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DEVELOPMENT OF IMMOBILIZED ENZYME SYSTEMS FOR ENHANCEMENT OF BIOLOGICAL WASTE TREATMENT PROCESSES

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by

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For the

WATER QUALITY OFFICE

ENVIRONMENTAL PROTECTION AGENCY

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ABSTRACT

In existing biological wastewater treatment systems, the degradation and utilization of wastewater nutrients are controlled by complex enzyme systems.

The objectives of this study were the biochemical fractionation, concentration, and evaluation of a lyophilized immobilized enzyme preparation for its effectiveness to enhance the biological degradation of domestic sewage.

A method was developed to biochemically fractionate the microbial enzymes from activated sludge, to concentrate and characterize their activity, and to immobilize this activity by entrapment in a polyacrylamide gel. The enzyme-gel preparation was tested for its effect on the biological degradation of a bench-scale batch activated sludge process.

The conclusions were: (1) the soluble enzymatic components of activated sludge can be readily separated from the particulate components of the cell; (2) the soluble system thereby obtained can be fractionated in such a manner as to maintain the activity of the catabolic enzyme systems of interest while removing nonessential components; (3) the enzymatically active preparation can then be immobilized within the matrix of a polyacrylamide gel; (4) the gel can maintain the activity during storage, repeated washings, and repeated exposure to substrate; and (5) the limited bench-scale activated sludge experiments failed to produce meaningful results due to possible incomplete polymerization of the polyacrylamide gel and an improper activated sludge culture.

The recommendations were: (1) utilize inorganic carriers such as porous glass or nickel oxide on nickel screening to insolubilize the enzymes from activated sludge organisms; (2) perform further work to substantiate whether or not insolubilized enzymes extracted from activated sludge organisms will enhance the biological degradation process; and (3) conduct further studies on a synthetic substrate representative of the constituents found in the waste flows being investigated.

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CONCLUSIONS

The overall objective of the research described in this report was to prepare, characterize, and evaluate an immobilized enzyme preparation from the material known as the microbial floc or zoogleal film, demonstrated to be the material responsible for the biological removal and degradation of sewage in the activated sludge process.

Specifically, the research concluded that:

- The soluble enzymatic components of activated sludge can be readily separated from the particulate components of the cell.

- The soluble system thereby obtained can be fractionated to maintain the activity of the catabolic enzyme systems of interest while removing nonessential components.

- The enzymatically active preparation can then be immobilized within the matrix of a polyacrylamide gel.

- The gel will maintain activity during storage, repeated washings, and repeated exposure to substrate.

- The limited bench-scale activated sludge experiments failed to produce meaningful results due to possible incomplete polymerization of the polyacrylamide gel and an improper activated sludge culture.

SECTION II

RECOMMENDATIONS

Inert inorganic carriers such as porous glass or nickel oxide on nickel screening should be utilized to insolubilize the enzymes extracted from activated sludge organisms.

In view of the tentative nature of the conclusion of the limited benchscale activated sludge experiments, it can only be recommended that further work be carried out to substantiate whether or not insolubilized enzymes extracted from activated sludge organisms will enhance the biological degradation process.

The studies should be carried out on a synthetic substrate representative of the constituents typically found in the waste flows being studied. It should be reproducible, and there should be a relatively easy method of separating the substrate from the cell material.

SECTION III

INTRODUCTION

NEED FOR STUDY

The proper treatment and disposal of sewage for small communities having up to 500 people and for individual isolated houses has been for many years one of the most difficult problems in the sanitary engineering field. For these isolated houses, septic tanks have long been the standard answer, but the maintenance required and the results produced have left much to be desired.

For the small communities, oxidation ponds -- either alone or in combination with an Imhoff tank or septic tank -- have failed to provide either the necessary protection from a public health standpoint or the aesthetic requirements now demanded by society. Maintenance costs and poor treatment process results, resulting in odor and pollution problems, have left the small community in a poor position to improve its sanitation standards.

The small "package plant" sewage disposal treatment has provided a partial solution to these problems. These treatment devices, such as activated sludge units and trickling filters, essentially are modifications of naturally occurring processes. The same biological phenomenon of microbial metabolism underlies the removal of waste materials. The major difference in the "package plant" and the naturally occurring purification process is that the man-made devices are designed for relatively short detention times in order to make them economically feasible. However, increasing the degree of treatment as measured by the percentage removal of waste matter requires longer detention times and increases the cost markedly. It follows that the increasing need for more complete treatment of wastewaters, which is projected for the future, will stimulate a search for cost/effectiveness in the design of treatment systems.

Design of conventional treatment facilities is based on an impressive accumulation of many years of practical experience. Within the context of these correlations many process design improvements have been developed and cost reductions achieved; it seems unlikely, however, that any major design improvement or cost reduction for conventional treatment facilities will be forthcoming from this approach in the future.

An alternate approach is to develop a more fundamental understanding of the underlying principles of biological treatment in the hope of arriving at new system design concepts or uncovering new ideas.

OBJECTIVES OF STUDY

The objectives of the research can be stated as follows:

- To prepare, characterize, and evaluate an immobilized enzyme preparation from the material known as the microbial floc or zoogleal

film, demonstrated to be the material responsible for the biological removal and degradation of wastewater effluents in the activated sludge process.

- To conduct limited studies of a bench-scale activated sludge system treating domestic sewage in order to determine the effects of the immobilized enzymes.

APPROACH RATIONALE

A review of the literature revealed no pertinent reference to the isolation, characterization, purification, and immobilization of enzymes from microorganisms in the microbial floc of activated sludge. Many references were found describing the addition of crude enzyme preparations, usually from yeast, to activated sludge.

These studies concluded that enzymes added in this manner were not effective in enhancing the efficiency of the activated sludge process. Two possible explanations for these failures are apparent: (1) the enzymes were crude preparations and were not isolated from the microorganisms involved in the activated sludge process, and (2) no attempt was made to protect the enzymes from instant destruction by the viable microorganisms in the activated sludge to which they were added.

Using this rationale, the investigation first developed a method to biochemically fractionate the microbial enzymes from activated sludge, to concentrate and characterize their activity and to immobilize this activity by entrapment in polyacrylamide gel. The enzyme-gel preparations were then tested for their enhancement effects on a bench-scale activated sludge process.

SECTION IV

LITERATURE REVIEW

INTRODUCTION

Domestic wastewater is composed of suspended solids, colloidal matter, and soluble organic substances. The settleable portions of the wastes are frequently removed prior to the aeration reactor in the activated sludge process. Other impurities are to be reduced or eliminated through various mechanisms during the detention of the wastewater in the aeration tank. To more fully understand these mechanisms, the ecology of activated sludge will be described.

The principal and most numerous biological "workmen" in the aeration tank are saprophytic microorganisms, including autotrophic bacteria and gelatinous masses constructed by bacteria with filamentous organisms such as Zooglea ramigera. Bacteria are responsible for the stabilization of the organic matter through biochemical processes. The suspended solids are removed by flocculation and enmeshment in the biological floc. The colloidal matter is absorbed on the biological surfaces. The protozoa present in the wastewater assist in removal of dispersed microorganisms to produce a clarified effluent in the final sedimentation tank.

The hypothetic biosorption mechanism to remove soluble organic constituents present in the wastes has been recently disproved by Krishnan and Gaudy (Ref1), Siddiqi (Ref 2), and many other investigators. It was found that soluble organic substances would be removed from biological systems primarily under enzymatic reactions. The study of organic transport into cell interiors has been reported by Siddiqi, et al (Ref 2). It was concluded that intracellular enzymes, permeases, and extracellular enzymes were the three systems to operate the whole biochemical reactions between organic substances and enzymes.

The intracellular enzymes comprise (a) hydrolases and (b) synthesis and respiration enzymes. Permeases facilitate the transport of exogenous substance into the cells. The extracellular hydrolases are secreted by the cells to hydrolyze long polymeric substrates into smaller units.

In the conventional activated sludge process, the sludge organisms are in contact with the waste for a period of three to six hours. This period is sufficiently long to permit the organisms to synthesize inducible enzymes which may be required for the utilization of particular waste, but which are not present initially in sufficient quantity. On the other hand, in the contact stabilization process, the sludge is in contact with the waste in the contact unit for only a short period of time, usually 0.5 to 1.0 hour. The sludge is then separated from the waste and returned to the stabilization unit in which the assimilated organic compounds are used for biosynthesis and maintenance of microbial life under the reaction of intracellular enzymes with the assimilated substance. Therefore, satisfactory operation of the contact-stabilization activated sludge process can be achieved when the sludge organisms process a complete set of preformed enzyme systems at the time they are introduced to the wastewater.

In the extended aeration process, rate of substrate removal per unit weight of activated sludge decreases as the aeration time prolongs. The loss of activity is due to inactivation of synthesis and respiration enzyme systems. The achievement of a high degree of purification is attributed to high concentrations of the mixed liquor suspended solids.

The transport of substrate into cells is the limiting step. Subsequent endogenous utilization of the substrate by hydrolysis, synthesis and respiration enzymes permits additional permeation of substrate into cells.

Recirculation of sludge to the aeration tank, of course, is intended to maintain microbial populations and to decrease the organic loading on a unit weight of sludge. In addition to the above, the assimilated organic matter within the cell cytoplasm are stabilized during detention in the final sedimentation tank prior to introduction into the inlet of the aerator to mix with incoming wastes. Such recirculated sludge possesses high ability for further permeation of exogenous soluble organic substance.

ENZYME ADDITIVES

A survey of the general field of enzyme additives to the biological waste degradation process revealed the following material.

Chamberlin (Ref. 3) presented a review of organic catalysts (enzymes) as well as laboratory work conducted to determine "in what quantities they appeared during the course of digestion and their effect on gasification and liquefaction of sewage solids."

The enzymes used in the initial study to determine the effect of enzymes on digestion when added to properly seeded mixtures, consisted of additions of trypsin, rennet, pepsin, lipase, and diastase with and without pH and temperature adjustment to 1 liter laboratory digesters. Results on digesters (with and without adjustment) shows in general that the addition of enzymes causes only a "slight" or no increase in gasification and no increase in solids or volatile matter. More specifically, of the enzymes used, only trypsin "has shown ... increase in reduction over the control (20%)." The author explains, "trypsin converts all of the solid proteins, proteoses and peptones to lower nitrogenous compounds. An enzyme like this demonstrates digestion much better by solid reduction than by gasification." With regard to lipase and pepsin which also reduce solids in a "similar manner ... both of the materials which they attack are not only solids but also heavy liquids." Lipase, he considers, "may not have been the type (of enzyme) which would attack the heavy oils, fats and grease." Trypsin, he considers to be the only enzyme that increased digestion "from the standpoint of liquefaction."

From the standpoint of gasification," lipase and diastase were the only enzymes that increased digestion unter optimal conditions, while lipase and rennet, "in the absence of fresh solids supernate" behaves similarly. For the first experiments the author concludes that the rate of digestion is not increased.

The second set of experiments to determine the "effect of liquefaction of fresh solids" by enzymes used basically the same experimental approach. The author concluded that liquefaction of fresh solids resulting from the action of added enzymes, "although slight in most instances, is appreciable in the case of lipase."

He notes that lipase gave a 12.6 per cent reduction in solids (per 100 cc fresh solids) over the control, whereas rennet gave 7.3 per cent and diastase 6.2 per cent. Liquefaction was determined by filtration, with ferric chloride added.

An evaluation of the effect of enzymes on swelling of fresh solids indicated that the great swelling is caused by lipase, pepsin and a mixture of the five enzymes used. The lowest swelling was caused by trypsin. Lipase, it is noted "hastens swelling immensely" at the beginning of the test and then "falls below the usual degree of swelling after a few hours." The author, nevertheless, concludes that with the exception of lipase and pepsin, the addition of enzymes does not affect the swelling of fresh solids.

The effect of enzymes on dewatering of fresh solids indicated that in the absence of ferric chloride there was no effect, but that in general ferric chloride together with the enzymes appeared slightly better than the ferric chloride alone. Trypsin was the only enzyme "in the presence of ferric chloride" which cause appreciable decrease in final moisture.

Heukelekian and Berger (Ref. 4) present a general discussion of the biochemical activities of bacteria in sludge digestion including their role in hydrolysis and liquefaction as well as a brief consideration and review of the "claims made for the addition of bacterial cultures or enzymes with regard to promoting digestion.

The authors consider that "if bacterial cultures or their enzymes are to prove of any value they might be used in two different ways. First, they might be used as initial cultures to shorten the "working-in" period required to establish digestion. Second, they might be added to a tank in which digestion has been established in order to accelerate the digestion. Heukelekian and Berger consider the first method as showing "greater promise."

The purpose of the investigation is to "produce experimental evidence to determine the value of some biological preparations consisting of enzymes and/or bacterial cultures on the digestion of sewage solids." The work, however, also includes an experiment to determine the effect of yeast additions on the digestion process and the addition of a preparation added to sewage "to determine whether an influent with a lower BOD could be obtained after sedimentation in settling or septic tanks..."

Parameters ("yardsticks") used to evaluate these experiments included for the raw sludge experiments, the BOD of the supernatant. For experiments using ripe and raw sludge mixtures gas production and solids reduction was used. In all the experiments, the concentration of additives were according to the instructions of the manufacturer, or "in many instances several times higher."

Preparations used included:

1. Pure preparations

   (a) Special Diastase HT concentrate - "obtained from a strain of Bacillus subtilis with considerable proteolytic activity in addition to diastatic activity converting starches and glycogen to maltose

(b) Clarase 900 - "A very concentrated diastatic enzyme obtained from Aspergillus oryzae and similar in its action to Special Diastase."

(c) Cellulolytic enzyme - from Rhizopus, "capable of hydrolyzing cellulose and pectin."

(d) Pectinase - "Stronger in its action on pectin than the cellulolytic enzyme, but free from cellulolytic activity."

(e) Pancreatin, "with a hydrolyzing action on fats, proteins, and starches."

2. Preparation A - "claimed to consist of more than 70 enzymes belonging to... esterases, sulfatases, carbohydrases, amylases, amidases, and proteolytic enzymes, for use in home septic tanks."
3. Preparation B - living bacteria and enzymes
4. Bakers yeast

Results from the addition of pure enzymes (Group 1, Pure preparations), to sterilized (autoclaved) fresh solids for two runs, shows in general a "definite increase in the BOD of the supernatant liquor after 12 hour incubation with each of the enzymes used separately and with the combination of the five enzymes. The minimum increase was obtained with pancreatin and the maximum with the combination of all five enzymes. Greater increases in BOD were obtained after 5 days of contact than after 12 hours with each of the enzymes, although in the case there is a question about the validity of the BOD value of the control." The results of the addition of enzymes to unsterilized fresh solids for the two runs indicates" no significant increase in the BOD of the supernatant in the first run with any of the enzymes or the combination of all five enzyme additions." In the second run, "only diastase and clarase gave appreciable increases in the BOD after 18 hours, but none of the other enzymes or the combination of enzymes gave an increase.

The addition of pure enzymes on the digestion of a "properly seeded fresh solids ripe sludge mixture" showed after 39 days that the "addition of enzymes did not increase the gas yield or affect the volatile matter reduction and pH values."

The results of the addition of preparation "A" to sterilized and unsterilized fresh solids indicates that for both sterile and unsterile fresh solids the addition of this enzyme preparation "did not give a significant change in BOD of the supernatant over the control." A similar study with "B" gave essentially the same results.

