Production of Biogas by Mesophilic Bacteria Isolated from Manure

Wardah Sharmeen Syed*, Muhammad Nadeem†, Ikram-ul-Haq‡, Farid Ahmed Khan§

1Government College University, Lahore, Pakistan; 2PCSIR Laboratories Complex, Lahore, Pakistan; 3University of Veterinary and Animal Sciences, Lahore, Pakistan
*Corresponding author: wardah_syed2002@yahoo.com

INTRODUCTION

Our lifestyle is basically based on most of the energy demanding processes. The total energy demand of the world is estimated to be 400EJ/Year (McKendry, 2002). Most of the recent reports have indicated that this would increase by the factor of two or three during this century (International Energy Agency, 2006). Globally the demand for energy has been encompassed to 88% by the use of fossil fuels. However, all the existing resources are scarce to fulfill the total energy claims.

Biofuel is the most promising alternative resource. The demand for bio-fuels had been enhanced by the past few decades to 30 billion (3x109) in 2003 (Stevens, 2004). This group includes bio–hydrogen, biodiesel, bio–ethanol and biogas (Kaparaju et al, 2009). Among these, biogas has to be used as a feedstock for producing a variety of materials and chemical, generation of electricity and heat (Weiland, 2010).

Biogas or bio–methane is a combination or a mixture of methane (60%), carbon dioxide (40%) so is “energy rich gas”. When CO2 is removed from the mixture, pure methane can be used in natural gas grid station and in the vehicles as a source of fuel. The remaining residue (after the removal of methane and carbon dioxide) is known as “digestate.” All of the nutrients like potassium, phosphorous and nitrogen which are essential plant nutrients are stored in this digestate and hence they can be used in agricultural fields as fertilizers (Barglund, 2006).

Biogas occurs widely in nature and it can be exploited for the production of biogas and reduction in CO2 emissions to intend decrease global warming (Claassen et al, 1999). In biogas synthesis selection of biomass is not only helpful in maintaining the microbial growth but also leads to the positive synergism (Mata–Alvarez et al, 2000). Cellulosic biomass, present excessively in nature, has high potential to cope up with the increasing energy demand but it cannot fulfill all the requirements to meet the energy demands. However, organic waste such as animal manure has been extensively demonstrated and practiced for such biogas production (El–Mashad and Zhang, 2010).

The biological anaerobic conversion of organic material is basically done in three evident steps. The first step i.e. hydrolysis, involves the transformation of the complex insoluble organic matter like fats, lipids, polysaccharides, nucleic acids, proteins etc. into easily soluble organic material i.e., fatty acids, monosaccharides and amino acids etc. This step is conducted by strict anaerobes including Bacteroides and Clostridia and facultative anaerobic bacteria like Streptococci etc. The second step is acidogenesis which includes microbial consortia which help in the breakdown of these simple, soluble organic materials into hydrogen, acetic acid, carbon dioxide and other lower weight simple volatile organic acids like butyric acid and propionic acid which later are converted into more simple form such as acetic acid (Yadvika et al, 2004). The acetotrophic archaea are responsible for converting acetate into methane. They are another category of obligate anaerobes (Ferry, 1992). The third step called “methanogenesis” includes some strict anaerobes like Methanosaeta spp., Methanococcus spp., Methanothrix spp. and Methanobacterium spp. which convert the products of second step into a mixture of methane and carbon dioxide and some amount of energy (Yadvika et al, 2004). Hydrogen might be proved as a limiting factor for the growth of methanogens when produced during the reaction (Bagi et al, 2007).

In the process of anaerobic digestion of the biomass only little information is available for the activity of hydrogenotrophic and acetogenotrophic methanogens (Demirel & Scherer, 2008). When the microbial populations were determined by composition, biomass and number in an anaerobic digester for one and two-stage processes under
continuous process, the concentrations of both acidogenic and methanogenic strains came out to be 99% and 26%, respectively (Solera et al., 2001). However the data on the composition and equilibrium between different strains of microbial strata isn't well understood in this two-stage anaerobic process (Lozano et al, 2009).

