

• • • • • ENZYMATIC DIGESTION OF ALGAL CELLS

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INTRODUCTION

It should not prove necessary in this report to reiterate all of the reasons why studies of the nutritional value of algae have become increasingly important. Suffice it to say, should algae be utilized in closed ecological systems in space travel it will be mandatory that maximum utilization be made of the nutritional potential of algal cells.

Algae of the genus Chlorella, which have been considered most often as components of life-support systems, consist of unicellular, spheroid organisms in which the cell contents are surrounded by a cell wall composed of protein, carbohydrate, and lipid material.

We have been studying a variety of enzymes in an effort to increase the degradation of the algal cell wall. This study is based on two assumptions, which are closely interrelated.

1. The algal cell wall is composed of material not readily digested under the conditions prevalent in the human digestive tract. In order to utilize these substances for nutrition, under conditions which preclude chemical or physical processing of the algae, an enzyme mixture which would degrade the cell wall and which would act within the human digestive tract would be highly desirable. To prove of value, the enzyme supplement would have to increase the utilization of the algae to an extent sufficient to compensate for the weight of enzyme being added to the diet.
2. The intact cell wall would act as a barrier to the digestion of the contents of algal cells by the normal human digestive enzymes. The cell wall should be ruptured in order to make the contents readily accessible. In addition, certain of the polysaccharides of the cell contents may not be digestible by human digestive enzymes, and the enzyme supplement would be necessary to increase their utilization.

Our interests thus encompass the structure and composition of the algal cell wall, enzymes which degrade cell walls and/or similar materials, and enzyme activities over the pH ranges of the gastrointestinal tract. Enzymes which are judged to be active by in vitro methods of analysis must still be shown to be effective in whole-animal feeding studies. The algae-enzyme foodstuffs must not only be shown to be adequate nutritionally but must also be shown to be free from toxic manifestations.

The experimental studies were supported by the Aerospace Medical Research Laboratories under Contract No. AF 33 (616)-7964.

BACKGROUND INFORMATION

Structure and Composition of the Algal Cell Wall

Two excellent studies of the structure and chemical composition of algal cell walls have been conducted by Northcote et al. (ref. 15) and by Cronshaw et al. (ref. 1). Northcote utilized differential centrifugation of mechanically disintegrated Chlorella pyrenoidosa cells to obtain his cell wall fraction. Cronshaw studied a variety of green, red, and brown algal species and investigated the fraction of ground frozen cells which was insoluble in boiling ethanol. Both groups utilized electron microscopy to supplement chemical and chromatographic analyses for specific components. Although there are naturally, some differences in interpretations of results, both groups of workers present a similar picture of the algal cell wall.

The wall appears to be composed of two distinct phases: an organized microfibrillar structure and a surrounding continuous matrix. In the Chlorella cell wall Northcote found the microfibrils to be irregularly interwoven in a continuous network over the wall. The microfibrils (30 to 50 A in diameter) lie in two main directions, at right angles to one another. No definite lamellae were found. In Cladophora and Chaetomorpha Cronshaw found many lamellae consisting of straight microfibrils arranged parallel to each other and surrounded by amorphous material. The direction of orientation of the microfibrils in adjacent lamellae was approximately at right angles. When the matrix was removed the microfibrils became easily separated. The cell walls of the other algal species investigated by Cronshaw showed a basic structure of randomly arranged microfibrils embedded in an amorphous matrix.

In Chlorella the cell wall is approximately 210 A thick. After digestion by an enzyme preparation from the snail, Helix pomatia (Keilin, ref. 9) the material left shows no microfibrillar structure but resembles the granular matrix. Sections of these digested walls have a laminated appearance. Two distinct layers approximately 50 A thick can be seen, one near the outer edge and one near the inner edge, separated by a space of approximately 100 A. This seems to indicate local concentrations of some of the materials of the matrix in these outer and inner lamellae. Northcote et al. identify the microfibrils with the α -cellulose fractions of the cell walls. They are composed of polymers of glucose, galactose, mannose, arabinose, and rhamnose. The protein, which may exist in part as a glycoprotein, is associated with the hemicellulose and with these polysaccharides makes up the greater part of the continuous matrix. The hemicellulose was isolated as an electrophoretically pure particle and upon acid hydrolysis yielded galactose, mannose, arabinose, xylose, and rhamnose.

