Interactive Metabolic Regulations During Biomethanation of *Leucaena leucocephala*

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ABSTRACT

Biomethanation of leaves of the legume *L. leucocephala* operated in batch reactors at different input volatile solids (12–18 g/L) proceeded in distinct metabolic phases. An initial cellulolytic phase of 4 d was followed by an early and active methanogenic phases (5–21 d) and a terminal phase of low-rate methanogenesis. Hydrolysis of cellulose was concentration-dependent and resulted in increased volatile acid levels. The trend of changes showed some variations at different input volatile solids. The changes in the levels of volatile acids followed an oscillatory pattern. The controlled rate of cellulose hydrolysis, levels of volatile acids, and steady-state levels of soluble carbohydrates and reducing sugars observed during active methanogenesis are indicative of interactive metabolic regulations.

Index Entries: Biomethanation and, —metabolic regulations; —kinetics; —cellulose degradation; —steady state parameters and —of *L. leucocephala;* anaerobic digestion of *L. leucocephala;* anaerobic digestion and, —kinetics in batch reactors; and —biomethanation efficiency.

INTRODUCTION

The relevance of anaerobic digestion of biomass has assumed greater significance in recent times, owing not only to the methane economy but

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also to the accompanying ecological balance. The development of biomass options as alternative sources for energy and chemicals has been discussed (*1–5*). The complexity of anaerobic digestion is inherently linked to the chemical nature of substrates and the metabolic action of the mixed consortium of microbial population involved in the process, and the terminal step is restricted to a specialized archaebacterial group, the methanogens.

Renewable biomass sources are classified into primary sources, generated specifically for energy or chemicals, secondary sources, such as agroforestry crop residues, and the tertiary sources, comprising all urban, industrial, and agricultural wastes. Sources for anaerobic digestion have largely been restricted to tertiary biomass substrates, such as agrowastes (*6*), domestic refuse (*7*), and other urban wastes (*8*). These studies relate to monitoring the levels of methane produced at different concentrations of input volatile solids and the effect of temperature thereon.

Leucaena leucocephala, a fast growing leguminous species, has been identified as a potential primary biomass substrate for large-scale biomethanation (*9*), and further studies have indicated its suitability for mixed feed formulations as well (unpublished data). Sea kelp, *Macrocystis pyrifera,* is the only other primary biomass source that has been investigated for biomethanation (*10*).

Attempts toward understanding the biochemical and microbiological aspects of anaerobic degradation have been restricted to studies under simulated environments using defined co- or tricultures of methanogens with cellulolytic or acidogenic bacteria on pure cellulosic substrates (*11–13*) and anaerobic degradation of cellulosic substrates using mixed cultures (*14*). However, in natural habitats, methane is generated by the synergistic action of a mixed bacterial population, not only on cellulose but also on various other biopolymers (including proteins and lipids), while striving for a metabolic coexistence. Thus methanogenesis in mixed cultures differs from that in pure cultures because of the interaction of methanogens with other organisms at metabolic and physiological levels (*15*). Studies on the intermediary metabolic events for possible control of methane generation by the microbial groups during anaerobic digestion of *L. leucocephala* are presented in this paper.

MATERIALS

All chemicals used were of AR grade. Distilled water was used throughout the studies. The feed substrate used in this study was leaves of *L. leucocephala.* Batch reactors of 2.5 L capacity were operated for a period of 45 d at varying levels of input volatile solids (VS) ranging from 12 to 28 g/L, using seed inocula from previously operated digesters. Reactor design, preparation of seed inoculum, and maintenance of anaerobic atmosphere were as described previously (*9*). In a typical reactor, the gas was collected

in a gas buret connected to a leveling water reservoir and provided with a port sealed with a butyl rubber stopper to enable periodic withdrawal of gas samples.

Estimations of total solids (TS), volatile solids (VS), and volatile acids (VA) were done by standard methods (*16*). Cellulose was determined by the method of Updegraff (*17*). The amount of total soluble carbohydrates (*18*) and reducing sugars (*19*) are expressed as glucose equivalents in g/L. The content of VA is reported as acetic acid equivalents in mmol/L. The output gas volume was monitored and evacuated daily for a period of 45 d, whereas gas analysis was carried out periodically using Tracor Model 540 Gas Chromatograph.

