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# Morphology analysis and microbial diversity in novel anaerobic baffled reactor treating recycled paper mill wastewater

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**Keywords:** anaerobic digestion (AD), novel anaerobic baffled reactor (NABR), recycled paper mill wastewater (RPMW), microbial diversity, microbial morphology

**Abstract:** The profile of microbial diversity in a NABR digesting RPMW was investigated using phylogenetic analysis of partial 16S rRNA sequences by a neighbor-joining-tree, supported by microbial morphology analysis by SEM. The results showed that microorganism inside NABR consisted of dominant Bacillus (25 strains) and Bacterium (1 strain) which were isolated from the settled sludge at the bottom of the reactor, whilst Bacillus (2 strains), Pseudomonas (2 strain) and Chryseobacterium (2 strain) were isolated from the biofilm formed on the packing material. It revealed that the microbial community strains, function, and structure changed simultaneously throughout the reactor system. The microscopic results showed rich biofacies, while the dominant microorganisms have various morphologies in every compartment of the system. It consisted of a long rod-shaped and filamentous bacterium composed majorly of bacilli of different sizes. Although the study successfully analyzed the microbial diversity and morphology in the system, the microbial communities reported in this study were different from other similar studies. This may be caused by the application of a culture-based technique that usually provides limited information due to the number of barely cultivated or uncultured strains.

## Introduction

Anaerobic digestion is a very promising method for the sustainable management of industrial effluents. It offers several advantages such as high total waste reduction, the possibility of operation at critical conditions, and final products of biogas and fertilizers (Kozłowski et al. 2019). The successful application of anaerobic digestion depends on many factors, one of them being a microbial community formed in the system. The microbial community of anaerobic digestion consists of various bacteria and archaea, and their populations differ under different digesters, operational procedures, substrate characteristics, and analytical methods (Zwain et al. 2017).

In anaerobic digesters, a complex microbial community and their competitive dynamics are still uncertain. Anaerobic metabolic processes involve hydrolysis of organic matter, fermentation of amino acids and sugars, generation of acetate, and the production of methane. The structure of microbial communities strongly depends on the nature of decomposing substrate. Close collaboration among microbial communities is based on tropical relationships and exchanges of growth factors which determine the hydrolytic, acidogenic, and methanogenic activities. The more complex composition of the digested substrates, the more diverted microbial community with a higher metabolic development would be formed (Świątczak et al. 2017).

The anaerobic baffled reactor (ABR) is a very effective system for the treatment of high strength effluents. The ABR design supports the growth of various microorganism species with long cell doubling periods, in which partial separation of acidogenesis and methanogenesis processes may occur (Zhu et al. 2015). The ABR consists of series of vertical baffles forcing the wastewater to flow up and down of them, where the anaerobic granular sludge in the system slowly rises and settles in each compartment, resulting in a relatively slow rate of horizontal moving with a high cell retention time of

100 days at an HRT of 20 h (Hassan et al. 2013). In the up-flow compartment of the ABR reactor, microbial communities with distinct structure and functions, high biological activity and strong resilience could be formed (Liu et al. 2008). However, the traditional ABR has several disadvantages that can be overcome by modifying the reactor configuration and allowing sufficient time for the slow growing methanogens.

On the other hand, recycled paper mill wastewater (RPMW) is considered as a complex industrial wastewater due to significant amounts of total solids, oxygen consuming organics, organic acids, and micro-organic pollutants (Zwain et al. 2021). However, due to readily biodegradable materials, RPMW could be anaerobically treated by different combinations of biological systems and microbial communities. In lagoon treatment system, a mixture of *Bacillus subtilis*, *B. megaterium*, *B. licheniformis*, *B. pumilus*, *B. thuringiensis*, *B. cereus*, *Chryseobacterium daecheongense*, and *Microbacterium sediminis* were identified in the treated pulp and paper mill wastewater (PPMW), whereas *B. cereus* was the best strain for cellulose and lipase activities (Bailon-Salas et al. 2018). Likewise, *Bacillus aryabhattai* isolated from PPMW had the ability to reduce color and lignin (Zainith et al. 2019). *Brevibacillus agri* was also reported to potentially degrade 62% of COD, 37% of color, 30% of lignin and 40% of adsorbable Organic Halides (AOX) semi-continuous reactor (Hooda et al. 2015). Gao et al. (2019) reported that *Methanolinea*, *Methanogenium*, *Porphyromonadaceae*, and *Fibrobacteraceae* were the predominant bacterial communities in an up-flow anaerobic sludge blanket (UASB) reactor during toilet blackwater treatment. Similarly, Tsavkelova et al. (2018) identified *Ruminiclostridium*, *Methanothermobacter* and *Methanosarcina* as the predominant archaeal genera in the anaerobic thermophilic reactor treating paper wastewater.

