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Role of Algal and Fungal Polysaccharides in the Formation and Hydrolysis of Lake Sediments

Vijay Sangar and Patrick R. Dugan
Department of Microbiology
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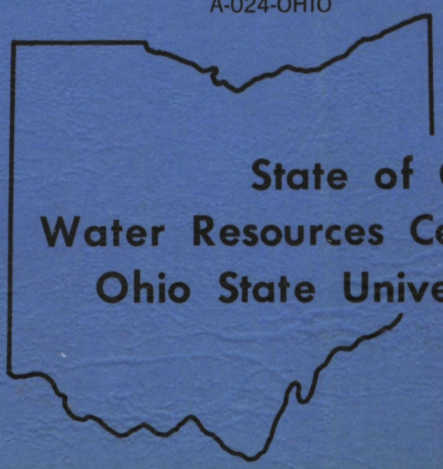

State of Ohio
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ROLE OF ALGAL AND FUNGAL POLYSACCHARIDES IN THE FORMATION AND HYDROLYSIS OF LAKE SEDIMENTS

BY

Vijay Sangar and Patrick R. Dugan
Department of Microbiology
The Ohio State University

WATER RESOURCES CENTER
THE OHIO STATE UNIVERSITY
COLUMBUS, OHIO 43210

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A. Introduction

Evidence has been reported which indicates that extracellular bacterial polymers in the form of capsules, slime layers or dissolved in the water, contributes significantly to sedimentation of dissolved and suspended microparticulate contaminants in a lake environment (Dugan, et al. 1971). The sediments consisting of organic-inorganic conglomerates then accumulate on the lake bottom and represent an oxygen demand as well as a high concentration of certain adsorbed chemicals.

Little information is available in this regard with respect to the role of non-bacterial microorganisms such as algae and fungi, although both algae and fungi are present in the lake and in contemporary sediments and both groups of microorganism produce extracellular polymer. Further, it is postulated that fungi, which are known to be active hydrolytically, could aid in the hydrolysis of the organic portions of sediments and thereby release inorganic chemicals from the physically or chemically bonded form. This in turn would allow the inorganic substances as well as the by-products of microbial action on the organic fraction to enter the water column by various mixing processes.

Blue-green algae are widely distributed in nearly all aquatic habitats. Some of the fresh-water species grow in such abundance that they result in serious losses of economic and aesthetic resources (See Holm-Hansen 1968, for a review).

At least three classes of organic compounds are known to be liberated by some species of fresh-water green and blue-green algae into their environments: 1) nitrogen compounds, such as polypeptides and free amino acids have been reported to be liberated by Anabaena sp. (Fogg, 1952); 2) organic acid production by six species of Chlamydomonas has been studied by Allen (1956) who noticed that the growth of a Chlamydomonas species in a sewage plant increased rather than decreased the amount of oxidizable organic material present; 3) extracellular polysaccharide material has been reported to be produced by several green and blue-green algae. There is considerable variation in the polysaccharides synthesized by algae and in the component monosaccharide units. Lewin (1956) reported on seven algal polysaccharides from Chlamydomonas species which were composed of only two units. Also, Lewin isolated a polysaccharide from Chlamydomonas sphagmorphila which contained seven monosaccharide units. Monosaccharide components of Nostoc muscorum, Nostoc commune and Anabaena

cylindrica contained seven, eight and six units respectively (Jones et al., 1952; Biswas, 1957; Bishop et al., 1956). The intracellular polysaccharides of Anabaena flos-aqnae consist mostly of glucose and xylose residues, with lesser amounts of glucuronic acid and ribose (Moore and Tischer, 1964). The type and amount of extracellular polysaccharide production depends upon cultural conditions and the species employed and quantitative data are available from papers of Lewin (1956) and Moore and Tischer (1964)a Species of green algae studied by Lewin (1956) produced extracellular polymer ranging from 3-113 mg per liter, while mucilaginous species of green and blue-green algae examined by Moore and Tischer (1964) produced the polymer in concentrations from 174 to 557 mg per liter of growth medium. About 25 percent of the carbohydrates of Tolypothrix tenuis consists of a non-reducing glucofructan which was tentatively identified as fructofaronosyl - (2 4)$_n$ - fructofuranosyl - (2 1) glucopyranoside (Tsusue and Yamakawa, in Holm-Hansen, 1968). Most blue-green algae which have been investigated are obligate photoautotrophs, in that they require light for continued growth and apparently cannot grow heterotrophically. Studies with C^{14}-labeled compounds demonstrate that some species can assimilate added substrates when grown in the light. Thus Carr and Pearce (1968) have shown that when cells of Anabaena variabilis or Anacystis nidulans are grown in light with U-C^{14} glucose or 2-C^{14}-acetate, 18-32% of the cellular dry weight is derived from the labeled organic substrate. Kiyohara et al. reported that Tolypothrix tenuis also shows photoassimilation of glucose; in the dark glucose was respired, whereas in the light it was polymerized into polysaccharides (In Holm-Hansen, 1968).

In spite of the frequent occurrence of filamentous fungi in aquatic ecosystems, most work on these organisms has been confined to their taxonomy and distribution and with little attention paid to their potential ecological significance in aquatic ecosystems. Jones (1964) found that the filamentous fungus Geotrichum candidum is an important sludge bulking organism. Pipes (1966) reviewed the published work on the relationship of the activated sludge process to the fungi and other microorganisms. Cooke et al. (1956) reported that fungi are among the active organisms in biological treatment of sewage in relation to BOD reduction and noted that their effectiveness is only slightly decreased in the colder months. Geotrichum candidum is capable of using hydrocarbons as food sources and of producing oils. Species of Penicillum and Aspergillus are able to add complex substances such as antibiotics

and organic acids to the environment (see Cooke, et al. 1956). Using a continuous enrichment system with sewage as the inoculum, Volesky and Zajic (1970) showed that a Graphium species is primarily responsible for the bio-oxidation of the gaseous hydrocarbon ethane, which the fungus uses as a sole source of carbon and energy. Species of Cladosporium, Alternaria, Cephalosporium and Fusarium are able to degrade cellulose. Several lower aquatic phycomycetous genera such as Pythium, Saprolegnia and the crayfish plaque fungus Aphanomyces, have been reported to hydrolyze pectin, cellulose and chitin to a considerable extent (Unestam, 1965). Meyers et al. (1960) have shown that several wood inhabiting marine ascomycetous and deutromycetous fungi developing on manila twine in a medium containing 0.1% yeast extract and sea water exhibited extensive cellulolytic activity accompanied by significant loss in the tensile strength of fiber.

