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Complete reutilization of fishery processing wastewater by biodegradation

by

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Complete reutilization of fishery processing wastewater by biodegradation (생물학적 분해에 의한 수산 가공 폐액의 완전 재활용)

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by

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A dissertation

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생물학적 분해에 의한 수산 가공 폐액의 완전 재활용

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요약

수산물은 단백질, 탄수화물 등 영양가가 풍부한 식품으로, 전 세계적으로 점차 그 소비량이 증가하여 2017년에는 1억 7238만 톤을 넘어서게 됨으로써, 수산물의 소비는 엄청난 양의 폐기물과 폐액을 생성하게 되었다. 수산물 가공 공장의 공정에 따라 폐기물 및 폐액의 생산량은 차이가 있지만, 어류 및 해조류의 가공 공정 과정에서 각각 약 60%와 10%의 폐기물이 생성된다. 따라서 수산물 가공 폐액의 재활용은 환경 문제를 해결하고 경제적인 이익을 가져올 수 있는 유익한 방법이 될 수 있다. 이 연구의 목적은 수산 가공 폐액을 생물학적으로 분해하여 고부가가치의 분해산물들을 생산함으로써, 재활용을 통한 실용화를 보여주고자 하였다.

먼저 수산폐기물의 발생 및 환경에 미치는 영향, 환경부하를 줄일 수 있는 효과적 처리 방법, 궁극적으로는 처리과정에서 추가적인 폐기물이나 폐액이 발생하지 않는 '무배출 시스템'의 실현 방법에 대해 알아보았고, 유용미생물을 이용한 수산가공폐액이 함유하는 유기물질들의 친환경적 생분해 방법으로 천연 생물활성물질들을 생산할 수 있음을 밝힘으로써, 수산가공폐액의 재활용가치를 높일 수 있었다. 최종적으로는 친환경적 생분해 방법으로 양질의 생물액체비료를 생산한 다음, 이것을 직접 상추 수경재배에 적용시켜 상추 성장이 향상되는 결과를 얻게 됨으로써, 수산가동폐액의 완전한 재활용이 실현 가능함을 보여주었다.

그 후, 우리나라에서 많이 소비되는 생선인 고등어의 폐액을 재활용하기 위해, 혼합 미생물을 사용하여 고등어 폐액의 호기성 생분해를 3l 반응기에서 수행하였다. 처음 24시간 동안 생분해가 활발히 일어나고 최대 균체량이 4.3×10^8 CFU ml^{-1}로 증가하였고 이에 따라, 용존 산소량과 pH 및 산화 환원 전위가 감소했으

며 생선 비린내가 점차 사라졌다. 42시간 배양 상등액은 높은 항산화 활성, 항균 활성 및 DNA 보호 활성, 그리고 유효한 항진균 활성을 나타내었다. 고등어 폐액의 높은 재활용 가치를 증명하였다. 또한, 고등어 폐액의 생분해 후 남은 배양액은 식물 독성 실험 결과 식물 독성이 없었으며, 팥과 보리의 수경 재배를 통해 바이오비료로 사용될 수 있음을 확인하였다. 결과적으로, 친환경 처리를 통한 고부가가치의 분해산물의 생산으로 고등어 폐액의 재활용 가치가 높아짐을 확인할 수 있었다.

고등어 폐액의 바이오비료로서의 재활용 가치를 확인한 후, 혼합 수산 폐액을 생분해하여 친환경적인 방법으로 바이오비료를 얻는 연구를 실시하였다. 고등어와 미역 수산 폐액의 다양한 혼합 비율을 테스트한 결과, 생분해에 가장 적합한 비율은 10 : 1이었다. 최적 혼합 비율에서 72시간의 생분해동안 혼합 미생물은 안정적인 protease, alginate lyase 및 laminarinase 활성을 나타내었고 그 결과 많은 가수분해로 화학적 산소 요구량 및 총질소가 감소했다. 또한, 24시간 배양 상등액은 높은 항산화 활성을 나타내었고, 72시간 배양 상등액의 총 아미노산 함량은 7715.7 μg ml^{-1}이었다. 그리고 72시간 분해산물은 질소, 인, 칼륨 및 중금속의 표준 함량 및 균체량에서 바이오비료로서의 조건을 잘 충족시켰다. 생산된 바이오비료를 사용한 수경재배에서 1개월 동안 성장한 상추는 엽록소와 카로티노이드 함량(각각 8.29 mg g^{-1}, 1.89 mg g^{-1})이 높았으며 높은 항산화 활성(DPPH 및 ABTS 라디칼 소거 활성)을 나타냄으로써 대조군과 비교하여 상당히 뛰어난 성장률을 보여주었다. 또한, 바이오비료 용액의 순환식 수경재배 기간 동안 병원성균은 바이오비료 용액 내에 존재하지 않았다. 이러한 결과는 혼합 수산 폐액의 완전한 재활용과 더불어 고품질 상추 생산을 위한 바이오비료로서의 가능성을 보여주었다.

본 연구를 통해 분리되어 폐기되기 어려운 혼합 수산 폐액의 혼합미생물에 의한 친환경적인 처리가 가능함을 확인하였으며, 분해된 혼합 수산 폐액은 높은 항산화 및 항균 활성, 그리고 유효한 항진균 활성을 나타내었다. 또한 식물 독성이 없고 높은 아미노산 함량을 가지며 질소, 인, 칼륨의 함량 및 균체량에서 바이오비료로서의 조건을 충족하였고, 바이오비료액이 순환되는 개방형 수경재배에서

병원성균의 침입이 없는 상태로 높은 성장률, 엽록소 함량 및 카르티노이드 함량을 가지는 상추를 재배할 수 있었다. 따라서 생물학적으로 분해된 혼합 수산 가공 폐액으로부터 다양한 고부가가치의 유용 자원을 생산함으로써, 혼합 수산 가공 폐액의 재활용 및 실용화 가능성을 보여주었다.

GENERAL INTRODUCTION

1. Background

As important foods, fisheries products have been steadily consumed. Globally, their consumption is expected to increase from 172.38 million tons in 2017 to 180.22 million tons in 2020, 190.79 million tons in 2025, and 196.44 million tons in 2028 according to the 'World Fisheries Consumption Forecast' recently released by the Organization for Economic Cooperation and Development (OECD) and the United Nations Food and Agriculture Organization (FAO) (OECD/FAO, 2019) (Table 1.). Therefore, Prompt measures should be taken to deal with the ever-increasing quantities of garbage.

Table 1. Expected amounts of human activities for fishery and its products.

	Average 2016-2018	2022	2025	2028
Production (kiloton)	172,268	184,508	191,143	196,324
Consumption (kiloton)	172,525	184,533	190,793	196,449

2. Fish Waste

The projected consumption of fish in 2020 is estimated to be 16.2 kg/capita/year for developing countries and 21.5 kg capita^{-1} year^{-1} for developed countries (Chowdhury et al., 2010), which indicates that the amount of fish waste to treat will increase gradually. Currently, across the world more than 20 million tons of fish are known to have been discarded as by-products, which corresponds to almost 25% of the total amount of fish captured by marine fisheries (Hayes et al., 2008). It is estimated that only 25-50% of the raw material captured is utilized for primary products. The remaining raw material is considered processing waste, which is utilized in low-value products or disposed of (Visvanathan et al., 2012). This demonstrates that the fish processing industry generates large quantities of solid waste and wastewater. The solid waste represents 20-60% of the initial raw material and contains various kinds of residues (head, viscera, skin, bones, blood, liver, gonads, and guts, etc.), which are of both environmental and economic concern (Rebah and Miled, 2013).

3. Seaweed waste

The worldwide consumption of seaweed has increased steadily due to its health benefits, and thus, seaweed aquaculture has increased. Seaweed is also used as a plant to clean inland sea areas. The seaweed industry provides a wide variety of products that have an

estimated total annual value of US$ 5.5–6 billion, and are derived from 7.5–8 million wet tons of seaweed annually (McHugh, 2003). China, Korea and Japan are the largest producers, of about 5 million, 800,000, and 600,000, wet tons per year, respectively. Approximately one fifth of the wet seaweed is used to produce seaweed meal (McHugh, 2003). In recent years, the amount of seaweed waste has been increasing, due to both the culturing of seaweed as an industrial resource, and its use to combat the environmental problems of eutrophication (Tang et al., 2009). A large amount of the seaweed waste is dumped on beaches by tides. The reutilization of seaweed waste is essential for the preservation of the marine environment and recycling of organic matter (Kim et al., 2013).

4. Treatment of fishery waste

In many countries, solid fishery waste is commonly recycled to produce fishmeal, or treated along with municipal waste, while liquid fishery waste is disposed of through the municipal sewage system, or directly into a waterbody. For the latter, the receiving waterbody has to be able to degrade the biological and chemical constituents present in the waste for there to be no detrimental effect on the aquatic fauna and flora (FAO, 2005). To design the appropriate measures for the efficient disposal of fishery wastewater, the evaluation of various physicochemical and biological parameters is required. The most important parameters are known to be solid content, pH, temperature,

odor, organic matter, biochemical oxygen demand (BOD), chemical oxygen demand (COD), oil and grease content, and nitrogen and phosphorous content (FAO, 2005).

5. Reutilization of fish waste

The recovery of marketable by-products from fishery wastes is important in industry. For the utilization of fishery waste, the most commonly used methods are the manufacture of fishmeal and oil, the production of silage and the manufacture of organic fertilizer; these products afford environmental and public benefits, besides reducing the cost of animal production. The hydrolyzed fishery wastes can be used for fish or pig meal (Arvanitoyannis and Kassaveti, 2008). It is well known that fish offal, as a valuable source of high-quality protein and energy, can be used as a feed ingredient, because protein-rich materials are frequently used as feed supplements for monogastric animals (Arvanitoyannis and Kassaveti, 2008). Composting of fish offal is also carried out to transform fishery waste into useful agricultural products (López-Mosquera et al., 2011). Besides these uses, fish bones heated to 600°C or 900°C can be utilized for chromium immobilization (Arvanitoyannis and Kassaveti, 2008). The reutilization of fish oil as biodiesel has also been reported, where it is processed using an ozone treatment with two catalysts (iron oxide and calcium phosphate monobasic) (Arvanitoyannis and Kassaveti, 2008).

6. Reutilization of seaweed waste

Seaweeds are often used in fertilizers, fungicides, herbicides, and phycocolloids, such as alginate, carrageenan, and agar (Kim et al., 2013). To reutilize seaweed waste, its complicated molecular structure must be hydrolyzed. Due to increasing worries about global climate change, seaweeds have gained particular attention as an alternative energy source for the production of bioethanol. As seaweeds are able to grow and convert solar energy to chemical energy at rates 3-4 times greater than terrestrial plants, they have a greater potential to generate and store sufficient carbon resources for biorefinery products (Uju et al., 2015). Seaweeds also have a high bonding affinity with heavy metals (Areco et al., 2012); the research on this suggests that seaweeds provide a potential alternative to replace the most widely applied industrial materials (activated carbon and ion-exchange resins), as they are economical, efficient and sustainable materials.

7. Challenge to enhance reutilization values

Fishery waste offers a huge wealth of biomolecules that have a diversity of potential uses. The use of eco-friendly processes, such as fermentation and enzymatic hydrolysis, minimize the use of hazardous chemicals. Through the eco-friendly treatment of fishery waste, the recovered bioactive molecules can be effectively utilized in aquaculture feeds, biomedical industries, as flavor precursors, or as colorants (Rai

et al., 2012). In the case of underutilized fish protein hydrolysates, these have gained great attention from food scientists, due to their high protein content, good amino acid balance and bioactive peptides (which have antioxidant, antihypertensive, immunomodulatory and antimicrobial properties), which has enabled their use in various industrial applications (Chalamaiah et al., 2012). Recently, the challenge to enhance the reutilization value of fishery waste has continued, with good prospects.

PURPOSE OF THIS STUDY

The worldwide amount of fishery consumption has been steadily increasing because of its benefit for human health. From this human activity, a large amount of fishery waste and wastewater is being generated, mostly from the fishery processing. So far, however, these large quantities of fishery waste and wastewater have not been efficiently utilized. Unutilized fishery waste and wastewater are often disposed of by landfill or incineration, or by dumping into the sea. Therefore, the result of current fishery waste and wastewater disposal causes large negative impacts on our environments. This situation urges us to find ecologically acceptable means for efficient reutilization of fishery waste and wastewater.

This doctoral dissertation is composed of three chapters. In chapter 1, a brief review of the current situation, problems and eco-friendly treatment of fishery waste, use of eco-fiendly products and the related regulations of fishery waste was provided. Chapter 2 demonstrated eco-friendly waste treatment of mackerel wastewater for enhancement of its reutilization value. For the reutilization of mackerel wastewater, aerobic biodegradation using mixed microorganisms was performed in a 3-l reactor. As index parameters for biodegradation parameters, dissolved oxygen level, pH, oxidation-reduction potential, cell number, chemical oxygen demand and total nitrogen were monitored during the biodegradation. Antioxidant, antimicrobial, antifungal and DNA protective activity were also determined for the biodegraded culture

supernatants, and thin layer chromatography was used to characterize the biodegraded compounds. Finally, the remaining culture broth after biodegradation was tested as a biofertilizer for zero-emission management, enhancing the reutilization value of mackerel wastewater.

Subsequently in chapter 3, the production of the biofertilizer from mixed fishery wastewater of mackerel and brown seaweed (*Undaria pinnatifida*) was attempted for its complete reutilization. The optimum mixing ratio for the mixed wastewater was first determined to produce a high-quality biofertilizer. For the produced biofertilizer, total amino acid content, antioxidant activity, contents of nitrogen, phosphorus, potassium and heavy metals, and number of viable cells were all determined to verify its quality. Finally, the produced biofertilizer was applied in lettuce hydroponic culture, and the result indicated that the biodegraded fishery wastewater is a good sustainable resource.

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CHAPTER I

Zero-emission management of organic fisheries' waste and its favorable impact on the environment

Abstract

This chapter provides a brief review of the zero-emission management of fisheries' waste and its favorable impacts on the environment. First, the current situation and problems facing fisheries' waste, and plans to overcome these issues, are discussed. Finally, microbes for efficient treatment, eco-friendly processes, uses, and the related regulations of fisheries' waste are addressed to alleviate the environmental impacts.

The annual amount of fisheries' waste has increased steadily due to increases in the consumption of marine products and their inefficient treatment. This situation has prompted the urgent review of waste policies with improved regulations, encouraging efficient recycling of fish-processing waste. Eco-friendly zero-emission management in which fisheries' waste is turned into valuable resources is discussed, and a zero-emission fisheries' waste management model is proposed. No additional production of waste and/or wastewater is generated during the entire treatment process through biodegradation using a microbial consortium. Eventually, this management of fisheries' waste will result in more favorable impacts on the environment, and the use of fisheries' waste as a valuable resource will expand.

1. Fisheries' waste

1.1. Current state and problems

The total global fisheries' production has increased gradually, and in 2014 it reached 167.2 million tons (excluding aquatic plants), combining 93.4 million tons by capture and 73.8 million tons by aquaculture (FAO, 2016). The amount of human consumption was 146.3 million tons with 20.1 kg of per capita food fish supply, while the non-food use was 20.9 million tons. Therefore, for the first time in history, global fish consumption was more dependent on aquaculture than capture. When including aquatic plants, global aquaculture production reached 101.1 million tons in 2014, representing 52% of total fisheries' production (FAO, 2016). This increase in the total global fisheries' production was caused by increased catch (more than 100 kilotons compared with 2013) by major fishing countries, such as China, Indonesia, Myanmar, Norway, Chile, and Peru (FAO, 2016). Furthermore, the annual global catch in inland waters has also continued to increase (37% increase over the past decade), and reached 11.9 million tons in 2014 (FAO, 2016). However, some of the major fishing countries (Tanzania, Egypt, Congo, Russia, and Brazil) have reported reduced catches due to environmental degradation and overfishing.

In recent years, awareness of the value of fisheries' products as foods that promote well-being has increased. Because of the gradual

increase in fish consumption, a large quantity of fish waste is also generated. Traditional methods for the disposal of fish waste have included landfill, incineration, or deep-sea dumping, while the wastewater generated during the treatment processes has been treated with raw sewage or discharged into a receiving water body. As one of the disposal means, all types of waste, including contaminated dredged material, industrial waste, and sewage sludge, were dumped before the London Convention (1972) and the London Protocol (1996) on deep-sea dumping were established (Tornero and Hanke, 2016). Up to a decade ago, 18-30 million tons of fisheries' waste were dumped globally every year, although they were recognized as harmful pollutants because of their high concentration of chemical oxygen demand (COD), fat-oil-grease, and total suspended solids, including pathogens (Sapkota et al., 2008). Today, the deliberate dumping of these harmful materials into the ocean is essentially regulated by the London Protocol. Under this regulation, a much-reduced amount of dumped fisheries' waste has been estimated, although data collection systems for ocean dumping in several countries are unreliable or non-existent. For the safe conservation of marine resources in an agreeable marine environment, more efforts are required to adhere to the regulations.

1.2. Overcoming schemes

To date, fisheries' waste has been treated for disposal, but not for

reuse. Solid fish waste is mostly recycled to produce fishmeal or treated together with municipal waste, while liquid fish waste is disposed of via the municipal sewage system or directly into a waterbody. In the case of disposal using a waterbody, the receiving waterbody has to be able to degrade the components present in the fisheries' waste to ensure no detrimental impact on the aquatic fauna and flora (FAO, 2005). To design appropriate criteria for the efficient disposal of liquid fish waste, important physicochemical and biological parameters must be evaluated, including solid content, pH, temperature, odor, biochemical oxygen demand (BOD), COD, oil and grease content, and N and P content (FAO, 2005).

1.2.1. Established methods

1.2.1.1. Treatments of solid fisheries' waste

Although fisheries' waste has not been reused efficiently so far, it contains valuable resources, such as carbohydrates, proteins, fats, and minerals (Arvanitoyannis and Kassaveti, 2008). In addition, the concentrations of toxic substances, including heavy metals, are relatively low in fisheries' waste. The most common destination for solid fish waste has been fishmeal production as animal feed. This partial recycling of fish waste results in the reduction in both animal production cost and environmental problems caused by waste pollution. For fishmeal production, fish waste is first ripened and pressed. Then,

the liquid part is used to produce oil, while the solid part (fish cake) is used to produce fishmeal. Therefore, fish oil production is another benefit of this treatment process. The fish cake is subsequently heated to reduce the moisture content and to ensure the microbiological quality (Arvanitoyannis and Kassaveti, 2008). To produce fishmeal as commercial product, paying for adequate heating is required. As an eco-friendly means to recover other useful products from fisheries' waste, solid state fermentation is used, because it is more stable, requires less energy input, and produces greater enzyme yields than submerged fermentation (Archana and Satyanarayana, 1997; Solis-Pereira et al, 1993). In solid state fermentation, the microorganisms use diverse solid materials as a source of nutrition, and secrete the necessary enzymes for the degradation of the available substrates under sufficient moisture (Tunga et al., 1999). This fermentation often results in improved product recovery with little wastewater generation (Uyar and Baysal, 2004).

Seaweed waste has usually been treated as chemical solid waste after the extraction of alginate, iodine, and mannitol. Although seaweed waste has high potential for use in diverse applications, it has not been efficiently reused because of the small range of applications and high reuse cost (Zhang et al., 2012). This results from the complex molecular structure of seaweed, and thus specific microorganisms must be developed to enable effective biodegradation for extensive reuse (Tang et al., 2009). As global shellfish aquaculture has grown steadily, shellfish processing activities generate large quantities of endoskeleton

shell parts from the crustacean peeling process. Crustacean waste has generally been used to produce chitin and chitosan. Without any pretreatment, dried, broken into pieces, and extracted by acid and alkaline treatment for chitin production (Rattanakit et al., 2002). Chitinase has been used for the hydrolysis of chitin (Rattanakit et al., 2003). Oysters, which are mainly cultured in Korea and Japan, are another shellfish that have created a serious waste problem. Approximately 50 - 70% of the oyster shell waste is dumped into public waters and reclaimed lands, and thus the recycling of waste shells has aroused much interest in the mariculture industry (Jung et al., 2012). Recently, oyster shell waste has been used as deodorant and calcium additive to basic fertilizer. A large amount of the tunics of sea squirts are generated as waste after their muscles are consumed as a favorite seafood, mainly in Korea and Japan. To date, the carotenoids contained in the tunic have been extracted and used as natural marine pigments. In recent years, some biofunctional compounds, including taurine, glycosaminoglycan, chondroitin sulfate, and dietary fiber, have been extracted from the tunic, and commercialized for medicine and food (Jung et al., 2003).

1.2.1.2. Treatment of fisheries' wastewater

Pretreatment prior to biological treatment is an efficient way of treating fisheries' wastewater. In the case of seafood-processing wastewater, screens, grit chambers, oil and grease removers, and

flotation units and equalization tanks are commonly used in the treatment process. A screen reduces the amount of solids present in the wastewater, and a grit chamber slows down the flow and allows any grit to fall out from the effluent wastewater (Visvanathan et al., 2012). To enhance the biological processes and oxygen diffusion, oil and grease is removed, and an equalization tank is used to equalize the flow and concentration of fisheries wastewater. Next, a sedimentation process removes additional solids, such as scales, muscle, and offal (Visvanathan et al., 2012), and is subsequently followed by coagulation-flocculation and clarification processes (FAO, 2005). After suitable pretreatment, various biological treatments are then applied to degrade the organic matter present in the wastewater (Chowdhury et al., 2010).

1.2.1.2.1. Aerobic process

Aerobic processes are useful to treat fish-processing wastewater. When oxygen is sufficiently available, aerobic decomposition of the organics present in the wastewater results in harmless, stable products, such as carbon dioxide, sulfate, orthophosphate, and nitrate.

(i) Activated sludge process

The activated sludge process is a suspended growth treatment that is preferred to attached growth treatment in the practical treatment of fish-processing wastewater (Battistoni and Fava, 1995). For system

stabilization, a higher amount of oxygen is needed in fish-processing wastewater compared with other food-processing wastewater (Carawan et al., 1979). Therefore, a long aeration time with low organic loading is recommended. The two important parameters in this process, ratio of food to microorganisms and retention time of solids, are 0.1-0.3 and 18-20 days, respectively. The performance efficiency of this system is significantly dependent on temperature (Carawan et al., 1979).

