Isolation and Identification of Phenylethyl Alcohol from *Pichia anomala* SKM-T

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Phenylethyl alcohol (PEA, GRAS 2858), which has a rose-like aroma, is an important fragrance in the cosmetic industry and possesses organoleptic characteristics that contribute to the quality of beverage and foods [Fabre *et al.*, 1998]. It has been estimated that approximately 700,000 kg of PEA is consumed annually as a food component [Maarse *et al.*, 2000].

The biologically synthesized PEA is 250- to 300-fold more expensive than chemically synthesized one [Etschmann *et al.*, 2002]. Flavors and fragrances that can be labeled "natural" both in the United States and Europe have to be produced from natural sources by physical, enzymatic, or microbiological processes [US Food and Drug Administration, 2001]. Scientifically, there is no difference between a flavor compound synthesized in nature and the same molecule produced in the laboratory; however, consumers are critical of things they regard as artificial and demand natural food additives [Anonymous, 1999]. Therefore increasing demand for natural flavors makes microbial production of PEA an interesting research field.

Pichia anomala is an ascomycetous heterothallic yeast that reproduces asexually by budding and sexually by the formation of hat-shaped ascospores [Kurtzman and Fell, 1998]. *P. anomala* is present in many types of environments and have been isolated from fruit and plant materials [Kurtzman and Fell, 1998], cereal grain [Lacey and Magan, 1991], maize silage [Kitamo *et al.*, 1999], high sugar food products [Lanciotti *et al.*, 1999], and wine [Mingorance-Cazorla *et al.*, 1999]. *P. anomala* is classified as a biosafety level 1 organism that is considered safe for healthy individuals [De Hoog, 1996]. In the literature, there are no reports on hazardous mycotoxin formation or the production of allergenic spores from yeast.

The most prominent microorganisms regarding production of natural PEA are yeasts. In this study, we have isolated PEA with proper identification from *P. anomala* SKM-T fermentation.

Cultivation. *Pichia anomala* SKM-T was isolated and identified by our research team [Mo *et al.*, 2004]. This strain was cultured on potato dextrose broth (PDB, Difco, Detroit, MI) in 5 *l*-Erlenmeyer flask. Initial pH of medium was adjusted with 0.1 N HCl to pH 4.5. Cultivation was conducted at 30°C and with agitation speed of 200 rpm for 24 h.

Extraction and isolation of PEA. The cultured broth (2 *l*) was centrifuged for 30 min at 3,000 rpm. The supernatant was extracted with 200 m*l* solvent (dichloromethane : pentane = 2 : 1, v/v) for five times, then solvent phase was evaporated with rotary vacuum evaporator (N-1000S-W, Eyela, Tokyo, Japan) and nitrogen gas.

The concentrated fraction was separated on thin layer chromatography (TLC, Silicagel 60, Art 5721, Merck, Darmstadt, Germany) and silica gel column (Kieselge 60, 70~230 mesh, Merck, Darmstadt, Germany) and eluted with a solvent (hexane : ethyl acetate : water = 12 : 8 : 1, v/v). The purified extracts were used for analytical procedure.

Analytical conditions. The analysis of PEA was performed on an Aglient 6890 N gas chromatograph equipped with flame ionization detector and a capillary column (HP-5, 30 m × 0.320 mm, thickness 0.25 μm). The injector and detector were held at 250°C and the oven temperature was increased as follows: 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.

Electron impact-mass spectrometry (EI-MS) was performed using a mass spectrometer (HP6890 GC, 5973 MSD, Hewlett Packard) under the same GC analytical condition. Spectra were obtained at 70 eV, ion species were normal ion (MF-Linear) and 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.

A UV absorption spectrum of the purified isolate in ethanol was recorded on a spectrophotometer (DU 650, Beckman, USA) at the range of 200 to 600 nm. FT-IR spectrum was taken on a FT-IR spectrometer (Bio-Rad,

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Cambridge, USA) as KBr discs and the absorbance frequency was expressed in cm^{-1}. The PEA fraction was dissolved in $CDCl_3$ (Sigma), and ^{1}H-NMR spectrum was recorded on an FT-NMR spectrometer (JNM-AL400, JEOL, Akishima, Japan) operated at 400 MHz.

