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# Removal of Eutrophic Nutrients from Wastewater and their Bioconversion to Bacterial Single Cell Protein for Animal Feed Supplements - Phase III

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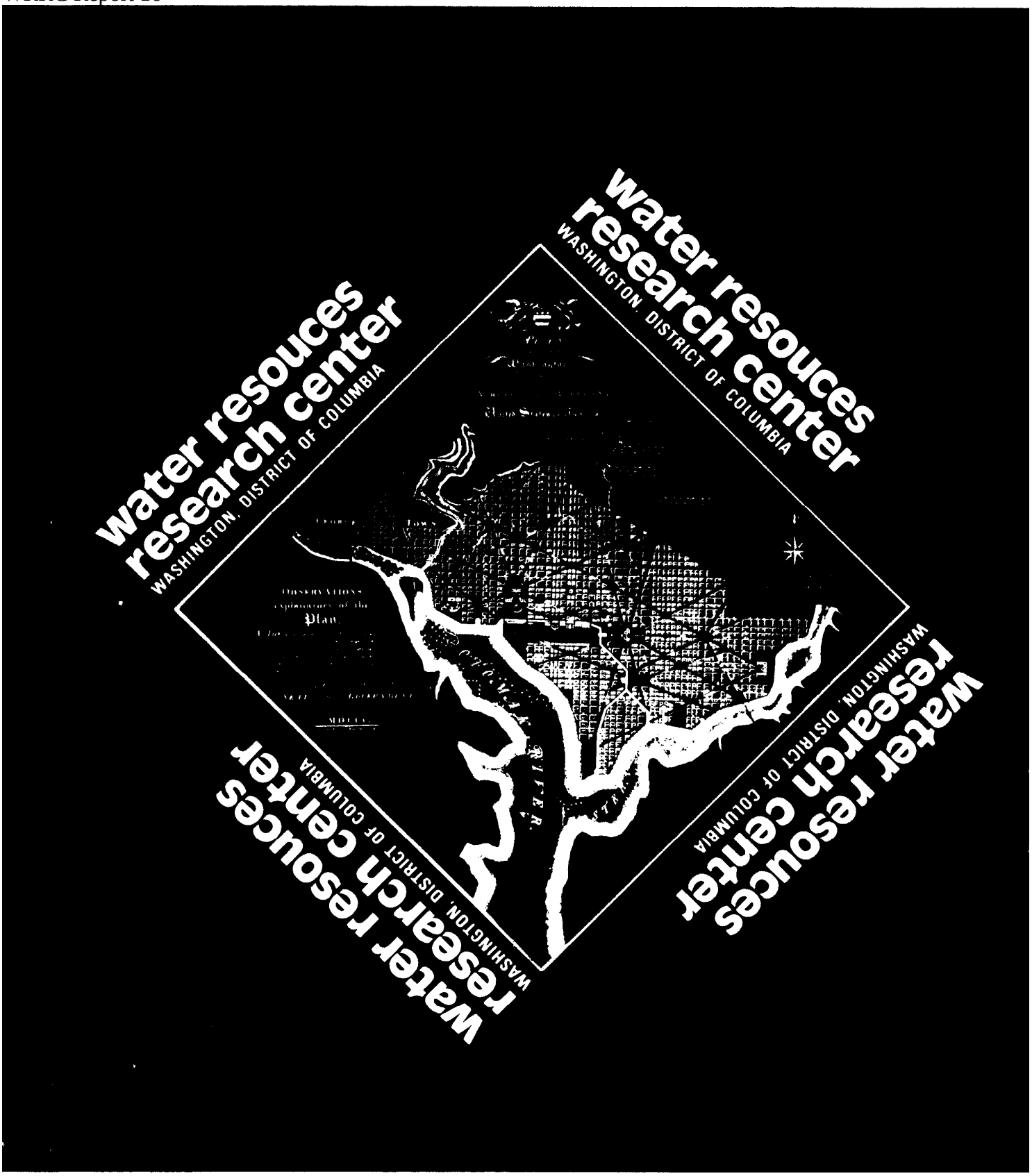

