BACTERIAL FLOCCULATION

By

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CHAPTER I

INTRODUCTION

The phenomenon of bacterial flocculation is extremely complex, and flocculation may depend upon a large number of factors. Any factor may predominate according to the peculiar and particular set of environmental conditions in which the bacteria exist. Regardless of many proposed theories made by different investigators, the exact mechanism of bacterial flocculation has not been agreed upon.

In this research, several microorganisms, both floc-producing bacteria and non floc-producing bacteria, were used. They were Pseudomonas aeruginosa, Serratia marcescens, Escherichia Coli, and three unknown organisms isolated from sewage (hereafter called the slime former, the blue organism, and the yellow organism). Glucose was selected as the substrate throughout the study. Pure cultures and mixed pure cultures of bacteria with different substrate concentrations were studied. The relationships among substrate removal, substrate concentration, bacterial capsule, metabolic intermediates and/or end products, bacterial specific growth rate, and bacterial flocculation were investigated. Bacterial flocculation was observed by using a Zeiss microscope.

CHAPTER II

LITERATURE REVIEW

A. Bacterial Growth

In traditional batch methods of growing cells, the developing population passes through the following phases:

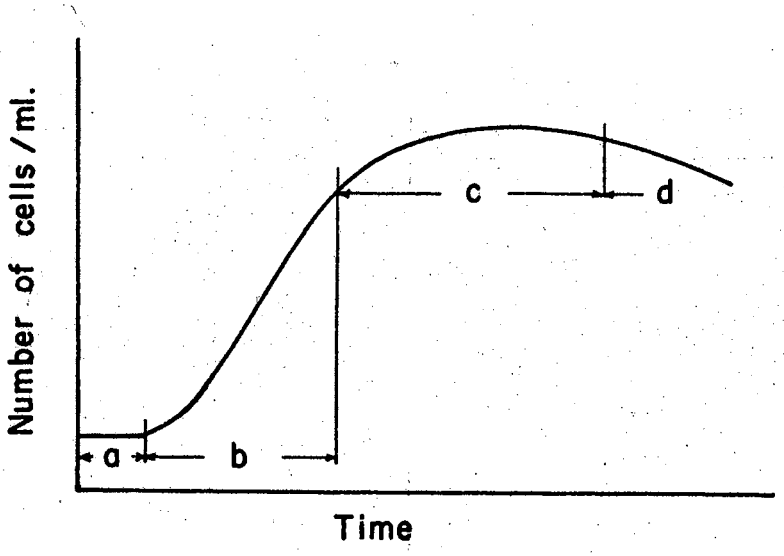

Figure 1. Schematic Representation of Bacterial Growth

Phase a: lag phase

In which cell mass increases but no division occurs.

Phase b: logarithmic phase

During this period the cells divide steadily and cell number increases at a constant growth rate.

Phase c: stationary phase

The population remains constant for a while, perhaps as a result of complete cessation of division or the balancing of reproduction rate by equivalent death rate.

Phase d: declining phase

Bacteria die at a rate which exceeds the rate of production of new cells (1).

In case of unicellular growth, the rate of growth can be expressed by a limiting substrate concentration as follows:

$$\frac{dx}{dt} = f(x,s)$$

x = mass of cells per unit value

s = concentration of growth-limiting substrate

The specific growth rate of the cells, μ, can be defined as

$$\mu = \frac{1}{x}\frac{dx}{dt} = \frac{d\ln x}{dt}$$

This is the so-called exponential growth equation which shows that growth is proportional to the mass of cells present.

Monod, in his study of bacterial growth in which the growth rate was limited by a single substrate, suggested the following relationship (2):

$$\mu = \mu_{max}\left(\frac{s}{K_s + s}\right)$$

μ_{max} = **maximum growth rate when the substrate is unlimited**

K_s = the substrate concentration at which the specific growth rate observed is $\frac{1}{2}$ the maximum μ .

B. Bacterial Surface

The surface of the bacteria may be the key to floc formation. For the most part, bacteria are regarded as colloids. Although the size of bacteria exceeds the limits normally set for colloids, the physical properties are predominately of a colloidal nature. So the principles of colloidal chemistry must be kept in mind whenever the bacterial surfaces are discussed.

Most bacteria are found to have definite capsules and it appears that bacteria which grow in chain form are jointed at the capsular surface. Dubos (3) stated that capsular production was not dependent on the bacteria strain but was a factor of the conditions under which the culture was growing. After carefully reviewing the work of other investigators, Dubos concluded that capsular material was a high molecular polysaccharide containing acidic, acetyl, and amino groups in varying amounts. Porter (4) and Knaysi (5) also came to the same conclusions as Dubos (3).

Abramson (6) found that bacteria had a definite electrokinetic potential and that the reduction of this potential resulted in agglutination of the bacteria. The reduction in electrokinetic potential was brought about by the addition of various salts to the solution. It was shown by

McCalla (7) in 1940, that bacteria adsorbed positive ions from solution as a result of their negative surface charge. E. Coli were found by Weiss (8) to be adsorbed onto silt in a stream as the result of electrical charge.

The chemical composition of the slime layer is believed to be responsible for the electrical charge on the bacteria. Very little is known about the chemical structure of capsular material. The conclusions on the capsular structure presented by Dubos (3), Porter (4), and Knaysi (5) were drawn on quite heavily, as they have collected the important data from almost every available source. These data and data obtained by Goebel (9) were composited, and a general capsular structure was set forth by McKinney (10). The chemical formula for this material is shown in Figure 2.

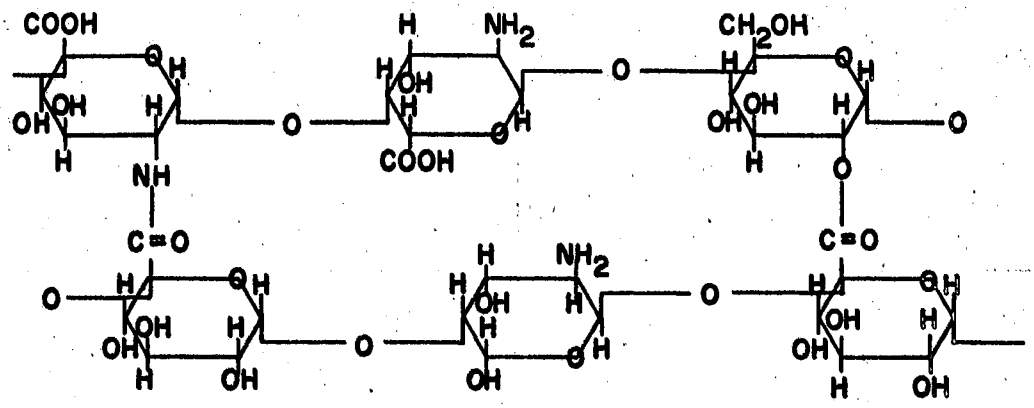

Figure 2. Chemical Structure of Capsular Material Composited from Various Bacteria

This structural formula does not represent the exact surface structure of any specific organism but does contain the general components of the capsular material of the organisms which might be found in activated sludge. Although

the material is predominantly polysaccharide, it does have the electrical characteristics of a protein, that is, amphoteric. This satisfied the experiments of Dyar (11).

In a neutral or basic solution the carboxyl group ionizes as follows:

$$-COOH \rightleftharpoons -COO^- + H^+$$

The ionized carboxyl group gives the bacterial surface a negative charge. It can be seen from the chemical structure that the negative charge is actually a point function on the bacterial surface. If the solution is acidic, the amino group is affected as follows by ionization.

$$-NH_2 + H^+ \rightleftharpoons -NH_3^+$$

This would give the surface a positive charge.

C. Floc-Producing Bacteria

Butterfield (12) and Wattie (13) found that the zoogloeal organisms active in the trickling filter were identical and interchangeable with the zoogloeal organism in activated sludge.

1. Buswell and Long (14) in 1923 proposed that activated sludge flocs were composed of a synthetic gelatinous matrix, in which filamentous and uncellular bacteria are imbeded and on which various protozoa feed and crawl. The purification is accomplished by digestion and assimilation by organisms of the organic matter in the sewage and its resynthesis into the living material of the organisms.

This process changes organic matter from the colloidal and dissolved states of dispersion to a state in which it will settle out. Buswell also stated that activated sludge is made up of zoogloeal floc and that the protozoa on this floc are responsible for the major portion of the purification of the sewage.

2. In 1935, Butterfield (12) was able to isolate from a sample of activated sludge, the organism Zoogloea ramigera. He later postulated that the floc was caused by particulate matter adsorbing to the large gellatinous capsule secreted by this organism. Also he pointed out that this organism, alone, was responsible for the removal of the soluble components of sewage in the activated sludge process.

3. McKinney and Horwood (15) in 1952 made the following observations:

"(1). It is possible to isolate from activated sludge other organisms than Z. ramigera which are capable of forming a floc similar to activated sludge when aerated in a suitable nutrient substrate.

(2). The floc-forming organisms isolated, other than Z. ramigera, included Escherichia intermedium, Paracolobactrum aerogenoides, Nocardia actinomorpha, Bacillus cereus, and a bacterium belonging to the genus Flavobacterium.

(3). Aerobacter arogenes was also found to form a floc of the same nature as the other bacteria, even though it was not isolated from activated sludge during any of the experiments."

D. Definition and Classification of Bacterial Flocculation

The fundamental mechanism of the process of bacterial flocculation has not yet been established. Therefore, it is impossible to give an exact definition of bacterial flocculation. However, bacterial flocculation can be classified into three types, which may involve physical, chemical, and biological factors. They are:

1. Natural flocculation - Natural flocculation has been attributed to the collision of bacteria with impurities present in the wastes. In 1914, the discovery of activated sludge by Arden and Lockett in the laboratory of the Manchester Sewage Works gave rise to the idea of natural flocculation. The biological slimes naturally developed in the aerated organic wastes and formation of zoogloeal flocs. These flocs are highly active centers of biological life, composed of living masses of organisms, food, and slime material (16, 17).

2. Auto-flocculation (or self-flocculation) - No one has clarified the apparent mystery of bacterial auto-flocculation, but many postulations have been made to explain this phenomenon. The mechanism is usually attributed to the colloidal characteristics of the cells; negative charges distributed over the bacterial surface set up a competitive force between electrostatic repulsion and Van der Waal's attraction. When the latter predominates, auto-flocculation can take place.

3. Chemical flocculation - From 1923 to 1925,

Theriault and Clark (18) and Miller (19) set forth the fundamental concept of chemical flocculation. They said that there must be present a certain minimum quantity of aluminum (Al^{+++}) or ferric (Fe^{+++}) cations; there should be present an anion of strong coagulating power; and pH must be carefully adjusted.

E. The Theory of Floc Formation

1. Previous Work

The theory of activated sludge was in considerable doubt before Butterfield (12) showed that it was possible to isolate a bacterium from normal activated sludge which was capable of producing a floc. Butterfield felt that this bacterium, Zoogloea ramigera, secreted a gelatinous matrix which was observed in photomicrographs of normal activated sludge.

Heukelekian and Littman (20) in 1939 confirmed the work of Butterfield. This was followed by more extensive work by Wattie (13). These investigators felt that the bacteria were bound together by a gelatinous material surrounding each cell. Also in 1939, Rudolfs and Gehm (21) reviewed the various theories of colloidal adsorption in the aerobic treatment process. The work of Dunbar, Dienert, Theriault, Cavel, Buswell, Baly, Lumb, and many others (22) showed that the colloidal matter in sewage was adsorbed by the slimes, but none agreed on the mechanisms involved. Theriault believed that the gelatinous matrix of activated sludge was a

biozeolitic substance and that the organic materials were adsorbed onto the sludge by an ion exchange process. Electrical charge was the theory of Cavel and others. Cavel felt that the slimes were positively charged, as they adsorbed negative colloids. This idea was disproved by Buswell, who showed that both the colloid and slime had negative charges.

In 1940, McCalla (7) measured the cation adsorption capacity of Escherichia Coli and other bacteria by four methods. He demonstrated that

(1) Escherichia Coli adsorbed cations and the magnitude of this capacity could be measured.

(2) Other bacteria adsorbed cations.

(3) The adsorbed cations were exchangeable.

He also postulated that the presence of protein polar groups in the cell or on the surface of the cell would produce an ionizable H^+ which could be replaced by any cation as indicated in the following equation

$$R\text{-}CH\text{-}NH_2\text{-}COOH \longrightarrow R\text{-}CH\text{-}NH_2COO^- + H^+$$

Such active spots might account for the presence of a cation adsorption complex in a living cell.

2. Microscopic Observations

McKinney (10) in 1952 made observations of pure bacterial cultures, with the microscope. The bacteria used in his work were Zoogloea ramigera, Escherichia intermedium,

Nocardia actinomorpha, Bacillus cereus, Aerobacter aerogenes, and others in addition to using sewage-seeded synthetic substrates. In all cases it appeared that the floc was formed in the same manner. The bacterial cells attracted each other and were joined together by their capsules. This capsular material did not expand in the early stage of floc development. New cells were soon nothing more than a large mass of bacteria separated by their capsules. This bears out the observations of Heukelekian and Littman (20) and Wattie (13). When using soluble substrates, there was not at any time the appearance that the bacteria were residing in a large zoogloeal or slime matrix. However, when sewage or synthetic substrates containing insoluble materials were used, the floc had the appearance of bacteria residing in a zoogloeal or slime matrix.

McKinney and Weichlein (23) in 1953, maintained that floc formation was correlated with the metabolic activities of the bacteria. The bacteria did not form floc as long as they were actively metabolizing organic matter and reproducing. It was only after the bacteria had ceased their metabolic activities that floc could be determined microscopically. Microscopic observations indicated that floc was the result of complete metabolism of the organic substrate and was not caused by special zoogloeal-producing bacteria.

3. Theory of Floc Formation

In 1952, McKinney (10) set forth a probable theory on

the formation of activated sludge floc. The floc formation was mainly due to the collisions between cells and electric charges of bacterial surface. The bacteria will either stick together as the result of collision, or bounce off in a new direction. There are several factors which will affect the probability of the two bacteria sticking on that first collision. The angle of collision, velocity of the bacteria, surface tension forces and electrical charge all play a part in whether or not the two bacteria will stick. If the surface charge on the cell is low, the angle of collision is large, the surface of contact is large, and the velocity of collision is large, the probability of the two bacteria sticking together is high. Once the two bacteria have stuck together, a larger surface area is available for collision with a third bacterium. The overall surface potential has been reduced by the proportion of surface area in contact. Thus, the probability of collision increases as the size of the floc becomes larger. As more bacteria collide and stick, the probability of a collision with a sewage colloid also increases. Bacterial flocculation was brought about merely by reducing the electrical charge below the critical level and allowing the cells to come into contact through agitation. It appears that the bacterial surfaces undergo direct chemical reactions, thus forming a solid bond between cells. The chemical structure of the bacterial surface offers many possibilities for chemical bonding. Because the bacteria contain the necessary enzymes

for synthesizing proteins from amino acids, a peptide linkage might exist. Direct salt linkage and ester linkage are also possible.

But in 1956 McKinney (24) presented a slightly revised version of his theory. He maintained that bacterial floc was primarily dependent on the energy of the bacteria rather than their surface charge. He proved that many bacteria have a surface charge of about 6-12 milivolts, whereas a charge of at least 20 milivolts is necessary to prevent floc. He maintained that the primary factor was the energy level of the bacteria. If the bacteria were able to get enough energy from substrate to overcome the Van der Waal's forces of attraction, floc would not occur. In the low energy system, Van der Waal's forces would predominate and floc would occur. He grew Serratia marseacens in nutrient media. Then he separated the bacteria from the nutrient media and resuspended them in a salt solution. As he predicted, floc occurred.

Tenney and Stumm (25) in 1964, proposed that biological self-flocculation results from the interaction of naturally produced polyelectrolytes which form bridges between individual microbial particles.

Crabtree, Boyle, McCoy, and Rohlich (26) in 1965 pointed out that bacterial flocculation is not absolutely related to slimes (capsules). They proposed that bacterial flocculation is due to the accumulation of the polymer poly-β-hydroxybutyric acid (PHB). They observed that the rapid

accumulation of PHB by Zoogloea ramigera was intimately associated with the flocculation of the organism. When Z. ramigera was grown in growth-limiting substrates, PHB did not accumulate and the culture did not flocculate. The addition of excess carbohydrate to the medium resulted in accumulation of the polymer by the organism and flocculation.

CHAPTER III

METHODS AND MATERIALS

A. Synthetic Waste

The composition of the synthetic waste used in this study is given in Table I.

TABLE I

COMPOSITION OF SYNTHETIC WASTE

Constituent	Concentration mg/l
Glucose	100 - 1,000
$CaCl_2$	7.5
$FeCl_3 \cdot 6H_2O$	0.5
$MnSO_4 \cdot H_2O$	10
$MgSO_4 \cdot 7H_2O$	100
$(NH_4)_2SO_4$	500
KH_2PO_4	526
K_2HPO_4	1,070

B. Bacterial Cultures

The pure cultures of bacteria used in this study were either isolated from sewage or known to be present in sewage. Three organisms were used for the major part of the

work. These were the slime former, Serratia marcescens, and Pseudomonas aeruginosa. Serratia marcescens and the slime former are floc-producers, whereas, Pseudomonas aeruginosa is a non floc-producer. To observe the relationship between growth rate and flocculation three additional organisms were used. They were Escherichia Coli, the yellow organism, and the blue organism.

C. Equipment Used in Obtaining Growth Curves

The shaker flasks used for growth curve experiments were of a special design, as shown in Figure 3 and were fitted to tubes which permitted light transmittance reading at 540 mμ by inverting and placing the tubes in a spectrophotometer (Bausch & Lomb Spectronic 20). The flasks were shaken at a constant speed of 90 strokes/min. in a water bath shaker apparatus operated at a constant temperature of 25^{o}C.

D. Equipment Used for Observation of Bacterial Flocculation

A Zeiss microscope with camera was used. Wet slides were observed with a green filter under 40x10 magnification; india ink slides were also observed under 40x10 magnification. The projected floc size was estimated by changing the irregular shape to an approximately equivalent circular one by eye.

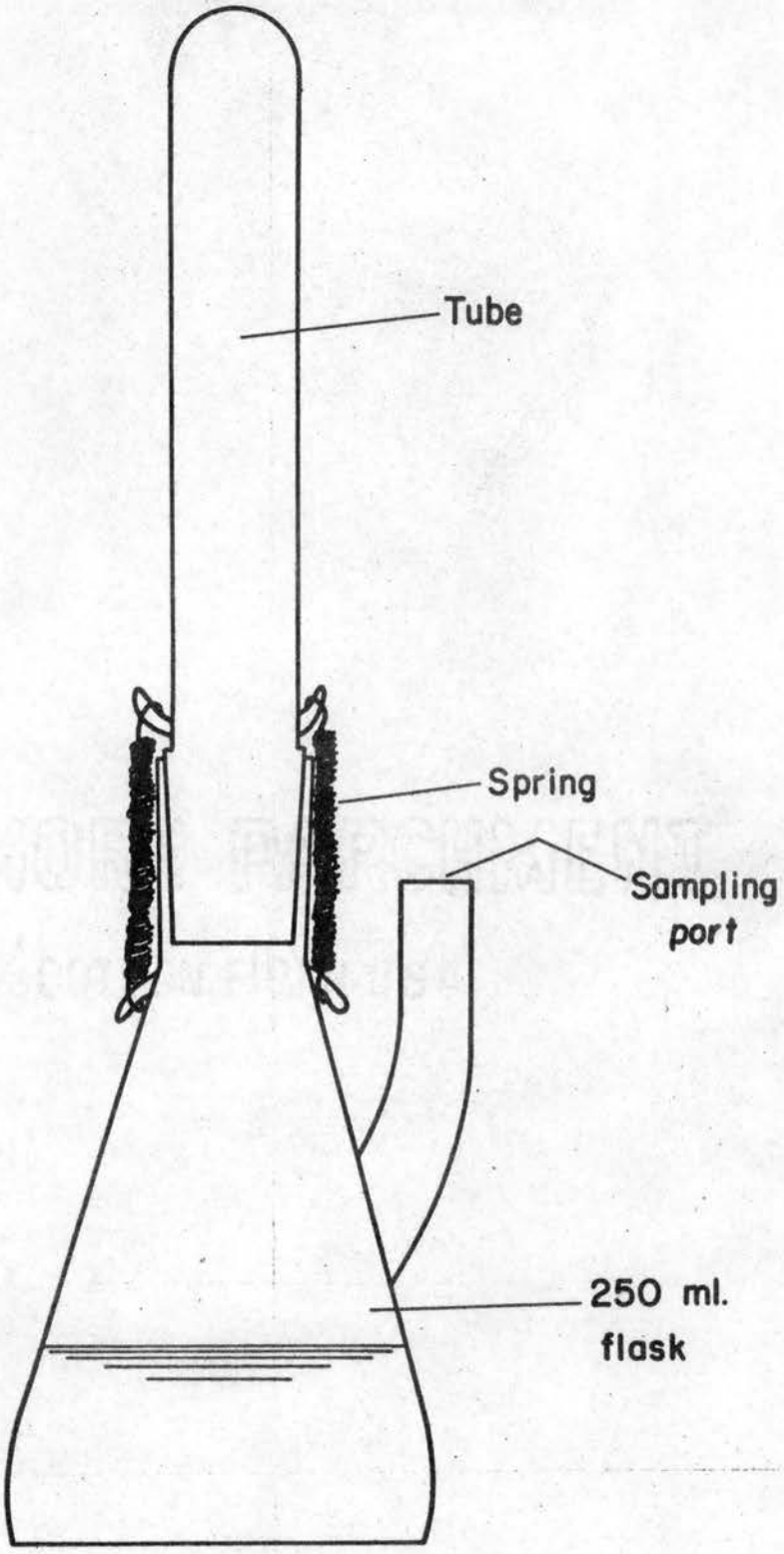

Figure 3. The Special Designed Shaker Flask

E. Analytical Techniques

1. Substrate Determination

(1) Chemical Oxygen Demand (COD)

COD determination is an important method to measure the oxygen equivalent of the organic matter in a sample.

