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MARINE FUNGI OF THE BAY OF FUNDY VI: GROWTH AND METABOLITES OF LEPTOSPHAERIA ORAEMARIS, SPHAERULINA ORAEMARIS, MONODICTYS PELAGICA AND DENDRYPHIELLA SALINA

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The growth of four lignicolous marine fungi on a variety of carbon sources was measured quantitatively. The fatty acids and sterols of the marine fungi were also investigated. The fungi grew on constitutive wood sugars and grew poorly on polysaccharides and hydrocarbons. The major sterol of the fungi tested was ergosterol. The relative amounts of the fatty acids produced was somewhat different than that of terrestrial fungi.

La croissance de 4 champignons marins lignicoles, exposés à diverses sources de carbone, a été mesurée quantatitivement. Les acides gras et les stérols de ces champignons marins ont aussi été étudiés. Les champignons croissaient en utilisant les sucres constitutifs du bois mais parvenaient difficilement a croître sur des polysaccharides et des hydrocarbones. Le principal stérol des champignons testés est l'ergesterol. Les quantités relatives d'acides gras produites étaient un peu différentes de celles des champignons terrestes.

Introduction

The number of studies on the carbon nutrition of higher marine filamentous fungi is surprisingly low. Kohlmeyer and Kohlmeyer (1979) list approximately 250 species of higher marine fungi and about 12 have been tested quantitatively for growth on a range of carbon compounds in liquid culture (Henningsson 1978; Meyers 1966; Meyers & Scott 1967; Sguros et al. 1973), and about 8 using agar plate cultures (Barghoorn & Linder 1944,; Johnson et al. 1959).

Chesters and Bull (1963) tested 6 lignicolous marine fungi for laminarase, five of which produced a low amount of the enzyme. Tubaki (1969) tested three lignicolous marine fungi for growth on laminarin, carrageenan, and Na-alginate. All of them produced "luxuriant" growth on laminarin, "moderate" to "good" growth on carrageenan (which form of carrageenan not stated) and "scanty" to "moderate" growth on Na-alginate.

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The ability of yeasts and filamentous terrestrial fungi to degrade petroleum under saline conditions is well known (Ahearn & Myers 1976; Ahearn & Crow 1980; Cerniglia & Perry 1973). The authors are not aware of any reports with respect to the ability of marine filamentous fungi to degrade petroleum or hydrocarbons. Based on circumstantial evidence, Kirk (1983) suggested that marine fungi may play a role in the degradation of hydrocarbons.

Very few reports exist concerning the sterols and fatty acids of higher marine fungi (Kirk & Catalfomo 1970; Kirk et al. 1974), although much data exist with respect to terrestrial fungi (Weete 1980).

The purpose of this study was to measure the growth of four higher marine fungi on a variety of carbon sources and to determine their sterols and fatty acids.

Materials and Methods

Growth studies

Cultures of *Leptosphaeria oraemaris* Linder, *Sphaerulina oraemaris* Linder, and *Monodictys pelagica* (Johnson) E.B.G. Jones were obtained from driftwood by placing ascospores or conidia from driftwood on seawater agar (Johnson & Sparrow 1961) containing 0.5 g/L penicillin G and streptomycin sulphate. *Dendryphiella salina* (Sutherland) Pugh et Nicot, was similarly isolated from an oil-contaminated weir pole. Fungi were isolated from wood collected on the New Brunswick coast of the Bay of Fundy as described in Miller and Whitney (1981a).

The growth of *L. oraemaris*, *S. oraemaris*, and *M. pelagica* on various carbon compounds from seaweeds (either from commercial sources or appropriate substitutes) was measured using gravimetric methods (Sguros et al. 1962). The fungi were tested for growth on the carbon sources in artificial seawater (ASW) ($NaNO_3$, 0.4 g/L; K_2HPO_4, 0.06 g/L) at 10 and 20°C (Churchland & McLaren 1976) adjusted to 28‰.