It might be best at this point to define the terms hemicellulose and α -cellulose. Hemicellulose refers to those cell-wall polysaccharides which may be extracted from plant tissues by treatment with dilute alkalis, either hot or cold, but not with water and which may be hydrolyzed to constituent sugar and sugar-acid units by boiling with hot dilute mineral acids (ref. 14). The term α -cellulose refers to that portion of the cell-wall polysaccharides which are insoluble in cold alkali (ref. 17).

From the yield of α -cellulose in the whole cell and in the cell walls Northcote calculated that the cell wall represents 13.6% of the dry weight of the cell. The composition of the cell wall and the degree to which each of these components is digested by the snail enzyme preparation is listed in Table 1.

Table 1

CONSTITUENTS OF CHLORELLA CELL WALLS*

<u>Constituent</u>	<u>% in Intact Cell Walls</u>	<u>% Liberated by Snail Enzyme</u>
Protein	27.0	—
Lipid	9.2	40
α -cellulose	15.4	70
Hemicellulose	31.0	13
Glucosamine	3.3	
Ash	<u>5.2</u>	
Total recovered	91.0	

* Data from Northcote et al. (ref. 15).

The data of Cronshaw et al. agree in the main with that cited for Chlorella. They too, consider the microfibrils to be cellulosic in nature. However, in the case of Cladophora and Chaetomorpha they consider the microfibrils to be pure Cellulose I, containing only glucose residues. In the other algal species they studied, glucose is still the major structural unit but it is invariably associated with other sugars, frequently xylose. Other hexoses, pentoses, and uronic acids may also be associated in the microfibrillar fraction and in the amorphous matrix. In Porphyra mannose appears to replace glucose as the basic building unit of the microfibrils.

The general conclusion that the cell walls contain material other than a classical glucosidic α -cellulose has been reinforced by investigations on other algal systems. Myers and Preston (ref. 12) report that the α -cellulose fraction of the cell wall of Rhodomenia palmata, a red alga, is composed of approximately equal portions of glucose and xylose residues. In other red alga galactose is also present, and in Porphyra they also found that glucose is replaced by mannose. Iriki and Miwa (ref. 8) isolated crude fiber from a green alga and found it to be composed of 91 to 93 % mannose. They believe this fiber from Codium to be a β -1, 4-mannan, but other Codiaceae yielded β -1,3-xylans (ref. 8). Peat (ref. 16) has recently published an

extensive analysis of the carbohydrate of the red alga *Porhyra umbilicalis* and reports a variety of hexoses, pentoses, methyl- and anhydro- derivatives, and sulfate esters. It should be kept in mind that complex polysaccharides of the cell interior may also be refractory to digestion by normal human digestive enzymes. Hirst (ref. 6), in a general review on polysaccharides of marine algae, lists the following components as being present in one or another species:

- D-glucose
- D-galactose
- L-galactose
- 3,6-anhydro-D-galactose
- 3,6-anhydro-L-galactose
- 6-o-methyl-D-galactose
- D-mannose
- L-fucose
- L-rhamnose
- D-xylose
- L-arabinose
- D-mannuronic acid
- L-guluronic acid
- D-mannitol
- Glycerol
- Pyruvic acid

What we hope to have shown by this listing of components is some indication of the complexity of the structural elements of the algal cell wall and the cell contents. It would appear obvious that a mixture of enzyme activities might prove more useful in degrading the cell wall than a purified enzyme capable of acting on a single class of substrates.

Enzymes of Potential Usefulness

Certain of the enzyme systems to be described in this section are included for their known cellulolytic activity; others, because algae forms part of the diet of the organisms in which they are found.

β -Glucosidase (Cellulase)

Murti and Stone (ref. 11) have recently reported a separation of two types of β -1,4-glucosidase activities found in *Aspergillus niger*. The difference in specificity appears related to the degree of polymerization of the substrates upon which they act. One fraction shown no activity toward cellobiose, slight activity toward cellotetraose, and high activity on cellohexaose, cellodextrin, and hydrated cellulose. The other fraction shown greatest activity on the lower polymers. These enzymes are thought to be endoglucosidases with random sites of attack.

