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Isolation of Sake Yeast Mutants Producing a High Level of Ethyl Caproate and/or Isoamyl Acetate

YUKIHIKO ARIKAWA,[1]* MASATO YAMADA,[2] MAKOTO SHIMOSAKA,[2] MITSUO OKAZAKI,[2,3] AND MIKIO FUKUZAWA[1]

Food Technology Research Institute of Nagano Prefecture, 205–1 Nishibanba, Kurita, Nagano City 380–0921[1] and Faculty of Textile Science and Technology,[2] Gene Research Center,[3] Shinshu University, 3–15–1 Tokida, Ueda 386–8567, Japan

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In the case of sake, ethyl caproate and isoamyl acetate are considered to be closely associated with flavor. Various mutant yeast strains producing a higher level of flavor compounds (ethyl caproate and /or isoamyl acetate) than the parent strain were isolated by ethyl methane sulfonate treatment followed by global selection. Two of the mutants obtained also showed a high malate productivity. These mutants would be promising for practical sake fermentation.

[**Key words:** sake fermentation, ethyl caproate, isoamyl acetate, malate, *Saccharomyces cerevisiae*]

The quality of flavor in alcoholic beverages is determined by a large number of compounds. In the case of sake, ethyl caproate and isoamyl acetate are considered to be closely associated with flavor. These two compounds are mainly produced by yeast (*Saccharomyces cerevisiae*) during fermentation. Sake yeast strains producing a high level of flavor compounds and organic acids were isolated by positive selection for mutants resistant to analogues of objective compounds (1–7). This procedure to isolate objective mutants is effective and labor-saving, however, it is not always applicable because suitable analogues cannot be obtained for all metabolites related to flavor compounds and organic acids.

In this study, we report the isolation of mutants having a high productivity for flavor compounds by global selection using the diploid sake yeast, *S. cerevisiae* Kyokai no. 901 (K901) as a parent strain. K901 cells were mutated with ethylmethane sulfonate (EMS) as described previously (8). A total of 4800 single colonies were obtained on YPD plates (2% peptone, 1% yeast extract, 2% glucose, and 2% agar) after mutagenesis with 0.4% survival rate. These colonies were transferred to 96-well titer plates contained YPD broth (200 μl), cultured at 30°C, and then stored at −80°C after adding an equal volume of 50% glycerol. This mutant bank of diploid cells stored in 96-well titer plates containing YPD broth will be valuable for isolating a small number of recessive mutants by negative selection because a large number of mutant cells can be easily replica-plated on the selection plates using a replicator. A preliminary test demonstrated the generation of 16 auxotrophic mutants (contents: *ura*$^-$, 2 strains; *lys*$^-$, 2 strains; *met*$^-$, 4 strains; *his*$^-$, 1 strain; *trp*$^-$, 3 strains; *leu*$^-$ *arg*$^-$, 2 strains; *val*$^-$, 1 strain; not identified, 1 strain) and 25 glycerol non-assimilating mutants from this mutant culture bank.

We selected mutants with a high productivity of flavor compounds from the bank as follows. Each of the 4800 mutant cells was grown in 20-ml test tubes containing 5 ml of YPD10 (1% yeast extract, 2% peptone, and 10% glucose) at 20°C for 5 d without shaking. After the cells were removed by centrifugation, the amounts of flavor compounds produced in the culture broth were determined by gas chromatography (Shimadzu GC 17A, Kyoto) using a headspace system (CTC Analytics, Switzerland). Figure 1 shows the distribution of concentrations of ethyl caproate and isoamyl acetate produced by the mutants tested. A total of 205 strains produced 1.5-fold higher level of both flavor compounds than the parent strain did (compared to the maximum value). Some of these mutants with a constantly high productivity were chosen, and used for laboratory-scale sake production (total amount of rice, 200 g) as described previously (8). Table 1 shows the properties of sake produced using seven mutants with an increasing ethyl caproate and/or isoamyl acetate productivity (more than 1.5-fold compared with the parent strain).

The productivity of ethyl caproate in mutants P6-53 and P18-53 was 2.7-fold and 3.2-fold higher, respectively, than that of the parent K901. Moreover, P6-53 showed an increased productivity of malate, and a decreased productivity of ethyl acetate and isoamyl acetate as compared to K901 (Table 1). As a result, the contents of malate, ethyl acetate and isoamyl acetate in sake produced using the mutant P6-53 were 2.8-fold higher, 2.3- and 2.9-fold lower, respectively, than those in sake produced using the parent K-901. The mutant P6-53 also showed a leaky uracil requirement phenotype.

