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OPTIMIZATION OF BIOGAS PRODUCTION FROM BREWERY WASTEWATER

By

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This work is dedicated to my husband and kids, who encouraged and supported me all through to this level of education. Above all to God, who provided strength, health and favour to enable me see this output.

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LIST OF ABBREVIATIONS

AAS	Atomic Absorption Spectrophotometer
AD	Anaerobic digestion
AIC	Alkaike Information Criterion
ARDRA	Amplified Ribosomal DNA Restriction Analysis
AT	Aerobic Thermophilic
APHA	American Public Health Association
BI	Biodegradability Index
BOD_5	Biological Oxygen Demand
Bp	Base pairs
C	Carbon
CH_4	Methane
C/N/P	Carbon Nitrogen Phosphorus
CIP	Clean in Place
CO_2	Carbon dioxide
COD	Chemical Oxygen Demand
DNA	Deoxyribonucleic Acid
EAC	East African Community
ECA	East African Community
EDTA	Ethylene diamine tetra-acetic Acid

FAO	Food Agricultural Organisation
GHG	Greenhouse Gas
GOK	Government of Kenya
IBR	Institute of Biotechnology Research
JKUAT	Jomo Kenyatta University of Agriculture and Technology
KIRDI	Kenya Industrial Research and Development Institute
KOH	Potassium Hydroxide
NEMA	National Environmental Management Authority
NCBI	National Centre of Biotechnology Information
O_2	Oxygen
OD	Optical Density
PCR	Polymerase Chain Reaction
pH	Hydrogen Potential
R^2	Coefficient of determination
rDNA	Ribosomal Deoxyribonucleic Acid
RSS	Residual Sum of Squares
rRNA	Ribosomal Ribonucleic Acid
SDS	Sodium Dodecyl Sulphate
SIM	Sulphur Indole Motility

SMA	Specific Methanogenic Activity
TDS	Total Dissolved Solids
TSI	Triple Sugar Iron
TSS	Total Suspended Solids
UV	Ultra Violet
WWTP	Waste Water Treatment Plant

ABSTRACT

The production of biogas from renewable resources is becoming a prominent feature of most developed and developing countries of the world. Food industries produce byproducts which contain high level of organic matter that could be converted into energy. Brewing is one such industry. It consumes large volumes of water that often ends up in the waste stream. A study was undertaken to optimize biogas production from brewery wastewater. The study characterized brewery wastewater, investigated the methanogenic community as a step towards optimal biogas production through isolation and identification using morphological, biochemical and molecular techniques. The performance of these isolates with regard to methane production were also studied and their population modeled to predict growth. Samples from brewing line, cleaning in place line and mixing line from two brewing industries in Kenya were analyzed for BOD_5, COD, TDS, TSS, sodium, total nitrogen and phosphorous using standard method as per American Public Health Association (APHA). There was a significant variation ($p<0.001$) in the physicochemical parameters between the industries and a significant interaction ($p<0.001$) between sampling point and the company. Analysis of the BOD to COD ratio showed the Biodegradability Index (BI) to range from 0.039 to 0.567 for brewing line, 0.177 to 0.766 for cleaning in place and 0.776 to 0.911 for mixing point, thus the wastewater was found to be easily degradable at the mixing point for all the industries. A model on the effect of change in the physicochemical parameters on the Biodegradability Index developed explained 73% of the variations ($R^2=0.7339$). Thirty-two isolates were obtained using brewer thyglycollate agar medium. 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). Only 16 isolates could be placed in the phylogenetic tree, the others had too low an identity to allow

for sensible alignment. 81.25% belonged to the Bacillus genus, within the Firmicutes in the domain bacteria with similarities between 70% and 100%. Among them were; Bacillus subtilis, Bacillus licheniformis, Lactobacillus casei, Bacillus methylotrophicus and Lysinibacillus sp. The genus Providencia, Ralstonia and Myroides each had 6.25% with similarities of 96%, 77%, and 98% respectively. The abilities of some of the isolates to ferment different sugars, hydrolyse starch, liquefy gelatin, split amino acid tryptophan, produce catalase enzyme and hydrogen sulphide gas suggests their involvement in biogas production. The two primary models provided high goodness of fit ($r^2 > 0.93$) for all growth curves for six isolates based on optical density, in approximately 33.3% of the cases. However, Gompertz model was accepted in 75% of the remaining cases based on the Alkaike Information Criterion (AIC) values and also supported by the RSS and R^2 values. The study has demonstrated that brewery waste water harbours diverse bacteria with potential biogas production at operating temperatures of 35 ºC and 37 ºC for all the pH ranges. The model provides knowledge to describe the growth of the methanogenic community in a bio-digester as a function of time, hence maximum utilization of the exponential phase of the microbial growth for production of biogas. This indicates the practicality of applying Gompertz model to actual anaerobic digestion of brewery waste. The model predicted the specific growth rate and lag time parameters for the microorganisms. The BI model developed guides on physicochemical parameters to be maximized as a step towards optimal biogas production and also to reduce environmental pollution.

Key words: *biodegradable, brewery, wastewater, methanogenic bacteria, physicochemical, pollution, anaerobic, Gompertz model, logistic model, environment, biogas, Kenya*

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background Information

Abundant availability of energy for domestic, agricultural and industrial purposes is the most captivating features of any civilized communities (Sayibu and Ampadu, 2015). Energy is the source of economic growth and its consumption reflects the state of development of a Nation. Renewable energy utilizes natural resources with technologies ranging from solar power, wind power, hydroelectricity, micro-hydro, biomass and biofuels. These sources are a feasible alternative to the problems relating to imminent fossil fuel shortage, the complicity of setting up hydroelectric and thermoelectric powers thus they are gaining significant attention. Increase in energy demand due to growth in the worlds' economies has resulted to change in energy consumption patterns, which in turn, vary depending on the source and availability of the energy source, conversion loss and end use efficiency (Martins das Neves *et al*., 2009). Most developed and developing countries have shown interest in the production of biogas from renewable resources. Biogas plays an important role in the domestic and agricultural life of the rural dwellers for its application in cooking, crop drying and soil fertilizing (Samuel, 2013).

Biogas is produced when bacteria degrade biological materials in the absence of oxygen, during anaerobic digestion (Weedermann *et al*., 2015) Anaerobic treatment involves breakdown of organic matter in the absence of oxygen and the stabilization of these materials by converting them to methane and carbon dioxide gases (Rabah *et al*., 2010). Biogas can be converted to heat and/or electricity, and its purified derivative, biomethane, which are suitable for every function

for which fossil natural gas is used. Varieties of diverse microbes, including members of the Eubacteria and Archaea degrade the complex molecules to a mixture of CH_4 and CO_2. The composition of this microbial consortium depends on various environmental and internal factors such as substrate ingredients, temperature, pH, mixing, or the geometry of the anaerobic digester (Bayer *et al.,* 2004; Cirne *et al*, 2007). The coexistence of different microbial populations as a result of change in the reactor operational conditions provides unprecedented control over their overall contribution to the degradation of the organic matter (Jalowiecki *et al*., 2016). Investigation of microbial methanogens can assist in not only their classification but also in the optimization of anaerobic digestion systems (Karakashev *et al.,* 2005). There is therefore need for molecular characterization to explore their full potential in biogas production.

Anaerobic digestion of waste from food and beverage industries can contribute positively to the environmental management since it combines both waste removal and stabilization with net fuel (Biogas) production. Effluent from food and beverage industries contain high level of organic matter that could be converted into energy as supplement for fossils. The use of biogas is capable of providing a special impetus in both rural and urban areas and the plant can be built using materials which are locally available in most developing countries (Martins das Neves *et al*., 2009). Therefore, the objective of the research was to optimize the production of biogas from brewery waste water through modelling the biodegradability index and microbial growth.

1.2 Statement of the problem

The issue of global warming and climate change is strongly receiving public attention and has become a major environmental concern both at National and International level. Culpable human activities including agricultural expansion (especially livestock husbandry, rice cultivation),

industrial activities, fossil-fuel exploitation and use, and waste production and management (landfills and animal wastes) contributes towards the increasing concentration of atmospheric greenhouse gases. In addition, the use of the traditional biomass mainly wood fuel, exacerbates the situation as the majority of Kenyans still live in rural areas where it is the leading source of energy for both cooking and lighting. However, the potential of biomass has not been effectively utilized in the provision of modern energy (Manyi-Loh *et al*., 2013). Continued over-dependence on unsustainable wood fuel and other forms of biomass as the primary sources of energy to meet household energy needs has contributed to uncontrolled harvesting of trees and shrubs with negative impacts on the environment (Githiomi and Oduor, 2012). The increasing population, developing science, technology and innovation, with a direct increase on human comfort and needs, further, increases the need for burning fuel. The technology of production of biogas is very important as it may combine the treatment of various organic wastes with the generation of an energy carrier, methane (Kovács *et al*., 2013), the most versatile applications with direct reduction in the production costs for processing industries. In contrast to the general biogas production technology, the complexity of the microbial communities involved is not well understood (Wirth *et al*., 2012). Brewery industry is one of the largest consumers of water which ends up into the waste stream and require vast quantities of energy for its normal operations. The amount of biogas produced by the breweries in Kenya is below the expected amount to power production. For this reason studies were undertaken to evaluate the effect of physicochemical parameters on the biodegradability index and to identify methanogenic microbial population and their growth parameters, from brewery wastewater by use of primary models for optimal biogas production.

1.3 Justification

There is need to constantly search for eco-friendly renewable energy which utilizes biological materials. Food processing comprises the methods and techniques used to transform raw ingredients into food; or to transform food into other forms for consumption by humans or animals, either at home or in the food processing industries (Kaushik, *et al.*, 2009). These processes often produce large amounts of byproducts, which have been evaluated in many studies for their potential utilization and their suitability for chemical and biological treatments. Brewery industry is one such industry with consumption of large volumes of water that end up to waste stream. This wastewater may be utilized in the production of energy. In industrial applications of anaerobic digestion for biogas generation, the focus is to stabilize and capitalize on biogas production. Thus the study builds on the analysis of the biodegradability index of the brewery wastewater and primary microbial growth models developed by Benjamin Gomperzt in 1938 and Logistic by Pierre-François Verhulst in 1838, to describe the growth of the methanogenic community in a bio-digester as a function of time, and to determine their growth parameters, hence maximum utilization of the exponential phase of the microbial growth for production of biogas.

1.4 General objectives of the study

To optimize biogas production from brewery wastewater

The specific objectives of the study were to:

a) Analyze the physicochemical characterization of the brewery wastewater

b) Isolate and characterize methanogenic anaerobic bacteria from brewery wastewater

c) Optimize biogas production from the isolated strains through modeling.

1.5 Research questions

a) Can methane producing bacteria be found in brewery environment?

b) Do the methanogens isolated differ from already known and isolated bacteria?

c) How does methanogen isolated contribute to the yield of biogas from anaerobic process?

1.6 Scope of research

The study focused on optimization of biogas production by the isolated and characterized bacteria strains from brewery wastewater, through modelling.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Introduction

This chapter provides an overview of the biogas technology as an alternate renewable energy source, by looking at the global context as well as the Kenyan situation. It begins with an overall review of the brewing process, water use and waste generation. The chapter also provides discussion on the biogas production process, methanogens, factors affecting biogas production and techniques for isolation and characterization of bacteria. Finally, it provides a critical review of microbial growth primary models have also been presented.

2.2 Brewing industry

Brewery has a significant economic value in the agro-food sector as one of the traditional industries. Beer is produced through the fermentation of sugars derived from the saccharification of starch from malted grains (such as barley, rice and wheat). It can be flavored using hops, herbs or fruits. As one of the oldest beverages produced by humans, a wide variety of beer has been cultivated and established and can vary in alcohol content, bitterness, pH, turbidity, color, and most importantly, flavor (Goldammer, 2008).

Beer is the most consumed alcoholic beverage in the world, and third most popular beverage after water and tea. Globally, a beer culture has been established and beer festivals, such as the widely known Oktoberfest in Munich, Germany, are held in a number of countries. Generally,

Kenya leads in beer production in the East African Community (EAC) region with production capacity of 2.8 million hectolitres for the year 2003, followed by Tanzania 2.1 million hectolitres and Uganda 1.3 million hectolitres (Export Processing Zones, 2005).

Processing of beer involves both chemical and biochemical reactions which include mashing, lautering, hops boiling, fermenting and maturation. In the mashing process, malts (germinated and dried grains) are mixed with adjunct flavorings and liquor (pure water) and heated to allow enzymes to break down starch into sugars. This process yields a mixture of malt and wort (sugar water) called mash for the lauter tun. In the lauter tun, the mash is separated into clear liquid wort and residual malt. Lautering consists of three steps: mashout, recirculation, and sparging. During mashout, the temperature is raised to stop the enzymatic conversion of starches to fermentable fluid. Recirculation consists of drawing off the wort from the bottom of the mash and adding it to the top. After recirculation, water is trickled through the grain to extract the sugars in the sparging process. Care has to be taken during sparging process, as wrong temperature or pH during sparging can extract tannins from the grain husks, which results in an unpleasant and extremely bitter taste. Once the mash is sparged, the resultant wort is sent to a hops boiler where hops are added for flavor and boiled according to a recipe hops schedule based on individual company. Eventually the wort is sent to a fermentor where the sugars undergo fermentation, via the glycolysis which has the overall chemical reaction as illustrated in equation [2-1].

$$C_6H_{12}O_6 \rightarrow 2CH_3CH_2OH + 2CO_2 \qquad [2\text{-}1]$$

The duration for fermentation depends on the desired final alcohol content of the beer. After fermentation the beer is drained and moved into bright tanks where it is allowed to condition, and

additional flavorings may be added during the aging process. Additional carbonation may also be added in the bright tanks. Once conditioning is completed, the yeast is filtered out, and the beer is either pumped into kegs or to the bottling line where it's generally exposed to a stream of hot water to kill any remaining yeast or microbes and to fix the flavor profile.

For every 1,000 tons of beer produced, 137 to 173 tons of solid waste may be created in the form of spent grain, trub from wort production and waste yeast (Caliskan *et al*., 2014). Water usage in brewing industries varies widely among breweries and is dependent upon specific processes and location. The main water using areas within the brewery include brewhouse, cellars, packaging and utilities such as boiler house, cooling and amenities. Water use attributed to these areas include the water used in the product, vessel washing, general washing and cleaning in place, which are of considerable importance, in terms of composition of the effluent that end up to waste stream (Zheng *et al*., 2015). In addition, the quantity and quality of the effluent can vary significantly depending on the process employed. The effluent must be disposed off or safely treated for reuse, which is often costly and problematic for most breweries, though water reuse in this type of industry is not common, due to public perception and possible product quality deterioration problems (Janhoappliedm *et al.*, 2009). However, many brewers are still investigating techniques to reduce water consumption during processing and an effective, low cost effluent treatment method with possibilities for reuse (Simate *et al*., 2011).

Currently, only one out of the two main brewing industries in Kenya is engaged in anaerobic digestion of the waste water with little biogas being produced. The other brewing industries discharge their untreated waste water to the municipal line, which in turn increases its loading. Efforts should be made towards providing waste water treatment options for these industries to

allow environmentally friendly disposal of their waste water with potential for bioenergy production.

Effluent characteristics play an important role in the selection of treatment process of the waste water (Rana *et al*., 2014; Ojoawo and Udayakumar, 2015). Biological oxygen demand (BOD_5), Chemical oxygen demand (COD), total suspended solids (TSS), total dissolved solids (TDS), total nitrogen and phosphorous are some of the physicochemical parameters used to characterize waste water. BOD_5 measures the amount of oxygen required by bacteria for breaking down to simpler substances, the decomposable organic matter present in any wastewater or treated effluent. It is a measure of the concentration of organic matter present in any water. The greater the decomposable matter present, the greater the oxygen demand and the greater the BOD_5 values (Singh *et al*., 2012). COD is a measure of the oxygen required to oxidize all organic material into carbon dioxide and water, and the values are always greater than BOD_5. The BOD_5 to COD ratio is commonly used as an indicator for biodegradability of the waste and is dependent on the characteristics of the waste (Samudro and Mangkoedihardjo, 2010; Zaher and Hammam, 2014). However, C/N/P is also an important parameter for the successful anaerobic degradation of organic wastes.

2.3 Biogas in Kenya

With the increase in Kenyan population, developing science, technology and innovation, several other national issues including but not limited to energy, food, environmental, water, transportation, are also emerging. Although the Kenya wants to transform into a newly industrialized middle income country, through its Vision 2030 program, she has only 2,150 MW of generation capacity to serve her population of more than 43 million, which is a constrain to

accelerated economic growth (Owiro *et al*., 2015). The realization of the Kenyas' overall national development objectives of accelerated economic growth, through increased productivity and enhanced agricultural and industrial production requires that quality energy services are available in a sustainable, cost-effective and affordable manner to people (The Ministry of Planning and Devolution, 2007). In order to uplift the broader adoption and use of renewable energy technologies and thus enhance their role in the country's energy supply matrix, Session Paper 4 of 2004 on energy proposes that the Government of Kenya will design incentive packages to promote private sector investments in renewable energy and other off-grid generation.

At National level, biomass (mostly wood fuel) accounts for about 68% of the total primary energy consumption, followed by petroleum at 22%, electricity at 9% and others at about less than 1%. In rural areas, the reliance on biomass is over 80% (Ministry of Energy, 2016). Access to affordable modern energy services is constrained by a combination of low consumer incomes and high costs. The scattered nature of human settlements further escalates distribution costs and reduces accessibility. The majority of Kenyans live in rural areas where traditional biomass (mainly wood fuel) has remained the leading source of energy (both for cooking, and at times for lighting) (Githiomi and Oduor, 2012). Biomass includes materials derived from plants, animals, humans as well as their wastes. Other sources of biomass waste are food processing, agro-industrial and industrial wastes. Similar cultivable and non-cultivable metabolically active microbial population exists within these wastes, and depending on the waste characteristics they can be transformed into energy/and or fuel by combustion, gasification, co-firing with other fuels or through anaerobic digestion.(Manyi-Loh *et al*., 2013).

The potential of biomass has not been effectively utilized in the provision of modern energy for a variety of reasons. One is the failure to exploit the opportunities for transforming wastes from agricultural production and processing into locally produced modern energy. Another constraint to shift from traditional to modern biomass energy utilization is high incidence of poverty. Continued over-dependence on unsustainable wood fuel and other forms of biomass as the primary sources of energy to meet household energy needs has contributed to uncontrolled harvesting of trees and shrubs with negative impacts on the environment (deforestation). Environmental degradation is further exacerbated by climate variability and unpredictable of rainfall patterns (NEMA, 2011). In addition, continued consumption of traditional biomass fuels contributes to poor health among users due to inhalation of excessive products of incomplete combustion and smoke emissions in the poorly ventilated houses common in rural areas (Owiro *et al*., 2015). Biogas is an energy technology that has the potential to counteract many adverse health and environmental impacts.

Although this study focused on the brewery waste water, as a source of biogas production, there are many other sources that can be exploited to produce biogas using methanogens (Fischer *et al*., 2010).

2.4 Biogas production process

Biogas originates from bacteria in the process of bio-degradation of organic material under anaerobic (without O_2) conditions. The natural generation of biogas is an important part of the biogeochemical carbon cycle. Methanogens (methane producing bacteria) are the last link in a chain of micro-organisms which degrade organic material and return the decomposition products to the environment, in this process biogas is generated, a source of renewable energy

(Budiyono and Kusworo, 2011). Biogas consists mainly of methane and carbon dioxide, but also contains several impurities, with specific properties as listed in Table 2-1.

Table 2-1: Properties of biogas

Composition	55 - 70% Methane (CH_4) 30 - 45% Carbon Dioxide (CO_2) and Traces of Other Gases
Energy content	6.0 – 6.5 kWh/m^3
Fuel equivalent	0.60 – 0.65 L oil/m^3 biogas
Critical pressure	75 – 89 bar
Critical temperature	− 82.5° C
Normal density	1.2 kg/m^3
Smell	Bad eggs (the smell of desulfurized biogas is hardly noticeable)
Molar Mass	16.043 kg/kmol

Source: Modified from Martins das Neves *et al*., 2009

Biogas is a clean and environmental friendly as the method of production does not utilize oxygen and it's burnt to give energy as a product, thus reducing uncontrolled greenhouse gas emission into the atmosphere (Clemens *et al*., 2006). The formation of biogas can occur either in natural environment or controlled conditions in constructed biogas plants. Areas where biogas is formed naturally include; swamps, marshes, river beds and rumen of herbivore animal. The same microbial activities are achieved in both natural and controlled conditions.

2.5. Anaerobic digestion

Anaerobic digestion (AD) is best suited to convert organic wastes from agriculture, livestock, industries, municipalities and other human activities into energy and fertilizer. It is the

degradation of organic materials by microorganisms in the absence of oxygen. It is a multi-step biological process where the organic carbon is mainly converted to CO_2 and CH_4 (Angelidaki and Ellegaard, 2003). Acid forming and the methane forming microorganisms vary broadly in terms of structure, nutritional needs, growth kinetics, and sensitivity to environmental conditions. Thus, failure to maintain the balance between these two groups of microorganisms is the primary cause of reactor instability (Chen *et al*., 2008). The limiting step in anaerobic digestion is defined as the step that causes process failure under imposed kinetic stress. In a continuous culture, kinetic stress is defined as the imposition of a constantly reducing value of the solids retention time until it is lower than the limiting value; hence resulting in a washout of the microorganism. In literature, the rate -limiting for complex organic substrate is reported as the hydrolysis step due to the formation of complex heterocyclic compounds which are considered to be toxic byproducts or non-desirable volatile fatty acids (VFA) formed during hydrolysis step whereas methanogenesis is the rate limiting step for easy biodegradable substrates (Adekunle and Okolie, 2015). In addition, the low growth rates and the susceptibility of the organisms to toxins enhances the difficulties in the optimization of methanogenesis (Karakashev *et al*., 2005). The process can be divided into four steps: hydrolysis, acidogenesis, acetogenesis and methanogenesis.

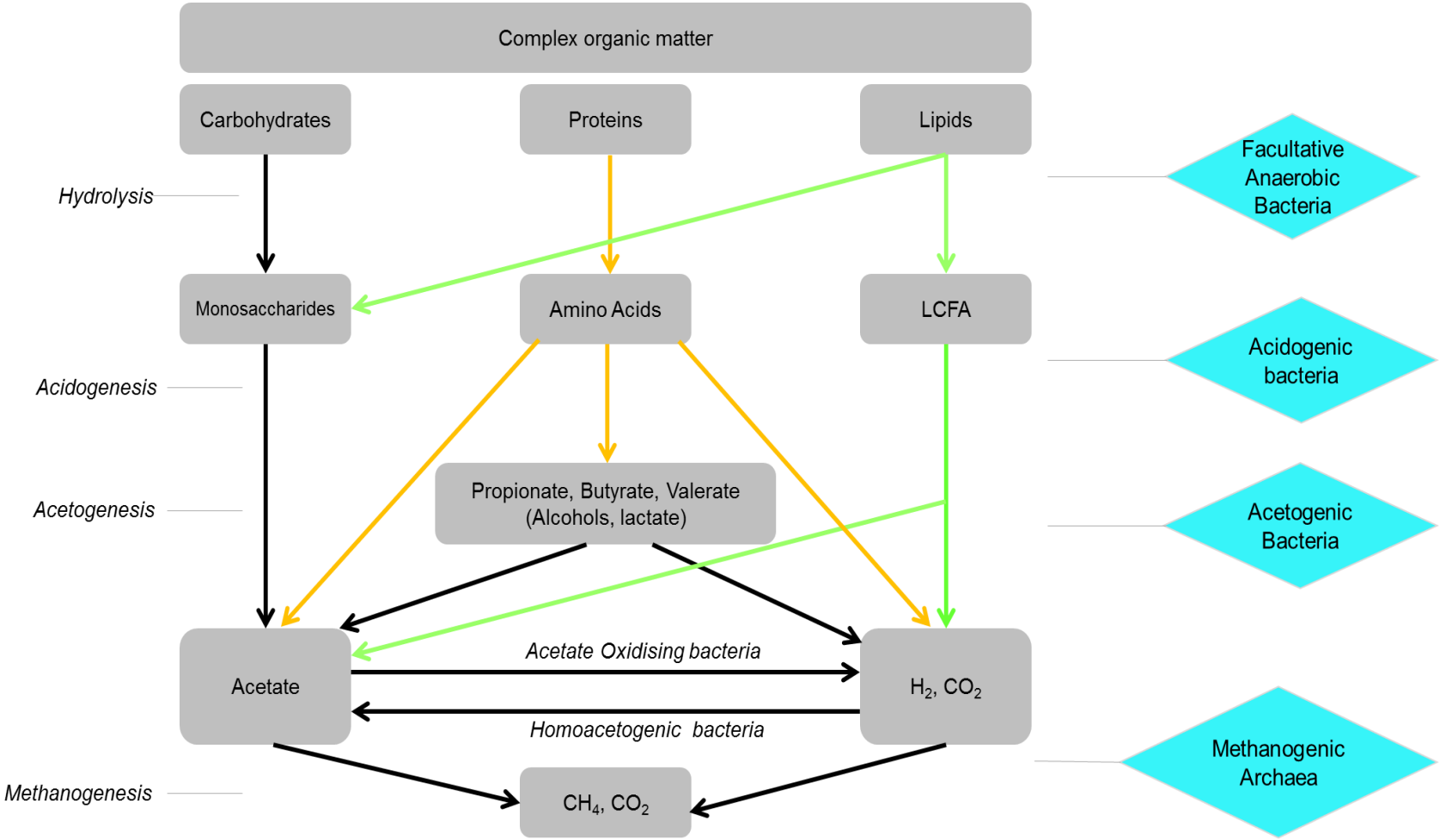

Figure 2-1: Generalized methane production process

Source: Modified from Batstone *et al.*, 2002

2.5.1 Hydrolysis

Hydrolysis is the first step in anaerobic digestion processes. During the hydrolysis step, complex organic matters, such as carbohydrates, proteins and lipids are hydrolyzed into soluble organic molecules such as sugars, amino acids and fatty acids by extracellular enzyme, i.e. cellulase, amylase, protease or lipase (Parawira *et al*., 2005). Insoluble carbohydrates are hydrolyzed to soluble and inert carbohydrates; proteins to amino acids and inert protein and lipids to fatty acids and glycerol as illustrated in equation [2-2], [2-3] and [2-4]. Hydrolytic bacteria, which hydrolyze the substrate with these extracellular enzymes, are facultative anaerobes, Figure 2-1. Hydrolysis can be the rate-limiting step if the substrate contains large molecules (particulates) with a low surface-to-volume ratio (Panico *et al*., 2014). For substrate that is readily degradable, the rate-limiting step is acetogenesis and methanogenesis (Björnsson *et al*., 2001). When the substrate is hydrolyzed, it becomes available for cell transport and can be degraded by fermentative bacteria.

$$(C_6H_{10}O_5)\,insoluble \rightarrow X_c\,(C_6H_{10}O_5)_{soluble} + (1 - X_C)\,(C_6H_{10}O_5)\,inert \quad [2\text{-}2]$$

$$(protein)_{insoluble} \rightarrow X_p\,(Aminoacids) + (1 - X_p)\,(Protein)_{inert} \quad [2\text{-}3]$$

$$C_{57}H_{104}O_6 + 3H_2O \rightarrow C_3H_8O_3 + 3C_{18}H_{34}O_2 \quad [2\text{-}4]$$

Where X_c and X_p represents the fraction of degradable carbohydrates and proteins.

