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Identification of Microbial Community in an Anaerobic Reactor Treating Brewery Wastewater

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Abstract**—**The study of microbial ecology and their function in anaerobic digestion processes are essential to control the biological processes. This is to know the symbiotic relationship between the microorganisms that are involved in the conversion of complex organic matter in the industrial wastewater to simple molecules. In this study, diversity and quantity of bacterial community in the granular sludge taken from the different compartments of a full-scale upflow anaerobic sludge blanket (UASB) reactor treating brewery wastewater was investigated using polymerase chain reaction (PCR) and real-time quantitative PCR (qPCR). The phylogenetic analysis showed three major eubacteria phyla that belong to *Proteobacteria, Firmicutes* and *Chloroflexi* in the full-scale UASB reactor, with different groups populating different compartment. The result of qPCR assay showed high amount of eubacteria with increase in concentration along the reactor's compartment. This study extends our understanding on the diverse, topological distribution and shifts in concentration of microbial communities in the different compartments of a full-scale UASB reactor treating brewery wastewater. The colonization and the trophic interactions among these microbial populations in reducing and transforming complex organic matter within the UASB reactors were established.

Keywords**—**Bacteria, brewery wastewater, real-time quantitative PCR, UASB reactor.

I. Introduction

MICROBIAL species involved in the conversion of organic material in anaerobic digesters are grouped based on their biochemical activities. These groups include hydrolytic, acidogenic, acetogenic and methanogenic organisms [1]. These organisms grow in a syntrophic manner when the digester is operated under optimum reaction conditions during anaerobic digestion process [2], [3]. For efficient functioning of an UASB reactor, it is vital to have an in-depth understanding of the microbial consortium and concentration for effective reactor operation and better effluent quality [4]. However, it is difficult to assess the diversity, colonization, topological distribution and the trophic interactions among the microbial populations within the UASB reactors using conventional methods due to the structural complexity of the granular sludge. Also most of these bacteria that help in bioconversion in bioreactor are not culturable [5]. Due to these facts, fluorescence in-situ hybridization (FISH), qPCR, denaturing gradient gel electrophoresis (DGGE) and next generation sequencing (NGS) have been used in investigate the shift in microbial community and their population size due to changes in environmental conditions in anaerobic reactor [6]-[9]. However, the bacterial composition within the different compartments of a UASB reactor treating brewery wastewater is still not well understood and investigated. Hence, the focus of this study was on the identification and quantification of bacterial at different compartments of a full-scale UASB reactor treating brewery wastewater using PCR and qPCR. This will help in understanding the bacterial interaction between the different compartments of an anaerobic reactor as they convert complex organic matter present in the brewery wastewater to simple monomer and by-products.

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II. Materials and Methods

A. Sample Collection

Well suspended granular samples were obtained from different compartments (C1, C2, C3, C4, and C5) of a full-scale industrial UASB reactor treating brewery wastewater for molecular analysis [9]. Before sample collection, sampling valves were opened for 5 min to flush the sampling tubes and valves. Then granular sludge samples were collected for molecular analysis in a pre-autoclaved screw-cap glass bottles from a sampling point at a fixed position and height of the reactor and flushed with nitrogen gas and closed immediately to maintain anaerobic condition during transportation to the laboratory at 4 °C for analysis. 2 mL of aliquot samples were centrifuged at 9,600 x g, 4 ˚C for 5 min. After discarding the supernantant, the pellets were washed using 1 x PBS (phosphate buffered saline). Sub-samples were combined together as a single time point and stored at -20 ˚C for DNA extraction.

B. Extraction of Total Genomic DNA

Total genomic DNA in the granular sludge samples was isolated according to phenol-chloroform extraction protocol described by [9]. Briefly, 700 µl lysis buffer (0.5 mol^{-1} Tris/HCl, 0.1 mol^{-1} NaCl, 0.5 mol^{-1} EDTA, at pH 8.0), 30-40 mg PVPP and 0.2% ß-mercaptoethanol were added to the samples. 0.5 mm sterile glass beads were added to solution and homogenised at 600 x g for 5 min using bead beater machine. The granules were further treated with 20 µl of Proteinase K, vortexed to mix and incubated for 30 min at 37 °C. The suspension was incubated for 2 h at 65 °C and the

cells were freeze-thaw in series (in dry ice: ethanol slurry) and thawed at 65 °C in a water bath (five times each). After cell lysis, two-step phenol–chloroform–isoamyl alcohol extraction (25:24:1) was carried out to separate protein and RNA from the aqueous phase containing the DNA followed by 24:1 chloroform–isoamyl alcohol. The solution was repeatedly centrifuged at 13,800 x g for 10 min to remove the phenol until a clean interface was seen. The genomic DNA was precipitated by the addition of 1 x volume of isopropanol and stored at -20 °C overnight for complete precipitation. The DNA was collected by centrifugation for 20 min at 13,800 x g and 90% ice-cold ethanol was used to wash the DNA twice followed by 70% ice-cold ethanol. The pellet was air dried and dissolved in TE buffer (100 µl), while the purity and yield of extracted genomic DNA were determined by Qubit fluorometer and Nanodrop Spectrophotometer (ND-1000). DNA samples were stored at -20 °C further analysis.

