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Isolation and characterization of methanogenic bacteria from brewery wastewater in Kenya

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The production of biogas from renewable resources is becoming a prominent feature of most developed and developing countries of the world. A study was undertaken to characterize methanogenic microbial community found in brewery waste water. Their performance with regards to methane production was also studied. Thirty-two isolates were obtained using brewer thyglycollate agar medium. Characterization of the isolates was done by culture and biochemical methods. 65% of the isolates were found to be positive with Gram staining reaction, while 35% were negative. The isolates were identified by method of polymerase chain reaction (PCR). From the phylogenetic analysis, thirteen isolates were clustered into genus *Bacillus* sp., isolate 9^{3b} was closely related to *Bacillus subtilis* strain, while isolates 20^{a1}, 17^{1} and 7 closely related to *Bacillus methylotrophicus* isolate 10 was grouped together with *Bacillus tequilensis,* isolate 31 was clustered together with *Bacillus licheniformis*, while isolates 13^{2}, 25^{2}, 15, 26^{2} and 18^{2} were closely related to *Lysinibacillus* sp. and isolate 19^{1} was clustered together with *Lactobacillus casei*. The study also shows that three isolates 3^{2}, 18^{1} and 4 were closely related to *Ralstonia pickettii*, *Providencia rettgeri* and *Myroides odoratimimus,* respectively. The presence of isolates 20^{1a}, 17^{1} and 7 with abilities to ferment different sugars, hydrolysis starch, liquefy gelatin, split amino acid tryptophan, produce catalase enzyme and hydrogen sulphide gas suggests their involvement in biogas production. The percentage methane content in the total gas produced at pH 8 varied significantly (p<0.001) for all the temperature ranges. The highest concentration of methane for most isolates was recorded at temperatures of 35 and 37°C for all the pH ranges.

Key words: Biogas, characterization, methanogenic bacteria, pH, temperature, wastewater.

INTRODUCTION

Readily available energy for domestic, agricultural and industrial applications defines the utmost attractive features of a developing community (Rabah et al., 2010). Energy is the source of economic growth and thus its consumption reflects the state of development of a nation. The growing interest in the search for cleaner source of energy globally, has been heightened by the allied harmful environmental, health and social effects of

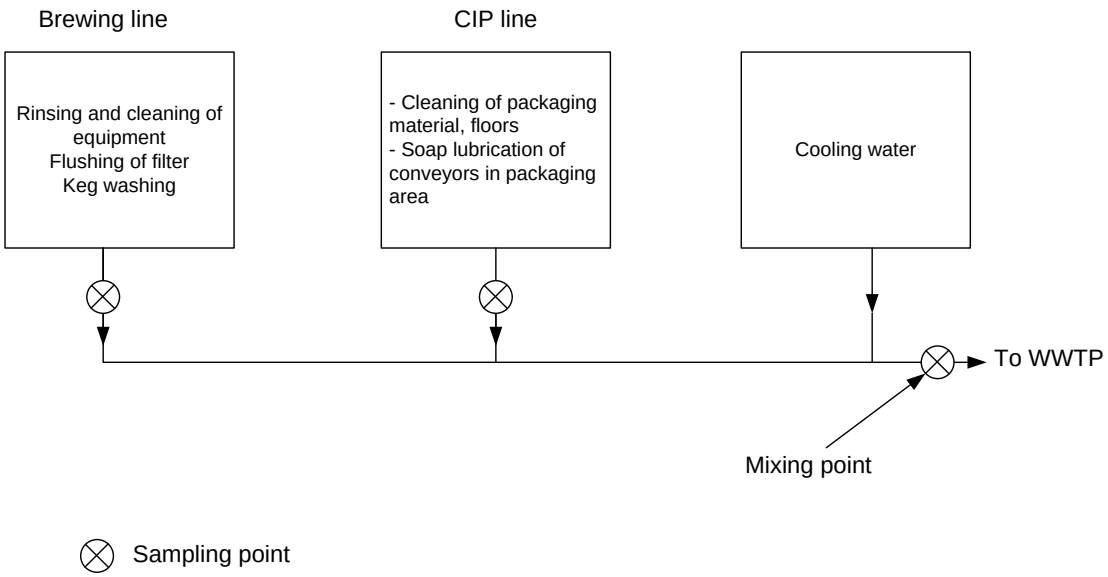

Figure 1. Sampling points.

dependence on fossil fuel (Sárvári Horváth et al., 2016; Sayibu and Ofoso, 2015). Biogas is a promising alternate energy source as the technology of its production may combine the treatment of various organic wastes with the generation of an energy carrier, methane, for the versatile applications with direct reduction in the production costs for processing industries. Most countries in the World have focused interest in the production of biogas from renewable resources. Biogas is produced when bacteria degrade biological materials in the absence of oxygen, in a process known as anaerobic digestion (Weiland, 2010; Horváth et al., 2016). The great varieties of diverse microbes that participate in the microbial food chain gradually degrade the complex molecules essentially to a mixture of CH_4 and CO_2 (Bayer et al., 2004). The environmental and internal factors usually control the actions of the various microbes, involving members of the Eubacteria and Archaea. In addition, the composition of the microbial consortium is determined by numerous factors, including substrate ingredients, temperature, pH, mixing or the biodigester geometry (Cirne et al., 2012). A lot has been done on the general biogas production technology, albeit, the microbial communities involved have not been fully documented (Kröber et al., 2009), indicating that various microorganisms in the analysed fermentation samples of the biogas plants are still unclassified or unknown. This study focused on the isolation and characterization of methanogenic bacteria from brewery wastewater and evaluation of the effect of temperature and pH on the quantity of methane produced.

