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Kinetics of Biomethanation of Solid Tannery Waste and the Concept of Interactive Metabolic Control

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ABSTRACT

Anaerobic digestion of calf skin collagenous waste was optimized for a batch process based on accelerated maximal methane yield per gram of input volatile solid. A kinetic analysis with respect to changes in the levels of volatile solid, collagen, amino sugars, amino acids, hydroxyproline, ammonium ions, and volatile fatty acid were followed for a period of 80 d. Distinct metabolic phases included an initial high rate collagenolysis for 4 d, with 50% degradation and was followed by an acidogenic phase between 4–12 d with voltatile fatty acids levels increasing to 215 mmol/L. Subsequently methanogenesis ensued and was maximal between 12–24 d when volatile fatty acids attained steady state levels. During the period of 80 d, the overall decrease in volatile solid level was 65%, whereas the collagen level declined by 85% with 0.45 L of methane yield/g of volatile solid degraded. Based on the levels of various metabolites detected, the concept of interactive metabolic control earlier proposed has been validated.

Index Entries: Biomethanation, of tannery waste; anaerobic digestion, skin collagen; metabolic control; pollution control; methanogenesis, mixed culture; tannery waste, skin collagen; biomethanation; anaerobic digestion; pollution control; bioenergy, metabolic control; mixed culture; methanogenesis, collagenolysis.

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INTRODUCTION

Research and development have been intensive in recent times in the area of nonpolluting renewable energy sources in response to the ever increasing global energy demands (*1–5*). Renewable biomass is classified into primary, secondary, and tertiary sources and among the conversion technologies available, the biological process of anaerobic digestion offers several advantages, such as:

1. Complete conversion of available organic matter, with concomitant energy recovery;
2. A stabilized solid sludge which does not undergo any further spontaneous degradation; and
3. Effective recycling of inorganic nutrients.

The process of anaerobic digestion is comprised of the conversion of biopolymers to gaseous products rich in methane and carbon dioxide in the absence of oxygen brought about by the simultaneous action of a complex microbial consortium (*6,7*). Anaerobic digestion of secondary and tertiary wastes, viz., of agricultural residues, industrial wastes, urban refuse, and sewage sludge has become important to control the release of toxic pollutants into the environment (*8–12*). A comparison of the composition and methane fermentation of some primary biomass, such as grasses and other traditional tertiary biomass substrates under mesophilic conditions, was reported by Klass (*13*).

Leucaena leucocephala, a drought-resistant legume, was identified in earlier studies from this laboratory as a high rate-biomethanating substrate and a viable energy farm based on a plantation of *L. leucocephala* was proposed (*14*).

Biomass substrates being complex, attempts have been made to explain some of the microbiological and biochemical aspects of cellulose degradation by culture enrichment method (*15–17*), and coculture studies of pure methanogen with fermenting bacteria by Lathan and Wolin (*18*) and Weimer and Zeikus (*19*). Even for pure cellulosic substrates, assessments were made only on the extent of degradation and biomethanation over specified periods. Studies on the kinetic analysis has been restricted to cow manure degradation by Hashimoto (*20*). In view of the complex chemical nature and the extent of biodegradability varying among different biomass substrates, the need for kinetic analysis and related chemical and biochemical investigations has become increasingly important, since such an approach has not been seriously attempted. In the present investigation the anaerobic digestion of a proteinaceous biomass is described for the first time, to understand the biochemical nature of the process. Indication of the regulatory nature of biomethanation earlier noted with the cellulosic substrate *L. leucocephala* (*21*) is confirmed in the present investigation and results are presented for the operation of interactive metabolic control during active biomethanation.

MATERIALS AND METHODS

Glucose, galactosamine, leucine, hydroxyproline, gelatin, and ninhydrin were from Sigma Chemical Co. (St. Louis, MO). All other chemicals used were of AR grade and solvents were distilled prior to use.

Substrate Feedstock

Calf skin shreddings procured from local tanneries were suitably desalted, scudded, dehaired by enzymatic treatment, cut into small pieces, dried, and pulverized before using as input feed stock.

Source of Inoculum

Anaerobic sediments from tannery effluents contained in a tank situated in a local tannery was the main source of inoculum. A suitable mixed inoculum was formulated using the tannery effluent and slurry from an ongoing semicontinuous digestor degrading leafy biomass. This inoculum was stored up to 2 mo at 4°C in airtight plastic cannisters.

Experimental Design

Laboratory studies were carried out in 2-L glass reactors equipped with input and output ports, gas collection tube with a pressure head to adjust the level of water in the collection tube, and provided with a gas vent tube. Batch reactors were operated at 30 ± 5°C with pulverized skin shreddings as input feed at four different volatile solid load rates, viz., 7.5, 15.1, 18.9, and 22.7 g vs per unit volume of the digester designated as 1, 2, 3, and 4, respectively. Mixed inoculum described above, was added to one-third of the digestor volume and adjusted to 1.8 L with 0.01*M* potassium phosphate buffer, pH 7.4. Gas production and methane yield was monitored for a period of 80 d. Kinetics of degradation of solid tannery waste was studied in 1-L saline bottles operated with one-third volume of mixed inoculum and skin material as input feedstock at 20 g (dry wt)/L medium. Total volume was made up to 0.9 L. Anaerobicity was maintained by purging nitrogen into the bottles and then sealing them airtight. Biochemical and gas analysis was carried out corresponding to the first 10 d, subsequently once in 2 d up to the 26th d, and then at 4-d intervals up to the 40th d.