Much attention has been paid to the extracellular cellulose of *Myrothecium verrucaria*. Two of the more recent studies are those of Halliwell (ref. 5) and of Selby (ref. 19). Unlike the parent organism which completely solubilized undegraded forms of cellulose the cell-free culture medium has negligible effect on the same substrate unless it has first been swollen. Halliwell noted that the cellulase activity and the carboxymethylcellulase activity of the filtrate

decay at different rates under varying assay conditions. The cellulase is strongly adsorbed on its insoluble substrate and is only slowly released as the cellulose itself is solubilized. Selby has been interested in the mechanism of action of the Myrothcium extracellular cellulase. He believes that the enzyme does not act randomly at all the β -glucosidic linkages. Selby suggests that the enzyme, being composed of large molecules with restricted mobility in the substrate, removes a number of adjacent glucose residues from each of a relatively few sites of attack.

Pectic Enzymes (Polymethylgalacturonase and Polygalacturonase)

The pectic enzymes are of two types: the polymethylgalacturonases, which preferentially attack pectin, and the polygalacturonases, which act on pectic acid. The prefixes "exo" and "endo" refer respectively to enzymes which attack terminally and those which attack randomly. An endopolygalacturonase was recently isolated from A. niger by Tuttobello and Mill (ref. 21) which rapidly lowered the viscosity of a solution of pectic acid and released reducing groups from it randomly. This preparation showed only slight activity toward pectin.

Other polygalacturonases have been isolated from Bacillus polymyxa by Nagel and Vaughn (ref. 13). The extracellular crude enzyme and fractions separated by paper chromatography were able to hydrolyze trigalacturonide and higher oligogalacturonides. From the nature of the products isolated after hydrolysis it appears that the enzyme attacks from the nonreducing end of the polymer.

Lysozyme

The mucopolysaccharide nature of the substrate attacked by lysozyme was established by Meyer et al. (ref. 10). The more recent studies of Salton (ref. 18) have been concerned with investigations of the nature of the products formed on digestion with lysozyme. The isolated cell walls of Micrococcus lysodeikticus, which have a mucocomplex nature, are directly attacked by this enzyme. The main component split off by lysozyme is not N-acetylglucosamine but an amino-sugar complex (possibly a disaccharide) of glucosamine and an unidentified amino sugar. The mode of linkage of this amino sugar to the other molecular components of the cell wall is not known.

Helix pomatia Enzyme

Northcote et al. have used a snail enzyme preparation based on the method of Keilin. This enzyme mixture is active against the cell wall of Chlorella pyrenoidosa. The preparation has been shown to contain only weak proteolytic activity but active lipases and carbohydrases. From the data in Table 1 it can be seen that the enzyme is much more active against cell wall α -cellulose and lipid than it is toward hemicellulose.

Diverse Enzymes from Marine Sources

Studies on the localization of enzymes capable of digesting algal material were conducted by Huang and Geise (ref. 7) on three marine herbivores. An intestinal extract of the purple sea urchin, Strongylocentrotus purpuratus, was incapable of digesting various algal carbohydrates. However, a

bacterial suspension from the gut of this organism could digest entire algae and their constituents. In another organism, Cryptochiton stelli, extracts derived from the stomach, the digestive tract, and the intestine each showed a different spectrum of activities toward a number of substrates derived from algal sources.

Charonia lampas, a marine gastropod, has been shown to be a good source of glucosulfatase, arylsulfatase, chondrosulfatase, cellulose polysulfatase, and steroid sulfatase. Takahashi and Egami (ref. 20) found these enzymes in crude extracts of the liver of this animal. They found that cellulose polysulfate containing glucose residues sulfated at positions 6 and 2 and/or 3 can be degraded to glucose. They believe this reaction to result from the activity of three enzyme entities: cellulose polysulfatase, which partially desulfates the molecule, particularly at positions 2 and 3; polysaccharase, which degrades the polysaccharide chain, yielding glucose and glucose monosulfate; and finally, glucosulfatase, which completes the conversion of the monosulfate to glucose.

Other carbohydrases from marine sources include agarase and carrageen-inase. These extracellular enzymes have been found in cell-free filtrates of Pseudomonas atlantica by Yaphe (ref. 23) and by Yaphe and Baxter (ref. 24).

The above listing is not meant to be exhaustive; rather, it is intended to indicate the variety of enzymes from a diversity of sources which yield material found to be constituents of algal cell walls.

EXPERIMENTAL STUDIES

When we began our investigations on enzyme degradation of the algal cell we assumed that the wall contained a glucosidic type of insoluble cellulose. For this reason commercially available cellulases were screened for activity over a wide pH range using a fairly well-purified wood cellulose, Solka-Floc, as substrate.