(ii) Aerated lagoon

Another suspended growth treatment for fish-processing wastewater is aerated lagoons, in particular, when it is not economically viable to use an activated sludge system. Either a completely mixed or facultative lagoon is commonly used for the treatment. The general characteristics of ponds are 2.4-4.6 m depth and 2-10 days hydraulic retention time (HRT), which conditions can result in approximately 90-95% BOD removal efficiency (Carawan et al., 1979).

(iii) Rotating biological contactor

A rotating biological contactor (RBC) is an attached growth process, and a multi-stage RBC is used in fish-processing wastewater treatment (Tay et al., 2004). The aerobic RBC has several advantages: short hydraulic retention time; high specific surface area; high biomass concentration; insensitivity to toxic substrates; less accumulation of sloughed-off biofilms; low energy consumption; and operational simplicity (Reynolds and Richards, 1996). The treatment efficiency is

mainly dependent on disc rotational speed, hydraulic retention time, loading rate, level of disk submergence, and temperature. Compared with the activated sludge process, the RBC provides more stability and requires less energy with no sludge recycling (Najafpour et al., 2006).

(ⅳ) Trickling filter

Another attached growth process used in fish-processing wastewater treatment is a trickling filter (Gonzalez, 1996). Stone and synthetic media are commonly used for biofilm formation, and the penetration of substrate into biofilm is primarily dependent on wastewater strength, flow rate, substrate use rate by the biofilm, and diffusivity of substrate in the biofilm (Benefield and Randall, 1980). A high liquid recirculation rate and forced air circulation are used to achieve better performance in the removal of organics (Carawan et al., 1979). As shown in all biological processes, low temperatures reduce the treatment capacity of a trickling filter.

(ⅴ) Effect of salinity on process performance

High salinity of wastewater can affect the performance of aerobic processes due to its inhibition of microbes. Some negative effects on aerobic treatment occur at chloride concentrations above 5 g l^{-1}. However, this issue can be overcome in the activated sludge process (Doudoroff, 1940; Pillai and Rajagopalan, 1948). Considerable reduction in BOD has also been reported, despite the combined effect of high salinity and high organic loading (Stewart et al., 1962).

1.2.1.2.2. Anaerobic process

Anaerobic processes are suitable to treat fish-processing wastewater because of the high removal of BOD at a significantly lower cost and sludge production than comparable aerobic processes (Johns, 1995). Furthermore, the methane-rich biogas end product can be used as a fuel.

(i) Anaerobic biofilm reactor

Anaerobic biofilm reactors have shown good performance in the treatment of fish cannery wastewater at low operating costs (Balslev-Olesen et al., 1990). The main reaction parameters are organic loading rate (OLR) and HRT, which have a great influence on the biodegradation of organics present in the wastewater. These are reflected in the profiles of volatile fatty acids and biogas production.

(ii) Upflow anaerobic sludge blanket reactor

Upflow anaerobic sludge blanket (UASB) reactors are applied to various types of industrial wastewater, even those containing toxic/inhibitory compounds (Weiland and Rozzi, 1991). The application of an UASB reactor system is therefore a promising treatment option for fish-processing wastewater (Palenzuela-Rollon et al., 2002). Good performance in COD removal has been shown in the UASB reactor system using a mixed type of tuna, sardine, and mussel-processing wastewater (Punal and Lema, 1999). For wastewater containing high

lipid content, a two-step UASB reactor system has been recommended.

(iii) Effect of pH and ammonia content on process performance

The fish condensate produced during the fishmeal manufacturing process has high ammonia content (approximately 2000 mg N L^{-1}) and pH 9-10 (Sandberg and Ahring, 1992). This high pH can therefore have an influence on the anaerobic degradation occurring in a UASB reactor, because the optimal pH for a mesophilic biogas reactor is 6.7-7.4 (Clark and Speece, 1971). Fish condensate was efficiently treated in a UASB reactor at pH 7.3-8.2, and COD removal reduced as the pH increased (Sandberg and Ahring, 1992). The methanogenic activity was reduced by high concentrations of ammonia resulting from protein degradation during the anaerobic treatment.

(iv) Effect of salinity on process performance

The wastewater generated during fish processing contains rich protein-based nitrogen, organic matter, and salts. The high salt concentrations resulting from the use of a large amount of salt for fish conservation can inhibit methanogenesis during anaerobic treatment of the wastewater (Lefebvre and Moletta, 2006). However, the treatment of fish-processing wastewater was performed in an upflow anaerobic filter, suggesting that methanogenic bacteria can be adapted to the salinity level of the wastewater (Omil et al., 1995). Furthermore, successful anaerobic digestion using marine methanogens to eliminate proteins and grease has been reported in the treatment of

fish wastewater that was mainly generated at the time of fish unloading (Aspe et al., 1997).

1.2.2. Zero-emission management

"Green growth" is a term that implies renewable energy production by moving away from fossil fuels as energy sources. To promote green investment, related policies have been designed and proposed to address global climate change (Danish Council of Environmental Economics, 2013; World Economic Forum, 2013). To achieve green growth by promoting resource recycling for waste, waste policies must include reuse of resources for security of raw materials, energy recovery by biomass, and advanced treatment for prevention of pollution. During resource recycling, natural resources must be efficiently handled in production, circulation, and consumption to reduce the amount of waste. The final product of the advanced treatment must be in a form that can be returned to nature without further environmental impacts.

Today, fish waste occurs in large quantities in the environment, and is considered a potential resource for value-added products. However, the waste is not yet efficiently reused, and additional waste and/or wastewater is produced during treatment. Leading nations have now realized the importance of waste management, and have encouraged ecologically acceptable means of waste treatment (Jung and Kim, 2016). This has resulted in changes to waste policies that emphasize

resource recycling and zero-emission processes (Mathews, 2012). In past waste treatments, the environment and economy have mainly been addressed by separate strategies from that of producing energy from waste. Today, energy is integrated with the environment and economy by obtaining novel materials from waste with potential energy development. The aim is therefore to achieve highly-efficient social-based resource recycling, moving toward zero-emission management. This idea is summarized in Fig. 1.

Zero-emission management for the efficient treatment of fish waste is proposed in Fig. 2. The biodegradation of fish waste or fish wastewater is carried out in a bioreactor to reuse the whole bulk (liquid and solids). During the biodegradation using mixed microbes, peptides and amino acids, fatty acids, and oligosaccharides are produced from proteins, lipids, and carbohydrates, respectively, which are the main polymers present in fish waste or fish wastewater. After biodegradation, solids, including mixed microbes, are separated from the culture broth by ultrafiltration. Several biofunctional materials can then be obtained by extraction from the culture broth. Finally, biofertilizer is manufactured by mixing together the solids remaining after the ultrafiltration and the culture broth remaining following the production of biofunctional materials (Jung and Kim, 2016). As a result, there is no additional generation of waste or wastewater during the process, with complete reuse of the fish waste or fish wastewater. An additional merit of this process is the recovery of value-added resources from the fish waste.

Fig. 1. Recent changes in the paradigms for waste policies.

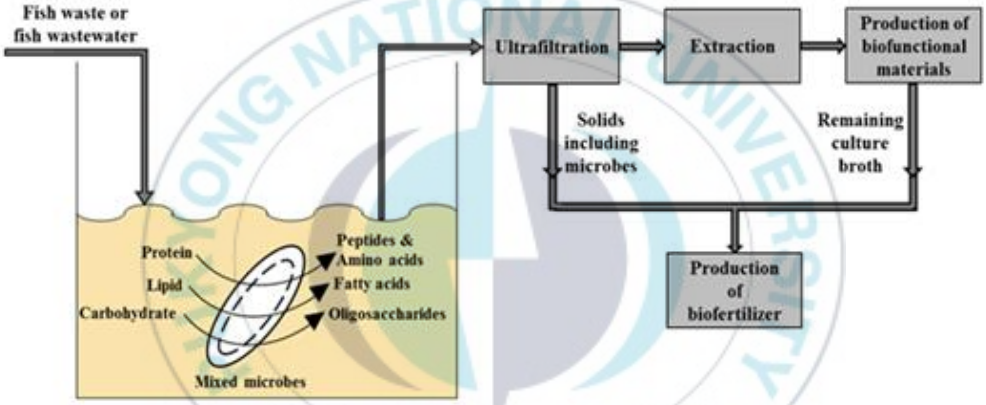

Fig. 2. Schematic proposal for zero-emission management of fish waste or fish wastewater.

2. Fisheries' waste management

2.1. Fisheries' waste and microbes for treatment

As the of quantity of fisheries' waste has gradually increased, its disposal has become a matter of concern. The process of reusing fisheries' waste that results in a reduction in quantity includes identifying the diverse components of the waste and then developing useful microbes for biodegradation.

2.1.1. Diversity of fisheries' waste

2.1.1.1. Fish waste

During fish processing, approximately 30% of solid fish waste, including head, tails, skin, gut, fins, and frames, is generated, and the extent of waste generation is dependent on the level of processing and type of fish (AMEC, 2003). However, fish waste can be a great source of value-added products, such as proteins and amino acids, collagen and gelatin, and oil and enzymes (Disney et al., 1977; Esteban et al., 2007). The waste contains proteins (58%), fat (19%), and minerals (especially iron, zinc, and calcium). Furthermore, monosaturated acids, palmitic acid, and oleic acid are also relatively abundant in the fish waste.

2.1.1.2. Seaweed waste

Seaweeds are classified into three groups; brown (*Phaeophyta*), red (*Rhodophyta*), and green (*Chlorophyta*), according to their pigmentation (Wei et al., 2013). They contain carbohydrates (up to 60%), proteins (10-47%), and lipids (1-3%) with mineral ash (7-38%). The differences in the content of each type is also the result of diverse species and seasonal influence (Pereira, 2011; Vassilev et al., 2010). Each seaweed not only has different carbohydrate content, but also different types of carbohydrate. The main carbohydrates contained in brown seaweed are alginate, laminaran, fucoidan, cellulose, and mannitol. In red seaweed, they are agar, carrageenan, cellulose, mannan, and xylan; in green seaweed, they are cellulose, mannose, xylan, and starch (Barbot et al., 2016). During seaweed processing, the average waste production is approximately 10% (Basic survey of actual biomass use in Korea, 2010). Seaweed waste contains approximately 20% crude protein, 50% crude fiber, and 3% ash content (Gan et al., 1999), as well as iodine, vitamins, minerals, dietary fiber, and active ingredients (Zhang et al., 2012).

2.1.1.3. Others

Shrimp processing results in 45% waste, consisting mainly of exoskeleton and cephalothorax. This waste contains valuable components, such as chitin, protein, and pigments, and the content is

mainly dependent on the processing conditions, the species, and seasonal variation (AL Sagheer et al., 2009; De Holanda and Netto, 2006; Palpandi et al., 2009; Rodde et al., 2008; Wang et al., 2011; Xu et al., 2008). In the case of *Styela clava*, approximately 40% of the body is composed of tunic, and thus large amounts of tunic waste are generated during processing (Lee et al., 2015). The tunic is composed of water, proteins, mucopoly-saccharides, and carbohydrates in diverse proportions (Welsch, 1984). Shellfish waste, including from oyster and bivalves, contains mainly $CaCO_3$ (> 90%), calcium phosphate (1-2%), and a trace amount of $MgCO_3$.

2.1.2. Microbes for treatment

2.1.2.1. For fish waste treatment

Fish waste has been treated by fermentation using mixed cultures of *Saccharomyces* sp. and *Lactobacillus plantarum* to convert it into a stable feed ingredient (Faid et al., 1997). In addition, to remove fish odor, a mixture of yeasts (*Saccharomyces cerevisiae* and *Candida* sp.) and lactic acid bacteria (*L. plantarum* and *Pediococcus acidilactici*) have been studied in alcohol-lactic acid fermentation (Faid et al., 1994). In the fermentation of fish offal waste using *L. acidophilus*, the addition of whey has enhanced the removal of harmful microbes, such as *Staphylococcus, Clostridium*, and coliform bacteria (Samaddar and Kaviraj, 2014). Thermophilic microbes (*Bacillus subtilis*, *Bacillus*

licheniformis, *Brevibacillus agri*, *Bacillus coagulans*, *Bacillus circulans*, *Bacillus anthracis*, and *Bacillus fusiformis*) have been proposed to degrade organics present in fishmeal wastewater and to produce liquid fertilizer (Kim et al., 2007). Useful microbes have also been isolated from earthworm viscera for the reuse of fish waste. They have been identified as *Brevibacillus agri*, *Bacillus cereus*, *Bacillus licheniformis*, *Bacillus licheniformis*, and *Brevibacillus parabrevis* (Kim et al., 2010).

2.1.2.2. For seaweed waste treatment

Seaweeds are diverse in both their content and type of carbohydrate. In the composting process for the disposal of Wakame (*Undaria pinnatifida*) waste, *Bacillus* sp. HR6 (Tang et al., 2007), *Gracilibacillus* sp. A7 (Tang et al., 2009), and *Halomonas* sp. AW4 (Tang et al., 2011) have been applied to produce fertilizer (Tang et al., 2009). To make use of *Laminaria japonica* waste, simultaneous saccharification and fermentation with mixed microorganisms (*Enterobacter* sp., *Pantoea agglomerans*, *Erwinia tasmaniensis*, *Candida lusitaniae*) and Nuruk (Lee and Lee, 2011) have been proposed. *Microbulbifer* sp. has been suggested for the degradation of Wakame thalli to single cell detritus particles (Wakabayshi et al., 2011). Furthermore, *Microbacterium oxydans* has been shown to be a good microorganism for the degradation of alginate and laminaran in brown seaweed waste (Kim et al., 2013). A *Bacillus* sp. SYR4 has also been isolated from a sandbar, and possesses high agarase and

carrageenase activity to degrade red seaweed waste (Kang and Kim, 2015). Recently, a halophilic *Bacillus licheniformis* TK3-Y has been isolated and showed good performance in the degradation of green seaweed waste with high salinity (Kang and Kim, 2015).

2.1.2.3. For other waste treatment

To efficiently treat crustacean waste, lactic acid fermentation has mainly been applied. For this bioprocess, several potential strains, such as *Lactobacillus plantarum*, *L. pentosus*, *L. salivarius*, *L. paracasei*, *L. casei*, *L. rhamnosus*, *Enterococcus faecium*, *Pediococcus acidilactici*, and *Lactococcus lactis*, are used as single microbe treatments (Gortari and Hours, 2013). In some cases, mixed microbes (*L. plantarum* and *Aspergillus niger*; *L. paracasei* and *Serratia marcescens*; *Lactobacillus acidophilus* and *Bacillus licheniformis*; *L. lactis* and *Teredinibacter turnerae*) are applied to shrimp and crab waste for chitin and/or chitosan production via demineralization and deproteinization (Gortari and Hours, 2013). For the biodegradation of shrimp shell waste, *Aeromonas hydrophila* SBK1 strain has been proposed (Halder et al., 2013), and the fungus *Mucorrouxii* has also been applied for chitosan production (Vázquez et al., 2013). To degrade squid pen waste, *Acinetobacter calcoaceticus* TKU024 has been used as a chitosanase-producing strain (Wang et al., 2011). Hyaluronic acid has been produced from the fermentation of mussel-processing wastewater with *Streptococcus* species (Vázquez et al., 2010).

2.2. Processes for fisheries' waste treatment

To date, fishmeal production has been widely used as a means for the partial treatment of fisheries' waste. The remaining waste is disposed of by incineration, landfill, or deep-sea dumping. However, current management practices of fisheries' waste are more positive, with efforts to recover materials and energy from the waste.

2.2.1. Eco-friendly processes

In the production process, unnecessary materials are often generated as byproducts or waste, whereas energy and resources are becoming more precious because of their limited supply. Therefore, green processes that minimize the environmental impact of manufacturing should be pursued as much as possible. These processes can develop clean products that are safer, more environmentally friendly, and of higher quality, which are advantages over the existing chemical processes. Avoiding the generation of waste or pollutants can often be more cost-effective than their control or disposal. As a result, eco-friendly processes involve minimizing waste, pollution, and natural resource depletion, which incorporates the concepts of pollution prevention and sustainability. The eco-friendly process is more than a method for addressing environmental problems, and can offer a framework for achieving innovation with environmental and economic returns. Fermentation is a biological method to recover useful materials

from fish offal waste and to recycle the waste as feed in an eco-friendly way (Mondal et al., 2007; Mondal et al., 2008). It has the benefit of protecting the environment from the disposal risk of polluting waste. In addition, fermentation maintains the cost of aquaculture feed production at an acceptable level (Samaddar and Kaviraj, 2014). Furthermore, enzymatic hydrolysis is often applied as an eco-friendly method to reuse the frame part of the fish waste, because the fish frame parts contain large amounts of muscle proteins that are highly nutritious and easily digestible (Venugopal et al., 1996).

2.2.2. Production and extraction of bioactive compounds

2.2.2.1. Peptides

Proteins are extracted from fish muscle. Several peptides are also included in the extract, which have diverse bioactivities, such as antihypertensive, antithrombotic, immune modulatory, and antioxidative properties (Kim et al., 2000). Moreover, some peptides show anticoagulant and antiplatelet properties (Je et al., 2004). Biologically active peptides can also be obtained by the enzymatic hydrolysis of fish muscle (Benkajul and Morrissey, 1997).

2.2.2.2. Oligosaccharides

As a useful resource, seaweed hydrolysates are often applied as

fertilizer, fungicides, herbicides, and phycocolloids. Different types of oligosaccharides can be produced from various seaweeds. To obtain the oligosaccharides, different methods of pretreatment prior to fermentation have been performed using enzymes (Choi et al, 2010), alkali (Harun et al., 2011), or acid at high temperatures because of the complex structure of the polymers contained in seaweed (Harun and Danquah, 2011). The direct application of a microorganism itself has been recently proposed, which results in a considerable reduction in the product cost incurred for enzymatic or chemical saccharification of the polysaccharides (Rabelo et al., 2009). To turn fisheries' waste into valuable resources with low energy consumption, microbes must be developed for the biodegradation. The oligosaccharides produced through biodegradation are also a good resource for the production of ethanol as an alternative fuel, and related studies are currently in progress.

2.2.2.3. Oils

After pressing, fish oil is extracted from the liquid parts of fish waste by chemical or enzymatic methods. Chemical extraction can be carried out using the hexane or petroleum method, the chloroform/methanol method, or the acid digestion method, whereas alcalase, neutrase, lecitase ultra, protex, and protamex are used for enzymatic extraction (Christie, 1993). In the presence of highly hydrophilic functional groups, fish tissues are initially extracted with

chloroform/methanol in the presence of calcium chloride, and then with 1 M HCl for better recovery of oil (Christie, 1993). To extract fish oil from a sample rich omega-3 (such as salmon oil) or certain impurities (such as some species of arsenic), supercritical fluid extraction with carbon dioxide as an extractive solvent moderate conditions (25 MPa and 313 K) is useful for reducing fish oil oxidation, compared with conventional extraction processes such as cold extraction, wet reduction, or enzymatic extraction (Rubio-Rodríguez et al., 2012). Furthermore, extraction coupled with the fractionation process has been proposed to remove free fatty acids and to improve fish oil quality, as an alternative to physical and chemical refining procedures.

2.2.2.4. Chitin

Crustacean shell waste is rich in chitin. For its commercial preparation, the shell waste has traditionally been processed first by mechanical grinding, then demineralized with strong acids, and finally deproteinized with alkali at 90-100°C (Khor, 2011; Naznin, 2005; Palpandi et al., 2009; Percot et al., 2003; Thirunavukkarasu and Shanmugen, 2009; Thirunavukkarasu et al., 2011). There are also chemical and enzymatic processes for chitin production. By acid treatment with HCl, raw material is obtained from crustacean shell waste, which is then hydrolyzed by commercial proteases at the optimal pH and temperature. After the hydrolysis, the solid and liquid fractions are separated. Pigment is extracted from the liquid fraction,

followed by lyophilization to obtain the decolorized protein hydrolysate. Crude chitin is obtained from solids following washing and drying processes. However, the same result can be attained when crustacean shell waste is fermented with lactic acid and proteolytic bacteria. After fermentation, crude chitin is obtained from the solids, while pigment and protein hydrolysate are obtained from the liquid fraction. Finally, chitin is produced when the crude chitin is bleached with H_2O_2 and then dried. Chitosan is produced from crude chitin after deacetylation using fungal chitin deacetylases (Wangetal.,2011).

2.3. Use of fisheries' waste

Based on various efforts to recover materials and energy from fisheries' waste, the use of fisheries' waste after suitable treatment has been expanding. This positive management of fisheries' waste can considerably reduce the environmental impacts produced from the waste itself.

2.3.1. Medicine

Fish bones are a good source of hydroxyapatite, which is mechanically stable and compatible (Larsen et al., 2000). It is also thermodynamically stable at physiological pH, and thus plays an active role in bone binding. It is widely applied in the medical and dental fields. Amino acids extracted from fish waste have extensive

nutritional value, taste, and medicinal and chemical properties. Some amino acids are used in protein pharmaceuticals as excipients for drug development: arginine in a human tissue plasminogen activator; glycine in a recombinant antihemophilic factor and human monoclonal antibody; glutamate varicella virus live vaccine; and histidine in coagulation factor IX (Larsen et al., 2000). Furthermore, hyaluronic acid extracted from the humor of the eyeball of fish (tuna, shark, and swordfish) has diverse physicochemical and biological properties and functions, such as lubricity, viscoelasticity, biocompatibility, angiogenicity, and immunostimulatory properties (Vázquez et al., 2013). It is also known to play important roles in embryogenesis, signal transduction and cell motility, and to be associated with cancer invasiveness and metastasis (Kogan et al., 2007). Chondroitin sulfate produced from marine waste plays a key role in several biological processes, such as the function and elasticity of the articular cartilage, hemostasis and inflammation, regulation of cell development, cell adhesion, proliferation and differentiation (Vázquez et al., 2013). Gelatin from shark cartilage has been used as a carrier of bioactive components, including antioxidants and antimicrobial substances (Gómez-Guillén et al., 2011). In addition, chitin, chitosan, and their derivatives obtained from crustacean shell waste are used in the pharmaceutical and medical sectors because of their antimicrobial and antitumor activities (Zhang et al., 2010). These compounds are also applied in biomedical fields, because they are used in tissue engineering, wound healing, drug delivery, and cancer diagnosis (Jayakumar et al., 2010). Astaxanthin, which is extracted

from shrimp or crawfish waste, can inhibit prostate cancer by modulating the immune responses against tumor cells (Guerin et al., 2003) and bladder carcinogenesis (Tanaka et al., 1994).

