

Assessment of Pathogen Reduction Potential of an Anaerobic Digester containing Winery Wastewater

Vincent I. Okudoh, Benjamin I. Okeleye, Ronny M. Ngwe and Seteno K.O. Ntwampe

Abstract: In this study, assessment of the pathogenic bacteria reduction capacity of an anaerobic digester (AD) containing winery wastewater (WWW) was carried out. Samples were collected from a winery drainage system (influent) and a bioreactor after digestion (effluent) at the Agricultural Research Council (ARC), Stellenbosch, South Africa. Qualitative analysis was by means of pH, optical density (OD) and most probable number (MPN) techniques. The isolates were identified using biochemical and polymerase chain reaction (PCR) methods. Results showed an increase in pH from 6.71 to 6.97 with MPN index per 100 mL reduction of presumptive coliforms from >1,100 to 313.3 after digestion at 95% confidence interval (CI), respectively. A 3 log removal efficiency (99.9%) of *Escherichia coli*, *Salmonella spp.*, *Azomonas spp.* and *Aeromonas hydrophila* was achieved after 30 days retention time in the bio-digester. The significant reduction of these surrogate pathogens during the process of biogas production is of importance to mitigate against environmental health concerns.

Keywords—Anaerobic digestion, biogas, methanogens, pathogen reduction

I. INTRODUCTION

Of all challenges faced by many countries of the world today, none has become more urgent than improper waste management practices and depletion of natural resources aggravated by increasing population and urbanization [1]. Domestic and industrial wastes contain toxicants and pathogenic microorganisms that are detrimental to ecosystem and human life [2]. Anaerobic digestion (AD) has been reported to be effective in wastes treatment with plausible economic potential of the biogas produced as a source of renewable energy and fertilizer [3].

Waste treatments consist of the removal of microorganisms, organic and inorganic components, using aerobic or AD oxidation ponds including trickling bed filters subsequent to filtration for removal of residual pollutants, and disinfection procedures using chlorine, UV radiation, or ozone for deactivation of microorganisms [2], [4]. All the downstream processes are highly dependent on the functionality of the AD, as a primary process for organic matter reduction. AD process is a fermentation process, through the action of anaerobes; that

decompose the decomposable organic matter in low redox potential, in a close system, without the supply of oxygen. Bacteria are therefore directly or indirectly linked in a symbiotic way; where the product of one provides the substrate for others. The four basic processes involved in anaerobic conversion of organic matter are hydrolysis, acidogenesis, acetogenesis and methanogenesis as shown in Fig. 1.

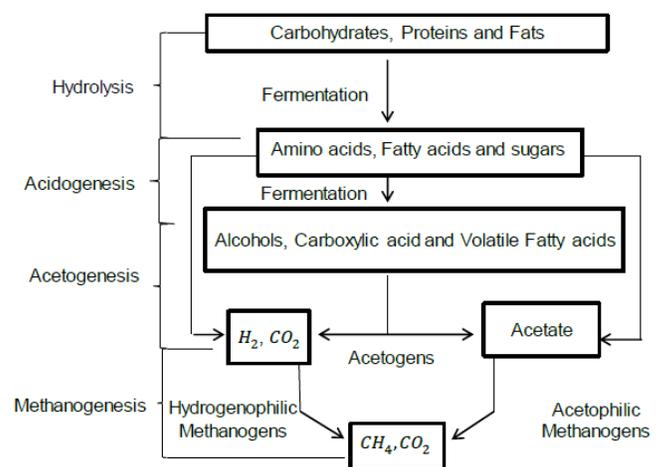


Fig. 1 Biochemical stages in anaerobic digestion [2], [5]

The survival of microbes in AD depends on this interaction, and the environmental factors in which they are exposed. Bacteria are classified into different groups, according to their ability to use the organic matter available, at varying temperature, pH range, and salinity. AD operates well at meso and thermophilic conditions within a pH range near neutral [5]. A low redox potential environment is required to stabilize growth of facultative anaerobes through the use of organic acids available. Microbes that are involved in the hydrolysis of organic matter can produce substrates suitable for acidogens. The acidogens also yield products that favoured the growth of acetogens that ferment organic acids and lower dissolved hydrogen (H_2) gas concentration to create a suitable environment for methanogens for H_2 , CO_2 and acetic acid consumption for the production of methane gas [1]. In AD, the proliferation of pathogens is high, as most constitute the sludge used in AD technologies.

