ISOLATION & CHARACTERIZATION OF METHANOGENIC BACTERIA FROM LOCAL ENVIRONMENT AND COMPARATIVE STUDY OF KINETICS WITH TRADITIONAL BIOGAS PRODUCERS.

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Abstract: In the present study the soil samples from paddy field (kalikiri Andhra Pradesh), Karnataka compost development corporation limited (KCDC, kudlu village, Bangalore) & spent waste from floating type biogas plant were collected and methanogens were isolated, characterized & subjected to growth under anaerobic conditions by providing the kitchen and vegetable market waste as the substrate the kinetic study was carried out. A total of 16 isolates were obtained from the sample. The morphology and phenotypic characteristics were studied by performing various biochemical tests which revealed that the organism were anaerobes. Biogas production was carried out by using anaerobic digester. Paddy field and Biogas sample showed maximum production of biogas than KCDC. But mixed consortia had a very high degrading ability in very short period of time up to 92.95%.

(*keywords- municipal solid waste, methanogens, anaerobic digestion, Modified Gompertz equation, biogas production*)

I. INTRODUCTION

Municipal Solid Waste (MSW), commonly known as trash or garbage and as refuse or rubbish, is a waste type consisting of everyday items that are discarded by the public. "Garbage" can also refer specifically to food waste, as in a garbage disposal. The composition of municipal solid waste varies greatly from municipality to municipality,[1] and it changes significantly with time. Biodegradable waste can be commonly found in municipal solid waste (sometimes called biodegradable municipal waste). Biodegradable waste: food and kitchen waste, green waste, paper (most can be recycled although some difficult to compost plant material may be excluded [2]). In the absence of oxygen, much of this waste will decay to methane by anaerobic digestion.[3] Biodegradable waste includes any organic matter in waste which can be broken down into carbon dioxide, water, methane or simple organic molecules by micro-organisms and other living things using composting, aerobic digestion, anaerobic digestion or similar processes. Anaerobic digestion is a collection of processes by which microorganisms break down biodegradable material in the absence of oxygen.[4] The process is used for industrial or domestic purposes to manage waste or to produce fuels. Much of the fermentation used industrially to produce food and drink products, as well as home fermentation, uses anaerobic digestion. Anaerobic digestion (AD) occurs naturally in some soils and in lake and oceanic basin sediments, where it is usually referred to as "anaerobic activity".[5][6] This is the source of marsh gas methane as discovered by Alessandro Volta in 1776.[7][8]The digestion process begins with bacterial hydrolysis of the input materials. Insoluble organic polymers,such as carbohydrates, are broken down to soluble derivatives that become available for other bacteria. Acidogenic bacteria then convert the sugars and amino acids into carbon dioxide, hydrogen, ammonia, and organic acids. These bacteria convert these resulting organic acids into acetic acid, along with additional ammonia, hydrogen, and carbon dioxide. Finally, methanogens convert these products to methane and carbon dioxide.[9] The methanogenic archaea populations play an indispensable role in anaerobic wastewater treatments.[10]Anaerobic digestion(AD) is used as part of the process to treat biodegradable waste and sewage sludge. As part of an integrated waste management system, anaerobic digestion reduces the emission of landfill gas into the atmosphere. The nutrient-rich digestate produced can be used as fertilizer. Methanogens are microorganisms that produce methane as a metabolic byproduct in anoxic {low level oxygen} conditions. They belong to the domain of archaea most of them have cell wall. They occur in 2 forms cocci, bacilli, consume H_2 & CO_2.Methanogens are responsible for marsh gas {bio gas} production. They are obligate anaerobes, extremophiles, living in the guts of cows, deep in swamps and even in the muck from sewage treatment plants. Methanogenesis or bio methanation is the formation of methane by microbes known as methanogens. Methanogenesis is sensitive to both high and low pH's and occurs between pH 6.5 and pH 8.[11] The remaining, indigestible material the microbes cannot use and any dead bacterial remains constitute the digestate.[12]

II. MATERIALS AND METHODS

A. Collection of samples

The samples from three different places, soil sample from paddy field(PD) of kalikiri Andhara Pradesh, fine manure sample from Karnataka compost development corporation limited(KCDC) and spent from floating type biogas plant(BG)of chikballapura were collected in sterile zip-lock plastic maintaining aseptic conditions. The collected samples were brought to the laboratory for isolation and characterization.

