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Production of volatile organic sulfur compounds (VOSCs) by basidiomycetous yeasts

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Abstract

Thirty-seven basidiomycetous yeasts belonging to 30 species of seven genera were grown on media containing L-cysteine or L-methionine as sole nitrogen sources with the objective of evaluating volatile organic sulfur compound (VOSC) production. The headspace of yeast cultures was analyzed by the solid-phase microextraction (SPME) sampling method, and volatile compounds were quantified and identified by GC-MS techniques. Ten strains assimilating L-methionine produced the following VOSCs: 3-(methylthio)-1-propanol, methanethiol, *S*-methyl thioacetate, dimethyl disulfide, dimethyl trisulfide, allyl methyl sulphide and 4,5-dihydro-3(2*H*)-thiophenone. Production was <1 mgl^{-1} except for 3-(methylthio)-1-propanol of which between 40 and 400 mgl^{-1} was synthesized. Higher alcohols (isobutyl alcohol, isoamyl alcohol and active amyl alcohol) and esters (ethyl acetate, ethyl propionate, *n*-propyl acetate, isobutyl acetate, *n*-propyl propionate, *n*-butyl acetate, isoamyl acetate, amyl acetate, isoamyl propionate, amyl propionate and 2-phenylmethyl acetate) were also sporadically produced. This is the first report of VOSCs production by basidiomycetous yeasts. Consequently, basidiomycetous yeasts may be considered an interesting new group of microbial VOSCs producers for the flavor industry.

Keywords: VOSCs; Basidiomycetous yeasts; Flavour industry

1. Introduction

Volatile organic compounds (VOCs) are highly volatile, low-molecular-weight organic substances that can interact with olfactory receptors [1]. Single molecules (''impact compounds'') or, more frequently, mixtures of particular flavouring compounds are responsible for bringing about natural or artificial aromas, while other VOCs generally provide only insignificant modifications of the final aroma [1]. Although many VOCs are known (e.g. aldehydes, alcohols, esters, lactones, terpenes and sulfur compounds) only a few are used by the flavor industry in chemicals, pharmaceuticals, cosmetics, or in food and animal feeds [1,2]. Among them, volatile organic sulfur compounds (VOSCs) are of particular interest since they are normally effective at very low concentrations (often ppb or less) [1,3,4], and may be essential for the aroma of many foods and beverages such as cheese [5–10], truffles [11–14], beer [15] and wine [4].

While chemical synthesis is currently the preferred technology for producing flavor compounds, increasing

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consumer demand for "natural flavors" has given impetus to the development of microbial systems for the production of VOSCs [1,16,17]. Although ascomycetous yeasts and bacteria have been shown to be good VOSC producers [5,10,18,19], this activity has never been critically evaluated in basidiomycetous yeasts. The objective of this study was to explore VOSC production by basidiomycetous yeasts.

2. Materials and methods

2.1. Yeast strains

Thirty-seven basidiomycetous yeast isolates belonging to 30 species of seven genera were investigated (Table 1). Each species was represented by the type strain [20] and in some cases also by other authentic strains of the same species. All strains were obtained from the Industrial Yeasts Collection DBVPG of the Dipartimento di Biologia Vegetale e Biotecnologie Agroambientali, Sezione di Microbiologia Applicata of the Università di Perugia (http://www.agr.unipg.it/dbvpg).

2.2. Culture conditions

Yeast cells were maintained on YEPG agar slants (yeast extract 10 $g l^{-1}$, peptone 10 $g l^{-1}$, glucose 20 $g l^{-1}$, agar 15 $g l^{-1}$) at 4 °C, in lyophilized form, or frozen at −80 °C in special cryopreservative vials provided by STC Ltd. (Lancashire, UK). Aliquots (0.2 ml) of 24-h

