The Mass Culture of *Porphyridium cruentum*

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Received for publication September 12, 1961

ABSTRACT

GOLUEKE, CLARENCE G. (University of California, Berkeley), AND WILLIAM J. OSWALD. The mass culture of *Porphyridium cruentum*. Appl. Microbiol. **10**:102–107. 1962.—A study was made of the effect of temperature, detention period, light intensity, and salinity on the growth rate and over-all light energy conversion efficiency of *Porphyridium cruentum* cultured on a medium consisting of concentrated sea water and sewage enriched with urea, chelated iron, and other additives. It was found that the optimal temperature was within the range of 21 to 26 C. Growth was retarded at temperatures less than 13 C, and completely inhibited above 31 C. Over-all light energy conversion efficiency increased from 2.24% at the 4-day detention period to 2.76% at the 10-day period. Conversion efficiency ranged from 5.8% at a light energy absorption rate of 8.2 cal:liter:min to 2.3% at 35 to 39 cal:liter: min.

At salt concentrations less than 3.5%, *Porphyridium* could not successfully compete with other algae in open cultures. Salt concentrations as high as 4.6% had no inhibitory effect on its growth.

In studies on nutrition, it was found that growth on a medium of salts used in formulating synthetic sea water dissolved in sewage was equal to that on a control medium consisting of concentrated sea water and sewage (see above). They showed that sewage contains a substance or substances essential for optimal growth. Vitamin B_{12} alone could not substitute for it.

The extraction of gel-forming substances from marine algae is the basis of a small but active industry in this country. The dependence upon a marine crop for raw material constitutes a definite disadvantage to the industry, however, because the location and the rate and type of growth of the crop cannot be readily controlled. Because of the unpredictability of the crop, a constant supply of raw material cannot be assured. On the other hand, the controlled culture of an alga providing the same type of gel-forming substance would ensure a constant and uniform supply of raw material, and would make possible the engineering design of an industry built around it.

P. cruentum has been recognized as an excellent source of carrageens, and many attempts have been made to establish mass cultures of the organism. The present paper describes a study made of the development and maintenance of continuous mass cultures of *P. cruentum* as well as of the principal environmental factors involved. Continuous cultures were used because they more closely approximate the type of operation conducive to economy of area and equipment in large scale production.

Variables studied included detention period, light intensity, temperature, salt concentration, and nutrient. During the course of the study, a new method of harvesting the alga was developed.

MATERIALS AND METHODS

Growth units. The experiments were conducted with the use of continuous automatic growth units, which have been described in detail by Oswald (1960). Four of the units were operated in parallel during each experiment. Each was equipped to maintain a preset detention period, temperature, light intensity, and rate of gas flow. They were designed so that the experiments could be continued indefinitely, although a period of 10 days was more than ample for the attainment of a "steady state."

Inoculum. The culture of *Porphyridium* used as the original source of inoculum in experiments concerned with the effect of environmental factors and in the greater part of those on nutrition, was obtained from the Botany Department algal collection of the University of California through the courtesy of G. F. Pappenfuss and Robert Burman. In experiments on nutrition involving a completely synthetic medium, the inoculum was obtained from a bacteria-free culture provided by R. A. Lewin, Scripps Institution of Oceanography, University of California.

Inoculum for the first of the experiments concerned with the effect of environmental factors was prepared by culturing *Porphyridium* in one of the growth units under the standard conditions to be described later. Inoculum for each of the succeeding experiments was obtained from the control culture of the preceding experiment. Thus, the control culture served as a stock culture. Unless free of bacteria, stock cultures in the form of stationary batch cultures, i.e., maintained in flasks or similar containers, were not practical because such cultures soon deteriorated despite suitable temperature and illumination. In many instances, after a period of a week or two, fungi appeared in the culture. On the other hand, when maintained as a continuous culture under proper conditions, the alga maintained itself indefinitely regardless of the presence of bacteria and other contaminants.

Although no measures were taken to limit the algal population to *Porphyridium*, and bacteria-free cultures were not used in most of the experiments, except on rare occasions the entire algal population consisted of *Porphyridium*, and its dry weight accounted for approximately 99% of the biomass of the cultures.