Chemical and Biochemical Analysis

Collagen was estimated from the hydroxyproline content of the acid hydrolysate of the remaining undigested solid waste. The contents of the saline bottle were centrifuged at 7000*g* for 20 min and the supernatant was stored and used for other analysis, whereas the pellet was suspended in 10 mL of 6*N* HCl, flushed with nitrogen and hydrolyzed at 100°C for 12 h. The acid hydrolysate was separated from the particulate matter by

centrifugation at 7000*g* for 10 min, evaporated to remove all the acid and reconstituted in 2 mL of deionized water, and hydroxyproline content was estimated using the method of Neuman (*22*). Protein was estimated by the Biuret method (*23*), using gelatin as the standard. Estimation of total solids (TS), volatile solids (VS), and volatile fatty acids (VFA) were done by standard methods (*24*). The VFA level is reported as acetic acid equivalents in mmole/L. Amino sugars (*25*), amino acids (*26*), and ammonium ions (*27*) were estimated in the clarified supernatant of the digestor slurry after a low spin centrifugation to remove undigested feed material and other particulate matter. Gas analysis was done using a Tracor (Austin, TX) model 540 gas chromatograph attached to a Nelson interface for online quantification of peak area. Methane was detected using flame ionization detector, whereas hydrogen and carbon dioxide were analyzed using the thermal conductivity detector.

RESULTS AND DISCUSSION

Proteinaceous wastes from tanning industry and slaughter houses form a major pollutant. Proteinaceous wastes have not so far been considered systematically for anaerobic digestion, whereas a report on gelatin degradation is related to studies on the effect of addition of other polymers on the extent of its degradation (*28*). Skin protein collagen is structurally unique compared to other proteins and occurs as an enmeshed network of collagen fibers embedded in a proteoglycan assemblage. Although proteins in general are degraded by specific classes of proteases, collagen along with its ground substance is degraded by collagenases and other accessory matrix degrading enzymes. The present study was undertaken to understand the nature and kinetics of anaerobic digestion of solid tannery waste. The sample was prepared carefully by removing extraneous matter in order to ensure meaningful correlations between the amount of input substrate and the course of degradation.

A preliminary assessment of the anaerobic digestion of solid tannery waste in a batch process and the effect of different mixed inocula on the biomethanation efficiency was presented earlier and a viable inoculum for degrading solid tannery waste has been formulated (*29*). Using this inoculum, results on the optimization of the input load rate for batch process are reported here. Also, at the optimal VS load rate, detailed kinetic analysis of proteinaceous waste has been done based on chemical and biochemical parameters.

For assessing the optimum VS load rate, batch reactors were operated at four different input VS level ranging from 7.5–23.0 g/L reactor and degradation over a period of 80 d was monitored by estimating the remaining VS in the digester and correlating with the total gas and methane content thereof. The gas yields from the four reactors designated as 1, 2, 3,

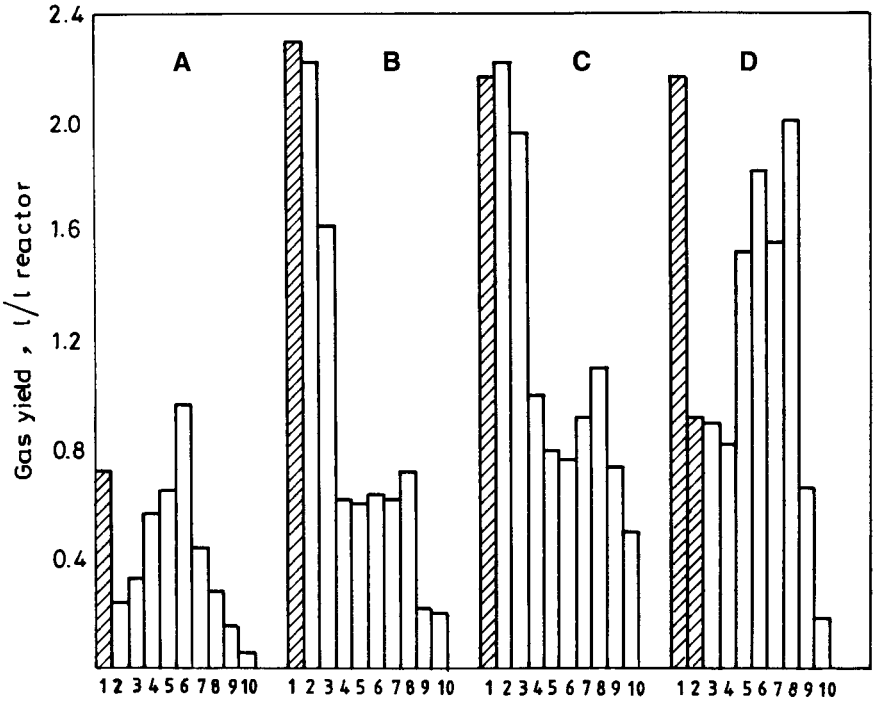

Fig. 1. Gas yields during anaerobic digestion of calf skin trimmings. Batch reactors were operated for 80 d at four different input VS load rates. **A,** 7.5; **B,** 15.1; **C,** 18.9; and **D,** 22.7 g per unit volume of the digester. One unit on *x*-axis represents 8 d. Shaded regions denotes carbon dioxide rich phase.

