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THESIS

IDENTIFICATION OF MICROORGANISMS FROM FROZEN SEAFOOD WASTEWATER TREATMENT PLANT BY USING MOLECULAR TECHNIQUES

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A Thesis Submitted in Partial Fulfillment of
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The majority of microorganisms in 6 units; influent, equalization tank, UASB tank, aeration tank, sedimentation tank and effluent form frozen seafood wastewater treatment plant investigated by polymerase chain reaction (PCR) techniques and sequencing analysis. The PCR techniques amplified approximately 1500 bp of 16S rDNA of each samples and fragment bands were confirmed by gel electrophoresis after PCR. The chemical properties especially BOD and COD indicated that lower efficiency of organic removal than the unit design criteria in the UASB tank and the aeration tank.

The results showed the majority of microorganisms belonged to Fermicute and Proteobacteria phylum. In the influent and the equalization tank found the Fermicute member were *Streptococcus sp*. and *Bacillus sp*. The other units were Proteobacteria member while the UASB tank was *Serratia marcescens*, *Enterobacter sp.*, *Klebsiella pneumoniae* and *Escherichia coli*. For the aeration tank belonged to Proteobacteria, was *Thiothrix sp.* and uncultured actinobacterium were found. For the sedimentation tank was *Escherichia coli*, *Shigella sp.*and *Salmonella typhimurium* while the effluent was found *Pseudomonas putida, Providencia sp., Aeromonas veronii* and *Alcaligenes faecalis.* The majority of microorganisms in each of units play the major role in biodegradable of different organic compounds this related to the chemical properties analysis in the study.

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Student's signature Thesis Advisor's signature

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Kandarat Lamchumchang

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LIST OF ABBREVIATIONS

%	=	Percentage
bp	=	Basepairs
BOD	=	Biochemical oxygen demand
°C	=	Degree celcius
COD	=	Chemical oxygen demand
CO_2	=	Carbondioxide
DNA	=	Deoxyribonucleic acid
dNTPs	=	Deoxynucleotide Triphosphates
DO	=	Dissolved oxygen
F	=	Forward
mg	=	Milligram
min	=	Minute
mL	=	Milliliter
mM	=	Millimolar
N	=	Nitrogen
P	=	Phosphorus
PCR	=	Polymerase chain reaction
pmol	=	Picomole
V	=	Voltage
PEG	=	Polyethylene glycol
R	=	Reverse
rpm	=	Round per minute
dsDNA	=	Double strand deoxyribonucleic acid
ssDNA	=	Single strand deoxyribonucleic acid
TSS	=	Total suspended solids
rDNA	=	Ribosomal deoxyribonucleic acid
UASB	=	Up flow anaerobic sludge blanket reactor
UV	=	Ultraviolet
µL	=	Microliter

IDENTIFICATION OF MICROORGANISMS FROM FROZEN SEAFOOD WASTEWATER TREATMENT PLANT BY USING MOLECCULAR TECHNIQUES

INTRODUCTION

During recent decades, frozen foods are become an important component of meals preparation, that has been widely favorite in many country therefore the frozen foods industries are greater expansion. However in the frozen foods processing require large amounts of water consumption per ton of product especially in washing and cutting process (Sandra *et al*., 2005) also discharge large volumes of wastewater with a high organic matter cause by contamination of blood, scraps, meat and fat so the characterization of wastewater are high chemical oxygen demand (COD) or biological oxygen demand (BOD), large amounts of total suspended solids, and various inorganic constituents including nitrogen and phosphorus (Contreas *et al*., 2000).

Microorganisms play the major responsible for degradation of organic matters in wastewater. Thereby several biological systems to treat wastewater from the food processing industry (Wei, 2006; Ioannis, 2008), however anaerobic digestion that followed by an aerobic process is an optimal process option for fish processing wastewater treatment (Chawdhury *et al.*, 2010).

The performance of biological removal of organic materials in wastewater treatment plants due to both of the activity of the microbes and the controlling parameters of system. The microbial community structure (diversity, populations, quantity, and distribution) alteration results many problems found in wastewater treatment plants that confirmed their role in the process. Therefore, the efficiency of bioreactor could be reflected by the microbial community structure. Accordingly, there are importance of bacteria assemblages to the proper functioning and maintenance of anaerobic and aerobic process treatment system.(Froster *et.al.,* 2003).

The understanding in condition, process, and the interaction in the microbial diversity helpful to design and operation of bioreactors (Briones and Raskin, 2003).

However, less than 1 % of microorganisms in the environment can be conventional cultivated by standard methods. The development of molecular techniques haves allowed the identification and monitoring of microbial communities with out requirement of isolation and cultivation of microorganisms.

Many molecular techniques to identification of microbial in the wastewater treatment plant such as amplified ribosomal DNA restriction analysis (ARDRA and also referred to as 16S-RFLP), ribosomal spacer analysis (RISA), terminal restriction fragment length polymorphism (t-RFLP), denaturing gradient gel electrophoresis (DGGE) and single strand conformation polymorphism (SSCP) have been used in many areas of microbial research ability to illustrate and monitor mixed populations. Full-cycle 16S rRNA analysis has allowed describe the diversity of individuals within populations and identify novel organisms. Also, the use of fluorescent in situ hybridization (FISH) and confocal laser scanning microscopy (CLSM) has provided a means to study microbial populations in a more quantitative way (Maukonen *et. al.,* 2003; Kirk *et. al*., 2004; Gilbride *et. al*., 2006; Sanz *et.al.,* 2007; Malik *et.al*., 2008)

The purpose of this study are identify of microorganisms comprising culturable and unculturable bacteria that are present in 6 units of UASB tank followed by activated sludge treatment plant that used to treat frozen seafood wastewater and monitor the changing of bacteria community and predominant in each units of this plant processing by amplification of 16S rDNA and sequence analysis.

OBJECTIVES

1. To identify the microorganisms in influent, equalization tank, UASB tank, aeration tank, sedimentation tank and effluent using PCR and sequence analysis.

2. To monitor the changing of bacteria community and predominant in influent, equalization tank, UASB tank, aeration tank, sedimentation tank and effluent.

LITERATURE REVIEW

1. Frozen seafood wastewater

Frozen seafood has the advantages of being very close in taste and quality to fresh seafood as compared with other preserved or processed. Frozen seafood are popular and accessible in most developed countries. Numerous types of seafood are processed such as mollusks (oysters, clams, scallops), crustaceans (crabs and lobsters), saltwater fishes, and freshwater fishes. However in the frozen seafood processing require large amounts of water consumption per ton of product, especially in washing and cutting processes (Sandra *et al*., 2005) and produce large amount of wastewater containing substantial contaminants in soluble, colloidal, and particulate forms. The degree of the contamination depends on the particular operation; it may be small (e.g., washing operations), mild (e.g., fish filleting), or heavy (e.g., blood water drained from fish storage tanks) (Wang *et al*., 2004).

There are many types of frozen seafood products depending on raw material. However the main processes are very similar. The typical flow diagram of the frozen seafood processing in Figure 1 represents the material and waste generate from the processes (Pollution Control Department [PCD], 2005).

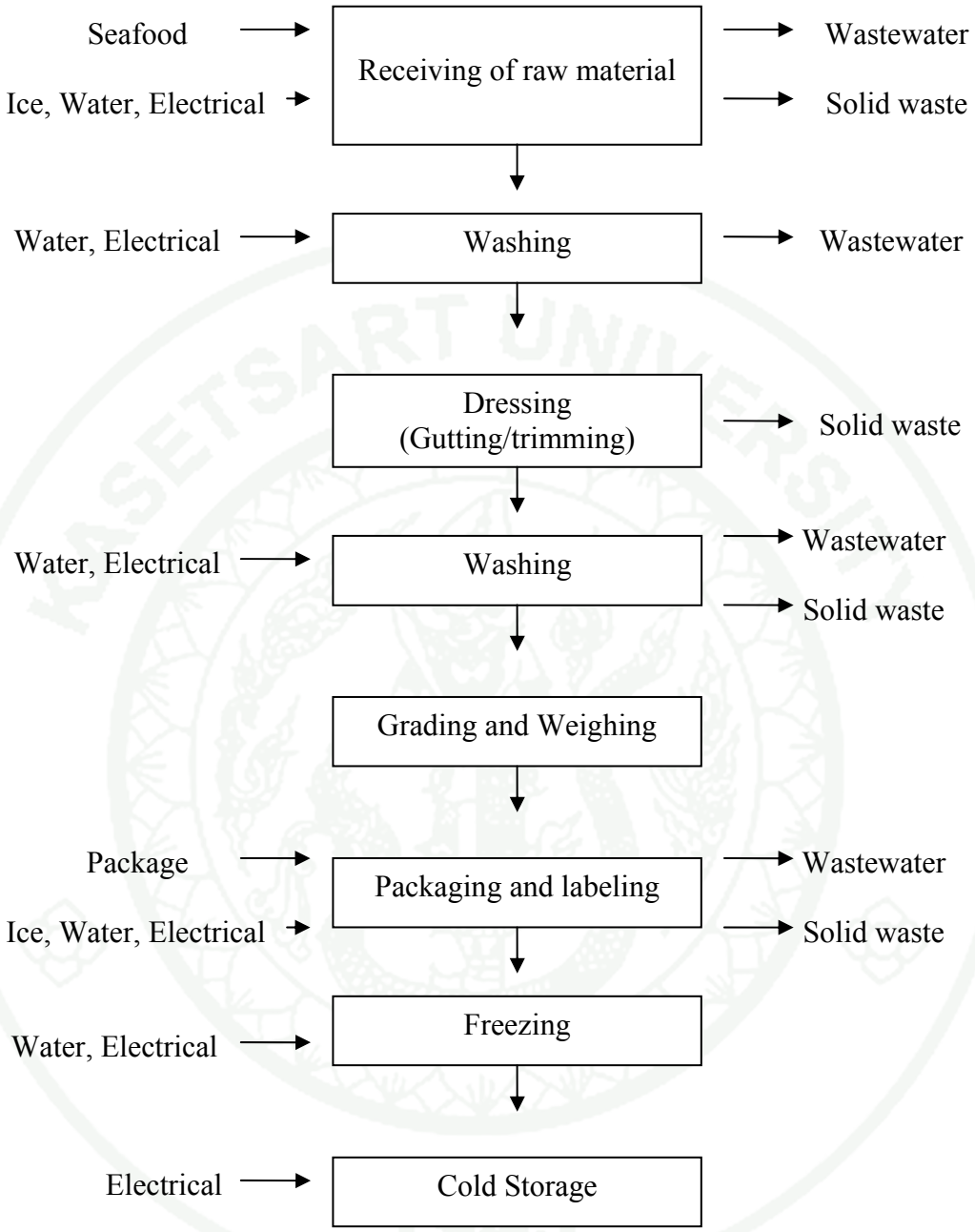

Figure 1 The typical flow diagram of the frozen seafood processing

Source: Adapted from PCD (2005)

1.1 Characterization of wastewater from frozen sea food plants

The volume and concentration of wastewater from fish processing depends mainly on the raw material composition, additive used, processing water source and the unit process. The main components of this wastewater are lipids and protein. In general, this wastewater can be characterized by its physico-chemical parameters, organics, nitrogen, and phosphorus contents. Important pollutant parameters of the wastewater are biochemical oxygen demand (BOD_5), chemical oxygen demand (COD), total suspended solids (TSS), fats, oil and grease (FOG), and water usage. (Wang *et al*., 2004).

1.1.1 pH

The pH levels generally reflect the decomposition of proteinaceous matter and emission of ammonia compounds. Effluent pH from seafood processing plants is usually close to neutral (Wang *et al*., 2004; Chawdhury *et al.*, 2010).

1.1.2 Solids content

In general, this processing wastewater contains high levels of suspended solids which are mainly proteins and lipids (Palenzuela-Rollon *et al*., 2002; PCD, 2005).

1.1.3 Organic content

The BOD_5 originates primarily from carbonaceous compounds and nitrogen containing compounds (protein, peptide and volatile amines). In a fish possessing industry, the effluent COD is usually higher than BOD_5. Depending on the types of seafood processing, the COD of the wastewater can range from 150 to about 42,000 mg/L (Wang *et al*., 2004).

1.1.4 Fats, Oil, and Grease

The fats, oil, and grease (FOG) are another important parameter of seafood processing wastewater. The presence of FOG in an effluent is mainly due to the processing operations .The FOG should be removed from wastewater because it usually floats on the water's surface and affects the oxygen transfer to the water; it is also objectionable from an aesthetic point of view. The FOG may also cling to wastewater ducts and reduce their capacity in the long term. The FOG of a seafood-processing wastewater varies from 0 to about 17,000 mg/L, depending on the seafood being processed and the operation being carried out (Wang *et al*., 2004).

1.1.5 Nitrogen and Phosphorus

The nitrogen and phosphorus concentration in the seafood processing wastewater are minimal in most cases. In the design criteria of wastewater treatment plant recommended that a ratio of N to P of 5:1 be achieved for proper growth of the biomass in the biological treatment. Phosphorus also partly originates from the seafood, but can also be introduced with processing and cleaning agents (Wang *et al*., 2004).

2. Biological treatment processes

During biological treatment, the objective is to remove or reduce the concentration of organic and inorganic compounds by using microorganisms. With biological treatment, the organic material in the wastewater is removed by the microorganisms through metabolic processes. The organic compounds may be used by the microorganisms to form new cellular material or to produce energy that is required to sustain the microorganisms.

2.1 Aerobic treatment process

The aerobic treatment process consists of a large variety of bacteria working side by side to degrade the organic compound. During aerobic degradation of organic compounds, aerobes and facultative anaerobes use free molecular oxygen to completely degrade organic compounds such as proteins to CO_2,H_2O, new bacterial cells (sludge), and inorganic compounds such as NH_4^+, HPO_4^{2-}, and SO_4^{2-}

$$\text{Organic compound} + O_2 \rightarrow CO_2 + H_2O + \text{cells} + NH_4^+ + HPO_4^{2-} + SO_4^{2-} \quad (1)$$

This microbial function as catalysts by the reaction. Aerobic bacteria are predominant in biological wastewater treatment processes such as activated sludge and trickling filters and other biological processes that utilize free oxygen for their biochemistry. During aerobic degradation of organic compounds, the carbon from the compounds is degraded completely and is incorporated in the end products CO_2 and new bacterial cells (equation 1). This is complete oxidation of the organic compounds or substrate (Gerardi, 2003).

2.2 Anaerobic treatment process

The microorganisms in anaerobic process utilize compounds such as sulfates and nitrates for energy, and their metabolism is substantially reduced. In order to remove a given amount of organic matters in an anaerobic environment, the organic matters must be exposed to a significantly higher quantity of bacteria and/or engaged for a much longer period of time.

The following reactions in equation 2 and 3 represent the anaerobic transformation by anaerobes common in wastewater treatment.

$$\text{Organics} + NO_3 \rightarrow \text{Anaerobes} + CO_2 + N_2 + \text{energy} \quad (2)$$

Which utilizes bounded oxygen in nitrate, or

$$\text{Organics} + SO^{2-}_4 \rightarrow \text{Anaerobes} + CO_2 + H_2S + \text{energy} \quad (3)$$

Which utilizes bounded oxygen in sulphate.

The anaerobic process consists of a large variety of bacteria working in sequence, that is, one after the other represent in Figure 2.

