

## Development of Fermentative Fertilizer from Fish-Meal Wastewater

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To degrade organic matters in wastewater generated during the process of fish-meal production (FMW), seven thermophilic microorganisms were newly isolated. In experiments of 100 ml- syringes, the degradation by the screened microorganisms showed more active mineralization of the organic matter under an aerobic condition. Faster biodegradation occurred with more diluted FMW, which resulted in faster removal rates of COD<sub>Cr</sub> and TN. The best maximum gas production rate and the best maximum cell number during biodegradation were obtained when 8 -folds diluted FMW was used as a substrate.

The amino-acid composition in the final broth of the biodegradation in a 5l- bioreactor starting with 8 -folds diluted FMW clearly showed that it was almost twice that of non-biodegraded FMW, and the levels of amino acids in the end product of biodegraded FMW were comparable to those in a commercial fertilizer. In phytotoxicity assays for final broth of biodegradation using 32-folds diluted FMW, a strong unpleasant smell noticeably disappeared in the end. According to the GI criterion, the final broth required only 2 -folds dilution to reach the stabilization. The addition of photosynthetic-bacterial culture broth or milk wastewater to FMW was not prominent effect on liquid-fertilization. From all results, reutilization of wastewater generated during the process of fish-meal production was feasible without the environmental problem of an offensive odor and is expected to yield high economic value.

**Key words :** Thermophilic microorganisms, Aerobic biodegradation, Fish-meal wastewater treatment, Fertilizer

### 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. The fish-meal manufacturing 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.

Biological treatment technologies of fish-processing wastewater have been studied to improve effluent quality<sup>1,2)</sup>. 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.<sup>3)</sup>. It has been reported that the organic wastes contain compounds, which are capable of prom-

oting plant growth, and seafood processing wastewaters do not contain known toxic or carcinogenic materials unlike other types of municipal and industrial effluents<sup>3,4)</sup>. Therefore, FMW could be a valuable resource for agriculture. However, potential utilization of this fish wastes has been limited because of its bad smell<sup>5)</sup>. 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<sup>6)</sup>. 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<sup>7)</sup>. 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 and diluted manure streams after biological treatment<sup>8,9)</sup>. Therefore, aerobic biodegradation is considered to be the most suitable alternative to treat FMW and realize a market for such a waste as fertilizer.

This paper presents the results of a study aimed to develop fermentative fertilizer from FMW. For this purpose, microorganisms were newly isolated and identified. With the isolates, the first trial for the biodegradation of FMW was then carried out in a bioreactor, and both analysis of amino-acid composition and tests of seed germination and root length were accomplished to examine the fertilizing value of the biodegraded end-product. The effect of addition of photosynthetic-bacterial culture broth or milk wastewater to FMW on the biodegradation was also examined.

## Materials and Methods

### Isolation of useful microorganisms

The potential aerobically degrading bacteria were isolated from compost and leachate collected at three different sites of composting plants in the suburbs of Busan, Korea. 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·l<sup>-1</sup> of yeast extract, 3 g·l<sup>-1</sup> of malt extract, 5 g·l<sup>-1</sup> of peptone, 10 g·l<sup>-1</sup> of glucose, and 0.05 g·l<sup>-1</sup> of ampicillin, pH 6.2) and Bennet's medium (1 g·l<sup>-1</sup> of yeast extract, 1 g·l<sup>-1</sup> of beef extract, and 10 g·l<sup>-1</sup> of glucose, pH 7.2). After one day of incubation at both of 45 and 55°C and at agitation speed of 180 rpm, cells in each tube were spread with a platinum loop on each solid agar medium which contained the same liquid medium and 1.5% nutrient agar, respectively.

To screen useful microorganisms among isolates, all isolates were spread on three 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·l<sup>-1</sup> of beef extract, 20 g·l<sup>-1</sup> of soluble starch, 10 g·l<sup>-1</sup> of tryptose, 5 g·l<sup>-1</sup> of NaCl, and 15 g·l<sup>-1</sup> of agar, pH 7.4) and cellulose agar (10 g·l<sup>-1</sup> of cellulose powder, 1 g·l<sup>-1</sup> of yeast extract, 0.1 g·l<sup>-1</sup> of NaCl, 2.5 g·l<sup>-1</sup> of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.25 g·l<sup>-1</sup> of K<sub>2</sub>HPO<sub>4</sub>, 0.125 g·l<sup>-1</sup> of MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.0025 g·l<sup>-1</sup> of FeSO<sub>4</sub>·7H<sub>2</sub>O, 0.025 g·l<sup>-1</sup> of MnSO<sub>4</sub>·4H<sub>2</sub>O, and 15 g·l<sup>-1</sup> of agar, pH 7.2) for detection of carbohydrate-degrading microorganisms, respectively.