Pathogens are microbes that have the ability to cause disease in hosts. They have a direct interaction with the surrounding environment, and represent a risk to plants and animals through oral and cutaneous pathways. Validation and efficacy of pathogen reduction or removal in the AD is therefore vital to accomplish a safe environment [6].

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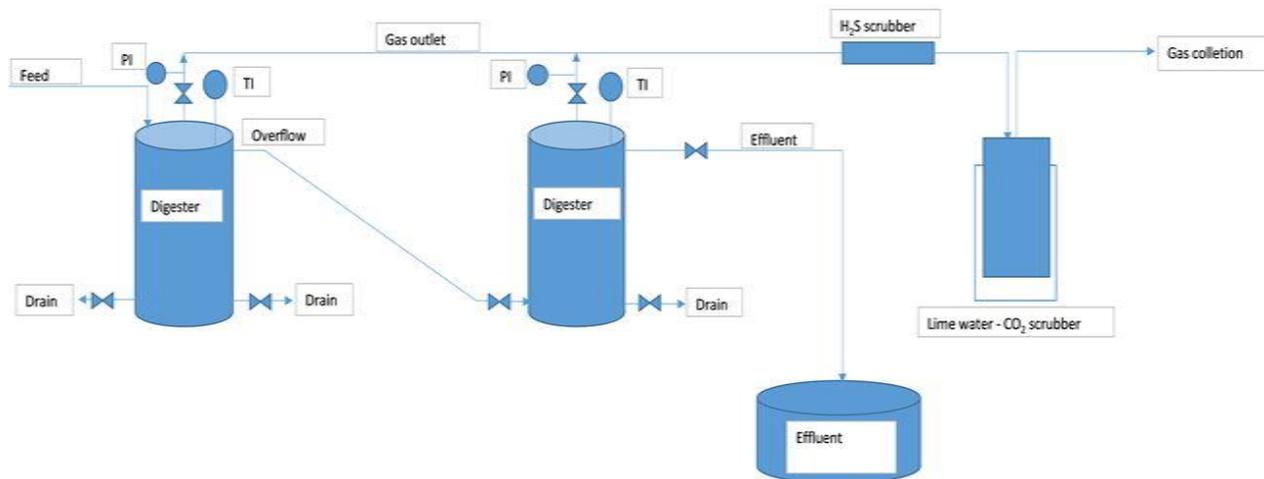


Fig. 2. Design of the anaerobic digester used for biogas production.

About 35% of municipal and 13% of agricultural waste were produced in the Western Cape Province South Africa in the year 2014. The indiscriminate disposal of untreated winery wastewater (WWW) directly into the environment by some winery farms constitutes a danger to public health due to its potential to support proliferation of pathogenic bacteria which can result in the outbreak of emerging diseases [7]. A technique that will effectively facilitate biogas production from the winery waste, reduce the density of microbial pathogen proliferation, as well as to contribute to the improvement of soil fertility used for cultivar growth and crop's yield, is therefore needed. To the best of our knowledge, there is no known study that reported on the pathogen surveillance of the winery wastewater generated on Agricultural Research Council (ARC), Nietvoorbij farm, Stellenbosch South Africa. The present study aims to measure the rate of reduction in the density of some specific pathogenic bacteria and to identify the microorganisms involved in AD containing WWW for biogas production.

II. MATERIALS AND METHODS

A. Design and Construction of Anaerobic Digester

Little Green Monster anaerobic digesters were commercially purchased from Sustainable Engineering Solutions (Pty) Ltd, and used in the AD plant design and construction in the Biotechnology and Chemical Engineering Departments, Cape Peninsula University of Technology. The AD was designed for the production of biogas (methane) and built at proximity to the winery wastewater (WWW) drainage system at Agricultural Research Council (ARC), Nietvoorbij farm, Stellenbosch South Africa (Fig. 2 and 3).