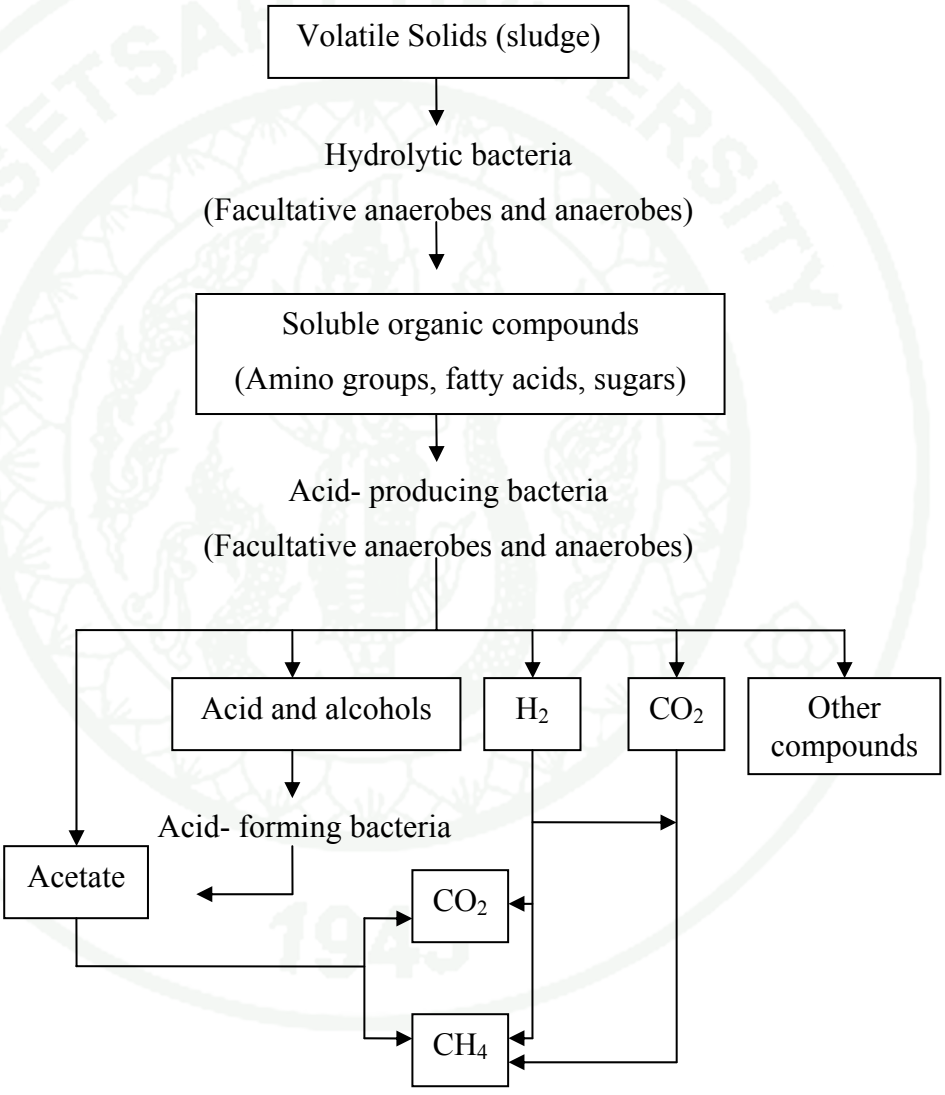

Figure 2 The bacteria working in sequence in anaerobic process

Source: Gerardi (2003)

Anaerobic bacterial activities are primarily founded in the digestion of sludge and wastewater lagoons. Anaerobic processes are normally biochemical inefficient and generally slow and produce complex end products some of which emit an obnoxious smell. In food and agricultural wastewater treatment, proteins are often degraded into amino acids and CO_2 (like aerobic degradation), H_2, alcohols, organic acids, methane, hydrogen sulphide, phenol, and indol.

Most of the bacteria that absorb the organic matters in a wastewater treatment system are facultative in nature. The facultative bacteria are living both in aerobic and in anaerobic environments. The nature of individual facultative bacteria is dependent upon the environment in which they live.

There are significant microbiological (Table 1) and operational differences between the degradation of organic compounds by aerobic and anaerobic digesters.

Table 1 Significant microbiological differences between aerobic and anaerobic.

Microbiological Feature	Aerobic Digester	Anaerobic Digester
Significant bacteria	Strict aerobic, including nitrifying bacteria Facultative anaerobic	Facultative anaerobic, anaerobic including methane-forming
Final electron carrier	Free molecular oxygen	Organic compounds, H_2, sulfur compounds, CO_2
Number of cells produced	Higher	Fewer
Products from reactions	CO_2, H_2O, cells, NH_4, NO_3, SO_4, HPO_4	CO_2, H_2O, cells, NH_4, CH_4, H_2, H_2S
Higher life forms	Numerous, ciliated protozoa, metazoa	Few, ciliated protozoa
Nitrification	Yes	No

Source: Gerardi (2003)

2.3 The UASB followed by activated sludge process

The activated sludge process has been widely applied for the treatment of domestic and industrial wastewaters, due to its high efficiency, operational flexibility, possibility for nutrient removal, among other advantages. However, there are some disadvantages, such as high mechanization level, high construction and operational costs, sophisticated operation and the need for treating a substantial amount of sludge.

In the anaerobic condition the UASB (Upflow Anaerobic Sludge Blanket) tanks are the most common high-rate anaerobic treatment process. The process works on the principle of promoting sludge granulation by proper seeding with a granular inoculums of anaerobic bacteria, and adjusting organic loading and upflow feed rates so that biomass is retained as a dense blanket with a clear liquid zone above. The UASB tank has shown to be a technology capable of overcoming some of the disadvantages of the mechanized aerobic systems, especially because of the absence of energy consumption and lower excess sludge generation. Nevertheless, the treated effluent is usually unable to comply with most existing discharge standards.

From the advantage and disadvantage of UASB tank and activated sludge system. Many study the performance of the wastewater treatment configuration composed of UASB (Upflow Anaerobic Sludge Blanket) tank followed by an activated sludge (AS) system.

The studied of Sperling *et.al.,(*2001) showed the combined system (UASB - activated sludge) to be a very good alternative for the treatment of municipal wastewaters, based on the performance of the system and the compactness of the treatment units (average COD removal efficiencies between 85% and 93%).

The combination of a UASB and AS system represented a very promising option for the treatment of combined dairy and domestic wastewater at a total HRT of 26 h (24 h for UASB and 2.0 h for the AS system). This combined system achieved an overall removal efficiency of 98.9% for COD_{total}, 99.6% for $BOD_{5\ total}$ and 98.9%

for oil and grease. The effluent quality of the combined system satisfies standards required for discharge into agricultural drains (Tawfik *et.al*., 2008).

Huang *et. al.,* (2005) conducted the Microbial activity in a combined UASB–activated sludge reactor system. The result showed that the combined UASB–AS reactor system achieved efficient removal of COD (95–97%), TKN (100%) and TN (54–55%) from suspended-solids pre-settled piggery wastewater. the combined UASB–AS reactor system should be regarded a promising alternative for the removal of organic carbon and nitrogen from piggery wastewater.

3. Microorganisms in wastewater

The common microorganisms found in biological wastewater treatment represent in Table 2.

Table 2 The common microorganisms found in biological wastewater treatment**.**

Species	Genera	Process Involved
Achromobacter	Bacteria	Biofilters and activated sludge
Acinetobacter	Bacteria	Biological phosphorous removal
Alcaligenes	Bacteria	Biofilters, activated sludge, and sludge digester
Chironomus	Metazoa	Stabilization ponds and sludge
Desulfovibrio	Bacteria	Sludge digesters
Flavobacterium	Bacteria	Activated sludge, biofilters, sludge digester
Geotrichum	Fungus	Activated sludge and biofilters
Gordonia	Bacteria	Activated sludge
Micrococcus	Bacteria	Activated sludge and biofilters
Microtrix	Bacteria	Activated sludge

Table 2 (Continued)

Species	Genera	Process Involved
Nitrobacter	Bacteria	Nitrification
Nitrosomonas	Bacteria	Nitrification
Pseudomonas	Bacteria	Denitrification
Rotifera	Metazoa	Activated sludge
Sphaerotilus natans	Bacteria	Activated sludge
Tubifex	Metazoa	Biofilters
Vorticella	Protozoa	Allaerobic processes and ponds
Zoogloea ramigera	Bacteria	Activated sludge and biofilters

Source: Liu (2007)

Many studied of microorganisms in wastewater treatment plant .The studied of Ahmed (2012) showed the microbial communities in nutrient removing membrane bioreactor which suggested in the denitrification process using methanol as carbon source was considered to carry out by facultative hetetrophic bacteria including *Streptococcus sp.* and *Bacillus sp.* in the absence of oxygen.

Gerardi (2003) explained the anaerobic bacteria in UASB tank mainly living in soil and water were capable of fermentation (species of *Aeromonas*, *Citrobacter*, *Klebsiella*, *Pasteurella*, *Proteus*, *Enterobacter*, *Escherichia* and *Serratia*). They accumulate and produce organic compounds such as lactic acid, succinic acid, propionic acid, butyric acid, acetic acid and ethanol during fermentation.

Keyser *et. al.,* (2006) studied of microorganism in the UASB tank using PCR-DGGE analysis in four different types of South African UASB granules that are used to treat winery, brewery, distillery and peach-lye canning wastewaters. This experiment represented the microorganism following genera: *Bacillus*, *Pseudomonas*,

Bacteroides, *Enterococcus*, *Alcaligenes*, *Clostridium*, *Shewanella*, *Microbacterium*, *Leuconostoc*, S*ulfurospirillum*, *Acidaminococcus*, *Vibrio*, *Aeromonas*, *Nitrospira*, *Synergistes*, *Rhodococcus*, *Rhodocyclus* and *Syntrophobacter*.

Xin *et.al.* (2008) studied bacteria diversity of activated sludge in wastewater treatment plant by using 16s rDNA gene clone library. The results indicated the dominant bacteria community was Proteobacteria, Nitrosomonas-like and Nitrospira-like bacteria, which have played important roles in ammonia and nitrate oxidizers in the system.

Ding *et.al*., (2011) investigated dynamic of bacteria community in a full scale wastewater treatment plant with anoxic-oxic configuration using 16S rDNA PCR-DGGE analysis found four major related to dominant bacteria were α, β, γ-*Proteobacteria* and the phylum Fermicutes.

4. Molecular techniques

The characterization of microbial community composition in contaminated soil and water has been limited to the ability to culture microorganisms from environmental samples. The culture-dependent characterization of microorganisms at contaminated sites may limit the scope of microbial biodiversity and the ecological importance of unculturable organisms at contaminated sites may go undetected.

The molecular techniques provide an exciting opportunity to overcome the requirement for culturing and have therefore greatly increased our understanding of microbial diversity and functionality in the environment (Gilbride *et al*., 2006).

3.1 16S rDNA

The 16S rDNA is prescribe to 16S rRNA gene that is a component of 30s subunit of ribosomal of prokaryotic cell can be use for identify microorganism because of its highly conservative within species of the same genus, its have length

of approximately 1500 base pairs can be relatively to sequence. This techniques become a popular caused by the DNA sequence information of the 16S rDNA gene data found in the National Centre for Biotechnology Information (NCBI) (Spratt, 2004). This molecular techniques mostly use to diagnostic clinical bacteria. However can be use in the environmental study such as indentification of bacteria in organophosphates treated agricultural soil (Sultan *et.al*, 2012), identification of capable microorganism of degrading trichloroethylene (Mitra and Roy, 2010).

3.2 Polymerase Chain Reaction (PCR)

PCR is molecular technique to amplify a single or a few copies of a piece of DNA to millions of copies of a particular DNA sequence.

PCR amplification of DNA occurs by repeated cycles of three temperature dependent steps (Figure 3 and 4)

3.2.1 Denaturation: the dsDNA is denatured by heating, typically to 94°C, to separate the complementary single strands.

3.2.2 Annealing: the reaction is rapidly cooled to an annealing temperature to allow the oligonucleotide primers to hybridize to the template. The single strands of the template are too long and complex to be able to reanneal during this rapid cooling phase. During this annealing step the thermostable DNA polymerase will be active to some extent and will begin to extend the primers as soon as they anneal to the template. This can lead to specificity problems if the annealing temperature is too low.

3.2.3 DNA synthesis: the reaction is heated to a temperature, typically 72°C for efficient DNA synthesis by the thermostable DNA polymerase.

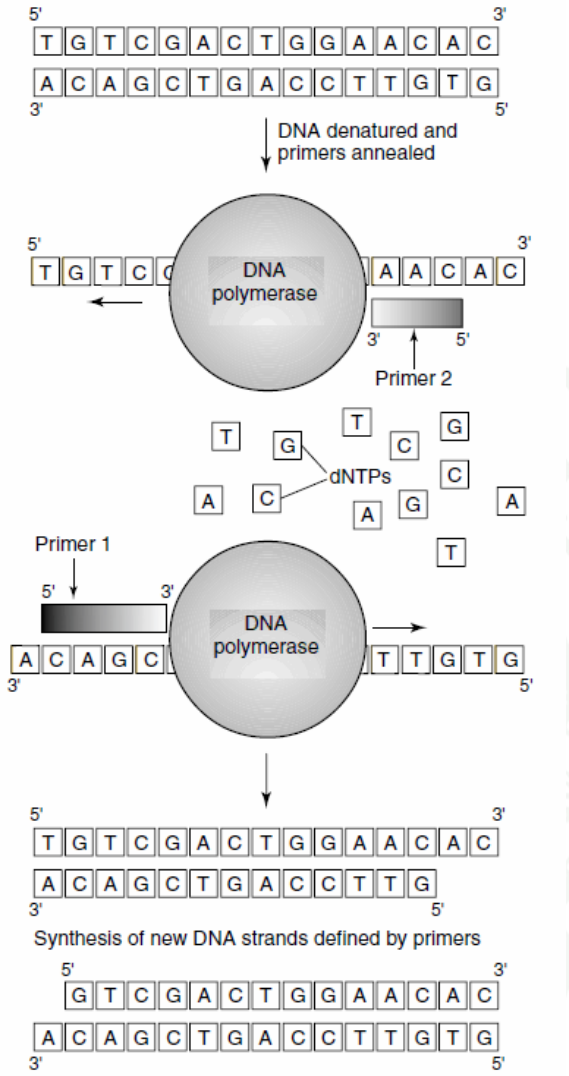

Figure 3 The first cycle of a PCR.

Source: Mcpherson and Møller (2006)

A double-stranded template molecule is denatured. Primers anneal to their complementary sequences on the single-stranded template. DNA synthesis is catalyzed by a thermostable DNA polymerase. The result of this PCR cycle is that two copies of the target sequence have been generated for each original copy.

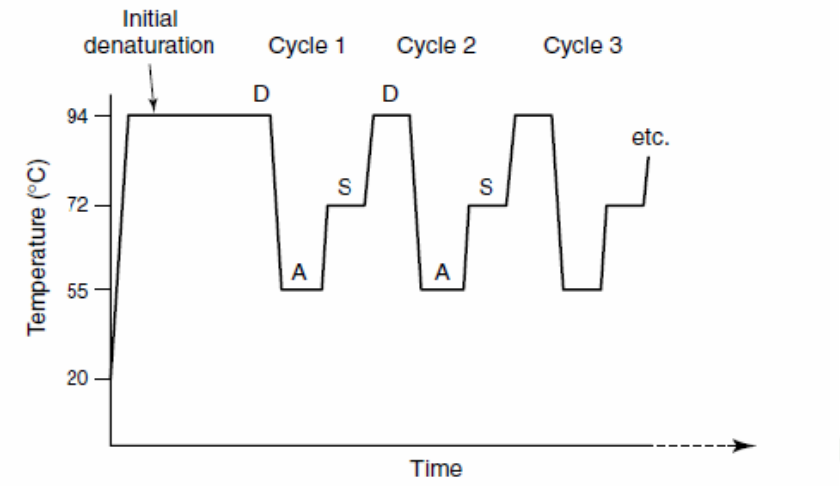

Figure 4 The thermal cycling during a PCR.

Source: Mcpherson and Møller (2006)

The reaction is heated from room temperature to an initial denaturation phase of around 5 min at 94°C to ensure the original template strands are now single-stranded. There then follows a series of repeated cycling steps through temperatures for denaturation of doublestranded molecules, annealing of primers to template and DNA synthesis from the primer.

