriction enzyme reaction. (a) shows prediction results that display differences between wild type and mutant types and (b) shows electrophoresis results on gel image with size difference and the number of bands. *R represented solution after enzyme reaction.

3.6. Sequencing analysis of all mutants

To examine how the CRISPR-Cas9 system works exactly,, sequencing of the *CAR1* gene should be performed finally. It was clear that mutants had a customized genome as opposed to the wild type reference genome. Fig. 2.14. and Fig. 2.15. represent the sequencing chromatogram results of the SNUws △ *CAR1* and alignment with the wild type results. It was examined to check through PCR for genotyping differences, which seemed to be about 1,000 bp shorter than the wild type length. With corresponding to PCR genotyping of putative SNUws△*CAR1* strain, sequencing showed the nucleotide aligned in the front and rear locations of the *CAR1* gene excepting the sequence that expressed the *CAR1* segment. It was also confirmed with Fig.2.14. that there were no side effects on sequence alignment. Except for the existence of the *CAR1* gene sequence, the sequencing results seemed to demonstrate no difference between the wild type and SNUws△*CAR1*. These findings indicated that the CRISPR-Cas9 system's gRNA successfully targets specifically the *CAR1* gene. And Fig. 2.16. shows the sequencing chromatogram of the Gln26stop type results and their comparison with wild type results. Sequencing the Gln26stop type strains appeared to compactly repair the altered custom genome that switched to a stop codon (5'-UAA-3') close to the start codon of the *CAR1* gene. And also, sequencing results showed that the sequence of the Gln26stop type was identical to the wild type except for only 4 bp nucleotide sequence difference. Thus, the sgRNA system is working effectively on the *CAR1* gene for inactivation function.

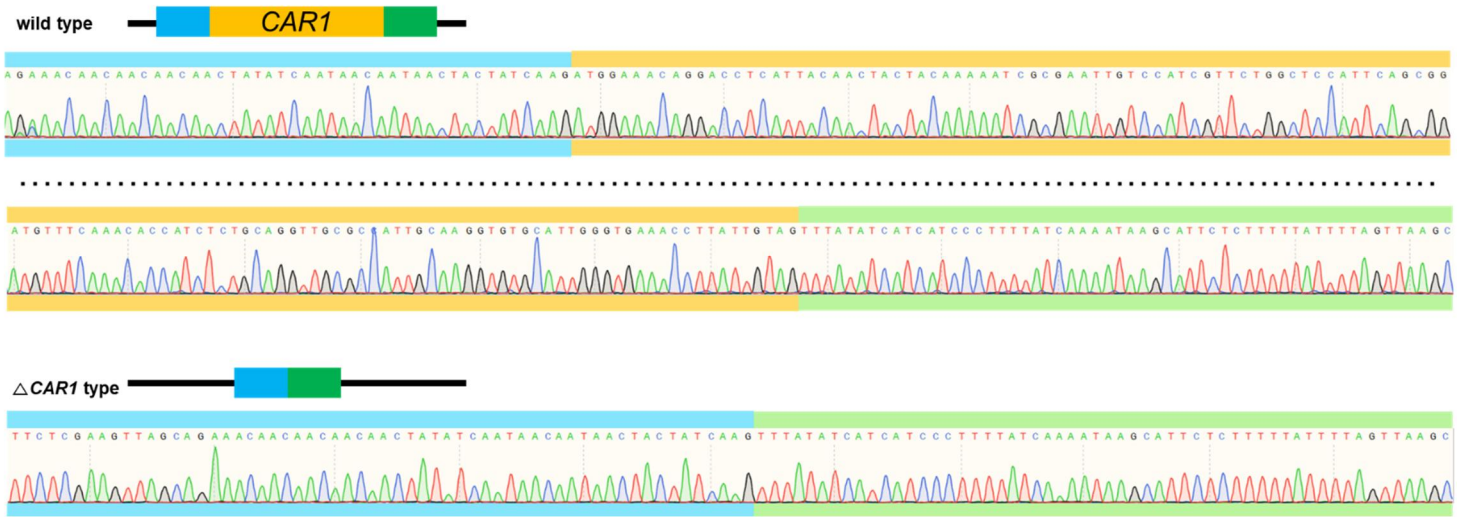

Fig. 2.14. Sequence analysis results of the SNUws△*CAR1*.

Fig. 2.15. Sequence alignment results of the del type SNUws△*CAR1*.

