Determination of Inhibitory Concentration of Oxytetracycline on Methanogenic Bacteria by In Vitro Study

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ABSTRACT

Introduction: Antibiotics have the potential to adversely affect the microbial community. For anaerobic digestion, a sufficient methanogenic population needs to be preserved in the system. The main aim of this study was determination of inhibitory concentration of oxytetracycline on methanogenic bacteria.

Methods: A 120 mL jacketed bioreactor with a 90 mL working volume was inoculated granular sludge from an anaerobic digester, substrate and different concentration of oxytetracycline with 10 days cycles and intermittent mixing. The reactor was operated at 35 ± 2 ° C. The inhibitory effect of antibiotic was evaluated by monitoring biogas production.

Results: Based on the findings from each batch, complete inhibitory concentration of oxy tetracycline was in concentration of 800 mg L^{-1}. Significant relation was seen between inoculated antibiotic concentrations and methane production (r=-0.86).

Conclusion: The addition of antibiotics to the biomass affected the utilization of fatty acids, resulting in unfavorable effects on methanogenesis. Thus, overusing of antibiotics can adverse effects of intestinal flora.

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Introduction

Oxytetracycline (OTC) is an extensive range antibiotic that is active against diversity of bacteria thus used in dairy cattle production primarily to treat or prevent diseases and to a lesser extent to increase milk production or improve feed efficiency. Between 17% and 76% of antibiotics are excreted by urine and feces in an unaltered form or as metabolites of parent compounds (1). The presence of antibitics in manure and waste milk warrants further consideration due to usage of these materials in anaerobic digestion which provides a method of reducing pollution from agricultural wastes and offers numerous significant advantages, such as low sludge production, low energy requirement, and possible energy recovery. The presence of antibiotic compounds has been reported to affect anaerobic digestion efficiencies, both at the laboratory and the field scale (2). Research has shown that low concentrations of many pharmaceuticals are detectable in municipal wastewater, surface water and ground water, and that there may be potential environmental effects from such residues. Recently, the anaerobic treatment of pharmaceutical wastewater containing antibiotics and synthetic drug-based effluents has been reported. It was reported that tetracycline and penicillin reduced the methane production of psychrophilican aerobic digestion of swine manure slurry in sequencing batch reactor by 25% and 35% respectively. Similarly, Alvarez *et al*. (2010) reported significant inhibition of an aerobic digestion of swine manure containing a combination of chlortetracycline (CTC) and OTC at concentrations of 10, 50 and 100 mg L^{-1}, where maximum methane production decreased by 64%in manure containing 100 mg L^{-1} of both CTC and OTC (3). The antibiotics that exist in waste streams directly inhibit substrate degradation and also have an influence on the composition of the microbial consortium. Antibiotic mixtures have an effect on homoacetogenic bacteria and methanogens, which may exert inhibitory effects on propionate and butyrate-oxidizing syntrophic bacteria, resulting in unfavorable effects on methanogenesis (4). Almost complete methane inhibition was observed for antimicrobial doses above 500 mgL^{-1}. The inhibition mechanism is well studied in the literature, mostly by enzyme analogy, conveniently associating it to different enzymatic mechanisms, i.e. competitive inhibition, where the inhibitor and substrate compete for the same reactive site on the enzyme, or non-competitive inhibition where the inhibitor can also bind with the enzyme and deactivate the bound enzyme sites (5). The effects of antibiotics on the bacteria can be examined through measuring the amount of biogas (CH_4) produced by methanogens. This study focuses on the oxy tetracycline effects on biogas production during anaerobic digestion process.