Extracellular polysaccharides with various degrees of structural complexity frequently occur in fungal cultures and some of these have been shown to be glucans (see Clark and Stone, 1963, for review). Bouveng et al. (1962) studied the structure and production of - -linked glucans produced in shake cultures by Pullularia pullulans from a variety of glycosidic substrates. Pullulan is an extracellular glucan elaborated by P. pullulans, the structure of which has been determined by Bender et al. (1961) as consisting of -maltotriose units polymerized in linear fashion through 1 - 6 linkages on the terminal glucose residues of the trisaccharide. Catley (1971) has described the utilization by P. pullulans of the nonglycosidic substrates, glycerol and acetate for cell growth and production pf pullulan. Davis, Rhodes and Shulke (1965) studied the production of glucans in shake culture by the Ascomycete fungi Plectania occidentalis and a Helotium species. Plectania occidentalis consumed up to 6% glucose with about 30% conversion to polymer in a medium composed of hydrolyzed soy proteins, salts and thiamine. Wallen, Rhodes and Shulke (1965) investigated the structure and physical properties of these glucans and found them to be branched polymers containing appreciable amounts of 1 - 3 linkages. Buck et al. (1968) studied the formation and structure of an extracellular polysaccharide by Claviceps fusiformis and reported yields of polysaccharide up to 4 g per liter of the culture fluid. The polysaccharide was shown to be a branched glucan with 1 - 3 linkages. Szaniszlo, Wirsen and Mitchell (1968) reported that the marine filamentous fungus, Leptoshaeria albopunctata produces a capsular polysaccharide composed mainly of glucose with

traces of mannose. Time course studies of the nutritional requirements for capsular polysaccharide production by these authors revealed that the capsular material was produced in large amounts and on wide variety of sugars, during the period of rapid growth, but was quickly degraded and presumably re-metabolized in older cultures. According to these authors an extracellular enzyme is quickly produced by L. albopunctata, either as constitutive enzyme or as an induced enzyme formed in response to the production of the extracellular polysaccharide capsular material. Polysaccharide production by Claviceps fusiformis, Plectania occidentalis and L. albopunctata was observed by Buck et al. (1964), Davis, Rhodes and Shulke (1965) and Szaniszlo et al. (1968) respectively, to be independent within a fairly wide range of the amount of substrate supplied.

Knowledge of the types and amounts of organic compounds secreted by microorganisms into aquatic environments is essential to the understanding and interpretation of dynamic processes in natural waters. For this reason studies were conducted to determine the extent and composition of polysaccharide produced by the blue-green alga, Anacystis nidulans and also to determine what nutritional factors might influence cell and polymer production because of its potential significance during algal blooms in Lake Erie. Since filamentous fungi frequently occur in aquatic exosystems, quantitative data for the extracellular polysaccharide production by some fungal species was obtained. Some of the carbohydrate and nitrogen requirements, different growth temperatures and pH leading to cell and polymer synthesis by Anacystis nidulans and Alternaria tenuis are also discussed. In addition to the extracellular polysaccharides, cell walls of Smittium culisetae were isolated by mechanical means and a quantitative and qualitative study was made. Smittium belongs to the class Trichomycetes, members of which live as endocommensals attached to the internal chitinous lining of the guts of aquatic diptera larvae. Lipid composition of a species of Alternaria was also studied. Alternaria is a deutromycetous fungus belonging to the order Moniliales and its species are found in all habitats. Conidia of some species are considered as to be the chief fungal cause of hay fever. The relative distribution of major lipid classes (steroids, steroid esters and hydrocarbs, free fatty acids, triglycerides and polar lipies) of A. tenuis isolated from the bottom muds of the western basin of Lake Erie are also presented in this report.

B. Materials and Methods

Cultures and Media

Several unsuccessful attempts were made to obtain axenic (bacteria free) cultures of the blue-green algae isolated as unialgal cultures from Lake Erie. The predominent blue-green alga obtained at the time of sampling Lake Erie was Anacystis nidulans. Therefore, a bacterial free isolate of this species was obtained from Dr. Jack Myers of the University of Texas. Further studies were performed in the bacterial free isolate of A. nidulans although it was not isolated from Lake Erie.

A bacteria-free, unialgal isolate of A. nidulans was cultured in the medium "c" of Kratz and Myers (1955) composed of: $MgSO_4 \cdot 7H_2O$, 0.25 g; K_2HPO_4, 1 g; $Ca(NO_3)_2 \cdot 4H_2O$, 0.025 g; KNO_3, 1 g; $Na_3H_5C_6O_7 \cdot 2H_2O$, 0.165 g; $Fe_2(SO_4)_3 \cdot 6H_2O$, 0.004 g; and distilled water to make 1 liter (final pH 7.5). Cultures were grown in 3 liter Fernbach flasks in a Psychrotherm shaker incubator (100 rev/min) at 25 or 40 C under the continuous illumination of 130 foot candles from a bank of cool, white fluorescent tubes and with 5% CO_2 in air added to the incubator.

A species of Alternaria identified as A. tenuis was isolated from the bottom surface mud of the Western basin of Lake Erie.

Alternaria tenuis was grown on a medium of the following composition per liter of deionized water: KH_2Po_4, 1.36 g; $Na_2HPO_4 \cdot 12H_2O$, 1.79 g; $MgSO_4$ $7H_2O$, 0.12 g; $(NH_4)_2SO_4$, 0.66 g together with the appropriate carbon source pH after autoclaving was 6.6 and unadjusted unless otherwise stated. Cultures were maintained in shake flasks containing the above nutrients together with 1% glucose. After 3-4 days, the growth was harvested, washed and homogenized in a Waring blender for 1 min and 1 ml of this homogenized suspension was used as inoculum in 250 ml flasks containing 100 ml of the medium to be subjected to analysis. Duplicate cultures were grown on a rotary shaker for 120 rev/min.

Both Anacystis nidulans and Alternaria tenuis were cultured on various nitrogen sources supplied at a level of 1 mg per ml of culture medium, for the purpose of determining effects of nitrogen sources on growth. Response to nitrogen source was measured both as dry weight of cells and as production of extracellular polysaccharide from dialyzed cell-free culture supernatant.

The following carbon sources at 1.0% concentration were also examined for influence on cell and extracellular polysaccharide production by Alternaria tenuis: cellobiose, galactose, glucose, lactose, maltose, mannose, starch, trehalose and xylose.