In this regards, scanning electron microscopy (SEM) has been widely used in environmental microbiology to characterize the surface structure of biomaterials and to measure cell attachment and changes in the morphology of bacteria. Moreover, SEM is useful for defining the number and distribution of microorganisms that adhere to surfaces. Traditionally, inability to provide phylogenetic or genetic information about microorganisms has been one limitation of SEM in environmental microbiology (Kenzaka and Tani 2012). Modern molecular studies based on DNA and RNA sequence analysis have led to an understanding of the microbial diversity and composition of bacterial communities in various environments. This includes nucleic culture-based techniques, acid-based analytical methods (denaturing and temperature gradient gel electrophoresis (DGGE/TGGE), specific and multiplex-PCR, Real-time quantitative PCR, and others), restriction fragment length polymorphism (RFLP), DNA fingerprinting, fluorescence in situ hybridization (FISH), and others (Banach-Wiśniewska et al. 2021, Tsavkelova et al. 2018). Nowadays, analysis of microbial communities is made by the combination of different molecular approaches. Combining morphological study by SEM with molecular studies has provided new insights into the understanding of the spatial distribution of target cells on various materials.

Therefore, it is important to study the microbial community shifts of the anaerobic processes within a system treating complex industrial wastewater under optimal operational conditions. Thus, the main objective of this study is to analyze the microbial diversity and morphology in a novel anaerobic baffled reactor (NABR) treating recycled paper mill wastewater (RPMW). It also aims to investigate the phase separation of anaerobic processes and microorganism's profile throughout the reactor system.

## Materials and Methods

### *Reactor set-up*

In this study, a laboratory-scale NABR was fabricated using plastic polypropylene, a detailed schematic diagram of which is shown in Figure 1 (Zwain et al. 2021). The basic reactor shape is rectangular at the top with an inverted pyramid or non-rectangular shape at the bottom, with a dimensions of 80 cm length, 30 cm height and 15 cm width (without water jacket). The reactor effective operational volume was 35 L. The system had five compartments in series, each compartment composed of an up-flow chamber and a down flow chamber. The compartments were separated by modified vertical baffles, while the lower part of the hanging baffles was bent in order to route the flow into the up-flow compartments. Each compartment contained three sampling ports: lower port for sludge, upper port for effluents, and top port for biogas. The modified baffles have the following characters and function:

- Compartment 1 (Inclined 45° baffle with sloped 45° edge): The top narrow part is to provide higher velocity flow at the top to avoid effluents clogging, while the wide bottom part is to avoid backpressure.
- Compartment 2 (Flat baffle with sloped 45° edge): Since all of the solid contents will accumulate in Compartment, the flat narrow baffle is to allow higher velocity flow from Compartment 1 to Compartment 2 in order to avoid effluents clogging.
- Compartment 3 (Zigzag baffle): The function of this baffle is to provide higher mixing capacity to ensure longer contact between the effluent and the biomass.
- Compartment 4 (Inclined 45° baffle + hanged ladder baffles): The baffle has similar function to the zigzag baffle.
- Compartment 5 (Flat baffle with sloped 45° edge): Flat baffle is to ensure a smooth flow and avoid any further mixing to minimize the sludge washout.

In addition, approximately 50% of the total volume of the second and third compartments was filled with 6 L (450 g) of polypropylene pall ring materials to support/develop the biofilm formation. The packing materials were placed at the top of Compartments 2 and 3.

### *Reactor start-up and Operation*

The substrate used in this study was real RPMW obtained from Muda paper mill in Simpang Ampat, Penang, Malaysia. The reactor was seeded using anaerobic sludge from lagoon treatment system treating palm oil mill effluent, obtained from Sungai Jawi, Penang, Malaysia. The system was operated by mesophilic condition at 37°C, and shortly started after 30 days. Details on the optimum start-up procedures have been previously reported, including the effect of feeding mode (Zwain et al. 2013), effects of inoculum source and effluent recycle (Zwain et al. 2016a), and effect of inoculum to substrate ratio (Zwain

et al. 2016b). After the reactor was started, it was then operated at different influent COD concentrations and hydraulic retention times for a period of 126 days; detailed information on the reactor performance, physiochemical parameters tested to evaluate the reactor performance, and composition of raw RPMW have been previously reported in Zwain et al. (2018). During the start-up and operation, effluents and biogas samples were tested every 2 days, and three repetitions were analyzed and only average values were reported. Thereafter, different sludge samples were taken from the system for microbial and morphological analysis.

