



## Original Research Article

### Reduction of microorganisms in thermophilic process of anaerobic digestion of cattle manure

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#### ABSTRACT

#### Keywords

Anaerobic digestion; thermophilic; mesophilic regime; test bacteria; decontamination

The changes in the quantities of microorganisms during continuous thermophilic anaerobic digestion of cattle manure at  $45 \pm 1,5^\circ\text{C}$  were studied. Test strains (TS) of *Pseudomonas aeruginosa* and *Staphylococcus epidermidis*, resistant to antibiotics from the groups of amphenicols and tetracyclines, were used. They were introduced in the material in quantities by  $10^6$  CFU/ml of its content. After 5 days TS of *P. aeruginosa* disappeared in the bioslime. By the 55th day of the experiments *Escherichia coli* disappeared too, and the amounts of the TS of *S. epidermidis* and of the other microorganisms monitored, which include pathogenic species such as coliforms, *Enterococcus* sp., *Clostridium perfringens* and *S. epidermidis* decreased to minimum values of  $10^2$ - $10^3$  CFU/ml. Similar amendments were established and in parallel conducted analogous experiment mesophilic process of anaerobic digestion of the same manure, but to a lesser degree.

#### Introduction

One of the important criteria for the ecological assessment of the effectiveness of anaerobic digestion of organic matter is the degree of microbial decontamination, as measured by the number of sanitary indicator microorganisms. Given the relevance of the problem related to the possibility to limit the spread of zoonoses a number of studies in this area have been made, including by use of test microorganisms (Popova *et al.*, 2007;

Petkov *et al.*, 2008). Results show decontamination in respect of certain basic groups of bacteria when the amount of them in the starting material is not very high (up to  $10^6$ CFU/ml). Studies of other groups (Philipp *et al.*, 2005, Gannoun *et al.* 2009) suggest that some pathogens remain somewhat in the final bioslime. One of the methods for sure decontamination of the final product is the thermophilic regime of anaerobic

digestion, which occurs in 54-55°C. Its use for decreasing of pathogenic microorganisms is more successful in comparison with the mesophilic regime of anaerobic digestion (Philipp *et al.*, 2005; Sahlström *et al.*, 2005; Popova *et al.*, 2010). Furthermore, that regime ensures most quantity of biogas with better quality in comparison with the mesophilic process (Vindis *et al.*, 2009; Gannoun *et al.*, 2009). Its disadvantage, however, is the fact that it consumes more energy for supporting of higher temperature. The knowledge about the survival of pathogenic bacteria like *E. coli*, *Salmonella enterica* and others in organic fertilizer and their heat resistance can serve to estimate risks in use of these manures on agricultural areas and optimize biotechnological treatments (Eling *et al.*, 2013).

Since maintaining a high temperature of about 55°C during the anaerobic degradation in thermophilic regime leads to significant energy consumption, the paper is aimed at assessing the microbiological decontamination of the final product obtained by the continuous thermophilic process of anaerobic digestion at 45°C - more lower temperature than normally used. Our purpose was to establish the surviving abilities of sanitary indicative microorganisms in cattle manure, and of pathogenic test-bacteria, introduced in materials undergoing at continuous thermophilic (at 45°C) and mesophilic (33°C) processes of anaerobic digestion with a view to evaluate the possibilities for decontamination of the final products.

## Materials and Methods

### Cattle manure

Fresh manure litter from dairy cows was examined.

### Microorganisms

Pure cultures of two pathogenic bacterial test strains were used in the investigations: *Pseudomonas aeruginosa* and *Staphylococcus epidermidis*. They were isolated from dogs with chronic infections and were selected by their poly-resistance *in vitro* to Gentamicin and tetracyclines (Tetracycline, Doxycycline and Oxytetracycline). An additional cultivation of these strains on nutrient media with antibiotics from these groups was performed to isolate and use in studies of branches, the most thriving in the presence of high concentrations of these antibiotics.