and 4 with an input VS of 7.5, 15.1, 18.9, and 22.7 g/L reactor, respectively, is shown in Fig. 1. During the initial period, which varied from 4 d for reactor 1, to 12 d for reactor 4, the output gas had a maximal carbon dioxide content (90%). Methanation commenced immediately after this period at a low rate, reaching maximal levels at different periods for the reactors at varying load rates. Reactors at the lowest and highest load rates attained maximal methanation between 40–48 d, with gas yields of 0.96 and 1.82 L/L reactor during this peak period, before declining gradually and these VS load rates were not at optimal level. In contrast, in reactors 2 and 3, after an initial phase of carbon dioxide generation that was 0.83–1.1 L/L reactor in the first 8 d, a high rate of methanation commenced immediately after, reaching maximal methane output within 24 d and thereafter attained a steady level of output per day for a prolonged period that was 24–60 d at 15.1 g of VS load rate, whereas methane output extended up to the entire period of observation of 80 d at 18.9 g of VS load rate. Subsequently, methanation declined abruptly to almost negligiable levels in these two reactors. The results, given in Table 1, indicate that the total gas and the methane yield increased proportionately with increasing load rate, except that at

Table 1
Anaerobic Digestion of Solid Tannery Waste
at Different Input Load Rates, Batch Process[a]

Evaluated parameters	Reactors with different input load			
	1	2	3	4
Input, g dry wt	8.0	16.0	20.0	24.0
TS, input, g	7.59	15.19	18.98	22.78
VS, input, g	7.56	15.12	18.9	22.68
VS, in inoculum, g	3.7	3.8	3.6	3.9
Decrease in VS, g	4.52	9.93	14.4	11.99
Input VS degraded, %	59.8	65.7	76.2	52.9
Total gas yield, L	3.9	9.8	12.0	12.6
Methane yield, L	2.8	6.2	8.4	7.9
Biomethanation efficiency, methane yield/g input VS	0.38	0.42	0.45	0.35

[a]Batch reactors of 2-L capacity were operated and monitored for 80 d.

the highest substrate input of 24 g/L reactor there was a decline in the methane yield. The biodegradation of the given input after 80 d, appropriately correlated with biomethanation efficiency (measured as methane yield per gram of VS input) with different reactors and was maximal for the initial VS input of 18.9 g for reactor 3. The biomethanation index dropped from 0.45 to 0.35 when the initial VS input was increased to 22.7 g in reactor 4. From these results, the optimum load rate for the batch process was confirmed at 18.9 g VS per unit volume of reactor, whereas reactors 1 and 2 were at suboptimal level and reactor 4 was at a slightly overloaded level.

Anaerobic digestion is known to be brought about by an initial hydrolysis of the biopolymer followed by a further acidogenic conversion, before proceeding to the terminal step of methanogenesis. The rate of hydrolysis of collagen and the proteoglycan matrix were followed by analyzing the hydrolysis products namely amino acids and amino sugars, respectively. Changes in the level of amino sugar during the anaerobic digestion of skin material is shown in Fig. 2. Within 1 h of the onset of hydrolysis, the amount of amino sugar released reached a maximum concentration of 1 g/L, which was followed by a rapid decrease in the level by 76% to 0.24 g/L within 24 h and by 96% by the 10th d, which remained steady at 0.04 g/L between 12–24 d. A similar observation with respect to soluble sugars, had been made during the anaerobic digestion of cellulose, which also showed an initial rapid decrease and a steady state during active methanation (*21*). The changes in the level of undegraded collagen present in the digestor, estimated from the hydroxyproline levels after acid hydrolysis is shown in Fig. 3. The degradation commenced after 24 h and 50% was degraded within 8 d. Significantly, free hydroxyproline in

