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THE EFFECTS OF ENZYMES ON ACTIVATED SLUDGE FLOC

BY

TERRY LEE STODDART

B.S. FLORIDA TECHNOLOGICAL UNIVERSITY, 1971

THESIS

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1977

ABSTRACT

Dialysed activated sludge was used as a substrate to test for enzymes which can hydrolyse activated sludge floc. Two hundred and fifty aerobic and anaerobic microorganisms were tested against activated sludge for the presence of hydrolytic enzymes. These included known genera and organisms obtained by various enrichment procedures. Anaerobic digester mixed liquor was contacted with activated sludge agar under anaerobic conditions. None of the microorganisms present in the digester liquor hydrolysed the floc. The following commercial enzymes were contacted with activated sludge singly, in combination, and sequentially under various physical and chemical conditions: protease, lipase, cellulase, pectinase, phospholipase C, trypsin, and glucuronidase. Although commercial enzymes and various microorganisms reacted with known substrate controls neither the enzymes nor microorganisms employed affected the activated sludge floc. Treatment of activated sludge floc with ethylenediaminetetraacetic acid resulted in gross deflocculation and release of humic substances. The floc particle, probably a combination of polymers bound in a complex manner, is resistant to enzymatic degradation. Several workers have reported on the enzymatic hydrolysis of polymers produced by axenic cultures isolated from activated sludge floc. It is unlikely that the results of their work can be extrapolated to the complex structure of activated sludge floc particles.

ACKNOWLEDGEMENT

I would like to express my sincere appreciation to Dr. Rudy Wodzinski for his guidance and assistance in the preparation of this document. I would also like to express my appreciation to the Orange County government for the use of their laboratory facilities. I also thank my wife for her patience and encouragement.

PREFACE

The nature of activated sludge floc particles has not been resolved. A complete knowledge of floc particle structure would provide information useful for improving the operation of waste-water treatment facilities. The enzymatic degradation of activated sludge floc could provide the following information:

1. The type of chemical bonds involved in floc formation.
2. The type (s) of chemical substances present in activated sludge floc.
3. The effectiveness of enzymes in releasing viable microorganisms from activated sludge floc.

The known facts about activated sludge floc can be summarized as follows:

1. Activated sludge floc particles appear to be an agglomeration of microbial cells, debris, and metabolic by-products of microorganisms, plants, and animals.
2. The agglomeration of these materials is held together by an undefined mechanism or substance.
3. Activated sludge floc particles persist in the activated sludge process and are somewhat resistant to degradation.
4. The position, type, and function of microorganisms bound to floc particles has not been determined.

5. The formation of activated sludge floc is a necessary event in the activated sludge treatment process.

The purpose of this work was to determine the effects of various enzymes on activated sludge floc or to isolate enzyme (s) capable of degrading floc particles. The nature of the enzymes capable of floc degradation should yield valuable information as to the structure, composition, biological content, and persistent nature of floc particles in the waste treatment process.

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INTRODUCTION

Since the biological content and activity of activated sludge is extremely important to the proper functioning of the activated sludge treatment system, any effort that would determine the position of microorganisms in the floc (as related to function) would provide a much needed understanding of the biological process involved. In order to provide the rationale for the events occurring during the secondary treatment of waste-water, there is a need for general knowledge about the biological content of activated sludge, the physical-chemical structure of activated sludge floc, theories of activated sludge floc formation, physical-chemical methods of deflocculation, and enzymatic methods of floc polymer hydrolysis.

The activated sludge process is a dynamic system. The interaction of organisms within the activated sludge system includes all the variables encompassed in a complex biological ecosystem. The excellent review by Pipes (50) recapitulates the complexities and interactions of the many organisms present in activated sludge. Pike and Carrington (46) and Pike and Curds (45) reviewed the bacteriology of activated sludge and emphasized the important functions of bacteria in the stabilization of wastes. These functions included the rapid oxidation of wastes with the concomitant formation of a microbial biomass possessing the capacity for flocculant growth.

The activated sludge process, when operated under optimum conditions, is suited to the economical removal of degradable organic materials from domestic and industrial waste. The activated sludge process operates in a manner analogous to a continuous flow chemostat. However, steady-state conditions are not always achieved in the activated sludge process due to the variability of substrate loading rates. Operation of the activated sludge process involves contacting microorganisms (in the form of sludge) with nutrients (in the form of wastes). The process includes a concomitant recycling of a portion of the biomass produced in the treatment process. The process when operated properly can remove up to 99% of the degradable organic material present in the untreated waste as measured by biochemical oxygen demand.

The flocculant growth of bacteria in the activated sludge process allows for rapid settling of the biological sludge and removal of suspended solids. The activated sludge process is a mineralization process involving the degradation of chemical substances in sewage by a myriad of microorganisms. Normally these substances are highly resistant to complete degradation by a single organism.

Biological Content. there are numerous reports (1,2,3,7,8,10,11,12, 16,17,24,27,34,35,36,38,41,52,59,60,61,62,63,64) which show that many different types of organisms are present in the activated sludge process (Tables 1-5). Bacteria, fungi, protozoa, metazoa, and higher organisms such as nematodes and insect larvae, have been isolated from activated sludge with each group possessing a different

function in the process. The high surface to volume ratio of bacteria permits high specific growth rates, allowing for the rapid exchange of nutrients and metabolites between cells and suspending medium. Therefore, bacteria are dominant in the system and play the major role in the stabilization of degradable organic waste. The non-bacterial organisms are not capable of the growth rates of bacteria and generally act as scavangers (predators), removing dispersed bacteria and suspended debris from the fluid medium.

A review of bacterial genera isolated from activated sludge (Table 1) reveals several genera that appear regularly (Table 2). It should be noted that these organisms are not among those abundant in raw sewage. The persistance of commonly encountered genera in the activated sludge process probably results from flocculant growth habits or their ability to metabolize a wide variety of organic compounds. Organisms present in untreated wastes are not capable of these growth characteristics and are subsequently washed out of the activated sludge process.

Several investigators (17,41,52) have reported on the function of protozoa in the wastewater treatment process and have determined that several genera of protozoans can serve as indicators of good treatment (Table 3). Generally, the motile ciliates predominate in the protozoan population when wastes contain large amounts of suspended matter such as bacteria or organic suspended solids. Their presence, therefore, indicates poor plant operations. Stalked ciliates predominate in the protozoan population when non-flocculated suspended

TABLE I

Genera Of Bacteria Isolated From Activated Sludge*

Genera*	Author
Flavobacterium Alcaligenes Pseudomonas	Unz and Dondero (62)
Zoogloea	Unz and Dondero (61)
Escherichia Bdellovibrio Enterobacter Corynebacterium (Coryneform group) Achromobacter (Alcaligenes) Azotobacter	Dias and Bhat (17)
Nocardia Micrococcus Achromobacter (Alcaligenes) Flavobacterium Corynebacterium (Coryneform group) Alcaligenes Lophomonas (Alcaligenes) Arthrobacter Zoogloea	VanGils (64)
Zoogloea	Heukelekian and Littman (24)
Salmonella Mycobacterium	Leclerc et al. (34)
Cytophaga Flavobacterium Achromobacter (Alcaligenes) Pseudomonas	Lighthart and Oglesby (35)
Alcaligenes Achromobacter (Alcaligenes) Pseudomonas	Lighthart and Loew (36)

* The generic names appearing in parentheses indicate current bacterial nomenclature as defined by Bergey's Mannual of Determinative Bacteriology (8). The other entries indicate the authors stated identification.