### Tests of antagonism

Screening of potential bacterial antagonists against other isolates was carried out by the use of perpendicular streak technique as described by Alippi and Reynaldi<sup>10)</sup>. 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.

### Identification of useful isolates

Identification of the screened isolates was carried out using 16S-rDNA sequence analysis. PCR amplification of the DNA using the 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') were performed with PCR thermal cycler DICE model TP600. The purified products were ligated into pGEM T-easy vector and then transformed into *E.coli* DH5 $\alpha$  MCR Competent Cells. The plasmid DNA was sequenced in the MacroGen, Ltd. The 5'- end and 3'- ends of the constructs were sequenced using M13 primers flanking the

cloning sites. These partial sequences were searched against GenBank using the Advanced BLAST similarity search option accessible from the homepage at the National Center for Biotechnology Information<sup>10)</sup>. BioEdit Sequence Alignment Editor version 5.0.9 was used to check alignment and remove all positions with gaps before calculating distances with DNAdist programme in PHYLIP<sup>12)</sup>.

### Aerobic biodegradation by screened isolates

The effects of O<sub>2</sub> and the dilution factor of wastewater on the biodegradation were examined in a 100 ml-syringe<sup>13)</sup>. 0.2 g (wet weight basis) of mixed isolates 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 effect of dilution ratio of the original FMW on biodegradation, 2-, 4-, and 8-folds diluted FMW were used with the original FMW as control. For faster biodegradation, the inoculated cells were previously acclimated in the original FMW for two days. The syringes prepared in this way were incubated in a shaking incubator at 45°C and 180 rpm. Oxygen was supplied into the syringe with sterile oxygen (85% purity) when oxygen was depleted.

A batch type of aerobic biodegradation was carried out in a 5 l- continuous stirred bioreactor, with the diluted FMW at two different dilution ratios (8- and 32-folds). The characteristics of the diluted FMW were tabulated in Table 1. The mixed isolates (20 g wet cell) were suspended in the bioreactor filled with 4 l of sterile diluted FMW, and the bioreactor was operated at 45°C and 200 rpm. The effect of addition of photosynthetic-bacterial cul-

ture broth or milk wastewater to FMW on the biodegradation was also carried out with the 8-folds diluted FMW.

### Analytical Methods

The concentrations of cations and anions were estimated by IC. The columns used in these analyses were Metrosep C 2 -150 and Metrosep Supp 5 -150 for cation and anion, respectively. COD<sub>Cr</sub> and TN concentrations were analyzed by the Water-quality Analyzer. BOD<sub>5</sub> was analyzed by the OxiDirect BOD-System. The numbers of viable cells were measured by counting colonies formed on the agar plate of the nutrient broth medium containing 1.5% (w/v) agar. DSW was determined by weighing the sludge after being dried in an oven at 105°C for 12 h.

For determination of gases, 20 µl samples were taken for GC/TCD analysis. The columns used were a 'molecular sieve 13X' and 'carboxen 1,000' for N<sub>2</sub> and CO<sub>2</sub>, 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<sup>-1</sup> and the injector and the detector temperatures were 100 and 200°C, respectively. However, the oven temperature for nitrogen gas was 40°C, and that for carbon dioxide gas was 40°C for 3 min initially then increased to 170°C with a rate of 30°C·min<sup>-1</sup>.

### Seed germination test

To evaluate the phytotoxicity of biodegraded FMW, seed germination test were carried out according to the method of Wong et al.<sup>10)</sup>. For tests of seed germination and root length, 5 ml of each aliquot of sample was pipetted

