Thesis for the Degree of Master of Engineering

# Fed-batch production of high-quality biofertilizer from the mackerel wastewater and its economic analysis for commercialization

by

Su Hyeon Park

School of Marine Fisheries and Life Science
(Major in Biotechnology)

The Graduate School

Pukyong National University

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(유가배양에 의한 고등어폐액의 생분해를 통한 고품질 바이오비료의 생산과 상업화를 위한 경제성 분석)

Advisor: Prof. Joong Kyun Kim

by

Su Hyeon Park

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A dissertation
by
Su Hyeon Park

Approved by

(Chairman) Gwi-Tack Jeong

(Member) Joong Kyun Kim

(Member) Jong-Min Lee

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# 유가배양에 의한 고등어폐액의 생분해를 통한 고품질 바이오비료의 생산과 상업화를 위한 경제성 분석

박수현

부경대학교 대학원 해양수산생명과학부 생물공학전공

## 요 약

유기물 폐기물과 폐수가 유용한 자원으로 인식이 되며, 생선 폐수로부터의 생물학적 비료 생산은 생선 폐수의 완전한 재활용뿐만 아니라 농업에 유익한 도움이 되는 방식으로 많은 주목을 받고 있다. 이러한 측면에서, 생물학적 비료의 핵심 성분은 공급-일괄 공정에서 생물 분해를 통해 수집되고 특성화되었다. 공급-일괄 공정에서는 높은 수($5.33 \times 10^9$ CFU/mL)의 생존 세포가 더 많은 저분자량 가수분해물을 더 높은 가수분해도(48.1%)로 생성하여, 2 kDa 미만의 가수분해물로부터 높은 항산화 활성(84.17%의 DPPH 및 98.45%의 ABTS)을 나타났다. 생물 분해에 사용된 미생물은 식물 성장 촉진 활동을 가지고 있으며, 밀 수경재배 동안 식물 뿌리에 위치하게 된다. 비료에 포함된 바이오매스의 수가 많을수록 잎 길이, 잎 무게, 그리고 뿌리 길이에서 보여지는 밀의 성장이 더욱 우수하였다. 이런 경향은 밀의 건강 상태와 잎의 항산화 활성에서도 나타났다. 마찬가지로, 비료의 이러한 효과는 30일 동안의 상추 수경재배에서도 발견되었는데, 이는 대조군보다 상당히 높고 상업적 비료와 비교할 수 있는 수준이었다. 모든 결과는 핵심 성분이 생물학적 비료의 품질에 중요한 영향을 미친다는 것을 보여준다. 생물학적 비료 생산의 상업적 실현 가능성을 확인하기 위한 경제적 분석에서는 생물학적

비료 생산 규모의 확대에 대한 명확한 효과가 있었고, 원료 고등어 폐수의 실질적인 재활용으로부터 예상되는 이익은 한 번의 생분해를 수행할 경우 150리터당 $308.25로, 이는 연간 $14,796.13에 해당하게 된다. 결과적으로, 고등어 폐수로부터의 생물학적 비료 생산은 환경 영향의 감소와 지속 가능한 농업에 기여할 수 있다.

# 1. Introduction

As fish consumption increases, a considerable amount of fish waste and wastewater is generated, requiring an efficient treatment to reduce environmental impact. Nowadays, biofertilizer production by biodegradation attracts much attention not only as a complete reuse of fish wastewater, but also as a useful contributor for agriculture. Therefore, in this study, key components in biofertilizer were explored.

Therefore, it is essential to reutilize these resources in a sustainable way without causing environmental pollution. In this study, we employed a fed-batch process using a mixed culture of protein-degrading bacteria and plant growth-promoting bacteria to biodegrade mackerel wastewater. After that, the mackerel hydrolysates were used as a biofertilizer in wheat sprout and lettuce hydroponics to evaluate their plant growth-promoting activity and their effect on the bioactive compounds present in the biofertilizer that affect plant growth and health. We confirmed the possibility of antioxidant plants by extracting plant leaves and conducting ABTS and DPPH radical scavenging assays. Finally, an economic analysis of the entire process was conducted to assess its commercial viability.

In economic analysis, effect of scale-up production was distinctly revealed, and the expected profitability from the practical reuse (as biofertilizer) of raw mackerel wastewater was estimated to be

$308.25 per a single biodegradation in 150 L, which corresponds to $14,796.13 per year. As a result, the complete reuse of mackerel wastewater could feasibly provide essential benefits with both reduction of environmental impact and sustainable agriculture.

# 2. Materials and Methods

## 2.1. Preparation of mackerel wastewater and microorganisms

Mackerel wastewater (MWW) for the production of biofertilizer was prepared using raw mackerel. The entire mackerel was cut into small pieces (<1 cm), and autoclaved at 121℃ for 15 min. The floating fish oil was removed after autoclaved mackerel parts were cooled down. After then, the mackerel parts were squeezed through a porous cotton, and centrifuged to remove insoluble components. For these soluble components, the concentration of chemical oxygen demand-chromium ($COD_{Cr}$) was measured, and $COD_{Cr}$ was adjusted to 20,000 mg/L with distilled water (DW). For experiments, the simulated MWW was prepared with pH adjustment at 7. To degrade MWW in high degree of hydrolysis, 11 microorganisms reportedly showing high activity of protein degradation without mutual antagonism were used in an equal amount (J.H. Kang et al., 2018).: *Bacillus subtilis* (DQ219358), *Bacillus coagulans* (AF466695), *Bacillus circulans* (Y13064), Bacillus anthracis (AY138279), Brevibacillus agri (AY319301), *Bacillus licheniformis* (AY468373), *Bacillus fusiformis* (AY548950), *Bacillus cereus* (DQ923487), *Brevibacillus agri* (AJ586388), *Bacillus licheniformis* (EF113324) and *Brevibacillus paravrevis*

(AB215101). During experiments, each strain maintaining on a 1.5% nutrient agar plate at 4℃ was transferred to a fresh nutrient agar plate not to lose cell activity.

## 2.2 Biodegradation in a fed-batch process

To produce biofertilizer from MWW, biodegradation of MWW was carried out using a 3 L bioreactor (Winpact fermenter, Major Science, USA). The biodegradation started after 240 mL of seed culture (10%, v/v) was inoculated into 2160 mL of autoclaved MWW in a 3 L bioreactor. Therefore, the bioreactor was operated with 2.4 L of the total working volume for 48 h under the conditions of 45℃, 140 rpm and 16.7 vvm of aeration. To analyze major reaction parameters, biodegradation samples were taken periodically.

When cells reached a stationary phase in the batch operation, 2160 mL of culture broth was drained out of bioreactor to carry out a fed-batch operation. Under the same culture conditions, the remaining 240 mL of culture broth in the bioreactor was used as seed culture (10%, v/v) for the fed-batch operation. The MWW was fed at 3, 7, and 11 h in accordance with the batch operation data of cell growth at early, mid, and late phases. Samples were taken periodically, and the fed-batch operation was terminated when cell activity decreased obviously after 48 h. All measurements were carried out in triplicate.

The biodegradation in fed-batch process was also performed in an unelaborate 150 L reactor installing only agitator, aerator and temperature controller to access commercial feasibility for the production of biofertilizer from MWW. Prior to biodegradation, the whole working room was cleaned using the detergent Terg-A-Zyme (Alconox, USA) to prevent contamination. For the sterilization of reactor, a chloroform solution at 3 mg/L was filled into the reactor and drained after 1 day. After then, autoclaved hot DW (>80℃) was filled and placed for 7 h to wash the remaining chloroform. When the sterilization of the reactor was completed, biodegradation was started with inoculation of seed culture. Considering the commercial production, the process of seed culturing was simplified with the preparation of only 3 microorganisms (B. subtilis, B. circulans and B. paravrevis) that have the most potential degradation ability of protein among 11 microorganisms. The 3 microorganisms at the base of 1:1:1 weight was cultivated for 12 h, and 9-L culture broth (10%, v/v) was seed into the 150 L reactor. The reactor was set at 45℃ and 200 rpm. The air from the air compressor (set at 2 $kgf/cm^2$) was supplied through the air filter packed with sterile glass wool into the reactor at 5 vvm, and air bubbles were coming out by three ceramic disk-typed diffusers (12-cm diameter) installed at the bottom of the reactor. The fed-batch was processed with pulse feeding at 3, 7, and 11 h according to the fed-batch data obtained from 3-L biodegradation. The final working volume was 90 L,

samples were taken periodically until the termination of biodegradation. The fed-batch biodegradation operation was terminated after 48 h when cell activity decreased considerably. All measurements were carried out in triplicate.

