PROTEINS FROM PHOTOSYNTHETIC BACTERIA

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CHAPTER I

GENERAL INTRODUCTION

THE IMPORTANCE OF RESEARCH IN THE AREA OF SINGLE-CELL PROTEIN

A growing concern for the acute food needs of the world's expanding population has led to the examination of a variety of unconventional food sources. A potentially important new food source is Single-Cell Protein (SCP). SCP has become, by definition, a generic term for crude or refined sources of protein whose origin is unicellular or simple multicellular organisms, i. e.: bacteria, yeasts, fungi, algae, and perhaps protozoa and even bacteriophages. Important advantages of SCP over conventional sources of protein are:

(1) Microorganisms are capable of rapid mass-production. Under optimal conditions, bacteria and some yeasts can double their mass 500 times faster than most agricultural crops and 1,000 - 5,000 times faster than most farm animals (Table 1-1).

(2) Microorganisms generally contain more protein than conventional protein-rich foods (Table 1-2). SCP provides an excellent source of proteins and B-vitamins which may be added to foods in significant amounts to improve their protein

TABLE 1-1

Maximum Rates of Mass Doubling (1)

Organism	Mass-doubling Time
bacteria and yeast	20-120 minutes
fungi and algae	2-6 hours
grass and some plants	1-2 weeks
poultry	2-4 weeks
swine	4-6 weeks
cattle	1-2 months
humans	3-6 months

TABLE 1-2

Proximate Composition Of Single-Cell Proteins And Other Protein Sources (2,3)

Type of Protein	Substrate	Protein %	Fat %	Carbohydrate %	Ash %
bacteria	carbohydrate	40-80	1-30	10-30	1-4
bacteria	n-paraffins	62-73	8-15	10	6-12
baker's yeast	carbohydrate	40-50	1-2	32-40	6-10
torula yeast	sulfite liquor	47	4-5	32	9
yeast	n-paraffins	44-54	10-19	22-26	9-10
yeast	gas-oil	40-50	1-2	12-20	6-12
algae	--	55-65	2-7	20-29	5-9
eggs	--	48.8	44.5	2.8	4.2
milk	--	26.9	30.0	37.7	5.4
wheat	--	13.6	1.5	84.1	1.4
corn	--	10.2	3.9	85.2	1.3
soybean meal	--	37-53	15-23	3-12	4-6
cottonseed meal	--	39-50	3.5-9.9	23.4-34.5	7.3-.5.5

value and supplement their vitamin content (Table 1-3).

(3) Microbial proteins normally contain significant amounts of the essential amino acids as shown in Table 1-4.

(4) SCP production does not depend upon agricultural or climatic conditions.

(5) The total land requirement for the production of SCP would be only a small fraction of that required for agricultural production. To further emphasize the importance of land requirement, A. E. Humphrey mentioned that it would be technologically possible to produce 150 million tons of protein, representing approximately fifty percent of the world's protein needs, in a facility requiring less than one square mile of land.

BACKGROUND INFORMATION ON THE DEVELOPMENT OF SINGLE-CELL PROTEIN

Historically, man has consumed microorganisms for centuries in alcoholic beverages, cheeses, yogurts, soya sauces and other fermented foods. As early as the fifth century B.C., Hippocrates was apparently aware of both the nutritional and curative properties of yeast. Spirulina maxima, a blue-green algae harvested from the surface of shallow ponds, has been used for centuries by peoples in the Chad Republic of Africa, and was probably used also by inhabitants of ancient Mexico. The first really extensive effort to manufacture

TABLE 1-3

Vitamin Content of SCP

Vitamin	Pseudomonas(4) µg/g	Candida(5) µg/g	Chlorella(6) µg/g	Torula(7) µg/g	Brewer's(7) µg/g
Thiamine(B_1)	2.88	5.6	6.28-23.2	5.3	50-360
Riboflavin(B_2)	10.50	44.8	21.0-43.5	45.0	36-42
Pyridoxine(B_6)	1.6	2.4	0.12-3.20	33.4	25-100
Niacin	180	146	105-176	417.3	320-1000
Pantothenic acid	7.0	111	3.17-9.3	37.2	100
Biotin	--	0.37	0.18-0.64	2.3	5-18
p-Aminobenzoic acid	--	--	4.10-25.1	17	9-102
Inositol	--	--	1510-2140	--	--
Folic acid	--	--	16.9-47.8	21.5	15-80
Choline	--	--	735-3020	--	--
Ascorbic acid(C)	--	100	475-3680	--	--
Vitamin D	--	1.2	--	--	--

TABLE 1-4

Essential Amino Acid Distribution in Various Types of Proteins (8)

Essential AminoAcid	Torula Yeast (g/16gN)	Brewer's Yeast (g/16gN)	Pseudomonas(9) (g/16gN)	Candida(10) (g/16gN)	Casein (g/16gN)	Soybean Meal (g/16gN)	Egg (g/16gN)
Lysine	7.9	4.5	4.3-9.9	8.1	8.1	6.6	6.4
Histidine	2.7	2.7	1.2-1.9	2.0	3.0	2.5	2.4
Arginine	7.2	4.9	4.7-5.0	4.2	4.1	7.2	6.6
Threonine	5.0	5.1	3.8-4.1	4.6	4.3	3.9	5.0
Valine	6.3	5.9	3.5-5.1	5.2	7.4	5.2	7.4
Methionine	2.4	3.0	0.8-1.3	1.3	3.5	1.1	5.5
Isoleucine	7.2	5.2	3.8-4.3	5.0	6.6	5.8	6.6
Leucine	8.0	7.0	6.6-6.7	6.9	10.2	7.6	8.8
Phenylal-anine	5.1	4.1	3.3-3.4	4.3	5.4	4.8	5.8
Tryptophan	1.4	1.5	0.9-1.2	0.9	1.3	1.2	1.6

microorganisms on a commercial scale for nutritional use occurred in Germany prior to the end of World War I. Yeasts commercially produced from waste sulfite liquor were used to supplement or replace other sources of protein in short supply and, during World War II, Germany actually incorporated as much as 16,000 tons of Torula yeasts annually into human foods (11).

Although yeasts have been used in food and animal feeds for many years, the potential of single cells as a source of protein has excited an extraordinary amount of industrial interest only during the last ten years. In 1967, the first international symposium devoted exclusively to the use of microorganisms as a means of meeting the demand for new sources of proteins was held at the Massachusetts Institute of Technology in Cambridge, Massachusetts. It was at this symposium that the generic name, "Single-Cell Protein" was adopted.

In June, 1971, the second meeting of the United Nations Protein Advisory Group on Single-Cell Protein convened in Moscow. In the series of meetings which followed, the ad hoc working group reviewed currently available information on yeasts, bacteria, fungi and algae; discussed aspects of general safety, nutritional evaluation of SCP, problems of nucleic acid toxicity, and methods for lowering the nucleic acid content of microbial cells; prepared guidelines for the production of SCP for human consumption; reviewed recent developments in the utilization of SCP in human foods and discussed potential uses of SCP

in food products.

In May, 1973, the Second International Conference on Single-Cell Protein was again held at M.I.T. Some of the major topics discussed at this recent symposium dealt with general process considerations such as fermentor design, process dynamics and control, and product recovery and purification; industrial processes utilizing agricultural by-products, petroleum fractions and petrochemicals; nutritional and toxicological studies; and finally, utilization and marketing considerations.

The future of SCP presently stands at a very critical point of development as a result of a number of large-scale ventures planned by governments and industrial organizations. The success or failure of these ventures will determine the future interest of a large number of groups and, ultimately, the impact that SCP will have on the world as a source of protein.

POTENTIAL ORGANISMS FOR SCP PRODUCTION

All classes of organisms should be considered as potential sources of protein, provided they are capable of meeting the criteria of safety, nutritive value and economics. Among the many factors involved in the choice of a suitable organism are: the nature of the available substrate, i. e., carbohydrates or hydrocarbons, in a particular locality where the microbial food is wanted; the growth rate of cells in the specific medium; the nature of the facilities required for

production; the ease of recovery, purification and processing; and the nature of the effluent as it relates to biochemical oxygen demand (BOD) and possible reutilization to avoid waste of nutrients. Organoleptic properties and feed and food uses are additional factors of vital importance. Desirable factors in considering a microorganism as a potential source of food are outlined in Table 1-5. Yeasts, bacteria, algae, and fungi are all under active consideration for pilot or commercial scale processes. Each, however, has its own relative advantages and disadvantages.

Yeasts. There are a number of classical and current processes for the production of SCP for food or animal feed from waste sources of carbohydrate such as beet and cane molasses, whey, starches and waste sulfite liquor (13-17). For many years, yeasts commercially-grown on these materials have been used as food flavoring agents and, in animal feeding, have been used as supplemental sources of vitamins and proteins. The development of yeasts capable of growing on various petroleum fractions now greatly expands the opportunities for increasing annual production (18,19). In addition to having reasonably high protein content, yeasts are easier to recover from fermentation media than are bacteria. They also have the advantage of being currently available in the open market since they are produced from a variety of substrates which are recognized as sources of food themselves.

Bacteria. From the ancient arts of brewing and baking to the modern fermentation processes for the production of various chemicals,

TABLE 1-5

Requirements for a Food Microorganism (12)

(I) Technical

(1) Rapid growth
(2) Simple media
(3) Submerged culture
(4) Simple separation
(5) Freedom from infection - stable fermentation
(6) Efficient utilization of energy source
(7) Disposable effluent

(II) Physiological and Organoleptic

(8) Capable of genetic modification
(9) Nontoxic
(10) Good taste
(11) Highly digestible
(12) High nutrient content
(13) Protein, fat, and carbohydrate content of high quality

vitamins, antibiotics and enzymes, extensive technological information is available concerning the mass-cultivation of bacteria (20, 21). Although the number of bacterial species that have been grown specifically for food purposes is limited, bacteria possess a number of advantages over other organisms. Bacteria generally have higher growth rates (Table 1-6) and much higher protein contents (Table 1-7) than most other types of microorganisms. They have the disadvantages of being susceptible to phage, having a higher content of nucleic acids, and are not well-known in human nutritional experience.

Algae. Algae represent additional sources of edible proteins which recently have been considered for production on an industrial scale (24-27). Algae are perhaps the most attractive form of microbial food on account of their photosynthetic ability which eliminates the provision of a carbon substrate. However, the tenacity of algal cell walls impose limitations on the digestibility and utilization of algal proteins. The primary limitations, in addition to palatability, appear to be economic factors connected with capital investment; land availability; local temperature and sunlight conditions; and the desirability of having a natural source of bicarbonate-rich water.

Fungi. The higher fungi have received little attention as potential protein sources because their generation time was thought to be too slow and no procedure had been devised to produce them in continuous culture. Recent work with mold cultures, however, indicates the

TABLE 1-6

Specific Growth Rate of Various Microorganisms (22)

Organism	Specific Growth Rate (day^{-1})
Bacteria:	
Escherichia coli	59.7
Pseudomonas flourescens	29.9
Azotobacter chroococcum	13.8
Yeast:	
Hansenula anomala	13.8
Algae:	
Chlorella pyrenoidosa	1.96
Chlorella vulgaris	1.13
Scenedesmus quadricauda	2.03
Scenedesmus costulus	1.05
Euglena gracilis	1.38

TABLE 1-7

Protein Contents of Microorganisms (23)

Organism	Protein Content (% dry weight)
Bacteria:	
Lactobacillus fermentans	87
Alcaligenes viscosus	84
Escherichia coli	82
Cornebacterium diphtheriae	81
Lactobacillus casei	47
Yeasts:	
Saccharomyces cerevisiae	56
Hansenula suaveoleus	53
Candida utilia	53
Candida arborea	46
Algae:	
Chlorella pyrenoidosa	44
Chlorella vulgaris	24-41

possibility of successful growth in continuous culture on inexpensive carbohydrate waste sources (28, 29). Molds as a group offer promise as sources of protein for the following reasons: the size and mycelial character of fungi facilitate their recovery from fermentation media; the microfungi include a wide variety of organisms with versatile metabolic properties so that they may be cultured on a variety of different substrates; fungi have been used in the preparation of certain home-processed foods eaten in some parts of the world; the filamentous nature of fungi should permit them to be manufactured into structured foods with a minimum of processing and to be used in simulated meat products. Although fungi might be of interest because of their potent enzyme capabilities and the ease of harvesting them from fermentation media, their nutritional adequacy remains to be proven because of their relatively thick and possibly indigestible cell wall structures.

CRITICAL FACTORS IN SCP PRODUCTION

The future of SCP as a useful source of protein as well as its ability to compete with more conventional sources of protein is dependent on three critical factors; (1) overall cost; (2) nutritional value, as reflected in its amino-acid profile and biological value; and (3) acceptability in terms of flavor, aroma, color and texture.

A listing of important factors which contribute to the economics of SCP production is given in Table 1-8. Although major factors consist

TABLE 1-8

Important Factors Contributing to the Economics

Of SCP Production (30)

(1) Raw material
   (a) ease of collection, delivery and storage
   (b) availability - seasonal fluctuations
   (c) bulk handling properties

(2) Sterility requirement

(3) Fermentation
   (a) dilution rate
   (b) maximum productivity
   (c) operating temperature (cooling water vs. refrigeration)
   (d) total oxygen requirement
   (e) power requirements for mass transfer
   (f) heat of reaction
   (g) foaming tendency

(4) Cell harvesting techniques
   high speed centrifuges vs. flocculating agents, separation vessels and filters.

(5) Washing and purification techniques for the removal of:
   (a) substrate residues
   (b) raw material impurities
   (c) nucleic acids
   (d) metabolic by-products

(6) Processing SCP
   (a) spray drying vs. drum drying
   (b) microbial cells vs. cell isolates
   (c) extrusion cooking

(7) Product value
   (a) protein content
   (b) amino acid profile
   (c) digestibility
   (d) acceptability in terms of flavor, aroma, color and texture

primarily of capital investment, raw material or substrate costs, operational costs, quality control, market development, and marketing costs, the most crucial of these are raw material costs and operational costs. In fermentations employing crude carbohydrates as substrates, raw material or substrate costs contribute as much as fifty to sixty percent to the total SCP production cost. In typical hydrocarbon fermentations, operational costs may exceed fifty percent of the total production cost.

POTENTIAL SUBSTRATES FOR SCP PRODUCTION

The number of possible substrates for producing SCP is as large as the number of species used for their digestion (Table 1-9). Sources of substrates for the production of potentially nutritive microorganisms include: food and agricultural products such as whey and starch; industrial by-products such as cellulosic fiber and sulfite waste liquor; hydrocarbons such as refined petroleum fractions and natural gas; alcohols; and recycled animal wastes. Raw materials such as sulfite waste liquor, whey, molasses and various sources of starch may be used to produce microbial proteins by conventional fermentation technology. Significant attention, in terms of world interest, has been drawn to processes which utilize hydrocarbons or derivatives thereof as the sole energy source for the production of SCP. The development of processes for growing microorganisms on various petroleum factions would greatly expand opportunities for increasing the world's food supply. Recommended substrates for applications involving human food and animal feed

TABLE 1-9

Potential Substrates for the Production of SCP (31)

(1) Food and agricultural products - beet and cane molasses; corn molasses; cull fruits; cereal grains; cereal starches; potato starch wastes; whey; fish meal; soybean, cottonseed, linseed and peanut oil meals; peptones; yeast autolysates

(2) Industrial by-products - waste sulfite liquor; corn steep liquor; cannery wastes; fruit wastes; distiller's solubles; milling wastes; tankage; slaughter and packing wastes

(3) Cellulosic wastes - wood wastes; corn cobs; oat hulls; bagasse; straw; livestock wastes

(4) Hydrocarbons - n-alkanes; n-paraffins; crude oil fractions; gas oil; natural gas (methane); flue gas

(5) Alcohols - methanol; ethanol

(6) Sewage - domestic sewage; recycled animal wastes; feedlot run-off

production are shown in Table 1-10.

Carbohydrates versus Hydrocarbons as Potential Substrates for SCP Production

For some time there has been active discussion as to the relative merits of carbohydrates versus hydrocarbons as potential carbon and energy sources for the production of microbial protein concentrates. The feasibility of either hydrocarbons or carbohydrates as substrates is largely governed by the cost associated with the substrate, local conditions and the size of the local market. In certain areas throughout the world, molasses and other sources of crude sugars are available at a cost often less than three cents per pound, while in many Middle Eastern countries, petroleum fractions are available at extremely low cost while carbohydrates and water are scarce. In many developing countries, there are large quantities of crude carbohydrate materials such as cassava and fruit and vegetable wastes, which are not presently fully utilized, and which could be available at low and even negative costs (waste disposal) for fermentation processes; however, the costs of collecting the wastes and converting them to a form suitable for microbiological utilization may make their use unattractive.

SCP from Cellulose

One carbohydrate source which is ubiquitous in nature and of potential interest in SCP production is cellulose (33-35). The major

TABLE 1-10

Recommended Substrates for SCP Production (32)

(1) Substrates for human food consumption

- sugars
- starches
- ethanol
- natural gas
- whey and other agricultural wastes
- molasses and other sugar industry wastes

(2) Substrates with potential for human food production

- methanol
- wood hydrolysates

(3) Substrates for animal feed production

- crude oil fractions
- n-paraffins
- domestic sewage and recycled animal wastes

problem associated with utilizing this material for the growth of microorganisms is the initial conversion of cellulose to metabolizible sugars. Presently it is not economically feasible to convert this material at costs competitive with other more readily available fermentable carbohydrates.

Critical Factors in SCP Production from Hydrocarbons

Petroleum fractions used as substrates in typical hydrocarbon fermentations are generally mixtures of various hydrocarbon components which are relatively inexpensive. Purified hydrocarbon compounds or refined hydrocarbons are generally more costly (Table 1-11). The distinct advantages of deriving microbial proteins from hydrocarbon fractions are lower raw material costs and comparatively greater yields than most carbohydrates. However, raw material cost advantages must be balanced against greater operational costs, greater cell-product separation difficulties, and higher capital investments. SCP production from hydrocarbons represents a possible solution for alleviating the world's food shortage; however, it is not without its serious problems. Major problems which contribute to the operational costs of hydrocarbon fermentation include:

(1) an excessive oxygenation requirement combined with problems in oxygen transfer:

Oxygen constitutes approximately twenty to thirty percent of the cell mass (see Table 1-12). Therefore, molecular

TABLE I-II

Costs of Hydrocarbon Substrates (36)

Material	Cost (¢/lb)*
Refined hydrocarbons (n-alkanes)	2.0-4.0
Partially refined hydrocarbons	1.0-2.0
Gas oil (20-35% n-alkanes)	0.5-1.0
Crude oil	1.0
Natural gas (methane)	0.2-0.4
Flue gas	0.4
Methanol	1.5-4.0
Ethanol	3.0

*Figures based on costs in 1969.

TABLE 1-12

Composition of Microbial Cell Mass (37)

Element	Bacteria (% composition)	Yeasts (% composition)
Carbon	57	47
Hydrogen	7.3	6.5
Oxygen	19	31
Nitrogen	12	7.5
Ash	8	8

oxygen must be supplied in order for the cells to grow. In addition, the oxygen requirement for hydrocarbon substrates is roughly three to five times higher than for carbohydrates as shown in Table 1-13. The power requirement associated with higher agitation rates along with the operation of high pressure aeration pumps add a considerable burden to the production cost.

(2) an excessive heat of fermentation:

Fermentation generates considerable heat that must be disposed of in order to maintain optimum temperatures for growth. The amount of heat generated during fermentation is largely dependent upon the substrate employed (see Table 1-14). Unfortunately, hydrocarbon fermentation generates much more heat than carbohydrate fermentation. Mechanical refrigeration employed to remove excess heat generated may contribute as much as fifteen percent to the total operational cost (40).

(3) problems related to hydrocarbon transfer:

The cultivation of microorganisms on liquid hydrocarbons introduces a relatively different type of problem not encountered with other substrates. Liquid hydrocarbons are relatively insoluble and tend to form tiny oil droplets in an aqueous solution. Microbial growth is generally limited to the surface of these oil droplets, thus creating problems in efficient oxygen

TABLE 1-13

Oxygen Transfer Rates for SCP Production (38)

Substrate	Doubling Time (hr)	Cell Concentration (g. cells/liter)	O_2 Required (gO_2/g cell)	O_2 Transfer Rate (g. mole O_2/liter·hr)
Carbohydrate	3	15	1.0	108
Methane	3	15	5.0	540
n-Alkanes	3	15	3.3	356

TABLE 1-14

Heat of Evolution for SCP Production on Various Substrates (39)

Substrate	Yield on Substrate (g cell/g substrate)	Heat of Fermentation (K cal/100 g cell)	Rate of Heat Evolution*	
			K cal/hr	Btu/gal•hr
Carbohydrate	0.5	380	13.2	198
n-Alkanes	1.0	780	27.0	405
Methane	0.6	1,860	64.3	965

* Doubling time = 3 hr.; cell concentration = 15g/liter

transfer and, most importantly, oil dispersion.

(4) problems existing with recovery and purification of cells: The presence of unused hydrocarbons closely associated with cellular membranes greatly hinders normal separation and purification procedures. If purified n-alkanes are used, unassimilated hydrocarbons associated with the cells must be removed by successive centrifugation followed by washings with hot water. Gas-oil substrates, however, require the most difficult recovery and purification procedures. Gas-oil fractions contain certain polynuclear aromatic compounds, several of which are known carcinogens, which must be completely removed in order to ensure a wholesome and safe product for use in either animal or human food supplements (41).

POTENTIAL FOOD USES FOR SCP

SCP functions as an excellent source of proteins and B-vitamins which may be added to foods in significant amounts to improve their protein value and fortify their vitamin content. Processed SCP of rather neutral color and bland taste has been successfully incorporated into a variety of food products such as breads, pastries, macaroni, dehydrated soups, sauces, sandwich spreads, reconstitutable beverages and imitation dairy products. The addition of SCP has resulted in substantial improvement of the nutritive value of these products without detriment to taste or texture (42-44).

As a potential food in itself, certain single-cell proteins are capable of a variety of textural modifications, including chewy and/or crispy structures. In this application, cell walls are broken, the soluble proteins are extracted and spun into protein fibers (45-48).

It should be recognized that SCP will be economically feasible only when food products can be prepared which are suitable for introduction into industrialized countries. This may require further processing of a crude SCP material into other products with more desirable functional properties. The number of potential food uses would seem to be limited only by imagination, safety and economics.

IMPORTANT CONSIDERATIONS RELATING TO THE ACCEPTANCE OF SCP

If microbial proteins are to contribute to man's food supply, two main criteria must be considered:

(1) There must be sufficient standardization and reproducibility of production technology to ensure a biomass with constant properties.

(2) There must be no toxic substances in the biomass which could in any way adversely affect human or animal health.

In the development of microbial proteins intended for human or animal consumption, it is essential that the resulting food product have the same nutritional quality, safety, and acceptability as conventional food sources. In commercial products, chemical and biological

properties must be the same as in control products used to determine the safety of the material. There must be assurance that consumption of the resulting food products will not lead to any adverse effects on metabolic processes and health including carcinogenesis, leucogenesis, mutagenesis, allergenicity or embryotoxic effects (49).

The operations following cell production should be closely monitored to ensure the required product quality and uniformity. Although the nature of these operations vary according to the particular process, cell washing and/or extraction and cell drying significantly influence the flavor, acceptability and nutritional value of SCP. Production facilities and SCP processing equipment should be of sanitary design. Special care should be exercised in all aspects of processing including raw material selection, quality control, sanitation, handling and packaging.

Safety Evaluation. There is adequate evidence which indicates that certain species of yeasts, algae and bacteria can be safe and useful sources of proteins, vitamins and minerals for animal and human consumption. The safety of such materials depends on the organism selected, the quality of the substrates utilized, and the conditions for growth. Whether or not a product continues to be safe depends on an extremely careful control of all factors involved in cell production and can be ensured only by a rigid program of frequent microbiological and biochemical analytical control and periodic biological re-evaluation of

the material. Any proposed use of SCP for human consumption must be preceded by extensive and properly designed toxicological testing in several species of experimental animals for sufficiently long periods of time to detect cumulative toxicity and the presence of carcinogens. Even the use of SCP in the feeding of domestic animals should be preceded by such tests in experimental animals. There must be adequate evidence showing that the feeding of SCP to domestic animals does not render unsafe the consumption of such animals or animal products such as eggs or milk (50).

Nutritional Evaluation. There are several important reasons for determining the nutritional value of the final food products containing SCP. Differences in processing procedures such as vacuum drying versus roller drying profoundly influence the acceptability as well as the nutritive value of SCP. The proteins of all SCP are susceptible to heat damage or to alteration by other improper processing procedures which may drastically reduce the nutritional value of such products. The nutritional quality of SCP is generally determined by the estimation of digestibility, biological value, and either net protein utilization or protein efficiency ratio in animals and by nitrogen balance studies in man. SCP generally contains nutrients other than protein and should be analyzed for these as well. The nucleic acid content, specific peculiarities of cell wall lipids and other unusual components must be taken into consideration when evaluating the nutritional value of SCP (51, 52).

Composition.

(1) Protein. Crude protein should be determined by N analysis (53). However, a constant conversion factor, i. e., N x 6.25, cannot be applied to total N to estimate true protein because some N-containing compounds such as nucleic acids, amines, urea and ammonium citrate may not contribute to actual protein content. The complete spectrum of amino acids should be determined in the product as produced under conditions comparable to those of commercial operation. The ratio of essential amino acids to total amino acids should be calculated.

(2) Nucleic Acids. Nucleic acids are generally not regarded as toxic substances but, rather, physiological ones with certain tolerance limits. Apart from toxicological considerations, there is a limit to the amount of nucleic acids which should be introduced into human diets since nucleic acid purines are excreted by man primarily as uric acid. A few susceptible individuals have a genetic tendency to overproduce uric acid and still a smaller number have renal defects in eliminating uric acids. In these individuals, elevated serum levels of uric acid increase the risk of gout and increased urinary concentrations of uric acid may result in the formation of kidney stones. Currently available information (54) suggests that there should be a limit of two grams per day on the amount of nucleic acid introduced by SCP into the diet of an adult and correspondingly less by weight for children. This

is the amount contained in approximately ten to thirty grams of microbial cells harvested in the logarithmic growth phase without special processing to lower the nucleic acid content. The use of larger quantities of SCP will require a reduction in the nucleic acid content by any of several methods available.

Ribosomal RNA comprises the greater part of the nucleic acid content of microbial cells. Therefore, the problem of lowering the nucleic acid content mainly becomes a problem of reducing the amount of ribosomal RNA in microbial cells. Several methods exist for the removal of RNA from intact cells without excessive loss of cell protein (55, 56). Regulation of culture conditions can be combined with the physical extraction of RNA with organic solvents or buffer solutions. Hydrolysis under acidic or basic conditions may also be useful. Although there are effective plasmolytic methods for isolating highly polymerized RNA, their suitability for food production is complicated by difficulties encountered in removing all traces of the extracting medium.

Other methods involve the activation of specific enzymes (ribonucleases) which hydrolyze RNA. One method described in a Japanese patent involves the use of high concentrations of phosphate salts (0.5M) to activate endogenous ribonucleases. Another method extensively developed at M.I.T. involves a specific heat-shock treatment of intact cells to activate endogenous ribonucleases (57,58). A third method, also developed at M.I.T., employs a commercial ribonuclease to treat

either cells or cell isolates. The cells are heated to make them permeable to the added enzyme, then incubated at a temperature which causes breakdown of the RNA to nucleotides. These nucleotides leak through the cell membrane and are washed free from the remaining cellular material.

(3) Lipids. Extracted lipid materials should be analyzed for the presence and content of triglycerides, steroids and phospholipids. Fatty acid profiles should be determined by gas chromatography, with special reference to the presence and identification of fatty acids of unusual structure.

Quality Control. In addition to quality control procedures associated with factors listed under "Composition," particular attention must be directed towards maintaining the integrity of the original strain of the microorganism. Appropriate microbiological and biochemical routines should be performed in order to demonstrate the stability of the organism and the absence of contaminants.

AREAS OF SCP PRODUCTION REQUIRING ADDITIONAL TECHNOLOGICAL RESEARCH

A few specific areas of single-cell protein production requiring further technological research are as follows:

(1) Elaboration of Standards. It is essential to determine standards for concentrations of materials in cells such as hydrocarbons,

nucleic acids, proteins, minerals, etc. At this time, insufficient information is available to set these standards; research is required to define acceptable limits for concentrations of important cell components as well as standard methods for analysis.

(2) Continued Research on the Selection and Testing of New Strains of Micro-Organisms for Possible Food and Feed Use. Growth characteristics, safety and nutritional value will be major considerations.

(3) Studies of the Effects of Dietary Nucleic Acids. Additional information must be obtained concerning safe plasma and urinary uric acid levels in normal individuals consuming various amounts of nucleic acids contained in SCP. Additional information must also be learned about the true frequency of genetic tendencies to gout in various human populations so as to more realistically appraise the extent of this problem in relation to increased nucleic acid ingestion in the form of SCP.

(4) Methods for Reducing the Nucleic Acid Content of SCP. Methods for reducing the nucleic acid levels in SCP may be accomplished by the control of fermentation procedures by physical or chemical means, or by the use of endogenous ribonucleases. Procedures for such a process may consist of a combination of methods, however, additional research is necessary to make these procedures generally more applicable and more effective.

(5) Cell Purification Procedures. It may be necessary to assure removal of components of the spent fermentation medium (hydro-

carbons, surface active agents, minerals, microbial metabolites, etc.) from the final product. No single procedure, however, would be adequate for all production methods. Purification processes could include solvent extraction for removal of lipids, solvent traces and residues, followed by washing and drying.

(6) SCP Isolates. It is desirable to improve methodology of production of protein isolates from SCP to have a more pure and more concentrated source of protein. The biological properties of these isolates should be investigated to supplement the testing of SCP per se.

(7) Application of SCP to Nutritional Needs. The addition of SCP to foods and diets at levels high enough to improve protein quality will require further food technological research. The optimum methodology for addition to a wide variety of foods must be determined, and the optimum method may vary from one food to another.

The Future of Edible Proteins Derived from Microorganisms

If single-cell proteins are to be used to supplement human foods or animal rations, the cost of production must be competitive with conventional food sources. The future of SCP, therefore, depends primarily on the economical solution of many of the aforementioned problems associated with cultivation, recovery, purification, processing and marketing. Once these solutions are at hand, SCP production can become a giant step towards relieving the world's hunger and malnutrition.

OBJECTIVES OF THIS RESEARCH

For nearly two decades, intensive research efforts have been directed toward the utilization of single-cell proteins derived from the mass-culture of algae, yeasts, bacteria and fungi as a source of edible protein. Man has consumed microorganisms such as photosynthetic algae for many centuries. Controlled large-scale production of microalgae is currently underway at many facilities throughout the world. There is much information which can be found in the literature concerning the large-scale production of algae. However, information relating to the mass-cultivation of photosynthetic bacteria is virtually non-existent.

The major concern of this research was to determine the feasibility of producing single-cell proteins from photosynthetic bacteria (The second chapter of this work is devoted to a review of photosynthetic bacteria). A marine strain of Rhodopseudomonas gelatinosa (family, Athiorhodaceae), isolated from sea water samples obtained near the Keelung Harbor in Northern Taiwan, was used in these investigations.

In the initial phase of investigations, numerous batch scale experiments were conducted in order to determine the effects of temperature, pH, salt concentration, substrate concentration, etc., on the batch growth of the organism. Results of these experiments are contained in Chapter III. A variety of readily-available, inexpensive raw materials (e. g., raw molasses, corn starch, potato starch waste, wheat

bran) was examined as potential substrates for the mass-cultivation of Rps. gelatinosa. Once a suitable substrate material was selected, additional experiments were conducted and biochemical analyses were performed on the harvested cellular products.

The second phase of investigations was concerned with the continuous cultivation of Rps. gelatinosa in a specially modified laboratory-scale bench fermentor; dilution rate, agitation, pH, temperature and illumination were carefully monitored. This phase is explained in Chapter IV. Routine analyses to measure cell concentration (optical density) and substrate concentration were performed in order to determine the effects of dilution rate on the growth of the organism. Harvested cellular material obtained throughout the duration of the continuous cultivation experiments was analyzed for protein, amino acids, and nucleic acids. Results of these analyses are necessary not only in evaluating the quality of the resulting product, but also in determining any effects of dilution rate on the protein content and/or nucleic acid content of growing cells.

The last phase of these investigations was devoted exclusively to the design of a commercial process for the production of SCP from photosynthetic bacteria, based upon data obtained from previous studies. Finally, an economic evaluation of the process was made in order to determine the feasibility of producing SCP from photosynthetic bacteria. This phase of the investigation is described in Chapter V.

