

# The Development of Aroma Components by Microbial Fermentation

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There have been remarkable developments in the fermentation industry, with cell fusion and gene engineering being put to practical usage in medicine and plant breeding. For the production of aromatic substances, good flavorful products are being developed, for example, by way of fusion of the cells of wine yeasts and the utilizing of sake (rice)—wine yeasts. Simultaneously, many studies are being conducted on the mechanisms, per se, through which aromas are formed.

On the other hand, as may be seen in the amino acid industry, microorganisms are used in the production of kiral products. It is now believed that the time has come for such techniques to be used in the production of aroma providing products. There have been many general reviews<sup>1-3</sup> on the formation of aroma products, but we will introduce the most recent research trends here. Many microorganisms are capable of absorbing and decomposing ammonia and hydrogen within themselves, and are utilized as deodorizing agents.<sup>4</sup>

## Available Microorganisms of the Food Industry

Even before knowledge of microorganisms existed, we had been separating, breeding, and utilizing microorganisms for the making of liquors and cheeses from the natural world. These microorganisms are being used not only by the brewing and the food industry to produce alcohol and lactic acid, but also to produce secondary metabolite aroma substances for application of their special characteristic flavors to products.

Although the yeasts used in bread, wine and sake are taxonomically of the same strain as *Sacc. cerevisiae*, their characteristics differ and make it impossible, for example, to make a good sake wine using bread yeast or vice versa. Whereas liquors are made using yeasts, by transformation of the saccharides of cereals and fruits to ethyl alcohol, various kinds of aromatic flavor substances such as higher alcohols, esters, aldehydes, etc., are created simultaneously.

Each one gives their unique characteristics to the finished liquors.

Whereas at the enzyme level the producing mechanisms of these aroma components are being studied, it has been reported that the quantity of  $\beta$ -phenyl acetate, ethyl caproate and isoamyl caproate increases when utilizing a yeast cultivated under conditions allowing a high level of isoamyl acetate formation.<sup>5</sup> Leucine, one of the amino acids converted to isoamyl alcohol by yeast, produces, by isomerizing yeast and obtaining resistant mutant leucine analogs,<sup>6</sup> three to four times more than the parent strain of isoamyl alcohol and isoamyl acetate.

From the same point of view, if yeast is made with lessened take up of arginine, three to four times more isoamyl acetate and  $\beta$ -phenyl acetate<sup>7</sup> than the parent may be obtained. A patent showing how to obtain much ethyl leucinate, an important component of Japanese sake flavor, by using a koji mould of high leucine acid producing capacity is also known.<sup>8</sup>

Methyl ketones are well known as key components of blue cheese aroma, and are made by the process of fat  $\rightarrow$  fatty acid  $\rightarrow$  methyl ketone by *Penicillium blue* mould. There are many reports and patents, now already in the industrial production<sup>3</sup> on methyl ketones for natural flavor use.

Fatty acids, esters, and lactones are important as flavor providing components of milk and cheese. Various lipases which are made by using moulds, yeasts and bacteria, can hydrolyze the fat, by reacting on milk, butter and cheeses. This results in fatty acids, and further produces esters as lactones are produced by ring formation of hydroxy fatty acids. By a delicate balancing of these aroma components, the characteristic aromas of milk and cheese are developed and used in large quantities in frozen products such as ice creams and confectionery products.

The lipases are produced by various microorganisms with their characters differing much according to their origins, and even though reacting on the same cream, the result differs by reason of positional

features of hydrolysis and the chain length of fatty acids<sup>9,10</sup> (Table I). Consequently, it is by making selective usage of various lipases most fitting to the purpose involved, and selecting the reactive conditions, that the quantitative ratio of fatty acids is presently being controlled. Furthermore, there is also a patent regarding combining lactic acid bacteria and inserting a bioreactor.<sup>11</sup>

Diacetyl and acetoin are found extensively in dairy foods, and are very important constituents of butter, sour cream and cheese. They are known to be produced from the citric acid in milk by lactic acid bacteria such as *St. diacetylactis*, *Leu. citrovorum*, etc. as a starter.<sup>9,10</sup>

By studying the enzyme level, control of the pH, and the addition of piruvic acid, the acetoin production is increased to 7 g/l.<sup>12</sup> There is a patent on increasing the diacetyl output by adding copper ions.<sup>13</sup> Since the volatility of diacetyl is high, there is much loss in heating, and a method is used to accumulate the  $\alpha$ -acetolactic acid which is a precursor of diacetyl.<sup>14</sup>

## Screening and Usage of Aromatic Substance Producing Microorganisms

Though each microorganism used in the food industry provides characteristic aromas, the volume produced by many is too small. Consequently, much study of microorganisms is being done for the aroma producing capabilities of materials from such natural sources as fruits, saps and soils.<sup>15</sup>

For microorganisms to produce aroma providing substances, one route is to produce ethyl acetate, a secondary metabolite, through several steps from saccharides using *Hansenula* yeast. Another is to convert precursors into aroma components by oxidation, reduction and degradation.

