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Amino Acid Composition and Microbial Contamination of *Spirulina maxima*, a Blue-Green Alga, Grown on the Effluent of Different Fermented Animal Wastes

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Tremendous energy is expended to make fertilizers used to grow food for livestock which in turn are slaughtered to feed human beings; yet large amounts of those nutrients are lost through animal wastes. The nutrient value of animal wastes has been verified repeatedly (ANTHONY 1971; BATTACHARYA & FONTENOT 1965; BRUGMAN et al. 1974; EL-SABBAN et al. 1970; YOSHIDA & HOSHII 1963). In the past, manure has been spread as a crop fertilizer, but land space is becoming increasingly less available for this type of waste disposal.

Recently, the possibility of reclamation of the nutrients from animal wastes as a resource for animal production has been studied (ANTHONY 1970, FLEGAL & ZINDEL 1971, NOLAND et al. 1955). There is a need for an indirect way to recycle nutrients from wastes. Anaerobic fermentation of manures provides methane as a useful energy source, but the residue contains N and inorganic elements. Utilization of the residues from methane generation as a nutrient source for the growth of algae may provide a means for accomplishing this objective.

The nutrient composition of Chlorella sp. has been investigated (COMBS 1952, DAM et al. 1965, MORIMURA & TAMIYA 1954, POWELL et al. 1961). Chlorella sp. and other algae can be grown on sewage (COOK 1962, KOSARIC et al. 1974, GOLUEKE & OSWALD 1965, GRAU & KLEIN 1957, HINTZ et al. 1966, OSWALD & GOTAAS 1957), swine waste (CHUNG et al. 1978, STANLEY & MADEWELL 1975) or poultry waste (RONALD 1972).

Arthrospira platensis has been consumed since ancient times of the Chad Republic and Spirulina maxima is used for human food in Mexico (KROGMANN 1981). Both species are well known for their high protein content (55-70% on a dry basis), high digestability, low toxicity, easy growing and easy harvesting (BOURGES et al. 1971, CLEMENT et al. 1967, GUERINDUMARTRAIT & MOYSE 1975, CHUNG et al. 1978; YAP et al. 1978). Differences exist in the amino acid sequence of ferridoxins in A. platensis and S. maxima (KROGMANN

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1981), illustrating that these two similar cyanobacteria are genetically different. The purpose of this research was to determine the chemical composition of fermented liquors of different animal wastes, and the amino acid content ot Spirulina maxima grown on different wastes. Also, the heterotrophic microorganisms associated with the lyophilized algae were enumerated.

MATERIAL AND METHODS

Fermentation process. Source and treatment of wastes. Swine, cattle and poultry feces were collected from Cornell research farms; sewage sludge was from the municipal sewage disposal plant. All waste samples were dried overnight in a 60 C oven. Swine blood slurry was kindly supplied by National Renderers' Association.

Fermentation. To each 20-L size o.d. polyethylene carboy was added 1.0 Kg of dry swine manure, cattle manure, dry sewage sludge or 0.7 Kg of poultry manure or 1.0 Kg of swine blood slurry. The carboy was then filled with water and shaken to mix well. After 14 days of anaerobic fermentation at 25 C, the supernatant liquor was filtered first through cotton cloth and then through Whatman No. 40 filter paper for use as the algae nutrient source and for chemical analysis.

Production of the algae. Growing conditions. Six algae tanks made of plywood and measuring 116 x 56 x 9 cm with available capacity of 54 L, were lined with polyethelene sheeting. Temperature was maintained at 30 + 2 C by circulating water through submerged glass tubing. Air was bubbled from an air pump into the culture medium to circulate the contents of the culture tank. The lighting from fluorescent lamps was kept at about 500 foot candles at the water surface.

Growth media. Each liter of S. maxima basal medium contained 2.500 g $NaNO_3$, 12.000 g $NaHCO_3$, 0.090 g $MgSO_4$, 0.100 g K_2HPO_4, 1.00 g NaCl, 0.001 g EDTA and 1.000 mL microelement solution. Each liter of microelement solution contained 2.500 g H_3PO_3, 0.800 g $FeCl_3.6H_2O$, 0.200 g $MnSO_4.H_2O$, 0.200 g $NaSeO_3$, 0.150 g $CoCl_2.6H_2O$, 0.020 g MoO_3, 2.500 g $CaCl_2$ and 0.100 g $ZnSO_4.2H_2O$. The initial composition (%, w/v) of various supplemental animal wastes as culture media is shown in Table 1.

