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Note

Bioconversion of Ferulic Acid to 4-Vinylguaiacol and 4-Ethylguaiacol and of 4-Vinylguaiacol to 4-Ethylguaiacol by Halotolerant Yeasts Belonging to the Genus *Candida*

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In order to examine the genesis of the characteristic flavors of soy sauce and miso, seven novel halotolerant yeast strains of two types, which showed convertibility of ferulic acid (FA) to 4-vinylguaiacol (4-VG) and to 4-ethylguaiacol (4-EG), were isolated from miso-koji and miso pastes. Two of these strains were identified as *Candida guilliermondii* (anamorph of *Pichia guilliermondii*), and *Candida fermentati* (anamorph of *Pichia caribbica*), based on sequence analyses of a partial 26S ribosomal RNA gene and the region of internal transcribed spacers 1 and 2, and the 5.8S ribosomal RNA gene. Moreover, we also found three *Candida etchellsii* strains which showed convertibility of FA to 4-VG, but not to 4-EG, and two atypical strains of *Candida versatilis* which showed no convertibility of FA to 4-VG, but did show convertibility of 4-VG to 4-EG from soy sauce mashes. The bioconversion pathway from FA to 4-EG *via* 4-VG in halotolerant yeasts and bacteria is discussed.

Key words: bioconversion of ferulic acid to volatile phenols; *Candida etchellsii*; *Candida fermentati*; *Candida guilliermondii*; *Candida versatilis*

In the soy sauce and miso fermentation processes, many flavor compounds, including alcohols,[1,2] esters, volatile phenols, and furanones, are produced by halotolerant yeasts. For example, *Zygosaccharomyces rouxii* mainly produces alcohols and furanones such as 4-hydroxyl-2(or5)-ethyl- 5(or2)-methyl-3(2H)- furanone (HEMF)[3] and *Candida etchellsii* and *C. versatilis* produce volatile phenols such as 4-ethylguaiacol (4-EG) and 4-ethylphenol (4-EP), which are characteristic flavors of soy sauce and miso.[4] It has also been reported that 1–2 ppm 4-EG in soy sauce gives a better evaluation as to the flavor quality of soy sauce.[4]

Previous papers[5,6] explain the following points by re-examination of the conversion of FA to volatile phenols under 15% NaCl by *Candida* yeast and the other bacteria isolated from soy sauce mashes and miso pastes: *C. versatilis* can convert ferulic acid (FA) to 4-EG, while *C. etchellsii* can neither convert FA to 4-vinylguaiacol (4-VG), nor 4-VG to 4-EG. The bioconversion pathway from FA to 4-EG consists of two steps: (i) FA is first converted to 4-VG by FA-decarboxylase, and (ii) the 4-VG thus derived is converted to 4-EG by 4-VG-reductase.[5] The other yeasts and bacteria isolated, *Pichia anomala*, *P. subpelliculosa*, *Debaryomyces hansenii*, *Bacillus subtilis*, and halotolerant *Staphylococcus* sp. (this was later identified as *Staphylococcus xylosus* by API STAPH kit, unpublished data), convert FA only to 4-VG. 4-VG is furthermore converted to 4-EG by halotolerant *C. versatilis*. Hence the results obtained indicate that *C. versatilis* is the only species that can produce a characteristic flavor, 4-EG, from FA in soy sauce and miso fermentation.

On further screening of FA converting yeasts from soy sauce mashes and miso pastes for improvement of flavor quality, unexpectedly, we isolated four kinds of novel strains: (i) 4-VG producing strains of *C. etchellsii*, (ii) 4-EG producing strains of *C. versatilis* that did not convert FA but did convert 4-VG, (iii) 4-VG producing strains of *C. guilliermondii*, and (iv) 4-EG producing strains of *C. fermentati*.

Here we report the convertibility of FA and 4-VG and the identification by sequence analysis of the D1D2 domain of the 26S ribosomal RNA gene (hereafter, the 26S rDNA sequence), the region of the internal transcribed spacer 1, 5.8S ribosomal RNA gene, and the

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Abbreviations: FA, ferulic acid; 4-VG, 4-vinylguaiacol; 4-EG, 4-ethylguaiacol; *p*-CA, *p*-coumaric acid; 4-VP, 4-vinylphenol; 4-EP, 4-ethylphenol

region of the internal transcribed spacer 2 (hereafter, the ITS sequence). Further, we discuss the conversion pathway of FA by new isolates.