2.5.2 Acidogenesis

In the acidogenesis, the soluble organic molecules from hydrolysis are utilized by fermentative bacteria or anaerobic oxidizers (Garcia-Heras, 2003). These microorganisms are both obligate and facultative anaerobes. In a stable anaerobic digester, the main degradation path way results

in acetate, carbon dioxide and hydrogen, Figure 2-1. The intermediates, such as volatile fatty acids and alcohols, play a minor role. This degradation path way gives higher energy yield for the microorganisms and the products can be utilized directly by methanogens. However, when the concentration of hydrogen and formate is high, the fermentative bacteria will shift the path way to produce more reduced metabolites (Angelidaki & Ellegaard, 2003). Soluble carbohydrates are degraded to acetate ($C_2H_4O_2$), propionate ($C_3H_6O_2$), butyrate ($C_4H_8O_2$), equation [2-5]; oleate ($C_{18}H_{34}O_2$) and glycerol ($C_3H_8O_3$) reduces to biomass ($C_5H_7NO_2$) and propionate ($C_3H_6O_2$), as illustrated in equations [2-6], [2-7] and [2-8]. The products from acidogenesis step consist of approximately 51% acetate, 19% H_2/CO_2, and 30% reduced products, such as higher VFA, alcohols or lactate (Weedermann *et al*., 2015). Acidogenesis step is usually considered the fastest step in anaerobic digestion of complex organic matter (Yu *et al*., 2013).

$$(C_6H_{10}O5)\,soluble + 0.1115NH_3 \rightarrow 0.1115C_5H_7NO_2 + 0.744C_2H_4O_2 + 0.5C_3H_6O_2 + 0.4409C_4H_8O_2 + 0.6909CO_2 + 0.0254H_2O$$

[2-5]

$$C_3H_8O_3 + 0.04071NH_3 + 0.0291CO_2 \rightarrow 0.04071C_5H_7NO_2 + 0.9418C_3H_6O_2 + 1.09305H_2O$$

[2-6]

$$C_{18}H_{34}O_2 + 15.2398H_2O + 0.1701NH_3 + 0.2500CO_2 \rightarrow 0.1701C_5H_7NO_2 + 8.6998C_2H_4O_2 + 14.500H_2$$

[2-7]

$$14.500H_2 + 0.0836NH_3 + 3.8334CO_2 \rightarrow 0.0836C_5H_7NO_2 + 3.4139C_3H_6O_2 + 7.4997H_2O$$

[2-8]

2.5.3 Acetogenesis

Intermediates formed during acidogenesis, consist of fatty acids longer than two carbon atoms, alcohols longer than one carbon atom and branched-chain and aromatic fatty acids. These products cannot be directly used in methanogenesis and have to be further oxidized to acetate and hydrogen H_2 in acetogenesis step by obligated proton reducing bacteria in a syntrophic relationship with hydrogen utilizers. This is illustrated in Figure 2-1. Propionate ($C_3H_6O_2$) and butyrate ($C_4H_8O_2$) are reduced to acetate ($C_2H_4O_2$) and hydrogen (H_2), as demonstrated in equations [2-9] and [2-10]. Low H_2 partial pressure is essential for acetogenic reactions to be thermodynamically favorable (Adekunle and Okolie, 2015; Schon, 2009). The products from acetogenesis are then the substrates for the last step of anaerobic digestion, which is termed methanogenesis.

$$C_3H_6O_2 + 1.764H_20 + 0.0458NH_3 \rightarrow 0.0458C_5H_7NO_2 + 0.9345C_2H_4O_2 + 2.804H_2 + 0.902CO_2 \quad [2\text{-}9]$$

$$C_4H_8 + 1.7818H_2O + 0.0544NH_3 + 0.0544CO_2 \rightarrow 0.0544C_5H_7NO_2 + 1.8909C_2H_4O_2 + 1.8909H_2 \quad [2\text{-}10]$$

2.5.4 Methanogenesis

In methanogenesis step, acetate and H_2/CO_2 are converted to CH_4 and CO_2 by methanogenic archaea. The methanogenic archaea are able to grow directly on H_2/CO_2, acetate and other one-carbon compound, such as formate and methanol (Ali Shah *et al*., 2014). Methane can be produced through different pathways in the methanogenic step. These could involve hydrogen-using methanogenesis as illustrated in equation [2-11] and equation [2-12], which are derived from the propinic step (equation [2-9] and the butyrate step equation [2-10]), respectively. It could also involve aceticlastic methanogenesis, a primary methanogenic step where acetate is

broken down to evolve CH_4 and CO_2 as illustrated in equation [2-13]. In the normal anaerobic digesters, acetate is the precursor for up to 70% of total methane formation while the remaining 30% originates from H_2/CO_2 (Panico *et al.*, 2014). Moreover, the inter-conversion between hydrogen and acetate, catalyzed by homoacetogenic bacteria, also plays an important role in the methane formation pathway. Homoacetogens can either oxidize or synthesize acetate depending on the hydrogen concentration in the system (Kotsyurbenko, 2005). Hydrogenotrophic methanogenesis functions better at high hydrogen partial pressure, while aceticlastic methanogenesis is independent on hydrogen partial pressure. Protein and lipid conversion to acetate involves sequential aceticlastic methanogenesis reactions as described by equation [2-13] (Yu *et al*, 2013). At higher temperatures, the acetate oxidation pathway becomes more favorable (Appels *et al*., 2008). Methane formation through acetate oxidation can contribute up to 14% of total acetate conversion to methane under thermophilic conditions (60°C) (Weedermann *et al*., 2015).

$$2.804H_2+0.01618NH_3+0.7413CO_2 \rightarrow 0.001618C_5H_7NO_2+0.6604CH_4+1.45H_2O \quad [2\text{-}11]$$

$$1.8909H_2+0.0109NH_3+0.4999CO_2 \rightarrow 0.0109C_5H_7NO_2+0.4452CH_4+0.9780H_2O \quad [2\text{-}12]$$

$$C_2H_4O_2+0.022NH_3 \rightarrow 0.022C_5H_7NO_2+0.945CH_4+0.066H_2O+0.945CO_2 \quad [2\text{-}13]$$

2.6 Methanogens

Methanogenic archaea are a phylogenetically diverse group of strictly anaerobic Euryarchaeota with an energy metabolism that is restricted to the formation of methane from CO_2 and H_2, formate, methanol, methylamines and/or acetate (Garcia *et al*., 2000). Methanogens can be

classified as Gram-positive or Gram-negative. They can also be motile (flagellated) or immotile bacteria. Usually, methanogens are coccoid (spherical), spirillum or bacilli (rod) in shape. Methanogens are classified into five orders (Figure 2-2) namely, Methanobacteriales, Methanococcales, Methanosarcinales, Methanomicrobiales and Methanopyrales. Changes in taxonomy and variation of the orthographic of the methanogens have contributes to different names of the methanogens of the same species.

There has been development in the biotechnology of biogas production technology and microbial community with recent studies on phylogenetic characterization of a biogas plant microbial community integrating clone library 16S-rDNA sequences and metagenome sequence data obtained by 454-pyrosequencing by Kröber *et al*., (2009). In his study, most of the bacterial 16S-rDNA sequences could be assigned to the phylum Firmicutes with Clostridia as the most abundant class and to the class Bacteroidetes, while the archaeal 16S-rDNA sequences were clustered close to Methanoculleus bourgensis. A large fraction of 16S-rDNAmetagenome reads could not be assigned to lower taxonomic ranks, demonstrating that numerous microorganisms in the biogas plant are still unclassified or unknown. In addition, literature on the contribution of each bacterial strain to the yield of biogas production is scanty, hence the knowledge gap.

A study by Sinbuathong *et al*., (2009) on the effect of sulfate on the methanogenic activity of a bacterial culture was increased and reached optimum values of 0.128 g methane gas COD/(g VSS x d) when biomass was in contact with sulfate at a ratio of 1:0.114 by weight. In a study on the enhancing effect of aerobic thermophilic (AT) bacteria on the production of biogas from anaerobically digested sewage sludge (Miah *et al*., 2005), it was concluded that addition of 5%

(v/v) AT1 bacterial culture closely related to Geobacillus thermodenitrificans increased biogas production by 2.2 times relative to that from the sewage sludge.

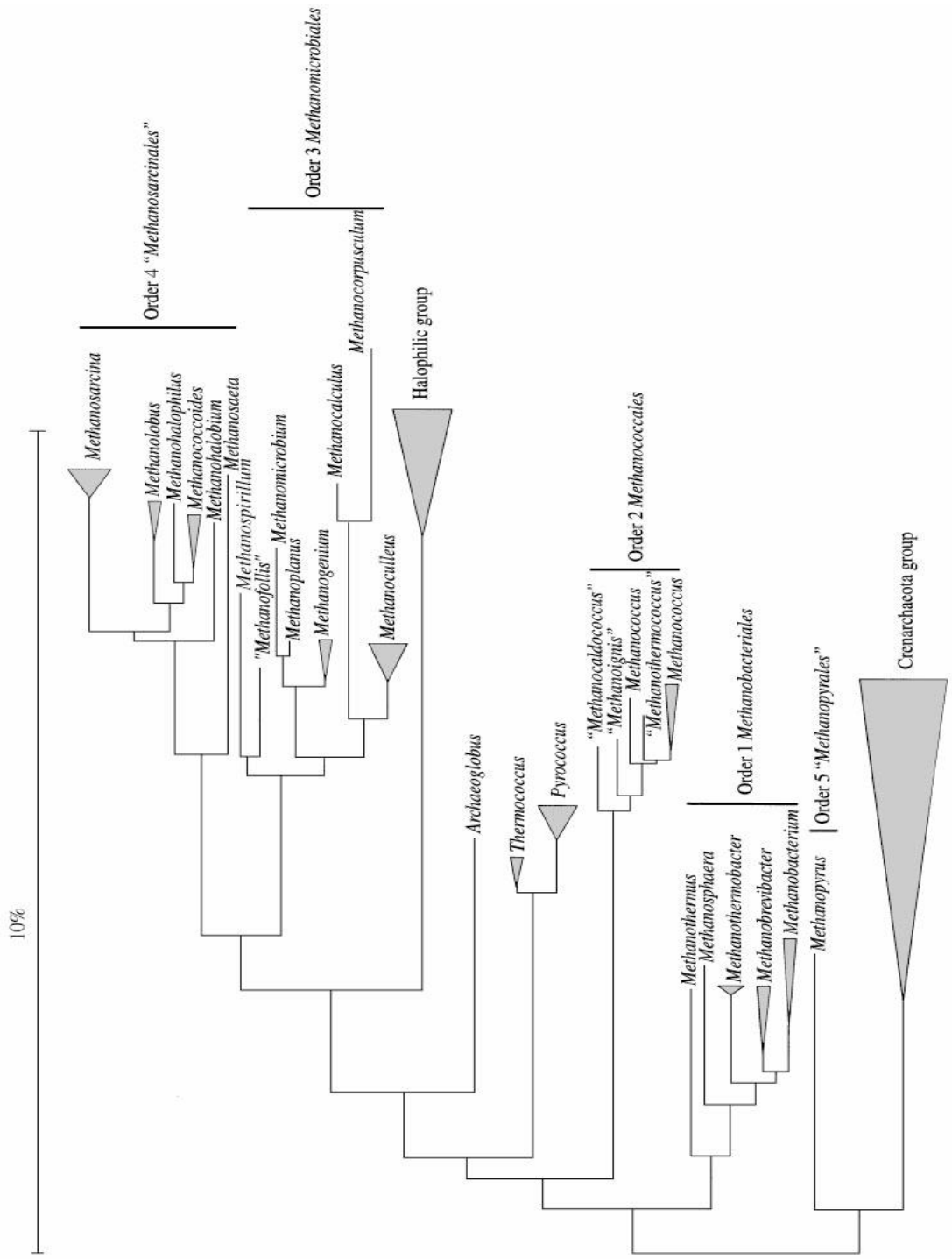

Figure 2-2: Biodiversity of Methanogens

Source: Classification of methanogenic bacteria by Zuo *et al.*, 2015

2.7 Factors affecting biogas production

Efficient utilization of brewery waste water offers an opportunity to produce renewable energy and also reduce greenhouse gas (GHG) emissions. Anaerobic digestion is an essential process for the production of biogas and the main parameters affecting methanogenic reactions in a biodigester include but not limited to nutrient concentration, pH value and temperature among others. These factors need to be controlled to allow maximum growth of the microorganisms involved in the AD.

2.7.1 Nutrient concentration

Organic matters, which are broken down by microorganisms without oxygen, often produces some quantities of methane. All biological process requires adequate nutrients supply particularly Carbon and Nitrogen as well as other elements are also required in trace quantities. The lack of specific elements required for microorganism growth will limit the production of biogas (Sorathia *et al*., 2012). Carbohydrates supplies Carbon which is a source of energy, while the proteins provide Nitrogen needed for the growth of microbial organisms. If the other operating conditions are made favourable for the production of biogas and maximum biological activity, a Carbon to Nitrogen ration of about 30:1 is reported to be ultimate for the raw materials with 2% Phosphorous fed into a biodigestor. A higher carbon to nitrogen ratio will result in excess carbon still available after complete consumption of the nitrogen starving some of the bacteria of this element, leading to the death and returning nitrogen to the mixture, with the net effect of slowing the process. Excess of nitrogen at the end of digestion, which stops when the carbon has been consumed, and reduce the quality of the sludge produced. Thus, nutrients like C, P and N_2 are to be maintained within the optimum range for accelerated fermentation and biogas production (Fillaudeau *et al*., 2006; Macias-Corral *et al*., 2008).

2.7.2 pH

Hydrocarbons are easier to acidify and no pH-buffering ions are released as with the degradation of proteins. Therefore, the pH-value decreases more easily. With the degradation of carbohydrates, the partial pressure of hydrogen increases more easily, as with other substances. This happens in combination with the formation of reduced acidic intermediate products. The pH optimum of the methane-forming microorganism is at pH of 6.8-7.2. Therefore, it is important to adjust the pH-value. Only *Methanosarcina* is able to withstand lower pH values (pH of 6.5 and below). With the other bacteria, the metabolism is considerably suppressed at pH <6.7 (Jayaraj *et al*., 2014).

2.7.3 Temperature

For maximum gas yield, different temperature ranges exist for which the mesophilic and thermophilic bacteria are most active. Two optimum temperature levels have been established the mesophilic level (35-40°C) and thermophilic level (50-65°C) (Jha *et al*., 2011), the choice of which is determined by the natural climatic conditions where the biodigestor is located. Most of the methanogenic microorganisms belong to the mesophilics. Only a few are thermophilic. Methanogenics are generally sensitive to rapid changes of temperature. Thermophilic methanogens are more temperature-sensitive than mesophilics. Even small variations in temperature cause a substantial decrease in activity. Therefore, the temperature should be kept exactly within a range of +/- 2°C. Under mesophilic operating conditions, the inhibition of ammonium is reduced because of the lower content of inhibiting free ammonia. It has to be established that the energy balance is better in the mesophilic range than in the thermophilic range (Cioabla *et al*., 2012).

2.8 Isolation and characterization of bacteria

A wide range of media has been used to estimate the size of the bacterial community of waste water treatment systems and to isolate representatives of these communities (Vieira and Nahas, 2005) although, only a small part of the total number of bacteria in the sample, are able to form colonies on microbiological media (Davis *et al.*, 2005).

There are many practical applications for identifying unknown bacteria. Primary identification involves morphological and biochemical characterization among others. Morphological characterization involves identification of bacteria using visible characteristics of the colony e.g colour, although it's not a reliable way to identify bacteria, as many different types of bacteria have similar colony morphology. Biochemical characterization however is based on the reaction of different microorganisms to biochemical test since each microorganism has a unique DNA that is able to synthesize different protein enzymes that catalyze all of the various chemical reactions. This, in turn, means that different species of bacteria must carry out different and unique sets of biochemical reactions. Molecular techniques are equally important in the analysis of microorganisms as they are effective and fast technology for identification of microbial diversity in different environments (Clarridge, 2004). Genetic diversity can identify individual organisms from some unique part of their DNA or RNA providing definitive information on its biodiversity. In molecular techniques, bacteria are generally identified by 16S ribosomal DNA (rDNA) sequencing. It is a well-established method for studying phylogeny and taxonomy of samples from various environments. 16S rDNA is the most conserved gene in all cells and portions of this rDNA sequence from distantly-related organisms are remarkably similar, indicating that, sequences from distantly related organisms can be precisely aligned, hence ease in estimating rates of species divergence among bacteria

(Janda and Abbott, 2007). The 16S rDNA sequence has hyper variable regions, where sequences have diverged over evolutionary time which are often flanked by strongly-conserved regions. Primers are designed to bind to conserved regions and amplify variable regions. The DNA sequence of the16S rDNA gene has been determined for an extremely large number of species. Sequences from tens of thousands of clinical and environmental isolates are available over the internet through the NCBI (National Centre for Biotechnology Information) (www.ncbi.nlm.nih.gov). These sites also provide search algorithms to compare new sequences to their database.

2.9 Modelling microbial growth

2.9.1 Microbial growth

A growing population of bacteria periodically doubles when grown in the laboratory under favorable conditions. They grow in geometric progression of 2^0, 2^1, 2^2, 2^3 ..... 2^n, where n is the number of generations during the exponential phase. Exponential phase in reality only forms part of the bacterial life cycle but does not represent the pattern of the normal growth of bacteria in nature (Prescott, 2002).

When a fresh medium is inoculated with a given number of cells and the population growth is monitored over a period of time. The growth curve is commonly expressed in terms of microbial numbers, but can also be expressed in terms of optical density as an indirect measurement. Optical density method uses absorbance measurement which is a rapid, nondestructive, inexpensive, and relatively automated method to monitor bacterial growth, as compared to many other techniques like classical viable count methods (Pla *et al*., 2015). Plotting the population growth verses time data yields a typical bacterial growth curve which is

usually divided into the lag phase, exponential phase and the stationary phase as illustrated in Figure 2-3 (Joanne *et al*., 2016).

Lag Phase.

In this phase, the population remains temporarily unchanged immediately after inoculation of the cells into fresh medium. Although there is no apparent cell division occurring, the cells may grow in volume or mass, synthesizing enzymes, proteins, RNA and increase in metabolic activity. The length of the lag phase depends on various factors, including, without limitation on the size of the inoculum, the time from physical damage or shock required to recover in the transmission, time required for synthesis of essential coenzymes or division factors, and time required for synthesis of new enzymes that are necessary to metabolize the substrates present in the medium. The growth is approximately equal to zero, thus;

$$dN/dt = 0 \qquad [2\text{-}14]$$

Exponential (log) phase.

The cells divide regularly by binary fission, and grow by geometric progression. They divide at a constant rate depending upon the composition of the growth medium and the conditions of incubation. The rate of exponential growth of a bacterial culture is expressed as generation time, also known as the doubling time of the bacterial population. The growth can be represented as;

$$2^0N \rightarrow 2^1N \rightarrow 2^2N \rightarrow 2^3N \rightarrow 2^4N \rightarrow 2^5N \rightarrow 2^nN$$

Where n represents the number of doublings occurred after some time interval.

Thus,

$$n = t/t_d. \quad [2\text{-}15]$$

Where; t_d is the doubling time in hours. It follows therefore that the number of cells present at time t, in relation to the initial population is given as;

$$N_t = N_0 2^n \quad [2\text{-}16]$$

Where, n= represents the number of generation,

N=Final number of cells

N_0=Initial number of cells

Substituting the value of n in equation [2-16] gives;

$$N_t = N_0 2^n = N_0 2^{t/t_d} \quad [2\text{-}17]$$

Similarly,

$$N_t/N_0 = 2^{t/t_d} \quad [2\text{-}18]$$

Taking logarithms,

$$\ln(N_t/N_0) = \ln 2^{t/t_d}$$

Which is the same as,

$$(\ln N_t - \ln N_0)/t = 0.693/t_d \quad [2\text{-}19]$$

Plotting the natural logarithm of the number of cells against time of incubation should yield as

straight line whose slope is equivalent to $0.693/t_d$. Thus,

$$d\,(lnN)\,dt = 0.683/t_d \qquad [2\text{-}20]$$

The specific growth rate constant (the rate of increase in the number of cells per unit time can be given by;

$$d\,(\ln N)\,/dN \times dN/dt$$

$$d\,(\ln N)\,/dN \times dN/dt = 1/N \times dN/dt = 0.683/t_d, \qquad [2\text{-}21]$$

Where;

$1/N \times dN/dt$ is the specific growth rate usually symbolically written as **μ** and the units are in reciprocal hours (h^{-1}).

Stationary phase.

Exponential growth cannot be continued forever in a batch culture (e.g. a closed system such as a test tube or flask). Population growth is limited by a number of factors including but not limited to exhaustion of available nutrients; accumulation of inhibitory metabolites or end products; and exhaustion of space. During the stationary phase, if viable cells are being counted, it cannot be determined whether some cells are dying and an equal number of cells are dividing, or the population of cells has simply stopped growing and dividing. The stationary phase, like the lag phase, is not necessarily a period of quiescence. Bacteria that produce secondary metabolites, such as antibiotics, do so during the stationary phase of the growth cycle. It is during the stationary phase that spore-forming bacteria have to induce or unmask the activity of

dozens of genes that may be involved in sporulation process.

Death phase.

If incubation continues after the population reaches stationary phase, a death phase follows, in which the viable cell population declines. The death phase cannot be observed if counting is done by turbidimetric measurements or microscopic counts. During the death phase, the number of viable cells decreases geometrically (exponentially), essentially the reverse of growth during the log phase.