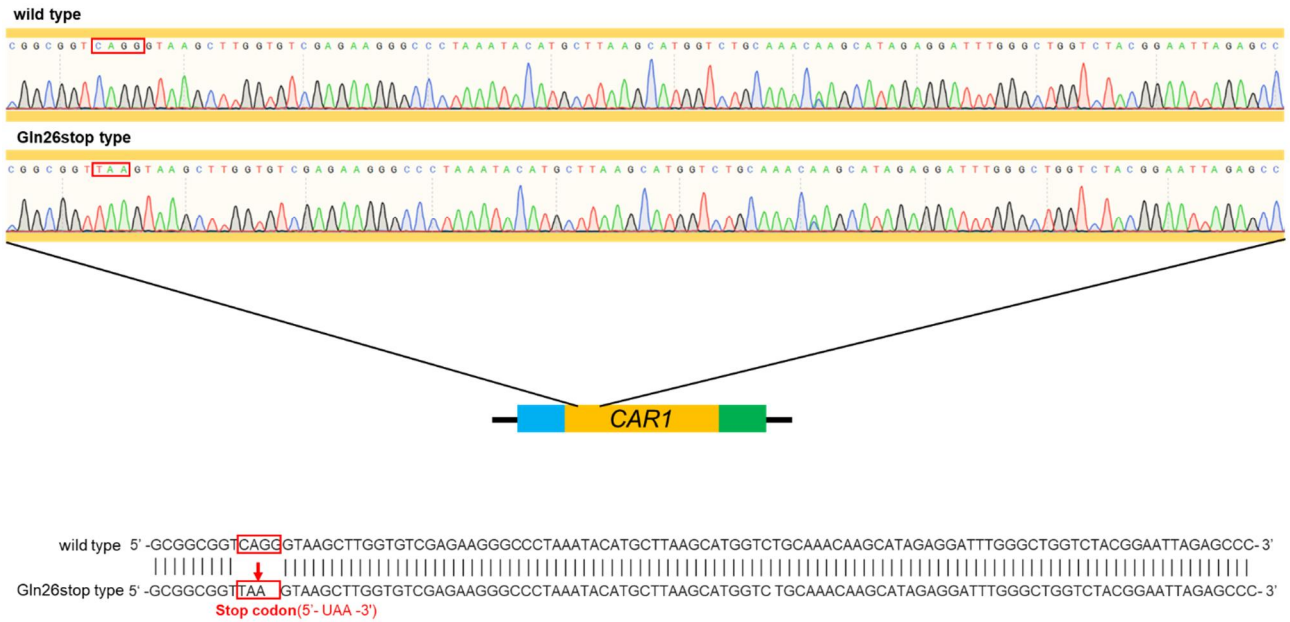

Fig. 2.16. Sequence analysis and alignment results of the Gln26stop type mutant.

3.7 Using a range of genetic data generated by the CRISPR-Cas9 technology

It resulted in establishing the genetic difference between yeast species. Thus a comparison of not only flavor level but fermentation capacity between a wild type SNUws and mutants of SNUws needed to be discussed. Additionally, wild type of other species of yeast such as the KCCM 51299 strain, and the SNUit strain were also compared with their mutant types called Gln26stop and△*CAR1*. In particular, concentrations of aroma compounds were closely examined in GC-MS results as our hypothesis may prove that inactivation of the *CAR1* gene leads to enhancement of rosy and tropical flavors.

3.7.1. Growth

In the SNUws strain, mutant of Gln26stop type grows better than wild type and SNUws△*CAR1* strain. However, the difference is only a growth curve. Likewise, there is little difference between the KCCM 51299 wild type, △*CAR1* type, and Gln26stop type strain. As shown in Fig. 2.19., the SNUit wild type strain is not differnent from its mutant strains. These results show that 3 types of yeast species have similar growth ability with CRISPR-engineered mutant strains. It is explained using data from the KCCM 51299 strain's whole genome sequencing, which revealed no differences and only customized sequences that performed similarly.. (Chin et al., 2021)..

3.7.2. Fermentation ability

The SNUws△*CAR1* has a greater capacity for fermentation than the wild type and Gln26stop type, as demonstrated in Fig. 2.17. But it is not statistically significant like the preceding growth ability. And in Fig. 2.18., other wile type of KCCM 51299 strain also has no difference with mutants. The other SNUit strain was not shown to be different from mutant strains in Fig. 2.19. Given that three different yeast species were used in the test, these findings might suggest that there are no variations in fermentation ability.

