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Balance of Activities of Alcohol Acetyltransferase and Esterase in *Saccharomyces cerevisiae* Is Important for Production of Isoamyl Acetate

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Isoamyl acetate is synthesized from isoamyl alcohol and acetyl coenzyme A by alcohol acetyltransferase (AATFase) in *Saccharomyces cerevisiae* and is hydrolyzed by esterases at the same time. We hypothesized that the balance of both enzyme activities was important for optimum production of isoamyl acetate in sake brewing. To test this hypothesis, we constructed yeast strains with different numbers of copies of the AATFase gene (*ATF1*) and the isoamyl acetate-hydrolyzing esterase gene (*IAH1*) and used these strains in small-scale sake brewing. Fermentation profiles as well as components of the resulting sake were largely alike; however, the amount of isoamyl acetate in the sake increased with an increasing ratio of AATFase/Iah1p esterase activity. Therefore, we conclude that the balance of these two enzyme activities is important for isoamyl acetate accumulation in sake mash.

Isoamyl acetate is a major and important determinant of sake flavor. This ester is assumed to be simultaneously synthesized from isoamyl alcohol and acetyl coenzyme A (CoA) by alcohol acetyltransferase (AATFase) (EC 2.3.1.84) (1, 2, 6, 12, 13, 15, 24) and hydrolyzed by esterases in sake mash (10, 18, 19, 21–23). To increase the amount of isoamyl acetate in sake mash, two approaches are possible: (i) overproduction of AATFase and (ii) disruption of the esterase(s) that acts on isoamyl acetate. The gene corresponding to AATFase (*ATF1*) of *Saccharomyces cerevisiae* has been cloned and sequenced (2). Transformants carrying the *ATF1* gene on a multicopy plasmid produced 27 times more isoamyl acetate than the control strain (2). We also cloned *IAH1* (formerly *EST2*), whose gene product is thought to be the major esterase that hydrolyzes isoamyl acetate (3). We constructed an *iah1*-deficient diploid sake yeast by a one-step gene disruption method using two different disruption plasmids sequentially, and the resulting mutant was completely deficient in Iah1p activity. These mutants accumulate more isoamyl acetate in sake mash than does the parent strain (4). Therefore, accumulation of isoamyl acetate appears to be controlled by a balance of these two enzyme activities. Our objectives were to construct yeast strains with various numbers of *ATF1* and *IAH1* sequences and to verify the relationship between the accumulation of isoamyl acetate and the ratio of these enzyme activities.

S. cerevisiae IFO 10506 (*MAT*α *trp1-*Δ*63 his3-*Δ*200 leu2-*Δ*1 lys2-801 ade2-101 ura3-52 IAH1*) and Kyokai no. 7, which is one of the most commonly used strains in sake brewing in Japan, were obtained from the Institute for Fermentation, Osaka, Japan, and the Brewing Society of Japan, respectively. *Escherichia coli* JM109 (Δ*lac-proAB recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1* [F′ *traD36 proAB*$^+$ *lacI*^{q}*Z*ΔM15]) was used as a host for plasmid amplification. The *ATF1* gene with 5′ and 3′ noncoding regions was amplified by PCR from genomic DNA of *S. cerevisiae* Kyokai no. 7 with the primers 5′-ACTTTG*GTCGAC*CAAAGCGTGTGAGGACTACTC-3′ and 5′-TCATAG*GTCGAC*CTAAACCAACCAAAGCCGAGG-3′. Both primers were designed to generate a *Sal*I site (shown in italics). The amplified *ATF1* gene fragment was inserted into the *Sal*I site of a multicopy plasmid, YEp24 (20), to yield YEp24-ATF1. Since deficiency of the *URA3* and/or *TRP1* gene affects the fermentation rate (4, 8), *S. cerevisiae* IFO 10506 was transformed with the 1.6-kb *Eco*RI-*Eco*RI (*TRP1*) fragment from YRp7 (20) to reverse the *TRP1* marker, and the resultant strain was designated YPHT. The strains YPHT (*trp1*::*TRP1*) and YPT1 (*iah1*Δ::*TRP1*) (4), transformed with YEp24, were used as control strains. The *IAH1* gene was also cloned into YEp24 to yield YEp24-IAH1, previously termed YEp24-BB (3), for overproduction of Iah1p. Yeast was transformed according to the method of Ito et al. (7).