TABLE I
(continued)

Genera	Source
Arthrobacter Bacillus Cytophaga Flavobacterium Vibrio Aeromonas	
Acinetobacter Alcaligenes Brevibacterium (incertae sedis) Caulobacter Comamonas (Pseudomonas) Cytophaga Flavobacterium Hyphomicrobium Microbacterium (Coryneform group, incertae sedis) Pseudomonas Sphaerotilus	Benedict and Carlson (8)
Achromobacter (Alcaligenes) Alcaligenes Bacillus Flavobacterium Micrococcus Pseudomonas Bacterium (no longer recognized) Corynebacterium (Coryneform group) Microbacterium (incertae sedis)	Jasewicz and Porges (27)
Zoogloea	Unz and Farrah (63)
Pseudomonas Zoogloea Vibrio Escherichia Enterobacter Achromobacter (Alcaligenes) Brevibacterium (incertae sedis) Paracolobactrum (no longer recognized)	Ueda and Earle (60)
Zoogloea	Amin and Ganapati (3)
Beggiatoa Sphaerotilus Actinomyces	Tomlinson (59)

TABLE I
(continued)

Organisms	Reference
Arthrobacter *Achromobacter* (*Alcaligenes*) *Pseudomonas* *Flavobacterium*	Adamse (1)
Zoogloea	Butterfield (10)
Achromobacter *Chromobacterium* *Pseudomonas*	Allen (2)
Acinetobacter *Comamonas* (*Pseudomonas*) *Flavobacterium* *Pseudomonas* *Micrococcus* *Aeromonas* *Spirillum* *Bacillus* *Achromobacter* (*Alcaligenes*) *Actinobacillus* *Pasterurella* *Vibrio*	Ministry of Technology (41)
Achromobacter (*Alcaligenes*) *Aerobacter* (*Enterobacter*) *Alcaligenes* *Bacillus* *Brevibacterium* (*incertae sedis*) *Corynebacterium* (*Coryneform group*) *Comamonas* (*Pseudomonas*) *Flavobacterium* *Micrococcus* *Pseudomonas* *Spirillum* *Zoogloea*	Dias and Bhat (16)
Escherichia *Aerobacter* (*Enterobacter*) *Klebsiella* *Pseudomonas* *Flavobacterium* *Alcaligenes* *Achromobacter* (*Alcaligenes*) *Zoogloea* *Bacillus*	McKinney and Werchlein (38)

TABLE I
(continued)

<u>Neisseria</u>
<u>Lactobacillus</u>
<u>Bacterium</u> (no longer recognized)
<u>Micrococcus</u>
<u>Paracolobactrum</u> (no longer recognized)

TABLE 2

Bacterial Genera Most Frequently Isolated From Activated Sludge

GENERA
Flavobacterium
Alcaligenes
Pseudomonas
Corynebacterium
Micrococcus
Zoogloea
Cytophaga
Arthrobacter
Bacillus
Brevibacterium

TABLE 3

Genera of Protozoa Commonly Isolated From Activated Sludge

Genera	Author
Vorticella	Ministry of Technology (41)
Opercularia	
Aspidisca	
Trachelophyllum	
Hemiophrys	
Euplotes	
Drepanomonas	
Chilodonella	
Carchesium	
Vorticella	Schofield (52)
Epistylis	
Aspidisca	
Hemiophrys	
Cochliopodium	
Amoeba	Barker (7)
Vahlkamfia	
Arcella	
Trinema	
Actinophrys	
Mastigamoeba	
Oikomonas	
Monas	
Pleuromonas	
Cercoboda	
Trepomonas	
Notosolenus	
Ciliophora	
Chilodon	
Colpidium	
Bodo	
Cercobodo	
Trichoda	
Urotricha	
Uronema	
Cyclidium	
Cinetochilum	
Vorticella	
Carchesium	
Opercularia	
Podophrya	

matter is present at low levels and are therefore indicators of good plant operations.

The metazoa commonly isolated from activated sludge (Table 4) generally acts as scavangers, consuming bacteria and small protozoa.

The function of fungi commonly found in activated sludge (Table 5) has not been determined.

Physical structure of activated sludge. Sládka and Zahrádka (53) reviewed the morphology of activated sludge floc. Their findings indicated that floc morphology was dependent on the species composition and type of waste being treated in the sewage treatment plant (STP). Pipes (51) reported that activated sludge floc particles ranged in size from 50 micrometers to 500 micrometers in diameter. Laubenberger and Hartman (33) described floc as an agglomeration of particles containing different quantities of bound water. They found that the settling characteristics of the sludge were dependent on the floc structure and quantities of bound water.

The physical structure of activated sludge floc, as I have observed it, is most frequently amorphous in nature. Occasionally, floc particles with finger-like projections are also observed. The amorphous floc from properly operating plants is dense and compact with encrustations of bacteria and detritus on the surface (figure 1). Waste treatment plants demonstrating bulking conditions posses a filamentous floc. Depending on environmental conditions the filamentous floc can be extremely light and lacy or contain a very dense filamentous mat.

TABLE 4

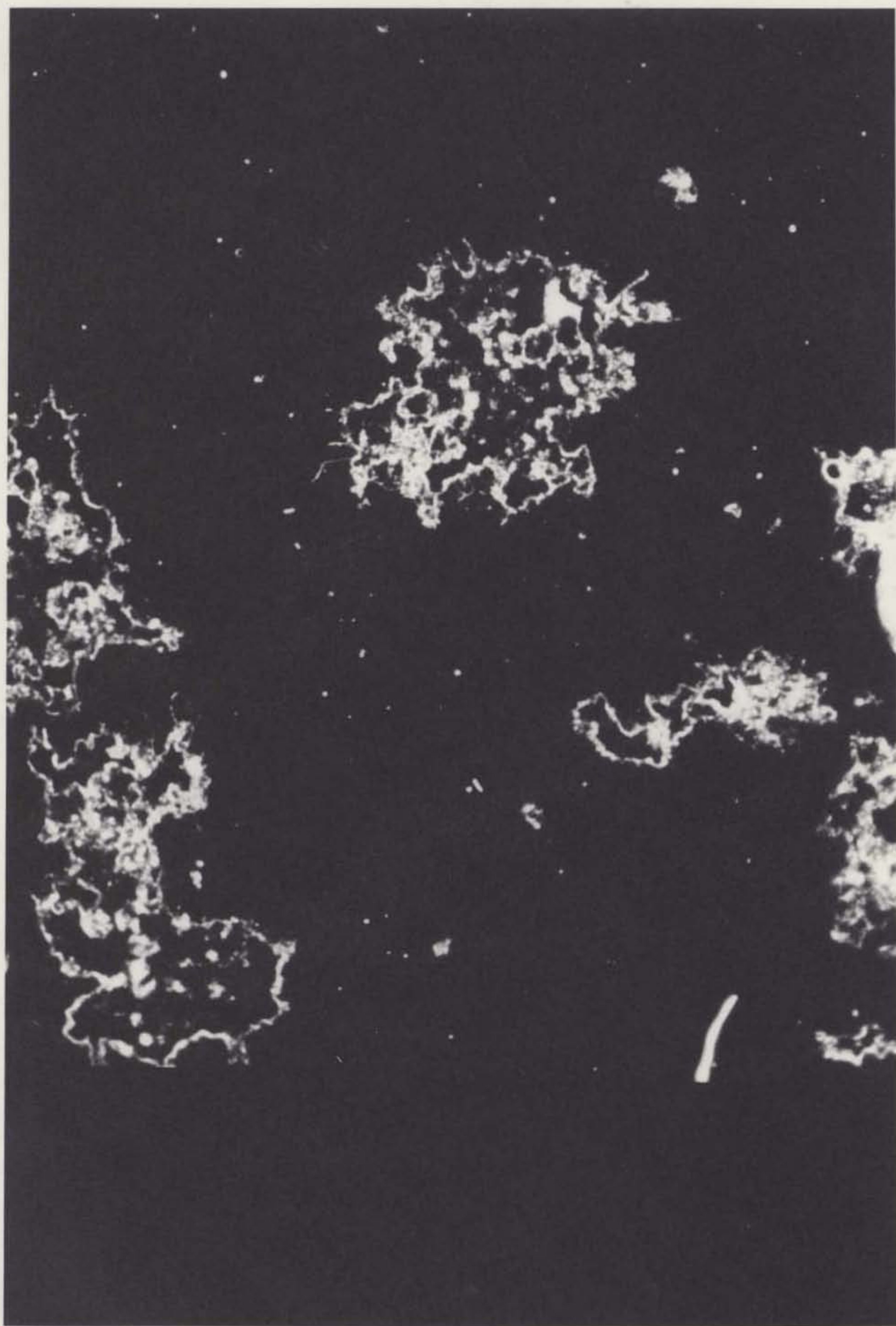
Metazoa - Rotifers Isolated From Activated Sludge