DNA synthesized during the first cycle has the 5' end of the primers and a variable 3' end. When these strands are denatured, the parental strand will rehybridize to the primer, so the product with a variable 3' end will continue to be synthesized during subsequent cycles of PCR. (Only one copy of each of the products with a variable 3' end will accumulate with each cycle.) The second cycle of denaturation, annealing, and primer extension produces discrete products with the 5' end of one primer and the 3' end of the other primer. Each strand of this discrete product is complementary to one of the two primers and thus acts as a template in subsequent cycles. Therefore, these products with defined 5' and 3' ends accumulate exponentially with each round of DNA amplification that is, for every *n* cycles of PCR there will be 2^n fold amplification of the specific DNA fragment.

More recently, 16S rDNA and PCR amplification and the other techniques has been widely used to study environmental microbial diversity.

Verma *et. al*., (2012) studied about bacteria in Indian jaggery manufacturing units using the amplified 16S rDNA and BLAST analysis showed 98–99 % homology with pathogenic bacteria *Acinetobacter baumannii, Stenotrophomonas maltophilia* and *Ralstonia pickettii.*

Deshmukh *et al.*(2011) studied bacterial diversity of Lonar Soda lake of India by using 27F and 1488R primers found the majorities were belonged to phylum *Firmicutes* and *Proteobacteria* and the bacteria were potential producers of industrially important enzymes, pigments, antibiotics as well.

Zufang *et. al.*,(2011) studied microbial community diversity of plckeld mustard tuber at low salinity processing and the optimal of SSCP profiles. The results found the SSCP analysis under 22 % of polyacrylamide gel concentration with 6 % glycerol in gel at 20 ° C for 18 h at 250 V 11 distinct dominant bands were obtained throughout the fermentation process of mustard tuber. Based on the sequence comparison the results showed that *Leuconostoc mesenteroides* was the predominant microorganism in the initial stage of fermentation. Then the *Lactobacillus plantarum* and *Lactobacillus brevis* appeared quickly. At the later stage, the predominant species were *Lactobacillus plantarum* and *Lactobacillus versmoldensis*.

Zhao *et.al.,* (2010) investigated the performance of a sulfidogenic bioreactor and the response of bacterial populations to influent alkalinity in the bioreactor reached 40% of sulfate removal efficiency (SRE) with 0 mg/L of alkalinity, and SSCP profiles showed that some membersof *Bacteroides, Dysgonomonas, Sporobacter, Quinella,* and *Citrobacter* became dominant populations. 16S rDNA gene library analysis indicated that the Actinobacteria group increased from 0% in seed to 23% in sludge. An increase in alkalinity to 1300 mg/L led to a rapid increase of SRE to 65% and changes in the bacterial community. Sequences representing D*ysgonomonas, Raoultella, Kluyvera,* and *Phascolarctobacterium* were now found. When alkalinity

was deceased to 0 mg/L, SRE dropped and the bands representing *Raoultella, Kluyvera,* and *Phascolarctobacterium* disappeared, while bands representing *Clostridium* appeared. A second cycle of low/high alkalinity did not result in obvious changes to the bacterialcommunity. These results indicate that the sulfidogenic bioreactor favored higher influent alkalinity and that the different functional microbial populations responded well to the alkalinity changes.

Eltaief *et.al.,* (2009) investigated the effects of increasing wastewater loading rates (WLRs) on the performance of an up-flow anaerobic fixed bed bioreactor and on the dynamics of the bacterial community of the sludge using polymerase chain reaction–single stranded conformation polymorphism (PCR–SSCP) methods. The analysis showed that WLRs variations influence the bacterial community structure and affect the bioreactor performance. The use of molecular and microbiological methods to recover bacterial populations involved in this anaerobic process showed that fermentative (*Clostridium* spp.) and sulphate-reducing bacteria (SRB) (*Desulfovibrio* spp.) were the prominent members of the bioreactor bacterial community.

The studied of Abid *et.al,* (2007) in involvement of microbial populations during the composition of olive mill wastewater sludge composed in a bench scale reactors by using PCR SSCP method . During the period of high respiration rate (7-24 days), cultivation method showed that thermophilic bacteria as well as actinomycetes dominated over eumycetes. During the composting process, the PCR SSCP method showed a higher diversity of the bacterial community than the eukaryotic one. After 60 days of composting, the compost exhibited a microbial stability and a clear absence of phytotoxicity.

MATERIALS AND METHODS

Materials

1. Glass bottles
2. Centrifuge
3. Filter funnels
4. Filter membranes
5. Microcentifuge
6. Pipette
7. Vortex and vortex adapter
8. Flask
9. Burette and Burette Clamp
10. Incubation bottles
11. Air incubator, thermostatically controlled at 20 ±1°
12. BOD bottles
13. Oven (Operate at 150 ± 2°C)
14. Drying oven, for operation at 103 to 105°C.
15. Evaporating dishes
16. Analytical balance
17. Desiccator
18. Suction pump
19. Kjeldahl flasks total capacity of 800 mL
20. Digestion apparatus
21. Distillation apparatus
22. Apparatus for ammonia determination
23. Electrophoresis apparatus
24. Water bath
25. Spectrophotometer
26. Ice bath
27. Water bath
28. Computer

Methods

1. Wastewater Samples

The wastewater samples were taken form frozen seafood industry wastewater treatment plant in Samutsakhon province, Thailand.

For chemical properties analysis, samples were collected from: influent (A) , wastewater before entering UASB reactor(B), wastewater before entering aeration tank (C), wastewater before entering sedimentation tank (D) and effluent (E) (Fig 1.)

For DNA extraction samples were collected from: influent (sample 1), equalization tank (sample 2), UASB reactor (sample 3), aeration tank (sample 4), sedimentation tank (sample 5) and effluent (sample 6).Samples were frozen until DNA extraction.

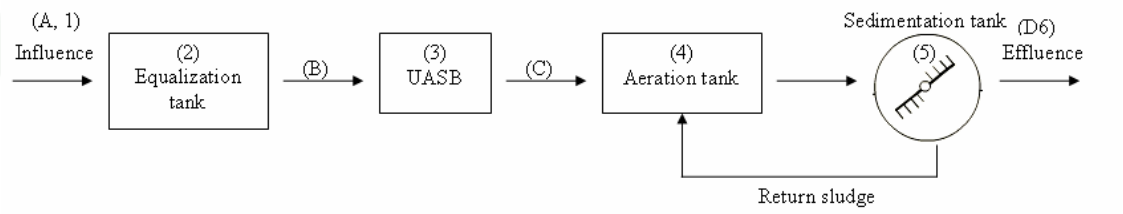

Figure 5 Wastewater samples selection: A, B, C, D& E were samples colleted for chemical properties analysis and 1, 2, 3, 4, 5 and 6 were samples colleted for DNA extraction.

2. Chemical Properties Analysis

The chemical parameters analysis of wastewater follow standard methods for the examination of water and wastewater 20th (ASHA *et.al.,* 1998) in each sample were pH by using pH meter, BOD belong to dilution methods, COD using close reflux titrimetric method, total phosphorus was ascorbic acid method**,** total Kjeldahl nitrogen using Kjeldahl nitrogen method , total suspended solids used of glass fiber filter disc, grease and oil using partition gravimetric method and DO was the Azide modification of the Winkler method.

3. DNA Extraction

All the DNA extraction experiments were performed in duplicate. DNA was extracted using NucleoSpin® Soil Genomic DNA extraction kit (Macherey-Nagel,Germany) according to the manufacturer's instructions

4. Primers and PCR amplification and Sequencing

16S rDNA fragments were amplified with primers 27F and 1488R which are specific for universally conserved bacterial 16S rDNA sequences. (Table 2),

Table 3 Primers sequences for PCR amplification.

Name	Sequence (5' → 3')	Size	Tm(°C)
27F	5' AGA GGT TGA TCA TGG CTC AG 3'	20	56.4
1488R	5' CGG TTA CCT TGT TAC GAC TTC ACC 3'	24	65.3

The PCR mixture contained 5 µL of 10x buffer, 2 µL of 2.5mMdNTPs, 1 µL of 20 pmol/ µL of each primers, 1 µL of 1 unit/ µL of Taq, 1 µL of DNA template and distilled water fill up to 50 µL of final volume.

The PCR amplification was carried out for initial denaturation at 94 °C for 5 min, 35 cycles of: denaturation at 94 °C for 1 min, annealing at 57.7 °C for 1 min and extention at 72 °C for 1 min. The final extention was 72 °C for 5 min.

The PCR products were run on a 1% agarose gel for 20 min at 100 V. The electrophoresis gels were stained to visualized with ethiduim bromine staining. Gel was photographed under UV light and recorded using gel documentation system (Gene Flash).

The products were purified for sequencing by the Polyethylene glycol (PEG) method (Rosenthal *et.al.*,1993) and confirmed with electrophoresis as mention above. The sequencing performed in the Macrogen. Ltd. (Seoul, Korea).The 16S rDNA sequences were analyzed using BLAST program (www.ncbi.nlm.nih.gov/blast/blast.cgi).

RESULTS AND DISCUSSION

Results

In this study, the experiments were set in the laboratory and were divided in to 2 parts which consist of chemical properties analysis and DNA extraction, PCR amplification and sequencing analysis.

1. Chemical Properties Analysis

The chemical properties from each sampling sites (Figure 5) represented in Table 3 which slightly increased in pH from 6.98 in the influent to 7.69 in the effluent. The COD was higher than the BOD were reduced from 3,200 mg/L to 880 mg/L and 1,485 mg/L to 318 mg/L, respectively. The total phosphorus, suspended solids, grease and oil were reduced in each site whereas total Kjeldahl nitrogen was increased from 70 mg/L to 250 mg/L when the wastewater pass through the UASB. The efficiency of BOD and COD removal for the UASB were 21.76 % and 28 % and the aeration tank were 70.95 % and 59.25 % (Table 4).

Table 4 The chemical properties from each sampling sites.

Parameters	Concentration (mg/L)			
	A	B	C	D
pH	6.98	6.67	7.57	7.69
BOD	1,485	1,400	1,095	318
COD	3,200	3,000	2,160	880
Total phosphorus	452	459	456	51
Total Kjeldahl Nitrogen	175	70	250	1
Suspended solids	522	360	260	51
Grease and oil	250	80	75	55

Table 5 The BOD and COD removal efficiency of the UASB and the aeration tank.

Parameters	UASB tank			Aeration tank		
	B	C	Efficiency (%)	C	D	Efficiency (%)
BOD (mg/L)	1,400	1,095	21.76	1,095	318	70.95
COD (mg/L)	3,000	2,160	28	2,160	880	59.25

The DO was analyzed of aeration tank and effluent found the concentration increased from 0.84 mg/L to 2.87 mg/L (Table 5).

Table 6 The DO of the aeration tank and the effluent.

Samples number	Sites	DO (mg/L)
4	Aeration tank	0.84
6	Effluent	2.87

2. DNA extraction, PCR amplification and Sequencing Analysis

The DNA was extracted form each samples were shown in Figure 6 that distinguishable between lane 4 and the other lanes however there was no major bands occurrence for all samples.

The PCR amplification with 27F and 1488R primers indicated 1 major band which approximately 1500 bp in length of 16s rDNA for each samples were amplified and fragment bands were confirmed by gel electrophoresis after PCR (Figure 7).

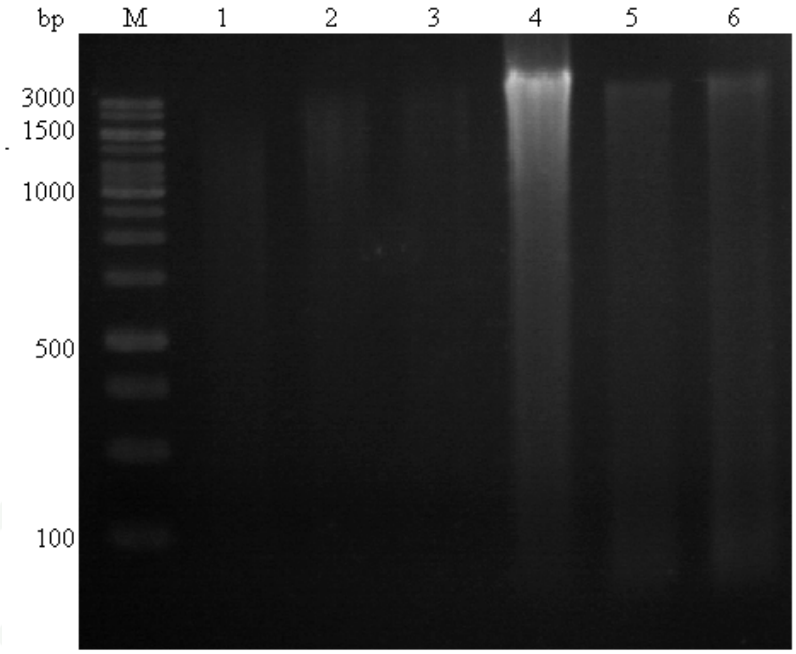

Figure 6 Gel electrophoresis of DNA extraction in 1% agarose. Lane M , 100 bp plus DNA ladder size maker; 1, influent; 2, equalization tank; 3, UASB reactor; 4, aeration tank ; 5,sedimentation tank; 6,effluent.

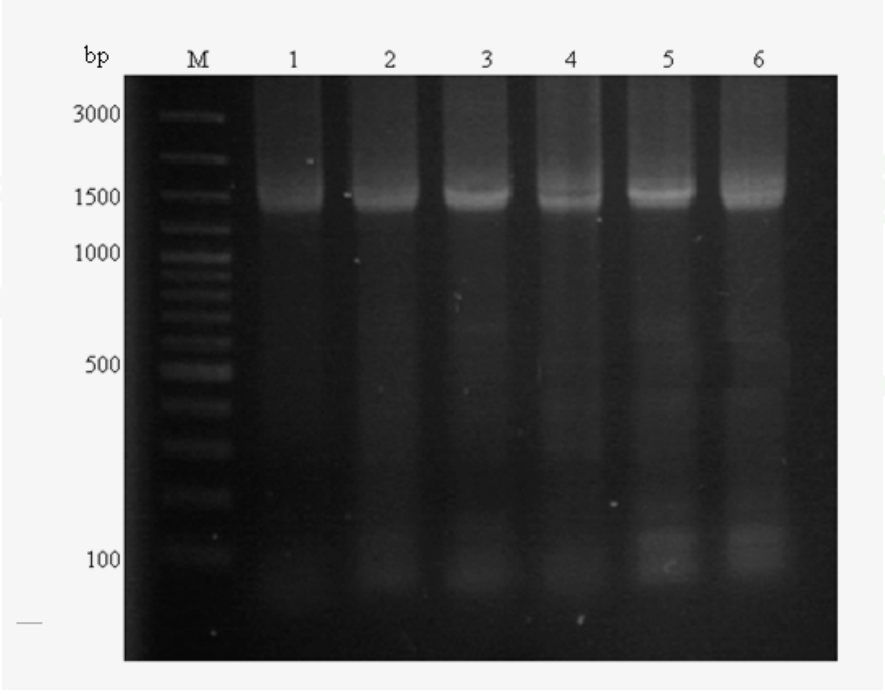

Figure 7 Gel electrophoresis of PCR amplification for 16s rDNA in 1% agarose. Lane M , 100 bp plus DNA ladder size maker; 1, influent; 2, equalization tank; 3, UASB reactor; 4, aeration tank ; 5,sedimentation tank; 6,effluent.

The 1% of gel electrophoresis was confirmed after PEG purification method which thickly and clearly major bands when compared which the result from PCR amplification (Figure 8).

