The Predominance of Bacillus Species and non-Culturable Microaerophilic/Anaerobic Microflora in the Aerobically Operated Predigester of Nisargruna Biogas Plant

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Research Article

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Abstract

In an attempt to convert biodegradable solid wastes into methane gas, the *Nisargruna* biogas technology is developed by the Bhabha Atomic Research Center, Mumbai and is functioning at more than 300 places in India. This plant is uniquely designed to allow microbiological degradation in a two-step process, where breakdown of waste material is facilitated under aerobic conditions prior to anaerobic digestion. Introduction of aerobic predigestion helps to overcome the drawbacks of solely anaerobic degradation like scum formation and high retention time of waste material. The aim of the present study was to identify the culturable as well as non-culturable microorganisms from the predigester of two phase *Nisargruna* biogas plant. Surprisingly, only *Bacillus* sp. were prevalent in the predigester and, among them, *B. subtilis* and *B. pumilus* were predominated. The spores, parasporal bodies and TEM micrographs of *Bacillus* sp. were also studied. Molecular techniques like PCR and RFLP analysis identified non culturable bacteria like *Citrobacter, Klebsiella, Cytophaga, Erwinia, Pediococcus, Geobacter, Brucella* and *Vibrio* sp. and clones of 14 different genera of anaerobic bacteria in the predigester slurry. The current study introduces the relatively unique microflora of two-phase *Nisargruna* biogas plant as compared to other anaerobic digesters, and relates its efficiency to diverse metabolic activity of *Bacillus* sp.

Introduction

The increasing population continuously challenges the waste management systems especially in countries with faster urban development. In spite of the radical measures undertaken, the millions of tonnes of waste collection and disposal annually is an arduous task. Moreover, the approaches of waste reduction, recycling and other socio-economic aspects of solid waste management become impractical in this scenario \[1, 2\]. Hence, effective solutions and improvement in existing technologies are continuously in demand. In addition, proper solid waste management practices can be economically favourable since wastes are nothing but vital reserve of precious biochemical elements \[3\]. If handled intelligently, it can lead to production of valuable bioenergy like methane, and manure that can be applied as a fertilizer and soil conditioner. The biodegradable waste generally consists of carbohydrates, proteins and lipids in variable proportions. These compounds contribute to more than half of the solid wastes generated and can be composted under appropriate conditions for its management. The kitchen wastes generated from household, restaurants and food industries can be repurposed for the above processes \[4\]. In this regard, the use *Nisargruna* biogas plant, introduced by Bhabha Atomic Research Centre, Mumbai, has proven to be beneficial. It combines the advancement in methods of biological treatment of wastes with biogas production technology to provide a productive and environment friendly approach for management of biodegradable solid waste.

The most remarkable feature of *Nisargruna* system is the aerobic decomposition of wastes in the predigester prior to anaerobic degradation in the main digester of the plant. The raw slurry is first collected in the predigester 1 and after initial decomposition and acquiring a flowing consistency, it moves to predigester 2. During the processing in this stage, the temperature is maintained between 40°C to 45°C to promote the degradation of waste material into their soluble monomeric components by
extracellular enzymes produced by bacteria naturally introduced in the predigester. The moderately high temperature further facilitates the conversion of degraded compounds into short-chain fatty acids, alcohols, CO$_2$ and H$_2$ by fermentative bacteria. Our previous study indicated that the raw waste containing proteins, carbohydrates and lipids are broken down to their respective monomers that are fermented to produce volatile fatty acids like formic acid, acetic acid, propionic acid and butyric acid along with liberation of CO$_2$. The volatile acids produced as a result of fermentation in the predigester remain in the aqueous phase and enter the main digester (anaerobic) to promote acetogenesis and methanogenesis [5]. The anaerobic digestion is a slow process and hence the hydrolysis and fermentation becomes a rate limiting step in methane production. The *Nisargruna* plant is designed to overcome this shortcoming by partitioning the reactor into predigester and main digester that operates under aerobic and anaerobic conditions respectively. This allows the initial hydrolysis and fermentation, to occur under aerobic conditions in the predigester. The above step not only reduces the retention time of wastes but also provides ready nutrients as raw materials for methanogenesis that occurs in the main digester [6].