Yeast additions to fresh solids (56 day runs) indicated that a "somewhat greater volume of gas is obtained from the fresh solids to which yeast was added than from the fresh solids alone. The yeast alone produced a considerable volume of gas. When the volume of gas produced from the yeast itself is subtracted from the fresh solids yeast mixture, the gas yield from the fresh solids portion of this mixture is not appreciably higher than the fresh solids digesting without yeast addition." They further note that pH values of the fresh solids-yeast mixture was "somewhat higher after 53 days," and indicate that the solids content of the yeast alone "decreased greatly, (67.5% reduction in volatile solids),

which probably accounts for the higher volatile solids reduction of the fresh solids-yeast mixture than the control." In summary, Heukelekian and Berger consider that "it does seem likely that the addition of yeast resulted in a greater reduction of volatile matter in fresh solids."

The addition of Preparation "A" to septic tanks which were then allowed to remain quiescent for six days (20°C) gave "no indication that the addition of enzyme preparation resulted in any improvement in BOD of the sewage."

The authors consider that the addition of biological preparations such as enzymes or bacterial and yeast cultures to promote the digestion of sludge in septic tanks or sludge digestion tanks "would be of value in initiating new tanks or in accelerating digestion of overloaded tanks if there were proof of their beneficial effect." The authors believe, however, that in general, laboratory investigations are "in disagreement with this line of reasoning."

On an a-priori theoretical basis Heukelekian and Berger consider that the use of enzymes "does not offer a promising outlook, and point out that while" it is generally true that of the two types of reactions - bacterial and enzymatic - enzymatic is less efficient than bacterial." The authors note that enzymes "as true catalysts" should theoretically not be consumed by the processes they are bringing about and "should be regenerated so that starting with a given quantity of an enzyme, the process should go to completion without their utilization or destruction. In bacteria-free preparations this may be true to a certain extent, although even under this condition the rate of reaction is not as high as with bacterial cultures because:

(a) Certain enzymes such as trypsin inactivate themselves;

(b) Enzymatic reactions are reversible and the products formed, which in bacteria-free enzyme reactions would naturally accumulate, bring this reaction to a stop by virtue of the equilibrium established; and

(c) Each enzyme has its own optimum pH value."

The authors therefore consider that in the presence of mixed bacterial cultures the inactivation, utilization, and destruction of the added enzymes would be greater than in pure enzyme reactions."

Heukelekian and Berger recognize that the above discussion is "from the standpoint of pure enzyme reactions in substrates containing relatively simple soluble, organic compounds." In sewage sludge, "sludge compounds do not exist in a similar state of subdivision. There are no soluble sugars, starches and proteins but rather complex polysacharides, cellulose, proteins and fats associated with large suspended particles." "Since hydrolytic enzyme reactions are apparently surface contact phenomena, it can readily be seen why an enzyme preparation cannot bring about as efficient a reaction when added to the substrate containing coarse solids. They further note that "in the case of hydrolysis,

brought about by a bacterial culture with coarse suspended particles, bacteria can attach themselves to these particles and secrete enzymes in-situ to hydrolyze and solubilize these particles in preparation for diffusion into and assimilation by cells." The authors further consider that in many of the hydrolytic reactions with complex organic materials more than one enzyme is involved, "which the bacteria produce readily in proper sequence for the specific reaction, whereas in the arbitarily selected enzyme preparations some of the important enzymes required may be overlooked."

The authors note that the hydrolytic phase of complex organic materials in the presence of bacteria proceeds rapidly and does not constitute the rate controlling factor. They note that "diffusion into and the breakdown and assimilation of the products of hydrolysis within the cell is the slower and rate controlling reaction." It is therefore reasoned that the addition of extracellular enzymes into a substrate "by no means can accelerate this phase nor can this phase be brought about by extra-cellular enzymes in the absence of living cells."

Other arguments advanced against the addition of bacterial culture preparations for materially improving digestion includes:

(1) "All bacteria necessary for digestion are present in the raw solids."

(2) "These bacteria are normally few in number in the raw solids," but soon multiply and establish themselves under a favorable environment.

(3) "Even during the initial period prior to the establishment of flora responsible for digestion, the addition of cultures of bacteria normally does not result in shortening the period of maturation and adjustment unless proper environmental conditions are established, and if these conditions are an optimum the rapid rate of multiplication of bacteria normally present in the raw sludge could establish the necessary flora without the aid of artifical additions of cultures. In other word, one can add a great number of bacteria artifically, but unless conditions are conducive for their multiplication, the benefit derived will be negligible and

if the environmental conditions are an optimum the organisms present in the raw sludge multiply and establish the necessary flora. The important consideration is the multiplication of the bacteria, with the resultant biochemical activity, rather than the existence of a large number of bacteria. Furthermore, it is unlikely that the diverse bacteria necessary for the digestion are included in the artificial culture because so far the exact types of organisms involved in the digestion have not been isolated or studied."

Heukelekian and Berger further consider "practical considerations," with regard to the additions of enzymes or bacteria to septic tanks. Generally these additions are made to the sewage before entering the tank. They consider that since these preparations are in a "highly dispersed state" it is " very probably that the major portion will pass out of the tank and will not settle to the sludge zone where the digestion takes place." They state that in order to bring these preparations in contact with the sludge, either the entire tank contents have to be disturbed by mixing, which is contradicted from the standpoint of septic tank operation, or some means have to be found to introduce and distribute these preparations in the sludge.

Heukelekian and Berger conclude that evidence available in the published literature seems rather " inconclusive and is not based on rigidly controlled experimental work." They consider that the results present in this paper on the basis of "controlled comparisons does not show any evidence of improvement due to the addition of any enzymes, preparation, of enzymes, bacterial cultures, and yeast to sterile and non-sterile fresh solids and to sewage, with a single exception. The exception is the indicated higher BOD values of supernatant liquor when pure and strong enzyme preparations were added to sterile fresh solids." They further note than "on the other hand when these same enzymes were added to the unsterilized fresh solids there was no increase in the BOD of the supernatant, ... (indicating) that when the bacteria and their enzymes are destroyed it is possible to get liquefaction of the complex organic materials by use of artifically added strong enzymes, "but in the presence of bacteria and their enzymes as found in fresh solids, artificial addition of enzyme does not increase liquefaction."

Ingols (Ref. 5) presents a general review of the literature of sewage treatment and more specifically the activated sludge process with regard to enzymes. The laboratory studies presented include methods of analysis for hydrolytic enzymes. The principal work concerns studies in activated sludge using the hydrolytic enzymes diastase, pepsin, trypsin and lipase. The presence of other hydrolytic enzymes such as cellulase, cellobiase, maltase and chitinase, although noted, require methods for study "so cumbersome as to render their inclusion (in this study) ... impossible."

The study includes enzymatic activities during the development of activated sludge, enzymatic activities of sludge mixtures during aeration, environmental effects (substrate, temperature, reactants, salts), effects of poisons (arsenate, copper and mercury at 1.5 and 6.0 mg/L), and a correlation between activity and clarification.

From these studies it is concluded that there is a "marked" increase in pepsin during the development of activated sludge from sewage. Lipase, pepsin and diastase are found only on the surface of the sludge floc whereas trypsin is also found in the liquor surrounding the floc.

The enzymatic activities of activated sludge and sewage are considered to "change little" during a 6-hour aeration period. "Slight" fluctuations in pH have little effect on the enzyme studies.

When activated sludge is aerated continuously for several weeks the "importance of enzyme is demonstrated because the enzyme is increased during the first three days and only after that decreases gradually."

Diastase may be increased 500 per cent by feeding the sludge organisms starch at 5°C. The rate of hydrolysis of a given substrate will be influenced by the rate of assimilation of its hydrolysate by the sludge organisms. The author considers that diastase requires a certain salt concentration for action.

Ingols (Ref. 6) also presented a review of the oxidation-reduction enzymes present in activated sludge, but also included references to studies using hydrolytic enzymes. The author considers that these enzymes are important in providing the energy "necessary for the other functions of the organism." An increase in number of cells brings a concurrent increase in activity in the oxidation-reduction enzymes "but that after a period of time the food supply is used up and the rate of cell metabolism decreases and the cells are said to be resting. As the cells approach the "resting" stage there is a decrease in the activity of the oxidation-reduction enzymes, but no decline in the number of organisms."

It is concluded that oxidation-reduction enzymes are necessary in the activated sludge process for purification of sewage and that for this process the activity of activated sludge is dependent upon the concentration of these enzymes.

McKinney and Sawyer (Ref. 7) present a general review of biocatalysts including a presentation of the "Fundamental Facts" of waste treatment describing the roles of enzymes in the metabolism of waste treatment. The authors point out that one "important" difference between catalysts of inorganic nature and biocatalyst is that the inorganic catalysts have a long life and need rejuvenation or replacement "only at infrequent intervals." Biocatalysts, on the other hand, "have a relatively short life owing to denaturation which occurs during use." The authors also point out that loss of enzymes released by bacteria are lost to the effluent making it "imperative" that regenerative capacity of the organism be kept at a satisfactory level." For this reason "emergency" addition of biocatalysts "has to be on a continuous or frequent basis to obtain results" and can hence "become an expensive measure."

The availability and contents of biocatalysts are discussed. The contents of "the commercial biocatalyst are basically either concentrated cultures of bacteria or concentrated enzymes and are used in specific treatment units such as digesters, trickling filters, Imhoff plants, activated sludge plants and septic tanks. Various claims for commercial biocatalysts are examined ("there is little specific information,") and evaluated in light of "what they have done." Although the authors consider that little information is available in this respect, "little by little information is becoming available directly from operators and from independent research studies."

The cost of commercial biocatalysts is discussed in light of claims made by manufacturers in terms of savings." The authors note that "where the products have been used with limited success there is some question as to the economics of use.

The authors consider that "at present, there is considerable doubt as to the value of these commercial products," and that "results have shown that these commercial biocatalysts can bring about improvement in certain operating problems, but that for the most part, it is not dramatic nor do they appear to afford an economical solution."

Rudolfs (Ref. 8) reports on a group of experiments in which pure enzymes were added to fresh solids, fine screenings and activated sludge. The enzymes used included trypsin, pepsin and lipase at various pH's and temperatures. Results are reported for both laboratory and plant-scale operation.

The author considers that the experiments conducted do not show that digestion time is decreased "even with the addition of comparatively large quantities of enzymes." However, using lipase "under optimum conditions" gave somewhat more gas per gram of volatile matter destroyed, but with trypsin the reverse occurred." The author noted that trypsin also "seemed to stimulate liquefaction," and notes that "with our present knowledge it would seem that the bacterial groups produce sufficient different enzymes during the digestion process that any further addition is unnecessary."

Evaluation of the results in terms of digestion time, gas production, material handled, drainability and odors indicates "very little practical difference, if any," as compared to controls.

Wooldridge and Standfast (Ref. 9) present their laboratory findings supporting previous work which considered that "the most important factor in sewage purification is a series of catalyzed reactions present in either living or dead bacterial cells or liberated by them into the fluid of the reaction system."

For oxygen absorption by sewage or sludge, a Barcroft microrespirometer was used in the studies, "simulating, on a small scale, those applying to the activated sludge process of sewage purification." Oxidation of the constituents of sewage and of sludge depends upon the presence of certain oxidative enzymes (dehydrogenases and oxidases) of microorganisms. These enzymes may be effective whether the organisms are alive or dead, "provided the method of

killing has not destroyed the enzymes." Although protozoa possess enzymes that can oxidize the constituents of sewage, "the greater proportion of the oxidation is brought about by bacterial enzymes, "the bacteria being both far more active."

Sewage oxidation is mainly dependent on the presence of bacterial enzymes which may be associated with either living or dead cells. It is "probable" that the activities of the living proliferating cells for certain oxidations is greater than that for dead cells. Dead cells may be considered to be important in oxidation in activated sludge since the sludge contains a high proportion of dead bacterial cells "many of which are probably enzymatically active."

The most active microorganisms as a source of enzymes in sewage oxidations are Bacillus alkaligenes, Proteus vulgaris, Pseudomonas pyocyanea, and Pseudomonas fluorescens. Bacillus coli appeared to be "less active."

Of the protozoa examined Polytomella uvella and Euglena gracilis had an oxidizing power less than B. coli, and "in general less active than bacteria."

SECTION V

EXPERIMENTAL PHASE

BIOCHEMICAL INVESTIGATIONS

Materials and Methods

The materials for this investigation are readily divided into two groups. The first group includes those compounds used as standards within the various assay protocols:

1. Crystallized Bovine Plasma Albumin, Armour Pharmaceutical Company, Chicago, Illinois.
2. Proteinase, Nutritional Biochemicals Corporation, Cleveland, Ohio.
3. Lipase Steapsin, Nutritional Biochemicals Corporation, Cleveland, Ohio, Lipase value 3.5 x USP.
4. Alpha Amylase, B. subtilis, Nutritional Biochemicals Corporation, Cleveland, Ohio, 1800° Lintner or 2500 SKB units/gm.
5. Acetylcholinesterase (horse serum), Nutritional Biochemicals Corporation, Cleveland, Ohio, 1 unit hydrolyzes 1μM acetylcholine/min at 25°C (4 units/mg).

The second major group consists of those specific reagents employed in the immobilization protocol:

1. Acrylamide, Eastman Organic Chemicals, Rochester, New York.
2. N, N' - Methylenebisacrylamide, Eastman Organic Chemicals, Rochester, New York.
3. Riboflavin, Eastman Organic Chemicals, Rochester, New York.
4. Ammonium Persulfate, A.C.S. crystals, Matheson Coleman and Bell, Cincinnati, Ohio.

The detailed procedures for the specific assays employed during the investigation are presented in the Appendix (Section VIII) of this report. A general description of each method is as follows:

1. Proteolytic Activity: Two different assays were used to quantitate the proteolytic activity of experimental samples. The first of these entailed the use of a substrate composed of an insoluble hide protein to which the dye, Remazolbrilliant Blue, is chemically coupled. During the attack of proteolytic enzymes on the substrate-dye complex, the dye molecules are released. Subsequently, the reaction is terminated by filtration, and the absorption of the filtrate, now containing dye molecules, is determined at 595 mμ . Initially enzyme activity was expressed as a function of absorption units, but later experiments converted these value into the equivalent milligrams of a reference proteinase required to obtain the identical absorption reading.

The second proteolytic assay entailed the use of casein as a substrate. Digestion of the latter compound released amino acids into the media. With termination of the assay via trichloracetic acid (TCA) precipitation, separation of the precipitated enzyme and non-digested substrate from the acid soluble components was accomplished by centrifugation. The increased absorption due to the release of soluble amino acids was then determined at 280 mμ with a Beckman spectrophotometer.

2. Amylytic Activity: Two different assays were employed. The first entailed the use of an insoluble substrate-dye complex similar to that employed in the proteolytic assay, but in this case the substrate was starch. The enzyme activity is measured as a function of the number of dye molecules released absorbing at 595 mμ.

The soluble substrate system used was that of the classic KI-I_2 interaction with starch molecules. The soluble starch substrate is incubated with an enzyme source. If amylase activity is present, the starch is broken down. Since iodine interacts with starch to form a blue complex absorbing at 650 mμ, activity is expressed as a function of absorption of the control (no digestion of starch) minus the experimental absorption. As with the other enzymes, these activities were converted into the equivalent mg of a standard alpha amylase preparation which would yield the same change in absorption.

3. Dehydrogenase Activity: Was measured fluorometrically using resazurin as the indicator. This assay is based on the conversion of the nonfluorescent resazurin to the highly fluorescent resorufin. The rate of change in fluorescence (ΔF/min) is then used as a measure of enzyme activity.

4. Lipase Activity: Was measured by the interaction of an enzyme source with the nonfluorescent substrate fluorescein dibutyrate. The rate of formation (ΔF/min) of the fluorescent product fluorescein is then converted into the equivalent mg of lipase steapsin required to yield the same enzyme activity.

