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Regulation of Growth and Metabolic Activities of *Chlorella fusca* by Release Products of Some Aquatic Fungi

S.K.M. Hassan*, E.M. Fadl-Allah*, I.A. Kobbia and M.A. Shoulkamy***
*Botany Department, Faculty of Science, *Minia and **Cairo University, Egypt*

수생균의 분비물질에 의한 *Chlorella fusca*의 성장 및 대사조절

ABSTRACT: The growth and biochemical activities of *Chlorella fusca* were studied in the presence of different concentrations of either filtrates or mycelial mats of *Saprolegnia ferax* and *Pythium graminicola*. Low concentrations of both fungal filtrates exerted increase in total count, dry weight and in the biosynthesis of photosynthetic pigments, carbohydrates and nitrogen content. High concentrations showed inhibitory effect on both growth and biochemical activities of *Chlorella fusca*. Supplementation with different concentrations of dry mycelial mats of either fungi the culture of *Chlorella* showed elevation in biomass, dry weight, and biosynthesis of carbohydrates and nitrogen content especially at low concentrations. The contents of photosynthetic pigment were inhibited only at low concentrations. Neither the culture filtrate of *Pythium* nor *Saprolegnia* had cellulolytic activity, although polygalacturonase enzymes were detected, whereas chloroform-extract of both fungal filtrates showed blue spots under long wave light (366 nm).

KEYWORDS: ***Chlorella fusca, Saprolegnia ferax, Pythium graminicola***

The phytotoxicity of *Pythium* and mycoparasitism of *Saprolegnia* has been demonstrated by Martin (1964) and Schneider (1979), respectively. Martin (1964) reported that *Pythium irregulare* produced non-specific phytotoxic substances of proteinaceous character, while Janardhanan and Husain (1974) reported that the culture filtrate of *Pythium butleri* induced wilting to higher plants.

Recently, it has been shown that *Saprolegnia declina* produces protease and lipase enzymes (El-Feki, 1987). Also, one of the main characteristic feature of many phytopathogenic organisms is their ability to produce an array of enzymes capable of degrading the complex polysaccharides of the plant cell wall (Albersheim *et al.*, 1969 and Wood, 1973) and membrane constituents (Tseng and Bateman, 1968). These enzymes usually are produced inductively, generally extracellular and highly stable. Pectic enzymes that cause maceration in plant tissues also cause injury and death of the cell (Mount *et al.*, 1970 and Wood, 1972). Clear evidence of killing the cells by pectic enzymes was reported by Basham and Bateman (1975).

The present investigation aimed to study the metabolic changes that might take place in a pure synchronons culture of *Chlorella fusca* in response to effect of extrametabolic filtrates and dry mycelial mats of *Pythium graminicola* and *Saprolegnia ferax* supplemented to the cultures. Particular emphasis was devoted for identification of the fungal extracellular enzymes and mycotoxins responsible for the metabolic changes in these algal cultures.

Materials and Methods

Fungi;
Pythium graminicola and *Saprolegnia ferax*

were obtained during the investigation work of Shoulkamy (1990).

Algae;

Chlorella fusca was obtained from the collection of Algal cultures, Gottingen, F.R.G.

Culturing and Treatments;

Synchronous culture of *Chlorella fusca* were performed according to Pirson and Lorenzen (1958) and Kuhl and Lorenzen (1964), and the nutrient culture solution used by Grimme and Boardman (1972) was applied. One m*l* of the dilute synchronous *Chlorella* cultures was transferred to 49 m*l* of the nutrient media which were containing the following series of concentrations prepared from either the fungal filtrates or dry matter of mycelial mats of the two tested fungi:
Filtrates: (m*l*/50 m*l* culture media)

a) *Saprolegnia ferax;* 0.5, 1, 2, 4 and 8 m*l*/50 m*l*

b) *Pythium graminicola;* 0.1, 0.4, 1.6, 6.4 and 25.6 m*l*/50 m*l*

All volumes in experimental flasks were completed to 50 m*l* with sterile media.
Mats: (gm/50 m*l*).

a) *Saprolegnia ferax;* 0.1, 0.2, 0.4, 0.8 and 1.6 gm/50 m*l*

b) *Pythium graminicola;* 0.025, 0.05, 0.1, 0.2 and 0.4 gm/50 m*l*

Control in two series was carried out parallel to treatments using (a) *Chlorella* grown on its medium without using any treatments, (b) by the addition of different level of the fungal medium comparable to that used in treatments.

