

Influence of Effective Microorganisms on Polluted Marine Sediment and Its Microbial Community

Sung-Cheol Koh† · Byung-Hyuk Kim* · Hwan-Jin Bae** · Sung-Hyun Kwon*** ·
Jung-Hye Choi**** · Jae-Woo Kim*****

†,****,***** Division of Civil and Environmental Engineering, Korea Maritime University, Busan, 606-792, Republic of Korea

* Environmental Biotechnology Research Center, Korea Research Institute of Bioscience and Biotechnology, Daejeon, 305-806,
Republic of Korea

, Department of Marine Environmental Engineering, Gyeongsang National University, Tongyeong, 650-150, Republic of Korea

Abstract : *Lactobacillus* sp., *Acetobacter* sp. and yeast were the most dominant organisms in the EM stock culture and subculture product. Lactic acid bacteria and yeast were able to grow in the fermentation process utilizing seawater. EM treatment of higher concentrations using EM stock culture and EM clay balls (1% or 4%) contributed to an early removal of malodor and an increase of DO in the polluted sediments, indicating an odor-removing activity of EM. The EM treatment of higher concentrations (1% or 4%) somewhat appeared to modify the microbial communities within the sediments, which was confirmed by existence of a few unique fragments from the stock culture based on PCR-DGGE. It still remains to be elucidated that EM cultures were directly involved in the malodor removal and potential sediment bioremediation.

Key words : effective microorganisms (EM) marine sediment bioremediation PCR-DGGE *Lactobacillus* sp.

1. Introduction

The microbial agent, Effective Microorganisms (EM), has been developed by Dr. Higa (University of Ryukyus, Japan) (Higa, 1991) and is known to include a complex group of microorganisms consisting of mixed cultures of photosynthetic bacteria, lactobacilli, Streptomycetes, filamentous fungi and yeasts (10 genera and 80 species) (Higa, 1996). These mixed cultures in EM can coexist in mutual benign interactions and in synergy to exert an antioxidation effect in soil, to suppress putrefaction malodor and to clean up the wastewater (EMRO, 1995; Higa, 1996). The culture has been widely used for agricultural and environmental application in South East Asia and the rest of the world (Higa, 1995 Koh et al., 1997 Oh et al., 2002). EM appeared to facilitate composting through the synergy between EM and soil flora (Heo et al., 2008). The synergy effect of EM and *Sphingobacterium* sp. WY on oil bioremediation was also recently observed (Yoon et al., 2008) and EM could enhance the degradation of crude oil at early stage of the oil pollution (Lee et al., 2008). Application of EM was able to enhance the efficiency of both organic

and mineral nutrient sources but the EM alone was ineffective in increasing the seed cotton yield (Khaliq et al., 2006). EM was proven to be effective in facilitating composting of food wastes and controlling putrefaction malodor through the potential action of lactic acid bacteria and yeasts (Koh et al., 1997).

EM has started to be tested in clinical practices (Higa, 1996; Higa, 2003). In medicine, EM-X, an antioxidant beverage EM-X derived from the fermentation of unpolished rice, papaya, and sea-weeds with effective microorganisms, inhibited the release of IL-8 at the transcriptional and also decreased the iron/ascorbate dependent peroxidation of ox-brain phospholipids, due to the antioxidant potential (Deiana et al., 2002). EM-X treated rats also showed a reduction in the overall levels of conjugated dienes (CD) in the kidney by 27% and in the liver by 19%, indicating inhibition effect of lipid peroxidation in these organs (Aruoma et al., 2002). Oral administration of EM-1[®] could attenuate asthmatic manifestations including airway hyper-reactivity (AHR) (Do et al., 2006), and beneficial effects of EM-X on increase of bone density in rat were also reported (Ke et al., 2008). However, there has been no

† Corresponding Author : skoh@hhu.ac.kr +82-51-410-4418

* tiger417@kribb.re.kr +82-42-860-4324

** lightgod717@naver.com +82-55-640-3162

*** shkwon@gsnu.ac.kr +82-55-640-3162

**** seawitch@bada.hhu.ac.kr +82-51-410-4984

***** kafkajw@naver.com +82-51-410-4984

research done regarding EM implementation for remediation of polluted sea sediments so far, to the best of our knowledge.

