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# Aroma Compounds Produced by the Yeast *Hansenula saturnus* var. *saturnus* Isolated from Soil

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**Abstract**

A yeast strain producing fruity-floral aroma was isolated from soil and identified as *Hansenula saturnus* var. *saturnus*. Glucose was found to be the best carbon source and sodium nitrate or phenylalanine as nitrogen source in terms of the nature and the intensity of the aroma produced by the isolated yeast. Seventeen compounds, mainly esters and alcohols, were identified in the ether-pentane extract of the culture broth by gas chromatography and/or coupled gas chromatography-mass spectrometry. Ethyl alcohol, isobutyl alcohol, isoamyl alcohol, phenethyl alcohol and their acetate esters together with ethyl caprylate were the major compounds in the aroma concentrate. Three unusual compounds, dibutyl disulfide, 3-methyl pentanoic acid and methyl pentanoate were also tentatively identified in the culture broth of the isolated yeast.

Key words: *Hansenula saturnus* var. *saturnus*, aroma, flavor, identification of volatiles

## Introduction

There has been a growing interest in the production of flavors or flavor compounds by using microorganisms since they are considered as natural products deserving GRAS status[1]. A wide variety of microorganisms are reported to produce interesting volatile flavor compounds such as esters, terpenes, lactones and pyrazines[2-4].

Kiuchi et al.[5] screened some *Saccharomyces* spp. and *Hansenula* spp. for their ability to produce strong flower and/or fruit-like odor. Koizumi et al.[6] tested a lot of tree exudate yeasts for aroma production, and reported *Geotrichum* spp., *Candida* spp. and *Hansenula* spp. as very fragrant aroma producers. Recently *Geotrichum* spp. has attracted attention in the production of esters[7] and apple aroma[8].

In this study, as a part of screening microorganisms which can produce valuable volatiles, we isolated and identified a yeast strain producing agreeable aroma and tested the effect of medium composition on the aroma formation. We also identified the volatile compounds produced by the isolated yeast which impart flower or fruit-like odor

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## Materials and Methods

### Sources for strain isolation

Sixty-five soil samples were collected from various mountains, seashores, flower gardens and farms in Korea. The soil samples were stored at 4°C until needed.

### Isolation and identification of yeast strain

A 5g of the soil sample was suspended in 45m*l* of distilled water and agitated for about 5min. After serial dilution, cultivation was carried out on YM agar containing 0.1% chloramphenicol at 25°C for 3 days. Single colonies were picked up and cultured in a liquid medium used by Hubball et al.[9] and the headspace vapors of culture broths were organoleptically tested to select interesting strains which produce agreeable aromas. A yeast strain producing the most strong and good aroma was selected for further experiments.

The identification of the selected yeast was carried out on its morphological, cultural and

physiological characteristics described by Kreger -Van Rij[10].

### Media and culture condition

The medium used by Hubball et. al.[9] was modified by replacing $Ca(NO_3)_2 \cdot 4H_2O$ and $Fe(NO_3)_3 \cdot 9H_2O$ with $CaCl_2 \cdot 2H_2O$ and $FeCl_3 \cdot 6H_2O$, respectively, and used as basal medium for the production of volatile compounds. Various carbon and nitrogen sources were added to the basal medium in quantities supplying carbon equivalent to 3% dextrose and nitrogen equivalent to 0.5% urea.

Cultivation was started on a reciprocal shaker at 30°C by inoculating 100m*l* of the medium in a 500m*l* Erlenmyer flask with 1m*l* of precultured cell and continued for 3 days.

### Characterization and identification of aroma compounds

The aromas of the culture broths were characterized by organoleptic test and the aroma compounds were identified by GC and GC-MS as previously described[11].

## Results and Discussion

### Identification of the isolated yeast

The morphological, cultural and physiological characteristics of the isolated yeast are shown in Table 1. The cell was oval shape and reproduced by multilateral budding. The strain was able to assimilate potassium nitrate and formed pseudo hyphae and endo spore.

