ORIGINAL PAPER 원저

# Benzalkonium Chloride가 돈슬러리의 바이오가스 생성에 미치는 영향

박혜림, 최홍림†, Arumuganainar Suresh
서울대학교 농생명공학부

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# Effect of Benzalkonium Chloride on Biogas Potential of Pig Slurry

H. R. Park, H. L. Choi†, A. Suresh

Department of Agricultural Biotechnology and Research Institute for Agriculture and Life Sciences, Seoul National University, Seoul, Republic of Korea, 151-921

## ABSTRACT

Benzalkonium chloride is most widely used in S. Korea as a disinfectant in livestock husbandry. Inhibition of biogas potentials were investigated with three different doses of benzalkonium chloride in swine slurry. The system was operated at batch mode. The inhibition rates were 10%, 30~40% and >70% at the dose of 10ppm, 40ppm and 80ppm, respectively assuming it was zero percent in case of no dose. Enzymatic activities were analyzed to determine the enzymatic type which was inhibited by benzalkonium chloride. The acid phosphatase, alkaline phosphatase and protease were shown negatively correlated with biogas potential. Correlation of $\alpha$-glucosidase and biogas potentials was observed not high ($p<0.01$, $r=-0.426$) while benzalkonium chloride ($r=-0.853$, $p<0.01$) and acid phosphatase ($p<0.01$) with biogas potentials were significantly and negatively correlated. The effect of benzalkonium chloride on *Escherichia coli* were also evaluated by disc diffusion method. As increase of benzalkonium concentration, inhibition zone of anaerobic bacteria was extended. It revealed that benzalkonium chloride significantly deteriorated biogas potential through inhibition of acetogenic bacteria.

Keywords : Benzalkonium chloride, Biogas, Methane, Enzymatic activity

## 초 록

본 연구에서는 benzalkonium chloride 처리에 따라 바이오가스 생산이 억제되는 정도를 평가하였다.

†Corresponding author(ulsoo8@snu.ac.kr)

바이오가스 생산 억제 수준은 10 ppm, 40 ppm, 80ppm의 benzalkonium chloride가 처리되었을 때 각각 10%, 30−40%, 70% 이상이었다. Benzalkonium chloride의 처리에 따라 저해되는 효소를 알아내기 위하여 효소 활성을 분석하였으며 산성/알칼리 포스파타아제, 프로테아제는 메탄 생산량과 음의 상관관계를 나타내었다. α−글루코시다아제는 실험기간 동안 메탄 생성량과 상대적으로 낮은 음의 상관성을 보였으며($p<0.01$, $r=-426$), 다른 효소와의 상관성도 상대적으로 낮았다. 메탄생성률(ml/day)은 benzalkonium chloride및 산성 포스파타아제와 유의한 상관성을 나타내었다. Benzalkonium chloride가 대장균에 미치는 영향을 원판확산법을 통하여 분석하였다. Benzalkonium chloride의 농도가 높을수록 세균증식 억제대가 확장되었으며, 이를 통하여 benzalkonium chloride가 초산생성균의 증식을 억제함으로써 혐기소화조에 영향을 미칠 수 있다는 것을 확인하였다.

핵심용어 : benzalkonium chloride, 바이오가스, 메탄, 효소활성

## 1. INTRODUCTION

In South Korea, the swine industry is growing rapidly as the intensive farming becomes prevalent. Almost 9.6 million pigs are raised and each pig produces 8.6 kg/day of wastewater, feces and urine. Although pig slurry is considered as wastewater, it can be used as potential organic resources through anaerobic digestion. Anaerobic digestion is biochemical transformation process decomposed biomass to methane and carbon dioxide under the several anaerobic stages with complex series of microbial interactions. Anaerobic digestion produces a useful energy, methane, and a stabilized residue that can be subsequently applied to land as a soil amendment[1]. However, anaerobic digestion usually requires some circumstances, modest temperatures, ambient pressures, neutral pH and absence of electron acceptors like oxygen, nitrate and sulfate[2]. In consideration of sensitivity of anaerobic digestion, operating parameters of anaerobic digestion are dependent on the influent slurry condition.

The characteristics of swine slurries are heterogeneous due to the condition of swine herds, type of housing, surrounding environment of farm and several addictives. A number of swine farms use artificial additives like disinfectants, antibiotics and cleaning agents which have negative influence on the wastewater treatment process[3]. Thus stabilization step as a pretreatment process of swine slurry can be important as swine slurry is irregular and changeable. Particularly, disinfectants usually applied to disinfect animal buildings or sprayed against infectious diseases several times in a year; appear intermittently in the liquid manure. Thus, this liquid manure may transfer rather high loads of inhibitors to biogas plants and this can cause inhibition of biogas production[4].