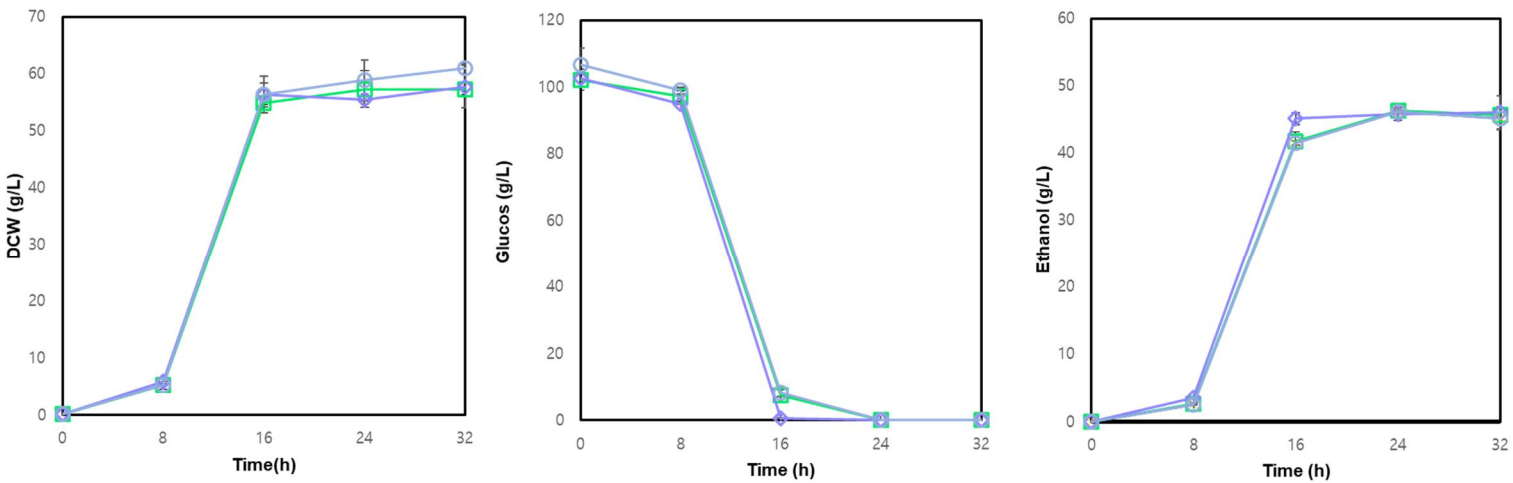

Fig. 2.17. Growth ability and fermentation features of the SNUws strain. (□), the SNUws wild type; (◇), the SNUws△*CAR1*; (○), the SNUws Gln26stop type.

