Microbial Functional Roles In Eco-friendly Treatment for Tannery Wastewater Using a Microbial Consortium

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Microbial Functional Roles In Eco-friendly Treatment for Tannery Wastewater Using a Microbial Consortium

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List of Abbreviations

BLAST: Basic local alignment tool

BM: Beneficial microorganisms

bp: Base pair

℃: Degree centigrade

COG: Clusters of orthologous groups

Ct: Threshold cycle

DGGE: Denaturing gradient gel electrophoresis

DO: Dissolved oxygen

DNA: Deoxyribonucleic acid

EC: European commission

GC: Gas chromatography

HRT: Hydraulic retention time

ICP-AES: Inductively coupled plasma-atomic emission spectrometer

KCTC: Korea collection for type cultures

KEGG: Kyoto encyclopedia of genes and genomes

kJ/mol: Kilojoule per mole

L: Liters

M^3: Meter cubed

mg/l: milligram per liter

MG-RAST: Metagenomics rapid annotation using subsystem technology server

ml: milliliters

NCBI: National center for biotechnology information

Nos Z: Nitrous oxide reductase

ng: Nanogram

OTU: Operational taxonomic unit

PCA: Principal component analysis

PCR: Polymerase chain reaction

pH: Hydrogen ion concentration

PVC: Poly vinyl chloride

rRNA: Ribosomal ribonucleic acid

RT-qPCR: Real-time quantitative polymerase chain reaction

sp: Species

Wt/v: Weight per volume

UP-GMA: Unweighted pair group method with arithmetic mean

UV: Ultravoilet

VFA: Volatile fatty acids

μ: Micro

μM: Micromole

초록

자연토양에서 분리된 복합미생물제(BM-S-1)를 활용한 피혁폐수 처리시스템을 국내의 경우 pilot scale system 에서 국외(중국)의 경우 현장규모에서 별도의 화학적 처리 공정없이 성공적으로 운전하였다. 처리수의 수질과 처리효율을 추적하기 위해 각 처리단계의 COD, 총질소, 총인, 크롬 및 MLSS 을 측정하였다. 처리중의 전체 미생물군집의 변화는 pyrosequencing 기법에 의해, 그리고 탈질균의 개체군변화는 realtime PCR 기법에 의해 nitrous oxide reductase gene (*nosZ*)을 정량적으로 측정함으로 평가하였다. pilot 규모의 처리시스템에서의 COD, 총질소 및 총인의 제거효과는 각각 91, 79 및 90%로 나타났으며, 현장규모의 처리시스템에서의 COD, 총질소, 총인 및 크롬의 제거효과는 각각 98.3, 98.6, 93.6 및 88.5%로 나타났다. pilot 규모의 처리시스템의 미생물의 주요 문(phyla)은 유량조정조(B), 1 차폭기조(PA), 2 차폭기조 (SA) 및 슬러지분해조(SD)의 경우 Proteobacteria, Firmicutes, Bacteroidetes, Planctomycetes 및 Deionococcus-Thermus 의 순으로 나타났으며, 현장규모의 처리시스템의 경우는 Proteobacteria, Firmicutes, Bacteroidetes, Chloroflexi 및 Deinococcus-Thermus 순으로 나타났다. pilot 규모의 처리시스템에서의 각처리 단계에 출현한 미생물종의 출현경향을 Unifrac distance 집괴분석(cluster analysis)법으로 분석한 결과 PA 는 SA 와 유사한 경향을 보였으며 B 는 SD 에 보다 유사한 경향을 보였다. 반면 현장규모의 처리시스템의 경우는 B 는 PA 에, SA 는 SD 에 보다 유사한 경향을 보였다. qPCR 기법을 활용하여 탈질유전자 *nosZ* genes 를 추적함으로서 각 처리 단계의 혐기적 탈질균 의 밀도를 추적한바, pilot 규모의 처리시스템에서는 유입수에 비해 B 의 경우가 734 배나 증가하는 경향을 보였으며, 현장규모의 처리시스템의 경우는 B 의 경우 유입수에 비해 약 195 배 증가하였다. pilot 규모의 처리시스템에서의 미생물군집변화를 pyrosequencing 기법으로 분석한 결과 AB430337_f_uc_s, Methyloversatilis_uc, 및 AB430336_s 가 우점하였으며, 반면에 현장규모의 처리시스템의 경우는

호기적 탈질균인 *Brachymonas denitrificans* 가 B 에서 우점하는 것으로 나타났다 (6-37.5%). 기타 고분자물질 분해균(*Clostridia)*, 황산염환원균(*Desulfuromonas palmitatis*), 및 황산화균 (uncultured *Thiobacillus*) 등도 SD 에서 우점하는 것으로 나타났다. 현장규모의 처리시스템에 있어서 피혁폐수의 처리와 관련된 미생물의 군집구조 및 잠재적 기능을 밝히기 위해 시료의 총 DNA 를 추출한 후, 총유전체 염기서열분석 (metagenome sequencing)을 실시하였다. 이 경우 가장 우점하는 4 가지 문은 Proteobacteria, Bacteriodetes, Firmicutes 및 Actinobacteria 로 나타났다. B and PA 에 있어서 탄수화물, 단백질 및 아미노산의 대사에 관련된 유전자의 출현빈도는 80%이상으로 나타난 반면, 지질, 지방산, 질소, 황 및 인의 대사와 관련된 유전자의 출현빈도는 70%이하로 나타났다. 질소의 대사경우를 보면, 암모니아, 질산염 및 아질산 동화에 관련된 유전자는 탈질 및 이화적 아질산 환원효소의 암호화 유전자에 비해 우점하는 것으로 나타났다. 이러한 질소대사에 관련된 주요 속은 *Burkholderia*, *Polaromonas*, *Albidiferax*, *Acidovorax*, *Geobacter*, *Dechloromonas*, *Pseudomonas*, 및 *Rhodopseudomonas* 로 나타났다. Glutamate synthase, glutamate dehydrogenase, nitrous oxide reductase 및 nitrate reductase 의 암호화에 관련된 유전자는 이들 속에 따라서 다양한 수준의 출현빈도를 보였다. 피혁폐수의 여러 처리단계에 있어서 glutamate synthase (GS) 및 glutamate dehydrogenase (GDH)를 암호화하는 유전자는 *Burkholderia*, *Delfia*, *Bordetella Albidiferax*, *Acidovorax*, *Cupriavidus*, *Thiobacillus*, *Methylibium*, *Azoarcus*, *Dechloromonas*, 및 *Aromatoleum* 에 특이적으로 우점한 반면에 nitric oxide reductase 및 nitrate reductase (NAR)를 암호화하는 유전자는 *Geobacter*, *Burkholderia*, *Acidovorax*, *Cupriavidus*, *Thiobacillus*, 및 *Chromobacterium* 에 특이적으로 우점하는 것으로 나타났다. 그리고 대부분의 대사과정은 상대적으로 PA 및 B 에 보다 활동적으로 나타나는 것으로 관찰이 되었는데 이는 화학적 분석자료(COD, T-N 및 T-P, 등)의 경향과도 일치하는 것으로 나타났다. 따라서 본 연구의 결과는

현장규모의 생태친화적인 피혁폐수의 처리에 있어서 주요한 역할을 하는 미생물의 군집구조와 그 고유한 대사적 특징을 이해하는 데 필요한 정보를 제공하여 줄 것으로 판단된다. 또한 이러한 정보는 향후 현장처리시스템의 설계에도 유용한 정보를 제공해 줄 것으로 사료된다.

ABSTRACT

A novel microbial consortium (BM-S-1) enriched from the natural soils was successfully implemented to treat the tannery wastewater from leather manufacturing industries in Korea in a pilot scale and full scale system in China without chemical pretreatment. The full scale tannery system was built after the pilot system has been proven effective. Chemical oxygen demand (COD), total nitrogen (TN), total phosphorus (TP), chromium (Cr) and mixed liquor suspended solids (MLSS) were measured to monitor treated water quality and treatment efficiency. Microbial population dynamics were analyzed using pyrosequencing and denitrifying bacteria was quantified using real-time PCR (RT-PCR) of the nitrous oxide reductase gene (*nosZ*) in both systems. Removal efficiencies for COD, T-N and T-P were more than 91%, 79%, and 90%, respectively for the pilot system while the removal efficiencies of COD, TN, TP, and Cr were 98.3%, 98.6%, 93.6%, and 88.5% in the full scale system. In the pilot system, the dominant phyla in buffering tank (B), primary aeration (PA), secondary aeration (SA) and sludge digestion tank (SD), were Proteobacteria, Firmicutes, Bacteroidetes, Planctomycetes and Deionococcus-Thermus while the full scale system contained Proteobacteria,

Firmicutes, Bacteroidetes, Chloroflexi and Deinococcus-Thermus. Cluster analysis based on unifrac distance of the species in the different stages showed that the microbial communities in PA is similar to SA while B is closer to SD in the pilot system, B is closely related to PA and SA to SD in the full scale. The qPCR of *nosZ* genes showed the highest abundance of denitrifiers in B increasing 734 folds compared to the influent (I) in the pilot system while the abundance of denitrifiers increased approximately 195 times in B, as compared to the influent of wastewater, and after the BM-S-1 consortium augmentation in the full scale treatment system. The pyrosequencing analysis of the pilot system showed AB430337_f_uc_s, Methyloversatilis_uc, AB430336_s, were the most abundant species while *Brachymonas denitrificans* was the most abundant in full scale especially in B (6-37.5%). Other polymeric substance degraders (*Clostridia)*, sulfate reducers (*Desulfuromonas palmitatis*), and sulfur oxidizers (uncultured *Thiobacillus*) were dominant in the sludge digestion (SD) tank. Whole-metagenome sequencing analysis using Illumina MiSeq Sequencer was done to determine microbial community structures and functional genes associated with treatment of tannery wastewater in the full scale treatment system. The metagenome sequencing data demonstrated that four phyla were Proteobacteria, Bacteriodetes, Firmicutes and Actinobacteria were dominant in the treatment process. The genes involved in metabolisms of carbohydrates, protein and amino acids were more than 80% compared to the genes associated with metabolisms of lipids and fatty acid, nitrogen, sulfur and phosphorus that were less than 70% in B and PA. For nitrogen metabolism, genes associated with ammonia, nitrate and nitrite assimilation were

more abundant than the genes responsible for denitrification and dissimilatory nitrite reductase. The dominant genera involved in nitrogen metabolism were *Burkholderia*, *Polaromonas*, *Albidiferax*, *Acidovorax*, *Geobacter*, *Dechloromonas*, *Pseudomonas*, and *Rhodopseudomonas*. Glutamate synthase, glutamate dehydrogenase, nitrous oxide reductase and nitrate reductase were distinctively observed depending on the kinds of these genera. Glutamate synthase (GS) and glutamate dehydrogenase (GDH) were abundant in *Burkholderia, Delfia, Bordetella Albidiferax, Acidovorax, Cupriavidu* genes such *s, Thiobacillus*, *Methylibium, Azoarcus, Dechloromonas,* and *Aromatoleum* while as nitric oxide reductase and nitrate reductase (NAR) showed high abundance in *Geobacter*, *Burkholderia, Acidovorax, Cupriavidus, Thiobacillus*, and *Chromobacterium* in the different stages of the treatment process. Most of the metabolic processes were relatively more active in PA and B which corroborated with the chemical data (COD, T-N and T-P, etc.) obtained from all the stages during the treatment process. These results provide a detailed understanding into the structures and functions of the microbial communities in the eco-friendly tannery wastewater treatment. Furthermore, these data would provide useful information for designing the full scale treatment system.

CHAPTER 1 INTRODUCTION

1.1 General introduction

The tannery industry has been in existence for many centuries but the technological processes have evolved and developed to a degree that it constitutes a major part of most developed and developing economies. Tannery processing and waste management technologies are still being developed to curb and eliminate the negative environmental impact generated by the waste produced in the process.

Some of the contents of tannery waste include dyes, surfactants, sulfonated oils, chromium salts, solid waste fragments, and waste skin trimmings, among others (Durai et al., 2011; Sharma et al., 2011; Zupancic et al.,2010) which inhibit microbial activities such as nitrification and denitrification (Farabegolia et al., 2004). High concentrations of organic nitrogen and ammonium, collagen, and toxic tanning agents make tannery wastewater more recalcitrant and inhibit biological treatment (El-Sheikh et al., 2011). In the European Union, extensive amounts of sludge are generated annually from leather processing, most of which are deposited into landfills (EC, 2011).

The conventional tannery wastewater treatment systems use physico-chemical and biological methods or a combination of both. Physico-chemical pretreatment removed 98.8% of chromium, 31% of COD, 25.8% of BOD, and 51.2% of TSS (Elsheikh et al., 2009) indicating overall low efficiency at removing organic wastes. Ganesh et al. (2006) reported that the use of a sequencing batch reactor in tannery wastewater biodegradation has removal efficiencies of 80–82% of COD, 78–80% of TKN, and 83–99% of NH_3-N, which are more efficient than those of conventional

aerobic systems. Lefebvre et al., 2005, reported 95%, 93%, 96% and 92% removal efficiencies of COD, PO_4^{3-}, TKN and SS, respectively, in a sequencing batch reactor treating tannery soak in liquor. Although this system was efficient, it showed high effluent turbidity (Lefebvre et al., 2006). An activated sludge pre-treatment process (Feo et al., 2009) applied to the tannery wastewater removed about 67% COD before the application of reverse osmosis (RO) to produce are usable effluent. Munz et al. (2008) compared the use of conventional activated sludge process (CASP) and membrane bioreactor (MBR) in tannery wastewater treatment, where CASP showed COD removal of 80% as opposed to 76% in MBR. CASP also showed more stable nitrification than MBR. Roca et al. (2007) used conventional pre-treatment to obtain 40% reduction of COD before combining ultrafiltration and reverse osmosis to obtain reusable water from tannery wastewater.

Conventional biological treatments for tannery wastewater are rigorous, inadequate, inefficient, and/or not cost-effective, due to large variations in tanning practices and the kinds of chemicals used in those processes (Durai, 2011; El Sheikh et al., 2011). Therefore, there is a need to develop effective treatment systems which are economical and environmental friendly, with the resulting effluent characteristics that meet the regulation standards in terms of COD, TN, TP, heavy metals, sludge and odor, etc. Bioaugmentation of *Brachymonas denitrificans* into tannery wastewater successfully enhanced biological nitrogen removal (Leta et al., 2005). Reducing oxygen supply in the wastewater treatment of a food processing factory significantly reduced sludge through selection of denitrifying bacteria (*Comamonadaceae*) including *Brachymonas denitrificans*) (Sadaie et al., 2007).

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Several microbial communities are involved in the degradation, detoxification and removal of nutrients in wastewater treatment systems. Such communities have been studied using the cultivation-based method (Maruf et al., 2012; Amann et al., 1998). This method has the limitation of culture medium selectivity and all microorganisms cannot be grown in a medium. This leads to bias when applied to microbial community analysis. However, culture- independent approach based on 16S rRNA genes and functional genes such as PCR-DGGE (Muyzer et al., 1993), qPCR-PCR (Zhang & Fang, 2006), microarray (Xia et al., 2010) etc., offered increased understanding to microbial community in wastewater; yet these methods are limited by PCR bias or low throughput. The advent of next whole genome sequencing of microbial community gives a comprehensive approach without PCR bias and allows for simultaneous exploration of both taxonomic and functional diversity, richness and evenness of the community (Shokralla et al., 2012). These new and cost effective sequence technologies have been used to analyze microbial diversity in the marine water (Gilbert et al., 2008), soil (Urich et al., 2008), lake water (Oh et al., 2011), human gut (Qin et al., 2010), swine (Lamenda et al., 2011) and food (Jung et al., 2011). However, only few metagenomics studies have been done on wastewater treatment systems (Albertsen et al., 2012; Yu & Zhang, 2012; Ye et al., 2012) but not on tannery wastewater. Bioaugmentation of tannery wastewater with BM-S-1 leads to a complex microbial ecosystem consisting of bacteria, eukaryotes, archaea and viruses in which bacteria are dominant and play profound role in pollutant degradation and nutrient removal.

Assimilation, nitrification, denitrication and dissimilatory nitrite reduction to ammonia (DNRA) are the major nitrogen-cycle processes in biological wastewater treatment systems. Biological nitrogen removal processes in wastewater follow specific pathways under specific environmental conditions depending on the

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microbial communities involved and the substrate available. The frequencies of glutamate synthase (GS), glutamate dehydrogenase (GDH), nitrous oxide reductase (NOOR), nitrate reductase (NAR), nitric oxide reductase (NIOR), NAD.P.H nitrite reductase (NNIR), and ferredoxin subunit nitrite reductase (FSNIR) genes in the different stages of the treatment could be used to determine the dominant process leading to the effective biological nitrogen removal. The microbial community structures and functions in the wastewater treatment systems were examined using pyrosequencing to identify the dominant taxa and their potential roles in the effectives treatment, malodor and sludge reduction. We also used metagenomic to identify the metabolic processes and the microbial communities present after bioaugmentation in the full scale, and also to assess the different genes encoding the nitrogen metabolism processes as biomarkers to study the functional genes relating to nitrogen removal in the treatment process.

1.2 Literature review

1.2.1 Tannery production and its wastewater

The major producers of raw hides and skin are USA, Argentina, the former USSR, New Zealand, Australia, the near East and the EU. The EU however, is the highest producer of leather (**Fig. 1**) and also the largest and most dynamic consumer of leather goods. 25% of the world`s leather are produced in EU and 62% of the EU leather comes from Italy (**Fig. 2**) Besides EU, the major leather production centers in the world are Mexico, Brazil, Japan, South Korea, China, India, and Pakistan. Each kilogram of hides processed, generates 30 liters of effluent and during the

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tanning process, about 300 kg chemicals are added per ton of hides. Indian industries produce about 50,000 metric tons of wastewater per day.

In Hazaribagh, a particularly large tanning region of Bangladesh that has over 200 tanneries, about 7.7 million liters of wastewater and 88 million tons of solid waste are disposed of annually. These pollutants are responsible for the contamination of all nearby surface and ground water systems with severely high levels of chromium and sludge. The large amount of sludge and malodor generated which pose tremendous ecological risks. The main environmental directive that directly affects the leather tanning industry is Directive 96/61/EC concerning integrated pollution prevention and control (IPPC): this requires that plants for the tanning of hides and skins where the treatment capacity exceeds 12 tons of finished products per day are subject to the IPPC directive yet several production lines are below capacity and many are found in developing countries where it is difficult to monitor the treatment and waste disposal. According to the information collected in Blacksmith's inventory of sites, South Asia, particularly India and Pakistan and also South America have the highest number of tanning industries, with high risk of large populations and ecosystems being exposed to contamination.

