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Economics

HPLC ANALYSIS OF SECONDARY METABOLITES IN *Calotropisgiganteа*DURING BIOMETHANATION.

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ABSTRACT The study is focused to assess the production of biogas and change in phytochemical and evaluation of toxicity reduction. Biogas was produced from cow dung mixed with Calotropis. The contents of secondary metabolites were measured by HPLC and there by toxicity reduction was evaluated. Among the three secondary metabolites during methanogenesis different degrees of degradation have been observed (Flavanoid -92%, Terpenoid – 80%, Alkaloid -55%). It is concluded that the degradation potential depends upon the structural integrity of the secondary metabolites.

KEYWORDS : *Calotropisgiganttea* , Biogas, HPLC,

INTRODUCTION

Energy is one of the major building blocks of modern society. Energy pervades all sectors of society: economic, labor, environment, international relations *etc,* in addition to our own personal livings i.e., housing, food, transportations recreation and more. Energy is simply the capacity for doing work (EIA, 2006). Organic carbon based materials of plants and animals are called biomass. This biomass may be transformed by physical, chemical and biological processes to **'biofuel'.**

Biogas technology refers to the production of a combustible gas (called biogas) and a value added fertilizer (called slurry or sludge) by the anaerobic fermentation of organic materials under certain controlled conditions of temperature, pH, C/N ratio, etc. Methane is a main constituent (63%) of biogas. The biogas slurry, rich in nutrients but low in carbon content, can be used as a natural biofertilizer, preferable to other inorganic fertilizers (Chakrabortyet *al.,* 2002).

The latex bearing plants *viz*, *Plumeriaalba*, *Calotropisprocera, Euphorbianerrifolia, Mimusopselengi* and*Neriumindicum* were evaluated as potential renewable sources of energy and chemicals (Kalita and Saikia, 2004). The plant parts (leaf, stem, bark) consist of oil, polyphenols, hydrocarbons, crude protein, α-cellulose, lignin and ash. For the successful utilization of any biomass as a source of biogas, its methanogenic potential alone is not sufficient. The residue left after the process must be properly managed for its sustainability. In order to implement this, the characteristic features of the residue, especially assessment of the toxicity is inevitable.

Hence, the present study is focused to assess the production potential of biogas (methane) and changes in some phytochemicals and evaluate the reduction of toxicity, of secondary metabolites of the plant *viz.,*terpenoid (γ-terpinene), flavonoid (catechin), steroid (β-stigmasterol) and alkaloid (histamine) quantification were estimated before and after biomethanation .

Materials and methods :

Collection and preparation of biomass

*Calotropis*twigs were collected from the campus playgrounds of Urumu Dhanalakshmi College, Tiruchirappalli, and authenticated. The leaf material was dried and ground to fine powder to be used as a substrate. Fresh cow dung was used as seeding material with*Calotropis*. Cow dung and water in the ratio 1:2 (w/v) served as a control substrate (Kasali, 1990 and Chakraborthy*et al*., 1996). The pH of the above two substrates were adjusted to 6.8.

The control and the experimental substrates were subjected to fermentation in separate batch fermentors assembled in the laboratory . The raise in the level of substrate and appearance of effervescence confirm the initiation of methanogenesis. The emitted biogas was collected by the downward displacement of water. Some phytochemical components of cow dung and *calotropis*biomas before and after biomethanation were assessed by standard procedures.

Qualitative and quantitative determinations ofsecondary metabolites :

The residue obtained after methanogenesis, was allowed to air dry in the sheltered area at 25-30˚C until moisture was exhausted. The sample was ground, and it was stored in a plastic bag for subsequent analysis. The*Calotropis* leaf powder and the biogas residue were subjected for qualitative identification of the following secondary metabolites with appropriate standards. **(**Flavonoid –Catechin**,** Terpenoid- γ-Terpinene, Alkaloid –HistamineSteroid -β-Stigmasterol]

The contents of secondary metabolites were qualitatively measured by high pressure liquid chromatography (HPLC).

RESULT AND DISCUSSION :

The quality of methane accumulated, from *Calotropis* biomass and the control [cowdung] through downward displacement system, were recorded at every 6 hourly intervals for 15 days.

Under the present experimental conditions, after inoculation, methanogenesis began on the first day itself. The quantity of biogas generated was significantly higher in the plant biomass throughout the experimental period .The higher potential of plant biomass to generate methane has been reported as early as 1996 by Mittal. The biomass of different plants *viz*., *Spirogyra* (Algae), *Ipomea*, *Eichhornea*; *Jatropa*and*Parthenium* were most effective in biomethanation (Chakraborty, 2002).