All flasks were incubated for 3 days in a large controlled illuminated incubator (Precision Model 818) at 22°C under continuous light (2000 Lux) by fluorescent lamps for a period of 10 hours in the light and 14 hours in the dark.

At the end of each experiment the algal masses were separated from media by filtration and washed with distilled water; then were dried by further suction. One set consisted of 3 replicates from each treatment as well as controls was dried at 800°C till constant weight. The second set was extracted with 90% acetone for pigment estimation. A third one was homogenized with 10 m*l* of borate buffer at pH 8 before centrifugation.

Pigments estimation

The photosynthetic pigments (Chlorophyll a, chlorophyll b, and carotenoids) were determined using spectrophotometric method recommended by Metzer *et al.* (1965).

Biochemical analysis

The algal masses were analysed for their total carbohydrate, total nitrogen and protein contents. All analyses were carried out on the unacidified extracts, except for the carbohydrate, where clearing was necessary.

1) Carbohydrate analysis

This was carried out according to the procedure recommended by Naguib (1969).

2) Nitrogen analysis

Total insoluble nitrogen (Naguib, 1969).
Total soluble nitrogen (Naguib, 1969).
Protein-nitrogen (Lowery *et al.*, 1951).
Nitrate-nitrogen (Paech and Tracy, 1956).

Cultural studies on production of cell wall degrading enzymes and mycotoxins by *Pythium graminicola* and *saprolegnia ferax*

The basal cultural medium for growth of zoosporic fungi was prepared according to Emerson's (1958). The two fungal isolates were grown separately in 500 m*l* Erlenmeyer flasks containing 50 m*l* of the basal medium. Each flask was sterilized and then inoculated with one mycelial disc (0.7 cm) cut from the margin of 3 day old cultures of each fungal isolate grown on M3-medium (Hasija and Miller, 1970) for the Chytridiomycetes. Fungal mycelia were removed by filtration through 3 layers of cheese cloth. Enzyme extracts were assayed for cellulase and protease activities.

1. The ability to produce extracellular enzymes

1) Assay of cellulase activity

Cellulase in culture filtrates was assayed viscometrically with CMC as a substrate in a locally fabricated viscometer as previously described by EL-Katathy (1984).

2) Assay of protease activity

Protease activity was assayed by a modification of the casein digestion method of Kunitz (1946, 1947) and Lowery *et al.* (1951).

3) Assay of polygalacturonase (PG)

Viscometric method: Polygalacturonase in the culture filtrate was assayed by viscometric method using 1% sodium polyacetate as sub-

Table I. Effect of various concentrations of fungal media on the biomass (cell count/ml) and pigment contents (μg/g d. wt) of *Chlorella fusca* after 3 days growth

Concentration (w/v) %	No. of cells		Chlorophyll (a)		Chlorophyll (b)		Carotenoids	
	Abs. Coundt.	% of increase or decrease	Abs. value	% of increase or decrease	Abs. value	% of increase or decrease	Abs. value	% of increase decrease
P.graminicola								
Control	2100	100	1.12	100	0.42	100	0.47	100
0.1	4000	+90	0.22**	−80	3.47**	+726	1.11	+136
0.4	8233**	+292	0.52**	−53	7.46**	+1676	3.37**	+617
1.6	5083**	+142	0.33**	−70	4.62**	+1000	2.16**	+359
6.4	3726**	+77	0.16**	−85	1.75*	+316	1.87**	+297
25.6	1866**	−11	0.11**	−90	0.82	+95	0.78	+65
L.S.D. 1%	371		0.58		2.12		1.44	
5%	264		0.41		1.51		1.03	
S. ferax								
Control	3266	100	0.59	100	2.54	100	0.68	100
0.5	3700	+13	0.93**	+57	4.45**	+ 75	0.76	+11
1	9383**	+187	1.58**	+167	6.72**	+164	3.55**	+422
2	8050**	+146	1.31**	+122	5.49**	+116	1.69**	+148
4	5730**	+75	1.05**	+77	4.2**	+65	0.88*	+29
8	1300**	−60	0.7	−23	2.57	−1	0.67	−1
L.S.D. 1%	732		0.21		0.71		0.39	
5%	522		0.15		0.51		0.28	

strate according to El-Katatny (1984).