RESULTS

Batch reactors were operated with input VS levels ranging from 12 to 28 g/L. Representative results are presented for input VS levels of 15.6 and 27.5 g/L, respectively designated as reactor I (lower VS input) and reactor II (higher VS input).

The total gas yield during batch digestion of *L. leucocephala* is shown in Fig. 1. During the initial period of 4 d, CO_2 content of the gas was maximal (95–98%). Methanation commenced after this period and accelerated to reach a level of 85–90% of CH_4 in the output gas in reactor I and 70–75% in reactor II in about 11 d. Active biomethanation lasted from 12 to 21 d, during which time the total gas yields in both reactors were equal. Subsequently, from 22 d till the end of the 45-d observation period, the total gas yield from reactor I declined sharply to a level of 0.1 L/d/L reactor volume and continued at this rate, whereas that from reactor II was at a moderate level of 0.2–0.3 L/d/L reactor volume.

Results presented in Table 1 indicate that, during the first 4 d of biodegradation, the initial cellulose levels (1.8 g/L in reactor II and 1.03 g/L in reactor I) declined to the extent of 66 and 25%, respectively. Prior to the onset of active biomethanation, cellulose levels were identical in both reactors, viz., 0.47 g/L (Figs. 2A and 2B). Attainment of this level corresponds to a degradation of 54 and 72% of input cellulose in reactors I and II, respectively. During the period of active biomethanation, viz., 12–21 d, the patterns of cellulose degradation were similar in both reactors. The level of cellulose, 0.36 g/L, attained on the 21st day in both reactors corresponds to 80% degradation in reactor II and 60% in reactor I. During the terminal phase of biomethanation, a sudden spurt in the rate of cellulolysis was noted in the reactor on lower VS input, and the cellulose level dropped to 0.16 g/L. In the reactor on higher VS input, cellulolysis continued at the same rate and reached a level of 0.26 g/L. These residual cellulose levels correspond to an overall cellulose degradation of 85% of the initial input in both of the reactors.

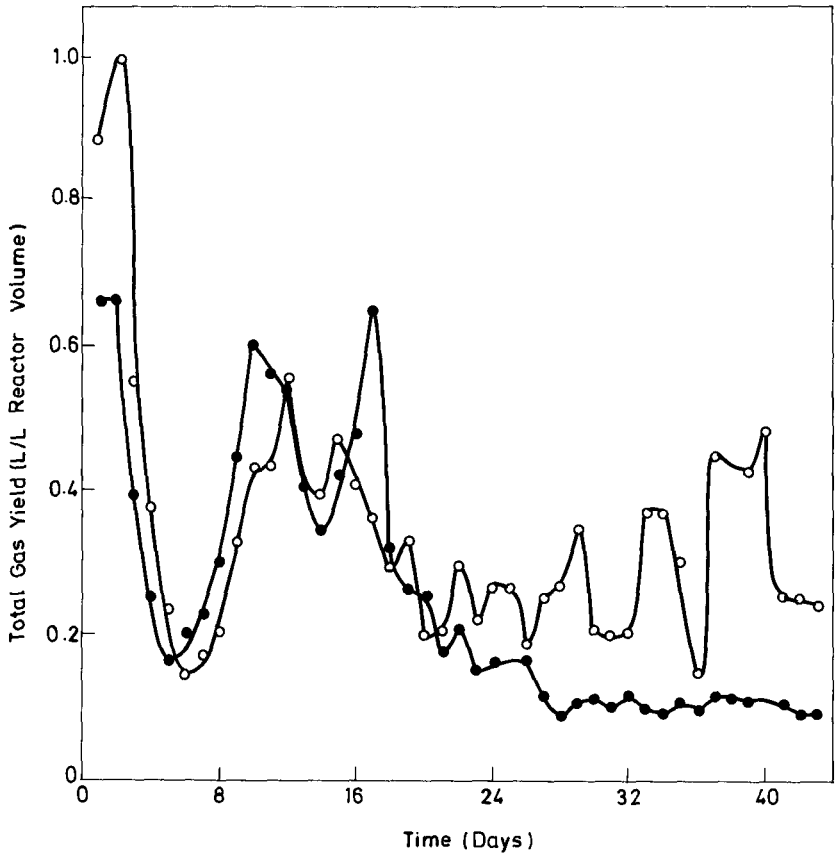

Fig. 1. Total gas yield during batch digestion of *L. leucocephala;* ●---●, reactor with lower VS input (15.6 g/L); ○---○, reactor with higher VS input (27.5 g/L).