2.3.2. Agriculture

Fish contains well-balanced amino acid compositions, including eight essential and eight nonessential amino acids, and biodegraded fish waste is suitable for use as fertilizer (Hamid et al., 2002). Shell waste is typically heated, crushed, and ground into a powder, and the final processed form is then used as a fertilizer (Gortari and Hours, 2013). In particular, chitin, chitosan, and their derivatives obtained from crustacean shell waste are widely applied in agriculture because of their biocompatibility and non-toxicity (Zhang et al., 2010). Drift-seaweed has been collected and used for centuries as a natural fertilizer in many coastal regions throughout the world (Mchugh, 2003). Seaweed can provide trace elements and growth activators that improve soil structure (Guiry and Blunden, 1991; López-Mosquera and Pazos, 1997; Verkleij, 1992). Shellfish waste, including oysters and bivalves, is also used as a calcium additive to basic fertilizer.

2.3.3. Other uses

2.3.3.1. Animal feed

Fish silage from fish waste is an excellent protein source, which has excellent biological properties for animal feed (Ghaly et al., 2013). The fishmeal manufactured from wild-collected whole fish and shellfish currently provides the main aquatic protein source for animal feed. Fishmeal production using fisheries' byproducts has increased significantly (FAO, 2012). Most of the body oils of fish, excluding salmon, are also used in aquaculture feeds (Arvanitoyannis and Kassayeti, 2008). Moreover, astaxanthin extracted from shrimp or crawfish waste is used in functional feed for crustaceans and salmon (De Holanda and Netto, 2006).

2.3.3.2. Enzymes

The enzymes in fish viscera, including pepsin, trypsin, chymotrypsin, and collagenase, show high catalytic activity at relatively low concentrations. These enzymes have been extracted on a large scale for commercial use (Byun et al., 2005; Kim and Mendis, 2005; Zhou, 2011). In addition, they have additional characteristics: good efficiency at lower temperatures, lower sensitivity to substrate concentrations, and greater stability over a wide range of pH. Proteases are industrially important enzymes that are used globally (Garcia-Carreño et al., 1994). Shrimp proteases partially purified from *Pandalus borealis* can be used at industrial scale in the food industry because of their effectiveness for beef meat tenderization (Aoki et al., 2004). They are active at low temperatures and inactive after mild heat treatment.

Therefore, this results in energy savings through operation at room temperature (Aoki et al., 2004). As an inexpensive alternative to rennet substitutes, milk-clotting enzymes have been also extracted from fish stomach mucosa for cheese manufacturing, which shows some potential (Arvanitoyannis and Kassayeti, 2008).

2.3.3.3. Oils

After pressing ripened fish waste, the liquid parts contain fish oil. Fish oil is a good material for the production of margarine, omega-3 fatty acids, and biodiesel (Ghaly et al., 2013). Biodiesel consists of the monoalkyl esters of vegetable oils, animal fats, or fish oils, and can therefore be synthesized from waste oils (Ghaly et al., 2013). The oil obtained from filtration after primary and secondary treatment of fish waste is known to have suitable properties for use in diesel engines (Arvanitoyannis and Kassayeti, 2008). This oil had better properties than methyl-esterified vegetable oil waste, with a higher heating value and density, lower flash and pour points, no sulfur oxide production, lower or no soot, and lower polyaromatic and carbon dioxide emissions.

2.3.3.4. Food and additives

Fish protein hydrolysates are obtained from the fish protein extracted from fish waste by chemical or enzymatic methods. The

hydrolysates are industrially used as milk replacers, protein supplements, stabilizers in beverages, and flavor enhancers (Ghaly et al., 2013). As a milk replacer, fish protein hydrolysate has a high protein efficiency ratio that is more cost effective than dried skimmed milk (Kristinsson and Rasco, 2000). Amino acids obtained from the biodegradation of fish waste are widely used in the food flavoring industry, including monosodium glutamate, alanine, aspartate, and arginine (Ghaly et al., 2013). The fish bones obtained from fish-processing waste can be used to provide soluble calcium as a mineral source (Ghaly et al., 2013).

2.4. Environmental impacts and related regulations

The environmental impacts of fisheries' waste have gradually increased. This is because the reuse of fisheries' waste has, to date, been inefficient, although the amount of waste has increased annually. Therefore, government regulations have been issued to address such environmental impacts.

2.4.1. Awareness of environmental impacts

If fisheries' waste is not treated appropriately, it can create aesthetic problems and strong odors because of bacterial putrefaction. For example, *Styela clava* waste often collects on the shore producing a foul smell (Lee et al., 2015). Oyster shell waste is often dumped into

public waters because large amounts of oyster shell waste are no longer accepted at landfill sites. Therefore, such waste often piles up in coastal areas and causes many environmental problems (Jung et al., 2012). Furthermore, the organic waste has a high oxygen demand when treated, which poses environmental and health problems if not managed properly. For this reason, the deep-sea dumping of diverse fisheries' waste has caused some environmental concerns, such as reduced oxygen levels in bottom waters of ocean basins; burial or smothering of living organisms; and introduction of disease or non-native and invasive species to the sea floor ecosystem (EPA, 2012).

With the onset of global environmental problems and the depletion of natural resources, the focus of waste management policies has changed during the late 20^{th} and early 21^{st} centuries. Paradigms have changed to pursue the concept of sustainability through reduce, reuse, and recycle policies, whereas previous policies focused on environmentally sound waste treatment to avoid local environmental pollution (Sakai et al., 2011). The integrated options for waste management mainly include reduction, reuse/recycle, and disposal (Archer, 2008). In general, source reduction is given the highest priority, followed by recovery for recycling and recovery for composting with or without energy capture. Finally, disposal of discards includes landfills, combustion with energy recovery, and incineration without energy recovery. Nevertheless, the environmental impacts of untreated and inefficiently treated waste have gradually

increased, leading to a governmental movement to establish new preventative regulations.

2.4.2. Related regulations

In connection with the marine environment, the London Convention 1972 was established, followed by the 1996 Protocol, to clarify the prohibition of dumping of fish waste or other matter specified therein (NOAA, 2012). According to this Convention, fish means both fish and shellfish, and the prohibited waste includes fish waste or waste generated during processing of wild and aquaculture resources, such as body, bone, viscera, skin, and viscous liquid; however, waste discharge during fishing is not included (MOF, 2008). Therefore, global efforts have gradually focused on the awareness of the environmental impact of ocean dumping. In Korea, the government has raised treatment cost burdens for landfill and incineration, encouraging recycling of fish-processing waste materials. The related industries have endeavored to follow government policy, and thus a large fraction of fish waste is now used for animal feed and fertilizer (MOF, 2008). However, several civil petitions have been raised during the process of reuse because of the emission of odors. Fish-processing byproducts are treated based on 'the law of waste management' and 'the law of saving resources and promoting recycling'. According to 'the law of eco-friendly agriculture-fisheries' rearing and support for organic foods management', established in 2012, the government has made efforts

toward the exploitation of resources with optimum treatment plans (Lee et al., 2013). Since then, 'the law of promoting a resource-recycling society' and 'the law of promotion of fish-processing byproduct recycling' have followed to prescribe the reuse of the fish-processing byproducts.

During the mid to late 1970s, related laws were established for the reuse of fish-processing byproducts in the USA and Japan, which have been used to recover and manage fish resources (Lee et al., 2013). In the USA, the disposal method and system for fish waste is different in each state, and ocean dumping is partially allowed with permission in state law. According to the Marine Protection, Research and Sanctuaries Act, referred to as the Ocean Dumping Act, a permit is required for the ocean disposal of fish waste if the disposal occurs in harbors, protected waters, enclosed coastal waters, or any location where such disposal can potentially endanger health, the environment, or ecological systems (EPA, 2012). Considering oyster shell waste, its landfill treatment has been prohibited in North Carolina since 2007, because oyster shell was recognized as a useful resource for, for example, construction and the beauty industry (Lee et al., 2013). Maryland adopted this regulation in 2013, and other states are planning to establish it. In Japan, fish waste is, by law, mostly disposed of through incineration, and not through ocean dumping. Certain parts, including viscera, are reused as pet feed and oil after processing. Fish oils extracted from sardine, in particular, are used to produce margarine, soap, and alcohol, and eicosapentaenoic and docosahexaenoic

acids, which have medicinal uses (MOF, 2008). Other valuable products from fish waste include taurine (from crab, squid, and octopus) and chitosan (from crab and crawfish shells). To promote these activities, 'the law of promotion of renewable resources use' was changed to 'the law of promotion of the effective use of resources' in 2000 (Lee et al., 2013). The law regarding the reuse of recycled food resources was also revised in 2007. The treatment of fish-processing byproducts has followed this law. The related laws promote recycling of fish-processing byproducts, such as fishmeal and fish soy sauce. Each country has gradually adopted national legislation to improve environmental regulations. Further effort is required to preserve both the marine environment and marine resources.

3. Conclusion

As the quantity of fisheries' waste increases annually, more efficient treatment is urgently required. Eco-friendly zero-emission management of fisheries' waste is a more positive way to turn fish waste into valuable resources with considerable reduction in the amount of waste, which greatly extends product use. To secure safe and stable marine products, marine resources have to be preserved by maintaining a clean marine environment. More efforts are required under strict policies and regulations for organic waste treatment.

4. References

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CHAPTER II

Eco-friendly waste management of mackerel wastewater for enhancement of its biological activities and biofertilizer value

Abstract

To reutilize mackerel wastewater (MWW) generated from mackerel processing plants, aerobic biodegradation of MWW was conducted in a 3-l reactor by using mixed microbes. During the first 24 h, biodegradation occurred actively, and the dissolved oxygen level, pH, and oxidation-reduction potential decreased with an increase in cell number. Then, the fishy smell started to disappear. The 42-h culture supernatant displayed high antioxidant activities, as indicated by the following results: 88.7% 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity, 99.7% 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging activity, 96% hydroxyl radical scavenging activity, and 0.35 reducing power at A_{700}. The high antioxidant activities were attributed to specific amino acids, and this was confirmed using two-dimensional thin-layer chromatography. Moreover, the culture supernatants displayed antimicrobial and DNA protective activities and weak antifungal activity, enhancing the reutilization value of MWW. The remaining culture broth after MWW biodegradation was found to be phytotoxin-free, and it could be used as a biofertilizer on the basis of hydroponic culture of red bean and barley. Overall, advanced waste management of MWW was demonstrated.

1. Introduction

In 2012, the worldwide supply of fish from capture fisheries and aquaculture was about 158 million tons, and approximately 86% was consumed as food (FAO, 2014). A major proportion of fishery products is used for human consumption, and a huge quantity of fishery wastes and by-products is being generated by fishery-processing industries. In addition, significant amounts of fishery wastes are also generated by the aquaculture industry, and these wastes pollute surface and ground water. The organic wastes contain nitrates and phosphates; they can stimulate algal blooms, resulting in oxygen depletion and destruction of aquatic life. Either the fishery wastes are used as low market-value products such as fishmeal, fish oil, and fertilizers or simply dumped into the sea, causing a serious environmental problem (Arvanitoyannis and Kassaveti, 2008). Therefore, appropriate management strategies for various fishery wastes are required.

Discarded wastes are rich sources of protein, and fish protein hydrolysates produced from fishery wastes by proteolytic hydrolysis are marketable as nutritional supplements, functional ingredients and flavor enhancers (Je et al., 2008). Therefore, underutilized fishery wastes could be used to produce value-added products such as proteins, amino acids, glycerol, fatty acids, meal, lactic acid and ethanol (Ramakrishnan et al., 2013). Fish proteins in fishery wastes can be hydrolyzed by chemicals in a relatively inexpensive and simple way. However, it is difficult to control the whole process specifically

and leads to products with variable chemical composition and functional properties, limiting their use as food ingredients. Besides, hydrolysis performed at extreme temperatures and pH conditions may degrade the nutritional qualities of the products, resulting in poor functionality (Kristinsson and Rasco, 2000). Enzymatic hydrolysis was the preferred method for reutilization of fishery wastes because no residual organic solvents or toxic chemicals are present in the products. Under moderate conditions of pH and temperature, enzymatic hydrolysis is performed using endogenous or/and exogenous enzymes that influence the molecular size and hydrophobicity of the hydrolysate, but this process compromises the nutritive quality of the hydrolysate (Kristinsson and Rasco, 2000). Because of the low hydrolysis rate and high cost of enzymatic hydrolysis, microbial hydrolysis was introduced, particularly for the production of bioactive peptides (Fang, 2010). Microbial hydrolysis can produce a wide range of peptides that contain different amino acid sequences when it was performed by microorganisms possessing with extracellular proteases (Wang et al., 2015). The degree of hydrolysis depends upon the microbial species, substrate composition, and reaction conditions; thus, interesting bioactive properties can be observed.

Reutilization of fishery wastes would be a fruitful strategy, as it would address the environmental issue and result in economic gain (Awarenet, 2004). Fishery wastes are known to be valuable sources of raw materials such as bioactive compounds that play an important role in metabolic regulation and modulation (Sheriff et al., 2014). For

instance, biologically inactive peptides that exist within the sequences of source proteins exhibited various physiological functions after being released by enzymatic hydrolysis (Vercruysse et al., 2005). This biofunctional activity was found to be dependent on the structural properties of the peptides; thus, their amino acid composition and sequences are required for diverse biological functions.

Use of antioxidants, especially food-derived natural antioxidants, has been recognized as feasible and potentially effective in overcoming radical-mediated damage, as synthetic antioxidants have restricted applications in food products because of safety and negative consumer perception (Park et al., 2001). Compared with synthetic antioxidants, antioxidants derived from marine resources are easy to obtain, cheap, and safe with no adverse effects. To date, antioxidant activity has been found in fish protein hydrolysates from diverse species: yellow stripe trevally (Klompong et al., 2007), yellowfin sole frame (Jun et al., 2004), herring (Sathivel et al., 2003), mackerel (Wu et al., 2003), muscles of threadfin bream and flyingfish (Shabeena and Nazeer, 2010), backbone of flyingfish (Shabeena and Nazeer, 2011), and backbones of great barracuda and hairtail (Nazeer et al., 2011). In addition, antimicrobial peptides secreted by fish have been known to play major roles in the innate immune system and provide protection against bacterial, fungal, viral, and other pathogenic infections (Smith et al., 2010). As fungal diseases have become a growing threat, many studies have been conducted in an attempt to isolate natural antifungal substances with potential pharmaceutical utility (Alcouloumre et al.,

1993). Consequently, recovery of bioactive compounds from fishery wastes is significant because it would alleviate the problems related to the treatment of fishery wastes.

Mackerel (*Scomber scombrus* or *Scomberomorus niphonius*) is one of three fish (mackerel, squid, and hairtail) consumed by most people in Korea (http://portal.nfrdi.re.kr/upload/all/all_2010_07.pdf). Of the three fish, protein content is the richest in mackerel (http://portal.nfrdi.re.kr/upload/all/all_2010_07.pdf). Mackerel has been known to contain approximately 15.57% protein in the whole body; 12.3%, head; 17.17%, skin; 14.16%, frame; 3.92%, bone; and 12.18%, viscera (Li et al., 2013; Ramakrishnan et al., 2013). Mackerel is processed to enhance its food value: frozen mackerel is thawed, sorted, eviscerated, washed, salted, and finally washed (http://chickenofthesea.com/company/know-your-seafood/mackerel). Similar to most processing industries, mackerel processing produces a large volume of wastewater that contains organic contaminants, oils, etc. (Garcia-Sanda et al., 2003). Recently, the Ministry of the Environment in Korea changed the paradigm for waste policy and emphasized resource recycling with zero emission (Mathews, 2012), urging ecologically acceptable waste management.

The objective of this study was to demonstrate a new advance in waste management of mackerel wastewater (MWW) for the enhancement of its reutilization value. For the advanced waste management of MWW, it was first degraded by mixed microbes possessing protease, lipase, and carbohydrate-degrading enzymes. Culture broths containing metabolites produced from MWW

biodegradation were examined for antioxidant, antifungal, antibacterial, and DNA protective activity in order to find the reutilization value of MWW. To pick out specific amino acids affecting antioxidant activity among those produced from the MWW biodegradation, two-dimensional thin-layer chromatography (2D-TLC) was used. Finally, possibility of use of the remaining culture broth as a biofertilizer was tested using hydroponic culture in order to find out an advanced way to reutilize waste culture broth.

2. Materials and Methods

2.1. Design for advanced waste management

Advanced waste management was designed to reutilize MWW (Fig. 1). Sterile MWW was fed into a 3-l bioreactor and degraded using the mixed microbes. Bioactive compounds produced after appropriate biodegradation were extracted from the culture broth and tested for use as antioxidant, antifungal, antibacterial, and DNA protective agents. The remaining culture broth, including the mixed microbes, was tested for use as a biofertilizer for plant growth. Use of this design for advanced waste management led to no additional generation of wastes during the whole process and enhancement of the reutilization value of MWW.

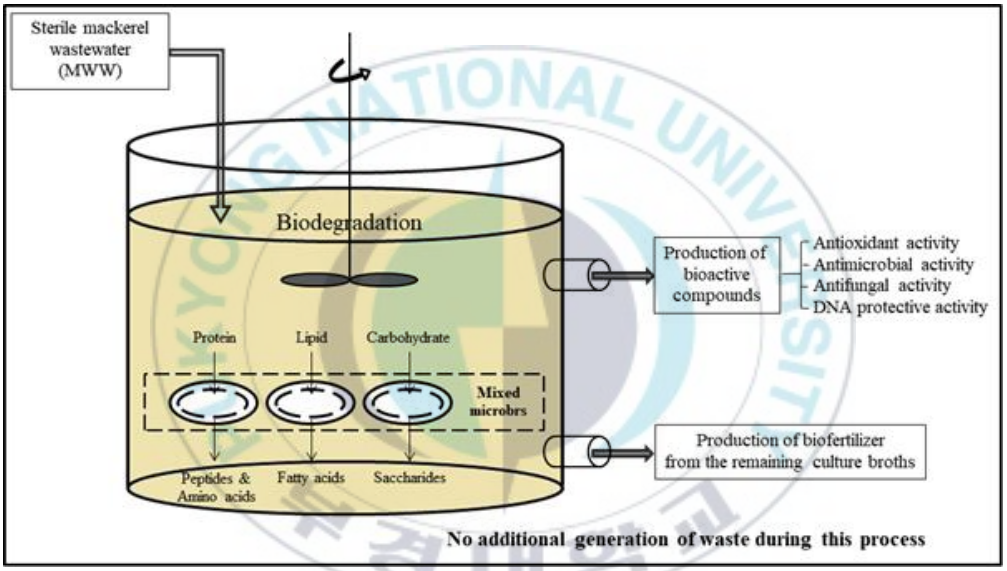


Fig. 1. Schematic design for efficient waste management of MWW.

2.2. Microorganisms and culture

The mixed microbes used in this study were composed of two patented bacteria, *Pknufermbacteria* (patent No. 10-0822239) and *Wormbacteria* (patent No. 10-1058245). *Pknufermbacteria* is a mixture of seven bacteria: *Bacillus subtilis* (DQ219358), *Bacillus licheniformis* (AY468373), *Bacillus coagulans* (AF466695), *Bacillus circulans* (Y13064), *Bacillus anthracis* (AY138279), *Bacillus fusiformis* (AY548950), and *Brevibacillus agri* (AY319301). *Wormbacteria* is a mixture of four bacteria: *Bacillus licheniformis* (EF113324, 99% similarity), *Bacillus cereus* (DQ923487, 99% similarity), *Brevibacillus agri* (AJ586388, 99% similarity), and *Brevibacillus parabrevis* (AB215101, 99% similarity). It was confirmed that there was no bacterial antagonist among them. For the preparation of seed culture, equal amounts of cells from each strain were first cultivated in a 100-ml flask containing 3-fold diluted MWW. Each cell was transferred to 0.5-fold diluted MWW and cultivated to adapt the cells to MWW to the maximum extent. Then, each cell proliferated until the late-log phase and was separately harvested and combined together for use as an inoculum for MWW degradation. Each pure strain was maintained on 1.5% nutrient agar plates at 4 ℃ until use and transferred to a fresh agar plate every two weeks.

2.3. Preparation of MWW

MWW was obtained from D company (Jangnim, Busan, Korea), which produces processed foods from mackerel. MWW (per liter) had the following characteristics: 29800 ± 2800 mg, chemical oxygen demand (COD_{Cr}); 3900 ± 350 mg, total nitrogen (TN); and 0.3 ± 0.01%, salt. When MWW was used in the biodegradation experiment, initial pH was adjusted to 7, and MWW was autoclaved at 121 °C for 15 min.

2.4. Biodegradation of MWW

The autoclaved MWW was aseptically transferred to a sterile 3-l bioreactor. Degradation of MWW was initiated just after 10% (v v^{-1}) seed culture of the mixed microbes was used for inoculation. Air (5 l min^{-1}) generated from an air compressor was supplied into the bioreactor through a 0.2-μm filter. The biodegradation was performed for 74 h, and samples were obtained periodically via a sampling port to measure changes in major reaction parameters (pH, dissolved oxygen [DO], oxidation-reduction potential [ORP], cell number, COD and TN) and protease activity. During the biodegradation, pH was adjusted between 6 and 8, and oxygen (80% purity) was additionally supplied four times through a 0.2-μm filter when the DO levels dropped to less than 1 mg l^{-1}.

2.5. Analyses

During the biodegradation, pH, DO, and ORP were measured using probes installed in the reactor. To measure the number of viable cells, samples obtained from the bioreactor were diluted appropriately and poured on nutrient agar plates. After the agar plates were incubated at 45 °C for 1 day, the number of colonies formed on the agar plates was counted. The number of viable cells was expressed as colony-forming units (CFU) per microliter of the sample. COD_{Cr} and TN in supernatant of the sample were analyzed using a Water-quality Analyzer (HS 2000; Humas Co., Ltd, Korea). For measuring protease activity, 5 μl of each sample obtained at 0, 24, 42, and 74 h was dropped at the center of skim-milk agar plates. After incubation at 45 °C for 1 day, the degree of protease activity was observed on the basis of the clear zones formed on the skim-milk agar plates. The confirmation of proteins and degradation products present in the culture supernatants was performed using SDS-PAGE and tricine SDS-PAGE, respectively.

