Thesis for the Degree of Doctor of Philosophy

Isolation and Characterization of Useful Microorganisms for Efficient Biological Treatment of Wastewaters and Their Application to Upscaled Processes

by

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효율적인 생물학적 폐수처리를 위한 유용미생물의 분리, 특성 연구 및 규모가 큰 공정으로의 적용

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by

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A Dissertation

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효율적인 생물학적 폐수처리를 위한 유용미생물의 분리, 특성 연구 및 규모가 큰 공정으로의 적용

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요약

인구의 증가와 산업의 발전으로 많은 산업 폐기물이 생성된다. 이러한 폐기물들은 물리적, 화학적 또는 생물학적인 방법으로 처리되는데, 생물학적 처리 방법은 물리 화학적 방법에 비해 간편하고 안정적이며, 친환경적인 방법이라고 이다. 본 연구에서 염색폐수, 피혁폐수 및 수산폐기물 폐수의 친환경 생물학적 처리를 위해, 각 폐수의 특성에 맞는 미생물을 새로 분리하고, 그 분리된 미생물을 이용하여 실험실 단위 및 대규모화를 통한 각 폐수의 생분해 특성을 알아보았다.

염색폐수는 난분해성 고색도(high chromaticity)를 가지는 화합물이 포함되어 있어 전통적인 방법으로는 분해가 어렵다. 본 연구에서는 염색폐수 처리장의 유입원수와 반송수로부터 분리된 두 종류의 곰팡이 (*Ascomycetes*와 *Basidiomycetes*)를 이용하여 그 분해 특성을 알아본 결과, 이 균주는 염료가 들어있는 tube 및 flask 단위 실험에서 약 80% 이상의 색도 분해 효율을 얻었다. 광합성세균과 fungi와의 친화성을 알아보기 위해 1:1 비율로 혼합하여 분해 실험에 이용한 결과 fungi 만을 이용하여 분해할 때 보다 색도 제거 효율이 높게 나타났고, 각각 다른 성분이 포함되어 있는 두 가지 배지 (BSM, ME) 에서 실험한 결과, ME 배지에서 배양할 경우 더 높은 색도 제거 효율을 나타내었다. 200-m^3/d 파일럿 단위에서의 실험에서 70일간 분리된 fungi를 폭기조 용량의 0.2% 만큼 매일 투입한 결과, 실험 15일 이후에는 fungi가 폭기조에 안정하게 잘 정착하여 기존의 색도 제거 효율(약 350) 보다 높은 색도 제거 효율을 나타내었으며, 배출수의 색도는 약 230으로, 그 기준치(400)를 만족한 결과를 얻었다.

피혁폐수는 높은 농도의 질소를 포함하고 있어 부영양화를 일으킬 수 있으므로 본 연구에서는 피혁폐수 속 질소처리를 위하여 8종의 bacteria를 새롭게 분리하였다. 8종의 bacteria 중 KH8 균주가 가장 높은 탈질 효율을 나타내었고, 동정결과 *Pseudomonas aeruginosa* R12와 100% 일치하였다. 실험실 단위의 실험에서 KH8과 다른 7종의 균주의 비율이 10:1 일 때 높은 탈질 효율을 나타내었고 또한 C/N비가 적절할 때 탈질 효율이 높음을 확인하여 기존의 실험에 추가적인 탄소원이 요구됨을 확인하였다. 실제 피혁폐수 중의 질소제거를 위해 10:1 비율의 bacteria와 추가 탄소원으로서 lactose를 9000-m^3/d 규모 피혁폐수 처리장 내 폭기조에 투입한 결과 기존의 질소 제거 효율보다 약 38% 높은 제거 효율을 나타내었다.

수산폐기물의 경우 생선, 해조류 및 어패류 폐기물 등 다양한 폐기물의 분리 수거가 어려워 다양한 성분을 처리하기 위해 혼합 미생물을 이용해 왔으나. 본 연구에서는 고염 조건에서도 견딜 수 있으며 다양한 효소를 생산할 수 있는 미생물인 *Bacillus licheniformis* TK3-Y 균주를 분리함으로서 효율적인 수산폐기물 처리에 이용하였다. TK3-Y 균주는 cellulase, protease 및 lipase를 동시에 생산하는 능력을 가지고 있으며, 17.5%의 높은 염도에서도 생장이 가능하였으며, 생산된 3가지 효소는 35% 염도에서도 활성이 유지되었다. Carboxymethyl cellulose, skim milk 및 olive oil이 함께 혼합되어 있는 배지에서 TK3-Y 균주에 의한 셀룰로오스, 단백질 및 지질의 동시 분해가 일어남을 확인하였으며, 또한 150-L 규모 파일럿 스케일 반응기에서 실제 멸치 및 파래 폐기물을 이용하여 TK3-Y 균주의 혼합 폐기물 분해 능력을 확인하였다.

본 연구에서의 염색폐수, 피혁폐수 및 수산폐액의 생물학적 처리 결과, 각 폐수의 성분특성에 맞는 유용미생물의 분리 및 그 특성 연구, 그리고 큰 규모 공정에의 적용은, 미생물을 이용한 산업적 폐수 처리로의 실용화 가능성을 알아볼 수 있었다.

GENERAL INTRODUCTION

Backgrounds

Rapid growth of population and development of industries have elevated the standard of living and quality of life. As a consequence it aggravated the release of domestic and industrial waste materials. These wastes and their wastewater are commonly disposed by incineration, landfill and dumping into ocean and river causing secondary pollution. According to the London Convention, dumping waste into ocean has been prohibited since 2014 (International Maritime Organization, 2006). Accordingly, eco-friendly treatment, and reutilization of wastes are necessary and lots of studies associated with advanced wastewater treatment have been proceeded. Wastewater treatment is a multi-stage process to clean and dispose the wastewater into natural water bodies without hazard or reuse as industrial water. The ultimate objective of wastewater treatment is to remove organic pollutants, nutrients, nitrogen before disposing into natural water bodies. This will improve the water quality of the effluent to be disposed into water bodies (UNIDO, 2011).

In this study, biological treatments of dyeing, leather and fishery industrial wastewater using specific microorganisms such as bacteria and fungi were studied. The industries generating such dyeing, leather and fishery waste and wastewater are located in Busan, especially,

the dyeing and leather industries are the second largest industries in Sinpyeong-Jangnim Industrial Complex and the fishery industry is also one of the biggest local industries near this area. Wastewater discharged from these industries caused water and soil pollution, and emission of effluents that limited self-purification also caused difficulty in using the water resource due to its bad odor. In 2006, Sinpyeong-Jangnim Industrial Complex was designated as odor management area. For these reasons, it became to be necessary to apply eco-friendly and economical wastewater treatment process in these areas. Therefore, as one of the eco-friendly biological treatment system, isolation of effective microorganisms and application to wastewater treatment system were performed for efficient treatment, improvement of water quality and reutilization of these wastes.

Wastewater treatment

Wastewater treatments are composed of physical, chemical, biological methods or combination of these processes. Filtration, carbon absorption, reverse osmosis, coagulation and precipitation are involved in the physicochemical treatment. However, these treatment processes have several disadvantages that are unstable, unsafe and that generate by-products. Hence, biological treatment is preferred in lots of wastewater treatment plants. Operation of biological wastewater treatment includes preliminary, primary, secondary,

tertiary and/or advanced treatment and disinfection. In the preliminary treatment, floating solid and large stuffs such as wood, cloth, paper, glass, metal, etc. are removed in screen tank and grit chamber. This treatment will help in prevention of contamination and sediment settling in effluent discharged zone. It will also help to smooth operation of treatment without damage to the machine and pump. In primary treatment, organic and inorganic solids and floating materials are removed by sedimentation and skimming. The 20–50% of BOD, 50–70% of suspended solids, and 65% of oil and grease are removed during primary treatment. The sludge from the primary treatment system are removed from the bottom of clarifier and pumped to sludge thickener. In the preliminary and primary treatment, only physical operation is used. Meanwhile, in secondary treatment in which biological and chemical processes are used to remove biodegradable dissolved residual organic and colloidal matters by this treatment system, with typical removals of BOD and SS by 85%, and of some harmful heavy metals (FAO, 1992). In tertiary/advanced treatment, remaining inorganic compounds and substances such as nitrogen and phosphorus are removed to improve treated–water quality before it is discharged to the natural environment or reused (Sidney Water Corporation, 2010). In disinfection stage, bacteria and viruses will be removed by injection of chlorine solution or ozone and UV irradiation (FAO, 1992).

Biological wastewater treatment

In wastewater treatment, biological process is an important and essential part. Pollutants are treated by various biological methods in sewage treatment system. To treat municipal and industrial wastewater by biological process, more than 30 species of microorganisms like protozoa, fungi, yeast and bacteria can be generally used due to their rapid growth, useful enzyme production abilities and their degrading abilities of organic substances. (Son et al., 2013; Daims et al., 2006). In operating microbiological treatment system, it should be considered that the cultural and environmental conditions such as temperature, pH, alkalinity, nutrients, growth rate, substrate uptake rate and characteristics of wastewater have some influence on the microbial growth and activities (Wang, 1994). Microbial communities existing in these treatment systems form a various ecosystems according to characteristics of the wastewater. Similarly, the treatment efficiencies are influenced by microbial biomass and their activities (Son et al., 2013). There are two classes of microbiological processes, anaerobic (absence of oxygen) and aerobic (presence of oxygen) treatments (Mittal, 2011). Anaerobic processes such as anaerobic filter, fluidized/expended bed reactor and upflow anaerobic sludge blanket (UASB) process are relatively slow process and the reaction products are carbon dioxide and methane gas with hydrogen sulfide. On the other hand, aerobic processes such as activated sludge processes, trickling filter and membrane bioreactor are faster and larger than anaerobic processes, and metabolize the organic matters in the

wastewater, producing more biomass and end-products like carbon dioxide and water (Mittal, 2011). However, like nitrogen removal, anaerobic and aerobic processes occur simultaneously or separately. In general, nitrification occurs under an aerobic condition and denitrification occurs under an anaerobic condition. Hence, in treating dyeing wastewater, combination of anaerobic/aerobic process system was used (van der Zee and Villaverde, 2005). The anaerobic-aerobic sequencing batch reactor was used for piggery wastewater treatment with anaerobic digestion of organic carbon where more organic carbon removal and ammonia oxidation occurred in aerobic process (Bernet et al., 2000).

Dyeing industry wastewater

The dyeing industry wastewater contains high alkalinity, chemical oxygen demand (COD), biological oxygen demand (BOD), total organic carbon (TOC), total suspended solids (TSS), extreme pH and color. As much as 50% of non-stained or unfixed high colored dyeing industry waste dissolve in water, damaging water quality and metabolism of ecosystem (Pearce et al., 2003; Kabra et al., 2013). The DIW was treated by conventional physicochemical methods such as flocculation, membrane filtration, electro-coagulation, ion-exchange and adsorption (Banat et al., 1996). However, these method were uneconomical and unsuitable for treatment in a broad range of dyeing wastewater due to the non-degradable characteristics of dyes (Fu and Viraraghavan, 2001).

Therefore, non-toxic biological methods using fungi, yeast microalgae and bacteria have been studied for replacement of physicochemical treatments (Rai et al., 2005; Saratale et al., 2011). The main purpose of dyeing degradation process is decomposition and mineralization of dyes into carbon dioxide and water without generation of toxic intermediates (Lade et al., 2015). In dyeing wastewater treatment, some enzymes are involved in the degradation and decolorization processes including laccase (EC 1.10.3.2), azoreductase (EC 1.7.1.6), lignin peroxidase (EC 1.11.1.14), manganese peroxidase (EC 1.11.1.13), etc. These enzymes are able to oxidize dyes and to perform a reductive breakdown of dyes (Lade et al., 2015a; Lade et al., 2015b). Some studies reported bacterial and fungal strains that produce the dye degrading enzymes. Fig. 1 and Fig. 2 showed the azo-dye (Congo Red and Trypan Blue) degradation pathway by azoreductase and laccase (Lade et al., 2015a; Lade et al., 2015b). Liu et al. (2006) performed dye-degradation by the photosynthetic bacterium, *Rhodopseudomonas palutstris* that possesses azoreductase that decolorizing azo dyes under an anaerobic condition. Similarly, Balan and Monteiro (2001), Harazono and Nakamura (2005) and Gomes et al. (2009) reported the white-rot fungi, *Basidiomycetes* strain producing lignin-degrading enzymes such as laccase, lignin peroxidase and manganese peroxidase play the roles of transformation and degradation of synthetic azo, heterocyclic, indigo, polymeric and reactive dyes. Ramalho et al. (2004) reported the *Ascomycetes* yeast strain, *Issatchenkia occidentalis* cleaved azo dyes reductively.

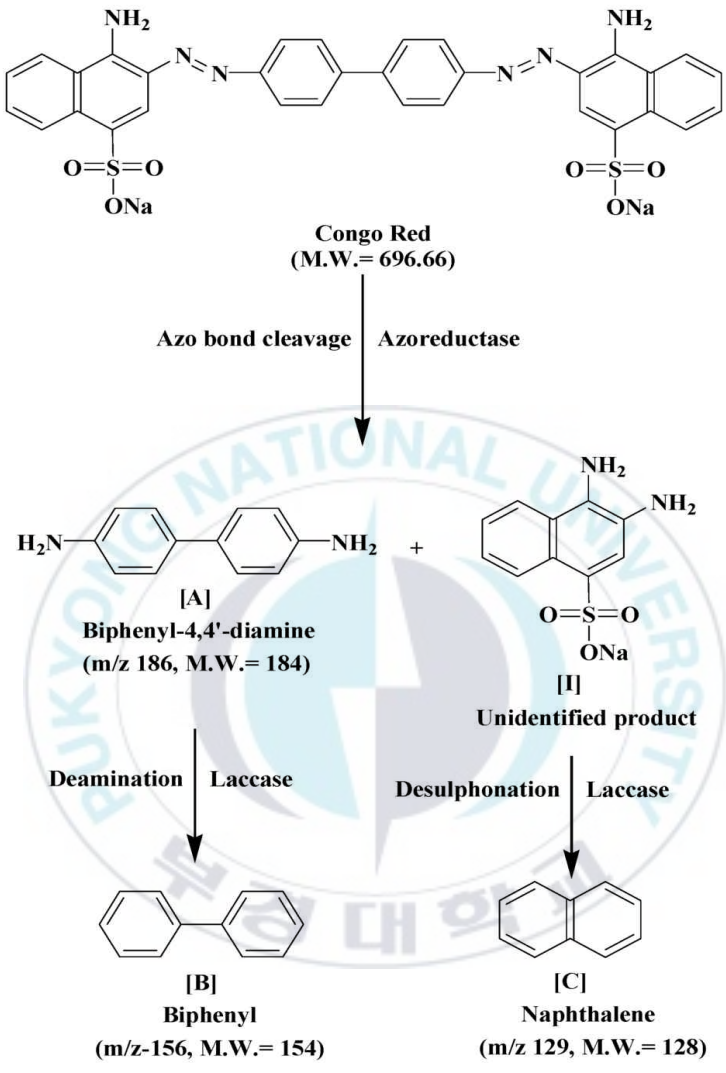

Fig. 1. A proposed biodegradation pathway of Congo Red by azoreductase and laccase produced by microbial consortium (Lade et al., 2015a).

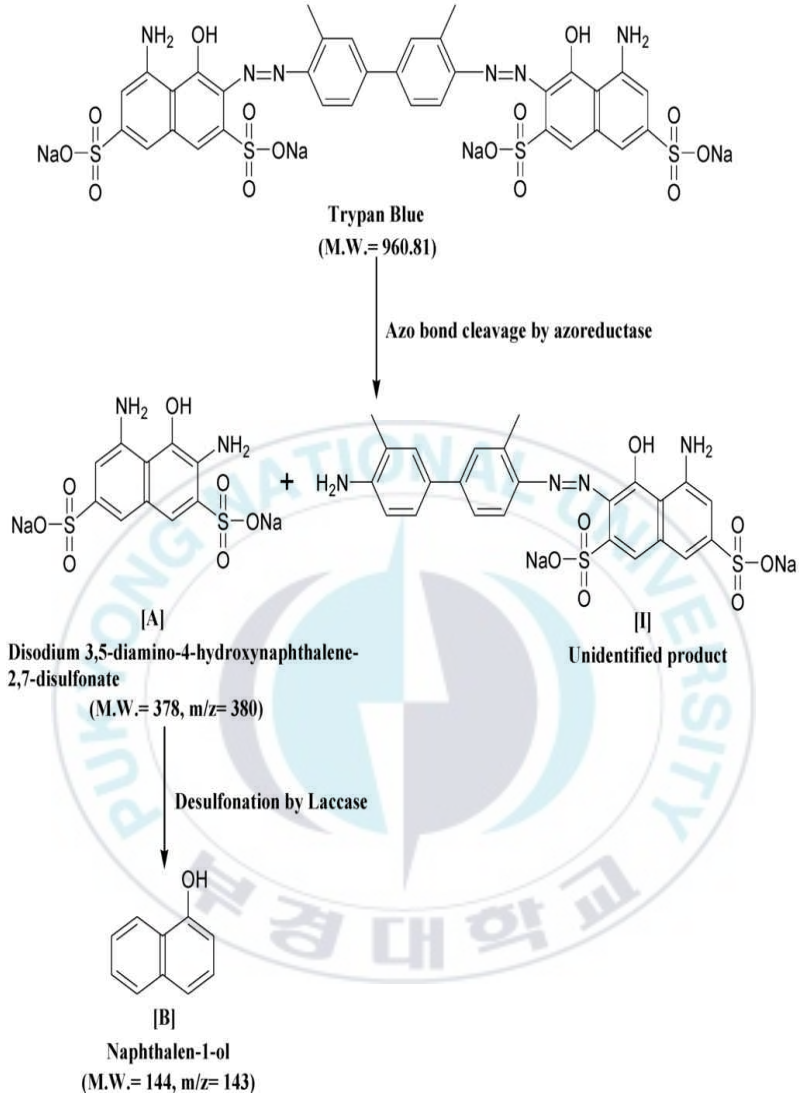

Fig 2. A possible mechanism of Trypan Blue by microbial consortium (Lade et al., 2015b).