Method of assaying reducing groups: Polygalacturonase in the culture filtrates was also assayed by measuring the increase in liberated reducing groups. After incubation for 7 hrs at 30°C, the increase in reducing groups was measured using Nelson's modification of Somogy's method (Nelson, 1944).

2. Preparation of crude toxins of the tested fungi

The toxins in the crude chloroform extract of tested fungi were extracted from mycelium and filtered culture medium and individually analysed as previously described by Hassan (1988).

Appropriate methods of statistical analysis were carried out for analysing the data obtained.

Results

It is necessary to report that administrations of fungal medium to the algal cultures with doses equal to those applied in experiments of fungal filtrates resulted generally in significant increases in pigment biosynthesis and gain in biomass of *Chlorella fusca;* an effect that was furthered up to moderate levels. At the highest concentrations, however the pigment content and algal biomass dropped significantly (Table I).

Effect of different concentrations of tested fungal filtrates on growth and biochemi-

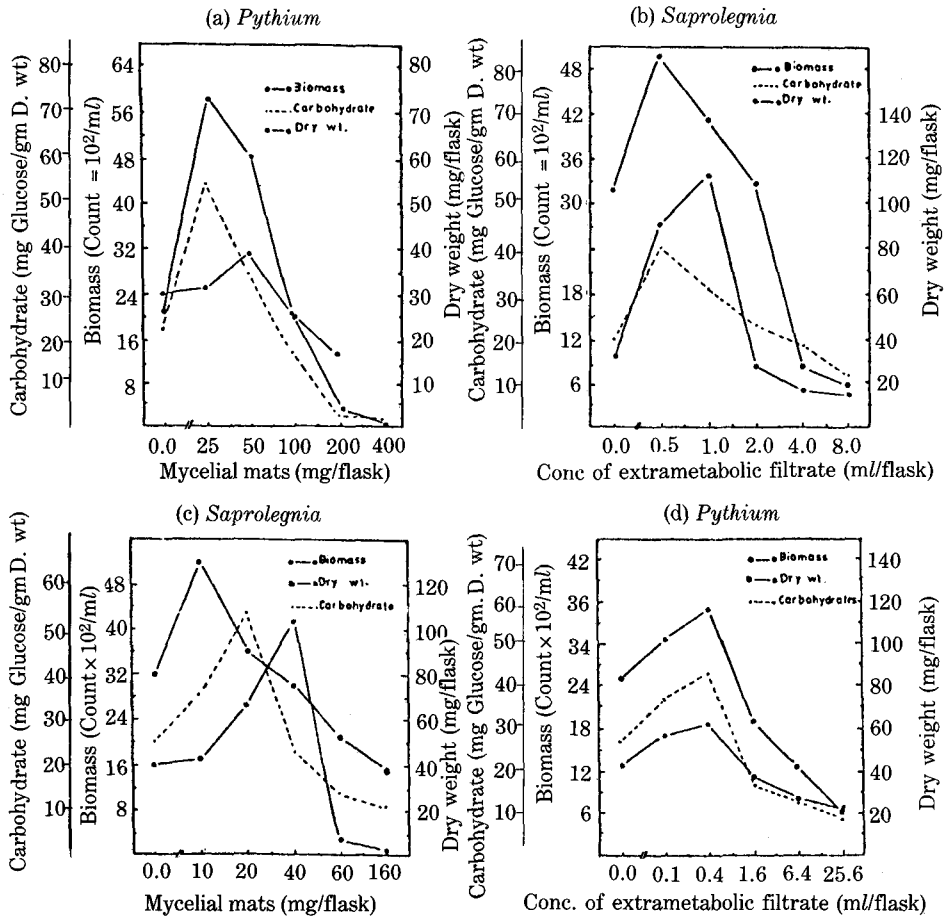

Fig. 1. Effect of various concentrations of dry mycelial mats (a,c) and extrametabolic filtrates (b,d) of *Pythium graminicola* and *Saprolegnia ferax*, respectively on different growth critera of *Chlorella fusca* of 22°C after 3 days.