**Table 1.** Characteristics of the diluted FMW

8-folds dilution		32-folds dilution	
Constituents	Concentration (mg·l <sup>-1</sup> )	Constituents	Concentration (mg·l <sup>-1</sup> )
COD <sub>Cr</sub>	17,846±2,413	COD <sub>Cr</sub>	4,634±459
TN	1,740±15	TN	421±8
BOD <sub>5</sub>	6,240±120	BOD <sub>5</sub>	2,749±856
NH <sub>4</sub> <sup>+</sup> -N	478±25	NH <sub>4</sub> <sup>+</sup> -N	59±40
NO <sub>3</sub> <sup>-</sup> -N	0	NO <sub>3</sub> <sup>-</sup> -N	0
NO <sub>2</sub> <sup>-</sup> -N	0	NO <sub>2</sub> <sup>-</sup> -N	0

into a sterile petri dish lined with Whatman #1 filter paper. Ten cress (*Lepidium sativum*) seeds were evenly placed in each dish. The plates were incubated at 25°C 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 72 h. The percentages of relative seed germination, relative root growth and germination index (GI) after expose to wastewater treated were calculated by the formula of Zucconi et al.<sup>[5]</sup>

## Results and Discussion

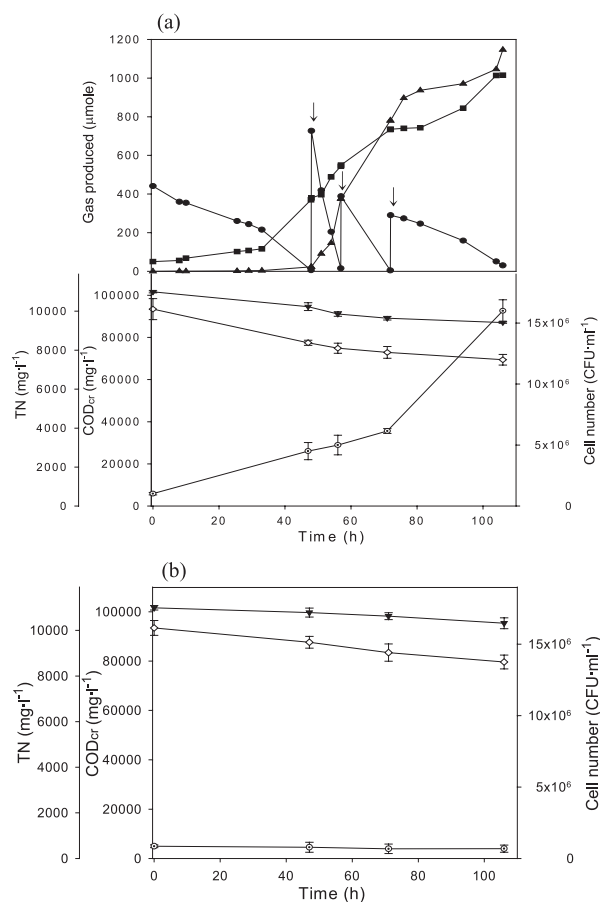
### Screening of useful microorganisms and their identification

Forty-six microorganisms were purely isolated, which showed at least one positive reaction on various solid media. Only seven microorganisms were found to produce no antagonistic substances against other microorganisms. Sequence analysis of the 16S-rDNA gene and BLAST sequence comparison confirmed that the isolated strains were *Bacillus subtilis*, *Bacillus licheniformis*, *Brevibacillus agri*, *Bacillus coagulans*, *Bacillus circulans*, *Bacillus anthracis* and *Bacillus fusiformis*, with similarity of 98-100%.

### Effects of oxygen and dilution ratio on biodegradation of FMW

Experiment for effect of oxygen on biodegradation was carried out in a 100 ml- syringe, and its result is presented in Fig. 1. Under the condition of O<sub>2</sub> supplement, the concentrations of COD<sub>Cr</sub> and TN were much more reduced by the degradation of the seven isolates, compared to those under the condition of no O<sub>2</sub> supplement. With supplement of O<sub>2</sub>, the production of CO<sub>2</sub> gas was increased with increase of N<sub>2</sub>, but only small bubble was produced in the syringe vessel without supplement of O<sub>2</sub>. This 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<sup>[6]</sup>. Thus, the degradation of the seven isolates showed more active mineralization of the organic matter under the aerobic condition.

As shown in Fig. 2, the experiment continued for 72 hours with original and 2-folds diluted FMWs (Fig. 2 a



**Fig. 1.** Effect of O<sub>2</sub> on biodegradation in the syringe experiment with the original FMW under an aerobic condition (a) and an anaerobic condition (b). Supplement of O<sub>2</sub> (↓); O<sub>2</sub> (●); N<sub>2</sub> (■); CO<sub>2</sub> (▲); COD<sub>Cr</sub> (◇); TN (▼); and cell number (○). Error bars: mean ± S.D. of three replicates.

