Original Article

Effect of Amoxicillin on Biogas Production and the *Eschericia coli* Population in Biogas Systems Treating Swine Wastewater

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Abstract

The effect of amoxicillin concentrations (0, 200 and 400 ppm) on biogas production and the *Eschericia coli* population in lab-scale biogas systems treating swine wastewater was examined. Three anaerobic fermentation reactors (with an available volume of 25 l), containing wastewater collected from a swine farm and free from antibiotics, were operated for eight weeks. After eight weeks, the cumulative yields of biogas and methane in the control group (0 ppm) were the highest (11,563 ml of biogas and 542 ml of methane, as 100%). The cumulative biogas yields in the group 1 (400 ppm) and group 2 (200 ppm) decreased to 70.5% and 90.04% compared to the control group, respectively. Moreover, group 1 and group 2 had cumulative methane yields decreased to 31.9% and 65.3% respectively, compared to the control group. The total suspended solids (TSS) and chemical oxygen demand (COD) were highest for the group 1 (352 and 930 mg/l, respectively) and subsequently decreased for the group 2 (308 and 570 mg/l, respectively) and the control group (121 and 275 mg/l, respectively). Volatile fatty acid (VFA) concentrations were the highest in the control group (563 mg/l), and decreased in other treatment groups (488 and 225 mg/l in group 2 and 1, respectively). The pH values did not change for any group. In addition, it was found that Escherichia coli decreased over time in all groups. Over a five week period, amoxicillin concentrations decreased each week from 400 to 178, 99.1, 88.23, and 2.12 ppm respectively (group 1) as well as from 200 to 140.6, 55.36, 36.23 ppm and no detection respectively (group 2). These results illustrate that although the concentration of detectable amoxicillin decreases over time, its presence results in a decrease in the cumulative biogas and methane produced. However, based upon the analytical assay, the concentration of detectable amoxicillin is decreasing in the biogas systems.

Keywords: amoxicillin, biogas, methane, *Eschericia coli*, swine wastewater

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บทคัดย่อ

ผลของยาอะม็อกซีซิลลินต่อผลผลิตก๊าซชีวภาพ และประชากรของ *เอสเชอริเชีย โคไล* (*E. coli*) ในการบำบัดน้ำเสียจากฟาร์มสุกรด้วยระบบก๊าซชีวภาพ