The goal of this study was to test the feasibility of EM application for remediation of the polluted sediments in the "Dongsam Seastream," Yeongdogu, Busan in terms of malodor removal and improvement of sediment quality. In order to accomplish this goal, three specific aims were implemented: 1) To figure out growth of EM in seawater 2) To analyze the microbial communities in EM stock culture and subculture for a QA/QC purpose; and 3) To study the EM treatment effects on the sediment quality in terms of malodor removal and physicochemical changes of the sediments.

2. Materials and Methods

2.1 Sampling of sediments

Marine sediments were sampled at the sites 1st, 2nd and 3rd Bridges of Yeongdo Seastream (sites 1, 2, and 3 in Fig. 1). Sampling using Van Veen grab was performed 2-3 times to get appropriate amount of sediment. The sample at the site 2 showed a strong H₂S smell. Samples were kept frozen -20°C until they were subjected to the analysis.

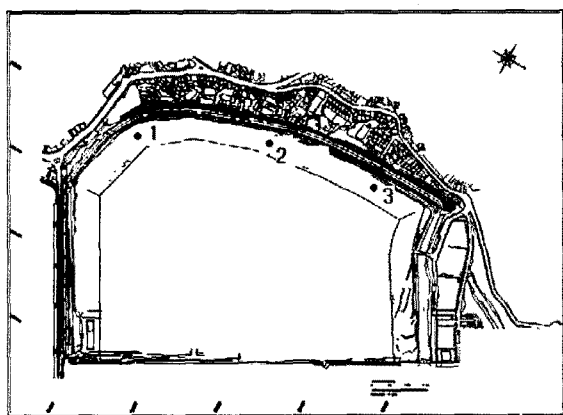


Fig. 1 Sampling sites of the polluted marine sediments in Seastream of Dongsamdong for this study

2.2 Analysis of sediment samples treated with EM

Chemical analysis of sediment was performed in terms of COD, T-N, T-P, and acid volatile sulfide (AVS) according to *Official Methods for Analysis of Marine Environments* (Ministry of Maritime Affairs and Fisheries, 2002). Measurements of DO and pH were also done using instruments YSI (Yellow Springs, Ohio) and pH meter (Istek, Inc., Korea), respectively. Malodor analysis of

sediment was based on the olfactory method in *Official Methods for Analysis of Air Pollution* (Ministry of Environment, 1998).

2.3 EM cultures used in this study and their isolation

Fresh EM stock culture, subculture and EM clay ball for the experiment were obtained from Environmental Protection Division in Yeongdogu Office, Busan, Republic of Korea. EM clay ball was manufactured by adding the EM subculture (7-10 days old) to the yellow clay (1:10 ratio) and drying at shading place for 3 months. Representative microbial populations of EM such as lactic acid bacteria, yeast, filamentous fungi, and photosynthetic bacteria were isolated and identified using MRS medium, yeast malt extract (YM) medium, potato dextrose agar (PDA) medium, and a photosynthetic bacterial medium, respectively. They were grown at ambient temperature (28-30°C) for at least 7 days to confirm their colony characteristics for enumeration.

2.4 Growth test of EM in seawater

EM was tested for their growth in the seawater according to the recipe shown in Table 1. Inoculation of stock culture and addition of molasses were 4% (w/v) respectively. The seawater was obtained from Yeongdo Island. Growth of EM here was performed at 32°C for 9 days. The four EM populations were monitored following the afore-mentioned isolation procedure.

