Metabolic Engineering of Wine Strain

Subjects: Microbiology

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The adaptive laboratory evolution (ALE) is a technique of strain optimization that assumes serial or continuous culturing of a particular yeast strain for many generations under selective pressure, such as high ethanol content, high osmolarity etc., thus directing the accumulation of mutants with desired phenotype. As compared to stochastic and laborious CSI techniques, ALE methods are more targeted and convenient . The power of this approach towards optimizing wine yeast is exemplified by generation of strains with altered production of important metabolites (ethanol, glycerol, succinic, and acetic acid) and more rapid sugar utilization, strains with increased sulfite tolerance and glycerol accumulation, strains with improved resistance towards KCL-induced osmotic stress with increased glycerol and reduced ethanol content, as well as enhanced viability and resveratrol production.

Keywords: winemaking, ; metabolic engineering, genomic editing

1. Introduction

Modern industrial winemaking is based on the use of starter cultures of specialized wine strains of *Saccharomyces cerevisiae* yeast. Commercial wine strains have a number of advantages over natural isolates, and it is their use that guarantees the stability and reproducibility of industrial winemaking technologies. For the highly competitive wine market with new demands for improved wine quality, it has become increasingly critical to develop new wine strains and winemaking technologies. Novel opportunities for precise wine strain engineering based on detailed knowledge of the molecular nature of a particular trait or phenotype have recently emerged due to the rapid progress in genomic and “postgenomic” studies with wine yeast strains.

2. CRISPR-Cas for Wine Yeast

For laboratory strains of *S. cerevisiae*, an extensive and diverse set of tools for genetic engineering and directed modification of the genome has been developed quite a long time ago and are widely used for research in the fields of functional genomics, synthetic biology, biotechnology, and metabolic engineering [1]. At the same time, the application of such approaches for industrial strains faces a number of difficulties. These strains are usually polyploids and aneupoloids, poorly sporulate, there are no convenient auxotrophic markers for them, etc. [2].

The use of CRISPR-Cas genome editing systems can successfully overcome these limitations. The first work on the application of the CRISPR-Cas system for *S. cerevisiae* was published back in 2013 [3] and the advantages of this approach for yeast, in which the system of homologous recombination was already well developed, were at first not obvious. However, after overcoming a number of technical difficulties aimed at optimizing the expression and delivery of CRISPR-Cas system components, the system quickly gained popularity and is now successfully used in areas such as multiplex genome engineering, reprogramming transcription, creating synthetic genomes, etc. [4].

Examples of the successful application of CRISPR-Cas systems for industrial yeast strains relate to such aspects as the production of bioethanol from lignocellulosic raw materials, metabolic engineering for the production of vitamins and antibiotics, the improvement of aromatic and taste properties of beer, and a number of others [5][6][7]. From the point of view of food safety, it is fundamentally important that the use of CRISPR-Cas genome editing methods does not carry the risk of introducing foreign genes and genetic elements, markers of antibiotic resistance into the genomes of food yeast strains, i.e., the resulting strains are safe according to regulatory restrictions adopted in some countries.

One recent study describes the use of the CRISPR-Cas system for producing wine strains with reduced urea production. A group of scientists from Canada and Italy constructed derivatives of wine strains EC1118 and AWRI1796 defective in both alleles of the *CAN1* gene [8][84]. The *CAN1* gene encodes arginine permease, which along with *GAP1* amino acids permease is responsible for the transport of arginine to yeast cells from the culture medium. During the subsequent stages of catabolism, arginine is cleaved by Car1p arginase to ornithine and urea, which is either excreted by Dur4p

permease or converted to carbon dioxide and ammonia by Dur1p/Dur2p urea amidolyase. The resulting recombinant strains were characterized by reduced urea production (18–36% compared to the initial ones) under experimental micro-winemaking with the ability to ferment a synthetic substrate, although at a slightly reduced growth rate. The authors believe that further verification of the strains is necessary under the conditions of industrial winemaking. The advantage of introducing a mutation into the *CAN1* gene compared to other methods of modifying arginine utilization pathways is that this technique is less sensitive to fluctuations in the content of nitrogen sources in the wort and less affects the growth parameters of yeast strains [7].

A promising area of application of genome editing methods is the directed change in the pathways of biosynthesis of aromatic compounds. Thus, in a recent work, yeast strains with increased production of phenylethyl acetate (PEA) were obtained using the CRISPR-Cas system [9]. PEA is an important aromatic compound that provides alcoholic drinks a pink and honey flavor. Genetic mapping methods first identified unique alleles of the *FAS2* genes (encodes the α subunit of fatty acid synthase) and *TOR1* (a growth regulator in response to the availability of a nitrogen source), linked to the trait of increased PEA production. Then, using CRISPR-Cas in commercial wine strains, wild alleles were replaced with mutant ones. As a result, the production of PEA increased by 70% [10].

In another work, the CRISPR-Cas system was used to reduce the production of 4-vinyl guaiacol (4VG) in a hybrid *S. pastorianus/bayanus* beer yeast strain [11]. It is known that 4VG is a sharp-tasting phenolic compound that spoils the organoleptic characteristics of beer. Formed from ferulic acid, 4VG is present in beer wort under the influence of yeast decarboxylase Fdc1p. Ale beer yeast strains do not produce 4VG due to the nonsense mutation in the *FDC1* gene. Using the CRISPR-Cas system, the authors introduced a mutation characteristic of ale strains into all four copies of the *FDC1* gene in the lager strain. The result was a strain containing a cis-gene mutation that lacks the ability to produce 4VG and has significant potential for use in the beer industry.

