Roles of Esterase and Alcohol Acetyltransferase on Production of Isoamyl Acetate in *Hansenula mrakii*

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Isoamyl acetate is a major determinant of the quality of Japanese sake. The amount of isoamyl acetate in the cultures of *Hansenula mrakii* and *Saccharomyces cerevisiae* Kyokai No. 7, which is industrially used in sake fermentation, and the isoamyl acetate-producing activities of each yeast strain were compared to investigate biochemical properties of the producing system of isoamyl acetate in these yeast strains. *S. cerevisiae* could not produce, or produced an extremely low level of, isoamyl acetate when the cells were cultured under aerobic conditions, while *H. mrakii* could produce a large amount of isoamyl acetate cultured at both 15 and 30 °C under aerobic conditions. Intact cells of *H. mrakii* cultured at 15 and 30 °C could produce isoamyl acetate from isoamyl alcohol and acetic acid. Alcohol acetyltransferase activity of *H. mrakii* was detected in insoluble fractions, while isoamyl acetate-synthesizing esterase was detected only in soluble fractions of the cell extracts. Isoamyl acetate-hydrolyzing esterase was detected in both soluble and insoluble fractions. Expression patterns of esterase were examined by native PAGE followed by activity staining by using 1-naphthyl acetate and Fast Blue B salt. *H. mrakii* is likely to have several esterases, and their expressions were varied depending upon the growth phase and temperature.

Keywords: *Hansenula yeast; isoamyl acetate; esterase; alcohol acetyltransferase*

INTRODUCTION

Sake (Japanese rice wine) is one of the traditional alcoholic beverages in Japan. In sake brewing, rice starch is saccharified by the glucoamylase of *Aspergillus oryzae*, and the liberated glucose is then fermented to ethanol by *Saccharomyces cerevisiae*. Both steps occur simultaneously (Kuriyama et al., 1986a,b). This fermentation system distinguishes sake brewing from other alcohol fermentations in the world, such as wine fermentation. In wine fermentation, grape contains a large amount of fermentable sugar (sucrose), and yeast can use it for fermentation without saccharification by other microorganisms.

The *ginjo*-sake is regarded as one of the high-quality Japanese sakes. The desirable features of the *ginjo*-sake are a clear taste and a fruitlike flavor. Fruitlike flavor of the *ginjo*-sake is owing to more than 100 molecular species of esters, alcohols, organic acids, carbonyl compounds, and sulfhydryl compounds. Among them, isoamyl acetate and ethyl caproate are known as major flavor components in sake and other alcoholic beverages (Kuriyama et al., 1986a,b). Isoamyl acetate has a fruitlike flavor and is recognized as an important factor for determining the flavor quality of sake. Therefore, much attention has been given to the improvement of the production of isoamyl acetate (Gillies et al., 1987; Thurston et al., 1981).

Acetate esters in *S. cerevisiae* are believed to be synthesized from alcohols and acetyl-CoA by alcohol acetyltransferase (AATFase, EC 2.3.1.84) (Howard and Anderson, 1976; Ishikawa et al., 1984; Kuriyama et al., 1986a; Malcorps and Dufour, 1992; Minetoki, 1992; Nordstorm, 1961, 1962a,b, 1963; Yoshioka and Hashimoto, 1981). This enzyme is a membrane-bound protein, is unstable at high temperature, and is strongly repressed when the yeast is cultured under aerobic conditions or by the addition of unsaturated fatty acids into the culture medium (Malcorps et al., 1991; Minetoki et al., 1993; Yoshioka and Hashimoto, 1981). The enzyme was purified and characterized by Minetoki et al. (1993). Recently, the *ATF1* gene encoding AATFase was cloned from *S. cerevisiae*, and its nucleotide sequence was determined (Fujii et al., 1994).