MATERIALS AND METHODS

Collection of waste water samples

Samples of brewery waste water from Keroche industries were used as an inoculum. The samples were collected in glass sampling bottles that were pre-treated by washing with 70% ethanol and later rinsed with distilled water and dried overnight in an oven at 105°C, for disinfection and drying of the sampling bottles (APHA, 2005; World Health Organization, 2008). They were stored in a refrigerator at 4°C without further treatment. The sampling points included the Brewing line; Clean in Place (C.I.P) and the Mixing point, as illustrated in Figure 1.

Isolation of waste water bacteria

Brewer thyglycollate media from Oxoid was used for cultivation of the anaerobic bacteria. It consisted of 1.0 g lab-Lemco' powder, 2.0 g yeast extract, 5.0 g peptone, 5.0 g glucose, 5.0 g sodium chloride, 1.1 g sodium thioglycollate, 0.002 g methylene blue and 1.0 agar at pH 7.2 ± 0.2 per litre. One millilitre of each sample was inoculated at the base of each sterilized test tube containing the medium using a sterile syringe and incubated at 37°C in anaerobic jar. Observations for growth were made after every 12 h. Serial dilutions of 12 h old bacteria culture in the ratios of 10^0, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} were transferred to Petri-dishes containing brewer thyglycollate media with modification and spread over the surface with a sterile glass spreading rod. Each dilution series was

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used to inoculate a series of plates with three plates at each dilution level and incubated in an anaerobic jar. Anaerobic jar was evacuated by placing a kindled candle, which quenches immediately the left over oxygen. The jar was incubated for a period of 72 h at 37°C. The colonies that emerged on the plates were sub-cultured repeatedly on fresh plates to obtain pure isolates.

Characterization of the isolates

Morphological and cultural characteristics of pure colonies were used to perform preliminary characterization (Holt et al., 1994). The cell shape and arrangement characteristics were observed under the compound microscope after standard staining of the isolates. Three percent (w/v) KOH test (Gregersen, 1978) was used to determine gram characteristics of isolates. Among the biochemical tests conducted were triple sugar iron, gelatine liquefaction, motility, starch hydrolysis, H_2S production, catalase test and indole production test. Molecular characterization was used to confirm the identity of the isolates. Total bacterial DNA was extracted according to the procedures described by Marmur (1961). Bacterial 16S rRNA genes of the pure isolates were amplified (Plate 2) and used as a template for amplification of 16S rRNA gene. PCR amplification was performed using PeQlab advanced Primus 96 Hamburg thermal cycler (Applied Biosystems), using universal primers pair combination of forward primer 8F forward 5'-AG (A/G) GTTTGATCCTGGCT-3') and 1492R reverse, 5'-CGGCTACCTTGTTACGACTT-3' according to the position in relation to *Escherichia coli* gene sequence (Lane, 1991).

DNA was amplified in a 50 µl mixture containing 0.30 µl of gene script Taq, 2.5 µl (10 pmol/ µl) of 8F forward primer, 2.5 µl (10 pmol/µl) of 1492R reverse primer, 10 µl of template DNA (10 ng/µl), and 6.0 µl of dNTP's mix (1.25 mM), 5.0 µl PCR 10 × buffer with $mgcl_2$ and 23.7 µl of PCR water. Reaction mixtures were subjected to the following temperature cycling profiles repeated for 32 cycles: Initial denaturation at 94°C for 5 min, denaturation at 94°C for 1 min, primer annealing at 49°C for 1 min, extension at 72°C for 2 min and a final extension at 72°C for 10 min (Roux, 1995). Agarose gel 1%, stained with ethidium bromide was used to confirm amplified PCR products. Successfully amplified PCR products were purified by QIAquick purification Kit protocol (Qiagen, Germany) according to manufacturer's instructions (Sambrook et al., 1982). Sequencing was performed by Macrogen through ABI prism big dye terminator. The 16S rRNA gene sequences were viewed and edited by Chromas pro software (www.technelysium.com.au). Aligning of the sequences was achieved using CLASTAL W 1.6 software, and was compared to the public databases through BLAST search program on the National Center for Biotechnology Information (NCBI) Website (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic relationship (Figure 2) was performed by the Maximum likelihood method using Mega 5 software (Tamura and Nei, 1993; Tamura et al., 2007).

Identification of methanogenic bacteria

Colonies of methanogenic bacteria were identified on Petri plates using Fluorescent test in which a blue-green fluorescence, characteristic to this metabolic group of bacteria (Dhadse et al., 2012) was observed and was distinct from the white-yellow fluorescence normally observed with non-methanogenic bacteria. To confirm this group, the isolates were sub-cultured in brewer thyglycollate broth media and incubated anaerobically in batch digesters for 7 days in an mrc laboratory equipment water bath Bo-200. Effects of temperatures and pH on the methane quantity were also studied under temperatures of 30, 35, 37 and 40°C and pH variations of 6, 7.2 and 8. The experiments were carried out three times The gas produced was analysed using Biogas 5000 analyser, with CH_4 and CO_2 accuracy of ±0.5% of measurement reading after calibration. The cumulative percentage of CH_4 produced was based on optical density (OD). The OD 600 nm for all the isolates were scaled down to OD of 1.0 for comparison. The OD values were determined using Eppend of Bio photometer AG 22331 Hamburg. Each experiment started after a preliminary operation of 5 min in order to minimize the effects of environmental changes and gas phase differences. The cumulative volume of the gas produced during the incubation period was estimated using ideal gas law (Equation 1). The Initial pressure was indicated on the KIF LAB Labotporp vacuum pump made in (France) during air evacuation and the final pressure was as indicated by barometer on the biogas analyzer 5000.