Materials and Methods

Glass vials of 120 ml were used to test every batch lasted 10 days. In order to mix the contents of reactor, some magnets were put into each vial making the sludge mix with the substrate and the antibiotics completely with their rotation. A specific volume of the sludge was taken from anaerobic digester and poured into vial. Then, a certain concentration of antibiotic was added to the vials after adding a specific amount of substrate and nutrients. The temperature was kept at about 35 °C. For the better activity of anaerobic biomass and better performance of methanogens, a substrate mixture of macronutrients and micronutrients was added to the reactor. Various compounds of the substrate were shown in Table 1. Furthermore, 36 mg/l of yeast extract and 36 mg/l of peptone were added to the reactor. Oxytetracycline (OTC) was purchased from Zigma Aldrich chemicals. Then, mixed to the biomass with different concentrations (10, 100, 200, 500 and 800 mg L^{-1}). pH of the reactor was adjusted with NaOH or KOH (1:1 molar ratio). During 10 months of the reactor operation, the methane gas produced from the anaerobic biomass activity was measured daily by replacing the gas with KOH (2 N) solution as an absorber of CO_2 and using bromine thymol blue as an indicator.

Table1. The compounds of substrate used in this study

Inorganic nutrients:	**COD, mg/l**
Acetic acid	76.45 COD
NH_4Cl	10 mg/g COD
KH_2PO_4	10 mg/g COD
Trace Elements:	
K_2HPO_4	25.32 mg/g COD
Fe Cl_3	1.021
$Cacl_2$, $2H_2O$	2.06
$MgSO_4$, 7 H_2O	2.14
$MnCl_2$, 2 H_2O	0.34
$CoCl_2$, 6 H_2O	0.092
$NiSo_4$, 6 H_2O	0.0793
$ZnSo_4$	0.0592
$NaMoO_4$, 2 H_2O	0.0822
$CuCl_2$, 2 H_2O	0.016
HBO_3	0.020

Results

At date, more than 5000 different pharmaceutical compounds intended for human and veterinary applications are used worldwide. Despite their benefits, a growing concern about potential adverse impacts on biota and human health has emerged as a result of their continuous release into the environment (6). According to the results obtained from each batch test, with increasing of OTC concentration, biogas production was decreased. So that, with addition of 10 mg L^{-1} OTC to the bioreactor, biogas production was not decreased significantly ($p>0.05$). With increasing of feed concentration step by step, biogas production was dropped significantly ($p<0.05$). Inoculation of 800 mg L^{-1}OTC was inhibited biogas production completely. So that in the end of 10 days reaction, biogas generation stopped. Overall view of methane production in bioreactor with addition of oxytetracycline is shown in Figure 1. Considering a 10-day period for each batch testing, the effect of different concentrations of oxytetracycline on biomass activity was studied. Pure anaerobic biomass activity and its methanogenic capability were studied in separate tests and the volatile suspended solids were determined in the laboratory. The anaerobic biomass methanogens activity was examined while no substrate or antibiotic was added to the reactor. In further experiments, some prepared substrate (about 5 ml) and 0.7 g of glucose were added to the biomass. As expected, methanogens activity was enhanced and the methane gas production increased. Regarding the biomass capability in producing methane gas and also the enhancement of the biomass with certain amount of nutrients, different concentrations of antibiotics were added in further processes. These processes started with adding low concentration and continued with higher concentrations in further tests. The effect of 800 mg/l concentration of antibiotic on anaerobic biomass activity was quite inhibitory, therefore, the experiments started with low concentrations until it reached the desired extent. The lowest concentration of the added antibiotic was 10 mg L^{-1}. Methanogens were tolerant to low concentrations and continued their activity easily. However, during the first days of the experiment, due to the compatibility of methanogens with the environment, the amount of produced gas was low and scarcely increased until it reached the pick point and then decreased again. The trend of biogas production in presence of different oxytetracycline concentration is shown in Figure 2. As shown in graphs of Figure 2, in presence of 10 mg L^{-1} OTC, maximum methane production was 7 ml CH_4. g $VSS.d^{-1}$. After 150 hrs it was stopped completely. Injection of 100 mg L^{-1} antibiotic to the bioreactor was led to production of 12 ml CH_4. g $VSS.d^{-1}$ after 12 hrs and after 120 hrs reached zero. Accordingly, with addition of 500 mg L^{-1} OTC biogas generation was increased and after 50 hrs reached to 20 ml CH_4. g $VSS.d^{1}$. In addition of 800 mg L^{-1} OTC to the biomass mixture, it was seen less methane. So that, maximum production was 0.5 ml CH_4. g $VSS.d^{-1}$ and after 60 hrs anaerobic reaction it was stopped completely. A similar study on the effect of erythromycin on anaerobic biomass showed that the gas production and COD (chemical oxygen demand) removal decreased to 5-10% and methanogens activity decreased from 3.64 to 0.64 ml/grVSS.d (8). Moreover, in this study, the antibiotic removal was 42-82% due to the long retention time and adsorbing to the flocks. Results on measurement of daily produced methane gas (ml/grVSS per day) showed that oxytetracycline had rather similar effects on methanogens activity in anaerobic biomass. Based on the findings from each batch, complete inhibitory concentration of oxy tetracycline was in concentration of 800 mg L^{-1}. So that, after 60 hr operation, methane production was declined to zero. Significant relation was seen between inoculated antibiotic concentrations and methane production (r =-0.86).