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CHAPTER II

PHOTOSYNTHETIC BACTERIA

INTRODUCTION

One of the most important biological processes on earth is photosynthesis - a process in which cells convert sunlight into chemical energy. The study of photosynthesis in algae, bacteria and plants indicates that photosynthesis consists of two series of reactions as shown in Figure 2-1. The first of these reactions, the "light reactions," involves the absorption of light by chlorophyll followed by the subsequent dissociation of water into an oxidizing fragment (OH^-) ultimately becoming oxygen, and a reducing fragment (H^+). In the second series of "dark reactions," the reducing fragment is used to reduce carbon dioxide into carbohydrate and molecular oxygen. In contrast to the light reactions of plant photosynthesis in which ATP and reduced NADP are synthesized, only ATP is synthesized during the light reaction of bacterial photosynthesis (2). Unlike algae which split water to provide reducing power for cellular synthesis, photosynthetic bacteria obtain their reducing power from constituents of their environment, such as H_2S and organic compounds, and therefore do not yield oxygen during

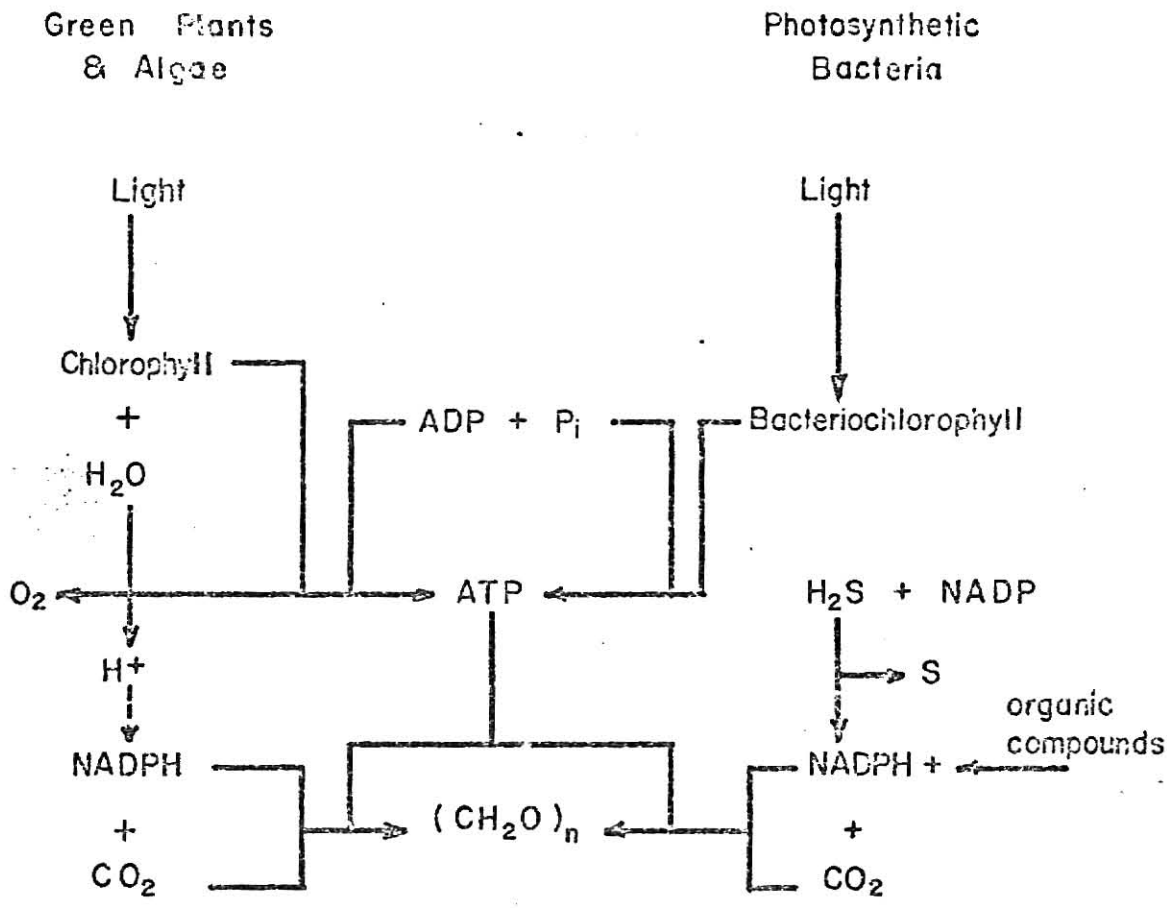

Figure 2.1 Comparison of Plant and Bacterial Photosynthesis (1).

photosynthesis (3).

Photosynthesis occurs only in those organisms which contain chlorophyll. Therefore, it seems reasonable to conclude that chlorophyll is directly related to the assimilation of light energy. There are several different kinds of chlorophyll which differ both chemically and structurally and are distinguishable by their various absorption spectra.

Many plants have more than one chlorophyll, but the most common are chlorophylls a and b. Blue-green algae also contain chlorophyll a, but photosynthetic bacteria have chlorophylls of different structure called "bacteriochlorophylls." In addition to chlorophylls, photosynthetic organisms usually contain a number of identical or closely related accessory photoactive pigments, enzyme systems and electron carriers which participate in photosynthesis (4).

LIGHT REACTIONS AND PHOTOPHOSPHORYLATION

When a chlorophyll molecule absorbs a quantum of light, the molecule undergoes a change in properties and becomes "excited." Excitation results in an electron of the chlorophyll molecule being driven off, and the chlorophyll molecule itself becomes positively charged:

$$Chl + light \rightarrow Chl^{+} + e^{-}$$

Released electrons then migrate through the photosynthetic unit to the reaction center, comprised of approximately forty chlorophyll molecules, and transfer energy to a special reaction center chlorophyll. At the reaction center, a charge separation equivalent to an oxidation-reduction reaction occurs. The electron is the energy source while the positively charged chlorophyll becomes the electron acceptor (5,6).

Cyclic Photophosphorylation

In photosynthetic bacteria (see Figure 2-2), the electrons flow through an electron transport system, being passed successively to ferredoxin, ubiquinone, cytochrome b and cytochrome f, and ending with a positively charged chlorophyll molecule. In the step between cytochrome b and cytochrome f, ATP synthesis occurs. This cyclic process by which light energy is converted into the chemical energy of ATP is termed "cyclic photophosphorylation" (8).

Noncyclic Photophosphorylation

In splitting water to provide reducing power for cellular synthetases, algae and plants require the cooperative action of light reactions in two electron transport systems: light system I, similar to that of photosynthetic bacteria; and light system II, operating at shorter wavelengths with light quanta of higher energy (see Figure 2-3). In light system II, light absorbed by the chlorophylls again leads to a charge

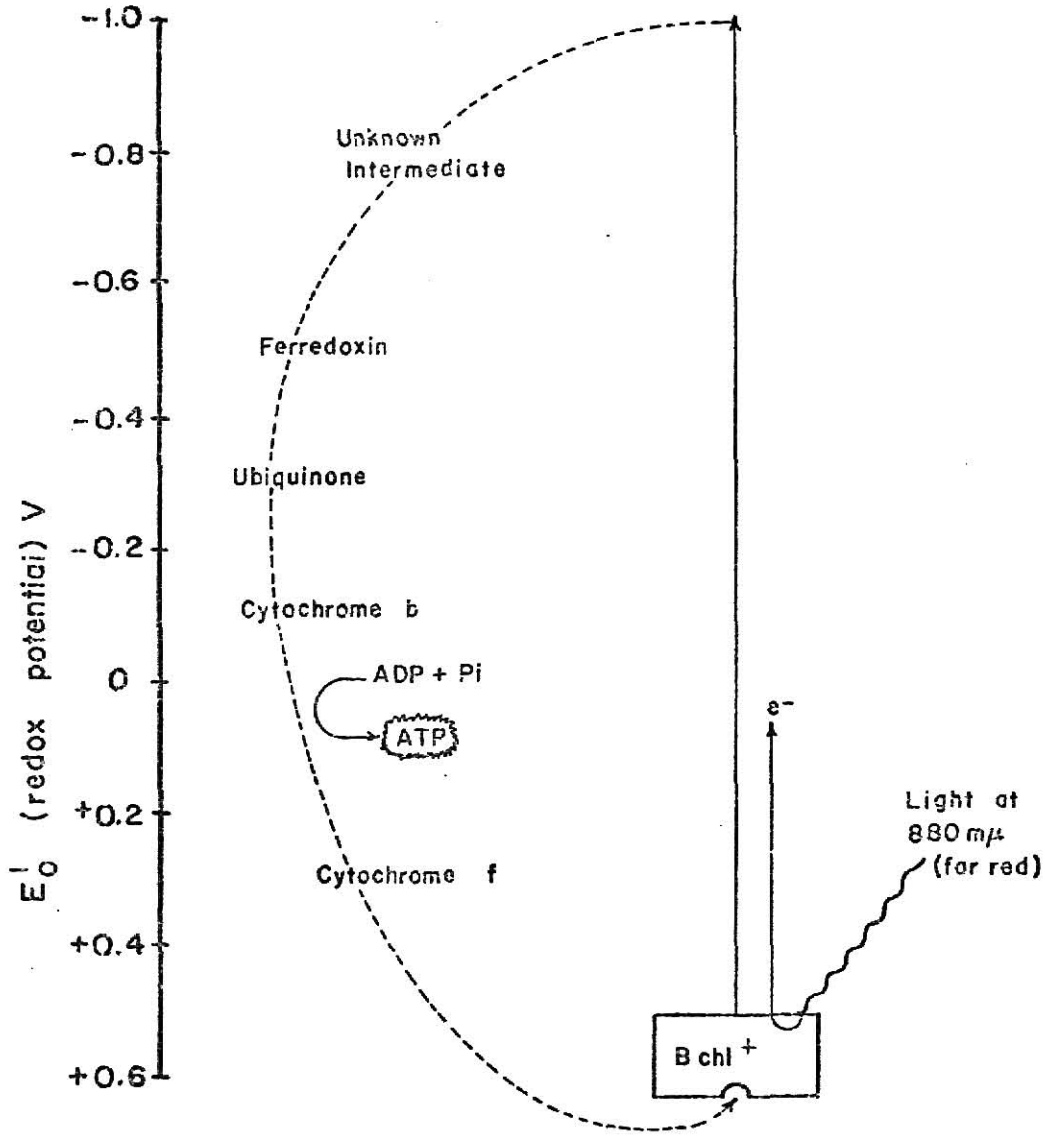

Fig. 2.2. Cyclic Photophosphorylation in Photosynthetic Bacteria (7).

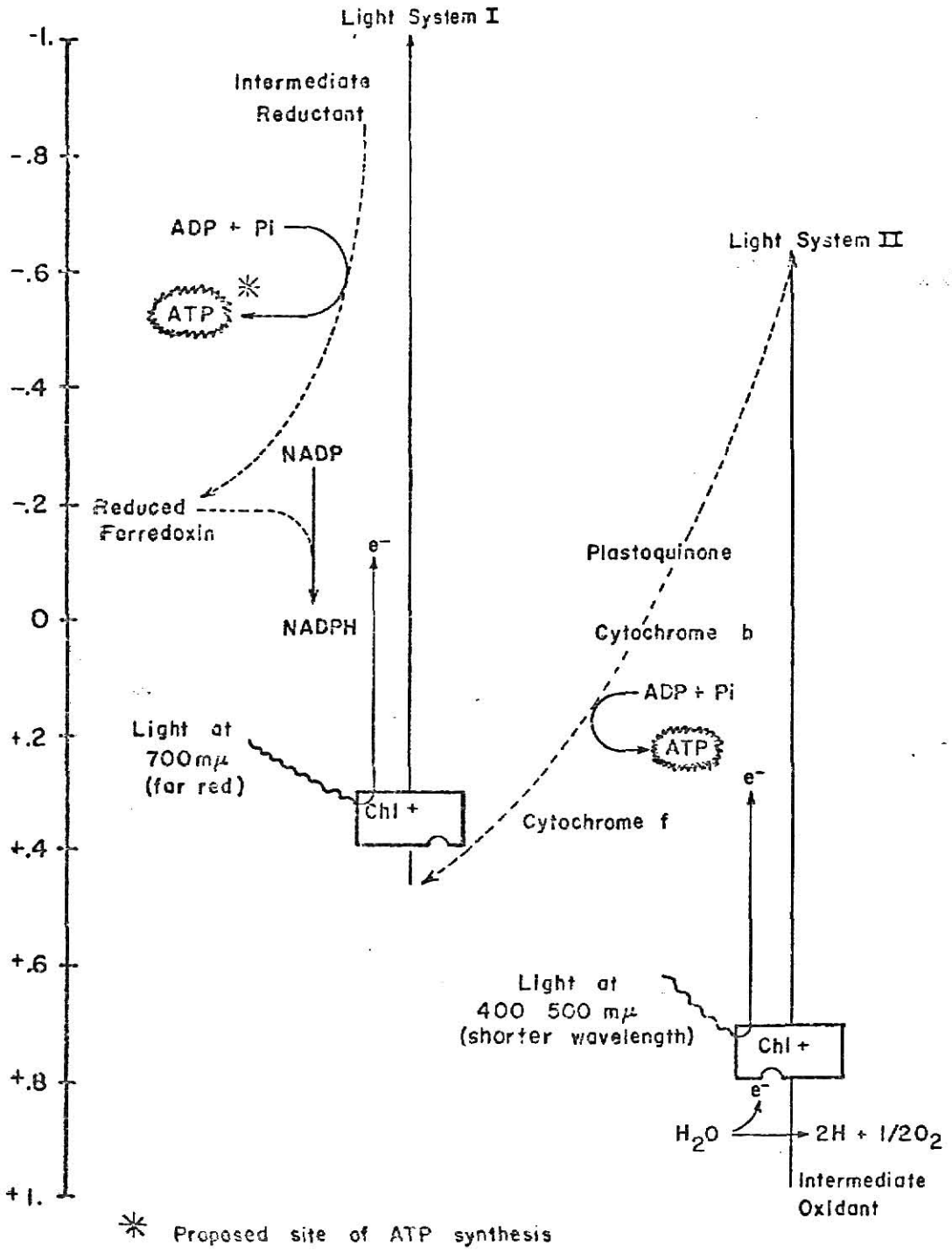

Fig.2.3 Noncyclic Photophosphorylation Pathway in Algae and Plants.

separation with an electron being transferred from the hydroxyl radical of water to the positively charged chlorophyll unit, leading to the formation of molecular oxygen. The electron derived from water migrates through the electron transport system until it reaches a specific photosynthetic cytochrome, cytochrome f. During the passage from cytochrome b to cytochrome f, one high-energy phosphate bond in ATP is synthesized. Reduced cytochrome f is reoxidized in light system I, with the electron from cytochrome f being transferred to NADP by way of the positive charge created in system I; however, the proposed site of phosphorylation is uncertain (10). In summary, photosynthesis in algae and plants requires the cooperation of two light reactions with the resulting synthesis of two molecules of ATP, one molecule of NADPH, and the cleavage of water with the formation of molecular oxygen. The one-directional flow of electrons together with ATP synthesis in light reactions I and II is termed "noncyclic photophosphorylation"(11).

TAXONOMY OF PHOTOSYNTHETIC BACTERIA

Photosynthetic bacteria are represented by a rather large number of species of different morphology, pigmentation and physiological-biochemical properties (12). On this basis, all photosynthetic bacteria are divided into three groups or families: Thiorhodaceae, Athiorhodaceae and Chlorobacteriaceae (see Table 2-1).

TABLE 2-1

Outline of Subdivisions of the Photosynthetic Bacteria (13)

	Green Sulfur Bacteria (Chlorobacteriaceae)	Purple Sulfur Bacteria (Thiorhodaceae)	Nonsulfur Purple Bacteria (Athiorhodaceae)
Major Pigment System	B Chl. c(abs. max. 747mμ) B Chl. d(abs. max. 725mμ)	BChl. a(abs. max. 820mμ) BChl. b(abs. max. 1025mμ)	B Chl. a(abs. max. 820mμ) B Chl. b(abs. max. 1025mμ)
Cell Morphology	motile or nonmotile rods, cocci, pleomorphic; some with gas vacuoles	motile or nonmotile rods, cocci, pleomorphic; some with gas vacuoles	motile rods or spirals; some multiply by budding
Photosynthetic Electron Donors	H_2S, thiosulfate, H_2(organic compounds by some strains	H_2S, thiosulfate, H_2(organic sompounds by some strains	H_2, organic compound (H_2S usually toxic)
Sulfur Deposition	always outside the cell	usually inside the cell, except Ectothiorhodaceae	none
Aerobiosis	obligate anaerobes	obligate anaerobes	facultative; grow in the dark aerobically, with photosynthetic growth anaerobically
Growth Factor Requirements	B_{12} or none	B_{12} or none	usually complex
DNA Base Composition %G ≡ C	48-58	46-67	61-73

Thiorhodaceae - Purple Sulfur Bacteria

The purple sulfur bacteria (Thiorhodaceae) are strict anaerobes, photoautotrophic, and are able to oxidize hydrogen sulfides and certain other inorganic compounds in the process of photosynthesis.

Athiorhodaceae - Purple Nonsulfur Bacteria

Nonsulfur purple bacteria (Athiorhodaceae) include representatives of photoheterotrophic purple bacteria, i. e., organisms requiring specific organic compounds for their growth. Some species of nonsulfur purple bacteria are facultative aerobes and can develop in the dark.

Chlorobacteriaceae - Green Sulfur Bacteria

Green sulfur bacteria (Chlorobacteriaceae) are easily distinguished from purple sulfur bacteria by the composition of their pigment. They resemble purple bacteria in being strict anaerobes; in being photoautotrophs; and in being able to oxidize hydrogen sulfide and other sulfur compounds in the process of photosynthesis.

PIGMENTS OF PHOTOSYNTHETIC BACTERIA

It has long been established that the pigment system is responsible for the important light-gathering function of photosynthesis. The pigment system of photosynthetic bacteria consists of a specific chlorophyll (bacteriochlorophyll) and accompanying carotenoids resembling

those of green plants and algae.

Bacteriochlorophyll

Structural Properties of Bacteriochlorophyll.

All known purple sulfur and nonsulfur bacteria contain the classical bacteriochlorophyll a which differs only slightly from chlorophyll a of plants and algae (14). Important structural differences between chlorophyll a and additional bacteriochlorophylls are shown in Table 2-2.

Composition of Bacteriochlorophyll.

All purple photosynthetic bacteria contain the same bacteriochlorophyll, the composition of which is expressed by the following (16):

formula: $C_{55}H_{74}O_6N_4Mg$

molecular weight: 910.50

percent composition: C - 72.47
H - 8.18
O - 10.53
N - 6.15

Extraction and Purification.

Bacteriochlorophylls may be readily extracted from intact cells of purple bacteria employing various organic solvents such as ethanol, methanol, ether, acetone and toluene. The bacteriochlorophylls may be purified by column chromatography on saccharose or cellulose on which a characteristic blue zone is produced. Solvents saturated with hydrogen sulfide are generally used during extraction to prevent oxidation of the bacteriochlorophyll. Bacteriochlorophylls extracted in this manner may be stored in darkness, at a low

Table 2-2 The Relationship between Chlorophyll-a and Bacteriochlorophylls*(15).

	R_1	3 , 4	R_2	R_3	R_4
Chlorophyll - a	$-CH=CH_2$		$-C(=O)-O-CH_3$	phytyl ester ($C_{20}H_{39}O-$)	- H
Bacteriochlorophyll - a	$-C(=O)-CH_3$	dihydro	$-C(=O)-O-CH_3$	phytyl ester	- H
Bacteriochlorophyll - c	$-C(=OH)(H)-CH_3$		- H	farnesyl ester ($C_{15}H_{25}O-$)	$-CH_3$
Bacteriochlorophyll - d	$-C(=OH)(H)-CH_3$		- H	farnesyl ester	- H
Bacteriochlorophyll - b	Structure unknown				

* Refer to porphyrin ring structure in Figure 2.4.

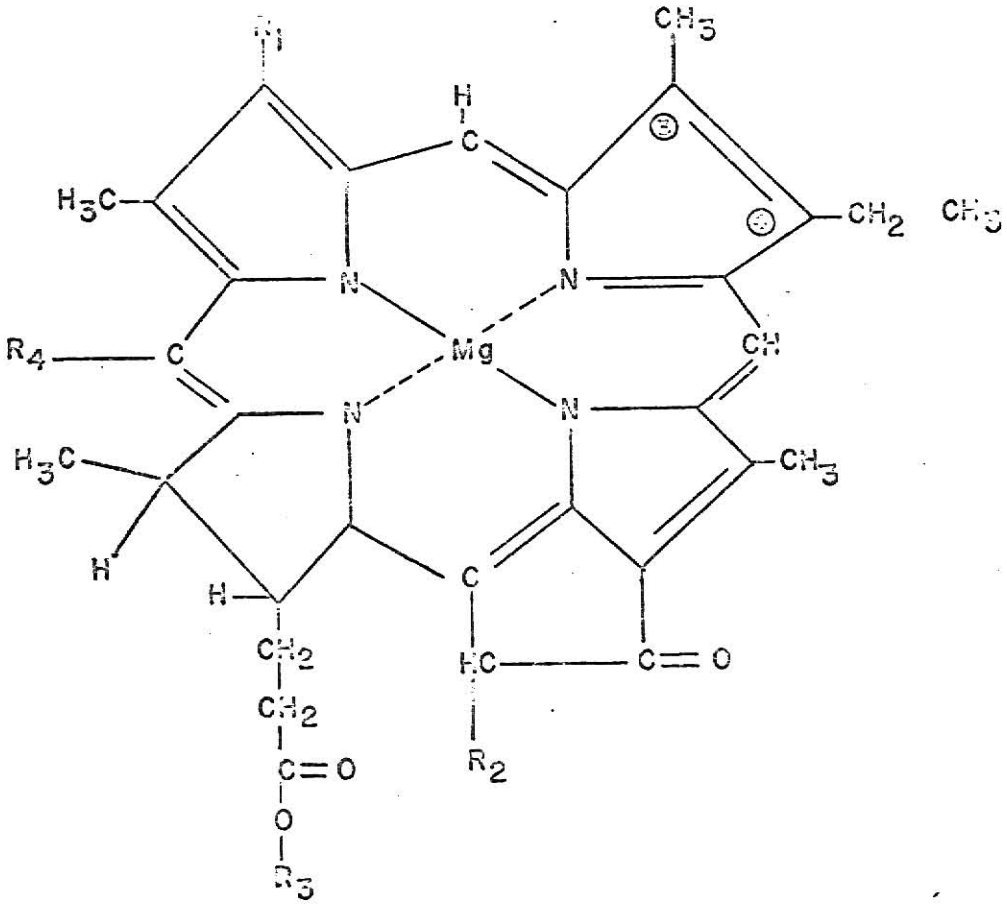

Fig. 2.4 Porphyrin Ring Structure of Plant Chlorophylls and Bacteriochlorophylls.

temperature, for a considerable length of time. Methods are also available for obtaining crystalline bacteriochlorophylls (17).

Absorption Spectra. Although the position of absorption maxima changes somewhat depending on the solvent used for extraction, the primary maximum absorption of bacteriochlorophyll in solutions is in the range of 770-780 nm.

Intracellular bacteriochlorophyll exhibits different spectral properties from extracted bacteriochlorophylls in solution. In intact cells, the bacteriochlorophylls present two or three absorption maxima instead of only the one noted in solution. As compared with absorption maxima of bacteriochlorophyll in solution, these maxima shift towards longer wave lengths. In some purple bacteria, the absorption maxima of intracellular bacteriochlorophyll are about 890, 850 and 810-800 nm; in others, significant absorption maxima are observed at about 880 and 800 nm or 850 and 800 nm (18).

Production of Bacteriochlorophyll. The bacteriochlorophyll concentration in photosynthetic bacteria is similar to that found in algae (Table 2-3), and may vary from traces to two to five percent of the cellular dry weight, depending on growth conditions such as: presence of oxygen, light intensity, medium composition and age of cultures.

Regarding age of culture, the highest bacteriochlorophyll concentration in cells is generally found during the logarithmic phase of growth (Table 2-4). Purple bacteria growing photoautotrophically

TABLE 2-3

Bacteriochlorophyll Content of Some

Photosynthetic Bacteria (19)

Organism	Carbon Source	Bacteriochlorophyll (n moles/mg. dry wt.)
Athiorhodaceae		
R. rubrum	malate	13
Rps. spheroides	malate	12
Rps. palustris	malate	11
Rps. capsulata	succinate	22
Thiorhodaceae		
Thiopedia sp.	carbon dioxide	16
Chromatium D	carbon dioxide	24
Chromatium D	succinate	33

TABLE 2-4

Bacteriochlorophyll Content (mg/g. cellular dry wt.)

Of Purple Bacteria Grown on Mineral and

Organic Media with Sodium Bicarbonate (20)

Medium Component	Rhodopseudomonas		Chromatium	
	logarithmic	stationary	logarithmic	stationary
Sodium sulfide	21	14	20	16
Sodium thiosulfate	21	15	-	-
Acetic acid	31	23	29	18
Propionic acid	30	26	11	13
Butyric acid	29	20	-	-
Pyruvic acid	33	20	29	21
Succinic acid	34	24	26	22

Note: Organic acids are added to the media in the form of Na salts.

contain less bacteriochlorophyll than the same species grown on media with various organic acids (Table 2-4).

Numerous investigators (21-23) have suggested that synthesis of photosynthetic pigments by cultures of nonsulfur photosynthetic bacteria is regulated by light intensity. Elegant experiments with Rps. spheroides have demonstrated that the rate of bacteriochlorophyll synthesis is inversely proportional to the light intensity and, the pigment content of cells grown in dim light (50 foot-candles) is about eight times higher than those grown in bright light (5000 foot-candles). On transfer from dim to bright light or vice versa cultures rapidly adjust their pigment level (Table 2-5) by preferential synthesis or by transient repression of pigment formation.

In nonsulfur purple bacteria, oxygen exerts a spectacular control over pigment synthesis as shown by the almost complete absence of bacteriochlorophyll and carotenoids in organisms grown aerobically in the dark (Table 2-5). Similarly, introduction of oxygen into cultures growing in the light results in an immediate arrest of pigment synthesis. When cultures are transferred to anaerobic conditions and exposed to light, bacteriochlorophyll synthesis is restored. These experiments suggest that absence of pigment in dark-aerobic cultures might be due to repression of their synthesis by oxygen rather than to an obligatory requirement for light. This was confirmed by showing that nonsulfur

TABLE 2-5

Bacteriochlorophyll Content of Nonsulfur

Purple Bacteria Growing

Under Different Environmental Conditions (24)

Rhodospirillum rubrum	
Photosynthetic growth at light intensity of (anaerobic conditions):	Bacteriochlorophyll content of cells (μg/mg cellular protein)
50 foot-candles	25.0
2000 foot-candles	10.2
6000 foot-candles	5.6
Respiratory growth conditions:	
Full aeration	0.2
Full aeration followed by limiting oxygen for 3 hr.	3.3
Rhodopseudomonas spheroides	
Photosynthetic growth at light intensity of (anaerobic conditions):	
50 foot-candles	66.0
9500 foot-candles	5.9
Aerobic growth	0.14

purple bacteria can indeed synthesize bacteriochlorophyll and carotenoids in the dark provided that the atmospheric oxygen tension is reduced to one to six percent (25). With suspensions of nonsulfur purple bacteria forming bacteriochlorophyll in the dark, the oxygen tension which permits synthesis is critical and must presumably be sufficient for general metabolism (e. g., to supply ATP by oxidative phosphorylation), yet insufficient to cause repression of pigment formation.

Carotenoids

Carotenoids are present in the cells of various photosynthetic organisms where they constitute the univariable components of plant chloroplasts and bacterial chromatophores. The carotenoids of photosynthetic bacteria, however, differ from those contained in the plastids of algae and higher plants. There are several reviews describing the function, distribution and biosynthesis of carotenoids in photosynthetic bacteria and other microorganisms (26-29).

The principle carotenoids in most purple bacteria are open-chain (aliphatic) compounds, which frequently bear one or two terminal methoxyl ($-OCH_3$) groups. A few purple bacteria contain aryl carotenoids, which have an aromatic ring at one end of the chain. The green bacteria always contain aryl carotenoids different from those in purple bacteria. The structures of some major carotenoids of

purple and green bacteria are compared in Figure 2.5 with the structure of B-carotene, a precursor of vitamin A, and a typical carotenoid of the alicyclic type common to oxygen-evolving photosynthetic organisms.

Function of Carotenoid Pigments

The close association of carotenoids to the photosynthetic system suggests that these pigments have a definite role in the process of photosynthesis. In investigations of factors inducing phototaxis in some photosynthetic bacteria, the activities of carotenoids during phototaxis have been interpreted as an indirect proof of their participation in photosynthesis. Carotenoids of purple photosynthetic bacteria (see Table 2-6), like those of algae, function in transmitting absorbed light energy to bacteriochlorophyll. Carotenoid pigments transfer about thirty to fifty percent of the absorbed light energy to bacteriochlorophyll, and are to such an extent functional as "light-harvesting" pigments. A more fundamental function of carotenoids may be their protective action against harmful photo-oxidation of sensitized bacteriochlorophyll (32).

Extraction of Carotenoids. Carotenoids are generally extracted from microorganisms by treating the cells with an organic solvent, e.g., anhydrous methanol, acetone, or diethyl ether. Extraction can often be hastened by use of a blender, several treatments usually being necessary to remove all pigment (33).

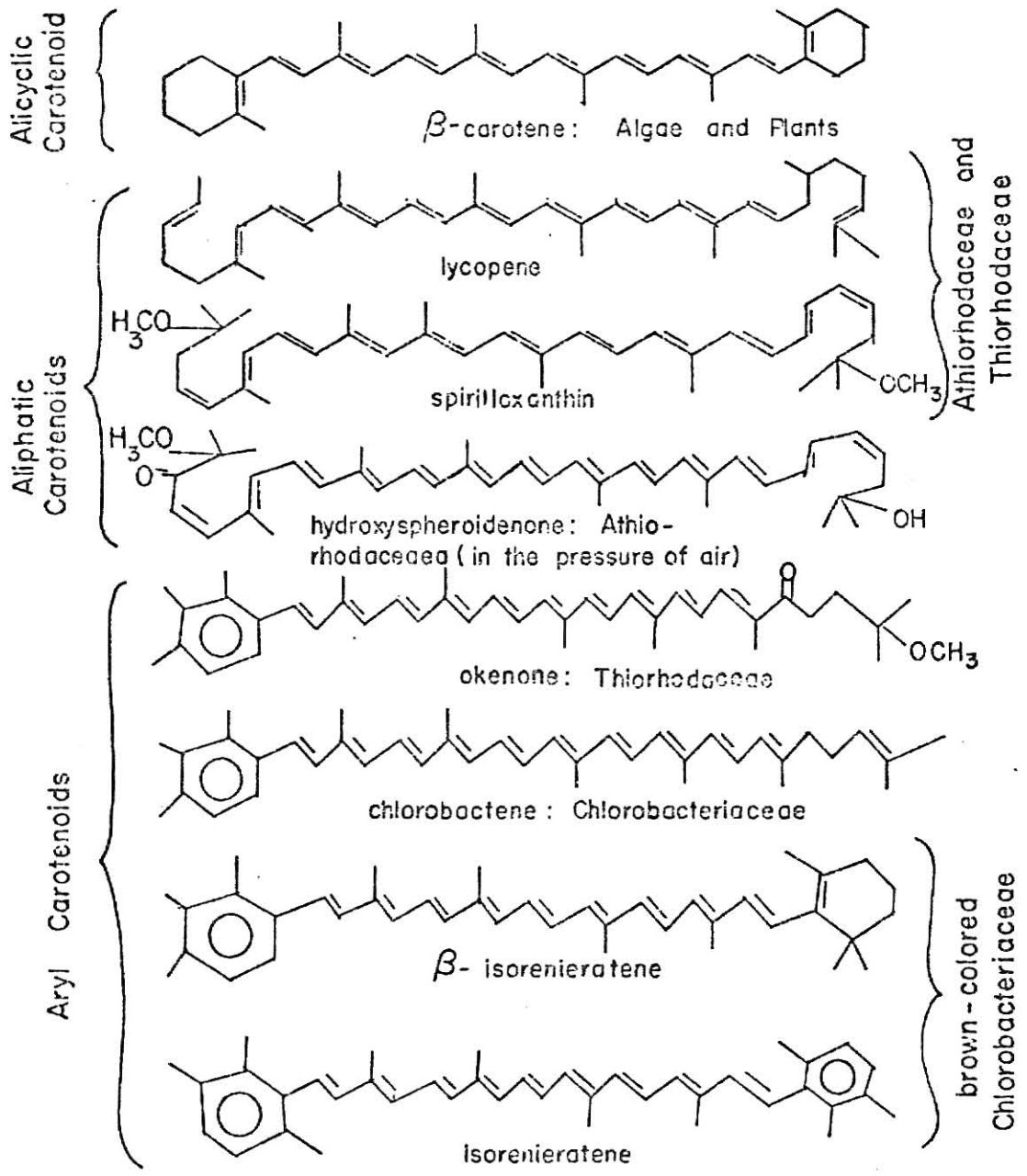

Figure 2.5 Representative Carotenoids of Photosynthetic Bacteria Compared with β-Carotene, the Major Carotenoid in most Algae and Plants(30).

TABLE 2-6

Absorption Maxima of the Main Carotenoids

Of Purple Bacteria (31)

Carotenoid	Pigment Absorption Maxima (nm) In Petroleum Ether			Color
Lycopene	445	470	502	brown-orange
P-481	454	481	514	purple-orange
Spheroidene	426	451	482	yellow
Spheroidenone	458	480	512	red-purple
Spirilloxanthin	463	491	525	purple
Rhodopene	445	470	501	brown-orange
Rhodovibrin	455	481	514	purple-orange
Hydroxy-spheroidene	426	452	482	yellow
P-512	485	512	543	dark purple
Hydroxy-spheroidenone	452	480	512	purple-orange
Demethylated spirilloxanthin	465	491	525	purple

Separation of Carotenoids. Mixtures of pigments can be resolved either by absorption chromatography or by first partitioning the pigments between ninety percent methanol and petroleum ether. Pigments separated by phase partition can be further separated by employing absorption chromatography with suitable solvents and absorbents.

Individual carotenoids are characterized by their behavior in partition tests, by their chromatographic behavior, and by their absorption spectra in various solvents. Crystallized pigments can be further analyzed for melting point, functional groups, unsaturation, optical rotation, and derivative formation. Many variations in methodology exist, depending upon the pigments to be analyzed (34).

Absorption Spectra of Various Carotenoids. Carotenoids of purple bacteria which have been separated on an aluminum oxide column, usually form yellow, orange and red zones. In solution, these pigments have three absorption maxima in the 425 to 550 nm band (Table 2-6). The intensity of maximum absorption of carotenoids depends on the solvent.

In intact cells and in chromatophores, purple bacteria carotenoids absorb light at the 400 to 600 nm band, where several maxima are detected. Unsaturated and partially saturated yellow carotenoids absorb light in the shorter wave band from 400-520 nm. Red carotenoids containing oxy- and methoxy groups have absorption maxima at

longer wave lengths of the spectrum (35).