Mixtures containing 200 mg of Solka-Floc and 1 mg of the enzyme under study in 10 ml buffer were incubated with shaking for 4 hr at 37 °C. At the end of the incubation period the mixtures were filtered and the filtrates were heated in a boiling water bath for 4 min. to inactivate the enzymes. Glucose determinations were performed by the glucose oxidase glucostat method (ref. 4). The results of this series of experiments are given in Table 2.

It is obvious that not only did the crude preparation yield the greatest activity but, equally as important, this material was active over a wide range of pH.

A similar type of screening using a sample of freeze-dried Chlorella 71105 as substrate was attempted. For this purpose 2 mg of enzyme was incubated with the algae at pH 2, 5, and 8. The filtrates which resulted from the digestion were varying shades of green. The presence of chlorophyll which was not readily extracted with benzene, petroleum ether, or chloroform precluded the use of the colorimetric determination for glucose. We

Table 2
LIBERATION OF GLUCOSE FROM 200 MG OF SOLKA-FLOC
AFTER INCUBATION AT 37 °C FOR 4 HR

pH	Glucose Liberated, $\mu\text{g/ml}$					
	Cellulase 35	Cellulase 36	Cellulase 4000	Mylase SA	Takamine Cellulase 4000	Crude Cellulase
1.2	1.1	1.47	4.4	0.0	0	3.03
2.1	1.1	2.94	4.4	0.0	3.3	25.0
3.2	0	2.94	2.94	0.0	3.3	37.8
4.2	2.27	10.3	17.6	4.4	19.7	60.5
5.2	7.95	11.8	25.0	13.2	25.0	66.5
5.8	12.5	8.82	19.1	10.3	16.7	56.0
6.8	2.27	1.47	2.94	4.3	4.54	34.8
7.8	0	1.47	1.47	1.47	1.51	
8.9	2.27	1.47	2.94	1.47	1.51	6.05
9.9	1.1	1.47	1.47	1.47	1.51	6.05

therefore chromatographed aliquots of the digestion mixtures in order to make a qualitative determination of whether there was any breakdown of the cell wall. Hot methanol extracts and aqueous extracts of the filtrates were spotted on Whatman No. 1 filter paper and chromatograms were run by a descending technique using a butanol-acetic acid-water mixture (5:1:2) as solvent. The reducing carbohydrates were detected by spraying with aniline oxalate. The digests containing enzymes did not appear to yield more reducing substances than the controls. It was noted that the extracts contained galactose as well as materials which had the mobilities suggestive of a disaccharide and trisaccharide.

Since it had become obvious that substances other than glucose residues were being produced, we next chromatographed a 5-hr acid hydrolysate of the algae (Fig. 1). The hydrolysate of *Chlorella* 71105 (Column 1) contained an unidentified disaccharide or trisaccharide (D), galactose (GA), glucose (GL), two other compounds which are probably hexoses or hexose derivatives such as deoxy-sugars (H), and three compounds which are pentoses or pentose derivatives (P). These results confirmed information from the literature which indicated the extreme diversity of algal cell wall polysaccharides.

We next examined the activities of enzyme preparations from the snail *Helix pomatia*, and the fungus *Myrothecium verrucaria*, as well as lyozyme and pectinase under conditions which simulated stomach and intestinal conditions. A new substrate, lyophilized *Chlorella* 71105 grown under sterile conditions and harvested by centrifugation, was used in this and subsequent experiments.

For the digestion 200 mg of dry *Chlorella* was incubated with shaking for 2 hr at 37 °C in 10 ml of pH 2 buffer containing 5 mg of pepsin. Phosphate buffer (10 ml) containing 10 mg of pancreatin, 0.03 mg of trypsin and 5 mg of the enzyme under test was then added. When pectinase was used it was added during the acid incubation phase. This mixture at pH 8 was then incubated for 4 hr at 37 °C. The samples were then heated for 5 min at 100 °C to inactivate the enzymes, centrifuged, and stored under refrigeration until analyzed.

Sugar determinations were made using a modification of the quantitative Kiliani method (ref. 3). Aliquots of the digest (4 ml) were passed through a mixed-bed ion-exchange resin and concentrated to dryness. The residue was dissolved in 3 ml H₂O and treated with 5 ml of 0.4 N acetic acid and 5 ml of 0.8 N KCN, stoppered, and heated for 3 hr at 39 °C. The contents of the flasks were then acidified and the excess HCN driven off by passing air through the solution. The resulting nitrile was hydrolyzed by the addition of 20% NaOH and the ammonia steam distilled into boric acid and determined titrimetrically.

