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FOREWORD

This program was conducted by IIT Research Institute, Chicago 16, Illinois, under the sponsorship of the 6570th Aerospace Medical Research Laboratories. The program was initiated by 1/Lt. E. G. Sander and monitored by 1/Lt. Paul A. Lachance and 1/Lt. E. W. Speckmann of the Biospecialties Branch, Physiology Division, of the Biomedical Laboratory. The work was conducted under Contract No. AF 33(657)-10066, Project No. 7164, "Space Biology Research," Task No. 716403, "Environmental Biology." At IIT Research Institute the program was designated Project L6002. Mr. Milton J. Becker, Mr. Alan M. Shefner, Dr. Bernice Kohn, and Mr. Charles Hagen planned and conducted the research.

This has been designated Report No. IITRI-L6002-4. The authors are M. J. Becker and A. M. Shefner. The studies performed and compiled into this report were conducted during the period of October 10, 1962 to August 31, 1963.

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ABSTRACT

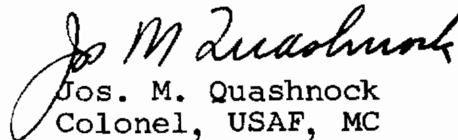
Cell walls of the alga Chlorella pyrenoidosa 7-11-05 were isolated and purified. The carbohydrates present as monosaccharides in the purified cell walls and in chemical fractions of the cell walls were analyzed by paper chromatography and thin layer chromatography (TLC).

Rhamnose, glucose and galactose were the predominant monosaccharides. Arabinose was not present in significant quantities in the cell walls or fractions thereof.

Conditions for the large scale heterotrophic growth of C. pyrenoidosa (7-11-05) were explored and found to be feasible. Studies on the growth and partial digestion of the algal cells by a fungus, Aspergillus oryzae, were found to be feasible. Such studies were oriented towards controlled feedings of the alga, the alga-fungus mixture and the fungus to young rats.

PUBLICATION REVIEW

This technical documentary report is approved.



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RESEARCH ON THE CHEMICAL COMPOSITION AND DIGESTIBILITY OF ALGAL CELL WALLS

I. INTRODUCTION

Algae cannot be fully utilized by humans because their digestive system is unable to degrade the algal cell wall. In order to determine what nutritive value might be expected from degraded cell walls, cell walls of the alga C. pyrenoidosa 7-11-05 were purified and the monosaccharide content was analyzed.

On the supposition that a fungus might digest the cell walls of algae and yield a more digestible mixture, several edible molds were studied. Aspergillus oryzae was found to be the most efficient fungus for partial digestion of the algal cell walls.

Optimum conditions for large-scale growth of C. pyrenoidosa 7-11-05 were established. Preliminary experiments were performed to determine optimum conditions for obtaining large quantities of A. oryzae and of a mixture of algae and fungi. These large quantities of materials will ultimately be used for nutritional studies in young rats.

II. GENERAL INFORMATION

A. Composition of Algal Cell Walls

The chemical composition of whole cells and cell walls of C. pyrenoidosa has been characterized by Northcote (ref 10) and Olaitan (ref 11). The sugars present were identified by paper and column chromatography and by paper electrophoresis. The whole cells were fractionated into soluble (hemicellulose) and insoluble (α-cellulose) polysaccharide fractions. The cell walls were also fractionated into soluble and insoluble polysaccharides. The monosaccharides found in the various acid-hydrolyzed fractions of the algae are listed in Table 1. The cell-wall material constituted 13.4% of the whole cells, (ref 10) and about 37% of this was recovered as purified cell walls. In the purified cell walls the soluble polysaccharides constituted 31% and the insoluble polysaccharides 15.4%. The two sugars which predominated were galactose in both soluble and insoluble fractions, and glucose in the insoluble fraction. The other monosaccharides were present in lesser amounts as determined by staining intensity (ref 10).

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Table 1

MONOSACCHARIDES OF C. PYRENOIDOSA CELLS AND CELL WALLS (from ref 10)

Whole-Cell Polysaccharides		Cell-Wall Polysaccharides	
Soluble	Insoluble	Soluble	Insoluble
Galactose	Glucose	Galactose	Glucose
Arabinose	Galactose	Mannose	Galactose
Mannose		Arabinose	Arabinose
Xylose		Xylose	Mannose
Rhamnose		Rhamnose	Xylose
			Rhamnose

B. Digestion of Algal Cell Walls

An enzyme preparation of the snail Helix pomatia digests 70% of the α-cellulose and 13% of the hemicelluloses of C. pyrenoidosa cell walls (ref 10). The total loss in cell wall weight due to the action of the enzyme is 21%. A similar enzyme preparation of H. pomatia partially digests whole cells of C. pyrenoidosa 7-11-05 (ref 4).

Cellulase of the mold Myrothecium verrucaria also produced utilizable material from 7-11-05 algae (ref 4). The edible mold Aspergillus oryzae has been used commercially for digestion of material containing algae.

C. Nutritional Studies

Gray (ref 3) considered the protein content and the role of fungi as a food source. The ability of fungi to form useful protein from excess carbohydrates and inorganic nitrogen salts is well documented. Thus the use of an organism such as A. oryzae to synthesize usable protein from algal sources may be feasible.

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Nutritional studies on C. pyrenoidosa 7-11-05 have been reported in a number of papers (refs 2,6,7,15). Feeding of algal diets alone caused growth deficiencies in young rats and chicks, but the deficiencies were overcome by supplementing the algal diets with other necessary ingredients (ref 6). Thus a diet supplemented with an edible fungus which has predigested some algal cells may be a good nutrient source for human **consumption.**

III. EXPERIMENTAL METHODS

The research was performed in the following sequence. First, C. pyrenoidosa 7-11-05 cell walls were isolated and purified. Then the carbohydrates in the cell walls were analyzed. Next heterotrophic large-scale growth of 7-11-05 was achieved. An edible fungus which can digest the algal cells was selected. Large quantities of the selected fungi and algae-fungi mixtures, were then grown.

A. Isolation and Purification of Algal Cell Walls

When a Virtis 45 or a Waring blendor was used to obtain large amounts of homogeneous cell walls of C. pyrenoidosa 7-11-05, the whole cells were not adequately broken and the cell walls contained contaminants which were difficult to remove. Consequently a Mickle disintegrator was used in the manner described by Northcote (ref 10).

Lyophilized cells were used. When examined under the microscope, the cells were essentially intact and had very little free cytoplasmic constituents and cell-wall materials. In order to maximize the yield of cell walls from the cells, the Mickle disintegrator was used for different periods of time, with various sizes of glass beads, in a medium of distilled water, phosphate buffer (pH 7.4), or phosphate buffer plus ethylene diamine tetraacetic acid. Maximum yields were obtained when the cells were agitated for 30 to 45 min at 4°C with 4 g of Ballotini No. 12 beads per 50 mg of cells in 10 ml of distilled water.

The purity of the cell walls was characterized by microscopy and by the color of heavy suspensions. Contaminating cytoplasm was stained with 1% aqueous solutions of safranin, congo red, iodine-potassium iodide, or methylene blue. Cell walls, however, were not stained by these dyes and appeared as distinct, dark layers surrounding the cytoplasm.

The centrifugation procedure used by Northcote (ref 10) to obtain cell walls of high purity had to be modified. Figure 1 outlines the Northcote procedure. The fractions and yields obtained are as noted in reference 10. In our hands, the residue 3R was not a white cell wall preparation but was heavily contaminated with other material. As depicted in Figure 2, the residue consisted of dark green lower portion, 3R-1, and an upper portion, 3R-2, containing pigmented cytoplasmic particles. Further purification