cal changes of *Chlorella fusca*

1. Growth

1.) Dry weight

The growth of *Chlorella fusca* (expressed as loss or gain in dry weight) was remarkably affected by both fungal filtrates (Fig. 1b, d). Thus a pronounced increase in dry weight was obtained at the lowest levels of either filtrate. Such stimulatory effect was furthered to reach maximum at concentrations of 0.4 and 1 ml/flask for *Pythium* and *Saprolegnia*, respectively. These maxima represented percentage of increases which accounted for 41 and 242% of control, respectively. However, these significant changes were more prominent in *Chlorella* cultures supplemented with *Saprolegnia* filtrate relevant to those of *Pythium*

2) Biomass

The data demonstrated in (Fig. (1b, d) clearly show that cell counts of *Chlorella* were significantly increased following supplementation of both fungal filtrates to the algal cultures; an effect that was furthered to reach maximum cell accumulation at 0.4 and 0.5 ml/flask levels of *Pythium* and *Saprolegnia*, respectively. Beyond these concentrations, the number of cells was significantly decreased to reach lowest yield at highest concentrations of both fungal filtrates. Again, these significant changes were more pronounced in the treatments of *Saprolegnia* than that of *Pythium*.

2. Biochemical components

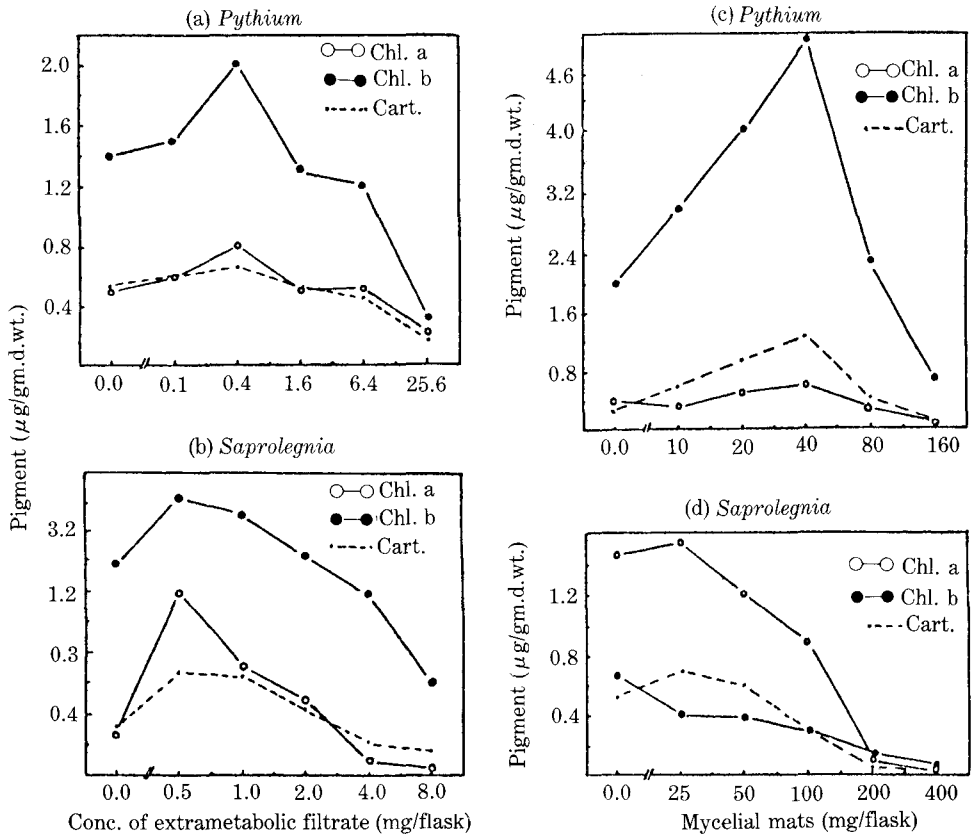

Fig. 2. Effect of various concentrations of extrametabolic filtrates (a,b) and dry mycelial mats (c,d) of *Pythium graminicola* and *Saprolegnia ferax* on chlorophyll and growth period at 22°C.