B. Sample Collection and Preparation

Samples were collected from the ARC winery wastewater (WWW) drainage system (influent) and the AD (effluent) designed for post organic matter reduction. About 120 mL of wastewater samples were collected between 6th and 20th April 2016, in triplicate, into 250 mL clean glass bottles and stored in ice box before transported to the Biotechnology laboratory for analysis. The samples were analyzed within 16 h of collection

and under aseptic conditions according to standard methods for the examination of water and wastewater [8].



Fig. 3. A, Winery drains (Influent) and **B,** AD (effluent) at ARC

C. Physicochemical Analysis

The physicochemical analysis was done according to the method of Igbinosa and Okoh [9], with some modifications. The pH analysis was carried out by means of standard pH meter (YSI, USA). About 60 mL of WWW was measured into a 100 mL beaker and stirred before measuring the pH. A spectrophotometer adjusted at 600nm wavelength was used to determine the optical density or turbidity of the samples. Distilled water was used as a blank and the sample readings were recorded.

D. Multiple-Tube Fermentation (MTF) Assay

Assay for faecal contamination and total coliforms were conducted with multiple-tube fermentation (MTF) technique and the results were expressed in terms of the most probable number (MPN), which provides statistical estimate of total coliforms present in the sample. A series of double strength Brilliant Green Bile (BGB) broth tubes were inoculated with 0.1 mL, 1.0 mL, and 10.0 mL dilutions of the WWW sample in triplicate. A positive presumptive test showed production of gas in the Durham tube after 24 h of incubation at 37 °C. Tubes with positive presumptive tests were subjected to confirmation test in 3 single strength BGB broth (and peptone water) containing Durham tubes. A positive confirmation tube was accompanied by formation of a gas in single strength BGB broth within 48 h

E. Cultural Counts and Preliminary Identification

Tenfold serial dilution up to 10⁻⁶ of the WWW was prepared in sterile water. Each dilution was plated onto Nutrient Agar (NA) plates and incubated at 37 °C for 24 h. The diversity of colony morphologies on NA was compared across the different dilution treatments and the number of colony-forming unit per mL (CFU/ mL) was determined after 2 days. Twelve colonies were randomly chosen from each plate and colony morphology was described based on size, elevation, form, and pigmentation. Gram stain, Kinyoun stain, and biochemical tests; i.e. carbohydrate alcohol fermentation, indole production, starch hydrolysis, methyl red and Voges proskauer tests, gelatin hydrolysis, oxidation fermentation production, lipid hydrolysis, litmus milk, lysine decarboxylase, urease test, citrate utilization and nitrate reduction were used as presumptive identification methods for pure isolates.

The log removal value (LRV) was calculated using the following equation 1:

$$LRV = \log_{10} \frac{\text{Influent Pathogen Concentration}}{\text{Effluent Pathogen Concentration}} \quad (1)$$

