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# STUDIES ON *ECTOCARPUS* IN CULTURE

## I. INTRODUCTION AND METHODS OF OBTAINING UNI-ALGAL AND BACTERIA-FREE CULTURES

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In recent years pure cultures of algae have been one of the main sources of material for research on plant metabolism. By far the greatest proportion of this research has been carried out on members of the Chlorophyceae—in fact mainly on members of the genus *Chlorella*. As members of the Chlorophyceae show close resemblances to the higher plants a knowledge of their metabolism is useful in suggesting metabolic pathways in higher plants. However, considerable variations in pigmentation and storage products of the algal classes suggest important differences in metabolism, and it is clearly desirable to have some knowledge of the metabolism of representative members of all the algal classes.

For a reliable investigation of algal metabolism pure cultures should be used whenever possible. Only two members of the Phaeophyceae have hitherto been reported in pure culture (Provasoli, McLaughlin & Droop, 1957) but their growth appears to be too slow to render them of value for physiological or metabolic studies. Species of *Ectocarpus* have been maintained in crude or uni-algal cultures by a number of workers for studies of life history and morphology. When this study was initiated a uni-algal culture of *E. confervoides* (Kütz.) Batt. was already being maintained at University College, London, by Dr J. M. Kain, and as this grew well it was decided to use members of the genus *Ectocarpus* for the present investigation.

Members of this genus are important ship-fouling algae. It is interesting that they show a marked resistance to anti-fouling paints containing copper (Harris, 1943).

The taxonomic position of many species of *Ectocarpus* is in some doubt, but material used for culture work was identified as far as possible from the works of Newton (1931), Hamel (1931–39), Kylin (1947) and Rosenvinge & Lund (1941).

### MATERIALS AND METHODS

Although a uni-algal culture of *E. confervoides* was already available it was thought advisable to have another species in uni-algal culture and then if *E. confervoides* proved to be exacting in its requirements a second and perhaps

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less exacting species would be available for purification. For this purpose material of *E. secundus* Kütz. (*Giffordia secunda* (Kütz.) Batt.) was collected from a rock pool at Beer, south-east Devon.

### *Culture vessels*

Cultures were grown in vessels of 'Pyrex' or similar quality glass, the necks of which were plugged with non-absorbent cotton wool. All culture vessels were cleaned by being soaked in chromic acid for 24 h and then being rinsed thirty times with tap water and three times with distilled water to remove traces of chromium.

### *Media*

Natural sea water was used as the basis for most of the culture media and was filtered through paper and stored in the dark for 1 month to 'age' before being used (Harvey, 1941). After storage the pH was 7·7–8·0. The basic culture medium (medium A) was this sea water enriched with potassium nitrate ($KNO_3$) 2·0 mM (202 mg/l.), potassium phosphate ($K_2HPO_4$) 0·2 mM (34·8 mg/l.), ferric chloride ($FeCl_3.6H_2O$) 0·01 mM (2·7 mg/l.) and manganese chloride ($MnCl_2.4H_2O$) 0·001 mM (0·2 mg/l.). The phosphate was autoclaved separately from the rest of the medium and added prior to inoculation to prevent precipitation (Kain & Fogg, 1958).

TABLE 1. COMPOSITIONS OF STERILITY-TESTING MEDIA

| Medium number | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| Aged natural sea water (l.) | 1 | 1 | 1 | 1 | 1 | 1 |
| B.D.H. Bacto-peptone (g) | 5·0 | 5·0 | 0·5 | 10·0 | 1·0 | 1·0 |
| Ferric phosphate (g) | 0·1 | 0·1 | — | — | — | — |
| B.D.H. Agar (g) | — | 20·0 | 20·0 | — | — | 20·0 |
| Soluble sodium caseinate (g) | — | — | 0·5 | — | — | — |
| Soluble starch (g) | — | — | 0·5 | — | — | — |
| Glycerol (ml.) | — | — | 1·0 | — | — | — |
| Potassium phosphate ($K_2HPO_4$) (g) | — | — | 0·2 | — | — | — |
| Magnesium sulphate ($MgSO_4.7H_2O$) (g) | — | — | 0·05 | — | — | — |
| Glucose (g) | — | — | — | 10·0 | 1·0 | 1·0 |
| Ferric chloride ($FeCl_3$) | — | — | Trace | — | — | — |

All media were adjusted to pH 7·5–8·0 before use. Media 1, 2, and 3 are modified from Spencer (1952), medium 4 from Fogg (1942) and media 5 and 6 adapted from ZoBell (1946).

Occasionally the medium was enriched with organic nutrients. 'Analar' grade chemicals were used where available. Culture materials were normally sterilized by autoclaving at 15 lb. for 15 min. Media containing thermolabile materials were sterilized by Seitz filtering. Six different media, three liquid and three solidified with agar, were used to test for bacterial contamination (Table 1). Not only the culture media but the algal material itself was tested. The test cultures were kept in a dark cupboard at room temperature, usually about 20° C—the optimum for marine

bacteria (ZoBell, 1946), and were examined every few days over a period of 3 weeks. Once pure cultures were obtained little bacterial contamination occurred but fungal contamination did occur on several occasions.