A culture of Smittium culisetae, which is a fungal species isolated from mosquito larvae, was obtained from Dr. Robert Lichtwart of the University of Kansas. Species of Smittium, characteristically, live within the hindgut of various aquatic diptera larvae.

Isolation of the polysaccharide material and measurement of cell dry weight.

Samples were withdrawn aseptically at 2 day intervals. Cells were removed by centrifugation and total soluble polysaccharide in the supernatant was measured using the anthrone procedure (Ashwell, 1966). Dry weights were determined after drying the cells in tared aluminum cups overnight at 100 C. At the end of growth period, cultures of both Anacystis nidulans and Alternaria tenuis were harvested, and the cell free extracts were concentrated to one tenth volume in a rotary evaporator at 60C. After dialysis against distilled water, the extracellular polysaccharides were precipitated by adding 3 volumes of cold 95% ethanol. The precipitate was collected by centrifugation, washed with absolute ethanol, dried and weighed. Yield was calculated as mg per liter of the growth medium and efficiency as percentage by weight of glucose utilized. Anacystis cells were killed with 0.5 ml of a 2:1:1 (v/v) mixture of chlorobutane:chlorobenzene:dichloroethane (Lewin, 1956) and capsular polysaccharides were extracted with distilled water at 5 C for 24 hrs., concentrated and then precipitated with 3 volumes of ethanol and weighed. In order to extract water soluble intracellular polysaccharides, the cells were ruptured by ultrasound (Branson Sonifier, Model S110) for 30 minutes. The supernatant was treated with 10% trichloroacetic acid to precipitate protein and the polysaccharides were precipitated with ethanol from the protein free solution.

Polysaccharide material was also extracted by the alkali extraction method of Bishop et al. (1956). The culture of Anacystis was made alkaline by adding 4:1 (v/v) sodium hydroxide. The mixture was boiled for 6 hours under reflux, filtered through sintered glass, adjusted to pH 4 with HCl and dialyzed against cold distilled water for 3 days at 4 C. The non-dialyzable material was isolated by freeze drying.

Extraction of lipid material. Harvested mycelial growth of Alternaria tenuis was extracted with 100 ml of chloroform:methanol (2:1, v/v) overnight at room temperature with continuous stirring and filtered through a sintered glass funnel. The filtrate was washed according to Folch, Lees and Sloane-Stanley (1957) and concentrated in a rotary evaporator.

Isolation and fractionation of cell walls. The harvested culture of Smittium culisetae was suspended in cold water and the suspension was homogenized in a Waring blender and the fragments were separated by centrifugation. The fragments were resuspended in water and subjected to sonic treatments and centrifuged. Centrifugations and washings were repeated until a preparation free from cytoplasm was obtained. Finally the clean walls were lyophilized. For infrared spectroscopy and chitinase digestion, cell wall Fraction IV was prepared following the method of Mahadevan and Tatum (1965), and was used for chitinase digestion and to obtain the infrared spectrum.

In order to evaluate their hydrolytic activities, several preliminary experiments were run on some of the filamentous fungi. Substrates chosen for this purpose were those found in relatively high concentrations in the lake sediments. Chitin, cellulose and pectin preparations were made according to Unestam (1965). Microparticulates (0.1-1 M) were obtained following the procedure of Pfister et al. (1968) from samples of lake water collected at the depth of 15 feet. Both extracellular carbohydrate polymer of the blue-green alga Anacystis nidulans and heat killed algal cells were also used as substrates for the fungi. Agar plates were prepared similarly with chitin, cellulose, microparticulates, algal cells and extracellular polysaccharides inoculated with fungi to detect any visible hydrolytic activities.

In a typical experiment, vigorously shaken cultures were harvested by centrifugations, washed and homogenized in a Waring blender. 1 ml of this suspension was used as an inoculum per 24 ml of the basal salts medium. The salts medium used throughout was that of Gleason (1968) without a carbon source. Suspensions of cellulose, etc., were added to the basal salt medium aseptically using different concentrations. For example, polysaccharide suspensions were added at concentrations ranging from 1 mg per 5 ml of the salts medium to 1 mg per ml. Three different nitrogen sources were used: $(NH_4)SO_4$, KNO_3 and L-arginine at concentrations of 0.66 g., 0.5 g and 1.0 g per liter respectively. Two kinds of controls were run: (a) without any

carbon source, (b) with glucose as carbon source. All experiments were carried out in duplicate for 10 days. Growth was estimated visually and by taking dry weights.

Hydrolysis and chromatography. 50 mg of the freeze-dried wall preparations of Smittium culisetae were treated with 1 ml of 72% H_2SO_4 at 25 C for 12 hrs. The acid was then diluted with water to 1 N and the sample hydrolyzed at 100 C to 12 hr in a sealed ampoule. The hydrolyzate was neutralized with $BaCO_3$ and precipitated $BaSO_4$ was removed by centrifugation to freeze-dried polysaccharide preparations of Anacystis nidulans and Alternaria tenuis were also hydrolyzed with H_2SO_4 and also with HCl and TFA. Enzymatic digestion of fraction IV of the cell walls of S. culisetae was performed with chitinase (Nutritional Biochemical Corp., Cleveland, Ohio). 5 mg of enzyme, 10 mg of cell wall fraction (IV) and 10 ml of 0.1 M citrate-phosphate buffer (pH 5.1) were incubated at 37 C on a shaker for various time intervals up to 96 hr. N-acetyl glucosamine was determined by the procedure of Reissig et al. (1955).

Monosaccharides in acid hydrolyzate were separated on individual descending chromatograms on Whatman No. 1 paper using the following different developing systems: Butanol-Pyridine-H_2O (6:4:3, v/v) ethyl acetate-Pyridine-H_2O (8:2:1, v/v) and Benzene-butanol-Pyridine-water (1:5:3:3, v/v). Sugar spots were located on dried, developed chromatograms by dipping in alkaline $AgNO_3$ and also by spraying with aniline hydrogen phthalate (Hough and Jones, 1962). Known sugars were included with each run. Molar ratios of the monosaccharides were determined by gas-liquid-chromatography (GLC) of the hydrolyzates following the procedure of Albersheim et al. (1967). The hydrolyzate was reduced with sodium borohydride and after aceylation with acetic anhydride, 10 ml were injected into a Varion GLC apparatus.