Leather industry wastewater

Leather industry wastewater contains high levels of organic and inorganic chemicals and high nitrogen concentration as well. Nitrogen is considered to be a limiting growth factor for plants and microbes. In wastewater, nitrogen exists to be organic nitrogen as uronic and amino acid or inorganic nitrogen as ammonia, ammonium ion, nitrite and nitrate, which causes water body impairment such as oxygen depletion, harmful algal blooming and turbidity of water due to nitrogen-related eutrophication (Nourmohammadi et al., 2013). As a conventional denitrification method, Mandal et al. (2010) and Chowdhury et al. (2013) reported that penton oxidation, filtration and UV/H_2O_2 and electro-coagulation were used for LIW treatment. However, this physicochemical method is efficient in treating organic nitrogen, but not in treating inorganic nitrogen. For this reason, biological nitrogen removal process have been recommended in previous studies. As shown in Fig. 3, nitrogen elimination from wastewater comprises three main processes; nitrification, denitrification and anaerobic ammonium oxidation (ANNAMOX) (Daims et al., 2006; Ni and Zhang, 2013). During nitrification, ammonia monooxygenase (EC 1.14.99.39), hydroxylamine oxidoreductase (EC 1.7.99.1) and nitrite oxidase (EC 1.7.1.4) produced by ammonium oxidation bacteria (AOB) are involved in the ammonium oxidation process. Nitrate reductase (EC 1.7.99.4), nitrite reductase (EC 1.7.1.4), nitric oxide reductase (EC 1.7.99.7) and

nitrous oxide reductase (EC 1.7.99.6) produced by denitrifiers play the denitrification process. In general, biological nitrogen removal involves both nitrification under un aerobic condition and denitrification under an anaerobic condition in two separate reactors, following equation 1−4:

Nitrification:

1) $NH_3 + O_2 + 2H^+ + 2e^- \rightarrow NH_2OH + H_2O$

2) $NH_2OH + H_2O \rightarrow NO_2^- + 5H^+ + 4e^-$

3) $2NO_2^- + O_2 \rightarrow 2NO_3^-$

Denitrification:

4) $NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$

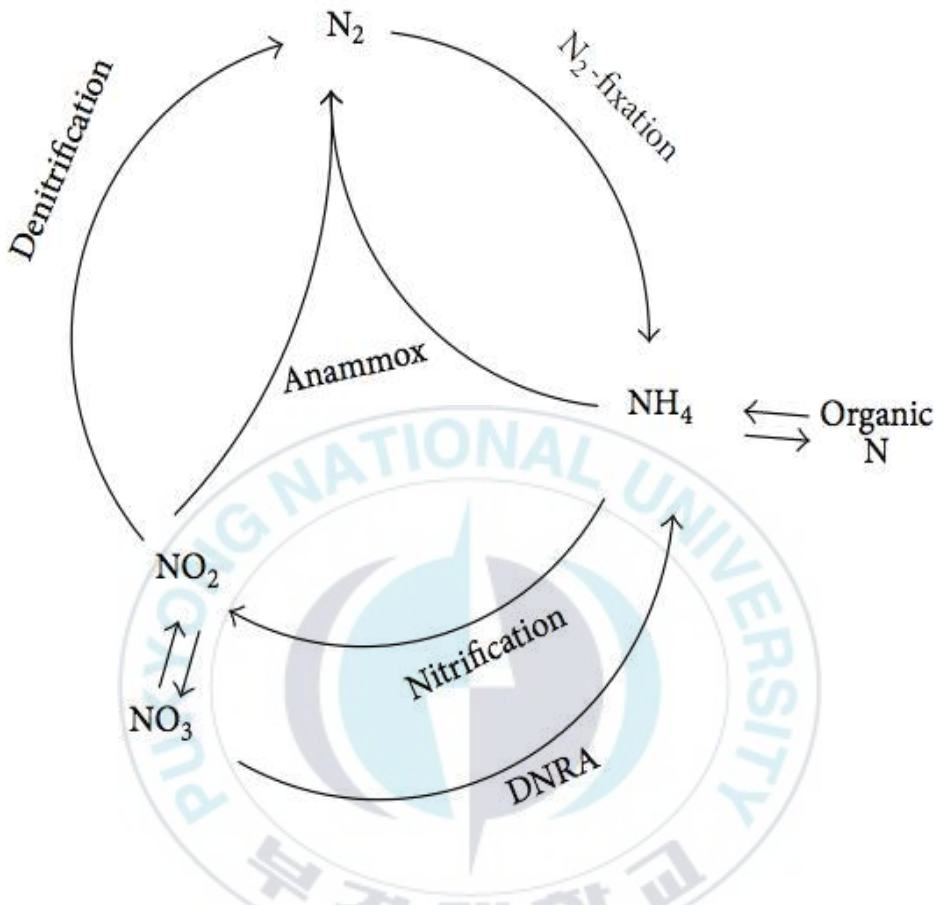

Fig. 3. A biological nitrogen cycle (Ni and Zhang, 2013).

However, the two stage nitrification and denitrification requires extra site area, costs for construction of two bioreactors, and external carbon source during denitrification. For these reasons, development of anaerobic ammonium oxidation/aerobic denitrification and one stage nitrification/denitrification process has been recommended and studied (Zhu et al., 2012).

Recently, lots of large-scale nitrogen removal systems have been studied. Activated sludge and trickling filter systems were performed by Nourmohammadi et al. (2013). Chung et al. (2004) and Ganesh et al. (2015) treated high nitrogen concentration contained tannery wastewater using anoxic/oxic membrane bioreactor and sequencing batch reactor. Wang et al. (2012) represented that characteristics of ammonia oxidizing *Nitromonas* sp. in a pilot-scale wastewater treatment plant, and Yao et al. (2013) reported bacterial consortium for nitrification and denitrification at a low temperature. The C/N ratio and substrates concentration are also important factors in denitrification, influencing the nitrogen removal efficiency.

Fishery wastes

The fishery wastes containing seaweeds have not been efficiently utilized to date and cause great influence on the local environment (Kim et al., 2014). It emits fishy odor and when the floated seaweeds accumulated on the beach, ruining the beach view. Due to increase of disposal cost, reutilization of fishery wastes have been issued. One of conventional method for reutilization of fishery wastewater is production of animal feed or composting (Kim et al., 2010). Recently, liquid fertilizer production for reutilization of fishery waste has been studied and for commercialization of liquid fertilizer in which biodegradation of fishmeal waste was operated in 1 m^3 bioreactor as shown in Fig. 4. (Kim and Lee, 2009; Gwon and Kim, 2012). Fishery wastewater contains biodegradable components such as carbohydrates, proteins and lipids that are able to be converted to useful compounds as bioenergy resources and bioactive substances by useful microbes. However, several non-separately collected fishery wastes have high concentration of salinity, especially wastewater generated from fish-sauce manufacture process (Cho et al., 2014), which causes some difficulty in their biodegradation. For high salinity fishery wastewater treatment, it is necessary to isolate salt-tolerant bacteria such as *Bacillus flexus* (Trivedi et al., 2011); *B. megaterium* (Mishra et al., 2011); *B. licheniformis* (Ghani et al., 2013); *B. ligniniphilus* (Zhu et al.,2014). Kim et al. (2009) and Toyokawa et al. (2010) also reported salt-tolerance *Bacillus* sp. and *B. licheniformis* RKK-04 isolated from fish sauce.

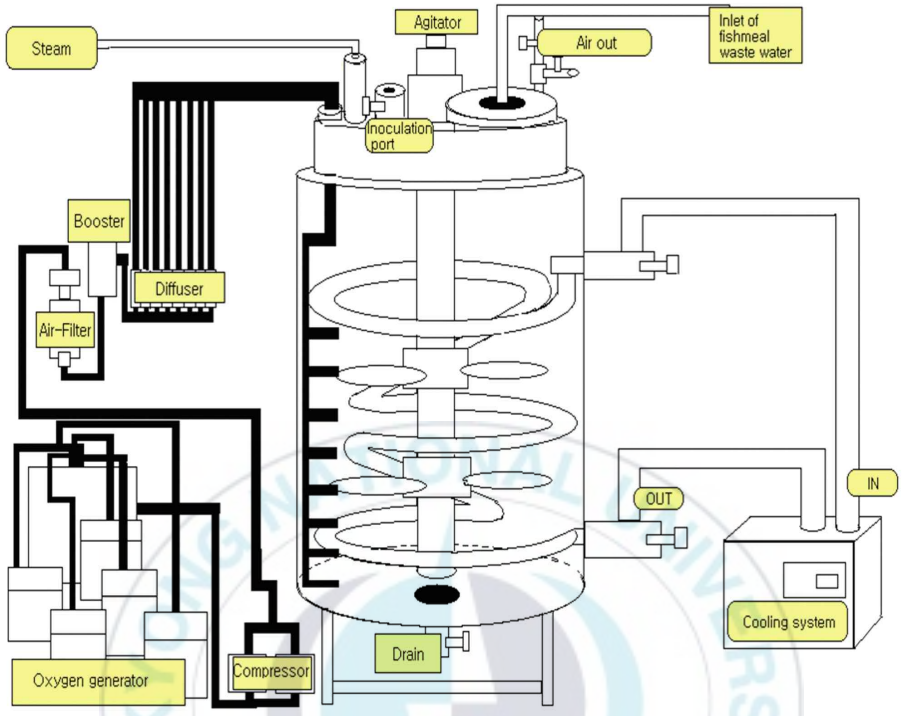

Fig. 4. A schematic diagram of 1 m^3 reactor for the biodegradation of fishmeal wastewater (Gwon and Kim, 2012).

Scale-up

Scale-up process proceeds from the laboratory- to pilot- or full-scale. Scale-up means increasing the dimension, while keeping same design of the system. Most biochemical studies and processes depend on the culture or reaction scale. Experimental conditions are adjusted and modified at the laboratory scale, and applied to pilot scale. Pilot-scale reactor is typically smaller than industrial, full-scale and commercial scale system. It is performed to provide information about the operation of the system and to reduce the risk that involves with the scale-up to large scale. Laboratory or bench-scale data are used for demonstration of pilot scale and commercial scale processes, and it is important to understand main parameters during the whole processes for scale-up from bench scale to pilot and commercial scale (Sitompul et al., 2013). In the wastewater treatment, mass transfer phenomena and hydraulic performance of the system are important and considered for operation of large scale system (Vassils, 2010).

In operating large-scale reactor or system, the results of reaction can be vary according to the operation type (mode) and methods. Batch type is a closed system, while continuous type is an open system. As shown in Table 1, batch and continuous system have advantages and disadvantages. As an alternative way to solve their disadvantages, semi-batch (fed-batch) is sometimes used for operation of bioreactor or treatment plant (Nielsen and Villadsen, 1994).

Table 1. Advantages and disadvantages in the different modes of operations (Nielsen and Villadsen, 1994)

Mode of operation	Advantages	Disadvantages
Batch	Versatile: can be used for different reactions every day. Safe: can be properly sterilized. Little lisk of infection or strain mutation. Complete conversion of substrate is possible.	High labor cost: skilled labor is required. Much idle time: Sterilization, growth of inoculum, cleaning after fermentation. Safety problems: when filling, emptying and cleaning
Continuous	Works all the time; low labor cost, good utilization of reactor. Often efficient: due to the autocatalytic nature of microbial reactions the productivity can be high. Automation many be very appearing Constant product quality	Often disappointing: promised continuous production for months fails due to infection or mutation of microorganisms. Very inflexible: can be rarely be used for other productions without substantial retrofitting. Downstream: all the downstream process equipment must be designed for low volumetric rate, continuous operation
Semi-batch	Combines the advantages of batch and continuous operation.	Disadvantages of both bath and continuous operation

Objectives

As mentioned before, it is necessary to apply eco-friendly and economical wastewater treatment processes for improvement of water quality and reutilization of the wastes. Therefore, in this study, isolation and characterization of useful microorganisms for treatment of ⅰ) chromaticity in dyeing industry wastewater, ⅱ) nitrogen in leather industry wastewater and ⅲ) polymeric component in high salinity fishery wastewater and the characteristics of lab-scale biodegradation were applied to upscaled processes performed for efficient biological treatment.

In chapter 1, for the removal of chromaticity in non-degradable dyeing industry wastewater, fungi and photosynthetic bacteria were used and the characterization of biodegradation was performed first in tube- and flask-scale. Then, the results of biodegradation were applied the microorganisms to 200 m^3/d pilot-scale wastewater plant in a continuous mode of operation for 70 days. During the treatment, chromaticity, COD and suspended solid (SS) were monitored as index parameters for degradation parameters.

In chapter 2, for the removal of high concentration of nitrogen in leather industry wastewater, a new strains were isolated. With the isolates, nitrogen removal efficiency was first evaluated in a 1 L five-neck flask. Then, the results of the biodegradation were

applied to 9000 m^3/d leather wastewater treatment plant. To improve nitrogen removal efficiency, additional lactose as a C-source was additionally added into the reactor. The wastewater plant was performed as continuous type operation, and during the treatment, change of nitrogen compound level (NH_4^+, NO_2^-, NO_3^- and TN), COD_{Cr}, BOD_5 and SS were monitored as index parameters for wastewater treatment.

In Chapter 3, NaCl-tolerant and multiple-enzyme possessing bacterium newly isolated and was used for treatment of high-salinity fishery waste. Firstly, Characterization of biodegradable activities using simulated fishery wastes were performed in a lab-scale operation. Then, the results of the biodegradation were applied to 150 L pilot scale bioreactor operating in a batch type. During the operation, cell growth, enzymes activities, pH, DO, COD, TN and ORP were monitored as index parameters for wastewater treatment.

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Chapter 1. Characterization of dye-degrading microorganisms used for treatment of dyeing industry wastewater (DIW)

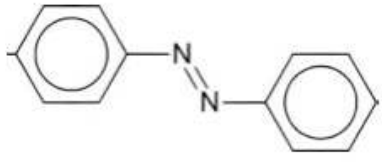
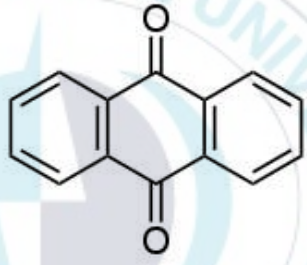
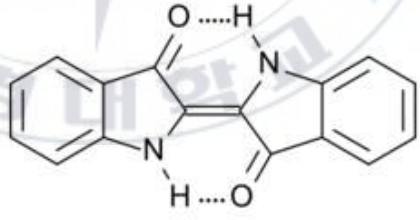
Abstract

To treat chromaticity contained in effluent of dyeing industry wastewater (DIW) efficiently, potent dye-degrading enzyme synthesizing microorganisms, *Ascomycetes* and *Basidiomycetes* were isolated from influent and recycled water of DIW treatment plant. When fungi were inoculated into the ME medium containing basal mixed-dyes, 80% of color was removed after 8 days of incubation. To test the synergetic effect between fungi and photosynthetic bacteria for more efficient DIW treatment, dye degradation experiment was carried out for 12 days. The 1:1 mixed-culture of composed two strains of fungus and photosynthetic bacterium exhibited 88% color removal in the dye mixture. The microorganisms showed their better ability when cultured in the ME medium than the BSM medium. This indicated that ME medium provided better nutrients than the BSM media for synthesis of dye-degrading enzymes. The results obtained from the lab-scale experiment were applied to a 200 m^3/d pilot-scale DIW treatment process. During 70 days operation, the chromaticity was well removed, and it reached approximately 230, which met the required standard. However, the action of microorganism community did not affect both the COD and SS removals.

1. Introduction

In dyeing processing industry including textile, paper, plastic and cosmetic, dye containing organic chromaticity and fluorescent bleaching agent are commonly used (Anliker, 1977; Crini, 2005), and during the processing 10-15% of unfixed dyes are wasted (Sponza and Isik, 2005). Dyeing industry wastewater (DIW) is characterized with high temperature, high alkalinity, extreme pH, contents of detergent and pigment concentrate compounds. These characteristics can block penetration of sunlight, inhibit the carbon anabolism, and deteriorate water system. Dyes are highly water soluble so that human eyes are able to detect reactive dyes above at the concentration of 0.005 mg/L in clear river water the level of which are not removed by conventional wastewater treatment system (Willmott et al., 1998). Table 1 showed these representative reactive dyes. Azo dye contains nitrogen in the azo group as part of their molecular structures, anthraquinone dye is an organic dye that has while anthraquinone structure and contains sulfonic acid group. Indigoid (indigo) dye is an organic compound, which is blue-colored.

Table 1. Chemical structures of three representative reactive dyes

Dye	Structure
Azo	
Anthraquinone	
Indigoid	

To treat DIW, physicochemical processes such as coagulation-precipitation and penton-oxidation, and biological process such as activated sludge process were commonly used (Banat et al., 1996). However, these processes are highly expensive, have low-efficiency and can generate some secondary pollutants. For these reasons, microbiological and enzymatic treatment methods are considered as a better option. So far conducted studies on microbiological treatment methods included the treatment of azo-dye by *Bacillus cereus* KWLC1 and KWLC2 (Kim et al., 2005), treatment of disperse Blue 79 and acid Orange 10 by *Bacillus fusiformis* KMK5 (Kolekar et al., 2008), the treatment of nitroaniline compound in dyeing wastewater by microorganism consortium (Khalid et al., 2009), and removal of Reactive Blue 21 and a sort of copper-phthalocyanine dye by *Aeromonas hydrophilis* (Fu et al., 2002). Enzymatic treatment methods also have been studied using laccase (Mukhopadhyay et al., 2012), lignin peroxidase (Parshetti et al., 2012) and alcohol oxidase (Phugare et al., 2011) for DIW treatment.

Recently, optimization of operation parameters in hybrid anaerobic baffled microbial reactor and two-phase UASB (Upflow Anaerobic Sludge Blanket) type pilot reactor has been conducted to remove COD and chromaticity (Chen et al., 2011; Senthilkumar et al., 2011). Similarly, large-scale wastewater treatment using PEMT fluidized carrier and biofilm and change of microbial community in

full-scale dyeing wastewater treatment system were studied (Park et al, 2011; Yang et al., 2012).

However, to obtain higher and more stable dyeing-degradation ability, further investigation for organization and fixation of effective microbial system in wastewater treatment system is needed. Also the development of new technologies for eco-friendly and economic chromaticity removal system is necessary. Therefore, in this study, we evaluated optimization and characterization of efficient dyeing wastewater treatment performed by effective microbial community acting in lab scale and 200 m^3/d pilot scale.

2. Materials and methods

2.1. Isolation of useful microorganisms

To obtain dyeing-degrading microorganisms samples were taken from influent, aeration tank, sedimentation tank and recycled water in 'B' dyeing wastewater treatment company in Busan, Korea. 1 mL of each sample was added into a sterile 9 mL 0.1% NaCl solution and spread and streaked them onto agar plates containing 0.01% (v/v) dyes. The agar plates were incubated at 30℃ for 12 days. After then, colonies that generated a clearance zone were isolated. Pure isolates were obtained by repeated streaking onto fresh agar plates.

2.2. Identification of the isolates

The isolated microorganisms were identified on the basis of color and morphology of colony, clamp connection, existence of septa of mycelia and structure and formation of fungi under microscopic examination. The characteristics of fungus were compared them with previously known fungal strain (Mishra, 2005).