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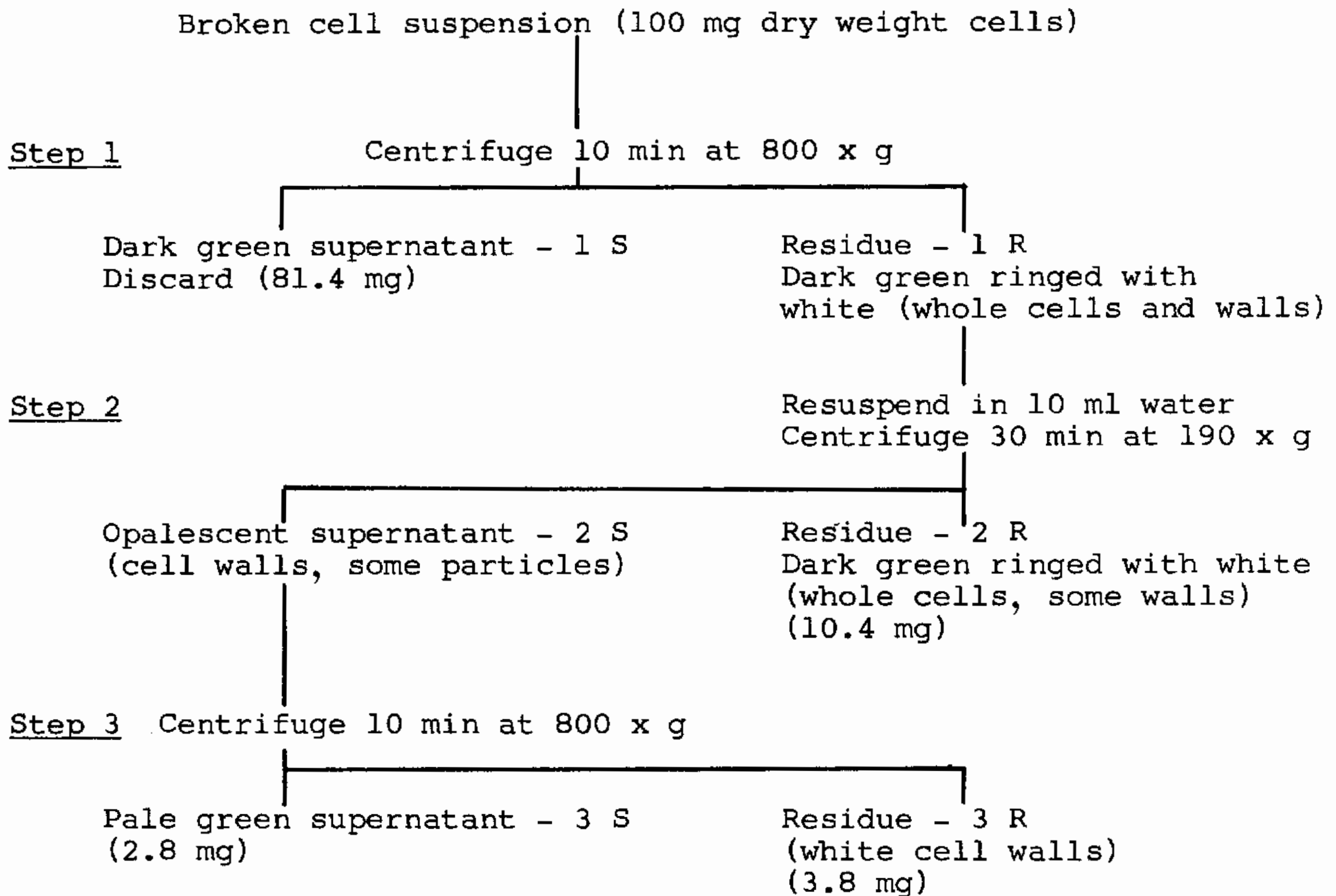

Figure 1

NORTHCOTE CENTRIFUGATION PROCEDURE FOR ISOLATION OF ALGAL CELL WALLS

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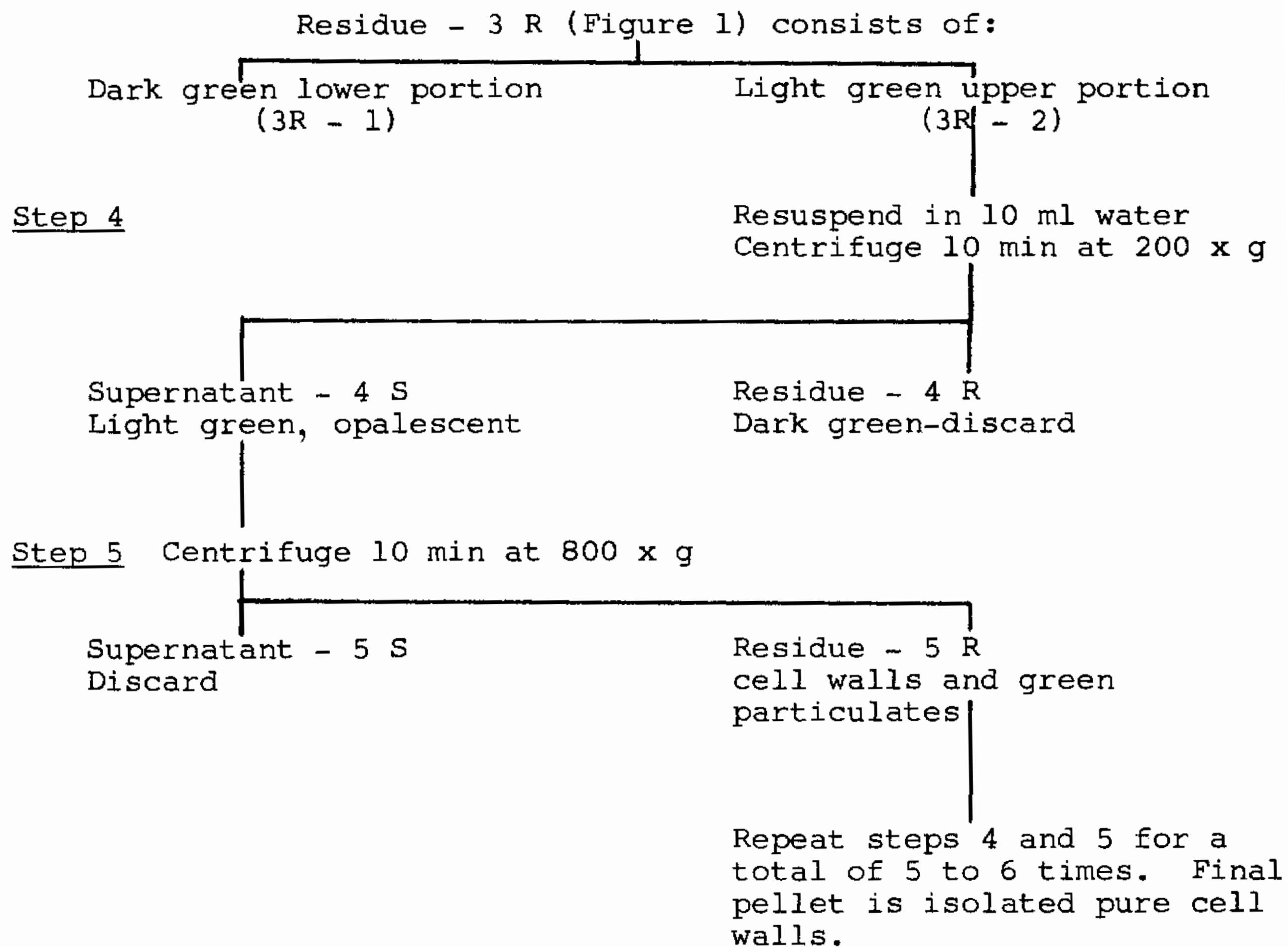

Figure 2

MODIFIED PROCEDURE FOR ISOLATION OF ALGAL CELL WALLS

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of cell walls beyond step 3 in Figure 1 was accomplished by 5 to 6 cycles of differential centrifugation of 3R-2, as denoted in Figure 2.

B. Chemical Analysis of Algal Cell Walls and Whole Cells

1. Sugar Composition

Potassium hydroxide-soluble and -insoluble polysaccharides of C. pyrenoidosa 7-11-05 cells and cell walls were prepared according to Northcote's procedure (ref 10). Total acid hydrolysis of isolated cell walls and soluble and insoluble polysaccharides from whole cells and cell walls was performed by heating for 6 hr at 100 to 105°C in a sealed combustion tube with 1 ml of 1 N sulfuric acid per 10 mg of material. Partial acid hydrolysis was performed by heating for 3 hr at 100 to 105°C with 3 ml of 0.3 N sulfuric acid per 10 to 15 mg of material.

The sulfate salts present in the hydrolysate were removed with di-N-octyl methyl amine by a simplified procedure (ref 5).* The hydrolysate was washed with about 15 ml of distilled water into a separatory funnel. To this solution was added 50 to 60 ml of 5% di-N-octyl methyl amine in chloroform. The resulting suspension was vigorously shaken and allowed to equilibrate for 1 to 2 hr. The hypophase was removed and discarded. The interface and epiphase were retained. The addition of di-N-octyl methyl amine was repeated twice more. After the second extraction, most of the white interface material was removed by centrifugation from the epiphase. The epiphase, which contained the sugars, was then extracted a third time. The final epiphase solution was concentrated under reduced pressure at approximately 30 to 35°C. Aliquots of the concentrated solutions were then applied to chromatographic systems for resolution of the monosaccharides present.

The primary chromatographic method used in preliminary analyses was thin-layer chromatography (TLC) as described by Stahl (ref 14). A new solvent system for TLC, tertiary amyl alcohol: water (11:2 v/v) was also used. Both systems allowed resolution of monosaccharides and disaccharides in a much shorter time than was possible by conventional paper chromatographic systems. Descending paper chromatography on Whatman No. 1 paper was performed with the same tertiary amyl alcohol solvent. This method was used to confirm the results and usually gave more defined resolution of the sugars present. Monosaccharides and disaccharides were located by spraying with anisaldehyde or aniline oxalate reagent.

2. Nitrogen Content

As a further check on the purity of the cell walls, the nitrogen content of various fractions was determined by a micro-Kjeldahl procedure (ref 16).

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*Personal communication.

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C. Heterotrophic Growth of Algae

A sterile stock culture of C. pyrenoidosa 7-11-05 cells was obtained from Dr. C. Sorokin of the University of Maryland. The cells were subcultured in medium formulated according to Vanderveen et al. (ref 15); potassium nitrate was used as the nitrogen source. The cultures were maintained at room temperature (~ 25°C) and at 37°C in the dark and under illumination (~ 75 foot-candles) for several weeks. The cultures were agitated occasionally and were subcultured into fresh medium as required. The wet weight yield of light- and dark-grown cells was determined.