Analytic procedures. Total reducing sugar in the hydrolyzate as well as in the cell free medium was measured as glucose equivalent (Nelson, 1944) and total glucose in the hydrolyzates was measured with glucose oxidase (Worthington Biochemical Corp., Freehold, N. J.). Hexosamine in cell wall hydrolyzate was purified by cation exchange according to Boas (1953) and quantitatively determined by a modified Elson-Morgan procedure as described by Davidson (1966). The identity of hexosamine as glucosamine was established by the Ninhydrin degradation method of Stoffyn and Jeanloz (1953). Protein, ash, carbohydrate

and lipid were determined on unhydrolyzed wall samples. Ash was determined by heating the wall sample in a porcelain crucible over a Bunsen burner until no more loss in the material could be seen. Amino acids were measured with ninhydrin (Rosen, 1957) using 20 mg cell wall samples hydrolyzed in 6 N HCl for 24 hr at 105 C. Soluble carbohydrates were measured by anthrone (Chung and Nickerson, 1954). Lipids of cell walls were extracted with ethanol-ether, and protein was measured by the procedure of Lowry et al. (1951). An attempt to detect hexuronic acids in the cell walls and extracellular polysaccharides was made using chromatography and the Carbazole method of Dische (1962).

Electron Microscopy. A drop of an aqueous suspension of the cell wall preparation was placed on formvar coated grids and shadowed with germanium at an angle of 40°. The grids were examined in a Philips 300 electron microscope.

Infrared Spectroscopy. The spectra of KBr pellets were obtained with a Beckman infrared spectrophotometer, model IR10, using fraction IV of Smittium cell walls and chitin (Nutritional Biochemical Corp., Cleveland, Ohio).

Cytochemistry. Fuller and Barshad's (1960) procedure was followed for detecting chitin and cellulose. Washed cells of Smittium were boiled in 4.5% KOH (w/v) to remove all cytoplasm and the cells were washed repeatedly by boiling distilled water until the resulting pH was 7. Following this step, the cells were treated with 2% acetic acid to remove any chitosan that might have formed and the acid was removed by washing with water. These cells were considered chemically cleaned and were suspended in 23 M KOH and autoclaved for 3 hr. IKI-H_2SO_4 tests were then performed as described by Tracey (1955).

Separation and identification of Lipid Components. Neutral and polar fractions of Alternaria were separated by using a 4 g column (0.8 x 12 cm) of silicic acid. Separation into lipid classes was effected by thin-layer chromatography on plates of silica gel. D.). For quantitation, individual lipid spots were removed from thin-layer plates. The neutral lipids were eluted with chloroform:methanol (4:1) and the polar lipids were eluted first with chloroform-methanol-acetic acid-water (25:15:4:2) and then with 90% methanol. Neutral lipids were quantitated by the dichromate oxidation method as described by Skipski and Barclay (1969) and the polar lipids were assayed by determining

their phosphorus content following Ames' (1969) procedure. For the identification of ninhydrin positive components, individual phospholipid fractions were hydrolyzed in 2N HCl and the water soluble portions were chromatographed on sheets of Whatman No. 1 paper separated in solvent systems of phenol:water (80:20) and butanol:acetic acid:water (4:1:5). Iodine vapors, ninhydrin and the potassium dichromate-sulfuric acid reagent of Skipski and Barclay (1969) were used routinely for visualization of lipids on the plates.

Fatty Acids. Individual neutral and polar lipids separated on thin-layer plates were eluted and saponified in 2N KOH in 50% ethanol at 100 C for 2 hrs. Methyl esters of the fatty acids were then prepared by the BF_3-methanol method of Dittmer and Wells (1969). The fatty acid methyl esters were fractionated by gas-liquid chromatography using a Varion Aerograph (Model 200) Dual Channel Chromatograph equipped with a flame ionization detector. Two stainless steel columns were employed separately: (a) 15% ethylene glycol succinate on chromosorb W (5' x 1/8"), and (b) 5% silicone SE-30 on chromosorb W (8' x 1/8"). The peaks were provisionally identified by a comparison of retention times with those of known standards.

RESULTS AND DISCUSSION

Anacystis nidulans

Analysis of spent growth medium, capsular and alkali extracted polysaccharides of Anacystis nidulans, all showed the presence of glucose, galactose and mannose as components. The average molar ratio for glucose:galactose:mannose was 66:14:20. Chromatographic and colorimetric analysis did not reveal hexuronic acids or ninhydrin positive components. The composition of polysaccharide is similar to the sugar moeity of the cell wall of Anacystis reported by Drews and Gollwitzer (1965), although these authors also tentatively identified fucose as a cell wall constituent. No fucose could be identified in the present study.

The amount of extracellular polysaccharide, determined gravimetrically after ethanol precipitation was 366 mg/L of growth medium (average of 5) after 21 days at 24 C. Capsular and water soluble intracellular polysaccharide accounted for 10 mg/L and 35 mg/L respectively. The extensive accumulation of extracellular polysaccharide as compared to capsular polysaccharide may be attributed to agitation during growth. This has been reported with Anabaena flos-aquae (Moore and Tischer, 1964). After extraction and removal of ethanol soluble as well as ethanol precipitable material, 0.0836 g of cell residue per Liter of medium remained.

Production of extracellular polysaccharide as determined with Anthrone reagent, is presented in Table 1 in comparison to cell growth at 25 C and 40 C over a period of 14 days. The total anthrone positive sugar values are considerably less than expected on the basis of the gravimetric determination of total alcohol precipitable polysaccharide. Although incubation time (21 vs. 14 days) might account for some of the difference, it is assumed that some polysaccharide is lost during our analytical procedure. Comparison of values shown in Table 1 indicates that cells grow much more rapidly at 40 C than at 25 C for the first eight days. Total cell growth is nearly the same after 12 days at both temperatures and could be attributable to depletion of a limiting nutrient in the growth medium. Production of total extracellular polysaccharide during the growth period was higher at 25 C than at 40 C. Also, the calculated rate of polysaccharide production per unit of cell synthesis ranged from 2 to 3 fold greater at 25 C than at 40 C. It appears that CO_2 is fixed and directed toward polysaccharide synthesis preferentially at the lower

Table 1. Increase of cell dry weight and extracellular polysaccharide of *A. nidulans* vs time at 25°C and 40°C.