## 2.3. Characterization of biodegradation

The change of cell growth was estimated by that of viable cell number. The sample taken from the bioreactor was poured on a nutrient agar plate under the appropriate dilution with sterile DW, incubated at 45℃ for 24 h, and counted the number of colonies forming on the nutrient agar plate. Considering the applied dilution, cell density was finally expressed as colony-forming units (CFU) per 1 mL of the sample. To determine the protease activity of the mixed culture, 10 μL of the culture supernatant was dropped at the center of a 1% skimmed milk agar plate and incubated at 45℃ for 24 h. The degree of protease activity was evaluated according to the diameter (in cm) of the clear zone appearing on the agar plate.

To determine the degree of MWW hydrolysis, the following procedure was executed: The sample taken from bioreactors was centrifuged at 15,000 × g for 15 min, 1 mL of culture supernatant was mixed with 5 mL of 0.5 N NaOH, 1 M Folin and Ciocalteu' s phenol reagent (Sigma-Aldrich, St. Louis, USA) was added to the mixture, the total mixed solution was incubated at 30℃ for 15 min

after vortexing, the incubated mixed solution was filtered using a cellulose acetate syringe filter (0.2 □m, Ministart NML, Sartorius, Germany), and finally 1.5 mL of the filtrate was used to measure its absorbance at 578 nm using a UV-Vis spectrophotometer (Opron 3000, Hanson Technology Co., Korea). L-tyrosine was used as a standard reagent for plotting a standard curve. With the absorbance values, the degree of hydrolysis (DH) value was calculated using the following formula:

$$DH\ (\%) = (A_0 - A)/A_0 \times 100$$

where $A_0$ and A are the absorbance of the autoclaved MWW sample and filtrate of the biodegraded MWW sample, respectively. Prior to investigation of the biodegraded MWW as a biofertilizer, the culture supernatant at the final stage of biodegradation was analyzed not only for its content of N, P and K content, but also for concentrations of heavy metals by Center for Research Facilities (Pukyong National University, Busan, Korea) to check its suitability as a biofertilizer.

## 2.4. Characterization of biofertilizer components

### 2.4.1 Culture supernatant

The culture supernatant is one of key biofertilizer components, and the biodegraded substances (mainly peptides) and non-biodegraded proteins are included in the culture supernatant. The biodegraded substances, hydrolysates present in the biofertilizer have molecular weights in diversity. Since antioxidant activity differs in molecular weight (Mponda and Kim, 2023), ultrafiltration was applied to the culture supernatant at the final stage of biodegradation to achieve different molecular-weight fractions, and consequently to investigate the effect of hydrolysates by the molecular weight on antioxidant activity. For ultrafiltration, a 5 kDa membrane (Vivaspin Turbo 15, VS15T11, Hanover, Germany) and 2 kDa membrane (Vivaspin 15R, VS15RH91) were used and centrifuged at 3,667 × g for 30 min. Finally, the biodegraded substances in the culture supernatant were classified into three groups depending on molecular weight. The collected molecular-weight fraction of each filtrate was used to determine antioxidant activity in plant leaves.

### 2.4.2. Cell pellet

Another key component of biofertilizer is cell pellet mainly composing of viable cells that were used for biodegradation. These microbial species were tested whether they show any plant-growth-promoting activity in hydroponics. Therefore, four plant-growth-promoting activities of microbial species were tested.

#### 2.4.2.1. Nitrogen fixation

To verify the activity of nitrogen fixation, each species was incubated in a nitrogen-free medium (known as Jensen's medium at 28° C for 7 days. Jensesn' s medium contained (g/L) 20 g of sucrose, 2 g of K2HPO4, 0.5 g of MgSO4, 0.5 g of NaCl, 0.1 g of FeSO4 · 7H2O, 0.005 g of Na2MoO4 and 15 g of agar. The nitrogen fixation ability was confirmed when microbial species forms colonies on the Jensesn' s medium (Das and De, 2018).

#### 2.4.2.2. Siderophore production

To detect whether each species produce siderophore, the modified chrom azurol S (CAS) assay was applied (Schwyn and Neilands, 1997). If a microorganism can produce siderophore, color change (blue to purple or dark purplish-red) occurs in the CAS-blue agar medium. The intensity of siderophore production activity is dependent upon the intensity of the color change. The CAS blue agar medium was prepared by the following procedure: 60.5 mg of CAS was dissolved in 50 mL of DW, the CAS solution was mixed with 10 mL of iron (III) solution containing 1 mM FeCl3 · 6H2Oand 10 mM HCl, the mixed solution was slowly added to 40 mL of hexadecyltrimethylammonium bromide (HDTMA) solution (72.9 mg HDTMA dissolved in DW) under stirring, the resultant dark-blue solution was autoclaved for 15 min, the autoclaved solution was gently mixed with 900 mL of autoclaved LB agar medium containing 10 g of tryptone, 5 g of yeast extract, 5 g of NaCl and 15 g of agar per L of DW (pH 6.8), and 15 mL of the mixed solution was poured into a petri dish to produce the CAS blue agar medium. After the CAS blue agar medium was prepared, 10 $\mu$L of each cultured species in a log-growth phase was spread on the CAS blue agar medium, and were incubated at 27° C for 7 days to observe the color change.

#### 2.4.2.3. Indole-3-acetic acid production

The ability of each species to produce indole-3-acetic acid (IAA; Sigma-Aldrich, St. Louis, USA) was tested using the method of Brick et al. (1991) with modification. The test was carried out as follows: The nutrient medium was enriched with 500 $\mu$g/mL of sterile L-tryptophan, each species was incubated on this medium at 30° C for 48 h, culture supernatant was collected after centrifugation at 15,000 × g for 10 min, two volumes of Salkowski reagent (2% 0.5 M FeCl3 dissolved in 35% perchloric acid) were added to a volume of the culture supernatant, the entire mixture was incubated at 28° C in the dark for 1 h, and finally the absorbance of resultant pink solution was measured at 530 nm using a UV spectrophotometer (Opron 3000). The IAA concentration was determined on a calibration curve where pure IAA was used as the standard.

### 2.4.2.4. Phosphate solubilization

The ability of each species to solubilize phosphate was initially tested on the Pikovskaya's (PVK) agar plate, (Gaur, 1990). The test was carried out as follows: Each species was incubated at 28° C for 7 days after inoculated in 25 mL of PVK broth, culture supernatant was collected after centrifugation at 15,000 × g for 30 min, 1 mL of the culture supernatant was mixed with 10 mL of chloromolibdic acid, DW was added into the mixture to make the total volume of 45 mL, 0.25 mL of chlorostannous acid was added to the diluted mixture, DW was added again into the final mixture to make the total volume of 50 mL, and finally the absorbance of resultant blue solution was measured at 600 nm using a UV spectrophotometer (Opron 3000). The soluble phosphate concentration was determined on a calibration curve where $KH_2PO_4$ was used as the standard.

If microorganisms affect plant growth, this effect would be different according to the number of viable cells. Therefore, the effect of viable-cell number on plant growth, health and functionality was also investigated. To collect viable cells, culture broth at the final stage of biodegradation was centrifuged at 3,667 × g for 30 min. The effect of viable-cell number on plant growth, health and functionality was investigated using viable cells at various cell numbers ($10^7-10^{10}$).

## 2.5. Phytotoxicty of biofertilizer

As a biofertilizer, phytotoxicity of final MWW culture broth was conducted to evaluate the toxic effect of the biodegraded MWW on plants. Cress (Lepidium sativum) seeds preliminarily incubated at 25° C for 12 h in the dark were used, and the phytotoxicity was evaluated according to the method described by Wong et al. (2001). A 5 mL sample was dropped on Whatman #1 filter paper (Sigma-Aldrich) placed in a sterile petri dish, 10 cress seeds were evenly distributed, and the petri dish was incubated at 25° C for 72 h in the dark under 75% humidity. A control group using DW was conducted in parallel. The sample is considered phytotoxic-free when the value of the germination index (GI) exceeds 50% (Mponda and Kim, 2023). The percentage of GI value was calculated as follows:

$$GI\ (\%) = RSG\ (\%) \times RRG\ (\%) / 100$$

where RSG (%) is the percentage of the number of seeds germinated in biodegraded MWW to the number of seeds germinated in control and RRG (%) is the percentage of mean root length in biodegraded MWW to mean root length in control.

## 2.6. Hydroponics

The effect of biofertilizer on plant growth was explored in hydroponics using wheat (Triticum aestivum) sprout for 7 days (short term) and lettuce (Lactuca sativa) seedling for 30 days (long term). To help the germination of wheat seeds, they were preliminarily washed with DW and incubated in darkness at 25° C for 2 days. After then, 20 wheat sprouts were cultivated in a mini-hydroponic culture pot (5 × 12 × 8 cm). The pot consisted of a glass vessel with a plastic screen inside where wheat sprouts were placed on top of the screen, and 300 mL of the 1000-fold diluted biofertilizer solution was supplied beneath the plastic screen. The wheat sprouts were cultivated under a 14 h light/10 h dark cycle and 60% relative humidity. A control group using DW was conducted in parallel. After 7 days, the length and weight of wheat leaves, and root shape of wheat were evaluated to explore the effect of each biofertilizer component (viable cells, small molecules or both components) on wheat growth. All measurements were carried out in triplicate.