The principle of the COD test is based upon the fact that all organic components with a few exceptions can be totally oxidized to CO_2 and H_2O by the action of the strong oxidizing agent, potassium dichromate, under acid conditions. In spite of the fact that the chief limitation of the COD test is its inability to differentiate between biologically oxidizable and biologically inert organic matter, it is widely used in research work because of the speed with which results can be obtained and its helpfulness in indicating the presence of biologically resistant organic substances.

The dichromate reflux method has been selected for the COD determination in the Bioengineering Laboratory of Oklahoma State University. Because it has advantages over other oxidants in reproducibility and applicability to a wide variety of samples and ease of manipulation. The detailed procedures for running the COD test is given in Standard Methods (27).

(2) Glucose Determination (Glucostat Test)

The Glucostat test which is specific for glucose was

utilized for glucose determination. This enzymatic determination was run in accordance with manufacturer's specifications (Worthington Biochemical Corp., Freehold, New Jersey, U.S.A.), and method 1-A was employed.

The calculation of glucose concentration was based on a standard curve plotted by measuring three or four different concentrations of the standard glucose covering the range from 0.05 to 0.30 mg glucose. Glucose COD may be obtained by the relationship between concentration and COD value of glucose:

$$\text{mg/l glucose} \times \frac{192}{180} = \text{mg/l glucose COD}$$

F. Experimental Protocol

All investigations were conducted in two phases:

1. Pure cultures of bacteria including:
   (1) The slime former,
   (2) Pseudomonas aeruginosa, and
   (3) Serratia marcescens
2. Mixed pure cultures of bacteria as shown below:
   (1) The slime former + Pseudomonas aeruginosa
   (2) The slime former + Serratia marcescens
   (3) Pseudomonas aeruginosa + Serratia marcescens,
   and (4) The slime former + Pseudomonas aeruginosa + Serratia marcescens.

In general, the experimental procedures were as follows:

1. The cells were grown on a glucose medium for 12-14 hours.
2. The shaker flasks and pipettes were sterilized in an oven at 350°F for 2 hours. The glucose medium, phosphate buffer, and salt growth medium were sterilized in an autoclave at 121°C, 15 psi for 15 minutes.
3. The required amount of salt growth medium and phosphate buffer were placed into the shaker flasks.
4. A 1:15 volumetric ratio of bacterial seed was added into the flask.
5. The required amount of glucose was then added.
6. The flasks were set in the water bath shaker, with constant temperature of 25°C and a constant oscillating velocity of 90 strokes/min.
7. Optical density and microscopic observations were made every 1 to $1\frac{1}{2}$ hours, also samples were collected every 1 to $1\frac{1}{2}$ hours for determination of COD and glucose.

The amount of salt growth medium, phosphate buffer, glucose, and acclimated seeds used in any one unit were dependent on the designed substrate concentration and total volume of the unit. For the unit of 500 mg/l glucose concentration and total volume of 60 ml, additions of 52.4 ml salt growth medium, 0.6 ml of 1 M phosphate buffer solution, 3 ml of 10 mg/ml glucose, and 4 ml of acclimated seeds were required. For other units, the addition of these materials

was made in the same proportion.

In order to find out whether the bacterial flocculation is proportional to the specific growth rate, six organisms were used. They were the slime former, Serratia marcescens, Pseudomonas aeruginosa, Escherichia Coli, the blue organism, and the yellow organism. Their maximum specific growth rates, μ_m's, and saturation constants, K_s's, were determined and their degree of flocculation were compared by microscopic observations.

In another study the slime former was observed to see whether or not the bacterial slime material was related to flocculation. The procedure used is given below:

1. The slime former was grown on a glucose medium until microscopic observations showed an abundance of slime.
2. The cells were centrifuged and resuspended in a phosphate buffer solution.
3. 30 ml of supernatant containing slime material (determined by microscopic observations of india ink slides) was placed into a shaker flask, and the rest centrifuged.
4. The cells were resuspended and the supernatant wasted.
5. Steps 2 and 4 were repeated until microscopic observations (india ink slides) showed that the bacteria contained no slime material.
6. 30 ml of the resuspended cells were placed into

another shaker flask.

7. 3 ml of 10 mg/ml glucose were added into each flask, and the salt growth medium was added to 60 ml volume.
8. Optical density and microscopic observations were made at every one hour interval.

CHAPTER IV

RESULTS

A. Typical Photomicrographs of Bacterial Flocculation

Figure 4 shows the growth curve of the slime former at a substrate concentration of 500 mg/l of glucose. During the period of growth, floc formation was observed using a microscope under 40x10 magnification. Photomicrographs of wet and india ink slides were taken at intervals marked on the curve. Figures 5 to 11 represent the photomicrographs of the wet slides of the slime former. The photomicrographs show that the slime former is a rapid floc-producer. Photomicrographs of india ink slides of the slime former are given in Figures 12 to 18. They reveal that the slime former produced a great deal of slime material and the individual cells, with their capsules tightly attracted together, formed the flocs.

The growth curve of Serratia marcescens is shown in Figure 19. Photomicrographs of wet slides at time 0, $1\frac{1}{2}$, 3, 4, 5, $6\frac{1}{2}$, and 8 hours are given from Figures 20 to 26. From these photomicrographs it can be seen that Serratia marcescens did form flocs. The photomicrographs of india ink slides, as shown in Figures 27 to 29, reveal that very little capsule-like material was produced by Serratia

marcescens. Repeated microscopic observations of its india ink slides showed that the produced capsule-like material was so small and so dim that it could hardly be distinguished whether it was capsular material or not.

The growth curve of Pseudomonas aeruginosa and its typical photomicrographs are represented in Figure 30 and in Figures 31 to 33 respectively. It may be seen that there was very little floc formed by Pseudomonas aeruginosa, hence it is classified as a non floc-producer. Van Gils (28) in 1964, reported that Pseudomonas aeruginosa did not flocculate. As shown in Figure 34, very little capsule-like material was produced by Pseudomonas aeruginosa. Repeated microscopic observations of its india ink slides revealed that the produced capsule-like material was also so small and so dim that it could hardly be distinguished whether it was capsular material or not.

B. Substrate Removal and Bacterial Flocculation

McKinney and Weichlein (23) in 1953, pointed out that floc formation was correlated with the metabolic activities of bacteria. They found that bacteria did not form floc as long as they were actively metabolizing organic matter and reproducing. Substrate removal can be used to predict the metabolic activity of bacteria. Substrate removal vs bacterial flocculation studies were conducted to determine if this was true with the pure cultures under study. Glucose removal was measured by employing the Glucostat test

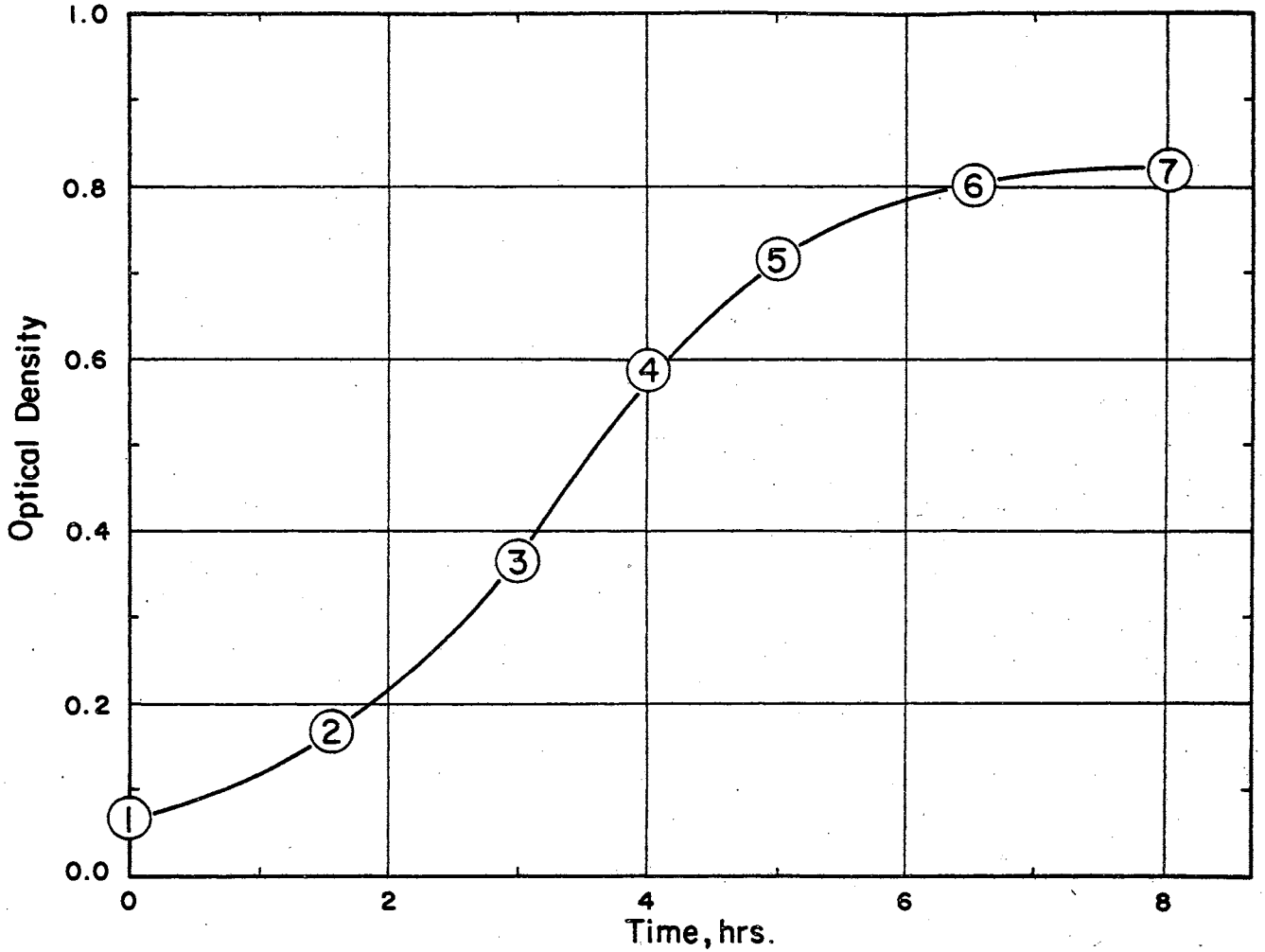

Figure 4. The Growth Curve of the Slime Former Showing the Time Periods at Which Photomicrographs Were Taken. 500 mg/l Glucose

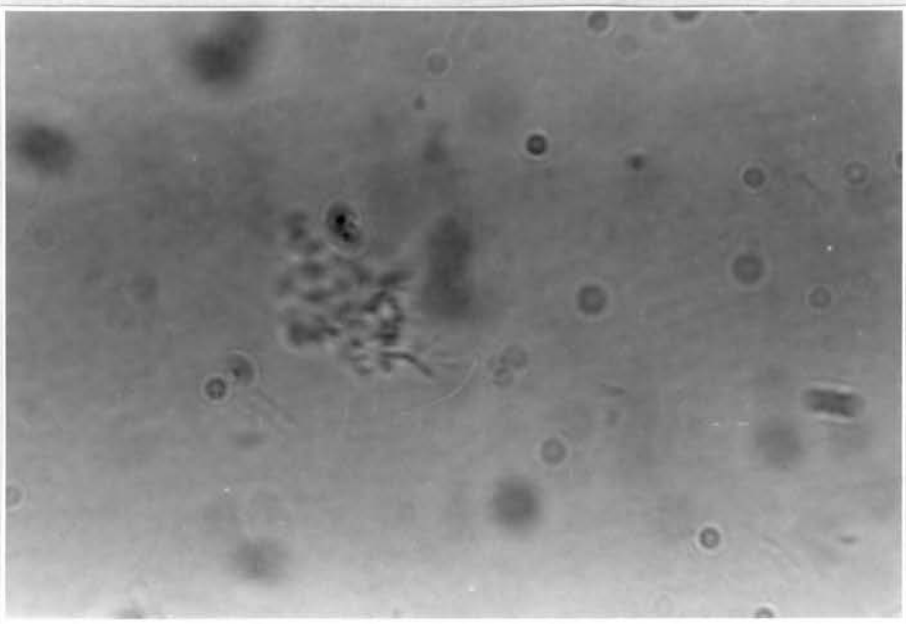

Figure 5. Photomicrograph of Wet Slide of the Slime Former at 0 hr.
Magnification = 40x10x3

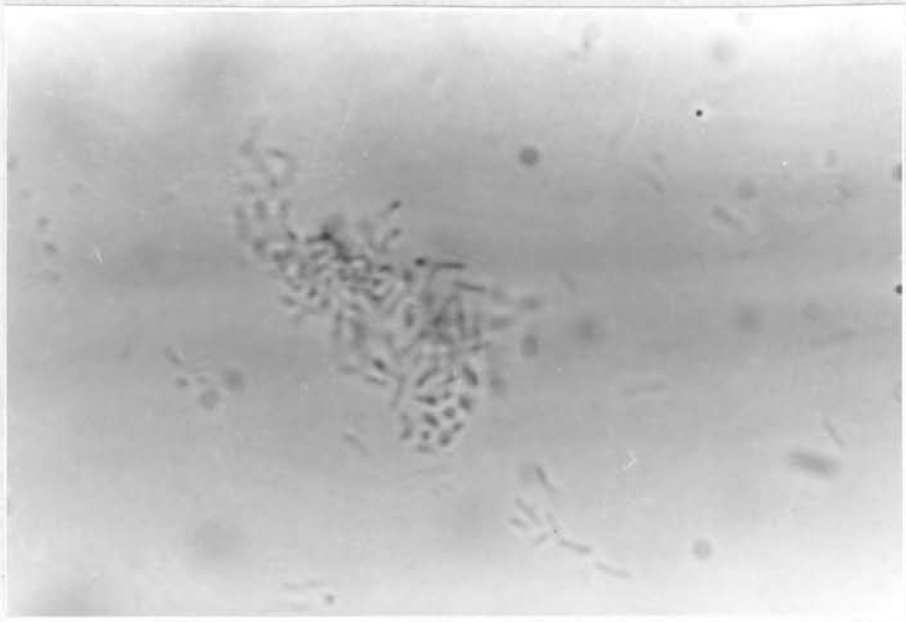

Figure 6. Photomicrograph of Wet Slide of the Slime Former at $1\frac{1}{2}$ hr.
Magnification = 40x10x3

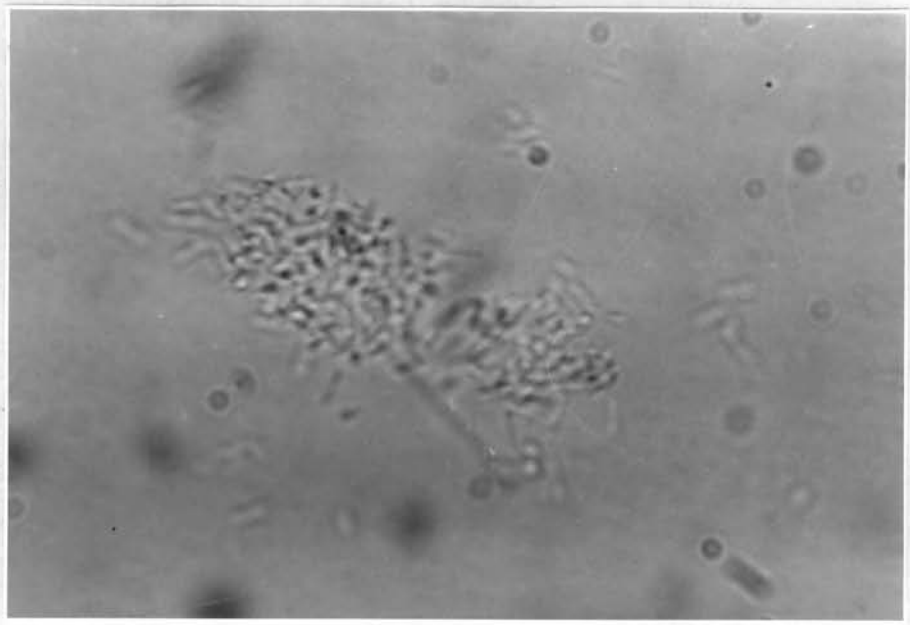

Figure 7. Photomicrograph of Wet Slide of the Slime Former at 3 hr.
Magnification = 40x10x3

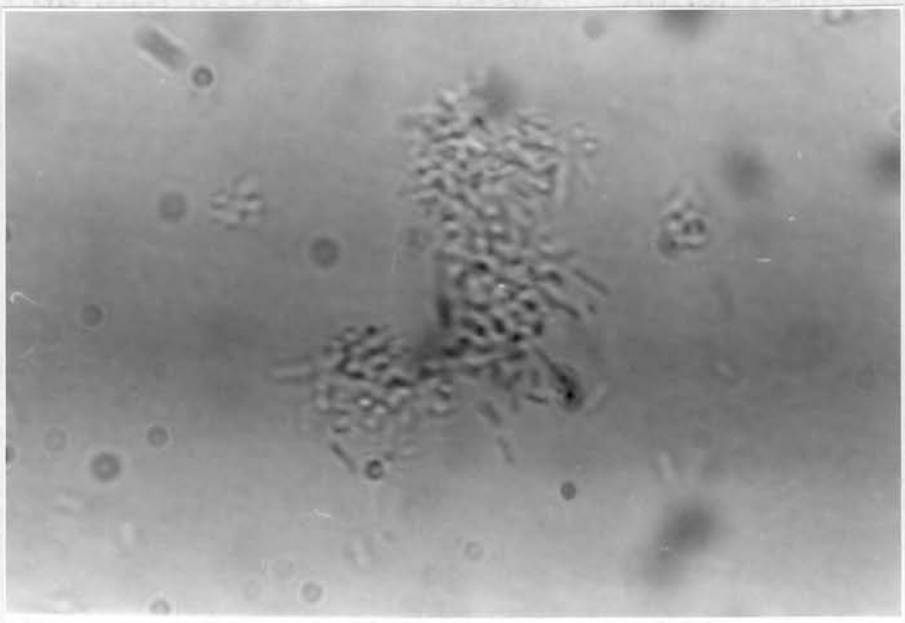

Figure 8. Photomicrograph of Wet Slide of the Slime Former at 4 hr.
Magnification = 40x10x3