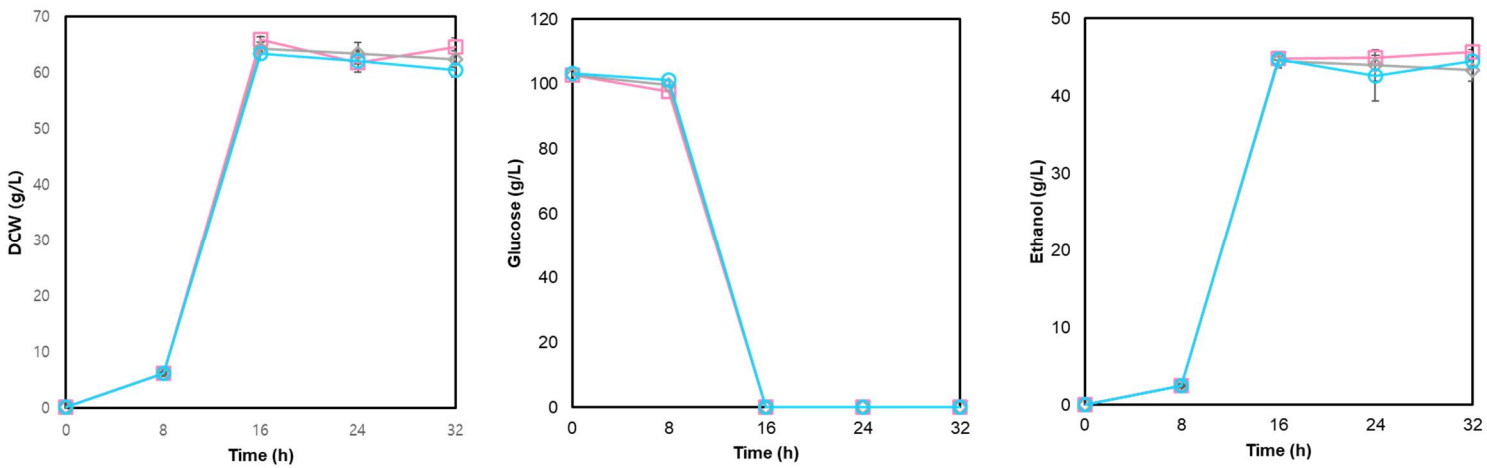

Fig. 2.18. Growth ability and fermentation features of the KCCM 51299 strain. (□), the KCCM 51299 wild type; (◇), the KCCM 51299△*CAR1* type; (○), the KCCM 51299 Gln26stop type.

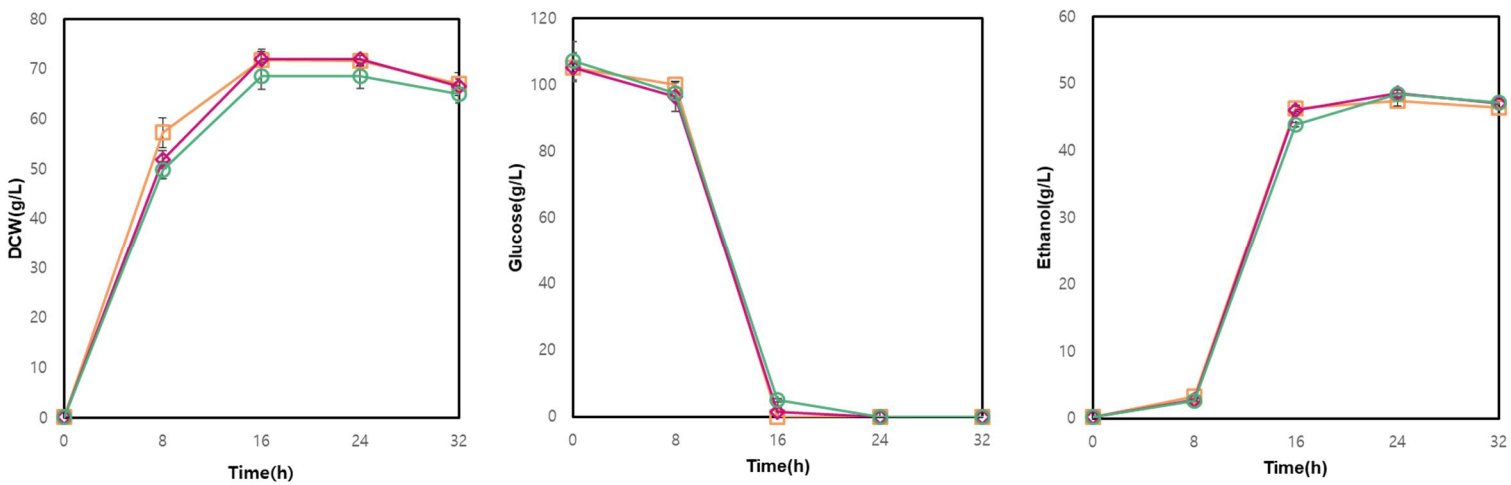

Fig. 2.19. Growth ability and fermentation features of the SNUit strain. (□), the SNUit wild type; (◇), the SNUit △*CAR1* type; (○), the SNUit Gln26stop type.

3.7.3. Flavor compound analysis

As shown in Fig. 2.20, it may be a constant pattern that all type mutants produce more isoamyl alcohol than the wild type of each species. Between SNUws wild type and SNUws mutants, This pattern is particularly obvious. In addition, showed the yeast species, the KCCM 51299 strain and the SNUit strain, also have a similar pattern. It could explain the apparent link between the improvement of isoamyl alcohol and the inactivation of the *CAR1* gene. According to Fig. 2.21., the output of phenethyl alcohol also has links with the inactivation of the *CAR1* gene. The production of phenethyl alcohol was also increased in all mutants, including different types and strain types. Although it is not a statically meaningful difference in KCCM 51299 mutants at (b), it could identify the tendency. The sole genetic similarity between Gln26stop type and △*CAR1* mutants was a trun-off the *CAR1* gene. Through GC-MS results of wild type and mutants in various yeast species, it is verified aroma compound enhancment such as isoamyl alcohol having a tropical flavor and phenethyl alcohol having a rosy flavor in various *CAR1* mutants.