YPG agar medium (1% yeast extract, 1% polypeptone, 2% glucose, and 2% agar) and the same medium with the addition of 15% NaCl (hereafter, YPG + 15% NaCl medium) were used for isolation of yeasts from soy sauce mashes and miso pastes. Table 1 shows a list of yeast strains used in this study. HPLC chromatograms of the bioconversion of FA to 4-VG and 4-EG are summarized in Fig. 1.

The results of convertibility of FA to 4-VG and to 4-EG and of 4-VG to 4-EG are summarized in Table 1. Twelve strains of *C. versatilis* (IFO 10056^T, JCM 5974, 8M2, 15M2, 25M2, 30M2, 33Z1, J5M2, Miso 19, Miso 22, Miso 83, and OH3G1) showed convertibility of FA to 4-EG, including that of 4-VG to 4-EG (designated type 1 FA conversion), in agreement with previous results[5] for *C. versatilis* strains. However, two strains (JCM 5958 and KS05) of *C. versatilis* did not show convertibility of FA to 4-EG, but did show convertibility of 4-VG to 4-EG (designated type 2 FA conversion). On the other hand, three strains of *C. etchellsii* (9Z1, 25Z1, and 33Z2) showed convertibility of FA to 4-VG, but did not show convertibility of 4-VG to 4-EG (designated type 3 FA conversion). This is the first finding of a *C. etchellsii* strain with convertibility of FA to 4-VG. The other four strains of *C. etchellsii* (IFO 1592^T, Miso 0201, Miso 0208, and Miso 0209) showed neither convertibility of FA to 4-VG or to 4-EG, nor convertibility of 4-VG to 4-EG (designated type 4 FA conversion) in agreement with previous results[5] for *C. etchellsii* strains. Five strains newly isolated from miso pastes and miso-koji, B-2, B-3, I-5, I-6, and K-3, showed convertibility of FA to 4-EG (corresponding to type 1 of FA conversion). However, two strains isolated from miso-koji, I-4 and K-2, showed convertibility of FA to 4-VG, but not of 4-VG to 4-EG (corresponding to type 3 of FA conversion).

The FA decarboxylase and 4-VG reductase activities of the strains tested (type 1 to type 4), which were examined by the method described in footnote (*4) in Table 1, and the bioconvertibilities are summarized in Table 1. *C. versatilis* IFO 10056^T, 8M2, and *C. fermentati* I-5 (type 1) had both FA decarboxylase and 4-VG reductase activities, while *C. versatilis* JCM 5958 and KS05 (type 2) had only 4-VG reductase activities. *C. etchellsii* 9Z1 and *C. guillermondii* K-2 (type 3) had only FA decarboxylase activites, while *C. etchellsii* IFO 1592^T and Miso 0208 (type 4) had neither FA decarboxylase nor 4-VG reductase activities. This result agrees with those for FA convertibility of the tested strains.

The results of molecular identification of the isolates carried out by analyses of the 26S rDNA sequence and the ITS sequence[7] are summarized in Table 1. The seven isolates were identified as *Picha guilliermondii*. Furthermore, according to the new classification of *Pichia guilliermondii* species complex proposed by Vaughan-Martini *et al.*,[8] they were divided into three species, (i) *P. guilliermondii* (its anamorph: *Candida guilliermondii*), (ii) *Candida carpophila*, and (iii) *Pichia caribbica* (its anamorph: *Candid fermentati*). Since the sex of the seven isolates could not be observed, the species names of the anamorph of the teleomorphic species are used in this study. The 26S rDNA sequences of the five strains, B-2, B-3, I-5, I-6, and K-3, were identical with that of the type strain of *C. fermentati* (AY187283), and their ITS sequences were also identical with that of the type strain of *C. fermentati* (AB032175).[8] Therefore, they were identified as *C. fermentati* (anamorph of *P. caribbica*). On the other hand, the 26S rDNA sequences of I-4 and K-2 were identical with that of the type strain of *P. guilliermondii* (U45709), and their ITS sequences were also identical with that of the type strain of *P. guilliermondii* (AY939792).[8] Therefore, they were also identified as *C. guilliermondii* (anamorph of *P. guilliermondii*).

