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Production of fusel alcohols in species of the family Ceratocystidaceae

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Abstract: Fungi in the family Ceratocystidaceae produce fusel alcohols and acetates, which have fruity and floral odours. These compounds are valuable additives of food products, perfumes and soaps. Our aim was to analyse the volatiles produced by the Ceratocystidaceae and to study their biosynthesis. All the strains included in our study produced high levels of isoamyl acetate while some members of *Bretziella, Berkeleyomyces, Ceratocystis* and *Huntiella* produced isobutyl acetate. The majority of Ceratocystidaceae also produced 2-phenylethyl acetate in low quantities. Fusel alcohols are produced in fungi from amino acids via the Ehrlich pathway in a three-step catabolic process. We searched in the available genomes of fungi in the Ceratocystidaceae for genes encoding enzymes involved in catalysing the first and second steps in the production of the fusel alcohols. We discovered three gene sequences encoding putative aromatic amino transferases, three gene sequences encoding putative branched-chain amino transferases and a single gene sequence encoding a putative pyruvate decarboxylase in each of the genomes. We also discovered that most fungi of the Sordariomycetes which do not produce fruity or floral smells have a similar number of copies of each of these genes. The amino transferases from *C. albifundus*, *C. manginecans*, *E. polonica* and *H. monilliformis* were heterologously expressed and functionally characterized by *in vitro* enzyme assays. All the assayed enzymes were either functional aromatic amino transferases accepting the substrates phenylalanine, tyrosine and tryptophan or branched-chain amino acid transferases accepting the substrates leucine, isoleucine or valine.

1. Introduction

Fusel alcohols and acetates are volatile chemical compounds that are widely used in food, cosmetic, pharmaceutical and biofuel industries (Fig. 1 and Table 1) (Hua & Xu, 2011). They play an essential role in the flavouring of food and drinks (Welsh *et al.*, 1989, Mauricio *et al.*, 2001, Etschmann *et al.*, 2002), as well as fragrance modification of cosmetic products like perfumes and soaps (Welsh *et al.*, 1989, Branduardi *et al.*, 2013). In addition, fusel alcohols are utilized as biofuels or biodiesels, bringing solutions to the problems associated with limited fossil resources and climate change (Keasling & Chou, 2008, Branduardi *et al.*, 2013). More than 10 000 tons of fusel alcohols and acetates are estimated to be produced and used throughout the world every year (Welsh *et al.*, 1989, Scognamiglio *et al.*, 2012).

Fusel alcohols and acetates can either have an aliphatic branched chain structure or they can be aromatic (containing a phenolic ring) (Fig. 1 and Table 1) (Nykänen & Nykänen, 1977). The branched chain fusel alcohols include isobutanol, 3-methyl butanol (isoamyl alcohol) and 2-methyl butanol while 2-phenylethanol is the most well-known and used aromatic fusel alcohol. Both branched chain and aromatic fusel alcohols and acetates can be produced artificially through chemical synthesis or naturally by plants and microorganisms (Spinnler & Djian, 1991, Goettmann *et al.*, 2006, Hazelwood *et al.*, 2008). Since chemical synthesis is environmentally unfriendly, natural

production of branched chain and aromatic fusel alcohols and acetates is more sustainable (Etschmann *et al.*, 2002).

Currently fusel alcohols are mostly produced by chemical methods in the industries (Jensen *et al.*, 2001, Carlini *et al.*, 2003, Goettmann *et al.*, 2006, Kang *et al.*, 2007, Uyttebroek *et al.*, 2015). However, these methods have a number of disadvantages such as high energy consumption, involvement of toxic chemical compounds used as catalysts and air pollution (Goettmann *et al.*, 2006). For this reason there is increasing interest by consumers for naturally synthesised products. However, producing these volatiles in plants is inefficient; plants are affected by environmental conditions, are prone to plant diseases, and only available at certain times of the year (Carlquist *et al.*, 2015). Alternatively, production of fusel alcohols and acetates by microorganisms has been proposed to be more advantageous (Hazelwood *et al.*, 2008).

Fungi are the most promising and well-studied microorganisms for production of fusel alcohols and acetates. Fungi produce fusel alcohols through a biosynthetic pathway known as the Ehrlich pathway (Fig. 2) (Ehrlich, 1907). The Ehrlich pathway removes the amino-group from amino acids by transamination to produce pyruvate-derivatives which are subsequently decarboxylated to aldehydes and finally dehydrogenated to fusel alcohols (Ehrlich, 1907, Hazelwood *et al.*, 2008). The first step of the Ehrlich pathway (transamination) is catalysed by transaminase enzymes encoded by *aro8, aro9* or *bat1* genes in *Saccharomyces cerevisiae.* The second step is catalysed by a decarboxylase enzyme that is encoded by the *aro10, pdc1, pdc5, pdc6, kidv* or *thi3* genes in yeast. The last step of the pathway is encoded by a dehydrogenase encoded by *adh1, adh2, adh3, adh4, adh5* and *sfa1* in *S. cerevisiae* to produce fusel alcohols (Ehrlich, 1907, Sentheshanmuganathan, 1960).

Once the fusel alcohols are produced they can be converted to fusel acetates that are formed through esterification. This process is catalysed by alcohol acyltransferase (AAT) enzymes and involves the transacylation from acetyl coenzyme A to a fusel alcohol (Beekwilder *et al.*, 2004). In *S. cerevisiae,* the AAT enzymes are encoded by the *atf1* and *atf2* genes (Yoshioka & Hashimoto, 1981). Landaud *et al.* (2001) showed the time frame in which fusel alcohols and acetates are produced during fermentation by *S. cerevisiae* is a three-phase timetable. The three phases were identified as the accumulation of alcohols alone, accumulation of both alcohols and acetates and acetates accumulating alone. The timing of each phase depends on the redox status of the cell, culture broth and the fermentation temperature (Vuralhan *et al.*, 2003, Vuralhan *et al.*, 2005, Yilmaztekin *et al.*, 2013).

The pleasant scent produced by fusel alcohols and acetates can be easily detected by the human nose (Lilly *et al*., 2006). Odour results from an interacting mixture of volatile chemical compounds (Leonardos *et al*., 1974). The human nose can detect and identify odourants and blends in concentrations impossible to detect with available instruments (Hellman & Small, 1974). Humans link an odour which they have experienced previously to other gustatory or olfactory experiences (Guadagni *et al*., 1963). Odours are inhaled through the nose and bind to olfactory receptors, which then relay the odour information via olfactory sensory neurons to the brain (Guadagni *et al*., 1963). For example, isoamyl acetate is characterized by a banana flavour and 2-phenylethyl acetate is associated with a rose like scent (Etschmann *et al.*, 2002, Vuralhan *et al.*, 2005, Saerens *et al.*, 2010, Kosseva *et al.*, 2016). Most fusel alcohol-acetate esters have fruity odour characteristics (Kosseva *et al.*, 2016). The instrumental approach for characterizing volatiles in emitted odour blends is gas chromatography coupled to various detectors, including mass spectrometry. These techniques evaluate the chemical composition of an odour blend and the concentration of individual components in the blend (Scott, 2003, Cooks *et al.*, 2006). Volatiles can be identified by mass spectrometry which can provide detailed structural information on the compounds (Scott, 2003, Cooks *et al*., 2006).

So far, most of the research on the production of fusel alcohols and acetates by fungi has focused on brewer's and baker's yeast, *S. cerevisiae* (Hazelwood *et al*., 2008). In order to fill an increasing demand for natural volatiles in the industries more research must be conducted on the biosynthesis of these valuable compounds by other fungi as well. In this study, we investigated fusel alcohol biosynthesis in the family Ceratocystidaceae. Fungi belonging to the Ceratocystidaceae are well known for producing enticing banana, fruity and rose scents which they use to attract insects vectors (Kile, 1993, Juzwik *et al.*, 2004, Birkemoe *et al.*, 2018). These fungi are often devastating plant

pathogens (Kile, 1993, Engelbrecht & Harrington, 2005). The most wide-spread genus in the Ceratocystidaceae is *Ceratocystis*, which contains currently 32 species which all have smooth non-ornamented ascomata and hat-shaped ascospores (De Beer *et al.*, 2014). These morphological features distinguish *Ceratocystis* from two other genera in the family, known as *Thielaviopsis* and *Huntiella* which have ornamented ascomata with different types of ornamentations and obvoid ascospores (De Beer *et al.*, 2014). Another genus belonging to the Ceratocystidaceae family is *Endoconidiophora* which is characterized by its symbiotic interactions with conifer-inhabiting bark beetles (Yamaoka *et al*., 1998, Wingfield *et al*., 2013). Three new genera were recently described in this family, named *Davidsoniella, Berkeleyomyces* and *Bretziella* that are pathogenic on woody and herbaceous hosts, respectively (De Beer *et al*., 2014, De Beer *et al*., 2017, Nel *et al*., 2018).

The primary aim of this study was to characterize fusel alcohol biosynthesis in the family Ceratocystidaceae. Firstly, fusel alcohols and acetates produced by fungal species in the family were analysed using gas chromatography and mass spectrometry. Secondly, in order to describe the fusel alcohol biosynthesis pathway in these fungi, amino acid sequences of putative enzymes catalysing the first and second steps of the Ehrlich pathway were identified in the genomes of the Ceratocystidaceae species available. The enzymes from the first step of the Ehrlich pathway were functionally characterized by cloning a subset of gene sequences encoding putative amino-transferases and expressing them in *Escherichia coli.* Furthermore, to determine how common fusel alcohol biosynthesis is in other fungi, amino acid sequences encoding Ehrlich pathway enzymes were also identified in other fungi in the Sordariomycetes with genome sequences deposited in the Joint Genome Institute (JGI).

2. Experimental Procedures

2.1. Fungal culture characterization

2.1.1. Fungal strains, medium and cultural conditions

Fungal cultures used in this study (Table 2) were obtained from the CMW culture collection at the Forestry and Agricultural Biotechnology Institute (FABI) at the University of Pretoria, Pretoria, South Africa. These cultures were grown and maintained at 25 °C on half-strength potato dextrose agar medium (PDA) (Merck, USA) supplemented with 0.8 % of pure agar (Merck, USA) and streptomycin antibiotic (Sigma-Aldrich, USA) to a final concentration of 50 µg/ml.

2.1.2. DNA extraction, PCR and sequencing

The Internal transcribed spacer (ITS) regions ITS1 and ITS4 were amplified and sequenced to verify whether all fungi used in this study were the correct species. The PrepMan kit (Applied Biosystems, USA) was used for genomic DNA extractions. DNA concentrations were determined using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, USA) and DNA was diluted to 150 ng/ µl for use in polymerase chain reactions (PCR).

DNA was amplified using primers ITS1 and ITS4 (White *et al.*, 1990). A 25 µl PCR reaction was prepared by using 5X MyTaq buffer (Bioline, UK), 0.5 µl (10 mM) forward and reverse primer, 0.25 µl (1U) MyTaq enzyme, deionized water, and 2 µl DNA template. The PCR thermal cycler (Applied Biosystems, USA) was programmed as follows: initial denaturation at 95 °C for 3 min, 35 cycles of denaturation at 95 °C for 30 s, annealing temperature of 55 °C for 30 s and elongation at 72 °C for 2 min and a final elongation step at 72 °C for 10 min.

PCR products were cleaned using Sephadex G650 (Sigma-Aldrich, USA). The cleaned DNA was sequenced using an ABI PRISM 3100™ automated Applied Biosystems DNA sequencer. A 10 µl sequencing reaction was set up using the following protocol; 1 µl (150 ng) DNA template, 2 µl Big Dye Terminator v3.1 (Applied Biosystems), 0.5 µl (10 mM) forward or reverse primer, 1 µl (5X) sequencing buffer (Applied Biosystems) and 5.5 µl deionized water. DNA sequences were compared to known DNA sequences using BLASTn on the National Centre for Biotechnology Information database (NCBI; https://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.2. Odour panel

In order to detect specific odours that resemble fusel alcohols and acetate scents, a sniffing panel was conducted. The odour of the volatile blend of each fungus on half strength PDA medium Petri dish plates (60 mm × 15 mm) was defined by questioning an odour panel, who voluntarily participated in the sensory tests. The fungus was grown on half strength PDA in a Petri dish and sealed with Parafilm. The panellists removed the Parafilm and slightly opened the Petri dish lid for smelling the volatiles. Once the Petri dish was opened to smell the fungus, it was not used again for sensory purposes. This was to maximize the concentration of odourants for the smelling test. The odour panel was conducted in a laboratory at FABI, University of Pretoria where there were no distractions.

Before the odour panel proceeded, panellists were taught and trained to make unbiased and objective decisions about odour characterization and identification (McGinley *et al.*, 2000). A panellist used experience and previous knowledge of odours to scale the odours they sniffed. They were reminded of individual odour experiences and memory referencing. Odours were put in different categories and this was done using referencing vocabulary of sense and odour descriptors (McGinley *et al.*, 2000). Questionnaire papers (Table 3), questioning various attributes that can be obtained utilizing the sense of smell were also given. The flavour descriptions of the panel members were recorded and then categorized according to the main flavour groups.

2.3. Analysis of fusel alcohols using gas Chromatography coupled with mass spectrometry

Fungal isolates that were included for this section of the study are shown in Table 4. These isolates were analysed using gas chromatography coupled to mass spectrometry (GC-MS) for presence of fusel alcohols and fusel acetates. Each fungus was grown on half strength PDA medium for 4 d at 25 °C. A block (approximately 4 mm^2) of growing mycelium was cut and put in a sterile 1.5 mL GC vial (Separations, SA). Fungal samples in vials were equilibrated at 28 °C for 90 min on a heating block. Volatiles were also analysed directly from whole fungal cultures grown on half strength PDA slants in test tubes incubated at 25 °C for 4d. Before analysis slants were also equilibrated at 28 °C for 90 min on a heating block.

To extract volatiles from the air above the fungal sample, a solid phase micro extraction device (SPME) was used (Fig. 3). The SPME fibre was coated with 50/30 μm Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) (Supelco, USA). Before sampling, fibre blanks were run to ensure that fibre coatings were free from contaminants prior to exposing the fibre to the given samples. During the sampling step, the SPME device needle was inserted through the vial lid septum or through the lid of a test tube containing an agar slant. The fibre was then released from the needle by depressing the SPME device plunger. Volatiles adsorbed onto the polymeric coating during this process. After 20 min of sampling the SPME fibre was retracted from the vial and injected into the injection port of a Shimadzu GCMS-QP2010 gas chromatography-mass spectrometry system (Shimadzu, Japan). A 230 °C temperature in the injection port thermally desorbed volatiles from the fibre coating and carried them into the column by a carrier gas pressure of 1 ml/min using a splitless injection. Individual volatiles adsorbed on the stationary phase of a J&W DB-5 15 m, 0.10 mm, and 0.10 μm GC column (Chemetrix, SA).

Individual compounds were separated based on their affinity to the stationary phase. As the GC temperature increased from 35 °C (initial temperature) to 230 °C (5 °C/min) volatiles were desorbed from the stationary phase. Volatiles that had a strong affinity to the stationary phase and low volatility were retained for a longer time period than those that bound weakly. Individual volatiles that desorbed from the stationary phase were carried into the mobile phase; an inert gas, helium, was used as the mobile phase at a flow rate of 1 ml/min. A column cleaning step was included where the column was heated to 300 °C for 10 min. Separated compounds moved from the GC column to a mass spectrometer where they were analysed and detected. The parameters of the mass spectrometer were set as follows: electron impact ionization potential was kept at 70 eV, ion source temperature was 230 °C, and the interface temperature was 250 °C. All the ions were detected (total ion chromatogram mode). Within the mass spectrometer, respective compounds are ionized and fragmented.

Fragmented ions were detected and mass spectra were visualized. Mass spectra show the mass to charge ratio of fragments from the ionized compounds. Compounds were identified by comparing them with spectra from the NIST library version 14 compiled by the National Institute for Standards and Technology (Boulder, USA).

2.4. Ehrlich pathway enzymes in the Ceratocystidaceae and other Sordariomycetes

Augustus gene predictions were conducted using the online Augustus programme (http://augustus.gobics.de/) to predict open reading frames from the genomes of fifteen (Table 5) sequenced fungal species from the family of Ceratocystidaceae. Ceratocystidaceae fungal species used in this study were: *C. albifundus, C. eucalypticola, Bretziella fagacearum, C. fimbriata, C. harringtonii, C. manginecans*, *Davidsoniella virescens, Endoconidiophora laricicola, E. polonica, Huntiella bhutanensis, H. decipiens, H. monilliformis, H. omanensis, H. savannae* and *Thielaviopsis punctulata* (there were two isolates from two of the species, *C. manginecans* and *T. punctulata*).

Putative amino acid sequences of enzymes catalysing reactions of the Ehrlich pathway were searched for in the genomes of members of the family of Ceratocystidaceae included in this study. This was conducted by doing local BLASTn searches in the BioEdit v 7.2.5 programme (www.mbio.ncsu.edu/BioEdit/bioedit.html) using *Saccharomyces cerevisiae S288C* amino acid sequences as references.

For the transaminase step, BAT1 (branched chain amino transferase enzyme) was searched for in fungi of the Ceratocystidaceae using *S. cerevisiae* BAT1 (NP_012078.3) as a reference (Table 6). The amino acid sequences of two aromatic aminotransferases (ARO8, NP_011313.1 and ARO9, NP_012005.1) from *S. cerevisiae* were also searched for in the Ceratocystidaceae fungi (Table 6).

Protein sequences of putative enzymes catalysing the second step of the Ehrlich pathway were also searched for in the Ceratocystidaceae using local blast searches in BioEdit. These included ARO10 (NP_010668.3), THI3 (NP_010203.1), PDC1 (NP_013145.1), PDC5 (NP_013235.1), and PDC6 (NP_011601.3) from *S. cerevisiae* and KIDV (AIS03677.1) from *Lactococcus lactis* (Table 7). All these protein sequences were used as references for BLAST searches in the genomes of the Ceratocystidaceae using local blast searches in BioEdit (this was done using the tBLASTn function). The InterProScan online programme was used to verify whether respective predicted proteins contain correct domains and if they belong to the amino transferase or pyruvate decarboxylase families.

All the fungal genomes studied produced similar BLAST hits for all amino acid sequences used as references. And thus all these fungi have genomes which potentially encode aromatic amino transferases or decarboxylase enzymes. For aromatic amino transferases, only the results from searches with ARO9 were selected for subsequent phylogenetic analysis. BLAST hits of all BAT1, ARO9 and pyruvate decarboxylase with exceptional value (E) less or equals to 0.000001 were selected and saved as separated fasta files for alignments and phylogenetic analyses. Files containing all blast hits from all fungi in the Ceratocystidaceae for specific proteins (BAT1, ARO9 or pyruvate decarboxylase) were aligned separately using MAFFT (http://mafft.cbrc.jp/alignment/server/). These alignments were used to construct maximum likelihood trees with the raxmlGUI 1.3 using LG model with 1000 bootstrapping replicates. MEGA7 (www.megasoftware.net) was used for model testing and editing of trees (Kumar *et al.*, 2016).

For comparison, putative amino acid sequences of enzymes of the Ehrlich pathway were also searched for in the genomes of other fungi in the Sordariomycetes with complete genomes deposited in the Joint Genome Institute (JGI). In total, thirty seven (37) Sordariomycetes in JGI (table 8A and 8B) were used to do blast searches using the above reference sequences. These included: *Acremonium alcalophilum, Beauveria bassiana, Chaetomium globosum, Colletotrichum acutatum, C. graminicola, C. higginsianum, Coniochaeta ligniaria, Cordyceps militaris, Cryphonectria parasitica, Daldinia eschcholzii, Eutypa lata, Fusarium fujikuroi, F. oxysporum, F. verticillioides, F. graminearum, Grosmannia clavigera, Hypoxylon sp., Ilyonectria radicicola, Metarhizium robertsii, Neurospora crassa, N. discreta, N. tetrasperma, Ophiostoma piceae, Phaeoacremonium aleophilum, Podospora anserina, Sodiomyces alkalinus, Sporotrichum*

thermophile, Thielavia appendiculata, Thielavia arenaria, T. hyrcaniae, T. terrestris, Trichoderma atroviride, T. harzianum, T. longibrachiatum, T. reesei, T. virens and *Verticillium dahliae.*

Blast hits with E. values of ≤ 0.005 were selected and putative amino acid sequences were exported as fasta files from the JGI website. Blast hits obtained in JGI for each protein (BAT1, ARO9, or PDC5) were combined with respective BLAST hits obtained from the Ceratocystidaceae to construct phylogenetic trees. Again, alignments for respective hits were made in MAFFT, resulting in three separate aligned files for the BAT1, ARO and pyruvate decarboxylase. These alignments were then used to construct maximum likelihood trees with RAxML using the same methods as above. Trees were edited in MEGA7.

2.5. Functional characterization of enzymes catalysing reactions in the Ehrlich pathway

2.5.1. RNA extraction, cDNA synthesis and PCR

Four fungal species, representing three genera in the Ceratocystidaceae, were selected for functional characterization studies. These fungi included *Ceratocystis* (*C. albifundus* and *C. manginecans*), *Endoconidiophora* (*E. polonica*) and *Huntiella* (*H. monilliformis*). Fungi were grown in sterile 2% potato dextrose (Merck, USA) broth medium in 10 ml urine sample containers (Boenmed, China) for 4 d. Growing fungal mycelium was recovered from the liquid broth using filter paper and a spatula was used to transfer the respective mycelium into 1.5 ml microcentrifuge tubes. Holes were pieced into the lids of the tubes with a needle. These were stored at -80 prior to freeze drying the following day using a SP Scientific Freeze Dryer (SP Scientific, USA) which was operated under 4 mbar pressure at a temperature of – 68 °C. Freeze dried mycelium was ground to powder in liquid nitrogen using a small plastic pestle. The plant total RNA kit (Stratec Molecular, Germany) was used to extract RNA from the mycelium following the manufacturer's protocols.

For cDNA synthesis, an RNA concentration between 100-500 ng was used. Concentrations that were above 500 ng were diluted with sterile RNase-free water. The Superscript™ IV first-strand synthesis protocol was utilized (Invitrogen, USA) for making complementary DNA from the RNA. RNA templates were annealed to 1 µl 10mM Oligo d (T) $_{20}$ primers. A reverse transcription mixture was prepared by using Superscript™ IV reverse transcriptase (200U/ µl) following the manufacturer's protocols (Invitrogen, USA) and incubated at 55°C for 10 min. cDNA was stored at -20°C for future use or used immediately for PCR reactions.

Aromatic aminotransferase primers designed to amplify the three genes designated ARO9-1, ARO9-2 and ARO9-3 and branched chain amino transferase primers designed to amplify the three genes designated BAT1-1, BAT1-2 and BAT1-3 (Table 9) were ordered from Metabion International AG (Germany). These primers were used to amplify respective cDNAs using GoTaq DNA polymerase (Promega, USA). The PCR reaction mixture was prepared as follows: buffer with final concentration of 1.5 mM $MgCl_2$, 0.2 mM dNTP mixture, 0.4 µM forward and reverse primers, 11.25 U GoTaq and 1 µl cDNA template with a concentration less than 500 ng in a total volume of 50 µl. Respective PCR reactions were amplified in a thermocycler for 35 cycles with initial denaturation temperature of 95 °C for 5 min, 35 cycles of denaturation at 95 °C for 15 s, annealing temperature of 56 °C for 15 s, extension at 72 °C for 2 min, and a final extension step at 72°C for 8 min.

2.5.2. Gateway cloning, transformation and enzyme assay

Luria Bertani (LB) agar and broth was prepared and the respective antibiotics were added after the medium had cooled down to 50 °C. Super optimal broth with catabolite repression (SOC) medium was prepared and 20 mM glucose was added after the medium was autoclaved and cooled down by passing it through a 0.2 µM filter. All reagents utilized to prepare the media were manufactured by Merck (USA).