5. Esterase Activity: Is measured using the very non-specific substrate indoxyl acetate. This nonfluorescent compound is hydrolyzed by esterase into highly fluorescent indoxyl. The rate of indoxyl formed (ΔF/min) is then converted into the equivalent mg of acetylcholinesterase required to yield the same fluorometric response.

6. RNA and DNA: Analyses were performed via the now classical colorimetric tests employing orcinol and diphenylamine as the color reagents.

7. Total Protein: Was determined via colorimetric Biuret test. By comparing the samples absorption at 540 mμ with the absorption by known quantities of bovine serum albumin, the results were readily converted to equivalent mg protein per ml sample.

Experimental Laboratory Studies

1. Determination Optimal Source. Authorization to obtain sludge samples was secured from the Leominster, Massachusetts, Plant. It was found advantageous to limit our initial concern to determining which of five strategically located sources within the plant would yield preparation of optimal enzymatic

activity. A diagramatic representation of the Leominster plant is depicted in Figure 1. Since biological variation is inherently severe during in-plant processing, it was deemed useless to define "daily" enzymatic activity. Theoretically it would be expected that aeration (which activates the sludge - Tank I) and exposure to sewage (which could potentially induce synthesis of specific enzymes - Tanks II, III and IV) might produce a source of increased activity. On this basis, probably only source V would prove less active enzymologically. However, preliminary determinations of dehydrogenase, amylytic and proteolytic activity (Figure 2) demonstrated that none of the five sources appeared to exhibit significantly less activity when compared to the other four. On the basis of these results, it was arbitrarily decided that future experiments would be conducted on the activated sludge obtained from Tank I. This source represents sludge derived from the final settling tank and which, through a process of vigorous aeration, is activated prior to recycling through the inlet sequence.

2. Preliminary Fractionation: Membrane Disruption Techniques. Our concern was now focused on determining the method whereby cell disruption was most efficiently achieved. Using amylytic and proteolytic activity as tracers, it was found (Figure 3) that sonication yielded a much higher release of enzymatic activity into the supernatant fraction than did either freeze-thaw or homogenization techniques. Dehydrogenase activity was lost by all cell disruption procedures. Although pressure bomb disruption would theoretically enhance the yield, we were unable at this time to procure the instrument from commerical sources.

3. Preliminary Fractionation: Storage Techniques. It was important to determine early in the program adequate means for storing the enzymatically active preparations derived from the original activated sludge source. Of the methods examined, freezing represented the simplest and fastest means of maintaining activity. In Figure 4, the 48 hours frozen sonicate exhibited complete retention of activity. Indeed, subsequent work has demonstrated stability after over 12 weeks of storage at $0^{o}C$. Lyophilization is well documented for its gentleness toward enzymatically active preparations and has the additional advantage of offering an opportunity to concomitantly concentrate activity. As might be predicted, on this basis, lyophilization did indeed prove to be an effective storage technique. Acetone powder preparation was found to have a detrimental effect upon amylytic activity and was, therefore, discarded from use in future protocols.

4. Preliminary Fractionation - Partial Enrichment Studies. The three fractionation steps proposed represent the simplest and most basic employed by investigators of microbial enzyme purification. Protamine sulfate precipitation is specifically used to remove nucleic acid contaminants from the remaining soluble components. The latter have been shown to interfere with many enzyme assay systems and, in any event, would not contribute to total biochemical activity during future immobilization attempts. Dilute acetic acid precipitation will fractionate on the basis of enzyme isoelectric points via variations in pH. Ammonium sulfate precipitation is perhaps the most widely applied of the fractionation techniques investigated, but entails a lengthly time lag to permit extensive dialysis of the preparation prior to enzymatic analysis. Ammonium sulfate fractionates proteins on the basis of their isoelectric point as determined by variations in ionic strength.

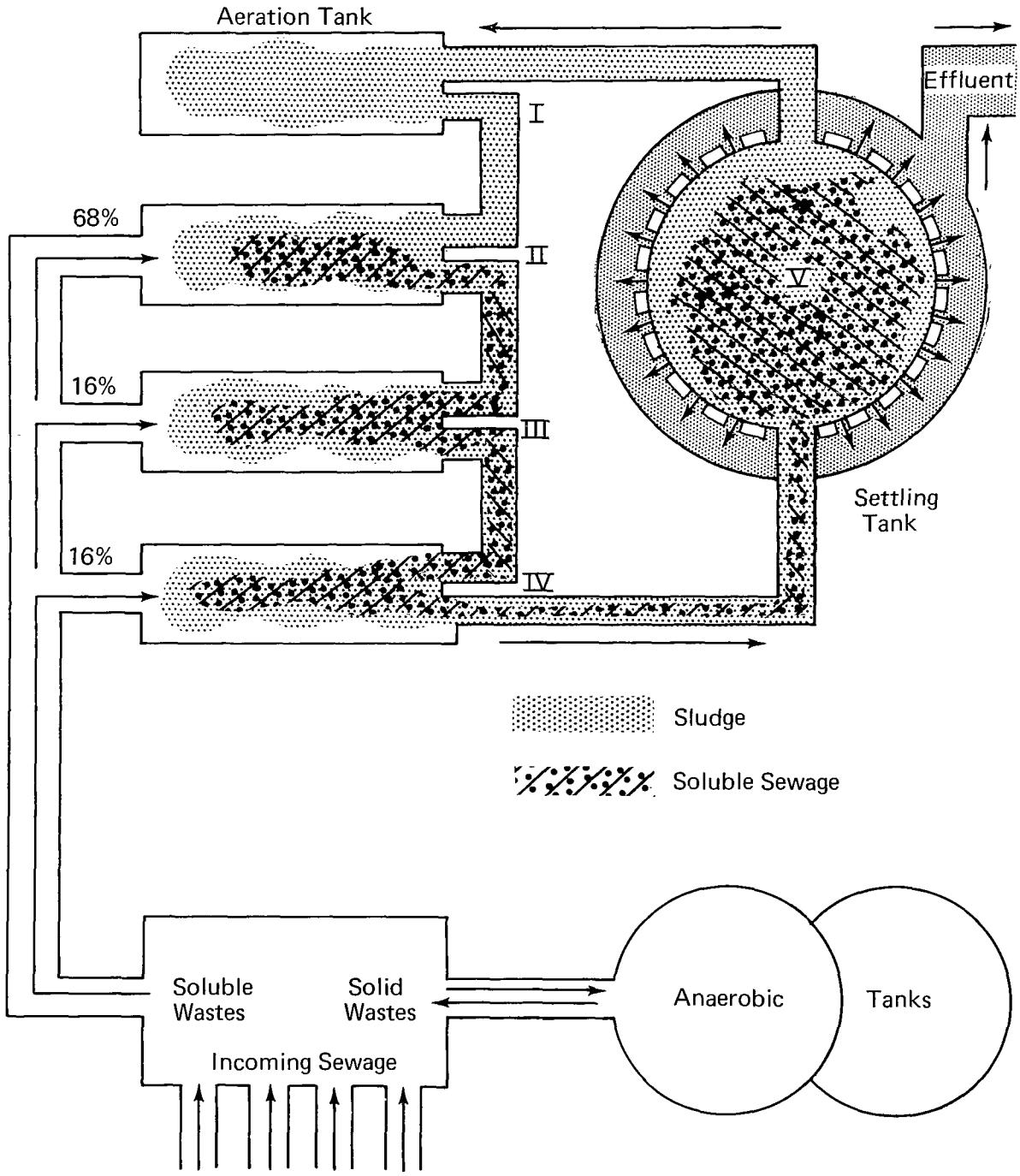
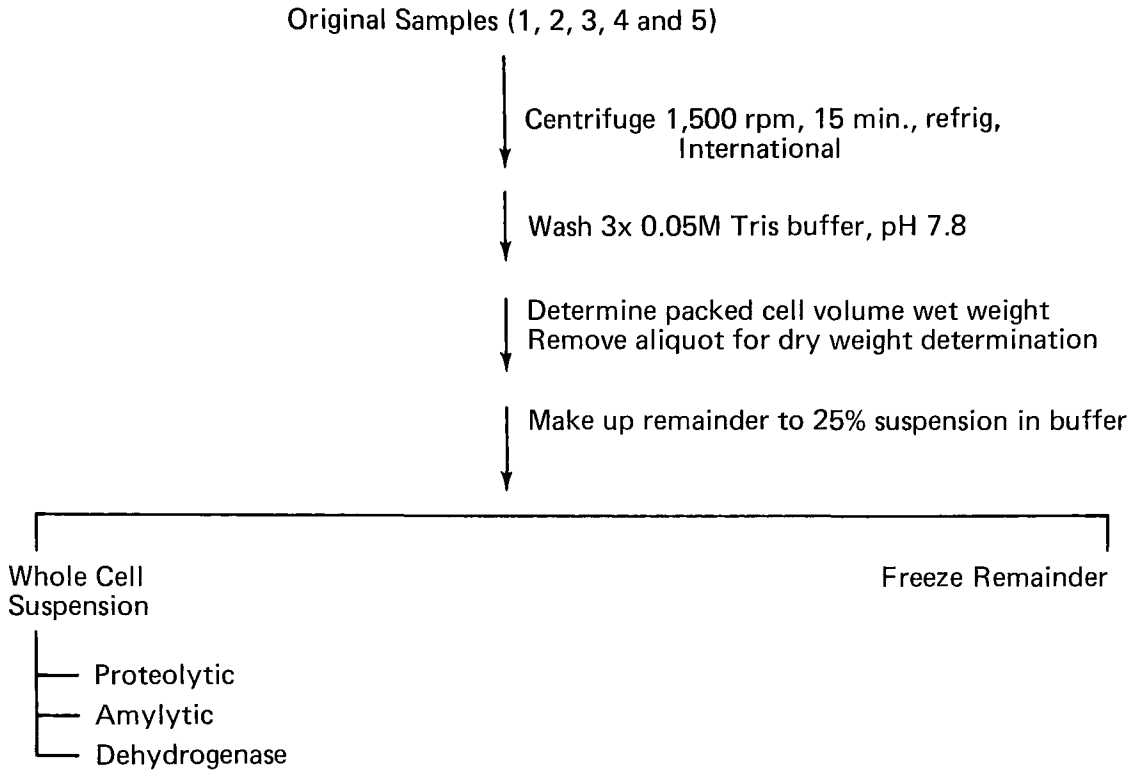


Figure 1. Leominster Plant



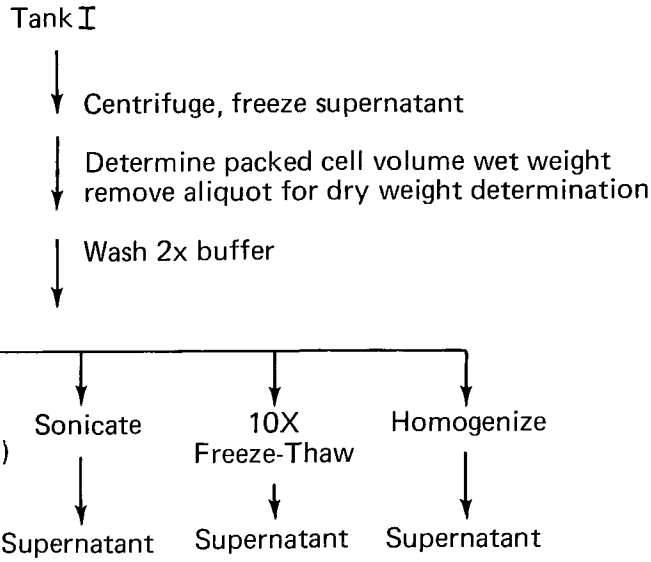
Description Whole Cells	Dehyrogenase Activity**	Amylase Activity***	Proteolytic Activity***
(1 ml 25% suspension)			
Tank I Derivative	0.323	0.119	0.161
Tank II Derivative	0.309	0.125	0.153
Tank III Derivative	0.352	*	0.135
Tank IV Derivative	0.298	*	0.157
Tank V Derivative	0.317	0.110	0.127

*Not Assayed

**Absorption values at 430 mμ

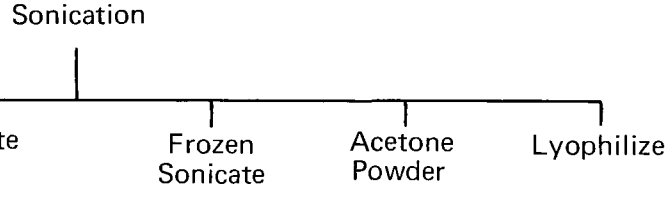
***Absorption values at 535 mμ

Figure 2. Determination of Optimal Source of Bacterial Sludge



Protease abs 0.595 mμ	0.377	0.398	0.109	0.117
Amylase abs 0.595 mμ	0.234	0.209	0.043	0.123

Figure 3 Preliminary Fractionation: Membrane Disruption Techniques



Protease*	3.25	3.09	3.16	4.12
	9.75	9.68	1.99	9.21

*abs 595 mμ/mg protein/hr

Figure 4. Preliminary Fractionation: Storage Techniques

Preliminary experiments indicated that the enzymatic activity of the acetic acid supernatant fraction exhibited maintenance of proteolytic and amylytic activity. Protamine sulfate precipitation encountered some problems due to the difficulty in determining the end point for nucleic acid removal. The addition of excess protamine sulfate to the suspension subsequently led to apparent loss in enzyme activity in the supernatant fraction. Ammonium sulfate precipitation similarly appeared to have a negative effect on amylytic activity although the precipitate demonstrated a 2-3x enrichment of proteolytic activity.

These preliminary results had several important ramifications upon the general investigational protocol. First of all, the already apparent efficiency of the fractionation systems selected obviously required the monitoring of additional enzymes to assure optimal maintenance of the hydrolytic bacterial enzymes of interest. Thus, both a general esterase and lipase assay were incorporated into the enzyme monitoring system. Secondly, it became apparent that further attempts at enrichment would entail the inevitable loss of component enzymes among the widely diverse catabolic enzymes derived from activated sludge. As a result of these conclusions, a series of experiments were performed to verify and improve upon the applicability of the aforementioned fractionation techniques. In Table I, a summary of the data obtained from these experiments is presented.

From these results, the decision was made to use a protamine sulfate precipitation followed by a dilute acid precipitation for the preparation of the sonicate derivatives to be used in immobilization. However, it was found that incomplete nucleic acid precipitation was obtained in fractions #1 and #2, and therefore, a second protamine sulfate precipitation was done on these two derivatives of the acetic acid fractionation. Subsequently, the preparation of large scale quantities of the soluble enzymatic derivatives was completed according to these procedures and a chart detailing the data obtained via the enzyme assay monitoring system is shown in Table II. The three final fractions (Protamine Sulfate supernatant Ib, Protamine Sulfate supernatant 2b, and Acetic Acid supernatant #3) were than combined (Mixed Supernatant Fraction) and tested to determine the biochemical characteristics of the final product. In Table III, a summary of these biochemical parameters for both the original sonicate and the final combined sludge derivatives is presented.

5. Immbolization in Polyacrylamide Gels. Initial attempts at immobilization of enzymatically active preparations according to the procedure of Updike and Hicks (Ref 10 and 11) yielded a very unsatisfactory product. Thus, experiments were designed to determine the co-polymer concentrations which would yield both maximal retention of enzymatic activity and optimal gel consistency for our particular application. The basic components of the polymerization system are as follows: 40% solution of acrylamide, 2.3% solution of N, N-Methylene-bisacrylamide (MBA) and solutions of riboflavin and potassium persulfate such that a 0.1 ml contained 0.03 mg. In all these solutions 0.1 M phosphate buffer, pH 7.4 was used as the solvent. The preparative protocol entailed the bubbling of nitrogen gas separately through the previously measured copolymer component for a minimum of fifteen minutes to remove any traces of dissolved oxygen. The latter solutions are then combined and both riboflavin and persulfate are added in the stipulated catalytic amounts. After thorough mixing, the enzyme preparation is added to the mixture, stirred and placed in an ice bath.