In South Korea, various disinfectants are used at swine farms such as phenols, halogens, aldehydes and quaternary ammonium compounds. Among these kind of disinfectants, quaternary ammonium compound covers a wide target range with bacteria, virus, fungi and algae, and used for several purposes like disinfecting animal houses, drinking water, facilities, implements and animal itself. Quaternary ammonium compounds are not readily biodegradable materials[5] and are not

affected much by pH or temperature. In practically, quaternary ammonium compounds have been used large amount in the swine farms (792 ton) among the various kinds of disinfectants in South Korea, 2006. In accordance with these reasons, quaternary ammonium compound has been selected for this study and observed the inhibition of nutrients breakdown pathway in anaerobic digestion.

Quaternary ammonium compounds can be clustered into 4 groups as alkyl or hydroxyalkyl substituted, non-halogenated benzyl substituted, di- and tri-chlorobenzyl substituted and quaternary ammonium compounds with unusual substitutes. However, alkyl dimethyl benzyl ammonium chloride (benzalkonium chloride) served as the model compound for pesticide[6].

Benzalkonium chloride is a clear yellow to straw colored liquid with an amine odor and is soluble in water and alcohols. It is hydrolytically stable under abiotic and buffered conditions over the pH 5-9 range[6]. It is commonly used in agricultural premise and equipment like swine farms, farrowing barn and animal housing facilities as various purposes like algicide, bactericide, fungicide, viricide, microbiocide, sanitizer and disinfectant. Eventually it enters into the slurry storage tank and inhibits further application of swine slurry such as biogas production.

The aim of this paper is to investigate the effect of benzalkonium chloride, a kind of quaternary ammonium compound, on the anaerobic digester productivity by applying biochemical methane potential (BMP) test.

## 2. MATERIALS AND METHODS

### 2.1 Experimental setup

Biochemical methane potential (BMP) was observed for swine slurry in serum bottles (125 ml). The swine slurry was collected from the test farm of Seoul National University, Suwon. The samples were seeded with sludge collected from anaerobic digester of Tan-cheon Sewage Treatment Plant. The compositions of inoculums were 2.5% total solids (TS) and 1.5% volatile solids (VS). Anaerobic digestion was conducted at 6 different concentration of benzalkonium chloride at 0, 5, 10, 20, 40 and 80 ppm. The samples for testing at each concentration were filled with 10% of inoculums[7] and swine slurry. The samples were flushed with $N_2$(70%) and $CO_2$(30%) to remove air contamination and closed with butyl rubber stoppers and aluminum seals. The experiments were carried out 70 days at 35 °C to evaluate anaerobic digestion progress and performed in duplicate for each concentration.

### 2.2 Kinetic tests

The total biogas yield was measured with an airtight glass syringe (100 ml) and the composition of biogas were analyzed by gas chromatography (Agilent, HP 6890N) using 60/80 Carboxen-1000 packed column with TCD detector at 250°C. The 0.6ml gas sample was injected, at 50°C inlet temperature, and oven temperature at 35°C (5 min) to 225°C at 20°C/min. The helium was used as carrier gas at 30 ml/min. The liquid samples were analyzed for pH, TS, and VS according to APHA (2005) method[8].

### 2.3 Enzymatic activity

The $\alpha$-glucosidase activity were analyzed[9] with 4 ml of 0.2M Tris-HCl buffer (pH 7.6), 2 ml of sample, 2 ml of substrate (0.1% $\rho$

-nitrophenyl α-D glucopyranoside) and incubated for 60 min at 37℃. The protease activity was measured by Ladd and Butler[9]. The 5ml of sample treated with 5 ml Tris-HCl buffer (0.05M, pH 8.1) and 5 ml sodium caseinate (2%) as substrate and samples were incubated in a water bath at 50℃ for 2 hours. Then 5 ml TCA (15%) and 7.5 ml alkaline reagent were added to samples followed by addition of Folin reagent (33%) and measured absorbance at 700 nm. Phosphatase activity was measured with the phosphomonoesterase at pH 6.5 and pH 11 and p-nitrophenyl phosphate as substrate described by Tabatabai and Bremner (1969) and Eivazi and Tabatabai (1977)[9].