The biodegradation of organic matter is a complex phenomenon involving diverse microorganisms exhibiting various enzyme activities. Thus, elaborate characterization of microbial flora in any microbiological process is essential for improvising the technology for better and wider applications. Hence the current study was carried out with an objective to analyze the metabolically dynamic system of the predigester by isolation and enumeration of its culturable and non-culturable microbial flora.

**Materials And Methods**

**Sample collection**

Representative samples were collected from two different compartments of the predigester of *Nisargruna* biogas plant located at Govandi, Mumbai, Maharashtra and labeled as predigester 1 and predigester 2. The homogenized samples were collected in sterile plastic bottles of 1 L capacity and processed immediately.

**Enumeration and identification of isolates from predigester**

Selective media were used for isolation and enumeration of bacteria (Nutrient agar), fungi (Rose Bengal potato dextrose agar) and actinomycetes (Kenknight Munaier’s and Wickerham’s agar). In addition, the presence of coliforms and pseudomonads were also screened on Mac Conkey’s and cetrimide agar plates respectively. All the isolation techniques were performed in triplicates and the plates were incubated for 24-48h at 45°C. This is because the temperature in predigester is maintained between 45°C to 50°C. After incubation, colonies were counted and colony-forming units per mL (cfu/mL) were estimated. All the isolates were maintained on nutrient agar slants.

A stepwise biochemical tests were performed for identification of *Bacillus* sp. as indicated in Table 1 since most of the isolates showed cultural and morphological characteristic of the genus *Bacillus* [7].
Spores were also observed under phase contrast microscope (Zeiss) [8-10]. The parasporal bodies are used for differentiation between *Bacillus thuringenesis* and *Bacillus cereus* by Ziehl-Neelson staining method [11]. In addition, the sporulating cells (48h old) of *Bacillus* were also observed under transmission electron microscope (Jeol JEM – 2000 FX) at 160KV, with 30,000 – 50,000 magnification and electron micrographs were documented.

**Molecular identification of culturable and non-culturable bacteria**

The confirmation of species was done by 16S rDNA sequencing technique. The primers used for identification of culturable and non-culturable bacteria are indicated in Table 2. PCR amplified products were purified using Genei quick PCR purification kit (Bangalore Genei, India) and sequenced bidirectionally specific primers. For this purpose, ABI PRISM 3100 (Applied Biosystems/Hitachi) was used as a sequencer. The sequence data thus obtained was aligned to obtain the complete fragment sequence and analyzed for homology in GenBank by BLASTn search to identify the bacteria. The phylogenetic trees were also constructed for the identified bacteria [12]. The PCR product was ligated into T/A vector and *E. coli* DH5α cells were transformed with the recombinant DNA. The T/A vector is a high copy number vector with pMB1 origin of replication and carries ampicillin resistance marker. The genetic map of T/A vector is represented in Supplementary data, Fig. 1. Plasmids were then isolated from the transformed cells and cloning was confirmed by checking the release of insert by treatment with NcoI. The clones that showed release of insert and hence confirmed cloning were selected for re-amplification and purified using plasmid purification kit (KT 61, Bangalore, Genei). The re-amplified inserts were subjected to RFLP using two restriction endonucleases- Sau3AI and Taq I. Clones that showed different RFLP patterns were selected for sequencing and identified using the ABI PRISM 3100 sequencer (Applied Biosystems/Hitachi). The data obtained after sequencing was aligned to get the complete sequence of the 16S rDNA gene. This was done by using Bioedit software. The sequences were then analyzed for homology in GenBank by using BLASTn search. Phylogenetic trees of all the isolates were constructed for their identification.