In a period of 4 d, the initial soluble carbohydrate levels of 11.4 g/L in reactor I and 22.3 in reactor II both decreased by about 98%. Subsequently, the decline in soluble carbohydrate levels during the period 5–11 d was minimal and was 0.13 and 0.33 g/L in reactors I and II, respectively. These levels remained steady till the end of 21 d. During the terminal phase, the decrease in soluble carbohydrates was negligible only in the reactor on lower VS input; in reactor II, the level was about 0.24 g/L at the end of 33 d.

The entire amount of reducing sugars (0.4 and 0.7 g/L present as a part of the initial VS input) in reactors I and II was utilized during the first 4 d. Reducing sugars were undetectable till 11 d, but during active biomethanation reached the steady-state level concentrations of 0.16 and 0.24 g/L in the two reactors. During the terminal phase, again, no detectable amount of reducing sugars was present.

In both reactors, I and II, the utilization of VA during the first 24 h was to the extent of 80% of the initial levels of 74 and 100 mmol/L, respec-

Table 1
Changes in Various Components of *L. leucocephala* During Biomethanation

Parameter	Reactor	Period of digestion, d					
		0	4	8	11	21	33
VS, g/L	I	15.6	12.4	9.0	6.5	4.3	3.
	II	27.5	18.0	15.0	14.5	13.8	13.3
Cellulose, g/L	I	1.03	0.77	0.63	0.47	0.39	0.16
	II	1.79	0.60	0.58	0.49	0.35	0.26
Soluble carbohydrates, g/L	I	11.3	0.19	0.15	0.13	0.13	0.04
	II	22.4	0.80	0.44	0.33	0.33	0.24
Reducing sugars, g/L	I	0.37	N.D.	N.D.	0.16	0.16	N.D.
	II	0.70	N.D.	N.D.	0.24	0.24	N.D.
Total gas yield	I		1.97		2.5	3.82	1.75
	II		2.8		1.52	3.61	3.09
Methane yield[a]	I		0		0.75	3.36	1.24
	II		0		0.46	2.53	2.16

Values are averages of three independent determinations.
[a]Total gas and methane yields are expressed in L/L reactor volume for the periods 0–4, 5–11, 12–21, and 22–33 d.
N.D., not detectable.

tively. The levels fluctuated from 100 mmol/L to a maximum in the range of 250–350 mmol/L in the reactors (Fig. 3). At the end of 21 d, the levels again declined, more markedly in the reactor on lower VS load rate. In general, an oscillatory pattern was observed throughout the process of biodegradation in both of the reactors.

The pattern of VS degradation presented in Fig. 4 indicates a marked decline in VS levels in a period of 8 d, corresponding to 45% degradation of the initial level in both reactors. Further degradation of VS was not significant in reactor II, whereas a slow rate of decline in the level was noted in reactor I. When compared to the original input levels, the total VS degraded at the end of batch digestion was only 59% in the reactor on higher VS input, whereas the extent of degradation was 94% in the reactor on lower VS input.

DISCUSSION

Considering the complexity of anaerobic digestion in natural habitats, comprising the biodegradation of polymeric and mixed substrates by a microbial consortium, the choice of the substrate, *L. leucocephala*, based on its chemical composition (*9*) and the development of a mixed inocula specifically to degrade this substrate were found appropriate for detailed