TABLE I. FRACTIONATION TECHNIQUES
(25% WHOLE CELL SUSPENSION - 11/10/69)

ACTIVITY	Sonicate						
	Sonicate	Protamine Sulfate Precipitation		Acetic Acid Precipitation		Ammonium Sulfate Precipitation	
		super.	ppt	super.	ppt	super.	ppt
Proteolytic							
Total Enzyme Units	5,340	5,400	*	5,850	330	840	3,260
Sp Activity	3.12	3.26	*	4.31	0.62	1.43	9.30
Amylase							
Total Enzyme Units	112.0	111.0	*	102.0	8.0	12.0	15.0
Sp Activity ($x10^{-2}$)	6.6	6.7	*	7.5	1.5	2.0	4.3
Esterase							
Total Enzyme Units	46.8	38.6	1.5	41.6	1.4	32.6	14.1
Sp Activity ($x10^{-2}$)	2.7	2.3	1.5	3.1	0.3	5.5	4.1
Lipase							
Total Enzyme Units	22.6	19.1	none	21.9	2.1	none	7.20
Sp Activity ($x10^{-2}$)	1.32	1.16	none	1.61	0.40	none	2.06
Total Protein	1,710	1,650	100	1,360	530	590	350

*Not Assayed

1. Enzyme Units:
   - Proteolytic -- equivalent to mg proteinase/ml/30 min
   - Amylase -- Equivalent to mg alpha amylase/ml/30 min
   - Esterase -- equivalent to mg acetylcholinesterase/ml/min
   - Lipase -- equivalent to mg lipase (steapsin)/ml/min
2. Specific Activity: equivalent enzyme units/mg protein

TABLE II. VALUES OBTAINED WITH SPOT MONITORING DURING FRACTIONATION OF 1/19/70 SONICATE

Sonicate (1-19-70)

proteolytic	7.00 mg proteinase
amylytic	0.300 mg alpha amylase
esterase	0.376 mg acetylcholinesterase
lipase	0.298 mg lipase (steapsin)

Protamine Sulfate Fractionation

Supernatant		Precipitate	
#1	Prot - 8.81 (8.63) Amyl- 0.201 (0.185) Ester 0.278 (0.366) Lip - 0.250 (0.292)	#1	Ester - 0.127 (0.151) Lip - 0.069 (0.084)
#2	Prot 8.48 (8.63) Amyl 0.160 (0.185) Ester 0.439 (0.366) Lip - 0.335 (0.292)	#2	Ester - 0.122 (0.151) Lip - 0.091 (0.084)
#3	Prot - 8.60 (8.63) Amyl - 0.195 (0.185) Ester - 0.374 (.366) Lip - 0.247 (0.292)	#3	Ester - 0.203 (0.151) Lip - 0.122 (0.084)

Acetic Acid Precipitation

Precipitate		Supernatant		2nd Protamine Sulfate PPT. (Super)	
#1	Ester -* Lip -*	#1	Prot - 5.88 (7.09) Amyl - 0.195 (0.153) Ester - 0.229 (0.230) Lip - 0.242 (0.241)	#1b	Prot - 6.52 (7.00) Amyl - 0.175 (0.144) Ester - 0.238 Lip - 0.235
				Protamine Sulfate #2 (Super)	
#2	Ester - 0.087 (0.105) Lip - 0.257 (0.350)	#2	Prot - 8.22 (7.09) Amyl - 0.155 (0.153) Ester - 0.271 (0.271) Lip - 0.242 (0.242)	#2b	Prot -7.28 (7.00) Amyl -0.136 (0.144) Ester -0.220 Lip -0.232
		#3	Prot - 7.19 (7.00) Amyl - 0.120 (0.144) Ester - 0.189 (0.230) Lip - 0.239 (0.241)		

* not assayed

Note: The numbers in parenthesis represent the average values obtained by the three different samples within the same fractionation step.

TABLE III. COMPARATIVE BIOCHEMISTRY OF THE SONICATE AND THE MIXED SUPERNATANT FRACTION

Enzyme Activity*	Sonicate	Mixed Fraction
Proteolytic Activity		
1. Equivalent mg Proteinase/hr	6.46	5.76
2. Specific Activity	1.46	2.11
Amylase Activity		
1. Equivalent mg Amylase/hr	0.331	0.295
2. Specific Activity	7.5×10^{-2}	10.8×10^{-2}
Lipase		
1. Equivalent mg Lipase (Steapsin)	0.343	0.268
2. Specific Activity	7.8×10^{-2}	9.9×10^{-2}
Total Protein/ml	4.41 mg	2.73 mg
Total Nucleic Acid/ml	38.3 mg	14.5 mg

*Numerical values represent the equivalent milligrams of a reference standard proteinase, and amylase, acetylcholinesterase and steapsin lipase required to obtain the enzymatic activity exhibited by each of the various fractions.

The area directly in front of the preparative flask is cleared of ice and the vessel moved as close as possible to the glass wall of the ice bath. A number 2 photo flood lamp is placed directly opposite the preparative flask and functions as the light source for the photocatalytic initiation of polymerization. During the immobilization process nitrogen is gently blown down on the experimental mixtures in order to both exclude oxygen from the system and to provide for slight agitation of the polymerization components. The latter requirement, in direct contrast to the procedure of Bernfeld (Ref 12 and 13) was found essential to obtaining a homogeneous consistency within the individual polyacrylamide gel preparations. During the procedure the temperature of the bath was also carefully maintained at 0-4°C in order to prevent possible thermal denaturation of the constitutent enzymes (Ref 14). Initially the resulting block of polymerized enzyme-gel was mechanically dispersed into particles by extrusion through a syringe. However, this procedure soon proved feasible only for the less firm gel produced in the presence of high concentrations of either sludge derivatives or MBA. The optimal gel consistency eventually selected required the use of

sieving devices (mesh screen or modified garlic press) to achieve the force necessary to obtain satisfactory particles. The particles prepared by the latter procedures were then thoroughly washed with liter quantities of fresh buffer and stored at 0-4°C prior to enzymatic analysis.

A summary of the results observed during experimental variation in co-polymer concentrations is shown in Table IV. No significant change in enzymatic activity was apparent once a homogeneous consistency was obtained (Sample 2-5). However, both the time required for the reaction to go to completion and the characteristic integrity of the gel (as measured by its ability to resist externally applied force) changed drastically as a function of co-polymer concentration (Ref 15). In addition, it was found in subsequent experiments that increasing the total reaction volume resulted in disproportionate increase in the required reaction time. Although the time lag had no visible effect on the resulting gel product characteristics, it was found to have drastically reduced recoverable enzyme activity (for example, 6.2 ml total volume-6 min. and 20% recoverable enzyme activity; 62.0 ml total volume-2.5 hours and less than 3% recoverable enzyme activity.)

It must be noted, however, that a more detailed investigation of possible modifications in batch preparation would probably permit large-scale gel synthesis feasibility. However, on the basis of the available data a proportion of 4 ml acrylamide and 1 ml MBA was selected as providing maximal firmness while still permitting division of the gel into small bead-like particles. Similarly, a maximum volume of 30 ml/preparation was selected as providing the optimal balance between needs for large scale preparation vs minimizing polymerization time.

Immobilizations of commercially obtained proteinase, acetylcholinesterase and lipase were initially done to affirm that these enzymes would indeed withstand the polymerization reaction. Subsequently, an attempt was made to immobilize an aliquot of sonicate, but no detectable enzyme activity was observed. However, upon concentrating the sonicate via resuspension of a lyophilized preparation, all four enzymatic parameters exhibited partial retention of activity. In Table V is found a summary of these initial results. Caution must be observed in interpreting these results since they represent only preliminary testing in which the emphasis was on the presence vs absence of enzyme activity rather than precise quantitation of the retention. However, it was established that: (1) immobilization of the activated sludge derivatives was indeed feasible; (2) that the gels were capable of retaining activity for at least three weeks at 4°C; and (3) that repetitive use of the same gel fraction exhibited continued enzymatic activity.

It was at this point that the two major phases of the preparative program met. The preparation of the soluble fraction derived from activated sludge and possessing optimal catabolic enzyme activity (Table III) was essentially completed, and it remained to be determined the degree of concentration necessary to obtain optimal recovery of enzymatic activity in the immobilized form. In essence, this consisted of lyophilizing a sample of the fractionated sonicate, resuspending at a concentration of about 50 mg protein/ml and making several dilutions

TABLE IV. VARIATION IN CO-POLYMER CONCENTRATION IN POLY-ACRYLAMIDE GEL PREPARATIONS

Sample No.*	ml Acrylamide	ml MBA	Approx Reaction Time	Gel Description	Proteolytic Activity Absorption 595 mμ
1	1	4	35 min	opaque, curds & whey consistency	0.197
2	2	3	25 min	opaque, junket-like consistency	0.284
3	3	2	15 min	transparent, orange jello consistency	0.264
4	4	1	10 min	transparent, orange firmer	0.273
5	4.8	0.2	5 min	transparent, orange rubber-like firmness	0.258
6	5 ml tris buffer		———	———	1.056

* All samples also contained 0.1 ml riboflavin and persulfate catalysts and 1 ml proteinase (20 mg/ml).

TABLE V. IMMOBILIZATION OF RESUSPENDED LYOPHILIZED SONICATE 11/9/69

Enzyme Units/ml	Sonicate	Immob Gel	Est % Recovery
Protease	1.062	0.186	17.5
Amylase	2.55	0.320	12.6
Esterase	0.25	0.01	4.0
Lipase	1.04	0.07	6.8

through 5 mg/ml and immobilizing each concentration. The gels were then compared on the basis of total activity in terms of the lipase and esterase assays, required time, and the final characteristic gel consistency.

It was predicted that, the rate of increase in enzymatic activity corresponding to increasing higher concentration of total enzyme immobilized would eventually reach a point of diminishing returns. Although this prediction might yet be valid, it was found that the physical characteristics of the gel itself proved to be the limiting factor. In Table VI the results of this experimental series are presented. At concentrations greater than approximately 12 mg of protein/ml (3x concentration of the mixed supernatant fractions) the consistency of the gel became increasingly viscous. At maximum concentrations, the gel had become so amorphous as to be resistant to breakdown into the desired particles and exhibited a high degree of adhesion for almost any surface. Also in Table VI the results of this experiment are summarized. It will be noted that with increasing enzyme concentration used for immobilization a corresponding increase in enzyme activity was indeed observed.

The experimental protocol also included an analysis of the protein content of the initial 10 ml wash of the gel particles containing immobilized enzymes. It was of interest to note that none of these five concentrations tested exhibited any release of protein into the wash water. In one of Bernfelds most recent papers (Ref 12), he has used a radioactive enzyme sample to verify that in this system all activity also remained associated with the polymerized gel. Bernfeld (Ref 16 and 17) has also reported in his original article that no leakage was detected into the buffer during storage of his immobilized gel. However, Kalchalski's review article (Ref 18) cites Bernfeld as reporting a continual low level of leakage. In any event, several spot checks of the buffers in which the immobilized sludge derivatives were stored revealed no leakage of protein within the sensitivity of the Biuret test. Immobilization of protein components within the gel matrix appears to be complete within the concentration ranges examined. Consequently, any loss of enzymatic activity is directly attributable to some aspect of the polymerization process rather than to a non-specific removal of unbound protein by multiple washings.

In addition, a series of experiments was done in an attempt to further delineate the causative factors in the formation of the heterogenous viscous gel occuring when high concentrations of the mixed supernatant fraction were used. The first of these entailed varying the co-polymer concentrations in a similar protocol as that described for Table IV. However, it was found that the characteristics of the various proportions described in Table IV were again reproduced but this time in combination with the constant viscous-gel type elicited by the use of the concentrated enzyme preparation. A highly concentrated sample of bovine serum albumin (75 mg/ml) was also subjected to immobilization and yielded a very firm clear gel. On the basis of these results it can be assumed that the difficulty experienced was not simply due to a need for different co-polymer proportions or too high a protein concentration. It appears, therefore, that the unknown causative agents were present in the fractionated derivatives of activated sludge.

TABLE VI. DETERMINATION OF CONCENTRATION OF MIXED SUPERNATANT DERIVATIVE YIELDING OPTIMAL RETENTION OF IMMOBILIZED ENZYME ACTIVITY

Description	mg protein per ml of enzyme source	Esterase Activity	Lipase Activity	Physical Characteristics of Gel
Lyophilized Concentration	61.2	330/ml	278/ml	
Dilution A	36.2	197/ml	180/ml	
Dilution B	27.6	113/ml	406/ml	
Dilution C	9.72	58.8/ml	52.0/ml	
Original Mixed Supernatants	2.85	41.2/ml	42.0/ml	
Gel Derivative of Concentrate		6.62/gm	6.00/gm	Viscous, sticky Heterogeneous
Gel Derivative of A		3.01/gm	2.56/gm	Viscous, sticky
Gel Derivative of B		1.59/gm	1.71/gm	Slightly less Viscous
Gel Derivative of C		1.33/gm	2.82/gm	Homogeneous Gels semi-firm
Gel Derivative of Original Mixed Supernatant		0.90/gm	1.62/gm	Characteristics Firm, Clear Gel

On the basis of these results, the mixed supernatant fraction was lyophilized and adjusted to final concentration of 12 mg protein/ml. The latter preparation was then used for immobilization according to the proportions 5 ml enzyme preparation, 5 ml MBA, 20 ml acrylamide, 0.5 ml persulfate and 1.0 ml riboflavin. As previously stated, attempts to increase the reaction volume beyond 30 ml had entailed a disproportional increase in the time required to complete polymerization and an observed loss in recoverable enzyme activity.

Using the above proportions for polymerization a total volume of 1 1/2 liters of immobilized enzyme gel was prepared over a period of 9 days. In addition, two different control polyacrylamide gels were prepared. The first of these consisted of the standard polymerization components to which distilled water was added instead of the enzyme source. The second control preparation entailed the substitution of bovine serum albumin (BSA, 12 mg/ml) for the enzymatic component. It is important to note that an absolutely valid control for the enzymatically active immobilized gel is extremely difficult to obtain. The boiling of a gel to destroy enzymatic activity presents two potential disadvantages. First of all the structural integrity of the gel itself may be altered and secondly, a structural alteration of the enzymes (denaturization) definitely occurs. The potential ramifications include loss of small (or even large) molecular weight components into the system, or conversely a possible increased absorption of soluble components from the assay system. The acrylamide gel alone can show possible non-enzymatic effects that the polyacrylamide has on the system. However, since the polymerization system lacks the "structuring effect" mediated by the sludge derivative, the gel type is not necessarily identical to that which entrapped enzymes. Similarly, the entrapped BSA can potentially serve to mimic an immobilized protein without enzymatic activity. On the other hand it also lacks the ability of the enzyme derivative to vastly effect gel structure.