Based upon the above results, experiments were conducted on a larger scale by a subcontractor (Amber Laboratories, Inc., Milwaukee, Wis.) to determine the optimum conditions for obtaining 35 to 40 lb dry weight of lyophilized cells. The studies by Amber Laboratories were performed in New Brunswick 14-liter glass fermentors. Cultures of sterile C. pyrenoidosa 7-11-05 cells were supplied to them, and variations in light intensity, temperature, agitation, and aeration were explored. Modifications in other physical factors, media composition, and methods of controlling contamination led to a final set of conditions which should produce maximum growth in larger tanks.

Light at the surface of the fermentors was provided by Tensor* lamps capable of producing 2,000 to 15,000 foot-candles using a General Electric 6-volt, No. 1133 incandescent white bulb. Temperature was controlled by immersing the fermentors in a constant-temperature water bath. The cultures were agitated at 350 to 450 rpm.

Aeration was found to be necessary for continuous growth of submerged algae. The optimum aeration rates with sterile air were 1/6 to 1/4 volumes per minute. When samples were removed for determination of growth rate, a positive pressure was produced within the fermentor by the addition of filtered air. This procedure led to irregularities in the growth curves. Addition of nitrate or phosphate to the culture did not eliminate these irregularities. Continuous aeration, however, produced continuous maximum growth.

The requirement of C. pyrenoidosa 7-11-05 cells for glucose was shown to be absolute even when agitation, illumination, and continuous aeration were used. Glucose was also required when the other ingredients of the medium were doubled in concentration.

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*Manufactured by Tensor Electric Development Co., Inc. Brooklyn, N.Y.

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An aerobic, spore-forming, granulated, mobile, gram-positive rod -- probably Bacillus subtilis -- contaminated most of the cultures by 50 hr. Various sterilization methods proved to be ineffective. As a consequence, a commercial preparation, Bacteriaban,* was tested and was found to inhibit the contaminant but have no effect on algal growth. Bacteriaban was subsequently used routinely, at levels of 2 g/liter. Growth of algal cells for 70 hr or longer was then possible.

Algae from several of the 14-liter fermentors were harvested by centrifugation in a DeLavall Laboratory continuous centrifuge at 12,000 rpm. Cells were obtained as a wet paste or a heavy wet suspension. The harvested cells were dried by either freeze-, spray-, or roller-drum-drying. The final, dried algal cells were compared to lyophilized C. pyrenoidosa 7-11-05 cells originally obtained from the Aerospace Medical Research Laboratory of the Aerospace Medical Division.

The optimum experimental conditions established for submerged fermentation of the algae in 14-liter fermentors, were adapted for large-scale production in 500- or 2000-gallon tanks.

D. Growth of Fungi and of Combined Algae-Fungi

Several edible molds were screened to determine which mold was best suited for the digestion of C. pyrenoidosa 7-11-05. These were Penicillium roquefortii, Aspergillus oryzae, and Rhizopus stolinifera. Aqueous suspensions of C. pyrenoidosa 7-11-05 adjusted to pH 4.5 were heavily inoculated with each of the three molds. The cultures were incubated at 30°C for 1 week on a rotary shaker. They were then incubated for 2 additional weeks without shaking.

Growth, pellicle formation, and deposition of alga-like sediment were observed visually in the flasks. A. oryzae not only grew better than the other molds tested, but also yielded the highest percentage of algal cell walls and broken cells. The overall growth of A. oryzae and the yield of broken algal cell walls, however, were not considered optimum.

1. Fungi

Slants were made in 100-ml milk dilution bottles. The slants contained 2% Difco agar-agar, 0.5% Oxoid peptone, 0.1% Difco yeast extract, and 1% glucose. They were inoculated with A. oryzae and grown at 27°C, as a heavy layer. Spores were removed as needed by the addition of a 0.1% solution of Tween 80.

*Product of Paul-Lewis Laboratories, Inc., Milwaukee, Wis.

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About 10^6 to 10^8 spores per ml of Tween 80 solution were present for the algae-fungi experiments.

A. oryzae was also grown in a liquid medium containing the same components as above. The mycelia and spores were harvested by centrifugation and washing in several cycles. The washed product was dried thoroughly, the total nitrogen was determined (ref 16), and the protein content calculated.

An estimate of the protein content of the edible mold gave some assurance that animals on a nutritional diet, consisting in part of the mold, were not limited in terms of available protein.

2. Combined Alga-Fungus Growth Studies

The combined organisms were grown both in flasks and in petri dishes under sterile conditions. Freeze-dried algal materials were washed by several cycles of suspension and centrifugation at 1500 rpm for 20 min at 5°C, and resuspended to 10 g% in sterile distilled water. This procedure eliminated residual nutrient ingredients and algal fragments.

a. Flasks

Each 125-ml flask contained 5 g% algae in an initial volume of 25 ml. In order to facilitate the development of enzymes by A. oryzae, the medium used in the initial experiments favored growth of A. oryzae. It contained basal salt and maltose solutions. All solutions added were sterilized by Seitz filtration. The sterile distilled water and the basal salt solutions were adjusted to pH 4.5 with sterile hydrochloric acid.

Two basal salt solutions were used, differing only in that one had 4 times more nitrogen than the other. The final concentration of salts was 0.5 g magnesium sulfate, 1.0 g potassium dihydrogen phosphate, and 0.1 or 0.4 g ammonium nitrate per liter (ref 12). Maltose was used at final concentrations of 5 and $\simeq$ 12 g%. The final pH of the flask system was checked and adjusted to pH 4.5. One ml of spore suspension was inoculated into appropriate flasks. The cotton-plugged flasks were placed on an Eberbach shaker at room temperature (25 to 30°C) and shaken for 7 days at moderate speed. The extent of mold growth was then observed. The flasks were incubated for another 7 to 10 days to allow for pellicle formation and any further changes in growth.

b. Petri Dishes

Two petri dishes with a 2% agar suspension containing 2 g% C. pyrenoidosa 7-11-05 cells were inoculated with 0.5 ml of the spore suspension of A. oryzae. One control petri dish had no nutrients added, while another had the basal salts with the higher nitrogen level and 5 g% maltose. They were both incubated at room temperature.

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IV. RESULTS

A. Characterization of Cell Walls

The final C. pyrenoidosa 7-11-05 cell walls obtained were examined microscopically at 450 and 1000 x magnification. They were estimated to contain about 10% contamination, chiefly in the form of pigmented cytoplasmic particles. Hemocytometer counts of cell-wall suspensions confirmed the preliminary microscopic findings.

The number of cells present in 100 mg dry weight of cells was calculated to be about 5×10^{10} on the basis of density and volume (ref 1,8). Hemocytometer counts showed that 100 mg dry weight of cells yielded 1.4 to 2×10^{8} cell walls for an average recovery of 0.34%.

The microscopic analyses of cell-wall recovery were compared with the dry weight of several preparations. When the dry weight due to contaminants is taken into account, the microscopic and the dry weight data agree. Thus, 0.68 mg of 90% pure cell walls was obtained from 100 mg of lyophilized whole cells. This yield is about 20% of the yield reported by Northcote, who used fresh wet-packed cells (ref 9)*. The data in Table 2 indicate that nitrogen content is a poor criterion of the purity of a cell wall preparation when it is correlated with the microscopic appearance.

B. Sugar Analysis of Algal Cell Walls and Whole Cells

A first representation of sugars present in acid hydrolysates of whole cells and cell walls is depicted in Figure 3. Cell walls contain rhamnose, glucose, and galactose with smaller quantities of materials which move in the area of mannose and xylose. Soluble polysaccharides of whole cells contain the same three monosaccharides and possibly disaccharides. The insoluble polysaccharides of whole cells contain essentially glucose-galactose sugars with a potential disaccharide or trisaccharide, but are lacking rhamnose. Subsequent TLC and paper chromatography of C. pyrenoidosa cell walls and of potassium hydroxide cell-wall fractions showed sugars to be distributed as depicted in Table 3.

Figure 4 is a diagrammatic representation of TLC results based upon measurements taken from representative chromatograms shown in Figures 5,6, and 7. Resolution of arabinose and glucose on the TLC system used is seen to be poor. However, upon staining with aniline oxalate, concentrations of arabinose equivalent to glucose stained dark green and glucose stained brown-beige. This differentiation showed that arabinose was not present in significant quantities in the cell walls. This is in contrast to the findings of Northcote (ref 10). With the tertiary amyl alcohol solvent system, paper chromatography (Figure 8) gave better resolution of glucose and arabinose than did TLC. In addition, the staining reaction for arabinose (pink) on paper was insignificant compared to the staining for glucose (brown-yellow), which was predominant.

*Personal communication.