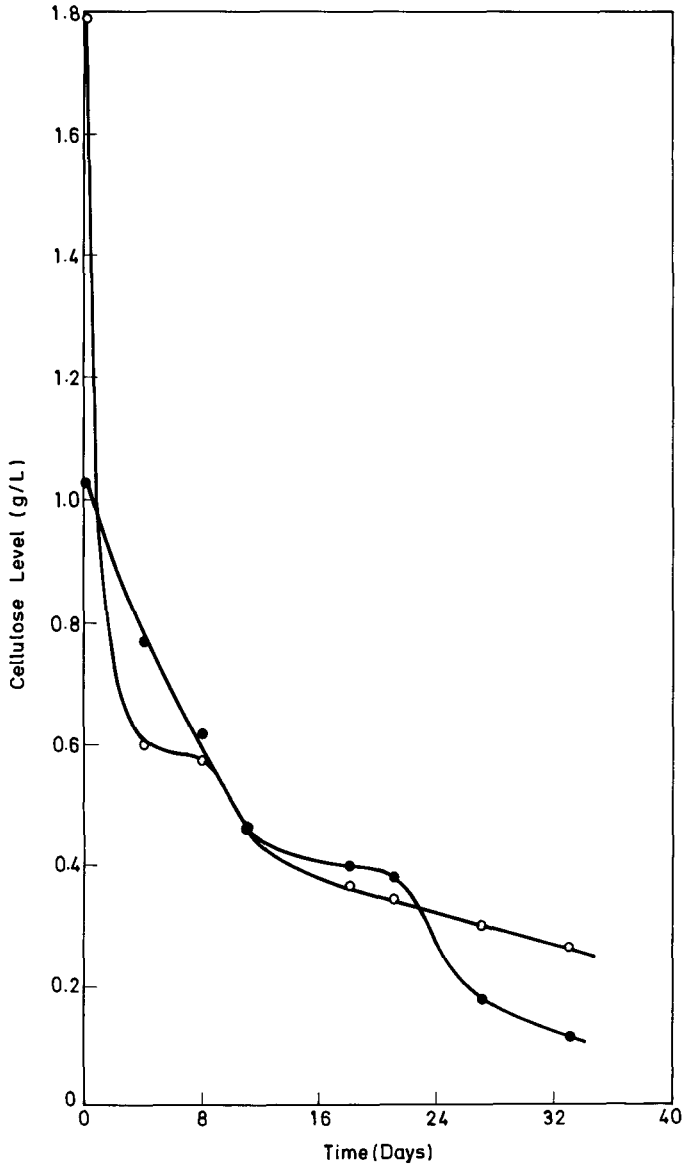

Fig. 2a. Changes in cellulose levels during anaerobic digestion of *L. leucocephala;* ●---●, reactor with lower VS input (15.6 g/L); ○---○, reactor with higher VS input (27.5 g/L).

kinetic investigations. Long-term studies established that biomethanation of *L. leucocephala* resulted in a methane yield of 0.66 L/g VS input, compared to that of cow manure, which was 0.22 L/g VS. The only other primary biomass substrate studied was *M. pyrifera,* yielding 0.28 L CH_4/g VS input (*10*). Preliminary studies on *L. leucocephala* revealed distinctly different phases of degradation.