2.3. Lab scale experiment

For the biodegradation of reactive dyes, the basal medium (NH_4Cl, 1 g/l; K_2HPO_4, 0.25 g/l; $MgSO_4 \cdot 7H_2O$, 0.1 g/l; $CaCl_2 \cdot 2H_2O$, 0.1 g/l, pH 7.0), the BSM medium (Bacto-peptone, 0.188 g/l; KH_2PO_4, 0.318 g/l; sucrose, 0.563 g/l; NH_4Cl, 0.344 g/l; $MgSO_4$, 0.049 g/l; $FeCl_3$, 0.011 g/l; yeast extract, 1 g/l; mineral solution, 1 ml/l; vitamin solution, 1 ml/l, pH 6.8) and ME medium (malt extract, 20 g/l; mineral solution, 1 ml/l; vitamin solution, 1 ml/l, pH 5.6) were used with 0.01% (v/v) of red, orange, blue, black and mixture dyes, respectively (Table 2). In tube, removal of chromaticity by two fungal strains and synergetic effect on dye degradation test between fungi and photosynthetic bacteria (PSB) were executed. PSB was inoculated into the fungi-cultured basal medium with 1:1 ratio and the culture tubes were incubated for 12 days. To test effect of medium on the synthesis of dye-degrading enzymes, cultivation of microorganisms in the BSM and the ME media was performed in a 250-mL flask.

Table 2. Dyes used in degradation experiments

Type	Dyes
Red (7 species)	Disperse red 73, Disperse Red 60, Comacid Red F-GS, Acid Red 336, Acid red 361, Acid Red 57, and Acid Red 138
Orange (4 species)	(Disperse orange 30, Acid Orange 67, Direct orange 26 and Acid Orange 7
Blue (5 species)	Comasol blue 3G, Acid Blue 62, Acid Blue 185, Disperse Blue 60 and Disperse Blue 354
Black (5 species)	Kemachrome black T, Mordant Black 11, Sinarcio Black BFGR, Disperse Black EX-NS and Acid Black 172

2.4. Pilot scale experiment

The size of the pilot plant was 200 m^3/d, and the influent was by-passed from chemical-reaction tank to pH control tank. The influent was equalized in influent equalization tank for 2 hours. After then it passed into aeration tanks. Finally, the treated effluent passed into settling tank. The microorganisms were seeded daily (0.2%, v/v) for adaptation and settlement of them into aeration tank. The treatment of wastewater in pilot scale was carried out for 70 days.

(A) (B)

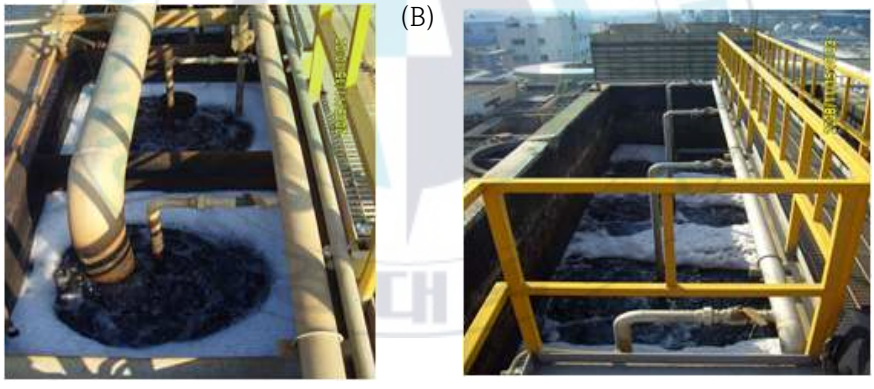


Fig. 1. A 200-m^3/d plant for DIW treatment: Influent tank (A) and aeration tank (B).

2.5. Analyses

The cell growth was observed under microscope, and the grown cells were centrifuged. The supernatant cultural sample was used for measurement of chromaticity. Removal of chromaticity was measured by UV-VIS spectrophotometer (OPRON-3000, Hansol Technology Co., Ltd) at the maximum absorbance wavelength (515 nm for Red; 621 nm for Blue; 490 nm for Orange; 535 nm for Black; 591 nm for mixture dyes). In pilot plant operation, COD, chromaticity, and SS were measured. The decolorization degree was calculated as the following formula:

$$Decolorization\,(\%) = \frac{(I-F)}{I} \times 100$$

where I = absorbance of initial medium solution, and F = absorbance of decolorized medium solution.

3. Results and discussion

3.1. Characterization of the isolates

Among the samples, two species from influent and recycled water produced the clearance zones around their colonies, and named IW and RW, respectively. Microscopic observation revealed that IW strain formed spore in the hyphae, while RW strain formed septa in the mycelia (Fig. 1). From these results, it can be confirmed that IW and RW strains matched up with *Ascomycetes* and *Basidiomycetes* species, respectively (Mishra., 2005). The *Ascomycetes* and *Basidiomycetes* are well-known dye-degrading-enzyme-producing species. It has been known that *Ascomycetes* strain, *Issatchenkia occidentalis* cleaves azo-dyes reductively (Ramalho et al., 2004), and white-rot fungi, *Basidiomycetes* strain that producing lignin-degrading enzymes such as laccase, lignin peroxidase and manganese peroxidase plays roles of transformation and degradation of synthetic azo, heterocyclic, indigo, polymeric and reactive dyes (Balan and Monteiro (2001); Harazono and Nakamura (2005); Gomes et al. (2009).

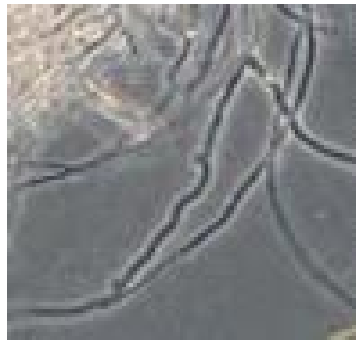
(A)  (B) 

Fig. 2. Micrographs of two fungi under microscope: *Ascomyoetes* (A) and *Basidiomycetes* (B).

3.2. Lab-scale dye biodegradation

For the 8 days experiment using isolated fungi, the degradation of dyes were shown in Fig. 3. Low degradation rate was shown in each dye. Only 20-30% of removal efficiency was found in each dye. In the dye mixture tube, however, degradation rate was higher than those cultivated in other tubes. In contrast, approximately 80% of dye was removed in the mixed-dye medium. Franciscon et al. (2009) reported that *Klebsiella* sp. showed higher removal efficiency of black and mixture dye wastewater than other dyes. In mixed culture of fungi and PSB, the chromaticity removal was higher in each dye used in this study than when used fungi alone (Fig. 4). However in mixed dye, the result using fungi was slightly higher than that using fungi and PSB. The removal efficiency was of 56% on red and 80% on black dye shown in the mixed-culture. However only 33% of removal efficiency in single culture of fungi, similar result of which was also observed in Hong and Otake (2003). It was reported that the PSB possessed azoreductase and decolorized azo dyes under anaerobic condition (Liu et al., 2006). This implys that PSB plays a pivotal role in effective microorganisms as well known as eco-friendly environmental microorganism consortium and promote other strains by providing their synthesized vitamins and nutritions. In the dye-degradation experiments using the BSM and the ME media, the removal was visually observed after 12 days. Especially, more than 95% of chromaticity of black dyes was

removed after 12 days (Fig. 5). It was higher than that of other dyes cultivated in both the BSM and the ME media. This removal efficiency was higher than that of the Reactive black 5 removal by *Phanerochaete chrysosporium* (90%) (Enavatizamir et al., 2011). In addition, the dye-degradation efficiency was higher in the ME medium than the BSM medium. This result indicates that the nutrient in ME media were better than BSM media for the synthesize of dye-degrading enzymes.

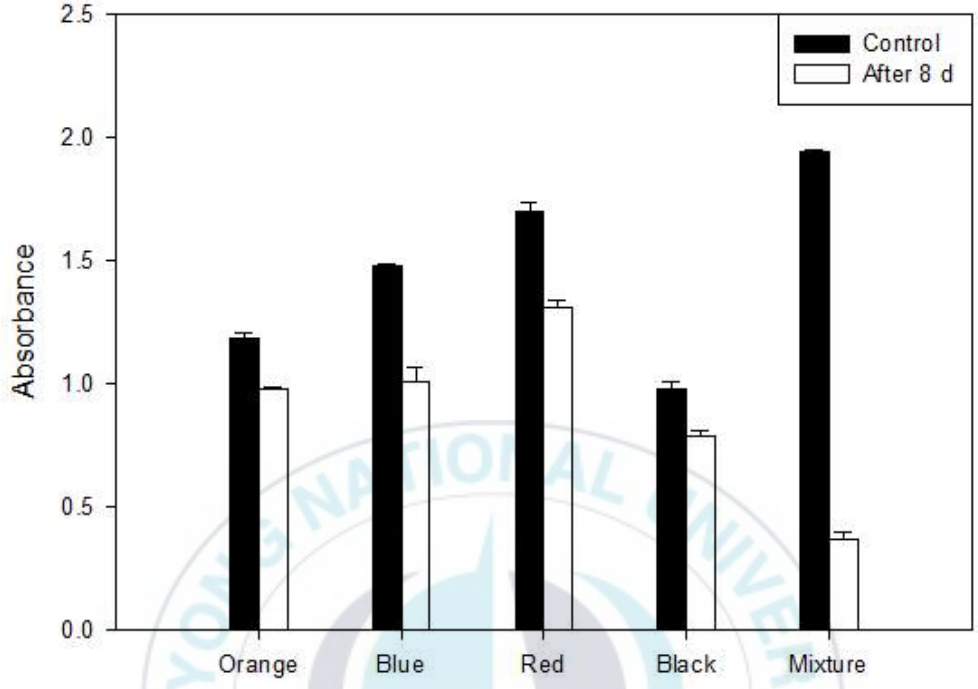


Fig. 3. Absorbances on various colors with 10-fold diluted sample. Error bar: mean ± S.D. of the three replicates.

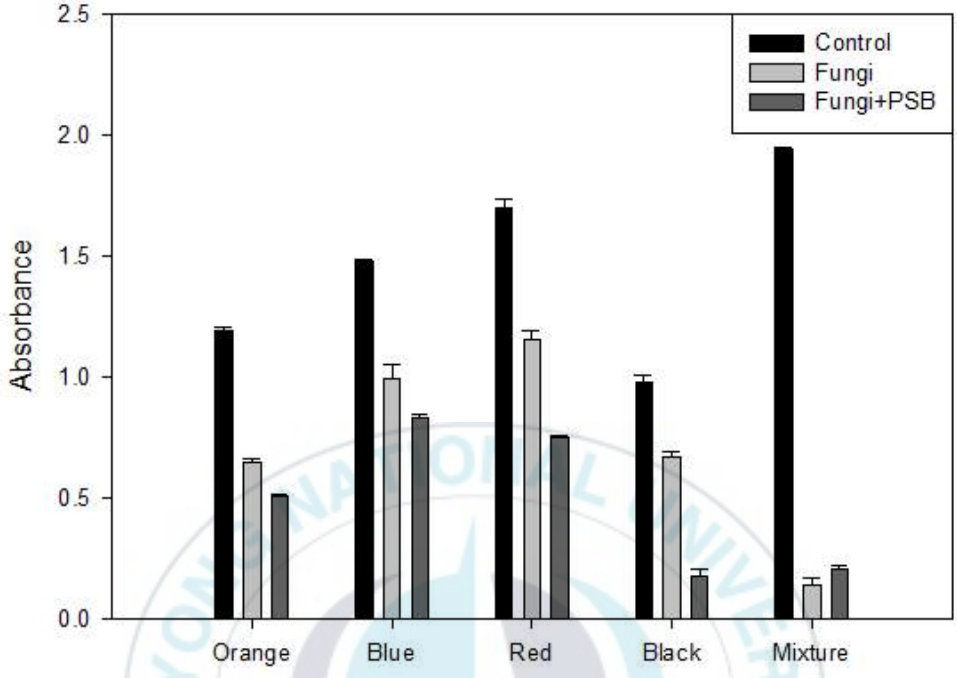


Fig. 4. Absorbances on various colors with 10-fold diluted samples of dye decolorization by fungi and photosynthetic bacteria. Error bar: mean ± S.D. of the three replicates.

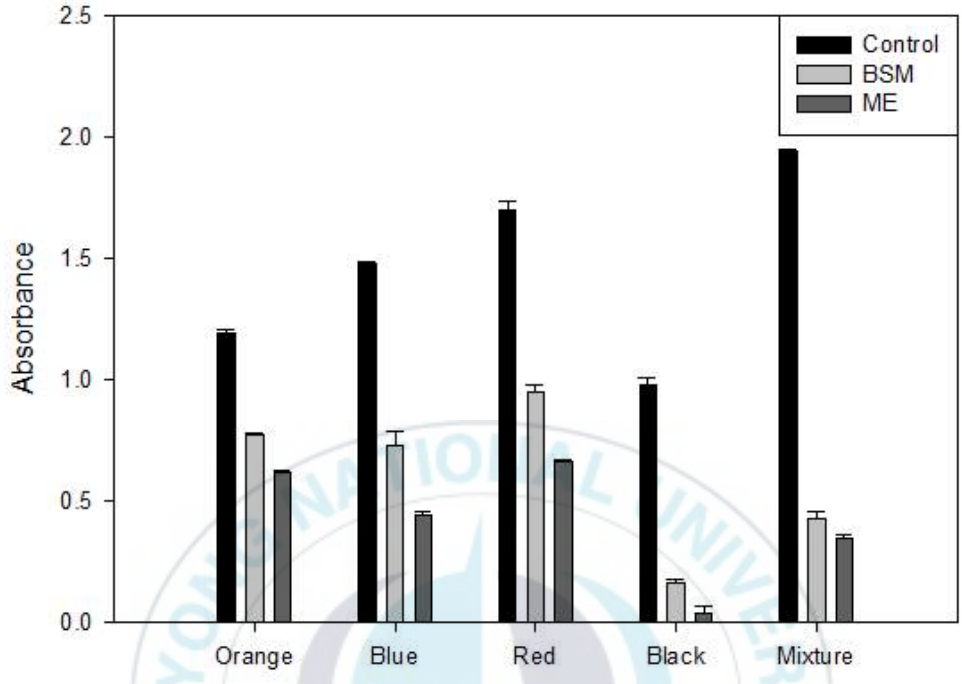


Fig. 5. Absorbances on various colors with 10-fold diluted samples of dye decolorization by fungi in BSM and ME media. Error bar: mean ± S.D. of the three replicates.

3.3. Pilot-scale DIW treatment

In a pilot plant experiment, COD, chromaticity and SS were measured to evaluate the degree of performance for dye degradation. Samples were collected from influent, aeration tank and effluent. In the early 15 days, the chromaticity was not degraded due to the poor adaptation and unstable settlement of microorganism community into the aeration tank. However, after 16 days, he chromaticity was well degraded as the microorganism community was adapted and settled well into the aeration tank. Similarly, the average chromaticity removal was approximately 53% and it reached 230, which met the required standard (400) (Fig. 6a). However, there was no effect on the removal of COD and SS as shown in Fig. 6b and Fig. 6c. It showed the COD and SS values were influenced on the concentration of influent. Although the water temperature of influent was high, the treatment plant operated at low temperature because the experiment was performed in winter (approximately 10-20℃). Therefore, the microorganisms were influenced by the low temperature, resulting in low degradation activity in the pilot-scale wastewater treatment system (Martin Jr et al., 2005). In operation of pilot plant maintenance of constant temperature is important to obtain high removal efficiency of chromaticity (Pearce et al., 2003). As an alternative means Su et al. (2009) reported that immobilization of microorganisms was an effective treatment method in treatment of non-degradable dyeing wastewater.

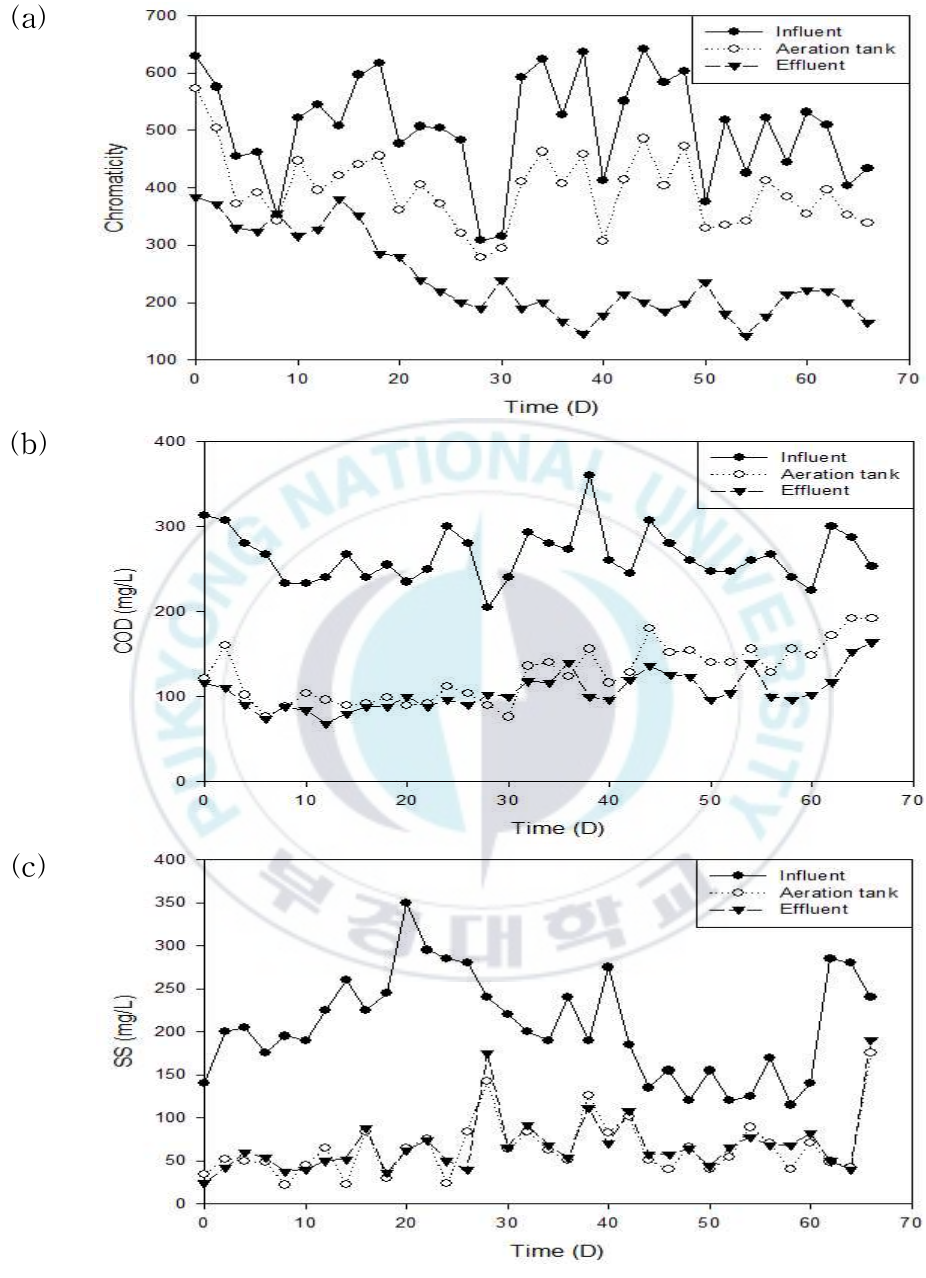


Fig. 6. Change of chromaticity (a), COD (b) and SS (c) in influent, aeration tank and effluent.