Time day	Cell dry wt. ug/ml		Extracellular polysaccharide (anthrone equivalent) ug/ml		Calculated milligram polysaccharide per gram of cells	
	25°C	40°C	25°C	40°C	25°C	40°C
2	60	258	15	22	250	85
4	112	293	27	23	241	78
6	163	324	47	25	288	77
8	292	384	55	27	188	71
10	439	396	59	29	134	73
12	455	493	73	38	160	77
14	500	325	64	43	128	132
Control	0	0	0	0	0	0

temperature. The 14 day values may be misleading because the low total cell weight at 40 C probably reflects cell lysis and this would bias the calculated amount of polysaccharide produced per gram of cells.

The influence of several sources of nitrogen on cell yield and polysaccharide production after 10 days at 25 C is presented in Table 2. Although all media contain less available nitrogen, and therefore supported less cell growth than the Kratz medium used for data in Table 1, relative influences can be compared. Nitrate supported more growth and polysaccharide production than either ammonium or urea, which indicates that nitrogen source. However, the amount of polysaccharide produced per gram of cells is significantly higher in the presence of the less metabolizable ammonium or urea nitrogen than in the presence of nitrate. This suggests that the available carbon source (CO_2) is directly toward polysaccharide production rather than toward cell material when either total available nitrogen is a limiting nutrient or when ammonium urea are the nitrogen sources. Therefore, nitrate concentration has more relevance than total available nitrogen in determining the fate of metabolically fixed CO_2. As an ecological consideration we would expect rapid growth of A. nidulans (potential bloom) only after the process of nitrification has converted reduced nitrogen (e.g. from organic pollutants)to nitrate.

The relative effect of different concentrations of KNO_3 in A. nidulans growth was determined after cultivation for 8 days in the Kratz mecium "C". Results of this experiment are shown in Table 3. Similarly, the effect of different concentrations of K_2HPO_4 on growth is shown in Table 4. Cell yield increased in each case with increasing concentration of either nitrate or phosphate. However, the cell weight response to increased nitrate appeared to be greater than for equivalent concentrations of phosphate which would tend to implicate nitrate as a nutrient with potential for stimulating growth of Anacystis nidulans to bloom proportions.

Anacystis is one of the blue-green algae responsible for algal blooms in fresh water and most fresh water is less than 30 C. It is therefore likely that soluble polysaccharides are produced which diffuse into the water causing a greater organic enrichment than would be accounted for on the basis of many procedures used to measure primary productivity. The report of Moore and Tischer (1964) on polysccharide production by several green and blue-green

algae in life-support systems also supports the present conclusion. The significance of bacterial extracellular polysaccharides in aquatic ecosystems has been reported (Dugan et al., 1971) and the algal polysaccharides undoubtedly have a similar influence.

Alternaria tenuis

The growth of Alternaria tenuis, production of extracellular polymeric material, culture pH and utilization of carbon substrate of a shake culture grown on a medium containing 0.5% glucose as sole carbon source are described in Fig. 1. Considerable polymer biosynthesis begins after 4-5 days, when almost all the supplied glucose in the medium is exhausted and the pH drops to about 3.0. Polysaccharide accumulation continues even after the cell dry weight reaches a stationary phase. When the culture medium was buffered with 0.2 M phosphate buffer (pH 7.2), the course (Fig. 2) of polymer production was depressed with respect to rate and maximal yield. No viscosity increase was observed at any stage of growth.

In addition to glucose, the carbon sources galactose, mannose, xylose, maltose, starch, cellobiose, trehalose, sorbose, sucrose and lactose were tested as substrates for polymer production. All the substrates allowed some growth. No polymer was synthesized either on starch or on sorbose. Xylose supported maximum polymer and cell dry weight formation (Table 5). Table 6 describes the effect of various concentrations of glucose on cell dry weight and polymer formation. Glucose concentrations between 2-4% were optimal for polymer production.

The effect of various nitrogen sources tested for cell and polymer production are given in Table 7. Ammonium sulfate, KNO_3 and $(NH_4)NO_3$ supported good growth and polymer production, while urea and ammonium acetate as sole nitrogen sources allowed little growth and essentially no polymer formation.

Of all the fungi isolated from Lake Erie during the present study, none of them showed appreciable hydrolytic activities on relatively recalcitrant biochemical compounds and the isolates were not further investigated.

A preliminary chemical analysis of the extracellular polysaccharide produced by Alternaria tenuis when grown on glucose, lactose, mannose, sucrose or xylose, revealed that it is primarily composed of glucose with traces of

Table 2. Cell weight and extracellular polysaccharide produced by A. nidulans grown on different nitrogen sources for 10 days at 25°C.

Nitrogen source at 1 mg/ml concn.	Calculated available nitrogen (mg/ml)	Cell dry wt[a] (ug/ml)	Extracellular polysaccharide[b] (ug/ml)	Calculated polysaccharide (mg/g of cell)
$NaNO_3$	0.165	72	7.2	100
$Ca(NO_3)_2 \cdot 4H_2O$	0.118	94	6.7	70
KNO_3	0.126	97	10.8	111
$(NH_4)_2SO_4$	0.217	22	4.0	182
Urea	0.465	33	5.2	160
Control, no nitrogen added	0.00	20	3.2	160

[a]Mean value of duplicate cultures.

[b]Anthrone equivalent.

Table 3. Effect of different concentrations of KNO_3 on cell dry weight of Anacystis nidulans.

KNO_3 concentrations in growth medium mg/L	Calculated available nitrate mg/L	Calculated available nitrogen mg/L	Dry weight cells[1] mg/L
125	77.5	17.3	95
250	155.0	34.6	300
500	310.0	69.2	348
750	465.0	103.8	780

[1]Average of duplicate cultures harvested after 8 days.

Table 4. Effect of different concentrations of K_2HPO_4 on cell dry weight of Anacystis nidulans.

K_2HPO_4 concentration in growth medium (mg/L)	Calculated available phosphate mg/L	Dry weight cells[1] (mg/L)
125	68.25	134
250	136.5	150
500	273.0	202
750	409.5	350
1000	556.0	440

[1]Average of duplicate cultures harvested after 8 days.

