Enhanced Formation of 3-(Methylthio)-1-propanol in a Salt-tolerant Yeast, *Zygosaccharomyces rouxii*, Due to Deficiency of *S*-Adenosylmethionine Synthase

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Mutants resistant to L-ethionine, an analogue of methionine, were derived from *Zygosaccharomyces rouxii* MA'1. Some of them produced large amounts of 3-(methylthio)-1-propanol (methionol); an important flavor component of soy sauce. One of these mutants, M33, produced 60-fold as much methionol as the parental strain MA'1 in a minimum medium without methionine. The intracellular methionine pool of this mutant was increased 5-fold. *S*-Adenosylmethionine (SAM) synthase (EC 2.5.1.6) in the mutant was reduced to 45% of that in the parent and a decrease in intracellular SAM level was also confirmed. The reduction of the SAM level due to the deficiency of SAM synthase, accompanied by derepression of enzymes of the methionine synthetic pathway, was considered to be responsible for the hyper-production of methionol in the mutant.

Soy sauce is a fermented soy bean food containing high concentrations of amino acids including methionine (about 10 mM) as well as a large amount (16%) of NaCl. 3-(Methylthio)-1-propanol (methionol) is one of the most important flavor components in soy sauce^{1–3)} and is produced by the salt-tolerant haploid yeast, *Zygosaccharomyces rouxii*, during the fermentation process. Generally, methionol is a metabolite from methionine, and it is scarcely produced in media without methionine. Hence the methionol biosynthetic route from methionine was assumed to be what is called the Ehrlich pathway, known for the production of several higher alcohols (fusel oils).^{1,4)}

Throughout attempts to control the production of higher alcohols in soy sauce fermentation,^{5,6)} we have established that the production of several higher alcohols by *Z. rouxii* was closely related to its amino acid metabolism and that it could be enhanced in mutants resistant to analogues of the corresponding amino acids. Methionol-hyper-producing yeast, however, has not been obtained so far. Now an unique strain that produces a large

amount of methionol in a medium without methionine was found among our ethionine-resistant mutants. In this report, we described the isolation of methionol-hyper-producing mutant and elucidation of the hyper-producing mechanism including estimation of the mutated enzyme.

Materials and Methods

Organisms. *Z. rouxii* NISL 3355 (wild strain) was obtained from the Noda Institute for Scientific Research. A methylamine-resistant strain, MA'1, was obtained from the wild strain as a mutant decreasing the uptake of ammonium ion.⁶⁾ *Saccharomyces cerevisiae* (ATCC4098) was a culture kept in the research division of Kikkoman Corporation.

Media and growth conditions. A basal medium (pH 5.2) contained 5% NaCl, 5% glucose, 0.87% K₂HPO₄, and 0.2% Difco nitrogen base without (NH₄)₂SO₄ and amino acids. The medium was supplemented with 2 mM L-proline (M.pro-medium) or 10 mM (NH₄)₂SO₄ (M.am-medium). *Moromi* juice medium (MJ-medium) was prepared as an amino acid-rich medium modeled after soy sauce *moromi* by the method of Kanbe and Uchida,⁷⁾ except for its pH (pH 5.2). Yeast cells were grown at 30°C under static or aerobic conditions using a reciprocal shaker (120 rpm). When *S. cerevisiae* was cultured, NaCl in the media was

removed.

Gas chromatographic analysis of methionol. After static culture of a yeast strain in a medium at 30°C for 7 days, cells in the broth were removed by centrifugation. The cell-free broth (3 ml) was mixed with an internal standard (dimethylmalonate 133 µg) and was saturated with NaCl (1 g). Volatiles, including methionol, in the broth were extracted with methylacetate (1 ml) by liquid-liquid extraction in a screw-capped tube for 20 min at 30°C using a shaker (Thermomixer TM121). The methylacetate layer was separated from the aqueous solution by centrifugation at 3500 rpm for 10 min at 5°C. Gas chromatography was done with a Shimadzu GC-14A chromatograph with a FID and a fused silica capillary column (Supelcowax 10, 15 m, 0.25 mm i.d. and 0.25 µm f.t., Supelco, Inc.): oven temp., 50–220°C, programmed 4°C/min; inlet temp., 240°C; carrier gas, He (1 ml/min); split rate, 1:30.