Table 1
Basidiomycetous yeasts used in the present study

Species	DBVPG Accession No.	CBS Accession No.	Locality and source of isolation	Status of the strain
Bulleromyces albus	6655	501	USA, dairy atmosphere	T[a]
Cryptococcus aerius	6001	155	Japan, air	T
Cryptococcus albidus	6110	142	Japan, air	T
Cryptococcus albidus	6237	4192	Hungary, soil of vineyard	T of *Torulopsis pseudoaeria* [b]
Cryptococcus amylolentus	7015	6039	South Africa, insect frass	T
Cryptococcus curvatus	6206	570	The Netherlands, sputum	T
Cryptococcus diffluens	6002	160	Austria, fingernail	T
Cryptococcus diffluens	6234	926	Unknown, air	T of *Torulopsis albida var. japonica* [b]
Cryptococcus diffluens	6240	6436	Uruguay, water	T of *Cr. diffluensvar. uruguaiensis* [b]
Cryptococcus elinovii	6685	7051	Russia, soil	T
Cryptococcus flavus	6004	331	Japan, air	T
Cryptococcus himalayensis	6242	6293	Bhutan, soil	T
Cryptococcus humicolus	6019	571	Unknown, soil	T
Cryptococcus magnus	6009	140	The Netherlands, air	T
Cryptococcus magnus	6692	4685	Portugal, human skin	T of *Cr. ater* [b]
Cryptococcus skinneri	6011	5029	USA, insect frass	T
Cryptococcus terreus	6012	1895	New Zealand, soil	T
Cryptococcus terricolus	6238	4517	Norway, soil	T
Filobasidium capsuligenum	6972	1906	Japan, sake	T
Filobasidium capsuligenum	6984	4736	South Africa, wine cellar	T of *Torulopsis capsuligenus* [b]
Filobasidium uniguttulatum	6129	1730	Austria, finger nail	T
Rhodosporidium toruloides	6739	349	Japan, soil	T
Rhodosporidium toruloides	6740	14	Sweden, wood pulp	T
Rhodotorula acheniorum	7024	6386	UK, fruits of strawberry	T
Rhodotorula acuta	7028	7053	Japan, grape must	T
Rhodotorula bacarum	7025	6526	UK, berries of *Ribes* spp.	T
Rhodotorula graminis	7021	2826	New Zealand, grass	T
Rhodotorula lactosa	7022	5826	Japan, air	T
Rhodotorula lignophila	7029	7109	Chile, wood of *Drimys* spp.	T
Rhodotorula minuta	7020	319	Japan, air	T
Rhodotorula mucilaginosa	7019	316	Unknown	T
Sporidiobolus salmonicolor	3782	483	France, leaf of *Citrus* sp.	T of *Sp. odorus* [b]
Sporidiobolus salmonicolor	6650	2873	France, extract of oak	T of *Sp. hispanicus* [b]
Sporobolomyces albo-rubescens	6649	482	France, leaf of bush	T
Sporobolomyces roseus	6197	486	The Netherlands, air	T
Sporobolomyces singularis	6620	5109	USA, frass of *Scolytus* spp.	T
Sporobolomyces tsugae	6619	5038	USA, frass of *Tsuga* spp.	T

[a] Type strain.
[b] Species considered to be synonyms of the lead listed species.

cell suspensions, calibrated to $A_{580} = 0.5$ (average cell concentration of 10^6 ml^{-1}), were used to inoculate 5 ml of 3% v/v Yeast Carbon Base (YCB) (Difco, Detroit, MI) supplemented with 0.5 g l^{-1} L-cysteine or L-methionine. Final pH was 5.0.

Cultures were grown at 25 °C for 72 h in a rotary shaker (40 rpm) after which cell growth was stopped by addition of 0.5 ml of a 100 ppm solution of nystatin (Serva, Heidelberg, Germany) in *N*,*N*-dimethylformamide (DMF). Five ml of each culture was transferred to a 25-ml glass vial which was sealed with a Viton rubber septum (Agilent Technologies, Palo Alto, CA) and closed with an aluminum crimp cap. Samples were frozen and stored at −20 °C until analysis.

