Thesis for the Degree of Doctor of Philosophy

Eco-friendly Upcycling of Spent Coffee Ground and Treatment of Recalcitrant Wastewaters Using Microbial Consortia

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Department of Civil and Environmental Engineering

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Korea Maritime and Ocean University

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Abstract

Spent coffee grounds (SCG), one of the world's most discarded wastes, could be an excellent resource to make a good organic fertilizer because of its richness in organic nutrients. SCG, poultry manure, and agricultural waste-derived biochar were used to manufacture functional composts through microbial bioaugmentation. The research objective is to employ fermentation utilizing biochar and beneficial microorganisms and manufacturing a functional compost product of an eco-friendliness and high quality. The highest yield of tomato stalk- based biochar (40.7%) was obtained at 450°C with a surface area of 2.35 m^2/g. Four pilot scale composting reactors were established to perform composting for 45 days. The ratios of NH_4^+-N/NO_3^--N, as an indicator of compost maturity, indicate a rapid and successful composting via microbial bioaugmentation and the biochar amendment. Moreover, germination indices for radish also increased by 14 -34% through the augmentation and the biochar amendment. The microbial diversity was also enhanced in the augmented and biochar-amended composts by 7.1-8.9%, where two species of Sphingobacteriaceae were dominant (29-43%). DPPH scavenging activities were enhanced

by 14.1% and 8.6% in fruits of pepper plants grown in presence of the compost TR-2 (augmentation only) and TR-3 (biochar amendment plus augmentation), respectively. Total phenolic content was also enhanced by 68% in fruits of the crop grown in TR-3. Moreover, the other compost TR-L (augmentation only) boosted DPPH scavenging activity by 111% in leek compared with the commercial organic fertilizer, while TR-3 increased the phenolic content by 44.8%. The composting facilitated by microbial augmentation and biochar amendment could shorten the composting time and enhance quality of the functional compost. These results indicate that the functional compost has a great potential to compete with the commercially available organic fertilizers and the novel composting technology could significantly contribute to eco-friendly recycling of organic wastes such as spent coffee grounds, poultry manure and agricultural wastes.

The next topic of the thesis was to investigate a synergistic relationship between carbon degradation and denitrification pathways in the full-scale tannery wastewater treatment plant bio-augmented with a microbial consortium BM-S-1. It was hypothesized that denitrification process closely related to the degradation of amino acids and fatty acids which could generate electron donors for the nitrate reduction, and that an efficient removal of nitrogen was linked to COD removal (and hence sludge reduction). The goal of this study was to elucidate the relationships between denitrification and degradation pathways for amino acids and fatty acids through functional metagenomic analysis. Shotgun metagenomic reads were mapped to 'ChocoPhlAn' pan-genome database and MetaPhlAn2 database for organism-specific functional profiling and formed as a gene abundance data using HUMAnN2. We then compared and selected the gene families and pathways using the extended databases UniProt Reference Clusters (UniRef90, http:/www.uniprot.org) and MetaCyc metabolic pathway database. During the functional gene analysis, 40,181 gene families and 196 pathways were revealed for the five treatment stages B, PA, SA, SD, and I. The metagenomics analysis of the microbial community showed that Brachymonas denitrificans, a known denitrifier, highly occurred in B, PA and SD. The occurrences of the amino acid degrading enzymes, alpha ketoglutarate dehydrogenase (α- KGDH) and tryptophan synthase, highly correlated with those of the denitrification genes such as *napA, narG, nosZ* and *norB*. The occurrence of glutamate dehydrogenase (GDH) also highly paralleled with those of the denitrification genes such as *napA, narG,* and *norZ*. The

denitrification genes (*nosZ, narG, napA, norB* and *nrfA*) and amino acid degradation enzymes (tryptophan synthase, α- KGDH and pyridoxal phosphate dependent enzyme) were dominantly observed in B. This indicates that degradation of the substrates (proteins) and denitrification of ammonium may occur actively in the treatment stage. The high abundance of fatty acid degradation enzyme groups (enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase and beta-ketotiolase) was observed together with the denitrification genes like *napA, narG* and *nosZ.* Like amino acid degradation enzymes, more diverse enzymes were dominantly observed in the stage B such as phospholipase/carboxylesterase, enoyl-CoA hydratase/isomerase, acyl-CoA dehydrogenase, phenylacetate degradation, and 3-hydroxyacyl-CoA dehydrogenase 2. All these results clearly state that the denitrification pathways could be linked with degradations of the amino acids and fatty acids whose degradation products go through TCA cycle to generate NADH that is used as electron donors for the denitrification. This understanding of nitrogen and carbon metabolic pathways in the tannery wastewater treatment system will undoubtedly contribute to an optimized and efficient operation of the treatment system and any other wastewater treatment systems.

The last topic of the thesis was to evaluate the degradation activities of pollutants in the dye wastewater treatment process in terms of treatment efficiency and to provide metagenomic understanding of the treatment process. Hypothesis is that the azo-dye could be degraded via pathways including azo-dye reduction, aromatic compound degradation, nitrification, denitrification, and TCA cycle. The activities of dye wastewater treatment plant were evaluated before and after the addition of CES-1. Total genomic DNA samples were extracted from different treatments with different time variables. The effect of CES-1 bioaugmentation on the microbial community structure and functional genes of each treatment step was explored by high-throughput sequencing and metagenomic analysis. The removal efficiencies (%) for COD, T-N, T-P, SS and color intensity before bioaugmentation were 94.9, 48.1, 91.6 63.9 and 66.3, respectively. By the way, the removal efficiencies (%) for these parameters 50 days after the bioaugmentation increased up to 97.8, 62.6, 95.8, 63.4 and 77.9, respectively. The sludge reduction rate over 20 months after the bioaugmentation was 26% (reduction from 6.18 to 5.00 in sludge per ton of influent COD). There appeared to be no clear delineation in the microbial communities between processes of influent (I)

(I_0310_18) and buffering (B) (B_0310_18). The buffering sample (B_0629_17) maintained quite a different community structure from the influent sample (I_0629_17), indicating the modifying effect of CES-1 bioaugmentation on the buffering process despite the very high domination of *Mesorhizobium soli* (98.5%) in the influent. The presence of FMN dependent NADH-azoreductase were dominant before treatment (B_0303_17), but a drastic decrease of the enzyme was observed in B after CES-1 treatment, indicating that a competition occurs between microbial communities from CES-1 and the indigenous organisms carrying azoreductase. Enzymes involved in aromatics degradation were generally present in the samples (B_0310_18, PA_0310_18, SA_0310_18 and SD_0310_18) 300 days after bioaugmentation while frequencies of these enzymes were quite low before and 50 days after augmentation, indicating the selection of these aromatic degradative enzymes by CES-1 augmentation over time. CES-1 augmentation also delineated samples into the two groups: the samples of control and early stage of bioaugmentation (B_0303_17 and I_0629_17) and the samples of later stage of bioaugmentation (PA_0310_18). Dominant amino acid degradative enzymes found in the later stage of the augmentation were 2-hydroxyglutarate dehydrogenase, tryptophan synthase, 2-oxoglutarate dehydrogenase and acetylglutamate kinase. The representative enzymes for TCA cycle found in later stage of the augmentation were succinate dehydrogenase (ubiquinone) iron-sulfur subunit, succinate dehydrogenase, triosephosphate isomerase and 2,3-bisphosphoglycerate-independent phosphoglycerate mutase. Enzymes involved in nitrogen metabolism dominantly found in the later stage of the augmentation were nitrite reductase, hydroxylamine dehydrogenase, nitrous oxide reductase, nitrate reductase/nitrite oxidoreductase, nitrite reductase, perisplasmic nitrate reductase, ammonia monooxygenase subunit A and nitrite reductase. The intrinsic relationships between microbial community structures and functions of enzymes for the dye metabolism need to be analyzed using more sophisticated algorithms. Since the successful performance in reduction of pollution and sludge in the dye wastewater treatment plant and the concomitant metagenomic analysis of the treatment process were accomplished, the future perspective of this research will be development of specific genes and enzymes as biomarkers used to monitor the functional properties of degradation and operate the novel dye wastewater treatment system in the optimal conditions.

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개요

세계에서 가장 많이 버려지는 폐기물 중 하나인 커피찌꺼기 (Spent coffee grounds; SCG)는 유기물영양소가 풍부하여 좋은 유기농 비료를 만드는 훌륭한 자원이 될 수 있다. SCG, 가금류 분뇨 및 농업 폐기물 유래의 바이오 숯을 사용하여 미생물 바이오 증강을 통한 기능성 퇴비를 제조 하였다. 토마토 줄기 기반 바이오 숯 (40.7 %)의 최고 수율은 450 ° C 에서 나타났고 2.35 m^2 / g 의 표면적이 확보되었다. 4 개의 파일럿 규모 퇴비화 반응기를 설치 후 45 일 동안 퇴비화를 수행하였다. 퇴비 성숙도의 지표 인 NH_4^+-N / NO_3^--N 의 비율은 미생물 생물증강 및 바이오숯처리를 통하여 빠르고 성공적인 퇴비화를 나타냈다. 또한, 무의 발아 지수는 증강 및 바이오숯 처리를 통해 14-34 % 증가했다. 또한 미생물 종다양성은 미생물이 증강된 바이오숯 처리 퇴비에서 7.1-8.9 %까지 향상되었으며, 여기서 Sphingobacteriaceae 내

2 종의 미생물이 우세했다 (29-43 %). DPPH 소거 활성은 퇴비 TR-2 (증강처리) 및 TR-3 (바이오숯 + 증강처리)의 존재 하에서 재배 된 고추 식물의 열매에서 각각 14.1 % 및 8.6% 증가되었다. TR-3 에서 자란 작물의 열매에서 총 페놀 함량도 68 % 향상되었다. 또한, 다른 퇴비 TR-L (증강처리)은 상업적 유기비료와 비교하여 볼 때 부추에서 DPPH 소거 활성을 111 % 증가 시켰으며, TR-3 은 페놀 함량을 44.8 % 증가시켰다. 미생물 증강 및 바이오처리에 의해 촉진 된 퇴비는 퇴비 시간을 단축시키고 기능성 퇴비의 품질을 향상시킬 수 있었다. 이러한 결과는 본 연구의 기능성퇴비가 상업적으로 이용 가능한 유기질 비료와 경쟁 할 수있는 큰 잠재력을 가지고 있으며, 본 새로운 퇴비화 기술은 커피찌꺼기, 가금류 분뇨 및 농업 폐기물과 같은 유기성 폐기물의 친환경 재활용에 크게 기여할 수 있음을 낸다.

다음 과정은 미생물 컨소시엄 BMS-1 로 생물 증강 된 본격적인 무두질 공장 폐수 처리장에서 탄소분해와 탈질경로 사이의 상승적 관계를 조사하기위한 목적으로 수행하였다. 탈질 공정은 질산염 제거를위한 전자 공여체를 생성 할 수있는 아미노산 및 지방산의 분해와 밀접한 관련이 있으며 질소의 효율적인 제거는 COD 제거 (및 슬러지 감소)와 관련이 있다는 가설을 세웠다. Shotgun metagenomic 판독 값은 유기체별 기능프로파일링을 위해 'ChocoPhlAn'pan-genome 데이터베이스 및 MetaPhlAn2 데이터베이스에 매핑되었으며 HUMAnN2 를 사용하여 유전자 풍부도 데이터로 구성되었다. 그런 다음 확장 데이터베이스 UniProt Reference Clusters (UniRef90, http : /www.uniprot.org) 및 MetaCyc 대사 경로 데이터베이스를 사용하여 유전자 패밀리와 경로를 비교하고 선택했다. 기능적 유전자 분석 동안 B, PA, SA, SD

및 I 의 5 가지 처리단계에서 40,181 개의 유전자패밀리와 196 개의 경로가 밝혀졌다. 미생물 군집의 metagenomics 분석은 알려진 탈질균인 *Brachymonas denitrificans* 가 B, PA 및 SD 에서 크게 발생했음을 보였다. 아미노산 분해효소인 alpha ketoglutarate dehydrogenase (α- KGDH) 및 tryptophan synthase 의 발생은 *napA*, *narG*, *nosZ* 및 *norB* 와 같은 탈질유전자와 밀접하게 관련되어 있었다. glutamate dehydrogenase (GDH) 효소의 발생은 또한 *napA*, n*arG* 및 *norZ* 와 같은 탈질 유전자의 것과 유사하다. 탈질 유전자 (*nosZ*, *narG*, *napA*, *norB* 및 *nrfA*) 및 아미노산 분해 효소 (tryptophan synthase, α- KGDH 및 pyridoxal phosphate dependent enzyme)가 B 에서 우세하게 관찰되었다. 이는 기질 (단백질) 및 탈질의 분해를 나타낸다. 처리 단계에서 암모늄이 능동적으로 발생할 수있다. *napA*, *narG* 및 *nosZ* 와 같은 탈질 유전자와 함께 지방산 분해 효소그룹 (enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase 및 beta-ketotiolase)이 풍부하게 관찰되었다. 아미노산 분해 효소와 같이, 단계 B 에서 phospholipase/carboxylesterase, enoyl-CoA hydratase/isomerase, acyl-CoA dehydrogenase, phenylacetate degradation 및 3-hydroxyacyl-CoA dehydrogenase 2 와 같은 보다 다양한 효소가 지배적으로 관찰되었다. 이러한 결과는 탈질경로가 아미노산 및 지방산의 분해경로와 연계될 수 있음을 의미하는바, 이러한 물질들의 분해산물이 궁극적으로 탈질의 전자공여체로서 사용되어 NADH 를 생성할 수 있기 때문이다. 피혁폐수 처리 시스템에서의 질소 및 탄소 대사 경로에 대한 이러한 이해는 의심할 여지없이 처리 시스템 및 다른 폐수처리 시스템의 최적화와 효율적인 운전에 기여할 것이다.

논문의 마지막 주제는 처리 효율 측면에서 염색폐수 처리 공정에서 오염 물질의 분해활성을 평가하고 처리공정에 대한 metagenomic 이해를 제공하는 것이었다.

가설은 아조염료가 아조염료 환원, 방향족 화합물 분해, 질화, 탈질 및 TCA 사이클을 포함한 경로를 통해 분해 될 수 있다는 가설이다. 염색폐수 처리장의 잠재적 분해활성을 CES-1 첨가 전후에 평가하였다. 상이한 시간변수를 갖는 상이한 처리로부터 총게놈 DNA 샘플을 추출 하였다. 각 처리 단계의 미생물 군집구조 및 기능성 유전자에 대한 CES-1 생물증강효과는 고도처리 시퀀싱 및 metagenomic 분석에 의해 검토되었다. 생물증강 전 COD, T-N, T-P, SS 및 색 강도에 대한 제거 효율 (%)은 각각 94.9, 48.1, 91.6 63.9 및 66.3 이었다. 그런데, 생물증강 50 일 후 이들 요인에 대한 제거효율 (%)은 각각 97.8, 62.6, 95.8, 63.4 및 77.9 까지 향상되었다. 생물증강 후 20 개월에 걸친 슬러지 감소율은 26 %였다 (유입 COD 톤당 슬러지 6.18 톤 에서 5.00 톤으로 감소). 유입수 (I) (I_0310_18)와 유량조정조(B) (B_0310_18) 단계 사이의 미생물 군집에서는 명확한 차이가 없는 것으로 관찰되었다. 유량조정조 샘플 (B_0629_17)은 유입수 샘플 (I_0629_17)과는 상당히 다른 커뮤니티 구조를 유지하여 유입수에서 *Mesorhizobium soli* (98.5 %)의 우점적 지배에도 불구하고 유량조정조에서 CES-1 생물증강의 효과를 나타냈다. FMN 의존성 NADH-azoreductase 의 존재는 처리 전에 우점하였으나 (B_0303_17), CES-1 처리 후 B 에서 효소의 급격한 감소가 관찰되었으며, 이는 CES-1 미생물 군집과 아조염료 환원 효소를 생성하는 토착미생물 사이에서 경쟁이 일어날 수 있음을 의미한다. 방향족물질 분해에 관여하는 효소는 일반적으로 생물증강 300 일 후 샘플 (B_0310_18, PA_0310_18, SA_0310_18 및 SD_0310_18)에 존재하는 반면, 이들 효소의 빈도는 증강 전 및 증강 후 50 일 후에 매우 낮게 나타났으며 이는 운전시간에 따라 CES-1 에 의한 이들 방향족물질 분해효소 생성 균주의 선택적 효과를 의미한다. CES-1 증강은 또한

샘플을 2 개의 그룹, 즉 대조군 및 초기 생물증강 단계 (B_0303_17 및 I_0629_17)의 시료 및 후기 생물증강 단계 (PA_0310_18)의 시료로 구분이 되었다. 증강의 후기 단계에서 발견되는 우점 아미노산 분해효소는 2-hydroxyglutarate dehydrogenase, tryptophan synthase, 2-oxoglutarate dehydrogenase 및 acetylglutamate kinase 였다. 증강의 후기 단계에서 발견되는 TCA 사이클에 대한 대표적인 효소는 succinate dehydrogenase (ubiquinone) iron-sulfur subunit, succinate dehydrogenase, triosephosphate isomerase 및 2,3-bisphosphoglycerate-independent phosphoglycerate mutase 였다. 증강의 후기단계에서 주로 발견되는 질소대사에 관여하는 효소는 nitrite reductase, hydroxylamine dehydrogenase, nitrous oxide reductase, nitrate reductase/nitrite oxidoreductase, nitrite reductase, perisplasmic nitrate reductase, ammonia monooxygenase subunit A 및 nitrite reductase 이었다. 상기에서 언급한 미생물군집 구조와 염색물질 대사관련 효소의 기능사이의 고유한 관계는 보다 정교한 알고리즘을 사용하여 분석할 필요가 있다.

염색폐수 처리장의 오염물질 및 슬러지 감소에 대한 성공적인 성취와 처리공정의 동시 metagenomic 분석이 달성되었으므로, 향후 이 연구의 방향은 분해의 기능적 특성을 모니터링하는데 사용되며 또한 최적의 조건에서 새로운 염색 폐수 처리시스템을 운전하기 위해 사용될 수 있는 biomarkers 로서 특정 유전자 및 효소의 개발이 될 것이다.

CHAPTER I - ECOFRIENDLY UPCYCLING OF SPENT COFFEE GROUND

1.1 Introduction

1.1.1 Motivation of work

Soils contaminated with hazardous chemicals worldwide are awaiting remediation activities; bioremediation is often considered as a cost-effective remediation approach. Potential bio approaches are bio stimulation, e.g. by addition of nutrients, fertilizer, and organic substrates, and bioaugmentation by addition of compound-degrading microbes or of organic amendments containing active microorganisms, e.g. activated sludge, or compost. In most contaminated soils, the abundance of the intrinsic metabolic potential is too low to be improved by bio stimulation alone, since the physical and chemical conditions in these soils are not conducive to biodegradation. In the last few decades, compost, or farmyard manure addition as well as composting with various organic supplements have been found to be very efficient for soil bioremediation. The spent coffee ground is considered as one of the world's most discarding waste. Reuse of the coffee ground could lead to a good quality of compost. Therefore, various studies have been going on to make value-added products such as enzymes, organic acids, flavor and aroma compounds and mushrooms from the by-products (Murthy and Naidu, 2012).

In this work, we focused on the composting using spent coffee ground wastes through bioaugmentation. Therefore, we first systemize the concepts of composting and compost addition, and then summarize the relevant physical, chemical and biotic factors and mechanisms for improved contaminant degradation triggered by compost addition. These factors and mechanisms are of interest since they are more

relevant and easier to determine than the composition of the degrading community. Due to the mostly empirical knowledge and the non-standardized bio waste or compost materials, the field use of these approaches is highly challenging, but also promising. Based on the huge metabolic diversity of microorganisms developing during the composting processes, a highly complex metabolic diversity is established as a 'metabolic memory' within developing and mature compost materials. Compost addition can thus be considered as a 'super-bioaugmentation' with a complex natural mixture of degrading microorganisms and high growth rate. It also improves the abiotic soil conditions, thus enhancing microbial activity in general.

1.1.2 Literature review

The poultry manure that containing organic matter, nitrogen, phosphorus, potassium, magnesium, and other micronutrients (copper, iron, manganese, and boron), if properly processed, could become an excellent organic quality fertilizer. The manure can increase soil carbon and nitrogen content, and soil porosity and enhance soil microbial activity. In South Korea, total manure production of the egg-laying chickens has been estimated to be 7815M/T/day in 2013 (Livestock Economic News, 2014), but there were little facilities to treat the manure in a sustainable way. Instead, most manure treatment facilities were publicly constructed to process piggery slurries. Hence it will be necessary to develop an efficient recycling system where the poultry manure (nitrogen sources) as well as the spent coffee ground (carbon source) and biochar (bulking agent) can be used as functional substrates to manufacture a quality functional compost product.

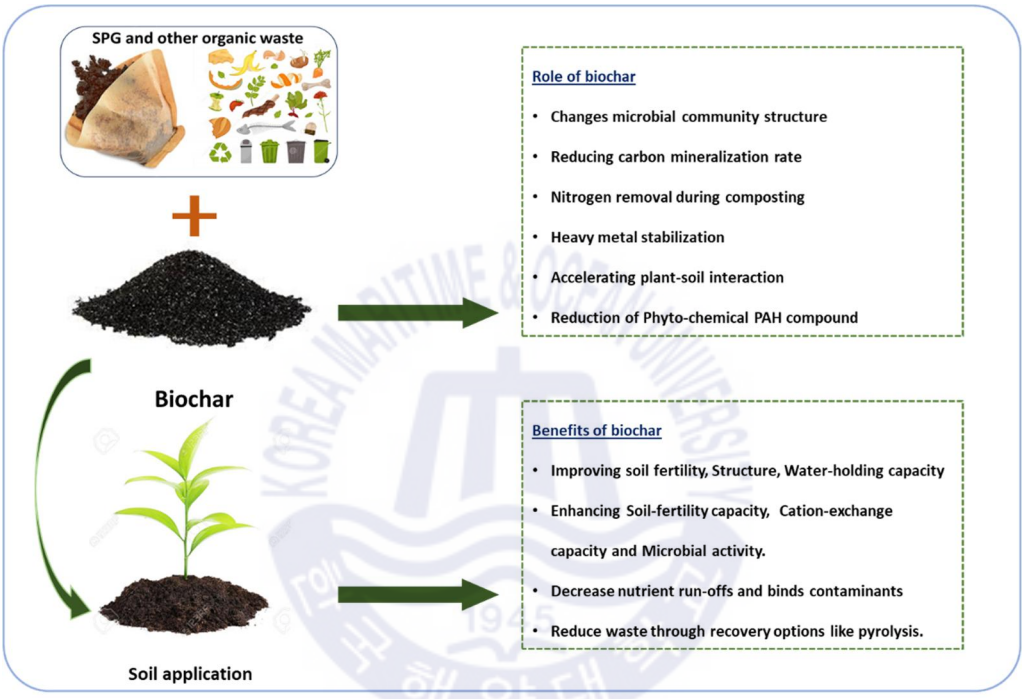

Figure 1-1 Roles and benefits of biochar in composting

The benefits of adding biochar to the composting process may include shorter compost times and reduced rates of malodor emissions. For the biochar material itself, undergoing composting helps to charge the biochar with nutrients without

breaking down the biochar substance in the process (Birk et al., 2009). The poultry manure–biochar mixture during composting favored a more generation of humic acids over fulvic acids, and poultry manure amended with biochar lessened the losses of nitrogen in the mature composting products (Dias et al., 2010; Jindo et al., 2016). Biochar addition during composting of tomato stalk and chicken manure composting allowed a reduced time to enter thermophilic phase, a higher temperature and longer duration of thermophilic stage. The addition also showed could significantly affect physico-chemical process and microbial community diversity (Wei et al., 2014). The highest amendment rate of biochar caused the max peak in temperature, and the biochar addition shortened the period of thermophilic phase and increased C-CO_2 emission (Czekała et al., 2016). A small amount of biochar (4%) as an amendment for composting was used successfully to improve the value of olive mill waste composts containing sheep manure by reducing N losses by 15% and increasing NO_3^- availability without affecting the amount of N2O released (López-Cano, et al., 2016). Addition of higher amount of biochar (40-80%, v/v) during composting of pig slurry stimulated seed germination and plant growth by decreasing the EC and available Cu and Zn contents, hence reducing phytotoxic effects, as well as reducing CO_2, NO and VOC emissions (Sáeza, et al., 2016).

The biochar is characterized by a sustainable enhanced fertility due to its high levels of soil organic matter and nutrients such as N, P and Ca (Glaser, 2007; Glaser & Birk, 2011). It provides a habitat for soil microorganisms which can degrade more labile soil organic and inorganic matter and maintains more moisture and nutrients useful for plant growth. In addition, the higher microbial activity enhances soil stabilization and fertility. Biochar generally causes a positive proliferation effect for several symbiosis microorganisms due to its structure providing an appropriate habitat for soil microbes. Steiner et al. (2004) observed a significant increase of microbial activity and growth rates by applying biochar to a Ferralsol. Furthermore, an increase of soil microbial biomass and a changed composition of soil microbial community were also observed after biochar amendments.

1.1.3 Development of functional compost

Coffee is the largest traded commodity only second to crude oil in the world (Mussatto et al., 2011) and brings about enormous amount of coffee by-products/residues during its processing from fruit to cup. The major by-products of coffee-industry are the coffee husks/peel/pulp and constitute nearly 45% of cherry. Interestingly, the by-products carry rich nutrients such as cellulose, hemicellulose, protein, sugars, chlorogenic acid and polyphenols (Table 1-1). The coffee ground also contains a fair amount of protein and is rich in sugar such as mannose and galactose (about 14%) and can be suitable as a material for good quality compost (Dam and Harmsen, 2010). Coffee waste management has been the focus of recent developments, aiming to implement ecological disposal approaches and develop value-added alternatives. These by-products are generated in the producing countries (e.g., coffee pulp, cherry husks, and parchment skin), by the roasting industries (e.g., coffee silver skin), in the soluble coffee industry, and after brewing up, the left-over by-products are designated generally as "spent coffee ground (SCG)" (Cruz et al., 2014). The spent coffee ground is considered as one of the world's most discarding waste. Reuse of the coffee ground could lead to a good quality of compost. Therefore, various studies have been going on to make value-added products such as enzymes, organic acids, flavor and aroma compounds and mushrooms from the by-products (Murthy and Naidu, 2012). However, implementation of the technology at pilot or field scale still needs to be accomplished. Moreover, traditionally, the by-products and residues have been limitedly applied as fertilizer, compost, livestock feed, etc. So, it will be highly necessary to develop an efficient technology which will enable more effective utilization of available quantity of the recyclable coffee wastes.

