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Research article

Bactericidal and virucidal mechanisms in the alkaline disinfection of compost using calcium lime and ash

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ABSTRACT

In the present study, the bactericidal and virucidal mechanisms in the alkaline disinfection of compost with calcium lime and ash were investigated. Two indicator microorganisms, *Escherichia coli* and MS2 coliphage, were used as surrogates for enteric pathogens. The alkaline-treated compost with calcium oxide (CaO) or ash resulted primarily in damage to the outer membrane and enzyme activities of *E. coli*. The alkaline treatment of compost also led to the infectivity loss of the coliphage because of the partial capsid damage and RNA exteriorization due to a raised pH, which is proportional to the amount of alkaline agents added. These results indicate that the alkaline treatment of compost using calcium oxide and ash is effective and can contribute to the safe usage of compost from a mixing type dry toilet.

1. Introduction

A mixing type composting toilet using an organic matrix is one of the established concepts of dry toilet. The mixture of human feces with organic matrix enhances aerobic degradation of feces with little odor, decreases the fecal volume by water evaporation, and contributes to internal heating generated by the activity of microorganisms. The dry toilet has been recognized as an improved sanitation facility (WHO and UNICEF, 2008) and has advantages for reducing the amount of water usage. It can be installed quickly and it saves public investment, since it does not require large-scale infrastructure such as a water distribution network and sewer piping system (Esrey et al., 1998; Lopez et al., 2002a). Furthermore, compost produced in a dry toilet promotes plant growth by providing nutrients and conditioning soil properties (Hijikata et al., 2011). These characteristics related to installability, usefulness, and nutrient usability are attractive for low-income countries, and rural areas, natural parks and emergency evacuation sites in developed countries, which often have a lack of improved sanitation facilities, incomplete water and sewer services, a restriction on wastewater discharge, and high demand for fertilizers.

From a hygienic perspective, feces should always be considered to contain pathogens that cause gastrointestinal infections. Although the composting process in dry toilets reduces concentrations of indicator microorganisms and pathogens to some extent (Sossou et al., 2011, 2014), the compost recovered from a dry toilet always has the potential to include pathogens derived from feces of infected persons, which pose infectious disease risks for users (Otaki et al., 2007). The highest infection risk most likely is posed during emptying the compost and exchanging the matrix (Nakagawa et al., 2006; Schönning et al., 2007). Potential hazard risks when the compost is removed from a composing toilet are caused by direct and/or indirect ingestion of enteric pathogens originating from human feces, which include bacteria, viruses, parasitic protozoa and helminthes (WHO, 2006). These pathogens may cause diarrhea, fever and cramps. According to the global burden of diseases study, diarrhea accounts for 89.5 million disability-adjusted life years (DALYs) and is still a large contributor to the burden, accounting for 3.6% of the global DALYs in 2010 (Murray et al., 2012). It is thus very critical to manage the infection risks of enteric pathogens associated with the use of composting

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toilets.

WHO (2006) and Schönning et al. (2007) have recommended storing mature fecal matter for 6–12 months to assure safety during handling. The recommendation mainly targets at pit-type dry toilets such as the urine-diversion dry toilet (UDDT), which has a double storage system. It may be thus difficult to apply this recommendation to the mixing type composting toilets as it is, because of their structure comprised of a single reactor. Kazama and Otaki (2011) showed that alkaline-treated compost with calcium lime was effective for the rapid inactivation of bacteria and virus indicators in sawdust used in a composting toilet. Lime and ash are often used in practical and scientific reports about the alkaline treatment of the UDDT, and the potential for pathogen inactivation has been described (Nordin et al., 2009a; Niwagaba et al., 2009). However, bactericidal and virucidal mechanisms in the alkaline disinfection of compost from a mixing type composting toilet have not been well investigated.

In the present study, the inactivation mechanisms of surrogate microorganisms (*Escherichia coli* and MS2 coliphage) in compost treated with varied quantities of alkaline agents was investigated. Three types of growth medium were used for cultivating *E. coli* from the alkaline-treated compost, which allows us to identify the most affected physiological function of the surrogate for the bacterial pathogen. The plaque assay and the enzymatic treatment using Protease K and RNase A coupled with reverse-transcription quantitative PCR (ET-RTqPCR) were employed to identify the effect of the alkaline treatment on the infectivity of MS2 coliphage. Based on the results from these disinfection tests, the efficacy of alkaline disinfection of compost was discussed.

2. Materials and methods

2.1. Compost preparation

Rice husks were used as a compost matrix and pig feces were used as a substitute for human feces since the characteristics of pig fecal matter are similar to those of human feces (Lopez et al., 2002b). Fresh pig feces (500–600 g) were mixed into 20 L (1429g-dry, approximately a half volume of a general composting toilet reactor for one family) of the matrix in a composting machine (Hitachi, BGD-120). Fresh pig feces (500–600 g) were put into the matrix in the composting machine everyday up to 38th day. At the end of the operation, the fecal load ratio (total input of feces [g-dry] per initial matrix [g-dry]) was 1.71, the fecal degradation (loss of the mass) ratio was 45.8%, and the water content was 48.5%, which is comparable with compost from a mixing type dry toilet. The obtained compost was stored in refrigerated room at 4 °C. The preparation process of the compost was depicted in Fig. 1S.