These results indicate that the strain could be classified as genus *Hansenula*. In addition, the strain was able to ferment glucose and sucrose, but could not assimilate maltose, and did not require vitamin as growth factor. From these results, the strain was identified as *Hansenula saturnus* var. *saturnus*.

Table 1. Morphological, cultural and physiological characteristics of the isolated yeast

| | | |
|---|---|---|
| Cell shape | oval | |
| Cell size | 3.4-4.5x5.4-6.8$\mu$m | |
| Reproduction | multilateral budding | |
| Endo spore | present | |
| Pseudo hyphae | present | |
| Formation of sediment | moderate | |
| Formation of pellicle | wrinkled pellicle | |
| Fermentation | | |
| Glucose + | Galactose + | Raffinose + |
| Saccharose + | Lactose − | Maltose − |
| Assimilation | | |
| Galactose − | Raffinose + | Erythritol − |
| Saccharose + | Soluble starch − | Ribitol − |
| Maltose − | Xylose + (slow) | Mannitol + (slow) |
| Cellobiose + | Arabinose − | Succinic acid + |
| Trehalose − | Ribose − | Citric acid+(slow) |
| Lactose − | Rhamnose+(slow) | Inositol − |
| Sorbose − | Inulin + | Lactic acid + |
| Melibiose − | Melezitose − | Salicin + |
| Assimilation of $KNO_3$ | + | |
| Vitamin requirement | − | |
| Acid production | + | |
| Ester formation | + | |
| Splitting of arbutin | + | |

### Effect of carbon and nitrogen sources on the aroma production

Of the factors influencing the production of volatile compounds by microorganisms, the composition of cultivation media such as carbon and nitrogen sources was first attempted.

When the carbon source was altered, the aromas of the culture broths were changed as shown in Table 2. Dextrose, fructose and sucrose were found to be good sources for the formation of aroma based on its nature and intensity. The analysis of headspace gas of the culture broths showed the presence of ethyl acetate, isobutyl acetate and isoamyl acetate as major components. But the cultures containing raffinose and rhamnose produced ammonia odor and showed slow growth compared to the above cultures. And these cultures didn't give any detectable peaks in the gas chromatogram obtained.

These findings indicate that the floral or fruity odor of the cultures is mainly due to the presence of the volatile esters of higher alcohols.

Table 2. Effect of carbon sources on growth and headspace odor of *H. saturnus*

| Carbon source | Growth* | Nature of odor | Intensity of odor** | Major components in headspace gas |
|---|---|---|---|---|
| Dextrose | 1.77 | fruity-floral | +++ | ethyl acetate<br>isobutyl acetate<br>isoamyl acetate |
| Fructose | 2.14 | floral | ++ | ethyl acetate<br>isoamyl acetate |
| Raffinose | 1.27 | ammonia | + | not detected |
| Sucrose | 2.10 | floral | ++ | ethyl acetate<br>isoamyl acetate |
| Rhamnose | 0.02 | ammonia | + | not detected |

Carbon equivalent to 3% dextrose
Nitrogen source; $NaNO_3$

* Cell numbers by haemacytometer($\times 10^8$ per ml)
** +++, very strong; ++, strong; +, weak

The variation of nitrogen source also affected the quality and intensity of the produced aroma(Table 3). Inorganic nitrogen sources such as sodium nitrate and ammonium chloride gave rise to a strong floral odor, which is contrary to the results with *Hansenula anomala*[11] where these nitrogen sources yielded yeast and ammonia odor, respectively. The influence of amino acids such as leucine, isoleucine, aspartic acid, glycine and phenylalanine were also tested as nitrogen source since these amino acids are known to easily converted into some higher alcohols important for aroma.