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Europe

Rest of the world
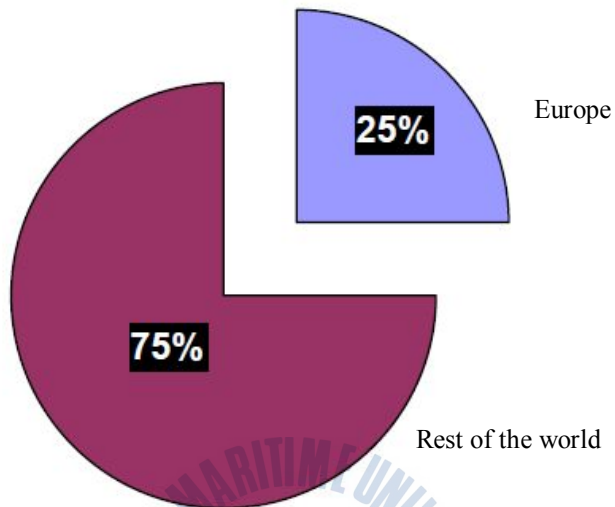
Fig. 1 Global leather production

(http://www.euroleather.com/french_brochure.htm)
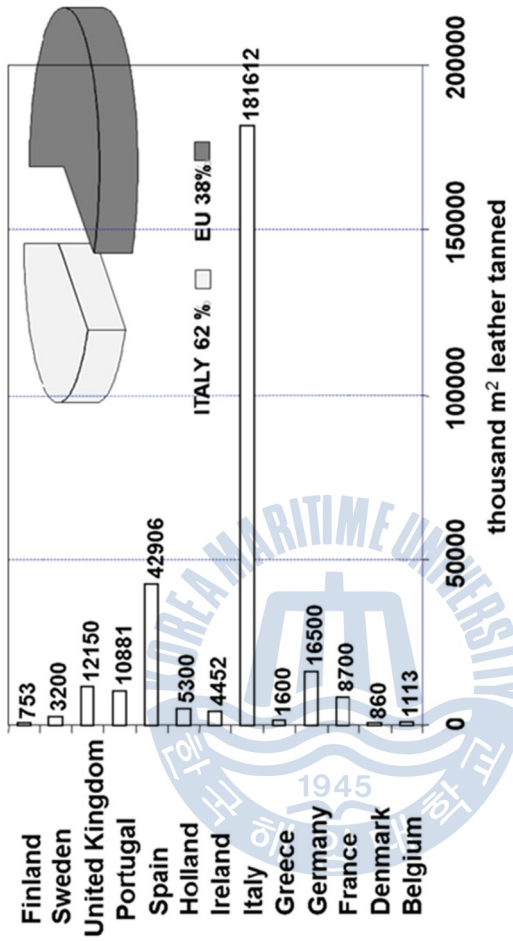
Fig. 2 EU standing on leather production (UNIC, 2013)

1.2.2 Details about BM-S-1 Consortium

BM-S-1 is novel microbial consortium enriched from the natural soils. This consortium could be used as probiotics, for agricultural purposes, and environmental remediation. The BM-S-1 patent culture (KCTC11789BP) has been deposited by BM, Inc. (Busan, Republic of Korea) at the Biological Resource Center, Korea Research Institute of Bioscience and Biotechnology (Daejeon, Republic of Korea). 16s rRNA pyrosequence analysis of BM-S-1 shows it is composed of 8 phyla, 19 classes and 49 genera. Firmicutes (70.6%), Bacteroidetes (23.9%), Proteobacteria (5.3%) and Actinobacteria (0.3%) were among the major phyla while Bacilli (67%) Bacteroidia (23.8%), Alpha proteobacteria (5.2%) and Clostridia (3.5%) were the major class. The genera Lactobacillus (59.6%), Prevotellaceae_uc (22.1%), Lactobacillaceae_uc (5.8%) and Acetobacter (5%) were also prevalent. The major species (≥5%) are *Prevotellaceae_uc_s* (22.1&), Lactobacillus_uc (13.7%), *Lactobacillus parabuchneri* (7.1%), *Lactobacillus paracasei* (7%) and unclassified species 4P001068_s (6%)(**Fig. 3**). A comparative study with another popular consortium known as effective microorganisms EM (Higa et al., 1998), reveals that BM-S-1 has a greater diversity and richness, and therefore could possess a wider range of applications (**Fig. 4** and **Table 1)** Other details of BM-S-1 relating the microbial composition would be found at Biological Resource Center, Korea Research Institute of Bioscience and Biotechnology (Daejeon, Republic of Korea).

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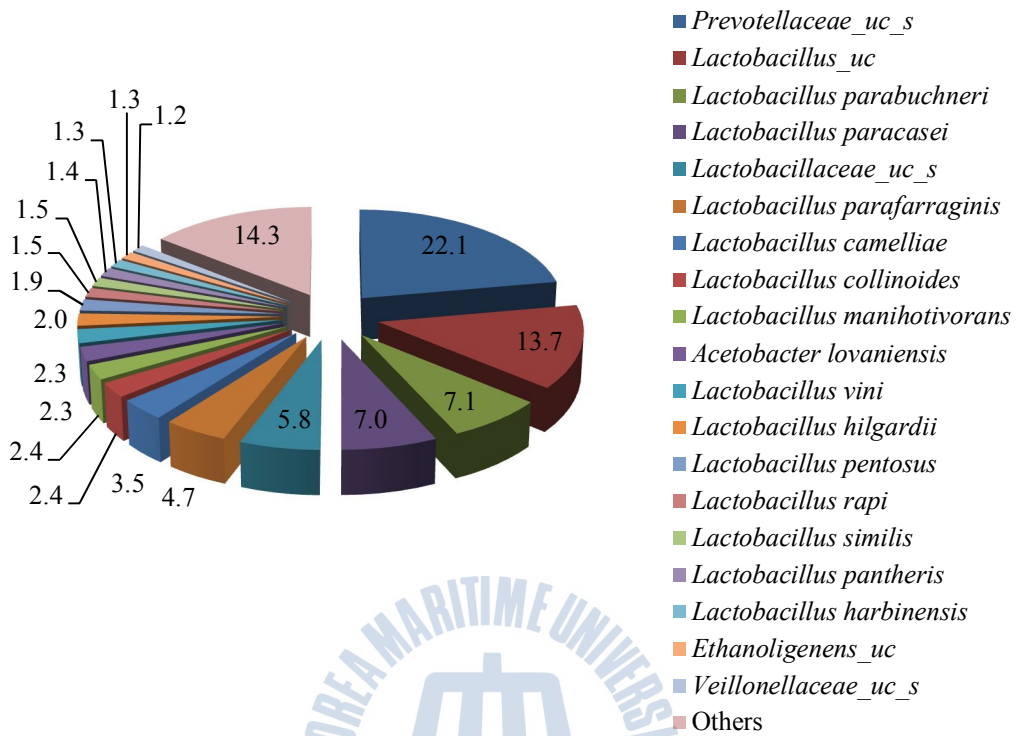
Fig. 3 The composition of major species in the mixed culture BM-S-1

Legend:
- *Prevotellaceae_uc_s*
- *Lactobacillus_uc*
- *Lactobacillus parabuchneri*
- *Lactobacillus paracasei*
- *Lactobacillaceae_uc_s*
- *Lactobacillus parafarraginis*
- *Lactobacillus camelliae*
- *Lactobacillus collinoides*
- *Lactobacillus manihotivorans*
- *Acetobacter lovaniensis*
- *Lactobacillus vini*
- *Lactobacillus hilgardii*
- *Lactobacillus pentosus*
- *Lactobacillus rapi*
- *Lactobacillus similis*
- *Lactobacillus pantheris*
- *Lactobacillus harbinensis*
- *Ethanoligenens_uc*
- *Veillonellaceae_uc_s*
- Others

Fig. 4 Rarefaction analysis of the consortia EM and BM-S-1

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Table 1 Comparison of diversity indices of EM and BM-S-1 consortia

	EM	BM-S-1
Analyzed reads	6172	6747
Normalized reads	6172	6172
Maximum length	459	489
Mean length	410.5	427.7
Observed OTUs	145	676
Chao1 estimation	223	1060.7
Shannon index	3.69	5.02
Goods coverage	0.99	0.96

Data obtained from the Mothur program Scholes et al. (2009) and based
on normalized reads of each sample

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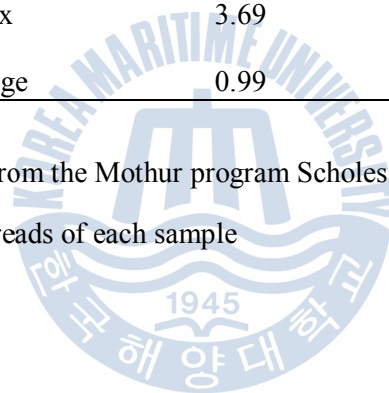
1.3 Overview of the nitrogen cycle

Nitrogen is a vital component of all biomolecules including DNA, protein, and chlorophyll (Bernhard et al., 2010). It is an element with the oxidation states of -3 to +5. All the oxidation states are possible in aqueous systems, and the inter-conversion of these molecules by different microorganisms under varied environment conditions forms the nitrogen cycle **Fig. 5** (Egli et al., 2003). Nitrogen fixation is the first reaction in the nitrogen cycle, a process in which molecular nitrogen is converted to ammonia by symbiotic nitrogen fixers (Miller et al., 2007) and made available to primary producers- plants (Bernhard et al., 2010). The ammonia thus generated is assimilated into the cells to form glutamine glutamate by glutamate dehydrogenase or glutamine synthase (Meti et al., 2011). Nitrification is the first process where ammonia is converted to nitrate by microbes. This chemoautotrophic process is carried out by ammonia oxidizing Bacteria (AOB) such as *nitrosomonas spp.* converting NH_3 to NO_2 and autotrophic nitrite oxidizing bacteria such as *nitrobacter spp.* converting NO_2 to NO_3 (Kowalchuk & Stephen, 2001). Heterotophic denitrifiers convert nitrates anaerobically to nitrogen gas (Zumft et al., 1997).
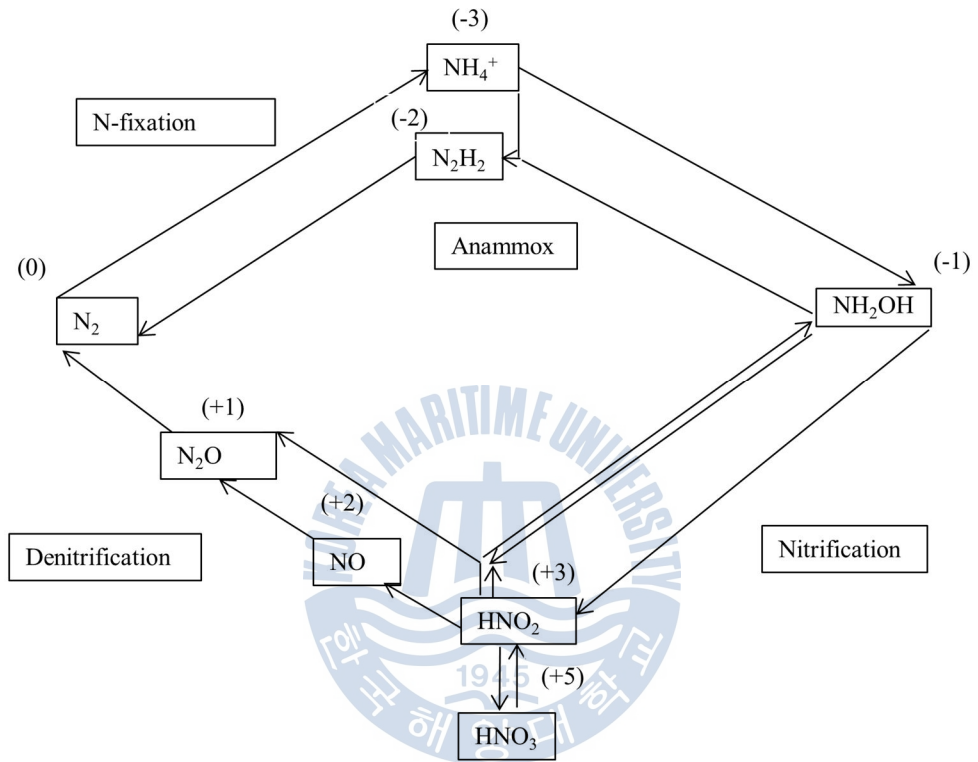
Fig. 5 The nitrogen cycle (Egli et al., 2003)

1.3.1 Nitrification pathways in wastewater

Nitrifying bacteria are chemoauthotrophs using carbon dioxide as carbon source for growth. Oxidation of ammonia to nitrite is a two-step process. This first step in nitrification is the oxidation of ammonia. Ammonia oxidation is the rate determining step in the nitrification (eqn. 1). It is usually done by ammonium oxidizing bacteria (AOB) belonging to the family nitrobacteraceae or ammonium oxidizing archaea (AOA). Nitrification plays important role in the removal of nitrogen in wastewater treatment plants. The enzymes ammonium monooxygenase (AMO) and hydroxylamine oxydoreductase (HAO) catalyze the oxidation of ammonia to nitrite. The presence of amoA genes as marker has helped to identify AOB and AOA in wastewater in treatment plants (Limpiyakorn et al., 2011). The growth of nitrifiers such as AOB and AOA in wastewater treatment plants depends on the aeration process. AOB population was known to increase while NOB decreased during anoxic exposure and gradually recovered when exposed to subsequent aerobic conditions (Ge et al., 2014). Other factor the affect nitrification are ammonia source, organic loading rate, pH, presence of inhibitors, UV light, ammonia concentration and nitrifying bacteria population.

Step 1: Conversion of ammonia to nitrite by ammonia oxidizing bacteria (AOB)

  (i) Ammonia to hydroxylamine carried out by Ammonia monooxygenase

$$NH_3 + O_2 + 2H + 2e^- \rightarrow NH_2OH + H_2O \ \dots\dots\dots\dots\dots\dots (1.1)$$

  (ii) Hydroxylamine to nitrite by hydroxylamine oxoreductase

$$NH_2OH + H_2O \rightarrow NO_2^- + 4H^+ + 4e^- \ \dots\dots\dots\dots\dots\dots (1.2)$$

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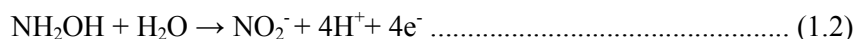
The second step is the oxidization of the nitrite to nitrate by nitrite oxidizing bacteria (NOB)

Step 2: Conversion of nitrite to nitrate by Nitrite oxidizing bacteria (NOB)

$NO_2^- + 1/2O_2 \rightarrow NO_3^-$ .... (1.3)

ΔG^o= -74KJmol^{-1}N

E^o= 434mV

Overall reaction:

$NH_3 + O_2 \rightarrow 2H^+ + NO_2^- + H_2O + 2e^-$ .... (1.4)
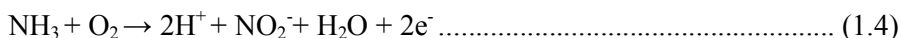
ΔG^o= -275KJmol^{-1}N

E^o= 343mV
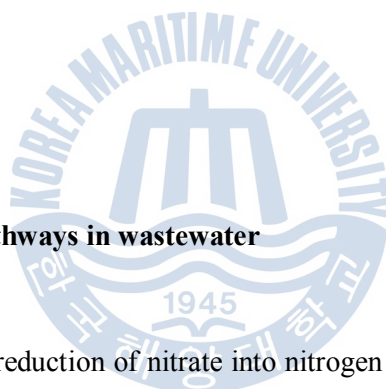
1.3.2 Denitrification pathways in wastewater

This is the microbial reduction of nitrate into nitrogen gas using nitrogen oxides (nitrate or nitrite) as terminal electron acceptors under anaerobic, microaerophilic, anoxic and occasionally under aerobic condition (**Fig. 6**). This is the process in the nitrogen cycle that reverses dinitrogen fixation, and is associated with chemolithotrophic, phototrophic, diazotrophic, or organotrophic metabolism. This process was discovered century ago and is believed to be exclusively a bacterial trait until recently that denitrification has been found in halophilic and hyper thermophilic archaea and in the mitochondria of fungi (Zumft et al., 1997). The biochemical characterization of denitrification and the associated genetics have been achieved with various organisms (like species from the genera *Pseudomonas*,

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Paracoccus, *Ralstonia*, *Bradyrhizobium*, *Rhodobacter*, *Magnetospirillum*, *Sphingobacterium* among others (Zumft et al., 1997; Ishii et al., 2011). The several steps in the denitrification processes are catalyzed different enzymes depending on the species nitrogen available. Functional genes such as nitrite reductase (*nirK* or *nirS*) or nitrous oxide reductase (*nosZ)* has been used as biomarkers to identify and study the distribution of denitrifiers in the ecosystem (Henry et al., 2008; Ishii et al., 2011). Numerous environmental factors can influence denitrification activity, such as nitrate and nitrite concentrations, pH, aeration, temperature, carbon availability, and relative activities of NO and N_2O reductases (Bergsma et al., 2002).

Nitrate (NO_3^-)

⬇ Nitrate

Nitrite (NO_2^-)

⬇ Nitrite

Nitric oxide (NO)

⬇ Nitric oxide

Nitrous oxide (N_2O)

⬇ Nitrous oxide

Dinitrogen (N_2)

Denitrification
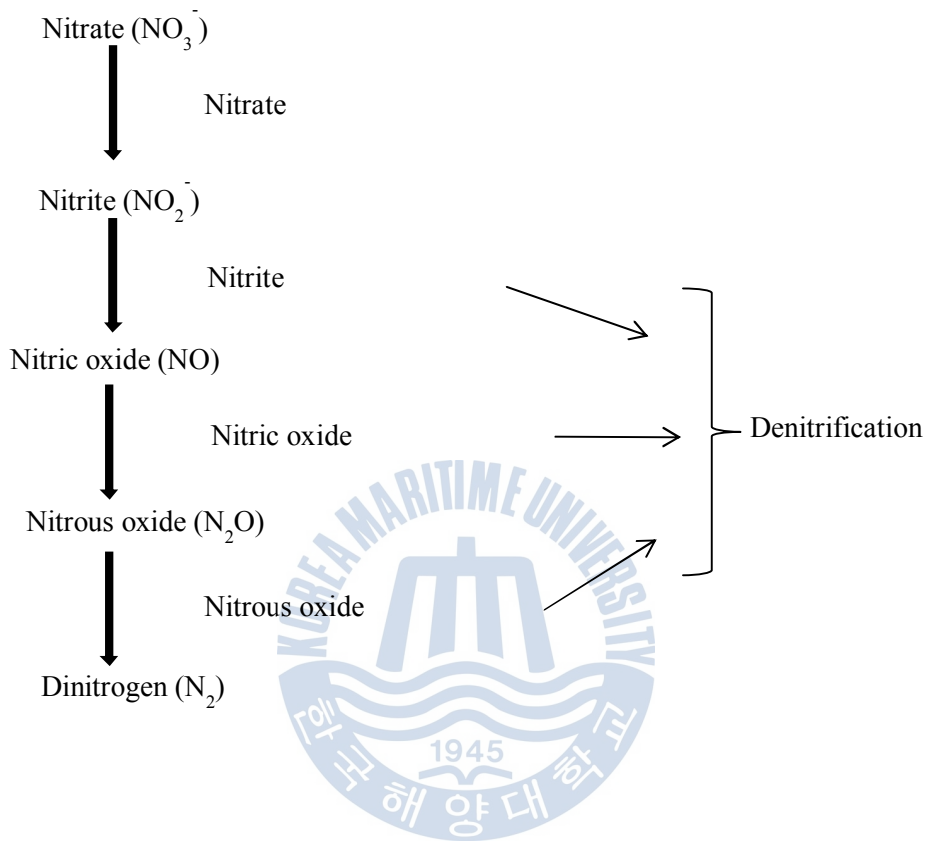
Fig. 6 The dissimilative nitrate reduction process (Brock et al., 2006)

1.3.3 Anaerobic ammonium oxidation in wastewater.

Anaerobic ammonium oxidation (anammox) is the conversion of ammonium as inorganic electron donor and nitrite as electron acceptor into nitrogen gas under anaerobic or anoxic condition. This reaction has lower free energy (-357 kJ/mol) than aerobic ammonium oxidation and therefore is thermodynamically more favorable. This process was first discovered in a pilot plant in Delft in the Netherlands (Mulder et al., 1995). It is known to contribute more than 50% of the nitrogen gas produced in the Oceans (Arrigo et al., 2005). Most bacteria that can perform anammox belong to the phylum planctomycetes and the five popular known genera include *Brocadia, Keunia, Anamoxoglobus, Jettenia and Scalindua* (Keunen et al., 2008). They are known for not having peptidoglycan in their cell wall and they contain anammoxone in their cytoplasm, a catalytic site for anammox reaction (Jetten et al., 2009). Anammox has the following advantages: (1) reducing energy which is used for aeration (2) reducing cost due to no organic need of organic carbon, (3) generating little sludge and (4) the emission of CO_2 is eliminated as it use as carbon source.

$$NH_4^+ + NO_2 \rightarrow N_2 + 2H_2O \dots\dots\dots\dots (5)$$
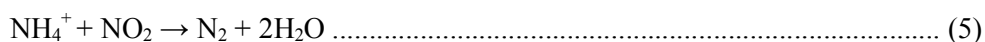
$\Delta G^o = -357 KJmol^{-1}N$

CHAPTER 2 MATERIAL AND METHODS

2.1 Design and operation of the pilot tannery wastewater treatment system

A treatment system using a kind of continuous flow reactor was employed to biologically treat the tannery wastewater. The built pilot treatment system consisted of tanks manufactured from PVC material and carried on steel elements as to ensure a firm fixation of the different units. The view of the pilot treatment system is shown in **Fig. 7**. Tannery wastewater was periodically supplied from a tannery company (Daejeon, Republic of Korea). This system consisted of a buffering tank (B), a sludge digestion tank (SD), a primary aeration tank (PA), a primary sedimentation tank (PS), a secondary aeration tank (SA), and a secondary sedimentation tank (SS)(**Fig. 8**). The BM-S-1 culture used in this experiment (KCTC11789BP) was obtained from the Biological Resource Center, Korea Research Institute of Bioscience and Biotechnology (Daejeon, Republic of Korea). It was grown a medium containing 0.3% w/v NaCl and 0.3% w/v molasses per liter of water, at an ambient temperature of $25^{o}C$ for ten days, reaching 10^{8}-10^{9} (cells/ml), and was then inoculated into B, SD, PA, and SA, daily, at a ratio of 0.01%, 0.01%, 0.002%, and 0.002%v/v, respectively.