4. Conclusion

In this study, the effect of useful microorganisms on efficiency of chromaticity removal was evaluated. The fungi were isolated from influent and recycled water of dyeing wastewater plant. The two isolated fungi were *Ascomycetes* and *Basidomycetes*. The fungi showed good dyeing-removal efficiency: especially 80% of color was removed in a dye mixture medium. The mixed culture of fungi and photosynthetic bacterium with 1:1 mixture ratio exhibited 88% of color removal. The mixed-culture showed better color removal for each single dye. The dye-degradation efficiency were higher in the ME medium than the BSM medium, indicating the nutrient for the synthesis of the related enzymes in the ME media were better than the BSM media. For 70 days operation of the pilot plant, removal of chromaticity was clearly viable and the final chromaticity was approximately 230, which met the required standard. The proper adaptation and stable settlement of microorganism community were necessary for the efficient chromaticity removal. Further, development of immobilized microorganisms would be possible for the more efficient and stable DIW treatment.

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Chapter 2. Characterization of an aerobic denitrifier used for treatment of leather industry wastewater (LIW)

Abstract

To treat leather industry wastewater (LIW) containing high nitrogen concentration, eight aerobic denitrifiers were isolated from sludge existing in an LIW-treating aeration tank. Among them, one strain named as KH8 had showed the great ability in denitrification under an aerobic condition, and it was identified as *Pseudomonas aeruginosa* R12. The aerobic denitrification ability of KH8 strain was almost comparable to the anaerobic denitrification ability. In lab-scale aerobic denitrifications performed in 1-L five-neck flasks for 48 h, nitrogen removal efficiency was found to be much improved as the KH8 strain held a great majority in the seeded cells. From the nitrogen balance at the cell-combining ratio of 10:1 (KH8 isolate to the other seven isolates) within the seeded cells, the percentage of nitrogen lost during the aerobic denitrification process was estimated to be 58.4, which was presumed to be converted to N_2 gas. When these seed cells with lactose we are applied to plant-scale aeration tank for 56 d to treat high-strength N in LIW, the removal efficiencies of COD_{Cr} and TN we are achieved to be 97.0 and 89.8%, respectively. Under this treatment, the final water quality of the effluent leaving the treatment plant was below the water-quality standard concentrations. Consequently, the isolated aerobic denitrifiers could be suitable for the additional requirement of N removal in a limited aeration-tank capacity.

1. Introduction

Nitrogenous substances present in the industrial and domestic wastewater have attracted attention because of the role of nitrogen in eutrophication of receiving waters. Biological nitrification-denitrification is known to be one of the most economical processes for nitrogen removal (Gupta and Gupta, 2001). During this process, aerobic nitrification and anaerobic denitrification are generally consisted of because the nitrogenous substances in wastewater are mostly in the form of ammonium ion. It has commonly been accepted that these two biochemical processes are separately conducted for the sake of their different requirements for the reaction parameters, such as dissolve oxygen, substrate sources and retention time (Third et al., 2005). Nowadays, however, a host of reports dealing with a potential way to combine them into an integrated one has been reported for savings in oxygen for nitrification and carbon requirements for denitrification: Ammonium oxidation (Schmidt et al., 2003); aerobic denitrification (Robertson and Kuenen, 1983; Su et al., 2001); nitrification-denitrification (Astrid et al., 1995); and nitrite nitrification (Eum and Choi, 2002). The utilization of these bacteria is apparently more cost-effective and manageable than the conventional process.

In the nitrogen removal process, various microorganisms

participate, and the nitrification process has been considered to be carried out mainly by ammonia- and nitrite-oxidizing bacteria that are obligately aerobic and chemoautotrophic. Nevertheless, a number of heterotrophic microorganisms have been reported to nitrify many types of nitrogen compounds (Focht and Verstraete, 1977). Thus, understanding the characteristics of bacteria used in the biological nitrification-denitrification process is very important in order to remain high efficiency of treatment at all times, and the possible microbial nitrogen conversions should be provided. Currently, aerobic denitrification becomes the center of public interest due to its potential application (Shi et al., 2013). Therefore, the isolation and characterization of aerobically denitrifying bacteria have been actively dealt, and the representatively reported bacteria were: *Acinetobacter calcoaceticus* (Zhao et al., 2010), *Pseudomonas stutzei* (Wan et al., 2011; Zheng et al., 2014), *Bacillus subtilis* (Yang et al., 2011), *Agrobacterium* (Chen and Ni, 2012), *Marinbacter* (Zheng et al., 2012), *Pseudomonas mendocina* (Zu et al., 2012), *Paracoccus versutus* (Shi et al., 2013), *Acinetobacter* sp., (Yao et al., 2013) and *Klebsiella pneumoniae* (Pahdi et al., 2013). It has been known that these bacteria can not only reduce nitrate under the aerobic condition but also convert ammonium to nitrogen gas via hydroxylamine, nitrite nitrate and nitrous oxide in the order which they occur. With the advancement of research in the characteristics of these bacteria, the heterotrophic

nitrification and aerobic denitrification processes have been clarified to some extent (Zhu et al., 2012). As a result, it is more feasible that the biological nitrogen removal could be conducted in one aerobic reactor (Shi et al., 2013).

For feasibility of commercialization, the aerobic denitrifiers have to be indispensably applied to the wastewater treatment plant and examined for their potential. The LIW is characterized to contain complex pollutants with low biodegradability, and its effluent has to meet the permissible discharge standard for total nitrogen (Chung et al., 2013). During the biological nitrogen removal process, the imbalance between carbon and nitrogen concentration in the influent is generally occurred. Hence, high nitrogen concentration contained in the LIW was taken into consideration in this study. In addition, biological properties depend on the size of reactor, although the metabolic patterns remain unchanged (Kim et al., 2005). For this reason, candidate aerobic denitrifiers were isolated from the sludge suspended in the aeration tank of LIW treatment plant, the characteristics of their aerobic denitrification in a lab-scale were examined from the nitrogen balance, and their potential ability for N removal in the plant-scale LIW treatment was performed in this study.

2. Materias and methods

2.1. Isolation of aerobic denitrifiers

The sludge used to isolate the denitrifying bacteria was taken from an aeration tank in the LIW treatment plant located in Busan, Korea. The sludge sample was agitated to obtain homogeneous suspension in sterile 0.2% NaCl. One ml of the suspended liquid was pipetted into a 10-ml tube that contained 'PYK medium': 5 g peptone, 3 g yeast extract and 2 g KNO_3 in 1 L tap water (pH 7.0). Another 1 mL of the suspended liquid was also pipetted into a 10-mL tube that contained 'Succinate medium': 4 g succinate, 1 g $(NH_4)_2SO_4$, 0.3 g KH_2PO_4, 0.1 g K_2HPO_4, 0.2 g $MgSO_4{\cdot}7H_2O$ and 2 g KNO_3 in 1 L tap water (pH 7.0). After 3 days incubation at 30℃ and 180 rpm, the liquid culture was spread with a platinum loop onto the same media solidified with 1.5% nutrient agar. The separated colonies formed on the agar plates were picked up serially, and a purified isolate was obtained by repeated streaking onto fresh agar plates. Each isolate was maintained on the agar plate at 4℃, and transferred to a fresh agar plate every 2 weeks until use.

The ability of aerobic denitrification for each isolate was tested by the use of the syringe technique under an aerobic condition (Kim et al, 2008). First, each isolate (5%, v/v) was inoculated into a 50

mL- glass syringe containing a 20 mL sterile PYK medium. At the beginning, 10 mL of pure O_2 was supplied using a Hamilton gastight syringe, after which the syringe reactor was incubated at 30℃ and 180 rpm for 72 h. Oxygen was supplied into the syringe reactor twice more before depleted. To measure the moles of gas produced by denitrification, 20 μl of gas sample was taken periodically using a 50 μl gas-tight syringe and analyzed by gas chromatography (GC). At the same time, a syringe reactor under an anaerobic condition was also tested in parallel. The number of moles of gas produced in the syringe reactor was calculated by the ideal gas law.

2.2. Identification of the isolate

The isolate that produced the largest amount of N_2 gas in the syringe test was primarily identified on the basis of colony morphology, Gram reaction, and microscopic examination.

The specific identification of this isolate was performed using 16S-rDNA sequence analysis. Chromosomal DNA of the isolate was extracted from cells grown in the given medium with AccuPrep® Genomic DNA extraction kit (Bioneer, Korea) as the manufacturer's instructions. PCR amplification of the DNA using the 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') primers was performed with a DICE model TP600 PCR thermal cycler (Takara, Japan) as described by Kim et al. (2008), and the 16S rDNA genes were determined by the Macrogen Company (Seoul, Korea). These sequences were compared with GenBank (National Center for Biotechnology Information, USA) entries using the Advanced BLAST similarity search option (Altschul et al., 1997), which is accessible from the homepage of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). BioEdit Sequence Alignment Editor version 5.0.9 was used to verify the alignment and remove all positions with gaps before calculating distances with DNAdist programme in PHYLIP (version 3.5c).

2.3. Lab-scale experiment

The characteristics of aerobic denitrification by the isolates were investigated in 1-L five-neck flasks. The flask was comprised of five necks, and at each neck, a thermometric sensor, a pH probe, tubing for sampling, oxygen-inlet tubing connected to a membrane filter (0.2-μm pore size), and oxygen-outlet tubing were installed. To avoid contamination from foreign microorganisms, two consecutively connected 1-L flasks containing 10 N NaOH were placed for the discharging gas. This flask set-up was placed in a hot stirring bath system (Eyela, Japan) and maintained at 45±0.2℃. Stirring the medium inside the flask was accomplished with the Variomag Telesystem (H+P Labortechnik AG, Germany) at 500 rpm. Oxygen (1.5 kgf/cm^2) was supplied continuously into the flask from an oxygen tank (80% purity), and 10-fold diluted Antifoam 204 was used when the foam occurred severely.

For the preparation of inoculums, each isolate was separately cultivated in a sterile PYK medium until a late-log growth phase. Then, equal amounts (5%, v/v) harvested from each of eight isolates were combined together and inoculated to the flask containing the PYK medium under an aseptic condition. To examine the effect of one potent isolate on the aerobic denitrification, a flask inoculated with the combined cells at the ratio of 10:1 (one potent isolate to other seven isolates) was cultivated in parallel. During the

experiments, the culture broth was sampled periodically from the flask by a peristaltic pump using Tygon tubing, and the changes of the reaction parameters were measured. From these measurements, the percentage of nitrogen loss to N_2 gas by the isolated aerobic denitrifiers was calculated as the following formula:

$$\frac{Initial\ TN - Final\ TN}{Initial\ TN} \times 100 = Nloss\,\%$$

where the concentration of final TN was the total concentrations of NH_4^+−N, NO_2^-−N, NO_3^-−N, organic N and N in biomass at the final stage of aerobic denitrification. The biomass composition was assumed to be $C_5H_7O_2N$.

2.4. Plant-scale experiment

To investigate the potential of the isolated aerobic denitrifiers in plant-scale treatment, they were applied to a LIW treatment plant (Busan, Korea) in which effluent quality was difficult to meet the N regulatory standard. The process flow diagram of the LIW treatment plant is shown in Fig. 1. The LIW treatment plant was comprised of collection tank (180 m^3), equalization tank (10,472 m^3), four relay tanks (758 m^3), three clarifiers (6,666 m^3), fermentation and synthesis tank (1,769 m^3), eight-stage aeration tank (18,572 m^3), two sludge thickeners (2,773 m^3), two biocontact tanks (1,144 m^3), silica tank (450 m^3), carbon tank (336 m^3), mineral tank (453 m^3) and industrial water tank (593 m^3). The maximal capacity of this plant for LIW treatment was 9,000 m^3 per day. The existing treatment for this LIW had been conducted by the use of microbial consortium (Korean patent: 10-1231977) consisting of *Bacillus*, *Paenibacillus*, *Leuconostoc*, *Kurthia*, *Sphingobacteirum*, etc. The microbial consortium was seeded daily into equalization tank (0.2%, v/v), fermentation and synthesis tank (0.04%, v/v), aeration tank (0.02%, v/v) and two sludge thickeners (each 0.04%, v/v), respectively. This treatment was set as a control experiment in this study.

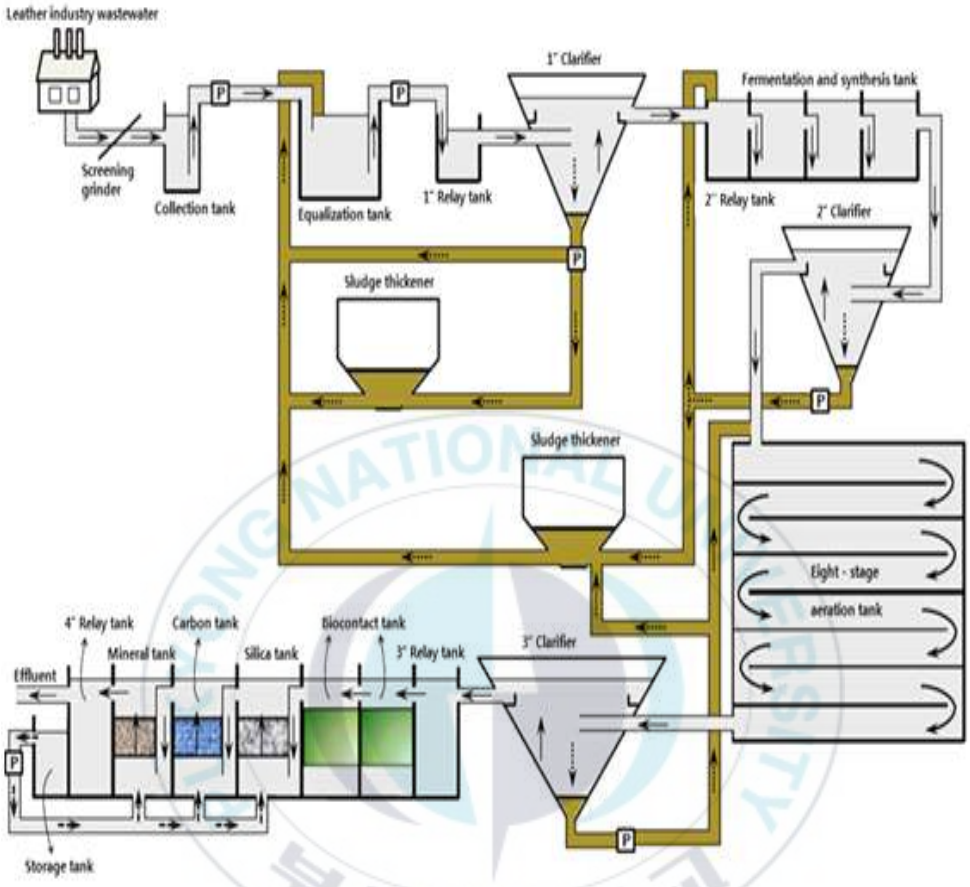

Fig. 1. Process flow diagram of the LIW treatment plant. Each arrow indicates flows of wastewater (⟶), sludge (⋯→) and backwash water (·—→), respectively.

Against the control, the experiments using additional input of isolated strains were performed for better N removal of this LIW to meet the N regulatory standard. For the preparation of seed culture, each isolated strain was actively cultivated at 30℃ and 180 rpm in a 1-L flask containing the PYK medium, and then cells were collected after centrifugation. The cells were combined in a 10:1 ratio of one potent isolate to other seven isolates, based on the result of lab-scale experiment. After 1 day acclimation in the PYK medium at 30℃ and 180 rpm under an aerobic condition, the actively grown cells were transferred to a 150 L reactor under an aseptic condition. With addition of 50-mg lactose/L, the seed culture for this plant-scale experiment was further cultivated in a 3-ton tank for 3 days under an aerobic condition. Finally, the proliferated cells prepared in this way were seeded into the aeration tank in two different ways: 1) The proliferated cells at a concentration of 0.1% (v/v) were seeded at the first day and then the cells of 300 mg/L were seeded daily from the second day to the end (designated as 'treatment 1'), and 2) The cells were seeded as the above strategy and moreover lactose (50 mg/L) as a carbon source was additionally put every 3 days (designated as 'treatment 2'). The aeration tank was divided into eight stages, and the hydraulic residence time was approximately 42.2 h (with maximal flow rate of 440 m^3/h). Samples were periodically taken from the aeration tank, and the changes of the reaction parameters were measured according to standard methods for the water pollution. Experiments were carried out for eight weeks.

2.5. Analyses

The dry-cell weight of cells (DCW) was determined by weighing the cell pellet after being dried in an oven at 100℃ for 12 h. The cell pellet was prepared by centrifuging a 5 mL sample of broth culture at 5000 rpm for 10 min and then by decanting the supernatant after washing twice with distilled water. To measure the nitrogen and carbon dioxide gases produced by aerobic denitrifiers in the syringe experiments, 20 μl samples (injection volume) were taken for GC/TCD (Perkin Elmer Instruments) analysis. The columns used were a 'molecular sieve 13X' and 'carboxen 1000' for nitrogen and carbon dioxide, respectively. In the analyses of both gases, the following conditions were equally applied: the carrier gas was helium at a flow rate of 30 ml/min while the injector and detector temperatures were 100 and 200℃, respectively. However, the oven temperature for nitrogen gas was 40℃, whereas that for carbon dioxide gas was initially 40℃ for 3 min and then increased to 170℃ at a rate of 30℃/min.

In the nitrogen removal experiments, the following parameters were analyzed: 5-day biological oxygen demand (BOD_5), chemical oxygen demand-dichromate (COD_{Cr}), total phosphorus (TP), suspended solids (SS), and total nitrogen (TN), total Kjeldahl nitrogen (TKN), ammonium, nitrite and nitrate. These analyses were carried out according to standard methods for the water pollution

(APHA, AWWA, 1995). The concentration of dissolved oxygen (DO) and the value of oxidation-reduction potential (ORP) were monitored with an YSI DO probe (Model 58) and Isteck ORP probe (Model 730P), respectively.