To explore the effect of biofertilizer on plant growth in a long term (30 days) cultivation, lettuce hydroponics was conducted in an open-flow mini-hydroponic system (Self Gardening LED Water Culture Pureun, Kunok, Korea) equipped with light-emitting diode (LED) lamps (average intensity of 200 mmol/$m^2$/s). This hydroponic system consisted of two layers, and in each layer, five

sites were arranged in two parallel rows (total 20 sites). Lettuce seedlings were positioned in each site (6.5 x 6.5 x 5 cm). After biofertilizer was filled into a storage tank (40 L) situated at the bottom of the system, the biofertilizer was pumped in and circulated at a flow rate of 1.2 L/min for 30 days. This lettuce hydroponics was also conducted in parallel for both the control group using DW and the positive control group using a commercial fertilizer (Roots Organics Oregonism XL, Aurora Innovations, USA) to assess the quality of biodegraded MWW. Samples were taken periodically to assess the growth and health of lettuce, antioxidant content of lettuce leaves, and possible infiltration of detrimental bacteria into the circulating biofertilizer was tested. The test bacteria used in this analysis were a faecal contamination indicator (faecal coliforms) and pathogenic bacteria (Listeria and Staphylococcus). The detection of pathogens at day 15th and 30th days was conducted by plating 1 mL of biofertilizer solution on 3 M Petrifilm (3M Centre, St. Paul, MN, USA) in duplicate (Han et al., 2007). All measurements were carried out in triplicate.

To check the health of plants, the chlorophyll (*chl*) and carotenoid (*car*) content in the leaves were determined. Extraction of *chl* and *car* was conducted from 0.05 g of plant leaves at 4° C overnight using 1 mL of 80% acetone. The supernatant of the extract solution was collected after centrifugation at 14,000 × g for 5 min, and its absorbance was measured using a UV spectrophotometer (Opron 3000) at 663, 645, and 470 nm. With

the measured absorbance (A) values, the contents (in mg per g of sample) of *chl a*, *chl b*, and *car* were determined as follows:

$$\text{chl a} = (12.72 \times A663) - (2.59 \times A645)$$

$$\text{chl b} = (22.88 \times A645) - (4.67 \times A663)$$

$$\text{car} = ([1000 \times A470] - [3.27 \times \text{chl a}] - [104 \times \text{chl b}])/229$$

All measurements were carried out in triplicate.

## 2.7. Antioxidant activity

Since small peptides are known to show antioxidant activity, antioxidant activity of the biodegraded MWW samples was analyzed. Antioxidant activity of plant leaves was also analyzed to investigate the effect of antioxidant content in biofertilizer on plant growth and health. The antioxidant activity of plant leaves grown in hydroponics was evaluated as follows: 1 g of plant leaves were crushed and immersed in 200 mL of 96% ethanol for 24 h, the mixture was filtered through a 0.2 □m acetate filter (Sartorius), and the resultant filtrate was used for assays. All assays were carried out in triplicate.

### 2.7.1. 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity was assayed as follows: 1 mL of the filtrate sample was mixed with 2 mL of 0.1 mM DPPH solution dissolved in 80% ethanol, the mixture was incubated in a dark room for 30 min, and the absorbance (A) of mixture was then measured at 517 nm using a UV spectrophotometer (Opron 3000) against a blank containing 2 mL of DPPH solution and 1 mL of 80% ethanol. Negative and positive controls were prepared by mixing 1 mL of 80% ethanol with 2 mL of 0.1 mM DPPH solution and 0.1 mM L-ascorbic acid with 2 mL of 0.1 mM DPPH solution, respectively. DPPH antioxidant activity of the filtrate sample was evaluated as follows:

DPPH antioxidant activity (%) = (A of control - A of sample) / A of sample × 100

### 2.7.2. 2,2' -azino-bis(ethybenzthiazoline-6-sulfonic acid) radical cation decolorization activity

The 2,2' -azino-bis(ethybenzthiazoline-6-sulfonic acid) (ABTS) radical cations decolorization activity was assayed as follows: ABTS radical cations reagent was first prepared by mixing 5 mL of 7 mM ABTS with 5 mL of 4.9 mM K2S2O8, the mixture was incubated in a dark room for 16 h, the absorbance (A) of incubated mixture was adjusted to 0.72 at 734 nm with 80% ethanol, 100 $\mu$L of the filtrate was mixed with 900 □L of the prepared ABTS reagent above, the mixed solution was tempered for 6 min, and finally A value was measured at 734 nm against a blank that was prepared by replacing the ABTS reagent with 80% ethanol. The control was prepared by replacing the filtrate with DW, while the positive control was prepared by replacing the filtrate with 0.3 mM L-ascorbic acid. The inhibition (%) of the filtrate sample was evaluated as follows:

$$\text{Inhibition (\%)} = (\text{A of control} - \text{A of sample}) / \text{A of sample} \times 100$$

## 2.8. Economic analysis

To decide whether the production of biofertilizer from MWW is commercially feasible, economic analysis was conducted for the entire production process. The economic analysis was performed for both 3 L and 150 L fed-batch processes to explore merits of scale-up for commercialization. Key factors in economic analysis are capital investment cost and operation cost. The investment cost was estimated by multiplying the capital investment by an annuity factor, $k= i/[1- (1+i)-t]$, that is composed of interest rate (i) and the economic lifer time (t). In a biodegradation process using typical bioreactor equipment, i and t were set to 7% and 10 years, respectively (Kang et al., 2018). The operation cost included cost parameters for: raw materials, chemicals, utilities, and others such as labor, maintenance and insurance. The amounts of raw MWW and chemicals were estimated according to the process mass balances. It was assumed that the mackerel processing was accompanied by biofertilizer production to efficiently treat raw MWW generated in a mackerel processing plant and chemicals was purchased from laboratory chemical suppliers. The cost of heating and pumping for sterilization, input and output of wastewater, and agitation was included in utility requirements. Electricity, water and labor costs were based on the standard (in 2022) of average seasonal power consumption rate provided by Korea Electric Power Corporation (KEPCO), the

standard (in 2022) of average regional unit price provided by Korea Water Resources Corporation (K-water), and the standard (in 2022) of the minimum wage provided by Ministry of Employment and Labor (MOEL), respectively. The costs of maintenance and insurance were counted as 10 and 15% of the annual capital investment cost, respectively (Kang et al., 2018). Credits in the economic analysis for biofertilizer production from MWW included sale revenue of biofertilizer, saving from MWW treatment cost, and government subsidy. The price of biofertilizer was fixed on the lowest price of organic fertilizers on domestic sale and saving cost from MWW treatment was fixed on the average domestic treatment price per a ton of both liquid (MWW) and solid (cells).

## 2.9. Statistical analysis

All experiments and measurements of samples were carried out in triplicate, and the measured values were presented as the mean ± standard deviation (SD). The standard deviation was calculated as follows: Every deviation was squared, and the sum of the squares was divided by (n − 1), where n symbolizes the sample size. Finally, the extraction of the square root retrieved the original scale of measurement. The normality and homogeneity of the variance were verified using SAS software (SAS Inst. Inc., Cary, NC, USA; https://www.sas.com/en_us/home.html). One-way analysis of variance was applied to evaluate differences in the mean values of measurement properties using PROC GLM in the SAS program, followed by Tukey's HSD test (Neter et al., 1996). A p-value of less than 0.05 was considered significant.

# 3. Results and discussion

## 3.1. Characterization of biodegradation in a fed-batch process

To produce a good-quality biofertilizer having a great number of viable cells, fed-batch MWW biodegradation was operated after the cell growth reached a stationary phase in batch process. In the batch process for 72 h, pH started at 6.97, slightly decreased to 6.87 after 6 h, and gradually increased to 7.24 at the end **(Fig. 1A)**. With the maintenance of protease activity, the cell number increased to $3.55 \times 10^8$ CFU/mL in 48 h and slightly decreased to $1.55 \times 10^8$ CFU/mL at 72 h. The concentrations of $COD_{cr}$ and total nitrogen (TN) were reduced by 34.7% and 34.2%, respectively with the C/N ratio in a range of 9.1-10.3 in a stable culture conditions as cells utilized them for cellular metabolism and proliferation. The DH value reached 35.8% at 72 h, indicating that hydrolytic enzymes were sufficiently synthesized by viable cells proliferated on active consumption of $COD_{cr}$ and TN.

