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**An endophytic *Myrothecium inundatum* producing volatile organic compounds**

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*Myrothecium inundatum* was isolated as an endophyte from a euphorbeacean herb, *Acalypha indica* in NE India. This fungus when grown in shake culture produced an abundance of foam. Contained in the foam was a mixture of volatile organic compounds (VOCs) some of which were hydrocarbons and hydrocarbon derivatives. The most prevalent compounds were 3-octanone, 3-octanol, and 7-octen-4-ol. Numerous other volatile organic compounds were also produced including many terpenes, organic acids, ketones, and alcohols. The VOCs of this fungus demonstrated growth inhibitory activity against a number of plant pathogenic fungi including *Pythium ultimum* and *Sclerotinia sclerotiorum*. However, when grown in microaerophilic conditions, the organism produced a number of hydrocarbons of interest as fuel related hydrocarbons including octane and tentatively identified- 1,4- cyclohexadiene, 1-methyl- and cyclohexane, (1-ethylpropyl) and others. An NMR method was used to measure VOC production which peaked at day 15 in a time course experiment. Numerous substrates can serve to support the production of VOCs by this fungus including potato broth and beet pulp extracts.

**Key words** – endophyte – rDNA – NMR – hydrocarbons – fungal foam – 3-octanone

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**Introduction**

Many bacteria and some fungi make one or more volatile organic compounds (VOCs) (Schulz & Dickschat 2007, Crespo et al. 2008, Korpi et al. 2009). However, the composition of VOCs produced by microorganisms varies with the microbial species and its growth environment. Recently, one organism of special interest is *Ascocoryne sarcoides* because when grown under microaerophilic conditions it produced many VOCs, the most interesting of which were the acetyl esters of alkanes including those of hexane, heptane, octane, and decane (Strobel et al. 2008). These alkanes

make up the backbone ingredients of diesel fuel and as such make this organism of interest as a Mycodiesel™ producer. Thus, it appears that microorganisms may have potential as sources of new liquid fuels or fuel additives. This provided encouragement to seek for other microbes that may make other hydrocarbons of interest.

While *Ascocoryne sarcoides* does make some desirable potential fuel components in its VOCs, the relative amount of each compound in the gas phase, in the air space above the culture, was only at the parts per billion (ppbv) level (Strobel et al. 2008). As a result of a

growing interest in biofuels, especially from microbial sources, a search is on for other microbes that have the genetic machinery for hydrocarbon production (Schirmer et al. 2010). In this communication an endophytic fungus of a euphorbeacean herb, *Acalypha indica* has been isolated and described; it makes hydrocarbon derivatives that were measured both qualitatively by GC/MS and quantitatively by NMR. The organism is identified with morphological and molecular techniques as *Myrothecium inundatum*. This organism also resists the VOCs of *M. albus*, possesses biological activity in its gas phase, and some of its gaseous components are of interest from the standpoint of being biofuels.

## Materials and methods

### Endophyte isolation and characterization

Stem pieces of *Acalypha indica* L. (local name Muktajhuri, Swetbasanta) were collected at Kalyani, India (22°58'10"N and 88°26'41"E). The procedures for isolating endophytes followed a previously described protocol (Worapong et al. 2001, Ezra et al. 2004). Briefly, external tissues were thoroughly exposed to 70% ethanol prior to excision of internal tissues which were cultured on standard Petri dishes of water agar and glycerol arginine medium (GAM). Endophytic fungi growing from the plant tissues were then picked and re-cultured on potato dextrose agar (PDA). A fungus of interest, because it was producing gases, was labeled IN-5. The fungus was stored by placing small plugs of PDA supporting mycelial growth in 15% glycerol at -70°C. An alternative storage method was also utilized in which the fungus colonized sterile barley seed, which was subsequently air dried and then stored at -70°C. The fungus is deposited in the living Montana State University mycological collection as acquisition number 2372.

### Growth of the Microorganism

The fungus, IN-5, was grown in 2L Erlenmeyer flasks with 600 ml of potato dextrose (PD) broth (EMD, Germany) at 23 °C for 15 days in a rotary shaker (120 rpm). The composition of the medium was 24 g of PD broth powder per liter of tap water. Four mm<sup>2</sup> mycelial mats (approx. dry weight 1 mg) from

15–20 days old agar plates were scraped from the agar surface and used as the inoculum for each flask.

### Fungal DNA isolation and acquisition of ITS- 5.8S rDNA phylogenetics

Mycelium grown on PD broth for 7 days was harvested and the genomic DNA extracted using DNeasy Plant and Fungi Mini Kit (Qiagen), according to the manufacturer's directions. The ITS region of the fungus was amplified using PCR with the universal ITS primers ITS1 (5' TCC GTA GGT GAA CCT GCG G 3') and ITS4 (5' TCC TCC GCT TAT TGA TAT GC 3') (White et al. 1990). All other procedures were carried out as previously described by Ezra et al. (2004). The DNA was sequenced at the W.M. Keck Facility at Yale University, and the sequence was submitted to GenBank (HQ165763). Sequences were compared to the GenBank data base using BLAST.