$$V_{gas} = \frac{(P_2 - P_1)V_r T_a}{P_a T_r} \quad (1)$$

Where, V_{gas} is cumulated volume of gas produced (mL); P_1 is the initial pressure in the digester as indicated by the vacuum pump (kPa); P_2 is the final pressure after incubation period (kPa); P_a is ambient pressure; T_a is ambient (Initial) temperature (K); T_r is temperature of the digester (K) and V_r is the capacity of the digester. Methane produced in mL was calculated according to Equation 2:

$$CH_{4(gas)} = \%CH_4 \times V_{gas} \quad (2)$$

Calibration of the equipment

Two calibration setups were performed once every week, the zero' and 'span'. Zero experiments. The zero experiment was the point at which the gas analyzer was calibrated when there was none of the methane gas present (in the open field). Span zero was at the point at which the gas analyser was calibrated when a known quantity of the methane gas was present (using cooking gas from Total Kenya). Zeroing of the gas analyser was undertaken at the start of each week's monitoring.

RESULTS

Most colonies were observed to grow within two to three days of incubation at 37°C. The colony morphology of the isolates obtained from brewery waste water ranged from circular, entire, flat to filamentous (Table 1). They were smooth or entire and the colour ranged from white to cream and bluish. 65% of the isolates were Gram positive, while 35% were Gram negative and they ranged from short to long rods (Plate 1). The biochemical characteristics of these isolates are given in Table 2. BLAST analysis of the partial sequences (Table 3) showed that 81.25% were from the genus Bacillus within the Firmicutes in the domain bacteria with similarities between 70 and 100%. Among these were *Bacillus subtilis, Bacillus licheniformis, Lactobacillus casei* and *Bacillus methylotrophicus.* Five isolates from the bacillus group belonged to genus *Lysinibacillus* sp. with percentage similarities between 95 and 97. Three isolates had 6.25% each and belonged to the genera *Ralstonia* (isolate 3^2), *Providencia* (isolate 11) and *Myroides* (3^2) with similarities of 77, 96 and 98%, respectively.

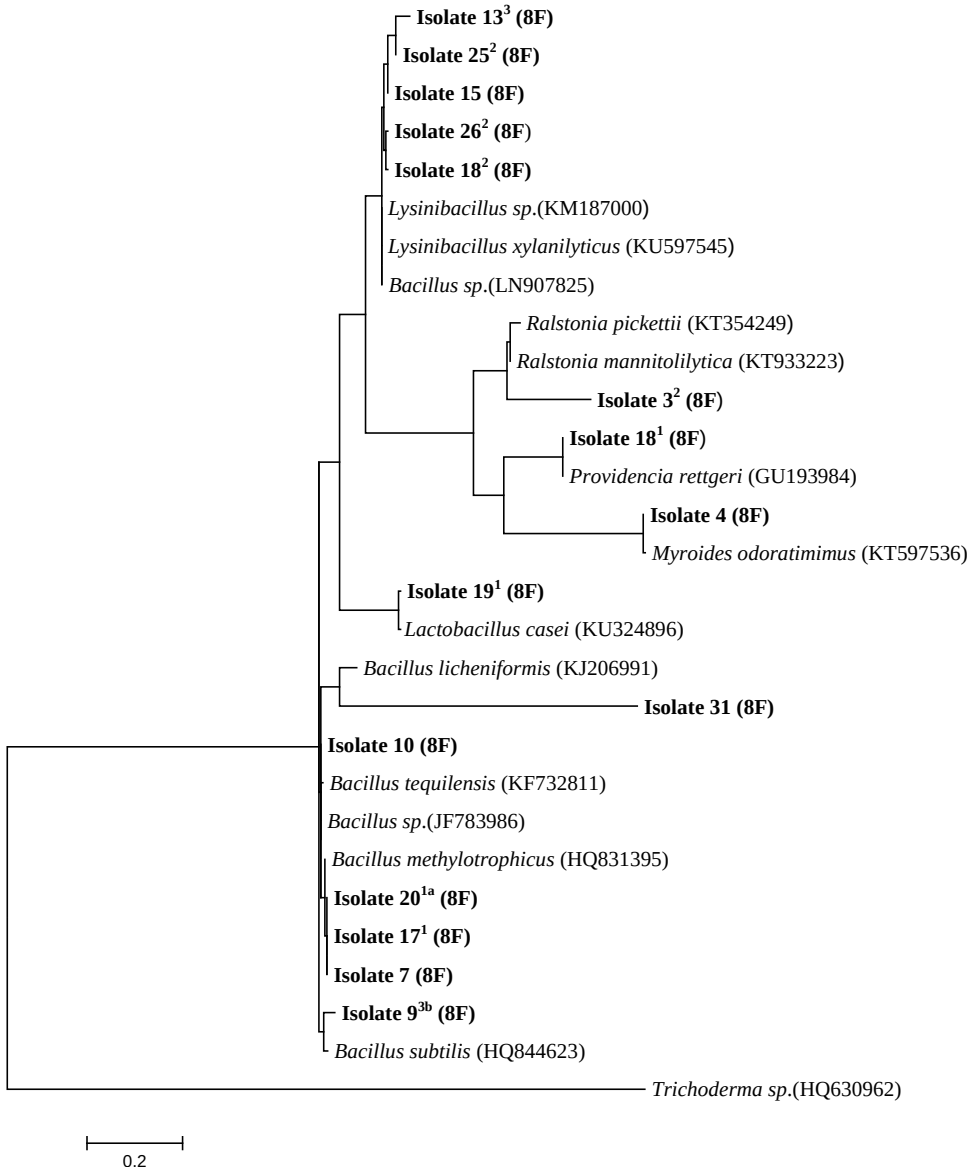

Figure 2. The evolutionary history inferred by using the Maximum Likelihood method based on the Tamura and Nei model (1993) and Tamura et al. (2007). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 29 nucleotide sequences. Evolutionary analyses were conducted in MEGA6. The gene sequence of *Trichoderma* sp. (HQ630962.1) was used as an out-group.