# REMOVAL OF EUTROPHIC NUTRIENTS FROM WASTEWATER AND THEIR BIOCONVERSION TO BACTERIAL SINGLE CELL PROTEIN FOR ANIMAL FEED SUPPLEMENTS PHASE III

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## INTRODUCTION

The effluent from municipal wastewater treatment plants can be a major source of the *inorganic* pollutant which enter waterways and lead to eutrophication. The form of these elements that are readily utilized by polluting microorganisms and which are commonly present in secondary wastewater effluents are ammonium ions, nitrate, and phosphate. These compounds are also the inorganic forms of nitrogen and phosphorus that are best utilized by some heterotrophic and chemolithotrophic bacteria for assimilation into organic cellular components. In typical bacteria, nitrogen constitutes 10-15% and phosphorous 1-2% of the cell biomass (dry weight). Since in final effluents there is frequently a paucity of utilizable organic substrates, the use of heterotrophic bacteria for N and P removal would require the addition of an organic substrate which is expensive and could lead to an increase in B.O.D. With most chemolithotrophic bacteria

the major disadvantages are slow growth rates and inhibition by organics. Exceptions are the hydrogen oxidizing chemolithotrophs, which are all facultatively autotrophic bacteria that utilize either hydrogen or a

wide range of organic molecules as carbon and energy sources. The utilizable organic substrates include most of the organic acids found as end products of anaerobic sludge digestion as well as many common cyclic amino acids such as histidine, proline, phenylalanine, tyrosine, and tryptophan (1). All of these compounds, plus benzoic acids and phenols are completely degraded to C02 and H2O by members of this group of bacteria (2).. In addition, any urea remaining from secondary treatment would be degraded and utilized. The known capacity of these organisms to remove traces of nutrients from media plus their ability to grow under alkaline *conditions* (to pH 9) makes them a logical choice for an advanced treatment system. Thus, in theory, this group of microorganisms could be employed to remove eutrophic inorganic compounds plus numerous residual organic molecules from wastewater. The hypothetical system would involve the addition of gaseous hydrogen, air and carbon dioxide to the wastewater medium, which would supply all other necessary nutrients. The end result of this advanced treatment system would be H2O and microbial biomass. The feasibility of this process was demonstrated in Phase I of this project.

A second objective of this research project was to evaluate the feasibility of utilizing the biomass produced from wastewater as a source of high quality protein suitable for animal feed supplements. The high cost of cereal grains coupled with the scarcity and high cost of mineral fertilizers have led to dramatic increases in animal feed prices. Both grain production and livestock grazing require the use of large amounts of increasingly scarce tillable land. These problems have led to a rekindling of interest in single cell protein (SCP) mainly using petroleum byproducts and methanol as substrates (3, 4). The major microorganisms employed in these procedures are bacteria and yeast. An alternative is to produce protein for animal feed by growing microorganisms on waste products such as wastewaters.

Of the hydrogen-oxidizing chemolithotrophic bacteria, several classified in the genera Pseudomonas and Alcaligenes seem to possess the physiologic characteristics suitable for wastewater treatment and as a source of useable SCP. The organism employed in these studied, Alcaligenes eutrophus, consists of approximately 70%

protein when grown in a synthetic medium and had previously been shown to possess protein of high nutritional quality (5, 6).

During phase I of this project it was demonstrated that the hydrogen oxidizing chemolithotrophic bacterium Aicaligenes eutrophus was an efficient remover of eutrophic nitrogenous and phosphorus compounds from final effluent and digester elutriate obtained from the Blue Plains Wastewater Treatment Plant, Washington, D.C. Average removal levels from final effluent were 98% for ammonia, 67% for nitrate, $83^{1-}$ for TO and 84°~ for total phosphorus. Mixtures of 1 part elutriate to 3-parts final effluent yielded 88% removal of both ammonia and total phosphorus. No pretreatment of elutriate or effluent was necessary to support growth, *and* sterilization of wastewater samples yielded results similar to unsterile samples; i.e., the chemolithotroph did not need to be grown in pure culture.