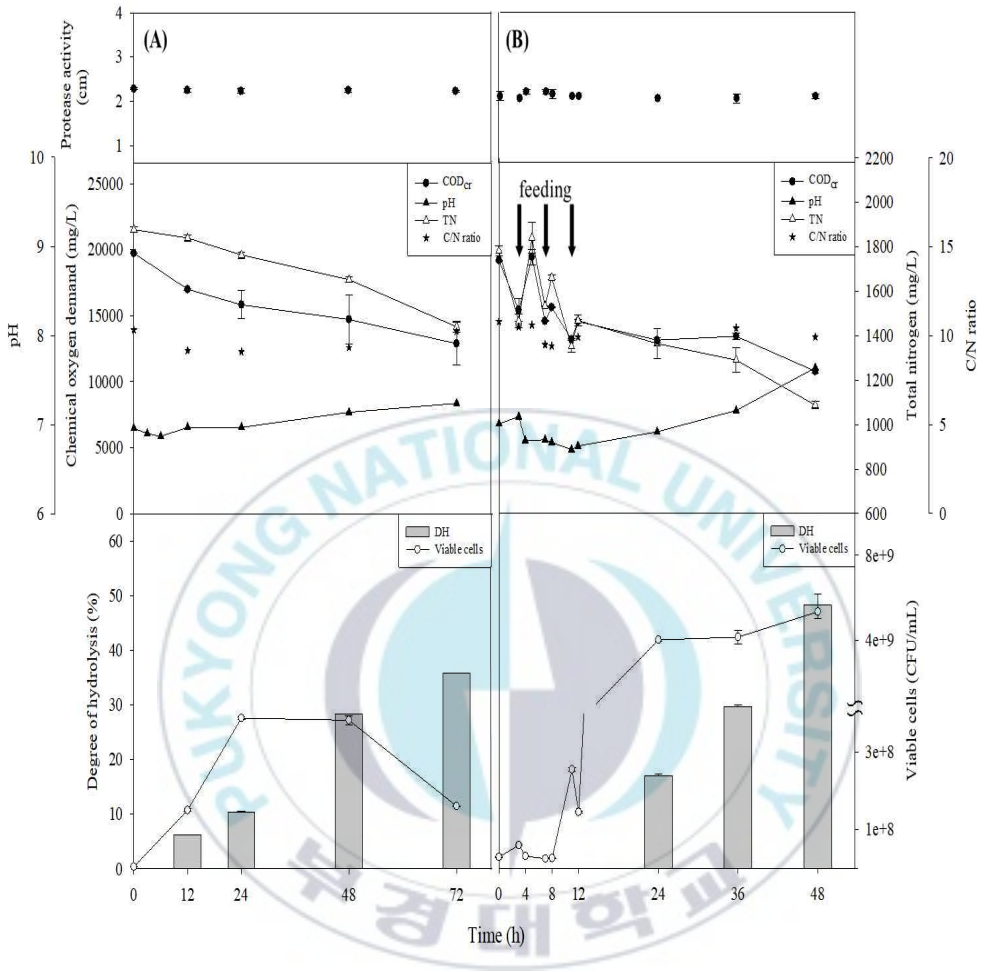


**Fig. 1.** Changes in reaction parameters during the biodegradation in batch process (A) and in fed-batch process (B).

In the fed-batch process, a pulse feeding strategy fitting cell growth was used and terminated at 48 h when cell activity was obviously reduced under microscopic observation. The main reaction parameters in **Fig. 1B.** During the biodegradation, pH started at 7.01, slightly decreased and after then, gradually increased to 7.46 at the end. The concentrations of $COD_{cr}$ and TN were reduced by 43.8% and 39.0%, respectively with the C/N ratio in a range of 9.5-10.8. The protease activity was maintained (in clear-zone sizes of 2.1-2.2cm) until the end, and the cell number gradually increased to $5.33 \times 10^9$ CFU/mL at 48 h. This increase in the viable cell number resulted in a higher degree of hydrolysis (maximally 48.1% at 48 h), indicating more production of small peptides and amino acids during the biodegradation. In conclusion, fed-batch process yielded higher viable cells than batch process, enabling a good-quality biofertilizer to be produced.

**Table 1.** The N, P and K contents and concentrations of heavy metals in the culture supernatant at a final stage of biodegradation.

| Element | Content (%) | Standard (%) |
|---|---|---|
| N | 0.11 | Sum of N, P and K >= 0.3 |
| $P_2O_5$ | 0.07 | |
| $K_2O$ | 0.19 | |
| **Heavy metal** | **Content (mg $kg^{-1}$)** | **Standard (mg $kg^{-1}$)** |
| As | 0.24 | 5 |
| Cd | n.d. | 0.5 |
| Ni | 0.01 | 5 |
| Cu | 0.11 | 30 |
| Cr | 0.03 | 30 |
| Zn | 0.57 | 130 |
| Pb | n.d | 15 |
| Hg | n.d | 0.2 |

Prior to investigation of the biodegraded MWW as a biofertilizer, the culture supernatant at the final stage of biodegradation was analyzed for N, P and K contents and concentrations of heavy metals to check its suitability (Table 1). The sum (0.37%) of N, P and K contents in the culture supernatant exceeded the standard (0.3%), and each individual concentration of all heavy metals was less than the standard, indicating the culture supernatant is eligible to be used as biofertilizer. In addition, the remaining non-biodegraded proteins present in biofertilizer would be slowly degraded by microorganisms, supplying some nutrition for plant. Therefore, the biofertilizer produced from MWW was adequately suitable for plant cultivation.

## 3.2. Characterization of biofertilizer components

As a key component of biofertilizer, hydrolysates present in culture supernatant are main factors to contribute to plant growth, health and functionality. Since proteases randomly break protein during biodegradation, the hydrolysates have many-sided molecular weights. Considering this fact, molecular-weight fractions of hydrolysates in biodegraded MWW were separated by ultrafiltration. The <2 kDa fraction (44.6%) of biodegraded MWW obtained from fed-batch process was more plentiful than that (21.8%) obtained from fed-batch process, while the >5 kDa fraction obtained from fed-batch process was considerably less (Table 2). This was because more hydrolysis (48.1%) took place in fed-batch process with a higher number of viable cells.

Table 2. Molecular-weight fractions of hydrolysates in biodegraded MWW in batch and fed-batch processes.

| Molecular weight (kDa) | Fraction (%) | |
|---|---|---|
| | In batch process | In fed-batch process |
| > 5 | 40.23 ± 1.72[a] | 26.06 ± 1.44[c] |
| 2-5 | 37.64 ± 0.86[ab] | 29.33 ± 3.17[bc] |
| < 2 | 21.84 ± 1.15[c] | 44.62 ± 1.74[a] |

The microorganisms included in biofertilizer are one of main factors to determine the quality of biofertilizer. Therefore, plant-growth-promoting activities of 11 microorganisms were explored. All microorganisms had nitrogen fixation activity, while siderophore production activity was detected only in 7 microorganisms (Table 3). The highest and the second highest IAA production activity was obtained from *B. agri* and *B. circulans*, while it was not detected in *B. subtilis*. The high levels of $PO_4^{-3}$ solubilization activity was obtained from *B. fusiformis*, *B. anthracis* and *B. cereus* in descending order. This result indicates 11 microorganisms had multi-functions, i.e., functions for MWW biodegradation and contribution to plant functionality. Therefore, 11 microorganisms used for biodegradation were found to be a key factor to determine the quality of biofertilizer.

Table 3. Plant-growth-promoting activities of 11 microorganisms.

| Microorganism | Nitrogen fixation | Siderophore production | IAA Production (μg/mL) | $PO_4^{-3}$ Solubilization (μg/mL) |
|---|---|---|---|---|
| *Brevibacillus agri* | + | + | 8.70 ± 1.52 | 28.44 ± 0.49 |
| *Bacillus cereus* | + | - | 1.82 ± 0.13 | 75.90 ± 1.79 |
| *Bacillus licheniformis* | + | + | 1.27 ± 0.55 | 57.57 ± 0.37 |
| *Brevibacillus paravrevis* | + | + | 1.15 ± 0.22 | 43.25 ± 0.25 |
| *Bacillus subtilis* | + | + | n.d.* | 46.15 ± 3.89 |
| *Bacillus licheniformis* | + | + | 3.18 ± 0.45 | 56.03 ± 2.29 |
| *Brevibacillus agri* | + | + | 13.18 ± 1.79 | 32.75 ± 2.84 |
| *Bacillus coagulans* | + | + | 1.36 ± 0.15 | 41.21 ± 2.28 |
| *Bacillus circulans* | + | - | 12.09 ± 1.18 | 30.10 ± 0.31 |
| *Bacillus anthracis* | + | - | 1.70 ± 0.15 | 78.74 ± 0.68 |
| *Bacillus fusiformis* | + | - | 2.82 ± 0.54 | 82.26 ± 5.68 |

*n.d.: not detected.