The former procedure features production of many kinds . . . with the cultured malt broth of *Fusarium* sp. mould with a strong fruity odor and with many lac-

tones, such as  $\gamma$ -decalactone, etc.<sup>16</sup> When cultivating *Neurospora* sp., the predominant components found were ethyl hexanoate and 3-methyl-1-butanol.<sup>17</sup> The patents for fruity aroma production are concerned with *Moniliella acetoabutans*<sup>18</sup> and *Monilia frukuticola*.<sup>19</sup>

Recently, there are numerous reports on cultured products of basidiomycetes fungi. These include vari-

**Table II. Production of aroma compounds by basidiomycetes fungi liquid cultures<sup>20-25</sup>**

Strain	Product	Reference
<i>Bjerkandera adusta</i>	3,4-Dimethoxybenzyl alcohol 3,4-Dimethoxybenzaldehyde 4-Methoxybenzaldehyde 5-Hexanolide	20
<i>Poria aurea</i>	2-Octen-4-olide 2-Methyl-1-propanol 3-Methyl-1-butanol 3-Phenyl propanol	20
<i>Tyromyces sambucens</i>	Benzyl alcohol, Benzaldehyde 4-Decanolide	20
<i>Polyporus durus</i>	3-Methyl-1-butanol 2-Octen-4-olide, 4-Octanolide 6-Octen-4-olide, 4-Hexanolide	21
<i>Pleurotus euosmus</i>	Coumarin, Linalool cis-Linalooloxide	22
<i>Ischnoderma benzolium</i>	Benzaldehyde, 2-Octen-4-olide 4-Methoxy benzaldehyde 3-Phenyl propanol	23
<i>Agaricus bisporus</i>	Tetrachloro-1,4-dimethoxy benzene 2,4-Nonadienal 2,4-Decadienal	24
<i>Agaricus Subrufecens</i>	Benzyl alcohol, Benzaldehyde Methyl benzoate 1-Octen-3-ol	25

**Table I. The profile of free fatty acids treated by various kinds of lipases<sup>9</sup>**

Fatty acid	Untreated	Calf pregastric esterase	Hog pancreatic lipase	<i>Candida cylindracea</i> lipase	<i>Alcaligenes</i> lipase 266	<i>Alcaligenes</i> lipase 679	<i>Rhizopus delamar</i> lipase	<i>Phycomyces nitens</i> lipase
C <sub>4</sub>	8.26	44.00	14.78	4.84	3.46	8.70	5.25	7.27
C <sub>6</sub>	3.59	13.03	6.19	3.19	1.60	3.43	3.96	2.89
C <sub>8</sub>	1.82	3.62	2.90	2.52	1.29	1.57	2.15	2.33
C <sub>10</sub>	3.77	6.75	4.22	5.14	2.41	2.51	2.76	2.98
C <sub>12</sub>	4.57	6.69	3.42	5.07	2.31	3.15	3.17	3.28
C <sub>14</sub>	9.96	8.38	7.59	9.85	8.75	7.47	8.14	8.94
C <sub>16</sub>	27.24	8.00	23.80	27.14	41.67	35.55	29.49	30.10
C <sub>18</sub>	8.29	0.89	8.22	4.33	10.62	7.52	10.97	10.51
C <sub>18:1</sub>	27.46	6.64	23.81	31.99	22.49	24.79	29.11	27.09
C <sub>18:2</sub>	4.81	1.60	4.89	5.93	5.40	5.31	5.00	4.01
Hydrolysis rate (%)	0	13.7	13.7	14.6	14.9	13.7	15.2	14.3

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ous kinds of volatile substances, such as phenols, alcohols, monoterpenes and sesquiterpenes, etc., listed in Table II and Figures 1 to 4.<sup>20-26</sup> As fungi are higher class microorganisms close to plants, their relation with the biosynthesis of volatile components of plant organisms is a matter of much interest. As to patents, applications for such mushroom aroma producing methods as 1-Phenyl-3-pentanone by *Mycoliptodanoides*,<sup>27</sup> mushroom<sup>28</sup> and 1-octen-3-ol by *Morchella* sp.<sup>29</sup> have been submitted.