The pilot treatment system treated approximately 40L of tannery wastewater, per day, with an HRT of 4.6days. The system was operated for six months before samples were collected for a consistent data analysis. The pH of the system was stably maintained between 7 and 9 during the operation. The system was designed such that the sludge from the sludge digestion tank was automatically returned to

the buffering tank at the rate of 16L/day while the settled materials from the secondary sedimentation tank was returned to the secondary aeration tank at the rate 4L/day using peristaltic pumps. This system did not work without BM-S-1. The accumulation of sludge after three weeks of running the control experiment without augmentation BM-S-1 almost broke down the system.

Fig. 7 Actual view of the pilot scale tannery wastewater treatment system

2.2 Growing the BM-S-1 for treatment of tannery wastewater

The stock culture was cultivated in a batch reactor carrying a medium (1.7 m^3; 3% wt/v molasses) using BM-S-1 (powder product; 10kg) as an inoculum at 20-25°C for 7days under an aeration (0.8-3.0 mg. DO/L). The stock culture was then sub-cultured in a batch reactor carrying a medium (50 m^3; 1% wt/v molasses) to reach 10^9 (cells/mL), and was then inoculated daily into B, PA, SA, and SD at ratios of 0.6% (culture: wastewater, v/v), 029%, 0.15%, and 2.00%, respectively.

2.3 Monitoring water quality of the tannery wastewater in the pilot scale system

The water quality of the tannery wastewater treatment processes was monitored in terms of COD, T-N, T-P, NH_4^+-N, NO_2^--N, NO_3^--N, alkalinity and mixed liquor suspended solids (MLSS). These parameters were measured following the Standard Methods for the Examination of Water and Wastewater (APHA, 2005). pH was measured using Neomet pH meter (Istek, Inc., Republic of Korea).

Fig. 8 Scheme of the pilot scale, tannery wastewater treatment plant, and hydraulic retention time for each treatment process

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2.4 Pyrosequencing analysis of microbial communities in the pilot scale treatment system

The profiles of bacterial community at each treatment tanks were analyzed using 16S rRNA gene-based pyrosequencing. Variable regions (V1□V3) of the 16S rRNA gene were amplified from genomic DNA of each sample from the pilot scale system, using fusion primers (Hur et al., 2011). Amplification, construction of the sequencing library, sequencing and bioinformatic analyses were according to previous studies (Chun et al., 2010; Kim et al., 2011; Kim et al., 2012) using 454 GSFLX junior Sequencing System (Roche, Brandford, CT, USA). Raw sequence reads were separated from their origins based on unique barcodes. Reads containing either two or more ambiguous nucleotides (with either average quality scores < 25 or short reads > 300bp) were removed any. After removing the low quality sequences, chimera checks was performed and taxonomic assignments of those reads was done using the extended EzTaxon data base (http://eztaxon-e.ezbiocloud.net/) (Kim et al., 2012). Statistical analysis of microbial communities was performed with the Mothur program, using a 3% difference cut-off value (Scholes et al., 2009). Random subtraction was used to normalize the different read numbers in each sample. Cluster analysis using Fast UniFrac distance was conducted with CL community software (Chunlab,Inc., Seoul, Republic of Korea).

2.5 Community analysis of denitrifying bacteria by real time qPCR in the full scale system

The abundances of denitrifying bacteria in I, B, PA, SA, and SD were monitored with quantitative PCR (qPCR) of the nitrous oxide reductase genes (*nosZ*), according to methods in Henry et al. (2006) with some modification. Standards for qPCR were generated by serial dilution of the plasmid that carries the *nosZ* gene, and the qPCR was performed with GoTaqq PCR Master Mix Green (Promega, Madison, WI) and a 7500 Real Time PCR System (Applied Biosystems, Foster City, CA). The conditions for qPCR cycle started with an initial denaturation step for 10 min at $95^{o}C$, followed by 40 cycles at $95^{o}C$ for 45 sec, $55^{o}C$ for 45 sec, $72^{o}C$ for 35 sec, and a measurement step at $80^{o}C$ for 35 sec.

2.6 Community analysis of denitrifying bacteria by real time qPCR in the pilot scale system

The treatment system located at Dungsung Inc. in People's Republic of China is a two stage activated sludge process with a sludge digestion tank. This system consisted of a buffering tank (B), a sludge digestion tank (SD), a primary aeration tank (PA), a primary sedimentation tank (PS), a secondary aeration tank (SA), and a secondary sedimentation tank (SS) (**Fig. 9**). The characteristics of the influent treated are outlined in **Table 2.** 750 (m^3/day) of sludge were generated from both PS and SS, and returned into PA and SA, alongside SD. About 750 (m^3/day) of sludge was treated in SD and the partially digested sludge was recycled into B. Hydraulic

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retention time for the whole system was 3.9 days. Aeration was provided to maintain DO levels of the system (1-5mg. DO/L). During steady state operation, the biorefractory residues (e.g. hair, skin, etc.) were routinely removed from the sludge with a micro-screen between B and PA, at a rate of 15% wt/wt (5m^3/day). The full scale system was implemented after the pilot scale treatment system was proved to be effective and efficient. It was proved that the full scale system could be feasible based on data from the pilot scale experiment. The full scale system was operated with augmentation of the microbial consortium BM-S-1, for more than six months. In all the four stages, there were little of aggressive odors typically observed in the conventional tannery wastewater treatment plant.
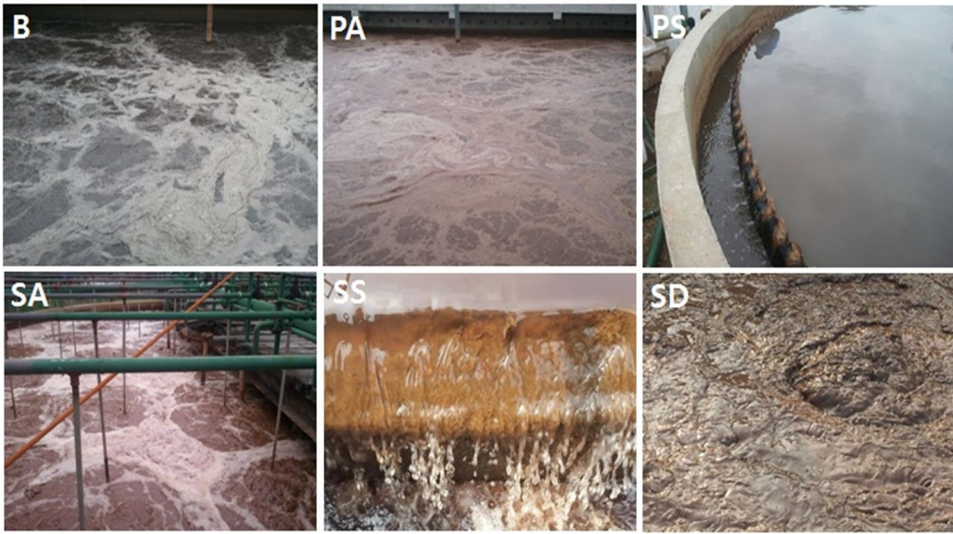
Fig. 9 View of the full scale tannery wastewater treatment plant (Dungsung Leather Inc., People's Republic of China)

Table 2 Characteristics of the influent used in the experiment

Parameter	Concentration range
pH	8 ~12
COD_{Cr}	4,500 ~7,700
BOD	1,800 ~ 2,400
SS	2,000 ~ 2,520
T-N	320 ~ 690
NH_4^+-N	260 ~ 380
NO_2^--N	1.28 ~ 1.86
NO_3^--N	0.062 ~ 0.094
T-P	4.82 ~ 6.84
S^{2-}	65 ~ 92
Cr^{3+}	6.4~8.8
Na	139.6
Mg	3.19
Al	0.20
Fe	0.28
Pb	0.08
Ni	0.02

* All values are in mg/L except pH
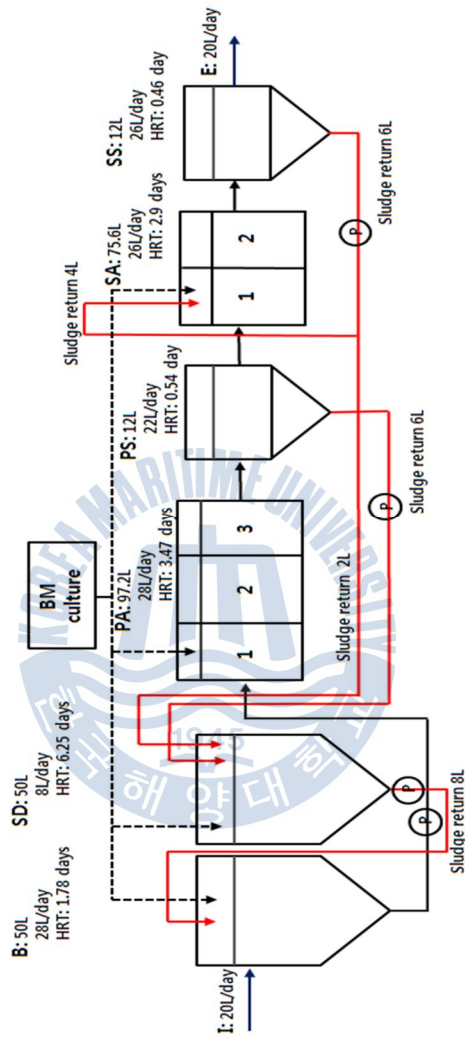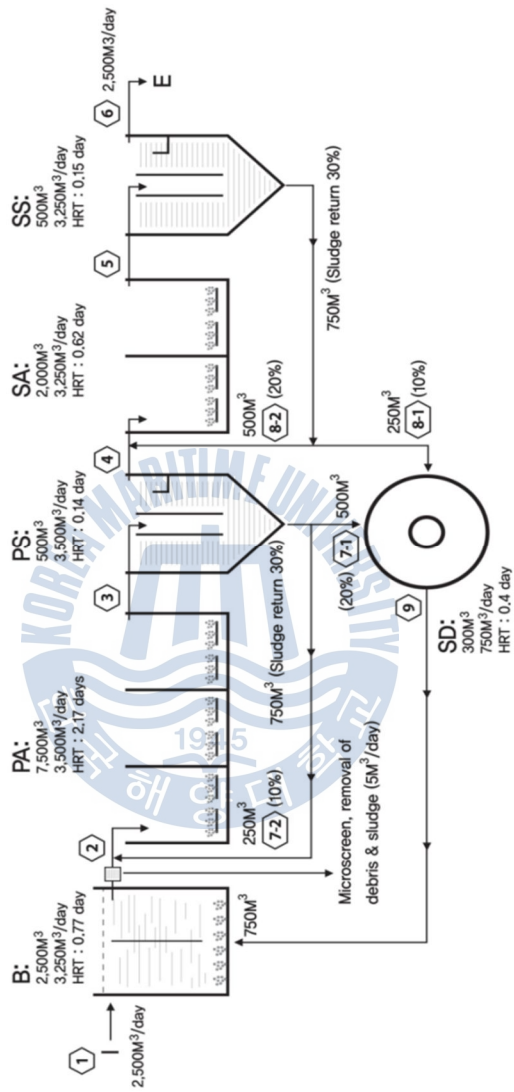
Fig. 10 Scheme of the full scale, tannery wastewater treatment plant, and hydraulic retention time for each treatment process

2.7 Analysis of wastewater and mass flow calculation in the full scale treatment system

The pH, COD, TN, TP, MLSS, and alkalinity, in both the pilot and the full scale wastewater treatment systems were measured following the standard methods (APHA, 2005).For total chromium analysis, 4.5mL of pretreated sample (APHA, 2005)was used in the inductively coupled plasma-atomic emission spectrometer (ICP-AES; OPTIMA 2100DV; Perkin Elmer Inc., Waltham, MA, USA) to determine total chromium. At the full scale plant, 2,500 m^3 of tannery wastewater was treated daily with an HRT range of 0.14—2.17 days per treatment process (**Fig. 10**). The average mass flow of each measurement (COD, TN, TP, and MLSS) was calculated by multiplying the aqueous phase concentrations with the corresponding average flow for each wastewater treatment process (I, B, PA, PS, SA, E, 7-1, 7-2, 8-1, 8-2, and SD):

$$m_{aq}= Q_{aq}C_{aq} \dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots \text{(2.1)}$$

where

m_{aq} (tonsday^{-1}) = the mass flow of the measurement calculated in the aqueous phase, Q_{aq} = m^3day^{-1} wastewater flow, and C_{aq} = the average concentration(mg/L) in each wastewater sample. COD, TN, and TP concentrations and removal efficiencies were statistically compared to the influent using paired t –test.

2.8 Pyrosequencing analysis of microbial communities in full scale treatment of wastewater

Bacterial community compositions in the different treatment tanks were examined using 16S rRNA gene-based pyrosequencing. Variable regions (V1-V3) of the bacterial 16S rRNA gene were amplified from genomic DNA of each sample from the full scale system, using fusion primers (Hur et al., 2009). Amplification conditions, construction of the sequencing library, sequencing, and analyses used the previously applied (Chun et al., 2010; Kim et al., 2011) 454 GS FLX Junior Sequencing System (Roche, Brandford, CT, USA). During the filtering process, we separated sequence reads that originated from different samples with unique barcodes, and we removed any reads containing either two or more ambiguous nucleotides (with either average quality scores < 25 or short reads > 300bp). Chimera checks and taxonomic assignments of those reads were performed using the extended Ez Taxon data base (http://eztaxon-e.ezbiocloud.net/) (Kim et al., 2012). Statistical analyses of microbial communities were performed with the Mothur program, using a 3% difference cut-off value (Scholes et al., 2009). To compare samples with different read numbers, the sizes of different samples were normalized, where applicable, by random subtraction. Principal coordinate analysis (PCoA) and fast Unifrac analysis were conducted with CL community software (Chunlab, Inc., Seoul, Republic of Korea). Comparisons of community structure between samples were based on Bray-Curtis and Jaccard similarity analyses, and their significance was determined by Libshuff analysis.

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2.9 Community analysis of denitrifying bacteria by real time qPCR in the full scale system

The abundances of denitrifying bacteria in I, B, PA, SA, and SD were measured with quantitative PCR (qPCR) of the nitrous oxide reductase genes (*nosZ*), according to methods in Henry et al. (2006) with some modification. qPCR standards was generated by serial dilution of the plasmid that carries the *nosZ* gene. The qPCR was performed with Go Taqq PCR Master Mix Green (Promega, Madison, WI) and a 7500 Real Time PCR System (Applied Biosystems, Foster City, CA). Terminal restriction fragment length polymorphism (T-RFLP) analysis of *nosZ* genes was conducted to compare denitrifying bacterial community structure among B, PA, SA, and SD. The primers, *nosZ*1F (6'-FAM label) and *nos* Z2R, were used to amplify *nosZ* genes in each sample, using PCR condition described in Henryet al. (2006). The purified amplicons (699bps) were digested with 10U of *HhaI* restriction enzyme (New England Biolab, Ipswich, MA, USA) and then precipitated with 75% isopropanol and placed into an ABI 3130xl automated genetic analyzer for fingerprint analysis (Applied Biosystems, Foster City, CA, USA). T-RFLP patterns were analyzed and compared using the Gene Mapper program (Applied Biosystems, Foster City, CA).

2.10 Sampling and metagenomic analysis of microbial communities

2.10.1 Sample collection and DNA extraction

Samples used in this analysis were collected from the different stages of the treatment process; influent (I), buffering (B), primary aeration (PA), secondary aeration (SA) and sludge digestion (SD) from Nantong Dongsung Leather Co., Ltd. (Nantong, People's Republic of China). These samples were immediately preserved with RNAlater buffer solution (Qiagen Inc. CA, USA) on ice and transported to the laboratory. DNA extraction was performed according to the user manual using MOBIOPower Soil DNA Isolation Kit (MOBIO Laboratories Inc. Carlsbed, CA, USA). The extracted DNA was quantified using Nanodrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). This system has been in operation for 3 months before sampling. The DNA sample concentrations were 64 ng/μL, 78 ng/μL, 63 ng/μL, 64 ng/μL and 58 ng/μL for I, B, PA, SA and SD respectively.

2.10.2 Whole metagenome sequencing

The extracted DNAs were fragmented using a NEBNextds DNA Fragmentase (NEB, MA, USA). The size of 300-400bp was excised and extracted from 2% agarose gel using an Qiaquick gel extraction kit (Qiagen Inc. CA, USA). Fragemented DNAs was afterwards processed according to the TruSeq DNA LT Sample prep kit (Illumina, San Diego, CA, USA). Paired end sequencing (2x150bp) was performed on an IlluminaMiSeq system following the manufacturer's protocol.

2.10.3 Metagenomic sequence analysis

Raw sequences from MiSeq machine were uploaded on the MG-RAST server (http://metagenomics.anl.gov), filtered by the QC pipeline of MG-RAST (Meyer et al., 2008). Functional annotation was assigned using the SEED, COG, and KEGG database (e-value < 1e-5 and alignment length $\geq$ 40bp). The *p*-value threshold of each gene cluster comparison was under 0.01. For taxonomic assignment of functional genes, we constructed a reference genome database containing COG information. The genome data was obtained from NCBI, and we annotated their function genes against the COG database with RPS-Blast (MArchler-Bauer et al. 2003), and constructed reference genome database containing COG information.

CHAPTER 3 ANALYSIS OF THE PILOT SYSTEM

3.1 Monitoring water quality of the tannery wastewater in the pilot scale system

The influent characteristics as well as the parameters monitored in the treatment process were shown in (**Table 3**). Like most tannery wastewater the influent has a high COD values. The high COD value of the influent might been as a result of high organic material content of the tannery wastewater and the chemicals used in the tannery process (Durai et al., 2011). Removal efficiencies of COD, T-N and T-P were 91.4%, 77.9%, and 89.4%, respectively. The removal efficiencies of COD, T-N and T-P were 68.6%, 51.3%, and 39.8% respectively in stage B and gradually decreased from PA to SA. This indicates that a significant amount of organic material could have been removed at the first stage of the treatment system due to effect of BM-S-1 augmentation and further reduced because BM-S-1 was added to the PA, SA and SD. The MLSS increased 7times in B compared to I, more than 14times in SD and decreases down the stages. Generally, B stage appeared to show higher microbial activity compared to the other stages of the treatment as reflected in the most of the parameter analyzed. Alkalinity also decreased down the stages compared to the influent following the similar trend with the pH of the system (reduced by 73%).

Other parameters such as NH_4^+, NO_2^- and NO_3^- also varied at the different stages but did not follow a specific pattern which might be due to the existence of these compounds in unstable and gaseous forms in the system. The results demonstrated that BM-S-1 augmentation was effective at reducing COD, T-N, and T-P. Although

much of the pollutants are removed at B, the system configuration ensured greater pollutant removal efficiency. The system was configured so that all processes except SA, included mild aeration (1□3 mg. DO/L), a condition that allows for effective performance of BM-S-1 consortium. Also, a two-stage treatment system that employs primary and secondary stages of aeration and sedimentation has been known for enhancing high pollutant removal, as previously shown (Calheiros et al., 2009). The system design therefore, must have helped in the efficiency of this system. The MLSS increase at B was probably due to increased biomass boosted by BM-S-1 augmentation and the partially digested sludge recycled from SD. The progressive increase in MLSS compared to the influent as in B and SD has also been known to contribute to sludge reduction (Low et al., 1999), as experienced in this system. It was not possible to treat the tannery wastewater successfully without the augmentation of BM-S-1, indicating that this treatment system was dependent on the augmentation.

