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Research article

ISOLATION AND IDENTIFICATION OF ACETOGENIC AND METHANOGENIC BACTERIA FROM ANOXIC BLACK SEDIMENTS AND THEIR ROLE IN BIOGAS PRODUCTION

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ABSTRACT: The increasing population and developing science and technology increased human comfort and needs which in turn increased need of burning fuel. Universal resource of this fuel in non-renewable therefore there is a constant search for renewable eco-friendly energy source. Biogas is one such energy which needs greater attention towards effective production of methane under anaerobic decomposition. In the light of this the present studies aimed at isolating the acetogenic and methanogenic bacterial species and understand their growth dynamics for enrichment of biogas production. *Acetobacterium woodii* and *Methanospirillum hungatei* were the acetogenic and methanogenic species which reported to be effectively involved in anaerobic decomposition. Present study was successfully identifying the species through specific media and characterizes them biochemically. Growth of acetogenic and methanogenic species found to be enhanced by yeast extract and increasing substrate at acidic pH for acetogenic species and alkaline pH for methanogenic species. However, growth was higher with methanogenic species when compared to the acetogenic species in concentration of yeast extract and substrate. During the biogas production enrichment of both the species increased the biogas production both at individual and combined levels. The present study would suggest that detailed study on this species at different concentration of inoculate, temperature, and other chemical and physical resources would further strengthen the attempt in effective production of biogas through anaerobic digestion.
Keywords: Biogas, *Acetobacterium woodii*, *Methanospirillum hungatei*, Growth kinetics, anaerobic digestion.

INTRODUCTION

Anaerobic decomposition is a process by which a complex mixture of symbiotic microorganisms transforms organic materials under oxygen-free conditions into biogas, nutrients and additional cell matter, leaving salts and refractory organic matter. Raw biogas typically consists of methane (60%), carbon dioxide (40%), water vapor and trace amounts of hydrogen sulfide [20]. Healy and Young (1979) first reported on the anaerobic degradation of vanillic acid and other substituted aromatic acids by methane forming enrichment cultures. Later on, the complete anaerobic oxidation of benzoate and phenyl substituted fatty acid by pure cultures of sulfate-reducing bacteria was demonstrated [25]. These results encouraged experiments with vanillic acid as substrate of anaerobic enrichment cultures for sulfate-reducing bacteria. Anaerobic mud and sediment samples of various freshwater environments served as inoculants. To our surprise, most of the cultures yielded well growing bacterial enrichments after 3 to 12 days of incubation. The formation of H_2S from sulfate was detected only in some of the enrichments and only after prolonged incubation. Anaerobic acetate-producing bacteria are generally referred to as acetogens [17]. Besides using polysaccharides, most of the known acetogens also fermented as single carbon compounds to acetate and under certain conditions, some produce butyrate and caproate [13]. Bacterial growth on single-carbon substrates has been termed unicarbonotrophy or methylotrophy [25]. Methylotrophic growth is possible only if a more oxidized co substrate, *i.e.* CO2 is presented. Most of the information on acetogen single-carbon transformations derives from analysis of the glucose catabolism of *Clostridium thermoaceticum* [17]. Later, *Eubacterium limosum* was shown to utilize H_2-CO_2, methanol–CO_2, and CO for growth [18].