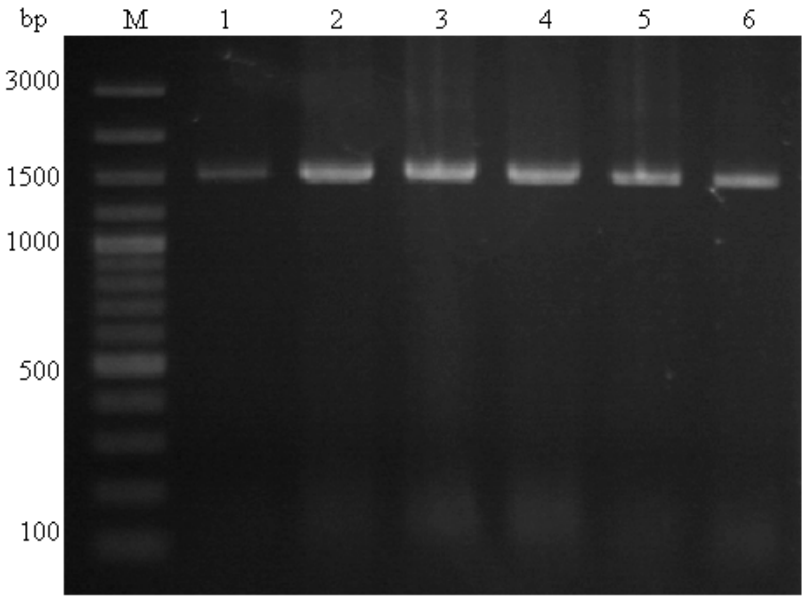

Figure 8 Gel electrophoresis of PEG method for 16s rDNA in 1% agarose. Lane M , 100 bp plus DNA ladder size maker; 1, influent; 2,equalization tank;3, UASB reactor; 4, aeration tank ; 5,sedimentation tank; 6,effluent.

The sequence chromatogram of aeration tank represented in Figure 9 with the first 23 bases give the round, crowded peak. The height was smaller at the beginning. At 25 to 260 bp, peaks were sharp, well defined, with even spacing between them. Peak height was higher than the earlier data with little or no background interference at the baselines.

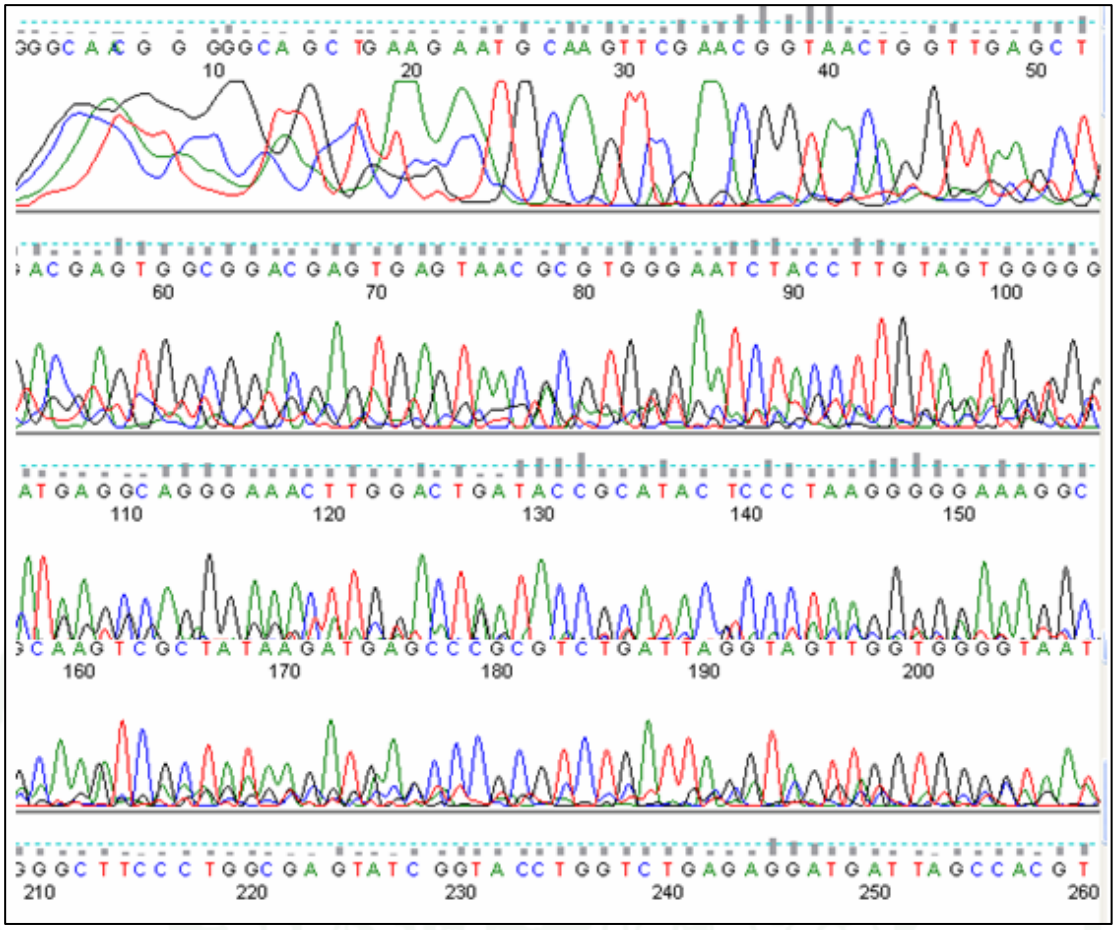

Figure 9 The sequences chromatogram of the aeration tank.

The Sequencing analysis of 16s rDNA and BLAST sequence revealed of identities of samples to several group of bacteria were presented in Table 6. The results consideration on the E value which represent the probability of the alignment occurring by chance and max identity (%) found majorities of bacteria in the influent was *Streptococcus sp.*, and *Bacillus sp* . For the equalization tank was *Bacillus sp.* and uncultured organism while the UASB was *Serratia marcescens*, *Enterobacter sp.*, *Klebsiella pneumoniae* and *Escherichia coli*. For the aeration tank, *Thiothrix sp.* and uncultured actinobacterium were found. For the sedimentation tank was *Escherichia coli*, *Shigella sp.*and *Salmonella typhimurium* while the effluent was found *Pseudomonas putida, Providencia sp., Aeromonas veronii* and *Alcaligenes faecalis*

Table 7 The closest relatives of microorganisms in each sample.

Samples Number	Sites	Closest relative	Similarity (%)	Accession number	Classifier	Phylum
1	Influent	*Streptococcus sp.*	77	NR036758	*Lactobacillales*	*Firmicutes*
		Bacillus sp.	77	FJ435217	*Bacillales*	*Firmicutes*
2	Equalization tank	Uncultured organism	81	JN485691		
		Bacillus sp.	78	AB301017	*Bacillales*	*Firmicutes*
3	UASB tank	*Serratia marcescens*	92	DQ182325	*Gammaproteobacteria*	*Proteobacteria*
		Enterobacter sp.	91	EF428999	*Gammaproteobacteria*	*Proteobacteria*
		Klebsiella pneumoniae	91	DQ379507	*Gammaproteobacteria*	*Proteobacteria*
		Escherichia coli	88	FJ997270	*Gammaproteobacteria*	*Proteobacteria*
4	Aeration tank	*Thiothrix sp.*	94	AB042537	*Gammaproteobacteria*	*Proteobacteria*
		Uncultured actinobacterium	77	EU283363		*Actinobacteria*
5	Sedimentation tank	*Escherichia coli*	80	GU594312	*Gammaproteobacteria*	*Proteobacteria*
		Shigella sp.	79	JQ315922	*Gammaproteobacteria*	*Proteobacteria*
		Salmonella typhimurium	79	U90316	*Gammaproteobacteria*	*Proteobacteria*

Table 7 (Continued)

Samples number	Sites	Closest relative	Similarity (%)	Accession number	Classifier	Phylum
6	Effluent	*Pseudomonas putida*	94	JN941324	*Gammaproteobacteria*	*Proteobacteria*
		Providencia sp.	93	GQ368699	*Gammaproteobacteria*	*Proteobacteria*
		Aeromonas veronii	93	AM937464	*Gammaproteobacteria*	*Proteobacteria*
		Alcaligenes faecalis	92	AY823619	*Betaproteobacteria*	*Proteobacteria*

Discussion

The analysis of chemical properties in influent (Table 3) indicated BOD, COD, SS, Grease and oil were 1485 mg/L, 3,200 mg/L, 522 mg/L and 250 mg/L, respectively this results agreed with Islam *et al*.(2004) who reported the characteristic of raw wastewater from preserved seafood processing industries to have BOD of 100-24,000 mg/L, COD of 150- 42,000 mg/L, TSS of 70- 20,000 mg/L, and FOG of 20-5,000 mg/L depending on the types of seafood processing (Islam *et al*.2004). The influent pH was 6.98 agreed with Wang *et al*.(2004) and Chawdhury *et al.*(2010) who reported the pH of food processing wastewater usually close to neutral.

The resulted total phosphorus (Table 3) almost the same concentration when the wastewater pass through the UASB, this explanation agreed with Aslan and Sekerdag (2008) who studied the performance of UASB reactors treating high-strength wastewater found the TKN and total phosphorus removal rates were quite low because of anaerobic treatment does not remove phosphate, ammonia, and sulfide.

In this study was successful to amplified the partial of 16s rDNA using 27F and 1488R primers which sequences revealed a range of identities to several bacteria groups. This primers set were amplified approximately 1,500 bp this supported by the reported of Deshmukh *et al.*(2011) who studied bacterial diversity of Lonar Soda lake of India by using 27F and 1488R primers found the majorities were belonged to phylum *Firmicutes* and *Proteobacteria* .

According to the bacteria the results of majority of microorganisms (Table 6) for all of samples were belonged to phylum *Firmicutes* and *Proteobacteria*. The bacteria found in the influent and the equalization tank belonged to phylum *Firmicutes* was *Streptococcus sp.* and *Bacillus sp.* The results accordant by Ahmed (2012) in the review of microbial communities in nutrient- removing membrane bioreactor which suggested in the denitrification process using methanol as carbon source was considered to carry out by facultative hetetrophic bacteria including *Streptococcus sp.* and *Bacillus sp.* in the absence of oxygen that were consistently

with the decreased of TKN from 175 mg/L to 70 mg/L when the wastewater pass though the equalization tank. However TKN in the effluent from UASB was 250 mg/L that higher than the influent (170 mg/L) because of the collecting points for effluent from UASB was closed system so the samples from this sampling site collected from influent pipe of aeration tank (Figure 6) therefore the samples contained high amount of sediment which high organic nitrogen and ammonia loading.

The bacteria belonged to phylum *Proteobacteria* represented in UASB tank (Table 6) was *Serratia marcescens*, *Enterobacter sp.*, *Klebsiella pneumoniae* and *Escherichia coli*. This results agreed with Gerardi (2003); Mara and Horan (2003) explained the degradation of wastes with in UASB tank , facultative anaerobic bacteria normally living in soil and water were capable of fermentation (species of *Aeromonas*, *Citrobacter*, *Klebsiella*, *Pasteurella*, *Proteus, Enterobacter*, *Escherichia* and *Serratia*). They accumulate and produce organic compounds such as lactic acid, succinic acid, propionic acid, butyric acid, acetic acid and ethanol during fermentation. Therefore it seems as though the anaerobic condition provides substances for the proliferation of aerobic phosphate-accumulating bacteria, which consistently with the results of total phosphorus (Table 3). However TKN in the effluent from UASB was 250 mg/L that higher than the influent (170 mg/L) because of the collecting points for effluent from UASB was closed system so the samples from this sampling site collected from influent pipe of aeration tank (Figure 6) therefore the samples contained high amount of sediment which high organic nitrogen and ammonia loading which related to BOD and COD removal found in UASB were 21.76% and 28%, respectively that less than 85% - 95% of expected design criteria (Tchobanoglous *et.al.,* 2004).

The samples from aeration tank showed the majority of *Thiothrix* sp. and uncultured actinobacterium (Table 6). The *Thiothrix* sp. was belonged to filament sulfur bacteria use readily biodegradation substrate present at moderate to high sludge retention time (SRT). The *Actinobacteria* had participation in processes leading to the biological removal of phosphate (Seviour *et.al*., 2008) corresponded to total phosphorus removal in aeration tank from 456 mg/L to 51 mg/L. However the

dominate or excessive growth of *Thiothrix sp.* and *Actinobacteria* caused activated sludge bulking that common found in activated sludge process growth well at low DO concentration relative to DO was 0.84 mg/L found in the aeration tank represented in Table 5 (Vaiopoulou *et.al.,* 2007; Seviour *et.al*., 2008). When the sludge bulking develop, it causes poor settle down of sludge that results in low of effluent quality get the efficiency of aeration tank to BOD and COD removal were 70.75% and 59.25%, respectively that less than the unit design to 80%-85% removal (Tchobanoglous *et.al.,* 2004) increased in costs of operation and loss of active biomass. The dominant of *Thiothrix sp.* in this aeration tank helpful to understand, predict condition of the unit and simplify to solve a bulking problem for the purpose of enhance effective of the unit.

The majority of *Escherichia coli*, *Shigella sp.* and *Salmonella typhimurium* found in the sedimentation tank and *Pseudomonas putida, Providencia sp., Aeromonas veronii* and *Alcaligenes faecalis* in the effluent were the pathogenic bacteria common found in wastewater usually resistant to hostile environments (Gerardi and Zimmerman, 2005). The disinfection processes absolutely necessary for eliminated pathogenic organisms in wastewater treatment plant before discharge to the environment.

The majority of bacteria were different in each of units. In the influent and the equalization tank were aerobic bacteria belonged to phylum Fermicutes when the aeration tank and the effluent were aerobic bacteria belonged to phylum Proteobacteria while the UASB tank and the sedimentation tank were facultative anaerobic Proteobacteria, this changing due to the conditions suitable for life especially environmental factors (source of energy, source of carbon, temperature, pH, etc.), therefore the majority of microorganisms in each of units play the major role in biodegradable of different organic compounds this related to the chemical properties analysis in the study (Ivanov,2011).

CONCLUSION AND RECOMMENDATION

Conclusion

From the experimental results and discussion of this study, the conclusion can be drawn as the majorities of bacteria belonged to Fermicutes and Proteobacteria phylum. The influent was found *Streptococcus sp.*and *Bacillus sp*. For the equalization tank was *Bacillus sp.* and uncultured organisms while the UASB tank was *Serratia marcescens*, *Enterobacter sp.*, *Klebsiella pneumoniae* and *Escherichia coli*. For the aeration tank, *Thiothrix sp.* and uncultured actinobacterium were found. For the sedimentation tank was *Escherichia coli*, *Shigella sp.*and *Salmonella typhimurium* while the effluent was found *Pseudomonas putida, Providencia sp., Aeromonas veronii* and *Alcaligenes faecalis*

The majority of bacteria were different in each of units. In the influent and the equalization tank were aerobic bacteria belonged to phylum Fermicutes when the aeration tank and the effluent were aerobic bacteria in phylum Proteobacteria while the UASB tank and the sedimentation tank were facultative anaerobic Proteobacteria, this changing related to the chemical properties analysis in each of units.

Recommendation

They are many molecular techniques using for bacteria identification. The different methods reflect different aspects of bacterial diversity or community structure. All of molecular techniques methods suffer from various limitations. No single method reflects the true diversity or species composition. The literature review helpful to select the proper methods for the sample in the experiment. In many case the PCR products were not well identity but when sent for sequencing they related mix population of bacteria. However to overcome the accuracy of mix population, the PCR and clone into cloning vector become more advantage.

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APPENDICES

Appendix A

Chemical Properties Analysis

1. DO; Dissolved Oxygen Analysis

The dissolved oxygen (DO) levels in natural and wastewaters depend on the physical, chemical, and biochemical activities in the water body. The analysis for DO is a key test in water pollution and waste treatment process control. The analysis in this studied was the Azide modification of Winkler method.

