# ESTIMATION OF MICROBIAL COMMUNITY STRUCTURE DURING COMPOSTING RICE BRAN WITH CHARCOAL

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## Abstract

Biomass charcoal made from bamboo was mixed with rice bran as a nutrient. Aerobic complex microorganisms (ACM) for composting biomass waste were added to seed the mixture. As incubation-time of the mixture went by, the ACM concentration increased. ACM proliferation rate was dependent on addition amount of the charcoal. Rod and short-rod typed microorganisms can be observed by electron scanning micrograph on the surfaces of the charcoals and in the mouths of the pores. We confirmed that charcoal functioned as a matrix for these microorganisms. Three peaks in the ATP concentration vs. the incubation-time were observed. Two major bands of 16S rDNAs from ACM were amplified by polymerase chain reaction (PCR), and analyzed by denaturing gradient gel electrophoresis (DGGE) method. By cloning some bands, deciding base sequence, and searching similarity, two kinds of microorganisms species, *Paenibacillus Sp.* and *Alcaligenes Sp.,* were recognized. In other bands, *Bacillus circulans* and *Bacillus cereus* were recognized.

## Introduction

Recently, two technologies have been receiving attention in the field of biomass waste recycling. One is the carbonization of biomass wastes such as waste construction materials, waste paper, and wood and bamboo forest thinnings, and another is the composting and use of garbage generated by homes, restaurants, and food industries and of livestock waste. It was reported that the addition of charcoal to farm soils had a proliferative effect on symbiosis microorganisms such as root nodule bacteria and mycorrhizae (Sugiura, 1984, Ogawa, 1984, 1987). It is well known that symbiosis microorganisms play important roles in growing plants. It was expected, therefore, that addition of charcoal to composting garbage enhances microorganisms proliferation, leading to the composting time shortened. Yoshizawa (2005) and Yoshizawa et al. (2005) previously verified the successful composting of a mixture of charcoal and garbage from 55 houses over a 2-month period in the city of Suwa, Japan. Aged compost was obtained after 1 or 2 months, and composting microorganisms were observed on and in the charcoal in the compost.

Wood and bamboo have pores that range from several to several tens of microns in diameter and originate from tracheae and charcoal prepared from carbonized wood and bamboo has pores of almost the same size. Tanaka et al. (2005) and Yoshizawa et al. (2006a, 2006b and 2006c) found that the proliferation of composting microorganisms was enhanced on and in bamboo charcoal as a medium to which rice bran had been added as a nutrient.

From April 29 to May 2, 2007, International Agrichar Initiative (AIA) 2007 Conference was held in Terrigal, New South Wales, Australia. There, It was discussed agriculture charcoal (agrichar or biochar) production and utilization can renew the world's soils through the addition of organic carbon, and can additionally help solve the pressing problem of global climate change (International Agrichar Initiative, 2007).

In this report, charcoal made from bamboo was added to rice bran with aerobic complex microorganisms (ACM) used for composting. We studied the proliferation of the microorganisms by measuring the cultivation time-dependence of the concentrations of adenosine triphosphate (ATP) from the microorganisms, and we analyzed 16S rDNAs of microorganisms in the compost by denaturing gradient gel electrophoresis (DGGE) after amplification by polymerase chain reaction (PCR) method.

## Experimental

### *Sample Preparation*

Charcoals were prepared from thinned Moso bamboo (*Phyllostachys pubescens* Mazel ex Houzeau de Lehaie). The raw material was carbonized at 650°C in a batch-type furnace (Venture Viser Inc., Type-IV). **Figure 1** shows SEM photograph of cross section of bamboo charcoal. The pH of the charcoals was 9.2. The specific surface area of the charcoal was estimated by the Brunauer-Emmett-Teller equation applied to $N_2$ adsorption isotherms for charcoals measured with an adsorption apparatus (BELSORP 18). The specific surface area of the charcoal was 420 $m^2/g$. The relative pore volume of the charcoal was measured by a mercury porosimeter (Shimadzu Corp., Autopore III 9420). The peak is centered in the range of 0.1 - 1 μm.