The optimization of the conditions (pH, temperature, oxygen concentration, humidity etc.), use of different chemical and biochemical additives, controlling the nutritional requirements of the microorganisms and by changing the feeding proportions are of keen interest while dealing with the production processes (Lettinga et al, 1980; Santosh et al, 2004; Azzar et al, 2008; Li et al, 2010; Wei et al, 2010). The use of biological agents (microorganisms) is one of the best methods used for enhancing the yield of biogas. Some fungal and bacterial strains have been found to increase the production of biogas by the range of 8.4–44% form cattle dung (Attar, 1998; Tirumale and Nand, 1994; Potivichayanon et al, 2011).

Temperature plays a very important role in the yield of biogas by these plants. In a recent study, the biogas yield was found out to be relatively decreased in the month of December at 24°C as compared to the yield observed in summer at 36°C in the month of April. This decreased ambient temperature led to the shift of microorganisms, as relatively a very diverse range of microbial community occupies these digesters (Rastogi et al, 2007).

Many countries are actively engaged in the fruitful improvement in this technology. Units for the methane gas production, using biomass had been employed in the rural areas of India and China, in order to meet their energy requirements (Levis, 1983) and most recently in Vietnam for the production of biogas efficiently.

MATERIALS AND METHODS
Isolation of the Desired Strain
The methanogens were collected and isolated from fresh manure samples collected from the PCSIR, Laboratories Complex Lahore, Pakistan. As the fresh manure is a reservoir of a number of bacteriological species so for isolation purpose the sample was serially diluted and the cells were allowed to grow on nutrient agar medium and on MRS agar medium. After the incubation period the bacterial growth was observed on the nutrient agar plates and not on the MRS agar medium. The incubation was provided in anaerobic gas chamber at 35°C for 48–72 hours. The culture was preserved at 35°C in Fluid thioglycolate medium.

The medium used for the purification of the culture was “Fluid thioglycolate medium” set at the pH of 6.9–7.3. The cells were allowed to grow for 24–48 hours at 37°C.

Production of Biogas
The production of biogas was studied first in the laboratory by setting a small practice which included 3 flasks with different feeding proportions are of keen interest while dealing with the production processes (Lettinga et al, 1980; Santosh et al, 2004; Azzar et al, 2008; Li et al, 2010; Wei et al, 2010). The use of biological agents (microorganisms) is one of the best methods used for enhancing the yield of biogas. Some fungal and bacterial strains have been found to increase the production of biogas by the range of 8.4–44% form cattle dung (Attar, 1998; Tirumale and Nand, 1994; Potivichayanon et al, 2011).

Temperature plays a very important role in the yield of biogas by these plants. In a recent study, the biogas yield was found out to be relatively decreased in the month of December at 24°C as compared to the yield observed in summer at 36°C in the month of April. This decreased ambient temperature led to the shift of microorganisms, as relatively a very diverse range of microbial community occupies these digesters (Rastogi et al, 2007).

Many countries are actively engaged in the fruitful improvement in this technology. Units for the methane gas production, using biomass had been employed in the rural areas of India and China, in order to meet their energy requirements (Levis, 1983) and most recently in Vietnam for the production of biogas efficiently.

**RESULTS**

The strain obtained was referred as WS1 strain in the laboratory. The strain was gram positive cocci, non-motile and non-spore forming. Creamy yellowish and smooth colonies were obtained. The strain was catalase and indole positive.

**Table 1: Effect of temperature on the growth of Methanosarcina WS1 strain**

<table>
<thead>
<tr>
<th>Temperature</th>
<th>OD&lt;sub&gt;600&lt;/sub&gt;</th>
<th>Observation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.00</td>
<td>--</td>
</tr>
<tr>
<td>25°C</td>
<td>1.23</td>
<td>Growth</td>
</tr>
<tr>
<td>30°C</td>
<td>1.26</td>
<td>Growth</td>
</tr>
<tr>
<td>35°C</td>
<td>1.90</td>
<td>Optimum growth</td>
</tr>
<tr>
<td>37°C</td>
<td>1.85</td>
<td>Growth</td>
</tr>
<tr>
<td>40°C</td>
<td>1.43</td>
<td>Growth</td>
</tr>
<tr>
<td>45°C</td>
<td>1.2</td>
<td>Growth</td>
</tr>
<tr>
<td>50°C</td>
<td>0.21</td>
<td>Mild growth</td>
</tr>
<tr>
<td>55°C</td>
<td>0.14</td>
<td>Mild growth</td>
</tr>
<tr>
<td>60°C</td>
<td>--</td>
<td>No growth</td>
</tr>
</tbody>
</table>