Factors Influencing the Carotenoid Composition of Photosynthetic Bacterial Cells. Purple bacteria are distinguished by considerable qualitative and quantitative changes of carotenoids in relation to the age of cultures and growth conditions (36).

Age of Culture. In the Athiorhodaceae, great changes in carotenoid composition are detected during development. Cells of young cultures contain, in addition to spirilloxanthin, considerable quantities of other carotenoids: P481, demethylated spirilloxanthin, rhodopene and lycopene. As the cultures age, a gradual decrease of all carotenoids occurs, except spirilloxanthin. The spirilloxanthin content, however, increases proportionately to the decrease of other carotenoids and, after 96 to 158 hours growth, the spirilloxanthin content comprise ninety to ninety-five percent of the total cellular carotenoid content (37).

Effects of Oxygen and Light on Carotenoid Synthesis. Oxygen elicits great changes in carotenoid synthesis by purple bacteria. In the presence of oxygen, certain cultures of Athiorhodaceae become red, whereas under anaerobic conditions they are brown. Subsequent investigations have shown that in these bacteria, in the presence of air, the content of yellow carotenoid Y drops considerably, whereas the amount of red carotenoid R increases.

The total carotenoid content of purple bacteria cells cultivated aerobically, both in light and in darkness, decreases significantly.

Carotenoid synthesis is almost completely inhibited as a result of vigorous aeration. Carotenoid synthesis is also inhibited by the simultaneous presence of light and oxygen, whereas carotenoids present a priori in cells are preserved under such conditions. Exposure to light leads to quantitative changes in carotenoids; as light intensity increases, carotenoid synthesis decreases (38).

There is a definite similarity in the effect of different environmental factors on the syntheses of carotenoids and bacteriochlorophyll of purple photosynthetic bacteria. This resemblance is due to the fact that bacteriochlorophyll and carotenoid syntheses depend on the oxidation-reduction system participating in the transport of electrons from organic compounds to their acceptors. The more this system is oxidized, the less pigments are produced by photosynthetic bacteria. The oxidation system may be determined by the speed of the primary photosynthetic reactions which increase with increasing light intensity and the effectiveness of assimilation of organic compounds as hydrogen donors.

THE DISTRIBUTION AND ROLE OF PHOTOSYNTHETIC BACTERIA

The growth and development of purple and green photosynthetic bacteria have been observed in various parts of the world and in many different climatic zones. Photosynthetic bacteria are encountered in

almost every body of water and also in soil. However, they are most prevalent in fresh and salt water containing hydrogen sulfide. In stagnant or polluted waters abundant in organic materials, purple and green photosynthetic bacteria often develop profusely, forming massive conglomerations (39).

Distribution of Purple Sulfur Bacteria

The development of purple sulfur bacteria generally depends on the presence of light, hydrogen sulfide concentration and adequate anaerobic conditions. Luxuriant growth of purple sulfur bacteria is frequently observed in habitats rich in hydrogen sulfide, primarily sulfur springs. In addition to mineral springs, purple sulfur bacteria are widespread in waters rich in organic substances. They are constant inhabitants of polluted ponds, reservoirs, sewers and drainage channels where decaying algae accumulate and where the water is relatively stagnant.

Distribution of Green Sulfur Bacteria

Green sulfur bacteria are often encountered in the same habitats as purple sulfur bacteria (40). Massive development of green sulfur bacteria has likewise been observed in shallow, polluted ponds, ditches, rivers and other waters abounding in decaying organic matter. Concurrent development of green and purple sulfur bacteria is explained by their similar physiology, the growth of both being conditioned by the

presence of light, hydrogen sulfide concentration and by sensitivity to oxygen. Green sulfur bacteria are able to grow at low light intensities, much like purple sulfur bacteria, and are, therefore, encountered at relatively great depths. Profuse growth of green sulfur bacteria is somewhat limited by high concentrations of hydrogen sulfide. Green sulfur bacteria also tend to be more sensitive to oxygen than purple bacteria, developing only in the complete absence of oxygen.

Distribution of Purple Nonsulfur Bacteria

In addition to purple and green sulfur bacteria, many different species of purple nonsulfur bacteria are often encountered in water sources polluted by sewage and other organic materials. Nonsulfur bacteria may accumulate in large masses and form pink layers in certain lakes at depths where oxygen is present, whereas purple and green sulfur bacteria develop at lower, oxygen-free levels. Mass accumulations of nonsulfur purple bacteria are rarely found in nature; this is even true for conditions in which they could be expected to constitute the predominant population of phototrophic bacteria, for example, in the presence of organic wastes such as in sewage plant and slaughter house waste ponds. The ecologically significant factor under these conditions is sulfate, which is rarely lacking in natural habitats. Under anaerobic conditions, sulfate-reducing bacteria are continuously forming sulfide from the reduction of sulfate. Sulfide inhibits the

growth of purple nonsulfur bacteria but establishes developmental conditions for green and purple sulfur bacteria. The latter organisms eventually reduce the sulfide concentration to such a low level that the purple nonsulfur bacteria can coexist beside them.

The Role of Photosynthetic Bacteria in Nature

In nature, the purple and green photosynthetic bacteria are constantly accompanied by a rich microflora developing concurrently in hydrogen sulfide-rich reservoirs. In many habitats abounding with purple and green photosynthetic bacteria, many sulfate reducing, as well as active septic and thionic bacteria are found. Although a massive growth of photosynthetic bacteria can fix carbon dioxide at a rate of about 1.15 mg/l daily, the main function of many photosynthetic bacteria in water reservoirs is related to their participation in the sulfur cycle (Figure 2.6).

Participation of Photosynthetic Bacteria in the Sulfur Cycle

There appears to be a most distinct and intimate relationship between the sulfate-reducing and photosynthetic bacteria. The principle metabolite of sulfate-reducing bacteria in most water reservoirs is hydrogen sulfide, which is required for the growth and development of photosynthetic and achromatic sulfur bacteria. Photosynthetic bacteria, in turn, may stimulate growth of the sulfate reducers by delivering a certain amount of organic matter and sulfates necessary for

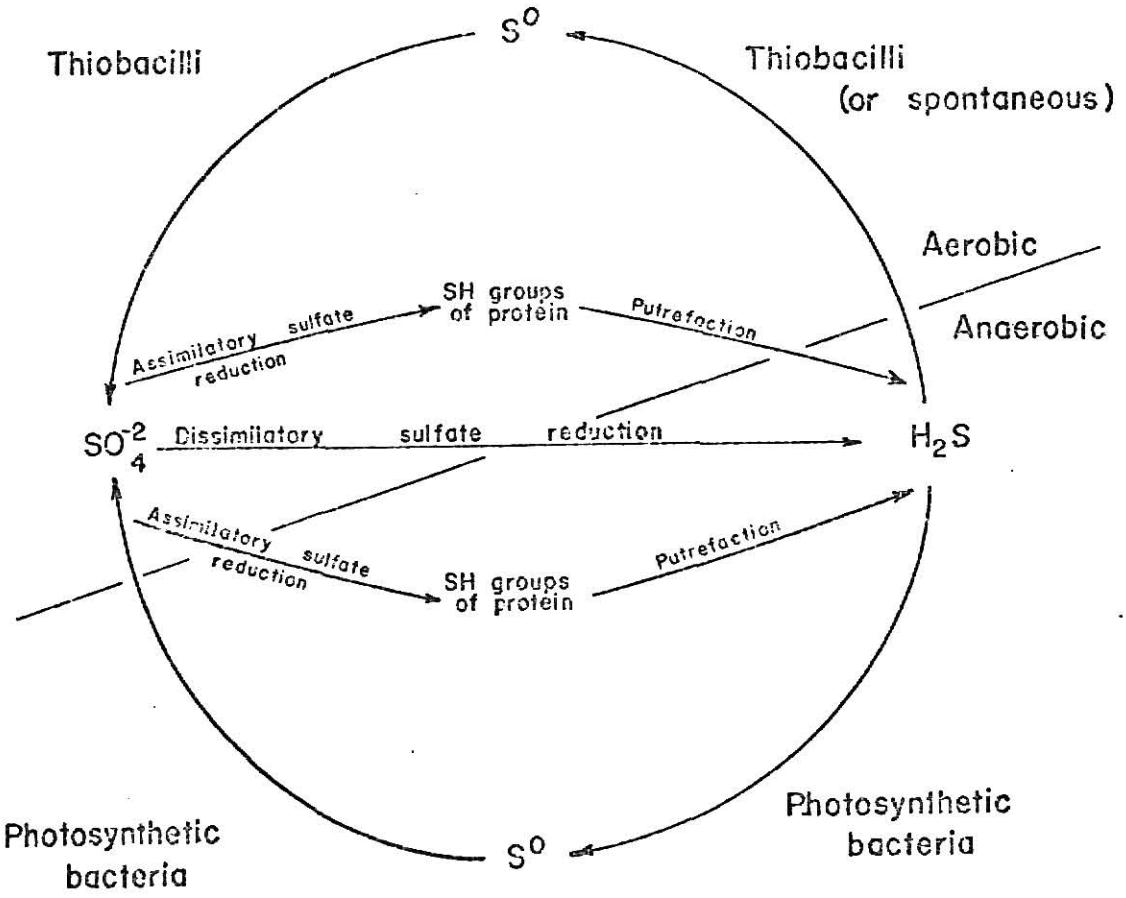

Fig. 2.6. The Sulfur Cycle.

growth. As a result of the activity of the photosynthetic, as well as the achromatic sulfur bacteria and thionic bacteria, natural water reservoirs are freed of hydrogen sulfide. By oxidizing the hydrogen sulfide, photosynthetic bacteria growing in the depths of the water reservoirs prevent this toxic compound from diffusing into the upper layers of the waters, thus enabling the development of many plant and animal organisms.

If an adequate amount of hydrogen sulfide is available, the purple and green sulfur bacteria oxidize it to sulfur which sometimes accumulates in the medium. The sulfur released by their metabolism may form large deposits which are of commercial value. Intensive sulfur formation as the result of bacterial activity by purple and green photosynthetic has been observed in a number of inland lakes throughout the world. Indication that numerous sulfur deposits are the result of microbial activity dating back to very early geological eras is substantiated by the fact that these sulfur deposits are often located at the sites of ancient sea beds. In addition, analysis of the isotope composition of sulfur in different areas confirms that many of these sulfur deposits are the results of early microbiological activity.

Since some countries are short of sulfur as a source of sulfuric acid, it has been frequently suggested that sulfur production in water be intensified by means of photosynthetic sulfur bacteria. To

achieve this it is evidently necessary to change the physiochemical conditions of the water so as to shift the sulfur cycle balance in the direction of sulfur accumulation (42).

Photosynthetic bacteria have also been suggested as being important in the production of therapeutic mud in lakes and estuaries. Photosynthetic bacteria, together with algae, accumulate organic matter as well as restore sulfur to the mud in the form of sulfates.

Finally, photosynthetic bacteria are able to fix nitrogen. Nitrogen fixation is important in that it enables them to grow in areas with little or no nitrogen compounds and to enrich the medium with bound nitrogen compounds.

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CHAPTER III

BATCH CULTIVATION OF PHOTOSYNTHETIC BACTERIA

INTRODUCTION

Within the past two decades an intensive world-wide research effort has been directed toward the production of Single-Cell Protein (SCP) from a vast number of microorganisms grown on an infinite variety of substrates. Presently, only a limited amount of research has been done concerning the production of edible proteins from photosynthetic bacteria (1-3). Most representatives of photosynthetic bacteria would be unsuitable for the production of SCP since many of the purple and green sulfur bacteria accumulate sulfur droplets within their cytoplasm as an end product of sulfur oxidation. Nonsulfur purple bacteria, however, utilize organic compounds as electron donors for photosynthesis and thus do not accumulate sulfur. The nonsulfur purple bacteria (Athiorhodaceae) could therefore serve as potential sources of edible protein. It is the intention of the present investigation to study the batch cultivation of one such organism as a means of determining the feasibility of producing SCP from photosynthetic bacteria.

CULTIVATION OF Rhodopseudomonas gelatinosa

Selection of Organism

For this study, a marine strain of the nonsulfur purple bacterium, Rhodopseudomonas gelatinosa, was generously supplied by Dr. Nganshou Wai, Institute of Chemistry, Academia Sinica, Taipei, Taiwan.

Maintenance of Stock Cultures

Stock cultures of Rps. gelatinosa were maintained by inoculation to slants of agar medium every two to three months. The composition of agar medium used for the preservation of pure cultures is shown in Table 3-1. After inoculating, the culture tubes were immediately filled with sterile hydrogen gas and tightly sealed with rubber stoppers. Cultures were incubated at 30°C under continuous illumination provided by two 75-watt incandescent light bulbs. Dark reddish-purple colonies were observed usually after twenty-four to thirty hours growth. After incubation for forty-eight hours, cultures were transferred to a refrigerator and stored at 4°C until needed.

Important Morphological, Physiological and Biochemical Properties

Throughout the duration of these investigations it was frequently necessary to microscopically determine the integrity of stock cultures. There are many important morphological, physiological and biochemical properties which are useful in determining the integrity of pure cultures

TABLE 3-1

Composition of Agar Medium for Preservation

Of Pure Cultures of Rhodopseudomonas gelatinosa

K_2HPO_4	1.0 g
$MgCl_2$	0.5 g
NaCl	23.0 g
Yeast Extract	5.0 g
Agar	20.0 g
Tap Water	1000 ml

Final pH adjusted to 7.2 ± 0.2.

of Rps. gelatinosa (4).

Morphological Characteristics.

(1) These short, rod-shaped, gram negative bacteria are approximately 0.5 μ wide and 1.0 to 2.0 μ in length. Older cells usually appear longer, up to 15.0 μ in length, and usually appear as irregularly curved rods, often swollen and gnarled.

Photomicrographs of Rps. gelatinosa taken during the growth cycle appear in Figures 3.1 to 3.3. Photographs were taken on Kodak 35 mm Plus-X Pan film with a Zeiss Photomicroscope equipped with microflash for Zernicke phase contrast and Nomarski interference optics.

(2) Young cells are actively motile by means of polar flagella. Motility is often difficult to ascertain due to the pronounced tendency of cultures to conglomerate; individual cells in clumps appear to be non-motile.

(3) Single cells are infrequent due to a copious mucus production in all media causing cells to clump together in extensive slime masses.

(4) Coloration is due to chlorophylls and carotenoid pigments. The coloration may vary from a pale, delicate pink to a dirty, faded brown.

Physiological Characteristics.

(1) Liquification of gelatin.

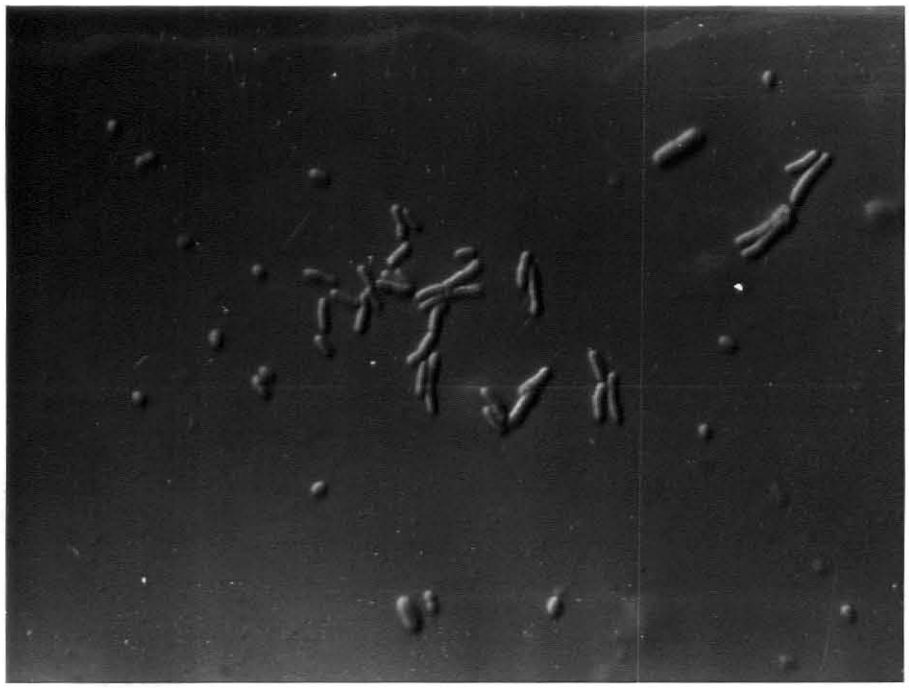

Fig. 3.1 Photomicrograph of Rps. gelatinosa Taken During the Logarithmic Growth Phase. Magnification 640 X.

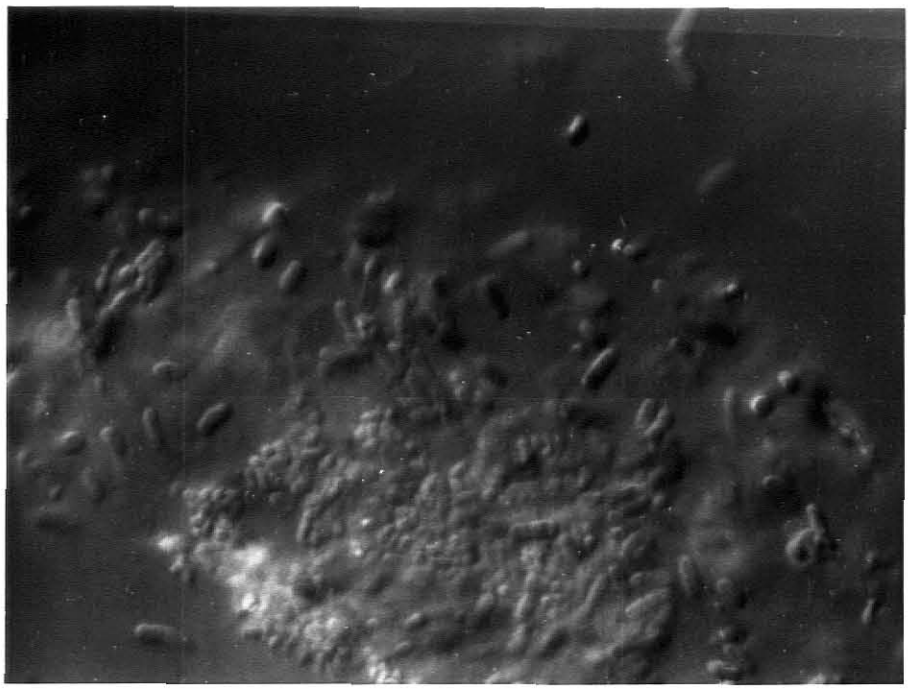

Fig. 3.2 Photomicrograph Showing Initiation of Clump Formation; Culture Approaching Stationary Growth Phase. Magnification 640 X.

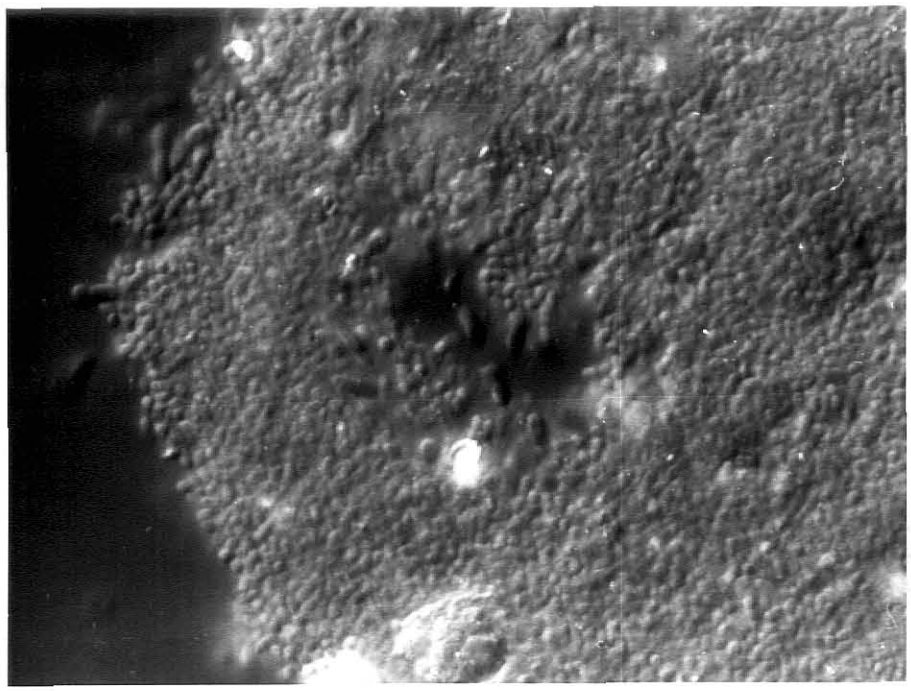

Fig. 3.3 Photomicrograph Showing a Clump of Immotile Cells of Rps. gelatinosa. Magnification 640 X.

(2) Production of a characteristic acrid odor.

Biochemical Properties.

(1) Oxidation of thiosulfate.

(2) Inability to assimilate 0.2 percent propionate, mannitol, sorbitol, glycerol or tartrate.

(3) The best oxidizable substrates appear to be ethanol, glucose, fructose, mannose, citrate, fumarate, succinate, malate and a variety of amino acids. The single amino acids, alanine, aspartic and glutamic acids generally appear as satisfactory substrates.

(4) The vitamins biotin and thiamin are reportedly required for growth.

(5) Microaerophilic cultures of Rps. gelatinosa are generally more oxygen-tolerant than other members of the genus Rhodopseudomonas. Growth under aerobic conditions occurs in the dark. Anaerobically, growth occurs in illuminated cultures by means of a photosynthetic metabolism.

(6) DNA base composition (useful for certain taxonomic purposes) is seventy to seventy-five moles percent guanine and cytosine.

(7) Major pigment system:

(a) Bacteriochlorophyll a (maximum absorption at 820mμ).

(b) Bacteriochlorophyll b (maximum absorption at 1025 mμ).

(c) Carotenoid pigments which function essentially as "light-gathering" pigments in transferring approximately thirty to fifty percent of the absorbed light to chlorophyll. A more fundamental function of carotenoids may be their protective action against harmful photo-oxidation of the chlorophylls. Carotenoids most commonly found are aliphatic intermediates of the normal spirilloxanthan series in which spheroidene and hydroxy-spheroidene are the predominating intermediates in the transformation of lycopene to spirilloxanthin.

PRELIMINARY GROWTH STUDIES

This phase of investigation was concerned with establishing optimal conditions for the growth of Rhodopseudomonas gelatinosa. Effects of such parameters as temperature, pH, substrate and salt concentration were examined in order to establish optical conditions for growth.

Experimental Procedures

Batch cultivations of Rps. gelatinosa were conducted in a thermistatically-controlled incubator-shaker (Psychrotherm, New

Brunswick Scientific Co.) at 100 rpm. Growing cultures were continuously illuminated with two 75-watt incandescent light bulbs.

One-liter Erlenmeyer flasks containing 900 ml of sterile media, the composition of which is shown in Table 3-2, were inoculated with 100 ml of a pure culture of Rps. gelatinosa and immediately sealed with rubber stoppers. Procedures followed for preparation of the inoculum are outlined in Appendix I.

Samples were periodically withdrawn from the culture flasks and the optical density (O.D.) of each sample was measured with a spectrophotometer (Spectronic 20, Bausch and Lomb) at 650 mμ. Optical density was converted to cellular dry weight using a standard plot of optical density versus cellular dry weight (Appendix II).

Results and Discussion

The Effects of Temperature on Growth. Experimental results showing the effects of growth at temperatures of 20, 25, 30 and 35°C are presented in Figure 3.4. In this study, only a limited amount of growth was observed at 20°C. Although the final cellular dry weights of cells harvested at 25, 30 and 35°C did not appear to vary, differences were observed in the length of time required for cultures to reach the stationary growth phase (Table 3-3).

During the exponential phase of growth, microorganisms divide at a constant rate (6). Thus:

TABLE 3-2

Composition of Basal Media

For the Cultivation of Rps. gelatinosa

KH_2PO_4	0.5 g
K_2HPO_4	0.5 g
$(NH_4)_2SO_4$	1.0 g
$MgSO_4 \cdot 7H_2O$	0.5 g
NaCl	23.0 g
Yeast Extract	2.0 g
DL-Malic Acid	2.0 g
Tap Water	1000 ml

Final pH adjusted to 7.2 ± 0.2.

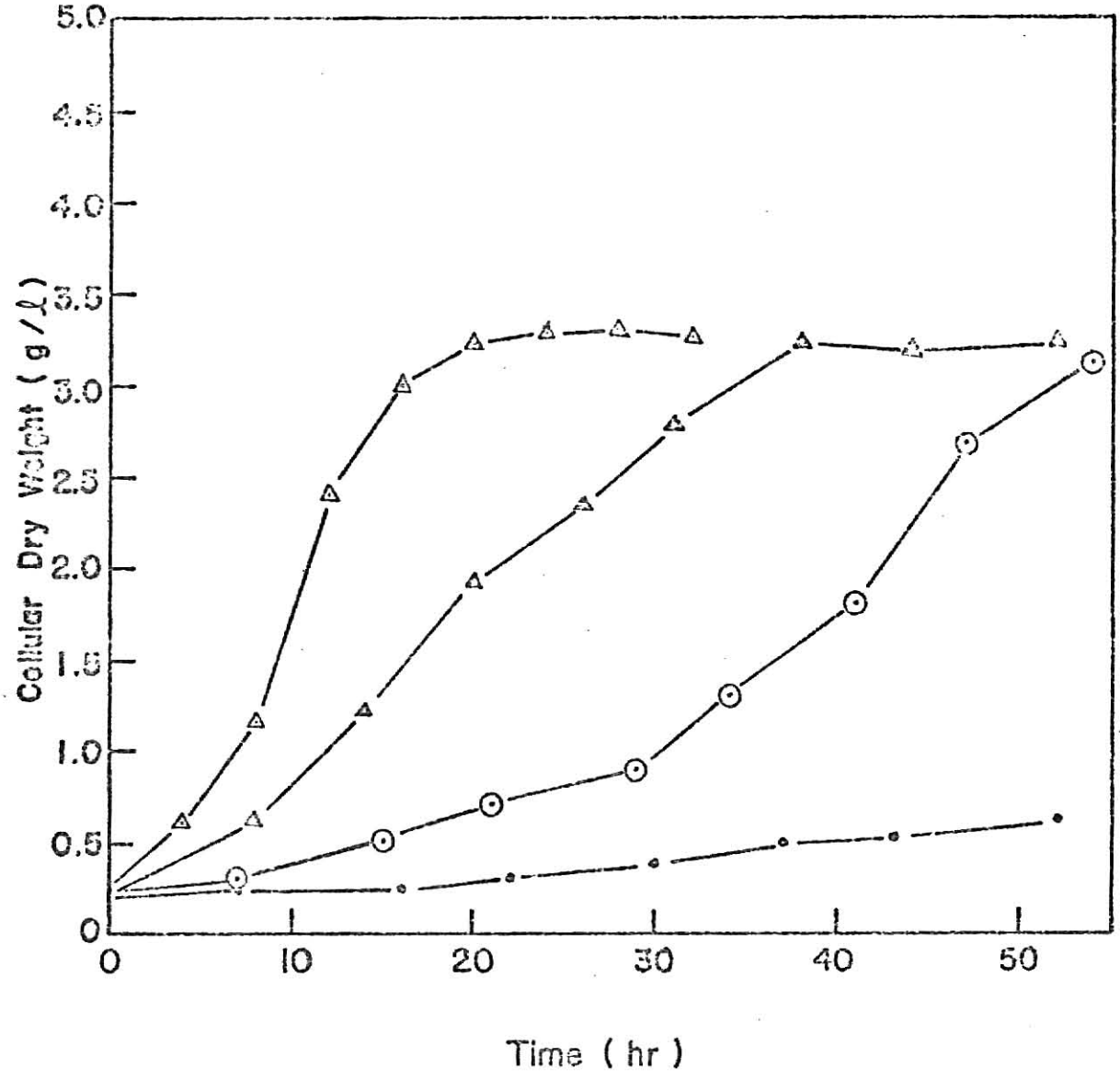

Fig.34 The Effect of Temperature on the Batch Growth of Rps. gelatinosa : (•) 20°C ; (⊙) 25°C ; (△) 30°C ; (△) 35°C .

TABLE 3-3

The Effect of Temperature on the Growth

Of Rps. gelatinosa

Temperature (°C)	Approximate Time Required To Reach Stationary Phase (hr)	μ (hr^{-1})	t_d (hr)	T (1/°K)
20	little growth observed	.039	17.8	.00341
25	54-56	.074	9.4	.00336
30	36-38	.076	9.1	.00330
35	16-18	0.175	4.0	.00325

$$\mu = \frac{1}{X}\left(\frac{dX}{dt}\right) = \frac{d(\ln X)}{dt} \qquad [1]$$

in which

X = concentration of organisms (g. dry wt./l)

t = time (hr)

μ = specific growth rate (hr^{-1})

Specific growth rate (μ) is also related to the generation time, i. e., the time required for an organism to double its mass, by the equation

$$t_d = \frac{\ln 2}{\mu} = \frac{0.693}{\mu} \qquad [2]$$

Values for specific growth rate (μ) and generation time (t_d) calculated from the data obtained at the various growth temperatures also appear in Table 3-3.

The dependence of specific growth rate on temperature may be expressed by the Arrhenius equation (7):

$$\frac{d(\ln \mu)}{dT} = \frac{E_a}{RT^2} \qquad [3]$$

in which

μ = specific growth rate (hr^{-1})

T = absolute temperature (^oK)

E_a = activation energy (k cal/mole·^oK)

R = gas constant (1.987 cal/mole·^oK)

Integration of Equation [3] gives

$$\ln \mu = \frac{-E_a}{RT} + C \qquad [4]$$

in which C is a constant. Upon converting the natural logarithm to the logarithm of base 10, Equation [4] becomes:

$$\log \mu = \frac{-E_a}{2.303RT} + C \qquad [5]$$

In an Arrhenius plot in which specific growth rate is plotted against temperature, the value $-E_a/2.303R$ represents the slope. A semi-log plot of experimental values of specific growth rate versus temperature appear in Figure 3.5. The linear relationship shown by this plot demonstrates the dependency of growth rate on temperature.

The growth activation energy (E_a) may be solved from Equation [4] or [5] as follows:

$$\log\left(\frac{\mu_2}{\mu_1}\right) = \frac{-E_a}{2.303R}\left(\frac{1}{T_2} - \frac{1}{T_1}\right) \qquad [6]$$

or

$$E_a = \frac{-2.303R \log\left(\frac{\mu_2}{\mu_1}\right)}{\frac{1}{T_2} - \frac{1}{T_1}} \qquad [7]$$

This implies that E_a can be obtained from the slope of the regression line in Figure 3.5. The result is:

$E_a = 16.5$ k cal/mole

The Effect of pH on Growth. Sterile culture media in this batch cultivation study ranged from pH 2.0 to 9.0 and was adjusted

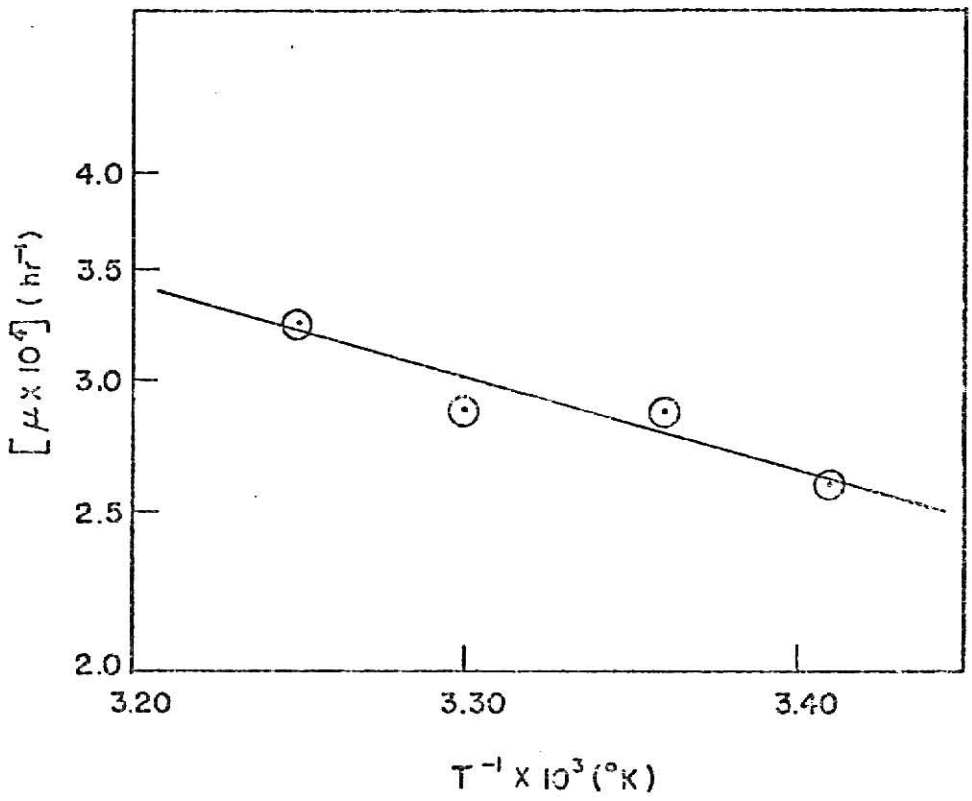

Fig. 3.5 Logarithmic Plot of Specific Growth Rate (μ) versus Temperature.

with 0.2N NaOH prior to inoculation. Cultures of Rps. gelatinosa were incubated at 30°C for forty-eight hours.

Experimental results showing the effects of initial pH on growth are shown in Table 3-4. Maximal growth, expressed as total cellular dry weight (g/l), was observed within a limited pH range of 7.0 ± 1.0 with a peak around pH 7.0. Experimental values and a normal curve fitted to the experimental data appear in Figure 3.6. Although a plot of the experimental data resembles a normal-type distribution, the pH range allowing maximal growth is much narrower than for a true normal distribution.