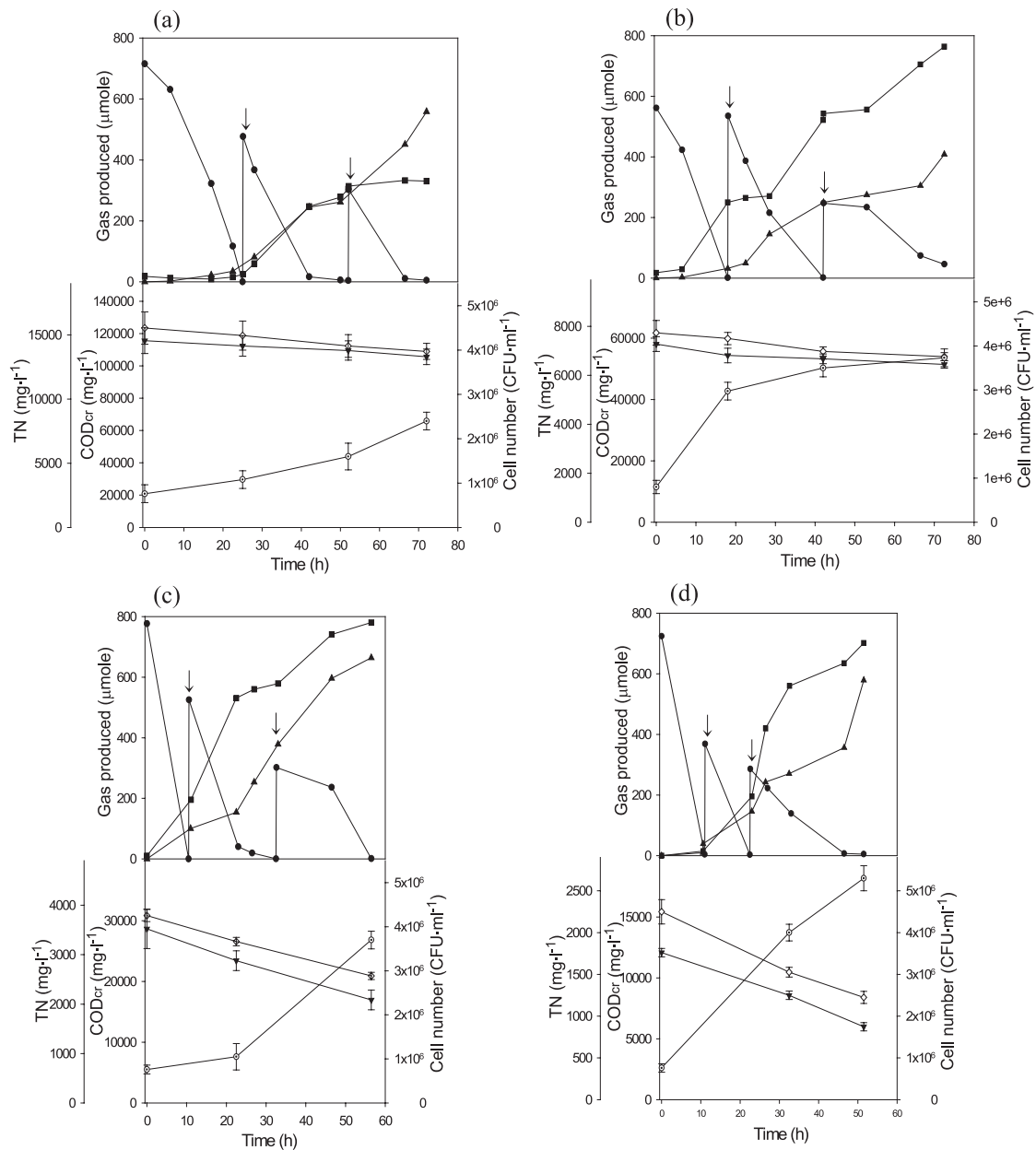
and 2 b). However, the experiment continued only for approximately 55 hours with 4-folds and 8-folds FMWs (Fig. 2 c and 2 d), since the gas produced in these experiments filled up the syringe vessel in shorter time as biodegradation proceeded. Thus, the oxygen consumption rate by the seven isolates in the syringe vessel tended to increase when more diluted FMW was used as substrate. This indicates that cellular metabolism is dependent on substrate concentration<sup>[7]</sup>. The maximum rates of gas productions of CO<sub>2</sub> and N<sub>2</sub> during biodegradation were the highest with 8-folds diluted FMW, which were measured to be 46.4 and 64.1 mg·l<sup>-1</sup>·h<sup>-1</sup>, respectively. The microbial population also tended to increase with more diluted FMW, and the maximum cell number was 5.4 × 10<sup>6</sup> CFU·ml<sup>-1</sup> when 8-folds diluted FMW was used. The mineralization of the organic matter by the seven isolates showed the best

result when 8 -folds diluted FMW was used as a substrate. Thus, this dilution ratio of FMW was selected for later experiments.