At the completion of these preparations, the gels were stored at 4^{o}C for an additional two weeks. During this time various biochemical assays were performed on the immobilized derivatives of activated sludge. The assays used were the standard esterase and liapse fluorometric assays, the casein test for proteolytic activity, and the starch-iodine test for amylase activity. In both the fluorometric and the amylytic assays the control used was a heat-inactivated sample of the immobilized enzyme gel. The latter was prepared by subjecting the gel to a boiling water bath for 20 minutes after which the gel was thoroughly washed to remove any components which might have initially been solubilized by this procedure. The caseinolytic assay provides for the control to consist of incubation of substrate and enzyme source separately and then combination of the two components in the presence of 10% trichloracetic acid (TCA). All assays also include a calibration curve for a commercially obtained enzyme source, and the gel activity was therefore computed in terms of activity relative to that of the standard enzyme. And finally, a preliminary "interference-curve" was also obtained to determine the effect (i.e., quenching of fluorescence, non-specific absorption of substrate or product, etc.) that the gel itself might have upon the particular assay system. The effect of the gel was determined by combining a constant concentration of the commercial enzyme with an assay system to which varying amounts (gms) of gel were also added. The fluorescence of absorption between the system to which no gel was added and those with increasing quantities of gel were then compared. The data which was obtained enabled the investigator to correct upwards the experimental values obtained for the enzymatic activity of the immobilized sludge derivative. An example of the effect of correcting for interferences is found in Figure 5.

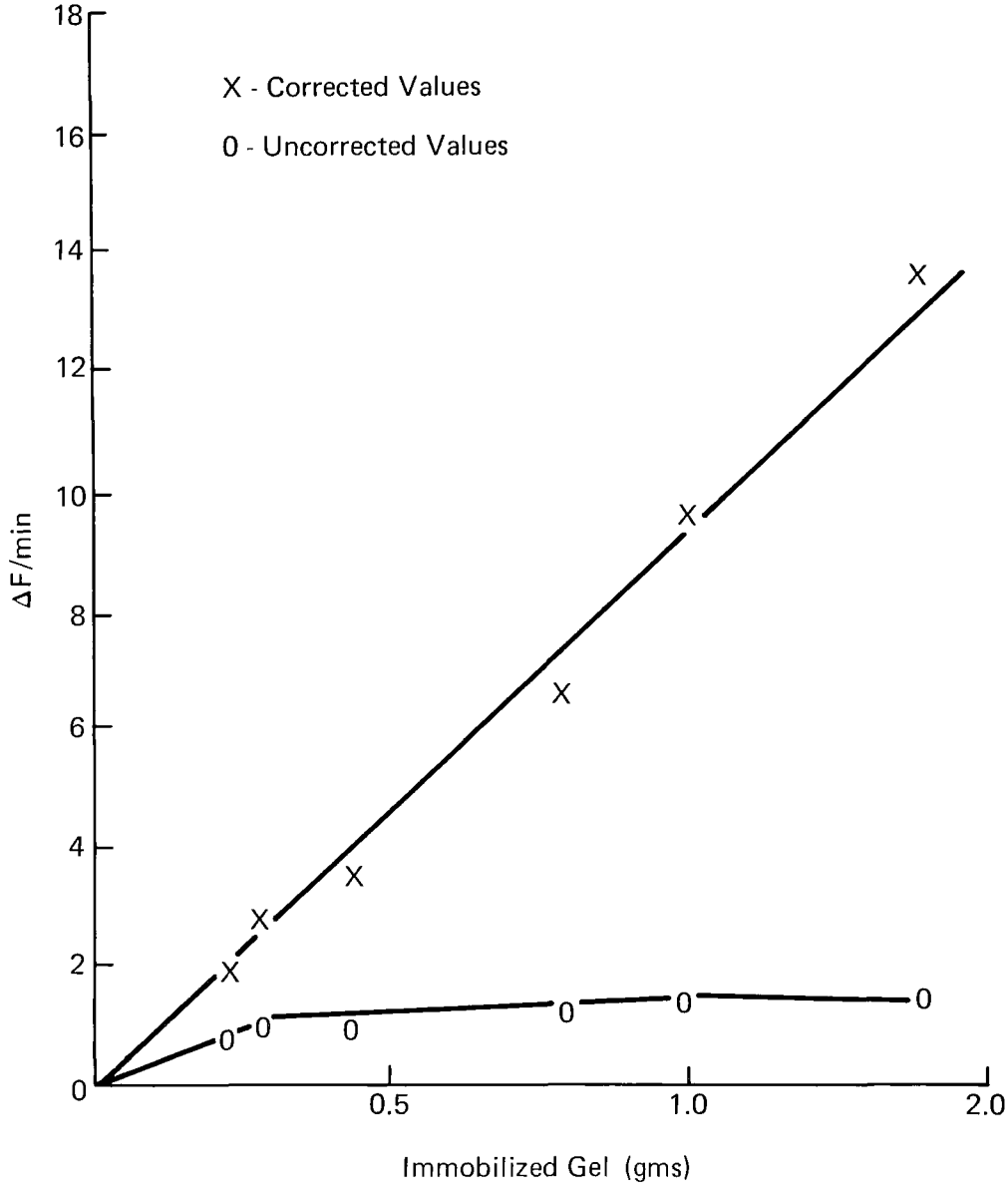


Figure 5. The Effect of Correcting for Non-Specific Interference Upon Detection of Esterase Activity Immobilized in Polyacrylamide Gels

However, it must be emphasized that even with this correction for interference one cannot directly equate the activity of a soluble system with that exhibited by the immobilized polyacrylamide gel. The inherent problem this system presents is one of access. The gel matrix presents an obstacle across which potential substrate molecules must pass. The difficulty with which passage is accomplished is primarily a function of the degree of cross-linking between the two copolymers and as previously stated is also tremendously influenced by the components of the preparation immobilized within the polyacrylamide gel. However, the further delineation of the effects of these factors and of their combinations upon accessibility represents a major investigational problem in itself, and is, therefore, far beyond the scope of this study.

With these reservations in mind, Table VII presents a summary of the results of experiments performed to determine the enzymatic activity retained by the gel. The activity of the gel was corrected for interference and then compared to the soluble system in order to determine an estimated percentage of activity retained. It is of interest that at least in Bernfelds (Ref 16) work only a 3-6% retention of enzyme activity among various enzymes was observed. The single disturbing factor is the low retention of proteolytic activity. In previous work this class of enzymes had proven resistant to any and all inactivational forces mediated by either fractionation or immobilization techniques. The investigation to determine rationale behind the observed loss in activity is beyond the immediate scope of this contract, and it is felt that it might prove to be an exception within the probable range of proteolytic enzyme recovery.

The final experiments conducted to complete this phase of the contract include analysis of a sample of the gel to determine the enzymatic response to repetitive use. In summary initial experiments using the lipase and esterase systems indicated that when the gel source was removed from a depleted assay system, thoroughly washed, and recombined with additional substrate, the maximal rate of enzymatic activity was once again observed. The immobilized gel samples were tested a total of 7 times within a two day period for these preliminary findings. In terms of storage, the enzymatic activity was retained for up to 5 weeks if the gel was maintained in a liquid (distilled water or buffer) environment and kept refrigerated. No attempts were made at this time to determine the effects of either lyophilization or freezing upon the enzyme activity or the physical integrity of the immobilized gel.

TABLE VII. COMPARATIVE SUMMARY OF ENZYME ACTIVITY RETAINED AFTER IMMOBILIZATION OF A POLYACRYLAMIDE GEL

Enzyme Activity	Commercial Enzyme Standard	mg Std Enzyme Per ml Lyophilized Prep*	mg Std Enzyme Per gm Immob Gel	Estimated Retention
Esterase	Acetylcholinesterase	0.0718	0.006	8.41%
Lipase	Lipase Steapsin	0.114	0.019	18.50%
Amylase	Alpha Amylase	0.173	0.010	5.78%
Proteolytic	Proteinase	0.912	0.008	0.88%

*The concentrated lyophilizate is diluted with buffer to duplicate the dilution to which it is subjected during the immobilization procedure.

PRELIMINARY BENCH-SCALE ACTIVATED SLUDGE INVESTIGATIONS

Construction and Operation of Apparatus

Completely mixed reactors, consisting of two ten gallon aquarium tanks for the Series 1 experiment and three ten gallon aquarium tanks for the Series 2 experiment; in which the liquid volume in each tank was maintained at 18.925 liters for Series 1 and 35 liters for Series 2. The reactor contents were mixed with 2-inch X 1-inch stirring paddles with a propeller on the end of the mixer shaft, driven at 150 rpm. Laboratory compressed air was introduced to the reactors at a rate of 1400 cc/min for Series 1 and 500 cc/min for Series 2 through porous stone diffusers after passing through an oil trap, an adsorber containing Purafil to remove any organics, a microbial filter, water scrubbers and rotameters.

A pictorial diagram of a typical reactor system is shown in Figure 6 (Series 2).

Substrate

The substrate for the laboratory experiments was raw sewage obtained from the diverter feeding Grumman's extended aeration sewage treatment plant. Raw sewage samples were obtained on different days for Series 1 and Series 2 experiments.

Activated Sludge

Activated sludge cultures were obtained directly from the return sludge line to the aeration tank in Grumman's extended aeration waste treatment plant. Activated sludge samples were obtained on different days for Series 1 and Series 2 experiments.

LABORATORY PROCEDURES AND ANALYSES

In the Series 1 experiment, 3.785 liters of sludge and 15.14 liters of raw sewage were added to each of the two tanks. A nylon stocking containing 400 ml of polyacrylamide gel was suspended in Tank I and a similar nylon stocking containing the immobilized enzymes in the polyacrylamide gel was suspended in Tank II.

The sludge and raw sewage were sampled both prior to and after being mixed. After suspending the nylon stockings in each tank, samples were taken every hour for 8 hours and at the end of 24, 28 and 32 hours. These samples were analyzed for chemical oxygen demand (COD) and suspended solids according to "Standard Methods". Soluble COD was used as the substrate parameter in these experiments. The soluble constituents were separated from cell material by centrifuging the mixed liquor sample at 15,000 rpm for 5 minutes and then analyzing the centrate for COD. Cell material in this study was determined as volatile suspended solids (VSS).

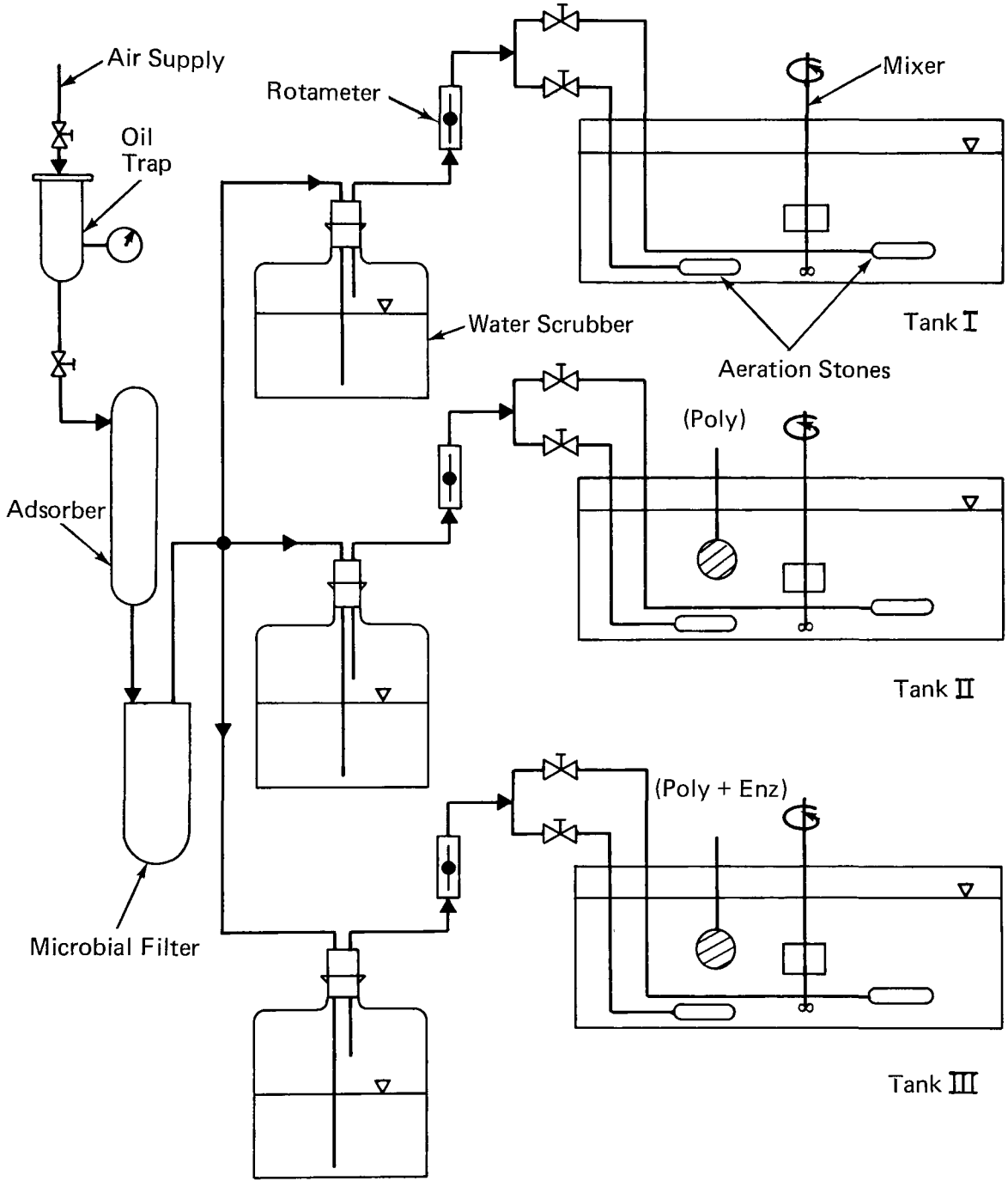


Figure 6. Series 2 Experimental Set-Up

A second laboratory scale study (Series 2) was conducted using three 10 gallon tanks equipped as in Series 1. The same polyacrylamides (with and without the enzymes) that were used in Series 1 were employed again because of insufficient time to acquire new supplies.

Seven liters of sludges and 28 liters of raw sewage were added to each of the three tanks. A nylon stocking containing 200 ml of the polyacrylamide gel with enzymes was suspended in Tank III, and a similar stocking containing 200 ml of polyacrylamide gel was suspended in Tank II. Tank I contained only the sludge and raw sewage.

Samples of the sludge, raw sewage and mixed liquor were obtained before suspending the nylon stockings. After suspending the nylon stockings containing polyacrylamide gel with and without enzymes, the three tanks were then sampled every hour for 8 hours and at the end of 25 hours and 31 hours. These samples were analyzed according to "Standard Methods" for pH, temperature, COD (soluble and mixed liquor), suspended solids and alkalinity.

Results

Series 1 experimental results are presented in Table VIII. Plots of COD and Volatile Suspended Solids (VSS) as a function of aeration time are shown in Figures 7 and 8. The difference in COD between the two tanks were noticeable and suspect since the COD of the tank containing polyacrylamide gel was steadily increasing over that of the tank containing polyacrylamide gel with enzymes. The polyacrylamide gel was removed from the tank and thoroughly rinsed with distilled water several times. The final rinse water was checked for soluble COD and found to exert a COD of approximately 15,000 ppm. Similar results were obtained from the polyacrylamide gel with enzymes. The cause of this could be due to the incomplete polymerization of the polyacrylamide gel thus allowing acrylamide to go into solution; increasing the COD and Volatile Suspended Solids. Since the samples were centrifuged at high speeds, it is unlikely that the high COD readings are due to gel in the sample tested.

It is also possible that the organic constituent, acrylamide may be resistant to biodegradation even though it is readily oxidized in the dichromate COD procedure. This material may not contribute measurably to the growth of the activated sludge organisms. There is an indication of this since the Volatile Suspended Solid did not appear to increase as might have been expected.