1.1.4 Role of microbial community in functional compost and the objective of the study

There were a few studies regarding composting facilitated by bioaugmentation. Efficacy test of white rot fungi bioaugmentation in composting of a flare pit soil was performed to select the best strain and bulking agent (Baheri and Meysami, 2002). Recently a study of selecting organisms involved in the effective composting bioaugmentation based on metabolic activities was performed and several high potential species were obtained like *Streptomyces albus, Gibellulopsis nigrescens, Bacillus licheniformis, Bacillus smithii* and *Alternaria tenuissima*. Interestingly, a combination with foliar sprays of plant growth promoting bacteria (beneficial microorganism) and humic substances appeared to boost yield of organic tomatoes. Moreover, it increased nitrate uptake and nitrate reductase activity, and stimulated the secondary metabolic phenylalanine ammonia lyase pathway (Olivares et al., 2015). This indicates that a combined amendment of mature compost, soluble organic matter and beneficial microorganisms may provide chances for effectively providing useful bioactive compounds to the sustainable organic food sources.

The objective of this study is to manufacture a quality functional compost that is based on spent coffee ground, sustainable/recyclable organic wastes, and biochar as composting raw materials, and on additions of a microbial agent and PGPB to speed up the composting process and to enhance the functional capacity of the compost. The manufactured functional compost proved a maturity enough to enhance the radish seed, improve soil fertility and stimulate antioxidant production in pepper and leek plants.

Parameters (%)	Coffee pulp	Coffee husk	Sliver skin	Coffee spent
Celluose	63.0 ± 2.5	43.0 ± 8.0	17.8 ± 6.0	8.6 ± 1.8
Hemicellulose	2.3 ± 1.0	7.0 ± 3.0	13.1 ± 9.0	36.7 ± 5.0
Protein	11.5 ± 2.0	8.0 ± 5.0	18.6 ± 4.0	13.6 ± 3.8
Fat	2.0 ± 1.6	0.5 ± 5.0	2.2 ± 1.9	ND
Total fiber	60.5 ± 2.9	24 ± 5.9	62.4 ± 2.5	ND
Total polyphenols	1.5 ± 1.5	0.8 ± 5.0	1.0 ± 2.0	1.5 ± 1.0
Total sugar	14.4 ± 0.9	58.0 ± 20	6.65 ± 10	8.5 ± 1.2
Pectic substance	6.5 ± 1.0	1.6 ± 1.2	0.02 ± 1.0	0.01 ± 0.005
Lignin	17.5 ± 2.2	9.0 ± 1.6	1.0 ± 2.0	0.05 ± 0.05
Tannins	3.0 ± 5.0	5.0 ± 2.0	0.02 ± 0.1	0.02 ± 0.1
Chlorogenic acid	2.4 ± 1.0	2.5 ± 0.6	3.0 ± 0.5	2.3 ± 1.0
Caffine	1.5 ± 1.0	1.0 ± 0.5	0.03 ± 0.5	0.02 ± 0.1

Table 1-1 Parameters of different nutrients present in different parts of the coffee plant

1.2 Experimental section

1.2.1 Composting raw materials

The spent coffee grounds were collected from several local coffee shops in Yoengdo, Busan, South Korea and dried in a shading place up to 40-50% of moisture. Dried poultry manures (35-45% in moisture) were collected from an egg production and distribution company in Yangsan, South Korea. The biochar was made of discarded tomato stems which were collected from a vinyl house farm in Gunwigun, South Korea following the modified method based on Angin et al., (2013). Fresh tomato stems were chopped up to around 10cm and dried at 110°C for at least 2 hrs. A measured amount of the dried stems was subjected to pyrolysis at the different temperatures (400℃, 450℃, 500℃and 550℃) with a heating rate (10℃/min) using a furnace for 3 hrs. The furnace was operated under an oxygen-limited condition in the presence of positive stream of N_2 gas. Here, the biochar yield was in the range of 26-41%.

1.2.2 Composting procedure

Four kinds of compost were manufactured at two pilot scales (25 kg and 200kg): TR-1 (25 kg), TR-2 (25 kg), TR-3 (25 kg) and TR-L (200kg). Composting vessels used for TR-1, TR-2 and TR-3 were rectangular plastic trays (L x W x H = 70cm x 50cm x 20cm) without a heat insulation, and vessel for TR-L was a designed reactor made of acryl plastic (L x W x H = 90cm x 60cm x 30cm) with a heat insulation wrap built-in. Composting materials for TR-1, TR-2, TR-3 and TR-L were spent coffee ground (SCG) (77.4 - 85%), chicken manure (15-20%) and biochar (2.6%) (Table 1-2). The C/N ratio for all the composts under these conditions was estimated to be 20. The microbial agent (MA-1) (0.2% of total amount of composting materials) was augmented to facilitate the composting process in cases of TR-2, TR-3 and TR-L. Composting was

performed under 20-25ºC (room temperature) and 50-55% in moisture for 4 weeks and then maturation lasted for 2 weeks. For the first ten days of the composting period, the mixture of composting materials was overturned every other day to maintain optimal composting conditions. The complete composts were dried to 40-50% in moisture and kept at room temperature until the subsequent experiments (Figure 1-2).

1.2.3 Physico-chemical analysis and maturity test of compost products

In the beginning, 50g of each compost was extracted in ionized water (500mL) on a rotatory shaker (10000 rpm) at room temperature for 1 h and then the extraction was used for physico-chemical analysis and maturity test. Physical analysis such as electrical conductivity (EC) and pH were measured using YSI Multi-Parameter Water Quality Sonde (6600 V2-4) and Istek pH meter (pH-200L), respectively. Moisture content was determined by weight loss during oven drying of 10g of compost, which was indicated as a dry matter in percentage (Ameen et al, 2016). Total organic carbon (TOC) was analyzed with the dilution of 30 x using TOC analyzer (TOC-L, Shimazu Inc, Japan). Total nitrogen (TN), ammonia (NH_4^+-N), nitrate (NO_3^--N), and available phosphorus (PO_4^{3-}) were analyzedusing pretreatment system (HS-R200), water quality analyzer (HS-3300) and appropriate water analysis kits (NS 3300, Humas Inc., South Korea). Maturity test for composts was performed based upon the germination index of the test plant. The leaf length and width were measured and calculated using the formula GI = GR × RE / 100, where GR was a growth rate and RE indicated root elongation (Selim et al., 2012).

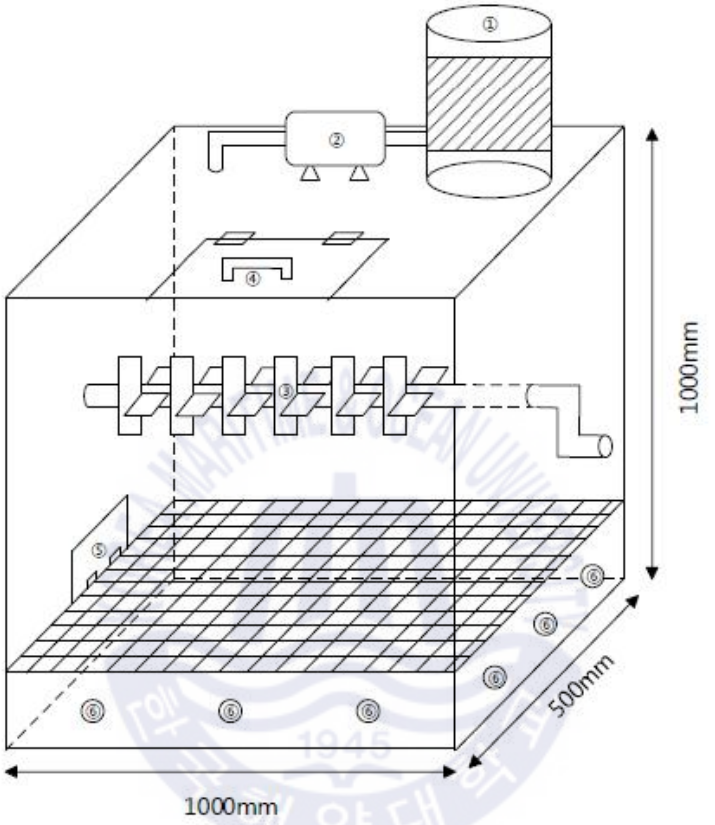

Figure 1-2 Overview of composting system at pilot level system

Table 1-2 The composition for the pilot scale level composting using spent coffee grounds, chicken manure, biochar, and the microbial agent MA-1.

Treatment	Spent coffee grounds (%)	Chicken manure (%)	Biochar (%)	Microbial agent (MA-1) (%)
TR-1	80	20	0	0.0
TR-2	80	20	0	0.2
TR-3	77.4	20	2.6	0.2
TR-L	85	15	0	0.2

1.2.4 Microbial community analysis of the complete compost

Microbial community structures in the composts were analyzed using 16S rRNA gene-based pyrosequencing since various microbial communities were involved in the composting process. The detail analysis procedures were described by Kim et al. (2014). In brief, total bacterial genomic DNA of each compost was isolated using PowerSoil® DNA Isolation Kit (Mo Bio Laboratories, Inc., U.S.A.). Variable regions (V1–V3) of the bacterial 16S rRNA gene were then amplified from the genomic DNA, and the library construction, sequencing and all subsequencing analyses were performed using 454 GS FLX Junior Sequencing System (Roche, Brandford, CT, USA) and the accompanying protocols. Statistical analyses of microbial communities were accomplished with the Mothur program, using a 3% difference cut-off value (Schloss, et al., 2009). Principal coordinate

analysis (PCoA) and fast Unifrac analysis were conducted using CLcommunity softwares (Chunlab, Inc., Seoul, Republic of Korea).

1.2.5 Crop growth test for the complete composts

As a part of efficacy test of the complete composts, effectiveness of the composts on growth of the commercial crops such as pepper and leek were studied at field and lab scale, respectively. The pepper plant growth test was performed on sandy loam soil in a commercial vinyl house farm in Milyang, South Korea. Four rectangular plots (0.9m x 4.5m = 4.1 m^2) were set up and designated CON, TR-1, TR-2 and TR-3 and the plots were amended with commercial chemical fertilizer (Perters Professional, 301010, 191919 and 230836, Everris International B.V., Netherland), TR-1, TR-2 and TR-3 (the three complete composts from this study), respectively. The water-soluble chemical fertilizer was amended according to the manufacturer's recommendation for duration of the experiment while the compost was amended once in the beginning of the experiment (1.1kg/m^2). Seven pepper seedling plants were then transplanted 60cm apart in a row in each plot. Growth effect examination was performed for the three representative plants in each plot, and leaf length and leaf width of second branch from the crown top were measured for ten leaves after 74 days, and number of all pepper fruits in each representative plant was also counted. The pepper leaves (ca. 200g) and fruits (ca. 300g) were taken for the analysis of antioxidant production.

The leek plant growth test was also performed using a round plastic pot (upper diameter 15cm, lower diameter 10cm, and height 12cm) carrying the sandy loam soil (1.1 kg) taken from the field under ambient indoor conditions (25^oC; 300-500 lux sunlight). Eight kinds of fertilizer. amendment conditions were set up: Control 0.00(kg/m^2), a commercial chemical fertilizer 0.10, a commercial organic fertilizer 0.12, TR-1 2.00, TR-2 2.00, TR-3 2.00, TR-L 2.00, and TR-L (2x) 4.00. Each amendment was carried out in three replicate pots each of which was planted with

three seedling plants of leek, and the growth experiment lasted for 76 days. All leaves of each pot were taken and pooled for the analysis of growth and antioxidant production.

1.2.6 Antioxidant activities of pepper and leek grown in the presence of the composts

A certain amount (1g) of dried pepper leaves and fruits was used to extract and prepare a sample solution for the antioxidant test. Assay of 1,1-diphenyl 2-picryl-hydrazyl (DPPH) radical scavenging activity was performed following the previous report (Chang et al., 2016). Briefly, one milliliter of ethanol extract and 5 mL of a freshly prepared DPPH ethanol solution (0.1 mM) were mixed thoroughly and kept in the dark. After 30 min of incubation at ambient temperature, the absorbance was read against a blank at 517 nm by using a UV–visible spectrophotometer (Model POP, Optizen, Inc. Seoul, South Korea). The percentage of free radical scavenging activity was calculated as follows: scavenging activity (%) = [1 − (A517 nm of sample/A517 nm of blank)] × 100.

Total phenolic content as an antioxidant material was determined by the Folin–Ciocalteu method and using gallic acid as the standard following the previous report (Chang et al., 2016). The extract was prepared from 1g of dried and macerated leaf or fruit with the incubation time of 24 hours and filtered. Due to the less addition of ethanol extract, the sample had concentrated for 4X and Briefly, 0.4 mL diluted ethanol extract solution was shaken for 1 min with 0.4 mL of the Folin–Ciocalteu reagent (1 M), and 0.8 mL of $Na_2 CO_3$ (20%, w/v) were mixed. After 8 min of incubation, the mixture was centrifuged at 15,000 g for 10 min. The absorbance of the supernatant was measured at 730 nm by using the spectrophotometer. The results expressed as the milligram equivalent of gallic acid per gram of extract (mg GAE/g) based on the standard curve for the gallic acid concentration versus absorbance (R = 0.873) (Chang et al., 2016).

1.2.7 Statistical analysis

The data from the experiments were subjected to analysis of variance for a completely random design by using SPSS statistical software (Jothy et al, 2011). The data were presented as the mean ± standard deviation of duplicate or triplicate determinations according to the test and treatment. Comparison of means was analyzed by Duncan's test of the SPSS system (IBM SPSS statistics 19) and differences were considered significant when p is < 0.05.

1.3 Result and discussion:

1.3.1 Physical and elemental characteristics of biochar made from tomato plant stems

The biochar to be used as an amendment material for the compost was manufactured using tomato plant stems as a raw material. Surface area yield and elemental compositions (C, H, O, N and S) were measured depending upon the different temperatures (Table 1-3). There was a gradual increase of surface area (BET) as the working temperature increased where the highest surface area (3.41 m^2/g) was achieved at 550ºC. However, the highest yield was obtained 450ºC in which 69% of the highest area (3.41 m^2/g) was achieved. For major elemental analysis, C content proportionally increased as the temperature increased while H and O decreased. The ratios of O/C and H/C were inversely proportional to the increasing temperature. In all, 450ºC was considered an optimal working temperature in which a quality biochar could be manufactured to be used as a compost amendment substrate.

1.3.2 Composting process monitoring

Temperature and moisture content were monitored for the composting treatments TR-1, TR-2, TR-3 and TR-L. TR-1 (25kg in total amount) and TR-2 (25kg) reached their highest temperatures, 51°C and 49°C, respectively after one day, and showed a gradual decrease of temperature up to the ambient room temperature (ca. 20°C) after 6 days. TR-3 (25kg; contained biochar),

Samples	BET (m^2/g)	Yield (%)	C (%)	H (%)	O (%)	N (%)	S (%)	O/C	H/C
Raw Material	0.49	-	36.2	5.67	42.85	1.85	1.57	1.18	0.15
400°C	2.19	30.7	42.88	2.9	21.75	2.17	1.58	0.50	0.06
450°C	2.35	40.7	44.05	2.16	21.55	2.13	1.58	0.48	0.04
500°C	2.64	34.8	47.57	2.08	20.59	2.14	1.57	0.43	0.04
550°C	3.41	25.6	50.02	1.97	20.56	1.65	1.38	0.41	0.03

Table 1-3 Physical and elemental characteristics of the biochar made from tomato plant stems

However, reached its highest temperature, 47°C after 2 days, and the temperature decreased up to the ambient room temperature after 9 days. By the way, TR-L (200kg) reached its highest temperature, 63°C after 2 days, and the temperature decreased up to the ambient room temperature after 4 weeks (about 28 days). It was not easy to maintain the composting temperature within the vessels

for TR-1, TR-2 and TR-3 since the vessels did not carry an insulation wrap and a proper cover in the winter season. Moisture content of all the compost was adjusted to 50-55% to maintain a good microbial activity for the composting. Temperature, one of the key indicators of composting, determines the rate of many biological processes and plays a significant role in evolution and succession of microbiological communities (Hassen et al., 2001). Amendment of biochar during the composting process can provide some benefits: helping to maintain useful nutrients onto the biochar in self (Birk et al., 2009). The biochar could also favor a more generation of humic acids over fulvic acids during composting of poultry manure–mixture and alleviate nitrogen loss in the mature composting products (Dias et al., 2010). Biochar addition during composting of tomato stalk and chicken manure composting allowed a reduced time to enter thermophilic phase, a higher temperature and longer duration of thermophilic stage, and the biochar addition shortened the period of thermophilic phase and increased C-CO_2 emission (Czekała et al., 2016). A longer duration of the thermophilic stage was also observed in TR-2 with the addition of biochar in our study.

1.3.3 Physico-chemical analysis and maturity quality test of the complete composts

Some important physical and chemical parameters were measured for extracts of the manufactured composts to figure out their quality (Table 1-4). Physical ones were EC, pH and salinity while chemical ones were NH_4^+-N, NO_3^--N, TOC and T-N. TR-2, TR-3 and TR-L showed a significantly higher EC than TR-1 (mS cm^{-1}) which was not bioaugmented with MA-1, indicating a significantly higher generation of ionic materials. Similarly, pH also showed a significantly higher trend in the composts (6.7-7.1) augmented with MA-1, indicating a neutralization of the composts facilitated by the microbial agent. There were no significant differences in salinity among the composts except TR-L which showed a bit lower than the rest. The highest TOC was observed in TR-3 (2977mg/kg^{-1})

while the lowest in TR-2 (1341mg/kg^{-1}), indicating effects of the biochar amendment and the bioaugmentation, respectively. TR-2 showed the highest T-N (548 mg/kg^{-1}) while TR-1 the lowest (423 mg/kg^{-1}), reflecting an active role of MA-1 in producing and releasing soluble nitrogen compounds like proteins, NH_4^+ and NO_3^- as opposed to TR-1 not augmented by MA-1. Ratios of TOC/T-N showed the highest (6.91) in TR-3 containing biochar while the lowest in TR-2 in contrast to TR-1. This indicates that the biochar addition contributed to higher TOC content in TR-3 considering the lowest TOC/T-N ratio in TR-2. TR-1 is considered as a control, because of absence of addition of biochar and microbial agent. Here the biochar appeared to survive the composting process mediating its reaction. Concentrations of NO_3^--N were significantly higher in TR-2, TR-3 and TR-L than TR-1. However, the ratios of NH_4^+-N/ NO_3^--N as an indicator of compost maturity in TR-2, TR-3 and TR-L were significantly lower compared with TR-1, showing that the former composts were more mature due to the presence of MA-1 and biochar which contributed to nitrification of NH_4^+ and sequestration of NO_3^-, respectively. Biochar has the capacity to improve the soil properties and it is desirable to prepare compost product by applying biochar as a composting amendment and has been recently found to decelerate the ammonification process in soil, with a build-up of organic nitrogen, while promoted soil ammonia-oxidizer populations and accelerated gross nitrification rates (Prommer et al., 2014). A recent study by Kammann et al. (2015) also found that biochar can capture and retain NO_3^- during composting. The captured NO_3^- was found to be largely protected against leaching and partially plant-available, although the mechanisms for NO_3^- capture in biochar are still unknown.

Hence the compost reaches maturity more rapidly when supplemented with biochar (Wang et al., 2014). Moreover, the presence of biochar in composting of the buffalo manure led to a protection of organic matter against biodegradation through chemical oxidation, suggesting that biochar could facilitate the carbon sequestration potential and organic phosphorus transformation into available inorganic phosphorus.

Parameter	TR-1	TR-2	TR-3	TR-L
EC (mS cm^{-1})	2.9 ± 0.2^{a}	3.8 ± 0.1^{b}	3.9 ± 0.2^{b}	4.2 ± 0.4^{b}
pH	6.5 ± 0.2^{b}	6.1 ± 0.2^{a}	6.6 ± 0.1^{b}	6.2 ± 0.1^{a}
Salinity	0.44	0.46	0.43	0.37
TOC (mg/kg^{-1})	$2,074\pm360^{b}$	$1,341\pm87^{a}$	$2,977\pm289^{c}$	$2,209\pm225^{b}$
T-N (mg/kg^{-1})	423 ± 9^{a}	548 ± 21^{c}	460 ± 14^{ab}	471 ± 39^{b}
NH_4^+-N (mg/kg^{-1})	51 ± 0.1^{a}	63 ± 1.1^{a}	56 ± 0.5^{a}	113 ± 10^{b}
NO_3^--N (mg/kg^{-1})	70 ± 2.5^{a}	184 ± 22.9^{b}	163.8 ± 7.6^{b}	280.1 ± 28.4^{d}
PO_4^- (mg/kg^{-1})	136 ± 4.5^{b}	118 ± 3.1^{a}	139 ± 1.4^{b}	145 ± 3.9^{b}
TOC/ T-N	5.22 ± 0.7^{b}	2.87 ± 0.1^{a}	6.91 ± 0.9^{c}	5.04 ± 0.3^{b}
NH_4^+-N/ NO_3^--N	0.73 ± 0.12^{b}	0.34 ± 0.17^{a}	0.33 ± 0.07^{a}	0.40 ± 0.93^{a}

Results are expressed as the means ± standard error. Values with different letters indicate significant differences at $p< 0.05$ according to Duncan's multiple range test.

Table 1-4 Physical and chemical characteristics of the composts made from spent coffee grounds, chicken manure, biochar through bioaugmentation of the microbial agent MA-1.

An official quality test for the manufactured composts was performed by a fertilizer analysis company officially approved by Office of Rural Development, a government of South Korea. All the data herein turned out to meet all the criteria required by the institution as shown in Table 4. This indicates that all the composting process, secure a successful manufacturing of quality organic compost to be used in the agricultural industry.

Compartive analysis of germination rate (GR), root elongation rate (RE) and germination index (GI) of extractions (80x dilution) from the four manufactured composts was presented in Fig 4. Germination index (GI) is a sensitive parameter for the evaluation of compost phytotoxicity. GI's for TR-2 and TR-3 were 14 and 34% more higher than that for TR-1, respectively. This indicates the microbial agent MA-1 could contribute to the increase of GI in TR-2 and TR-3, and biochar also could increase even more of GI (20%) in TR-3, showing the efficacy of biochar amendment in the composting process (Figure 1-3). TR-L is assumed that these GI increase effects could mean a potential increase of crop yield in the field test to be accomplished later. The GI test was used to evaluate phytotoxic substances contained in the compost, which was one of the most sensitive parameters accounting for low toxicity affecting root growth (Gu et al., 2011). The GI values of all treatments started at higher than 90%, indicating that amending soil with compost mixture might increase the fertility of soil. The increase of germination index comparing with control clearly indicates that the growth has been stimulated by the growth factors (putatively NO_3^- and other micronutrients) present in the compost and the amended biochar able to efficiently carry the useful nutrients and microbes as well as moisture.

Based on the compost result given by Dias et al., 2010, the compost with addition of biochar provides the higher value, which the data is similar as we provided. The total nitrogen provided the different value by increase because of the addition of microbial agent. Compaing the different treatment of compost with control either Biochar added compost or compost with microbial agent provided the higher value in all forms of elemental analysis. The germination index of the SCG compost amended by biochar and bioaugmented by *Trametes versicolor* was higher by 20% compared with the control (Hachicha et al., 2012), which clearly indicated the biochar may accelerate the composting and induce the plant growth.

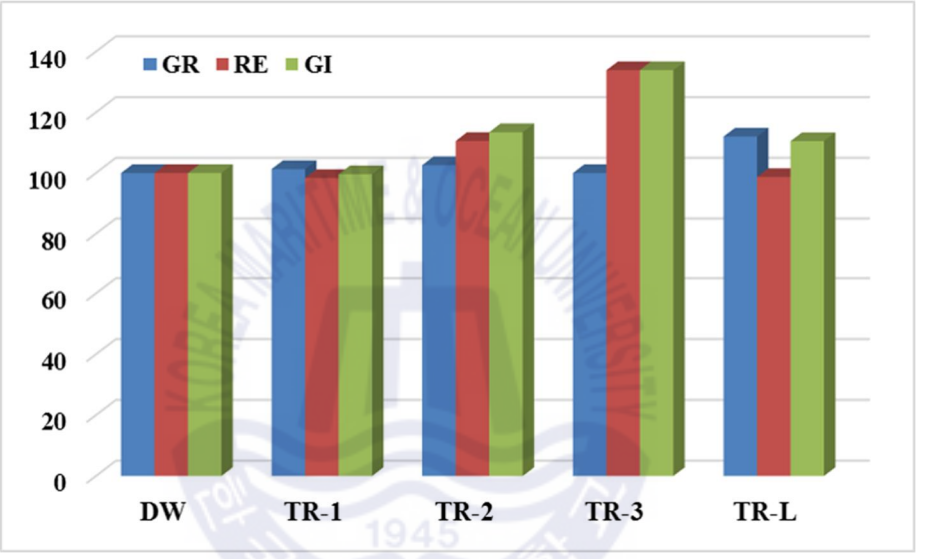

Figure 1-3 Comparative analysis of germination rate (GR), root elongation rate (RE) and germination index (GI) of the manufactured composts (TR-1, TR-2, TR-3 and TR-L).

$$GI = GR \times RE / 100$$

1.3.4 Microbial community analysis of the manufactured composts

Microbial communities of the manufactured composts were analyzed based on 16S rRNA gene pyrosequencing method after 6 weeks to elucidate how the bioaugmentation and composting conditions could affect dynamics of the microbial communities in the composts. The valid read sequences were assigned to the range of 2683-7670. The operational taxonomic units (OTUs) at a 97% sequence identity cut-off, species richness and species diversity destimations were calculated for each compost samples. The Good's coverage showed that the libraries represented the majority of bacterial 16S rRNA gene sequences present in each compost sample, with values ranging from 88% to 97%. A species richness based operational taxonomic units (OTUs) was higher in order of TR-L, TR-3, TR-2 and TR-1. Other species richness indices as Ace and Chao1 were higher in order of TR-L, TR-3, TR-1, and TR-2. The higher richness of the compost TR-L (200kg) was probably because it appeared to possess higher contents of available organic matter (45.3%; Table 1-5), NH_4^+-N and NO_3^--N compared with the other composts so that the microbial population density and activity might be higher. Species diversity indices (Shannon indices) of TR-2, TR-L and Tr-3 (4.96 – 5.04) were higher at similar level than that of TR-1(4.63), indicating that microbial augmentation and biochar amendment might enhance the species diversity in the basal composting materials (spent coffee ground and poultry manure). TR-1 gave the lowest species diversity probably because of no bioaugmentation of MA-1 whose species could survive in the compost.