Genera	Author
Dicranophorus	Calaway (11)
Lepadella	
Bdelloidae	
Adineta	
Rotaris	
Philodina	
Habrotrochea	
Lecane	

TABLE 5

Fungal Genera Commonly Isolated From Activated Sludge

Genera	Author
Geotrichum	Cooke and Pipes (12)
Trichosporon	
Penicillium	
Cladosporium	
Alternaria	
Candida	
Cephalosporium	
Fusarium	
Trichoderma	
Mucor	
Phoma	
Phialophora	
Aureobasidium	
Aspergillus	
Rhodotorula	



The chemical composition of activated sludge floc. Although the chemical composition of activated sludge floc has not been studied in great detail, the majority of investigations concerned with composition have limited their studies to polymers produced by pure cultures of microorganisms isolated from activated sludge. Crabtree et al. (14) isolated extracellular material from Zoogloea sp. (I-16-M). This polymer, isolated from a hot-water extract, contained glucose, xylose, arabinose, and a hexoseamine. Parsons and Dugan (43) isolated an extracellular polymer from Zoogloea ramigera (115) that contained glucose and galactose. Friedman et al. (19) identified a polyglucose polymer similar to cellulose from Zoogloea ramigera (115) and Zoogloea sp. (I-16-M). The polymer was hydrolyzed by treatment with cellulase. Tezuka (58) isolated Zoogloea sp. from activated sludge. The organism produced an extracellular polymer containing N-acetyl glucosamine and N-acetlyl fucosamine. Walen and Davis (65) found glucose, mannose, and galactose in exopolymer produced by a Zoogloea sp. (NRRL B3669M). Farrah and Unz (18) reported isolations of extracellular polymer from Zoogloea strains (MP6, 106) and from activated sludge. The polymers isolated appeared to be similar and contained glucosamine and an amino sugar similar to a methyl pentose amine. The work by these two investigators is the first effort relating the zoogloeal polymers to activated sludge polymers.

Hunter (26) documented the presence of numerous organic compounds in activated sludge and in activated sludge process effluents: amino acids, fatty acids, volatile fatty acids, pyrene, non-ionic

surfactants, sterols, vitamins, uric acid, alkylbenzene sulfonates, polysaccharides, and humic acids. Kolattukudy and Purdy (32) isolated large quantities of cutin from sewage sludge. They estimated that cutin comprised 12 to 28% (w/w) of the sludge organic matter. It is probable that many other organic compounds are present in the activated sludge process. Their presence is dependent on the nature of the waste being treated and the activity in a community at the time a sample is obtained.

Formation of activated sludge floc. Several diverse theories have been promulgated regarding the formation of floc in the activated sludge treatment process. The earliest theories, typified by those of Butterfield (10), attributed flocculation and reduction of biochemical oxygen demand solely to *Zoogloea* sp. However, later investigations conducted by McKinney and Weichlien (38) and McKinney (39) demonstrated that several bacterial genera isolated from activated sludge were capable of forming floc under certain environmental conditions. Kiuchi et al. (30,31) and Kato et al. (29) have also identified a number of bacterial genera isolated from activated sludge that were capable of forming floc. Their work reaffirms the earlier investigation conducted by McKinney (38,39); that most probably no single organism in activated sludge is responsible for floc formation. Pillai and Subrahmanya (49) and Hardin (22) theorized that protozoans present in activated sludge are responsible for floc formation. However, these investigators did not present a clear explaination as to the exact function of protozoa in floc formation.

Several investigators (13,14,19,37,43,44,55) have implicated organic polymers as mediators of activated sludge floc formation. Crabtree et al. (14,13) have identified an intracellular polymer, poly -B-hydroxybutyric acid (PHB), from Zoogloea sp. They correlated the accumulation of PHB to flocculation and proposed that a specific chemical bond, an ester linkage, was responsible for floc formation. Friedman et al. (19) and Parson and Dugan (43) isolated a polyglucose extracellular material from Zoogloea sp. and related this polymer to floc formation. Peter and Wuhrmann (44) suggested that flocculation could be explained via principles of colloid chemistry. They theorized that naturally occuring humic acids in activated sludge would coat the surface of bacterial cells and that they would act as a polyelectrolyte in conjunction with divalent cations to form bridges between cells or particles. Tenney and Stumm (55) proposed that charged biological polymers could act as bridging agents (polyelectrolytes) and therefore prompt floc formation. McKinney (37) explained floc formation in activated sludge as being related to the surface charge of bacterial cells and the velocity of the charged particles at the time of collision. He explained further that as the agglomeration of cells became larger there would be greater particle surface area available for additional collisions and subsequent floc formation.

Several investigators have reported on the effects of the various chemical parameters on the floc forming ability of microorganisms. Tezuka (56) reported that magnesium ion inhibited flocculation in a Zoogloea type organism. By contrast Tezuka (57) demonstrated the

cation dependent flocculation of a Flavobacterium sp. isolated from activated sludge. By pH reduction, Angelbeck and Kirsh (4) induced aggregative growth in a non-slime-forming strain of Zoogloea ramigera. They also demonstrated that cations reversed aggregative growth. They postulated that flocculation was dependent on the cation metabolism of the test organism. Harris and Mitchel (23) cultivated Leuconostoc mesenteroides on media containing sucrose substrate; flocculant growth was inhibited. In contrast, cultures grown on glucose demonstrated flocculant growth characteristics but did not produce dextran.

Although numerous theories of floc formation have been advanced, many questions remain unanswered regarding the mechanism of floc formation in activated sludge.

Physical and chemical methods of floc disruption. Several investigators have attempted to develop methods of disrupting or deflocculating activated sludge floc. The primary purpose of this work was to establish a method of releasing microorganisms from the activated sludge floc without destroying viable cells. Table 6 is a synopsis of methods employed to obtain total counts of viable microorganisms by disrupting floc polymer.

Several methods have been developed to extract and hydrolyse activated sludge floc polymer. However, these methods were employed to study the chemical composition of the polymer without regard for preserving the viability of bound microorganisms. Therefore a review of the literature is not necessitated for the purpose of investigation.

TABLE 6

Methods Reported to Release Bacteria or Hydrolyse Polymer Produced by Activated Sludge Bacterial Isolates

Method	Reference
Homogenization Cream Making Machine	Allen (1944) (2)
Reciprocal Shaker	Dias and Bhat (1964) (16)
Chemical Dispersants	Yin and Moyer (1968) (68)
Vibro Stirrer Homogenization	Adamse (1968) (1)
Waring Blender Homogenization	Lighthart and Oglesby (1969) (35)
Oscillating Wire Ultra Sonic Dispersal	Williams et al. (1970) (66)
Silverson Mixer Homogenization	Gayford and Richards (1970) (21)
Chemical Dispersion	Gayford and Richards (1970) (21)
Waring Blender Homogenization	Benedict and Carlson (1971) (8)
Tissue Homogenizer	Pike et al. (1972) (47)
Braun MSK Homogenizer	" " " "
Dow Soniprobe	" " " "
Kerry Ultrasonic Cleaning Bath	" " " "
Enzymatic	Pike (1973) Personal Communication
Enzymatic	Friedman et al. (1969) (20)
Enzymatic	Kato, et. al. (1971) (29)
Enzymatic	Steiner, et al. (1976) (54)

Enzymatic deflocculation of activated sludge floc. Since one of the objectives of this work is to isolate or develop a method for the sequential release of microorganisms from activated sludge floc it is appropriate to consider enzymatic hydrolysis of the activated sludge floc polymer.

Several attempts have been made to hydrolyse activated sludge floc with enzymes. Pike, et al. (47) included cellulase, B-Glucuronidase, hyaluronidase, pectinase, and Helix pomatia digestive juice in the diluent to use in ultrasonic dispersal techniques. The diluent containing pronase was the only enzymatic treatment that increased viable bacteria counts beyond those obtained in a sonicated control without enzyme. Kato, et al., (29) deflocculated pure cultures of microorganisms (isolated from activated sludge) employing cellulase, pectinase and protease enzymes. Kilattukudy and Purdy (32) isolated bacteria and fungi that produced stable extracellular enzymes that hydrolyzed purified cutin obtained from settled activated sludge. Friedman et al. (20) isolated several polarly flagellated gram-negative bacteria capable of flocculant growth. The bacteria appeared to flocculate due to the presence of extracellular fibrils that were susceptible to cellulase treatment. Stiener, et al. (54) contacted washed activated sludge with cellulase and lysozyme. Their results indicated a substantial release of glucosamine and glucuronic acid from the treated activated sludge floc.