Thirteen isolates were clustered into genus *Bacillus* sp., in the phylogenetic analysis with isolate 9^{3b} being closely related to *B. subtilis* (HQ844623) strain while isolates 20^{a1}, 17^{1} and 7 closely related to *B. methylotrophicus* (HQ831395), isolate 10 was grouped together with *Bacillus tequilensis,* isolate 31 was clustered together with *B. licheniformis* (KJ206991) while isolates 13^{2}, 25^{2}, 15, 26^{2} and 18^{2} were closely related to *Lysinibacillus* sp. (KM187000) and isolate 19^{1} was clustered together with *L. casei* (KU324896). The study also showed that three isolates including 3^{2}, 18^{1} and 4 closely related to *Ralstonia pickettii* (KT354249), *Providencia rettgeri* (GU193984) and *Myroides odoratimimus* (KT597536), respectively. Isolates 4, 17^{1}, 18^{1}, 18^{2}, 19^{1}, 20^{1a}, 25^{2}, 26^{2} and 31 were isolated from brewing line sample while isolates 3^{2}, 7, 9^{3b}, 10, 13 and 15^{1}, were from the mixing point (Figure 1).

Effect of temperature and pH on the quality of methane production

Table 4 shows the means of the quality of methane gas produced by different isolates with variations in

Table 1. Morphological characteristics of bacteria isolates obtained from brewery waste water.

Isolate	Colony characterization				Cell characterization	
	Color	Form	Elevation	Margin	Gram reaction	Arrangement
1	Cream	Oval	Flat	Entire	+	Rods
2	Cream	Oval	Slightly raised	Entire	+	Rods
3^{1}	Bluish/clear	Oval	Slightly raised	Entire	-	Rods
3^{2}	Clear/Bluish	Oval	Slightly raised	Entire	-	Rods
4	White	Irregular	Flat	undulated	-	Rods
5	White	Filamentous	Flat	Filiform	-	Rods
6	Cream	Oval	Raised	Entire	+	Rods
7	Cream	Oval	Raised	Entire	+	Rods
8	White	Oval	Flat	Entire	+	Rods
9^{2}	Clear/bluish	Oval	Raised	Entire	+	Rods
9^{3a}	Clear/Bluish	Oval	Raised	Entire	+	Rods
9^{3b}	Clear/Bluish	Oval	Raised	smooth	-	Rods
10	Bluish/clear	Oval	Slightly raised	Entire	+	Rods
11	Bluish/clear	Oval	Raised	Entire	-	Rods
12	Bluish/clear	Oval	Raised	Entire	-	Rods
13^{2}	White	Irregular	Flat	Undulated	+	Short rods
13^{3}	White	Irregular	Flat	Undulated	+	Rods
14	White	Irregular	Flat	Undulated	-	Rods
15^{1}	Cream	Oval	Raised	Entire	+	Short rods
16	White	Irregular	Flat	Lobate	+	Rods
17^{1}	Clear/Bluish	Oval	Raised	Entire	+	Short rods
17^{2}	Clear/bluish	Oval	Raised	Entire	+	Short rods
18^{1}	Cream	Oval	Raised	Entire	-	Rods
18^{2}	Cream	Oval	Raised	Entire	+	Rods
19^{1}	Clear/bluish	Oval	Raised	Entire	+	Rods
20^{1a}	Cream	Oval	Raised	Entire	+	Rods
25^{2}	Cream	Oval	Raised	Entire	-	Rods
26^{1}	Clear/bluish	Oval	Raised	Entire	-	Rods
26^{2}	Clear/Bluish	Oval	Raised	Entire	+	Rods
27^{1}	Clear/bluish	Oval	Raised	Entire	+	Rods
28^{2}	Clear/bluish	Oval	Raised	Entire	+	Rods
31	Clear/bluish	Irregular	Flat	Undulated	+	Rods

temperature and pH. Generally, the percentage methane content in the total gas produced at pH 8 varied significantly (p<0.001) for all the temperature ranges with reduction in the total volume of gas produced with increase in temperature. Most isolates were observed to float in the digesters at temperature 40°C, with a corresponding least quality of methane and volume of the total gas produced (Figures 3 and 4). The highest concentration of methane for most isolates was recorded at temperatures of 35 and 37°C for all the pH ranges. The methane concentration for isolate 17^{1} increased from temperature 30, 35 to 37°C followed by a drop of temperature 40°C. In addition, its best quality was observed at pH 8 for at least 75% of the temperatures studied. Isolate 18^2 had its best quality at temperatures 35 and 37°C, at pH 7. There was no significant difference (p< 0.001) in the quality of methane for isolates 26^{2}, 25^{2} and 20^{1a} for pH 6 and 8 at temperatures 35 and 37°C (Table 4).