On the other hand, some yeasts can synthesize esters using the reverse reaction of esterase in the absence of acetyl-CoA (Suomalainen, 1981). Schermers et al. (1976) suggested a role of esterase in the production of esters by brewing yeast, since they found a positive correlation between the activity of esterase and the level of acetate esters in different strains of *S. cerevisiae*. Kuriyama et al. (1986b) have studied the esterase activity of sake yeast in synthesis and hydrolysis of ethyl caproate and found two pathways; one was performed by esterase and the other by AATFase. However, the accumulation of esters is widely believed to be dependent on the ratio of activities between AATFase for ester production and esterase for hydrolysis of esters (Yanagiuchi et al., 1989b). Yanagiuchi et al. (1989a) derived mutants deficient in esterase activity from sake yeast, and the resultant mutants showed high productivity of isoamyl acetate and isobutyl acetate compared with the parent strain.

It has been reported that *Hansenula* yeasts are one of the potent producers of esters (Bedford, 1942; Davies et al., 1951; Gray, 1949). Nevertheless, few detailed studies have been done on the biochemistry of ester production by *Hansenula* yeasts (Yoshioka and Hashimoto, 1981; Inoue et al., 1994). Thus, we focused our effort on analyzing the system for production of isoamyl

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acetate in *Hansenula* yeasts to apply it in sake brewing. Previously we screened several *Hansenula* yeasts for resistance against isoamyl alcohol, a substrate of isoamyl acetate formation, and found that *H. mrakii* IFO 0895 showed higher resistance to isoamyl alcohol among the *Hansenula* yeasts examined (Inoue et al., 1994).

In this report, we describe the roles of esterase and AATFase which contribute to the production of isoamyl acetate by *H. mrakii*.

MATERIALS AND METHODS

Chemicals. Acetyl-CoA (trilithium salt) was purchased from Wako Chemical, Kyoto, Japan. Isoamyl alcohol and isoamyl acetate were obtained from Nacalai Tesque, Kyoto, Japan. Fast Blue B salt was purchased from Merck, Darmstadt, Germany. Combination acetate kit (F-kit) was from Boehringer Mannheim-Japan, Tokyo, Japan.

Microorganisms and Culture. *H. mrakii* IFO 0895 and *S. cerevisiae* Kyokai No. 7 were obtained from the Institute for Fermentation, Osaka (IFO), Japan. *S. cerevisiae* Kyokai No. 7 was originally named by the Brewing Society of Japan (Nippon Jozo Kyokai).

A nutrient medium for *H. mrakii* (HM medium) contained 1% glucose, 0.5% peptone, 0.2% yeast extract, 0.03% K_2HPO_4, 0.03% KH_2PO_4, and 0.01% $MgCl_2$ (pH 5.5). A nutrient medium for *S. cerevisiae* (YPD medium) contained 2.0% glucose, 1.0% yeast extract, and 2.0% peptone (pH 5.5).

Determination of Isoamyl Acetate and Isoamyl Alcohol. Cells were cultured in a 2-L Sakaguchi flask containing 1 L of nutrient medium at 15 or 30 °C with reciprocal shaking (300 rpm, 30-cm stroke). The cells at log phase ($OD_{610} = 0.8-1.0$), early stationary phase ($OD_{610} = 3.0-4.0$), and stationary phase (3-day culture) were collected by centrifugation (5600*g*, 20 min, 4 °C). Optical density of culture at 610 nm (OD_{610}) was measured by a Shimadzu UV-160 spectrophotometer (Shimadzu, Kyoto, Japan). Resultant supernatants (3.0 mL) were put into a 50-mL vial, and 50 ppm (final concentration) of *n*-butanol (Nacalai Tesque, Kyoto, Japan) was added as an internal standard. The vial was tightly sealed by a silicon rubber cap and aluminum cap and heated at 50 °C for 30 min, and then the mixture was subjected to head space gas chromatography as described below.