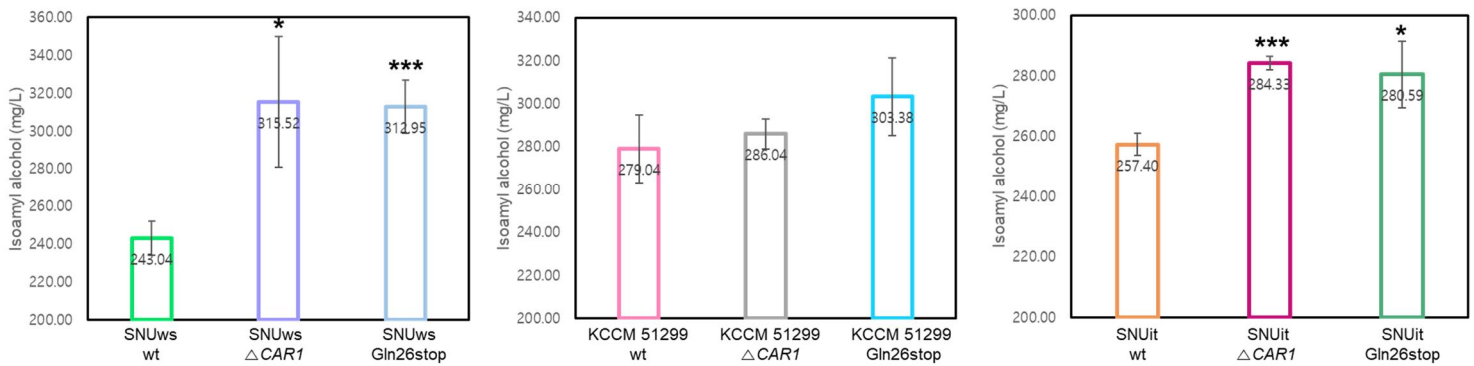

Fig. 2.20. Isoamyl alcohol analysis in 3 type of yeast strains. (a): GC-MS results in the SNUws strain; (b): GC-MS results in the KCCM 51299 strain; (c): GC-MS results in the SNUit strain. Significant variations between each mutant's concentration value and the wild type are indicated by an asterisk (p value ≤ 0.05). And two asterisk report p-value less than 0.01 (p value ≤ 0.01). and three asterisk report p-value less than 0.001 (p value ≤ 0.001).

