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ANAEROBIC DIGESTION IN SANITIZATION OF PIG SLURRY AND BIOMASS IN AGRICULTURAL BIOGAS PLANT

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ABSTRACT

Pig slurry is one of the production manure, which should be managed properly because of environmental threats it can cause. Pig slurry contains a wide range of microorganisms, most of which are opportunistic or obligatory pathogens for people and animals. Spreading it on fields without control can cause microbial contaminations of water and soil. Use of pig slurry as substrate in anaerobic digestion can be an effective way of sanitization. In this work role of methanogenic fermentation in pig slurry sanitization was laboratory examined. Study materials were biological samples: 1 sample of raw slurry and 3 samples of fermented biomass from different stages of fermentation. Total number of coliforms was determined by MPN (most probable number) method, and presence of enterococci was verified in each sample. Study have shown that anaerobic digestion reduced total number of coliforms from initial amount of 7.0 x 10^6 [MPN/ml] in raw slurry to 3.7 x 10^4 [MPN/ml]. Total reduction was 99.47%. Moreover, after first fermentation, enterococci in the sample were undetectable. Results of this study proved anaerobic fermentation to be an affective way to neutralize microbial threat, that is pig slurry.

Keywords: Anaerobic digestion, pig slurry, sanitization

INTRODUCTION

Nowadays, there is a constant need to increase food production due to still growing human population. It causes new techniques and methods of intensive agriculture and animal husbandry to be developed. This necessary progress causes an important problem, which is a huge amount of waste products, including pig slurry which is produced by pig farms.
Pig slurry is a liquid mixture of pig faeces and urine, which is produced by dilution of manure in livestock production process (**Zimny, 2003**). According to Polish Law of 10 July 2007 about fertilizers and fertilization pig slurry is valuable natural organic fertilizer, but it should be managed in suitable way because it is a potential threat for environment due to high content of nitrogen (1.5 – 9.2 g/dm^3) and phosphorus (0.2 – 2.5 g/dm^3) compounds. The excessive emission of these substances to the environment can cause a pollution of water and soil (**Marszałek *et al.*, 2013**). Slurry also contains a large number of microorganisms, mainly from animal feces, but also from external sources. The main slurry bacteria is *Enterobacteriaceae* family, especially *Enterobacter* genus, and fecal coliforms. Most of them are opportunistic pathogens for people and animals and can cause infections. In slurry also obligatory pathogens, such as *Salmonella typhi*, can be found (**Iannotti *et al.*, 1982; Podkówka, 2012**).
For those reasons proper management of pig slurry is very important. Annual production of pig slurry in Poland is estimated at 35-38 millions of m^3 (**Stankiewicz, 2010**). Most of it is poured out on fields as a fertilizer, but 30% of wastes is a supplement for anaerobic digestion in biogas production process.
The aim of this study was to determine the role of anaerobic digestion in sanitization, especially in the aspect of fecal bacteria, of biomass from biogas plant, which contains pig slurry produced by industrial pig farm located in the West Pomerania in Poland.

MATERIAL AND METHODS

Biological samples – characteristics, source and preparation

The source of material for the study was an agricultural biogas plant, which uses a pig slurry from nearby pig farm and maize silage to produce biogas. As a fuel for generator and stove, biogas is immediately converted to heat and electricity, which is defined as a high-efficiency cogeneration. Technological process runs in a continuous system with hourly addition of substrates. During one cycle defined infeed is prepared and added to the fermentation tank (FT), where the first fermentation in mesophilic conditions take place (30-42°C). In the same time volume of biomass equal to the infeed from FT is automatically transported to the post-fermentation tank (PT), where second, additional fermentation takes place and biogas is stored. Simultaneously the same volume of fermented biomass is transported from PT to the lagoon (L) - external, isolated tank which is a storage for biomass.
The material of the study were 4 biological samples representing all stages of technological process of biogas production: 1 sample of raw pig slurry (ca. 1 dm^3) – signed as I, collected from initial tank (IT), which is reservoir for slurry; 1 sample of fermenting biomass (ca. 1 dm^3) – labeled as II, collected from FT; 1 sample of fermented biomass (ca. 1 dm^3) – labeled as III – collected from PT; 1 sample of biomass from lagoon – labeled as IV. Samples I and IV was collected according to polish norm **PN-B-12098 (1997)** – directly from tanks by hand-made sterile sampler. Samples II and III were collected from the sampling valves installed on the outputs of the FT and PT. After collecting, samples were transported to the laboratory cold storage, and stored for analysis in 4°C for 1 day.
Before microbial analysis, a serial dilutions to 10^{-6} of each sample were made, according to **PN-EN ISO 6887-1 (2000)** norm.