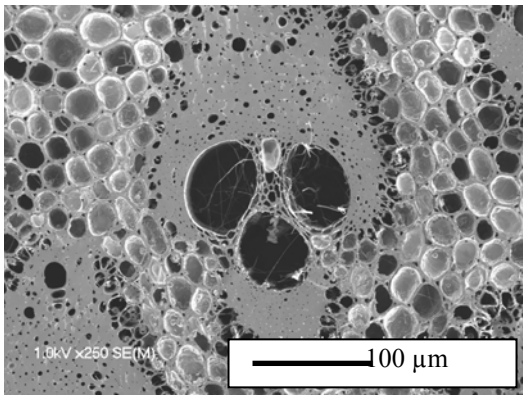


**Figure 1.** SEM photograph of cross section of bamboo charcoal.

Flow chart of sample preparation is shown in **Figure 2**. Charcoal pulverized and sifted into particles 1 to 3 mm in diameter was used as a medium. Rice bran (17.8 g) as nutrient was added to 15.5 g charcoal powder in a 300-ml flask, giving a weight ratio of charcoal to rice bran of 1:1.15 (Imanishi and Sakawa, 2003) The moisture content of the mixture was adjusted to 65% by adding distilled water. The mixture was then treated at 120 °C for 60 min in a high-pressure sterilizer. ACM of 0 g, 0.01 g, 0.1 g 0.5 g 1 g and 5 g were added to seed the mixture, respectively. The samples were incubated in an cultivation chamber with a relative humidity (RH) of 53% at 23 °C and stirred vigorously with a spatula once a day for aeration.

***Measurement***

A part of the mixture was periodically sampled for measurement. Microorganisms that proliferated on the surface of the charcoal were observed by SEM. After freeze-drying of the sample in liquid nitrogen in a vacuum, the sample was fixed by osmic acid evaporation. The surface was then coated with a thin film of sputtered Pt–Pd alloy.

The concentration of microorganisms was estimated by measuring the ATP concentration in the sample (Meidensha Corp., Luminometer UPD-4000) (Inamori, 1988). Because ATP exists in mitochondria in the cytoplasm, the concentration of

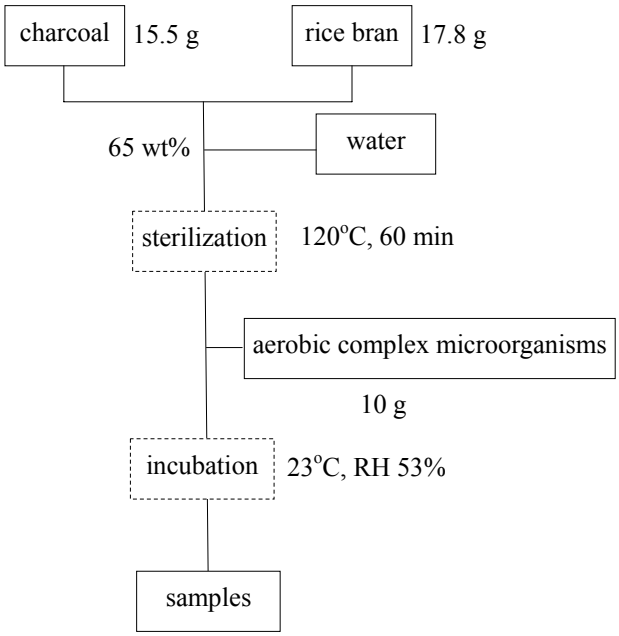


**Figure 2.** Flow Chart of Sample Preparation.

ATP can be used as an indication of microorganism activity. When ATP to which d-luciferin has been added changes to adenosine monophosphate in the presence of luciferase and $Mg^{2+}$, light at a wavelength of 560 nm is emitted. Distilled water (20 ml) was added to 2 g of the sample and stirred with a tube mixer at 2500 rpm for 1 min. Then 250 µl of this suspension was withdrawn with a micropipette and an ATP measuring kit (Meidensha Corp., Lucifer AS) added.

DNAs was extracted from 2 g of sample, and 16S rDNAs was amplified with PCR method using primers with GC cramps, and the PCR products were analysed by DGGE method with denaturing agent concentration of 0–80% at 200 V for 7 h.