Determination of Acetic Acid. Yeasts were grown aerobically in a nutrient medium, the cells were removed by centrifugation at 5600*g*, 4 °C for 20 min, and the supernatant (1.0 mL) was used for assay of acetic acid by using the acetic acid assay kit (Boehringer Mannheim-Japan, Tokyo, Japan). Detailed procedures were followed to the vendor's specification.

Production of Isoamyl Acetate by Intact *H. mrakii* Cells. *H. mrakii* was cultured in HM medium to log phase, or stationary phase under aerobic conditions, and cells were collected as described above. Cells were washed twice with 0.85% NaCl solution; then a small portion of the cells (100 mg as wet weight) was transferred into the reaction mixture (3.0 mL) containing 15 mM isoamyl alcohol and 100 mM acetate buffer (pH 5.0), or 46 mM isoamyl alcohol, 1.0 mM acetyl-CoA, and 50 mM potassium phosphate buffer (pH 7.0). The reaction was carried out at 25 °C for 60 min in a 50-mL vial which was tightly packed with a silicon rubber cap and aluminum cap. Isoamyl acetate formed in each reaction system was measured by head space gas chromatography as described below.

Preparation of Cell Extracts. *H. mrakii* was cultured aerobically in HM medium to each growth phase, and cells were collected by centrifugation. Cells were washed twice with 0.85% NaCl solution, resuspended in an appropriate amount of ice-chilled 10 mM potassium phosphate buffer (pH 7.0), and disrupted with glass beads (0.4−0.5 mm diameter) using a Braun homogenizer (B. Braun HOM-MSK type, Melsungen, Germany) at 0 °C for 2 min. The homogenates were centrifuged at 15000*g* for 30 min at 4 °C, and the supernatants were used as cell extracts.

Fractionation of AATFase. Cell extracts were prepared from the cells (1 g as wet weight) cultured at 15 °C to log phase and centrifuged at 200000*g*, 4 °C for 2 h. Insoluble materials were used as total membrane fractions (Tran et al., 1993). Unless otherwise stated, all procedures were carried out at 0−4 °C. The resulting pellets were suspended in 1 mL of 10 mM potassium phosphate buffer (pH 7.0), and the suspension was kept in a refrigerator for 2 h with gentle shaking. The suspension was centrifuged at 200000*g* for 2 h. Soluble fractions were subjected to the AATFase assay as described below. Insoluble materials were resuspended in 1 mL of 10 mM potassium phosphate buffer (pH 7.0) containing 2 M NaCl, kept for 2 h, and then centrifuged at 200000*g* for 2 h. Pellets were resuspended in 1 mL of 10 mM potassium phosphate buffer (pH 7.0) containing 0.1% Triton X-100, kept for 2 h, and then centrifuged at 200000*g* for 2 h. After centrifugation, the pellets were treated with 2% Triton X-100 and then centrifuged at 200000*g* for 2 h. Insoluble materials were then resuspended in 1 mL of 10 mM potassium phosphate buffer (pH 7.0) containing 5.0% Triton X-100 and centrifuged. Resultant supernatant was assayed for AATFase activity as described below.

Enzyme Assays. *Esterase.* Isoamyl acetate-synthesizing activity from isoamyl alcohol and acetic acid was measured according to the method of Yoshioka and Hashimoto (1981). The reaction mixture (3.0 mL) contained 15 mM isoamyl alcohol, 100 mM acetate buffer (pH 5.0), and enzyme. The reaction was carried out in a 50-mL vial which was tightly packed with a silicon rubber cap and aluminum cap. The vial containing the reaction mixture was left to stand at 25 °C for 60 min. The reaction was stopped by the addition of 0.6 g of solid NaCl into the mixture, and then 50 ppm (as a final concentration) of *n*-butanol was added as an internal standard. The reaction vial was left at 50 °C for 30 min, and then the synthesized isoamyl acetate was measured by head space gas chromatography under the following conditions: gas chromatograph, Shimadzu GC-15A gas chromatography with dual flame ionization detectors (Shimadzu, Kyoto, Japan); column, glass column (3 mm × 3.3 m i.d.) packed with 10% DNP on celite 545 SK DMCS (80/100 mesh) (GL Sciences, Tokyo, Japan); column temperature, 82 °C; injection temperature, 200 °C; sample size, head space gas, 3 mL; carrier gas, N_2 (50 mL/min); hydrogen flow rate: 45 mL/min (hydrogen pressure, 0.6 kg/cm^2); air flow rate, 500 mL/min (air pressure, 0.5 kg/cm^2).