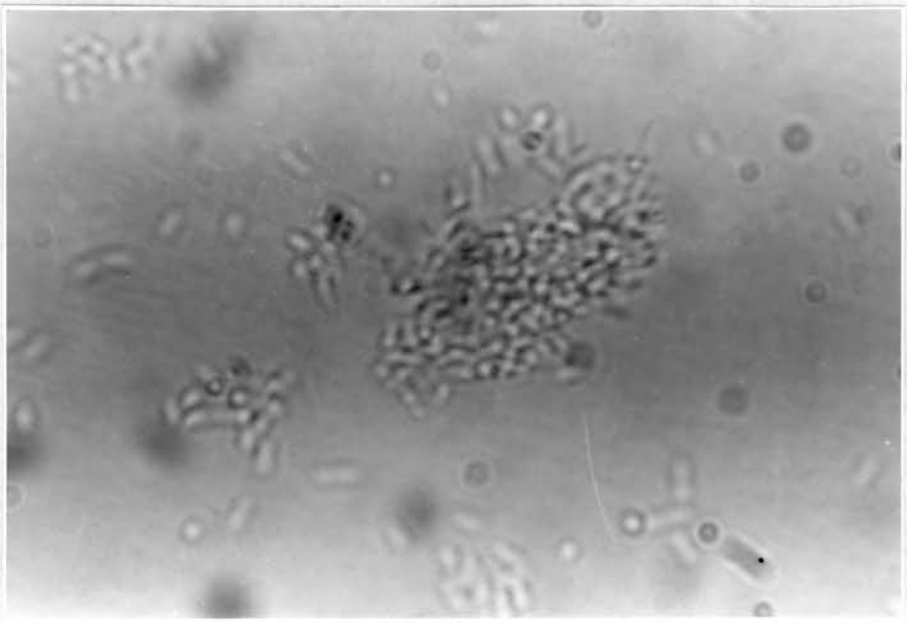

Figure 9. Photomicrograph of Wet Slide of the Slime Former at 5 hr.
Magnification = 40x10x3

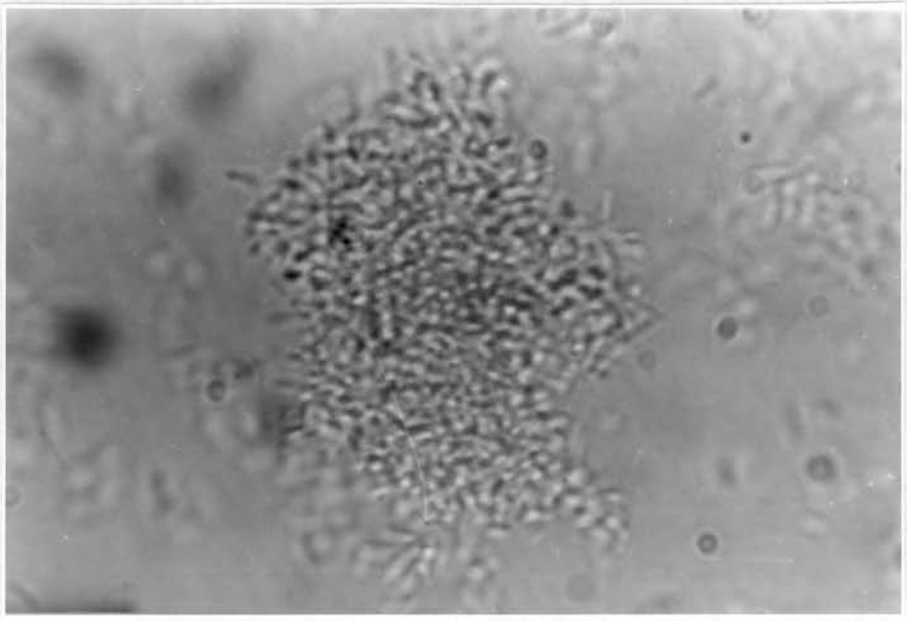

Figure 10. Photomicrograph of Wet Slide of the Slime Former at $6\frac{1}{2}$ hr.
Magnification = 40x10x3

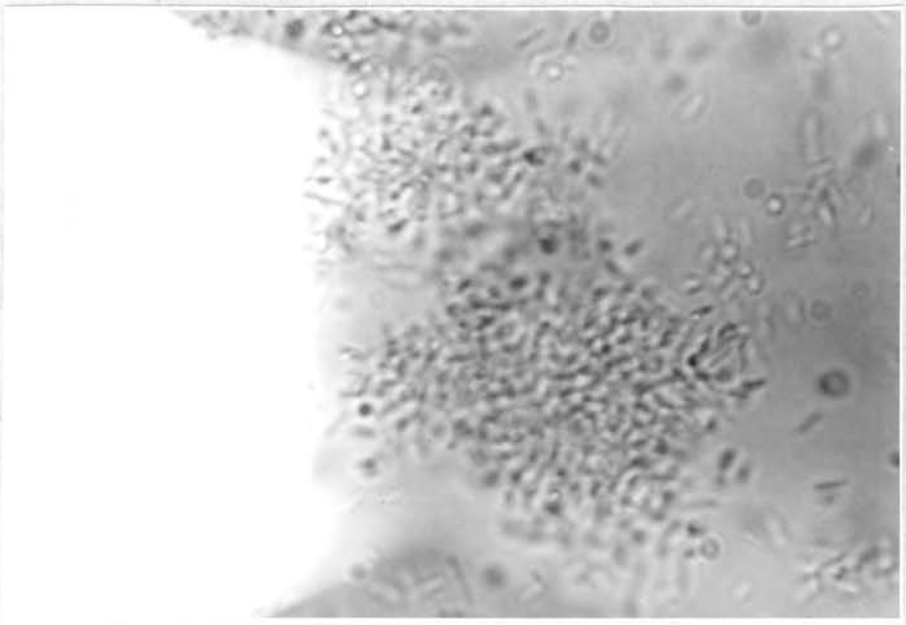

Figure 11. Photomicrograph of Wet Slide of the Slime Former at 8 hr.
Magnification = 40x10x3

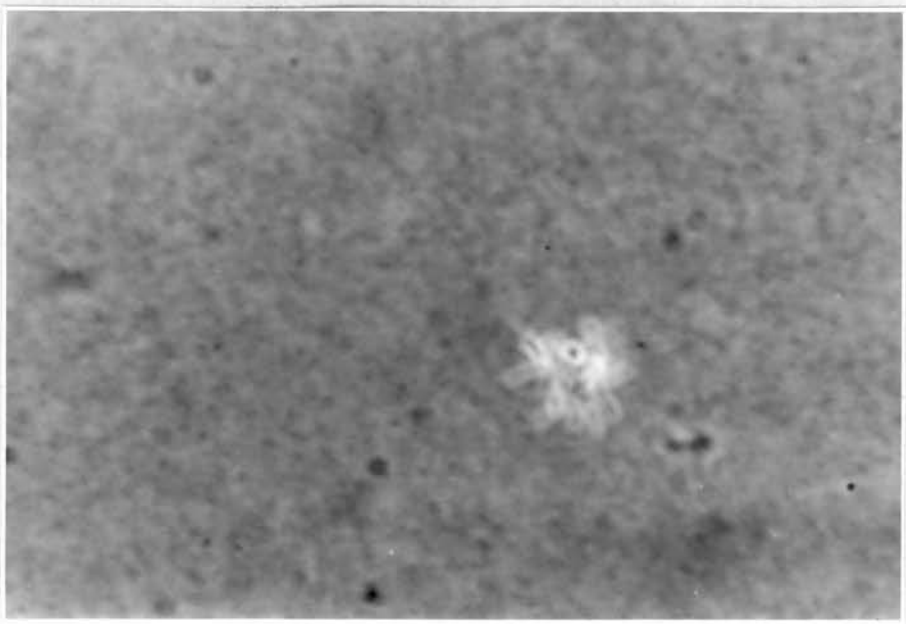

Figure 12. Photomicrograph of India Ink Slide of the Slime Former at 0 hr. Magnification = 40x10x3

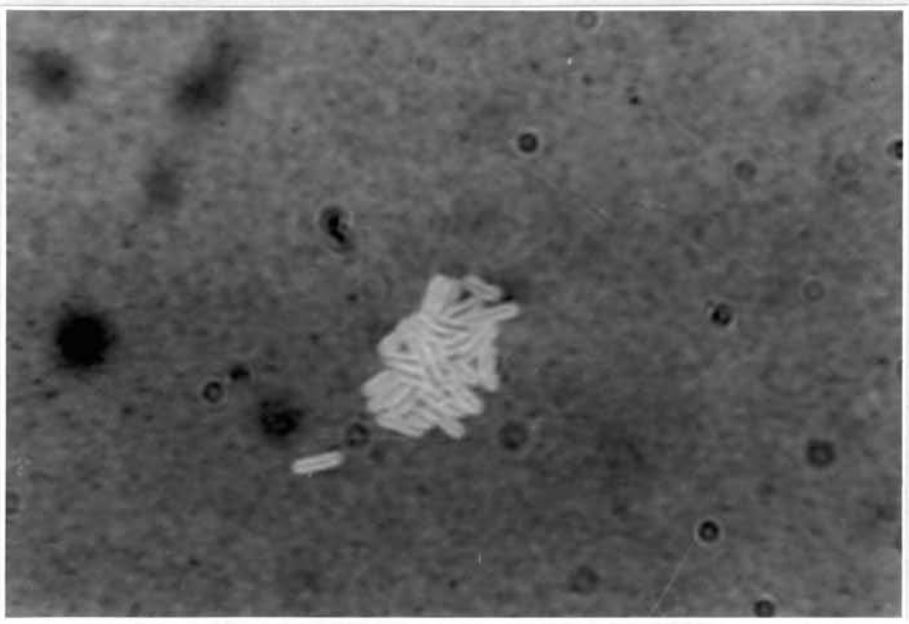

Figure 13. Photomicrograph of India Ink Slide of the Slime Former at $1\frac{1}{2}$ hr. Magnification = 40x10x3

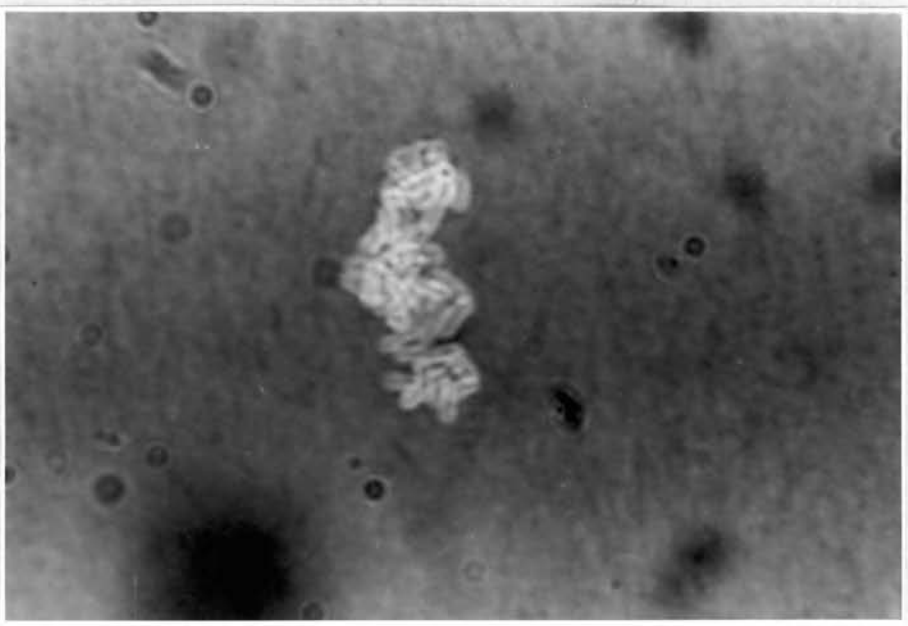

Figure 14. Photomicrograph of India Ink Slide of the Slime Former at 3 hr. Magnification = 40x10x3

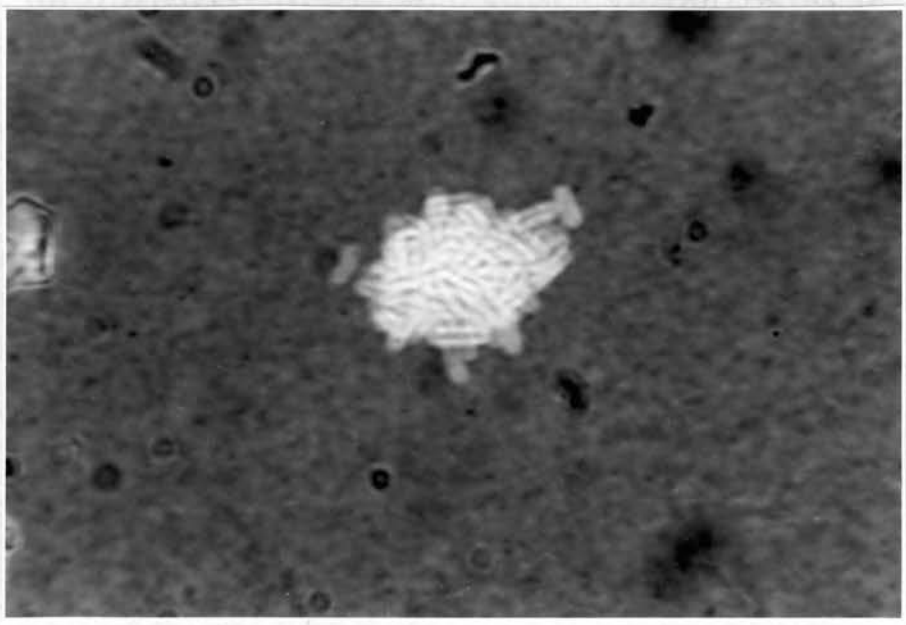

Figure 15. Photomicrograph of India Ink Slide of the Slime Former at 4 hr. Magnification = 40x10x3

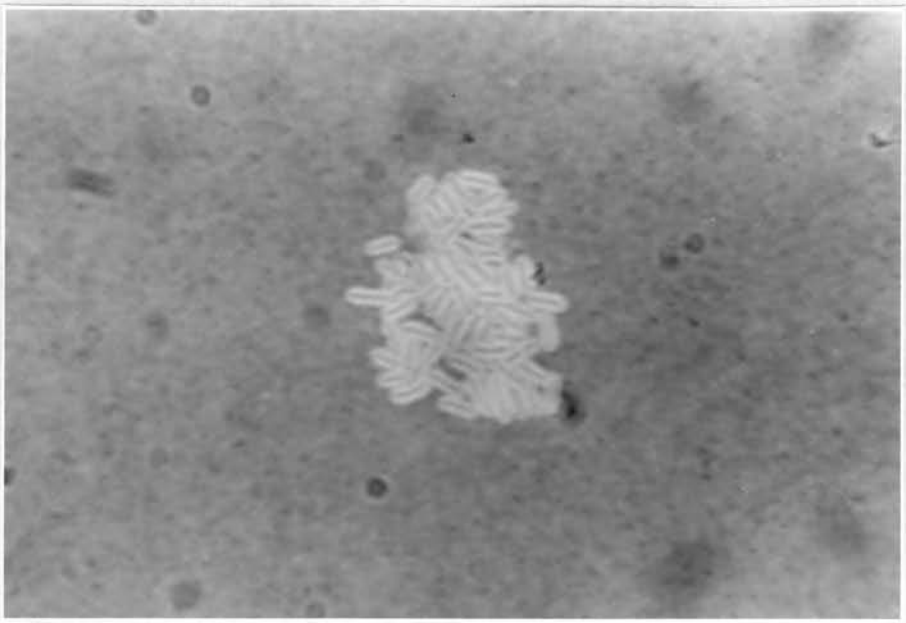

Figure 16. Photomicrograph of India Ink Slide of the Slime Former at 5 hr. Magnification = 40x10x3

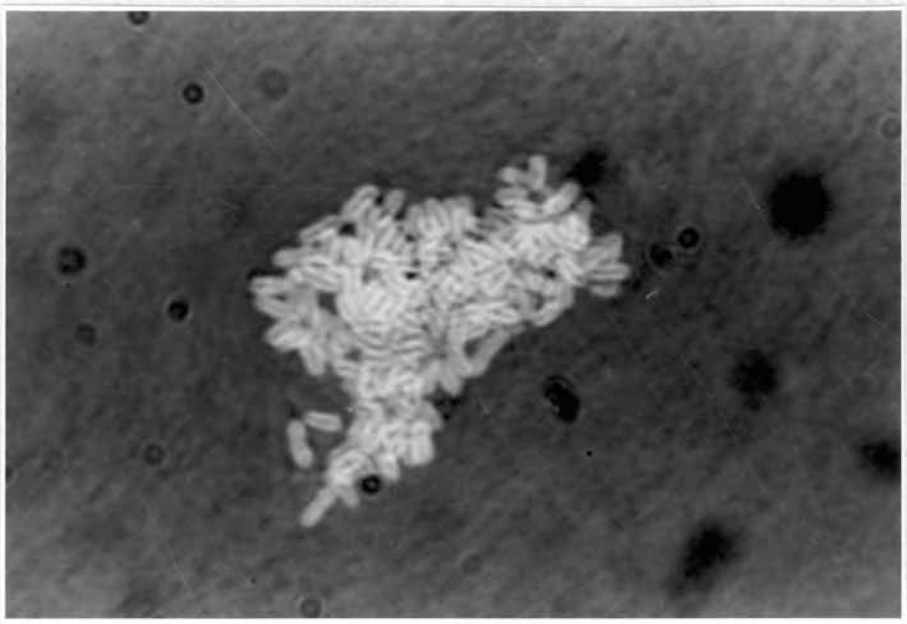

Figure 17. Photomicrograph of India Ink Slide of the Slime Former at $6\frac{1}{2}$ hr. Magnification = 40x10x3

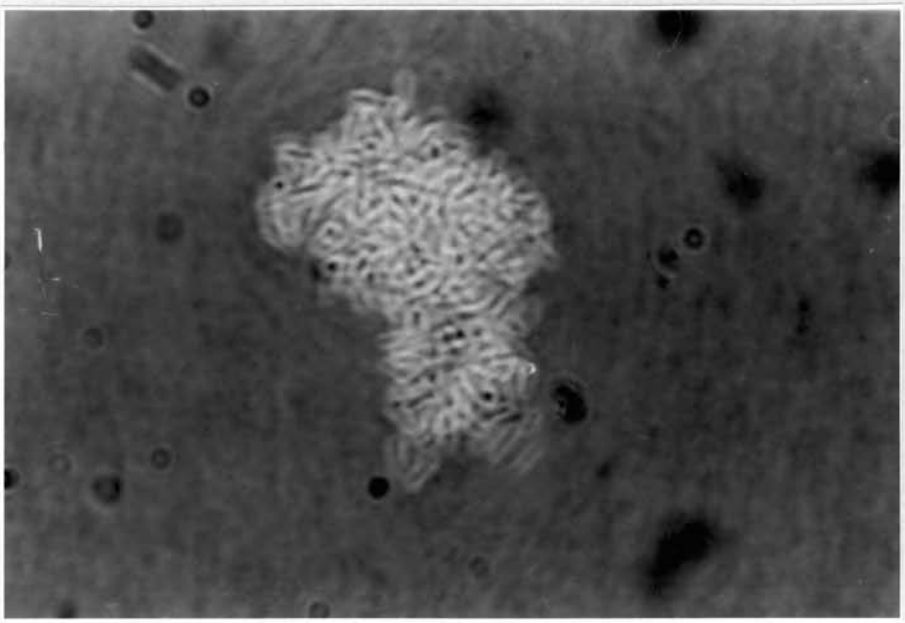

Figure 18. Photomicrograph of India Ink Slide of the Slime Former at 8 hr. Magnification = 40x10x3