Medium. The medium used in determining the effect of variables other than nutrient consisted of a mixture of sewage and sea water concentrated to one-half its former volume by evaporation. Before mixing, each was passed through a Seitz filter to remove suspended coagulum and particles that might impart turbidity to the culture. Sterile technique was not used in the filtration process. Sufficient filtered sewage was added to the concentrated sea water to bring the specific gravity of the resultant solution to 1.030 (25 C). Because the heat used in evaporating the sea water resulted in the precipitation of a large part of the salts essential to algal growth, it was necessary to replace them. This was done by adding 1 ml of a stock solution of microelements as modified from Arnon by Krauss (1953), but at one-half the concentration listed by Krauss. In addition, $MgSO_4$ was added in the amount of 250 mg per liter of culture, $RhCl_3$ at 0.01 mg per liter, and 1 ml per liter of a ferric potassium EDTA stock solution prepared according to the method of Jacobson (1951). The medium was then enriched with 300 mg of urea per liter of culture.

Standard conditions. Unless otherwise stated in later descriptions of individual experiments, each experiment was initiated as follows. One liter of the control culture of the previous experiment was poured into an 11-liter Pyrex bottle. Three liters of the concentrated sea water-sewage medium were then added. An aliquot of the resulting suspension was placed in each of the four growth units. Each culture was illuminated by a single fluorescent lamp (daylight, 30 w) for a period of 4 days to permit the cultures to become adjusted to the radiation of the fluorescent lamp and to build up the concentration of the cultures. At the end of this period, the light was increased or diminished according to the conditions of the experiment. The cultures were also placed on detention period at this time, i.e., feeding was initiated. They were then subjected to the conditions of the experiment. Each experiment was allowed to continue for a period of 10 to 25 days, depending upon the length of the detention period.

Except for the factors being varied, "standard conditions" for the experiments were detention period, 4 days; CO_2 concentration, 1%; incident light energy, 28 to 30 cal:liter:min (1,000 to 1,200 ft-c); and temperature, 26 C. Gas was passed through each culture at 0.15 ft^3:liter:hr. Although no attempt was made to control the pH of the cultures, it rarely varied beyond the range of 6.5 to 7.0.

Parameters. Parameters used in determining the effect of a variable included either one or all of the following: cell number, viscosity of the culture medium, and the over-all light energy conversion efficiency. Determination of cell number was made with the use of a hemocytometer. Viscosity was determined by comparing the time required for a given volume of the liquid being tested to pass through a given orifice with that required by an equal amount of distilled water at the same temperature. Values for the viscosity of water were obtained from the Handbook of Chemistry and Physics (1956). Viscosity was used as a measure of relative carrageen production on the assumption that increase in viscosity of the liquid was due to the formation of the substance.

Values for light energy conversion efficiency presented in this paper are based upon the ratio of chemical energy permanently fixed in the form of algal material each 24-hr period to the visible light energy absorbed by the cultures during the same period. Determinations of the amount of visible light energy absorbed were made according to the procedure described by Oswald (1960). The caloric value of the energy contained in the algal material was based upon its heat of combustion as determined with a bomb calorimeter. The average value for the heat of combustion was found to be 4.8 cal per mg of volatile cell matter (SD $\pm$ 0.2), and this is the value used in determining the energy fixed by the algae.

To arrive at a true estimate of the over-all conversion efficiency of the cultures, it was necessary not only to determine the caloric value of the cell material produced, but also that of photosynthate which may have gone into solution. This posed a problem not easily solved. Normally, the dry weight of an algal culture can be obtained by centrifuging a sample of the culture to separate the cells from the culture medium. Separation of the cells in cultures of *Porphyridium* by centrifugation proved extremely difficult and uncertain, however, because of the high degree of salinity and viscosity of the culture medium.

Moreover, the increase in viscosity of the medium that accompanied growth of *Porphyridium*, and the continued high viscosity after centrifugation, indicated that some of the carrageens produced by the cells were in solution. Consequently, a method had to be found for determining the caloric value of this fraction.

Harvesting technique. The problem of removing both cells and dissolved photosynthate was solved to a great extent by the development of a technique for simultaneously removing both. This was done by adding to an aliquot of the culture, an equal volume of 80 to 90% ethyl alcohol. The mixture was then stirred vigorously (1 to 2 min) until a stringy coagulum was formed. The coagulum was wrapped around a straight inoculating wire and removed from the liquid, as is shown in Fig. 1. The resultant mass was squeezed between the fingers until it was freed of most of the liquid clinging to it. Dewatering the coagulum in the manner described was found more satisfactory than filtration with a Büchner funnel. In the latter procedure, some of the coagulum adhered to the

filter medium in such a manner as to render impossible the complete recovery of the material. On the other hand, material sticking to the fingers could be removed readily.