F. Molecular Identification Using PCR

Deoxyribonucleic acid (DNA) extracted from a 10-12 h old isolate was used for polymerase chain reaction (PCR) analysis according to the method reported by Deshmukh et al. [10]. Briefly, two to three colonies suspended in 200 µL of Lysis buffer with 0.3g of sterile glass beads were vortexed (3 min) and incubated in water-bath at 37 °C for 1 h. After cooling and centrifugation at 10000 x rpm (5min) the supernatant was treated with isopropanol and incubated for 10min. The pellet was suspended in Tris-EDTA (TE, 400 µL) and Bovine Pancreatic Ribonuclease A (RNase A, 3µL) after centrifugation, subsequent to incubation for 5 min (37 °C). The extracted DNA was precipitated with 100 % ethanol (1 mL) and the pellet was further washed twice with 70 % ethanol before re-suspension in 50µL of TE prior to PCR analysis. The PCR assays were carried out using a MyCycler™ thermal cycler, with a reaction mixture of 2.5 µL of DNA sample, 2.5 µL of 10 x amplification buffers, 0.5 µL of 20 µM of forward and reverse primers 16F27 (5'-CCAGAGTTTGATCMTGGCTCAG-3') and 16R1488 (5'-AGAGTTTGATCMTGGCTCAG-3'), 0.5 µL of 20 mM solution of four dNTPs, 18.375 µL of H₂O, 0.125 µL of 1-5 units thermostable DNA polymerase. The cycle number was set according to 1 cycle with denaturation for 2 min (95 °C), 35 cycles with denaturation for 1 min (95 °C), annealing for 30 sec (50 °C) and polymerization for 2 min (72 °C). About 5-10 µL of the mixture and molecular marker was withdrawn to visualize the DNA under Ultra Violet (UV) light through agarose gel with ethidium bromide.

III. RESULTS AND DISCUSSION

A. Physicochemical Characterization

Winery wastewater (WWW) samples collected from the inlet (influent) and outlet (effluent) of an anaerobic digester was analyzed for their acidity and basicity (pH). The influent water indicated pH of 6.73, 6.71 and 6.69 (3 samples). The samples were acidic due to the presence of organic acids found in pressed grapes (malic and tartaric acid) that lowered the pH of the wastewater used during the process of cleaning the materials in the winery farm [11]. However, the effluent showed an increase in pH to 6.95 (sample 1) and 6.98 (sample 2 and 3) after digestion. This could be as a result of acetogenesis, which is the acidic substrate conversion of bacteria that produced ammonia; hence, an increase in the pH close to neutral (pH 7.00) [12]. The spectrophotometric analysis of turbidity of the WWW revealed that light was absorbed more in the influent (1.7-1.88) than in effluent (1.45-1.57) samples as shown in Fig. 4 and 5. This gave an indication of high organic matter concentration in the influent and a decrease in the bacteria density of the effluent. The turbidity at the exit port of the anaerobic digester decreased due to the possible inhibitory products generated during the AD process. The optical density therefore showed the reduction of suspended solids and matter contributing to the organic load in the effluent port. This corroborates the differentiation observed in the bacteria cell concentration for the influent and effluent in this study. Generally, high cell concentration of a sample, results in high turbidity [13], albeit suspended matter is also known to contribute to high turbidity, therefore the AD bed can alter a bio-filter.

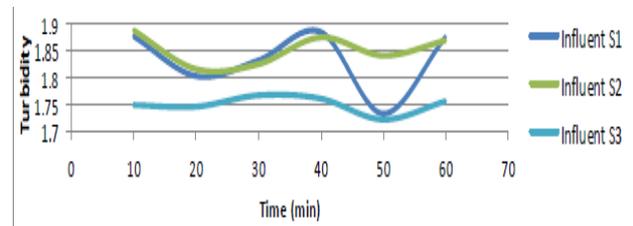


Fig. 4 Turbidity of the influent

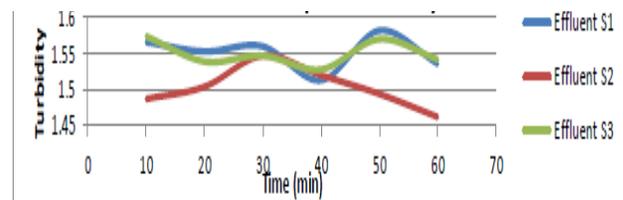


Fig. 5. Turbidity of the effluent

B. Most Probable Number of Coliforms

Most probable number (MPN) index per 100 mL reduction of presumptive coliforms was recorded from >1,100 (influent) to 313.3 (effluent) after digestion at 95% confidence interval (CI). One of the common target microorganisms that confirmed faecal contamination in water is *E. coli*. MPN of 376 per 100 mL (peptone water) and 349 per 100 mL (single strength BGB agar) were detected in the influent from the presumptive