Following a review of the appropriate literature the objectives

of this work were established to isolate or identify an enzyme or enzymes capable of hydrolysing activated sludge floc.

MATERIALS AND METHODS

Cultures. The following axenic cultures were employed in this study. *Fusarium solani* was supplied by Linda Brown, Washington State University; the *Cellumonas* sp. was supplied by Dr. Srinivasan, Louisiana State University; and *Serratia marcessans* (BF-37) was supplied by Dr. Rudy Wodzinski, Florida Technological University. Stock cultures of *F. solani* were maintained on potato dextrose agar (Difco). *Cellulomonas* sp. was maintained on nutrient agar (Difco) supplimented with 0.1% yeast extract (Difco). Cultures of *Serratia* (BF-37) were maintained on tryptone-glucose-yeast extract medium (Difco) supplemented with 0.005 M cysteine (Sigma Chemical Co.) and 1.0% caseine (Difco). All cultures were maintained at ambient temperature (22-26°C).

Activated sludge agar. Activated sludge was obtained from the McLeod Road sewage treatment plant, Orlando, Florida. Whole activated sludge agar (WASA) was prepared by adding 1.0% agar (Difco) to well mixed activated sludge. Sterilization was accomplished by autoclaving at 121°C for 20 min.

Enzymes. Several types of proteolytic enzymes were obtained from the Sigma Chemical Company, St. Louis, Mo. These were type 2 from *Aspergillis oryzae*, type 6 from *Streptomyces griseus*, crude ficin from fig tree latex, type 5 from *Streptomyces griseus*, and type 3

trypsin from bovine pancreas. Celluolytic enzymes were obtained from Sigma (type 1 practical from Aspergillus niger) and from the Wallerstein Company (cellzyme 225 lot k8117). Pectinolytic enzymes were obtained from Sigma (polygalacturonase from Aspergillis niger) and from the Wallerstein Co.(Klearzyme H.T. liquid lot k8059x). The lipolytic enzyme employed was obtained from the Sigma Chemical Company (type 2 from hog pancreas). Phospholipase C (lecithinase C type 1 from Clostridium welchii) was obtained from the Sigma Chemical Co. A crude commercial enzyme preparation (TS-3) containing proteolytic and lipolytic activity was obtained from the Wallerstein Company. Glucuronidase from Helix pomatia containing high sulfatase activity (Type H-2) was obtained from the Sigma Chemical Co.

Spot plate enzyme assay. Whole activated sludge agar (WASA), pH 6.5, was employed as a test substrate to assay commercial enzyme preparations for hydrolytic activity. Large amounts (several mg/ml) of each of the commercial enzymes were suspended in approximately 10 ml of 0.03 M phosphate buffer, pH 7.2. Filter paper discs (Gelman) were saturated with the enzyme solutions. The saturated pads were then placed on the surface of the WASA and incubated at ambient temperature for 48 h. Following incubation plates were examined for enzymatic activity. A positive result would be recorded if an area of clearing (halo) appeared around the filter paper disc.

Spot plate procedure-multiple enzyme. WASA, pH 6.5, was employed as a substrate to detect lytic activity of several enzyme combinations. Several mg/ml of Sigma protease type VI, Sigma lipase, Sigma

pectinase and Sigma cellulase were individually suspended in approximately 10 ml of 0.03 M phosphate buffer pH, 7.2. Filter paper discs were then saturated with the enzyme solutions and the discs placed circumferentially on the WASA agar plate, pH 6.5. The WASA was incubated at ambient temperature for 48 h and then observed to detect any hydrolytic activity produced by the various enzyme combinations (zones of identity).

Enzyme single deletion study. Two different (1:50) dilutions of fresh activated sludge were prepared in 0.05 M acetate buffer, pH 4.5 and in 0.03 M phosphate buffer, pH 7.2. Stock enzyme solutions were prepared as follows: trypsin (4 mg/ml), pectinase (4 mg/ml), protease type 6 (4 mg/ml), ficin (8 mg/ml), protease type III (8 mg/ml, lipase (4 mg/ml) and cellulase (8 mg/ml). All enzymes with the exception of cellulase and pectinase were suspended in 0.03 M phosphate buffer, pH 7.2. The cellulase and pectinase enzyme were suspended in 0.05 M acetate buffer, pH 5.0 and 0.5 M acetate buffer, pH 4.0. The final reaction mixture consisted of 2 ml of the diluted (1:50) activated sludge and 2 ml each of all enzymes for a final volume of 16 ml. The assay was conducted employing a single deletion protocol; i.e., different enzyme was removed from each reaction mixture progressively until all enzymes had been deleted. The reaction vessels were incubated at ambient temperature for 24 h. Periodically aliquots were removed from the reaction mixture and subjected to microscopic examination to determine if the activated sludge floc was being attacked by the enzyme system.

Release of Amino Acids and Reducing Sugars from activated sludge. Fresh activated sludge was dialysed against triple distilled water at 10°C. for 24 h. Four (1:100) dilutions of the dialysed activated sludge were prepared in 0.5 M acetate buffer, pH 4.0, 0.05 M acetate buffer, pH 4.5, 0.03 M phosphate buffer, pH 7.5 and 0.85% NaCl, pH 8.0 respectively. Stock solutions of the proteolytic enzyme group (Type II, Type VI, and ficin) were prepared at a concentration of 1 mg/ml in 0.03 M phosphate buffer, pH 7.5. Cellulase Type I was prepared at a concentration of 1 mg/ml in 0.05 M acetate buffer, pH 4.5. Pectinase was prepared at a concentration of 1 mg/ml in 0.5 M acetate buffer, pH 4.0. Lipase was prepared at a concentration of 1 mg/ml in 0.03 M phosphate buffer, pH 7.5.

Proteolytic activity against the activated sludge was determined by monitoring the release of aromatic amino acids following protein precipitation (42). A Coleman 124D Spectrophotometer (275 nm) was used to detect the aromatic amino acids released. The final reaction mixture for the proteolytic enzyme assay consisted of 1 ml of diluted (1:100), dialysed activated sludge, pH 7.5, 1.0 ml of 0.03 M phosphate buffer, pH 7.5, 0.2 ml of 0.15 M cysteine and 1 ml of the stock enzyme solution.

Cellulytic activity was assayed via the method described in the Worthington Enzyme Mannual (67) and reducing sugars determined by the method of Nelson (5). Absorbance readings were taken at 540 nm on a Coleman 124D Spectrophotometer. The final reaction mixture contained 1.0 ml of diluted (1:100), dialysed activated sludge, pH 4.5, 1.0 ml

of 0.05 M acetate buffer, pH 4.5, 1.0 ml of 0.1 M NaCl, and 1.0 ml of stock enzyme solution. Pectinolytic activity was determined by contacting the activated sludge with the enzyme and monitoring the release of reducing sugars via Nelson's method (5). Absorbance readings were taken at 540 nm on a Coleman 124D Spectrophotometer. The final reaction mixture contained 1.0 ml of diluted (1:100), dialysed activated sludge, pH 4.0, 1.0 ml of 0.5 M acetate buffer, pH 4.0, 1.0 ml of 0.1 M NaCl, and 1.0 ml of the stock enzyme solution. Aliquots were taken at time 0, 2 h and 4 h and submitted to reducing sugar analysis.

Lipolytic activity was determined via the method published in the Worthington Enzyme Mannual (67). The final reaction mixture contained 12 ml of diluted (1:100), dialysed activated sludge pH 8.0, 12 ml of 3.0 M NaCl, 6 ml of 0.075 M $CaCl_2$ and 12 ml of stock enzyme solution.

Initial activity measurements for all assays were taken at the time 0 and were followed by assays at 2 h and 4 h.