Extraction and assay of L-methionine and S-adenosyl-methionine (SAM). A yeast strain was cultured aerobically in a medium. Cells were harvested and washed twice with cold water. L-Methionine and SAM were extracted with hot water or cold perchloric acid (1.5 N) by the method of Chrest *et al.*⁸⁾ and measured by an amino acid analyzer (Hitachi Model 8500) or a high performance liquid chromatograph,⁹⁾ respectively.

Preparation of crude extracts. After aerobic culture of a yeast strain in M.am-medium, cells were harvested, washed twice with ice-cold 0.05 M potassium phosphate buffer (pH 7.5) by centrifugation at 4°C, resuspended in the buffer, and disrupted with glass beads (diameter 0.25 to 0.5 mm) in a grinder container for 3 min at 0°C. The cell debris was removed by centrifugation at 18,000 rpm for 20 min at 4°C, and the supernatant was dialyzed against the buffer and used as a crude extract.

Enzyme assays. Some enzyme activities were measured with the crude extracts. SAM synthase (ATP-L-methionine-S-adenosyltransferase, EC 2.5.1.6) activity was assayed by the method of Peter *et al.*¹⁰⁾ Homocysteine synthase and cysteine synthase were assayed as described by Wiebers and Garner¹¹⁾ at 30°C and the homocysteine or the cysteine formed was estimated by the method of Kredich and Tomkins.¹²⁾ Protein was measured by a Pierce protein assay kit using bovine serum albumin as the standard.

SAM uptake assay. Yeast was cultured statically in M.am-medium at 30°C to a cell density of 40 to 60 Klett units. Cells were harvested by centrifugation at 30°C and resuspended in prewarmed M.am-medium to the cell density of about 100 Klett units. After an additional culture at 30°C for 3 hr, a 2.45-ml sample of the culture was transferred to a test tube containing 50-µl of 0.5 mM ¹⁴C-SAM (specific activity, 10 µCi/µmol). The tube was

then incubated at 30°C, and 400-µl of the culture were removed at 30-sec intervals for 2.5 min and collected each on a membrane filter (0.45-µm pore diameter). The filter was then washed three times with 4 ml of ice-cold water, placed in 5 ml of aqueous scintillation fluid (Filter-count, Packard Company), and the radioactivity was measured by a Beckman LS-9000 liquid scintillation counter. SAM retention on the washed filter was negligible in this method.

Results and Discussion

Isolation of Ethionine-resistant mutants in Z. rouxii

To isolate ethionine-resistant mutants, *Z. rouxii* MA^r1 was mutagenized by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. Colonies grown on plates of the M.pro-medium supplemented with 2% agar and 100 µg/ml L-ethionine at 30°C for 7 days were selected. The parent MA^r1 could not grow on the plate. These ethionine-resistant mutants produced 1.3- to 9-fold methionol in MJ-medium, as compared with the parent strain. Especially, mutant M33 showed the largest production of methionol (about 108 ppm), hence the mutant was used throughout following experiments. Mutant M33 showed resistance also to >0.01 mM of seleno-L-methionine, another analogue of methionine, in M.pro-medium. These results suggested that analogues of methionine were useful to select mutants hyper-producing methionol.

Production of methionol in the parent and the mutant M33 strains

Parent MA^r1 produced very large amounts

Table I. EFFECTS OF L-METHIONINE OR SAM ON PRODUCTION OF METHIONOL BY *Z. rouxii* MUTANT

Strain	Production of methionol (ppm)		
	None ^a	L-Methionine (20 mM)	SAM (10 mM)
Parent	0.5	790.0	ND ^b
M33	30.0	785.0	25.0

Each compound was added to M. am-medium. The cells were statically cultured at 30°C for 5 days in the medium.

