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# Identification of Phenylethyl Alcohol and Other Volatile Flavor Compounds from Yeasts, *Pichia farinosa* SKM-1, *Pichia anomala* SKM-T, and *Galactomyces geotrichum* SJM-59

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**Abstract** Three strains of yeasts, *Pichia farinosa* SKM-1, *Pichia anomala* SKM-T, and *Galactomyces geotrichum* SJM-59, produced volatile flavor compounds during fermentation. To investigate these volatile flavor compounds, the liquid culture broth of the three yeast strains were extracted with methylene chloride, and then GC and GC-MS analyses were conducted. Flavor analyses revealed that 5, 12, and 15 kinds of volatile compounds were isolated, and 4, 8, and 11 volatile flavor compounds were identified, respectively. Phenylethyl alcohol was identified with the common volatile flavor compound of *Pichia farinosa* SKM-1, *Pichia anomala* SKM-T, and *Galactomyces geotrichum* SJM-59. 1H-indole-3-ethanol, a precursor of plant growth hormone, was identified from *Pichia anomala* SKM-T.

**Key words:** *Pichia farinosa* SKM-1, *Pichia anomala* SKM-T, *Galactomyces geotrichum* SJM-59, phenylethyl alcohol, 1H-indole-3-ethanol, flavor volatile compounds

According to a survey of the US Food Market Institute, flavors and fragrances constitute a yearly worldwide market of 7 billion U.S. dollars and 91% of consumers consider flavors to be the most important factor when they choose and purchase foods [2]. There has been a growing interest in the production of flavors by using microorganisms, since they are considered as natural products deserving GRAS (generally recognized as safe) status and are economic alternatives to expensive extraction from raw materials like plants [3]. Numerous yeasts are reported to produce volatile flavor compounds like ester, alcohols, and apple aroma. Ester formation results from enzymatic activities such as alcohol acetyltransferase and carboxylic ester hydrolases, including lipase and esterases.

Phenylethyl alcohol is known to have a mild-warm and rose-honey-like odor. This material is one of the most widely used among all perfume chemicals and enters perfume compositions at the rate of 5–20% or even more [2]. It is almost never out of place in a perfumery composition, be it floral, balsamic, oriental, mossy, herbaceous, or modern-aldehydic, and has some use in food flavor composition, mainly in imitation butter, strawberry, raspberry, caramel, honey, and melon flavoring, as well as in various fruit complexes. Phenylethyl alcohol can be considered as an analogue of the amino acids phenylalanine and tyrosine, and of the aromatic neurotransmitter phenylethylamine, and to be originated from phenylalanine [6]. Fukuda *et al.* [8] reported that *Saccharomyces cerevisiae* overproduced phenylethyl alcohol when the activity of prephenate dehydrogenase (PDG) was decreased, whereas that of 3-deoxy-D-arabinoheptulosonate-7-phosphate synthase was increased. Also, *Kluyveromyces marxianus* is a dairy yeast that accumulates 2-phenylethyl alcohol in liquid culture, and *Hansenula saturnus* var. *saturnus* produces volatile phenylethyl alcohol when phenylalanine is used as a nitrogen source [7].

The halotolerant yeast *Pichia farinosa* produces a sodium mediated killer toxin that is a heterodimer consisting of α and β subunits [7, 19], and is known to have resistance to cadmium ion and forms cadmium binding complexes via a synthesized cadmium-binding protein that was similar to the cadmium metallothionein produced by *Saccharomyces cerevisiae* [11]. *Pichia anomala*, referred to as *Candida pelliculosa*, is known to have an antagonistic property against a range of spoilage molds including *Penicillium roquefortii* [4] and *Botrytis cinerea* [16], and to have alternative oxidase activity [18], D-ribose secretion [23],

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invertase production [17], and degradation of anthraquinone [12]. In addition, *Pichia anomala* killer toxin has been demonstrated to have a specific inhibitory effect on the *in vitro* attachment of *Pneumocystis carinii* that is responsible for pneumonia in immunocompromised individuals [22].

*Galactomyces geotrichum*, often referred to as a fungi *Geotrichum candidum*, produces fatty acid esters and a specific fruit aroma. Its lipolytic and proteolytic activities may form flavor compounds and have been partly characterized [5].

Nevertheless, no report could be found to show that *Galactomyces geotrichum* produced phenylethyl alcohol, and *Pichia farinosa* and *Pichia anomala* have never been reported to produce volatile flavor compounds as well as phenylethyl alcohol. To investigate flavor compounds, three kinds of yeasts were inoculated into potato dextrose broth and incubated for 24 h at 30±1°C. The volatile flavor compounds were extracted with methylene chloride, and the extracts were then analyzed by GC and GC-MS. Butyl benzene (Sigma, St. Louis, MO, U.S.A. ACS gr.) and benzeneethanol (Sigma, St. Louis, MO, U.S.A. ACS gr.) were used as an internal and/or external standard, respectively.