DISCUSSION

From the detailed BLAST analysis, the genus Bacillus were found to be the most prominent indicating a possibility of this group playing an important role in biogas production process as discussed by Horváth et al. (2016), Li et al. (2013) and Kröber et al. (2009). This is also comparable to the results obtained by Rabah using abattoir waste as the inoculum (Rabah et al., 2010) and

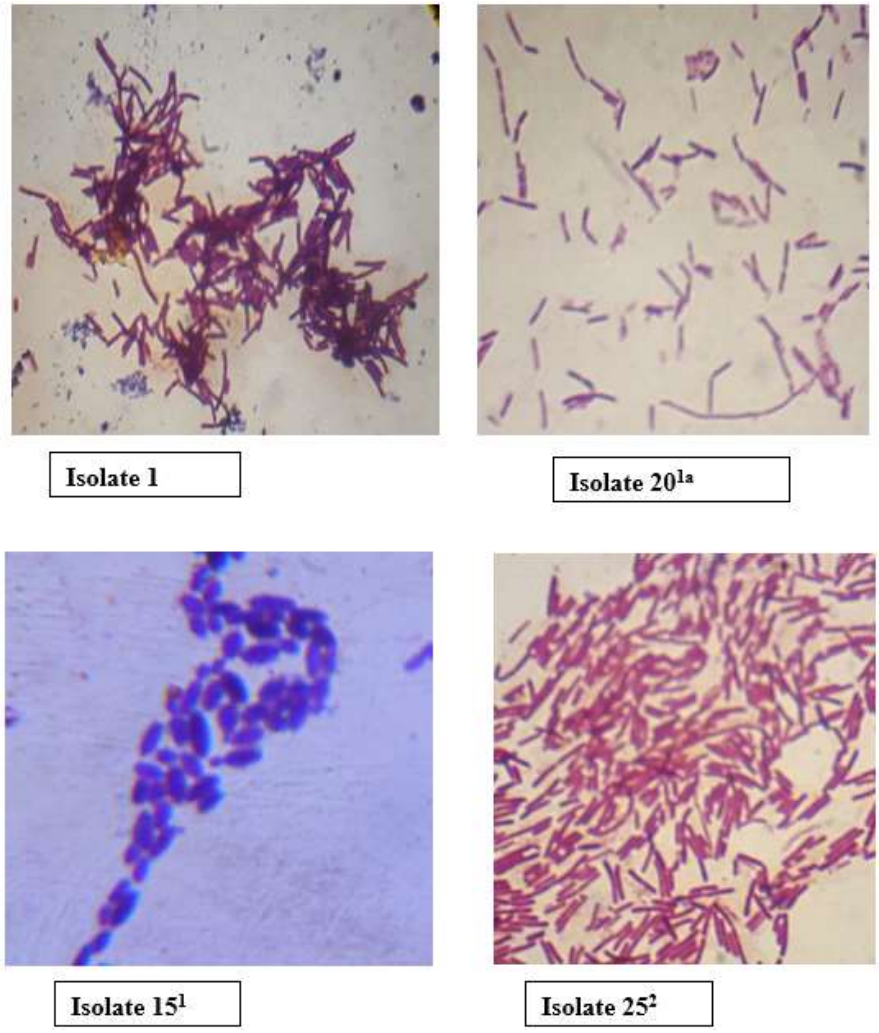

Plate 1. Gram reaction of the selected bacterial isolates. Gram positive rods (1), gram positive rods (20 ^{1a}), gram positive short rods (15^{1}), gram negative rods (25^{2}).

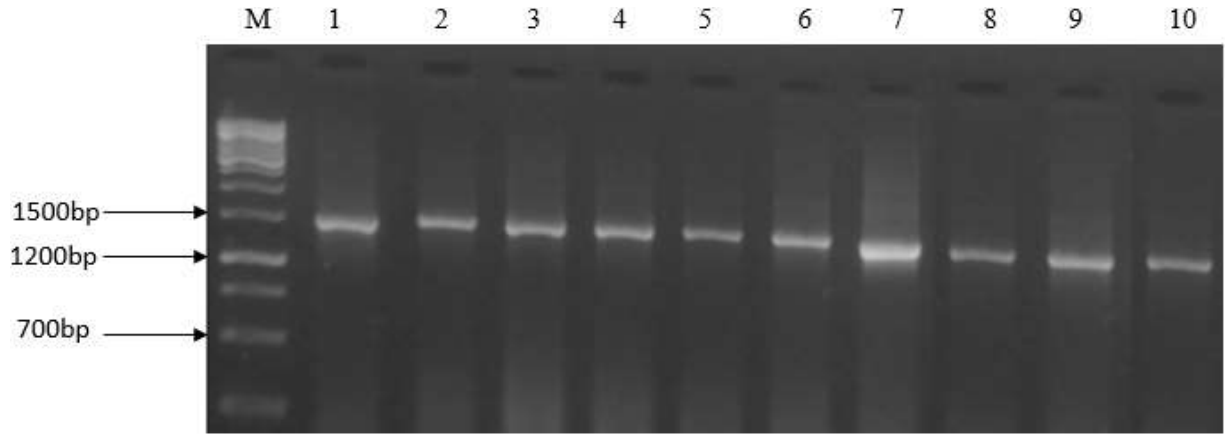

Plate 2. PCR amplified 16S rDNA products from representative isolates among the isolates brewery waste water using universal primers bac 8F and bac 4392R. Lanes 1 (9^{3b}*), 2 (13^{3}*), 3 (18^{1}*), 4 (25^{2} *), 5 (26^{2}*), 6 (15^{1}*), 7 (18^{2}*), 8 (3^{2}*), 9 (20^{1a}*), 10 (17^{1}*) and (M*) M-1500 bp, Molecular marker size. *The figures within the brackets are the isolate numbers.

Table 2. Biochemical characteristics of bacterial isolates obtained from brewery waste water.