จักรกริศน์ เนื่องจำนงค์[1] พิชญ รัชฎาวงศ์[2] ธงชัย เฉลิมชัยกิจ[3]*

การศึกษานี้มีวัตถุประสงค์ เพื่อศึกษาผลของยาอะม็อกซีซิลลินที่ระดับความเข้มข้นต่างๆ (0 200 และ 400 พีพีเอ็ม) ต่อปริมาณก๊าซชีวภาพที่ผลิตได้และประชากรแบคทีเรีย *เอสเชอริเชีย โคไล* ในการบำบัดน้ำเสียจากฟาร์มสุกรด้วยระบบก๊าซชีวภาพในระดับห้องปฏิบัติการ ทำการทดลองในถังปฏิกรณ์จำนวน 3 ถัง โดยมีความจุถังละ 25 ลิตร บรรจุน้ำเสียจากฟาร์มสุกรที่ไม่ได้รับยาปฏิชีวนะใดๆ ทำการศึกษาเป็นเวลาทั้งสิ้น 8 สัปดาห์ หลังจาก 8 สัปดาห์ พบว่า ปริมาณก๊าซชีวภาพสะสมและก๊าซมีเทนสะสมที่เกิดขึ้นในกลุ่มควบคุม (0 พีพีเอ็ม) มีค่าสูงที่สุด (11,563 และ 542 มล. ตามลำดับ : คิดเป็นร้อยละ100) ปริมาณก๊าซชีวภาพสะสมในกลุ่มที่ 1 (400 พีพีเอ็ม) และกลุ่มที่ 2 (200 พีพีเอ็ม) มีปริมาณลดลงเมื่อเปรียบเทียบกับกลุ่มควบคุม คิดเป็นร้อยละ 70.5 และ 90.04 ตามลำดับ อย่างไรก็ดีกลุ่มที่ 1 และกลุ่มที่ 2 มีปริมาณก๊าซมีเทนสะสมคิดเป็นร้อยละ 31.9 และ 65.3 ตามลำดับ เมื่อเปรียบเทียบกับกลุ่มควบคุม ค่าของแข็งแขวนลอยทั้งหมดและค่าซีโอดี พบว่ามีค่าสูงที่สุดในกลุ่มที่ 1 (352 และ 930 มก/ล. ตามลำดับ) และลดลงในกลุ่มที่ 2 (308 และ 570 มก/ล. ตามลำดับ) และกลุ่มควบคุม (121 และ 275 มก/ล.ตามลำดับ) ส่วนค่ากรดไขมันระเหย พบว่ามีค่าสูงที่สุดในกลุ่มควบคุม (563 มก/ล.) และลดลงในกลุ่มที่ 2 (488 มก/ล.) และกลุ่มที่ 1 (225 มก/ล.) ตามลำดับ แต่ไม่พบการเปลี่ยนแปลงของค่าความเป็นกรด-ด่างในทุกกลุ่มทดลอง นอกจากนี้พบว่าแบคทีเรีย*เอสเชอริเชีย โคไล* (*Escherichia coli*) ในน้ำเสียจากฟาร์มสุกรมีปริมาณลดลงจากตอนเริ่มต้นในทุกกลุ่มทดลอง ส่วนการลดลงของยาอะม็อกซีซิลลินในสัปดาห์ที่ 5 หลังเติมยาเข้าสู่ระบบ ได้ผลการลดลงของยาในแต่ละสัปดาห์ดังนี้ กลุ่มที่ 1 ลดลงจาก 400 พีพีเอ็ม เหลือ 178 99.1 88.23 และ 2.12 พีพีเอ็ม กลุ่มที่ 2 ลดลงจาก 200 พีพีเอ็ม เหลือ 140.6 55.36 36.23 พีพีเอ็ม และไม่สามารถตรวจพบได้ตามลำดับ จากผลดังกล่าวนี้ พบว่า แม้ความเข้มข้นของยาอะม็อกซีซิลลินที่ตรวจพบได้จะลดลงตามระยะเวลา ซึ่งมีผลต่อการลดลงของปริมาณก๊าซชีวภาพและก๊าซมีเทนสะสมที่ผลิตได้ อย่างไรก็ตามผลจากการตรวจวิเคราะห์หาปริมาณยา พบว่า ความเข้มข้นของยาอะม็อกซีซิลลินที่ตรวจพบได้สามารถลดลงในระบบก๊าซชีวภาพ

คำสำคัญ: ยาอะม็อกซีซิลลิน ก๊าซชีวภาพ ก๊าซมีเทน *เอสเชอริเชีย โคไล* น้ำเสียจากฟาร์มสุกร

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Introduction

Anaerobic digesters are one of the most efficient methods for treating swine wastewater, and the produced biogas can be useful as an alternate energy resource. Biogas is produced by large numbers of strict and facultative anaerobic bacteria. The composition of biogas is generally 50-70% CH_4, 30-50% CO_2, 5-10% H_2, 1-2% N_2, 0.3% H_2O, and a trace of H_2S (Taoturee, 2004; Yadav and Hesse, 1981).

In order to efficiently operate biogas systems, parameters such as pH and temperature need to be controlled, however, some parameters cannot be regulated, particularly the quantity of residual antibiotics that are commonly used to control swine diseases. Antibiotics are administered orally either by water medication or in-feed medication (Burch, 2003). Consequently, a percentage of the administered antibiotics may be excreted in the manure or urine as the parent compound or as a metabolite and could affect the bacterial community and population in the biogas system (Campruhi et al., 1988; Masse et al., 2000).

Amoxicillin is one of the broad-spectrum antibiotics widely used in swine farming to treat several diseases (Agerso and Friis, 1998; Jensen et al., 2004). One main property of this antibiotic is that it unaltered passing through the digestive tract and excreted unchanged (Anfossi et al., 2002). As a result, its effect on biogas performance can be studied by directly spiking amoxicillin into swine wastewater without having to be first consumed by swine. Theoretically, amoxicillin will kill both circular gram negative and cubical gram positive forms of bacteria as well as anaerobic bacteria (Plumb, 2005). Therefore, the objective of this study was to investigate whether amoxicillin, if present in swine wastewater, would affect the biogas and methane produced as well as the E. coli population within the biogas system.