1) Pigment content

The results demonstrated in (Fig.2a, b) reveal that the biosynthesis of pigment fractions (Chlorophyll a, b and total carotenoids) was remarkably and significantly stimulated at lower and moderate levels of either fungal filtrate. Maximum pigment content was detected at 0.4 and 0.5 ml/flask of *Pythium* and *Saprolegnia* filtrates, respectively. Thereabove, the biosynthesis of all fractions was inhibited with rise of filtrate concentrations. Such inhibition was statistically highly significant at the highest concentrations of the two fungal filtrates.

2) Total carbohydrate content

The biosynthesis of total carbohydrates in *Chlorella* cells was favoured in the presence of either *Pythium* and *Saprolegnia* filtrate as shown in (Fig. 1b, d). At 0.4 and 0.5 ml/flask of *Pythium and Saprolegnia* filtrates, maximum accumulation of total carbohydrates was displayed representing increases which accounted for 57.5 and 15% of control, respectively.

3) Nitrogen content:

Fig. (3-d) clearly show that addition of *Pythium* filtrate to culture media of *Chlorella* resulted generally in a significant increase in total nitrogen content of algal cells. The maximum total nitrogen content was recorded at 1.6 ml/flask. Such increase was accounted for 578% of control.

At the lowest concentrations of *Saprolegnia* filtrte (Fig. 3c) augmented to culture of *Chlorella,* the total nitrogen content slightly increased. Elevation in concentration resulted in conspicuous drop in total nitrogen control of the algal cells. Maximum inhibitation of total nitrogen accumulation was attained at the highest concentration, being accounted for 85% decrease of control.