BLAST results having full species identification were downloaded from Genbank. After noting the order of the organisms, a representative from each genus in the order was also downloaded (see Supp. Table 1) and aligned with IN-5 and aligned using MUSCLE (Edgar 2004). Gaps were cleaned with Phyutility (Smith & Dunn 2008) and ends truncated in Jalview (Waterhouse et al. 2009) leaving 489 characters. Trees were assembled using a GTR+Γ+I model in MRBAYES 3 (Ronquist & Huelsenbeck 2003) with 1,000,000 generations for each of two runs of four chains. The first 25% of the trees were discarded as the burn-in and the rest reported as posterior probability scores for each node.

### Scanning electron microscopy (SEM and ESEM)

The fungus was grown on PDA, or autoclaved gamma irradiated carnation leaves for several weeks until fungal fruiting structures were observed and then was processed for SEM. The samples were slowly dehydrated in ethanol as previously described and then critically point dried, coated with gold and examined with a Philips XL 30 ESEM FEG scanning electron microscope (Strobel et al. 2007).

Because the spores and other fruiting structures of the fungus appeared fragile and

easily disrupted, the organism was subjected to the relatively unique microscopic application that preserves the fruiting bodies intact (spores attached). Thus, fresh or non-treated specimens were examined by environmental scanning microscopy (ESEM) using the Phillips instrument (Strobel et al. 2007). The temperature was 4°C with a chamber pressure that ranged from 5 to 6 Torr providing humidity up to 100% at the sample.

**Supplementary Table 1** GI numbers of organisms used to generate the phylogenetic tree for IN-5.

Genus	species	GI number
IN-5		
Acremonium	alternatum	237769572
Acrostalpmus	luteoalbus	294612745
Cephalosporium	caerulens	50401961
Cylindrocladiella	camelliae	12963070
Fusarium	boothii	91719228
Gliocladiopsis	irregularis	12963095
Gliocladium	cibotii	254681496
Lanatonectria	flocculenta	134134062
Melanospora	pascuensis	5102758
Myrothecium	inundatum	11877346
Myrothecium	inundatum	225547727
Myrothecium	inundatum	37956611
Myrothecium	roridum	197283754
Myrothecium	lachastrae	37956618
Myrothecium	carmichaelii	37956609
Myrothecium	leucotrichum	11877343
Myrothecium	roridum	237872425
Myrothecium	gramineum	209779008
Myrothecium	roridum	11830875
Myrothecium	roridum	11830873
Myrothecium	roridum	11830872
Myrothecium	gramineum	256259698
Myrothecium	roridum	255098794
Myrothecium	gramineum	227460832
Myrothecium	roridum	209360973
Myrothecium	roridum	32165599
Myrothecium	roridum	53125402
Myrothecium	verrucaria	296279058
Myrothecium	togaense	37956616
Myrothecium	roridum	11830871
Myrothecium	verrucaria	289718955
Myrothecium	verrucaria	209779009
Myrothecium	roridum	37956614
Myrothecium	roridum	294335294
Myrothecium	roridum	240248322
Myrothecium	roridum	259186989
Myrothecium	roridum	122888928
Myrothecium	roridum	73697710
Myrothecium	roridum	122888929
Myrothecium	roridum	119567599
Myrothecium	masonii	37956612
Myrothecium	setiramsum	37956615

Genus	species	GI number
Myrothecium	roridum	300076914
Myrothecium	roridum	262410750
Parasarcopodium	ceratocaryi	37962701
Pochonia	suchlasporia	66796177
Pochonia	suchlasporia	32328884
Polycephalomyces	ramosus	53748163
Pteris	ensiformis	161725012
Rotiferophthora	agustispora	13160430
Rubrinectria	olivacea	152126061
Sarcopodium	araliae	199599780
Sesquicillium	microsporum	281307458
Solheimia	costispora	74027014
Stachybotrys	echinata	162415949
Stachybotrys	echinata	11602959
Stachybotrys	echinata	225675532
Stachybotrys	echinata	11692064
Stachybotrys	echinata	11692063
Stachybotrys	echinata	11692062
Stachybotrys	echinata	162415952
Stachybotrys	echinata	11692070
Stachybotrys	echinata	11692069
Stachybotrys	echinata	11692068
Stachybotrys	echinata	162415955
Stachybotrys	echinata	11692067
Stachybotrys	echinata	11692066
Stachybotrys	echinata	11692065
Stachybotrys	echinata	11692071
Stachybotrys	oenanthes	3420915
Stilbella	aciculosa	296410163
Ustilnoidea	virens	269996996
Verticillium	bguttatum	157072458
Xenocylindrocladium	guianense	12658332

### Qualitative analysis of fungal gases

The airspace on an 18-day-old culture grown under microaerophilic conditions was assayed by exposing a pre-baked Solid Phase Micro Extraction (SPME) syringe (Supelco) consisting of 50/30 divinylbenzene/carboxen on polydimethylsiloxane on a Stable Flex™ fiber. In addition, the organism was also grown from 9 to 31 days at 23 °C on 600 ml of PD broth in 2 liter flasks with shaking at 125 rpm to give maximum aeration. The foam that was produced was harvested and placed in sealed 1 L bottle and frozen at -70 °C for 1 hr to compress the foam and release the trapped gases. A SPME fiber was placed through a small hole drilled in the septum of the 1 L bottle and exposed to the vapour phase for 45 min. In all cases the SPME fiber was then inserted into the splitless injection port of a Hewlett Packard 6890 gas chromatograph where the contents were thermally desorbed onto a 30 m × 0.25 mm I.D. ZB Wax capillary column with a film thickness of 0.50 μm. The

column was temperature programmed as follows: 30°C for 2 min and increased to 220 °C at 5°C/min. Details for all other aspects of running the gas analysis have been previously published (Strobel et al. 2001). Data acquisition and data processing were performed on the Hewlett Packard ChemStation software system. All chemical names in this report follow the nomenclature of this database. Data are presented as the percent of each compound represented of the total in each analysis done at days 9, 12, 15, 18, 21, 25 with each test being replicated one or more times.