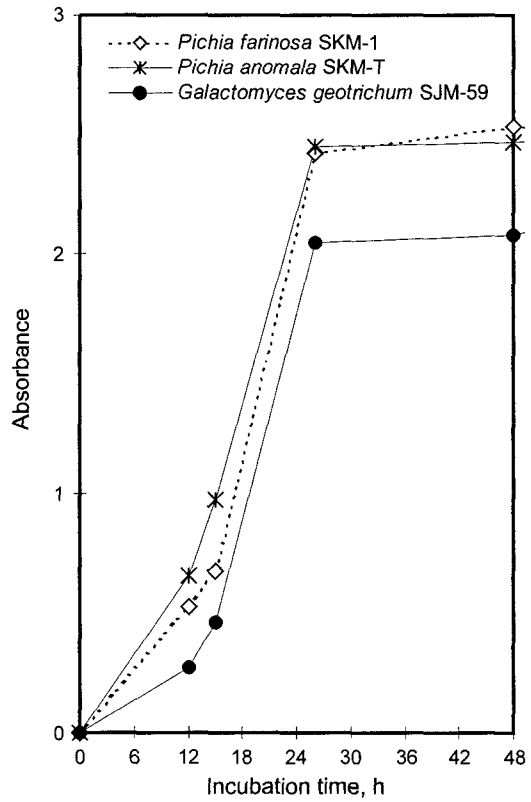


**Fig. 1.** The growth curves of *Pichia farinosa* SKM-1, *Pichia anomala* SKM-T, and *Galactomyces geotrichum* SJM-59.
*Pichia farinosa* SKM-1, *Pichia anomala* SKM-T, and *Galactomyces geotrichum* SJM-59 were inoculated into 1.5-l potato dextrose broth in 3-l Erlenmeyer flask with baffles and the 3 samples were placed on the shaker at 140 rpm for 48 h at 30±1°C.

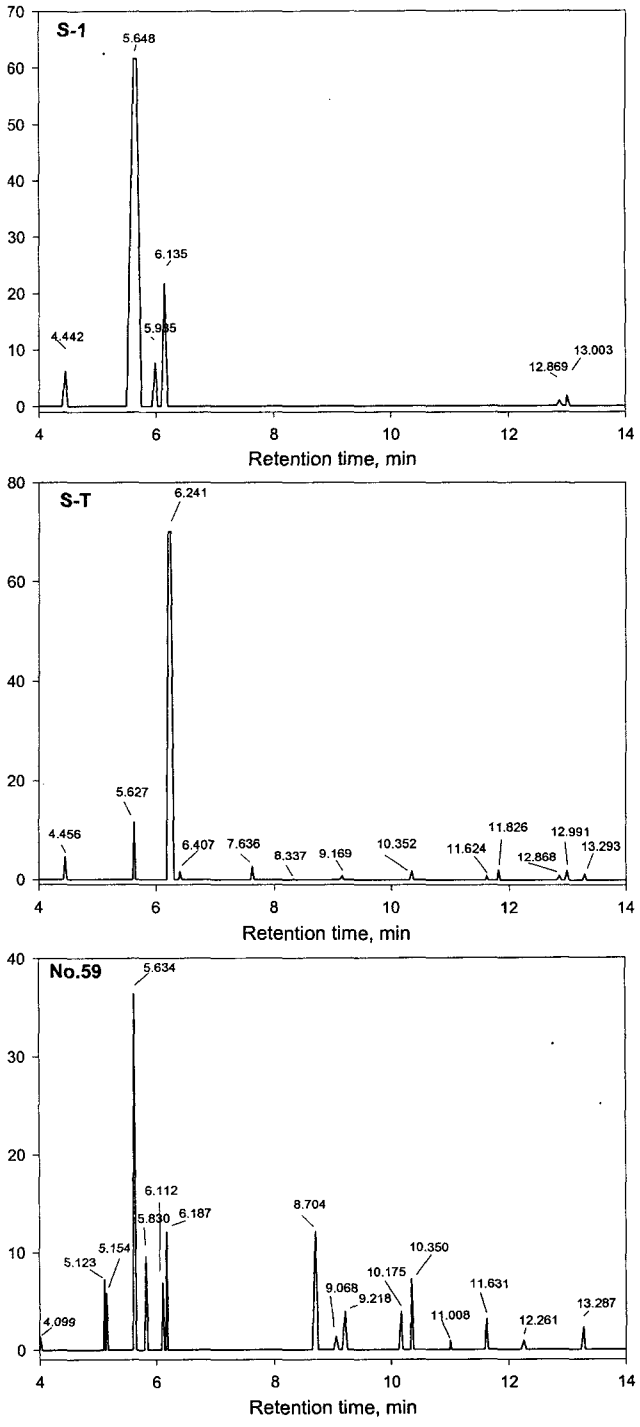


**Fig. 2.** Chromatograms of volatile flavor compounds from *Pichia farinosa* SKM-1, *Pichia anomala* SKM-T, and *Galactomyces geotrichum* SJM-59.
The analysis of the volatile flavor compounds was performed on a Hewlett-Packard 5890A gas chromatograph equipped with flame ionization detector and a capillary column (HP-1, 30 m×0.25 mm i. e.×0.33 mm). The injector and detector were held at 250°C, the oven temperature was increased with the following program: hold at 50°C for 1 min, raised from 50°C to 230°C at 15°C/min, hold at 230°C for 1 min. A volume of 1 μl was injected in all analyses. S-1 is *Pichia farinosa* SKM-1, S-T is *Pichia anomala* SKM-T, and No. 59 is *Galactomyces geotrichum* SJM-59.