Isolate	Starch	Catalase	Indole	Motility	Gelatin	Butt	Slant	Fluorescence	H_2S
1	+	+	-	+	-	++	++	+	+
2	+	-	-	+	+	++	++	++	+
3^1	+	-	-	+	+	++	++	++	+
3^2	-	+	-	+	-	++	++	+	-
4	+	+	-	-	-	++	++	+	+
5	-	+	-	-	+	++	++	+	+
6	+	+	-	+	-	+++	++	++	+
7	+	+	-	+	+	++	+++	+	+
8	+	+	-	+	+	++	++	+	+
9^2	+	+	-	+	+	++	++	+	+
9^{3a}	+	+	-	+	+	+ +	+ +	+	+
9^{3b}	+	+	+	+	+	+ +	+ +	+	+
10	+	+	+	+	+	+++	++	-	+
11	+	+	-	+	+	++	++	++	+
12	+	-	-	+	+	++	++	+	+
13^2	+	-	-	-	+	++	+++	+	-
13^3	+	-	-	+	-	+++	+++	++	+
14	-	+	-	+	-	++	+++	+	-
15^1	-	+	-	-	-	++	++	+	+
16	+	+	-	+	+	++	++	+	+
17^1	+	-	-	+	+	+++	+++	+++	+
17^2	+	+	-	-	+	++	+++	++	+
18^1	+	+	-	+	+	++	+	+	-
18^2	+	-	-	+	+	+	+++	++	+
19^1	+	-	-	-	+	+	+++	+	+
20^{1a}	+	+	-	+	+	++	++	++	+
25^2	+	+	-	-	-	+++	+++	+	+
26^1	-	-	-	+	-	++	++	++	+
26^2	-	-	-	+	+	++	++	+++	+
27^1	+	+	-	+	+	++	++	++	+
28^2	+	-	-	+	+	++	+++	+	+
31	+	-	-	+	+	++	++	++	+

Table 3. BLAST analysis results of the isolates from brewery waste water nearest neighbours in the data bank and their percentage relatedness.

Isolate	Next neighbour	Accession number	Similarity (%)
9^{3b}-(bac 8F)	*Bacillus subtilis strain AIMST 7.Os.2*	HQ844623.1	94
	Bacillus licheniformis strain BNR143	KT074465.1	94
	Bacillus tequilensis strain HS10	KP743123.1	94
18^1-(bac 8F)	Bacillus thuringiensis serovar indiana strain HD521	CP010106.1	100
	Bacillus cereus strain S2-8	JF838294.1	100
	Bacillus anthracis strain Ames_BA1004	CP009981.1	100
25^2-(bac 8F)	*Bacillus sp.* MSB1-25E	KT030900.1	96
	Lysinibacillus fusiformis strain L13	KU179364.1	96
	Lysinibacillus sphaericus strain C2-37c-8	JX517244.1	96
	Lysinibacillus xylanilyticus strain 11W6RMR3-2	KT728728.1	96

Table 3. Contd.

26^{2}-(bac 8F)	*Lysinibacillus boronitolerans* strain KnMuC3-2	KF032677.1	97
	Lysinibacillus sp. BFE17K1	KM187000.1	97
15-(bac 8F)	*Lysinibacillus sp.* DB14515	KP670240.1	97
	Lysinibacillus xylanilyticus strain RD_AZIDI_12	KU597545.1	97
18^{2}-(bac 8F)	*Lysinibacillus xylanilyticus* strain MA	KT030900.1	95
	Lysinibacillus fusiformis strain L13	KU179364.1	95
20^{1a}-(bac 8F)	*Bacillus amyloliquefaciens* strain Y1	KJ616752.1	97
	Bacillus methylotrophicus strain Nk5-1	HQ831395.1	97
	Bacillus subtilis strain yxw4	KF278950.1	97
	Bacillus methylotrophicus strain NMTD14	HQ844484.1	97
17^{1}-(bac 8F)	*Lysinibacillus boronitolerans* strain KtTA1-2	KF025654.1	97
	Lysinibacillus sp. Je33-2	HF563553.1	97
10-(bac 8F)	*Bacillus subtilis* strain F111	HQ647257.1	98
	Bacillus tequilensis strain ADIP3	KF732811.2	98
	Geobacillus sp. CRRI-HN-1	JQ695928.1	98
3^{2}-(bac 8F)	*Ralstonia mannitolilytica* strain 4903	KT933223.1	77
	Ralstonia pickettii	KT354249.1	77
	Uncultured bacterium clone Ap.ba-F-DM-HN-1-46	KT354249.1	77
11-(bac 8F)	*Providencia rettgeri* strain IITRP2	GU193984.1	96
	Uncultured Providencia sp. clone F2jun.39	GQ417423.1	96
	Uncultured bacterium clone PB16	GU166190.1	96
16-(bac 8F)	*Bacillus licheniformis* strain RTS	EF644417.1	95
	Bacillus sp. J26	JF783986.1	95
	Bacillus tequilensis strain EB-95	KU258071.1	95
	Bacillus subtilis strain 1201	EU982509.1	95
19^{1}-(bac 8F)	*Lactobacillus casei* strain L1	KM350161.1	95
	Lactobacillus casei strain MSJ1	KU324896.1	95
	Lactobacillus casei strain EM2	KM350160.1	95
3^{2}-(bac 8F)	*Myroides odoratimimus* strain LZ1306-2-5	KT597536.1	98
	Myroides odoratimimus strain YRL08	EU373415.1	98
7-(bac 8F)	*Bacillus amyloliquefaciens* subsp. *plantarum* strain L09	JN700139.1	98
	Bacillus methylotrophicus strain CR1	KP851947.1	98
	Bacillus subtilis strain EPP2 2	JQ308548.1	98
31-(bac 8F)	*Bacillus licheniformis* strain R2	KJ206991.1	70
	Bacillus licheniformis strain SMR1	KF600749.1	70
	Bacillus subtilis strain VJJS-01	DQ872516.1	70

in line with that of Onwuliri et al. (2016) in which *Bacillus, Yersinia* and *Pseudomonas* species were found to be responsible for biogas production from cow dung. In the literature, *Bacilli* are described as aerobic or facultative