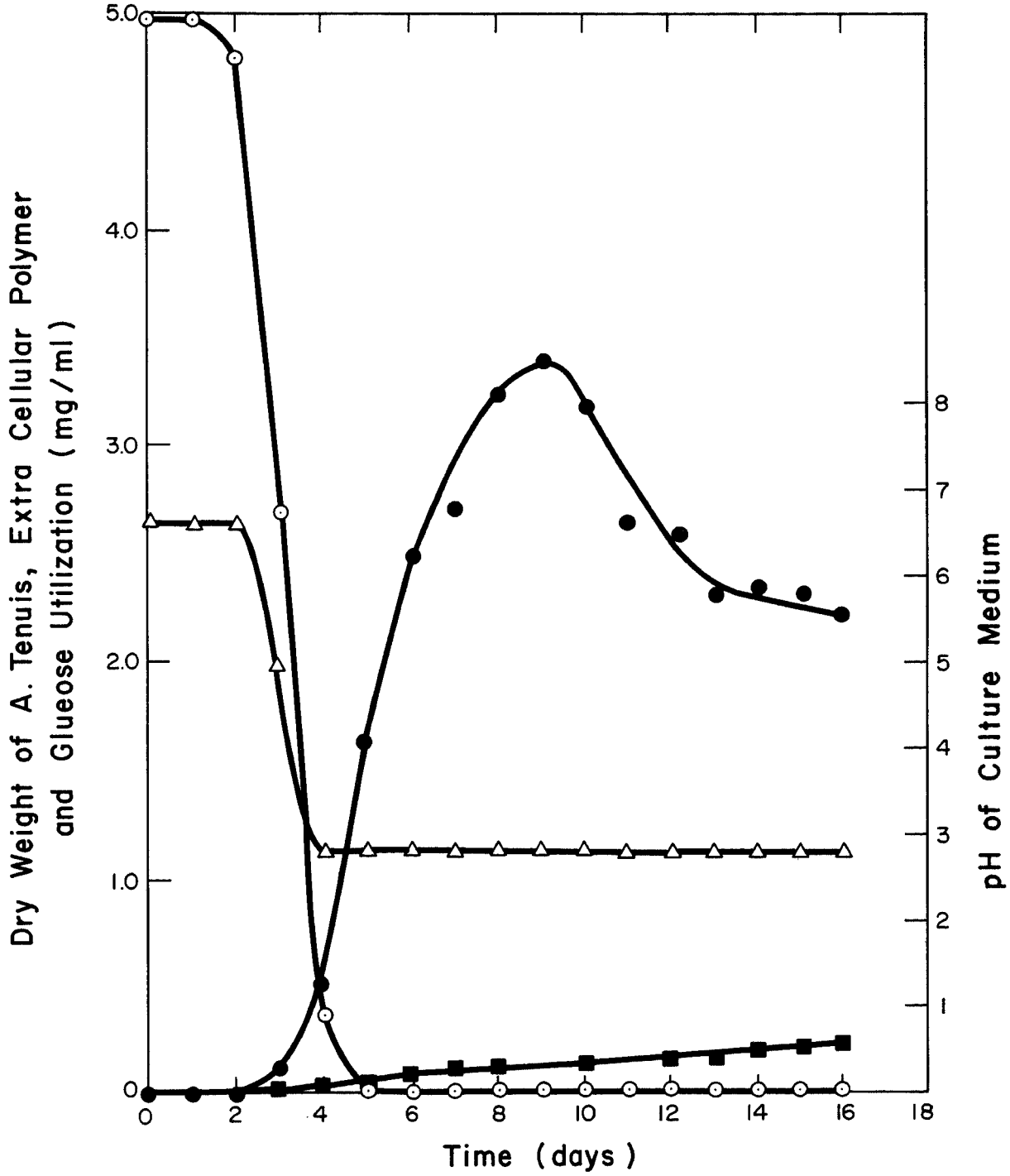

Figure 1. Growth of Alternaria tenuis cells, ● ; production of extracellular polymer, ■ ; culture pH, △ ; and glucose utilization, ○ ; versus time.