Nutrient media (Antisel, Sharlau Chemie S. A., Spain). Selective nutrient media with added together doxycycline (50 µg/ml) and Gentamicin (16 µg/ml) were used for isolation and cultivation of the test bacteria. Cetrimide agar for *P. aeruginosa* and Chapman Stone agar for *S. epidermidis* were selected. On these media, other bacteria were not grown, except the test strains selected by the resistance to the antibiotics pointed out. The total number of microorganisms in the studied materials was reported on Mueller Hinton agar without antibiotics. For isolation and quantitative determination of the microorganisms from the other studied groups the following media have been chosen: Eosin Methylene Blue agar for *E. coli* and Gram-negative aerobic bacteria (coliforms), Cetrimide agar for bacteria of the genus *Pseudomonas*, Chapman Stone agar for those of the genus *Staphylococcus*, Sabouraud agar for fungi, selective medium for enterococci, Salmonella-Shigella agar for *Salmonella enterica*. The content and quantities of *Clostridium perfringens* on selective agar (Merck-Bio Lab, Bulgaria) were also tracked.

## Anaerobic digestion

Anaerobic digestion was carried out using a plastic syringes with a volume of 20-50 ml, of the cone of which were placed a closing tubes, wherein the amount of gas is read directly on the scale on the syringe. These adapted by us laboratory equipments for modeling methane fermentation refer to the type of micro-ecosystem or microcosm according to the classification of Odum (1986). Anaerobic process occurs at continuous thermophilic regime at  $45\pm 1.0^{\circ}\text{C}$  and at continuous mesophilic temperature regime at  $33\pm 1.0^{\circ}\text{C}$ , 7 % dry substance, pH 6.2 and downtime in the thermostat up to 55 days. The test strains were introduced in the examined material each in concentration  $10^6$  CFU/ml. Samples from the bioslime were taken on 5<sup>th</sup>, 20<sup>th</sup>, 35<sup>th</sup> and 55<sup>th</sup> days.

## Quantitative determination

Quantitative determination of microorganisms was carried out using the classical method in serial (10 times) increasing dilutions of the examined materials in a sterile physiological solution. Cultures on the selected media were prepared from these dilutions, by three for each medium and dilution. After incubation at  $37^{\circ}\text{C}$  for 24–72 h under aerobic and anaerobic conditions (with Anaerocult<sup>®</sup> A mini – Merck-Bio Lab, Bulgaria), the mean arithmetical number of the developed colonies was calculated and the colony forming units (CFU) in 1 ml of the initial material were determined.

## Statistical analysis

Statistical analysis of the results was made using the classic method of Student-Fisher.

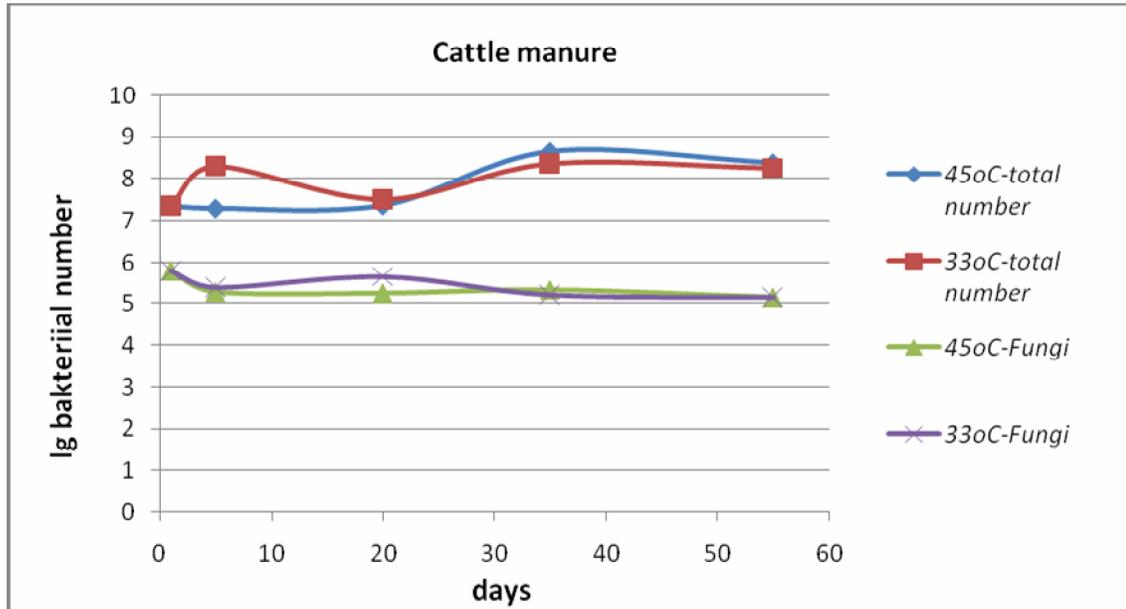
## Result and Discussion

The changes in the quantities of the total number of microorganisms and the quantitative changes of the fungi in the bioslime during the processes of anaerobic digestion at thermophilic and mesophilic conditions are presented in Figure. 1.

