Thesis for the Degree of Master of Engineering

Aerobic biodegradation of fish-meal wastewater from lab scale to large scale

by

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Department of Biotechnology and Bioengineering,

The Graduate School

Pukyong National University

February 2008

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(어분 제조공정에서 발생되는 폐수를

실험단위에서부터 대규모 단위로의 생물학적 처리)

Advisor : Prof. Joong Kyun Kim

by

Soo Kyoung Jeong

A thesis submitted in partial fulfillment of the requirement for the degree of

Master of Engineering

in the Department of Biotechnology and Bioengineering, Graduate School,
Pukyong National University

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A Dissertation

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February 28, 2008

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Abstract

To reutilize the wastewater generated during the process of fish-meal production (FMW), seven thermophilic microorganisms were newly isolated and their characteristics of aerobic biodegradation of FMW were examined in a lab-scale bioreactor. It clearly showed that the amino-acid composition (12.54 $g \cdot 100g\ sample^{-1}$) in the final broth of the biodegradation using 8-fold diluted FMW was almost twice that of non-biodegraded FMW. The levels of amino acids in the final broth were also comparable to those in a commercial fertilizer. When more (32-fold) diluted FMW was used as a substrate, phytotoxicity of biodegraded final broth was further reduced with disappearance of a strong unpleasant smell in the end. The results of the lab-scale biodegradation suggest the promising potential of biodegraded FMW for the production of fertilizer.

From the laboratory results, a large-scale biodegradation using the original FMW was designed and carried out in a 1-ton reactor. During the biodegradation, the concentration of DO in the liquid broth was closely related to pH and the oxidation reduction potential (ORP) as well. Keeping a low level of DO resulted in both the increase of pH and the decrease of ORP with strong smell of ammonia. Under the maintenance of DO level over 1 $mg \cdot l^{-1}$,

the initial fishy smell from the FMW was converted to a pleasant smell in the end with production of fairly good content of amino acids. Therefore, the DO level in the liquid broth was found to be decisive influence on the quality of final fermented broth, and ORP to be a key operation parameter in biodegradation of FMW. The final broth taken from the bioreactor is required to be maintained its quality as a liquid fertilizer, during the period of circulation in market. The addition of 3% lactic acid could preserve the final broth well for six months, whereas the addition of lower concentrations of lactic acids could not preserve properly and resulted in putrefaction in the end. When a good-quality final broth was used, the addition of 1% lactic acid could preserve it for six months with amino-acids enriched soy sauce-like smell. From the results of the large-scale biodegradation, the reutilization of FMW is expected to yield high economic value.

I. INTRODUCTION

The amount of fisheries waste generated in Korea is expected to increase with a steady increase in population to enjoy taste of slices of raw fish. The fisheries waste is reduced and reutilized through the fish meal production. Depending on the raw material used, there are basically two types of fish-meal manufacturing processes: those that use fish wastes, such as heads, bones or other residues, and those that use the whole fish. The process, using fish wastes, is the commonest used in the Korean industries. The first step of the fish-meal manufacturing processes is the compression and crushing of the raw material, which is then cooked with steam, and the liquid effluent is filtered off in a filter press. The solids obtained are introduced to a rotating drier and finally cut and crumbled to obtain the commercial fish-meal product. The liquid stream contains oils and a high content of organic suspended solids. After oil separation, the fish-meal wastewater (FMW) is generated with stinky odor and shipped to wastewater treatment place.

FMW has been customarily disposed of by dumping into the sea, since direct discharge of FMW can cause serious environmental problems. Besides, bad smell, which is produced during fish-meal manufacturing processes, causes civil petition and stricter regulations for this problem come into force every year in Korea. Therefore, there is an urge to seek for an effective treatment to remove the organic load from the FMW; otherwise the fish meal factories will be forced to shut down.

Biological treatment technologies of fish-processing wastewater have been studied to improve effluent quality (Battistoni and Fava, 1995; Park et al., 2001). The common feature of the wastewaters from fish processing is their diluted protein content, which after concentration by a suitable method would enable the recovery and reuse of this valuable raw material, either by direct recycling to the process or subsequent use in animal feed, human food, seasoning, etc. (Afonso and Borquez, 2002). It has been reported that the organic wastes contain compounds, which are capable of promoting plant growth (Day and Katterman, 1992), and seafood processing wastewaters do not contain known toxic or carcinogenic materials unlike other types of municipal and industrial effluents (Afonso and Borquez, 2002). Therefore, FMW could be a valuable resource for agriculture. However, potential utilization of this fish wastes has been limited because of its bad smell (Martin, 1999). There is an increasing need to find ecologically acceptable alternatives to overcome this problem.

Aerobic biodegradation has been widely used in treatment of wastewaters, and recently references to the use of meso- and thermophilic microorganisms have become increasingly frequent (Cibis et al., 2006). During the biodegradation, the organic matter is biodegraded mainly through exothermic aerobic reactions, producing carbon dioxide, water, mineral salts, and a stable and humified organic material (Ferrer et al., 2001). There have been few reports that presented the reutilization of biodegraded waste products as liquid-fertilizer:a waste product of alcoholic fermentation of sugar beet (Agaur and Kadioglu, 1992) and diluted manure streams after biological treatment (Kalyuzhnyi et al., 1999). Therefore, aerobic biodegradation is considered to be the most suitable alternative to treat FMW and realize a market for such a waste as fertilizer. Moreover, the reutilization of FMW can create the high additional value, since FMW is collected from the industry with security for cost of FMW treatment.

Scale-up is the study of the problem associated with transferring data obtained in laboratory equipment to industrial production. It is clear that problems of scale-up in a bioreactor are associated with the behavior of liquid in the bioreactor and the metabolic reactions of the organisms. Biological properties, especially various constants involved in kinetic equations depend on scale-up, although the metabolic patterns remain unchanged. The typical differences of bioreactions have been known between large-scale and lab-scale reactors: i) the biomass yield is reduced at large-scale; ii) more metabolic by-products are produced at large-scale; and iii) limiting substrate gradients are present at large-scale as measured at different heights in the bioreactor (Bylund et al., 1998; Xu et al., 1999). All these phenomena are classified under the term ‘scale-up effect’. Although scale-up is still regarded more as an art than a science (Humphrey, 1998), transport limitation is considered as one of the major factors responsible for phenomena observed at large-scale. For this reason, it is necessary to investigate both the biological and technological aspects of the system in the large scale.

Prevention of slowing down deteriorative processes is required after liquid fertilizer was produced by aerobic biodegradation in order to maintain its quality during the period of circulation in market. Generally, the lower the pH, the less the chance that microbes will grow and cause spoilage. It has been known that organic acids can lower the pH and have a bacteriostatic effect (Zhuang et al., 1996). A number of other methods have also reported for microbial control (Agarwal et al., 1986; Stratham and Bremner, 1989; Curran et al., 1990). However, information of the preservation of liquid-fertilizer has been lacking so far. A means of a more long-term preservation of the liquid fertilizer is required.

In this study, biodegradation of FMW was attempted in a large scale and suitability of biodegraded product was determined as a fertilizer. For this purpose, microorganisms were newly isolated and their characteristics of biodegradation were investigated in lab scale. Based on the lab-scale data, biodegradation of FMW was then carried out in a 1-ton bioreactor. To examine the fertilizing value of the biodegraded end product, analysis of amino-acid composition and tests of seed germination and root length were accomplished. The long-term preservation of the end-product was also studied.

II. MATERIALS AND METHODS

1.Isolation of useful microorganisms

The potential aerobically-degrading bacteria were isolated from commercial good-quality humus and from compost and leachate collect ed at three different sites of composting plants. The soil and compost samples (0.5 g each), and 0.5 ml of raw leachate sample were added into 5 ml of sterile 0.2% NaCl and agitated to obtain homogeneous suspension. One ml of each suspended liquid was pipetted into various 10 ml-tubes that contained 0.8% nutrient broth (pH 6.8), yeast-maltose medium (3 $g{\cdot}l^{-1}$ of yeast extract, 3 $g{\cdot}l^{-1}$ of malt extract, 5 $g{\cdot}l^{-1}$ of peptone, 10 $g{\cdot}l^{-1}$ of glucose, and 0.05 $g{\cdot}l^{-1}$ of ampicillin, pH 6.2) and Bennet's medium (1 $g{\cdot}l^{-1}$ of yeast extract, 1 $g{\cdot}l^{-1}$ of beef extract, and 10 $g{\cdot}l^{-1}$ of glucose, pH 7.2). After one day incubation at both of 45℃, the liquid culture was spread onto the agar plate of each medium. The separated colonies formed on the plates were serially picked up and inoculated onto the fresh agar plates repeatedly until a pure isolate was obtained.