For measuring isoamyl acetate-hydrolyzing activity, isoamyl acetate was used as a substrate and potassium phosphate buffer (pH 7.0) was used in the reaction mixture as described above. One unit of esterase activity is defined as the amount of enzyme forming or hydrolyzing 1.0 ppm of isoamyl acetate/h.

AATFase. AATFase was measured by the method of Minetoki et al. (1993) with some modifications. The reaction mixture (3.0 mL) contained 46 mM isoamyl alcohol, 1.0 mM acetyl-CoA, 2.0% Triton X-100, 20% glycerol, and 50 mM potassium phosphate buffer (pH 7.0). The reaction was carried out in a 50-mL vial which was tightly packed with a silicon rubber cap and aluminum cap. The vial containing the reaction mixture was kept at 25 °C for 60 min. The reaction was stopped by the addition of 0.6 g of solid NaCl into the mixture, and then 50 ppm (as a final concentration) of *n*-butanol was added as an internal standard. The reaction vial was left at 50 °C for 30 min, and then the synthesized isoamyl acetate was measured by head space gas chromatography as described above. One unit of AATFase activity is defined as the amount of enzyme forming 1.0 ppm of isoamyl acetate/h.

Protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Activity Staining (Diazo Staining) of Esterase. To investigate the expression pattern of esterase activity, native polyacrylamide gel electrophoresis (PAGE) was carried out with 12.5% polyacrylamide gel at 4 °C using the buffer system of Laemmli without SDS (Laemmli, 1970). The amount of protein applied on each lane was adjusted to 100 mg. After electrophoresis, the gel was stained for esterase activity by emerging it into an appropriate amount of 10 mM potassium phosphate buffer (pH 7.0) containing 0.025% 1-naphthyl acetate and 0.04% Fast Blue B salt, and the gel was kept at room

Table 1. Volatile Compounds in Yeast Cultures (Aerobic Conditions)[a]

compounds (ppm)	log		early stationary		stationary	
	15 °C	30 °C	15 °C	30 °C	15 °C	30 °C
H. mrakii IFO 0895						
isomyl alcohol	2.7	64.7	37.7	153	278	198
acetic acid	7.0	19.3	16.3	36.5	3.23	1.46
isoamyl acetate	3.6	5.0	7.8	10.6	40	0.01
S. cerevisiae Kyokai No. 7						
isoamyl alcohol	9.3	30	21.6	56.8	48.3	87.9
acetic acid	13	35.3	19	56	12.9	11.8
isoamyl acetate	0.05	ND[b]	ND	0.02	0.06	0.03

[a] Results are a summary of three independent experiments. [b] Not detected.

Table 2. Production of Isoamyl Acetate by Intact *H. mrakii* Cells

culture temp (°C)	growth phase	activity (ppm/h/10 g of cells)[a]	
		esterase	AATFase
15	log	8.3	2.6
	stationary	33.9	0.7
30	log	4.5	3.6
	stationary	9.0	1.1

[a] Assay was carried out using 100 mg of cells for 1 h, and the activity is expressed as ppm/h/10 g of cells according to Yoshioka and Hashimoto (1981).

temperature for 15 min with gentle shaking to visualize the esterase band (Wakai and Yanagiuchi, 1989).