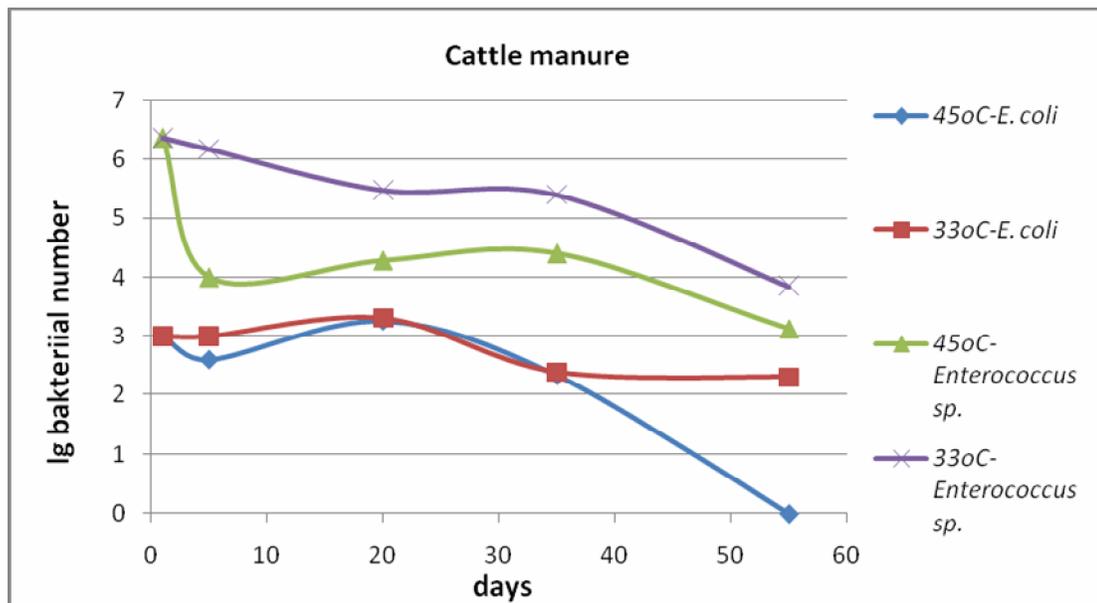
As seen from the graphs, the total number of microorganisms after decreasing around the 20th day slightly increased by 1 lg (about 10 times) in both monitored process of digestion ( $P < 0.001$ ). The fungi in both modes slightly decreased in quantities and up to 55th day were less with about 0.5 lg (five times),  $P < 0.01$ .

The dynamics of the basic sanitary indicative bacteria *E. coli* and *Enterococcus* sp. in manure at the thermophilic anaerobic mode compared to the mesophilic process is shown in Figure 2. The figure shows that, after a slight initial reduction *E. coli* marked a slight increase at the end of the third week of both processes, but in the thermophilic mode then gradually decreased and disappeared. During the mesophilic process after a slight reduction in the second half of the study this species was kept in a small amount ( $3 \cdot 10^2$  CFU/ml at the end of the process). The number of enterococci in the thermophilic mode was dropped significantly with over 2 lg (hundredfold) in the first week ( $P < 0.001$ ), then was preserved at the level of about  $10^4$  CFU/ml up to 20<sup>th</sup> day and after some increasing to 40<sup>th</sup> day, significantly decreased to  $10^3$  CFU/ml at the end of the process. In the mesophilic mode after small reduction in the second half of the study it remained at a low level ( $3 \cdot 10^2$  CFU/ml at the end of the process).