Quantitative analysis of coliforms

Analysis of coliforms number in collected samples was performed according to **PN-EN ISO 9308-2 (2014)** standard. Number of microorganisms was determined by the most probable number (MPN) method, using McCrady's tables for 2 tubes in 3 dilutions. The method is well explained by **Oblinger & Koburger (1975)**. To the prior prepared, sterile test tubes with 10 ml of liquid Brilliant Green Bile liquid medium (Biomed Sp z o. o., Warszawa) and Durham tubes placed inside, 1 ml inoculums from all dilutions of each sample was added (4 samples x 6 dilutions x 2 tubes). Prepared cultures were incubated for 48h in 37°C. Afterwards cultures were examined by observing the presence of 3 characteristic features in microbiological medium: color change, turbidity, and the presence of gas in the Durham tubes. When all 3 features were observed it was considered as

positive result – presence of coliforms in examined culture. Lack of at least one trait was considered as negative – absence of coliforms in examined culture. For each sample (I,II,III,IV) three last dilutions in which growth was observed were taken into account. Numbers of positive results was compared with McCrady's tables for 2 tubes variant, and the MPN was obtained from them.
In order to confirm the results and obtain single colonies of coliforms for further microscopic analysis, cultures were prepared by streaking method, from two tubes of each sample (I, II, III, IV) that were considered as positive for coliforms presence. A slightly selective and differential medium for isolation of coliforms was used – ENDO Agar (BTL Sp. z o.o., Łódź). Cultures were incubated for 24 h in 37°C. After incubation colony morfology was examined, and 2 selected characteristic colonies was prepared on microscopic slides, stained by Gram staining procedure (**Wawrzkiewicz *et al.*, 1983**), and examined in optical microscope (Olympus, Japan) enabling taking pictures under immersion (x100).

Detecting faecal streptococci

Presence of group D streptococci was examined by direct streaking biological material from each sample (I,II,III,IV) in two variants on Petri plates with selective and differential for enterococci Slanetz & Bartley medium (BTL Sp. z o.o., Łódź). Cultures was incubated for 48 h in 34°C. After incubation in each plate presence or lack of characteristic for enterococci growth colonies was examined. Then, for confirmation of results, 10 selected characteristic colonies from plates marked as positive were screened on selective and differential for *Enterococcus* genus medium – Bile Esculin Agar (BTL Sp. z o.o., Łódź). Cultures were incubated for 24h in 37°C. Afterwards, from selected single colonies identified as *Enterococcus* genus 2 microscopic slides were prepared and stained by Gram method (**Wawrzkiewicz *et al.*, 1983**). Slides were examined with optical microscope (Olympus, Japan) and photographed.

RESULTS AND DISCUSSION

Most probable number of faecal coliforms

The results of the analysis indicate significant reduction of faecal coliforms number in each sample from subsequent stages of biogas production process. In the first fermentation in FT the most probable number of bacteria was reduced from 7.0 x 10^6 [MPN/ml] in raw pig slurry (I) to 2.4 x 10^6 in fermenting biomass (II). Another fermenting process in PT reduced number of coliforms to 2.4 x 10^5 in fermented biomass (III). In sample collected from lagoon (IV) the most probable number of bacteria was 3.7 x 10^4. The results of each sample are presented in detail in Table 1. The total reduction of coliforms was 99.47% of the initial value from raw slurry. The percent reduction from each stage is shown in Figure 1.

Table 1 Number of positive results of each sample and most probable number calculated according to McCrady's table for 2 tubes

Sample	Serial dilution						MPN/ml
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	
I	2	2	2	2	2	1	7.0 x 10^6
II	2	2	2	2	0	0	2.4 x 10^6
III	2	2	2	0	0	0	2.4 x 10^5
IV	1	2	2	0	0	0	3.7 x 10^4

Legend: I – raw pig slurry, II – fermenting biomass, III – fermented biomass, IV – biomass from lagoon. Highlighted values - dilutions taken into account in determination of the most probable number of bacteria.

