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Flavour production by *Saprochaete* and *Geotrichum* yeasts and their close relatives

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ABSTRACT

In this study, a total of 30 yeast strains belonging to the genera *Dipodascus*, *Galactomyces*, *Geotrichum*, *Magnusiomyces* and *Saprochaete* were investigated for volatile organic compound production using HS-SPME-GC/MS analysis. The resulting flavour profiles, including 36 esters and 6 alcohols compounds, were statistically evaluated by cluster and PCA analysis. Two main groups of strains were extracted from this analysis, namely a group with a low ability to produce flavour and a group producing mainly alcohols. Two other minor groups of strains including *Saprochaete suaveolens*, *Geotrichum marinum* and *Saprochaete gigas* were diverging significantly from the main groups precisely because they showed a good ability to produce a large diversity of esters. In particular, we found that the *Saprochaete* genus (and their closed relatives) was characterized by a high production of unsaturated esters arising from partial catabolism of branched chain amino-acids. These esters were produced by eight phylogenetically related strains of *Saprochaete* genus.

1. Introduction

Geotrichum and *Saprochaete* yeasts are filamentous yeast like fungi which belong to the Ascomycota division, Saccharomycetes class and Saccharomycetales order (De Hoog & Smith, 2011a, 2011b). *Saprochaete* and *Geotrichum* species are very closely related. This is best illustrated by the fact that some species like *Saprochaete suaveolens* and *Saprochaete clavata* have a separate *Geotrichum* name (synonym) (De Hoog & Smith, 2004). These microbial eukaryotes are cosmopolitan and widespread and are often found in soil, manure, fruits, dairy products, human skin and digestive tract, insects as well as in other environments such as decaying plants and industrial effluents (Damasceno, Cereda, Pastore, & Oliveira, 2003; Suh & Blackwell, 2006). De Hoog and Smith (2011a) listed 11 species of *Geotrichum* of which one has a

teleomorphic state in the genus *Dipodascus* de Lagerh, three in the genus *Galactomyces* Redhead and Malloch and seven for which a sexual state has not been found. *G. ghanense* (Nielsen, Jakobsen, & Jespersen, 2010) was the most recent *Geotrichum* species found in nature. In the *Saprochaete* genus, 13 species were described by De Hoog and Smith (2011b) of which, only three have a teleomorphic state in the genus *Magnusiomyces*. Within this genus, some species like *S. suaveolens*, *S. clavata*, *S. gigas* and *S. ingens* have their synonym in the *Geotrichum* genus (*G. fragrans*, *G. clavatum*, *G. gigas* and *G. ingens*, respectively). With respect to safety, strains such as *Saprochaete capitata*, *S. clavata* or *G. candidum* are described as human pathogens (Camus et al., 2014; Garcia Ruiz et al., 2013).

Geotrichum candidum is one of the most studied species among this group of yeasts. It is well known in food industry for cheese ripening (Marcellino, Beuvier, Grappin, Guéguen, & Benson, 2001) and enzyme production (Ayed, Assas, Sayadi, & Hamdi, 2005; Brabcova et al., 2013; Hang & Woodams, 1990). *Geotrichum candidum*, as well as *S. suaveolens*, *G. klebahnii*, *Gal. geotrichum*, *Gal. reessii*, *D. aggregatus*, *D. albidus*, *D. armillariae*, *M. capitatus* and *M.*

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magnusii are also well known for flavour production (Bonnarme et al., 2001; Buzzini, Martini, Cappelli, Pagnoni, & Davoli, 2003; Damasceno et al., 2003; De Oliveira et al., 2013; Farbood, Morris, & Seitz, 1987; Fischer, Senser, & Grosch, 1983; Jollivet, Chataud, Vayssier, Bensoussan, & Belin, 1994; Neto, Pastore, & Macedo, 2004; Shimizu, Kataoka, Kizaki, & Yasohara, 2004; Sinha, 2007; Wu, Xu, & Chen, 2012).

Flavours are secondary metabolites produced by living cells through some specific pathways such as Ehrlich (Hazelwood, Daran, van Maris, Pronk, & Dickinson, 2008), β oxidation (Maggio Hall & Keller, 2004) and glycolytic pathways (Liu, Holland, & Crow, 2004; Ugliano & Henschke, 2009). Among yeasts, *Saccharomyces cerevisiae* is probably the best known species for its applications in the field of food flavouring (Saerens, Delvaux, Verstrepen, & Thevelein, 2010). Other non conventional yeasts, like *Saprochaete suaveolens*, have also been described as excellent producers of flavours including unsaturated esters such as ethyl tiglate (ethyl (*E*) 2 methylbut 2 enoate), an interesting top note flavour characterized by a strong fruity odor (Grondin, Shum Cheong Sing, Caro, de Billerbeck, et al., 2015; Grondin, Shum Cheong Sing, Caro, Raherimandimby, et al., 2015). Numerous publications deal with the production of flavours from both an academic and applied perspective, but comparatively few of them use flavours as a tool to discriminate between species and/or strains. Chemotaxonomy, according to the definition given by Frisvad, Andersen, and Thrane (2008) using flavours as a taxonomic tool was first reported for the filamentous fungi *Penicillium* (Larsen & Frisvad, 1995).