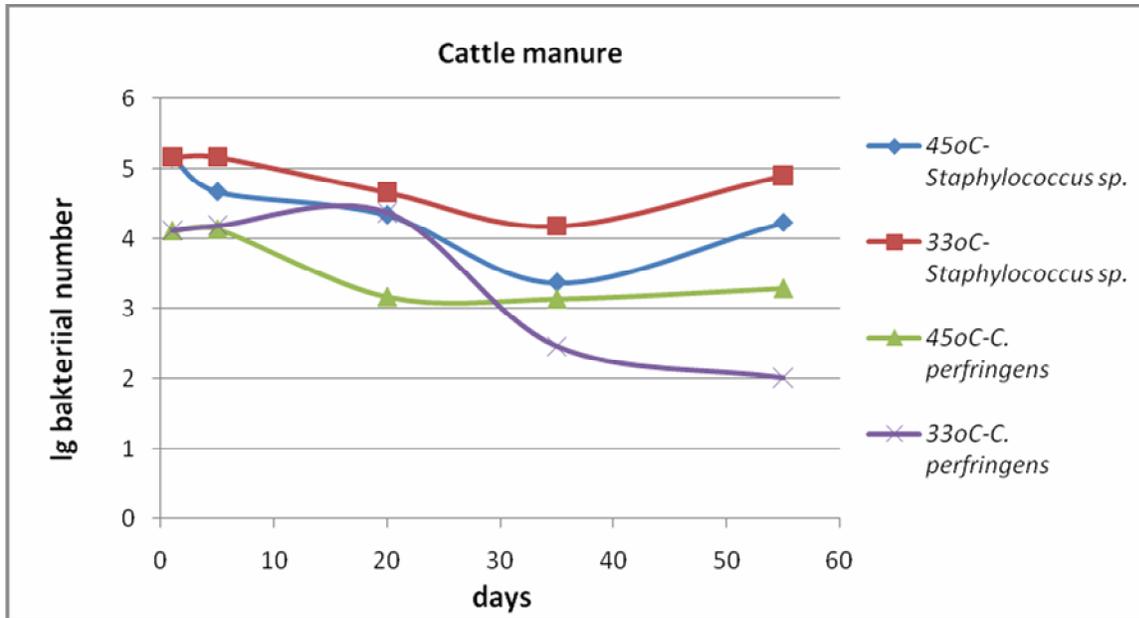
**Figure.1** Changes in the total number of microorganisms and fungi in cattle manure in a continuous thermophilic (45°C) and mesophilic (33°C) process of anaerobic digestion



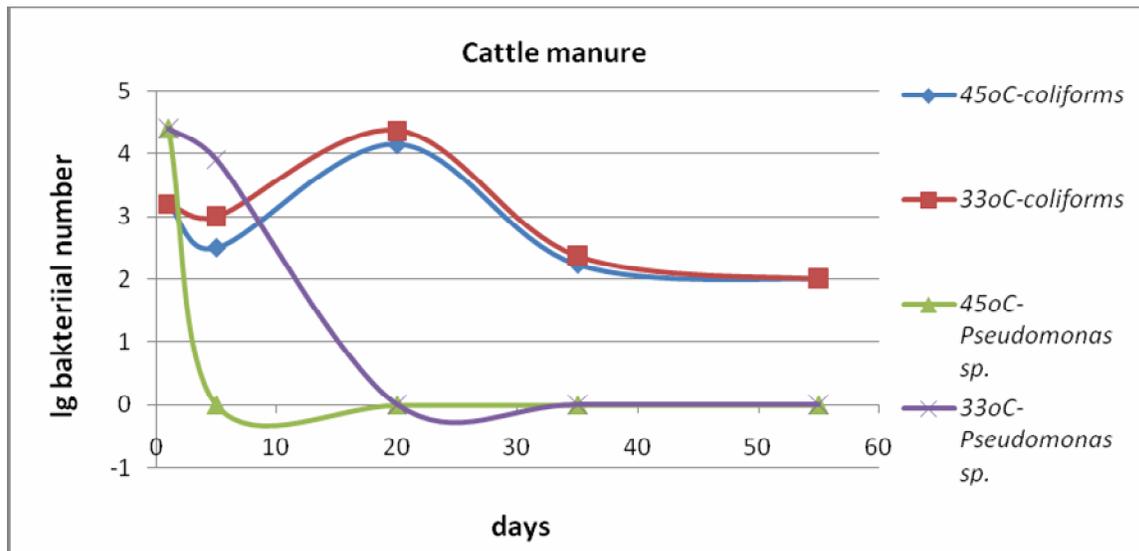
**Figure.2** Quantitative changes of *E. coli* and *Enterococcus* sp. in continuous thermophilic (45°C) and mesophilic (33°C) processes of anaerobic digestion