The CRISPR-Cas system is an extremely convenient tool for research in the field of functional genomics of wine strains. Until recently, the vast majority of experiments in the field of functional genomics of yeast were performed using laboratory strains. Nevertheless, according to the latest information from the SGD database (27 June 2020), when classified in terms of gene ontology, a significant number of yeast genes remain “unknown” (in the category “Biological Process”-1768 genes, 2548 genes in the category “Molecular Function” and 1298 genes in the cell compartment category). Such uncertainty is partly determined by the lack of specific conditions in which these genes are important. At the same time, these unknown genes experience regular changes in expression during many technological processes, including at different stages of wine fermentation (see, for example, [12]).

Characteristic changes in the expression pattern of a number of “unknown” genes were revealed in our recent work during the transcriptome analysis of the sherry strain at different stages of film formation [13]. CRISPR-Cas mediated genetic inactivation of “unknown” genes, allele replacement in wine strains of yeast can significantly clarify their role in various winemaking processes, and will help to create strains with improved characteristics(Table 1).

Table 1. Selected metabolically-engineered yeast strains and their oenology-related phenotypes.

Strain	Genetic Modification	Oenology-Related Trait	Ref.
ML01	Overexpression of *S.pombe mae1* gene *O.oeni mleA* gene	Malolactic fermentation	[14]
ECMo01	Overexpression of *S.cerevisiae DUR1,2* gene	Reduced ethyl carbamate content	[15]
AWRI 1631	Deletion of *MFA2* gene	Improved fermentation efficiency under nitrogen limitation	[16]
C911D	Deletion of *ECM33* gene	Improved fermentation efficiency under nitrogen limitation	[17]
S288C	Overexpression of *S.cerevisiae YOL155c* and *YDR055w* genes	reduced haziness during fermentation	[18]

EC1118	Deletion of *KNR4* gene	reduced haziness during fermentation, retaining good fermentation performance	[19]
VIN13	Overexpression of *Butyrivibrio fibrisolvens end1* gene, *Aspergillus niger xynC* gene	decrease in wine turbidity, increase in colour intensity, increase in phenolic compounds	[20]
VIN13	Overexpression of *Erwinia chrysanthemi pelE* gene, *Erwinia carotovora peh1* gene	decrease in phenolic compounds	[20]
ICV16, ICV27	Overexpression of *S. cerevisiae HSP26* and *YHR087W* genes	Improved Stress resistance and fermentation efficiency	[21]
PYCC 5484	Overexpression of 925–963 segments of *TDH1* and *TDH2/3* ORFs	Secretion of AMPs, inhibiting D.bruxellensis growth	[22]
Sigma1278	Overexression of *A. niger GOX* gene	Reduction of sugar content in juice	[23]
V5.TM6*P.	Overexpression of chimeric *HXT1-HXT7* gene in a *hxt* null strain	decreased ethanol production, increased biomass under high glucose conditions	[24]
MC42	Deletion of *ADH1, ADH3,ADH4* genes, *ADSH2* gene mutations	66% reduction of ethanol yield, increased glycerol production	[25]
CEN.PK 113-7D	Deletion of *TPI1* gene	Unable to grow on glucose, growth on mixed substrates	[26]
YSH l.l.-6B	Deletion of *PDC2* gene, overexpression of *GPD1* gene	Reduction of glucose catabolism, 6-7-fold increase in glycerol formation	[27]
AWRI1631	*GPD1* overexpression, *ALD6* deletion *	Decreased ethanol production	[28]
BY4742, VIN13	Screening of EOROSCARF deletion collection, weak *TPS* overexpression	10% reduction in ethanol yield, increased glycerol, trehalose production	[29]
CMBS33, BY4742	Analysis of ATF1,2 knockouts in the lab strain, constitutive *ATF1,2* overexpression in lager strains	Reduction in acetate esters production in ATF1,2 deletion strains, enhanced production of volatile esters in overexpression strains	[30]
T73-4	Overexpression of *Ocimum basilicum* (sweet basil) geraniol synthase (*GES*) gene	Increased geraniol production during fermentation, 230-fold increased total monoterpene content	[31]

VIN13	Overexpression of *A. awamori* arabinofuranosidase, *A. kawachii* β-glucosidase.	increased release of citronellol, linalool, nerol and α-terpineol.	[32]
WY1	Overexpression of *BDH1,2* genes	Decreased diacetyl, increased acetoin, butanediol contents	[33]
AWRI	Overexpression of *RtPAL, AtC4H, At4CL, RtBAS* genes for frambion biosynthesis	Frambion production at 0.68 mg/L simultaneously with chardonnay wine fermentation	[34]
CEN.PK 113-7D	Overexpression of *AtPAL2, AtC4H, At4CL, VvVST1* gene for resveratrol biosynthesis, complex strain and cultivation optimization strategy	Yeast-based de novo resveratrol production from glucose at 800 mg/l level	[35]
133d	Overexpression of *FLO11* gene using different promoter variamts	Improved velum formation	[36]
P3-D5	Deletion of *CCW14, YGP1* genes in a flor strain	Impaired velum formation	[37]
FJF206, FJF414, B16	Overexpression of *SOD1, SOD2, HSP12* in flor strains	increased superoxide dismutase, catalase, gluthathione peroxidase activities, increased oxidative stress resistance, quicker velum formation,slight decrease in ethanol and increase in acetaldehyde content	[38]
EC1118, AWRI1796	Crispr-cas9 mediated inactivation of *CAN1* gene	Reduced ethyl-carbamate formation	[9]
BTC.1D	Crispr-cas9 mediated allele exchange for *FAS2* and *TOR1* genes in wine strain	Increased phenyl-ethyl acetate formation	[10]
W34/70	Crispr-cas9 mediated allele exchange for FDC1 gene in lager strain	Decreased 4-vinyl guaiacol formation	[11]

* other modifications had non-significant effects.

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