All purified PCR products of the genes from the four fungi in the Ceratocystidaceae encoding enzymes catalysing the transamination step of the Ehrlich pathway (ARO9-1, ARO9-2, ARO9-3, BAT1-1, BAT1-2 or BAT1-3) were cloned using the Gateway cloning procedure from Invitrogen (USA). The Gateway cloning system is based on a two-step process (Marsischky & LaBaer, 2004). The

DNA fragment of interest is first cloned into pDONR 207 (Invitrogen, USA), a general donor plasmid. The respective ligation reactions were set up by mixing 6 µl of the respective PCR products with 2 µl of TE buffer (pH 7.8), 1 µl pDONR vector (150ng) and 1 µl BP clonase enzyme (Invitrogen, USA). These were incubated overnight at 25 °C and were used for transforming chemically competent Top 10 *Escherichia coli* cells (Invitrogen, USA) through heat shock. Competent Top 10 cells were prepared with two washes with 80 mM $CaCl_2$-50 mM $MgCl_2$ solution and a single wash with 100mM $CaCl_2$. 100 ul aliquots of cells suspended in 100 mM $CaCl_2$ and 20% glycerol and were immediately flash frozen in liquid nitrogen. These cells were stored at -80 °C for later use. Cells transformed with the respective plasmids were incubated overnight on LB agar with gentamycin. Positive colonies were screened by colony PCR. Colonies with plasmids containing the correct insert sizes were grown in LB broth overnight and plasmids were extracted using the NucleoSpin plasmid EasyPure kit (Macherey Nagel, Germany).

Inserts in the pDONR plasmids were transferred to the destination plasmids (PH9) using 150 ng pDONR plasmid (with the insert), 150 ng PH9 plasmid, 1 µl LR clonase enzyme mixture and TE buffer. Respective cloning reactions were incubated at room temperature overnight and were used to transform Top 10 competent cells. Transformed bacterial cells were grown on kanamycin LB agar plates in the 37°C incubator overnight. Colonies were screened by colony PCR using the appropriate primers (for ARO9-1, ARO9-2, ARO9-3, BAT1-1, BAT1-2 or BAT1-3). All of the cloned products were sequenced using the protocols above.

PH9 plasmids each containing either of the transaminase genes (*aro9-1, aro9-2, aro9-3, bat1-1, bat1-2* or *bat1-3*) were used to transform BL21 *E. coli* cells (Invitrogen, USA). Transformed cells were inoculated in 5 ml of LB broth with kanamycin and incubated overnight in a shaking incubator at 200 RPM speed at 37 °C. Cultures were transferred into Erlenmeyer flasks with 45 ml kanamycin-amended auto induction terrific broth (Merck, USA) and were incubated for 48 h in a shaking incubator at 200 RPM speed at 18 °C. Cells were harvested and centrifuged in an Eppendorf 5810R Centrifuge (Eppendorf, Germany,) for 20 min at maximum speed (4000 RPM) at 4°C to pellet the cells. Respective pellets were resuspended on ice in 8 ml 100 mM Tris HCl buffer (pH 8) with 10 % glycerol. Pelleted bacterial cells were lysed by sonication for 2 min in a QSONICA ultrasonic processor (Qsonica, USA) using 10 s pulses and 65 % amplitude. Lysed cells were further centrifuged for 30 min at a maximum speed and proteins in the supernatant were used for enzyme assays, from which 500 µl was added in the enzyme assay reaction which contained 60 µl 10 mM oxoglutarate, 10 µl 0.05 mM pyridoxal phosphate, 30 µl 3 mM amino acid substrate (either phenylalanine, tryptophan or tyrosine in aromatic amino transferase enzyme assays; either leucine, isoleucine or valine in the branched chain aminotransferase enzyme assays). The enzyme assay reactions were incubated for 2h at 30°C. There were three replicates for each substrate per enzyme. After incubation, 500 µl methanol was added to the enzyme assay reaction which was centrifuged for 30 min at a maximum speed 14 000 rpm and 10 µl of supernatant was analysed using high-performance liquid chromatography coupled to mass spectrometry (LC-MS; branch-chain amino acids) or a UV detector (LC-UV; aromatic amino acids).

Compounds from the enzyme assays were separated on a Nucleodur Sphinx RP18ec reversed phase column (dimensions 250 × 4.6 mm and a particle size of 5 µm; Macherey Nagel, Germany) using an Agilent 1100 series HPLC (Agilent Technologies, USA). 0.2 % aqueous formic acid (A) and acetonitrile (B) were used at a flow rate of 1.0 ml/min. The column temperature was maintained at 25°C. The proportion of B was increased from 5% to 85 % in a linear gradient of 22 min. The column was washed for 5 min with 95 % B and re-equilibrated to 5% B for 5 min. The HPLC was coupled to an Esquire 3000 electrospray ionization ion trap mass spectrometer (Bruker Daltronics, Germany). Pyruvic acids originating from the enzyme assays with branched-chain amino acids were analyzed in negative mode scanning from 100 to 300 Da (skimmer voltage, 60 V; capillary exit potential, -121 V; capillary voltage, 4000 V; drying gas,11 ml-/l, 330 °C; nebulizer gas, 35 psi). In addition, the instrument was coupled to a Bruker Daltronics Prototype UV detector at 280 nm. Compounds were identified by comparison with commercial standards (3-phenyl-2-oxopropanoate, 3-hydroxyphenyl pyruvate, 3-methyl-2-oxopropanoate, 4-methyl-2-oxopropanoate, 3-methyl-2-oxobutanoate were

kindly provided by the Department of Neuroethology, Max Planck Institute for Chemical Ecology (Jena, Germany). Compounds were quantified by manually integrating their peak areas. Specific activity was calculated for each enzyme (the amount of product produced within one hour). The relative activity was determined by calculating the percentage conversion of the primary ligand (e. g. phenylalanine) relative the secondary ligands (e. g. tyrosine and tryptophan for ARO enzymes).

3. Results

3.1. Odour panel

In total there were 16 panellists who voluntarily participated in an odour panel. These panellists sniffed 21 fungal isolates from the 41 isolates used in this study. These included 2 *Bretziella*, 2 *Berkeleyomyces*, 7 *Ceratocystis*, 1 *Davidsoniella*, 4 *Endoconidiophora*, 4 *Huntiella* isolates and 1 *Thielaviopsis* isolate. Overall, feedback given by the panellists show that the majority of the fungal isolates had a sweet, pleasant and fruity smell (Table 3) and that most fungi smelled similar to banana.

Two isolates within the *Bretziella (B. fagacearum* CMW 2039 and *B. fagacearum* CMW 2656) genus were used in the odour panel. *Bretziella fagacearum* CMW 2039 produced a smell resembling very ripe apples as the major component of the smell and a banana or flower mix smell as the minor component. At a scale of 1-5 (1- barely related, 2-slightly related, 3-moderately related, 4-related and 5-very related), the odour character relatedness of this isolate to the major component of the smell was 4; the smell was pleasant with a strong intensity. On the other hand, *B. fagacearum* CMW 2656 had a sweet fruity smell as the major component and no minor components were smelled. Odour character relatedness to the fruity smell was moderate and the smell was moderately pleasant with a strong intensity.

Two Isolates within the *Berkeleyomyces* genus produced mainly a banana scent (major components of the smell); there were no minor components smelled in these two isolates, the odour character relatedness of these two isolates to the smell of banana was 4 (Table 3). However, *Be. basicola* CMW 7065 had a lower odour intensity compared to *Be. basicola* CMW 14219.

Within the *Ceratocystis* genus, *C. albifundus*, *C. eucalypticola*, *C. fimbriata* and *C. harringtonii* were used for odour panel tests. Two isolates out of three *C. albifundus* (CMW 42433 and CMW 43584) had a pleasant banana-like smell and there were no minor components of the smell (Table 3). These isolates had moderate odour relatedness to banana and their odour intensity was also moderate. However, *C. albifundus* CMW 17620 had a fruity smell and this isolate had odour character relatedness of 4. The smell was pleasant and the odour intensity was strong (Table 3).

Ceratocystis eucalypticola isolate CMW 9998 had a sweet banana odour and an odour character relatedness scale of 4. This isolate had a pleasant smell and its odour intensity was moderate. Similar to the *C. eucalypticola* CMW 9998 isolate, *C. fimbriata* CMW 15075 also had a banana smell; the odour character relatedness was scaled at 4. This isolate also had a pleasant smell and the odour intensity was very strong. The last two isolates belonging to the *Ceratocystis* genus were *C. harringtonii* CMW 14789 and CMW 26381. The former had a sweet fruity smell while the latter had a rotten or alcohol smell. Both isolates had either a musky, spicy or an insect like minor component to the smell. These two isolates had moderate odour character relatedness and were moderately pleasant. Their odour intensity was also moderate.

There was only one isolate of the *Davidsoniella* genus that was used in the odour panel test. *Davidsoniella virescens* smelled like a very ripe banana and there were no minor components to the smell. The odour character relatedness was good and was scaled at 4. The smell was pleasant and the odour intensity was strong.

The *Endoconidiophora* genus contained 5 isolates (3 *E. laricicola* isolates and 2 *E. polonica* isolates). The three *E. laricicola* isolates with CMW number 26374, 23746 and 20928 had a sweet smell. Isolates CMW 26374 and CMW 23746 had a sweet banana smell; the minor odour component of *E. polonica* CMW 23746 was more similar to a flower mixture. *Endoconidiophora laricicola* CMW 20928 also had a sweet flowery or fruity smell; the minor component of this smell was banana-like. All of the isolates within *E. laricicola* had odour character relatedness of 4; the smell was very sweet and pleasant and

their odour intensity was strong. Furthermore, one isolate of the *E. polonica* (CMW 20930) had a banana smell with odour character relatedness of 3. The smell was pleasant and had strong odour intensity. However, *E. polonica* CMW 39742 had an earthly smell, with a mushroom minor component to the smell. The smell was unpleasant with moderate odour intensity.

Among all the isolates tested thus far, only isolates within the *Huntiella* genus did not produce the banana smell. For example, *H. bhutanensis* smelled like a rotten fruit powder and the minor component of the smell was similar to fermented cereal. This isolate had a slightly pleasant odour with strong odour intensity. In contrast, *H. decipiens* smelled similar to a pineapple, the smell was very pleasant with a strong intensity. Lastly, *H. savannae* had a sweet smell with strong odour intensity. Within the *Huntiella* genus, *H. decipiens* produced the strongest odour intensity compared to other isolates. Finally, in the *Thielaviopsis* genus, there was one isolate which had a mouldy pineapple smell and the minor component of the smell was of apple. This isolate was moderately pleasant with low odour intensity (Table 3).

3.2. Analysis of fusel alcohols using gas Chromatography coupled with mass spectrometry

3.2.1. Analysis of the volatiles produced by whole fungal cultures over four days

Ceratocystidaceae fungi were grown on 50% PDA for four days before quantitative analysis of volatiles from whole cultures by gas chromatography coupled with mass spectrometry (GCMS). Fusel alcohols and acetates identified in cultures grown on agar slants were isoamyl alcohol, isobutyl acetate, isoamyl acetate, 2-phenylethanol and 2-phenylethyl acetate (Table 4).

Most of the Ceratocystidaceae produced the branched-chain fusel acetates, isoamyl acetate and isobutyl acetate, in large quantities (Table 4). Isobutyl acetate was produced by *Bretziella, Berkeleyomyces, Ceratocystis* and *Huntiella* but not by *Davidsoniella, Endoconidiophora,* and *Thielaviopsis* (Table 4). *Bretziella, Berkeleyomyces*, *Davidsoniella* and *Thielaviopsis* produced isoamyl alcohol in large quantities. This volatile was also produced by a few isolates of *Ceratocystis* and one isolate of *Huntiella*. *Berkeleyomyces* and *Thielaviopsis* produced 2-phenylethanol, but in very low quantities. This volatile was also produced in low amounts by two isolates of the genus *Huntiella* and one isolate of the genus *Ceratocystis*. Similar to 2-phenylethanol, 2-phenylethyl acetate was also produced in low quantities by *Berkeleyomyces, Davidsoniella, Endoconidiophora, Huntiella* and a few isolates from the *Ceratocystis* genus (Table 4).

We found significant quantitative as well as qualitative variation in the volatiles produced by different isolates within a single species. For example, the *Berkeleyomyces* genus included three isolates from *Be. bassicola* (CMW 26479, 7065 and 14213). These isolates produced both aliphatic and aromatic fusel alcohols and acetates. *Berkeleyomyces bassicola* CMW 26479 produced all of the volatiles in significantly larger quantities compared to the other *Be. bassicola* isolates. This isolate produced isobutyl acetate as the major component, while isoamyl alcohol and isoamyl acetate were produced as second major components; 2-phenylethanol and 2-phenylethyl acetate were produced as minor components. *Berkeleyomyces bassicola* CMW 7065 produced isoamyl alcohol as the major component, while isobutyl acetate and 2-phenylethanol were produced as minor components. Lastly, *Be. bassicola* CMW 14216 produced isoamyl acetate as the major component whereas isobutyl acetate, 2-phenylethanol and 2-phenylethyl acetate were produced as minor components.

Species within the *Ceratocystis* genus, on the other hand, produced relatively similar volatile profiles. We included *C. albifundus, C. eucalypticola, C. fimbriata, C. harringtonii and C. manginecans* in the analysis. All species produced mainly aliphatic fusel acetates (isoamyl acetate and isobutyl acetate). For example, *C. albifundus* isolates (CMW 43584, 18784, 24887 and 42433) produced isoamyl acetate as the major component and isobutyl acetate as the second major component with 2-phenylethyl acetate as a minor component. Most *C. fimbriata* isolates showed a similar profile, producing only isoamyl acetate as the major component and isobutyl acetate as the second major component. On the other hand, *C. manginecans* isolates, with the exception of CMW 28232, showed the opposite profile, with isobutyl acetate as the major component and isoamyl acetate as the second major component of the emitted volatile blend. Interestingly, isolates of *C. eucalypticola* (CMW, 9998,

15045 and 43694) produced isobutyl acetate as the major component and isoamyl acetate as the second major component. However, *C. eucalypticola* CMW 4799 produced isoamyl acetate as the major component while isobutyl acetate was produced as the second major component and isoamyl alcohol as the minor component.

In the genus *Endoconidiophora*, *E. laricicola* isolates (CMW 20928, 23746, and 26374) and *E. polonica* (CMW 20930) produced only isoamyl acetate as the major component and 2-phenylethyl acetate as the minor component. Among these isolates, *E. laricicola* CMW 23746 produced larger quantities of the major volatile component and *E. polonica* produced the lowest quantities of volatiles.

Three species of the genus *Huntiella* (*H. decipiens* CMW 30859, *H. monilliformis* CMW 10134 and *H. omanensis* CMW 11056) produced isoamyl acetate as the major component, isobutyl acetate as the second major component and 2-phenylethyl acetate as the minor component. From these three isolates, only *H. decipiens* CMW 30859 produced 2-phenylethanol as a minor component. The fourth species, *H. bhutanensis* CMW 8215, produced isoamyl alcohol as the major component and isobutyl acetate and 2-phenylethanol as minor components. This isolate did not produce isoamyl acetate and 2-phenylethyl acetate like the other *Huntiella* species. *Huntiella decipiens* CMW 30859 produced the highest quantities of these volatiles compared the other species, illustrating the large qualitative and quantitative differences in volatile emission between isolates of the same species or the same genus.

Only one species each from the genera *Thielaviopsis* and *Bretziella* were included in volatile analysis of whole cultures. *Thielaviopsis punctulata* 26389 produced isoamyl alcohol as a major component, while isoamyl acetate and 2-phenylethanol were produced as minor components. Similarly, *Bretziella fagacearum* 2656 produced isoamyl alcohol as a major component. However, this isolate produced isobutyl acetate and isoamyl acetate as minor components

3.2.2. Analysis of the volatiles produced de novo by fungal plugs

In a second experiment, volatiles were analysed from fungal plugs harvested from the edges of actively growing cultures of single isolates from each species to determine which volatiles are produced *de novo* during fungal growth. For each isolate, three plugs were harvested and the volatiles produced during 90 minutes were analysed by GCMS. When the different genera of the Ceratocystidaceae were compared with each other, they were found to produce unique volatile bouquets (Fig. 4) due to their production of different ratios of isobutyl acetate (A), 2-phenylethyl acetate (B), isoamyl acetate (C) and isoamyl alcohol (D). For instance, isobutyl acetate was only produced by *Berkeleyomyces, Ceratocystis* and *Davidsoniella* and 2-phenylethyl acetate was only produced by isolates of genera *Davidsoniella, Endoconidiophora* and *Thielaviopsis* (Fig. 4 and 5). However, all fungi in the Ceratocystidaceae produced isoamyl acetate. Lastly, isoamyl alcohol was produced by almost all fungi in the Ceratocystidaceae except fungi of the genera *Davidsoniella* and *Thielaviopsis* (Fig. 4 and 5).

The three biological replicates (fungal plugs harvested from the edge of the actively growing mycelium) from each fungal species produced similar volatiles and grouped closely to each other when plotted on a heat map (Fig. 5). The only exception was *C. manginecans,* where one isolate grouped with *C. fimbriata*, while the other two isolates grouped closely to *E. polonica* and *C. albifundus.* Even though, the three *C. manginecans* isolates in the heat map grouped separately from each other, they all produced isoamyl acetate, isobutyl acetate and isoamyl alcohol. But the *C. manginecans* that grouped with the *C. fimbriata* produced lower levels isoamyl acetate and isobutyl acetate compared to other two *C. manginecans* isolates (Fig. 5).

Dark brown colour in the heat map correlates to larger quantities of the volatiles produced while dark blue colour correlate significantly lower quantities of the volatiles (Fig. 5). Apart from this, the heat map also shows that species within the different genera of the Ceratocystidaceae produce similar odour bouquets except for *Davidsoniella,* where *D. eucalypti* produced a blend dominated by isoamyl and isobutyl acetate (similar to the genus *Ceratocystis*) whereas *D. virescens* produced mainly 2-phenylethyl acetate (similar to the genus *Thielaviopsis*). *Ceratocystis*, *Huntiella* and *Berkeleyomyces* produced mainly aliphatic fusel acetates but no 2-phenylethyl acetate (Fig. 5). In contrast,

Endoconidiophora and *Thielaviopsis* produced higher levels of 2-phenylethyl acetate. Overall, *Huntiella* produced lower levels of fusel alcohols and acetates than the other genera.

3.2.3. Comparisons of volatiles produced de novo by fungal plugs and volatiles produced by whole cultures

Taken together, results of the two experiments involving (1) quantitative analysis of volatiles of whole fungal cultures incubated for 4 days (Table 4) and (2) quantitative analysis of *de novo* synthesised volatiles from fungal plugs harvested from the edges of actively growing cultures (Fig. 4 and 5) showed that all fungi in the Ceratocystidaceae produced high levels of isoamyl acetate. *Berkeleyomyces, Ceratocystis* and *Huntiella* produced isobutyl acetate from whole fungal cultures (Table 4) while this compound was produced by *Berkeleyomyces, Ceratocystis and Davidsoniella de novo*. Furthermore, all fungi except *Davidsoniella* and *Thielaviopsis* produced isoamyl alcohol *de novo* (Fig. 4 and 5) but when analysed from whole cultures (Table 4) only *Davidsoniella* and *Thielaviopsis* produced isoamyl alcohol and *Endoconidiophora* did not. In addition to this, several isolates of the genus *Berkeleyomyces*, one isolate of the genus *Ceratocystis*, and isolates of the genera *Huntiella* and *Thielaviopsis* produced 2-phenylethanol from whole fungal cultures (Table 4), but none produced this compound *de novo* within 90 min in sufficient quantities to be detected by the mass analyser. Furthermore, more genera produced 2-phenylethylacetate compared to 2-phenylethanol from whole fungal cultures (Table 4), including isolates of the genera *Davidsoniella, Endoconidiophora, Berkeleyomyces, Ceratocystis* and *Huntiella* while *Ceratocystis, Berkeleyomyces* and *Huntiella* did not produce this compound in sufficient quantities *de novo* (Fig. 4 and 5).

3.3. Ehrlich pathway enzymes in the Ceratocystidaceae and other Sordariomycetes

In *Saccharomyces cerevisiae,* the first step of Ehrlich pathway is catalysed by two aromatic amino transferase enzymes (ARO8 and ARO9) and two branched chain amino transferase enzymes (BAT1 and BAT2). The second step of the fusel alcohol biosynthesis pathway is catalysed by six decarboxylase enzymes (ARO10, PDC1, PDC5, PDC6, KIDV and THI3) in *S. cerevisiae*.

In total, genomes of 17 species of the Ceratocystidaceae were available. Homologous amino acid sequences for the amino transferases and pyruvate decarboxylases were identified from these genomes through local tBlastn searches in BioEdit using *S. cerevisiae* protein sequences. Three aromatic aminotransferases with homology to ARO8/9, three branched-chain amino transferases with homology to BAT1/2, and a single pyruvate decarboxylase with homology to ARO10, PDC1, PDC5, PDC6, KIDV and THI3 was identified from each of the 17 species of the Ceratocystidaceae (Table 6 and 7).

3.3.1. Aromatic amino transferases

In total 51 sequences for aromatic amino transferase enzymes (ARO9) were identified from 17 genomes of species in the Ceratocystidaceae. The aligned ARO9 sequences contained a conserved region, which was characterised by 11 invariant amino acid residues that are thought to contain the active site which binds to pyridoxal 5′ phosphate and the substrates phenylalanine, tyrosine and tryptophan (Iraqui *et al.*, 1998). These amino acid sequences also shared other conserved residues. In comparison to the protein structure of ARO9 from *S. cerevisiae* (Bulfer *et al.*, 2013) the three ARO9 copies identified from the Ceratocystidaceae showed that they possess a similar common fold for interacting with pyridoxal 5′ phosphate. Analysis with InterProScan revealed that they all belong to a superfamily of homologous proteins whose conserved domain includes a number of other pyridoxal-5′-phosphate-dependent enzymes.

A maximum likelihood tree of the ARO9 gene region of the Ceratocystidaceae (Fig. 6) revealed that the three copies of this gene identified in the Ceratocystidaceae grouped into three different clades. Three copies of this enzyme were also identified in *S. cerevisiae*, with two copies grouping in clade 1 and the other copy grouping in clade 3. Within each clade, *Ceratocystis* species grouped together into a sub-clade and the *Davidsoniella* species grouped with the *Endoconidiophora* species into

a sister clade. Similarly, the *Huntiella* species grouped into their own clade, with *B. fagacearum* forming a sister clade. Lastly, the amino acid sequences from the two *T. punctulata* genomes grouped together.

The copy number of the ARO9 genes from the Ceratocystidaceae was compared with other Sordariomycetes using available genomes on JGI MycoCosm. *Acremonium alcalophilum, Colletotrichum acutatum, Colletotrichum graminicola, Cordyceps militaris, Neurospora crassa, Neurospora discreta, Neurospora tetrasperma, Thielavia arenaria* and *Verticillium dahliae* had a similar copy number of ARO genes compared to the Ceratocystidaceae (Table 9). Most of the other Sordariomycetes in JGI had a lower copy number with the majority of fungi containing two copies of ARO while some fungi only contained a single copy (Table 9).

On the other hand, *Trichoderma harzianum* had 4 copies of ARO; this suggests that one of the ARO1 group genes was duplicated

Maximum likelihood analysis of the combined ARO9 copies of the species from JGI MycoCosm and the Ceratocystidaceae showed that the Sordariomycetes species from JGI grouped together with the Ceratocystidaceae into 3 clades. In clade 1 the Sordariomycetes grouped into two separate sub-clades (Fig. 7). The first sub-clade contained species from *Colletotrichum*, species from *Trichoderma* which formed two sister clades and *Cordyceps militaris* which grouped alone. Species from the *Acremonium* and *Sodiomyces* grouped separately into a sister clade in the second sub-clade, with *Neurospora* and *Podospora* forming a second sister clade. Furthermore, the Ceratocystidaceae also formed a distinct sub-clade which consistently grouped similar to the ML tree containing only the Ceratocystidaceae shown above. A single copy of ARO 8 of *S. cerevisiae* grouped separately from the Ceratocystidaceae and Sordariomycetes species from JGI MycoCosm.

The expanded clade 2 of the combined tree (Fig. 8) of the Ceratocystidaceae and JGI Sordariomycetes included the two copies of *S. cerevisiae* ARO 9 sequences (13102 and 12362). However, there were more JGI Sordariomycetes species that grouped in clade 2 than in clade 1 (Fig. 8). For some species from JGI MycoCosm clade 2 contained more than one gene copy, for example, two *Neurospora crassa* (21415 and 2141), two *Fusarium verticillioides* (22295 and 21418) and two *Fusarium fujikuroi* (*Fusarium fujikuroi* 1610 and 11169). Furthermore, a few species from JGI MycoCosm which did not have an amino acid sequence grouping in clade 1, but had sequences that fell into clade 2. Similar to the expanded clade 1 (Fig. 7), the Ceratocystidaceae grouped separately from the other Sordariomycetes and into the respective *Ceratocystis, Thielaviopsis, Huntiella, Davidsoniella* and *Endoconidiophora* sister clades observed in clade 1.