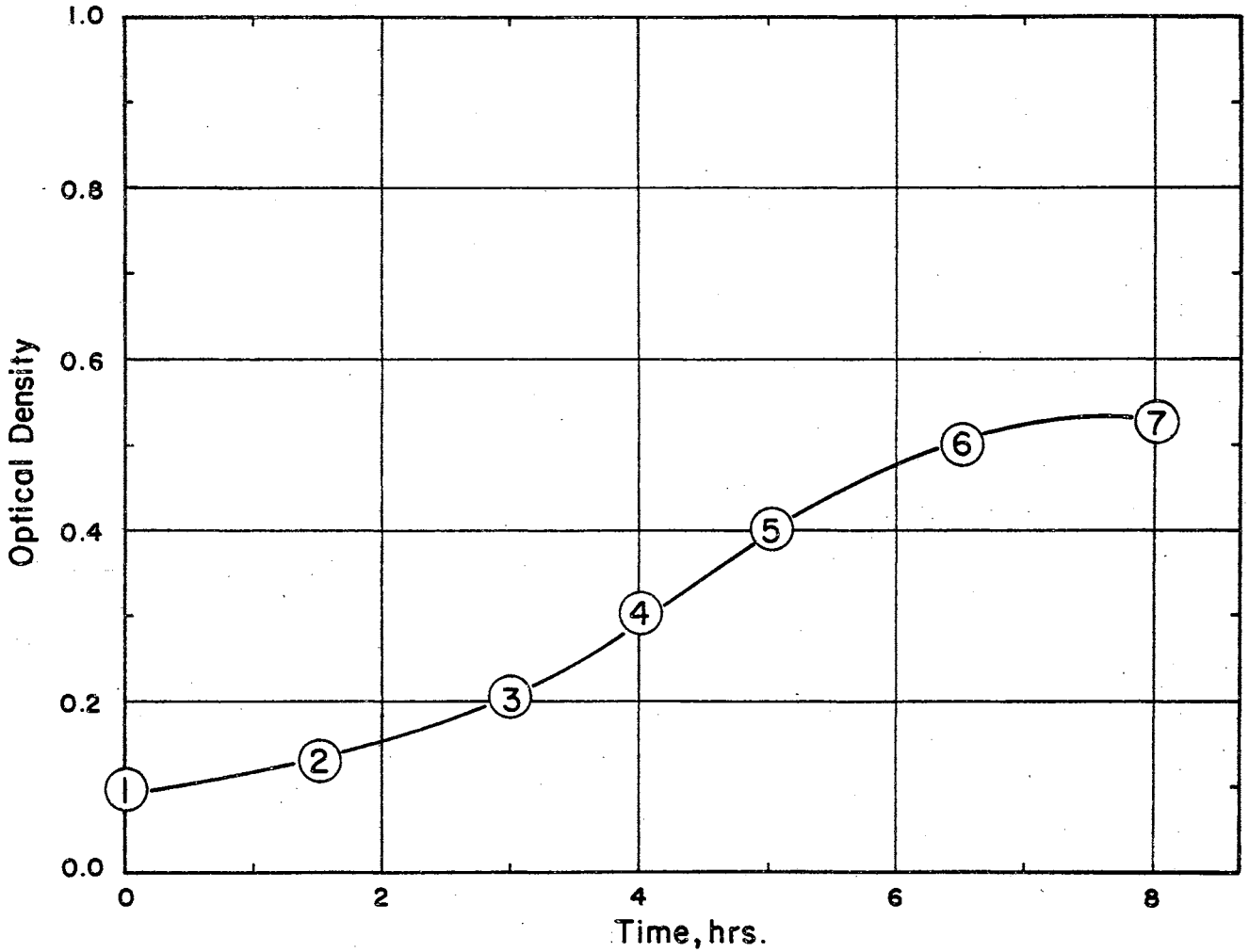

Figure 19. The Growth Curve of Serratia Marcescens Showing the Time Periods at Which Photomicrographs Were Taken. 500 mg/l Glucose

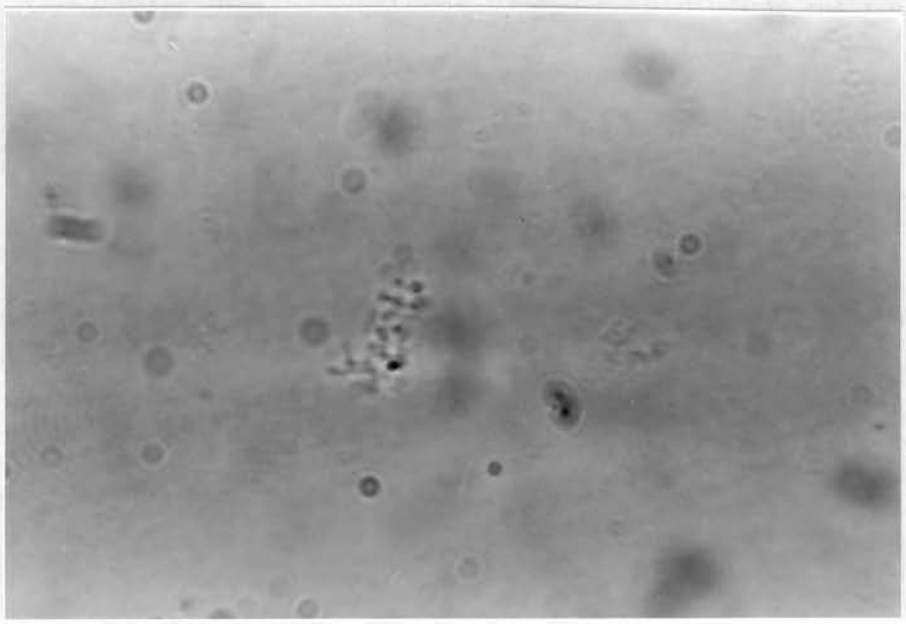

Figure 20. Photomicrograph of Wet Slide of Serratia Marcescens at 0 hr. Magnification = 40x10x3

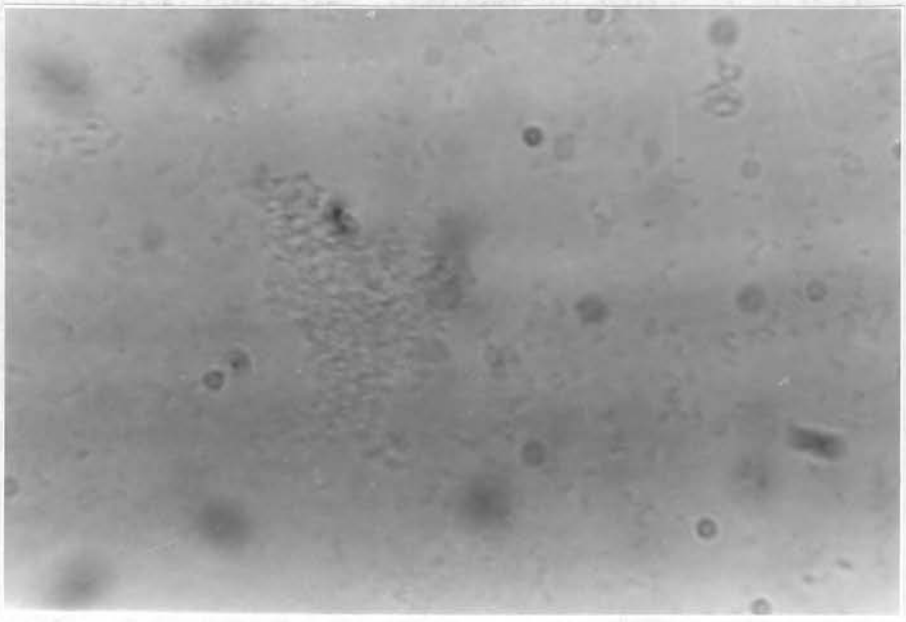

Figure 21. Photomicrograph of Wet Slide of Serratia Marcescens at $1\frac{1}{2}$ hr. Magnification = 40x10x3

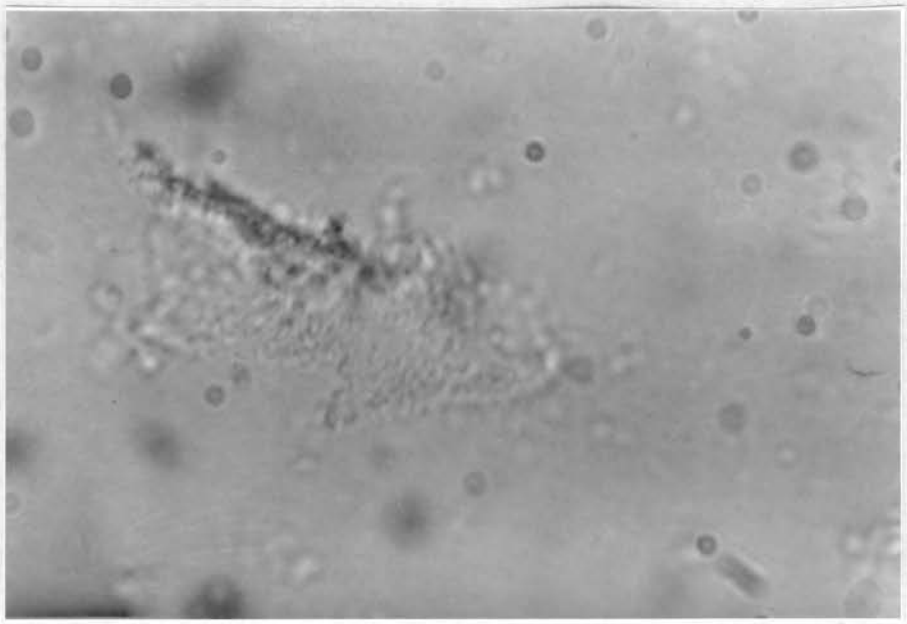

Figure 22. Photomicrograph of Wet Slide of Serratia Marcescens at 3 hr. Magnification = 40x10x3

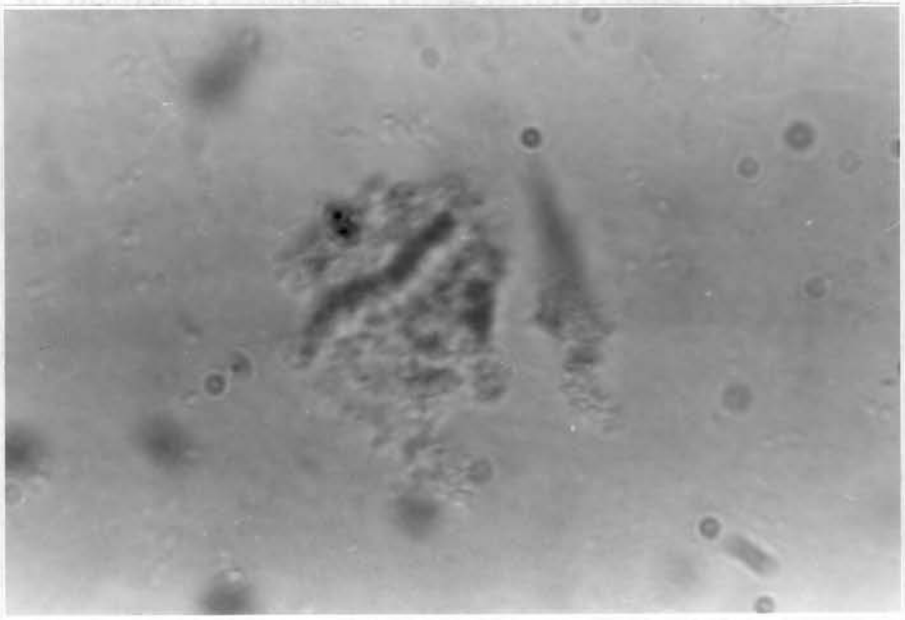

Figure 23. Photomicrograph of Wet Slide of Serratia Marcescens at 4 hr. Magnification = 40x10x3

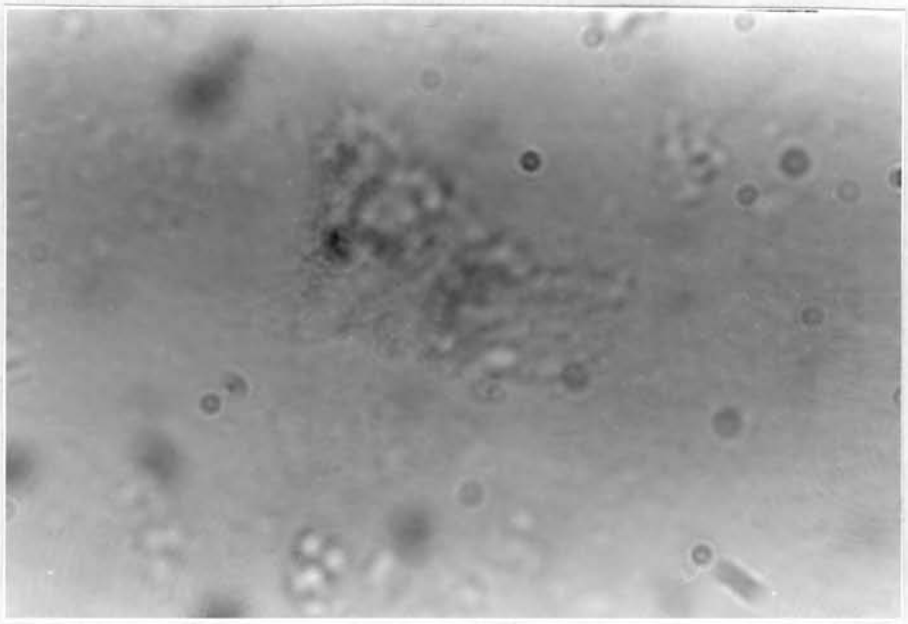

Figure 24. Photomicrograph of Wet Slide of Serratia Marcescens at 5 hr. Magnification = 40x10x3

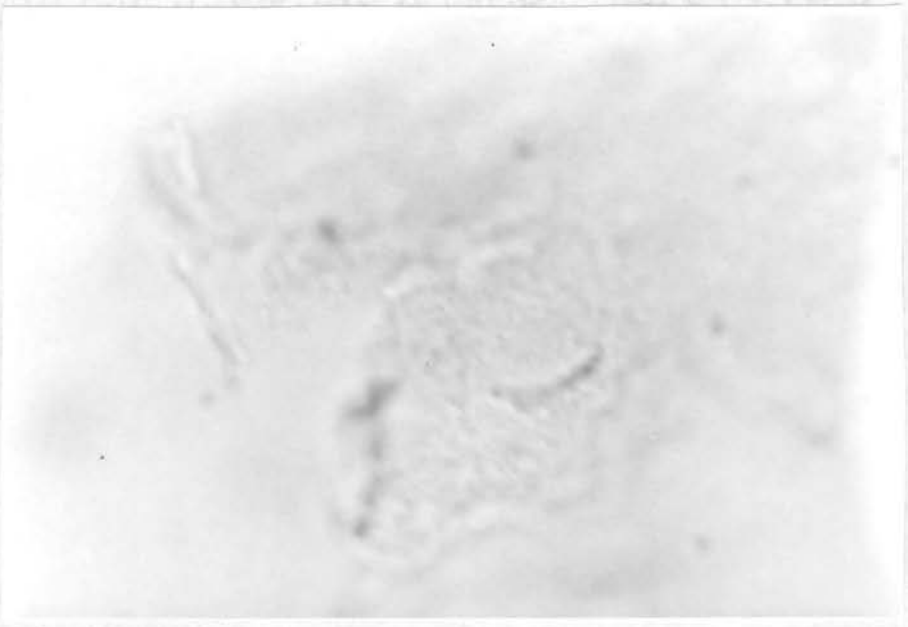

Figure 25. Photomicrograph of Wet Slide of Serratia Marcescens at $6\frac{1}{2}$ hr. Magnification = 40x10x3

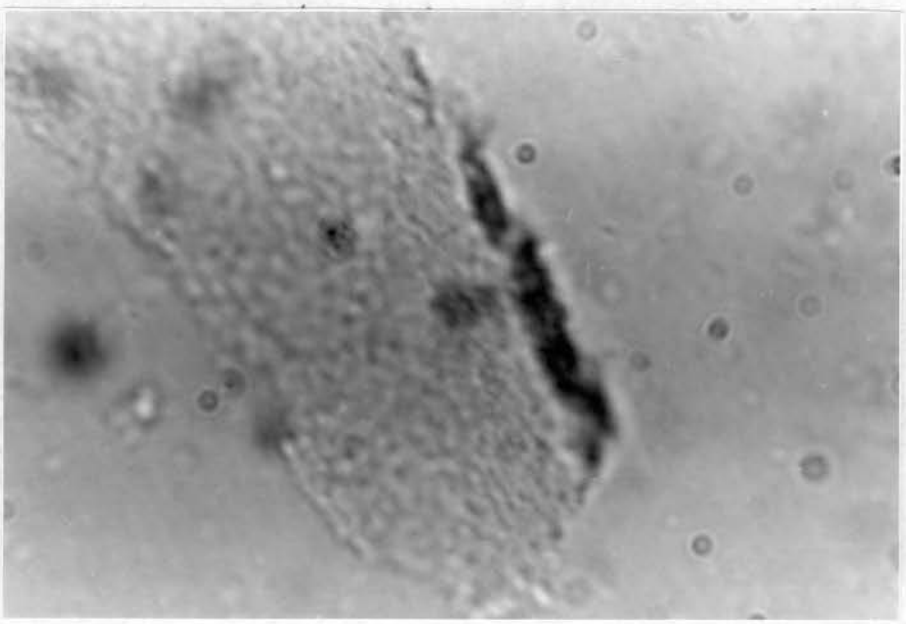

Figure 26. Photomicrograph of Wet Slide of Serratia Marcescens at 8 hr. Magnification = 40x10x3

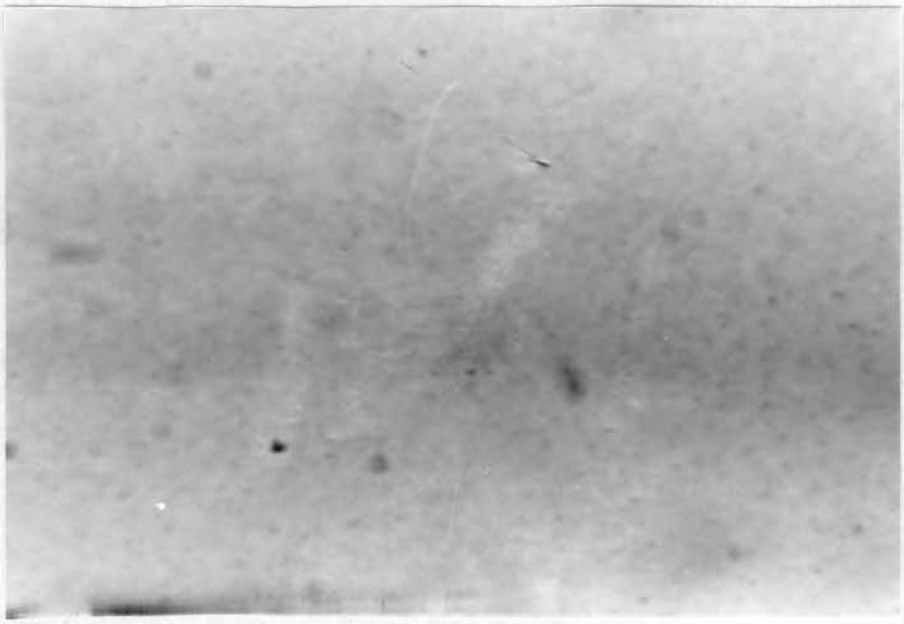

Figure 27. Photomicrograph of India Ink Slide of Serratia Marcescens at 0 hr. Magnification = 40x10x3

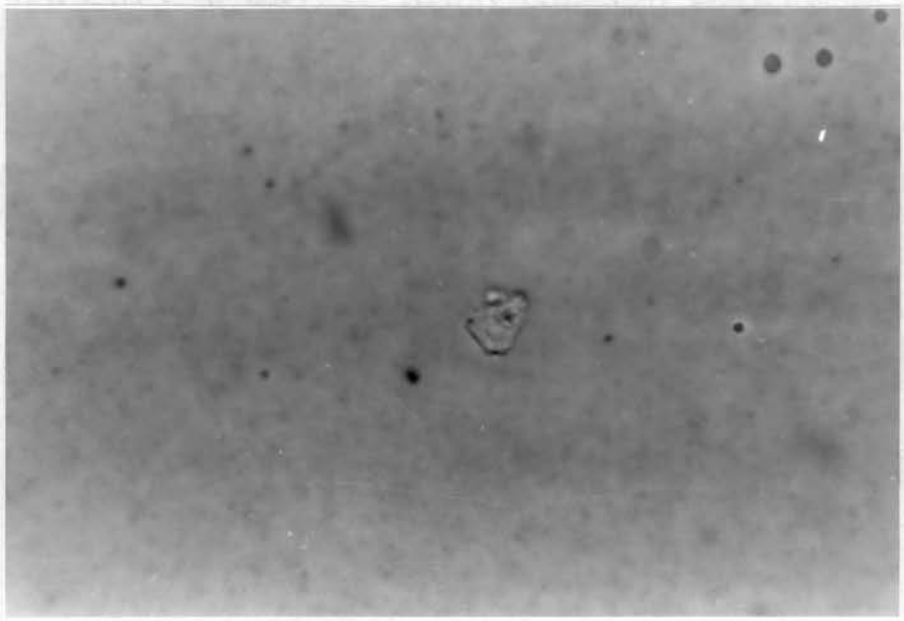

Figure 28. Photomicrograph of India Ink Slide of Serratia Marcescens at $1\frac{1}{2}$ hr. Magnification = 40x10x3