Effect of different concentrations of

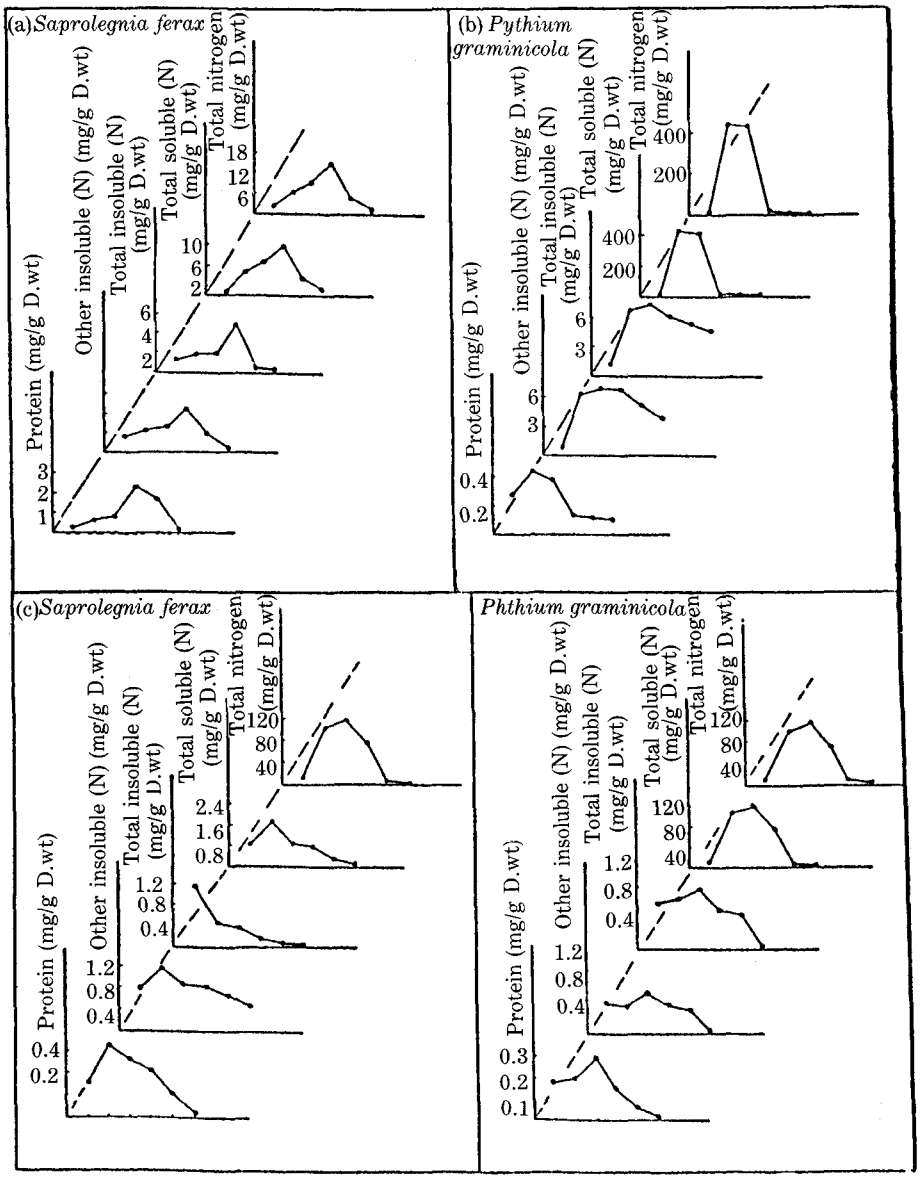

Fig. 3. Effect of various concentrations of dry mycelial mats (a,b) and extrametabolic filtrates (c,d) of *Saprolegnia ferax* and *Pythium graminicola* on the nitrogen content of *Chlorella fusca* after growth of 3 days at 22°C (mg nitrogen/g.d.wt.).

mycelial mats of tested fungi on growth and biochemical changes of *Chlorella fusca*

1. Growth

1) Dry weight

The dry weight of *Chlorella* cells was found to be appreciably increased under the lower levels of either *Saprolegnia* or *Pythium* mycelial mats Fig. 1a, c. Maximum inhibition in the dry weight gain was detected at 0.2 and 1.6 gm/flask of *Pythium* and *Saprolegnia*, respectively. *Chlorella* was found to be more sensitive to the treatment with mycelial mats of *Saprolegnia* than *Pythium*.

2) Biomass

As shown in Fig. 1a, c, the lower concentrations of either fungal mats of *Pythium* and

Table II. Production of polygalacturonase, cellulase, protease enzymes and fungal metabolite by *Pythium graminicola* and *Saprolegnia ferax* in culture medium

Organisms	Enzyme activity				Fungal metabolites	
	PG		Cellulase	Protease g tyrosin/ml	$R_{f\times 100}$	Max. abs
	V.M. (%)	R.E.A. (g/ml/h)	Viscometric (% reduction)	Lowry *et al* (1951)		
Pythium graminicola	11.0	2.0	0.0	85.0	22	268
Saprolegnia ferax	12.5	8.5	0.0	102.5	60	259

Where: P.G. = Polygalacturonase, V.M. = Viscometric method,
R.E.A. = Relative enzyme active, Max. abs. = Maxmum absorption.

Saprolegnia exerted stimulatory effects on the biomass yield of *Chlorella fusca*. The magnitude of increase in cell count under such condition accounted for 129 and 128% increase of control in the cultures supplemented with *Pythium* and *Saprolegnia* mycelia, respectively.

Another additive explanation for accelerating growth at lower concentrations of extracellular products of fungi or mycelial mats may be due to the beneficial role maintained by supplying *Chlorella* with limiting CO_2, which would be in agreement with the findings of Lange (1971) on studying the effect of bacteria on algal growth.

2. Biochmical components

1) Pigment content

The biosynthesis of all pigment fractions was significantly suppressed under the effect of all doses of mycelial mats, both except at the lowest concentration of *Pythium* mats, where a significant increases in chlorophyll a and total carotenoids contents were established (Fig. 2c, d). Maximum inhibition of pigment accumulation was observed at the highest concentration of both mycelial mats. In the mean time, a highly significant increases in all pigment fractions were harvested at 0.04 gm/flask of *Pythium* mats.

2) Total carbohydrate content

Both fungal mats of tested fungi at lower and moderate concentrations exerted an stimulatory effect on the total carbohydrate accumulation in *Chlorella* cells, (Fig. 1a, c). Beyond these levels, the biosynthesis of total carbohydrate was gradually decreased; a trend that was furthered to reach maximum drop at the highest levels of both fungal mats.

3) Nitrogen content

A glance to the data illustrated in Fig. 3a, b, clearly indicates that administration of dry mycelial mats of either *Pythium* or *Saprolegnia* increased the total nitrogen content of *Chlorella* cells. At the highest level of mycelial mats, the total nitrogen content was significantly decreased. Apart of some minor fluctuations the ratios of total insoluble nitrogen to total soluble were generally increased, whereas the total insoluble nitrogen fraction participated in total nitrogen content was remarkably decreased, a trend that was associated with enhancement of protein accumulation.