Series 2 experimental results are presented in Table IX. Plots of soluble COD, mixed liquor COD and Volatile suspended solids (VSS) are shown in Figure 9, 10 and 11.

The sludge had a low pH indicating that it was acidic. This condition tends to inhibit the biological activity of the sludge. Studies have shown that a sludge having a pH of 7.5 to 8.0 gives the best results in an activated sludge digestion system.

A comparison of the COD and VSS data from the three tanks is inconclusive as to the effect of immobilized enzyme on the biodegradation of sewage. The

erratic results obtained may be attributed in part to an improper activated sludge sample, incomplete polymerization of the polyacrylamide gel and possible denaturation or inhibition of the enzymes.

TABLE VIII. SERIES 1 EXPERIMENTAL RESULTS

Date	Time	Identification	COD (ppm)	Total Suspended Solids (ppm)	Volatile Suspended Solids (ppm) Bio-Mass	Fixed Suspended Solids (ppm)
4/8	0815	Sludge	106	3896	3504	392
		Raw Sewage I	267	468	436	32
		Raw Sewage II	288	428	400	28
		Mixed Liquor I	352	756	676	80
		Mixed Liquor II	338	796	700	96
	0915	Tank I (Poly)	354	792	680	112
		Tank II (Poly + Enz)	356	852	736	116
	1015	Tank I	348	772	672	100
		Tank II	327	756	672	84
	1115	Tank I	395	796	728	68
		Tank II	333	772	692	80
	1215	Tank I	426	764	696	68
		Tank II	300	716	684	32
	1315	Tank I	420	752	664	88
		Tank II	343	756	Lost	---
	1415	Tank I	441	784	712	72
		Tank II	331	732	668	64
	1515	Tank I	462	744	668	76
		Tank II	352	700	656	44
	1615	Tank I	472	752	688	64
		Tank II	320	700	668	32
4/9	0815	Tank I	644	728	708	20
		Tank II	370	680	652	28
	1215	Tank I	602	700	748	52
		Tank II	370	720	644	76
	1615	Tank I	544	652	612	40
		Tank II	814	704	672	32

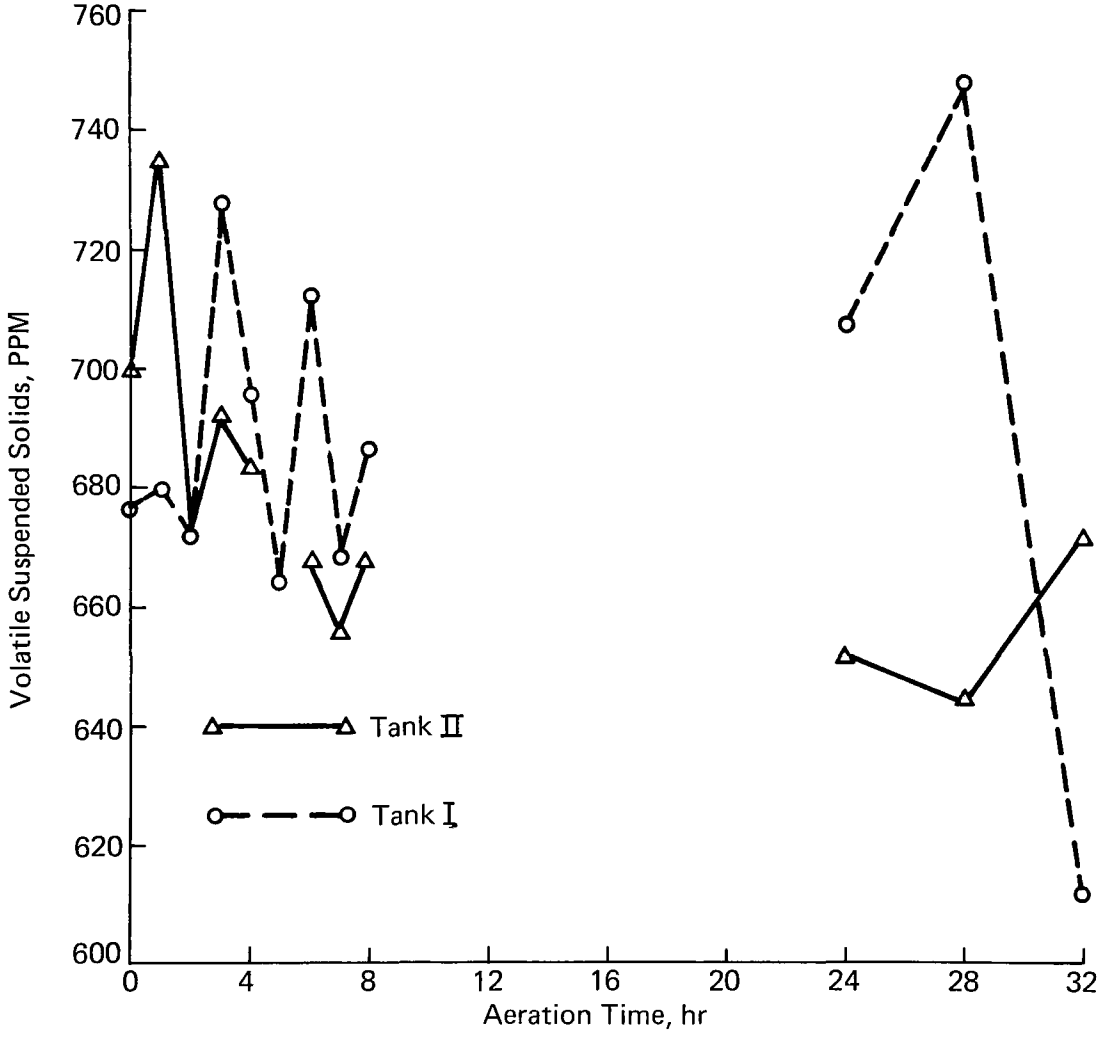


Figure 7. Series 1 Experimental Results, Volatile Suspended Solids vs Aeration Time

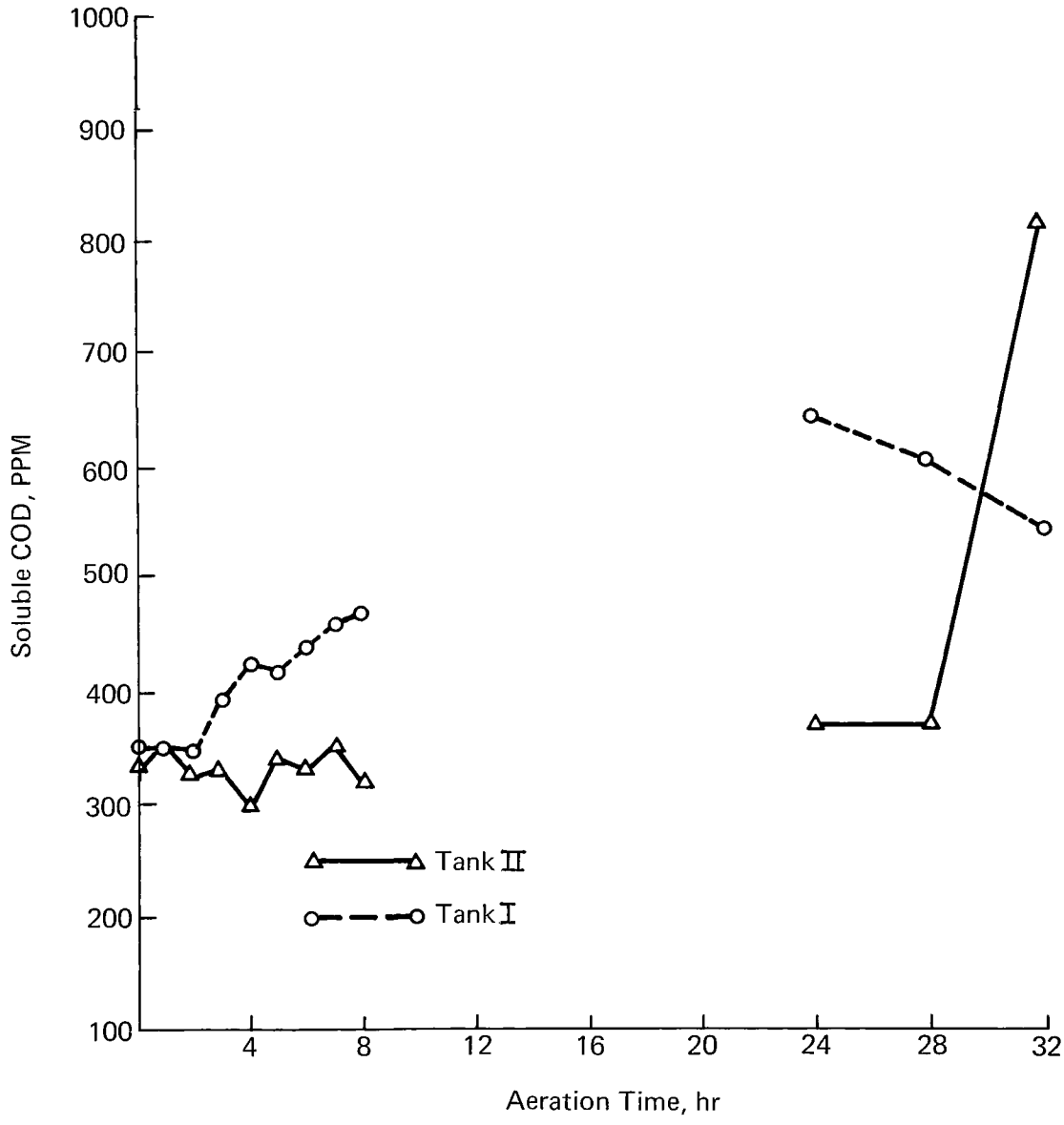


Figure 8. Series 1 Experimental Results, Soluble COD vs Aeration Time

TABLE IX. SERIES 2 EXPERIMENTAL RESULTS

Identification	Date	Time	pH	Alka-Linity (ppm Ca CO3)	Temp. (°C)	COD Super-Natant (ppm)	COD Mixed Liquor (ppm)	Suspended Solids (ppm)		
								Total	Volatile	Fixed
Sludge	4/21	0730	5.90	27.5		3760	8600	2952	2712	240
Raw Sewage I			7.75	212.8		168	500	280	244	36
Raw Sewage II			7.89	208.9		160	688	396	372	24
Raw Sewage III			7.72	209.5		176	500	256	224	32
Mixed Liquor I		0800	7.21	167.0		908	2280	1100	1024	76
Mixed Liquor II		0800	7.30	172.0		880	2372	908	824	84
Mixed Liquor III			7.25	163.4		992	2440	1300	1220	80
Tank I (Plain)		0815	7.21	169.4	23.4	872	2292	1128	1060	68
II (Poly)			7.33	168 1	23.2	900	2432	1008	960	48
III (Poly + Enz)			7.24	164.9	23.4	980	2420	1004	968	36
I		0915	7.21	172.1	23.4	840	2352	1068	1036	32
II			7.28	176.5	23.3	832	2320	964	908	56
III			7.25	168.3	23.5	960	2392	1012	936	76
I		1015	7.23	168.0	23.5	840	2352	1052	1032	20
II			7.28	174.4	23.5	852	2268	1084	1028	56
III			7.24	172.0	23.6	960	2328	1040	984	56
I		1115	7.24	171.1	23.7	852	2300	1260	1180	80
II			7.30	176.3	23.6	860	2288	1044	940	104
III			7.27	172.5	23.8	960	2368	1012	916	96
I		1215	7.27	177.6	23.9	840	2328	1104	988	116
II			7.30	171.5	23.8	852	2400	1176	1144	32
III			7.28	172.4	24.0	956	2328	1094	948	96
I		1315	7.23	174.6	24.0	840	2300	960	892	68
II			7.30	177.8	23.9	860	2408	1016	916	100
III			7.25	174.5	24.1	960	2408	944	856	88
I		1415	7.27	180.0	24.0	840	2308	976	900	76
II			7.31	180.0	23.9	860	2388	1084	984	100
III			7.31	176.6	24.1	920	2388	1012	908	104
I		1515	7.28	176.6	23.9	812	2308	1028	968	60
II			7.24	180.5	23.8	836	2280	1048	956	92
III			7.25	176.9	24.0	928	2448	992	940	52
I		1615	7.24	176.5	23.8	820	2212	984	908	76
II			7.27	182.5	23.6	820	2112	1260	1172	88
III	4/21		7.25	177.5	24.0	940	2304	988	892	96
I	4/22	0930	7.24	176.0		780	2212	996	908	88
II	4/22		7.28	183.2		840	2192	1008	892	116
III	4/22		7.26	178.0		820	2212	900	840	60
I	4/22	1530	7.21	182.9		748	2112	1188	1100	88
II	4/22	1530	7.14	181.2		800	2168	1016	920	96
III	4/22	1530	7.16	176.5		800	2300	1024	952	72