Table 4. Means for quality of % methane gas produced by different isolates.

pH	Temperature (°C)	15^1	17^1	18^1	18^2	20^1[a]	25^2	26^2	3^2	9^3b
6.0	30	0.52±0.02^{cd}	0.72±0.02^{d}	0.29±0.07^{a}	0.48±0.02^{c}	0.33±0^{a}	0.31±0^{b}	0.41±0.03^{b}	0.29±^{ab}	0.24±0.02^{a}
6.0	35	0.64±0.05^{d}	0.93±0.03^{e}	0.62±0.07^{c}	0.67±0.07^{d}	0.69±0.06^{d}	0.5±0.03^{cd}	0.79±0.03h	0.28±0^{a}	0.74±0.01^{bc}
6.0	37	0.38±0.01^{abc}	1.12±0.02^{f}	0.49±0.03^{b}	0.42±0.03^{bc}	0.58±0^{c}	0.47±0.01^{c}	0.29±0^{a}	0.29±0.01^{ab}	0.35±0^{a}
6.0	40	0.33±0.01^{ab}	0.34±0.01^{b}	0.23±0^{a}	0.26±0^{a}	0.28±0^{a}	0.3±0^{b}	0.25±0^{a}	0.33±0^{ab}	0.29±0.01^{a}
7.2	30	0.32±0.01^{ab}	0.21±0^{a}	0.33±0.01^{a}	0.73±0^{d}	0.48±0.02^{b}	0.21±0^{a}	0.44±0.06^{bc}	0.38±0.01^{bc}	0.43±0.04^{a}
7.2	35	0.24±0.02^{a}	0.33±0.01^{b}	0.43±0.01^{b}	1.12±0.03^{f}	0.48±0.02^{b}	0.21±0.01^{a}	0.47±0.03^{bcd}	0.43±0.01^{c}	0.63±0.04^{b}
7.2	37	0.44±0.02^{bc}	0.51±0.08^{c}	0.72±0.08^{d}	1.15±0.03^{f}	0.5±0^{b}	0.78±0.01^{e}	0.5±0^{cde}	0.6±0^{d}	0.62±0^{b}
7.2	40	0.47±0.01^{bc}	0.25±0.02^{ab}	0.52±0^{bc}	0.75±0.02^{d}	1.3±0^{f}	0.76±0.02^{e}	0.51±0.04^{cde}	0.59±0.09^{d}	0.61±0.01^{b}
8.0	30	0.86±0.14^{e}	0.75±0.01^{d}	1.1±0^{e}	0.97±0.08^{e}	0.55±0^{bc}	0.55±0^{d}	0.55±0.02^{def}	0.52±0.03^{d}	0.83±0.19^{c}
8.0	35	0.45±0.01^{bc}	2.3±0h	2.2±0^{f}	0.77±0^{d}	0.77±0^{e}	0.52±0.03^{d}	0.58±0^{ef}	0.73±0^{e}	2.3±0^{d}
8.0	37	0.42±0^{bc}	2.1±0^{g}	1.08±0.01^{e}	0.42±0^{bc}	0.7±0de	0.51±0.01^{cd}	0.66±0.02^{g}	2.1±0^{f}	0.7±0^{bc}
8.0	40	0.37±0.02^{ab}	0.71±0.05^{d}	1.1±0^{e}	0.34±0.02^{ab}	0.5±0.03^{b}	0.51±0.01^{cd}	0.63±0^{fg}	0.77±0^{e}	0.77±0^{bc}
LSD	-	0.133	0.093	0.104	0.107	0.069	0.045	0.080	0.092	0.189
CV%	-	17.1	6.3	7.8	9.2	6.6	5.5	9.2	7.8	14.4

Means with same letter are not significantly different.

anaerobic; rod shaped, Gram positive, motile, flagellated bacteria, either catalase positive that belongs to the division Firmicutes with varying ecological diversity. They are most commonly found in soil, waste water, milk, dust and plant surfaces.

The pH ranges of brewery wastewater is reported as 6.5 to 8.2 (Janhoappliedm et al., 2009; Caliskan et al., 2014; Zheng et al., 2015). The study considered pH values of 6, 7.2 and 8 in order to investigate the effect of pH on the growth of the bacteria which has a direct impact on the concentration of methane gas produced by the different isolates (Harris et al., 1984). This could be attributed to different adaptations levels by individual isolate. The low pH of 6 and temperature of 30°C, and high pH of 8 and temperature 40°C, may have inhibited the growth of some isolates, resulting in low concentrations of the methane produced. Production of methane at low pH is essential for digestion to progress from the anaerobic acid phase to the methane production phase. Presence of isolate 25^{2}, 26^{2} and 20^{1a} which were acid tolerant is consistent with literature (Ladapo et al., 1997). At pH 7.2, most of the isolates were able to adjust and increase in numbers especially at temperatures between 35 and 37°C. The isolates observed floating in the digesters could indicate a possibility of death for these isolates as they could not adapt easily to the high pH and temperature of 40°C.

CONCLUSIONS AND RECOMMENDATIONS

The study demonstrated that brewery waste water harbour diverse bacteria species with potential biogas production. Biochemical properties of some isolates like ability to ferment different sugars, hydrolysis of starch, liquefying of gelatin, amino acid tryptophan split, and production of catalase enzyme and hydrogen sulphide gas suggests their involvement in biogas production. Since most isolates adapted easily at temperatures of 35 and 37°C, with the highest quality of methane gas, these conditions could be exploited as optimal working conditions.

More research is required to assess whether the isolates in this study possess unique physiological characteristics to explore their full potential.