Evaluation criteria	Unit	Commercial quality standard	TR-1	TR-2	TR-3	TR-L
Organic matter N	-	< 45	24.3	15.3	18.1	16.7
NaCl	%	< 2	0.54	0.49	0.51	0.55
Misture	%	< 55	52.1	51.5	53.5	46.3
As	Mg/kg	< 45	ND	ND	ND	ND
Cd	Mg/kg	< 5	ND	ND	ND	ND
Hg	Mg/kg	< 2	ND	ND	ND	ND
Pb	Mg/kg	< 130	1.08	1.27	1.07	0.94
Cr	Mg/kg	< 200	3.99	4.33	3.31	3.33
Cu	Mg/kg	< 360	5.8	48.22	43.62	43..99
Ni	Mg/kg	< 45	5.18	5.79	4.85	4.5
Zn	Mg/kg	< 900	137.0	133.4	93.76	97.43
Organic matter	%	> 30	38.3	37.0	38.0	45.3
E. Coli	ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND

*The analysis was performed by AT Analysis Center Co., Ltd., Incheon and was officially approved by the Office of Rural Development, a part of the South Korean government; ND, no detection; **Soiltek, Inc., Jeju, South Korea.

Table 1-5 Quality evaluation of the manufactured composts (TR-1, TR-2, TR-3, and TR-L) according to the criteria required by the Office of Rural Development, South Korea*

For the analysis of phylum and species, the dominant taxa (greater than 0.5-1% abundance) of each sample were demonstrated in Figure 1-4A. The dominant phyla of MA-1 were Firmucutes (80%), Proteobacteria (13.7%), Bacteroidetes (3.6%) and Actinobacteria 1.3% (Figure 1-4A). Most of Firmucutes were composed of *Lactobacillus sp.* (68%). TR-1, a compost without bioaugmentation, carried the dominant phyla Bacteroidetes (77.1%), Proteobacteria (20.2%), Actinobacteria (0.6%) and Firmicutes (0.2%). Bacteroidetes (73-85%) and Proteobacteria (13-25%) were dominant in the composts augmented with MA-1 (TR-2, TR-3 and TR-L). The microbial agent MA-1 appeared to increase rate of Proteobacteria somewhat, but amendment of additional biochar tended to decrease the population of Proteobacteria. The highest distribution of Actinobacteria was observed in TR-L.

The dominant species in MA-1turned out to be *Lactobacillus brevis* (21%), *Lactobacillus plantarum* (12.4%), *Lactobacillus acidipiscis* (8.9%) and *Lactobacillus coryniformis* (7.1%). Other dominant *Lactobacillus sp.* were *Lactobacillus coryniformis* group, *Lactobacillus vaccinostercus,* and *Lactobacillus*_uc which were ranging 3-8% (Figure 1-4A & B). All *Lactobacillus* sp. most dominant in MA-1 were barely observed in all the composts, indicating that this species could act dominantly in the early stage of the composting process, but its population density significantly decreased in the later stage. Other representative dominant species in MA-1 were *Oenococcus oeni* (4.1%) and *Chitinophaga terrae* (1.7%) but also little of them survived in the complete composts. The two species of Sphingobacteriaceae (*Sphingobacteriaceae_uc_s and JF237857_s*) were the most dominantly distributed in all the composts but not in MA-1: TR-L (42.5%), TR-3 (29.3%), TR-1 (25.2%) and TR-2 (23.3%).

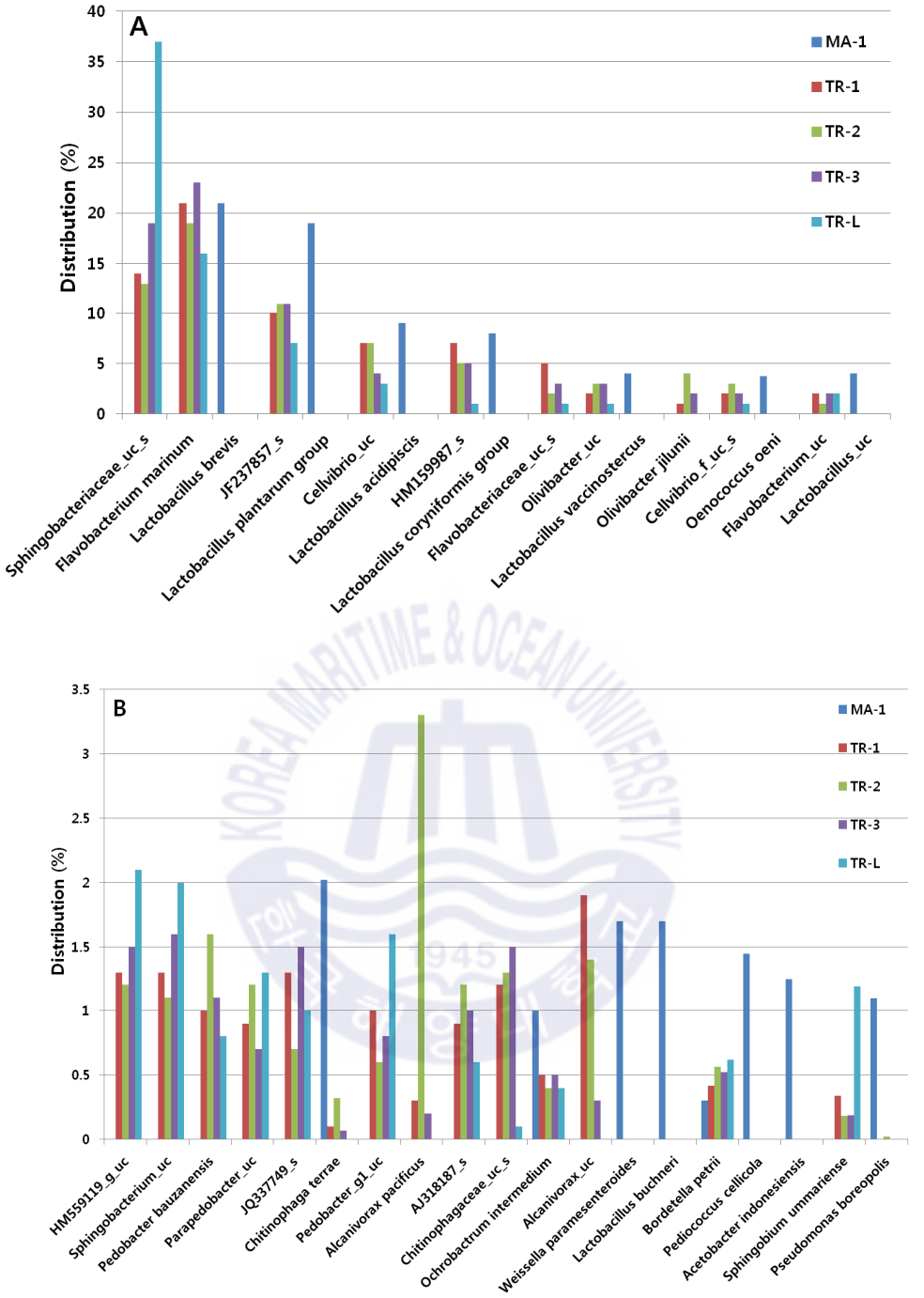

Figure 1-4 Microbial community structures at species level in the complete composts based on pyrosequencing analysis

Flavobacterium marinum was the next most dominant species in all the composts (16.8-23.3%) with highest density in TR-L and the lowest in TR-2. The data indicates that microbial augmentation and biochar amendment can increase the densities of species of Sphingobacteriaceae and *Flavobacterium marinum* by 4% and 3%, respectively (Figure 1-3). Interestingly, population of *Alcanivorax pacificus* increased 10 times in TR-2 (3.0%). Density of *Olivibacter jilunii* increased by 2.0% in TR-2 and 0.9% in TR-3, indicating efficacy of the bioaugmentation and biochar amendment.

A beta-diversity was further investigated using Fast Unifrac analysis method to find a similarity of microbial communities of the composts and the microbial agent MA-1 in species distribution (Figure 1-3 B). MA-1 was remotely located from the rest of the samples, indicating that little populations of microbes of MA-1 survived the augmented complete composts (TR-2, TR-3 and TR-L). TR-1 and TR-2 were most closely clustered, and TR-3 was a little distantly clustered from the TR-1 and TR-2 group likely due to the amendment of biochar and bioaugmentation of MA-1. TR-L was quite distantly clustered from TR-1, TR-2 and TR-3 since TR-L possessed higher contents of available organic matter, NH_4^+-N and NO_3^--N compared with the other composts, leading to selection of different microbial microbial communities. Each compost has different microbial community profiles and nutritional chemical compositions will be affecting the plant growth and useful plant components of commercial use.

1.3.5 Analysis of crop growth and antioxidant production in crops

A growth analysis of pepper plant was performed to test efficacy of the composts (TR-1, TR-2 and TR-3) on crops, and leaf length, leaf width and fruit number were measured (Figure 1-5) after 3 months. Length of the leaf is highest in TR-2, and TR-1 and TR-3 were not different from the control (CON) that was not amended with any compost. Leaf width of TR-1 and TR-2 was significantly higher than the control and TR-3. This means that TR-2 was more effective in stimulating leaf

growth compared with CON and TR-1, indicating a potential efficacy of bioaugmentation of MA-1 on the basal compost (TR-1). TR-1 showed the highest fruit yield than all the others. However, TR-2 produced a lowest yield of pepper fruit in contrast to the highest growth of the leaves. It was seemed that a possible reason for the lower fruit yield in TR-2 was due to a relatively intense pest damage to the young pepper plants on the testing plot located nearest to the neighboring vinyl house already contaminated by pests. TR-3 made no difference from CON in leaf growth and fruit yield. In all, there was no clear correlation between plant leaf growth and fruit yield in this experiment.

Samples of the pepper leaf and fruit were taken after the 3 month period, and antioxidant properties of their ethanolic extracts were examined for DPPH radical scavenging activity (Figure 1-6). The scavenging activity of catechin as a positive control was 92.8% ± 2.5. There were no signifcant differences of the activities of leaf and fruit among TR-1, TR-2 and TR-3 in comparison of the control (CON). However, TR-3 showed a significant higher activity than TR-1 in the leaf while TR-2 showed a significant higher one than TR-1. This means that bioaugmentation or bioaugmentation combined with bioachar amendment could enhance the antioxidant activity at this time of sampling. Moreover, it will be worthwhile to investigate change of the activity over time even after the 3 month period to see if the application of the composts could induce more antioxidant compounds in comparison with the control. Comparable increase in the TR-2 with 22.9% with other samples and the lower value in of scavengimg activity of TR-1 sample of 21.7% at the concentration tested (1 mg/ml) and scavenging activity in fruit is quite similer to the pepper leaf, where TR-2 gives 50.1% of activity and percentage of TR-1 gives 44.4% of scavenging activity. Even though control had the second higher value, the addition of microbial agent in TR-2 showed the higher value and addition of biochar in TR-3 showed more a ctivity than control ad TR-2.

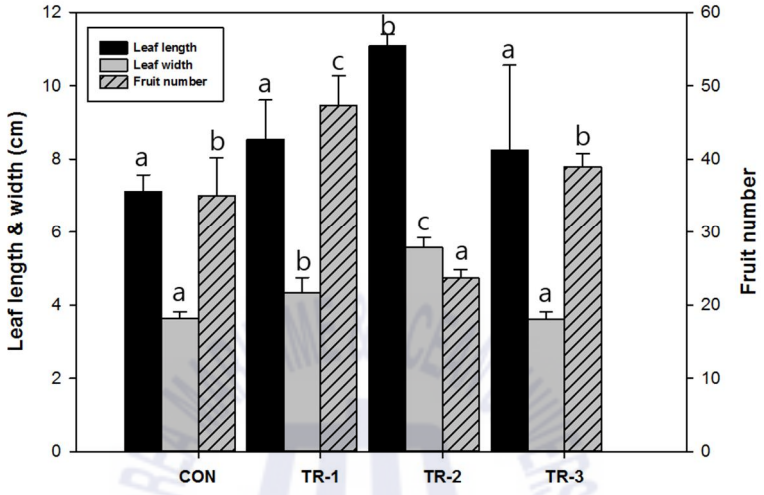

Figure 1-5 Growth analysis of pepper plants grown in soil amended with the complete composts (TR-1, TR-2 and TR-3). CON indicates no treatment of any compost. Bars with different letters indicate significant differences at $p< 0.05$ according to Duncan's multiple range tests.

The anitoxidant activity seemed to give an equivalent performance to colour and pigment amount that was the presence of medium-high percentages of spent coffee ground's bioactive quality. Regarding DPPH radical scavenging effect, a gradual decrease in the inhibition value was observed in plant grown in substrate with increasing fresh-SCG percentage. The reducing power outcomes and an identical pattern to radical scavenging activity was observed for dry weight of plant treatments (Cruz et al., 2014).

Total phenolic content was also monitored as an indicator of antioxidant activity in the pepper crop (Figure 1-7). The plant phenolics are known to be able to scavenge reactive oxygen species due to their electron exchanging properties. Essentially there were no differences in total phenolic contents of leaf among TR-1, TR-2 and TR-3 in comparison of the control (CON). However, TR-3 and TR-1 showed a significant higher content than CON in the fruit, showing an increase of 4.5% and 3.9%, respetively. This means that an application of the basic compost (TR-1) containing SCG and poultry manure only or bioaugmentation of the basic compost combined with bioachar amendment (TR-3) could enhance the phenolic content. Here the results indicate that bioaugmentation combined with bioachar amendment could increase the anitoxidant activity of the edible portion of pepper plant and hence increase the quality of crops. The growth of the leek plant has been done in laboratory scale and the antioxidant activity was measured. Comparing with other samples TR-L shows the higher scavenging activity of 20.7% and the next is TR-L(2X) with 16.8% and chemical fertilizer gives lower scavenging actiity with 9.4%. TR-2 and TR-L is comparabily higher than control. The total phenolic data retrives TR-1 gives 16.9 mg GAE/L antioxidant activity. TR-3, TR-1 and the organic fertilizer provides higher value than the control. The rest of the samples are quite similer with each other exept TR-L provides low activity of 6.6 mg GAE/L.

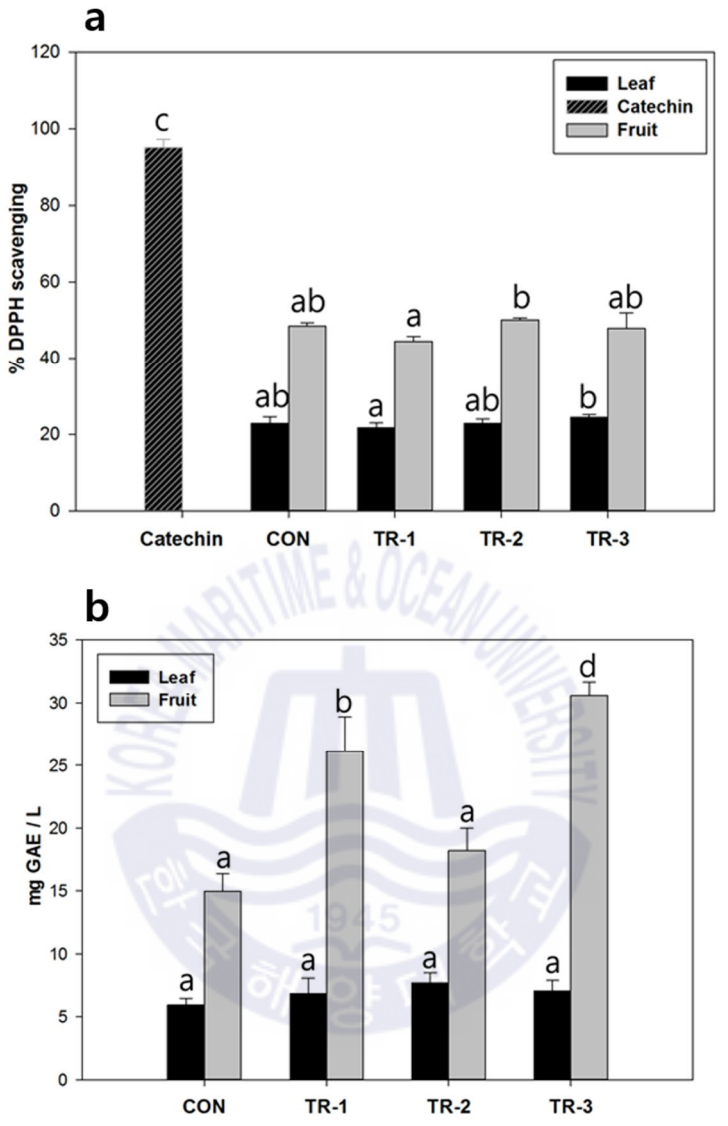

Figure 1-6 DPPH scavenging activity of extracts of pepper plants grown in soil amended with the complete composts (TR-1, TR-2 and TR-3) (a). Total phenolic content of extracts of pepper plants grown in soil amended with the complete composts (TR-1, TR-2 and TR-3)(b). CON indicates no treatment of any compost.Bars with different letters indicate significant differences at $p< 0.05$ according to Duncan's multiple range test.

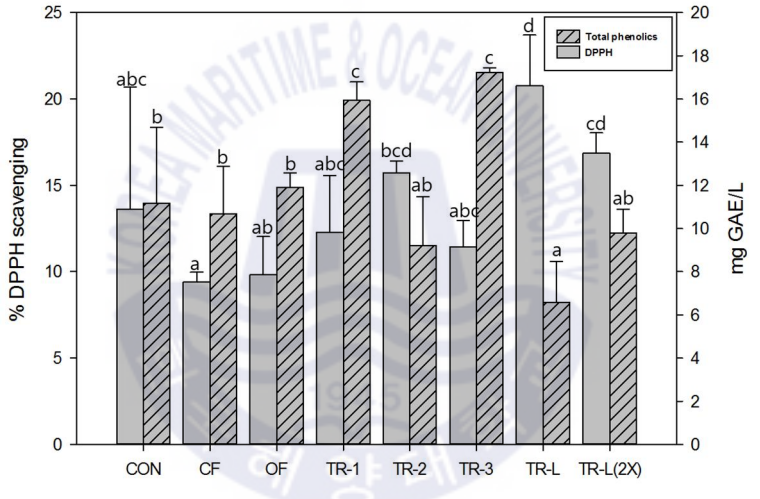

Figure 1-7 DPPH scavenging activity and total phenolic content of extracts of leek plants (leaf) grown in soil amended with the complete composts (TR-1, TR-2, TR-3 and TR-L) together with the commercial fertilizers. Bars with different letters indicate significant differences at $p< 0.05$ according to Duncan's multiple range tests.

Phenolic compounds are abundantly present in plants and play a fundamental role in human diet, mainly due to their antioxidant properties. As per knowing that the Folin–Ciocalteu method is not specific for these substances, this assay is regularly used to estimate total phenols content indirectly in several vegetable matrices, allowing the estimation of total reducing capacity of the extracts. Thus, it was selected to investigate if changes in plant's antioxidant profile with plant observance from the compost which are related to their phenolic content (Cruz et al., 2014). The major phenolics in pepper and leek plant exhibit a wide range of physiological properties, such as anti-inflammatory, antimicrobial, cardio protective, hepatoprotective, hypoglycemic and antiviral effects (Navarro-González et al., 2011). The DPPH and the total phenolic test done with the procedure provided by Chang et al., 2016 and the result has been compared with the similar article and the plant is differentiated by providing pepper and leek plant instead of welsh onion plant. The DPPH raidical scavenging ability quite lower than the welsh onion plant still there is an increase in the addition of biochar comparing with control. The incerase in the percentage ability of DPPH in pepper plant provides the difference in addition of biochar. As same as in DPPH; total phenolic content also provided higher antioxidant activity in the composting treatment with the addition of biochar.

1.4 Conclusion

SCG, poultry manure, and agricultural waste-derived biochar were used to manufacture functional composts through microbial bioaugmentation. Tomato stalk-based biochar was successfully obtained at 450C with the highest yield (40.7%) and a surface area of 2.35 m^2/g^{-1}. Radish germination indices for the composts manufactured through augmentation and/or biochar amendment increased by 14– 34%. Bacterial diversity was also enhanced in the augmented and biochar-amended composts by 7.1–8.9%, where two species of Sphingobacteriaceae were dominant (29– 43%). The scavenging activities for

DPPH were enhanced by 14.1% and 8.6% in the fruits of pepper plants grown in the presence of the TR-2 (augmentation applied only) and TR-3 (both augmentation and biochar amendment applied) composts, respectively. Total phenolic content was alsoenhanced by 68% in the fruits of the crop grown in TR-3. Moreover, the other compost, TR-L (augmentation applied only), boosted DPPH scavenging activity by 111% in leeks compared with commercial organic fertilizer, while TR-3 increased the phenolic content by 44.8%. Therefore, this novel technology can provide eco-friendly recycling of organic wastes, such as spent coffee grounds, poultry manure, and agricultural wastes. The high availability of the low-cost composting materials and the benefits of the functional compost should allow commercialization of the compost as a marketable product. Therefore, it will be worthwhile to evaluate the economic and business feasibility of the overall composting technology in terms of compost resource provisions and the effectiveness of processing at an industrial scale in future studies.

1.5 Acknowledgment

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CHAPTER II – METAGENOMIC ANALYSIS OF ECO-FRIENDLY TREATMENT PROCESS OF TANNERY WASTEWATER AT A FULL-SCALE SYSTEM

2.1 Introduction

2.1.1 Motivation of work

Communities were identified that had the ability to colonize such harsh niches with desired catabolic traits, and these provided an opportunity to develop specialised inocula that could be exploited by bioaugmentation (Van der Gast et al., 2002). Bioaugmentation is using added microorganisms to "reinforce" biological waste treatment populations so that they can effectively reduce the contaminant load by transforming it into less dangerous compounds. Conventional biological treatments for wastewater are rigorous, inadequate, inefficient, and/or not cost-effective, due to 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 environmental friendly with the resulting effluent characteristics that meet the regulation standard in the terms of COD, TN, TP, heavy metals, sludge, odor etc.

2.1.2 Tannery wastewater treatment using microbial consortium

The tanning industry also has one of the highest toxic intensities per unit of output (Khan et al., 1999). Tannery wastewater carries waste skin trimmings, dyes, surfactants, sulfonated oils, chromium salts, and solid wastes, representing a serious environmental risk (Durai and Rajasimman., 2011 and Sharma and Adholeya.,

2011). Tannery wastewater is characterized by high concentrations of nitrogen (especially ammonium) and organic compounds, including proteins and fats, heavy metals (Cr_3^+, Pb and Ni) and various solutions (Durai and Rajasimman., 2011, Sharma and Adholeya, 2011 and Jianto et al., 2018). Wastewater is more recalcitrant and inhibits biological treatment because of its high concentrations of organic nitrogen and ammonium, collagen, and toxic tanning agents (El-Sheikh et al., 2011).

At the core of wastewater treatment is the biodegradation, oxidation, and reduction of organic macromolecules and inorganic chemical species, primarily by complex microbial communities (Ferrera and Sanchez., 2016). The complex nature and high toxicity of wastewater compositions can have a significant impact on nitrogen metabolism, including assimilation and nitrification. Ammonium (NH_4^+), nitrate (NO_3^-) and nitrite (NO_2^-) are usually effectively removed by nitrification and denitrification depending on the microbial community and environmental conditions (Mohan et al., 2004 and Juretschko et al., 2002). Denitrification is facilitated by a sufficient supply of unstable organic carbon, but anammox is more feasible when organic carbon is limited. Denitrification and anabolism were extensively studied in wastewater treatment systems (Lu et al., 2014 and Yu et al., 2014). Biological nitrogen removal implementations in tannery wastewater treatment plants or systems have been relatively well documented (Fiehn et al., 1994, Goltara et al., 2003, Chung et al., 2004, Leta et al., 2004 and Szpyrkowicz and Santosh., 2004). However, the mechanisms of the microbial removal of nitrogen from full-scale tannery wastewater treatment plants have not yet been addressed.

2.1.3 Literature review

The diversity of microbial communities and the nitrogen metabolism activities in wastewater treatment systems were effectively monitored by pyrosequencing and metagenomic methods (Wang et al., 2017 and Fang et al.,

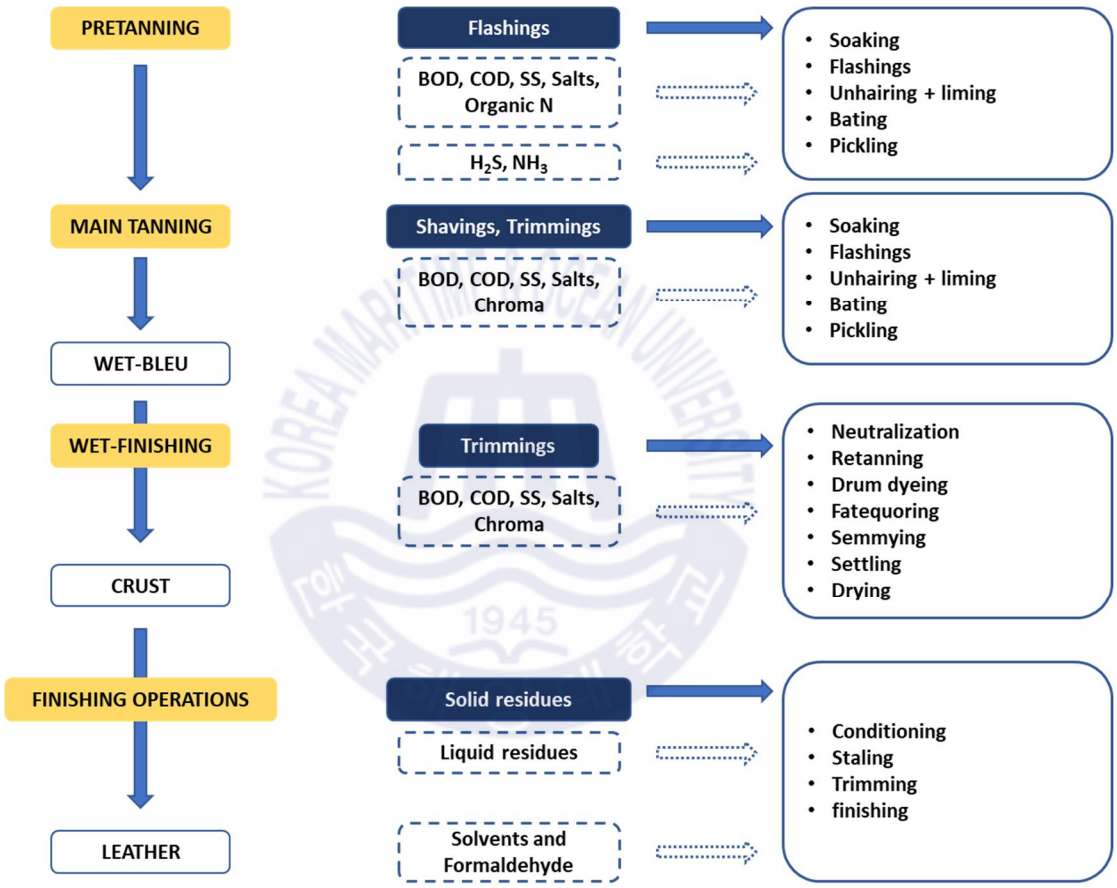


Figure 2-1 Process of tanning to generate tannery wastewater

2018). The metagenomic and metatranscriptomic techniques have revealed the global patterns of microbial community structure and gene expression in activated sludge along with the enzymes involved in ammonia oxidation, nitrification, denitrification and nitrogen fixation, and amino acid and fatty acid metabolism processes in wastewater treatment plants (Yu and Zang., 2012 and Shchegolkoval et al., 2016). Recently, the analysis of community structures and functional genes of a variety of bacterial nitrifiers and denitrifiers was conducted in tannery wastewaters using high-throughput sequencing techniques (Wang et al., 2014). The biological process of nitrogen removal in wastewater follows a path under certain environmental conditions, depending on the microbial communities involved and the substrates available (Shchegolkoval et al., 2016, Wang et al., 2014 and Ye and Zang., 2013).