**Results**

**Isolation and enumeration of microorganisms**

Gram staining of the predigested slurry showed presence of variety of gram-positive rods in singles, pairs and chains. All the cells were in their vegetative form and no spores were observed. This was reflective of fast growing cells in nutritionally rich environment. The total bacterial count of slurry samples collected from predigester 1 and 2 was found to be 2.5x10^7 and 1.96 x10^7 respectively, suggesting extensive growth of bacteria. Few colonies were developed on Kenknight Munai'er's and Wickerham's agar. However, they showed characteristics of *Bacillus* sp. and not of actinomycetes. No growth was observed on Rose Bengal potato dextrose agar plates even after 7 days of incubation. The MacConkey’s and cetrimide agar plates also showed absence of colonies. These observations indicated that fungi, coliforms and *Pseudomonas* sp. were absent in the predigested slurry.
Cultural and morphological characteristics of organisms isolated from the predigester

Twenty colonies from predigester 1 (Supplementary data, Table 1) sample and twenty four colonies from predigester 2 (Supplementary data, Table 2) sample showing different morphological and cultural characteristics were selected for identification in our study. Gram staining and spore staining were also performed for each isolate and all of them occurred as Gram-positive and spore forming rods of variable sizes (Supplementary data Fig. 2). Thus, based on morphological and cultural characteristics, it was concluded that all isolates belonged to the genus *Bacillus*.

In *Nisargruna* biogas plant, the biodegradable wastes mainly consist of kitchen wastes from houses, restaurants and hotels, and other green wastes like foliage, papers etc. are treated. These materials are mainly composed of carbohydrates, proteins and lipids. Thus, the heterogeneous nature of the waste allows the introduction of diverse microorganisms in the predigester of *Nisargruna* biogas plant. However, the direct microscopic examination of the predigested slurry showed only one type of organism i.e. gram-positive rods of various dimensions. All the isolates were identified as *Bacillus* species in our study. Similar to our study, Ghosh *et al*. [13] also indicated the predominance of *Bacillus* sp. in the pre-digester of *Nisargruna* biogas plant in a previous study. The selective enrichment of *Bacillus* sp. was presumed to be the result of moderately high temperature of the predigester (between 45°C to 50°C) that killed pathogenic bacteria but supported the growth of spore-bearing *Bacillus* sps.

Biochemical characteristics of organisms isolated from the predigester

Surprisingly, microbiological analysis of the predigested slurry revealed that the entire aerobic degradation process was carried out exclusively by ten different species of genus *Bacillus* and their innumerable variants isolated from predigester 1 (Supplementary data, Tables 3) and predigester 2 (Supplementary data, Tables 4). For identification of *Bacillus*, the morphology of the rods, spore shape, structure and its location have considerable significance. The preliminary identification was done based on their gram nature and morphology (Supplementary data, Fig. 2). The phase contrast microscopy was particularly helpful in the study of spores. A simple identification key consisting of commonly used biochemical tests was helpful in easy identification of *Bacillus* sp. with precision [7]. Specially prepared and stained sections of different species of *Bacillus* were viewed under transmission electron microscope (TEM) to illustrate their ultrastructure and thus reveal structural differences within different species. Fig. 1 demonstrates the TEM micrographs observed for different *Bacillus* sp.

Studies on the microbiological analysis of anaerobic digesters have been reported as early as 1967. In a study undertaken by Toerien [14], specific emphasis was given to determine the aerobic and facultative anaerobic participants of early anaerobic digestion process, in the degradation of cellulose, starch, casein, peptone and sunflower oil. In their study, the facultative anaerobic bacteria such as *Bacillus* sp. were suggested to play a major role in the primary liquefaction of macromolecules. Later in the same year, Toerien et al. [15] extended the scope of their study towards several laboratory-scale digesters and concluded that aerobic and facultative anaerobic bacteria occur in all digesters at all stages. However, they are negligible in number in the further stages of digestion. These observations thus indicated the
importance of obligate anaerobic bacteria in acid and gas production. It also indicated the importance of aerobic bacteria in initial decomposition of waste material.