In all cases, uninoculated control flasks were also analyzed and the compounds found therein were subtracted from those appearing in the flask supporting fungal growth. Tentative identification of the fungal volatiles was based on observed mass spectral data as compared to those in the WILEY database. Final confirmatory identification was made on many of the compounds of this fungus by comparing GC/MS data of authentic standards with the comparable GC/MS data of the fungal products. The experiments were repeated at least twice with comparable results. Identities of fungal compounds whose authentic standards were not available were made on the basis of the percentage identity match in the GC/MS data at a level of 60% or greater. Thus, some compounds that are listed may actually be entirely different compounds and all MW 136 and 204 compounds are simply listed as numbered monoterpenes and sesquiterpenes, respectively. This was arbitrarily done for these terpenes since final identification of these molecules could not be ascertained as authentic standards were not available.

### Estimating fungal VOCs by NMR analysis

Foam from each fermentation vessel was collected in a flask and an equal volume of methylene chloride was added to it. The flask was placed in an ice bucket for 10 min and shaken vigorously. Eventually, after 10–15 min, the foam disintegrated to produce an aqueous slimy layer at the top of organic phase. The organic phase was then poured from the flask and flash evaporated in a rotary evaporator (RE47, Yamamoto Scientific Co., Japan) at 30 °C, 18–20 mm Hg, 90–120 rpm rotor speed. A water trap (250 ml) between the vacuum

pump and the rotary evaporator contained a 75 ml water-methylene chloride mixture (20:1 v/v) in order to maintain the vapor pressure of methylene chloride. When the evaporation of the methylene chloride ceased, the evaporation flask was immediately separated from the system, sealed and placed in a refrigerator (0–4 °C). After an hour the liquid condensed at the bottom of the flask and was collected and analyzed in a Bruker DRX500 NMR spectrometer. It is to be noted that much of the condensed and collected liquid was the methylene chloride solvent containing the fungal VOCs. Calculations were then made for the actual amount of total hydrocarbons per treatment for each experimental flask. This was done by integrating the total proton signal in the hydrocarbons and the total signal of protons of protons in methylene chloride. The PD broth itself has VOCs associated with it, but an exact foam control flask could not be processed since no foam developed in PD broth alone. However, a volume of sterilized PD broth equal to that of the aqueous phase of the fungal cultured foam mentioned above was extracted with an equal volume of methylene chloride and processed in an identical manner. The amount of organic products detectable with identical settings on the NMR was negligible. Thus, the contribution to the organic products from the media alone in the final calculation of organic fungal products was not factored into the calculation. Finally, the calculation for the actual amount of hydrocarbons present (HC) was as follows:

$$HC = V(P_{CH_2Cl_2}/P_{IN-5}) (Area_{IN-5}/Area_{CH_2Cl_2})$$

HC = fungal VOCs

V = volume of concentrate

$P_{CH_2Cl_2}$  = number of protons in  $CH_2Cl_2$

$P_{IN-5}$  = number of protons in fungal volatiles obtained by averaging the protons per molecule of those compounds detected by GC/MS estimated at 8.6.

Area = NMR integral area for sample HC or  $CH_2Cl_2$  as indicated

P = number of protons in sample or  $CH_2Cl_2$

### Screening of different media for hydrocarbon production

The following media were used for hydrocarbon production: 1) mashed potatoes

(Albertsons, USA) 24 gm/L; 2) corn starch (Albertsons, USA) 24 gm/L; 3) cellulose (0.5%), cellobiose (0.5%) in basal salts; 4) glucose (1%, w/v) in basal salts; 5) sucrose (1%, w/v) in basal salts; 6) soytone peptone (Difco, USA) (0.1 %), cellulose (0.5%) in basal salts; 7) beet pulp (collected from local market) 24 gm/L; 8) Cyanobacteria (collected as algal bloom from Canyon Ferry Reservoir and air dried) 24 gm/L. The composition of the basal salts medium was (g/L): (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 2 gm; KH<sub>2</sub>PO<sub>4</sub>, 1 gm; KCl, 500 mg; MgSO<sub>4</sub>, 500 mg; FeSO<sub>4</sub>, trace; yeast extract (trace).

### Estimation of biomass

After removal of the fungal generated foam, the fungal mycelium was collected in a flask and equal volumes of distilled water was added and shaken vigorously. The process was repeated thrice in order to remove unwanted mucoidial materials. The mycelial suspension was centrifuged 10,000 x g for 5 min and the mycelium was collected in a pre-weighed aluminum sheet, dried at 40 °C for 48h and weighed. For estimation of biomass on agar plates, the mycelial mat was scraped from a measured area of the plate surface and the same process was followed.