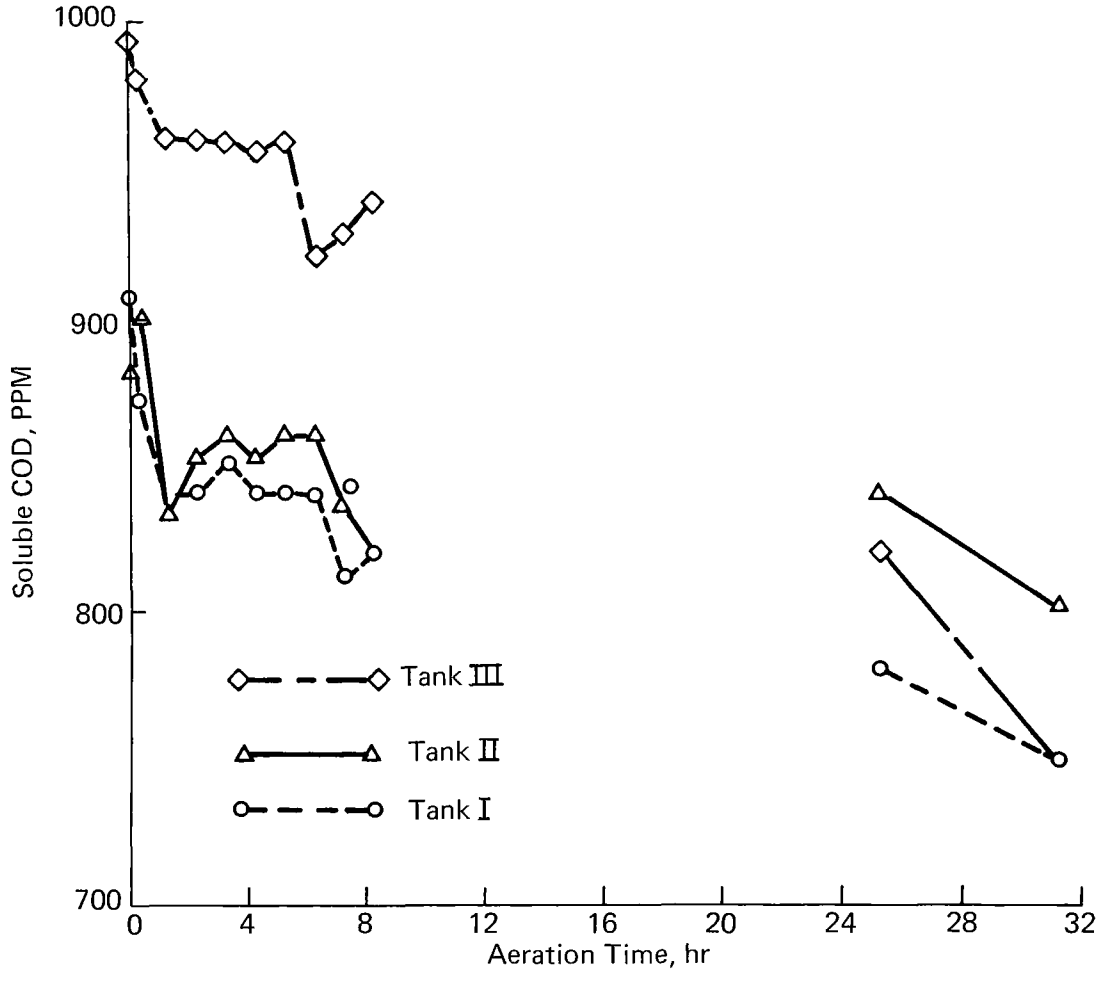


Figure 9. Series 2 Experimental Results, Soluble COD vs Aeration Time

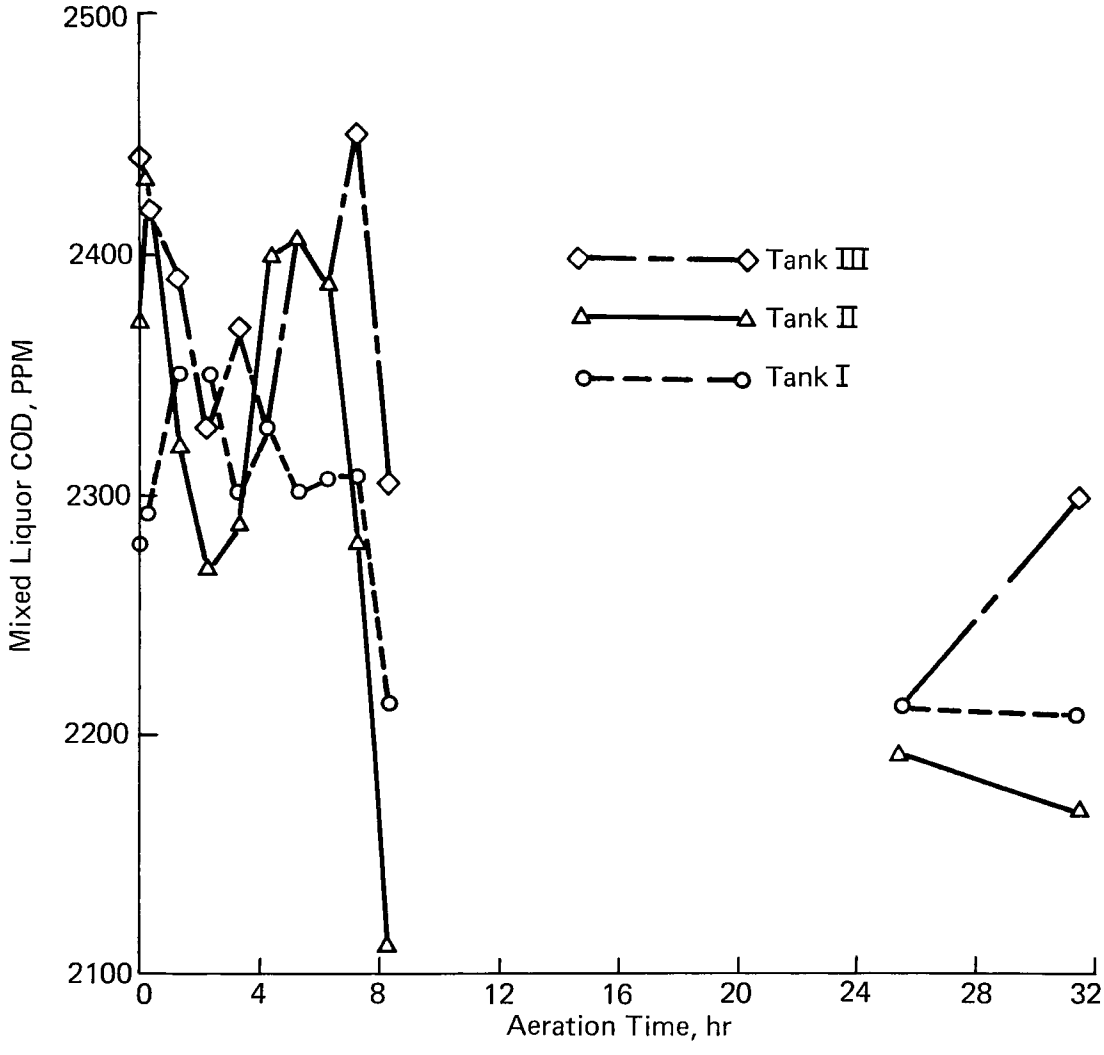


Figure 10. Series 2 Experimental Results, Mixed Liquor COD vs Aeration Time

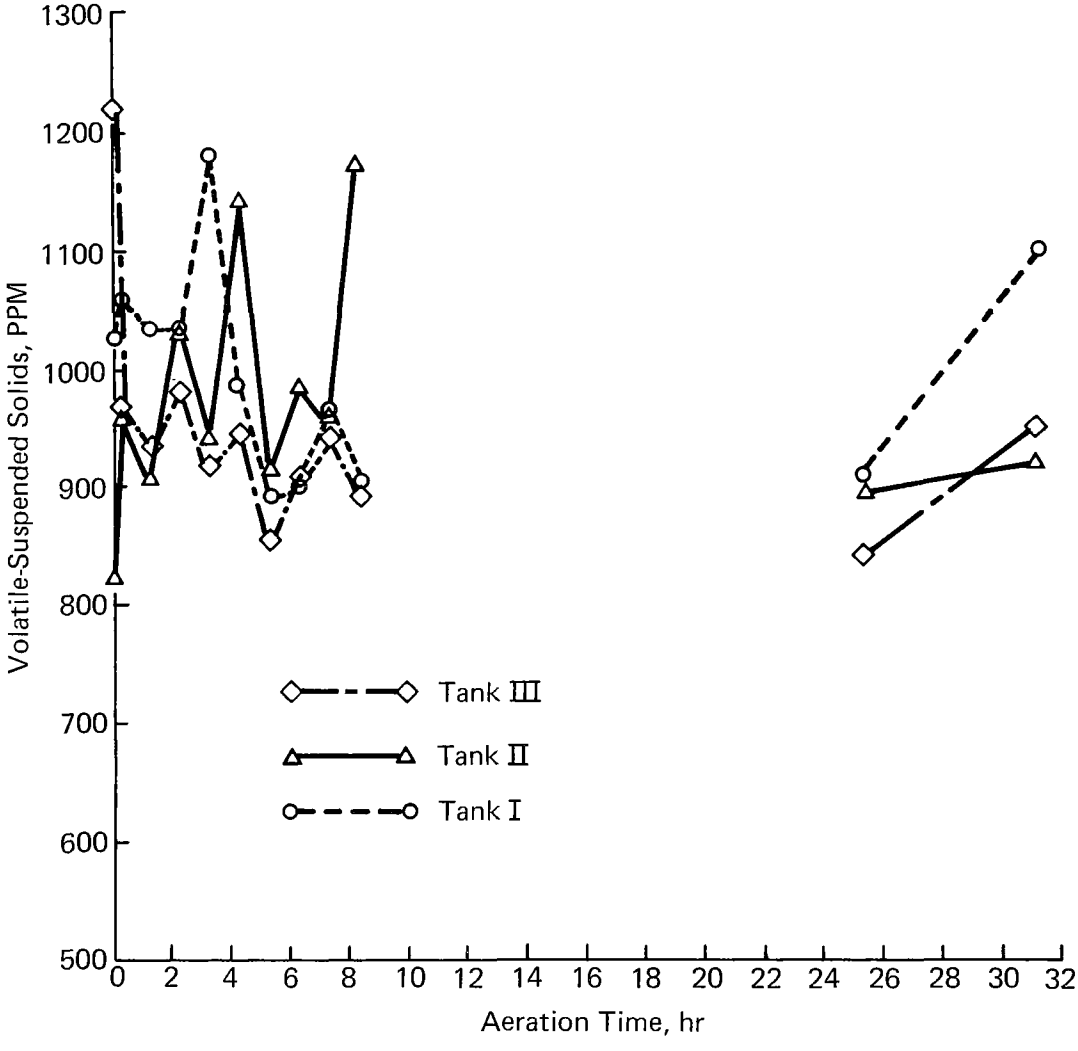


Figure 11. Series 2 Experimental Results, Volatile Suspended Solids vs Aeration Time

SECTION VI

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The overall project was under the direction of Dr. Lawrence Slote, Advanced Civil Systems of Grumman Aerospace Corporation.

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SECTION VII

REFERENCES

1. Krishnan, P. and Gaudy, A.F., "Mechanisms and Kinetics of Substrate Utilization at High Biological Solids Concentrations," Proceedings 21st Industrial Waste Conference, Purdue University Extension Series, 1966

2. Siddiqi, R.H., et. al., "The Role of Enzymes in the Contact Stabilization Process," The 3rd International Conference on Water Pollution Research II-15, Munich, Germany 1966

3. Chamberlin, N.S., "Action of Enzymes on Sewage Solids," New Jersey Agriculture Experimental Station Bull. 500, 1930

4. Heukelekian, H. and Berger, M., "Value of Culture and Enzyme Additions in Promoting Digestion," Sew. Ind. Wastes, 25(11):1259 1953

5. Ingols, R.S., "A Study of Hydrolytic Enzymes in Activated Sludge," New Jersey Agriculture Experimental Station Bull. 66, 1939

6. Ingols, R.S., "Oxidation-Reduction Enzymes in Activated Sludge," Sew. Wks. J., 12(5):862 1940

7. McKinney, R.N. and Sawyer, C.N., "Wonder Drugs - Enzymes - Will They Cure Waste Treatment Ills? A Critical Look at the Modern 'Panaceas' for Sewage Treatment," Eng. News Record, 151(14):34 Oct. 1, 1953

8. Rudolfs, W., "Enzymes and Sludge Digestion," Sew. Wks. J., 4(5):782-89 1932

9. Wooldridge, W.R. and Standfast, A.B., "The Role of Enzymes in Activated Sludge and Sewage Oxidations," Biochem. J., 30:1542-53, 1942

10. Updike, S.J. and Hicks, G.P., "The Enzyme Electrode," Nature, 214:986-88 June 3, 1967

11. Hicks, G.P. and Updike, S.J., "The Preparation and Characterization of Lyophilized Polyacrylamide Enzyme Gels for Chemical Analysis," Anal. Chem., 38(6):727-30 May 1966

12. Bernfeld, P. and Bieber, R.E., "Water-insoluble Enzymes:Kinetics of Rabbit Muscle Enolase Embedded within an Insoluble Carrier," Arch. Biochem. Biophysics, 131:587-78 1969

13. Bernfeld, P., et. al., "Kinetics of Water-insoluble Phosphoglycerate Mutase," Biochim. Biophysica Acta, 191:570-78 1969

14. Goodfriend, T., et. al., "Antibody in Polyacrylamide Gel, A Solid Reagent for Radioimmunoassay," Immunochemistry, 6(3):481-4, 1969

15. Bauman, E.K., et.al., "Preparation of Immobilized Cholinesterase for use in Analytical Chemistry," Anal. Chem., 37(11):1378-81, Oct. 1965

16. Bernfeld, P. and Wan, J., "Antigens and Enzymes Made Insoluble by Entrapping Them into Lattices of Synthetic Polymers," Science, 142:678-79, Feb. 1963

17. Bernfeld, P., et. al., "Water-insoluble Enzymes:Arrangement of Aldolase with an Insoluble Carrier," Arch. Biochem. Biophysics, 127:779-86 1968

18. Silman, I.H. and Katchalski, E., "Water-insoluble Derivatives of Enzymes and Antibodies," Ann. Rev. Biochem., 35(2):873-908 1966

19. Rinderknecht, H., et. al., "A New Ultrasensitive Method for the Determination of Proteolytic Activity," Clin. Chim. Acta, 21:197-203 Aug. 1968

20. Rosenkrantz, H. and Kirdani, E., "Proteolytic Enzymes of Canine Prostatic Fluid," Cancer Chemotherapy Report, No. 15, 9, 1961

21. Rinderknecht, H., et. al., "A New Method for the Determination of α amylase," Experientia, 23(10):805 1967

22. Banerji, S.K., et. al., "Kinetics of Removal of Starch in Activated Sludge Systems," Journ. Water Pollution Control Federation, 40(1):16-29, Jan. 1968, 40 (2):161-73 Feb. 1968

23. McCready, R.M., et. al., "Determination of Starch and Amylase in Vegetables," Anal. Chem., 22(9):1156-58 Sept. 1950

24. Guilbault, G.C. and Kramer, D.N., "Fluorometric Procedure for Measuring the Activity of Dehydrogenases," Anal. Chem. 37(10):1378-81 Oct. 1965

25. Guilbault, G.C., et. al., "Evaluation of Fluorometric Substrates for Lipase," Anal. Letters, 1(9):551-63, 1968

26. Guilbault, C.G., et. al., "N-Methylindoxyl esters as Substrates for Cholinesterase," Anal. Letters, 1(6):365-79 1968

27. Ashwell, G., Methods in Enzymology, Colowick and Kaplan, editors, Academic Press, New York, 3:87, 1957

28. Dische, Z., "Color Reactions of Nucleic Acid Components," The Nucleic Acids:Chemistry and Biology, Chargaff, E. and Davidson, J.N., editors, Academic Press, New York, 1:285-305 1955

29. Gornall, A.G., et. al., "Determination of Serum Proteins by Means of the Biuret Reaction," J. Biol. Chem., 177:751-66 1948

30. Kingsley, G.R., "The Determination of Serum Total Protein, Albumin and Globulin by the Biuret Reaction," J. Biol. Chem., 131:197-200 1939

SECTION VIII

APPENDIX

PROTEOLYTIC ACTIVITY #1 (INSOLUBLE SUBSTRATE) (REF. 19)

Reagents: RBB-Hide Protein (Calbiochem)
Tris buffer, 0.05M, pH 7.8
Proteinase (2 mg/ml Tris buffer)
Enzyme Source (unknowns)

Standard Curve

1. Weigh out 20 mg RBB-Hide Protein for each of a series of duplicate 25 ml Erlenmeyer flasks .

2. Add Tris-buffer and proteinase solutions to the flasks as stipulated below:

Final Enzyme Conc	ml Proteinase	ml Tris Buffer
10 mg	5.0	-
8 mg	4.0	1.0
6 mg	3.0	2.0
4 mg	2.0	3.0
2 mg	1.0	4.0
1 mg	0.5	4.5
0.2 mg	0.1	4.9

3. Incubate flasks in a Dubanoff shaker at 37°C for 30 Minutes.

4. The reaction is stopped by filtering the suspension through glass wool.

5. The absorption of the individual samples is then determined using a Coleman Junior Spectrophotometer - 595 m μ.

Unknowns

1. Set up duplicate flasks for each concentration of the unknown to be tested and add 20 mg RBB-Hide to each flask.

2. The addition of buffer and the enzyme source are added as previously described such that the final concentration totals 5 ml.

3. Unknown samples previously boiled for 10 minutes are used as blanks, in addition to one set of flasks to which no enzyme is added.

4. Run standard enzymes (proteinase) at 5, 2 and 1 mg total concentration. Incubate samples as described and stop reaction by filtration.

5. Absorption is determined at 595 mμ

6. Substrate saturation is verified by the observation of increases in enzyme concentration yielding proportional absorption increases.