As the culture with phenylalanine produced

Table 3. Effect of nitrogen sources on growth and headspace odor of *H. saturnus*

| Nitrogen source | Growth* | Nature of odor | Intensity of odor** | Major components in headspace gas |
|---|---|---|---|---|
| $(NH_4)_2SO_4$ | 1.43 | esteric | ++ | ethyl acetate<br>ethyl alcohol |
| $NaNO_3$ | 1.65 | fruity-floral | +++ | ethyl acetate<br>isobutyl acetate<br>isoamyl acetate |
| $NH_4Cl$ | 0.66 | floral | ++ | ethyl acetate<br>isobutyl acetate |
| Urea | 3.63 | yeast odor | + | not detected |
| Casamino acid | 2.53 | yeast odor | - | not detected |
| Leucine | 0.73 | esteric | +++ | ethyl acetate<br>ethyl alcohol<br>isobutyl acetate<br>isoamyl acetate |
| Isoleucine | 0.26 | fusel oil | ++ | ethyl acetate<br>active amyl acetate<br>active amyl alcohol |
| Aspartic acid | 0.23 | fruity-floral | + | ethyl acetate<br>isobutyl acetate<br>isoamyl acetate |
| Glycine | 2.08 | acetone, ether | +++ | ethyl acetate |
| Phenylalanine | 0.41 | fruity-floral | +++ | ethy alcohol<br>isobutyl alcohol |

Nitrogen equivalent to 0.1% urea
Carbon source; dextrose

* Cell numbers by haemacytometer($\times 10^8$ per ml)
** +++, very strong; ++, strong; +, weak

most intense fruity-floral aroma among the amino acids used, it was selected as nitrogen source for further experiments as well as sodium nitrate.

### Identification of volatile compounds

Two cultures grown on dextrose-sodium nitrate and dextrose-phenylalanine media were chosen to investigate the compounds responsible for their aromas because they give more agreeable and intense odors than those of other cultures. The ether-pentane extracts of these cultures contained a number of compounds as shown in Fig. 1. and Table 4. Seventeen compounds; nine esters, six alcohols, one acid and one sulfur compound were tentatively or positively identified in the aroma concentrate of the dextrose-sodium nitrate culture. Ethyl alcohol, propyl alcohol, isobutyl alcohol, isoamyl alcohol, phenethyl alcohol and their acetate esters were found to be major constituents in the volatile fraction of the culture broth. All these alcohols and esters are frequently found both in the culture of the yeast *Hansenula anomala* and in that of the mold *Geotrichum penicillatum*. The

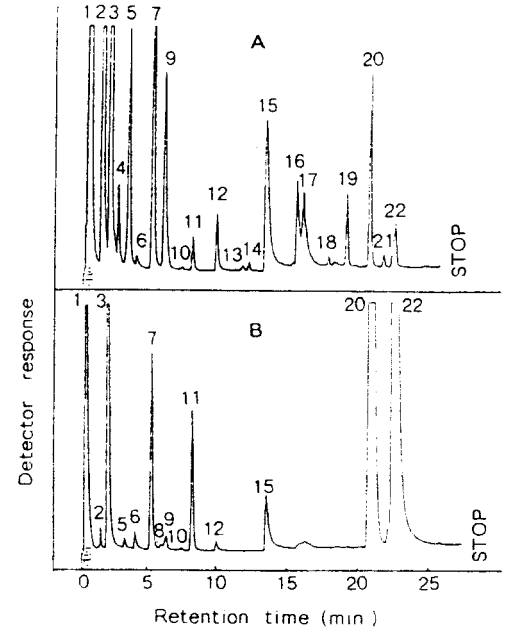


Fig. 1. Gas chromatograms of the ether-pentane(2:1) extracts from cultures of *H. saturnus* grown on dextrose-sodium nitrate(A) and dextrose-phenylalanine(B) medium.