Table 1 Growth of EM in the presence of seawater and rice-wash water* within reactor (100L)

Culture	Seawater (L)	Rice Wash Water (L)	Molasses (L)	Stock Culture (L)
Culture -1	0	92	4	4
Culture -2	46	46	4	4
Culture -3	92	0	4	4

* Fermentation at 32°C for 9 days

2.5 Treatment of polluted marine sediment with EM stock culture and EM clay balls

This experiment was performed to see if EM could remediate the polluted sediments and hence deodorize them. Stock culture of EM was inoculated according to the recipe as shown in Table 2. In case of Treatment 5, 0.3% (w/v) of

molasses was added to stimulate EM growth. EM clay ball was also used to treat the polluted sediment for its remediation effect. For this, 4 different treatments of clay balls were made into 1.600 L of the sediment: 0.1% (w/v) clay ball, 1.0%, 4.0%, and 0.1% with 0.16% (w/v) molasses. All the experiments were performed in triplicate mode. The additional EM clay ball treatments were made 8 and 16 days after the first treatment (0 day).

2.6 Statistical analysis

Statistical analysis was performed by one way ANOVA test and Duncan's new multiple range test using SPSS (V. 12.0)

Table 2 Experimental design for EM treatment in the polluted marine sediment

Treatment*	EM stock culture % (w/v)	Molasses % (w/v)	Final volume (L)
A (Control)	0.00	0.0	1.700
B	0.01	0.0	1.700
C	0.10	0.0	1.702
D	1.00	0.0	1.717
E	0.10	0.3	1.710

* Performed three replicates for each treatment

2.7 Molecular analysis of EM community in stockculture, subculture and the treated sediment

The representative populations of EM were analyzed by constructing clone libraries and sequencing at least 50 clones of each library. The procedure was based upon the previous method (Duhamel et al., 2006). 16S rRNA gene sequences (over 1300 base pairs for bacteria) were assembled with SeqMan II (DNASTar) using the best match sequence of each operational taxonomic unit on Genbank (www.ncbi.nlm.nih.gov/BLAST) as a template. Microbial communities in the treated sediments were analyzed by the PCR-DGGE technique as described before (Kim et al., 2008).

3. Results and Discussion

3.1 Analysis of EM community in stockculture, molasses subculture and seawater subculture

Clone library analysis showed that the dominant populations of the stockculture were *Lactobacillus*

parabuchneri (56%), *Clostridium* sp. (13%), and *Brevibacillus* sp. (12%) (Fig. 2). However, *Lactobacillus farraginis* (38%), *Acetobacter* sp. (31%), and *Lactobacillus parafarraginis* (12%) were dominant in the molasses subculture (Fig. 3). Population of *Lactobacillus parabuchneri* in the subculture was diminished to 6% as opposed to the stock culture. This indicates that the stock culture of EM could be significantly variable according to culture conditions including medium used. Viable population density of *Lactobacillus* sp. was maintained at least 10⁸ (c.f.u./ml) in the medium containing seawater up to 92%. Yeast population was also maintained at least 10⁸ (c.f.u./ml) in the seawater containing medium.

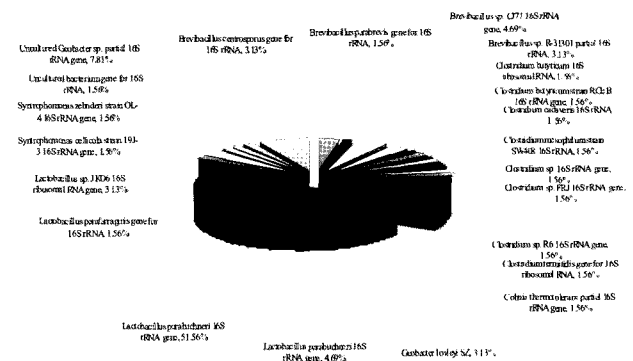


Fig. 2 Analysis of microbial community of the EM stock culture based on clone library sequencing