Acetobacterium woodii possesses a single-carbon substrate range similar to that of *Bacillus methylotrophicum* and *E. limosum* but does not produce butyric acid [4]. All three organisms contain high levels of corrinoids [26]. The reason for this is that these micro-organisms perform, in their energy metabolism, the transformation of unicarbon groups. Evidence indicates that *Acetobacterium* can oxidize substrates other than hydrogen, but the substrate range is narrow, being limited to fructose, glucose, lactate, methanol and glycerate [26]. The only major product detected is acetate as a sole fermentation product and for this reason; the organism is referred to as a homoacetogen. The nutritional requirements of an acetogen recently isolated from sea sediments and classified as *Acetobacterium* sp. Inoue *et al*., 1992 has been studied using methanol–CO_2 , but other unicarbon mixtures might be proposed [3, 16]. In view of this, it may be reasonable to suppose that formate may be another possible carbon substrate for *Acetobacterium* sp. as it is an important intermediate in the Acetyl CoA pathway [18, 6]. The catabolic and anabolic pathways are closely linked in methanol-utilizing anaerobes and the presence of a catabolic intermediate, such as formate, might be expected to create energetic effects other than that of acting as an extra source of energy, as reported for certain aerobic [2] and anaerobic micro-organism [16]. *Methanospirillum hungatei* was first found in sewage sludge and was named in honor of R.E. Hungate. This genus and species name was first proposed in by Ferry *et al.*, 1974 [11]. The cultures are usually yellow in color, circular in shape, and convex with lobate margins. The cells are spiral shaped (curved rods) and range from 0.5-7.4 microns in diameter and 15 to several hundred microns long and have tufts of polar flagella that provide a small amount of motility. The optimum habitat for these organisms has a temperature range of 30-37° Celsius (mesophilic) and a pH range of 6.6-7.4 [11]. *Methanospirillum hungatei* is motile, can grow either as single cells or in chains of cells, and possesses a complex cell envelope; thus, this archaebacterium is a unique microorganism in which to study locomotory properties. These cells possess an encompassing cell wall, which in turn is bounded by a resilient sheath structure and two spacer plugs, one at each pole of the cell. It was of interest to determine whether the flagella would span all of these layers and still maintain movement without some architectural modification to either its component parts or the insertion site within the cell envelope. Since both the sheath and spacer (or end) plug have a paracrystalline structural format, the existence of holes for flagella may introduce localized crystal defects that could affect the integrity of each structural layer. In addition, the plasma membrane possesses novel diether- and tetraether-linked phospholipids, which chemically bond the bilayer together. Therefore, lipid packing and movement within the membrane are affected by more than hydrophobic forces. Thus, these characteristics made it important to study the structural organization and insertion site of *M. hungatei* flagella into this novel and interesting cellular envelope. Flagella of archaea have not been extensively studies either biochemically or ultrastructurally. From a limited number of observations, the occurrence of multiple flagellins in archaebacterial flagella appears to be more common than in eubacterial flagella. In *Halobacterium* halobium, recent evidence indicates there are five different but highly homologous genes coding for the flagellins, which are sulfated glycoproteins. All five genes are expressed, and the five gene products have been shown to be integrated into the flagella, although their distribution has not been determined. Therefore the present studies aims at isolating the Acetobacterium woodii and Methanospirillum hungatei and understand their biochemical characters, growth dynamics and their efficiency in biogas production.

MATERIALS AND METHODS

Isolation

The anoxic black sediment samples were collected from domestic sewage at Rasipuram. Acetogenic bacterium was isolated through basal medium [4]. The compositions of basal medium for 1000ml were ammonium chloride1.0g, magnesium chloride 0.1g, potassium di-hydrogen phosphate 0.4g, di-potassium hydrogen phosphate 0.4g, resazurin 0.0001g, cysteine HCl 0.5g, sodium sulphide 0.5g, sodium bicarbonate 7g, calcium carbonate 10g, yeast extract 2g, vitamin solution 10ml, mineral solution 10ml, and agar 20g. The pH of the medium was 6.7. A Methanogenic bacterium was isolated through enriched medium [11]. The compositions of enriched medium for 1000ml were sodium benzoate 0.2g, ammonium chloride 0.075g, di-potassium hydrogen phosphate 0.04g, magnesium chloride 0.01g, resazurin 0.0001g, sodium carbonate 0.15g, Sodium sulphide 0.025g, agar 20g. The pH of the medium was 7.2.