### The Conversion of Aroma Precursors by Microorganisms

In the 1960s, by way of efficiently utilizing petroleum, many studies were done on petroleum based proteins to cope with the coming world food crisis. Consequently, with the renewed recognition of metabolizing various compounds by microorganisms, reports appeared that bacteria resistant compounds such as plant essential oils could be degraded, as well, by certain special microorganisms. One accomplishment has been that presently large quantities of

brassylic acid, the raw material for synthetic musk, is being produced by yeast from n-paraffin.

An attempt to convert aroma substances to more valuable matter was realized in the production of tobacco flavors made from  $\beta$ -ionone, with similar studies progressing on  $\alpha$ -Ionone, etc., as well. As shown in

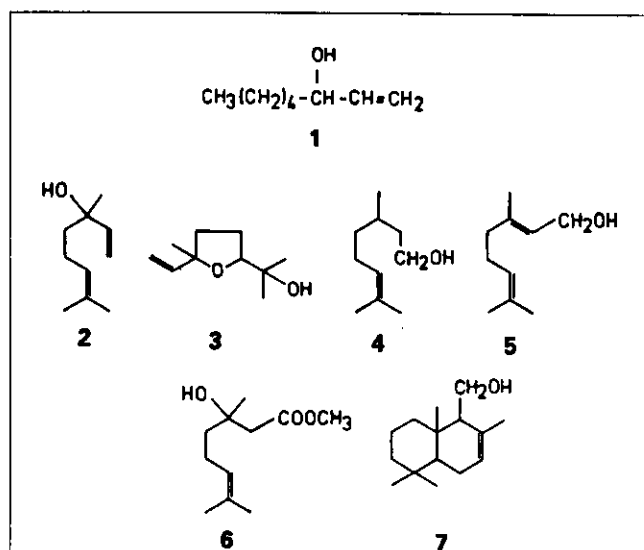


Figure 1. Volatile metabolites from liquid cultures of *Gloeophyllum odoratum* CBS 444.61 grown on glucose-asparagine-mineral salt medium (for culture conditions, see Experimental section)<sup>26</sup>

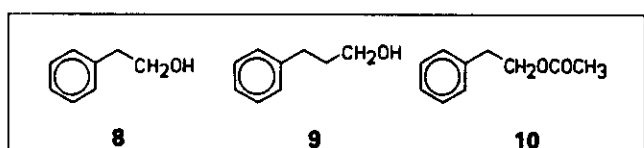


Figure 2. Volatile metabolites with aromatic structure from liquid cultures of *Gloeophyllum odoratum* CBS 444.61 grown on glucose-phenylalanine-mineral salt medium (for culture conditions, see Experimental section)<sup>26</sup>