An experiment was conducted to determine the reproducibility of values of dry weights obtained through the use of the alcohol method of separation. In the experiment, 100 ml of 95% ethyl alcohol solution were added to each of ten 100-ml aliquots removed from a single *P. cruentum* culture. The algae were precipitated and removed as described previously. The dry weight of the solids obtained from each of the aliquots was determined. Maximal deviation from the average dry weight was 9%, whereas the average deviation was 3.8%. Not all of the variation can be attributed to the removal process, because some undoubtedly was due to the handling and weighing procedures.

The alcohol used in coagulating the algal mass was recovered from the medium by fractional distillation. The extent of alcohol recovery was determined by comparing the volume and density of the alcohol added to those of the alcohol recovered. In one series of experiments conducted to determine the extent of recovery of alcohol, 300 ml of ethyl alcohol solution, having a density of 0.8187 and therefore containing 261.6 ml of 100% alcohol, were added to a 300-ml aliquot of *P. cruentum* culture to precipitate the algae. After precipitation and removal of the algae, the remaining alcohol-medium solution was subjected to fractional distillation. At the completion of the distillation process, the reclaimed alcohol was added to a second aliquot of culture. This process was repeated until six such passes were completed. At the end of the sixth run, the volume of recovered alcohol amounted to 285 ml and its density was 0.8126 (256.6 ml of 100% alcohol). According to these results, 5.1 ml of 100% ethyl alcohol were lost upon treating 1,800 ml of culture. A second run consisting of six passes showed a loss of 11.2 ml alcohol; and a third, 8.2 ml. Thus, from 0.28 to almost 0.62 ml of alcohol was lost per 100 ml of culture. The loss is attributable to handling, because a certain amount of the solution was unavoidably removed with the precipitated algae. In terms of milliliters of alcohol per mg (dry weight) of precipitated solids, the average ratio was 1 ml of alcohol per 150 to 327 mg of volatile solids; and 1 ml per 300 to 657 mg of total solids removed.

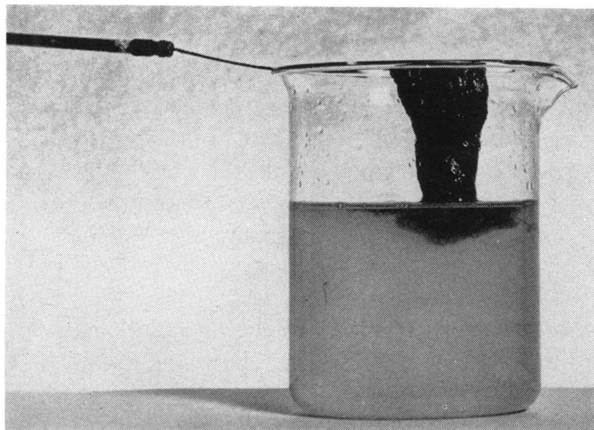

FIG. 1. *Removal of a coagulum of algae and other precipitated materials formed by adding ethyl alcohol to a culture of Porphyridium cruentum.*

RESULTS

Temperature. The effect of temperature on the growth of *P. cruentum* was studied in two experiments. In both, the temperatures applied were 13, 21, 26, 31, and 35 C. In the first series, the effect of temperature was judged on the basis of differences in the over-all light energy conversion efficiency of the cultures. In the second experiment, the effect of temperature was estimated from the rate of increase in number of cells. In this experiment, the volume of the inoculum used in setting up the cultures was reduced to 100 ml per liter of culture and the cultures were operated as batch cultures. Cell counts were begun on the day of initiating the cultures and were made daily thereafter. The experiment was ended when the number of cells either remained constant or began to decline.