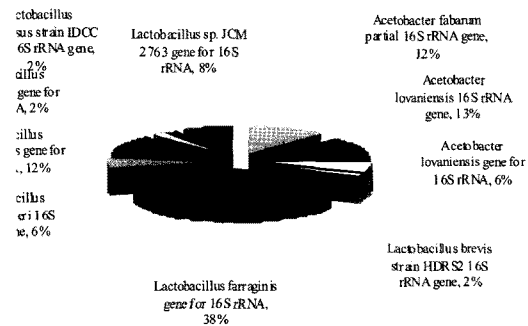


Fig. 3 Analysis of microbial community of the EM subculture grown on rice-wash (92%) water and molasses (4%) with the stock culture (4%) based on clone library sequencing

3.2 Analysis of chemical changes of sediment samples treated with EM

Chemical changes in the sediments treated with EM stock culture were monitored in terms of COD, T-N, T-P and acid volatile sulfide (AVS) to see if EM could remediate the sediments (Table 3). In case of COD, all the treatments showed a higher value than the control in 11 days. This may indicate that EM culture and the organic compounds (i.e., molasses and its metabolites) within the culture were still present in the sediments and left over for their degradation. The treatment (EM 0.1% + molasses 0.3%) showed the highest value of COD where degradation (fermentation) of carbon source (sugars) was actively going on in 11 days.

There was little significant difference in total nitrogen (T-N) among all the treatments including control. This may reflect that COD was mostly attributed by sugars from culture or molasses added rather than the reducible nitrogen (NH_4^+ or NO_2^-) because the treatment carrying molasses showed a similar level of T-N to other treatments.

Like T-N, there was also little significant difference in T-P among all the treatments except the treatment carrying molasses (0.3%) as a growth substrate which showed the highest level of T-P. This similar trend appeared to be due to the culture inoculated.

Table 3 Effect of the EM stock culture treatment on changes of chemical parameters (COD, T-N, T-P and acid volatile sulfide) in the polluted marine sediment (11 days).¹

Treatment ²	COD	T-N	T-P	AVS
A (Control)	21.3±2.3 ^a	8.70±0.08 ^a	1.99±0.01 ^a	3.16±0.40 ^a
B	46.7±1.2 ^b	12.59±0.01 ^b	2.89±0.01 ^b	2.72±0.49 ^{ab}
C	36.0±2.0 ^c	13.14±0.03 ^c	2.53±0.05 ^c	3.08±0.40 ^{ab}
D	64.7±1.2 ^d	9.75±0.05 ^d	2.96±0.02 ^d	3.12±0.24 ^{ab}
E	223.3±3.1 ^e	14.25±0.02 ^e	3.26±0.02 ^e	3.67±0.31 ^{bc}

¹ Values are means of triplicate groups, values in the same column not sharing a common superscript are significantly different ($p < 0.05$)

² Refer to Table 1 for the detail recipe.

In case of AVS, there was no significant difference among all the treatments including the control. However, the trend was different from the cases in T-N and T-P in that AVS level in the control was quite similar to those of all

the rest of treatments. Particularly, the AVS level of the treatment carrying molasses (0.3%) was relatively low compared with other parameters (COD, T-N, and T-P). This may indicate that the occurrence of AVS can be suppressed by the EM treatment, one of the ways to deodorize the polluted sediment. In fact, EM treatment (0.01%) showed the lower concentration of AVS than the control, indicating the possibility of EM action on the sediment. An extensive incubation period (beyond 11 days) would have more decreased the AVS level. Another experiment of our research group in which a polluted sediment from an industrial complex in Busan, Republic of Korea was treated with 1% of the EM subculture showed a significant removal of malodor from the sediment in 4 weeks (data not shown).

3.3 Effect of EM clay ball treatment on physical changes and malodor removal in the polluted marine sediments

Effect of EM clay ball treatment on changes of physical parameters (DO and pH) and malodor removal in the polluted marine sediment was investigated. Treatment of EM clay ball (0.1, 1.0, and 4.0 %) caused a higher level of dissolved oxygen (DO) than the control (Fig. 4). Higher concentrations of EM treatment (1% and 4%), in particular, showed a rapid increase in DO compared with other treatments. EM treatment (0.1%) with molasses (0.1%) showed a lower level of DO than the control probably due to the active degradation of molasses remaining. Overall the enhanced DO increase over time would result from a degradation of substrate by EM culture.