C. PCR Assay and 16S rRNA Gene Sequences

PCR amplification was performed to detect the bacterial gene using forward primer 27F 5'- AGAGTTTGATCMTGGC TCAG-3' and reverse primer 1492r 5'-TACGGYTACCTTGTT ACGACTT-3' primer set [10]. The PCR mixture contained 0.3 µl of Taq DNA polymerase (5 U/ml), 2.5 µl of PCR reaction buffer, 1 µl of each of the primer (10 µM), 0.5 µl of dNTPs (10 mM), 2 µl of the extracted DNA (10 ng – 20 ng) and PCR-grade water to a final volume of 25 µl. Modified PCR amplification of [11] was used as: Initial denaturation was performed at 94 °C for 5 min; followed by 40 cycles of denaturation at 92 °C for 1 min; primer annealing at 53 °C for 1 min, elongation at 72 °C for 1 min and a final extension was performed at 72 C for 5 min on automatic thermal cycler Veriti (Applied Biosystems). The PCR amplified products were analysed on 1.0% (w/v) agarose-Tris-borate EDTA gel (ABgene, UK), visualized and photographed under the BioDoc-It transilluminator system. Purification of the PCR products and cloning were performed with a commercial kit (Thermo Scientific, InsTAclone PCR Cloning Kit) following manufacturer's instructions. Positive clones were randomly selected and sequenced usinfig forward primer on Genetic Analyzers with 24-capillaries, 3500xL System (Applied Biosystems, USA). Sequences generated were analysed and subjected to detailed phylogenetic analyses.

D. Bioinformatics for Bacterial Community Identification

The obtained bacterial sequences were analyzed using the FinchTV, v 1.4.0 software (Geospira Inc., Seattle, WA) and similarity search for the sequences were carried out using the Basic Local Alignment Search Tool (BLAST) program to search the National Centre for Biotechnology Information (NCBI) sequence database (http://www.ncbi.nlm.nih.gov/ BLAST) for bacteria identification.

E. Amplification of Genomic DNA Using qPCR

Quantification was performed with primer targeting all bacteria (P388f and P518r) [12] on real-time PCR machine (C-1000 Touch, CFX 96, Biorad Laboratories Pty Ltd, USA). The reaction mixture contained 10 µl of Master Mix (the Sso fast Eva green by Bio-Rad Laboratories Pty Ltd. USA), 1 µl of each primer (10 µM), 4 µl of template DNA (2 ng) and PCR-grade water to a final volume of 20 µl. DNA amplification was carried out as follows: initial denaturation at 94 °C for 3.5 min followed by 40 cycles of 30 s at 95 °C, annealing temperature of 55 °C for 30 s and final extension with image capturing at 72 °C for 30s [9]. For melting curve analysis, the temperature was increased at 0.5 °C every 10 s from 40 to 95 ˚C. Each qPCR assay was conducted in duplicates with appropriate negative control. Purified plasmid was used for preparing the standard curve. Samples were performed in duplicates with negative controls. The results were analyzed using the 1000 Touch, CFX 96 qPCR software.

III. Result and Discussions

A. Bacterial Diversity in the Granular Sludge Obtained from the Reactor Using PCR

Degradation of organic matter requires many biochemical processes that are catalysed by different microbial populations in a bioreactor [13]. Amplification of eubacteria 16S rDNA in the granular sludge samples taken from the different compartments using PCR were positive and yielded the expected base pair (Fig. 1). The obtained PCR products were cloned and selected positive clones were sequenced to determine the bacterial diversity in each compartment.