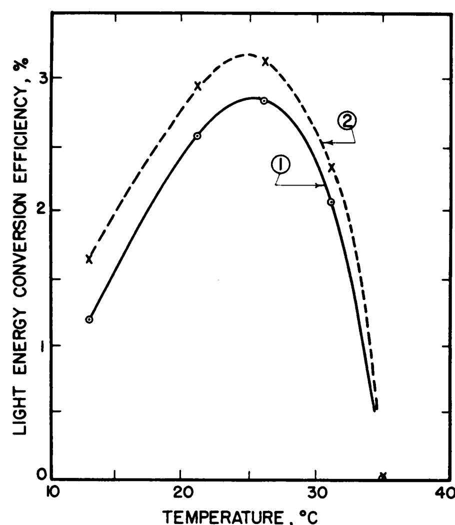

FIG. 2. *Relation of temperature to the over-all light energy conversion efficiency of Porphyridium cruentum. Curve 1 indicates the conversion efficiency at a 4-day detention period; curve 2, at a 10-day period.*

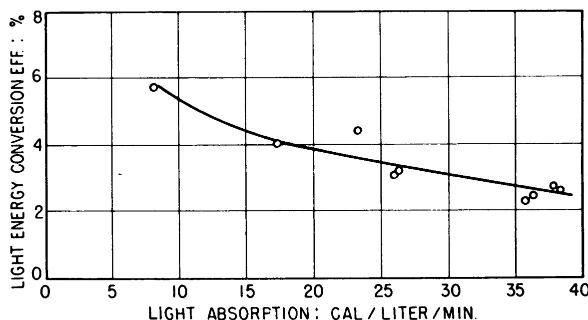

FIG. 3. *Over-all light energy conversion efficiency of Porphyridium as a function of rate of light energy absorption.*

The effect of temperature on over-all light energy conversion efficiency at detention periods of 4 and 10 days is shown by the curves in Fig. 2. According to the curves, optimal temperature at both detention periods was within the range of 21 to 26 C, and probably was closer to 26 C. No growth was observed at 35 C; hence, efficiency at this temperature was nil.

The effect of temperature on the growth rate of *Porphyridium* was similar in that the optimal temperature again was between 21 and 26 C. The generation times at 21 and 26 C were 1.4 and 1.5 days, respectively. At 13 C, it was 2 days; and at 31 C, 4 days. No increase in cell number took place at 35 C. With the exception of the culture held at 13 C, all reached maximal cell concentration within 5 days. A lag period of 3.5 days characterized the 13 C culture. However, its active period lasted until the ninth day, so that its ultimate cell concentration approximated that at the higher temperatures.

Detention period. Detention periods of 4, 6, 8, and 10 days were applied in the experiments on detention period. The experiments were repeated three times. In the first experiment, it was noted that the conversion efficiency of the alga increased from 2.24% at the 4-day detention period to 2.76% at the 10-day period. Since the increase with lengthening of detention period, albeit small, was contrary to that observed in cultures of green algae (Oswald, 1960; Golueke, 1960), it was decided to repeat the run. In the second and third experiments, the results were similar to those obtained in the first, in that a consistent increase in efficiency accompanied increase in detention period within the range tried. The difference in values between the 4-day and the 10-day period in the two experiments also was small, viz., 0.45% and 0.69%.

Light intensity. In the experiments on light intensity, incident light energy ranged from 8.2 to 39.5 cal:liter:min. Parameters used in the experiment were change in viscosity of the medium and over-all light energy conversion efficiency.

The viscosity of the culture medium increased with increase in rate of light energy absorption. At a light absorption rate of 9 cal:liter:min, the viscosity of the culture was 10.10 η (millipoises); at 36.4 cal:liter:min, it was 10.56 η; and at 39.5 cal:liter:min, 10.57 η. The viscosity of the medium prior to algal growth was 10 η in all cases.

The effect of increase in rate of light energy absorption on the over-all light energy conversion efficiency of the cultures is indicated by the curves in Fig. 3. As the curve indicates, efficiency of the cultures decreased with increase in amount of light energy received by the cultures. At an absorption rate of 8.2 cal:liter:min, the conversion efficiency was 5.8%; whereas, at rates within the range 35 to 39 cal:liter:min, it varied from 2.3 to 2.7%.

Salinity. In the experiments on the effect of salt concentration on the growth of *Porphyridium,* salt concentrations were 2.5% (sp gr 1.010, 25 C), 3.08% (sp gr 1.017), 3.5% (sp gr 1.020), 3.9% (sp gr 1.025), 4.6% (sp gr 1.030), and 5.2% (sp gr 1.033).

