Inactivation of Pathogenic Bacteria by Addition of Thermophilic Bacteria in the Thermophilic Aerobic Oxidation(TAO) System

Lee, W. I., Tsujii, H., Maki, T.* and Lee, M. G.**

Science of Biological Production, Shinshu University, Kamiina-gun, Minamiminowa-mura, Nagano-ken 399-4598, Japan

고온호기산화장치의 고온미생물 첨가에 의한 병원성 미생물의 불활성화

이원일 · Tsujii Hirotada · Maki Takaaki* · 이명규**

일본 나가노현 카미이나군 신주대학 생물생산과학

Summary

This study analyzed temperature increase, microorganism changes, and inactivation of pathogenic microorganisms in pig slurry when treated with thermophilic microorganisms in Thermophilic Aerobic Oxidation(TAO) system. An amount of 6 m^3 of pig slurry was treated in an 18 m^3(3.0 × 2.5 × 2.4 m) reactor for 5 to 7 days in two groups: the control of pig slurry only and the treatment of pig slurry with 6 liters of thermophilic microorganism(*Bacillus* sp.). To study the microorganism changes in the reactor, the populations of aerobic mesophilic microorganisms, thermophilic microorganisms and general pathogens were analyzed. To study the inactivation of pathogenic microorganisms, the levels of *E. coli*, *Salmonella* sp, *Cryptosporidium parvum* and *Giardia lamblia* were analyzed. The temperature inside the reactor ranged from 18 to 62℃ for the control while for the treatment group it ranged from 18 to 66 ℃, showing a slightly higher array. With regard to changes in microorganisms, both mesophilic and thermophilic organisms decreased from 3.1×10^6 to 1.2×10^2 CFU/mℓ and from 1.0×10^4 to 8.0×10^1 CFU/mℓ, respectively, in the control. In the treatment, on the other hand, mesophilic organisms decreased from 3.0×10^8 CFU/mℓ to 8.6×10^5 CFU/mℓ while thermophilic organisms increased sharply from 2.0×10^6 to 1.2×10^8 CFU/mℓ. For pathogens, *Salmonella* and *Giardia* were not detected either before or after the treatment, while *E. coli* and *C. parvum* were found to be 10^5 CFU/mℓ each before treatment and negative after it. From this experiment, it was concluded that thermophilic microorganisms could effectively sanitize liquid compost by generating high temperature in the TAO system, which in turn would inhibit the growth of pathogenic organisms.

(Key words : Pig slurry, Pathogenic microorganisms, Temperature, Thermophilic microorganisms, Thermophilic aerobic oxidation system)

* Matumoto Institute of Microorganism Co. Ltd, Ooaza-niimura, Matumoto-shi, Nagano-ken 390-1241, Japan

**한국 강원도 원주시 우산동 상지대학교 환경공학과 220-702,

(Environmental Engineering, Sangji University, Woosan-dong, Wonju City, Kangwon-do 220-702, Korea)

Corresponding author : Dept. of Environmental Engineering, Sangji University, Woosan Wonju, Kangwon-do 220-702, Korea, E-mail : mglee@mail.sangji.ac.kr, Fax : +82-33-746-0443.