Isoamyl acetate-hydrolyzing activity was measured by monitoring the production of acetate or isoamyl alcohol from isoamyl acetate. A reaction mixture (1.5 ml) containing 100 ppm of isoamyl acetate, 50 mM sodium phosphate buffer (pH 7.0), 7 mM $MgCl_2$, and enzyme in a 25-ml vial sealed with a silicone rubber septum was incubated at 25°C for 1 h. Solid NaCl (1.0 g) was added to the reaction mixture to stop the reaction, and the supernatant of the reaction mixture was withdrawn to measure acetate with an F-kit (Boehringer, Mannheim, Germany) or the reaction mixture stopped by the addition of NaCl was directly analyzed by headspace gas chromatography, as described by Yoshioka and Hashimoto (24). One unit of isoamyl acetate-hydrolyzing activity was defined as the amount of enzyme forming 1 ppm of acetate or isoamyl alcohol per h at 25°C. AATFase activity was measured by the method of Minetoki et al. (15). One unit of AATFase activity was defined as the amount of enzyme forming 1 ppm of isoamyl acetate per h at 25°C. Protein was measured by the method of Lowry et al. (11).

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TABLE 1. AATFase and Iah1p activities in cells grown in YPD medium

Strain/plasmid	Activity (U/mg of protein)[a]		No. of copies[b]	
	AATFase	Iah1p	*ATF1*	*IAH1*
YPHT/YEp24	1.2 ± 0.4	0.42 ± 0.09	1	1
YPT1/YEp24	1.2 ± 0.3	0.013 ± 0.03	1	0
YPHT/YEp24-ATF1	18 ± 6.5	0.44 ± 0.09	16	1
YPT1/YEp24-ATF1	20 ± 6.5	0.012 ± 0.02	17	0
YPHT/YEp24-IAH1	1.1 ± 0.3	3.3 ± 0.33	1	9

[a] One unit of AATFase activity is defined as the amount of enzyme forming 1 ppm of isoamyl acetate per h at 25°C (4). One unit of Iah1p activity is defined as the amount of enzyme forming 1 ppm of acetate per h at 25°C (3). Values are means ± standard deviations from four different tests.
[b] Copy number is the sum of the number of plasmid copies and endogeneous copies.

Isoamyl acetate-hydrolyzing esterase and AATFase activities of transformants were compared after culturing of the cells in YPD medium (2% glucose, 2% peptone, 1% yeast extract [pH 5.5]) at 20°C under static conditions for 2 days (Table 1). Isoamyl acetate-hydrolyzing esterase activities in strains YPT1/YEp24 and YPT1/YEp24-ATF1 were approximately 4 to 6% of that of the YPHT/YEp24 control. Isoamyl acetate-hydrolyzing esterase activity in strain YPHT/YEp24-IAH1 was eight times higher than that of control strain YPHT/YEp24. Specific activities of the AATFase of YPHT/YEp24-ATF1 and YPT1/YEp24-ATF1 were approximately 15 and 18 times higher than those of the control strains YPHT/YEp24 and YPT1/YEp24, respectively. The numbers of copies of the *ATF1* and *IAH1* genes were estimated by Southern blot analysis. Hybridization signals were compared with the endogenous *ATF1* and *IAH1* genes on the genome by using an AE-6920M densitometer (ATTO, Tokyo, Japan). Increases in copy number were correlated with increases in activity (Table 1). Transformants with YEp24-ATF1 and YEp24-IAH1 maintained multiple copies of each plasmid even when grown in YPD medium under static conditions for 2 days at 20°C (Table 1).

To determine the effect of *ATF1* overexpression and *IAH1* disruption on sake brewing, small-scale sake brewing was performed with these transformants. Laboratory-scale sake brewing and ester detection were carried out as described by Namba et al. (16). General components of the sake were analyzed by standard methods established by the National Tax Administration Agency of Japan (17). Evolution of CO_2 was measured to evaluate the fermentation profile; the fermentation rates of the transformants were almost the same. Analysis of general components in the brewed sake showed that there were no differences in transformants except for acidity and the concentration of acetate esters (Table 2). As shown in Table 2, acidity values of the sakes produced by the *iah1*Δ mutants were lower (5.7 ml) than those of *IAH1*$^+$ strains (6.9 to 7.1 ml). Acidity represents total acid, which consists mainly of organic acids in sake. If the esterase activity is decreased, ester hydrolysis is blocked and the carboxylic acid level decreases. Therefore, we interpreted lower acidity in sake produced by the *iah1*Δ mutants to indicate decreased concentrations of organic acids due to lack of esterase activity. The sake brewed by YPT1/YEp24-ATF1 accumulated approximately 50 times more isoamyl acetate than the sake brewed by the control strain YPHT/YEp24. Concentrations of isoamyl alcohol were slightly reduced in sakes produced by YPHT/YEp24-ATF1 and YPT1/YEp24-ATF1. We attributed this reduction to consumption of isoamyl alcohol by AATFase, since it is one of the substrates used by AATFase to produce isoamyl acetate.