PROTEOLYTIC ACTIVITY #2 (SOLUBLE SUBSTRATE SYSTEM) (REF. 20)

Reagents

1. Saturated casein solution was prepared by dissolving 1.3 gm Hammerstein grade casein in 100 ml 0.05M Tris-buffer, pH 7.8 and heating in a boiling water bath for 20 minutes. The suspension is then centrifuged at 2,000 X G for 15 minutes to remove any excess particulate matter remaining. (Can be refrigerated for up to 5 days).

2. Tris buffer - 0.05M, pH 7.8.

3. Enzyme source (unknown and protease at 10 mgm/ml).

4. 10% Trichloracetic Acid (TCA)

Calibration Curve and Unknown Assay

1. Duplicate sets of four test tubes are set up for each enzyme concentration and to each of three is added 2 ml of the casein solution.

2. Tris buffer is then added to each of three of the test tubes such that, upon addition of enzyme, the total volume would be 7 ml.

3. A standard enzyme preparation is then added to two of the tubes containing the casein, and the same quantity is added to the as yet empty tube remaining.

4. The tubes are incubated at 37° for one hour in a Dubanoff shaker.

5. To the three enzyme containing tubes 3 ml of 10% TCA is added to stop the reaction. Subsequently, the tube containing the buffer and casein is combined immediately with the one containing enzyme and TCA alone.

6. The suspensions are then centrifuged, the ppt discarded, and the absorption of the supernatants read at 280 mμ on the Beckman DU Spectrophotometer to determine the amino acids enzymatically released.

7. The running of a blank in this manner allowed for an accurate determination of both the possible non-enzyme partial breakdown of casein and also the possible non-specific release of entrapped proteins from the gel during the hour long incubation.

8. The protocol adopted essentially represents that employed in the paper cited (Ref. 20) with only the above modification for blank determinations and also an adjustment upward in total assay volume.

AMYLYTIC ACTIVITY #1 (INSOLUBLE SUBSTRATE) (REF. 21)

Reagents
RBB - Starch (Calbiochem)
Tris buffer (0.05M, pH 7.0) - 0.5M NaCl
Amylase (1 mg/ml)
Enzyme Source (unknowns)

Standard Curve

1. 2 ml of a 2% suspension of RBB-starch (0.05M Tris buffer, pH 7.0 - 0.5M NaCl) is pipetted into a series of duplicate 25 ml Erlenmeyer flasks.

2. Add Tris buffer and amylase to the flasks as stipulated below:

Final Conc	ml Amylase	ml Tris buffer
5	5.0	-
4	4.0	1.0
3	3.0	2.0
2	2.0	3.0
1	1.0	4.0
0.5	0.5	4.5
0.1	0.1	4.9

3. The flasks are incubated in a Dubanoff shaker for 30 minutes at 37°C.

4. Initially the reaction was stopped by the addition of acetic acid followed by filtration but later filtration alone via S. and S. prefolded filters was used. The absorption of the samples at 595 mμ was determined with the use of a Coleman Jr. Spectrophotometer.

Unknowns

1. Set up duplicate flasks for each concentration of the unknown to be tested and add 20 mg RBB-Hide to each flask.

2. The addition of buffer and the enzyme source are added as previously described such that the final concentration totals 5 ml.

3. Unknown samples previously boiled for 10 minutes are used as blanks, in addition to one set of flasks to which no enzyme is added.

4. Run standard enzyme (alpha amylase) at 5, 2 and 1 mg total concentration. Incubate samples as described and stop reaction via filtration.

5. Absorption is determined at 595 mμ.

6. Substrate saturation is verified by the observation of increases in enzyme concentration yielding proportional absorption increases.

AMYLYTIC ACTIVITY #2 (SOLUBLE SUBSTRATE) (REF. 22 and 23)

Reagents

1. 1 mg/ml starch (soluble starch for Iodometry) in 0.05M Tris - 0.05M NaCl pH 7.0.

2. 0.05M Tris buffer - 0.05 NaCl, pH 7.0.

3. KI-I_2 color reagent.

Calibration

1. From 0.1 ml to 7 ml of the starch solution is added to duplicate test tubes.

2. Using the Tris buffer, the total volume is adjusted to 7 ml.

3. The tubes are incubated for 15 min at 37° and 0.3 ml of KI-I_2 color reagent is added.

4. The tubes are incubated an additional 15 min and the absorption at 650 mμ determined with a Coleman Jr. Spectrophotometer.

Assay

1. 2 ml of the starch solution is added to each tube.

2. The duplicate unknowns are assayed according to the same protocol as described above at concentration of 0-5 ml.

3. The final volume of all tubes is adjusted to 7 ml with Tris buffer.

4. Blanks are run using enzyme preparations previously heated in a boiling water bath for 15 minutes.

5. In addition, standard amylase concentrations of 5, 3, 1 and 0.5 mg are run with all assay systems.

6. Problems inherent in the system include the inability to easily stop reaction at the end of the 15 min. incubation, and also the occasional presence of contaminating substances in the sonicate derivatives which interfered with the reaction.

DEHYDROGENASE ASSAY (Ref. 24)

Reagents

1. Krebs acid intermediate soln (substrate) ($10^{-2}PO_4$, pH 8)
2. DPN,$^+$ TPN$^+$ soln (confactor) ($10^{-2}H_2O$ soln)
3. PMS soln (electron acceptor) (light sensitive) (10^{-3}, H_2O)
4. Resazurin (fluorescent source) (10^{-2}M in cellosolve)
5. PO_4 buffer, 0.1M, pH 8.0
6. Isocitric DH. in glycerol
7. Liver Homogenate - 25% in PO_4 buffer, 0.1M, pH 8.0

Substrate Mixture

1 ml DPN, TPN
1 ml Resazurin
10 ml PMS
20 ml Kreb intermed.

} Ues 2 ml/assay

KEEP PROTECTED FROM LIGHT!!

Fluorometer Setting

Ex 560 mμ
Em 580 mμ

} Scalar 10

Protocol

1. Obtain reading on substrate mixture alone
2. Add enz., invert to mix, replace for reading
3. Obtain reading on enzyme-substrate mixture rate

Series Procedure

1. Substrate + liver homogenate 1 (0.1ml 25%)
2. Substrate + liver homogenate 2 (0.2 ml 25%)
3. Substrate + isocitric DH (0.5 ml)
4. Substrate + sample conc. 1 (0.1)
5. Substrate + sample conc. 2 (0.2)
6. Substrate + sample conc. 3 (0.5)
7. Substrate + conc. 1,→ 2,→ 3 boiled WC preparation 0.1, +0.1, +0.3 ml

LIPASE ASSAY (Ref. 25)

Reagents

1. Fluorescein Dibutyrate (1×10^{-6}M, cellosolve)
2. PO_4 buffer 0.1M, pH 6.5

Substrate Mixture — 1 ml fluorescein dibutyrate; 29 ml PO_4 buffer, 0.1M, pH 6.5 } Use 2 ml/assay

Fluorometer — 1"/min chart speed; 10 Scalar

Protocol

1. Obtain reagent blank reading $\bar{c}$ 2 ml substrate mixture
2. Add enz. prep., invert to mix, replace
3. Obtain rate on substrate-enzyme interaction

Series Procedure

1. Substrate mixture + 0.1 ml lipase
2. Substrate mixture + 0.3 ml lipase
3. Substrate mixture + 0.1 ml fraction sample
4. Substrate mixture + 0.3 ml fraction sample
5. Substrate mixture + 0.5 ml fraction sample
6. Substrate mixture + boiled enzyme $\rightarrow$0.3 $\rightarrow$0.5 (0.1ml; + 0.2 ml; + 0.2 ml)

Note: Both the standard enzyme and the fluorescein dibutyrate are made fresh daily.

ESTERASE ASSAY (Ref. 26)

Reagents

1. Indoxyl acetate (0.02M in cellosolve)
2. PO_4 buffer, 0.1M, pH 7.5
3. ENZ - acetylcholinesterase, alkaline phosphatase, Lipase (all mg/ml)

Substrate Mixture — 1 ml indoxyl acetate; 29 ml PO_4 buffer pH 7.5 } Use 2 ml/assay

Fluorometer — Ex 395 mμ 1"/ min chart speed

Em 470 mμ Scalar 1

Protocol

1. Obtain reading on substrate mixture
2. Remove, add enz., invert to mix, return
3. Obtain reading on enzyme-substrate mixture

Series Procedure

1. Acetylcholinesterase +2 ml substrate mix (0.1 ml)
2. Acetylcholinesterase +2 ml substrate mix (0.2 ml)
3. Alkaline phosphatase +2 ml substrate mix (0.5 ml)
4. Lipase + 2 ml substrate mix (0.5 ml)
5. Fraction sample + 2 ml substrate mix (0.1)
6. Fraction sample + 2 ml substrate mix (0.2)
7. Fraction sample + 2 ml substrate mix (0.5)
8. Boiled fraction sample + 2 ml substrate mix (0.1, +0.1, +0.3)

RNA ANALYSIS (Ref. 27)

Reagents

1. 10% TCA, 5% TCA
2. ETOH: ether (3:1)
3. Abs ETOH, 95% ETOH
4. ETOH: Chloroform (3:1)
5. Orcinol reagent - 6% orcinol in 95% ETOH
6. Ferric chloride reagent (10%) 1 gm $FeCl_3 . 6H_2O$ in 10 ml H_2O
7. RNA Sources (Std. yeast 10μ g/ml and Unknowns)

Preparation of RNA Fraction from Unknowns

1. Precipitate a 5 ml aliquot of sonicate and/or derivative with an equal volume of 10% cold TCA and allow to stand overnight at 4°C.
2. Centrifuge the suspension for 15 min at 2,000 rpm - 5°C (IEC) and discard the supernatant.
3. Wash the pellet 3 times with 2 ml of 95% ETOH.
4. Wash the pellet 2 times with 2 ml ETOH: chloroform (3:1)
5. Wash once with 2 ml of ETOH: ether (3:1) and once with ether alone
6. The solvent is removed after washing by a 10 min centrifugation at 2,000 rpm, -5°C (IEC)
7. The pellet is dried in an open vacuum dessicator for 3 min, the vacuum valve is then closed, the sample pellets stored overnight in the cold room (5°C)
8. To each of the dry pellets, 3 ml of hot (50°C) 5% TCA is added and the tubes maintained at 90°C for 15 minutes. The tubes are centrifuged for 15 min at 2,000 rpm, and the supernatant decanted and saved.
9. To the pellet an additional 1 ml of hot 5% TCA is added and the above extraction repeated. The supernatant is added to that obtained in the previous step. The pellet is discarded.

Assay (Standards and Unknowns)

1. Duplicate 1.5 ml samples of the unknowns are placed in 19 x 105 Coleman pH tubes and 0.5 ml distilled water added.

2. A reagent blank employeng 2.0 ml distilled water is prepared.

3. Known concentrations of RNA are added to Coleman tubes and the volume adjusted to a total of 1.5 ml with distilled water.

4. 0.2 ml of orcinol reagent and 3 ml ferric chloride solution are added to all tubes and the samples incubated in a boiling water bath for 10 min.

5. The samples are allowed to cool for 5 min at 0°C and 1.5 ml absolute ETOH is added to prevent turbidity.

6. The absorption of the samples at 670 mμ is then determined with a Beckman DU Spectrophotometer.

DNA ANALYSIS (Ref. 28)

Reagents

1. 10% TCA, 5% TCA
2. Abs ETOH, 95% ETOH
3. ETOH: ether (3:1)
4. ETOH: chloroform (3:1)
5. DNA sources (Std. Salmon sperm 100 μ g/ml) in 5% TCA and unknowns)
6. Diphenylamine - 1% solution in glacial acetic acid to which 2.75 ml H_2SO_4 is added

Preparation of DNA Fraction from Unknown

(Same protocol as that followed for RNA extraction)

Assay Standards and Unknowns

1. Standard DNA concentrations of from 5-100 μ g and a maximum volume of 1 ml are pipetted into Coleman test tubes.
2. Duplicate 1 ml samples of the unknowns are added to the test tubes.
3. 2.0 ml of diphenylamine reagent is added to each tube and the samples heated in a boiling water bath for 10 minutes.
4. After cooling the absorption of the samples are determined at 595 mμ with a Coleman Spectrophotometer.

TOTAL PROTEIN - BIURET REACTION (Ref 29 and 30)

Materials:

Volumetric flask - 25 ml
0.1 ml pipettes, graduated to top
15 x 125 mm test tubes
B&L calibrated 4-inch tube type cuvette

Reagents:

Harleco Biuret Reagent

Standard

Albumin Bovine (Armour & Co., Chicago)
Std Soln: 100 mg/ml
Weigh carefully 2.5 gm albumin and dilute with water in 25 ml volume flask. Store in refrigerator

Standard Curve:

To prepare a standard curve:

1. Pipette in duplicate into clean 15 x 125 mm test tubes as follows:

Conc	10 mg/ml Std	Saline H_2O
10 mg	1.0 ml	0
5	0.5	0.5
2.5	0.25	0.75
1	0.10	0.90
Blank	0	1.0

2. Continue procedure with the addition of 4 ml Biuret Reagent

3. Follow assay protocol as described below:

Procedure for Unknown Samples

1. Set up 2 test tubes in a rack for each sample to be analyzed plus 1 tube for a blank.

2. Pipette an appropriate amount of sample into the tubes (See Table below). Do not use blow out pipettes.

3. To each sample add 0.85% saline to bring volume to a total of 1.0 ml. Pipette 1.0 ml of saline into blank tube.

4. Using a volumetric pipette add 4.0 ml of Biuret Reagent to all tubes.

5. Mix thoroughly on vortex mixer and allow to stand for 15-20 min. at room temperature.

6. Read tubes in Spec. 20 at 540 mμ against blank. Record O.D. and calculate protein concentration using the formula:

$$\frac{\text{O.D.}}{2.8} \times \text{dilution} = \% \text{ protein}$$

7. If O.D. is greater than 0.500 repeat test using a higher dilution of sample.

Dilution	Sample (ml)	Saline (ml)	Biuret Reagent (ml)
1:10	0.5	0.5	4.0
1:25	0.2	0.8	4.0
*1:50	0.1	0.9	4.0

*Dilution used for protein determination of whole serum.

BIBLIOGRAPHIC:

Lawrence Slote, Eng. Sc. D., Grumman Aerospace Corporation, Development of Immobilized Enzyme Systems for Enhancement of Biological Waste Treatment Processes, Final Report FWQA Contract No. 14-12-562, July 1970

ABSTRACT

A method was developed to biochemically fractionate the microbial enzymes from activated sludge, to concentrate and characterize their activity, and to immobilize this activity by entrapment in a polyacrylamide gel. The enzyme-gel preparation was tested for its effect on a bench-scale batch activated sludge process.

The conclusions were: (1) the soluble enzymatic components of activated sludge can be readily separated from the particulate components of the cell; (2) the soluble system thereby obtained can be fractionated in such a manner as to maintain the activity of the catabolic enzyme systems of interest while removing non-essential components; (3) the enzymatically active preparation can then be immobilized within the matrix of a polyacrylamide gel; (4) the gel will maintain activity during storage, repeated washings, and repeated exposure to substrate; (5) the limited bench-scale activated sludge experiments failed to produce meaningful results due to possible incomplete polymerization of the polyacrylamide gel and an improper activated sludge culture.

ACCESSION NO.

KEY WORDS:

Enzymes

Biological Treatment

Biochemical Oxygen Demand

Sanitary Engineering

Research and Development

Waste Water Treatment

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1 Accession Number	2 Subject Field & Group	SELECTED WATER RESOURCES ABSTRACTS INPUT TRANSACTION FORM
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