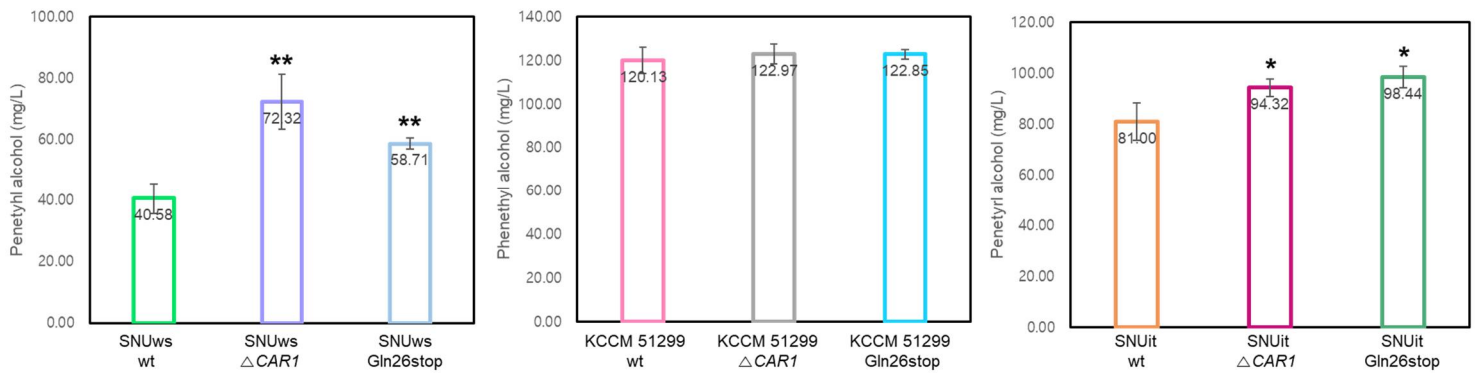

Fig. 2.21. Phenethyl alcohol analysis in 3 type of yeast strains. (a): GC-MS results in the SNUws strain; (b): GC-MS results in the KCCM 51299 strain; (c): GC-MS results in the SNUit strain. Each mutant's concentration value is significantly different from the wild type's concentration, as indicated by an asterisk (p value ≤ 0.05). And two asterisk report p-value less than 0.01 (p value ≤ 0.01). and three asterisk report p-value less than 0.001 (p value ≤ 0.001).

4. Conclusion

In chapter 2, the CRISPR-Cas9 system was applied to SNUws yeast strains isolated from Korean fermented foods for the establishment of reference data about the enhancement of flavors. To make a strain line of similar form to prior prediction, the design of sgRNA targets was an identical gene, *CAR1*. One type of 2 types of genotypes was a complete deletion of a gene, called the △*CAR1*, and the other was the introduction of a stop codon sequence into a gene, called the Gln26stop type. sgRNA targeting the *CAR1* gene was transcribed *in vitro* and co-transformed with a customized repair template to the Cas9 expressed transformants. Then the transformants of △*CAR1* were genotyped for PCR to image on the gel by size difference. Primer set (ttaa_115F & R) was used to amplify the region including the "modification part of *CAR1* upstream". Due to having a MseI flanking site in the Gln26stop type template, two bands were observed on the gel electrophoresis (Fig. 2.6.). Transformants of Gln26stop type were imaged on electrophoresis gel after processing with a restriction enzyme that works only on a specific site (5'-TTAA- 3'). Finally, shotgun sequencing was used to study the site adjacent to the *CAR1* gene. Additionally since mutants which inactivated the *CAR1* gene did not produce arginase encoded the *CAR1* gene and mutants were resistant to canavanine. Unlike wild types, which deplete arginines of canavanine analogue, they could live as a toxic agent to yeast through positive selection in a specific canavanine concentration of 150 μg/L. This selection was used to SNUws wild type and SNUws△*CAR1* that confirmed by PCR. Finally, colonies sorted in CAO medium

could be verified. By sequencing the PCR products and genomic DNA, it was confirmed that the desired mutation was induced for all type mutants of SNUws strain. First, to check for correction to growth rate, wild type and all mutants were fermented for 40 hours. Although a slight increase was observed, there was no difference in the stationary phase and it was not significant. By analyzing the glucose metabolic process using HPLC, the ability to fermentation could be evaluated. Between wild type and two mutant kinds of three different yeast species, glucose decomposition with ethanol production was not different significantly. However, when aroma compounds like flavor agents were analyzed using GC-MS, both isoamyl alcohol, which has a tropical flavor, and phenethyl alcohol, which has a rosy flavor, were found to be higher in each $\triangle CAR1$ mutant and Gln26stop type mutant than in the wild type of all species.

5. Discussion

A Strategy for genome editing has been developed to embody prior studies (Chin et al., 2016b; Chin et al., 2021). It is important to use the application of the CRISPR-Cas9 system safely without off-target effects. The PAM site and adjacent base pair of the target DNA sequence occur in situations based on Cas9 interrogation and off-target effects, which have been investigated using numerous sequencing-based approaches (Ebrahimi & Hashemi, 2020; Herai, 2019; Newton et al., 2019). In living eukaryotic cells, the mechanism between on/off target binding and Cas9 binding complex chromatin environment has not been verified yet (Knight et al., 2015). Previous studies have clearly revealed to not have off-target DNA cleavage of the CRISPR-Cas9 system by sequencing the whole genome. And as described in chapter 2, though the SNUws strain is not subjected to the whole genome sequencing, it is confirmed to have no effects near the target gene by partial sequencing. In addition, CRISPR-Cas9 was established with transcription of sgRNA *in vitro* to the SNUws strain. It was understood that sgRNA delivered directly into strain presented fewer bioethical issues than plasmiplasmid-based RNA insertion.