During phase II, A.eutrophus was grown-in both batch and semi continuous culture in a fermentor using phosphorus supplemented digester elutriate as the growth medium. Gaseous hydrogen, oxygen and carbon dioxide were supplied as carbon and energy sources. Ammonia and phosphate removal averaged 87% and 93%, respectively, at the time of cell harvest. The cellular biomass was washed, dried, chemically analyzed. and employed. as the protein component of chick diets. The biochemical composition of the wastewater grown biomass was 8.7% RNA, 1.3% DNA, 56% protein, 3.2% carbohydrate, and 30.8% lipid and inert materials. Chick feeding experiments demonstrated that the bacterial biomass had a protein quality of 88% compared to reference casein.

In addition, mixtures of undigested sludge and final effluent were treated under various conditions. In sterilized samples of sludge effluent at dilutions of 1:4, 1:8 and 1:20 under a H2, 02, C02 atmosphere, growth of A. eutrophus_ was substantial and was proportional to the sludge concentration, reaching $8 \times 10^9$ organisms/ml in the

1:4 mixture. When the gas atmosphere was replaced by air, growth was substantially less, showing that the autotrophic atmosphere was essential for optimal growth. This was confirmed by chemical analyses of the treated mixtures, showing 39-92% ammonia removal and 98-99v phosphate removal from gassed cultures. The cultures

incubated under air showed increases in ammonia levels or 25-670 and phosphate reductions of approximately 90%.

During this final phase (III) of this project, A. eutrophus cells grown in the fermentor were treated in various ways to find conditions promoting aggregation. Growth in digester elutriate also was compared when the elutriate was unsterile, sterile or heated at different temperatures; this determined the need to remove some heat sensitive inhibitory component.(s)of the elutriate. Digester elutriate and sludge were tested using the Ames bacterial assay for the presence of mutagenic substances. The results of these studies are described below. .

## MATERIALS AND METHODS

The major microorganism employed in this study is the facultative chemolithotrophic bacterium Alcaligenes eutrophus (formerly named Hydrogenomonas eutrophus). This organism has been maintained in our laboratory for many years on a sodium *pyruvate-inorganic* salts agar medium.

Wastewaters employed throughout these studies were obtained at regular intervals from the Blue Plains Wastewater Treatment Plant, Washington, D.C. These include final effluent, sludge digester elutriate or wash water, undigested and digested sludge. Collected samples were used immediately or stored for brief periods at 4°C until used.

Samples were dispensed normally in 100 ml amounts into 300 ml baffled Erlenmeyer flasks. When sterilization was employed, samples were autoclaved at 121°C for 15 minutes. After coming to room temperature samples were withdrawn for chemical analyses. Flasks to be incubated under an autotrophic gas mixture were connected via a gassing manifold to a gas reservoir, which was maintained at a pressure slightly greater than one atmosphere. Flasks incubated under air contained styrofoam stoppers. All flask

cultures were incubated at 30-32°C with shaking at 200 rpm. For fermentor cultures, 8-9 liters of undiluted elutriate *(containing* approximately 300 mg/L ammonia nitrogen) were supplemented with small amounts of potassium phosphate to produce the desired ratio of N: P. The medium was sterilized in the 14 liter fermenter vessel and inoculated with A. eutrophus. Gaseous hydrogen, oxygen and carbon dioxide were added individually or in pairs via separate flow meters to yield a ratio of 18H2:1 0,:1 $C0_2$. The temperature was maintained at 30-32°C.

Growth was monitored by reading optical densities at 540 rm. With A. eutrophus an OD540 of 1.0 corresponds to 0.32 mg dry weight of cells per ml.

Chemical analyses were performed as outlined in Standard Methods for the Examination of Water and Wastewater (7). Samples were analyzed for total Kjeldahl nitrogen (TKN), total phosphorus (TP) and ammonia nitrogen. TKN was determined by digestion of samples, collection of distillate in indicating boric acid and titration in standard sulfuric acid. Ammonia nitrogen was assayed after pretreatment using Nessler's reagent. Total phosphorus was determined using the ascorbic acid method after preliminary digestion with persulfate-sulfuric acid. After inoculation with A. Eutrophus, the cultures were incubated until growth ceased; the cells were then harvested by centrifugation and samples of supernatant were assayed for each of the indicated substances.