In previous works, we confirmed the occurrence and potential functions of denitrifiers in tannery wastewater treatment (Kim et al., 2014 and Kim et al., 2013). COD and total nitrogen (N) removal in a tannery wastewater treatment plant were significantly enhanced by the bioaugmentation of the novel microbial consortium BM-S-1. Pyrosequencing analysis showed that the dominant phyla were Proteobacteria, Bacteroidetes and Firmicutes in the buffering tank, primary aeration tank, secondary aeration tank, and sludge digestion tank, whereas Firmicutes was the most dominant in the influent (Kim et al., 2014). Furthermore, a metagenomic analysis was able to identify the dominant taxa responsible for dissimilatory N metabolism in the tannery wastewater treatment system undergoing bioaugmentation (Sul et al., 2016). The augmentation increased the abundance of the denitrification genes found in genera such as *Ralstonia* (*nirS, norB*, and *nosZ*), *Pseudomonas* (*narG, nirS*, and *norB*) and *Escherichia* (*narG*) in the buffering tank and primary aeration tank. This corroborated the higher total N removal in these two stages.

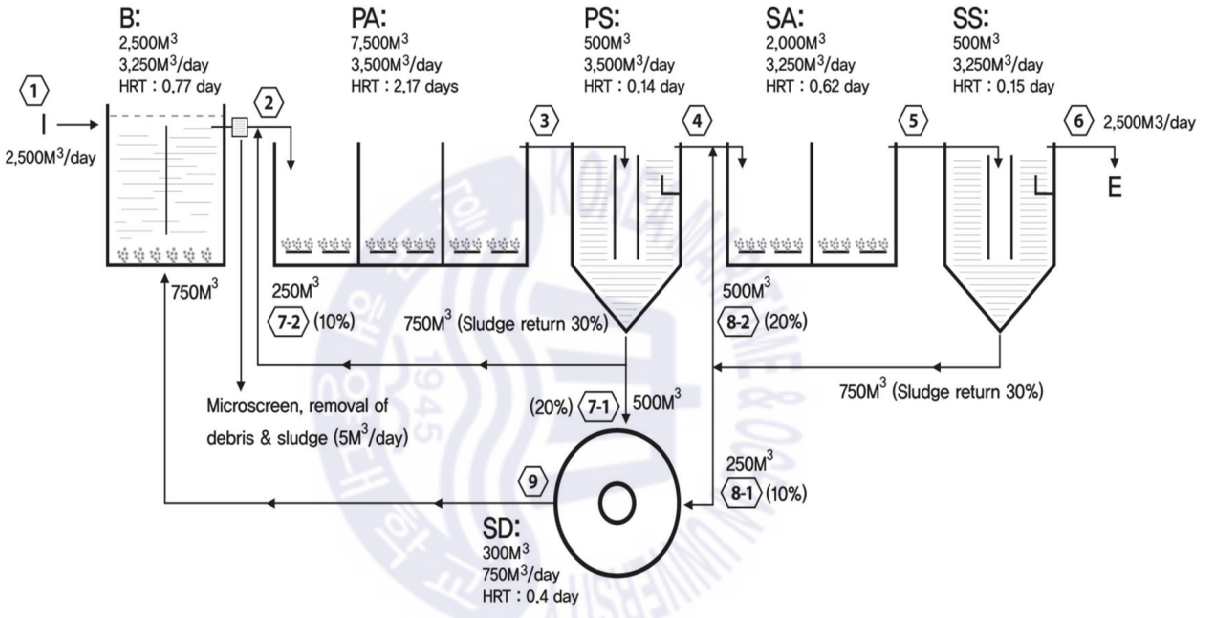


Figure 2-2 Full-scale biological treatment of tannery wastewater treatment

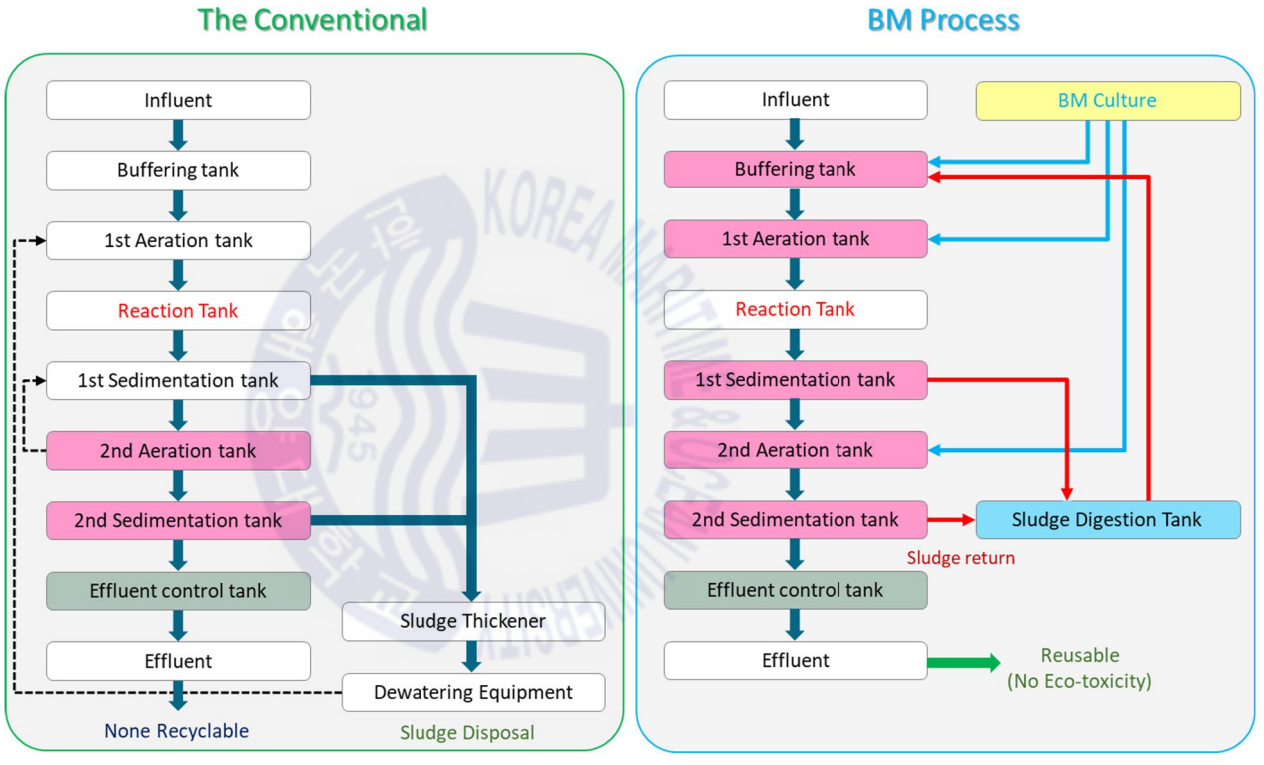


Figure 2-3 The workflow of conventional and BM process of tannery wastewater system (Kim et al., 2014).

2.1.4 Metagenomic insight in tannery wastewater treatment and its goals

The denitrification process could be closely related to carbon catabolic pathways, such as the degradation of amino acids, fatty acids, and carbohydrates, which could generate electron donors for nitrate reduction, and the efficient removal of nitrogen could be linked to COD removal (and hence sludge reduction) (Sul et al., 2016). Shotgun metagenomic reads were mapped specific functional profiling, and then the gene abundance data were analyzed using HUMAnN2 (Franzosa et al., 2018). The degradative pathways of the 20 amino acids from proteins lead to a specific point of entry into the citric acid cycle, such as acetyl-CoA, α-ketoglutarate, succinyl-CoA, fumarate, and oxaloacetate (Nelson and Cox., 2018). Some amino acids are degraded to acetyl-CoA via pyruvate, while others are converted into acetyl-CoA and acetoacetyl-CoA, which are cleaved to form acetyl-CoA (Nelson and Cox., 2018). Fatty acids are degraded to acetyl-CoA via the β-oxidation cycle, which enters the citric acid cycle, where itis completely oxidized to CO2 and water (Stryer., 1995). The TCA cycle generates the reducing agent NADH that is used in the denitrification process. It is therefore highly likely that the degradative pathways of amino acids and fatty acids prevalent in tannery wastewater could relate to the denitrification process via electron carriers (e.g., NADH) (Figure 2-4). To date, there have been no studies on the mechanistic basis of denitrification in relation to amino acid or fatty acid degradation based on high-throughput sequencing techniques.

The connections were analyzed in view of the three different aspects: 1) comparative analysis of microbial communities depending on the different treatment stages; 2) analysis of the correlation between denitrification genes and amino acid or fatty acid degradation enzymes based on pathway abundance analysis; and 3) comprehensive analysis of the overall roles of denitrification, amino acid degradation and fatty acid degradation in the different treatment stages of tannery wastewater.

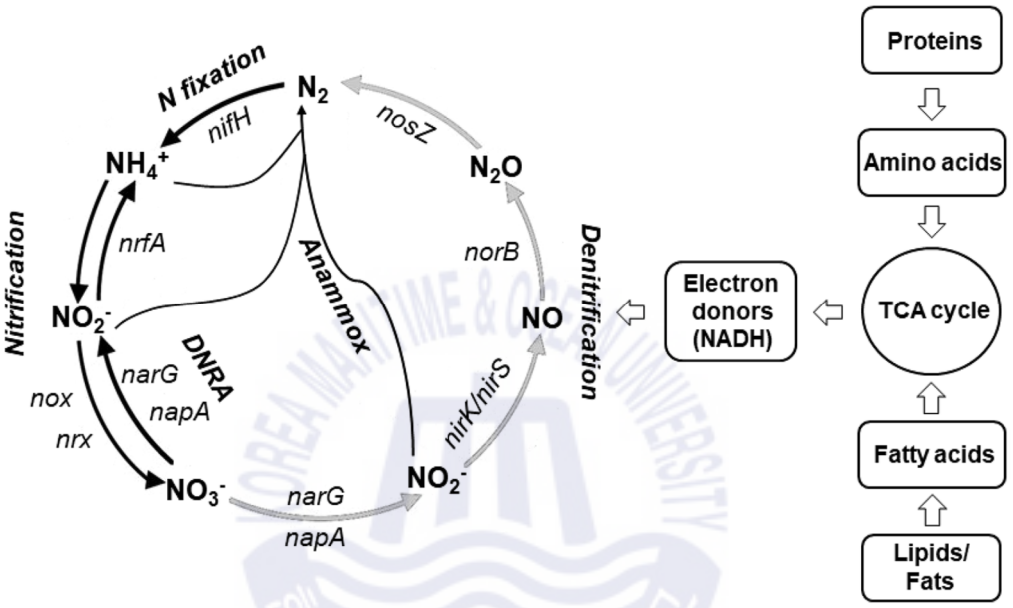


Figure 2-4 The hypothetical relationship between denitrification and degradation pathways for amino acids and fatty acids (lipids); the nitrogen cycle was constructed based on the previous report (Cardenas et al. 2018)

2.2 Experimental section

2.2.1 Library preparation, sequencing, and gene assembly

The extracted DNAs were fragmented using the NEBNext dsDNA Fragmentase kit (New England Biolabs, Beverley, MA, USA). A fragment size of 300–400 bp was excised from 2% agarose gel and extracted using a QIAquick Gel Extraction Kit (Qiagen Korea Ltd., Seoul, South Korea), and then processed using the TruSeq DNA LT Sample Prep Kit (Illumina, San Diego, CA, USA). Paired-end sequencing (2-150 bp) was performed on an Illumina MiSeq system (ChunLab, Inc., Seoul, South Korea) following the manufacturer's protocol.

Raw sequences from the MiSeq machine were uploaded to the rapid metagenomic annotations using subsystems technology (MGRAST) server (http://metagenomics.anl.gov) and filtered by the QC pipeline of MG-RAST. General functional gene annotation was assigned using the SEED database for complete microbial genomes, Cluster of Orthologous Groups of proteins (COG), and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases (at a cut off e-value <10-5). Artificial replicates representing 2.4%, 1.4%, 4.9%, 4.8% and 5.9% of the total reads of each sample from influent (I), buffering (B), primary aeration (PA), secondary aeration (SA) and sludge digestion (SD) tanks, respectively, were removed. The P-value threshold of each gene cluster comparison was under 0.01. For the taxonomic assignment of functional genes, we constructed a reference genome database containing COG information. After the genome data was obtained from the NCBI, we annotated their functional genes using the COG database with RPS-BLAST and constructed a reference genome database containing COG information.

2.2.2 Gene prediction from the tannery wastewater treatment stages

The denitrification enzymes were represented as genes whose sequence reads were filtered by the QC pipeline of MG-RAST and transformed to FASTA format by FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). To annotate the query genes against nitrogen metabolism, reference genes were collected from the FunGene database. A BLAST database was built with all protein seed sequences of the denitrification genes (*napA, narG, nrfA, nirS, nirK, norB, and nosZ*), and whole queries were annotated by BLASTx from BLASTC (2.2.26v) with an e-value lower than 1x e-5 and over 70% identity. After BLASTx analysis, the annotated genes were grouped according to four nitrogen metabolism enzymes as follows: nitrate reductase (*napA* and *narG*), nitrite reductase (*nirK, nirS*, and *nrfA*), and nitric oxide reductase (*norB*) and nitrous oxide reductase (*nosZ*). Nitrogen metabolism pathways were assigned via KEGG analysis, and each enzyme of the pathway was identified based on NCBI and BRENDA.

2.2.3 Microbial community analysis in the different treatment stages

The microbial community analysis began by selecting the organisms based on the criteria of an abundance value of more than one. The analysis was then performed using the following procedures. First, operational taxonomical units (OTUs) were analyzed for each sample with a 97% sequence similarity cut off limit (Regina et al., 2011). Second, the OTU data were analyzed and converted through MetaPhlAn (Segata et al., 2012). The phylogenetic relationship was elucidated using GraPhlAn (Nicola et al., 2013). Finally, taxonomical units were aligned according to their hierarchy.

2.2.4 Occurrence and abundance profiles of amino acid degradation enzymes in the treatment stages

The enzymes involved in amino acid and fatty acid degradation were selected based on the abundance value and named according to their functions. The correlation analysis was performed to find the exact connection between denitrification genes and degradation enzymes functioning in the different treatment stages. The relationships between specific targets were predicted and visualized with heatmap and correlation maps prepared using R studio software (R Development Core Team 2011). The correlations between denitrification and amino acid or fatty acid degradation were analyzed separately.

2.2.5 Correlation between denitrification genes and amino or fatty acid degradation enzymes

The bioinformatic frameworks for the correlation between denitrification genes and the amino or fatty acid degradation enzymes were illustrated based on different online databases and software. These analyses were based on the gene abundance, enzyme pathway abundance, and their origin. We demonstrated the connection between denitrification genes and the degradation enzymes in the two different categories. First, all quality-filtered metagenomic data were used in the selection of high potential enzymes found in the various treatment stages. The amino acid and fatty acid degradation enzymes were directly identified based on reads, which provided very reliable annotation results in all five treatment stages using heatmap. The BLAST hit outputs were also considered for the selection of enzymes. Second, the selected enzymes were correlated with each other and used to identify relationships with denitrification genes through heatmap and the correlation plot using R studio software (R Core Team 2018).

2.2.6 The overall correlation of the selected enzymes in the treatment stages

The predicted relationships between the denitrification genes and the amino acid or fatty acid degradation enzymes were compared under each of the five different treatment stages and visualized using Circos, as described in previous reports (Krzywinski et al., 2009, Jianhua et al., 2017, Itzair et al., 2018 and Wang et al., 2017). The different levels of clusters were analyzed, and the enzymes and the treatment stages were compared with logarithmic calculations using the abundance values. The selected amino acid or fatty acid degradation enzymes were also validated with online databases by finding the sequences in UniProt, and all sequence reads were aligned to the reference sequences of the following enzymes: α-ketoglutarate dehydrogenase (P0AFG5), tryptophan synthase (P0A879), glutamate dehydrogenase (P39633), aminotransferase (P76473), aspartate transaminase (C3SN02), pyridoxal phosphate (P37528), lipase (P13266), acyl CoA dehydrogenase (P33224), enoyl CoA hydratase (P77399), 3-hydroxy acyl CoA dehydrogenase (P21177), and beta-ketothiolase (Q0KBP1).

2.3 Result and Discussion

2.3.1 Microbial community analysis in the different treatment stages through phylogenetic analysis

The compositions of the microbial communities in the five different treatment stages were analyzed. The microbial communities from B, SA, and SD were diverse and occupied three-fifths of the cladogram (Figure 2-5). The dominant phylum, order and species are highlighted, and the phylogenetic analysis of the bioaugmented BMS-1 consortium was also performed to the compare the microbial communities of each treatment stage. Flavobacteriales, Bacteroidetes and Bdellovibrionales were dominant in the two treatment stages PA and SA. At the family level, Anaerolinaceae was dominant in the band Carnobacteriaceae in the

BMS-1 consortium. The thirty commonly occurring species from the different treatment stages were identified using abundance values. All the treatment stages except the influent and BMS-1 consortium showed the presence of Brachymonas denitrificans which commonly occurred in B (band A), PA (band G) and SD (band D).Our previous study showed that B. denitrificans was dominant in the B (37.5% of total reads), SD (19.3%) and PA (16.9%) orders based on the pyrosequencing analysis. Nitrate (NO3−) was efficiently removed by B. denitrificans as a denitrifier in a pilot plant that was augmented with this species (leta et al, 2005). The dominant species of the BMS-1 consortium, *Lactobacillus vini, Lactobacillus ghanesis, Acetobacter lovaniensis* and *Acetobacter oeni* were rarely observed in all treatment stages in this metagenomics analysis, as shown in the previous study (Kim et al, 2014). This indicates that the bioaugmented consortium may facilitate the wastewater treatment process in cooperation with the indigenous microbial communities.

2.3.2 Occurrence correlations between denitrification genes and amino acid degrading enzymes

The corrplot showed the correlations between the denitrification genes and the amino acid degrading enzymes, in which the dark blue circle indicated the highest correlation and the dark maroon circle the lowest correlation (Figure 2-5). The correlations between the same enzymes showed the highest values and points in the matrix point. The correlations between denitrification genes were high in the *narG* - *napA* pair and *nosZ* paired with *napA* and *narG*. The genes *narG* and *napA*, involved in nitrate reduction, were also found in the B, PA, SA, and SD treatment stages in a similar frequency pattern. The presence of *nosZ* (nitrous oxide reductase gene) appeared to be linked with the presence of the *napA* and *narG* genes (Sul et al., 2018). Alpha ketoglutarate dehydrogenase (α- KGDH) and tryptophan synthase were highly correlated with denitrification genes such as *napA, narG, nosZ* and *norB* while glutamate dehydrogenase (GDH) was highly

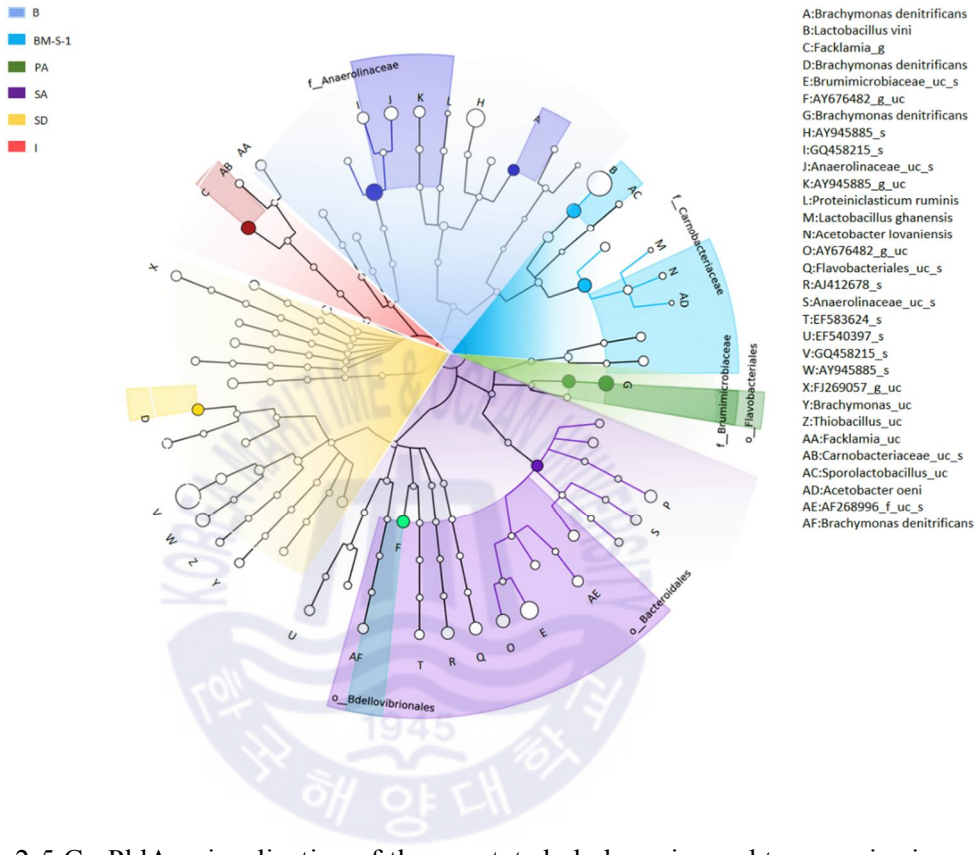


Figure 2-5 GraPhlAn visualization of the annotated phylogenies and taxonomies in each of the different treatments. A taxonomic cladogram reports all clades present in one or both cohorts with the abundance value. Circle size is proportional to the log of average abundance, and each color represents the microbial consortium (BM-S-1) and the different treatment stages.

correlated with denitrification genes such as *napA*, *narG*, and norZ (Figure 2-5). α-KGDH is a TCA cycle enzyme that catalyzes a non-equilibrium reaction by converting α-ketoglutarate, coenzyme A and NAD+ to succinyl-CoA, NADH and CO2 (Tretter and Vera 2005 and Mailloux et al, 2009). GDH is an enzyme that converts glutamate to α-ketoglutarate, NH3+, NADH and H+, and vice versa (Mailloux et al, 2009) Tryptophan synthase is an enzyme that catalyzes the final two steps in the biosynthesis of tryptophan (Dunn et al., 2008). The reversible formation of indoleand glyceraldehyde-3-phosphate (G3P) from indole-3-glycerol phosphate (IGP) is catalyzed by the α subunits of tryptophan synthase. The β subunits of tryptophan synthase catalyze the irreversible condensation of indole and serine to synthesize tryptophan in the presence of a pyridoxal phosphate (PLP). However, in our study, the reason for the high correlation of tryptophan synthase with the denitrification genes (*napA, narG, nosZ* and *norB*) was not clearly understood (Figure 2-6). A three- step pathway of aerobic L-tryptophan degradation to anthranilate (anthranilate pathway) was reported in several bacteria, such as Bacillus cereus, Pseudomonas aeruginosa and Ralstonia metallidurans (Kurnasov et al., 20013).