The purpose of this study was to determine the flavour production profiles of the *Saprochaete*, *Geotrichum* and closely related yeasts. Experimental measurement of flavour production profiles using HS SPME CG/SM and processing of the data using descriptive statistical methods were performed to characterise the volatile organic compounds (VOCs) of these yeasts. Multivariate statistical methods were applied to find out whether the flavour production by these yeast species and their genomic classification using the ribosomal internal transcribed spacer (ITS) sequences were correlated.

2. Materials and methods

2.1. Yeast strains

The list of strains used in this study is presented in Table 1. Most of them were purchased from CBS (Utrecht, The Netherlands) and from BCCM (Brussels, Belgium) strain collections. The *Saprochaete suaveolens* strain (GEC0) used in this study was isolated from Pitaya fruits collected in Reunion Island as described elsewhere (Grondin, Shum Cheong Sing, Caro, de Billerbeck, et al., 2015; Grondin, Shum Cheong Sing, Caro, Raherimandimby, et al., 2015). The rDNA D1/D2 sequence of the isolated strain showed a difference of one nucleotide with the *G. fragrans* reference strain CBS 152.25.

2.2. Culture media

Cells were stored in a rich medium containing glycerol in cryogenic vials at -80°C and refreshed on autoclaved YEPD agar slants containing 20 g/L of glucose (Sigma), 20 g/L of peptone (Becton, Dickinson and Co.), 10 g/L of yeast extract (Biokar Diagnostics) and 15 g/L of agar (Merck). Cells were refreshed at 28°C for 48 h prior to their utilization.

2.3. VOCs analysis

Isolation and characterization of volatile metabolites was performed using solid phase micro extraction (SPME), followed by

Table 1
Strains of *Saprochaete*, *Geotrichum* and teleomorphs used in this study.

Strain number	Species	Collection number	GenBank accession number of ITS sequences
S1	<i>Dipodascus albidus</i>	CBS 766.85	AY788342
S2	<i>Dipodascus armillare</i>	CBS 834.71	AY788350.1
S3	<i>Galactomyces candidus</i>	CBS 11176	KJ579946 ^a
S4	<i>Galactomyces citri-aurantii</i>	CBS 176.89	AY788296.1
S5	<i>Galactomyces geotrichum</i>	CBS 774.71	JN974293.1
S6	<i>Galactomyces pseudocandidus</i>	CBS 10073	JN974292.1
S7	<i>Geotrichum candidum</i>	CBS 615.84	KJ608128 ^a
S8	<i>Geotrichum carabidarum</i>	CBS 9891	DQ143888.1
S9	<i>Geotrichum cucujoidarum</i>	CBS 9893	DQ143890
S10	<i>Geotrichum erienze</i>	CBS 694.83	This study
S11	<i>Geotrichum europaeum</i>	CBS 866.68	AY788351
S12	<i>Geotrichum fermentans</i>	CBS 625.85	AY788319
S13	<i>Geotrichum ghanense</i>	CBS 11010	This study
S14	<i>Geotrichum histeridarum</i>	CBS 9892	DQ143889
S15	<i>Geotrichum klebahnii</i>	CBS 179.30	AY788298
S16	<i>Geotrichum marinum</i>	MUCL 42958	This study
S17	<i>Geotrichum phurueaensis</i>	CBS 11418	HE663403
S18	<i>Geotrichum restrictum</i>	CBS 111234	EF126738.1
S19	<i>Magnusiomyces ingens</i>	CBS 101346	This study
S20	<i>Magnusiomyces magnusii</i>	CBS 151.30	AY788290.1
S21	<i>Magnusiomyces ovetensis</i>	CBS 192.55	AY788303.1
S22	<i>Saprochaete chiloensis</i>	CBS 8187	AY788349
S23	<i>Saprochaete fungicola</i>	CBS 625.85	AY788333.1
S24	<i>Saprochaete gigas</i>	CBS 126.76	AY838940.1
S25	<i>Saprochaete ingens</i>	CBS 524.90	AY788326.1
S26	<i>Saprochaete japonica</i>	CBS 100158	AY788287.1
S27	<i>Saprochaete psychrophila</i>	CBS 765.85	AY788341.1
S28	<i>Saprochaete quercus</i>	CBS 752.85	This study
S29	<i>Saprochaete saccharophila</i>	CBS 412.95	AY788316.1
S30	<i>Saprochaete suaveolens</i>	GEC0	This study

^a Sequence of another strains of the same species.

gas chromatography mass spectrometry (GC MS) analysis. The VOCs extraction and the chromatographic conditions were described in our previous studies (Grondin, Shum Cheong Sing, Caro, de Billerbeck, et al., 2015; Grondin, Shum Cheong Sing, Caro, Raherimandimby, et al., 2015).