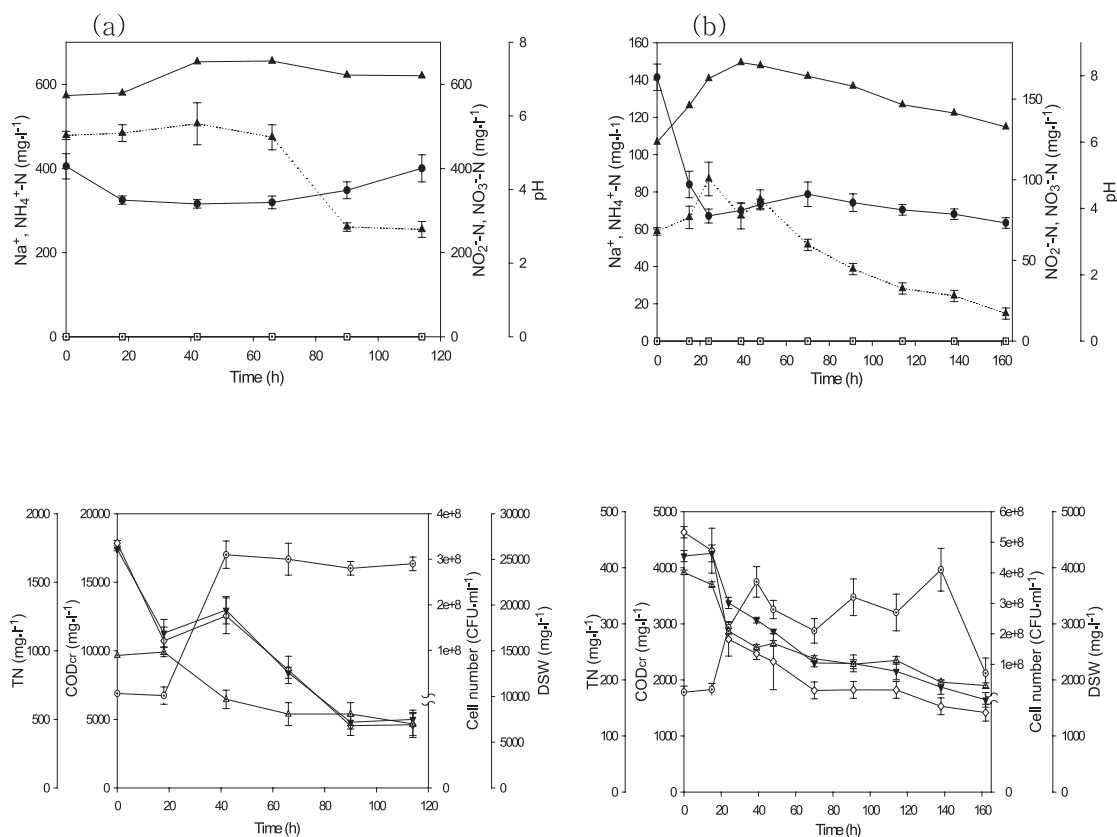
### Biodegradation of FMW in a bioreactor and properties of final broth

The experiment of biodegradation in 5l- bioreactor was started with 8 -folds diluted FMW, based upon the result of syringe experiment. The FMW was characterized by its

high organic content with suspended solids, and the result of biodegradation is shown in Fig. 3. The average removal percentages of  $COD_{Cr}$  and TN were 74.1 and 71.3%, respectively. The removal percentages were more prominent, compared with the results of the syringe experiment using the same diluted FMW. The concentration of  $Na^+$  and pH were not changed considerably and the cell number increased up to  $2.9 \times 10^8$   $CFU \cdot ml^{-1}$ . DSW decreased from 14,500 to 7,010  $mg \cdot l^{-1}$ , which means that some fractions of



**Fig. 2.** Effect of dilution ratio of the original FMW on biodegradation in the syringe experiment with the original FMW (a), 2 -folds diluted FMW (b), 4 -folds diluted FMW (c), and 8 -folds diluted FMW (d). Supplement of  $O_2$  ( $\downarrow$ );  $O_2$  ( $\bullet$ );  $N_2$  ( $\blacksquare$ );  $CO_2$  ( $\blacktriangle$ );  $COD_{Cr}$  ( $\diamond$ ); TN ( $\blacktriangledown$ ); and cell number ( $\odot$ ). Error bars: mean  $\pm$  S.D. of three replicates.