^a Nothing was added.

^b Not measured.

of methionol in M.am-medium supplemented with 20 mM L-methionine, but not in M.am-medium (Table I). This result suggested that the production of methionol by the parent strain was suppressed in a medium without methionine, although the strain potentially had a pathway for production of methionol from methionine. Mutant M33 produced a larger amount of methionol in M.am-medium than the parent, with no accumulation of methionine. This was also shown in minimum media containing L-glutamate or L-proline as a sole nitrogen source (data not shown). These results suggested that the mutant could produce methionol by *de novo* synthesis.

Intracellular pool of methionine and methionol production

The possibility of *de novo* synthesis of methionol by mutant M33 was examined by

Table II. EFFECTS OF L-METHIONINE ON INTRACELLULAR POOL OF METHIONINE AND SAM IN *Z. rouxii* MUTANT

Strain	Methionine ^a		SAM ^a	
	None ^b	L-Methionine (20 mM)	None	L-Methionine (20 mM)
Parent	1.9	58.2	0.96	31.44
M33	9.3	81.7	1.35	2.23

The cell were aerobically cultured at 30°C for 3 days in M. am-medium supplemented with or without L-methionine.

^a $\mu\text{mol/g}$ of dry cell weight.

^b Nothing was added.

measurement of intracellular methionine. The pool of free methionine in the mutant cells grown in M.am-medium was about 5 times larger than that of the parental strain (Table II), so production of intracellular methionine through *de novo* synthesis was probably significantly increased in the mutant. In both the parent and the mutant strains, accumulation of a very large amount of intracellular methionine due to L-methionine supplementation in the M.am-medium was accompanied by significant hyper-production of methionol (Tables I, II). This result indicated that the hyper-production of methionol by the mutant strain is due to the increase in intracellular methionine.

Estimation of mutated enzyme in mutant M33

At least three classes of methionine-hyper-producing mutant have been known among methionine-resistant mutants of yeasts, according to the activity of SAM synthesis.^{8,13)} Therefore, the SAM pools in the cells grown in M.am-medium with or without addition of 20 mM L-methionine were measured (Table II). The pooled amount of SAM in the parent strain was greatly increased when the cells were cultured in methionine-supplemented medium, but that in the strain M33 was increased only slightly. Since SAM synthesis was reduced in the mutant, activity of the synthetic enzyme, SAM synthase, was measured (Table III). The activity of SAM synthase in mutant M33 was reduced to 45% of that in the parent strain. Therefore the deficiency of SAM synthase in

Table III. REPRESSION OF SAM SYNTHASE, CYSTEINE SYNTHASE AND HOMOCYSTEINE SYNTHASE BY L-METHIONINE IN *Z. rouxii* MUTANT

Strain	Addition	Specific activity (nmol/min/mg protein)		
		SAM synthase	Cysteine synthase	Homocysteine synthase
Parent	None	0.0380	1.92	5.06
	L-Methionine	0.0137	0.90	0.05
M33	None	0.0171	2.01	6.65
	L-Methionine	0.0074	2.17	7.65

Enzyme activities were assayed with crude extracts prepared from cells grown in M. am-medium supplemented with or without 20 mM L-methionine.

Table IV. UPTAKE OF SAM BY *Z. rouxii* MUTANT AND *S. cerevisiae* ATCC4098

Strain		Specific activity of SAM-uptake (nmol/min/mg dry cell weight)
<i>Z. rouxii</i>	Parent	0.008
	M33	0.061
<i>S. cerevisiae</i>	ATCC4098	1.220

Uptake of SAM (0.005 mM) was measured by the cells grown in M. am-medium as described in the text.

mutant M33 was considered to be a reason for the accumulation of intracellular methionine.

