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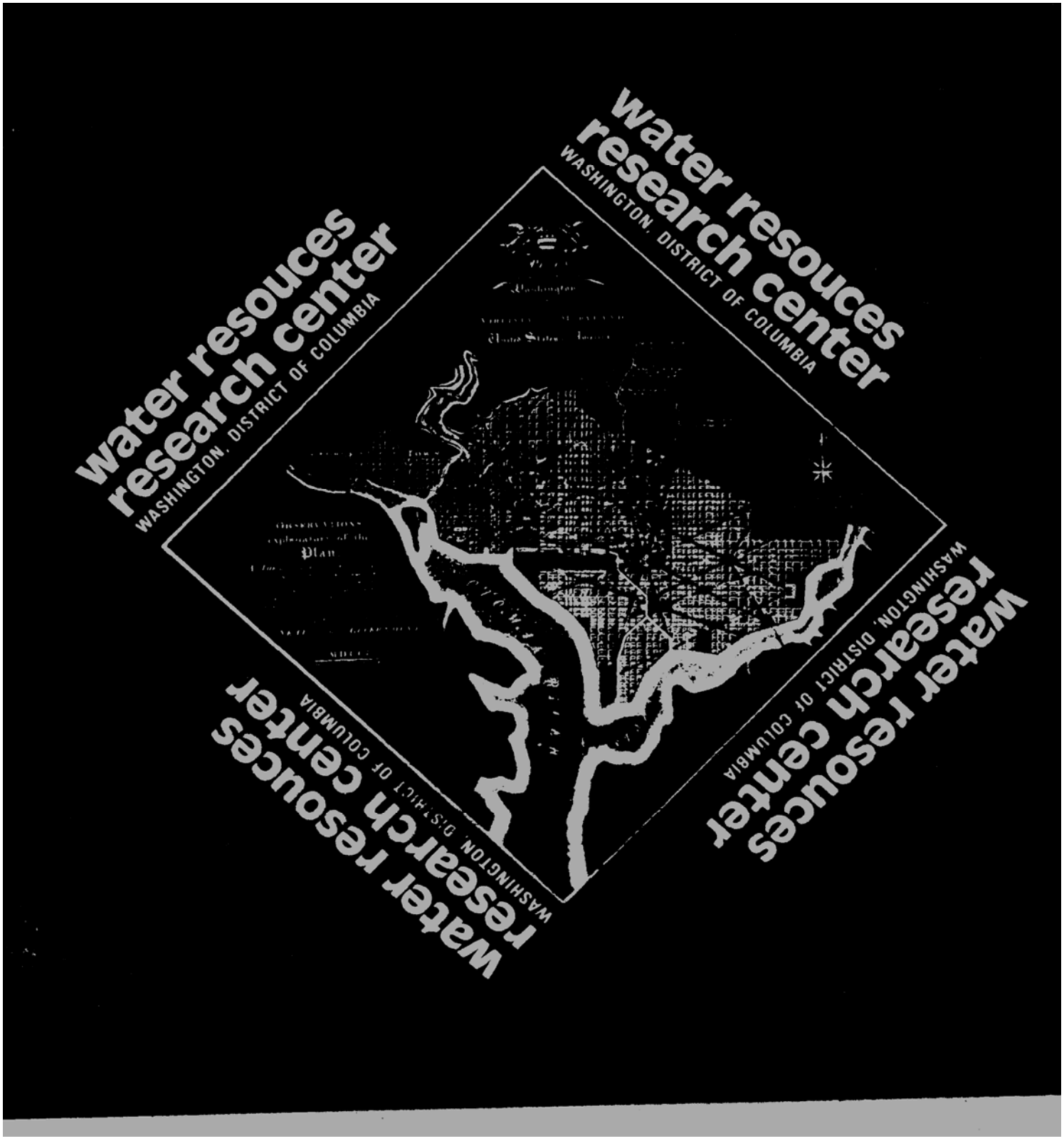

REMOVAL OF EUTROPHIC NUTRIENTS FROM WASTEWATER AND THEIR BIOCONVERSION TO BACTERIAL SINGLE CELL PROTEIN FOR ANIMAL FEED SUPPLEMENTS PHASE II