The Effect of Ammonium Sulfate Concentration on Growth. In order to evaluate the effects of nitrogen source on growth, Rps. gelatinosa was cultured in media containing varying concentrations of ammonium sulfate. In this study, culture media was supplemented with concentrations of ammonium sulfate varying from 0.5 to 5.0 g/l. Experimental results showing the effects of ammonium sulfate on growth are depicted in Figure 3.7. Maximum cellular dry weights obtained for varying ammonium sulfate concentrations are shown in Table 3-5. Growth appeared to increase with increasing concentration until reaching a maximum at 0.2 percent ammonium sulfate (Figure 3.8).

TABLE 3-4

The Effects of pH on the Batch Growth

Of Rps. gelatinosa

Initial pH	Maximum Optical Density (650 mμ)	Cellular Dry Weight (g/l)
2.0	0.18	0.17
3.0	0.21	0.20
4.0	0.24	0.23
5.0	0.38	0.36
5.5	0.69	0.65
6.0	2.32	2.18
6.5	2.70	2.53
7.0	2.86	2.68
7.5	2.64	2.48
8.0	2.51	2.35
8.5	1.40	1.31
9.0	0.45	0.42
10.0	0.23	0.22
11.0	0.20	0.19

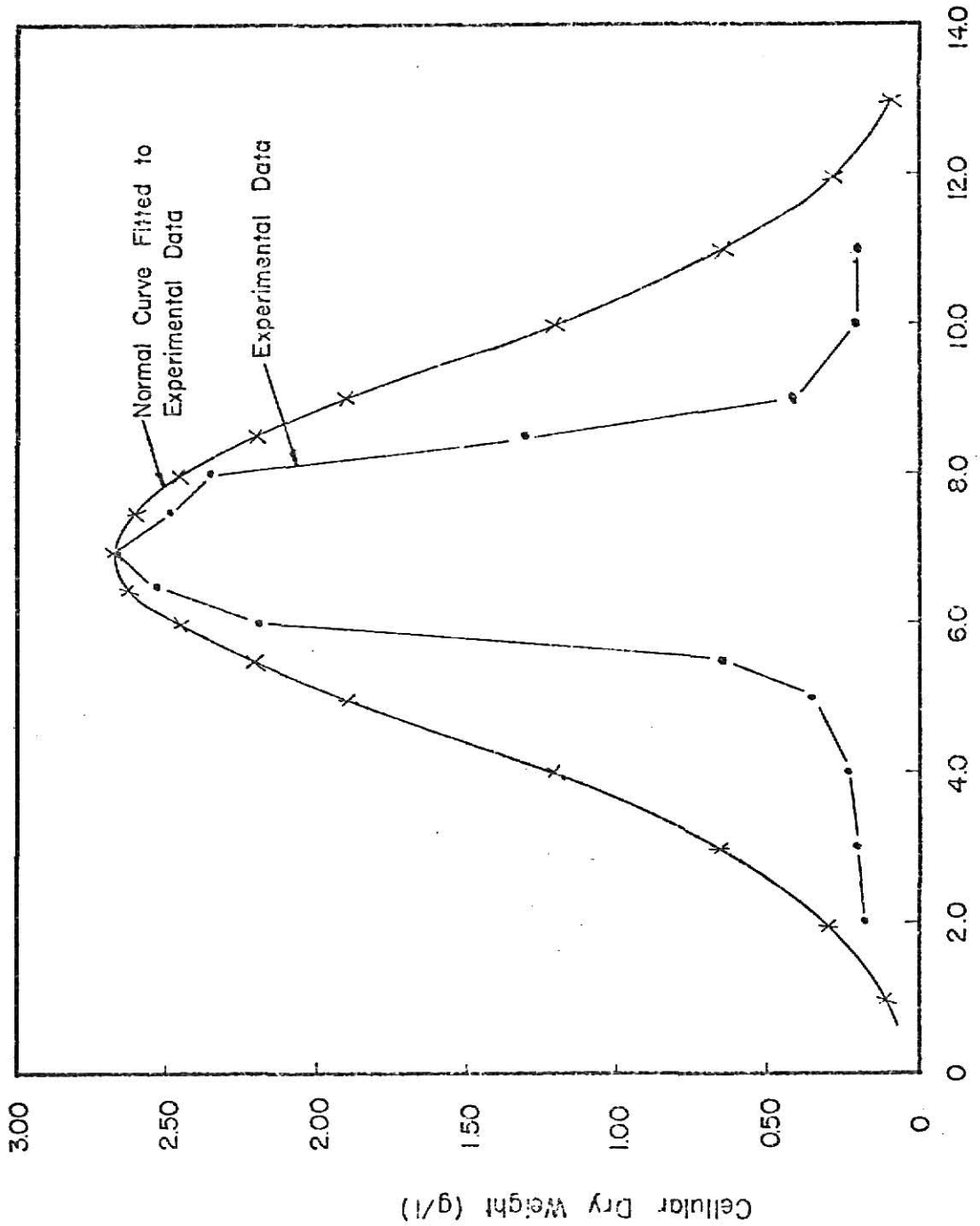

Fig. 3.6 Plot of Cellular Dry Weight versus pH.

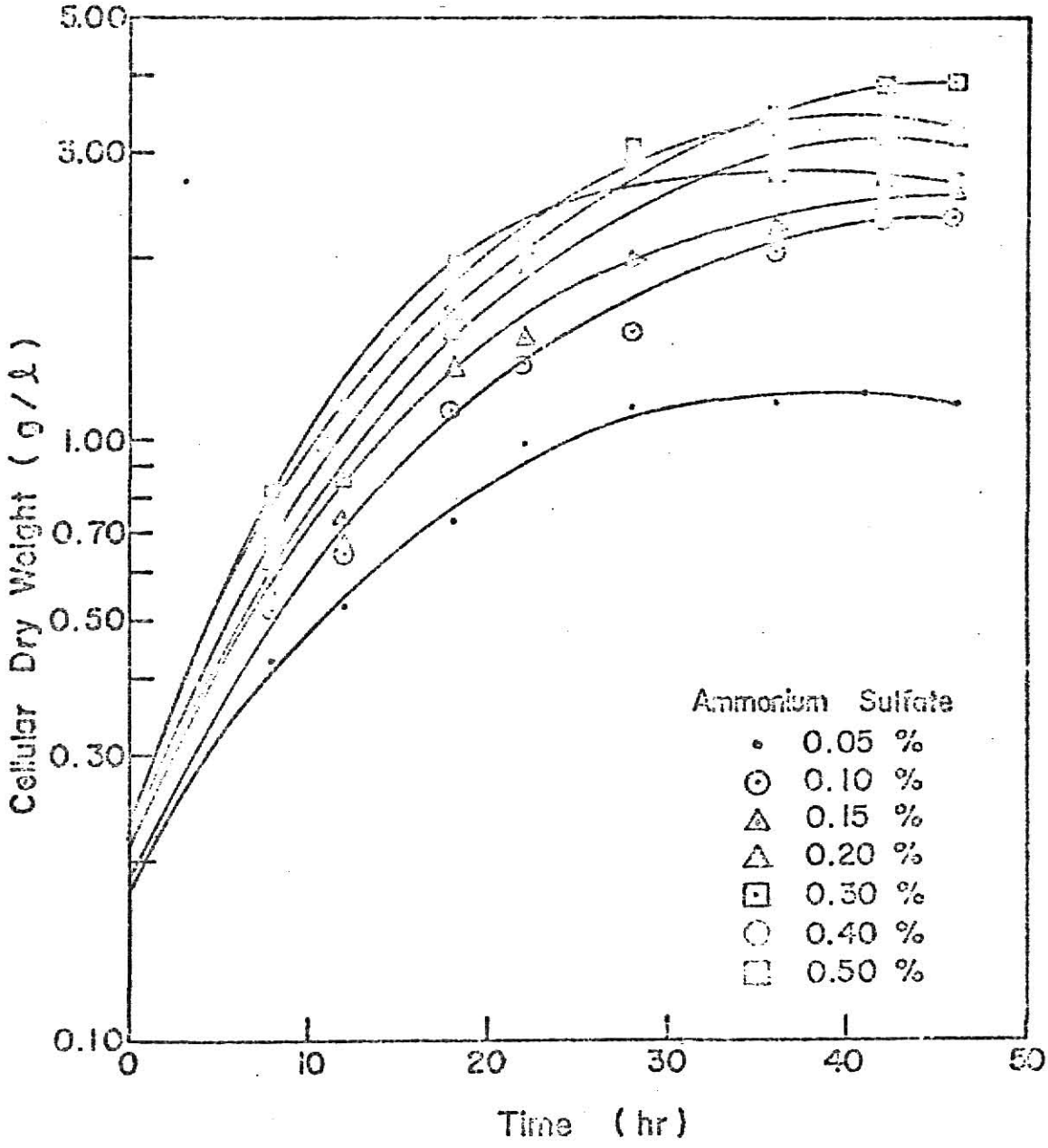

Fig. 3.7 The Effects of Initial Ammonium Sulfate Concentration on the Batch Growth of Rps. gelatinosa.

TABLE 3-5

The Effect of Ammonium Sulfate Concentration

On Maximum Cellular Dry Weight

And Specific Growth Rate (μ)

Initial Ammonium Sulfate Concentration (%)	Maximum Cellular Dry Weight (g/l)	μ (hr^{-1})
0.05	1.29	0.109
0.10	2.33	0.126
0.15	2.56	0.126
0.20	3.91	0.137
0.30	3.31	0.110
0.40	3.30	0.137
0.50	2.76	0.152

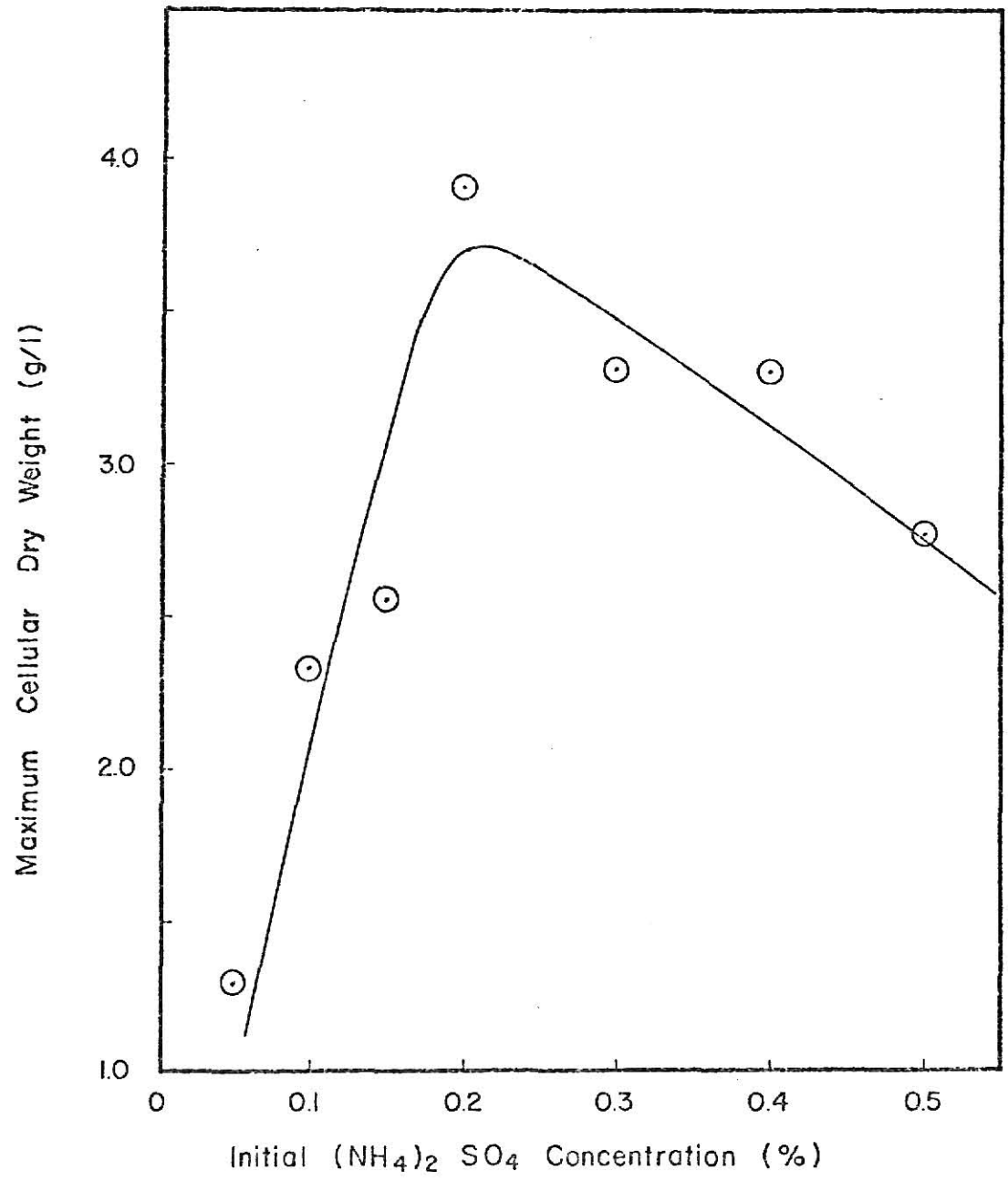

Fig. 3.8 Plot of Maximum Cellular Dry Weight versus Ammonium Sulfate Concentration.

The Effect of Malic Acid Concentration on Growth. Malic acid, a dicarboxylic acid and an important intermediate in the metabolism of many microorganisms, is an ideal substrate for culturing Rps. gelatinosa. As a substrate, malic acid not only provides the essential carbon skeleton (CO_2) but in addition, supplies the necessary reducing power (H_2) required for photosynthetic growth.

Growth of Rps. gelatinosa in culture media, supplemented with DL-malic acid (Fisher) in concentrations varying from 0.5 to 10.0 g/l, is shown in Figure 3.9. Maximum cellular dry weights obtained for each substrate concentration and calculated values for specific growth rate (μ) appear in Table 3-6. Maximum cellular dry weight plotted against malic acid concentration in Figure 3.10 shows growth increasing maximally with increasing substrate concentration. However, at concentrations beyond 0.5 percent malic acid, growth decreases as the organisms become saturated with substrate. This same phenomenon may also be shown by plotting specific growth rate against malic acid concentration (Figure 3.11).

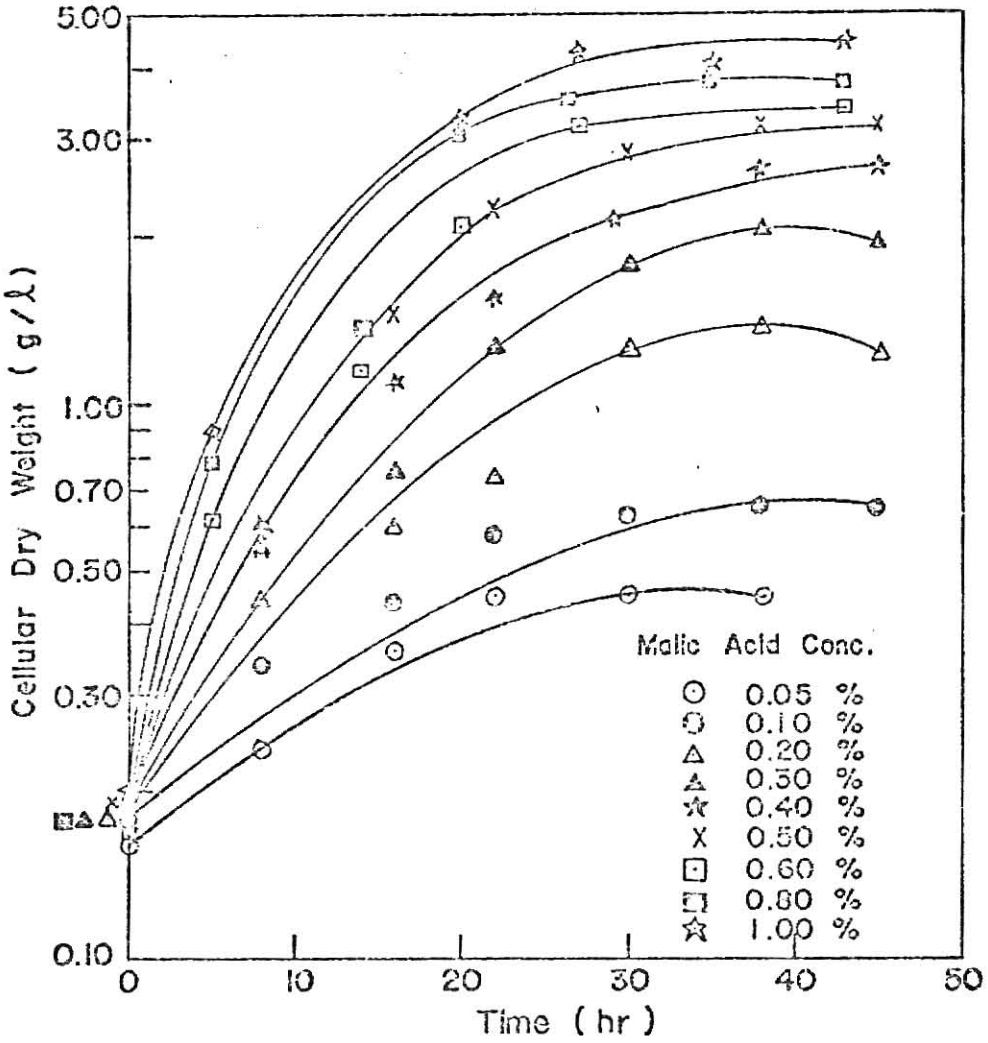

Fig. 3.9 The Effect of Initial Malic Acid Concentration on the Batch Growth of Rps. gelatinosa.

TABLE 3-6

The Effect of Malic Acid Concentration

On Maximum Cellular Dry Weight

And Specific Growth Rate (μ)

Initial Malic Acid Concentration (%)	Maximum Cellular Dry Weight (g/l)	μ (hr^{-1})
0.05	0.47	0.056
0.10	0.66	0.073
0.20	1.39	0.114
0.30	2.03	0.139
0.40	2.68	0.144
0.50	3.27	0.133
0.60	3.37	0.259
0.80	3.77	0.293
1.00	4.32	0.298

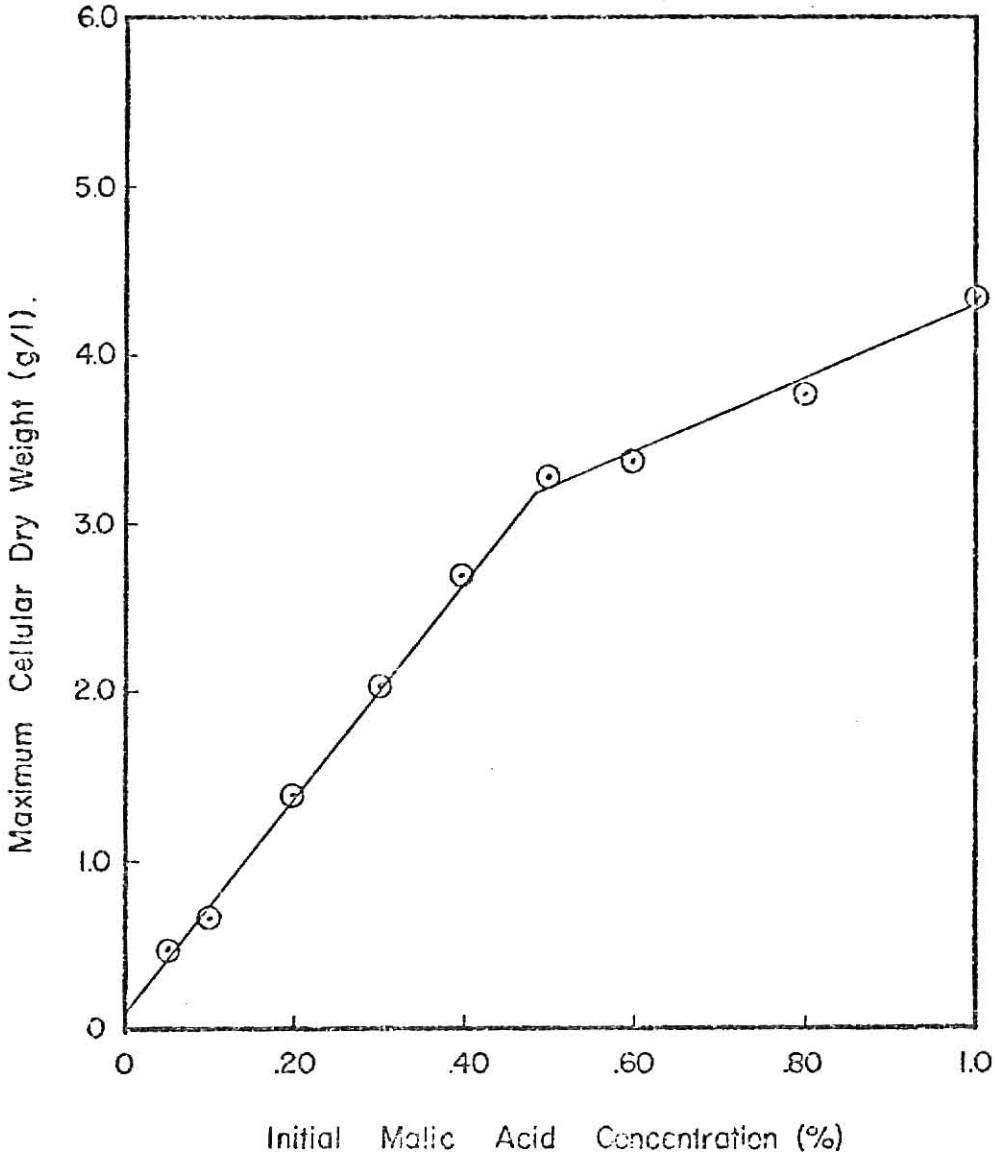

Fig. 3.10 Plot of Maximum Cellular Dry Weight against Initial Malic Acid Concentration.

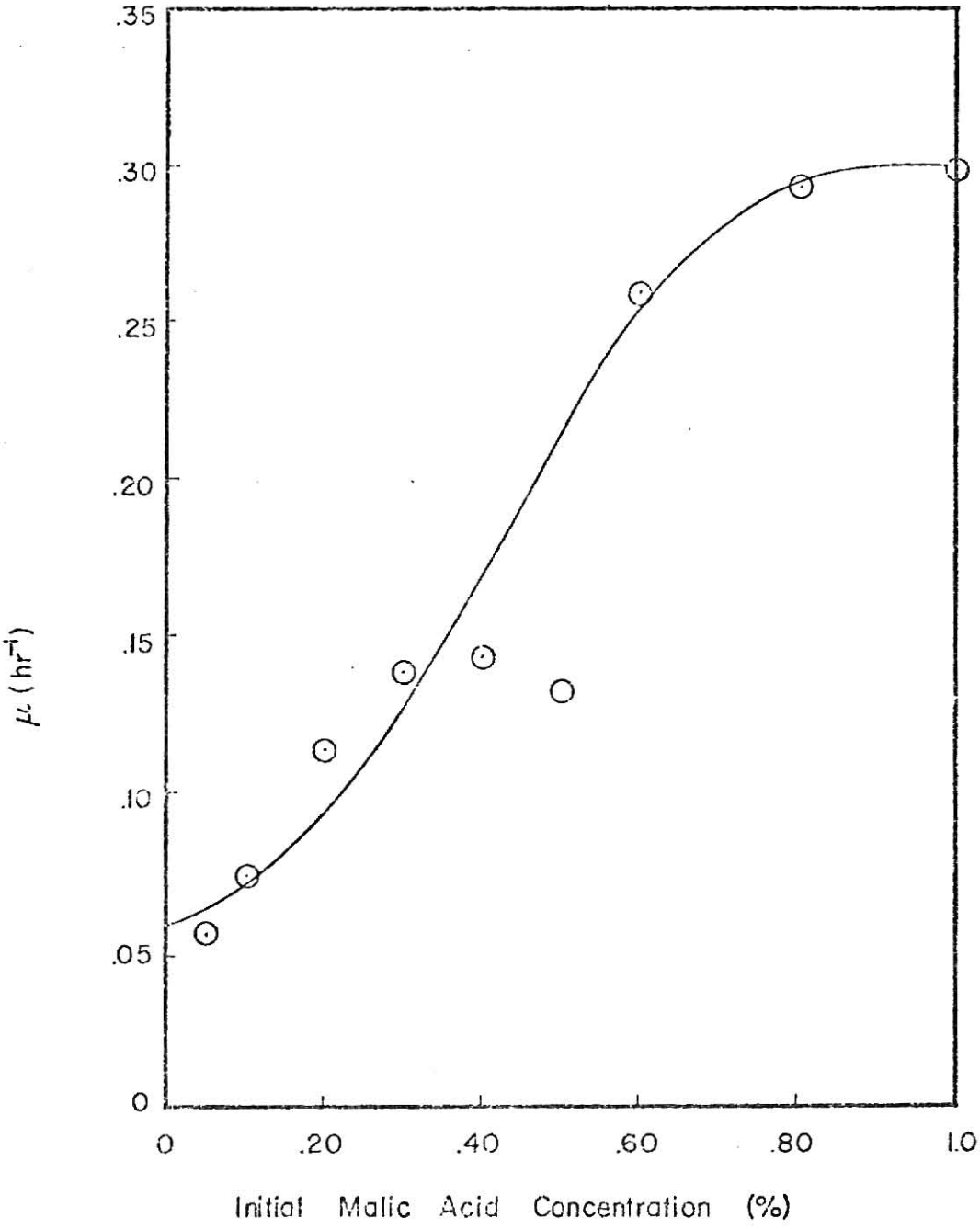

Fig. 3.11 Plot of Specific Growth Rate (μ) versus Initial Malic Acid Concentration.

The Effect of Yeast Extract Concentration on Growth.

Supplementation of culture media with yeast extract reportedly enhances the growth of Rps. gelatinosa (5). Yeast extract not only provides an additional substrate source, i. e., amino acids, peptides, etc., but also provides a rich source of vitamins and other unknown factors which promote the growth of nonsulfur photosynthetic bacteria.

In these investigations, Rps. gelatinosa was cultured in media supplemented with yeast extract (BBL) varying from 0.4 to 30.0 g/l. Results of this study appear in Figure 3.12. Maximum cellular dry weight and values for specific growth rate (μ) are shown in Table 3-7. Growth appeared to increase with increasing yeast extract concentration; however, beyond a concentration of 1.0 percent, little increase in growth was observed (Figure 3.13). Similar results were obtained by plotting specific growth rate (μ) against yeast extract concentration (Figure 3.14). These results suggest that yeast

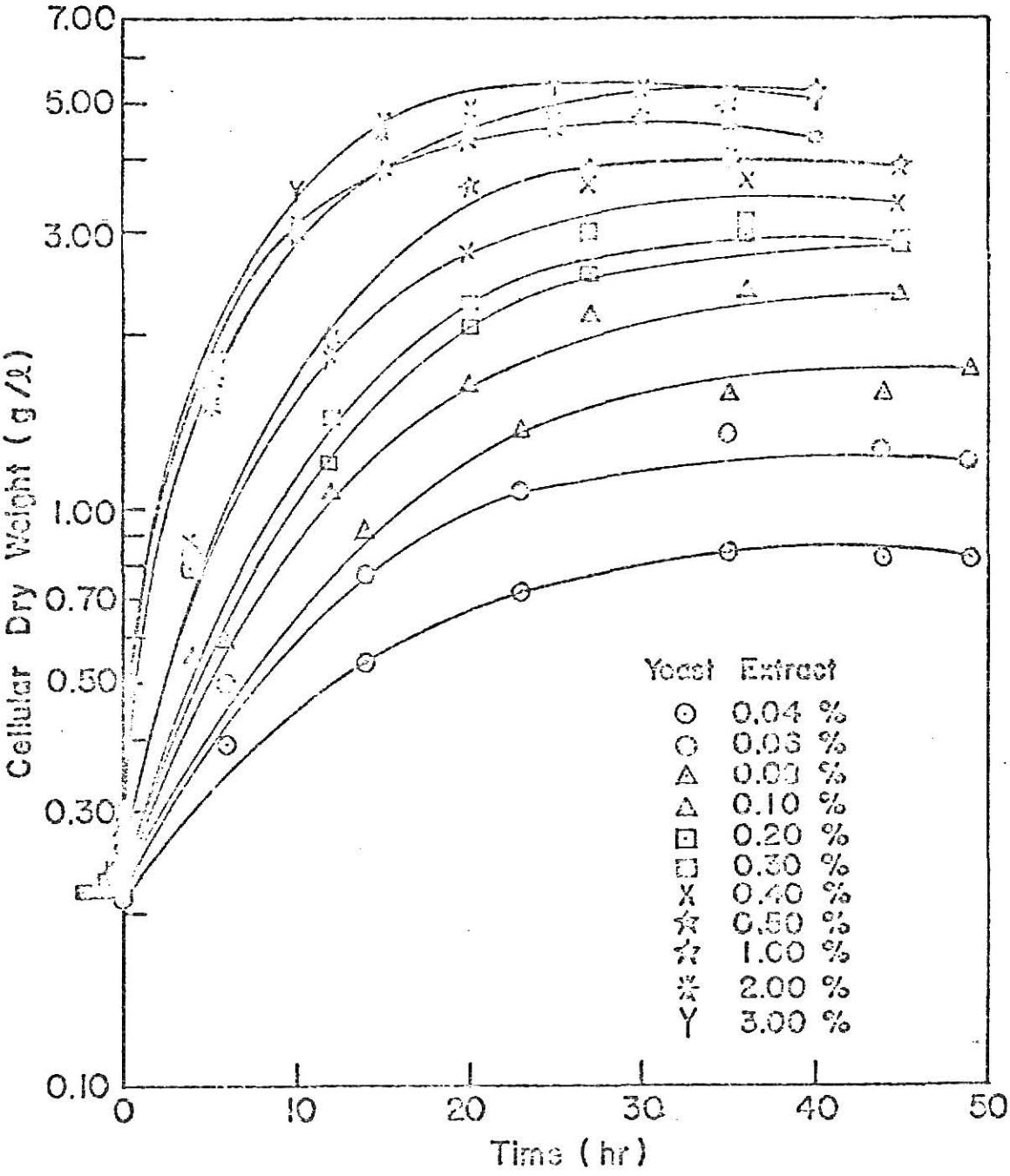

Fig. 3.12 The Effect of Initial Yeast Extract Concentration on the Batch Growth of Rps. gelatinosa .

TABLE 3-7

The Effect of Yeast Extract Concentration

On Maximum Cellular Dry Weight

And Specific Growth Rate (μ)

Initial Yeast Extract Concentration (%)	Maximum Cellular Dry Weight (g/l)	μ (hr^{-1})
0.04	0.84	0.103
0.06	1.31	0.143
0.08	1.72	0.157
0.10	2.39	0.234
0.20	2.93	0.308
0.30	3.10	0.335
0.40	3.66	0.312
0.50	3.92	0.307
1.00	5.16	0.353
2.00	5.09	0.350
3.00	5.19	0.358

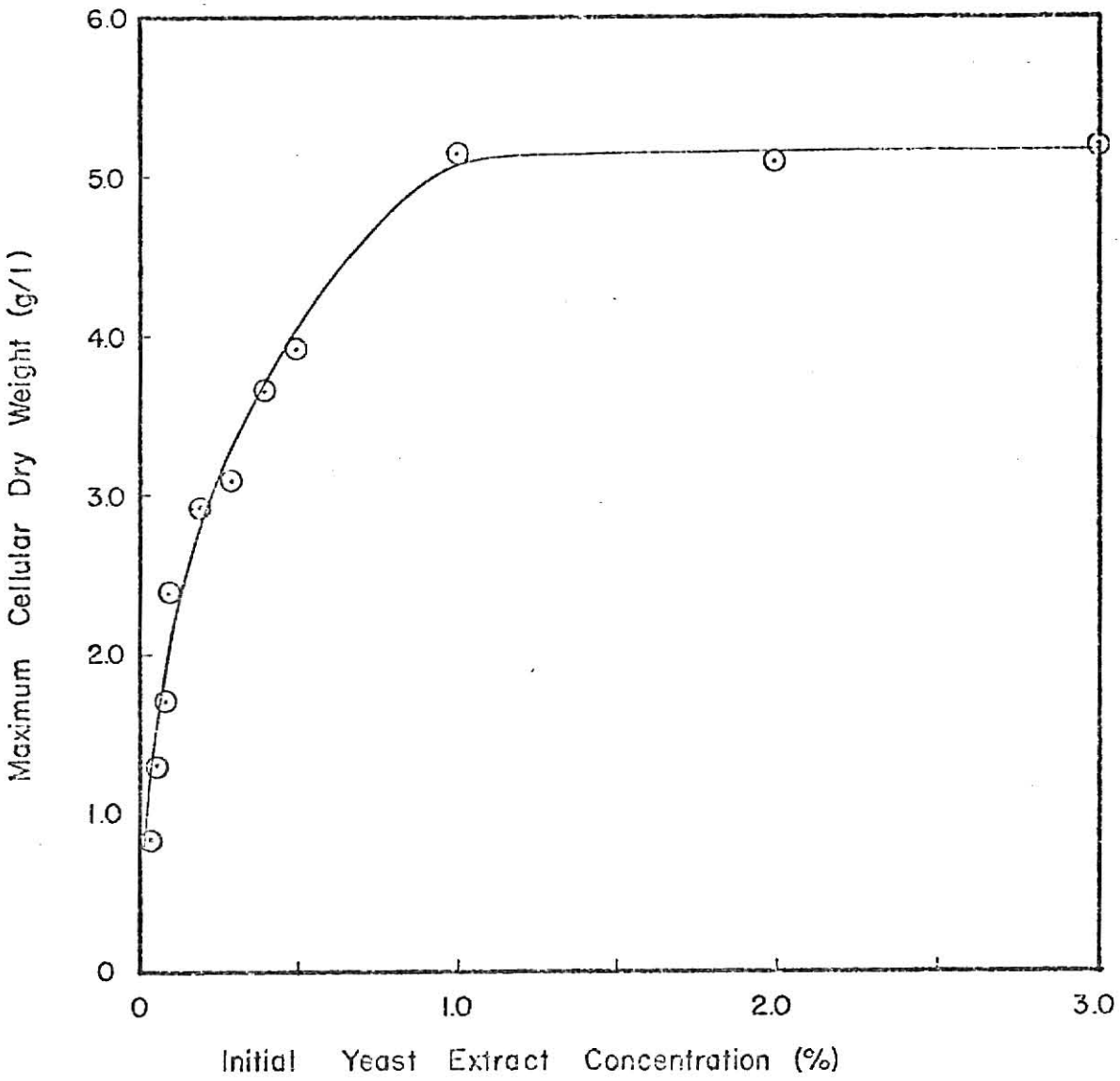

Fig. 3.13 Plot of Maximum Cellular Dry Weight versus Initial Yeast Extract Concentration.