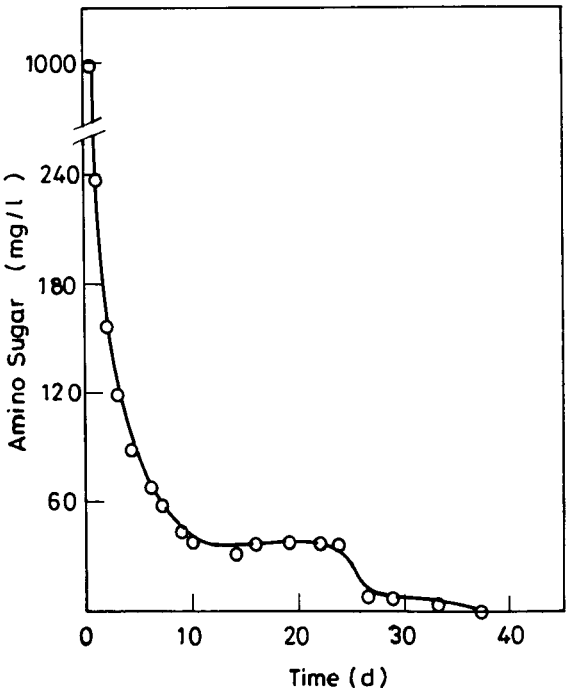

Fig. 2. Changes in the level of amino sugars during anaerobic degradation of skin collagen matrix.

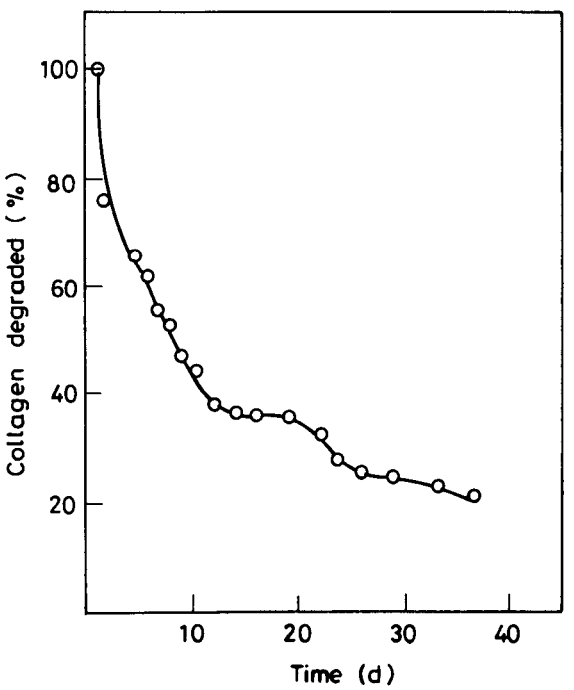

Fig. 3. Rate of collagenolysis of calf skin trimmings in anaerobic batch reactors.

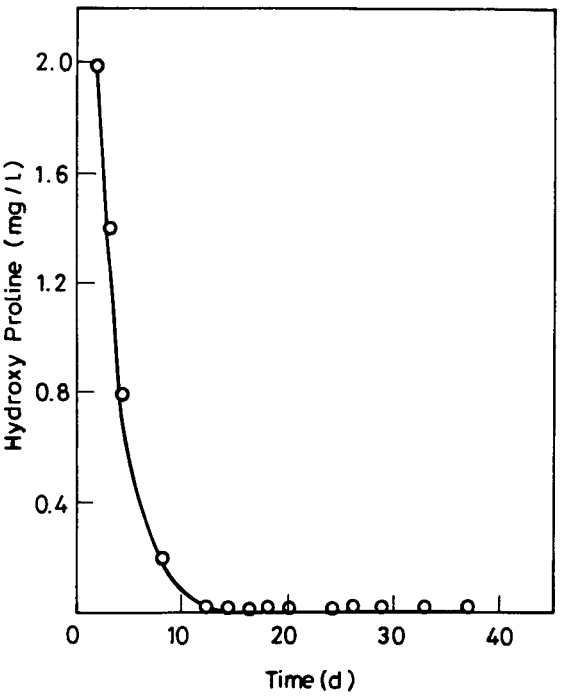

Fig. 4. Change in the free hydroxyproline concentration during the degradation of skin collagen.

the medium shown in Fig. 4, was at low levels and not detectable after 4 d, suggesting a faster turnover of this amino acid. Subsequently, collagenolysis became decelerated and by 20th d, 65% of the initial collagen had been degraded. While correlating collagenolysis with the pattern of gas yield it was observed that when methane yield declined, there was a renewed spurt in the rate of collagenolysis around the 22nd d. The trend of degradation of the skin macromolecule is biphasic and is similar to the earlier report on controlled hydrolysis of cellulose observed during the anaerobic digestion of *L. leucocephala* (*21*), wherein a negligible rate of cellulolysis during active methanation, increased during the late methanogenic phase, resulting in the hydrolysis of the small amounts of polymer remaining.

The degradation of the input VS is given in Fig. 5. During the first 8 d, there was a 38% decline in the input VS and during the next 30 d, the rate declined with an overall decrease of 60% of input VS during this period. This trend in the decrease of the total input VS is very similar to the decrease in the VS of *L. leucocephala* during anaerobic degradation at optimal load rate in batch reactors (*21*).