The FADH2-utilizing monooxygenase of a hydroxylase system in *Geobacillus thermodenitrificans* can convert anthranilate to 3-hydroxyanthranilate (3-HAA), which is further degraded to acetyl-CoA, a key intermediate of the TCA cycle (Liu et al., 2010) that generates NADH. It appears, therefore, that the potential tryptophan synthesis by tryptophan synthase observed in our study could eventually contribute to denitrification requiring NADH. The nitrate reductases encoded by *napA* and *narG*, the nitric oxide reductase encoded by *norB*, and the nitrous oxide reductase encoded by *nosZ* reductase require electrons from NADH for their functions (Zumft and Walter., 1997). To determine the occurrence patterns of various amino acid degradation enzymes in the different stages of treatment, 28 dominant enzymes were selected, and their profiles were demonstrated using a heatmap (Figure 2-7A). The dominant enzymes were classified into six groups (amino transferase, α-KGDH, pyridoxal phosphate-dependent enzyme, aspartate

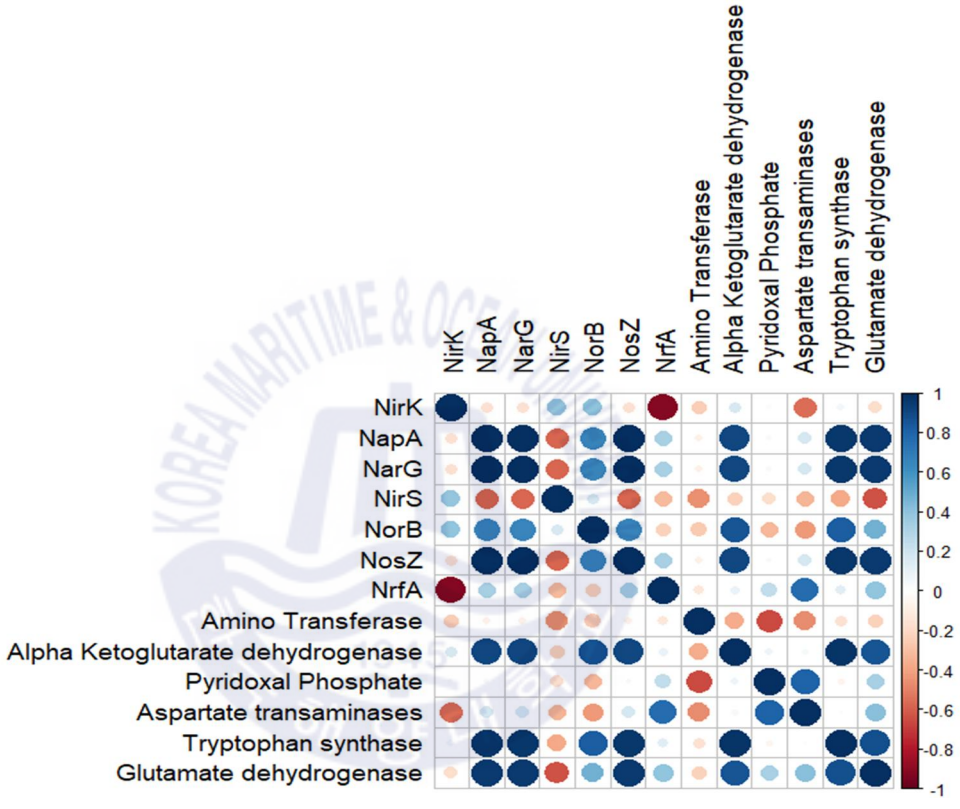
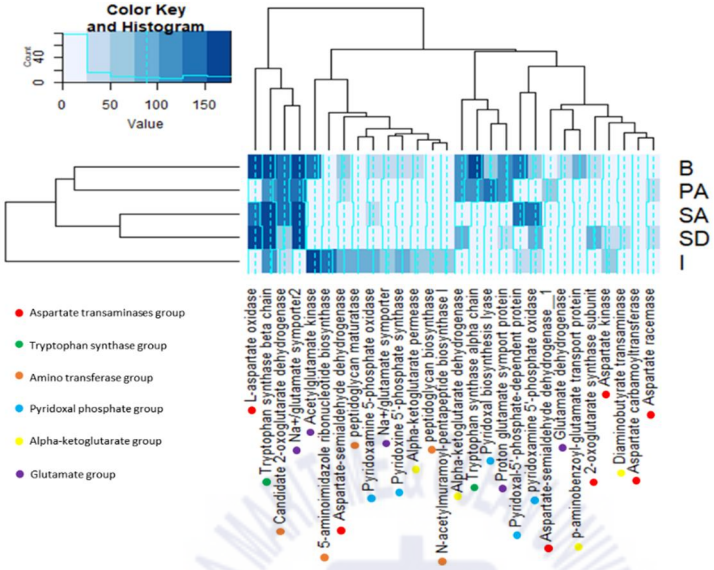


Figure 2-6 Matrix of Pearson's correlations for the occurrence and abundance of denitrification genes and representative amino acid degrading enzyme groups obtained with the corrplot package in the R environment. Only correlations with $p < 0.05$ are displayed.

transaminase, tryptophan synthase and GDH) (Figure 2-7B). Overall, there were two groups of the enzyme occurrence patterns mostly due to the bioaugmentation of BM-S-1 into the influent wastewater (I): the pattern of I and the pattern of the remaining stages (B, PA, SA, and SD). Increased enzyme diversity was shown in stage B, where L-aspartate oxidase, tryptophan synthase beta chain, candidate 2-oxoglutarate dehydrogenase, Na+/glutamate symporter, acetylglutamate kinase, tryptophan synthase alpha chain and pyridoxal-5'-phosphate-dependent protein were dominant. L-aspartate oxidase, tryptophan synthase beta chain, candidate 2-oxoglutarate dehydrogenase and Na+/glutamate symporter were also frequently observed in the PA, SA and SD stages. The enzyme profiles of stage I, however, were quite different from those of all the other stages, except for the profile of acetylglutamate kinase, which was also observed in B.

The occurrence patterns of denitrification genes and amino acid degrading enzymes are shown together grouped by the different treatment stages (Figure 2-7B). The occurrence patterns of these genes and enzymes were distinct among the treatment stages. The relative level of denitrification genes and amino acid degrading enzymes in each treatment stage was as follows: B (highest), PA, SA and SD (lowest). The denitrification genes (*nosZ, narG, napA, norB* and *nrfA*) and amino acid degradation enzymes (tryptophan synthase, α- KGDH and pyridoxal phosphate dependent enzyme) were relatively dominant in B. This indicates that the degradation of the substrates (proteins) and the denitrification of nitrogen may actively occur in B. In fact, the removal efficiencies for COD and total nitrogen were 88% and 81%, respectively, indicating that most of the substrates were removed in stage B (Kim et al, 2014). However, all of these metabolic genes and enzymes were observed at the lowest level in I, but the bioaugmentation of the BM-S-1 consortium was able to successfully facilitate the treatment of tannery wastewater due to the higher presence of these functional genes and enzymes. Amino acid degradation enzymes such as α-KGDH, tryptophan synthase and GDH

had an equally high abundance of all genes responsible for the denitrification process.



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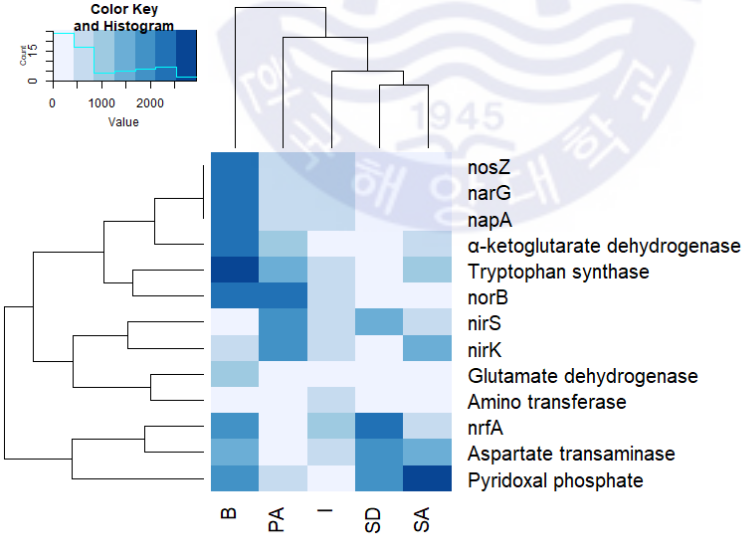


B

Figure. 2-7 Occurrence and abundance of denitrification genes and representative amino acid degrading enzymes (A) and enzyme groups (B) in the different stages of the tannery wastewater treatment.

2.3.3 Occurrence correlations between denitrification genes and fatty acid degrading enzymes

The overall occurrence correlations between denitrification genes and fatty acid degrading enzymes are shown as a corrplot (Figure 2-8). High abundances of fatty acid degradation enzyme groups (enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase and beta-ketothiolase) were observed together with denitrification genes such as *napA, narG* and *nosZ*. High levels of each of these fatty acid degradation enzymes were also correlated with each other. Enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase and beta-ketothiolase are enzymes that are involved in the production of the metabolites L-hydroxyacyl CoA, ketoacyl CoA and acyl (-2C) CoA, respectively, during the metabolism of fatty acids in Escherichia coli (DiRusso et al., 1999).

To determine the patterns of various fatty acid degradation enzymes in the different stages of treatment, 14 representative dominant enzymes were selected, and their profiles were visualized as a heatmap (Figure 2-9A). The dominant enzymes were classified into five groups (lipase, acyl CoA dehydrogenase, enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase and beta-ketothiolase) (Figure 2-9B). Lipase catalyzes the hydrolysis of fats (lipids) to produce monoglycerides and two fatty acids (DiRusso et al., 1999). Acyl-CoA dehydrogenase catalyzes the initial step of each cycle of fatty acid β-oxidation, introducing a trans double-bond between C2 and C3 of the acyl-CoA thioester substrate (Thrope and Kim., 1995). Overall, there were two groups of enzyme occurrence patterns due to the bioaugmentation of BM-S-1 into the influent wastewater (I):the pattern in B and the pattern in the remaining stages (SD, SA, PA and I).The occurrence of the fatty acid degradation enzymes was much lower than that of the denitrification genes and amino acid degradation enzymes. Like the amino acid degradation enzymes, increased enzyme diversity

was observed in stage B, where phospholipase/carboxylesterase, enoyl-CoA hydratase/isomerase, acyl-CoA dehydrogenase, phenylacetate degradation and 3-hydroxyacyl-CoA dehydrogenase 2 were dominant. There was a high level of lysophospholipase and phospholipase D in PA. There was a lower level of these enzymes in I than in the other treatment stages. Phospholipase C catalyzes the cleavage of the linkage between the phosphate and glycerol of phosphatidyl choline The occurrences of denitrification genes and fatty acid degrading enzymes are shown together grouped by the different treatment stages (Figure 2-9B). The occurrence patterns of these genes and enzymes were distinct among the treatment stages. The relative levels of denitrification genes and fatty acid degrading enzymes were as follows: B (highest), PA SD, SA and I (lowest). The denitrification genes (*nosZ*, *narG*, *napA*, *norB* and *nrfA*) were relatively dominant in B, (Flores-Diaz et al., 2016). Carboxylesterases are a group of esterases that catalyze the hydrolysis of carboxyl ester molecules in the presence of water to form alcohols and carboxylic acids (Sood and Tek., 2016).

while the five fatty acid degradation enzyme groups were observed at lower levels in all treatment stages than in B. However, the degradation of the substrates (fatty acids) and the denitrification of nitrate may occur actively in treatment stage B. In fact, the removal efficiencies of COD and total nitrogen were 88% and 81%, respectively, indicating that most of the substrates, including fatty acids, were removed in stage B. However, all of these metabolic genes and enzymes were observed at the lowest density in I, but the bioaugmentation of the BM-S-1 consortium could induce the efficient treatment of tannery wastewater due to the higher presence of these functional denitrification genes and fatty acid degradative enzymes. comprehensively visualized by using Circos (Figure 2-9 and Figure 2-10). For convenient identification and comparison, Circos plots of the treatment stages and denitrification genes are shown in the bar formats, while the amino acid or fatty acid degradation enzymes are shown in a scatter format. The scatter Circos plots

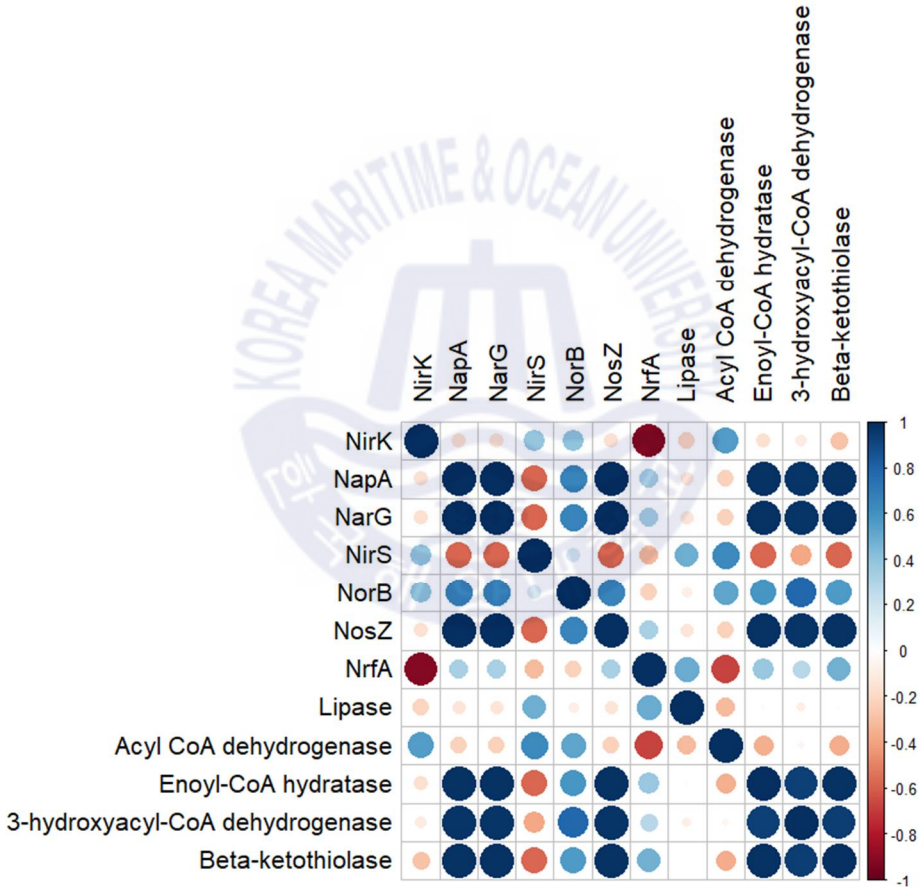


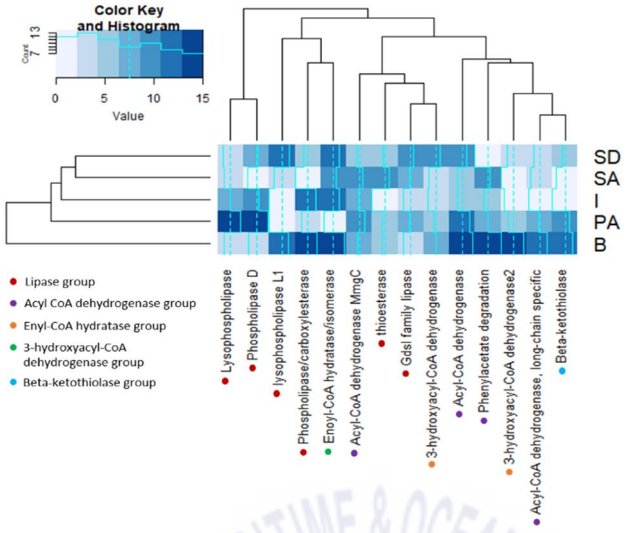
Figure 2-8 Occurrence and abundance correlations between denitrification genes and representative fatty acid degrading enzyme groups

representing amino acid and fatty acid degradation enzymes are shown in two perspectives.

First, an increase or decrease in the y-axis shows a common abundance value with the starting point at zero. Second, the scattered points represent the correlation points in the different treatment stages of tannery wastewater. In general, the overall relative abundances of the enzymatic classes were higher in the buffering and secondary aeration tanks, followed by the primary aeration tank and influent. The denitrification genes were variably distributed according to the percent abundance in each treatment stage (in the sequential order of *nirK, napA, narG, nirS, norB, nosZ* and *nrfA*). *nirK* and *nirS* were the dominant genes observed in stage B, while *nirK, napA* and *narG* were generally the dominant genes in all the remaining treatment stages. *napA* was observed at a relatively higher level in influent than in the rest of the treatment stages.

2.3.4 Overall correlation and relationships of nitrogen and carbon metabolism in different treatment stages

The correlations between the treatment stages and occurrences of the denitrification genes and degradation enzymes for amino acids or fatty acids were The connections among amino acid degradation enzymes or fatty acid degradation enzymes in the five different stages of tannery wastewater are illustrated by a ribbon form (Figure 2-11 and Figure 2-12). This figure shows the multiple correlations among the degradation enzymes of the different treatment stages, indicating the possible connections and occurrences of common enzymatic reactions among the treatment stages. These connections reflect the possibility that enzymatic reactions can occur in the same species that could be commonly present in the different treatment stages. Furthermore, the Circos plot provides different possibilities that amino acid or fatty acid degradation enzymes are present specifically during the denitrification process.



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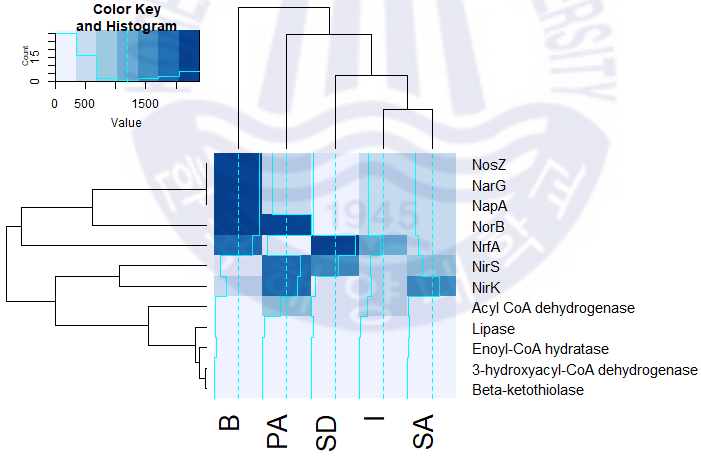

B

Figure 2-9 Occurrence and abundance of denitrification genes and representative fatty acid degrading enzymes (A) and enzyme groups (B) in the different stages of tannery wastewater treatment.

The amino acid degradation enzymes are represented by six numbers (Figure 2-8). In the treatment stages B and SA, tryptophan synthase and glutamate dehydrogenase had higher abundance values than the other amino acid degradation enzymes. It was determined that α-KGDH, tryptophan synthase and GDH were highly correlated with the *napA, narG* and *nosZ* genes regardless oftreatment stage, as shown in Figure 2-10. The fatty acid degradation enzymes were also represented by five numbers (Figure 2-10). The degradation enzymes did not have a high abundance compared to the amino acid degradation enzymes. Nevertheless, enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase and beta-ketothiolase were highly correlated with buffering and sludge digestion tanks. These three enzymes were also highly correlated with *napA, narG* and *nosZ*, while lipase did not have a high correlation with most of the denitrification genes (Figure 2-9). This indicates that *napA*, *narG* and *nosZ* had more specific connections with these amino acid and fatty acid degradation enzymes. Therefore, this clearly indicates that the denitrification pathways encoded by the three selected genes could be linked with the degradation of the amino acids and fatty acids whose degradation products go through the TCA cycle to generate NADH that is used as electron donors for denitrification.

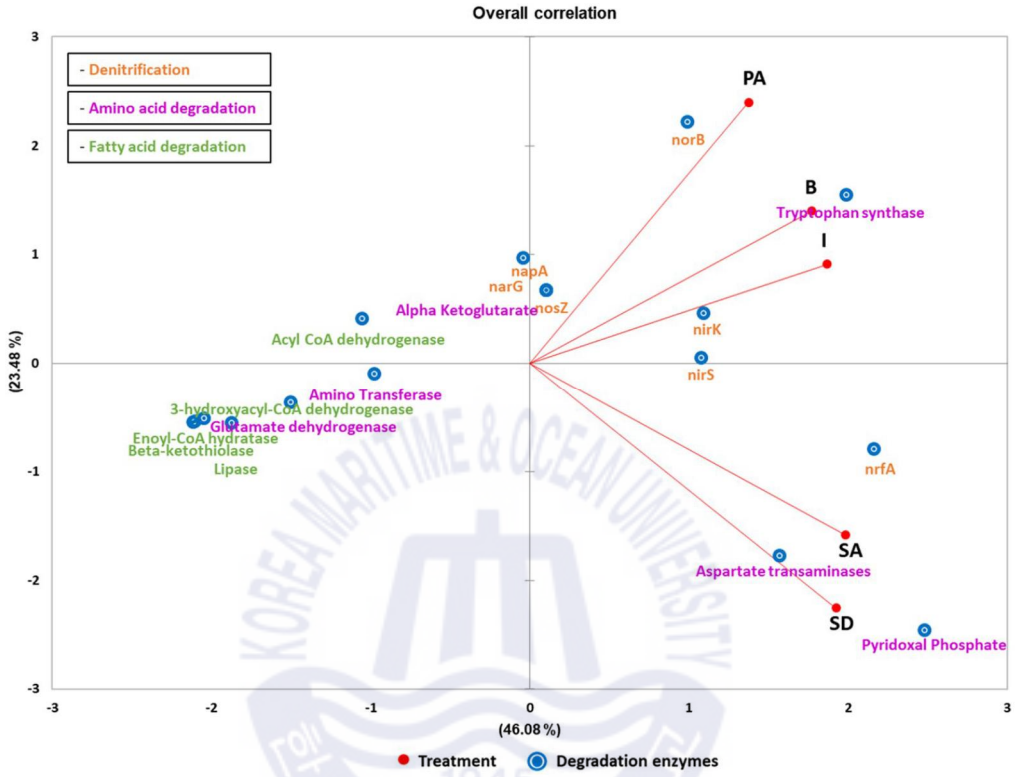

Figure 2-10 Principal component analysis (PCA) of denitrification genes and the degradation enzymes according to different quantitative variables. Treatment stages indicated in the red dot, denitrification genes and degradation enzymes indicated in blue dot. Each genes and enzymes were differentiated according to their group by different colored texts

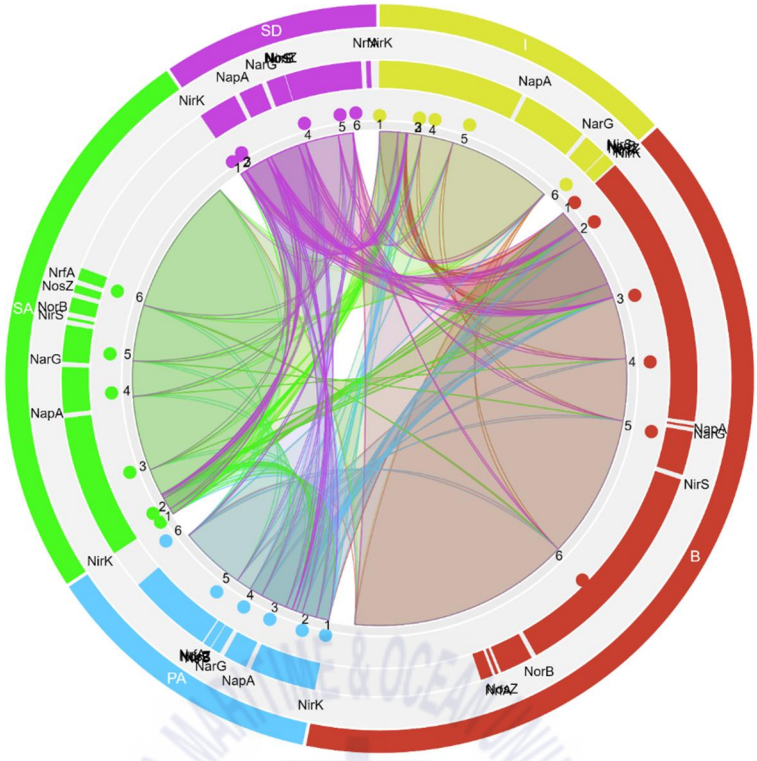

Figure 2-11 Distributions of denitrification genes and amino acid degradation enzymes of each of the different treatment stages of tannery wastewater. The data are visualized using a Circos plot. The outer circle represents the different treatment stages, the middle layer represents the denitrification gene occurrences, and the inner circle represents the occurrences of amino acid degradation enzymes. The length of the bars represents the relative abundances of total genes and denitrification genes. The interconnecting ribbons within the innermost circle indicate a concomitant presence of the amino acid degradation enzymes in the different treatment stages. 1, amino transferase; 2, alpha ketoglutarate dehydrogenase; 3, pyridoxal phosphate-dependent group; 4, aspartate transaminases; 5, tryptophan synthase; 6, glutamate dehydrogenase

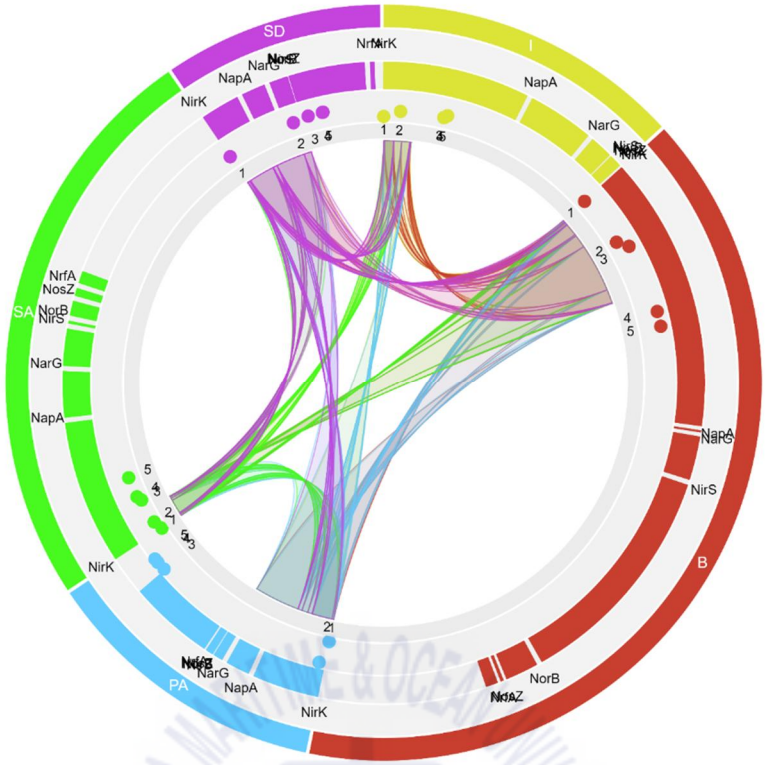

Figure 2-12 Distributions of denitrification genes and fatty acid degradation enzymes in each of the different treatment stages of tannery wastewater. The data are visualized using a Circos plot. The outer circle represents the different treatment stages, the middle layer represents the denitrification gene occurrences, and the inner circle represents the occurrences of fatty acid degradation enzymes. The length of the bars represents the relative abundances of total genes and denitrification genes. The interconnecting ribbons within the innermost circle indicate a concomitant presence of the fatty acid degradation enzymes in the different treatment stages. 1, lipase; 2, acyl CoA dehydrogenase; 3, enoyl-CoA hydratase; 4, 3-hydroxyacyl-CoA dehydrogenase; 5, beta-ketothiolase

2.4 Conclusion

Tannery wastewater may contain proteins and fats as major organic pollutants that degraded to amino acids and fatty acids. Our previous metagenomics study has shown that nitrogen metabolisms such as denitrification, DNRA and ammonium assimilation contributed to the effective simultaneous removal of nitrogen source and COD (carbon source), and hence sludge reduction during the tannery wastewater treatment. The denitrification process appeared too linked to removal of COD. It hypothesized in a study that denitrification process closely related to the carbon pathways such as degradation of amino acids, fatty acids and carbohydrates which could generate electron donors for the nitrate reduction, and that an efficient removal of nitrogen linked to COD removal (and hence sludge reduction). The metagenomics analysis of the microbial community showed that Brachymonas denitrificans, a known denitrifier, highly occurred in B, PA, and SD. The occurrences of the amino acid degrading enzymes, alpha ketoglutarate dehydrogenase (α- KGDH) and tryptophan synthase, highly correlated with those of the denitrification genes such as *napA*, *narG*, *nosZ* and *norB*. The occurrence of glutamate dehydrogenase (GDH) highly paralleled with that of the other denitrification genes such as *napA*, *narG*, and norZ. The denitrification genes (*nosZ*, *narG*, *napA*, *norB* and *nrfA*) and amino acid degradation enzymes (tryptophan synthase, α- KGDH and pyridoxal phosphate dependent enzyme) dominantly observed in B. This indicates that degradation of the substrates (proteins) and denitrification of ammonium may occur actively in the treatment stage. The high abundance of fatty acid degradation enzyme groups (enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase and beta-ketotiolase) observed together with the denitrification genes like *napA*, *narG* and *nosZ*. Overall, the 14 kinds of fatty acid degradation enzymes grouped in two clusters like the stage B and the rest stages (SD, SA, PA and I). Like amino acid degradation enzymes, more diverse enzymes were dominantly observed in the stage B such as phospholipase/carboxylesterase, enoyl-CoA hydratase/isomerase, acyl-CoA dehydrogenase, phenylacetate degradation, and 3-hydroxyacyl-CoA dehydrogenase 2.