Morphological and Biochemical parameters

Morphological and Biochemical characters of acetogenic and methanogenic bacteria was determined using gram staining and IMViC, triple sugar ion, lipid hydrolysis, starch hydrolysis and mannitol test.

Growth kinetics

Growth kinetics of acetogenic and methanogenic bacteria was studied under different pH of 6.2, 6.7, 7.2, 7.7, and 8.2 and 6.0, 6.7, 7.4, and 8.1with 0.5% and 0.2% concentration fructose, glucose, sodium formate and sodium benzoate as substrates for 48 hrs incubation respectively.

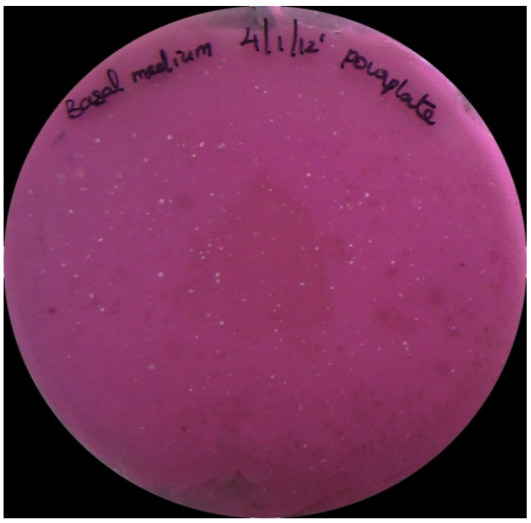
The growth was also recorded at different concentration of substrates specifically for acetogenic bacteria (0.04, 0.08, 0.12, 0.16, 0.20gm/ml) and methanogenic bacteria (0.05, 0.10, 0.15, 0.20gm/ml) under both with and without yeast extract for 48 hrs incubation. Growth was recorded through optical density in spectrophotometer at 660 nm and 550 nm.

Biogas production

The isolated acetogenic and methanogenic organisms were inoculated into laboratory scale biogas units where sewage water used as a substrate as sole (10%) and combined (10%) for 45 days gas samples were drawn for every 15 days and analyzed in GC-MS with porapak QS column for methane, carbon dioxides, hydrogen sulfide gases.

RESULTS

1 ml of anoxic black sediment samples at10^{-5} dilution inoculated into basal medium showed 77 colonies. The colonies appeared white in colour initially and growth produced yellow pigments.1ml of sewage water sample at10^{-7} dilution inoculated into enriched medium showed 66 lemon yellow colonies (Plate 1 & 2).



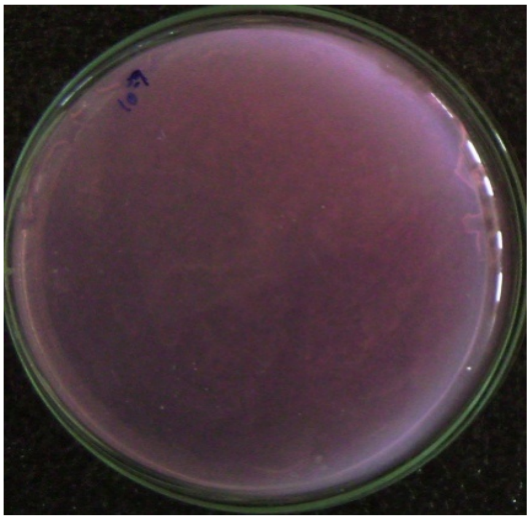


Plate1. Isolated *Acetobacterium woodii* **Plate 2. Isolated *Methannospirillum hungatei***

Gram staining of acetogenic bacteria showed gram positive, oval shaped, short rods (pairs) and appeared in purple color and methanogenic bacteria showed gram negative, spiral shaped and pink coloured colonies Biochemical test revealed that both the micro-organisms were positive for methyl red, citrate and triple sugar ion tests and negative for catalase, lipid and starch hydrolysis. Differences were found in indole test where acetogenic bacterium is negative and methanogenic bacterium is positive (Table 1). The morphological and biochemical parameters revealed that acetogenic bacterium was *Acetobacterium woodii* and the methanogenic bacterium was *Methanospirillum hungatei*.