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Table 2

NITROGEN CONTENT AND MICROSCOPIC APPEARANCE
OF ALGAL CELL-WALL PREPARATIONS

Fraction	Color of Suspension	Microscopic Observations	% N (dry weight basis)	Designation or Disposition
3R-1 (Fig. 2)	Dark green	Many whole cells, very few walls	7.85	Discard
3R-2 (Fig. 2)	Green, opalescent	About 70% whole cells and 30% walls	2.46	Discard or recycle
5R (Fig. 2)	White-gray, opalescent (tint of green)	About 90% walls, some pigmented cytoplasmic particles	4.10	Final cell walls
3R (Fig. 1)	White	Cell Walls	4.6	Northcote's cell walls

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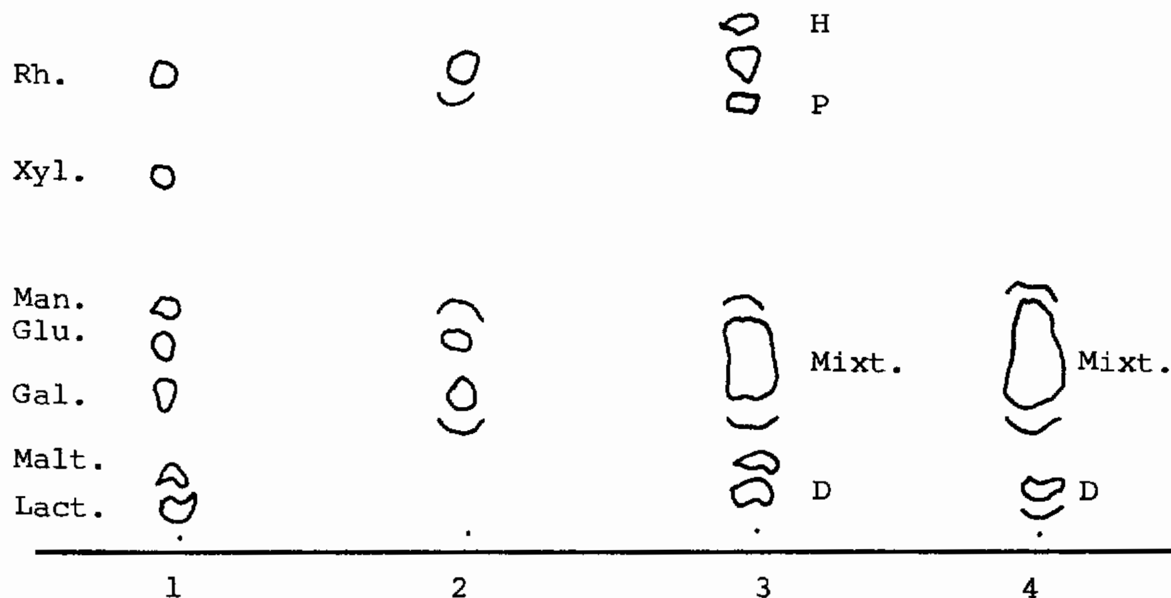

1 Mixture of standard sugars: rhamnose, xylose, mannose, glucose, galactose, maltose, and lactose

2 Cell wall: rhamnose, glucose, and galactose

3 Soluble polysaccharides of whole cell: a hexose (H), rhamnose, a pentose (P), glucose-galactose (mixt.), and one or possibly two disaccharides (D)

4 Insoluble polysaccharides of whole cell: glucose-galactose mixture, and a potential disaccharide or trisaccharide.

Figure 3

TLC RESULTS REPRESENTING SUGARS IN ACID-HYDROLYZED C. PYRENOIDOSA 7-11-05 FRACTIONS

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Table 3

SUGAR ANALYSIS OF CELL WALLS OF C. PYRENOIDOSA 7-11-05

	Total Cell Wall	Soluble Fraction		Insoluble Fraction
	Total Hydrolysis	Total Hydrolysis	Partial Hydrolysis	Total Hydrolysis
Major conc.	Rhamnose	Glucose	Glucose	Rhamnose
	Glucose	Rhamnose	Rhamnose	Galactose
	Galactose			Glucose
Trace conc.		Galactose	Galactose	
	Xylose	Xylose		Xylose
	Mannose	Mannose		Mannose
			Lactose	

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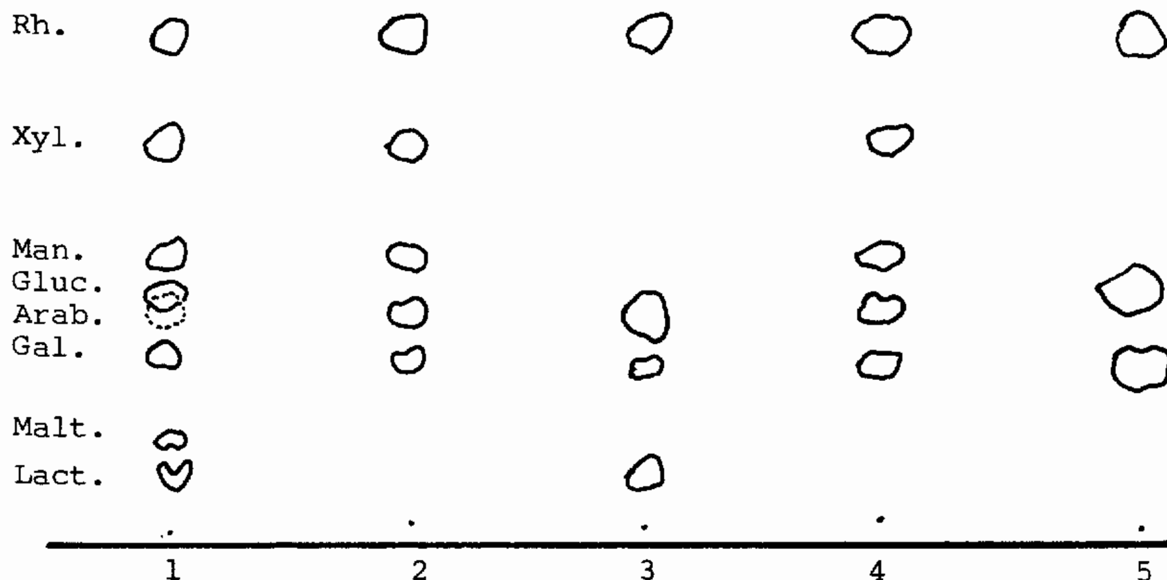

1 Mixture of standard sugars: rhamnose, xylose, mannose, glucose, arabinose, galactose, maltose, and lactose.

2 Cell wall, soluble fraction, total hydrolysis: rhamnose, xylose, mannose, glucose, and galactose.

3 Cell wall, soluble fraction, partial hydrolysis: rhamnose, glucose, galactose, and lactose.

4 Cell wall, insoluble fraction, total hydrolysis: rhamnose, xylose, mannose, glucose, and galactose.

5 Gum acacia, total hydrolysis: rhamnose, arabinose, and galactose.

Figure 4

TLC RESULTS REPRESENTING SUGARS
IN ACID-HYDROLYZED C. PYRENOIDOSA 7-11-05 CELL-WALL FRACTIONS

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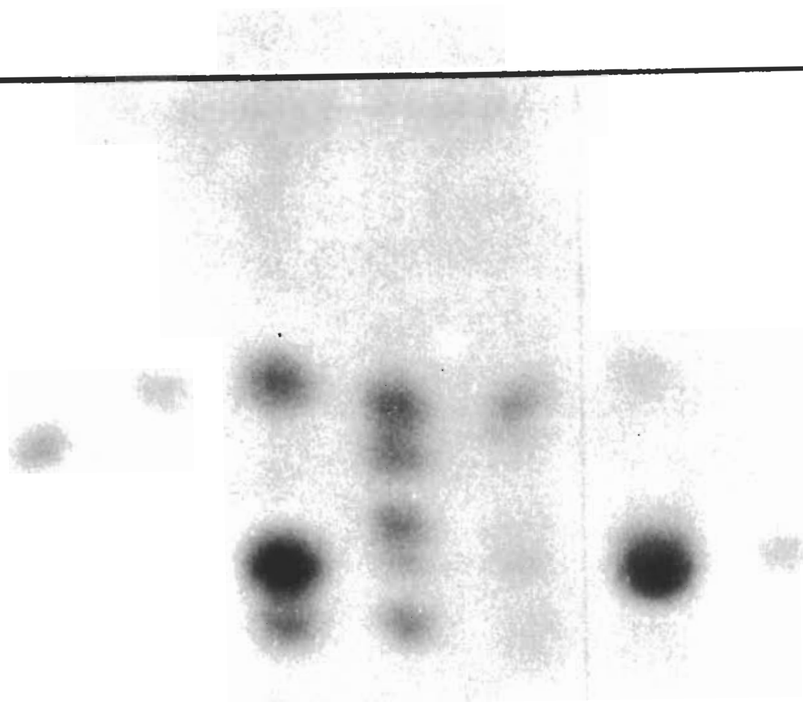

1 2 3 4 5 6 7 8 9 10

Column	Samples
1	Galactose - 3.5 μg
2	Glucose - 3.5 μg
3	Arabinose - 3.5 μg
4	Xylose - 3.5 μg
5	Rhamnose - 3.5 μg
6	Mixt. of rhamnose, glucose, arabinose and galactose - 3.5 μg each
7	Mixt. of rhamnose, xylose, mannose, glucose and galactose - 3.5 μg each
8	Cell wall, insol. fraction; total hydrolysis - 5 λ
9	Cell wall, sol. fraction; total hydrolysis - 5 λ
10	Arabinose - 3.5 μg