This understanding of nitrogen and carbon metabolic pathways in the tannery wastewater treatment system will undoubtedly contribute to an optimized and efficient operation of the treatment system and any other wastewater treatment systems.

2.5 Acknowledgment

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CHAPTER III – METAGENOMIC ANALYSIS OF ECO-FRIENDLY TREATMENT PROCESS OF DYE WASTEWATER AT A FULL-SCALE SYSTEM

3.1 Introduction

3.1.1 Bioaugmentation in dye wastewater

Textile industry is considered as one of the largest water consumers in the world. It is rapidly expanding, and dyes are also continuously being upgraded and replaced by superior compounds that have enhanced fastness, stability, brightness and resistant to natural degradation such as due to sunlight and microbes. Therefore, the need to develop new technologies which can cope with the treatment of TW has been well recognized (Wijetunga et al., 2010).

Color is the first contaminant to be recognized in wastewater and has to be removed before discharging into waterbodies or on land. The presence of very small amounts of dyes in water (less than 1 ppm for some dyes) is highly visible and affects the aesthetic merit, water transparency and gas solubility in lakes, rivers and other waterbodies (Ibrahim et al., 1996).

It is noteworthy that some dyes are highly toxic and mutagenic, and decrease light penetration and photosynthetic activity, causing oxygen deficiency and limiting downstream beneficial uses such as recreation, drinking water and irrigation (Przystas et al., 2012).

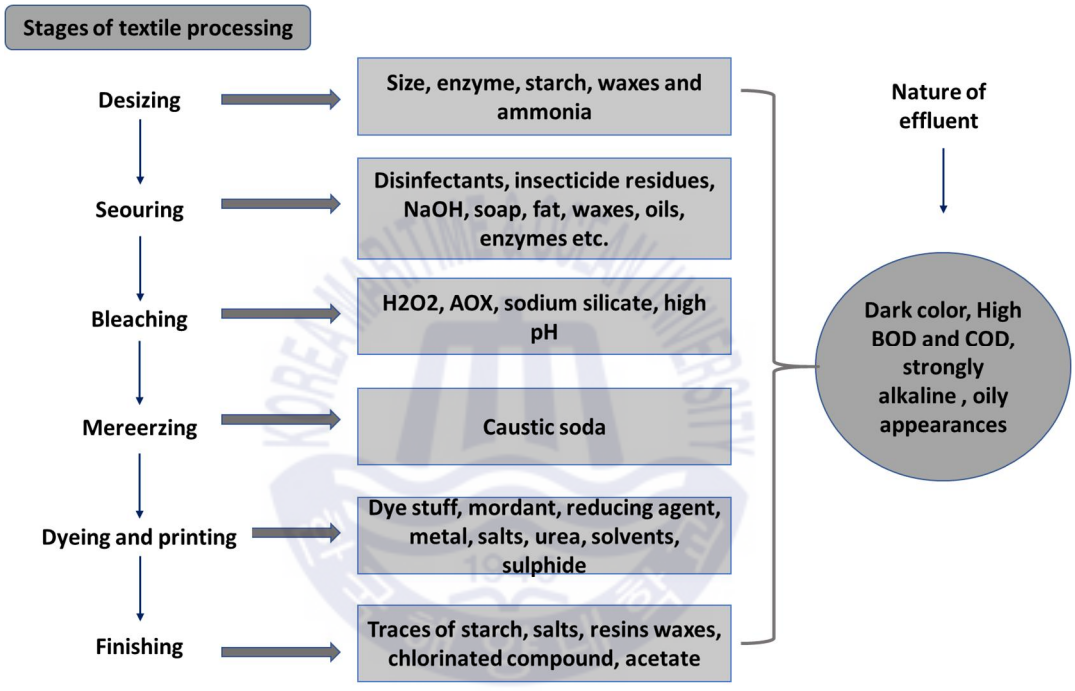


Figure 3-1 Typical process for dye wastewater generation and its kinds of pollutants

Based on the chemical structure of the chromophoric group, synthetic dyes are classified as azo, nitro, anthroquinone, triarylmethane, xanthene, heterocyclic or indigo dyes. A high proportion of industrially used dyes are azo-derived and many of these are produced from the arylamines such as benzidine (Bzd, e.g., Direct Black 38), 3,3′-dimethylbenzidine (DMBzd, e.g., Direct Red 2 (DR2)) and 3,3′-dimethoxybenzidine (DMOBzd, e.g., Direct Blue 15). benzidine and other aromatic compounds, all of which might be reformed because of microbial metabolism (Clarke & Anliker., 1980). It has been shown that azo- and nitro-compounds are reduced in sediments (Weber & Wolfe., 1987) and in the intestinal environment (Chung et al., 1978), resulting in the regeneration of the parent toxic amines (Ibrahim and Poonam., 1996).

Imperfection of color removal with standard activated sludge relates to changes in concentration and dyes composition, their toxicity as well as sludge adaptation to such unstable conditions. Therefore, there is still a growing need in finding an effective and low-cost method for the removal of dyes from wastewater (Przystas et al., 2012).

Controlling organic contaminants and ammonia are key objectives of many wastewater treatment plants. Ensuring the healthy growth of beneficial microorganisms is critical to the efficient operation of secondary treatment systems, which are designed to remove organic contaminants and sometimes nitrogen.

3.1.2 Metagenomic insight in dye wastewater treatment

In contrast, in-depth study of the microbial composition and metabolic potentials of treatment with bioaugmented CES-1 with culture-independent high-throughput sequencing technology will undoubtedly help to further reveal the mechanism underlying the operating process, and thereby achieve the goal of improving treatment efficiency and reducing treatment cost. Recently,

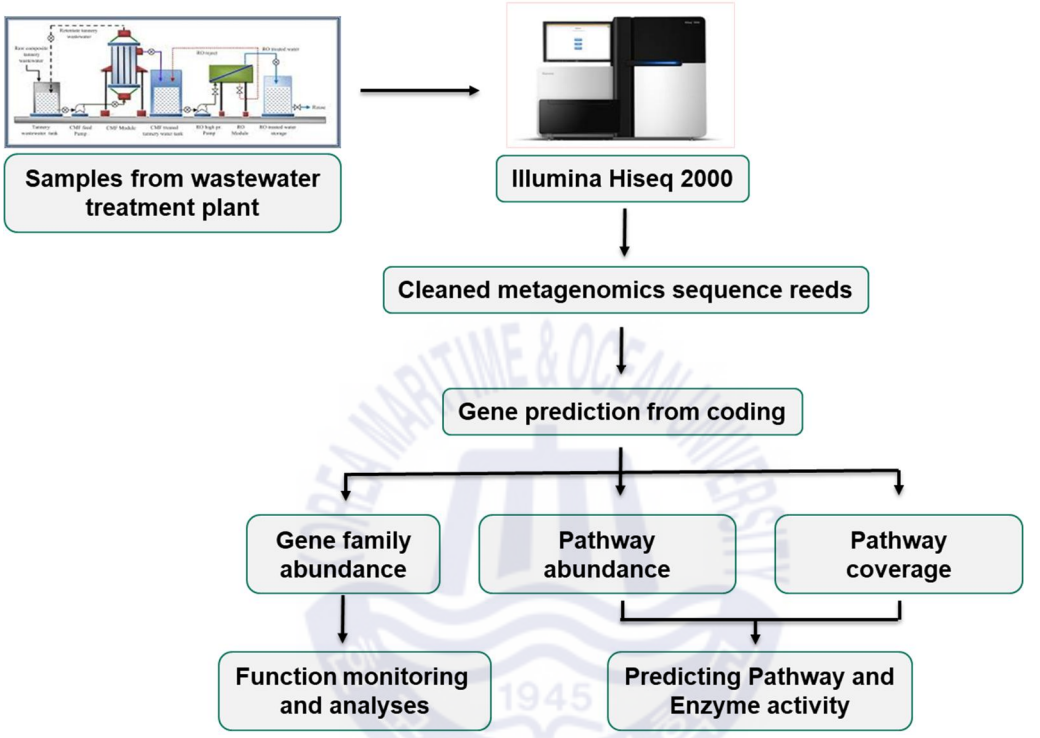


Figure 3-2 Procedures for metagenomic analysis of dye wastewater samples in terms of functional gene annotations and metabolic pathway predictions.

metagenomic approaches based on high-throughput sequencing have been widely used to explore the structures and functions of microbial communities (Feng et al., 2013) (Figure 3-2). This promising approach will provide a great opportunity and new insights to reveal composition of microbial communities, diversity of functional genes and enzymes responsible for the enhancement of the treatment. Guo, J. et al., used metagenomic approach to unravel the community diversity and functional profiles within activated sludge from a full-scale SNPR WWTP and found that various key enzymes involved in metabolisms degrading various chemicals could be annotated in different treatments. However, it was hard to distinguish the effects of each divergent variable on microbial community in a full-scale dye wastewater treatment plant since a lot of uncontrollable or even undetectable influential factors were involved in such a pollutant-removing process (Feng et al., 2014). Thus, it is necessary to evaluate the adverse effects of enzyme involvement and variation on the microbial community structure and the activities of activated sludge under controllable processing parameters.

In this study, the activities of dye wastewater treatment plant were evaluated before and after the addition of CES-1. Total genomic DNA samples were extracted from different treatments with different time variables. The effect of temperature variation on the microbial community structure and functional genes of each treatment was further explored by high-throughput sequencing and metagenomic analyzes. The intrinsic relationship between community structure, function of enzymes and its metabolism was proposed by metagenomics analysis. This study provides novel insights into the adverse effects of bioaugmented CES-1 on the microbial community structure and the enzymes responsible for the degradation of toxic chemicals in dye wastewater treatment plant.

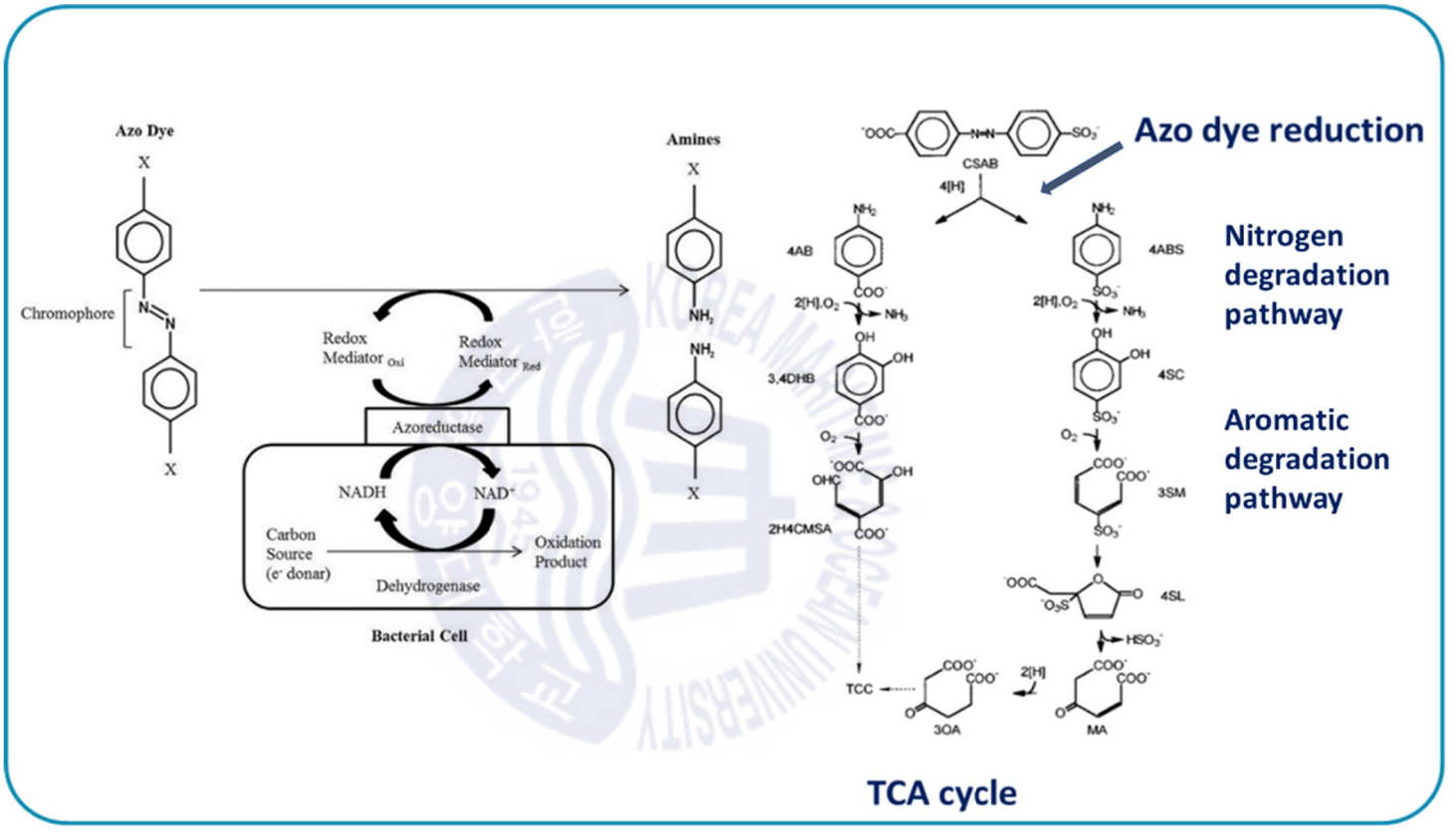


Figure 3-3 The typical degradation pathway of azo dyes and its key steps.

3.2 Experimental section

3.2.1 Description of wastewater treatment system

The treatment system was a two-stage activated sludge process with a sludge digestion tank and located at dye wastewater treatment plant in in Daegu City, South Korea. The process was essentially composed of the multiple steps including the influent tank (I), the buffering tank (B), primary aeration tank (PA), primary sedimentation tank (PS), secondary aeration tank (SA), secondary sedimentation tank (SS), tertiary sedimentation tank (TS) and sludge digestion tank (SD) (Figure 3-3). The main physical and chemical characteristics of the influent were shown in Table 3-1. The influent wastewater was alkaline (pH 11.7-12.4), warm (30-41^{o}C), and showed 4.8-10.2 mg/L of DO and 104-126 mV of ORP, COD_{Mn}, T-N, T-P and SS were 208-1,623 mg/L, 12-96 mg/L, 2.0-7.0 mg/L and 81-1,098 mg/L, respectively. The effect of bioaugmentation on the wastewater treatment was monitored in terms of COD, T-N, T-P, NH_3-N, NO_2^--N, NO_3^--N, PO_4^{3-}, SS and color intensity of the wastewater. COD, T-N, T-P, NH_3-N, NO_2^--N, NO_3^--N and PO_4^{3-} were measured using Humas' pre-treatment equipment (HS-R200, Humas, Inc. Daejon, South Korea), total water quality Analyzer (HS-3300, Humas, Inc.) and their measurement kits (HS-COD (Mn) -L, HS-TN-L, HS-TP-L, HS- NO_2^-(N) -L, HS- NO_3^-(N) -L and HS- PO_4^{3-} (P) -L) were used. Suspended solid (SS) and color intensity were measured according to the method presented in Standard Methods for the Examination of Water and Wastewater (APHA, 2005). Water temperature, pH and dissolved oxygen (DO) were measured using the meters YSI550A and YSI63 (YSI, Inc., OH, U.S.A.) and ORP was measured using ORP meter (Orion Star A212, Thermo Fisher Scientific Solutions LLC, Seoul, South Korea).

Parameter	Range
pH	11.7-12.4
Temperature (°C)	30-41
DO (mg/L)	4.8-10.2
ORP (mV)	104-126
COD_{Mn} (mg/L)	208-1,623
SS (mg/L)	81-1,098
T-N (mg/L)	12-96
NH_3-N (mg/L)	3.2-18.4
NO_2^--N (mg/L)	0.5-3.9
NO_3^--N (mg/L)	3.5-11.7
T-P (mg/L)	2.0-7.0
PO_4^{3-} (mg/L)	2.6-4.8
Color intensity (PCU*)	270-364

Table 3-1 Physical and chemical characteristics of the dye wastewater influent in this study

*Pt-Co Unit

3.2.2 System operation conditions

The dye wastewater treatment plant in Daegu City, South Korea treated 15,000 (m^3 per day) of wastewater at 25–35°C (Figure 3-4). 1,000(m^3/day) of sludge was generated from primary, secondary, and tertiary sedimentation tanks and transported to the sludge thickening tank. 400 (m^3/day) of the sludge was then transported into SD (900m^3) after filtration through a sieve screen and digested through bioaugmentation of the active culture of CES-1 (15 tons per day), and the

remaining sludge (600 m^3/day) was subjected to dehydration, generating dehydrated sludge (50-60 tons/D; 60% moisture). The digestion was performed at 35-42°C and under 0.5–3.5mg/L of DO for 54 hours, and then the digested sludge was recycled into B. The consortium culture CES-1 was enriched from the natural soils and was originally deposited by Bayo, Inc. (Jinju, Republic of Korea) as a proprietary culture (KCTC 12579BP) at the Biological Resource Center, Korea Research Institute of Bioscience and Biotechnology (Daejeon, Republic of Korea). Major genera of the culture based on the pyrosequencing analysis were *Clostridium* (58.0%), *Lactococcus* (13.5), *Gluconacetobacter* (11.0), *Acidomonas* (4.9), *Klebsiella* (2.9), *Enterobacter* (1.9), *Lactobacillus* (1.2), *Chitinophaga* (1.1), *Dyella* (1.0), *Acidocella* (0.5), *Citrobacter* (0.5), *Lacibacter* (0.4) and *Burkholderia* (0.4). The stock culture of CES-1 was cultivated in a batch reactor carrying a mixture of inoculum and medium (7 kg of CES-1 powder product dissolved in 1.0 m^3 of water) at 40°C for 3 days under an aeration (50L/min). The stock culture reached 10^8-10^9 (cells/mL), and was then diluted 30 times before being augmented into the sludge digestion tank (SD) at 0.1% (v/v) of the total influent treated daily (15,000 m^3 x 0.1%=15,000L). Hydraulic retention time for the whole wastewater treatment system was 46.2hours as shown in Figure 3-4. Traditionally the sludge generated from the primary, secondary, and tertiary sedimentation tanks was transported to the sludge thickening tank and then the thickened sludge was dehydrated to reach 60% of moisture content (50-60 tons produced per day).

3.2.3 DNA isolation and sequencing of metagenomes

DNA extraction procedures have been shown previously (Kim et al., 2014), and detailed processes for library preparation, sequencing and bioinformatic analysis of the metagenomes have been previously described (Sul et al., 2016).

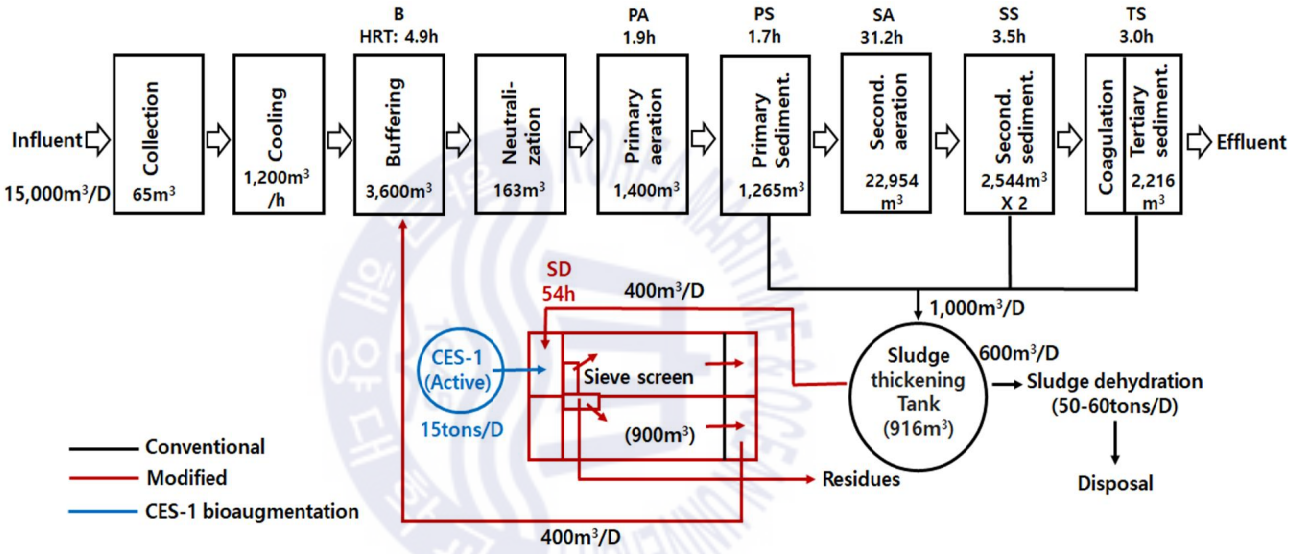


Figure 3-4 The full-scale treatment system of dye wastewater and the sampling sites. Sampling sites: I, influent; B, buffering tank; PA, primary aeration tank; SA, secondary aeration tank; SD, sludge digestion tank

Briefly, DNA extraction was performed on 1-2 milliliters of each Sample, which was accomplished using the PowerSoil DNA Isolation Kit (Mo Bio Laboratories, Inc.). The extracted DNA was fragmented using the NEBNext dsDNA. Fragmentase kit (New England Biolabs, Beverley, MA, USA). Fragments sizing from 300 to 400 bp were excised from 2% agarose gel, and extracted using QIAquick Gel Extraction Kit (Qiagen, Inc.), and then processed using TruSeq DNA LT Sample Prep Kit (Illumina, San Diego, CA, USA).

3.2.4 Contig assembly and binning

The raw reads were trimmed using Trimmomatic (version 0.36) with default option to filter low quality reads. *De novo* assembly was performed with Megahit (version 1.1.1) for each sample under meta-sensitive option (min-contig-length equal to or greater than 500bp). Metagenome-assembled genomes (MAGs) were obtained with Metabat2 (version 2.2.4) and quality of MAGs was evaluated by CheckM (version 1.0.8). MAGs were compared and dereplicated with dRep (version 2.3.2) with default parameter. Only MAGs with completeness equal to or greater than 50 % and contamination less than 10 were considered and classified using GTDB-TK.

3.2.5 Microbial community analysis with metagenome reads

Quality trimmed reads were used for taxonomic classification using Kraken2 with a standard database. This database consisted of the bacterial, archaeal, and viral genome sequences from RefSeq, along with the human genome and UniVec. Because the bacterial genome of species (genus) level is nearly identical, Kraken 2 results may mislead the results. Therefore, we re-estimated the relative abundance of each taxonomic level using Bracken for more accurate taxonomic composition analysis.

3.2.6 Metabolic pathway analysis with family level taxon bins

To compensate for poor assembly and binning results of individual samples, contigs of all samples were clustered with identity equal to or greater than 99% (cd-hit-est v.4.7) to obtain a representative contig catalogue. Each contigs were binned to family level from Kraken2 results and predicted coding sequences using Prodigal. Genes were annotated to KEGG Orthology (KO) with Ghost KOALA and were reconstructed to metabolic pathways using MinPath1.4 for each taxon bin. Families with a KO of less than 100 were excluded from the pathway construction. Reads from all samples were mapped to the contig catalogue using bwa-mem (default) to calculate the abundance of each gene. The abundance of genes was normalized to copies per million (CPM) unit (sum to 1 million, the same concept of transcript per million (TPM) unit in RNA-seq). Metabolic pathway abundances were calculated for the median of the constitutive genes of each pathway.

3.3 Results and Discussion

3.3.1 Wastewater treatment monitoring during bioaugmentation

The efficiency of the dye wastewater treatment 67 days before the bioaugmentation of CES-1 was shown in Table 3-2, which was used as a control for the treatments after the bioaugmentation. The removal efficiencies (%) for COD, T-N, T-P, SS and color intensity were 94.9, 48.1, 91.6 63.9 and 66.3, respectively. These parameters met the regulation standards for the pollution control. By the way, the removal efficiencies (%) for COD, T-N, T-P, SS and color intensity 50 days after the bioaugmentation were 97.8, 62.6, 95.8, 63.4 and 77.9, showing the increase of COD, T-N, T-P and color intensity removal efficiencies (%) by 2.9, 14.5 4.2 and 11.6, respectively (Table 3-2).

Parameter (mg/L)	Influent [I]	Buffering [B]	Primary Sedimentation [PS]	Secondary Sedimentation [SS]	Effluent [E]	Removal Rate (%)*
COD	664.0±6.1^{e}	584.0±8.5^{c}	370.5±2.5^{b}	20.6±0.4^{a}	34.0±4.7^{a}	94.9
T-N	-	36.2±1.3^{b}	35.3±0.4^{b}	18.8±0.8^{a}	-	48.1
NH_3-N	-	14.8±0.5^{b}	28.1±2.0^{c}	8.9±0.4^{a}	-	39.9
NO_2^--N	-	1.7±0.1^{c}	1.3±0.0^{b}	0.3±0.0^{a}	-	82.4
NO_3^--N	-	7.7±0.2^{c}	3.5±0.1^{b}	0.80±0.07^{a}	-	89.6
T-P	-	5.1±0.5^{b}	0.54±0.01^{a}	0.43±0.01^{a}	-	91.6
PO_4^{3-}	-	3.4±0.0^{c}	1.5±0.1^{b}	0.20±0.01^{a}	-	94.1
SS	-	122	124	44	-	63.9
Color intensity**	323.9±13.9				108.9±1.5	66.3

*All the parameters except COD and color intensity were measured using the data from buffering and secondary sedimentation tanks; **Influent (samples of Year 2018; n=5) and effluent (samples of Year 2016; n=5)

Table 3-2. Characteristics of the dye wastewater of the different treatment stages 67 days (March 2, 2017) before bioaugmentation (May 9, 2017) of the composite microbial consortium CES-1

This indicates that the bioaugmentation of CES-1 may be effective in facilitating the dye wastewater treatment. Moreover, the removal efficiencies (%) for COD, T-N, T-P, SS and color intensity 300days (531days) after the bioaugmentation were 95.5 (97.3), 80.9 (75.9), 96.2 (70), 44.1 (81.3) and 90 (94) (Tables 3-3). Overall, the removal efficiencies for COD, T-N, T-P, SS and color intensity after the augmentation were higher than the control data (before augmentation).