Isoamyl acetate is synthesized by AATFase and hydrolyzed by esterase(s), and we have speculated that isoamyl acetate accumulation is dependent on the activity ratio of these two enzymes. Thus, we altered the Iah1p/AATFase ratio by disrupting the *IAH1* gene (4, 22). We also constructed yeast strains with various numbers of copies of the alcohol acetyltransferase gene (*ATF1*) and the isoamyl acetate-hydrolyzing esterase gene (*IAH1*). Fermentation profiles as well as components of the resulting sake of these strains were similar; however, the amount of isoamyl acetate accumulated in the resulting sakes increased with the ratio of AATFase/Iah1p esterase activity. In the *iah1*Δ mutant, the relative amount of isoamyl acetate in sake increased approximately 1.2-fold (Table 2), which is statistically significant. On the other hand, in the *IAH1*-overexpressing strain, the amount of isoamyl acetate decreased (Table 2). In this case, the decrease in isoamyl acetate was attributed to the overproduction of Iah1p, which hydrolyzes isoamyl acetate. The effect of *ATF1* overexpression on isoamyl acetate production was more significant than was the *IAH1* copy number. However, varying the number of *IAH1* copies in the *ATF1* multicopy background still affected the isoamyl acetate production (Table 2). From these results, we conclude that the balance of these two enzyme activities in yeast is important for the accumulation of isoamyl acetate in sake.

Minetoki (14) reported that AATFase of *S. cerevisiae* is extremely labile and that 55% of AATFase activity is lost if the enzyme is kept at 20°C for 30 min. Laboratory-scale sake fermentations were performed at 15°C, the authorized temperature for evaluation of the parameters listed in Table 2, and AATFase activity might gradually be lost during fermentation (9, 22). Iah1p activity does not decrease during fermentation (22). Constructing a yeast strain with a thermostable AATFase might increase isoamyl acetate production. Recently, we found a yeast, *Hansenula mrakii* IFO 0895, that has a thermostable AATFase (6) and produces isoamyl acetate at a level comparable to that produced by industrial sake yeasts (5). Thus, the

TABLE 2. Properties of sake brewed on a small scale

Strain/plasmid	Sake meter	Glucose (%)	Ethanol (%)	Acidity[a] (ml)	Amino acidity[b] (ml)	Ethyl acetate (ppm)	Isobutyl acetate (ppm)	Isobutyl alcohol (ppm)	Isoamyl acetate (ppm)	Isoamyl alcohol (ppm)	Ethyl caproate (ppm)
YPHT/YEp24	−55	6.4	12	7.1	2.1	28 ± 3[c]	0.05 ± 0.07	130 ± 6	0.27 ± 0.05	110 ± 4	0.38 ± 0.00
YPT1/YEp24	−56	6.7	12	5.7	2.1	35 ± 6	0.13 ± 0.03	170 ± 8	0.33 ± 0.00	120 ± 6	0.41 ± 0.04
YPHT/YEp24-ATF1	−57	6.9	11	7.1	2.2	85 ± 6	0.77 ± 0.07	130 ± 5	6.5 ± 0.55	94 ± 5	0.38 ± 0.02
YPT1/YEp24-ATF1	−57	6.8	11	5.7	2.1	140 ± 24	3.0 ± 0.56	160 ± 11	13 ± 2.2	84 ± 3	0.44 ± 0.01
YPHT/YEp24-IAH1	−57	6.8	11	6.9	2.2	35 ± 11	0.07 ± 0.06	130 ± 9	0.21 ± 0.04	110 ± 4	0.41 ± 0.03

[a] Acidity indicates the volume of 0.1 N NaOH which titrates a 10-ml sample of sake.
[b] Amino acidity indicates the volume of 0.1 N NaOH which titrates formol nitrogen in a 10-ml sample of sake.
[c] Values are means ± standard deviations from three or four different tests.

ATF1 gene of *H. mrakii* might be used to increase isoamyl acetate production in sake. In addition to the balance of AATFase and esterase activities in yeast cells, we must consider the concentrations of substrate for AATFase, i.e., isoamyl alcohol and acetyl CoA, for production of isoamyl acetate. The K_m of AATFase for isoamyl alcohol was reported to be fairly high (29.8 mM) compared with that for acetyl CoA (0.19 mM) (15); therefore, a major rate-limiting factor for isoamyl acetate production is the amount of isoamyl alcohol in the sake mash (1, 9, 15). Low levels of isoamyl alcohol in sake mash (2 to 3 mM) (1, 9) and relatively low levels of active AATFase may result in poor production of isoamyl acetate. Efficient production of isoamyl acetate requires an adequate supply of both substrates (isoamyl alcohol and acetyl CoA) and an appropriate balance of enzyme activities (AATFase and esterase).

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