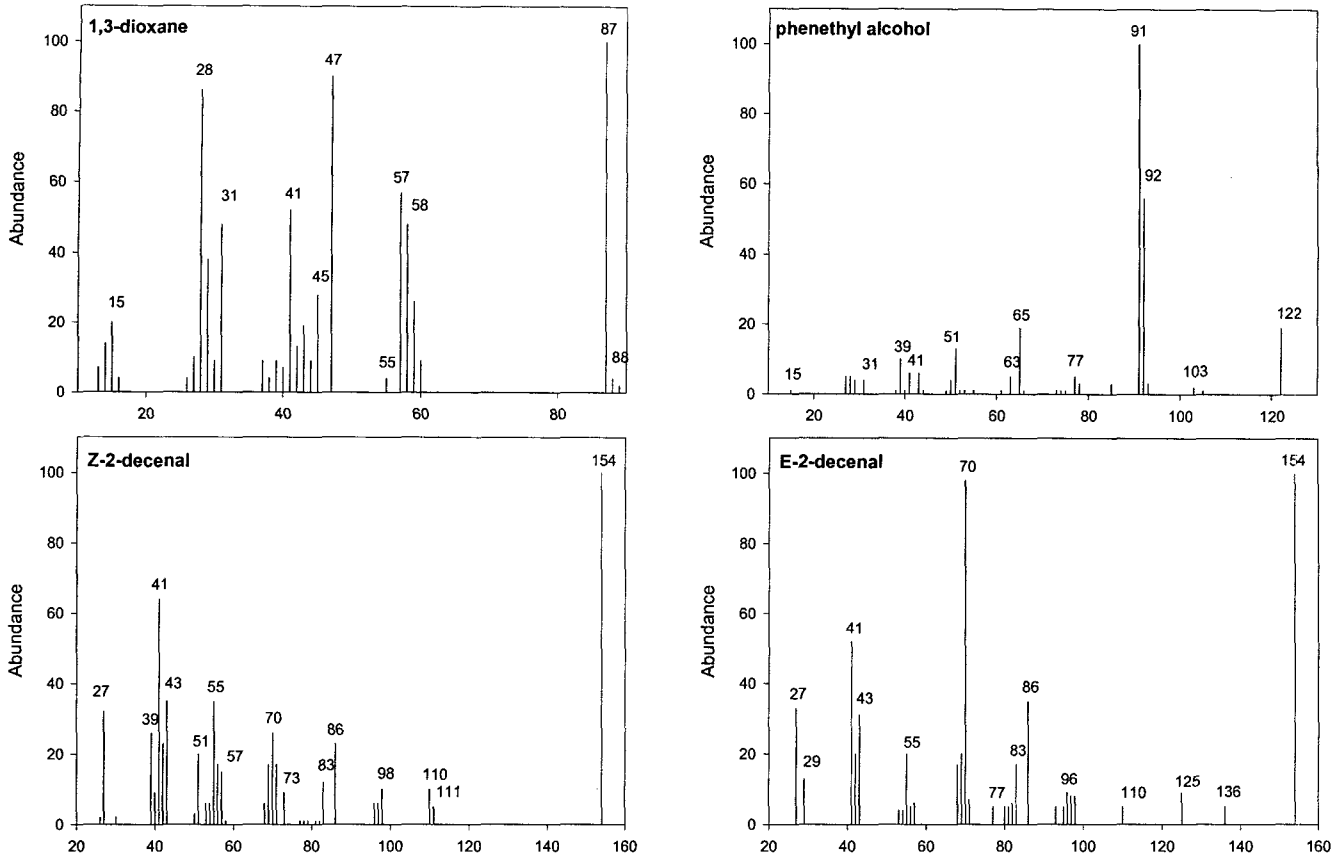


**Fig. 3.** GC-MS spectrum of identified volatile flavor compounds from the liquid culture broth of *Pichia farinosa* SKM-1.
Mass spectra were obtained by electron impact ionization at 70 eV, ion species were normal ion (MF-Linear), and the TIC range was m/z 0 to 600. The spectrometric data were compared with those from the NIST Hewlett-Packard 59942C original library mass-spectra.

As shown in Fig. 1, volatile flavors were not detected at the lag phase as well as at inoculation by organoleptic evaluation, therefore, it was considered that the concentration of volatile flavor compound was too low to be detected. When the incubation time reached 24 h, *Pichia farinosa* SKM-1, *Pichia anomala* SKM-T, and *Galactomyces geotrichum* SJM-59 were in the logarithmic phase. It was the best time for sensory evaluation of the volatile flavor that did not produce unpleasant odors. The flavor of *Pichia farinosa* SKM-1 was changed depending on incubation time. *Pichia farinosa* SKM-1 have had the best flavor at the logarithmic phase, but diffused unpleasant odor at the stationary phase. The flavors of *Pichia anomala* SKM-T and *Galactomyces geotrichum* SJM-59 were not related to incubation time. *Pichia farinosa* SKM-1, *Pichia anomala* SKM-T, and *Galactomyces geotrichum* SJM-59 diffused their own flavors at the logarithmic phase and were classified by organoleptic evaluation.