TABLE III: BIOCHEMICAL TEST REACTION

	II 1*	II 2	II 3	II 4	II 5	EI 6	EI 7	EI 8	EI 9	<i>E. coli</i>
Carbohydrate alcohol fermentation	Glucose	-	-	+	+	+	-	+	+	+
	Sucrose	-	-	+	-	+	-	+	+	-
	Maltose	-	-	+	+	+	-	+	+	+
	Xylose	-	-	-	+	+	-	-	-	+
	Gas	No	No	Yes	No	Yes	Yes	No	Yes	Yes
Starch agar	-	-	+	-	-	+	-	-	-	-
MRVP	MR	-	+	+	+	-	+	-	-	+
	VP	-	-	-	-	-	-	-	-	-
OF	Aerobe/Facultative anaerobe	+	+	+	+	+	-	+	+	+
Citrate		+	+	-	-	-	+	+	+	-
Lipid		-	-	+	-	-	-	-	-	-
Litmus		Acid	Acid	Alkaline	Alkaline	ACR	APR	Alkaline	Acid	Acid
Nitrate		-	-	+	+	+	-	+	+	+
Gelatin		-	-	+	-	-	-	-	-	-
Urea		-	-	-	-	-	-	-	-	-
Lysine		-	-	+	+	+	-	-	-	-
Indole		-	-	+	-	+	-	-	-	+

*II, Influent Isolate; EI, Effluent Isolate; ACR, Acid Curd Reduction; APR, Alkaline Peptonization Reduction; +, Positive; -, Negative

coliforms test in VRB agar plate counts as shown in Table I. These findings revealed that the drainage water from the winery farm had been contaminated with enteric pathogens, which is an indication of possible faecal contamination or poor water sanitation [10], similar to the study conducted by Kayembe et al. [14]. It was observed that *E. coli* was only identified in the influent and not in the effluent, which confirmed the reduction of *E. coli* colonies during the anaerobic process.

TABLE I: COMPARATIVE PRESUMPTIVE COLIFORMS TO THE MPN TABLE

Influent samples	MPN index per 100 mL	95% confidence limits	
		Lower	Upper
1	>1, 100	>150	>4, 800
2	>1, 100	>150	>4, 800
3	>1, 100	>150	>4, 800
Effluent samples			
1	240	36	1, 300
2	460	71	2, 400
3	240	36	1, 300

C. Viable Bacteria Counts and Biochemical Test

Bacteria in the ten-fold serially diluted WWW were enumerated on both pour and spread agar plates and expressed as colony forming unit per mL (CFU/ mL). Nine (9) colonies were randomly selected, from both influent and effluent, during spread plate (SP) and pour plate (PP) counts. Presumptive bacteria isolates were detected by means of staining techniques and biochemical reactions (Table III). These are the recommended methods to identify and enumerate frank and opportunistic bacterial pathogens in water and biofilms [15]. As shown in Table II, effluent bacterial concentration (EBC) was reduced to 2.5×10^5 (SP) and 2.3×10^5 (PP) from 6.7×10^5 (SP) and 7.6×10^5 (PP) of influent bacterial concentration (IBC) at 10^{-4} dilution factor (DF), respectively. This is in line with the study conducted by Di Pinto et al. [6], who reported the isolation of *Aeromonas* spp. in the seafood as a result of water contamination. They

were isolated in 70.3% of seafood, which in recent years has received increasing attention as an agent of food-borne diseases in healthy people.