B. Construction of anaerobic chamber

The chamber was constructed by using polycarbonate material & anaerobic condition was maintained by complete removal of oxygen.

C. Isolation of methanogens

Methanogenic organisms were grown in Thioglycollate media [13] by serial dilution of sample and by using pour plate & spread plate method. (Basic Practical Microbiology A Manual by Society for General Microbiology (SGM)). Identification of bacterial isolates was done on the basis of their colony characteristics on the basal media & Gram staining [13].

D. Fluorescence test

Fluorescence is presumptive evidence for methanogenic bacteria, but definitive proof requires further characterization. [13]. To observe the presence of fluorescence, the isolated colony plates were directly placed in UV Trans-illuminator & checked for blue green fluorescence.

E. Agar deep culture

An agar deep culture produced by a deep inoculation into a solid medium (thioglycollate agar media) that is used especially for the growth of anaerobic bacteria (Harley J.P. And Prescott Lansing L.M. 2002. Laboratory Exercises in Microbiology, 5th Ed. McGraw-Hill Higher Education, New York, NY, USA). The inoculation was carried out using a sterile needle loop. The obtained isolates were subculture in liquid BM3 media. [14]

F. Cell count and motility test

The cell count was performed by using hemocytometer and the results were recorded. Motility test was carried out using hanging drop technique to identify motile & non-motile isolates. [13] Gram's staining was carried out for the obtained isolates in order to study their morphology ("Pioneers in Medical Laboratory Science: Christian Gram 1884"). [15]

G. Biochemical tests

Biochemicals tests such as IMViC, urease, TSI, catalase, carbohydrate fermentation test, starch test were performed in order to identify bacterial species based on the differences in the biochemical activities of bacteria.

H. Antibiotic sensitivity test

Antibiotic sensitivity test was carried out by disc diffusion technique using streptomycin (10mcg) and the results were recorded.

I. Waste collection and processing

Kitchen Waste (KW) was collected from houses of residential area (H S R layout) & vegetable market waste were collected from Madiwala vegetable market. The waste along with inoculum (3:1 ratio) was fed to screw capped pet bottles and the setup was left at room temperature for the production of biogas through AD. Cow Manure (CM) was collected from Mallela (Pileru, Andhra Pradesh). The wastes were cut into small size & blended in order to reduce size to ease the process of digestion.

J. Liquid displacement setup

A simple lab-scale experiment was fabricated using digesters. Each digester was made of plastic pet bottles. In this study the volume of produced gas was measured by water displacement method considering the volume of the generated gas equal to that of expelled water in the water collector. Each digester was connected to water chamber (plastic bottles) by a plastic pipe (gas pipe) which was used to pass the produced gas into water chamber. Another glass pipe (water pipe) was used to take the displaced water from the water chamber to the water collector which was fitted & air sealed. Both the ends of the gas pipe were inserted just at the top of the digester and the water chamber. The water pipe was inserted just bottom of the water chamber and top of water collector. The set up is illustrated in figure 1.

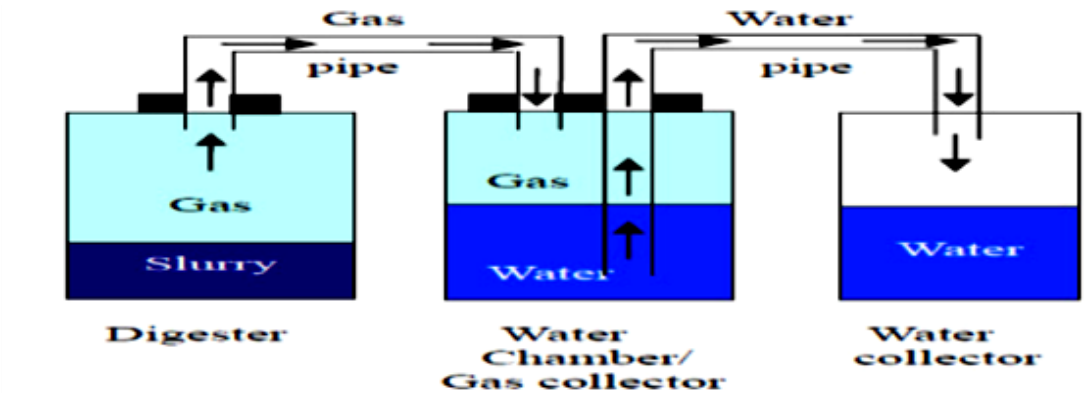


Fig 1: schematic diagram of the lab-scale experimental set-up [16]