Division of the isolate*s* into two species, *C. fermentati* and *C. guilliermondii*, based on the results of the 26S rDNA and the ITS sequence analyses, is closely correlated to typing among convertibility of FA to 4-VG and those of the both compounds to 4-EG, as shown in Table 1. Thus, since the seven strains can be clearly divided into the two species by the convertibility of FA as well as by the results of 26S rDNA and ITS sequence analyses, the bioconvertibility of FA can be a useful tool for differentiation between *C. fermentati* and *C. guilliermondii*, which are indistinguishable on the basis of conventional phenotypical criteria.[8]

Conversion of FA by microorganisms under NaCl-free conditions has frequently been reported. For example, it is well known that some microorganisms,[9] including wine and beer yeasts[10,11] and yeasts isolated from frozen concentrated orange juice,[12] can convert FA to 4-VG. Chatonnet *et al.*[10,11] found that only three species, *Brettanomyces intermedius*, *Dekkra intermedius*, and *Brettanomyces lambicus* produce both cinnamate decarboxylase and vinylphenol reductase under NaCl-free conditions such as wine making. However, under high NaCl conditions such as the soy sauce and miso fermentation processes, convertibility of FA by microorganisms besides *C. versatilis* and *C. etchellsii* and the degradation pathway have rarely been studied. In this study, we first obtained four novel isolates with different functions for conversion of FA and 4-VG in the presence of 15% NaCl: (i) 4-VG producing strains of *C. etchellsii*, (ii) 4-EG producing strains of *C. versatilis* that did not convert FA but did convert 4-VG, (iii) 4-VG producing strains of *C. guilliermondii*, and (iv) 4-EG producing strains of *C. fermentati*. From success in obtaining novel isolates and from previous studies,[5,6] four types of conversion mode of FA were identified: type 1, 4-EG production by conversion of FA and 4-VG, in most *C. versatilis* strains and *C. fermentati*; type 2, 4-EG production by conversion of 4-VG, in atypical

Table 1. Tested Strains, Bioconvertibility of FA to 4VG and 4EG, and Identification by 26S rDNA and ITS Sequence Analysis