### Test for inhibition by *Muscodor albus*

A 1 cm wide agar strip from the center of a PDA plate was removed and *M. albus* 620 was inoculated and allowed to grow on one half of the plate for 10–12 days and then IN-5 was inoculated on the other side of the plate, using a small plug of agar. Growth was then observed after two days and recorded. Similarly IN-5 was first grown for 7 days and then test organisms were placed as small plugs on the opposite half moon of agar. Growth measurements of the test organisms were made after 2 days of incubation at 23°C (Strobel et al. 2001).

## Results and discussion

### Identification of the endophytic isolate IN-5 as *Myrothecium inundatum*

On PDA, the fungal colony turned off-white with a pink sheen after 7 days. The organism produced sporodochia with irregular margins at 100–500 µm diam. (Fig. 1A, C).

These structures, when adjacent, eventually coalesce to form rosette structures, light to dark green, slimy, sessile, and cushion-like. Eventually, green, shiny, slimy masses of spores are produced that turn darker with time and with the subsequent loss of their shine. Conidia are cylindrical, swollen and convex at the base and are 1.7–2.0 x 3–5 µm and ellipsoidal (Fig. 1E, F). The conidiophores are 50–80 µm long and are tightly positioned within the sporodochia (Fig. 1C, D, E). The collective morphological characteristics of this organism associate it most closely to *Myrothecium inundatum* (Preston 1961, Watanabe et al. 2003). This assignment agrees with a phylogenetic analysis comparing the ITS sequence to deposited sequences in GenBank showing *M. inundatum* as the closest relative (Fig. 2). All morphological and molecular data support the conclusion that the organism is *M. inundatum* (Fig. 1A). *Myrothecium inundatum* is a member of the family Bionecteriaceae in the class Sordariomycetes.

### Biological effects of *Myrothecium inundatum* volatiles on test fungi

*Myrothecium inundatum* inhibits the growth of several plant pathogenic fungi including *Fusarium solani* (15%), *Trichoderma viride* (5%), *Pythium ultimum* (100%), and *Sclerotinia sclerotiorum* (70%) with a 2 day exposure to its VOCs. Interestingly, *M. inundatum* grows well, showing no inhibition, in presence of *Muscodor albus* (strains 620, GBA, E-6 –Strobel's lab isolates) which produces volatile antibiotics and has been shown to be a useful tool to select fungi that produce VOCs (Strobel et al. 2001, Ezra et al. 2004, Strobel et al. 2007, Mitchell et al. 2010). Thus, *M. inundatum*, also as a gas producer, seems to conform to the growing list of VOC producing fungi making a wide mix of gaseous compounds that are insensitive to the VOCs of *M. albus*.

### Chemical composition of the volatiles

The volatile compounds of *M. inundatum* were assayed using the SPME fiber technique. The organism was grown for 18 days under microaerophilic conditions on solid PDA as previously described. This was done by growing the organism for 6 days under aerobic

**Table 1** GC/MS analysis of the VOCs produced by *Myrothecium inundatum* after 18 days incubation in a 250 ml bottle at 23 °C on 80 ml PDA medium under microaerophilic conditions as described in the methods section. Total dry weight of the mycelial mat was 27.5 mg. Compounds found in the control PD broth are not included.