RESULTS

Concentration of Volatile Compounds in Yeast Cultures. Cells of *H. mrakii* and *S. cerevisiae* Kyokai No. 7 were cultured in nutrient medium for various periods at 15 and 30 °C, respectively, under aerobic conditions. As shown in Table 1, *S. cerevisiae* could not produce, or produced only a small amount of, isoamyl acetate under aerobic conditions. Contrary to this, a large amount of isoamyl acetate was detected in the culture of *H. mrakii* at both 15 and 30 °C at any of the growth phases studied under aerobic conditions, except for the culture of stationary phase at 30 °C.

Production of Isoamyl Acetate by Intact Cells of *H. mrakii*. To examine the possibility that some esterases may be involved in the synthesis of isoamyl acetate, the intact cells of *H. mrakii* cultured under aerobic conditions were subjected to the *in vitro* synthesis of isoamyl acetate. As shown in Table 2, the cells cultured at 15 °C to stationary phase produced 33.9 ppm of isoamyl acetate/h/10 g of cells from isoamyl alcohol and acetic acid. Isoamyl acetate concentrations in the culture at this stage were also high as shown in Table 1. Cells cultured at 30 °C could also produce isoamyl acetate from isoamyl alcohol and acetic acid. These results strongly suggested that some esterases contribute to the production of isoamyl acetate in *H. mrakii*.

In addition to this, when the intact cells were incubated with the substrate for AATFase, i.e., isoamyl alcohol and acetyl-CoA, isoamyl acetate was also synthesized. The amount of isoamyl acetate produced by such a system was lower compared with those synthesized by esterase (Table 2). However, it is controversial whether acetyl-CoA can enter the cells; therefore, we then measured the esterase and AATFase activities in the cell extracts. Before the enzyme assay, we investigated the distribution of esterase and AATFase in *H. mrakii* cells.

Distribution of Esterase and AATFase in *H. mrakii*. The AATFase in *S. cerevisiae* was reported to be bound to the cytoplasmic membrane (Yoshioka and Hashimoto, 1981; Minetoki et al., 1993). Cell homogenates of *H. mrakii* cultured under aerobic conditions were subjected to ultracentrifugation at 200000*g*, and esterase and AATFase were assayed using the resultant supernatants and pellets. As shown in Table 3, a large amount of AATFase activity was detected in the pellet and only a trace of activity was detected from the soluble fraction in the log-phase cells grown at 15 °C. On the other hand, when the cells entered the stationary phase, the AATFase activity in the pellets decreased to approximately 24% compared with the log-phase cells. AATFase activity was also detected in the pellets prepared from the cells cultured at 30 °C (log phase, 0.46 unit/mg of protein; stationary phase, 0.27 unit/mg of protein), and the activity also decreased at the stationary phase. However, specific activities of AATFase in the cells cultured at 15 °C were approximately 4-fold (log phase) and 1.7-fold (stationary phase) higher, respectively, compared with those at 30 °C. These results suggested that AATFase of *H. mrakii* was also somewhat labile at higher temperature, although it was still able to produce isoamyl acetate. These properties of AATFase in *H. mrakii* are different from those of *S. cerevisiae* (Minetoki et al., 1993; Minetoki, 1992).

Esterase that synthesizes isoamyl acetate from isoamyl alcohol and acetic acid was found mainly in the soluble fraction. On the other hand, isoamyl acetate-hydrolyzing activity was detected in the insoluble fraction of the cells at the stationary phase at both 15 and 30 °C.

Fractionation of AATFase. To confirm whether or not the AATFase of *H. mrakii* binds to the membrane, total membrane fractions, which were obtained by ultracentrifugation of the cell extracts prepared from the cells cultured at 15 °C to log phase, were treated by high concentrations of NaCl and detergent (Table 4). Insoluble fractions were first treated by 10 mM potassium phosphate buffer (pH 7.0) at 4 °C for 2 h, and then the suspension was centrifuged at 200000*g* for 2 h. AATFase activity in the resultant supernatants was measured, although only a trace of activity was detected (recovery, 0.291%). Insoluble fractions were then treated with 2 M NaCl, although approximately 1.5% of the activity was recovered. These results suggested that AATFase was not associated with membrane by ionic interaction. Insoluble materials after NaCl treatment were treated with 0.1% Triton X-100; however, only 1.2% of AATFase was solubilized. Almost all AATFase (approximately 97%) was recovered from the pellets by treatment with 2.0% Triton X-100, and only a trace amount of AATFase was extracted by a higher concentration (5.0%) of Triton X-100. These results indicated that AATFase of *H. mrakii* was tightly bound to the biological membrane.