K. Solid Analysis

- **Moisture content(MC):** The MC is the water content that is present in the reaction mixture. The water present in the reaction mixture was measured by the direct method of determination.

$$\text{Moisture Content (\%)} = \frac{W2-W3}{W2-W1} * 100$$

Where
W1= weight of the container; W2= weight of the container and sample before drying; W3= weight of the container and sample after drying.

- **Total solids(TS):** Total solids include both the suspended solids and the dissolved solids which are obtained by separating the solid and liquid phase by evaporation. These solids are the substrate components that will be utilized for the production of the biogas.

$$\text{Total Solids (\%)} = 100 - \text{Moisture (\%)}$$

- **Volatile Solids(VS):** the solids that remain after drying, evaporating or filtration were then ignition at 600° C. the sample turn to ash.

$$\text{Volatile solids(\%)} = \frac{W2-W3}{W2-W1} * 100$$

Where
W1= weight of the dish; W2= weight of the dried residue and dish; W3= weight of the residue and dish after ignition.

III. Result and Discussion

The samples were successfully collected in sterile zip-lock plastic maintaining aseptic conditions. An anaerobic chamber was constructed and anaerobic condition was maintained. The isolation of methanogens was carried out by using different dilutions of sample (10^{-2}, 10^{-3}, 10^{-4}). Pure cultures were selected by streaking the individual colony on TG agar plates. Pentagon streak was carried out in order to check whether the culture consists of only one organism. A total of 16 bacterial strains were identified by standard bacteriological identification procedure from the three samples. They were named as KC1-KC5 (obtained from KCDC), BG1-BG5(biogas plant) and PF1-PF6(paddy field) respectively. Microscopic examinations were carried out by gram staining and motility test. The results obtained are given in table 1.

Table1: result for cell count, gram's staining, motility test

Sl. No.	Strain No.	Cell Count(Cells/ml)	Gram's Staining	Shape	Motility
1.	KC1	$35*10^4$	Gram Negative	Cocci	Motile
2.	KC2	$27*10^4$	Gram Negative	Cocci	Motile
3.	KC3	$49*10^4$	Gram Negative	Cocci	Non -Motile
4.	KC4	$50*10^4$	Gram Negative	Cocci	Motile
5.	KC5	$50*10^4$	Gram Negative	Cocci	Non -Motile
6.	BG1	$19*10^4$	Gram Negative	Bacilli	Non- Motile
7.	BG2	$27*10^4$	Gram Negative	Bacilli	Motile
8.	BG3	$24*10^4$	Gram Negative	Bacilli	Motile
9.	BG4	$48*10^4$	Gram Negative	Bacilli	Motile
10.	BG5	$26*10^4$	Gram Negative	Bacilli	Motile

11.	PF1	$21*10^4$	Gram Negative	Cocci	Non-Motile
12.	PF2	$48*10^4$	Gram Negative	Bacilli	Non-Motile
13.	PF3	$31*10^4$	Gram Negative	Bacilli	Non-Motile
14.	PF4	$28*10^4$	Gram Negative	Bacilli	Motile
15.	PF5	$48*10^4$	Gram Negative	Bacilli	Non-Motile
16.	PF6	$23*10^4$	Gram Negative	Cocci	Non-Motile

a. Fluorescence test

Fluorescence test was carried out for the identification of methanogenic bacteria containing the F420 coenzyme which shows blue-green fluorescence by methanogenic bacteria and is readily distinguishable from the white-yellow fluorescence occasionally observed in non-methanogenic colonies. The strains showed no fluorescence indicating the absence of F240 co-enzyme production

b. Butt Culturing

The isolates were found to be anaerobic & few isolates developed cracks in agar indicating the gas production.