All isolates were spread on four different agar plates: 1% skim milk agar for detection of proteolytic microorganisms; 3.215% spirit blue agar for detection of lipolytic microorganisms and starch hydrolysis agar (5 $g{\cdot}l^{-1}$ of beef extract, 20 $g{\cdot}l^{-1}$ of soluble starch, 10 $g{\cdot}l^{-1}$ of tryptose, 5 $g{\cdot}l^{-1}$ of NaCl, and 15 $g{\cdot}l^{-1}$ of agar, pH 7.4) and cellulose agar (10 $g \cdot l^{-1}$ of cellulose powder, 1 $g \cdot l^{-1}$ of yeast extract, 0.1 $g \cdot l^{-1}$ of NaCl, 2.5 $g \cdot l^{-1}$ of $(NH_4)_2SO_4$, 0.25 $g \cdot l^{-1}$ of K_2HPO_4, 0.125 $g \cdot l^{-1}$ of $MgSO_4 \cdot 7H_2O$, 0.0025 $g \cdot l^{-1}$ of $FeSO_4 \cdot 7H_2O$, 0.025 $g \cdot l^{-1}$ of $MnSO_4 \cdot 4H_2O$, and 15 $g \cdot l^{-1}$ of agar, pH 7.2) for detection of carbohydrate-degrading microorganisms, respectively. All agar plates were incubated under same conditions until change of color or a clear zone around each colony appeared.

2. Tests of antagonism and salt effect on growth

Screening of potential bacterial antagonists against other isolates was carried out by the use of perpendicular streak technique as described by Alippi and Reynaldi (2006). Of all forty-six isolates, several isolates were arbitrarily selected and streaked on nutrient agar plates first. The surface of agar plate streaked by each isolate was divided into four sections, and then, four other isolates were streaked perpendicular to the original streak. Each plate was incubated at both of 45 and 55°C for three days to allow the production of antagonistic substances and then checked for any growth inhibition of each isolate.

After the antagonism test, the effect of salt concentration on cellular growth was investigated with the screened microorganisms. Each screened cell was spread on three different agar plates containing various concentrations of 1, 2 and 3.5% NaCl additionally. All agar plates were incubated under the same conditions, and the effect of salt on the growth of each cell was verified by measuring difference in change of color or size of a clear zone around each colony.

3. Identification of useful Microorganisms

Chromosomal DNA of the isolate was extracted from cells grown in the given medium with AccuPrep®Genomic DNA extraction kit (Bioneer), according to the manufacturer's instructions. Amplification of the DNA using the 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) were performed with a PCR thermal cycler DICE model TP600 (Takara) as described by Kim et al. (2007), and the 16S rDNA genes were determined by the Macrogen Company (Seoul, Korea). Related sequences were searched against GenBank (National Center for Biotechnology Information, USA) using the Advanced BLAST similarity search option (Altschul et al., 1997) accessible from the homepage, http://www.ncbi.nlm.nih.gov/. BioEdit Sequence Alignment Editor version 5.0.9 was used to check alignment and remove all positions with gaps before calculating distances with DNA dist programme in PHYLIP (version 3.5c).

4. Lab-scale Aerobic Biodegradation

Using the screened microorganisms, aerobic biodegradation was carried out in a 100 ml-syringe that served as the reaction vessel (Cho et al., 2006). Under supply of sterile oxygen, 0.2 g (wet weight basis) of mixed isolates (5% inoculums) were suspended in the syringe with 40 ml of the original FMW (pH 6.5±0.2) obtained from a fish-meal factory. To examine the possibility of combined wastewaters as a substrate for the production of liquid-fertilizer, milk wastewater (MW) generated from milk-processing factory or wasted broth (WB) generated after the cultivation of photosynthetic bacteria for biomass production was used with the original FMW at the dilutions of 10-fold and 32-fold. The effect of combined microorganisms (isolated microorganisms with a photosynthetic bacterium (PSB), *Rhodobacter capsulatus*) on biodegradation was also investigated. For faster biodegradation, the inoculated cells were previously acclimated for two days in the original FMW under an aerobic condition. The syringes prepared in this way were incubated in a shaking incubator at 45℃ and 180 rpm. The gas produced by the mixed microorganisms during incubation was analyzed by gas chromatography (GC). At the same time liquid broth was taken from the syringe to measure the concentrations of chemical oxygen demand-dichromate (COD_{Cr}) and total nitrogen (TN).

5. Large-scale Aerobic Biodegradation

Large-scale biodegradation using original FMW was executed twice in a 1-ton bioreactor. The characteristics of the FMW are given in Table 1. The pH of FMW was not adjusted because it was always measured to be 7.0±0.3, and 30 l of isolated mixed microorganisms were seeded into the bioreactor filled with 600 l of original FMW. The bioreactor was operated at 42±4℃, and air from the blower was supplied into the bioreactor through ceramic disk-typed diffuser. Two blowers and 12 disks were used to supply adequate oxygen into the bioreactor. Ten-fold diluted 'Antifoam 204' was used when severe foams occurred during biodegradation. Samples from the bioreactor were collected periodically, and the concentration of dissolved oxygen (DO), oxidation-reduction potential (ORP) and pH were measured.

Table 1

Characteristics of the original FMW

Constituents	Concentration ($mg \cdot l^{-1}$)
COD_{Cr}	115,000±13,000
TN	15,400±1,300
BOD_5	68,900±7,600
NH_4^+-N	2,800±600
NO_3^--N	0
NO_2^--N	0

6. Seed germination test

To evaluate the phytotoxicity of biodegradated FMW, seed germination test were carried out according to the method of Wong et al. (2001). Ten milliliters of biodegraded FMW sample taken from 5l-bioreactor were centrifuged at 8,000 rpm and filtered through 0.45 μm membrane filters before being introduced into a 10 ml-tube and kept at 4℃ until tested. For tests of seed germination and root length, 5 ml of each aliquot of sample was pipetted into a sterile petri dish lined with Whatman #1 filter paper. Ten cress (*Lepidium sativum*) seeds were evenly placed in each dish (three replicates for each sample). The plates were incubated at 25℃ in the dark at 75% of humidity. Distilled water was used as a control. Seed germination and root length in each plate were measured at 72h. The percentages of relative seed germination (RSG), relative root growth (RRG) and germination index (GI) after expose to wastewater treated were calculated as the following formula (Zucconi et al., 1981 Hoekstra et al., 2002):

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

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

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

7. Preservation of Final Broth

Various concentrations (0.5, 1 and 3%) of lactic acid were used to preserve four different types of biodegradated wastewaters. The preservation was carried out for six months in a 1 l plastic bottle, which was stored at room temperature. During the preservation, samples were periodically taken under a clean bench for the odor evaluation by panel. The panel was composed of twenty persons. The change of level of amino acids was also analyzed.

8. Analyses

The concentrations of cations (NH_4^+) and anions (NO_2^- and NO_3^-) were estimated by IC (Metrohm 792 Basic IC). The columns used in these analyses were Metrosep C2-150 and Metrosep Supp 5-150 for cation and anion, respectively. The concentrations of COD_{Cr} and TN concentrations were analyzed by the Water-quality Analyzer. The five days biological oxygen demand (BOD_5) was analyzed by the OxiDirect BOD-System. The composition of amino acids in samples was analyzed at Feeds and Foods Nutrition Research Center in Pukong National University by our request.

For determination of nitrogen and carbon dioxide gases, 20 $\mu\ell$ samples (injection volume) were taken for GC/TCD (Perkin Elmer Instruments) analysis. The columns used were a 'molecular sieve 13X' and 'carboxen 1,000' for nitrogen and carbon dioxide, respectively. In analyses of both gases, the following conditions were equally applied: the carrier gas was helium at a flow rate of 30 ml·min^{-1} and the injector and the detector temperatures were 100 and 200℃, respectively. However, the oven temperature for nitrogen gas was 40℃, and that for carbon dioxide gas was 40℃ for 3 min initially then increased to 170℃ with a rate of 30℃·min^{-1}.

III. RESULTS AND DISCUSSION

USEFUL MICROORGANISMS FOR BIODEGRADATION OF FMW

1. Screening of useful microorganisms

In our previous study (Kim et al., 2007), we finally developed seven isolates, which were able to produce no antagonistic substances against other microorganisms among forty-six isolates. The seven isolates were given the names as JB1 to JB7, respectively. All the cells were very motile in vegetative state and Gram-positive rods measuring 0.5-0.7 ㎛ in width and 3-5 ㎛ in length.

In the experiment for examination of the salt effect on cellular growth, there was no effect on cellular growth of each microorganism at the concentrations of 1 and 2% of NaCl. However, the effect was distinct at 3.5%. The concentration of salt in a raw material(fish wastes) used for the production of fish meal varies, and thus the salt concentration of the original FMW can also vary. The salt concentration of the original FMW used in this study was measured to be much less than 1% (0.6±0.1%). It is concluded that the low salt concentration of FMW cannot affect the growth of the seven isolates.