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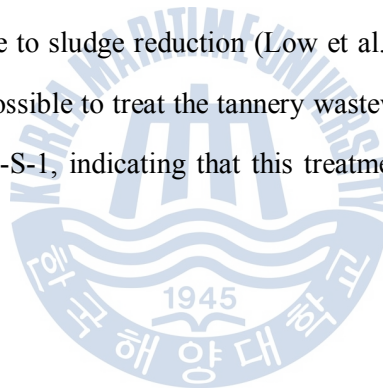
Table 3 Pilot scale system water quality measurements of tannery wastewater undergoing bioaugmentation with the BM-S-1 microbial agent

Treatment Stage	I	B	PA	PS	SA	E	SD	Removal (%)
COD (mg/L)	1334	419*	336*	361*	288*	115**	1029	91.4
T-N (mg/L)	114.3	55.7	43.5	49.4	40.8*	25.3*	36.6*	77.9
T-P (mg/L)	12.7	7.65	4.37	4.03	3.43	1.35**	4.87	89.4
NH_4^+-N	43.15	33.3	26.7	42.4	31.24	21.2	30.65	50.9
NO_2^--N	0	0.52	1.48	1	1.71	1.94	1.4	
NO_3^--N	0	0.23	0.18	0.22	0.07	0	0.3	
(mg/L)	700	5050	4600	0	1120	360	10130	
Alkalinity	445.6	191	163	185	160.8	121.7	160.9	
pH	7.9	7.8	7.8	7.6	7.4	7.3	7.6	

All data were measured in duplicate or triplicate. I, Influent; B, Buffering tank; SD, Sludge digestion; PA, Primary aeration; PS, Primary sedimentation; SA, Secondary aeration; E, Effluent.* $p < 0.05$ when t-test for the treatment stages was performed against influent(I).** $p < 0.01$ when t-test was performed against influent(I)

3.2 Pyrosequencing analysis of microbial communities in the pilot scale system

A total of 47,576, reads (average length = 462.9bp) were obtained from the GS Junior System **(Table 4).** 41,911 sequences from total raw reads were analyzed in this study, after trimming sequences of either short length or low quality, and removing chimeras. Different analyzed read numbers of samples were normalized to 5000 reads per sample, as described at previous work (Kim et al., 2012; Gihring et al., 2012; Price et al., 2009). The lowest number of OTUs was observed in influent sample (1684 OTUs) and the highest number of OTUs in primary aeration (2,448 OTUs). The highest value of Shannon diversity index and estimated Chao1 were obtained from PA compared to the other stages. The coverage values of PA and SA were lower than those in other samples because their diversity values were higher. The high diversity of bacterial communities in PA and SA were also reflected in rarefaction curves (**Fig. 11**).
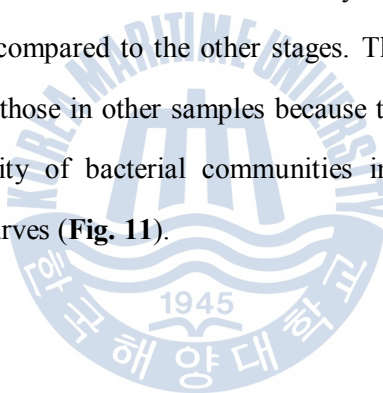
Table 4 Summary of pyrosequencing analysis of the pilot system

Samples	Total reads	Analyzed reads	Normalized reads	Mean length (bp)	Maximum length (bp)	Observed OTUs	Chao1 estimation	Shannon index	Goods coverage
I	5,563	5,278	5,000	474.5	536	1,684	11,238.60	5.57	0.72
B	8,687	7,340	5,000	462.2	549	1,858	14,564.30	5.73	0.67
PA	19,231	17,003	5,000	459.4	541	2,448	30,202.20	6.32	0.55
SA	8,260	7,220	5,000	459	533	2,226	21,769.90	6.17	0.6
SD	5,835	5,070	5,000	459.5	542	1,724	11,027.90	5.6	0.7

Diversity indices were obtained from the Mothur program (Scholes et al., 2009) based on normalized reads of each sample
* bp; base pair

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The influent I consists of four major phyla (phyla > 2%) of the total analyzed reads; Firmicutes (51.3% of total reads), Proteobacteria (14.4%), Bacteroidetes (12.8%) and Tenericutes (16.2%). The relative abundance of phyla from the BM-S-1 inoculates of each process, were Firmicutes (87.8% of analyzed reads) and Proteobacteria (12.2%). The influent microbial community changed starting from B stage (HRT = 0.89days) due to the inoculation of BM-S-1 (**Fig. 12**). The dominant phyla from B were Proteobacteria (54%), Bacteroidetes (19.6%), Planctomycetes (10.4%), Deionococcus-thermus (7.2%) and Cynobacteria (3.6%), respectively. The relative abundance of the population in PA was Proteobacteria (57.4%), Cynobacteria (4.4%), Bacteroidetes (20.2%), Planctomycetes (10.4%) and Deionococcus-thermus (3.4%).

The microbial phylum populations in SA were quite very similar to PA that in except that Cynobacteria increased about 6%. The dominant phyla in SD were Proteobacteria (55.1%), Bacteroidetes (21.6%), Planctomycetes (7.7%), Deionococcus- thermus (5.8%) and Cynobacteria (3.8%). Actinobacteria (2.6%) were also present in SD in higher amount than all the stages. Firmicutes were present in both B and SD 0.6% each while they were present in PA and SA 0.9% each, indicating their significant reduction from the influent and inoculums BM-S-1. This is one of the challenges in this treatment application, the population of the major constituents of consortium decreased instead of increasing although the BM-S-1 is being applied daily. This might have been due to the fact we did not analyze the population after seeding and before the treatment started. It could also be that the BM-S-1 augmentation enhanced the succession of microbial population capable of degrading the influent since the influent communities also did not increase.
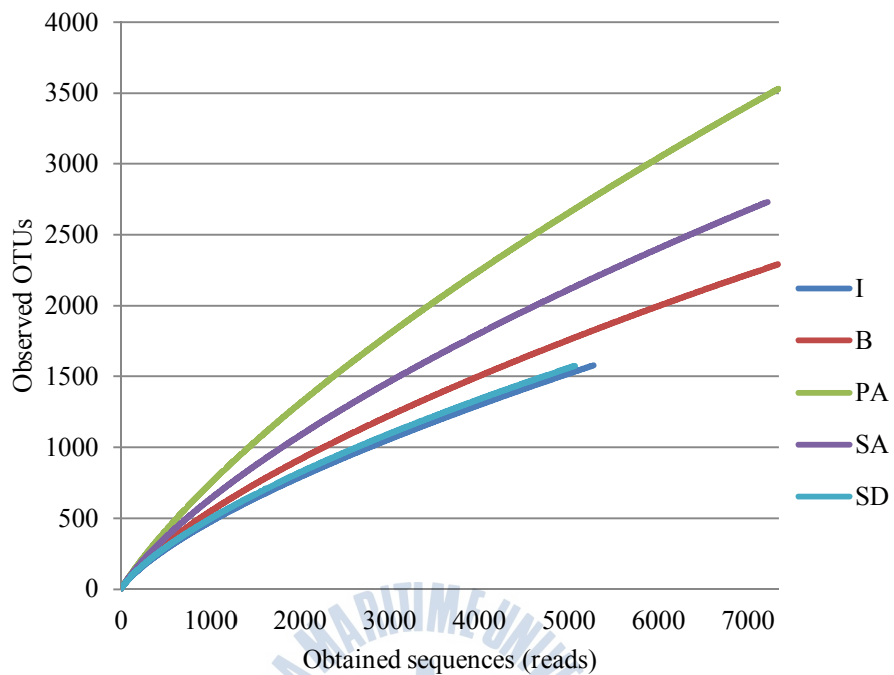
Fig. 11 Rarefaction curves of the five different samples from the pilot wastewater treatment system. Sample size was normalized by random subtraction. I, influent; B, buffering tank; PA, primary aeration tank; SA, secondary aeration tank; SD, sludge digestion tank

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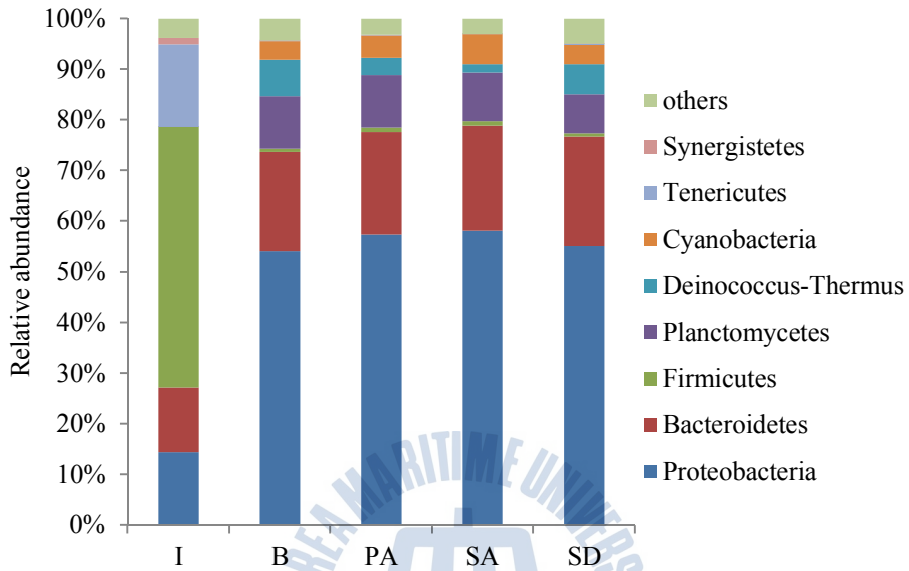
Fig. 12 Bar charts showing the percentage abundances of the phyla microbial communities of the different stages from the pilot scale tannery wastewater treatment system. I, influent; B, buffering tank; PA, primary aeration tank; SA, secondary aeration tank; SD, sludge digestion tank

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The genus *Dokdonella* was significantly present in B (7.3%), SD (5.9%), PA (5.6%) and SA (5.0%) while the uncultured genus AB430337_f_uc was also dominant B (6.9%), SD (4.9%), PA (6.2%) and SA (5.8%). *Methyloversatilis,* FJ529971, and EF648038_f_uc_g were present in all the stages in close proportions (**Fig. 13**). The genus *Sphingomonas* was more dominant in PA and SA, while EU283558_g was dominant in B and SD. *Paracoccus* and FJ230926_g were highly present in SD compared to other stages. Other significant genera (genus > 2%) in B, PA, SA and SD include *Xanthomonadaceae*_uc*, Arenimonas, Thauera*_uc*, Sterolibacterium*_f_uc, *Arenibacter, Phycisphaerales*_uc_g and *Bordetella.* We also investigated the bacterial community at the genus level; Genera of *Lactobacillus* (86% of total reads) and *Acetobacter* (9.6%) were the major groups in the BM-S-1 consortium. *Acholeplasma* (12.1%) from the phylum Tenericutes while Clostridiales_uc_s (6.6%), Proteiniclasticum, (9.7%) and GU196243_s (7.2%) were the dominant genera within Firmicutes in the influent sample.
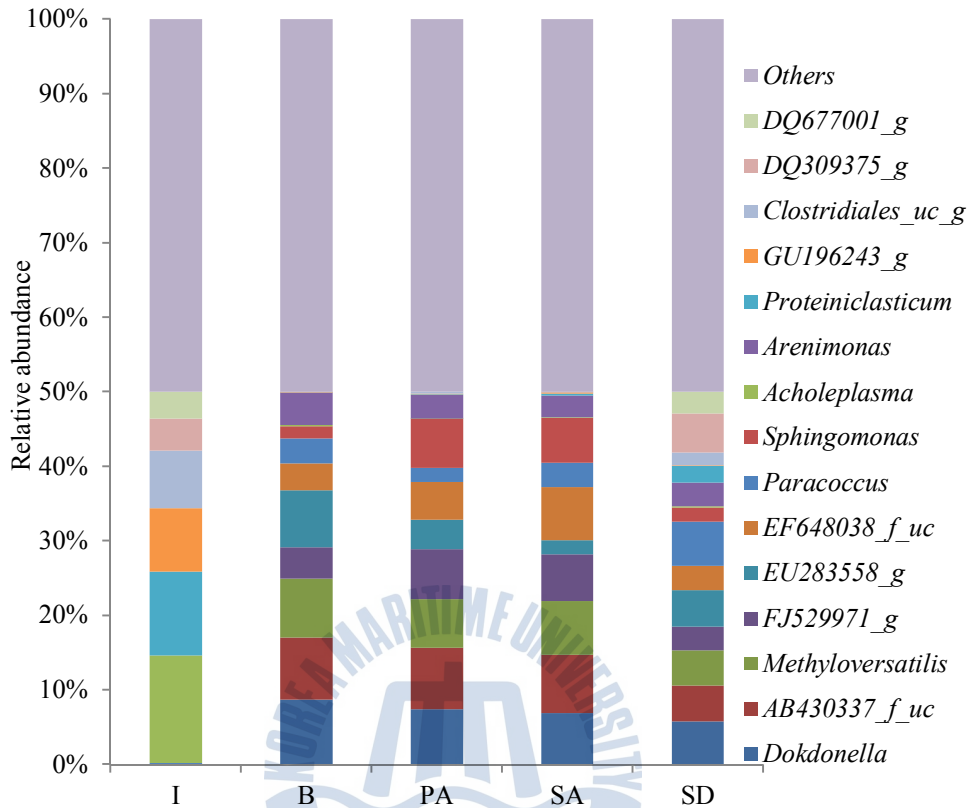
Fig. 13 Bar charts showing the percentage abundances of the genus microbial communities of the different stages from the pilot scale tannery wastewater treatment system. Genera representing more than 3% of total reads, from each treatment, were selected for the analysis. I, influent; B, buffering tank; PA, primary aeration tank; SA, secondary aeration tank; SD, sludge digestion tank

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The species composition was also identified in the various stages (**Fig. 14**) AB430337_f_uc_s, *Methyloversatilis*_uc, AB430336_s, 4P000382_s, Xanthomonadaceae_uc_s, Flavobacteriaceae_uc_s were abundant in all the stages. The uncultured species AB430337_f_uc_s (4.9-6.9%) from the class Phycisphaerales were present in all the stages and some species in this class were facultative anaerobic heterotrophs known for their denitrifying activities (Fukunaga et al., 2009) AB430336_s, belonged to the genus *Dokdonella*, a gram-negative, aerobic, non-spore-forming bacterium which has been isolated from soil and wastewater treatment plant (Li et al., 2012). However, AB430336_s, *Arenimonas donghaensis*, *Dokdonella*_uc and Xanthomonadaceae_uc_s all belong to the family Xanthomonadaceae, a well known group of denitrifiers and possessing *NosZ genes* (Torrentó*et al*., 2011).Other dominant species were *Flavobacteriaceae_uc_s, Vitellibacter_uc, Arenibacterechinorum,* and *Muricauda_uc* were all member of the family Flavobacteriaceae which are known for odor removal and denitrificationacivities. *Muricauda* and *Vitellibacter* were both carbohydrate utilizers while *Vitellibacter*is known for nitrate reduction (Nedashkosvkaya et al., 2003a; Nedashkosvkaya et al., 2003b). *Thauera aromatica* was also present especially in PA. This species is known for its ability to degrade aromatic compounds.

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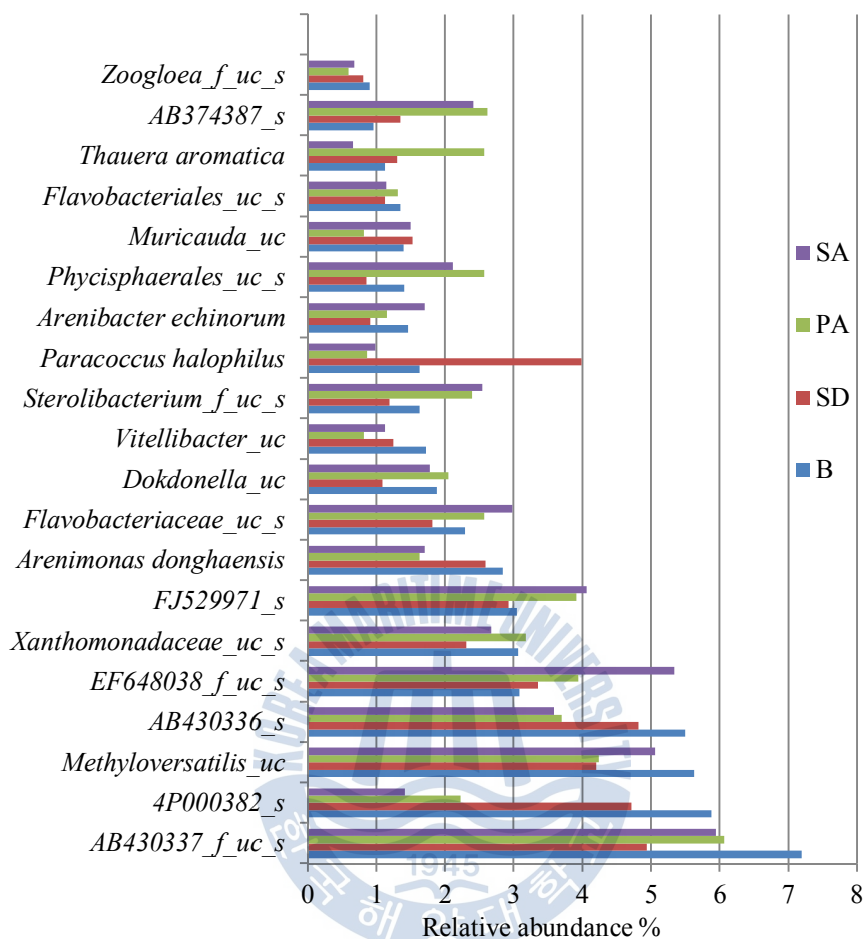
Fig. 14 Comparative analysis of the dominant species in the different stages of the pilot scale treatment system. Dominant species representing more than 1% of total reads, from each treatment, were selected for the analysis

Cluster analysis based on Unifrac distance of the species in the different stages of the treatment process showed that PA was similar to SA while B is closer to SD. The community structure similarity between PA and SA might be a result of the similar aeration conditions and the closeness HRT between these stages (**Fig. 15**). B also showed a similar community structure to SD despite the difference in aeration conditions probably because the sludge in SD was continuously recycled to B. Though there were some similarities in the species present at the different stages, a comparison based on Taxon Exclusive Analysis (Chunlab), a program that compared the presence/absence of the normalized Taxons hierarchy at the different stages showed that 5.7% of the species in I are not in B, 3.4% of the species in B were absent in PA, while 2.5% of the species community in PA were not SA. Also, 2.6% of the species communities in SA were not in SD while 3.1% of species in SD are not in B. This shows how the initial species community has adapted or evolved due to the BM-S-1 bioaugmentation thereby enhancing the tannery wastewater treatment.
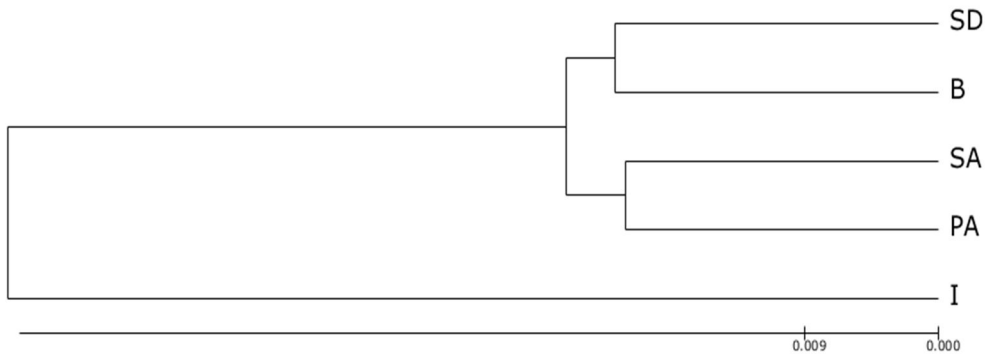
Fig. 15 Unweighted pair group method with arithmetic mean (UP-GMA) cluster analysis for the pyrosequenced community data of the different processes of the pilot scale treatment system. I, influent; B, buffering tank; PA, primary aeration tank; SA, secondary aeration tank; SD, sludge digestion tank

### 3.3 Denitrifying bacteria genes by real time qPCR in pilot scale system

The *nosZ* genes known as nitrous oxide reductase genes are responsible for the conversion of nitrous oxide (N_2O) to nitrogen (N_2) gas. This is the last step in denitrification process. The quantitative PCR (qPCR) of the nitrous oxide reductase genes (*nosZ*), showed that *nosZ* genes were dominant in the buffering stage B (596,666.7 ± 263,333.3 copy no./ng DNA), followed by PA (147,454.3 ± 76,363.7), SA (82,898.6 ± 51598.6) and SD (20,745.7 ± 1258.1)(**Fig. 16**).