Figure 5

TLC PLATE (NO. 4-43) OF MONOSACCHARIDES AND CELL WALL FRACTIONS

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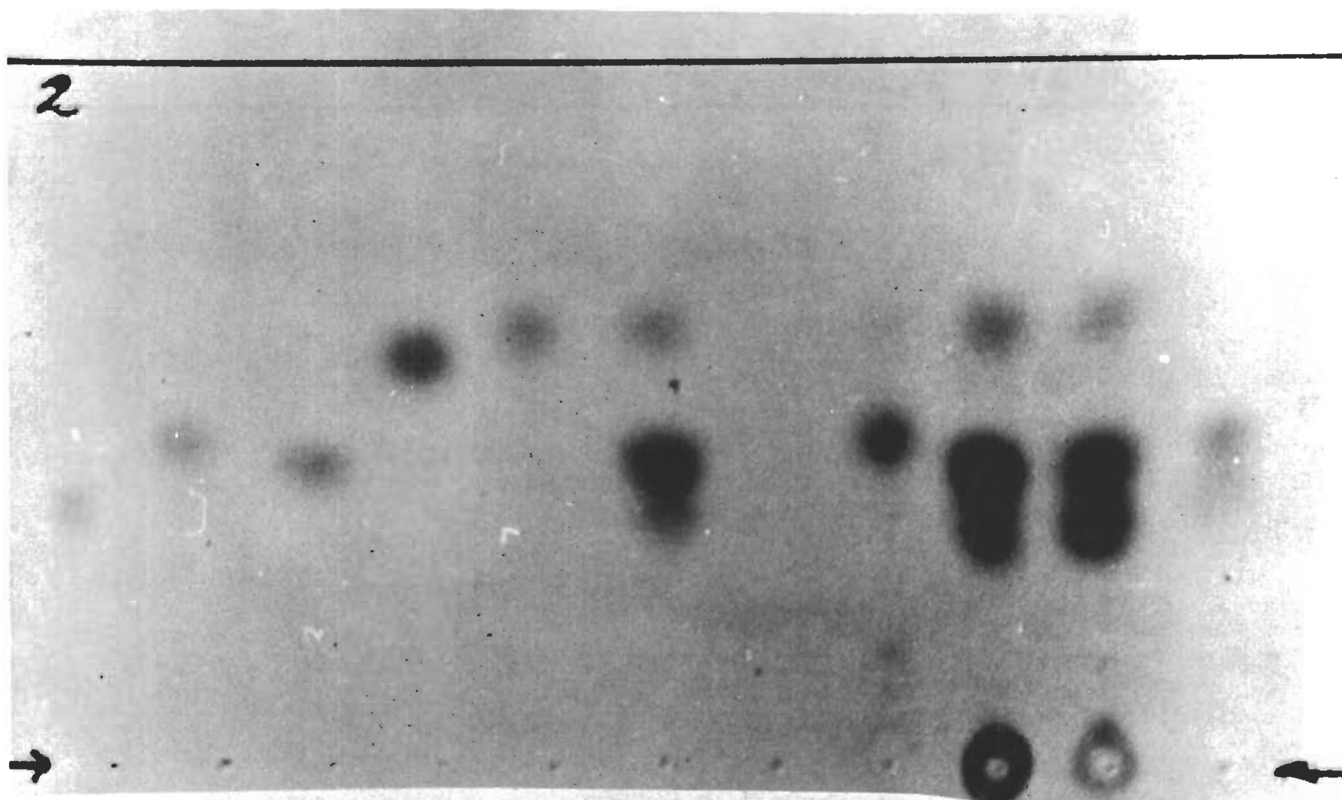

1 2 3 4 5 6 7 8 9 10 11

Column	Samples
1	Galactose - 3.5 μg
2	Glucose - 3.5 μg
3	Arabinose - 3.5 μg
4	Xylose - 3.5 μg
5	Rhamnose - 3.5 μg
6	Mixt. of rhamnose, glucose, arabinose and galactose - 3.5 μg each
7	Cell wall, insol. fraction; partial hydrolysis - 10 λ (lost)
8	Cell wall, sol. fraction; partial hydrolysis - 10 λ
9	Gum acacia, partial hydrolysis - 10 λ
10	Gum acacia, total hydrolysis - 10 λ
11	Arabinose and galactose - 3.5 μg each

Figure 6

TLC PLATE (NO. 2-46) OF MONOSACCHARIDES, CELL WALL FRACTIONS AND GUM ACACIA

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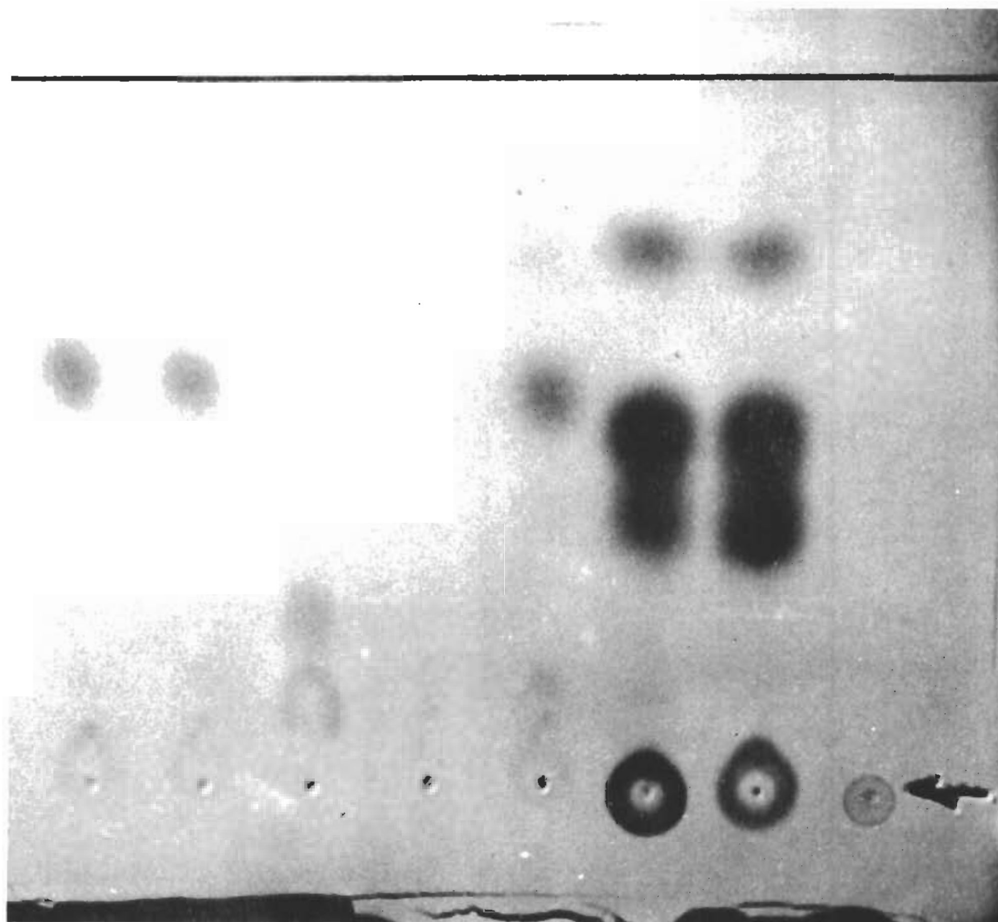

Column	Samples
4	Cell wall, sol. fraction, total hydrolysis - 5 λ
5	Cell wall, sol. fraction; partial hydrolysis - 5 λ
6	Maltose, lactose - 3.5 μg each
7	Cell wall, Insol. fraction; partial hydrolysis - 8 λ (lost)
8	Cell wall,sol. fraction; partial hydrolysis - 8 λ
9	Gum acacia, partial hydrolysis - 8 λ
10	Gum acacia, total hydrolysis - 8 λ
11	Gum acacia, non-hydrolyzed - 5 λ

Figure 7

TLC PLATE (NO. 6-46) OF DISACCHARIDES, CELL WALL FRACTIONS AND GUM ACACIA

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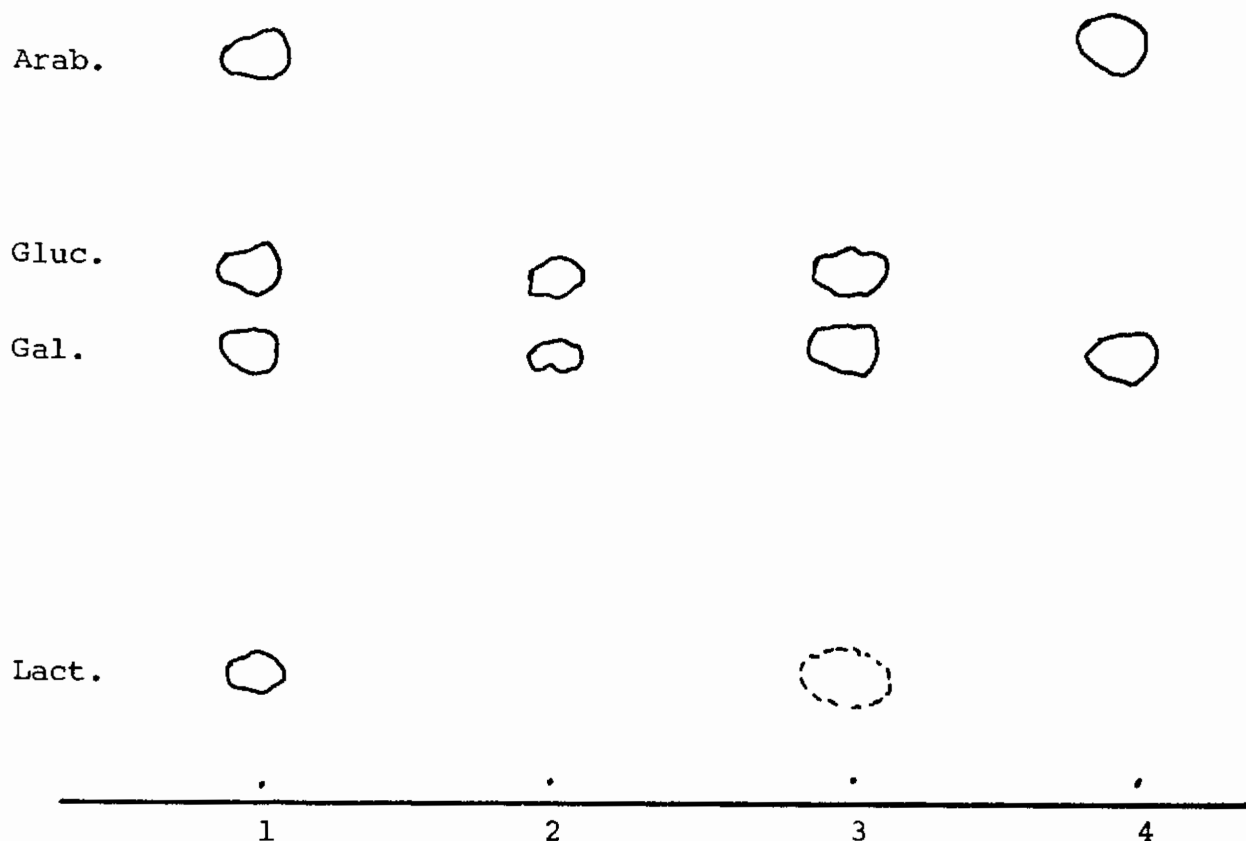