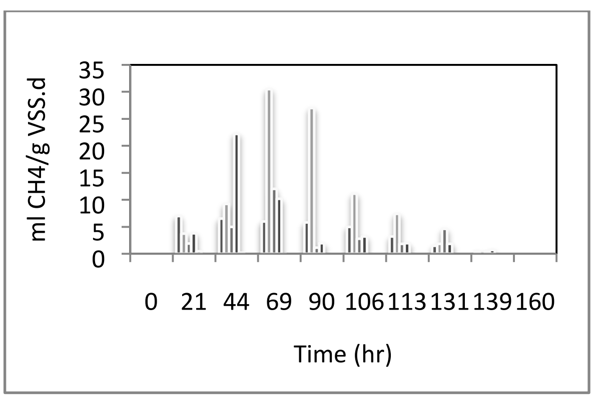
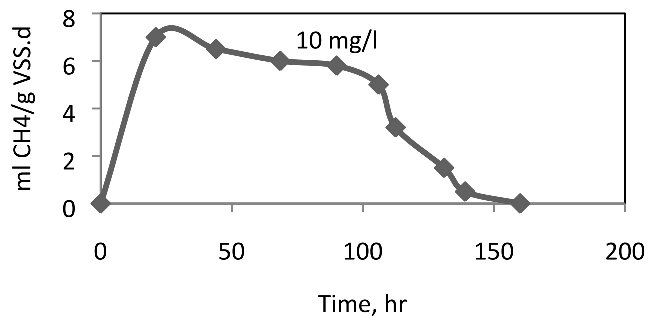
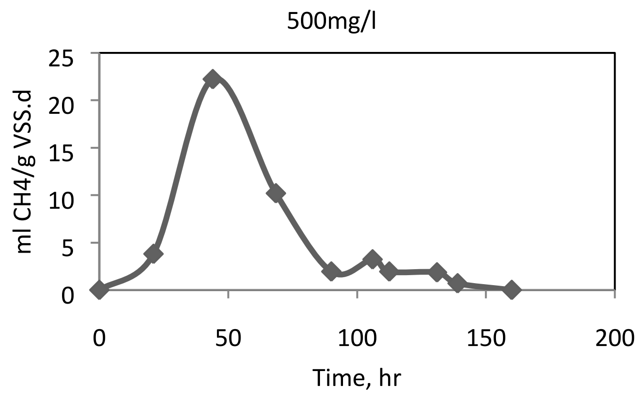
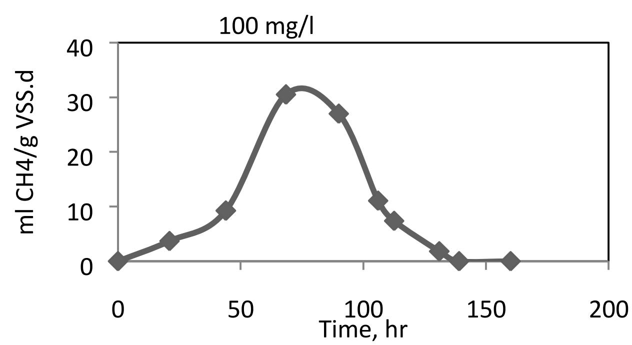
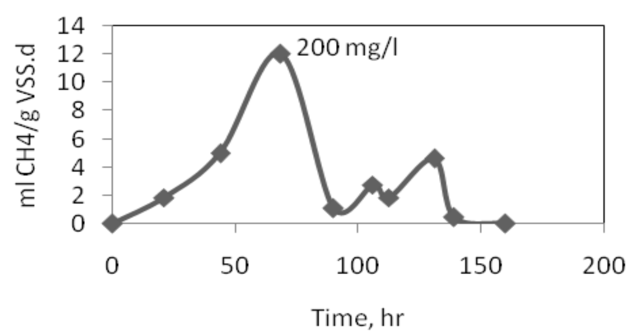