**Table 2: Effect of salt concentration on Methanosarcina WS1 strain**

<table>
<thead>
<tr>
<th>Salt concentration M</th>
<th>Absorbance OD&lt;sub&gt;600&lt;/sub&gt;</th>
<th>Observation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>--</td>
</tr>
<tr>
<td>0.1</td>
<td>1.23</td>
<td>Growth</td>
</tr>
<tr>
<td>0.2</td>
<td>1.48</td>
<td>Growth</td>
</tr>
<tr>
<td>0.3</td>
<td>1.87</td>
<td>Optimum growth</td>
</tr>
<tr>
<td>0.4</td>
<td>1.43</td>
<td>Growth</td>
</tr>
<tr>
<td>0.5</td>
<td>1.09</td>
<td>Growth</td>
</tr>
<tr>
<td>0.6</td>
<td>0.64</td>
<td>Mild growth</td>
</tr>
<tr>
<td>0.7</td>
<td>0.51</td>
<td>Mild growth</td>
</tr>
<tr>
<td>0.8</td>
<td>0.47</td>
<td>Mild growth</td>
</tr>
<tr>
<td>0.9</td>
<td>0.39</td>
<td>Minute growth</td>
</tr>
<tr>
<td>1</td>
<td>0.23</td>
<td>No growth</td>
</tr>
</tbody>
</table>

WS1 had the tendency to grow within the range of 25–60°C, but the optimum growth was observed at 35°C (Table 1). The salt
range of the strain WS1 was observed between 0.1–0.5 M NaCl
but the strain showed optimum growth in terms of turbidity of
the medium at 0.3 M concentration of the NaCl (Table 2).
Under these set of conditions the organism was termed as
Methanosarcina WS1 according to Bergey’s Manual of
Determinative Bacteriology.

Table 3: study of different parameters for biogas production

<table>
<thead>
<tr>
<th>Days</th>
<th>Feed Kg</th>
<th>Temperature °C</th>
<th>pH</th>
<th>Humidity %</th>
<th>Inoculums ml</th>
<th>Pressure Psi</th>
<th>Digester height Cm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>30</td>
<td>23</td>
<td>8</td>
<td>40</td>
<td>1000</td>
<td>0</td>
<td>12.2</td>
</tr>
<tr>
<td>2</td>
<td>30</td>
<td>24</td>
<td>8</td>
<td>43</td>
<td>–</td>
<td>2.5</td>
<td>15.2</td>
</tr>
<tr>
<td>3</td>
<td>30</td>
<td>26</td>
<td>8</td>
<td>43</td>
<td>1000</td>
<td>4</td>
<td>19.7</td>
</tr>
<tr>
<td>4</td>
<td>30</td>
<td>25</td>
<td>8</td>
<td>48</td>
<td>–</td>
<td>5</td>
<td>25</td>
</tr>
<tr>
<td>5</td>
<td>30</td>
<td>26</td>
<td>8</td>
<td>47</td>
<td>1000</td>
<td>6</td>
<td>30</td>
</tr>
<tr>
<td>6</td>
<td>30</td>
<td>30</td>
<td>8</td>
<td>46</td>
<td>–</td>
<td>8.5</td>
<td>34.5</td>
</tr>
<tr>
<td>7</td>
<td>30</td>
<td>26</td>
<td>8</td>
<td>49</td>
<td>1000</td>
<td>10</td>
<td>36.8</td>
</tr>
</tbody>
</table>

Syed et al (2013). Production of Biogas By Mesophilic Bacteria
DISCUSSION

As a matter of fact, the production of biogas can be accomplished cozily, economically and with no harms. But despite of its practical implications, in a developing country like Pakistan, the masses are not much aware of its beneficial aspects. Keeping this thing in consideration, the present study was conducted that aimed at the isolation of the mesophilic strains which were isolated from animal muck. Serial dilution method was employed for the isolation of methanogens followed by spread plate. A single colony was selected and designated as isolate WS1. Fluid thioglycolate medium was used for the growth of WS1 to confirm its anaerobic nature. The presence of thioglycolate provides a complete oxygen free environment, as the oxygen present in the head space of the vessel was reduced completely. Moreover, this medium contained all the necessary nutrients and a resazurin dye, the color of which varies from red–dark pink (in presence of oxygen), pale yellow (in the absence of oxygen) and is an indicator for the anaerobic environment. When selected isolate WS1 was grown in this medium, it turned to pale yellow.