Table 1. Biochemical tests of *Acetobacterium woodii* and *Methanospirillum hungatei*

S. No.	TEST	*A. woodii*	*M. hungatei*
1	Indole	-ve	+ve
2	Methyl red	+ve	+ve
3	Citrate	+ve	+ve
4	Triple sugar ion	+ve, acid, gas production	+ve, acid, gas production
4	Lipid hydrolysis	-ve	-ve
5	Starch hydrolysis	-ve	-ve
6	Catalase	-ve	-ve
7	Mannitol	-ve	-ve

In the growth kinetics of *A. woodii* with different pH and substrate, it showed increased growth in pH 6.7 with all the substrates used and decreased growth was observed in pH 6.2 with fructose as substrate and pH 8.2 with the other substrates shows decreased growth. In *M. hungatei* with different pH and substrate, it showed increased growth in pH 6 with fructose and sodium formate as substrates, pH 7.4 with glucose as substrate and pH 8.1 with sodium benzoate as substrate. Decreased growth was observed at pH 6 with glucose and sodium benzoate as substrate and pH 6.7 with sodium formate as substrate, pH 7.4 with fructose as substrates (Figure 1 & 2).

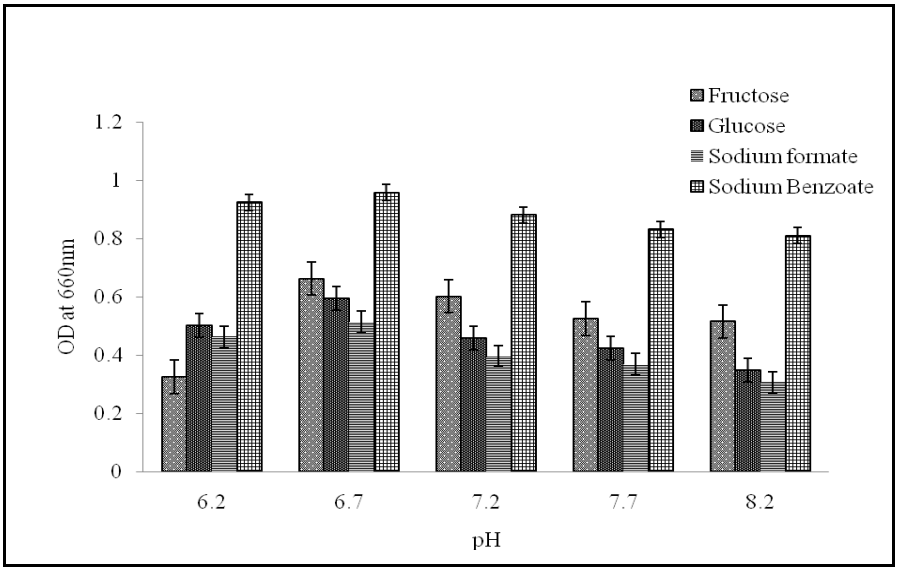


Fig: 1Growth kinetics of *A.woodii* in different pH with different substrate

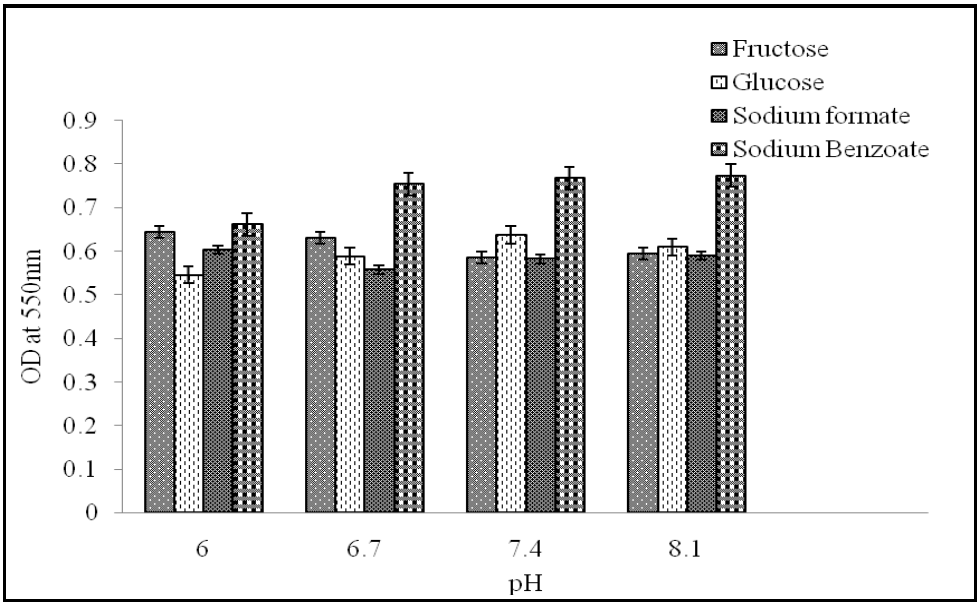


Fig: 2 Growth kinetics of *M.hungatei* in different pH with different substrate

In the growth kinetics of *A. woodii* with different substrate concentration with yeast extract, showed increased growth in 0.2% concentration of all the substrates used and decreased growth was observed in 0.04 %. The same without yeast extract showed increased growth in 0.2% concentration of all the substrates used and decreased growth was observed in 0.04%. However, increased growth in the medium of substrate with yeast extract showed increased growth comparing to the substrate without yeast extract (Figure 3 & 4).