2.4. Phylogenetic analysis

The ribosomal internal transcribed spacer (ITS) sequences were retrieved from GenBank (NCBI database) under the accession numbers indicated in Table 1. Where sequences were not available in the database, analysis was performed according to James et al. (2014). Yeast cells were broken using microwaves (Panasonic, 800 W) for 30 s in 50 μL of water to obtain cell extracts. The ITS region was amplified by PCR directly from whole yeast cell extracts, amplified using primers ITS5 and ITS4 and sequenced using these primers as well as internal primers ITS2 and ITS3. The amplified DNA was checked by 1% agarose gel electrophoresis, purified and concentrated using QIAquick PCR purification spin columns (Qiagen) and sequenced using a Life Technologies 3730XL sequencer at the Genome Analysis Centre (TGAC), Norwich, UK. Pairwise alignments and phylogenetic analysis were conducted using Geneious 7.1 software created by Biomatters. A phylogenetic neighbour joining tree was then generated using the distance Tamura Nei model. Confidence values for branch nodes were estimated from bootstrap analyses of 1000 replicates

Table 2

Volatile organic compounds (VOCs) produced by the representative yeasts of the genus *Geotrichum*, *Saprochaete* and teleomorphs during growth on YEPD and identified by SPME-GC/MS.

Volatils compounds	RRI EXP ^a	RRI TH ^b	Yeast producing strains
Alcohol			
2-Methylpropan-1-ol (isobutanol)	637	628	<i>G. pseudocandidus</i> ; <i>G. europaeum</i> ; <i>S. fungicola</i>
Butan-1-ol	661	662	<i>G. candidum</i> ; <i>G. carabidarum</i> ; <i>G. restrictum</i> ; <i>M. magnusii</i> ; <i>S. suaveolens</i>
2-Methylbutanol (active amyl alcohol)	770	739	<i>G. pseudocandidus</i> ; <i>G. candidum</i> ; <i>G. cucujoidarum</i> ; <i>G. europaeum</i> ; <i>G. histeridarum</i> ; <i>G. marinum</i> ; <i>G. restrictum</i> ; <i>M. magnusii</i> ; <i>S. fungicola</i> ; <i>S. quercus</i> ; <i>S. suaveolens</i>
3-Methylbutanol (isoamyl alcohol)	731	734	<i>D. armilliare</i> ; <i>G. candidus</i> ; <i>G. citri-aurantii</i> ; <i>G. geotrichum</i> ; <i>G. pseudocandidus</i> ; <i>G. candidum</i> ; <i>G. cucujoidarum</i> ; <i>G. europaeum</i> ; <i>G. fermentans</i> ; <i>G. histeridarum</i> ; <i>G. marinum</i> ; <i>G. phurueaensis</i> ; <i>G. restrictum</i> ; <i>M. magnusii</i> ; <i>S. fungicola</i> ; <i>S. gigas</i> ; <i>S. japonica</i> ; <i>S. quercus</i> ; <i>S. suaveolens</i>
2-Phenylethanol	1105	1114	<i>G. candidus</i> ; <i>G. citri-aurantii</i> ; <i>G. pseudocandidus</i> ; <i>G. candidum</i> ; <i>G. eriense</i> ; <i>G. europaeum</i> ; <i>G. fermentans</i> ; <i>G. ghanense</i> ; <i>G. histeridarum</i> ; <i>G. marinum</i> ; <i>G. phurueaensis</i> ; <i>M. magnusii</i> ; <i>M. ovetensis</i> ; <i>S. fungicola</i> ; <i>S. gigas</i> ; <i>S. japonica</i> ; <i>S. quercus</i> ; <i>S. suaveolens</i>
2-Ethylhexanol	1021	1030	<i>G. histeridarum</i> ; <i>M. magnusii</i> ; <i>S. fungicola</i> ; <i>S. quercus</i>
Ester			
Methyl 2-methylbutanoate	774		<i>G. marinum</i>
Methyl 3-methylbutanoate (methyl isovalerate)	774		<i>G. pseudocandidus</i>
Ethyl ethanoate (ethyl acetate)	620	612	<i>G. carabidarum</i> ; <i>G. europaeum</i> ; <i>G. marinum</i> ; <i>G. restrictum</i> ; <i>S. quercus</i> ; <i>S. suaveolens</i>
Ethyl propanoate	709	709	<i>G. marinum</i> ; <i>S. gigas</i> ; <i>S. suaveolens</i>
Ethyl 2-methylpropanoate (ethyl isobutyrate)	755	756	<i>G. pseudocandidus</i> ; <i>G. europaeum</i> ; <i>G. histeridarum</i> ; <i>G. marinum</i> ; <i>S. gigas</i> ; <i>S. suaveolens</i>
