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Inactivation mechanisms of pathogenic bacteria in several matrixes during composting process in composting toilet

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Abstract: *This study aimed to compare the inactivation rate and the mechanisms of pathogenic bacteria in three matrixes (sawdust, rice husk and charcoal) during composting process. The inactivation rate was evaluated with Escherichia coli strain and the damaged parts and/or functions were evaluated with three different media. Normalized inactivation rate constant in three media and from three matrixes had no significant difference in each process (pure, 1 month and 2 month). The value in rice husk was relatively increased during 2 month but there was no significant difference. The inactivation rate constants of TSA and C-EC in pure sawdust and rice husk were relatively lower than that of DESO, but increased in 2 month. This indicated that damaging part was changed from outer membrane to enzymes and metabolisms during 2 month composting process. In the case of charcoal, only the TSA value in pure matrix was relatively lower than that of others, but it increased in 2 month. This indicated that damaging part was changed from outer membrane and enzyme to metabolisms during the composting process. Composting matrix and composting process did not significantly affect inactivation rate of pathogenic bacteria during process but affected damaging part of the bacteria.*

Key words: *alkaline pH, composting matrixes, composting toilet, inactivation mechanism, pathogenic bacteria*

Introduction

World-wide, 2.6 billion people are estimated to defecate in open or unsanitary places (WHO and UNICEF, 2010). The lack of adequate sanitary installations and proper sanitary waste disposal systems leads to serious implications on human and environment health (Schaefer, 2008). Improved sanitation has become an issue global importance, highlighted by the UN Millennium Development Goal (MDG) who target to 'halve by 2015 the proportion of people without access to basic sanitation'. However, one speculation has shown a difficulty of the MDG achievement in developing countries (WHO and UNICEF, 2010). In order to overcome the difficulty, small foot printed and resource recycling based sanitation system such as On-site Wastewater Differentiable Treatment System (OWDTS) (Lopez *et al*, 2002a) and Ecological Sanitation (Esrey, 1998) has been proposed. In these concepts, wastewater from a household is fractioned to faeces, urine and grey water to reuse as agricultural materials after treatment with simple facilities.

A composting toilet, which is one of a key technology of the OWDTS, has many advantages from the viewpoint of preserving water resources, allowing nutrient recycling (Winblad *et al*, 2004) and introducing with inexpensive investment (Ushijima *et al*, 2011). Taking these advantages, the practical application has been attempted in rural Japan under mesothermal climate (Ito *et al*, 2006), urban slum in Indonesia Under tropical rainy climate climate (Ushijima *et al*, 2007) and rural in Burkina Faso under tropical dry climate (Ushijima *et al*, 2012). In the composting toilet, sawdust has been frequently used as composting matrix. The matrix plays a role of giving gas phase for aerial fecal decomposition with little odor (Otaki *et al*, 2007). Besides, this aerobic decomposition subsequently raises temperature in the compost heap and the bio-generated heat accelerates evaporation of water derived from feces. To adapt the toilet all over the world, alternative matrix is necessary because of limitation of sawdust availability. It has been reported that chopped corn stalk, rice husk and charcoal, which were regarded as a model of the alternative matrix, showed well fecal decomposition rate in the composting toilet (Hijikata *et al*, 2011a) and their composts promoted vegetable growth (Hijikata *et al*, 2011b). However, hygienic aspect in these alternative matrixes has not been observed, so far.

A used matrix in the composting toilet has a potential to trap pathogens derived from infected persons (Sossou *et al*, 2011), which raises the possibility for other users or farmers to become infected (Otaki *et al*, 2007). Therefore, care must be taken when handling the used matrix. In the case of pathogenic bacteria, their inactivation is affected by temperature, moisture content and pH in the used matrix (Redlinger *et al*, 2001; Kazama and Otaki, 2011). Furthermore, pure matrix itself contains various anti-bacterial substrates such as polyphenols, phenolic substrates and quinones (Lynch, 1993). These physical and chemical parameters might differ from matrix to matrix and stage of composting process. Therefore, we hypothesized that inactivation and its mechanism of pathogenic bacteria would differ with the type of matrix and the composting process. To investigate the hypothesis, inactivation rate and estimated damage part of pathogenic bacteria in three different matrixes, which are sawdust, rice husk and charcoal from rice husk, during composting process were compared in the present study. For the investigation of bacteria, *E. coli* was used as an indicator of fecal pathogenic bacteria. Three types of media were simultaneously studied to estimate the damaging part of *E. coli*, followed by a report of Kazama and Otaki (2011).