3. Results and discussion

3.1. Characterization of aerobic denitrifiers

By repeated streaking on agar plates, eight strains were purified. The eight isolates were given the names KH1 to KH8, and their characteristics of colonies and morphology were tabulated in Table 1. Each isolate exhibited its own characteristics of colony and morphology distinctively. Among these strains, the KH8 strain exhibited the most prominent ability of gas production in the syringe experiment. For the efficient treatment of nitrous concentration contained in LIW, N_2 gas as an end metabolite must be produced from the nitrous compounds at a higher conversion rate. Hence, the aerobic denitrification ability of the KH8 strain was further examined against an anaerobic condition. As shown in Table 2, 72.1-μmole N_2 gas was produced anaerobically for 72 h of cultivation with 28.3-μmole CO_2 gas. Under the aerobic condition, N_2 production was not active at initial stage. After then, however, N_2 was steadily produced, and 64.2-μmole was produced after 72 h with a much higher amount of 411.9-μmole CO_2. This indicates that the KH8 strain needed sometime to adapt to anaerobic condition but its denitrification ability under anaerobic condition was almost comparable to the anaerobic denitrification ability. For this reason, the KH8 strain was identified and characterized in later experiments.

Table 1. Characteristics of each isolated strain

Strain	Colony		Cell	
	Color	Diameter (mm)	Shape	Size[a](μm)
KH1	White	1.0−2.0	Short rod	L:1.0, W:0.5
KH2	Ivory	0.1	Short rod	L:1.0, W:0.5
KH3	Ivory	0.3	Rod	L:1.5−2.0, W:0.8−1.0
KH4	Semi−transparent	0.1	Coccus	L:1.0, W:0.5
KH5	Ivory	0.5	Rod	L:1.5−2.0, W:0.5−0.8
KH6	White	0.5	Rod	L:1.5−2.0, W:1.0−1.2
KH7	White	0.5	Coccus	L:1, W:0.5
KH8	Ivory	1.0	Rod	L:3, W:1

[a]L and W indicate the length and the width of each cell, respectively.

Table 2. Comparison of characteristics of denitrification caused by the isolate KH8 between aerobic and anaerobic conditions

Reaction condition	N_2 production after 8 h (μmole)	N_2 production after 72 h (μmole)	CO_2 production after 72 h (μmole)
Anaerobic	37.0±0.8	72.1±1.2	28.3±0.6
Aerobic	10.2±0.4	64.2±0.9	411.9±5.2

The species-specific identification of the isolate was performed using 16S rDNA sequence analysis. The 1,418 bp-sized fragment of the 16S rDNA gene of the isolate was amplified and sequenced. Homology searches revealed that the isolate KH8 was closely related to *Pseudomonas aeruginosa* R12 (DQ073454; 100% similarity) and P. aeruginosa LCD12 (FJ194519; 99% similarity). The phylogenetic tree based on the partial 16S rDNA gene showed the relation of the KH8 strain and other related strains (Fig. 2). From these distinctive features, the isolate KH8 was designated as *P. aeruginosa* R12.

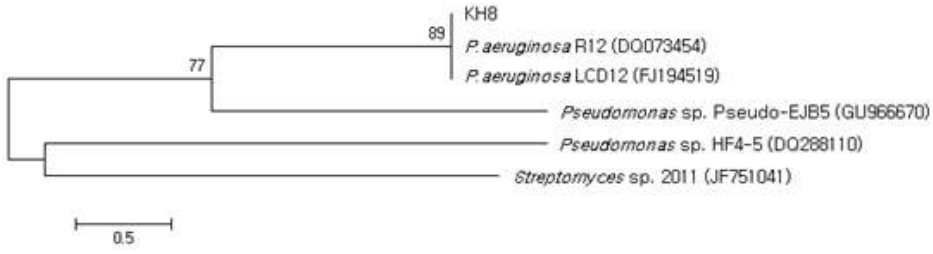

Fig. 2. Phylogenetic tree based on a partial 16S rRNA gene of KH8 strain and other related species.

3.2. Lab-scale aerobic denitrification

The changes of reaction parameters were examined during the aerobic denitrification using different compositions of isolated strains as seed culture. The characteristics of aerobic denitrification starting with equal amounts of isolated strains under an aerobic condition are shown in Fig. 3a. The initial DO was measured to be 5.8 mg/L under the continuous supply of oxygen. As the aerobic denitrification started, DO levels decreased to 0.3 mg/L in 2 h, after this fluctuated, and maintained over 0.9 mg/L after 32 h. This implies that the supplied oxygen did not match the amount of DO consumed by isolates during the active reaction, probably due to the low solubility of oxygen (Nielsen et al., 1994). As DO levels dropped, ORP decreased from 56.7 to - 44.0 mV in 1 h, decreased further to −148.4 mV after 4 h, then increased and maintained at positive values after 10.5 h. This indicates that the decrease in ORP value was related to the decrease in DO. The pH was 7.24 at the beginning, and decreased to 7.09 in 1 h, and then increased to 8.01 after 5 h. After then, the pH decreased steadily to a final value of 6.20. During the experiment carried out for 48 h, the concentration of COD_{Cr} reduced from 10695 to 1632 mg/L, the concentration of TN reduced from 2355 to 1767 mg/L, and the concentration of TKN reduced from 1333 to 588 mg/L, respectively. From these data, the removal efficiencies of COD_{Cr}, TN and TKN were estimated to be 84.7, 25.0 and 55.9%, respectively. The resulting COD_{Cr}/TN ratio

decreased from 8.6 (at the beginning) to 2.0 (at the end). It is known that the C/N ratio may influence the metabolic pathway of organic matter utilization (Ruiz et al., 2006). Accordingly, the decrease in the C/N ratio in a later stage of aerobic denitrification implies that external carbon may be necessary for high efficiency of N removal. Along with these reaction parameters, profiles of NH_4^+−N, NO_2^-−N and NO_3^-−N concentrations are also shown in Fig. 3a. The concentration of NH_4^+−N increased from 73.7 to 354.0 mg/L for first 12 h, and then decreased slowly to the end. On the other hand, the concentration of NO_3^-−N decreased from 270.7 to 104.6 mg/L for first 12 h, and then increased slowly to the end. The concentration of NO_2^-−N as an intermediate metabolite in aerobic denitrification maintained low for first 12 h, then increased to 202.7 mg/L after 24 h, but decreased to almost zero at the end. From the analyses of all parameters, it was found that active aerobic denitrification took place in the first 12 h when equal amounts of eight isolates were used as inoculums.

At the cell−combining ratio of 10:1 (the KH8 isolate to the other seven isolates), the characteristics of the aerobic denitrification are shown in Fig. 3b. From the beginning of the experiment, DO levels maintained below 1 mg/l for the first 5.5 h even under the continuous supply of oxygen. After then, the DO levels increased and maintained over 2.5 mg/L until the end. Due to active denitrification, the ORP values decreased as DO levels dropped. The

ORP decreased to -182 mV after 3 h, then increased steadily, and maintained at positive values after 11 h. This was followed by a slight increase to 113.3 mV measured at 48 h. The initial pH was 7.24, and decreased slightly to 7.20 in 1 h. After then, the pH increased steadily to 8.34 after 24 h. After 34 h, the pH somewhat decreased to a final value of 7.75. In particular, severe foams were generated between 1 and 6 h. As a result, the changes in DO, ORP and pH appeared mostly in the first 12 h. During the reaction performed for 48 h, the concentration of COD_{Cr} reduced from 7381 to 1099 mg/L, the concentration of TN reduced from 3513 to 1464 mg/L, and the concentration of TKN reduced 1471 to 728 mg/L, respectively. From these data, the removal efficiencies of COD_{Cr}, TN and TKN were estimated to be 85.1, 58.3 and 50.5%, respectively. Although there was no significant difference in the COD_{Cr} removal, the TN removal doubled. Thus, this result indicates that the increase in the composition of KH8 isolate in the seeded cells resulted in improvement of N removal efficiency. The initial ratio of COD_{Cr}/TN was 5.5, but it decreased to 3.3 after 12 h. After then, the C/N ratio continued to decrease, and was calculated to be 2.2 at the end. This result indicates that the aerobic denitrification efficiency was influenced by C/N ratio (Kim et al., 2008). Along with these reaction parameters, changes in NH_4^+-N ,NO_2^--N and NO_3^--N concentrations were also examined. The concentration of NH_4^+-N increased from 101.1 to 524.0 mg/L for first 12 h, and then decreased to 144.9 mg/L attheend. On the other hand, the

concentrations of NO_3—N and NO_2—N decreased from 202.3 to 18.0 mg/L and from 24.5 to 0 mg/L for first 12h, respectively. After then those concentrations increased slowly to 82.9 and 477.0 mg/L at the end, respectively. Therefore, the final concentration of NO_3^-—N was almost nine times lower, compared with the result shown in Fig. 3a, indicating that aerobic denitrification was adequately taken place by the dominant KH8 isolate.

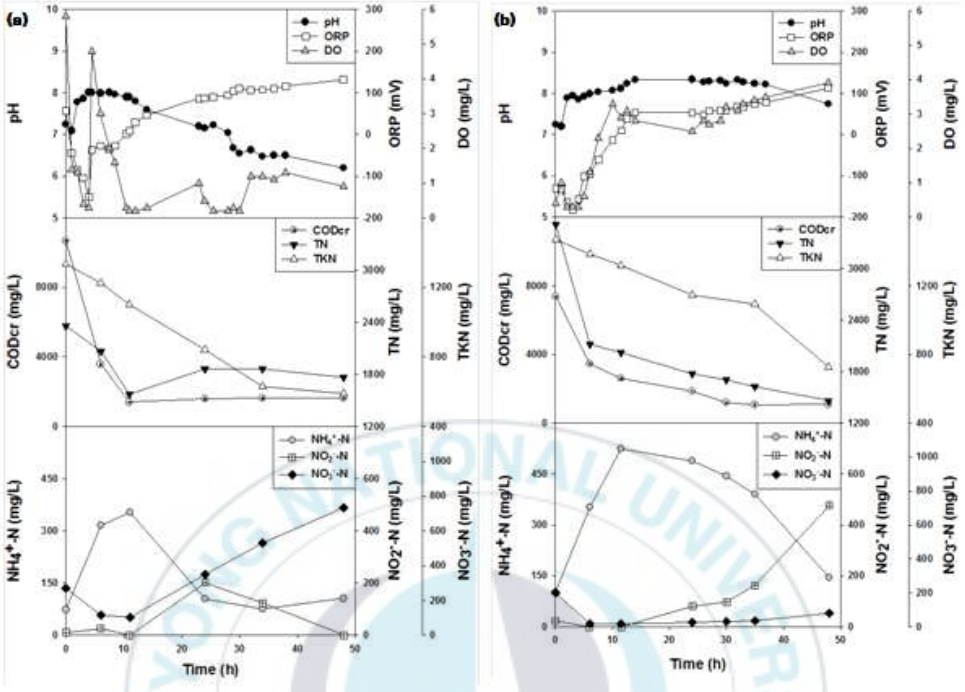

Fig. 3. Changes in reaction parameters during aerobic denitrifications in 1-L 5-neck flasks at different compositions of the seeded cells. The combining ratios of the strain KH8 to other seven isolates in the seeded cells were 1:1 (a) and 10:1 (b), respectively.

To examine the characteristics of denitrifying isolates under an aerobic condition, nitrogen balances for their aerobic denitrifications were investigated at different compositions of seeded cells (Table 3). At the cell-combining ratio of 1:1, N lost was estimated to be approximately 32.8% during the 12-h aerobic denitrification, with cells of 9 mg/L. This result was presumed that 32.8% of the initial N in the culture medium was converted to N_2 gas by the denitrifying activities of isolated strains under anaerobic condition. However, this aerobic denitrification slowed down during the next 36 h. The N lost taking place in the aerobic denitrification was somewhat improved when the composition of KH8 isolate was increased within the seeded cells (at the cell-combining ratio of 10:1). This percentage of N lost was 42.5 during the first 12 h, and it was 58.4 during an entire period of this experiment. From these results, the potential of KH8 isolate was found to be obvious for aerobic denitrification, and the other seven isolates were involved in the nitrification. The similar results were found in the previous studies using potential aerobic denitrifiers (Zhao et al., 2010; Yang et al., 2011; Kim et al., 2005).

Table 3. Nitrogen balances for aerobic denitrifications at different compositions of seeded cells

Cell-combining ratio[a]	Duration (h)	Initial TN[b]	Final				N in Biomass[b]	N lost (%)
			NO_3^--N[b]	NO_2^--N[b]	NH_4^+-N[b]	Org-N[b]		
1:1	12	2350	104.6	0	354.0	1120.28	1.12	32.8
	48		735.4	0	105.9	927.46	1.24	24.7
10:1	12	3510	18.0	0	524.0	1476.51	1.49	42.5
	48		82.9	477.0	144.9	753.96	1.24	58.4

[a]Combining ratio of KH8 isolate to other seven isolates within the seeded cells.

[b]Values are in mg/L.

3.3. Plant-scale LIW treatment

To examine the potential ability of the isolates for better TN removal, plant-scale aerobic denitrification using the isolates was performed in the aeration tank under three different types of treatments (control, treatment 1 and treatment 2). In this experiment, we used lactose which was shown more effective both in cell growth and denitrification than other c-sources such as glucose, fructose and methanol (data is not shown). Fig. 4 shows the concentration profiles of the influent and effluent during the entire course of the experimental study. The LIW flowed into this treatment plant had the following characters: pH of 8.5, DO of 1.0 mg/L, COD_{Cr} of 3,000 mg/L, BOD_5 of 2,500 mg/L, TN of 450 mg/L, NH_4^+-N of 400 mg/L, TP of 50 mg/L, SS of 3,000 mg/L, and undetectable NO_2^--N and NO_3^--N. The LIW passed through equalization tank, fermentation and synthesis tank, and then flowed into the eight-stage aeration tank, as shown in Fig. 1. The influent and effluent water quality of the aeration tank under each treatment was tabulated in Table 4. From the control experiment, the influent and effluent concentrations of DO, BOD_5, TP and SS were found to be 1.0 and 2.4 mg/L (DO), 1248 and 71 mg/L (BOD5), 33 and 20 mg/L(TP), and 5174 and 2976 mg/L (SS), respectively. The increase in SS concentration was due to the return sludge, and this outcoming SS settled down in tertiary clarifier. When the method of treatment 1 (additional input of isolates, as described in the section

of 2.4) or treatment 2 (additional input of isolates and C-source, as described in the section of 2.4) was applied, DO levels increased lightly and the removal efficiency of BOD_5 also increased slightly. However, the removal efficiencies of TP and SS were least different by these treatments. During the experimental period, the variances in pH, COD_{Cr} and TN were obvious among the different types of treatments. The best removal efficiencies of COD_{Cr} (97.0%) and TN (89.8%) were achieved by the treatment 2. This TN removal efficiency was much higher than that (51%) obtained from the control experiment. Along with higher TN reduction, a higher pH value (7.34) revealed in the effluent at day 56 under the treatment 2, because more active denitrification occurred under anaerobic condition. In all the treatments, NO_2^--N was not detected in the effluent at day 56, implying that it converted to N_2 by denitrification. The lowest concentrations of NH_4^+-N (20 mg/L) and NO_3^--N (8.9 mg/L) in the effluent at day 56 were achieved by the treatment 2, in proportion to the TN removal efficiency.

Table 4. Average values of reaction parameters indicating water quality of influent at day 0 and effluent at day 56 in the aeration tank under different types of treatments

Parameter	Control[a]		Treatment 1[b]		Treatment 2[c]	
	Influent	Effluent	Influent	Effluent	Influent	Effluent
pH	7.20	7.03	7.18	7.19	7.26	7.34
DO[d]	1.0	2.4	1.0	2.9	1.0	3.0
COD[d]	1147	83	1122	49.8	1154	35
BOD[d]	1248	71	1212	42.2	1203	31
TN[d]	300	147	303	48	295	30
NH_4^+-N[d]	255	40	251	35	247	20
NO_2^--N[d]	0	0	0	0	0	0
NO_3^--N[d]	53	121.6	49	20.1	49	8.9
TP[d]	33	20	31	21	32	21
SS[d]	5174	2976	5153	2947	5169	2931

[a]Existing treatment by microbial consortium.

[b]Treatment by isolated aerobic denitrifiers in addition to the existing treatment.

[c]Treatment by lactose input in addition to the treatment 1.

[d]Values are in mg/L.

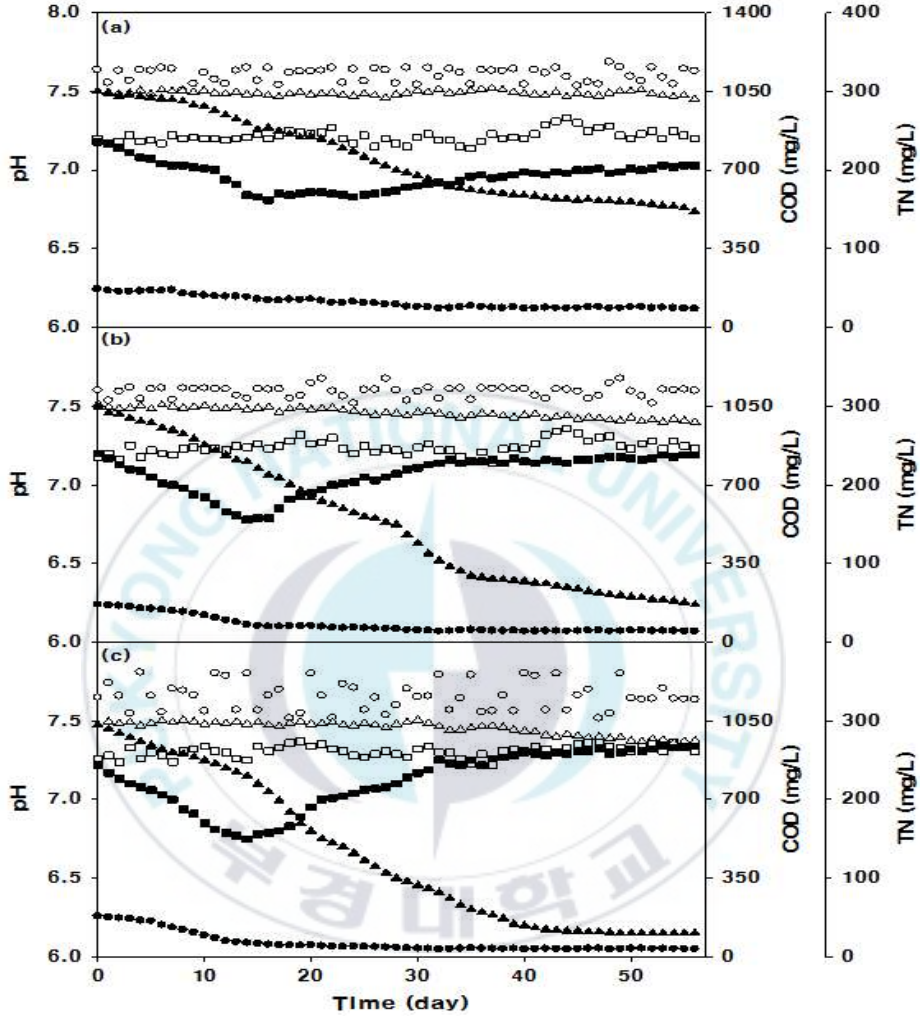

Fig. 4. Changes in pH (■), COD (●) and TN (▲) values of plant-scale operation under different types of treatments. (a) Control, (b) Treatment 1, and (c) Treatment 2. Open and closed symbols represent influent and effluent of aeration tank, respectively.