Lastly, representative species from Sordariomycetes in JGI that contained the third copy of ARO grouped into a third cade (expanded in Fig. 9). These included species from the genera *Cordyceps, Colletotrichum, Phaeoacremonium, Coniochaeta, Podospora, Chaetomium, Sporotrichum, Thielavia, Neurospora, Daldinia* and *Eutypa.* This expanded clade shows three separate sub-groupings, including a sub-clade containing only two Sordariomycetes species (*Acremonium alcalophilum* and *Verticillium dahliae*) from JGI. All other Sordariomycetes grouped in sub-clade 1, while the Ceratocystidaceae again formed a separate sub-clade, showing similar clustering as in the other two clades (Fig. 7 and 8).

3.3.2. Branched chain amino transferases

In total 51 sequences for branched chain amino transferase enzymes (BAT) were identified from 17 genomes of species in the Ceratocystidaceae. The aligned BAT sequences contained a conserved region that is thought to contain the active site which binds to the cofactor pyridoxal 5′ phosphate and the substrates leucine, isoleucine and valine. Analysis with InterProScan revealed that they all belong to phosphate dependent enzyme class IV (PLPDE_IV) superfamily. Pyridoxal 5'-phosphate forms an internal aldimine bond (Schiff base linkage) with the catalytic residue lysine, which is also found in the conserved region of all the BAT copies of the Ceratocystidaceae. In comparison to the protein structure of BAT from the human mitochondrial branched chain aminotransferase (Bertrand *et al.*, 2015, Pavkov-Keller *et al.*, 2016, Anderson *et al.*, 2017), the three BAT copies identified from the Ceratocystidaceae showed that they possess a similar common fold for interacting with pyridoxal 5′ phosphate.

A maximum likelihood tree of the BAT1 gene region of the Ceratocystidaceae (Fig. 10) revealed that the three copies of this gene identified in the Ceratocystidaceae grouped into three different clades. Two copies of this enzyme were also identified in *S. cerevisiae*, which grouped in clade 1. Within each clade, *Ceratocystis* species grouped together into a sub-clade and the *Davidsoniella* species grouped with the *Endoconidiophora* species into a sister clade. Similarly, the *Huntiella* species grouped into their own clade, with *B. fagacearum* forming a sister clade. Lastly, the amino acid sequences from the two *T. punctulata* genomes grouped together.

The copy number of the BAT1 genes from the Ceratocystidaceae was compared with other Sordariomycetes using available genomes on JGI MycoCosm. Most of Sordariomycetes in JGI had a similar copy number of BAT genes compared to the Ceratocystidaceae (Table 9). However, *Acremonium alcalophilum* (22042 and 2202), *Ophiostoma piceae* (1638 and 12878), *Thielavia arenaria* (17825 and 18344) and *Trichoderma longibrachiatum* (31438 and 3133) only had two copies of the BAT1 gene. The two copies of *Acremonium alcalophilum* and *Ophiostoma piceae* grouped into the first and second clade, while copies of *Thielavia arenaria* and *Trichoderma longibrachiatum* grouped into the first and third clade (Fig. 10, 11 and 12).

Maximum likelihood analysis of the combined BAT1 gene copies of the species from JGI MycoCosm and the Ceratocystidaceae showed that the Sordariomycetes species from JGI grouped together with the Ceratocystidaceae into 3 clades. In clade 1 the Sordariomycetes grouped into two separate sub-clades (Fig. 11). The first sub-clade contained species from *Fusarium* and *Cordyceps* which formed a sister clade with species from *Beauveria.* Species from *Sporotrichum, Thielavia, Chaetomium, Neurospora, Podospora, Grosmannia, Ophiostoma, Cryphonectria, Eutypa, Daldinia, Hypoxylon, Trichoderma, Phaeoacremonium, Verticillium, Colletotrichum, Sodiomyces, Acremonium,* and *Ilyonectria* grouped separately in the second sub-clade. However, *Thielavia arenaria* did not contain an amino acid sequence that grouped in clade 1. Furthermore, the Ceratocystidaceae also formed a distinct sub-clade which consistently grouped similar to the ML tree containing only the Ceratocystidaceae shown above.

The expanded clade 2 of the combined tree (Fig. 12) of the Ceratocystidaceae and JGI Sordariomycetes showed that the Sordariomycetes species from JGI grouped into a single sub-clade. This sub-clade contained species from *Sporotrichum, Thielavia, Chaetomium, Neurospora, Podospora, Grosmannia, Ophiostoma, Cryphonectria, Eutypa, Daldinia, Hypoxylon, Trichoderma Phaeoacremonium, Verticillium, Colletotrichum, Sodiomyces, Acremonium,* and *Ilyonectria Fusarium, Beauveria* and *Cordyceps.* However, *Trichoderma longibrachiatum* did not have an amino acid sequence grouping in clade 2. Similar to the expanded clade 1 (Fig. 11), the Ceratocystidaceae grouped separately from the other Sordariomycetes into respective *Ceratocystis, Thielaviopsis, Huntiella, Davidsoniella* and *Endoconidiophora* sister clades also observed in clade 1.

Lastly, representative species from Sordariomycetes in JGI that contained the third copy of BAT grouped into the third cade (expanded in Fig. 13). Similar to the expanded clade 2 in Fig. 12, species from JGI MycoCosm grouped into a single sub-clade, which contained species from *Sporotrichum, Thielavia, Chaetomium, Neurospora, Podospora, Grosmannia, Cryphonectria, Eutypa, Daldinia, Hypoxylon, Trichoderma, Phaeoacremonium, Verticillium, Colletotrichum* and *Sodiomyces. Ilyonectria, Fusarium, Beauveria* and *Cordyceps* grouped into one sub-clade in clade 3 (Fig. 13). However, *Acremonium alcalophilum* and *Ophiostoma piceae* did not contain amino acid sequence that grouped in clade 3.

3.3.3. Pyruvate decarboxylase

In total, the Ceratocystidaceae encoded a single pyruvate decarboxylase gene when all of the pyruvate decarboxylases (ARO10, KIVD PDC1, PDC5, PDC6 and THI3) were used in blast searches of all the 17 genomes of species in the Ceratocystidaceae. All of the sequences obtained from blasting all of the pyruvate decarboxylases (ARO10, KIVD PDC1, PDC5, PDC6 and THI3) contained similar blast hits (Table 7), The aligned pyruvate decarboxylase sequences contained a conserved region with a pyrimidine (PYR) binding domain found in many key metabolic decarboxylase enzymes which use thiamine pyrophosphate (TPP) as a cofactor, thus belonging to the TPP family.

A maximum likelihood tree of the pyruvate decarboxylase gene region of the Ceratocystidaceae (Fig. 14) revealed that a single copy of this gene region identified in the Ceratocystidaceae grouped into single clade. Within this clade, *Ceratocystis* species grouped together into a sub-clade and the *Davidsoniella* species grouped with the *Endoconidiophora* species into a sister clade. Similarly, the *Huntiella* species grouped into their own clade, with *B. fagacearum* forming a sister clade. Lastly, the amino acid sequences from the two *T. punctulata* genomes grouped together. Included in this ML phylogeny is a single copy of *S. cerevisiae* ARO10 which formed a sister clade with *Lactococcus lactis* KIVD copy and grouped separately from a single copy of *S. cerevisiae* PDC1, PDC5, PDC6 and THI3 sister clade.

The copy number of the pyruvate decarboxylase gene from the Ceratocystidaceae was compared with other Sordariomycetes using available genomes on JGI MycoCosm. All of Sordariomycetes in JGI had a single copy of the pyruvate decarboxylase gene compared to the Ceratocystidaceae (Table 9). Maximum likelihood analysis of the combined pyruvate decarboxylase dataset of the species from JGI MycoCosm and the Ceratocystidaceae showed that all of the Sordariomycetes species from JGI grouped separately into a sub-clade (Fig. 15). Furthermore, the Ceratocystidaceae and *S. cerevisiae* also formed distinct sub-clades that consistently grouped similar to the ML tree containing only the Ceratocystidaceae shown above.

3.4. Enzyme assay of Ehrlich pathway enzymes in the Ceratocystidaceae

3.4.1. Aromatic amino transferases

The three aromatic amino transferase enzymes with the closest identity to ARO8 and ARO9 from *S. cerevisiae* were assayed for their enzyme activity using the full complement of enzymes identified from four fungal species (*C. manginecans, C. albifundus, E. polonica* and *H. monilliformis*). All the tested enzymes accepted the substrates phenylalanine, tryptophan and tyrosine which resulted in production of 2-oxo-3-phenylpropanoate, indole-3-pyruvate and 3-(4-hydroxyphenyl) pyruvate, respectively (Table 10, Table 11 and Fig. 16). Overall, ARO9 -1 enzyme activity was higher than the activities of ARO9-2 and 9-3 within each species with the substrate phenylalanine (Fig. 16). Between the species, the specific activities of enzymes of *C. manginecans* with phenylalanine were lower than the specific activities of the enzymes from other species. The enzyme activities with tyrosine relative to their activities with phenylalanine were more variable, but on average only 50% compared to phenylalanine. The highest relative activity with tyrosine was found for ARO 9-2 enzymes across all species (Table 10). Interestingly, the activity of ARO 9-3 from *C. albifundus* and *H. monilliformis* were significantly lower than all the other enzymes tested (5% of its activity with phenylalanine in *C. albifundus* and 0% in *H. monilliformis*), indicating that this enzyme poorly transforms tyrosine to 3-hydroxyphenylpyruvate in these fungi. The activity of ARO 9-3 from *H. monilliformis* was also significantly lower with all of the substrates (phenylalanine, tyrosine and tryptophan). Activities of the fungal ARO9 enzymes (except ARO 9-3 of *H.* monilliformis) with the substrate tryptophan relative to phenylalanine were more than 100%, indicating that these enzymes have higher activities with tryptophan than with phenylalanine. The relative activities with this substrate were higher for enzymes from the genus *Ceratocystis* than for *Huntiella* and *Endoconidiophora* (Table 11). The highest relative activity with tyrosine was recorded for ARO 9-2 from *C. albifundus* and ARO 9-3 from *C. manginecans.* All other enzymes showed fairly similar relative activities with tyrosine.

3.4.2. Branched chain amino transferases

The three branched chain amino transferase enzymes identified from the Ceratocystidaceae with the closest identity to BAT1 from *S. cerevisiae* were assayed for their enzyme activity using a subset of enzymes from three fungal species (*C. albifundus, E. polonica* and *H. monilliformis*). All the tested enzymes accepted the substrates valine, leucine and isoleucine which resulted in production of 3-methyl-2-oxobutanoate, 4-methyl-2-oxopentanoate and 3-methyl-2-oxopentanoate (Table 11, Table 12 and Fig. 17), respectively. However, enzyme activities of BAT1-3 (*C. albifundus),* BAT1-2 and 3 (*E. polonica*) and BAT1-1 and 2 (*H. monilliformis*) were not determined due to equipment failure.

Overall, BAT1-1 enzyme activity was higher than the activity of BAT 1-2 in *C. albifundus*, while BAT 1-1 enzyme activity was lower than the activity of BAT 1-3 in *H. monilliformis* with the substrate leucine (Fig. 17). Between the species, the specific activity of BAT 1-1 from *C. albifundus* with the substrate leucine was higher. The enzyme activities with valine relative to their activities with leucine were mostly evenly distributed and the enzymes were 80% as active as with leucine. BAT 1-1 enzymes across all species had the highest activity when incubated with valine as the substrate (Table 11). All BAT1 enzymes were 80% as active with isoleucine relative to their activities with leucine and were also mostly evenly distributed (Table 13). Activities of the fungal BAT1 enzymes with the substrate leucine were higher compared to those with isoleucine and valine. In addition, the activities with leucine, valine and isoleucine were higher for enzymes from the genus *Ceratocystis* than for *Huntiella* and *Endoconidiophora* (Fig. 17, Table 11 and 12). The highest relative activity with leucine was recorded for BAT 1-1 from *C. albifundus.* BAT 1-3 enzyme activities of *H. monilliformis* with the substrate valine and isoleucine were higher compared to BAT 1-2 enzyme activities of *C. albifundus* (Fig. 17).

4. Discussion

4.1. The Ceratocystidaceae encode multiple enzymes involved in the first step and a single enzyme of the second step of the Ehrlich pathway

We were able to identify fusel alcohols and acetates produced by fungal species in the family Ceratocystidaceae using gas chromatography mass spectrometry. Our findings showed that fungi in the Ceratocystidaceae produced fruity, banana or floral scents consisting of mixtures of volatile chemical compounds, including fusel alcohols and acetates. This led us to hypothesise that these volatiles are produced via biochemical reactions catalysed by enzymes in a pathway, known as the Ehrlich pathway, which was previously described in *S. cerevisiae* (Ehrlich, 1907, Sentheshanmuganathan, 1960). Consequently, we identified amino acid sequences of the enzymes catalysing the first and second steps of the Ehrlich pathway in the genomes of the Ceratocystidaceae and some of these putative enzymes were functionally characterized by assaying their enzyme activities *in vitro*.

In this study, we showed that the genomes of fungi in the Ceratocystidaceae contain three functional branched chain amino transferase (BAT1) enzymes which catalyse the first step in aliphatic fusel alcohol biosynthesis. This is different from what was found in *S. cerevisiae* where only two functional BAT1 enzymes were recorded in the genome (Colón *et al.*, 2011). On the other hand, the *S. cerevisiae* (Iraqui *et al.*, 1998, Rząd & Gabriel, 2015, Ohashi *et al.*, 2017) and the Ceratocystidaceae genomes both encode three functional copies of the aromatic amino transferase (ARO8/9) enzymes that catalyse the first step in aromatic fusel alcohol biosynthesis. Most of the representative Sordariomycetes included in this study also have similar numbers of copies of each gene family as the Ceratocystidaceae. All of the identified *bat1* and *aro8/9* genes contained a conserved region, with an active site that binds to the co-factor pyridoxal 5′ phosphate and the respective amino acid substrates (Bertrand *et al.*, 2015, Pavkov-Keller *et al.*, 2016, Anderson *et al.*, 2017).

It is possible that the three *aro8/9* and *bat1* copies in the Ceratocystidaceae might have resulted from whole genome duplication in an ancestral species of the Sordariomycetes. Whole genome duplication (WGD) has been confirmed in *S. cerevisiae* (Wolfe & Shields, 1997, Kellis *et al.*, 2004) and the two gene copies of *bat* in *S. cerevisiae* form part of a duplicated chromosomal block (Wolfe & Shields, 1997, Kellis *et al.*, 2004, Colón *et al.*, 2011). It is thought that the two paralogous *bat* genes (*bat1* and *bat2*) in *S. cerevisiae* arose from an ancestral gene *klbat1* from *Kluyveromyces lactis* (Colón *et al.*, 2011). The lineage which gave rise to *K. lactis* diverged before the WGD event and as a result, *K. lactis* does not contain the duplication block present in *S. cerevisiae* (Kellis *et al.*, 2004).

Aminotransferases constitute an interesting model to study diversification of paralogous genes carrying out two functions since amino transferases are part of both biosynthetic as well as catabolic pathways whose opposed action relies on a single catalytic site (Colón *et al.*, 2011). In *S. cerevisiae* the biosynthetic and catabolic roles of the branched chain amino transferase have been allocated as

follows: BAT1 is preferentially involved in branched chain amino acid biosynthesis while BAT2 is involved in branched chain amino acid catabolism, indicating functional diversification (Colón *et al.*, 2011). The original gene present in *K. lactis* performs both functions and therefore could not be independently improved. However, after duplication each gene copy can be driven by positive selection to improve one of the two functions (Hough & Stevens, 1961). It is therefore possible, that each of the gene copies present in the Ceratocystidaceae has a unique function and is regulated differently during the fungal life cycle. Enzymes of the Ceratocystidaceae that grouped in clade 2 have highest sequence similarity to BAT2 of the *S. cerevisiae* (Fig. 10). On the other hand, the other two additional copies of both the Ceratocystidaceae that grouped in clade 1 and 3 were also related to BAT2 sequences of *S. cerevisiae* but in lower sequence similarity compared to sequences that grouped in clade 2. Sequences that grouped in clade 3 had the lowest sequence similarity to the *S. cerevisiae* two copies of BAT2.

We further identified a single copy of a pyruvate decarboxylase enzyme in the Ceratocystidaceae, catalysing the second step of the Ehrlich pathway (Ehrlich, 1907, Sentheshanmuganathan, 1960, Hazelwood *et al.*, 2008). The pyruvate decarboxylase gene encodes a thiamine diphosphate-dependent decarboxylase enzyme belonging to the TPP family. In contrast to the Ceratocystidaceae with only one gene encoding a pyruvate decarboxylase enzyme, the genome of *S. cerevisiae* encodes five functional pyruvate decarboxylases which are all involved in the Ehrlich pathway (ARO10, PDC1, PDC5, PDC6, and THI3) (Hohmann & Meacock, 1998, Vuralhan *et al.*, 2003, Vuralhan *et al.*, 2005). The five pyruvate decarboxylase isoenzymes in *S. cerevisiae* (Hohmann, 1991) are involved in the catabolism of the branched-chain amino acids, isoleucine, leucine and valine (Vuralhan *et al.*, 2003). THI3, PDC1, PDC5 and PDC6 catalyse the decarboxylation of all three branched chain amino acids in *S. cerevisiae,* while ARO10 has higher specificity for isoleucine (Dickinson *et al.*, 2000).

Based on mutation studies, two members of the pyruvate decarboxylase gene family (*aro10* and *thi3*) are also essential in the decarboxylation of phenylpyruvate for production of the aromatic fusel alcohol, 2-phenyethanol (Dickinson *et al.*, 2000, Dickinson *et al.*, 2003, Vuralhan *et al.*, 2003). In contrast to the catabolic activities of the enzymes identified from *S. cerevisiae,* a pyruvate decarboxylase KIVD was identified in *Lactococcus lactis* (De La Plaza *et al.*, 2004, De La Plaza *et al.*, 2009) which showed highest specificity and activity for α-ketoisovalerate, an intermediate metabolite in valine and leucine biosynthesis. The roles of the single pyruvate decarboxylase enzyme in the Ceratocystidaceae is still unclear, however, a role in both anabolism and catabolism is probable.

The phylogenetic relationships of the aromatic amino transferase, branched chain amino transferase and decarboxylase amino acid sequences from all 17 available fungal genomes of the Ceratocystidaceae revealed that the amino acid sequences from each enzyme-group closely followed the species trees shown by De Beer *et al.* (2014). However, *B. fagacearum* did not group consistently within the same clade in our analysis and either grouped separately from the other genera or together with *Huntiella* or closely together with *Endoconidiophora*. This confirms that none of the four genera (*Ceratocystis*, *Endoconidiophora*, Thielaviopsis or *Huntiella*) are able to accommodate *B. fagacearum* (De Beer *et al.*, 2014, De Beer *et al.*, 2017). Therefore, the taxonomy of *B. fagacearum,* formerly known as *C. fagacearum,* was recently revised (De Beer *et al.*, 2017). Genes encoding enzymes therefore might also be used to infer phylogenetic relationships between fungal lineages.

4.2. The Ceratocystidaceae produce sweet smells to attract insects

This study demonstrated that the Ceratocystidaceae produced fruity, banana or floral scents consisting of mixtures of volatile chemical compounds, including fusel alcohols and acetates. Most Ceratocystidaceae produced aliphatic fusel alcohols and acetates (isoamyl acetate, isobutyl acetate and isoamyl alcohol) in larger quantities. This accounts for the distinct banana and fruity scent detected in the majority of these cultures (Etschmann *et al.*, 2002, Vuralhan *et al.*, 2003, Vuralhan *et al.*, 2005, Kosseva *et al.*, 2016). On the other hand, 2-phenylethanol and 2-phenylethyl acetate (Etschmann *et al.*, 2002), were produced in lower quantities and as a result a rose-like smell was not detected in many of these fungi.

The banana smell is associated isoamyl acetate, while isobutyl acetate is associated with a fruity smell and 2-phenylethyl acetate has a rose like odour (Etschmann *et al.*, 2002, Vuralhan *et al.*, 2005, Saerens *et al.*, 2010, Kosseva *et al.*, 2016). Some of the Ceratocystidaceae produced these scents with strong intensity. However, there were a few fungi that also produced a pleasant smell but with lower odour intensity, for example *C. albifundus* and *T. musarum*. By analysing volatiles using gas chromatography-mass spectrometry (GC-MS), we could show that a relation exists between odour intensity and the concentration of odourants affecting the odour (Grosch, 1994, Stettler & Axel, 2009) and that the stronger intensities of odours are related to larger quantities of odourants present in a sample (Ferreira *et al.*, 2000).

Microorganisms produce fusel alcohols and acetates to specifically interact with insects (Kandasamy *et al.*, 2016, Kandasamy *et al.*, 2019). Fungi can use these volatiles to attract their vectors (Gibson & Hunter, 2010, Sumby *et al.*, 2010). For example, the common fruit fly *Drosophila melanogaster*, is attracted to the baker yeast *S. cerevisiae* on which it feeds through fusel acetates (Becher *et al.*, 2012). Yeast odours represent the critical signal to establish the fly on ripe fruits (Becher *et al.*, 2012). The *Drosophila* flies gain nutrition and detoxification of harmful chemicals from *S. cerevisiae* thereby enhancing larval development, while feeding. On the other hand the yeast strains are vectored by flies which then mediate dispersal and outbreeding (Starmer & Fogleman, 1986, Reuter *et al.*, 2007).

Our results support the idea that the pleasant smells produced by fungi in the Ceratocystidaceae represent an adaptation for insect dispersal which is typical for this group of fungi (Kile, 1993, Juzwik *et al.*, 2004, Birkemoe *et al.*, 2018). The genera of the Ceratocystidaceae share similar morphological features, which include long necked ascomata and ascospores that are surrounded by sticky sheaths (De Beer *et al.*, 2014). These morphological features indicate the adaptation of these fungi to dispersal by insect vectors. *Endoconidiophora* species are attractive to bark beetles. These fungi produce more 2-phenylethyl acetate and thus have a rose scent (Redfern *et al.*, 1987, Harrington & Wingfield, 1998). Species belonging to the genera *Bretziella* and *Ceratocystis* attract nitidulid beetles with their fruity and banana smell (Appel *et al.*, 1990, Juzwik *et al.*, 2008). Furthermore, *Huntiella* with a scent that is similar to the *Ceratocystis* isolates also attracts nitidulid beetles, flies and ambrosia beetles (Kile, 1993).

The ecological roles of volatiles produced by *E. polonica*, and other fungi associated with the European bark beetle, *Ips typographus,* have been studied intensively. These fungi use fusel alcohols to mediate their interactions with bark beetles and their host, Norway spruce (Kandasamy *et al.*, 2019). It is thought that fungal volatiles released from trees that are already attacked by bark beetles and their associated fungi can repel other incoming beetles by signalling to them that the host tree or an area on the host tree is already under attack by a sufficient number of beetles to overwhelm host tree defences. Fungal volatiles may therefore repel newly arriving competing beetles (Cardoza *et al.*, 2008, Kandasamy *et al.*, 2016). Additionally, fungi use volatiles to ensure that they are maintained in the insects' galleries (Kandasamy *et al.*, 2019). Insects recognize and differentiate among different microbes based on the volatile profiles they emit, thereby preferentially maintaining beneficial microbes (Mansourian & Stensmyr, 2015). Bark beetles create conditions in their galleries in which microbes that are important to them survive. However, in this scenario, some harmful microbes, may also take advantage of the same conditions and reduce insect fitness (Hofstetter *et al.*, 2006). The response of insects to microbial volatile chemical signals through specialized sensory pathways represents one mode of evolutionary coadaptation that maintain interactions in beneficial symbioses (Kandasamy *et al.*, 2019). Since all the fungi in the Ceratocystidaceae produce attractive volatiles, we hypothesise that the biosynthesis of these compounds is specifically evolved to interact with insects, which can vector them from host to host.