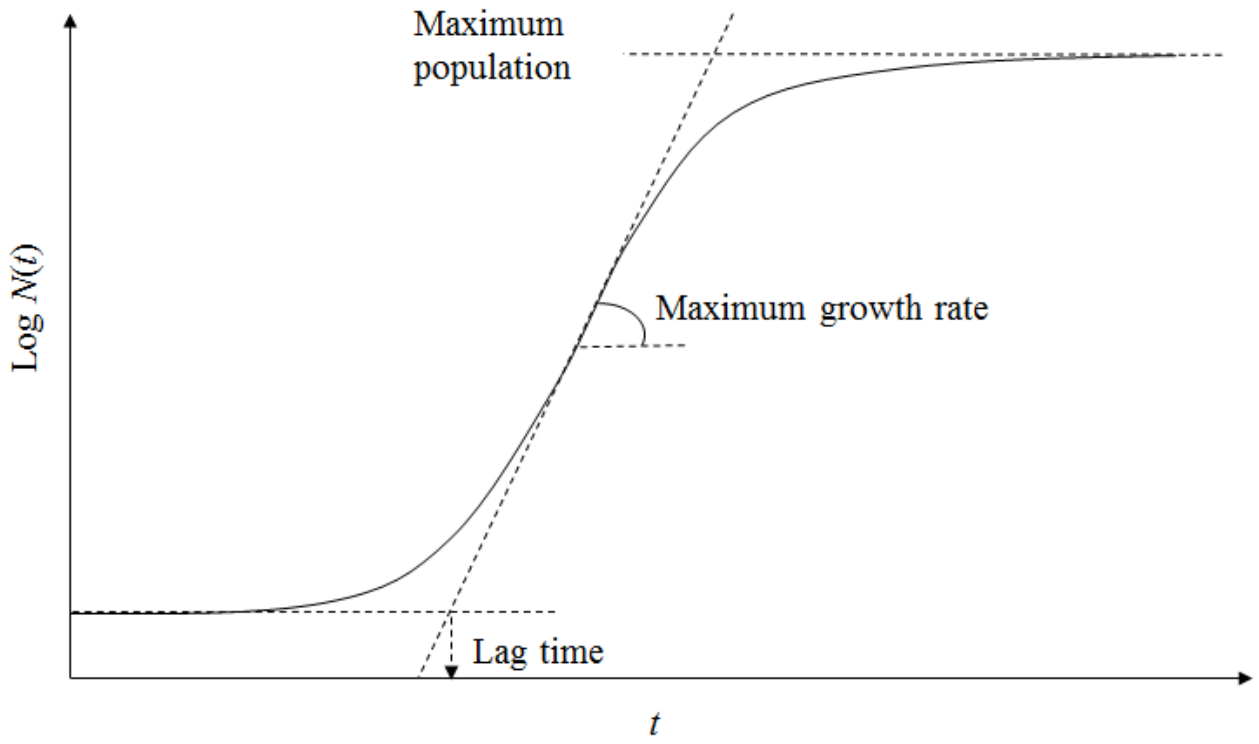

Figure 2-3: Microbial growth curve

Source: Modified from Joanne *et al*., 2016

2.9.2 Microbial growth models

Microbial models are mathematical expressions that describe the number of microorganisms in a given system, as a function of relevant intrinsic or extrinsic variables, generally on a macroscopic scale. Modeling is an important tool for understanding microbial growth in the processes such as safe food production, wastewater treatment, bioremediation, or microbe-mediated and mining among others (Esser *et a.l*, 2015; Marks, 2008; Mitchell *et al.*, 2004) and could also be used as virtual laboratories to optimize experimental design (Pla *et al*, 2015). They can be classified as primary, secondary or tertiary. Primary models describe how the number of microorganisms in a population changes with time under specific conditions. Secondary models relate the primary model parameters to environmental or intrinsic variables such as temperature or pH. Tertiary models combine primary and secondary models with a computer interface providing a complete prediction tool (Marks, 2008).

For the models to be constructed, growth has to be monitored and modelled. Several primary growth models exist in the literature, such as the models by Gompertz, Richards, Stannard *et al*., Schnute, and the logistic model among others (Longhi *et al*., 2013; Zwietering *et al*.,1990). Table 2-2 shows their mathematical and modified forms, which gives biological meaning to the parameters.

This mathematical equations and their modified biological form differ in “ease of use” and number of parameters in the equation (DaSilva *et al*., 2012). Model selection seems to be biased though, Gompertz, Richards, and logistic, are the most commonly used (Pla *et al*., 2015).

Table 2-2: Sigmoidal microbial growth models and their biological modified forms

MODEL	MATHEMATICAL EQUATION	MODIFIED EQUATION
Logistic	$y = \frac{a}{[1+\exp(b-cx)]}$	$y = y_o + \frac{A}{\{1+\exp[\frac{4\mu_{max}}{A}(\lambda - t)+2]\}}$
Gompertz	$y = a - \exp[-\exp(b-cx)]$	$y = y_o + A\exp\{-\exp[\frac{\mu_{max}.e}{A}(\lambda - t)+1]\}$
Richards	$y = a\{1+\upsilon.\exp[k(\tau - x)\}^{(-\frac{1}{\upsilon})}$	$y = y_o + A\{1+\upsilon.\exp(1+\upsilon).\exp[\frac{\mu_{max}}{A}.(1+\upsilon)(1+\frac{1}{\upsilon}).(\lambda - t)]\}^{(-\frac{1}{\upsilon})}$
Stannard	$y = a\{1+\exp[-\frac{(1+kx)}{p}]\}^{(-p)}$	$y = y_o + A\{1+\upsilon.\exp(1+\upsilon).\exp[\frac{\mu\max}{A}.(1+\upsilon)(1+\frac{1}{\upsilon}).(\lambda - t)]\}^{(-\frac{1}{\upsilon})}$
Schnute	$y = \{\mathbf{y}_1^b + (y_2^b - y_1^b).\frac{1-\exp[-a(t-\tau_1)]}{1-\exp[-a(\tau_2-\tau_1)]}\}^{\frac{1}{b}}$	$y = y_o + (\mu\max\frac{(1-b)}{a})[\frac{1-b.\exp(a.\lambda+1-b-at)}{1-b}]^{\frac{1}{b}}$

Source; Zwietering, 1990

Legend; [a] a, b, c are mathematical parameters, A is the asymptote of growth curve when population reaches maximum, μ_{max} is the maximum of specific growth rate, λ is the lag time

The models have different number of growth parameters defined by the growth curve thus there may be existence of difference in the results obtained using different growth models (Zwietering *et al*.,1990). The growth of bacterial usually goes through a phase in which the specific growth rate (the tangent in the inflection point) starts at a value of zero and then accelerates to a maximal value (μ_{max}) in a certain period of time, resulting in a lag time (λ), (the x-axis intercept of the tangent in the inflection point). The asymptote is the maximal log10 N value reached illustrated as the point at which there is maximum population in Figure 2-3, resulting in sigmoidal curves. The models with four parameters also contain a shape parameter (v).

The behaviour of different growth models have been compared in literature ranging from different mathematical measures of goodness of fit and/or other statistical criteria (Pla *et al*., 2015; Tsoularis and Wallace, 2002). Direct comparisons of specific growth parameters as predicted by various models have also been explored with different conclusions, hence, there is substantial disparity in literature on which is the best-fitting model for predicting microbial growth (Longhi *et al*., 2013; Perni *et al*., 2005).The modified stannard equation appears to be the same as the modified Richards equation (Table 2-2),with four growth parameters (A, $\mu_{max,}$ λ and ν).The ν, in the four parameter models represents the shape parameter which is difficult to explain biologically, and are significantly better to use when a large number of datum points are collected (Zwietering *et al*., 1990). However, three parameter models have more degrees of freedom for the parameter estimates which can very useful when growth curves of small number of measured points is used.

2.10 Overview of methodology adopted for the study

The accurate and definitive identification of microorganisms, including bacteria, is important wastewater treatment, food safety, bioremediation, mining among others. Identification is based upon the labelling of bacteria, parasites, and fungi with appropriate binomial names of Latin or Greek origin (Janda and Abbott, 2002). Various techniques for bacteria identification exist ranging from the rapid analysis to the use of molecular methods to provide genus and species identification (Petti, 2007). Bacterial can be identification can using genotypic techniques based on profiling an organism's genetic material (primarily its DNA) and phenotypic techniques based on profiling either an organism's metabolic attributes or some aspect of its chemical composition. Genotypic techniques have the advantage of being independent of the physiological state of an organism unlike the phenotypic methods. Phenotypic techniques, however, can yield more direct functional information that reveals what metabolic activities are taking place to aid the survival, growth, and development of the organism (Emerson *et al*., 2008).

Genotypic microbial identification methods can be classified into two broad categories including the pattern or fingerprint-based techniques and sequence-based techniques. Pattern-based techniques typically use a systematic method to produce a series of fragments from an organism's chromosomal DNA. These fragments are then separated by size to generate a pro-file, or fingerprint that is unique to that organism and its very close relatives. With enough of this information, researchers can create a library, or database, of fingerprints from known organisms, to which test organisms can be compared. When the profiles of two organisms match, they can be considered very closely related, usually at the strain or species level. Sequence-based techniques rely on determining the sequence of a specific stretch of DNA, usually, but not always, associated with a specific gene (Mcdonald *et al*., 2012; Stackebrandt *et al*., 2012). The

degree of similarity, or match, between the two sequences is a measurement of how closely related the two organisms are to one another. A number of computer algorithms have been created that can compare multiple sequences to one another and build a phylogenetic tree based on the results. Sequence-based techniques have proved effective in establishing broader phylogenetic relationships among bacteria at the genus, family, order, and phylum levels, whereas fingerprinting-based methods are good at distinguishing strain or species level relationships but are less reliable for establishing relatedness above the species or genus level (Emerson *et al.*, 2008).

2.11 Summary of literature review

As the utmost common process for the biological treatment of wastewater and biogas production, AD has gained significant importance, albeit problems such as low methane yield and process instability, preventing this technique from being widely applied. This process requires synergistic efforts of various microorganisms in various steps as discussed, where the products of one step are utilized in the next one finally culminating in the production of biogas. Acid forming and the methane forming microorganisms vary broadly in terms of structure, nutritional needs, growth kinetics, and sensitivity to environmental conditions. Thus, failure to maintain the balance between these two groups of microorganisms is the primary cause of reactor instability. Among the important methanogenis processes within AD are the hydrogenotrophic methanogenesis and aceticlastic methanogenesis, which, when they are inhibited, the digestion is effectively blocked at acidogenesis. In addition, the low growth rates and the susceptibility of the organisms to toxins increases the difficulties in the optimization of methanogenesis.

Methanogenic reactions are usually affected by various factors including microbial growth, nutrient concentration, pH value and temperature among others. These factors need to be controlled to allow maximum growth of the microorganisms involved in the AD. The growth of microorganisms can be monitored over a period of time when a given number of cells have been inoculated in afresh medium, and these growths have been modelled for different operations using both primary and secondary models.

Investigation of methanogens can assist in not only the classification but also the optimization of the AD systems. Thus, the study builds on the analysis of the biodegradability index of the brewery waste water and primary microbial growth models developed by Gomperzt and Logistic to describe the growth of the methanogenic community in a bio-digester as a function of time. In industrial applications of anaerobic digestion for biogas generation, the focus is to stabilize and capitalize on biogas production.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Introduction

This chapter summaries the research methodology adopted. It elucidates the wastewater streams which guided the sampling process and the design of the study including the sampling points. The experimental set up for characterization of wastewater, isolation of the bacteria, morphological, and biochemical characterization have been presented. A detailed description on the process for the identification of the pure isolates through molecular characterization is included. In addition, this chapter describes studies undertaken during biogas optimization and modelling of the population growth of the isolated strains. Data analysis tools used are also outlined.

3.2 Sample collection and preservation

Waste water was collected from two different brewing industries here in referred to as 1 and 2 in Kenya at different times. The samples were collected in 2litre glass sampling bottles. The bottles were pre-treated by washing with 70% ethanol and then rinsed with distilled water and dried overnight in an oven at 105 ^{0}C, for disinfection and drying of the sampling bottles (World Health Organization, 2008). The sampling points included the Brewing line; Clean in Place (C.I.P) and the Mixing point between brewing line and C.I.P (Figure 3-1). These was done to evaluate the possibility of waste stream separation during treatment. The samples were immediately transported in cooler boxes to Kenya Industrial Research and Development Institute (KIRDI) and the Institute of Biotechnology Research (IBR) at Jomo Kenyatta University of Agriculture and

Technology (JKUAT) laboratories and stored at 4^0C without further treatment, until they were analyzed.

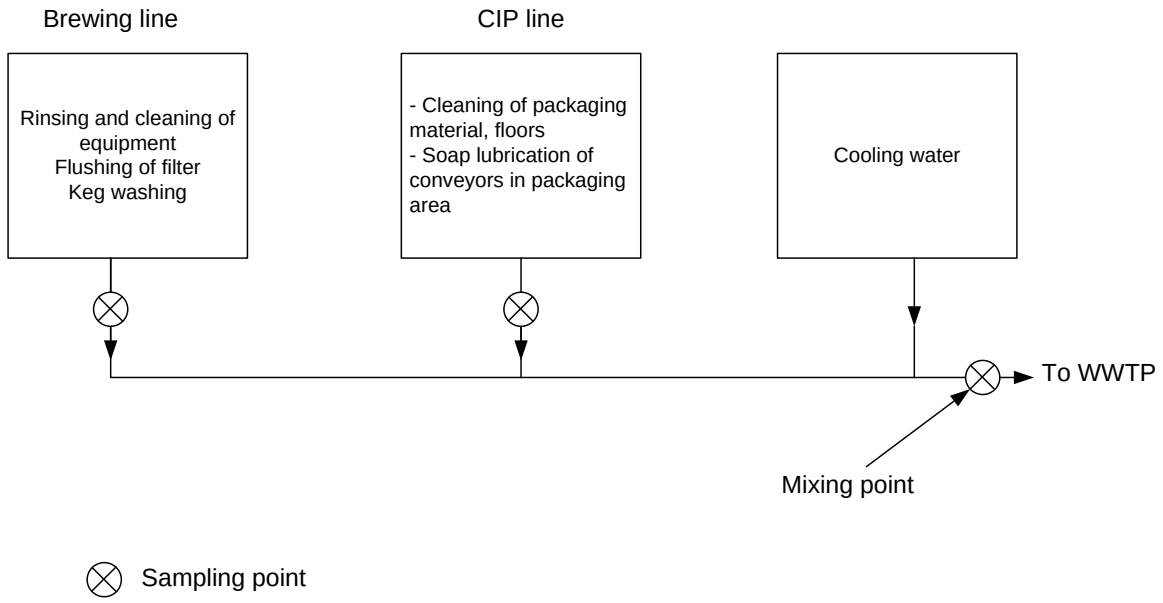

Figure 3-1: Sampling points

3.3 Characterization of Brewery waste water and evaluation of its potential for Biogas Production

Analyses of the samples were all carried out at KIRDI Laboratories. The samples were analyzed as per Standard Method for the examination of water and wastewater (APHA, 2005). The physicochemical characteristics measured include: BOD_5, COD, TDS, TSS, TS, sodium, Total nitrogen and Total phosphorous.

3.3.1 Laboratory analysis

Total solids, Total dissolved solids (TDS), Total suspended solids

Total solids were determined by gravimetric method and then suspended solids were calculated by using equation [3-1].

$$TS = TSS + TDS \quad [3\text{-}1]$$

Biochemical Oxygen Demand (BOD)

BOD was estimated by preparing required volume of dilution water with the addition of nutrients namely phosphate buffer, magnesium sulfate, calcium chloride and ferric chloride. The diluted sample was transferred to BOD bottles. After determining initial DO, final DO was estimated of the bottles kept for incubation period of five days. The bottles kept for DO determination and blank were fixed by adding 2ml manganese sulfate ($MnSO_4$), 2ml of alkali iodide azide ($NaOH + KI + NaN_3$).

Chemical Oxygen Demand (COD)

COD determination was carried out with dichromate reflux method with the addition of 10 ml of 0.25 N potassium dichromate ($K_2Cr_2O_7$) and 30 ml $H_2SO_4 + Ag_2SO_4$ reagent in 20 ml diluted sample. The mixture was refluxed for 2 hours and was cooled to room temperature. The solution was then diluted to 150ml by using distilled water and excess $K_2Cr_2O_7$ remained was titrated with ferrous ammonium sulfate (FAS) using ferroin indicator. The COD values were determined using equation [3-2]

$$COD = ((A - B) \times N \times 100 \times 8)/Volume of sample \quad [3\text{-}2]$$

Where A is the ml of FAS used for blank; B is the ml of FAS used for sample, N is the normality of FAS and 8 is milli equivalent weight of oxygen

Total Nitrogen

The Nitrogen levels were determined using Kjeldahl method, developed by Johan Kjeldahl, where the sample was weighed, digested, neutralized and the nitrogen estimated by titration.

Sodium

The sodium levels were determined using Atomic Absorption Spectrophotometer equipment (AAS). Prior to measurement, the sample were digested in 1:1HCL on a sand bath. After digestion, distilled water was used to take to mark and the sample subjected to AAS.

Phosphorous

The phosphorus levels were determined using UV spectrophotometer. Prior to measurement, the samples were digested in 1:1 HCL on a sand bath. After digestion, distilled water was used to take to mark and a complexing reagent (molybdate) added and color change observed. The sample was then read on the instrument.

3.4 Isolation and Characterization of Methanogenic bacteria from brewery wastewater

To evaluate the microbial population of the brewery wastewater, methane producing bacteria were isolated as a step towards optimization of biogas production. Both morphological and molecular techniques were used during the identification of the isolates. Samples from Brewing Industry 1 were used as inoculum.

3.4.1 Isolation of wastewater 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. Twenty grams of the medium was suspended in 1 litre of distilled water, brought to boil, mixed well and allowed to stand until completely dissolved. 40 ml were dispersed into 100 ml screw cap glass test tubes and sterilised by autoclaving at 121°C for 15 minutes. One millilitre of each sample was inoculated at the base of each test tube using a sterile syringe and incubated at 37^0C in an anaerobic jar. Observations for growth were made after every 12 hours. Serial dilutions of 12 hours 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 used to inoculate a series of plates with two plates at each dilution level and incubated in an anaerobic jar (Plate 3-1). 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 hours at 37°C. The colonies that emerged on the plates were sub-cultured repeatedly on fresh plates to obtain pure isolates.

3.4.2 Morphological characterization.

Preliminary characterization of the isolates involved the examination of colony morphology, (Plate3-2 (1a-1d)). Culture features such as colour, pigmentation, elevation, shape, size and growth form, of safranin-stained bacterial isolates were observed under the dissecting and compound microscope using the slide procedure (Holt *et al*., 1994).

3.4.2.1 Gram staining of the isolated bacteria

Smears of bacteria cultures were prepared and heat-fixed. Slides were placed on the staining rack and flooded with crystal violet and allowed to stand for 30 seconds. The slides were then rinsed with water for 5 seconds followed by covering with Gram's iodine mordant. They were then

allowed to stand for 1 minute and rinsed with water for 5 seconds. Decolonization was done with 95% ethanol for 15 to 30 seconds followed by rinsing with water for 5 seconds. Counterstaining was done using Safranin for about 60 to 80 seconds and the slides were rinsed again with water for 5 seconds.

Blot drying was carried out using a bibulous paper and examined using microscope at x100 under oil immersion (Srinivasan *et al.*, 2012). Gram-positive organisms stained blue to purple; Gram-negative organisms stained pink to red. Gram staining results were confirmed by using the 3% KOH test (Chandra and Mani, 2011). This test was performed by adding a drop of 3% KOH on a slide. A loop full of the bacteria was introduced and mixed thoroughly. Positive results were observed if the culture pulled along with the wire loop when raised up and negative results were recorded if nothing was pulled along the wire loop.

Plate 1a

Plate 1b

Plate 1c

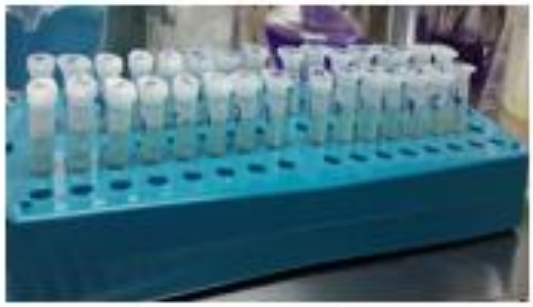

Plate 1d

Plate 3-1: (1a) Autoclaved media on plates and tubes for inoculation. (1b) incubation in anaerobic jar. (1c) Growth on plates. (1d) Pure strains sub-cultured in Eppendorf tubes

Plate 1a

Plate 1b

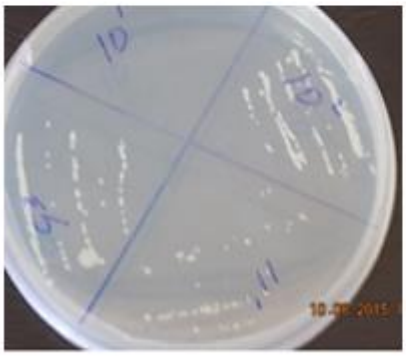

Plate 1c

Plate 1d

Plate 3-2: Morphological characterization under compound microscope depicting different isolates

3.4.3 Biochemical characterization of isolated bacteria

Biochemical tests were carried out as described in Cappuccino and Sherman (2001) and Harold (2002). The biochemical tests conducted were triple sugar iron, gelatine liquefaction, motility, starch hydrolysis, H_2S production, catalase test and indole production test.

3.4.3.1 Triple Sugar Iron Agar Test

Triple Sugar Iron contains three carbohydrates namely, glucose, sucrose and lactose. The media also contains beef extract, yeast extract and peptones which are sources of Nitrogen, vitamins and minerals. Agar and phenol red are also used to solidify the medium and pH indicator respectively. The tubes containing molten agar were angled during preparation and using aseptic technique, the TSI slant was inoculated by first stabbing the butt down to the bottom and then streaking the surface of the slant with appropriate bacterium. The caps on the tubes were tightly screwed not to permit access of air. Incubation was done anaerobically for 18 to 24 hours at 35°C for changes in the butt and on the slant.

A change in the original colour of the medium (reddish-orange) to yellow indicates fermentation of any of the carbohydrates, a red colour in both slant and butt indicates neither glucose, lactose nor sucrose has been fermented. Presence of bubbles in the butt indicates the ability of the bacteria to produce gas. Hydrogen sulfide production from thiosulfate is indicated by a blackening of the butt as a result of the reaction of hydrogen sulfide with ferrous ammonium sulfate to form a black ferrous sulfide.

3.4.3.2 Catalase test

Catalase test detects the activity of enzyme catalase that is present in most cytochrome-containing aerobic bacteria. These microbes produce hydrogen peroxide during the aerobic breakdown of sugars. The test was performed to determine the ability of the isolates to produce catalase enzyme which microorganisms living in oxygenated environment use to neutralise toxic forms of oxygen metabolites (hydrogen peroxide). Catalase enzyme neutralises the bactericidal effects of hydrogen peroxide and thus protecting them. This enzyme catalyses the

breakdown of hydrogen peroxide into water *et al* and oxygen. Therefore, Catalase-negative bacteria may be anaerobes, or they may be facultative anaerobes that only ferment and do not respire using oxygen as a terminal electron acceptor (Manimegalai., 2014). Catalase test was carried out by scooping a colony of a 24-hour culture, placing it on a glass slide and adding a drop of 3% hydrogen peroxide solution. A positive reaction was indicated by the formation of bubbles, while the absence of air bubbles indicated a negative catalase test.

3.4.3.3 Gelatine liquefaction/hydrolysis

Gelatine liquefaction detects the breakdown of gelatine to polypeptides and amino acids by enzyme gelatinase. Gelatine protein is produced by hydrolysis of a component of the connective tissues and tendons of animals known as collagen. Gelatine is solid at room temperature but above 25^{o}C it turns into liquid. When gelatinase hydrolyses this protein into amino acids, it remains liquefied even at the low temperatures of an ice bath. The bacterial isolates were inoculated onto nutrient broth supplemented with 12% gelatine and 1.5% agar, to demonstrate hydrolytic activity of gelatinase. One uninnoculated tube was used as control for each isolate. After incubation, cultures that remained liquefied totally or partially when placed in refrigerator at 4 ^{o} C for 30 minutes were considered positive for gelatine hydrolysis.

3.4.3.4 Indole, Motility and Hydrogen sulphide production tests

The test identifies isolates with the ability to produce the enzymes tryptophanase that removes the amino group from tryptophan to form Indole, pyruvic acid and ammonia, and cysteine desulfurase, that produces pyruvate, ammonia and hydrogen sulphide from sulphur containing amino acids. Indole reacts with Kovacs reagent (*p*-dimethylamino-benzaldehyde) to form a

deep red colour while the Iron in the medium reacts with hydrogen sulphide to produce a black precipitate.

The isolates were inoculated in Sulphur-Indole Motility (SIM) agar media by stabbing method in duplicate for replication, and then incubated at 37 ^{o}C for 48 hours. Two uninnoculated tubes were used as controls. Kovac's reagent was the added to each of the 48-hour culture. The presence of a cherry red layer in the media indicated positive result for Indole production while negative results were indicated by colour remaining brown. The presence of a black coloration in the media after incubation indicated lack of hydrogen sulphide in the media. Lack of motility was detected by the confinement of the bacteria along the line of inoculation.

3.4.3.5 Starch Hydrolysis

Using the wax pencil, starch agar plate was divided into two straight sections. Labelling was done each with the bacterium to be inoculated. Using aseptic technique streaking was done with the respective bacteria onto the plate in a straight line within the section. Incubation of the plate for 24 to 48 hours at 37 ^{o} C was done. Drops of Gram's iodine were placed on each of the line streaked on the starch agar plate. If the area around the line of growth was clear, starch had been hydrolysed, and the test was positive; if it was not clear or the entire medium turned blue, starch has not been hydrolysed, and the test was negative.

3.4.4 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. Pure isolates were subcultured on the plates and incubated anaerobically

in anaerobic jar at 37^{o} C for three days. The cultures were then subjected to excitation by long wave ultraviolet light at 312nm using Uvidoc BTS-20.M. The cultures that exhibited a bluish green fluorescence were considered positive. 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. The gas produced was analysed using Biogas 5000 analyser, with CH_4 and CO_2 accuracy of ±0.5% of measurement reading after calibration.

3.4.5 Molecular characterization

Molecular characterization was used to confirm the identity of the isolates. Total bacterial DNA was extracted according to the procedures described by Sambrook *et al*. (1982) and Magarvey *et al*., (2004). Bacterial 16S rDNA genes of the pure isolates were amplified and used as a template for amplification of 16S rDNA gene.