To understand the characteristics of the isolated aerobic denitrifiers in the aeration tank during the plant-scale operation, the major parameters were monitored. The profiles of pH, COD_{Cr} and TN values under three different types of treatments are shown in Fig. 4, and those of NH_4^+-N, NO_2^--N and NO_3^--N are shown in Fig. 5. As shown in Fig. 4a (in case of control experiment), the pH was 7.20 at the beginning and it ended at 7.03 without significant variance. This result indicates that poor denitrification did not result in pH increase at the end (Pan et al., 2012). The influent concentration of COD_{Cr} fluctuated because it was dependent upon daily LIW content. The influent TN was in a range of 290 - 300 mg/L, and was removed steadily. The removal efficiencies of COD_{Cr} and TN were 92.8 and 51%, respectively. The influent C/N ratios were in a range of 3.7 - 4.0. Hence, the composition of carbons and nitrogens in the influent was not significantly fluctuated. These removal efficiencies were improved when the isolated aerobic denitrifiers were additionally seeded into the aeration tank (in case of treatment 1, as shown in Fig. 4b). Those efficiencies were estimated to be 95.6 and 84.2%, respectively. The intense reductions in COD_{Cr} and TN started after 14 d, with the increase of pH. The influent pH at 7.18 decreased to 6.78 after 14 d due to strong nitrification. After then, the pH increased steadily to 7.19, and was recovered by denitrification. These results indicate that the isolated aerobic denitrifiers needed at least 14 d to be established in the aeration tank. The influence of the isolated aerobic

denitrifiers on denitrification in the aeration tank was more obvious when these cells were seeded with lactose as carbon source (in case of the treatment 2, as shown in Fig. 4c). With addition of a carbon source, the influent C/N ratios (3.9 to 4.2) increased lightly. In this treatment, the pH decreased during the first 14 d, and then it increased by the strong denitrification. The effluent pH revealed 7.34, which was higher by 0.3 than that (7.03) obtained from the control experiment. The similar results could be found in the previous report of heterotrophic nitrification and aerobic denitrification for treatment of high-strength ammonium (Shoda and Ishikawa, 2014). By this improved denitrification, the removal efficiencies of COD_{Cr} (97.0%) and TN (89.8%) increased further.

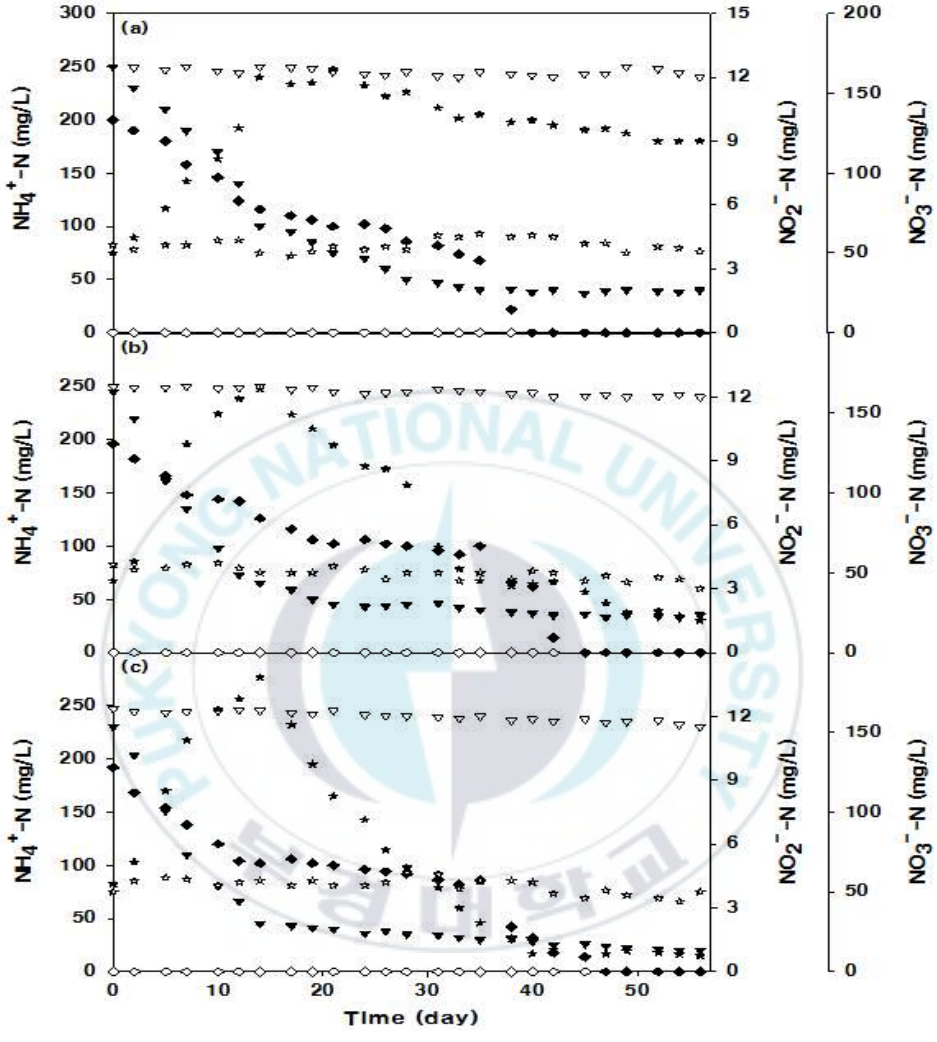

Fig. 5. Change in NH_4^+−N(▼), NO_2^-−N(◆) and NO_3^-−N(★) values of plant−scale operation under different types of treatments. (a) Control, (b) Treatment 1, and (c) Treatment 2. Open and closed symbols represent influent and effluent of aeration tank, respectively.

As shown in Fig. 5a, the concentrations of NH_4^+−N, NO_2^-−N and NO_3^-−N were changed in the control experiment. The concentration of influent NH_4^+−N in a range of 240−255 mg/L converted to NO_2^-−N and NO_3^-−N by nitrification, and final concentration of effluent NH_4^+−N was 40 mg/L. Although the influent did not contain NO_2^-−N, but its concentration in the effluent at day 0 was 10 mg/L due to the effect of nitrification. However, NO_2^-−N was not detected after 40 d. The NO_3^-−N was detected first in the fermentation and synthesis tank. As a result, the initial concentration of influent NO_3^-−N into the aeration tank was 53 mg/L. The initial NO_3^-−N of 50 mg/L in the effluent increased rapidly to 165 mg/L during the first 14d, but it did not decrease adequately after 56 d, implying that active denitrification did not occur to offset the nitrification. This reduction of NO_3^-−N after 14 d was obvious in the result of treatment 1 (Fig. 5b). The effluent NO_3^-−N of 165 mg/L at 14 d decreased steadily to 20.1 mg/L until 56 d, implying that active denitrification occurred in the aeration tank. Along with NO_3^-−N, the concentration of the effluent NH_4^+−N decreased from 251 to 35 mg/L in parallel. Similarly, the effluent NO_2^-−N also decreased, and was not detected after 45 d. This trend was more obvious in the result of the treatment 2 (Fig. 5c). The concentration of effluent NO_3^-−N increased from 49 to 184 mg/L during the first 14 d, and then decreased rapidly to 8.9 mg/L after 56 d. At the same time the effluent NH_4^+−N decreased from 247 to 20 mg/L for 56 d. The effluent NO_2^-−N of 9.6 mg/L at the beginning decreased slightly, and

was not detected after 47 d. Therefore, more active denitrification by the treatment 2 resulted in the best N removal efficiency among the three different types of treatments. Besides, variances in the values of parameters were not significant after 42 h operation. This result implies that the seeded aerobic denitrifiers for the removal of excess N existing in LIW were stabilized in the aeration tank in 6 weeks. It is important that operation under a stabilized system is a key to reliable N treatment (Yubo et al., 2008). Suneethi and Joseph (2011) reported that a large-scale anaerobic ammonium oxidation (ANAMMOX) process was stabilized in 130 d after ANAMMOX bacteria were seeded. Compared with this result, the stabilization of the seeded isolates in this study was completed in a shorter time although the LIW contained refractory organic N compounds to some extent (Lofrano et al., 2013). Under the treatment 2, the final water quality of the effluent leaving the treatment plant was as follows: pH 7.3, DO of 3.0 mg/L, COD_{Cr} of 30 mg/L, BOD_5 of 24 mg/L, TN of 18 mg/L, NH_4^+-N of 12 mg/L, NO_2^--N of 0 mg/L, NO_3^--N of 6 mg/L, TP of 5mg/L, and SS of 15 mg/L, all of which were below the water-quality standard concentrations. As a result, efficient LIW treatment was achieved by additional input of isolated aerobic denitrifiers with a carbon source. Therefore, the isolated aerobic denitrifiers could be an attractive candidate for application to wastewater treatment plants in which the N removal is difficult after nitrification due to poor denitrification or advanced treatment is required for N under the existing aeration-tank capacity.

4. Conclusion

In this study, eight aerobic denitrifiers were newly isolated to treat high-strength N contained in LIW efficiently. When one potent isolate (KH8 strain) held a great majority (at 10:1 of the cell-combing ratio) within the seeded cells, TN removal was much improved and 58.4% of initial N was presumed to be converted to N_2 gas, based on the N balance obtained from the lab-scale aerobic denitrification. When these seed cells with lactose were applied to plant-scale aeration tank for the treatment of high-strength N in LIW, the system could be stabilized after 42 d and approximately 38% higher efficiency of N removal was achieved than that of the existing process. Consequently, the isolated aerobic denitrifiers could be an attractive candidate for application to the efficient removal of high-strength N from wastewater.

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Chapter 3. Characterization of *Bacillus licheniformis* TK3-Y used for treatment of high-salinity fishery wastewater (HFW)

Abstract

For reutilization of high salinity fishery waste (HFW), a strain was isolated from a coastal area in Busan. It was identified as *Bacillus licheniformis* TK3-Y. From plate assay and 500-mL flask experiments, it was revealed that the isolate simultaneously possessed cellulolytic, proteolytic and lipolytic activities with salt tolerance. With 10% (v/v) inoculums, the biodegradation characteristics of the isolate was examined on the carboxymethylcellulose, skim milk and olive oil media, respectively. The optimum conditions of pH, temperature, agitation speed and NaCl concentration on each 1% substrate were equally 6, 50℃, 180 rpm, and 17.5%, respectively. Under the optimum conditions, the isolate showed 1.07 U/mL cellulolytic, 1426 U/mL proteolytic and 6.45 U/mL lipolytic activities. All enzyme activities were stable within a range of 17.5-35%. Therefore, the salt-tolerant ability of the strain TK3-Y was superior to other related strains. In degradation of a mixed medium containing all three substrates, both cellulolytic and proteolytic activities were somewhat lower than those on each single substrate, while lipolytic activity was somewhat higher. In 150 L pilot-scale bioreactor, the isolate degraded a mixture of anchovy and green seaweed wastes, and the enzyme activities were shown 0.45 U/mL of cellulase, 1014 U/mL of protease and 5.8 U/mL of lipase, respectively. From the above results, the *Bacillus licheniformis* TK3-Y strain seemed to be a good candidate for use in the efficient treatment of fishery wastewater whose component is not collected separately.

1. Introduction

The consumption of seafood such as fish and seaweeds has been increased every year in the world. Consequently, large amounts of fishery wastes during industrial seafood process are generated. This trend also has been shown in Korea and large amounts of fishery wastes (2,100 tons/day) generated from lots of seafood processing plants and restaurants have been reported (Kim et al., 2010). Fishery wastes such as by-products of seafood and non-separated waste cause environmental problem. Large quantities of fishery waste including seaweeds, have not been efficiently utilized to date and cause great effect on the local environment (Kim et al., 2014). In Jeju island, green seaweeds waste such as *Ulva pertusa* and *Enteromorpha prolifera* floated over costal area ruin view of beach and give out a bad odor every summer.

The fishery wastes were disposed by landfill, composting, and ocean dumping (Gwon and Kim, 2012), and reutilized for animal feeds. According to the London Convention, the sea dumping of fishery wastes were prohibited since 2014 (International Maritime Organization, 2006), Therefore, biodegradation for reutilization has been newly issued.

There are some advantages of biodegradation: less dangerous and simpler than physicochemical reaction, saving of resources, reutilization of wasted materials and production of various useful materials by

development of useful microorganisms (Kim, 2011). Using purified enzymes over intact bacteria has some advantages: high catalytic activity, the avoidance of undesirable side reactions and bacterial reproduction. However, purification of a specific enzyme is likely to be expensive, and a purified enzyme may be more sensitive to inactivation than an enzyme within an intact cell (Kim et al., 2013). Therefore, it is necessary to use bacterial cell for treatment of wastes or degradation of polymers effectively.

Fishery wastes have lots of biodegradable components such as carbohydrates, especially cellulose in green laver, proteins and lipids, as tabulated in Table 1 (NFRDI, 2009), which are able to be converted to useful compounds like bioenergy resources and bioactive substances by the diverse potential microbes such as cellulolytic strain; *Acremonium strictum* (Goldbeck et al., 2013); alginate- and laminarin-degrading strain; *Microbacterium oxydans* (Kim et al., 2013); proteolytic strain; *Bacillus pseudofirmus* (Raval et al., 2014); and lipolytic strain; *Aneurinibacillus thermoaerophilus* strain HZ (Masomian et al., 2013). Currently, reutilization of fishery waste into liquid fertilizer by mixed microorganisms (Kim et al., 2007; Kim et al., 2010), and treatment of brown and red seaweed wastes by *Microbacterium oxydans* (Kim et al., 2013) and *Bacillus alcalophilus* (Kang and Kim, 2014), respectively have been reported.

Table 1. Chemical composition of fishery products in Korea (NFRDI, 2009)[a]

Fishery product	Water	Carbohydrate	Protein	Lipid
Brown seaweed (*Undaria pinnatifida*)	91.0	4.2[b]	1.1	0.2
Red seaweed (*Porphyra tenera*)	90.6	4.6[c]	3.5	1.7
Green seaweed (*Enteromorpha compressa*)	88.4	5.2[d]	2.0	0.7
Mackerel (*Scomber japonicus*)	68.2	0.0	20.2	10.0
Anchovy (*Engraulis japonicus*)	73.4	0.3	17.7	5.4
Flatfish (*Paralichtys olivaceus*)	75.2	0.3	20.9	2.1
Rockfish (*Sebastes chlegeli*)	79.2	0.1	19.1	1.1

[a]Weight %

[b]Consisting mostly of alginate and laminarin

[c]Consisting mostly of agar and carrageenan

[d]Consisting mostly of cellulose, pectin and sulfate ulvan

Bacillus strains, especially *B. licheniformis* have been verified themselves as multi-functional and multi-enzyme-producing ones that enable to degrade various substrates and to grow under various environmental conditions (Ghani et al., 2013; Parrado et al., 2014). Table 2 showed the multiple-enzyme-producing *B. licheniformis* strains. However, no *B. licheniformis* strain has been reported for simultaneous degradation of polysaccharides, proteins and lipids so far.

When large amounts of fishery are wasted, practically, it is unable to collect them separately. In fish-sauce manufacturing industry, high concentration of salt (20-25%) are added during fish sauce processing (Cho et al., 2014). Due to the high salinity, it is difficult for the microorganisms to survive and degrade the fish sauce waste. Therefore, it is essential to use a multi-enzyme-possessing and high NaCl-tolerant strain.

In this study, we evaluated biodegradation and reutilization of HFW containing green seaweed by a novel multi-enzyme-possessing *Bacillus licheniformis* TK3-Y strain isolated from the coastal area in lab-scale and 150 L pilot scale operations for its industrial uses.

Table 2. Multiple-enzyme producing *B. licheniformis*

Strains	Enzymes					References
	Amylase	Cellulase	Xylanase	Protease	Lipase	
B. licheniformis NH1	+	-	-	+	-	Hmidet et al., 2009
B. licheniformis KJ-9	-	+	-	+	-	Seo et al., 2010
B. licheniformis VSG1	-	-	-	+	+	Sangeetha et al., 2010
B. licheniformis KIBGE-IB3	+	-	-	+	-	Ghani et al., 2013
B. licheniformis JK7	-	+	+	-	-	Seo et al., 2013
B. licheniformis ATCC 21415	-	-	-	+	+	Parrado et al., 2014
B. licheniformis TK3-Y	-	+	-	+	+	This study

2. Materials and methods

2.1. Isolation of useful microorganisms

The potential fishery-wastes-degrading-microorganisms were isolated from marsh located near at coastal area in Busan. One gram of marsh sample was added into a sterile 250 ml flask which contains (per L): 10 g of green seaweed powder, 1 g of NH_4Cl and 35 g of NaCl (pH 7) in 100 ml of distilled water. The flask was incubated at 37℃ and 180 rpm for 3 weeks. After that, 10 ml of each liquid suspension was transferred to fresh media, and they were incubated again under the same conditions. Subsequently, the cells in each flask were spread with a platinum loop onto the solidified with 1.5% nutrient agar plates. A purified isolate was obtained by repeated streaking onto fresh agar plates. The strain was maintained on the agar plate at 4℃, and it was transferred to fresh agar plates every two weeks.