Cellobiose, dextrose, fructose, galactose, maltose, mannitol, mannose, sucrose and trehalose were added as 1 mL volumes of filter-sterilized ASW in 125 ml Erlenmeyer flasks to a concentration of 1.0 g/L. Agar (Difco, special purified), iota carrageenan, kappa carrageenan, lambda carrageenan, alginic acid, sodium alginate, ammonium-calcium alginate (Sigma Chemical Co.), gelatin (Difco)[1], starch (potato)[2], and Tween 20[3] were mixed each in a separate flask into ASW at a concentration of 0.5 g/L. Then 24 mL were dispensed into flasks and autoclaved. All flasks were stoppered with non-absorbent cotton wool.

Inoculum was prepared as described by Sguros et al. (1962). Inoculum suspensions were adjusted to deliver 2.0 mg dry weight in a 1 mL volume of ASW. All flasks were inoculated with 1.0 mL of inoculum for a final volume of 25 mL per flask.

Three replications of each treatment plus controls (carbon-free solutions) were done. With careful inoculum preparation, accurate dispensing of all solutions and the use of identical 125 mL flasks (Parberry 1971), standard deviations were about 1% of the mean. Flasks at both temperatures were incubated for two weeks.

At the end of the incubation period, cultures grown on the algal polysaccharides, gelatin, and Tween 20 were brought just to boiling (2 min) to reduce viscosity prior to filtration through pre-weighed HA 0.45 μm Millipore filters. Filters and filtered material were washed with warm distilled water. All filters were dried at 100°C and weighed.

The fungi above and *Dendryphiella salina*[4] were grown on a series of hydrocarbons in 25 mL sterilized ASW (28‰) in 125 mL flasks each containing one of

[1] a protein source.

[2] Percival (1968) suggests that potato starch is similar to Floridean starch.

[3] Tween 20 is a convenient lipid source.

[4] Data on the growth of an isolate of *D. salina* on the other carbon compounds are given in Miller and Whitney (1981b).

n-undecane, *n*-dodecane, *n*-tetradecane, *n*-hexadecane, *n*-octadecane, or *n*-eicosane, added aseptically to give 0.5 g/L. The methods were as described above. There were 3 trials with hydrocarbons and carbon-free controls. Filtered material and filters were washed with chloroform (2-3 mL) to ensure that no hydrocarbon remained on the filter. Filters were dried and weighed.

Analysis of selected metabolites

Cultures of *L. oraemaris, S. oraemaris, M. pelagica* and *D. salina* were grown in three 2.8 L Fernbach flasks containing 560 mL ASW (28‰, $NaNO_3$, 0.4 g/L; K_2HPO_4, 0.06 g/L; dextrose, 5 g/L) at 20°C for three months. At the end of the incubation period, cultures were filtered through HA 0.45 μm Millipore filters. The hyphae were scraped from the filters and homogenized in chloroform. The filtrate was separately extracted with chloroform.

The total crude extracts were each hydrolyzed in 10 ml of IN HCl for 3 h at 100°C. The reaction mixture was cooled and extracted with chloroform (3x15 mL). The chloroform extract was washed with water (2x15 mL) and then evaporated under reduced pressure after drying with anhydrous sodium sulphate.

The residue was treated with a mixture of anhydrous methanol (18 mL), chloroform (1 mL), concentrated hydrochloric acid (2 mL) and heated under reflux for 2 h. After cooling to room temperature, the reaction mixture was concentrated under vacuum, diluted with water (50 mL) and extracted with chloroform (3x20 mL). The chloroform extract was washed with water (2x50 mL), dried over anhydrous sodium sulphate and evaporated under vacuum. The oil was subjected to preparative TLC on Kieselgel $60F_{254}$, (hexane:ether:glacial acetic acid, 80:20:1). The fatty acid methyl ester fractions (R_f = 0.5 - 0.55) thus separated (Table I) were analysed (Tables II, III) on a fused silica capillary column, SP-2330, i.d. 0.32 mm x 30 m, film thickness 0.20 μm, using temperature programmed runs, 40°-160° at 10°/min, 160°-200° at 5°/min and isothermal at 200°C using helium gas (16 psi back pressure) until all components emerged.