Uni-algal cultures were maintained in medium A to which soil extract was added at 20 ml./l., although it seems likely that soil extract is not necessary for the growth of *Ectocarpus confervoides* or *E. secundus*. Pure cultures of both species were maintained in medium A to which 'Oxoid' dehydrated liver infusion was added at 0·1 g/l.; liver extract was not necessary for growth but did help to indicate the presence of bacterial contamination (Droop, 1954).

### *Culture apparatus*

Three types of culture apparatus were used in all of which the illumination was artificial and continuous.

In culture apparatus 1 cultures were grown around fluorescent lamps at room temperature.

A constant temperature water-bath was used for culture apparatus 2 with illumination from fluorescent tubes in glass sleeves passing through the bath. The cultures were subjected to a constant illumination of 6000–8000 lux and a constant temperature but were not shaken. In order to overcome differences in illumination in different parts of the apparatus the cultures were moved around in the bath at least twice a week.

Apparatus 3, a unit consisting of four thermostatically controlled tanks, has been described fully by Fogg, Smith & Miller (1959). In this, cultures were subjected to controlled temperature and illumination and could be shaken at different speeds.

## EXPERIMENTS TO OBTAIN BACTERIA-FREE CULTURES

Various methods for obtaining uni-algal and bacteria-free cultures of marine algae have been described by Schramm (1914), Bold (1942), Chu (1946), Pringsheim (1946), Fish (1950), Provasoli, Pintner & Packer (1951), Spencer (1952), Droop (1954) and Kain & Fogg (1960). The application of these methods to *Ectocarpus* will now be described.

### UNI-ALGAL CULTURES

### *Isolation of single cells*

*Ectocarpus* with its long branching filaments cannot be isolated with micropipettes as is usual in this method. However, filaments of *Ectocarpus* were broken up into short lengths with glass needles and clean lengths isolated. These fragments did not grow, since presumably, as they were only one or two cells long, they did not have active meristematic regions. It was found possible to start cultures from short lengths of filaments with at least one plurilocular sporangium on them, but it was not possible to isolate these sporangia without carrying over epiphytes, especially diatoms, which soon overgrew the *Ectocarpus*. In an attempt to isolate the zoospores, material of *Ectocarpus* bearing plurilocular sporangia and not too densely covered with diatoms was allowed to stand overnight on a moist filter-paper in a sterile Petri dish. The algal material was then transferred to a boiling tube of sterile-

enriched sea water and the spores were soon released giving a suspension of zoospores and diatoms. These zoospores were picked up with micro-pipettes but as soon as they touched the walls of the pipette they lost their mobility and settled down on the inside of the pipette making transfer difficult.

*Phototactic isolation*

The phototactic isolation method of Droop (1954) is a modification of the previous method. Papenfuss (1935) and Kylin (1943) asserted that the zoospores from the plurilocular sporangia of *Ectocarpus* showed positive phototaxis, but in spite of repeated attempts I found the spores did not show any marked phototaxis.

*Dilution*

This method did not prove suitable for zoospores of *Ectocarpus* partly because of the ease with which the spores settled on the sides of vessels during the dilution and partly because it was found difficult to obtain a suspension in which spores were more numerous than diatoms.

*Plating*

As it was unlikely that the zoospores of *Ectocarpus* would germinate on an agar surface the method as normally used (Pringsheim, 1946) was not attempted. Some *Ectocarpus* was left overnight on a moist filter-paper in a Petri dish and the next morning was flooded with sterile culture medium to bring about the release of spores. After 15 min the algal material was removed, a little more culture medium added, and the covered dish placed in the light. Ten days later sporelings of *Ectocarpus* were growing on the filter-paper. Colonies of diatoms were also present but were not spreading very rapidly over the rough filter-paper. The sporelings were examined with a binocular microscope and one which was free from epiphytic diatoms was removed to a tube of sterile medium. In this way a uni-algal culture of *E. secundus* was obtained.

## BACTERIA-FREE CULTURES

Although useful preliminary work on algal nutrition may be carried out with uni-algal cultures, for many purposes bacteria-free cultures are essential. Attempts were therefore made to obtain bacteria-free cultures of *Ectocarpus* before any detailed investigation was begun.

The methods for obtaining bacteria-free cultures can be divided into two groups. In the first group the bacteria are eliminated by washing them off the cells or in some way transferring the cells to a bacteria-free medium. The second group of methods aims at killing the bacteria, or at least suppressing their activity whilst the alga continues to grow. Methods of the first type are only successful if there are no bacteria attached to the algal cell walls.

The second group of methods is by far the more effective as these not only remove bacteria in the medium but also allow new cells to grow up without attached bacteria.

*Single cell washing*

This technique as described by Pringsheim (1946) should be applicable to the zoospores of *Ectocarpus*, but as was previously recorded these proved to be difficult to transfer with micro-pipettes.

*Phototactic isolation*

The method of Droop (1954) could not be applied because under the conditions employed in this investigation the zoospores were not sufficiently phototactic.