Therefore, the cultured broths of *Pichia farinosa* SKM-1, *Pichia anomala* SKM-T, and *Galactomyces geotrichum* SJM-59 were harvested after 24 h of inoculation, and then extracted with methylene chloride. The major problem of the batch solvent extraction method is the formation of emulsion in the separatory funnel [10], hence the centrifugation

**Table 1.** Characteristics of identified volatile flavor compounds from *Pichia farinosa* SKM-1.

| No. | R.T.* | Compounds | M.W. | Characteristics |
|---|---|---|---|---|
| 1 | 4.442 | 1,3-dioxane | 88 | very sweet, mild-ethereal odor |
| 2 | 5.648 | internal standard** | | |
| 3 | 5.985 | unknown | | |
| 4 | 6.135 | phenylethyl alcohol | 122 | rose-honey odor |
| 5 | 12.869 | *Z*-2-decenal | 154 | waxy-orange-like, sweet-aldehydic odor |
| 6 | 13.003 | *E*-2-decenal | 154 | waxy-orange-like, sweet-aldehydic odor |

* R.T. is retention time; ** is butyl benzene.

was conducted to break the emulsion at 4°C. The lower phase was collected carefully and filtered to remove moisture in the solvent fraction. The filtrated and concentrated extracts were used in GC and GC-MS analyses.

As can be seen in Fig. 2 and Fig. 3, the batch solvent extract of *Pichia farinosa* SKM-1 contained five volatile compounds. Coupled with mass spectrometry, this led to the identification of four kinds of volatile flavor compounds. Table 1 shows the flavor characteristics of the identified flavor components. 1,3-Dioxanes are chemically formed in the apples and cider from natural apple ingredients and appropriate aldehydes and ketones, which are produced from either apples or yeast during fermentation [14]. Phenylethyl alcohol has been known to have a mild-warm and rose-honey-like odor, and it has been used in food flavor composition, mainly in imitation butter, strawberry, raspberry, caramel, honey, and melon flavoring as well as in various fruit complexes. Decenal has a very powerful, waxy-

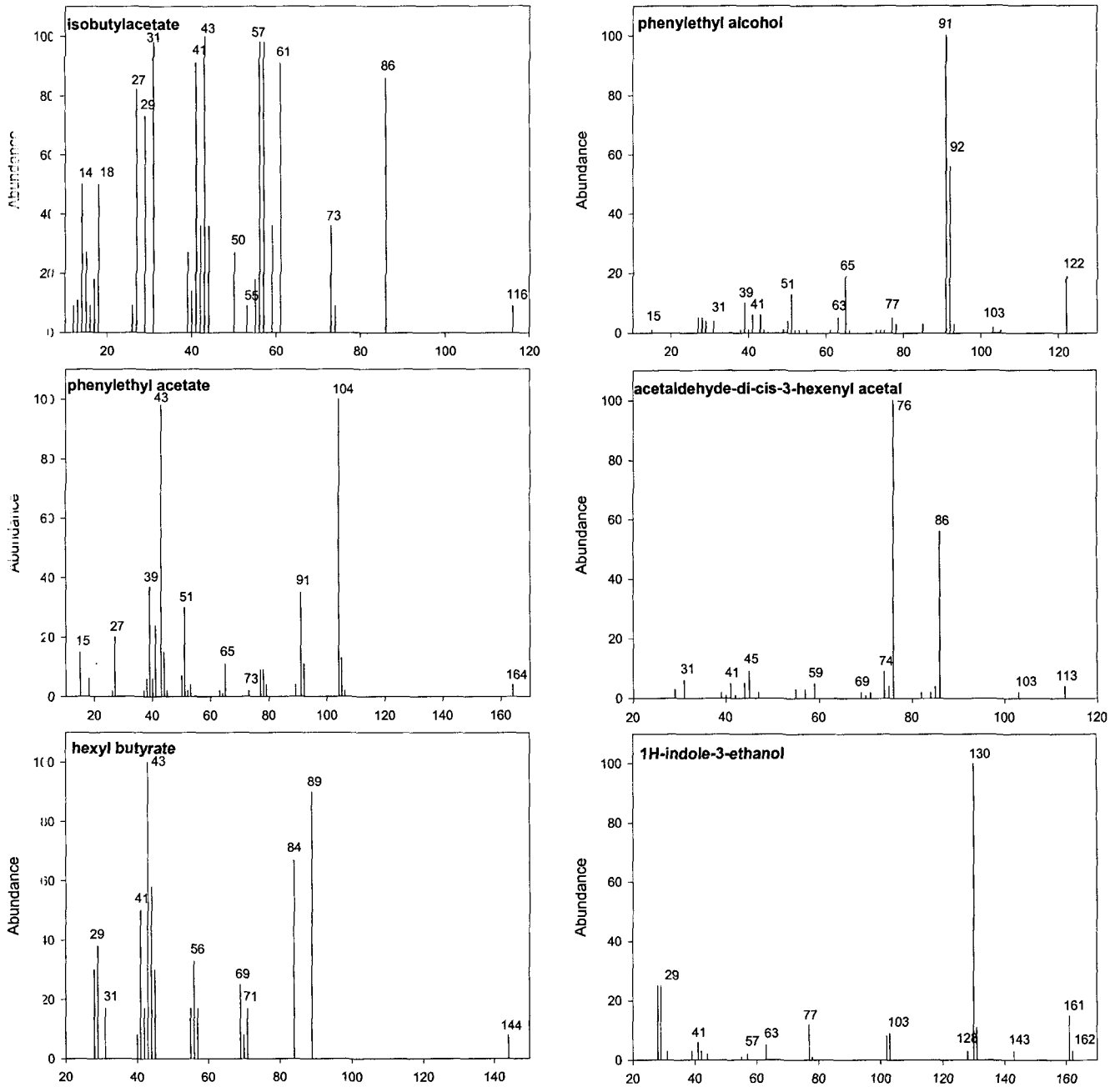


**Fig. 4.** GC-MS spectrum of identified volatile flavor compounds from the liquid culture broth of *Pichia anomala* SKM-T.
Mass spectra were obtained by electron impact ionization at 70 eV, ion species were normal ion (MF-Linear), and the TIC range was m/z 0 to 600. The spectrometric data were compared with those from the NIST Hewlett-Packard 59942C original library mass-spectra.