By

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INTRODUCTION

The entrance into waterways of both organic and inorganic compounds can lead to the undesirable growth of microorganisms. The polluting materials can originate in numerous categories of point or nonpoint sources, but one of the major point sources of microbial nutrients, especially of the inorganic variety, is the final effluent from wastewater treatment plants. The two major eutrophic nutrients present in effluents from municipal treatment plants are nitrogen and phosphorus compounds which, when present in significant concentrations, can lead to algal growth and pollution. The forms 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 10-1596 and phosphorus 1-296 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 chemolithotrophic bacteria the major disadvantages are slow growth rates and inhibition by organics. Exceptions are the hydrogen oxidizing chemolithotrophs, which are all facultative 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 CO_2 and E20 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) make 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 $C0_2$, H_2O and microbial biomass.

A second objective of this research project is 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). An alternative is to produce protein for animal feed by growing microorganisms on waste products such as wastewaters. The major microorganisms employed in these processes are bacteria and yeast.

Some, if not all, hydrogen-oxidizing chemolithotrophic bacteria consist of approximately 70% protein. The one strain studied to date has been shown to possess protein of high nutritional quality, comparable to that of casein (milk protein). These studies were performed with rats (5) and more recently at Catholic University with mice (6). The protein from the strain studied contained significant proportions of lysine, methionine and tryptophan, three amino acids that are required by animals and are deficient in cereal grain proteins.

It is not difficult to envision microbial fermentations similar to those now occurring in' the brewing and pharmaceuti-cal industries taking place in plants, adjacent to and fed by wastewater treatment plants. Conditions would be optimized for the removal of organic and inorganic pollutants by the microbes and hydrogen and oxygen generated electrolytically would supplement organic substrates as energy sources. Since the end products of chemolithotrophic growth are water and cell biomass, no soluble toxic metabolic products are formed. This overcomes a major disadvantage to SCP production from petroleum products, where there is great difficulty in separating the biomass from the petroleum substrate. The cells would be harvested, dried and packaged for animal feed and the concomitant removal of nutrients from the wastewater (and possibly sludge) would constitute an advanced form of wastewater treatment.

During phase I of this project it was demonstrated that the hydrogen oxidizing chemolithotrophic bacterium Alcaligenes 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% for TKN and 84% for total phosphorus. Mixtures of l 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 we have grown A. eutrophus in batch and semi-continuous culture in a fermentor using digester elutriate as the growth medium. The cells were harvested, washed, dried chemically analyzed and employed in diets for chick feeding experiments. Two measures of protein quality were computed from the experimental results. In addition, undigested sludge was employed as a growth medium for A. eutrophus and nutrient removal was determined.

MATERIALS AND METHODS

The major microorganism employed in this study is the facultative chemolithotrophic bacterium Alcaligenes eutrophus (formerly named Hydrogenomonas eutropha). 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 wastewater, and undigested sludge. Obtained samples were used immediately or stored for brief periods at 4^0C until used.

Samples were dispensed normally in 100ml amounts into 300ml baffled Erlenmeyer flasks. When sterilization was employed, samples were autoclaved at 121^oC 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 30-32^0C with shaking at

200rpm. For fermentor cultures, 8-9 liters of undiluted elutriate (containing approximately 300mg/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 fermentor 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_2:1 C02. The temperature was maintained at 30-32°C.

Growth in effluent, elutriate, or effluent-elutriate mixtures was monitored by reading optical densities at 540nm. With A. eutrophus an OD54O of 1.0 corresponds to 0.32mg dry weight of cells per ml. All readings were corrected for initial turbidities present in samples before incubation.

Chemical analyses were performed as outlined in Standard Methods for the Examination of Water and Wastewater Samples were analyzed 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 persulfatesulfuric 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 cellular biomass produced in the fermentor was harvested in a large capacity centrifuge, washed repeatedly with distilled water and lyophilized (freeze dried). The resulting material was a fluffy beige powder; in one case the lyophilized material was darker in color and had a fibrous consistency, possibly due to some thawing during the drying process. This darker material was kept separate from the lighter colored batches.