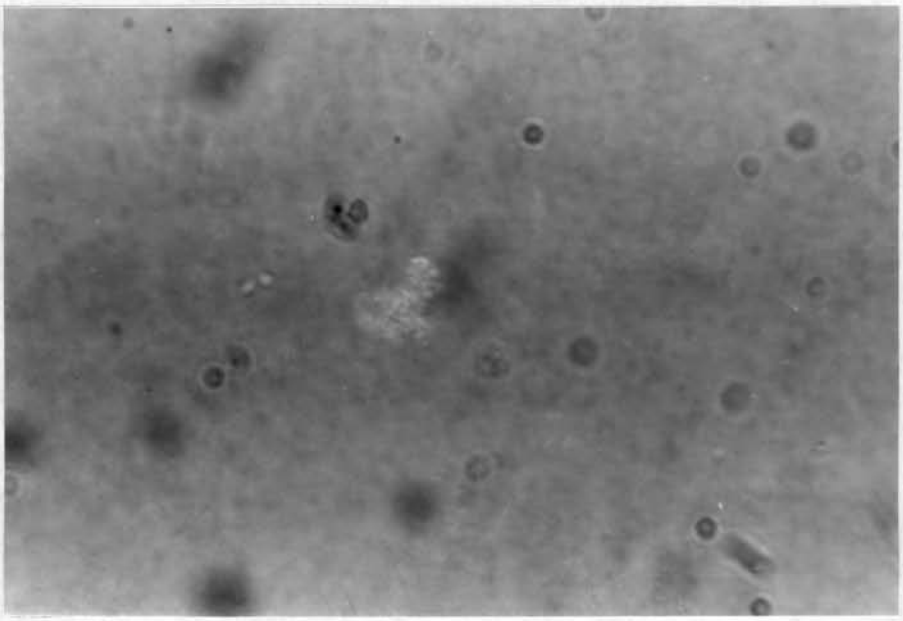

Figure 29. Photomicrograph of India Ink Slide of Serratia Marcescens at 4 hr. Magnification = 40x10x3

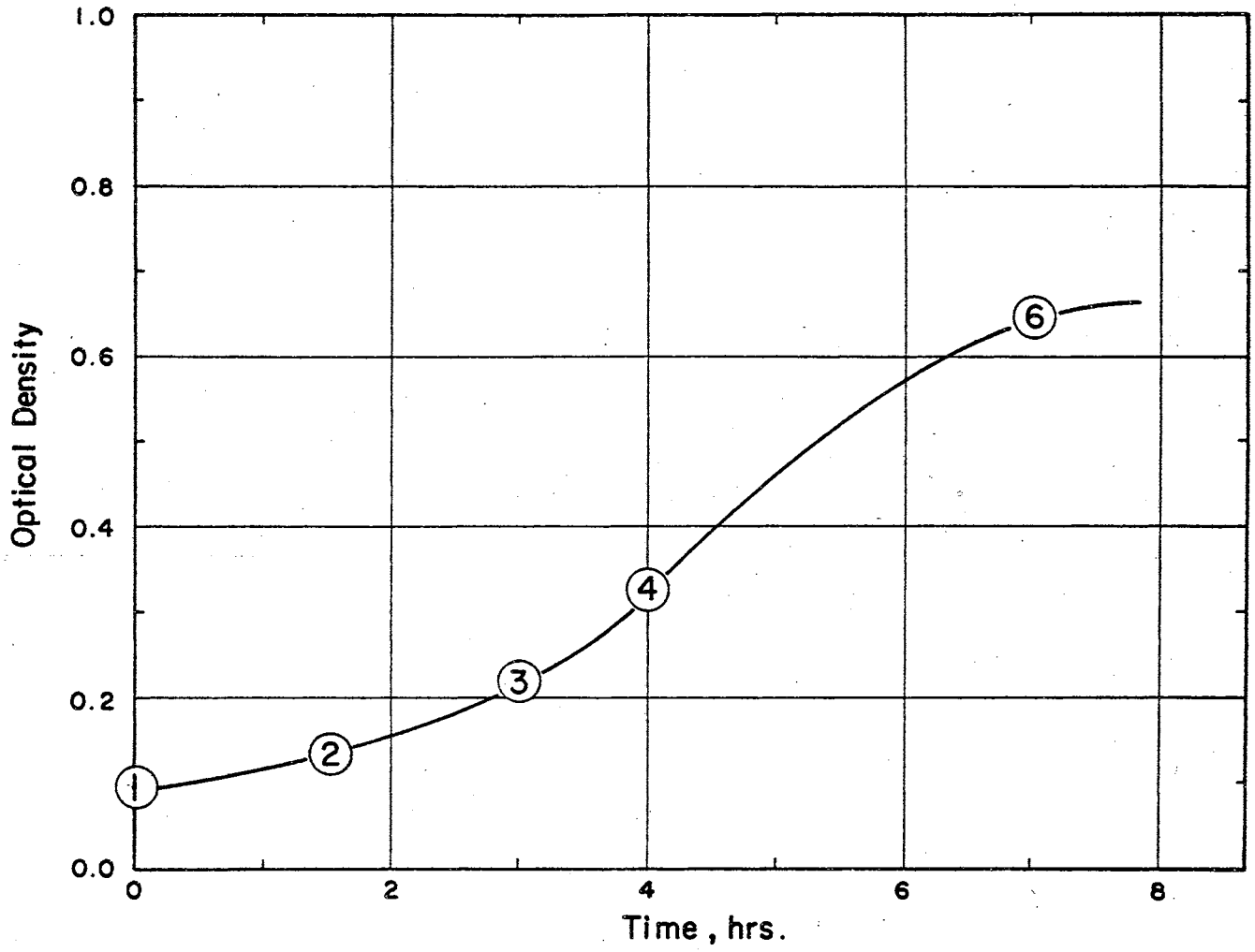

Figure 30. The Growth Curve of Pseudomonas Aeruginosa Showing the Time Periods at Which Photomicrographs Were Taken. 500 mg/l Glucose

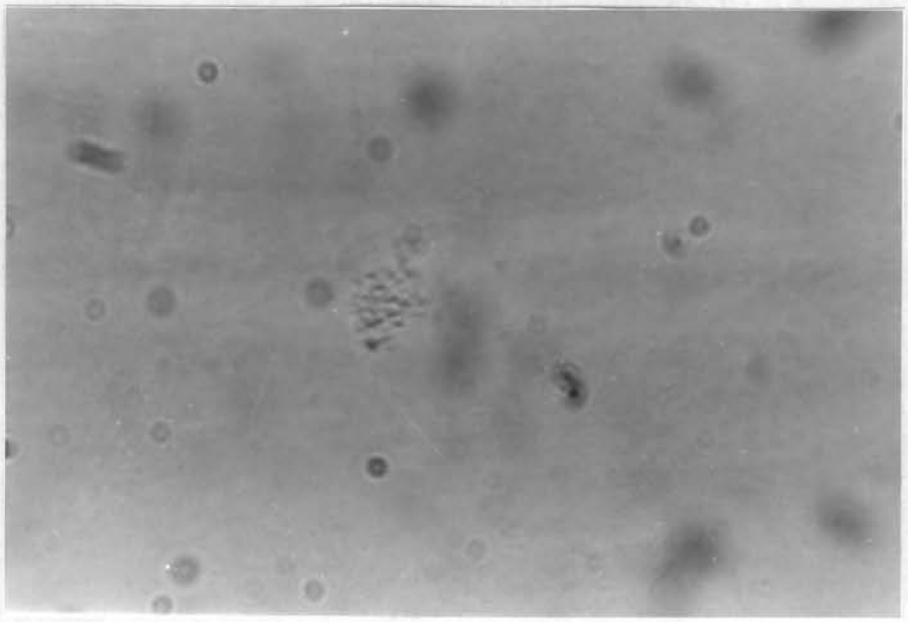

Figure 31. Photomicrograph of Wet Slide of Pseudomonas Aeruginosa at 0 hr.
Magnification = 40x10x3

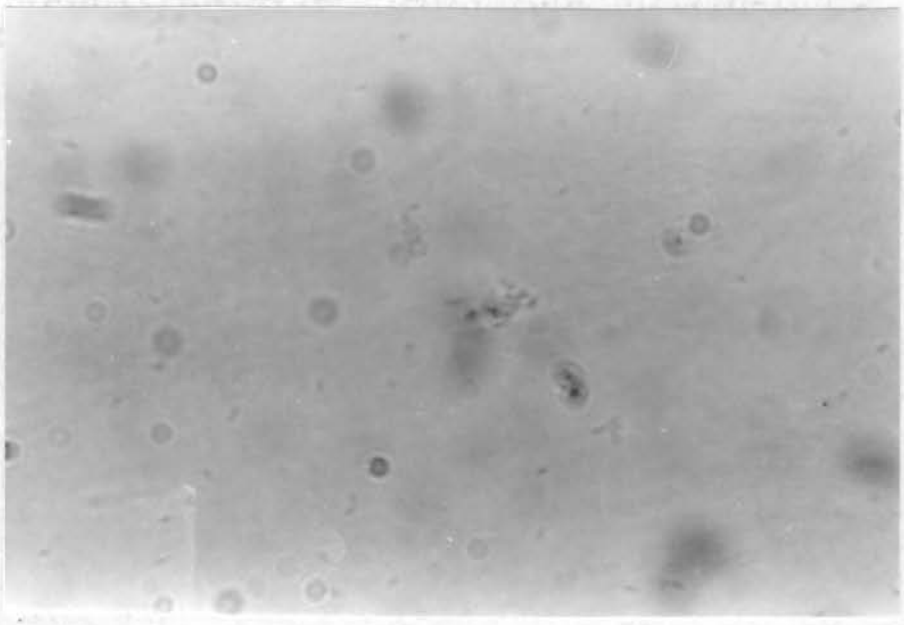

Figure 32. Photomicrograph of Wet Slide of Pseudomonas Aeruginosa at 4 hr.
Magnification = 40x10x3

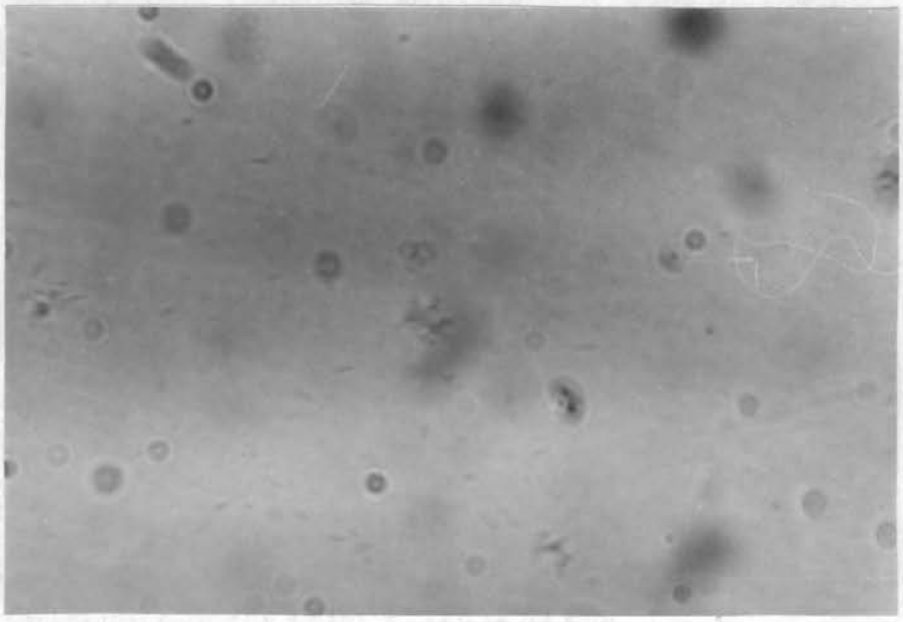

Figure 33. Photomicrograph of Wet Slide of Pseudomonas Aeruginosa at 7 hr.
Magnification = 40x10x3

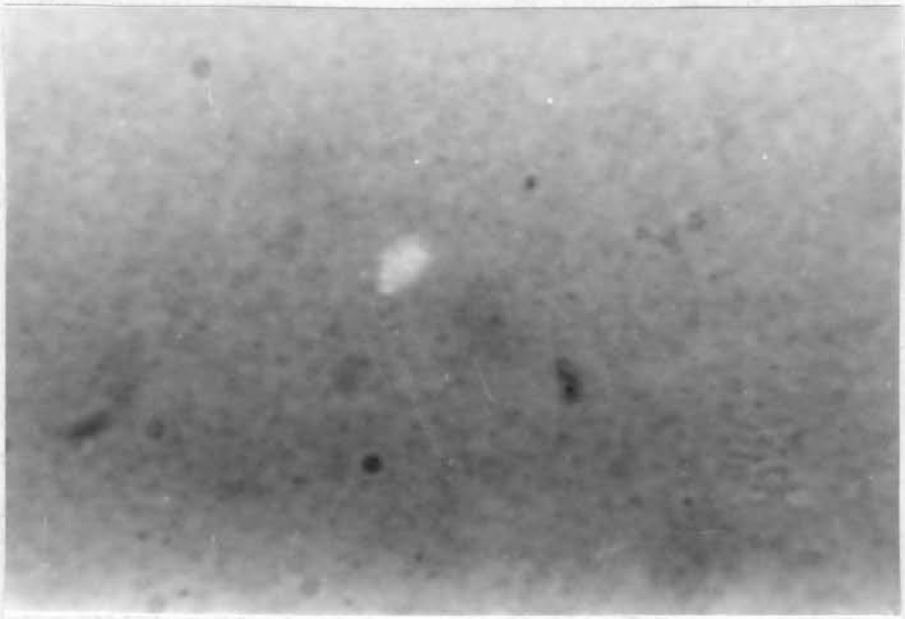

Figure 34. Photomicrograph of India Ink Slide of Pseudomonas Aeruginosa at $1\frac{1}{2}$ hr.
Magnification = 40x10x3

technique and flocculation was determined by microscopic observations. It should be noted however that in these studies the COD determination was not made, therefore there is no way of drawing if all of the carbon source was removed at the time of glucose removal.

The substrate removal of both pure cultures and mixed pure cultures are represented by Figure 35 and Figure 36 respectively. For pure cultures, glucoses were essentially removed after $6\frac{1}{2}$ hours of aeration for Pseudomonas aeruginosa, and $4\frac{1}{2}$ hours for both the slime former and Serratia marcescens. At the beginning, the rate of substrate removal for the slime former is slower than the other two, however its rate is faster after 3 hours of aeration. As shown in Table II, the slime former reached its optimum flocculation before substrate was exhausted. The floc size and floc number of Serratia marcescens increased during the course of substrate removal, but did not reach its optimum flocculation until the substrate was exhausted. Pseudomonas aeruginosa did not exhibit any floc formation during or after substrate removal. Twenty-four hours after the glucose had been removed, microscopic observations were also made. It was found that most of the floc particles of the slime former were dispersed, however the floc particles of Serratia marcescens had increased in size. Pseudomonas aeruginosa showed no change in floc size or number.

For mixed pure cultures, as shown in Figure 36 and Table III, the rate of substrate removal of the slime

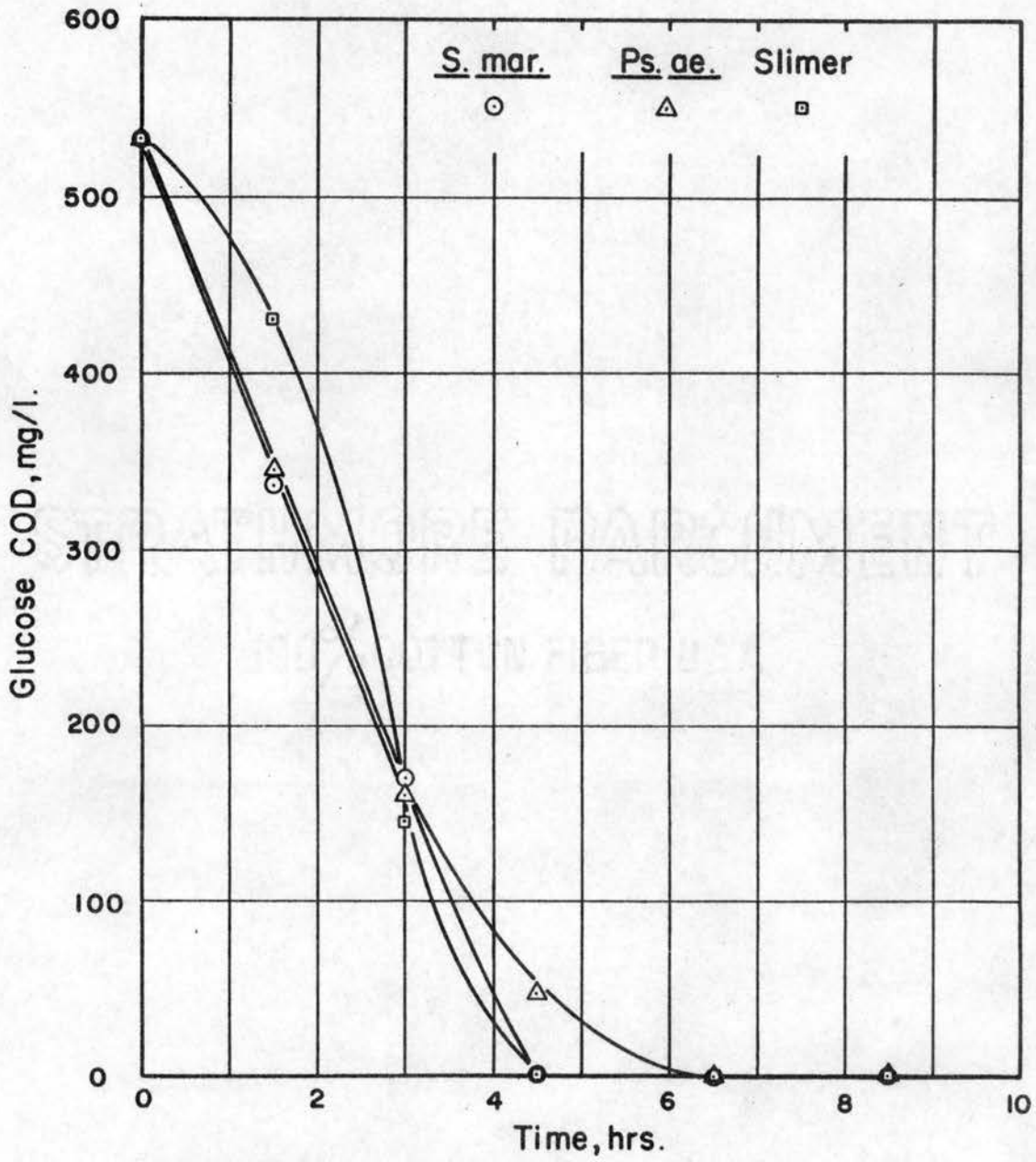

Figure 35. Substrate Removal Curves of Pure Cultures. 500 mg/l Glucose

TABLE II

FLOC FORMATION OF PURE CULTURES - 500 mg/l GLUCOSE

Unit \ Time (Hrs.)		0	1	2	3	4	5	6	7
S. Mar.	1.	Few	Few	A few	Several	Several	Several	Several	Several
	2.	<1.5	<1.5	<2	<3	< 5	< 6	< 6	< 8
Ps. Ae.	1.	Few	Few	Few	Few	Few	Few	Few	Few
	2.	≐1	≐1	≐1	< 1	< 1	≐1	< 1.5	≐1
Slimer	1.	Few	Several	Many	Many	Many	Many	Many	Many
	2.	≐1	1-1.5	< 2.5	Branched	Branched	Branched	Branched	Branched

Magnification = 40x10

1 = Number of flocs.