### 2.4 Statistical analysis

Microsoft Office Excel 2007 was used to analyze the experimental data for graphing purposes. Pearson product-moment correlation coefficients (r) were calculated using Statistical Package for the Social Sciences software ver. 13.0 (SPSS, 2004)[10] to show the relationship between parameters.

### 2.5 Growth of *E. coli* and mixed culture on plate

Inhibitory effects of benzalkonium chloride on cell growth were analyzed by a diffusion disk method. Tryptic soy Agar (DIFCO) plates and broth tubes were prepared and the plates were air-dried for overnight. *E. coli* culture was isolated from swine slurry by Chromocult coliform Agar (DIFCO) and a mixed culture was isolated by inoculating swine slurry in a 5ml respective broth tube and was incubated at 37°C for 24h. Diffusion disks were prepared with Whatman filter papers and treated with different concentration of benzalkonium chloride and chloramphenicol. *E. coli* and mixed culture were spread over separate plates by sterile swaps and the plates were dried for 10min. The disks were placed by sterile forceps subsequently and were incubated at 37°C for 24h.

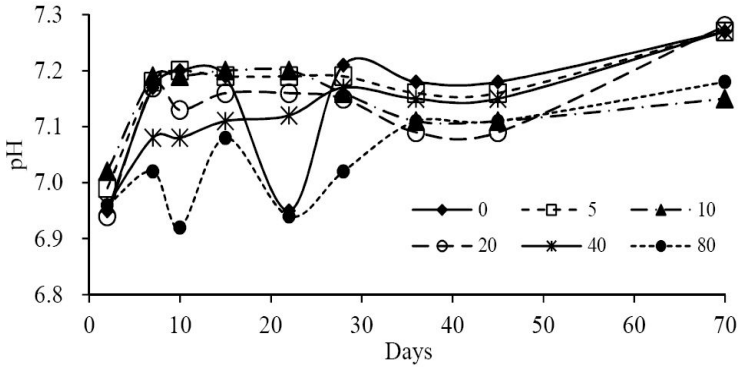
## 3. RESULTS AND DISCUSSION

### 3.1 Kinetic changes of pH and Biogas

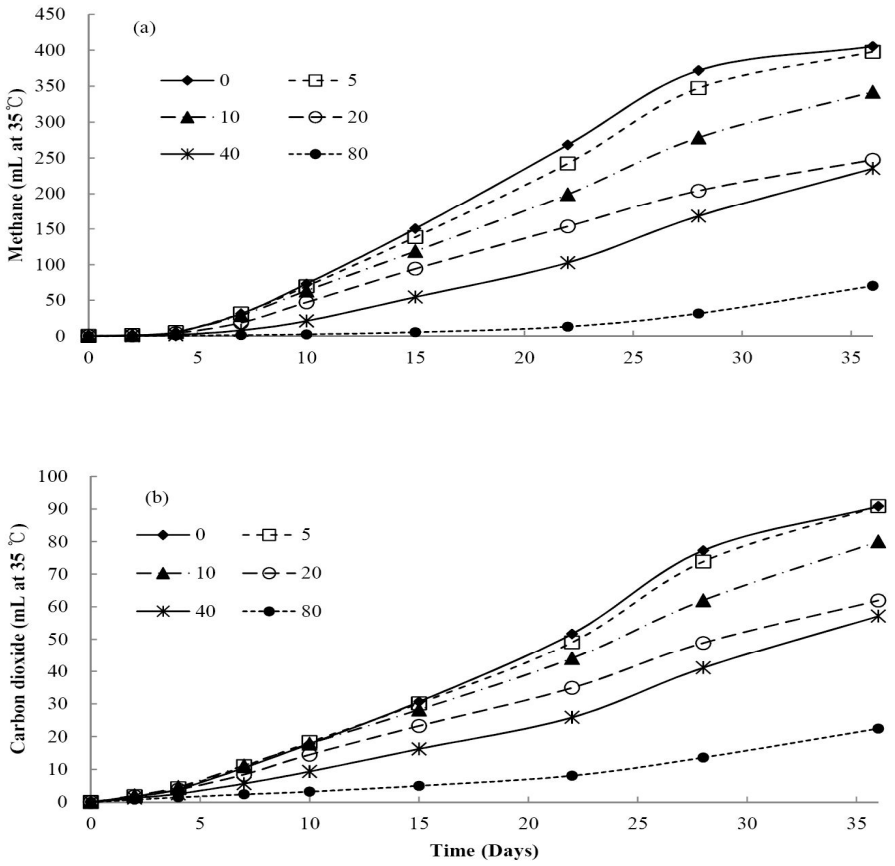
The effect of benzalkonium chloride on the pH is shown in **[Fig. 1]**. The differences in the pH were not obvious among the different experimental conditions. As the optimal pH range of methanogen is 6.4 to 7.4[3], this pH condition did not affect significantly to the growth of relative microbes.