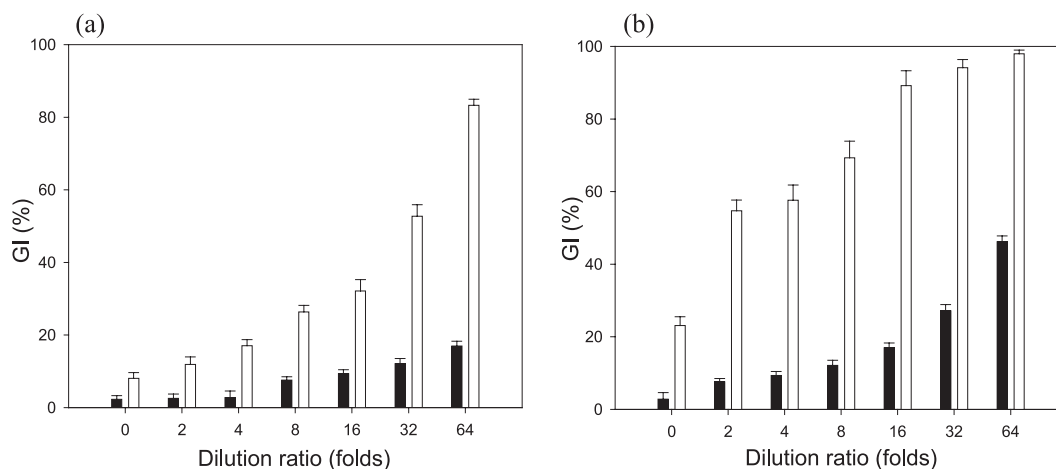
**Fig. 3.** Results of biodegradation in a 5 l-bioreactor with 8-folds diluted FMW (a) and 32-folds diluted FMW (b).  $\text{Na}^+$  ( $\bullet$ );  $\text{NH}_4^+-\text{N}$  ( $\blacktriangle$ );  $\text{NO}_2^--\text{N}$  ( $\square$ );  $\text{NO}_3^--\text{N}$  ( $\boxplus$ ); pH ( $\blacktriangle$ );  $\text{COD}_{\text{cr}}$  ( $\diamond$ ); TN ( $\blacktriangledown$ ); cell number ( $\odot$ ); and DSW ( $\triangle$ ). Error bars: mean  $\pm$  S.D. of three replicates.

suspended solids were biodegraded.

Sufficient aeration promotes the conversion of organic matters into nonobjectionable, stable end products. However, an incomplete aeration may result in accumulation of organic acid, thus giving trouble to plant growth if the fertilizer is incorporated into the soil<sup>18</sup>. To examine the fertilizing value of the final broth of the experiment, phytotoxicity assays were accomplished, and the result is shown in Fig. 4. The effect of the final broth on the germination was not pronounced. 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<sup>19</sup>. Instead, GI has been reported to be the most sensitive parameter used to evaluate the toxicity<sup>13</sup>. As shown in Fig. 4 a, the average GI of original final-broth, which was not diluted with DW, 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<sup>20,21</sup>. 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<sup>22</sup>. According to this GI criterion, the final broth of biodegradation using 8-folds diluted FMW required more mineralization to reach stabilization. Consequently, more diluted FMW was required for the further stabilization of the organic matter to maintain the long-term fertility in soil.

Amino acids are an essential part of the active fraction of organic matter in a fertilizer. The amino-acid composition of the biodegraded FMW was analyzed, and its comparison with those of other products is reported in Table 2. The results clearly showed that the amino-acid composition in final broth ( $12.54 \text{ g} \cdot 100\text{g sample}^{-1}$ ) was almost twice that of non-biodegraded FMW, and each amino-acid level in the final broth except for arginine, serine and ala-



**Fig. 4 .** Percentages of germination index (GI) for final broths in a 5 l- bioreactor starting with 8 -folds diluted FMW (a) and 32-folds diluted FMW (b). Control: diluted liquids with D.W. from non-biodegraded original FMW (■); and diluted liquids with D.W. from each final biodegraded broths (□). Error bars: mean  $\pm$  S.D. of three replicates.