Reagents

1.1.1 Manganous sulfate solution: Dissolve 480 g $MnSO_4 \times 4H_2O$, 400 g $MnSO_4 \times 2H_2O$, or 364 g $MnSO_4 \times H_2O$ in distilled water, filter, and dilute to 1 L. The $MnSO_4$ solution should not give a color with starch when added to an acidified potassium iodide (KI) solution.

1.1.2 Alkali-iodide-azide reagent: Dissolve 500 g NaOH (or 700 g KOH) and 135 g NaI (or 150 g KI) in distilled water and dilute to 1 L. Add 10 g NaN_3 dissolved in 40 mL distilled water. Potassium and sodium salts may be used interchangeably. This reagent should not give a color with starch solution when diluted and acidified.

1.1.3 Sulfuric acid, H_2SO_4, concentration : 1 mL is equivalent to about 3 mL alkali-iodide-azide reagent.

1.1.4 Starch: Use either an aqueous solution or soluble starch powder mixtures. To prepare an aqueous solution, dissolve 2 g laboratory-grade soluble starch and 0.2 g salicylic acid, as a preservative, in 100 mL hot distilled water.

1.1.5 Standard sodium thiosulfate titrant: Dissolve 6.205 g of $Na_2S_2O_3 \cdot 5H_2O$ in distilled water. Add 1.5 mL 6N NaOH or 0.4 g solid NaOH and dilute to 1000 mL. Standardize with bi-iodate solution.

1.1.6 Standard potassium bi-iodate solution, 0.0021M: Dissolve 812.4 mg $KH(IO_3)_2$ in distilled water and dilute to 1000 mL.

Procedure

To the sample collected in a 250 to 300 mL bottle, add 1 mL $MnSO_4$ solution, followed by 1 mL alkali-iodide-azide reagent. When precipitate has settled sufficiently (to approximately half the bottle volume) to leave clear supernate above the manganese hydroxide floc, add 1.0 mL conc H_2SO_4. Restopper and mix by inverting several times until dissolution is complete. Titrate a volume corresponding to 200 mL original sample after correction for sample loss by displacement with reagents. Thus, for a total of 2 mL (1 mL each) of $MnSO_4$ and alkali-iodide-azide reagents in a 300 mL bottle, titrate $200 \times 300/(300 - 2) = 201$ mL

1.2.2 Titrate with 0.025M $Na_2S_2O_3$ solution to a pale straw color. Add a few drops of starch solution and continue titration to first disappearance of blue color. If end point is overrun, back-titrate with 0.0021M bi-iodate solution added dropwise, or by adding a measured volume of treated sample. Correct for amount of bi-iodate solution or sample. Disregard subsequent recolorations due to the catalytic effect of nitrite or to traces of ferric salts that have not been complexed with fluoride.

Calculation

For titration of 200 mL sample, 1 mL 0.025M $Na_2S_2O_3$ = 1 mg DO/L.

2. BOD; Biological Oxygen Demand Analysis

The method consists of filling with sample, to overflowing, an airtight bottle of the specified size and incubating it at the specified temperature for 5 day. DO is measured initially and after incubation, and the BOD is computed from the difference between initial and final DO. Because the initial DO is determined shortly after the

dilution is made, all oxygen uptake occurring after this measurement is included in the BOD measurement.

Reagents

Phosphate buffer solution: Dissolve 8.5 g KH_2PO_4, 21.75 g K_2HPO_4, 33.4 g $Na_2HPO_4 \cdot 7H_2O$, and 1.7 g NH_4Cl in about 500 mL distilled water and dilute to 1 L The pH should be 7.2 without further adjustment.

Magnesium sulfate solution: Dissolve 22.5 g $MgSO4 \times 7H2O$ in distilled water and dilute to 1 L

Calcium chloride solution: Dissolve 27.5 g $CaCl_2$ in distilled water and dilute to 1 L.

Ferric chloride solution: Dissolve 0.25 g $FeCl3 \cdot 6H2O$ in distilled water and dilute to 1 L.

Acid and alkali solutions, 1N, for neutralization of caustic or acidic waste samples. For acid, slowly and while stirring, add 28 mL concentration of sulfuric acid to distilled water. Dilute to 1 L or alkali, dissolved 40 g sodium hydroxide in distilled water and dilute to 1 L.

Sodium sulfite solution: Dissolve 1.575 g $Na2SO3$ in 1000 mL distilled water. This solution is not stable; prepare daily.

Glucose-glutamic acid solution: Dry reagent-grade glucose and reagent-grade glutamic acid at 103°C for 1 h. Add 150 mg glucose and 150 mg glutamic acid to distilled water and dilute to 1 L. Prepare fresh immediately before use.

Ammonium chloride solution: Dissolve 1.15 g NH_4Cl in about 500

mL distilled water, adjust pH to 7.2 with NaOH solution, and dilute to 1 L. Solution contains 0.3 mg N/mL.

Dilution water

Procedure

Preparation of dilution water: Place desired volume of water in a suitable bottle and add 1 mL each of phosphate buffer, $MgSO_4$, $CaCl_2$, and $FeCl_3$ solutions/L of water.

Determination of initial DO: If the sample contains materials that react rapidly with DO, determine initial DO immediately after filling BOD bottle with diluted sample. If rapid initial DO uptake is insignificant, the time period between preparing dilution and measuring initial DO is not critical but should not exceed 30 min.

Incubation: Incubate at 20°C ± 1°C BOD bottles containing desired dilutions.

Determination of final DO: After 5 d incubation determine DO in sample dilutions.

Calculation

$$BOD\ mg/L = (D_1 - D_2) \times P$$

Where:

D_1 = DO of diluted sample immediately after preparation, mg/L,

D_2 = DO of diluted sample after 5 d incubation at 20°C, mg/L,

P = Dilution factor of sample used

3. COD; Chemical Oxygen Demand Analysis

Chemical oxygen demand (COD) is defined as the amount of a specified oxidant that reacts with the sample under controlled conditions. The quantity of oxidant consumed is expressed in terms of its oxygen equivalence. For this studied the COD analysis was closed reflux titrimetric method.

Reagents

Standard potassium dichromate digestion solution, 0.01667M: Add to about 500 mL distilled water 4.903 g $K_2Cr_2O_7$, primary standard grade, previously dried at 150°C for 2 h, 167 mL conc H_2SO_4, and 33.3 g $HgSO_4$. Dissolve, cool to room temperature, and dilute to 1000 mL.

Sulfuric acid reagent: Add Ag_2SO_4, reagent or technical grade, crystals or powder, to conc H_2SO_4 at the rate of 5.5 g Ag_2SO_4/kg H_2SO_4. Mix and lLet stand 1 to 2 day to dissolve.

Ferroin indicator solution: Dissolve 1.485 g 1,10-phenanthroline monohydrate and 695 mg $FeSO_4 \cdot 7H_2O$ in distilled water and dilute to 100 mL. Dilute this reagent by a factor of 5.

Standard ferrous ammonium sulfate titrant (FAS), approximately 0.10M: Dissolve 39.2 g $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ in distilled water. Add 20 mL conc H_2SO_4, cool, and dilute to 1000 mL. Standardize solution daily against standard $K_2Cr_2O_7$ digestion solution as follows:

Pipet 5.00 mL digestion solution into a small beaker. Add 10 mL reagent water to substitute for sample. Cool to room temperature. Add 1 to 2 drops diluted ferroin indicator and titrate with FAS titrant.

$$\text{Molarity of FAS solution} = \frac{\text{Volume 0.01667M } K_2Cr_2O_7 \text{ solution in titration, mLx 0.1}}{\text{Volume FAS used in titration in titration, mL}}$$

Potassium hydrogen phthalate (KHP) standard: Lightly crush and then dry KHP to constant weight at 110°C. Dissolve 425 mg in distilled water and dilute to 1000 mL.

Procedure

Wash culture tubes and caps with 20% H_2SO_4 before first use to prevent contamination.

Place sample in tube and add digestion solution. Tightly cap tubes and invert each several times to mix completely.

Preheated to 150°C and reflux for 2 h behind a protective shield.

Titration: Add 0.05 to 0.10 mL (1 to 2 drops) ferroin indicator and stir rapidly on magnetic stirrer while titrating with standardized 0.10M FAS. The end point is a sharp color change from blue-green to reddish brown, although the blue-green may reappear within minutes. In the same manner reflux and titrate a blank containing the reagents and a volume of distilled water equal to that of the sample.

Calculation

$$\text{COD as mg } O_2/L = \frac{(A-B) \times M \times 8000}{\text{mL Sample}}$$

Where:

A = mL FAS used for blank,

B = mL FAS used for sample,

M = molarity of FAS, and

8000 = milliequivalent weight of oxygen × 1000 mL/L.

4. TSS; Total Suspended Solid Analysis

Solids refer to matter suspended or dissolved in water or wastewater. Solids may affect water or effluent quality. In this studied was total solids dried at 103–105°C method

4.1 Procedure

4.1.1 Preparation of evaporating dish: If volatile solids are to be measured ignite clean evaporating dish at 550°C for 1 h in a muffle furnace. If only total solids are to be measured, heat clean dish to 103 to 105°C for 1 h. Store and cool dish in desiccator until needed. Weigh immediately before use.

4.1.2 Sample analysis: Choose a sample volume between 2.5 and 200 mg. and well-mixed sample, during mixing, to a pre weighed dish. Evaporate to dryness on a steam bath or in a drying oven. When evaporating in a drying oven, lower temperature to approximately 2°C below boiling to prevent splattering. Dry evaporated sample for at least 1 h in an oven at 103 to 105°C, cool dish in desiccator to balance temperature, and weigh. Repeat cycle of drying, cooling, desiccating, and weighing until a constant weight is obtained, or until weight change is less than 4% of previous weight or 0.5 mg, whichever is less. When weighing dried sample, be alert to change in weight due to air exposure and/or sample degradation. Analyze at least 10% of all samples in duplicate. Duplicate determinations should agree within 5% of their average weight.

4.2 Calculation

$$\text{mg total solids/L} = \frac{(A-B) \times 1000}{\text{Sample volume, mL}}$$

Where:

A = weight of dried residue + dish, mg, and
B = weight of dish, mg.

5. TKN; Total Nitrogen Analysis

The Kjeldahl methods determine sum of organic nitrogen and ammonia nitrogen. In the presence of H_2SO_4, potassium sulfate (K_2SO_4), and cupric sulfate ($CuSO_4$) catalyst, amino nitrogen of many organic materials is converted to ammonium. Free ammonia also is converted to ammonium. After addition of base, the ammonia is distilled from an alkaline medium and absorbed in boric or sulfuric acid. The ammonia may be determined colorimetrically, by ammonia-selective electrode, or by titration with a standard mineral acid.

5.1 Reagents

5.1.1 Digestion reagent: Dissolve 134 g K_2SO_4 and 7.3 g $CuSO_4$ in 800 mL water. Carefully add 134 mL conc H_2SO_4. When it has cooled to room temperature, dilute the solution to 1 L with water. Mix well. Keep at a temperature close to 20°C to prevent crystallization

5.1.2 Sodium hydroxide-sodium thiosulfate reagent: Dissolve 500 g NaOH and 25 g $Na_2S_2O_3 \cdot 5H_2O$ in water and dilute to 1 L.

5.1.3 Borate buffer solution

5.1.4 Sodium hydroxide, NaOH, 6N.

5.2 Procedure

5.2.1 Selection of sample volume and sample preparation: Place a measured volume of sample in 800mL Kjeldahl flask.

5.2.2 Ammonia removal: Add 25 mL borate buffer and then 6N NaOH until pH 9.5 Add a few glass beads and boil off 300 mL. If desired, distill this fraction and determine ammonia nitrogen.

5.2.3 Digestion: Add 50 mL of digestion reagent in Kjeldahl flask Boil until the volume is reduced to 25 mL and white fumes are observed Then continue to digest for an additional 30 min. As digestion continues, colored or turbid samples will become transparent and pale green. After digestion, let cool, dilute to 300 mL with water, and mix. Add 50 mL sodium hydroxide-thiosulfate reagent to form an alkaline layer at flask bottom. Connect flask to a steamed-out distillation apparatus and swirl flask to insure complete mixing.

5.2.4 Distillation: Distill and collect 200 mL distillate. Use 50 mL indicating boric acid as absorbent solution when ammonia is to be determined by titration. Use 50 mL 0.04N H_2SO_4 solution as absorbent for manual phenate or electrode methods.

5.2.5 Final ammonia measurement: Use the titration with standard 0.02N H_2SO_4 until indicator turn pale lavender.

5.3 Calculation

$$\text{mg } NH_3\text{-N/L} = \frac{(A-B) \times 280}{\text{mL of Sample}}$$

Where:

A = Volume of H_2SO_4 titrated for sample, mL, and

B = Volume of H_2SO_4 titrated for blank , mL.

6. Total Phosphorus Analysis

The total phosphorus analysis in this studied was ascorbic acid method. In this method ammonium molybdate and potassium antimonyl tartrate react in acid medium with orthophosphate to form a heteropoly acid that is reduced to intensely colored molybdenum blue by ascorbic acid.

6.1 Reagents

6.1.1 Potassium antimonyl tartrate solution: Dissolve 1.3715 g $K(SbO)C_4H_4O_6 \cdot 1/2H_2O$ in 400 mL distilled water in a 500 mL volumetric flask and dilute to volume.

6.1.2 Sulfuric acid, H_2SO_4, 5N: Dilute 70 mL conc H_2SO_4 to 500 mL with distilled water.

6.1.3 Ammonium molybdate solution: Dissolve 20 g $(NH_4)6Mo_7O_{24}$ $4H_2O$ in 500 mL distilled water.

6.1.4 Ascorbic acid, 0.1M: Dissolve 1.76 g ascorbic acid in 100 mL distilled water. The solution is stable for about 1 week at 4°C.

6.1.5 Combined reagent: Mix the above reagents in the following proportions for 100 mL of the combined reagent: 50 mL 5*N* H2SO4, 5 mL potassium antimonyl tartrate solution, 15 mL ammonium molybdate solution, and 30 mL ascorbic acid solution. Mix after addition of each reagent. If turbidity forms in the

combined reagent, shake and let stand for a few minutes until turbidity disappears before proceeding. The reagent is stable for 4 h.

6.1.6 Stock phosphate solution

6.1.7 Standard phosphate solution: Dilute 50.0 mL stock phosphate solution to 1000 mL with distilled water; 1.00 mL = 2.50 μg P.

6.2 Procedure

6.2.1 Treatment of sample: Pipet 50.0 mL sample into a clean, dry test tube or 125 mL flask. Add 0.05 mL (1 drop) phenolphthalein indicator. If a red color develops add 5N H_2SO_4 solution dropwise to just discharge the color. Add 8.0 mL combined reagent and mix thoroughly. After at least 10 min but no more than 30 min, measure absorbance of each sample at 880 nm, using reagent blank as the reference solution.

6.2.2 Correction for turbidity or interfering color: Natural color of water generally does not interfere at the high wavelength used. For highly colored or turbid waters, prepare a blank by adding all reagents except ascorbic acid and potassium antimonyl tartrate to the sample. Subtract blank absorbance from absorbance of each sample.

6.2.3 Preparation of calibration curve: Prepare individual calibration curves from a series of 6 standards within the phosphate ranges Use a distilled water blank with the combined reagent to make photometric readings for the calibration curve. Plot absorbance versus phosphate concentration to give a straight line passing through the origin. Test at least one phosphate standard with each set of samples.

6.3 Calculation

$$\text{Mg P/L} = \frac{\text{mg P (in approximately 58 mL final volume)} \times 1000}{\text{mL of Sample}}$$

7. Grease and Oil Analysis

The partition gravimetric method for grease and oil analysis was using in this experiment.

7.1 Reagents

7.1.1 Hydrochloric or sulfuric acid, 1:1: Mix equal volumes of either acid and reagent water.

7.1.2 n-Hexane, boiling point 69°C. The solvent should leave no measurable residue on evaporation.