### *Isolation and Identification of Microorganism*

Microbial isolation was performed to extract a pure strain of microorganism from a mixed culture. Six different sludge samples (i.e. collected from the bottom of Compartments 1, 2, 4 & 5, the upper of Compartment 3, and flocculate palm oil mill effluent (POME) sludge) were used for isolation process. The DNA isolation was conducted according to the basic protocol by Ausubel et al. (2003). Nutrient enrichment technique was used to isolate potential microbial strains. First of all, the essential requirements and necessary glassware were sterilized in the autoclave at 121°C for 1 h. Nutrient agar media were obtained from Merck (pH 7.0, 20 g/L, $H_2O$, 37°C), and used for the isolation of microorganisms. The sludge samples were serially diluted (10-fold) with distilled water. About 1 mL of viable bacteria count was spelled out on an agar plate containing the supplemented nutrient agar, followed by incubation at 32 ± 2°C for 24 h. The morphologically and phenotypically different colonies developed on the plates were selected, picked up and placed on the same medium. These inoculated agar plates were constantly incubated in an incubator at 32°C for one day. Repeated streaking of every single colony on agar media was implemented until pure colonies of each culture were obtained. Thereafter, pure colonies were placed in well closed tubes that contained agar medium, and then were sent for DNA analysis.

The genomic DNA from isolated microorganisms was extracted following the method described by Atashpaz et al. (2010). The extracted DNA was examined on 0.8% agarose gel, which contained 1 µg/mL ethidium bromide and the bands were observed on UV transilluminator. The 16S rRNA gene was amplified using 5 µL of genomic DNA (as template DNA) and universal eubacterial primers used for the PCR analysis were 27F 5′(AGA GTT TGA TCM TGG CTC AG)3′ and 1492R 5′ (TAC GGY TAC CTT GTT ACG ACT T)3′ (Gobi and Vadivelu 2015). The obtained sequences were analyzed using the National Centre for Biotechnology Information (NCBI) online nucleotide BLAST tool and ribosomal database-II to identify the taxonomic hierarchy of the sequences (U.S. National Library of Medicine 2021). Taxonomically related 16S rRNA gene sequences were obtained from the NCBI nucleotide database. The collected sequences were aligned using the muscle multiple sequence alignment algorithm.

Besides, MEGA6 software was used to analyze the phylogenetic affiliation based on 16S rDNA sequences. The Neighbor-Joining method was used to infer the evolutionary history. Accordingly, the evolutionary history of the taxa analyzed, presented by the bootstrap consensus tree, was inferred from 500 replicates. Hence, branches related to partitions with less than 50% bootstrap replicates were eliminated. The percentage of the replicate trees, where related taxa clustered simultaneously in the bootstrap test of 500 replicates, are shown above the branches. The Kimura 2-parameter method was used to compute the evolutionary distances, in the units of the number of base substitutions per site. Gamma distribution (with shape parameter =2) was applied to model the rate variation among sites. At the same time, the analysis of the evolutionary history involved 31 nucleotide sequences. For this reason, all positions containing missing data and gaps were excluded. The results showed a total of 569 positions in the final dataset.

### *Morphology Analysis of Microorganisms*

The scanning electron microscope (SEM) examinations were performed with Supra 50 VP (Carl Zeiss model) microscope at the School of Biological Science, USM, to obtain surface morphologies of biomass formed in the NABR. Therefore,