Ethyl butanoate	797	800	<i>G. pseudocandidus</i> ; <i>G. candidum</i> ; <i>G. histeridarum</i> ; <i>G. marinum</i> ; <i>G. restrictum</i> ; <i>S. suaveolens</i>
Ethyl but-2-enoate (ethyl crotonate)	841	833	<i>G. marinum</i> ; <i>S. suaveolens</i>
Ethyl 2-methylbutanoate	847	846	<i>G. pseudocandidus</i> ; <i>G. candidum</i> ; <i>G. europaeum</i> ; <i>G. histeridarum</i> ; <i>G. marinum</i> ; <i>S. suaveolens</i>
Ethyl (<i>E</i>)-2-methylbut-2-enoate (ethyl tiglate)	935	936	<i>G. geotrichum</i> ; <i>G. pseudocandidus</i> ; <i>G. europaeum</i> ; <i>G. marinum</i> ; <i>M. magnusii</i> ; <i>S. gigas</i> ; <i>S. quercus</i> ; <i>S. suaveolens</i>
Ethyl 3-methylbutanoate (ethyl isovalerate)	850	849	<i>G. geotrichum</i> ; <i>G. pseudocandidus</i> ; <i>G. candidum</i> ; <i>G. europaeum</i> ; <i>G. fermentans</i> ; <i>G. marinum</i> ; <i>S. gigas</i> ; <i>S. suaveolens</i>
Ethyl (<i>E</i>)-3-methylbut-2-enoate (ethyl 3-methylcrotonate)	919	920	<i>G. marinum</i> ; <i>S. gigas</i> ; <i>S. suaveolens</i>
Ethyl pentanoate (ethyl valerate)	895		<i>G. marinum</i>
Ethyl hexanoate (ethyl caproate)	991		<i>G. marinum</i>
Ethyl hex-2-enoate	1037		<i>G. marinum</i>
Ethyl octanoate (ethyl caprylate)	1186		<i>G. carabidarum</i> ; <i>G. restrictum</i> ; <i>S. quercus</i>
2-Methylpropyl ethanoate (isobutyl acetate)	771	753	<i>S. suaveolens</i>
2-Methylpropyl 2-methylpropanoate (isobutyl isobutyrate)	909	910	<i>G. marinum</i> ; <i>S. gigas</i> ; <i>S. suaveolens</i>
2-Methylpropyl butanoate (isobutyl butyrate)	951	950	<i>G. marinum</i> ; <i>S. suaveolens</i>
2-Methylpropyl 2-methylbutanoate (isobutyl 2-methylbutanoate)	995	998	<i>G. marinum</i> ; <i>S. gigas</i> ; <i>S. suaveolens</i>
2-Methylpropyl (<i>E</i>)-2-methylbut-2-enoate (isobutyl tiglate)	1084	1086	<i>G. europaeum</i> ; <i>G. marinum</i> ; <i>S. gigas</i> ; <i>S. suaveolens</i>
2-Methylpropyl 3-methylbutanoate (isobutyl isovalerate)	997	1002	<i>G. pseudocandidus</i> ; <i>G. marinum</i> ; <i>S. gigas</i> ; <i>S. suaveolens</i>
Butyl 2-methylbutanoate	1035		<i>S. suaveolens</i>
Butyl (<i>E</i>)-2-methylbut-2-enoate (butyl tiglate)	1125	1128	<i>S. suaveolens</i>
Butyl 3-methylbutanoate (butyl isovalerate)	1039	1040	<i>S. gigas</i> ; <i>S. suaveolens</i>
2-Methylbutyl ethanoate	903	874	<i>S. suaveolens</i>
2-Methylbutyl 2-methylpropanoate	1009		<i>G. marinum</i> ; <i>S. suaveolens</i>
2-Methylbutyl butanoate	1052	1052	<i>G. marinum</i> ; <i>S. suaveolens</i>
3-Methylbutyl ethanoate (isoamyl acetate)	873	871	<i>G. candidum</i> ; <i>G. histeridarum</i> ; <i>G. marinum</i> ; <i>S. suaveolens</i>
3-Methylbutyl propanoate (isoamyl propanoate)	964	964	<i>G. marinum</i> ; <i>S. suaveolens</i>
3-Methylbutyl butanoate (isoamyl butanoate)	1049	1050	<i>G. marinum</i> ; <i>S. suaveolens</i>
3-Methylbutyl 2-methylbutanoate (isoamyl 2-methylbutanoate)	1091		<i>G. marinum</i> ; <i>S. suaveolens</i>
3-Methylbutyl (<i>E</i>)-2-methylbut-2-enoate (isoamyl tiglate)	1185	1253	<i>G. marinum</i> ; <i>S. suaveolens</i>
3-Methylbutyl 3-methylbutanoate (isoamyl isovalerate)	1096	1101	<i>G. citri-aurantii</i> ; <i>G. fermentans</i> ; <i>G. marinum</i> ; <i>S. gigas</i> ; <i>S. suaveolens</i>
Pentyl propanoate (amyl propanoate)	964	964	<i>S. gigas</i>
Pentyl 3-methylbutanoate (amyl isovalerate)	1098	1103	<i>G. marinum</i> ; <i>S. gigas</i> ; <i>S. suaveolens</i>
Octyl ethanoate	1199		<i>G. carabidarum</i> ; <i>G. restrictum</i>