7.1.3 Methyl-tert-butyl ether (MTBE), boiling point 55°C to 56°C. The solvent should leave no measurable residue on evaporation.

7.1.4 Sodium sulfate, Na_2SO_4, anhydrous crystal.

7.1.5 Solvent mixture, 80% n-hexane/20% MTBE, v/v.

7.2 Procedure

7.2.1 Determination of sample volume and adjust pH lower than 2.

7.2.2 Transfer sample to a separatory funnel. Carefully rinse sample

bottle with 30 mL extracting solvent and add solvent washings to separatory funnel. Shake vigorously for 2 min. Let layers separate. Drain aqueous layer and small amount of organic layer into original sample container. Drain solvent layer through a funnel containing a filter paper and 10 g Na2SO4, both of which have been solvent rinsed, into a clean. tared distilling flask and the gain in weight of the tared distilling flask is due to oil and grease. Total gain in weight, A.

7.2.3 Repeat step if emulsion persists.

7.2.4 When visible solvent condensation stops, remove flask from water bath. Cover water bath and dry flasks on top of cover, with water bath still at 85°C, for 15 min. Draw air through flask with an applied vacuum for the final 1 min. Cool in desiccator for at least 30 min and weigh (less calculated residue from solvent blank, B).

7.3 Calculation

$$\text{mg grease and oil/L} = \frac{(A-B) \times 1000}{\text{mL of Sample}}$$

Where:

A = Total gain in weight, and

B = Less calculated residue from solvent blank.

Appendix B

Molecular Techniques Procedure

1. DNA Extraction

DNA was extracted using NucleoSpin® Soil Genomic DNA extraction kit (Macherey-Nagel,Germany) according to the manufacturer's instructions following:

Prepare sample: Transfer 250–500 mg fresh sample material to a bead tube containing the ceramic beads. Add 700 µL Buffer SL1 or Buffer SL2.

Adjust lysis conditions: Add 150 µL Enhancer SX and close the cap.

Sample lysis: Vortex the samples at full speed at room tempera ion (18 – 25 °C) for 5 min.

Precipitate contaminants: Centrifuge for 2 min at 11,000 x *g* to eliminate the foam caused by the detergent. Add 150 µL Buffer SL3 and vortex for 5 s. Incubate for 5 min at 0– 4 °C. Centrifuge for 1 min at 11,000 x *g*.

Filter lysate: Place a NucleoSpin® Inhibitor Removal Column (red ring) in a Collection Tube (2 mL, lid). Load up to 700 µL clear supernatant of step 4 onto the filter. Centrifuge for 1 min at 11,000 x *g*.

Adjust binding conditions Add 250 µL Buffer SB and close the lid. Vortex for 5 s.

Bind DNA Place a NucleoSpin® Soil Column (green ring) in Collection Tube (2 mL). Load 550 µL sample onto the column. Centrifuge for 1 min at 11,000 x *g*. Discard flow-through and place the column back into the collection tube. Load the remaining sample onto the column. Centrifuge for 1 min at 11,000 x *g*. Discard flow-through and place the column back into the collection tube.

Wash and dry silica membrane

Add 500 µL Buffer SB to the NucleoSpin® Soil Column.
Centrifuge for 30 s at 11,000 x *g*. Discard flow-through and place the column back into the collection tube.

Add 500 µL Buffer SW1 to the NucleoSpin® Soil Column.
Centrifuge for 30 s at 11,000 x *g*. Discard flow-through and place the column back into the collection tube.

Add 700 µL Buffer SW2 to the NucleoSpin® Soil Column.
Close the lid and vortex for 2 s. Centrifuge for 30 s at 11,000 x *g*. Discard flow-through and place the column back into the collection tube.

Repeat step 1.8.3

Dry silica membrane: Centrifuge for 2 min at 11,000 x *g*.

1.10 Elute DNA: Place the sample into anew microcentrifuge tube. Add 100 µL Buffer SE to the column. Do not close the lid and inclubate for 1 min at room temperature. Close the lid and centrifuge for 30 s at 11,000 x *g*.

2. PCR (Polymerase chain reaction)

The PCR is using to amplify a specific region of a DNA strand (the DNA target).The protocol of PCR following:

2.1 The extracted DNA is use as the template for PCR amplification.

2.2 The first step is prepare a PCR master mixture which consist of;

2.2.1 589 µl of Molecular grade water (nuclease –free water)

2.2.2 75 µl of 50 mM MgCl

2.2.3 22.5 µl of dNTPs (dATP,dGTP,dTTP,dCTP)

2.2.4 15 µl of Forward primer

2.2.5 15 µl of Reverse primer

2.2.6 15 µl of Taq DNA Polymerase

2.2.7 3 µl of PCR buffer (10x ammonium)

2.3 For each sample, 49 µl of master mix and 1 µl of DNA extract add in to a small microcentrifuge tube. A negative control (reagents only) include in each PCR amplification round to check for the contamination.

2.4 The 16s rDNA gene fragments is amplify in a PCR reaction mixture using a PCR reaction mixture using thermo Hybrid PX2 Thermal cycles. PCR perform for 40 cycles and thermal cycle programs by the following cycling parameters:

2.4.1 The initial denaturation of at 94 ^{o}C for 5 min.

2.4.2 35 cycles of amplification consist of denaturation at 94 ^{o}C for 1 min, primer annealing at 57.7 ^{o}C for 1 min, and primer extension at 75 ^{o}C for 1 min s.

2.4.3 The final primer extension at 72 ^{o}C for 5 min.

2.4 When the PCR completed, the PCR products store at -20 ^{o}C until PEG purification method.

3. Polyethylene glycol (PEG) Purification of PCR Product

The purify your PCR fragments for sequencing by PEG precipitation. After running PCR, ethanol precipitate fragment as:

3.1 Dissolve the precipitated fragment in 32 μL of reagent grade (milli-Q) water

3.2. Add 8 μL of 5.0 M NaCl (final concentration 0.5 M)

3.3 Add 40 μL of 22% PEG 8000 and mix (11% PEG will precipitate all DNA fragments larger than 180 bp)

3.4 Incubate on ice at least 20 min.

3.5 Centrifuge at 4°C for 5 to 10 min.

3.6 Aspirate and discard the supernatant.

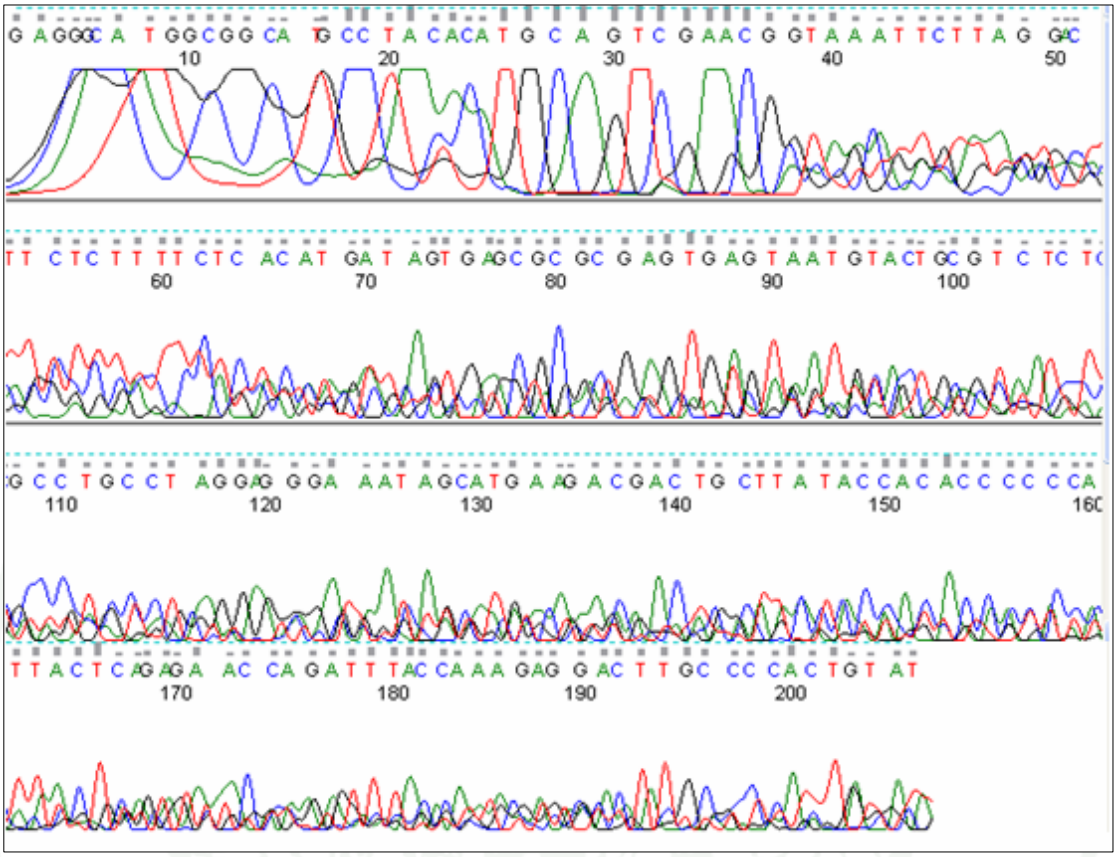
3.7 Dissolve the pellet in 20 μL 0.3 M NaOAc, and add 2.5 volumes of 95% ethanol mix and leave on ice for about 15 min, then spin in the microfuge for 15 min.

3.8 Carefully aspirate and discard the supernatant. Rinse the pellet with 250 μL of 70% ethanol. Spin for 5 min. in the microfuge, then carefully aspirate and discard the supernatant. Dry the pellet for 3 min. under vacuum, or air dry.

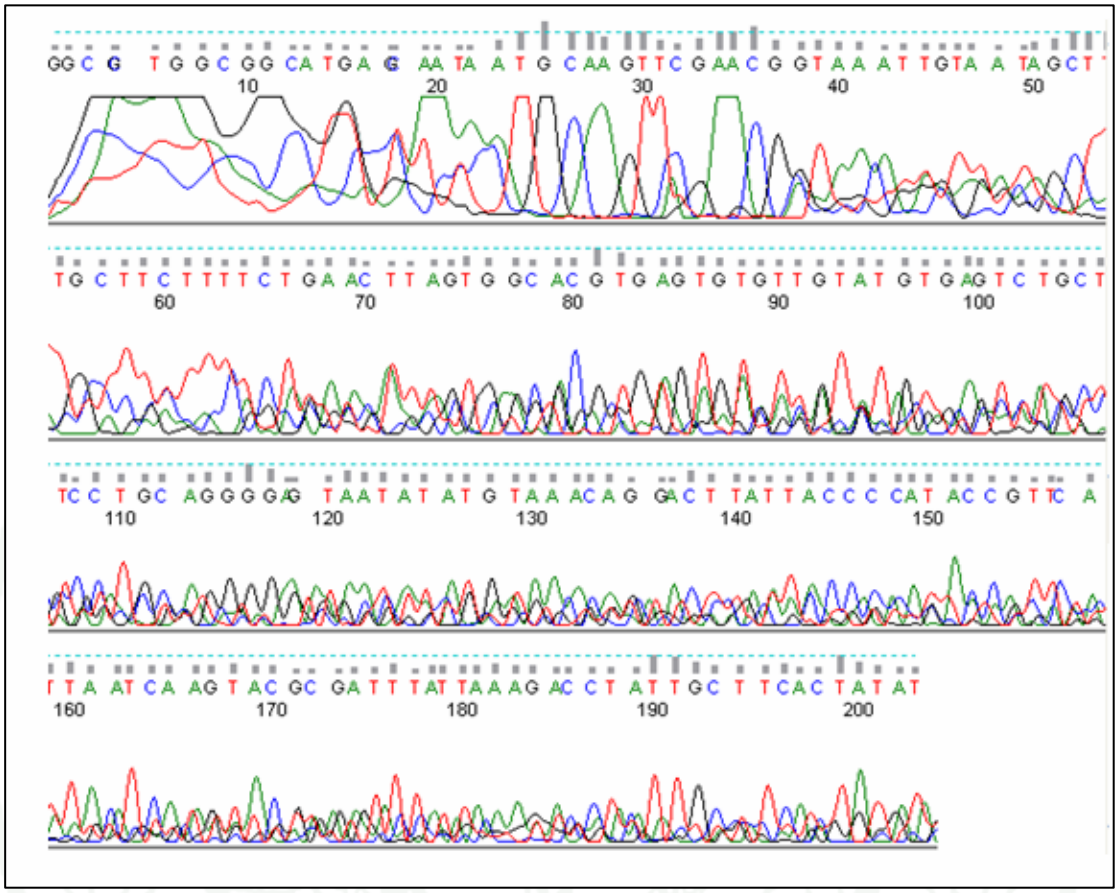
3.9 Resuspend the pellet in 20 μL of deionized water, and store at – 20°C.