The harvested cellular biomass was treated with a wide variety of conditions including temperature, pH, and ion exchange resins (cationic and anionic) in order to find conditions which promoted cellular aggregation

or clumping. This was evidenced by a sharp decrease in optical density and the presence of a pellet in the tube.

Samples of elutriate and sludges were tested for the presence of mutagenic substances by using the Salmonella mutagenicity test (8). Sludge samples consisted of 60% secondary digested sludge and 40% primary undigested sludge. Some samples were sterilized by autoclaving while others were treated with 95% ethanol and then frozen and dried under vacuum. Unsterilized samples could not be used since the large numbers

of microorganisms present in untreated elutriate or sludges would interfere with the proper reading of the test plates. Either four or five tester strains of S. typhimurium were used; and the test was run both with and without activation by rat liver S-9 microsomal enzymes. This activation will detect compounds that become mutagenic after metabolic conversions by mammalian enzyme systems. Rat liver S-9 fraction induced with Aroclor 1254 (PCB's) was purchased from Litton Bionetics, Inc., and was used at a concentration of 40 µl per plate. All tests were performed as recommended by Ames et al (8), with minor modifications. Negative controls employed either water or the vitamin niacin. Positive controls employed N methyl N'nitro nitrosoguanidine (MNNG).

## RESULTS AND CONCLUSIONS

*In* order to obtain a large biomass of A. eutrophus grown in wastewater, eight liters of digester elutriate were used as the growth medium *in* a 14 liter fermentor. Elutriate was used *since* it contains much higher concentrations of nitrogenous compounds than secondary effluent and therefore, supports greater cell densities. However, elutriate also contains a much higher ratio of nitrogen-phosphorus than the 7:1 ratio that we previously determined to be optimum for growth and nutrient removal. Thus, since the initial concentrations were: ammonia-nitrogen, 336 mg/L; TKN, 340 mg/L; and phosphate phosphorus, 16.2 mg/L; 32 mg/L of phosphate phosphorus (as KH2P04) were added to produce the *desired* ratio. The supplemented elutriate was adjusted to pH 5.5 to prevent ammonia loss, sterilized by autoclaving and, after cooling, was inoculated with A. eutrophus and supplied with hydrogen, oxygen and carbon dioxide. Growth was rapid and reached an optical density of

24.0. This was by far the highest cell density obtained to date; the previous high cell yield was 18.75. The increase in optical density and decrease in pH with time are shown in Figure 1. Note that when the culture was harvested at an optical density of 24, growth had not yet leveled off. Thus if the culture had not been harvested

at this point the biomass probably would have increased still further, although, as indicated by the low level of remaining nutrients, cell density must have been near its maximum. The initial concentrations of 336 mg/ L ammonia nitrogen and 48 mg/L phosphate phosphorus were reduced to 1.1 mg/L, nitrogen and less than 0.3

mg/L phosphorus by the bacterial treatment. Thus over 99% of both the ammonia and phosphate were removed from the treated elutriate.

We believe that the high cell density and extremely efficient removal of nutrients achieved during this run were due to the use of higher gas flow and agitation rates than used previously in the fermentor system.

The treated elutriate, consisting mainly of cellular biomass, was observed for its ability to form cellular aggregates by varying temperature and pH and by adsorption with ion exchange resins. The addition of 1 N NaOH produced-no observable change in the physical nature of the biomass until the pH was raised to 11, at which point the cells formed a tight aggregate. As the pH was raised further the aggregated biomass expanded and became gelatinous until at pH 12 the aggregate appeared as a large gelatinous mass surrounded by a clear liquid phase. As the cells clumped, the optical density of the suspension was reduced. The relationship between O.D. and pH is shown in Table 1.