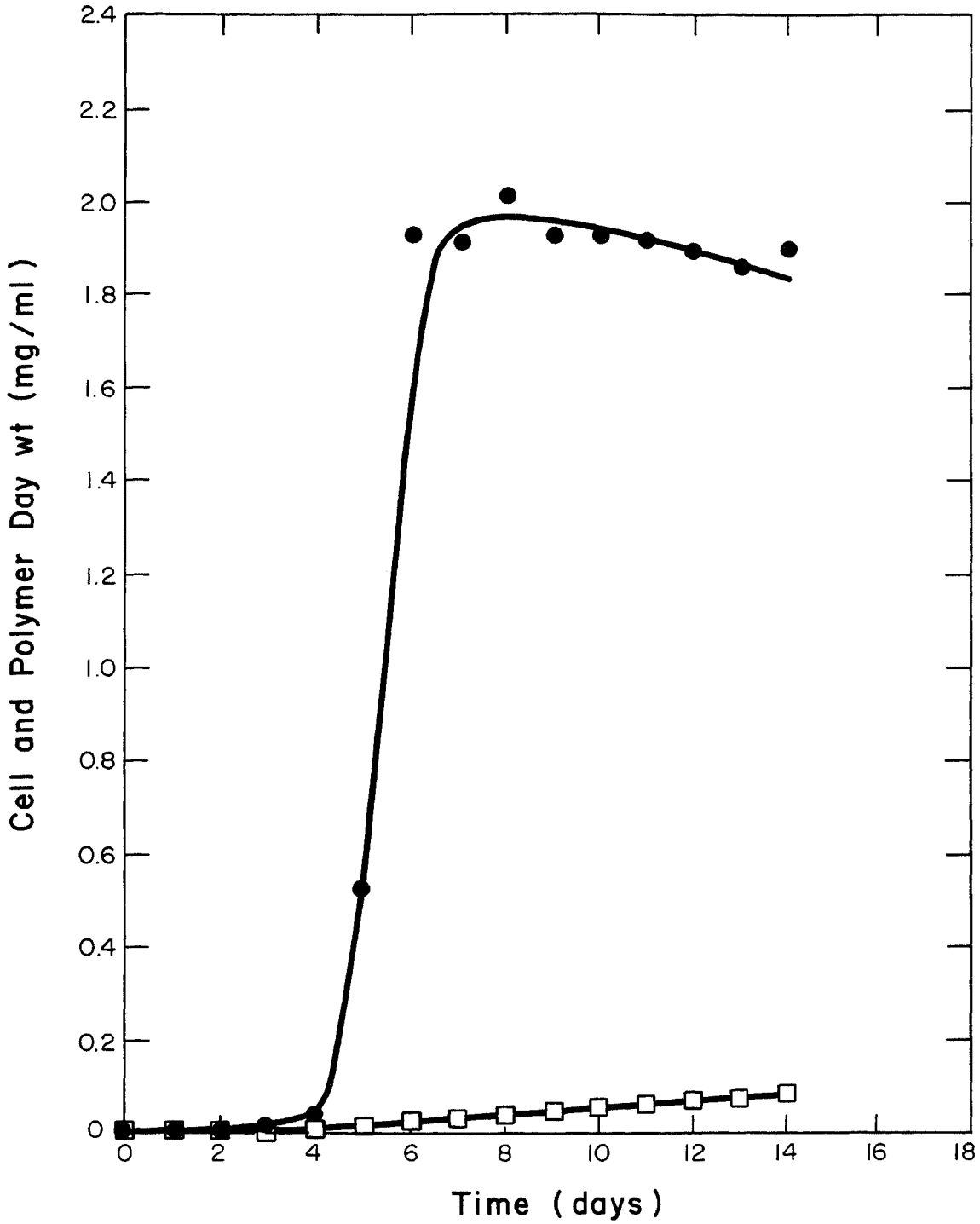

Figure 2. Growth of A. tenuis cells, ●; production of extracellular polymer, □; in 0.5% glucose medium buffered with phosphate at pH 7.2.

Table 5. Carbohydrate utilization by Alternaria tenuis.[1]

Carbohydrate[2]	Mycelium g/100 ml	Carbohydrate used[3] mg/100	Polymer synthesized mg/L	% of sugar	Final pH
Lactose	0.3422	98.4	208	2.7	3
Galactose	0.3284	85.6	242	2.8	3
Xylose	0.3410	95.6	357	3.7	2.8
Mannose	0.3150	92.8	150	1.6	2.8
Starch	0.1420	--	--	--	2.7
Sucrose	0.3030	97.4	165	1.7	--
Maltose	0.3130	97.8	235	2.3	2.8
Cellobiose	0.2690	86.7	200	1.1	3.0
Trehalose	0.3340	85.6	152	1.7	3.0
Sorbose	0.1550	--	trace	--	3.0
Glucose	0.2850	85	188	2.2	2.8

[1] Mean values of duplicate cultures harvested after 8 days.

[2] Initial concentration, 10 g/L.

[3] By reducing power as glucose equivalent.

Table 6. Effect of glucose concentrations on polysaccharide production by A. tenuis.

Initial glucose concentration in medium (%)	Mycelium[1] g/100 ml	Polysaccharide mg/liter	Final pH
0.1	0.035	40	5.8
0.25	0.092	52	3.5
0.5	0.178	156	3.0
1.0	0.215	172	2.8
2.0	0.280	265	2.8
3.0	0.225	325	2.8
4.0	0.209	320	2.8
5.0	0.201	265	2.8

[1] Duplicate cultures harvested after 8 days at 27°C.

Table 7. Effect of nitrogen-source on cell and polysaccharide production by Alternaria tenuis.[1]

N source in medium (1 g/L)	Calculated available nitrogen mg/L	Mycelium (mg/100 ml)	Polysaccharide (mg/L) glucose equivalent	Final pH
$(NH_4)_2SO_4$	0.217	0.1100	225	3.0
KNO_3	0.126	0.1830	120	7.6
$(NH_4)NO_3$	0.35	0.1490	150	2.7
Ammonium acetate	0.18	0.0660	trace	7.3
Urea	0.05	0.0510	50	8.5

[1]Mean value of duplicate cultures harvested after 8 days. Medium composition same as used in Fig. 1.

galactose and mannose and in all probability, is quite similar in composition to the capsular polysaccharide produced by Leptosphaeria albopunctata, (Szaniszlo, et al. 1968) and extracellular polysaccharides produced by Heoltium sp., Plectania occidentales (Davis, et al. 1965) and Pullularia pullulans (Buck, et al. 1968).

A. tenuis can utilize a wide variety of carbohydrate precursers for cell and polymer synthesis and in this respect is quite similar to the marine fungus L. albopunctata, but differs notably when compared with Helotium sp. and Claviceps fusiformis. Davis, Rhoades and Shulke (1965) reported that no polysaccharide was produced by Helotium sp. when grown on maltose as the carbon source and Buck et al. (1968) found no polysaccharide synthesis by Claviceps fusiformis when grown on xylose as energy source. A. tenuis produces polysaccharide from both xylose and maltose as well as with a number of other sugars (Table 5).

Adjusting the culture medium to pH 7 with 0.2 M phosphate buffer results in lower cell and polymer yield with A. tenuis. This situation is in contrast to several other fungi studied. In both Helotium sp. and Leptosphaeria albopunctata, the pH immediately began to decrease during the period of rapid growth, but quickly rose and stabilized. This subsequent sharp rise in pH signals maximal glucan formation in Helotium sp. and L. albopunctata (Davis, et al. 1965). Also, the maximal yield of the extracellular polysaccharide material in terms of percent of glucose conversion obtained from A. tenuis is very low as compared to the maximal yields of the polysaccharide reported for cultures of Helotium sp., Plectania occidentales (Davis, et al., 1965), and for Claviceps fusiformis (Buck, et al., 1968). Although, the efficiency of glucose conversion to polymer is also quite low in L. albopunctata (Szaniszlo, et al., 1968). These authors argue that such lower yields of the capsular meterial are due to its enzymatic degradation. Presumably, the organism produces an exocellular glucanase in response to the capsular polysaccharide material. In the present study, the polysaccharide produced by Alternaria tenuis showed no decline in quantity for 15 days.

An examination of the lipids of Alternaria tenuis showed that almost all the major classes of lipid were present in its mycelium, and their percent relative distributions are given in Tables 8 and 9. Gas chromatography of methyl esters of the fatty acids obtained from the individual lipid classes revealed that the major fatty acids found in this organism were linoleic,

Table 8: Neutral Lipid Components of Alterniara Sp.

Lipid Class	Percent Distribution of Total Combined Lipids
Triglycerides	47.0
Free fatty acids	4.0
1,3-diglyceride	4.5
1,2-diglyceride	3.0
Steroid ester + hydrocarbons	20.6
Steroids	15.5
Monoglycerides	5.3

Table 9: Polar Lipid Components of Alternaria Sp.