In the growth kinetics of *M. hungatei* with different substrate concentration with yeast extract, showed increased growth in 0.1% concentration of the fructose and glucose, 0.05% concentration of sodium formate and sodium benzoate, and decreased growth was observed in 0.15% concentration of sodium formate and 0.2 %concentration of fructose, glucose and sodium benzoate. The same with different substrate concentration without yeast extract, showed increased growth in 0.1% concentration of the fructose, 0.05% concentration of glucose and sodium benzoate, 0.2% concentration of sodium formate and decreased growth was observed in 0.15% concentration of fructose, glucose and sodium formate and 0.2% concentration of sodium benzoate. This also showed increased growth in the medium of substrate with yeast extract comparing to the substrate without yeast extract (Figure 5 & 6). Estimation of biogas characteristics with GC-MS showed increased methane concentration in all the treatments when compared to control, further this increase was observed only up to 30 days in all the treatments where as in control increase in methane concentration was observed up to 45 days in control (57.29 ± 0.86 %). However high methane concentration (83.16 ± 0.72 %) was recorded when both Acetogenic and Methanogenic microorganisms were substituted into the biogas plant followed by substitution of only methanogenic microorganism (81.38 ± 0.08 %) and acetogenic microorganism substitution (79.3 ± 0.9 %). High carbon dioxide and hydrogen sulfide was recorded in the control samples and less in Methanogenic microorganism substitution (Figure 7).