Analysis of extracellular metabolites of *Pythium graminicola* and *Saprolegnia ferax*

The results of physico-chemical and chromatographic analysis of the culture filtrates of both *Pythium* and *Saprolegnia* are shown in Table (II). Both culture filtrates showed no cellulytic activity, although polygalacturonase enzymes were detected to be found in either *Pythium* or *Saprolegnia* as was determined by Viscometric and Relative Enzyme Activity methods of bioassay.

However, the enzyme activity tended to be higher in *Saprolegnia* filtrate, than that of

Pythium. Estimating the efficiency of protease enzyme in the filtrates of both fungi revealed the high proteolytic activity of *Saprolegnia* as compared with that of *Pythium*.

Discussion

The present investigation clearly shows that, the most enhancement of both fungal filtrantes or mats appeared in enhancement of growth of *Chlorella* at lower concentrations and its supperssion at higher ones. Such effects were extended to comprise also chlorophyll contents, which indicates retardation of the photosynthetic activity of *Chlorella* under these conditions. This could be explained in the light of the fact that fungi are important nutrient regenerators and the phytoplankton response most likely reflected this process. It is axiomatic that the fungal filterates or mats have a wide variety of degradative enzymes, which at lower levels, would have accelerated the breakdown of some organic matter in the medium directly through mineralization. Accordingly nutrient availability would have increased and their losses through sedimentation decreased, as a result. In turn, the growth and metabolic activity of *Chlorella* would certainly have been stimulated.

Alternative hypothesis for the stimulatory response of *Chlorella* at the lower doses of both fungal filtrates or mats supplemented to the culture media appear less plausible may lay in vitamin enrichment induced by both fungal extracellular products or mycelia. In this respect, it was reported that although many algal species are auxotrophic (Provasoli and Carlucci, 1974) for vitamins released by various aquatic microorganisms (Niewolak and Sobierajska, 1971), yet in situ vitamin enrichments did not substantially alter the phytoplankton community (Smith *et al.*, 1984). The excessive production of exocellular enzymes or mycotoxins at higher doses, may mask the beneficial role played by vitamins.

The drastic effects on the growth and metabolic activities of *Chlorella* induced at higher concentrations of fungal mats or filtratees may be attributed to high polygalacturonic and proteolytic activities of these fungal enzymes. In this connection, it should be mentionl that the extracellular protease hyphomycetes has been explored by Suberkropp *et al.* (1983). They reported proteolytic activity for six species namely; *Flagellospora curvula, Heliscus lugduensis, Arguillsspora pseudolongissima, Clavariopsis aquatica and Lemonniera terrestris,* Samir and Saad (1989) listed 19 species of aquatic hyphomycetes which showed proteolytic activity.

The absence of cellulase enzymes from either fungal filtrates or mats, as observed in this investigation, may confirm again, that the inhibitory effects induced at higher doses, could be mainly attributed to the mycotoxins and not enzymatic activities (Canter, 1974, 1979).

It must be mentioned here, that not all phycomycetes are able to produce cellulases. It was reported by Singh and Yadava (1983) that *Helicoma dennisii, Helicoon farinosum* and *H. pluriseptatum* are not cellulase producing tropical aquatic hyphomycetes.

The relatively high proteolytic and polygalacturonic activities showed in this investigation are consistent with the findings of (El-Feki, 1987), who reported that *Saprolegnia diclina* produces protease and lipase enzymes capable of decaying the protein and lipid in fish.

Generally, the data presented in this investigation support the idea that relationships between these two groups of organisms, in natural environments are far more complex than it could be expected. In this connection, Parker and Bold (1961) studying the biotic relationships between soil algae and other microorganisms, reached to the conclusion that a-stimulatory effects occur naturally between remotely related groups such as autotrophs and heterophs, b- in soils deficient in nitrogen stimulatory and inhibitory relationships involving algae may center about the availability of nitrogen. c-stages in life cycles of different soil algae are evoked by activities of their neighbours; the rate of cell division, longevity of cells, induction and duration of motile stages, zoospore formation, sexuality, and other aspects of life cycles of algae may be influenced by associated organisms.

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