INTRODUCTION

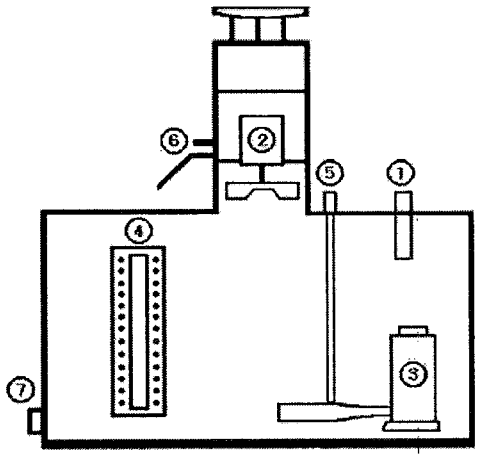
Traditional slurry treatment systems, whether aerobic or anaerobic, are not an effective measure in sanitation and pathogenic aspects and cannot meet the acceptable pathogen levels set by the US Environmental Protection Agency(EPA)(De Bertoldi et al., 1988; EPA 1990, 1992; Juris et al., 1992, 1993; Pagilla et al., 1996). Many European and North American countries are regulating the level of pathogenic organisms contained in human and/or animal wastes when these are discharged into soil or the ocean, necessitating the development of an aerobic thermophilic digestion system(EPA 1992; Droffner and Brinton 1995). The thermophilic aerobic digestion system has been known to have many advantages including fast digestion rate, rapid degeneration of pathogenic organisms, low slurry yields, simple processing units, safety, and absence of heat energy requirement for fermentation(Timothy et al., 1999; Himoto et al., 1988). We reported that the Thermophilic Aerobic Oxidation(TAO) system is very effective at slurry volume reduction, production of a pathogen-free liquid fertilizer, and the removal of the malodor(Lee and Lee. 2000 a, b). TAO system is a biological composting process that can be applied to a large spectrum of organic wastes, by utilizing auto-heating above 55℃.

In this study, the effects of thermophilic microorganisms when they are used to treat pig slurry were analyzed in TAO system, a branch of thermophilic aerobic digestion system. The analysis covered four aspects: effective temperature; changes in microorganisms; changes in pathogenic organisms *E. coli*, *Salmonella*, *Cryptosporidium parvum* and *Giardia lamblia*.

MATERIALS AND METHODS

1. Operating condition and sampling

The TAO system for the experiment was installed at a pig farm in Omachi city, Nagano prefecture, Japan with an 18 m^3 volume stainless steel reactor whose size was $3.0 \times 2.5 \times 2.4$ m. The reactor was insulated to reduce heat loss. An air pump(LG PDV-A400M), a foam cutter used to get rid of foams caused by aeration, and a gas neutralizer used to reduce odors generated during the fermentation process were also installed. The scheme of the TAO system used in Experiment is shown in Fig. 1. About 6.3 tons of pig slurry was put into the control group and the same amount was put in the treatment group. Then 6 ℓ of *Bacillus* was added in the treatment group when the temperature reached 60℃ inside the TAO system, and the system was operated for 5 to 7 days. Samples were collected from the reactors to analyze microorganism content.



① Input of sample, ② Foam Cutter, ③ Pump,
④ Indicator of water level, ⑤ Input of air,
⑥ Output of efflux, ⑦ Output of sample

Fig. 1. The scheme of the TAO system used in Experiment.

Table 1. Medium and incubation method of microorganism

Microorganism	Medium	Incubation condition	
Aerobic bacteria	Standard agar medium	Mesophilic	28℃, 48hr
		Thermophilic	55℃, 48hr
E. coli	BTB agar		37℃, 24hr
Salmonella sp.	MLCB, SS agar		37℃, 18℃, 24hr

BTB : Bromthymol blue agar; MLCB : SS : *Salmonella-Shigella* agar.

Samples were collected before the fermentation, at the point of 45℃ and 60℃, when the temperature began to decline, and when the test was complete. For microorganism counts, standard agar medium with an adjusted pH of 8.5, which is the average pH inside the system, was used. Microflora in the slurry was analyzed by the media and culture as shown in Table 1. Temperature was measured continuously through a sensor installed inside the reactor 40 cm high from the bottom.

2. *Cryptosporidium parvum* and *Giardia lamblia* analysis

C. parvum and *G. lamblia* were concentrated and purified from the sample, then treated with fluorescent antibody staining to be counted. Concentrate continued as follows Samples were first vortexed, and then 50 mℓ of it was taken and mixed with 2 mℓ of 10% formalin, and centrifuged again. Finally, 12 mℓ of sediment was transferred into a 15 mℓ centrifugal tube for a second centrifugation of 2,500 rpm for 5minutes.