Furthermore, growth was observed at the bottom of the tube which is a characteristic of strict anaerobic growth. A simple experiment using distillation flasks with attached balloons containing synthetic medium was carried out to analyze the production of gas by the Methanosarcina WS1. This medium contained all the macro and micro nutrients essential for the growth of methanogens. After incubation of 7–8 days, the balloons swelled to show the presence of the gas formation inside the flask. The gas produced was analyzed by Gas Chromatography and Mass Spectrometry (GCMS). Three peaks were obtained. The first peak showed similarity with carbon dioxide (CO₂), which is basic component of biogas or natural gas (Berglund, 2006). The second peak obtained showed similarity with fluoroacetylene. Fluoroacetylene when combined with air it becomes highly explosive with explosion limits in air of 2.4 to 13%. This compound has auto–ignition temperature of 365°C and the flash point of 6°C. It is highly heat sensitive having flammability of 3. (Pohanish and Greene 2009) (ChemSpider, the free chemical database). The specifications of methane gas include the auto–ignition temperature of 537°C and explosive limits in air being 5–15%. It’s highly flammable gas. (IPCS, 2000) The third peak obtained showed 23.19% similarities with trichloromethane (chloroform). This however isn’t flammable when in liquid state. The fourth peak obtained showed 25.77% similarities with benzene (C₆H₆). This is highly flammable and explosive as well having a flash point of –11°C and the auto–ignition temperature of 498°C. Its flammability is 3. (Hook et al., 2006)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>Experimental</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inoculum</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Time interval</td>
<td>11:21am–12:45pm</td>
<td>11:00am–02:00pm</td>
</tr>
<tr>
<td>Duration of flame burning</td>
<td>1 hr 24 min</td>
<td>3 hours</td>
</tr>
</tbody>
</table>

Figure 4: Effect of temperature and digester height

Figure 5: Effect of pressure on the digester height

Syed et al (2013). Production of Biogas By Mesophilic Bacteria
Later, the biogas was produced in a large digester located in PCSIR Laboratories, Lahore. The digester was fed daily with 30 kg of animal manure and with the prepared inoculums at alternate days. The assembly was given 9 days of incubation. The prescribed parameters like temperature, pressure, pH, humidity, digester height etc were determined on daily basis. The external environment was proved favorable for mesophilic conditions at that time so the research was conducted with quite ease. After the given incubation time, when the height of the digester was considerably high, the flame was burnt to study the duration to which it can withstand. Both the control and experimental digesters were burnt at the time. The control digester was the one, which lacked the inoculum, while the experimental was the one which contained inoculum diluted to a great extent. The burner from the control digester was burnt for almost 1 hr and 24 min while, the burner of the experimental digester was kept on burning for 3 hrs approx. This indicated that the added inoculum served the purpose of enhancing the effect of the biogas which usually too was produced, but by the addition of prepared inoculums it went far away in context of burning.

CONCLUSION
In present work mesophilic methanogen was isolated from animal manure on nutrient agar using anaerobic jar. The isolate was gram positive, non–spore former, non–motile, spheroid in shape, indole and catalase positive with optimum growth temperature 35°C and NaCl concentration of 0.3M. Thus it was identified as Methanosarcina sp. following the Bergey’s Manual and was designated as Methanosarcina WS1. This isolate was used for the production of biogas in a digester of 15 feet that was fed with 30 kg animal manure on daily basis and 1000 ml of inoculum (5 days old) on alternate days. Incubation was carried out for 9 days and gas produced was used to burn the Bunsen burner. The flame of the burner attached with the experimental digester burned for 3 hrs 1 hr and 36 min more compared to the burner attached with control digester (without the addition of inoculum). This study would help to find new solutions of energy crises in Pakistan.