Appendix C

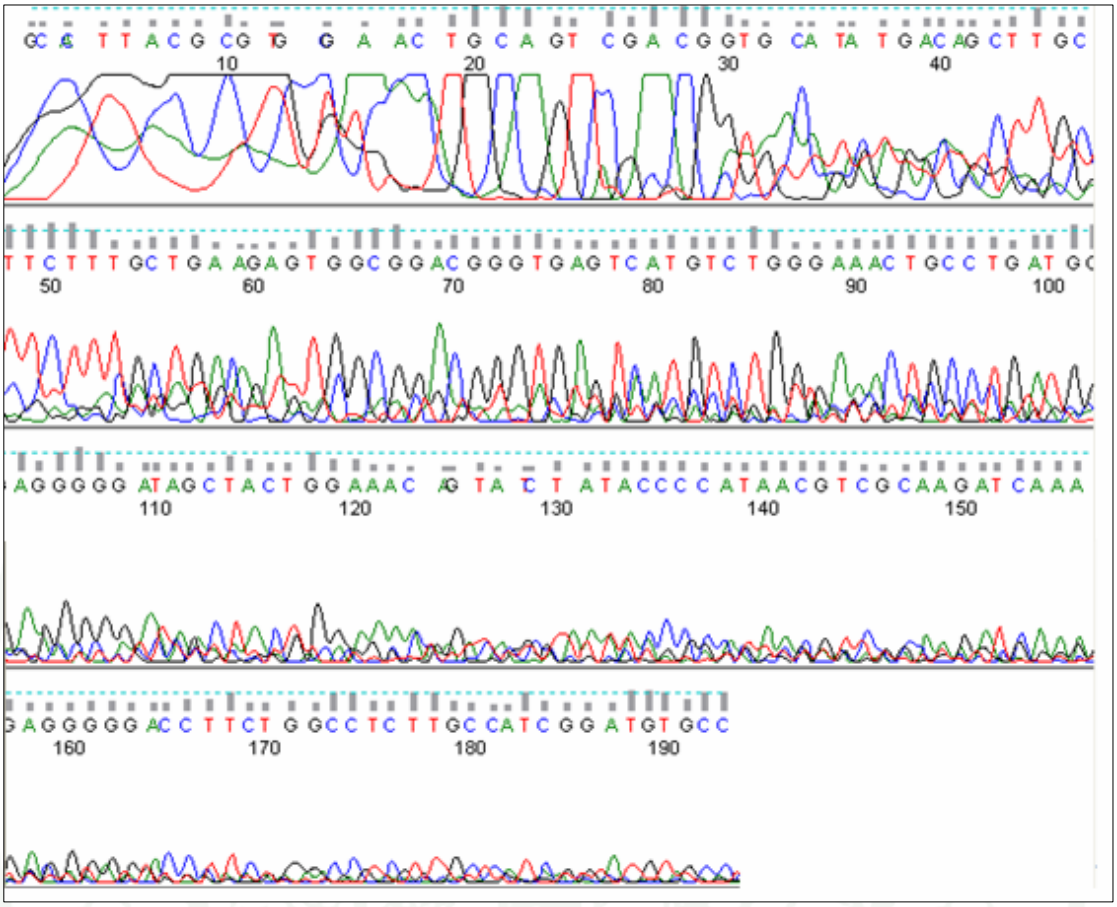
The Sequences Chromatograms



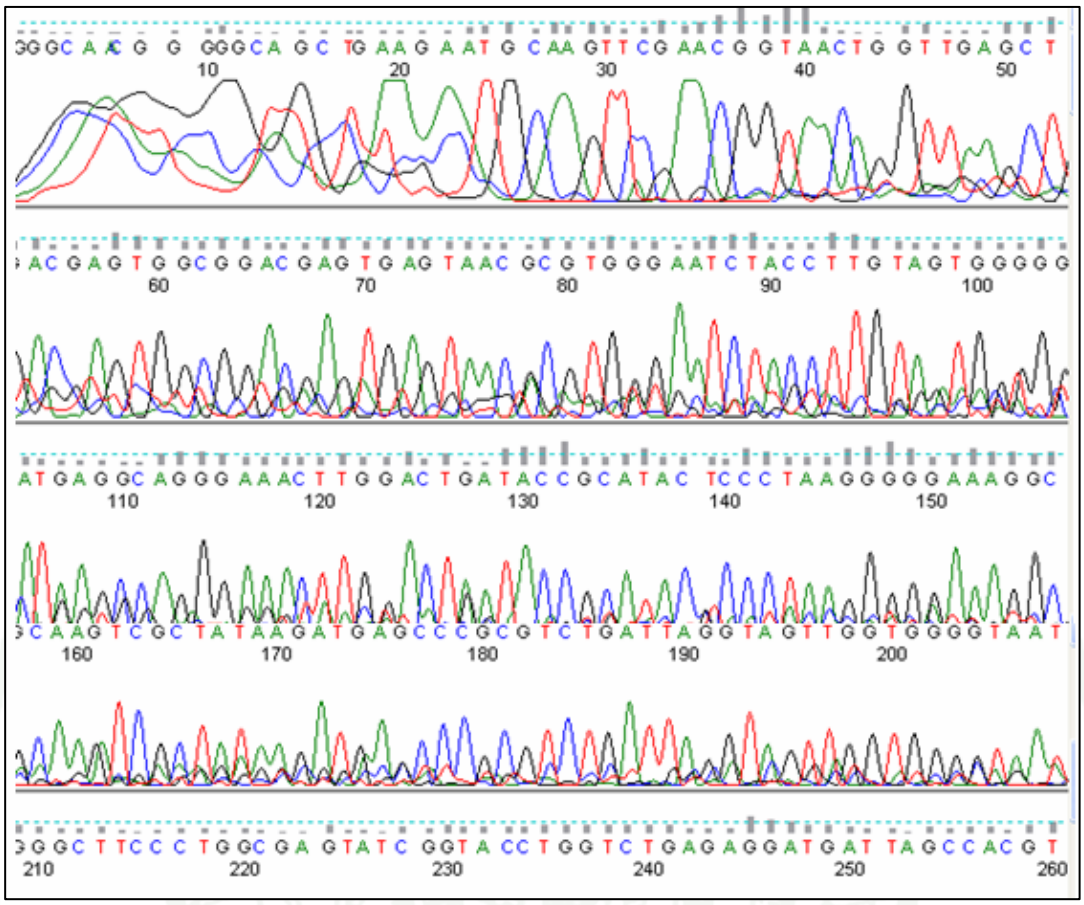
Appendix Figure C1 The Sequences Chromatogram of the influent.



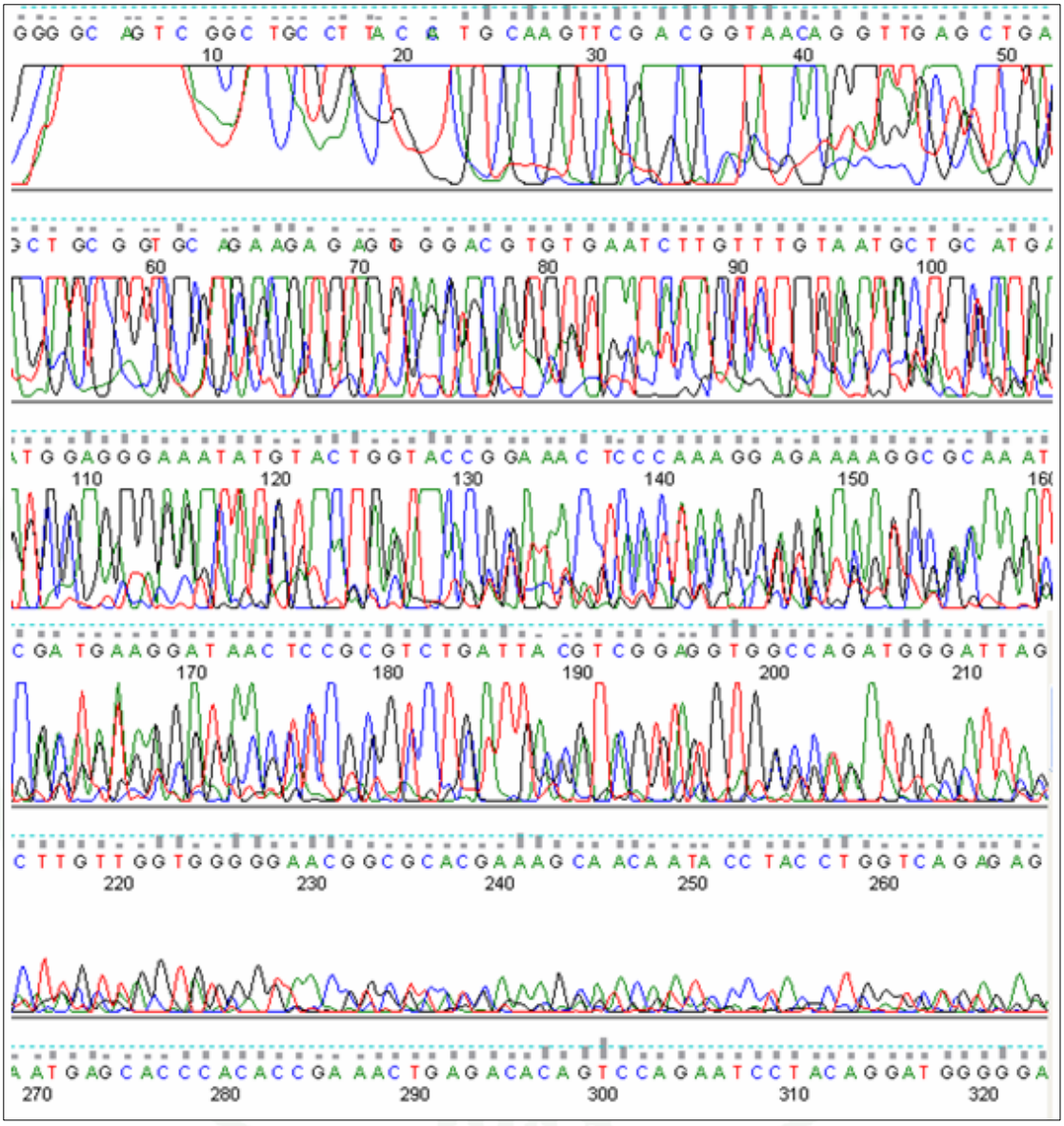
Appendix Figure C2 The Sequences Chromatogram of the equalization tank.



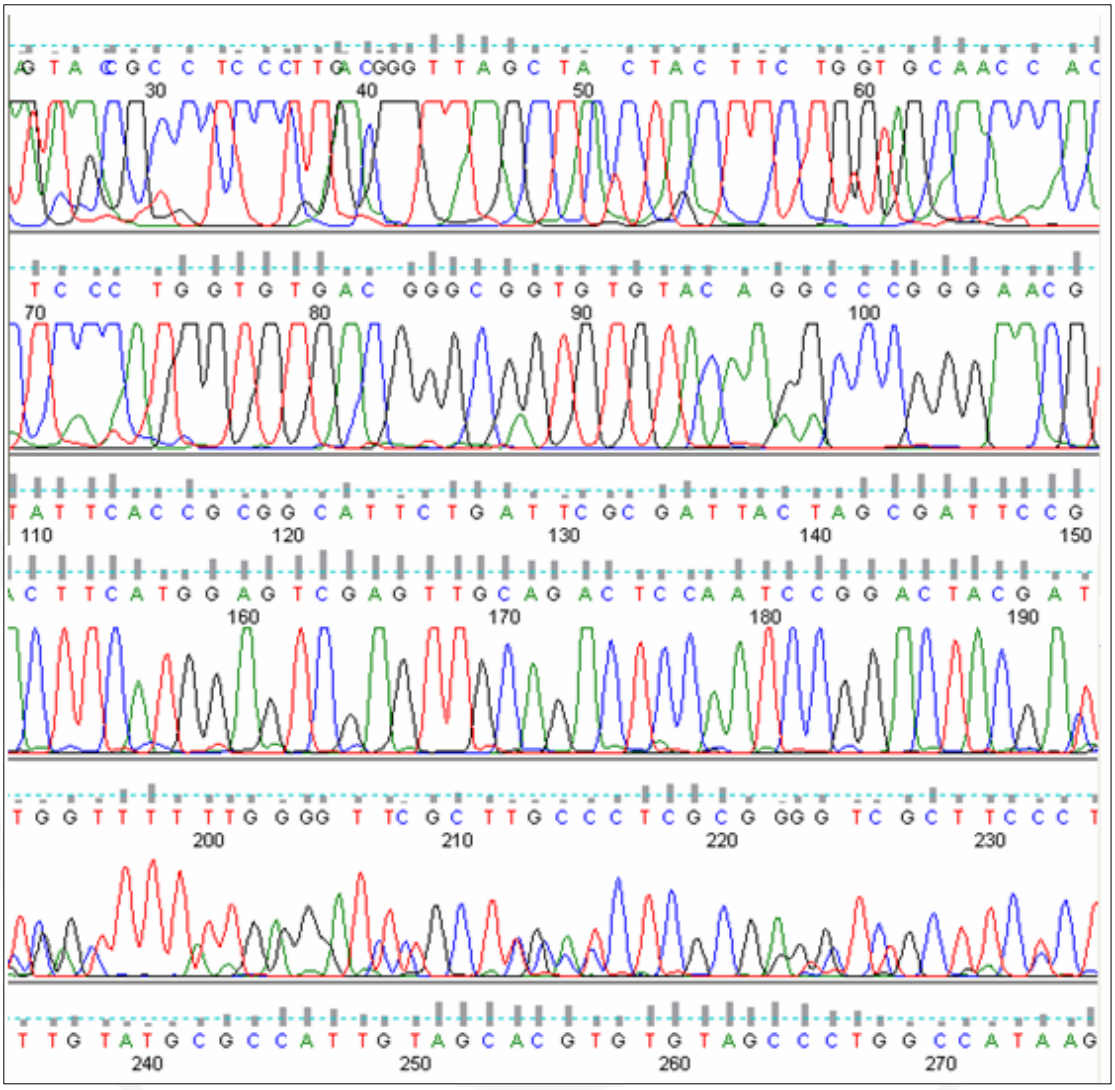
Appendix Figure C3 The Sequences Chromatogram of the UASB tank.



Appendix Figure C4 The Sequences Chromatogram of the aeration tank.



Appendix Figure C5 The Sequences Chromatogram of the sedimentation tank.



Appendix Figure C6 The Sequences Chromatogram of the effluent.

Appendix D

The Sequences Producing Significant Alignment Results

Appendix Table D1 The Sequences producing significant alignment for the influent.

Accession No.	Description	Max score	Query coverage	E value	Max identity
NR_036758.1	*Streptococcus equi subsp. zooepidemicus* strain Streptococcus equi DNA for 16S rRNA, strain ATCC 43079	116	95%	5.00E-23	77%
FJ435217.1	*Bacillus cereus* strain 3 16S ribosomal RNA gene, partial sequence	98.7	87%	1.00E-17	77%
FJ393296.1	*Bacillus cereus* strain TCCC11197 16S ribosomal RNA gene, partial sequence	98.7	87%	1.00E-17	77%
JF302672.1	*Enterococcus thailandicus* strain S56 16S ribosomal RNA gene, partial sequence	95.1	89%	2.00E-16	76%
AY854143.1	*Bacillus sp.* HPC504 16S ribosomal RNA gene, partial sequence	95.1	82%	2.00E-16	76%
HQ234328.1	*Bacillus subtilis* strain Bs12 16S ribosomal RNA gene, partial sequence	93.3	87%	5.00E-16	76%
GQ280034.1	*Bacillus anthracis* strain BJ-24 16S ribosomal RNA gene, partial sequence	93.3	83%	5.00E-16	76%
CP002904.1	*Streptococcus equi subsp. zooepidemicus* ATCC 35246, complete genome	98.7	95%	1.00E-17	75%
FM204883.1	*Streptococcus equi subsp.* equi 4047, complete genome	98.7	95%	1.00E-17	75%
FJ755949.1	*Bacillus sp.* RCT8 16S ribosomal RNA gene, partial sequence	98.7	87%	1.00E-17	75%

Appendix Table D1 (Continued)

Accession No.	Description	Max score	Query coverage	E value	Max identity
CP001129.1	*Streptococcus equi subsp. zooepidemicus* MGCS10565, complete genome	98.7	95%	1.00E-17	75%
JN836929.1	*Brevibacillus* sp. IICDBZ12 16S ribosomal RNA gene, partial sequence	96.9	91%	4.00E-17	75%
AP012336.1	*Streptococcus mutans* LJ23 DNA, complete genome	95.1	87%	2.00E-16	75%
AE014133.2	*Streptococcus mutans* UA159, complete genome	95.1	87%	2.00E-16	75%
AP010655.1	*Streptococcus mutans* NN2025 DNA, complete genome	95.1	87%	2.00E-16	75%
AY773148.1	Bacterium SM16-19 16S ribosomal RNA gene, partial sequence	95.1	87%	2.00E-16	75%
DQ105975.1	*Bacillus anthracis* 16S ribosomal RNA gene, partial sequence	95.1	95%	2.00E-16	75%
AF083593.1	Amazonian soil bacterium P24a 16S ribosomal RNA gene, partial sequence	93.3	91%	5.00E-16	75%
CP003332.1	*Bacillus amyloliquefaciens* Y2, complete genome	91.5	82%	2.00E-15	75%

Appendix Table D1 (Continued)

Accession No.	Description	Max score	Query coverage	E value	Max identity
JQ734537.1	*Bacillus amyloliquefaciens* strain BCL9 16S ribosomal RNA gene, partial sequence	91.5	82%	2.00E-15	75%
JQ734535.1	*Bacillus amyloliquefaciens* strain BGP20 16S ribosomal RNA gene, partial sequence	91.5	82%	2.00E-15	75%
CP003492.1	*Bacillus* sp. JS, complete genome	91.5	82%	2.00E-15	75%
HE617159.1	*Bacillus amyloliquefaciens* subsp. plantarum CAU B946 complete genome	91.5	82%	2.00E-15	75%
HE586891.1	*Bacillus* sp. ESC-str.50 partial 16S rRNA gene, strain ESC-str.50	91.5	82%	2.00E-15	75%
JN897282.1	*Bacillus* sp. KC8(2011) 16S ribosomal RNA gene, partial sequence	91.5	82%	2.00E-15	75%
GQ340490.1	*Bacillus* sp. M42(2010) strain M31 16S ribosomal RNA gene, partial sequence	113	95%	6.00E-22	74%
GQ340496.1	*Bacillus* sp. M41(2010) strain M41 16S ribosomal RNA gene, partial sequence	98.7	95%	1.00E-17	74%

Appendix Table D2 The Sequences producing significant alignment for the equalization tank.