Different type of strains, called $\triangle$*CAR1* type and Gln26stop type mutants, would have only one genetic modification that was expected not to produce arginase. But this special genetic part could cause unintended changes to other abilities, particularly flavor agents. Uniform flavor changes were discovered at a subsequent

test of prior study. Isoamyl alcohol with a tropically banana flavor and phenethyl alcohol with a rosy flavor, in particularly, were noticeably improved in mutants over wild type. In this study, though the *CAR1* gene has not yet been uncovered to be connected to flavor production, mutants of various strains showed equal increases without strain factor. Additionally, the *CAR1* gene was inactivated by two different courses. Both mutants were very different genetically, except that what they have in common is that the *CAR1* gene was inactivated. The iterative results of GC-MS at △*CAR1* type and Gln26stop type revealed that isoamyl alcohol and phenethyl alcohol were more produced than the wild type. It was sure to have effects specific flavor metabolisms. This unknown cycle will be expected to be studied metabolically. It is speculated that this is a further effect of promoting amino acid metabolism, especially decomposition, to regulate homeostasis. Ornithine will not be generated in mutants cells if arginase is not. In yeast, ornithine amino acid had fewer production pathways in biosynthesis. One path to making ornithine is using glutamate biosynthesis. There are numerous metabolic routes for glutamate, including those for the breakdown of phenylalanine and the leucine amino acid. Leucine amino acid was degraded to α-keto isocaproate and finally made isoamyl alcohol. Also, phenylalanine was degraded to phenylpyruvate and then phenethyl alcohol. Two pathways share a glutamate reward pathway. So it could be assumed that final compounds such as isoamyl alcohol and phenethyl alcohol have something to do with glutamate product-relation. But further research is needed to support the evidence.

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Abstract in Korean

세계적으로 효모는 인간에게 유용한 에탄올과 다양한 부산물들을 생산하는 데 사용돼 왔다. 특히 효모가 생산하는 2 차 대사산물 중 하나로 향미 그룹이 있다. 현대사회에서 소비자들은 다양한 향미 관련 시장에 높은 관심을 보이고 있으며, 화학합성 보다 친환경을 선호하는 경향을 보였다. 따라서 효모에서 생합성된 향성분은 소비자들을 만족시킬 수 있는 화장품, 향수, 및 케어산업에 이용될 수 있을 것이다. 연구실에서 CRISP-Cas9 시스템을 이용해 *CAR1* 유전자를 불활성화한 논문을 출판한 이후, 만들어진 균주를 분석하던 중 특별한 향이 증가된 경향을 보였다. 바로 트로피칼한 향과 장미향이다. 이전연구는 또한 *CAR1* 유전자를 다른 서열을 가진 유전자 서열로 불활성화 시켰기 때문에 두 가지의 뮤턴트 타입이 존재한다. 이 두 가지 뮤턴트에서 일관적으로 증가한 두 아로마 화합물의 증가는 CRISPR-Cas9 시스템을 이용해 특정 향미를 증진할 수 있다는 가설을 세울 수 있게 하였다. 이 연구에서 종특이적 결과인지 가설의결과인지 확인하기 위해 동일한 방식의 CRISPR-Cas9 을 이용해 같은 유전자인 *CAR1* 을 다른 효모 종에서 불활성화 시켜 동일한 구축 모델을 형성하였다. 추가로 이 연구에서는

sgRNA 를 *in vitro* 로 합성하여 플라스미드를 넣어주지 않는 방식으로 연구를 발전시켰다. 테스트 결과 3 가지 이스트 종 모두에서, *CAR1* 유전자가 유전적으로 다르게 불활성화 된 두 균주에서 트로피컬 향미를 나타내는 Isoamyl alcohol 과 장미향미를 나타내는 Phenethyl alcohol 이 모두 야생 균주에 비해 증가하는 경향을 GC-MS 를 통해 관찰 할 수 있었다. *CAR1* 유전자와 특정향미인 Isoamyl alchol 과 Phenethyl alcohol 은 현재까지 아무런 연관관계도 밝혀진 바 없고 보고된 바가 없다. 그러나 일관적인 연구 결과를 바탕으로 *CAR1* 유전자의 불활성화와 특정 향미의 증가는 연관이 되어있는 것으로 설명할 수 있다. 그러므로 연관관계에 대한 실타래를 풀기 위해 더 많은 연구가 미래에 필요할 것으로 보인다. 그리고 이것은 미래에 자연으로부터 합성된 원료로, 향미 관련 시장에서 중요하게 쓰일 것으로 기대된다.