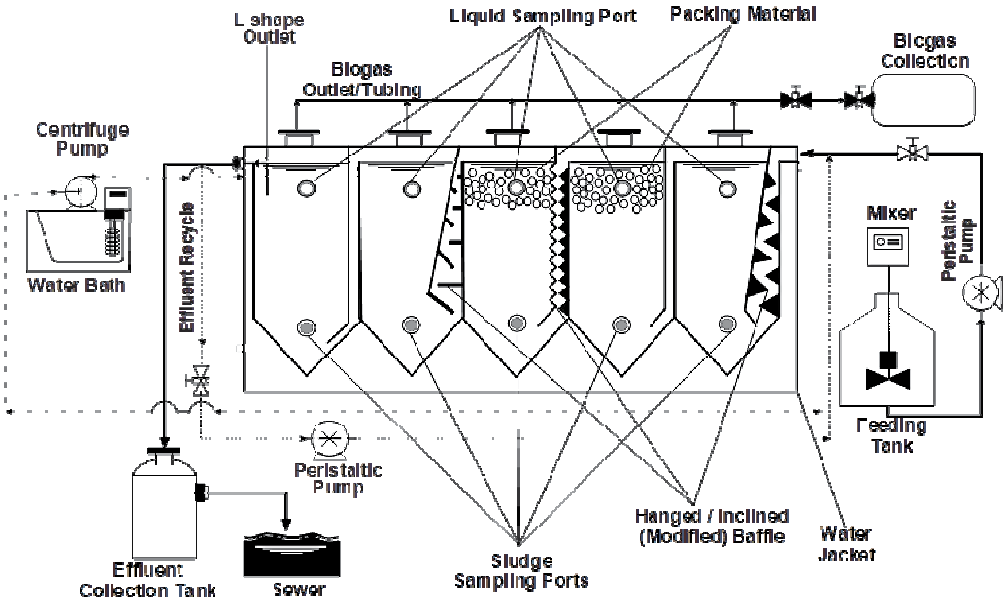


**Fig. 1.** Schematic diagram of novel anaerobic baffled reactor (NABR)

sludge samples describing the microbial content within the reactor were collected using a Perspex sampling tube, at near steady-state, and prepared for SEM analysis. Seven samples were collected, five of them were from the bottom side of each of the five compartments of the NABR, one from the top of Compartment 2, and one raw POME sludge.

According to the hexamethyldisilazane (HMDS) method (Araujo et al. 2003), each sludge sample was centrifuged for 15 minutes for pellet formation and the supernatant discards. Samples were buffer washed three times with McDowell--Triump fixable in 0.1 M phosphate buffer at pH 7.2 for at least 2 hours. The washed samples were decanted and fixed in 1% osmium tetroxide prepared in 0.1M phosphate buffer above for 1 hour. The fixed samples were then repeatedly washed with distilled water to remove excess fixative and dehydrated in a graded alcohol series (50, 75, 95, and 100%) and hexamethyldisilazane for every 10 minutes. Finally, the samples were sputter-coated with gold with the help of a sputtering gun. Then, the gold-coated sludge samples were examined by Supra 50 VP (Carl Zeiss model) microscope, and the images were recorded digitally.

## Results and Discussion

### *System Novelty and Performance*

First, the novel ABR is developed by modifying the vertical baffles to control the hydraulic condition and to facilitate a greater mixing and better contact between the wastewater and biomass in the system. This is believed to support its ability to partially separate between the various phases of anaerobic catabolism. Second, the system novelty further comprised the combination of attached growth with suspended growth microorganisms in one system to take advantage of both biomass types which theoretically advantageous to reactor kinetics and process optimization. The most significant advantages of this design were its ability to realize the staged multi-phase anaerobic theory, allowing different bacterial groups to develop under more favorable conditions, low costs and no associated control problems. Other advantages include reduced sludge bed expansion, no special gas or sludge separation required and high stability to organic, hydraulic and toxic shock loads. Moreover, the reactor was operated at wide range of organic loading rate (OLR) of 0.33–4 g COD/L day. At the highest OLR (4 g COD/L day), the system successfully removed 83% of COD and yielded 0.179 L $CH_4$/g COD removed, while the pH level was fluctuating above 6.

In addition, the compartmental study showed that most of the COD, BOD, and lignin was removed in Compartments 1 and 2. When the OLR was gradually increased to 4 g COD/L day, an accumulation of substrates in Compartments 1 and 2 occurred, then subsequently decreased in the following compartments. The NABR plays an important role in substrates removal by settlement and degradation processes. The formation of suspended (Compartments 1 and 2) and attached (Compartments 3 and 4) microorganisms were clearly observed from concentrations profiles. Furthermore, partial separation was also observed by low pH levels, low alkalinity concentrations and high volatile fatty acids (VFA) concentrations in the initial compartments, and conversely in the last compartments. This clearly confirms the occurrence of hydrolysis and acidogenesis processes in the front, acetogenesis process in the middle, and methanogenesis process in the rear part of the system. Moreover, detailed system performance and compartmental study can be found in Zwain et al. (2018).

### *Identification of Microbial Community*

Isolation of microbes on agar plate approach has been generally applied in environmental microbiology to investigate the structures of the microbial community in complex processes, including anaerobic reactors. Six sludge samples were cultured for 24 hours on an agar plate. Four of the samples were withdrawn from the bottom of Compartments 1, 2, 4, and 5, while one sample was taken from the upper part of compartment 3 (from packing material) and another sample was obtained from raw POME sludge. These samples were selected to cover as much as possible of the microbial community developed in the suspended and attached growth mechanisms, in addition to the seeding microorganisms' source. After culturing, thirty-six bacterial strains were isolated on an agar plate. They were designated on testing tubes and sent for subsequent sequencing analyses. Out of these thirty-six isolates, six bacterial strains were from Compartment 1, four bacterial strains were from Compartment 2, six bacterial strains were from Compartment 3, eight bacterial strains were from Compartment 4, eight bacterial strains were from Compartment 5 and four bacterial strains were from POME sludge. Table 1 presents the isolated bacteria which were determined by comparing a sequence to the GenBank database (Yu et al. 2014). From Table 1, it is noticed that the system consisted of dominant *Bacillus* (25 strains) and *Bacterium* (1 strain) which were isolated from the settled sludge at the bottom of the reactor, whilst *Bacillus* (2 strains), *Pseudomonas* (2 strain) and *Chryseobacterium* (2 strain) were isolated from the biofilm formed on the packing material. With regard to the raw sludge, it was observed that POME sludge majorly contains four types of microbes identified as *Stenotrophomonas* (2 strains) and *Bacillus* (2 strains).