2.6. Antioxidant activity

For measuring antioxidant activities, samples were obtained at 0, 24, 42, and 74 h of biodegradation. The samples were centrifuged, and the supernatants were used for the measurement. Further, peptides present in the sample were extracted and concentrated, and their antioxidant activities were measured to examine the level of enhancement.

2.6.1 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

DPPH free radical scavenging ability of the supernatant was determined according to the method described Blois (1958) with some modifications. First, 2 ml of 0.1 mM DPPH solution in 80% ethanol was added to 1 ml of the supernatant. The mixture was maintained at room temperature in the dark for 30 min, and the absorbance was measured at 517 nm (Opron-3000® UV/VIS Spectrophotometer, Hanson Technology Co., Ltd., Korea) against a blank sample. The blank sample was prepared by replacing DPPH with 80% ethanol. DPPH radical scavenging activity was calculated using the following formula:

$$\text{Scavenging activity (\%)} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

The control sample was prepared by mixing 1 ml of 80% ethanol with 2 ml of 0.1 mM DPPH, and 0.1 mM L-ascorbic acid was used as the positive control under the same assay conditions. The experiment was repeated three times.

2.6.2 2,2′-Azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical cation decolorization assay

ABTS radical cation decolorization assay was performed according to the method described by Re et al. (1999), with some modifications.

The ABTS radical cation (ABTS reagent) was prepared by mixing 5 ml of 7 mM ABTS with 5 ml of 4.9 mM potassium persulfate ($K_2S_2O_8$). The mixture was maintained at room temperature for 16 h in the dark. The absorbance of the ABTS reagent was then adjusted to 0.72 ± 0.02 at 734 nm with 80% ethanol. To determine scavenging activity, 900 μl of the ABTS reagent was added to 100 μl of the supernatant. After a 6-min interval, the absorbance was measured at 734 nm, and 0.3 mM L-ascorbic acid was used as the positive control. Percentage inhibition of the sample was calculated using the following equation:

$$\% \text{ inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

The control sample was prepared by replacing the supernatant with distilled water (DW), and the blank sample was prepared by replacing the ABTS reagent with 80% ethanol. The analysis was repeated three times.

2.6.3 Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity was measured according to the method described by Beara et al. (2009), with some modifications. A half-milliliter of the culture supernatant was mixed with 250 μl of ortho-phenanthroline (7.5 mM), 1.25 ml of phosphate buffer (0.2 M, pH 6.6), 250 μl of ferrous sulfate (7.5 mM), and 250 μl of H_2O_2 (0.5%). The mixed solution was diluted to a final volume of 6.25 ml with DW

and incubated at room temperature for 30 min after vigorous mixing. Then, the absorbance was measured using a 96-well microplate at 490 nm with the enzyme-linked immunosorbent assay. The scavenging percentage (P%) was calculated using the following formula:

$$P\ \% = \frac{A - A1}{A2 - A1} \times 100$$

where A is the absorbance value of all solutions, including H_2O_2 and the sample; A1 is the absorbance value without the sample; and A2 is the absorbance value without H_2O_2 and the sample.

2.6.4 Reducing power assay

A reducing power assay was performed according to the method described by Wu et al. (2010), with some modifications. One milliliter of the culture supernatant was mixed with 1 ml of 0.2 M phosphate buffer (pH 6.6) and 1 ml of 1% potassium ferricyanide. The mixed solution was incubated at 50 °C for 20 min in a shaking incubator. After incubation, the reaction was stopped by adding 1 ml of 10% (w v^{-1}) trichloroacetic acid to the reaction mixture, and the mixture was centrifuged at 3000 rpm for 10 min. From the upper layer, 2 ml of the solution was obtained and mixed with 2 ml of DW and 0.4 ml of 0.1% $FeCl_3$. The mixture was incubated for 10 min at room temperature, and then the absorbance of all sample solutions was measured at 700 nm. An increase in absorbance represented an increase in reducing power. The control sample was prepared by replacing the culture

supernatant with DW. The test was repeated three times.

2.7. Antimicrobial activities

To assess the antimicrobial activities of the culture supernatants in this study, one Gram-positive bacterium, three Gram-negative bacteria and one fungus were used. The test microorganisms were purchased from the Institutes of the Korean Culture Center of Microorganisms (KCCM, Seoul, Korea) and Korean Collection for Type Cultures (KCTC, Daejeon, Korea): *Staphylococcus aureus* (KCCM12256), *Escherichia coli* (KCCM11234), *Shigella flexneri* (KCCM409483), *Vibrio vulnificus* (KCCM41665) and the fungus (*Candida albicans* (KCTC7270)). The culture medium for *Staphylococcus aureus* and *V. vulnificus* was Brain-heart infusion broth (pH 7.4) containing (g l^{-1}) 5 g, beef heart; 12.5 g, calf brains; 2.5 g, Na_2HPO_4; 2 g, glucose; 10 g, peptone; and 5 g, NaCl. The culture medium for *E. coli* was Luria-Bertani broth (pH 7.0) containing (g l^{-1}) 10 g, tryptone; 5 g, yeast extract; and 10 g, NaCl. The medium for *S. flexneri* was Nutrient broth (pH 7.5) containing (g l^{-1}) 1 g, glucose; 15 g, peptone; 6 g, NaCl and 3 g, yeast extract. The medium for *Candida albicans* was YM broth (pH 6.2) containing (g l^{-1}) 3 g, yeast extract; 3 g, malt extract; 5 g, soybean peptone; and 10 g, glucose.

The paper-disc diffusion method was used to assay the antimicrobial activities of the culture supernatants. Five milliliters of soft agar (0.7%) was mixed with 50 μl of culture broth incubated for 12 h with

each test strain, and the mixture was poured into a petri dish. The incubation temperatures of each test strain were 27 ℃ for *S. aureus*, 37 ℃ for *E. coli*, 37 ℃ for *S. flexneri*, 30 ℃ for *V. vulnificus* and 24 ℃ for *C. albicans*, respectively. After agar solidification, sterile 6-mm paper discs (ADVANTEC®, Japan) were placed equidistantly in each petri dish. Then, 5 ㎕ of each culture supernatant was loaded onto the paper discs. The culture supernatant was prepared by centrifugation at 7000 rpm for 15 min, followed by filtration of the supernatant through a 0.2-㎛ cellulose acetate filter (CORNING®, Germany). The petri dishes were turned then upside down and incubated at the optimum temperatures for 24 h. After incubation, the diameter of the inhibitory zone formed around each disc was measured in millimeters. All assays were performed in du-plicate.

2.8. DNA protective activity

DNA protective activity of the culture supernatant was examined according to the method described Kim et al. (2012). λ DNA (4 ㎍) was exposed to hydroxyl radicals generated by a mixture of L-ascorbic acid (1 mM) and copper (II) sulfate (0.1 mM) in the presence and absence of the culture supernatant. One hundred microliters of each culture supernatant collected at 0, 6, 24, 42 and 74 h was used to evaluate DNA protective activity, and DW was used as the control. The whole mixture was incubated at 37 ℃ for 1 h. An aliquot of 10 ㎕ was loaded onto a 1% agarose gel in 1 × TAE

(mixture of Tris base, acetic acid and EDTA) buffer, and electrophoresis was performed at 100 V for 20 min. DNA bands were visualized using ethidium bromide under a UV transilluminator and documented using a Polaroid webcam (Vilber Lourmat, Marne-la-Vallee).

2.9. One-dimensional (1D)- and 2D-TLC

To examine the effect of culture supernatants on antioxidant activity, both 1D-TLC and 2D-TLC were performed using the samples collected during biodegradation. For both analyses, known amino acids that affect antioxidant activity were used as standard markers.

2.9.1. 1D-TLC

Degradation products of MWW were analyzed using TLC. One and a half microliters of the culture supernatant collected from the bioreactor at 0, 24, 42 and 74 h was applied once onto the TLC Silica Gel 60 plate (plate, 15 cm × 10 cm; Sigma-Aldrich, Germany), and then chromatography was performed using a mobile phase of 3:1:1 (v v^{-1} v^{-1}) n-butanol:acetic acid:DW. The products were stained using ninhydrin solution (0.2% w v^{-1} in acetone), followed by baking at 100 °C for 4 min. A mixture (0.2%) of glutamic acid, glutamine, histidine, leucine, lysine, methionine, tryptophan, phenylalanine, and tyrosine was run alongside as the marker.

2.9.2. 2D-TLC

Degradation products of MWW were analyzed using TLC. One and a half microliters of the culture supernatant collected from the bioreactor at 0, 24, 42 and 74 h was applied once onto TLC Silica Gel 60 plates (plates, 10 cm × 10 cm, 15 cm × 15 cm, 17 cm × 17 cm, and 20 cm × 20 cm; Sigma-Aldrich, Germany). Chromatography for each culture supernatant was performed using a mobile phase of 3:1:1 (v v^{-1} v^{-1}) n-butanol:acetic acid:DW. Then, the plate was picked up using a pincette and dried at room temperature for 1 h. For the second chromatography, the plate was turned by 90° in a counterclockwise direction and run using a mobile phase of 7:3 (v v^{-1}) phenol:DW. The products were stained using ninhydrin solution (0.2% w v^{-1} in acetone), followed by baking at 100 °C for 4 min. Like 1D-TLC, a mixture (0.2%) of glutamic acid, glutamine, histidine, leucine, lysine, methionine, tryptophan, phenylalanine, and tyrosine was run alongside as the marker.

2.10. Seed germination test

To evaluate the phytotoxicity of biodegraded MWW, the seed germination test was performed using the method reported Wong et al. (2001). Ten milliliters of the 74-h biodegraded MWW sample collected from the 3-l bioreactor was centrifuged at 8000 rpm, filtered through a 0.45-μm membrane filter, and maintained at 4 °C until use. For tests

of seed germination and root length, 5 ml of the prepared filtrate was pipetted into a sterile petri dish lined with Whatman #1 filter paper (Sigma-Aldrich, St. Louis). In parallel, 5 ml of the 74-h biodegraded MWW sample containing mixed microbes was pipetted into a petri dish to test its use as a biofertilizer. In this study, seeds of cress (*Lepidium sativum*) were used, and they were preliminarily tempered at 25 °C for 12 h in the dark. Then, 10 cress seeds were evenly placed in each petri dish, and DW was used as the control. The plates were incubated at 25 °C in the dark at 75% humidity. Seed germination and root length in each plate were measured after 72 h of incubation. All measurements were performed in triplicate.

Percentages of relative seed germination (RSG), relative root growth (RRG), and germination index (GI) after exposure to the treated wastewater were calculated as follows.

$$\text{RSG (\%)} = \frac{\text{Number of seeds germinated in biodegraded wastewater}}{\text{Number of seeds germinated in control}} \times 100$$

$$\text{RRG (\%)} = \frac{\text{Mean root length in biodegraded wastewater}}{\text{Mean root length in control}} \times 100$$

$$\text{GI (\%)} = \frac{\text{RSG} \times \text{RRG}}{100}$$

2.11. Hydroponic culture

To test the efficacy of biodegraded MWW as a fertilizer produced, a hydroponic culture system was used to cultivate red bean (*Vigna*

angularis) and barley (*Hordeum vulgare* L.) in a mini-hydroponic culture pot (5 × 12 × 8 cm^3) against the control (DW). Tests were performed using MWW culture broth containing mixed microbes at a 1000-fold dilution. Efficacy of the 1000-fold diluted culture supernatant as a fertilizer was also performed in parallel to examine how the presence and absence of mixed microbes affects the growth of the plants during hydroponic culture. The hydroponic culture pot used in this study was composed of a glass vessel with a plastic screen inside. In each pot, 10 seeds of red bean or 25 seeds of barely were placed on top of the plastic screen, and a 300-ml solution of the 1000-fold diluted MWW culture broth was added underneath the plastic screen. The seeds were previously incubated at 25 °C for two days in the dark, and they were soaked throughout the period; roots grew through the pores of the plastic screen. After the seeds germinated, the pots were placed by the window throughout the day so that the plants could obtain necessary sunlight for growth. Day and night air temperatures were maintained at 22 ± 2 °C and 18 ± 2 °C, respectively, by natural ventilation and heating. Water temperature was 15 ± 3 °C, and relative humidity in the room was 60 ± 5%. The fertilizer solution was refreshed every three days. Growth of the plants was observed periodically, and height, thickness of the stem, number of leaves, and length of a leaf from each plant were measured. All measurements were performed in triplicate.

2.12. Statistical analysis

Statistical analysis was performed on measurements obtained from this study. Because the sample observations were not arranged in a frequency distribution, the standard deviations were computed by the following procedures: each deviation was squared, the sum of the squares was divided by (n-1), one less than the sample size (n), and then extraction of the square root recovered the original scale of measurement. The comparison of means was accomplished by the Tukey method (Neter et al., 1985) using the SAS program, because all sample sizes were equal. The difference was considered significant at $P < 0.05$.

3. Results and Discussion

Heterotrophic microorganisms have been reported to be involved in the degradation of synthetic and natural polymers as potential substrates (Gu et al., 2000). Due to the action of enzymes secreted by mixed microbes, natural polymers present in MWW were transformed into smaller molecules. However, biodegradation of polymer substrate can rarely reach 100%, since material carbons couple with microbial growth (Gu, 2003). Therefore, some of carbons originally present in MWW incorporate into cell component and the others incorporate into various types of metabolic products. In this study, MWW was first degraded. Then, characteristics of culture broths containing bioactive compounds produced by the biodegradation were analyzed. The remaining culture broth at the end of biodegradation was tested for use as a biofertilizer.

3.1. Biodegradation of MWW

The biodegradation characteristics of MWW were examined using a 3-l bioreactor for 74 h (Fig. 2A). The mechanism of MWW degradation involved utilization of the polymer by mixed microbes as a source of carbon and energy. It has been reported that the degradation mechanism is related to the chemical structures (molecular composition), molecular weights, the participating microorganisms and environmental conditions, determining the extent of biodegradation

(Sheik et al., 2015). In this study, major mechanism of MWW degradation was the depolymerization of protein. With the cleavage of peptide bonds, the depolymerization of protein yielded small-size peptides and amino acids, especially by extracellular proteinases (Zhang et al., 2014, Wang et al., 2015). As shown in Fig. 2A, DO level in the bioreactor immediately after inoculation started to decrease because of active biodegradation by the mixed microbes. During the biodegradation, oxygen was supplied when active biodegradation occurred, so the DO level was maintained at more than 2mg l^{-1}. After 42 h, the DO level increased gradually, and the final value was 5.9 mg l^{-1}. DO level should be maintained at more than 1 mg l^{-1} for aerobic biodegradation (Tohyama et al., 2000). This is because oxygen is a key substrate in aerobic biodegradation because of its low solubility. Therefore, a continuous transfer of oxygen from the gas phase to the liquid phase is required to maintain the oxidative metabolism of the mixed microbes. In this study, it seemed that the additional supply of oxygen in the 3-l bioreactor met the demand of the mixed microbes.

The pH value was adjusted to 7.02 in the beginning, and the pH decreased as the biodegradation started. For the maintenance of stable biodegradation, pH was adjusted between 6 and 8 from 6 to 42 h. ORP was 130.2 mV in the beginning, and it deceased rapidly after 6 h and reached the minimum value at -243.4 mV after 30 h. Then, ORP increased to 38.5 mV after 42 h and slowly increased to the final value (71.2 mV). Cell number increased from 0.67 × 10^8 CFU ml^{-1} to 4.3 × 10^8 CFU ml^{-1} after 24 h. Then, it decreased gradually to 2.2 ×

10^8 CFU ml^{-1} in the end. On the basis of the changes in the reaction parameters, active biodegradation of MWW occurred between 6 and 24 h. In this period, DO level, pH, and ORP decreased, while cell number increased. These changes in the reaction parameters were consistent with the result obtained by Gwon and Kim (2012), who studied the biodegradation of fishmeal wastewater. After 24 h, a pleasant smell was noted. Disappearance of the fishy smell after active biodegradation was also reported by Kim and Lee (2009), who studied fishmeal wastewater treatment. Fish oil in the culture broth was also gradually degraded.

Concentrations of COD_{Cr} and TN decreased as the mixed microbes grew on the organic matter of MWW. After 74 h of biodegradation, concentrations of COD_{Cr} and TN were reduced to 7460 mg l^{-1} and 550 mg l^{-1}, respectively. The removal percentages of COD_{Cr} and TN were 75.4% and 66.2%, respectively. During the biodegradation, COD_{Cr}/TN was in a range of 10.2-18.8. This COD_{Cr}/TN ratio was rather high, when compared with that (5.2-7.5) obtained by Kim and Lee (2008), who studied the biodegradation of fishmeal wastewater. In general, a high C/N ratio stimulates cells to produce metabolites, while a low C/N ratio leads to biomass formation. Accordingly, diverse degradation products could be obtained from the organic compounds present in MWW, instead of an increase in biomass.

To acquire evidence of the breakdown of proteins initially present in MWW to low molecular weight oligomers over time in culture, culture supernatants were analyzed by gel electrophoresis. As seen in Fig. 2B,

the degradation products migrated down as proteins were biodegraded. The degradation products displayed several single bands on Tricine gel electrophoresis (Fig. 2C), indicating the production of small peptides and amino acids.

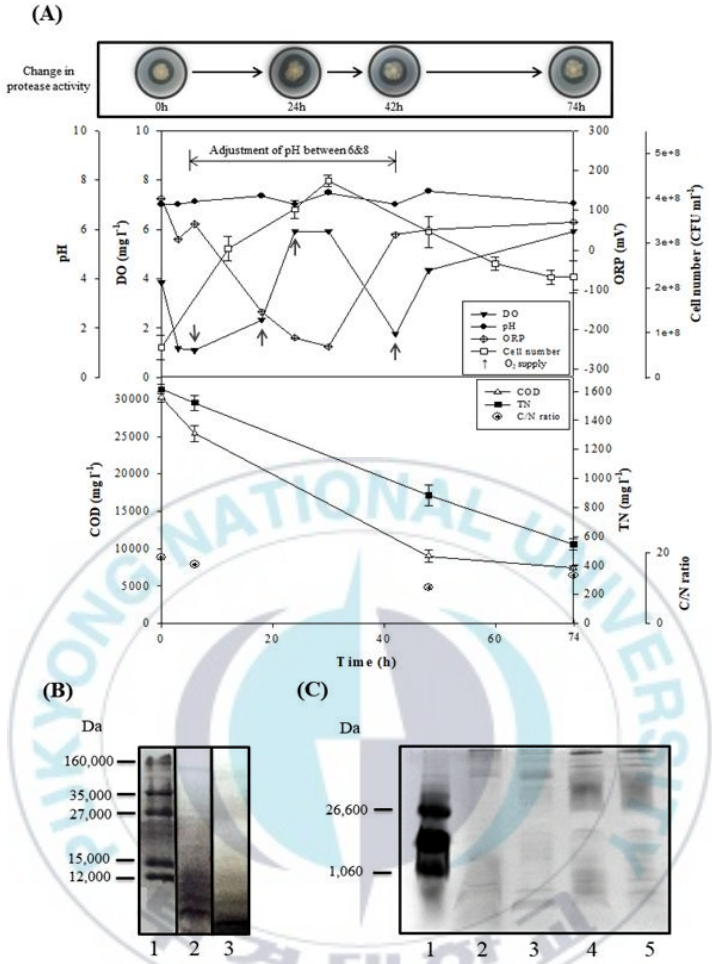


Fig. 2. (A) Profiles of the reaction parameters and change in protease activity during the biodegradation of MWW under optimal conditions. Error bar: mean ± S.D. of three replicates. (B) Confirmation of protein by SDS-PAGE: Lane 1, marker proteins; and Lane 2-3, degradation products present in culture supernatants at 0 h and 24 h, respectively. (C) Confirmation of oligomers by tricine SDS-PAGE: Lane 1, marker peptides; and Lane 2-5, degradation products present in culture supernatants at 0 h, 24 h, 42 h and 74 h, respectively.

3.2. Bioactivities of MWW culture supernatant

During MWW biodegradation, the culture broths were sampled to check whether they exhibited antioxidant, antimicrobial, antifungal, and DNA protective activities.

3.2.1. Antioxidant activity of the culture supernatants

Free radicals are highly reactive oxygen species. They are unstable and react rapidly with other substances in the body, causing cell or tissue damage (Wang et al., 2008). Thus, an imbalance between generation and exclusion of reactive oxygen species inside the human body results in the oxidative modification of cellular macromolecules and diverse health disorders such as diabetes mellitus, cancer, and neurodegenerative and inflammatory diseases (Butterfield et al., 2002). Recently, there has been a strong need for effective antioxidants from natural sources as alternatives to synthetic antioxidants in order to prevent free radical-induced diseases (Ramakrishna et al., 2012). Natural antioxidants are well known for scavenging the free radical chain of oxidation and forming stable free radicals, which prevent further oxidation (Azam et al., 2014). Therefore, the antioxidant potential of biodegraded MWW was evaluated using the DPPH free radical scavenging assay, ABTS radical cation decolorization assay, hydroxyl radical scavenging activity, and reducing power assay. A natural antioxidant, L-ascorbic acid, was used as the positive control.

Ability of the culture supernatant to scavenge long-lived deep violet organic nitrogen radicals of DPPH to pale yellow hydrazine was measured at 517 nm, and the low absorbance of the reaction mixture indicated high free radical scavenging activity. As shown in Table 1, the DPPH free radical scavenging ability of the culture supernatant was between 73.7% and 88.7% during the 74 h of biodegradation. The scavenging ability on DPPH was increased up to 88.7% when the culture was incubated for 42 h, while L-ascorbic acid (0.1 mM) showed 82.3% DPPH scavenging activity. This indicates that metabolic products with DPPH free radical scavenging activity were produced during MWW biodegradation. The DPPH radical is extensively used to measure the free radical scavenging capacity of antioxidants (Halliwell and Gutteridge, 1999). Hydrolysates of the blue mackerel (*Scomber australasicus*) (Wu et al., 2003) and backbone of the Indian mackerel (*Rastrelliger kanagurta*) (Sheriff et al., 2014) have been reported to have 60-77% and 46% DPPH radical scavenging activity, respectively. The MWW culture supernatant used in this study displayed a higher level of DPPH scavenging activity.