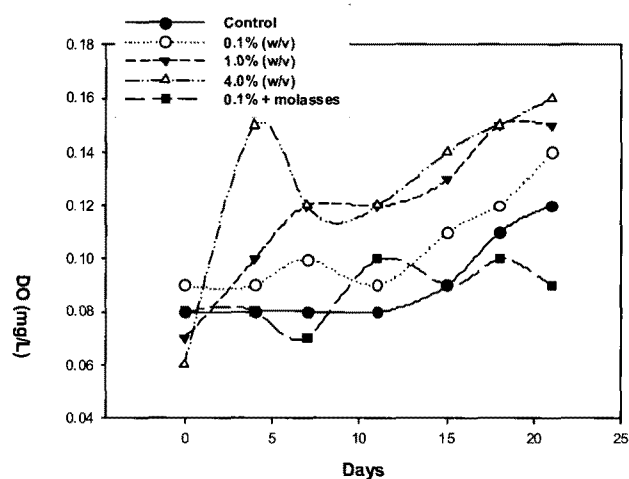


Fig. 4 Effect of EM clay ball treatment on DO of the polluted marine sediment over the incubation period.

Changes of pH were also monitored over the experimental period (Fig. 5). The pH of the treatments except the EM treatment with molasses generally more rapidly decreased than the control where the descending rate of pH appeared to be proportional to the amount of EM clay ball treatment. This would indicate the EM treatment may render the sediment neutralized from an alkaline condition (pH ~ 8.0), due to a potential activity of EM culture. In fact, a lactic acid bacterial population was observed at 10^{-3} dilution level after 7 days (data not shown) even though the species did not match one of the lactic acid bacteria isolated from the stock culture. However, the yeast population was not observed at the same period along with other EM cultures.

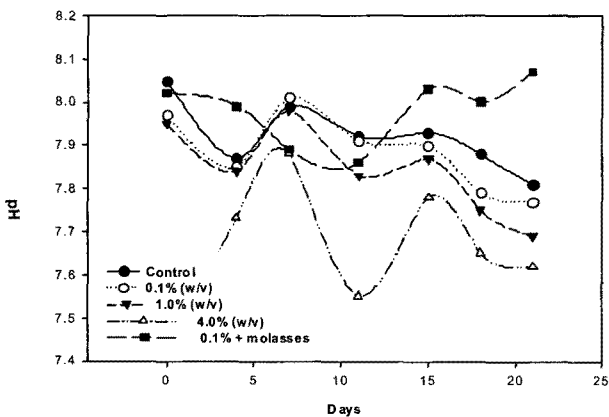


Fig. 5 Effect of EM clay ball treatment on pH of the polluted marine sediment over the incubation period.

Higher amounts of the EM clay ball treatments (1 and 4.0%) more effectively contributed to an early removal of malodor including H_2S measured based on the direct olfactory method (Fig. 6). This corroborated the results from the previous EM stock culture treatment experiment (data not shown).

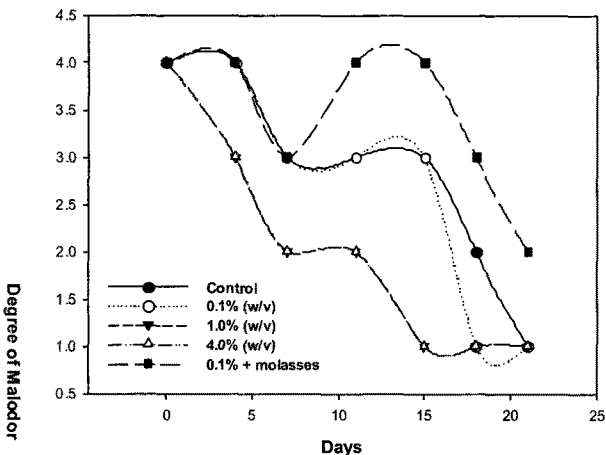


Fig. 6 Effect of EM clay ball treatment on removal of malodor in the polluted marine sediment over the incubation period.