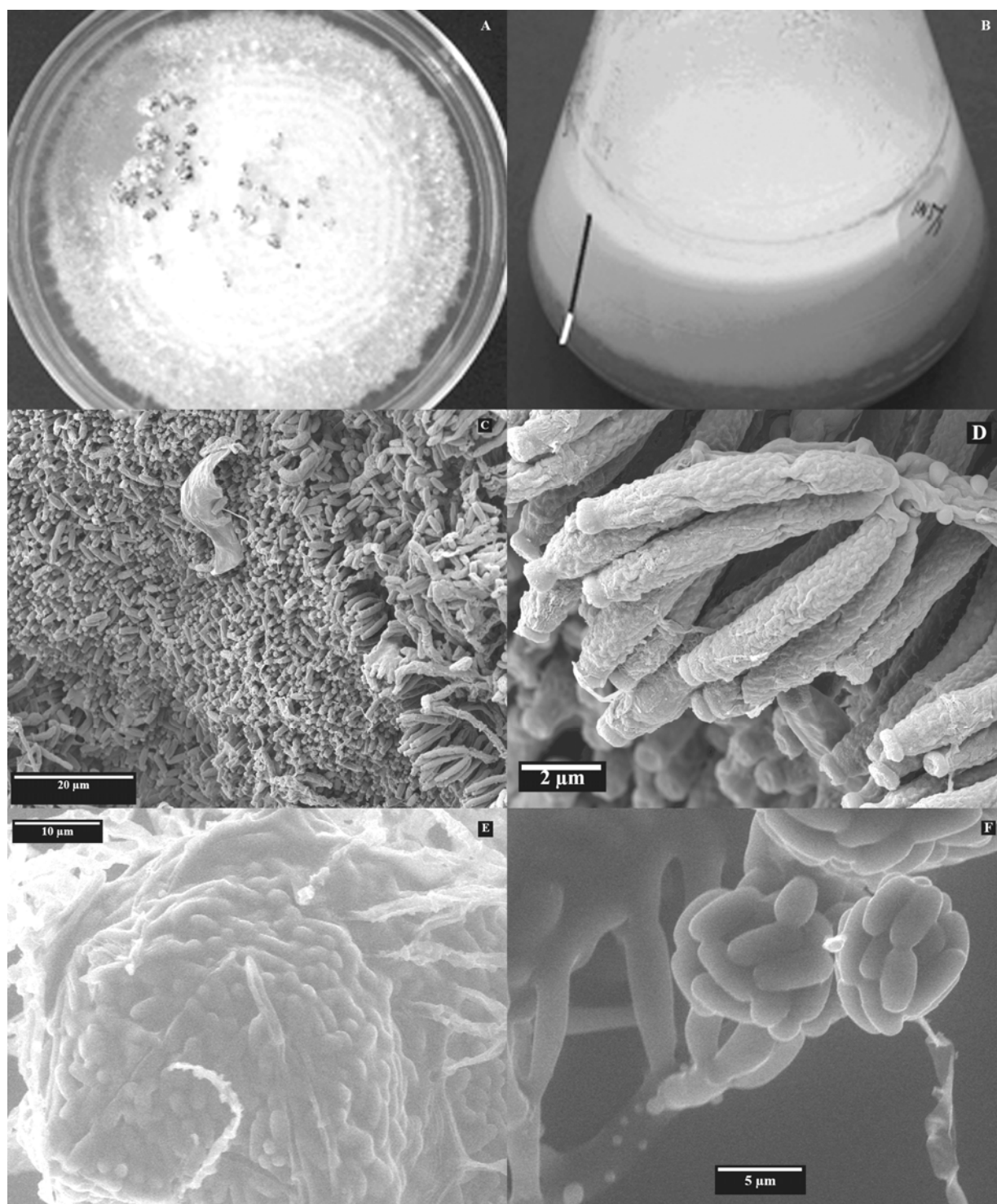
Time (min)	Possible Compound	Std	Relative %	Molecular mass (Da)
1.8	Methanethiol		0.64	48
1.89	2-Butanamine, 3-methyl-		0.69	87
2.33	Octane	*	0.68	114
3.95	Ethanol	*	1.18	46
4.22	Furan, 2-5-dimethyl-		1.29	96
4.57	2-Pentanone	*	0.71	86
4.96	Butane, 2-iodo-		2.12	184
5.13	1,4-Cyclohexadiene, 1-methyl-		6.26	94
5.4	C <sub>7</sub> H <sub>10</sub> ?		3.67	94
5.48	C <sub>8</sub> H <sub>14</sub> ?		1.08	110
7.45	Monoterpene 1		0.65	136
8.22	1-Butanol, 3-methyl-	*	7.86	88
9.01	Methyl ester of 4-methylene-hexanoic acid		1.45	142
10.95	2-Nonanone	*	5.24	142
11.19	Cyclohexane, (1-ethylpropyl)-		8.13	154
12.09	Acetic acid	*	8.05	60
12.27	Sesquiterpene 1		0.74	204
13.5	Sesquiterpene 2		1.33	204
13.57	Sesquiterpene 3		2.84	204
13.61	2,10-Bornanediol, exo-		1.37	170
13.91	Monoterpene 2		5.30	136
14.18	Sesquiterpene 4		1.18	204
14.55	Sesquiterpene 5		16.15	204
14.83	Sesquiterpene 6		4.62	204
15.2	Sesquiterpene 8		1.89	204
15.28	Sesquiterpene 9		1.32	204
15.55	Sesquiterpene 10		0.61	204
15.71	Sesquiterpene 11		0.57	204
15.79	Benzeneacetic acid, methyl ester		0.79	150
15.88	Ethanone, 1-(1,3-dimethyl-3-cyclohexen-1-yl)-		0.69	152
16.45	Benzene, 1,3,5-trichloro-2-methoxy-		2.48	210
17.49	Benzeneethanol	*	1.59	122

\* Standard authentic compounds having the same RT and MS as the fungal product.