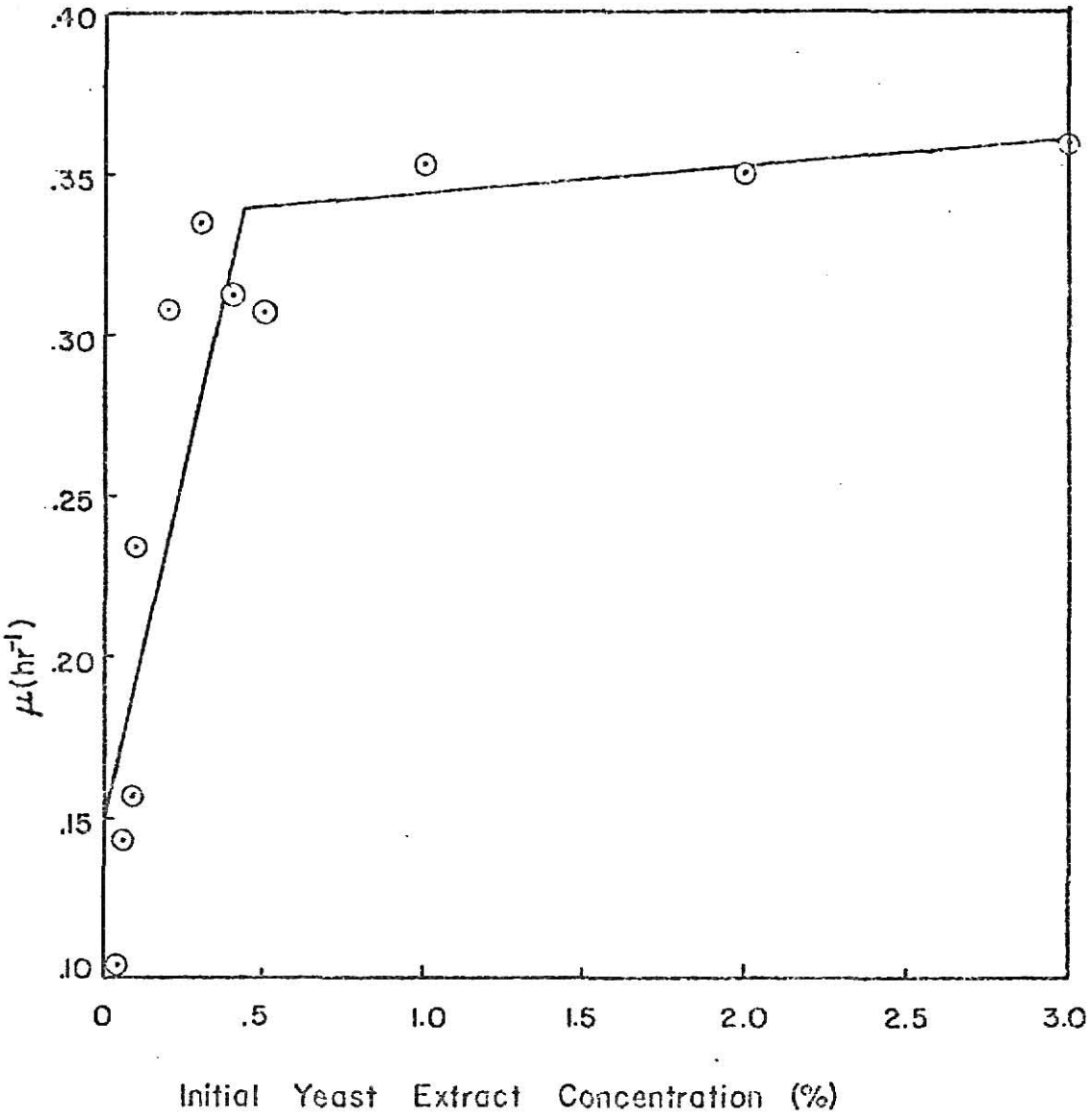

Fig. 3.14 Plot of Specific Growth Rate (μ) versus Initial Yeast Extract Concentration.

extract concentrations, beyond 0.5 percent, exhibit a saturation effect, in which the organisms become saturated with substrate.

Growth of Rps. gelatinosa in a Synthetic Media. Several experiments were conducted in which thiamine • HCl (Sigma Chemical Co.) and biotin (Sigma Chemical Co.) were substituted in place of yeast extract as supplemental sources of vitamins. Cultivations were performed under similar conditions as preceeding experiments in order to evaluate the feasibility of culturing Rps. gelatinosa in a synthetic media. Further studies designed to examine the effects of vitamin concentration on growth were inconclusive, nevertheless, it was determined that biotin (5.0 μg/l) and thiamine•HCl (10 mg/l) would provide adequate growth.

Experimental results comparing the growth of Rps. gelatinosa in synthetic media to growth in yeast extract-supplemented media appear in Table 3-8. Although cellular growth in synthetic media appeared somewhat less than in media containing yeast extract, the enhanced growth of the latter most probably resulted from additional metabolizable compounds, i. e., proteins and carbohydrates, provided by yeast extract (see Table 3-9). In addition to supplying essential vitamins required for growth, i. e., thiamine and biotin (8), yeast extract is also rich in other vitamins which may have a stimulating effect on the growth of Rps. gelatinosa (see Table 3-10).

TABLE 3-8

Growth of Rps. gelatinosa in Synthetic Media

And Yeast Extract-Supplemented Media

Malic Acid Conc. (g/l)	Maximum Cellular Dry Weight (g/l) 1% Yeast Extract	Synthetic*
10.0	4.28	3.66
5.0	3.16	2.02
1.0	0.58	0.32

*Synthetic media contained biotin (5 μg/l) and thiamine·HCl (10 mg/l) in place of yeast extract.

TABLE 3-9

Composition of Yeast Extract (9)

Constituent	% W/W
Moisture	29.5
Ash	19.0
Lipid	trace
Carbohydrate	7.7
Crude Protein	43.8
Amino Acids:	
Lysine	3.5
Tryptophan	0.6
Phenylalanine	2.1
Methionine	0.9
Threonine	2.5
Leucine	3.5
Isoleucine	2.8
Valine	2.6

TABLE 3-10

Vitamin Content of Yeast Extract (10)

Vitamin	Concentration (μg/g)
Thiamine	10
Riboflavin	20
Nicotinic Acid	400
Pantothenic Acid	50
Pyridoxine	25
Biotin	1.0
Inositol	1500
Choline	1500

POTENTIAL SUBSTRATES FOR THE MASS CULTIVATION OF Rps. gelatinosa

In laboratory-scale fermentations, malic acid and yeast extract are excellent substrates for culturing Rps. gelatinosa; however, malic acid and yeast extract are relatively expensive and thus economically unfeasible for large-scale production. Consequently, when large quantities of culture medium are required, it is necessary to find alternative sources of nutrients which are cheaper and readily available. The following study was undertaken to screen a variety of available carbon sources which would suffice as economical substrates for the mass cultivation of Rps. gelatinosa. In these investigations, corn starch, potato starch, molasses and wheat bran were examined as potential substrates.

Corn starch and potato starch provide a rich source of carbohydrates; however, when heated in the sterilization process, starch solutions gelatinize forming very viscous liquids. Concentrations less than 2.0 percent can usually be incorporated without modification. Gelatinized corn starch may be thinned either by using a mineral acid or an enzyme such as alpha-amylase. Acid-hydrolyzed starch solutions unfortunately can contain by-products which are toxic to yeasts and other microorganisms and therefore are unsatisfactory as substrates.

Different forms of molasses are obtained as by-products from the sugar-manufacturing industry. Available forms of molasses include

refiner's cane, blackstrap and beet molasses. Refiner's cane molasses is the residue remaining after refined white sugar has been recrystallized from crude, unrefined brown sugar. Blackstrap is the mother liquor from which crude sugar has been initially recovered. Beet molasses is the by-product from sugar beets. Although the sugar content of molasses is often expressed as total invert, not all sugars present are sucrose or invert. Molasses can have a high nitrogen content, being rich in asparagine; however, inorganic nitrogen forms such as betaine are not metabolized by microorganisms. In addition to being a rich source of biotin, molasses usually contain numerous other vitamins. The composition of molasses is shown in Table 3-11.

Wheat bran, an important by-product from the wheat milling industry containing substantial quantities of protein, fat and vitamins, for many years has been incorporated into many bakery products and cereals and has been an important component of many animal feeds. The composition of wheat bran is shown in Table 3-12.

Experimental Procedures

In order to evaluate the above-mentioned carbon sources as potential substrates for mass cultivation of Rps. gelatinosa, 1-liter Erlenmeyer flasks containing 900 ml of sterile media at pH 7.0 were inoculated with 100 ml of the seed culture. Batch cultivations were conducted at 30°C in an incubator-shaker at 100 rpm. Cultures were

TABLE 3-11

The Composition of Beet and Cane Molasses (11)

% Composition	Beet	Cane
Sucrose	48.5	33.4
Raffinose	1.0	-
Invert Sugars	1.0	21.2
Ash	10.8	9.8
Organic Non-Sugars	20.7	19.6
Water	18.0	16.0
Nitrogen	1.5-2.0	.08-0.5
Vitamins (µg/g)		
Thiamine	0.8	0.8
Riboflavin	-	-
Nicotinic Acid	35	15
Pantothenic Acid	50	20
Pyridoxine	-	-
Biotin	.04-.13	1.5-1.8
Inositol	5000	2000
Choline	-	-

TABLE 3-12

Composition of Wheat Bran (12)

Constituent	% Composition
Crude Protein	15.0
Amino Acids:	
Arginine	0.90
Lysine	0.60
Methionine	0.20
Cystine	0.25
Tryptophan	0.25
Fat	4.0
Crude Fiber	10.0
Ash	6.5

Vitamins	Composition(µg/g)
Pantothenic Acid	26.4
Riboflavin	2.6
Niacin	154.0
Choline	1040.0

continuously illuminated by two 75-watt light bulbs. Growth was monitored by the gravimetric method outlined in Appendix II.

Substrate Preparation. Special procedures used in the preparation of media were as follows:

(1) corn starch (acid-hydrolyzed)

(i) addition of starch to tap water

(ii) acidification to 2.0 N with conc. HCl

(iii) heat treatment; forty minutes at 121°C and fifteen psi steam

(iv) cooling

(v) neutralization of starch solution to pH 7.0 with 2.0 N NaOH.

(2) potato infusion

(i) addition of sliced whole potatoes ($\frac{1}{4}$" thickness) to tap water

(ii) heat treatment: ten minutes at 121°C and fifteen psi steam

(iii) cooling

(iv) separation

(v) filtration of liquid infusion

(3) wheat bran infusion (WBI)

(i) addition of wheat bran to tap water

(ii) heat treatment: fifteen minutes at 121°C and fifteen psi steam

(iii) cooling

(iv) separation

(v) filtration of liquid infusion

Results and Discussion

The experimental results of batch cultivations of Rps. gelatinosa on various substrates appear in Table 3-13. With the exception of wheat bran infusion, media supplementation with yeast extract appeared to enhance growth.

At this point a meaningful evaluation of the various substrates would be difficult since neither substrate utilization nor rate of consumption was investigated. These results, however, do indicate that complex carbohydrates such as wheat bran or potato infusion, commonly found as waste by-products of the food and milling industries, could serve as potential nutrient sources for the mass cultivation of Rps. gelatinosa.

CULTIVATION OF Rps. gelatinosa IN WHEAT BRAN INFUSION MEDIA

In the following study, Rps. gelatinosa was cultured in a wheat bran infusion media. In addition to providing excellent growth, wheat bran was selected as a suitable substrate material for these investigations primarily because it was abundant, inexpensive and easily handled.

TABLE 3-13

The Effect of Substrate on the Growth
Of Rps. gelatinosa

Media Constituents	Maximum Cellular Dry Weight (g/l)
0.2% glucose, 0.1% y.ext.[1]	1.69
1.0% corn starch (acid hydrolyzed), 0.1% y. ext.	2.70
0.2% corn starch, 0.1% y. ext.	1.18
0.2% corn starch, vitamins[2]	1.04
0.2% sucrose, 0.1% y. ext.	1.66
1.0% molasses (RJR Foods Inc.), 0.1% y. ext.	2.46
0.2% molasses, 0.1% y. ext.	1.22
0.2% molasses, vitamins	0.84
5.0% potato infusion,[3] 0.1% y. ext.	4.92
5.0% potato infusion, vitamins	4.32
2.0% potato infusion, 0.1% y. ext.	2.65
2.0% potato infusion, vitamins	2.07
5.0% wheat bran infusion,[4] 0.1% y.ext.	6.60
5.0% wheat bran infusion, vitamins	6.54
2.0% wheat bran infusion, 0.1% y. ext.	3.62
2.0% wheat bran infusion, vitamins	3.65

[1] y. ext. - yeast extract

[2] vitamins - biotin (5 µg/l) and thiamine·HCl (10 mg/l)

[3] potato infusion - weight of whole potatoes used in preparation of infusion

[4] wheat bran infusion - weight of wheat bran used in preparation of infusion

Procedures for the preparation of wheat bran infusion were relatively simple and could easily be carried out on an experimental scale.

Experimental Procedures

Batch Cultivation of Rps. gelatinosa in Wheat Bran Infusion Media.

In these studies, 1-liter Erlenmeyer flasks containing 900 ml of sterile media at pH 7.0 were inoculated with 100 ml of a pure culture of Rps. gelatinosa. Cultivations were performed at 30°C in an incubator-shaker at 100 rpm under continuous illumination. Growth was monitored by determining cellular dry weights according to methods outlined in Appendix II.

Batch Cultivation in a Completely-Stirred Jar Fermentor. In this study, Rps. gelatinosa was cultured in a 4-liter bench-type fermentor (Fermentation Design Inc.) equipped with agitation and temperature control as well as ports for inoculation and aseptic sampling. The fermentor vessel was constructed of Pyrex glass, approximately 7-8 mm in thickness. Illumination was provided by positioning four 75-watt incandescent light bulbs at the periphery of the fermentor vessel.

To 3600 ml of sterile 3.0 percent wheat bran infusion medium at pH 7.0 were added 400 ml of a pure culture of Rps. gelatinosa. Photosynthetic cultivation was allowed to proceed at 30°C until growth reached the stationary phase. Batch growth was continuously monitored through the use of optical density measurements and dry weight

determinations (Appendix II). The rate of substrate assimilation was determined by measuring the chemical oxygen demand (COD) as described by methods outlined in Appendix III.

Photosynthetic batch cultivation was terminated shortly after cultures had reached the stationary growth phase. At this time a 0.5 l aliquot of culture broth was withdrawn from the fermentor, centrifuged, resuspended in distilled water, and recentrifuged. Harvested photosynthetic cells were oven-dried overnight at 95°C. Total protein was determined by the micro-Kjeldahl method described by Mitchell (13). An outline of this method is described in Appendix IV.

Results and Discussion

The Effect of Wheat Bran Infusion (WBI) Concentration on Growth.

The experimental results obtained from culturing Rps. gelatinosa in concentrations of WBI media varying from 0.5 to 6.0 percent are shown in Figure 3.15. Maximum cellular dry weights and values of specific growth rate (μ) calculated for differing concentrations of wheat bran infusion appear in Table 3-14. Concentrations of wheat bran infusion greater than 6.0 percent were not used in this study due to increasing difficulties in separation arising from starch gelatinization and from filtration of the wheat bran infusion. In a plot of cellular dry weight versus concentration of wheat bran infusion (Figure 3.16), growth was observed to increase proportionately with substrate concentration. The

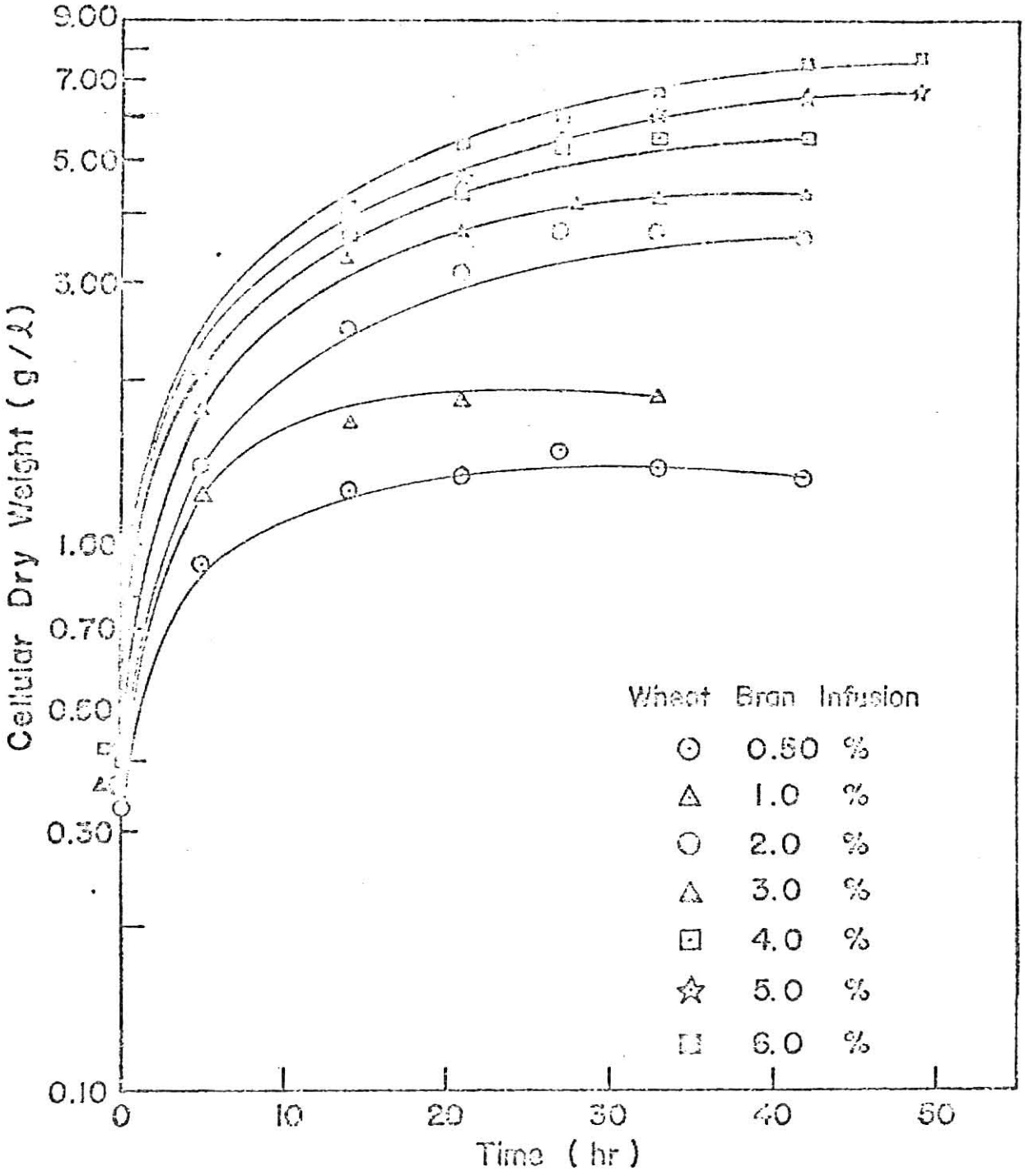

Fig. 3.15 The Effect of Wheat Bran Infusion Concentration on the Batch Growth of Rps. gelatinosa.

TABLE 3-14

The Effect of Wheat Bran Infusion (WBI) Concentration

On Maximum Cellular Dry Weight

And Specific Growth Rate (μ)

Initial WBI Concentration (%)	Maximum Cellular Dry Weight (g/l)	μ (hr^{-1})
0.5	1.48	0.205
1.0	1.91	0.249
2.0	3.70	0.271
3.0	4.24	0.318
4.0	5.47	0.330
5.0	6.62	0.318
6.0	7.45	0.320

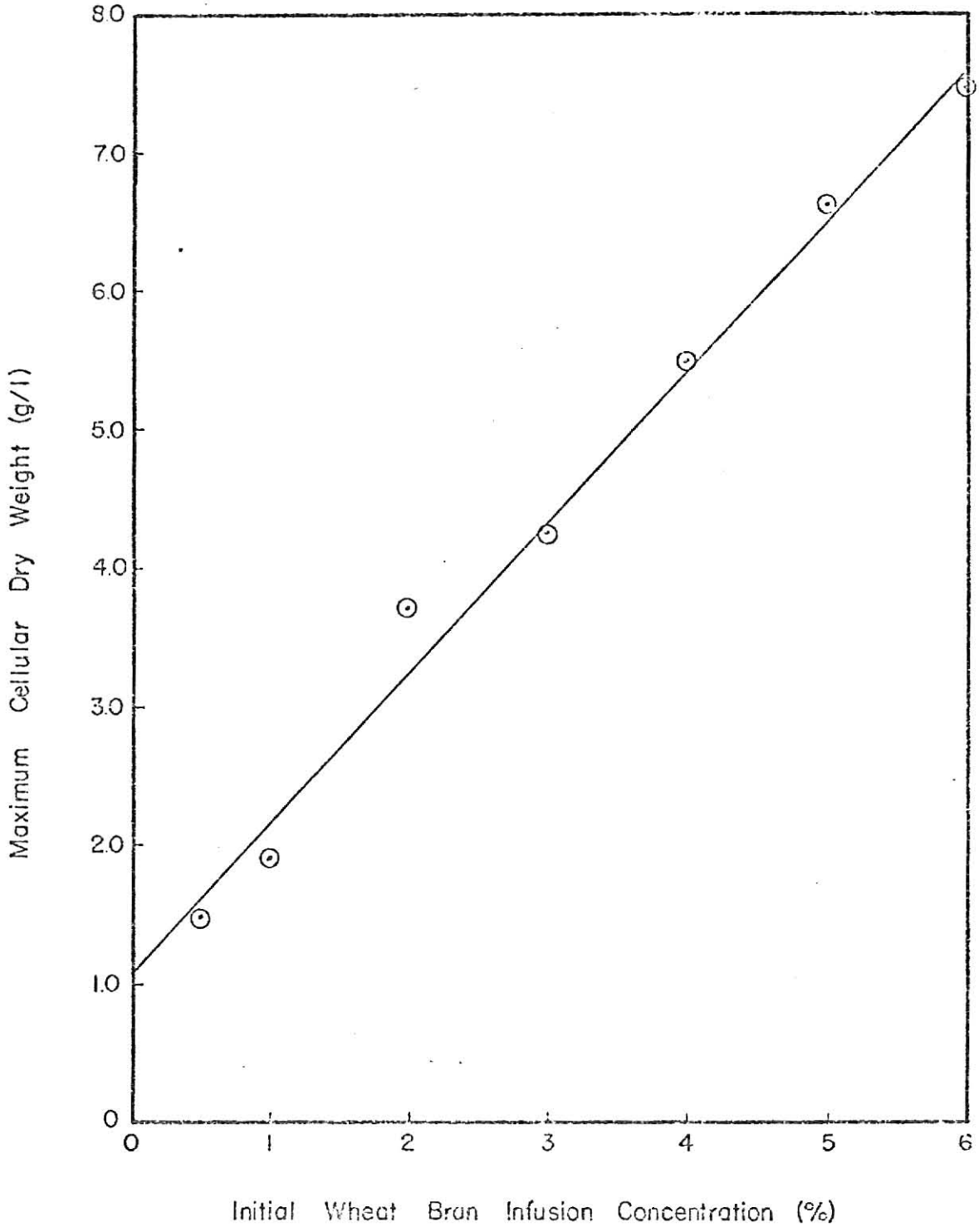

Fig. 3.16 Plot of Maximum Cellular Dry Weight against Initial Wheat Bran Infusion Concentration.

maximum cellular dry weight attained was 7.45 g/l in 6.0 percent WBI media. In Figure 3.17, specific growth rate (μ) was plotted against initial concentrations of WBI. At concentrations below 3.0 percent, specific growth rate (μ) appeared to be dependent on substrate concentration, while concentrations of WBI greater than 3.0 percent did not appreciably change values for μ.

The Effect of Sodium Bicarbonate on Growth. In previous experiments, addition of sodium bicarbonate, when added to wheat bran infusion medium, significantly enhanced the photosynthetic growth of Rps. gelatinosa. The results of bicarbonate supplementation, varying from 0.1 to 0.5 percent, on growth in 3.0 percent WBI media appear in Table 3-15. In general, bicarbonate addition appeared to enhance photosynthetic growth with concentrations of 0.2 percent sodium bicarbonate appearing to promote maximal growth.

Photosynthetic Cultivation in a Completely-Stirred Jar Fermentor. Substrate utilization and cellular growth of Rps. gelatinosa in 3.0 percent WBI media in a completely-stirred fermentor appear in Figure 3.18. A maximum cellular dry weight of 4.33 g/l was observed after approximately thirty-five hours of photosynthetic cultivation. Substrate utilization monitored by standard COD determinations resulted in approximately sixty-seven percent reduction in initial COD (Table 3-16). Harvested photosynthetic cells averaged 54.7 percent crude protein.

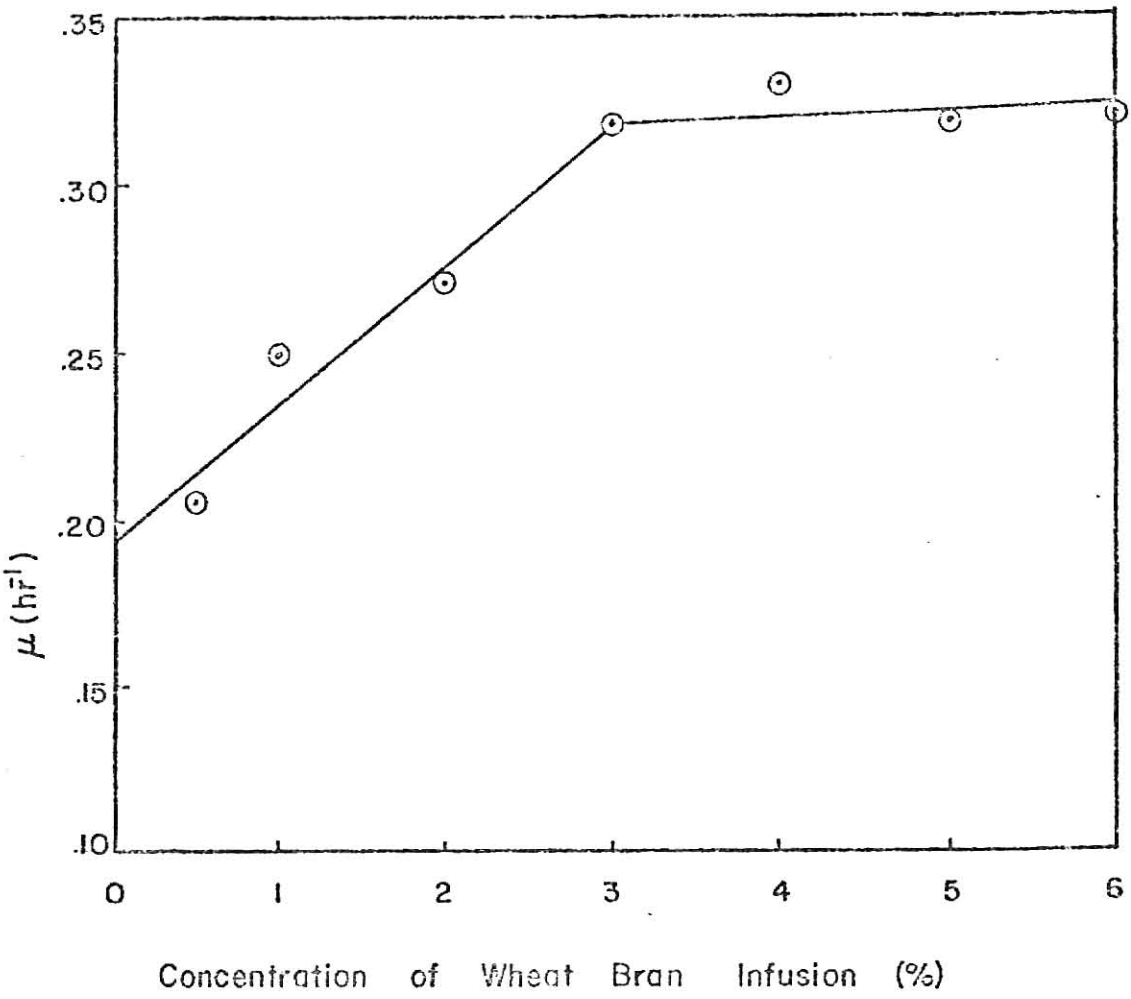

Fig. 3.17 Plot of Specific Growth Rate (μ) versus Wheat Bran Infusion Concentration.

TABLE 3-15

Effects of Sodium Bicarbonate Concentration

On the Growth of Rps. gelatinosa

Sodium Bicarbonate (g/l)	Maximum Cellular Dry Weight (g/l)
0	1.32
1.0	1.78
2.0	1.93
3.0	1.90
4.0	1.88
5.0	2.05

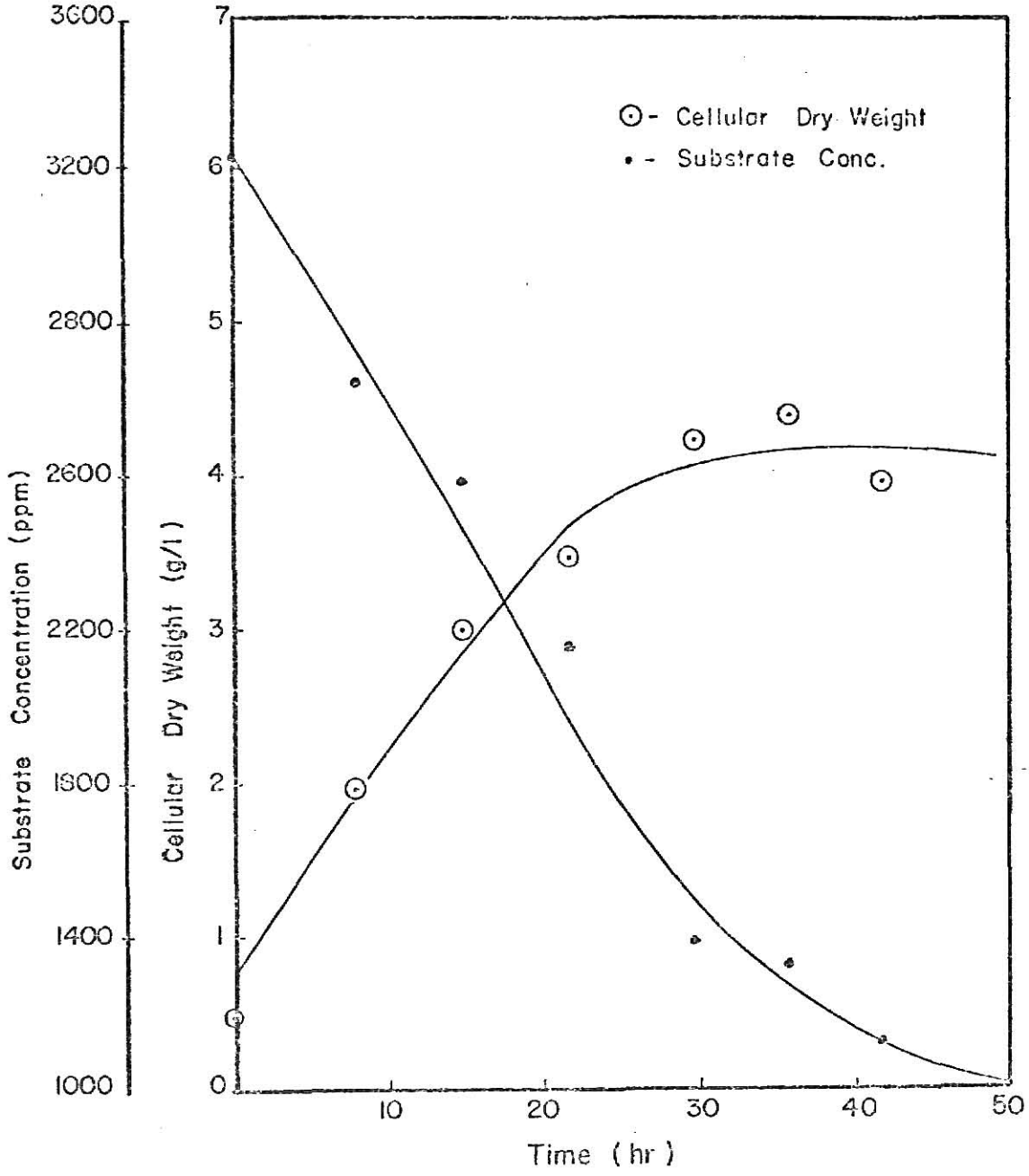

Fig. 3.18. Batch Growth of Rps. gelatinosa in 3.0 % Wheat Bran Infusion Medium.