Lytic affect of enzyme combinations on activated sludge. Fresh activated sludge was dialysed against triple distilled water, pH 6.8, at 10°C. for 24 h. A 1:100 dilution of the dialysed activated sludge was prepared in 0.1 M Phosphate buffer, pH 7.7. Stock solutions of Sigma protease Type VI, Sigma Lipase Type II, and Sigma Cellulase Type I were prepared at a concentration of 1 mg/ml in 0.1 M phosphate buffer, pH 7.7.

The final reaction mixture for these combined enzymes consisted of 2.0 ml of diluted (1:100), dialysed activated sludge pH 7.7,

1.0 ml of 0.1 M phosphate buffer pH 7.7, 1.0 ml of 3.0 M NaCl, 0.6 ml of 0.075 M $CaCl_2$, 0.4 ml of 0.15 M cysteine and 1 ml each of the three stock enzyme solutions.

Assays for proteolytic activity and release of reducing sugars were performed as described above. Initial activity measurments were taken at time 0 and were followed by assays at 2 h and 4 h.

Release of reducing sugars from activated sludge floc via the action of sequentially added enzymes. A (1:50) dilution of dialysed activated sludge was prepared in 0.1 M phosphate buffer, pH 7.0. Stock solutions of Sigma Protease Type VI, Sigma Lipase, and Sigma Cellulase Type II were prepared at a concentration of 2 mg/ml. The stock enzymes were added sequentially every 15 min, until all possible orders and combinations had been achieved. The final reaction mixture following the addition of all enzymes consisted of 2.0 ml of diluted (1:50), dialysed activated sludge, pH 7.0, 1.0 ml of 0.1 M phosphate buffer, pH 7.0, 1.0 ml of 3.0 M NaCl, 0.4 ml of 0.15 M cysteine and 1.0 ml each of the stock enzyme solutions. Aliquots removed from the reaction mixture at intervals of 1 h, 2 h, and 4 h were subjected to reducing sugar and microscopic analysis.

Effects of heat treatment on activated sludge floc. A (1:100) dilution of dialysed activated sludge was prepared in 0.1 M phosphate buffer, pH 7.7. The diluted activated sludge was heated at 56°C. for 5 min, cooled to ambient temperature, and assayed with the protease-lipase-cellulase combination described earlier. Assays were performed at time 0, 2 h and 4 h.

Effects of reduced pH on activated sludge floc. The hydrogen ion concentration of dialysed activated sludge was adjusted to pH 2 for 5 min. A (1:100) dilution of the acidified sludge was then prepared in 0.1 M phosphate buffer, pH 7.7 and assayed with the protease-lipase-cellulase enzyme combination previously described. Assays for reducing sugars and amino acids were performed at time 0, 2 h and 4 h.

Effects of chelating agents on activated sludge floc. A (1:100) dilution of dialysed activated sludge was prepared in 0.1 M phosphate buffer, pH 7.7, containing 2 mM ethylenediaminetetraacetic acid (EDTA). The EDTA-treated sludge was submitted to assay with the protease-lipase-cellulase enzyme system previously described. Aliquots were removed and submitted to reducing sugar and amino acid analysis at time 0, 2 h and 4 h. A (1:100) dilution of dialysed activated sludge was prepared in 0.1 M phosphate buffer, pH 7, containing 2 mM dipicolinic acid and assayed with the protease-lipase-cellulase enzyme system previously described. At time 0, 2 h, and 3 h aliquots were removed from the assay mixture and submitted to reducing sugar and amino acid analysis previously described.

Release of reducing substances from activated sludge via the action of glucuronidase. Fresh activated sludge was dialysed against triple distilled water at 10°C. for 96 hours. A (1:100) dilution of the dialysed activated sludge was prepared in 0.1 M phosphate buffer, pH 7.0, containing 0.001 M EDTA. Sigma glucuronidase from *Helix pomatia*, with high sulfatase activity was used as supplied. The final reaction mixture consisted of 2.8 ml 0.1 M phosphate buffer,

with 0.001 M EDTA, pH 7.0, 2.0 ml diluted (1:100), dialysed activated sludge and 0.1 ml glucuronidase. The reaction mixture was incubated at 37°C. for 4 h. Aliquots were removed from the reaction mixture at time 0, 2 h and 4 h then subjected to reducing sugar analysis via Nelson's method.

Release of reducing sugars from whole activated sludge via the action of cellulase. Whole activated sludge was prepared by washing a well mixed 15 ml aliquot once in 0.5 M acetate buffer, pH 4.5. The washed sludge was then sedimented by centrifugation, the supernatant decanted, and the washed sludge resuspended in 0.05 M acetate buffer, pH 4.5 to the original volume. The reaction mixture was prepared by adding Sigma cellulase (type 2) to 10 ml of washed activated sludge, pH 4.5, to a final concentration of 1 mg/ml. The reaction mixture was incubated at 45°C. for 24 h. Aliquots were removed at time 0, 2 h, 4 h, 5 h, and 24 h then subjected to reducing sugar analysis.

Release of reducing sugars and amino acids from whole activated sludge via the activity of combined enzymes. Whole activated sludge was dialysed against triple distilled water, pH 6.8, for 72 h. The dialysed activated sludge was contacted with the protease-lipase-cellulase enzyme system previously described. The final reaction mixture contained 20 ml whole activated sludge, 10 ml 0.1 M phosphate buffer, pH 7.65, 2 ml of 0.15 M cysteine, 12 mg Sigma lipase type 2, 12 mg Sigma cellulase type 2, 12 mg Sigma protease type 6, and 4 ml of 3.0 M NaCl. Aliquots were removed from the reaction mixture at time 0, 1 h, and 2 h and submitted to reducing sugar and amino acid analysis

procedures previously described.

Deflocculation of activated sludge floc by EDTA. EDTA was dissolved in whole activated sludge to obtain a final concentration of 1.0%. The mixture was stirred manually for 5 min and allowed to settle. Aliquots were examined visually and microscopically for evidence of deflocculation.

Lytic activity of microorganisms on whole activated sludge. Two hundred cultures were obtained from Dr. Rudy Wodzinski, Florida Technological University. These microorganisms were isolated on a basal salts medium containing different organic compound as the sole source of carbon. The carbon sources included cellulose, pectin, 2'4'5'T, phaltan, sevin, chitin, napthalene, and skim milk. In addition to these microorganisms *Fusarium solani*, *Cellulomonas* sp. and *Serratia marcessans* (BF-37) were assayed by streaking onto WASA, pH 6.5. The WASA plates were incubated at ambient temperature for 30 days. Plates were inspected daily for evidence of activated sludge floc degradation.

Isolation of lytic organisms/enzymes from waste water treatment plant. Several samples of waste liquor were obtained from the McLeod Rd. sewage treatment plant. Samples were taken from the aeration chamber, re-aeration chamber and from a separate "Complete-mix" system. Aliquots from each sample were diluted in 0.1% (w/v) peptone water (Difco). Appropriate aliquots of each dilution were plated on WASA and incubated at ambient temperature for 7 days. WASA plates were inspected daily for evidence of lytic activity. A 100 ml

aliquot from each sample was placed in an ice bath and homogenized with an ultra sonic generator (Artek-Moded 300) at 75% maximum power for 1 min and allowed to settle. Supernatant from the sonicated sample was filtered through a 0.45 µm, 47 mm Gelman GN-6 membrane filter. Approximately 2 ml of the filtrate were transferred to sterile filter paper discs and the discs then placed on the surface of WASA. These plates were incubated at ambient temperature for 48 h and then inspected for evidence of activated sludge floc hydrolysis.

Isolation of activated sludge floc degrading bacteria via enrichment. A broth consisting of whole activated sludge (pH 6.8) and 2500 ug/l Fungizone® was employed as an enrichment medium for the isolation of floc-degrading bacteria. A solid medium of identical composition was prepared by adding 1.0% agar. The Fungizone® solution was sterilized via membrane filtration and added aseptically to the cooled agar preparation. Five grams wet weight of soil and aerobically digested sludge samples were introduced into 100 ml aliquots of the enrichment broth. The enrichment broth was incubated at ambient temperature for 48 h. Aliquots were withdrawn from the enrichment culture, diluted and spread on the surface of the solid medium. The solid medium was incubated for 7 days at ambient temperature and inspected daily for evidence of floc-degrading microorganisms.