3.4.5.1 DNA Extraction

Genomic DNA was extracted from bacteria cells at exponential growth phase grown anaerobically in Brewer Thyglycollate broth media. Prior to extraction, bacterial cells were harvested from broth by centrifuging 1 ml of culture in a 1.5 ml Eppendorf tube at 13,000 rpm for 10 minutes. The pellet was washed by re-suspending the cells in equal volume of TE buffer, centrifuged for 5 minutes at 13,000 rpm and the supernatant discarded. The cells were re-suspended in 200 µl of solution 1(A) [50 mM Tris pH 8, 50 mM EDTA pH 8 and 25% sucrose solution), 20µl of lysozyme (20 mg/ml) and 10µl of RNase A (20 mg/ml) then mixed gently. The mixture was then incubated at 37 ^{o}C for 1 hour. After 1hr, 600 µl of solution 2 (B) (10 mM Tris pH 8, 5mM EDTA pH 8 and 1% sodium dodecyl sulphate] and 10 µl of proteinase K (20 mg/ml)

were added and mixed gently (appendix 2). The solution was incubated at 55 ^{o}C for 1 hr. The DNA was extracted using equal volume of phenol chloroform (1:1) and mixed by inversion for 5 minutes, followed by centrifuging for 10 minutes at 10000 rpm. The aqueous layer was carefully transferred into a 1.5 ml Eppendorf tube. This step was repeated twice before adding 500 µl of chloroform: Isoamylalcohol (24:1). This step was to wash off the excess phenol.

The mixture was then mixed by inversion for 5 minutes followed by centrifuging at 10000 rpm for 10 minutes and the supernatant carefully transferred into a clean Eppendorf tube. This step was repeated twice. The DNA was precipitated by adding an equal volume of ice cold absolute ethanol and 1/10 µl of sodium chloride and incubated overnight at -20^{o} C. It was then centrifuged at 13,000 rpm for 10 minutes and the supernatant discarded. 500 µl of 70% ethanol was added and centrifuged at 13,000 rpm for 10 minutes. The supernatant was discarded carefully. The pellets were then air-dried at room temperature and re-suspended in 100 µl of TE buffer and kept at −20^{o}C for future use. The DNA was visualised on a 1% agarose gel in 1XTBE buffer under UV after staining with ethidium bromide.

3.4.5.2 DNA amplification

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'-GGCTACCTTGTTACGACTT-3' according to the position in relation to Escherichia coli gene sequence (Bergmann *et al.*, 2010). 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 X buffer with mgcl2 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.

3.4.5.3 Restriction analysis of the PCR products

The preliminary genetic diversity was determined by amplified ribosomal DNA restriction analysis (ARDRA) of 16S rDNA using a modified procedure (Desaint *et al*, 2000). Aliquot of 8 µl of the PCR product was digested in a final volume of 30µl for 12 hours at 37^{o} C with 0.5 µl of a restriction endonuclease (*RsaI*) according to the manufacturer's specifications (Sigma Aldrich, Steinheim, Germany). Digested DNA fragments were separated by electrophoresis in 1.5 (w/v) agarose gels (Sigma Aldrich, Steinheim, Germany) for 2 hours at 80 V. The gel was stained with ethidium bromide and DNA fragments visualised under UV illumination (BTS-20.M, EEC, Taiwan). Similarity among strains was estimated from the proposition of shared restriction fragments bands generated by *RsaI* digestion.

3.4.5.4 Agarose gel electrophoresis

Preparation of Agarose gel (w/v) at 1% was done by dissolving 1.0 g of agarose powder into100 ml of 1XTBE buffer. The gel solution was stirred, brought to boil in a microwave for 3 minutes to completely dissolve the powder, the cooled gel solution was poured in a casting tray having combs and left for sometimes to gel (polymerise). Ethidium bromide (3 µl) was incorporated in the gel to facilitate visualisation of DNA under UV light. The PCR products (7 µl) was mixed

with 3 µl of loading dye (Bromophenol blue) and loaded into the well and subjected to electrophoresis at 80 V for 45 minutes.

3.4.5.5 Purification of PCR products

The PCR products were purified using the QIAquick PCR purification Kit protocol (Qiagen, Germany) according to manufacturer's instructions. Five volumes of buffer PB (binding buffer) (Qiagen, Germany) was added to one volume of the PCR sample and thoroughly mixed. The mixture was then transferred to QIAquick spin column and then centrifuged for three minutes at 8000 g. The flow-through was discarded, and the QIAquick column placed back into the same tubes. To wash the DNA, 740µl buffer PE (washing buffer) was added to the QIAquick column twice and centrifuged for one minute. The flow-through was discarded and the column centrifuged again for an additional one minute at 8000 g to remove residual ethanol from buffer PE. The QIAquick column was placed in a 1.5 ml microcentrifuge and 30 µl of buffer EB (elution buffer) (10 mM Tris-Chloride, pH 8.5) added to elute DNA. The tubes were then centrifuged for one minute, the spin column removed and DNA. The tubes were then centrifuged for one minute at 8000 g, the spin column removed and DNA stored at -20^{o} C.

3.5 Optimization of biogas from isolated strains

Optimization of the environmental conditions involved variations in temperature and pH. In addition, modified biological equations from mathematical models were used to predict the growth of the isolated methanogenic stains at a constant temperature of 37°C.

3.5.1 Effect of temperature and pH on methane production

Anaerobic digestion experiments to measure the methane produced were carried out using Brewer Thyglycollate media (appendix 1), 250 ml vacuum flasks were used as batch digesters

and the temperatures studied included 30 ºC, 35 ºC, 37 ºC and 40 ºC, with a pH range of 6.0,7.2 and 8. The methane yields from each experimental setup, Plate 3-3, were measured with Biogas 5000 gas analyzer with CH_4 and CO_2 accuracy of ±0.5%of measurement reading after calibration. Each experimental set up were done and recorded in triplicates. Both methane yield and biogas quality (CH_4, CO_2, O_2 and H_2S,) were investigated and were recorded at the end of the incubation period of seven days. The cumulative volume of the gas produced during the incubation period was estimated by measuring the initial and final pressure in the 250 ml vacuum flasks and applying the ideal gas law to calculate the volume as illustrated in equation [3-4]. The Initial pressure was indicated on the KIF LAB Labotporp vacuum pump during air evacuation and the final pressure was as indicated by barometer on the biogas analyzer 5000.

$$V_{gas} = \frac{(P_2 - P_1)V_r T_a}{P_a T_r} \qquad [3\text{-}4]$$

Where;

V_{gas} = volume of cumulated gas produced (mL);

P_1 = Initial pressure of the as indicated by the vacuum pump (kPa);

P_2 = Final pressure after incubation period (kPa);

P_a = ambient pressure

T_a = ambient (Initial) temperature (K);

T_r = temperature of the reactor (vacuum flask) (K);

V_r = capacity of the vacuum flask (250mL).

Methane production (ml) =Total gas produced (ml) × percentage methane in the sample

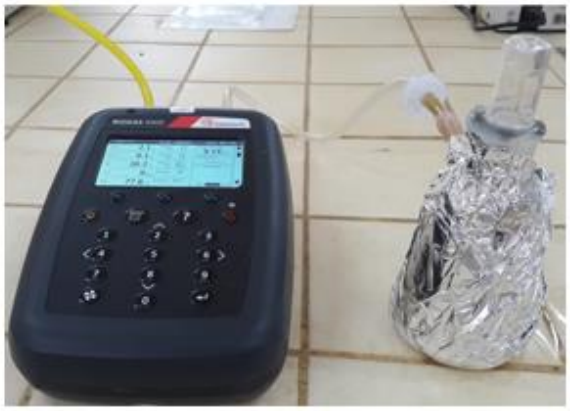

Plate 1a

Plate 1b

Plate 3-3: Experimental set up for biogas analysis in the bio-digester

3.5.2 Calibration of the equipment

The BIOGAS 5000 gas analyzer was calibrated 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.

3.5.3 Monitoring growth rate of the isolates at 37°C

Growth can be defined as an increase in cellular constituents and may result in an increase in a microorganism's size, population number, or both (Prescott, 2002). Freshly cultured 12 hour old

cells of pure isolates were used in this experiment. 200µl of the cells were inoculated in a closed culture vessel of 800µl brewer Thyglycollate single batch media after autoclaving at 121ºC. The contents were then incubated in a Labtech dhaihan shaking incubator at 250 rpm. No fresh medium was provided during incubation. The initial turbidity was observed by taking the OD 600 nm values using a spectrophotometer at time zero for all the isolates. Incubation was done at 37ºC and the OD values were taken at the time intervals of 0, 1, 2, 4, 6, 15, 18, 22, 24, and 28 hours respectively in triplicates. To reduce effect of cell multiplication during turbidity determination, the samples were placed in a freezer until the readings were recorded.

3.5.4 Mathematical model and non-linear regression analysis.

Using non-linear regression in *R* programming language and Marquardt algorithm, microbial population growth data were fitted to the modified logistic function and Gompertz function (Table 2-2) as illustrated in equation [3-1] and [3-2] respectively.

$$\log 10N(t) = \log 10No + \frac{A}{(1+\exp[\frac{4\mu\max}{A}(\lambda - t)+2])} \quad [3\text{-}1]$$

$$\log 10N(t) = \log 10No + A\exp\{-\exp[\frac{\mu\max.e}{A}(\lambda - t)+1]\} \quad [3\text{-}2]$$

Where A is given by equation [3-3]

$$A = \log 10\frac{N\max}{No} \quad [3\text{-}3]$$

$\mu_{\max}$ is as illustrated in equation [2-21], while λ and t represent the lag time and time of incubation respectively.

Marquardt algorithm is a search method for minimizing the sum of the squares of the differences between the predicted and measured values. The algorithm automatically calculates starting values by searching for the steepest gradient of the curve as illustrated in equation [2-21] between four datum points (estimation of μ_{max}), by intersecting this line with the x axis (estimation of λ), and by taking the final datum point as estimation for the asymptote (A) as illustrated in equation [3-2] (Pla *et al.,* 2015). The algorithm then calculates the set of parameters with the lowest residual sum of squares (RSS) and their 95% confidence intervals. The fitting of the growth models for Gompertz and Logistics are described in algorithm in appendix 3. The nls function is used for nonlinear least squares fitting of these models.

3.6 Statistical analysis

All the tests were replicated three times. Data was analysed using SPSS version 21 and Genstat 12th edition. Analysis of the observations on the physicochemical parameters of the brewery waste water were carried out to establish the variations in concentration of these parameters with respect to different sampling points as illustrated in Figure (3-1) irrespective of the industry from which the sample was taken. Analysis to determine the interaction between the sampling point and the industry were also computed. In order to evaluate the similarities (or dissimilarities) of the datasets based on the physicochemical parameters, hierarchical clustering using Bray-Curtis dissimilarity was performed. The single linkage (nearest neighbor) based on Euclidean distances was used to display the clusters combined at each stage and the distances at which this merger takes place. Computation of hierarchical clustering and heat map of the datasets were carried out using *R* programming language and Vegan package (*R* Development Core Team, 2011). A Principal Component Analysis was used to assess the factor composed of the physicochemical parameters measured that best explain the greatest variability in the brewery effluent quality

regardless of the industry. In addition, a model representing the relationship between the biodegradability index and the other physicochemical parameters has also been developed.

3.6.1 Sequencing and phylogenetic analysis

Sequencing of purified PCR products was done by a commercial service provider, Macrogen Nertherlands Branch, due to unavailability of the same service in the country, 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 was performed by the Maximum likelihood method using Mega5 software (Appendix 3). To show the evolutionary relationships of these taxa, the evolutionary history was inferred using the Neighbour-Joining method (Tamura *et al*., 2007). Bootstrap analysis using Mega 5 software, was performed to attach confidence estimates for the tree topologies. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the maximum composite likelihood method and were in the units of the number of base substitutions per site.

3.6.2 Model comparison

Data fits obtained by using the growth models were compared statistically by the use of Akaike Information Criterion (AIC) (Kahm *et al*., 2010) based on information theory, r^2,and RSS (Longhi *et al*., 2013; Pla *et al.,* 2015),with 95% confidence limits. The AIC is defined by equation [3-3].

$$AIC = N \ln \frac{SS}{N} + 2K \quad [3\text{-}3]$$

Where N represents the number of data points, K is the number of parameters fit by the regression plus one, since regression is estimating the sum of squares as well as the values of the parameters, and SS is the sum of square of the vertical distances of the points from the curve. An AIC value can be positive or negative and the sign doesn't have a meaning since it can be changed using different units to express data. Models were compared by evaluating the difference between the AIC values in which the model with the smallest AIC values was taken as the most likely to be correct.

3.6.3 Model validation

The effect of the physicochemical parameters on the biodegradability index model was validated by computing the R^2 values.

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1 Introduction

This chapter presents the physicochemical characteristics of the brewery waste water in which the variations in the concentration levels of these parameters and their relationships have been discussed. A model representing their effect on the biodegradability index has been presented. Isolated strains of methanogenic bacteria from the brewery waste water have been characterized and identified. The environmental effect on the biogas production have been analysed, the optimal conditions established and primary models predicting the population growth presented. To conclude, the chapter examines the study's contribution to knowledge.

4.2 Characterization of Brewery waste water and evaluation of its potential for Biogas Production

4.2.1 Variation in the sampling points in the industries

Table 4-1 shows the means of physicochemical parameters of the untreated brewery waste water without considering the industry from which the sample was taken. Samples from the brewing line had the highest concentrations for all the parameters measured except sodium concentrations and were significantly different (p<0.001) in concentration levels from the rest of the sampling points. The levels of COD were found to be very high with levels reaching 50,966 ± 20,146.67mg/l at brewing line. The BOD, Nitrogen, TS, TDS and TSS concentrations followed a trend in which the brewing line had the highest concentrations followed by the mixing line and lastly CIP. The concentrations for these parameters were found to be significantly different from each other at p<0.001.

4.2.2 Interaction between sampling point and the industry

From Table 4-2, COD levels for Brewing line for Industry 1 had the highest value which was significantly ($p<0.001$) different from those of Brewing line for Industry 2, CIP and Mixing lines for both industries. COD levels for Industry 2 brewing and mixing lines were not significantly different from each other; while the levels of CIP in both industries and Industry 2 mixing line were found to be not significantly different from each other. BOD results indicate that there were significant differences in values obtained, between the industries and sampling points, with the exception of Brewing line for industry 1 and mixing line Industry 2, which were not significantly different from each other. Generally, brewing line for industry 1 had the highest concentrations in all the physicochemical parameters, (Figure 4-1), a part from sodium which was not significantly different from other sampling lines in these industries with the exception of mixing line in industry 2.

The brewery wastewater in this study was characterized by large variations in the parameters mentioned in Table 4-1 and 4-2 and Figure 4-2. The high values of COD in the brewery wastewater from the industries indicates heavy load of organic components (sugars, soluble starch, ethanol and volatile fatty acids). The values of COD levels at the mixing line are comparable to the obtained values in literature of 2000 mg/l to 6000 mg/l (Caliskan *et al*., 2014; Simate *et al*., 2011). The discharge of this wastewater to the environment without any treatment would contribute to a significant risk for public health and environmental pollution. High BOD and COD levels could accelerate bacterial growth which consumes the oxygen levels when discharged untreated into the river. The oxygen may be diminished to levels that are lethal for most fish and many aquatic insects (Akpor *et al*., 2011).

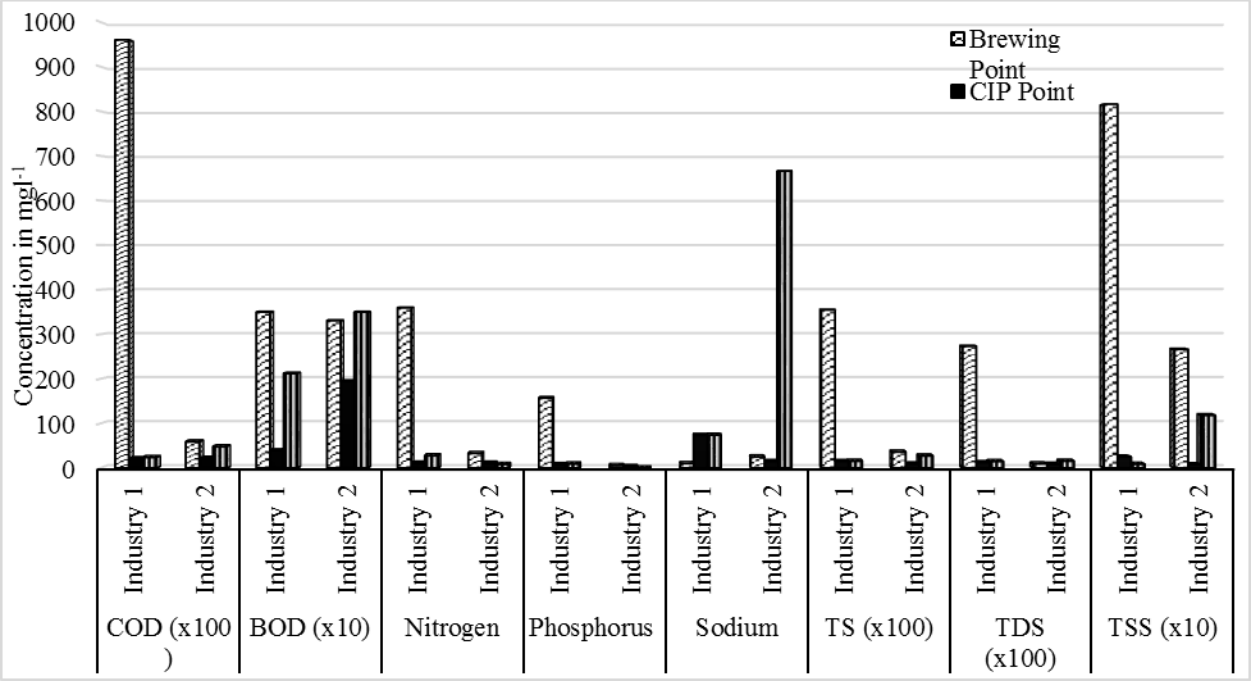

Figure 4-1: **Interaction between sampling point and industry for physicochemical parameters**

Nitrogen and Phosphorus levels were found to be high at the brewing lines for both industries with industry 1 values being significantly different from that of brewing line for industry 2. The high levels of Nitrogen and Phosphorous could be contributed by the difference in the raw material used by these industries for instance barley or rice during brewing (Goldammer, 2008). Fermentation and maturation step duration and variations in the amount of yeast present in the effluent could also increase the nitrogen and Phosphorous levels as reported by Caliskan *et al.*, 2014. The differences in phosphorous levels could also be contributed by heavy use of phosphorous containing chemicals used during cleaning and rinsing of brewing equipment and at the keg washing stage. Degradation of nitrogen during anaerobic digestion results into the production of ammonia which is considered toxic for the methanogens (Gerardi, 2003). The total

suspended solids were found to be 636 mg/l which was slightly higher than the literature values of 600 mg/l.

Table 4-1: Means for the physicochemical parameters for sampling points

Sampling point	COD	BOD	Nitrogen	Phosphorus	Sodium	Total solid	TDS	TSS
Brewing line	50966±20146.67 a	3403±53.52 a	196.23±53.52 a	81.54±33.94 a	17.7±3.35 b	19546±7120.59 a	14135±5891.45 a	5411±1235.4 a
CIP line	3610±541.51 b	1170±348.83 c	11.2±348.83 c	5.92±0.97 b	44.3±13.22 b	1216±126.78 c	1053±92.83 c	163±47.5 c
Mixing line	2167±61.46 c	2812±307.9 b	18.2±307.9 b	4.74±2.04 b	370.2±133.67 a	2157±261.63 b	1521±16.53 b	636±245.16 b
LSD	1035	64.1	3.498	3.407	35.93	142.2	157.5	251.1
CV%	4.4	2.1	3.7	8.8	19.8	1.5	2.2	9.6
p value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Note: LSD means least significant difference; CV means coefficient of variance

Table 4-2: Means for physicochemical parameters for the sampling point and industry

Point & Industry	COD	BOD	Nitrogen	Phosphorus	Sodium	Total solid	TDS	TSS
Brewing 1	96000±1154.7 a	3500±57.74 a	359.33±3.37 a	157.33±3.71 a	10.2±0.21 b	35467±156.78 a	27308±162.81 a	8159±275.33 a
Brewing 2	5931±34.06 b	3307±40.55 b	33.13±2.03 b	5.75±0.87 bc	25.2±0 b	3625±26.09 b	963±17.33 cd	2662±9.17 b
CIP 1	2133±66.67 c	390±0 e	11.2±0 c	8.1±0 b	73.9±0.08 b	1499±6.67 d	1249±64.92 bc	249±61.33 d
CIP 2	2200±115.47 c	1950±0 d	11.2±0 c	3.75±0 bc	14.7±0 b	933±15.25 e	857±17.98 d	76±3.06 d
Mixing 1	2400±38.68 c	2123±14.53 c	28±0 b	9.27±0.38 b	74.1±0.12 b	1572±4.93 d	1484±2.96 b	88±6.23 d
Mixing 2	4820±11.84 b	3500±0 a	8.4±0 c	0.2±0 c	666.3±40.39 a	2742±0 c	1558±0 b	1184±0 c
LSD	1463.7	90.6	4.946	4.818	50.81	201.1	222.7	355.1
CV%	4.4	2.1	3.7	8.8	19.8	1.5	2.2	9.6

Note: LSD means least significant difference; CV means coefficient of variance

4.2.3 Hierarchical clustering of the physicochemical parameters

From the six sampling points, the Clean in Place for industry 2 (CIP2), Mixing point for industry 1 (Mixing1) and Clean in Place for industry 1 (CIP1) were clustered in one group while Mixing point for industry two (Mixing 2) and brewing line for industry 2 (Brewing 2) were shown to form another group as illustrated in Figure 4-2. This implies that the sample points clustered together have similar physicochemical attributes; hence the waste water from these streams could be mixed prior to treatment without significantly affecting the treatment process. From the six sampling points, brewing line for industry one (Brewing 1) was observed to be distantly related from all the others. Thus the characteristics of waste water at this point have different characteristics in terms of concentration levels from the rest. It could also be attributed to the difference in processing method and raw materials employed by industry 1 in relation to industry 2. This was also supported by the heat map showing the similarities between the sampling points and physicochemical parameters, Figure 4-3. From the heat map additional information can also be extracted about the physicochemical parameters. In which case, total solids and TDS were in one cluster while BOD and TSS were in another. This clustering of the physicochemical parameters insinuates similar pattern of concentrations of the parameters across the sampling points. The pattern of concentrations of Sodium, total Nitrogen and Phosphorus were shown to be similar according to the heat map. COD on the other hand was observed to be an outlier from other parameters as the values obtained for both industries were high, implying that this waste water could be digested using anaerobic digestion (Metacalf & Eddy, 2004).

