Accumulation of Volatile Flavour Compounds in Liquid Cultures of *Kluyveromyces lactis* Strains

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The accumulation of volatile flavour constituents in liquid cultures of three *Kluyveromyces lactis* strains was studied after cultivation on defined culture media containing glucose as carbon source, yeast extract, vitamines, and different additional nitrogen compounds. Besides short-chain alcohols and esters (fruit esters), 2-phenylethyl alcohol, phenylacetaldehyde, and 2-phenylethyl esters could be identified by gas chromatography and coupled gas chromatography-mass spectrometry. Although the composition of these compounds was qualitatively comparable within the three strains investigated, quantitative differences were significant and strain-dependent reactions to the culture medium could be observed.

Introduction

Under suitable culture conditions, many yeasts produce an intensive and fruit-like aroma. Such fragrant odours have been reported from strains of *Sporobolomyces odorus* [1, 2], *Kluyveromyces lactis* [3], *Pityrosporum species* [4], *Saccharomyces fermentati* [5], *Eremothecium ashbyii* [6], and *Dipodascus magnusii* [7]. The volatiles identified from cultures of these yeasts being responsible for the aroma-impression comprise fruit esters, 2-phenylethyl alcohol and the corresponding acetate, lactones, and monoterpenes.

Our own studies on asco- and basidiomycetes [8, 9] show that the formation of volatile constituents by these organisms can strongly be influenced by the culture conditions in a strain-dependent manner. Especially, the variation of the nitrogen source or the addition of certain nitrogen compounds affected the production of volatiles by some higher fungi distinctly. For comparison with these previous results, we intended to include some yeasts known to produce fruit-like odours in our investigations.

In the present communication, the identification and accumulation of volatile metabolites in cultures of three *Kluyveromyces lactis* strains is described. These constituents are at least in part responsible for the fragrant odour of the yeast cultures. For these comparative studies, the authors made use of several culture media containing glucose as main carbon source, yeast extract, certain vitamines, but differing in an additional nitrogen compound. In consideration of previous investigations mentioned above, our special intention was to evaluate whether closely related yeasts would show distinct strain-dependent reactions to these different culture conditions.

Materials and Methods

Three strains of *Kluyveromyces lactis* (CBS 2359, CBS 4372, and CBS 5670) were obtained from Centralbureau voor Schimmelcultures (CBS), Baarn (NL).

After inoculation, yeasts were grown in a liquid culture medium with 5% glucose, 0.25% yeast extract, and vitamines (thiamine and biotin; each 4 mg/l culture broth) in 250 ml Erlenmeyer flasks containing 50 ml of culture medium. The additional compounds were altered as described below. Cultures were shaken at 135 rpm (8 h/d) at 22 °C.

The volatiles were determined five to six times during each culture period. In each case, they were obtained from ten cultures (500 ml culture broth) by circular steam distillation (10) in 2 ml pentane. The distillation residue was centrifuged at 3000 rpm for 20 min. The sediment was washed twice with distilled water, and dried at 80 °C to constant weight.

Glucose and amino acids were determined semi-quantitatively by TLC [11].

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The identification of individual components resulted from GC/MS data compared with those of the literature [12, 13] and with those of authentic reference substances, and comparison of retention time R_F values.

GLC analyses were performed with a Perkin Elmer PE F 22 model equipped with a flame ionization detector (FID; range 1, attenuation 1:4; split 1:30) and a computing integrator (Autolab System I, Spectra Physics). N_2 was used as carrier gas at a flow rate of 1 ml/min. The WG 11 (FFAP) capillary column (WGA; i.d. 0.3 mm) had a length of 22 m. For identification a temperature programme (70–200 °C; rate 2 °C/min) and various isothermic conditions were applied. Injection port temperature: 180 °C. Detector temperature: 180 °C. Injection volume: 1.0 µl.

MS analyses were performed on a Varian-MAT 111 (GNOM) mass spectrometer at 80 eV using a 3-m Carbowax (3%) column under various isothermic conditions or using a temperature programme as described above.