Strain	Substrate*2 / Product*2	FA / 4-VG	FA / 4-EG	4-VG / 4-EG	FA Bioconversion type*3	FA-Decarboxylase activity*4	4-VG Reductase activity*4	Identification by the 26S rDNA sequence	DBBJ accession no.	Identification by the ITS sequence	DBBJ accession no.
Candida versatilis IFO 10056^T		−	+	+	type 1	+	+	*C. versatilis*	AB196199	*C. versatilis*	AB196228
C. versatilis JCM 5974		−	+	+	type 1			*C. versatilis*	AB196201	*C. versatilis*	AB196230
C. versatilis 8M2		−	+	+	type 1	+	+	*C. versatilis*	AB196202	*C. versatilis*	AB196231
C. versatilis 15M2		−	+	+	type 1			*C. versatilis*	AB196203	*C. versatilis*	AB196232
C. versatilis 25M2		−	+	+	type 1			*C. versatilis*	AB196204	*C. versatilis*	AB196233
C. versatilis 30M2		−	+	+	type 1			*C. versatilis*	AB196205	*C. versatilis*	AB196234
C. versatilis 33Z1		−	+	+	type 1			*C. versatilis*	AB196206	*C. versatilis*	AB196235
C. versatilis J5M2		−	+	+	type 1			*C. versatilis*	AB196207	*C. versatilis*	AB196236
C. versatilis Miso 19		−	+	+	type 1			*C. versatilis*	AB196209	*C. versatilis*	AB196238
C. versatilis Miso 22		−	+	+	type 1			*C. versatilis*	AB196210	*C. versatilis*	AB196239
C. versatilis Miso 83		−	+	+	type 1			*C. versatilis*	AB196211	*C. versatilis*	AB196240
C. versatilis OH3G1		−	+	+	type 1			*C. versatilis*	AB196212	*C. versatilis*	AB196241
C. versatilis JCM 5958		−	−	+	type 2	−	+	*C. versatilis*	AB196200	*C. versatilis*	AB196229
C. versatilis KS05		−	−	+	type 2	−	+	*C. versatilis*	AB196208	*C. versatilis*	AB196237
Candda etchellsii 9Z1		+	−	−	type 3	+	−	*C. etchellisii*	AB196188	*C. etchellisii*	AB196218
C. etchellsii 25Z1		+	−	−	type 3			*C. etchellisii*	AB196189	*C. etchellisii*	AB196219
C. etchellsii 33Z2		+	−	−	type 3			*C. etchellisii*	AB196190	*C. etchellisii*	AB196220
C. etchellsii IFO 1592^T		−	−	−	type 4	−	−	*C. etchellisii*	AB196184	*C. etchellisii*	AB196214
C. etchellsii Miso 0201		−	−	−	type 4			*C. etchellisii*	AB196191	*C. etchellisii*	AB196221
C. etchellsii Miso 0208		−	−	−	type 4	−	−	*C. etchellisii*	AB196192	*C. etchellisii*	AB196222
C. etchellsii Miso 0209		−	−	−	type 4			*C. etchellisii*	AB196193	*C. etchellisii*	AB196223
B-2 (isolated from a miso paste)*1		−	+	+	type 1			*C. fermentati**5	AB260130	*C. fermentati**7	AB260137
B-3 (isolated from a miso paste)*1		−	+	+	type 1			*C. fermentati**5	AB260131	*C. fermentati**7	AB260138
I-5 (isolated from a miso-koji)*1		−	+	+	type 1	+	+	*C. fermentati**5	AB260132	*C. fermentati**7	AB260139
I-6 (isolated from a miso-koji)*1		−	+	+	type 1			*C. fermentati**5	AB260133	*C. fermentati**7	AB260140
K-3 (isolated from a miso-koji)*1		−	+	+	type 1			*C. fermentati**5	AB260134	*C. fermentati**7	AB260141
I-4 (isolated from a miso-koji)*1		+	−	−	type 3			*C. guilliermondii**6	AB260128	*C. gulliermondii**8	AB260135
K-2 (isolated from a miso-koji)*1		+	−	−	type 3	+	−	*C. guilliermondii**6	AB260129	*C. gulliermondii**8	AB260136

*1 B2, B-3, I-4, I-5, I-6, K-2, and K-3 strains were isolated from miso-koji or miso pastes in the same brewing factory.

*2 FA, ferulic acid; 4-VG, 4-vinylguaiacol; 4-EG, 4-ethylguaiacol

*3 FA bioconversion types: type 1, FA to 4EG; type 2, 4-VG to 4-EG; type 3, FA to 4-VG; type 4, not convert

*4 Each strain was cultivated in YPG + 15% NaCl medium containing 50 ppm FA or 20 ppm 4VG at 30 °C, at 120 rpm for 2 d, and crude enzyme extract was obtained by Zymolyase 20T treatment at pH 7.0. 10 µl of 10,000 ppm FA or 10,000 ppm 4-VG in ethanol, and about 3 mg NADH were added to 0.5 ml of crude enzyme extract, and incubated for 2 h at 30 °C, and the reactions were stopped by addtion of 0.5 ml methanol. FA decarboxylase and 4-VG reductase activity were detected produced 4-VG and 4-EG by HPLC analysis.

*5 *C. fermentati*: They have an identical 26S rDNA sequence with that of *Candida fermentati* (AY187283).

*6 *C. guilliermondii*: They have an identical 26S rDNA sequence with that of *Pichia guilliermondii* (U45709).

*7 *C. fermentati*: They have an identical ITS sequence with that of *C. fermentati* (AB032175).

*8 *C. giulliermondii*: They have an identical ITS sequence with that of *P. guilliermondii* (AF022717).