As the authors reported before [Mo *et al.*, 2003], PEA was a main volatile flavor compound in *P. anomala* SKM-T. Thereby, in order to isolate the PEA among volatiles produced from the tested strain, cultured broth was extracted and performed TLC. As a consequence of open column chromatography, we obtained several fractions. To examine the isolation of PEA, separated fractions were performed GC-MS, and one fraction showed desirable molecular weight (MW 122; Fig. 1). Therefore, to confirm the isolation of PEA, UV absorption was observed at 200~300 nm for the separated compound with the maximum absorption shown at 259 nm (Fig. 2). Hence, we assumed that separated compound has an aromatic ring. Figure 3 shows the FT-IR spectrum of the separated compound. FT-IR spectrum revealed the presence of 3322 cm^{-1} (OH), 2944~2876 cm^{-1} (C-H stretching), 1810~1940 cm^{-1} (w, combination), 1495~1603 cm^{-1} (aromatic), 1045 cm^{-1} (CO), and 698~747 cm^{-1} (s,

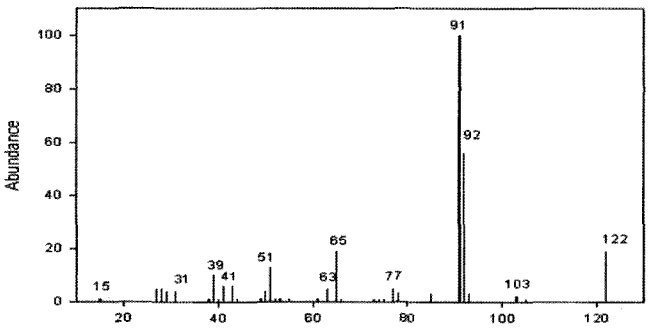

Fig. 1. EI-MS spectrum of the isolated compound from *Pichia anomala* SKM-T. Mass spectra were obtained by electron impact ionization at 70 eV. The ion species were of a normal ion (MF-Linear) and the TIC range was 0 m/z to 600.

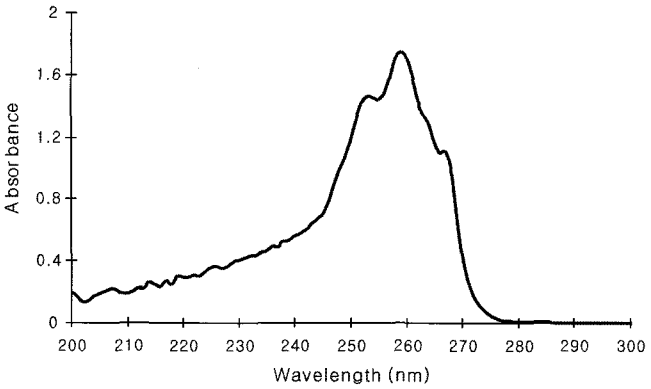

Fig. 2. UV spectrum of the isolated compound from *Pichia anomala* SKM-T.

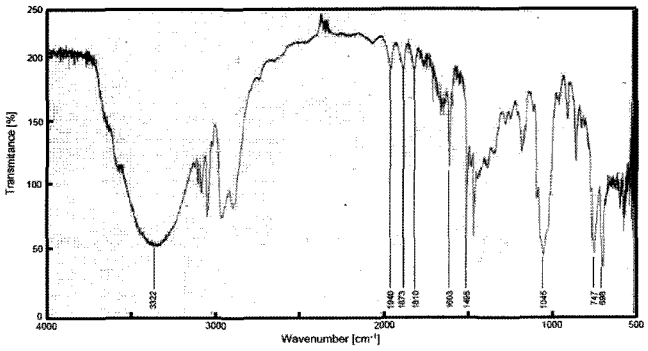

Fig. 3. FT-IR spectrum of the isolated compound from *Pichia anomala* SKM-T.

monosubstituted). Therefore, we considered that the separated compound has a hydroxyl group in its structure. In order to obtain high familiarity for structure elucidation, ^{1}H-NMR analysis was performed. ^{1}H-NMR spectrum of separated compound, in which mono-substitute phenol ring was verified from the 5 proton signals between δ7.19 and δ7.29. In addition, a hydroxyl group was observed at δ3.80 (2H, t, H-8) and a proton adjacent aromatic ring was monitored at δ2.82 (2H, t, H-7).

Consequently, the isolated compound from *P. anomala* SKM-T was identified as a phenylethyl alcohol based on the experimental results described above. This study confirmed that *P. anomala* SKM-T is PEA producing strain with PEA isolation and/or identification. With regard to an industrial process and/or economic aspects, further studies are needed to establish the optimum culture condition of PEA production and *in situ* product recovery technique.

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