The abundance of the denitrifiers increased approximately 734 times in B, as compared to the influent of wastewater, after the BM-S-1 consortium was augmented and acclimated. This indicated that a most significant amount of the microbial activities including denitrification might have occurred in B. The abundance of this gene corroborated with the high COD removal which took place at this stage of the system, leading to a great reduction of sludge through the denitrification process. It was reported that a denitrification stage added to the aerobic treatment caused about 25% sludge reduction in sludge production (McClintock et al., 1988). The true yield (yield in the absence of maintenance energy requirements) of heterotrophic microorganisms using nitrate as a terminal electron acceptor is supposed to be less than 78% of that realized under aerobic conditions (Payne, 1981). This shows that the sludge reduction in the treatment system could be attributed to the denitrification activity.
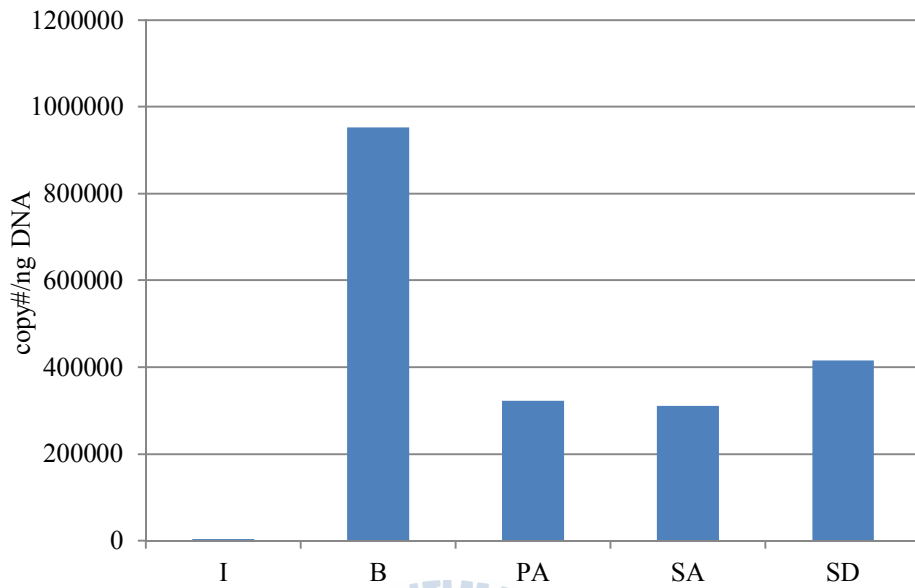
Fig. 16 Copies of *nosZ gene* quantified by RT-QPCR at the different stages of the treatment of the pilot scale system
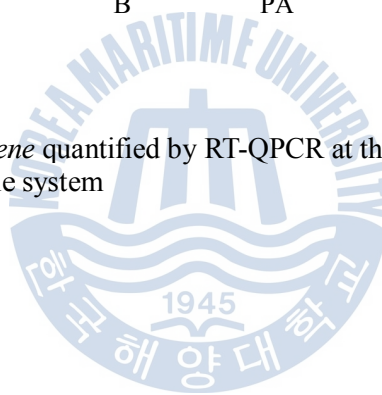
CHAPTER 4 ANALYSIS OF THE FULL SCALE SYSTEM

4.1 Monitoring water quality of the tannery wastewater in the full scale system

The full scale treatment of the tannery wastewater, with consortium BM-S-1 augmentation, was performed for more than six more months before taking samples for mass flow analysis (**Table 5**). The pH was stabilized from 9.0 (influent) to 7.0 (effluent) during the biological treatment. Average removal efficiencies of COD, TN, TP, and Cr were 98.3%, 98.6%, 93.6%, and 88.5%, respectively. The concentrations of COD, TN, and TP maintained similar patterns, as their values drastically decreased from the buffering stage (B) and became stable from the secondary aeration process (SA) to the effluent (E) as was in the pilot system. COD, TN, and TP in the influent were removed by up to 88%, 81%, and 75%, at B. The highest microbial activities appeared to be at B, since organic materials (presumably tannery-derived ones and the lysed cell biomass recycled from SD) were rapidly degraded. The tannery biomass composition (mostly lipids and proteins from the influent) caused high COD, TN, and TP that were associated with the tannery waste.

The augmented BM-S-1 appeared to enhance the degradation and utilization of tannery organic wastes, thereby decreasing the COD level. The removal efficiencies for COD, NH_4^+-N and TP were 84.1%, 97.5% and 97.5%, respectively in the tannery wastewater treatment plant of Nantong Dongsung Leather Co., Ltd. where the conventional traditional chemical treatment system (before renovation) was applied. This chemical treatment technology formally used by the company required a significant amount of ferrous sulfate, sodium hydroxide and coagulant polymers to precipitate the pollutants. The dewatered sludge cake (60 $m^3 day^{-1}$; 80% in

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moisture) is generated for each daily amount of influent (2,500 m^3/day) (communication from Nantong Dongsung Leather Co., Ltd). This indicates that the chemical treatment technology is environmentally unfriendly and costly.

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Table 5. Concentrations and mass flows for COD, TN, TP, and MLSS in the full scale, tannery wastewater undergoing bioaugmentation with the BM-S-1 microbial agent

Treatment Stage	COD Conc (mg/L)	COD Mass (ton/day)	T-N Conc (mg/L)	T-N Mass (ton/day)	T-P Conc (mg/L)	T-P Mass (ton/day)	Conc (mg/L)	MLSS Mass (ton/day)
1 (I)	6910.1	17.275	487.2	1.218	5.54	0.0138	3210	8.025
2 (B)	796.4*	2.588	92.6*	0.301	1.39*	0.0045	11200	36.4
3 (PA)	634.4*	2.22	87.7*	0.307	2.20*	0.0077	9500	33.25
4 (PS)	226.4*	0.792	22.9**	0.08	1.57*	0.0055	trace	trace
5 (SA)	163.9**	0.532	31.2**	0.101	0.47**	0.0015	10830	35.2
6 (E)	117.1**	0.293	10.5**	0.026	0.36**	0.0009	2	0.05
01-7	126	0.063	99.1	0.05	2.5	0.0013	35280	17.64
02-7	126	0.032	99.1	0.025	2.5	0.0006	35280	8.82
01-8	168	0.042	143.2	0.036	3.41	0.0009	41560	10.39
02-8	168	0.084	143.2	0.072	3.41	0.0017	41560	20.78
9 (SD)	1730	1.298	81.5	0.061	3.05	0.0023	27740	20.805
% Removal	98.3		98.6		93.6			

*$p < 0.05$ when t-test for treatment stages 2–6 was performed against the influent (I).
** $p < 0.01$ when t-test was performed against the influent (I)

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Previous studies utilizing the activated sludge process have shown that removal efficiencies of COD (880-1,900 mg/L) varied from 80 to 90% depending primarily on the biodegradability of the particular influent (Ahn et al., 1996; Munz et al., 2008; Ramteke et al., 2010). Moreover, a higher COD level of influent (2,000-7,600 mg/L) gave a poor removal rate (approx. 67%) for an activated sludge system as a pretreatment process (Feo et al., 2009). However, the full scale system in our study achieved COD removal efficiency (98%) for the level of influent (6,910 mg/L). The BM-S-1 augmentation and the other indigenous microbes might have facilitated the degradation of 14,687 kg COD day^{-1} and led to increase of 25,400kg MLSS day^{-1} (**Table 5)**. Therefore, F/M in B might be 0.59 (=14,678 kg COD/25,400 kg MLSS). A comparison between the BM-S-1 system and other conventional treatment systems (**Table 6**) shows that the BM-S-1 system was more effective in the removal of COD, T-N and T-P. In terms of efficiency, the BM-S-1 system was better also, having a lower HRT and average MLSS and MLVSS.

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Table 6 Comparison of various treatment process for different tannery wastewaters

Process	COD (mg/l)	TKN/TN* (mg/l)	TCr (mg/l)	HRT(Hrs)	MLVSS/ MLSS**(g/l)	SVI (mg/l)	COD (%)	TKN/ TN***(%)	TCr(%)	References
BM-S-1	6,910±55.5	487.2±10.3	32±16	3.9days	9.5-12**	45-50	98.3	98.6***	88.5	Kim et al., 2014
SBR+ Respirometry	4,800±350	225 ± 18	95± 55				80–82	78–80		Ganesh et al., 2006
AS	1,412-1,454	311-369		10--38			97-81			Roš et al., 1998
UASB	2,500±900			5days	9.3--44.9**		78			Lefevre et al., 2006
FO + Thiobacillus ferrooxidans	2,533		253				96		5	Mandal et al., 2010
CETP	6,240	168/327*	13.3				74			Durai et al., 2010
CETP	12,466±321	578±63*	.16				86.9	58.1***	87.9	2011
AS	880-12,132	65.8		12	2.9-3.8	90-100	72-87			Ahn et al., 1996

AS; activated sludge, CETP common effluent treatment plant, SBR; sequence batch reactor, FO; Fenton oxidation, UASB; Upflow anaerobic sludge blanket

4.2 Sludge reduction in the full scale system

One of the challenges in tannery wastewater treatment is excess sludge production. The BM-S-1 augmentation allowed a significant reduction of sludge compared with other traditional treatment systems. In our system, 28,000kg MLSS day^{-1} were entering SD and 20,800kg MLSS/day were leaving, showing a sludge reduction of 25.7% in SD, at an ambient temperature of 28-35°C and an HRT of 9.6h. However, the overall sludge reduction rate was about 83% at HRT of 3.85days compared with the non-renovated system (communication from Nantong Dongsung Leather Co., Ltd). The system was configured so that all processes, except SA (3-5 mg. DO/L), included mild aeration (1-3 mg. DO/L), a condition which favors less sludge production and BM-S-1 consortium function. Reduced excess sludge production was accomplished by implementing an oxic-settling-anoxic process which could reduce sludge yield by 21–56% at a sludge retention time of 6.75 h in an anoxic tank (Ye et al., 2010). Bioaugmentation of *Brevibacillus sp.* could enhance thermophilic sludge digestion by about 12% where they could degrade EPS (exopolysaccharides), promote decay of cells and inhibit the growth of certain kinds of microorganisms (Li et al., 2009). For our study, optimal conditions for sludge digestion (reduction) in the various stages still need to be determined considering initial MLSS, HRT and degradation products.

4.3 Pyrosequencing analysis of microbial communities in the full scale system

A total of 31,636 reads were obtained (average length = 464.1bp) from the GS Junior System (**Table 7**). To statistically compare diversity values across samples, sample sizes were normalized to 1,312 reads per sample, as highly recommended in previous work. The numbers of observed OTUs increased from influent materials (263 OTUs) to sludge digestion (629 OTUs). Estimated Chao1 was also the highest at the SD stage (2368.6). Rarefaction curves also showed a higher increase in diversity (**Fig. 17**). This result indicates that bacteria richness and diversity increased throughout the treatment process, along with calculated diversity indices. The number of phyla present at SA (14 phyla; > 0.5% of the total analyzed reads) was higher than those in other processes. The relative abundance of phyla and genera, from the BM-S-1 inoculates of each process (**Fig. 18**), was analyzed and two dominant phyla Firmicutes (87.8% of analyzed reads) and Proteobacteria (12.2%), were found in the BM-S-1 consortium, while Firmicutes (94.6%) was dominant in the influent. The influent microbial community shifted from the B stage due to 0.77 days of incubation and BM-S-1 inoculation (**Fig. 10**). The dominant phyla from the buffering process were Proteobacteria, Firmicutes, Bacteroidetes, and Chloroflexi, whose proportions shifted from the SA stage. The relative abundances of Proteobacteria (49.5% of total reads at B and 36.1% at SA) and Firmicutes (18.6% at B and 1.51% at SA) decreased from B to SA, while their abundance increased again at SD (53.3% of Proteobacteria and 8.6% of Firmicutes).

Table 7 Summary of pyrosequencing analysis of the full scale system

Samples	Total reads	Analyzed reads	Normalized reads	Mean length(bp)	Maximum length(bp)	Observed OTUs	Chao1 estimation	Shannon index	Goods coverage
BM-S-1	11,053	5,387	1,312	485.1	531	134	231.5	3.19	0.95
I	6,290	4,292	1,312	458.1	514	263	874.2	3.26	0.85
B	1,705	1,313	1,312	462.8	510	263	746.6	4.15	0.88
PA	2,027	1,538	1,312	456.2	542	299	546.7	4.71	0.88
SA	6,956	4,926	1,312	456.8	523	505	1372.6	5.43	0.74
SD	3,605	2,982	1,312	465.8	528	629	2368.6	5.6	0.62

Diversity indices were obtained from the Mothur program (Scholes et al., 2009) and based on normalized reads of each sample
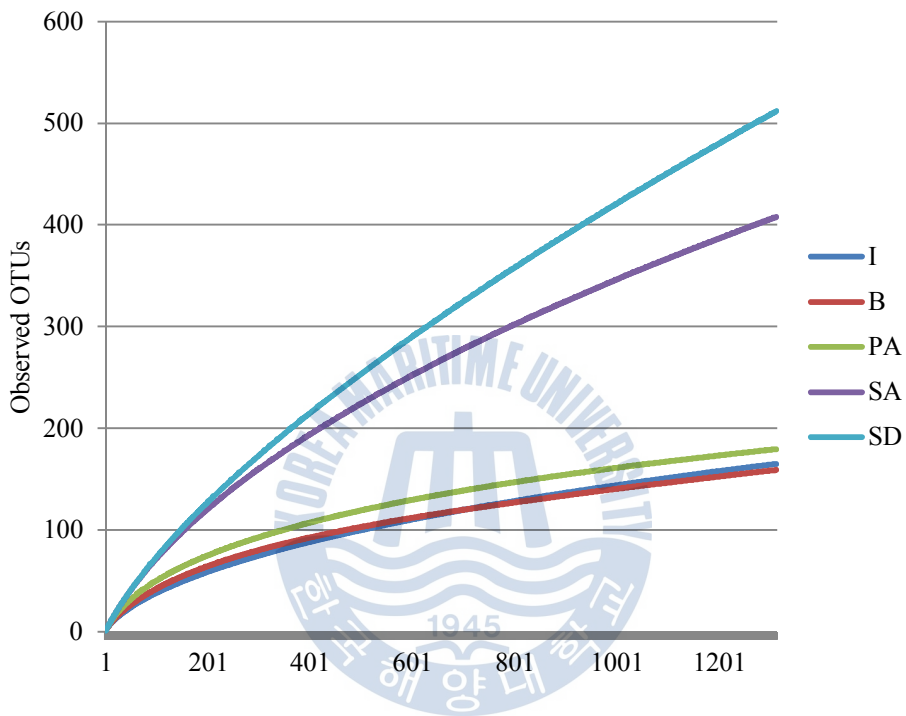
Fig. 17 Rarefaction curves of the five different samples from the full scale tannery wastewater treatment system. I, influent; B, buffering tank; PA, primary aeration tank; SA, secondary aeration tank; SD, sludge digestion tank; and sample size was normalized by random subtraction

Community shifts were also investigated at the genus level (**Fig. 18**). Genera of *Lactobacillus* (86% of total reads) and *Acetobacter* (9.6%) were the major groups in the BM-S-1 mixed culture. *Proteiniclasticum* (31.1% of total reads), uncultured *Carnobacteriaceae* (17.2%), and *Alkalibacterium* (12.2%) were dominant in the influent. The relative abundance of *Proteiniclasticum* was significantly reduced at B (from 31.1% to 2.4%), suggesting (1) significant reactions occurred in B due to a physical buffering reaction, and (2) recycled materials from SD. *Brachymonas* were dominant in B (37.7% of total reads), SD (22.1%), and PA (17.3%). The species composition in SA was different from those of B, PA and SD which had more similarity (**Fig. 19**). The bacterial community in SD was similar to those of B and PA, most likely due to the treatment process and the amount of treated water in that process. The treated water from PS was aerated in SA for 0.62 days. 500m^3 of sludge from PS entered SD, and 250m^3 of sludge from SS entered SD. Digested materials in SD were recycled to B. This process appeared to make the bacterial communities of B, PA, and SD more similar than that of SA Despite addition of BM-S-1 to B, PA, SA and SD, the dominant species in the culture (grown on molasses) such as *Lactobacillus* and *Acetobacter* were barely detected in the four treatment stages. This indicates that the bioaugmentation of BM-S-1 may not necessarily contribute to a significant increase of a population density of its composing species. *B.denitrificans* was the dominant species in B (37.5% of total reads), SD (19.3%), and PA (16.9%) samples, but was reduced to 1.55% in SA. *B. denitrificans* efficiently removed nitrogen (NO_3^-) in a pilot plant that was augmented with this species (Leta et al., 2005).

Phylum
- Firmicutes(37.9%)
- Proteobacteria(32.18%)
- Bacteroidetes(15.62%)
- Chloroflexi(6.8%)
- Deinococcus-Thermus(2.42%)
- Actinobacteria(1.19%)
- TM7(0.66%)
- Bacteria_uc(0.64%)
- Gemmatimonadetes(0.49%)
- Tenericutes(0.39%)
- Acidobacteria(0.34%)
- EF688356_p(0.25%)
- Thermotogae(0.18%)
- Chlorobi(0.14%)
- Planctomycetes(0.14%)
- EU245879_p(0.11%)
- Verrucomicrobia(0.1%)
- Spirochaetes(0.1%)
- OD1(0.08%)
- Synergistetes(0.06%)
- GN02(0.05%)
- SR1(0.05%)
- Cyanobacteria(0.04%)
- EU246057_p(0.04%)
- WS5(0.01%)
- Lentisphaerae(0.01%)
- OPB7(0.01%)
- NKB19(0.0%)
- OP11(0.0%)

Genus
- WS3(0.0%)
- Lactobacillus(14.34%)
- Facklamia(13.75%)
- AY945885_g(3.59%)
- Carnobacteriaceae_uc(0.97%)
- Proteiniclasticum(0.93%)
- Alkalibacterium(0.41%)
- Aerococcaceae_uc(0.41%)
- Clostridiales_uc_g(0.35%)
- Trichococcus(0.29%)
- DQ309375_g(0.28%)
- GU196243_g(0.26%)
- GU196243_f_uc(0.22%)
- Enterococcus(0.2%)
- EU864476_g(0.18%)
- Lactobacillales_uc_g(0.17%)
- Clostridium_g17(0.16%)
- Sporolactobacillus(0.15%)
- Halolactibacillus(0.12%)
- EU844484_f_uc(0.11%)
- Brachymonas(13.14%)
- Comamonas(1.97%)
- Acetobacter(1.61%)
- Thiobacillus(1.05%)
- DQ836749_g(0.96%)
- AY957890_g(0.92%)
- Bdellovibrionales_uc_g(0.87%)
- Thauera(0.79%)
- Alcanivorax(0.66%)
- AB186842_g(0.64%)
- Desulfuromonas_g1(0.54%)
- Comamonadaceae_uc(0.47%)
- Catellibacterium(0.28%)
- Desulfuromonas_g2(0.27%)
- Methylibium(0.27%)
- Burkholderiales_uc_g(0.25%)
- Steroidobacter(0.23%)
- EF632934_g(0.23%)
- Gluconacetobacter(0.23%)
- Dinoroseobacter(0.22%)
- Sterolibacterium_f_uc(0.21%)
- Roseomonas(0.2%)
- Derxia(0.15%)
- Betaproteobacteria_uc_g(0.15%)
- Phenylobacterium(0.13%)
- Pseudofulvimonas(0.13%)
- Alteromonadaceae_uc(0.13%)
- Gluconobacter(0.12%)
- AF170421_g(0.12%)
- Desulfococcus(0.12%)
- Thiobacillus_f_uc(0.12%)
- Zoogloea_f_uc(0.12%)
- AY792265_g(0.12%)
- Rhodobacteraceae_uc(0.12%)
- AB425068_g(0.12%)
- Oceanospirillales_uc_g(0.11%)
- Parvibaculum(0.11%)
- AY570558_g(0.11%)
- Methylophilaceae_uc(0.1%)
- AJ306739_g(3.86%)
- Brumimicrobiaceae_uc(1.61%)
- EU644166_g(1.43%)
- FJ269057_g(1.24%)
- Flavobacteriales_uc_g(0.97%)
- AY197415_g(0.82%)
- Bacteroidales_uc_g(0.75%)
- Sphingobacteriales_uc_g(0.56%)
- FJ437992_f_uc(0.51%)
- EF174263_g(0.45%)
- AY197415_f_uc(0.41%)
- Ferruginibacter(0.37%)
- EU592366_g(0.36%)
- AB355052_g(0.3%)
- Paludibacter(0.29%)
- DQ677001_g(0.2%)
- EU644166_f_uc(0.19%)
- EU234264_g(0.13%)
- Bacteroidia_uc_g(0.11%)
- AB195892_f_uc(0.1%)
- AJ009469_g(2.19%)
- Anaerolinaceae_uc(2.05%)
- AM933658_g(0.35%)
- EU542451_g(0.28%)
- Anaerolineales_uc_g(0.28%)
- AF280867_o_uc_g(0.24%)
- EU234165_g(0.19%)
- AF395429_f_uc(0.17%)
- AJ306798_g(0.14%)
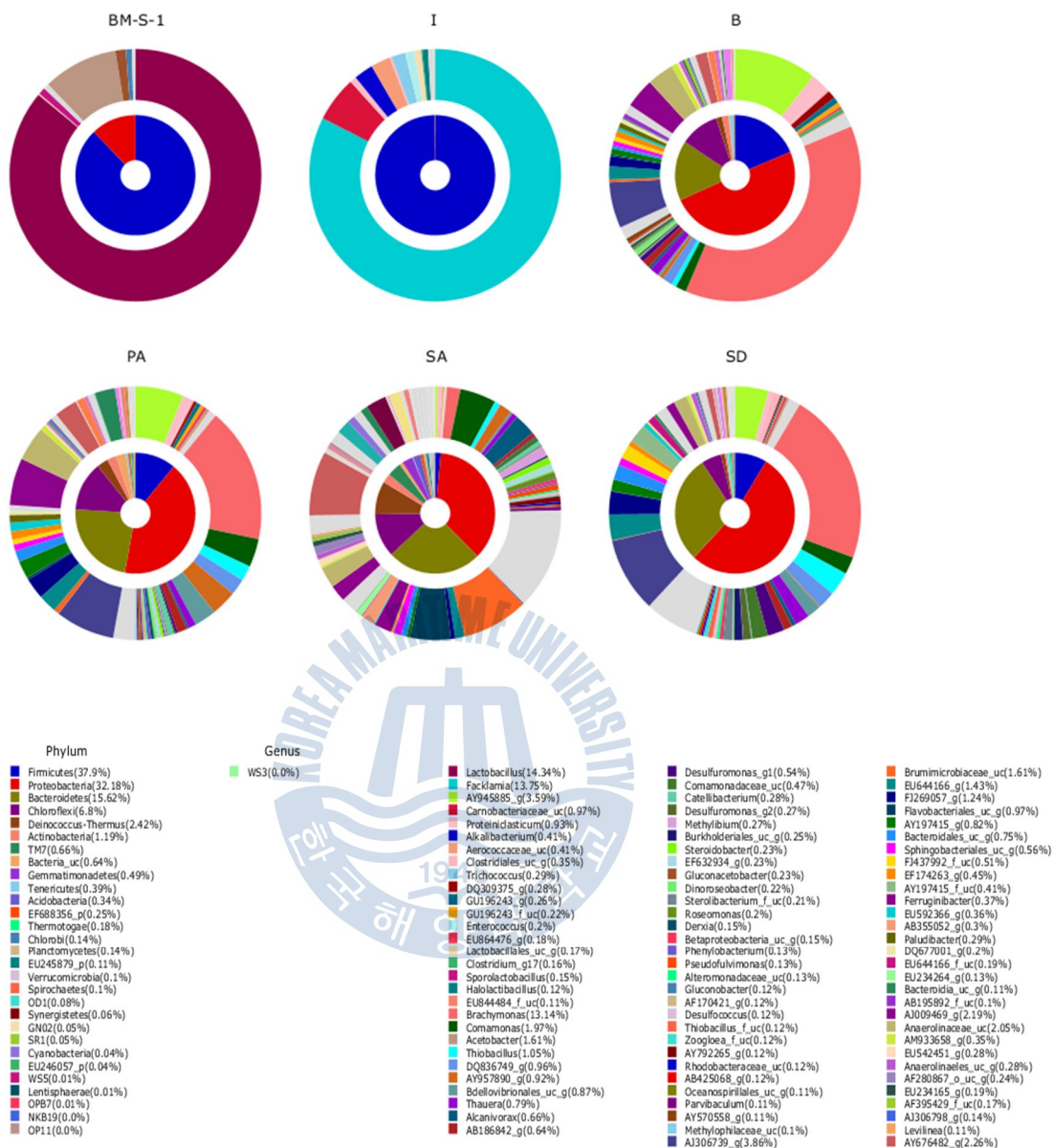- Levilinea(0.11%)
- AY676482_g(2.26%)