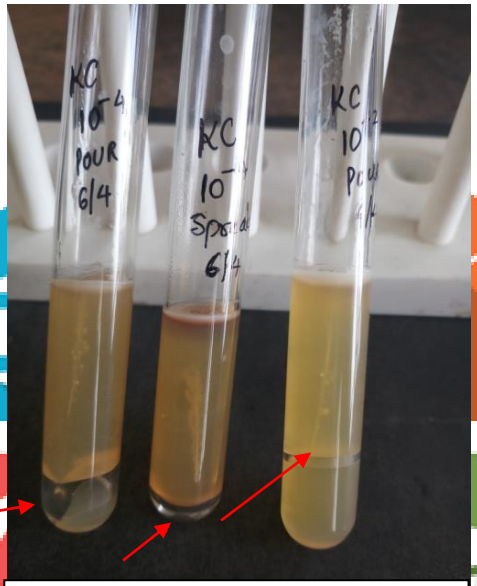


Fig2a: results for butt culture technique with gas production

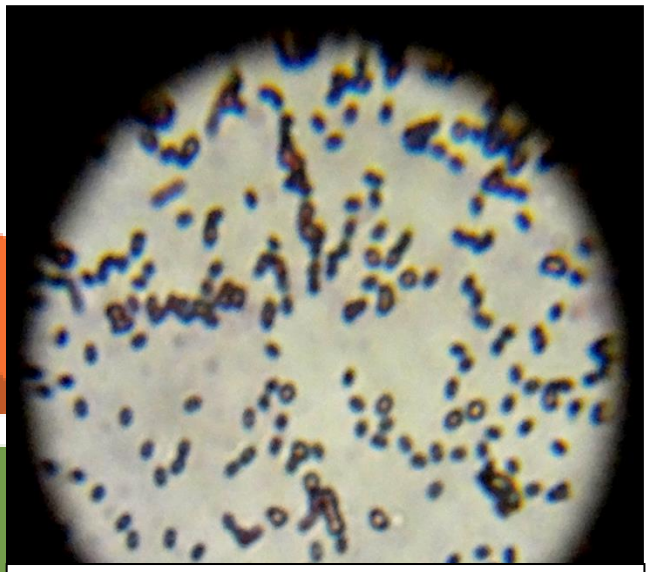


Fig 2b: results for grams staining indicating gram negative cocci shaped microbe.

c. Biochemical tests

Table 2: result for biochemical tests

SL.NO	STRAIN NO	I	M	V	CI	C	TSI	U	CHF	SHT
1	KC1	-ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve
2	KC2	-ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve
3	KC3	-ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve
4	KC4	-ve	-ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve
5	KC5	-ve	+ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve
6	BG1	-ve	-ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve
7	BG2	-ve	+ve	-ve	-ve	+ve	+ve	-ve	+ve	+ve
8	BG3	-ve	+ve	-ve	+ve	+ve	+ve	-ve	+ve	+ve
9	BG4	-ve	-ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve
10	BG5	-ve	+ve	-ve	+ve	+ve	+ve	-ve	+ve	+ve
11	PF1	-ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve
12	PF2	-ve	+ve	-ve	+ve	-ve	+ve	-ve	+ve	+ve

13	PF3	-ve	+ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve
14	PF4	-ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve
15	PF5	-ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve
16	PF6	-ve	-ve	-ve	+ve	-ve	+ve	-ve	+ve	+ve

I-Indole test
M-Methyl red test
V-Vogues Proskauer test
CI-Citrate test
C-Catalase test
TSI-Triple Sugar Iron test
U-Urease test
CHF-Carbohydrate Fermentation Test
SHT-Starch Hydrolysis test

d. Antibiotic sensitivity test

The results of antibiotic sensitivity test are given in table3.