Methods

Compost

Rice husk and charcoal were used as an alternative matrix of sawdust in composting toilet. In the present study, pig feces were used because its characteristic was similar to human feces (Lopez *et al*., 2002b). The feces were continuously input into matrixes in composting machines (Hitach, Ltd. BGD-120, Kinbhoshipuro GN-120) every weekday and continued the input for 1 month and 2 months. The pure matrix or compost pH was measured after mixing with deionized water at the ratio of 1:20 (w: v).

Bacteria incubation and sampling

Escherichia coli (NBRC 3301) was used as a model microorganism of pathogenic bacteria. *E.coli* incubation was done in a growth medium Tryptic Soy Broth (Difco) and incubated in a shaking water bath at 37°C overnight. This *E. coli* suspension was used as a source of inoculums. For sample preparation an adequate aliquot of *E.coli* (0.3 mL about 10^8 CFU/mL) was inoculated to 3 g of autoclaved pig feces and 50 g of sterilized pure matrix or compost. The inoculated mixture was incubated at 37°C after adjusted moisture content at 50% with sterilized deionized water. The mixture was sampled at adequate time (0-8 hours after *E. coli* inoculation).

Bacteria extraction and measurement

The bacteria were extracted from the pure matrix or compost using a 3% (w/v) beef extraction solution (Kazama and Otaki, 2011). Beef extract (MP Biomedicals) was dissolved in deionized water, adjusted to pH 9.6 with NaOH, and sterilized. Three g of the pure matrix or compost sample was added to a 20 mL volume of extraction solution and agitated for 3 minutes to extract microorganisms. After adequate dilution (10-10^4 times) with phosphate buffer, each extracts were inoculated in three types of agar media which were commonly used for *E. coli* detecting: Tryptic Soy Agar (TSA) (Difco), Desoxycholate Agar (DESO) (Eiken Chemical Co., Japan), and X-Gluc and Magenta-GAL (C-EC) (Compact Dry EC, Nissui pharmaceutical Co., Japan). These inoculated media were incubated at 37°C for one day, and then, *E. coli* colonies were counted.

Inactivation rate

In activation rate of *E. coli* was separately calculated with result of colony count on three types of media. According to previous studies (Nakagawa *et al*., 2006; Otaki *et al*., 2007), the inactivation of microorganisms followed a first order reaction, expressed as follows: $\ln(N/N_0) = -kt$. N= concentration of microorganism at time t; N_0 = concentration of microorganism at time 0; k= inactivation rate constant; t= retention time.

Bacteria damage estimation

In order to estimate the inactivation mechanism of *E.coli*, 3 types of media were used. According to their detection principles, the damage to *E.coli* can be assumed as shown in Table 1 (Kazama and Otaki, 2011).

Table 1 here

Using TSA a non selective agar, the *E coli* which can metabolize proteins (casein and soy bean) and grow, can be detected. Therefore, when the *E.coli* growth cannot be detected on TSA, it assumed that its nucleic acid and/or its metabolic function has been damaged. DESO, a selective agar selects for *E.coli* that can grow by metabolizing lactose in the presence of desoxycholic acid because they lack an outer membrane and their growth is inhibited by its surface-active effects. Therefore, when *E. coli* cannot be detected on DESO, this indicates that its membrane outer membrane and/or, its nucleic acid and/or its metabolic function has been damaged. C-EC, a selective agar, selects for *E coli* that produce beta-glucuronidase (the enzyme involved in the metabolism of peptone, pyruvic acid and lactose). Therefore, when *E. coli* cannot be detected on C-EC, it is assumed that its enzyme activity and/or, its nucleic acid and/or its metabolic function have been damaged. By comparing the degree of inactivation on each medium, the damage part of *E. coli* could be estimated.

Statistical analysis

Analysis of variance (ANOVA) test was applied for data analysis using the StatView software version 5.0 (SAS Institute Inc., Cary, NC, USA).

Results

Inactivation rate of *E. coli* in three matrixes

Figure 1 shows the inactivation rate of *E. coli* in three matrixes (sawdust, rice husk, charcoal) during composting period (pure, one month and two months). The inactivation rate of *E. coli* in sawdust increased from pure matrix (- 2.2 to -1.8) to one month compost but this decreased in rice husk (-1.2 to -2.6) and charcoal (-1.4 to -1.6). This change of inactivation rate of *E. coli* was reversed in 2 month compost reaching - 2.2; -2.4 and -1.6 for sawdust, rice husk, charcoal respectively.