To purify a sample, centrifugal sedimentation and density gradient centrifugation techniques were applied. For the former, the concentrated sediment was added with phosphate buffered saline(PBS) to make a total volume of 6 mℓ which was then agitated. Ethyl acetate(2 mℓ) was added, and the solution was vortexed for 30 seconds. Finally, the mix was centrifuged at 2,600 rpm for 5 minutes.

For the density gradient centrifugation technique, PBS was added to the sediment harvested from the centrifugal sedimentation to make a total volume of 4 mℓ which was then agitated. A total of 2 mℓ sucrose of 1.2 specific gravity was added to form layers. This was centrifuged at 2,500 rpm for 10 minutes. Each layer was taken into 50 mℓ centrifugal tubes, and PBS (seven times of sucrose) was again added for a second centrifugation. The harvested sediment was analyzed with Indirect Fluorescent Staining. For this part, 44 μℓ of cell suspension was placed on a glass slide then mixed with 6 μℓ of new methylene blue. The slide was covered with a cover glass, and the four sides were sealed with paraffin to prevent dehydration. The prepared slide was screened with 400 × microscope for oocysts of 4 ~ 6 μℓ in size, which were examined with 1000 × microscope to view their internal structures.

RESULTS AND DISCUSSION

1. Change in temperature and bacteria

Temperature is the most important indicator of the efficiency of the composting process and is

dependent on aeration rate(Lau et al., 1993). Also, the temperature is required to destroy the harmful microbes and viruses contained within excretions, thus making the production of a sanitary and stable fertilizer possible(Heinonen et al., 1998; Phae et al., 1999; Timothy et al., 2000). The change of temperature to total solid content was shown in Fig. 2. The samples used in this study had 5.5% or above total solid concentration, sufficient to reach temperature higher than 55℃. Lee and Lee(2000a) reported that the minimum total solid concentration in pig slurry should be greater than 5.5% in order to maintain atemperature of 50℃ or above in the TAO system to effectively treat it. In addition, Matsuda et al.(2000) reported that the temperature rose to 54.7℃ with 5.2% of total solid concentration, and only 33.3℃ with 2.8%. Fig. 3. shows the temperature change, a significant sign to compositing. The temperature variation of the treatment group ranged from 18 to 62℃, while that of the control group ranged from 19 to 66℃, showing the treatment group to have a wider range. However, after 30 hours of operation, the temperature of both groups reached above 55℃, with the highest point at 62℃ for the control and 66℃ for the treatment thereafter. The outside air temperature was between 10℃ and 20℃ when the results were obtained. Nevertheless, tests performed below the freezing point during winter showed similar tendencies. Since not only the microorganisms but also the pumpused in the tests could generate the heat, water was added into the system and the temperature variation was traced. The results were negligible, showing around 18 to 24℃. This confirms that the heat was generated mostly by the microorganisms' fermentation process.

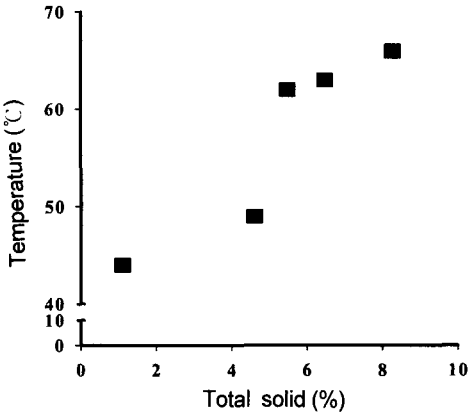


Fig. 2. The change of temperature by the total solid content.

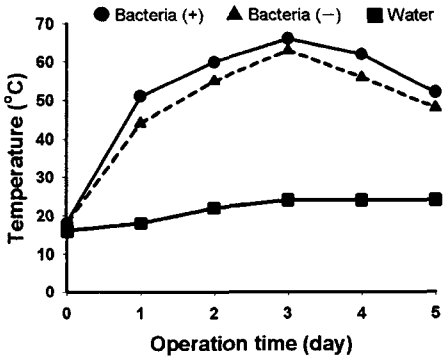


Fig. 3. Thermal increasing effect by addition of thermophilic bacteria in the TAO system.