2.3. SPME and GC-MS analyses

Vial headspace was analyzed according to a standard protocol [21,22] by GC-MS using the solid-phase microextraction (SPME) sampling technique. Sealed vials containing the yeast suspensions were thawed by immersion in a silicon oil bath at 35 °C for 15 min. Headspace was analyzed using a 2-cm needle containing a fiber coated with 50/30 μm divinylbenzene/Carboxen on polydimethylsiloxane bonded to a flexible fused silica core (Supelco, Bellefonte, PA). The needle was inserted into the vial through the septum and the fiber was exposed to headspace volatiles for 5 min at 30 °C. After direct desorption into the injector port at 280 °C for 10 min, VOCs were analyzed using a Hewlett Packard G1800C Series II gas chromatograph-mass spectrometer equipped with a HP-5 column (25 m × 0.2 mm, 0.5 μm film thickness) coated with (5%)-diphenyl-(95%)-dimethylpolysiloxane copolymer.

Compounds were identified on the basis of their respective mass fragmentation patterns (EI, 70 eV) by comparison with the database library NIST98.1 (MS Library Software Varian, Palo Alto, CA). Headspace volatiles were measured quantitatively by an internal standard method in which thawing vial contents were spiked with 50 μl of a freshly prepared chlorobenzene solution (0.1 mg ml^{-1} in deionized water). As a control, isoamyl alcohol, isoamyl acetate, dimethyl disulfide, and 3-methylthio-1-propanol levels were measured by headspace analysis of vials containing nystatin-supplemented media without cultures to which a known amount of the tested compounds were added. To determine whether VOC formation occurred in the absence of yeast cells, blank vials were analyzed at various times for up to a week.

2.4. Statistical analyses

Statistical evaluation of VOSC and non-sulfur-containing VOC profiles produced by different yeast strains was carried out by one-way ANOVA. Each result represented the average of three separate determinations. The above data matrix was used to calculate a correlation data matrix.

3. Results and discussion

Sixteen strains grew in media containing L-cysteine as the sole nitrogen source, while only 10 were able to grow on L-methionine. Only strains assimilating L-methionine produced VOSCs in the following classes: thiols (methanethiol – MTL), thioalcohols (3-(methylthio)-1-propanol – MTP), thioesters (*S*-methyl thioacetate – MTA), sulfides (dimethyl disulfide – DMDS; dimethyl trisulfide – DMTS; allyl methyl sulphide – AMS) and thiophenones (4,5-dihydro-3(2*H*)-thiophenone – DTP) (Table 2). On the basis of these results, it appears that L-methionine is the essential precursor of the VOSCs detected,

Table 2
Production of VOSCs by basidiomycetous yeasts

Species	DBVPG Accession No.	VOSCs (mg l^{-1} culture)						
		MTL	MTA	DMDS	DMTS	MTP	DTP	AMS
		0.01[a]	0.002[a]	0.003[a]	0.003[a]	0.1[a]	0.008[a]	0.01[a]
Bulleromyces albus	6655			0.03^{A}		84^{B}	0.02^{A}	0.48^{A}
Cryptococcus magnus	6692			0.03^{A}		101^{B}	0.06^{A}	
Cryptococcus curvatus	6206	0.08^{AB}	0.03^{A}	0.11^{B}	0.01^{A}	229^{C}		
Cryptococcus diffluens	6234					399^{D}	0.03^{A}	
Cryptococcus terreus	6012	0.01^{A}	0.02^{A}	0.12^{B}			0.64^{C}	
Rhodosporidium toruloides	6739	0.12^{B}	0.02^{A}	0.07^{A}	0.01^{A}			
Rhodosporidium toruloides	6740	0.03^{A}		0.03^{A}		57^{A}	0.06^{A}	
Rhodotorula acuta	7028			0.05^{A}		40^{A}	0.38^{B}	
Sporobolomyces albo-rubescens	6649	0.12^{B}		0.05^{A}	0.01^{A}			
Sporobolomyces roseus	6197	0.11^{B}	0.02^{A}	0.04^{A}	0.03^{A}		0.01^{A}	

MTL: methanethiol; MTA: *S*-methyl thioacetate; DMDS: dimethyl disulfide; DMTS: dimethyl trisulfide MTP: 3-(methylthio)-1-propanol; DTP: 4,5-dihydro-3(2*H*)-thiophenone; AMS: allyl methyl sulpfide.

Superscript capital letters (A, B, C, D) indicate significant ($p < 0.01$) differences.