Isolation of activated sludge floc-degrading fungi via enrichment. A broth consisting of 1 liter of whole activated sludge, pH 4.5, 20 mg/l chloramphenicol, 20 mg/l streptomycin sulfate, 50,000 units/l penicillin G and 35 mg/l rose bengal was employed to enrich sludge and

soil samples for fungal isolation. An identical solid medium was prepared by adding 1.0% agar to the above broth and autoclaving at 121^{0}C. for 20 min. The antibiotic mixture was filter sterilized and aseptically added to the cooled agar medium. Five grams wet weight of aerobically digested sludge and soil samples were added to 100 ml aliquots of the enrichment broth. The enrichment broth was incubated at ambient temperature for 96 h. Aliquots were withdrawn from the enrichment medium, diluted, and spread on the surface of the agar plates. These isolation plates were incubated for 7 days at ambient temperature. Plates were inspected daily for evidence of hydrolytic activity against activated sludge floc.

Release of reducing sugars from activated sludge floc via the lytic action of anaerobic bacteria. Twenty anaerobic bacteria isolates were obtained from Mr. Mark Himes, Florida Technological University. The microorganisms were isolated from the aerobic digester of the wastewater treatment plant at Florida Technological University.

Isolates were maintained on Brewers Anaerobic Agar (Difco). A thioglycollate broth medium (TGB) consisting of casitone (Difco) 15 g/l, yeast extrat (Difco) 5 g/l, glucose 1.0 g/l, NaCl 2.5 g/l, L-cysteine (Sigma) 0.25 g/l, Na_2SO_3 0.1 g/l, and Sodium thioglycollate 5.0 g/l was employed to culture the microorganisms prior to assay.

Whole activated sludge was diluted (1:100) in 0.1 M phosphate buffer, pH 7.7. The diluted activated sludge was dispensed into tubes and utilized as a substrate in the anaerobic bacteria assay. All materials for the anaerobic bacteria assay were placed in an anaerobic

chamber (Germ-free laboratories) for 13 h and allowed to equilibrate. The anaerobic chamber was maintained at less than -300 mv as measured by the Orion redox electrode. Isolates were transferred from maintainence medium to TGB cultures and incubated at 26° for 24 h. One ml aliquots of the 24 h TGB cultures were transferred to tubes of diluted activated sludge and incubated at 26°C for 48 h. Replicate tubes were removed from the anaerobic chamber at time 0, 2 h, 4 h and 48 h and analysed for reducing sugar. In addition to the reducing sugar analysis, pH measurements and microscopic examinations were performed on tubes incubated for 48 h.

Lytic activity of microorganisms isolated from anaerobic digester fluid. Anaerobic digester liquor was obtained from the Bennett Road waste water treatment plant, Orlando, Florida. The liquor, drawn from the mid point of the digester into a glass stoppered bottle, was placed in an anaerobic chamber to protect the microorganisms from oxygen toxicity. WASA plates, prepared as previously described and supplemented with 0.05% sodium thioglycollate, were placed in an anerobic chamber maintained at -300 mv, 26°C and allowed to equilibrate for 24 h. Appropriate dilutions of the anaerobic digester liquid were prepared in 0.1% peptone water (Difco). Spread plates were prepared at the appropriate dilutions and incubated at 26°C for 10 days. The plates were observed daily for evidence of lytic acitvity produced by the growth of the anaerobic microorganisms.

RESULTS

Several different procedures were employed to evaluate the effects of commercial enzymes on activated sludge floc. Early exploratory experiments were performed to qualitatively evaluate the effects of enzymes or enzyme combinations. Later work included analytical determinations to quantitate the amount of end-products produced by various enzyme activities on activated sludge floc. This later work included several types of controls depending on the specific assay being performed. The following controls were generally included to ascertain the validity of test results:

1. Substrate inactivation control (heated activated sludge)
2. Enzyme inactivation control (heated enzyme)
3. Enzyme dependent control (enzyme omission)
4. Standard additions of substrate and reaction end-product (internal standards)

Additionally, several isolation and enrichment techniques were employed to identify microorganisms capable of degrading activated sludge floc.

Spot plate enzyme assay. The thirteen commercial enzymes that were applied singly to activated sludge agar did not produce visible hydrolytic effects after 48 h incubation. Various combinations of protease, pectinase, lipase, and cellulase enzyme preparations used in the

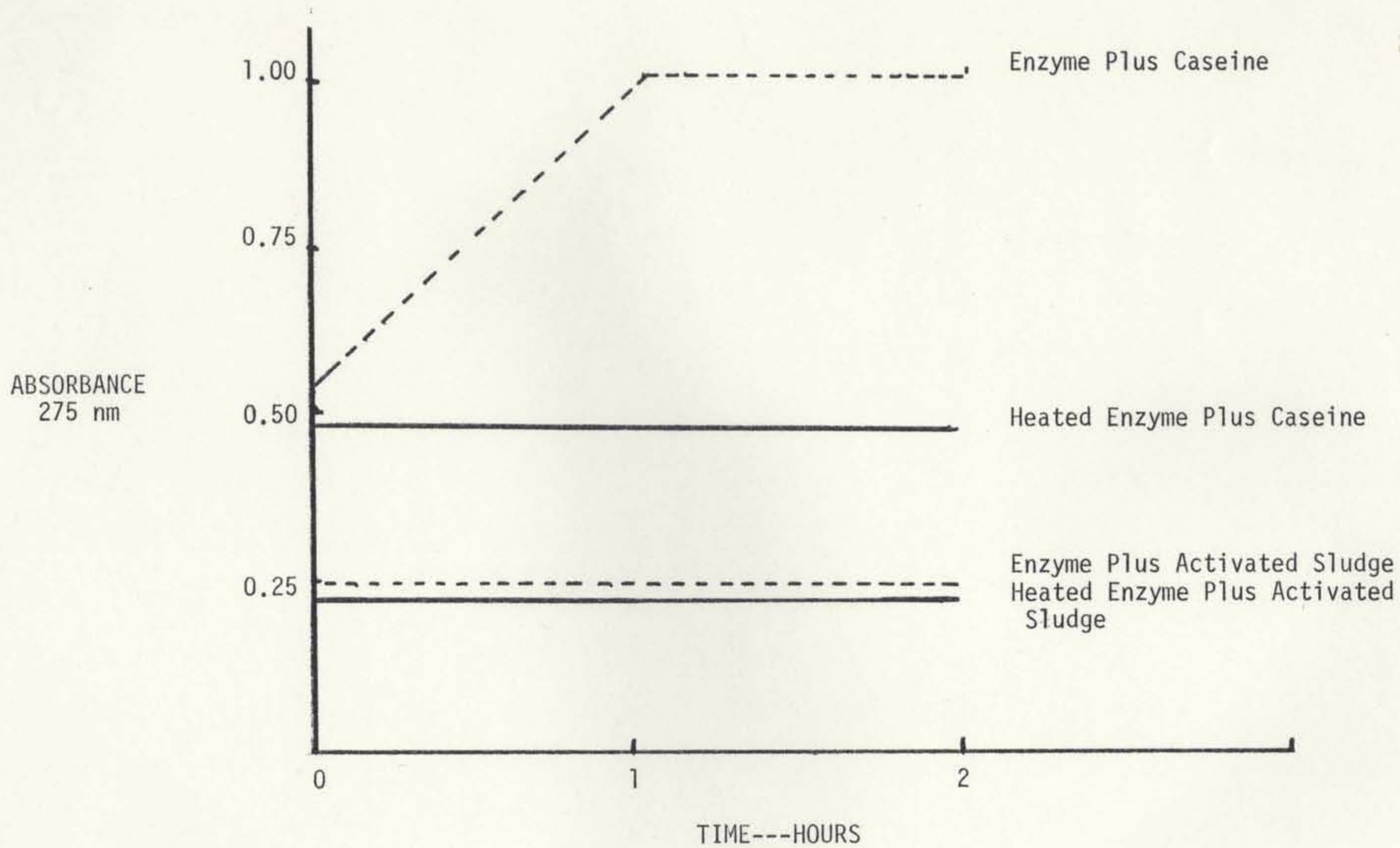
enzyme study were contacted with activated sludge agar. The combined enzymes did not produce any "zones of identity" which would indicate cooperative action between enzymes.