All the analyses were performed using a Finnigan 4000 series GC/MS, equipped with the Finnigan 9610 GC and INCOS data system.

The sterol fractions (R_f = 0.2 - 0.25) were separated (Table I) and analysed as their TMS ethers on a fused silica capillary column DB-1, I.D. 0.32 mm x 30 m, film thickness 1.0 μm, using temperature programmed runs, 40°-180° at 40°/min and isothermal at 280° until all the components emerged (35 min) using helium gas (20 psi back pressure).

Results

Growth studies

The results of the tests for growth of the marine fungi on the 20 algal-derived carbon compounds are shown in Table IV. Most of the fungi grew well on the sugars and mannitol, except *M. pelagica*, which showed limited growth on mannitol. None of the fungi showed any growth on agar or on the carrageenans. Alginic acid supported some growth of all three fungi, whereas Na-alginate and NH_4 Ca alginate did not. Gelatin was used by *M. pelagica* but not by the other fungi. Starch was not used. Tween 20 was used by *L. oraemaris, S. oraemaris* and *M. pelagica.*

In general the marine fungi tested did not grow on n-hydrocarbons but *Sphaerulina oraemaris* did grow on dodecane, tetradecane and hexadecane. Although the isolate of *D. salina* used was obtained from petroleum-contaminated wood, no growth was observed on any of the hydrocarbons tested.

Table I Amount of fatty acids and sterols of marine fungi.

Name	Weight of hyphae (g)	Weight of fatty acid methyl esters (g)	Weight of sterol (g)
Monodictys pelagica	0.256	0.003	0.001
Spaerulina oraemaris	0.403	0.005	0.002
Leptosphaeria oraemaris	0.782	0.008	0.003
Dendryphiella salina	1.316	0.013	0.007

Table II Fatty acids of marine fungi (% composition).

Fatty Acid[1]	*Monodictys pelagica*	*Sphaerulina oraemaris*	*Leptosphaeria oraemaris*	*Dendryphiella salina*
dodecanoic	0.2	0.1	0.2	0.2
tridecanoic	-	0.07	0.1	0.1
12-methyl tridecanoic	-	-	-	0.1
tetradecanoic	3.5	3.0	2.4	8.3
12-methyl tetracanoic	-	0.4	0.1	1.8
pentadecanoic	0.7	1.3	4.5	2.4
12-methyl pentadecanoic	-	0.1	-	-
hexadecanoic	42.4	42.2	53.5	39.8
11-hexadecenoic	5.1	7.7	-	3.2
15-Methyl hexadecenoic	0.8	0.8	6.8	1.9
octadecanoic	7.2	12.9	17.7	-
10-octadecenoic	17.8	9.0	1.6	22.3
hexadecadienoic[2]	0.3	0.8	0.2	1.0
octadecadienoic[2]	0.6	-	0.4	-
nonadecanoic	-	0.1	-	-
eicosanoic	1.1	0.6	0.8	1.0
$\Delta^{8,11}$octadecadienoic	4.7	2.4	1.1	2.5
$\Delta^{11,14}$octadecadienoic	8.0	4.4	3.1	1.3
docosanoic	-	0.5	1.1	1.3
tricosanoic	-	-	0.3	-
tetracosanoic	-	0.4	2.3	3.2
unidentified[3]	6.6	12.8	3.3	8.5

[1]Analysed as their methyl esters and identified tentatively on the basis of comparison with data in the mass spec. library of the Finnigan 4000 series GC/MS instrument.
[2]Position of the double bonds not confirmed.
[3]see Table III.