The relative biogas production at different concentrations of benzalkonium chloride in the digestion of swine slurry is shown in **[Fig. 2]**. Relative methane production volume to control with 5, 10, 20, 40 and 80ppm of benzalkonium chloride was 98.9%, 91.8%, 71.2%, 64.6% and 25.6% **[Table 1]**. With the 5 to 10ppm of benzalkonium chloride, the methane production inhibited slightly; approximately 90% of the methane produced at control. Meanwhile, the decrease in methane was considerably large at benzalkonium chloride between 20ppm and 80ppm. Methane production decreased gradually during all experimental period. These decreases were coupled with decreases in volatile solids **[Table 1]**.

The accumulated methane volume during 70 days incubation was similar between 20ppm and 40ppm, but peak time with 40ppm of benzalkonium chloride treated was relatively delayed and the maximum methane volume was only 63% in compare with the control.




[Fig. 1] Changes of pH in swine slurry with different concentration of benzalkonium chloride (ppm) in anaerobic digestion.




[Fig. 2] Methane. (a) and carbon dioxide (b) production profile during the batch inhibition assay with benzalkonium chloride at different concentration (0–80 mg/L).

[Table 1] Effects of Benzalkonium Chloride on Methane Production (day 45)

| | Gas production volume (ml) | | Comparing to control (%) | | Solid reduction rate (%) | |
|---|---|---|---|---|---|---|
| | $CH_4$ | $CO_2$ | $CH_4$ | $CO_2$ | Total solids | Volatile solids |
| Control | 415.86 | 96.37 | 100.0 | 100.0 | 26.8 | 40.6 |
| 5ppm | 411.26 | 97.89 | 98.9 | 101.6 | 25.5 | 39.9 |
| 10ppm | 381.65 | 91.24 | 91.8 | 94.7 | 25.4 | 38.5 |
| 20ppm | 296.08 | 74.68 | 71.2 | 77.5 | 21.0 | 31.9 |
| 40ppm | 268.84 | 66.77 | 64.6 | 69.3 | 20.3 | 30.3 |
| 80ppm | 106.50 | 30.02 | 25.6 | 31.2 | 9.4 | 14.2 |

With the 80ppm concentration of benzalkonium chloride reduced the biogas production significantly, approximately 75%, and the methane production was not recovered until the end of the experiment. Therefore, the high benzalkonium chloride concentrations over than 80ppm would break down the anaerobic digestion system. The productions of carbon dioxide were similar with methane productions profile and it reveals that the fermentative microbes were also inhibited.

### 3.2 Enzymatic activity and statistical analysis

Main organic materials of swine slurry are proteins, carbohydrates and lipids. To degrade organic matters and to produce methane, hydrolysis, acidogenesis and methanogenesis steps should be proceeding and the rate-limiting process is hydrolysis[11),12)]. Though a number of enzymes are involved in degradation of organic matters such as $\alpha$-glucosidase, protease and phosphatase, those enzymes are specific for carbohydrates, proteins and phosphate group, respectively. This enzymatic activity could provide a measure of the velocity in the initial process to break macromolecules down to methane and carbon dioxide[13)]. Especially, phosphatase analyzed 2 types as its optimal pH range, acid phosphatase and alkaline phosphatase.

Correlation analysis between all the variables evaluated in this study was carried out and observed significant correlation at $p<0.01$ **[Table 2]**. As substrates were degraded during the incubation period, acid phosphatase, alkaline phosphatase and protease showed negative correlation values with accumulated methane volume. The $\alpha$-glucosidase was shown considerably low negative correlation ($p<0.01$, $r=-0.426$) **[Table 2]**.