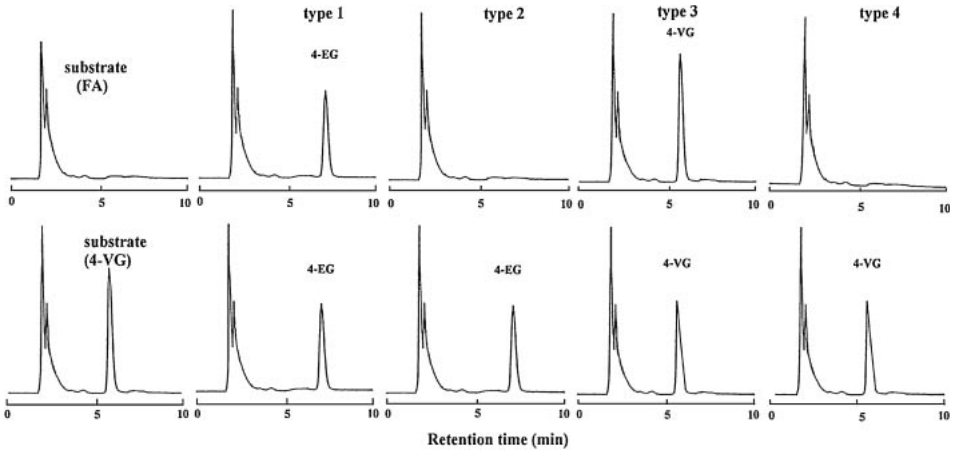
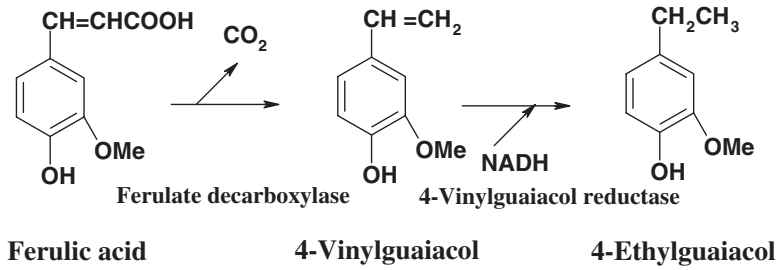


Fig. 1. HPLC Chromatograms of Products from FA (top) and 4-VG (bottom) by Halotolerant Yeasts (types 1, 2, 3, and 4; see text).
Analytical procedure: 0.1 ml of suspension of yeast cells, precultivated in YPG medium with the addition of 15% NaCl for 7 d at 30 °C in a tube, was added to 15 ml of the same YPG medium containing 50 ppm FA or 20 ppm 4-VG in a 50-ml Erlenmeyer flask, and incubated statically for 7 d at 30 °C. Cell suspension (0.1 ml) diluted with 0.9 ml of the mobile phase for HPLC chromatography described below, was filtrated by membrane filters, and subjected to determine 4-VG and 4-EG by the HPLC condition described below. HPLC condition: column, Wakosil-II 5C18 RS (i.d. 4.6 × 150 mm); column temp., 50 °C; mobile phase, 10 mM phosphate buffer (pH 2.5)/methanol (50/50, v/v); flow rate, 1.0 ml/min.; detector, fluorescence (ex 280, em 320); injection volume, 10 µl.



Candida versatilis **(type 1)**
C. fermentati* **(type 1)**
C. etchellsii* **(type 3)**
C. guilliermondii* **(type 3)**
Debaryomyces hansenii **(type 3)**
Pichia anomala **(type 3)**
P. subpelliculosa **(type 3)**
Bacillus subtilis **(type 3)**
Staphylococcus xylosus **(type 3)**

C. versatilis **(type 1 and type 2*)**
C. fermentati* **(type 1)**

Fig. 2. Conversion Pathway of Ferulic Acid to 4-Vinylguaiacol and 4-Ethylguaiacol by Microorganisms.
The result of bioconversion of *p*-coumaric acid was same as that of ferulic acid. *Original finding in this study.

C. versatilis strains; type 3, 4-VG production only by conversion of FA, in atypical *C. etchellsii* strains, *C. guilliermondii*, *P. anomala*, *P. subpelliculosa*, and *Debaryomyces hansenii*, and also 4-VG producing bacteria such as the *Bacillus subtilis* and *Staph. xylosus* strains; and type 4, no conversion of FA or 4-VG, in some *C. etchellsii* strains. It was also found that *C. versatilis* is not the only species with convertibility of FA to 4-EG, and that *C. fermentati* as well as *C. versatilis* can convert FA to 4-EG. Conversion pathway of FA to 4-VG and 4-EG by microorganisms is shown in Fig. 2.