Table3: results of antibiotic sensitivity test

Sl.no	Strain no	Antibiotic sensitivity	MIC(cm)
1	KC1	+ve	1.8
2	KC2	+ve	1.0
3	KC3	+ve	2.0
4	KC4	-ve	1.7
5	KC5	+ve	2.05
6	BG1	+ve	1.1
7	BG2	+ve	1.2
8	BG3	+ve	2.0
9	BG4	+ve	0.9
10	BG5	+ve	1.1
11	PF1	+ve	1.7
12	PF2	+ve	1.5
13	PF3	+ve	1.8
14	PF4	+ve	1.5
15	PF5	+ve	1.5
16	PF6	+ve	0.8

e. Liquid displacement

The biodegradable waste was fed into the screw capped PET bottles along with crude sample in 3:1 ratio (vegetable waste: inoculums), 4%NH4OH, 1.5%NaOH, 4g of cow manure(CM) was added to enhance the process of gas production.1.5%NaOH is used to maintain the alkaline (6.8 to 7.2) condition. The setup was left at room temperature for 20 days. The results are tabulated as follows

Amount of liquid displaced is tabulated as

Table4: Amount of liquid displaced in ml (crude sample)

Duration(days)	Sample 1 (PF)	Sample2(KC)*	Sample3(BG)
0	860	0	30
5-10	35.6	0	0
10-15	62.95	0	542
15-20	10	57	90

*- after 20th day KCDC produced gas but other samples were already converted into organic matter.

Table 5: Amount of liquid displaced in ml (result for isolated strains)

Sl	Strain no	Duration(days)	Liquid displaced(ml)

no			
1	BG1	0-5	-
		5-10	-
		10-15	-
		15-20	-
2	BG2	0-5	-
		5-10	-
		10-15	-
		15-20	6.2
3	BG3	0-5	-
		5-10	-
		10-15	-
		15-20	-
4	BG4	0-5	-
		5-10	-
		10-15	100
		15-20	250
5	BG5	0-5	-
		5-10	-
		10-15	-
		15-20	45
6	PF1	0-5	-
		5-10	-
		10-15	-
		15-20	-
7	PF2	0-5	-
		5-10	-
		10-15	
		15-20	-
8	PF3	0-5	-
		5-10	-
		10-15	-
		15-20	-
9	PF4	0-5	
		5-10	-
		10-15	-
		15-20	-
10	PF5	0-5	67
		5-10	
		10-15	-
		15-20	-
11	PF6	0-5	-
		5-10	-
		10-15	-
		15-20	-

f. Solid analysis

Moisture content and Total solids(TS) were determined gravimetrically after drying in oven at 105˚C. Volatile solids (VC) content was analysed by ignition dried sample at 600˚C for 2 hours and determining the ash free dry weight.

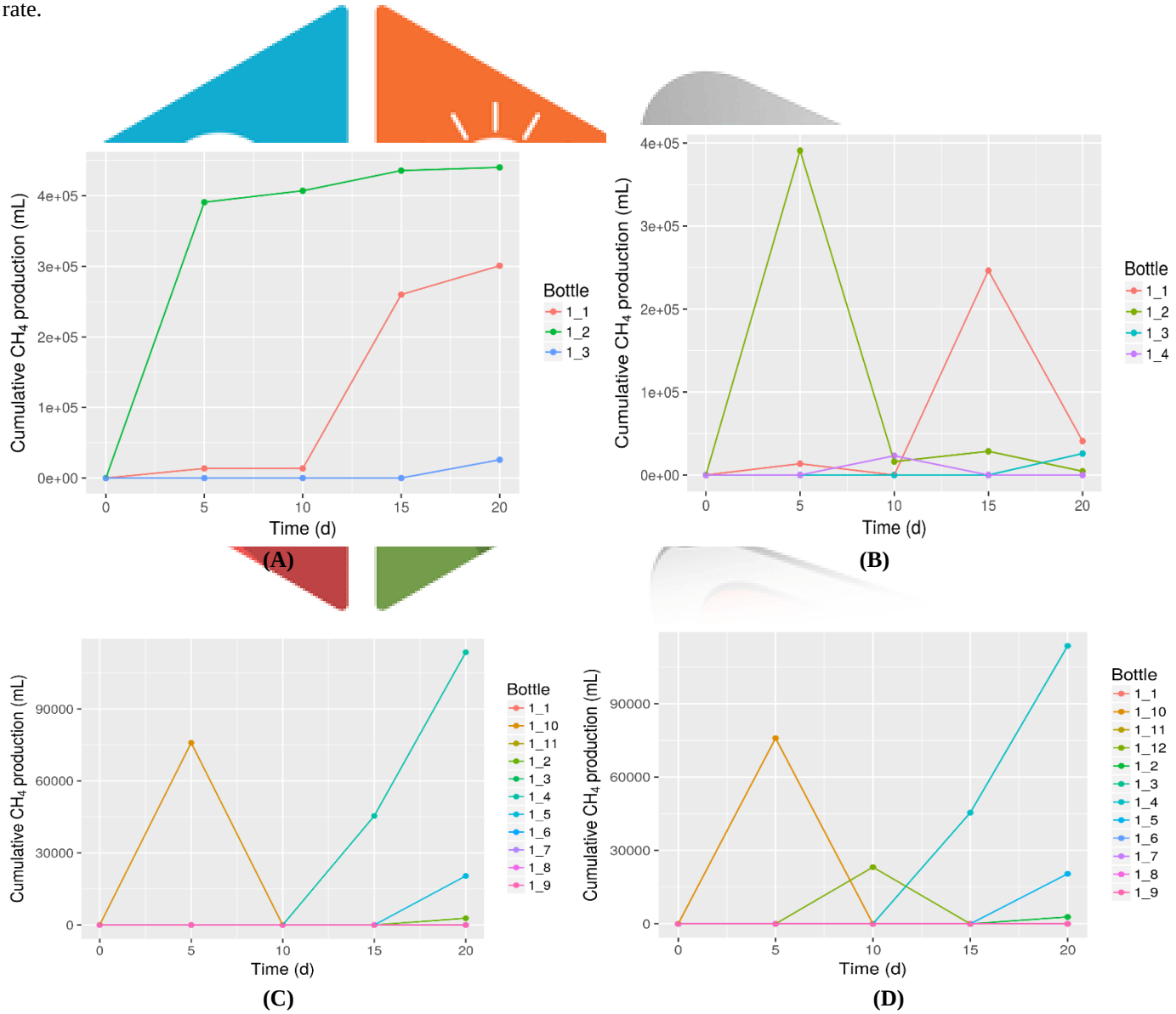
Table 6: Solid analysis of the crude sample