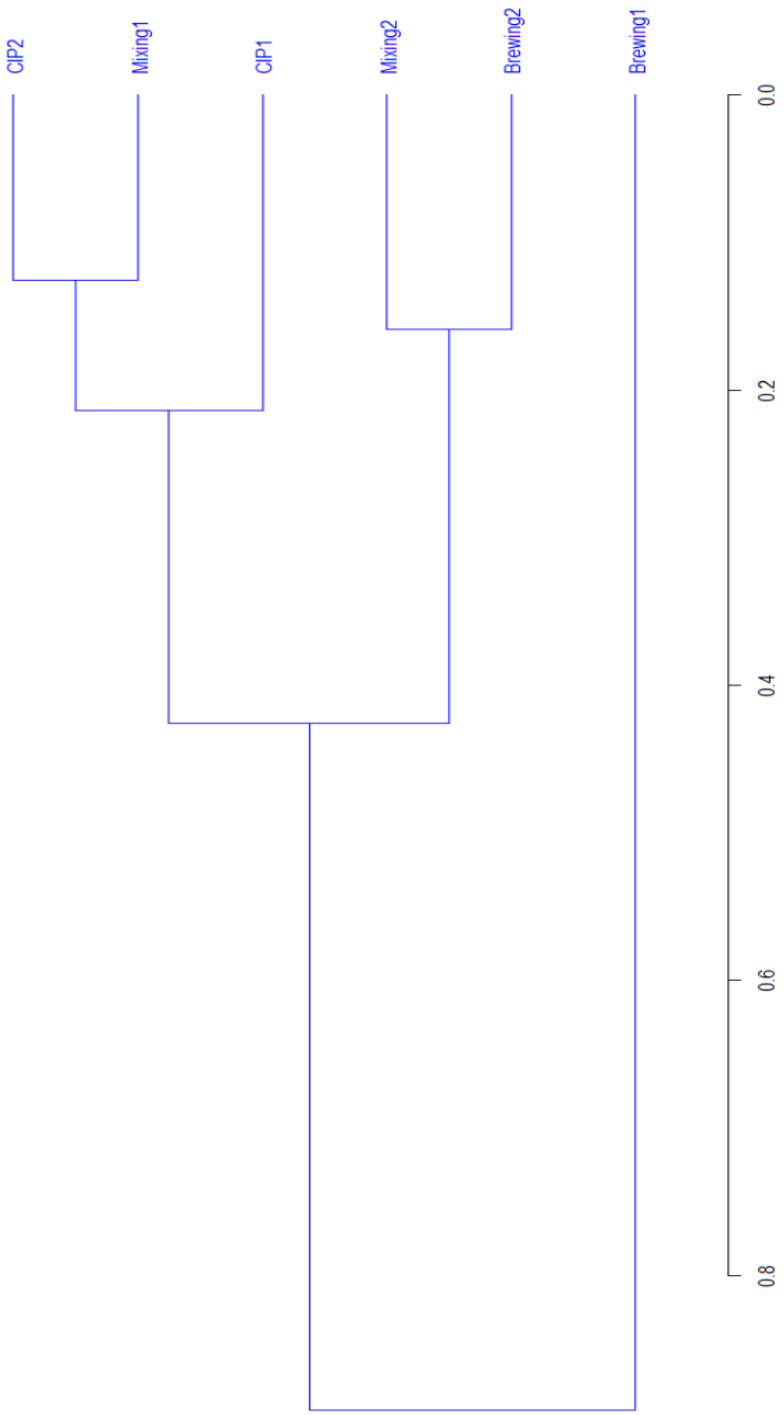

Figure 4-2: Physicochemical parameter clustering for Industries 1 and 2

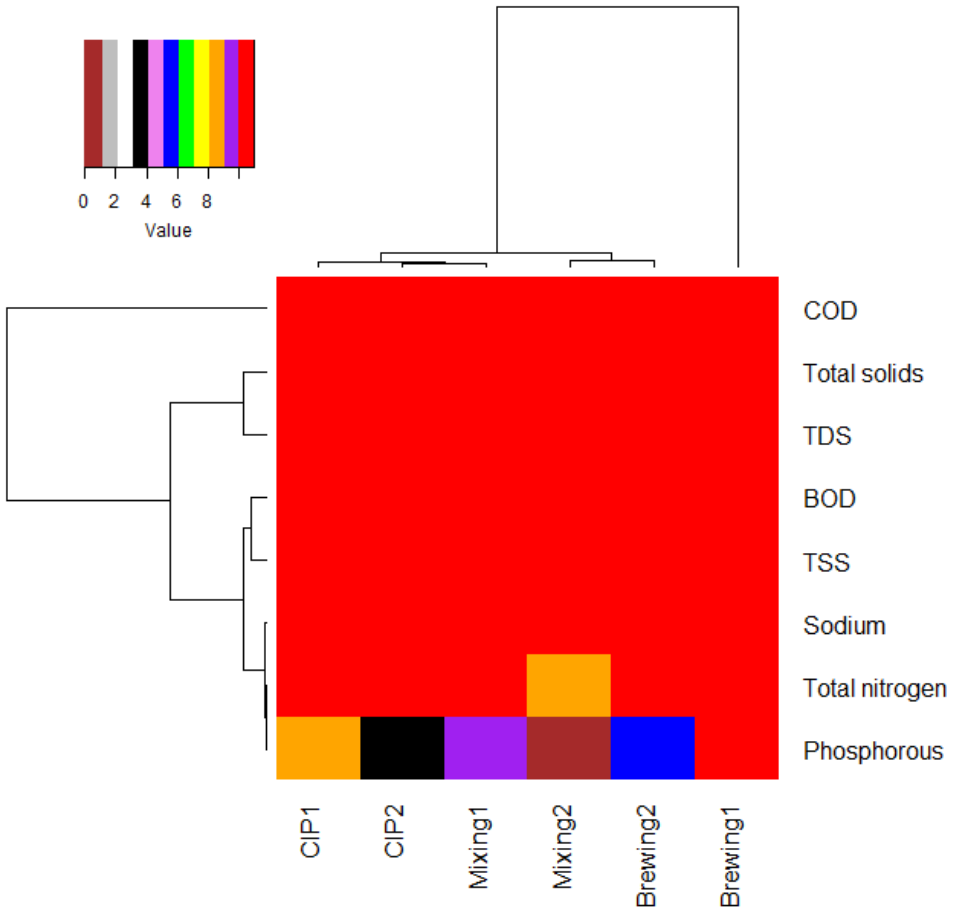

Figure 4-3: Heat map showing similarities between the sampling points and physicochemical parameters

The principal Component Analysis established that apart from Sodium and BOD, the rest of the parameters had a high correlation with factor 1, Table 4-3. Sodium and BOD showed a high correlation with factor 2. The amount of variability explained by the two principal components was 94%, thus the waste water from these industries could best be described using Total nitrogen content, total solids, Total phosphorus content, TDS, COD, BOD and TSS. These parameters explain the water quality by 76.60%. Sodium and BOD content explained water quality by 16.00%.

Table 4-3: Variability in wastewater quality

Physicochemical parameter	Component	
	Factor 1	Factor 2
Total Nitrogen	.996	
Total Solids	.997	
Phosphorous	.989	
TDS	.991	
COD	.996	
TSS	.977	
Sodium		.877
BOD	.502	.739
Eigen values	6.206	1.339
Proportional total	77.60%	16.00%
Extraction Method: Principal Component Analysis. Rotation Method: Varimax with Kaiser Normalization.		

4.2.3 Biodegradability of untreated brewery waste water

The study established the. BOD to COD ratio of the industries to be 0.039, 0.177, 0.911 for Brewing line, CIP line and Mixing line respectively for industry 1 and 0.567, 0.766, 0.766 for brewing line, CIP line and Mixing line respectively for industry 2. In all the two industries the B.O.D to C.O.D ratio for the brewing line was found to be the least with the mixing point having a range of 0.766 to 0.911. The low BOD to COD values obtained for the brewing line could be

attributed to high levels of C.O.D in this line contributed by heavy organic components, (Simate *et al*., 2011).These are close to literature values obtained by Zheng *et al*., (2015) in which the biodegradability of the influent waste water was found to be about 0.45. This waste water was used as inoculum to cultivate bacterial species with potential for biogas production.

A bivariate correlational analysis was performed to evaluate the level of association between the physicochemical parameters of the two industries. Although there was a 44.4% correlation between COD and BOD as depicted in Table 4-4, it could not be considered credible as it was not significant. Sodium level did not report a significant correlation with any of the water quality variables evaluated in this study, depicting that an increase or decrease in sodium concentration in the water would not correspond to a significant change in other variables and vice-versa. The highest correlation was between total solids and COD (99.9%), depicting an almost perfect positive relationship between the two parameters. Similarly, TDS and total solids had a strong association (99.7%) and this relationship was significant at (p<0.001). The other variables also had a highly significant association with other as shown in the table 4-4. BOD and TSS had a 58.6% association with each other and the association was significant at p=0.011.

A correlation analysis was also performed to evaluate the level of association between the BOD to COD ratios and the other physicochemical parameters. It was found that an increase in the Nitrogen, Sodium and total Solids levels in the waste water could result to a positive effect on the BOD to COD ratio while an increase in the phosphorous levels would have a negative effect, as per the general model described by equation [4-1].

$$Y = 0.533 + 0.033N - 0.025P + 0.00081Na + 0.00024TS \qquad [4\text{-}1]$$

- Where Y= BOD to COD ratio, N, P, Na and TS represents Nitrogen, Phosphorous, Sodium and total Solids respectively.

Since the effect of phosphorous to the ratio was not significant at $p<0.05$, the model was reduced to equation [4-2]

$$Y = 0.533 + 0.033N + 0.00081Na + 0.00024TS \quad [4\text{-}2]$$

Table 4-4: Pearsons correlation between the physicochemical parameters

		COD	BOD	NITROGEN	PHOSPHORUS	SODIUM	TOTALSOLID	TDS	TSS
COD	Cor	1							
	Sig. (2-tailed)								
BOD	Cor	.444	1						
	Sig. (2-tailed)	.065							
NITROGEN	Cor	.998**	.433	1					
	Sig. (2-tailed)	.000	.073						
PHOSPHORUS	Cor	.997**	.383	.998**	1				
	Sig. (2-tailed)	.000	.117	.000					
SODIUM	Cor	-.238	.339	-.287	-.290	1			
	Sig. (2-tailed)	.341	.169	.248	.242				
TOTALSOLID	Cor	.999**	.460	.997**	.994**	-.231	1		
	Sig. (2-tailed)	.000	.055	.000	.000	.357			
TDS	Cor	.998**	.417	.996**	.997**	-.236	.997**	1	
	Sig. (2-tailed)	.000	.085	.000	.000	.345	.000		
TSS	Cor	.960**	.586*	.957**	.942**	-.202	.967**	.945**	1
	Sig. (2-tailed)	.000	.011	.000	.000	.421	.000	.000	

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

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

Cor - Pearson correlation

Table 4-5: Pearsons correlation between the physicochemical parameters

	NITROGEN	PHOSPHOROUS	SODIUM	TOTAL SOLIDS	TDS	TSS	BOD/COD
NITROGEN	1						
PHOSPHOROUS	0.99755	1					
SODIUM	-0.28716	-0.290347652	1				
TOTAL SOLIDS	0.997099	0.994301722	-0.2309	1			
TDS	0.996403	0.997116136	-0.23644	0.997136852	1		
TSS	0.956625	0.942072428	-0.20221	0.966731443	0.94462 1	1	
BOD/COD	-0.67728	-0.695369964	0.250596	-0.693196169	-0.68544	-0.68967	1

4.2.4 Model validation

The model equation 4 was used to predict the BOD to COD values as tabulated in Table 4-6. The sum of squared difference was obtained as 0.530 and a mean squared error of 0.029.The model also explained 73% variations in the BOD_5 to COD ratio (r^2=0.7339).

Table 4-6: Fitted and experimental values for Biodegradability index

Experimental value	Fitted value
0.03829787	-0.005915492
0.03469388	-0.089775058
0.03645833	0.180407143
0.17727273	0.406972006
0.17727273	0.402207690
0.19500000	0.406839400
0.85123632	0.890627948
0.89583333	0.915195173
0.90870124	0.925443116
0.55091820	0.480278422
0.55177112	0.642845197
0.56988703	0.793166274
0.88636364	0.605503210
0.81250000	0.593154731
0.97500000	0.597434555
0.72614108	0.6597434555
0.72916667	0.764026128
0.72299112	0.672167216

4.3 Isolation and characterisation of methanogenic anaerobic bacteria from brewery wastewater

The incubation was carried out at 37^{o} C and observations were made as from day two of growth (Plate 4-1a-d). Isolation was done to explore the methanogenic community in the brewery wastewater. The isolates were characterized in order to understand their identity as a step towards optimization of biogas production. A total of thirty-one pure isolates were obtained from Brewery waste water.

4.3.1 Morphological characterization of bacterial isolates

4.3.1.1 Colony and Cell morphology

Morphological characterization was based on classical macroscopic attributes of colour, form, shape, and elevation of pure colonies. Most colonies were able to grow within 2-3 days of incubation at 37°C. The colony morphology of the isolates obtained from Brewery wastewater ranged from oval, entire, flat and filamentous. They were smooth or entire and the colour ranged from white to cream to bluish (Table 4-7). 65% of the isolates were Gram positive while 35% were Gram negative (Plate 4-2). All isolates were rod shaped.

Plate 1 a

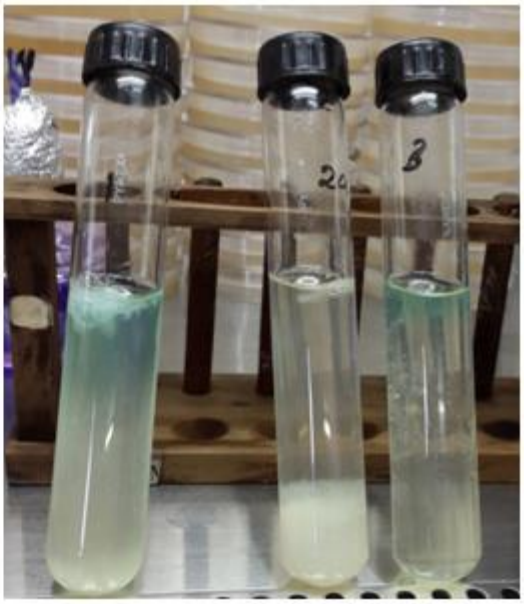

Plate 1 b

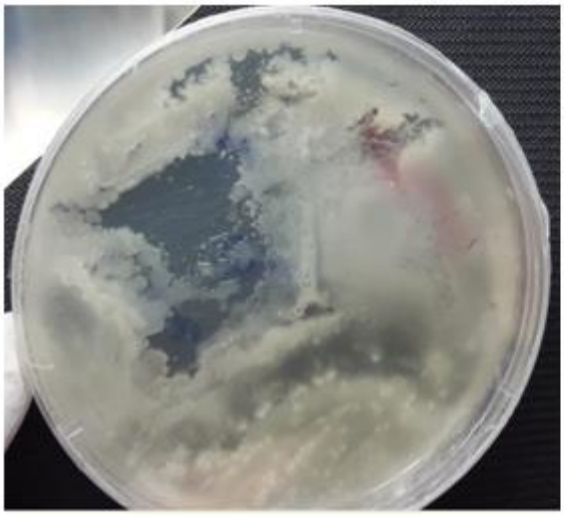

Plate 1 c

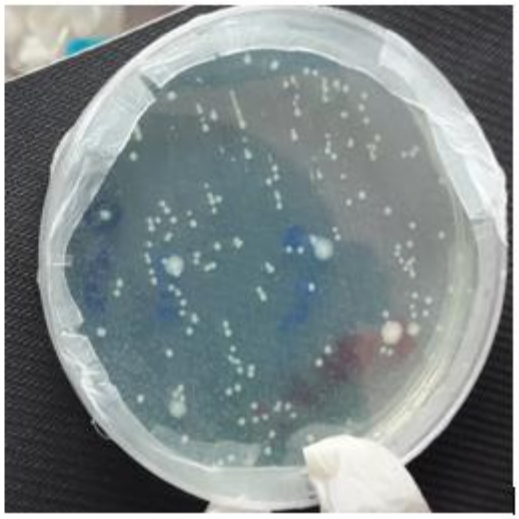

Plate 1 d

Plate 4-1: **(1a) Thyglycollate broth medium in anaerobic jar, turbidity and gas bubbles showing growth. (1b) Thyglycollate broth medium with different growth characteristics before sub-culturing on agar plates. (1c) Thyglycollate agar medium with different colonies**

Table 4-7: Morphological characteristics of bacterial 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

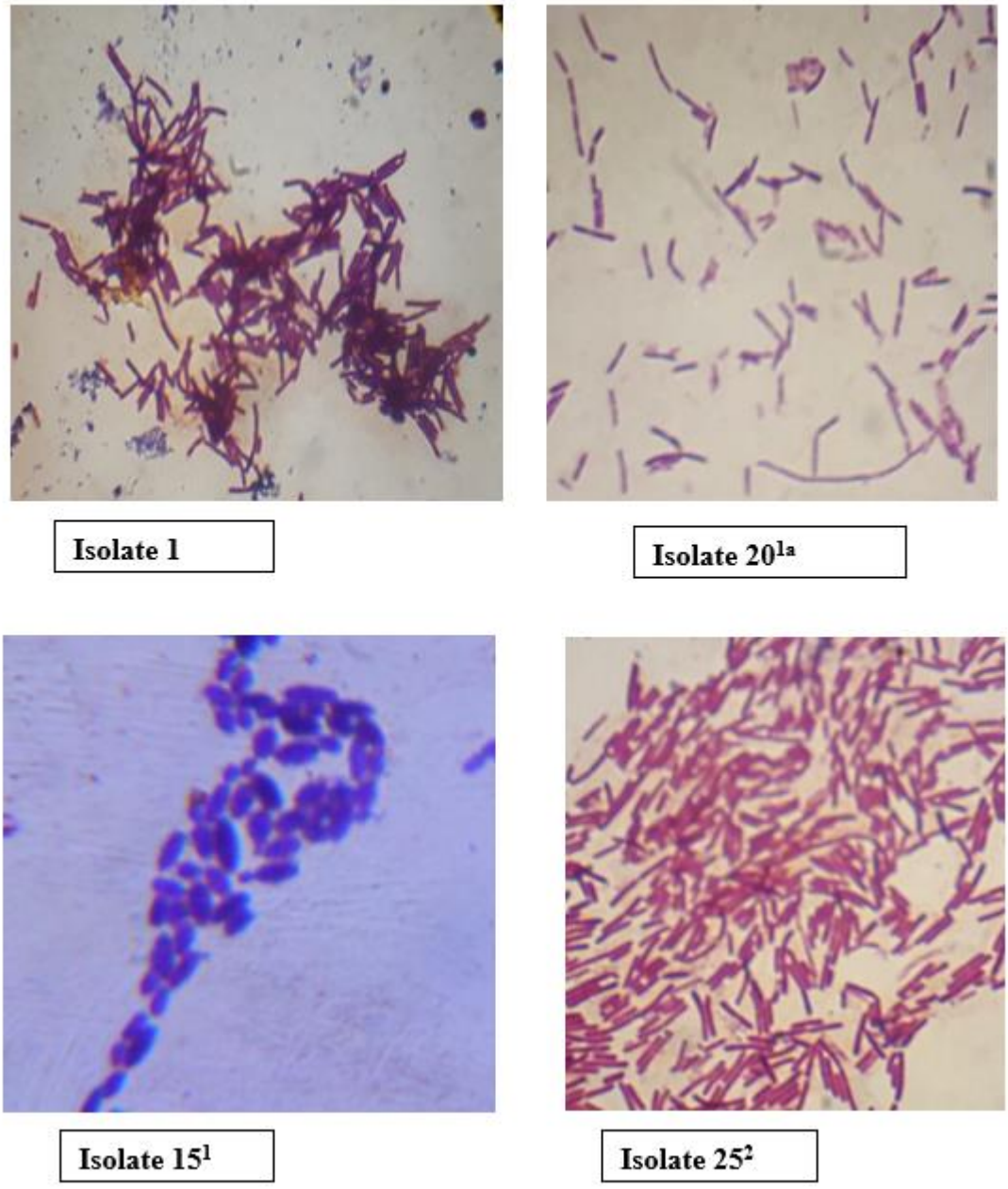

Plate 4-2: Gram reaction of the selected bacterial isolates.

Legend: Gram positive rods (1), Gram positive rods (20 [1a]), Gram positive short rods (15[1]), Gram negative rods (25[2])

4.3.2 Biochemical tests of the isolates

Results for the various biochemical assays namely, catalase test, fluorescent test, indole test, starch hydrolysis, motility test, triple iron sugar utilization test, gelatine hydrolysis and hydrogen sulphide gas production are shown on Table 4-8. Motility test done on SIM medium showed that some of the isolates were motile with the presence of flagella. Isolates 5, 17^2, 18^1, $19^1$and 25^2 were negative for motility test.

The ability of the isolates to excrete intracellular enzymes was determined through tests on catalase reaction. Catalase test revealed that isolates 1, 5, 6, 8, 9^2, 11, 14, 15^1, 16, 17^2, 20^{1a}, 25^2 and 27^1 were positive with the ability for production of hydrogen peroxide as an end product of oxidation of sugars.

All isolates were positive for Triple Iron Sugar utilization test indicating the ability of the isolates to ferment glucose and produce a lot of mixed acids as end products of fermentation (plate 4-3). The medium used, (triple sugar iron agar) is formulated to differentiate bacteria based on the glucose, lactose and sucrose fermentation (Dhadse *et al*., 2012; Issazadeh *et al*., 2013). The medium is prepared as a shallow agar slant with deep butt, this provides both the aerobic and anaerobic growth environment. Therefore, the bacteria that are able to ferment glucose and lactose and/or sucrose were able to turn the medium yellow throughout. Since lactose and sucrose concentrations are more than that of glucose, both the butt and slant remained yellow after 24 hours. An organism that does not ferment any of the carbohydrates but utilizes animal proteins will alkalinize the medium and turn it red. If the organism can use the proteins aerobically and anaerobically, both the slant and butt will appear red. An obligate aerobe will turn only the slant red (Amon *et al*., 2007; Chen *et al*., 2008). The ability to produce hydrogen

sulphide gas is the main function of anaerobic respiration during biogas production (Norrell and Messley, 1996).

Indole generation by reductive deamination from tryptophan via the intermediate molecule indole pyruvic acid was tested for the isolates. Tryptophanase enzyme catalyzes the deamination reaction, during which the amine ($-NH_2$) group of the tryptophan molecule was removed and final products of the reaction are indole, pyruvic acid, ammonia (NH_3), hydrogen sulphide and energy. The test was performed to show the ability of bacteria to split the amino acid tryptophan to indole, pyruvic acid and ammonia with the help of tryptophanase enzyme. Positive for indole test represented the bacterial isolates which were able to act on amino acids by causing deamination and hydrolysis leading to the formation of pyruvic acid and ammonia. Pyruvic acid and ammonia are precursors for methane and CO2 production, the main function of methanogenic bacteria. The negative results showed that isolates were unable to produce indole as a result of amino acid tryptophan breakdown attributed to lack of tryptophanase in the cell (Rezwan *et al*., 2004). The presence of indole can be detected by the addition of Kovacs' reagent which reacts with the indole, producing a bright red compound on the surface of the medium. All isolates were negative for this test but positive for hydrogen sulphide gas prodcution. The test is important in differentiating members of family Enterobacteriaceae and genus bacillus (Prescott, 2002).

The starch molecule consists of two constituents: amylose, a straight chain polymer of 200 to 300 glucose units, and amylopectin, a larger branched polymer with phosphate groups. Thus, bacteria that hydrolyze starch produce amylase enzyme that yield molecules of maltose, glucose and dextrin. Most isolates were positive for the test except isolate 5, 10, 14, 15^1, 17^1, 26^1 and 26^2 were negative for the test (Table 4-8). 1, 4, 6, 13^3, 14, 15^1, 25^2, 26^1, and 31 were negative for

extracellular gelatinase enzyme. Starch hydrolysis test is used to differentiate bacteria based on their ability to hydrolyze starch with the enzyme a-amylase or oligo-l, 6-glucosidase, starch is a polysaccharide consisting of a- D-glucose subunits that exists in two forms amylose (straight chain polymer) and amylopectin (a larger branched polymer with phosphate groups). Since starch is too large to pass through bacterial membrane, these enzymes are needed to hydrolyze it into smaller fragments of glucose molecules making it available for bacteria uptake (Harold, 2002). Therefore, when the bacteria that produce these enzymes are cultivated on starch agar, they hydrolyze the starch around the area of growth. But since both starch and its sugar subunits are invisible in the medium, iodine reagent is used to detect the presence or absence of starch in the around the bacterial growth. Iodine reacts with starch and produces a blue or dark brown color; therefore, any microbial starch hydrolysis was revealed as a clear zone surrounding the growth (Cappuccino and Sherman, 2001; Joanne *et al*., 2016).

Gelatin is a collagenous protein a component of animal connective tissues. Gelatin hydrolysis is a test that was used to detect the ability of the isolates to produce proteolytic enzyme (gelatinase) which causes the breakdown of this complex protein derivative to polypeptides. These polypeptides are further converted into single amino acids that bacteria can easily use for their metabolic process. Therefore, that hydrolyzed gelatin indicated the presence of gelatinase enzymes. This test is used to identify and differentiate different species of Bacillus, Clostridium, Pseudomonas and family Enterobacteriaceae (Prescott, 2002).

The characterization of the bacterial isolates by fluorescent technique, depends on the response of a target, detailing the fluorescence intensity as a function of both excitation and emission wavelength, that is provided by an Excitation-Emission Matrix (EEM). An EEM is generated by recording multiple emission spectra from the target as the excitation wavelength is incremented

across a broad range. All isolates showed positive fluorescence apart from 10 and 18^1 which were negative.

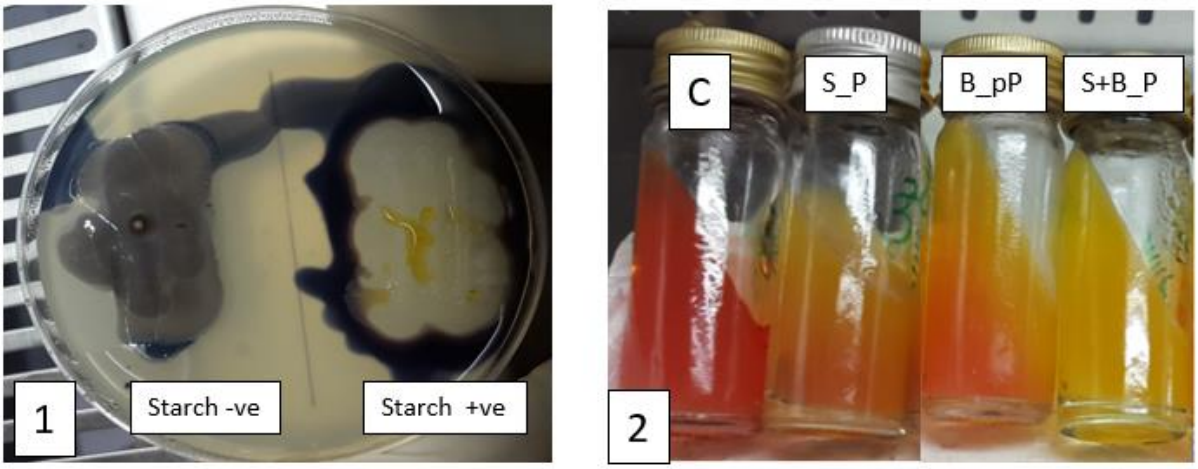

Plate 4-3: Starch Hydrolysis test (1), Triple Sugar iron test (2) as part of biochemical tests carried out.

Legend: C-control, -ve-negative, +ve- positive, S_P- slant positive, B_pP-butt partial positive, S+B_P- slant plus butt positive

Table 4-8: Biochemical characteristics of bacterial isolates

						TSI			
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	+	-	-	+	+	++	++	++	+

Key: (+) Positive/ less colour, (-) Negative, (++) partial colour (+++) intense colour and H_2S: Hydrogen Sulphide gas.