Figure 1 here

Normalized inactivation rate constant

Figure 2 shows normalized inactivation rate constant of the three matrixes in 2 months composting. This was calculated from the average of inactivation rate constant in three media to compare the inactivation degree. The result shows that, the inactivation rate constant was varied in each pure matrix [0.11; 0.17] h^{-1}, compost for one month [0,12 ; 0,23] h^{-1} and compost for two months [0.15 ; 0.24] h^{-1}. There was no significant difference in the three matrixes (sawdust, rice husk, charcoal) during each process (pure, 1 month and 2 months). On the other hand, the value in rice husk had relatively increased (from 0.11 to 0.24 h^{-1}) during 2 months but no significant difference (ANOVA, p=0.07) was also observed.

Figure 2 here

Mechanism of damaging part of *E. coli* during matrix change

Table 2 shows the estimated damage of *E. coli* based on relative comparison of detection by three media. The damage was estimated using the difference of the inactivation rate constant by each media in figure 3. In the comparison among types of matrixes, the value of TSA and C-EC in pure sawdust and pure rice husk were relatively lower than that of

DESO, and the value of TSA in pure charcoal was relatively lower than that of DESO and C-EC. This indicated that damage part in pure sawdust and pure rice husk was outer membrane but in pure charcoal was outer membrane and /or enzyme activity. This estimation suggested that pure charcoal made *E. coli* more lethal damage than pure sawdust and pure rice husk. In the comparison during composting process, on the other hand, the value of C-EC in pure sawdust [0.17] and pure rice husk [0.07] increased in 2 months compost and TSA value in 2 months compost of sawdust and rice husk was relatively lower than that of C-EC and DESO. This indicated that damaging part changed from outer membrane to enzymes and/or metabolisms. In the case of charcoal, TSA value [0.03] in pure matrix is lower than that of DESO [0.18] and C-EC [0.19], but the value increased in 2 months compost. This indicated that damaging part changed from outer membrane and enzymes into nucleic acid and/or metabolism.

Table 2 here
Figure 3 here

Mechanism of damaging part of *E. coli* during pH change

The pH in each matrix (sawdust, rice husk, charcoal) during composting process (figure 4) was varied and increased into alkaline zone from pure to 2 months. Pure charcoal pH was 8.32, originally higher than pure sawdust pH (7.35) and pure rice husk pH (6.34). Composted matrix pH was higher than pure matrixes and the value was [9.08-9.71].

Figure 4 here

Discussion

Inactivation rate constant of *E. coli* had no significant difference among sawdust, rice husk and charcoal during composting process (pure, one month and two months compost). It has been known that indigenous microflora of compost destroyed pathogenic bacteria (Pietronave *et al*, 2004). Therefore, the value of inactivation rate constant in the present study might be underestimate, since we used sterilized compost to measure *E. coli* on non-selective TSA media. Compared to a previous report with the same method (Kazama and Otaki, 2011), the values were similar to that of the report, which was 0.2 - 0.25 in sawdust compost at 37°C and 50% moisture content. This similarity implied our measurement was successfully conducted. In this present study, therefore, the comparisons in the same experimental condition let us conclude that the kinetic inactivation of pathogenic bacteria is same among the three matrixes and during composting process. However, the previous study also showed that low moisture content, high temperature and alkaline treatment caused the inactivation more significantly. Therefore, adequate inactivation treatment should be done in any type of matrixes, when the composted matrix is exchanged from the composting toilet. The way of inactivation treatment for composting toilet should be further investigated from the viewpoint of risk assessment.

On the other hand, estimated damage part was different among types of matrixes and composting process. The present results showed that pure charcoal led the bacteria to more lethal damage than pure sawdust and pure rice husk. Furthermore, composted sawdust and rice husk led the bacteria to more lethal damage than those of pure matrix. Some research works have observed re-growth of enterococci in composting toilet (Sossou *et al*, 2011)

and *E. coli*, fecal coliform and *Enterococcus faecalis* in source-separated household waste compost (Christensen *et al*, 2002). The re-growth is considered to be caused by un-lethal damage of the bacteria. Therefore, present results suggested that adequate composting process led the pure matrix to more secure sanitation, since composted matrix showed more lethal damage than that of pure.