**Molecular identification of Bacillus sp. by 16S rDNA sequencing technique**

Twenty three cultures that were identified by morphological, cultural and biochemical characterization were selected for 16S rDNA sequencing and the sequences of these organisms were submitted with the GenBank. The PCR amplification of 16S rDNA gene of 23 Bacillus sp. are represented in Fig. 2. The accession numbers of these identified organisms are given in Table 3. The different species of Bacillus identified were *B. subtilis*, *B. pumilus*, *B. megaterium*, *B. thuringenesis*, *B. cereus*, *B. licheniformis*, *B. velezensis*, *B. amyloliquifaciens*, *B. silvestris* and *B. firmus*. All these species belong to the *B. subtilis* cluster group I [7].

In almost all cases the molecular identification matched the conventional one which used morphological, cultural and biochemical characterization. However, there were some exceptions and the identification by the two methods did not match. A culture identified as *B. laterosporus* (PI15) by conventional methods was recognized as *B. pumilus* by molecular method. Similarly, *B. subtilis* (PI11) isolate was identified as *B. velezensis* and *B. sphaericus* (PII13) as *B. silvertis*. Among these, *B. velezensis* is a recently known species of Bacillus, closely related to *B. subtilis* [7]. In addition, the molecular identification by 16S rDNA gene could not distinguish between the closely related species like *B. thuringenesis* and *B. cereus*. The only differentiating character between these two species was the presence of parasporal bodies observed with the help of Ziehl Neelsen staining method (Supplementary data Fig. 3). *B. thuringenesis* produced the crystalline parasporal bodies which were absent in *B. cereus*. A special staining procedure thus proved valuable in identification of Bacillus sp. microscopically. These observations promptly suggests that identification of an organism should be considered authentic only when it is done by cultural as well as molecular methods, and not by either of the two alone.

**Molecular identification of unculturable bacteria**

The 16S rDNA gene was successfully amplified from DNA isolated from predigested slurry, ligated and cloned into T/A vector. Over 500 colonies were transformed and screened for clones. Around 100 clones confirmed the presence of desired inserts, when 16S rDNA fragments were amplified using insert specific primers. These clones were grouped into different categories based on their RFLP profile. Thirty nine sequence of clones obtained in this culture independent molecular identification study of predigester were deposited with the Genebank (Table 4). Figures 3 and 4 indicate representative RFLP profiles of PCR amplified fragments digested with TaqI and Sau3A respectively.

The PCR and RFLP analysis of mixed populations of predigested slurry showed presence of many species of *Lactobacillus* and *Leuconostoc*. Three species of the genus Enterobacter and two species of Pseudomonas were also detected. In addition, single species of genera viz., *Citrobacter*, *Klebsiella*, *Cytophaga*, *Erwinia*, *Pediococcus*, *Geobacter*, *Brucella* and *Vibrio* were identified. Although isolation of these bacteria was attempted on selective media in the present study, none of them showed
characteristic growth. Hence, it could be suggested that these organisms were present in the raw waste. However, the addition of hot water and maintenance of predigester at moderately high temperature (45°C) eliminated these bacteria during processing of wastes. Development of acidic conditions in 96 h and the predominance of spore bearing *Bacillus* sp. may also be responsible for these observations. During the culture independent approach, clones of anaerobic bacteria were also identified. They included *Pantoea, Eubacterium, Methanosarcina, Caloramator, Asteroplasma, Exiguobacterium, Clostridium, Ochrobacter, Butyrivibrio, Acinetobacter, Sphingomonas, Syntrophomonas, Megasphaera* and *Olsenella*. These bacteria may be present as non-viable forms in the predigester slurry due to its strong aerobic character. Presence of these anaerobic clones in the aerobic predigester slurry was presumed to be as a result of recirculation of water separated from manure pit into predigester tank. Our previous study suggested low BOD (100ppm) and absence of coliforms, fungi and other microorganisms in the liquid obtained on dewatering the manure collected after complete digestion of wastes in the *Nisargruna* biogas plant. Hence this liquid was recycled back into the predigester to maintain the consistency of slurry and, at the same time, save water [5].