4.3.3 Molecular characterization

4.3.3.1 PCR amplification of 16s rDNA genes from isolates

Genomic DNA was extracted from all the selected 31 isolates using the phenol/chloroform method. Amplification of 16S rDNA gene with bacterial universal primers bac 8F and bac 1492R (Hogg and Lehane, 1999) yielded an amplification product of approximately 1500 bp as shown in Plate 4-4. The amplicons were then stained with ethidium bromide and visualised under UV light on 1 % agarose gel.

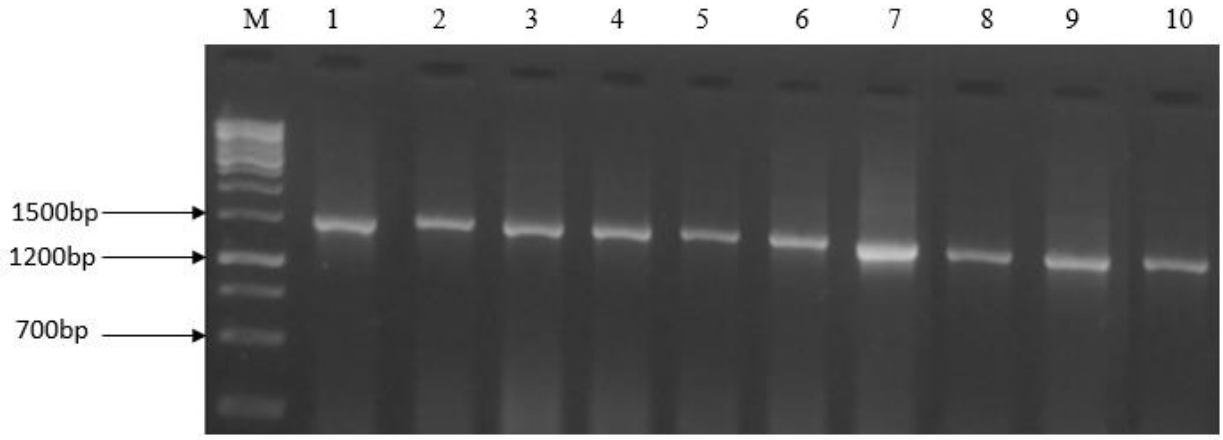

Plate 4-4: PCR amplified 16S rRNA products from representative isolates using universal primers bac 8F and bac 1492R

Legend: 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

4.3.3.2 Restriction analysis

The restriction enzyme analysis provided an initial clustering of strains into six groups with three or more members with the same restriction profile. Fragment restriction using *RsaI* produced six clusters of the isolates which were generated based on fragment sizes as illustrated in Plate 4-5.

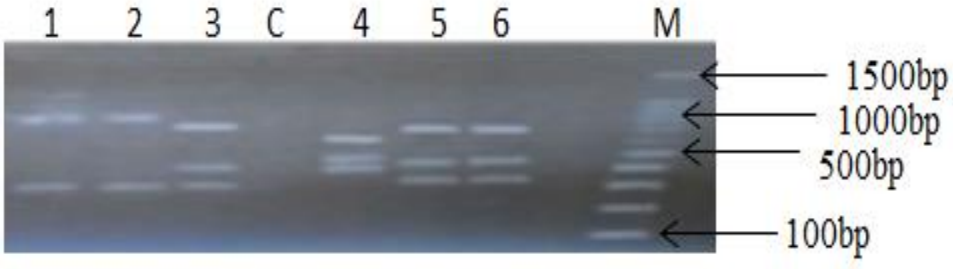

Plate 4-5 Restriction products as generated by Rsa I digestion run in 1.5% (W/V) agarose gel.

Legend: Lanes 1(18[1]*), 2(26[2] *), 3(15*), 4(20[1a] *), 5(17[1]*), 6(13[3]*), (C*) negative control and (M*) M-1500 bp Molecular marker size

*The figures within the brackets are the isolate numbers

The amplification of ribosomal DNA restriction analysis (ARDRA) was carried out using this enzyme. The restriction fragment Cluster profiles obtained from isolates showed patterns with distinct similarities allowing distinguishing six different groups. These were isolates 1, 2, 3, 5 and 6; which were clustered together generating fragments ranges 300-400bp; isolates 3, 4, 5, and 6 clustered to generate fragments ranges 400-500bp: isolate 4 generated fragment range 500-600bp; isolates 4, 5 and 6 were clustered together generating fragments range 600–700bp. Isolate 3 had one restricted fragment of 800-900bp. Finally isolates 1 and 2 were clustered together generating fragments range 900–1000bp. Sequences of PCR fragments from isolates were in close agreement with the phylogenetic correlations predicted with the ARDRA approach. ARDRA thus provided a quick assessment of the diversity in a strain collection.

4.3.3.3 Phylogenetic analysis of the sequences

Though a total of 20 isolates were sequenced, only 16 could be placed into the phylogenetic trees. The rest were not placed into the tree either because of they had too low an identity to allow for sensible alignment or they had sequence of less than 320 base pairs according to

Rezwan et al., 2004. The taxonomic classification of the isolates performed using morphological characteristics, biochemical tests and 16S ribosomal RNA sequences of their genomic DNA placed the isolates to the genera Bacillus, Lysinibacillus, Lactobacillus, Ralstonia, Myroides and Providencia. Bacilli are described as aerobic or facultative anaerobic; rod shaped, Gram positive, motile, flagellated bacteria that belongs to the division Firmicutes with varying ecological diversity. They are most commonly found in soil, waste water, dust, milk and plant surfaces. Different bacillus species have been described including bacillus subtilis, which supports plant growth by producing antibiotics that kill other harmful microorganism that may harm the plant (Lin *et al.*, 2011) bacillus cereus, Bacillus licheniformis and Bacillus tequilensis have been associated with food spoilage and poisoning.

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 of 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 32), Providencia (isolate 11) and Myroides (32) with similarities of 77, 96 and 98%, respectively.

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^{1a}, 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). 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 as illustrated in Figure 4-4. 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 (Figure3-1).

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); Kröber *et al*., (2009) and Li *et al*., (2013). This compares to the results obtained by Rabah *et al*., (2010) using abattoir waste as the inoculum. The results are also 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. Bacilli are described as aerobic or facultative 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, wastewater, milk, dust, and plant surfaces.

Phylogenetic analysis of isolate 93b suggested that it was closely related to Bacillus subtilis with 94% rDNA sequence analysis similarity. The morphological and biochemical tests supported this since the isolate was Gram positive rod, catalase, starch hydrolysis and sugar fermentation positive and did produce hydrogen sulphide gas. Bacillus subtilis is widely used in the industrial production of amino acids, recombinant proteins and fine chemicals (Straight *et al.*, 2006). It is also known for generation of bioenergy, production of industrial metabolites and bioremediation (Novak *et al*., 2013; Porwal *et al*., 2015). Isolates 201a, 171 and 7 closely related to Bacillus methylotrophicus with 97-98% rDNA sequence analysis similarity (Table 4-9).

Table 4-9: BLAST analysis results of the isolates 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
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

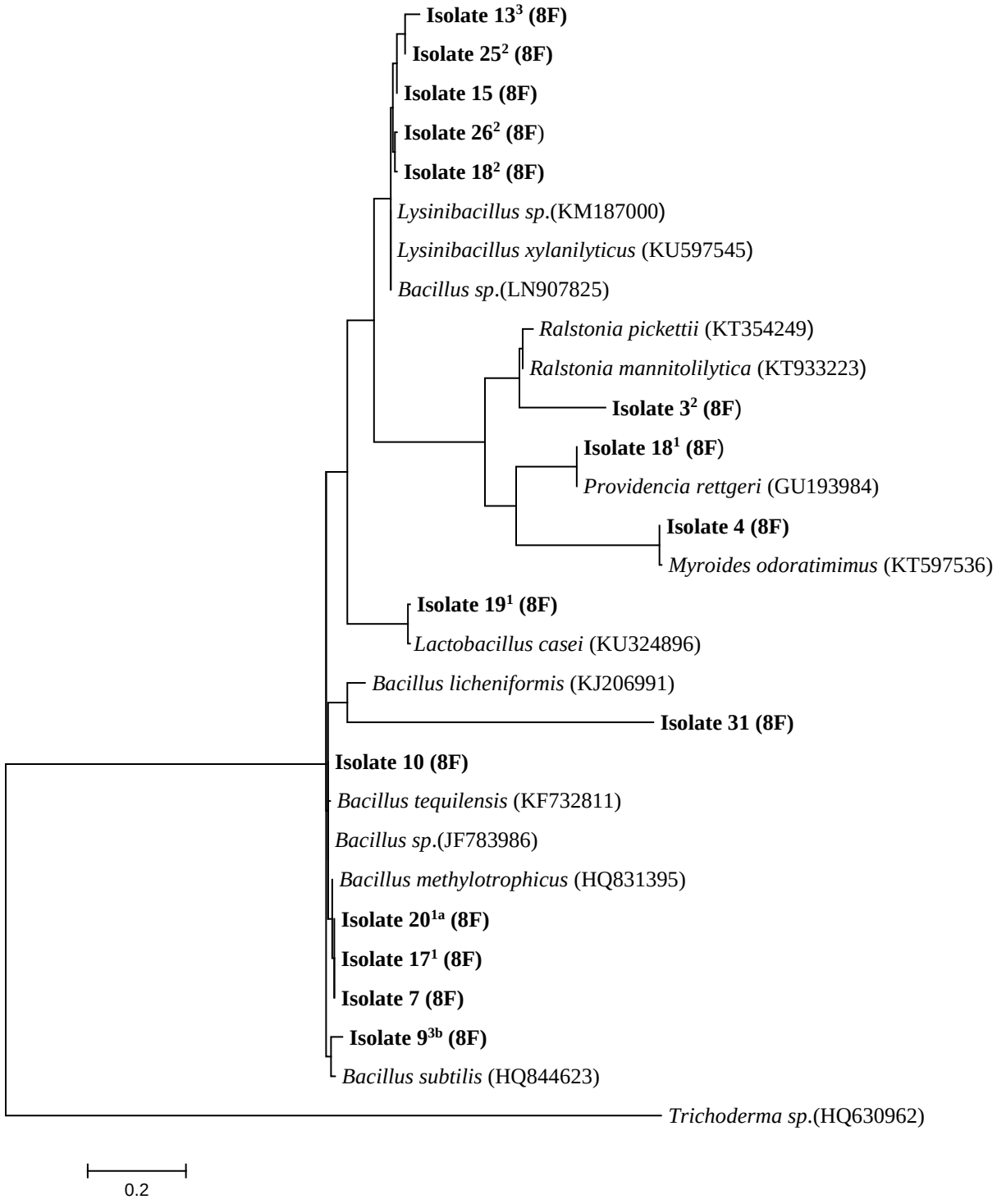

Figure 4-4: Phylogenetic analysis of 16S rDNA gene sequences using the Maximum Likelihood method based on the Tamura-Nei model (2007).

The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 29 nucleotide sequences. The gene sequence of Trichoderma sp. (HQ630962.1) was used as an out-group.

Phenotypic characteristics revealed that Bacillus methylotrophicus is Gram positive, rod shaped, endospore former, catalase positive and motile (Table 4-7). Various methylotrophs have also been described, including methanotrophs, some of which are obligate methane users, those that use methanol and a few that are able to grow on carbon containing compounds (Dedysh *et al.*, 2005).

Morphological and biochemical signatures of isolate 10 indicated that it was highly closely related to Bacillus tequilensis, with 98% rDNA sequence analysis similarity. It's a Gram positive motile rod, indole and catalase positive, liquefied gelatine, hydrolysed starch and fermented all three sugars through TSI test. It was first isolated from 2000-year-old tomb but generally it's found in the soil and most environmental samples, (Gatson *et al*., 2006; Rooney *et al*., 2009). Studies shows Bacillus tequilensis can produce an enzyme pectinase with many industrial applications such as pre-treatment of waste water, (Shah *et al*., 2013). Isolate 31 was closely related to Bacillus licheniformis with 16SrDNA sequence analysis of 70% and was Gram positive, motile rod, catalase positive, aerobic or facultative anaerobic. Bacillus licheniformis, has been associated with spoilage of food, under different clinical conditions (Ghani *et al*., 2013). The rapid development of industrial enzymology and fermentation technology have allowed the exploitation of this species for production of different enzymes.

Lysinibacilli are diverse group with different species, they are naturally found in various environments including farming soil and factory wastewater. Many Lysinibacillus species have been described including; Lysinibacillus boronitolerans, and also reclassified Bacillus sphaericus and Bacillus fusiformis to the genus Lysinibacillus. Several other species have also been added to this genus, Lysinibacillus parviboronicapiens, (Miwa *et al*., 2009). Sequence analysis by

BLAST search system on the NCBI website and phylogenetic analysis (Figure 1) showed that the isolates 13^2, 25^2, 1^5, 26^2 and 18^2 was phylogenetically most closely related to Lysinibacillus sp (KM187000) with 95-99% rDNA sequence similarity (Table4- 9). These isolates adhere to the phenotypic and biochemical characteristics of Lysinibacillus sp which is Gram positive, rod shaped motile or non-motile bacteria and were positive for starch hydrolysis, sugar fermentation and were negative for indole production (Table 4-8).

Isolate 19^1 displayed the typical characteristics and biochemical properties similar to Lactobacillus casei. It was Gram positive, non-motile, rod shaped catalase negative, and facultative anaerobic. It is believed to be a normal inhabitant of the oral cavity and the digestive tract in humans. It has been found to be also involved in the reduction of Chemical Oxygen Demand and colour from dark effluent, (Shibu *et al*., 1999). Sequence analysis by BLAST search systems on NCBI website showed that the isolate 191 was phylogenetically most closely related to Lactobacillus casei with 95 % rDNA sequence similarity (Table 4-9). Ralstonia pickettii belongs to family Burkholderiaceae and was first isolated from soil and water it can degrade various chlorophenol compounds, aromatic hydrocarbons 2,4-dichlorophenoxyacetic acid and pentacyclictriterpeniod compounds, (Seal *et al*., 1993).

The morphological and biochemical signatures for isolate 3^2 indicated that they are closely related to Ralstonia pickettii. It was Gram negative and motile rod, starch hydrolysis, indole, gelatin liquefaction and hydrogen sulphide production negative and catalase positive and fermented sugars through TSI test. The 16S rDNA sequence analysis suggested the isolate 3^2 is phylogenetically closely related to Ralstonia pickettii with 77% sequence similarity (Table 4-9).

Members of genus Providencia are Gram negative motile rods belonging to family Enterobacteriaceae, they are positive for catalase and acid production from glucose fermentation, without production of H_2S gas. They are mostly isolated from water and clinical (urine, faeces, blood, wounds and throat) human and animal samples, (Holt *et al*., 1994). Morphological and biochemical characteristics showed that isolate 18^1 was closely related to Providencia rettgeri being a Gram- negative, motile rods, catalase, starch hydrolysis positive and was negative for indole and hydrogen sulphide gas production, (Table 4-8). Sequence analysis by BLAST search systems on NCBI website showed that the isolate 18^1 was phylogenetically most closely related to Providencia rettgeri with 96% rDNA sequence similarity (Table 4-9).

Myroides species, which are formerly classified as Flavobacteriun odoratum, are Gram-negative, non-fermentative, obligately aerobic, yellow-pigmented, non-motile rods and are commonly found in the soil and water. This genus of Myroides comprises of, Myroides odoratus and Myroides odoratimimus, (Vancanneyt *et al*., 1996). Isolate 4 displayed the typical characteristics and biochemical properties similar to Myroides odoratimimus. It was Gram negative, non-motile and rod shaped, catalase, TSI, starch hydrolysis and hydrogen sulphide gas positive and was negative for indole, gelatin liquefaction. It was closely related to Myroides odoratimimus with 98% rDNA sequence analysis similarity (Table 4-9).

4.4 Optimization of biogas from isolated strains

4.4.1 Effect of temperature and pH on the quality of methane production

The pH ranges of brewery wastewater is reported as 6.5 to 8.2 (Caliskan *et al*., 2014; Janhoappliedm *et al*., 2009; 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 (Ward *et al*., 2008). 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 (Figure 4-5). However, 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 and Barlaz, 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.

Table 4-10 shows the means of the quality of methane gas produced by different isolates. Generally, 88.9% of the isolates had the highest percentage quality of methane gas at the temperature range of 35 ºC - 37 ºC for all the pH ranges. However, the quality produced by isolate 15^{1} at temperature 30 ºC and pH 8 was significantly different from that produced at higher temperatures and low pH. This isolate was observed to have the highest concentration at this pH and temperature and was significantly different ($p<0.001$) from that produced by the other isolates as illustrated in Figures 4-5, 4-6, 4-7, 4-8 and 4-9. The results didn't show any significance difference in the volume of the gas generated for different temperatures, although slight variations were observed with temperatures 30 ºC and 40 ºC recording the highest volume of gas but low in quality. Similarly, temperatures 35 ºC and 37 ºC had the lowest volumes of gas produced with better quality with high methane concentrations as indicated in Figure 4.6.

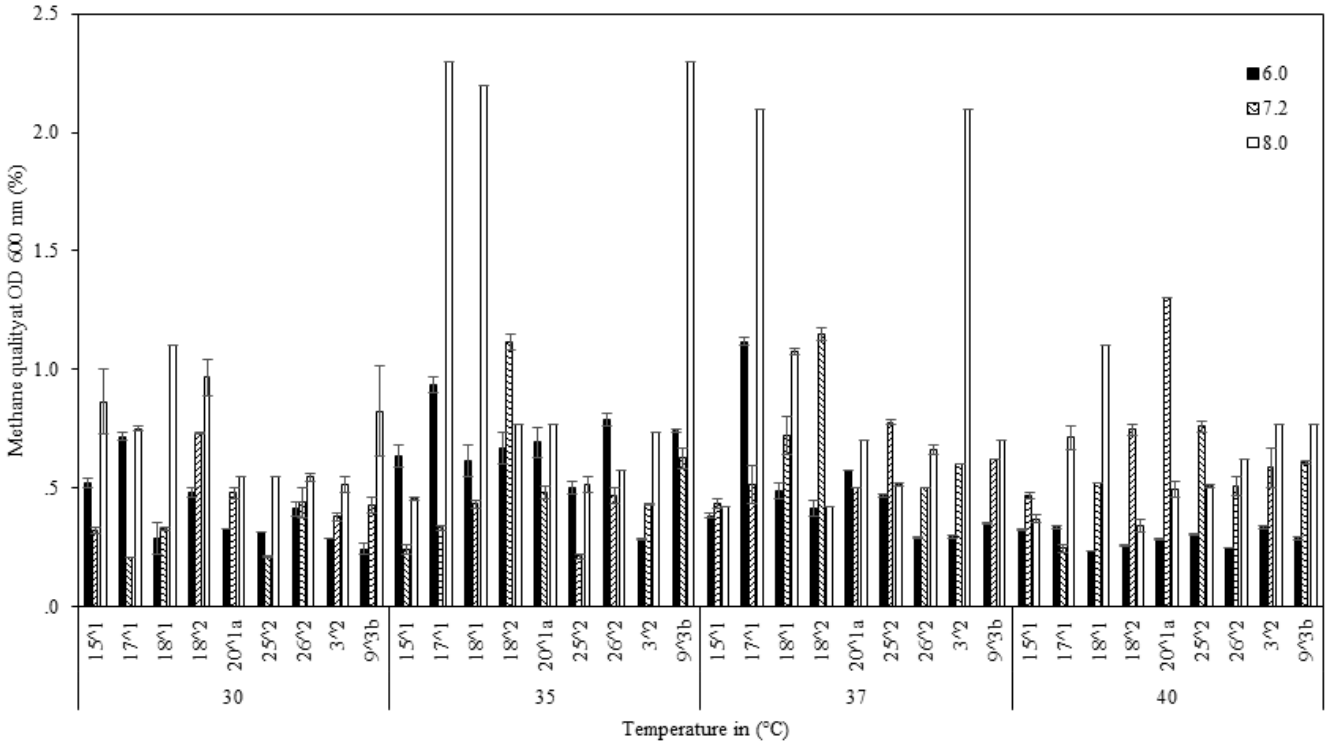

Figure 4-5: Effect of temperature and pH on the quality of the methane produced at OD 600 nm

Table 4-10: Means for quality of methane gas produced by different isolates

pH	Temperature (°C)	15^1	17^1	18^1	18^2	20^1ª	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