2. Identification of the screened isolates

Species-specificfor the seven isolates could be derived using 16S-rDNA sequence analysis, since each species possesses one or more unique 16S-rDNA nucleotide regions. Approximately 1,500 bp sized-fragment of the 16S-rRNA gene of each isolate was amplified and sequenced. Each fragment band was confirmed by electrophoresis after performance of PCR. Sequence analysis of the 16S-rDNA gene and BLAST sequence comparison confirmed that the isolated strain JB1 was Bacillus subtilis(GenBank Accession Number: DQ219358), JB2 was Bacillus licheniformis (GenBank Accession Number: AY468373), JB3 was Brevibacillus agri (GenBank Accession Number: AY319301), JB4 was Bacillus coagulans (GenBank Accession Number: AF466695), JB5 was Bacillus circulans(GenBank Accession Number: Y13064), JB6 was Bacillus anthracis(GenBank Accession Number: AY138279) and JB7 was Bacillus fusiformis (GenBank Accession Number: AY548950) with similarity of 98-100%.

3. Metabolic characteristics of the isolates

Metabolic characteristic of each isolate was characterized in a 100 ml syringe reactor and is tabulated in Table 2. Among the seven isolates, the isolate JB7 had the highest values of maximum O_2 consumption rate and maximum N_2 production rate under an aerobic condition, which resulted in increase of pH during biodegradation. The isolate JB2 showed the similar characteristic with high maximum CO_2 production rate. However, JB3, JB4 and JB5 had relatively lower O_2 consumption rate with lower N_2 and CO_2 production rates. Interaction always occurs between diverse microbial populations, and both of them may benefit from the interactions. Coexistence in the mixed culture has been reported to occur only if between species competition is weaker than within species. Recently, a new model with introduction of mutualism between competitive species has been proposed (Zhang, 2003). Thus, mutualism among isolated microorganisms could promote coexistence and enhance the carrying capacity of the system, since they did not show any antagonism.

Table 2

Metabolic characteristics of isolated microorganisms

Isolated microorganism	Max. O_2 consumption rate (mole·h-1)	Max. N_2 production rate (mole·h-1)	Max. CO_2 production rate (mole·h-1)
JB1	2.77	1.84	0.25
JB2	4.27	2.50	0.83
JB3	1.45	1.00	0.21
JB4	1.80	0.22	0.20
JB5	1.44	0.76	0.23
JB6	2.39	0.10	0.18
JB7	4.38	3.06	0.62

LAB-SCALE BIODEGRADATION

1. Effects of oxygen and dilution ratio on biodegradation of FMW

The experiment was carried out in a 100 ml syringe. In our previous study (Kim et al., 2007), it was found that the concentrations of COD_{Cr} and TN in original FMW were much more reduced by the seven isolates under the condition of O_2 supplement, compared to those under the condition of no O_2 supplement. With supplement of O_2, the production of CO_2 gas was increased with increase of N_2, but only small bubble was produced in the syringe vessel without supplement of O_2. The result indicates that the greater mineralization of the organic matter occurred under an aerobic condition. It is known that oxygen consumption is a general index of microbial metabolism (Tomati et al., 1996). Thus, the seven isolates showed more active mineralization of the organic matter under an aerobic condition.

The different content of organic matter in FMW can affect the biodegradation, since cellular metabolism is dependent on substrate concentration (Maria et al., 2000). For this reason, the effect of dilution ratio of FMW on biodegradation was also examined in our previous study using a 100 ml syringe (Kim et al., 2007). The oxygen consumption rate by the seven isolates in the syringe vessel tended to increase when more diluted FMW was used as substrate, i.e., faster biodegradation occurred with more diluted FMW, which resulted in faster removal rates of COD_{Cr} and TN. The maximum rates of gas productions of CO_2 and N_2 during biodegradation were the highest with 8-fold diluted FMW, and the microbial population also tended to increase with more diluted FMW.

2. Biodegradation of FMW in a lab-scale bioreactor

In our previous laboratory study (Jeong bo Kim, 2006; Kim et al., 2007), we examine the characteristics of aerobic biodegradation of FMW in a 5l- bioreactor starting on 8-fold diluted FMW. The removal percentages of COD_{Cr} and TN were more prominent with slight decrease of COD_{Cr}/TN ratio, compared with the results of the syringe experiment using the same diluted FMW. This is because oxygen is able to be supplied more sufficiently into a bioreactor, but not in the syringe vessel. It has been known that the COD/N ratio may influence biomass activity, and therefore on the metabolic pathways of organic matter utilization (Ruiz et al., 2006). Based on this information, the cell activity and metabolic pathways of the seven isolates may be maintained steadily during the period of this experiment. The change of pH was not considerable and the weight of sludge decreased approximately half, which means that some fractions of suspended solids were biodegraded.

Reduced phytotoxicity of final FMW broth was attained from aerobic biodegradation in a 5l-bioreactor when more diluted (32-fold) FMW was used. The trend in reduction of COD_{Cr} or TN was not much different, and pH was also not changed considerably. However, a noticeable result in this experiment was that a strong unpleasant smell (mainly a fishy smell) mostly disappeared in the end.

3. Phytotoxicity of final broth

Sufficient aeration promotes the conversion of organic matters into nonobjectionable, stable end products such as CO_2, SO_4^{2-}, NO_3^-, etc. An incomplete aeration may result in accumulation of organic acid, thus giving trouble to plant growth if the fertilizer is incorporated into the soil (Jakobsen, 1995). To examine the fertilizing value of the final broth taken from a 5l-bioreactor, phytotoxicity assays were accomplished in our previous study (Kim et al., 2007). The effect of the final broth on the germination was found to be not pronounced, since all cress seeds used in the experiment were germinated in one day. However, it has been reported that seed germination is regarded as a less sensitive method than root elongation when used as a bioassay for the evaluation of phytotoxicity (Wang and Kentri, 1990). Instead, the germination index (GI), which combines the measure of relative seed germination (RSG) and relative root growth (RRG) of cress seed (Lepidium sativum), has been reported to be the most sensitive parameter used to evaluate the toxicity (Zucconi et al., 1981). When phytotoxicity of the final broth of original FMW was assayed, its average GI was only 8.0%. The reduction in GI indicates that some characteristics existed had an adverse effect on root growth. This may be attributed to the release of high concentrations of ammonia and low molecular weight organic acids (Wong, 1985; Fang and Wong, 1999), since cress used in this study is known to be sensitive to the toxic effect of these compounds (Fuentes et al., 2004). The values of GI (%) tended to increase with increase of dilution ratio of the final broth. At the dilution ratio of 32, the average value of GI was found to be over 50%. A GI of 50% has been used as an indication of phytotoxin-free compost (Zucconi et al., 1985). According to this GI criterion, the final broth of biodegradation using 8-fold diluted FMW required more mineralization to reach stabilization. Phytotoxicity caused by organic compounds can be remedied by increasing the period of aerobic decomposition (Wong et al., 2001). Instead of increasing the period of biodegradation, the concentration of substrate had to be reduced to remedy the phytotoxicity, since cellular metabolism is dependent on substrate concentration (Maria et al., 2000). Consequently, more diluted FMW was required for the further stabilization of the organic matter to maintain the long-term fertility in soil.

Phytotoxicity was also assayed on the final FMW broths at various dilution ratios in our previous study (Kim et al., 2007). The GI value of the final broth biodegraded on 32-fold diluted FMW was higher than that of the final broth biodegraded on 8-fold diluted FMW at the same dilution ratio. This indicates that the degree of mineralization was higher in the biodegradation using 32-fold diluted FMW and this resulted in high value of GI, i.e., GI tended to increase as the content of mineralized organic matter in FMW increased by biodegradation (Fuentes et al., 2004). The final broth of biodegradation using 32-fold diluted FMW was found to require only two-fold dilution to reach stabilization.

4. Composition of amino acids in final broth

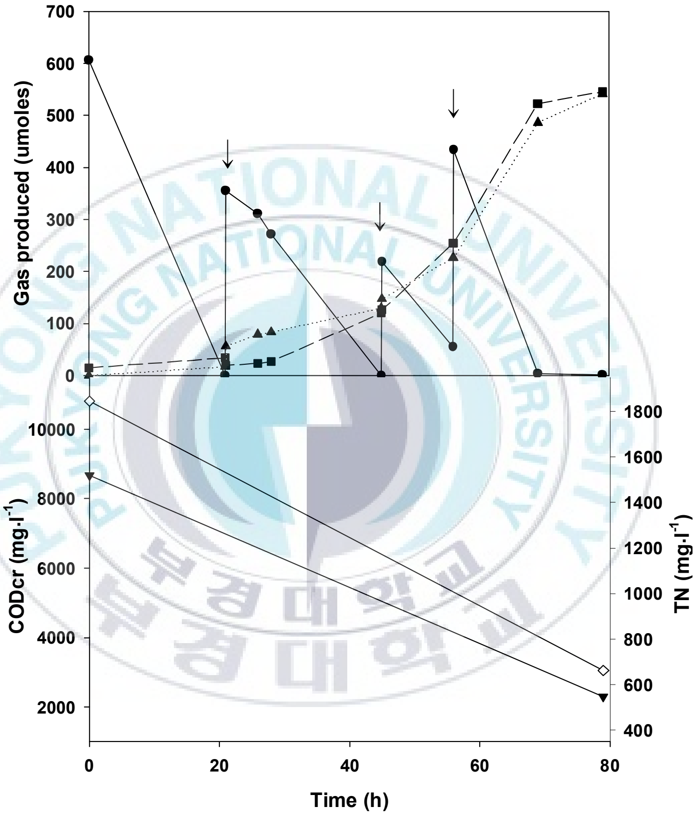
Amino acids are an essential part of the active fraction of organic matter in a fertilizer. The growth of plants depends ultimately upon the availability of a suitable balance of amino acids, and their composition might also be used as a means of assessing biodegradation. From this point of view, the amino-acid composition of the biodegraded FMW taken from a 5-l bioreactor was analyzed. In our previous study (Kim et al., 2007), the results clearly showed that the amino-acid composition in the final broth (12.54 g·100g sample-1) was almost twice that of non-biodegraded FMW. The higher content of amino acids in the final brothis probably due to the higher degree of mineralization of FMW, which indicates release of more nutrients available for plants. The amino-acid composition in the final broth was also higher in comparison with that of a commercial fertilizer for horticultural plants. Moreover, the content of sulfur-containing amino acids, cysteine and methionine, were much higher. It has been reported that the sulfur-containing amino acid, methionine is a nutritionally important essential amino acid and is the precursor of several metabolites that regulate plant growth (Amir et al., 2002). Consequently, the levels of amino acids in the final broth are comparable to those in a commercial fertilizer.