To confirm the mutation that resulted in this leaky uracil requirement phenotype, we screened a genomic library (9) of sake yeast to obtain a gene which complemented the uracil requirement phenotype of P6-53. As a result of screening, two plasmids (pAY6531 and pAY6532) were isolated, which respectively contained the 4.8- and 4.2-kb fragments necessary for complementation of the *ura*$^-$ phenotype. The restriction maps of the two plasmids indicate that a gene (*URA5*) coding for orotate phosphoribosyltransferase is located in the 1.7-kb fragment, which was determined by referring to the genomic DNA sequence database of *S. cerevisiae*. To confirm a possible mutation in the *URA5* gene, we synthesized two primers; sense, 5′-CTCGAATTCGTTCCAACTTT ACGTTCC-3′, and antisense, 5′-CTCGGATCCTGCATG TATCGTAGTAAC-3′, based on the sequence of both

* Corresponding author.

TABLE 1. Composition and flavor compound content of crude sake

Strains	Sake-meter	Acidity	Amino acidity	Ethanol (w/v%)	Ethyl acetate (mg/l)	Isoamyl acetate (mg/l)	Isoamyl alcohol (mg/l)	Ethyl caproate (mg/l)	Malate (mg/l)	Succinate (mg/l)	Lactate (mg/l)
K901	+4.5	2.6	2.4	17.0	155	7.2	145	0.54	275	687	403
P 6-53	−2.0	4.2	1.9	15.3	66	2.5	119	1.47	759	752	483
P11-58	−3.5	2.6	2.4	16.6	128	7.7	129	0.90	284	503	397
P18-53	+4.5	2.4	2.0	17.4	130	7.3	120	1.75	274	500	412
P28-36	−13.0	2.4	2.5	15.4	146	11.1	125	0.99	351	483	476
P30-29	−9.0	2.8	1.9	15.6	185	15.5	122	0.96	376	601	501
P33-37	+3.5	2.3	1.9	18.2	191	17.8	149	0.48	452	608	488
P43-14	−3.0	3.6	1.9	16.3	181	16.6	149	0.58	1264	392	464

ends of *URA5* ORF. Then, the DNA region containing *URA5* ORF was amplified using chromosomal DNAs of the mutant P6-53 and the parent K-901 as templates. Agarose gel electrophoresis revealed an amplification of a single PCR product of the expected size (1.0 kb). This PCR product was directly sequenced using an automated DNA sequencer (model 310, PE Applied Biosystems) and a BigDye Terminator Cycle Sequence kit (PE Aplied Biosystems), confirming that the 1.0-kb fragment indeed contained the *URA5* gene. The DNA sequence of the mutant *URA5* ORF had one base change (G to A) at position 496 (A of the start codon, ATG, is +1), resulting in replacement of the glutamate residue at position 157 with lysine. This base replacement was observed homogenously in DNA sequences of the PCR-amplified products from the diploid mutant. Therefore, the same mutation (G to A base change) must have occurred in two homologous loci (*URA5*) in the mutant P6-53. This high malate productivity of the mutant P6-53 could have been possibly induced by *URA5* mutation, because the strain P6-53 harboring pAY6532 (containing the wild-type *URA5*) showed a productivity similar to that of the parent K901 (data not shown).

S. cerevisiae possesses another gene (*URA10*) coding for orotate phosphoribosyltransferase which exhibited 20% activity in comparison with that coded by the *URA5* gene (10). This would explain why the mutant strain P6-53 showed a leaky phenotype of uracil auxotrophy. Previously, we reported that *URA3* mutation resulted in a high productivity of malate and succinate in *S. cerevisiae* (8) for unknown reasons. The mutant P6-53 also showed a high malate productivity, although succinate productivity was unchanged. This discrepancy would be caused by a different extent of damage in the pyrimidine pathway. Interestingly, the mutant P6-53 harboring plasmid pAY6532 recovered its uracil auxotrophic characteristic but still retained a high productivity of ethyl caproate and a low productivity of ethyl acetate and isoamyl acetate (data not shown). This result indicates that the mutant P6-53 might possess other mutation(s) leading to a change in productivity of flavor compounds.