Accession No.	Description	Max score	Query coverage	E value	Max identity
JN485691.1	Uncultured organism clone SBYW_396 16S ribosomal RNA gene, partial sequence	73.4	43%	5.00E-10	81%
JN530571.1	Uncultured organism clone SBZO_1998 16S ribosomal RNA gene, partial sequence	75.2	46%	1.00E-10	80%
JN529288.1	Uncultured organism clone SBZO_e410 16S ribosomal RNA gene, partial sequence	71.6	44%	2.00E-09	80%
JN536521.1	Uncultured organism clone SBZP_2242 16S ribosomal RNA gene, partial sequence	69.8	46%	6.00E-09	79%
JN536289.1	Uncultured organism clone SBZP_1982 16S ribosomal RNA gene, partial sequence	69.8	46%	6.00E-09	79%
AB301017.1	*Bacillus amyloliquefaciens* gene for 16S rRNA, partial sequence, strain: GH14	78.8	49%	1.00E-11	78%
AY854136.1	*Bacillus* sp. HPC507 16S ribosomal RNA gene, partial sequence	75.2	52%	1.00E-10	78%
JN477417.1	Uncultured organism clone SBYT_1058 16S ribosomal RNA gene, partial sequence	68	46%	2.00E-08	78%

Appendix Table D2 (Continued)

Accession No.	Description	Max score	Query coverage	E value	Max identity
JN539825.1	Uncultured organism clone SBZP_6730 16S ribosomal RNA gene, partial sequence	66.2	46%	8.00E-08	78%
JN492805.1	Uncultured organism clone SBYZ_782 16S ribosomal RNA gene, partial sequence	66.2	46%	8.00E-08	78%
JN485432.1	Uncultured organism clone SBYW_3690 16S ribosomal RNA gene, partial sequence	66.2	46%	8.00E-08	78%
JN524211.1	Uncultured organism clone SBZI_1911 16S ribosomal RNA gene, partial sequence	64.4	43%	3.00E-07	78%
JN507292.1	Uncultured organism clone SBZC_3330 16S ribosomal RNA gene, partial sequence	64.4	43%	3.00E-07	78%
JN456456.1	Uncultured organism clone SBYH_3495 16S ribosomal RNA gene, partial sequence	64.4	43%	3.00E-07	78%
EF608163.1	*Bacillus subtilis* strain HT2 16S ribosomal RNA gene, partial sequence	80.6	53%	3.00E-12	77%
JN535867.1	Uncultured organism clone SBZP_1527 16S ribosomal RNA gene, partial sequence	62.6	46%	9.00E-07	77%

Appendix Table D2 (Continued)

Accession No.	Description	Max score	Query coverage	E value	Max identity
JN534470.1	Uncultured organism clone SBZP_2673 16S ribosomal RNA gene, partial sequence	62.6	46%	9.00E-07	77%
JN501432.1	Uncultured organism clone SBZA_2478 16S ribosomal RNA gene, partial sequence	60.8	43%	3.00E-06	77%
JN532617.1	Uncultured organism clone SBZO_5661 16S ribosomal RNA gene, partial sequence	50	43%	0.006	75%

Appendix Table D3 The Sequences producing significant alignment for the UASB tank.

Accession No.	Description	Max score	Query coverage	E value	Max identity
DQ182325.1	*Serratia marcescens* strain RS-10 16S ribosomal RNA gene, partial sequence	55.4	51%	4.00E-05	92%
EF428999.1	*Enterobacter* sp. GIST-CPan2 16S ribosomal RNA gene, partial sequence	60.8	58%	9.00E-07	91%
DQ379507.1	*Klebsiella pneumoniae* strain TSL-4 16S ribosomal RNA gene, partial sequence	60.8	58%	9.00E-07	91%
FJ997270.1	*Escherichia coli* strain DSPV 284T 16S ribosomal RNA gene, partial sequence	69.8	77%	2.00E-09	88%
FJ997269.1	*Escherichia coli* strain DSPV 247T 16S ribosomal RNA gene, partial sequence	69.8	77%	2.00E-09	88%
EU557341.1	*Serratia* sp. 136-2 16S ribosomal RNA gene, partial sequence	69.8	77%	2.00E-09	88%
EU557339.1	*Serratia* sp. 25N 16S ribosomal RNA gene, partial sequence	69.8	77%	2.00E-09	88%
EF489445.1	*Enterobacter* sp. GIST-NKst3 16S ribosomal RNA gene, partial sequence	69.8	77%	2.00E-09	88%

Appendix Table D3 (Continued)

Accession No.	Description	Max score	Query coverage	E value	Max identity
EF446895.1	*Acinetobacter radioresistens* strain Philippines-11 16S ribosomal RNA gene, partial sequence	69.8	77%	2.00E-09	88%
EF429007.1	*Enterobacter* sp. GIST-OutAn2 16S ribosomal RNA gene, partial sequence	69.8	77%	2.00E-09	88%
FJ204050.1	Uncultured bacterium clone HK154 16S ribosomal RNA gene, partial sequence	68	77%	6.00E-09	88%
EU675856.1	*Enterobacter hormaechei* strain PSB3 16S ribosomal RNA gene, partial sequence	68	77%	6.00E-09	88%
HM640288.1	*Pantoea* sp. YUST-DW9 16S ribosomal RNA gene, partial sequence	64.4	77%	7.00E-08	86%
HM640283.1	*Pantoea* sp. YUST-DW4 16S ribosomal RNA gene, partial sequence	64.4	77%	7.00E-08	86%
AM502985.1	*Pantoea* sp. WF2 5 partial 16S rRNA gene, clone WF2 5	62.6	75%	2.00E-07	86%
DQ481467.1	*Serratia grimesii* strain VTan-6 16S ribosomal RNA gene, partial sequence	69.8	87%	2.00E-09	85%

Appendix Table D3 (Continued)

Accession No.	Description	Max score	Query coverage	E value	Max identity
HQ123487.1	*Serratia marcescens* strain RS8101 16S ribosomal RNA gene, partial sequence	66.2	79%	2.00E-08	85%
HM640285.1	*Pantoea* sp. YUST-DW6 16S ribosomal RNA gene, partial sequence	66.2	87%	2.00E-08	83%
EU239467.1	*Enterobacter hormaechei* strain XJUHX-4 16S ribosomal RNA gene, partial sequence	60.8	90%	9.00E-07	83%
FJ588708.1	*Enterobacter* sp. SAVR 16S ribosomal RNA gene, partial sequence	59	95%	3.00E-06	81%
HQ123487.1	*Serratia marcescens* strain RS8101 16S ribosomal RNA gene, partial sequence	66.2	79%	2.00E-08	85%

Appendix Table D4 The Sequences producing significant alignment for the aeration tank.

Accession No.	Description	Max score	Query coverage	E value	Max identity
AB042537.1	Thiothrix sp. B2-8 gene for 16S ribosomal RNA, partial sequence	719	38%	0	94%
AB042534.1	Thiothrix sp. B2-7 gene for 16S ribosomal RNA, partial sequence	719	38%	0	94%
AB042533.1	Thiothrix sp. B4-1 gene for 16S ribosomal RNA, partial sequence	719	38%	0	94%
AB042536.1	Thiothrix sp. B5-1 gene for 16S ribosomal RNA, partial sequence	717	38%	0	94%
HQ823669.1	Thiothrix disciformis strain DSM 14473 16S ribosomal RNA gene, partial sequence	715	38%	0	94%
AB042538.1	Thiothrix sp. OS-F gene for 16S ribosomal RNA, partial sequence	713	38%	0	94%
AB042535.1	Thiothrix sp. SCM-A gene for 16S ribosomal RNA, partial sequence	711	38%	0	94%
NR_024756.1	Thiothrix disciformis strain B3-1 16S ribosomal RNA, partial sequence >dbj|AB042532.1| Thiothrix sp. B3-1 gene for 16S ribosomal RNA, partial sequence	711	38%	0	93%

Appendix Table D4 (Continued)

Accession No.	Description	Max score	Query coverage	E value	Max identity
EU104075.1	Uncultured bacterium clone M0111_78 16S ribosomal RNA gene, partial sequence	673	39%	0	91%
AB042542.1	Thiothrix eikelboomii gene for 16S ribosomal RNA, partial sequence, strain:COM-A	673	37%	0	92%
AB042541.1	Thiothrix eikelboomii gene for 16S ribosomal RNA, partial sequence, strain:T2-1	673	37%	0	92%
EU104180.1	Uncultured bacterium clone N1512_07 16S ribosomal RNA gene, partial sequence	670	37%	0	92%
EU283396.1	Uncultured gamma proteobacterium clone AS99 16S ribosomal RNA gene, complete sequence	670	37%	0	92%
EU283389.1	Uncultured gamma proteobacterium clone AS76 16S ribosomal RNA gene, complete sequence	670	37%	0	92%
EU283363.1	Uncultured actinobacterium clone AS33 16S ribosomal RNA gene, complete sequence	670	37%	0	77%
AY962323.1	Uncultured bacterium clone P008_H3 16S ribosomal RNA gene and 16S-23S ribosomal RNA intergenic spacer, partial sequence	670	37%	0	92%

Appendix Table D4 (Continued)

Accession No.	Description	Max score	Query coverage	E value	Max identity
AB042539.1	Thiothrix eikelboomii gene for 16S ribosomal RNA, partial sequence, strain:KR-A	670	37%	0	92%
NR_024758.1	Thiothrix eikelboomii strain AP3 16S ribosomal RNA, partial sequence >dbj|AB042819.1| Thiothrix eikelboomii gene for 16S rRNA	668	35%	0	94%
EU104260.1	Uncultured bacterium clone N1512_95 16S ribosomal RNA gene, partial sequence	664	37%	0	91%
EU104250.1	Uncultured bacterium clone N1512_84 16S ribosomal RNA gene, partial sequence	664	37%	0	91%
EU104231.1	Uncultured bacterium clone N1512_63 16S ribosomal RNA gene, partial sequence	664	37%	0	91%
EU104229.1	Uncultured bacterium clone N1512_61 16S ribosomal RNA gene, partial sequence	664	37%	0	91%
EU104222.1	Uncultured bacterium clone N1512_53 16S ribosomal RNA gene, partial sequence	664	37%	0	91%

Appendix Table D4 (Continued)

Accession No.	Description	Max score	Query coverage	E value	Max identity
EU104214.1	Uncultured bacterium clone N1512_45 16S ribosomal RNA gene, partial sequence	664	37%	0	91%
EU104201.1	Uncultured bacterium clone N1512_30 16S ribosomal RNA gene, partial sequence	664	37%	0	91%
EU104178.1	Uncultured bacterium clone N1512_05 16S ribosomal RNA gene, partial sequence	664	37%	0	91%
EU283358.1	Uncultured gamma proteobacterium clone AS24 16S ribosomal RNA gene, complete sequence	664	35%	0	94%
EU283349.1	Uncultured gamma proteobacterium clone AS7 16S ribosomal RNA gene, complete sequence	664	37%	0	91%
AB042540.1	Thiothrix eikelboomii gene for 16S ribosomal RNA, partial sequence, strain:T1-4	664	37%	0	91%
JQ624269.1	Uncultured Thiothrix sp. clone OTU-28-AW 16S ribosomal RNA gene, partial sequence	661	37%	0	91%

Appendix Table D5 The Sequences producing significant alignment for the sedimentation tank.

Accession No.	Description	Max score	Query coverage	E value	Max identity
GU594312.1	Escherichia coli strain E36 16S ribosomal RNA gene, partial sequence	499	44%	3.00E-137	80%
GU594294.1	Escherichia coli strain A26 16S ribosomal RNA gene, partial sequence	499	44%	3.00E-137	80%
GU594315.1	Escherichia coli strain E10 16S ribosomal RNA gene, partial sequence	495	44%	4.00E-136	80%
GU594309.1	Escherichia coli strain AB6 16S ribosomal RNA gene, partial sequence	495	44%	4.00E-136	80%
GU594305.1	Escherichia coli strain B50 16S ribosomal RNA gene, partial sequence	495	44%	4.00E-136	80%
GU594301.1	Escherichia coli strain A5 16S ribosomal RNA gene, partial sequence	495	44%	4.00E-136	80%
GU594300.1	Escherichia coli strain B2 16S ribosomal RNA gene, partial sequence	495	44%	4.00E-136	80%
GU594296.1	Escherichia coli strain B6 16S ribosomal RNA gene, partial sequence	495	44%	4.00E-136	80%

Appendix Table D5 (Continued)

Accession No.	Description	Max score	Query coverage	E value	Max identity
JQ315922.1	Shigella flexneri strain Inspire86 16S ribosomal RNA gene, partial sequence	495	44%	4.00E-136	79%
JQ315921.1	Shigella flexneri strain Inspire85 16S ribosomal RNA gene, partial sequence	495	44%	4.00E-136	79%
JQ315920.1	Shigella flexneri strain Inspire84 16S ribosomal RNA gene, partial sequence	495	44%	4.00E-136	79%
JQ315919.1	Shigella sonnei strain Inspire83 16S ribosomal RNA gene, partial sequence	493	44%	1.00E-135	79%
JQ315918.1	Shigella sonnei strain Inspire82 16S ribosomal RNA gene, partial sequence	493	44%	1.00E-135	79%
JQ315917.1	Shigella sonnei strain Inspire81 16S ribosomal RNA gene, partial sequence	493	44%	1.00E-135	79%
JQ315916.1	Shigella sonnei strain Inspire80 16S ribosomal RNA gene, partial sequence	493	44%	1.00E-135	79%
JN596117.1	Shigella sonnei strain M13 16S ribosomal RNA gene, partial sequence	493	44%	1.00E-135	79%

Appendix Table D5 (Continued)

Accession No.	Description	Max score	Query coverage	E value	Max identity
JN596117.1	Shigella sonnei strain M13 16S ribosomal RNA gene, partial sequence	493	44%	1.00E-135	79%
FR870445.1	Shigella sonnei partial 16S rRNA gene, type strain CECT 4887T	493	44%	1.00E-135	79%
HQ407262.1	Shigella flexneri strain T87 16S ribosomal RNA gene, partial sequence	493	44%	1.00E-135	79%
EU857633.1	Shigella flexneri 16S ribosomal RNA gene, partial sequence	493	44%	1.00E-135	79%
U90316.1	Salmonella typhimurium Stm1 16S ribosomal RNA gene, complete sequence	489	44%	2.00E-134	79%
JN596117.1	Shigella sonnei strain M13 16S ribosomal RNA gene, partial sequence	493	44%	1.00E-135	79%
FR870445.1	Shigella sonnei partial 16S rRNA gene, type strain CECT 4887T	493	44%	1.00E-135	79%
HQ407262.1	Shigella flexneri strain T87 16S ribosomal RNA gene, partial sequence	493	44%	1.00E-135	79%

Appendix Table D6 The Sequences producing significant alignment for the effluent

Accession No.	Description	Max score	Query coverage	E value	Max identity
JN941324.1	*Pseudomonas putida* strain LF2-1 16S ribosomal RNA gene, partial sequence	57.2	45%	1.00E-05	94%
GQ368699.1	*Providencia* sp. AR8 16S ribosomal RNA gene, partial sequence	64.4	53%	7.00E-08	93%
AM937464.1	*Aeromonas veronii* partial 16S rRNA gene, isolate REI_044	64.4	53%	7.00E-08	93%
AY823619.1	*Alcaligenes faecalis* strain MT1 16S ribosomal RNA gene, partial sequence	82.4	80%	3.00E-13	92%
FN668390.1	*Yersinia ruckeri* partial 16S rRNA gene, strain 8386	71.6	80%	5.00E-10	86%
GQ407267.1	*Aeromonas hydrophila* strain MZQ-CS01 16S ribosomal RNA gene, partial sequence	68	80%	6.00E-09	83%
JN941324.1	*Pseudomonas putida* strain LF2-1 16S ribosomal RNA gene, partial sequence	57.2	45%	1.00E-05	94%
GQ368699.1	*Providencia* sp. AR8 16S ribosomal RNA gene, partial sequence	64.4	53%	7.00E-08	93%

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