Effects of Growth Phase and Temperature on Expression of Esterase. As described above, some esterases seemed to contribute to the production of isoamyl acetate in *H. mrakii*. Cell extracts were applied onto a native PAGE at 4 °C, and the gel was stained for esterase activity by 1-naphthyl acetate and Fast Blue B salt to see the expression pattern of esterase in the cells cultured at 15 and 30 °C, respectively. Figure 1A shows the expression pattern of esterase in the cells cultured at 15 °C at various growth phases in HM medium. Figure 1B shows the same data for cells cultured at 30 °C.

Only one esterase band was observed in the cell extracts prepared from the log-phase cell (Figure 1A,

Table 3. Esterase and AATFase Activities in *H. mrakii*

		activity (unit/mg of protein)							
		log phase				stationary phase			
		15 °C		30 °C		15 °C		30 °C	
product	enzyme	supernatant	pellet	supernatant	pellet	supernatant	pellet	supernatant	pellet
isoamyl acetate	AATFase	0.004	1.99	0.05	0.46	0.042	0.47	0.06	0.27
	esterase	0.039	ND[a]	0.0034	ND	0.014	ND	0.002	ND
isoamyl alcohol	esterase	0.30	ND	0.13	ND	1.8	0.29	0.89	0.064

[a] Not detected.

Table 4. Fractionation of AATFase

step	activity (unit)	recovery[a] (%)
buffer (10 mM potassium phosphate, pH 7.0)	0.01	0.291
buffer + 2 M NaCl	0.05	1.45
buffer + 0.1% Triton X-100	0.04	1.16
buffer + 2.0% Triton X-100	3.33	96.8
buffer + 5.0% Triton X-100	0.01	0.291

[a] Sum of the activity of each step was relatively taken as 100%.

lane 1). However, in the cell extracts of early stationary phase and stationary phase (lanes 2 and 3), two additional bands appeared. Isoamyl acetate-hydrolyzing activity was 0.3 unit/mg of protein at log phase, whereas it increased to 1.8 units/mg of protein (Table 3). This is presumably due to the additional esterases that appeared in the early stationary and stationary phases.

When the cells were cultured at 30 °C, the expression pattern of esterases was different from those at 15 °C. As shown in Figure 1B, one esterase band was observed at log phase (lane 1). This is a similar pattern of expression at 15 °C (Figure 1A, lane 1). When the cells entered the stationary phase, three bands were observed at the position of the esterase having the highest mobility (Figure 1B, lane 3). In addition to this, another esterase band appeared at the stationary phase; however, the mobility of this esterase was different from those of esterases observed in the stationary-phase cells at 15 °C (Figure 1A, lane 3). An additional esterase in the stationary phase at 30 °C had much higher mobility than those at 15 °C.

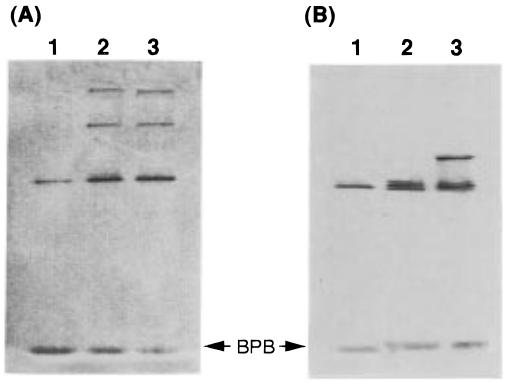

Figure 1. Activity staining of esterase from *H. mrakii*. *H. mrakii* was cultured in HM medium at 15 °C (A) and 30 °C (B) under aerobic conditions. Cell extracts were prepared as described in the text; 100 mg of protein was applied onto each lane: lane 1, cell extracts of log-phase cells; lane 2, cell extracts of early stationary-phase cells; and lane 3, cell extracts of stationary-phase cells. Arrows indicate the position of BPB (bromophenol blue).