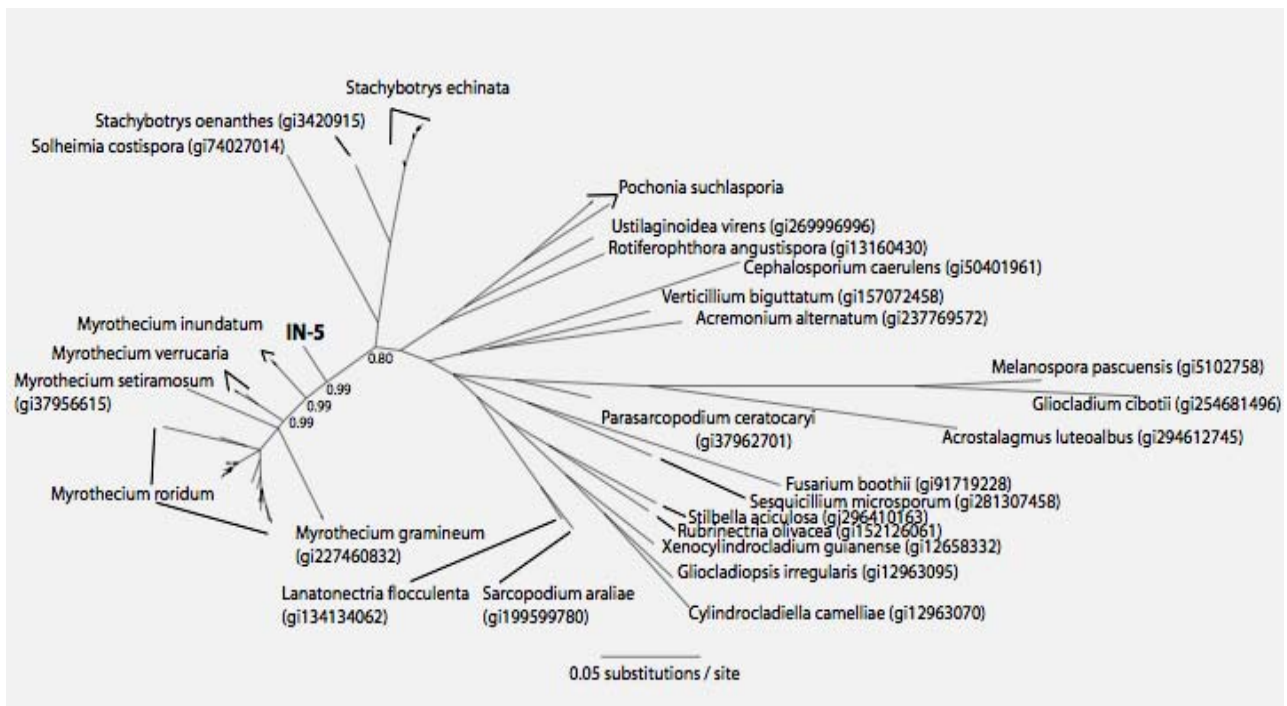
? A question remains as to the identity of the compound. All compounds listed, without appropriate standards, are only identified with respect to their match to the NIST database with a quality of 60% or greater.

conditions and then restricting air availability by sealing the fermentation bottle for 12 days. Due to the limited availability of oxygen the organism was expected to produce a number of compounds such as ethanol and acetic acid, however 1-butanol, 3-methyl also appeared (Table 1). Among others octane, ethanol and 1-butanol, 3-methyl are of interest from a potential fuel perspective (Table 1). Also of

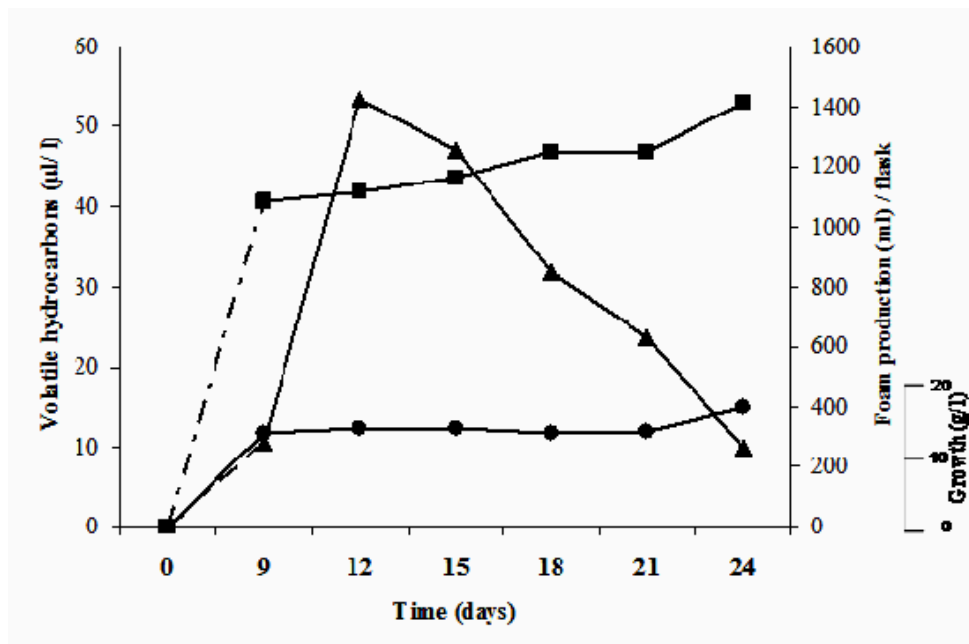
note is the presence of a wide range of compounds in the molecular weight range of 136 and 204 representing a mixture of mono- and sesquiterpenes of uncertain identity, most in relatively low abundances (Table 1). Other volatile compounds were also present including 1,4-cyclohexadiene, 1-methyl- and cyclohexane (1-ethylpropyl)- as well as some benzene derivatives, and ketones (Table 1).



**Fig. 1** – *Myrothecium inundatum* (IN-5) is a member of Bionectriaceae. A) On a PDA plate 2-week-old. B) liquid broth culture 12-days-old (black and white bar indicates foam and growth respectively). SEM photos C) sporodochium, D) conidiophores and ESEM photos spore clusters. E–F) two spore clusters on conidiophores.



**Fig. 2** – The unrooted phylogenetic tree showing the placement of IN-5 within the order Hypocreales. *Melanospora pascuensis* (gi 5102758) from the order Melanosporales is included in the outgroup. Nodes that distinguish IN-5 from its neighbors are labeled with Bayesian posterior probabilities.



**Fig. 3** – Time course of growth, foam and hydrocarbon production by *Myrothecium inundatum* (IN-5) (growth ●; foam production ■; VOCs ▲).