Figure 1. Overall view of methane production in bioreactor with addition of oxytetracycline









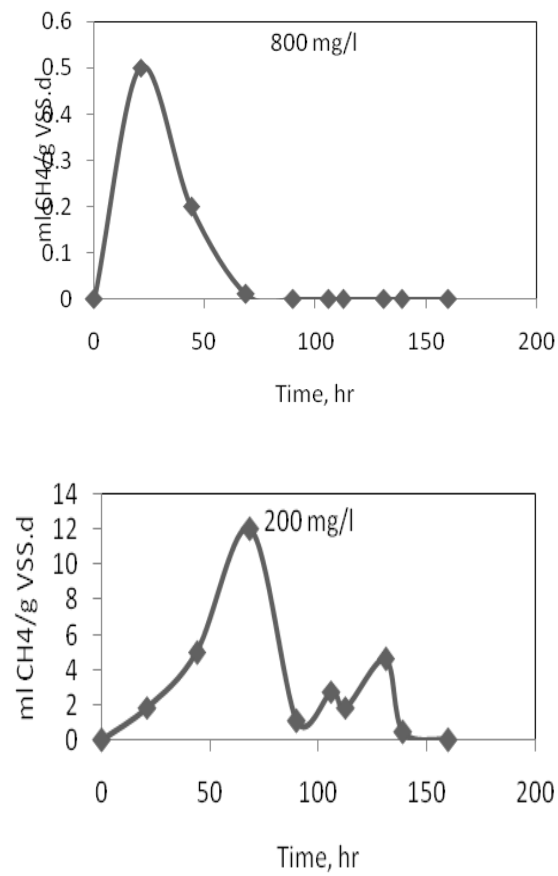


Figure 2. The trend of biogas production in different oxytetracycline concentration