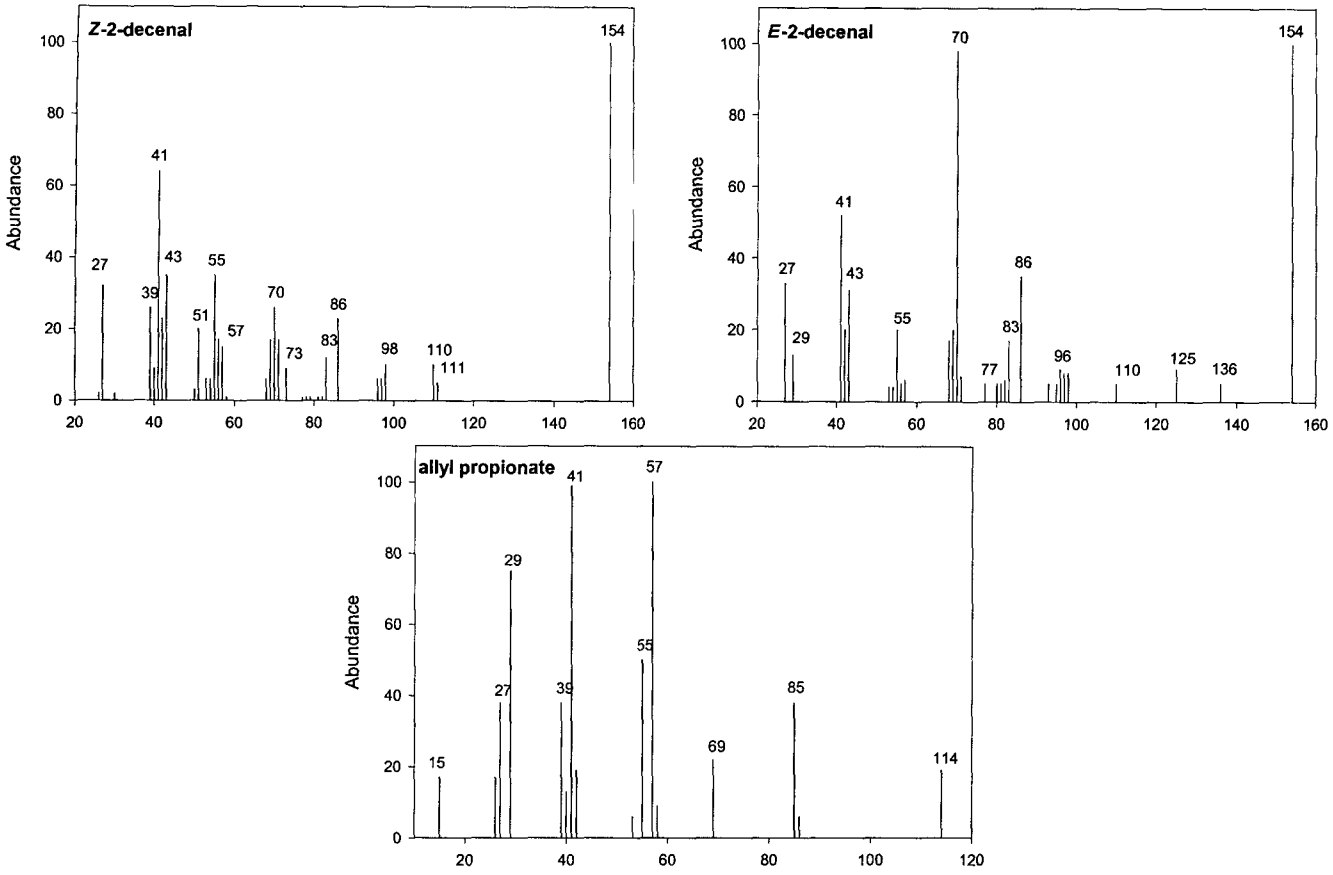


**Fig. 4.** Continued.

orange-like, sweet aldehydic odor, and it has been used in fruit flavors [2]. Fermenting cells of *Pichia farinosa* SKM-1 overall produced apple flavor. Therefore, 1,3-dioxane was regarded as the major flavor component of *Pichia farinosa* SKM-1 during fermentation on potato dextrose broth.

As can be seen in Fig. 2 and Fig. 4, the batch solvent extract of *Pichia anomala* SKM-T contained 12 volatile compounds and mass spectrometry led to the identification of eight kinds of volatile flavor compounds and one physiologically active compound. Table 2 shows the flavor characteristics of the identified flavor components. Ethyl