The difference in the quantity of biogas generated by cow dung and biomass throughout the experiential period (15 days) are presented in **Table .**1. The flux in the biogas, expresses a steep increase on the second day, maintains at the highest on the 3rd and 4th day gradually declines thereafter till the end, with significant statistical difference.

The raw material for biogas production evidently is higher in plant biomass since it is not subjected to any microbial degradation earlier. Cow dung is a product of microbial degradation in the ruminated digestive tract of the animal which harbors enormous microbial population *viz,Lactobacillus*, *Bifidobacterium, Propionobacterium, Clostridium* and *Coriobacterium*(Collado and Sanz, 2007). They maybe the cause for the synthe;sis of more biogas by the plant biomass.

Table 1. Difference in the quantity of biogas generated by the Cow dung and Biomass

S.No.	Day	Flux in Biogas (ml)
1.	1st	22
2.	2nd	71
3.	3rd	74
4.	4th	74
5.	5th	69
6.	6th	67
7.	7th	65
8.	8th	65

9.	9th	64
10.	10th	60
11.	11th	60
12.	12th	56
13.	13th	49
14.	14th	23
15.	15th	05

Table 2. DIFFERENCE IN THE QUANTITY OF BIOGAS GENERATED BY THE COW DUNG AND BIOMASS

t-Test

Samples	N	Mean	Std. Deviation	Std. Error Mean
COWDUNG	10	115.60	60.16	19.02
BIOMASS	10	183.50	63.90	20.21

Independent Samples Test

t	df	Statistical inference	Sig. (2-tailed)
2.446	18	P<0.05 **Significant**	.025

In support of the above notion, in the present investigation all the organic compounds studied, viz., cellulose, hemicellulose, organic carbon, volatile fatty acids, total nitrogen and C: N ration have been found to be significantly higher in plant biomass .Moreover *Calotropis* is a laticiferous plant and the latex contains 3.47 per cent carbon, which can also be transformed into biogas. Biomethanation brings in highly significant variations statistically in all the cell wall components studied [Cellulose, Hemicellulose and Lignin]

Among the 3 cell wall components studied, cellulose and hemicellulose are relatively more in the *Calotropis* biomass than the dung sample. But the quantity of lignin is more in cow dung than the plant biomass.

Lignin is basically a polymer of phenolic compounds; cellulose and hemicellulose are carbohydrate polymers. Generally carbohydrates are immediately utilized by the microbes as respiratory substrate unlike the phenolics (Yu-Sheng *et al*., 1994). This could be a reason for the decrease in cellulose and hemicellulose content during fermentation.

Moreover, hemicellulose degradation is relatively lesser than the cellulose degradation during biomethanation both in biomass and cow dung. The relative utility of cellulose more than hemicellulose could be because of its structural complexity. Cellulose is a long chain homopolymer of glucose, where as hemicellulose is a long chain heteropolymer having branched structure (World watch institute, 2007).

Volatile fatty acids are the major end products of microbial fermentation in the ruminant digestive tract. The major volatile fatty acids are acetate, propionate and butyrate. Callaghan *et al*., (2002) suggested that degradation of sugars gives a mixture of equal volumes of carbondioxide and methane, whereas degradation of lipids gives a greater percentage of methane.The content of volatile fatty acids before biomethanation is relatively higher in cow dung than biomass which is similar to the lignin. But unlike lignin the reduction of volatile fatty acids during biomethanation is highly significant.

Beforebiomethanation, the volatile fatty acid content in plant biomass is 5.23 per cent and cow dung is 8.1 per cent. After biomethanation the level of VFA is remarkably reduced to 2.1 per cent in biomass and 3.9 per cent in cow dung (Table 3).

Table 3. Changes in some Biochemical compounds and some macronutrients during biomethanation

S. No.	Compounds	Cow dung		Biomass	
		Biomethanation induced changes (%)	Level of significance	Biomethanation induced changes (%)	Level of significance
1.	Volatile fatty acids	-4.2 (51)	P<0.0001	-3.1 (60)	P<0.0001
2.	Cellulose	-7 (39)	P<0.0001	-15 (43)	P<0.0001
3.	Hemicellulose	-3.6 (32)	P<0.0001	-5.1 (33)	P<0.0001
4.	Lignin	+2.2 (43)	P<0.0001	+1.8 (46)	P<0.0001
5.	Phosphorus	+0.02 (5)	P<0.05	+0.07 (18)	P<0.0001
6.	Potassium	+0.03 (8)	P<0.01	+0.13 (18)	P<0.0001
7.	Carbon	-9.0 (32)	P<0.0001	-11 (31)	P<0.0001
8.	Nitrogen	-0.1 (5)	P<0.05	-0.13 (6)	P<0.0 001
9.	C:N ratio	-4.2 (29)	P<0.0001	-4.4 (26)	P<0.01