The level of amino sugars and the rate of collagenolysis observed during the anaerobic digestion of solid tannery waste indicate that it is the matrix proteoglycan that is amenable for initial hydrolytic action by the

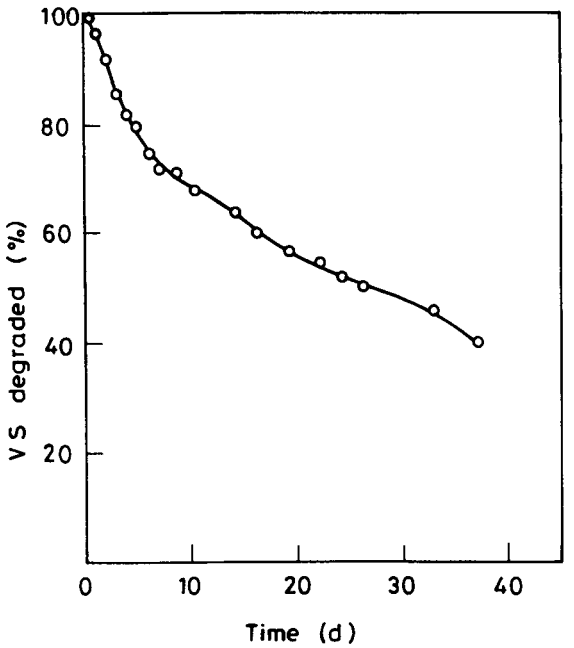

Fig. 5. Overall decline in input VS during the anaerobic digestion of calf skin trimmings.

microbial consortium mediated probably by glycosidases and neutral proteases (*30*). This is not surprising, since the metabolic requirements for the cells would be provided by the immediate energy readily recoverable from the sugars. It is imperative that the microbial consortia in the inoculum used had specific enzymes capable of mediating such sequential hydrolysis. The matrix depolymerization effected by these enzymes enables the collagen fibers to be exposed for the action of collagenases and other proteases, resulting in the formation of oligopeptides that would then be converted to amino acids.

The time course of changes in aminonitrogen level reflecting the total concentration of both amino acids and amino sugars and that of ammonium ion formed subsequently during amino acid fermentation are presented in Fig. 6. There was an initial decrease within 24 h in the level from 1.16 to 0.5 g/L for aminonitrogen and 1.4 to 0.4 g/L for ammonium ion, indicating their rapid utilization by the hydrolytic bacteria. During the next 7 d, although aminonitrogen increased to reach a maximum of 1.42 g/L, the level of ammonium ion was almost steady. This is probably because of continued collagenolysis and further growth of the acidogenic and methanogenic bacteria. The level of aminonitrogen decreased by the 9th d, whereas that of ammonium ion increased to 0.74 g/L during the same period. This is when collagenolysis decelerated, whereas organisms

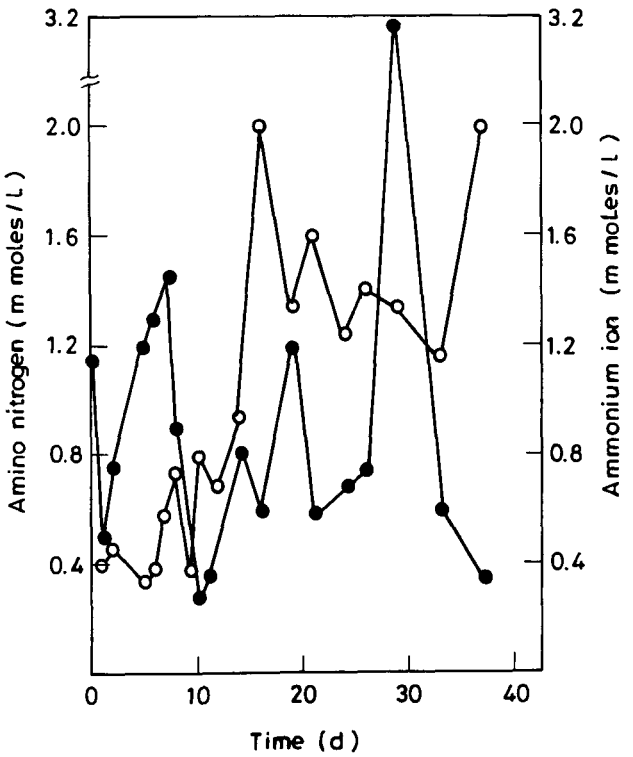

Fig. 6. Changes in the level of aminonitrogen and ammonium ions during the anaerobic degradation of skin collagen matrix. —●—, aminonitrogen; —O—, ammonium ion.

utilizing amino acids increased in number. Subsequently, during the period between 12–26 d, there was a constantly alternating pattern of oscillation between aminonitrogen and ammonium ion levels in an inverse proportion indicating that ammonium ion probably controlled the utilization of amino acids by the bacteria. The level of ammonium ions reached a peak value on the 16th d, followed by an immediate decrease, indicating effective amino acid fermentative conversion. There was a renewed spurt in the level of aminonitrogen reaching 3.2 g/L on the 30th d coinciding with the second phase of collagenolysis on the 24th d. The ammonium ions did not accumulate in the medium during the initial period up to 12 d, probably because of their utilization for generating bacterial biomass. The inverse proportion of ammonium ion to aminonitrogen level during the period between 12–26 d, explains the rate of amino acid fermentation being metabolically regulated, perhaps by ammonium ions among other things, and studies reveal the controlled formation of amino acids and their utilization thereof.