5. Biodegradation on combined wastewater

The dairy industry generates a large amount of effluent ranging from 0.2 to 10 L per L of processed milk.

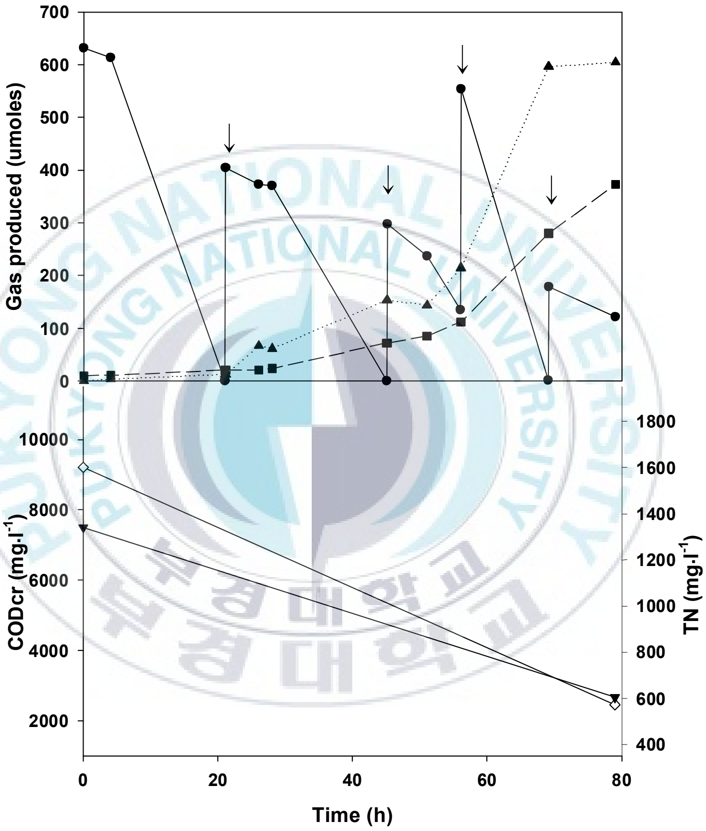
Wastewaters from the dairy industry are polluting chiefly because of the organic matter they contain and they should be treated before discharge into surface waters. Conventional treatment of dairy wastewater involves aerobic processes, since fats, lactose and proteins are all easily degraded by bacterial populations (Samkutty et al., 1996). For industrial reutilization of milk waste (MW), biodegradation on the combined wastewater of FMW and MW was investigated in a 100-ml syringe. In the experiment 10-fold and 32-fold diluted combined wastewaters were used, and the results are shown in Fig. 1 and Fig. 2. As the mixing ratio of FMW to MW decreased, the organic strength of the combined wastewater was weaker. In both experiments, the increase of the amount of MW in the combined wastewater did not give any remarkable effect on the production of N_2 or CO_2. Removal of COD_{Cr} and TN was rather reduced, compared to that of control. This indicates that isolated microorganisms seem to be not fit for biodegradation of MW, probably due to its different organic component. To reutilize FMW with MW together, further study is required to develop some useful microorganisms, especially for biodegradation of FMW.

(A)



Gas produced (umoles)
700
600
500
400
300
200
100
0
10000
8000
6000
4000
2000
CODcr (mg·l-1)
1800
1600
1400
1200
1000
800
600
400
TN (mg·l-1)
0
20
40
60
80
Time (h)

(B)



(C)

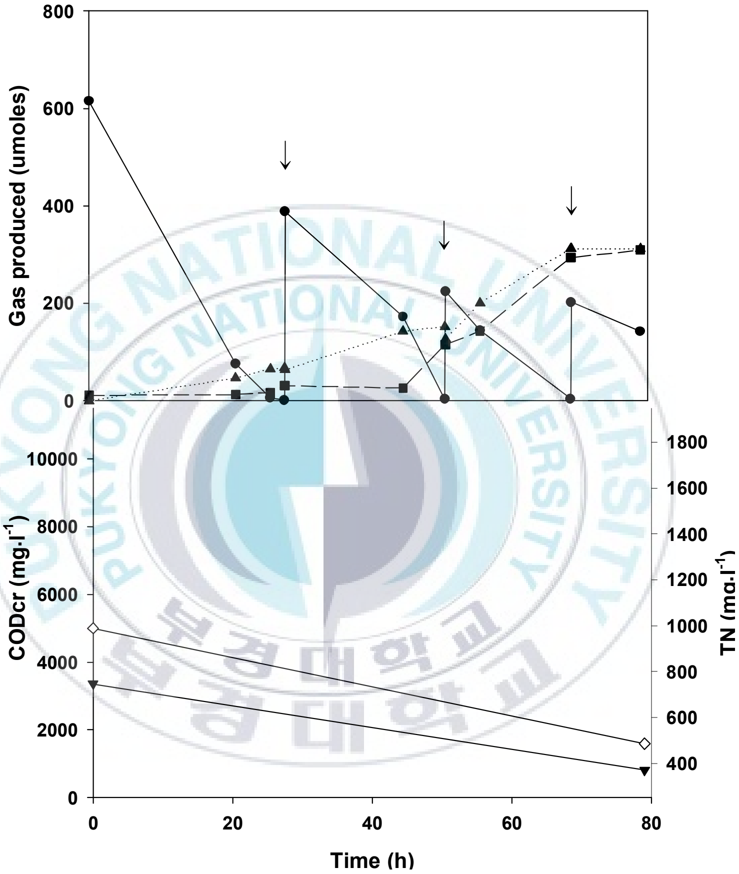
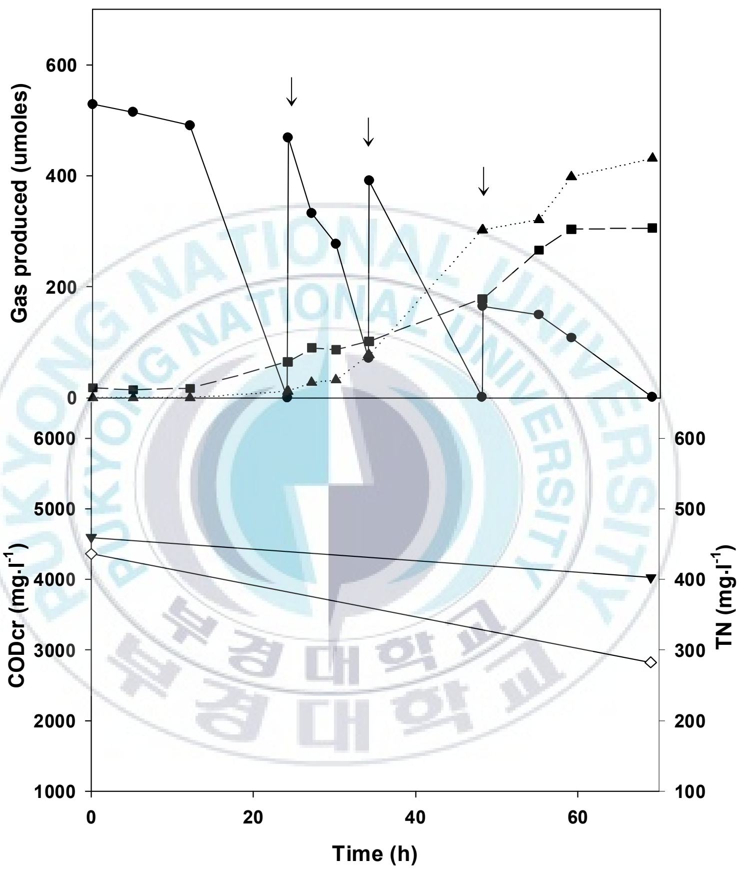
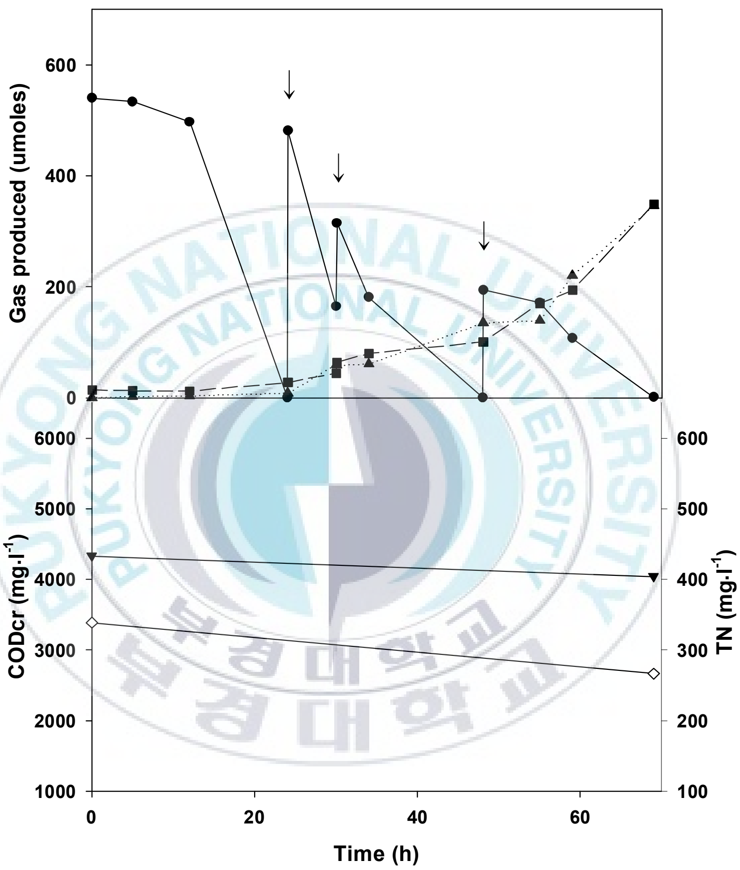