**Table 2.** Characteristics of identified volatile flavor compounds from *Pichia anomala* SKM-T.

| No. | R.T.* | Compounds | M.W. | Characteristics |
|---|---|---|---|---|
| 1 | 4.456 | isobutyl acetate | 116 | ethereal, fermented odor |
| 2 | 5.627 | internal standard** | | |
| 3 | 6.241 | phenethyl alcohol | 122 | rose-honey-like odor |
| 4 | 6.407 | unknown | | |
| 5 | 7.636 | phenylethyl acetate | 164 | very sweet, rose-fruity, honey-like |
| 6 | 8.337 | unknown | | |
| 7 | 9.163 | unknown | | |
| 8 | 10.352 | acetaldehyde-di-*cis*-3-hexenylacetal | 226 | oily-green odor with a sweet taste |
| 9 | 11.624 | hexyl butyrate | 172 | fruity, heavy odor |
| 10 | 11.826 | 1H-indole-3-ethanol | 161 | not flavor, auxin precursor |
| 11 | 12.868 | *Z*-2-decenal | 154 | waxy-orange-like, sweet-aldehydic |
| 12 | 12.991 | *E*-2-decenal | 154 | waxy-orange-like, sweet-aldehydic |
| 13 | 13.293 | allyl propionate | 114 | sour-fruity odor |

*R.T. is retention time; ** is butyl benzene.

acetate is probably one of the most used of all flavor chemicals by volume, and forms a major part of the imitation flavors of many berry types, banana, grape, pineapple, peach, lemon, tutti-frutti, whisky, rum, almond, butterscotch, cream soda, butter, mint, pear, and melon flavoring [2]. Phenylethyl acetate might be formed either from phenylethyl alcohol and acetyl CoA or from phenylethyl alcohol and acetic acid, depending on the catalytic action of alcohol acety transferase or by the reverse reaction of esterase [1]. Acetaldehyde-di-cis-3-hexenylacetal, often referred to as leaf alcohol acetal, has been used in soaps because of its stability in mild alkaline media [2]. Allyl propionate has a somewhat sharp, sour-fruity odor, and has been used in imitation fruit flavors of the sour type such as apple, apricot, and peach [2]. In general, the fermenting flavor of *Pichia anomala* SKM-T was very sour at the first step and then sweet and alcoholic at the end of sniffing. Therefore, it was considered that allyl propionate and phenylethyl

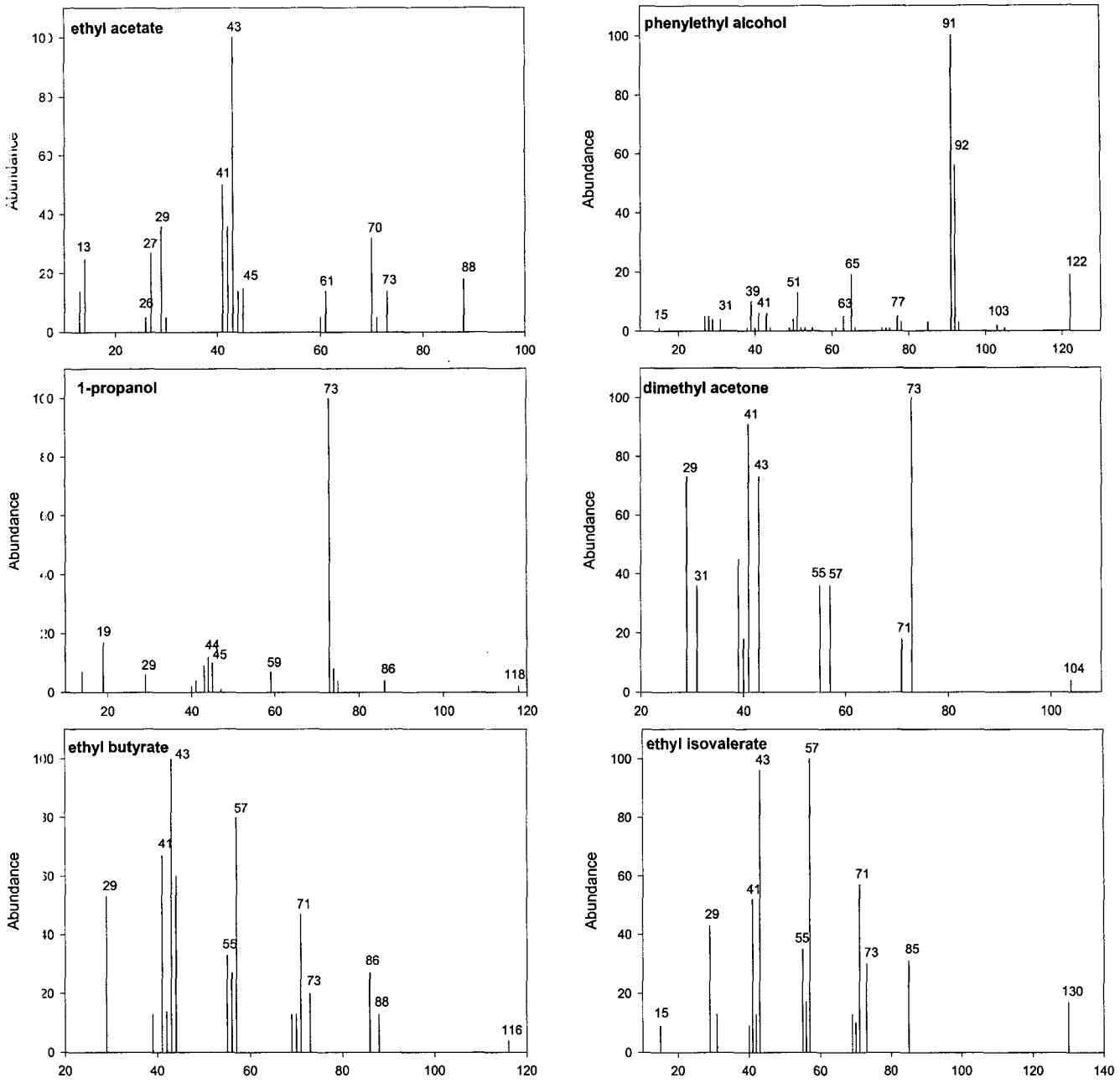


**Fig. 5.** GC-MS spectrum of identified volatile flavor compounds from the liquid culture broth of *Galactomyces geotrichum* SJM-59. Mass spectra were obtained by electron impact ionization at 70 eV, ion species were normal ion (MF-Linear), and the TIC range was m/z 0 to 600. The spectrometric data were compared with those from the NIST Hewlett-Packard 59942C original library mass-spectra.