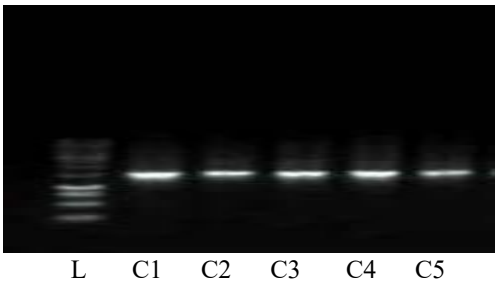

Fig. 1 Bacterial fragments from the samples obtained at different compartments of the full-scale UASB reactor using 27f/1492r primer set. L is the 1 kb DNA marker and C1-C5 is the expected fragments obtained from the reactor's compartments for bacterial domain

Abundance of three major bacterial phyla belonging to *Proteobacteria, Firmicutes* and *Chloroflexi* were identified within the reactor. The other major phylum was the uncultured candidate of WS6. Class Gamma and Deltaproteobacterium were present in abundance in the samples with *Syntrophorhabdus aromaticivorans, Cronobacter sakazakii* and *Dehalogenimonas* sp. as the abundant group. Similar pattern of diverse phylogenetic presentation of bacteria at phylum and genus level were reported during anaerobic degradation of brewery wastewater, corn straw, as well as birch and conifer pulp [14]. Detection of four major similar phyla (Bacteroidetes, Chloroflexi, Firmicutes and Proteobacteria) with relative differences of bacterial population in AD system was previously reported [15]-[17]. The abilities of *Syntrophorhabdaceae* bacteria to digest recalcitrant compounds of spent wash during anaerobic degradation has been reported especially, in brewery

wastewater treatment plant [18].

Among the clones that were selected from compartment 1 in this study, B1 showed similarity with cellulose, amylase and protease enzyme-producing bacterium P618 (JX120100) in the GenBank. The enzymes are excreted by hydrolytic and fermentative bacteria during the hydrolysis stage for anaerobic digestion of complex organic matter in the wastewater to soluble monomers [19]. Clone B8 was closely related to uncultured bacterium belonging to phylum Proteobacteria of *Enterobacteriaceae*. It shows 98% sequence similarity to *E. coli* and *Escherichia ferusonii* deposited at the GenBank. Acidogenic bacteria, members of alpha and gammaproteobacteria and the low G+C Gram positive classes that help in converting sugars, fatty acids and amino acids to organic acids (formic, acetic, propionic, butyric, lactic acids), ketones and alcohols in anaerobic reactors were well represented in this reactor. These intermediate products are utilized by obligate hydrogen–producing bacteria during acetogenesis process [19], [7].

Major groups of bacteria that are closely related to the clones isolated in the granular sample obtained in compartment 3 belong to class Gammaproteobacteria and uncultured *Enterobacteriaceae* bacterium clone (JQ516439). *Cronobacter sakazakii* formerly known as *Enterobacter sakazakii*, a Gram-negative, non-spore forming, motile and peritrichous rods of the Enterobacteriaceae family was also found to be closely related to these clones. *Cronobacter* strains affiliated to environmental samples and enumeration of *Cronobacter sakazakii* from sewage sludge have been reported. Keyser [7] demonstrated the importance of *C. sakazakii* strain in the treatment of winery effluent and its ability to degrade recalcitrant compounds in anaerobic digesters [20]. Relevance of *C. sakazakii* in the production of hydrogen as a metabolite that can be used by methane producing Archaea during dark fermentation were reported by [21]. *Cronobacter sakazakii* (JF690890) isolated from forest musk deer intestine and uncultured prokaryote (GU208330) bacterial communities in sediments of the shallow Lake Dongping, fiber degrading bacteria from pig feces (FJ753832) from the GenBank were found to be similar to the clones obtained from the compartment. Furthermore, the sequence similarity of clones obtained from compartment 4 was closely related to uncultured eubacterium WCHB1-06 (AF050595) of class *Clostridia*. Genus *Clostridium* are efficient in converting complex organic matter to metabolites that can be used directly by the methanogenic Archaea [7], [9], [22], [23]. Genus *Clostridium* is highly diverse with most of them showing acetogenic properties [22]. In addition, uncultured *Dehalogenimonas* sp. (JN540166) in phylum Chloroflexi, toluene-degrading methanogenic consortium bacterium (AF423183), uncultured prokaryote clone (GU208330), marine bacterium (HM100738), and other uncultured bacteria were affiliated to clones with 96% sequence similarity.

Diverse bacteria communities were detected as towards the last compartment with the largest proportion of bacteria belonging to class Delta and Gammaproteobacteria. Sequence similarity (99%) with known sequences in the GenBank showed that the clones from this compartment belong to class Deltaproteobacteria (formally known as Deltaproteobacteria group TA) of family *Syntrophorhabdaceae*. This family contains well-known species of syntrophic substrate-degrading anaerobes such as those of the genera *Syntrophus, Smithella* and *Syntrophobacter* [24], [25]. They are known as amino acid degraders and sulphate-reducing bacteria [26], [27]. *Syntrophobacter* species use sulphate as an external electron acceptor with slow growth during sulphate reduction [28].