Analysis of selected metabolites

Ergosterol accounted for 99.9% of the sterols of *Monodictys pelagica, Sphaerulina oraemaris* and *Leptosphaeria oraemaris.* However, in the case of *Dendryphiella salina,* ergosterol accounted for only 70.7% of the sterols. Two isomers of ergosterol, not yet identified, accounted for most of the remainder (5.2% and 22.4%). Cholesterol and cholestanol were not found.

The fatty acid methyl esters of the various fungi are listed in Table II, with the relative retention times (to C_{16}) of unidentified esters listed in Table III. The fatty

acids of these marine fungi were generally similar. However, the relative percentages of tetradecanoic, 12-methyltetradecanoic, 10-octadecenoic, octadecadienoic and tetracosanoic acids of *D. salina* were distinct from the other three fungi.

Discussion

The growth of the marine fungi on the various sugars tested revealed patterns similar to those reported by Henningsson (1978), Johnson et al. (1959) and Sguros et al. (1973). A difference was the relative growth of the fungi on cellobiose relative to glucose. Henningsson (1978) and Johnson et al. (1959) report more equal (but variable) values for the use of the two sugars by marine fungi. Meyers and Scott (1967) reported that *Corollospora maritima* Werdermann grew on mannitol as did *L. oraemaris* and *S. oraemaris* in this study, whereas *M. pelagica* did not. Henningsson (1978) and Meyers and Scott (1967) demonstrated that variability can exist with respect to the use of carbon compounds between strains of the same marine fungus, thus some of these responses may be variable.

Pisano et al. (1964) showed that several marine fungi can grow on gelatin. In the present study, only *M. pelagica* grew on gelatin. None of the isolates tested grew on potato starch. By contrast Meyers (1966), Meyers and Scott (1967) and Johnson et al. (1959) report the growth of several higher marine fungi on "starch" undefined with respect to source.

Generally, there was no growth of the marine fungi on the algal polysaccharides, which supports the observations of Chesters and Bull (1963), but is partly in conflict with the observations of Tubaki (1969). However, Tubaki's methods involved the growth of the marine fungi on agar media made up with laminarin, carrageenan or Na-alginate. This is an inadequate method for this purpose as growth may have resulted from carbon compounds in the inoculum or impure agar.

The fungi were able to grow on alginic acid but not on alginates. This demonstrates the importance both of testing the growth of fungi on the pure constituent polysaccharides as opposed to crude commercial preparations and also of using a number of salts of alginic acid to allow for possible cation toxicity (Hughes 1975).

The tests for growth of the marine fungi on hydrocarbons showed that generally they were unable to grow on these compounds. To the authors' knowledge there are no comparable published results. Marine fungi were less common in areas with oil pollution in the region surveyed by Miller and Whitney (1981a, cf. Kirk, 1983). We did not test whether these marine fungi are able to co-metabolize hydrocar-

Table III Unidentified fatty acid methyl esters.

M. pelagica		*S. oraemaris*		*L. oraemaris*		*D. salina*	
RRT[1]	%	RRT	%	RRT	%	RRT	%
1.13	0.3	0.76	0.4	1.60	0.4	0.63	0.1
1.29	0.2	0.85	0.1	1.90	0.3	0.75	0.4
1.34	0.6	1.14	0.3	1.94	0.5	0.86	0.6
1.52	0.5	1.34	0.1	2.15	0.1	1.26	5.9
1.71	0.8	1.66	1.4	2.89	2.0	1.78	0.2
1.78	0.3	1.89	0.4			1.82	0.1
1.84	1.2	2.57	7.3			1.85	0.6
2.05	0.1	2.89	2.8			2.90	0.6
2.11	0.5						
2.90	2.1						

[1]RRT = Relative Retention Time with respect to hexadecanoic acid methyl ester.