## 3.3. Test of biofertilizer components in short-term hydroponics

Prior to application of biofertilizer to hydroponics, phytotoxicity of biofertilizer was investigated. The phytotoxicity was affected by dilution, and original culture supernatant and culture broth (as biofertilizer) were phytotoxic (**Fig. 2**). At 100-fold dilution, the GI value (79.0%) of culture supernatant exceeded the standard GI value (50%) determining phytotoxicity, but that of culture broth did not exceed due to high viscosity derived from cells and remaining non-biodegraded proteins (Mponda and Kim, 2023). The culture broth was perfectly phytotoxic-free (> 100% GI) over 500-fold dilution. Since fertilizers are typically applied to plants at 1,000-fold dilution (Kim et al., 2021), biofertilizer produced from mackerel wastewater was eligible to meet the standard of non-phytotoxicity.

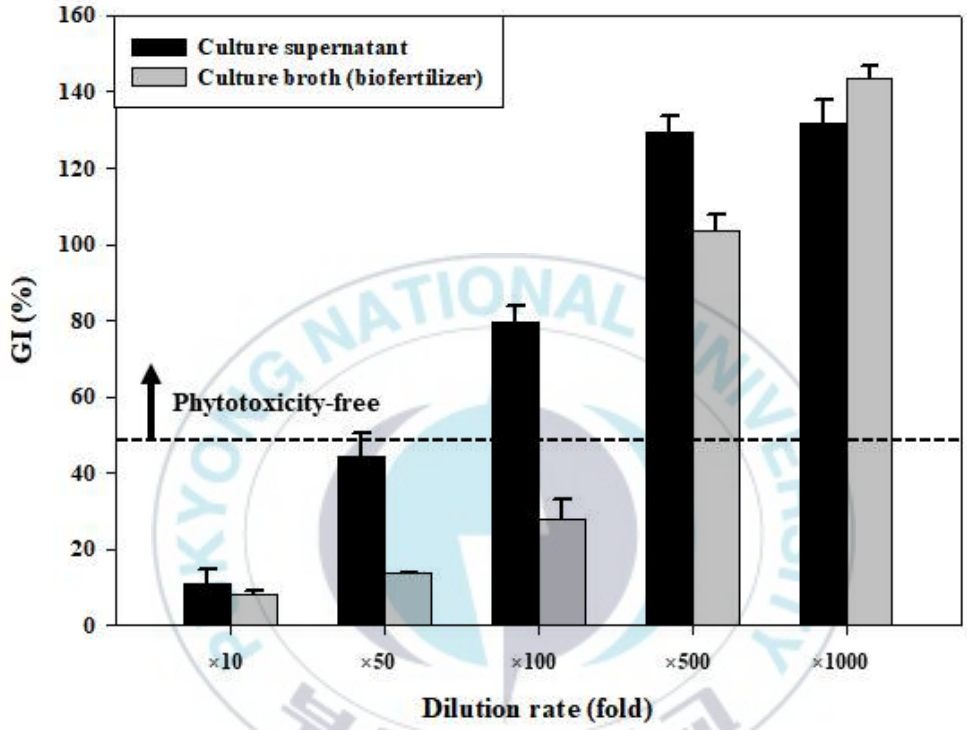


Fig. 2. GI values of the culture supernatant and culture broth at different dilution.

In this study, biofertilizer was produced from the MWW biodegradation, and the key components of the biofertilizer are culture supernatant and cell pellet after biodegradation. The investigation for the effect of key components of biofertilizer on plant growth is indispensable to assess the biofertilizer quality. To investigate this effect in a short term, wheat hydroponics was carried out for 7 days. In this hydroponics, culture supernatant, cell pellet and culture broth (combined two components) were used to assess the effects of hydrolysates, viable cells and synergy of both components, respectively against control. Each component of biofertilizer showed different effect on wheat growth. In the length of wheat leaf, the effect of culture supernatant or cell pellet was not significant, but that of cell broth was significant in comparison with control (Table 4). This indicates that there was a synergy effect of two components. However, this significantly synergistic effect was not observed in leaf weight. On the other hand, the effect of cell pellet on the length of wheat root was comparable to that of control, but culture supernatant or culture broth yielded a contrary result. This reason can be found in previous reports. Plant-growth-promoting microorganisms play their roles with positioning on the plant root (Moen et al., 2020; Adeleke et al., 2023), which was also observed in this study under the observation of scanning electron microscope (Fig. 3A). The root morphology of wheat was noticeably different after 7 days of hydroponics between DW (as control) and culture broth where

wheat roots were longer with more root hairs in DW (**Fig. 3B and 3C**). This is because wheat elongated more roots to efficiently secure limited nutrients available in DW, resulting in slower growth. This phenomenon was well observed in nitrogen-deficient environments (Li et al., 2016).

Table 4. Effect of each component in biofertilizer on wheat after 7 days of hydroponics

| Parameter | Control | Component in biofertilizer | | |
|---|---|---|---|---|
| | DW | Culture supernatant | Cell pellet | Culture broth |
| Growth indicators | | | | |
| Leaf length (cm) | 14.13 ± 0.01$^{b}$ | 15.19 ± 0.25$^{ab}$ | 14.61 ± 0.34$^{b}$ | 16.20 ± 0.12$^{a}$ |
| Leaf weight (g) | 2.30 ± 0.19$^{a}$ | 2.45 ± 0.10$^{a}$ | 2.36 ± 0.14$^{a}$ | 2.20 ± 0.17$^{a}$ |
| Root length (cm) | 15.96 ± 0.37$^{a}$ | 10.77 ± 0.31$^{b}$ | 14.83 ± 0.09$^{a}$ | 10.49 ± 0.59$^{b}$ |
| Root weight (g) | 3.73 ± 0.14$^{a}$ | 2.74 ± 0.18$^{ab}$ | 2.73 ± 0.28$^{ab}$ | 2.58 ± 0.09$^{b}$ |
| Health indicators | | | | |
| *chl a* (mg/g) | 3.10 ± 0.61$^{b}$ | 6.43 ± 0.62$^{ab}$ | 10.83 ± 0.23$^{a}$ | 10.85 ± 1.37$^{a}$ |
| *chl b* (mg/g) | 0.92 ± 0.66$^{b}$ | 3.47 ± 1.20$^{ab}$ | 6.70 ± 1.25$^{a}$ | 3.65 ± 0.55$^{ab}$ |
| *car* (mg/g) | 0.80 ± 0.33$^{a}$ | 1.34 ± 0.13$^{a}$ | 1.52 ± 0.51$^{a}$ | 1.97 ± 0.21$^{a}$ |
| Antioxidant activity in leaves (%) | | | | |
| DPPH activity | 40.94 ± 5.40$^{b}$ | 58.71 ± 1.57$^{ab}$ | 74.05 ± 4.71$^{a}$ | 59.06± 0.17$^{ab}$ |
| ABTS activity | 71.29 ± 2.21$^{a}$ | 76.44 ± 0.80$^{a}$ | 73.76 ± 3.48$^{a}$ | 74.57 ± 2.01$^{a}$ |

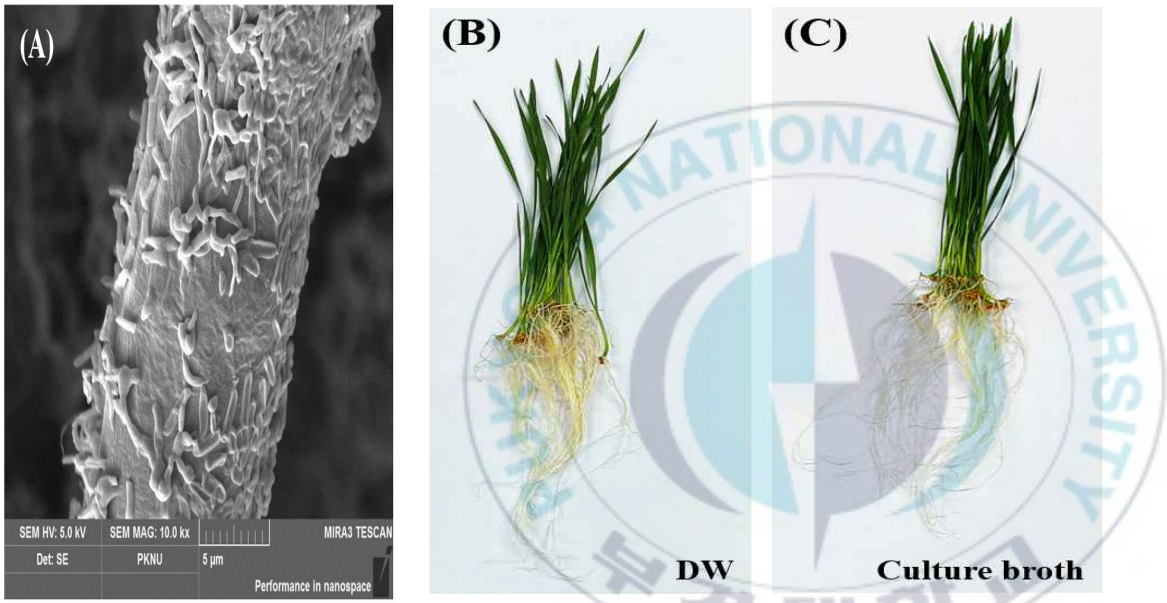


Fig. 3. Schematic diagram of scanning electron microscope of microorganisms positioning on wheat root (A) and the morphology of wheat root cultivated on DW (B) and culture broth (C). Samples were taken after 7 days of hydroponics.