These results revealed that the microbial community strains, function, and structure changed simultaneously throughout the reactor system. The NABR combined suspended sludge at the bottom of each compartment and the biofilm attached to the packing materials. In an anaerobic baffled reactor, complete phase separation means that the fermenting bacteria are dominant in the anterior compartments while methanogens are dominant in the latter compartments (Zwain et al. 2017). In this study, the results showed that there was no dominant microorganism in any compartment of the system. Hence, a complete separation of acidogenic and methanogenic phases did not occur within the NABR. This was proved by the observation of *Bacillus* strains in all compartments. A similar finding was also observed in ABR treating algae-laden water, where complete separation has not occurred (Yu et al. 2014).

*Bacillus* and *Pseudomonas* species are acid phase bacteria belonging to facultative anaerobes, their mechanism creates favorable conditions for the development of obligatory anaerobes (Shah et al. 2014). *Bacillus* and *Pseudomonas* species were also found efficient for resin acid degradation in paper mill wastewater (Thompson et al. 2001). The application of different bacterial communities could efficiently reduce the COD and BOD. This can be assigned to the biological difference, leading to differences in initial activity and substrate

adaptation. Therefore, different microbial populations will show a difference in the ability to convert the substrate to methane. For instance, a combination of the three bacteria *Bacillus megaterium* (MTCC 6544), *Pseudomonas aeruginosa* (DSMZ 03504) and *Pseudomonas aeruginosa* (DSMZ 03505) have been applied successfully to reduce about 76% of COD, 7% of TDS and as well as the BOD, AOX, color, and the toxicity within the pulp and paper mill effluents (PPME) (Tiku et al. 2010).

In this study, the NABR has been successfully removed up to 94% of COD and 97% of BOD with the presence of *Bacillus cereus* and *Pseudomonas aeruginosa*. Similarly, Chandra (2001) has reported the efficient removal of 97% of color, 96.63% of BOD, 96.8% of COD, 96.92% of phenolics, and 96.67% of sulfide using activated sludge process which contains the mixture of *Pseudomonas putida*, *Enterobacter sp.*, and *Citrobacter sp.* strains. Mehta et al. (2014) investigated the removal of COD from PPME using the mixture of *Alcaligenes faecalis* and *Bacillus cereus*. They revealed that bacterial strain *Bacillus cereus* was found more efficient in removing COD (63.2%) than bacterial strain *Alcaligenes faecalis*, due to its high capability of adaptation to the PPME.

As a result of the growth of specific bacteria species, mainly *Bacillus* and *Pseudomonas* strains, in this study, NABR could achieve a high methane production. Similarly, Duran et al. (2006) indicated that bioaugmenting the anaerobic system of biosolids with ancillary organic compounds containing various micronutrients along with selected strains of *Actinomycetes*, *Pseudomonas* and *Bacillus* led to improved methanogenesis and odor control. Sonakya et al. (2001) studied the metabolic activities of *Bacillus subtilis*, *Bacillus licheniformis,* and *Aspergillus niger* with respect to $H_2$ and $CH_4$ production from damaged wheat grains. They found that *Bacillus subtilis* and

**Table 1.** Identity of the genomic sequences from the isolated microbes obtained by sequencing and basic local alignment search tool (BLAST) analysis