High (82°C) and low (I°C) temperatures produced no significant cell aggregation by themselves. As mentioned above alkaline pH below 11 produced no aggregation while a low pH (2.7) produced cell lysis. The addition of a cationic exchange resin (Cellex-CX, Bio-rad Labs) produced some aggregation but anionic exchange resins were more effective. Two of these, Cellex-D (Bio-rad Labs) and Pencol 728 (the resin used to promote

aggregation at Blue Plains) were effective in producing cellular aggregation. Cellex-D produced a 90% reduction in the optical density of

elutriate grown cells at pH 7.3'and at either 25°C or 72°C. Lower temperatures or pH values far from neutrality reduced slightly the aggregation of the cellular biomass. Thus anionic exchange resins are effective in producing cellular clumping and would be one means o separating cells from supernatant after treatment with the bacterial system.

Earlier studies by us had shown that when A. eutrophus was inoculated into *elutriate-effluent* mixtures, growth was similar in both sterilized and unsterilized samples. However, more recent studies with two

recently obtained samples of elutriate yielded no growth when unsterilized samples were inoculated in the fermentor. This suggested that either viruses were present in the elutriate which were *infecting* and killing the bacteria or some heat labile inhibitor was present which was destroyed in sterilized samples. After repeated assays no viruses were found which infected A. eutrophus. In order to see if a heat labile inhibitor could be demonstrated, samples of elutriate were heated to different extents and compared to an unheated sample. All four samples were inoculated with A. eutrophus and culture densities were followed with time. The results are shown in Figure 2. The results indicate that an inhibitor was partially inactivated by heating at 5a°C for 10 min while 70°C for 10 min produced results similar to elutriate that was sterilized by autoclaving. We don't know yet whether the heat labile inhibitor is an undetected virus, competing bacteria, or some toxic compounds.

Mutagenicity tests using the Salmonella system were employed to determine whether mutagenic substances were present in sludge mixtures and in digester elutriate from the Blue Plains Wastewater Treatment Plant.

Table 2 shows the results of two analyses performed in the absence of S-9 metabolic activation. Table 3 *contains* the results of an analysis which included a rat liver S-9 activation system induced with Aroclor 1254. Since the sludge and elutriate contained large numbers of live microorganisms, the use of unsterilized sludge and elutriate produced contaminated test plates which obfuscated the results. Thus some treatment of the

samples was necessary to kill the microbes present. This complicated the analysis since the sterilizing treatments could inactivate or volatilize some mutagenic substances and lead to false negative results. Sterilization by autoclaving presents this objection, but a more gentle treatment with ethanol followed by freeze-drying did not produce sterile samples, probably due to the .survival of spores, and therefore could not be employed.

Results both without and with metabolic activation revealed no mutagenic activity in the tested samples with the possible exception of strain 1537. Ames suggests that reversion rates that are twice or more the negative control-rate should be considered positive, and Table 2 shows that the results with sludge are borderline in this regard, in that 0.1 ml samples yielded reverting rates which varied from 2-3 times the control rate; however 0.5 ml samples ranged, from double the control rate to values below the control. Samples tested using metabolic activation (Table

3) also showed minimal, if any, mutagenic effects. Thus we can conclude that the tested samples of sludge and digester elutriate contained at most a marginally detectable quantity of mutagens as evidenced by the Salmonella test system.˙ This system has been shown to be able to detect 90 % of the compounds that are confirmed animal carcinogens (9).

**Table 1**

Effect of the Addition of Alkali (1 N NaOH) on the Optical Density and Appearance of Elutriate- grown A. eutrophus

| Tube # | PH | OD540 | Appearance of Suspension |
|---|---|---|---|
| 1 | 6.3 | 22 | Normal turbid suspension |
| 2 | 7.3 | 21 | Normal turbid suspension |
| 3 | 8.8 | 21 | Normal turbid suspension |
| 4 | 9.4 | 20 - | Normal. turbid suspension |
| 5 | 9.9 | 19.5 | Normal turbid suspension |
| 6 | 10.9 | 12 | Less turbid -- at bottom |
| 7 | 11.2 | 13 | . Less Turbid -- larger: loose pellet |
| 8 | 11.7 | 11 | Less turbid -- gelatinous pellet |
| 9 | 11.8 | 12 | No pellet – gelatinous suspension |
| 10 | 12.0 | 13 | No pellet -- gelatinous suspension |
| 11 | 12.1 | 6 | Gelatinous mass surrounded by clear supernatant |

## Table 2

Analysis of Digester Elutriate and Mixtures of Primary and Secondary Sludge for Mutagenic Compounds using the Salmonella Bacterial Test without Metabolic Activation.