Quantities of volatile constituents were calculated *via* internal standard (6-methyl-5-hepten-2-one) using FID-specific substance factors [14].

Results and Discussion

The aroma concentrates obtained from three *Kluyveromyces lactis* strains consist of at least 60 to 70 volatile compounds. By GC retention time comparison and GC/MS-coupling the major constituents could be identified as short-chain alcohols and esters, and 2-phenylethyl alcohol and derivatives (Table I). The identified compounds are common to all strains and can be traced under nearly all tested culture conditions (see below). Monoterpenes previously identified in *Kluyveromyces lactis* CBS 2359 [3] could not be detected under our culture conditions.

The growth kinetics as well as the accumulation kinetics of short-chain alcohols and esters, and volatile constituents with aromatic structure, respectively, of strain CBS 5670 using additionally 0.05% phenylalanine as supplementary N-source are demonstrated in Fig. 1. The exponential growth phase is accompanied by a strong increase in fruit esters and short-chain alcohols with a maximum on the 8th day, while the accumulation of 2-phenylethyl derivatives reaches its maximum later (14 d). About 40% of the initial glucose concentration is detectable by the 8th culture day. After 11 days, no glucose can be traced in the culture medium, whereas phenylalanine as sole amino acid can be detected even after a culture period of 14 days. Such a bimodal distribution of volatile flavour constituents could be observed for all strains and nearly all tested culture media.

The accumulation data for individual components are displayed in Table I. Predominating constituents are ethyl acetate, isobutyl and isoamyl alcohol, and 2-phenylethyl acetate. This roughly outlined scheme applies qualitatively to the other strains as well.

Table I. Accumulation of volatile metabolites in cultures of *Kluyveromyces lactis* CBS 5670 during a culture period of 22 days on a glucose (5%)-yeast extract (0.25%)-phenylalanine (0.05%)-liquid medium (in mg/500 ml culture broth).

Compound	Culture age [days]					
	4	8	11	14	17	22
Ethyl acetate	9.91	23.49	15.09	0.25	0.24	0.13
Isopropyl alcohol	0.05	0.07	0.09	0.09	0.08	0.08
Isobutyl acetate	0.03	0.03	0.04	0.04	0.04	0.03
Isobutyl alcohol	0.21	1.74	1.61	1.49	1.18	0.99
n-Propyl alcohol	0.02	0.06	0.05	0.04	0.01	0.02
Isoamyl acetate	0.09	0.14	0.13	0.05	0.05	0.03
Isoamyl alcohol	3.24	5.54	5.10	4.34	4.17	3.46
Phenylacetaldehyde	0.05	0.06	0.06	0.42	0.24	0.12
2-Phenylethyl acetate	0.14	9.72	14.30	20.41	15.74	12.63
2-Phenylethyl isobutyrate	–	0.10	0.14	0.45	0.68	0.82
2-Phenylethyl alcohol	0.01	0.08	0.08	0.10	0.12	0.11
2-Phenylethyl butyrate	–	0.01	0.14	0.10	0.03	0.04

Detection limit: 10 µg/500 ml culture broth.

Besides phenylalanine, tyrosine (0.07%), asparagine (0.1%), leucine (0.1%), and – for comparison – peptone (0.5%) were used as supplementary N-sources. A compilation of maximum cell weight and accumulation of volatile metabolites is presented in Table II. As expected, an accelerated growth leading to highest cell dry weight could be observed with the peptone-containing culture medium, but it was not accompanied by an increased production of volatile constituents. The addition of phenylalanine led to a strong formation of 2-phenylethyl derivatives. This applies especially to the strains CBS 4372 and 5670. Obviously, 2-phenylethyl alcohol is formed from phenylalanine by decarboxylation and diamination [15]. The same authors could observe a formation of this aromatic alcohol by decomposition of tyrosine by yeasts. In our studies, cultivation on a tyrosine-containing medium led to significantly decreased amounts of 2-phenylethyl alcohol and related constituents in all strains. Further studies must elucidate whether a direct inhibition or repression by this amino acid may be the cause for these results. The relatively high yields of short-chain alcohols and esters obtained with leucine are due to an increased accumulation of isoamyl alcohol.