| Sample | Gene | Accession No. in GenBank | Identification (%) |
|---|---|---|---|
| C1B1 | *Bacillus thuringiensis* strain VCRC B544 | JN377783.1 | 99 |
| C1B2 | *Bacillus anthracis* strain TRTYP13 | KC734533.1 | 99 |
| C1B3 | *Bacillus subtilis subsp. Subtilis* 6051-HGW | CP003329.1 | 99 |
| C1B4 | *Bacillus subtilis* strain RB-114 | JQ085400.1 | 99 |
| C1B5 | *Bacillus thuringiensis* strain GS2 | JN816401.1 | 99 |
| C1B6 | *Bacillus anthracis* strain IHB B 2660 | KF475846.1 | 99 |
| C2B1 | *Bacillus cereus* strain L-05 | KJ534398.1 | 98 |
| C2B2 | *Bacillus thuringiensis* strain AHBR13 | KF241526.1 | 99 |
| C2B3 | *Bacillus sp.* OU-73 | FN663628.1 | 99 |
| C2B4 | *Bacterium* CulalnoE420 | KC484964.1 | 99 |
| C3U1 | *Bacillus sp.* OU-73 | FN663628.1 | 99 |
| C3U2 | *Bacillus cereus* strain JBE0002 | FJ982656.1 | 99 |
| C3U3 | *Pseudomonas aeruginosa* strain CZO | JX441328.1 | 99 |
| C3U4 | *Pseudomonas aeruginosa* strain M10 | JQ927361.1 | 99 |
| C3U5 | *Chryseobacterium sp.* Pp2f | FJ870662.1 | 99 |
| C3U6 | *Chryseobacterium sp.* THG A8 | JN196133.1 | 99 |
| C4B1 | *Bacillus cereus* strain VCRC B540 | JN377787.1 | 99 |
| C4B2 | *Bacillus anthracis* strain TRTYP13 | KC734533.1 | 99 |
| C4B3 | *Bacillus cereus* strain moh2 | KF021536.1 | 99 |
| C4B4 | *Bacillus thuringiensis* strain HEN2 | KF026329.1 | 99 |
| C4B5 | *Bacillus subtilis* strain MZA75 | HM101166.1 | 99 |
| C4B6 | *Bacillus subtilis* strain ASC4 | GU227615.1 | 99 |
| C4B7 | *Bacillus licheniformis* strain EdyKolBl23 | JX625991.1 | 97 |
| C4B8 | *Bacillus licheniformis* strain A10 | KC310461.1 | 95 |
| C5B1 | *Bacillus sp.* U2 | KC434993.1 | 99 |
| C5B2 | *Bacillus cereus* strain JBE0002 | FJ982656.1 | 99 |
| C5B3 | *Bacillus sp.* S22716 | KF956696.1 | 99 |
| C5B4 | *Bacillus sp.* TS1(2011) | JN944551.1 | 99 |
| C5B5 | *Bacillus sp.* NCCP-155 | AB576863.1 | 99 |
| C5B6 | *Bacillus megaterium* strain MSC 2 | HQ694774.1 | 99 |
| C5B7 | *Bacillus cereus* strain BAC-B2 | DQ884352.1 | 99 |
| C5B8 | *Bacillus cereus* strain JBE0002 | FJ982656.1 | 99 |
| S1 | *Stenotrophomonas maltophilia* strain VSS4 | KJ528947.1 | 99 |
| S2 | *Stenotrophomonas maltophilia* partial | FN395263.1 | 99 |
| S3 | *Bacillus thuringiensis* strain VCRC B544 | JN377783.1 | 99 |
| S4 | *Bacillus cereus* strain JBE0002 | FJ982656.1 | 99 |

*Bacillus licheniformis* could produce 45 to 64 L $H_2$/kg total solids and 155 to 220 L $CH_4$/kg total solids. In addition, the use of *Bacillus subtilis* and *Bacillus licheniformis* led to an increase in $CH_4$ production capacities from as low as 17% to as high as 110%.

In contrast, other studies reported different microbial communities in anaerobic systems. Zwain et al. (2017) reported that modified anaerobic inclining-baffled reactor was dominated by methanogenic *Methanosaeta Concilii*, *Candidatus Kuenenia Stuttgartiensis*, and anaerobic ammonium oxidation (ANAMMOX) microorganisms. Gao et al. (2019) have shown that *Methanolinea*, *Methanogenium*, *Porphyromonadaceae*, and *Fibrobacteraceae* are the predominant bacterial communities found in an up-flow anaerobic sludge blanket (UASB) reactor during toilet blackwater treatment. Likewise, Tsavkelova et al. (2018) reported that *Ruminiclostridium*, *Methanothermobacter* and *Methanosarcina* were the most predominant archaeal genera in the anaerobic thermophilic reactor treating paper wastewater. Notably, different anaerobic systems reported different microbial communities. This is due to the characteristics of treated substrates, system operational condition, and microorganisms' identification technique.

For further understanding, the particular microbial community profiles at different compartments and phylogenetic composition of bacteria were examined through phylogenetic analysis. Figure 2 shows the phylogenetic analysis of partial 16S rRNA sequences conducted by a neighbor-joining tree. The phylogenic tree was obtained to identify the evolutionary mutual relations among different bacterial isolates occurring in the system. Phylogenetic tree of 16S rRNAs sequences has indicated that the species of similar genera were aligned in one branch. The results conclude that the evolutionary relations showed by the species consist of five major branches, where they belong to the same eubacterial group. Figure 2 also shows that a significant proportion of bacteria clones are closely related to a large part of various *Bacillus* strains, while another part of clones is related to *Pseudomonas*, *Stenotrophomonas* and *Chryseobacterium* genus. The Phylogenetic tree in Figure 2 indicated that the 36 clones obtained from six samples were affiliated with thirty-one different bacterial divisions after sequencing and alignment. Most of them are gram-negative (*Bacillus*), and only a few clones assigned to *Pseudomonas*, *Stenotrophomonas* and *Chryseobacterium* are gram-negative.