## Results and Discussion

***Proliferation of Microorganisms***

The incubation time dependence of ATP concentration of the samples was measured. In **Figure 3**, in the system mixed with charcoal, rice bran and ACM, the ATP concentration increases accompanied with three concentration peaks with increase of incubation time. In the systems, the mixture of charcoal and rice bran without ACM and the single component, ACM alone, charcoal alone, and rice bran alone, no increase of the ATP concentration is observed.

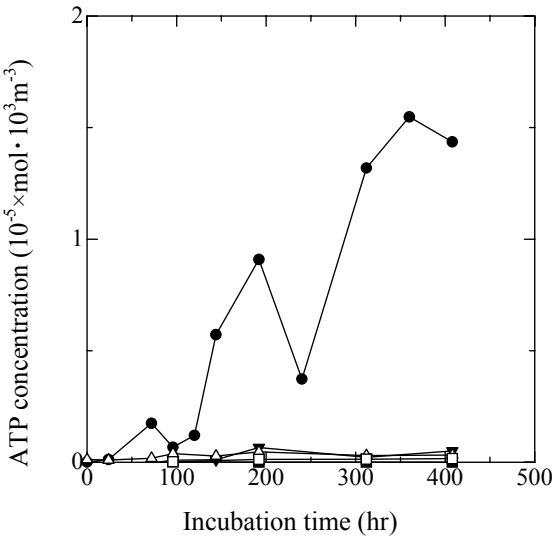


**Figure 3.** Incubation time dependent of ATP concentration of the systems. ●; ACM, charcoal and rice bran, ▼; charcoal and rice bran, △; ACM, ■; charcoal, and □; rice bran.

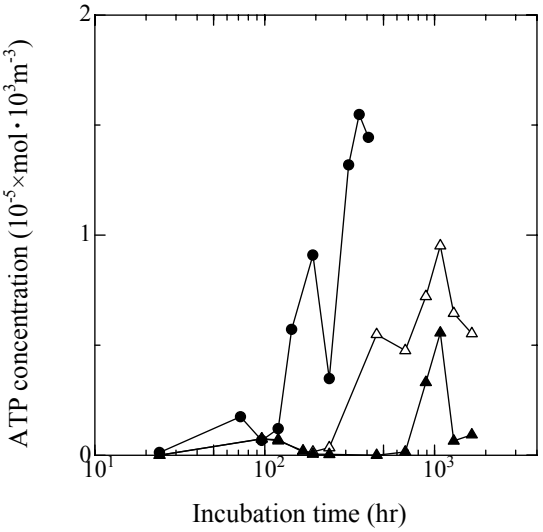


**Figure 4.** Incubation time dependent of ATP concentration of the systems. Charcoal amount : ▲; 1.0 g , △; 5.9 g and ●; 15.5 g.