The effect of biofertilizer component was also observed in wheat health (Table 4). Unlike the effect of biofertilizer component on wheat growth, each biofertilizer component on photosynthetic pigments (*chl a* and *chl b*) as indicators of wheat health was considerably effective. Especially, the effect of cell pellet was higher than that of cell supernatant. However, the effect of each biofertilizer component on carotenoid was not significantly different. In case of antioxidant activity in wheat leaves, the effect of cell pellet was the most effective in DPPH radical scavenging activity, while the effect of biofertilizer components was not significantly different against control (Table 4). As a result, each component of biofertilizer improved plant growth, plant health and antioxidant activity in leaves.

In this study, cell pellet had some beneficial effect on plant growth and health. Therefore, effect of viable cell number in biofertilizer on plant was inquisitive. The experiment connected with this issue was carried out in wheat hydroponics for 7 days. The greater number of viable cells biofertilizer included, the better wheat growth exhibited in leaf length, leaf weight and root length (Table 5). This trend also appeared in wheat health, especially in contents of chl a and chl b, while there was no significant difference in car content. Likewise, the effect of viable cell number was clearly shown in antioxidant activity in wheat leaves. This indicates that biofertilizer containing greater number of viable cells can be qualified as a quality biofertilizer, and the MWW biodegradation in fed-batch process is suitable to meet this standard.

Table 5. Effect of viable cell number in biofertilizer on wheat after 7 days of hydroponics

| Parameter | Control | Viable cells in biofertilizers (CFU/mL) | | | |
|---|---|---|---|---|---|
| | DW | $10^7$ | $10^8$ | $10^9$ | $10^{10}$ |
| Growth indicators | | | | | |
| Leaf length (cm) | 15.30 ± 0.40$^{b}$ | 16.46 ± 0.27$^{ab}$ | 16.32 ± 0.68$^{ab}$ | 16.83 ± 0.39$^{a}$ | 17.02 ± 0.07$^{a}$ |
| Leaf weight (g) | 2.31 ± 0.10$^{b}$ | 2.43 ± 0.10$^{ab}$ | 2.56 ± 0.05$^{ab}$ | 2.61 ± 0.06$^{a}$ | 2.57 ± 0.05$^{a}$ |
| Root length (cm) | 12.89 ± 0.4$^{b}$ | 13.28 ± 0.04$^{ab}$ | 13.48 ± 0.35$^{ab}$ | 13.64 ± 0.04$^{a}$ | 13.74 ± 0.09$^{a}$ |
| Root weight (g) | 3.82 ± 0.16$^{a}$ | 3.62 ± 0.12$^{a}$ | 3.63 ± 0.18$^{a}$ | 3.57 ± 0.08$^{a}$ | 3.44 ± 0.11$^{a}$ |
| Health indicators | | | | | |
| *chl a* (mg/g) | 4.78 ± 0.92$^{b}$ | 5.75 ± 1.04$^{ab}$ | 7.31 ± 1.07$^{ab}$ | 7.63 ± 1.20$^{ab}$ | 8.52 ± 1.23$^{a}$ |
| *chl b* (mg/g) | 1.76 ± 0.22$^{c}$ | 2.11 ± 0.30$^{b}$ | 2.68 ± 0.25$^{ab}$ | 2.69 ± 0.33$^{ab}$ | 3.13 ± 0.24$^{a}$ |
| *car* (mg/g) | 0.81 ± 0.11$^{a}$ | 1.12 ± 0.36$^{a}$ | 1.30 ± 0.34$^{a}$ | 1.46 ± 0.12$^{a}$ | 1.60 ± 0.11$^{a}$ |
| Antioxidant activity in leaves (%) | | | | | |
| DPPH activity | 70.76 ± 0.67$^{c}$ | 76.34 ± 0.45$^{b}$ | 76.12 ± 0.23$^{b}$ | 79.02 ± 0.89$^{ab}$ | 81.03 ± 0.23$^{a}$ |
| ABTS activity | 86.60 ± 0.42$^{c}$ | 88.25 ± 0.64$^{c}$ | 91.04 ± 0.33$^{b}$ | 91.87 ± 0.64$^{ab}$ | 93.60 ± 0.11$^{a}$ |

## 3.4. Application of biofertilizer to lettuce hydroponics

The effect of biofertilizer components on plant growth, health and functionality was confirmed in short-term hydroponics. For the commercialization of biofertilizer, application of the biofertilizer to relatively long-term hydroponics is indispensable. In this respect, lettuce cultivation in open-flow hydroponics was carried out for 30 days. The number of lettuce leaves increased from 4.00 at the beginning, and gradually increased 8.89 after 30 days, which was significantly higher than that of the control group, but not significant difference from that of a commercial fertilizer (Table 6). This number of lettuce leaves was almost like previous report for leaves of lettuce after 30-day hydroponics (Jung and Kim, 2020). The length of lettuce seedling was 6.71 cm and was 6.53 cm after 30 days. The decrease of lettuce length was due to sprouting of new leaves. This indicates that a better fertilizer results in more active spouting of new leaves. In this growth indicator, the effect of biofertilizer was bigger than those of control and a commercial fertilizer. Overall, real effect of biofertilizer on plant growth can be reflected in leaf weight. The leaf weight after 30 days exhibited the effect of biofertilizer was significantly higher than that of control and comparable to that of a commercial fertilizer.

**Table 6.** Growth indicators, health indicators and antioxidant activity in lettuce leaves after 30 days in open-flow lettuce hydroponics

| Parameter | Control | Fertilizer solution | |
|---|---|---|---|
| | DW | Biofertilizer | Commercial fertilizer |
| Growth indicators | | | |
| Leaf number | 8.00 ± 0.44$^{b}$ | 8.89 ± 0.10$^{a}$ | 8.93 ± 0.09$^{a}$ |
| Leaf length (cm) | 5.60 ± 0.16$^{b}$ | 6.53 ± 0.18$^{a}$ | 5.73 ± 0.18$^{b}$ |
| Leaf weight (g) | 5.07 ± 0.09$^{b}$ | 5.69 ± 0.09$^{a}$ | 6.12 ± 0.38$^{a}$ |
| Health indicators (mg/g) | | | |
| *chl a* | 10.57 ± 1.09$^{a}$ | 10.60 ± 1.34$^{a}$ | 11.98 ± 1.54$^{a}$ |
| *chl b* | 9.63 ± 0.18$^{a}$ | 9.16 ± 0.21$^{a}$ | 8.94 ± 1.07$^{a}$ |
| *car* | 0.39 ± 0.06$^{b}$ | 0.93 ± 0.06$^{a}$ | 1.18 ± 0.18$^{a}$ |
| Antioxidant activity in leaves (%) | | | |
| DPPH activity | 78.46 ± 0.94$^{a}$ | 81.56 ± 1.73$^{a}$ | 80.48 ± 2.38$^{a}$ |
| ABTS activity | 61.59 ± 1.14$^{b}$ | 73.91 ± 0.64$^{a}$ | 79.09 ± 1.36$^{a}$ |

The effect of biofertilizer on the levels of photosynthetic pigments chl a and chl b was not significant, but the effect on the level of car pigment was significantly from that of control group.