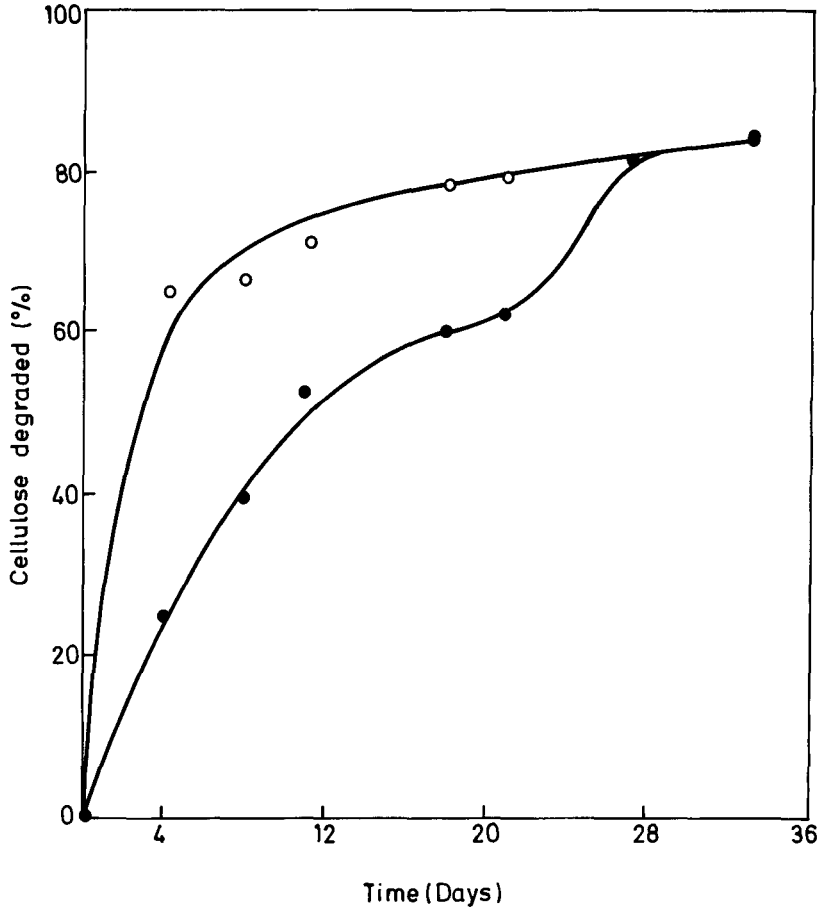

Fig. 2b. Degradation of cellulose during anaerobic digestion of *L. leucocephala;* ●---●, reactor with lower VS input (15.6 g/L); ○---○, reactor with higher VS input (27.5 g/L).

In batch reactors with different input VS, the chemical parameters were monitored periodically in relation to the total gas yield and the methane content thereof. At different concentrations of input VS, the degradative pattern related to these parameters showed different patterns with distinct variations. Results are indicative of specific metabolic phases during anaerobic digestion of *L. leucocephala.*

In the initial period of 4 d, all the available reducing sugars were completely utilized, and the degradation of total soluble carbohydrates was over 90%. A substantial decrease in the levels of cellulose, to the extent of 60%, was observed only in the reactor on higher VS input, which contrasted with a decrease of 25% in the reactor on lower VS input. Hydrolysis of cellulose thus proceeded in a concentration-dependent manner.

Subsequently, in the period from 5 to 11 d, celluloysis was more controlled, reaching almost identical levels in all reactors irrespective of input

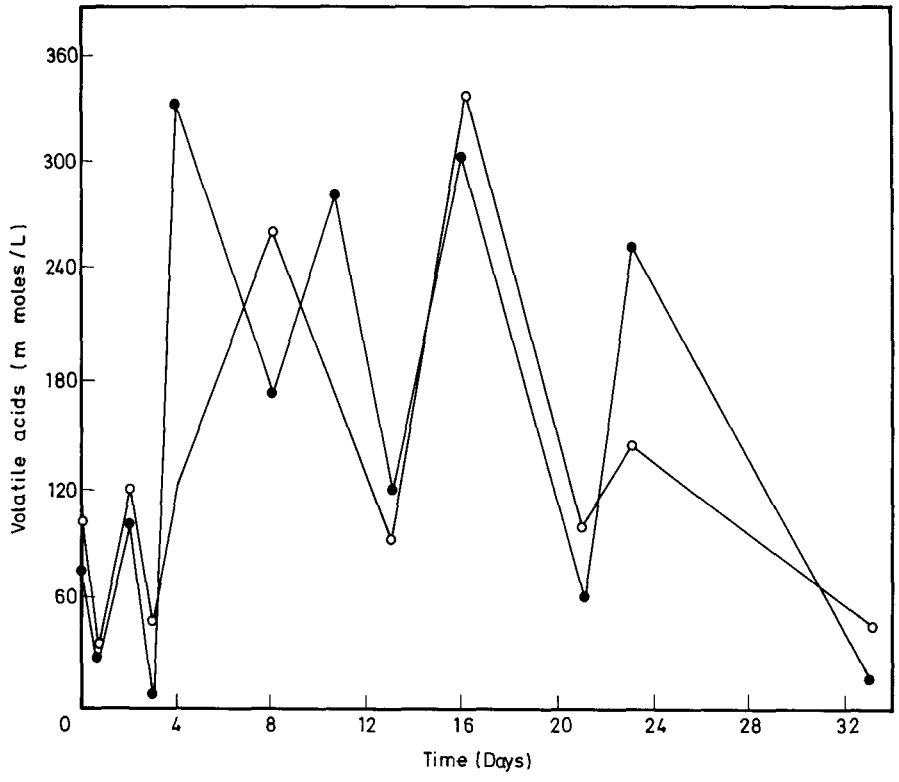

Fig. 3. Volatile acid levels during biomethanation of *L. leucocephala;* ●---●, reactor with lower VS input (15.6 g/L); ○---○, reactor with higher VS input (27.5 g/L).