3.4 Effect of EM stock culture treatment on microbial communities in the polluted marine sediment

Microbial community patterns based on PCR-DGGE for the treatments, stock culture and subculture were shown in the Fig. 7. There seemed to be little difference between the control and the lower amounts of treatment (0.01 and 0.1%) in the community patterns. However, the higher amount of treatment (1.0%) and the treatment with molasses shared a few potential EM cultures (lanes D1, D2, E1, and E2 indicated by the arrow heads in Fig. 6) which needed to be characterized further.

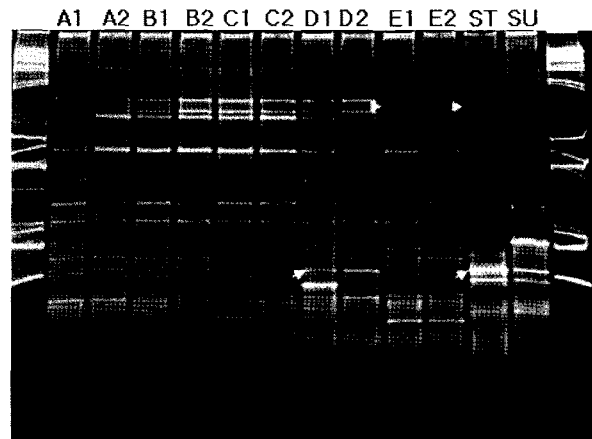


Fig. 7 Effect of the EM stock culture treatment on microbial communities in the polluted marine sediment (11 days). A1, A2 control; B1, B2 0.01% EM stock; C1, C2 0.1%; D1, D2 1.0%; E1, E2 0.1% + 0.3% molasses; ST, stock culture; SU, subculture; Arrow heads indicate the potential populations present in EM stock culture.

4. Conclusion

The dominant populations of the stock culture were *Lactobacillus parabuchneri*, *Clostridium* sp., and *Brevibacillus* sp. while *Lactobacillus farraginis*, *Acetobacter* sp., and *Lactobacillus parafarraginis* were dominant in the EM subculture. Higher concentrations of EM clay ball treatment (1% and 4%) contributed to an early removal of malodor and an increase of DO, indicating an odor removing activity of EM. It was, however, unknown that EM microbial community was directly involved in the malodor removal since EM cultures were not isolated from the treated sediments using spread plate counting method. It was necessary to further characterize EM community dynamics within treated sediments in relation to the remediation process.

Acknowledgment

This work supported by the Year 2008 grant from the Environmental Protection Division, Yeongdo-gu Office, Busan, Republic of Korea.