Materials and Methods

In this study, three lab-scale biogas systems were assembled with each using a cylindrical PVC reactor with a volume of 25 l. Each had a diameter of 28 cm and 49 cm height and was built as illustrated in figure 1. Twenty liters of swine wastewater from The Swine Research Unit farm, Department of Animal Husbandry, Faculty of Veterinary Science, Chulalongkorn University, Nakornpathom province was added into each reactor and mixed thoroughly. Each reactor was allowed to sit for 3 weeks in order to equilibrate the microbiological communities within the system (Taoturee, 2004). After 3 weeks, amoxicillin was added at a concentration (10-20 mg/kg; twice a day) similar what is encountered in normal agricultural practice (Plumb, 2005). Since 100 kg of swine will excrete approximately 8-10 kg of waste a day (Taoturee, 2004), the amount of amoxicillin in the manure should be 2,000-4,000 mg per 8-10 kg of waste (or 200-500 ppm). In this study, three reactors were established by adding amoxicillin into the wastewater at the concentration of 400 ppm (group 1), 200 ppm (group 2), and 0 ppm (group 3) as a control group. Sampling and analyses of wastewater were conducted at every phase of the study, i.e. before the wastewater was added into the system, during the operation before and after adding amoxicillin, as well as after the digestion at the end of eight weeks.

Table 1 listed the chemical parameters analyzed according to standard procedures (APHA et al., 1998; Teerachark, 2005). *E. coli* was determined by means of Most Probable Number (MPN) method (APHA et al, 1985). Before starting the experiment, the swine wastewater was tested with a CM-Test™ method (Chalermchaikit et al., 2003) and a European Four Plate Test (EFPT) method (Okerman and Hoof, 1998) in order to confirm that there was no antibiotic contamination. After amoxicillin addition to the swine wastewater, the amoxicillin concentration was determined every week using the Antimicrobial Susceptibility Test (Clinical and Laboratory Standards Institute, 2008). In this method, 15 µl of wastewater from each group was added into a disc with 0.6 cm diameter and placed on Muller Hinton Agar (MHA) containing 0.5 McFarland of *Bacillus subtilis*. The disc was then incubated at 37^{o}C for 24 hr. The concentration of amoxicillin was determined using an inhibition zone compared to a standard curve that made by the preparation of various amoxicillin trihydrate concentrations (i.e. 5120, 2560, 1280, 640, 320, 160, 80, 40, 20, 10, 5, 2.5, 1.25, 0.625, 0.3125 ppm) using two-fold dilution.

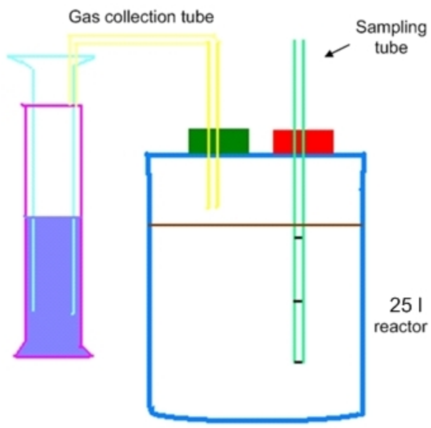

Figure 1 Schematic of a lab-scale biogas system

Table 1 Methods and frequencies of the analyses for each parameter

Parameter	Unit	Analysis methods	Frequencies
Biogas production	ml	Inverted glass cylinder method.	Everyday
CH_4	%	Gas chromatography	2 times/week
pH	-	pH meter	2 times/week
COD	mg/l	Open reflux method	Before and after the study
TSS	mg/l	Filtration through GC/F paper	Before and after the study
VFA	mg/l	Titration method	2 times/week
ORP	mg/l	SMWW 2580B	2 times/week

Results

Cumulative biogas and methane production: According to Figure 2 and 3, the control group produced the greatest accumulative amount of biogas, whereas the group 1 produced the lowest amount. Group 1 had 29.5% less cumulative biogas produced and 68.1% less cumulative methane produced than the control group, meanwhile group 2 had 10.0% and 34.7% less produced for each parameter, respectively.

pH: It was founded that there was only a minor difference of the pH values from each group. The pH of group 1 was in a range of 7.63-8.46 (mean value 8.01±0.3), whereas it was 7.63-8.60 (mean value 7.98±0.18) for group 2 and 7.56-8.84 (mean value 8.00±0.37) for the control group.

Total suspended solids (TSS) and Chemical Oxygen Demand (COD): The TSS and COD values before adding amoxicillin was approximately 2,760 and 3,400 mg/l respectively in every reactor. Upon completion of the experiment, TSS decreased to 352, 308, 121 mg/l in group 1, 2, and control, respectively. For the COD, the final concentration for each group was 930, 570, and 275 mg/l respectively.