TABLE II: BACTERIAL COLONY COUNTS AND CONCENTRATION

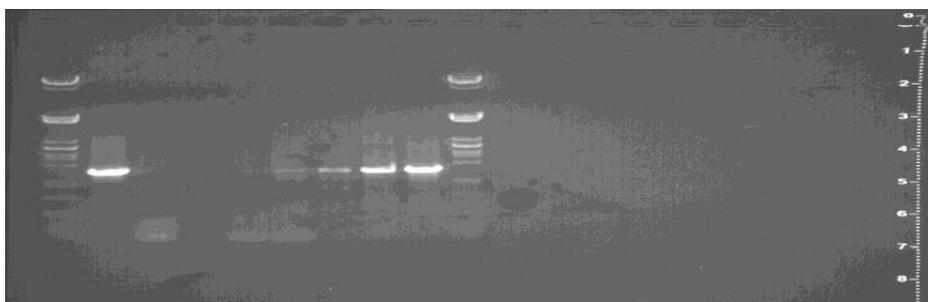
DF*	SP* (CFU/ mL)			PP (CFU/ mL)		
	10^{-2}	10^{-3}	10^{-4}	10^{-2}	10^{-3}	10^{-4}
IS	382	89	79	331	163	71
1*						
IS 2	374	101	73	313	152	83
IS 3	348	122	49	334	156	74
IBC	3.68×10^4	1.04×10^5	6.7×10^5	3.27×10^4	1.57×10^5	7.6×10^5
ES 1	246	44	23	220	91	17
ES 2	273	62	18	189	86	28
ES 3	288	47	34	194	66	24
EBC	2.69×10^4	5.1×10^4	2.5×10^5	2.01×10^4	8.1×10^4	2.3×10^5

*DF, Dilution Factor; SP, Spread Plate; PP, Pour Plate; IS, Influent Sample; IBC, Influent Bacterial Concentration; ES, Effluent Sample, EBC, Effluent Bacterial Concentration.

D. Genotypic Identification

Biochemical test assisted in the identification of bacteria isolated from the inlet and outlet of the anaerobic digester. Their interaction on different medium and preview of previous studies were significant to classify, and determine the identity of each colony [16]. The PCR analysis therefore confirmed the identity of the isolated strains. Molecular methods can identify DNA originating from many genera, containing pathogenic species and virulence genes [17]. Four (4) pathogenic bacteria strains (*Azomonas* spp., *Aeromonas hydrophila*, *Salmonella* spp., *E. coli*) were identified from influent WWW sample and one (1) from the effluent (*Enterococcus faecalis*) (Table IV, Fig. 6 and 7). According to Yergeau et al. [17], bacterial pathogens of concern that may be present in sewage are *Salmonella* spp., *E. coli* pathogenic strains, and *C. jejuni*. The sludge was less contaminated with large number of harmful bacteria colonies; albeit abundant with methanogens responsible for biogas

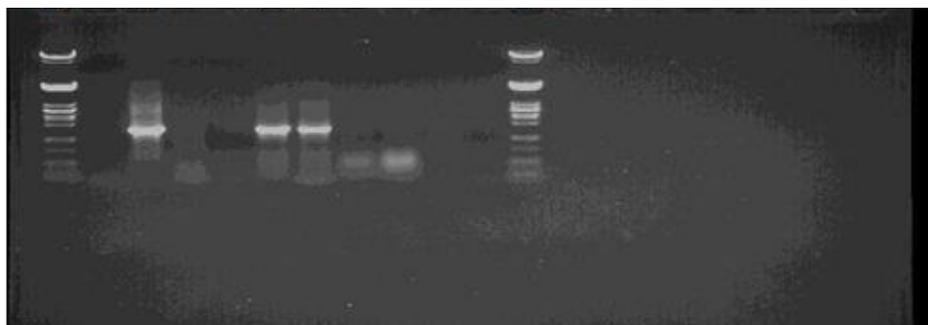
M 1a 2a 3a 4a 8a M



Gel 1 Legend:
 M: Molecular weight marker
 1a: Strain 1
 2a: Strain 3
 3a: Strain 4
 4a: Strain 5
 8a: *E. coli* VRBA

Fig.6. PCR for detection of bacteria from inlet of anaerobic digester

M 5b 6b 7b M



Gel 2 Legend:
 M: molecular weight marker
 5b: Strain 6
 6b: Strain 7
 7b: Strain 9

Fig.7. PCR for detection of bacteria from outlet of anaerobic digester

production. The rRNA based analysis is a fundamental technique in exploring the microbial diversity and to identify of strains [18], [19].