Fig. 1. Results of biodegradation on 10-fold diluted combined wastewaters against control. (A) Control (FMW:MW= 10:0); (B) FMW:MW= 9:1; and (C) FMW:MW= 5:5. Supplement of O_2 (↓); O_2 (●); N_2 (■); CO_2 (▲); CODcr (◇); and TN (▼).

(A)


Gas produced (umoles)
600
400
200
0
6000
5000
4000
3000
2000
1000
CODcr (mg·l-1)
TN (mg·l-1)
600
500
400
300
200
100
0
20
40
60
Time (h)

(B)


Gas produced (umoles)
600
400
200
0
6000
5000
4000
3000
2000
1000
CODcr (mg·l-1)
600
500
400
300
200
100
TN (mg·l-1)
0
20
40
60
Time (h)

(C)

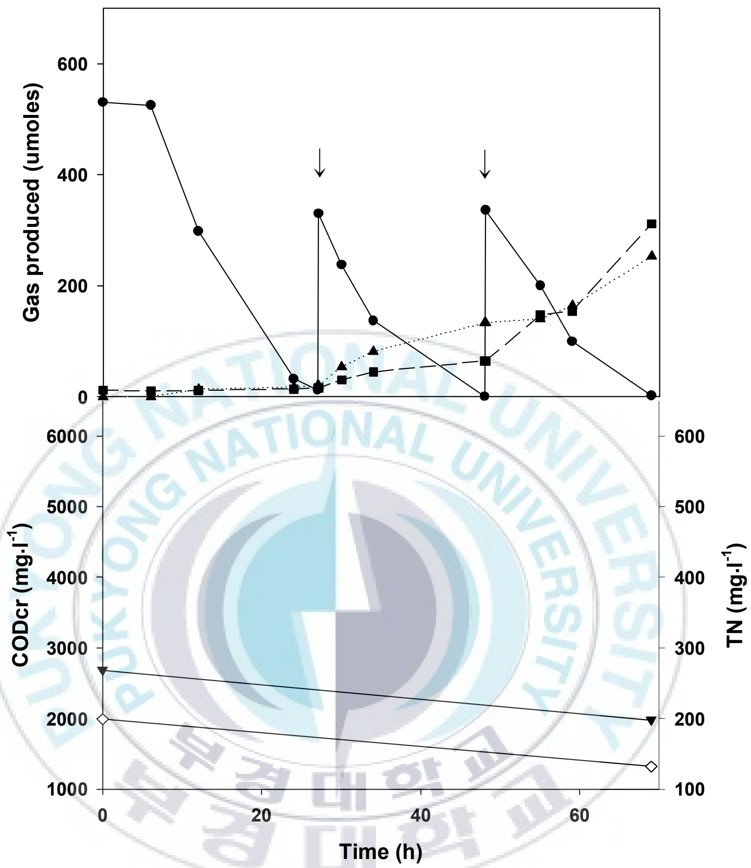
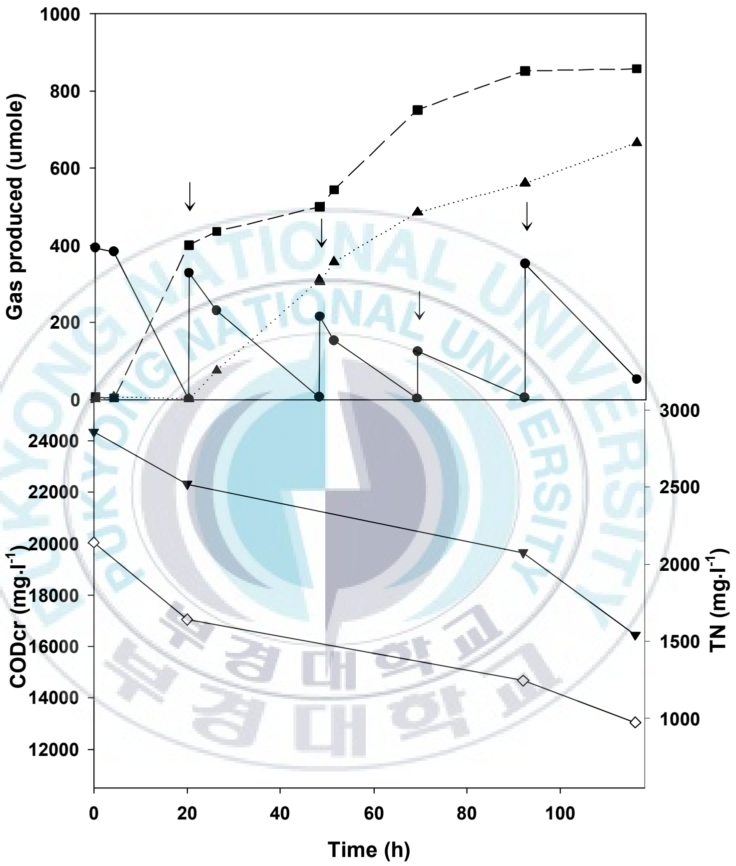


Fig. 2. Results of biodegradation on 32-fold diluted combined wastewater against control. (A) Control (FMW:MW= 10:0); (B) FMW:MW= 9:1; and (C) FMW:MW= 5:5. Supplement of O_2 (↓); O_2 (●); N_2 (■); CO_2 (▲); CODcr (◇); and TN (▼).

6. Biodegradation by combined microorganisms

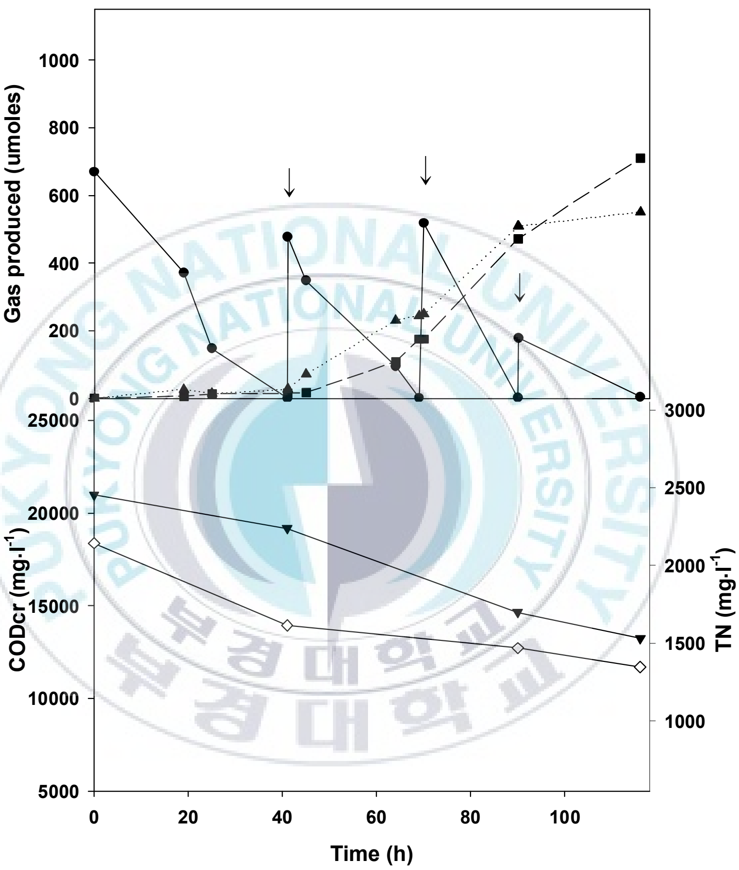
It has been reported that photosynthetic bacteria (PSB) can consume various types of organic substrates, nitrogenous and phosphorous compounds simultaneously, with a relatively high growth rate (Sasaki et al., 1998). For this reason, the effect of the combined microorganisms (by the addition of PSB to isolated microorganisms) on biodegradation was investigated in a 100-ml syringe, and the result is shown in Fig. 3. To maintain high activity of PSB during biodegradation, the experiment was carried out on the combined wastewater (1:1 mix of FMW and wasted broth (WB) generated after the cultivation of PSB), since PSB has been reported to excrete growth-related metabolites to the abiotic phase (Kim et al., 2001). The increase of the amount of PSB in the combined microorganisms resulted in reduced production of N_2 and CO_2 and reduced removal of COD_{Cr} and TN as well, compared to that of control. It has been known that cell interaction can be very complex in mixed culture (Purtschert and Gujer, 1999). The best use of PSB was tried, but the advantage of PSB addition was not shown in this case.