The lyophilized biomass and associated contaminant particulate material was chemically analyzed for DNA, RNA, protein and total carbohydrate (8). Lipid and inert material was determined by difference.

The lyophilized material was used to prepare diets for chick feeding experiments. Because these studies required the use of newly hatched chicks, which required heated brooder pens with special feeding troughs, the feeding experiments were performed at the U.S.D.A. facility in Beltsville, Md. with the cooperation of Dr. Edward Robel. Complete chick diets were prepared containing 1396 protein, all of which was supplied by the bacterial biomass. Control diets contained 1386 casein protein, 13% casein protein plus arginine, or no protein. Separate test diets were prepared using the lighter and darker colored biomass in order to ensure that any abnormalities present in the darker batch did not affect the outcome of the studies (we were concerned that vacuum pump oil or some other contaminating substance, which may be toxic, may have entered the darker batch during the vacuum drying process).

The five different diets were fed to Cornish crossbred chicks for eleven days, with the exception of the darker experimental diet, which was in sufficient quantity for seven days only. The amount of food consumed and the weight of the chicks was determined at seven and eleven days. These values were used to calculate two measures of protein quality and utilization, the Protein Equivalency Ratio (PER) and the Protein Retention Efficiency (PRE). PER is defined as the gain in weight/protein intake. Using this method Protein *Quality* is calculated as (sample PER/Reference casein PER) x 100. PRE is defined as (test gain - protein free gain/test protein intake) x 18.0.

The effect of treating undigested sludge with A. eutrophus was determined in the following manner. Three dilutions of sludge (1:4, 1:8, 1:20) and three different treatment methods were employed for a total of nine separate experimental *conditions.* Sludge dilutions were made using final effluent as diluent. The three treatment methods were as follows:

Set 1. Autoclaved, inoculated with A. eutrophus, incubated under an atmosphere of

70% H_2, 20% 02, 10% C02

Set 2. Autoclaved, inoculated with A. eutrophus and incubated under air

Set 3. Unsterilized, uninoculated, incubated under H_2, 02, C02.

Samples were removed after four and nine days and were analyzed for numbers of microorganisms, pH, and ammonia and phosphate concentration. Since the heavy concentration of particulate matter *in* the sludge suspensions precluded the use of optical density as measure of bacterial growth, the number of bacteria present *in* sludge media at different time intervals was determined by plate counts. Sludge media samples taken at various time intervals were diluted and 0.1 ml samples were spread onto trypticase soy agar. Plates were incubated at 30^0C and colonies were counted after 36-48 hours.

Results and Conclusions

During Phase II of this project eleven batches of digester elutriate from Blue Plains Treatment Plant were utilized in the fermentor as a growth medium for A. eutrophus. Elutriate was used as the growth medium rather than final effluent because of the need for sizeable quantities of biomass for the chick feeding experiments. In effluent the levels of nutrients present are such that unwieldy quantities of media would be needed in order to obtain the necessary cell mass. In digester elutriate, since the nitrogen levels are approximately twenty times greater than in final effluent, the cell yields would be proportionately higher. The elutriate was used in undiluted form; however, since undiluted elutriate as currently produced at Blue Plains contains an imbalance of N: P, small amounts of potassium phosphate were added to produce N:P ratios of 8:1 in earlier runs and 7:1 in later runs. These ratios are approximately the proportions in which the bacteria will remove and assimilate nitrogen and phosphorus from the medium.

Of the eleven fermentor batches, the amounts of ammonia nitrogen and phosphate phosphorus remaining after growth were determined in eight. Table 1 shows the initial N: P ratio as well as the cell density and percent removal of ammonia nitrogen and phosphate phosphorus at time of harvest. Based on the elemental composition of bacteria, an approximately 8:1 ratio of N:P should be assimilated by the bacteria; thus if the elements are supplied in this ratio, and if all the N and P are in useable form, there should be essentially complete conversion of both elements to cell biomass. The data in Table l show that ammonia removal averaged 8596 and phosphorus removal averaged 92% when an 8:1 ratio of N: P was supplied. Since our previous studies using shake flasks and effluent had shown

ammonia removal to average 9896 when ammonia was the limiting element, we ran the last three fermentor batches with a 7:1 ratio to see if ammonia removal could be increased by reducing the proportion of N:P. This was the case since the average removal levels for ammonia were 89.396 and for phosphorus were 93.7% in the last three batches.