One reason of different damage of *E. coli* would be different pH conditions in matrixes and composts. Kazama and Otaki (2011) has reported that high pH led the pathogenic bacteria to more lethal damage. Followed by this report, pH of pure charcoal was higher than that of pure sawdust and rice husk, and pH of composted sawdust and rice husk were also higher than that of pure matrixes, in the present study. It has been known that the pure charcoal has an originaly high pH because mineral is remained on its surface after carbonization (Blackwell *et al*, 2009). In the composting process, nitrogen derived from feces is ammonificated (Hotta and Funamizu, 2007) and this ammonification increases the compost pH. Anearobical fecal decomposition, which is caused by inadequate air gap or too much moisture content, leads to organic acid production and this would reduce the compost pH. Therefore, choosing adequate matrix and remaining optimal condition for aerobical fecal decomposition might be essential from the viewpoint of hygienic aspect.

In this study, we investigated only *E coli* as a model of pathogenic bacteria. Other pathogens, especially virus, those in form of spore or eggs may be less affected by inactivation in composting process. Therefore, inactivation of those more resistant pathogens must be considered.

Conclusion

This study was undertaken to compare the inactivation rate and the mechanisms of pathogenic bacteria in three different matrixes during composting process.
The results suggested that composting matrixes (sawdust, rice husk and charcoal) and composting process did not significantly affect inactivation rate of pathogenic bacteria, however, these differences affected damaging part of the bacteria. Composting process, accompanied with pH increase, changed the damage part of bacteria more lethally.
This result could help to choose matrix and time from the viewpoint of hygienic aspect in composting toilet.

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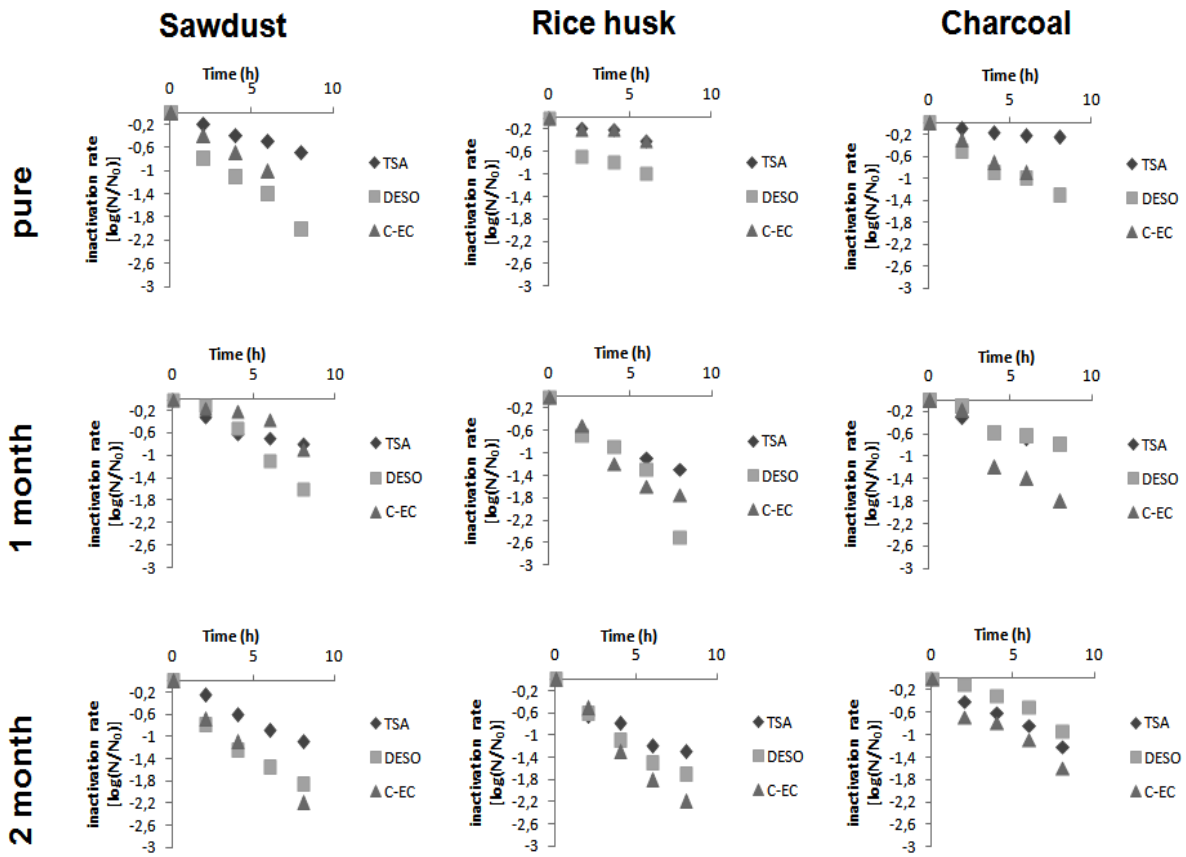