Benzalkonium chloride effects could be evaluated by methane production rate (ml/day), at $r=-0.853$ ($p<0.01$) **[Table 3]**. The activity of acid phosphatase was slightly influenced by benzalkonium chloride ($p<0.05$) and showed a significant correlation value with methane production rate (ml/day, $p<0.01$, $r=-0.519$) and methane contents ($p<0.01$, $r=-0.667$). The $\alpha$-Glucosidase showed low level of correlation with benzalkonium chloride concentration ($p<0.01$, $r=0.394$) and methane production rate ($p<0.01$, $r=-0.365$), but it did not show any relationship with other enzymatic activities **[Table 3]**.

Thus, when the pH was at about 7, the acid phosphatase can show activity in an anaerobic digester. Moreover as pH increasing, the

[Table 2] Correlation Value Between Kinetic Parameters and Enzymatic Activity

| | | Methane (accumulation) | Carbon dioxide (accumulation) | TS | VS | Acid Phosphatase | Alkaline Phosphatase | Glucosidase | Protease |
|---|---|---|---|---|---|---|---|---|---|
| Methane (accumulation) | Pearson Correlation | 1 | .994(**) | −.888(**) | −.941(**) | −.779(**) | −.717(**) | −.426(**) | −.766(**) |
| | Sig. (2−tailed) | | .000 | .000 | .000 | .000 | .000 | .000 | .000 |
| Carbon dioxide (accumulation) | Pearson Correlation | | 1 | −.893(**) | −.945(**) | −.798(**) | −.754(**) | −.424(**) | −.787(**) |
| | Sig. (2−tailed) | | | .000 | .000 | .000 | .000 | .000 | .000 |
| TS | Pearson Correlation | | | 1 | .514(**) | .490(**) | .489(**) | .402(**) | .514(**) |
| | Sig. (2−tailed) | | | | .000 | .000 | .000 | .000 | .000 |
| VS | Pearson Correlation | | | | 1 | .758(**) | .660(**) | .459(**) | .716(**) |
| | Sig. (2−tailed) | | | | | .000 | .000 | .000 | .000 |
| Acid Phosphatase | Pearson Correlation | | | | | 1 | .880(**) | .444(**) | .931(**) |
| | Sig. (2−tailed) | | | | | | .000 | .000 | .000 |
| Alkaline Phosphatase | Pearson Correlation | | | | | | 1 | .532(**) | .905(**) |
| | Sig. (2−tailed) | | | | | | | .000 | .000 |
| Glucosidase | Pearson Correlation | | | | | | | 1 | .534(**) |
| | Sig. (2−tailed) | | | | | | | | .000 |
| Protease | Pearson Correlation | | | | | | | | 1 |
| | Sig. (2−tailed) | | | | | | | | |

** Correlation is significant at the 0.01 level (2−tailed).

alkaline phosphatase showed higher correlation than the acid phosphatase. Such a result is on common ground with that phosphatase activity in anaerobic digesters, and it appears to be rapid biochemical test for predicting the instability or failure of anaerobic sludge digestion[14].

### 3.3 Benzalkonium chloride effects on *E. coli* and mixed culture

Benzalkonium chloride affects the growth of *E. coli* (acetogen) under anaerobic condition in swine slurry was significant as observed reduced cell number. The *E. coli* counts were almost the same (100 CFU/ml) on day 0 **[Fig. 3]** in control, 10ppm and 80ppm. However, the second day samples showed drastic decrease in the counts as 30, 15, and 10 CFU/ml in control, 10 ppm and 80 ppm, respectively. Whereas *E. coli* was observed in control only at 20 CFU/ml at day 3. It revealed that the benzalkonium chloride affected *E. coli* to some extent. To evaluate the impact of benzalkonium chloride on *E. coli* and mixed culture from