**Table 2 .** The comparison of amino-acid composition of biodegraded FMW with those of others<sup>a</sup>

Amino acid	Protein source (g·100g sample <sup>-1</sup> )		
	Diluted FMW <sup>b</sup>	End product of FMW <sup>c</sup>	Commercial product <sup>d</sup>
Lysine	0.43	2.04	1.54
Histidine	0.25	0.90	0.28
Arginine	0.37	0.18	0.23
Aspartic acid	0.54	1.21	0.90
Threonine	0.21	0.24	0.23
Serine	0.23	0.21	0.21
Glutamic acid	0.89	0.98	2.96
Proline	0.71	1.94	0.09
Glycine	1.25	1.38	0.31
Alanine	1.02	0.66	1.00
Valine	0.23	0.24	0.39
Cysteine	0.04	0.15	N.D.
Methionine	0.01	0.61	N.D.
Isoleucine	0.13	0.20	0.28
Leucine	0.26	0.31	0.42
Tyrosine	0.05	0.38	0.17
Phenylalanine	0.19	0.91	0.22
Total	6.81	12.54	9.23

<sup>a</sup>On dry weight basis.

<sup>b</sup>8- folds diluted liquid of the original FMW, which was diluted with D.W.

<sup>c</sup>Biodegraded FMW in 5 l- bioreactor. The biodegradation started with 8- folds diluted FMW.

<sup>d</sup>Liquid fertilizer which was used for horticultural plants in Korea.

nine was also high. The higher content of amino acids in the final broth is 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. Consequently, the levels of amino acids in the final FMW broth are comparable to those in a commercial fertilizer.

To reduce phytotoxicity of final FMW broth further (Fig. 4 a), aerobic biodegradation using 32-folds diluted FMW were carried out, and the result is shown in Fig. 3 b. The average removal percentages of  $\text{COD}_{\text{Cr}}$  and TN were 69.4 and 60.8%, respectively with decrease of  $\text{COD}_{\text{Cr}}/\text{TN}$  ratio in the end. The final concentration of  $\text{NH}_4^+\text{-N}$  averaged  $15 \text{ mg}\cdot\text{l}^{-1}$ , and  $\text{NO}_2^-\text{-N}$  and  $\text{NO}_3^-\text{-N}$  were not accumulated. The final concentration of  $\text{Na}^+$  and pH were not changed considerably. The cell number increased up to  $4.1 \times 10^8 \text{ CFU}\cdot\text{ml}^{-1}$ , and somewhat decreased to  $7.1 \times 10^7 \text{ CFU}\cdot\text{ml}^{-1}$  in the end. DSW decreased from 3,920 to 1,900  $\text{mg}\cdot\text{l}^{-1}$ , with noticeable disappearance of strong unpleasant smell in the end.

Phytotoxicity was also assayed on the final FMW broths at various dilution ratios, and the assay result is shown in Fig. 4 b. Although RSG of the final broth in a 5 l- bioreactor showed 100%, the average GI of original final-broth, which was not diluted with DW, was 23.0% with low root elongation. This GI value was higher than that (17.0%) obtained from the biodegradation of 8-folds diluted FMW at the dilution ratio of 4, although the overall dilution ratio of FMW from the original FMW was same (32 folds). This indicates that GI tended to increase as the content of mineralized organic matter in FMW increased by biodegradation<sup>23)</sup>. As shown in Fig. 4 b, the average value of GI was over 50% at the dilution ratio of 2 or more. Thus, the final broth of biodegradation using 32-folds diluted FMW required only two-folds dilution to reach stabilization, probably due to the presence of more degraded organic compounds with the low concentration of  $\text{NH}_4^+\text{-N}$  ( $15 \text{ mg}\cdot\text{l}^{-1}$ ) in the final broth<sup>14)</sup>. At the dilution ratios of more than 16, the average GI reached over 90%. At the appropriate dilution ratio, the biodegraded FMW appears to be comparable to a commercial fertilizer.