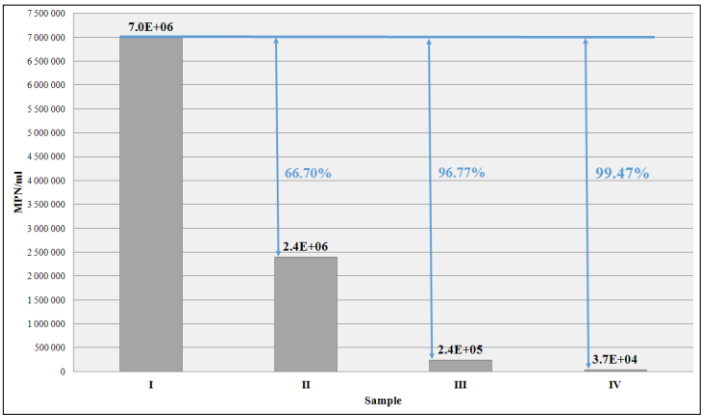

Figure 1 The most probable number of coliform in each sample with percentage reduction of microorganisms in all stages of the process

On plates with ENDO agar medium growth of small, opalescent colonies, which had a colour from pink to dark red was observed. Growth like this is characteristic for lactose positive bacteria from *Enterobacteriaceae* family. Two selected single colonies were prepared and examined by microscopic analysis (Figure 2).

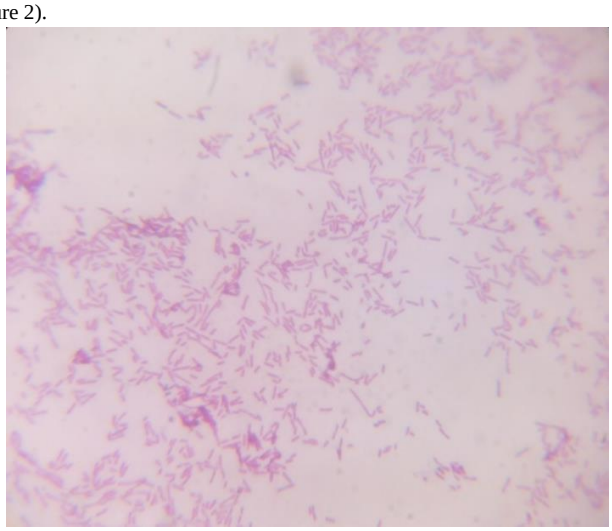

Figure 2 Microscopic view of material - Gram-negative, rod - shaped bacteria, which morfology indicates the possible presence of genus such as *Escherichia* and *Enterobacter* (Gram staining, x100, immerse).

The presence of enterococci

Streaking on plates with Slanetz & Bartley medium gave a result in presence of growth round, convex, not transparent, pink colonies, which is characteristic for faecal enterococci. The growth occurred in all variants of I and II sample cultures. On plates with III and IV sample cultures the growth was not observed. It may indicate a reduction of enterococci number to a value below the detection limit after first fermentation in FT.
Selected single, typical for group D streptococci colonies from Slanetz & Bartley medium which was streaked on Bile Esculin Agar gave a result in light brown, round colonies with surrounding dark areas, due to esculine degradation and variegated component diffusion to the medium. This is typical for enterococci growth on this medium.
On the microscopic slides small, spherical, Gram-positive cocci arranged into characteristic chain structure (Figure 3).

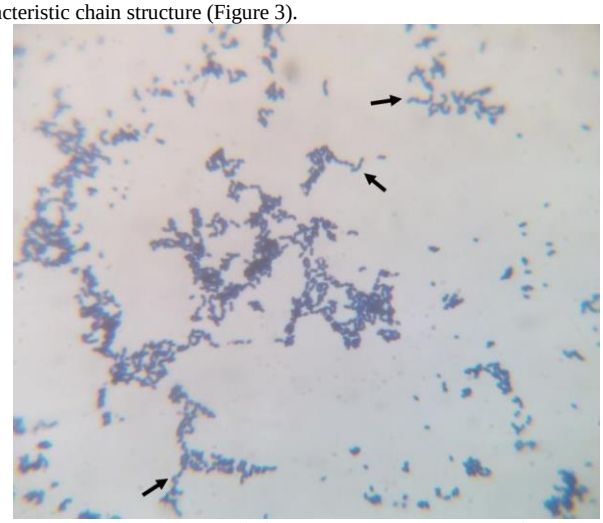

Figure 3 Microscopic view of faecal enterococci isolated from examined biomass. Arrows (↑) shows characteristic for streptococci, chain-like arrangement of bacteria.

DISCUSSION

Côté *et al.* (2006) examined indicator microorganisms and opportunistic pathogens reduction efficiency in pig slurry during the anaerobic digestion, and similar results were obtained. Samples were collected from industrial pig farms of Canada, and the fermentation was performed in laboratory scale in a continuous process at 20°C for 20 days without feeding. It was observed that the anaerobic digestion resulted in the reduction of faecal coliforms by 97.94-99.99%. Such a high level of reduction at such a low temperature of the process was achieved probably due to small-scale experiments, and the lack of a constant supply of fresh manure, which always delivers new inoculum of microorganisms. For that reason level of reduction of bacteria in industrial scale installation examined in current work was a bit lower.