TABLE 3-16

Cultivation of Rps. gelatinosa

In a Completely-Stirred Fermentor

Cellular growth:	
Maximum cellular dry weight -	4.33 g/l
Substrate utilization:	
Initial substrate concentration (COD) -	3260 ppm
Final substrate concentration (COD) -	1083 ppm
Effective COD reduction -	67%
Chemical composition:	
Crude protein content of harvested cells (%N X 6.25) -	54.7%

NOMENCLATURE

E_a = activation energy (k cal/mole)

R = gas constant (1.987 cal/mole·°K)

T = absolute temperature (°K)

t = time (hr)

t_d = mass-doubling time (hr)

X = cell concentration (g/l)

Greek Letters

μ = specific growth rate (hr^{-1})

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CHAPTER IV

CONTINUOUS CULTIVATION OF *Rhodopseudomonas gelatinosa* IN A SINGLE-STAGE FERMENTOR

INTRODUCTION

During the exponential phase of growth of a microorganism in batch culture, nutrients are continuously depleted from the medium while waste products of metabolism accumulate. Either or both of these factors result in a decreased rate of growth and the culture eventually enters into a stationary phase. However, if fresh nutrients are supplied to the culture and exhausted media and by-products removed at a constant rate, microorganisms can be maintained indefinitely in the exponential phase of growth (1). Continuous culture has become an important tool in maintaining microbial growth at a constant rate in a constant environment. Factors such as pH, concentrations of nutrients, metabolic by-products and oxygen which continuously change during the "growth cycle" of batch growth are constantly maintained in continuous culture and may be independently controlled by the experimenter. Continuous culture is thus an important investigative technique for determining and controlling specific kinetic parameters (2,3).

Continuous culture is industrially important since the productivity of continuous fermentation is usually higher than for batch

culture. There are some notable disadvantages often ascribed to large-scale continuous processes (4). These include: (1) the possibility that the microbial species will undergo deleterious mutations; (2) technical difficulties of running aseptically for long periods of time; and (3) lack of knowledge on the dynamic aspects of microbial behavior.

The experiments described in the following section were concerned with the continuous cultivation of Rps. gelatinosa and establishing conditions providing for maximum productivity. In addition, harvested cellular material was analyzed for nucleic acids, crude protein and amino acids.

EXPERIMENTAL PROCEDURES FOR THE CONTINUOUS CULTIVATION OF Rps. gelatinosa IN A SINGLE-STAGE FERMENTOR

Experimental Design

In this study, a single 14-liter magnetically-driven jar fermentor (Fermentation Design Inc.) equipped with agitation and temperature control was used. The fermentor was also equipped with exhaust, inoculation and sampling ports. Illumination was provided by four 75-watt incandescent light bulbs positioned at the periphery of the culture vessel. The experimental set-up for the continuous cultivation study is shown in Figure 4.1.

A variable-speed tubing pump (Sigma) was used to transfer sterile media from the media reservoir into the fermentor. A flowmeter

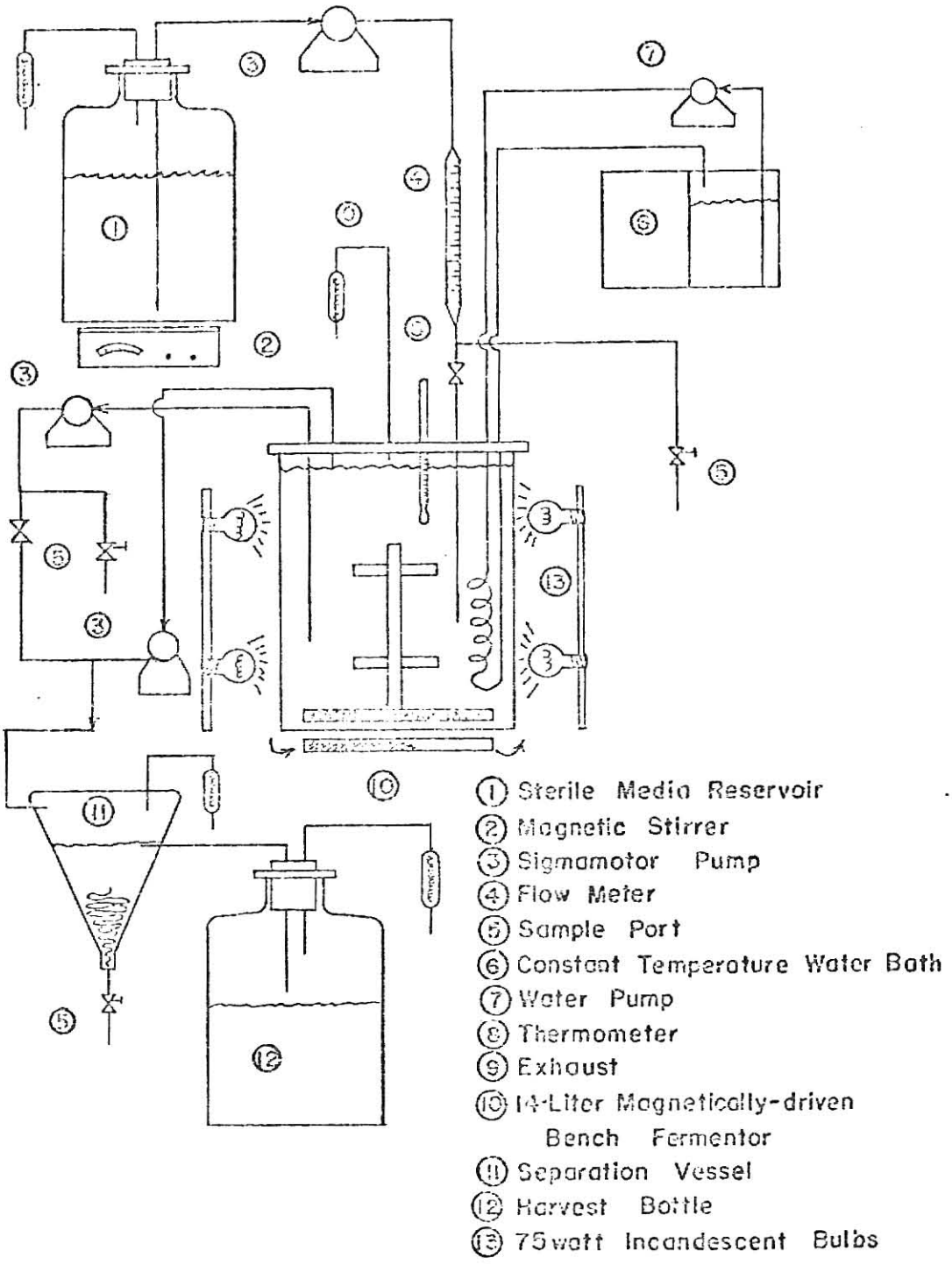


Fig.4.1 Experimental Set-up for the Continuous Cultivation of Photosynthetic Bacteria.

installed in the inlet line was used to measure and regulate the flow rate. The inlet line was also provided with a sampling port to obtain samples of media. Two variable-speed Sigma pumps operating at a slightly lower rate than the inlet feed were used to maintain a constant liquid level within the fermentor (see Figure 4.1). The media discharge line was also provided with a sampling port in order to sample from the effluent stream. In addition, the experimental unit employed a separation vessel, constructed from a 6-liter Erlenmeyer flask, which facilitated the recovery of the cellular product. A photograph of the experimental unit in operation is shown in Figure 4.2.

Equipment Sterilization

The assembled fermentation vessel, feed and effluent lines, reservoir and recovery bottles were all steam sterilized at 121°C for twenty-five minutes.

Culture Media

The composition of WBI medium used throughout this continuous cultivation study is shown in Table 4-1. Twelve-liters of culture media per batch were adjusted to pH 7.6 with 2N NaOH and transferred to five-gallon Pyrex sterilization bottles.

Media Sterilization

Twelve liters freshly prepared media was steam sterilized for

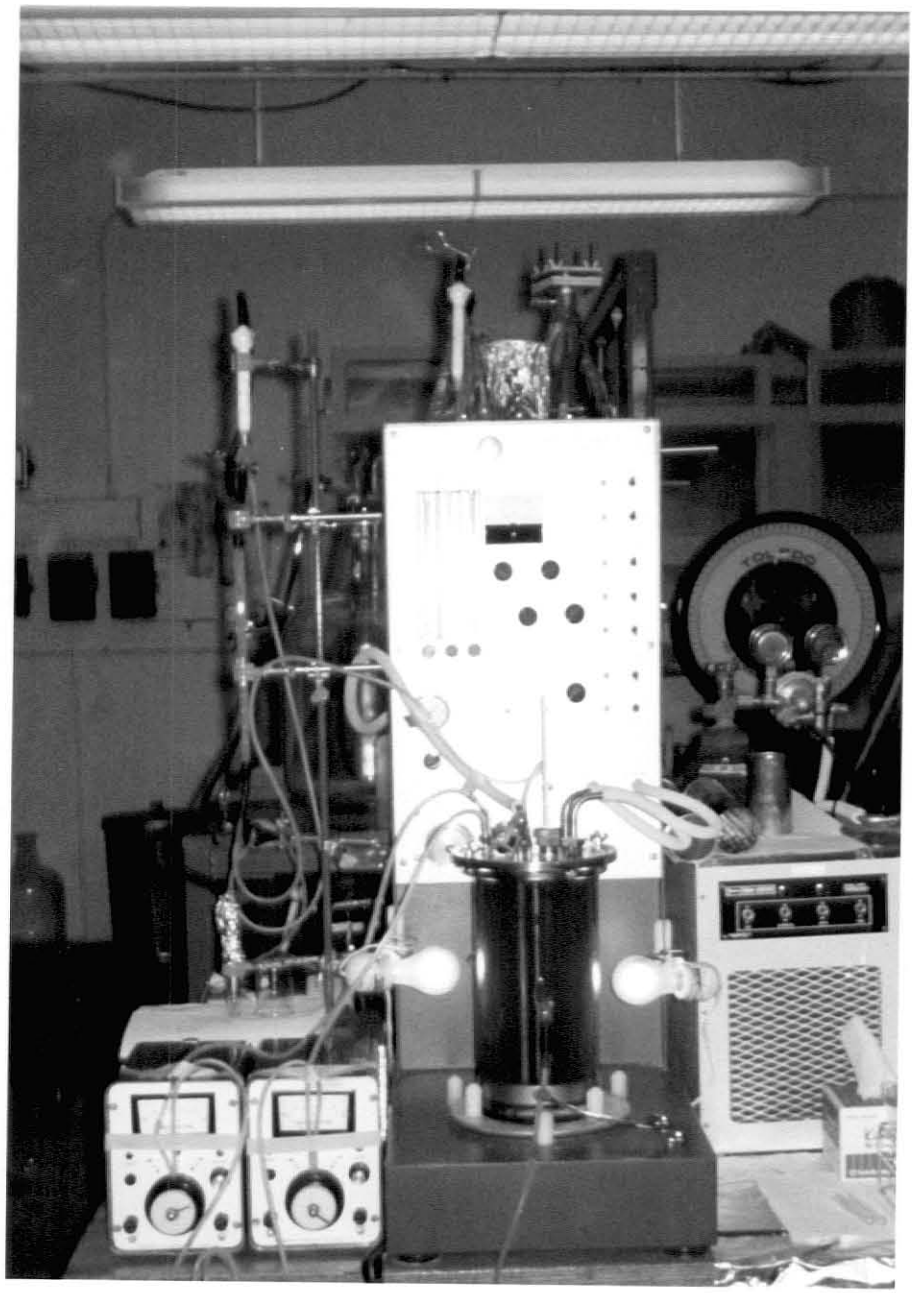


Fig. 4.2 Photograph of Experimental Photosynthetic Unit During Continuous Operation

TABLE 4-1

Composition of Wheat Bran Infusion Culture Medium

KH_2PO_4	0.5 g
K_2HPO_4	0.5 g
$(NH_4)_2SO_4$	2.0 g
$MgSO_4 \cdot 7H_2O$	0.5 g
$NaHCO_3$	2.0 g
Biotin	5.0 µg
Thiamine·HCl	10.0 mg
Wheat Bran (infusion from)	30.0 g
NaCl	23.0 g
Tap Water	1000 ml

Final pH adjusted to 7.2 ± 0.2

twenty minutes at 121°C in an autoclave, then cooled by immersing in a water bath of running tap water. Sterile media was aseptically transferred to a twelve-gallon reservoir bottle. No further pH adjustment of media was required since the final pH following sterilization usually ranged from 7.2 to 7.5.

Inoculum

A pre-culture was prepared by transferring 1.4 liters of a pure culture of Rps. gelatinosa to 12.6 liters of media contained within the fermentor. The impeller agitation speed was adjusted to 100 rpm and a batch cultivation was initiated at 30°C.

Continuous Cultivation

At the end of the exponential growth phase (approximately twenty hours) the continuous cultivation was started by actuating the Sigma pumps which supplied fresh media to the fermentor and discharged spent media from the fermentor at a constant rate.

Analyses

Periodically, the following analyses were made of samples taken from the effluent stream:

(1) The optical densities of samples were measured in a spectrophotometer at 650 mμ. From the O.D. measurements, cell concentrations were obtained from a previous standard plot of optical density

versus cell concentration (see Appendix II).

(2) Samples were centrifuged at 3,000 rpm for ten minutes and the supernatant chemical oxygen demand (COD) determined by the dichromate method outlined in Appendix III.

(3) The pH of effluent samples was measured.

(4) Frequent microscopic examinations of effluent samples were made in checking for possible microbial contamination.

After several volumes of media had passed through the fermentor, steady-state was confirmed by constant optical density readings in the effluent stream. Having acquired the necessary data for each steady-state, the flow rates were adjusted to another dilution rate. Continuous cultivation of Rps. gelatinosa was performed at dilution rates of 0.012, 0.025, 0.028, 0.035, 0.048, 0.059, 0.081, 0.098 and 0.136 hr^{-1}.

Biochemical Analysis

The protein and nucleic acid compositions of microbial cells are significantly influenced by specific growth rate. In general, an increase in the specific growth rate of a microorganism results in an increased protein and nucleic acid content. Photosynthetic bacterial cells harvested at dilution rates of 0.028 and 0.081 hr^{-1} were collected by centrifugation, washed with several aliquots of distilled water, and freeze-dried.

Proteins. Crude protein of harvested cells was determined by the method outlined in Appendix IV.

Nucleic Acids. Nucleic acids of microbial cells are generally comprised of ribonucleic acid (RNA). Therefore, harvested cells were analyzed for RNA employing a method outlined in Appendix V.

Amino Acids. The amino acid content of harvested cells was determined with a Beckmann Model 120-C Amino Acid Analyzer (Beckman Instruments, Palo Alto, California).

RESULTS AND DISCUSSION

Continuous Cultivation of Rps. gelatinosa

Data compiled throughout the course of this continuous cultivation study are presented in Figure 4.3. Steady-state values for the continuous cultivation of Rps. gelatinosa are shown in Table 4-2.

As mentioned previously in the experimental description of this section, the fermentor was not equipped with a pH-controller. However, the pH within the fermentor, ranging from pH 6.00 to 8.10, remained relatively stable throughout the duration of the experiment. The inlet Sigma pump was carefully regulated throughout the continuous run in order to ensure a constant flow rate. Simultaneously using two variable-speed Sigma pumps to maintain a constant liquid level within the fermentor also proved satisfactory.

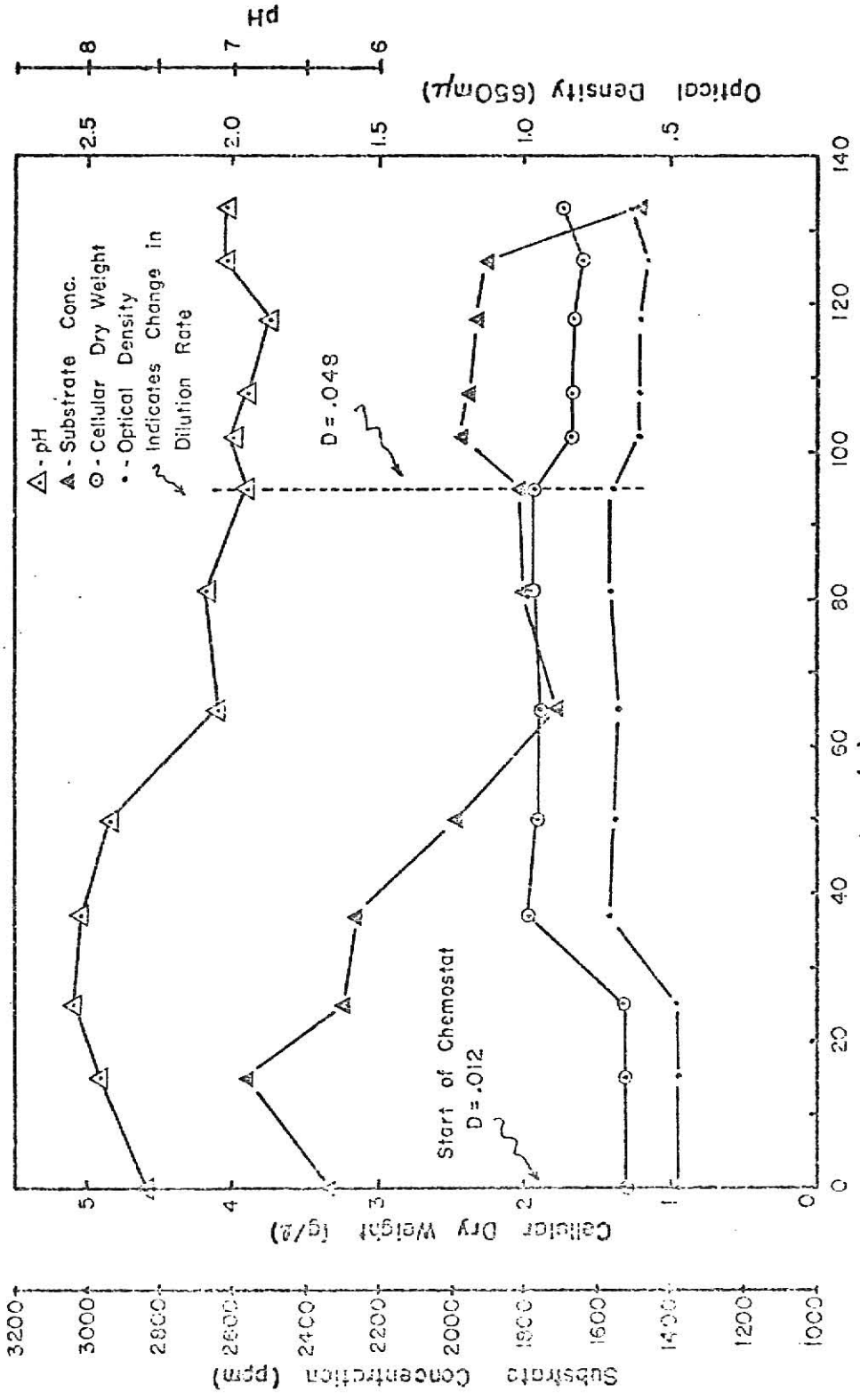


Fig.4.3 Plot of Experimental Data for the Continuous Cultivation of Rps. gelatinosa in a Single Stage 14-L Jar Fermentor.

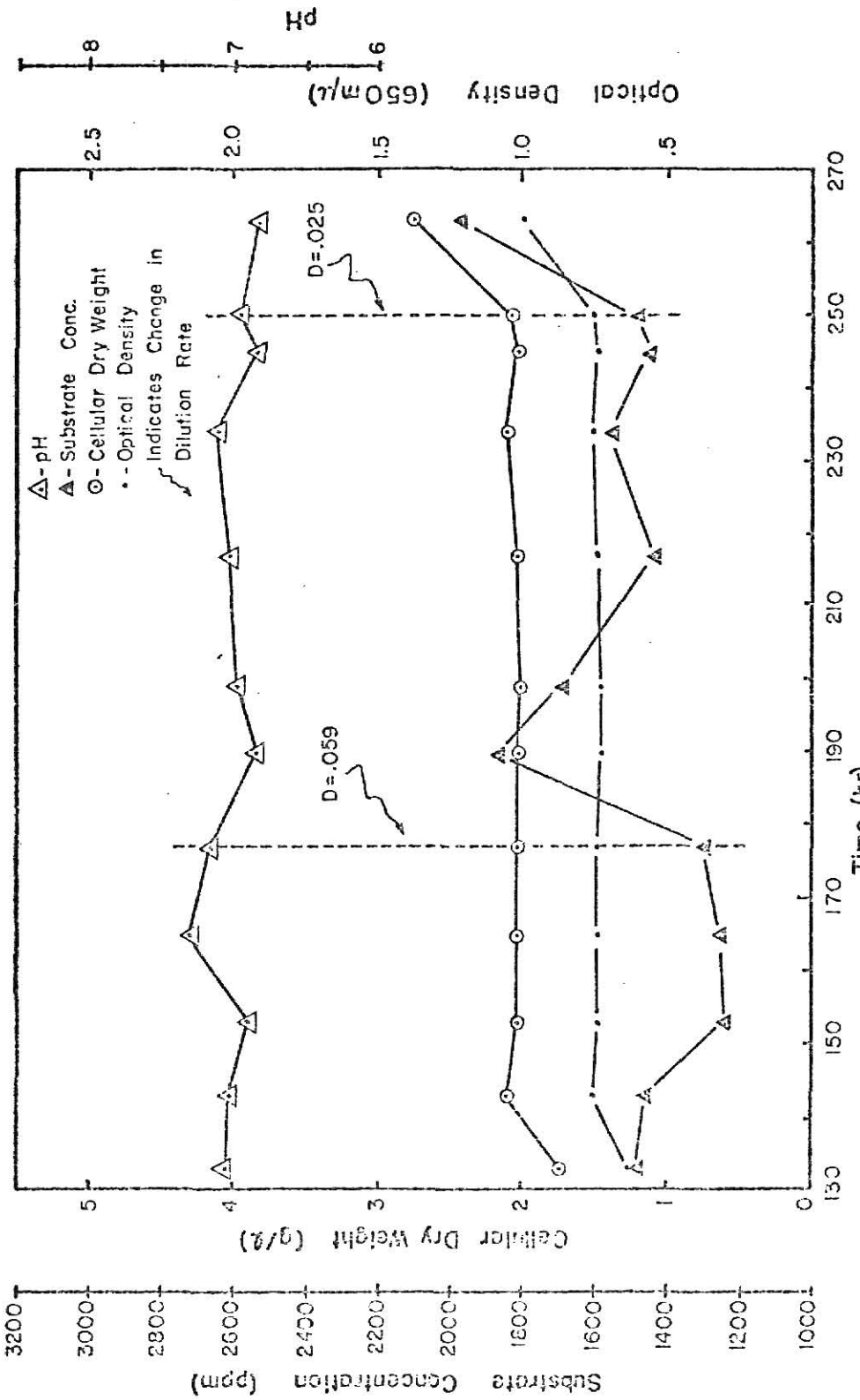
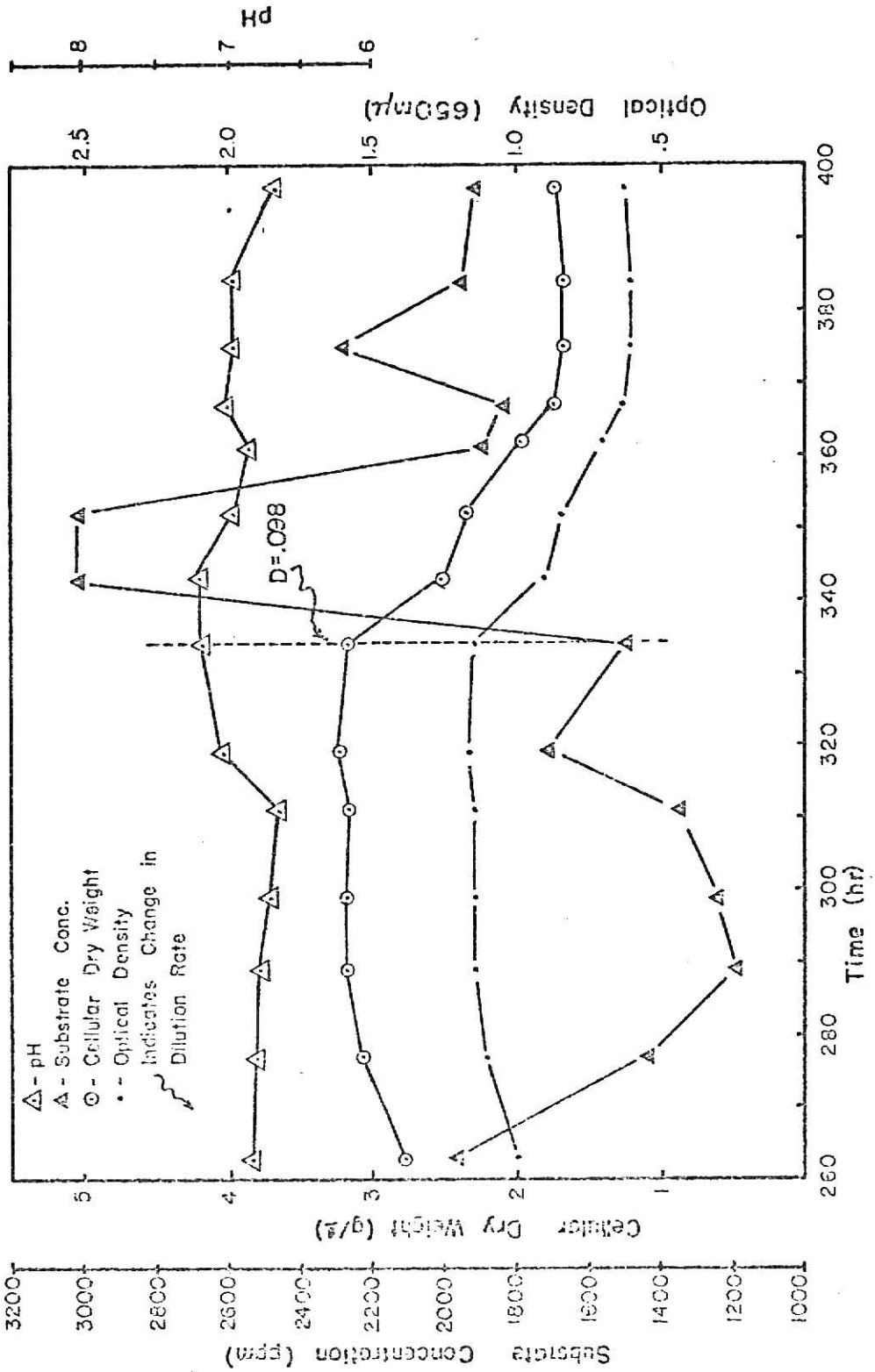


Fig. 4.5 (continued)



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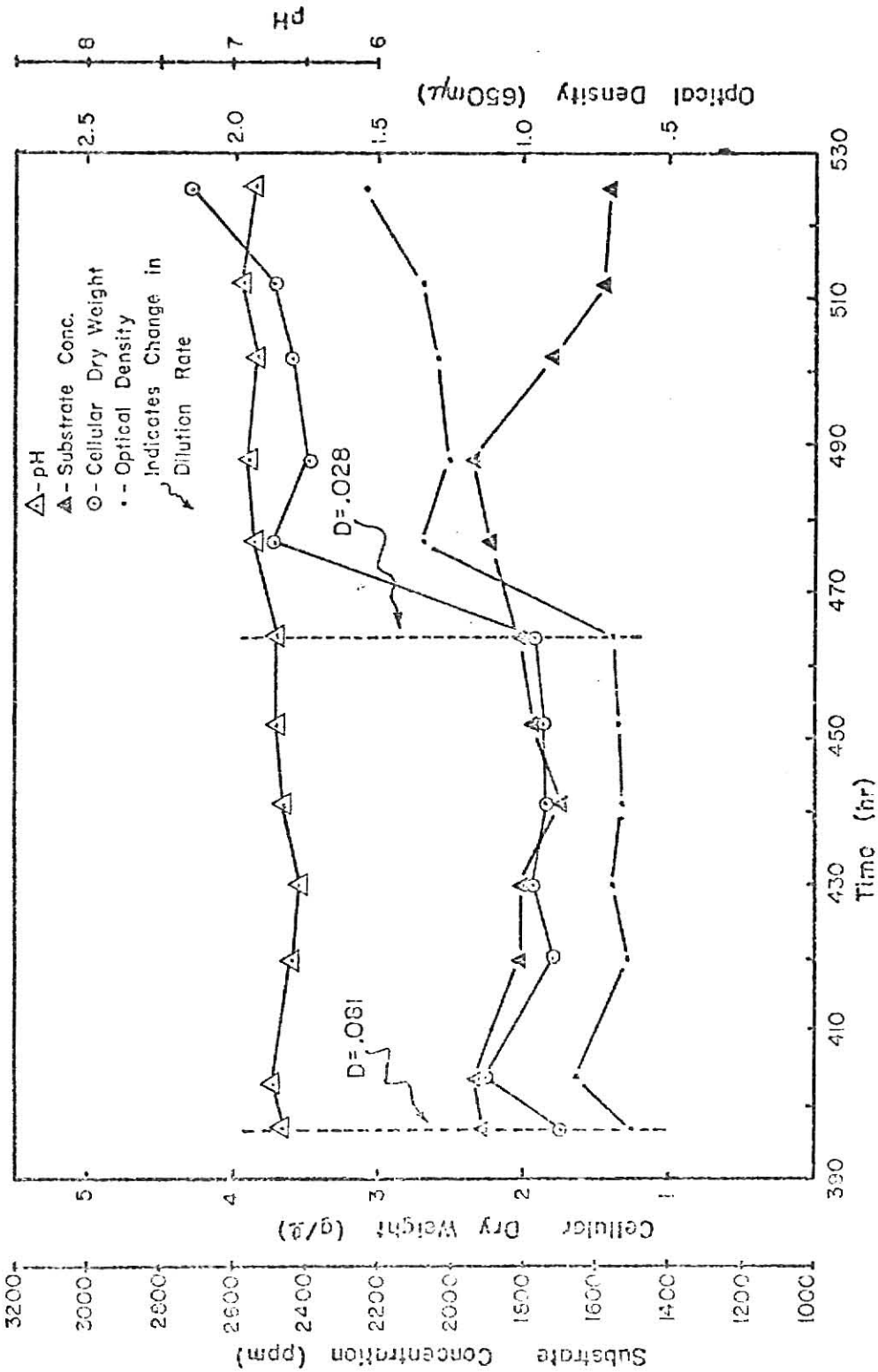

Fig.4.3 (continued)

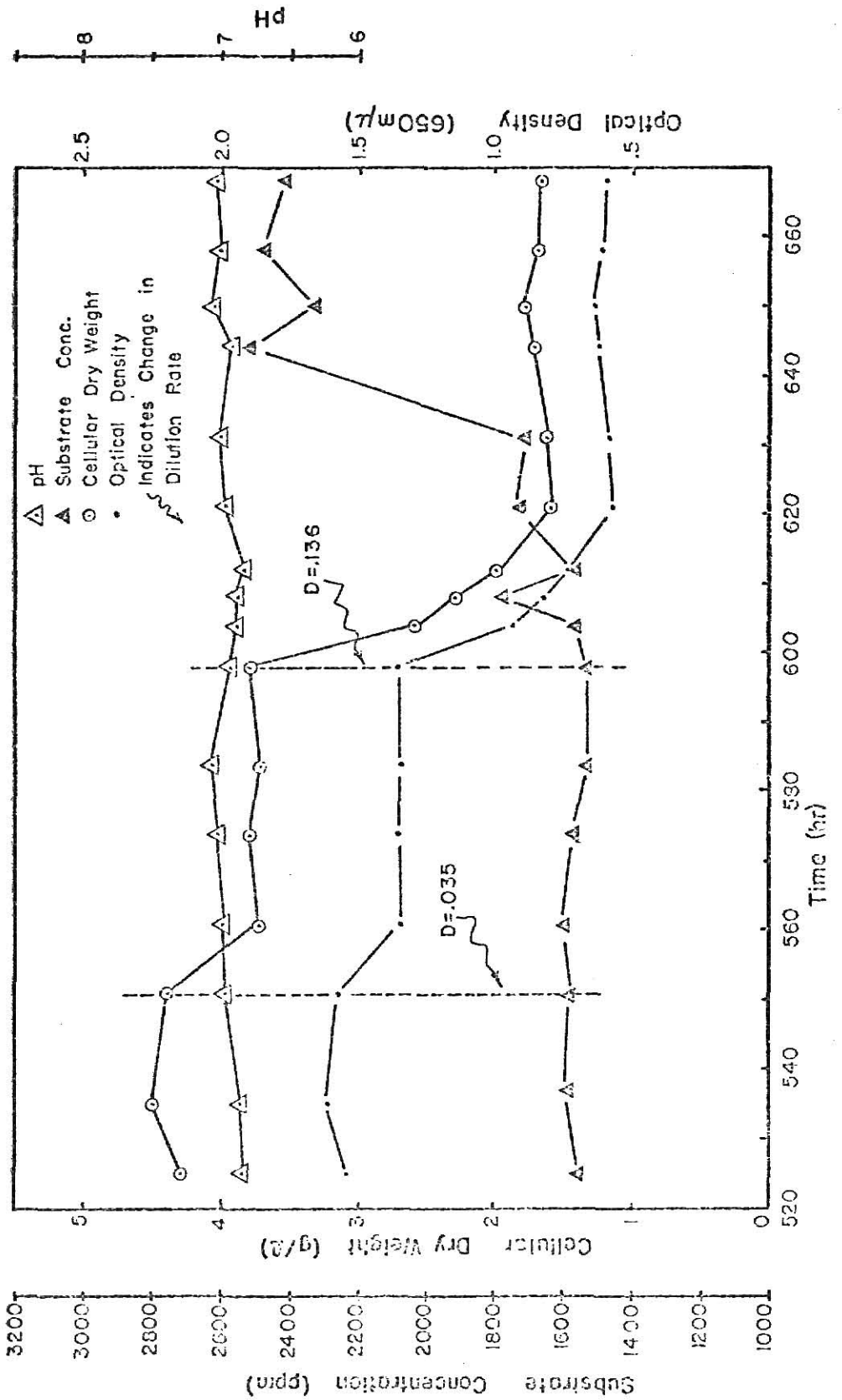

Fig. 4.3 (continued)

TABLE 4-2

Steady-State Values for the Continuous Cultivation Of Rps. gelatinosa in 3.0% Wheat Bran Infusion Medium

Dilution Rate (hr^{-1})	pH	Cell Concentration (g/l)	Outlet Substrate Concentration (ppm)	Productivity (mg/l·hr)
0.012	7.51±0.59	1.38	1877	17
0.025	6.91±0.26	2.29	1401	57
0.028	6.87±0.07	3.15	1574	88
0.035	6.99±0.05	2.69	1556	94
0.048	7.02±0.26	1.48	1327	71
0.059	6.99±0.16	1.48	1494	87
0.081	6.70±0.05	1.35	1769	109
0.098	6.97±0.22	1.24	1993	121
0.136	6.94±0.11	1.21	2196	165

The cultures exhibited little tendency to clump or flocculate; however, a slight amount of wall growth was observed. Several times daily, in order to minimize wall growth, the impeller speed was momentarily increased to 400 rpm. Considerable wall growth was also observed within effluent lines. It was necessary to remove any accumulated growth prior to sampling the effluent stream.