Discussion

Oxytetracycline has an aromatic structure which inhibits the activity of bacteria by affecting their RNA and cause ribosome 235 methylation (adding CH_3) in RNA. The sensitivity of gram-positive bacteria to the antibiotics is more than that of gram-negative bacteria (3). These antibiotics do not tend to be absorbed by soil and enter the groundwater through layers of earth. Therefore, it is essential to eliminate antibiotics from pharmaceutical, municipal, agricultural, and industrial wastewater. According to reports from the United States Geological Survey (USGS), the presence of antibiotics in surface water shows its contamination by wastewater causing potential effects on surface water. Researchers were measured the antibiotics in surface water in the concentration of 1.7 µg/l (7). The concentration of absorption and excretion of antibiotic depends on the type of used antibiotic (8). When antibiotics and its metabolites enter the environment and water resources cause toxic effects on organisms. The absorption of oxytetracycline after oral using is 60-70%. A portion of the administered oral dose of tetracycline remains in intestine changes the intestinal flora and is excreted with the feces. Renal clearance of oxytetracycline is more than other tetracycline antibiotics (90 ml/min). Oxytetracycline is a short-effect tetracycline with serum half-life of 6-8 hours (9). Widespread use of tetracycline for mild disease causes resistance among other sensitive groups. The concentration of tetracyclines in urine was reported more than 300 µg/ml, 2 hours after drug absorption and remains constant until 12 hours after absorption. It was reported that, antibiotic treatments can lead to a disruption of the human microbiota. This disruption can lead in 5–35% of the cases to Antibiotic-Associated-Diarrhea (AAD), with Amoxicillin and Clindamycin having the highest impact.Such correlations between microbiota composition and health disorders can be detected from in-vivo studies (6). The antibiotics that exist in waste streams directly inhibit substrate degradation and also have an influence on the composition of the microbial community. Synergistic effect was observed in nearly all the antibiotic mixtures that included tetracycline as a component (4). Antibiotics disturb and inhibit the performance of bacteria degrading volatile fatty acids. Oxytetracycline is a very strong inhibitor in anaerobic digestion stopping the methanogenic bacteria by affecting cell wall.

Conclusion

This study showed that with increasing concentrations of Oxytetracycline, the produced biogas volume from biomass per unit weight is decreased. With increasing OTC concentration, COD removal was decreased significantly. The addition of antibiotics to the biomass affected the utilization of fatty acids, resulting in unfavorable effects on methanogenesis. Thus, overusing of antibiotics can adverse effects of intestinal flora.

Conflict of interest

None declared conflicts of interest.

References

1. Beneragama N, Lateef SA, Iwasaki M, *et al.* The combined effect of cefazolin and oxytertracycline on biogas production from thermophilic anaerobic digestion of dairy manure. *Bioresource Tech* 2013; **133**: 23-30.
2. Mishra R. Antimicrobial Activity of Flower Extracts of Coli Forms Sphaeranthus Indicus on. *J of Experimental Bio* 2011; **2** (3): 513-6.
3. Chelliapan S, Wilby T, Sallis PJ. Performance of an up-flow anaerobic stage reactor (UASR) in the treatment of pharmaceutical wastewater containing macrolide antibiotics. *Water res* 2006; **40**: 507 – 516.
4. Aydin S, Cetecioglu Z, Arikan O, *et al*. Inhibitory effects of antibiotic combinations on syntrophic bacteria, homoacetogens and methanogens. *Chemosphere* 2015; **120**: 515–520.
5. Doorslaer XV, Dewulf J, Langenhove HV, *et al*. Fluoroquinolone antibiotics: An emerging class of environmental micropollutants. *Sci of The Total Environ* 2014; 250–269.
6. Ladirat SE, Schols HA, Nauta A, *et al*. High-throughput analysis of the impact of antibiotics on the human intestinal microbiota composition. *J of Microbiol Meth* 2013; **92**: 387–397.
7. Cetecioglu Z, Ince B, Orhon Derin, *et al*. Acute inhibitory impact of antimicrobials on acetoclastic methanogenic activity. *Bioresource Tech* 2012; **114**: 109–116.
8. Amin MM, Hashemi H, Ebrahimi A, *et al*. Effects of oxytetracycline, tylosin, and amoxicillin antibiotics on specific methanogenic activity of anaerobic biomass. *Int J of Environ Health Engineering* 2012*;* **1**(5): 3-4.
9. Heidari M, Nabavi BF, Saffari H, *et al*. Inhibition effects of antibiotics ampicillin and gentamycin on the methanogenic activity of anaerobic biomass. *Int J of Environ Health Engineering* 2012; **1** (3): 2-4.