[a] Minimal detectable level (mg l^{-1}). There is no entry for compounds that were present at a level below the level of detection.

as none were produced in culture media lacking this nitrogen source. No VOSCs were detected in the blank vial controls over a seven-day period, suggesting that spontaneous L-methionine degradation or volatile release by the rubber septum is not a source of VOSCs.

Three basic VOSC profiles were observed (Table 2): (i) *Bulleromyces albus* DBVPG 6655, *Cryptococcus magnus* DBVPG 6692 and *Rhodotorula acuta* DBVPG 7028 did not produce MTL or MTA, (ii) *Cryptococcus diffluens* DBVPG 6234 produced only MTP and DTP, and (iii) the remaining strains generally had a broader biosynthetic ability. Different strains produced significantly different amounts ($p < 0.01$) of MTL, DMDS, MTP, and DTP. MTP concentration was generally 100 times greater (from 40 to 400 mgl^{-1}) than any other VOSC (Table 2). The correlation matrix calculated on the basis of the VOSC quantitative data matrix indicated significant relationships ($p < 0.01$) between MTL and MTA ($r = 0.69$), MTL and DMDS ($r = 0.75$), MTL and DMTS ($r = 0.93$), as well as between DMDS and DMTS ($r = 0.78$) (Table 3).

Quantitative data of lesser produced VOSCs were aggregated into three different chemical classes, according to current literature [23–25]: (a) = thiols + thioesters (MTL + MTA); (b) = sulphides (DMDS + DMTS + AMS) and (c) = thiophenones (DTP). Accordingly, several strains exhibited a significantly ($p < 0.01$) higher biosynthetic potential (Fig. 1): *Cryptococcus curvatus* DBVPG 6206, *Rhodosporidium toruloides* DBVPG 6739, *Sporobolomyces alborubescens* DBVPG 6649 and *Sporobolomyces roseus* DBVPG 6197 for the class A (average value 0.13 mgl^{-1}), *B. albus* DBVPG 6655 for the class B (0.51 mgl^{-1}) and *Cryptococcus terreus* DBVPG 6012 and *Rh. acuta* DBVPG 7028 for class C (0.64 and 0.38 mgl^{-1}, respectively).

Some non-sulfur-containing VOCs (isoamyl alcohol, active amyl alcohol, isobutyl alcohol and acetaldehyde) were also produced in L-methionine-containing medium (Table 4), whereas esters, such as isobutyl acetate, *n*-propyl propionate, *n*-butyl acetate, amyl acetate, isoamyl propionate, amyl propionate, ethyl propionate, *n*-propyl acetate, isoamyl acetate, 2-phenylmethyl acetate, and ethyl acetate were only observed in *B. albus* DBVPG 6655 cultures (Fig. 2). On the contrary, traces of non-sulfur-containing VOCs were only occasionally detected in yeast strains grown on L-cysteine-containing medium.