^a Relative Retention index on non-polar column determined experimentally.

^b Relative Retention index on non-polar column (<http://webbook.nist.gov/chemistry/>).

Table 3Classification of the VOCs produced by the strains belonging to the genus *Geotrichum*, *Saprochaete* and teleomorphs.

Name of species	Concentration of VOCs per chemical class (µg/L)			Number of VOCs per chemical class			Number of VOCs per type of hypothetical pathway								
	[Al]	[Es]	[Ctotal]	Al	Es	Total	GP	MP	PP	BP	Pep	B1P	B2P	EP	
<i>Dipodascus albidus</i>	nd	nd	nd	0	0	0	0	0	0	0	0	0	0	0	0
<i>Dipodascus armillariae</i>	5	nd	5	1	0	0	0	0	0	0	0	0	0	0	1
<i>Galactomyces candidus</i>	85	Nd	85	2	0	2	0	0	0	0	0	0	0	0	2
<i>Galactomyces citri-aurantii</i>	48	7	55	2	1	3	0	0	0	0	0	0	0	0	4
<i>Galactomyces geotrichum</i>	9	39	49	1	2	3	2	0	0	0	0	0	1	3	3
<i>Galactomyces pseudocandidus</i>	305	37	342	4	7	11	5	1	0	1	0	0	1	11	11
<i>Geotrichum candidum</i>	437	8	445	4	4	8	4	0	0	2	0	0	0	6	6
<i>Geotrichum carabidarum</i>	46	121	167	1	3	4	3	0	0	1	0	1	0	0	0
<i>Geotrichum cucujoidarum</i>	75	nd	75	2	0	2	0	0	0	0	0	0	0	2	2
<i>Geotrichum eriense</i>	11	nd	11	1	0	1	0	0	0	0	0	0	0	1	1
<i>Geotrichum europaeum</i>	1164	165	1329	4	6	10	5	0	0	0	0	0	2	10	10
<i>Geotrichum fermentans</i>	82	11	93	2	2	4	1	0	0	0	0	0	0	5	5
<i>Geotrichum ghanense</i>	14	nd	14	1	0	0	0	0	0	0	0	0	0	1	1
<i>Geotrichum histeridarum</i>	247	30	277	4	4	8	4	0	0	1	0	1	0	6	6
<i>Geotrichum Klebahnii</i>	nd	nd	nd	0	0	0	0	0	0	0	0	0	0	0	0
<i>Geotrichum marinum</i>	375	3159	3534	3	27	30	13	1	2	5	1	2	5	29	29
<i>Geotrichum phuruaensis</i>	109	Nd	109	2	0	2	0	0	0	0	0	0	0	2	2
<i>Geotrichum restrictum</i>	244	169	414	3	4	7	4	0	0	2	0	2	0	2	2
<i>Magnusiomyces ingens</i>	nd	nd	nd	0	0	0	0	0	0	0	0	0	0	0	0
<i>Magnusiomyces magnusii</i>	151	1	152	5	1	6	1	0	0	1	0	1	1	4	4
<i>Magnusiomyces ovetensis</i>	13	nd	13	1	0	0	0	0	0	0	0	0	0	1	1
<i>Saprochaete chiloensis</i>	nd	nd	nd	0	0	0	0	0	0	0	0	0	0	0	0
<i>Saprochaete fungicola</i>	238	nd	238	5	0	5	0	0	0	0	0	1	0	4	4
<i>Saprochaete gigas</i>	48	1753	1801	2	13	15	5	0	1	1	1	0	3	19	19
<i>Saprochaete ingens</i>	nd	nd	nd	0	0	0	0	0	0	0	0	0	0	0	0
<i>Saprochaete japonica</i>	47	nd	47	2	0	2	0	0	0	0	0	0	0	2	2
<i>Saprochaete psychrophila</i>	nd	nd	nd	0	0	0	0	0	0	0	0	0	0	0	0
<i>Saprochaete quercus</i>	79	24	103	4	3	7	4	0	0	0	0	2	1	4	4
<i>Saprochaete saccharophila</i>	nd	nd	nd	0	0	0	0	0	0	0	0	0	0	0	0
<i>Saprochaete suaveolens</i>	268	3464	3732	4	28	32	12	0	2	9	1	0	5	33	33

VOCs were classified in three categories (Concentration of VOCs per chemical class, Number of VOCs per chemical class and Number of VOCs per type of hypothetical pathway) for cluster and PCA analysis. [Al]: concentration of alcohols; [Es]: concentration of esters; [Ctotal]: Concentration of VOCs produced; nd: not detectable; Al: number of alcohols; Es: number of ester; Total: number of VOCs produced; Number of molecules derived from GP: Glycolysis pathway; PP: Propanoate pathway; BP: Butanoate pathway; Pep: Pentanoate pathway; B1P: β -oxidation pathway; B2P: unsaturated compounds from β -oxidation pathway; EP: Ehrlich pathway.

(Cardinali et al., 2012; Urubschurov, Janczyk, Souffrant, Freyer, & Zeyner, 2011).

2.5. Statistical analysis

Principal Component Analysis and cluster analysis (Ward's method) were performed on yeast flavours using XLSTAT (Addin soft) (Grondin, Shum Cheong Sing, Caro, de Billerbeck, et al., 2015; Grondin, Shum Cheong Sing, Caro, Raherimandimby, et al., 2015).

3. Results and discussion

3.1. Yeast strain selection

Yeasts were selected in accordance to De Hoog and Smith (2011a, 2011b) who described all currently accepted yeast species in these genera. *Saprochaete clavata*, which is categorized as a Class 2 microorganism, was arbitrarily withdrawn because of associated safety problems. VOCs analysis was extended to some strains of *Geotrichum*, which are not described by De Hoog and Smith (2011a, 2011b) namely *G. marinum*, *G. phuruaensis* and *G. ghanense*. Given that anamorphic and teleomorphic species are genetically very close (Liu et al., 2001), we also selected some yeasts which have an anamorphic state in the genera *Geotrichum* and *Saprochaete*, such as *Galactomyces citri aurantii* (*Geotrichum citri aurantii*), *Gal. pseudocandidus* (*Geotrichum pseudocandidum*), *Gal. candidus* (*Geotrichum candidum*), *Magnusiomyces magnusii* (*Saprochaete ludwigii*), *M. capitatus* (*Saprochaete capitata*), *M.*

ovetensis (*Saprochaete sericea*) and *Dipodascus armillariae* (*Geotrichum decipiens*).