VS concentration. During this period, the total soluble carbohydrates continued to decrease, with a concommitant increase in VA levels, whereas the reducing sugars were undetected in the medium. This was indicative of the establishment of acidogens and acetogens. The gaseous product during the first 4 d was mostly CO_2 (90%); the methane content subsequently increased steadily from 5 to 11 d, attaining a maximum in both reactors on the 11th day. The period between 12 and 21 d was marked by high-rate biomethanation, with the product gas uniformly rich in methane (90%) in reactor I, compared to 60–70% CH_4 in reactor II.

Changes in the concentration of cellulose were independent of initial VS input, with identical patterns of decrease from 0.47 to 0.35 g/L in both reactors. Interestingly, independent of VS input, the concentrations of total soluble carbohydrates and reducing sugars in all reactors attained steady-state levels during active biomethanation. In reactor I, reducing sugars appear to account for all the available soluble carbohydrates present, whereas in reactor II, the reducing sugars (0.26 g/L) formed 80% of the total soluble carbohydrates. Results are suggestive of the presence of a small proportion of intermediary di- or oligosaccharides at higher VS input

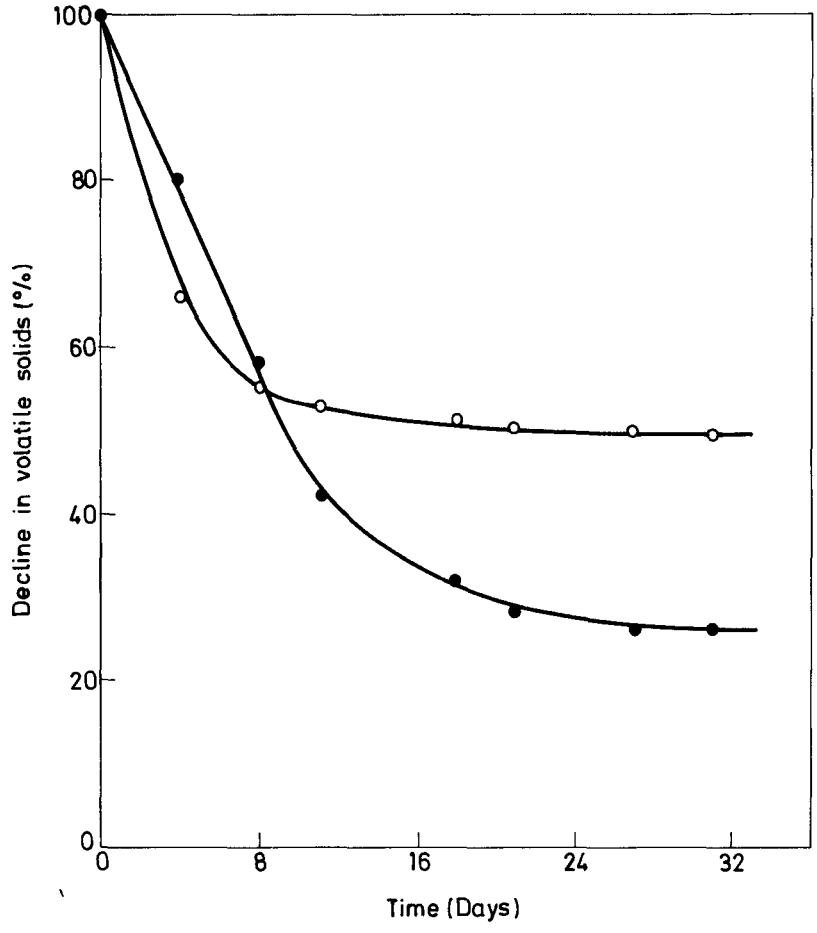

Fig. 4. Utilization of volatile solids during biomethanation of *L. leucocephala*; ●---●, reactor with lower VS input (15.6 g/L); ○---○, reactor with higher VS input (27.5 g/L).

reactors, mediating the availability of reducing sugars as ready energy sources for the microbes. The utilization of input VS, by the 33rd day, was almost complete in the reactor on lower VS input, compared to only 59% in the reactor with higher VS input. Throughout the study, the changes in the levels of VA followed an interesting oscillatory pattern.