The bioaugmentation of CES-1 appeared to increase the total nitrogen removal efficiencies (62.6-80.9%) of the system compared with the control (before bioaugmentation) (48.1%). Removal of T-N appeared to be mostly active in the

secondary aeration tank (SA)(46.7%) before the bioaugmentation, and this kind of trends were maintained until 111days after the bioaugmentation (removal activity of T-N in SA: 55.7-86.9). However, higher T-N removal activities (24.6-55.5%) were observed in the secondary aeration tank (SA) afterwards (112-531days after the bioaugmentation). Moreover, the overall T-N removal efficiencies generally increased after the augmentation (75.9 – 80.9%) compared with before augmentation (48.1%). TN removal is an important indication in the simultaneous denitrification process because a significant amount of nitrogen reduction is the premise for the subsequent denitrification, so the removal rate of TN was investigated in each treatment with different time durations. Nitrite is highly accumulated in SD, which was mainly ascribed to a high level of *nrfA* and *nirK* copies. Additionally, nitrite-N and ammonia was also affected by *AmoA* that is conducive to the accumulation of nitrite. As mentioned above, the whole concept of interconnecting the metagenomics analyses with TN removal is to understand the metabolism of enzymes involved in nitrogen cycle (Dong et al., 2017). Therefore, based on these results, the following nitrogen removal pathway was promoted in the dye wastewater treatment by augmenting CES-1: ammonium was oxidized to nitrite, and then the produced nitrite was rapidly reduced to N2 due to the bioaugmentation in SD. As far as other nitrogen transformations like anammox and reduction of nitrate to nitrite are concerned, they must exist and function since there are anoxic zones within the system, but they should play a marginal role under the treatments (Yu and Zhang, 2012). In summary, the high abundance of *nirK* and *nrfA* might be the primary reason for the drastic removal of TN and T-P removal appeared to be mostly active in the primary aeration tank (PA)(89.4%) before the bioaugmentation, and this kind of trends were mostly maintained until 531days after the bioaugmentation (removal rate of T-P in SA: 51.4-91.6).

	Influent [I]	Buffering Tank [B]	1st Sedimentation [FS]	2nd Sedimentation [SS]	Effluent [E]	Sludge digestion [SD]	Removal Rate (%)
SS (mg/L)	860	-	-	-	187	-	**78.2**
COD (mg/L)	674±51.6^{d}	466±24.7^{c}	222.5±3.2^{b}	16.5±3^{a}	14.5±0.3^{a}	141.5±57^{b}	**97.8**
T-N (mg/L)	23±0^{c}	33.4±0.8^{d}	52.1±0.2^{e}	6.8±0.4^{a}	8.6±0.4^{b}	22.5±0.1^{c}	**62.6**
T-P (mg/L)	2.4±0.1^{b}	1.9±0^{b}	0.4±0^{a}	0.1±0^{a}	0.1±0^{c}	6.0±1.2^{a}	**95.8**
NO2 (mg/L)	1.43^{d}	1.01^{b}	0.68^{a}	1.38^{c}	1.4^{cd}	0.9^{b}	**2**
NO3 (mg/L)	7.1±0.5^{d}	5.4±0^{c}	0.7±0^{a}	2.6±0^{b}	2.8±0.4^{b}	14.6±0.2^{e}	**60.5**
NH3 (mg/L)	10.5±0.1^{c}	9.6±0.1^{b}	28.7±2^{d}	6.2±0.3^{a}	6.1±0.2^{a}	9.5±0.1^{b}	**41.9**
PO4 (mg/L)	3.54±0.2^{d}	4.3±0.05^{e}	1.14±0.1^{b}	0.07±0^{a}	0.16±0^{a}	3.41±0.05^{c}	**95.4**

*Pt-Co Unit

Table 3-3 Characteristics of the dye wastewater of the different treatment stages 50 days after bioaugmentation of the composite microbial consortium CES-1 (June 29, 2017)

In addition, a considerable amount of the sludge reduction in the wastewater treatment process was observed after bioaugmentation of CES-1 (Table 3-4). The average sludge reduction rate over 20 months after the bioaugmentation was 26% (reduction from 6.18 to 5.00 in sludge per ton of influent COD).

In the previous study about tannery wastewater treatment (Kim et al., 2014), the degradation of the organic substrates (proteins, lipid or fatty acids) and the denitrification of nitrogen appeared to occur concomitantly in buffering (B) and sludge digestion (SD). In here, the removal efficiencies for COD and total nitrogen were 96% and 76%, respectively, indicating that most of the substrates were removed in B and hence a significant sludge reduction occurred. In this case, *Brachymonas denitrificans*, a denitrifier, was dominant in B and SD. The partially digested sludge, recycled from SD, could be rapidly utilized by populations of *B. denitrificans* and other communities present in B.

Sampling period	Influent (m^3)	Influent COD (g/m^3)	Effluent COD (g/m^3)	Sludge (ton)	Total Influent COD (ton)	Sludge per ton of influent COD	Sludge reduction*** %
2017. 1- 4*	411,588	664	34	1,690	273	6.18	-
2017. 5- 2018. 12**	385,158	560	33	1,007	217	5.00	26

*Monthly average before bioaugmentation (n=4); **Monthly average after bioaugmentation (n=20); ***Sludge reduction per ton of influent COD

Table 3-4 Effect of CES-1 bioaugmentation on the sludge reduction in the full-scale dye wastewater treatment plant

Compliance with nitrogen effluent standards affects a wide variety of dyes. Nitrification is well known for its process instability due to the requirement for the close linking of the bacterial species responsible for different parts of the removal process (Philips et al., 2002). Bioaugmentation has been shown to offer the potential to stabilize nitrification and to deal with transient treatment problems. Abeysinghe et al. (2002) demonstrated the ability of bioaugmentation to improve ammonia removal during stress conditions. Similarly, Ma et al., (2009) demonstrated the improved capability of a bioaugmented treatment might increase the COD removal 5% higher than any other method. Compliance can also be problematic for priority pollutants which are persistent and toxic, as the biomass not only requires acclimation, but it can still be negatively impacted by a sudden shock load of the toxic compound. As with nitrogen, bioaugmentation has been demonstrated to have some success in the treatment of such compounds. Qu et al., (2011) observed improved long-term stability of treatment systems for treating aromatic compounds.

3.3.2 Microbial community analysis in the dye wastewater treatment process

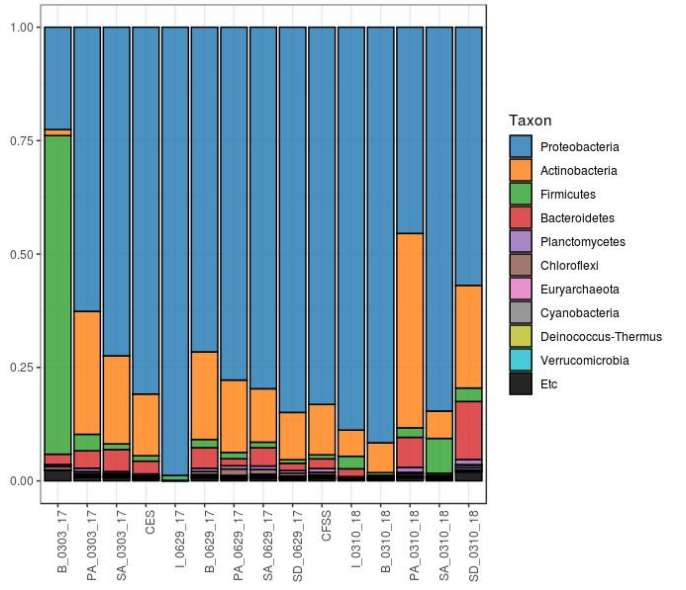
The taxonomic analysis based on Kraken2 showed that Bacteria were dominant (99.6%, 39 phyla in average), while Archaea (0.2%, 5 phyla) and Viruses (0.01%) were present in very small proportions (Figure 3-5A). Overall, the dye wastewater treatment system was dominated by Proteobacteria and Actinobacteria with small amounts of Firmicutes and Bacteroidetes. Mesorhizobium, Gluconacetoabcter, Burkholderia, Clostridium and Komagataeibacter were the most dominant genera in all samples (23.9% in mean abundance). Mesorhizobium is the only dominant genus (98.4%) in the influent (I_0629_17) and not in the rest of samples (mean abundance 0.71%). Acidovorax and Burkholderia were present in more than 5% for each of the five samples (mean value 9.5% of 5 sites (SA_0303_17, CES, SA_0629_17, SD_0629_17, CFSS) and 6.7% for each of the five samples (PA_0303_17, SA_0303_17, CES, B_0629_17, PA_0629_17,

SD_0629_17) (Figure 3-5B). The most dominant species *Mesorhizobium soli* is Gram-negative and aerobic, and often forms nitrogen-fixing nodules on the roots of a limited range of legume plants (Laranjo et al., 2014). The role of denitrification in nodules covers rhizobial denitrification as an energy-producing mechanism and rhizobial denitrification as a nitrite and nitric-oxide-detoxifying mechanism (Delgado et al., 2007). *Burkholderia vietnamiensis* strain G4 (R1808) is the best trichloroethene (TCE) co-oxidizing strain yet discovered (Fries et. al., 1997).

Strain G-4 was isolated for its TCE oxidizing ability from an industrial waste treatment facility at Pensacola Naval Air Station, Florida (Nelson et. al., 1987).Fresh human sewage was examined from a sewage treatment plant for the presence of members of the *Burkholderia cepacia* complex (BCC) of bacterial organisms and confirmed the presence of viable *B. cenocepacia* and *B. vietnamiensis*, by a combination of cultural, phenotypic and genotypic techniques (McNeely D et al., 2009).

Nitrification activity of *A. xylosoxidans* was demonstrated for the first time, while interestingly, the distinctive anaerobic denitrification property was preserved in S18. Ks values were determined as 232.13 ± 1.5 mg/l for COD reduction and 2.131 ± 1.9 mg/l for NH4+-N utilization (Kundu et al., 2011).

Gluconacetobacter diazotrophicus is a bacterium known for stimulating plant growth and being tolerant to acetic acid (Eskin et al., 2014). Significant nitrogenase activity was recorded among the isolates of *Gluconacetobacter diazotrophicus* and all the isolates produced IAA in the presence of tryptophan. All the four isolates efficiently solubilized phosphorus (Madhaiyan et al., 2004). S. marcescens CL1502 had good ability to remove NH_4^+-N, NO_3^--N, and NO_2^--N. High average NH_4^+-N, NO_3^--N, and NO_2^--N removal rates of 4.57 mg/(L·h), 4.60 mg/(L·h), and 4.42 mg/(L·h) were observed.



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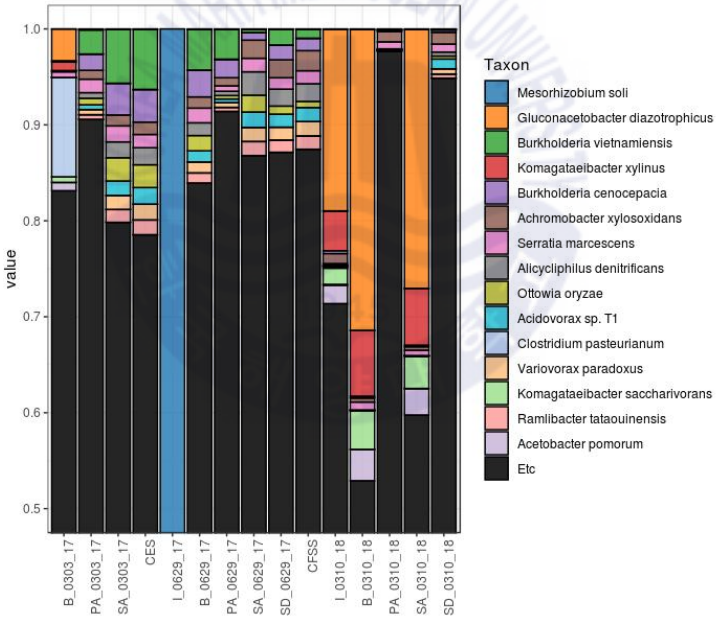


B

Figure 3-5 Comparative analysis of the microbial communities at phylum (A) and species (B) levels in the dye wastewater samples before and after bioaugmentation

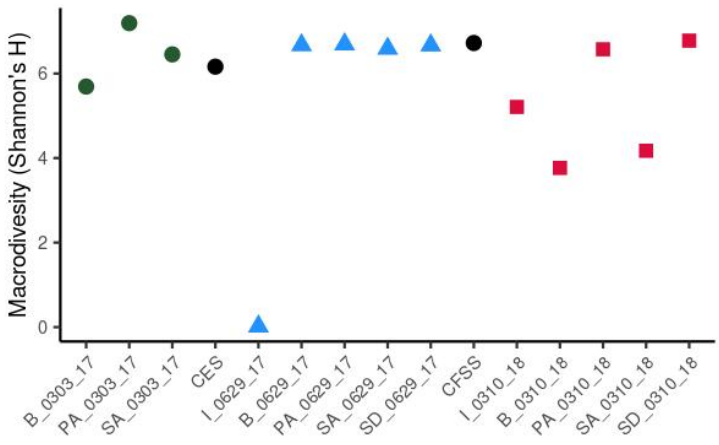
Application of strain CL1502 in actual tannery industry wastewater resulted in chemical oxygen demand (COD), NH4+-N, and TN removal efficiencies of 63.1%, 76.7%, and 72.9%. This is the first report of S. marcescens carrying out the process of simultaneous nitrification and denitrification (Huang, et al., 2017). *Alicycliphilus denitrificans* gen. nov., sp. nov., a cyclohexanol-degrading, nitrate-reducing beta-proteobacterium was isolated from a municipal sewage plant on cyclohexanol as sole carbon source and nitrate as electron acceptor (Mechichi et al., 2003). The species belongs to a separate lineage of the family Comamonadaceae in the beta-subclass of Proteobacteria. *Ottowia oryzae* of the family Comamonadaceae was isolated from an edible drink (Heo et al., 2018) but *Ottowia thiooxydans* gen. nov., sp. nov. strain K11T was isolated from activated sludge of a municipal wastewater-treatment plant (Spring et al., 2004). This strain was capable of aerobic chemolithoheterotrophic growth using thiosulfate as an additional substrate, and its facultative anaerobic growth was possible with nitrate and nitrite as electron acceptors, but not with ferric iron, sulfate or by fermentation. The sole product of the denitrification was N2O; nitrite accumulated only transiently in small amounts. *Acidovorax caeni sp.* nov., a denitrifying species with genetically diverse isolates from activated sludge was obtained from the activated sludge of an aerobic-anaerobic wastewater treatment plant in Belgium (Heylen et al., 2008). *Variovorax paradoxus* could be facultative lithoautotrophic and chemoorganotrophic. Many of the species are associated with important catabolic processes including the degradation of toxic and/or complex chemical compounds including sulfolane, 3-sulfolene, 2-mercaptosuccinic acid, 3,3'-thiodipropionic acid, aromatic sulfonates, alkanesulfonates, amino acids and other sulfur sources, polychlorinated biphenyls, dimethyl terephthalate, linuron, 2,4-dinitrotoluene and homovanillate (Satola et al., 2012). *Ramlibacter tataouinensis*, a β-proteobacterium strain isolated from an arid environment, has been reported to mediate a biologically controlled matrix-mediated calcification (Benzerara et al., 2004).

PCoA analyses based on the species level compositions showed a certain level of differences between the samples of before or early bioaugmentation stage

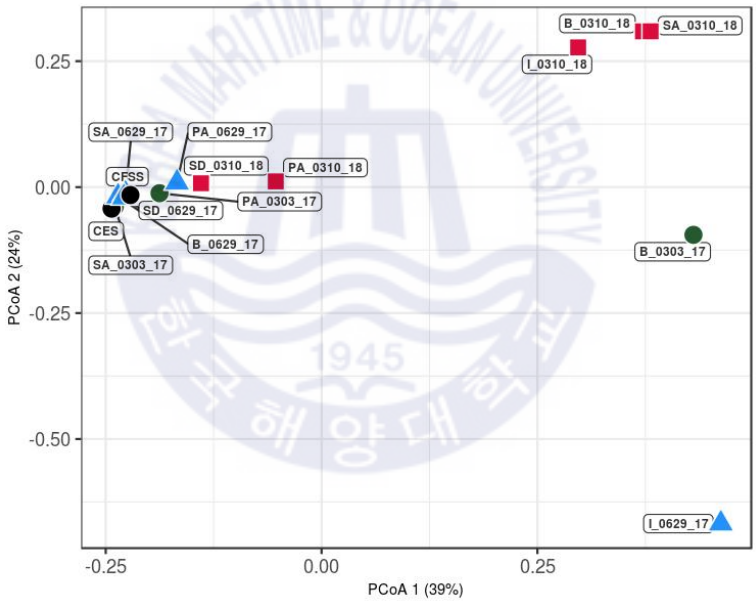
(PA_0302_17 and I_0629_17) and the samples of later bioaugmentation stage (SD_0310_18) shown in Figure 3-6, However, some samples (B_0302_17, PA_0310_18 and SD_0310_18) clustered against this general trend. There appeared to be no clear delineation in the microbial communities between processes of influent (I) (I_0310_18) and buffering (B) (B_0310_18). However, the buffering sample (B_0629_17) maintained quite a different community structure from the influent sample (I_0629_17), indicating the modifying efficacy of CES-1 bioaugmentation in the buffering process despite the very high domination of *Mesorhizobium soli* (98.5%) in the influent.

3.3.3 Analysis of enzyme profiles for the most dominant metabolic pathways

Heat map analysis of catabolic enzyme profiles for the most dominant metabolic pathways (the top 10% pool) before and after bioaugmentation was shown in Figure 3-7. Samples of the early stage bioaugmentation (B_0629_17) except I_0629_17 possessed similar enzyme profiles ranging from ATP-dependent Clp protease 1 down to tryptophan synthase beta chain. The typical catabolic enzymes relevant to dye wastewater treatment appeared to be various proteases (ATP-dependent Clp protease 1, membrane protease, modulator of *FtsH* protease, putative proteasome type protease and serine protease), amino acid degradative enzymes (tryptophan synthase ph chain, L-aspartate oxidase, tryptophan synthase beta chain and acetyl glutamate kinase), fatty acid degradative enzymes (acyl CoA dehydrogenase and enoyl-CoA hydratase) and aromatic hydrocarbon degradative enzyme (3,4-dihydroxyphenyl acetate 2,3-dioxygenase). 3,4-dihydroxyphenyl acetate 2,3-dioxygenase might be involved in degradation of the aromatic metabolic products from azo dyes (Diaz et al., 2001). This enzyme was present in both CES-1 and SA_0302_17 in a higher frequency. The dominant proteases (ATP-dependent Clp protease, ATP-dependent Hsl UV protease and regulator of sigmaE protease, etc.) and 2-oxoglutarate dehydrogenase (for amino acid degradation) and phospholipase C (for lipid degradation) were observed in the later stage



A



B

Figure 3-6 Analysis of alpha (A) and beta (B) diversities of the microbial communities in the dye wastewater samples before and after bioaugmentation

of bioaugmentation (B_0310_18 and SA_0310_18). The azo bond cleaving enzyme, FMN-dependent NADH-azoreductase, was found relatively in a higher frequency in the influent samples (I_0629_17 and I_0310_18) than CES-1, indicating the enzyme could be present in the incoming raw wastewater. The enzyme profiles for all the samples before and after bioaugmentation basedon NMDS analysis showed a similarity to their corresponding microbial community patterns as shown in Figure 3-7.

3.3.4 Analysis of azoreductase profiles for the dye wastewater samples

The FMN-dependent NADH-azoreductase was observed to surprisingly be highest in B before the bioaugmentation and its higher frequency was still maintained compared with other treatment stages after the bioaugmentation. However, the azoreductase frequencies in B, SA and SD were higher in 300 days after bioaugmentation compared with 50 days after the augmentation by 18%, 425% and 57%, respectively (Figure 3-8). Presence of the azoreductase in all the different stages might be due to induction of the enzyme by the azo dye materials in these stages.

The enzyme genes related to the azo dye degradation had a highest abundance despite the presence due to bioaugmentaion, especially for the gene encoding *AcpD* and *AzoR*. However, functional changing characteristics of associated enzyme genes are not been entirely explored in different treatments. In the current study, FMN-dependent NADH-azoreductase was identified after the bioaugmentation and shows the increasing abundance of azoreductase enzymes in different time of buffering treatment. The Fermicutes are highly present in B_0303_17 indicates the significance between the taxon and the high abundance of enzymes involved in the azo dye degradation. Hence, the specific enzymes involved in the pathway were enhanced due the presence of microbial community involved in the bioaugmentation.

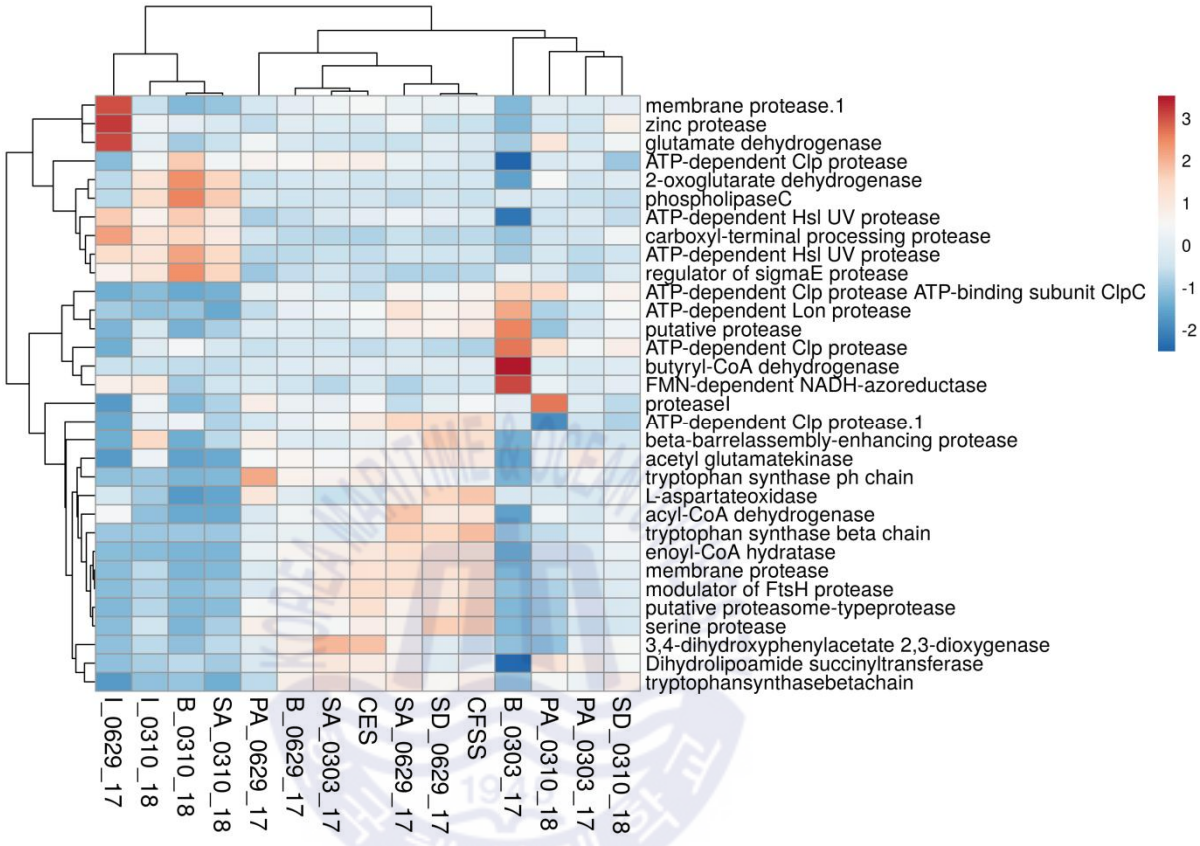


Figure 3-7 Heat map analysis of enzyme profiles for the most dominant metabolic pathways (the top 10% pool) in the dye wastewater samples before and after bioaugmentation

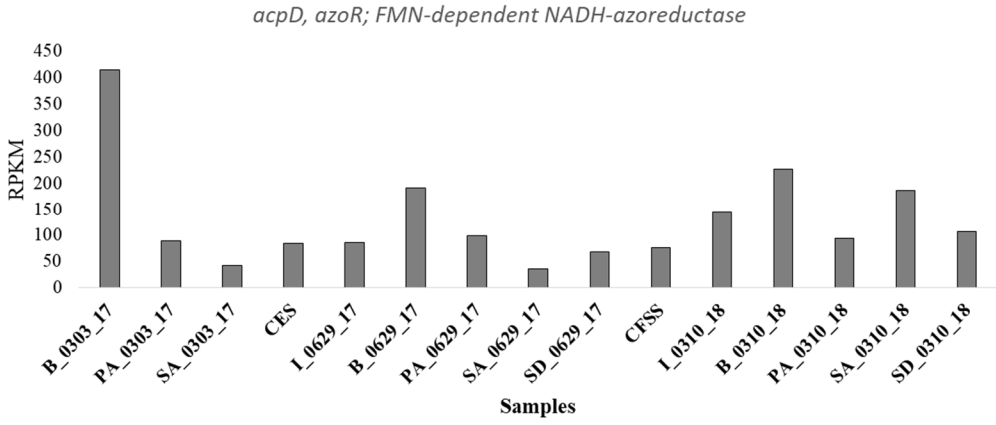


Figure 3-8 FMN dependent NADH-azoreductase profiles for the dye wastewater samples before and after bioaugmentation

3.3.5 Analysis of enzyme profiles for the aromatic compound degradative pathways

Heat map analysis of enzyme profiles for the aromatic compound degradative pathways was demomstrated in Figure 3-9. The augmented culture CES-1 carried various aromatic compound degradative pathways such as vanillate monooxygenase, catechol 1,2-diooxygenase, 2-hydroxymuconate-semialdehyde hydrolase, salicylate 5-hydroxylase small subunit, 3,4-dihydroxyphenylacetate dioxygenase, salicylate 5-hydroxylase large subunit, benzoate/toluate 1,2-diooxygenase subunit alpha, protocatechuate 4,5- dioxygenase alpha chain and gentisate 1,2- dioxygenase. These enzymes were generally present in the samples (B_0310_18, PA_0310_18, SA_0310_18 and SD_0310_18) 300 days after bioaugmentation while frequencies of these enzymes were quite low before and 50 days after augmentation, indicating the selection of these aromatic degradative enzymes by CES-1 augmentation over time.