**Figure.3** Dynamics of *Clostridium perfringens* and species from the genus *Staphylococcus* in continuous thermophilic (45°C) and mesophilic (33°C) modes of anaerobic digestion



**Figure.4** Variations in quantities of coliforms and species of the genus *Pseudomonas* in continuous thermophilic (45°C) and mesophilic (33°C) anaerobic digestion process



**Figure.5** Dynamics of test bacteria in cattle manure during a continuous thermophilic (45°C) and mesophilic (33°C) modes of anaerobic digestion.

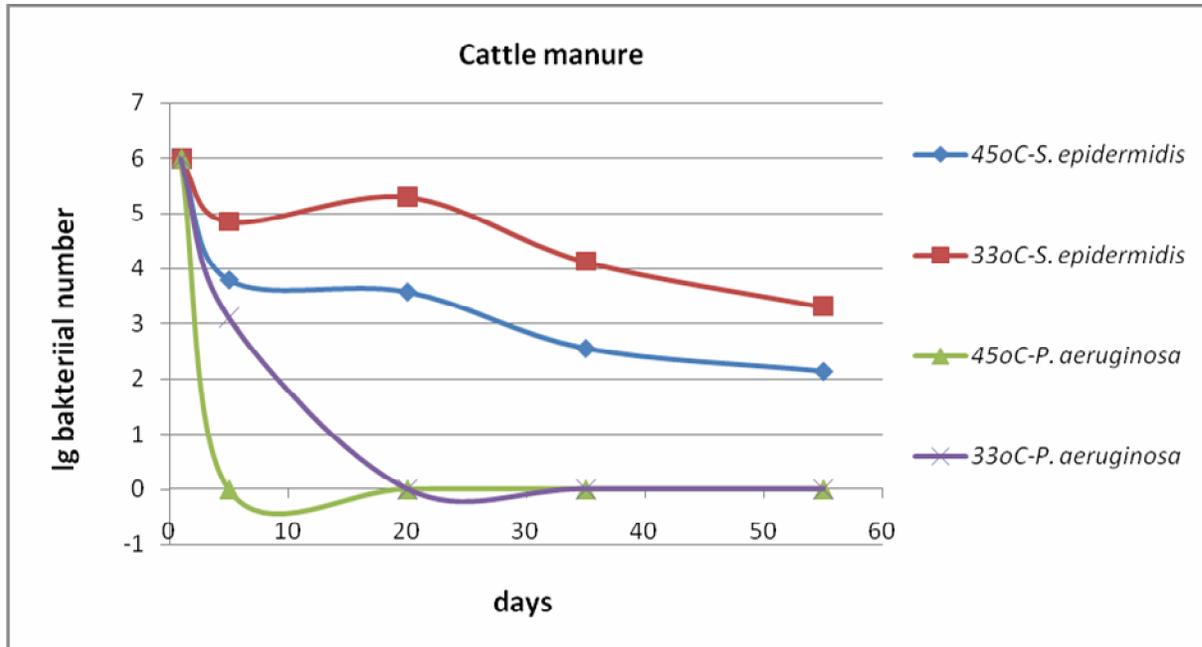


Figure 3 presents the changes in the quantities of the tested Gram-positive bacteria (staphylococci and *C. perfringens*) in both processes of anaerobic digestion. The graph shows the reduction of the quantities of staphylococci to the 35<sup>th</sup> day of anaerobic digestion, which was better expressed in the thermophilic process ( $P < 0.001$ ). Followed an increase in the two processes, but did not reach the baseline. *C. perfringens* decreased tenfold until the 20<sup>th</sup> day at the thermophilic process and to the end was retained in an amount of less than  $10^3$  CFU/ml ( $P < 0.05$  compared to baseline). In the mesophilic mode their number increases slightly during the first three weeks ( $P > 0.05$ ), but then substantially reduced to  $10^2$  CFU/ml at the end of the process ( $P < 0.001$  compared to the initial quantity).