Note: LSD means least significant difference; CV means coefficient of variance

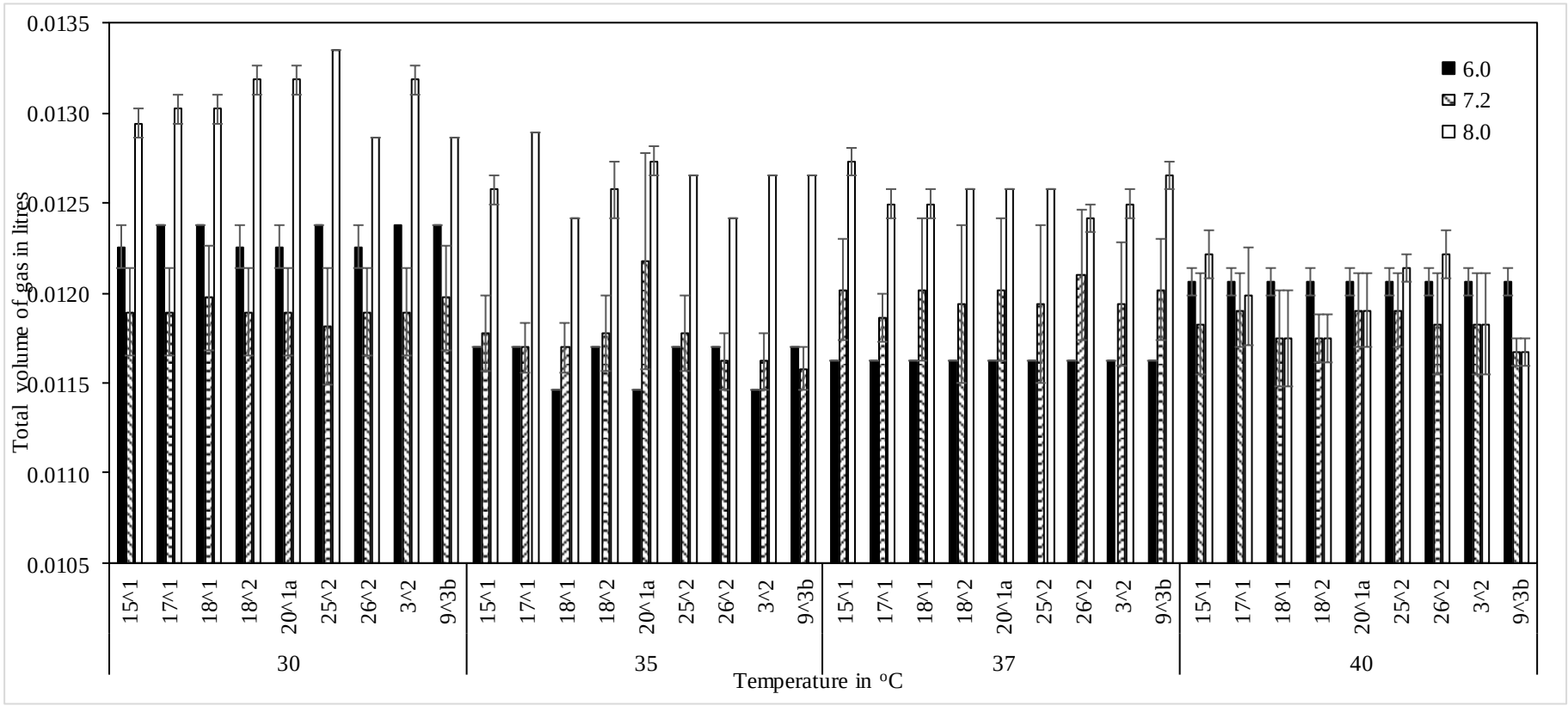

Figure 4-6: Effect of temperature and pH on the quantity of gas produced

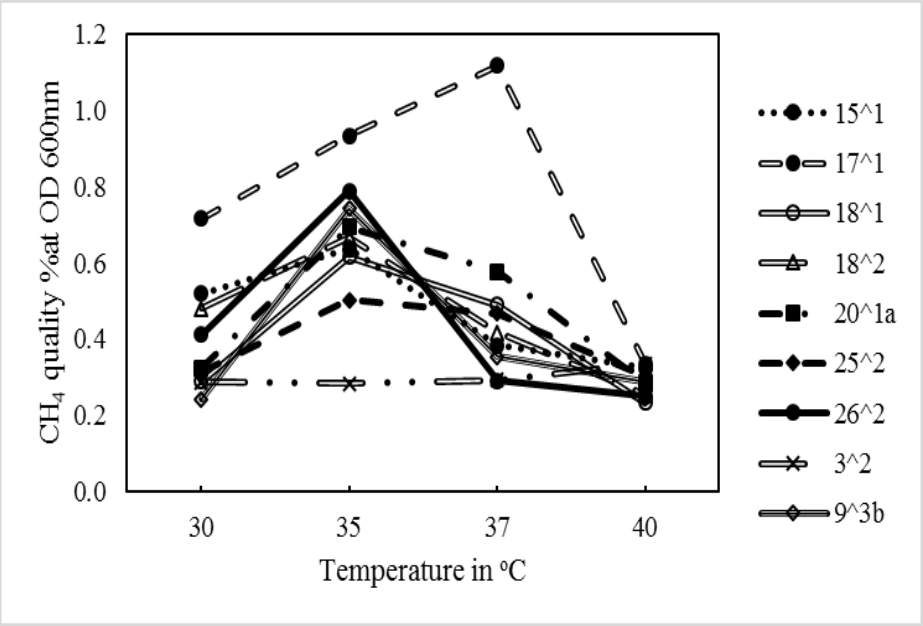

Figure 4-7: Methane quality at pH 6

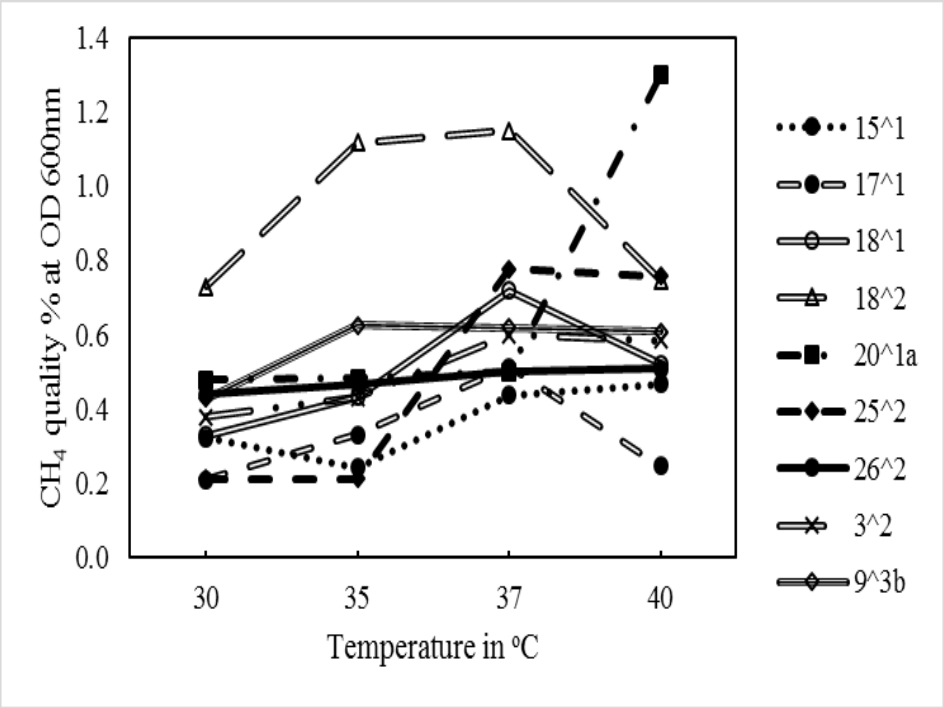

Figure 4-8: Methane quality at pH 7.2

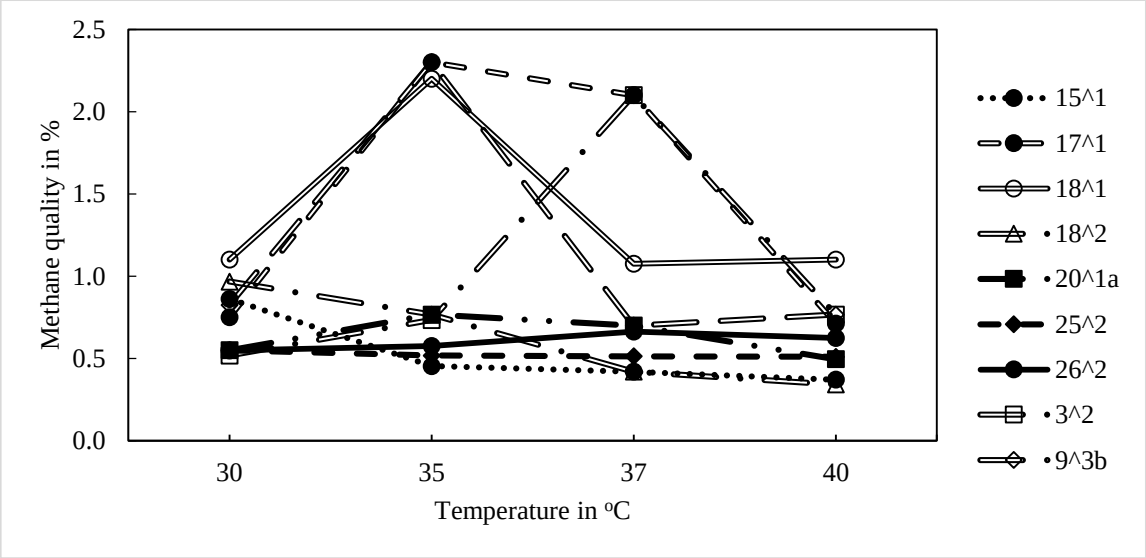

Figure 4-9: Methane quality at pH 8

4.4.2 Growth curves of the isolates at 37ºC

Figure 4-10 shows the sigmoidal shaped curves obtained for the isolates; 3^2, 9^{3b}, 15^1, 18^1,18^2, 20^{1a}, 17^1, 25^2 and 26^2. The curves clearly showed different stages of growth, including the lag phase, exponential phase and the stationary phase. Variation in the lag time for the isolates was observed for most of the isolates. The growth was not observed during the first 4 hours of incubation, but increased with increase in incubation period until 6^{th} hour for isolate 9^{3b}, and 18^2, while isolates 201a, 17^1, 25^2, 18^1,26^2, 15^1, increased till the 15^{th} hour, with isolate 3^2 reaching the 18^{th} hour, after which the growth was either stagnant or decreased as shown in Figure 4-10.

The growth curve of a microbial culture for the nine isolates was used to analyse their population growth. From the sigmoidal curve obtained Figure 4-10, the growth of most of the isolates could not be detected during the first four hours. This could be attributed to the lag phase in which the microorganisms were adapting to the new conditions having been inoculated in a fresh medium

and possibly the microorganisms have been injured and required time to recover (Klocke *et al*., 2008; Kotsyurbenko, 2005). After the 4th hour the growth of these isolates could be compared to the exponential phase in which case the growth increased with increase in incubation period. At this stage the isolates are growing and dividing at the maximal rate possible as they have adjusted to the new environment and that the microorganisms are dividing and doubling in number at regular intervals. From Figure 4-10, the curves for most of the isolates rose smoothly than discrete jumps indicating that the individual isolate divides at a slightly different time. The population of the microorganism at this stage is uniform with regards to the chemical and physical properties (Koch, 2001; Panikov, 1995) thus the maximum production of the methane gas could be linked to this stage. To maximize methane production, the exponential phase could be pro-longed by using continuous culture system, in which constant environmental conditions are maintained through continual provision of nutrients and removal of wastes. In this system, cells are supplied with nutrients and grow at a constant rate.

The decrease in the population growth observed could be attributed to nutrient limitation and accumulation of toxic waste products, which seems to limit the growth of many anaerobic cultures, (Cooper, 1991) although other factors could inhibit their growth.

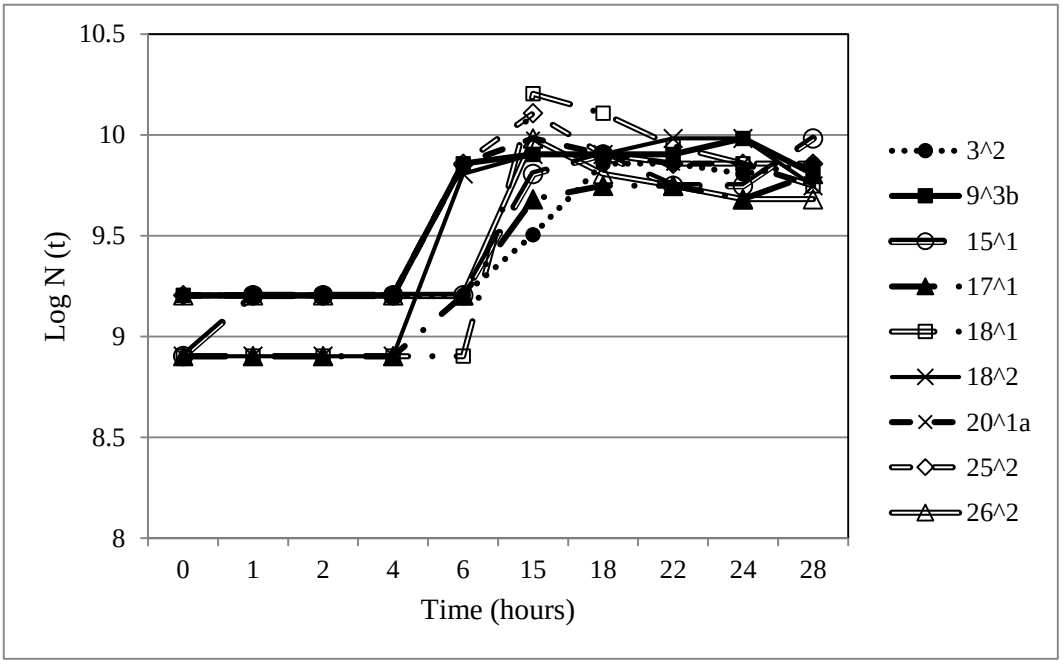

Figure 4-7: Monitored growth of the isolates at temperature 37^{0}C

4.4.3 Comparing growth Models for predicting microbial growth.

Table 4-12 shows the growth parameters as estimated by the Gompertz and logistic models for the growth curves of isolates 3^{2}, 9^{3b}, 25^{2},17^{1},18^{2}, and 20^{1a}, plotted with the log of N values as shown in Figure 4-11. Both models provided the values that could be expected for growth parameters of the selected six microorganism.

Table 4-11: Estimated growth parameters and their 95%confidence limits of fit obtained with growth curves for isolates3^{2}, 9^{3b}, 25^{2}, 17^{1}, 18^{2} and 20^{1a}

Isolate	Growth Model	Initial Concentration (No)	Asymptote A(cells)	Growth rate(μmax)cells h-1	Lag time (λ)(h)	Akaike information criterion (AIC)	r2	SSE
3^{2}	Gompertz	9.204 (9.191 - 9.211)	9.845 (9.832 - 9.854)	1.402 (0.222 - 1)	1.478 x 101 (13.717 - 14.715)	-46.783	0.998	0.002
	Logistic	9.204 (9.191 - 9.218)	9.845 (9.829 - 9.854)	0.9519 (0.683 - 1.532)	1.468 x 101 (14.545 - 14.809)	-46.982	0.985	0.014
9^{3b}	Gompertz	9.204 (9.141 - 9.232)	9.9 (9.863 - 9.933)	0.743 (0.355 - 2.680)	4.702 (3.763 - 4.725)	-26.253	0.986	0.016
	Logistic	9.204 (9.158 - 9.227)	9.9 (9.863 - 9.933)	1.548 (0.397 - 1.890)	5.468 (3.982 - 5.588)	-25.252	0.979	0.026
25^{2}	Gompertz	9.204 (9.144 - 9.252)	9.916 (9.880 - 9.980)	7.05 x 10-1 (0.257 - 2.217)	4.719 (3.171 - 4.809)	-15.212	0.962	0.047
	Logistic	9.204 (9.145 - 9.258)	9.916 (9.876 - 9.979)	1.64 (0.245 - 2.014)	5.523 (3.183 - 5.620)	-15.212	0.944	0.087
17^{1}	Gompertz	8.901 (8.865 - 8.930)	9.733 (9.704 - 9.759)	0.238 (0.117 - 0.637)	4.733 (3.648 - 5.599)	-29.625	0.993	0.011

	Logistic	8.903 (8.865 - 8.935)	9.733 (9.705 - 9.766)	0.971 (0.167 - 1.939)	5.693 (4.252 - 5.873)	-29.588	0.981	0.031
18^{2}	Gompertz	8.903 (8.799 - 8.955)	9.904 (9.842 - 9.951)	0.977 (0.534 - 2.549)	4.765 (3.831 - 4.884)	-17.743	0.985	0.037
	Logistic	8.903 (8.850 - 8.942)	9.904 (9.841 - 9.951)	2.322 (0.443 - 2.737)	5.545 (3.976 - 5.647)	-17.742	0.983	0.038
20^{1a}	Gompertz	9.204 (9.123 - 9.250)	9.87 (9.836 - 9.916)	0.797 (0.357 - 2.592)	4.469 (3.760 - 4.782)	-20.101	0.973	0.029
	Logistic	9.204 (9.133 - 9.241)	9.87 (9.842 - 9.924)	1.567 (0.364 - 2.036)	5.366 (5.733 - 5.633)	-20.001	0.937	0.069

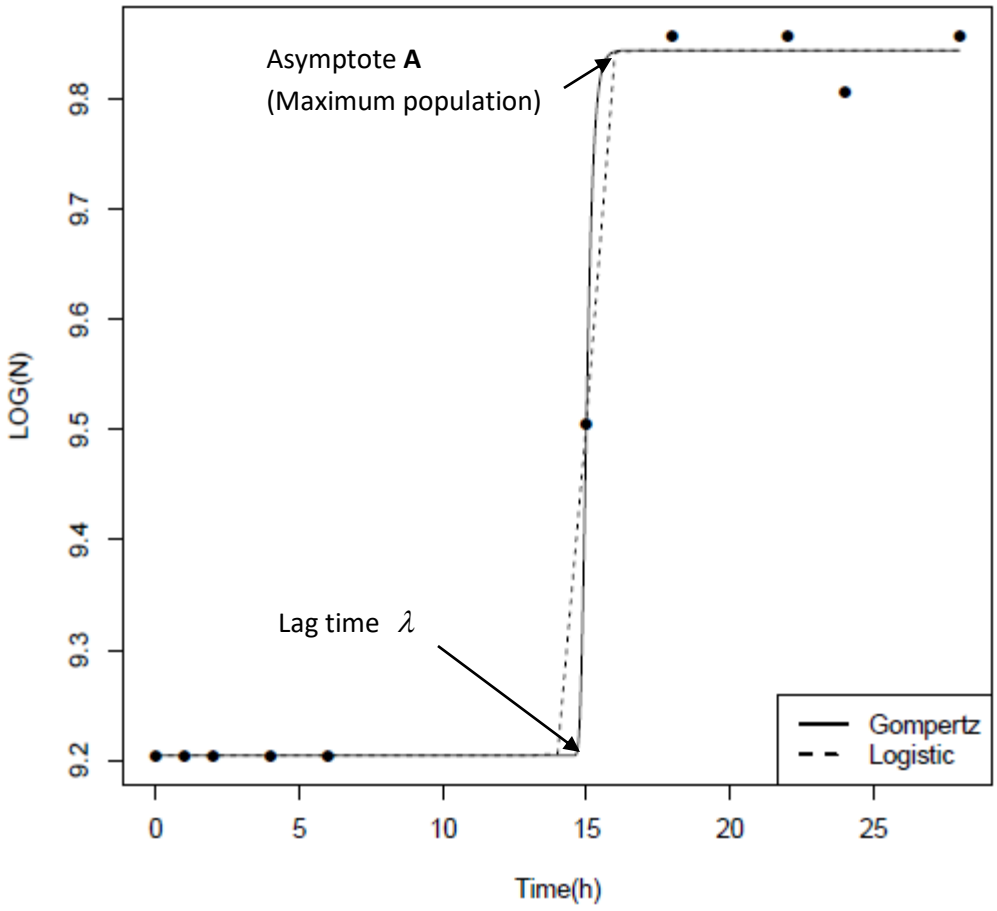

Figure 4-8: Growth curve for isolate 3^2 at 37C, pH 7.2 fitted with the Gompertz and logistic model

Both models provided a high goodness of fit (R^2> 0.93) for all growth curves for three isolates, in approximately 33% of the cases. The differences between the Gompertz and logistic models were not significant, which is in line with the findings of Longhi *et al*., 2013. However, Gompertz model was accepted in 75% of the remaining cases based on the AIC values and also supported by the R^2> 0.95 values and small RSS values (Table 4-12). For all the isolates, Gompertz and Logistic models gave different estimates of the growth parameters. For isolate 3^2 the models could not accurately estimate the growth parameters, for instance the lag time was

over estimated to close to 14.78 hours by Gompertz and 14.68 hours for logistic model, Figure 4-11. The estimated growth rate of 1.402 cells per hour by Gompertz model was outside the confidence interval limits. However, logistic model was found to have a lower AIC value as compared to Gompertz model, and the r^2 value of 0.986 was also lower as compared to 0.998 given by the Gompertz model, although based on the AIC value, the logistic model is likely to be correct.

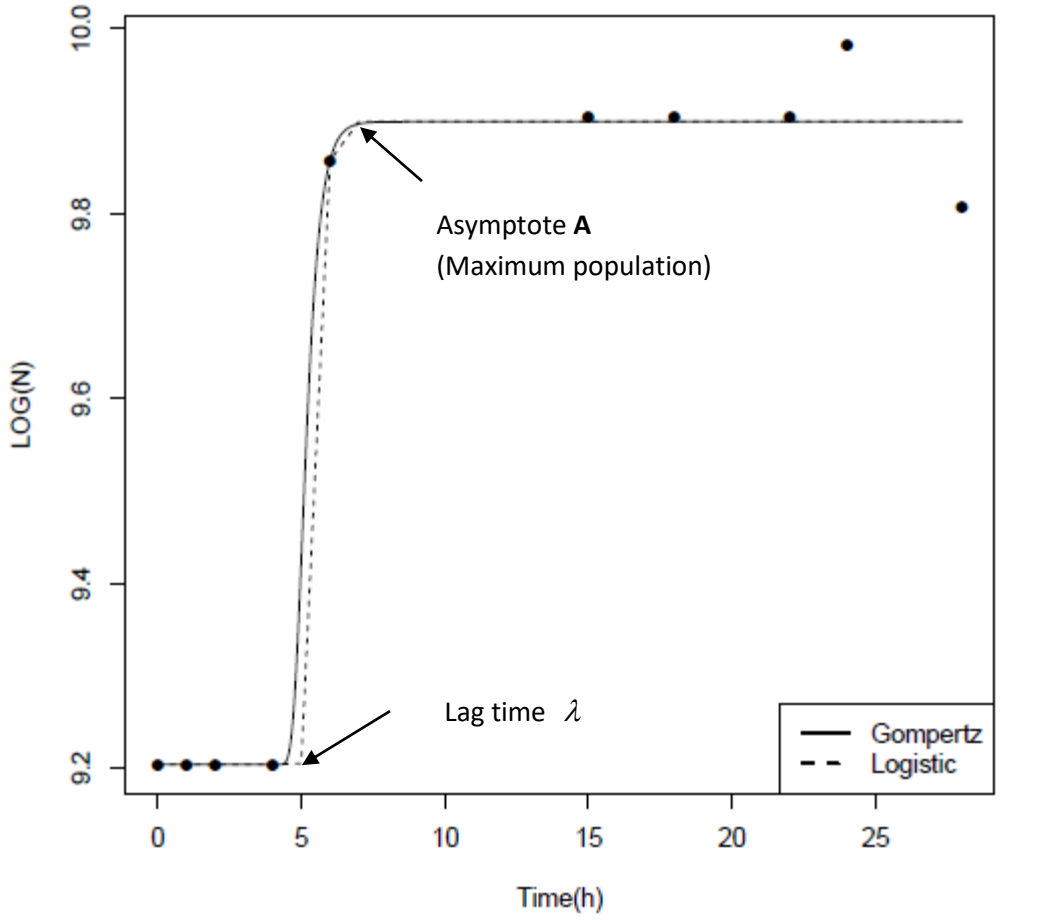

Figure 4-9: Growth curve for isolate 9^{3b} at 37C, pH 7.2 fitted with the Gompertz and logistic model

Figure 4-12 shows the growth curve fitted with both Gompertz and logistic models for isolate 9^{3b}.When comparing the growth rate values given by the two models Table 4-12, Gompertz gave the lowest growth rate (0.355-2.680) cells h^{-1} with a lag time of (3.762-4.725) hours while logistic had a growth rate of (0.397-1.890) cells h^{-1} with a lag time of (3.982-5.588) hours. The r^2 value for the logistic model was as low as 0.979, with a high SSE value of 0.026.This model was also found to have a high AIC value thus, Gompertz model was found to be the most likely model to be correct as it had the best fit for the growth curve of isolate 9^{3b}.

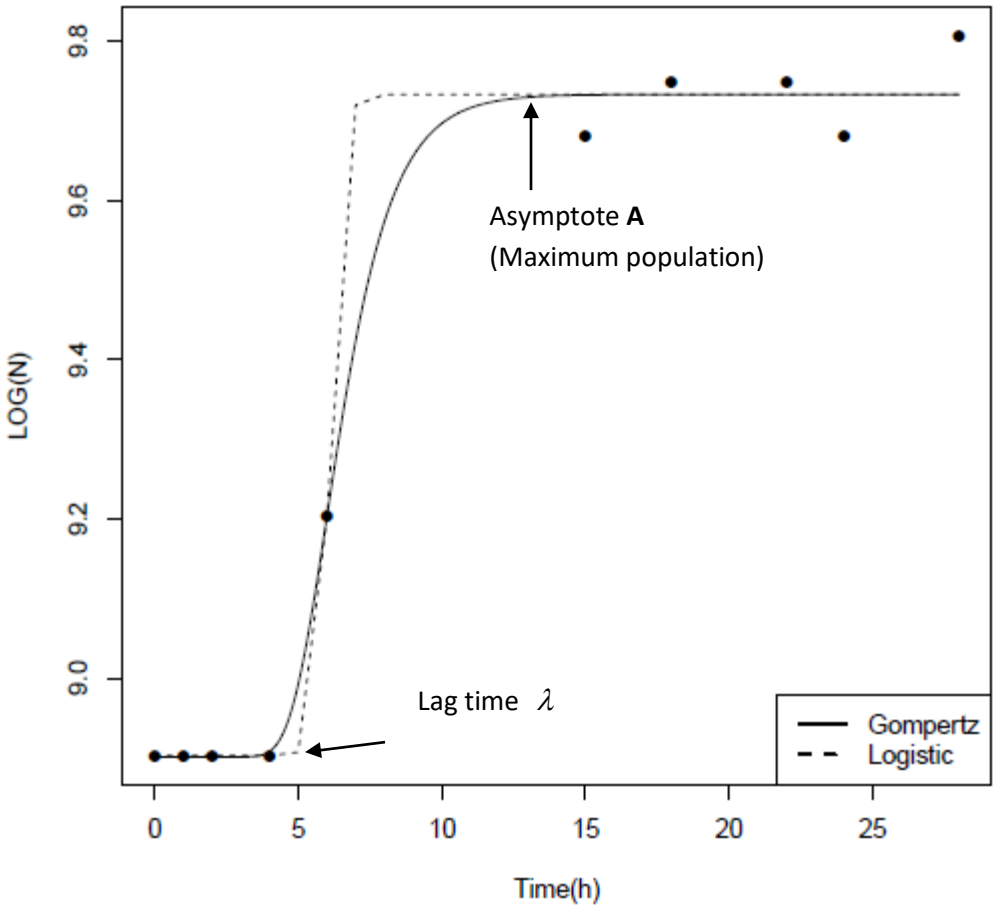

Figure 4-10: Growth curve for isolate 17[1] at 37C, pH 7.2 fitted with the Gompertz and logistic model

Gompertz model had the lowest values for the estimated growth parameters as (0.117-0.637) cells h^{-1} for growth rate and lag time of (3.648-5.599) hours as compared to (0.167-1.940) cells h^{-1} and (4.252-5.873) hours respectively for logistic model.(Figure 4-13).The AIC value and RSS value for this model were also observed to be lower. However, the r^2 value for all the models were below 0.995. Basing on the AIC value, the Gompertz model was found to be the most likely to be correct.