2. Change of bacteria

In general, most aerobic bacteria that cause fermentation are either mesophilic(20~40℃) or thermophilic(50~70℃). Fig. 4. shows the growth of mesophilic and thermophilic bacteria in the TAO system. The control was inoculated into standard agar medium, and the treatment group onto the same medium of pH 8.5. In the control, mesophilic and thermophilic organisms decreased from 3.1×10^6 to 1.2×10^2 CFU/mℓ and from 1.0×10^4 to 8.0×10^1 CFU/mℓ, respectively. In the treatment, mesophilic organisms decreased from 3.0×10^8 to 8.6×10^5 CFU/mℓ, but ther-

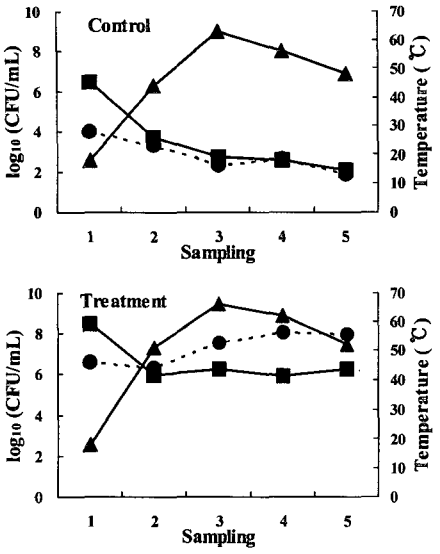


Fig. 4. The change of temperature and bacteria in reactor during the experimental period.
■ : Mesophilic bacteria(28℃), ● : Thermophilic bacteria(55℃), ▲ : Temperature. Sampling time: (1) at the start of operation of the reactor; (2) at 45℃, (3) at 60℃, (4) when the temperature dropped, (5) when the experiment was finished.

mophilic organisms increased from 2.0×10^6 to 1.2×10^8 CFU/mℓ. Concentration of mesophilic bacteria of control and treatment decreased 30% with 67%, respectively, according to temperature of system increasing. However, the thermophilic bacteria decreased in 53% in case of control, but the treatment was increased in 30%.

3. Inactivation of pathogenic microorganisms

The enteric protozoan parasites *Cryptosporidium* and *Giardia*, causing acute gastroenteritis in humans, have become significant waterborne pathogens in the developed world. There are three known species of *Giardia* and eight of *Cryptosporidium*, of which *Giardia intestinalis* and *Cryptosporidium parvum* are the species responsible for most human and mammal infections (Fayer, 1994). The waterborne transmission of these parasites has been well documented and over 160 outbreaks of cryptosporidiosis and giardiasis have been reported worldwide. The parasites multiply in several host animal species, including human beings, which excrete infective forms, *Cryptosporidium* oocysts and *Giardia*cysts, into the environment. The infective forms survive in aquatic environment and are resistant to disinfectants used in drinking water treatment. Anywhere from 0.8 oocysts/ℓ up to 112.0 oocysts/ℓ of *C. parvum*are present in exposed streams, posing a risk of mass diarrhea to residents (Tsushima et al., 2001; LeChevallier et al., 1991; Rose et al., 1991).

Table 2 shows the degradation of pathogenic microorganisms, namely, *E. coli*, *Salmonella*, *C. parvum*, and *G. lamblia*. *E. coli* and *C. parvum* were 10^5 CFU/mℓ and were positive before treatment but became negative after treatment. *Salmonella* and *G. lamblia*were negative both before and after treatment. Fig. 5 shows the phases of differential interference of *C. parvum* by before treatment of TAO system. The population of *E. coli*, used as an indicator of harmful bacteria before the fermentation, was 10^5 CFU/mℓ.

Table 2. TAO thermal effect for inactivation of pathogenic bacteria

	E. coli	*Salmonella*	*Cryptosporidium parvum*	*Giardia lamblia*
Before treatment	10^5	ND	+	-
After treatment	ND	ND	-	-

(+) : Oocyst positive; (-) : Oocyst negative; ND : Not Detected.