The predominance of different types of bacterial profiles at different stages of microbiological process has also been observed by other researchers. For this purpose, the PCR-RFLP analysis of 16S rDNA was first attempted by Hiraishi et al. [16] to identify methanogenic bacteria from mixed populations of anaerobic sludge. They amplified the 16S rDNA fragments of 0.4 kb size from the bulk DNA extracted from anaerobic digester using methanogen-specific primers and cloned it directly using the T/A cloning vector. It proved to be a rapid culture independent approach and has since become a popular method for microbiological analysis of anaerobic digesters. Using similar technique, a study identified over 1129 bacterial operational taxonomic units from anaerobic digester fed with dairy manure and wheat distillery is reported. They reported dominance of different genus during different phases of their analysis regardless of the raw material used, and indicated the prevalence of *Clostridium* sp. by day 7 and *Acetivibrio* related sp. by day 35 [17]. These observations were reported to be true not only the anaerobic digesters but also for wastewater treatment systems, where significant difference was observed in enriched cultures (depending on the phase of wastewater treatment), irrespective of the microbial profiles of inoculums [18].

**Conclusion**

The current study indicated that the complicated degradation process that takes place in the predigester of *Nisargruna* biogas plant is essentially carried out by ten species of the genus *Bacillus*. The cultures obtained from predigester 1, where the waste is first collected showed less variation in the *Bacillus* sp. Here, only four different sp. were found and amongst them *B. subtilis* and *B. pumilus* were predominant. In the predigester 2, where partially hydrolyzed waste from predigester 1 is concentrated, showed much more variation in identified *Bacillus* sp., as compared to that in predigester 1. This observation proposes that the first attack on the complex biomolecules is done primarily by *B. subtilis* and *B. pumilus*. Subsequently, the breakdown steps are then taken over by other *Bacillus* sp., leading to generation of mixtures of acids.
Declarations

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Tables

Table 1 Biochemical tests for identification of Bacillus sp.
| Test No. | Test                                   | Observation   | Next test (no.) performed | Identification               |
|---------|----------------------------------------|---------------|----------------------------|------------------------------|
| 1       | Catalase                               | Positive      | 2                          | -                            |
|         |                                        | Negative      | 17                         | -                            |
| 2       | Voges- Proskauer test                  | Positive      | 3                          | -                            |
|         |                                        | Negative      | 10                         | -                            |
| 3       | Growth in anaerobic agar               | Positive      | 4                          | -                            |
|         |                                        | Negative      | 9                          | -                            |
| 4       | Growth at 50°C                         | Positive      | 5                          | -                            |
|         |                                        | Negative      | 6                          | -                            |
| 5       | Growth in 7% NaCl                      | Positive      | -                          | Bacillus licheniformis       |
|         |                                        | Negative      | -                          | Bacillus coagulans           |
| 6       | Fermentation of glucose with acid and gas production | Positive | - | Bacillus polymyxa |
|         |                                        | Negative      | 7                          | -                            |
| 7       | Reduction of NO₃ to NO₂                | Positive      | 8                          | -                            |
|         |                                        | Negative      | -                          | Bacillus alvei               |
| 8       | Parasporal body in sporangium          | Positive      | -                          | Bacillus thuringenesis       |
|         |                                        | Negative      | -                          | Bacillus cereus              |
| 9       | Hydrolysis of starch                   | Positive      | -                          | Bacillus subtilis            |
|         |                                        | Negative      | -                          | Bacillus pumilus             |
| 10      | Growth at 65°C                         | Positive      | -                          | Bacillus sterothermophilus   |
|         |                                        | Negative      | 11                         | -                            |
| 11      | Hydrolysis of starch                   | Positive      | 12                         | -                            |
|         |                                        | Negative      | 15                         | -                            |
| 12      | Fermentation of glucose with acid and gas production | Positive | - | Bacillus macerans |
|         |                                        | Negative      | 13                         | -                            |
|   | Width of rod | 1µm | Bacillus megaterium |
|---|-------------|-----|---------------------|
|   |             |     | Longer 14 -         |
| 14| pH in VP broth | < 6 | Bacillus circulans |
|   |             | > 6 | Bacillus firmus     |
| 15| Growth in anaerobic agar | Positive | Bacillus laterosporus |
|   |             | Negative 16 - |
| 16| Fermentation of glucose with acid and gas production | Positive | Bacillus brevis |
|   |             | Negative - |
| 17| Growth at 65°C | Positive | Bacillus sterothermophilus |
|   |             | Negative 18 - |
| 18| Decomposition of casein | Positive | Bacillus larvae |
|   |             | Negative 19 - |
| 19| Parasporal body in sporangium | Positive | Bacillus popillae |
|   |             | Negative - |