(A)



Gas produced (umole)
1000
800
600
400
200
0
CODcr (mg·l-1)
24000
22000
20000
18000
16000
14000
12000
TN (mg·l-1)
3000
2500
2000
1500
1000
0
20
40
60
80
100
Time (h)

(B)


Gas produced (umoles)
CODcr (mg·l-1)
TN (mg·l-1)
Time (h)
1000
800
600
400
200
0
25000
20000
15000
10000
5000
3000
2500
2000
1500
1000
0
20
40
60
80
100

(C)

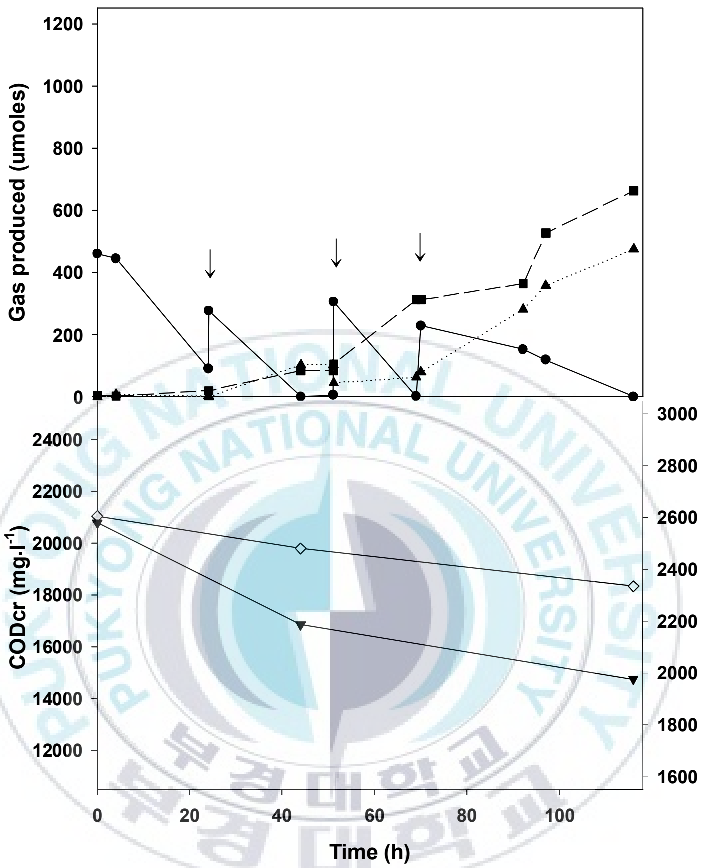


Fig. 3. Results of biodegradation by combined microorganisms against control. Biodegradation was carried on 10-fold diluted combined wastewaters (FMW:WB= 1:1). (A) Control (Isolates:PSB= 6:0); (B) Isolates:PSB= 4:2; and (C) Isolates:PSB= 3:3. Supplement of O_2 (↓); O_2 (●); N_2 (■); CO_2 (▲); CODcr (◇); and TN (▼).

LARGE-SCALE BIODEGRADATION

1. Biodegradation of FMW in a large-scale bioreactor

The large-scale FMW biodegradation from its lab-scale were attempted in a 1-ton bioreactor twice, and the results are shown in Fig. 4. In first trial (Fig. 4A), DO level in the bioreactor was maintained low (less than 1 $mg \cdot l^{-1}$), and pH increased up to 8.5. The ORP during biodegradation showed negative values with steady decrease from the beginning of the experiment. This implies that the supply of oxygen cannot meet the demand of oxygen by isolated microorganisms as they grew. Generally, it has known that DO level in a bioreactor should be maintained over 1 $mg \cdot l^{-1}$ for aerobic fermentation (Tohyama et al., 2000). The problem is to estimate the proper aeration rate in the large vessel.

To increase DO concentration level in the bioreactor, additional blower and oxygen diffuser were installed. Thus, the aeration rate was increased from 320 to 1,280 $l \cdot min^{-1}$. The result of the second trial is shown in Fig. 4B. Under the improved condition of aeration, DO level in the bioreactor could be maintained over 2 $mg \cdot l^{-1}$ during two days biodegradation, but thereafter it decreased. The pH was maintained in a range of 6.2-7.0, and the values of ORP were mostly positive during biodegradation. This trend showed different from that of the first trial, and the odor disappeared greatly. From these results, maintenance of DO level found to be very important and ORP could be a key parameter to operate biodegradation of FMW in a plant-scale. ORP was reported to be used as a controlling parameter for regulation of sulfide oxidation (Khanal and Huang, 2003), and on-line monitoring of ORP has been proved to be a practical and useful technique for process control of wastewater treatment systems (Yu et al., 1997). In aerobic processes, oxygen is a key substrate and because of its low solubility in aqueous solutions a continuous transfer of oxygen from the gas phase to the liquid phase is decisive for maintaining the oxidative metabolism of the cells. Thus, process optimization is required in a large-scale operation, especially aeration rate in this case. The reduction of COD_{Cr} or TN in the both trials was not much different.

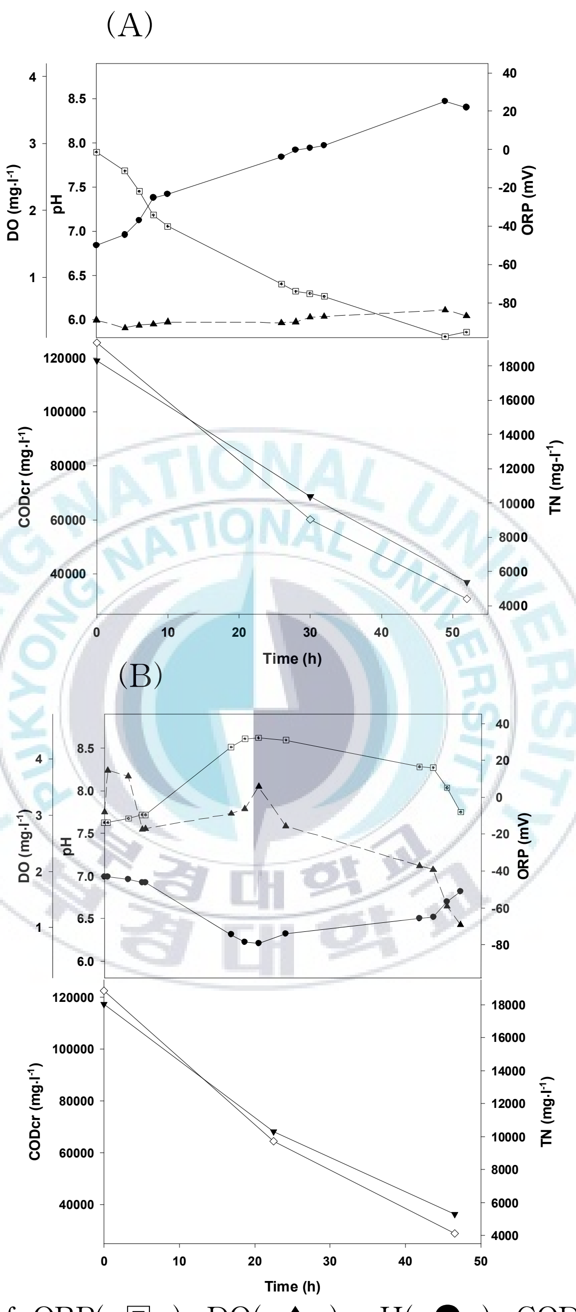


Fig. 4. Changes of ORP(▣), DO(▲), pH(●), CODcr(◇) and TN(▼) during biodegradation in a 1-ton bioreactor under supply of insufficient oxygen (A) and of relatively sufficient oxygen (B).