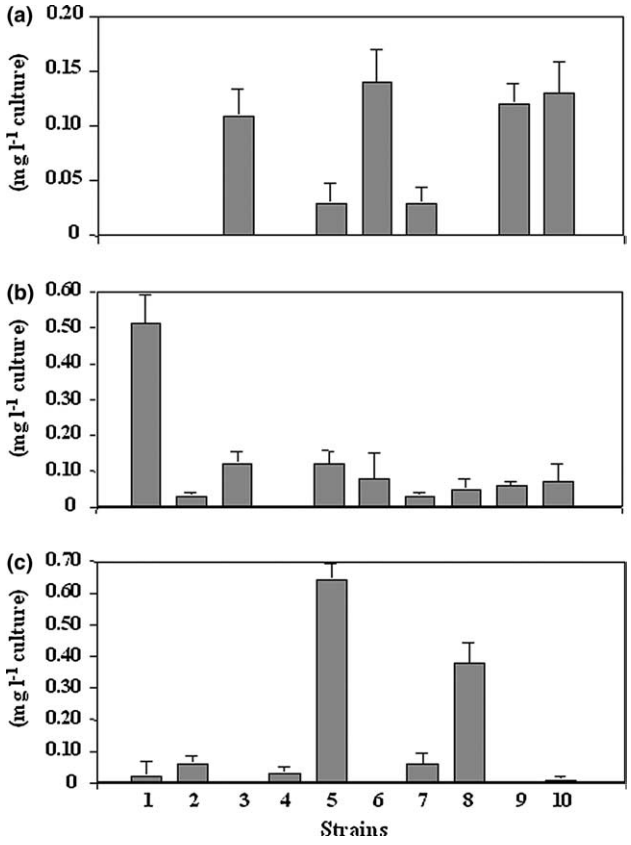

Fig. 1. Production of different classes of VOSCs by basidiomycetous yeasts. (a) = thiols + thioesters; (b) = sulfides; (c) = thiophenones. Strains: 1 = *Bulleromyces albus* DBVPG 6655; 2 = *Cryptococcus magnus* DBVPG 6692; 3 = *Cryptococcus curvatus* DBVPG 6206; 4 = *Cryptococcus diffluens* DBVPG 6234; 5 = *Cryptococcus terreus* DBVPG 6012; 6 = *Rhodosporidium toruloides* DBVPG 6739; 7 = *Rhodosporidium toruloides* DBVPG 6740; 8 = *Rhodotorula acuta* DBVPG 7028; 9 = *Sporobolomyces alborubescens* DBVPG 6649; 10 = *Sporobolomyces roseus* DBVPG 6197. Error bars represent standard deviations calculated on the average value of three separate determinations.

Table 3
Correlation coefficient matrix among VOSCs produced by basidiomycetous yeasts

Correlation coefficient[a]							
VOSCs	MTL	MTA	DMDS	DMTS	MTP	DTP	AMS
MTL	1						
MTA	**0.69**	1					
DMDS	**0.75**	0.09	1				
DMTS	**0.93**	0.54	**0.78**	1			
MTP	−0.36	−0.09	−0.31	−0.28	1		
DTP	−0.45	0.47	0.10	−0.37	−0.28	1	
AMS	−0.31	−0.26	−0.21	−0.22	−0.02	−0.20	1

MTL: methanethiol; MTA: *S*-methyl thioacetate; DMDS: dimethyl disulfide; DMTS: dimethyl trisulfide MTP: 3-(methylthio)-1-propanol; DTP: dihydro-3(2*H*)-thiophenone; AMS: allyl methyl sulfide.

[a] Values reported in bold character indicate a high correlation coefficient ($p < 0.01$).

Table 4
Production of non-sulfur VOCs by basidiomycetous yeasts

Species	DBVPG Accession No.	Aldehydes and alcohols (mgl^{-1} culture)				Total (mgl^{-1} culture)
		ACA	IBA	IAA	AMA	
		0.02[a]	0.01[a]	0.01[a]	0.01[a]	
Bulleromyces albus	6655			3.03^{C}	1.65^{B}	4.68
Cryptococcus magnus	6692	0.17^{A}	0.19^{A}	4.94^{D}	3.29^{C}	8.59
Cryptococcus curvatus	6206		1.04^{B}	3.34^{C}	0.56^{A}	4.94
Cryptococcus diffluens	6234		0.34^{A}	0.40^{A}	3.02^{C}	3.76
Cryptococcus terreus	6012			0.23^{A}	0.15^{A}	0.38
Rhodosporidium toruloides	6740			1.28^{B}	0.27^{A}	1.55
Rhodotorula acuta	7028		0.15^{A}	0.56^{A}	0.36^{A}	1.07
Sporobolomyces albo-rubescens	6649			0.14^{A}	0.09^{A}	0.23
Sporobolomyces roseus	6197			0.33^{A}	0.23^{A}	0.56

ACA: acetaldehyde, IBA: isobutyl alcohol; IAA: isoamyl alcohol; AMA: active amyl alcohol.
Superscript capital letters (A, B, C, D) indicate significant ($p < 0.01$) differences.
[a] Minimal detectable level (mgl^{-1}). There is no entry for compounds that were present at a level below the level of detection.