**Table 2** Primers used for 16S rDNA gene amplification of bacteria

| Bacteria                  | Primers                                      | PCR Program                                                                 |
|---------------------------|----------------------------------------------|------------------------------------------------------------------------------|
| *Bacillus* sp.            | F-5’-GTTTGATCCTGGCTCAG -3’ R-5’-CTCTCGTGTTGACG -3’ | Initial denaturation- 94°C/ 3min, 30 cycles of: Denaturation- 94°C/ 45sec; Annealing- 55°C/ 45sec; Extension- 72°C/ 2mins Final extension- 72°C/ 5mins |
| Non culturable bacteria   | F-5’-AGAGTTTGATCCTGGCTCAG -3’ R-5’-AAGGAGGTGATCCAGCGCA -3’ | Initial denaturation- 94°C/ 5min, 35 cycles of: Denaturation- 94°C/ 30sec; Annealing- 55°C/ 30sec; Extension- 72°C/ 90sec Final extension- 72°C/ 15mins |

**Table 3** Molecular identification of culturable *Bacillus* sp. from predigester and their accession numbers submitted with GenBank
| Sr. No. | Culture number | Biochemical Identification | Molecular Identification | Accession Number |
|---------|----------------|---------------------------|--------------------------|------------------|
| 1       | PI1            | B. subtilis               | B. subtilis              | EU366369         |
| 2       | PI2            | B. cereus                 | B. cereus / B. thuringenesis | EU366370       |
| 3       | PI3            | B. pumilus                | B. pumilus               | EU366363         |
| 4       | PI5            | B. laterosporus           | B. pumilus               | EU366364         |
| 5       | PI6            | B. pumilus                | B. pumilus               | EU366365         |
| 6       | PI10           | B. cereus                 | B. cereus / B. thuringenesis | EU366366       |
| 7       | PI11           | B. subtilis               | B. velezensis            | EU366367         |
| 8       | PI12           | B. subtilis               | B. subtilis              | EU366368         |
| 9       | PII1           | B. licheniformis          | B. licheniformis         | EU366371         |
| 10      | PII2           | B. subtilis               | B. subtilis              | EU366372         |
| 11      | PII3           | B. subtilis               | B. subtilis              | EU366373         |
| 12      | PII4           | B. firmus                 | B. firmus                | EU707377         |
| 13      | PII5           | B. thuringenesis          | B. cereus / B. thuringenesis | EU366375       |
| 14      | PII6           | B. cereus                 | B. cereus / B. thuringenesis | EU366376       |
| 15      | PII8           | B. subtilis               | B. subtilis              | EU366385         |
| 16      | PII11          | B. subtilis               | B. subtilis              | EU366377         |
| 17      | PII12          | B. cereus                 | B. cereus / B. thuringenesis | EU366384       |
| 18      | PII13          | B. sphaericus             | B. silvestris            | EU366378         |
| 19      | PII15          | B. licheniformis          | B. licheniformis         | EU366383         |
| 20      | PII17          | B. thuringenesis          | B. cereus / B. thuringenesis | EU366379       |
| 21      | PII18          | B. thuringenesis          | B. cereus / B. thuringenesis | EU366380       |
| 22      | PII22          | B. megaterium             | B. megaterium            | EU366381         |
| 23      | PII24          | B. subtilis               | B. subtilis              | EU366382         |