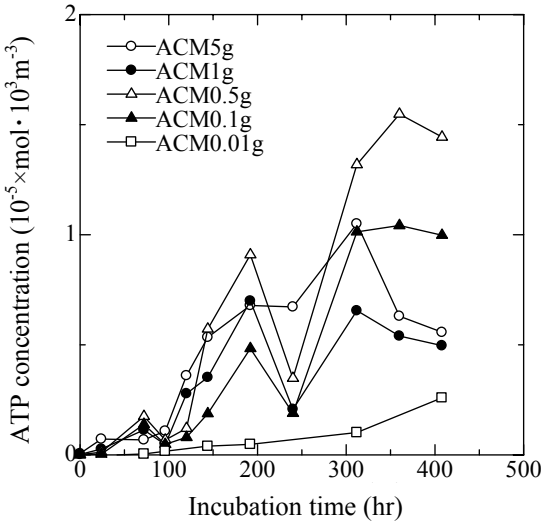


**Figure 5.** Incubation time dependent of ATP concentration of the systems with different ACM from 0.01 g to 5.0 g.

For studying influence of the charcoal amount on the microorganisms proliferation in the mixture of charcoal, rice bran and ACM, the logarithmical incubation time dependence of the ATP concentration in the mixture with different amount of the charcoal, 1.0 g, 5.9 g and 15.5 g, is shown in **Figure 4**. Increase rate and extent of the ATP concentration are dependent on the charcoal amount in the system. The result that ATP concentration increases in the mixture system with charcoal, rice bran and ACM, means that the charcoal can proliferate microorganisms in the system. There are found several peaks of the ATP concentration; two peaks at about 100 hr and 1,000 hr in the system with 1.0 g of charcoal, and three peaks at 100 hr, 500 hr and 1,000 hr with 5.9 g of the charcoal, and three peaks at 70 hr, 200 hr and 400 hr with 15.5 g of the charcoal. The peak shifts in the incubation time dependent on the amount of the charcoal. It is found that as the amount of the charcoal increases, the microorganisms proliferation is accelerated remarkably. From those results, there should be at least three kinds of microbial community in the system whose proliferation rate is different. It is suggested that this comes from difference of the proliferation rate of adaptive microorganisms as a specific response to the presence of nutrient such as glucide, protein and lipid contained in rice bran as a main component. The proliferation rate may reflect the biodegradation property of the nutrients.

The cultivation time-dependence of ATP concentration of the samples is shown in **Figure 5**. In the systems that used charcoal as a medium, the ATP concentration increases showing three peaks around 70 h, 190 h and from 300 to 350 h, respectively. This suggests that there are some kinds of microbial community whose proliferation rate is different.

***SEM Observation of Microorganisms***

SEM photographs were taken in a pore of the charcoal in the mixture, which were sampled near the peak of the ATP concentration. **Figures 6**, **7** and **8** show the SEM photographs of the samples with the charcoal amount of 1.0 g, 5.9 g and 15.5 g, respectively. Many kinds of microbial community can be observed; cocci, rod and short rod bacteria. We confirmed that charcoal functioned as a matrix for these microorganisms and that the composting microorganisms on the charcoal were morphologically diverse.

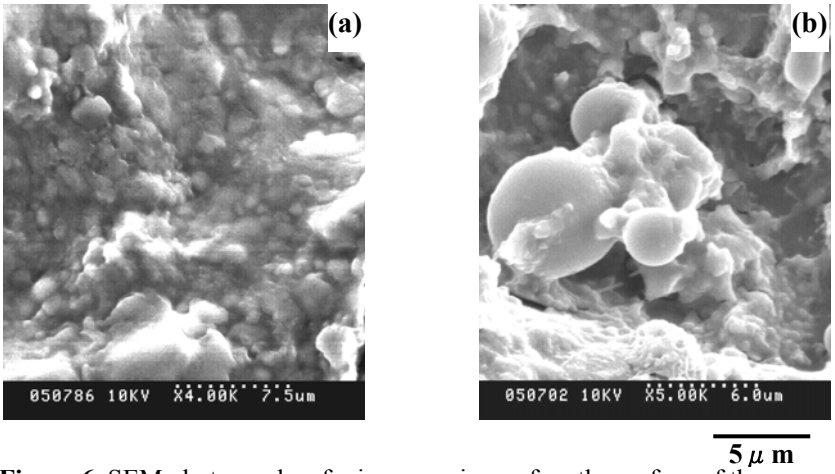


**Figure 6.** SEM photographs of microorganisms of on the surface of the charcoal. (a) after 216 hr and (b) after 1,056 hr. The charcoal amount; 1.0 g.