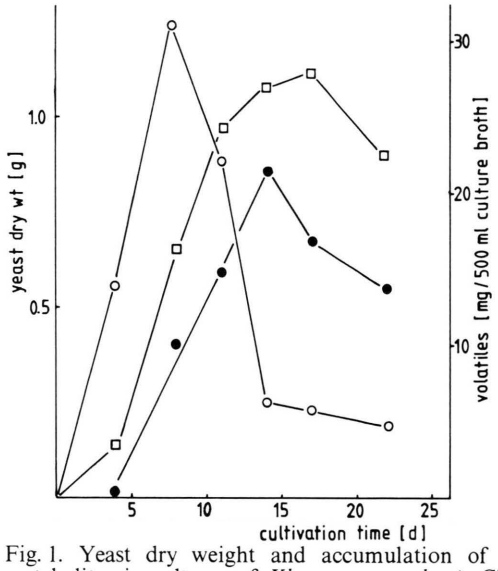

Fig. 1. Yeast dry weight and accumulation of volatile metabolites in cultures of *Kluyveromyces lactis* CBS 5670 over a culture period of 22 days on a glucose (5%)-yeast extract (0.25%)-phenylalanine (0.05%)-liquid medium. □—□ Yeast dry weight; ○—○ short-chain alcohols and esters; ●—● 2-phenylethyl derivatives.

Calcium nitrate cannot be assimilated by *Kluyveromyces lactis* as a nitrogen source. The addition of this compound (0.5%) led to a poor growth of all strains, relatively high amounts of 2-phenylethyl acetate, however, were accumulated by strain CBS 2359 under these culture conditions (maximum yield for all tested culture media; Table II).

Although the qualitative differences between these *Kluyveromyces lactis* strains were mostly insignificant, the results confirm and complement

Table II. Maximum cell mass and accumulation of volatile metabolites in cultures of three *Kluyveromyces lactis* strains grown on glucose (5%)-yeast extract (0.25%)-liquid media differing in supplementary N-additives.

Strain	N-additive (concentration [%])					
	phenylalanine (0.05%)	tyrosine (0.07%)	asparagine (0.1%)	leucine (0.1%)	peptone (0.5%)	calcium nitrate (0.5%)
Yeast dry wt. (g/10 cultures)						
CBS 5670	1.12 (22)	1.28 (23)	1.24 (12)	1.19 (21)	2.95 (12)	0.59 (23)
CBS 4372	1.31 (24)	1.13 (19)	0.73 (25)	1.49 (24)	3.39 (10)	1.02 (19)
CBS 2359	1.25 (25)	1.19 (28)	1.43 (23)	1.30 (27)	3.62 (18)	0.91 (30)
Short-chain alcohols and esters (mg/500 ml culture broth)						
CBS 5670	31.07 (8)	20.82 (7)	21.47 (8)	46.24 (21)	6.75 (8)	13.90 (11)
CBS 4372	93.32 (10)	73.25 (12)	46.66 (11)	157.58 (6)	9.80 (7)	25.17 (12)
CBS 2359	28.90 (7)	9.31 (28)	31.62 (12)	102.82 (14)	24.66 (7)	15.02 (22)
Phenylethyl derivatives (mg/500 ml culture broth)						
CBS 5670	21.48 (14)	1.26 (23)	1.79 (15)	1.77 (21)	3.00 (14)	1.46 (11)
CBS 4372	15.22 (7)	0.62 (15)	1.59 (18)	2.47 (14)	5.25 (15)	3.96 (19)
CBS 2359	6.05 (7)	0.63 (28)	2.75 (23)	2.62 (20)	3.25 (18)	8.08 (22)

( ): Culture age in days indicating maximum yeast dry weight and maximum accumulation of volatile metabolites, respectively, during a cultivation time of 35 days.

our previous findings with other fungi that even closely related strains can show distinct reactions to defined culture conditions. Further studies with other yeast strains will be undertaken to obtain a clearer picture of flavour formation by these economically important organisms.

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