Cell Growth and Substrate Utilization in Continuous Culture

The cultivation of microorganisms in which the rate of growth is limited by a single substrate may be described by the typical Monod model (5):

$$\mu = \frac{\mu_{max} S}{K_s + S} \qquad [1]$$

in which $\mu < \mu_{max}$ and where

μ_{max} = maximum value of the specific growth rate (hr^{-1})

K_s = saturation constant

The microbial mass-balance equation representing continuous growth in a completely stirred fermentor is,

$$\text{Increase} = \left[\begin{array}{l}\text{Growth of micro-}\\ \text{organisms in fer-}\\ \text{menter}\end{array}\right] - \left[\begin{array}{l}\text{Output of micro-}\\ \text{organisms in}\\ \text{effluent}\end{array}\right]$$

or

$$\frac{dX}{dt} = \frac{\mu_{max} SX}{K_s + S} - DX \qquad [2]$$

At steady-state conditions,

$$\left[\begin{array}{l}\text{Growth of micro-}\\\text{organisms in fer-}\\\text{mentor}\end{array}\right] = \left[\begin{array}{l}\text{Output of micro-}\\\text{organisms in}\\\text{effluent}\end{array}\right]$$

or

$$\frac{\mu_{max} SX}{K_s + S} = DX \qquad [3]$$

Similarly, the substrate mass-balance in which,

$$\text{Increase of substrate} = [\text{Input}] - [\text{Output}] - \left[\begin{array}{l}\text{Consumption of substrate}\\\text{by microorganisms}\end{array}\right]$$

Under steady-state conditions this leads to,

$$\frac{dS}{dt} = DS_o - DS - \frac{\mu_{max} SX}{Y(K_s + S)} \qquad [4]$$

in which the yield constant (Y) is defined as the ratio between the amount of microbial cell mass formed and the decrease of substrate.

Determination of Yield Constant (Y)

In determining the yield constant, Equation [4] may be rearranged to:

$$D(S_o - S) - \frac{\mu_{max} SX}{Y(K_s + S)} = 0 \qquad [5]$$

At steady-state,

$$D = \mu = \frac{\mu_{max} S}{K_s + S}$$

thus,

$$Y = \frac{X}{S_o - S} \qquad [6]$$

Yield constants appearing in Table 4-3 were calculated from steady-state values. Generally the yield constants for microbial growth on a single substrate is a constant value (6). However, the calculated yield constants for growth of Rps. gelatinosa on a complex substrate such as wheat bran infusion appeared to vary substantially in this study.

Determination of Substrate Affinity Constant (K_s)

In the Monod expression for microbial growth in a media containing a single organic substrate, the saturation constant (K_s) is equivalent to that substrate concentration at which the specific growth rate is equal to half the maximum growth rate (7). In an attempt to use the Monod equation to define photosynthetic growth on a complex substrate, K_s shall be redefined as a substrate affinity constant representing a microorganism's affinity for growth on a complex substrate such as wheat bran infusion.

Accurate values of K_s are not readily obtained from batch culture experiments; however, K_s may be determined from continuous culture experiments (8). Once μ_{max} has been determined, either from batch or continuous culture experiments, the steady-state substrate concentration (S) at any dilution rate (D) allows K_s to be easily calculated from Equation [3].

TABLE 4-3

Yield Constants, Substrate Affinity Constants
And Effective COD Reduction
At Steady-State Conditions

Dilution Rate (hr^{-1})	Yield Constant (mg/ppm)	Substrate Affinity Constant (ppm)	Effective COD Reduction (%)
0.012	0.68	5312	52
0.025	1.83	1905	47
0.028	3.84	1905	34
0.035	3.46	1509	33
0.048	1.26	955	47
0.059	1.37	867	42
0.081	1.95	743	28
0.098	2.11	698	23
0.136	2.23	549	20

Solving Equation [3] for K_s:

$$K_s = S\left(\frac{\mu_{max}}{D} - 1\right) \qquad [7]$$

For the case where $\mu_{max} \gg D$, Equation [7] may be reduced to:

$$K_s = \frac{S\mu_{max}}{D} \qquad [8]$$

Substrate affinity constants calculated from steady-state data appear in Table 4-3. In a plot of substrate affinity (K_s) versus dilution rate (Figure 4.4), the value of K_s appeared to decrease at different rates depending on whether the dilution rate was greater or less than $0.034 hr^{-1}$. The dilution rate of $0.034\ hr^{-1}$ above represents a factor of approximately 0.1 of the value calculated for μ_{max} in the previous chapter.

Determination of Effective COD Reduction

The utilization of 3.0 percent WBI media by Rps. gelatinosa may also be determined by calculating the effective COD reduction as follows:

$$\text{Effective COD Reduction (\%)} = \frac{\text{Inlet COD - Outlet COD}}{\text{Inlet COD}} \times 100$$

In a plot of effective COD reduction versus dilution rate (Figure 4.5), the COD reduction efficiency decreases with increasing dilution rate.

Determination of D_{max}

At steady-state, bacterial productivity (DX) may be obtained by combining Equations[3] and [6] as:

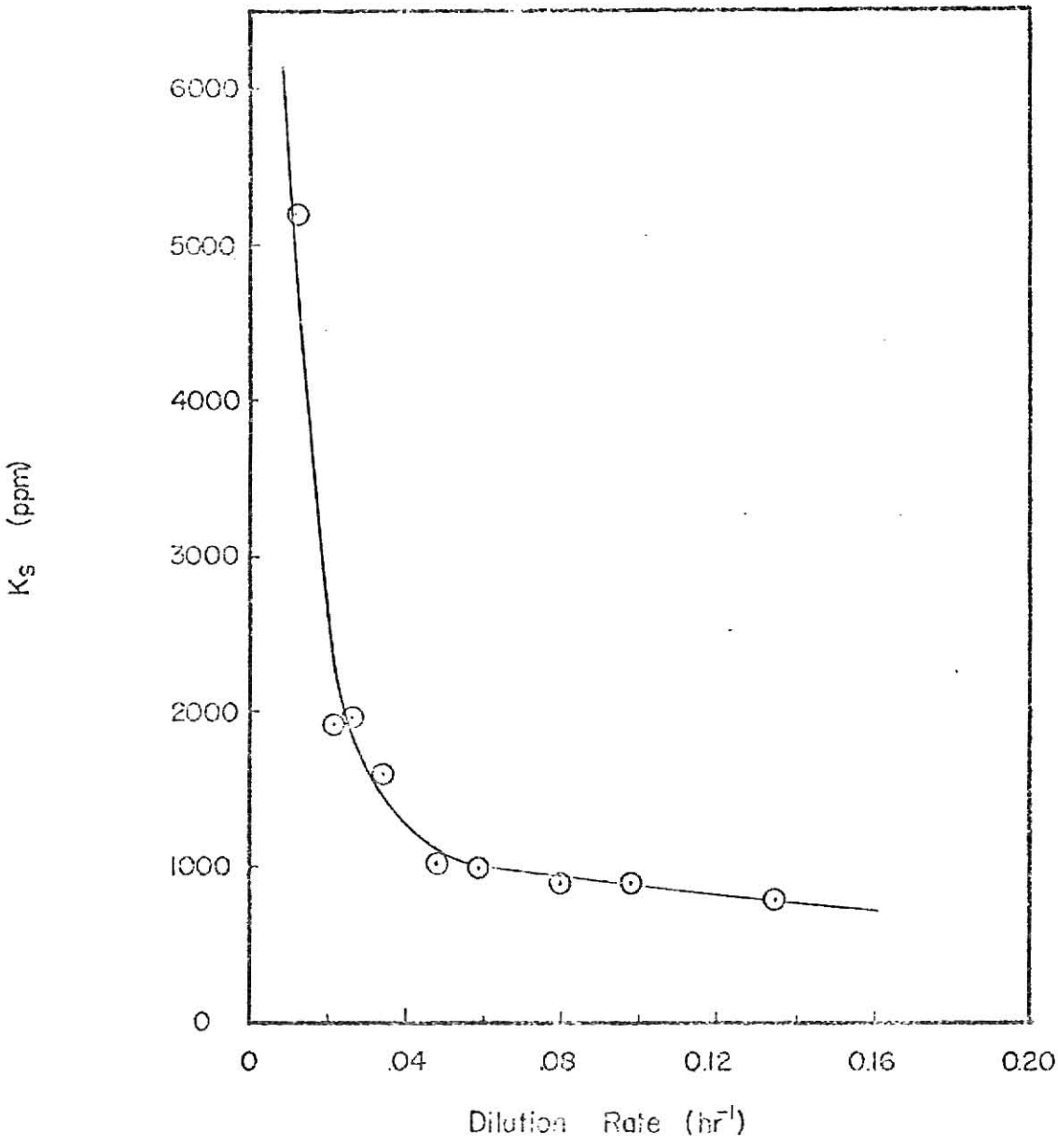

Fig. 4.4 Plot of Substrate Affinity (K_s) <u>versus</u> Steady-State Dilution Rate.

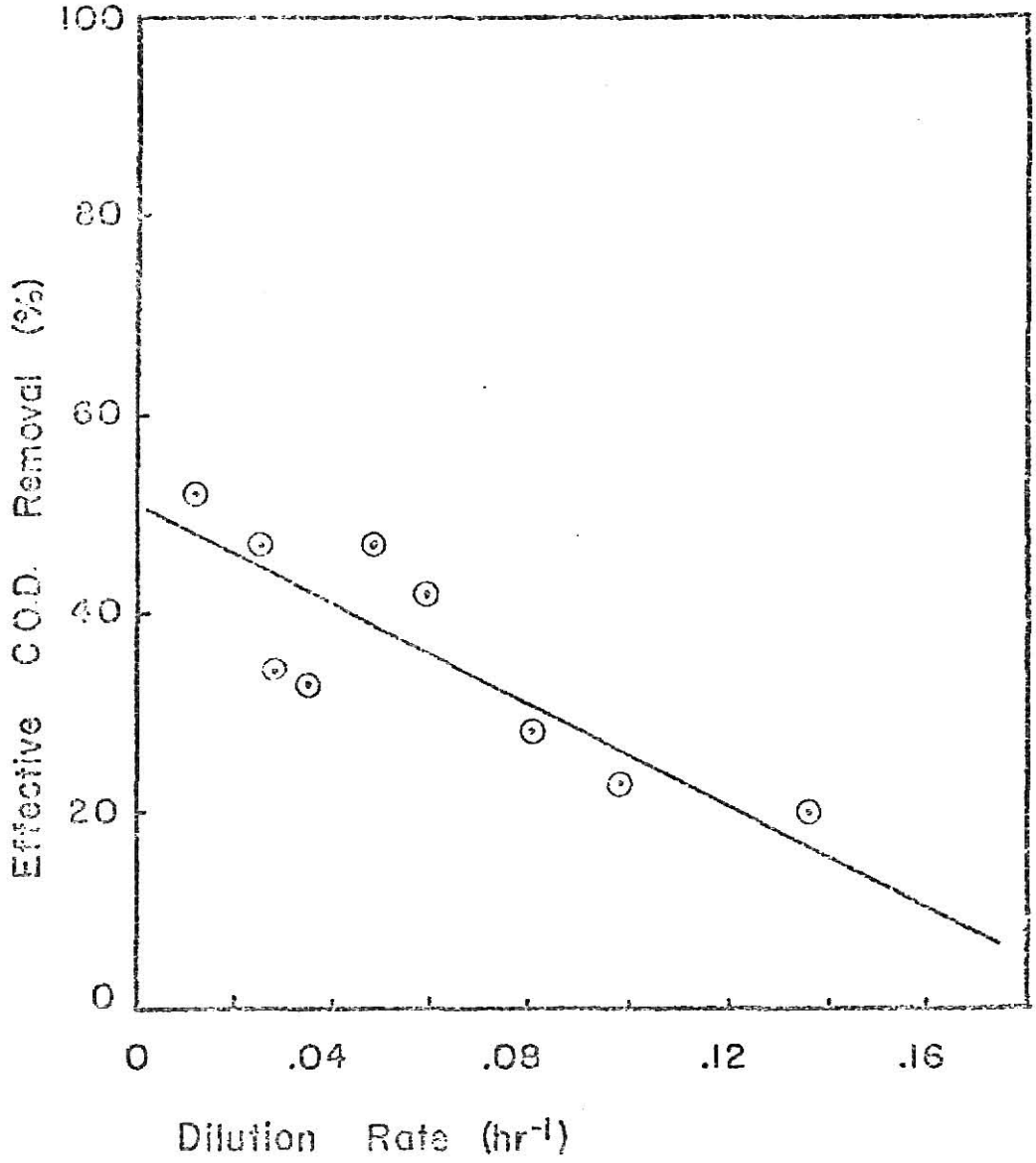

Fig. 4.5 Plot of Effective COD Removal versus Steady-State Dilution Rate.

$$DX = DY\left(S_o - \frac{K_s D}{\mu_{max} - D}\right) \qquad [9]$$

To find the dilution rate (D_{max}) at which productivity is maximum (9,10), let:

$$\frac{d(DX)}{dD} = 0 \qquad [10]$$

Solving the requlting equation for D

$$D = D_{max} = \mu_{max}\left(1 - \sqrt{\frac{K_s}{K_s + S_o}}\right) \qquad [11]$$

Calculation of D_{max}

The dilution rate providing maximum productivity (D_{max}) may be calculated by substituting the following parameter values into Equation [11]:

$\mu_{max} = 0.34\ hr^{-1}$ (value obtained from batch cultivation study in previous chapter)

$S_o = 2,500$ ppm (average inlet substrate concentration from continuous cultivation study)

$K_s = 1,000$ ppm (value obtained from Figure 4.4)

$$D_{max} = \mu_{max}\left(1 - \sqrt{\frac{K_s}{K_s + S_o}}\right)$$

$$= 0.34\ hr^{-1}\ (1 - 0.236)$$

$$= 0.16\ hr^{-1}$$

Biochemical Analysis

The crude protein and nucleic acid (RNA) contents of the cellular product harvested at the various steady-states appear in Table 4-4. The crude protein content ranged from 57.6 to 61.4 percent. The RNA content ranging from 4.28 to 5.93 did not appear significantly different for the different steady-states. The amino acid contents of Rps. gelatinosa harvested at steady-state dilution rates of 0.028 and 0.081 hr^{-1} are shown in Table 4-5. The amino acid profile of Rps. gelatinosa compares favorably with that of many conventional protein-rich foods (Table 4-6).

TABLE 4-4

Composition of Rps. gelatinosa

Harvested at Varying Dilution Rates

Dilution Rate of Harvested Cells (hr^{-1})	Crude Protein (%)	Crude Protein Moisture-Free (%)	RNA (%)
0.012	61.36	66.07	4.28
0.025	59.05	62.83	4.62
0.028	59.90	63.93	5.59
0.035	57.57	62.90	4.67
0.048	60.72	65.84	5.93
0.059	58.14	63.11	4.43
0.081	60.30	66.70	5.26
0.098	58.56	63.27	5.03
0.136	59.30	65.13	4.67

TABLE 4-5

Amino Acid Composition

Of Rps. gelatinosa

Amino Acid (%of crude protein)	Dilution Rate of Harvested Cells $0.028hr^{-1}$	$0.081hr^{-1}$
Lysine	5.53	6.07
Histidine	3.40	3.88
Arginine	7.13	8.20
Aspartate	10.25	9.88
Threonine	2.87	4.36
Serine	4.25	3.85
Glutamate	13.98	13.69
Proline	5.19	4.42
Glycine	5.52	5.09
Alanine	8.73	8.19
Cysteine[1]	1.06	0.81
Methionine[2]	3.02	3.04
Valine	6.97	6.47
Isoleucine	4.33	4.11
Leucine	7.87	7.43
Tyrosine	3.34	3.38
Phenylalanine	4.60	4.32

[1] Cysteine by oxidation

[2] Methionine by oxidation

TABLE 4-6

Amino Acid Composition of Food Products

Of Animal and Plant Origin (11)

Amino Acid (% protein)	Eggs	Cheese (cheddar)	Soybeans
Histidine	2.4	3.3	2.4
Isoleucine	6.6	6.9	5.4
Leucine	8.8	9.9	7.7
Lysine	6.4	7.5	6.3
Methionine	3.1	2.2	1.3
Phenylalanine	5.8	5.5	4.9
Threonine	5.0	3.8	3.9
Tryptophan	1.6	1.4	1.4
Valine	7.4	7.3	5.2

NOMENCLATURE

D = dilution rate (hr^{-1})

DX = productivity ($/l \cdot hr$)

D_{max} = dilution rate at which productivity is maximum (hr^{-1})

K_s = saturation constant; affinity constant (ppm)

S = substrate concentration (ppm)

S_o = inlet substrate concentration (ppm)

t = time (hr)

X = cell concentration (g/l)

Y = yield (mg/ppm)

Greek letters

μ = specific growth rate (hr^{-1})

μ_{max} = maximum value of specific growth rate (hr^{-1})

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CHAPTER V

PRODUCTION OF SCP FROM PHOTOSYNTHETIC BACTERIA: PROCESS DESIGN AND ECONOMIC ANALYSIS

INTRODUCTION

This study is concerned with the technical and economical feasibility of producing Single-Cell Protein from photosynthetic bacteria. The batch and continuous growth of the photosynthetic bacterium Rhodopseudomonas gelatincsa, examined in preceeding chapters, served as basis for the design of the following SCP process. In addition to design, an economic analysis of construction and operation of the proposed facility is also described.

PROCESS DESIGN

The proposed photosynthetic SCP process illustrated in Figure 5.1 is basically a continuous process with a mean production capacity of three tons SCP per day. In view of potential microbial strain improvement or process improvements, a production capacity slightly higher than actual experimental findings was assumed. Raw material mass balances calculated on a daily production basis appear in Table 5-1. Major processing steps include media preparation, sterilization, photosynthetic cultivation, recovery and purification. A

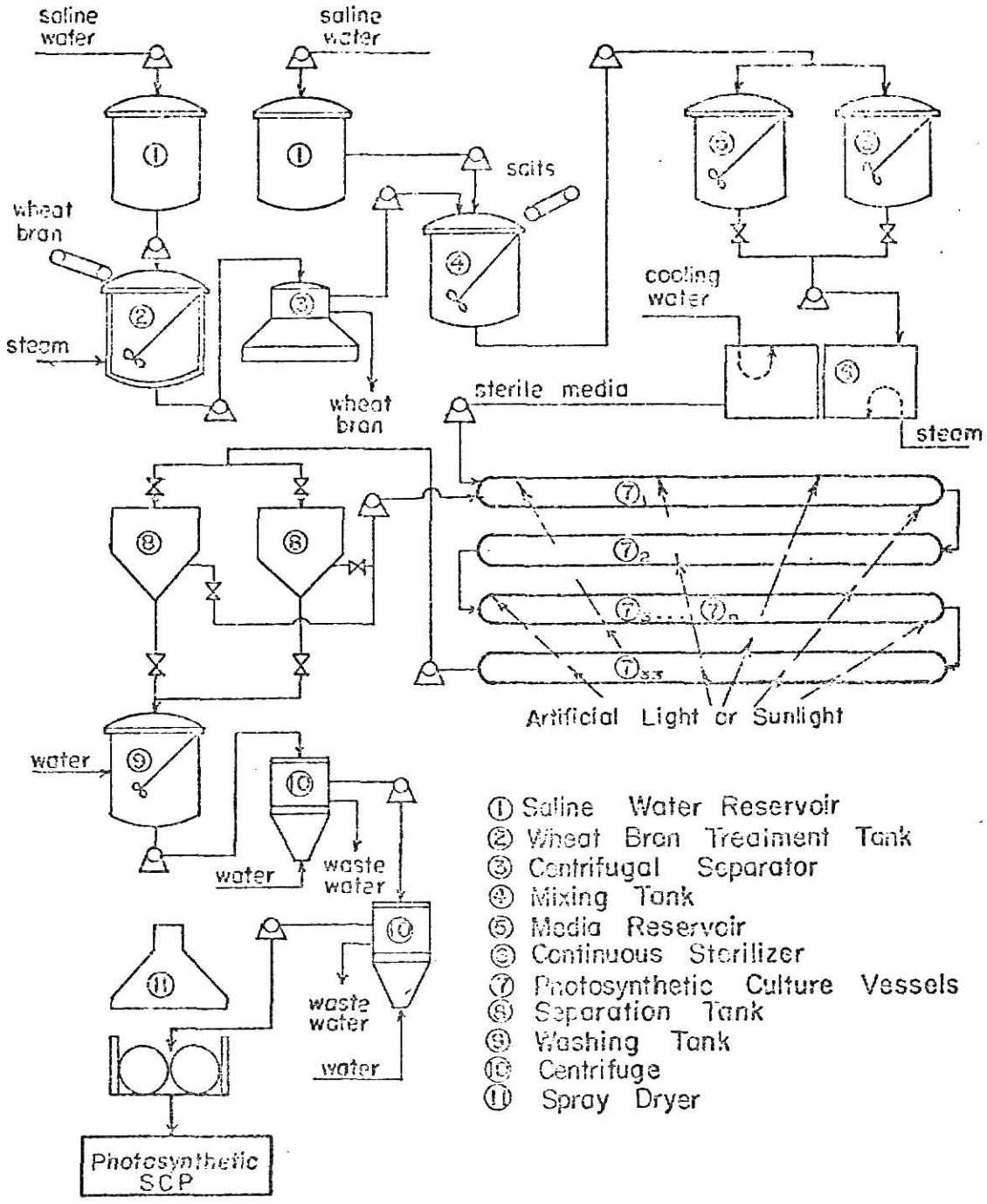

Fig.5.1 Flow Diagram for the Production of SCP from Photosynthetic Bacteria.

TABLE 5-1

Daily Raw Material Balances

Item	Weight/day
Wheat Bran	14.0 tons
Sea Water (saline water)	470.0 tons (liquid)
$(NH_4)_2SO_4$	0.94 tons
KH_2PO_4	0.47 tons
$NaHCO_3$	0.94 tons
Thiamine.HCl	4.7 kg
Biotin	2.35 g

more detailed description of the actual process is outlined in Figure 5.2.

Process Description

The initial processing steps including substrate pretreatment and media preparation are essentially a semi-continuous process in which wheat bran infusion is separated from a heated slurry of wheat bran and sea water. Wheat bran infusion is mixed together with additional sea water and salts, then transferred to a storage reservoir prior to sterilization. Fresh wheat bran infusion media fed through a continuous plate-type heat exchanger at a rate of 19.6 tons per hour is elevated to a temperature of 126°C then flash cooled to 30°C using process feed water as a coolant. After sterilization and cooling, vitamins are added and the pH is adjusted to neutrality with sodium bicarbonate. Photosynthetic cultivation is carried out at 30°C in a series of transparent PVC (polyvinyl chloride) tanks. Sunlight provides the necessary illumination for photosynthetic growth during the daytime while artificial illumination is provided by 400-watt sunlamps during nighttime operation. Retention time in the photosynthetic cultivation tanks is thirty-three hours. Photosynthetic bacterial cells are separated in a natural sedimentation process while remaining cells are cycled back to the cultivation tanks. Recovered cells then undergo a series of washing and centrifugation steps. Final processing in

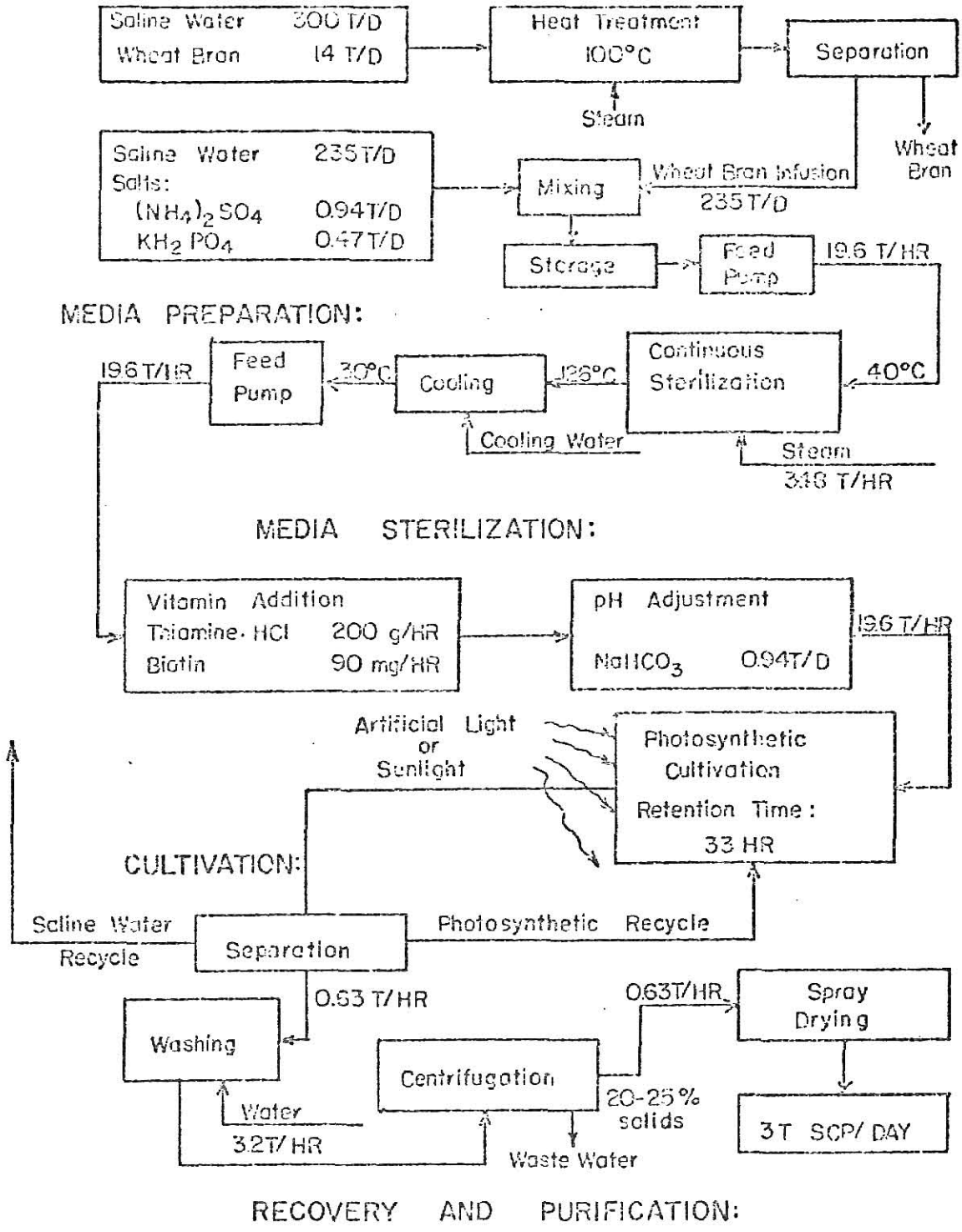

Fig. 5.2 Quantitative Flow Diagram for the Photosynthetic SCP Process.

which cells are spray-dried results in a product containing approximately sixty-five percent protein.

Processing Equipment

The capacities of processing equipment were calculated on the basis of previously determined daily production capacities and raw material balances. Major pieces of equipment required for mixing, storage, sterilization, photosynthetic cultivation, recovery and purifications are described in Table 5-2.

ECONOMIC ANALYSIS

Purchased Equipment Costs

Total purchased equipment costs for the photosynthetic SCP process appearing in Table 5-3 were estimated from available data on similar items of equipment, cost indices, and available cost-capacity factors. The total purchased equipment cost includes the costs for delivery but does not include installation.

Capital Investment

The fixed capital investment required for the construction of the facility and complete process operation was estimated with the aid of total purchased equipment cost and cost factors for items included in direct and indirect capital costs (1). The working capital necessary for plant operation was estimated at ten percent of the fixed capital

TABLE 5-2

Major Processing Equipment

Item	Quantity	Description
Mixing and Storage Tanks	5	capacity: 50 m^3 type: carbon steel
Reservoir Tanks	2	capacity: 100 m^3 type: carbon steel
Sedimentation Tanks	2	capacity: 40 m^3 type: carbon steel dimensions: 5.0 m(dia.) X 3.7m (depth)
Automatic Batch Centrifugal Separator	1	basket dia.: 1.08 m
Horizontal Transparent PVC Pipe	33	dimensions: 61 cm(dia.) X 67.1 m(length) volume: 19.6 m^3
High Capacity Transfer Pumps	20	type: cast iron, rotary-type pumping capacity: 20 m^3/hr
Sunlamps	200	400-watts; 110-115 volts
Basket-Type Centrifuges	2	basket dia.: 1.08 m max. capacity: 5m^3/hr
Spray Dryer	1	evaporative rate: 4m^3/hr dia. 16.4 m
Continuous Plate-Type Heat Exchanger	1	dia. of pipe: 15.5 cm length of pipes: 50 m
Steam Generator	1	operating pressure: 250 psig capacity: 5.0 m^3 steam/hr

TABLE 5-3

Major Purchased Equipment Cost

Item	Cost($)
Large Capacity Steel Mixing and Storage Tanks (@$8,500)	17,000
Steel Mixing and Storage Tanks ($6,600)	33,000
Steel Sedimentation Tanks (@$5,500)	11,000
Horizontal Transparent PVC Pipe (@$18.8/linear ft.)	136,500
Automatic Batch Centrifugal Separator	36,000
High Capacity Transfer Pumps (@$1,200)	24,000
Sunlamps (@$6)	1,200
Basket-Type Centrifuges (@$12,000)	24,000
Spray Dryer	22,000
Continuous Plate-Type Heat Exchanger	24,000
Steam Generator	4,500
Total Purchased Equipment Cost	$333,200

investment. The sum of the fixed capital investment and working capital is designated as the total capital investment. The total capital investment for the proposed SCP process comprised of direct costs, indirect costs and working capital, is summarized in Table 5-4.

Product Cost

The total product cost involving production costs and general expenses was determined on an annual production basis of 330 days using standard procedures outlined by Peters and Timmerhaus (2). Raw material costs for wheat bran were estimated at approximately twenty dollars per ton. Cost figures for ammonium sulfate, potassium phosphate, sodium bicarbonate and vitamins were obtained from "Chemical Marketing Reporter"(3). The operating labor costs for continuous operation were determined on the basis of three to four operators per shift representing an average annual income of $8,420 per man. Depreciation figures were based on an annual depreciation of ten percent of the fixed capital investment. Financing was estimated at one-third of the total capital investment at six percent for ten years as suggested by Walawender et. al.(4). The total annual product cost estimated for the proposed photosynthetic SCP process is summarized in Table 5-5.

A breakdown of total product cost per ton of SCP appears in Table 5-6. The reclamation and resale of wheat bran, which should account for an overall reduction in processing costs, was not assumed

TABLE 5-4

Estimated Capital Investment

Item		Cost($)
I. Fixed Capital Investment		
A. Direct Costs:		
(1)	Purchased Equipment (delivered)	333,200
(2)	Equipment Installation	78,000
(3)	Instrumentation and Control (installed)	33,000
(4)	Piping (installed)	77,000
(5)	Electrical (installed)	24,000
(6)	Buildings and Services	66,500
(7)	Yard Improvements	33,300
(8)	Service Facilities (installed)	122,200
(9)	Land	11,100
	Total Direct Plant Costs	$777,300
B. Indirect Costs:		
(10)	Engineering and Supervision	111,000
(11)	Construction Expense	89,000
(12)	Contractor's Fee	33,300
(13)	Contingency	100,000
	Total Indirect Plant Costs	$333,300
	Total Fixed Capital Investment	$1,110,600
II.	Working Capital	$ 123,400
III.	Total Capital Investment	$1,234,000

TABLE 5-5

Total Annual Product Cost

Item	Cost($)
I. Production Costs	
A. Direct Production Costs	
(1) Raw Materials	
(i) Wheat Bran (@$20/ton)	92,400
(ii) $(NH_4)_2SO_4$, K_2HPO_4, $NaHCO_3$	31,500
(iii) Vitamins	8,500
(2) Operating Labor	84,200
(3) Supervisory Labor	9,600
(4) Utilities	84,000
(5) Maintenance and Repairs	22,200
(6) Operating Supplies	2,000
(7) Laboratory Charges	12,800
Total	$347,200
B. Fixed Charges	
(8) Depreciation	111,000
(9) Local Taxes	11,100
(10) Insurance	5,600
	$127,800
C. Plant Overhead Costs	$ 56,100
Total Production Costs	$531,100
II. General Expenses	
A. Administrative Costs	14,000
B. Distribution and Selling Costs	28,000
C. Interest	2,500
Total	$44,500
III. Total Annual Product Cost	$575,600

TABLE 5-6

Total Product Cost

	Cost ($/ton SCP)
I. Production Costs	
A. Raw Materials	
(1) Wheat Bran	93.0
(2) Salts and Vitamins	40.4
B. Labor (Operating and Supervisory)	94.8
C. Utilities	85.0
D. Other	222.8
Total Production Costs	$536.0/ton SCP
II. General Expenses	45.0
III. Total Product Cost	$581.0/ton SCP

in the process. In any event, the total product costs amounted to $581 per ton SCP. The profitability of the proposed venture may be shown by the estimated gross earnings resulting from the sale of SCP (see Table 5-7).