SAMPLE	MC (%)	TC (%)	VC (%)
B1	94.4	5.6	88.14
B2	92.03	7.97	89.71
P1	92.7	7.3	77.05
P2	95.60	4.4	74.29
F2	94.9	5.1	75.55
F3	94.7	5.3	68.42

Table7: Solid analysis of the isolated sample

STRAIN	MC (%)	TC (%)	VC (%)
BG2	92.54	7.46	82.78
BG4	78.93	6.16	80.59
PF5	88.2	11.8	88.35
MIX	92.95	7.05	78.93

It was seen that after isolation the strain BG2 and mixed consortia (mix) had the ability to produce more amount of biogas at faster rate.



Graphs: Cumulative biogas production of (A) crude sample(1_1-BG,1_2-PF,1_3-KC) ;(B) crude sample with mix consortia(1_1-BG,1_2-PF,1_3-KC,1_4-Mix) ;(C) isolated sample (1_1to 1_5-BG,1_6 to 1_11-PF) ;(D) isolated sample with mix consortia (1_1to 1_5-BG,1_6 to 1_11-PF, 1_12-mix).

IV. Discussion

The study of biogas production from different substrate were conducted in anaerobic digester. The organisms were grown anaerobically using thioglycolate media. The morphology and phenotypic characteristics were studied by performing various biochemical tests which revealed that the organism were anaerobes. The biogas production was monitored and measured by liquid displacement study at regular intervals. A good substrate characterization is important in prediction of biogas potential from different substrates. The above graph indicates that mixed consortia had high degrading ability when compared to crude samples and isolated strains. Thus, speeding up the process of conversion of waste into organic matter. The moisture content of substrates ranged from 88% to 94%, this indicated that the substrate had enough moisture content for anaerobic digestion. The volatile solid of substrates ranged from 76% to 88%, this indicated that the substrate was rich in organic solid content that was to be converted to biogas.

V. Conclusion

It is concluded that individual strains had a potential to degrade the waste to organic matter in short period of time indicating a faster degradation rate compared to crude samples. The crude sample showed high amount of water displacement with increasing time, indicating the slow rate of degradation of waste into organic matter but higher rates of biogas production. From the graph, it was found that the mixed consortia have a high degrading ability in a short period of time. So, it can be concluded that mixed consortia gave a better result than the individual strains and can be used for speeding up the process.

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