4.3. Cultures of the Ceratocystidaceae produced a variety of fusel alcohols and acetates

In this study, we observed from GC-MS analysis that some of the Ceratocystidaceae produced different bouquets of fusel alcohols and acetates when whole fungal cultures accumulating volatiles for four days were analysed, compared to the volatiles produced by fungal plugs harvested from the edges of actively growing cultures. Volatiles produced *de novo* from the fungal plugs, were

synthesised in the early stages of fungal growth, while volatiles produced from whole fungal cultures were produced in the late stationary phase of fungal growth. It is, therefore, expected that there is a variation in timing of production of fusel alcohols and acetates in different fungi (Inoue *et al.*, 1997). Most volatiles are produced in fungi in larger quantities during the late stationary phase of growth (Inoue *et al.*, 1997). However, from our analysis, *de novo* production of volatiles in young fungal plugs in the Ceratocystidaceae was in some cases only slightly lower than the volatiles emitted from the stationary phase cultures.

All of the Ceratocystidaceae in this study produced fusel acetates in larger quantities than fusel alcohols when analysed from whole cultures as well as from fungal plugs. The reason for this could be the timing of the analysis in relation to fungal growth. Fusel alcohols are converted to fusel acetates after a certain time period before they are released from the culture. Landaud *et al*. (2001) showed that there is a three-phase timetable of accumulation of fusel alcohols and fusel acetates in fermentations of *S. cerevisiae*: 1, when fusel alcohols accumulate alone in fermentation, 2, when both fusel alcohols and fusel acetates accumulate at the same time and 3, when fusel acetates accumulate alone. Shorter fermentation times usually allowed higher accumulation of alcohols, while longer fermentation times allowed higher accumulation of acetates. However, in the case of the Ceratocystidaceae, the production did not depend on the incubation time, as both actively growing plugs as well as stationary phase mycelium produced similar volatile profiles. Conversely, production of fusel alcohols and acetates can depend also on the redox status of the cell (Vuralhan *et al.*, 2003, Vuralhan *et al.*, 2005). Further studies on the timing and dynamics of alcohol versus acetate production are therefore required in the Ceratocystidaceae. This includes determining which pH favours production of fusel alcohols and acetates, appropriate temperature for alcohols or acetates and effect of carbon dioxide pressures on alcohol or acetate production (Renger *et al.*, 1992). Furthermore, the regulation of acyl transferase enzymes, catalysing fusel acetate production, still needs to be studied to explain the specific volatile emission patterns of the Ceratocystidaceae.

4.4. Enzymes in the early steps of the Ehrlich pathway have a broad substrate specificity

A subset of the three aromatic amino transferase and branched chain amino transferase enzymes identified in the Ceratocystidaceae were tested for their enzyme activity in enzyme assay reactions. The aromatic amino transferase enzymes showed enzyme activity with the substrates phenylalanine, tryptophan and tyrosine. However, more enzyme activity was observed with tryptophan as a substrate compared to the substrates phenylalanine and tyrosine. In contrast to our results, enzyme assay studies using aromatic amino transferases from *Escherichia coli* and *S. cerevisiae* showed that aromatic amino transferases had more substrate specificity towards phenylalanine compared to tryptophan and tyrosine (Mavrides & Orr, 1975, Kradolfer *et al.*, 1982, Iraqui *et al.*, 1998). The results from the branched chain amino transferases in our study were consistent with results from studies conducted by Ichihara & Koyama (1966). These researchers used BAT from the submandibular gland of rats, in which more activity for the degradation of leucine was observed than was shown for isoleucine and valine.

While the enzymes characterized in this study we able to use all the substrates offered to them, the volatiles produced by the fungi when analysed using GC-MS did not include all the possible volatiles that could be formed from the amino acid substrates. Indole-3-ethanol, tyrosol and 2-methylbutanol were not produced *in vivo* by any species of the Ceratocystidaceae used in this study. We found that the enzymes from these fungi produced the intermediates indole-3-pyruvate, 3-(4-hydroxyphenyl) pyruvate and 3-methyl-2-oxopentanoate, for the production of these volatiles (Table 10, table 11, table 12 and table 13). This result is not easily explained as very little is known about amino acid metabolism in the Ceratocystidaceae.

Amino acids in fungi are involved in many other functions of the cell, as they are essential for growth. Tryptophan, for example is one of the least abundant amino acids in fungal cells and its biosynthesis requires more energy than the other standard protein amino acids (Hrazdina & Jensen, 1992). It could therefore be possible, that the three amino acids, tryptophan, tyrosine and isoleucine are not as abundant in the Ceratocystidaceae and thus volatiles produced from these amino acids are

much rarer. Future studies should focus on analysing the amino acid contents in the cells of the Ceratocystidaceae to understand the role of substrate availability in the production of fusel alcohols in these fungi.

There might be other pathways in the fungi which use the products from ARO 9/8 (2-oxo-3-phenylpropanoate and indole-3-pyruvate) and BAT1 (3-(4-hydroxyphenyl) pyruvate) to make compounds with different metabolic functions. For example fungi use indole-3-pyruvate to produce indole-3 acetic-acid (auxin) used normally as a plant growth hormone (Gruen, 1959, Strzelczyk & Pokojska-Burdziej, 1984, Contreras-Cornejo *et al.*, 2009). Apart from this, fungi degrade tryptophan to 2-acetyl-CoA molecules through the L-tryptophan degradation III pathway, which is subsequently used in the citric acid cycle (Colabroy & Begley, 2005, Caspi *et al.*, 2007). Furthermore, tryptophan can also be degraded to amino-3-carboxymuconate semi aldehyde through a completely different catabolic pathway (Caspi *et al.*, 2007)

Tyrosine is also involved in other essential processes of fungal cells such as synthesizing black or brown pigments. Many fungi are able to synthesize black or brown pigments derived from L-tyrosine through dihydroxyphenylalanine (DOPA) (Wheeler & Bell, 1988, Tsai *et al.*, 1999, Langfelder *et al.*, 2003, Rhodes & Brakhage, 2006). This might result in depleting most or the tyrosine in the cell. Many of the Ceratocystidaceae are darkly coloured fungi, which might be using DOPA polymerization for their pigmentation.

Another possible explanation of the lower diversity of volatiles produced by the Ceratocystidaceae could be that the single pyruvate decarboxylase available in the Ceratocystidaceae only accepts a few substrates in the decarboxylase step of the Ehrlich pathway. However, Dickinson and co- workers in several studies demonstrated that a single pyruvate decarboxylase could accept all the products of the amino transferase (Dickinson *et al.*, 1997, Dickinson *et al.*, 1998, Dickinson *et al.*, 2000, Dickinson *et al.*, 2003). In these studies it was demonstrated that *S. cerevisiae* triple pyruvate decarboxylase mutants (*pdc1, pdc5* and *pdc6)* produced several branched chain fusel alcohols; therefore any single isozyme of pyruvate decarboxylase is sufficient for the formation of fusel alcohols. Mutants in which all three PDC genes were knocked out, and which either only contained a functional ARO 10 or THI3, still produce branched-chain and aromatic 2-oxo aldehydes, depending on the nitrogen source in the media (ter Schure *et al.*, 1998, Dickinson *et al.*, 2003, Vuralhan *et al.*, 2003).

4.5. The biochemical and physiological mechanisms underlying the production of specific volatiles by the Ceratocystidaceae

Production of fusel alcohols by fungi result in the release of ammonium from amino acids, which can further be utilized as a nitrogen source by the cell. The carbon skeleton of the resulting 2-oxo-acid is left intact, which often cannot be used in other metabolic pathways in fungi. The Ehrlich pathway therefore metabolizes these 2-oxo-acids further, in order to excrete the waste products in the form of volatiles. However, there are a few exceptions, where the carbon atoms from the degraded amino acids are converted to metabolic intermediates of other essential biosynthetic pathways of the fungal cell such as glycolysis or the citric acid cycle (Bräsen *et al.*, 2014). These examples include alanine, which is directly transformed into pyruvate, and arginine, which can be catabolized into the citric acid cycle intermediate succinyl-CoA. It is possible, that the Ceratocystidaceae produce ammonium solely through deamination of amino acids, with fewer alternative amino acid degradation pathways available than in other fungi.

Some microorganisms such as *S. cerevisiae* use fusel alcohols to induce filamentous structures that are essential in searching for nutrients through cell elongation, formation of hyphae and invasive growth. Da Silva *et al.* (2007) showed that isoamyl alcohol induced filamentation at concentration between 0.3-0.5 % while isobutanol was only effective at 1 % concentration. Some fungi in the Ceratocystidaceae produce endoconidia, which have a yeast-like form; one possibility is that fusel alcohols inhibit the formation of endoconidia. This might explain why fungi such as *E. polonica* rarely produce endoconidia in the amino-acid rich culture medium on which they are commonly grown.

Fusel alcohols and acetates can be used by fungi to interact with other microbes. For example, volatile chemical compounds, such as 3-methyl-1-butanol, 2-methyl-1-butanol, 2-phenylethylanol

and 2-phenylethyl acetate produced by *S. cerevisiae* inhibited mycelial growth of the fungus *Guignardia citricarpa*, which causes black spot on citrus (Fialho *et al.*, 2010). Furthermore, 2-phenylethanol is a well-known bacteriostatic agent and is known to cause inhibition of the synthesis of bacterial deoxyribonucleic acids at low concentrations (Berrah & Konetzka, 1962). In addition to this, *S. cerevisiae* produces tryptophol in wines which affects the viability of *Campylobacter jejuni* bacteria (Naz *et al*., 2013) and tyrosol showed inhibitory effects against *Moraxella catarrhalis* and *Pseudomonas aeruginosa* pathogens (Cueva *et al.*, 2012). Microorganisms, therefore, produce volatile chemical compounds to affect the physiology of their competitors, causing them to be disadvantaged within a community (Mackie & Wheatley, 1999, Wheatley, 2002). Fusel alcohols might also be used by the Ceratocystidaceae to affect the life cycle of their competitors negatively while competing for nutrients (Sharma *et al.*, 2009), such as for example in bark and ambrosia beetle galleries, but further studies will have to be conducted to confirm this.

4.6. Conclusions

This study has expanded our knowledge on fungi that could be potential candidates for production of fusel alcohols and acetates. It demonstrated that the family Ceratocystidaceae that include a wide variety of genera (*Ceratocystis, Berkeleyomyces, Bretziella, Davidsoniella, Endoconidiophora, Huntiella* and *Thielaviopsis*) produced large quantities and a wide variety of fusel alcohols and acetates. So far, fusel alcohols and acetates are produced in the industries through chemical synthesis. However, these methods are very undesirable due to production of toxic chemical compounds that further contaminate the environment, high energy and pressure consumption. Thus scientists and industries are exploring various alternative methods for production of fusel alcohols and acetates, including natural synthesis of these compounds. The yeast *S. cerevisiae* is well known for its production of fusel alcohols and acetates. However, compared to the Ceratocystidaceae, *S. cerevisiae* produces significantly lower quantities of these volatiles. With this great potential, the fungi in the Ceratocystidaceae could be prime candidates to augment the supply of fusel alcohols and acetates for industrial use. With their high copy number of genes encoding the transamination step of the Ehrlich pathway, these fungi are also excellent candidates for genetic engineering of strains producing even higher levels of these compounds without affecting other metabolic processes. Further studies based on the overexpression of Ehrlich pathway genes in the Ceratocystidaceae will provide more insights for using these fungi for industrial production of fusel alcohols and acetates.

5. References

1. Anderson LC, Håkansson M, Walse B & Nilsson CL (2017) Intact protein analysis at 21 tesla and X-ray crystallography define structural differences in single amino acid variants of human mitochondrial branched-chain amino acid aminotransferase 2 (BCAT2). *Journal of the American Society for Mass Spectrometry* 28: 1796-1804.
2. Appel DN, Kurdyla T & Lewis Jr R (1990) Nitidulids as vectors of the oak wilt fungus and other *Ceratocystis spp*. in Texas. *European Journal of Forest Pathology* 20: 412-417.
3. Becher PG, Flick G, Rozpędowska E, Schmidt A, Hagman A, Lebreton S, Larsson MC, Hansson BS, Piškur J & Witzgall P (2012) Yeast, not fruit volatiles mediate *Drosophila melanogaster* attraction, oviposition and development. *Functional Ecology* 26: 822-828.
4. Beekwilder J, Alvarez-Huerta M, Neef E, Verstappen FWA, Bouwmeester HJ & Aharoni A (2004) Functional characterization of enzymes forming volatile esters from strawberry and banana. P*lant Physiology* 135: 1865-1875.
5. Bertrand SM, Ancellin N, Beaufils B, Bingham RP, Borthwick JA, Boullay A-B, Boursier E, Carter PS, Chung C-W & Churcher I (2015) The discovery of in vivo active mitochondrial branched-chain aminotransferase (BCATm) inhibitors by hybridizing fragment and HTS hits. *Journal of Medicinal Chemistry* 58: 7140-7163.
6. Birkemoe T, Jacobsen RM, Sverdrup-Thygeson A & Biedermann PHW (2018) Insect-fungus interactions in dead wood systems. *Saproxylic Insects* 1: 377-427.
7. Branduardi P, Longo V, Berterame NM, Rossi G & Porro D (2013) A novel pathway to produce butanol and isobutanol in *Saccharomyces cerevisiae. Biotechnology for Biofuels* 6: 68-80.

8. Bräsen C, Esser D, Rauch B & Siebers B (2014) Carbohydrate metabolism in Archaea: current insights into unusual enzymes and pathways and their regulation. *Microbiology and Molecular Biology* 78: 89-175.
9. Bulfer SL, Brunzelle JS & Trievel RC (2013) Crystal structure of *Saccharomyces cerevisiae* Aro8, a putative α-aminoadipate aminotransferase. *Protein Science* 22: 1417-1424.
10. Caspi R, Foerster H, Fulcher CA, Kaipa P, Krummenacker M, Latendresse M, Paley S, Rhee SY, Shearer AG & Tissier C (2007) The MetaCyc Database of metabolic pathways and enzymes and the BioCyc collection of Pathway/Genome Databases. *Nucleic Acids Research* 36: 623-631.
11. Cardoza YJ, Moser JC, Klepzig KD & Raffa KF (2008) Multipartite symbioses among fungi, mites, nematodes, and the spruce beetle, *Dendroctonus rufipennis*. *Environmental Entomology* 37: 956-963.
12. Carlini C, Macinai A, Marchionna M, Noviello M, Galletti AMR & Sbrana G (2003) Selective synthesis of isobutanol by means of the Guerbet reaction: part 3: methanol/n-propanol condensation by using bifunctional catalytic systems based on nickel, rhodium and ruthenium species with basic components. *Journal of Molecular Catalysis A: Chemical* 206: 409-418.
13. Carlquist M, Gibson B, Karagul Yuceer Y, Paraskevopoulou A, Sandell M, Angelov AI, Gotcheva V, Angelov AD, Etschmann M & de Billerbeck GM (2015) Process engineering for bioflavour production with metabolically active yeasts–a mini-review. *Yeast* 32: 123-143.
14. Colabroy KL & Begley TP (2005) Tryptophan catabolism: identification and characterization of a new degradative pathway. *Journal of Bacteriology* 187: 7866-7869.
15. Colón M, Hernández F, López K, Quezada H, González J, López G, Aranda C & González A (2011) Saccharomyces cerevisiae Bat1 and Bat2 aminotransferases have functionally diverged from the ancestral-like *Kluyveromyces lactis* orthologous enzyme. *Public Library of Science One* 6: 1-13.
16. Contreras-Cornejo HA, Macías-Rodríguez L, Cortés-Penagos C & López-Bucio J (2009) *Trichoderma virens*, a plant beneficial fungus, enhances biomass production and promotes lateral root growth through an auxin-dependent mechanism in Arabidopsis. *Plant Physiology* 149: 1579-1592.
17. Cooks RG, Ouyang Z, Takats Z & Wiseman JM (2006) Ambient mass spectrometry. *Science* 311: 1566-1570.
18. Cueva C, Mingo S, Muñoz-González I, Bustos I, Requena T, Del Campo R, Martín-Álvarez PJ, Bartolome B & Moreno-Arribas MV (2012) Antibacterial activity of wine phenolic compounds and oenological extracts against potential respiratory pathogens. *Letters in Applied Microbiology* 54: 557-563.
19. Da Silva PC, Horii J, Miranda VS, Brunetto HG & Ceccato-Antonini SR (2007) Characterization of industrial strains of *Saccharomyces cerevisiae* exhibiting filamentous growth induced by alcohols and nutrient deprivation. *World Journal of Microbiology and Biotechnology* 23: 697-704.
20. De Beer Z, Duong T, Barnes I, Wingfield B & Wingfield M (2014) Redefining *Ceratocystis* and allied genera. *Studies in Mycology* 79: 187-219.
21. De Beer ZW, Marincowitz S, Duong TA & Wingfield MJ (2017) Bretziella, a new genus to accommodate the oak wilt fungus, Ceratocystis fagacearum (Microascales, Ascomycota). *MycoKeys* 27: 1-19.
22. De La Plaza M, Fernández de Palencia P, Peláez C & Requena T (2004) Biochemical and molecular characterization of α-ketoisovalerate decarboxylase, an enzyme involved in the formation of aldehydes from amino acids by *Lactococcus lactis*. *FEMS Microbiology Letters* 238: 367-374.
23. De La Plaza M, Peláez C & Requena T (2009) Regulation of α-ketoisovalerate decarboxylase expression in *Lactococcus lactis* IFPL730. *Journal of Molecular Microbiology and Biotechnology* 17: 96-100.
24. Dickinson JR, Lanterman MM, Danner DJ, Pearson BM, Sanz P, Harrison SJ & Hewlins MJE (1997) A 13C nuclear magnetic resonance investigation of the metabolism of leucine to isoamyl alcohol in *Saccharomyces cerevisiae*. *Journal of Biological Chemistry* 272: 26871-26878.
25. Dickinson JR, Harrison SJ & Hewlins MJE (1998) An investigation of the metabolism of valine to isobutyl alcohol in *Saccharomyces cerevisiae*. *Journal of Biological Chemistry* 273: 25751-25756.
26. Dickinson JR, Harrison SJ, Dickinson JA & Hewlins MJE (2000) An investigation of the metabolism of isoleucine to active amyl alcohol in *Saccharomyces cerevisiae*. *Journal of Biological Chemistry* 275: 10937-10942.
27. Dickinson JR, Salgado LEJ & Hewlins MJE (2003) The catabolism of amino acids to long chain and complex alcohols in *Saccharomyces cerevisiae*. *Journal of Biological Chemistry* 278: 8028-8034.

28. Ehrlich F (1907) Über die Bedingungen der Fuselölbildung und über ihren Zusammenhang mit dem Eiweissaufbau der Hefe. *European Journal of Inorganic Chemistry* 40: 1027-1047.
29. Engelbrecht CJB & Harrington TC (2005) Intersterility, morphology and taxonomy of *Ceratocystis fimbriata* on sweet potato, cacao and sycamore. *Mycologia* 97: 57-69.
30. Etschmann M, Bluemke W, Sell D & Schrader J (2002) Biotechnological production of 2-phenylethanol. *Applied Microbiology and Biotechnology* 59: 1-8.
31. Ferreira V, López R & Cacho JF (2000) Quantitative determination of the odorants of young red wines from different grape varieties. *Journal of the Science of Food and Agriculture* 80: 1659-1667.
32. Fialho MB, Toffano L, Pedroso MP, Augusto F & Pascholati SF (2010) Volatile organic compounds produced by *Saccharomyces cerevisiae* inhibit the in vitro development of *Guignardia citricarpa*, the causal agent of citrus black spot. *World Journal of Microbiology and Biotechnology* 26: 925-932.
33. Gibson CM & Hunter MS (2010) Extraordinarily widespread and fantastically complex: comparative biology of endosymbiotic bacterial and fungal mutualists of insects. *Ecology Letters* 13: 223-234.
34. Goettmann F, Fischer A, Antonietti M & Thomas A (2006) Chemical synthesis of mesoporous carbon nitrides using hard templates and their use as a metal-free catalyst for Friedel–Crafts reaction of benzene. *Angewandte Chemie International Edition* 45: 4467-4471.
35. Grosch W (1994) Determination of potent odourants in foods by aroma extract dilution analysis (AEDA) and calculation of odour activity values (OAVs). *Flavour and Fragrance Journal* 9: 147-158.
36. Gruen HE (1959) Auxins and fungi. *Annual Review of Plant Physiology* 10: 405-440.
37. Guadagni DG, Buttery RG & Okano S (1963) Odour thresholds of some organic compounds associated with food flavours. *Journal of the Science of Food and Agriculture* 14: 761-765.
38. Harrington TC & Wingfield MJ (1998) The *Ceratocystis* species on conifers. *Canadian Journal of Botany* 76: 1446-1457.
39. Hazelwood LA, Daran J-M, van Maris AJA, Pronk JT & Dickinson JR (2008) The Ehrlich pathway for fusel alcohol production: a century of research on *Saccharomyces cerevisiae* metabolism. *Applied and Environmental Microbiology* 74: 2259-2266.
40. Hellman TM & Small FH (1974) Characterization of the odor properties of 101 petrochemicals using sensory methods. *Journal of the Air Pollution Control Association* 24: 979-982.
41. Hofstetter RW, Cronin JT, Klepzig KD, Moser JC & Ayres MP (2006) Antagonisms, mutualisms and commensalisms affect outbreak dynamics of the southern pine beetle. *Oecologia* 147: 679-691.
42. Hohmann S (1991) Characterization of PDC6, a third structural gene for pyruvate decarboxylase in *Saccharomyces cerevisiae*. *Journal of Bacteriology* 173: 7963-7969.
43. Hohmann S & Meacock PA (1998) Thiamin metabolism and thiamin diphosphate-dependent enzymes in the yeast *Saccharomyces cerevisiae*: genetic regulation. *Biochimica et Biophysica Acta (BBA)-Protein Structure and Molecular Enzymology* 1385: 201-219.
44. Hough JS & Stevens R (1961) Beer flavour. IV. Factors affecting the production of fusel oil. *Journal of the Institute of Brewing* 67: 488-494.
45. Hrazdina G & Jensen RA (1992) Spatial organization of enzymes in plant metabolic pathways. *Annual Review of Plant Biology* 43: 241-267.
46. Hua D & Xu P (2011) Recent advances in biotechnological production of 2-phenylethanol. *Biotechnology Advances* 29: 654-660.
47. Ichihara A & Koyama E (1966) Transaminase of branched chain amino acids. *The Journal of Biochemistry* 59: 160-169.
48. Inoue Y, Trevanichi S, Fukuda K, Izawa S, Wakai Y & Kimura A (1997) Roles of esterase and alcohol acetyltransferase on production of isoamyl acetate in *Hansenula mrakii*. *Journal of Agricultural and Food Chemistry* 45: 644-649.
49. Iraqui I, Vissers S, Cartiaux M & Urrestarazu A (1998) Characterisation of *Saccharomyces cerevisiae* ARO8 and ARO9 genes encoding aromatic aminotransferases I and II reveals a new aminotransferase subfamily. *Molecular and General Genetics* 257: 238-248.
50. Jensen KB, Thorhauge J, Hazell RG & Jørgensen KA (2001) Catalytic asymmetric Friedel–Crafts alkylation of β, γ-unsaturated α-ketoesters: Enantioselective addition of aromatic C− H bonds to alkenes. *Angewandte Chemie International Edition* 113: 164-167.
51. Juzwik J, Skalbeck TC & Neuman MF (2004) Sap beetle species (Coleoptera: Nitidulidae) visiting fresh wounds on healthy oaks during spring in Minnesota. *Forest Science* 50: 757-764.