**Test #1**

Strain # and # of Revertants/ Plates

| Material Tested/ Plate | TA 98 | TA 100 | TA 1535 | TA 1537 | TA 1538c |
|---|---|---|---|---|---|
| (negative control) | 48 | 190 | 16 | 21 | 41 |
| Negative Control [Literature(8)] | 40 | 160 | 20 | 7 | 25 |
| MNNG a (positive control) | - | +++ | +++ | + | NT[d] |
| 0.1 ml Elutriate | 73 | 190 | 16 | 7 | 14 |
| 0.5 ml Elutriate | 47 | 133 | 18 | 26 | 1$ |
| 0.1 ml Sludge b | 30 | 158 | 15 | 39 | 9 |
| 0.5 ml Sludge | 22 | 160 | 29 | 12 | 16 |

**Test #2**

| Material Tested/ Plate | TA 98 | TA 100 | TA 1535 | TA 1537 |
|---|---|---|---|---|
| H20 | 17 | 167 | 14 | 6 |
| 0.5 ml Elutriate | 31 | 202 | 23 | 13 |
| 0.1 ml Sludge | 43 | 180 | 17 | 19 |
| 0.5 ml Sludge | 28 | 168 | 24 | 12. |

a. N-methyl-N'-nitro-N-nitrosoguanidine, 2 µg was spotted onto each positive control plate; results were scored as negative (-) if no increase above background was noted as one, two or three + ’for increases from slightly above background to TNTC.

b. Consisted of 60%secondary digested and 40% primary undigested sludge sterilized by autoclaving.

c. TA 1538 is an optional strain for screening purposes.

d. Not tested.

**Table 3**

Analysis of Digester Elutriate and Mixtures of Primary and Secondary Sludge for Mutagenic Compounds using the Salmonella Bacterial Test with Metabolic Activation[a]

| | Strain # and # of Revertants/ Plates | | | |
|---|---|---|---|---|
| Material Tested/ Plate | TA 98 | TA 100 | TA 1535 | TA 1537 |
| H20 | 30 | 148 | 13 | 3 |
| 0.1 ml Elutriate | 28. | 172 | 13 | 6 |
| 0.1 ml Sludge | 23 | 135 | 7 | 6 |

Metabolic activation employed Rat Liver Homogenate (S-9) induced with Aroclor 1254, a polychlorinated biphenyl mixture, at a concentration of 10 microliters per plate.

Fig. 1 Growth of A. eutrophus in Phosphate-Supplemented Digester Elutriate in the Fermentor