Table 4 Molecular identification of non culturable bacteria from predigester and their accession numbers submitted with GenBank
| Sr. no. | Clone number | Identification       | Accession number |
|---------|--------------|----------------------|------------------|
| 1       | 11a          | *Pantoea sp.*        | EF593045         |
| 2       | 12a          | *Eubacterium sp.*    | EF593046         |
| 3       | 16a          | *Lactobacillus sp.*  | EF593047         |
| 4       | 20a          | *Leuconostoc sp.*    | EF593048         |
| 5       | 4c           | *Lactobacillus sp.*  | EF593049         |
| 6       | 6b           | *Caloramator sp.*    | EF593050         |
| 7       | 17d          | *Acinetobacter sp.*  | EF593051         |
| 8       | 56b          | *Asteroplasma sp.*   | EF593052         |
| 9       | 36b          | *Exiguobacterium sp.*| EF593053         |
| 10      | 57b          | *Leuconostoc sp.*    | EF593054         |
| 11      | 32a          | *Pseudomonas sp.*    | EF593055         |
| 12      | 86b          | *Citrobacter sp.*    | EF593056         |
| 13      | 51c          | *Pseudomonas sp.*    | EF593057         |
| 14      | 26a          | *Pseudomonas sp.*    | EF593058         |
| 15      | 47a          | *Lactobacillus sp.*  | EF593059         |
| 16      | 41b          | *Leuconostoc sp.*    | EF593060         |
| 17      | 46c          | *Pseudomonas sp.*    | EF593061         |
| 18      | 59b          | *Butyrivibrio sp.*   | EF593062         |
| 19      | 2c           | *Lactobacillus sp.*  | EF593063         |
| 20      | 12c          | *Ochrobactrum sp.*   | EF593064         |
| 21      | 15c          | *Lactobacillus sp.*  | EF593065         |
| 22      | 31c          | *Megasphaera sp.*    | EF593066         |
| 23      | 73c          | *Sphingomonas sp.*   | EF593067         |
| 24      | 96c          | *Pseudomonas sp.*    | EF593068         |
| 25      | 86c          | *Pseudomonas sp.*    | EF593069         |
| 26      | 20b          | *Klebsiella sp.*     | EF593070         |
| 27      | 4d           | *Acinetobacter sp.*  | EF593071         |
| 28      | 59c          | *Pseudomonas sp.*    | EF593072         |
|   |   | Species          |   |
|---|---|------------------|---|
| 29 | 27d | Lactobacillus sp. | EF593073 |
| 30 | 30d | Pantoea sp.      | EF593074 |
| 31 | 47d | Olsenella sp.    | EF593075 |
| 32 | 63d | Syntrophomonas sp. | EF593076 |
| 33 | 75d | Pseudomonas sp.  | EF593077 |
| 34 | 8e  | Leuconostoc sp.  | EF593078 |
| 35 | 38e | Lactobacillus sp. | EF593079 |
| 36 | 45e | Enterobacter sp. | EF593080 |
| 37 | 55e | Enterobacter sp. | EF593081 |
| 38 | 2a  | Enterobacter sp. | EF593082 |
| 39 | 2b  | Pantoea sp.      | EF593083 |

**Figures**

**Figure 2**

PCR amplification of 16Sr DNA gene of Bacillus sp. (L- 500 bp ladder)
Figure 3

RFLP profile of non culturable bacteria in predigester slurry using TaqI digestion (1L - 100 bp ladder; 2L – 500 bp ladder)
Figure 4

RFLP profile of non culturable bacteria in predigester slurry using Sau3A digestion (1L - 100 bp ladder; 2L – 500 bp ladder)