Puchajda & Oleszkiewicz (2003, 2004) examined reduction of pathogens in two different installations: one- and two-stage anaerobic digestion. Both processes were carried out in a continuous system, and the cyclic addition of the slurry. It was found that the two-stage process carried out in a system of two fermenters – first in thermophilic conditions and second, mesophilic - was significantly more effective in reducing coliforms than one-stage process. For this reason two-stage installation (both fermenters, FT and PT, in mesophilic conditions) examined in current work obtained so effective reduction of coliforms.

Peu *et al*. (2006) also obtained results showing reductive effects of anaerobic digestion, although only the liquid fraction of the pig slurry after separating off the solid effluent was studied. Biomass was stored under anaerobic conditions, and the fermentation occurred spontaneously, without the industrial use of the produced biogas. Molecular methods used in the experiment allowed for a very detailed analysis of the microorganisms population in fermenting slurry. Analysis have shown that faecal indicator bacteria number (enterococci and coliforms) significantly decreased during the first 20 days of anaerobic digestion. Moreover, soil that was fertilized with fermented slurry was examined. Number of enterococci and coliforms in there was below the detection limit.

Cao *et al.* (2013) in laboratory conditions examined influence of anaerobic digestion on number of microorganisms in pig slurry. 180 days of continuous fermentation of pig slurry samples collected from Jiangsu Academy of Agricultural Sciences pig farm showed significant reduction of the total number of microorganisms in pig slurry used in experiment. The initial viable count of 1.73 x 10^7 CFU/ml was reduced to 5.75 x 10^5 CFU/ml after fermentation. In the study the temperature of the process was highlighted as one of the main reducing factors, therefore reduction of *Bacillus* is such a small level because this genus is able to produce spores, and survive high temperature conditions.

Similar results were obtained by **Rui *et al.* (2014)**. Collected from 13 industrial biogas plants located in different regions of China, biological samples of fermenting biomass were tested. Material was analyzed for different bacteria content and the content of ammonium and phosphate ions. It has been found that the ratio of the content of these ions is the main factor conditioning composition of the microbial community in tested biomass. Authors pointed out that high proportion of ammonium ions to phosphate ions in biomass is largely beneficial for genera such as *Clostridium* and *Bacillus*. Probably this is one of the reasons of small reduction of these microorganisms showed in a current work.

Anaerobic digestion shows reducing effect of coliforms not only in pig slurry. **Manyli-Loh *et al.* (2014)** investigated the survival rates of bacterial pathogens before, during and after mesophilic anaerobic digestion of a dairy manure from one of a dairy farms in South Africa. Investigation showed that pathogens such as *Campylobacter*, *E. coli* and *Salmonella* inoculated to the digester were reduced from the initial level of 10.1 x 10^3, 3.6 x 10^5, 7.4 x 10^3 CFU/g to concentration below the detection limits, as same as in the current work. Authors obtained similar value of 90-99% reduction of a health importance pathogens. **Pandey & Soupir (2011)** also examined reduction of *Escherichia coli* in cattle manure used in production of biogas. Comparing conditions of anaerobic digestion: psychrophilic, mesophilic, and termophilic fermentation showed that *E. coli* inactivation during the termophilic process was 15-17 times more effective than in other thermal conditions, which confirms that the temperature has the biggest impact on sanitization efficiency in examined biogas plant.

CONCLUSION

The use of pig slurry as a feedstock in the production of biogas is an effective method of sanitizing it, due to significant coliforms reduction effect of anaerobic digestion. Moreover, research has indicated that fermentation can reduce number of enterococci in pig slurry to value below the detection limit. Determining the exact value of this reductions would require further quantitative analyzes conducted in this regard. What factors, and to what extent, cause reduction of the total number of microorganisms in pig slurry should also be examined.

Production of biogas from pig slurry in agricultural biogas plants has important environmental meaning, because it reduces emission of faecal opportunistic pathogens by intensive livestock production.

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REFERENCES

CAO, Y., CHANG, Z. C., WANG, J., MA, Y., FU, G. 2013. The Fate of Antagonistic Microorganisms and Antimicrobial Substances during Anaerobic Digestion of Pig and Dairy Manure. *Bioresource Technology*, 136, 664–671. http://dx.doi.org/10.1016/j.biortech.2013.01.052.