52. Juzwik J, Harrington TC, MacDonald WL & Appel DN (2008) The origin of *Ceratocystis fagacearum*, the oak wilt fungus. *Annual Review of Phytopathology* 46: 13-26.
53. Kandasamy D, Gershenzon J & Hammerbacher A (2016) Volatile organic compounds emitted by fungal associates of conifer bark beetles and their potential in bark beetle control. *Journal of Chemical Ecology* 42: 952-969.
54. Kandasamy D, Gershenzon J, Andersson MN & Hammerbacher A (2019) Volatile organic compounds influence the interaction of the Eurasian spruce bark beetle (*Ips typographus*) with its fungal symbionts. *The ISME journal -In Press*.
55. Kang Q, Zhao ZA & You SL (2007) Highly enantioselective Friedel− Crafts reaction of indoles with imines by a chiral phosphoric acid. *Journal of the American Chemical Society* 129: 1484-1485.
56. Keasling JD & Chou H (2008) Metabolic engineering delivers next-generation biofuels. *Nature Biotechnology* 26: 298-299.
57. Kellis M, Birren BW & Lander ES (2004) Proof and evolutionary analysis of ancient genome duplication in the yeast *Saccharomyces cerevisiae*. *Nature* 428: 617-624.
58. Kile G (1993) Plant diseases caused by species of *Ceratocystis sensu stricto* and *Chalara*. *Ceratocystis* and *Ophiostoma: taxonomy, ecology and pathogenicity* 173-183. APS Press, St Paul Minnesota.
59. Kosseva M, Joshi V & Panesar PS (2016) Science and technology of fruit wine production. 1-756. Academic Press, Amsterdam.
60. Kradolfer P, Niederberger P & Hütter R (1982) Tryptophan degradation in *Saccharomyces cerevisiae*: characterization of two aromatic aminotransferases. *Archives of Microbiology* 133: 242-248.
61. Lambrechts MG & Pretorius IS (2000) Yeast and its importance to wine aroma - a review. *South African Journal for Enology and Viticulture* 21: 97-129.
62. Landaud S, Latrille E & Corrieu G (2001) Top pressure and temperature control the fusel alcohol/ester ratio through yeast growth in beer fermentation. *Journal of the Institute of Brewing* 107: 107-117.Langfelder K, Streibel M, Jahn B, Haase G & Brakhage AA (2003) Biosynthesis of fungal melanins and their importance for human pathogenic fungi. *Fungal Genetics and Biology* 38: 143-158.
63. Leonardos G, Kendall D & Barnard N (1974) Odor threshold determination of 53 odorant chemicals. *Journal of Environmental Conservation Engineering* 3: 579-585.
64. Lilly M, Bauer FF, Styger G, Lambrechts MG & Pretorius IS (2006) The effect of increased branched-chain amino acid transaminase activity in yeast on the production of higher alcohols and on the flavour profiles of wine and distillates. *FEMS Yeast Research* 6: 726-743.
65. Mackie AE & Wheatley RE (1999) Effects and incidence of volatile organic compound interactions between soil bacterial and fungal isolates. *Soil Biology and Biochemistry* 31: 375-385.
66. Mansourian S & Stensmyr MC (2015) The chemical ecology of the fly. *Current Opinion in Neurobiology* 34: 95-102.
67. Marsischky G & LaBaer J (2004) Many paths to many clones: a comparative look at high-throughput cloning methods. *Genome Research* 14: 2020-2028.
68. Mauricio JC, Valero E, Millán C & Ortega JM (2001) Changes in nitrogen compounds in must and wine during fermentation and biological aging by flor yeasts. *Journal of Agricultural and Food Chemistry* 49: 3310-3315.
69. Mavrides C & Orr W (1975) Multispecific aspartate and aromatic amino acid aminotransferases in *Escherichia coli*. *Journal of Biological Chemistry* 250: 4128-4133.
70. McGinley CM, McGinley M & McGinley D (2000) Odour Basics, understanding and using odour testing 1-15: *In the 22nd Annual Hawaii Water Environmental Association Conference*. Parker House Publishing, Honolulu, 6-7 June.
71. Naz S, Cretenet M & Vernoux JP (2013) Current knowledge on antimicrobial metabolites produced from aromatic amino acid metabolism in fermented products. *Microbial pathogens and strategies for combating them: science, technology and education* 337-346. Formatex Research Center, Badajoz.
72. Nel WJ, Duong TA, Wingfield MJ, Wingfield BD, Hammerbacher A & de Beer ZW (2018) Heterothallism revealed in the root rot fungi *Berkeleyomyces basicola* and *B. rouxiae*. *Fungal Biology* 122: 1031-1040.
73. Nykänen L & Nykänen I (1977) Production of esters by different yeast strains in sugar fermentations. *Journal of the Institute of Brewing* 83: 30-31.

74. Ohashi K, Chaleckis R, Takaine M, Wheelock CE & Yoshida S (2017) Kynurenine aminotransferase activity of Aro8/Aro9 engage tryptophan degradation by producing kynurenic acid in *Saccharomyces cerevisiae*. *Scientific Reports* 7: 12180-12188.
75. Pavkov-Keller T, Strohmeier GA, Diepold M, Peeters W, Smeets N, Schürmann M, Gruber K, Schwab H & Steiner K (2016) Discovery and structural characterisation of new fold type IV-transaminases exemplify the diversity of this enzyme fold. *Scientific Reports* 6: 38183-38195.
76. Redfern DB, Stoakley JT, Steele H & Minter DW (1987) Dieback and death of larch caused by *Ceratocystis laricicola* sp. nov. following attack by *Ips cembrae*. *Plant Pathology* 36: 467-480.
77. Renger RS, Hateren SHv & Luyben KCAM (1992) The formation of esters and higher alcohols during brewery fermentation; the effect of carbon dioxide pressure. *Journal of the Institute of Brewing* 98: 509-513.
78. Reuter M, Bell G & Greig D (2007) Increased outbreeding in yeast in response to dispersal by an insect vector. *Current Biology* 17: 81-83.
79. Rhodes JC & Brakhage AA (2006) Molecular determinants of virulence in *Aspergillus fumigatus*. *Molecular Principles of Fungal Pathogenesis* 333-345. American Society of Microbiology Press, Washington DC.
80. Rząd K & Gabriel I (2015) Characterization of two aminotransferases from *Candida albicans*. *Acta Biochimica Polonica* 62: 903-912.
81. Saerens SMG, Delvaux FR, Verstrepen KJ & Thevelein JM (2010) Production and biological function of volatile esters in *Saccharomyces cerevisiae*. *Microbial Biotechnology* 3: 165-177.
82. Scognamiglio J, Jones L, Letizia C & Api A (2012) Fragrance material review on 2-phenoxyethanol. *Food and Chemical Toxicology* 50: 244-255.
83. Scott RPW (2003) Principles and practice of chromatography. *Chrom-Ed Book Series* 1-106 http://www.library4science.com/eula.html (Accessed February 3rd, 2014).
84. Sentheshanmuganathan S (1960) The mechanism of the formation of higher alcohols from amino acids by *Saccharomyces cerevisiae*. *Biochemical Journal* 74: 568-576.
85. Spinnler HE & Djian A (1991) Bioconversion of amino acids into flavouring alcohols and esters by *Erwinia carotovora* subsp. atroseptica. *Applied Microbiology and Biotechnology* 35: 264-269.
86. Starmer WT & Fogleman JC (1986) Coadaptation of Drosophila and yeasts in their natural habitat. *Journal of Chemical Ecology* 12: 1037-1055.
87. Stettler DD & Axel R (2009) Representations of odor in the piriform cortex. *Neuron* 63: 854-864.
88. Strzelczyk E & Pokojska-Burdziej A (1984) Production of auxins and gibberellin-like substances by mycorrhizal fungi, bacteria and actinomycetes isolated from soil and the mycorrhizosphere of pine (*Pinus silvestris* L.). *Plant and Soil* 81: 185-194.
89. Sumby KM, Grbin PR & Jiranek V (2010) Microbial modulation of aromatic esters in wine: Current knowledge and future prospects. *Food Chemistry* 121: 1-16.
90. ter Schure EG, Flikweert MT, van Dijken JP, Pronk JT & Verrips CT (1998) Pyruvate decarboxylase catalyzes decarboxylation of branched-chain 2-oxo acids but is not essential for fusel alcohol production by *Saccharomyces cerevisiae*. *Applied Environmental Microbiol*ogy 64: 1303-1307.
91. Tsai H-F, Wheeler MH, Chang YC & Kwon-Chung KJ (1999) A developmentally regulated gene cluster involved in conidial pigment biosynthesis in *Aspergillus fumigatus*. *Journal of Bacteriology* 181: 6469-6477.
92. Uyttebroek M, Van Hecke W & Vanbroekhoven K (2015) Sustainability metrics of 1-butanol. *Catalysis Today* 239: 7-10.
93. Vuralhan Z, Morais MA, Tai S-L, Piper MDW & Pronk JT (2003) Identification and characterization of phenylpyruvate decarboxylase genes in *Saccharomyces cerevisiae. Applied and Environmental Microbiology* 69: 4534-4541.
94. Vuralhan Z, Luttik MAH, Tai SL, Boer VM, Morais MA, Schipper D, Almering MJH, Kötter P, Dickinson JR & Daran J-M (2005) Physiological characterization of the ARO10-dependent, broad-substrate-specificity 2-oxo acid decarboxylase activity of *Saccharomyces cerevisiae*. *Applied and Environmental Microbiology* 71: 3276-3284.
95. Welsh FW, Murray WD, Williams RE & Katz I (1989) Microbiological and enzymatic production of flavor and fragrance chemicals. *Critical Reviews in Biotechnology* 9: 105-169.

96. Wheeler MH & Bell AA (1988) Melanins and their importance in pathogenic fungi. *Current Topics in Medical Mycology* 2: 338-387.
97. White TJ, Bruns T, Lee S & Taylor JL (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR protocols: A Guide to Methods and Applications*, 18. 315-322. Academic Press, San Diego CA.
98. Wingfield BD, Van Wyk M, Roos H & Wingfield MJ (2013) Ceratocystis: emerging evidence for discrete generic boundaries. *The Ophiostomatoid Fungi: Expanding Frontiers,* 90. 57-64. CBS-KNAW Fungal Biodiversity Centre, Utrecht.
99. Wolfe KH & Shields DC (1997) Molecular evidence for an ancient duplication of the entire yeast genome. *Nature* 387: 708-713.
100. Yamaoka Y, Wingfield MJ, Ohsawa M & Kuroda Y (1998) Ophiostomatoid fungi associated with I*ps cembrae* in Japan and their pathogenicity of Japanese larch. *Mycoscience* 39: 367-378.
101. Yilmaztekin M, Cabaroglu T & Erten H (2013) Effects of fermentation temperature and aeration on production of natural isoamyl acetate by *Williopsis saturnus var. saturnus. BioMed Research International* 2013: 1-6.
102. Yoshioka K & Hashimoto N (1981) Ester formation by alcohol acetyltransferase from brewers' yeast. *Agricultural and Biological Chemistry* 45: 2183-2190.

6. List of Tables and Figures

Table 1. List of amino acids with derived products (Ehrlich, 1907, Sentheshanmuganathan, 1960).

	Transamination	**Decarboxylation**	**Reduction**
Amino Acid	alpha- Keto Acids	Fusel Aldehyde	Fusel Alcohol
Isoleucine	3-methyl-2-oxo-pentanoate	2-Methylbutanal	2-Methylbutanol
Valine	3-Methyl-2-oxo-butanoate	2-Methylpropanal	Isobutanol
Leucine	4-methyl-2-oxo-butanoate	3-Methylbutanal	Isoamyl alcohol
Methionine	2-oxo-4-Methylthiobutanoate	3-Methylthiopropanal	Methionol
Phenylalanine	3-phenyl-2-oxo-propanoate	2-Phenylethanal	2-Phenylethanol

Table 2. Isolates of Ceratocystidaceae fungi used in this study were obtained from the CMW culture collection in the Forestry and Agricultural Biotechnology Institute, University of Pretoria.

Species	**Isolate ID[1]**	**Host**	**Locality[2]**	**Collector**	**Year**
Bretziella sp.					
B. fagacearum	CMW 2039	*Quercus rubra*	USA	RA Blanchette	-
B. fagacearum	CMW 2656	*Quercus rubra*	USA	S Seegmueller	1991
Berkeleyomyces sp.					
Be. bassicola	CMW 26479	*Styrax sp.*	Indonesia	MJ Wingfield	2007
Be. bassicola	CMW 14219	*Carrot/Eucalyptus regnans*	Chile	J Roux and R Ahumada	2007
Be. bassicola	CMW 7068	*Lathyrus odouratus*	Netherlands	GA van Arkel	-
Ceratocystis sp.					
C. albifundus	CMW 25576	*Acacia mearnsii*	Tanzania	RA Heath	2004
C. albifundus	CMW 17620	*Terminalia serilia*	SA	J Roux	2013
C. albifundus	CMW 42433	*Protea cyanaroides*	SA	J Roux	2013

C. albifundus	CMW 43584	*Acacia mearnsii*	SA	DH Lee , J Roux	2012
C. eucalypticola	CMW 15056	*Eucalyptus grandis*	SA	J Roux	2004
C. eucalypticola	CMW 43694	*Eucalyptus grandis*	Uruguay	R Reyna	2013
C. eucalypticola	CMW 4799	Eucalyptus sp.	Congo	J Roux	1998
C. eucalypticola	CMW 9998	*Eucalyptus grandis*	SA	M van Wyk	2002
C. fimbriata	CMW 46145	*Ipomoea batatas*	China	SF Chen, MR Zhang	2015
C. fimbriata	CMW 46120	*Ipomoea batatas*	China	SF Chen	2015
C. fimbriata	CMW 14799	*Impomoea batatas*	USA	D McNew	1998
C. fimbriata	CMW 15075	*Eucalyptus grandis*	South Africa	J Roux	2004
C. harringtonii	CMW 14789	*Populus sp.*	Netherlands	PW Crous	2001
C. harringtonii	CMW 26381	*Populus tremuloides*	Canada	G Smalley	1993
C. manginecans 2	CMW 28232	*Mango sp.*	Oman	-	-
C. manginecans 2	CMW 15315	*Mangifera indica*	Oman	AO AL Adawi	2003
C. manginecans 2	CMW 17570	*Prosopsis cineraria*	Oman	AO AL Adawi	2005
C. manginecans 2	CMW17567	*Mangifera indica*	Pakiston	AO Al Adawi	2005
C. manginecans 2	CMW 23640	*Mangifera indica*	Pakistan	AO Al Adawi	2006
C. manginecans 2	CMW 46461	*Acacia mangium*	Malaysia	R Rauf	2013
C. manginecans 2	CMW 22563	*Acacia mangium*	Indonesia	M Tarigan	2006
Davidsoniella sp.					
D. virescens	*CMW 17339*	*Acer saccharum*	USA	D Houston	1987
D. virescens	CMW 11165	*Acer saccharum*	USA	D Houston	1987
Endoconidiophora sp.					
E. laricicola	CMW 26374	*Larix pinaceae*	Japan	Y Yamaoka	1995
E. laricicola	CMW 23746	*Larix sp.*	SA	T Kiniits	1997
E. laricicola	CMW 20928	*Larix sp.*	Scotland	D Redfern	1997
E. polonica	CMW 20930	*Picea abies*	Norway	H Solheim	1990
E. polonica	CMW 39742	*Piceae abies*	Poland	W Siemazko	1938
Huntiella sp.					
H. bhutanensis	CMW 8215	*Picea pinulosa*	Bhutan	M J Wingfield	2007
H. decipiens	CMW 30859	*Eucalyptus saligna*	SA	G Kamgan	2008
H. decipiens	CMW 30855	*Eucalyptus saligna*	SA	G Kamgan	2008
H. monilliformis	CMW 10134	*Eucalyptus grandis*	SA	M van wyk	2002
H. monilliformis	CMW 27015	*Terminalia catappa*	SA	D Begoude and J Roux	
H. omanensis	CMW 11056	Mangifera indica	Oman	AO Al Adawi	2003
H. savannae	CMW 17300	*Acacia nigrescens*	SA	G Kamgan	2005
Thielaviopsis sp.					
T. punctulata	CMW 26389	*Lawsonia inermis*	Mauritania	J Brun	1967

[1] Culture Collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa. [2] SA-South Africa, USA-United States of America.

Table 3. Average feedback given by 16 panellists who participated on the odour panel to five questions regarding the volatiles produced by fungi of the Ceratocystidaceae based on a rating scale.

Species	Isolate ID	Major component	Minor component	Odour character[1]	Hedonic tone[2]	Odour intensity[3]
Bretziella sp.						
B. fagacearum	CMW 2039	Very ripe apples	Banana or flower mix	4	4	4
B. fagacearum	CMW 2656	Sweet fruit smell	N/A	3	3	4
Berkeleyomyces sp.						
Be. basicola	CMW 14219	Banana	N/A	4	4	4
Be. basicola	CMW 7065	Banana	N/A	3	3	2
Ceratocystis sp.						
C. albifundus	CMW 42433	Banana	N/A	3,5	3,5	3,5
C. albifundus	CMW 17620	Fruity	N/A	4	3	4
C. albifundus	CMW 43584	Banana	N/A	3,5	3,5	3
C. eucalypticola	CMW 9998	Banana	N/A	4	4	3
C. fimbriata	CMW 15075	Banana	N/A	4	4	4,5
C. harringtonii	CMW 14789	Fruity, sweet	Musky spicy	3	3	3
C. harringtonii	CMW 26381	Rotten, alcohol	Insect	3	3	3
Davidsoniella sp.						
D. virescens	CMW 11165	Very ripe banana	N/A	4	4	4
Endoconidiophora sp.						
E. laricicola	CMW 26374	Sweet smell, banana	N/A	4	4	4
E. laricicola	CMW 23746	Sweet smell, banana	Flower mix	4	4	4
E. laricicola	CMW 20928	Sweet flower mix or fruity	Banana	4	4	4
E. polonica	CMW 39742	Earthly	mushroom	3	1	3
E. polonica	CMW 20930	Perfume	Banana	4	4	4
Huntiella sp.						
H. bhutanensis	CMW 8215	Rotten fruit powder	Fermented cereal	3,5	2,5	3
H. decipiens	CMW 30855	Pineapple	Musky spicy	4	5	4
H. savannae	CMW 17300	Yeast	Dust	4	3	3
H. savannae	CMW 17300	Sweet smell	N/A	4	4	4
Thielaviopsis sp.						
T. punctulata	CMW 26389	Mouldy pineapple	Apples	3	3	2

*Odour Panel Questions.
Question1: Which major components can be smelled?
Question 2: What is the minor component of the smell?
Question 3: Scale the relatedness of the smell to its character?
Question 4: Hedonic tone: pleasant or unpleasant?
Question 5: Odour intensity.
Rating scale : 0-5.
[1]Odour character 5 point scale.
0=no odour, 1=barely related, 2=slightly related, 3=moderately related, 4=related and 5=very related.
[2]Hedonic tone 5 point scale.

0=no odour, 1=un-pleasant, 2- slightly pleasant, 3= moderately pleasant, 4=pleasant and 5=very pleasant.
[3]Odour intensity 5 point scale.
0=no odour, 1=barely perceptible, 2=slight, 3= moderate, 4=strong and 5= very strong.

Table 4. Relative quantities of fusel alcohols and acetates produced by whole cultures of different isolates in the Ceratocystidaceae grown on PDA slants, which analysed by gas chromatography coupled to a mass spectrometer.

Species	Isolate ID[1]	Isoamyl alcohol[2]	Isobutyl acetate[2]	Isoamyl acetate[2]	Phenyl-ethanol[2]	2-phenylethyl acetate[2]
Bretziella sp.						
B. fagacearum	CMW 2656	73,59	18,21	31,02	-	-
Berkeleyomyces sp.						
Be. bassicola	CMW 26479	261,56	398,22	283,9	2,33	0,69
Be. bassicola	CMW 7065	93,37	2,17	-	1,07	-
Be. bassicola	CMW 14219	-	2,18	229,95	1,25	8,12
Ceratocystis sp.						
C. albifundus	CMW 43584	-	434,51	681,41	-	0,69
C. albifundus	CMW 18784	-	261,08	514,74	-	0,53
C. albifundus	CMW 24887	-	169,02	498,66	-	0,34
C. albifundus	CMW 42433	-	148,12	302,64	-	1,03
C. eucalypticola	CMW 15045	-	293,26	278,12	-	-
C. eucalypticola	CMW 43694	-	94,51	41,79	-	-
C. eucalypticola	CMW 4799	14,61	173,19	272,08	-	-
C. eucalypticola	CMW 9998	-	126,44	74,37	-	-
C. fimbriata	CMW 46145	15,86	446,81	659,61	-	0,56
C. fimbriata	CMW 46120	-	161,41	240,97	-	-
C. fimbriata	CMW 4265	-	1265,77	1541,2	4,32	2,13
C. fimbriata	CMW 14799	143,22	141,15	38,84	-	-
C. harringtonii	CMW 14789	-	4,87	376,79	-	-
C. manginecans 2	CMW 15315	-	2351,88	606,24	-	-
C. manginecans 2	CMW 23640	-	481,8	53,1	-	-
C. manginecans 2	CMW 17567	-	256,3	155,75	-	-
C. manginecans 2	CMW 17570	3,47	37,3	23,26	-	-
C. manginecans 2	CMW 28232	-	116,03	138,67	-	-
C. manginecans 2	CMW 46461	-	995,01	74,08	-	-
Davidsoniella sp.						
D. virescens	CMW 17339	54,41	-	31,83	-	1,58
Endoconidiophora sp.						
E. laricicola	CMW 20928	-	-	25,76	-	7,29

E. laricicola	CMW 23746	-	-	365,42	-	12,3
E. laricicola	CMW 26374	-	-	31,2	-	1,12
E. polonica	CMW 20930	-	-	12,82	-	0,76
Huntiella sp.						
H. bhutanensis	CMW 8215	44,81	4	-	0,67	-
H. decipiens	CMW 30859	-	71,51	331,71	0,46	0,7
H. monilliformis	CMW 10134	-	65,07	289	-	0,38
H. omanensis	CMW 11056	-	30,89	62,74	-	0,77
Thielaviopsis sp.						
T. punctulata	CMW 26389	116,01	-	8,86	0,77	-

[1]CMW: Culture collection of the Forestry and Agricultural Biotechnology Institute (FABI). University of Pretoria South Africa. [2]Numbers denote relative peak area per ng naphthalene used as internal standard.

Table 5. Isolates from the Ceratocystidaceae, culture collection numbers and genome sequence information for the species used in for identifying genes involved in fusel alcohol biosynthesis.

Species [1]	Isolate number	GenBank Accession n umber	References
Be. bassicola	CMW49352	MH522765	Nel *et al* 2018
C. albifundus	CMW13980	JSSU000000000	Van der Nest *et al* 2014a
C. eucalypticola	CMW11536	LJOA00000000	Wingfield *et al* 2015b
C. fimbriata	CMW15049	APWK00000000	Wilken *et al* 2013
C. harringtonii	CMW14789	MKGM0000000	Wingfield *et al* 2016b
C. manginecans 2	CMW17570	JJRZ000000000	Van der Nest *et al* 2014b
D. virescens	CMW17339	LJZU000000000	Wingfield *et al* 2015b
E. laricicola	CMW20928	LXGT00000000	Wingfield *et al* 2016a
E. polonica	CMW20930	LXKZ00000000	Wingfield *et al* 2016a
H. bhutanensis	CMW8217	MJMS00000000	Wingfield *et al* 2016b
H. decipiens	CMW30855	NETU00000000	Wingfield *et al* 2017
H. monilliformis	CMW10134	JMSH00000000	Van der Nest *et al* 2014b
H. omanensis	CMW11056	JSUI000000000	Van der Nest *et al* 2014a
H. savannae	CMW17300	LCZG00000000	Van der Nest *et al* 2015
T. punctulata 1	BPI 893173	LAEV00000000	Wingfield *et al* 2015a
T. punctulata 2	CMW1032	MJMR01000000	Wilken *et al* 2018

Table 6. BLAST hits obtained from tBLASTn using local blast searches in BioEdit v 7.2.5 using the genomes of fungi in the Ceratocystidaceae and the *Saccharomyces cerevisiae* amino transferases amino acid sequences.

Species	Gene-ARO			Gene-BAT		
	Group 1	Group 2	Group 3	Group 1	Group 2	Group 3

B. fagacearum	g3637.t1	g151.t1	g256.t1	g409.t1	g4331.t1	g1139.t1
C. albifundus	g660.t1	g6640.t1	g5975.t1	g2579.t1	g2051.t1	g1764.t1
C. eucalypticola	g5165.t1	g1168.t1	g4302.t1	g6497.t1	g2343.t1	g3643.t1
C. fimbriata	g3992.t1	g4050.t1	g6985.t1	g4731.t1	g2395.t1	g3211.t1
C. harringtonii	g6180.t1	g1073.t1	g825.t1	g5108.t1	g4154.t1	g3865.t1
C. manginecans 1	g3677.t1	g271.t1	g1986.t1	g6441.t1	g4965.t1	g3564.t1
C. manginecans 2	g6002.t1	g2995.t1	g926.t1	g3043.t1	g5810.t1	g1162.t1
D. virescens	g897.t1	g4667.t1	g5741.t1	g5209.t1	g3766.t1	g3045.t1
E. laricicola	g5100.t1	g5569.t1	g2137.t1	g6144.t1	g5589.t1	g6775.t1
E. polonica	g1375.t1	g1124.t1	g4762.t1	g5519.t1	g3790.t1	g1975.t1
H. bhutanensis	g692.t1	g2875.t1	g1538.t1	g3372.t1	g4977.t1	g2674.t1
H. monilliformis	g2568.t1	g7598.t1	g8078.t1	g2736.t1	g5094.t1	g2240.t1
H. omanensis	g143.t1	g218.t1	g3456.t1	g1077.t1	g5294.t1	g1377.t1
H. savannae	g4719.t1	g529.t1	g2750.t1	g1702.t1	g3613.t1	g1411.t1
H. decipiens	g5551.t1	g7085.t1	g3531.t1	g3358.t1	g2126.t1	g6663.t1
T. punctulata 1	g705.t1	g6275.t1	g3956.t1	g1686.t1	g756.t1	g784.t1
T. punctulata 2	g1848.t1	g6242.t1	g5429.t1	g1519.t1	g5233.t1	g1237.t1

Blast hits were conducted using the genomes of fungi in the Ceratocystidaceae and the *Saccharomyces cerevisiae* ARO 8 or ARO 9 and BAT1 amino acid sequences. Both *S. cerevisiae* genes resulted in the same list of BLAST hits.