To test for the isolate strain, it was spread on CMC agar (2 g/L of peptone, 5 g/L of yeast extract, 1 g/L of carboxymethylcellulose sodium salt (CMC) and 15 g/L agar, pH 6.8), skim milk agar (10 g/L of skim milk powder, 8 g/L of nutrient broth and 15 g/L agar, pH 6.8) and spirit blue agar (31.25 g/L of spirit blue agar and 10 g/L of tributyrin), respectively. The CMC used in this study was purchased from Sigma Aldrich Company (St.Louis, MO, USA). All

agar plates were incubated at 37℃ for 24 h. After that, each isolated colony formed on the CMC, skim milk and spirit blue agar plates. To test cellulose-degrading ability, 10 mL of Gram's iodine solution was poured onto CMC agar plate (Kanasa et al., 2008) for staining and check a clearance zone around each colony. To test protein- and lipid- degrading abilities, generation of clearance zone and blue colonies was examined on the skim milk and spirit blue agars, respectively.

2.2. Identification of the isolate

A cellulose-, protein- and lipid-degrading potential bacterium was identified on the basis of colony morphology, the Gram reaction of a bacterial smear, catalase test, spore formation by staining with malachite green and microscopic examination. The specific identification of the screened isolate was carried out using 16S rRNA sequence analysis. DNA was extracted using the AccuPrep® Genomic DNA extraction kit (Bioneer, Korea), according to the manufacturer's instructions. PCR amplification of the DNA using the 518F (50-CCAGCAGCCGCGGTAATACG-30) and 800R (50-TACCAGGGTATCTAATCC-30) primers was performed with a DICE model TP600 PCR thermal cycler (Takara, Japan). The 50 mL reaction mixture was obtained from primers (10 pmol/mL), 2.5 mM dNTPs, 10x reaction buffer, 2.5 U Taq polymerase (Takara, Japan), 1 mg DNA template and sterilized water. PCR reactions were performed under the following conditions: initial denaturation at 95℃ for 5 min; 30 cycles of 9 5℃ for 30 s, 55℃ for 30 s, and 72℃ for 30 s; and a final extension step at 72℃ for 10 min. The DNA sequencing was done our request to Macrogen, Ltd (Seoul, Korea). The 50- and 30- ends of the constructs were sequenced using M13 primers that flanked the cloning sites. Then these sequences were compared with GenBank (National Center for Biotechnology Information, Rockville Pike, Bethesda, MD) entries using the Advanced Basic

Local Alignment Search Tool (BLAST) similarity search option, accessible from the homepage of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). The BioEdit Sequence Alignment Editor (version 5.0.9) was used to verify the alignment and to remove all positions using gaps before calculating distances with the DNAdist program in PHYLIP (version 3.5c; distributed by J. Felsenstein, University of Washington, Seattle). Phylogenetic analysis of the given sequences and their close relatives were performed using the neighbor joining method with 1,000 bootstrap replicates using MEGA version 5.2 software (Tamura et al. 2011).

2.3. Lab-scale experiment

To characterize and optimize the culture conditions for degradation of cellulose, protein and lipid by the isolate, flask experiments (with a 125 mL working volume) were carried out. In a shaking incubator, each flask was incubated in parallel at various pHs (5, 6, 7, 8 and 9), temperatures (30, 37, 45, 50 and 55℃), agitation speeds (100, 120, 150, 180 and 200 rpm) and various NaCl concentrations (0, 3.5, 7, 10, 14, 17.5, 20, 25, 30 and 35%). At each culture condition, flasks were incubated in parallel at 180 rpm for 5 d. The best profiles of biodegradation for cellulose, protein and lipid were achieved after the optimal conditions were determined. The isolate was cultivated on the culture medium containing 5 g/L of yeast extract, 2 g/L of peptone, 1 g/L of K_2HPO_4, 1 mL/L of mineral solution, 1 mL/L of vitamin solution and 10 g/L of carboxymethylcellulose sodium salt, 10 g/L of skim milk or 10 mL/L of olive oil for degradation of cellulose, protein or lipid , respectively. To test simultaneous actions of multi-enzymes activities, the experiment was also executed on the mixed culture medium containing all 10 g/L of carboxymethylcellulose sodium salt, 10 g/L of skim milk and 10 mL/L of olive oil. The pHs of the culture media were adjusted to 6.0 before autoclaving. The mineral solution contained: 3 g/L of $FeSO_4 \cdot 7H_2O$, 0.01 g/L of H_3BO_3, 0.01 g/L of $Na_2MoO_4 \cdot 2H_2O$, 0.02 g/L of $MnSO_4 \cdot H_2O$, 0.01 g/L of $CuSO_4 \cdot 5H_2O$, 0.01 g/L of $ZnSO_4$, and 0.5 g/L of ethylenediamine tetraacetic

acid. The vitamin solution contained: 0.2 g/L of nicotinic acid, 0.4 g/L of thiamine-HCl, 0.2 g/L of nicotinamide, and 0.008 g/L of biotin. Except for the vitamin solution, the culture media were sterilized at 121℃ for 15 min. The vitamin solution itself was filtered through a 0.2 mm membrane and separately added to the main culture medium after autoclave. During the experiments, samples were taken every 24 h from the flasks to measure changes of cell concentration (optical density and colony forming unit), viscosity, pH and enzyme activities.

2.4. Pilot-scale experiment

A batch type of HFW treatment was performed in a 150-L fermenter. For biodegradation, 40 L of sterilized anchovy wastewater obtained from "C company" (Goseong, Gyeongsangnam-do) and 1% (w/v) seaweed powder were prepared, 10 g of bacteria (TK3-Y strain) paste (wet weight basis) was seeded into the reactor. The reactor was operated at 50℃, 100 rpm and aeration rate of 2 vvm. During the experiment, sample was taken every 24 h from the reactor, and the changes of cell density (cfu/mL), COD, pH, DO, ORP and enzyme activities were measured.

2.5. Analyses

Cell growth of the isolate was measured using a VIS/UV spectrophotometer at 600 nm or colony forming unit (cfu). Viscosity of the culture medium was measured in a Brookfield (Middleboro, MA, USA) Series LVDV-II + Pro Viscometer with a SC4 chamber and 31/13R spindle, by measuring shear stress within the 0.3-100 rev/min range. Standardized single value was obtained for each sample by inter- or extra-polation on a log-log scale to a standard rate of shear (100 rev/min).

Thin-layer chromatography (TLC), was used to verify the depolymerized products from cellulose by a modified method of Kim et al.(2013). A supernatant of culture broth (1 μL) was spotted on silica gel 60 TLC plates (E. Merck, Darmstdt, Germany) and n-butanol:isopropanol:ethanol:water (2:3:3:2, v/v/v/v) solution was used to develop spots of the cellulose decomposition products. The spots were visualized by spraying with 12-N H_2SO_4 in ethanol and then by baking at 105℃ for 15 min. Standard materials used in this method were glucose, cellobiose, cellotriose, and cellotetraose.

Measurements of cellulolytic, proteolytic and lipolytic activities were done by the methods described by Ghose (1987), Meyers and Ahearn (1977), and Sharma et al. (2012), respectively. Each enzyme activity was measured at 540, 660 and 420 nm, respectively

using a VIS/UV spectrophotometer. One unit of each cellulase, protease or lipase activity was defined as the concentration of enzyme to release 1 μmole of glucose, tyrosine or ρ-nitrophenol per minute per mL under standard conditions, respectively.

3. Results and discussion

3.1. Characterization of the isolate

By repeated streaking on agar plates containing green seaweed powder, we purified a pure strain and named it TK3-Y. From plate assays, it was revealed that the TK3-Y strain produced clearance zones both on carboxymethylcellulose agar and skim milk agar, and it formed blue colony on the spirit blue agar plate (Fig. 1).

Based on microscopic observation, TK3-Y was very motile in the vegetative state and showed Gram-positive rods measuring 0.5-1 μm in width and 3-4 μm in length. It occurred mostly in random groups, and it was catalase-positive and formed endospores. From 16s rDNA sequence analysis, species-specific identification was cleary done for the isolate. The 1662 bp-sized fragment of the 16S rDNA gene of the isolate was amplified and sequenced. Sequence analysis of the 16S rDNA gene and BLAST sequence comparison confirmed that the isolate was *Bacillus* sp. PT 101 (GenBank Accession No.: AB374301.1) with 98% similarity. Furthermore, the phylogenetic tree based on the partial 16S rRNA gene exhibited the relation between the strain TK3-Y and other related strains (Fig. 2). As shown in the phylogenetic tree TK3-Y strain was closely related to other *Bacillus licheniformis* strains.

Fig. 1. Plate assay for the identifications of cellulolytic (A), proteolytic (B) and lipolytic activity (C).

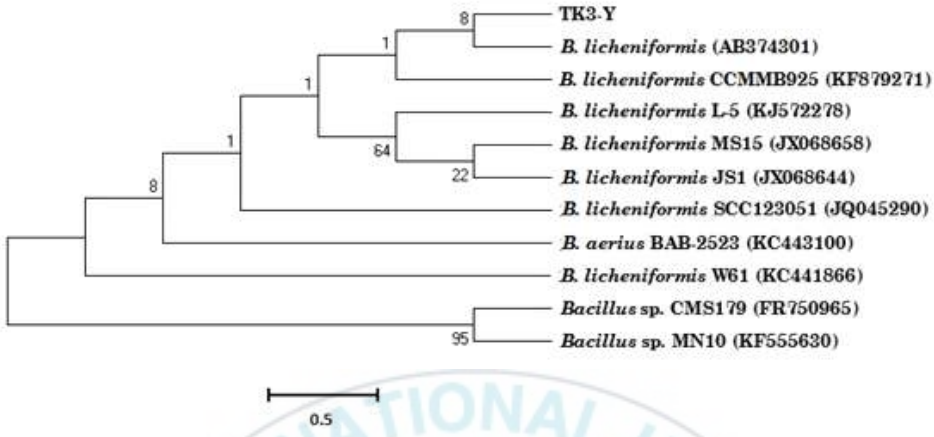

Fig. 2. Phylogenetic tree based on the partial 16S rRNA gene sequence of the strain TK3-Y and other related *Bacillus* species.

As shown in Table 2, many *Bacillus licheniformis* strains have ability to degrade diverse polymers; however, no strain was reported its degradation on all cellulose, protein and lipid simultaneously. From this feature, we had applied for a Korean patent (No.: 10-1453389) for the isolate, as a novel strain exhibiting distinct degradation ability on three polymers after depositing it into the Korean Agricultural Culture Collection (KACC) as KACC 91709P.

3.2. Lab-scale HFW biodegradation

To determine the optimal pH, temperature and agitation speed for cell growth of TK3-Y and its multiple enzymes, experiments were executed in 250 mL flasks. Both the highest cell growth and enzyme activities were obtained all from the TK3-Y cultivation on 1% carboxymethylcellulose, skim milk and olive oil at initial pH 6, 50℃ and 180 rpm (data is not shown). The effect of NaCl concentration on cell growth and the enzyme activities of the strain TK3-Y was also determined. Among various concentrations of NaCl, the highest cell density and the enzyme activities were obtained from the cultivation on 10% NaCl and 17.5% NaCl, respectively (Fig. 3.).

(a)

(b)

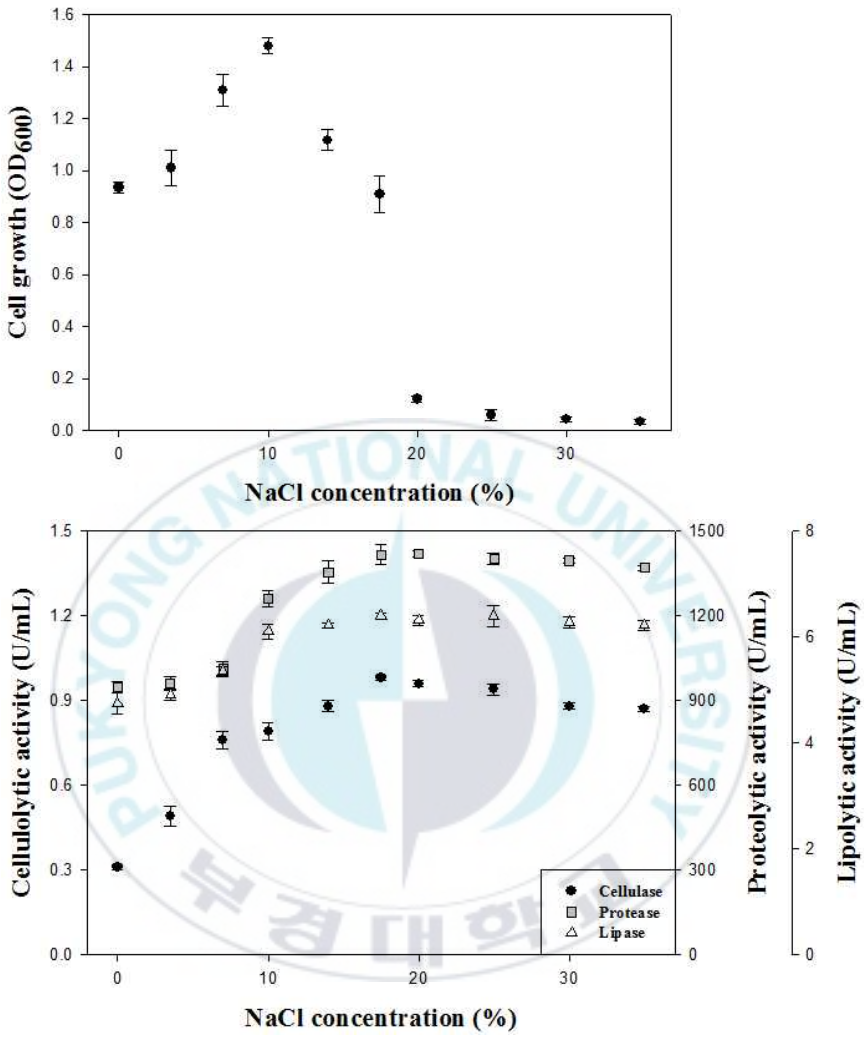

Fig. 3. Cell growth (a) and the enzymes stabilities (b) on CMC, skim milk and olive oil media by TK3-Y cultivated at various NaCl concentrations.

Some NaCl-tolerant *Bacillus* strains such as *B. flexus* (Trivedi et al., 2011); *B. megaterium* (Mishra et al., 2011); *B. licheniformis* (Ghani et al., 2013); and *B. ligniniphilus* (Zhu et al.,2014) have been reported. In addition to these strains, Kim et al. (2009) and Toyokawa et al. (2010) reported *Bacillus* sp. and *B. licheniformis* RKK-04 isolated from fish sauce, respectively. Annamalai et al. (2011) and Annamalai et al. (2014) reported that *B. licheniformis* AU01 cellulase and *Bacillus firmus* CAS7 protease could activate at 30% of NaCl concentration. The strain TK3-Y reported in this study had survival ability even on 17.5% NaCl, the salt concentration of which was the salt higher than those of other strains mentioned above, and all the enzyme synthesized by TK3-Y also had maximum activities at 17.5% NaCl as well. These enzyme activities could be maintained on high concentrations of NaCl (17-35%).

Under optimal conditions, the enzymes activities of the strain TK3-Y were examined during the cultivation in 500-mL flasks using 1% carboxymethylcellulose, skim milk, olive oil and mixture of the three substrate, respectively. In CMC degradation, the optical density of the cells (OD_{600}) increased to 0.926 within the 4 d. The pH increased steadily, and ended up at 7.6 after 5 d. The maximum concentration of reducing sugar (0.98 mg/mL) and cellulolytic activity (1.07 U/mL) were achieved at 4 d. The viscosity of the culture broth decreased as CMC was degraded and approached a

plateau with the CMC degradation degrees (Fig. 4). To get further evidence for the biodegradation ability of the isolate over time in culture, culture supernatant was analyzed by TLC at 0, 1, 2, 3, 4 and 5 days. The degraded products migrated on the TLC plate as CMC was degraded over time in culture. Dimers and monomers were started to be produced after 1 day and they were observed clearly after 4 days (Fig. 5). For degradations of protein and lipid, the growth of cells increased to approximately $2.3x10^8$ cfu/mL and $2.0x10^8$ cfu/mL, respectively. Increasing in pH had same trend in cultivation of CMC, increased steadily during cultivation on skim milk and olive oil, ended up at 7.83 and 7.44 after 5 d, respectively. The maximum proteolytic activity (1426 U/mL) and lipolytic activity (6.45 U/mL) were achieved at 5 d and 4 d, respectively (Fig. 6).

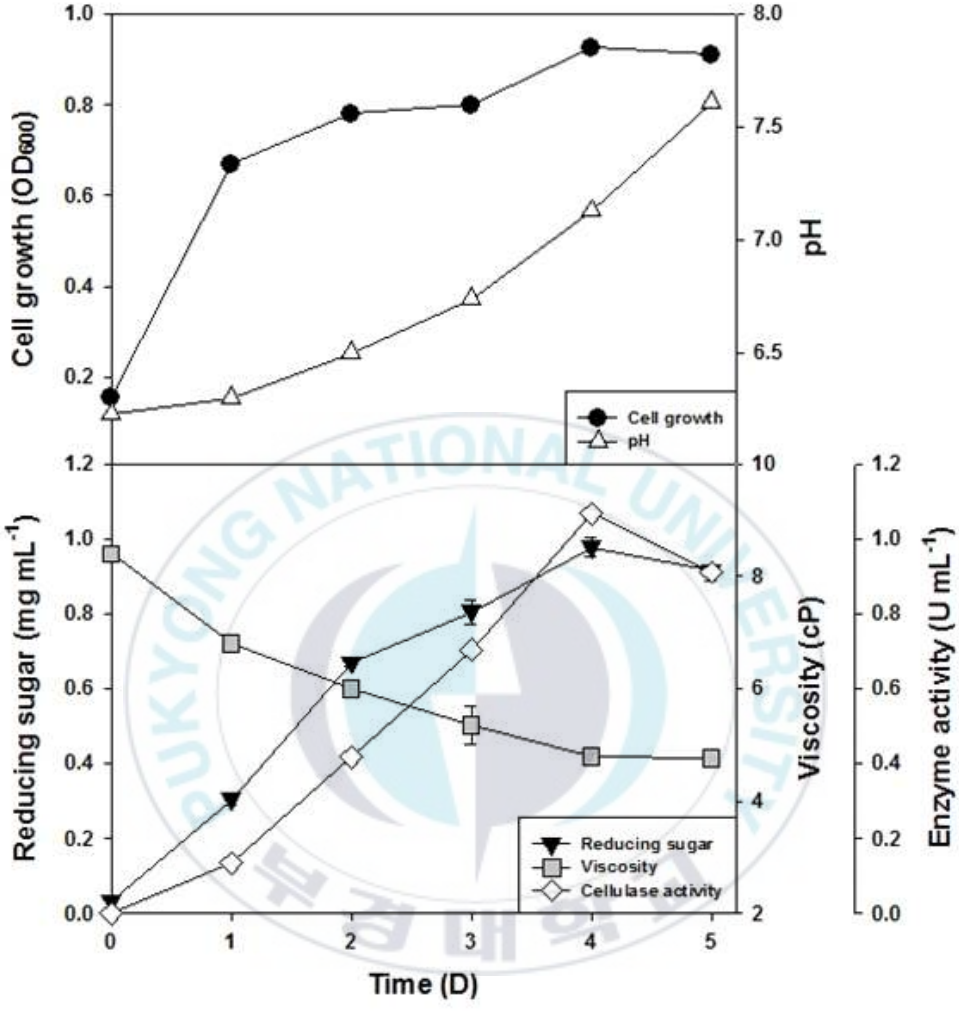

Fig. 4. Profiles of the reaction parameters for CMC degradation by TK3-Y under optimal conditions. Error bar: mean ± S.D. of the three replicates.