Table IV Growth of lignicolous fungi on various carbon sources in ASW at 10 and 20 C.

Compound		*L. oraemaris*	*S. oraemaris*	*M. pelagica*	*D. salina*[1]
cellobiose	10°[2]	5.4[3]	10.3	5.7	
	20°	7.1	10.9	9.7	
dextrose	10°	7.7	18.7	18.7	
	20°	14.4	35.6	36.2	
fructose	10°	7.8	10.6	10.6	
	20°	13.5	17.4	9.9	
galactose	10°	6.1	11.9	8.7	
	20°	12.7	21.7	21.8	
mannitol	10°	4.4	12.6	-	
	20°	11.4	25.6	-	
maltose	10°	8.2	7.9	11.6	
	20°	12.2	14.9	24.0	
mannose	10°	4.4	5.9	6.1	
	20°	11.2	12.4	6.8	
sucrose	10°	4.0	3.6	4.3	
	20°	7.1	6.6	5.2	
trehalose	10°	9.1	8.0	5.4	
	20°	13.1	10.0	9.3	
xylose	10°	4.7	8.7	11.9	
	20°	7.5	17.1	15.3	
agar	10°	-	-	-	
	20°	-	-	-	
i carrageenan	10°	-	-	-	
	20°	-	-	-	
L carrageenan	10°	-	-	-	
	20°	-	-	-	
K carrageenan	10°	-	-	-	
	20°	-	-	-	
alginic acid	10°	3.7	4.1	5.2	
	20°	6.5	5.3	5.4	
Na-alginate	10°	-	-	-	
	20°	-	-	-	
Ca-alginate	10°	-	-	-	
	20°	-	-	-	
gelatin	10°	-	-	4.9	
	20°	-	-	3.6	
starch	10°	-	-	-	
	20°	-	-	-	
Tween 20	10°	3.6	5.4	4.6	
	20°	6.5	7.3	12.3	
n-undecane	10°	-	-	-	-
	20°	-	-	-	-
n-dodecane	10°	-	-	-	-
	20°	-	5.2	-	-
n-tetradecane	10°	-	4.8	-	-
	20°	-	4.9	-	-
n-hexadecane	10°	-	4.5	-	-
	20°	-	4.2	-	-
n-octadecane	10°	-	-	-	-
	20°	-	-	-	-
n-eicosane	10°	-	-	-	-
	20°	-	-	-	-

1. See note in text.
2. Flasks incubated for two weeks.
3. mg dry weight hyphae per flask, n = 3, standard deviation ca. 1% of mean value.
4. - equals weight less than 3 mg.

bons with glucose as *Zalerion maritimum* (Linder) Anastasiou has been shown to do with pesticides (Sguros & Quevedo, 1978).

As expected, the predominant sterol of the marine fungi tested was ergosterol, which is the dominant sterol of Ascomycetes and Fungi Imperfecti (Weete 1980). Kirk et al. (1974) reported that *Leptosphaeria oraemaris* R-13 did not contain ergosterol.

To the authors' knowledge, there are no reports of the fatty acids of marine filamentous fungi, with the exception of a qualitative estimate of some fatty acids of *Corollospora maritima* (Kirk et al. 1974). The fatty acids of terrestrial Ascomycetes and Fungi Imperfecti are discussed by Weete (1980). In several respects, the fatty acids of terrestrial filamentous fungi differ from those of the four marine fungi reported here. The marine fungi contain more tetradecanoic, hexadecanoic, hexadecenoic and eicosanoic and less octadecenoic and octadecadienoic fatty acids (cf. Weete 1980).

The difference in the sterol and fatty acid composisiton of *D. salina* compared to the other marine fungi may have some ecophysiological significance. This fungus primarily occurred in intertidal regions in New Brunswick (isolate used obtained at Station 5, Miller & Whitney 1981a) and generally has a lower salinity tolerance than some other higher marine fungi (Jones & Bryne 1976).

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