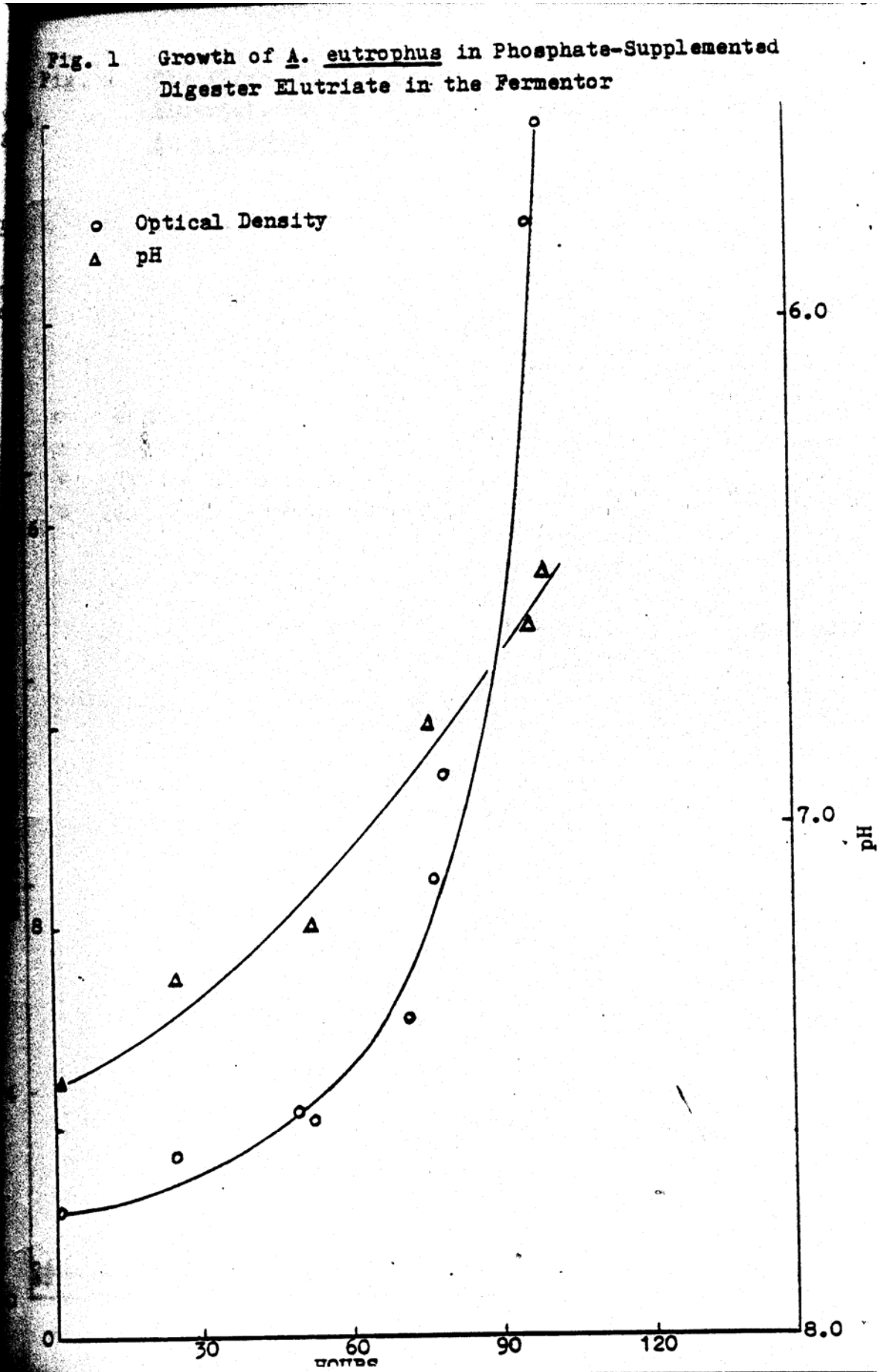
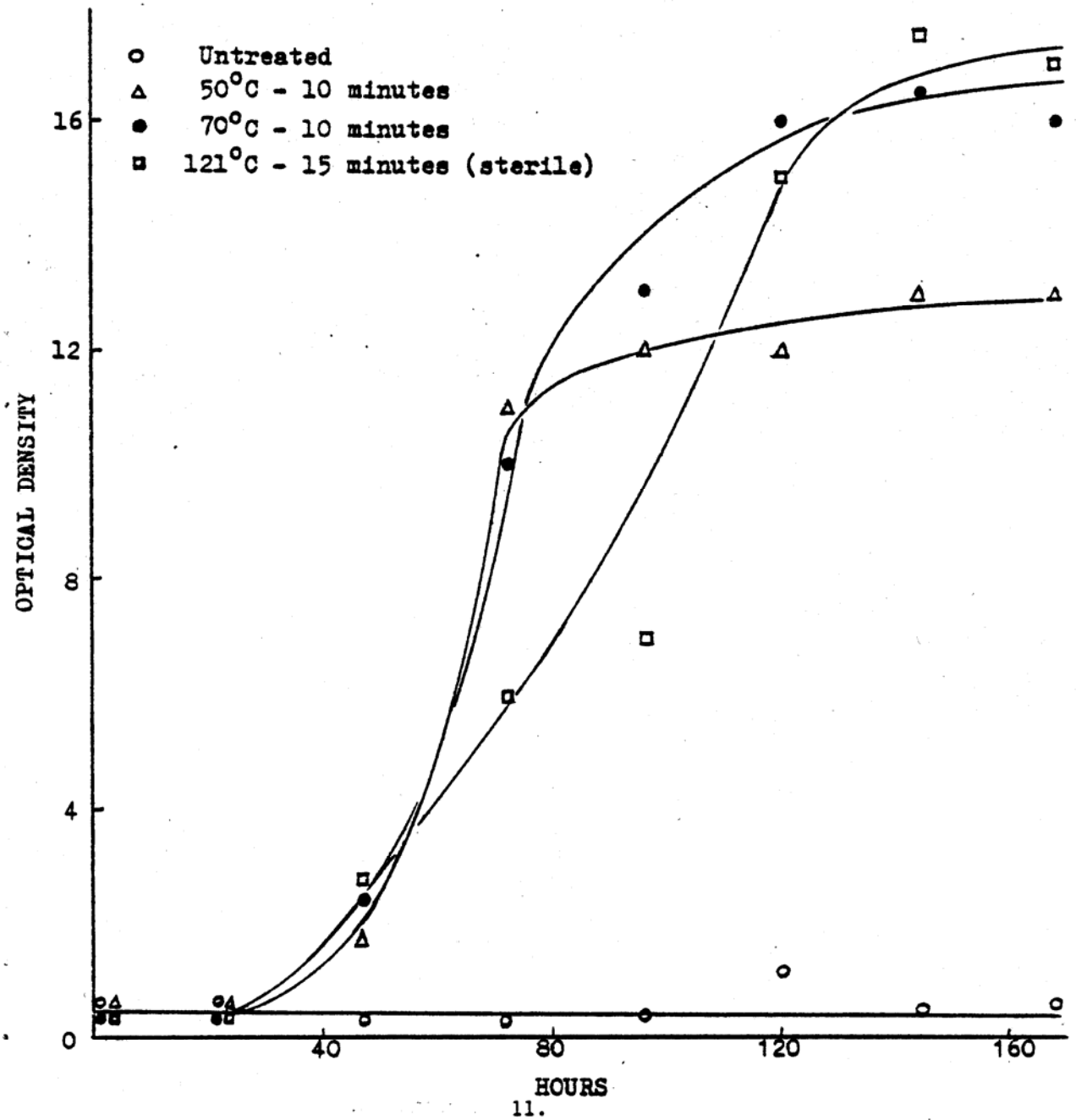