DISCUSSION

Isoamyl acetate is one of the most important factors that determine the quality of Japanese sake. In the case of wine brewing, flavor compounds are thought to occur from grape, but in sake brewing important flavor compounds are derived from metabolism of several organic compounds by *S. cerevisiae* during the fermentation. To increase the flavor quality of a special grade of sake, the improvement of yeasts to produce high concentrations of flavor components is important (Wakai and Yanagiuchi, 1989). Recently, one of our colleagues cloned and sequenced the *EST2* gene whose gene product was a carboxyesterase that corresponded to the hydrolysis of isoamyl acetate in *S. cerevisiae* (Fukuda et al., 1996). The *est2* deficient mutant accumulated an approximately 19-fold higher level of isoamyl acetate compared with the parent strain.

Synthesis of isoamyl acetate by sake yeast (*S. cerevisiae*) is performed by the action of AATFase in the presence of isoamyl alcohol and acetyl-CoA. The *ATF1* gene encoding AATFase was cloned from *S. cerevisiae* and brewery lager yeast (*Saccharomyces uvarum*) (Fujii et al., 1994). They reported that the concentration of isoamyl acetate in the culture was increased by overexpression of the *ATF1* gene in *S. cerevisiae*. However, the AATFase of *S. cerevisiae* is labile at high temperature (30 °C), and 55% of activity is lost if the enzyme is kept at 20 °C for 30 min (Minetoki, 1992). The enzyme activity was also inhibited in the presence of unsaturated fatty acids (Malcorps et al., 1991; Minetoki et al., 1993; Yoshioka and Hashimoto, 1981). When *S. cerevisiae* is cultured under aerobic conditions, molecular species of fatty acid involved in membrane phospholipid are changed; i.e., the population of unsaturated fatty acids is increased (Minetoki, 1992). Therefore, the AATFase activity is lowered when the cells are cultured under aerobic conditions that lead to decreased concentrations of esters. As shown in Table 1, *S. cerevisiae* Kyokai No. 7 could not produce, or produced only a small portion of, isoamyl acetate under aerobic conditions. On the other hand, *H. mrakii* could produce a large amount of isoamyl acetate even though the yeast was cultured at either 15 or 30 °C under aerobic conditions.

It has been reported that the bottle neck of isoamyl acetate production in sake brewing is the amount of isoamyl alcohol in sake mash (*moromi*) (Minetoki, 1992). Since the K_m value of AATFase for isoamyl alcohol is high (meaning that the affinity of AATFase to isoamyl alcohol is low), a large amount of isoamyl alcohol is required for the production of isoamyl acetate (Minetoki et al., 1993). However, the ratio of isoamyl acetate to isoamyl alcohol in the culture of *H. mrakii* in log phase at 15 °C was 1.33 (3.6 vs 2.7 ppm), while that of *S. cerevisiae* at the same growth phase was 0.0054 (0.05 vs 9.3 ppm). Isoamyl acetate-synthesizing esterase activity in the cells at this stage was higher (0.039 unit/mg of protein, Table 3) compared with those in other cells (log phase at 30 °C, 0.0034 unit/mg of protein; stationary phase at 15 °C, 0.014 unit/mg of protein; stationary phase at 30 °C, 0.002 unit/mg of protein).

Actually, intact cells of *H. mrakii* could produce isoamyl acetate from isoamyl alcohol (Table 2). These results suggested that *H. mrakii* cells in log phase at 15 °C might have a system for producing isoamyl acetate other than AATFase, which may be a reverse reaction of esterase.