CÔTÉ, C., MASSÉ, D.I., QUESSY, S. 2006. Reduction of Indicator and Pathogenic Microorganisms by Psychrophilic Anaerobic Digestion in Swine Slurries. *Bioresource Technology*, 97(4), 686–91. http://dx.doi.org/10.1016/j.biortech.2005.03.024.

IANNOTTI, E.L., FISCHER, J.R., SIEVERS D.M. 1982. Characterization of Bacteria from a Swine Manure Digester. *Applied and Environmental Microbiology*, 43(1), 136–43.

MANYI-LOH, C.E., MAMPHWELI, S.N., MEYER, E.L., OKOH, A.I., MAKAKA, G., SIMPSON, M. 2014. Inactivation of Selected Bacterial Pathogens in Dairy Cattle Manure by Mesophilic Anaerobic Digestion (Balloon Type Digester). *Int J Environ Res Public Health*, 11(7), 7184–7194. http://dx.doi.org/10.3390/ijerph110707184.

MARSZAŁEK, M., BANACH, M., KOWALSKI, Z. 2011. Utilization of pig slurry by anaerobic and areobic digestion - production of biogas and compost. *Czasopismo Techniczne. Chemia*, 103(2), 143-58.

OBLINGER, J. L., KOBURGER, J.A. 1975. Understanding and Teaching the Most Probable Number Technique. *J. Milk Food Technol*, 38(9), 540–45.

PANDEY, P.K., SOUPIR, M.L. 2011. *Escherichia Coli* Inactivation Kinetics in Anaerobic Digestion of Dairy Manure under Moderate, Mesophilic and Thermophilic Temperatures. *AMB Express*, 1(18). http://dx.doi.org/10.1186/2191-0855-1-18.

PEU, P., BRUGÈRE, H., POURCHER, A.M., KÉROURÉDAN, M., GODON J.J., DELGENÈS, J.P., DABERT, P. 2006. Dynamics of a Pig Slurry Microbial Community during Anaerobic Storage and Management. *Applied and Environmental Microbiology*, 72(5), 3578–85. http://dx.doi.org/10.1128/AEM.72.5.3578-3585.2006.

PKN. 1997. *Polish Standards:* Slurry. Collecting samples - polish version. PN-B-12098:1997.

PKN. 2000. *Polish Standards:* Microbiology of food and animal feed. Preparation of the samples, initial suspension and decimal dilutions for microbiological examination. General rules for the preparation of the initial suspension and decimal dilutions - polish verison. PN-EN ISO 6887-1:2000.

PKN. 2014. *Polish Standards:* Water quality - Quantification of *Escherichia coli* and coliform bacteria - Part 2: Method for Most Probable Number - english version. PN-EN ISO 9308-2:2014-06.

PODKÓWKA, W. 2012. Biogaz Rolniczy - Odnawialne Źródło Energii [Agricultural biogas - renewable source of energy]. Warszawa: Powszechne Wydawnictwo Rolnicze i Leśne. 263 p. ISBN 83-09 010-89-3.

PUCHAJDA, B., OLESZKIEWICZ, J. 2003. Single and Two-Stage Anaerobic Digestion: Hydrolysis, Acidification and Pathogen Inactivation. *Proceedings of the Water Environment Federation* (WEFTEC: Session 61 through Session 70), 284-301. http://dx.doi.org/10.2175/193864703784641801.

PUCHAJDA, B., OLESZKIEWICZ, J. 2004. Pathogen Inactivation through Organic Acids in Anaerobic Digestion. *Proceedings of the Water Environment Federation* (WEFTEC 2004: Session 41 through Session 50), 314-21. http://dx.doi.org/10.2175/193864704784138764.

RUI, J., LI, J., LI, J., WANG, Y., KE, L., ZHANG, S., LI, X. 2014. Prokaryotic community structures in biogas plants with swine manure. *Huagong Xuebao/CIESC Journal*, 65(5), 1868-1875.

STANKIEWICZ, D. 2010. Biomass as energy source in Poland. *Studia BAS* 1(21), 237–66.

WAWRZKIEWICZ, J., BUCZEK, J., WAWRZKIEWICZ, K.H. 1983. Mikrobiologia Weterynaryjna [The veterinary microbiology]. Warszawa: Państwowe Wydaw. Naukowe. 475 p. ISBN 97-88 301-04-9607.

ZIMNY, L. 2003. Encyklopedia Ekologiczno-Rolnicza [Ecological and Agricultural Encyclopedia]. Wrocław: Wydawnictwo Akademii Rolniczej we Wrocławiu. 230 p. ISBN 83–87 866–79–2.