Fig. 5. TLC of the CMC degradation products in the CMC culture broth. M1 – M5 indicate standard markers. M1, glucose; M2, cellobiose; M3, cellotriose; M4, cellotetraose; M5, CMC; lane 1, day 0; lane 2, day 1; lane 3, day 2; lane 4, day 3; lane 5, day 4 and lane 6, day 5 of cultivation.

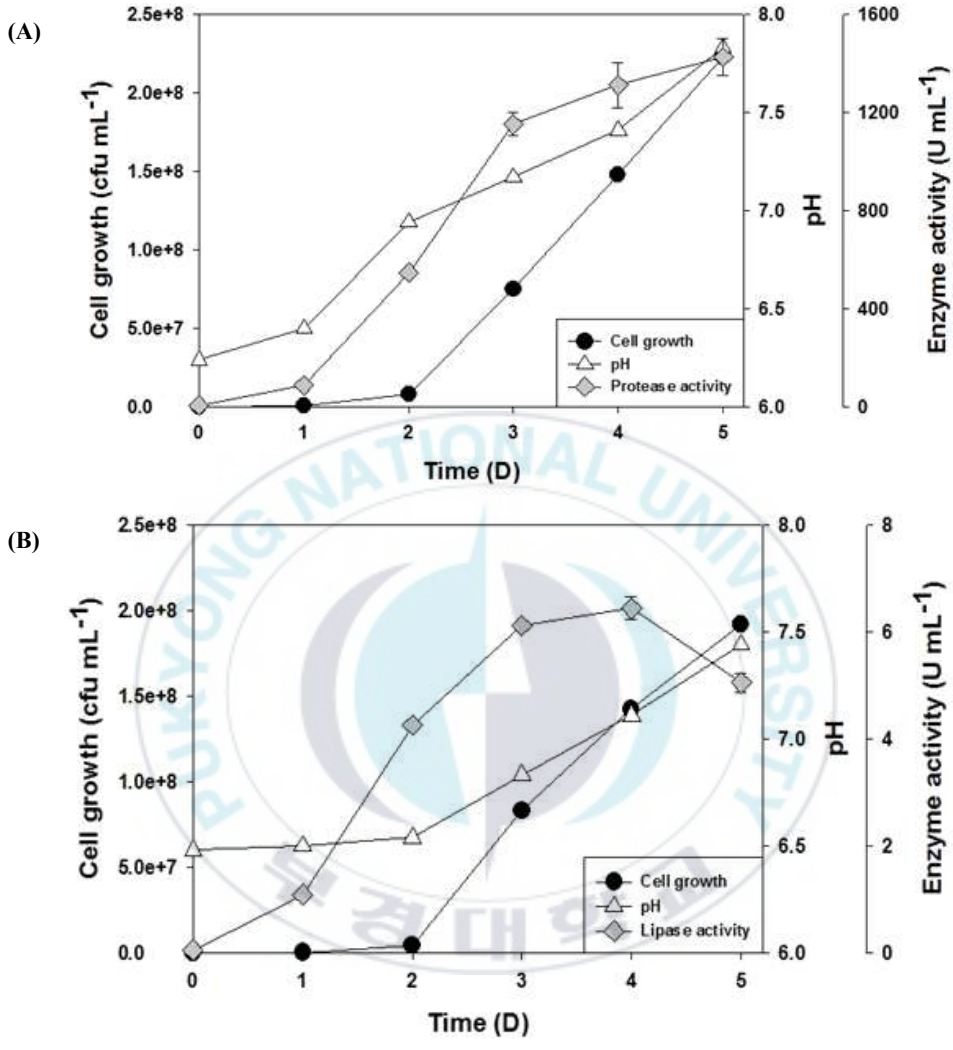

Fig. 6. Profiles of the reaction parameters for biodegradation of protein (skim milk) (A) and lipid (olive oil) (B) by TK3-Y under optimal conditions. Error bar: mean ± S.D. of the three replicates.

For degradation in mixture of three substrates as a simulated fishery waste, the cell growth increased to $6.2x10^7$ and pH increased steadily same as cultivation in CMC, skim milk and olive oil (Fig. 7). The final pH was 7.79 after 5 d and the maximum cellulolytic, proteolytic and lipolytic activities were 0.833, 1394 and 7.12 U/mL at 4 d, respectively. This result indicates biodegradation of cellulose, protein and lipid by the strain TK3-Y occurred simultaneously and consistently. In this experiment, the cellulolytic and proteolytic activity were lower than that obtained from degradation of CMC or skim milk as a sole carbon source. However, lipolytic activity was much higher than that obtained from the degradation of olive oil as a sole carbon source.

During the biodegradation of carboxymethylcellulose, the profiles of reaction parameters showed that the viscosity decreased as cellulose was degraded by the strain TK3-Y and accompanied by a reducing sugar production. Kang and Kim (2014) reported the relationship between viscosity and reducing sugar production from red seaweed waste. In the TLC analysis at different culture times, the migration of degraded oligosaccharides was revealed as cellulose was degrade by the strain TK3-Y over time in culture. Increasing in pH coincided with the cell growth in every experiment, which showed a similar tendency toward the cell cultivation of *B. licheniformis* SVD1 (van Dyk et al., 2009).

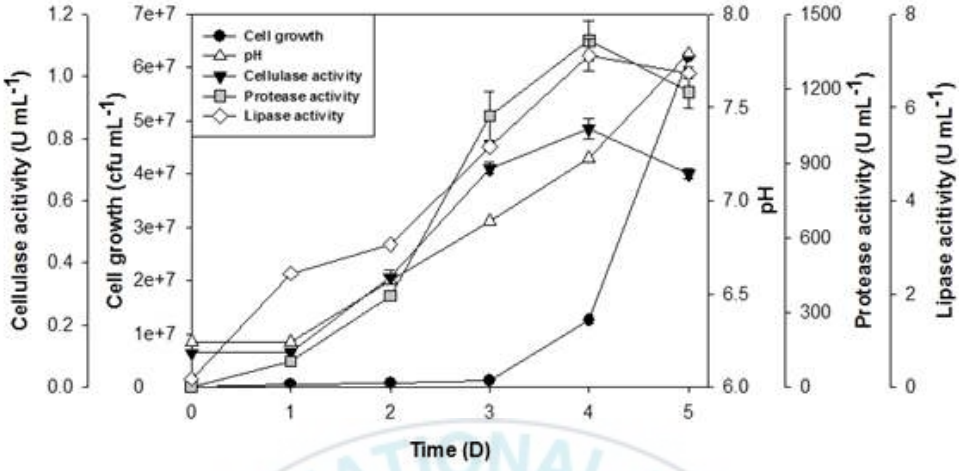

Fig. 7. Profiles of the reaction parameters for mixed-substrate degradation by TK3-Y under optimal conditions. Error bar: mean ± S.D. of the three replicates.

3.3. Pilot-scale HFW treatment

Anchovy wastewater including green seaweed powder was used for the 150-L pilot scale experiment. As shown in Figure 8, the cell growth increased to 1.75×10^8 cfu/mL and the cellulolytic, proteolytic and lipolytic activities were 0.448, 1009 and 19.24 U/mL, respectively. Each enzyme activity was lower than when that on CMC, skim milk or olive oil used for lab-scale experiments. It seemed that the polymers contained in fishery wastewater were less utilized by the strain TK3-Y than those contained in CMC, skim milk and olive oil. At 7 d, the end of the experiment, the pH increased to 8.31, and DO decreased to 4.54. The ORP increased and COD decreased as the HFW degradation proceeded.

(A)

(B)

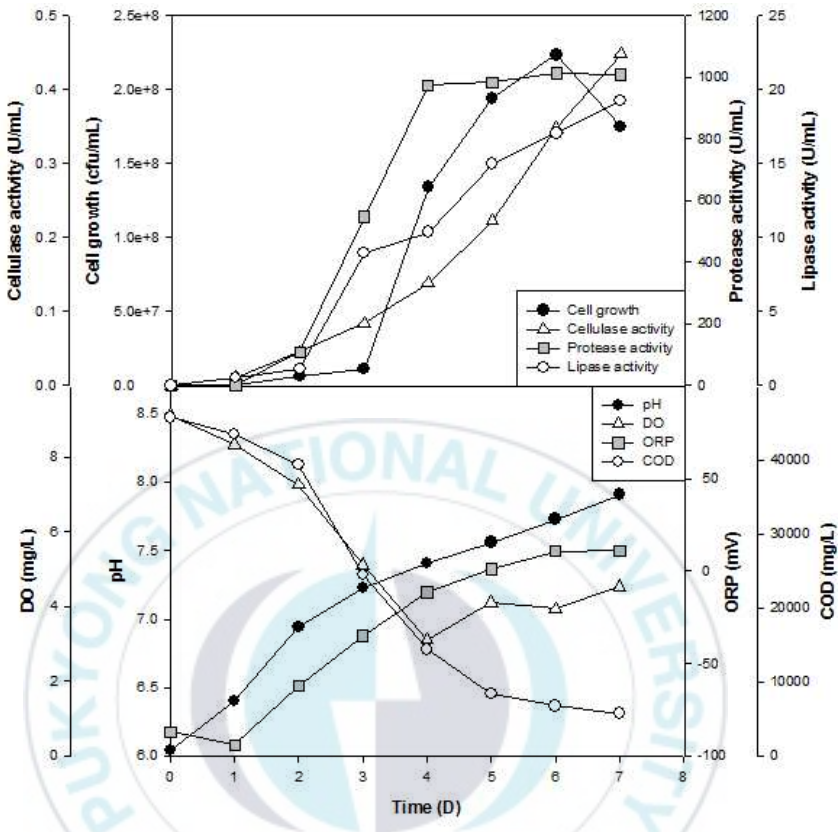

Fig. 8. Profiles of the reaction parameters for fishery wastewater. Cell density and enzyme activities (A) and pH, DO, ORP and COD (B) in degradation by TK3-Y under optimal conditions. Error bar: mean ± S.D. of the three replicates.

Lin et al. (2011) reported that mixed-cell culture was able to utilize substrates mixture containing various chemical composition. Single cell culture has range-limit in utilization of substrate; however, mixed-cell culture has some benefits when there is amicable interaction between cultural condition and microbial community. In other words, optimum microbial or enzyme activities may be different when whole strain have optimum cultural conditions diversely. Against to this demerit, cultivation of multi-enzyme -possessing single strain has some merits: easier control of the culture medium compositions and cultural conditions, and avoidance of domination of specific strain that may spoil the biodegradation.

In Table 3, the activity of enzyme synthesized by the strain TK3-Y is compared with those obtained from other strains. Cellulolytic and lipolytic activities of the strain TK3-Y both on single substrate and mixed substrate were higher than those of other strains. However, the proteolytic activity of the strain TK3-Y was lower than those of other proteases. It was reported that the additional supply of sugar or mannitol did not affect the production of lipase synthesized by *Candida rugosa* (Dalmau et al., 2000). On the contrary, the production of lipase synthesized by *Burkholderia cepacia* could be increased three-fold by addition of glucose to mustard-oil culture medium (Rathi et al., 2001).

Table 3. Comparison of enzyme activities between TK3-Y and others

Enzyme	Strain	Enzyme activity (U/mL)	References
Cellulase	TK3-Y	1.07 (CMC)	**This study**
		0.83 (mixture substrate medium)	
		0.45 (fishery wastewater)	
	Enterobacter cloacae	0.20	Vasan et al., 2011
	Aneurinibacillus thermoaerophilus WBS2	0.43	Acharya and Chaudhary, 2012
	B. licheniformis MVS1	0.54	Acharya and Chaudhary, 2012
	Geobacillus sp.	0.80	Rastogi et al., 2010
Protease	TK3-Y	1426 (skim milk) 1394 (mixture substrate medium) 1014 (fishery wastewater)	**This study**
	Bacillus sp. MPTK 6	1450	Veerabadran et al., 2012
	Bacillus firmus CAS7	2478	Annamalai et al., 2014
Lipase	TK3-Y	6.4 (olive oil) 7.1 (mixture substrate medium) 5.8 (fishery wastewater)	**This study**
	B. licheniformis MTCC-10498	2.0	Sharma et al., 2012
	B. subtilis	5.0	Song et al., 2013

4. Conclusion

In this study, we demonstrated the degradation abilities of cellulose, protein, lipid, and fishery waste, by a newly isolated microorganism, *Bacillus licheniformis* TK3-Y. The strain TK3-Y could synthesize cellulolytic, proteolytic and lipolytic enzymes simultaneously, which resulted in a good biodegradation on the mixed substrates. This result indicates that the strain TK3-Y would be a good candidate to degrade fishery waste the component of which are not often collected separately. Furthermore, the degradation characteristics of the strain TK3-Y could be applicable to the reutilization of high-salinity fishery waste and food wast. Consequently, this biodegradation method can give a good solution to answer to the critical problems regarding the prohibition of ocean dumping and help to preserve the environment.

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CONCLUSION

In this study, isolation and characterization of useful microorganisms were performed and the isolates were applied for wastewater treatment system. The microorganisms were screened and isolated depending on the characteristics of the wastewaters. The scaling-up processes confirmed the possibilities of industrialization of the microorganisms.

In Chapter 1, for removal of chromaticity in the dyeing industry wastewater, two fungi strains (*Ascomycetes* and *Basidiomycetes*) were isolated from recycled water of wastewater treatment plant. The microorganisms were applied from lab scale to 200 m^3/d pilot scale plant. After 70 days of treatment, chromaticity in the effluent met the required standard. And, in Chapter 2, eight strains of bacteria were isolated from sludge in aeration tank, among the isolates KH8 showed the best denitrification ability. The lab scale experiment provided that importance of mixing ratio between KH8 and other isolates and control of C/N ratio. The isolates were applied and lactose was added into aeration tank to evaluate efficient nitrogen removal in real leather industry wastewater. The nitrogen removal was 38% more than existing results. Lastly, in Chapter 3, for biodegradation of high salinity fishery wastewater, *Bacillus licheniformis* TK3-Y strain was isolated from marsh at the coastal area. The isolate was able to survive at 17.5% NaCl

concentration and produce cellulase, protease and lipase. The three enzymes degraded carboxymethyl cellulose, skim milk and olive oil simultaneously in the lab-scale experiments. In the 150-L pilot scale bioreactor operation, anchovy and green-seaweed wastewater was well degraded by the TK3-Y strain.

In the dyeing and leather industry wastewater, the treatment plants were operated as continuous type but in the fishery wastewater treatment, it was operated as batch type culture. The mixed-cell culture of microorganisms were performed in both of DIW and LIW, while the single-cell culture was performed in the HFW.

In this study, the isolation of useful microorganisms, based on the characteristics of wastewater and application of these microorganisms into suitable wastewater plant for the efficient wastewater treatment was investigated. Application of suitable microorganisms and determination of operation type are important factors for biological wastewater treatment. Moreover, the wastewater treatment systems will be more stable and efficient by microorganism immobilization in carrier and augmentation into treatment plant.

Isolation and Characterization of Useful Microorganisms for Efficient Biological Treatment of Wastewaters and Their Application to Upscaled Processes

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Abstract

With the population increase and industrial development, lots of industrial wastewater were generated. They were physically, chemically or biologically treated. Biological treatment of wastewater by microorganisms are less dangerous, and simpler than physicochemical reaction. In this study, we demonstrated characteristics of biological treatment of dyeing, leather and fish industry wastewater by newly isolated microorganisms in lab scale and pilot scale.

Dyeing industry wastewater (DIW) has non-degradable high-colored compounds which was not able to be treated by conventional methods. In this study, 2 fungi (*Ascomyteces* and *Basidiomycetes*) newly isolated from influent and recycled water were used in the treatment of dyeing industry wastewater. Approximately 80% of chromaticity removal was shown in the dye-contained tube and flask scale experiment. In the affinity test between photosynthetic bacteria and fungi, the 1:1 mixed culture of them exhibited 88% of color removal in the dye mixture. Also the microorganisms degraded the dyes when cultured in ME medium better than BSM medium. In the 70 days operation of 200 m^3/d pilot scale DIW treatment plant, the fungi were seeded into the aeration tank in a daily basis (0.2%, v/v). the chromaticity was well removed, it reached approximately 230 and it met the required standard.

Leather industry wastewater (LIW) was containing high nitrogen concentration which was effectively removed by 8 isolated bacteria. Among them, one strain named as KH8 had showed the great ability in denitrification under an aerobic condition, and it was identified as *Pseudomonas aeruginosa* R12. From the nitrogen balance at the cell-combining ratio of 10:1 (KH8 isolate to the other seven isolates) within the seeded cells, the percentage of nitrogen lost during the aerobic denitrification process was estimated to be 58.4, which was presumed to be converted to N_2 gas. When these seed cells with lactose we are applied to plant-scale aeration tank for 56 d to treat high-strength N in LIW, the removal efficiencies of COD_{Cr} and TN we are achieved to be 97.0 and 89.8%, respectively. Under this treatment, the final water quality of the effluent leaving the treatment plant was below the water-quality standard concentrations.

High salinity fishery wastewater (HFW) was treated by NaCl-tolerant bacterium. For reutilization of fisheries waste, a strain was isolated from a coastal area in Busan. It was identified as *Bacillus licheniformis* TK3-Y. From plate assay and 500-mL flask experiments, it was revealed that the isolate simultaneously possessed cellulolytic, proteolytic and lipolytic activities with salt-tolerance (survivability at 17.5% NaCl concentration). And the TK3-Y strain showed 1.07-U/mL cellulolytic, 1426-U/mL proteolytic and 6.45-U/mL lipolytic activities. Each enzyme activity could be stable within a range of 17.5-35%. And in 150-L pilot scale bioreactor, TK3-Y strain degraded the mixture of anchovy and green seaweed wastes and the enzyme activities were shown 0.45-U/mL of cellulase, 1014-U/mL of protease and 5.8-U/mL of lipase.

The results of DIW, LIW and HFW treatments indicated that the importance of using suitable microorganisms according to the characteristics of wastewaters and proved the possibility to apply to biological treatment of large-scale industrial wastewater by microorganisms.

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