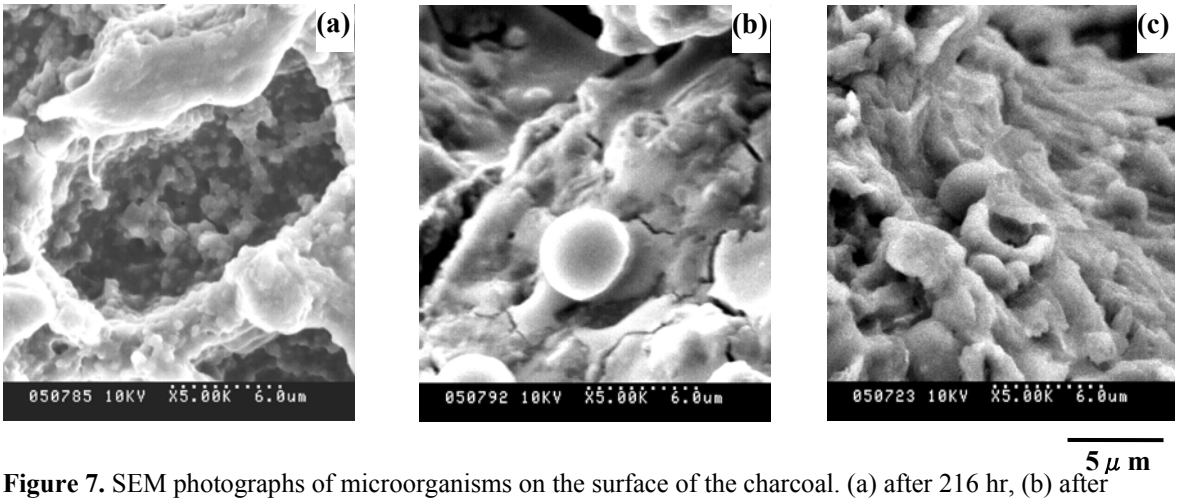


**Figure 7.** SEM photographs of microorganisms on the surface of the charcoal. (a) after 216 hr, (b) after 432 hr, and (c) after 1,056 hr. The charcoal amount; 5.9 g.

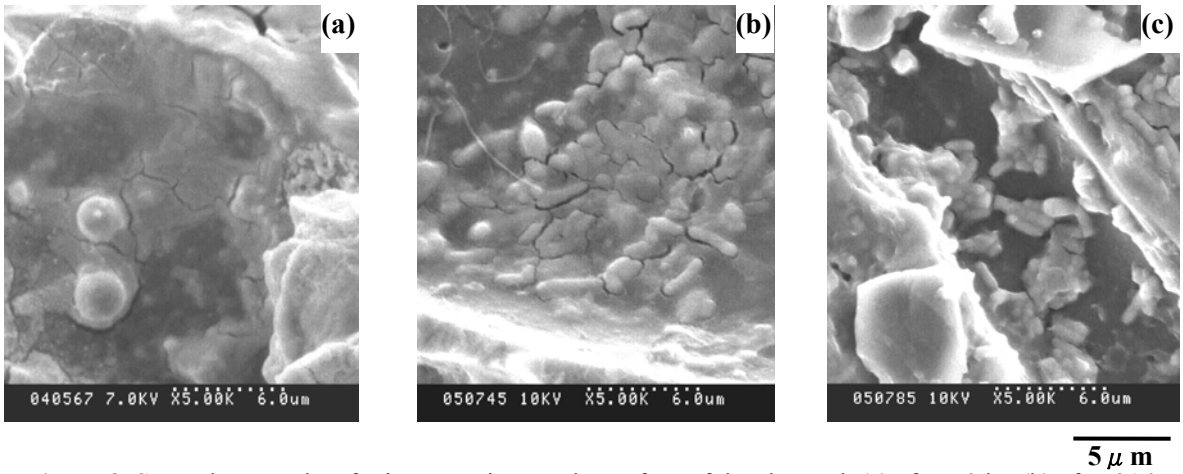


**Figure 8.** SEM photographs of microorganism on the surface of the charcoal. (a) after 72 hr, (b) after 216 hr, and (c) after 408 hr. The charcoal content; 15.5 g.

***Estimation of Microbial Community***

Because SEM observation is not considered sufficient to characterize fully the microbial communities, DNA fragments isolated from the composting microbial communities was studied. **Figure 9** shows photograph of DNA-bands of the ACM and the composts sampled at the peaks 1, 2 and 3 containing ACM 5g and 0.5 as shown in **Figure 5**. In electrophoresis