TABLE IV: BACTERIA STRAINS IN THE INLET AND OUTLET OF ANAEROBIC DIGESTER

Strains	Influent	Effluent
1	<i>Azomonas spp</i>	-
2	<i>Azomonas spp</i>	-
3	<i>Aeromonas hydrophila</i>	-
4	<i>Salmonella spp</i>	-
5	<i>Escherichia coli</i> and VRBA	-
6	-	<i>Enterococcus faecalis</i>
7	-	<i>Methanobrevibacter spp</i>
8	-	<i>Methanosarcina spp</i>
9	-	<i>Methanosarcina spp</i>

E. Removal Efficiency of Pathogens in Anaerobic Digester

The identity of bacteria found in the influent and effluent (Table IV), helped in measuring the efficiency in the reduction of surrogate bacteria. It was observed that process validation of the anaerobic digester designed and used in this study, was well established in the removal of some waterborne pathogens in the influent; meanwhile the result obtained were less satisfactory due to the presence of one gastrointestinal indicator, *Enterococcus faecalis* found in the effluent. Fig. 8 and 9 showed the effectiveness in the reduction of pathogen concentration on both the influent and effluent. In 1 mL, higher reduction was noticed between the last 2 dilutions; with a 1.36 of margin. Although in 0.1 mL, approximately 0.67 of margin removal was

observed between each dilution with efficiency results. The 3log removal of *E. coli*, *Salmonella spp.*, *Azomonas spp.*, and *Aeromonas hydrophila* was obtained after 30 days of the process operation. However, strains of *Enterococcus faecalis*, *Methanobrevibacter spp.*; and *Methanosarcina spp.* were observed in the effluent sludge. *Enterococcus spp.* was low in concentration and its reduction was estimated at 1log with *Methanosarcina spp.* colonies being observed. Although further process such as recycling of the sludge will be needed to completely remove these pathogenic organisms (*Enterococcus spp.*).

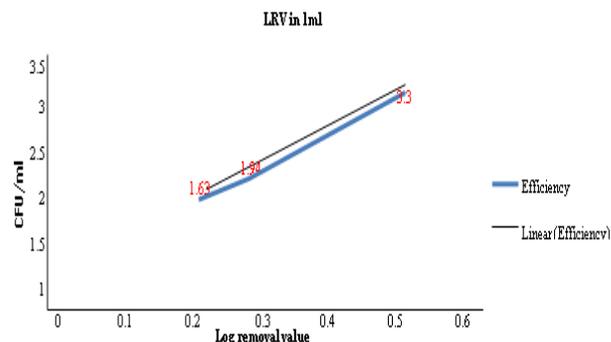


Fig. 8. Log removal value efficiency in 1ml of influent versus effluent

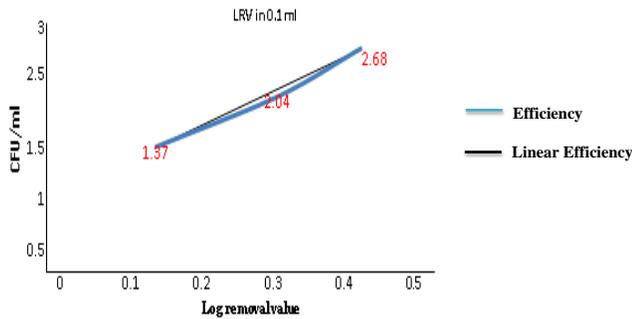


Fig. 9. Log removal value efficiency in 0.1ml of influent versus effluent

IV. CONCLUSION

In this study, the reduction of target pathogen indicator microorganisms, such as *E. coli*, *Enterococcus faecalis*, and *Salmonella* spp. were studied. It can be concluded from this study that the pathogen reduction was achieved in the anaerobic digestion plant at ARC Nietvoorbij farm, Stellenbosch. The concentration of these surrogate microorganisms has been significantly reduced during the process of biogas production using the designed anaerobic digester. Overall, 3log removal efficiency of *Escherichia coli*, *Salmonella* spp., *Azomonas* spp. and *Aeromonas hydrophila* was obtained after 30 days.

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