2. Final broth as liquid fertilizer

The success of the scale-up process for the production of liquid fertilizer from FMW can be verified by determining the composition of amino acids. The composition of amino acids between the final broths taken from first and second trials was analyzed and compared, and its result is tabulated in Table 3. The amino-acid composition of the second trial was higher than that of the first trial. This implies that better biodegradation under supply of sufficient oxygen resulted in increase of content of amino acids. Thus, steady supply of sufficient oxygen into large-scale bioreactor is very important. The level of total amino acids was lower, compared to that in commercial liquefied fertilizer. Especially the levels of apartic acid, glutamic acid, alanine and lysine were short, whereas those of proline and glycine were higher. The difference may be due to the limitation of oxygen, since the DO level could not be steadily maintained. Or the composition of the original FMW may be much different, since it was found to depend on the nature of the raw material processed in the factory (Kim et al., 2007).

Table 3

The comparison of amino-acid composition between final broths of first and second trials[a]

Amino acid	Source of final broth	
	First trial	Second trial
Aspartic acid	0.32	0.49
Threonine	0.18	0.17
Serine	0.19	0.21
Glutamic acid	0.54	0.78
Proline	0.50	0.50
Glycine	0.47	1.06
Alanine	0.32	0.60
Valine	0.15	0.10
Isoleucine	0.10	0.14
Leucine	0.20	0.24
Tyrosine	0.07	0.06
Phenylalanine	0.19	0.09
Histidine	0.09	0.20
Lysine	0.29	0.24
Arginine	0.31	0.24
Cystine	0.02	0.04
Metionine	0.13	0.06
Total	**4.07**	**5.22**

[a]composition of amino acids was based on dry weight (g•100g $sample^{-1}$)

Phytotoxicity assays were accomplished for liquid-broths taken at different times of biodegradation during the second trial. As shown in Fig. 5, the values of GI tended to increase with increase of dilution ratio of the liquid broths. The value of GI also tended to increase with more biodegraded broth as cultivation time elapsed. It has reported that GI tends to increase as the content of mineralized organic matter increases (Fuentes et al., 2004). Organic materials hold great promise due to their local availability as a source of multiple nutrients and ability to improve soil characteristics. Thus, it implies that more degraded organic compounds were present as cultivation time elapsed. In the case of experiment with liquid broth degraded for 3 days, only the half of cress seeds was germinated when 10-fold diluted broth was used, and its GI value was less than 10% with low root elongation. More diluted (100-fold) liquid broth resulted in increase of GI, and GI reached over 80%. This is enough to reach stabilization of the organic matter to maintain the long-term fertility in soil, according to the GI criterion (Zucconi et al., 1985). In case of commercial liquid-fertilizer, more than 100 dilutions are general for use in soil. A field experiment concerning the effect of final broth on soil fertility will be proceeding after this work.

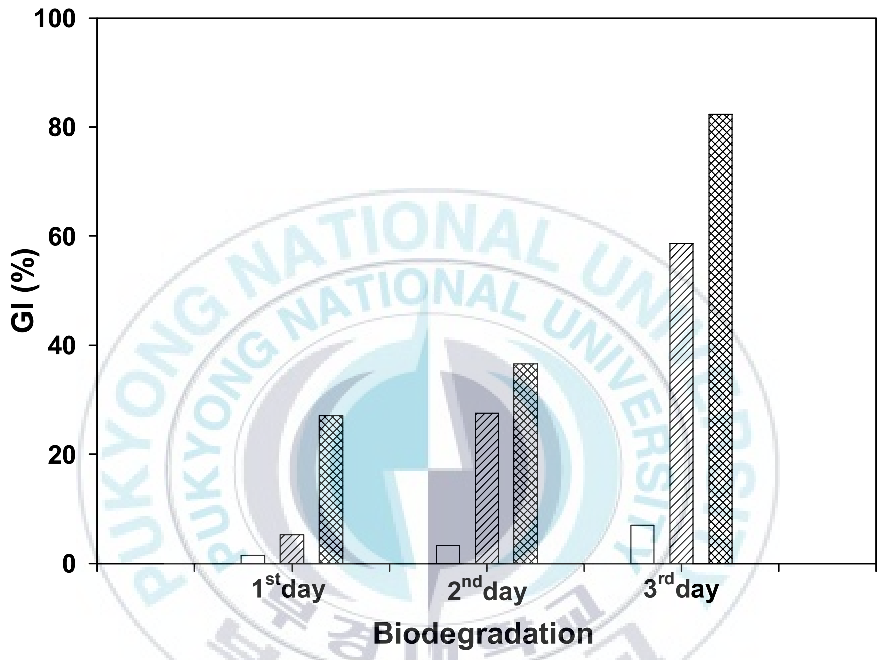


Fig. 5. Percentages of germination index (GI) for diluted liquid broths (10-fold (□), 50-fold (▨) and 100-fold (⊠)) taken from a 1-ton bioreactor starting on the original FMW under the supply of relatively sufficient oxygen.

PRESERVATION OF FINAL BROTH

1. Effect of lactate on preservation

The effect of lactate addition on the quality of final broths was investigated, and the results are tabulated in Table 4. Four different types of biodegraded wastewaters were stored by the various concentrations of lactate as a reagent for preservation. At the beginning of the experiment, FMW-1 emitted ammonia smell, whereas FMW-2 emitted relatively light ammonia smell. The smell seemed to depend on the quality of the biodegraded wastewater. In cases of control and addition of 0.5% lactate, unacceptable odors were produced from all types of wastewaters within 45 days by putrefaction. When 3% lactate was added to each biodegraded wastewater, the soy sauce-like smell could be emitted and retained for six months whether the quality of the biodegraded wastewater was good or not. The addition of 1% lactate could preserve the biodegraded FMW-2 for six months with the soy sauce-like smell. It has been reported that sweet-smell soy sauce had eighteen different free amino acids (Jingtian et al., 1988). This indicates that good-quality FMW-2 could be preserved for six months without any putrefaction. However, FMW-1, which was degraded poorly due to deficiency of oxygen, produced strong odor after two months. Addition of 1% lactate caused the change of pH from 7.8 to 5.7, with increase of ORP from - 10 to 73.2 mV. Consequently, lactate prevented the growth of spoilage microorganisms keeping their microbial counts steady and pH values within the acid region.

Table 4

Effect of lactate addition on the quality of biodegraded final-broths during their preservation at room temperature

Type of wastewater[a]	Addition of lactate (%)	Storage time (day)										
		0	10	20	30	45	60	75	90	120	150	180
FMW-1	0 (control)	A[b]	A	A	O[b]							
	0.5	A	A	A	O							
	1	A	A	A	A	A	O					
	3	A	L[b]	S[b]	S	S	S	S	S	S	S	S
FMW-2	0 (control)	L	A	A	O							
	0.5	L	L	A	A	O						
	1	L	S	S	S	S	S	S	S	S	S	S
	3	L	S	S	S	S	S	S	S	S	S	S
COMB-1	0 (control)	A	A	A	O							
	0.5	A	A	A	O							
	1	A	A	A	A	A	O					
	3	A	L	S	S	S	S	S	S	S	S	S
COMB-2	0 (control)	A	A	A	O							
	0.5	A	A	A	O							
	1	A	A	A	A	A	O					
	3	A	L	S	S	S	S	S	S	S	S	S

[a]biodegraded wastewater: ‘FMW-1’ was degraded under deficiency of oxygen (Fig. 4A); ‘FMW-2’ was degraded under relatively adequate supply of oxygen (Fig. 4B); COMB-1 was degraded on the combined wastewaters of 90% FMW-2 and 10% MW; and COMB-2 was degraded on the combined wastewaters of 90% FMW-2 and 10% MW containing EM (effective microorganisms).

[b]symbols represent different smell: ‘A’ means ammonia smell; ‘O’ means odor by putrefaction; ‘L’ means relatively light ammonia smell; and ‘S’ means soy sauce-like smell.

The effect of lactate addition on preservation of the combined wastewaters was investigated. Use of effective microorganisms (EM) inoculum along with organic materials has been known to be an effective technique for stimulating supply and release of nutrients from these nutrient sources. Some studies have shown that the inoculation of agro-ecosystems with EM cultures can improve soil and crop quality (Daly and Stewart, 1999 Hussain et al., 1999 Khaliq et al., 2006). Thus, EM is known to be an additive for optimizing all other amendments and practices used for crop production. Our study showed that better preservation was not found on the combined wastewater, COM-1 or COM-2 at the same concentration of lactate added. This implies that the role of EM in preservation was very weak. In conclusion, 1% lactate is a good preserving reagent for good-quality liquid fertilizer.

2. Change of amino-acid composition

The change of amino-acid composition was investigated along with the storage time, and the results are tabulated in Table 5. The levels of several amino acids slightly increased as storage time elapsed. Due to the production of amino acids, the pH of the liquid broth decreased further to 5.3 after 6 months. This implies that lactate could make the isolated microorganisms maintained at their minimum activity, which resulted in emission of soy sauce-like smell.