2.2. Test microorganisms

E. coli NBRC 3301 and MS2 coliphage were used as a surrogate for pathogenic bacteria and viruses, respectively. *E. coli* NBRC 3301 was incubated with 10 mL of 4% (w/w) of Tryptic Soy Broth (Difco Laboratory Inc., USA) in a shaking water bath at 37 °C for 4–6 h, and then the cultured *E. coli* was used as an inoculum for inactivation tests. *E. coli* NBRC 13965 was used as a host for MS2 coliphage. MS2 coliphage was co-incubated with precultured *E. coli* NBRC 139651 in 10 mL of liquid LB medium in the shaking water bath at 37 °C for 20–24 h. The culture was centrifuged at 6000 rpm for 10 min at 4 °C and the supernatant was filtered with a sterilized disposal filter (0.20 μm pore, DISMIC-25CS, ADVANTEC). The filtered liquid was used as an MS2 coliphage inoculum for inactivation tests.

2.3. Inactivation test of indicator microorganisms in the composting toilet

Calcium lime (CaO, Wako chemical, Japan) was used as the calcium oxide reagent, according to the previous study (Kazama and Otaki, 2011). Three types of ash, including wood ash, grass ash made from rice straws, and mixed ash (a mixture of wood grasses, crushed oyster shell and wood ash obtained from an Italian restaurant in Sapporo City, Japan) were also used as alkaline agents. The volatile solid [%, w/w] of these alkaline agents was obtained from a loss of weight by the incineration at 600 °C for 3 h. The chemical compositions of ash are indicated in Table 1.

Stored compost (20 g) was transferred to a glass bottle and autoclaved at 121 °C for 15 min, and then the water content was adjusted to 50% using sterilized deionized water to uniform the water content condition in the inactivation tests. After pre-incubation of the water content-adjusted compost at 37 °C for 1 h, an adequate amount of CaO or ash and 1 g of autoclaved pig feces were simultaneously added and mixed in the bottle, which can create a sterile but very similar condition with actual compost from a mixing type dry toilet. The pH value of compost mixture was measured by an electrode method using a suspension of compost mixture and pure water at a ratio of 1:20 (w:w), which was shaken for 30 min. The pH of compost mixture was adjusted to pH 9.5, 10.0, 10.5 and 11 by adding the alkaline agents. *E. coli* inoculum (0.5 mL, approximately 10^8 CFU/mL) was spiked into the compost mixture and shaken by hand for 1 min, which gave the experimentally highest *E. coli* concentration, enabling us to follow the inactivation profile very efficiently. The compost mixture was incubated at 37 °C, the optimum temperature for the *E. coli* growth, which may attenuate the disinfection efficiency of alkaline treatments. One gram of the compost mixture was sampled in an appropriate time (0–8 h) and *E. coli* was extracted by suspending it in 40 mL of 3% (w/v) beef extraction solution adjusted to pH 9.5 with an NaOH solution (Otaki et al., 2002). The previous study reported that the total recovery efficiency using this method is 70–100% (Otaki et al., 2002). After dilution (10^1–10^4 folds) with an autoclaved phosphate buffer (pH 7.5 adjusted with the NaOH solution), 1 mL of each extract was separately inoculated on three types of agar media: Tryptic Soy Agar (TSA, Difco Laboratory Inc., USA), Desooxycholate Agar (DESO, Eiken Kagaku Inc., Japan), and Compact Dry EC (C-EC, Nissui Inc., Japan). TSA is a non-selective agar that contains two peptones (casein and soy) as nutrition for microorganisms. The agar can isolate and cultivate a wide variety of organisms that metabolize the peptones and grow. DESO is a selective agar that inhibits to grow gram-positive bacteria. The agar contains general growth requirements with sodium desoxycholate, sodium citrates, lactose, and neutral red. Sodium desoxycholate, a surfactant, lyses gram-positive bacteria because they do not have outer membrane unlike gram-negative bacteria. Bacteria that ferment lactose produce acid and form red colonies in the presence of neutral red. Bacteria that do not ferment lactose form colorless colonies. C-EC, a selective agar for *E. coli* and coliform, includes general growth requirements with two chromogenic substrates: X-GLUC and Magenta-GAL. The chromogenic substrates are hydrolyzed with enzymes: β-glucuronidase and β-galactosidase that *E. coli* produces during fermenting lactose and make colonies blue or blue purple color. These agar media were incubated at 37 °C for 24 h and the colonies formed were counted.

The alkaline-treated compost identical with that used in the *E. coli* inactivation test was also prepared for the inactivation test of MS2 coliphage. Five milliliters of MS2 coliphage inoculum were spiked into 20 g of the compost and incubated at 37 °C. One gram of the incubated compost mixture was sampled in an appropriate time (0–8 h) and suspended in 40 mL of the autoclaved phosphate