The ABTS radical cation scavenging activity of the culture supernatant was recorded to be between 90.1% and 99.7% during the 74 h of fermentation (Table 1), while L-ascorbic acid (0.3 mM) displayed 71.4% ABTS radical scavenging activity. On the basis of the 99.7% scavenging measured in the 42-h culture supernatant, the ABTS radical scavenging activity of the culture supernatant was stronger than the DPPH radical scavenging activity. DPPH has been reported to

be more specific for lipophilic antioxidants and ABTS, for both lipophilic and hydrophilic antioxidants (Prior et al., 2005). Accordingly, high ABTS radical scavenging activity in this study implies that the antioxidant compounds present in the culture supernatant were most likely hydrophilic.

The hydroxyl radical scavenging activity of the culture supernatant was between 69.8% and 96% during the 74 h of biodegradation, while L-ascorbic acid (0.1 mM) displayed 45.5% scavenging activity (Table 1). Maximum scavenging activity was observed in the culture supernatant fermented for 42 h. The hydroxyl radical is a highly reactive oxidizing species that can react with most biomolecules. It is responsible for the formation of other radicals. Certain peptides or free amino acids were found to be partly responsible for the total antioxidant activity (Bellaaj et al., 2012). The abovementioned results suggest that the culture supernatant had a strong ability to donate electrons to reactive free radicals, converting them into more stable products and terminating the free radical chain reaction. Consequently, it could be concluded that a good candidate for a natural antioxidant was produced by the biodegradation of MWW with mixed microbes.

The reducing power assay was used to evaluate the ability of any antioxidative compound present in the culture supernatant, and it serves as a significant indicator of potential antioxidant activity (Gao et al., 2012). The antioxidative effect is related to the development of reductones (Yen and Duh, 1993) and directly correlated with the reducing power of certain bioactive compounds (Bellaaj et al., 2012).

Thus, the presence of reductants in the culture supernatant caused the reduction of the ferric cyanide complex to a ferrous form during the assay. As shown in Table 1, the reducing power of the culture supernatant was between 0.30 and 0.35 during the 74 h of incubation, while the reducing power of L-ascorbic acid (0.1 mM) was 0.02 at 700 nm. Reducing power increased with an increase in the absorbance. Maximum reducing power of the culture supernatant was reached after 42 h of incubation.

Antioxidants hydrolyzed from various fish proteins have health promoting potential for nutritional, pharmaceutical, cosmetic, and nutraceutical applications as functional ingredients (Chalamaiah et al., 2012). As such antioxidants also have no adverse effects, many studies on antioxidants from marine resources are being conducted. Table 2 lists the antioxidant activities of various types of mackerel hydrolysates. Collagen hydrolysates obtained from pepsin in mackerel skin and hydrolysates obtained from mackerel flesh displayed higher reducing power than other types of hydrolysates. Hydrolysates obtained after MWW biodegradation in this study displayed higher DPPH radical scavenging, ABTS radical scavenging, and hydroxyl radical scavenging activities than hydrolysates obtained from other parts of the mackerel. The proteins used in each experiment had different amino acid compositions, and proteases randomly hydrolyzed them into peptides. Thus, the sizes of peptides degraded from various proteins were different and had diverse amino acid compositions, resulting in the discrepancy in antioxidant activities. Therefore, MWW

and the mixed microbes used in this study are a good resource for producing potential antioxidant peptides.

Table 1. Changes in various antioxidant activities of culture supernatants during the biodegradation of MWW[1].

Culture time (h)	Antioxidant activity			
	DPPH radical scavenging activity (%)	ABTS radical scavenging activity (%)	Hydroxyl radical scavenging activity (%)	Reducing power (A_{700})
0	73.7±2.13^{c}	90.1±2.52^{c}	69.8±3.33^{c}	0.30±0.01^{c}
24	84.6±1.50^{b}	98.1±1.03^{a}	85.2±2.01^{b}	0.32±0.01^{b}
42	88.7±1.14^{a}	99.7±1.52^{a}	96.0±1.10^{a}	0.35±0.01^{a}
74	87.6±1.07^{a}	94.4±1.45^{b}	83.0±2.22^{b}	0.34±0.01^{ab}

[1]Means in the same column with different superscript are significantly different ($P < 0.05$). Values represent mean ± S.D. of three replicates.

Table 2. Comparison of antioxidant activities of various mackerel hydrolysates.

Source		DPPH radical scavenging activity (%)	ABTS radical scavenging activity (%)	Hydroxyl radical scavenging activity (%)	Reducing power (A_{700})	Reference
Backbone	Pepsin hydrolysate	46	n.d.[a]	n.d.	0.24	Sheriff et al. (2014)
	Papain hydrolysate	36	n.d.	n.d.	0.19	
Collagen hydrolysates from skin		65.7	n.d.	77.0	2.87	Chi et al. (2014)
Hydrolysates from flesh		78	n.d.	n.d.	0.67	Wu et al. (2003)
Hydrolysates from the biodegradation of MWW		88.7	99.7	95.9	0.35	This study

[a]n.d.: not detected.

3.2.2. Effects of specific amino acids on antioxidant activity

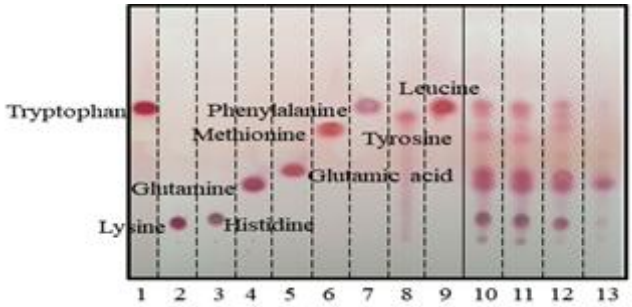
TLC is used to separate non-volatile mixtures, and it can be used to monitor the progress of MWW biodegradation and identify compounds present in the culture broth. In this study, diverse low-molecular-weight peptides and amino acids were produced during MWW biodegradation. Low-molecular-weight peptides have been reported to exhibit better radical scavenging activities than their high-molecular-weight counterparts (Ajibola et al., 2011). Amino acids such as histidine, tyrosine, methionine, and cysteine also have antioxidant activity, and histidine exhibits an especially strong radical scavenging activity because of the decomposition of its imidazole ring (He et al., 2012). Hydrophobic amino acids, including valine, leucine, phenylalanine, and tryptophan, exhibit high antioxidant potential (Virtanen et al., 2007). This is because these amino acids possess excess electrons that can be donated and used to quench free radicals or reduce metal cations (He et al., 2012). In addition, metal-chelating amino acid residues, such as methionine, glutamic acid, glutamine, lysine, or arginine, within the sequences of these peptides were reported to contribute to the superior Fe^{2+}-chelating ability of the antioxidant peptides as well as their high radical scavenging potential (Zhu et al., 2014). Thus, in this study, nine amino acids that affect antioxidant activity were selected as markers, and the accumulation of these amino acids in the culture broth along with culture time was examined.

First, 1D-TLC was performed using the culture supernatant and marker amino acids (Fig. 3A). The position of each marker amino acid was not directly related to its structure, molecular weight, or hydrophilic/hydrophobic characteristics; this indicates that it is very difficult to conclude how high a specific amino acid would be drawn up the plate via capillary action. Besides, the color of each amino acid on the TLC plate was not clearly differentiated. When the supernatant samples collected at different culture times were used, some migration of the degraded products was observed on the TLC plate. However, amino acids or small peptides could overlap in a band. On the basis of these results, it was concluded that 1D-TLC was not able to clearly identify each amino acid in the culture supernatant.

Then, 2D-TLC was performed (Fig. 3B). Unlike 1D-TLC, each marker amino acid was positioned separately on the TLC plate, with horizontal and vertical differences. It was clearly shown that each culture supernatant had a different amino acid composition. The dominant amino acids contained in the supernatants were as follows: lysine, glutamic acid, methionine, and tyrosine at 0 h; lysine, glutamic acid, glutamine, methionine, leucine, and tyrosine at 24 h; lysine, glutamic acid, methionine, leucine, tryptophan, phenylalanine, and tyrosine at 42 h; and lysine, glutamic acid, glutamine, and methionine at 74 h. Among the four culture supernatants, the 42-h culture supernatant showed the highest number of amino acids. Especially, phenylalanine and tryptophan were identified in only the 42-h culture supernatant. Various antioxidant activities were the highest in the 42-h

culture supernatant (subsection 3.2.1). The high antioxidant activity was probably attributable to these hydrophobic amino acids (Virtanen et al., 2007). Thus, 2D-TLC was useful in monitoring the progress of MWW biodegradation and identifying amino acids present in the culture broth.

(A)



(B)

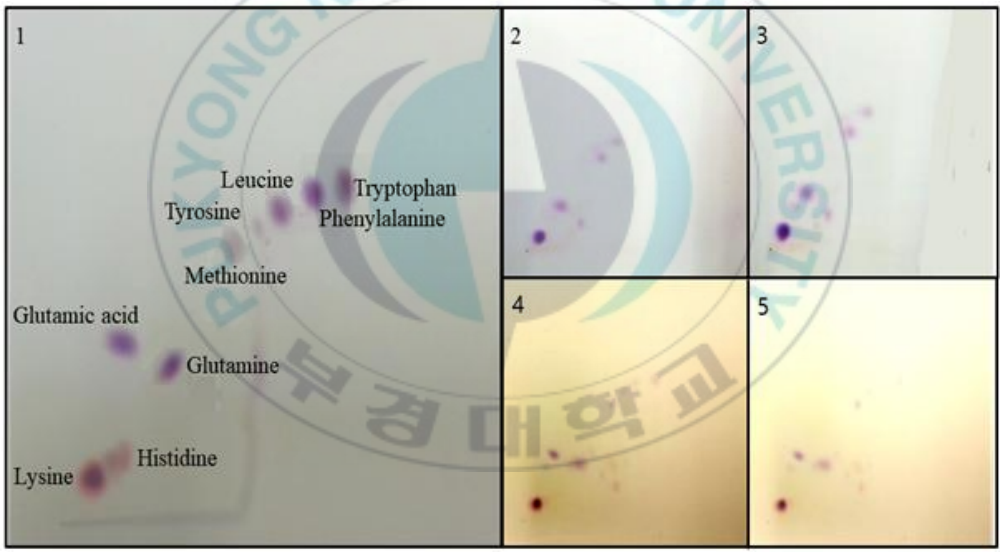


Fig. 3. TLC of the degradation products present in the biodegraded MWW. (A) 1D-TLC. Lane 1-9: marker amino acids and Lane 10-13: 0-, 24-, 42- and 74-h of culture supernatants, respectively; and (B) 2D-TLC. Box 1: marker amino acids and Box 2-5: 0-, 24-, 42- and 74-h of culture supernatants, respectively.

3.2.3. Other bioactivities of the culture supernatants

Antimicrobial, antifungal, and DNA protective activities of the MWW culture supernatants at various culture times were examined to enhance the reutilization value of MWW. Antimicrobial peptides have been reported to play a major role in native immunity by interacting directly with bacteria; thus, a wide variety of organisms produce such peptides (Zhang et al., 2008). In general, antimicrobial peptides have less than 50 amino acids, about half of which are hydrophobic. These peptides can be generated by enzymatic hydrolysis (Shahidi and Zhong, 2008). Like other organisms, fish exude various antimicrobial peptides, which are positively charged, short-chain molecules. To examine the antimicrobial function of the degradation products, the culture supernatants at various culture times were assayed using four pathogenic bacteria. The culture supernatants displayed antimicrobial activity only against *Staphylococcus aureus*, a gram-positive bacterium, while no activity against all gram-negative bacteria was observed (Fig. 4A). Sizes of the clear zones formed around the paper discs were 0.6, 0.6, 0.8, and 0.4 cm for 0-h, 24-h, 42-h and 74-h culture supernatants, respectively. Thus, the antimicrobial activity increased with the increase in MWW culture time until 42 h; then, the culture supernatant showed lower antimicrobial activity. A similar result of antimicrobial activity against only *S. aureus* was obtained by Trabulsi and Atterthum (2005), who studied the antimicrobial activity of a compound extracted from tilapia liver. These effects may be due to the

cell wall structure in bacteria. Gram-positive bacteria possess a thick outer layer, while gram-negative bacteria have a thin layer and an outer cell wall. Thus, this outer cell wall acts as an additional barrier for obstructing the entry of some antibacterial agents. Therefore, fermented hydrolysates may be used in the food industry as natural preservatives against foodborne pathogens.

To examine the antifungal activity of metabolic products obtained after MWW biodegradation, the culture supernatants at various culture times were assayed using a pathogenic fungus, *Candida albicans*. As shown in Fig. 4B, the culture supernatants at different culture times displayed some reaction around the paper discs, but no distinct clear zones were observed. The function of antifungal peptides is to either disrupt the membrane structure or interfere with the biosynthesis of essential cellular components (Debono and Gordee, 1994). Polysaccharide complexes in *Chlorella pyrenoidosa* have been reported to inhibit the proliferation of *C. albicans* (Mata et al., 2010). The growth of *C. albicans* was also inhibited by an antifungal sponge peptide containing a large number of D-amino acids and unusual amino acids such as *tert*-leucine, cysteinoic acid, and sarcosine (Fusetani, 2010). However, to the best of our knowledge, there have been no studies on antifungal peptides from fish hydrolysates. Similarly, antifungal activity of the culture supernatant against *C. albicans* was not prominently observed in this study.

In this study, to assess the DNA protective effect of the culture supernatant, hydroxyl radical-induced DNA damage was generated

both in the presence and absence of the culture supernatant. In this assay, significant DNA protective action by each culture supernatant (100 μl) was clearly visible on a 1% agarose gel. As shown in Fig. 4C, the λ DNA treated with hydroxyl radicals displayed a clear band in the presence of each culture supernatant, whereas the DNA band was smeared in the absence of the culture supernatant. Recently, there has been great interest in evaluating the protective activity of natural antioxidant compounds against damage to important cellular components. Free radical-induced damage to DNA was explained by the reaction of hydroxyl radicals with guanine, which led to mutation (Saenjum et al., 2010) and, eventually, cell death (Kim et al., 2012). Therefore, this result suggests that the culture supernatant has a strong protective effect against oxidative DNA damage.

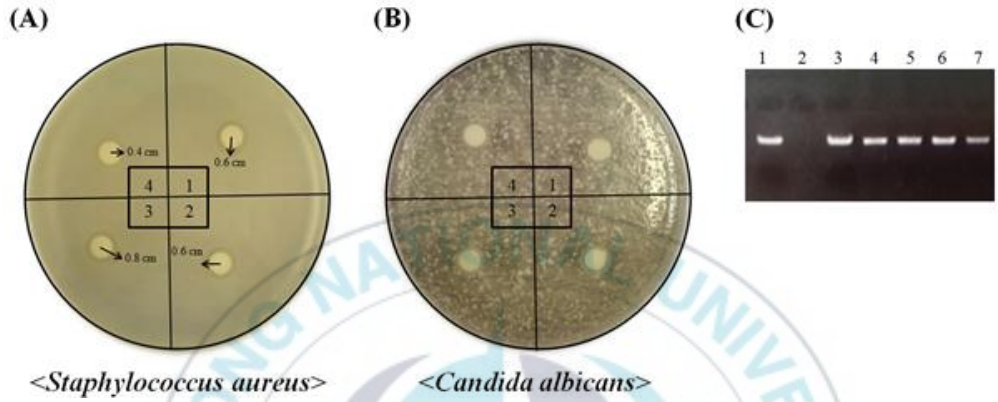


Fig. 4. Various biofunctional activities of MWW culture supernatants. (A) Antimicrobial activities of culture supernants at 0 h (1), 24 h (2), 42 h (3) and 74 h (4); (B) Antifungal activity of culture supernants at 0 h (1), 24 h (2), 42 h (3) and 74 h (4); and (C) Electrophoresis of λ DNA demonstrating protective effect of the biodegraded MWW. Lane 1: undamaged DNA, Lane 2: DNA exposed to Cu (II) and ascorbic acid, and Lane 3-7: DNA exposed to Cu (II) and ascorbic acid in the 0-, 6-, 24-, 42- and 74-h of culture supernatants, respectively.

3.3. Utilization of the remaining MWW culture broth

The remaining culture broth at the end of the biodegradation had to be utilized for advanced waste management of MWW. In this study, the efficacy of the remaining culture broth containing microorganisms as a biofertilizer was examined.

3.3.1. Phytotoxicity of biodegraded MWW

As biodegraded MWW contains compounds useful for plants, it could be used as a fertilizer; however, its application depends on the absence of any toxicity. Thus, the final culture supernatant collected after 74 h of biodegradation was assayed for phytotoxicity by using cress seeds, the 74-h culture broth containing mixed microbes was assayed in parallel (Fig. 5A). The cress seeds did not germinate when the original culture supernatant and culture broth were used without dilution. As the dilution ratios of both the culture supernatant and culture broth increased, GI tended to increase. Low GI values imply that certain compounds in the culture supernatant had an adverse effect on the root growth of cress seeds. Cress is sensitive to the toxic effects of ammonia and low-molecular-weight organic acids (Fuentes et al., 2004). At 50-fold dilution, the GI value was approximately 75% and 76% for the culture supernatant and culture broth, respectively. A GI value of 50% has been used as an indication of phytotoxin-free compost (Zucconi et al., 1985). According to this GI

criterion, the culture supernatant or culture broth of MWW required a more than 50-fold dilution to achieve stabilization of organic matter and maintain long-term fertility in the soil (Kim et al., 2010). Both the culture supernatant and culture broth that were diluted by more than 500-fold showed a GI value of over 100%, indicating the absence of any toxic compounds. Phytotoxicity caused by organic compounds has been reported to be remedied by aerobic decomposition (Wong et al., 2001). Moreover, the GI value of the culture broth was higher than that of the culture supernatant at dilution ratios of more than 500-fold. In horticulture, liquid fertilizer at 1000-fold dilution was reported to be often used for general purposes (Kim and Lee, 2009). On the basis of the abovementioned results, the possibility of using the culture broth as a biofertilizer appeared to be high.

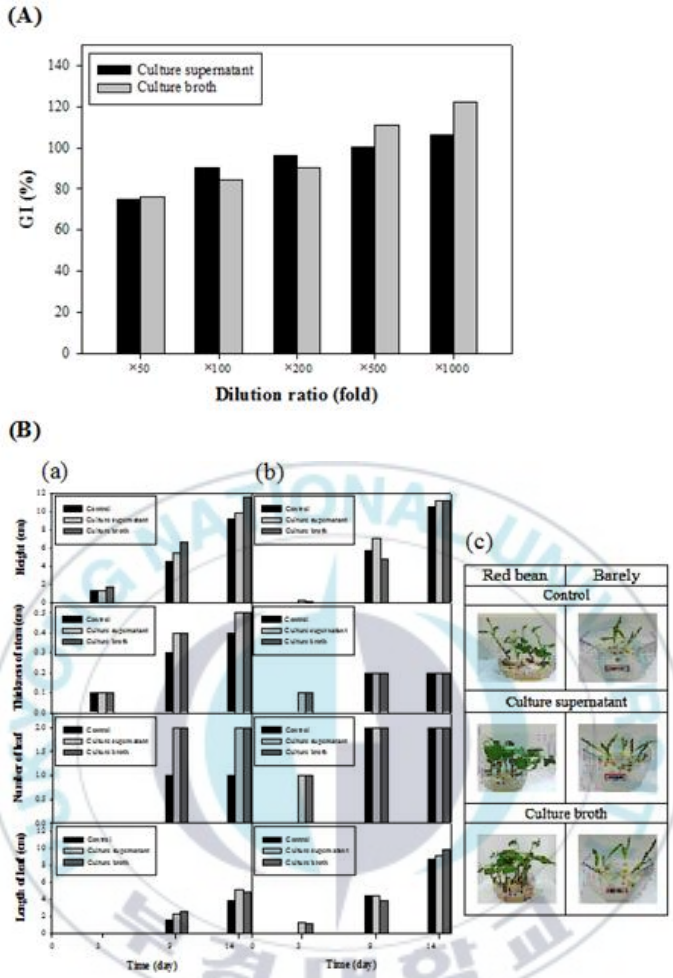


Fig. 5. Results of phytotoxicity (A) and hydroponic culture (B) for 1000-fold diluted final culture broth collected after 74-h biodegradation. During the hydroponic culture, increase in height, thickness of stem, number of leaf, and length of leaf are shown for red bean (a) and barely (b); and the photos (C) of the resultant hydroponic culture after 14 d are shown against control. Different letters on the bars indicate significant difference ($P < 0.05$) between groups.

3.3.2. Efficacy of biodegraded MWW as a biofertilizer

Biodegraded MWW culture broth was found to contain amino acids, and no phytotoxicity was observed at a high dilution ratio. Amino acids participate in many metabolic networks that control growth and adaptation to the environment. In young plants, amino acid biosynthesis is regulated by a compound metabolic network that links nitrogen assimilation with carbon metabolism. Accordingly, use of the culture broth as a fertilizer is an attractive application. In this study, efficacy of the 1000-fold diluted culture broth containing microorganisms as a biofertilizer was tested using hydroponic cultures of red bean and barley. As roots elongated, stems and leaves of both plants grew (Fig. 5B(a) and 5B(b)). For both plants, hydroponic culture using either the culture broth (containing microorganisms) or the culture supernatant (excluding microorganisms) resulted in better height, thickness of the stem, number of leaves, and length of a leaf than the control. Moreover, the results showed that plant growth in the culture broth was adequately comparable to that in the culture supernatant for both plants, indicating the possible use of the culture broth as a biofertilizer. The growth of both plants after 14 days of hydroponic culture is shown in Fig. 5B(c). In the cultivation of red bean or barley, both the culture supernatant and the culture broth displayed better growth than the control.