Since 4-VG reductase appears to be produced only by the two species, *C. versatilis* and *C. fermentati*, in the presence of 15% NaCl, the enzyme activity and the product, 4-EG, might be a useful tool to distinguish roughly the two species from other halotolerant yeasts. Therefore, it is desirable to investigate the convertibility of FA by the many other strains of *P*/*C. guilliermondii*, *C. fermentati*, and *C. carpophila*.[8] Furthermore, biochemical and genetic studies of the volatile phenol producing enzymes, FA decarboxylase and 4VG reduc-

tase, and of the coding genes, and a deep understanding of roles of halotolerant *Candida* yeasts in miso and soy sauce making should be promoted.

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References

1) Aoki, T., and Uchida, K., Enhanced fermentation of 2-phenyl-ethanol in *Zygosaccharomyces rouxii* due to prephenate hydrogenase deficiency. *J. Agric. Chem.*, **54**, 273–274 (1990).
2) Nakamura, M., Isolation of mutants of miso yeast, *Zygosaccharomyces rouxii* with high productivity of C3 to C5 alcohols. *Nippon Shokuhin Kôgyô Gakkaishi* (in Japanese), **37**, 981–983 (1990).
3) Hayashida, Y., Flavor-active furanones. The formation mechanism by micro-organisms and enhancement techniques in brewing industry. *Nippon Jôzô Kyôkaishi* (in Japanese), **98**, 89–95 (2003).
4) Yokotsuka, T., Asao, Y., and Sakasaki, T., Studies of the flavorous substances in shoyu Part XXVII. The production of 4-ethylguaiacol during shoyu fermentation, and its role for shoyu flavor. *Nippon Nôgeikagaku Kaishi* (in Japanese), **41**, 442–447 (1967).
5) Suezawa, Y., Bioconversion of ferulic acid and *p*-coumaric acid to volatile phenols by halotolerant yeasts. *Nippon Nôgeikagaku Kaishi* (in Japanese), **69**, 1587–1596 (1995).
6) Suezawa, Y., Yoshioka, N., and Mori, H., Bioconversion of ferulic acid and *p*-coumaric acid to volatile phenols by *Aspergillus* spp. and bacteria found in soy sauce koji and mashes. *Nippon Nôgeikagaku Kaishi* (in Japanese), **72**, 43–49 (1998).
7) Suezawa, Y., Kimura, I., Ioue, M., Gohda, N., and Suzuki, M., Identification and typing of miso and soy sauce fermentation yeasts, *Candida etchellsii* and *C. versatilis*, based on sequence analyses of the D1D2 domain of the 26S ribosomal RNA gene, and the region of internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2. *Biosci. Biotechnol. Biochem.*, **70**, 348–354 (2006).
8) Vaughan-Martini, A., Kurtzman, C. P., Meyer, S. A., and O'Neill, E. B., Two new species in the *Pichia guilliermondii* clade: *Pichia caribbica* sp. nov., the ascosporic state of *Candida fermentati*, and *Candida carpophila* comb. nov. *FEMS Yeast Res.*, **5**, 463–469 (2005).
9) Rosazza, J. P. N., Huang, Z., Dostal, L., Volm, T., and Rousseau, B., Biocatalytic transformations of ferulic acid: an abundant aromatic natural product. *J. Indust. Microbiol.*, **15**, 457–471 (1995).
10) Chatonet, P., Dubourdieu, D., Boidron, J.-N., and Pons, M., The origin of ethylphenols in wines. *J. Sci. Food Agric.*, **60**, 165–178 (1992).
11) Chatonet, P., Dubourdieu, D., Boidron, J.-N., and Lavigne, V., Synthesis of volatile phenols by *Saccharomyces cerevisiae* in wines. *J. Sci. Food Agric.*, **62**, 191–202 (1993).
12) Sutherland, J. B., Tanner, L. A., Moor, J. D., Freeman, J. P., Deck, J., and Williams, A. J., Conversion of ferulic acid to 4-vinylguaiacol by yeasts isolated from frozen concentrated orange Juice. *J. Food Protec.*, **59**, 1260–1262 (1995).