[Table 3] Enzyme Activity related with Methane Production Rate from day 7 to 28

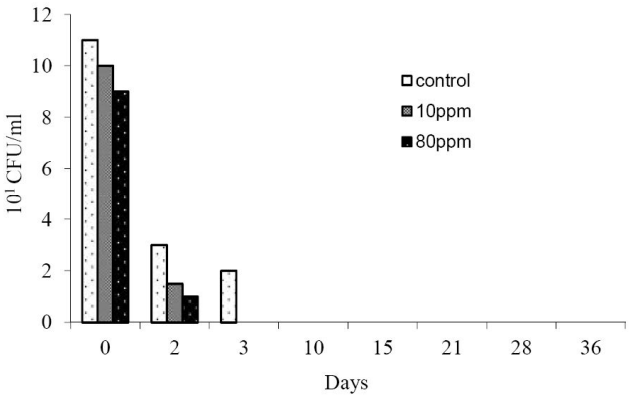
| | | ppm | ml/day | Methane (%) | Acid Phosphatase | Alkaline Phosphatase | Glucosidase | Protease |
|---|---|---|---|---|---|---|---|---|
| ppm | Pearson Correlation | 1 | −.853(**) | −.746(**) | .244(*) | −.144 | .394(**) | .097 |
| | Sig. (2−tailed) | | .000 | .000 | .021 | .176 | .000 | .363 |
| ml/day | Pearson Correlation | | 1 | .874(**) | −.519(**) | .058 | −.365(**) | −.371(**) |
| | Sig. (2−tailed) | | | .000 | .000 | .587 | .000 | .000 |
| Methane (%) | Pearson Correlation | | | 1 | −.667(**) | −.057 | −.258(*) | −.529(**) |
| | Sig. (2−tailed) | | | | .000 | .592 | .014 | .000 |
| Acid Phosphatase | Pearson Correlation | | | | 1 | .438(**) | −.063 | .770(**) |
| | Sig. (2−tailed) | | | | | .000 | .555 | .000 |
| Alkaline Phosphatase | Pearson Correlation | | | | | 1 | −.092 | .549(**) |
| | Sig. (2−tailed) | | | | | | .390 | .000 |
| Glucosidase | Pearson Correlation | | | | | | 1 | −.017 |
| | Sig. (2−tailed) | | | | | | | .872 |
| Protease | Pearson Correlation | | | | | | | 1 |
| | Sig. (2−tailed) | | | | | | | |

** Correlation is significant at the 0.01 level (2−tailed).
* Correlation is significant at the 0.05 level (2−tailed).

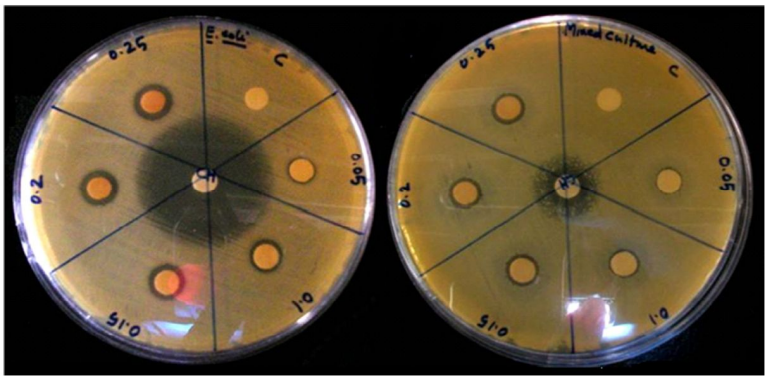
swine slurry, those cultures were spread on tryptic soy agar plates and placed different concentrations (50, 100, 150, 200, and 250 mg) of benzalkonium chloride disk. Both the mixed culture and *E. coli* growth were significantly affected as the inhibition zones were measured in diameters of 7, 7.5, 8.5, 9, and 9.5 mm on mixed cultures and 7.5, 8.5, 9, 9.5, and 10mm on *E. coli* culture according to benzalkonium chloride concentrations **[Fig. 4]**. The inhibition zone represents the sensitivity of cultures to a particular concentration of benzalkonium chloride. The known antibiotic chloramphenical showed 16 and 30 mm diameter of inhibition zone on mixed culture and *E. coli*, respectively.

## 4. CONCLUSION

Significant effects were observed in biogas production with addition of benzalkonium chloride in swine slurry. With 5−10ppm of benzalkonium chloride inhibited biogas production about 10% of total volume, 20−40 ppm inhibited 30% to 40% comparing with control treatments, whereas 80 ppm inhibited >70%. The biogas recovery was noticed at 20−40 ppm after 30 days. Enzymatic activity was analyzed for decomposition process of organic matters. The acid phosphatase,




[Fig. 3] Benzalkonium chloride effects on E. coli growth.




[Fig. 4] Plates of E.coli and mixed culture showing inhibitory zones at different concentrations of benzalkonium chloride.

alkaline phosphatase and protease showed a significant correlation with accumulated methane volume. The methane production rate (ml/day) and methane contents were influenced significantly by acid phosphatase. *E. coli* and mixed cultures were shown sensitivity against benzalkonium chloride as inhibition zone increased with concentration of benzalkonium chloride. This result suggests benzalkonium chloride could affect acidogenic bacteria in the anaerobic digester and inhibit the biogas production.

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