Table 5

Change in amino-acid composition during preservation by addition of lactate[a]

Amino acid	Storage time (day)		
	0	30	180
Aspartic acid	0.49	0.49	0.51
Threonine	0.17	0.18	0.21
Serine	0.21	0.21	0.21
Glutamic acid	0.78	0.79	0.84
Proline	0.50	0.51	0.52
Glycine	1.06	1.07	1.08
Alanine	0.60	0.61	0.62
Valine	0.10	0.11	0.14
Isoleucine	0.14	0.15	0.18
Leucine	0.24	0.25	0.28
Tyrosine	0.06	0.07	0.10
Phenylalanine	0.09	0.10	0.15
Histidine	0.20	0.21	0.22
Lysine	0.24	0.24	0.28
Arginine	0.24	0.24	0.24
Cystine	0.04	0.04	0.06
Metionine	0.06	0.06	0.07
Total	**5.22**	**5.33**	**5.71**

[a]composition of amino acids was based on dry weight ($g \cdot 100g\ sample^{-1}$)

IV. CONCLUSIONS

To reutilize the wastewater generated during the process of fish-meal production (FMW), seven thermophilic microorganisms were newly isolated and their characteristics of aerobic biodegradation of FMW were examined in a lab-scale bioreactor. It clearly showed that the amino-acid composition (12.54 g·100g sample-1) in the final broth of the biodegradation using 8-fold diluted FMW was almost twice that of non-biodegraded FMW. The levels of amino acids in the final broth were also comparable to those in a commercial fertilizer. When more (32-fold) diluted FMW was used as a substrate, phytotoxicity of biodegraded final broth was further reduced with disappearance of a strong unpleasant smell in the end. The results of the lab-scale biodegradation suggest the promising potential of biodegraded FMW for the production of fertilizer.

From the laboratory results, a large-scale biodegradation using the original FMW was designed and carried out in a 1-ton reactor. During the biodegradation, the concentration of DO in the liquid broth was closely related to pH and the oxidation reduction potential (ORP) as well. Keeping a low level of DO resulted in both the increase of pH and the decrease of ORP with strong smell of ammonia. Under the maintenance of DO level over 1 mg·l-1, the initial fishy smell from the FMW was converted to a pleasant smell in the end with production of fairly good content of amino acids. Therefore, the DO level in the liquid broth was found to be decisive influence on the quality of final fermented broth, and ORP to be a key operation parameter in biodegradation of FMW. The final broth taken from the bioreactor is required to be maintained its quality as a liquid fertilizer, during the period of circulation in market. The addition of 3% lactic acid could preserve the final broth well for six months, whereas the addition of lower concentrations of lactic acids could not preserve properly and resulted in putrefaction in the end. When a good-quality final broth was used, the addition of 1% lactic acid could preserve it for six months with amino-acids enriched soy sauce-like smell. From the results of the large-scale biodegradation, the reutilization of FMW is expected to yield high economic value.

V. 국문초록

Fish meal 제조 공정에서 발생하는 폐수(wastewater)를 재이용하기 위해, 새로이 분리된 7종의 호열성 미생물을 사용하여 Fish meal wastewater(FMW)의 호기적 생분해를 lab-scale 생물반응기에서 실험하였다. 8배 희석된 FMW를 이용한 생분해 반응의 마지막 배양액의 아미노산 성분(12.54g/100 sample)은 생분해를 하지 않은 FMW보다 거의 두 배로 나타났다. 마지막 배양액의 아미노산 수준은 상업적인 비료와 거의 대등하였다. FMW를 32배로 더 희석하여 기질로써 사용되어졌을 때, 생분해된 마지막 배양액에서 나던 강한 불쾌한 냄새가 보다 더 줄어듦을 알 수 있었다. Lab-scale 생분해의 결과는 생분해된 FMW을 비료의 생산으로써 기대되는 가능성을 제공하였다.

실험실 단위 결과를 토대로, 원액의 FMW를 설계된 1-ton reactor에서 large-scale 생분해 반응을 수행하였다. 생분해반응을 하는 동안에, 액체 배양액 속의 DO 농도는 pH, ORP와 밀접한 관련이 있었다. DO값을 낮게 유지했을 때, pH가 증가하고 ORP는 감소했으며 암모니아 냄새가 강하게 나는 결과를 보였다. DO가 1mg/L 수치 이상 유지되는 상태에서는 FMW로부터 나던 처음의 생선 비린내가 꽤 괜찮은 냄새로 바뀌었다.

그러므로 액체 배양액의 DO 수치는 마지막 발효된 배양액의 특성에 결정적인 영향을 주는 것을 알 수 있었고, ORP는 FMW의 생분해에 있어서 작용 요소의 열쇠임을 알 수 있었다. Bioreactor로부터 얻은 마지막 배양액은 시장에 유통되는 기간 동안 액체비료로써 품질 유지가 요구되어졌다. 3% lactic acid의 첨가는 6개월 동안 잘 보존 할 수 있었던 반면, lactic acid를 낮은 농도로 첨가한 경우는 결국 부패작용으로 알맞게 보존 할 수가 없었다. 좋은 상태의 마지막 배양액을 사용했을 때는 1% lactic acid의

첨가는 6개월동안 간장 냄새가 나고 풍부한 아미노산과 함께 보관 할 수 있었다. Large-scale 생분해반응의 결과로부터, FMW의 재이용은 높은 경제적 가치를 야기함을 기대할 수 있다.

VI. ACKNOWLEDGEMENT

지난 3년 간의 실험실 생활동안 부족한 저를 참고 기다려 주시고 인생의 밑거름이 되는 큰 가르침을 주시며, 깨우칠 수 있도록 지도해주신 김중균 교수님께 감사와 존경의 마음을 전합니다. 많은 고민 속에서 방황하고 있던 저를 받아주시고 하고 싶던 분야의 실험을 할 수 있게 기회를 주셔서 정말 감사합니다.

그리고 전공공부에 관심을 가지고 석사학위를 받기까지 많은 가르침을 주신 홍용기 교수님, 박남규 교수님, 공인수 교수님, 김성구 교수님, 이형호 교수님께 감사드립니다.

지난 3년 동안 많은 일들이 있었고 힘들었던 적도 많지만, 그럴 때마다 옆에서 힘이 되어주고 함께 고민해 준 실험실원들이 있어 끝까지 해낼 수 있었습니다. 석사 과정을 무사히 졸업 할 수 있게 옆에서 도와준 경주언니, 타지에서 고생하고 있는 경숙이언니 고마워요^^ 언니의 유머는 따라갈 수가 없어요. 그리고 실험실 생활을 하면서 항상 옆에서 모르는 부분을 채워주고 가르쳐주었던 정보언니 정말 고마워요~ 항상 긍정적이고 미소를 잃지 않았던 정현선배~ 보고싶어요. 저와 함께 실험실 생활을 하면서 나의 모든 성격을 받아주고 이해해주었던 Van에게도 감사의 마음을 전합니다. 마지막으로 항상 적극적이고 열심히 하는 Kien에게도 감사드립니다.

그 누구보다 석사 과정 2년을 함께한 동기 동균선배, 수정이, 남희, 지영이에게도 감사의 마음을 전합니다. 옆에서 힘이 되어주고 많은 이야기를 주고 받았던 그 시간들은 잊지 못 할 것입니다. 그리고 부족한 나에게 싫어하는 내색없이 도움을 주었던 무상선배, 상중선배, 문경이, 지혜에게도 고마운 마음을 표합니다.

창원에 자주 갈 수 없어서 1년에 3번 정도뿐이 못 만났지만 언제나 든든한 "조폭" 멤버들(미갱♡너에겐 매력이 넘쳐~, 영예♡의리로 똘똘 뭉친

영어쌤~, 행선♡너의 은근한 유머가 날 웃게 해~ , 희진♡치대학원합격축하해), 그리고 모임 때마다 늦었지만 실험실 생활을 이해해주고 기다려 주웠던 사랑하는 “반가워21기” 멤버들(뉴요커 영인♡, 옛 룸메이트 미정♡, 항상 즐거운 유빈♡, 애교쟁이 미림♡, 기대고 싶은 재윤♡, 옛룸메&항상 미소 문옥♡, 미국에서 공부 중인 유경♡) 고맙고 사랑해요! 우리는 돈으로 묶여있는 사이니깐 평생 함께하자^^ㅋ

18년지기 친구 정민, 호주에서 잘 지내고 있니? 연락도 잘하지 않는 이 친구를 항상 마음 속에 담아줘서 고맙다. 그리고 대학생활의 많은 추억을 남기게 해준 남천로타랙트 선후배와 02학번 동기들, 밀양 이쁜이 혜민, 재미있는 혜연이, 한 달 동안 여행을 함께한 지민, 다른 학교에서 열심히 하고 있는 미진에게도 감사의 인사를 전합니다. 내가 지치고 힘들어 포기하려 할 때마다 항상 나의 편에 서서 힘이 되어주고 용기를 주었던 남자친구에게도 감사의 마음을 전합니다.

마지막으로 저를 나아주시고 키워주신 사랑하는 부모님께 진심으로 감사의 마음을 전하고, 언제나 믿어주시고 저의 의견을 존중해주셔서 고맙습니다. 그리고 세상에 하나 뿐이 없는 할머니, 남동생 상혁이와 다른 가족에게도 감사드립니다.

VII. REFERENCES

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