Lipid Class	Percent Distribution of Total Combined Lipids
Phosphatidyl serine	66.2
Phosphatidyl ethanolamine	23.0
Lysophosphatidyl ethanolamine	trace
Lysophosphatidyl serine	trace
Unidentified phospholipid (ninhydrin positive)	10.9

oleic and palmitic with smaller amounts of stearic and palmitoleic. The extractable material separated on a silica acid column consisted of 25.8% neutral and 74.2% polar lipids. The value of polar lipid fraction does indeed seem high and perhaps could be attributed to the presence of large amounts of extractable pigments in this fraction. Alternaria tenuis had an unusually high content of linoleic (67.2 and 45.1% in the polar and neutral lipid fractions, respectively). This fatty acid, although found in smaller concentrations in other groups of fungi, has been shown to be a predominent fatty acid in the Fungi Imperfectii (Hartman et al., 1960, 1962; Tyrell, 1969). Hartman et al. (1960, 1962) in an analysis of fatty acids from spores and mycelium of Pithomyces charatarum and Stemphylium dendriticum reported high concentrations of linoleic acid (41-59%) together with substantial amounts of palmitic and oleic acids. Alternaria, like Pithomyces and Stemphylium belongs to the family Dematiceae of the Moniliales, and our results are consistent with the other Fungi Imperfectii studied. Earlier work has suggested that, in general, increased amounts of linoleic acid in fungi are associated with decreased amounts of palmitic or oleic acids. A conversion of oleic into linoleic acid was postulated by Ballance and Crombie (1961) in the imperfect fungus Trichoderma viridae, and is known to operate in the yeast Torulopsis utilis (yuan and Block, 1960). The fatty acids of the Alternaria do indeed show such a pattern, i.e. the high linoleic acid content is accompanied by a reduction in the amount of oleic acid. However, in view of the variety of factors involved such broad generalization does not yet seem justified.

Other major fatty acids found in the mycelium of Alternaria sp. besides linoleic were oleic, and palmitic, with smaller amounts of palmitoleic and stearic (see Table 10). These fatty acids have been reported in Fungi Imperfectii, (Tyrell, 1969) as well as in other groups of fungi (Sumner, 1970; Sumner and Colotelo, 1970). In the present study higher concentrations of linoleic acid were found in the polar than in the neutral lipid fraction while the reverse was true for oleic and palmitic acids. Shaw (1966) found no differential distribution of specific fatty acids in some Phycomycetes. Both polar and neutral lipid fractions had similar fatty acid compositons. However, the different lipid fractions of Pythium ultimum were shown by Bowman and Mumma (1967) to have different fatty acid compositions. The latter situation has been reported for a majority of fungi, e.g. in the Phycomycete, Choanephora cucurbitarum (White and Powell, 1966), in several Ascomycetes,

(Sumner and Colotelo, 1970), in Fungi Imperfectii, (Tyrrell, 1969) and in the basidiomycete, Tricholoma nudum (Leegwater et al., 1962).

The most abundant phosphatides found in fungi are phosphatidyl choline, phosphatidyl serine and phosphatidyl ethanolamine (Leegwater et al., 1962). Phosphatidyl serine and phosphatidyl ethanolamine (66.2% and 23.0%, respectively) were identified in addition to an unidentified ninhydrin positive phosphatide in Alternaria tenuis.

Steroids and steroid esters and hydrocarbons were found in considerably high quantities in Alternaria (15.5 and 20.6% respectively, of the neutral lipid fraction). In Pithomyces and Stemphylium also Hartman et al. (1962), reported high concentrations of total steroids and free steroids.

The chemical composition of the isolated walls are presented in Table 11. The difference between the neutral polysaccharides and the reducing sugar values indicates that some destruction of monosaccharides occurred during the hydrolysis. Smittium cell walls have 30% carbohydrate, 15% protein, 13% lipid and 3% ash.

The cell walls contain glucose, galactose, mannose and glucosamine. The molar ratios are given in Table 12. The Rg values for the known (control) and sugars obtained from cell walls in all the chromatographic systems agreed very well. Hexuronic acids were not detected on paper chromatograms or by colorimetric analyses.

Glucosamine is the predominant component in Smittium cluisetae cell walls, accounting for 35% of the total weight. Identification of this hexosamine as glucosamine and the absence of other amino sugars was confirmed by separating the amino sugars from neutral sugars by cation exchange (Boas, 1953), and subsequent ninhydrin degradation and paper chromatography of this isolated amino sugar according to Stoffyn and Jeanloz (1953) showed only arabinose.

When the chemically cleaned cells were subjected to IKI-1% H_2SO_4 test a strong violet color was obtained indicating the presence of chitin in the walls. In the cells which were washed with 2% acetic acid and then treated with IKI-1% H_2SO_4 no violet color could be seen, since chitosan is soluble in acetic acid. IKI-70% H_2SO_4 test did not show any blue colour indicating lack of cellulose in Smittium walls.

Table 10: Percent Fatty Acid Composition of Individual Lipid Fractions. Listed in Tables 8 and 9.

Fatty acid chain length	Triglycerides	Free fatty acid	1,3-diglyceride	1,2-diglyceride	PE	Unknown phospholipid	PS
12.0	0.4	1.5	5.6	1.5	-	-	-
14.0	0.8	0.8	8.3	10.5	6.9	6.5	3.9
15.0	0.3	0.7	-	1.2	2.7	-	-
16.0	18.0	18.7	16.7	30.2	10.6	7.5	7.0
16.1	4.5	trace	2.0	1.8	5.9	1.8	1.6
17.0	-	0.4	-	-	-	trace	-
18.0	2.6	18.3	3.6	3.1	2.1	2.7	2.5
18.1	19.9	21.4	8.4	6.8	7.4	11.2	8.9
18.2	45.9	32.4	52.7	40.0	61.2	69.2	69.1
18.3	7.5	5.7	2.8	4.9	3.2	0.9	5.8
20.0	-	-	-	-	-	-	1.2

PE = Phosphatidyl ethanolamine

PS = Phosphatidyl serine

Table 11: Composition of Smittium Cell Walls

Component	Percent of dry weight
1. Total carbohydrate (glucose equivalent)[a]	30
2. Reducing sugar (glucose equivalent)[b]	22.4
3. Glucose (enzymatic determination)[b]	13
4. Glucosamine[b]	35
5. Protein	15
6. Amino acids (glycine equivalent)[b]	13.8
7. Lipids	13
8. Ash	3
Sum of 1, 4, 5, 7 and 8	96

[a] Determination made on unhydrolyzed wall samples

[b] Determinations made on hydrolyzed samples

Table 12: Ratio of Monosaccharides in Cell Walls of Smittium

Monosaccharide	Percent	Molar ratios
Glucose	13	3.3
Galactose	4	1.0
Mannose	5.5	1.4
Glucosamine	35	8.7
Total recovery	88.5	
Compared to Controls		

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