The effect of biofertilizer was also reflected in raising the functionality of lettuce. ABTS radical scavenging activity (81.56%) in lettuce leaves by hydroponics using biofertilizer was higher than that by hydroponics using DW (as control), and comparable to that of a commercial fertilizer. This value of ABTS radical scavenging activity was significantly higher than that (51.9%) of previous report for leaves of lettuce after 30-day hydroponics (Jung and Kim, 2020). However, this effect was not significantly different in DPPH radical scavenging activity. In this study, the value of DPPH radical scavenging activity was 81.56%, which was almost approximate value (83.1%) of previous report for leaves of lettuce after 30-day hydroponics (Jung and Kim, 2020). The level of antioxidant activity has respect to the quantity of antioxidant present in the fertilizer solutions, resulting in a discrepancy in antioxidant activity. Moreover, ABTS radical scavenging activity is mainly dependent upon both lipophilic and hydrophilic antioxidants, whereas DPPH radical scavenging activity is more specific for lipophilic antioxidants (Prior et al., 2005). Considering the above facts, it was concluded that hydrophilic antioxidants were richer in biofertilizer from more hydrolyzed MWW due to increased viable cells produced in fed-batch process. Therefore, the fed-batch process can provide biofertilizer with improvement of plant growth,

health and functionality. During the hydroponics, the pathogen infiltration into the circulating biofertilizer was investigated, since it can depreciate the ability of 11 microorganisms as beneficial bacteria, i.e., reduction in the biodegradation of remaining protein (lower supply of nutrition) and lower contribution to plant growth as well. however, any pathogen was not detected in circulating biofertilizer during the hydroponics. Therefore, well maintained biofertilizer quality during long-term hydroponics is important for lettuce yield. The test results are shown in Table 7 in which none of the test pathogens were detected in the flowing biofertilizer solution. This result may be possible not only due to the characteristics of members of the genus Bacillus used for the MWW biodegradation, but also antimicrobial hydrolysates resulted from the biodegradation (Jung and Kim, 2020).

The genus Bacillus is known to possess antimicrobial property with the production of antibiotics or non-modified bacteriocins (Lee and Kim, 2011). Moreover, mackerel hydrolysates exhibit antimicrobial activity against Gram-positive (*Listeria innocua*) and Gram-negative (*Escherichia coli*) bacteria (Ennaas et al., 2015).

Table 7. Result of the number of pathogens infiltrating into the circulating biofertilizer during lettuce hydroponics

| Duration of hydroponics (day) | Infiltrated pathogen | | |
|---|---|---|---|
| | *E. coli*[a] | *Staphylococcus*[b] | *Listeria*[c] |
| 15 | 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 show red-violet; and 1, all of colonies show red-violet.

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

## 3.5. Economic analysis

In this study, the quality of biofertilizer produced from mackerel wastewater was analyzed based on the component of biofertilizer, and the quality of biofertilizer was acceptable as a consequence of the result on plant growth, health and functionality. What comes next is scale up production and economic analysis to seek the commercialization feasibility. Therefore, the MWW biodegradation in fed-batch process was also carried out in a 150-L reactor using the data obtained in a 3-L reactor and the result is shown in **Fig. 4**.

During the biodegradation, the protease activity steadily maintained in a range of 1.9-2.2 cm (represented as clear zones formed by proteases), resulting in 45.9% of DH after 48-h biodegradation. The number of viable cells reached $1.4 \times 10^9$ CFU/mL at 48 h, which was not significantly different from that obtained from 3-L fed-batch biodegradation. The initial pH (6.5) coming from the seed culture increased to 6.97 after pulse feedings, and it gradually increased to 7.64 at the end. As a result of biodegradation, the concentrations of $COD_{cr}$ and TN were reduced by 44.3% and 41.5%, respectively under C/N ratios at 9.5-10.4.

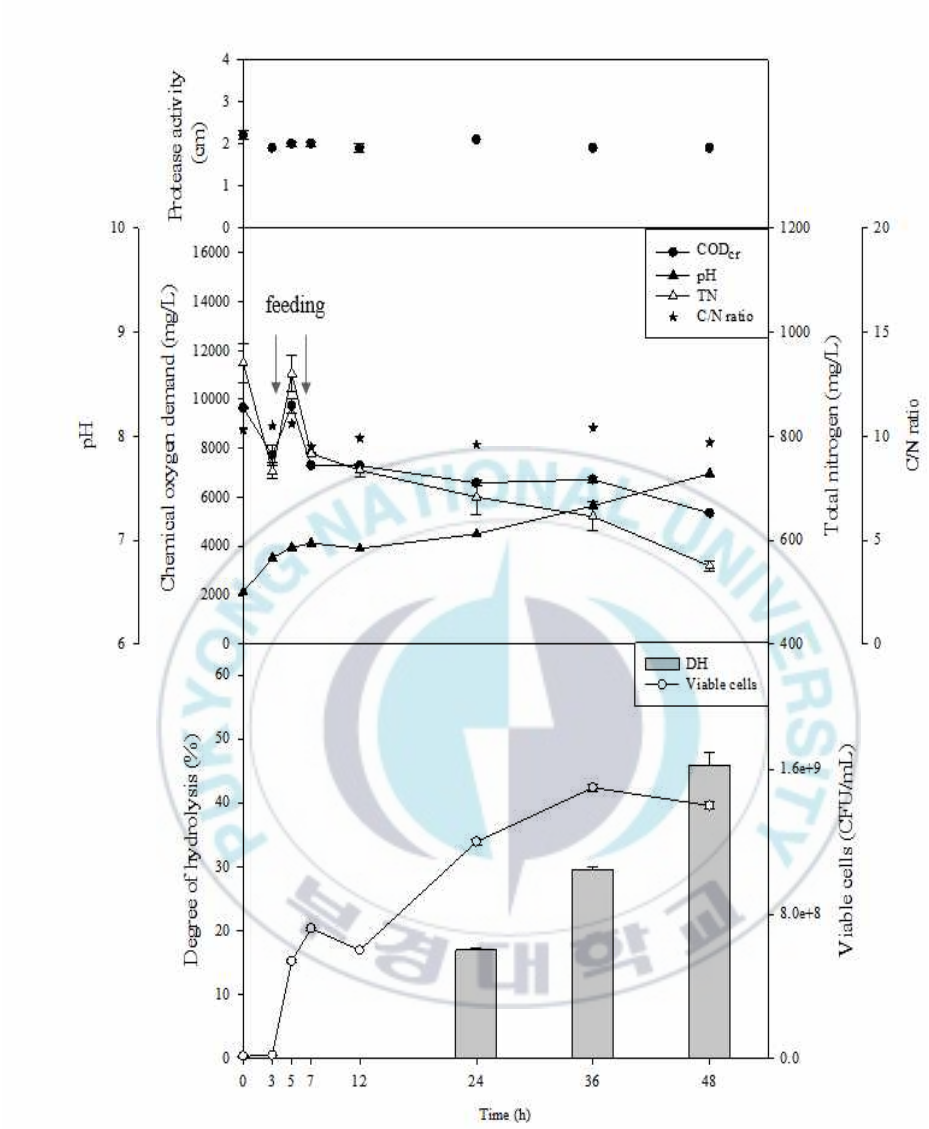


**Fig. 4.** Changes in reaction parameters during the biodegradation in 150 L reactor operating in fed-batch process.

With data of both 3-L reactor and 150-L reactor, economic analysis was applied to the entire process of biofertilizer production from MWW, and the result is shown in Table. 8. In this study, key components of biofertilizer produced from MWW were investigated and the biofertilizer was confirmed its potential use in hydroponics. Therefore, the next step is commercialization feasibility analysis based on economic analysis to practically reutilize MWW. Economic analysis is worthwhile to assess process feasibility of MWW reutilization and identify bottlenecks, although uncertainty can remain in economic analysis because reliable calculation of such process expenses in detail is not usually allowed in an early development stage (Tufvesson et al., 2011). Economic analysis was based on the evaluation for cost of the biofertilizer production in fed-batch process, and credits were also considered for the treatment of both MWW and sludge (mainly cells) remaining after biodegradation.

**Table 8.** Cost evaluation to produce biofertilizer by a single biodegradation process of mackerel wastewater in both 3-L and 150-L reactors.