2 = Projected floc size in cm ø. Actually floc shape is irregular, the so called projected size was determined by changing the irregular shape to an approximately circular one by eye.

This remark will be used throughout the tables.

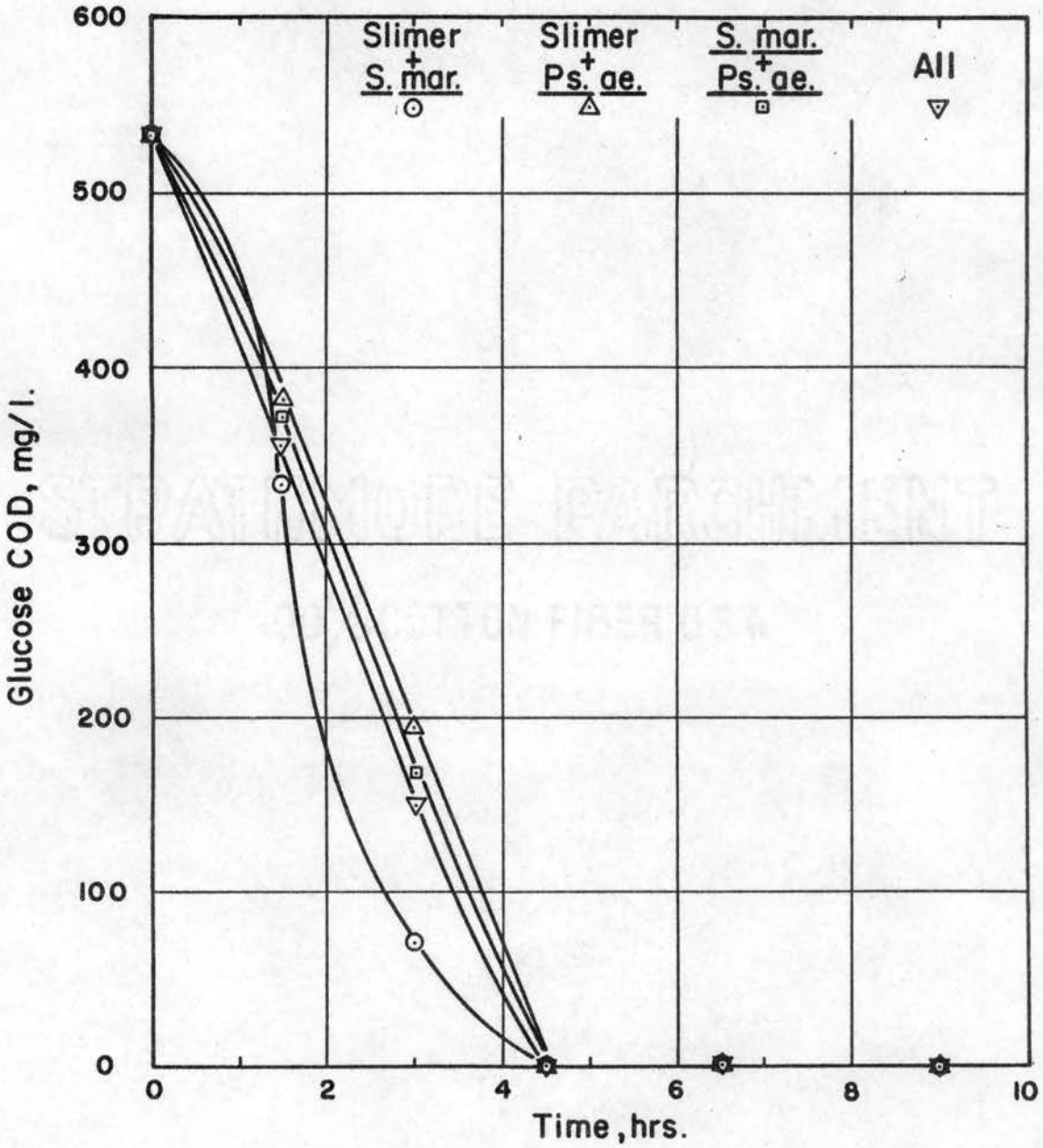

Figure 36. Substrate Removal Curves of Mixed Pure Cultures. 500 mg/l Glucose

TABLE III

FLOC FORMATION OF MIXED PURE CULTURES - 500 mg/l GLUCOSE

Time (Hrs.) / Unit		0	1½	3	4	5	6	7	9
S. Mar. & Slimer	1.	Few	A few	Several	Many	Many	Many	Many	Reduced
	2.	< 3	< 3	3-8	3-8	3-8	3-9	3-10	3-8
Slimer & Ps. Ae.	1.	Few	A few	Several	Many	Many	Many	Many	Reduced
	2.	< 1	< 3	2-7	2-6	2-7	1-7	2-6	2-5
Ps. Ae. & S. Mar.	1.	Few	Several	Several	Several	Several	Several	Many	Many
	2.	< 3	< 3.5	< 6	2-6	2-8	1-8	3-8	4-8
Slimer & S. Mar. & Ps. Ae.	1.	Few	Several	Many	Many	Many	Many	Many	Many
	2.	< 3	< 3	2-8	2-8	2-7	1-7	2-8	2-10

former + Serratia marcescens was slower at the beginning of the experiment than the other three units. Yet $1\frac{1}{2}$ hours later, its rate was the fastest. The substrate removal rates for the other three units were about the same. The time of glucose exhaustion was $4\frac{1}{2}$ hours for all mixed units. From Table III, it can be seen that the units containing the slime former flocculated earlier than the other unit. They reached their optimum flocculation approximately at the time of glucose removal. Microscopic observations showed that during the period of substrate removal, only flocs due to the slime former were present, and about three hours after the exhaustion of glucose, Serratia marcescens' flocs were present also. For the unit containing Serratia marcescens + Pseudomonas aeruginosa, floc size and floc number increased during the period of substrate removal and reached its optimum flocculation after the glucose was exhausted. Twenty-four hours after glucose removal, microscopic observations showed that most of the flocced material in the units containing the slime former were dispersed, only flocs due to Serratia marcescens were present.

C. Substrate Concentration and Bacterial Flocculation

Figure 37 shows growth and substrate removal and Table IV represents the flocculation of Serratia marcescens under initial substrate concentrations of 300, 500, 800, and 1000 mg/l of glucose. Microscopic observations showed that the floc formation in these four units was almost equivalent.

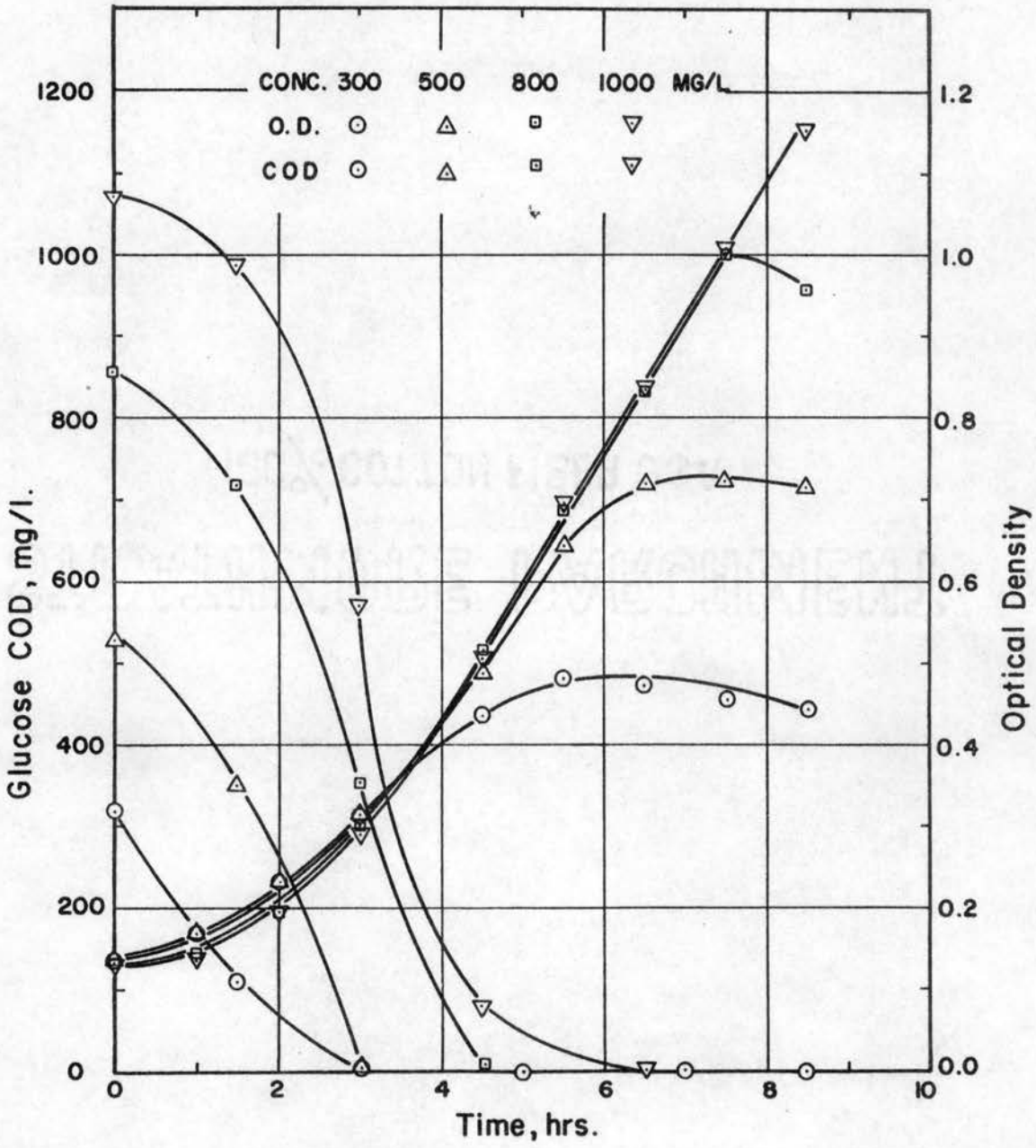

Figure 37. Growth Curves and Substrate Removal of Serratia Marcescens

TABLE IV

FLOC FORMATION OF SERRATIA MARCESCENS

Unit \ Time (Hrs.)		0	$1\frac{1}{2}$	3	$4\frac{1}{2}$	$5\frac{1}{2}$	$6\frac{1}{2}$	$7\frac{1}{2}$	$8\frac{1}{2}$
300 mg/l Glucose	1.	Few	A few	Several	Several	Several	Several	Several	Several
	2.	< 3	1-4	1-4	1-6	4-7	4-6	3-6	2-10
500 mg/l Glucose	1.	Few	A few	Several	Several	Several	Several	Several	Many
	2.	< 3	1-5	1-6	1-4	4-6	4-6	3-7	2-10
800 mg/l Glucose	1.	Few	A few	Several	Several	Several	Several	Many	Many
	2.	< 2.5	2-4	2-4	2-5	4-5	≐ 5	3-6	3-8
1000 mg/l Glucose	1.	Few	A few	Several	Several	Several	Several	Many	Many
	2.	< 2.5	2-4	2-6	2-7	3-7	2-5	3-8	3-10

The units with higher substrate concentrations, such as 800 and 1000 mg/l, did produce more flocs. Also the flocs produced in the lower substrate concentration units, 300 and 500 mg/l, reached optimum flocculation earlier than that for the higher concentration units. This may be attributed to the fact that in the lower substrate concentration units, substrates were exhausted earlier than the higher substrate concentration units. It may be seen in Figure 37 that in the 300 and 500 mg/l glucose concentration units, glucoses were totally removed within 3 hours, however for the 800 and 1000 mg/l units, glucoses were not exhausted until $4\frac{1}{2}$ hours and $6\frac{1}{2}$ hours respectively.

Figure 38 shows growth and substrate removal and Table V shows the floc formation of Pseudomonas aeruginosa with initial glucose concentrations of 300, 500, 800, and 1000 mg/l. Regardless of the initial substrate concentration, Pseudomonas aeruginosa did not floc.

Results for the slime former with initial glucose concentrations of 300, 500, 800, and 1000 mg/l are shown in Figure 39 and Table VI. The number of floc particles in the 800 and 1000 mg/l glucose concentration units was more than in the 300 and 500 mg/l glucose concentration units, however, the floc sizes were about the same. For all systems, the time of optimum flocculation was about 4 hours of aeration, also after glucose removal, the flocs of these four units began to disperse. This phenomenon is the opposite of what has been stated by either McKinney (24) and Heukelekian and

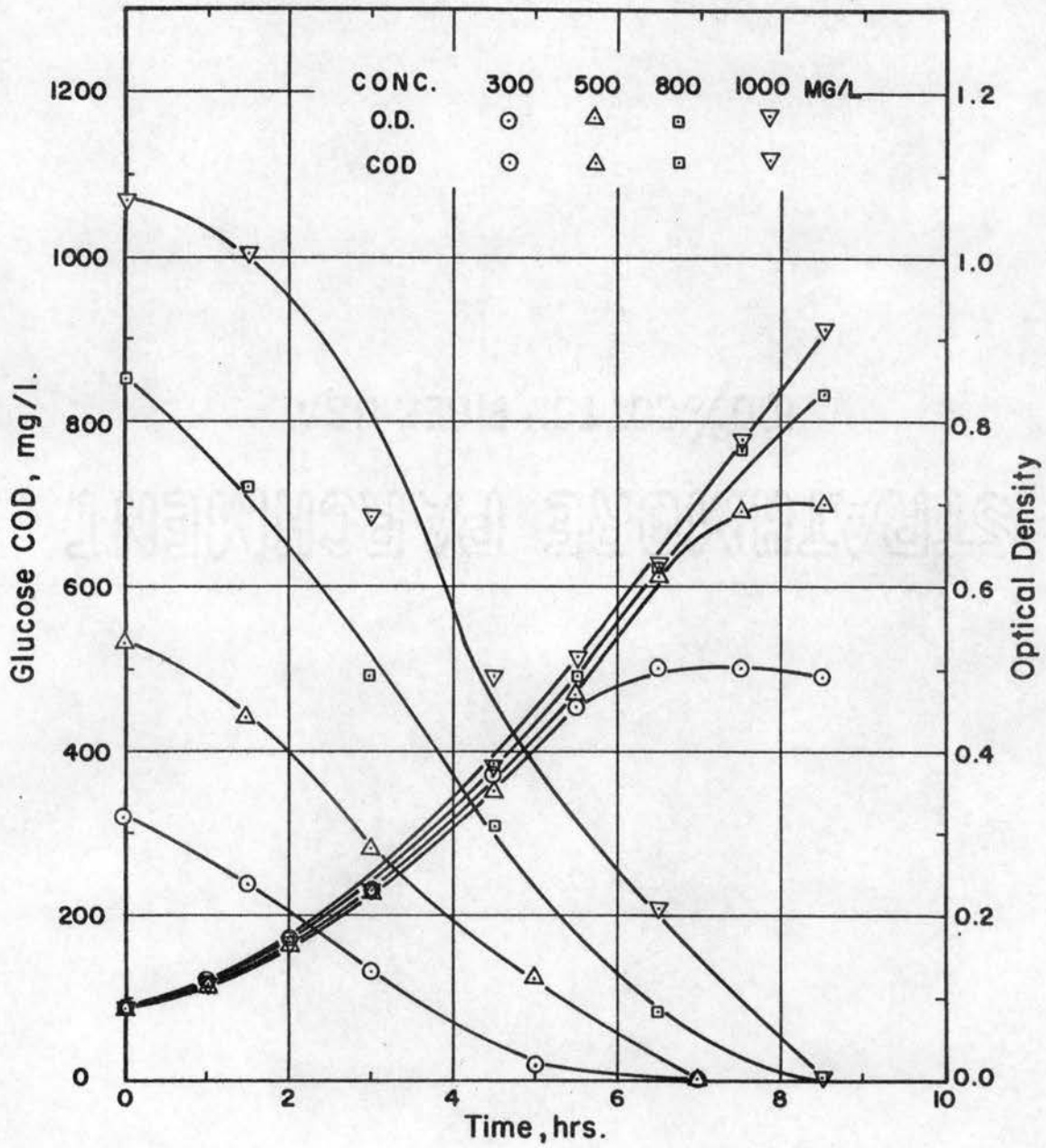

Figure 38. Growth Curves and Substrate Removal of Pseudomonas Aeruginosa

TABLE V

FLOC FORMATION OF PSEUDOMONAS AERUGINOSA

Unit \ Time (Hrs.)		0	$1\frac{1}{2}$	3	$4\frac{1}{2}$	$5\frac{1}{2}$	$6\frac{1}{2}$	$7\frac{1}{2}$	$8\frac{1}{2}$
300 mg/l Glucose	1.	Few	Few	Few	Few	Few	Few	Few	Few
	2.	< 1	< 1	< 1	<1.5	< 1	≐1	<1.5	< 1
500 mg/l Glucose	1.	Few	Few	Few	Few	Few	Few	Few	Few
	2.	< 1	< 1	< 1	< 1	<1.5	≐1	< 1	< 1
800 mg/l Glucose	1.	Few	Few	Few	Few	Few	A few	A few	Few
	2.	< 1	< 1	<1.2	< 1	≐1	< 1	≐1	<1.5
1000 mg/l Glucose	1.	Few	Few	Few	Few	Few	Few	A few	Few
	2.	< 1	< 1	<1.2	< 1	< 1	≐1	<1.5	< 1

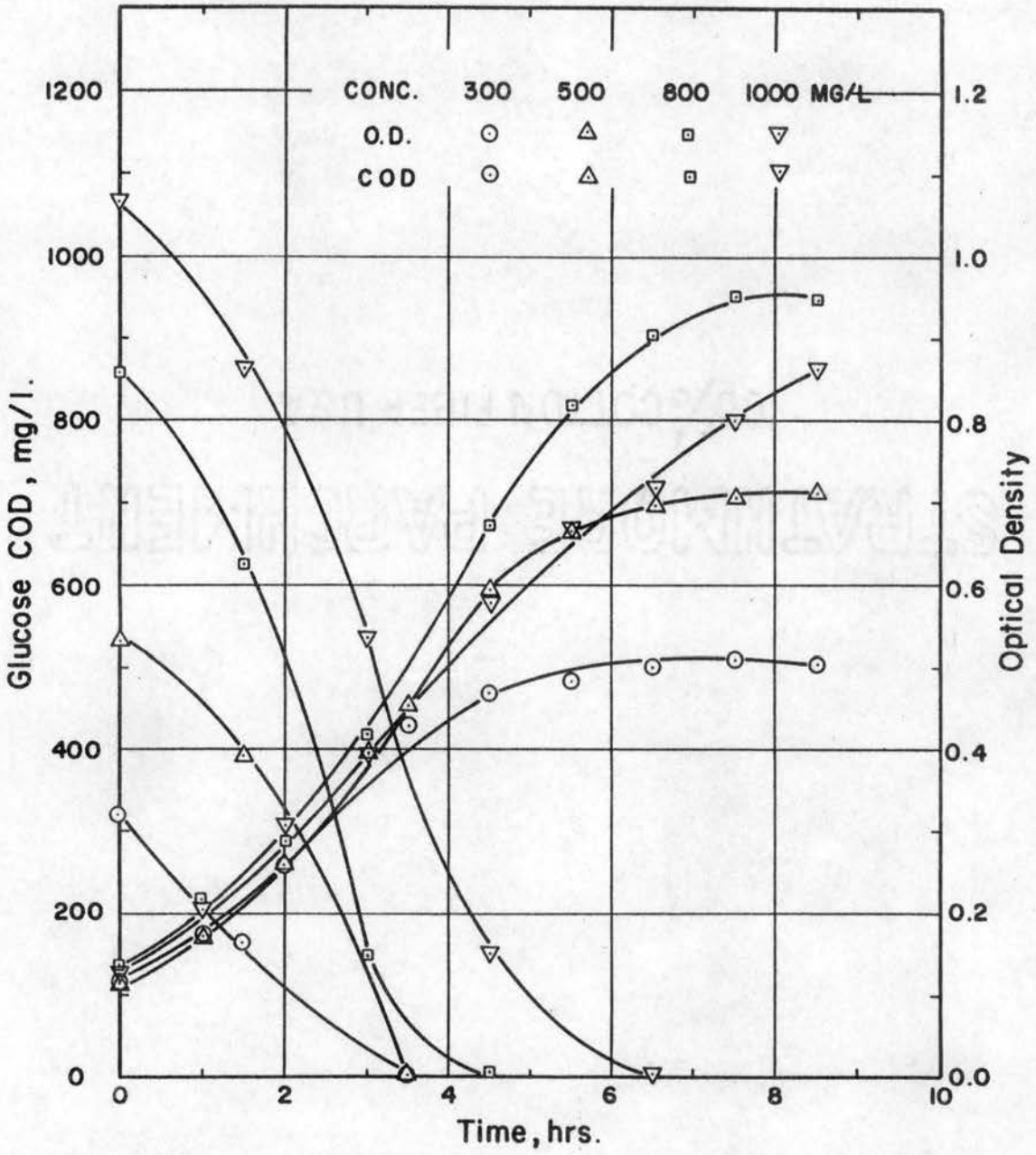

Figure 39. Growth Curves and Substrate Removal of the Slime Former

TABLE VI

FLOC FORMATION OF THE SLIME FORMER

Unit \ Time (Hrs.)		0	1½	3	4½	5½	6½	7½	8½
300 mg/l Glucose	1.	Few	A few	Several	Several	Several	Several	Several	Several
	2.	< 3	2-3	Branched	Branched	Branched	Branched	Branched	Branched
500 mg/l Glucose	1.	Few	A few	A few	Increased	Increased	Increased	Many	Many
	2.	< 3	< 5	Branched	Branched	Branched	Branched	Branched	Branched
800 mg/l Glucose	1.	Few	A few	Several	Many	Many	Many	Many	Many
	2.	<2.5	< 5	Branched	Branched	Branched	Branched	Branched	Branched
1000 mg/l Glucose	1.	Few	A few	Several	Many	Many	Many	Many	Many
	2.	<2.5	< 5	Branched	Branched	Branched	Branched	Branched	Branched

Littman (20).

