Note

Breeding of a Sake Yeast with Improved Ethyl Caproate Productivity

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Flavor components, especially isoamyl acetate and ethyl caproate, are important for the quality of sake. A special technique called *ginjōzukurī*, which uses highly polished rice and low temperature fermentation, is usually used to brew sake rich in these esters. It is not easy, even with this technique, to brew rich-flavored sake. It is therefore important to breed a yeast strain producing flavor components at high levels in the sake mash. We have isolated a mutant producing sufficient isoamyl acetate by selecting 5,5,5-trifluoroleucine-resistant mutants.¹⁾

The synthesis of ethyl caproate in sake mash was catalyzed by enzymes in yeast; esterase and alcohol acyltransferase.²⁾ The substrates of these enzymes for the synthesis of ethyl caproate are ethanol and caproic acid or caproyl-CoA. As there is sufficient ethanol in the sake mash, either caproic acid or caproyl-CoA is a rate-limiting factor for the synthesis. The production of caproic acid is important in producing sake with a large amount of ethyl caproate. In this study we isolated mutant yeasts that produce sufficient caproic acid and ethyl caproate from sake yeast as strains resistant to cerulenin, a specific inhibitor of fatty acid synthetase.³⁾

The pathway of caproic acid synthesis is not known in sake yeast. It has been reported, however, that medium-chain fatty acids in beer are produced by fatty acid synthesis, and not introduced by degradation of long chain fatty acids.⁴⁾ Although detailed data were not shown, we have been suggested that caproic acid synthesis by sake yeast is inhibited by cerulenin. From these results, we assumed that caproic acid is produced by fatty acid synthesis in sake yeast. Fatty acid synthesis of the yeast is catalyzed by fatty acid synthetase, a multifunctional enzyme with seven different catalytic activities.⁵⁾ Alteration in balance of the individual catalytic activities in fatty acid synthetase changes the chain length of synthesized fatty acids.⁶⁾ Since cerulenin is an inhibitor of fatty acid synthetase, it was expected that caproic acid productivity of a cerulenin-resistant mutant would be different from that of the wild strain.

Haploid yeast (G1103), isolated from the sake yeast (*Saccharomyces cerevisiae*) Kyokai No. 7, was mutagenized by ethylmethane sulfonate as described previously¹⁾ and spread on YEPD agar plates (1% yeast extract, 2% peptone, 2% glucose, 2% agar) containing 25 μ M cerulenin at 30°C. Fifty-four colonies that developed on the plates were isolated as mutants resistant to cerulenin. The mutants were inoculated into the medium containing 0.67% Difco yeast nitrogen base without amino acid, 1% casamino acid, and 5% glucose, and the cultures were incubated for 3 days at 30°C. Then the broth was centrifuged and the resultant supernatant was used for analysis of caproic acid. Samples of the supernatant (100 ml) were adjusted to pH 1.0 with HCl and total lipids were extracted once with 100 ml of ethyl ether. The extract was washed once with diluted HCl and dried over anhydrous Na₂SO₄. After this was concentrated with a Kderna-Danish apparatus, the sample was adjusted to 1.0 ml with ethyl ether and analyzed by gas chromatography under the following conditions; column, 10% FON on Celite 545; column temperature, 200°C; nitrogen flow rate 50 ml/min. Among fifty-four mutants, three produced more than three times as much caproic acid as G1103 did, while the other mutants produced the same level of caproic acid as G1103

Fig. 1. Changes in Caproic Acid and Ethyl Caproate Concentrations in Sake Mash. (A), caproic acid; (B), ethyl caproate; ○, G1103; ●, C-8.

Table I. COMPONENTS OF SAKE FERMENTED WITH G1103 AND C-8

Component	G1103	C-8
Sake meter ^a	-1.0	-3.5
Alcohol (%) ^a	17.4	17.1
Acidity (ml) ^a	2.72	2.93
Amino acidity (ml) ^a	2.50	2.72
Isobutyl alcohol (μg/ml)	110	102
Isoamyl alcohol (μg/ml)	257	207
Isoamyl acetate (μg/ml)	6.8	5.7
Ethyl caproate (μg/ml)	1.4	7.4
Ethyl caprylate (μg/ml)	0.5	1.5

^a Measured by the method of the National Research Institute of Brewing (Tax Administration Agency, Japan).

Table II. ORGANIC ACID COMPOSITION OF SAKE WITH G1103 AND C-8

Organic acid	Concentration (μg/ml)	
	G1103	C-8
Lactic acid	452	423
Acetic acid	22	18
Citric acid	52	60
Succinic acid	243	216
Malic acid	427	463
Caproic acid	4.4	24.9
Caprylic acid	1.7	4.9
Capric acid	0.7	1.7

accumulated, C-8 strain, which produced the highest amount of caproic acid, was used for further tests.

Fermentation tests with G1103 and C-8 were done as described previously.¹⁾ A regular grade of rice (Nipponbare) was used with the polishing ratio of 72%. Sake mash was composed of 1040 g of steamed rice, 240 g of koji, and 1400 ml of water, and fermented at a constant temperature of 15°C. The changes in the concentrations of caproic acid and ethyl caproate in sake mash are shown in Fig. 1. The concentration of caproic acid and ethyl caproate in C-8 mash was much higher than that in G1103 mash. The components of sake are shown in Table I. The sake meter,

alcohol concentration, acidity, and amino acidity in sake brewed with C-8 were similar to those in sake brewed with G1103. However, the concentration of ethyl caproate was more than five times as high as that of G1103, and the concentration of ethyl caprylate also increased. Table II shows the components of organic acids. C-8 produced caproic acid about five times as high as G1103 and also caprylic acid and capric acid increased. There was no significant differences in other organic acids. In a sensory evaluation, the sake brewed with C-8 had an excellent fruity flavor.

Hybrid strains between C-8 and a cerulenin-sensitive strain were resistant to cerulenin. This indicated that cerulenin resistance was dominant and that a similar mutant was obtainable from industrial sake yeast. K-9-3 strain, a mutant with improved caproic acid productivity, was also isolated from sake yeast Kyokai No. 9 (K-9) by the method as used for G1103. C-8, K-9-3, and K-9 were used for sake fermentation tests under the same conditions described above except that 1300 ml of water was used. C-8, K-9-3, and K-9 produced 8.1, 4.7, and 1.1 μg/ml of ethyl caproate, respectively; and 17.5%, 18.5%, and 18.7% alcohol, respectively. K-9-3 produced four times as much ethyl caproate as K-9, and sake brewed with K-9-3 had a superior flavor in the sensory evaluation. Taking alcohol tolerance and fermentation ability into consideration, K-9-3 seems more suitable for sake brewing than C-8.

Although highly polished rice and low temperature fermentation have been required to brew rich-flavored sake, the mutant isolated in this study enabled us to produce sake with sufficient concentration of ethyl caproate by conventional brewing techniques.

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