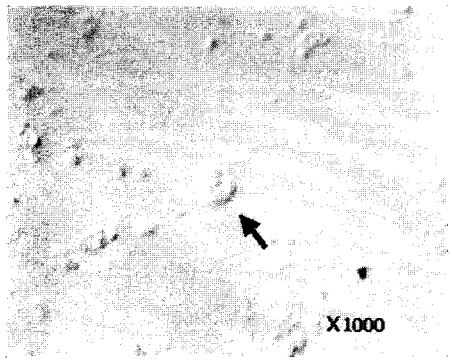


Fig. 5. The phases of differential interference of *Cryptosporidium parvun* by before treatment of TAO system.

This corresponded to what Okamoto et al.(1995) measured in animal slurry, which was $10^4 \sim 10^5$ CFU/mℓ. This became negative after the aerobic thermophilic fermentation process, which is similar to the study of Ugwuanyi et al.(1999). In their study, between 55℃ and 60℃, and between pH 7.0 and pH 8.0, *E. coli* became most quickly inactivated under pH 8.0 at 60℃, compared with the results of any other combination.

There are several methods for the inactivation of *Cryptosporidium* and *Giardiad.* UV disinfection systems produce no hazardous by-products and are easy to maintain. The UV systemis considered one of the more effective disinfection techniques for bacteria and viruses in drinking water and wastewater. Although coagulation sedimentation and rapid sand filtration can remove *C. parvumoocysts* and reduce their numbers by 2 to 3 log units(Hashimoto et al., 2002; LeChevallier and Norton 1995), the expected rate of removal may not sufficiently reduce the risk of infection to an acceptable level in cases where the source water is highly contaminated. Chlorinehas been used as a disinfectant in many water supplies; however, *C. parvumoocysts*are insensitive to the concentrations routinely used(Gyurek et al., 1997; Hirata et al., 2000; Korich et al., 1990). Thus, there is interest in developing an alternative, more effective disinfectant for inactivating these recalcitrant microorganisms. *C. parvum* oocysts, in particular, are very strong against chlorine. However, *C. parvum* and *G. lamblia* are vulnerable to heat. *C. parvum* dies within 30 seconds when heated at 55℃, and in 15 seconds at 60℃(Fujino et al., 2002). In this study, thermophilic bacteria added into the pig slurry in the TAO system generated heat above 60℃ for more than 48 hours through which *C. parvum* and *G. lambliawere* killed. This shows that TAO systems are effective in treating pig slurry in terms of biochemical degradation and disinfecting infectious pathogens such as *C. parvum, G. lamblia,* or *Salmonella.*

CONCLUSION

Pathogenic microorganisms in animal waste should be eliminated to produce fertilizer and liquid fertilizer helpful to soil and crops. Temperature can be considered as one of the inactivation methods of pathogenic microbes. However, at the usual fermentation, content of organic matter and active temperature of bacteria are important, and, also, the temperature is 45 ~ 50℃. Therefore, the thermophilic bacteria were added and the temperature change in a TAO system was investigated.

The temperature inside the reactor ranged from 18 to 62℃ for the control while for the treatment group it ranged from 18 to 66℃, showing a slightly higher array. The inactivation of harmful microorganisms such as *E. coli, Salmonella, C. parvum* and *G. lamblia* was accomplished and the production of more sanitary and safe liquid fertilizer using TAO system was possible.

적 요

본 연구는 양돈 분뇨를 고온호기산화장치(TAO)를 이용하여 처리하였을 때, 고온 미생물의 첨가에 의한 온도 상승과 시스템의 내부 미생물 변화 그리고 유해 미생물의 불활성화에 대하여 연구하였다.

실험은 총 용량 18 m^3(3.0 × 2.5 × 2.4 m)의 반응기에 양돈 분뇨 6 m^3을 투입하고 5~7일간 운전하였다. 대조구는 양돈 분뇨만을 투입하였고 처리구는 6 ℓ의 고온 미생물(*Bacillus.* sp)을 투입하였다. 반응기의 내부 미생물의 변화를 검토하기 위해서 호기성 중온균, 고온균 그리고 일반 세균을 분석 하였다. 또한, 유해 미생물의 불활성화를 검토하기 위하여 *E. coil*, *Salmonella.* sp, *Cryptosporidium parvum*, *Giardia lamblia*를 분석하였다.