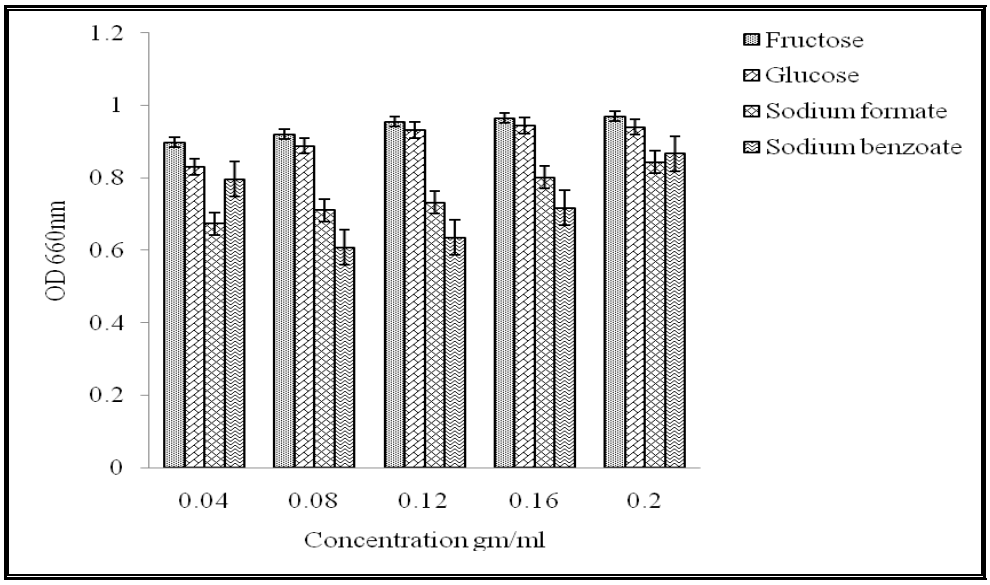


Fig: 3 Growth kinetics of *A.wodii* at different concentration of carbon sources with yeast extract

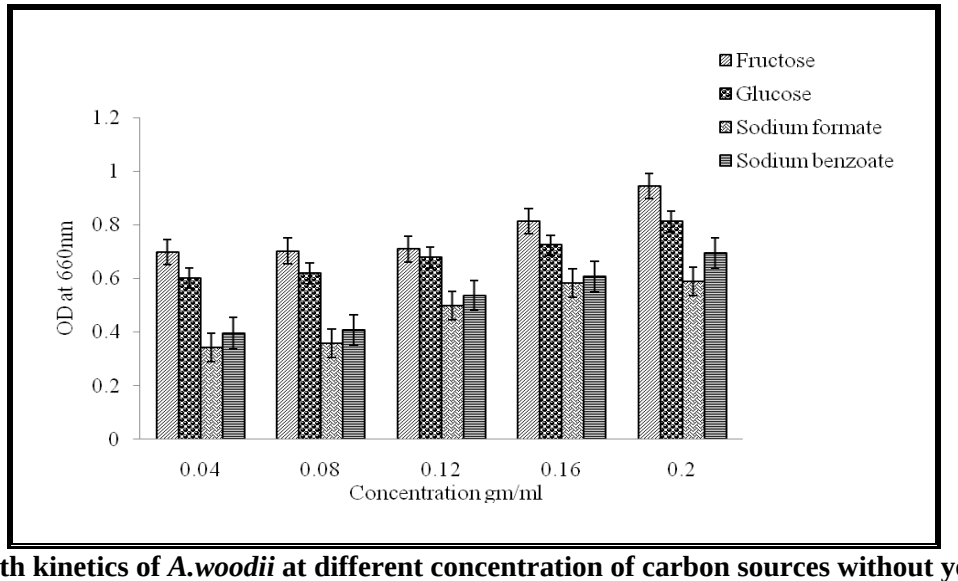


Fig: 4 Growth kinetics of *A.woodii* at different concentration of carbon sources without yeast extract