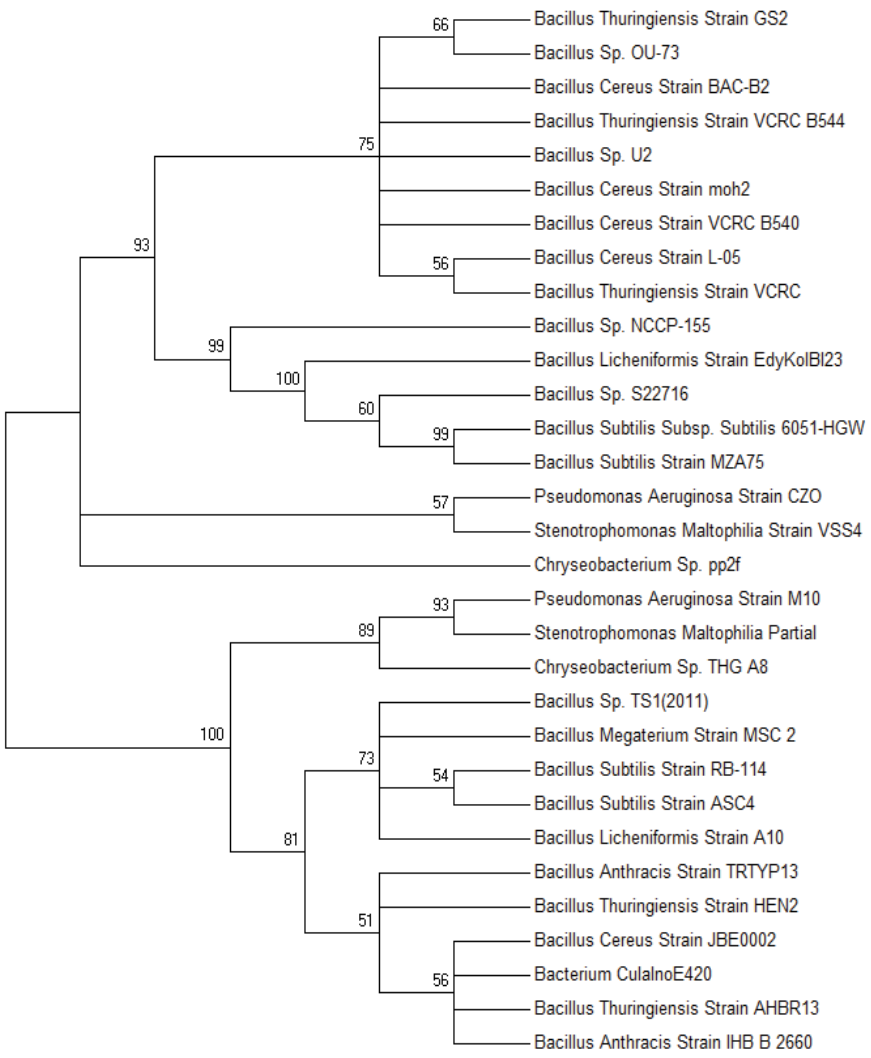


**Fig. 2.** Evolutionary relationship of the bacteria 16S rRNA gene partial sequences received from sludge of five compartments and POME. The phylogenetic tree was constructed with the neighbor-joining method. Bootstrap values smaller than 50% (based on 500 bootstrap re-samplings) were hidden at nodes.

### *Analysis of Microbial Morphologies*

Microbial morphology study has been widely used in environmental microbiology to characterize the biomaterials surface structure, measure cell attachment, and observe changes in morphology of bacteria. It is useful for defining the number and distribution of co-existed microorganisms. In combination with molecular studies, it has led to a wide understanding of the microbial diversity and composition of bacterial communities in various setups. In order to study the morphology of microorganisms inside the NABR, samples from different compartments were further subjected to SEM analysis. The microorganism from the bottom of compartments 1, 2, 4 and 5, upper part of compartment 3 and POME were tested by SEM to observe their structure and morphology. The results are shown in Figure 3 (a–f). The microscopic results of the microorganism showed rich biofacies, while the dominant microorganism occurred to have various morphologies in every compartment of the system (Zwain et al. 2017). Figure 3 (a) shows that the microbial structure in Compartment 1 consists of a long rod-shaped and filamentous bacterium composed majorly of bacilli of different sizes. This appearance suggests that these shapes might be attributed to hydrolyzing and acid-producing bacteria. Ran et al. (2014) reported that filamentous bacteria and shot rod-shaped in the initial compartment of ABR were in charge of hydrolysis and acidification.