Table 7. BLAST-hits obtained from tBLASTn using local blast searches in BioEdit v 7.2.5 using the genomes of fungi in the Ceratocystidaceae and the *Saccharomyces cerevisiae* pyruvate decarboxylase amino acid sequences.

Species name	Gene-ARO10	Gene-PDC1	Gene-PDC5	Gene-PDC6	Gene-THI3	Gene-Kivd
C. albifundus	g6196.t1	g6196.t1	g6196.t1	g6196.t1	g6196.t1	g6196.t1
C. eucalypticola	g3135.t1	g3135.t1	g3135.t1	g3135.t1	g3135.t1	g3135.t1
Bretziella fagacearum	g3391.t1	g3391.t1	g3391.t1	g3391.t1	g3391.t1	g3391.t1
C. fimbriata	g5125.t1	g5125.t1	g5125.t1	g5125.t1	g5125.t1	g5125.t1
C. harringtonii	g2075.t1	g2075.t1	g2075.t1	g2075.t1	g2075.t1	g2075.t1
C. manginecans 1	g6348.t1	g6348.t1	g6348.t1	g6348.t1	g6348.t1	g6348.t1
C. manginecans 2	g6082.t1	g6082.t1	g6082.t1	g6082.t1	g6082.t1	g6082.t1
D. virescens	g6494.t1	g6494.t1	g6494.t1	g6494.t1	g6494.t1	g6494.t1
E. laricicola	g2522.t1	g2522.t1	g2522.t1	g2522.t1	g2522.t1	g2522.t1
E. polonica	g4093.t1	g4093.t1	g4093.t1	g4093.t1	g4093.t1	g4093.t1
H. bhutanensis	g2661.t1	g2661.t1	g2661.t1	g2661.t1	g2661.t1	g2661.t1
H. monilliformis	g8169.t1	g8169.t1	g8169.t1	g8169.t1	g8169.t1	g8169.t1
H. omanensis	g1364.t1	g1364.t1	g1364.t1	g1364.t1	g1364.t1	g1364.t1
H. savannae	g1424.t1	g1424.t1	g1424.t1	g1424.t1	g1424.t1	g1424.t1
H. decipiens	g3185.t1	g3185.t1	g3185.t1	g3185.t1	g3185.t1	g3185.t1
T. punctulata 1	g6327.t1	g6327.t1	g6327.t1	g6327.t1	g6327.t1	g6327.t1
T. punctulata 2	g3861.t1	g3861.t1	g3861.t1	g3861.t1	g3861.t1	g3861.t1

Blast searches were conducted using the genomes of fungi in the Ceratocystidaceae and the *Saccharomyces cerevisiae* ARO10, PDC1, PDC5, PDC6, THI3 and *Lactococcus lactis* KIVD amino acid sequences. All *S. cerevisiae* proteins and *L. lactis* protein resulted in the same list of BLAST hits.

Table 8. A: Copy number of aromatic amino transaminase (ARO), branched chain amino transferase (BAT) and pyruvate decarboxylation enzymes in representative Sordariomycetes fungi in JGI MycoCosm.

Species	ARO1	BAT1	Pyruvate decarboxylase
Acremonium alcalophilum	3	2	1
Beauveria bassiana	1	3	1
Chaetomium globosum	2	3	1

Colletotrichum acutatum	3	3	1
Colletotrichum graminicola	3	3	1
Colletotrichum higginsianum	2	3	1
Cordyceps militaris	3	3	1
Cryphonectria parasitica	1	3	1
Daldinia eschcholzii	2	3	1
Eutypa lata	2	3	1
Fusarium fujikuroi	2	3	1
Fusarium graminearum	1	3	1
Fusarium verticillioides	2	3	1
Grosmannia clavigera	1	3	1
Neurospora crassa	3	3	1
Neurospora discreta	3	3	1
Neurospora tetrasperma	3	3	1
Ophiostoma piceae	1	2	1
Podospora anserina	2	3	1
Sodiomyces alkalinus	2	3	1
Sporotrichum thermophile	1	3	1
Thielavia appendiculata	2	3	1
Thielavia arenaria	3	2	1
Thielavia hyrcaniae	2	3	1
Thielavia terrestris	2	3	1
Trichoderma atroviride	2	3	1
Trichoderma harzianum	4	3	1
Trichoderma longibrachiatum	2	2	1
Trichoderma reesei	2	3	1
Trichoderma virens	2	3	1
Verticillium dahliae	3	3	1

Table 8. B: Summary to show aromatic amino transaminase (*aro*), branched chain amino transferase (*bat*) and pyruvate decarboxylation gene numbers in representative Sordariomycetes fungi in JGI MycoCosm.

Species	**Gene-ARO1**			**Gene-BAT1**			**Pyruvate decarboxylase**
	Group 1	**Group 2**	**Group 3**	**Group 1**	**Group 2**	**Group 3**	**Group 1**
Acremonium alcalophilum	22050	21054	21038	22042	2202	-	21066
Beauveria bassiana	-	18058	-	16262	15890	12995	1985
Chaetomium globosum	-	1114	11697	11881	11688	11105	1102
Colletotrichum acutatum	26564	26010	26471	26091	27217	25709	25778
Colletotrichum graminicola	17431 13642	17223	12392	17438	14500	17781	14888
Colletotrichum higginsianum	2615	2489	-	2241	2972	21234	2114
Coniochaeta ligniaria	-	1790	1354	-	-	-	1710
Cordyceps militaris	1427C	16969	13789	14677	19553	16831	1122
Cryphonectria parasitica	-	24670	-	22950	22961	23476	2347
Daldinia eschcholzii	-	3191	12131	12168	12175	12131	1
Eutypa lata	-	17025	14805	18159	12042	14124	1500
Fusarium fujikuroi	-	1610 11169	-	13054	11185	12296	16033
Fusarium graminearum	-	11494	-	11102	13429	16610	1138
Fusarium oxysporum	-	-	-	22435	21299	2344	22669
Fusarium verticillioides	-	22295	-	21496	27071	21786	21654

Grosmannia clavigera	-	21418 14118	-	15646	12568	11694	1593
Hypoxylon sp.	-	-	-	CI-4A154	CI-4A154	CI-4A195	CI-4A195
Ilyonectria radicicola	1348	1449	-	-	-	-	11722
Metarhizium robertsii	-	12825	-	-	-	-	-
Neurospora crassa	26716	21415 21416	2732	28725	28289	21918	21029
Neurospora discreta	11923	16172	27321	11671	11599	16666	18350
Neurospora tetrasperma	21458	-	27698	45987	18457	19902	1
Ophiostoma piceae	-	1339	-	1638	12878	-	173
Phaeoacremonium aleophilum	-	15972	11720	14343	1297	16817	-
Podospora anserina	32037	-	32050	32072	32049	32017	32084
Sodiomyces alkalinus	12867	12976	-	13173	1328	13405	13268
Sporotrichum thermophile	-	22294	22302	22306	22302	22122	21121
Thielavia appendiculata	-	15609	1641	16937	16014	16396	15712
Thielavia arenaria	-	-	-	-	17825	18344	17186
Thielavia hyrcaniae	-	15641	15215	17825 14946	14187	14548	14288
Thielavia terrestris	-	22121	14769	22110	21629	22122	22116
Trichoderma atroviride	21950	22997	-	21573	2155	21460	21500
Trichoderma harzianum	15142 1704	1814 17537	-	1779	17793	17787	11266
Trichoderma longibrachiatum	31324	31434	-	31438	-	3133	31400
Trichoderma reesei	1126	19922	-	24545	21238	27529	301
Trichoderma virens	265	24082	-	27658	23335	2172	292
Verticillium dahliae	-	19725	14337	14637	16511	18010	15791

Table 9. Sequences of primers used for amplifying aromatic and branched chain amino transferase transcripts of selected species in the Ceratocystidaceae.

Gene	***Ceratocystis albifundus* Oligo Sequence**
*aro1*_F	5′- GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATGACTGCTCATTCTTCGGTCGAC-3′
*aro1*_R	5′ -GGGGACCACTTTGTACAAGAAAGCTGGGTTTTACAGCTTGAATGACTTCTTGATAGCC-3′
*aro2*_F	5′ -GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATGGACGCCCTCCACACTAC-3′
*aro2*_R	5′ -GGGGACCACTTTGTACAAGAAAGCTGGGTTTCAGCAATGCATGGGGGATG-3′
*aro3*_F	5′- GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATGGCTACCACCAAGATCCCC-3′
*aro3*_R	5′ -GGGGACCACTTTGTACAAGAAAGCTGGGTTCTACCGCCAATCGCGAGTAG-3′
*bat1*_F	5′- GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATGCGGCGCACTATCTCTAG-3′
*bat1*_R	5′- GGGGACCACTTTGTACAAGAAAGCTGGGTTTTATTTGGCCACGTATGACCAC-3′
*bat2*_F	5′ -GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATGTCTCCTGCCGTAACATCTGC-3′
*bat2*_R	5′ -GGGGACCACTTTGTACAAGAAAGCTGGGTTCTACTCCTGTTCTGTCACCACCAC-3′
*bat3*_F	5′ -GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATGGCGTCTTTCCCCCCTCC-3′
*bat3*_R	5′ -GGGGACCACTTTGTACAAGAAAGCTGGGTTTCATGCCTCTCGCCAAACATGTG-3′
Gene	*Endoconidiophora polonica* Oligo Sequence
*aro1*_F	5′ -GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATGTCTGCTCCAGCTTCTAAAGCC3′
*aro1*_R	5′ GGGGACCACTTTGTACAAGAAAGCTGGGTTTTACATCTTGTAAGACTTTCTAATAGCAGCA CC3′
*aro2*_F	5′ GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATGAATATCTTTACACGTAACCGATCATTT TC3′
*aro2*_R	5′ -GGGGACCACTTTGTACAAGAAAGCTGGGTTTTAACAGCCCATCAACGAGCC3′
*aro3*_F	5′ - GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATGGCAACCAAACAAAAGAAGAATCTG3′
*aro3*_R	5′ -GGGGACCACTTTGTACAAGAAAGCTGGGTTTCATCTCCAATCATCCATTGACTTGG3′
*bat1*_F	5′ -GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATGCGCCGCACTGCCTCC-3′

*bat1*_R	5′ -GGGGACCACTTTGTACAAGAAAGCTGGGTTTTAATTGGCGACGTAGGACCACTGATG-3′
*bat2*_F	5′ -GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATGTCCTCTGAAGCACCACCG-3′
*bat2*_R	5′ -GGGGACCACTTTGTACAAGAAAGCTGGGTTCTAAGGGCTTAGGTTTTCGGTGAC-3′
*bat3*_F	5′ -GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATGTCGGGTTTAAGGTTCGCGAG-3′
*bat3*_R	5′ -GGGGACCACTTTGTACAAGAAAGCTGGGTTTTACTCCTTCTGCACCTTCTCGC-3′
Gene	*Huntiella monilliformis* Oligo Sequence
*aro1*_F	5′ GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATGTCTACATCTGCCGATTCCACC -3′
*aro1*_R	5′ GGGGACCACTTTGTACAAGAAAGCTGGGTTCTAAATCCCAAAAGACTTACGAATAGCC-3′
*aro2*_F	5′ GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATGTCTATAGCCATGGATTCCTCTTATTTCACAG-3′
*aro2*_R	5′ GGGGACCACTTTGTACAAGAAAGCTGGGTTTCAACATCCCATCTCCATCATTGATCC-3′
*aro3*_F	5′ GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATGAACAAACCCACCAAAAACCCC-3′
*aro3*_R	5′ GGGGACCACTTTGTACAAGAAAGCTGGGTTTTAGCGCCAGTCGCCCATTGAC-3′
*bat1*_F	5′ GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATGATGCGGCGTACTGCCTCTTC-3′
*bat1*_R	5′ GGGGACCACTTTGTACAAGAAAGCTGGGTTTTAGTTGGCAATGTACGACCACTTGTGC-3′
*bat2*_F	5′ GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATGACCATCGCCGTCTCCACC-3′
*bat2*_R	5′ GGGGACCACTTTGTACAAGAAAGCTGGGTTTTAAGCAGATTCGGCAACCTTGACG-3′
*bat3*_F	5′ GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATGGCAGCCACTTTCCCCCC-3′
*bat3*_R	5′ GGGGACCACTTTGTACAAGAAAGCTGGGTTCTACTCGTCCTTGACAGCAGCAACATC-3′

Table 10. Mean percentage of aromatic amino transferase activity toward tyrosine as substrate in relation to its activity towards phenylalanine in *in vitro* enzyme assays.

Species	Enzyme	Mean activity %	STD
C. albifundus	ARO9-1	39,7	1,9
	ARO9-2	58,6	18,8
	ARO9-3	5,2	0,8
C. manginecans 2	ARO9-1	39,2	1,9
	ARO9-2	77,9	16,6
	ARO9-3	70,9	10,0
E. polonica	ARO9-1	48,1	2,1
	ARO9-2	46,8	1,6
	ARO9-3	46,5	2,3
H. monilliformis	ARO9-1	45,4	1,6
	ARO9-2	62,5	4,8
	ARO9-3	0,0	0,0

Products from enzyme assay, which were carried out in triplicate were analysed by high performance liquid chromatography. *In vitro* enzyme assays with 3 mM phenylalanine and 3 mM tyrosine as substrates.

Table 11. Mean percentage of aromatic amino transferase activity toward tryptophan as substrate in relation to its activity towards phenylalanine in *in vitro* enzyme assays.

Species	Enzyme	Mean activity %	STD
C. albifundus	ARO9-1	193,5	9,6
	ARO9-2	323,7	183,4
	ARO9-3	334,9	42,3
C. manginecans 2	ARO9-1	288,5	13,8
	ARO9-2	323,1	225,2
	ARO9-3	242,3	40,0
E. polonica	ARO9-1	233,3	5,7
	ARO9-2	264,5	4,1
	ARO9-3	257,7	11,8
H. monilliformis	ARO9-1	261,0	16,1
	ARO9-2	234,4	29,8

	ARO9-3	43,1	6,8

Products from enzyme assay, which were carried out in triplicate were analysed by high performance liquid chromatography. *In vitro* enzyme assays with 3 mM phenylalanine and 3 mM tryptophan as substrates.

Table 12. Mean percentage of branched chain amino transferase activity toward valine as substrate in relation to its activity towards leucine in *in vitro* enzyme assays.

Species	Enzyme	Mean activity %	STD
C. albifundus	BAT1-1	61,2	2,0
	BAT1-2	63,5	3,1
E. polonica	BAT1-1	63,1	1,9
H. monilliformis	BAT1-1	64,3	2,3
	BAT1-3	60,5	3,3

Products from enzyme assay, which were carried out in triplicate were analysed by high performance liquid chromatography. *In vitro* enzyme assays with 3 mM leucine and 3 mM valine as substrates.

Table 13. Mean percentage of branched chain amino transferase activity toward isoleucine as substrate in relation to its activity towards leucine in *in vitro* enzyme assays.

Species	Enzyme	Mean activity %	STD
C. albifundus	BAT1-1	13,4	1,0
	BAT1-2	14,0	0,4
E. polonica	BAT1-1	13,5	1,0
H. monilliformis	BAT1-1	13,2	0,1
	BAT1-3	11,8	0,3

Products from enzyme assay, which were carried out in triplicate were analysed by high performance liquid chromatography. *In vitro* enzyme assays were conducted with 3 mM leucine and 3 mM isoleucine as substrates. .

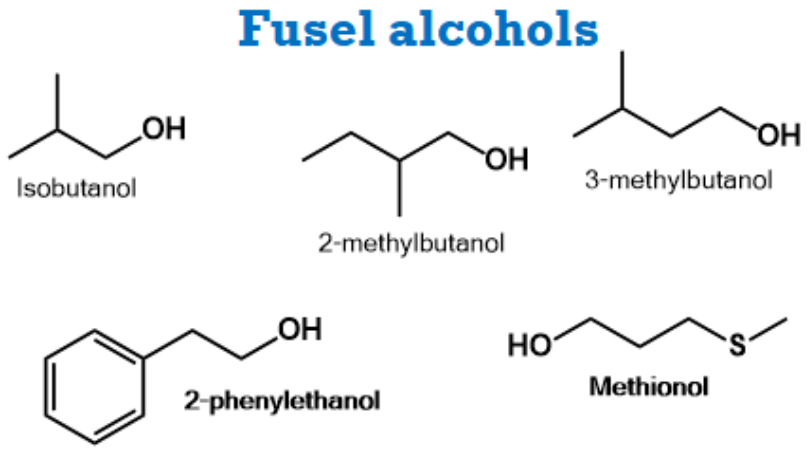

Figure 1. Chemical structures of fusel alcohols. These are volatile chemical compounds essential in food, cosmetic, pharmaceutical and biofuel industries (Hua & Xu, 2011). They all have a hydroxyl group (alcohol group) (Etschmann *et al.*, 2002, Perpete *et al.*, 2005, Hazelwood *et al.*, 2008, Choi *et al.*, 2014).

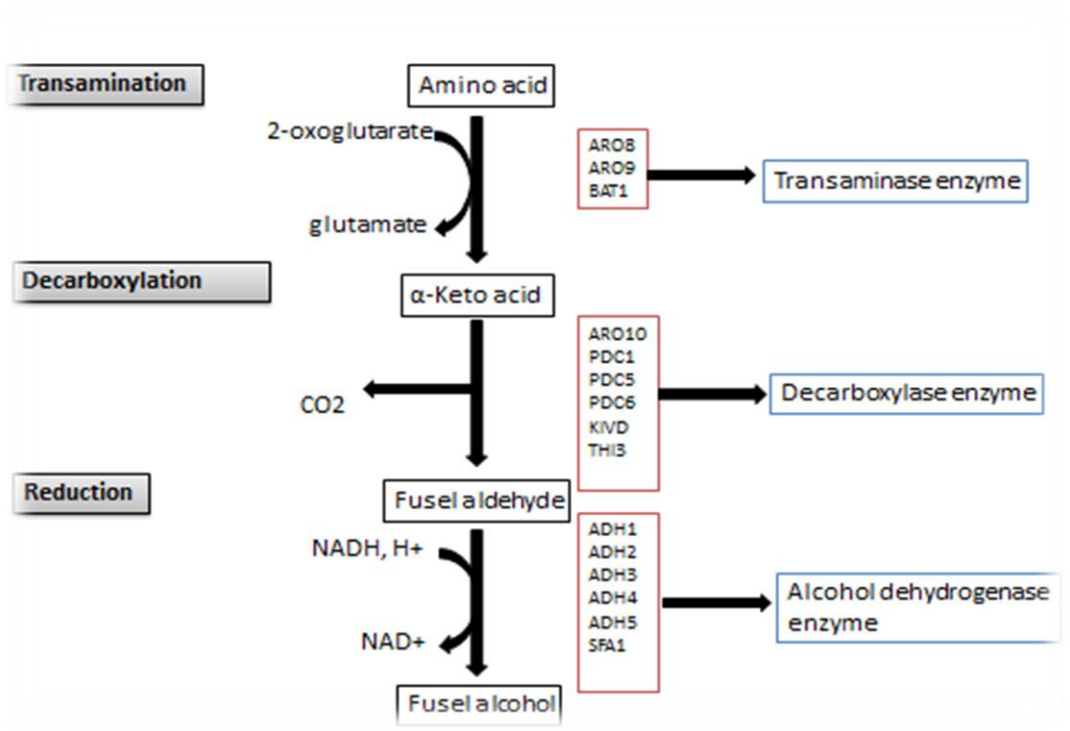

Figure 2. An outline of the Ehrlich pathway, a biosynthetic pathway for the production of fusel alcohols in yeast (*Saccharomyces cerevisiae*) (Ehrlich, 1907, Sentheshanmuganathan, 1960). ARO: aromatic amino transferase; BAT: branched chain amino transferase; PDC: pyruvate decarboxylase; KIVD: α-ketoisovalerate decarboxylase; THI: keto-isocaproate decarboxylase; ADH: alcohol dehydrogenase; SFA: sensitive to formaldehyde; NAD: nicotinamide.