Biofertilizers are composed of microorganisms that colonize the rhizosphere or the interior of the plant. Biofertilizers provide nutrients through the nitrogen fixation process, solubilizing phosphorus and

stimulating plant growth through the synthesis of growth-promoting substances (Vessey, 2003). Organisms that are commonly used as biofertilizers are nitrogen fixers, potassium solubilizers, and phosphorus solubilizers. The representative nitrogen-fixing bacteria are *Clostridium pasteurianum*, *Azotobacter*, *Rhodobacter*, cyanobacteria, and some methanogens. *Bacillus mucilaginosus* is known to be a potassium solubilizer, while *Bacillus megaterium*, *Bacillus circulans*, *Bacillus subtilis*, and *Pseudomonas striata* are phosphorus solubilizers (Mohammadi and Sohrabi, 2012). Most bacteria included in biofertilizers have a close relationship with plant roots and help in increasing plant height, number of leaves per plant, and plant dry matter. In this study, mixed microbes were used for the biodegradation of MWW, and most of them were *Bacillus* species. During hydroponic culture, these *Bacillus* species would form a relationship with plant roots, resulting in better plant growth than the control.

Amino acids (tryptophan, methionine, and cysteine) as antioxidants have been reported to have good effects on the growth of the sunflower (*Helianthus annuus* L.) and to improve growth characteristics such as plant height, stem diameter, number of leaves per plant, and total leaf area per plant (Al-Qubaie, 2012). In this study, amino acids and small peptides were produced by MWW biodegradation, and they showed high antioxidant activities. In addition, the degradation products showed antimicrobial and antifungal activities. Therefore, it was concluded that the remaining culture broth could be a good candidate for use as a biofertilizer.

Biofertilizer efficacy of the remaining culture broth was important for applications in agriculture. Therefore, the quality of the final culture broth was compared with that of other culture supernatants produced by the biodegradation of fishmeal wastewater and fish waste. As the length of a leaf was found out to be a prominent indicator for growth in red bean or barley in hydroponic culture (Dao and Kim, 2011), leaf lengths after 10 days of hydroponic culture were compared (Table 3). In this study, either red bean or barley grew slowly for the first six days, and after they grew faster. After 10 days, the leaf lengths of red bean and barley were found to be 3.5 cm and 6.2 cm, respectively, and the final leaf lengths of red bean and barley after 14 days were measured to be 4.9 cm and 9.7 cm, respectively. The 10-day values of leaf lengths for both plants were lower than those observed using the culture supernatant of either fishmeal wastewater or fish waste, although the GI value at 1000-fold dilution was higher. This discrepancy may be caused by the quantity and composition of nutrients, culture conditions such as amount of sunshine, etc. In the hydroponic cultures using the supernatant of biodegraded fishmeal wastewater, the initial concentration of COD was more than twice that of MWW. This indicates that larger amounts of amino acids and small peptides could be produced and be present in the supernatant by biodegradation with a higher substrate concentration. These larger amounts of amino acids and small peptides would result in faster plant growth. Thus, faster plant growth may be achieved using a lower dilution of the MWW culture broth. However, it should be emphasized

that these two culture supernatants produced from fishmeal wastewater and fish waste were found to be comparable to commercial fertilizers (Kim et al., 2010; Gwon and Kim, 2012). Based on the results, the remaining MWW culture broth could be used as a biofertilizer.

Table 3. Comparison of fertilizing quality among liquid fertilizers produced from different sources.

Source	Initial COD (mg/L)	GI at 1,000-fold dilution (%)		Length of leaf after 10-d hydroponics (cm)				Reference
				Red bean		Barely		
Fishmeal waste water	68,900	90		4.5		9.4		Gwon et al. (2012)
Fish waste	n.r.[a]	89		4.7		9.8		Kim et al. (2010)
MWW	29,800	Culture supernatant	Culture broth	Culture supernatant	Culture broth	Culture supernatant	Culture broth	This study
		106	122	3.4	3.5	6.2	6.2	

[a]n.r.: not reported. The known is 5% (w/v) of fish waste was initially used.

4. Conclusion

Eco-friendly waste management of MWW was demonstrated, and complete reutilization was observed. MWW degraded by mixed microbes produced diverse metabolic products, and the fishy smell disappeared. Culture broths containing the degradation products showed antioxidant, antimicrobial, and DNA protective activities and weak antifungal activity, indicating the production of high value-added products obtained from MWW biodegradation. The remaining culture broth containing the microbes at the end of biodegradation showed good efficacy as a fertilizer, resulting in advanced waste management. Consequently, this advanced waste management could be an economic method for extracting valuable products from MWW and solving the environmental pollution problem. Further studies are required for the development of value-added products from MWW in order to enhance its reutilization value.

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CHAPTER III

Complete reutilization of mixed mackerel and brown seaweed wastewater as a high-quality biofertilizer in lettuce hydroponics

Abstract

Biodegradation using *Bacillus* species can convert mixed fishery wastewater into biofertilizer in a clean manner. Herein, fishery wastewater from mackerel and brown seaweed was tested, and the ratio most suitable for biodegradation was 10:1. During the 72-h biodegradation at the optimum mixing ratio, 36.4% hydrolysis occurred due to stable protease, alginate lyase and laminarinase activities, which chemical oxygen demand decreased by 69.1% and total nitrogen by 62.0%. The highest antioxidant activities toward 2,2-diphenyl-1-picryl-hydrazyl (DPPH) and 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radicals were 84.9% and 93.1% respectively at 24 h, and the total amino acid content at 72 h was 7715.7 μg ml^{-1}. As a biofertilizer, the 72-h culture broth also met the requirements for standard content of nitrogen (N), phosphorus (P), potassium (K) and heavy metals, and the number of viable cells. In open-flow hydroponics using the produced biofertilizer, 1-month-old lettuce plants exhibited significantly enhanced growth rate compared with controls, with high chlorophyll and carotenoid content and high antioxidant activity. Moreover, there was no permeation of pathogens in the circular biofertilizer solution. These results indicate the potential of the biofertilizer for the production of high-quality lettuce, and demonstrate the complete reutilization of mixed fishery wastewater.

1. Introduction

Fishery products are consumed as nutritious foods, and their consumption has gradually increased in recent years, exceeding 146 million tons in 2014, with seaweed production reaching 28.5 million tons (FAO, 2016). Consumption of fishery products generates a huge amount of waste and wastewater. Approximately 60% (Dekkers et al., 2011) and 10% (IPET, 2010) of fish and seaweed wastes are typically generated from tissues during processing, respectively, and generation of waste and wastewater is dependent on configurations and processes in processing plants (Sirianuntapiboon and Srikul, 2006). Therefore, steady growth in fishery processing industries should be accompanied by reutilization of waste and wastewater to maximize returns on those raw materials, and this is mainly reliant on converting organics into diverse marketable products such as bait, fertilizers, fish oil and organic acids (Mathur et al., 1988). The reutilization of fishery waste and wastewater as fertilizers has been proposed, fertilizers made from fish waste are now commercially available, and some are authorized for use in organic agriculture due to the high content of nutrients including N, P and calcium (Ca) (Radziemska et al., 2018). Various protein hydrolysates can act as biostimulants for a wide range of horticultural and agronomic crops (Yakhin et al., 2017), and even fish-farm effluent has been used to irrigate cherry tomato plants (Castro et al., 2006). Meanwhile, macro- and microelement nutrients, amino acids, vitamins, cytokinins, auxins, abscisic acid, and so on,

known components of commercial seaweed products (Crouch and van Staden, 1993) or microalgae (Stirk et al., 2003), can also enhance plant growth and crop yield, indicating that seaweeds are potential sources of fertilizers.

Nowadays, much nitrogenous chemical fertilizer is used in agriculture; this may be converted to nitrous oxide by processes in soil microbes, and nitrous oxide is an important greenhouse gas (Fagodiya et al., 2017). This worldwide issue urgently requires a sustainable solution. The production of biofertilizer from organic wastewater has been suggested (Kang et al., 2018), which would facilitate considerable reduction in the use of chemical fertilizer. Biofertilizers supplement nutrients through the natural processes of nitrogen fixation, phosphorus solubilization and plant growth stimulation (Yosefi et al., 2011). As alternatives to chemical and organic fertilizers, liquid biofertilizers have aroused great interest. This interest has arisen because the use of biofertilizers leads not only to increased crop yield, by up to 50% (Ansari et al., 2015), but also to greatly reduced damage to soil texture and other environmental problems that arise from the use of chemical fertilizers (Buragohain et al., 2018). Therefore, the use of biofertilizers offers economic and ecological benefits, and supports the growing effort to intensify sustainable agricultural systems (Xiang et al., 2012). The complete reuse of liquid resulting from the biodegradation of fishery wastewater would be economically beneficial. However, if the culture supernatant is exclusively used as fertilizer, the remaining solid waste (mainly

cells) must be treated. Another benefit is that microorganisms used for biodegradation may enhance plant growth and assist the creation of healthy rhizospheres (Hoe and Rahim, 2010). Some plant growth-promoting rhizobacteria have been studied in hydroponic systems for use in agriculture as biofertilizers, biocontrol agents, and bioremediators (Hibar et al., 2006).

Hydroponics is a method of growing plants using direct feeding of nutrients, light and water solutions, often in a soil-free environment (Xydis et al., 2017). This can reduce water loss and increase crop productivity per unit area, compared with conventional agriculture (Alshrouf, 2017). Moreover, soil culture is an open system with low efficiency in terms of water and fertilizer use, whereas water can be conserved in a closed recirculating hydroponic system (Hagin and Lowengart, 1995). Therefore, hydroponics can be advantageous, although it requires electricity, water and heating to simulate a natural environment suitable for plants. To achieve high plant growth and crop yield, microbial pretreatment of organic fertilizer is performed before incorporation into hydroponic solutions (Atkin and Nichols, 2004), as the direct use of organic fertilizer is detrimental to plant growth due to the presence of phytotoxic organic compounds (Garland et al., 1997).

Discharge of untreated wastewater considerably impacts environments in diverse respects, and thus its reduction, or recycling or/and safe reuse of the water is required (Khan et al., 2019). Recently, improvement of wastewater treatment has aroused our

interest, since it is important for sustainability and cleaner production in diverse industries. A more efficient, economical, and environmentally friendly reductive process is required for wastewater treatment without the generation of secondary pollutants (Akbari et al., 2019). Hence, complete reutilization of organic wastewater has recently been suggested (Kang et al., 2018). To date, conversion of fishery wastewater into a biofertilizer has been reported in only a few studies of mackerel wastewater (Jung and Kim, 2016) and fish processing waste (Sahu et al., 2014); assessment of feasibility is in the early stages, and problems concerning the security of raw materials and production of high-quality biofertilizer for commercialization require attention. To develop a fishery wastewater-based biofertilizer for hydroponics, fishery wastewater supply must be reliable. However, the separate collection of waste by different fishery species is impractical, resulting in poor reutilization for biofertilizer production. Moreover, careful control of biodegradation is required for the production of high-quality biofertilizer that not only provides nutritive substances essential for plants, but also eliminates phytotoxic substances detrimental to plants (Michalak and Chojnacka, 2013). Considering these considerations, mixed fishery wastewater from mackerel and brown seaweed (*Undaria pinnatifida*) may be a substrate for production of biofertilizer due to large-scale consumption in Korea. Herein, we investigated the feasibility of complete reuse of mixed fishery wastewater (MFWW) as a biofertilizer to aim at sustainable, cleaner organic wastewater treatment. The optimum mixing ratio for

mackerel and *U. pinnatifida* wastewater was first determined for production of high-quality biofertilizer. Properties [total amino acid content, antioxidant activity, content of nitrogen (N), phosphorus (P), potassium (K) and heavy metals, and number of viable cells] of the biofertilizer were examined to determine its suitability, and simulation was carried out using open-flow lettuce hydroponics to demonstrate its potential.

2. Materials and Methods

2.1. Microbes and seed culture

Microbes used in this study were mixed strains of *Bacillus anthracis* (GenBank accession no. AY138279), *B. fusiformis* (GenBank accession no. AY548950) and *B. licheniformis* (GenBank accession no. EF113324) (Kim et al., 2007), and all synthesise multiple enzymes (protease, alginate lyase and laminarinase) suitable for the degradation of polymers present in fishery wastewater (Kang and Kim, 2015). There is no antagonism among them. Each strain was maintained on 1.5% nutrient agar plates at 4 °C until used and transferred to a fresh agar plate every 2 weeks. The seed culture for biodegradation was prepared in by cultivating each strain individually in a 100 ml flask containing fishery wastewater; each strain was proliferated until late-log phase, cells were harvested, and an equal number of cells ($2.8 \pm 0.29 \times 10^8$) from each strain was combined together to prepare an inoculum. Each strain was incubated at 45 °C for 24 h after spreading on 1% skim milk agar, alginate agar, and laminarin agar plates to check its ability to degrade protein, alginate and laminarin, respectively. Degradation ability was judged by measuring the size of the clear zone formed around colonies.

2.2. Preparation of MFWW

For MFWW preparation, mackerel wastewater and *U. pinnatifida* wastewater were collected from a local D company (Jangnim, Busan, Korea) and K company (Gijang, Busan, Korea), respectively. The properties of the mackerel wastewater were 38,000 ± 3000 mg l^{-1} chemical oxygen demand (COD_{Cr}), 3700 ± 350 mg l^{-1} total nitrogen (TN), and 0.3 ± 0.01% salt, compared with 16,000 ± 1300 mg l^{-1}, COD_{Cr}; 400 ± 30 mg l^{-1}, TN; and 1.3 ± 0.01%, salt, for *U. pinnatifida* wastewater. All prepared MFWW solutions were autoclaved at 121 °C for 15 min after adjustment to pH 7.

2.3. Production of biofertilizer from MFWW

To seek a suitable substrate for the production of high-quality biofertilizer, biodegradation was conducted in a 250 ml flask (with a 100 ml working volume) using mackerel wastewater and *U. pinnatifida* wastewater at various mixing ratios of 1:1, 1:5, 1:10, 5:1 and 10:1. The 72-h MFWW culture supernatants were removed from each flask according to previous analysis (Jung and Kim, 2016), reaction parameters (COD_{Cr}, TN and C/N ratio) were measured, and properties (amino acid composition, antioxidant activity, and the number of viable cells) were recorded. Biodegradation was then carried out in a 3-l bioreactor using MFWW at the optimum mixing ratio. Biodegradation was initiated with 10% (v v^{-1}) seed culture and continued for 72 h. During biodegradation, the bioreactor was agitated at 250 rpm, oxygen (80% purity) was supplied through a 0.2 μm filter at 5 l min^{-1}, and the

pH was controlled between 6 and 7 using 2 M NaOH. Samples were taken periodically to assess the reaction parameters, properties, degree of hydrolysis, the number of viable cells, and the fertilizing ability (elemental and heavy metal content, total amino acid content and antioxidant activity).

2.4. Analyses

The pH of bioreactor samples was measured by probes installed in the body of Winpact Bench-Top Fermenter (Major Science, USA). The number of viable cells was estimated by counting the number of colonies formed on the agar plate incubated at 45 °C for 1 day, and expressed as colony-forming units (CFU) per microliter of sample. COD_{Cr} and TN values of samples were analysed using a HS 2000 Water-quality Analyzer (Humas Co., Ltd, Korea). For measuring protease ability, 10 μl of sample was placed at the centre of a skim milk agar plate, the plate was incubated at 45 °C for 24 h, and the size of the clear zone formed around colonies was measured. The alginate degradation ability was measured by placing 10 μl of sample at the centre of an alginate agar plate, the plate was incubated at 45 °C for 24 h, 10 ml of 10% cetylpyridinium chloride monohydrate was added, the plate was washed twice with distilled water (DW) after 10 min, and the size of the transparent ring formed around colonies was measured. In the same way, the laminarin degradation ability on was measured by placing 10 μl of sample at the centre of a laminarin agar

plate, incubating at 45 °C for 24 h, adding 10 ml of 0.5% Congo Red, washing twice with 1 M NaCl after 15 min, and the size of yellow-coloured rings formed around colonies was measured. To examine the properties of biodegraded MFWW, the amino acid composition, content of N, P and K, and concentrations of microelements were determined by Scientec Lab Centre Co., Ltd. (Korea).

2.5. Degree of hydrolysis (DH)

To estimate the abundance of small molecules produced by biodegradation, the DH of biodegraded MFWW was determined. Samples were taken at 0, 24, 48 and 72 h of biodegradation, centrifuged at 15,000×g for 15 min, supernatant (1 ml) was added to 5 ml of 0.5 M NaOH, followed by the addition of 1 M Folin & Ciocalteu's phenol reagent (Sigma-Aldrich, USA). After vortexing, the mixture was incubated at 30 °C for 15 min, and then filtered through a 0.2 μm acetate filter (Minisart NML, Sartorius, Germany). After collection, the absorbance of 1.5 ml of filtrate was measured at 578 nm using an Opron-3000 UV/VIS Spectrophotometer (Hanson Technology Co., Korea). L-tyrosine was used to plot a standard curve, and DH was estimated based on the following formula:

$$\text{Degree of hydrolysis (\%)} = \frac{A_0 - A}{A_0} \times 100$$

where A_0 and A are the absorbance of autoclaved MFWW and filtrate from biodegraded MFWW samples, respectively.

2.6. Hydroponics

Prior to hydroponic analysis, the phytotoxicity of the 72-h biodegraded MFWW as biofertilizer was tested against cress (*Lepidium sativum*) seeds according to the method described by Wong et al. (2001). After confirming no phytotoxicity, red bean (*Vigna angularis*) was cultivated in a hydroponic culture pot (5 × 12 × 8 cm^3) using 72-h biodegraded MFWW for 14 days to examine the fertilizing ability of the biofertilizer. In each culture pot, 10 red bean seeds previously incubated for 2 days in a dark room were placed on top of the plastic screen and 300 ml of 1000-fold diluted culture broth was added underneath the plastic screen to completely soak the seeds into the solution. After seeds had germinated, all culture pots were placed by a window throughout the day to ensure adequate sunlight. Each fertilizer solution was refreshed every 3 days, and temperatures of solutions and the air during the day and night were maintained at 15 ± 2 °C and 22 ± 2 °C, respectively, by natural ventilation and heating. The relative humidity was 60 ± 5%. The height, thickness of the stem, number of leaves, and length of leaves were periodically measured. DW was used as a control under the same culture conditions. All measurements were performed in triplicate.

After confirming the fertilizing ability of the biofertilizer, hydroponic

analysis of lettuce was performed in an open-flow mini-hydroponic system (Self Gardening LED Water Culture Pureun, Kunok, Korea) using light-emitting diode lamps (average intensity of 200 μmol m^{-2} s^{-1} based on blue and red light integrated at 1:1) for 30 days. To investigate the effect of vertical and horizontal hydroponics on lettuce growth, a mini two-layer 20-site hydroponic system was employed (Fig. 1). In each layer of the mini-hydroponic system, five sites were arranged in parallel in two rows. Biofertilizer was fully loaded into a 40-l storage tank placed at the bottom of the system. Lettuce sprouts used for hydroponics were previously incubated for 7 days, and lettuce seedlings were placed in each pot (6.5 × 6.5 × 5 cm^3). After lettuce seedlings were placed in each pot, biofertilizer was pumped in and continuously circulated at 1.2 l min^{-1}. Control hydroponics experiments were performed with DW. Samples were taken at 15, 20 and 30 days to analyse the growth rate and antioxidant content of lettuce plants, and permeation of pathogen into the circular biofertilizer. The total content of phenols, flavonoids, chlorophyll (*chl*) and carotenoids (*car*) in lettuce leaves grown for 30 days was also estimated. All measurements were performed in triplicate.

Fig. 1. Photo of a mini two-layer 20-site hydroponic system.

2.7. Permeation of pathogen into the circular biofertilizer

During the open-flow hydroponic analysis, the circular biofertilizer solution was periodically sampled to check the possible permeation of detrimental bacteria incoming from outside. The test bacteria used in this assay were a faecal contamination indicator (faecal coliforms) and pathogenic bacteria (*Listeria* and *Staphylococcus*). Assays were performed by plating 1 ml of biofertilizer solution on 3M Petrifilm (3M Centre, St. Paul, MN, USA) in duplicate (Han et al., 2007).

2.8. Antioxidant activity

Antioxidant constituents in plants are known to increase resistance to oxidative damage (Cao et al., 2009). For this reason, the antioxidant activity of biodegraded MFWW as a biofertilizer was estimated, since antioxidant substances may be produced during biodegradation. Culture supernatant samples were taken at 0, 12, 24, 48 and 72 h, and antioxidant activity was also estimated for lettuce leaves cultivated for 30 days in hydroponics experiments. For estimation of antioxidant activity, 20 g of lettuce leaves were macerated into 200 ml of 96% ethanol for 24 h, filtered through a 0.2 μm acetate filter, and the filtrate was used for analysis. All measurements were performed in triplicate.

2.8.1. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical

scavenging assay

DPPH free radical scavenging activity was assayed as using 1 ml of sample mixed with 2 ml of 0.1 mM DPPH solution in 80% ethanol. The mixture was incubated for 30 min in a dark room, and the absorbance was measured at 517 nm using an Opron-3000 UV/VIS Spectrophotometer (Hanson Technology) against an appropriate blank prepared by replacing DPPH with 80% ethanol, while controls were prepared by mixing 1 ml of 80% ethanol with 2 ml of 0.1 mM DPPH solution. The positive control used in this assay was 0.1 mM L-ascorbic acid. After measuring the absorbance, the DPPH radical scavenging activity was calculated using the following formula:

$$\text{Scavenging activity (\%)} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

2.8.2. 2,2′-Azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical cation decolourisation assay

Antioxidant activity against ABTS radical cations was assessed using a decolorization assay. ABTS radical cation reagent was first prepared by mixing 5 ml of 7 mM ABTS with 5 ml of 4.9 mM potassium persulfate ($K_2S_2O_8$). The mixture was incubated for 16 h in a dark room, and the absorbance at 734 nm was adjusted to 0.72 with 80% ethanol. To measure ABTS radical scavenging activity, 100 μl of

sample was mixed with 900 μl of ABTS reagent, and the absorbance of the mixture was measured at 734 nm after 6 min. The control was prepared by replacing the supernatant with DW, while the blank was prepared by replacing the ABTS reagent with 80% ethanol. The positive control used in this assay was 0.3 mM L-ascorbic acid. After measuring the absorbance, the percentage inhibition was calculated using the following formula:

$$\% \text{ inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

2.9. Measuring the total content of phenols and flavonoids

The total content of phenols and flavonoids in lettuce leaves was estimated, since these substances are known to be responsible for antioxidant activity. Extract powder was prepared from lettuce leaves as described in the previous section, diluted in DW to 1 mg ml^{-1}, and a 0.5 ml aliquot was mixed with both 2.5 ml of 10-fold diluted Folin-Ciocalteu reagent and 2 ml of 7.5% (w/v) $NaHCO_3$. The mixture was incubated at 45 °C for 15 min, and the absorbance was measured at 765 nm using a spectrophotometer against a blank sample. Gallic acid (GA) was used to draw a standard curve.