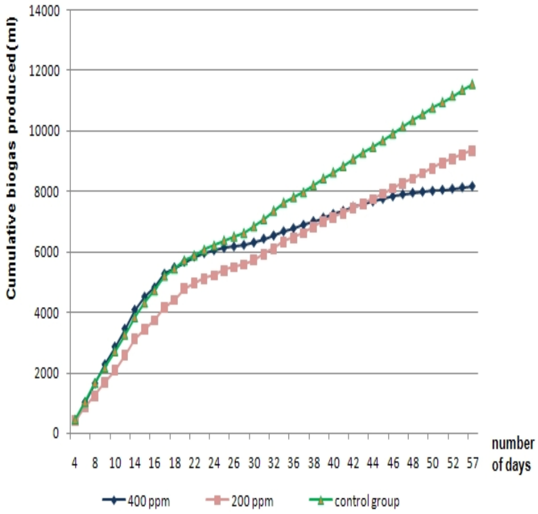

Figure 2 Cumulative biogas produced

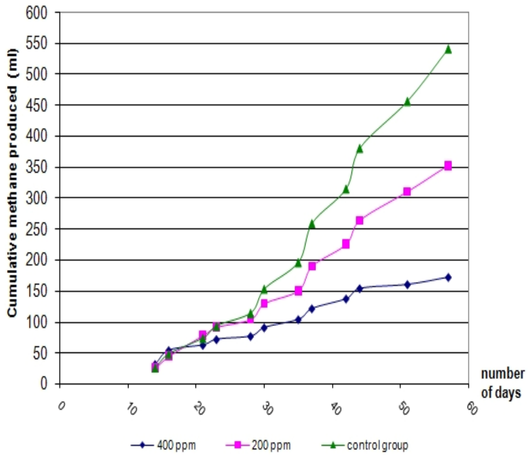

Figure 3 Cumulative methane produced

Volatile fatty acid (VFA): The reactors experienced significant decrease of VFA prior to the amoxicillin addition, with initial VFA values of more than 10,000 mg/l to approximately 140-525 mg/l by the second week of the equilibrium period. Once reaching this VFA range, VFA values remained fairly constant even after the addition of amoxicillin. The VFA values at the end of the experimental period in the control group (563 mg/l) were slightly higher than those in the group 2 (488 mg/l) and group 1 (225 mg/l) respectively.

Oxidation-Reduction potential (ORP): The ORP values before adding amoxicillin were similar for all three reactors, ranging from -182 to -164 mV. At completion of the experiment, the ORP in the control group was the lowest (-162 mV), followed by group 2 (-82 mV) and group 1 (-66 mV), respectively.

E. coli: As shown in table 2, the change in the amount of *E. coli* in the wastewater over the three week equilibrium period decreased by 97.9%, 93.8%, and 91.4% in group 1, 2, and control group respectively. After eight weeks in the presence of amoxicillin, the amount of *E. coli* decreased further by 98.3%, 97.8%, and 99.3% in group 1, 2 and control group respectively.

The decrease of amoxicillin concentration: One week after introduction of amoxicillin, its concentration in group 1 and 2 decreased from 400 ppm to 178 ppm (removal rate of 31.7 ppm/d) and from 200 ppm to 140.6 ppm (removal rate of 8.4 ppm/d) respectively. The removal rates of both groups were 11.2-12.1 ppm/d in the second week and 1.8-2.1 ppm/d in the third week. At the end of the study, amoxicillin was founded to be 2.1 ppm in group 1 and none in group 2, representing the efficiency of 97.5% and 100% respectively.

Table 2 *E. coli* in the wastewater before entering the system (1), before an addition of amoxicillin (2), as well as after leaving the system (3)

Group	*E. coli* (log MPN/100 ml)		
	(1)	(2)	(3)
1 (400 ppm)	7.73	6.04	5.97
2 (200 ppm)	7.73	6.52	6.08
3 (control)	7.73	6.66	4.60

Discussion

At the beginning of the experiment, the control group showed a sharp increase of cumulative biogas produced compared to the cumulative methane produced. It was due to the organic degradation in anaerobic process, i.e. hydrolysis phase and acidogenesis phase, which produced high amount of VFA, H_2 and CO_2 in order to be used as substrates for subsequent phases. In this study, no more wastewater was added into the system during these phases, resulting in an eventual decrease of biogas production.

