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IDENTIFICATION OF FLAVOUR VOLATILE COMPOUNDS PRODUCED BY *KLUYVEROMYCES LACTIS*

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Summary

More than 30 compounds were identified in *Kluyveromyces lactis* culture, including 5 aromatic hydrocarbons, 9 alcohols, 6 carboxylic acids, 8 esters, 2 ketones and 1 fuanone. Most of them have not been previously reported in the culture of *K. lactis*. The predominant components are isoamyl alcohol, 2-phenylethanol, and acetoin (73, 72 and 22 mg/L broth, respectively). 2-Phenylethyl acetate, isobutanol, isobutyric and isovaleric acids were also detected in significant amounts.

Introduction

A growing demand for natural products is stimulating the development of biotechnological techniques in the manufacture of flavours and fragrances. Among the major techniques used, i.e. tissue/cell culture, microbial and enzymatic processes, microbial fermentation seems to be the most promising for flavour production (Janssens *et al.*, 1992). *Kluyveromyces lactis* is one of the few GRAS microorganisms with potential to be used in flavour production. In an early study, the monoterpenes citronellol, linalool and geraniol were identified among 150 compounds detected in the *K. lactis* culture broths (Drawert and Barton, 1978). However, other research workers could only find some short-chain alcohols/esters and 2-phenylethanol derivatives, but failed to detect any of the monoterpene compounds previously reported in the cultures of this organism (Hanssen *et al.*, 1984 and Lee *et al.*, 1991).

In this study, a number of carboxylic acids, ketones, furanone, aromatic hydrocarbons and more alcohols/esters were identified in the *K. lactis* cultures, in addition to those alcohols/esters reported by Hanssen *et al.* (1984) and Lee *et al.* (1991).

Materials and Methods

Microorganism culture - *Kluyveromyces lactis* CBS 5670 was purchased from Centralbureau voor Schimmelcultures (Baarn, The Netherlands) and inoculated into 250 ml flasks, each of which contained 50 ml of medium consisting of glucose (5%), yeast extract (0.25%) and vitamins (B_1 and H, each 4000 ppm). The inoculated media were cultured at 30°C, 160 rpm in a shaking incubator. The broth was analyzed for its volatile composition at the 5th day after inoculation.

Sample preparation - the broth was centrifuged at 10,000 rpm for 5 min and the supernatant (25 ml) was extracted with dichloromethane (2x25 ml) in a 250 ml separating funnel. The funnel was vigorously shaken for about 2 minutes and then placed onto a rack for the dichloromethane layer to separate from the aqueous layer. When the two layers were clearly separated, the dichloromethane (bottom) layer was carefully collected into a glass tube and subsequently concentrated to a final volume of 0.1 ml by flushing nitrogen gas.

GC-MS analysis - the concentrated sample was analyzed on a Unicam Automass 150 GC-MS system (Unicam, Argenteuil, France). The gas chromatographic conditions were as follows: an HP-20M capillary column (Carbowax 20M 50m x 0.2μm, Hewlett Packard, USA), column temperature from 60 to 190°C at a rate of 5°C/min, injector temperature 200°C. The mass spectrometric conditions were as follows: EI mode, ionization voltage 70 *eV*, interface temperature 195°C and source temperature 150°C.

Identification and quantification - positive identifications of unknown compounds were achieved through library searches on an NBS Mass Spectra Library. Final confirmations of peak identities were made by comparing spectral and retention data obtained from the sample and authentic chemicals. Quantification of the major components were made by using standard solutions.

Results and Discussion

More than 40 peaks were detected and 31 identified in the dichloromethane-extracted sample from *K. lactis* cultures. The identified compounds, together with their retention and spectral data, are summarized in Table 1. Majority of the compounds were identified by comparing both retention and spectral data obtained from the sample and authentic chemicals while 5 of them were positively identified by the close matches between unknown spectra and NBS Library spectra.