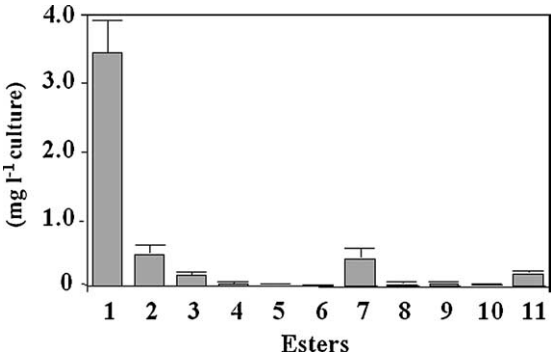

Fig. 2. Production of esters by *Bulleromyces albus* DBVPG 6655. Esters: 1 = ethyl acetate; 2 = ethyl propionate; 3 = *N*-propyl acetate; 4 = isobutyl acetate; 5 = propyl propionate; 6 = butyl acetate; 7 = isoamyl acetate; 8 = amyl acetate; 9 = isoamyl propionate; 10 = amyl propionate; 11 = 2-phenyl methyl acetate. Error bars represent standard deviations calculated on the average value of three separate determinations.

To the best of the authors' knowledge, this is the first report of VOSC production by basidiomycetous yeasts. All 30 tested species, represented at least by their type strains [20], are currently considered as not pathogenic to humans as they are classified at "biosafety level 1" (http://www.cdc.gov/od) and, therefore, molecules synthesized by these yeasts may be acceptable in pharmaceuticals and in food products for human consumption. Accordingly, most of these species have already been studied extensively for various biotechnological applications [26–33].

The ability of ascomycetous yeasts to produce VOSCs is well known. *Geotrichum candidum*, *Kluyveromyces lactis*, *Debaryomyces hansenii*, *Saccharomyces cerevisiae* and *Yarrowia lipolytica* can produce appreciable quantities of MTL, dimethyl sulfide (DMS), DMDS and DMTS [18,19,34]. In *G. candidum* the amount of MTA and other VOSCs produced depends on the strain evaluated [5]. The catabolism of L-methionine in *G. candidum* has been exhaustively studied and is described as a two-step degradation pathway involving firstly an aminotransferase which, in the presence of an amino acceptor such as α-ketoglutarate, leads to the transient accumulation of the intermediate 4-methylthio-2-oxobutyric acid (KMBA) [18,35]. Subsequently, KMBA is converted to MTL by a suitable demethiolase [34]. MTL represents the key precursor of most sulfur-containing volatiles. In particular, non-enzymatic transition metal-catalyzed auto-oxidation of MTL is known to yield DMDS and DMTS [36], whereas enzymatic or spontaneous reaction with acetyl-CoA affords MTA in *G. candidum* [18,35,37]. In this respect, the significant correlation coefficients observed in the present study between MTL and sulfides (both DMDS and DMTS), and between MTL and MTA (Table 3), could be consistent with their biosynthetic relationships and might suggest that, in close analogy to the situation in *G. candidum* [5,37], MTL could acts as the precursor of DMDS, DMTS and MTA in basidiomycetous yeasts as well (see Table 3).

The VOSC profiles observed in this study exhibited a number of differences to those reported for ascomycetous yeasts [5]. In particular, there is no report on the production of AMS and DTP by ascomycetous yeasts. Both of these VOSCs, which are included on the list of accepted flavoring agents of the Joint FAO/WHO Expert Committee on Food Additives (http://jecfa.ilsi.org), are currently used at low concentrations as flavor-enhancers in savory foods [23–25]. Consequently, *B. albus* DBVPG 6655, which produced 0.48 $mgl^{-1}$of AMS, as well as *Cr. terreus* DBVPG 6012 and *Rh. acuta* DBVPG 7028, which produced 0.64 and 0.38 $mgl^{-1}$of DTP, respectively, may be of interest to the flavor industry as new VOSC producers.

Although many microbial systems can produce interesting flavor compounds, the number of industrial applications is at present limited primarily because of low

yields resulting in high costs for downstream processing [16]. Nevertheless, some of these costs might be recoverable given the market price of natural aromas, which is estimated to be 10–100 times higher than that of the same compounds produced by chemical synthesis [1,16]. Since the European Community and United States legislations label a ''natural flavor'' as any compound produced by a biological system (e.g. microbial cells or enzymes derived from them) [38], the selection of useful microorganisms and the development of biotechnological processes for the production of ''natural flavor'' compounds could represent a strategic microbial challenge for the flavor industry.

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