To confirm the effects of a reduced SAM level in the methionine biosynthesis, activities of the two enzymes, homocysteine synthase and cysteine synthase, were assayed. The activities of both the enzymes in the parent strain were repressed by the addition of methionine to the medium, but not in mutant M33. Therefore it was suggested that the reduced SAM-level in mutant M33 induced derepression of the enzymes for methionine biosynthesis, although the mutant strain accumulated much intracellular methionine. Then, we examined the decrease in production of methionol due to repression of these derepressed enzymes by addition of SAM. Addition of SAM to M.am-medium could reduce the hyper-production of methionol, but the production was still large (Table I). Since it was doubted if mutant M33 could incorporate SAM, the uptake of SAM (0.005 mM) by both the *Z. rouxii* strains were compared with that by a strain of *S. cerevisiae*, with which the uptake of SAM had been established^{8,14)} (Table IV). The SAM-uptake by both the *Z. rouxii* strains was very much smaller than that by *S. cerevisiae*. These results suggested that the small effect of SAM addition in the hyper-production of methionol by mutant M33 was caused by the deficiency of SAM uptake in the mutant strain.

Hayashibe *et al.*⁴⁾ reported that the productivity of methionol in a medium containing methionine as a sole nitrogen source differed by yeast species and strains. We could

not find, however, a large difference in methionol productivity among some soy-yeast species and strains in MJ-medium or M.am-medium (unpublished data). We have bred various yeast strains producing relatively large amounts of some higher alcohols such as isoamyl alcohol, isobutyl alcohol, 2-phenylethanol, or methionol. In the future, it will be feasible to produce a variety of flavor-rich soy sauces with these strains.

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References

- 1) S. Akabori and T. Kaneko, *Nippon Kagaku Kaishi*, **57**, 832 (1936).
- 2) N. Nunomura, M. Sasaki, Y. Asao and T. Yokotuka, *Agric. Biol. Chem.*, **40**, 485 (1976).
- 3) S. Mori, N. Nunomura and M. Sasaki, Abstracts of Papers, the Annual Meeting of the Japan Society for Bioscience, Biotechnology, and Agrochemistry, Kyoto, April, 1982, p.36.
- 4) M. Hayashibe, S. Katoda, H. Owada, H. Yoshida, A. Katayose and T. Terashima, *Hakkokogaku Kaishi*, **48**, 22 (1970).
- 5) T. Aoki and K. Uchida, *Agric. Biol. Chem.*, **54**, 273 (1990).
- 6) T. Aoki and K. Uchida, Abstracts of Papers, the Annual Meeting of the Japan Society for Bioscience, Biotechnology, and Agrochemistry, Tokyo, April, 1987, p. 137.
- 7) C. Kanbe and K. Uchida, *Nippon Nōgeikagaku Kaishi*, **58**, 487 (1984).
- 8) H. Chrest, Y. Surdin-Kerjan, J. Antoniewski and H. Robichon-Saulmajster, *J. Bacteriol.*, **115**, 1084 (1973).
- 9) N. Shiomi, H. Fukuda, H. Morikawa, Y. Fukuda and A. Kimura, *Appl. Microbiol. Biotechnol.*, **29**, 302 (1988).
- 10) K. C. Peter and L. C. Giulio, *J. Biol. Chem.*, **252**, 4506 (1977).
- 11) J. Wiebers and H. R. Garner, *J. Biol. Chem.*, **242**, 5644 (1967).
- 12) N. M. Kredich and G. N. Tomkins, *J. Biol. Chem.*, **241**, 4955 (1966).
- 13) E. Morzycka, D. Sawnor-Korszynska, A. Paszewski, J. Gral and K. Raczynska-Bonjanowska, *Appl. Environ. Microb.*, **32**, 125 (1976).
- 14) J. T. Murphy and K. D. Spence, *J. Bacteriol.*, **109**, 949 (1972).