In this study, the 16S rDNA gene sequences of clones obtained in C5 were closely related to *Syntrophorhabdus aromaticivorans* strain UI of group TA isolated in granular sludge taken from an UASB reactor treating manufacturing wastewater [24]. *Syntrophorhabdus aromaticivorans* is an obligate anaerobic, syntrophic substrate-degrading mesophilic organism that is capable of oxidizing benzoate, phenol, p-cresol, 4-hydroxybenzoate and isophthalate in the presence of H_2-scavenging methanogen (hydrogenotrophic methanogen). They are long-chain fatty acid degrading microbes that have a syntrophic relationship with methane-producing microorganisms [29].

Detection of sulphate-reducing bacteria (SRB) explained the low to no sulphate in the brewery effluent (treated wastewater) from the UASB reactor. Reference [30] also noticed about 15% of SRB in a methanogenic reactor, even in the absence of sulphate in the reactors influent. Investigation on the competition and coexistence between SRB, acetogens and methanogens in an anaerobic reactor was reported by [31] at high organic loading rates where they compete for the available electrons and acetate [32]. A high amount of SRB was quantified in sludge taken from methanogenic environments especially in UASB reactors [33], [17]. The important role of *Syntrophorhabdus aromaticivorans* in degrading aromatic compounds during the treatment of industrial wastewater have been reported [24]. Hence, all the bacterial identified using the clones obtained from the reactor samples showed that major bacterial communities needed for the degradation of organic matter present in the brewery wastewater are well represented. This will help in reactor performance, thereby produced better quality effluent before the discharge into municipal treatment plants or receiving water bodies.

B. Shift in Bacterial Concentration along the Compartments of a UASB Reactor Using qPCR

Known concentrations of standard DNA were used to validate all qPCR assays with determination coefficients (R^2) values of 0.991 with no significant differences in the slopes of the standard curves at 95% confidence interval. In average, the values of intercept and slope that were used to quantify the eubacteria in the samples were 41.052 and -3.485 respectively. Average amplification efficiencies for bacteria (97.6%) show the consistency in the qPCR assay and melting curve analysis was used to determine the primer dimer at the end of each run. DNA copy numbers in the reactor sample was reported in 16S rDNA genes per nanogram of genomic DNA isolated.

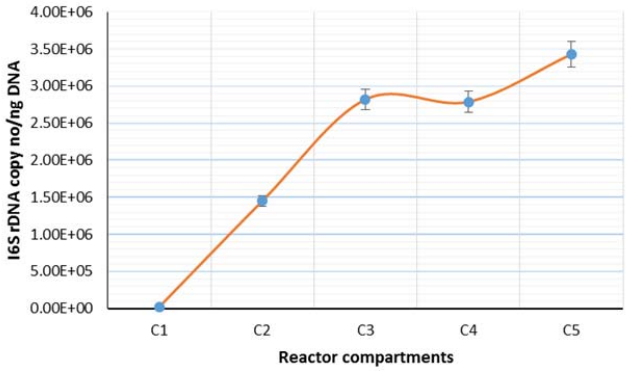

Fig. 2 Abundance of bacterial community along the different compartments of a full-scale UASB reactor using universal bacterial primer

In this study, the compartments showed a noticeable disparity in terms of total bacterial 16SrDNA copies in each compartment. The concentration increased along the compartments from compartment C1 to C5 as shown in Fig. 2. The total concentration of bacteria in this study ranged between 2.58×10^4 and 3.43×10^6 copies/ng DNA. The result shows that bacteria community is dominant and abundant in compartment C5 and decreases down the compartments (Fig. 2). Compartment 1 has a relatively low concentration of bacterial copy number, followed by an increase and fluctuation in cell number at the middle compartments. Fluctuation in the quantity of bacterial concentration might be as a result of production of some metabolites and the inhibitory activities of other bacteria group or the impact of temperature on the digestion process in anaerobic reactor [16]. Different metabolites are also produced during anaerobic fermentation and certain bacteria are unable to utilize the metabolite or compete with the fast-growing ones. Another rationale for increase and decrease of bacteria in the compartments could be that the metabolites produced might have inhibited the growth of other bacteria or possibly the production of organic monomer by the hydrolytic and acidogenic bacteria as previously explained [27].

IV. Conclusions

Characterization of bacterial community in the granular sludge samples collected from the different compartment of a full-scale UASB reactor suggested that hydrolysis to acetogenic bacteria were present in the UASB reactor investigated. Different group of bacteria were found at the different levels of the studied UASB reactor with *Proteobacteria, Firmicutes, Chloroflexi* and uncultured candidate division WS6 as the most dominant phyla. Also, the trend of the bacteria fingerprint down the compartments using qPCR showed that bacterial concentration increases with increase in compartment. Lower concentration of bacteria in compartment 1 was observed, thereafter bacteria increases as the compartment increases with little fluctuation.

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