3.2. VOCs production by different yeast species

Qualitative analysis of VOCs were carried out using HS SPME GC/MS on 30 representative strains belonging to the anamorphic genera *Saprochaete* (9 species), *Geotrichum* (12 species) and related teleomorphic genera *Magnusiomyces* (3 species), *Dipodascus* (2 species) and *Galactomyces* (4 species) after 24 h growth on YEPD (Table 2). A total of 42 different compounds were identified and classified into alcohols and esters (6 and 36 molecules, respectively). Due to their aromatic properties, esters are valuable molecules and are of relevance to industry. These molecules usually impart a characteristic fruity note to fermented beverages such as beer and wine (Schradler, 2007).

Among VOCs, 3 methylbutanol, 2 phenylethanol and 2 methylbutanol, which arise from the degradation of leucine, phenylalanine and isoleucine respectively by the Ehrlich pathway (Hazelwood et al., 2008), were the most frequently encountered compounds and were produced by 19, 18 and 11 different strains, respectively. In contrast, some VOCs such as methyl 2-methylbutanoate and butyl 2-methylbut 2-enoate were produced by only 1 yeast strain (*G. marinum* and *S. suaveolens*, respectively). Strains *S. suaveolens*, *G. marinum*, *S. gigas* and *G. europaeum* showed the best ability to produce qualitatively (more than 10 different compounds) and quantitatively (relative concentration higher than 1000 µg/L) flavour compounds (Table 3). Strain *Gal. pseudocandidus* was also found to produce a large number of VOCs (11 compounds) but quantitatively, the overall production of VOCs was significantly

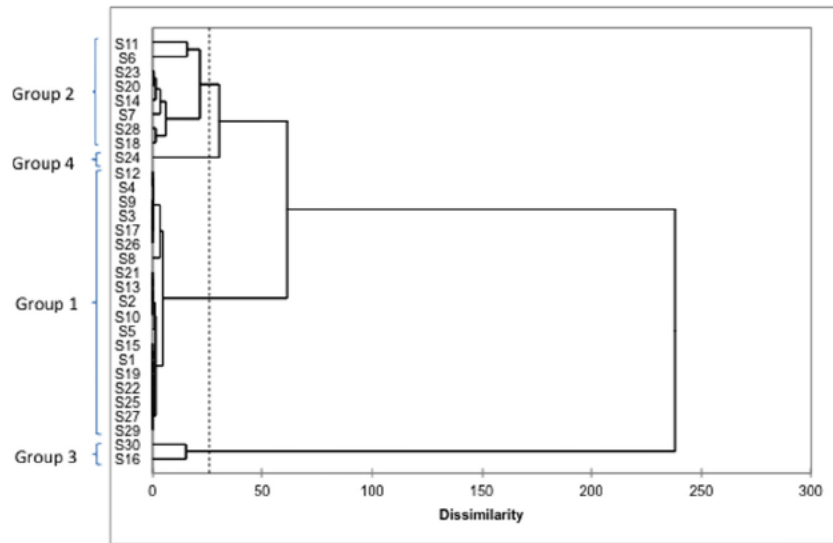


Fig. 1. Cluster analysis dendrograms from 30 yeasts based on flavour production. Dendrograms were calculated using ward's method and performed using XLSTAT (Addinsoft). Based on entropy, automatic truncation (dotted line) allowed identifying four consistent groups of yeasts among the strains S1 to S30 (Table 1).

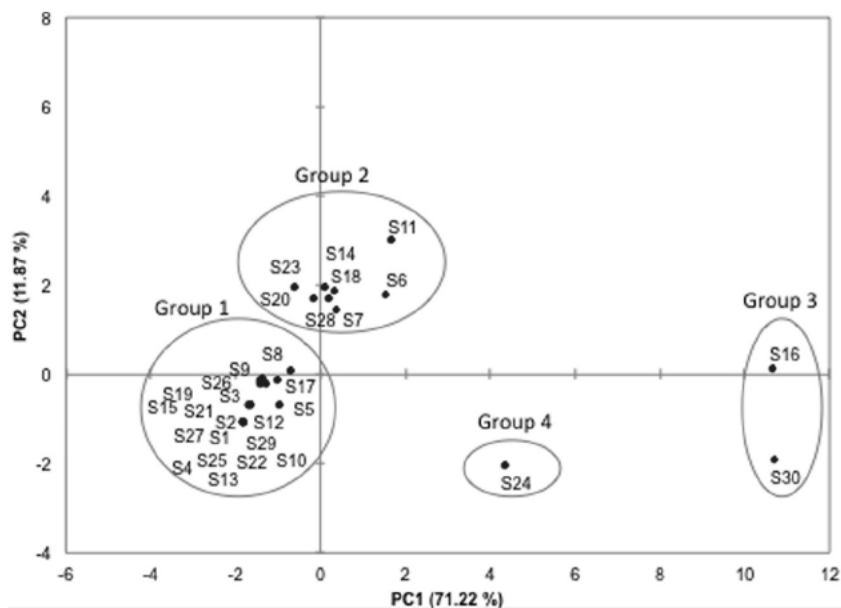


Fig. 2. Score plot of PC2 versus PC1 for yeast flavour analyzed by HS-SPME-GC/MS. Principal Component Analysis was performed using XLSTAT (Addinsoft). Variables including the relative concentration of VOCs ($\mu\text{g/L}$), number of VOCs per chemical classes and per hypothetical metabolic pathway (Table 3). S1 to S30 are the analyzed strains. Group 1 included strains that have a low ability to produce VOCs, group 2 strains that produced mainly alcohol and Group 3 and 4 strains that produced mainly esters.