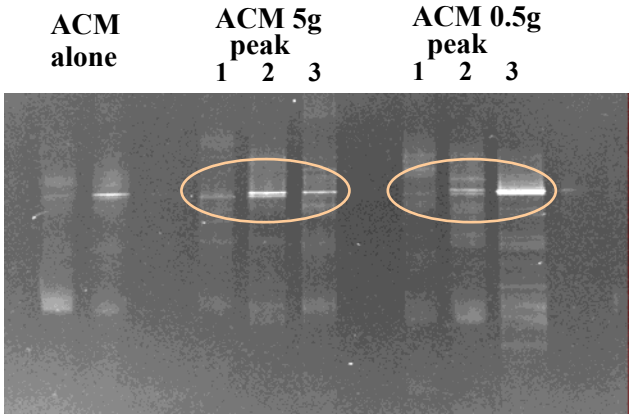


**Figure 9.** Photograph of DNA-bands of the ACM and the Composts Sampled at the Peak 1, 2 and 3 Containing ACM 5g, 0.5 g and 0 g

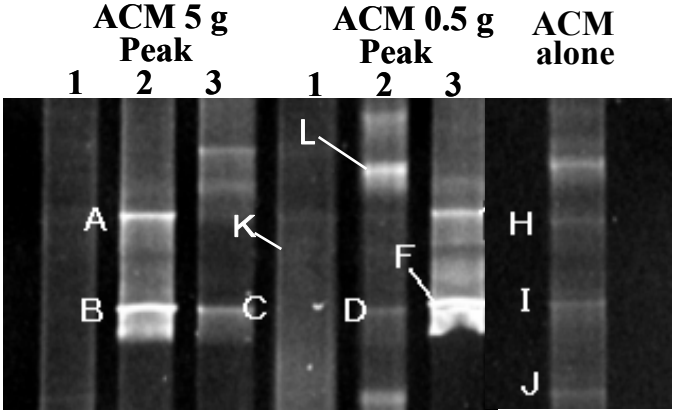


**Figure 10.** Photograph of DNA-bands of the ACM and the composts sampled at the peak 1, 2 and 3 containing ACM 5g and 0.5 g compared with ACM alone.

patterns of DNAs from the compost added with ACM 5 g and 0.5 g, the main band is separated into two sub-bands. In the DNAs sampled at the peak 1 of the compost with ACM 5 g, the lower sub-band is observed, and two sub-bands emerge at the peak 2. The upper sub-band is diminished at the peak 3. In the compost with ACM 0.5g, two sub-bands are observed at the peak 2 and 3. This suggests that ACM proliferation rate is dependent on addition amount of ACM.

**Figure 10** shows photograph of electrophoresis patterns of DNA-bands of the ACM and the composts sampled at the peaks 1, 2 and 3 containing ACM 5g and 0.5 g. Several bands are corresponding to the main bands of ACM alone. DGGE analysis result of the DNA in the extracted bands as follows; by cloning band A, deciding base sequence, and searching similarity, two kinds of microorganisms species, *Paenibacillus Sp.* and *Alcaligenes Sp.,* were recognized. In other bands, *Bacillus circulans* and *Bacillus cereus* were recognized. *Paenibacillus* genus is gram-positive, facultative anaerobic, rod shaped bacterium. *Alcaligenes* genus is gram-negative, aerobic, coccus or rod shaped bacterium, having non-polar flagella. The reason why those two genera usually isolated from cow feces were found in the systems comes from using compost made from cow feces and house garbage as a seed.

## Conclusions

Charcoal made from bamboo was mixed with rice bran as a nutrient. Aerobic complex microorganisms (ACM) for composting biomass waste were added to seed the mixture. As incubation-time of the mixed system went by, the ACM concentration increased. Three peaks in the ATP concentration vs. the incubation-time were observed. Two major bands of 16S rDNAs from ACM were amplified by PCR, and analyzed by DGGE. By cloning some bands, deciding base sequence, and searching similarity, two kinds of microorganisms species, *Paenibacillus Sp.* and *Alcaligenes Sp.,* were recognized. In other bands, *Bacillus circulans* and *Bacillus cereus* were recognized.

## Acknowledgements

This work was partly supported by the “Open Research Center” Project for Private Universities, with a matching fund subsidy from MIXT (Ministry of Education, Culture, Sports Science and Technology of Japan), 2004–2008.

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