Results obtained at salt concentrations less than 3.5% were inconclusive because of contamination by a single-celled blue-green alga having the general morphological characteristics of *Synechocystis.* The blue-green alga competed so successfully with *Porphyridium* that regardless of detention period, it completely took over a culture within 8 to 9 days. At salt concentrations greater than 3.5%, however, the blue-green alga was unable to establish itself. No difference in rate of growth of *Porphyridium* or in its over-all light energy conversion efficiency was observed when it was cultured at salt concentrations ranging from 3.5 to 4.6%. The cells became brownish in color and growth was somewhat inhibited at a salt concentration of 5.2%.

Nutrition. The investigation on nutrition was directed toward the development of a synthetic medium for *Porphyridium.* Nutritional characteristics of each medium used in the study were evaluated on the basis of degree of resemblance of growth pattern to that of a culture of *Porphyridium* grown in the concentrated sea water-sewage medium described earlier. The latter culture served as the control.

In one experiment, the medium was formulated by dissolving in sewage, the salts listed by Harvey (1957) for making artificial sea water. To the resulting solution were added urea (300 mg per liter of culture), $MgSO_4$, $CaCl_2$, and trace elements in the concentrations indicated under Materials and Methods. Rate of growth of *Porphyridium* cultured in the sewage-synthetic sea water medium was almost identical with that of the control culture. Color was the only tangible difference between the two; that of the former being orange, whereas that of the latter, a deep maroon. After 5 days, however, the color of both cultures was the same, viz., deep red. The performance of the two cultures was almost identical in other respects. The over-all light energy conversion efficiency of the control culture averaged about 3.1%, and that of the varied culture was 3.2%. The viscosity of the medium increased about 5% in both cultures.

Another study involved the determination of growth characteristics of *Porphyridium* cultured in media not containing sewage. In these experiments, the media consisted of natural sea water enriched with urea, with NH_4NO_3, or with alanine.

The investigation with each nitrogen source was conducted as two sets of experiments, each in turn, consisting of two series of experiments. In the first set, the specific gravity of the media was that of sea water (1.025, 25 C). In the second set, the specific gravity was increased to 1.030 by adding NaCl. In the first series of each set of experiments, the cultures were started by adding to 900 ml of the medium to be tried, 100 ml of inoculum obtained from the control culture used in earlier experiments. The culture used with a given medium in the first series served

as the source of inoculum for the second series of each set. Only 50 ml of inoculum were used per culture in the second series. Thus, the carry-over of nutrient elements from the original control culture to the second series was at the most $1/200$ of the original.

Appreciable growth was obtained only in the first series of each set of experiments, i.e., those in which there was an appreciable carry-over of the sewage-sea water medium with the inoculum. Within 24 hr, cell concentration increased from an initial count of 8.1×10^4 cells per ml to 3.4×10^5 in all of the media, including the control. By the end of the second day, the cell concentration had increased to 1×10^6 in the control, and ranged from 8.4 to 9.5×10^5 per ml in the remaining media. With the exception of the control, cell concentration reached its maximum in all of the media by the end of the third day. Cell concentration continued to increase in the control until the experiment was terminated.

Growth was very limited in the variable media in the second series of experiments, in that the cell concentration increased from approximately 4×10^4 at the start to a maximum of only 4.5 to 4.7×10^5 by end of the sixth and final day of the series.

A completely synthetic medium was tried in a final experiment. It consisted of synthetic sea water formulated according to the specifications of Chu (1949), and enriched with NH_4NO_3 (300 mg per liter of culture) and vitamin B_{12} in concentrations ranging from 1 to 100 mg per liter of culture. Bacteria-free inoculum was used. Extent of growth was negligible regardless of vitamin concentration.

Discussion

Values obtained for over-all light energy conversion efficiency of cultures of *Porphyridium* in the present study generally were much lower than those to be expected from cultures of *Chlorella* and *Scenedesmus* grown under similar conditions of CO_2 concentration and light (Golueke, 1960). A contributing factor to the low values for *Porphyridium* was its low heat of combustion. The heat of the volatile material in *Porphyridium* was only 4.8 cal per mg as compared to 5.8 and 6.0 for *Chlorella* and *Scenedesmus*. The lower heat of combustion of *Porphyridium* may have been due to the inclusion of some volatile inorganic salts with the algal cells in the harvesting process. However, they accounted for only about 3.3% of the total volatile material. Even were a heat value equivalent to that of *Chlorella* and *Scenedesmus* used in arriving at a conversion efficiency for *Porphyridium*, the resulting value nevertheless would have been lower than that for the green algae.