Fig. 18 Double pie charts of microbial communities of different samples from the BM-S-1 and full scale tannery treatment system. The inner pie represents the compositions of phyla, and the outer pie shows the compositions of genera. I, influent; B, buffering tank; PA, primary aeration tank; SA, secondary aeration tank; SD, sludge digestion tank
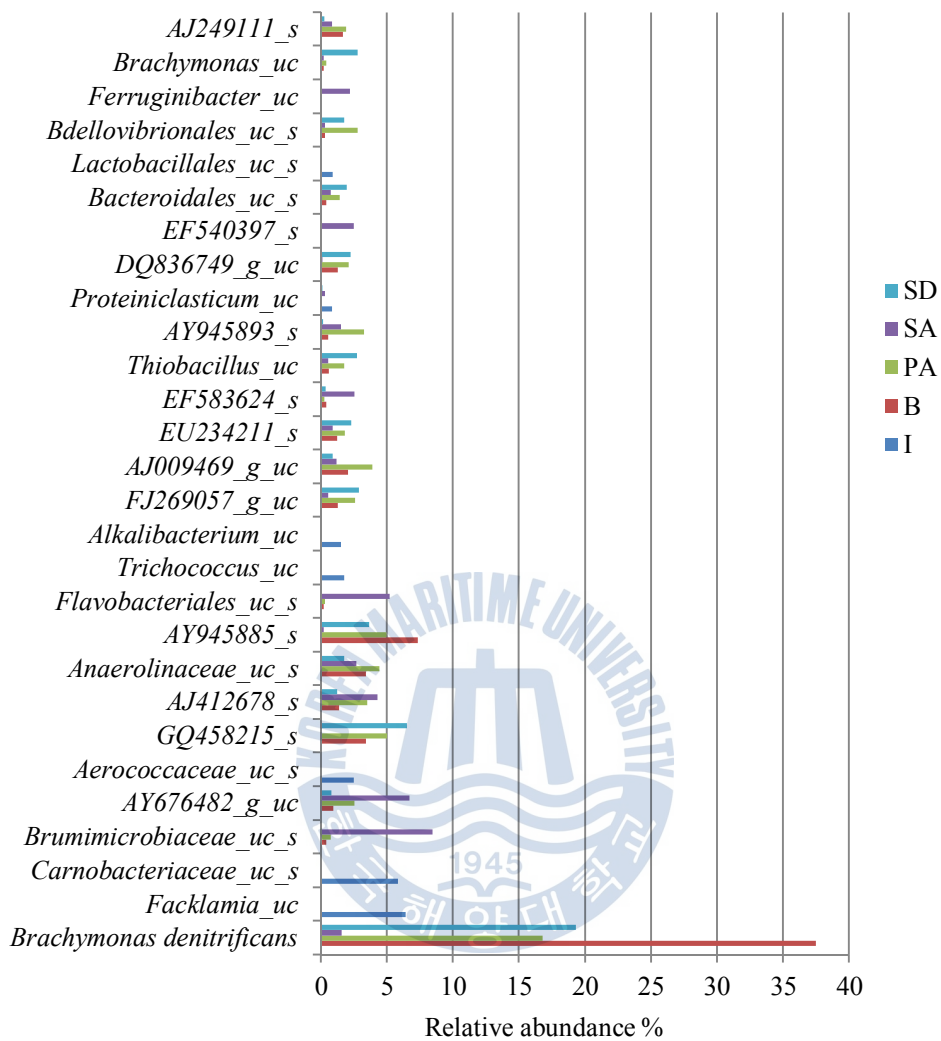
Fig. 19 Comparative analysis of the dominant species in the different stages of the full scale treatment system. Dominant species representing more than 2% of total reads, from each treatment, were selected for the analysis

Uncultured bacteria similar to AY945885, which have been shown to be involved in bioreactor denitrification (Liu et al., 2006), were also dominant in B, PA, and SA of this study. The higher proportions of *B.denitrificans* and AY945885_s, in B, PA, and SD, might be related to the denitrification reactions in our system. Bioaugmentation of *B.denitrificans* significantly enhanced denitrification in the anoxic tank of a pilot tannery effluent treatment plant (Leta et al., 2005). The *Brachymona*s genus has been shown to be dominant in raw wastewater and adjusting buffering tanks with DO < 1 mg. DO/L (Sadaie et al., 2007)where nitrate reduction and sludge reduction could be performed. Our study supports these previous findings since *B.denitrificans* were dominant in B and SD. The partially digested sludge, recycled from SD, could be rapidly utilized by populations of *B.denitrificans,* AY945885_s, GQ458215_s, uncultured *Anaerolinaceae*_s, and uncultured AY945885_g, which were all present in B. Moreover, all other communities might be also involved in the degradation and utilization of sludge, and denitrification, leading to effective sludge reduction in a treatment system. An uncultured *Brumimicrobiaceae*, similar to AF289153, EU803487 and available in rivers and lakes (Shaw et al., 2008; Zwart et al., 2002), was abundant in SA. Presence of this population could be an indication of effective and efficient treatment, with BM-S-1 augmentation, in the tannery wastewater treatment. The high abundance of denitrifying bacteria appeared to decrease of NO_2^- concentration in B, SD, and PA (data not shown).This shows denitrification-related reactions could be active in B, SD, and PA.

In this study, the dominant species at SD, which was more anoxic compared to PS and SS, were *Brachymonas denitrificans*, uncultured *Thiobacillus*, uncultured *Clostridia*_s, uncultured *Brachymonas*, and *Desulfuromonas palmitatis* (**Fig. 19**). *Firmicutes* and *Clostridia* may excrete extracellular enzymes, and generate volatile

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fatty acids (VFAs) and alcohols through the consumption of polymeric proteins and lipids (Cirne et al., 2007). *Desulfuromonas palmitatis*, a sulfate reducer, generates H_2S, which can be subsequently utilized by uncultured *Thiobacillus*, a sulfur oxidizer contributing to odor removal. Moderate DO (1-2 mg. DO/L) and nutrient depletion in SD could lead to changed microbial physiology, death, and decomposition. Dead and lysed cell biomass could then support the growth of BM-S-1 and coexisting indigenous microbes. Flow of activated sludge from an aerobic tank into an anaerobic one may promote sludge destruction and degradation due to the activities of Firmicutes and Clostridia (Quan et al., 2012). Organic substances could then be released and further degraded into smaller and lower molecular weight materials (VFAs, alcohols, etc). These metabolites could then be utilized by various aerobes and anaerobes present in the subsequent aerobic tank, leading to cryptic growth of sludge.

Similarities in bacterial communities among samples of different treatment processes were further investigated, using Unifrac distances of species distribution (**Fig. 20**). Influent (I) was different from the rest of the samples, showing the diverse change in community caused by bioaugmentation. B and PA were closely clustered, not too far from SD, likely due to the nutrient availability and recycling similarities among these stages. SA was quite distant from B, PA, and SD, which appeared to be directly related to low carbon and nitrogen sources, as reflected in the COD, TN, and TP of SA (**Table 5**).
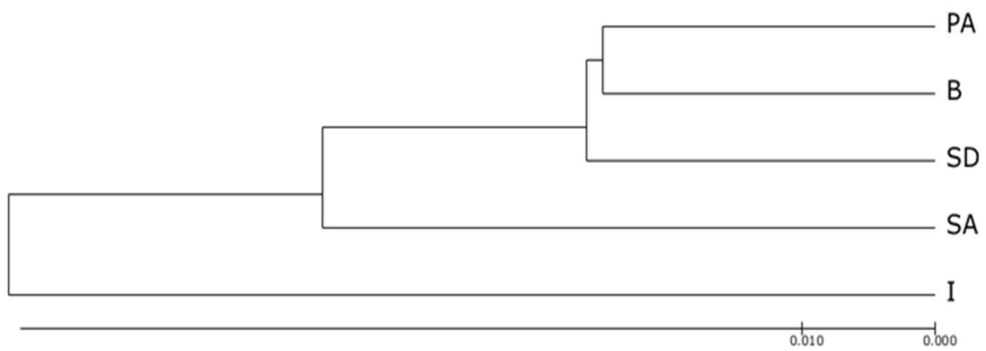
Fig. 20 Unweighted pair group method with arithmetic mean (UP-GMA) cluster analysis for the pyrosequenced community data of the different processes of the full scale tannery wastewater treatment system. I, influent; B, buffering tank; PA, primary aeration tank; SA, secondary aeration tank; SD, sludge digestion tank

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4.4 Quantification of denitrifying bacteria using qPCR in the full scale system.

The *nosZ* qPCR showed the highest abundance of denitrifiers in B (442,788 ± 374,673 copy no./ng DNA), followed by PA (105,050 ± 105,952). The abundance of denitrifiers increased approximately 195 times in B, as compared to the influent of wastewater, and after the BM-S-1 consortium was augmented and acclimated. One third of the denitrifiers remained in PA (68,978 ± 24,656) and SA (16,940 ± 6,710), since treated wastewater flowed throughout the treatment processes.
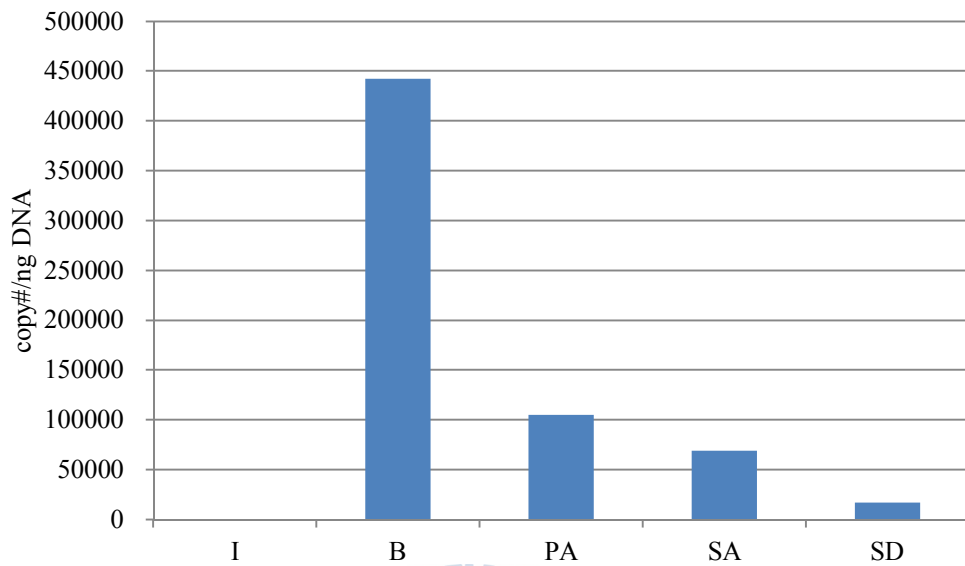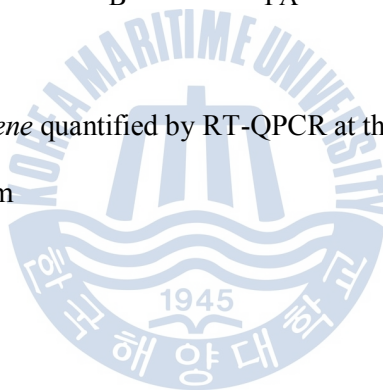
Fig. 21 Copies of *nosZ gene* quantified by RT-QPCR at the different stages of the

full scale treatment system

CHAPTER 5 METAGENOMIC ANALYSIS OF THE FULL SCALE SYSTEM

5.1 Metagenome library construction of the microbial samples

The treatment system of tannery wastewater with BM-S-1 has been in operation for 3months before this study. The microbial communities present in the various stages of the tannery treatment system including the influent (I, B, PA, SA and SD) was analyzed. A total of 2,1312,471, 1,801,377, 2,054,185 and 1,100,089 reads averaging 151 base pairs each for I, B, PA, SA and SD respectively were analyzed (**Table 8**). Artificial replicates representing 2.4%, 1.4%, 4.9%, 4.8%and 5.9% of the total reads of each sample I, B, PA, SA and SD were removed. Genes annotated as specific genes or functional groups by MG-RAST were less than 50% of obtained reads. This low annotated genes phenomenon is known to be common in metagenomic studies (Urich et al., 2008; Frias-Lopez et al., 2008; Poroyko et al., 2010), although we generated high quality reads which allowed us to compare the metagenomic data, in terms of function. However, annotated functional gene can be limited by the data already sequenced and characterized genes. This low annotations effect are possible also for a diverse and not well studied systems like the tannery wastewater (Antonopoulos et al., 2011).

5.2 Microbial community structure in the full scale system

The taxonomic classification of 786,326 (I), 1,101,022 (B), 1,030,124 (PA), 971,980 (SA) and 509,537 (SD) reads were assigned using the SEED data base (MG-RAST). Sequences representing bacteria were the most abundant ($\square$ 95% of

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the annotated proteins) in all the stages **Table 8**. The dominant phyla in I were Firmicutes 60% and Proteobacteria 21%. In B, the major phyla were Proteobacteria 58%, Firmicutes 11.3% and Bacteriodetes 16.5%. The major sequence reads in PA represented Proteobacteria 51%, Bacteroidetes 17% and Firmicutes 10.9% as the dominant phyla. The phyla sequence reads in SA were Proteobacteria 55.3%, Bacteroidetes 13.5%, Firmicutes 5.8% and Actinobacteria 6.8% while SD consisted of Proteobacteria 58.5%, Bacteroidete 6.9%, Actinobacteria 6.8%, Firmicutes 6.9% and Chloroflexi 8.4% as the major phyla **Fig. 22**. Previous metagenomics studies have been done on different wastewater treatment systems (Albertsen et al., 2012; Yu & Zhang 2012; Ye et al., 2012) but this study was focused on tannery wastewater. Besides the influent (before treatment), Proteobacteria constituted more than 50% of the total bacteria in all the stages. Other metagenomic analysis reports on activated sludge (AS) have revealed a similar dominance of Proteobacteria (45-50%) (Yu et al., 2012; Albertson et al., 2012). Bacteroidetes, Actinobacteria, Firmicutes and Chloroflex which were dominant phyla in this treatment system, have also been reported (Gomez-Alvarez et al., 2012; Yu et al., 2012). Although the dominance of these phyla has been reported in other treatment systems, the percentage composition in this case was a little higher compared to the previous reports. The higher values might be as a result of the composition of tannery wastewater, the reactor design and the influence of bioaugmentation of BM-S-1. The domain consisted mainly of bacteria and very little Achaea, virus and eukaryote (**Table 8**), which is common in wastewater treatment plant (Gomez-Alvarez et al., 2012). Annotated proteins representing eukaryotes and Achaea increased from B to SD. This also might be due to the enhancement caused by the augmentation leading to a wider diversity. Phages can be observed in any environment including wastewater as was in this case but the amount are usually low compared to the

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bacteria population distribution (Weinbauer, 2004). The Phages were higher in PA compared to I while they remained much lower and stable in the rest of the stages.