Table 4. Composition of the aroma constituents produced by *H. saturnus*

| Peak No. (Fig. 1) | Compound | Means of * identification | Composition (%)** | |
|---|---|---|---|---|
| | | | A | B. |
| 1 | Solvent | | | |
| 2 | Ethyl acetate | GC | 6.03 | 0.08 |
| 3 | Ethyl alcohol | GC:GC-MS | 48.25 | 4.35 |
| 4 | Propyl acetate | GC:GC-MS | 1.01 | - |
| 5 | Isobutyl acetate | GC:GC-MS | 6.02 | 0.08 |
| 6 | Propyl alcohol | GC:GC-MS | 0.30 | 0.12 |
| 7 | Isobutyl alcohol | GC:GC-MS | 12.45 | 1.08 |
| 8 | Unknown | | | |
| 9 | Isoamyl acetate | GC:GC-MS | 7.49 | 0.08 |
| 10 | Unknown | | | |
| 11 | Isoamyl alcohol | GC:GC-MS | 1.15 | 0.73 |
| 12 | Dibutyl disulfide | GC-MS | 1.61 | 0.07 |
| 13 | Hexanol | GC | 0.11 | - |
| 14 | Unknown | | | |
| 15 | Ethyl caprylate | GC | 3.51 | 0.38 |
| 16 | 3-methyl pentanoic acid | GC-MS | 1.53 | - |
| 17 | Unknown | | | |
| 18 | Ethyl caprate | GC:GC-MS | 0.41 | - |
| 19 | Methyl pentanoate | GC-MS | 1.28 | - |
| 20 | Phenethyl acetate | GC:GC-MS | 2.95 | 39.38 |
| 21 | Ethyl laurate | GC | 0.19 | - |
| 22 | Phenethyl alcohol | GC:GC-MS | 1.09 | 53.30 |

* GC=Gas chromatography; GC-MS=Gas chromatography/Mass spectrometry.

** A,B: Same as described in Fig. 1.

higher alcohols might be formed before esters via Ehrlich pathway and/or synthetic pathway as known in *Saccharomyces cerevisiae*[12].

However, the acetate esters are thought to be synthesized by the catalytic action of alcohol acetyltransferase from the corresponding alcohols and acetyl-CoA[13] or by the reverse reaction of esterase from the corresponding alcohols and acetic acid[14]. In addition to these compounds, three unusual compounds in yeast or mold volatiles were found in the culture extract and tentatively identified as dibutyl disulfide, 3-methyl pentanoic acid and methyl pentanoate. Of these compounds, it is possible that methyl pentanoate does some role in the aroma of the culture because it is found as a volatile compound of fruit or flower[15]. However, it is doubtful that dibutyl disulfide, which was reported as volatile compound of beef[16], and 3-methyl pentanoic acid play any positive role in the fruity or floral odor.

Fig. 1 and Table 4 also show that the culture using phenylalanine as nitrogen source produced two dominant compounds, phenethyl acetate(39.4%) and phenethyl alcohol(57.3%) in its volatile. Similar result was observed in the culture of *Saccharomyces cerevisiae* by authors[17].

Phenethyl alcohol is known to have an odor of rose and to be originated from phenylalanine through Ehrlich pathway. Phenethyl acetate might be formed either from phenethyl alcohol and acetyl-CoA or from phenethyl alcohol and acetic acid according to the mechanisms described above for ester formation.

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# 토양에서 분리한 *Hansenula saturnus* var. *saturnus*에 의한 휘발성 방향성분의 생성

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미생물에 의한 방향성 화합물의 생성을 목적으로 토양으로부터 꽃향 또는 과일향의 생성능력이 강한 효모를 분리하여 이화학적 특성을 조사한 결과 *Hansenula saturnus* var. *saturnus*로 동정되었다. 방향 생성에 영향을 미치는 배지조성을 검토한 결과 탄소원으로는 포도당이 질소원으로는 질산 나트륨과 페닐알라닌이 가장 좋은 방향을 생산하는 것으로 나타났다. 배양액의 농축물로 부터 GC 또는 GC-MS를 이용하여 17개의 화합물을 분리, 동정하였으며 이중 ethyl alcohol, isobutyl alcohol, isoamyl alcohol, phenethyl alcohol과 이들의 acetate 에스터 및 ethyl caprylate가 주된 성분으로 밝혀졌다. 또한 미생물에 의한 생성물로 보고된 바가 없는 것으로 조사된 dibutyl disulfide, 3-methyl pentanoic acid 및 methyl pentanoate가 분리, 동정되었다.