Among those compounds, $NaNO_3$ (or Urea), $NaHCO_3$, K_2HPO_4, and NaCl were of commercial grade and the rest were of analytical grade. Tap water was used as the solvent.

Growth and harvest. All experimental culture tanks were inoculated with 1000 mL of algal growth with a light transmittance of 10% at wavelength of 560 nm. The liquor of fermented wastes was continuously delivered into the culture tank by an automatic pumping machine to maintain a continuous growth. Algae were harvested when the cloudiness of growth had a transmittance of less

TABLE 1. Initial Composition (%,w/v) of Supplemented Animal Wastes for the Growth of Spirulina maxima

Nutrients	Waste				
	Cattle Manure	Sewage Sludge	Swine Manure	Swine Blood Slurry	Poultry Manure
KH_2PO_4	0.03	0.03	0.01	0.03	0.01
NaCl	0.08	0.08	0.04	0.08	0.04
$NaHCO_3$	0.30	0.30	0.30	0.30	0.30
Urea	0.03	0.03	0.00	0.02	0.00
Fermented Liquor	3.70	7.40	3.33	5.56	3.15

than 30%. It was then passed through screen mesh, washed with distilled water several times to remove salts, and collected in polyethylene bags for lyophilization.

Determination of chemical elements. Samples were analyzed for orthophosphate-phosphorus by following Standard Methods of Water and Waste (APHA 1971). K, Na, Ca, Fe, Cu, Zn, Co, Mn, and Cr were measured by atomic absorption spectrophotometry.

Chemical analysis of protein. Nitrogen was determined by the Kjeldahl method. Protein was assumed to contain 16% nitrogen and consequently a factor of 6.25 was used to convert nitrogen value to the protein content in the samples. For the determination of amino acid composition, algal samples were hydrolyzed in constant boiling 5.7 N HCl in sealed tubes at 110 C for 24 h. The hydrolysates were filtered and HCl was evaporated under vacuum at 40 C, brought to 5 mL of volume with pH 2.2 sodium citrate buffer and the amino acid concentrations were analyzed with an Amino Acid Analyzer.

Microbiological analyses. Algal dilution: One g of freeze-dried algae was transferred to 99 mL sterile water and the mixture was vortexed. Ten-fold serial dilutions were then made and kept in an ice bath until use.

Plating method. Aliquots of 5 drops (0.025 mL per drop) from microdropper of each dilution prepared were placed on algae plates in triplicate and incubated at 37 C for various times (SHARPE & KILSBY 1971). Isolates were identified by gram stain to confirm morphology and standard biochemical reaction (BREED et al., 1957). All counts reported were the average obtained from the appropriate triplicate set.

Enumeration of total heterotrophic aerobes and anaerobes was obtained by plating aliquots on 10% cow blood agar (Triptic Soy Base, Difco) and then counting the bacterial colonies 2 days later. Anaerobiosis was achieved by incubation of plates in BBL GasPak Jar with added "GasPak hydrogen and carbon dioxide producer envelope".

For spore-forming bacteria (Bacilli and Clostridia), cow blood agar was also employed. Ten mL of each algal suspension were transferred to sterile test tubes and placed in a boiling water bath for 10 min. The tubes were then cooled to room temperature and plated. Bacilli were enumerated 2 days later by presence of catalase decomposing H_2O_2 solution into water and oxygen. Anaerobic spore-forming bacteria were enumerated 6 days later and identified by absence of catalase.

Eosin-Methylene Blue Agar (Difco) ws used for detecting coli-aerogenes organisms. The plates were incubated for 24 and 48 h and typical coli-aerogenes colonies enumerated. Selected plates were placed in lactose broth to confirm the production of gas. For Salmonella and Shigella, MacConkey agar was used in conjunction with Salmonella Shigella agar.