Table 8 Characteristics of 454 pyrosequence of the microbial community
from the tannery wastewater

| Stages	I	B	PA	SA	SD
Reads	2131471	1801377	2054185	1656914	1100089
Avg reads (bp)	151	151	151	151	151
Data size (10^8bp)	3.2	2.7	3.1	2.2	1.6
Reads for analysis	1627480	1775996	1952852	1577330	1034241
MG-RAST					
Reads matching taxa	786326	1101022	1030124	971980	509537
Reads matching subsystem	567898	698905	685690	611164	318282
No of subsystems (function level)	3796	3753	3401	3436	3325
Annotated proteins (%) (SEED)					
Bacteria	97	97	95.7	96.3	95.5
Archaea	0.7	1	1.1	0.9	1.3
Virus	0.9	0.2	1.3	0.3	0.2
Eukaryota	1.3	1.6	1.7	2.2	2.7
Unclassified	0.1	0.2	0.2	0.2	0.3
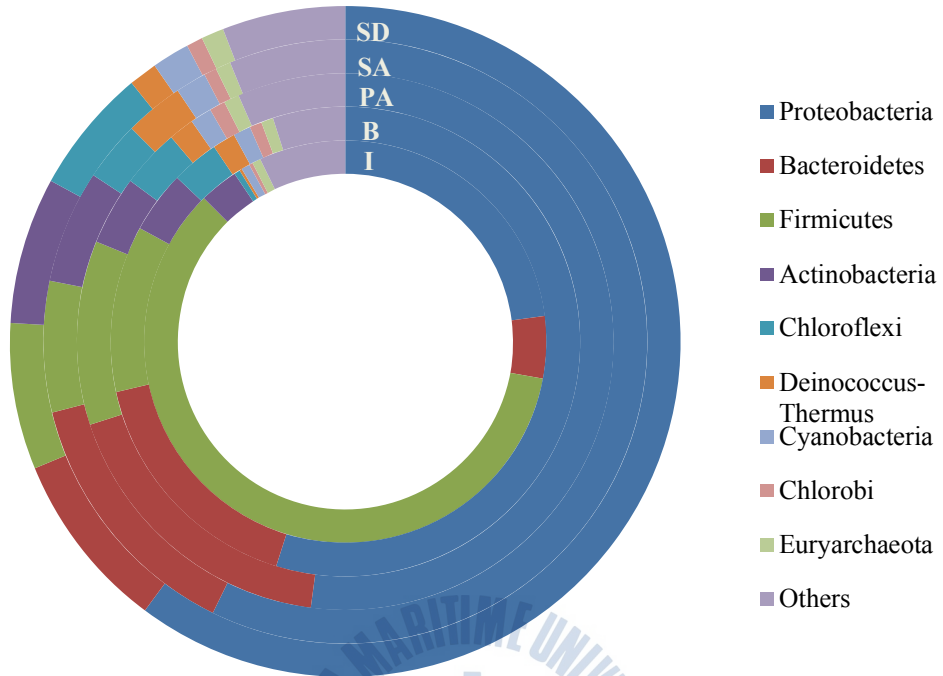
Fig. 22 The dominant phyla observed in the treatment stages. I, influent ; B, buffering tank ; PA, primary aeration tank ; SA, secondary aeration tank ; SD, sludge digestion tank

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5.3 The SEED subsystem distributions in the microbial communities

The SEED subsystem 1 result (MG-RAST) showed the metabolisms identified in the treatment stages and their normalized abundances **Fig. 23**. Metabolisms for carbohydrates, protein, nitrogen, phosphorus, sulfur etc. were observed. The abundance of carbohydrates metabolism gene was higher than that of nitrogen in all the stages, while the abundance of nitrogen metabolism genes was higher than that of phosphorus. For carbohydrate metabolisms, the genes associated with central carbohydrate, organic acids, one-carbon metabolisms etc were prevalent in all the stages (**Fig. 24**). Gene sequences representing protein metabolism were also obtained. Among these sequences, the genes responsible for protein biosynthesis and degradation were more abundant in than the rest the stages selenoproteins were the least (**Fig. 25**). Sulfur metabolism gene sequences were also identified. Inorganic sulfur assimilation, sulfate reduction and sulfur oxidation genes were among the abundant processes represented while thioredoxin-disulfide reductase showed the least abundance (**Fig. 26**). Regarding nitrogen metabolism, gene sequences representing ammonia assimilation, nitrate and nitrite assimilation processes were more abundant than the genes responsible for denitrification and dissimilatory nitrite reduction (**Fig. 27**). Ammonium assimilation showed the normalized abundances of I (89%), B (87%), PA (81%), SA (78%) and SD (76%) respectively. Also for denitrification and dissimilatory nitrite reduction genes, the abundances relatively followed the same trend as ammonium assimilation (**Fig. 28**). Phosphorus metabolism genes sequences were also present. Akalyphosphate utilization genes were the most abundant while phosphonate metabolism genes frequencies were the least.
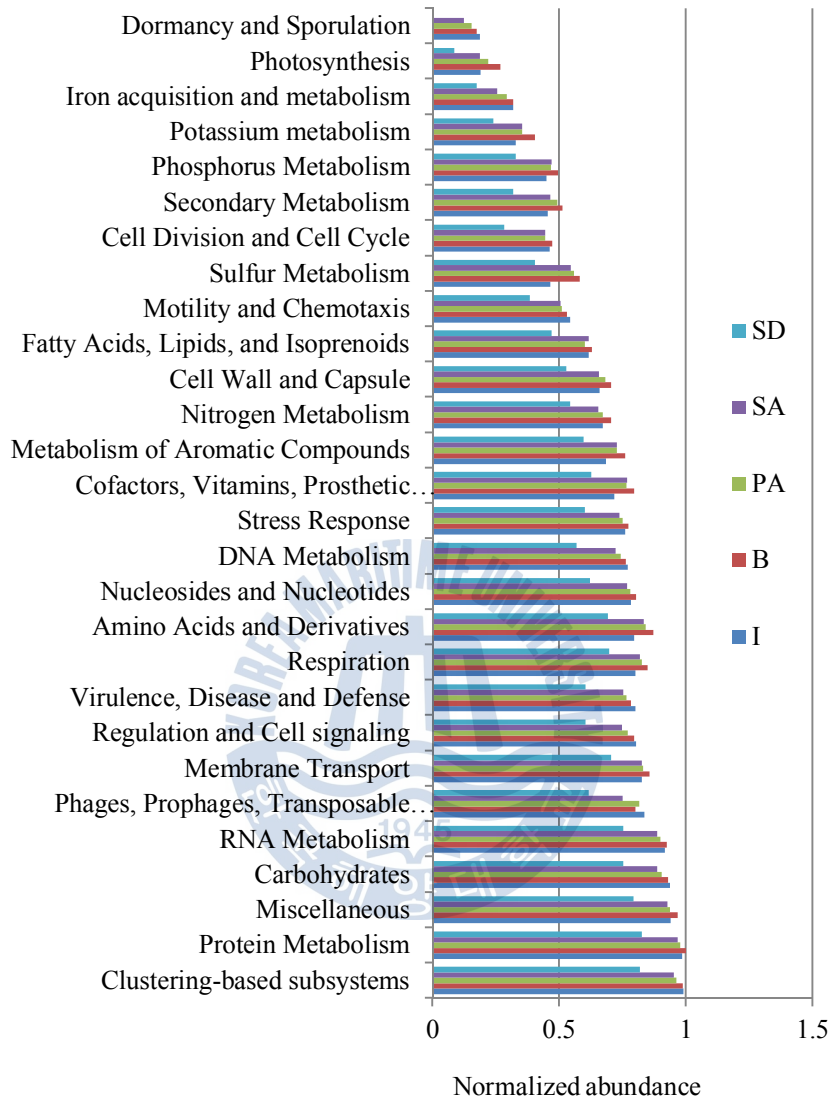
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Fig. 23 SEED subsystem showing occurrence of the different metabolisms in the full scale treatment system
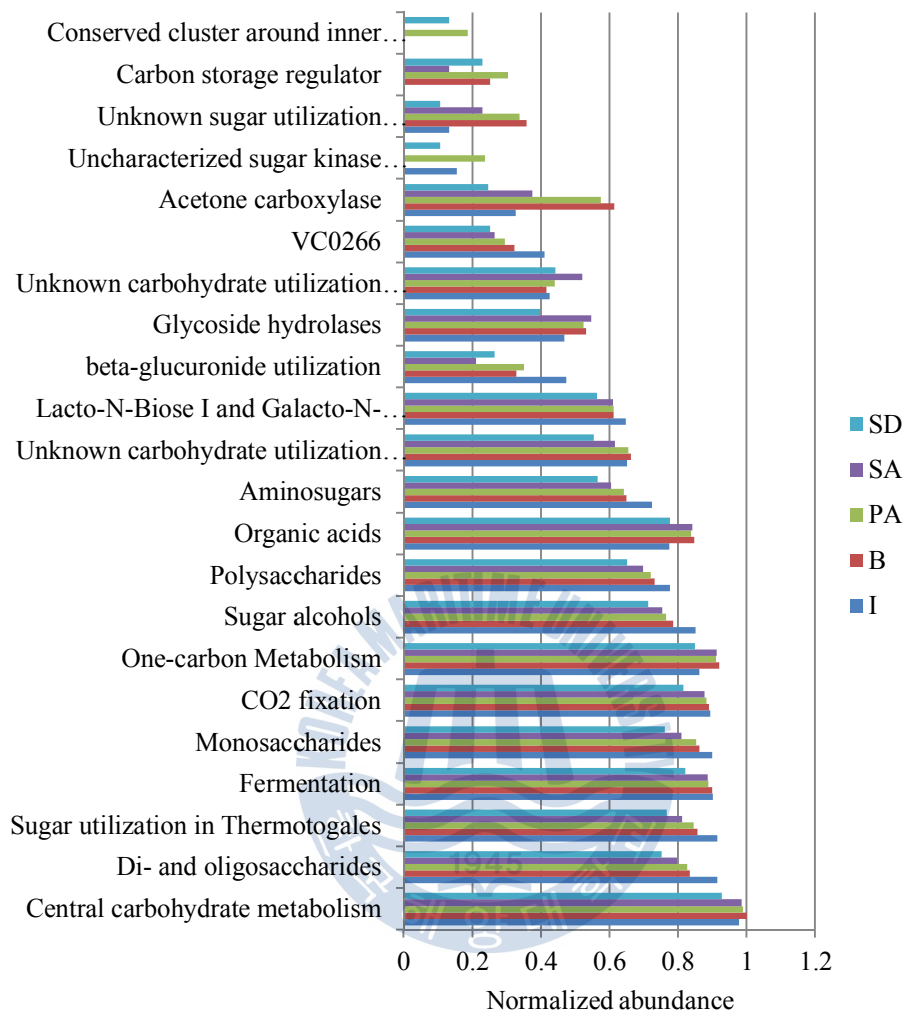
Fig. 24 Bar charts showing occurrence of the carbohydrate metabolism in the different stages of the full scale treatment system

Fig. 25 Bar charts showing occurrence of the protein metabolism in the different stages of the full scale treatment system

Fig. 26 Bar charts showing occurrence of the sulfur metabolism in the different stages of the full scale treatment system

Fig. 27 Bar charts showing occurrence of the phosphorus metabolism in the different stages of the full scale treatment system
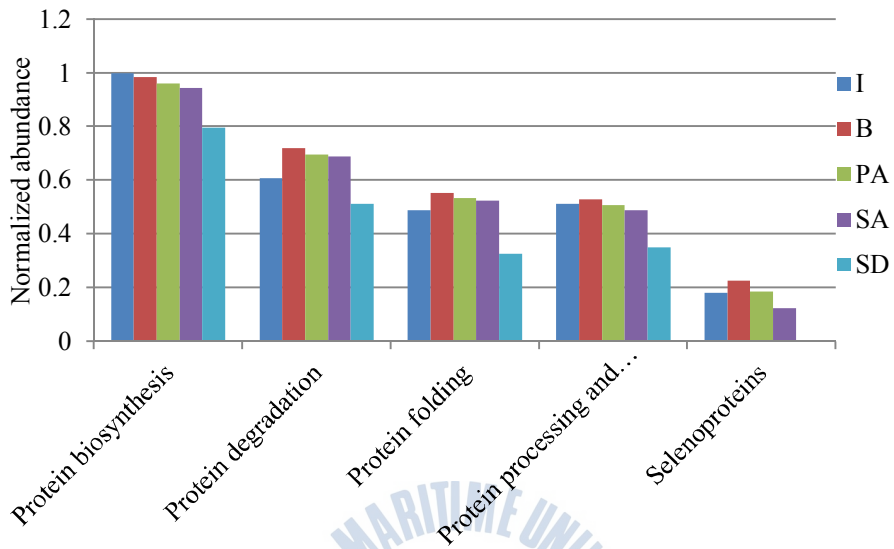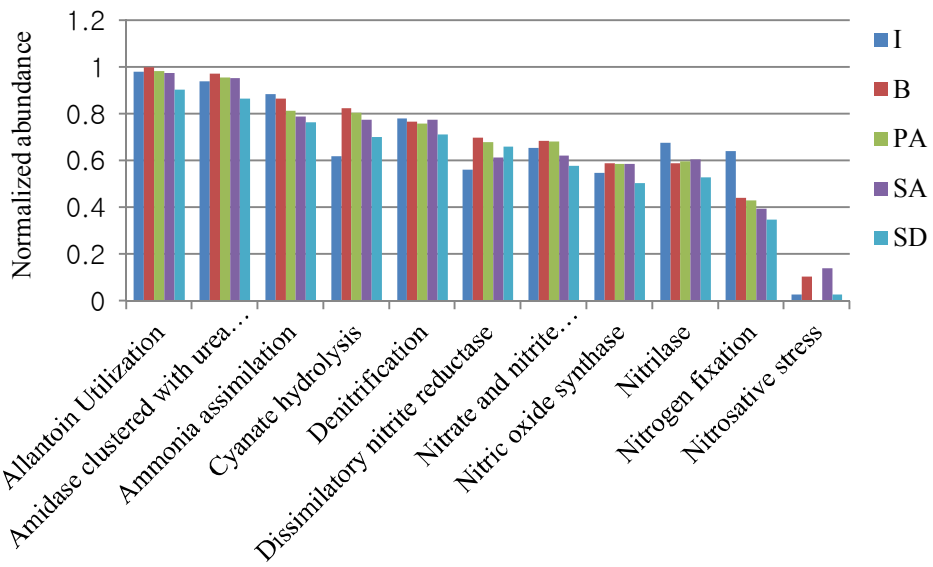
Fig. 28 Bar charts showing occurrence of the nitrogen metabolism in the different stages of the full scale treatment system
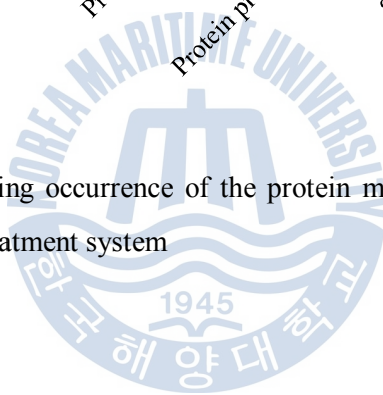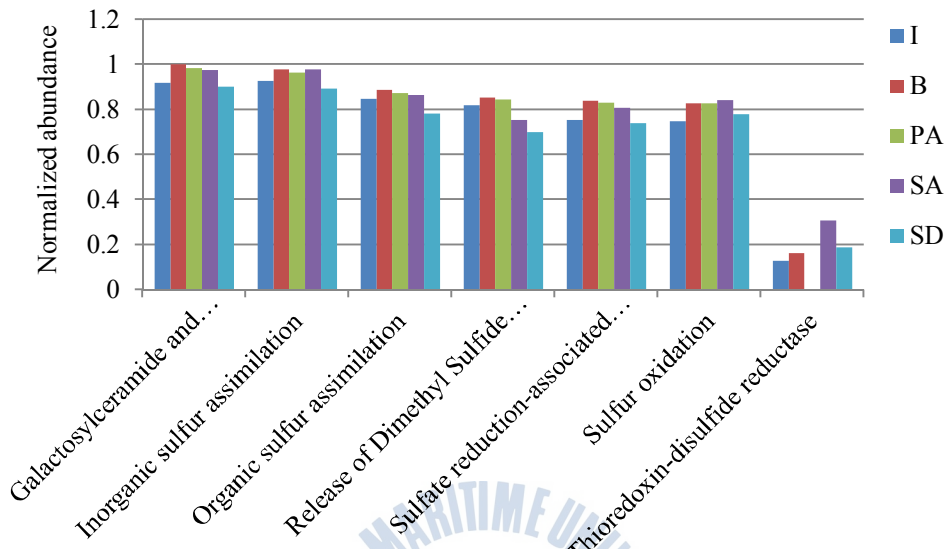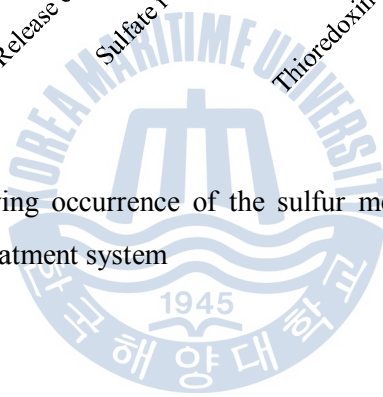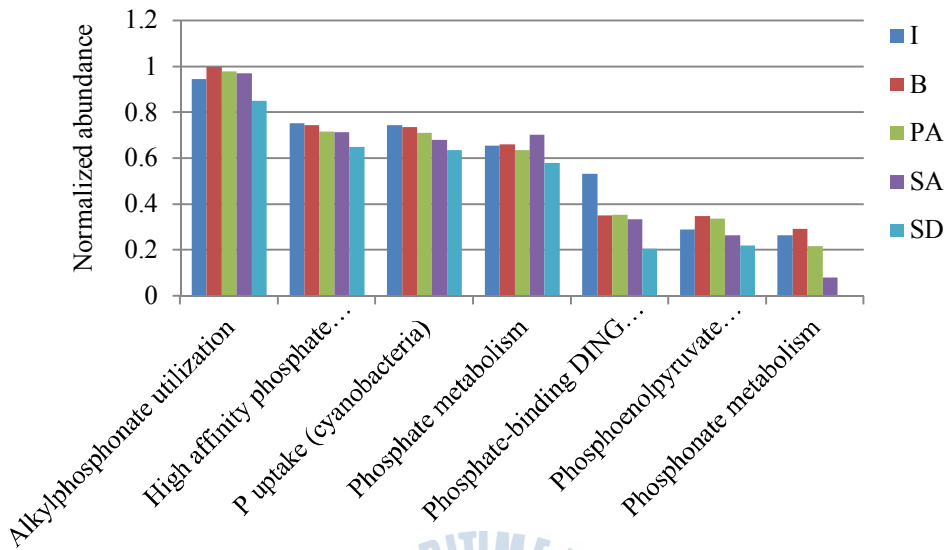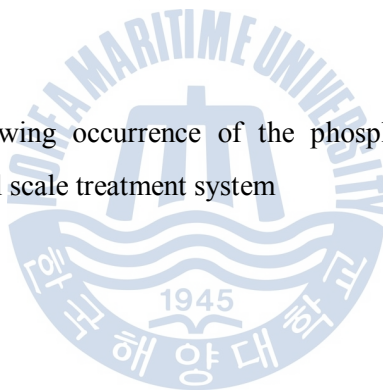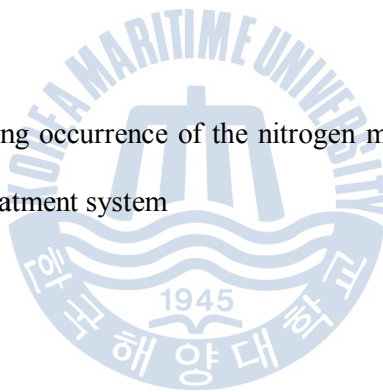
5.4 Comparative study of total nitrogen metabolic functions

We analyzed the nitrogen related functional genes from MG-RAST using the COG annotation subsystem based on the total abundance of the genes. The heat map analysis of metagenome sequencing data showed that glutamate synthase, glutamate dehydrogenase, NAD.P.H nitrite reductase, nitric oxide reductase, nitric oxide reductase, nitrate reductase and ferredoxin subunit nitrite reductase were the common nitrogen metabolism genes (**Fig. 29**) ferredoxin subunit nitrite reductase was moderately abundant in SD, SA and PA, but absent in I. Formate dependent nitrite reductase was abundantly present in B, SA and SD yet absent in I also. Nitric oxide reductase and nitrous oxide reductase were abundant in PA and SD, but little present in SA, nitrate reductase and nitrate/TMAO reductase were rather abundant in SD, SA and B, moderately present in PA yet absent in I. Glutamate synthase was abundant in all the stages except I and B while glutamate dehydrogenase was abundant in only I, SA and SD but slightly present in PA, and absent in B. The presence of nitrous oxide reductase in all the stages may indicate occurrence of a prolonged denitrification leading to a higher sludge reduction.

Fig. 29 Heat-map analysis showing the various nitrogen functions at different stages of the full scale system. I, influent ; B, buffering tank ; PA, primary aeration tank ; SA, secondary aeration tank ; SD, sludge digestion tank

5.5 Comparative study of the dominant nitrogen functions

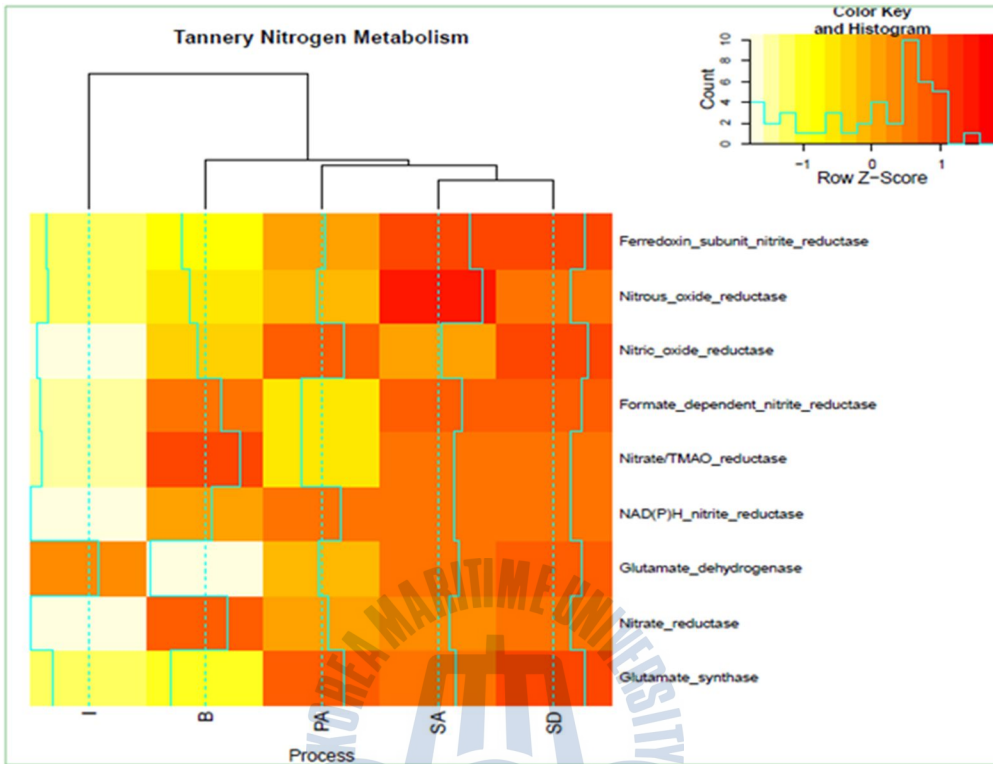The removal efficiency of COD, T-N and T-P in the treatment system were 98.3%, 98.6%, and 93.6%, respectively (Kim et al., 2014). Out of these values, 88%, 81.1% and 75%, of the removal of the COD, T-N and T-P from the influent occurred in B. Due to this significant efficiency and the essential role of nitrogen metabolism in wastewater treatment, we analyzed the various genes relating to nitrogen metabolism. The analysis was done using the hierarchical classification of microbial communities and the COG function level (MG-RAST) **Fig. 29**. The files generated were exported and sorted based on function and total abundance. The nitrogen metabolism related functional genes observed were GS, GDH, NNIR, NIOR, NOOR, NAR, FSNIR, nitrate/TMAO reductase (NTR) and formate dependent nitrite reductase (FDNR).