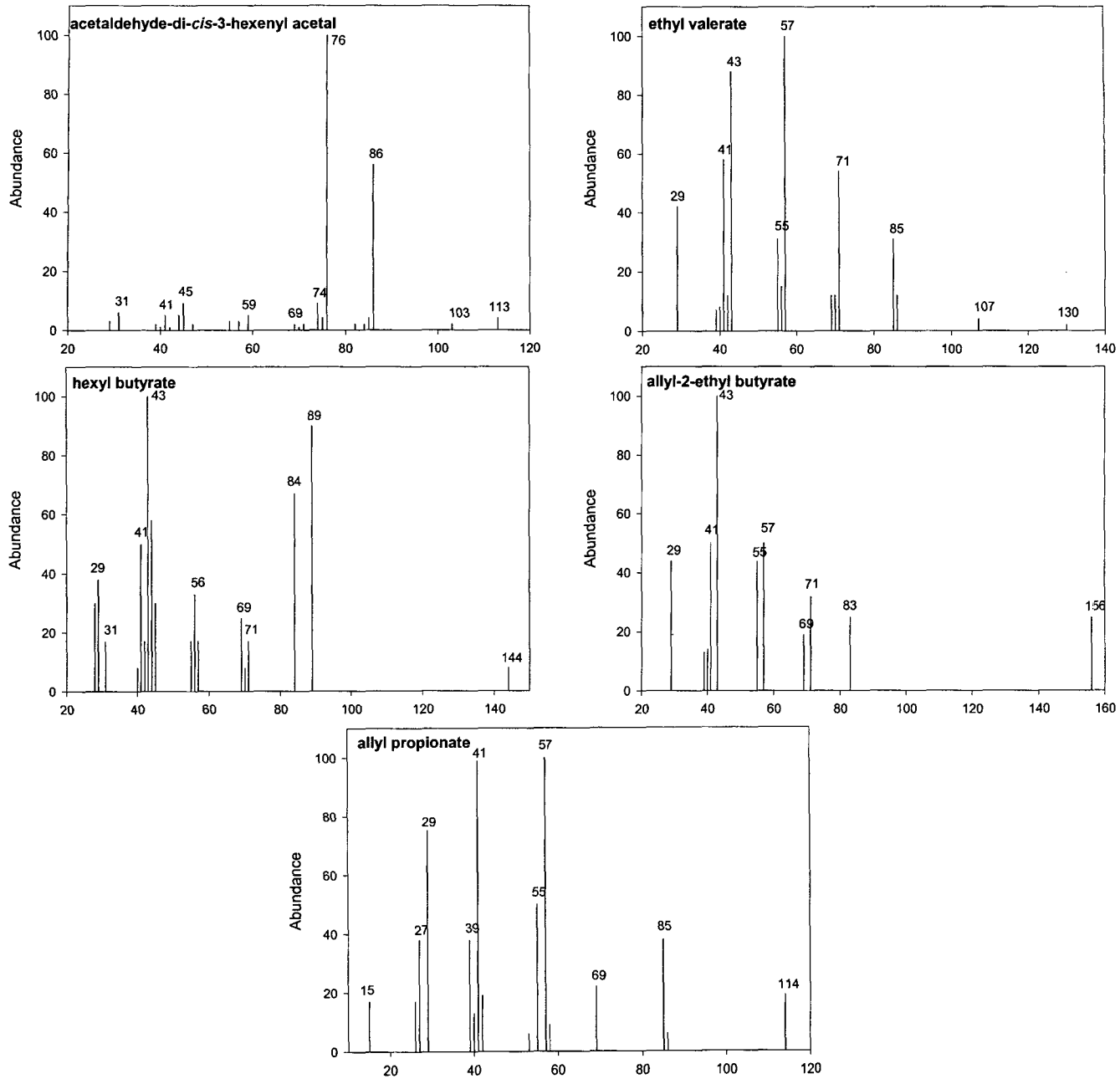


**Fig. 5.** Continued.

alcohol were major flavor compounds of *Pichia anomala* SKM-T during fermentation on potato dextrose broth. Of special note, 1H-indole-3-ethanol was detected, even though it is not a flavor component but an auxin precursor. Auxin, a plant growth hormone, has been known to have activities such as stimulation of cell division, enlargement and shoot growth, induction of root growth, control of vascular tissue development, inhibition of somatic embryo development, apical dominance, delay of senescence promotion of flowering, and fruit set and ripening. The stimulation of stem and root growth in sprouted beans and the inhibition of the softening change in strawberry were observed, when treated with *Pichia anomala* SKM-T (data not shown).