To estimate the total content of flavonoids, 1.0 ml of extract powder solution at 1 mg ml^{-1} was mixed with 1.0 ml of 2.0% (w/v) aluminium chloride ($AlCl_3 \cdot 6H_2O$) solution, reacted for 10 min at room

temperature, and the absorbance was measured at 430 nm using a UV/VIS spectrophotometer. Quercetin (QE) was used to draw a standard curve. All assays were carried out in triplicate.

2.10. Measuring the content of *chl* and *car*

The content of *chl* and *car* in lettuce leaves grown for 30 days was determined to estimate plant health. Using 1 ml of 80% acetone, *chl* and *car* were extracted from 0.05 g of lettuce shoots at 4 ℃ overnight. The extractant was centrifuged at 13,000×g for 5 min, and the absorbance of the supernatant was measured using a spectrophotometer at 663, 645, and 470 nm. The concentrations (in g per g of sample) of *chl* a, *chl* b, and *car* were estimated using the following formula:

$$chl\ a = 12.72 \times OD_{663} - 2.59 \times OD_{645}$$

$$chl\ b = 22.88 \times OD_{645} - 4.67 \times OD_{663}$$

$$car = (1000 \times OD_{470} - 3.27 \times chl\ a - 104 \times chl\ b)/229$$

2.11. Statistical analysis

Standard deviations were estimated by first calculating the squared values, and the sum of the squares was divided by (n-1), where n is the sample size. The square root was then derived to retrieve the original measurement scale. The normality and homogeneity of variance were preliminarily verified using SAS software (SAS Inst.

Inc., Cary, NC, USA; https://www.sas.com/en_us/home.html). Differences in mean values of properties (COD_{Cr}, TN and antioxidant activity) of the 72-h biodegraded MFWW at various mixing ratios of fishery wastewater, the difference in DPPH or ABTS antioxidant activity of the culture supernatants of MFWW and mackerel wastewater at different cultivation times, horizontal or vertical effects on lettuce growth in two-layer open-flow hydroponics at different cultivation times, and effects of biofertilizer and DW on lettuce growth at different cultivation times, were evaluated by one-way analysis of variance using PROC GLM in SAS. Comparison of means was performed using Tukey's method (Neter et al., 1996) at the 5% significance level.

3. Results and Discussion

3.1. Determination of a suitable MFWW source for biofertilizer production

3.1.1. Properties of biodegraded MFWW at various mixing ratios

Flask-scale biodegradations were carried out at various mixing ratios of raw MFWW to seek a suitable substrate for production of high-quality biofertilizer. Culture broth samples were taken at 72 h, based on previous analysis (Jung and Kim, 2016), and the properties of the 72-h MFWW culture broths at various mixing ratios were evaluated. Biodegradation of MFWW at various mixing ratios resulted in differences in COD_{Cr}, TN and C/N ratio (Table 1). At mixing ratios of 1:1, 1:5, 1:10, 5:1 and 10:1, the percentage of COD_{Cr} removed was 64.8, 75.7, 75.4, 54.3, and 69.3%, compared with 41.2, 60.2, 56.0, 29.1 and 46.8%, respectively, for removal of TN after 72 h of biodegradation. The C/N ratios were 7.9, 12.7, 14.4, 7.0 and 6.1 at mixing ratios of 1:1, 1:5, 1:10, 5:1 and 10:1, respectively, covering the typical range observed in biodegradation of fish wastewater (Jung and Kim, 2016). MFWW biodegradations at different mixing ratios also resulted in different antioxidant activities; antioxidant activities at mixing ratios of 1:1, 1:5, 1:10, 5:1 and 10:1 were 71.2, 57.3, 23.4, 78.8 and 80.1% for DPPH radical scavenging activity, and 98.8, 65.7, 58.0,

93.6 and 96.3% for ABTS radical scavenging activity, respectively (Table 1). This was because the production of low-molecular-weight substances responsible for antioxidant activity differs according to the composition of MFWW. After 72 h of biodegradation, the number of viable cells was 9.7×10^7, 8.9×10^7, 7.4×10^7, 1.26×10^8 and 1.62×10^8 CFU ml^{-1} at mixing ratios of 1:1, 1:5, 1:10, 5:1 and 10:1, respectively. Therefore, a higher cell density was obtained when fish wastewater was in greater abundance in MFWW. This indicates that protein abundant in fish wastewater had a strong influence on cell proliferation. The number of cells present in all biodegraded MFWW samples exceeded established standards ($>1 \times 10^6$ CFU ml^{-1}) for biofertilizer products (Rural Development Administration, 2018).

Table 1. Properties of 72-h biodegraded MFWW at various mixing ratios of fishery wastewater[a].

Mixing ratio[b]	COD_{Cr} (mg l^{-1})	TN (mg l^{-1})	C/N ratio	Antioxidant activity		Viable cells (CFU ml^{-1})
				DPPH (%)	ABTS (%)	
1:1	9498.1±279[c]	1206.4±41[c]	7.9	71.2±1.27[b]	98.8±1.56[a]	9.7×10^7
1:5	4782.4±430[d]	378.0±21[d]	12.7	57.3±2.25[c]	65.7±1.64[c]	8.9×10^7
1:10	4432.0±470[d]	308.9±23[d]	14.4	23.4±4.3[d]	58.0±1.58[d]	7.4×10^7
5:1	15690.4±256[a]	2232.0±33[a]	7.0	78.8±1.03[a]	93.6±1.45[b]	12.6×10^7
10:1	11052.1±269[b]	1809.1±39[b]	6.1	80.1±1.12[a]	96.3±1.34[ab]	16.2×10^7

[a]Data represent mean ± standard deviation of three replicates. Different letters on bars indicate significant differences ($p < 0.05$) between groups.

[b]Mixing ratio of mackerel and *U. pinnatifida* wastewater.

3.1.2. Amino acid composition of biodegraded MFWW at various mixing ratios

Amino acids are an essential part of the active fraction of organic matter in a fertilizer. As the growth of plants ultimately depends upon the availability of a suitable balance of amino acids, their composition might also be used as a means of assessing biodegradation (Dao and Kim, 2011). The amino acid composition of the 72-h biodegraded broth from different mixing ratios of mackerel and *U. pinnatifida* wastewater was therefore analysed (Table 2). Total amino acid content increased with increasing abundance of mackerel wastewater in MFWW. The highest total amino acid content was 4585.3 μg ml^{-1} at a mixing ratio of 10:1. The abundance of amino acids in the 72-h MFWW culture broth decreased in the order His > Arg > Trp > Lys > Leu > Phe > Met > Val > Tyr > Ile > Glu > Pro > Thr > Gln > Gly > Ala > Ser > Asp > Cys. Biofertilizers participate in nutrient cycling and can enhance crop productivity (Kavi Kishor and Sreenivasulu, 2014). Amino acids are known to perform various functions and to be essential for growth of garlic (El-Shabasi et al., 2005), pea (Ghaith and Galal, 2014), lettuce (Lee et al., 2013) and boreal forest plants (Näsholm et al., 1998). For example, Trp and Met can enhance nitrogen absorption; Gly, Ala, Cys and Arg give can increase total yield; Glu, Asp and Arg are nitrogen sources for growth; Cys and Met contribute to overcoming damage from cold or insufficient sunshine; Arg helps to control the growth of

pathogens; and Pro has some influence on reproductive growth. Therefore, a higher content of amino acids in biofertilizers can improve its quality. Based on the above results, a mixing ratio of 10:1 was optimal for biodegradation to produce high-quality biofertilizer containing a high amino acid content and high DPPH and ABTS radical scavenging activities. The use of this high-quality biofertilizer in hydroponics could be a cost-effective and environmentally friendly way to supply sufficient macro- and microelements for healthy plant nutrition, and may be particularly suitable for short-cycle crops such as lettuce (Medeiros and Lopes, 2006).

Table 2. Amino acid composition in 72-h MFWW culture supernatants at various mixing ratios[a].

Amino acid	Content (μg ml^{-1})				
	1:1	1:5	1:10	5:1	10:1
Cysteine	0.0	0.0	0.0	2.9	2.6
Aspartic acid	0.0	0.0	0.0	3.6	9.7
Glutamic acid	28.6	7.0	3.6	64.6	120.7
Asparagine	0.0	0.0	0.0	0.0	0.0
Serine	0.0	0.0	0.0	2.2	12.0
Glutamine	19.0	5.7	4.1	32.8	38.6
Glycine	0.0	0.0	0.0	7.2	23.4
Histidine	726.3	198.8	88.2	1097.2	1113.9
Arginine	564.0	157.7	100.4	938.9	1004.3
Threonine	0.0	0.0	0.0	0.0	56.6
Alanine	5.6	1.6	1.2	12.8	21.9
Proline	3.2	0.0	0.0	0.0	119.3
Tyrosine	94.5	22.0	14.3	152.4	176.7
Valine	129.6	0.0	0.0	189.7	190.1
Methionine	136.4	29.9	3.2	211.4	201.3
Cystine	0.0	0.0	0.0	0.0	0.0
Isoleucine	68.1	0.0	0.0	116.7	146.9
Leucine	190.1	4.9	0.0	268.5	306.0
Phenylalanine	130.9	38.4	32.0	194.3	203.5
Tryptophan	16.4	0.0	5.2	33.3	432.1
Lysine	260.2	62.2	45.6	414.0	405.7
Total	2372.9	528.2	297.8	3742.5	4585.3

[a]Mixing ratio of mackerel and *U. pinnatifida* wastewater.

3.2. Production of biofertilizer from MFWW

After determining the optimum mixing ratio for MFWW, biodegradation of MFWW at an optimum mixing ratio was executed in a 3-l bioreactor for 72 h. During biodegradation, the activities of protease, laminarinase and alginate lyase were steadily maintained (Fig. 2A). The protease activity was higher than that of the other two enzyme activities due to the higher content of protein in MFWW. This maintenance of enzyme activities indicates steady MFWW degradation by the *Bacillus* species.

Changes in COD_{Cr} and TN during biodegradation are shown in Fig. 2B. Concentrations of COD_{Cr} and TN decreased as the *Bacillus* species biodegraded the organic matter in MFWW. After 72 h, concentrations of COD_{Cr} and TN were reduced to 11,052 and 1309 mg l^{-1}, respectively, equating to 69.1 and 62.0%. During biodegradation, the COD_{Cr}/TN ratio was in the range of 7.3-10.4. This value is intermediate between those of fishmeal wastewater (5.2-7.5) (Kim and Lee, 2008) and mackerel wastewater (10.2-18.8) (Jung and Kim, 2016). Cells generally produce metabolites at a high C/N ratio, while they increase biomass at a low C/N ratio. In this study, MFWW was biodegraded during balanced growth, resulting in a small variation in C/N ratio. Therefore, the performance of the three *Bacillus* species in the biodegradation of MFWW overcame the difficulty associated with a single cell type for the utilization of a mixture of substrates of various composition (Lin et al., 2011).

During biodegradation of MFWW at a mixing ratio of 10:1, DH was estimated to check the extent of the production of low-molecular-weight substances responsible for antioxidant activity (Fonseca et al., 2016). Values of DH were 28.1, 34.3 and 36.4% at 24, 48 and 72 h, with a decreasing number of viable cells (4.1×10^8 to 2.0×10^8 CFU ml^{-1}), although differences were not significant (Fig. 3). The DH value at 72 h was higher than those (17.1-31.3%) reported previously for the hydrolysis of mackerel flesh or viscera by commercial enzymes (Morales-Medina et al., 2016; Wang et al., 2018). This indicates that the *Bacillus* species proliferating in MFWW could synthesise adequate amounts of hydrolyzing enzymes, resulting in high DH. The efficiency of *Bacillus* species for hydrolysis of proteins (Thazeem et al., 2016) and polysaccharides (Priest, 1977) has been reported in previous studies.

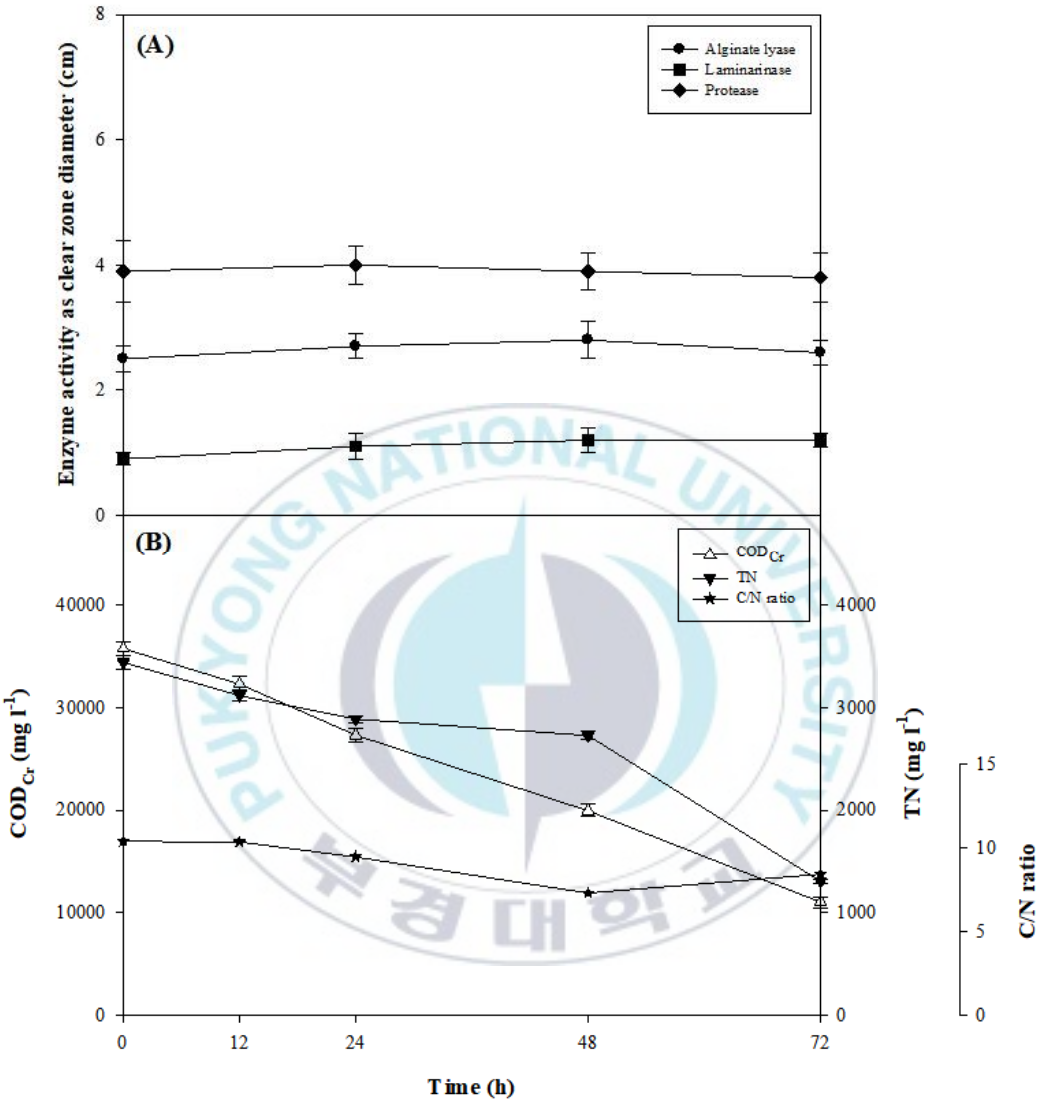

Fig. 2. Changes in enzyme activities of alginate lyase, laminarinase, and protease (A) and values of COD_{Cr}, TN and C/N ratio (B) during biodegradation of MFWW at a mixing ratio of 10:1. Error bars represent the standard deviation of three replicates.

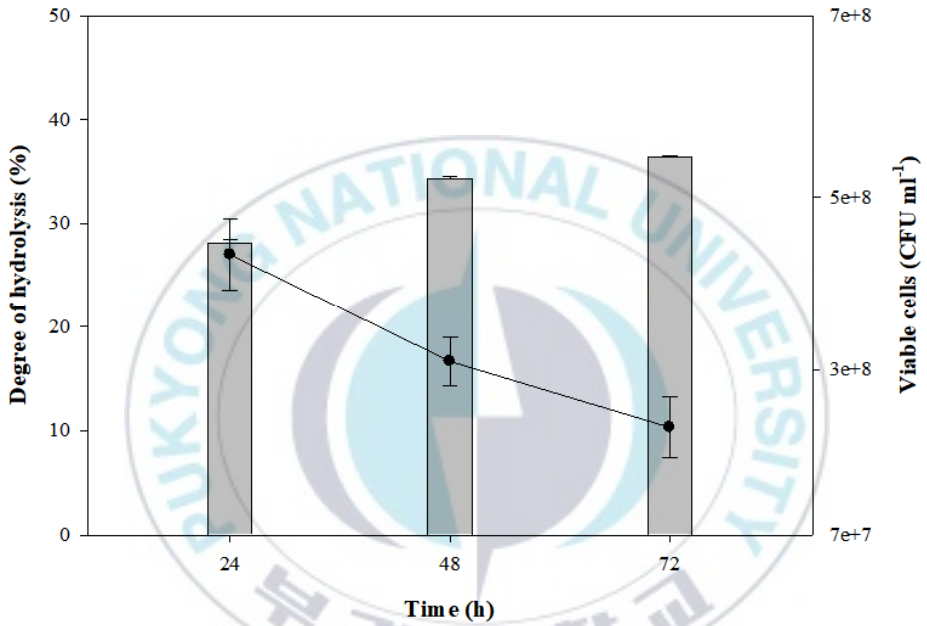

Fig. 3. Changes in the degree of hydrolysis (■) and the number of viable cells (-●-) during biodegradation of MFWW at a mixing ratio of 10:1. Error bars represent the standard deviation of three replicates.

3.3. Fertilizing values of biofertilizer

3.3.1. Elemental and heavy metal content of biofertilizer

The quantity of fertilizer needed for a given application is dependent on the constituent nutrients, which affects crop growth and yield (Thazeem et al., 2016). To augment the efficiency of the nutrients, organic and inorganic elements must be suitably combined (Xiaochuang et al., 2013). Moreover, toxic compounds aggravating plant growth should ideally be decreased (Masarirambi et al., 2010). In this study, the produced biofertilizer contained N, P and K at concentrations of 0.28, 0.08 and 0.17%, respectively, all higher than standard values (Table 3). Additionally, concentrations of all heavy metals were lower than the standard concentrations allowed by domestic law (Seo et al., 2017). These results indicate minimal potential for damage from the produced biofertilizer on plants.

Table 3. Elemental and heavy metal content in the 72-h MFWW culture supernatants.

Element	Content (%)	Standard[a] (%)
N	0.28	
P_2O_5	0.08	Sum of N, P and K ≥ 0.3
K_2O	0.17	
Heavy metal	**Content (mg kg^{-1})**	**Standard[a] (mg kg^{-1})**
As	0.83	5
Cd	N.D.[b]	0.5
Ni	0.35	5
Cu	1.67	50
Cr	0.23	30
Zn	0.24	130
Pb	N.D.	15
Hg	0.004	0.2

[a]Based on fertilizer made from animal manure (Seo et al., 2017).

[b]N.D., not detected.

3.3.2. Amino acid composition of biofertilizer

Changes in the amino acid composition of the MFWW culture broth at a 10:1 mixing ratio were analysed during biodegradation in a 3-l bioreactor (Table 4). The total amino acid content was 4074.1 μg ml^{-1} initially, and it reached 7715.7 μg ml^{-1} after 72 h. The total amino acid content at 72 h was higher than that reached by flask biodegradation (4585.3 μg ml^{-1}). The abundance of amino acids in the 72-h MFWW culture broths decreased in the order His > Arg > Trp > Leu > Ala > Glu > Phe > Lys > Ile > Val > Gly > Met > Tyr > Pro > Thr > Gln > Asp > Ser > Cys > Asn. Although the three most abundant amino acids (His, Arg and Trp) in the 72-h biodegraded culture broths were the same, there were some differences in the ratios of the others, compared with the results obtained from flask biodegradation. This discrepancy may be due to the different culture conditions (Kim and Lee, 2007). Therefore, the bioreactor was superior for biodegradation, resulting in increased amino acid content with slightly different composition.

Table 4. Changes in amino acid composition of biofertilizer during biodegradation[a].

Amino acid	Content (μg ml^{-1})			
	0 h	24 h	48 h	72 h
Cysteine	12.7 ± 1.1	3.6 ± 1.3	3.8 ± 0.8	10.9 ± 0.7
Aspartic acid	123.9 ± 0.9	4.8 ± 0.9	3.9 ± 1.1	35.7 ± 0.8
Glutamic acid	220.6 ± 13.8	145.2 ± 28.1	122.5 ± 10.0	320.6 ± 14.3
Asparagine	5.9 ± 0.6	0.0 ± 0.0	1.3 ± 1.1	0.0 ± 0.0
Serine	96.3 ± 11.0	32.5 ± 6.4	3.6 ± 0.9	33.4 ± 6.1
Glutamine	34.9 ± 6.5	13.3 ± 2.3	14.2 ± 2.1	39.3 ± 7.0
Glycine	95.3 ± 14.7	110.4 ± 21.5	126.0 ± 10.6	212.2 ± 14.5
Histidine	1014.2 ± 98.3	2904.1 ± 497.8	3160.2 ± 255.5	1728.1 ± 101.5
Arginine	1005.7 ± 102.2	1114.9 ± 207.6	1245.5 ± 103.7	1271.5 ± 82.7
Threonine	106.1 ± 10.7	123.1 ± 24.9	122.1 ± 10.3	159.3 ± 9.5
Alanine	176.8 ± 11.2	265.8 ± 49.8	217.6 ± 15.4	384.3 ± 16.2
Proline	79.3 ± 5.4	100.1 ± 20.7	134.7 ± 10.6	167.4 ± 13.3
Tyrosine	107.0 ± 15.9	65.9 ± 15.2	82.9 ± 9.1	175.9 ± 14.1
Valine	142.3 ± 23.6	136.4 ± 25.0	152.5 ± 12.0	239.1 ± 10.8
Methionine	79.5 ± 20.4	100.1 ± 19.0	136.5 ± 9.9	180.8 ± 9.0
Cystine	8.8 ± 1.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Isoleucine	121.9 ± 13.0	104.8 ± 19.7	127.0 ± 12.5	245.3 ± 20.2
Leucine	221.0 ± 40.9	255.7 ± 43.3	309.0 ± 25.4	509.0 ± 32.9
Phenylalanine	114.6 ± 31.2	153.9 ± 33.1	206.4 ± 18.8	307.5 ± 21.4
Tryptophan	96.3 ± 10.6	586.9 ± 127.7	679.3 ± 68.3	1217.8 ± 106.4
Lysine	211.0 ± 38.8	244.8 ± 46.7	294.3 ± 24.5	477.6 ± 27.5
Total	4074.1	6466.3	7143.3	7715.7

[a]Data represent mean ± standard deviation of three replicates.