1 Mixture of standard sugars: arabinose, glucose, galactose, and lactose.

2 Cell wall, soluble fraction, total hydrolysis: glucose and galactose.

3 Cell wall, soluble fraction, partial hydrolysis: glucose, galactose, and lactose.

4 Gum acacia, total hydrolysis: arabinose and galactose.

Figure 8

DIAGRAM OF PAPER CHROMATOGRAM RESULTS REPRESENTING SUGARS* IN THE SOLUBLE FRACTION OF C. PYRENOIDOSA 7-11-05 CELL WALLS

[*Rhamnose not shown since it ran off paper in these trials.]

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The soluble fraction of the cell walls is the hemicellulose fraction (ref 10). Glucose and rhamnose were the predominant sugars determined by total and partial hydrolysis. A small but detectable amount of a disaccharide moving in the area of lactose was present after partial hydrolysis. In order to confirm the presence or absence of arabinose, a sample of gum arabic was hydrolyzed totally and partially. TLC showed the presence of arabinose, galactose, and rhamnose in decreasing order.

The insoluble fraction of the cell wall is the α-cellulose fraction (ref 10). Rhamnose was the predominant sugar, with other sugars present in lesser quantities. The amounts of sugars present were estimated from their staining intensity with aniline oxalate. Appropriate control sugars and equivalent concentrations were used.

C. Growth of Algae

Optimum heterotrophic growth was found to require light. This finding is in agreement with Sorokin (ref 13). The wet weight yield at 25°C with 75 foot-candles of light (room light) was 7.5 g/liter in contrast to only 1.5 g/liter at 37°C in the dark.

The first results of growing C. pyrenoidosa 7-11-05 in 14-liter fermentors are shown in Figure 9. After an initial lag period, the growth entered the exponential phase and proceeded at a rapid rate. After 48 to 50 hr, the exponential rate of growth ceased and the optical density of the suspension at 650 mμ remained constant. Addition of fresh sterile glucose or complete medium did not increase the growth. The total wet weight yield was 14 g/liter. By 50 hr, the culture was contaminated with B. subtilis.

Algae were grown in the fermentors at three light levels. Calculations of the growth curves during the exponential phase yielded the results shown in Table 4. Growth was not inhibited at any level of light. The results are shown graphically in Figure 10.

Table 4

EFFECT OF LIGHT ON GROWTH RATE OF C. PYRENOIDOSA 7-11-05

Fermentor	Light at Surface foot-candles	Rate of Growth per Hour, Optical Density Units
1	25 to 50	0.010
2	5000	0.036
3	10,000*	0.050

*Two 5000 foot-candle Tensor lamps at opposite sides.

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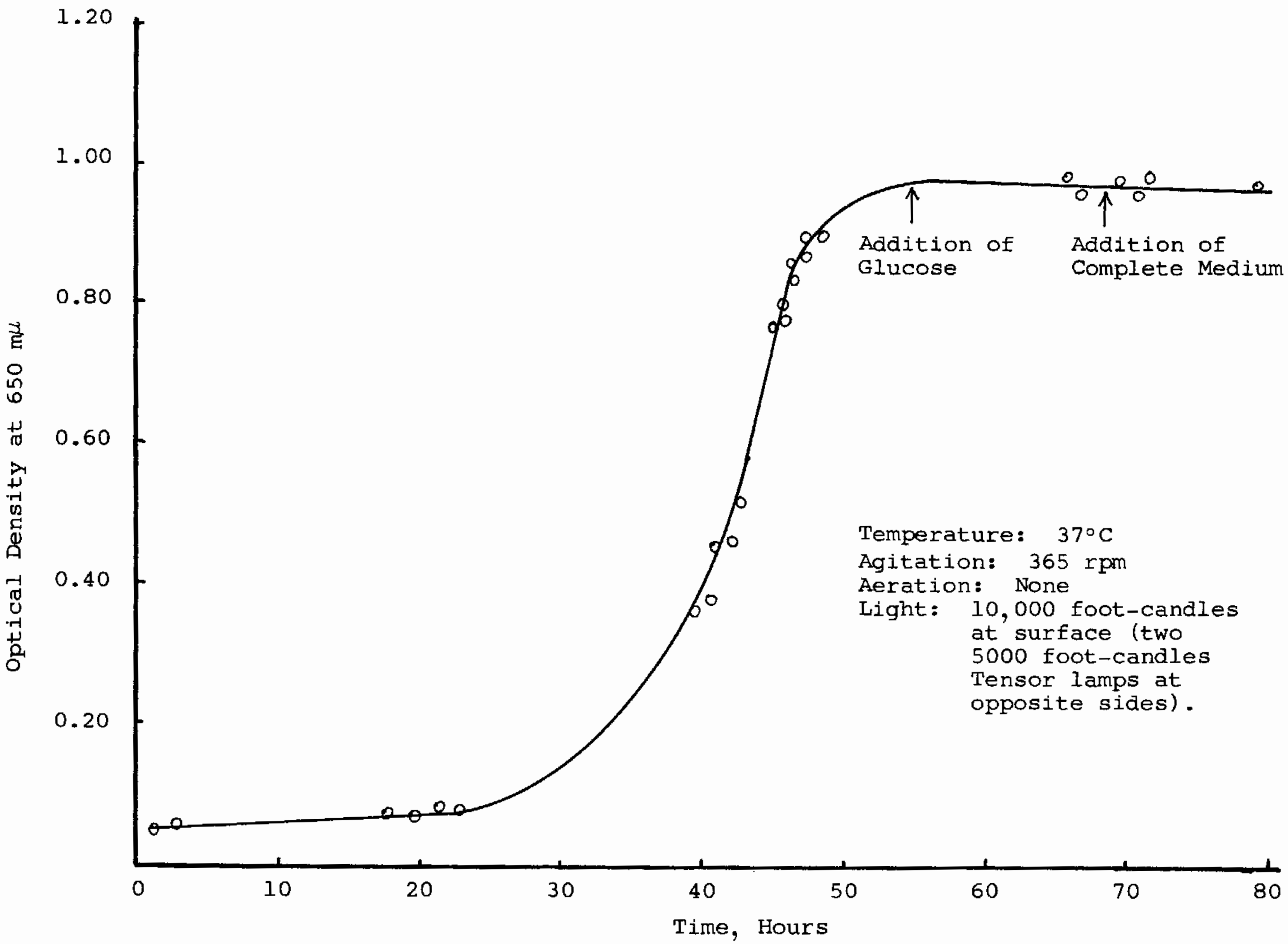