The addition of photosynthetic-bacterial culture broth or

milk wastewater to FMW was expected to give a better result, since it may have some nutritional compounds in each solution<sup>24,25)</sup>. However, it was not prominent effect on liquid-fertilization as shown in Fig. 5.

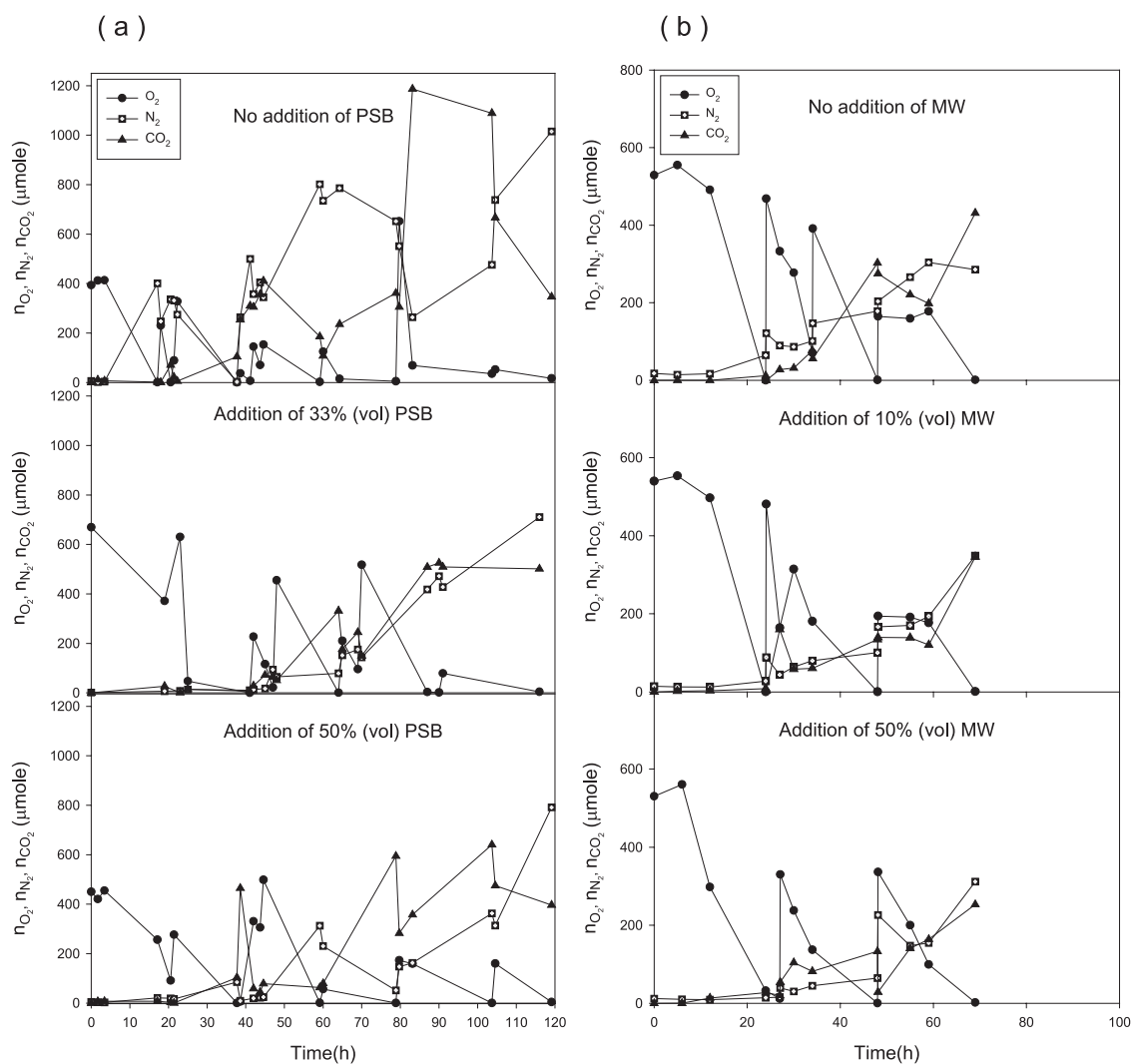
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**Fig. 5.** Results of biodegradation with the mixed solution of photosynthetic-bacterial culture broth and 8-folds diluted FMW at various mixing ratios of photosynthetic bacteria (PSB) (a) and with 8-folds diluted FMW at various mixing ratios of milk waste (MW) in the medium.

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