GS genes were most abundant in SD (3039) and least abundant in I (885) compared to the rest of the stages. GDH was also most abundant in SD (776) and least abundant in B (360). GS and GDH were the major enzymes involved in ammonia assimilation (Meti et al., 2011). GS genes were known to catalyze the reduction of amide group from L-glutamine to 2-oxoglutarate providing electrons (vav den Heuvel et al., 2004). GDH genes on the other hand are known for connecting carbon and nitrogen metabolism, and catalyzing the reductive amination of 2-oxoglutarate by ammonia using NADPH or NADH as cofactors. These two enzymes could be activated or derepressed intermittently depending on ammonia concentration in the system thereby helping the two to remain active (Perysinakis et al., 1995). The ability of these enzymes to function interchangeably under variable ammonia concentration must have led to their abundance in all or most of the stages of the tannery wastewater treatment in this study. FSNIR genes showed greater

abundance in SA (182), moderate abundance in PA (150), and least in I (112). Potential assimilatory nitrite reduction genes were identified by the presence of enzymes FSNIR. FSNIR genes are responsible for assimilatory nitrite reduction of NO_2^- to ammonia. It is more abundant in SD, SA and PA compared to B.

NOOR genes were most abundant in SA (596), slightly present in all the other stages and least in I. NIOR was abundant in SD (586), PA (379) and least in I (22) also. The presence of NIOR and NOOR genes sequences represent the last steps in denitrification process (Zumft 1997; Cabella et al., 2004; Wallenstein et al., 2006). Denitrification is an alternative to oxygen respiration used by bacteria under low oxygen or anoxic conditions (Kraft et al., 2011). NIOR genes catalyze the reduction of NO to N_2O while NOOR genes catalyze the reduction of NO to N_2 gas. NIOR and NOOR genes frequencies were higher in PA, SA and SD compared with B. PA, SA and SD have low DO (1-3 mg. DO/L) and also contained lesser carbon source (lower COD) which favor denitrification rather than dissimilatory nitrate reduction to ammonia (Bergsma et al., 2002).

NAR genes have the highest abundance in B (813) compared to the other stages and 10 times higher than I. NTR genes followed the same pattern as NAR genes except it showed a slight abundance in I. NNIR genes were most abundant in PA (110) but completely absent in I. FDNR genes were moderately present in B (80) and least abundant in I (15) also. Different reduction steps in dissimilatory nitrate reduction to ammonia (DNRA) were catalyzed by the enzymes NAR, NNIR, NTR and FDNR. These enzymes were most abundant in B. Besides the reducing (low DO) nature (Yin et al., 2002; Page et al., 2003) of B, other factors such as high C/NO_3 ratio (Fazzolari et al., 1998) and the low abundances of NIOR and NOOR genes (Bergsma et al., 2002) in this stage could have led to their high abundance.

However, nitritification related genes such as ammonia monooxygenase and

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hydroxylamine oxoreductase were not detected. This could be due to the complex composition of tannery wastewater unlike municipal wastewater. Chromium concentration was reported to have less influence on denitrification bacteria than on nitrification bacteria (Farabegoli et al., 2004). Novotnik et al, 2014 reported that Cr(III) higher than 50 mg/L negatively affected nitrification while Cr(VI) concentrations above 1.0 mg/L exhibited a negative effect on nitrification in an activated sludge system. Apart from chromium, other factors such as ammonia and nitrate concentration (Baribeau, 2006), light (Guerrero and Jones, 1996), temperature and pH (Holt et al., 1994) and dissolved oxygen concentration (USEPA, 1993) may also play some roles in the nitrification inhibition. In all, the sequence reads representing denitrification, DNRA and ammonium assimilation increased in abundance after bioaugmentation with BM-S-1 (Kim et al., 2014) except glutamate dehydrogenase in B, although abundance did not necessarily reflect activity.

5.6 Relationship between the different treatment stages in terms of nitrogen metabolic functional genes

The various nitrogen related functions were compared based on the PCA analysis to ascertain which stages were clustered together in terms functional properties **Fig. 30.** The result showed that I is remotely located from the other stages. B did not show any relationship in nitrogen functional genes composition with I. This slight closeness to PA could be attributed to fact that B was parallel to PA and the treated wastewater from B was transported directly to PA. PA, SA and SD were located on the same dimension in the PCA chart but SA was closer to PA than SD. SD might have a different environmental conditions compared to SA. The

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characteristics such as low oxygen concentration, nutrient availability and digested sludge amount could have contributed to the genetic profiles in SD.

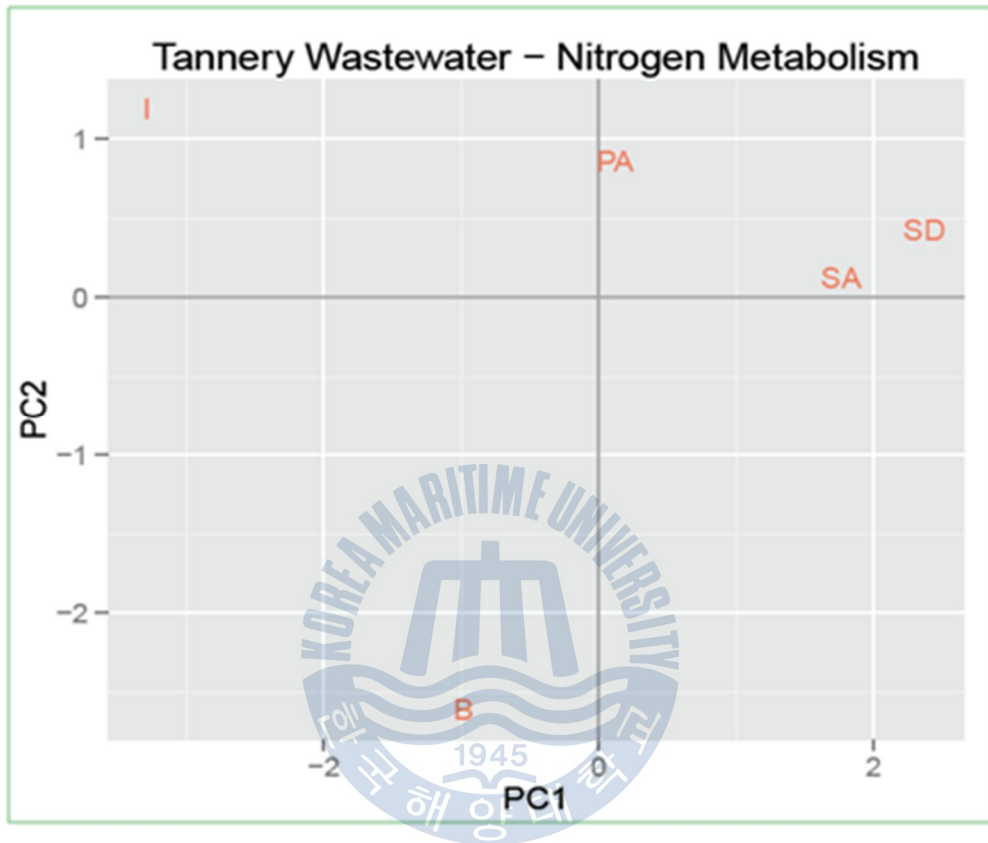**Fig. 30** PCA analysis showing the relationships between the nitrogen functions in the various stages of the full scale system. I, influent ; B, buffering tank ; PA, primary aeration tank ; SA, secondary aeration tank ; SD, sludge digestion tank

5.7 Analysis of the specific nitrogen metabolic functional genes using biomarkers

Using best hit classification from SEED annotation (MG-RAST) at max *e*-value cutoff of 10^{-5}, minimum percentage identity of 60 and minimum alignment cut off of 15 sequences. The dominant genera were extracted and sorted out using 70% cutoff of the total gene abundance. The files were exported to the work bench (MG-RAST) and the display annotated hits command was used to down load all the function files related to each genus **(Fig. 31)**. The following results were obtained; GS, GDH, NNIR, NIOR, NOOR, NAR and FSNIR genes were the common nitrogen metabolic functional genes found within this limit. Although gene abundance might not reflect activity, GS, NOOR and GDH genes were abundant among these genera especially in B and PA. NAR showed higher abundance in SA and SD and in some genera and in others the abundance was higher in PA and SA. For genera such as *Burkholderia, Polaromonas, Albidiferax and Acidovorax*, the GS genes abundance were high in B, PA, SA and SD while other genera such *Thiobacillus, Methylibium, Azoarcus, Dechloromonas* and *Aromatoleum* have high GS genes values in B, PA, SA not SD. *Bradyrhizobium, Opitutus, Magnetospirillum* and *Rhodopseudomonas* have more abundance of GS genes in SA and SD than in B and PA. Several genera contained GS genes in all the stages compared to the other nitrogen metabolism related genes. GDH genes unlike GS genes were abundant in a few genera such as *Acidovorax* and *Bordetella*. Their abundances decreased from B, PA, SA to SD, except in genera such as *Pseudomonas, Burkholderia* and *Cupriavidus*.
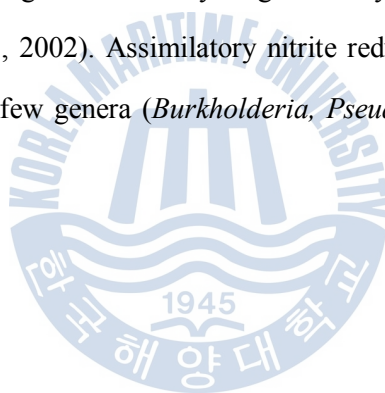
However, the abundances for NOOR genes were high in B and PA, and in genera such as *Albidiferax, Acidovorax, Cupriavidus,* and *Thiobacillus*. In some

genera such as *Methylibium, Azoarcus, Dechloromonas* and *Aromatoleum,* NOOR genes abundance was uniquely high in PA compared to the other stages. A distinct characteristic of NAR genes is its great abundance on *Chromobacterium* in the B and *Escherichia* in PA. Genus such as *Burkholderia* showed high abundance of NAR genes in all the stages decreasing from B down to SD. NAR gene abundances in *Bordetella, Azoarcus* and *Cupriavidus*, followed the same trend as *Burkholderia* although the values were lower. Other unique property of NAR genes in the analysis was their abundances in *Albidiferax, Delfia* and *Geobacter* in SD, which decreased from SD to B. NAR genes from *Meiothermus,* and *Acidovorax* was also abundant in SD. In all, the peculiar activity of NAR genes from few genera could have some special implication that needs to be uncovered. Some of these phyla and genera have been identified in the processes using genes as biomarkers.

Denitrification, one of the major processes in this study is associated with bacteria, archaea and eukaryotes and most of the denitrifiers belonged to the phylum Proteobacteria (Green et al., 2010; Hayatsu et al., 2008; Heylen et al., 2006) which was dominant in the system of this study. Biochemical and genetic characterization have been used to identify denitrification potentials in species from the genera *Pseudomonas*, *Paracoccus*, *Ralstonia*, *Bradyrhizobium*, *Rhodobacter*, *Magnetospirillum, Sphingobacterium* and others (Zumft et al., 1997; Ishii et al., 2011). These genera were possessing NOOR and NIOR denitrification gene in various stages **Fig. 21**. NOOR and NIOR genes were also abundant in *Albidiferax, Acidovorax, Cupriavidus, Thiobacillus*, *Methylibium, Azoarcus, Dechloromonas,* and *Aromatoleum*.

For ammonia assimilation, GS genes showed higher abundance in most of the stages than GDH genes except in *Burkholderia, Delfia and Bordetella.* This implies that most of these genera responded more to low ammonia concentration,

expressing GS genes rather than GDH genes (Perysinakis et al., 1995). On the other hand, DNRA has been found in Gamma-, Delta- and Epsilon proteobacteria (Smith et al., 2007), members of the Bacteroides (Mohan et al., 2004) and sulfate reducing Delta proteobacteria (Dannenberg et al., 1992; Pereira et al., 1996). Among the dominant genera, sequences representing NAR genes showed the highest abundance, but those encoding NNIR did not. The genes encoding NTR and FDNR were not detected within the cut off limit. The higher abundance of NAR genes at the different stages of the treatment was a unique characteristic of this system. This might be because of the bioaugmentation with BM-S-1, variable DO, and carbon source availability resulting from the recycling of decayed polymeric compounds from SD (Bergsma et al., 2002). Assimilatory nitrite reduction sequence encoding FSNIR was observed in few genera (*Burkholderia, Pseudomonas*, *Acidovorax* and *Bordetella*).

Fig. 31 Heat map showing the abundance of various nitrogen functions encoding genes from the dominant genera in the different stages of the full scale system during the treatment process

5.8 Proposed nitrogen metabolic pathways in the full scale system

From the metagenomic data generated and analyzed, it was observed that nitrification related biomarkers such as ammonia monooxygenase and hydroxylamine oxidoreductase genes sequences were not identified. This implies that the nitrogen removal process must have followed an alternative pathway other than nitrification because of the system conditions and the dominant microbial communities present in the system. Besides nitrification being influenced by factors outlined in the last paragraph of section **5.5,** most nitrifiers are aerobes and therefore thrives well in environments with non-limiting oxygen while denitrifiers could be aerobic or anaerobic as well (Robertson et al., 1994). Although the BM-S-1 system can be described as aerobic because of the aeration process applied, the sludge from SD which is re-circulated in the system could lead to the generation of flocs or biofilms with anoxic or even anaerobic conditions thereby affecting nitrification. Since denitrification genes were high in abundance as shown in the frequencies of their various biomarkers, this system could be said to be undergoing simultaneous nitrification and denitrification (SND) consisting of pre-denitrifcation and post-denitrification processes. Nitrification is an oxidizing process which leads to increase in alkalinity. SND is known for generating NH_3-N and NO_3-N which balances the alkalinity leading to a stable pH. This process could have led to the pH being stabilized in this system without physical adjustment. The pathways shown in **Fig. 32** depict our predicted pathways leading to nitrogen removal based on the analyzed data.
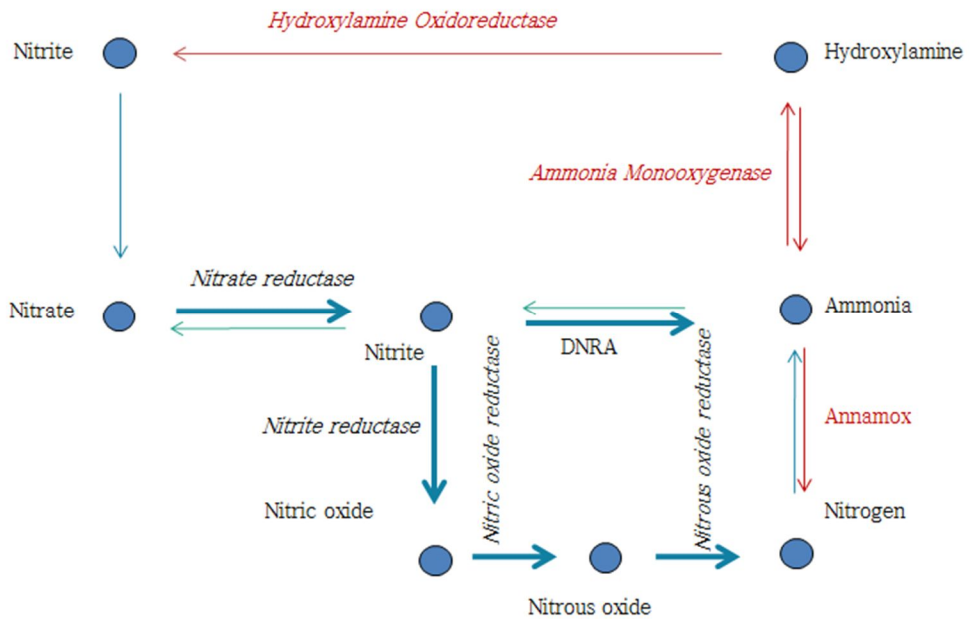
Fig. 32 Proposed nitrogen metabolic pathways in the full scale system; red color shows the missing pathways and enzymes catalyzing the process, the light green color shows the identified pathways while thick green color shows the highly identified pathways from the analysis

CHAPTER 6   CONCLUSIONS

Bioaugmentation of a novel microbial consortium BM-S-1 works and was successfully implemented to the pilot scale and full scale tannery wastewater treatment system. Specific microbial communities formed due to the bioaugmentation of BM-S-1 appeared to be actively involved in the treatment of the wastewater. Various denitrifiers, and other selected microbial communities played important roles in the eco-friendly treatment of the tannery wastewater. Microbial communities that occupy a different niche facilitated by the augmented BM-S-1 were actively involved in the treatment of the tannery wastewater. The denitrifiers (*Brachymonas denitrificans*), high molecular weight substance degraders (*Firmicutes* and *Clostridia)*, sulfate reducers (*Desulfuromonas palmitatis)*, and sulfur oxidizers (uncultured *Thiobacillus)*, appeared to play important roles in the biological and environmentally friendly treatment of the tannery wastewater.

Futhermore, the augmentation of BM-S-1 into the treatment system resulted into diverse microbial community with the capacity to degrade various contaminants available in the influent and leading to COD, T-N and T-P removal. Each of the different stages of the treatment process offered the necessary environment to promote the augmentation of BM-S-1 to function at optimum level. We provided detailed insight into nitrogen processes which may have contributed to the effective augmentation by identifying the genes sequences associated with them and their potential functions. The nitrogen metabolism analysis showed that the abundance of genera associated with different nitrogen metabolism processes at the different stages of the treatment could be used to predict the processes present or dominating each stage of the treatment. The dominant nitrogen cycle processes in the system

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were denitrification, DNRA and assimilatory nitrite reduction but unlike other metagenomic report on wastewater the absence of nitrification enzymes showed that the nitrogen removal process followed different pathways. The microbial community profiles reflected most of the phyla and genera of which their activities in wastewater treatment were already known from other previous studies but their proportions and diversity varied significantly at the different stages of the treatment. Most of the metabolic processes were relatively more active in B and PA which corroborated with the chemical data (COD, T-N and T-P, etc.). Heat map data also showed that PA and B possessed similar microbial functions while SD and I were different from those of PA and B. To the best of our knowledge, this is the first metagenomic analysis on tannery wastewater.
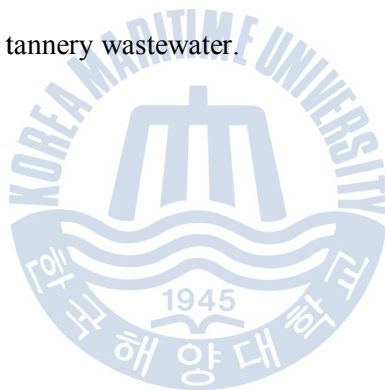
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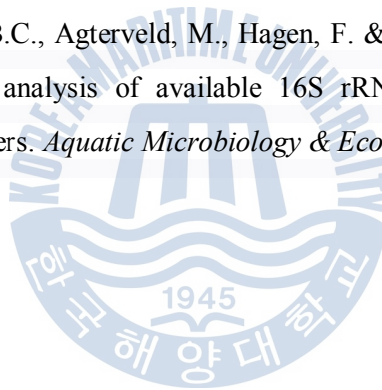
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