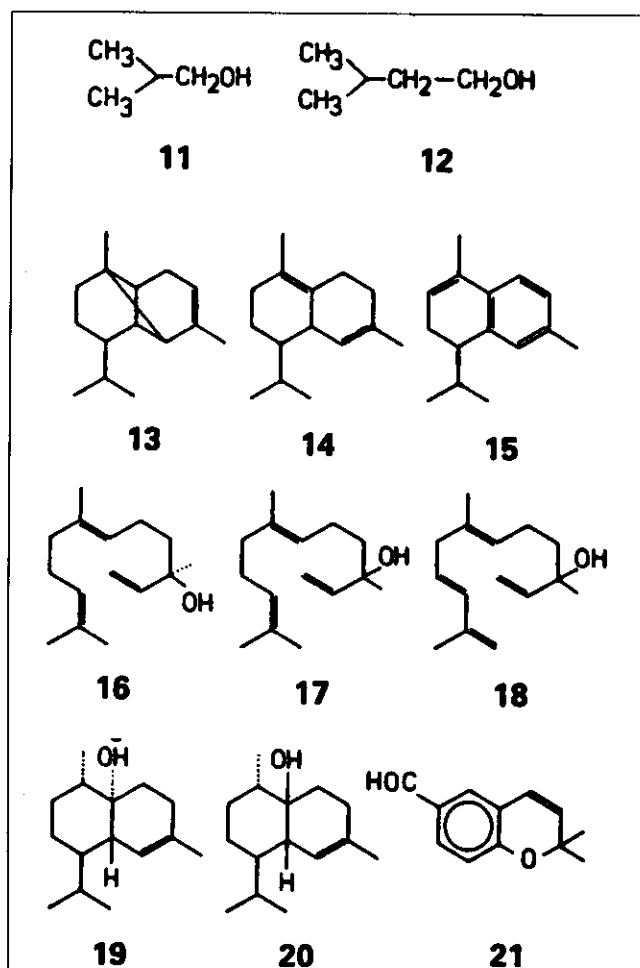


Figure 3. Volatile metabolites from liquid cultures of *Lentinellus cochleatus* CBS 201.47 grown on glucose-asparagine-malt extract-mineral salt medium (for culture conditions, see Experimental section)<sup>26</sup>

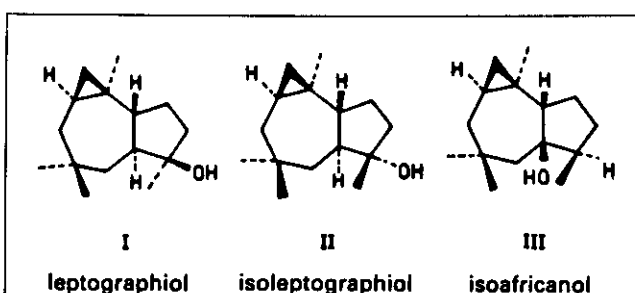


Figure 4. Africanols from *Leptographium lundbergii* Ha 2/82: leptographiol (I), isoleptographiol (II), and isoafrikanol (III)<sup>26</sup>

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Figure 5,  $\alpha$ -Ionone, after hydroxidation by *A. niger*, a black kouji mould,<sup>30</sup> was dehydrated and converted to products such as ketones. As an important tobacco flavor, there is also Ambroxide. There is also a patent submission on production of an intermediate of abroxide, by hydroxidation of raldane compounds by *Hypozyrna roseonige*.<sup>31</sup>

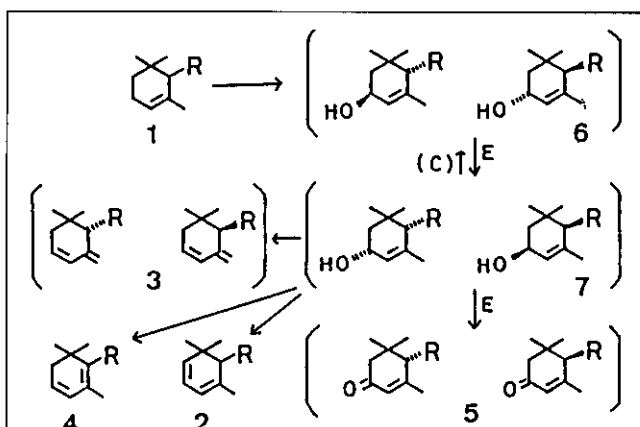
In addition, other than the aforementioned, as examples of utilizing oxidation-reduction of microorganisms, there is the reaction of reducing acids to

alcohols by *Cl. acetobutylicum*<sup>32</sup> and *Groeosporum orivarum*.<sup>33</sup> As to *Cl. thermosaccharolyticum*, as indicated in Table III, reactions whereby alcohol  $\rightarrow$  aldehyde and ketone oxidation or reverse reactions occur.<sup>34</sup> As for *Gluconobacter roseus*, the isobutanol in fusel oil is oxidized to isobutyric acid,<sup>35</sup> whereas, secondarily alcohol is oxidized to ketones by *Corynebacterium*.<sup>36</sup> There are reports of processes including production of photoactivated phenylmethoxymethoxyxylene by epoxide producing bacteria such as *Arthrobacter*,<sup>37</sup> the conversion of castor oil to  $\gamma$ -decalactone by *Sporoboromyces odoros* and others<sup>38</sup> and production of (R) butyrolactone- $\gamma$ -propionic acid ester by *Candida* yeast.<sup>39</sup>

There are, as well, reports on conversion of 1-methone to 1-menthol by *Cellulomonas bacteria*<sup>40</sup> and reduction of 2,2,6-trimethyl-1,4-cyclohexadione by *Bacillus bacteria*<sup>41</sup> and, as shown in Figure 6, oxidative decomposition of stilbene, a component of the inner bark of *P. Glehni* (pine tree), by *Pseudomonas bacteria* to vanillin<sup>42</sup> and production of ramnose, a raw material of furaneol, by *Acinetobacter bacteria*,<sup>43</sup> along with patent submissions on synthesizing lipase reactions on lactones<sup>44</sup> and terpene alcohol esters.<sup>45</sup>

### Summary

Though there have been many reports on the prospective use of microorganisms for the production of aroma providing substances, industrial production has actually been attained on but a small number . . . such as the above mentioned milk flavor, brassylic acid as a raw material for musk aroma, and natural esters, etc., used for tobacco flavor and food flavors.