Most of the identified compounds have not been previously reported in *K. lactis* cultures. The results obtained in this study were partially in agreement with the published reports by Hanssen *et al.* (1984) and Lee *et al.* (1991), in which most of the major volatile components, i.e. isoamyl alcohol, isobutanol, 2-phenylethanol and 2-phenylethyl acetate, were found in *K. lactis* culture. Furthermore, as reported by the two research groups, this study also failed to detect any of the terpene compounds previously found in *K. lactis* culture by Drawert and Barton (1978). However, there were some major differences in both qualitative and quantitative data on the volatile composition between this study and previous studies. 2-Phenylethanol was detected as one of the predominant compounds in this study while its acetate was a predominant compound in the previous study (Hanssen *et al.*, 1984). Some compounds newly identified in this study, such as 3-hydroxy-2-butanone (acetoin), isobutyric acid and isovaleric acid, were also found in significant quantities (Table 2). Undoubtedly, these compounds should have contributed to the overall flavour of *K. lactis* cultures, due to their strong sensory activities and relatively large quantities. The different results from these research groups may have been largely caused by such factors as different media, incubation temperature/time and even extraction procedures applied by the different laboratories.

Isoamyl alcohol and 2-phenylethanol, previously identified in *K. lactis* culture by Hanssen *et al.* (1984) and Lee *et al.* (1991), were the predominant components, accounting for 37.13% and 38.04%, respectively, of the total volatile compounds extracted by dichloromethane. Following the two alcohols, acetoin and 2-phenylethyl acetate accounted for 9.26% and 6.28%, respectively, of the total volatiles, indicating important contributions of these compounds to the pleasant aroma of *K. lactis* cultures. Acetoin is an important flavour compound and extensive studies have been conducted to explore the possibility of producing this compound by bacterial fermentation, using such species as *Bacillus polymyxa* (Serebrennikov *et al.*, 1992), *Bacillus subtilis* (Dettwiler *et al.*, 1989), *Klebsiella oxytoca*

Table 1: Compounds identified in *Kluyveromyces lactis* culture

Name	CAS#	Retention** STDS	Retention** UNKN	MS data
<u>Hydrocarbons</u>				
methylbenzene	108-88-3	4.00	3.59	91(100), 92(60), 65(14), 63(10), 39(7)
ethylbenzene	100-41-4	5.33	5.33	91(100), 106(67), 77(24), 51(10)
1,3-dimethylbenzene	108-38-3	5.49	5.49	91(100), 106(52), 77(14), 65(9), 51(10)
1-ethyl-3-methylbenzene	620-14-4	7.39	7.40	105(100), 120(47), 91(14), 77(19)
naphthalene	91-20-3	20.32	20.29	128(100), 102(5), 51(3)
<u>Alcohols</u>				
2-butoxy-1-ethanol	111-76-2	12.07	12.08	57(100), 87(21), 75(9), 45(33), 41(27)
3-ethoxy-1-propanol	111-35-3	*,**	11.09	59(100), 31(88), 45(59), 71(50), 75(37), 86(13)
isobutanol*	78-83-1	4.46	4.45	43(100), 41(65), 42(63), 33(31), 31(29), 74(24)
3-methyl-3-buten-1-ol	763-32-6	8.12	8.16	56(100), 41(71), 68(95), 86(41), 53(33), 31(24)
2-methyl-2-buten-1-ol	4675-87-0	*,**	10.02	71(100), 86(24), 67(24), 53(30), 41(39), 43(16)
isoamyl alcohol*	123-51-3	7.14	7.18	55(100), 70(80), 42(71), 43(59), 41(60), 31(22)
phenylmethanol	100-51-6	*,**	23.32	79(100), 77(75), 107(70), 108(77), 91(21), 51(21)
2-phenylethanol*	60-12-8	24.18	24.17	91(100), 92(63), 122(39), 77(7), 65(22)
2,3-butanediol	513-85-9	15.32	15.33	45(100), 57(13), 43(13)
<u>Acids</u>				
acetic acid	64-19-7	13.06	13.08	43(100), 45(94), 60(83)
2-hydroxy-2-methylpropanoic acid	594-61-6	*,**	10.36	59(100), 43(74), 41(35), 31(33)
butyric acid	107-92-6	17.38	17.40	60(100), 73(36), 45(14), 42(18), 41(17), 39(10)
isobutyric acid	79-31-2	16.09	16.09	43(100), 73(67), 88(17), 55(9), 41(53), 39(17)
isovaleric acid	503-74-2	18.40	18.41	60(100), 74(67), 87(35) 41(46), 43(36), 45(25)
hexanoic acid	142-62-1	22.44	22.45	60(100), 73(48), 87(15), 41(20)
<u>Esters</u>				
isobutyl acetate*	110-19-0	3.33	3.34	43(100), 56(46), 73(35)
isoamyl acetate*	123-92-2	5.22	5.25	43(100), 70(77), 55(51), 61(15), 73(16), 87(12)
ethyl propionate	105-37-3	2.54	2.55	57(100), 45(11), 74(8), 102(7), 56(7)
2-phenylethyl acetate*	103-45-7	22.09	22.11	104(100), 43(40), 91(17), 78(9), 65(9)
2-phenylethyl isobutyrate*	103-48-0	23.28	23.39	104(100), 43(49), 91(9), 77(8), 71(15)
1,2-ethanediol monoacetate	542-59-6	17.53	17.51	43(100), 74(22), 87(8), 61(8)
1,1-ethanediol diacetate	542-10-9	11.16	11.30	43(100), 87(22)
1,2-ethanediol diacetate	111-55-7	15.41	15.42	43(100), 73(10), 86(24), 116(11)
<u>Ketones</u>				
3-hydroxy-2-butanone (acetoin)	513-86-0	9.10	9.11	45(100), 43(76), 88(29)
1-hydroxy-2-propanone (acetol)	116-09-6	9.28	9.30	43(100), 74(17), 31(12)
<u>Furanones</u>				
dihydro-4-methyl-2(3H)-furanone	1679-49-8	*,**	20.05	42(100), 41(83), 56(77), 70(16), 100(30)