### VOC production in liquid-shake culture

In liquid-shake culture the organism grows fast and produces a viscous biomass within 6 days. Frothing begins after 8 days and this holds for at least 2 weeks. If distilled water is used then no frothing occurs which is related to the absence of cations. About 800 ml of

froth had accumulated at the top of the culture vessel after 15 days (Fig. 1B). The froth was collected in a bottle and analyzed after freezing and thawing over a time course (9–25 days at 3 day intervals) with a bottle for each designated time. The gas trapped in the froth is mainly carbon dioxide and volatile organic compounds.



The composition and concentration of different components varies with time (Table 2). The compound in the greatest abundance, or relative percentage, from days 9–21 was 3-octanone (Table 2). As expected, it was not detected in the flask grown under micro-aerophilic conditions neither was 3-octanol or octanone (Table 1). Both benzene, methyl- and naphthalene, diesel related compounds, were

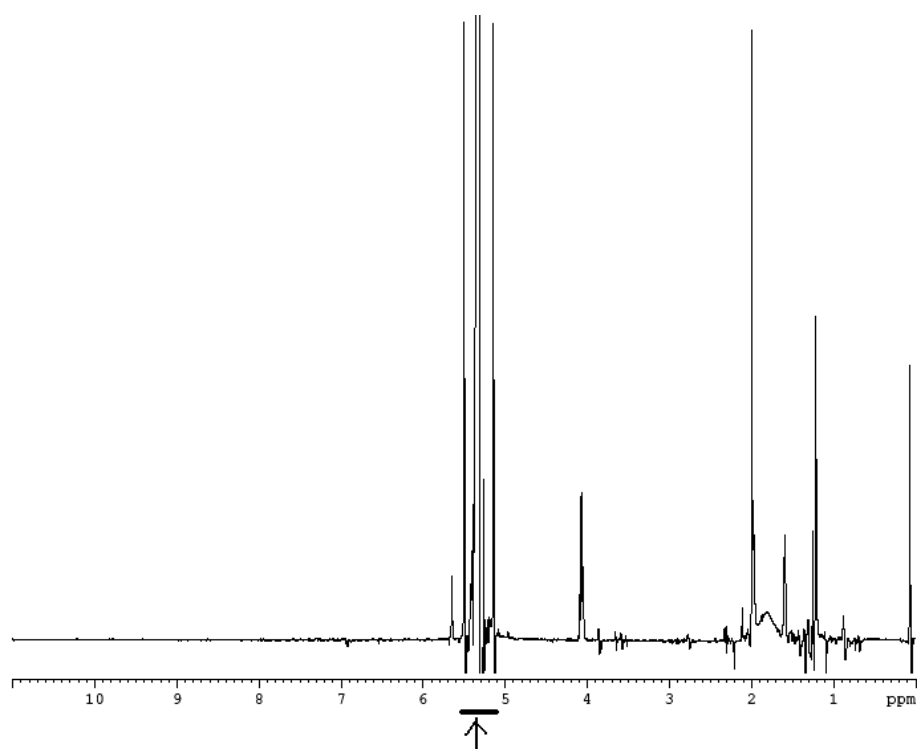
present in small quantities in the gas mixtures of this fungus at mid point and the terminal point of the fermentations (Table 2). Many terpenes were present, in relatively small quantities, as noted throughout the fermentation process with a maximization of production in mid-course of fermentation cycle (Table 2). In addition to 3-octanone many other ketones were produced including 1-octen-3-one,

**Table 2** GC/MS analysis of the volatile compounds produced by *Myrothecium inundatum* at different time intervals in a 2 l Erlenmeyer flask at 23 °C on PD broth medium at 120 rpm shaking speed. (Compounds found in the control PD broth are not included.)

Retention Time (min)	Compound	Std	Mol Mass (Da)	Relative abundance of the compound (%)					
				Collection time					
				9 day	12 day	15 day	18 day	21 day	25 day
4.17	Bicyclo [4.1.0] heptan-2-one		110	10.8	11.8	0	0	0	0
4.20	5,6- Undecadiene		152	0	0	0	4.1	0	14.5
4.21	1-Cyclohexen-1-al		110	0	0	9.1	0	6.3	0
5.56	Benzene, methyl-	a,b	92	0	1.11	0	0.6	0	0
6.40	1,3-Trans-5-cis-Octatriene		108	0.2	0.3	0	0	0	0
7.98	Monoterpene 1		136	0.3	0.5	0	0	0.2	0
8.23	1-Butanol, 3-methyl-	b	88	0.3	0.7	0	0	0	0
8.57	Hexanoic acid, ethyl ester		144	0	11.7	0	2.9	2.5	0
8.98	3-Octanone	b	128	62.7	41.8	36.8	57.8	64.8	0
9.68	1-Octen-3-one	b	126	0.9	0	0	0.4	1.1	18.8
10.00	5-Hepten-3-one, 5-methyl-, cis/trans		126	0.2	0	0.3	0	0	0
10.91	3-Octanol	b	130	19.2	2.2	0	12.9	18.9	0
11.42	1-Hexadecene		224	0	0	3.4	0	0.3	1.6
11.51	2-Octenal, (E)-	b	126	0	0	17.0	0	0	19.1
11.71	7-Octen-4-ol		128	0	21.1	2.0	10.3	0	2.4
12.62	Monoterpene 2		136	0.3	0	7.4	0.7	0.5	0.9
13.48	Sesquiterpene 1		204	0	0.2	0	0.6	0.4	0
13.53	Sesquiterpene 2		204	0.3	0	2.9	0.2	0	0
13.58	Cyclopentene, 3-ethylidene-1-methyl		108	0	0.6	0	1.4	0.8	0
13.87	Monoterpene 3		136	0.7	0.9	0	0	0	0
14.14	Sesquiterpene 3		204	0.3	0.5	0	0.8	0.8	0
14.51	Sesquiterpene 4		204	0.3	4.9	9.7	3.3	0.9	3.3
14.72	Sesquiterpene 5		204	0	0	10.5	1.1	0	0
14.89	4 -Nonyne		124	0	0	0	0.4	0	3.5
15.15	Sesquiterpene 6		204	0	0.3	0	0.4	0.2	0
15.61	Naphthalene	a,b	128	0	0	0	0	0	1.2
15.85	Ethanone, 1-(1,4-dimethyl-3-cyclohexen-1-yl)-		152	0	0.2	0.3	0.4	0.2	0
17.45	Benzeneethanol	b	122	2.5	0.4	0	0.8	0.8	14.9
17.78	Heptanoic acid	b	130	0	0	0	0	0	1.4
19.49	Dimethyl cyclopentadiene dimer		160	0	0	0	0	0	11.2
20.16	2-Octanoic acid		142	0	0	0	0	0	1.5