Figure 4 shows the quantitative changes of the Gram-negative bacteria *Pseudomonas* sp. and the coliforms in the thermophilic anaerobic digestion process compared to the mesophilic. As can be seen from the graph, the amount of coliforms decreased during the first week, but over the next two weeks increased tenfold from baseline in both modes ( $P < 0.001$ ). However, a significant reduction of these bacteria followed, which at the end of both modes of anaerobic digestion were reduced to  $10^2$  CFU/ml ( $P < 0.001$  compared to baseline). More rapidly and significantly was the reduction of the bacteria of the genus *Pseudomonas*, which in thermophilic process were decreased below the detecting minimum yet during the first week, and at mesophilic - several days later.

This also applies to the test strain of *Pseudomonas aeruginosa*, which can be seen in Figure 5. On it are presented the changes in the quantities of the imported into manure test bacteria in both compared anaerobic modes. The introduced *Staphylococcus epidermidis* decreased more rapidly and significantly in the thermophilic anaerobic digestion process, reaching a level of  $2,14 \cdot 10^2$  CFU/ml at the end of the study, which is over 10 times lower than that in the mesophilic process ( $P < 0.001$ ).

The results of these studies show a significant decontamination in anaerobic digestion of cattle manure for 55 days, more pronounced at the thermophilic process, occurring at 45° C, compared to the mesophilic at 33° C. Especially indicative in this respect is the dynamics of monitored sanitary indicative microorganisms, as well as that of the used test bacteria. The decontamination at 45° C, however, was in a lesser extent than that in the thermophilic anaerobic digestion of sewage sludge at 55° C, observed in our previous studies (Popova *et al.*, 2010) when the test strains of the species *E. coli*, *P. aeruginosa* and *S. aureus* were killed within 24 h. Other authors also were found that many types of pathogenic bacteria such as fecal coliforms, *Salmonella* sp. and enterococci die at thermophilic anaerobic digestion of sewage sludge (Watanabe *et al.*, 1997). Wagner *et al.* (2008) reported that none of the studied by them species of pathogenic bacteria (*Listeria monocytogenes*, *Salmonella enterica*, *Escherichia coli* and *Campylobacter jejuni*) was not able to survive for more than 24 h in the sewage sludge in a thermophilic biogas reactor at 55° C, which indicates that the temperature and the physicochemical conditions effectively inhibit survival of mesophilic

microorganisms. According Sahlstrom (2003) *Salmonella* sp. and *Mycobacterium paratuberculosis* were inactivated for 24 h under conditions of thermophilic anaerobic digestion of organic materials, while at the mesophilic process for this were required weeks or even months. Obviously at 45° C can't be achieved a rapid and complete decontamination, as it occurs at 55° C, but leads to a substantial reduction of the quantity of microorganisms from most of the studied by us groups to minimum values of  $10^2$ - $10^3$  CFU/ml, and some as *E. coli* and *Pseudomonas* sp, including *P. aeruginosa*, disappear to the end of the thermophilic process. Undoubtedly anaerobic digestion at 45° C is more economical than at 55° C. However, when the manure comes from animals with infections, from the epidemiological point of view should be preferred its anaerobic digestion at 55° C.

In continuous mode of thermophilic anaerobic digestion at 45° C complete decontamination was achieved in respect to some important pathogenic bacteria such as *E. coli* and the species of the genus *Pseudomonas*, including *P. aeruginosa*. The other groups of bacteria, which include pathogenic species such as coliforms, *Enterococcus* sp., *Clostridium perfringens* and *Staphylococcus epidermidis* decreased to minimum values of  $10^2$ - $10^3$  CFU/ml.

In continuous mesophilic anaerobic digestion for 55 days was achieved a significant, but not complete decontamination of cattle manure in terms of pathogenic microorganisms. *Pseudomonas* sp., including *P. aeruginosa*, disappeared to the end of the third week from the start of the process, and other groups of bacteria including pathogenic species such as

coliforms, *E. coli*, *S. epidermidis*, and *C. perfringens* were reduced to minimum values of  $10^2$ - $10^3$  CFU/ml.

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