Yeasts and molds were enumerated on Potato Dextrose Agar (Difco) for which the pH was adjusted to 3.5 with lactic acid. The plates were counted 3 days later.

RESULTS

The comparison of the nutrients from different animal excreta-fermented liquors and synthetic medium is shown in Table 2.

TABLE 2. Comparision of the Chemical Composition of Fermented Liquors and Synthetic Medium (mg/L)

Item	Waste					
	Cattle manure	Sewage sludge	Swine manure	Swine blood slurry	Poultry manure	Synthetic medium[a]
K	116	12	366	265	592	287
Na	38	31	111	84	214	4400
Mg	60	32	225	20	54	14
Ca	171	267	174	72	42	0.7
Fe	9.1	3.0	38.0	0.7	2.5	0.2
Cu	0.04	0.02	0.02	0.02	0.04	0.03
Zn	0.44	0.16	0.08	0.02	0.10	0.03
Co	0.02	0.12	0.09	0.12	0.12	0.04
Mn	0.12	0.26	1.15	0.01	0.10	0.07
Cr	0.002	0.012	0.050	0.002	0.047	0.011
PO_4-P	106	18	350	19	22	228
NH_3-N	71	25	570	178	1480	330
pH	6.5	6.7	6.7	5.6	6.2	8.2

[a] Theoretical value

Large differences among fermented liquor sources were noted in concentrations of all nutrients measured. For example, poultry manure contained almost 60 times as high a concentration of K as sewage sludge and 3 to 60 times higher concentration of NH_3-N than any of the other media.

There were no important differences among the amino acid compositions of algae grown on different nutrient sources (Table 3). All were low in methionine and rich in glutamic acid, aspartic acid, arginine, alanine and leucine. The crude protein content ranged from 71.8to 60.1% with an average of 63.5%.

All of the waste-grown algae were contaminated with various microorganisms (Table 4). Yeast, fungi and spore-forming bacteria were present in significant numbers in algae grown on all fermented wastes as well as on synthetic medium using urea as a nitrogen source. Shigella and Salmonella were only found in algae grown.

DISCUSSION

In anaerobic fermentation, the microorganisms can transform the waste materials into useful nutrients, which vary with the composition of the waste sources and the duration of fermentation time. The value of manure lies not only in its mineral content, but also, more importantly, in its organic matter content. The concentration of trace elements present in the fermented liquors may be a benefit or detriment in algal production. The data in Table 2 indicate that the mineral concentrations in different fermented liquors were extremely variable. All of the elements were much lower than the tolerance doses for Spirulina maxima. The highest NH_3-N concentration was found in poultry liquor due to the high uric acid content in poultry manure. Since the nitrogen content seems to be the most important limiting factor for the growth of algae, poultry manure is a good source of the nutrient for commercial production of the algae. Swine fermented liquors were high in concentration of inorganic phosphorus, ammonia nitrogen, magnesium and manganese and would serve as a satisfactory nutrient source for algal growth. The nutrients from fermented liquors of dry municipal sewage sludge, swine blood slurry and cattle manures were all limited in P and N.

The composition of Chlorella sp. may depend on the methods of growth, supporting media, strain as well as assay procedure (SCHIELER et al. 1953, SPOEHR & MILNER 1949). The crude protein content of S. maxima grown on different fermented liquors in this study ranged from 60.1% in cattle manure-grown algae to 71.8% in poultry manure-grown algae. It appeared that the protein content of algae was positively correlated with the N content of the supporting medium. The above finding agrees with that of KOSARIC et al. (1974). The amino acid pattern found in this study was also similar to that of CLEMENT et al. (1967).