D. Bacterial Flocculation and Bacterial Capsule

The slime former which produced a great deal of capsular material was used in this phase of investigation. The slime former was grown on a glucose medium until microscopic observations showed an abundance of slime. It was then centrifuged. The cells were resuspended in a buffer solution. Thirty ml of its supernatant containing slime material were placed into a shaker flask. The rest was centrifuged and resuspended in about eighty ml of buffer solution. This procedure was conducted repeatedly until microscopic observations showed no slime material (india ink slides); thirty ml of buffer solution containing slime free cells were placed into another flask. After adding 3 ml of 10 mg/ml glucose into each flask, salt growth medium was added into each flask and the volume brought to 60 ml.

As shown in Table VII, the unit with capsular material added contained a few flocs within the first $7\frac{1}{2}$ hours. The second unit without capsular material did not floc during the first $9\frac{1}{2}$ hours. From Figure 40, it may be seen that the cells of both units did not grow within these periods. After these periods, their growth curves were parallel to each other. But both the number and size of floc in the unit in which no capsular material was added was much less than in the unit where capsular material was added.

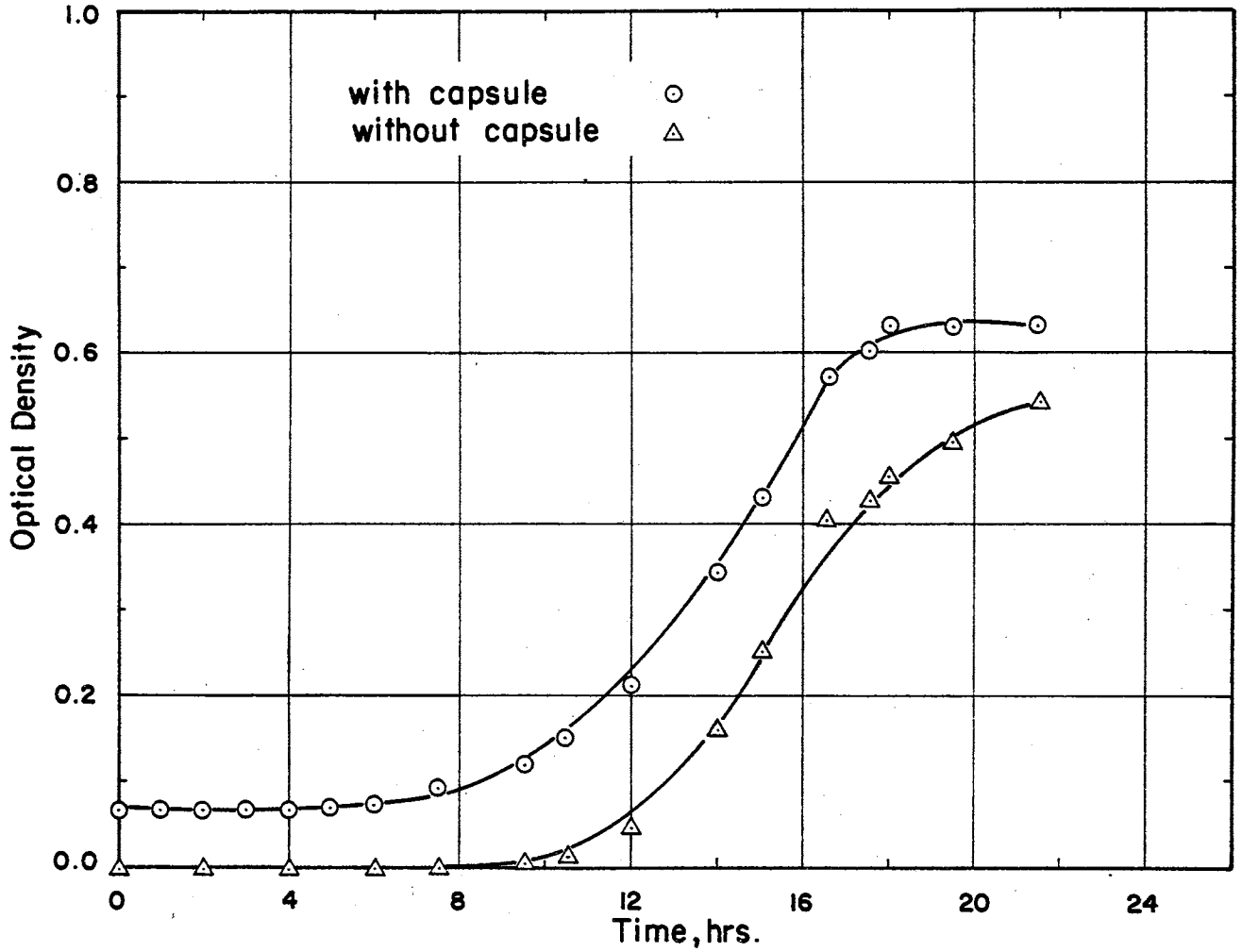

Figure 40. Growth Curves of the Slime Former. 500 mg/l Glucose

TABLE VII

THE EFFECT OF CAPSULE ON FLOC FORMATION - 500 mg/l GLUCOSE

Time (Hrs.) / Unit		0-7½	9½	10½	12	14	15	17	19
Slimer With Capsule	1.	A few	A few	Several	Several	Several	Several	Several	Several
	2.	< 5	3-6	5-8	3-8	5-10	5-8	< 10	3-8
Slimer Without Capsule	1.	None	Very few	Very few	Few	A few	Several	Many	Many
	2.		< 1	< 1.5	< 2.5	< 2.5	<2.5	< 3	< 3

E. Bacterial Growth Rate and Bacterial Flocculation

In order to find out whether bacterial flocculation is related to the specific growth rate, six organisms were studied. Those used were the slime former, Pseudomonas aeruginosa, Serratia marcescens, the blue organism, the yellow organism, and Escherichia Coli. Their maximum specific growth rates, μ_m's, and saturation constants, K_s's, were determined, and their relative degrees of flocculation were compared employing microscopic observations.

The kinetic constants for these organisms were determined by applying Monod's equation (2),

$$\mu = \mu_m \left(\frac{S}{K_s + S} \right) ,$$

which can be inverted as

$$\frac{1}{\mu} = \frac{K_s}{\mu_m \cdot S} + \frac{1}{\mu_m} .$$

Where μ_m = maximum specific growth rate when the substrate is unlimited, K_s = the saturation constant; i.e., the substrate concentration at which the specific growth rate observed is $\frac{1}{2}$ the maximum specific growth rate, and S = substrate concentration. Both μ_m and K_s can be determined graphically. For instance, in order to determine the μ_m and K_s of Serratia marcescens, five units with glucose concentrations of 100, 300, 500, 800, and 1000 mg/l were used. Optical density readings were taken every $\frac{1}{2}$ to 1 hour. The optical density vs time was plotted on semi-logarithmic

paper as shown in Figure 41. The specific bacterial growth rates at different substrate concentrations can be calculated as follows:

For 100 mg/l glucose concentration, the specific bacterial growth rate is

$$\mu_{100} = \frac{\ln \frac{0.230}{0.175}}{4 - 3} = 0.273 \text{ hr}^{-1}$$

Similarly

$$\mu_{300} = \frac{\ln \frac{0.270}{0.200}}{4 - 3} = 0.300 \text{ hr}^{-1}$$

$$\mu_{500} = \frac{\ln \frac{0.275}{0.202}}{4 - 3} = 0.308 \text{ hr}^{-1}$$

$$\mu_{800} = \frac{\ln \frac{0.292}{0.214}}{4 - 3} = 0.310 \text{ hr}^{-1}$$

$$\mu_{1000} = \frac{\ln \frac{0.292}{0.214}}{4 - 3} = 0.310 \text{ hr}^{-1}$$

The reciprocals of these computed specific bacterial growth rates vs the reciprocals of the corresponding glucose concentrations were then plotted as shown in Figure 42. The intersection of the connected straight line and ordinate represents the reciprocal of μ_m, that is

$$\mu_m = \frac{1}{3.17} = 0.315 \text{ hr}^{-1} .$$

K_s may be estimated either from the slope and ordinate

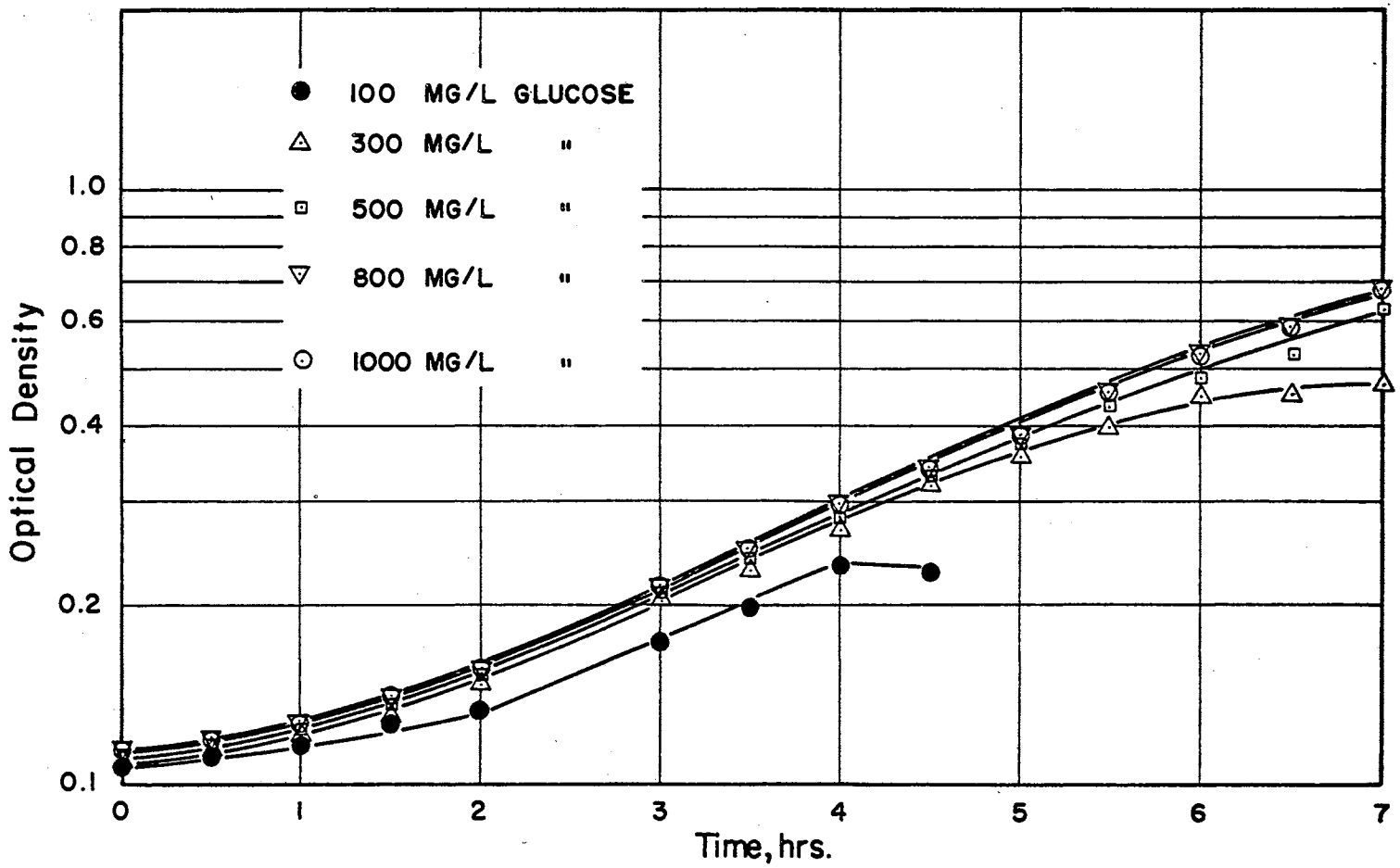

Figure 41. Growth Curves of Serratia Marcescens

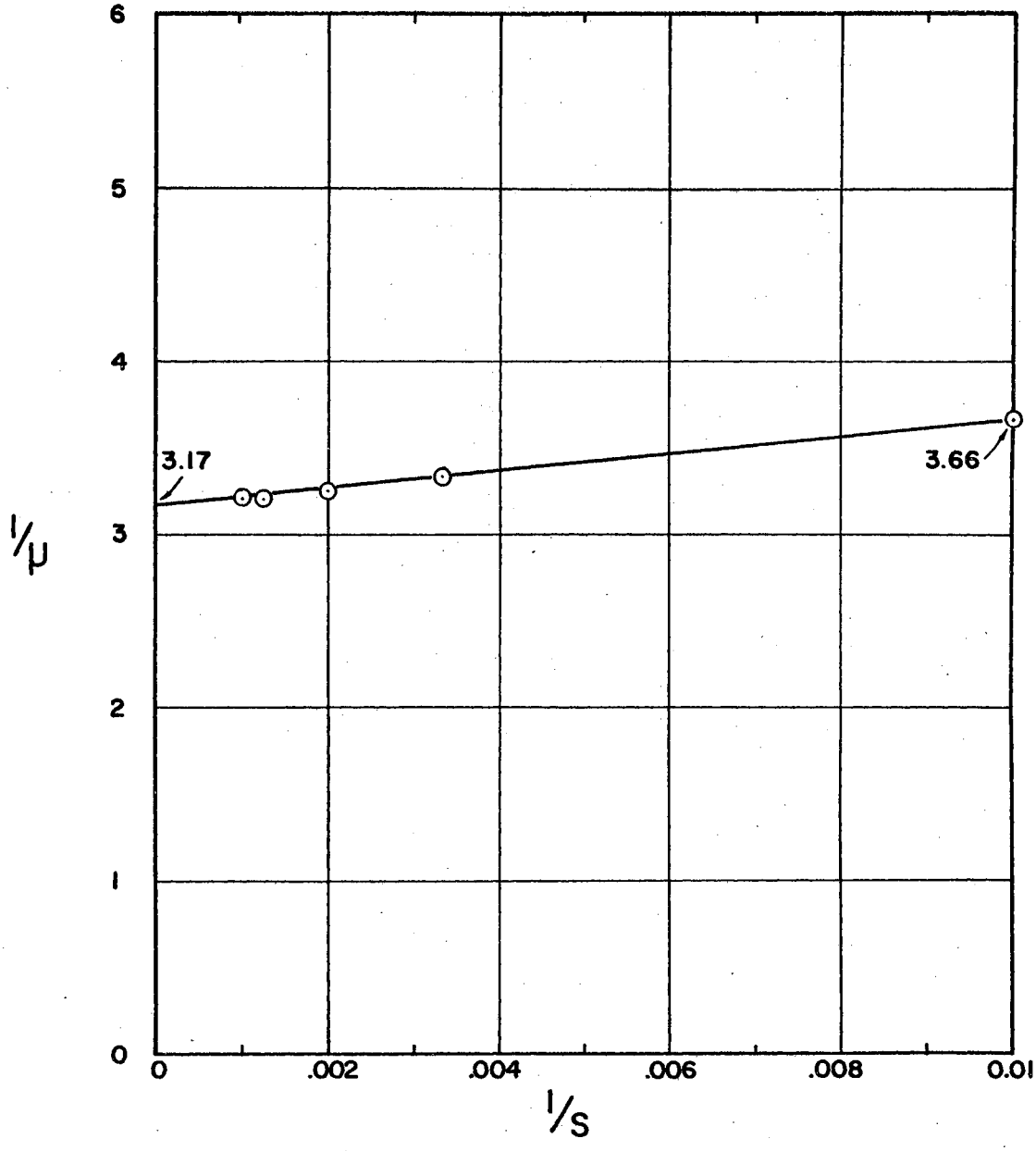

Figure 42. Graphical Determination of μ_m and K_s for Serratia Marcescens

intercept or from the negative abscissa intercept, that is

$$\frac{1}{K_s} = \left(\frac{3.66 \times 10}{3.66 - 3.17} - 10\right) \times 0.001 = 0.0647 \quad ,$$

and

$$K_s = \frac{1}{0.0647} = 15.4 \text{ mg/l} \quad .$$

It can be seen in Table IX that there is a possible relationship between maximum specific growth rate (μ_m) and bacterial flocculation. The order of flocculation in decreasing degree was (1) the slime former, (2) the blue organism, (3) Serratia marcescens, and (4) the yellow organism. Pseudomonas aeruginosa and Escherichia Coli did not produce floc particles. It can also be seen in Table VIII that the maximum specific growth rates (μ_m's) decreased in the same order as the degree of flocculation, that is, the slime former the fastest μ_m (0.581 hr^{-1}), and the yellow organism the slowest μ_m (0.220 hr^{-1}). Pseudomonas aeruginosa which did not flocculate had a μ_m value of 0.366 hr^{-1} which falls between the blue organism and Serratia marcescens. Escherichia Coli did not flocculate either and had the lowest μ_m (0.170 hr^{-1}). The saturation constants (K_s's), as shown in Table VIII, do not appear to have any relationship to bacterial flocculation.

F. Metabolic Intermediates and/or End Products and Bacterial Flocculation

Metabolic intermediates and/or end products produced

TABLE VIII

KINETIC CONSTANTS FOR SELECTED ORGANISMS

Organisms	μ_m (hr^{-1})	K_s (mg/l)	Comparable order of flocculation
Slime former	0.581	13	1
Blue organism	0.375	22	2
Pseudomonas aeruginosa	0.366	40	-
Serratia marcescens	0.315	15.4	3
Yellow organism	0.220	230	4
Escherichia Coli	0.170	20	-

TABLE IX

FLOC FORMATION OF VARIOUS ORGANISMS - 500 mg/l GLUCOSE

Unit \ Time (Hrs.)		0	1	2	3	4	5	6	7
Slime former	1.	Few	Several	Many	Many	Many	Many	Many	Many
	2.	< 1	1-1.5	< 2	Branched	Branched	Branched	Branched	Branched
Blue organism	1.	Few	A few	Several	Several	Many	Many	Many	Many
	2.	< 2	< 2.5	< 3	3-6	3-6	3-10	>10	>10
Ps. Ae.	1.	Few	Few	Few	Few	Few	Few	Few	Few
	2.	≐ 1	≐ 1	≐ 1	< 1	< 1.5	≐ 1	≐ 1	≐ 1
S. Mar.	1.	Few	Few	A few	Several	Several	Several	Increased	Increased
	2.	< 1.5	< 1.5	< 2	< 2	< 5	< 6	< 6	< 8
Yellow organism	1.	Few	A few	A few	Many	Many	Many	Many	Many
	2.	< 2	< 3	< 4	< 4	< 4	< 5	< 6	< 5
E. Coli	1.	Very few	Few	Few	Few	Few	Few	Few	Few
	2.	< 1	≐ 1	≐ 1	< 1	< 1	< 1	≐ 1	< 1

by bacteria can be determined by measuring total Chemical Oxygen Demand, COD, and glucose COD simultaneously. The metabolic intermediates and/or end products produced is the arithmetic difference of total COD and glucose COD. It was of interest to determine if flocculation characteristics and intermediate production were related. Total COD was determined by Standard Method, and glucose COD by the Glucostat test.