The micro-Kjeldahl method was used for nitrogen determination. Aliquots of 4 ml of the digests were treated with an equal volume of 10% trichloroacetic acid and centrifuged; 5 ml of the supernate was then digested for 4 hr with 1.5 ml H₂SO₄, 40 mg HgO, 0.5 g K₂SO₄ and 50 mg sucrose. The resulting ammonia was liberated by the addition of 50% NaOH, steam distilled into boric acid, and determined titrimetrically.

These analytical methods were selected in the belief that they would reflect the formation of material which would be absorbed in the human digestive tract.

Contrails

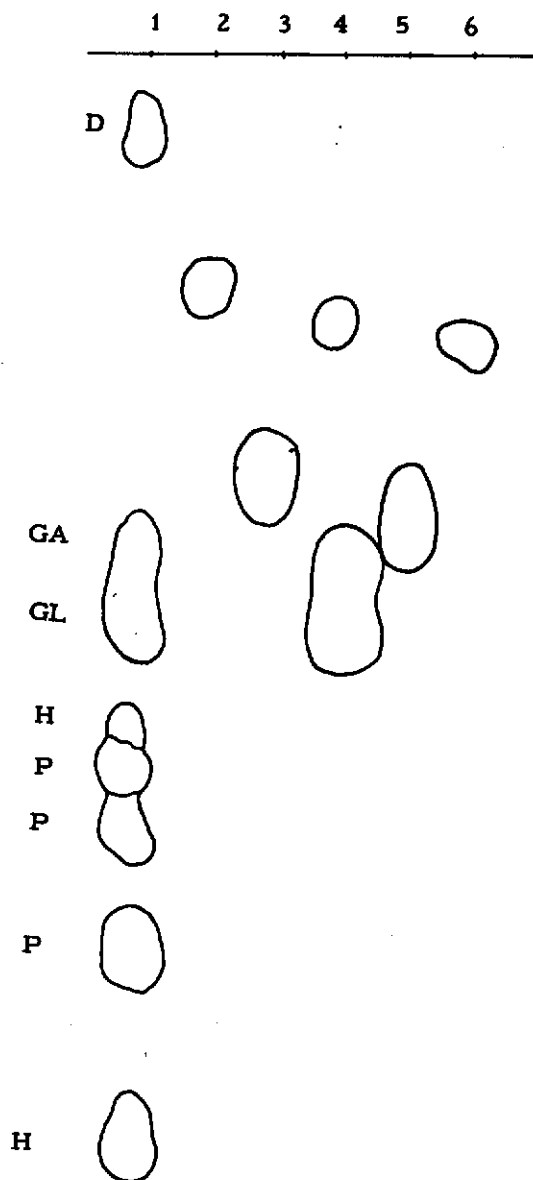


Figure 1

DRAWING OF CHROMATOGRAM OF ACID HYDROLYSATE CHLORELLA 71105

- Col. 1 Chlorella Hydrolysate
- Col. 2 Lactose
- Col. 3 Glucosamine-HCl
- Col. 4 Cellobiose, Galactose, Glucose from top to bottom.
- Col. 5 Sodium Glucuronate
- Col. 6 Maltose

Thus they measure amino acids and peptides too small to be precipitated by trichoroacetic acid as well as the increase in reducing groups in soluble carbohydrate substances. We have called the material measured by these analyses "utilizable."

Crude cellulase was isolated from the snail Helix pomatia by Karrer's method (ref. 2). The snails were removed from their shells and the gastrointestinal tract and its contents collected. This material was placed in a mortar with toluene, ground with fine sand, covered with water, and filtered through asbestos. The resulting suspension was dialyzed against distilled water, lyophilized, and stored in the freezer. About 1 g of the crude preparation was obtained from 20 snails.

Cellulase from Helix pomatia was purified by a modification of the method of Grassman et al. (ref 2). The crude lyophilized enzyme was dissolved in distilled water and adjusted to pH 3.5 with acetate buffer. This mixture was treated with activated alumina and centrifuged, and the precipitate was discarded. The supernatant was dialyzed against distilled water and lyophilized. The residue was stored in the freezer until used. The purified enzyme contained 66.4% protein, in contrast to 56.9% for the crude starting material.