The major product of amino acid utilization by the action of acidogenic bacteria results in the formation of VFA, carbon dioxide, and ammonia.

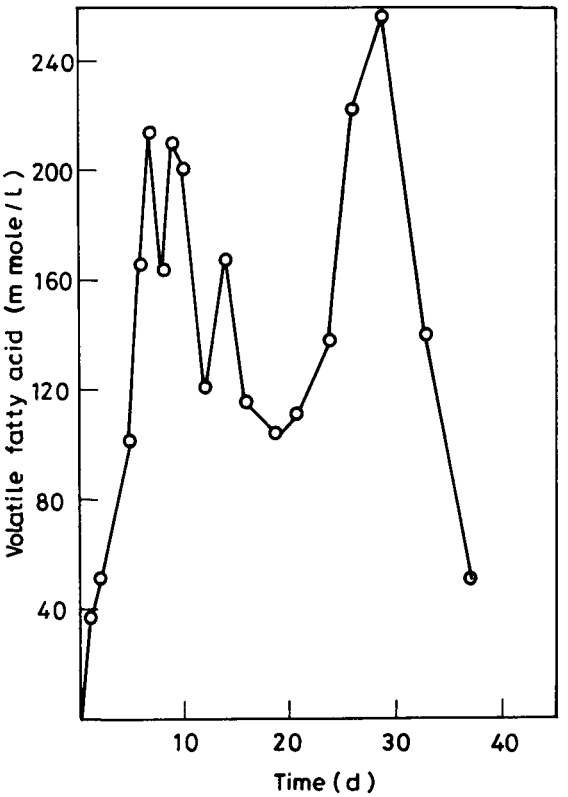

Fig. 7. Volatile fatty acid levels during biomethanation of skin collagen matrix.

Variations in the level during the anaerobic digestion of solid tannery waste is given in Fig. 7. There was a gradual increase in the level of volatile fatty acid during the first 4 d, reaching 80 mmol/L, and then a steep rise to 215 mmol/L during the next 5 d, indicative of the increase in the acidogenic bacterial population. Subsequently, the level of volatile fatty acid declined to 120 mmol/L by the 12 d, which was indicative of the growth of the methanogens and remained steady during the period between 12–24 d, coinciding with active methanation with methane yield around 70 mmol/L. This phase denotes coexistence of the different groups of bacteria deriving maximal energy by substrate conversion. Following a decrease in methane yield around the 25th d, the VFA levels started to increase and reached up to 260 mmol/L by the 29th d, which is owing to further collagenolysis during the same period. The results show that the level of VFA during active methanation attain a steady state, in contrast to the oscillatory pattern of earlier reported during cellulose degradation (*21*).

The results with respect to the changes in the concentration of amino sugar, amino acid, ammonium ion and VFA are given in Table 2. It can be seen from these results that collagenolysis was biphasic, which, after an

Table 2
Summary of the Changes in the Levels of Biochemical Parameters During Anaerobic Digestion of Calf Skin Trimmings

	Period of digestion, d							
Parameter	0	4	8	12	16	19	24	33
VS, g	18.98	13.78	12.43	12.05	10.45	9.97	9.34	7.88
Amino sugar, g	0.054	0.09	0.052	0.04	0.04	0.04	0.04	0.01
Hydroxyproline in pellet, g	0.72	0.48	0.38	0.26	0.26	0.25	0.20	0.15
Aminonitrogen, g	1.16	1.03	0.70	0.35	0.59	1.2	0.67	1.16
Ammonium ion, g	1.4	0.38	0.74	0.68	2.0	1.34	1.24	0.6
Volatile fatty acids, mmol	2.0	84.0	166.0	122.0	116.0	104.0	138.0	150.0
Methane yield, mmol	0	22.0	38.0	56.0	60.0	78.0	63.0	58.0

initial high rate of degradation, became controlled during the later period before a renewed spurt during the late methanogenic phase. The level of amino acids released during the hydrolysis showed an oscillatory pattern during the period of active methanation. The decrease in aminonitrogen coincided with the increase in VFA, indicative of the start up of the acidogenic phase. Methanogenesis was found to ensue when the VFA level was between 120–160 mmol/L. Active consumption of VFA by the methanogens results in a drop in its levels that triggers further conversion of aminonitrogen thus ensuring its steady state concentration during active methanation. The level of aminonitrogen itself may be regulated by ammonium ions, thus ensuring the effective and complete digestion and biomethanation of skin material.