* Compounds previously reported in *K. lactis* cultures.
** STDS = standard chemicals; UNKN = unknown peaks.
*,** Standards not available for these compounds, so their identification were positively made by the close matches between unknown spectra and NBS Library spectra.

(Afschar *et al.*, 1990), *Lactococcus lactis/Lactobacillus casei* (Meiji-Milk, 1991) and *Enterobacter aerogenes* (Zeng *et al.*, 1990a, 1990b). Limited studies have also been carried out to produce acetoin using fungus/yeast fermentation, such as *Neurospora sp.* (Yamauchi *et al.*, 1991) and *Candida boidinii* (Matsumura *et al.*, 1991). The relatively high yield of acetoin by *K lactis* in this study indicates that there may be a good potential in using this yeast for the production of acetoin.

The hydrocarbons identified in this study are aromatic compounds present in very small quantities. Although existing in very small amounts, these aromatic hydrocarbons may have important relationships to the formation and high contents of 2-Phenylethanol and its derivatives. It was interesting that two diacetates were found in the culture of *K. lactis*. Furanones are a group of important flavour and

Table 2: Contents of the major volatile compounds in *Kluyveromyces lactis* culture

Name	Content (mg/L broth)	% of total*
isobutanol	8.66	3.80
isoamyl alcohol	73.33	37.13
2-phenylethanol	72.05	38.04
2,3-butanediol	1.78	0.50
isobutyric acid	3.85	2.10
isovaleric acid	2.28	0.88
isoamyl acetate	1.85	0.57
2-phenylethyl acetate	9.97	6.28
3-hydroxy-2-butanone (acetoin)	22.34	9.26

* The proportions of individual peak areas to the total peak area including the unidentified peaks. Compounds with peak area less than 0.2% of the total peak area were not quantified and thus not included in the table.

fragrance compounds, and microbial production of certain furanones has been patented, such as 4-hydroxy-2,5-dimethyl-3(2H)-furanone by *Macrophomopsis sp.* (Kanebo, 1989) and 4-hydroxy-2-ethyl-5-methyl-3(2H)-furanone by *Zygosaccharomyces rouxii* (Kikkoman, 1991). Production of 3-hydroxy-4,5-dimethyl-2(5H)-furanone using a lipase from *Saccharomyces cerevisiae* and *Candida cylindracea* has also been explored (Fronza *et al.*, 1992). In this study, dihydro-4-methyl-2(3H)-furanone was detected for the first time in *K. lactis* cultures, but unfortunately in a very small amount. Further studies on the kinetics of volatile formation and the effects of cultural conditions are necessary to determine whether the yeast has the potential to produce the furanone in a significant quantity.

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