It was reported that a cerulenin-resistant mutant of yeast overproduced ethyl caproate (2). Therefore, five mutants (P6-53, P11-58, P18-53, P28-36 and P30-29) producing a high level of ethyl caproate were examined whether they were resistant to cerulenin or not. The mutant cells were cultured on YPD agar plates containing various concentrations of cerulenin, and the minimum cerulenin concentration that inhibits growth of the parent K901 was determined to be 10 μM, whereas the concentrations of 3 μM and 25 μM were determined for the mutants P6-53 and P18-53, respectively. The other three mutants (P11-58, P28-36, P30-29) showed the same minimum concentration requirement as the parent did. Inokoshi *et al.* reported that the cerulenin-resistant mutant of *S. cerevisiae* had one base change (G to A) at position 1257 (A of the start codon, ATG, is +1) in the fatty acid synthetase gene (*FAS2*) (11). Then, we synthesized two primers; sense, 5′-CTCGAATTCTGCCAC TTTATACATTCC-3′, and antisense, 5′-CTCAAGCTTC AAGATGGTTTCTACACC-3′, based on the sequence of *FAS2*. The corresponding DNA fragments were amplified by PCR using chromosomal DNAs of P6-53, P18-53, P11-58, P28-36 and P30-29 as templates, and were directly sequenced. All of the amplified DNAs but one (P18-53) had the same sequence as that of the wild-type *FAS2*. Thus, a high ethyl caproate productivity of these mutants seems to be induced by a mechanism different from cerulenin resistance. One exception is mutant P18-53 which had the same base change (G to A, at posi-

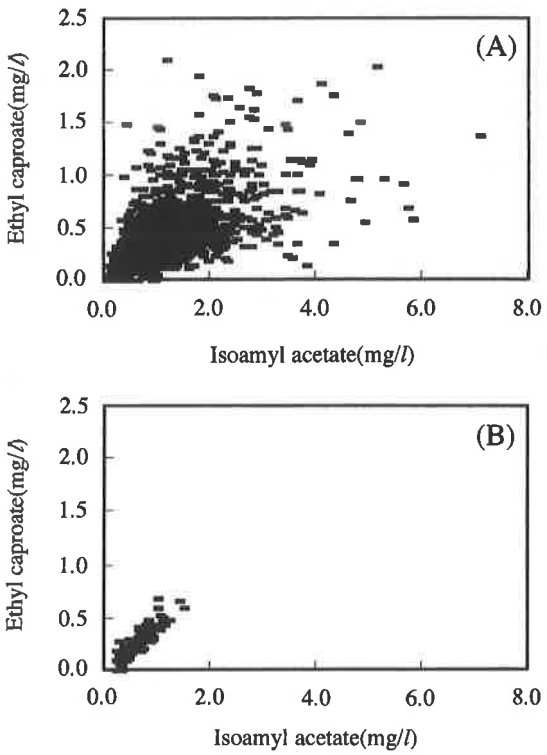

FIG. 1. Concentrations of isoamyl acetate and ethyl caproate in the liquors fermented using mutants or the parent strain. (A) Mutants, n=4800; (B) the parent strain (K901), n=50.

tion 1257) on at least one *FAS2* locus of two homologous chromosomes, which was reported to confer the cerulenin resistance phenotype (12).

The productivities of isoamyl acetate by the three mutants, P30-29, P33-37 and P43-14, were over two fold higher than that by strain K901 (Table 1). Overproduction of isoamyl acetate might be caused by a decrease in esterase activity in these mutants, because ethyl acetate was also overproduced by these mutants (13). The productivities of malate and succinate by the mutant P30-29 were 4.6-fold higher and 1.8-fold lower, respectively, than those of the parent strain. The reason for this difference in productivity of organic acids cannot be explained at present. The metabolic flow balance of acetyl-CoA may be important, since it is a common precursor for biosynthesis of ethyl caproate, isoamyl acetate and organic acids.

We constructed a bank of diploid mutants from sake yeast by EMS treatment, and selected strains that produce a higher level of ethyl caproate and/or isoamyl acetate than the parent strain by global screening. Furthermore, two mutants overproducing malate were also isolated. Unexpectedly, one of the mutants possessed a mutation in the *URA5* gene and showed a leaky phenotype of uracil requirement. These mutant strains will be valuable for practical sake fermentation.

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