**Figure 5. Proposed pathway of conversion of  $\alpha$ -ionone,  $\alpha$ -methylionone, and  $\alpha$ -isomethylionone by *A. niger* JTS 191. The number of each structural formula corresponds to the compound numbers in Tables I, II, and III.**

For  $\alpha$ -ionone, R is  $-\text{CH}=\text{CH}-\text{CO}-\text{CH}_3$ ;

for  $\alpha$ -methylionone,

R is  $-\text{CH}=\text{CH}-\text{CH}_2-\text{CH}_3$ ;

for  $\alpha$ -isomethylionone,

R is  $-\text{CH}=\text{C}(\text{CH}_3)-\text{CO}-\text{CH}_3$ .<sup>30</sup>

**Table III. Oxidation-reduction by *Cl. thermosaccharolyticum* (strain = 86EA-1)<sup>34</sup>**

Exam. No.	Substrate	Product	Yield (mg/ml/12 hr)	Optical purity (%)
1	2-butanol	methylethylketone	0.30	
2	ethanol	acetaldehyde	0.01	
3	propanol	propionaldehyde	0.14	
4	butanol	butylaldehyde	0.10	
5	2-propanol	acetone	0.25	
6	1,2-propanediol	acetol	0.22	
7	1,3-butanediol	4-hydroxy-2-butanone	0.10	
8	cyclohexanol	cyclohexanone	0.10	
9	acetaldehyde	ethanol	0.46	
10	methylpropylketone	sec-amylalcohol	0.47	> 80 cc, R
11	cyclopentanone	cyclopentanol	0.13	
12	cyclohexanone	cyclohexanol	0.55	
13	2-methylcyclohexanone	2-methylcyclohexanol	0.47	
14	3-methylcyclohexanone	3-methylcyclohexanol	0.42	
15	acetone	2-propanol	0.98	
16	propionaldehyde	propylalcohol	0.81	
17	methylethylketone	2-butanol	0.96	> 80 cc, R

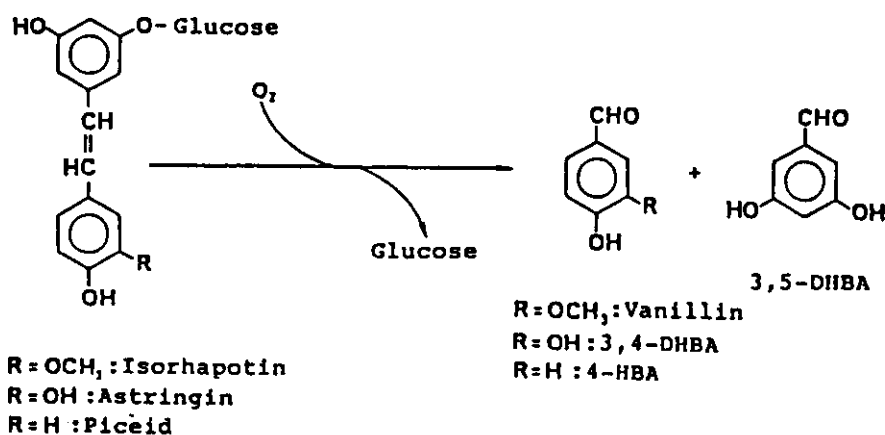


Figure 6. Enzymatic conversion of stilbenes<sup>42</sup>

To date, with regard to production via fermentation methods, since the concentration of many of the substances produced has been low, the costs in separation and purifying has been too high, constituting a barrier to actual application.

However, with progress in bioreactors, reacting of organic solvents, sensors and computer-assisted cultivation control technology, many actual industrial applications in the future may be anticipated. Improvements in membrane separation techniques, absorbents and extractive methods will contribute tremendously in technical advances peripheral to fermentation.

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