References

- [1] Aruoma, O., Deiana, I.M., Rosa, A., Casu, V., Piga, R., Peccagnini, S., Dessi, M.A., Ke, B., Liang, Y.F., and Higa, T. (2002), "Assessment of the ability of the antioxidant cocktail-derived from fermentation of plants with effective microorganisms (EM-X) to modulate oxidative damage in the kidney and liver of rats *in vivo*: studies upon the profile of poly- and monounsaturated fatty acids", *Toxicology Letters*, Vol. 135, pp. 209- 217.
- [2] Do, J.S., Choi, Y.H., Seo, H.J., Ryoo, J.H., and Nam, S.Y. (2006), "Effective Microorganism (EM) Fermentation extract attenuates airway hyperreactivity and lung inflammation in a mouse model of asthma", *Journal of Bacteriology and Virology*, Vol. 36, pp.1 - 10.
- [3] Duhamel, M. and Edwards, E.A. (2006), "Microbial composition of chlorinated ethene-degrading cultures dominated by *Dehalococcoides*", *FEMS Microbiol. Ecol.*, Vol. 58, pp.538-549.
- [4] EM Research Organization. (1995), "EM Application Manual for APNAN Countries", 1st Ed., pp1-7.
- [5] Heo, S.U., Moon, S.Y., Yoon, K.S., Kim, Y.J. and Koo, Y.M. (2008), "Enhanced compost maturity by effective microorganisms", *Abstracts / Journal of Biotechnology* Vol. 136: S65.
- [6] Higa, T. (1991), "Effective microorganisms: A biotechnology for mankind. *In Proceedings of the First International Conference on Kyusei Nature Farming*", Parr JF, Hornick SB, and Whitman CE (Ed.), U.S. Department of Agriculture, Washington, D.C., . pp 8-14.
- [7] Higa, T. (1995), "Use of microorganisms in agriculture & their positive effects on environmental safety", Nobunkyo, Tokyo, Japan.
- [8] Higa, T. (1996), "An earth saving revolution", Sunmark Publishing, Inc., Tokyo, Japan.
- [9] Higa, T. (2003), "Latest development of EM technology", *In Abstracts for the Second International EM Medical Conference, International EM Medical Conference Executive Committee Secretariat, Okinawa, Japan*, pp 7-9.
- [10] Ke, B., Xu, Z., Ling, Y., Qiu, W., Xu, Y., Higa, T., and Aruoma, O. I. (2008), "Modulation of experimental osteoporosis in rats by the antioxidant beverage effective microorganism-X (EM-X)", *Biomedicine & Pharmacotherapy*, in Press.
- [11] Khaliq, A., Abbasi, M. K., and Hussain, T. (2006), "Effects of integrated use of organic and inorganic nutrient sources with effective microorganisms (EM) on seed cotton yield in Pakistan", *Bioresource Technology*, Vol. 97, pp. 967 - 972.
- [12] Kim, B.H., Lee, S.E., Song, M.Y., Choi, J.H., Ahn, S.M., Lee, K.S., Cho, E., Chon, T.S., and Koh, S.C. (2008), "Implementation of artificial neural networks (ANNs) to analysis of inter-taxa communities of benthic microorganisms and macroinvertebrates in a polluted stream", *Science of Total Environment*, Vol. 390, pp. 262 - 274.
- [13] Koh, S.C., Song, Y.C., and Kim, I.S. (1997), "Effective treatment of food wastes by Effective Microorganisms (EM) and their recycling", *J. Korea Solid Wastes Eng.* Vol. 14, pp. 729-740.
- [14] Lee, E.J., Lee, S.M., Lee, G.T., Kim, I.S., and Kim, Y.H. (2008), "Application of effective microorganisms for bioremediation of crude oil spill in Taean, Korea", *J. of Environmental Sciences* Vol. 17, pp. 795-799.
- [15] Ministry of Environment (1998), "Official Methods for Analysis of Air Pollution".
- [16] Ministry of Maritime Affairs and Fisheries, (2002), "Official Methods for Analysis of Marine Environments".
- [17] Monica, D., Dessi, M.A., Ke, B., Liang, Y.F., Higa, T., Gilmour, P.S., Jen, L.-S., Rahman, I., and Aruoma, O. I. (2002), "The antioxidant cocktail effective microorganism X (EM-X) inhibits oxidant-induced interleukin-8 release and the peroxidation of phospholipids *in vitro*", *Biochemical and Biophysical Research Communications*, Vol. 296, pp. 148 - 1151.
- [18] Oh, J.S., Lee, J.S., Kang, K.H., Kim, H.T., Chung, W.B. and Jeong, S.J. (2002), "Effect of Microbial Fermentation Compost by Cultivating Area", *Kor. J. Org. Agricul.*, Vol. 10, pp. 87-97.
- [19] Yoo, K.S., Moon, S.Y., Heo, S.U., Yun, H.S., Kim, Y.J. and Koo, Y.M. (2008), "Synergy effect on bioremediation by using co-culturing effective microorganisms with *Shingobacterium* sp. WY", *Abstracts / Journal of Biotechnology*, Vol. 136: S704.

Received 4 December 2008

Revised 10 February 2009

Accepted 25 March 2009