lower than the above strains (less than $400 \mu\text{g/L}$). Comparatively, our results were in good agreement with other published data. For instance, production of 2 methylbutanol, 3 methylbutanol, 2 phenylethanol, ethyl acetate, ethyl propanoate, 3 methylbutyl ethanoate, ethyl 2 methylbut 2 enoate and 2 methylbutyl ethanoate were also detected in *G. candidum*, *S. suaveolens*, *G. klebahnii*, *Gal. geotrichum* and *M. magnusii* (Buzzini et al., 2003; Damasceno et al., 2003; Farbood et al., 1987; Fischer et al., 1983; Jollivet et al., 1994; Sinha, 2007; Wu et al., 2012). However, some results differed from other published data. Buzzini et al. (2003) reported a production of 3 methylbutanol and pentan 1 ol for *G. klebahnii* (S13) during growth in shaken flasks containing 10 g/L yeast extract, 10 g/L $(\text{NH}_4)_2\text{HPO}_4$ and 20 g/L glucose (pH 5.0) at 25°C for 72 h. In our study (YEPD standard agar medium), no VOCs could

be detected for this strain. This apparent contradiction might either come from differences in the design of the experiment (e.g., liquid vs. solid media, presence of phosphate buffer, ...) or from the genetic background of the strains. It should be noted however that one of the five *G. klebahnii* strains isolated by Buzzini et al. (2003) was also unable to produce VOCs. Such a difference between strains from the same species was previously reported, for example by Berger, Khan, Molimard, Martin, and Spinnler (1999) who observed differences in the production of sulphur compounds by ten isolates of the same species, *G. candidum*.

As previously shown by Grondin, Shum Cheong Sing, Caro, de Billerbeck, et al. (2015), Grondin, Shum Cheong Sing, Caro, Rahrerimandimby, et al. (2015), *Saprochaete suaveolens* produced several unsaturated ester compounds such as ethyl but 2 enoate,

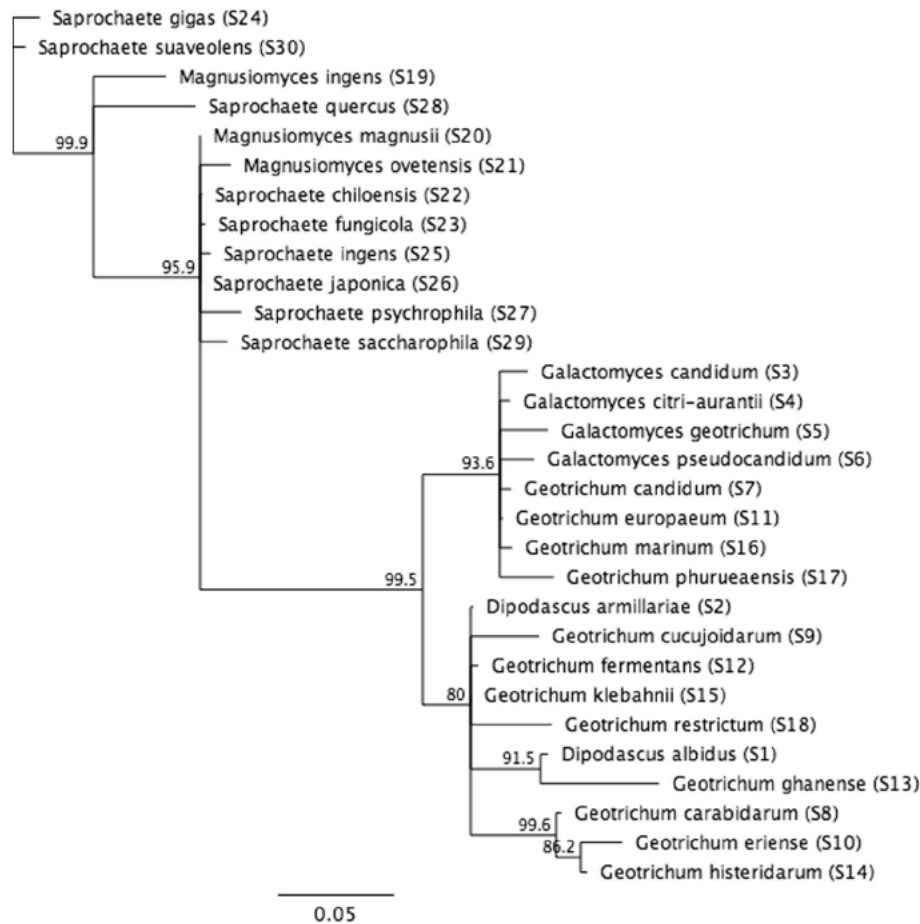


Fig. 3. Phylogenetic tree of species of the genus *Saprochaete*, *Geotrichum* and some teleomorphs strains. The tree was constructed using Geneious 7.1 (Biomatters) according to neighbour joining method with the ITS1–5.8S–ITS2 sequences collected from NCBI or determined in the laboratory. Bootstrap values (>80%) are from 1000 replicates. Scale Bar represents the number of substitution per site.