At the end of 21 d, the level of VA reached a minimum in both reactors. In the reactor with higher VS input, methane generation continued at a reduced rate even after 21 d, with controlled levels of VA as a result of a continued and regulated rate of cellulolysis mediated by the available di- and oligosaccharide fractions, most probably by cellobiose (*20*). On the contrary, in reactor I, when the VA level decreased after 21 d, the availability of soluble carbohydrates became limiting, thereby impairing the metabolic balance of bacterial groups. This triggered a renewed active cellulolysis, further lowering the level of cellulose. The resultant sugars contribute to

Table 2
Comparative Assessment of Biomethanation
of *L. leucocephala* at Different Levels of Input VS

Parameters	Reactor I	Reactor II
VS in inoculum, g/L	3.7	3.7
VS input, g/L	11.9	23.8
Decrease in VS, g/L	11.6	14.1
Input VS degraded, %	94	59
Total gas yield, L/L reactor volume	10.04	11.02
Methane yield, L/L reactor volume	5.34	5.15
Biomethanation efficiency, methane yield/VS input	0.34	0.18

acidogenesis, with the VA increasing to 250 mmol/L. The increase in VA during the terminal phase was detrimental to further methanation. The nature of VA needs to be assessed, in view of the fact that higher fatty acids have been reported to be inhibitory to methanogenesis (*21*).

From these results, it is tempting to suggest that the di- and oligosaccharide fractions (the nature of which is to be ascertained) may have a role in the overall metabolic control for interactive balance and maintenance of the various groups of bacteria.

An overall assessment of the biomethanation of *L. leucocephala* in batch reactors for a period of 33 d is presented in Table 2. Almost complete degradation in terms of VS input was achieved in reactor I, the remaining VS being that of the inoculum biomass, which contrasted with the biodegradation of 59% in the reactor II during the same period. The residual cellulose levels represent 85% degradation in both reactors. Obviously, the remaining organic fraction in reactor II includes the residual cellulose as well as the other components. Along with these constituents and with remnant sugars available, methane generation continued in reactor II, though on a lower rate, for a period of 45 d. The total gas yield for the period of 33 d and the CH_4 content thereof indicate a biodegradability of 0.46 and 0.36 L/g VS degraded and a biomethanation efficiency of 0.34 and 0.18 L/g VS input in the lower and higher load systems.

Reports on methane yield per unit mass of VS degraded indicate wide variations between different sources, which could be ascribed to the input load rate, conditions, and duration of studies. Thus, a gas yield of 0.87 L/g VS input with methane content of 0.66 L was obtained from biodegradation of *L. leucocephala* observed over a period of 3 mo. Methane yield per unit mass of VS degraded has been reported to range from 0.33 to 0.56 L/g (*22*).

Studies on biomethanation point to the breakdown of cellulosic and other polymeric material into intermediates, such as acetic acid and other

one-carbon compounds, that, along with CO_2, form the necessary precursors for methane (*1,23*). In this context, the coexistence of nonmethanogenic and methanogenic microbes assumes considerable significance toward achieving maximal energy conservation for their growth through interactive mechanisms. The results of the present investigation are suggestive of four distinct metabolic phases, viz., a cellulolytic phase, followed by premethanogenic, active, and late methanogenic phases operative during biodegradation of *L. leucocephala.* Attainment of steady-state levels of reducing sugars and total soluble carbohydrates during the period of active methanation is supportive of such a regulatory control for the overall process, particularly for cellulose degradation. Based on the results, the choice of *L. leucocephala* as a primary source for biomethanation is amply substantiated.

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