대조구와 처리구의 운전기간 동안 반응기 내부의 온도 범위는 18~66℃로 55℃ 이상의 높은 온도를 유지하였다. 미생물 변화에 있어서 대조구의 중온균과 고온균은 $3.1 \times 10^6 \sim 1.2 \times 10^2$ CFU/mℓ, $1.0 \times 10^4 \sim 8.0 \times 10^1$ CFU/mℓ로 감소하였으나 처리구의 경우, 중온균은 $3.0 \times 10^8 \sim 8.6 \times 10^5$ CFU/mℓ로 감소하였으나 고온균은 $2.0 \times 10^6 \sim 1.2 \times 10^8$ CFU/mℓ로 증가하는 경향을 보였다. *Salmonella*와 *Giardia*는 처리 전·후에 검출되지 않았으며 *E. coil*와 *Cryptosporidium*은 처리전 양성반응을 나타내었으나 처리 후 불활성화 되었다.

이상의 결과를 통해서, 우리는 TAO system에 고온 미생물을 첨가함으로써 유해한 미생물이 사멸된 액상비료를 생산할 수 있었고 분뇨로부터 기인하는 2차 오염을 방지할 수 있을 것으로 판단된다.

(핵심단어 : 양돈분뇨, 유해 미생물, 온도, 고온 미생물, 고온호기산화장치)

REFERENCES

1. De Bertold, M., Zucconi, F. and Civilini, M. 1988. Temperature, pathogen control and product quality. Biocycle. 2(88):43-50.
2. Droffner, M. L. and Brinton, W. F. 1995. Survival of *E. coli* and *Salmonella* populations in aerobic thermophilic compost as measured with DNA gene probe. Zentralblatt fuer Hygiene und Umweltmedizin. 197:387-397.
3. Fayer, R. 1994. Effect of high temperature on infectivity of Cryptosporidium parvum oocysts in water. Appl. Environ. Microbiol. 60:2732-2735.
4. Fujino, T., Matsui, T., Kobayashi, F., Haruki, K., Yoshino, Y., Kajima, J. and Tsuji, M. 2002. The effect of heating against Cryptosporidium oocysts. J. Vet. Med. Sci. 63(4): 199-200.
5. Gyurek, L. L., Finch, G. R. and Belosevic, M. 1997. Modeling chlorinc inactivation of Cryptosporidum parvum oocysts. J. Environ. Eng. 9:865-875.
6. Hashimoto, A., Kunikane, S. and Hirata, T. 2002. Prevalence of Cryptosporidium oocysts and Giardia cysts in the driking water supply in Japan. Water Res. 36:519-526.
7. Heinonen, T. H., Niskanen. E. N., Salmela, P. and Lanki, E. 1998. *Salmonella* in animal slurry can be destroyed by aeration at low temperatures. J. Appl. Microbiol. 85:277-281.
8. Himoto, J., Iwabuchi, K. and Matsuda, J. 1988. Aerobic composting of liquid animal waste. 1. Design of an Experimental Apparatus. Memories of Faculty of Agriculture Hokkaido University. 15:257-265.(Japanese)
9. Hirata, T., Chikuma, A., Shimura., Hashimoto, A., Motoyama, N., Takahashi, K., Moniwa, T., Kaneko, M., Saito, S. and Maede, S. 2000. Effects of ozonation and chlorination on viability and infectivity of Cryptosporidum parvum oocysts. Water Sci. Technol. 41:39-46.
10. Juris, P., Plachy, P., Dubinsky, P., Venglovsky, J. and Toth, F. 1993. Effect of aerobic stabilization of pig slurry on vitality of model pathogens. Veterinary Medicine. 9:553-558.
11. Juris, P., Plachy, P., Toth, F. and Venglovsky,