Single deletion assay of commercial enzymes. The trypsin, pectinase, protease, lipase, and cellulase enzymes which were combined and singly deleted from an activated sludge floc reaction mixture failed to demonstrate floc degrading capabilities as observed by microscopic examination.

Release of amino acids and reducing sugars from activated sludge floc. The several enzyme classes (protease, cellulase, lipase, and pectinase) that were contacted singly with activated sludge floc showed no indication of floc degrading capability as evidenced by monitoring of reducing sugars, aromatic amino acids and microscopic examination. The combined activity of the same enzymes also produced no detectable effect on activated sludge floc. Figure 2 is typical of the reaction kinetics of the combined enzyme system.

Physical and chemical effects on enzyme activity. Neither low pH, heat treatment, nor treatment with chelating agents (low concentration) produced conditions favoring the combined action of protease, lipase and cellulase on activated sludge floc.

Deflocculation of activated sludge floc by EDTA. Visual examination of the activated sludge following EDTA treatment indicated that gross deflocculation had occurred. A brown color appeared in the activated sludge supernatant concomitantly with the evolution of an "earthy odor", indicative of "humic substances". A microscopic examination



Enzyme Plus Caseine
Heated Enzyme Plus Caseine
Enzyme Plus Activated Sludge
Heated Enzyme Plus Activated Sludge
ABSORBANCE
275 nm
1.00
0.75
0.50
0.25
0
1
2
TIME---HOURS

of the activated sludge liquor revealed that a complete deflocculation had not occurred. Small particulate aggregates, encrusted with debris and microorganisms were evident. Prior to EDTA treatment the activated sludge supernatant was clear.

Effects of glucuronidase on activated sludge floc. Glucuronidase from Helix pomatia did not hydrolyse activated sludge floc in the presence of 2 mM EDTA at pH 7.0.

Effects of cellulase on whole activated sludge at elevated temperature. Sigma cellulase, type 2, was contacted with whole activated sludge at 45°C., pH 4.5. No reducing substances were released as monitored by Nelson's method for reducing sugars.

Effects of sequential enzyme addition on activated sludge floc. The addition of cellulase, lipase, and protease sequentially and in all combinations to activated sludge floc suspensions did not hydrolyze the floc as monitored by the release of reducing sugars and microscopic examination.

Isolation of activated sludge floc-degrading microorganisms. None of the 250 aerobic bacteria isolates nor any of the 35 anaerobic bacteria isolates demonstrated hydrolytic activity against activated sludge floc. Additionally, numerous other microorganisms produced by enrichment procedures did not degrade activated sludge floc.

DISCUSSION

The results of over 1500 enzyme assays and analytical determinations indicate that activated sludge floc particles are composed of substances not affected by common hydrolytic enzymes. The commercial enzymes employed in this study represent a broad spectrum of activity in that these enzymes or enzyme combinations are capable of hydrolysing a wide variety of naturally-occurring polymeric substances (Table 7). Theoretical calculations derived from published (26) and unpublished data (figures 3 and 4) were made to provide information about the maximum amount of protein and carbohydrate polymers available as substrate for enzymatic hydrolysis. These caluclations indicate a maximum of 1.78×10^{18} (407 μg amino acids) peptide bonds per milliliter of reaction mixture (figure 3). If only thirty percent of those peptide bonds were actually present the minimum sensitivity limits (18 μg) were adequate to detect enzymatic activity. Theoretical calculations of total organic carbon (figure 4), expressed in terms of glucose equivalents, indicate a maximum of 3.97×10^{17} molecules or 118 ug of glucose per milliliter of reaction mixture. If only 2.1% of the glucose equivalents present were actually available as substrate, detection limits (2.5 μg) were adequate to monitor their release via enzymatic hydrolysis. The presence of many organic compounds documented in the literature review indicates the availability of these

TABLE 7

Specificity Of Enzymes Employed
To Hydrolyse Activated Sludge Floc

BOND OR SUBSTRATE ATTACKED	ENZYME CLASS	ENZYME NAME
peptide	endopeptidase	trypsin
peptide	endopeptidase	ficin
peptide	non-specific peptidase	Sigma protease type 6
peptide	non-specific peptidase	Sigma protease type 2
peptide	non-specific peptidase	Wallerstein TS-3
beta-1,4 glucan	beta-1,4-glucan glucanohydrolase	Sigma cellulase type 1
hemicellulose	hemicellulase	Sigma cellulase type 1
beta-1,4 glucan	beta-1,4 glucan glucanohydrolase	Wallerstein cellzyme 225
pectin	poly-alpha-1,4-galacturonide-glycanohydrolase	Sigma pectinase type 1
pectin	poly-alpha-1,4-galacturonide-glycanohydrolase	Wallerstein klearzyme HT
phosphatidyl choline	choline phosphohydrolase	Sigma phospholipase C type 1
poly glucuronic acid	beta-D-glucuronide-glucuronohydrolase	Sigma glucuronidase type H-2

TABLE 7

(continued)

aryl sulfate	aryl sulfate sulfohydrolase	Sigma glucuronidase type H-2
glycerides	triacylglycerol acylhydrolase	Sigma lipase type 1
glycerides	triacylgylcerol acylhydrolase	Wallerstein TS-3

(1) 231 mg amino acids/gm mixed liquor suspended solids (mlss) (26)

(2) 3.7 gm mlss/L in McLeod Road Sewage Treatment Plant (STP) activated sludge (AS)

(3) 3.7 gm mlss/L x 231 mg amino acids/gm mlss = 854.7 mg amino acids/L AS.

(4) $\frac{(20\ \text{ml AS}) \times (854.7\ \mu\text{g amino acids/ml})}{42\ \text{ml final reaction mixture}} = \frac{407\ \mu\text{g amino acids}}{\text{per ml final reaction mixture}}$

(5) $\frac{407\ \mu\text{g amino acids/ml reaction mixture}}{137\ \mu\text{g}/\mu\text{M of average amino acid}} = \frac{2.97\ \mu\text{Moles}}{\text{per ml reaction mixture}}$ amino acids

(6) $\frac{(2.97\ \mu\text{Moles amino acids}) \times (6.022 \times 10^{23})}{1 \times 10^{6}} = \frac{1.79 \times 10^{18}}{\text{maximum possible peptide bonds/ml reaction mixture}}$

(1) 200 mg/L total organic carbon (TOC) present in activated sludge

(2) molecular weight of glucose is 180

(3) glucose is 40% carbon

(4) 72 mg carbon in 1 mM glucose

(5) assume that 50% of the total organic carbon is present in amino acids and not in reducing sugars

(6) $\frac{\text{200 mg TOC-100 mg TOC}}{\text{72 mg carbon/1 mM glucose}}$ = 1.38 mM glucose equivalents/L activated sludge or 1.38 μM/Ml

(7) (1.38 μM glucose equivalents) x (180 μg/μM glucose) = 248.4 μg glucose equivalents/ml activated sludge

(8) $\frac{\text{(20 ml activated sludge) x (248.4 }\mu\text{g glucose equivalents/ml)}}{\text{42 ml final reaction mixture volume}}$ =

118.3 μg glucose equivalents per ml final reaction mixture volume

(9) $\frac{118.3\ \mu\text{g glucose equivalents}}{180\ \mu\text{g}/\mu\text{M glucose}}$ = 0.66 μM glucose equivalents per per ml final reaction mixture

(10) $\frac{(0.66) \times (6.022 \times 10^{23})}{1 \times 10^{6}}$ = 3.97×10^{17} molecules glucose equivalents/ml final reaction mixture

materials as substrate for enzymatic activity. For those specific classes of organic compounds challenged with hydrolytic enzymes or microorganisms, the results of this study indicate that either none of them served a key role in floc formation or the enzymes used were unable to contact specific substances.

The inability of common enzymes to hydrolyse activated sludge floc gives ample reason for the persistence of floc particles in the activated sludge process. From an ecological view point the floc particles would have to be resistant to enzymatic degradation or they could not survive the myriad of enzymatic activities present in the activated sludge process.