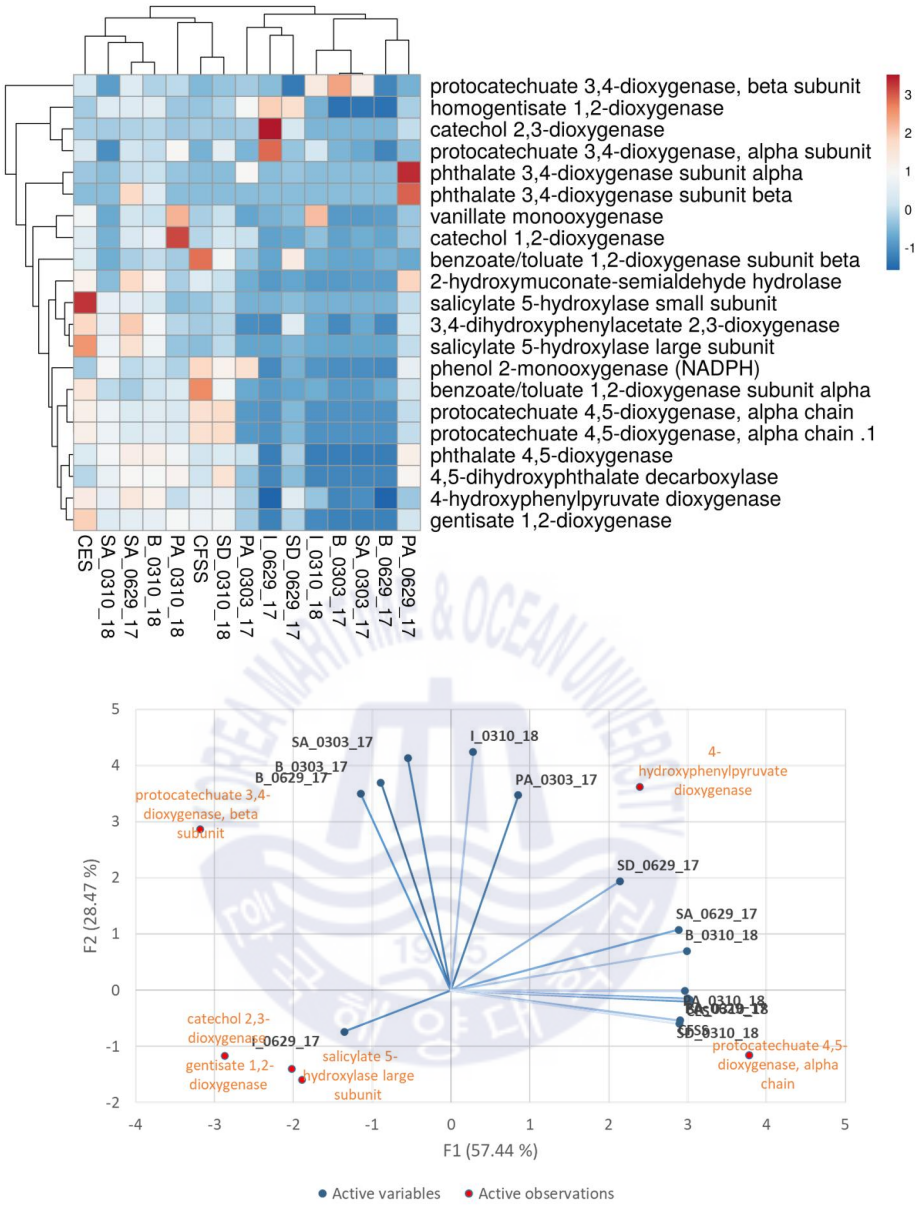


Figure 3-9 Heat map and PoCA analysis of enzyme profiles for the aromatic compound degradative pathways in the dye wastewater samples before and after bioaugmentation

3.3.6 Analysis of enzyme profiles for the amino acid degradative pathways

Metabolic enzyme profiles for the amino acid degradative pathways before and after bioaugmentation were shown in Figure 3-10. There were two essential groups in terms of kinds of samples and the amino acid degradation enzyme profiles. The samples clustered in two groups like the samples of control and early stage of bioaugmentation (B_0303_17 and I_0629_17) and the samples of later stage of bioaugmentation (PA_0310_18). The former group carried amino acid degradation enzymes including 2,5-dioxopentanoate dehydrogenase, D-alanine transaminase, glutamate-5-semialdehyde dehydrogenase, glutamate dehydrogenase (NADP), 2-oxoglutarate reductase, tryptophan 4-dimethylallyltransferase, pyridoxal phosphate-dependent aminotransferase and L-aspartate. The latter group contained the enzymes like 2-hydroxyglutarate dehydrogenase, tryptophan synthase, 2-oxoglutarate dehydrogenase, acetylglutamate kinase, N-acetyl-gamma-glutamyl-phosphate reductase and bifunctional N-acetylglutamate synthase/kinase.

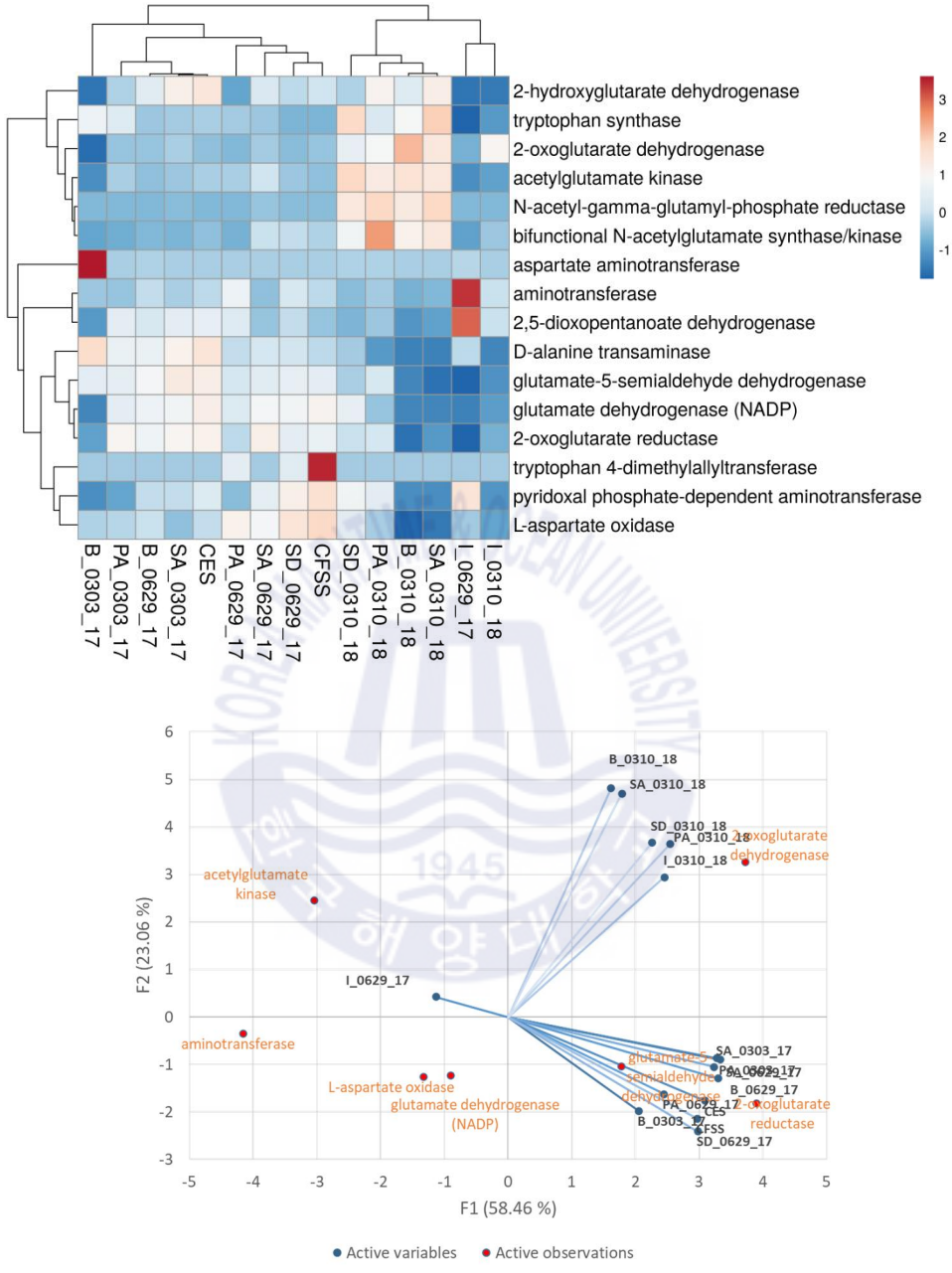


Figure 3-10 Heat map and PoCA analysis of enzyme profiles for the amino acid degradative pathways in the dye wastewater samples before and after bioaugmentation

3.3.7 Analysis of enzyme profiles for the TCA cycle pathways

Metabolic enzyme profiles for TCA cycle pathways before and after bioaugmentation were shown in Figure 3-11.

There were two essential groups in terms of kinds of samples. One group carried the samples like B_0303_17, I_0310_18, B_0310_18 and SA_0310_18. This group carried succinate dehydrogenase (ubiquinone) iron-sulfur subunit, succinate dehydrogenase, triosephosphate isomerase, 2,3-bisphosphoglycerate-independent phosphoglycerate mutase and glyceraldehyde-3-phosphate dehydrogenase. The other group contained all the rest including most of PA_0303_17 and B_0629_17.

The representative enzymes found in a higher frequency in the second group were succinate dehydrogenase, enolase, pyruvate kinase, fructose-biphosphate alolase class II, probable phosphoglycerate mutase, isocitrate dehydrogenase, malate dehydrogenase (*MaeB*), succinyl-CoA synthetase beta subunit and malate dehydrogenase (*Mdh*). Not surprisingly, CES-1 also carried this kind of enzyme patterns.

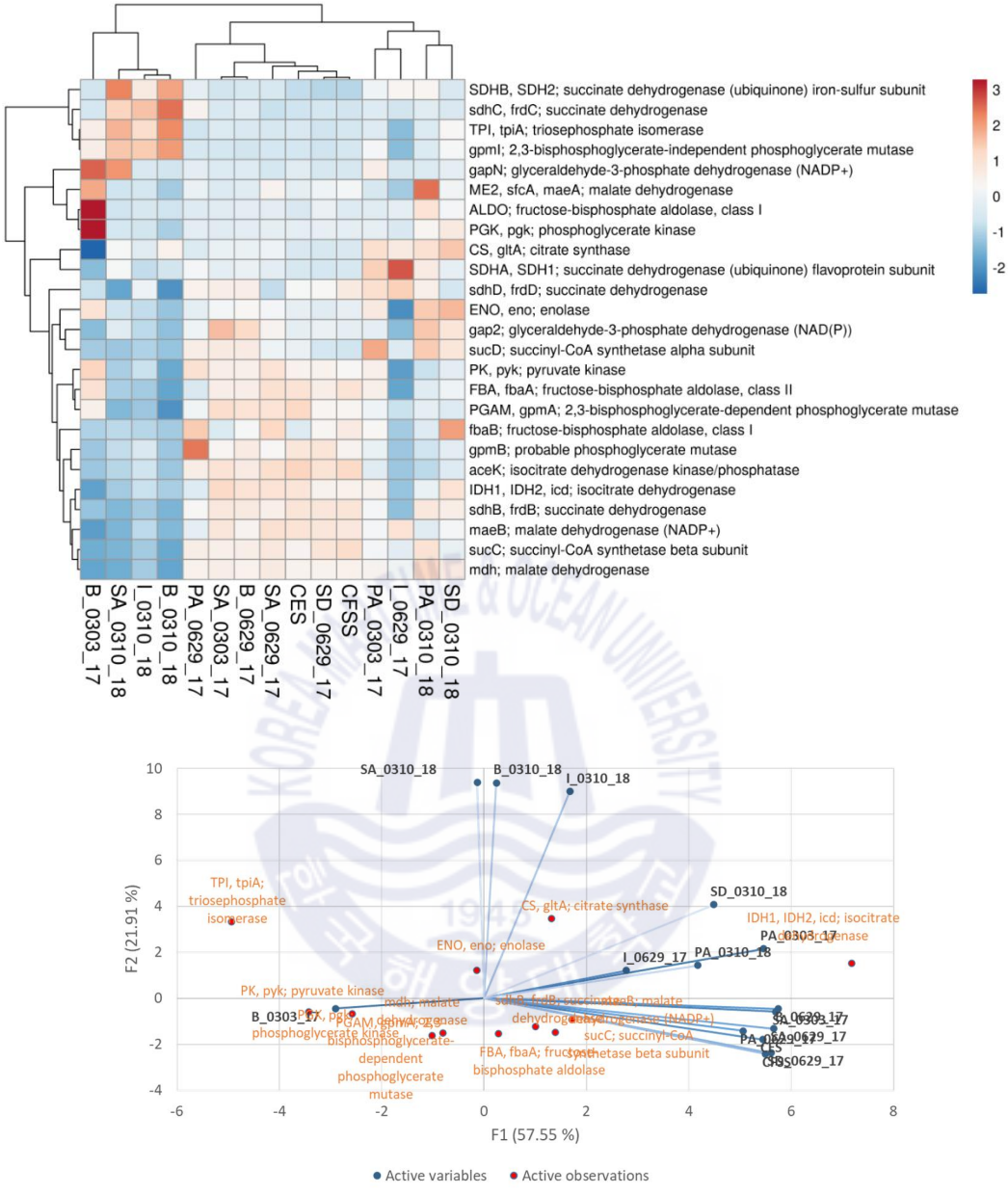


Figure 3-11 Heat map and PoCA analysis of enzyme profiles for the TCA cycle pathways in the dye wastewater samples before and after bioaugmentation

3.3.8 Analysis of enzyme profiles for the nitrogen cycle pathways

Heat map analysis of enzyme profiles for the nitrogen cycle pathways before and after bioaugmentation was shown in Figure 3-12. The profiles were clustered in two groups. One group carried most samples 50 and 330 days after augmentation in which nitrite reductase, hydroxylamine dehydrogenase, nitrous oxide reductase, nitrate reductase/nitrite oxidoreductase, nitrite reductase, perisplasmic nitrate reductase, ammonia monooxygenase subunit A and nitrite reductase were in a higher frequency. The other group carried nitrogenase iron protein (*NifH*) and nitrate/nitrite transporter. The influent samples (I_0629_17 and I_0310_18) showed a low frequency of all the above enzymes in the nitrogen cycle pathway.

Carbon source as electron donor would promote denitrification, the system might be short of carbon source due to the rapid growth of microorganisms. Thereby, microorganisms can only consume the inorganic compounds as carbon source, which is consistent with the decline of the TN (Peng et al., 2019). As the removal of nitrogen increases, the abundance of enzyme genes related to nitrogen metabolism was raised.

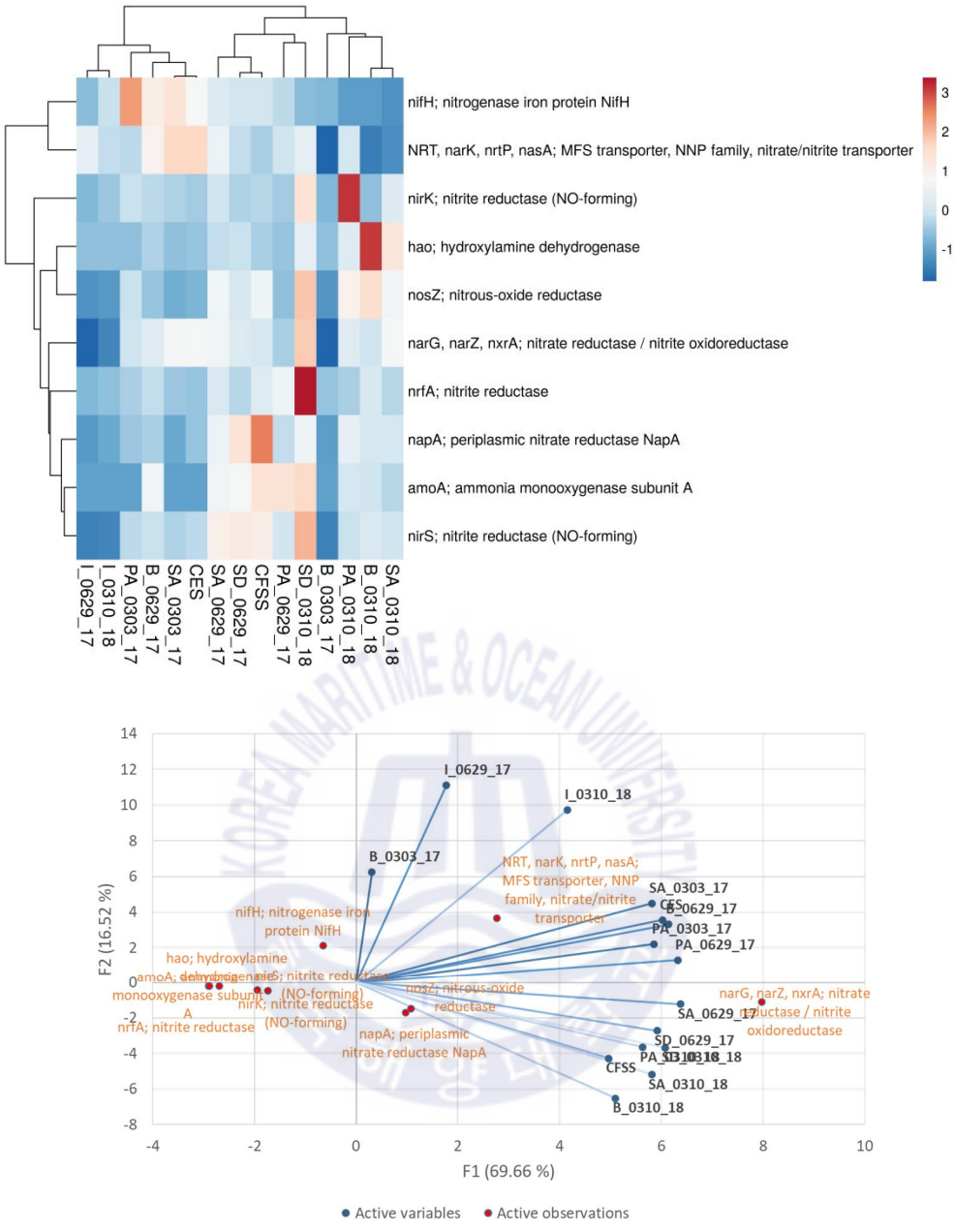


Figure 3-12 Heat map and PoCA analysis of enzyme profiles for the nitrogen cycle pathways in the dye wastewater samples before and after bioaugmentation

The abundance of nitrite reductase (*nrfA*) which is involved in the reduction of nitrite to ammonia raised by three times, and hydroxylamine dehydrogenase (hao) related to the reduction of hydroxylamine to ammonia also raised, which might lead to a decline in the NH4+-N removal rate (Aslam et al., 2018). Since the expression of functional genes of either single strain or microbial consortium were altered by various physicochemical parameters. Therefore, the high activity of enzymes is significant to the involvement of nitrogen cycle pathways.

3.3.9 Analysis of enzyme profiles for the phosphorus cycle pathways

Heat map analysis of enzyme profiles for the phosphorus cycle pathways before and after bioaugmentation was shown in Figure 3-13. The profiles were clustered in two groups, but all the samples were not segregated according to non-bioaugmentation and the bioaugmentation period. Acid phosphatase (class A) and 3-phytase were dominant in the sample after the bioaugmentation (PA_0310_18) while acid phosphatase (class B) and 4-phytase were in the sample (PA_0629_17 and SD_0629_17). The samples from the treatments of after 300 days were scattered compared to the samples taken in 90 days. B_0303_17 and B_1310_18 ware correlated each other and B_0629_17 is not significant with the other buffering treatments. This shows the presence of enzymes for the phosphorus cycle pathways was high in 90 days treatment and the removal rate of TP is almost same in both the treatments. Hence, there is no increase in the removal of TP in treatment of 300 days (Figure 3-13 & 3-14).

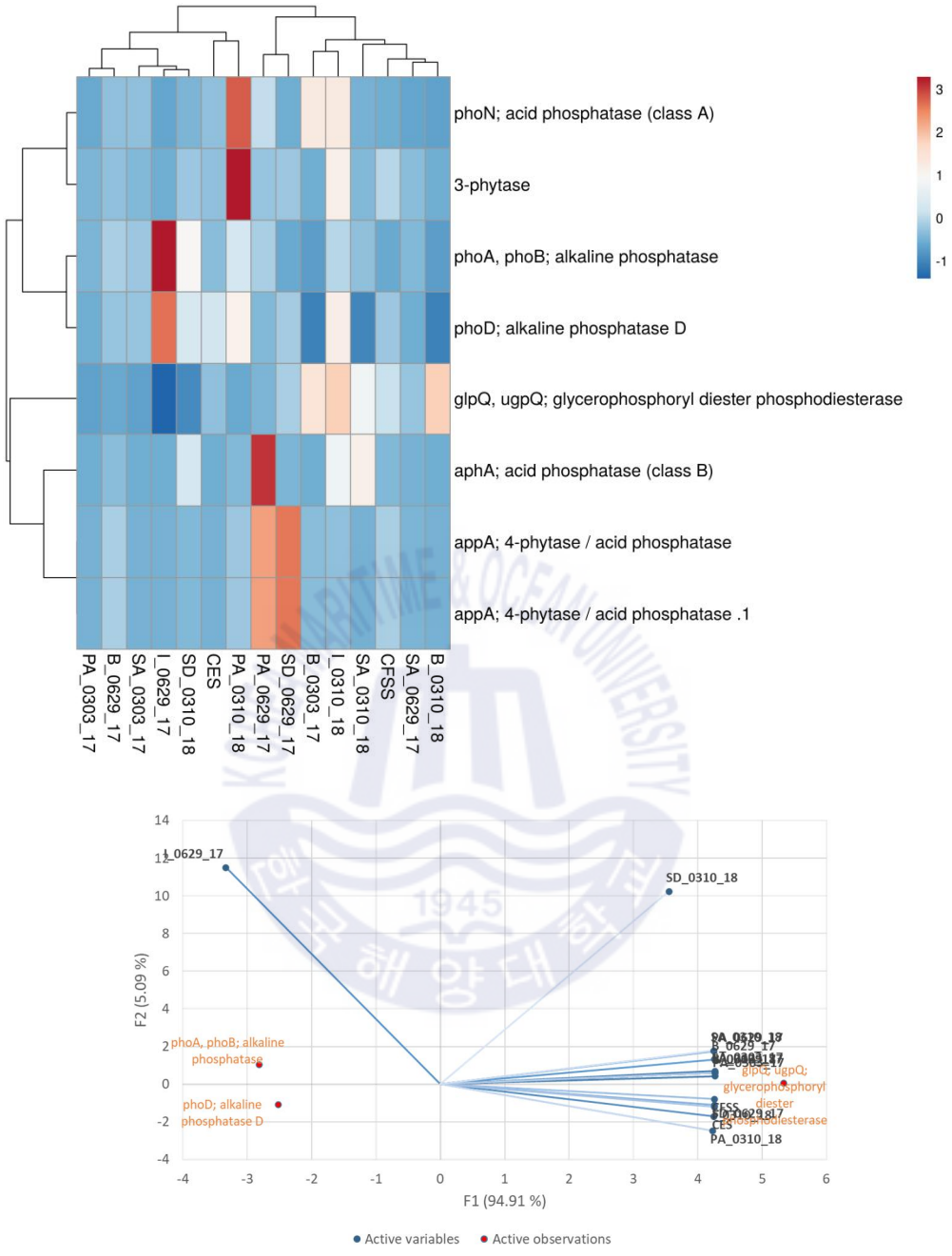


Figure 3-13 Heat map and PoCA analysis of enzyme profiles for the phosphorus cycle pathways in the dye wastewater samples before and after bioaugmentation.

3.4 Conclusion

The activities of dye wastewater treatment plant were evaluated before and after the addition of CES-1. The overall process showed a certain level of differences between the samples of before or early bioaugmentation stage (PA_0302_17 and I_0629_17) and the samples of later bioaugmentation stage (SD_0310_18), However, some samples (B_0302_17, PA_0310_18 and SD_0310_18) clustered against this general trend. There appeared to be no clear delineation in the microbial communities between processes of influent (I) (I_0310_18) and buffering (B) (B_0310_18). However, the buffering sample (B_0629_17) maintained quite a different community structure from the influent sample (I_0629_17), indicating the modifying efficacy of CES-1 bioaugmentation in the buffering process despite the very high domination of *Mesorhizobium soli* (98.5%) in the influent. The intrinsic relationships between microbial community structures and functions of enzymes for the dye metabolism need to be analyzed using more sophisticated algorithms. Since the successful performance in reduction of pollution and sludge in the dye wastewater treatment plant and the concomitant metagenomic analysis of the treatment process were accomplished, the future perspective of this research will be development of specific genes and enzymes as biomarkers used to monitor the functional properties of degradation and operate the novel dye wastewater treatment system in the optimal conditions.

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PUBLICATIONS

INTERNATIONAL

Emmanuel, S.A., Sul, W.J., Seong, H.J., Kim, H.G., Koh, S.C. Metagenomic Analysis of Full-scale Dye Wastewater Treatment Plant Undergoing Bioaugmentation of the Composite Microbial Culture CES-1 (in process).

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BOOK CHAPTER

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PATENT

MANUFACTURING METHOD OF NANO-METAL BIOCHAR FOR REMOVING PHOSPHORUS AND NANO METALS

IPC: B01J 20/30|C02F 1/28 | Inventers: Jae Soo Chang | Jin Sung Lee| S. Aalfin Emmanuel

CONFERENCES

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Chaeyoung Rhee, **Aalfin Emmanuel .S**, Woo-Jun Sul, Hoon-Je Seong, Sung-Cheol Koh, Metagenomic Analysis of Relationships between Carbon and Denitrification Metabolisms in Tannery Wastewater Treatment Plant Bioaugmented with Novel Microbial Consortium BMS-1, 9th International Conference on Environmental Science and Technology, Houston, Texas, United States, Jun 25-29th, 2018

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INTERNSHIPS AND AWARDS

- Chung Ang University (one month)
- National Centre for Cell Science (four months)
- National Facility for Marine Cyanobacteria (six months)
- Indian Academy of Science fellowship award