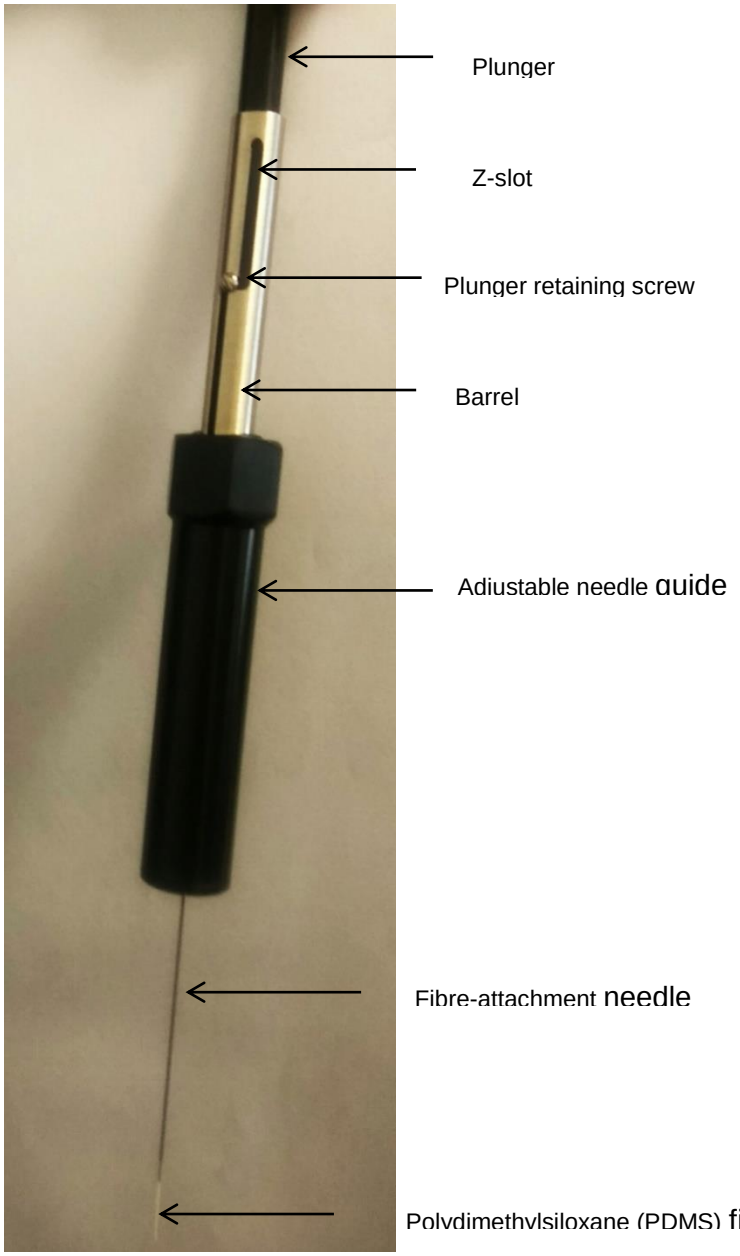

Figure 3. Solid phase micro extraction (SPME) device is used to extract volatiles from samples. A plunger is used to release the fibre into and out of the needle. When extracting volatiles, the plunger is twisted to release the plunger retaining screw which will move from the top of the z-slot to the middle of the z-slot as shown in the Fig. The fibre is coated with stationary phase which acts as sponge, concentrating organic analytes on its surface during the extraction and absorption of the sample (Kataoka *et al.*, 2000). After extraction, the fibre is retracted back into the needle, which is further injected into the gas chromatography and after injection the fibre is extended out of the needle for thermal desorption and analysis

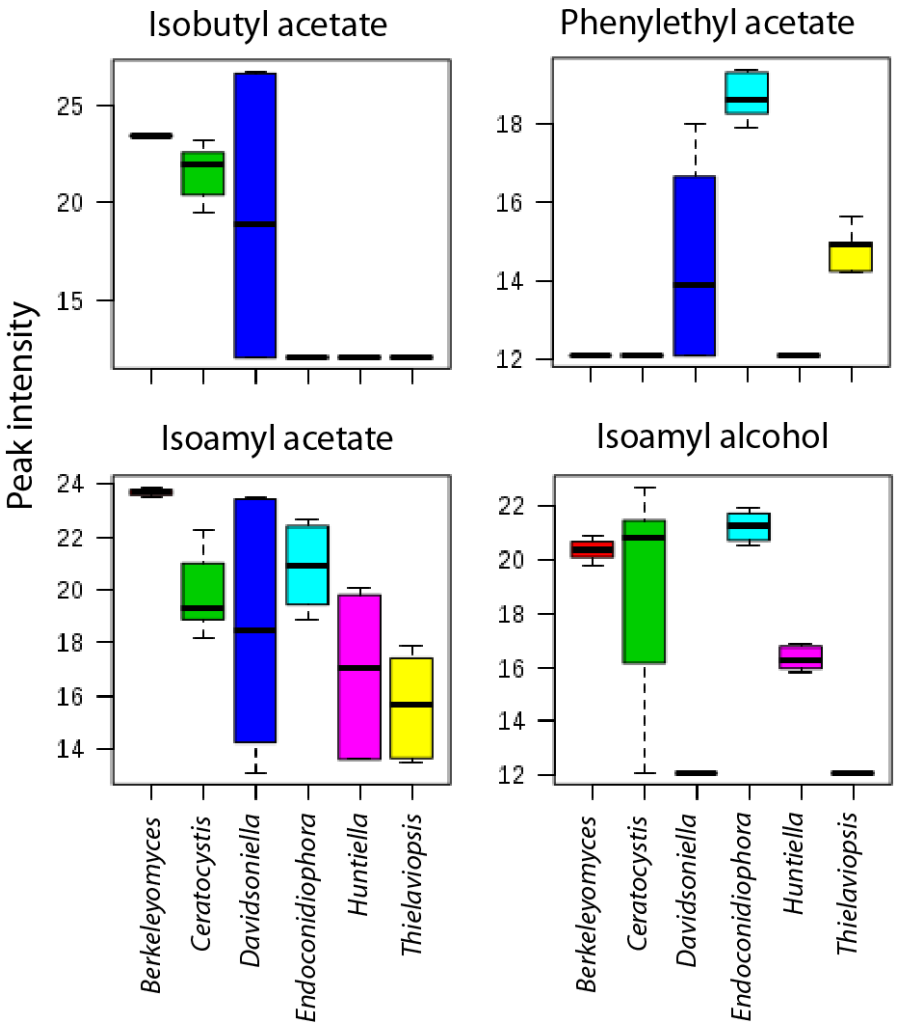

Figure 4. Box plot showing the relative abundance (peak intensity) of volatiles produced by species in different genera of the Ceratocystidaceae. Each genus represents many species within this family. Volatile samples were sampled from 5 mm diameter fungal plugs harvested from the edges of young cultures, placed in an airtight vessel and sampled for 40 min. Volatiles were analysed using GCMS.

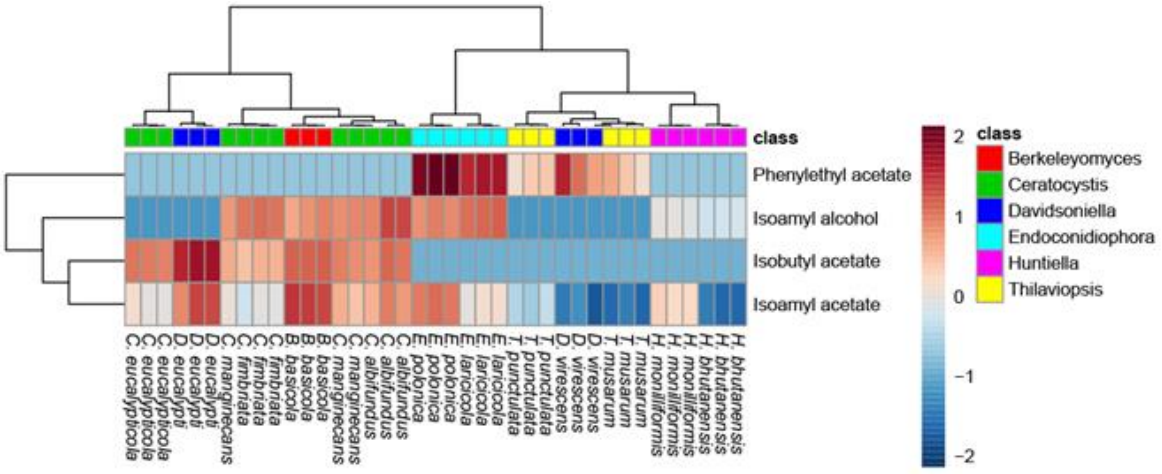

Figure 5. Species within the different genera of the Ceratocystidaceae produce similar odour bouquets. Volatiles were measured by GC-MS and the heat map of relative volatile abundance was calculated using data that was normalized using the natural logarithm. Volatile samples were

sampled from 5 mm diameter fungal plugs harvested from the edges of young cultures, placed in an airtight vessel and sampled for 40 min. Volatiles were analysed using GCMS.

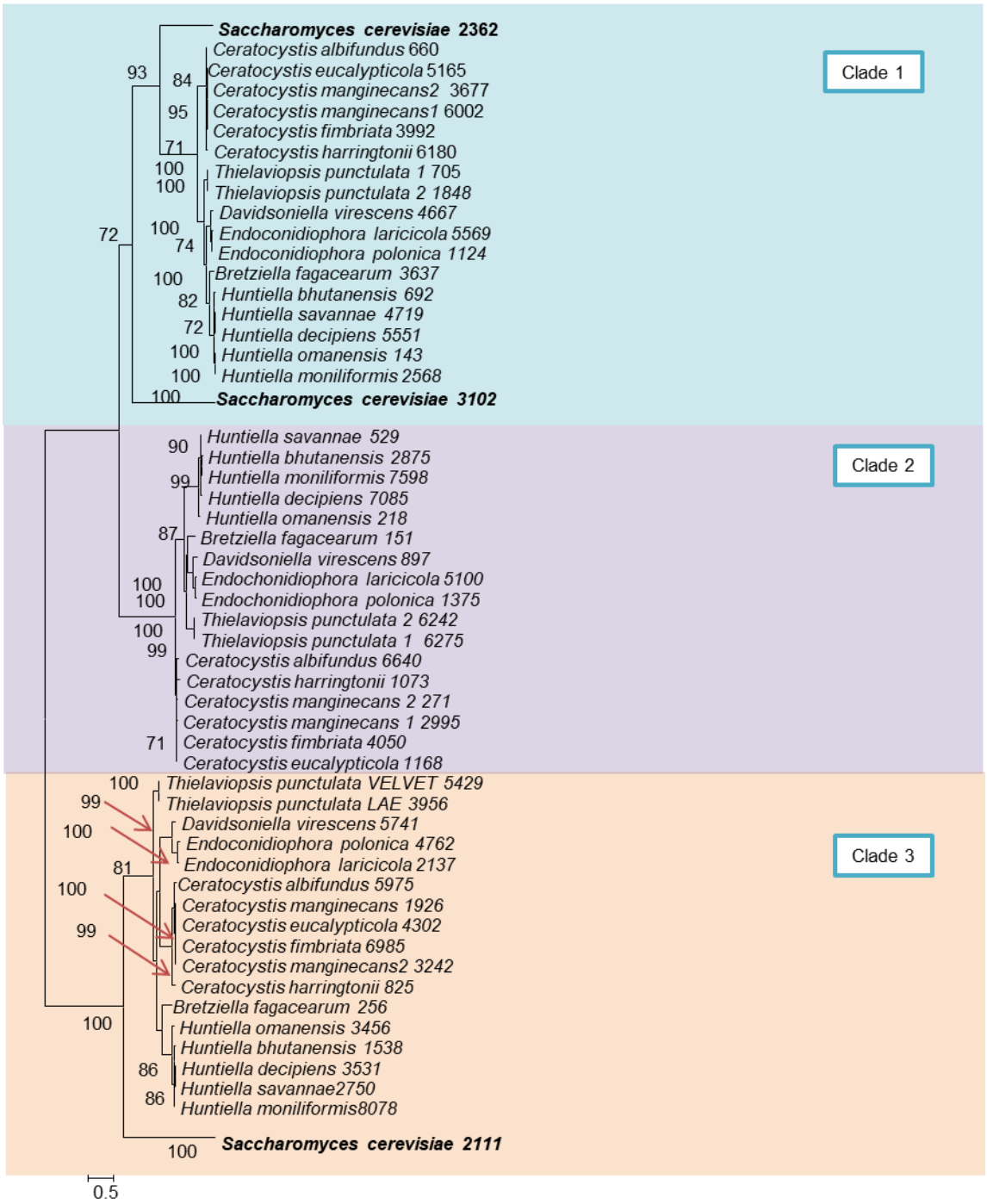

Figure 6. Maximum likelihood (ML) tree obtained from the conserved region of the aromatic amino transferase (ARO9) amino acid sequences of fungi in the Ceratocytidaceae. *Saccharomyces cerevisiae* amino acid sequences are indicated in bold. Bootstrap values are indicated at the branching points based on 1000 iterations; values below 70% were removed. This tree was constructed using the RAxMLGUI, edited in Mega v.7 and is a midpoint rooted tree. .

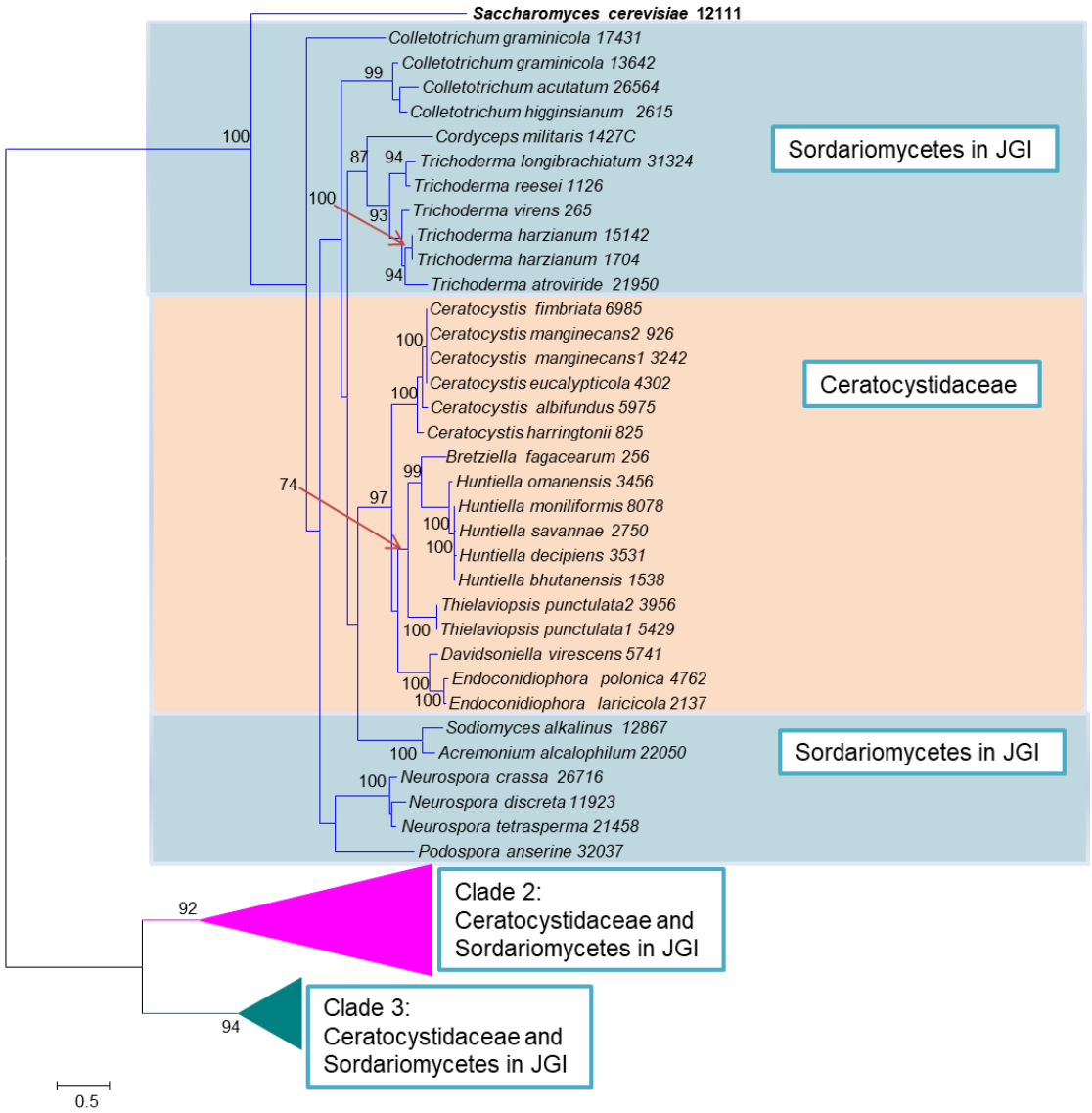

Figure 7. Phylogenetic tree based on the maximum likelihood (ML) analysis showing an expanded clade 1 of the ARO tree. This clade consists of Sordariomycetes species from JGI in the two sub-clades that grouped separately from the Ceratocystidaceae. The *S. cerevisiae* copy is indicated in bold. This tree was constructed using the RAxMLGUI, edited in Mega v.7 and is a midpoint rooted tree. Bootstrap values based on 1000 iterations below 70% were removed.

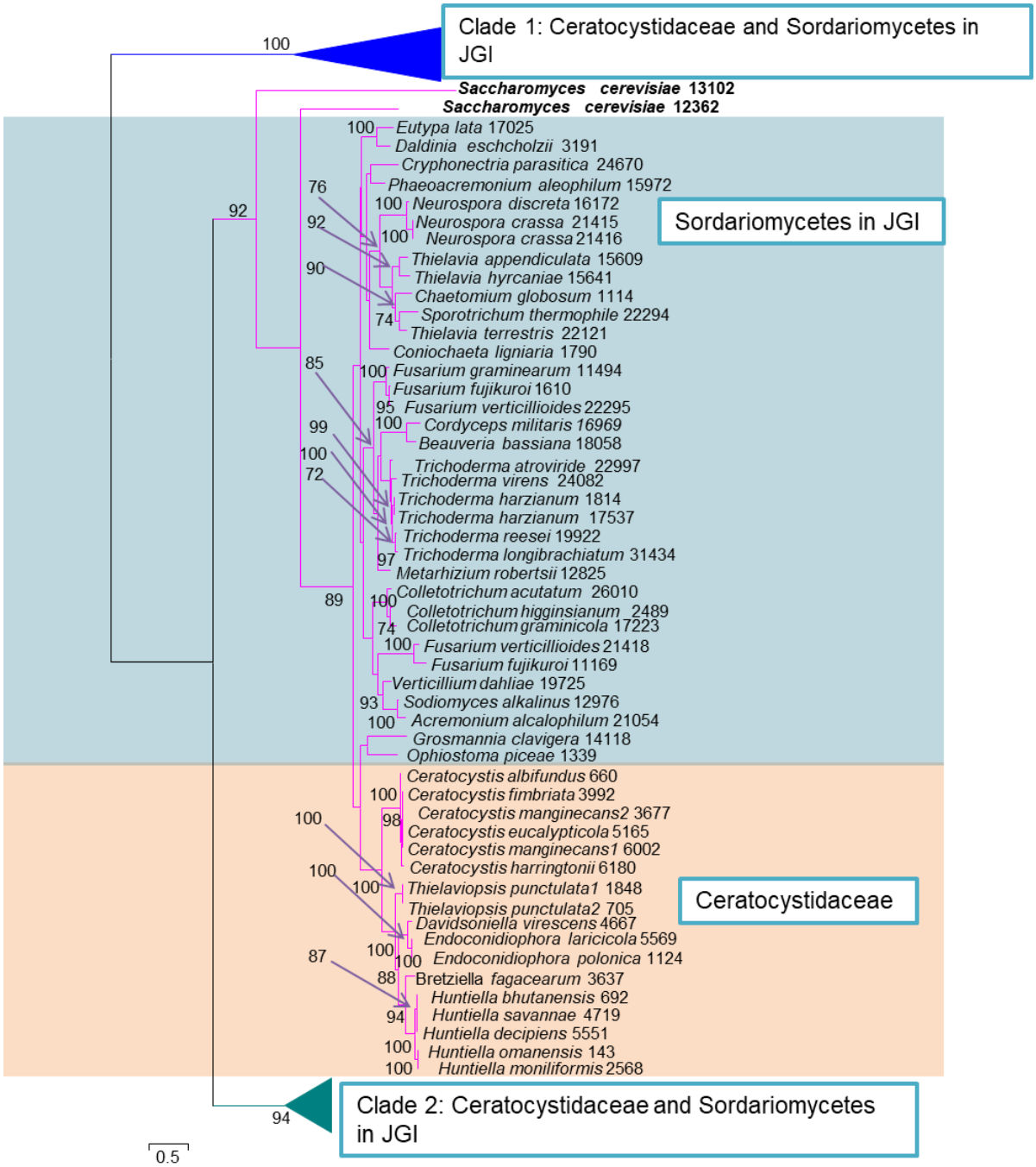

Figure 8. Phylogenetic tree based on the maximum likelihood (ML) analysis showing an expanded clade 2 of the ARO tree. This clade consists of two sub-clades which include Sordariomycetes species from JGI and the Ceratocystidaceae sub-clade. *Saccharomyces cerevisiae* copies are indicated in bold. This tree was constructed using the RAxMLGUI, edited in Mega v.7 and is a midpoint rooted tree. Bootstrap values based on 1000 iterations below 70% were removed.

Figure 9. Maximum likelihood (ML) analysis showing an expanded clade 3 of the ARO tree. This clade consists of a Sordariomycetes sub-clade and a Ceratocystidaceae sub-clade. Ceratocystidaceae fungi sub-grouped separately from the two sub-clades of species from JGI. This tree was constructed using the RAxMLGUI, edited in Mega v.7 and is a midpoint rooted tree. Bootstrap values based on 1000 iterations below 70% were removed.

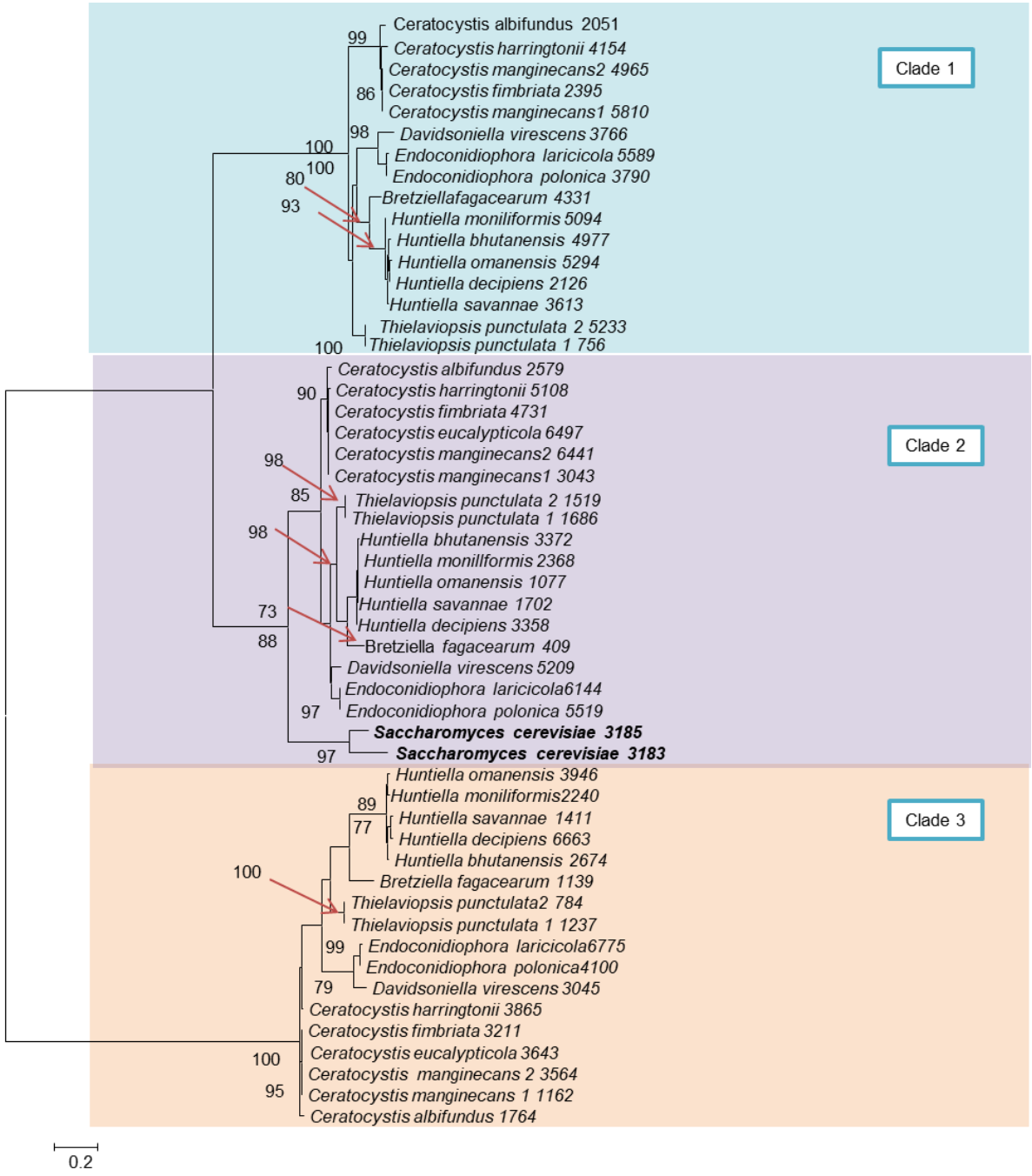

Figure 10. Maximum Likelihood (ML) tree obtained from the conserved region of the branched chain amino transferase amino acid sequences of fungi in the Ceratocystidaceae. Two known copies of *S. cerevisiae* are indicated in bold. This maximum likelihood tree was constructed using RAxMLGUI and the tree was edited using Mega v.7. Bootstrap values based on 1000 iterations below 70% were removed. This is a midpoint rooted tree.