Another reason for the low conversion efficiency of *Porphyridium* may have been the relatively long detention period required because of its slow growth. It should be remembered, however, that the growth rates obtained in the present study may not necessarily be characteristic of the species; and that perhaps, under optimal conditions, they would match those of the unicellular green algae. The light intensities used in the study also may account for the low conversion efficiency. Judging from the shallow slope of the curve in Fig. 3, it is quite likely that higher conversion efficiencies would have been obtained had light of lower intensity been applied. The shape of the curve in the figure is characteristic of that for the light saturation zone for the green algae.

The studies on nutrition, although far from complete, furnish sufficient information for practical application. They indicate that sewage contains a substance or substances essential to the successful culture of the alga. Lack of the substance was not compensated through the activity of bacteria in media not containing sewage; nor did the addition of vitamin B_{12} make up for it in synthetic media. This was demonstrated in the experiments with natural sea water enriched with nitrogen and in those involving the use of synthetic media, because growth was limited in all cases to that expected from the amount of control medium introduced with the inoculum. It is quite possible that the essential substance is identical with the growth factor in soil extracts found necessary for the normal growth of many groups of algae (Harvey, 1957).

The large scale culture of *Porphyridium* may be patterned according to the procedures developed by Oswald (Oswald, Golueke, and Gee, 1959) for the reclamation of waste waters through the production of green algae. *Porphyridium* can be cultured under such conditions, because its ability to thrive at relatively high salinities will enable it to compete with other algal types in large-scale ponds. The desired degree of salinity can be obtained either by concentrating the water through evaporation or by using blow-down from desalinization plants. The tolerance of high salinity by the organism would be of advantage in arid areas in which brackish waters are frequently found.

The economics of the large-scale production of *Porphyridium* will depend more upon that of the harvesting process than upon its culture, since little expense is involved in providing the raw materials to grow the alga. Brackish water is plentiful along coastal lands and in many arid regions. Similarly, wastes are always available. Hence, the only expense involved in culturing the plant is that of bringing the wastes to the ponds.

If the method of harvesting described in this paper is used, initial expenditure for alcohol and the cost of recovering it will constitute the major expense in harvesting the algae. This is true because the alcohol must be added in volumes ranging from two-thirds to the equivalent of that of the culture medium being processed.

The required volume of alcohol need only be equal to that of a 2-hr flow, since the alcohol used in precipitating the algae can be recovered within that time. Probably, under optimal conditions, the amount could be reduced to that of a 1-hr flow. Since the recovery process will be continuous, no equivalent reserve supply is needed. The

amount could also be reduced by lengthening the detention period of the pond culture, which would in turn result in higher concentrations of algal material and in a lower hourly flow. The required volume could also be reduced by decreasing the volume of the culture to be treated through the use of evaporation.

Although, theoretically, all of the alcohol can be recovered, in practice, some will inevitably be lost. According to the rate of loss in the experiments described in this paper, about 1 gal of alcohol was lost in producing 2.5 lb (dry weight) of volatile algal material. At 50 cents per gal, this would indicate a cost of 20 cents per lb (dry weight) of volatile algal material. Total dry weight of culture precipitate recovered per gal of alcohol lost would be about twice as much, and the cost per lb correspondingly lower. A large-scale, well designed operation undoubtedly would reduce this loss drastically.

Additional expenditure would be required for the heat used in recovering the alcohol. A heat exchanger using waste heat from the dryers would reduce this cost considerably. Despite the savings and economies possible, however, the process would be too expensive for the production of *Porphyridium* as a feedstuff alone. The recovery and sale of carrageen produced by the alga would be needed to bring the process within the realm of economic feasibility.

Although some salt is removed from the medium with the coagulated algae, the process is not a practical means for desalinizing water. At a 10-day detention period, only about 1/10 of the salts would be removed with each crop of algae and most of this would be due to the adhering of liquid to the coagulum. The improvement in methods of dewatering the coagulum that would accompany the full-scale production of *Porphyridium*, would drastically lower the amount of salt removed.

ACKNOWLEDGMENT

This study was supported in part by the National Institutes of Health of the U. S. Public Health Service.

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