+ Enhanced **-Reduced**

Degradation of some secondary metabolites during biomethanation

Terpenoids in *Calotropis*

It is inevitable to asses the quantity of terpenoids that are decomposed during the biomethanation process of Calotropisin order to manage the residues after biomethanation appropriately. In the present study, the terpenoid content in terms of one of the prominent terpenoids in Calotropis biomass (γ- terpinene) has been determined .

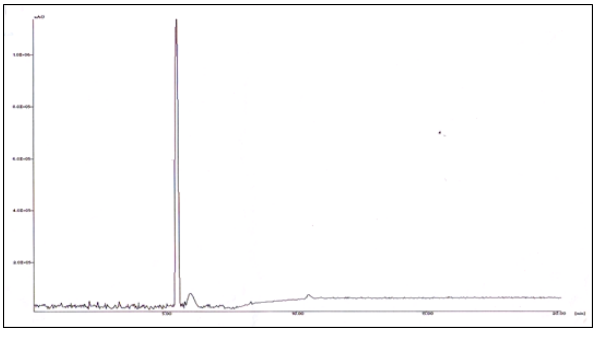
γ- Terpinene

The HPLC results indicate the reduction in the terpene levels significantly during biomethanation, when compared to the rawCalotropis biomass .The reduction is 92.9 per cent .The γ-terpinene is a volatile compound. Under the anaerobic condition, presence of methanogenes constant temperature (37˚C) and the acidic environmental condition would have favored the conversion of terpene to methane and CO_2.

Catechin

The flavonoid content in terms of one of the prominent flavonoids in Calotropis biomass (catechin) has been determined. (Fig 1.)

The HPLC detection of catechin in Calotropis before biomethanations is 0.8 mg/ml and after biomethanation it is only 0.059 mg/ml. About 80 percent degradation has thus been observed in catechin during biomethanation.



Retention time (minutes)→

Fig. 1.Chromatogram showing flavonoid in *Calotropis* Biomass (Before biomethanation)

β-stigmasterol

In the present study, the steroid content in terms of one of the prominent steroid in *Calotropis* biomass (β-stigmasterol) has been determined.Steroid degradation will occur at high temperature (Biedermann*et al.,* 1996). The fermentor under anaerobic condition at 37˚C in the present experimental condition would have favored the degradation of steroids through the enzymes of the methanogens.

The HPLC detection of steroid in *Calotropis* before biomethanation is

1.2 mg/ml; after biomethanationit is only 0.58 mg/ml . About 53 per cent degradation has thus been observed in β-stigmasterol during biomethanation.. In the present study, the alkaloid content in terms of one of the prominent alkaloid in *Calotropis* biomass (histamine) has been determined.

Histamine

The HPLC detection of histamine in *Calotropis* before biomethanationis 1.32mg/ml; after biomethanation it is only 0.6 mg/ml. About 48 per cent degradation has thus been observed in catechin during biomethanation.

The mechanism of degradation of secondary metabolites depends on both the structure of the compound and the microorganism species (Donova, 2007).

According to Ermavathi*et al.* (2006) oxidation is a powerful reaction that can attack C=C in aromatic heterocyclic and estrogenic substances.

Among the three secondary metabolites estimated during methanogenesis in this study different degrees of degradation have been observed (Flavanoid-92 per cent, Terpenoid-80 per cent, Alkaloid-55 per cent. Flavonoids>Terpenoids>Alkaloids.

The structural variations of these four secondary metabolites would have differently reacted with the available enzyme system during biomethanation process. Flavonoids are highly water soluble due to the glycoside linkages. This is also very unstable (Heneidak*et al*., 2006). Hence, possibly the maximum degradation.Terpenoids are highly volatile and heat labile. So at the temperature of fermentor (37˚C) the volatilization and degradation would have occurred. True alkaloids are heterocyclic ring containing nitrogen. The degradation of these structures is highly complicated. Thus the four secondary metabolites respond differently to the experimental conditions. Steroids are secondary alcohols with a very long carbon skeleton consisting of a tetracyclic steroid molecule.

Hence it is concluded that the degradation potential depends upon the structural integrity of the secondary metabolites.Compounds with simpler structure degrade faster than the compound with complex structure.

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