Figure 1- Normalized inactivation rate constant of three matrixes

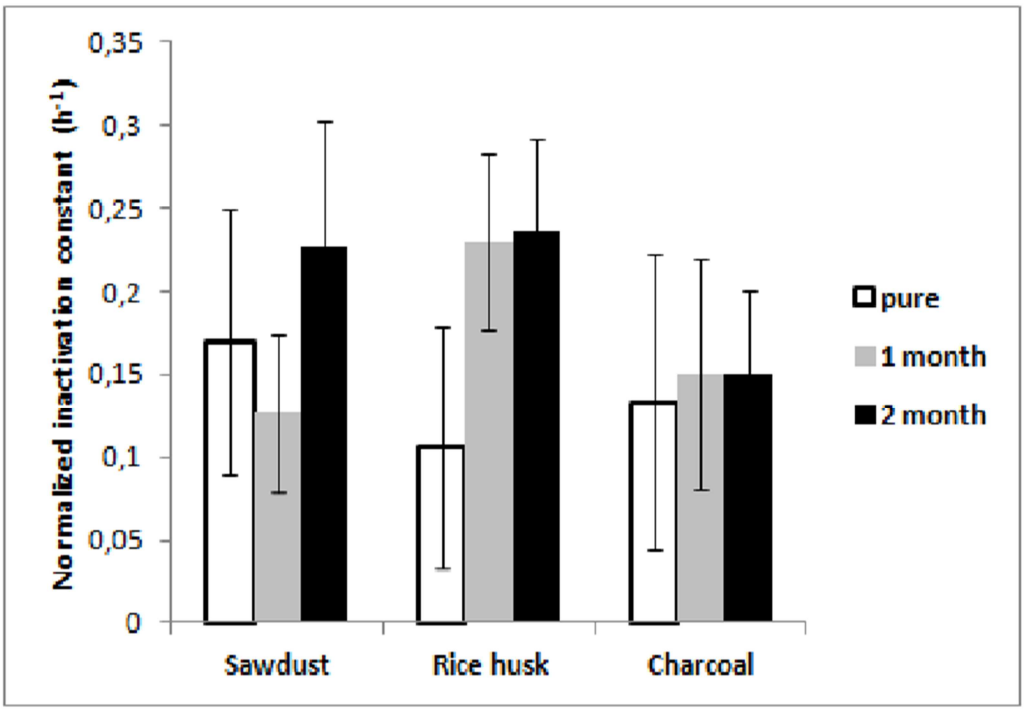

Figure 2- Normalized inactivation rate constant of three matrixes

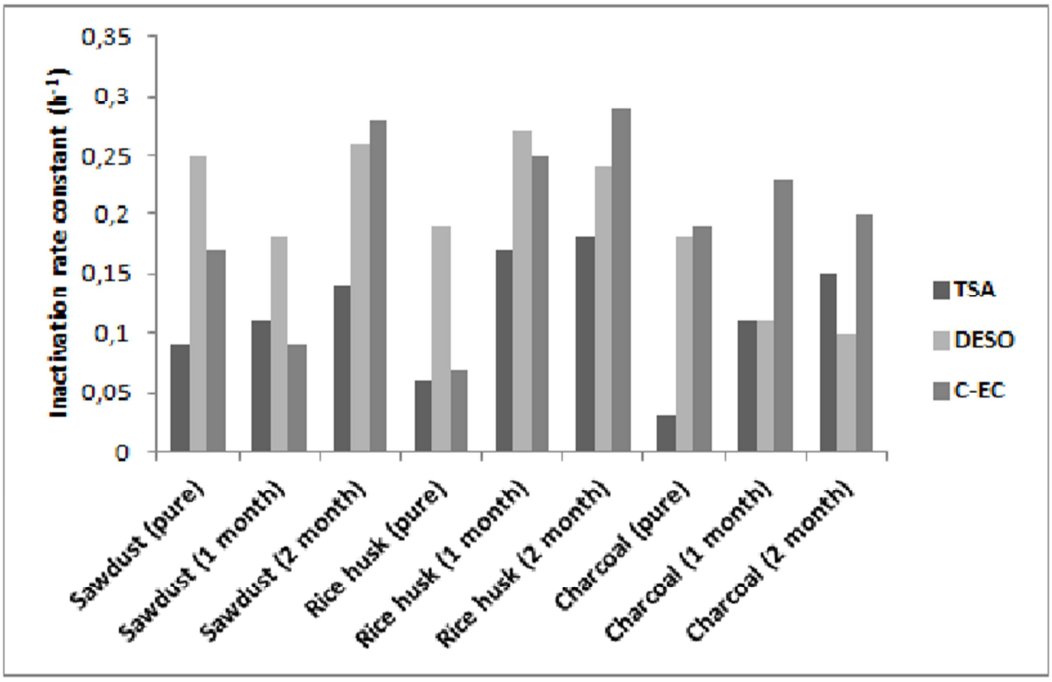

Figure 3- Inactivation rate constants by each media in different matrixes

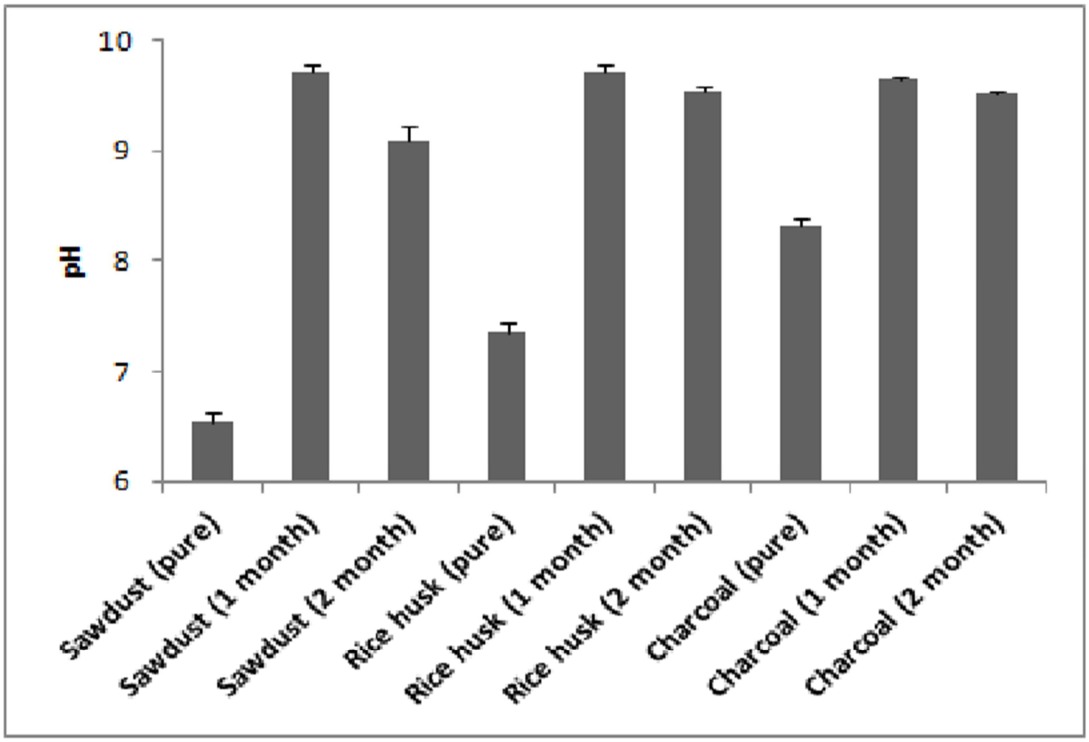

Figure 4- pH in each matrix (sawdust, rice husk, charcoal) during composting process

Table 1- Assumed damages on *E. coli* which result in undetection for each medium (Summarized by Kazama and Otaki, 2011)

Media	Damages assumed
TSA	Nucleic acid and/or Metabolism
DESO	Membrane and/or Nucleic acid and/or Metabolism
C-EC	Enzyme activity and/or Nucleic acid and/or Metabolism

Table 2- Estimated parts of *E. coli* damage according to the detection differences among the three media (Summarized by Kazama and Otaki, 2011)

Media			Estimated parts which were damaged
TSA	DESO	C-EC	
X	X	X	➔ Nucleic acid and/or metabolism
O	O	O	➔ Enzyme activity
O	X	O	➔ Membrane
O	X	X	➔ Membrane and/or enzyme activity