*Dilution and plating*

For the dilution method to be successful it was necessary that zoospores should be more abundant than bacteria in the suspension to be diluted, a situation that was never obtained. As the zoospores did not survive very long on the moist filter-paper in a Petri dish it seemed unlikely that they would grow on an agar surface.

Pringsheim (1946) has commented that contaminating bacteria are always attached to the walls of members of the Phaeophyceae. Tests made on material of *Ectocarpus* showed a number of bacterial types in the medium and additional types on the alga itself.

*Ultra-violet irradiation*

Prolonged exposure to ultra-violet irradiation is lethal to most living organisms, but the lethal dose varies from one organism to another and is difficult to predict for any specific organism. It has been shown that doses of ultra-violet irradiation below the lethal level may have very marked effects on living organisms (Redford & Myers, 1951). However, ultra-violet irradiation has been successfully used to obtain pure cultures of algae and does not appear to have had any deleterious effects. This technique has been successful with a filamentous blue-green alga (Fogg, 1951) and was therefore tried with *Ectocarpus*.

A young uni-algal culture of *E. confervoides*, consisting mainly of sporelings 5–7 mm long, was transferred to a quartz boiling tube which was fixed 15 cm from a 'Hanovia' 12 in. jacketed ultra-violet lamp (wavelength 2537 Å). The lamp was switched on and the quartz tube well shaken every $\frac{1}{2}$ min. After 5 min of irradiation samples were taken aseptically from the quartz tube and culture tubes of each of eight different nutrient sea water media were inoculated. Further samples were taken after 10, 20 and 30 min irradiation. After

8 days illumination all media containing organic nutrients showed bacterial growth. Thirty minutes irradiation had not killed all the bacterial contaminants and tests showed at least three bacterial types were still present. Although this experiment had not produced a pure culture even the longest period of irradiation appeared to have had no ill effects on *Ectocarpus*, and it was thought worth while subjecting this material to even longer periods of irradiation. The times of irradiation in the next experiment were 25, 35 and 45 min and the material was a culture of sporelings grown up from material subjected to 30 min irradiation in the previous experiment. After 45 min irradiation the tips of the filaments of the sporelings showed a very marked bleaching. All cultures grew but the material subjected to 45 min irradiation gave positive contamination tests and the contaminants appeared to be the same bacterial types as were present at the beginning of the experiment. *E. secundus* was also subjected to ultra-violet irradiations. The experimental procedure was the same as in the previous experiments. Total irradiation times were 20, 30 and 40 min. All material subjected to 40 min irradiation died and all media containing organic nutrients showed bacterial growth, even those in which the alga had died. Forty minutes irradiation had killed the alga but not all the contaminants, although the number of contaminating types was again reduced.

*Antibiotics*

As with ultra-violet irradiations there have been some reports that a sub-lethal dose of antibiotics can have mutagenic effects. Nevertheless, several workers (Fish, 1950; Provasoli *et al.*, 1951; Spencer, 1952; and Provasoli *et al.*, 1957) have used antibiotics singly or in mixtures to obtain bacteria-free cultures of marine algae.

A sterile solution was prepared from 'Glaxo' crystalline sodium penicillin G and was added to sterile culture medium A to give a final penicillin concentration of 4000 units/ml. Material of *E. confervoides* subjected to 30 min ultra-violet irradiation was subcultured and the young sporelings from this culture transferred to the penicillin solution. Subcultures were made from the penicillin after 9, 21 and 45 days and subcultures made into media containing organic nutrients did not show any bacterial growth. Before these cultures could be thoroughly tested for contamination it was important that all traces of penicillin should be removed. Further subcultures were made every 7–10 days and at each subculture only a small number of young sporelings was used as inoculum. After five such serial subcultures the cultures were tested for bacterial contamination and both culture medium and algal material gave negative results. *E. secundus* was treated in a similar way. In this experiment a range of concentrations of penicillin was used. Once again a culture from an ultra-violet irradiation experiment was used to provide sporelings for treatment with penicillin. The concentrations of penicillin and lengths

of treatment are given in Table 2. Bacteria-free cultures of *E. secundus* were successfully obtained by this method.

The strain of *E. confervoides* arising from material subjected to 4000 units/ml. of penicillin for 9 days has been used for investigations to be described in subsequent papers.

TABLE 2. TREATMENT OF *ECTOCARPUS SECUNDUS* WITH PENICILLIN

c, contaminated; F, bacteria-free

| No. of days in penicillin | Penicillin concentration (units/ml.) 1000 | 5000 | 10,000 |
|---|---|---|---|
| 1 | c | c | c |
| 2 | c | c | c |
| 4 | c | c | F |
| 8 | c | F | F |
| 15 | F | F | F |

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## SUMMARY

Details are given of the culture apparatus and methods found successful for the laboratory culture of species of *Ectocarpus*. In attempts to obtain uni-algal cultures of this genus several common methods were tried but only one of them, a modification of the plating technique, proved successful. Methods of washing and dilution were unsuccessful in giving bacteria-free cultures, presumably due to bacteria closely adhering to the cell walls. Ultra-violet irradiation was also unsuccessful but treatment with penicillin gave bacteria-free cultures of *E. confervoides* and *E. secundus* (*Giffordia secunda*).

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