PROCESS APPRAISAL AND RECOMMENDATIONS

Estimating the current selling price of commercial yeast protein at thirty to thirty-five cents per pound (5), the proposed photosynthetic production scheme has economic potential. However, this investigator's experimental findings indicate that improvements in production resulting from microbial strain improvement or process improvements would be necessary before initiating such a venture.

One of the most evident advantages of the proposed photosynthetic process would be the utilization of photosynthetic SCP for human food supplementation. At the present, however, raw material costs account for nearly forty-two percent of the total product cost. Utilization of the residual wheat bran could result in additional income which would offset the total product cost. Potential residual wheat bran utilization schemes could include either drying the residue and marketing the wheat bran by itself as a livestock feed or supplementation of wheat bran with SCP and marketing the product as a protein-enriched cereal food or livestock feed. Anaerobic and/or aerobic treatment of the processing waste water may be used to produce methane

TABLE 5-7

Estimated Gross Earnings from the Sale of SCP

Selling Price for SCP ($/lb.)	Gross Earnings ($/ton SCP)
.30	20.0
.35	120.0
.40	220.0
.45	320.0
.50	420.0

gas (anaerobic digestion) and additional SCP to be incorporated into animal feeds.

Another possible scheme for the production of photosynthetic SCP, which would alleviate high substrate costs, would be the cultivation of photosynthetic bacteria in sewage, animal manure, feedlot wastes, etc. Kobayashi, et. al. (6) describe the construction and operation of a sewage treatment plant in which photosynthetic bacteria are harvested as a by-product of the purification process. Although SCP grown on these materials would be unsuitable for human food supplementation, microorganisms harvested from sewage and animal wastes could be used to supplement animal feed (7,8). The cultivation of photosynthetic bacteria on alternate sources of nutrients such as sewage and animal wastes should be considered in future studies.

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CHAPTER VI

CONCLUSIONS AND RECOMMENDATIONS

In evaluating photosynthetic bacteria as potential sources of SCP suitable for human consumption or animal feed supplementation, a series of investigations were undertaken to establish important growth parameters such as temperature, pH, and substrate concentration. Preliminary growth studies in which Rps. gelatinosa was cultured in a series of small-scale batch cultivations indicated that optimal growth occurred at a temperature of 30°C and a pH of 7.0 $\pm$ 0.5. Growth was observed to increase with ammonium sulfate concentration until reaching a maximum at 0.2 percent ammonium sulfate. Growth in malic acid increases maximally with substrate concentration, however, beyond a concentration of 0.5 percent malate, the rate of growth decreases as the organisms become saturated with substrate. Yeast extract was shown to enhance the growth of Rps. gelatinosa. Yeast extract concentrations greater than 1.0 percent, in any event, did not significantly increase growth. Studies involving the cultivation of Rps. gelatinosa in a synthetic medium indicated that this organism could be successfully grown in a completely defined medium, however, supplementation with yeast extract significantly enhanced growth. At

this point, the author recommends that future studies be concerned with isolating and cultivating other faster-growing strains of photosynthetic bacteria; re-examining and evaluating various growth parameters such as temperature, pH, salt and substrate concentration and their effects on growth and, finally, investigating the effects of light intensity or wavelength on the growth of photosynthetic bacteria.

Studies concerned with the examination of various raw materials as potential substrates for the mass cultivation of photosynthetic bacteria, indicated that materials such as food processing wastes, milling wastes, or other sources of crude sugars could serve as satisfactory nutrient sources for the growth of photosynthetic bacteria. Future studies in the area of waste utilization should involve the isolation of photosynthetic bacteria from such sources as raw sewage, processing wastes, animal wastes, etc. Isolated species of photosynthetic bacteria should also be subjected to conditions promoting adaptation to growing in a wide variety of waste materials.

The growth of Rps. gelatinosa in wheat bran infusion media was observed to increase with substrate concentration; the highest cellular dry weight obtained for growth in 6.0 percent wheat bran infusion media was 7.46 g/l. A maximum specific growth rate of $0.33 hr^{-1}$ reemphasizes the need for selecting faster growing strains of photosynthetic bacteria. Photosynthetic growth in a completely-mixed fermentor, resulting in a net COD reduction of sixty-seven percent,

indicates the possibility of incorporating photosynthetic bacteria in the treatment of wastes; however, considerable work is needed in this area.

Continuous cultivation studies in which Rps. gelatinosa was cultured in a completely-mixed fermentor indicate the possibility of culturing photosynthetic bacteria in a continuous-type system. The typical Monod expression for growth was used in an attempt to model the growth of photosynthetic bacteria. A maximum specific growth rate (μ_{max}) of 0.33 hr^{-1}, obtained from batch cultivation studies, was 9.7 times greater than a μ_{max} value of .034 hr^{-1} obtained from continuous cultivation. The value for K_s, redefined as a substrate affinity constant, appeared to vary with the dilution rate.

A K_s value of 1000 ppm was used to calculate the dilution rate providing maximum productivity (D_{max}) at 0.016 hr^{-1}. The results of the continuous cultivation study are yet inconclusive. Factors such as light intensity, which were not examined in these investigations, apparently play a significant role in photosynthetic growth and remain to be examined. Much work still remains to be done in this area of continuous cultivation.

The protein content of harvested photosynthetic cells ranged from approximately 57.6 to 61.4 percent. A change in dilution rate did not result in any significant changes in protein content or nucleic acid (RNA) content. An amino acid analysis of proteins extracted

from harvested cells indicated that photosynthetic proteins are relatively rich in essential amino acids and are comparable to those of many conventional foods. The nucleic acid content of harvested cells, ranging from 4.28 to 5.93 percent RNA, at this point would not appear to represent any significant toxicity problems resulting from high nucleic acid levels. Further studies relating to safety and nutritional evaluation are recommended.

A study evaluating the technological and economical feasibility of SCP production from photosynthetic bacteria indicates that a photosynthetic process could have economic potential if one considers the current selling price of commercial food yeasts at thirty to thirty-five cents per pound. Raw material cost, comprising approximately forty-two percent of the total production costs, is the major contributor to the total product cost in the proposed photosynthetic SCP process. The cultivation of photosynthetic bacteria on alternate sources of nutrients such as sewage and animal waste as a possible solution in alleviating high substrate costs should be considered in future studies. In addition, the design and optimization of photosynthetic SCP processes with a more efficient utilization of solar energy should also be considered.

APPENDIX I

PREPARATION OF INOCULUM

Seed cultures used in the growth studies of Rps. gelatinosa were prepared by aseptically transferring several loopfuls from stock cultures of Rps. gelatinosa to flasks of sterile nutrient media. The composition of the nutrient media is as follows:

K_2HPO_4	1.0g
$MgCl_2$	0.5g
NaCl	23.0g
Yeast Extract (BBL)	5.0g
DL-Malic Acid	2.0g
Tap Water	1000ml

Final pH adjusted to 7.2 ± 0.2

The culture flasks were tightly sealed with rubber stoppers. Photosynthetic growth was allowed to proceed at 30°C in an incubator-shaker (Psychrotherm, New Brunswick Scientific Co.) at 100 rpm. Growing cultures were continuously illuminated with two 75-watt incandescent light bulbs. After 24 to 48 hours of photosynthetic growth, the culture media usually appeared deep red in color. At this time, the optical density at 650 mμ was measured approximately every four to six hours. Cultures were not used for inoculation purposes until the optical density measurement read at least 2.60.

APPENDIX II

PROCEDURES FOR PREPARING A STANDARD PLOT OF CELLULAR DRY WEIGHT VERSUS OPTICAL DENSITY FOR Rps. gelatinosa

Optical density measurement is a rapid, simple and accurate technique widely employed for quantitatively measuring cellular growth(1). Briefly, cells suspended in their respective growth medium are diluted to a turbidity range that can be quantitatively measured in a spectrophotometer. The amount of light deflection caused by microbial cells in suspension is read as optical density. Optical density measurements are usually standardized against some other procedure such as gravimetric analysis. Cellular dry weights can therefore be easily determined by using a standard plot of optical density versus dry weight.

Optical Density Measurement. Concentrated cellular suspensions were diluted with distilled water to an optical density (O.D.) less than 0.7 prior toO.D. measurement with a spectrophotometer (Spectronic 20, Bausch and Lomb, Inc.). The optical density of the original non-diluted sample was obtained by multiplying the optical density of the diluted sample by its respective dilution factor.

Gravimetric Analysis. A 10 - 15ml suspension of photosynthetic cells was harvested by vacuum filtration through a preweighed bacteriological membrane (Millipore) having a pore diameter of 0.45 microns. The membrane was washed with three aliquots of distilled water and

oven-dried to a constant weight.

Standard plots of cellular dry weight versus optical density for Rps. gelatinosa cultured in malate media and wheat bran infusion appear in Figures A.1 and A.2 respectively.

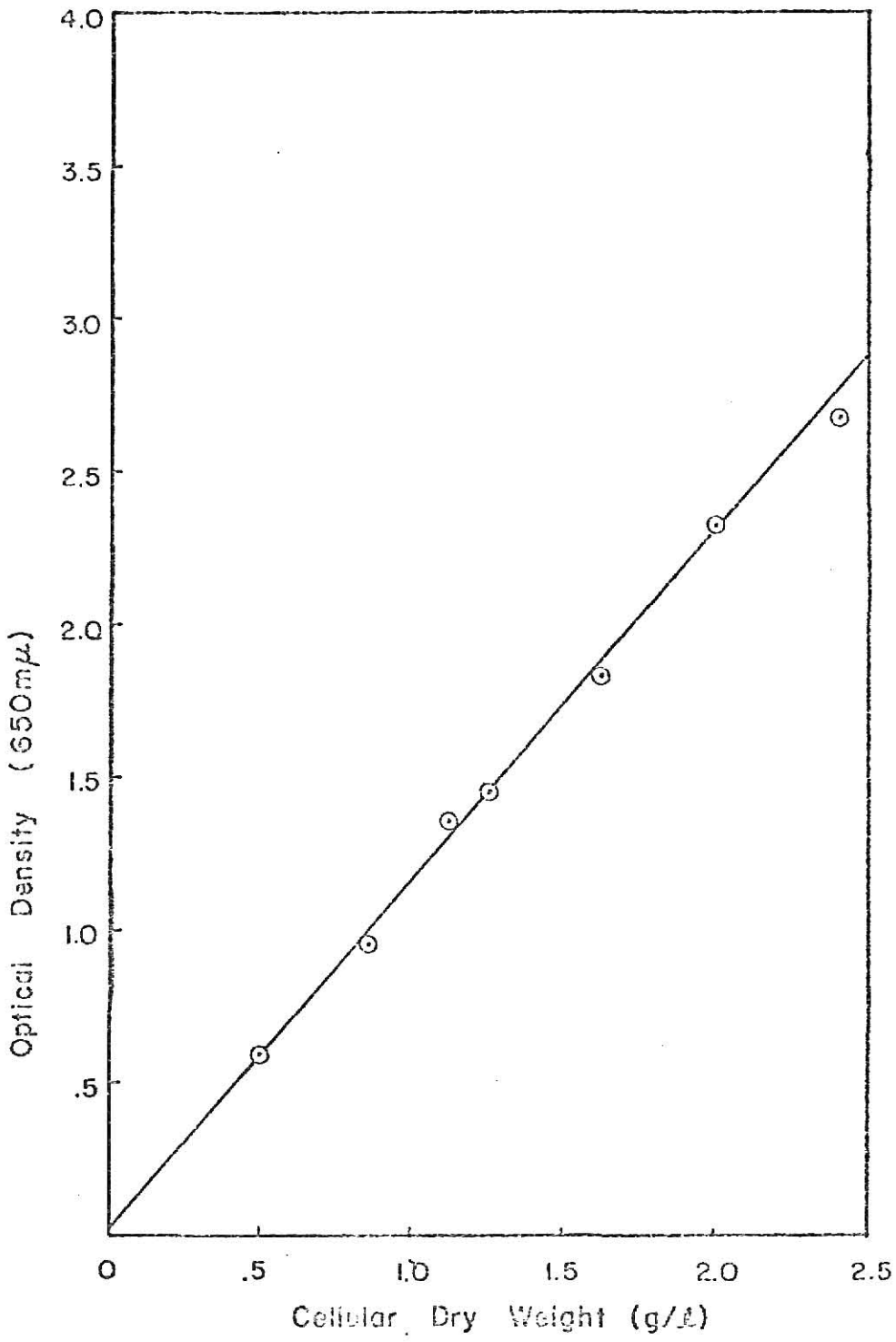

Fig. A.I Standard Plot of Optical Density versus Cellular Dry Weight for Growth in Malate Media.

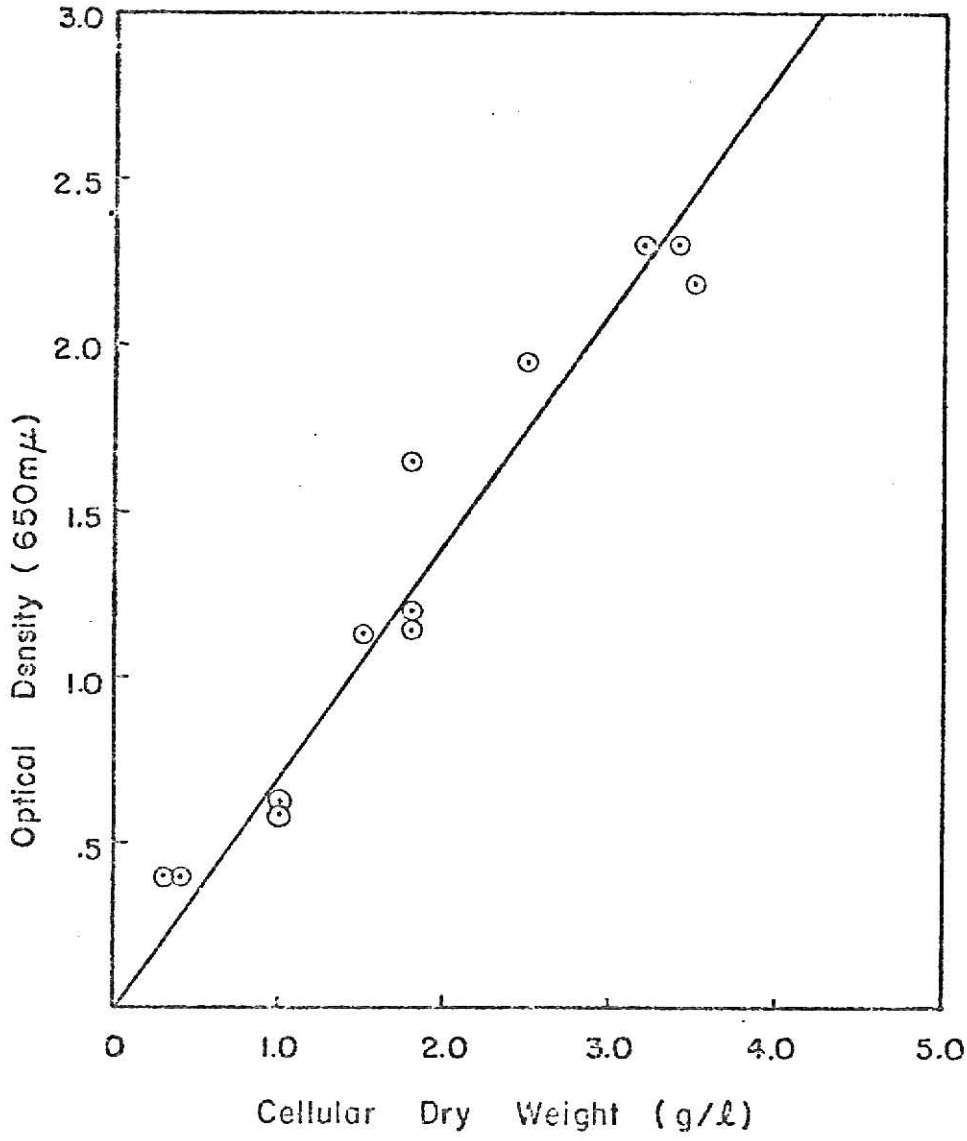

Fig.A.2 Standard Plot of Optical Density versus Dry Weight for Growth in Wheat Bran Infusion Media.

APPENDIX III

COD DETERMINATION

Chemical oxygen demand (COD) determination is a useful technique for measuring the oxygen equivalent of the total amount of organic matter in food processing wastes, sewage and other sources, susceptible to complete oxidation by a strong chemical oxidant. The dichromate reflux method (2) of COD determination, briefly outlined below, was used to measure the rate of substrate utilization.

Procedures: Effluent samples withdrawn from the fermentor were centrifuged at 3,000 rpm for ten to twelve minutes. Approximately 10 ml of supernatant was then withdrawn and used for COD determination.

To a 250 ml Erlenmeyer refluxing flask (Pyrex) were added:

(1) 0.4 g $HgSO_4$

(2) an aliquot of supernatant diluted to 10.0 ml with distilled water

(3) 5 ml of 0.25 N standard $K_2Cr_2O_7$ solution

(4) 15 ml of conc. H_2SO_4; carefully added

(5) 2-3 glass boiling beads

The mixture was thoroughly mixed, a condenser was attached to the flask and the mixture was refluxed for two hours on an electrical heating plate. A blank consisting of 10.0 ml of distilled water instead of sample, together with reagents, was refluxed in the same manner. After refluxing, the mixture was allowed to cool and the condenser was rinsed with distilled water which was added to the reflux mixture. To this

mixture was added two to three drops of ferroin indicator (Fisher). The solution was then titrated to a sharp reddish-brown endpoint with 0.5N standard ferrous ammonium sulfide.

Calculation: The chemical oxygen demand from dichromate was calculated as follows:

$$\text{mg/l COD} = \text{ppm} = \frac{(a-b)c \times 8,000}{\text{ml of sample}}$$

where

COD = chemical oxygen demand

ppm = parts per million

a = ml Fe $(NH_4)_2(SO_4)_2$ used for blank

b = ml Fe $(NH_4)_2(SO_4)_2$ used for sample

c = normality of standard Fe $(NH_4)_2(SO_4)_2$

APPENDIX IV

PROTEIN DETERMINATION

The following method of protein determination is based on a modification of the official micro-Kjeldahl method for nitrogen as suggested by Mitchell (3).

Procedures: Photosynthetic bacterial cells were harvested from culture medium by centrifugation. Harvested cells were washed twice by resuspending cells in distilled water and recentrifuging. Washed cells were dried overnight at 95°C in an air-circulating drying oven (Precision Scientific).

To a 30 ml Kjeldahl flask were added: 50 mg of dried photosynthetic bacterial cells, 1 ml conc. H_2SO_4 and 1 g catalyst. The mixture was heated on a digestion rack until the solution was clear. After digestion the solution was diluted to 250 ml and 5 ml of digest was transferred to a 100 ml volumetric flask. In addition, the following reagents were added to the volumetric flask:

(1) 1 ml EDTA

(2) 5 ml Solution A

(3) 10 ml Solution B

(4) 10 ml Solution C

(5) distilled water to volume.

Reagents:

(1) Catalyst - 2g $CuSO_4 \cdot 5H_2O$ and 30g K_2SO_4

(2) EDTA (ethylenediaminetetraacetic acid) - 1g EDTA suspended in 100 ml water; pH adjusted to 10.0 with conc. NaOH.

(3) Solution A - 4.8g NaOH dissolved in water and diluted to 1.0 l.

(4) Solution B - 5g phenol and 25mg sodium nitroprusside dissolved in water and diluted to 500 ml.

(5) Solution C - 2.5g NaOH, 1.87g anhydrous Na_2HPO_4, 15.9g $Na_3PO_4 \cdot 12H_2O$ and 5ml sodium hypochlorite (5.25%) dissolved in water and diluted to 500ml.

A standard curve (Figure A.3) was prepared by adding varying amounts of ammonium sulfate in place of sample in the above procedure. The crude protein content was determined by multiplying total nitrogen (percent) by 6.25.

Calculation. The crude protein content (N X 6.25) was determined as follows:

$$\text{Crude Protein (\%)} = \frac{\text{total N in sample } (\mu g) \times 50}{\text{sample dry weight (mg)}} \times 100 \times 6.25$$

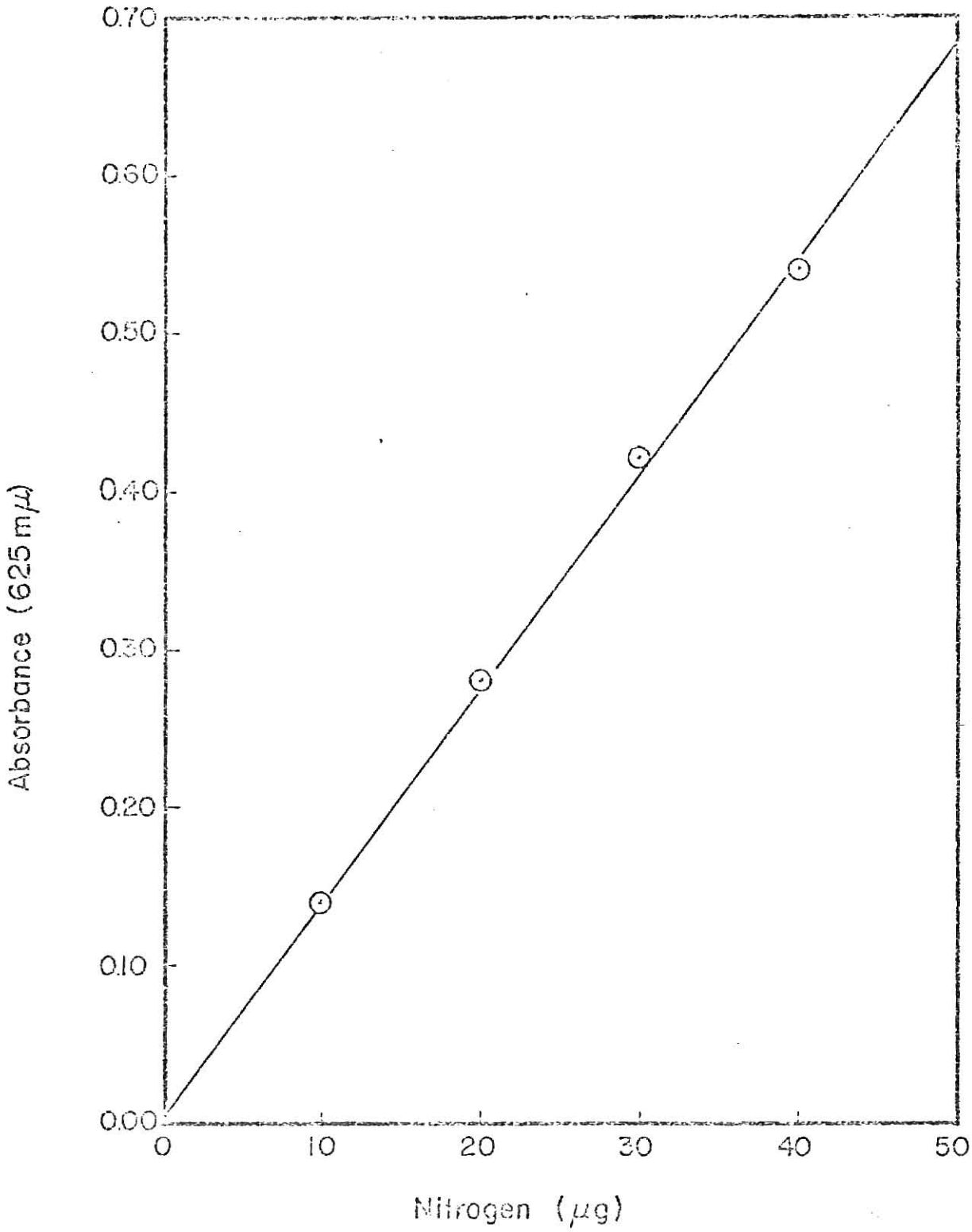

Fig. A.3 Standard Plot of Absorbance versus Total Nitrogen.

APPENDIX V

RIBONUCLEIC ACID (RNA) ANALYSIS

Among various methods which have developed for the analysis of RNA, the determination of RNA content of microbial cells by sugar analysis has been widely used (4). The major steps involved in this analysis are:

(1) Extraction of Acid Soluble Materials with Cold Trichloroacetic Acid (TCA) - 0.5ml of ice cold fifty percent trichloroacetic acid (TCA) was added to 5ml chilled culture broth containing approximately 50mg/ml of RNA. The samples were allowed to stand in an ice water bath for thirty minutes with occasional shaking. Samples were then centrifuged at 12,000 rpm for ten minutes. The supernatant was discarded and the pellets were treated for the extraction of RNA.

(2) Extraction of RNA - 2.5ml of five percent TCA was added to the abovepellets, the mixture was vigorously shaken, then placed in a water bath at 90°C for fifteen minutes. The contents were centrifuged at 12,000 rpm for fifteen minutes. The supernatants were analyzed for purine-bound ribose with orcinol reagent.

(3) Sugar Analysis (Purine-Bound Ribose) - The RNA extract was analyzed for its sugar content (purine-bound ribose) with orcinol reagent prepared as follows:

orcinol reagent - 20mg orcinol/ml Fe-HCl mixture where

Fe-HCl mixture = 4mg $FeNH_4(SO_4)_2 \cdot 12H_2O$/ml conc. HCl.

Although the Fe-HCl mixture is quite stable, the orcinol reagent must be prepared each time just prior to its use.

The orcinol reaction was carried out with 1ml of RNA extract and with standard sugar solution (10 µg arabinose/ml of solution). The reaction mixtures were prepared as follows:

Test Tube No.	Standard Sugar Solution(ml)	RNA Extract (ml)	Orcinol Reagent (ml)	Water (ml)
1	0.0	-	2.0	2.0
2	0.5	-	2.0	1.5
3	1.0	-	2.0	1.0
4	1.5	-	2.0	0.5
5	2.0	-	2.0	0.0
6	-	1.0	2.0	1.0

The test tubes were placed in a water bath at 100°C for twenty minutes and the absorbance was read at 660 mµ with a spectrophotometer (Spectronic 20, Bausch and Lomb, Inc.). A standard plot of absorbance versus arabinose content was prepared (Figure A.4) and the sugar content of RNA extract

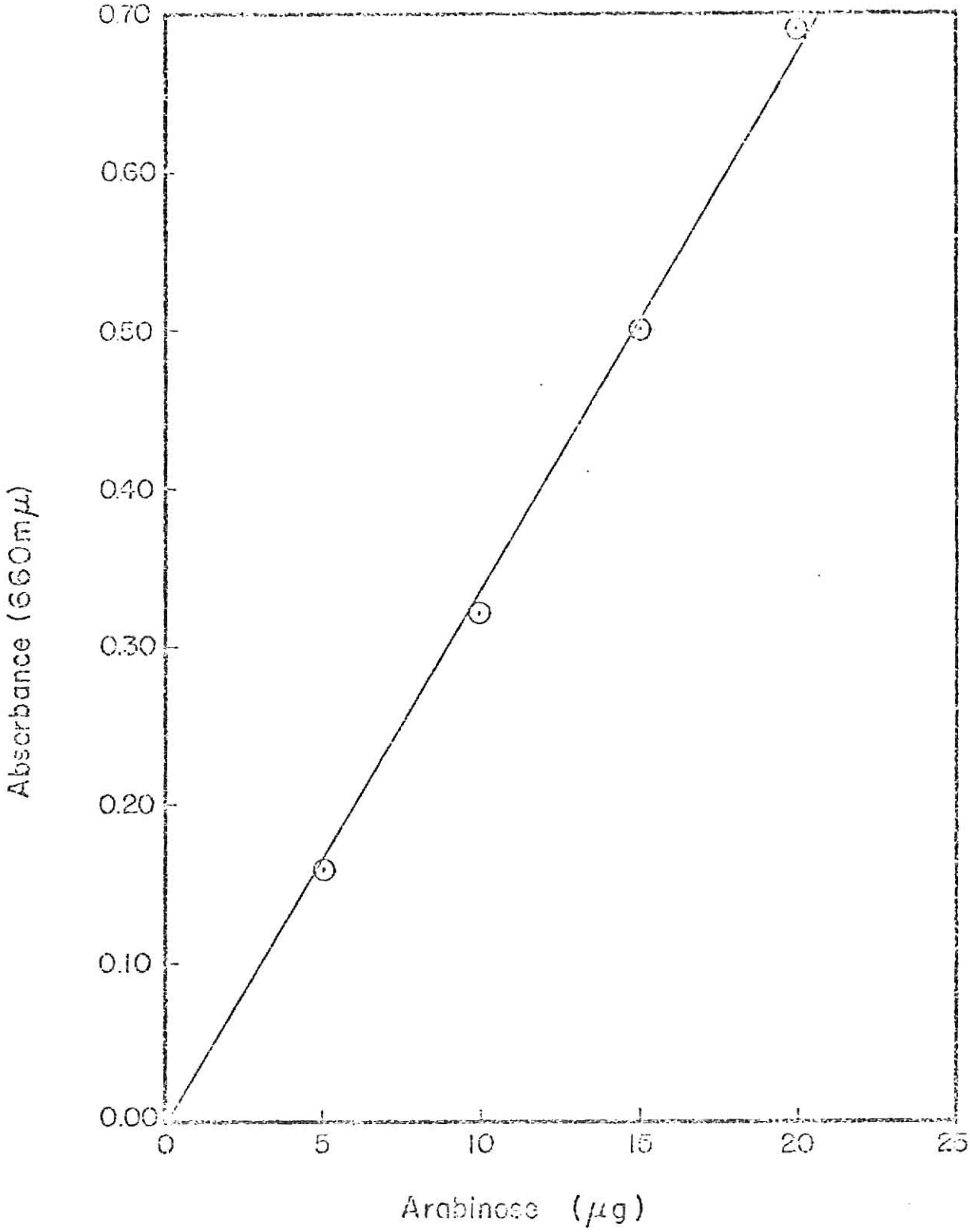

Fig. A.4 Standard Plot of Absorbance versus Arabinose Concentration.

was obtained from this plot. RNA content was obtained by multiplying the sugar content by a factor of 3.61.

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PROTEINS FROM PHOTOSYNTHETIC BACTERIA

by

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AN ABSTRACT OF A MASTER'S THESIS

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ABSTRACT

In light of the world-wide fight against hunger, intensive research activities have been directed toward the production of Single-Cell Protein (SCP) from microorganisms as a means of meeting the demand for new sources of edible proteins. Important features of SCP are that they are fast-growing, have a high protein content of relatively good quality, can be derived from sources independent of an agricultural base, are not dependent in their production upon climatic conditions, can be easily manipulated from a genetic point of view, and can flourish on simple inorganic nutrients and a variety of carbon sources. Problems related to the production of SCP center around factors affecting cell grwoth, cell recovery, protein isolation, toxicity, and general acceptability. The long-term success of SCP is largely dependent upon the ease by which microorganisms may be manipulated to produce custom protein isolates used in synthetic foods of the future.

Until recently, photosynthetic bacteria have long gone unrecognized as potential sources of edible proteins. In an attempt to evaluate the feasibility of producing SCP from photosynthetic bacteria, a series of laboratory-scale batch experiments were designed to investigate the effects of certain parameters such as temperature, pH, salt concentration and substrate concentration on the growth of Rhodopseudomonas gelatinosa.

Preliminary studies involving the batch cultivation of Rps. gelatinosa indicated optimal growth occurring at a temperature of 30°C and a pH of 7.0±0.5. Concentrations of 0.2 percent ammonium sulfate were shown to be optimal for growth. Growth in DL-malic acid increased maximally with substrate; at concentrations greater than 0.5 percent, the rate of growth decreases as the organisms become saturated with substrate. Yeast extract appeared to enhance growth, however, beyond a concentration of 1.0 percent yeast extract, a significant increase in growth was not observed.

Studies concerned with the examination of various raw materials as potential substrates for large-scale mass cultivation indicated that materials such as food processing wastes, milling wastes, or other sources of crude sugars could serve as satisfactory nutrient sources for the cultivation of photosynthetic bacteria. The growth of Rps. gelatinosa in wheat bran infusion media appeared to increase with substrate concentration; the highest cellular dry weight obtained for growth in 6.0 percent wheat bran infusion media was 7.46 g/l.

Continuous cultivation experiments in which Rps. gelatinosa was cultured in a specially-modified laboratory-scale bench fermentor indicate the possibility of cultivating photosynthetic bacteria, however, many of the results obtained are inconclusive at this point. Many factors, including light intensity, which play a significant role in photosynthetic growth, remain for further examination.

Harvested photosynthetic cells contained from 57.6 to 61.4 percent protein. The amino acid content of photosynthetic proteins was shown to be comparable to that of many conventional foods. Neither the nucleic acid (RNA) content, which ranged from 4.28 to 5.93 percent, nor the protein content of harvested photosynthetic cells appeared to vary significantly with changes in dilution rate.

A technological and economical feasibility study indicated that a process for production of SCP from photosynthetic bacteria could have economic potential if one considers the current selling price of commercial food yeasts at thirty to thirty-five cents per pound.

Major areas of photosynthetic SCP production recommended for future study include: selection and isolation of faster growing strains of photosynthetic bacteria, mass-cultivation on alternate sources of nutrients such as sewage and animal wastes as a possible solution to alleviating high substrate costs, safety and nutrition studies, and the design and optimization of photosynthetic SCP processes with more efficient utilization of solar energy.