J. 1992. Effect of biofermentation of pig slurry on Ascaris suum eggs. Helminthologia. 29:155-159.

12. Korich, D. G., Mead, J. R., Madore, M. S., Sinclair, N. A. and Sterling, C. R. 1990. Effects of ozone, chlorinc dioxide, chlorinc, and monochloraminc on Cryptosporidium parvum oocyst viability. Appl. Environ. Microbiol. 56:1423-1428.
13. Lau, A. K., Liao, P. H. and Lo, K. V. 1993. Evaluation of swine waste composting in vertical reactors. J. Environ. Sci. Health. Part A. 28:1889-1901.
14. LeChevallier, M. W. and Notron, W. D. 1995. Giardia and Cryptosporidium in raw and finished water. J. Am. Water Works Assoc. 87:54-78.
15. LeChevallier, M. W., Norton, W. D. and Lee, R. G. 1991. Occurrence of Giardia and Cryptosporidium spp. in surface water supplies. Appl. Environ. Microbiol. 57(9):2610-2616.
16. Lee, M. G. and Lee, W. I. 2000a. Operation condition and characterisitics for treatment of piggery slurry using Thermophilic Aerobic Oxidation System. Journal of Livestock Housing and Environment. 6:161-168.(Korean)
17. Lee, W. I. and Lee, M. G. 2000b. Reducing technique for nitrogen and phosphorus in piggery slurry by the Thermophilic Aerobic Oxidation(TAO) System. Journal of Livestock Housing and Environment. 6:185-190.(Korean)
18. Matsuda, J., Abe, Y., Okamoto, E. and Maeda, T. 2000. The effects of dilution onnitrogen decomposition and ammonia emission in slurry during aerobic treatment. 9th International conference on management strategies for organic waste use in agriculture.
19. Okamoto, E., Miyagawa, E., Matsui, Y. and Matsuda, J. 1995. Change of microbial population on liquid composting of dairy cattle slurry. J. Rakuno Gakuen Univ. 20(1):81-90.
20. Pagilla, K. R., Craney, K. C. and Kido, W. H. 1996. Aerobic thermophilic pretreatment of mixed sludge for pathogen reduction and Nocardia control. Water Environ. Res. 68: 1093-1098.
21. Phae, C. G. and Sim, H. S. 1999. Treatment of Human Manure by Thermophilic Aerobic Digestion System Using Thermophilic Bacteria. Korean Journal of Solid Wastes Engineering Society. 16:526-535.
22. Rose, J. B., Gerba, C. P. and Jakubowski, W. 1991. Survey of potable water supplies for Cryptosporidium and Giardia. Environ. Sci. Technol. 25:1393-1400.
23. Timothy, M. L. and James, E. A. 1999. Thermophilic aerobic biological wastewater treatment. Wat. Res. 33(4):895-908.
24. Timothy, M, L., Allan, K., Cindy, H, N. and James, E, A. 2000. Thermophilic aerobic wastewater treatment in continuous-flow. J. Environ. Eng. 126:739-744.
25. Tsushima, Y., Karanis, P., Kamada, T., Nagasawa, H., Xuan, X., Igarashi, I., Fujisaki, K., Takahashi, E. and Mikami, T. 2001. Detection of Cryptosporidium parvum Oocysts in Environmental Water in Hokkaido, Japan. J. Vet. Med. Sci. 63(3):233-236.
26. Ugwuanyi, J. O., Harvey, L. M. and McNeil, B. 1999. Effect of process temperature, pH and suspended solid contents upon pasteurization of a model agricultural waste during thermophilic aerobic digestion. Appl. Environ. Microbiol. 87:387-395.
27. U.S. Environmental Protection Agency. 1990. Autothermal Thermophilic Aerobic Digestion of Municipal Wastewater Sludge. EPA, 625, 10-90-007 September. Washington, D.C.: Government Printing Office.
28. U.S. Environmental Protection Agency. 1992. Control of Pathogens and Vector Attraction in Sewage Sludge. EPA, 625, R-92-013 December. Washington, D.C. Government Printing Office.