The results of the present study are suggestive of four distinct phases operative during the anaerobic digestion of solid tannery waste, viz., a hydrolytic phase followed by acidogenic phase, then active methanogenic and late methanogenic phases. During active methanation, there is a steady state level of VFA indicative of comparable rates of its formation and utilization thereof. Normally, a steady state with respect to substrate concentration is characteristic of a chemostat, whereas in semicontinuous operations of a biostat the substrate level decreases for a short period, although some of the intermediary metabolites assume oscillatory or steady state levels. In a batch process, on the other hand, the substrate is utilized over a prolonged period and steady state concentrations have never been considered nor reported for such situations. Interestingly, the prevalence of steady state concentration with respect to some of the key intermediary metabolites and the oscillatory levels of certain other metabolites within a narrow range of concentration has been reported for the first time in studies on anaerobic digestion of a cellulosic substrate, *L. leucocephala* (*21*), and also in the present investigation involving the degradation of proteinaceous skin collagen matrix. These results signify that optimal yield of a product by a mixed culture will necessarily be achieved only when all the bacterial population involved in the biochemical conversions grow efficiently. This occurs only at the optimal VS load rate and not at extremely low or high VS load rates, owing to either lack of a metabolite or attainment of inhibitory

concentration of an intermediate being the cause for the disbalance in the growth of the microbial population concerned. Our investigations reveal that at the optimum input concentration sustained growth of the bacterial population ensued, and the interesting feature of the present study is the revalidation of the concept of interactive metabolic control proposed earlier (*21*). Metabolic control mechanisms operative in eukaryotic species have been understood in a limited context and these mechanisms comprise of many levels of organization, but mainly hormonal and genetic levels, involving expressions and control of various enzymes mediated by signal transduction. But the concept of steady state levels mediated outside the cellular milieu and also involving the interaction of entirely different groups of bacterial population reported here is novel and very interesting. It is tempting to speculate that some of the intermediate metabolites themselves could function as metabolic control elements binding to or signaling at the individual cellular surface level. It is also likely that certain of these metabolites can also act as regulators in the extracellular interactions between metabolites themselves or with some of the extracellular enzyme thereof.

During the anaerobic digestion of solid tannery waste, the rate of collagenolysis was controlled by the aminonitrogen level, which itself was regulated by ammonium ion, formed during the further conversion of amino acids to VFA. Significantly, the level of VFA itself, was at a steady state during methanogenesis. It emerges from these results that an interactive metabolic control regulates the overall rate of collagenolysis, which is operative only at the optimal VS load rate. In contrast, at load rates slightly above optimal, the concentration of short chain fatty acids produced is high, resulting in the delayed onset of the methanogenic phase. If the system is overloaded, it results in reactor instability, manifested by a marked and rapid increase in VFA concentration, which is detrimental to the methanogenic population (*31*). In addition, the elevated ammonia concentration can also be a major cause for operational failure. A digestor that has not been previously acclimated to high ammonia loadings or a reactor operating near the limits of its design capacity, shock loadings of high ammonia concentration were reported to cause rapid production of VFA such that the buffering capacity of the system is unable to compensate for the decrease in pH (*32*). These may be some of the irreversible consequences resulting in depletion in one or more of the bacterial populations involved. The study thus underscores the importance of initially assessing the optimal load rate for any biomass substrate per unit volume of the reactor. The present investigation has highlighted some of the interesting biochemical parameters that are of importance and useful in assessing and understanding the course of the process of anaerobic digestion and of the interacting species thereof.

Studies on the nature of anaerobic digestion in order to rationalize the design and optimization of biological waste treatment systems have been made by many researchers. Some of the studies included: acidogenesis of glucose and sewage waste (*33*), VFA conversion to methane in ana-

erobic upflow sludgebed (*34*), and generalized kinetic treatment involving a fermentative and methanogenic bacterial groups based on the Monod's model or the application of Contois equation, to a complex substrate, cow manure (*35–38*). The biochemical nature of the intermediary metabolites during conversion of various wastes has not been considered by these researchers in general. But in a limited context, defined cocultures of a methanogen or cellulolytic organism and/or acidogenic bacteria was studied (*15–17*), which may explain individual interaction but have limited relevance to the natural anaerobic habitats or biogas reactors, wherein many different groups of bacteria thrive with maximal economic efficiency in utilizing varied complex biopolymers.

Interspecies hydrogen transfer mechanisms have been shown to be responsible for the controlled flow of reducing equivalents in well-studied synthrophic methanogenesis as reported for ethanol conversion by *M. omelianskii* mixed culture (*39*). Further, Thiele and Zeikus have suggested that, during methanogenesis of complex organic matter, interspecies electron transfer was mediated via formate and not through hydrogen (*40*). However, the apparent importance of interspecies transfer of metabolites related to syntrophic methanogenesis in a natural and artificial ecosystem has not been taken into serious consideration. It is in this context that the present study assumes significance in demonstrating the operation of an interactive metabolic control during anaerobic digestion mediated by the organisms themselves, which results in steady state levels of some metabolites and interesting alternating oscillations with respect to certain others, reflected outside the cellular environment. More studies are needed in clarifying the nature of these interactions between the microbial communities and the regulating metabolites. The study revealed the existence of control mechanisms in the environment related to coexistence of different groups of bacteria which assumes ecological significance.