Figure 9

HETEROTROPHIC GROWTH OF <u>C. PYRENOIDOSA</u> 7-11-05

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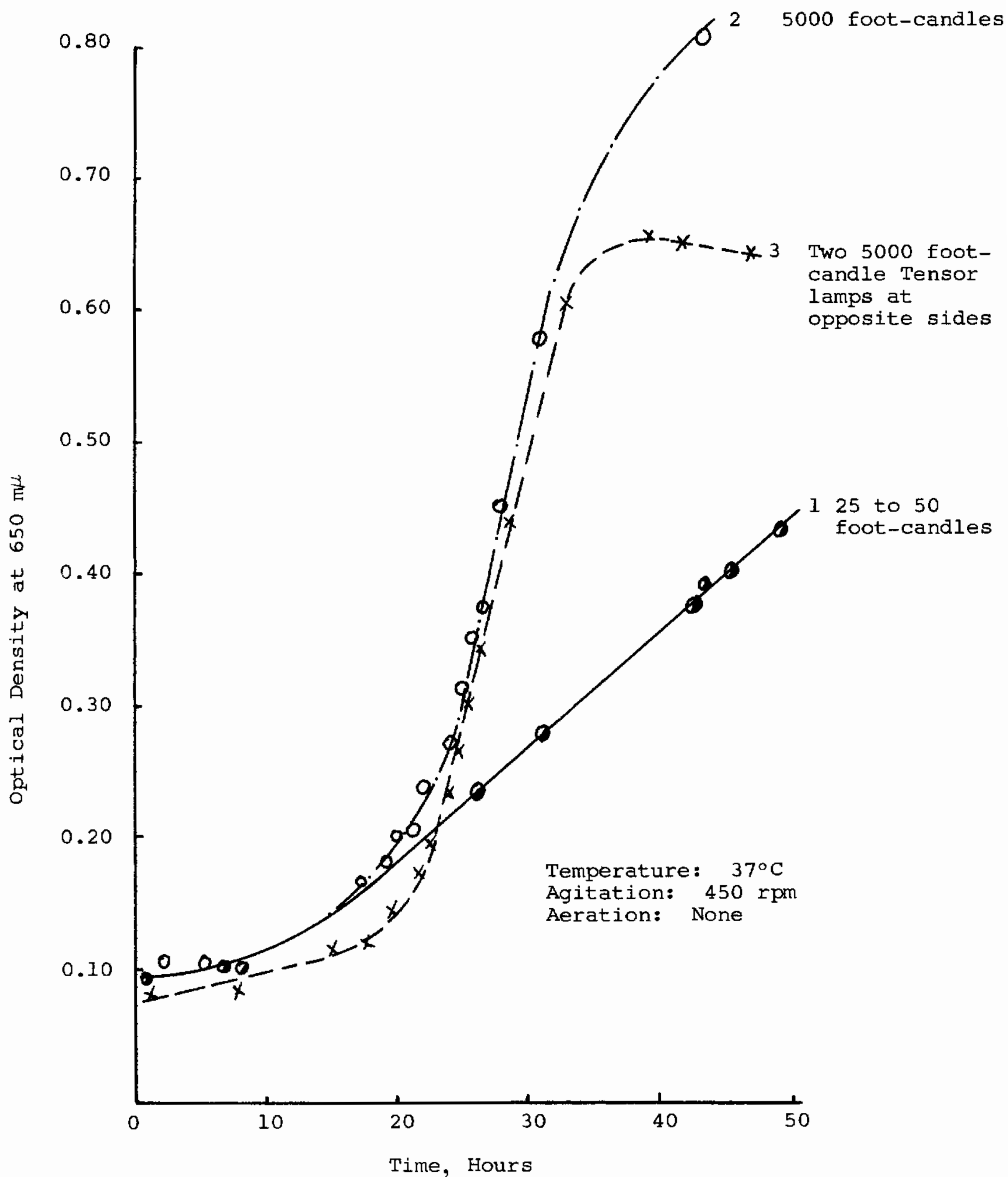

Figure 10

EFFECT OF LIGHT ON HETEROTROPHIC GROWTH
OF <u>C. PYRENOIDOSA</u> 7-11-05

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Autotrophic growth is shown in Figure 11. The culture medium had no glucose but was provided with continuous aeration and a relatively high light intensity. A slight initial increase in growth occurred, but there was no rapid growth until glucose was added, at about 67 hr, at which time a rapid increase of 0.064 optical density units per hour resulted. At the end of 92 hr, the wet weight yield was 17.5 g/liter. The use of a double concentration of each ingredient when glucose was present yielded the same growth curve as in Figure 9.

The effects of aeration and of media modifications are noted in Figure 12. Two fermentors were used at two light intensities without continuous aeration. At the lower intensity the initial growth rate was slower, otherwise both growth curves were similar. Addition of nitrate or phosphate did not influence the character of the curves.

The stepwise nature of both curves resembled a synchronous-type growth. The area on Figure 12 marked "day" is when samples were removed for determination of cell growth. It was concluded that the shape of the growth curves was due to the entry of sterile air under pressure before the samples were removed for growth assay. This conclusion was confirmed by the results found under continuous aeration as shown in Figure 13. The growth rate was constant regardless of when samples were taken. Figure 13 also shows the effect of adding Bacteriaban to the medium. In the presence of Bacteriaban the wet weight yield was 20 g/liter versus 7.5 g/liter in its absence. Further, contamination was practically eliminated.

Samples of freeze-dried and roller-drum-dried cells were inspected microscopically. Freeze-dried cells were considered superior to the roller-drum-dried cells because they had intact walls, less aggregation, and less cytoplasmic debris. The protein content of the final, dried cells was 53.13%, determined by the method of Willits (ref 16).

D. Growth of Fungi and of Combined Algae-Fungi

The liquid media described in Section IIID and in a basal yeast media* gave luxurious growth of *A. oryzae*, amounting to 20 to 25 g/liter. The protein content of the organism was 25 and 30 %, respectively.

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*Amber Laboratories, Milwaukee, Wis.

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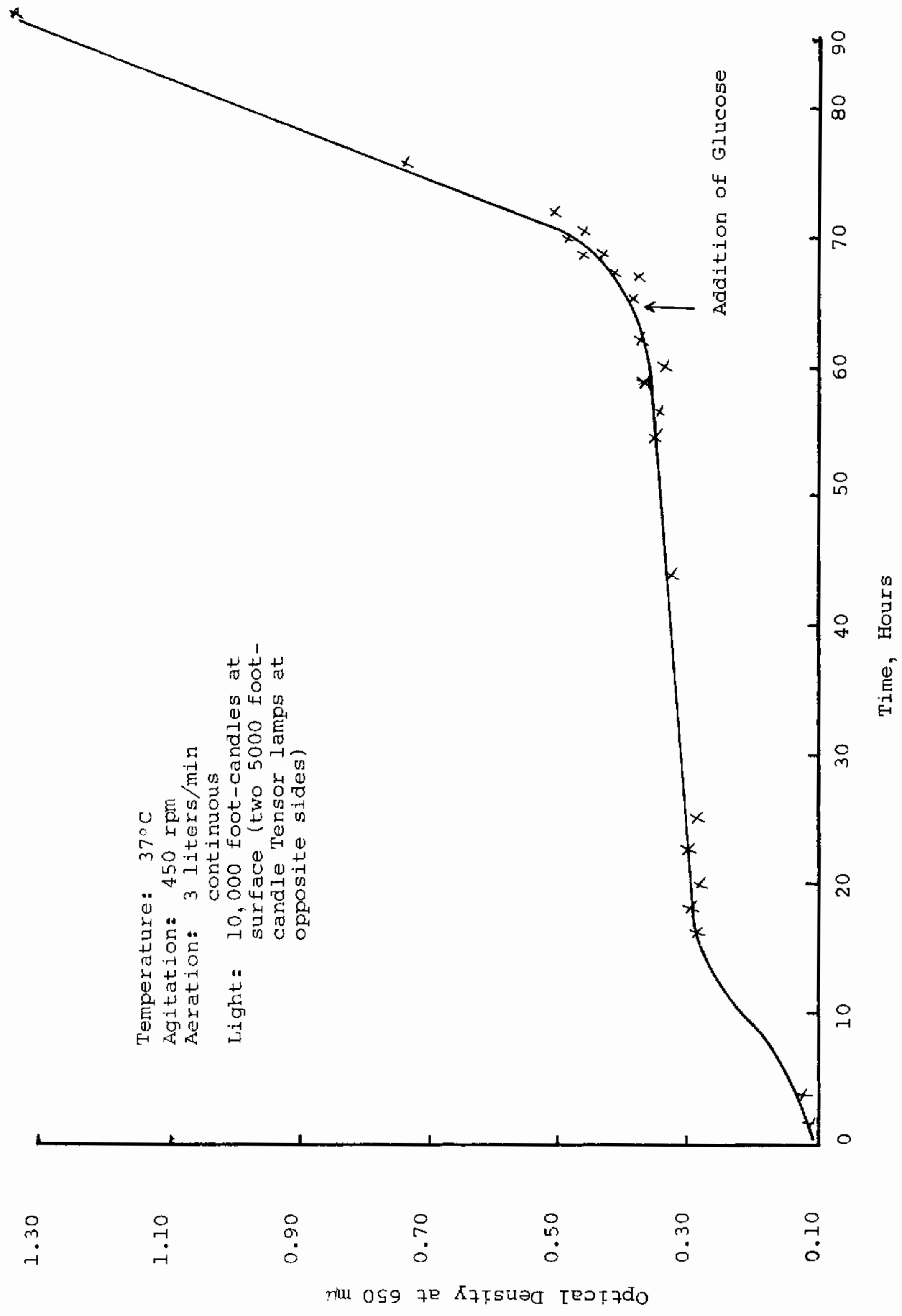