It is important to note that growth in the fermentor had the primary objective of producing cell biomass for the nutritional experiments rather than of optimizing removal of nitrogen and phosphorus. Thus once the cell' density of a fermentor batch seemed to be peaking, the run was halted and the cells were harvested. With the last fermentor run, a significantly higher cell density was obtained and concurrently, ammonia and phosphate removal were 96% and 92%, respectively. In conclusion it appears as if both 8:1 and 7:1 ratios of NO yielded high cell densities and high removal rates for both ammonia and phosphate but the 7:1 ratio may produce slightly higher utilization of ammonia.

Table 2 shows the biochemical composition of the lyophilized bacterial biomass. The lighter and darker batches were analyzed separately in order to determine if any differences were detectable. The compositions of both batches were almost identical, with both containing 56% protein. While 56% protein content is quite high, it is lower than we expected, based on earlier analyses with cells grown in chemically defined media, where the protein content was approximately 70% of dry weight (6). We are rather certain that the reason for the lower protein content of wastewater grown cells is that these cells were supplied with an excess energy and carbon supply (as the gases H , 0_2, CO_2) while the nitrogen supply was limiting. Under these conditions it has been shown that A. eutrophus synthesizes and accumulates large amounts of a lipid-type polymer, polybetahydroxybutyric acid. We believe the presence of this polymer is responsible for the high lipid content and lower protein level in the elutriate grown biomass.

Tables 3 and 4 summarize the results of the feeding experiments using newly hatched chicks. Table 3 shows the results after 7 days while Table 4 shows 11 day results. With chicks, the nutritional value of a food is normally determined by measuring weight gain with time and comparing experimental feeds with complete reference diets. In these experiments a. protein free diet was constructed based on the nutritional requirements of chicks (9) and protein

was then added to a final concentration of 13% using the dried bacterial biomass, reference casein, or casein supplemented with arginine. Protein quality of the bacteria as compared to reference Casein was determined by the ratio of the PER values, converted to percent. Both the light and the dark bacterial biomass yielded similar values of 88% and 87% of the value obtained with casein and slightly less than the 94% observed with casein supplemented with arginine. The chicks fed a protein free diet lost weight over the course of the experiment.

Table 4 shows the results after 11 days. No result with the darker biomass are shown because that feed was consumed after seven days the light wastewater grown biomass again yielded a protein value of 88% as compared to casein.

In order to study the ability of A. eutrophus to degrade the organic components in sludge, remove and assimilate its eutrophic nutrients, and modify its appearance and odor, mixtures of undigested sludge and final effluent were treated under various conditions. The mixtures employed were 1:4, 1:8 and 1:20 ratios of sludge to effluent. Sets of the 3 mixtures were treated as follows: Set 1 was sterilized by autoclaving, inoculated with A. eutrophus and incubated under an atmosphere of 70% H2, 20'6 0 an o $C0_2$. Set 2 was sterilized and inoculated as in set i but was incubated under air. Set 3 was neither sterilized nor inoculated with A. eutrophus and was incubated under the same gas atmosphere as set 1. Samples were removed after 4 and 9 days and were analyzed for numbers of microorganisms, ammonia, phosphate and pH. In the sterilized samples incubated under the gas mixture, A. eutrophus cells increased from $_9$5 x 10^7 orgs/ml in each of the sludge dilutions to 1.2 x 10 /ml in the 1•20 mixture, 4.1 x 10 in 1:8 and 8 x 10^9 in the 1:4 mixture. Thus growth was substantial and was proportional to the concentration of sludge. In set 2, with the air atmosphere, growth of A. eutrophus was significantly less than in set 1 and was not related to the concentration of sludge, reaching 2.0 to 6.4 x 10^8 orgs/ml in all 3 concentrations. In set 3 microbial populations reached lower levels than in sets 1 and 2; presumably no hydrogen utilizing bacteria capable of efficient growth in the time allowed were present in the sludge-effluent mixtures. This latter set also shows that organisms naturally present in sludge and effluent are inefficient utilizers of sludge-effluent nutrients.