Cellulase was obtained from filtrates of two M. verrucaria cultures grown on a mineral medium. In one culture, 30 g of Solka Floc, a highly purified wood cellulose, was added to 1 liter of the mineral medium. In the other culture 30 g of lyophilized pasteurized Chlorella ellipsoidea was added to 1 liter of the medium. Flasks containing 150 ml of the culture medium were inoculated with the spores and incubated for 2 weeks at 28°C with shaking. At the end of the incubation period the contents of the flasks were centrifuged and the residual solutions purified by a modification of the method of Whitaker (ref. 22). The filtrates were adjusted to pH 6.5 with phosphate buffer and brought to 30% saturation with ammonium sulfate. This mixture was refrigerated overnight. It was then centrifuged, and the precipitate was discarded. The supernatant was dialyzed at 4°C against distilled water to remove the ammonium sulfate and lyophilized. The residue was stored in the freezer until used. The preparation from the material grown on cellulose contained 71.9% protein and that from the material grown on the Chlorella contained 47.2% protein.

The results of a large number of individual experiments are recorded in Table 3.

Each value in the material-recovered columns represents the quantity of "utilizable" protein or carbohydrate recovered minus the quantity found in the digestive enzyme control for that particular experiment. Calculations in this fashion were found necessary since the material recovered from the digestive enzyme controls showed considerable variation from one experiment to the next.

Careful examination of Table 3 shows that certain trends are evident. Those single-enzyme additives that lead to large increases in the quantity of material recovered include purified snail enzyme, mold cellulase (cellulose-grown) and pectinase. The highest yields of "utilizable" material result from combinations of a cellulase and pectinase. It should be brought out that the enzyme preparations derived from Helix and Myrothecium include a variety of enzyme activities.

Table 3

RECOVERY OF UTILIZABLE PROTEIN AND CARBOHYDRATE
FROM ENZYME-DIGESTED ALGAE

Enzyme Added ^a	mg	Material Recovered ^b		
		Protein, %	Carbo- hydrate, %	Total, mg
Crude snail cellulase alone	5	2.2	0.9	6
+ lysozyme	10	2.8	1.1	8
+ pectinase	10	5.3	5.6	22
+ double pepsin + pectinase	15	5.3	5.1	21
+ double pancreatin + pectinase + double trypsin	20	5.5	3.2	17
Purified snail cellulase alone	5	2.8	2.5	11
+ lysozyme	10	3.4	2.1	11
+ pectinase	10	3.8	4.4	16
Mold cellulase (<i>Chlorella</i> -grown) alone	5	-1.8	0	-4
+ pectinase	10	- .7	2.5	4
Mold cellulase (cellulose-grown) alone	5	4.1	4.4	17
+ lysozyme	10	2.3	4.2	13
+ pectinase	10	3.9	6.1	21
+ double pepsin + pectinase	15	3.1	7.2	21
+ double pancreatin + pectinase + double trypsin	20	5.9	8.9	30
Pectinase alone	5	4.2	3.0	14

^aEnzymes added to basic digestive enzyme system.

^bDifference between test enzymes and controls.

The yield of additional utilizable substances surpasses the weight of enzyme additives for a number of additive mixtures, thus fulfilling the requirements stated earlier; that is, the return appears to more than adequately compensate for the additional input required.

CONCLUSIONS

It is evident that the algal cell wall is a highly complex structure. Not only does it contain carbohydrate residues other than glucose, but many of the bonds in the various polysaccharides of which it is partially composed are not susceptible to human digestive carbohydrases. The variety of basic carbohydrate residues which have been recovered from algal material makes it unlikely that a single highly purified enzyme would be able to cause extensive degradation.

Two main lines of research, which are in reality dependent upon each other, appear to offer the greatest likelihood for successfully degrading algal cell walls.

The first is a continued study of cell wall polysaccharides, including identification of simple residues and fragments derived through acid and enzymatic degradation of cell wall preparations.

The second is a study of cell wall degrading enzymes derived from natural sources in which algae constitute a normal part of their diet. The survey of enzymes of potential usefulness indicated that a number of marine organisms, or the bacteria of their gastrointestinal tract, elaborated a large number of such enzymes.

The use of enzyme additives in algal diet for human nutrition does appear to offer a way of obtaining maximal nutritional benefit from the food which is to be consumed. The usefulness of these additives must be demonstrated in whole-animal experiments.

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ACKNOWLEDGEMENT

Mr Joseph Kyle ably assisted in the performance of the experimental program. We should like to thank 6570th Aerospace Medical Research Laboratories for providing us with quantities of sterile lyophilized Chlorella 71105.