3.3.3. Antioxidant activity of biofertilizer

Antioxidant activities of MFWW culture supernatants were estimated during biodegradation. As biodegradation proceeded, antioxidant substances were produced. The highest scavenging activities for DPPH and ABTS radical were 83.8-84.9% and 93.1-94.1% between 12 and 24 h, respectively (Table 5). These antioxidant activities were higher than those obtained from flask-scale experiments, further demonstrating the superiority of the bioreactor culture conditions for the production of low-molecular-weight substances such as amino acids. Moreover, these antioxidant activities were comparable with those obtained for DPPH (85.6%) and ABTS (99.2%) with L-ascorbic acid as a positive control. After 24 h, there was a slight decrease in antioxidant activities, possibly resulting from the utilization of amino acids by microorganisms, which is ordinarily observed during the latter stages of fish wastewater biodegradation (Kang et al., 2018). Antioxidant activities of the MFWW culture supernatants were lower than those for DPPH (88.7%) and ABTS (99.7%) with mackerel wastewater culture supernatants previously reported by Jung and Kim (2016) (Table 5). These differences in antioxidant activities may be due to differences in the composition of substrates from different sources. Therefore, mixing with *U. pinnatifida* wastewater that has lower protein content would reduce the total antioxidant activities of MFWW culture supernatants. Moreover, high antioxidant activity accompanied high DH, resulting in high production of low-molecular-weight

substances responsible for antioxidant activity (Kong et al., 2015).

Table 5. Comparison of antioxidant activities of culture supernatants between different substrate sources for biodegradation[a].

Substrate source	Biodegradation (h)	Antioxidant activity		Reference
		DPPH (%)	ABTS (%)	
Mackerel wastewater	0	73.7 ± 2.13^{d}	90.1 ± 2.52^{c}	Jung and Kim (2016)
	24	84.6 ± 1.50^{ab}	98.1 ± 1.03^{ab}	
	42	88.7 ± 1.14^{a}	99.7 ± 1.52^{a}	
	74	87.6 ± 1.07^{ab}	94.4 ± 1.45^{bc}	
MFWW at 10:1	0	70.9 ± 2.3^{d}	65.4 ± 2.1^{d}	This study
	12	83.8 ± 2.1^{b}	94.1 ± 1.0^{bc}	
	24	84.9 ± 1.5^{ab}	93.1 ± 1.2^{c}	
	48	78.7 ± 1.2^{c}	91.7 ± 1.2^{c}	
	72	73.6 ± 1.0^{d}	90.6 ± 1.4^{c}	

[a]Values (mean ± standard deviation of triplicate samples) in the same column with different superscript letters are significantly different ($p < 0.05$).

3.4. Fertilizing ability of the produced biofertilizer in a simple hydroponic experiment

To examine the fertilizing ability of the biofertilizer produced from MFWW, red beans were cultivated using 1000-fold diluted culture broth for 14 days. The hydroponic results showed that the growth of red beans was clearly better than controls in terms of height, thickness of the stem, number of leaves, and length of leaves (Fig. 4). These results demonstrate the potential of the produced biofertilizer for application to lettuce cultivation.

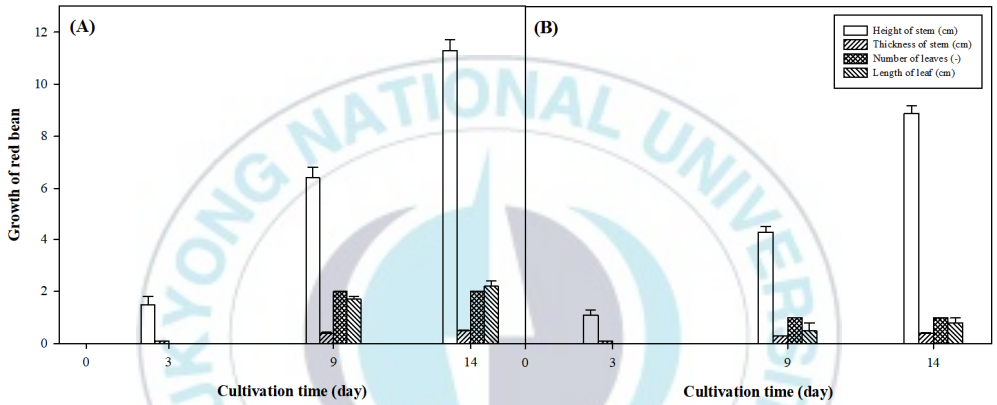

Fig. 4. Fertilizing ability of a 1000-fold diluted MFWW culture broth as a biofertilizer (A) vs. controls (B) in red-bean hydroponic experiments. Fertilizing ability was estimated by height of stem, thickness of stem, number of leaves and length of leaf. Error bars represent the standard deviation of three replicates.

3.5. Hydroponics in an open-flow system using biofertilizer

Hydroponic growth of lettuce in an open-flow two-layer system was examined for 30 days to assess the quality of the produced biofertilizer. The number of lettuce leaves increased during the experiment, although the average length of leaves decreased due to the sprouting of new leaves, and the growth of lettuce plants fed on biofertilizer was significantly better than that of plants fed on DW as a control (Fig. 5A and B). The number of lettuce leaves after 30 days (9.0-10.3) was larger than the number obtained from a 49-day hydroponics experiment using various nutrient solutions (7.6-8.2) (Charoenpakdee, 2014), indicating the competitiveness of the produced biofertilizer.

The effect of pot location on lettuce growth was also examined in the open-flow hydroponics experiment, and neither horizontal nor vertical effects on lettuce growth were significant (Fig. 5 A and B). This indicates that the feeding of biofertilizer according to the circulation pattern did not significantly affect lettuce growth over 30 days. Recently, vertical farming methods have been introduced aiming to discover an engineering solution to create a complete sustainable food system, while large-scale hydroponics set-ups (up to 52,000 m^2) have been developed for a higher yield (Jin et al., 2018). Touliatos et al. (2016) reported the advantage of vertical systems over a conventional horizontal system in lettuce hydroponics. However, the result may be different depending on hydroponic system conditions,

such as scale, lighting, source and circulation pattern of fertilizer, root zone volume, and planting density. In conclusion, our results provide basic information applicable to large-scale hydroponics with multiple rows and layers.

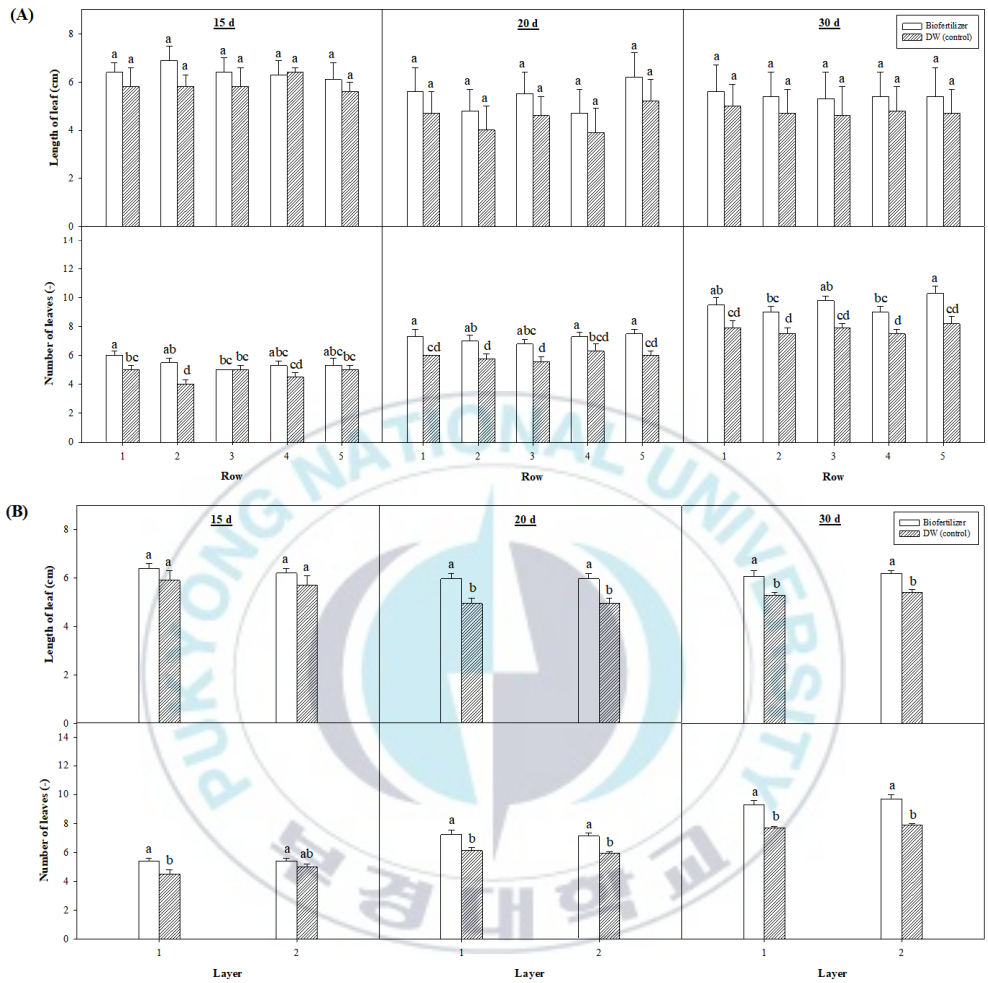

Fig. 5. Horizontal (A) and vertical (B) effects on lettuce growth in a two-layer open-flow hydroponic system at different cultivation times. The lettuce growth was estimated by number of leaves and length of leaf. Different letters on bars indicate significant differences ($p < 0.05$) between groups.

3.6. Maintenance of biofertilizer quality during hydroponics

Maintenance of biofertilizer quality during long-term hydroponics is important for lettuce yield. For example, detrimental bacteria could enter the flowing biofertilizer solution, and this could disrupt the beneficial bacteria present in the original biofertilizer. To examine this possibility, the presence of pathogens incoming from outside was tested, and the results are shown in Table 6. During hydroponics for 30 days, none of the test pathogens were detected in the flowing biofertilizer solution, despite remaining present. This may reflect the characteristics of members of the genus *Bacillus*, which are known to produce antimicrobial substances such as lantibiotics and non-modified bacteriocins (Lee and Kim, 2011). Antimicrobial peptides exhibit broad-spectrum antimicrobial activity against bacteria, viruses and fungi. In particular, mackerel hydrolysates display antimicrobial activity against Gram-positive (*Listeria innocua*) and Gram-negative (*Escherichia coli*) bacteria (Ennaas et al., 2015). Therefore, antimicrobial peptides can enhance plant defences against diseases, thereby preventing deterioration in quality and ensuring the safety of agriculture products (Jung and Kang, 2014).

The utilization of sewage sludge in agriculture is not considered economical for sludge disposal, since toxic heavy metals, organic contaminants and pathogens present in sewage sludge limit its direct use as a fertilizer (Zhou et al., 2017). Despite this weakness, Kominko et al. (2018) reported production of organomineral fertilizers from dried

sewage sludge, proposing that use of sewage sludge as a component in fertilizer production is the best sustainable option for its disposal. Moreover, the utilization of wastewater in agriculture for irrigation purposes is challenging because pollutants and pathogens limit its direct use on crops intended for direct or indirect human consumption (Heaton and Jones, 2008). Meanwhile, biodegraded fish wastewater was successfully applied to field cultivation of lettuce and Chinese cabbage (Kang et al., 2018). In this study, biofertilizer was tested as a supplementary component for crop management, not only for enhancing the base fertility of plants, but also for the control of pathogens, and helping to ensure sustainable production (Sahoo et al., 2013). Biological control is now considered an attractive alternative to chemical control due to health concerns for both producers and consumers (Compant et al., 2005). Therefore, the results of the present study indicate that the produced biofertilizer could assist the improvement of plant growth by stimulating defences against plant pathogens (García-Fraile et al., 2015).

Table 6. Detection of pathogens present in biofertilizer solution during lettuce hydroponic experiments.

Hydroponics duration (day)	Pathogen		
	E. coli[a]	*Staphylococcus*[b]	*Listeria*[c]
0	0	0	0
15	0	0	0
20	0	0	0
30	0	0	0

[a]Detection limit: 0, none of red colonies form bubbles around them; and 1, all of red colonies form bubbles around them.

[b]Detection limit: 0, none of colonies present red-violet; and 1, all of colonies present red-violet.

[c]Detection limit: 0, none of colonies are formed; and 1, all of colonies are formed.

3.7. Quality of lettuce plants grown under open-flow hydroponic conditions

To estimate the fertilizing ability of biofertilizer on lettuce plants in an open-flow hydroponic system, the health and antioxidant activity of leaves grown for 30 days were examined. The content of *chl* a (6.34 mg g^{-1}), *chl* b (1.95 mg g^{-1}) and *car* (1.89 mg g^{-1}) in lettuce leaves grown with biofertilizer were significantly higher than controls (Table 7). The pigment *chl* confers plants their green colour and is crucial for photosynthesis. It also assists plants in the activation of enzymes for growth, and serves protein synthesis (PROMIX, 2018). The amount of *chl* formed in plants is strongly influenced by the cultivation environment (Kleinhenz et al., 2003), hence total *chl* content in lettuce leaves is affected by the composition of fertilizer (Fallovo et al., 2009). Moreover, the ratio of *chl* a/*chl* b is closely correlated with the developmental stage of photosynthetic tissues and leaf maturity (Schoefs et al., 1998). The values of *chl* a and *chl* b obtained from the present work were much higher than those obtained in previous lettuce hydroponic studies (1.64 for *chl* a and 0.63 for *chl* b) (Phibunwatthanawong and Riddech, 2019). The *chl* a/*chl* b value (3.25) calculated in this study was reasonable, compared with values obtained for lettuce (*Lactuca sativa* L.) grown under different ratios of red light to blue light (3.00-3.28) (Wang et al., 2016).

Lettuce is an important source of vitamins, minerals, fibre and other health-promoting substances, such as carotenoids and polyphenols. The

carotenoid composition is influenced by cultivation conditions (Rodriguez-Amaya, 1993). In this study, the value of *car* was 1.89 mg g^{-1}, significantly higher than controls (1.02 mg g^{-1}). Therefore, the results indicate that the health of lettuce plants was satisfactory (Evans, 1988). The content of total phenols and total flavonoids was 45.5 mg GA g^{-1} and 21.4 mg QE g^{-1}, respectively, both significantly higher than controls (Table 7). Flavonoids and phenolic acids are secondary metabolites and bioactive compounds in plants (Kim et al., 2003). These antioxidant substances provide certain health benefits (Hodgson and Croft, 2006), and increasing their levels in food plants has potential for improving human health. Lettuce is a good dietary source of natural antioxidants, and its antioxidant properties are attributed to polyphenols and flavonoids (Heimler et al., 2007). In this study, DPPH and ABTS radical scavenging activities were 83.1 and 51.9%, respectively. The DPPH radical scavenging activity of lettuce leaves in this study was better than values reported in previous studies (74.4-84.2%) (Liu et al., 2007). Thus, the high content of these substances was reflected in the DPPH activity, but less by ABTS activity. This could also be due to differences in the scavenging activities of antioxidants for specific radicals (Fonseca et al., 2016). Moreover, it was reported that most flavonoids from plants are glycosides that significantly reduce antioxidant activity (Kim et al., 2014), while polyphenols exert synergistic effects with other antioxidants present in plants (Graversen et al., 2008). The low-molecular-weight substances present in the produced biofertilizer

exhibited high antioxidant activity, and their bio-accumulation in lettuce resulted in effective improvements in lettuce quality (Heaton and Jones, 2008). Thus, biofertilizer produced from raw MFWW possesses great potential for lettuce hydroponics.

Table 7. Health and antioxidant activity of hydroponic lettuce leaves grown for 30 days.

Parameter	Content	Biofertilizer	Control
Leaf health	*chl* a (mg g^{-1})	6.34 ± 1.02	3.09 ± 0.75
	chl b (mg g^{-1})	1.95 ± 0.21	1.16 ± 0.39
	chl a/*chl* b	3.25	2.66
	car (mg g^{-1})	1.89 ± 0.08	1.02 ± 0.13
Antioxidant activity	DPPH radical scavenging activity (%)	83.1 ± 2.3	80.5 ± 1.4
	ABTS radical scavenging activity (%)	51.9 ± 0.5	41.5 ± 0.4
	Total phenols (mg GA g^{-1})	45.5 ± 3.2	16.4 ± 1.7
	Total flavonoids (mg QC g^{-1})	21.4 ± 2.5	12.1 ± 1.2

4. Conclusion

Raw mixed fishery wastewater is an eco-friendly resource that is biodegraded using *Bacillus* species for reutilization as a biofertilizer. Herein, high-quality biofertilizer was produced from mixed mackerel and *Undaria* wastewater at a mixing ratio of 10:1. During the 72-h biodegradation at the optimum mixing ratio, 36.4% hydrolysis occurred due to stable protease, alginate lyase and laminarinase activities. Due to the high degree of hydrolysis, various low-molecular-weight substances were present in the biofertilizer, and high amino acid content (7715.7 μg mL^{-1}) and high antioxidant activities (84.9% DPPH and 93.1% ABTS) were observed. Moreover, the biofertilizer also met the requirements for standard content of N, P, K and heavy metals, and the number of viable cells. In open-flow hydroponic experiments, the growth rate was enhanced and high antioxidant activities (83.1% DPPH and 51.9% ABTS) was observed in 1-month-old lettuce plants, without the permeation of pathogens into the circular biofertilizer solution. Useful low-molecular-weight substances included in the biofertilizer might bioaccumulate in lettuce leaves. The lettuce hydroponics demonstrated herein achieved complete reutilization of mixed fishery wastewater as an economically attractive biofertilizer. Sustainable, cleaner production from wastewater with no secondary pollution is still developing, and thus the result of this study could be a promising solution not only for wastewater treatment, but also for reduction in use of chemical fertilizer.

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Complete reutilization of fishery processing wastewater by biodegradation

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Abstract

As a lot of people enjoy fishery products as nutritious foods, their worldwide consumption is steadily increasing. After all, the consumption exceeded 172 million tones in 2017. This human activity, on the other hand, inevitably generates a huge amount of waste and wastewater. The extents of waste generation from fish and seaweed bodies are approximately 60% and 10%, respectively, although the extent is different from fishery processing procedures. Thus, the reutilization of fishery processing waste is urgent to solve environmental problems and to earn an economic benefit as well. For this reason, feasible production of high value-added products from fishery processing wastewater in eco-friendly way was demonstrated in this study and evaluated the related matters for the practical use.

First, we briefly reviewed the current status of fishery waste generation, treatment methods and reutilization methods, and explored the characteristics and reutilization methods of products produced by eco-friendly treatment. We also identified the challenges to enhance the reutilization value of fishery wastes.

After reviewing fishery waste treatment methods, mackerel wastewater was selected as a sustainable resource, since mackerel is largely caught and

consumed in Korea. For the reutilization of mackerel wastewater, its biodegradation was carried out in a 3 l reactor using mixed microorganisms. In the first 24 h, biodegradation occurred actively and the maximum amount of cell number increased to 4.3×10^8 CFU ml^{-1}, thereby reducing dissolved oxygen content, pH and oxidation-reduction potential, and fishy smell gradually disappeared. The 42-h biodegraded supernatant indicated high antioxidant, antimicrobial and DNA protective activity, and weak antifungal activity. Moreover, the remaining culture broth after biodegradation of mackerel wastewater showed non-phytotoxicity, and it was confirmed by its use as a biofertizer in hydroponic culture of red bean and barley. As a result, the production of high value-added products in an eco-friendly treatment increased the reutilization value of mackerel wastewater.

After achieving satisfactory reutilization value of mackerel wastewater, it was extended to reutilize mixed fishery wastewater difficult to be separated and disposed of. In this study, various mixing ratios of mackerel and *Undaria* were tested, and the optimum mixing ratio for biodegradation was 10:1. During 72 h of biodegradation at the optimum mixing ratio, the mixed microorganisms showed stable protease, alginate lyase and laminarinase activity, resulting in reductions in chemical oxygen demand and total nitrogen with 36.4% hydrolysis. High antioxidant activities (84.9% DPPH and 93.1% ABTS) was obtained from the 24-h culture supernatant, and the total amino acid content of the 72-h culture supernatant was 7715.7 μg ml^{-1}. As a biofertilizer, the quality of 72-h biodegraded culture broth well met the standard of commercial biofertilizer on the basis of contents of nitrogen, phosphorus, potassium and heavy metals, and viable cell number. Application of the produced biofertilizer to lettuce hydroponic culture turned out satisfactory. The lettuce grown for one month showed a significantly superior growth rate with high chlorophyll (8.29 mg g^{-1}), and carotenoid contents (1.89

mg g^{-1}), compared with the control. Moreover, there was no permeation of pathogens in the circular biofertilizer solution during in open-flow hydroponics. Consequently, the production of high-quality lettuce using a biofertilizer manufactured from biodegradation of mixed fishery wastewater was feasible, and this result demonstrated complete reutilization of mixed fishery wastewater as a sustainable resource.

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