Conflict of Interests

The authors have no affiliations with or involvement in any organization or entity with any financial or non-financial interests in the materials

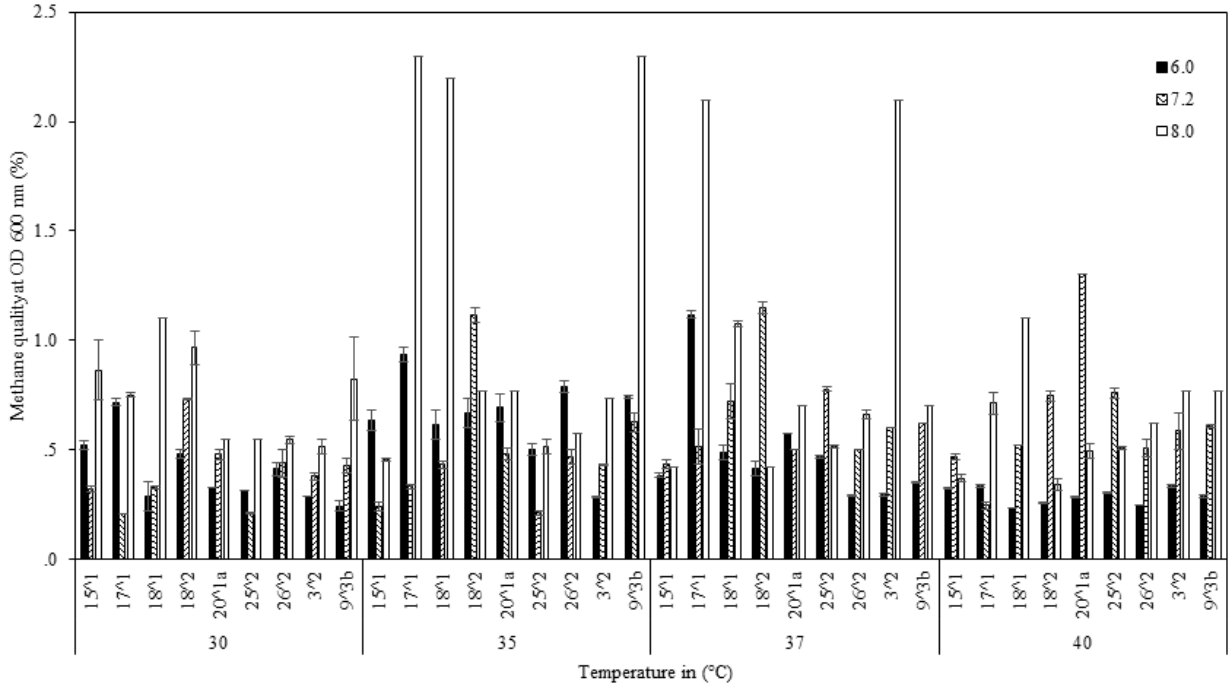

Figure 3. Effect of temperature and pH on the quality of the methane produced at OD 600 nm.

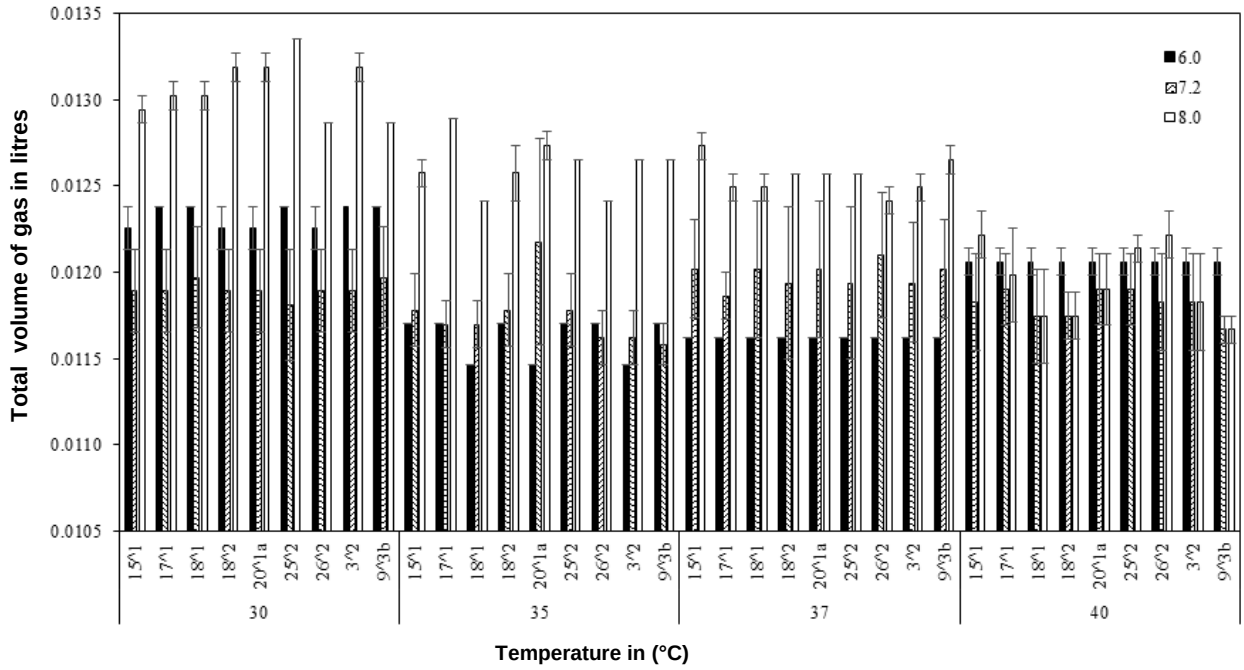

Figure 4. Effect of temperature and pH on the total volume of gas produced.

discussed in this manuscript.

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