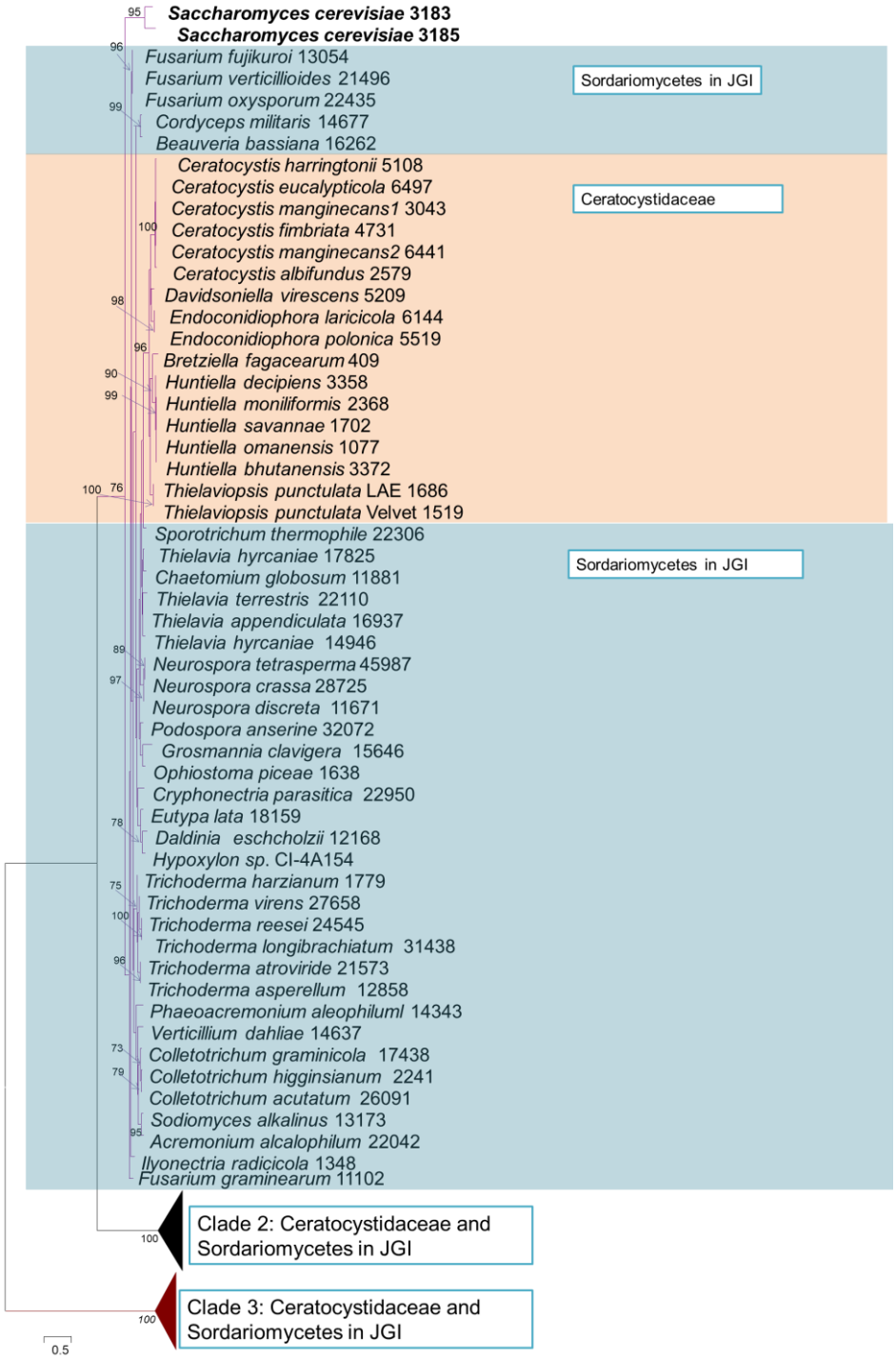

Figure 11. An expanded clade 1 of a phylogenetic tree based on the maximum likelihood (ML) analysis of the conserved amino acid sequences of the branched chain amino transferase (BAT) enzymes of the Ceratocystidaceae and Sordariomycetes from JGI. *S. cerevisiae* copies are included in bold. This tree was constructed using the RAxMLGUI, edited in Mega v.7 and is a midpoint rooted tree. Bootstrap values based on 1000 iterations below 70% were removed.

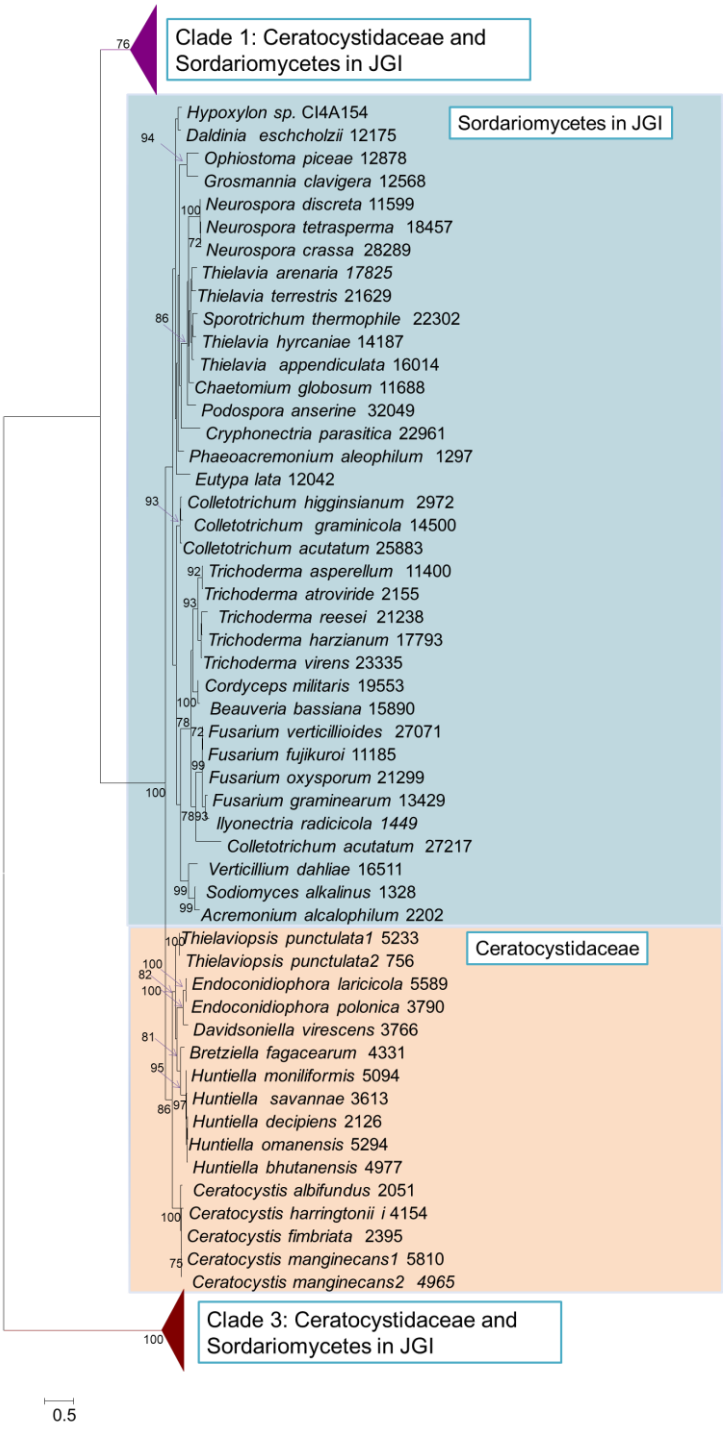

Figure 12. An expanded clade 2 of a phylogenetic tree based on the maximum likelihood (ML) analysis of the conserved amino acid sequences of the branched chain amino transferase (BAT) enzymes of the Ceratocystidaceae and Sordariomycetes from JGI. This is a midpoint rooted tree constructed using the RAxMLGUI and edited in Mega v.7 and Bootstrap values based on 1000 iterations below 70% were removed.

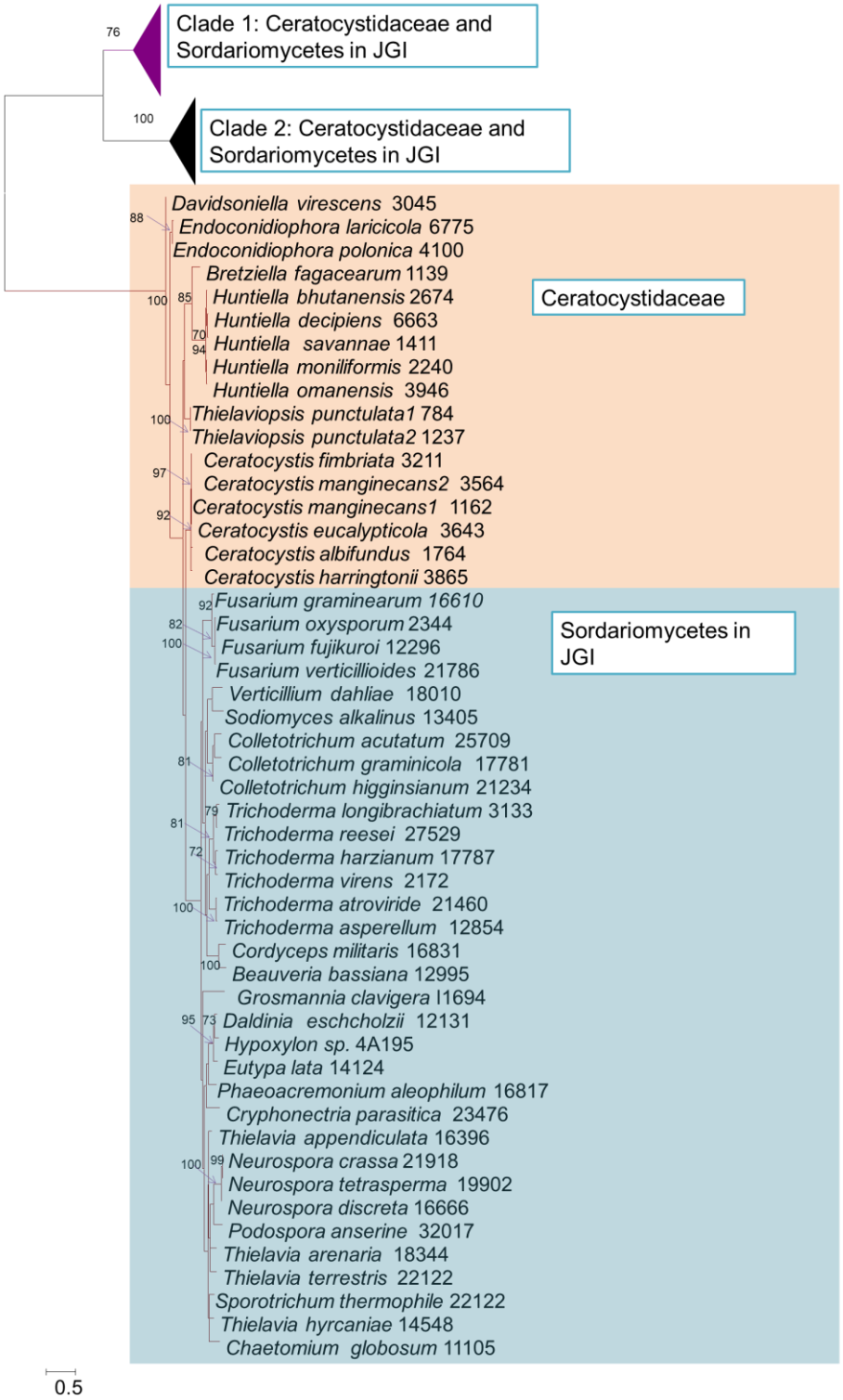

Figure 13. An expanded clade 2 of a phylogenetic tree based on the maximum likelihood (ML) analysis of the conserved amino acid sequences of the branched chain amino transferase (BAT) enzymes of the Ceratocystidaceae and Sordariomycetes from JGI. This is a midpoint rooted tree constructed using the RAxMLGUI and edited in Mega v.7. Bootstrap values based on 1000 iterations below 70% were removed.

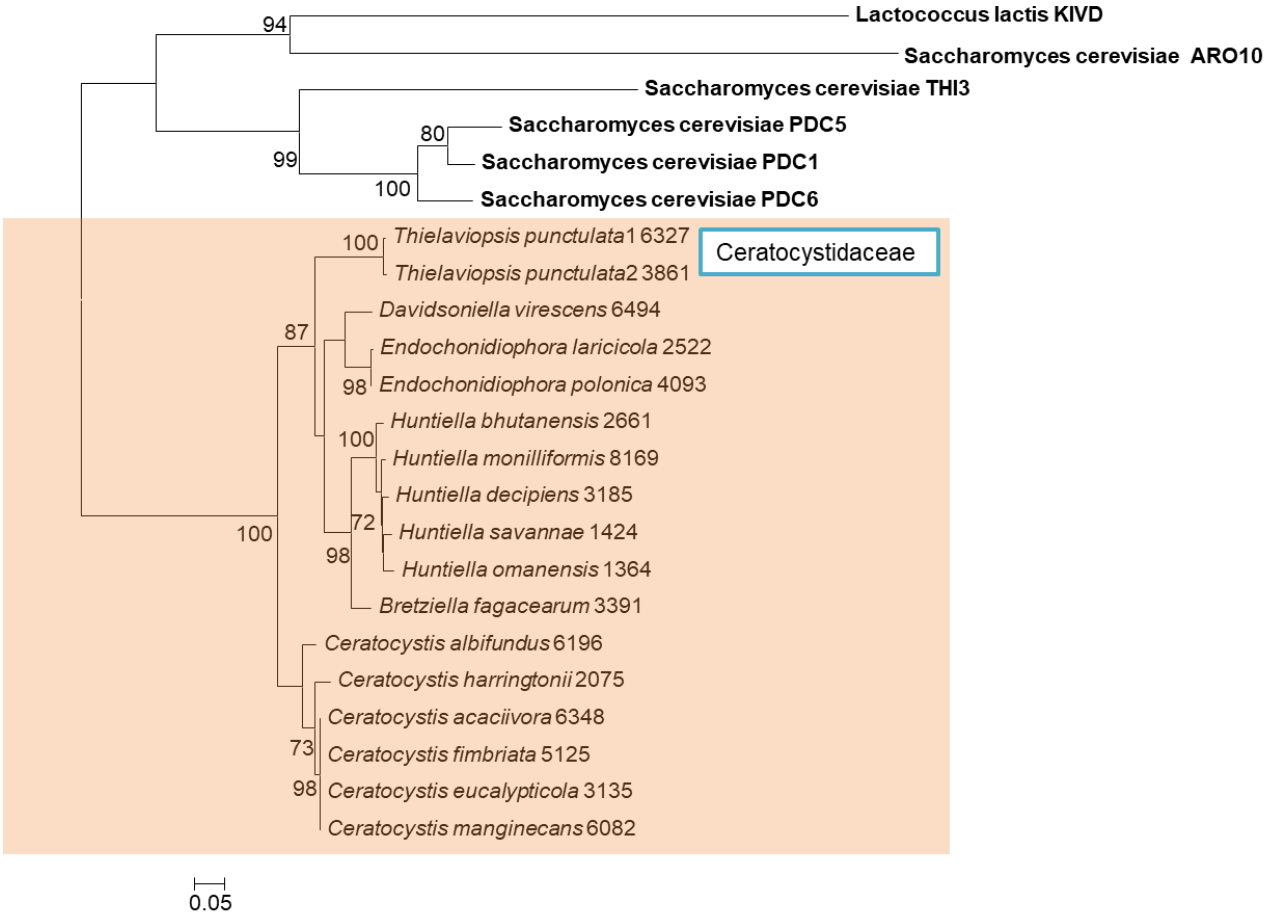

Figure 14. Maximum likelihood (ML) tree obtained for the conserved amino acid region of the pyruvate decarboxylase (PDC) enzymes of the Ceratocystidaceae. Included in this tree are decarboxylase enzymes of the *S. cerevisiae* and *L. lactis* shown in bold. This tree was constructed using RAxMLGUI, edited in Mega v.7 and is midpoint rooted. Bootstrap values based on 1000 iterations below 70% were removed.

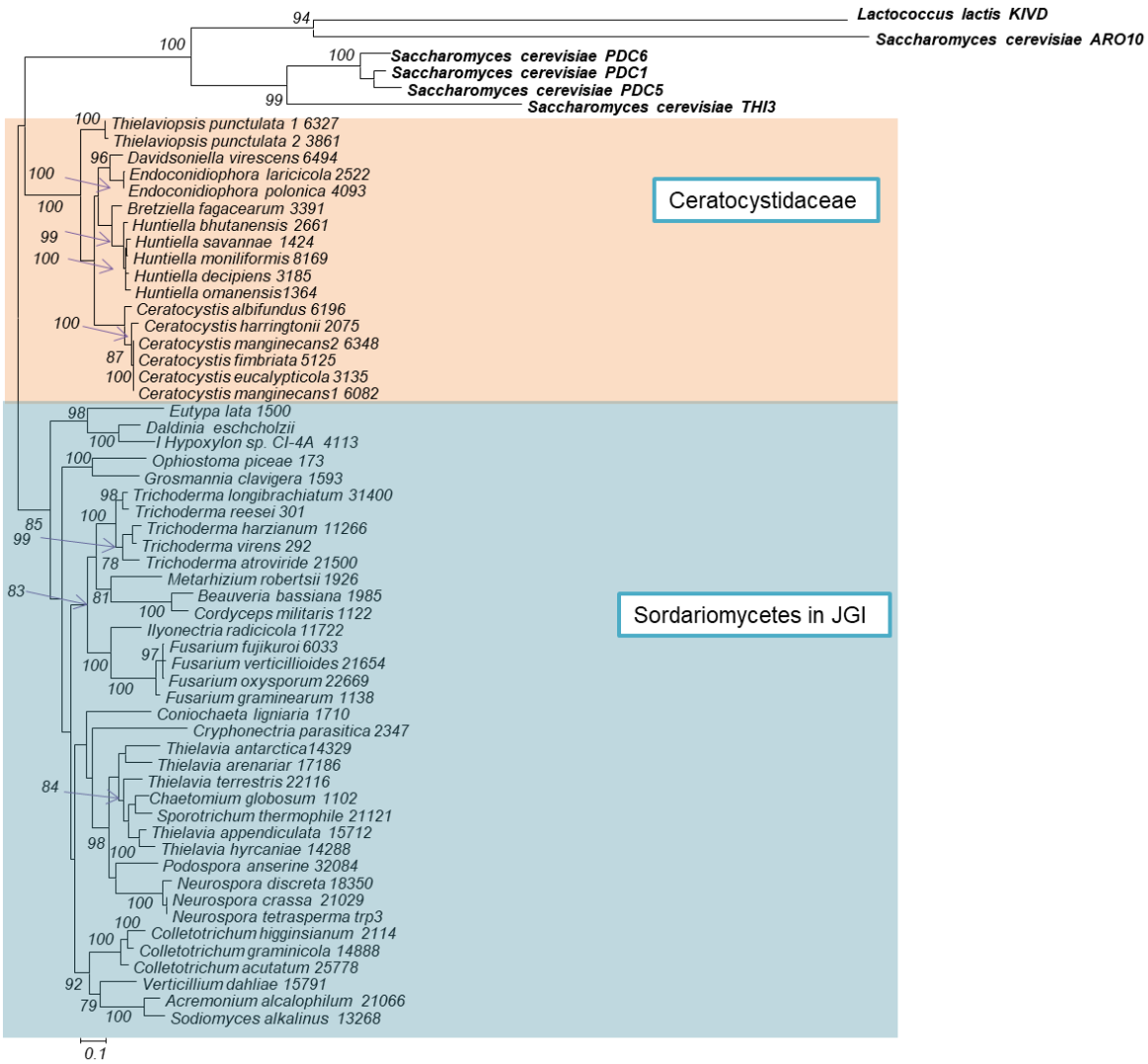

Figure 15. Maximum likelihood (ML) tree for the conserved amino acid sequence of the pyruvate decarboxylase (PDC) enzymes of the Ceratocystidaceae and Sordariomycetes from JGI. Included in the tree are pyruvate decarboxylase enzyme of the *S. cerevisiae* and *L. lactis* shown in bold. This tree was constructed using the RAxMLGUI, edited in Mega v.7 and is a midpoint rooted tree. Bootstrap values based on 1000 iterations below 70% were removed.

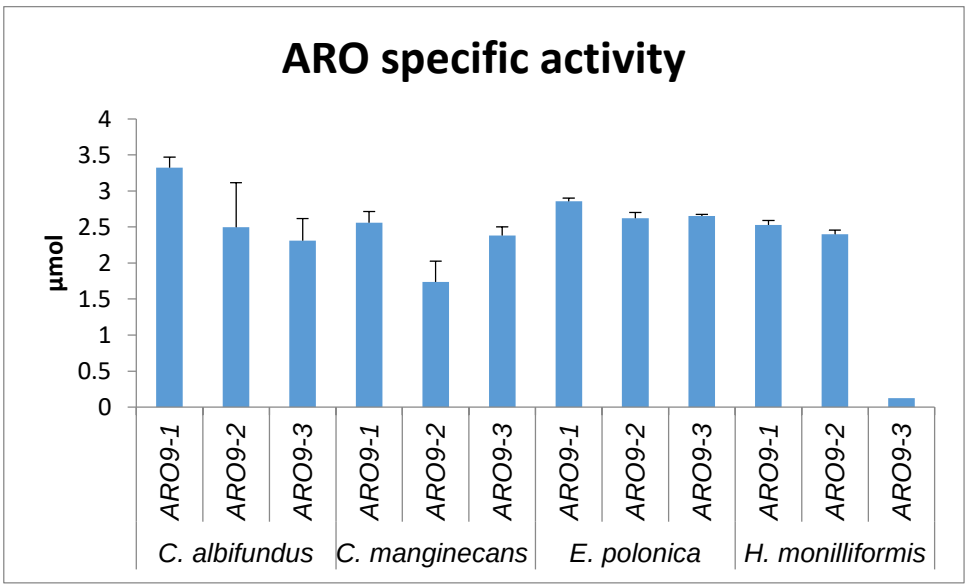

Figure 16. Aromatic amino transferase enzymes from four fungal species which were used as representatives of the *Ceratocystis*, *Endoconidiophora* and *Huntiella* genera showed high specific activity for phenylalanine in an *in vitro* enzyme assay using proteins which were heterologously expressed in *E. coli*. Three replicates were made of each assay. Absolute quantification of the product, 2-oxo-3-phenylpropanoate, was carried out using high performance liquid chromatography. Peak areas of 2-oxo-3-phenylpropanoate were determined manually, and the specific activity was calculated for each enzyme per hour (umol product/hour).

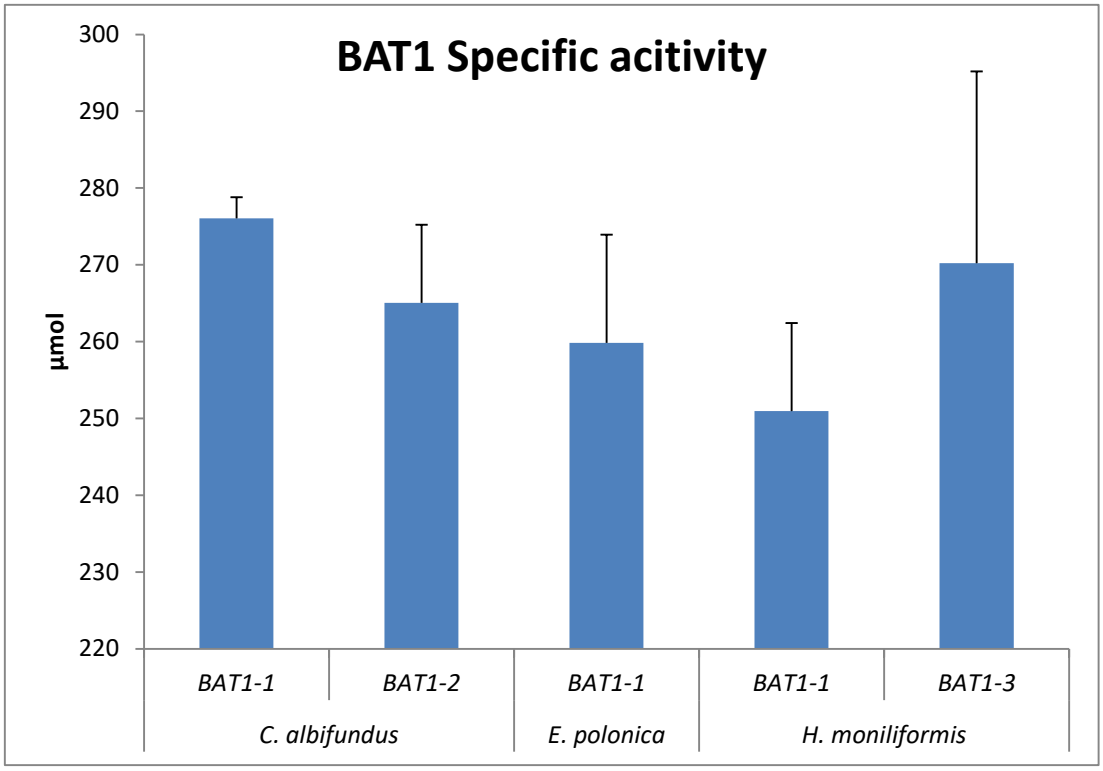

Figure 17. Branched chain- amino transferase enzymes from three fungal species which were used as representatives of the *Ceratocystis*, *Endoconidiophora* and *Huntiella* genera showed high specific activity to leucine in an *in vitro* enzyme assay using proteins which were heterologously expressed in *E. coli*. Three replicates were made of each assay. Absolute quantification of the product, 4-methyl-2-oxopentanoate, was carried out using high performance liquid chromatography. Peak areas of 4-methyl-2-oxopentanoate were determined manually, and the specific activity was calculated for each enzyme per hour (umol product/hour).