Figure 11

EFFECT OF GLUCOSE ON AUTOTROPHIC GROWTH OF C. PYRENOIDOSA 7-11-05

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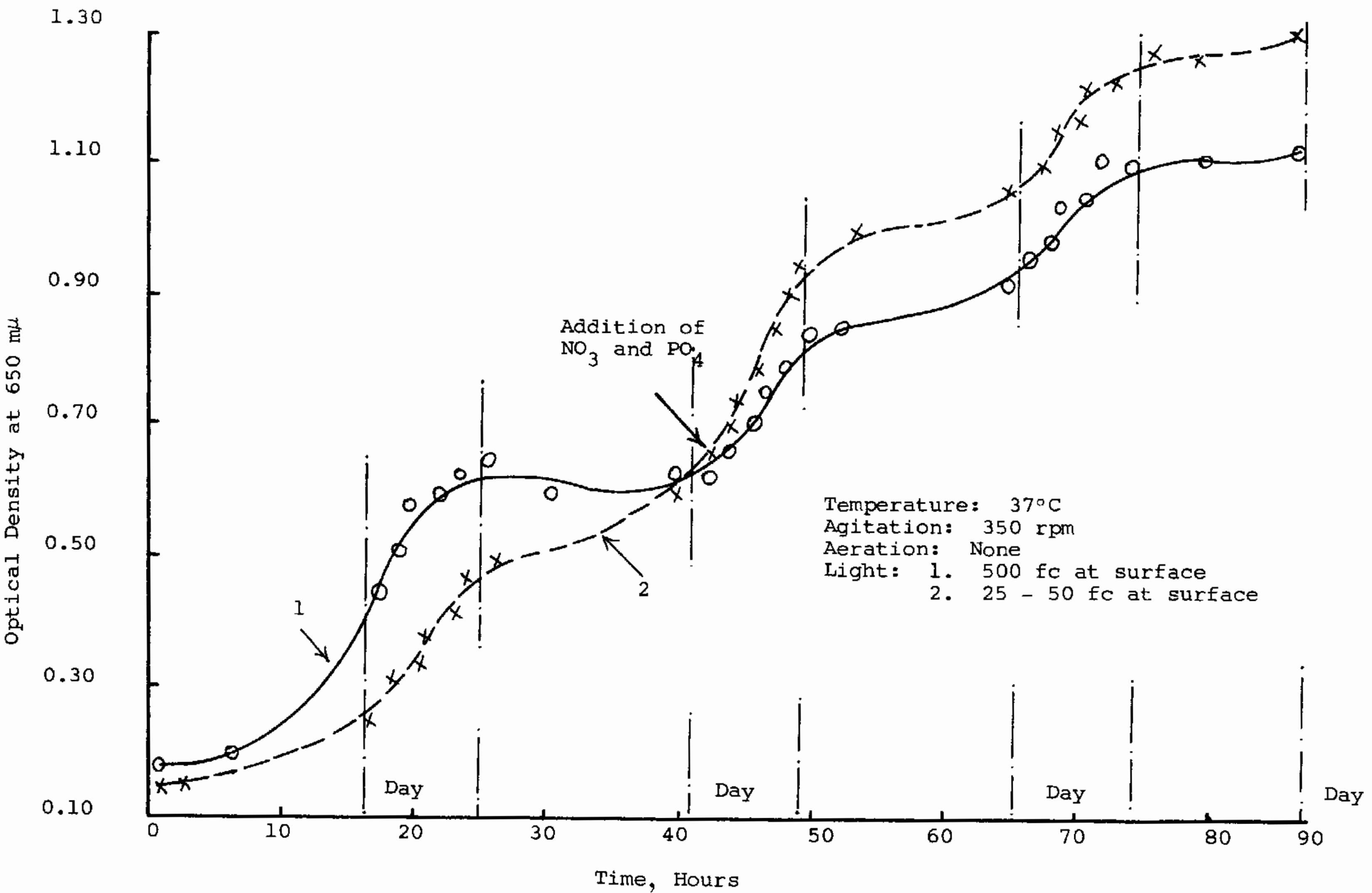

Figure 12

EFFECTS OF AERATION AND OF MEDIA MODIFICATION
ON GROWTH OF C. PYRENOIDOSA 7-11-05

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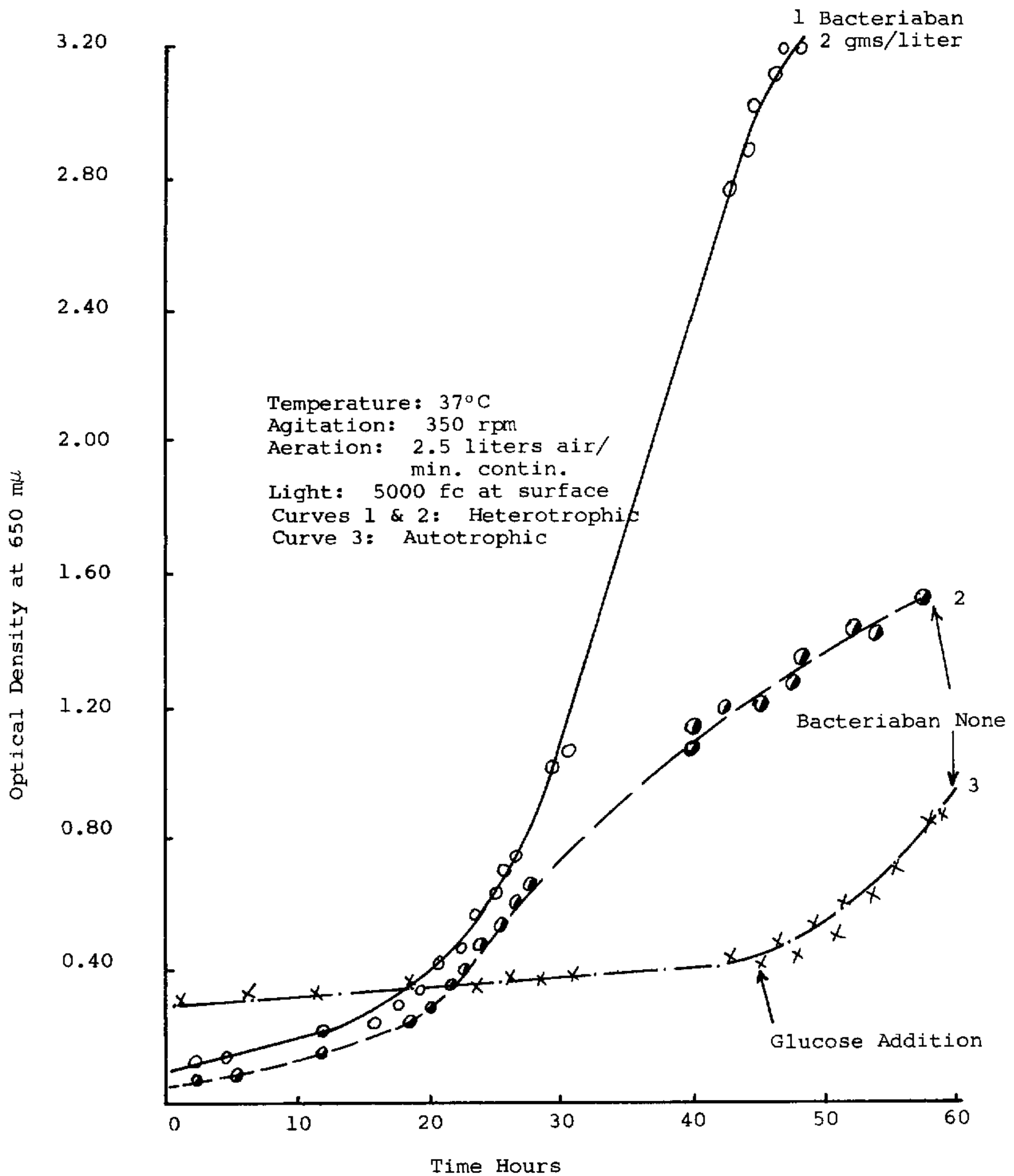

Figure 13

EFFECTS OF CONTINUOUS AERATION AND
OF BACTERIABAN ON GROWTH
OF <u>C. PYRENOIDOSA</u> 7-11-05

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Only general conclusions can be drawn from the preliminary studies on the combined growth of algae and fungi. Significant fungal growth was obtained when a sugar source was present to supplement the basal salts at either high or low levels of ammonium nitrate. The presence of the high concentration of sugar, of ammonium nitrate, and of salt nutrients in the same flask did not necessarily provide the best conditions for fungal growth. When sufficient nutrients were present, growth of the fungus was increased slightly by shaking the culture. Good growth of the fungus was generally accompanied by a sludgelike appearance of the algae and a clearing of areas of the algae. These preliminary observations will be used to determine optimum methods of growing approximately a 10-lb, dry weight, optimum mixture of algae-fungi.

V. CONCLUSIONS

Totally hydrolyzed cell walls of C. pyrenoidosa 7-11-05 were shown by chromatographic techniques to contain rhamnose, glucose, and galactose as the three major monosaccharides. Xylose and mannose were present in trace quantities. The totally and the partially hydrolyzed soluble cell-wall fractions contained glucose and rhamnose as the major monosaccharides and a trace of galactose. A trace of disaccharide, similar perhaps to lactose, was also present in the partially hydrolyzed fraction. The totally hydrolyzed insoluble cell-wall fraction contained rhamnose, galactose, and glucose as the major sugars. Arabinose appears to be absent from the cell wall and fractions thereof. The presence of glucose, rather than arabinose in fractions of 7-11-05 cell walls may make the cell walls more utilizable for human nutrition, if the walls are adequately hydrolyzed.

Relatively uncontaminated C. pyrenoidosa 7-11-05 cells were grown under heterotrophic conditions on a scale large enough to provide sufficient material for nutritional studies. Preliminary studies indicated that combined growth of A. oryzae - C. pyrenoidosa cells is feasible; wherein the fungus digests some of the algal cell wall.

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