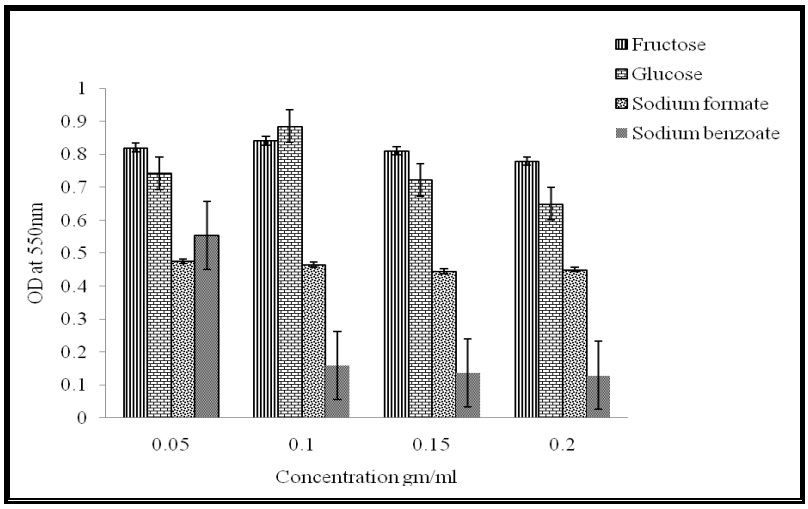


Fig: 5 Growth kinetics of *M.hungatei* at different concentration of carbon sources with yeast extract

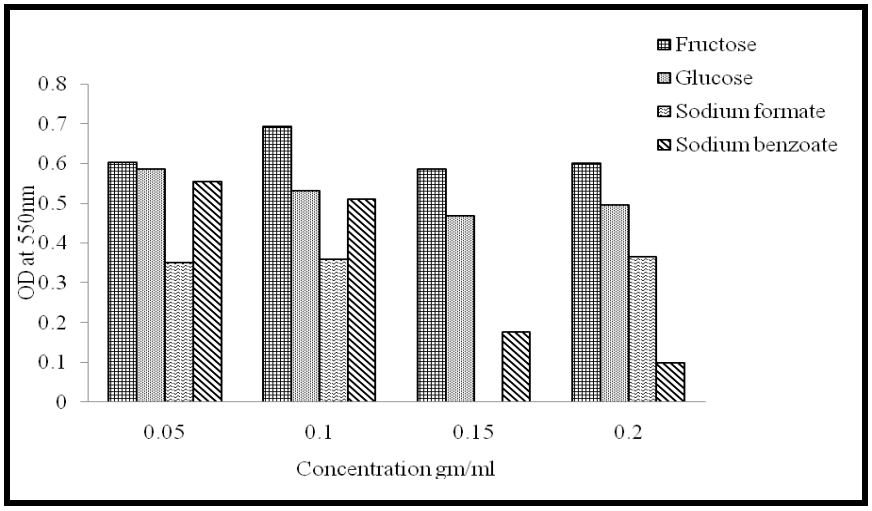


Fig-6: Growth kinetics of *M.hungatei* at different concentration of carbon sources without yeast extract