Considering the effect of amoxicillin, it was revealed that the amounts of cumulative biogas and methane gas were lower in groups 1 and 2 relative to the control group. Group 2 showed higher cumulative amounts than group 1. This result is in accordance with the result of Lallai et al. (2002) who found that as the amoxicillin concentration increased, methane yield decreased in the anaerobic digestion of swine waste slurry. Moreover, it might be related to the finding that amoxicillin could inhibit *Bacteroides spp.* (Elman et al., 1992) and *Clostridium spp.* bacteria (Plumb, 2005) by inhibiting the biosynthesis of cell wall mucopeptide (Lohsiriwat et al., 2009). These bacteria were the predominant species in the biogas digester (Nagamani and Ramasamy, 1999).

All three reactors showed that pH values were in the base range and remained fairly constant over the course of the entire experiment. This might be one of the reasons that methane yield in this study was not highly efficient. The pH between 7.0-7.2 was optimal for increasing biogas yield, though it was satisfactory between pH 6.6-7.6 (Nagamani and Ramasamy, 1999).

According to the result, the TSS and COD of the final effluent were the lowest in the control group (121 and 275 mg/l, respectively), followed by group 2

(308 and 570 mg/l, respectively) and group 1 (352 and 930 mg/l, respectively). This result suggests that amoxicillin affected the anaerobic decomposition process. As stated previously, the presence of amoxicillin could inhibit several bacteria required for efficient hydrolysis and acidogenesis.

The initially high VFA value, as well as its substantial decrease during the first two weeks of the equilibrium period could be due to the cession of the acidogenesis phase caused by the disruption to the microbial community resulting from setting up and establishing the reactors. Subsequently, VFA decreased by approximately the same volume in every group. At the end of the experimental period, VFA was found to be the highest in the control group (563 mg/l), but decreased in group 2 (488 mg/l) and group 1 (225 mg/l) respectively leading to the assumption that the activity of acidogenic bacteria was partly inhibited by amoxicillin.

From the experiment, the control group showed the most reducing environment (-162 mV), followed by group 2 (-82 mV), and group 1 (-66mV) respectively. As such, the more reducing the ORP, as in the control group, the more cumulative methane produced. Similarly, Allen (2007) also observed a negative relationship between the ORP and the amount of methane produced.

During the three week equilibrium period, the amount of *E. coli* in swine wastewater decreased by 91.4-97.9% due to the anaerobic processes occurring within the biogas system. This suggests that biogas systems may effectively decrease this pathogen because of high competition among the bacteria in the system (Smith, et al., 2005). The decrease of the amount of *E. coli* occurred further by an additional 97.8-99.3% after amoxicillin was added into the system. Nevertheless, it was still inconclusive whether amoxicillin could contribute to the pathogen decrease as there was no significant difference in the impact among the three groups.

Based on the results, it could be concluded that the amoxicillin concentration in the effluent wastewater can be efficiently decreased under anaerobic condition, which corresponds well with other studies (Giger et al., 2003; Kakimoto et al., 2007; Morse and Jackson, 2004). This could partially be explained by the high pH of swine wastewater. Amoxicillin undergoes degradation by base-catalyzed dimerization and hydrolysis (Bundgaard, 1977; Connors et al., 1986). However, since the pH values were similar for all three reactors, and since in the first week group 1 had a much higher rate of amoxicillin compared to group 2, it is unlikely that abiotic base-catalyzed hydrolysis is the primary removal mechanism. More likely, removal of amoxicillin is due to enzymatic hydrolysis by protease, amylase, and lipase produced by *Bacilli* bacteria which is widely presented in wastewater (Deshpande et al., 2004). Moreover, amoxicillin could also be degraded by β-lactamase, an enzyme produced by gram-negative bacteria (i.e., *Pseudomonas spp., Proteus spp., Enterobacter spp.*) presented in wastewater (Plumb, 2005). As such, our results demonstrate that anaerobic bioreactors treating swine wastewater can decrease amoxicillin concentrations to a level considered environmentally safe. However, it would be interesting to investigate the effects of amoxicillin in the biogas system over great operational periods since amoxicillin can impact bioreactor performance which could result in having to run the bioreactors longer due to their lower efficiency.

It was shown that addition of amoxicillin into the lab-scale biogas reactors negatively affected the cumulative biogas and methane produced. The amoxicillin caused an adverse impact on acidogenic bacteria. For the organic matter and solid parameters, it also contributed partly by decreasing the efficiency. Every group had an efficient decrease of *E. coli*. For the treatment groups, the decrease of amoxicillin was highly efficient. In conclusion, the amoxicillin, apart from the negative effects to treatment performance and cumulative biogas and methane produced, could be effectively removed from effluent by the biogas system.

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