Figure 2 and Fig. 5 show that the batch solvent extract of *Galactomyces geotrichum* SJM-59 contained 15 volatile compounds, and mass spectrometry led to the identification of 11 kinds of volatile flavor compounds. As can be seen in Table 3, *Galactomyces geotrichum* SJM-59 produced many short-chain ethyl esters like ethyl acetate, ethyl butyrate,

**Table 3.** Characteristics of identified volatile flavor compounds from *Galactomyces geotrichum* SJM-59.

| No. | R.T.* | Compounds | M.W. | Characteristics |
|---|---|---|---|---|
| 1 | 4.099 | unknown | | |
| 2 | 5.123 | unknown | | |
| 3 | 5.154 | ethyl acetate | 88 | ethereal, fruity, brandy-like odor |
| 4 | 5.634 | internal standard** | | |
| 5 | 5.830 | unknown | | |
| 6 | 6.112 | unknown | | |
| 7 | 6.187 | phenylethyl alcohol | 122 | rose-honey like odor |
| 8 | 8.704 | 1-propanol | 60 | alcoholic, sweet odor |
| 9 | 9.068 | dimethyl acetone | 104 | ethereal odor |
| 10 | 9.218 | ethyl butyrate | 116 | fruity, pineapple, banana, ethereal |
| 11 | 10.175 | ethyl isovalerate | 130 | fruity, apple on dilution odor |
| 12 | 10.350 | acetaldehyde-di-*cis*-3-hexenylacetal | 226 | oily-green odor with a sweet taste |
| 13 | 11.008 | ethyl valerate | 130 | fruity, apple odor |
| 14 | 11.631 | hexyl butyrate | 172 | fruity, heavy odor |
| 15 | 12.261 | allyl-2-ethyl butyrate | 156 | oily-fruity odor |
| 16 | 13.287 | allyl propionate | 114 | sour-fruity odor |

*R.T. is retention time; ** is butyl benzene.

ethyl isovalerate, and ethyl valerate. Such ethyl esters are recognized as a typical fruity aroma. Alcohol acetyltransferase, lipases, esterases, and proteases are responsible for the formation of esters and other volatile flavor compounds during fermentation of *Galactomyces geotrichum* [9, 13, 19]. *Galactomyces geotrichum* SJM-59 had powerful protease activity (data not shown), and the formation of volatile flavor compounds was regarded as its proteolytic activity; fermenting cells of *Galactomyces geotrichum* SJM-59 generally produced pineapple and apple odors. Therefore, short-chain esters and phenylethyl alcohol were major flavor compounds of *Galactomyces geotrichum* SJM-59 during fermentation on potato dextrose broth.

**Table 4.** Concentration* of identified volatile flavor compounds from *Pichia farinosa* SKM-1, *Pichia anomala* SKM-T, and *Galactomyces geotrichum* SJM-59.

| No. | *Pichia farinosa* SKM-1 | *Pichia anomala* SKM-T | *Galactomyces geotrichum* SJM-59 |
|---|---|---|---|
| 1 | 0.99 | 3.93 | 0.39 |
| 2 | 10.00 | 10.00 | 1.99 |
| 3 | 1.25 | 60.30 | 1.59 |
| 4 | 3.53 | 1.41 | 10.00 |
| 5 | 0.16 | 2.25 | 2.65 |
| 6 | 0.29 | 0.18 | 2.43 |
| 7 | | 0.71 | 3.32 |
| 8 | | 1.52 | 3.33 |
| 9 | | 0.71 | 0.37 |
| 10 | | 1.75 | 1.07 |
| 11 | | 0.79 | 1.06 |
| 12 | | 1.51 | 1.99 |
| 13 | | 0.99 | 2.61 |
| 14 | | | 0.86 |
| 15 | | | 0.26 |
| 16 | | | 0.62 |

*Concentration is ppm.

The amount of identified volatile flavor compounds are shown in Table 4. Phenylethyl alcohol was a major and common flavor compound of *Pichia farinosa* SKM-1, *Pichia anomala* SKM-T, and *Galactomyces geotrichum* SJM-59. Phenylethyl alcohol was also identified with the external standard. It was also detected when the three kinds of yeasts were fermented in YPD using GC-MS (data not shown), but not detected by sniffing. Only PDB and/or YPD extracts did not show the peak of phenylethyl alcohol.

Fukuda *et al.* [8] reported that *Saccharomyces cerevisiae* overproduced phenylethyl alcohol when the activity of prephenate dehydrogenase (PDG) was decreased, while that of 3-deoxy-D-arabinoheptulosonate-7-phosphate synthase was increased when the *TYR*1 gene encoding PDG was genetically mutated. Also, *Hansenula saturnus* var. *saturnus* produced volatile phenylethyl alcohol, when phenylalanine was used as a nitrogen source [1]. *Kluyveromyces marxianus* is a dairy yeast that accumulates 2-phenylethyl alcohol in its liquid culture when carbon and nitrogen sources are modified [7]. As mentioned above, aroma compound production by microorganisms was influenced by environmental conditions as well as strains [15, 20].

Furthermore, the volatile flavor volume produced is dependent on the analytical method used. The batch solvent extraction method is a very simple and basic skill, but extraction yield is low in comparison with the dynamic head space method, solid phase extraction, and/or simultaneous distillation extraction [10]. In conclusion, *Pichia farinosa* SKM-1 and *Galactomyces geotrichum* SJM-59 were investigated, since they are phenylethyl alcohol-producing yeasts. In particular, *Pichia anomala* SKM-T was found to be an effective strain in phenylethyl alcohol production.

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