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REFERENCES

1. Starr, C., Searl, M. F., and Alpert, Sy, (1992), *Nature* **256,** 981.
2. Carlson, D. E. (1990), *Annu. Rev. Energy* **15,** 85.
3. Twidell, J. W. and Weir, A. D. (1986), *Renewable Energy Resources,* University Press, Cambridge, UK.
4. Grubb, M. J. (1990), *Energy Policy* **6,** 525.
5. Richardson, S. W. and White, A. A. L. (1980), *Nature* **286,** 103.
6. Hobson, P. N. (1983), *J. Chem. Tech. Biotechnol.* **33B,** 1.

7. Gujer, W. and Zehnder, A. J. B. (1983), *Water Sci. Technol.* **15,** 127.
8. Hill, D. W. and McCarty, P. L. (1967), *J. Water Polut. Control Fed.* **39,** 1259.
9. Raghu, K. and Macrae, I. C. (1966), *Science* **29,** 263.
10. Sharma, S. K. (1990), *Rural Tech. J.* **7,** 17.
11. Hobson, P. N. (1982), *Adv. Agri. Microbiol.* Subha Rao, N. S., ed., Butterworth Scientific, London, p. 523.
12. Dent, C. G. and Krol, A. A. (1990), *Biomass* **22,** 307.
13. Klass, D. L. (1984), *Science* **223,** 1021.
14. Narayanaswamy, V., Sankar, K., Chandrasekaran, P. M., and Lalitha, K. (1986), *Fuel* **65,** 1129.
15. Khan, A. W. and Trottier, T. M. (1978), *Appl. Envirin. Microbiol.* **35,** 1027.
16. Khan, A. W., Trottier, T. M., Patel, G. B., and Martin, S. M. (1979), *J. Gen. Microbiol.* **112,** 365.
17. Khan, A. W. and Mes-Hartree, M. (1981), *Appl. Microbiol.* **50,** 283.
18. Latham, M. J. and Wolin, M. J. (1977), *Appl. Environ. Microbiol.* **34,** 297.
19. Weimer, P. J. and Zeikus, J. G. (1977), *Appl. Environ. Microbiol.* **33,** 289.
20. Hashimoto, A. G. (1982), *Biotechnol. Bioeng.* **24,** 2039.
21. Krishnan, S. and Lalitha, K. (1990), *Appl. Biochem. Biotechnol.* **26,** 73.
22. Neuman, R. E. and Logan, M. A. (1950), *J. Biol. Chem.* **184,** 299.
23. Herbert, D., Phillips, P. I., and Strange, R. E. (1971), in *Methods in Microbiology,* Norris, J. R. and Ribbons, D. W., eds., Academic Press, New York, 5B, p. 244.
24. *Standard Methods for the Examination of Water and Waste Water,* 14th ed., American Public Health Association, New York (1975).
25. Davidson, E. A. (1966), in *Methods in Enzymology,* Neufeld, E. F. and Ginsberg, V. eds., Academic Press, New York, vol. 8, p. 56.
26. Moore, S. and Stein, W. H. (1948), *J. Biol. Chem.* **176,** 367.
27. Johnson, M. J. (1941), *J. Biol. Chem.* **137,** 575.
28. Breure, A. M., Mooijman, K. A., and Van Andel, J. G. (1986), *Appl. Microbiol. Biotechnol.* **24,** 426.
29. Swaminathan, K. R., Padma Bai, R., and Lalitha, K. *International Conference on Agriculture and Forestry,* Indian Agricultural Research Institute, New Delhi (1993), Abstract No. C-VI-1.
30. Weiss, J. B., Sedowfia, K., and Jones, C. (1980), *Biology of Collagen,* Viidik, A., and Vuust, J., eds., Academic Press, New York, p. 113.
31. Hobson, P. N. and Shaw, B. J. (1976), *Water Res.* **10,** 849.
32. Stronach, S. M., Rudd, T., and Lester, J. N. (1986), *Biotechnology Monographs,* Aiba, S., Fan, L. T., Fiechter, A., and Schiigerl, K., eds., vol. 2, Springer-Verlag, Berlin, Heidelberg, p. 73.
33. Ghosh, S. (1981), *Biotech. Bioeng. Symp.* **11,** 301.
34. Bhatia, D., Vieth, W. R., and Venkatasubramanian, K. (1985), *Biotechnol. Bioeng.* **27,** 1192.
35. Andrews, J. F. (1978), *Mathematical Models in Water Pollution Control,* Jammes, A., ed., Wiley, Chichester, UK.
36. Hill, D. T. and Barth, C. L. (1977), *J. Water Pollut. Control Fed.* **49,** 2129.
37. Chen, Y. R. and Hashimoto, A. G. (1980), *Biotechnol. Bioeng.* **22,** 2081.
38. Barathakur, A., Bora, M., and Singh, H. D. (1991), *Biotechnol. Prog.* **7,** 369.
39. Bryant, M. P., Wolin, E. A., Wolin, M. J., and Wolfe, R. S. (1967), *Arch. Microbiol.* **59,** 20.
40. Thiele, J. H. and Zeikus, J. G. (1988), *Appl. Environ. Microbiol.* **54,** 20.