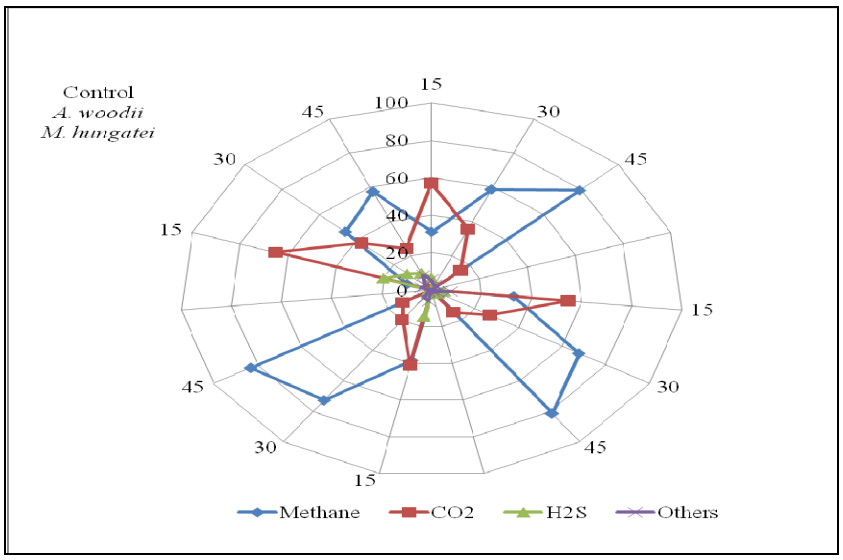


Fig: 7 Biogas Characteristics during the experiment

DISCUSSION

Biogas is an anaerobic decomposition technology in which biochemical reaction carried out by several types of microorganisms that require little or no oxygen to live. During this process a gas that is mainly composed of methane and carbon dioxide is produced. The amount of gas produced varies with biochemical characteristics of organic wastes, consortia of microorganisms, pH, temperature, etc. However, anaerobic digestion progresses with several complex sequential and parallel biological reactions with many intermittent products through action of sequential of microorganisms producing substrates for other [22, 23, 8]. These reactions were consolidated into four phases as Hydrolysis/liquefaction, acidogenesis, acetogenesis and methanogenesis and proposed a model anaerobic digestion [8]. In the present research acetogenic (*A. woodii*) and methanogenic (*M. hungatei*) species were isolated from sewage waste were substituted into biogas plant to understand their role in biogas production. Biochemical study of *A.Woodii* showed they are gram positive which indicates peptidoglycan in the cell wall with DD-transpeptidase indicates that it act upon the products of proteins from hydrolysis phase and peptides are being degraded to release more propionic acid, CO_2 and hydrogen in the substrate for methanogenesis which was the major function of acetogenic organisms [28]. *M. hungatei* is the gram negative organism, which contains a very thin layer peptidoglycan lacking in degrading peptide therefore they depend on the acetogens [19]. *M. hungatei* showed positive to indole test indicating that it acts upon the amino acids and carbon dioxide for forming methane and other gases in the presence triptophanase of which is the functions of methanogens [12, 9]. Both species are positive to methyl red which shows their presence and growth in acid phase during the biogas production. Positive to citrate test shows the presence of citritase which breaks down citrate to oxaloacetate and acetate and further oxaloacetate into pyruvate and carbon dioxide which also produces ammonia with sodium citrate. Similar production of ammonia and carbon dioxide are reported during the biogas production [1, 14, 27]. Positive to triple sugar test of both the species indicate that they utilize sugar and produce hydrogen in the media which is reported during the biogas production [15, 24]. Negative to Lipid hydrolysis, starch hydrolysis, catalase and Mannitol by both species is possible since they are thriving in acidic and anaerobic. Growth kinetics of both species were similar with growth in acetogenic in acidic pH and methanogenic species ranged between acidic and at litter higher pH in sodium benzoate as substrate indicating the adaptation nature where *A. woodii* is acetogenic and *M. hungatei* is methanogenic. However there are reports that the *A. woodii* do not grow in sodium benzoate [5] the may be due to change in pH conditions. Both species in different substrates without yeast extract growth was less indicating that both the species growth in enhanced by addition of yeast extracts particularly by fructose however, the growth of *A. woodii* is little higher than the *M. hungatei* similar observations were made by Braun,1981 [5]. GC-MS analysis of the gases evolved during the 45 days showed high concentration of methane production when both acetogenic and methanogenic organisms were used this is as expected since both are the important phase in methane production where the acetogenic produced suitable substrates for methanogenic organisms to produce methane [21]. However the 83% of methane is quite high in 30 days time therefore it could be deduced that both *A. woodii* and *M. hungatei* were compatible consortia. At individual level the *M. hungatei* produced higher methane concentration 81% which is enhanced by addition of *A. woodii.*

CONCLUSION

It is evident the both the *A. woodii* and *M. hungatei* were functioning as acetogenic and methanogenic from their biochemical and growth characteristics. Consortium of these two microbes was also successful in increasing the methane yield and reducing the time however more consortia's could be tested for large scale applications.

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