In Compartment 2, there was no predominant microorganism. As shown in Figure 3 (b), bacilli and shot rod-shaped microorganisms were observed. Depending on their bionomics, the acidogenic and methanogens microorganisms may likely coexist in this compartment. These results are similar to the morphology obtained by Yu et al. (2014) who found that a heterogeneous colonization pattern bacteria were developed in their compartment. In Compartment 3, the biofilms developed on the packing material were found to be dense and various kinds of microorganisms were highly compacted as shown in Figure 3 (c): some colonies of cocci, rods and bacilli were seen on the surface of packing material.

The microorganisms in Compartments 4 and 5 of the NABR were mixed, with bacilli and rods mainly found, as illustrated in Figure 3 (d) and Figure 3 (e). Generally, the number of bacilli and rods microorganism decreased from Compartments 2 to 5. Numerous gaps in the sludge were observed. With respect to POME sludge, Figure 3 (f) shows a variety of filamentous, small shots and small cocci microbes. It is clearly observed that the POME sludge contains various microorganisms. This is one of the reasons for the efficient performance of the NABR. The unique flow characteristics of the NABR allow different bacterial populations to grow in every compartment, which resulted in the variation of microbial morphology in each compartment.

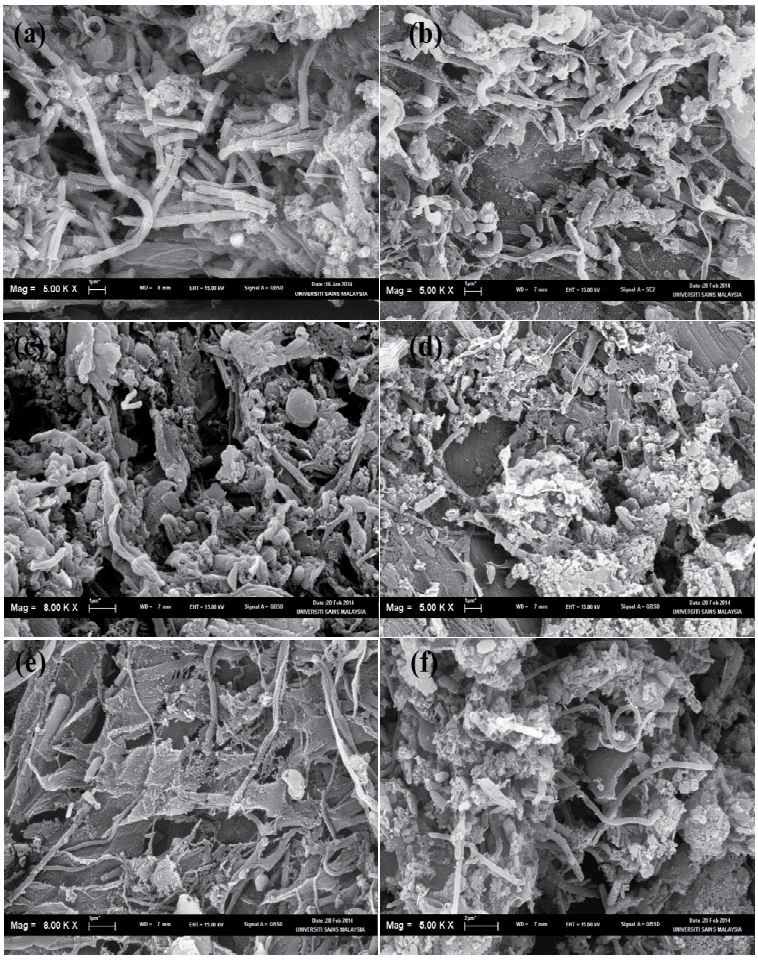


**Fig. 3.** Scanning electron microscopy (SEM) showing various types of bacteria in the biomass from Compartments 1 to 5 and POME, respectively. (a) Compartment 1; (b) Compartment 2; (c) Upper of compartment 3; (d) Compartment 4; (e) Compartment 5 and (f) POME.

## Conclusion

The study successfully identified the microbial community in NABR responsible for the digestion of RPMW. A partial phase separation of anaerobic processes was noticed throughout the system compartments. The microbial morphology showed that the microorganisms' structure shifted from one compartment to another with a long rod-shaped and filamentous bacterium found in Compartment 1 and bacilli and rods found in Compartment 5. Nevertheless, the accuracy of culture--based technique depends on several technical factors and the selection of proper clones. Hence, in order to comprehensively understand the composition and dynamics of microorganisms within the microbial community, molecular techniques are recommended as a method that provides more information.

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