The metabolic intermediates and/or end products produced by pure cultures and mixed pure cultures with an initial glucose concentration of 500 mg/l are shown in Figures 43 and 44. In the pure culture systems, both the slime former and Serratia marcescens produced much more metabolic intermediates and/or end products than Pseudomonas aeruginosa. When making microscopic observations, it was observed that the units which produced the greater amount of metabolic intermediates and/or end products flocculated better than the other units. As shown in Figure 43, the slime former produced the most metabolic intermediates and/or end products, with Serratia marcescens the next largest producer. Figure 44 shows that when good floc-producing bacteria such as the slime former and Serratia marcescens were mixed together, they produced more metabolic intermediates and/or end products than the other units.

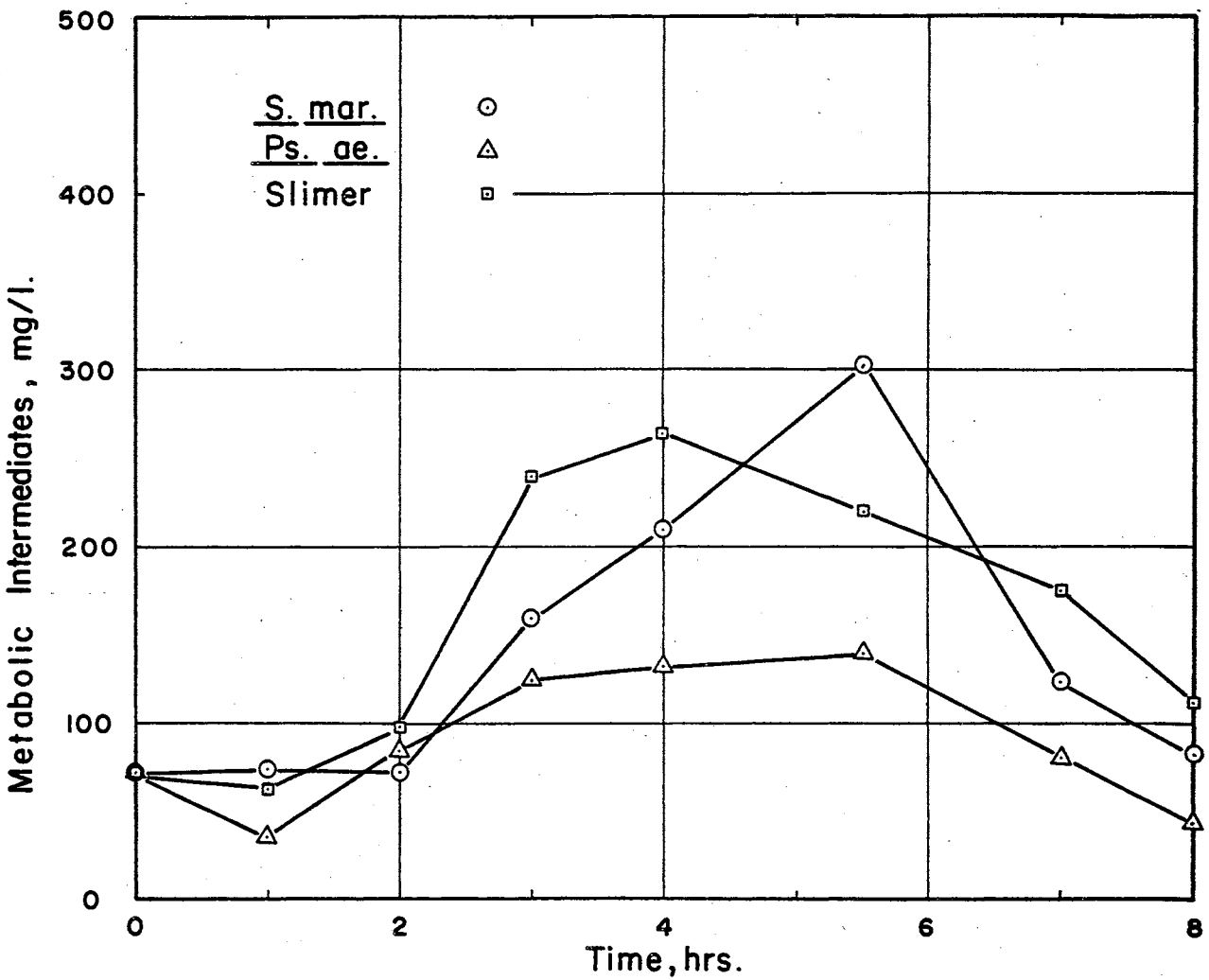

Figure 43. Curves of Metabolic Intermediates and/or End Products of Pure Cultures. 500 mg/l Glucose

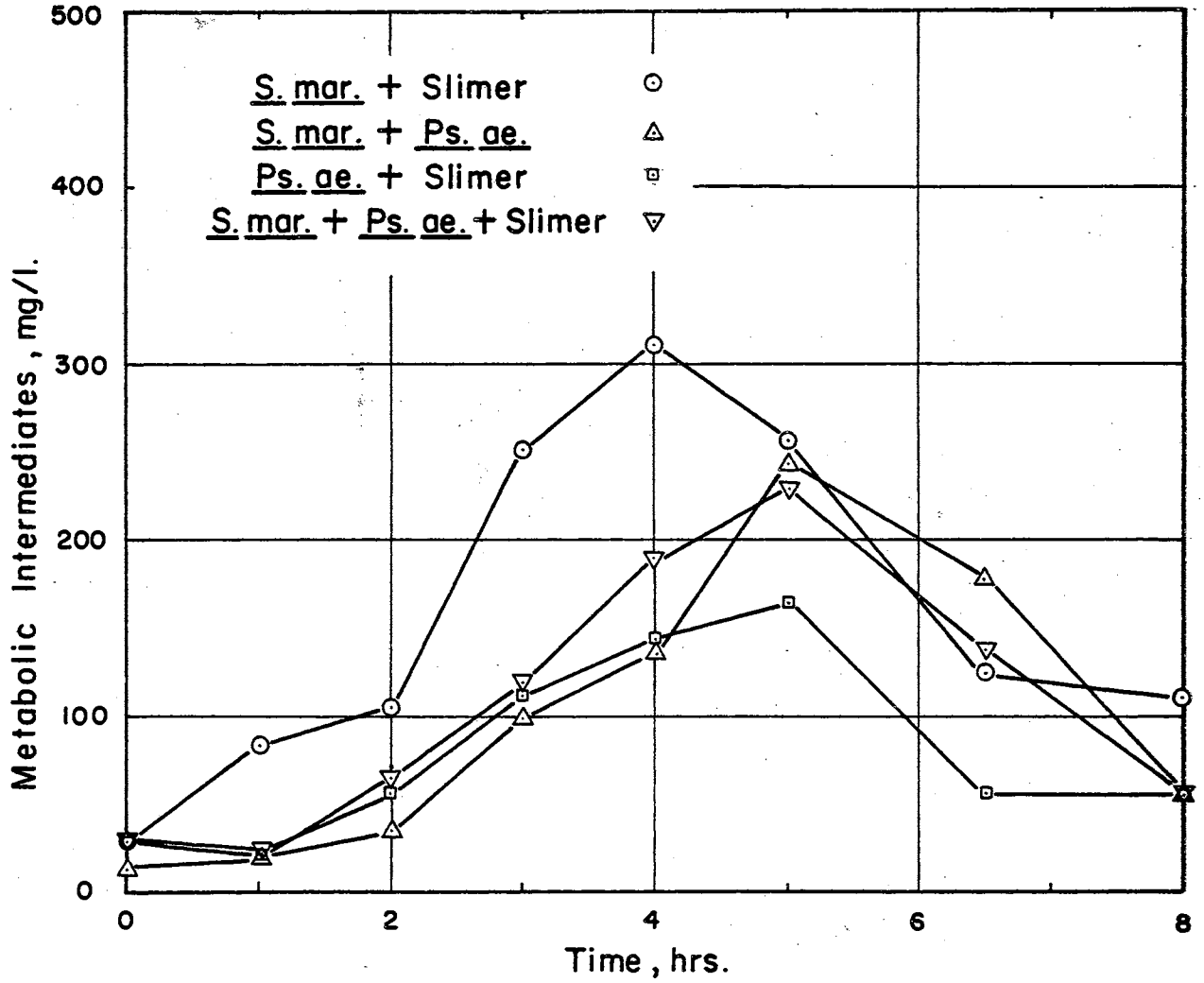

Figure 44. Curves of Metabolic Intermediates and/or End Products of Mixed Units. 500 mg/l Glucose

CHAPTER V

DISCUSSION

A. Significance of Bacterial Flocculation

Since the activated sludge process was developed some fifty years ago, there has been some question as to which microorganisms participated in the waste water purification brought about by this process.

Activated sludge is a flocculated mass composed mainly of bacteria and protozoa. The bacteria are considered to be the most important group forming the heterogeneous flora. Taylor (29) in 1930 stated that the bulk of the activated sludge was composed of a jelly-like mass in which bacteria were present in large numbers. This was thought to be a typical zoogloeal formation. Butterfield (12) was the first to obtain a zoogloeal-forming bacterium from activated sludge in pure culture. This microorganism, identified as a variety of Zoogloea ramigera, produced flocs resembling activated sludge. The supposed importance of this bacterium in the purification process was confirmed later by experiments of Butterfield, et al in 1937 (30). Heukelekian and Littman (20) in 1939 examined Zoogloea ramigera as described by Butterfield (1935). It seemed that Zoogloea ramigera, because of its ability to form flocs and to mineralize

nutrient substrates, should be considered as the principal microorganisms in activated sludge. Yet in 1962, McKinney (31) observed that several types of bacteria could make up activated sludge, and that the nature of the organic compounds in the waste supplied determined which bacteria would predominate. Thus the opinion that Zoogloea ramigera plays the only important role in activated sludge flora has been changed. The fact that floc formation is apparently not a special property of any particular group of bacteria explains the ease with which activated sludge is formed in various industrial wastes.

As stated by McKinney (24), one of the fundamental mechanisms essential to the activated sludge process is the formation of floc. It is on the basis of floc formation that the success of the activated sludge process depends. Without flocculation only a portion of the organic matter contained in waste waters would be removed; most of it would merely be transformed from one form to another form.

McKinney and Gram (32) showed that various pure cultures of bacteria utilized soluble organic matter, produced new cells, and underwent flocculation in a manner similar to normal activated sludge. But unlike normal activated sludge, the pure bacterial floc did not clarify the effluent completely upon quiescent settling. The motile actively metabolizing bacteria remained in the supernatant after the floc had settled. These motile bacteria were found to be responsible for the major portion of the effluent BOD.

Buswell and Long (14) in 1923 proposed that bacterial slime was a cause of floc formation. McKinney (10) in 1952 proposed that bacterial floc was dependent on surface charge, however, in 1956, he presented a slightly revised version of his theory. He maintained that bacterial flocculation was primarily dependent upon the energy of the bacteria rather than their surface charge. Tenney and Stumm (25) in 1964 stated that biological self-flocculation resulted from the interaction of naturally produced polyelectrolytes which form bridges between individual microbial particles. Crabtree, et al (26) in 1965 pointed out that bacterial flocculation is not absolutely related to slimes. He proved that the flocculation was due to the accumulation of a polymer poly-β-hydroxybutyric acid (PHB). Reviewing these proposals, it can be seen that there is no one mechanism to explain bacterial flocculation.

B. Possible Mechanisms for Bacterial Flocculation

Based upon observations made during the present research, two possible mechanisms of bacterial flocculation seem prominent.

1. Bacterial flocculation might be brought about by the bacterial capsule.

As previously mentioned, the slime former reached its optimum flocculation during the course of substrate removal. After the glucose was exhausted, the flocs began to disperse and microscopic examinations (india ink slides) showed that

the amount of capsular material around the cell had decreased. The flocculation of the blue organism was similar to the slime former. It reached its optimum flocculation during the period of substrate removal. Microscopic observations revealed that capsular material was also produced by this organism. From this it might be proposed that the flocculation of both the slime former and the blue organism was based upon their capsular material, rather than the energy level.

2. Bacterial flocculation might be brought about by Van der Waal's forces of attraction.

Serratia marcescens flocculated gradually during the course of substrate removal and reached its optimum flocculation after the glucose was exhausted. From this study no definite statement regarding this mechanism of flocculation can be made. However, from observations by McKinney (24) and from some of the observations made in this study, it appears that the energy level may have played a role in the flocculation of Serratia marcescens. It may be reasoned that at the beginning of the experiment there was enough energy from the substrate to overcome the Van der Waal's forces. Consequently, very few floc particles were observed. During substrate removal the energy level was reduced and an increase in floc particles was observed. After the substrate was exhausted the energy was at its lowest level and the maximum level of flocculation was observed. Therefore, it is possible that as the substrate was removed,

the Van der Waal's forces began to predominate and cause the bacterial cells to flocculate.

C. Relationship Between Substrate Concentration and Bacterial Flocculation

As mentioned earlier, McKinney proposed that floc formation was based on energy level. He said, "If the bacteria were able to get enough energy from the substrate to overcome the Van der Waal's forces of attraction, floc would not occur. In the low energy system, Van der Waal's forces would predominate and floc would occur." Serratia marcescens was the only organism studied that might have followed this mechanism.

The slime former exhibited a reverse phenomenon. Either under high or low substrate concentrations, it flocculated well during the course of substrate removal and reached its optimum flocculation before substrate was completely exhausted. After the glucose was exhausted, the flocs gradually dispersed. Heukelekian and Littman (20) pointed out an interesting relationship between bacterial flocculation and availability of food, "If a large food source is present, there will be a large number of free swimming bacteria. If the food source is extremely limited, then the bacteria will exist mostly in floc." This statement is, of course, incomplete. In the present study, it was found that for Serratia marcescens, cell flocculation in the low substrate concentration unit such as 300 mg/l was

somewhat less than in the 500 mg/l unit. However, in the 800 mg/l and 1000 mg/l units, very little difference was observed. Accordingly, it would appear that the observations of Heukelekian and Littman do not represent a general phenomenon. The slime former seemed to floc best when the initial glucose concentration was 500 mg/l. When the food source was extremely limited, the flocs dispersed. Thus, flocculation seems to be related to the metabolic activity of the bacteria. During the course of substrate removal, metabolic activity caused the synthesis of capsular material which appeared to produce flocculation. After the substrate was exhausted, the capsules were gradually reduced and the flocs dispersed. It was observed by microscopic examinations that the size of the individual capsular material was much greater during substrate removal than after substrate exhaustion. It seems possible that the capsular material may have served as a carbon source, or since it was easily removed by simple centrifugation, it may have been reduced by attraction of the cells with continued agitation.

D. Relationship Between Maximum Specific Growth Rate and Bacterial Flocculation

Another interesting observation made during the course of this study was the relationship between the maximum specific growth rate of each organism and its degree of flocculation. It was observed that the faster the maximum specific growth rate the better the flocculation. Since only a

limited number of organisms were studied, no definite statement regarding this can be made. However, it appears that there is a relationship between the maximum specific growth rate of the bacteria and its flocculating capabilities.

E. Bacterial Surface Charge

A very simple method was utilized to gain some insight into the surface charge of the bacteria studied.

Under microscopic observations, a 1.5 volt dry battery with two electric wires welded on its ± electrodes respectively, was used to test whether the flocs would migrate to the anode or to the negative pole.

The migration of the slime former's floc particles was very fast. In fact, the movement was so fast that the floc particles were dispersed during the migration.

The floc particles of Serratia marcescens migrated much slower than the slime former. No migration of Pseudomonas aeruginosa cells could be observed. It would be very difficult to make any conclusion from this very simple study regarding surface charge. However, it is important to note that the best flocculating organisms had the faster rate of migration which might indicate a higher surface charge.

F. Some Relationship of These Findings to Those of Others

In this research, it was found that both the slime former and Serratia marcescens were floc-producing bacteria,

whereas, Pseudomonas aeruginosa was a non floc-producing bacterium. Van Giles (28) also reported that Pseudomonas aeruginosa did not flocculate. But Crabtree, et al (26) pointed out that Pseudomonas aeruginosa was a floc-forming bacterium and Serratia marcescens was a non floc-forming organism. However, McKinney (24) in 1956, used Serratia marcescens to prove his theory that bacterial floc was primarily based on energy level, as he predicted, Serratia marcescens did floc. Bacterial slime as a cause of floc formation was first proposed by Buswell and Long (14). Also as observed by McKinney (10), Zoogloea ramigera, Escherichia intermedium, Nocardia actinomorpha, Bacillus cereus, Aerobacter aerogenes, and some others flocced in the same manner, i.e., the bacterial cells attracted each other and were joined together by their capsules. In the present study, the slime former flocced well and produced capsules (or slimes). Contrarily, Crabtree, et al (26) proved that capsules were not involved in the flocculation he observed. Therefore, it seems possible that different experimental methods may result in different observations. Crabtree, et al (26) set forth a mechanism of floc formation for Zoogloea ramigera which stated that bacterial flocculation was due to the accumulation of the polymer poly-β-hydroxybutyric acid (PHB). The accumulation of PHB preceded flocculation, and floc formation was prevented by metabolic blocking of PHB synthesis. Also endogenous dissimilation of PHB resulted in deflocculation. It is very interesting that in the

present study, the floc formation of Serratia marcescens was similar to that noted by McKinney in 1956, and the floc formation of the slime former was very similar to that of Zoogloea ramigera used by Crabtree, et al in 1966. But without any identification, it cannot be said that the slime former examined in the present study was Zoogloea ramigera simply because it was isolated from sewage.

Furthermore, it should be noted that more precise definition of the nature of the bacterially synthesized floc-causing material is needed. Tenney and Stumm (25) proposed that biological self-flocculation resulted from naturally produced polyelectrolytes; McKinney in 1952 observed that floc formation was brought about by capsules; Crabtree, et al pointed out that floc formation of Zoogloea ramigera was due to the accumulation of PHB; in the present study, the floc formation of the slime former was believed to be due to slimes (or capsules). In no study except that of Crabtree, et al has any attempt been made to associate the presence of a particular compound with the occurrence of flocculation. Valuable information could be obtained by studying the chemical composition of bacterial slimes or capsules other than that of Zoogloea ramigera which are present when flocculation occurs.

CHAPTER VI

CONCLUSIONS

The phenomenon of bacterial flocculation is extremely complex. Flocculation may depend upon a large number of factors, anyone of which may predominate according to the peculiar and particular set of environmental conditions in which the bacteria exist. However, the results of the present study support the following conclusions:

1. There are at least two mechanisms of bacterial flocculation.

2. The time for optimum flocculation occurred during substrate removal for the slime former and after the substrate had been exhausted for Serratia marcescens.

3. The initial substrate concentration has very little effect on bacterial flocculation.

4. It appears that for floc-producing bacteria, the faster the maximum specific growth rate, the better the bacterial flocculation.

5. When two floc-producing bacteria are mixed together, flocculation proceeds in a manner similar to flocculation of the one which has the faster growth rate.

6. The floc-forming bacteria studied produced more metabolic intermediates and/or end products than the non

floc-producing bacteria studied.

CHAPTER VII

SUGGESTIONS FOR FUTURE WORKS

All the experiments of the present research were performed using only glucose as substrate. It would be worthwhile to study bacterial flocculation with different substrates to determine any possible relation between substrate structure and floc formation.

It would also be of interest to isolate as many organisms as possible which are present in waste waters for individual study to determine which ones are floc producers and which are not floc-producing organisms.

A zeta potential meter should be used for measuring the zeta potential during the course of flocculation.

In order to determine if bacterial flocculation is a function of the bacterial surface charge, a special instrument for the observation and measurement of bacterial charge should be designed. This might be a microscope slide with a circular concavity at its center. To the opposite sides of the concave rim must be attached two electrodes connected to an adjustable voltage meter. It would be easy to observe the migration of cells and flocs to the electrodes under the microscope by adjusting the voltage.

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