| Category and its parameters | Cost ($) per unit† | | Cost ($) | |
|---|---|---|---|---|
| | 3 L | 150 L | 3 L | 150 L |
| Capital investment cost | | | | |
| • Biodegradation equipment | 541.51/y | 2,904.00/y | 11.28 | 60.50 |
| • Auxiliary equipment | 909.71/y | 1,435.55/y | 18.95 | 29.91 |
| Operating cost | | | | |
| • Seed culture | | | (0.24 g required) | (9 g required) |
| - Culture chemicals | 0.26/g | | 0.11 (0.44 g) | 4.10 (16 g) |
| - Electricity | 0.07/KWh | | 1.93 (26 h) | 4.53 (61 h) |
| - Water | 0.56/ton | | 0.0003 (0.6 L) | 0.0003 (1.8 L) |
| - Labor | 7.15/h | | 171.55 (24 h) | 450.84 (63 h) |
| • Raw mackerel wastewater | | | | |
| - Preheating | 0.07/KWh | | 0.15 (2 h) | 0.22 (3 h) |
| - Pumping into reactor | 0.07/KWh | | 0.22 (3 h) | 0.89 (12 h) |
| - Labor | 7.15/h | | 42.94 (6 h) | 107.34 (15 h) |
| • Reactor preparation | | | | |
| - Washing | 0.56/ton | | 0.01 (10 L) | 0.09 (160 L) |
| - Sterilization | 0.07/KWh | | 0.04 (0.5 h) | 2.38 (32 h) |
| - Labor | 7.15/h | | 7.16 (1 h) | 250.47 (35 h) |
| • Biodegradation | | | (total vol.: 2.4 L) | (total vol.: 90 L) |
| - Electricity | 0.07/KWh | | 1.43 (0.4 kW, 48 h) | 3.56 (1 kW, 48 h) |
| - Labor | 7.15/h | | 350.66 (49 h) | |
| - Water (for cooling) | 0.56/ton | | 0.0006 (1 L) | - |
| • Final product (as biofertilizer) | | | | |
| - Preservation (1% lactate) | 0.26/kg | | 0.01 (0.024 kg) | 0.23 (0.9 kg) |
| - Bottling and packing | 0.56/ton | | 0.37 (2.4 L) | 13.99 (90 L) |
| - Labor | 7.15/h | | 14.31 (2 h) | |
| • Maintenance | 90.86/y | | 3.02 | 9.04 |
| • Fixed operation | 136.3/y | | 4.54 | 13.56 |
| Credits | | | | |
| • Product value as biofertilizer‡ | 9.36/L | | -22.50 (2.4 L) | -843.75 (90 L) |
| • Saving the treatment cost | | | | |
| - Wastewater | 0.05/L | | -0.13 (2.4 L) | -4.70 (90 L) |
| - Cell | 0.11/kg | | -0.0003 (0.0024 kg) | -0.01 (0.09 kg) |
| • Government subsidy | | | -372.78 | -776.41 |
| Total | | | 233.46 | -308.25 |

The fixed capital is the capital required for installation of process equipment including all the accessories for start and operation of biodegradation. The cost of equipment for biodegradation (in a reactor installing stirring, heating and cooling systems, sampling and drain ports and sensors) was evaluated to be US$3,813.44 (for 3 L) and US$20,450.71 (for 150 L). According to this cost, the equivalent annual cost was calculated to be US$541.51 (for 3 L) and US$2,904.00 (for 150 L) by multiplying this cost by an annuity factor (k = 0.142). Considering the frequency of operation of single biodegradation (one run per week) and break-in period in labor, the biodegradation equipment cost was calculated to be US$11.28 (for 3 L) and US$60.50 (for 150 L) per biodegradation. For the commercial production of biofertilizer, auxiliary equipment is necessary, such as pumps, oxygen and steam generators, MWW reservoirs, packing machinery, etc. This equipment cost was calculated to be US$6,460.38 (for 3 L) and US$10,109.50 (for 150 L), which yielded - US$909.71 (for 3 L) and US$1,435.55 (for 150 L) of the annual cost when an annuity factor (k = 0.142) was applied. Accordingly, the auxiliary equipment cost became - US$18.95 (for 3 L) and US$29.91 (for 150 L) per biodegradation. In the analysis for operating cost, it includes costs for the preparation of seed culture, raw MWW and reactor, biodegradation and the treatment of final product as biofertilizer, maintenance and fixed operation. Prior to biodegradation, seed culture must be prepared: hence, 0.24 g and 9 g cells are required for 3 L and

150 L fed-batch processes. To calculate cost for seed culture, culture chemicals, electricity, water and labor were considered. Considering all these parameters, the costs required for seed culture preparation were US$173.79 and US$459.47 for 3 L and 150 L, respectively. For the biodegradation, raw MWW source must be prepared by preheating and pumping into reactors and handling (counted as labor charge) for these works was individually calculated for 3 L and 150 L considering different sizes of reactors. The cost was calculated to be US$43.31 and US$108.46 for 3 L and 150 L, respectively.

The next consideration in calculation of operating cost was cost for reactor preparation. Reactor must be washed and sterilized before biodegradation and handling for these works was individually calculated for 3 L and 150 L. The cost was calculated to be US$7.20 and US$252.93 for 3 L and 150 L, respectively. When all these works were completely prepared, biodegradation could be started. In the biodegradation, electricity for stirring, heating, use of tap water for cooling to control reactor temperature (not applied to unelaborate 150 L reactor), and handling for these works were considered and calculated to be US$352.08 and US$354.22 for 3 L and 150 L, respectively. After the collection of final culture broth (cell pellet and supernatant), preservation (by 1% lactate), bottling and packing are required. Considering the handling for these works, the process cost was calculated to be US$14.69 and US$28.54 for 3 L and 150 L, respectively.

In addition, maintenance costs and fixed operating costs (including depreciation, taxes, insurance, etc.) cannot be neglected in the calculation of operating cost. These costs were calculated to be US$362.80 and US$1,084.89 for 3 L and 150 L, respectively (equivalently US$7.56 for 3 L and US$22.60 for 150 L per single biodegradation) as 10 and 15% of the annual capital investment cost, respectively (Tufvesson et al., 2011). Based on the above calculations, the total production costs of biofertilizers (2 L and 120 L) per single batch operation were estimated to be US$598.63 and US$1,226.22 for 3L and 150 L, respectively, which corresponds to US$28,734.35 and US$58,858.50 per year. Consequently, the greatest contribution to production costs was labor charge (US$586.81 as 98.0% of the total cost – 3L) (US$1,173.63 as 95.7% – 150L), and next was equipment (US$30.23 as 5.05% – 3L) (US$90.41 as 7.37% – 150L). On the other hand, utility costs (US$11.82 in 3 L and US$52.59 in 150 L) were not influential, although the impact of the individual cost varies greatly with scale.

The production of biofertilizer produced from raw MWW can be considered as credits in the economic evaluation due to economic benefits by selling. Compared with the lowest price of organic fertilizer on the market, the biofertilizer selling is worth – US$22.50) and US$843.75 for 3 L and 150 L, respectively. Moreover, reuse of MWW brings the treatment effect of wastewater and sludge, saving disposal fees for them. Saving costs

for wastewater were calculated US$0.13 (3 L) and US$4.70 (150 L) per biodegradation, and those for sludge were calculated US$0.0003 (3 L) and US$0.01 (150 L) per biodegradation, based on the current disposal fees that are annually increasing under strict limitations from the government. Accordingly, government encourages reuse of fish waste/wastewater to efficiently conserve environment. Therefore, the biological MWW treatment earns a credit (subsidy) from the government, and the current rate of financial aid from the government is 60% (Ministry of Environment, 2023). The government subsidy was evaluated to be and −US$372.78 and −US$776.41 for 3 L and 150 L, respectively per single biodegradation, and thus the total financial profits from MWW reuse were US$22.63 and US$848.46 for 3 L and 150 L, respectively per biodegradation. Therefore, a merit of scale−up was revealed in this economic analysis, and thus, the scale−up effect will be more considerable in industrial scales (Lam et al., 2014).

In the economic analysis for biofertilizer production in 150 L, the expected profitability from reuse of raw MWW was estimated to be US$308.25 per biodegradation, which corresponds to US$14,796.13 per year. This implies that the production of biofertilizer from raw MWW would be profitable. It is not economically attractive so much, since this project is in the early stages of development. It will become more interesting project by: increase of the selling biofertilizer price by recognition of

biofertilizer quality; reduction of the capital investment and operational costs by scale– up (in an industrial scale); increase of disposal fees for organic waste and wastewater by stricter government policy; and consideration of crop and sustainability effect in agriculture, as a substitute for chemical fertilizers.

# 4. Conclusion

Since organic waste and wastewater are recognized as useful resources, biofertilizer production from fish wastewater attracts much attention not only as a complete reuse of fish wastewater, but also as a useful contributor for agriculture. In this respect, key components of biofertilizer were collected from biodegradation in fed-batch process and characterized. In fed-batch process, a higher number (5.33 × 109 CFU/mL) of viable cells yielded more low-molecular-weight hydrolysates with higher degree of hydrolysis (48.1%), resulting in high antioxidant activities (84.17% for DPPH and 98.45 for ABTS) from hydrolysates <2 kDa. Microorganisms used for biodegradation possessed plant-growth-promoting activities, and positioned on the plant root during wheat hydroponics. The greater number of viable cells biofertilizer included, the better wheat growth exhibited in leaf length, leaf weight and root length. This trend was also exhibited in both wheat health and antioxidant activity in wheat leaves. Likewise, this effect of biofertilizer was found in lettuce hydroponics for 30 days, which was significantly higher than that of control and comparable to that of a commercial fertilizer. All the results indicate that key components significantly affect the quality of biofertilizer. In the economic analysis to access commercial feasibility of biofertilizer production, there was clear effect of scale-up in biofertilizer production, and the expected profitability

from the practical reuse of raw mackerel wastewater was estimated to be $308.25 per a single biodegradation in 150 L, which corresponds to $14,796.13 per year. As a result, the production of biofertilizer from mackerel wastewater can contribute to reduction of environmental impact and sustainable agriculture as well.

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