ACKNOWLEDGMENTS
This work was supported by the keen interest and thought provoking ideas of Prof. Dr. Ikram-ul-Haq. The authors would like to thank Dr. Sakhawat, Dr. Uzma Hameed and Dr. Qurat-ul-Ain Syed for many thoughtful discussions and critical reading of the manuscript.

REFERENCES
Biogas technology in the third


Kaparaju PLN and Rintala JA. (2003). Effects of temperature on post-
anaerobic digestion of dairy cow manure, in a large scale biogas

Kapdi SS, Vijay VK, Rajesh SK and Prasad R (2003). Biogas scrubbing,
compression and storage: perspective and prospects in Indian

Kendall M, Liu Y, Sieparkska-Lupa M, Stetter KO, Whitman WB and
Boone DR (2006). Methanococcus aeolicus sp. nov., a mesophilic,
methanogenic archaean from shallow and deep marine sediments.
International journal of systematic and evolutionary microbiology,
(56): 1525–1529.

Kennedy KJ and Berg LVD (1982). Anaerobic digestion of pig pitty

Lettinga G, Velsen AFMW, Hobna SW, De Czeuw W and Klapwijk A
(1980). Use of upflow sludge blanket reactor for biological wastewater
treatment, especially for anaerobic treatment. Biotechnol Bioeng.,

effects of dry anaerobic codigestion of cow dung with waste water
sludge on biogas yield and biodegradability. International journal
of physical sciences, 6(15): 3679–3688.

Li R, Chen S and Li X (2010). Biogas production from anaerobic codigestion
of food waste with dairy manure in a two-phase digestion system.

Li R, Chen S, Li X, Lur JS, He Y and Zhu B (2009). Anaerobic codigestion of
kitchen waste with cattle manure for biogas production. Energy and fuels,
(23): 2223–2228.

Lier JBV, Tilche A, Ahring BK, Acarie H, Meletta D, Rohanyo M, Hulshof

production from dairy manure filtrate using conventional and fixed-

Lozano CIJS, Mendez MV, de Arteaga MC and Monroy EFC (2009).
Microbiological characterization and specific methanogenic activity of
anaerobe sludges used in urban solid waste treatment. Waste

Maestrajuan GM, Boone JE, Mah RA, Menaia JAGF, Sachs MS and Boone DR
(1992). Taxonomy and halotolerance of mesophilic Methanocarcina
strains, assignment from strains to species, and synonym of
Methanocarcina maezi and Methanocarcina frisia. International journal

barkeri MST and 227/Methanocarcina maezi 5–6T, and
Methanocarcina vacuolata 7–8T. International journal of systematic

solid wastes: an overview of research achievements and perspectives.

McKendry P (2002). Energy production from biomass (part 1) overview of

of a methanogen from deep marine sediments that contain methane
hydrates and description of Methanocellulus submarinus sp. nov.

Moller HB, Sommer SG and Ahring B (2004). Methane productivity of
manure, straw and solid fractions of manure. Biomass Bioenergy,
(20)(5): 485–495.

Mshandete A, Bjornsson L, Kaiwa S, Rubindamayugi MST and
Angelidaki I (2009). Effects of dry anaerobic codigestion of
kitchen waste from dairy and deep marine sediments that contain
methane hydrates and description of Methanocellulus submarinus sp. nov.

Moliner HB, Sommer SG and Ahring B (2004). Methane productivity of
manure, straw and solid fractions of manure. Biomass Bioenergy,
(20)(5): 485–495.

Mshandete A, Bjornsson L, Kaiwa S, Rubindamayugi MST and
Angelidaki I (2009). Effects of dry anaerobic codigestion of
kitchen waste from dairy and deep marine sediments that contain
methane hydrates and description of Methanocellulus submarinus sp. nov.

Moller HB, Sommer SG and Ahring B (2004). Methane productivity of
manure, straw and solid fractions of manure. Biomass Bioenergy,
(20)(5): 485–495.

Mshandete A, Bjornsson L, Kaiwa S, Rubindamayugi MST and
Angelidaki I (2009). Effects of dry anaerobic codigestion of
kitchen waste from dairy and deep marine sediments that contain
methane hydrates and description of Methanocellulus submarinus sp. nov.