ethyl 2 methylbut 2 enoate, ethyl 3 methylbut 2 enoate, 2 methylpropyl 2 methylbut 2 enoate, butyl 2 methylbut 2 enoate and 3 methylbutyl 2 methylbut 2 enoate. Some of them were also produced by other strains, namely *Gal. geotrichum*, *Gal. pseudocandidus*, *G. europaeum*, *G. marinum*, *M. magnusii*, *S. gigas*, *S. quercus* and *S. suaveolens* (Tables 1 and 2). Like *S. suaveolens*, *G. marinum* was found to produce 5 out of the 6 unsaturated esters. Ethyl 2 methylbut 2 enoate (ethyl tiglate) was produced by the 8 strains whereas some unsaturated esters like ethyl hex 2 enoate or butyl 2 methylbut 2 enoate (butyl tiglate) were produced by only one strain (*G. marinum* and *S. suaveolens*, respectively). Since four out of unsaturated esters were derived from 2 methylbutanoyl CoA or tiglyl CoA, we could argue according to Grondin, Shum Cheong Sing, Caro, de Billerbeck, et al. (2015), that these esters arose from the catabolism of isoleucine that partially halted at the dehydration step due to low enoyl CoA hydratase activity.

As reported in Table 2, *G. marinum* can produce ethyl butanoate, ethyl but 2 enoate, ethyl hexanoate and ethyl hex 2 enoate, whereas *S. suaveolens* was limited to the production of the first two esters. This result may suggest that either *S. suaveolens* cannot further condense a acetyl CoA to elongate the fatty acid chain, or alternatively, butanoyl CoA is generated by the butanoate pathway that exist in human and some bacteria (<http://www.genome.jp/kegg/pathway.html>) but to our knowledge had never been reported in fungi. This pathway can lead to either butanoate as well as to butanol. Together with intermediates in catabolism of isoleucine, this pathway may account for the production of butyl

2 methylbutanoate, butyl 2 methylbut 2 enoate and butyl 3 methylbutanoate.

3.3. Comparison of the VOC profile of the strains by cluster and multivariate analysis and correlation with a taxonomical analysis

Multivariate statistical analysis was used in order to group yeast species based on their flavouring characteristics. To this end, each strain was associated with variables described in Table 3. Cluster analysis (Fig. 1) and PCA analysis (Fig. 2) both suggested the occurrence of two main and two minor groups of yeasts. The first group seems to be characterized by strains which have a low ability to produce VOCs and the second by strains that produced mainly alcohols. Three strains, namely *G. marinum*, *S. suaveolens* (group 3) and *S. gigas* (group 4) deviated from these groups because of their ability to produced esters (Fig. 2). Among group 2, *G. europaeum* appeared to be the best producer of alcohols with 1164 µg/L. In other experiments, we found *Debaryomyces nepalensis* to be the best strain for alcohol production (985 µg/L of alcohol was produced when tested in the same experimental conditions; Grondin, Shum Cheong Sing, Caro, Raherimandimby, et al., 2015). With a production of 15, 30 and 32 different VOCs respectively, *S. gigas*, *G. marinum* and *S. suaveolens* were by far the best producers of esters, and more specifically unsaturated esters among all strains analyzed (Table 3). *Geotrichum marinum* has the particularity to produce methyl esters like methyl 2 methylbutanoate and methyl 3 methylbutanoate (Table 2).

The fact that 4 genera (8 species) produced these unsaturated compounds suggested phylogenetic belonging. Then, only *Saprochaete* and related genera *Geotrichum*, *Galactomyces* and *Magnusiomyces* were found to produce these unsaturated esters, and in particular ethyl tiglate which could reach 113 mg/l in *S. suaveolens* culture (Grondin, Shum Cheong Sing, Caro, de Billerbeck, et al., 2015). The presence of unsaturated compounds in eight species of these neighbour genera indicated high metabolic similarities between these yeasts.

In order to establish a genomic link between these strains, a phylogenetic tree was generated using rDNA sequences either retrieved from the NCBI and GenBank databases or determined in our laboratory (Fig. 3). The internal transcribed spacer (ITS) region was selected because it is widely used to identify a broad range of different fungi (Schoch et al., 2012). As we can see, the strains under study seem very closed genetically (0.3 substitution per site between the most distant strains) and could explain the metabolic similarity for VOCS production (Fig. 3).

To summarize, this work was a first approach to study VOCS from the yeast of the genus *Saprochaete*, *Geotrichum* and close relative strains. Statistical analysis allowed us to classify the strains according to their flavour production and four groups of strains were highlighted by this approach. While we identified the *Saprochaete* genus as exhibiting an unusual capacity to produce a large variety of unsaturated esters such as ethyl tiglate, we could not find any relationship between flavours profiles and genomic classification of these yeast strains, suggesting that the metabolic activities underlining the flavour production has been shaped by their ecological niche.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.foodchem.2017.06.009>.

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