Samples from set 1 showed 89-92% ammonia removal after growth of A. eutrophus. Set 2 showed net increases in ammonia concentrations from 25-67% above the original level while set 3 samples ranged from an increase' of 3% to

a decrease of 42% in ammonia levels. The results seen in sets 2 and 3 show that the presence of both A. eutrophus and the H2, 0_2, CO2 gas mixtures are essential to efficient nutrient removal from sludge-effluent mixtures.

Phosphorus results paralleled those with ammonia. Concentrations of phosphate in set 1 ranged from 2% to a fraction of 1% of the original level. Phosphate concentrations in sets 2 and 3 were 10-20 times higher than in set 1.

Table 1

Removal of Ammonia Nitrogen and Phosphate Phosphorus from Elutriate ring Growth of A. eutrophus in the Fermentor

Batch #	Final OD	Initial N:P	% Ammonia N Removed	% Phosphate P Removed
1	12.5	8:1	85	94
2	11.3	8:1	84	85
3	11.3	8:1	84	90
6	13.8	8:1	92	98
7	15	8:1	80	93
8	12.5	7:1	86	93
9	13.8	7:1	86	96
11	18.8	7:1	96	92

Table 2

Biochemical analysis of the dried bacterial biomass grown on digester elutriate

Component	Light Material (% of Dry Weight)	Dark Material (% of Dry Weight)
RNA	8.7	8.8
DNA	1.3	1.2
Protein	56	56
Total Carbohydrate	3.2	3.2
Non-nucleic Acid Carbohydrate	Not detectable	Not detectable
Lipid and Inert Material	30.8	30.8

Table 3

Nutritive quality of A. eutrophus biomass grown in wastewater (digester elutriate) as a protein source in chick feed

- 7 day results

Protein Source	No. Chicks	Feed Intake gms/ chick	Weight Gain gms/chick	Protein Intake gms/chicks	PER b	Protein Quality (%)[b]	PER (%)[b]
Bacteria (dark)	2	88	42	11.44	3.66	88	71.7
Bacteria (light)	4	68	33	8.84	3.64	87	74.5
Casein	21	81	44'	10.53	4.17	100	81.3
Casein + Arginine	21	94	49	12.22	3.93	94	77.4
None	21	41	-3.6	-	-	-	-

a.. The 4 protein supplemented diets each contained 13% protein

b. See Materials and Methods for the definition of these values

Table 4

Nutritive quality of A. eutrophus biomass grown in wastewater (digester elutriate) as a protein source in chick feed

- 11 day results

Protein Source	No. Chicks	Feed Intake gms/chick	Weight Gain gms/chick	Protein Intake gms/chick	PER	Protein Quality (%)	PER (%)
Bacteria (light)	4	108	41	14.04	2.92	88	57.5
Casein	21	162.7	70.4	21.1	3.33	100	63.4
Casein + Arginine	21	174	79.3	22.6	3.33	100	62.2
None	21	108.5	-3.9	-	-	-	-

SUMMARY

The bacterium Alcaligenes eutrophus was grown in phosphorus supplemented digester elutriate from the Blue Plains Wastewater Treatment Plant using a microbial fermentor. 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.

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, 0_2 CO_2 atmosphere, growth of A. eutrophus was substantial and was proportional to the sludge concentration, reaching 8 x 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 89-92% ammonia removal and 98-99% phosphate removal from gassed cultures. The cultures incubated under air showed increases in ammonia levels of 25-67% and phosphate reductions of approximately 90%.

The bacterial system employed in these studies appears to be an efficient means of removing nitrogenous and phosphorus pollutants from final effluent, digester elutriate and undigested sludge resulting from wastewater treatment. The bacterial biomass produced during elutriate treatment appears to possess high nutritive value as a protein source for chick feed.

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