Fig. 2 The Effect of Differential Heating of Digester Elutriate on its Ability to Support Growth of A. eutrophus



## SUMMARY

We previously have demonstrated that the bacterium Alcaligenes eutrophus is an efficient and effective remover of nitrogenous and phosphorus compounds from municipal wastewaters. Further, the biomass generated from growth on wastewater proved to be a source of high quality protein for the growth of chickens. During this phase a quantity of biomass grown in the fermentor on digester elutriate was treated with a variety of conditions in order to find conditions which promoted cellular aggregation, since this would allow easier separation of cellular biomass from the treated wastewaters. Two treatments produced sedimentation of the cells; either addition of alkali to pH 11 or the addition of an anionic exchange resin (Cellex-D) produced aggregation of the biomass *and* clarification of the wastewaters.

Elutriate samples that were unheated and then inoculated with A. eutrophus yielded little growth, while samples heated to 50° and 70°C produced cell densities that were 74% and 94% respectively, as high as a sample sterilized by autoclaving. Thus a heat labile inhibitor of A. eutrophus was present in elutriate.

Samples of digester elutriate, secondary digested sludge and primary undigested sludge were analyzed for the presence of mutagenic substances, using the Ames Salmonella mutagenicity test. Samples were assayed both with and without metabolic activation with rat liver S-9 mix. Tests were negative with all samples and bacterial strains with the possible exception of a marginally positive response with both sludge and elutriate samples tested with strain TA 1537.

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