Not only is activated sludge floc degraded anaerobically, it is also degraded when land spread. However, the isolation and enrichment procedures employed in this work failed to identify microorganisms capable of hydrolysing activated sludge floc. An explanation of these results would include the possibility that physical, chemical and environmental factors present in these experimental techniques did not adequately approximate the same factors present in soil or anaerobic digesters. A comparison between a mineralization process and the process required to degrade activated sludge floc provides some insight to the possible mechnaism involved in the decomposition. For example, several organisms or enzymes contacting the activated sludge floc in sequence may be responsible for the decomposition. In contrast to the results presented in this work, Steiner, et al. (54) reported the release of large quantities of reducing sugars from washed

activated sludge following treatment with lysozyme and cellulase. An attempt was made in this laboratory to duplicate the work reported by these investigators. This attempt did not meet with success. Personal communications with these investigators failed to resolve the disparity in results. The disparity in results might be explained by differences in composition of the activated sludge employed or by differences in Steiner's washing procedure which might have rendered the activated sludge floc more susceptible to degradation.

Although the physical and chemical structure of activated sludge floc is undefined, it is possible to propose a theory to explain the nature of the activated sludge floc particle since other theories already discussed do not explain available data adequately. However, certain foregoing information must first be taken into account. One consideration is that several investigators (29, 30, 31, 38, 39) have isolated numerous organisms from activated sludge capable of flocculant growth. These data dispell the theory that floc formation results from a single organism. A second consideration is that other investigators (14, 18, 19, 29, 43, 65) have isolated numerous polymers produced by microorganisms isolated from activated sludge, thus indicating their availability as agents of floc formation. A third consideration is that Peter and Wuhrman (44) reported the deflocculation of activated sludge floc with concomitant release of "humic acids" following EDTA treatment; thus indicating the role of "humic acids" as bridging agents involved with floc structure. A fourth consideration is that the work reported here confirms and expands the data presented

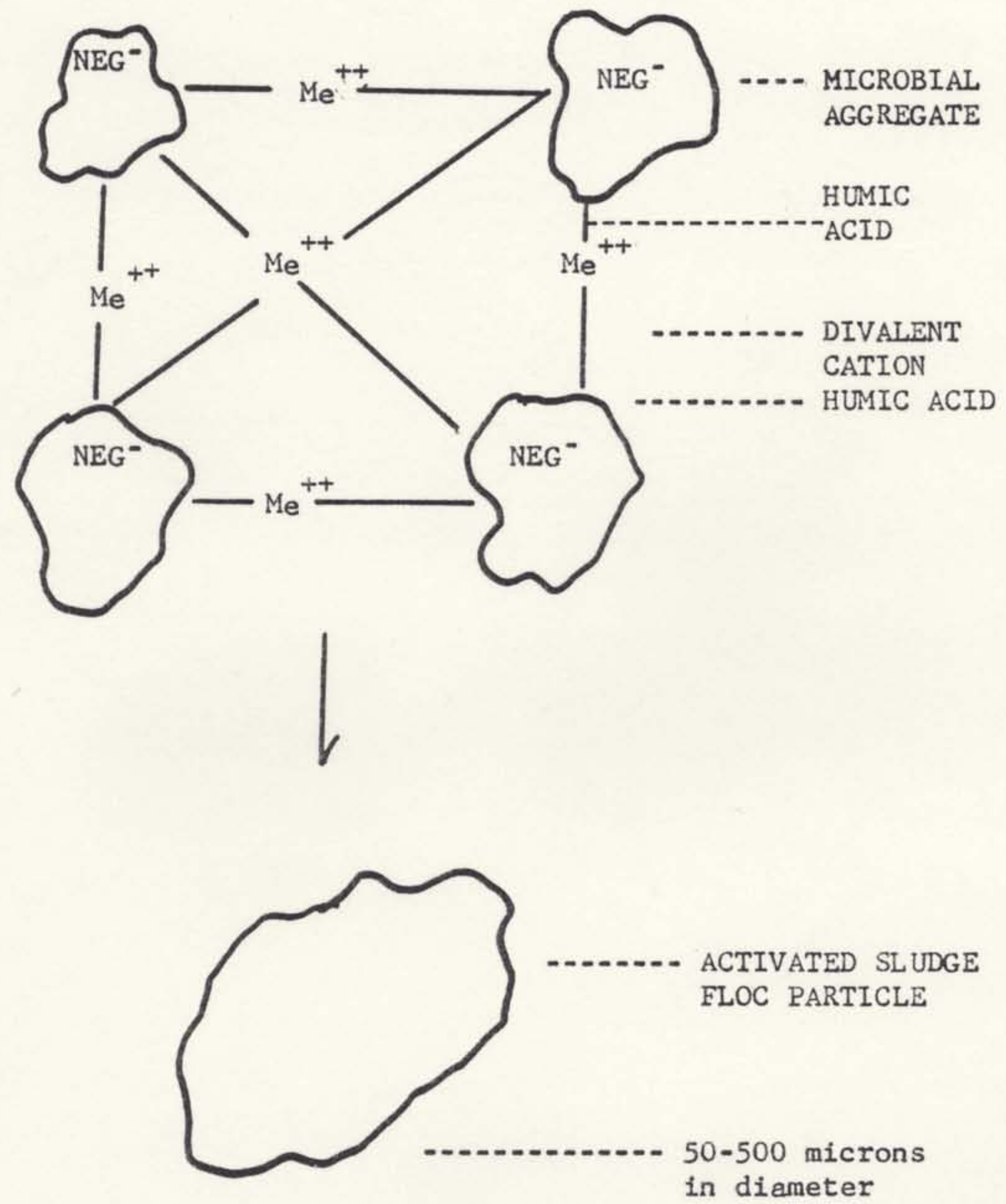
by Peter and Wuhrman (44) in that EDTA treatment does not completely disrupt all particulate structures in activated sludge; thus indicating a sub-structure within the floc aggregate.

Having reviewed the work of others and taking into consideration the work reported here, I propose the following as an explanation of floc formation:

1. Numerous microorganisms produce small aggregates comprised of single polymeric substances. These small aggregates bind cells, detritis, and metabolic by-products.
2. These small aggregates are linked together via the action of humic acids mediated by divalent cations; thus forming a floc particle.

Although other types of bonding can occur in activated sludge, I believe that the theory proposed above (figure 5) provides answers to several questions concerning floc formation. For instance, humic acids may not be just the primary force responsible for floc formation but may also be involved in shielding the single polymer aggregates from enzymatic attack. The "humic" compounds are known for their resistance to microbial degradation and for their ability to chelate metals.

Divalent cations have been implicated as a necessary factor for floc formation; lack of these ions favors dispersed growth in the activated sludge process. A crude example of the role played by humic acids in floc formation is the deep brown color observed in activated


NEG⁻
Me⁺⁺
NEG⁻
MICROBIAL AGGREGATE
HUMIC ACID
Me⁺⁺
Me⁺⁺
Me⁺⁺
DIVALENT CATION
HUMIC ACID
NEG⁻
NEG⁻
Me⁺⁺
ACTIVATED SLUDGE FLOC PARTICLE
50-500 microns in diameter

sludge when floc formation is optimum. Although this observation is not precise it does suggest some minimum concentration of "humic" substances is required before floc formation can occur.

A word of caution should be conveyed to investigators conducting research on cultures isolated from activated sludge, based on the work presented here. The information derived from pure culture studies may not be easily extrapolated to explain phenomena in the highly complex activated sludge treatment process.

Several additional areas of investigation that should be pursued to shed some light on the composition and strucutre of activated sludge are outlined below:

1. The use of humic acids as substrate to enrich for organisms capable of deflocculating activated sludge floc.
2. Duplication of anaerobic digester environmental factors in the laboratory to allow for the isolation of anaerobic bacteria capable of hydrolysing activated sludge floc.
3. Contacting activated sludge with low concentrations of surfactants in combination with commercially available enzymes.
4. The use of immunofluorescence techniques to identify the location of various microorganisms in activated sludge.

The work reported here emphasized the complexity of activated sludge floc structure and its resistance to degradation via enzymatic methods in combination with mild physical and chemical treatment.

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