<sup>a</sup> A product found in diesel fuel.

<sup>b</sup> Standard authentic compounds having the same RT and MS as the fungal product. All compounds listed, without appropriate standards, are only identified with respect to their match to the NIST database with a quality of 60% or greater.



**Fig. 4** – The NMR difference spectrum of volatiles produced by *Myrothecium inundatum* after 12 days. Arrow indicates the position of methylene chloride. This NMR difference spectrum was produced by subtracting the spectrum taken after drying the fungal foam extracted sample from the original sample yielding a spectrum of the evaporated materials (VOCs). The major signals in the upfield region (1.2–1.5 ppm) are consistent with those of 3-octanone and related octane derivatives, for at least 3 weeks, relatively the most abundant product in the foam (Table 2).

**Table 3** Growth (dry weight), foam and VOC production by *Myrothecium inundatum* on different media.

Medium	Growth <sup>*</sup>	Foam production <sup>+</sup>	Volatile hydrocarbons (µl/l foam) <sup>@</sup>
PD Broth	Good	Good	ca.50
Mashed potato	Medium	Medium	NA
Corn starch + MIN <sup>#</sup>	Medium	Medium	ca.7
Cellobiose+ MIN	Poor	Poor	NA
Glucose + MIN	Medium	Medium	NA
Sucrose+ MIN	Medium	Medium	NA
Cellulose+ MIN	Poor	Poor	NA
Beet pulp	Good	Good	ca.30
Blue green algae	Medium	Poor	NA

<sup>#</sup>MIN, mineral solution.

<sup>\*</sup>good, more than 7 g/l; medium, 3–7 g/l; poor, less than 3 g/l.

<sup>+</sup>good, equal volume of broth; medium, ¼ to ½ volume of broth; poor, less than ¼ volume of broth.

<sup>@</sup>indicating the approximate value of hydrocarbons produced by the NMR method.

NA, not applicable since little or no foam was produced.

5-hepten-3-one, 5 methyl-cis trans and bicyclo [4.1.0] heptan-2-one and they seem to appear earlier in the fermentation cycle (Table 2). The fluctuation in relative amounts of individual compounds (Table 2) is most likely due to the inability of the SPME fiber to behave in a

predictable manner when doing analyses of this type. The data simply represent an approximation of the production of the various VOCs being produced by the fungus as a function of time.

### Quantification of fungal VOCs

Components of the foam were collected and extracted with methylene chloride which was reduced in volume to less than 1.0 ml and then subjected to NMR for estimation of fungal products associated with the foam. A corresponding volume of PD culture broth was used as a control and the amount of product obtained from it by methylene chloride extraction method was minimal, yielding only trace signals in the NMR and it was therefore not considered in the calculations. The organism reached its maximal growth, more or less, at 12 days whereas the production of foam became maximal at 24 days (Fig. 3). The production of VOCs peaked at 15 days with the production of about 50 µl hydrocarbons per liter of foam (Fig. 3). As a check on the NMR method used to acquire these data an NMR difference spectrum was obtained (Fig. 4). First the spectrum (original) was acquired and then all solvent and fungal VOCs were removed by complete drying. The minimal residue was taken up in methylene chloride and the NMR spectrum acquired on it was subtracted from the original spectrum (Fig. 4). The NMR data are internally consistent with each other.

### Effect of different media composition on fungal VOC production

Different media were prepared and tested for VOC production by *M. inundatum* (Table 3). A number of products can serve as carbon sources for the production of VOCs by this fungus including beet pulp and corn starch (Table 3). It was found, however, that PD broth is most suitable for VOC production. Synthetic medium with cellulose and cellobiose is unable to support the production of any VOCs. This may be due to the inability of the organism to breakdown cellulose within the time frame of the experiment or the prerequisite production of various substances needed to make the foam itself, which provides the trapping structures for the fungal VOCs.

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