AATFase activity in *H. mrakii* cells cultured at 15 °C to log phase was also high even though the isoamyl alcohol level was low (2.7 ppm, Table 1). AATFase activity declined when the cells entered stationary phase, although the concentration of isoamyl alcohol increased in the stationary-phase culture indicating that production of isoamyl acetate in the stationary-phase culture may be due to the AATFase. Interestingly, the ratio of isoamyl acetate to isoamyl alcohol was higher in the log-phase culture of *H. mrakii* at 15 °C compared with those in other cultures of *H. mrakii* at 15 °C. As shown in Table 3, isoamyl acetate-hydrolyzing esterase activity increased when the cells entered stationary phase (log phase, 0.30 unit/mg of protein; stationary phase, 1.8 units/mg of protein); thus once isoamyl acetate was synthesized, which might be catalyzed by AATFase, it was thought to be hydrolyzed by esterase in the cells at stationary phase.

The *ATF1* gene of *S. cerevisiae* consists of a 1575-bp open reading frame encoding 525 amino acids with a calculated molecular weight of 61 059 Da. According to the amino acid sequence deduced from the DNA sequence, the *ATF1* gene product does not have the hydrophobic regions to be considered as a membrane-penetrating region (Fujii et al., 1994), although the AATFase of *S. cerevisiae* was purified from membrane fractions (Minetoki et al., 1993). As shown in Table 4, AATFase of *H. mrakii* was thought to be tightly bound to the cell membrane. On the other hand, isoamyl acetate-synthesizing esterase was detected only in the soluble fractions. Isoamyl acetate-hydrolyzing activity was detected in both soluble and insoluble fractions. *H. mrakii* was found to have AATFase which is resistant against heat and aerobic conditions, and the AATFase seemed to be a major producer of isoamyl acetate in this yeast. The AATFase of *H. mrakii* may be applicable for the isoamyl acetate production in sake brewing. Since AATFase of *S. cerevisiae* is labile at high temperature, brewing of the *ginjo*-sake must be carried out at low temperature. Though the AATFase activity in *H. mrakii* cells also seemed to be lowered by increasing temperature, it still maintained the activity. Cloning of the *ATF1* gene from *H. mrakii* to breed sake yeast possessing the temperature resistant AATFase is in progress.

Expression patterns of esterases in *H. mrakii* were changed by the growth phase and temperature. Log-phase cells had one esterase at 15 and 30 °C, although when the cells entered the stationary phase, several esterases were detected by activity staining. However, species of esterases appearing at the stationary phase at 15 °C were different from those appearing at 30 °C. Isoamyl acetate-hydrolyzing activity increased when the cells entered the stationary phase; therefore, some esterases appearing at stationary phase by activity staining might correspond to the hydrolysis of isoamyl acetate. However, it is still controversial whether such esterases actually hydrolyze isoamyl acetate because the activity staining was done using 1-naphthyl acetate as a substrate. Wakai and Yanagiuchi (1989) reported that isoamyl acetate-hydrolyzing activity could be detected by the diazo-coupling method using 1-naphthyl acetate as a substrate. Yanagiuchi et al. (1989b) reported that *S. cerevisiae* had four esterases which could be separable by DEAE-cellulose column chromatography, and one esterase (*EST2* gene product) was thought to correspond to the hydrolysis of isoamyl acetate. Recently, we proved that the *EST2* gene product was the isoamyl acetate-hydrolyzing esterase. Overexpression of the *EST2* gene in *S. cerevisiae* led to increased isoamyl acetate-hydrolyzing activity, while the amount of isoamyl acetate increased in culture of the *est2* mutant (Fukuda et al., 1996). Regulation of the esterase gene expression in *H. mrakii* seemed to be complicated. Further studies, including the gene-cloning experiments, must be done to identify the esterase whose activity corresponds with synthesizing and hydrolyzing isoamyl acetate.

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