Although anaerobic fermentation will inhibit most of the aerobic bacteria in wastes, surviving aerobic microorganisms in fermented liquors may proliferate in the culture pond. The competition between S. maxima and bacteria or fungi is not fully known. Exotoxins or metabolites from some microorganisms might inhibit algal growth. Furthermore, a change in the pH value in

TABLE 3. Amino Acid Composition of Spirulina maxima Cultivated from Different Fermented Wastes (g of amino acid/16 g N)

Amino Acid	Waste						
	Cattle manure	Sewage sludge	Swine manure	Swine blood slurry	Poultry manure	Synthetic medium[a/]	Ave.
Alanine	8.05	6.88	8.02	7.70	7.72	7.92	7.72
Arginine	7.19	7.63	7.13	6.82	7.03	7.36	7.19
Aspartic	9.67	10.4	10.6	9.44	9.47	9.94	9.92
Glutamic	13.4	12.0	14.2	13.3	13.5	14.6	13.5
Glycine	4.5	4.2	5.1	4.8	5.0	4.7	4.7
Histidine	1.7	1.5	1.8	1.6	1.7	1.9	1.7
Isoleucine	6.2	5.4	6.3	5.9	5.9	6.2	6.0
Leucine	9.1	8.1	9.3	8.7	8.8	8.1	8.7
Lysine	3.9	3.7	4.1	3.9	3.9	4.9	4.1
Methionine	1.4	2.0	2.0	2.1	2.3	2.0	2.0
Phenylalanine	5.0	4.3	5.0	4.7	4.9	5.2	4.9
Proline	4.0	3.6	4.1	3.7	3.9	4.1	3.9
Serine	4.6	4.2	4.8	4.5	4.6	4.0	4.5
Threonine	5.0	4.5	5.2	5.2	4.9	4.8	4.9
Tryptophan	1.3	1.3	1.1	1.2	1.1	1.2	1.2
Tyrosine	4.2	3.7	4.1	4.0	3.6	4.1	4.0
Valine	6.2	6.3	7.2	6.9	4.9	5.9	6.3
Total Amino Acid	95.6	89.8	99.9	94.5	93.3	96.8	94.9
Dry Matter(%)	94.7	94.1	94.0	92.8	92.7	93.5	93.6
Crude Protein(%)	60.1	60.6	63.2	62.1	71.8	63.1	63.5

a/ Nitrate as Nitrogen source.

TABLE 4. Microbial Flora of the Lyophilized *Spirulina maxima* Grown on Different Fermented Liquors (Colony Counts/Gram of Dry Algae)

	Coli-Aerogenes	Salmonella and Shigella	Total Heterothrophic		Spores		Yeast and Fungi
			Aerobes	Anaerobes	Bacillus	Clostridia	
Cattle Manure	5.80×10^3	absent	2.91×10^5	1.60×10^5	7.28×10^3	1.08×10^4	1.45×10^4
Sewage Sludge	3.17×10^4	absent	7.21×10^4	2.90×10^4	7.32×10^3	8.70×10^2	4.73×10^3
Swine Manure	4.73×10^4	5.02×10^3	5.46×10^5	4.08×10^5	1.45×10^4	8.00×10^3	4.37×10^4
Swine blood slurry	1.09×10^4	7.28×10^2	2.91×10^6	1.75×10^6	1.30×10^4	7.64×10^3	2.55×10^3
Poultry Manure	9.10×10^3	absent	4.00×106	1.31×10^6	3.13×10^5	2.18×10^3	2.08×10^3
Synthetic Medium[a/]	1.45×10^2	absent	3.64×10^5	1.45×10^4	5.75×10^2	4.37×10^2	2.18×10^2
Synthetic Medium[b/]	9.10×10^1	absent	2.18×10^4	1.05×10^3	absent	absent	absent

a/ Urea as Nitrogen source.
b/ Nitrate as Nitrogen source.

the pond due to the growth of microorganisms might affect the growth of S. maxima. Most of the bacteria can survive the lyophilization process; fungi are more susceptible than are bacteria to this process.

The microflora remained at low levels in the algae cultivated in synthetic chemical media with nitrate as the N source. In contrast, a much higher content of microorganisms (especially spore-forming bacteria and fungi) was detected in those grown on fermented animal wastes with urea as the N source. This suggests that ammonia might be favoring the growth of microorganisms in the algae culture tank. On the other hand, the growth of Salmonella and Shigella was independent of the nitrogen source or concentration in the tank. Due to the high protein content of the algae and the concept of energy recycling, the use of fermented waste to grow algae for animal feed or human food is of considerable potential importance. Additional studies must be done to determine the public health hazard in using waste-grown algae as a nutrient source for animals and humans.

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