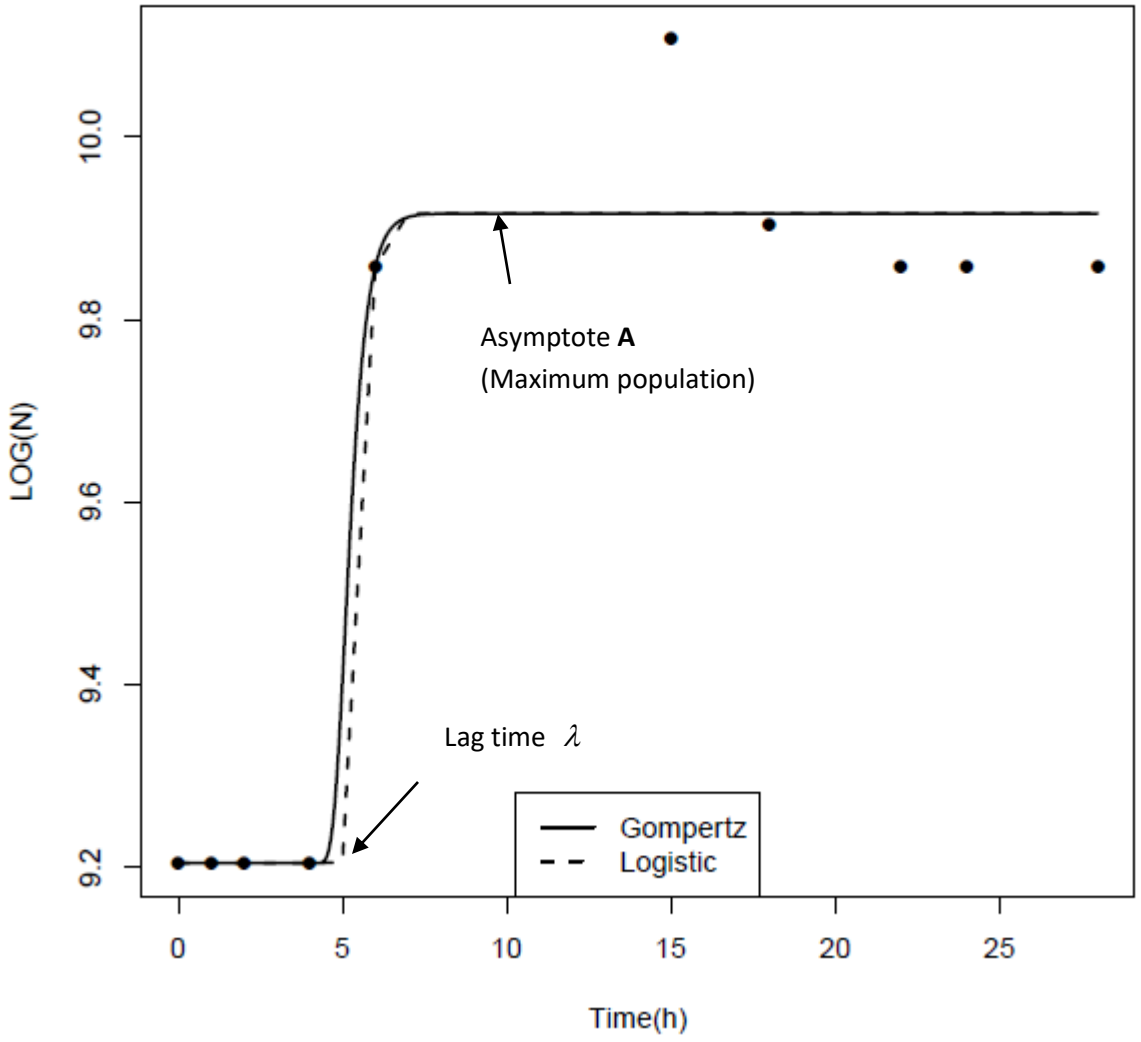

Figure 4-11: Growth curve for isolate 25^2 at 37C, pH 7.2 fitted with the Gompertz and logistic model

Figure 4-14 shows the growth curve for isolate 25^2, fitted with both Gompertz and logistic models. Logistics model gave the lowest growth rate values of (0.245-2.014) cells h^{-1} with a lag time of (3.183-5.620) hours while Gompertz had a growth rate of (0.257-2.217) cells h^{-1} with a lag time of (3.172-4.809) hours. The r^2 value for the logistic model was as low as 0.944, with a high RSS value of 0.087 as compared to r2 and RSS value of 0.962 and 0.0447 respectively for

the Gompertz model. Gompertz model however had a lower AIC value thus, it was found to be the most likely model to be correct for isolate 25^2.

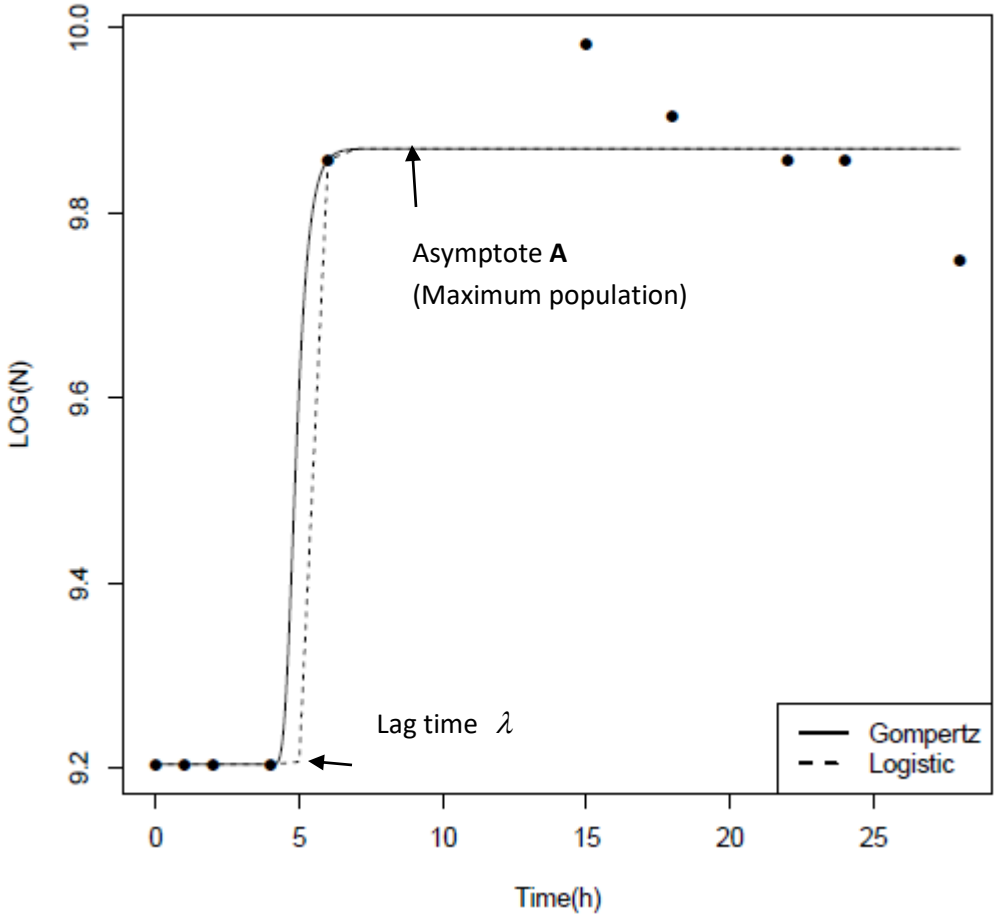

Figure 4-12: Growth curve for isolate 18^2 at 37C, pH 7.2 fitted with the Gompertz and logistic model

Growth curve for isolate18^2, fitted with both Gompertz and logistic models Figure 4-15 above displayed both Logistics model and Gompertz model to estimate relatively similar values for the growth parameters, Figure 4-15 and Table 4-12.The RSS value of 0.037 for Gompertz and 0.038 for logistic were also observed with little or no difference. The same observation was made for

the AIC values, thus, both models were likely to be correct although their r^2 values were below 0.995 (Table 4-12).

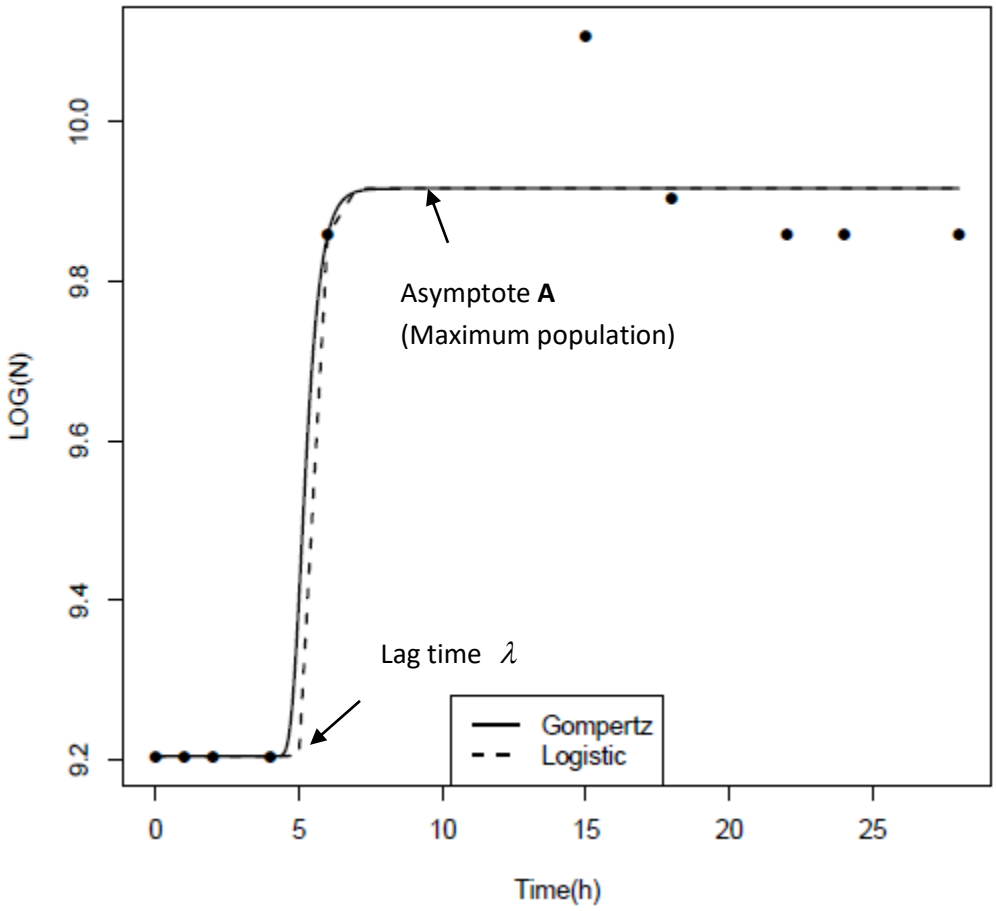

Figure 4-13: Growth curve for isolate 20^{1a} at 37C, pH 7.2 fitted with the Gompertz and logistic model

Figure 4-16 shows the growth curve for isolate20^{1a}, fitted with both Gompertz and logistic models. Gompertz model gave the lowest growth rate values of (0.357-2.592) cells h-1 with a lag time of (3.760-4.782) hours while Logistic had a growth rate of (0.364-2.036) cells h-1 with a lag time of (3.733-5.633) hours. The r^2 value for the logistic model was as low as 0.937, with a high RSS value of 0.069 as compared to r^2 and RSS value of 0.973 and 0.0029 respectively for the

Gompertz model. Gompertz model however had a lower AIC value thus, it was found to be the most likely model to be correct for isolate 20^{1a}.

Generally, data on growth curves are necessary to define and construct predictive models in anaerobic digestion. For reduction of measured data to important growth parameters in microbial growth, models play an important role as opposed to using linear regression. The lag stages, slopes and constant growth stages for all the growth curves representing the growth of these microorganism were in line with, lag, exponential and stationary growth phases as reported in literature (Esser *et al*., 2015; Perni *et al*., 2005; Pla *et al*., 2015; Zwietering *et al*., 1990). The lag time and growth rates for the microorganisms were also different although they were grown in the same conditions, depicting different adaption times for different microorganisms (Koch, 2001; Panikov, 1995).This information could be useful in the determination of the sludge retention time for the methanogenesis step in order to allow maximum contact of the feedstock and the bacterial mass and to minimize transport problems related to toxins with respect to substrate compounds, intermediate and end products (Y. Chen *et al*., 2008; Karakashev *et al*., 2005).

4.5 Contribution to knowledge

Anaerobic digestion of bio-waste is the most predictable way to produce biogas with high methane content, which has great potential to replace fossil fuel in various application. The models presented provides knowledge to describe the growth of the isolated and identified methanogenic community in a bio-digester as a function of time, hence maximum utilization of the exponential phase of the microbial growth for production of biogas. This indicates the practicality of applying Gompertz model to actual anaerobic digestion of brewery waste as a step

towards optimal methane production. The specific growth rate and lag time parameters were obtained from the models. The BI model developed guides on physicochemical parameters to be maximized to ensure proper levels of COD and BOD for both higher biodegradability ratio for optimal biogas production and also reduce environmental pollution. The study also provides information on the characteristics of the brewery wastewater before treatment, thus cautioning direct discharge to the environment.

CHAPTER FIVE

5.0 Conclusions and recommendations

5.1 Conclusions

1. The untreated brewery wastewaters generally had high levels of COD of 50966±20146.67mg/l and BOD of 3403±53.52 mg/l in both industries. The variations were prominent between industries. These industries have high organic loadings, thus the demand for environmental investments is high but, the organic material available in brewery wastes also has very high potential for bioenergy production allowing an environmentally friendly disposal solution. However, pretreatment prior to the digestion is necessary for improved biogas production. The low ratio of BOD to COD at the brewing line and CIP line could have a negative effect on the biogas production thus anaerobic digestion of the wastewater from these streams separately could be uneconomical.

2. The study has demonstrated that brewery wastewater harbour diverse bacteria species with potential biogas production. 31 isolates were obtained; 16 isolates were characterized and identified. Biochemical properties of some isolates for instance ability 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. Molecular characterization of the bacterial isolates indicates that 50 % belong to genus *Bacillus* within the Firmicutes in the domain bacteria, 32 % belong to the genus *Lysinibacillus sp*. The genus *Ralstonia and Myroides* both had 6 % each. Ten (10) isolates showed identity of 95 - 97 % similarity with the previously known sequences in

the GenBank database. Two (2) isolates showed identity of 70 -93 % similarity, representing novel genera of organisms within brewery waste water.

3. Comparisons of the behavior of the two primary growth models (Gompertz and Logistic) for different isolates at the same growth conditions showed that Gompertz model was the best-fitting model. These results provided insight into predicting microbial growth using proper primary growth predictive model, in anaerobic digestion as a step towards optimizing methane production.

5.2 Recommendations

The obtained bacterial isolates could be adopted for commercial production of methane but for full potential, further studies could be undertaken on:

1. Effect of seasonal changes on the quality of the brewery wastewater.
2. Continuous system as opposed to batch system with variation of the substrate concentration.
3. Unique physiological characteristics of the isolates to be to explore the applicability and full potential of the isolates.

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APPENDICES

Appendix 1: Media preparation

The nutrient composition of the Brewer Thyglycollate media is as follows;

Typical Formula*	gm/litre
`Lab-Lemco' powder	1.0
Yeast extract	2.0
Peptone	5.0
Glucose	5.0
Sodium chloride	5.0
Sodium thioglycollate	1.1
Methylene blue	0.002
Agar	1.0

pH 7.2 ± 0.2

Sulphur-indole mortility (sim) agar media

Preparation: 3.6% SIM, 4% Nacl, 1% Na_2NO_3.

Use; to test for the production of tryptophanase enzyme and the ability to produce hydrogen sulphide from substrates, such as sulphur containing amino acids and organic sulphur

Appendix 2: DNA extraction reagents

Solution 1

50mM Tris pH 8.5

50mM EDTA pH 8.0

25% Sucrose solution

Solution 2

10mM Tris pH 8.5

5mM EDTA pH 8.0

1% SDS

Lysozyme 20 mg/ml

RNase A 20 mg/ml

Proteinase K 20 mg/ml

Phenol

Chloroform

Absolute ethanol.

3M NaCl

Isopropanol

Electrophoresis buffer Working Concentrated stock

TBE buffer 10 ×

Chemical	Volume
Tris	108 g
Boric Acid	55 g
$Na_2EDTA.2H_2O$	9.3 g

Adjust the volume to 1 liter with ddH_2O and divide into 500ml bottles

Running conditions: use 1× **TBE** as the running buffer. Pre run the gel at 40W for 30 minutes. Load 2µl of sequencing reactions/well making sure to low out wells with a syringe first then Run the gel at 60W for 1.5-2h interval.

EDTA 0.5 M pH 8.0

Dissolve 186.1 g of disodium ethylenediaminetetra-acetate (EDTA $.2H_2O$ Sigma ED2SS mw 372.2) in 800 ml of double distilled water. Stir vigorously and adjust the pH to 8.0 with sodium hydroxide pellets (EDTA will not go into solution until the pH is near 8.0, so add some of the pellets before trying to adjust the pH. Bring it to a final volume of 1000 ml. Divide into 100 ml aliquots and autoclave.

Ethidium Bromide l0X

Dissolve 1.0 g of EtBr in a final volume of 100 ml double distilled water. Wrap the bottle in aluminum foil and stir several hours to get a true solution. Store at 4°C.

To make the 1X stock used to stain gels take 10 ml of the 10× stock and bring to a final volume of 100 ml using double distilled water. Wrap bottle in aluminum foil and store at room temperature.

Proteinase K

To 1 ml of ddH_2O add 20 mg of Proteinase K (Promega # 52066). This gives a 20mg /ml stock.

SDS 10%: Dissolve 100 g of electrophoresis-grade SDS in 800 ml double distilled water. Heat the solution to dissolve. Bring to a final volume of 1000 ml using double distilled water. Do not autoclave.

TE pH 7.4 or pH 8.0

Chemical	Volume
1 M Tris pH 8.0	2 ml
0.5 M EDTA pH 8.0	400 µl

Bring it to a final volume of 100ml using double distilled and Autoclave.

Tris 1 M p H 7.4 : Dissolve 121.1 g of Tris base in 800 ml of double distilled water and adjust the pH to 7.4 with concentrated hydrochloric acid. Bring the final volume to 1000 ml with double distilled water. Divide into 100 ml bottles and autoclave.

Appendix 3: Polymerase Chain Reaction standard operation procedure

Principles

The polymerase Chain Reaction (PCR) is a method of oligonucleotide primer directed enzymatic amplification of a specific DNA sequence of interest. This technique is capable of amplifying a

sequence 105 to 106–fold from Nano gram amount of template DNA within a large background of irrelevant sequences (e.g. from total genomic DNA). A prerequisite for amplifying a sequence using PCR is to have known, unique sequences flanking the segments of DNA to be amplified so that specific oligonucleotides can be obtained. It is not necessary to know anything about the intervening sequence between the primers.

The PCR product is amplified from the DNA template using a heat-stable DNA polymerase such as that from *Thermus aquaticus* (*Tag* DNA polymerase) and using an automated thermo cycler to put the reaction through 30 or more cycles of denaturing, annealing of primers, and polymerization. After amplification by PCR, the products are separated by agarose gel electrophoresis and are directly visualized by after staining with ethidium bromide. The use of the so-called Multiplex PCR has been adopted in order to allow analysis of two or more targets simultaneously.

Equipment Reagents:

- A Thermo cycler.
- A horizontal gel electrophoresis system.
- Photographing equipment and UV transilluminator.
- Sterile thin-wall 0.2ml Thermo cycler microfuge tubes (Eppendorf)
- Pipette tips; 10 µl, 100 µl, 200 µl, 1000 µl.

Materials/Reagents:

- Synthetic oligonucleotide primer pair flanking the sequence to be amplified, store at -20^{o}C.

- 10X PCR Buffer (250 mM KCl, 50 mM Tris-HCL pH 8.3), store at 4^{o}C or -20^{o}C.
- A stock solution with a concentration of 10mM for each dNTP: dATP, dTTP, dCTP and, store at -20^{o}C.
- Tag DNA polymerase, store at -20^{o}C.
- $MgCl_2$ (25 mM), store at -20^{o}C.
- Agarose, store at room temperature.
- Ethidium Bromide (10mg/ml, store at 4^{o}C.
- Distilled water, DNase-RNase free.

Procedure

Template DNA isolation

Day 1

Grow cells at 37^{o}C overnight in 2ml broth medium.

Day 2

- Pellet cells from 500 µl of bacterial culture by centrifugation at 13,000 g for 30 seconds and remove spent media. Resuspend pelleted cells in 500 µl of sterile distilled water.
- Heat in a water bath at 95^{o}C for 5 minutes.
- Freeze at -80^{o}C for 30 minutes then take for PCR.
- Use 10µl of the sample (template DNA) for PCR.

- The template can then be stared at 80^{o} or -20^{o}C until further analysis.

Polymerase chain reaction

PCR Amplification:

The protocol below is carried out in a 50 µl reaction volume.

Components	**Volume (50 µl)**	**Final**
Distilled water	38.8	Variable
10XPCR Buffer (with 1.5mm of $MgCl_2$)	4.0	5 µl
dNTPs mix (1.25mM each nucleotide)	4.0	200µl
Primer	1 µl -Forward 1 µl - Reverse	0.1-1.0µl
Taq DNA polymerase (Gene script	0.2	1 Unit/µl
Genomic DNA template	1	1 µl

Because of the small volume involved, it is convenient to make a cocktail of the six ingredients for each primer pair to be used. For instance, if 10 PCR reactions are to be performed from 10 different genomic DNA templates using one primer pair, then a cocktail may be made (including a slight excess) for eleven reactions by mixing together each of the volumes above multiplied by 11 . Add a 49 µl aliquot of the cocktail to each tube. 1.0 µl of template DNA is then added to each tube.

Steps:

- Plan your experiment before adding any reagents (number of primer pairs to be used, number of Template DNA).After doing so, make the appropriate cocktail/s and ensure complete mixing by tapping the tube and quick spinning.

- Pippete 49.0µl of the appropriate cocktail directly into the bottom of a sterile microeppendorf tube for each reaction. The tubes should be labelled using a permanent marker.

- Add 1.0 µl of the DNA directly into the drop of cocktail in each tube and ensure adequate mixing. Quick spin to collect the reaction mixture in the bottom of the tube.

- Place the tightly capped tubes in the temperature block and make sure each is firmly seated by pressing on the tubes individually.

The PCR machine must now be programmed for the specific reaction conditions desired. Each cycle in the polymerase chain reaction involves three steps (denaturing, primer annealing, and Polymerization), and the products are amplified by performing many cycles one after the other with the help of the automated thermal cycler. The Taq polymerase is heat stable, and remains active despite the high denaturing temperature of each cycle. A representative set of reaction condition for 32 cycles is:

Name	**Temperature**	**Time**
First Denaturation	94º C	5 Minutes
Denaturation	94ºC	1 Minute
Annealing	49ºC	1 Minute

Extension	72^{o}C	5 Minutes
Final Extension	72^{o}C	10 Minutes

5. Create or select a program from memory and proceed to 'RUN' the program. Refer to the Thermo cycler manufacturer's manual for detailed operating instructions.

6. After completion remove the tubes and ensure labelling markings are still clearly visible.

7. The reaction products are conveniently separated according to size by electrophoresis through a 1.2% agarose gel (Appendix 3) at 80 Volts for 45 minutes-1hour, and visualized after staining the gel with ethidium bromide.

Appendix 4: Gel electrophoresis

Gel electrophoresis is an important molecular biology tool. DNA sequencing, fingerprinting (or "profiling"), and genetic engineering are based upon it. Gel electrophoresis separates DNA fragments by their size or molecular weight. The agarose gel acts like a sieve, separating different sized fragments while the electric current provides electrodes; the voltage determines how fast the DNA will travel through the gel. Larger molecules or DNA fragments become entangled in the gel and travel more slowly, while smaller one pass through more easily and travel farther down the gel. Similar-sized DNA fragments travel at the same rate and form a tight bunch called a "band". The DNA in the gel must be stained in order to see the bands.

Preparing the gel

DNA gels are made of agarose, a highly purified agar, which is heated and dissolved in a buffer solution. The agarose molecules form a matrix with pores between them. The more concentrated the agarose, the smaller the pores. 100 ml of 1.2% agarose gels was used (1.2 grams of agarose per 100ml of TBE buffer).

Procedure:

- Weigh 1.2 grams of agarose powder and place it in a conical flask.
- Add 100 ml of 1X TBE buffer into the flask. Swirl to mix the solution.
- Place the flask in the microwave. Heat on the high until the solution is completely clear and no small floating particles are visible (1-2 minutes). Swirl the flask frequently to mix the solution and prevent the agarose from burning.
    - Do not allow the agarose to boil over.
    - Use hot mitts when handling the flask because it will be very hot.
    - Evaporation during boiling may have caused a reduction in the volume thus increase in concentration. Add water to compensate for the loss and maintain the concentration.
    - Cool the solution to 55 ^{O}C before pouring the gel into the plastic casting tray. Higher temperatures will melt the plastic tray. Ethidium bromide can be added at this point to a final concentration of 0.5 µg/ml; 2 µl of EtBr added per 100 ml of buffer.

Care! Ethidium bromide is an irritant, mutagen, toxic and may cause heritable genetic damage. Always wear gloves when handling the powder, solutions and all gels that contain ethidium bromide. Remember to clean working surface, gloves and equipment using 10% bleach solution.

- Place the plastic comb in the slots on the side of the gel tray. The comb teeth should not touch the bottom of the tray. Push any air bubbles to the side farthest from the wells.

- Allow the agarose gel to cool until solidified. The gel will appear a cloudy white colour and will feel cool to touch (about 20 min). Gels can be stored, wrapped in plastic wrap, in the refrigerators for a few days.

- Once the gel is completely solidified, lift the tray out of the chamber, turn it 90^{O}, and replace it in the chamber with first comb closest to the cathode side of the chamber. The running position exposes the open ends of the agarose to the buffer. The standard agarose should solidify completely in about 30 min.

- Pour the buffer into the unit to fill chamber and completely cover and submerge the gel. A "Fill Line" is located on each unit clearly mark the correct buffer level.

NB: Too little buffer may cause the gel to dry out during the run, while excess buffer may slow down DNA migration in the gel.

Loading and Running an Agarose Gel

- Remove the comb from the wells by pulling straight up on the comb. Be careful not to tear the wells as you remove the comb.

- Place the gel box with the wells closest to the negative (black) electrode.

- Add 1X TBE buffer to fill the buffer tank and submerge the gel about ¼ inches.

- Cut a piece of parafilm and place 2 µl of gel loading dye onto the waxy side for each sample to be loaded. Dispense 10 µl of the sample and mix the solution by pipetting the dye up and down into the sample. The gel loading dye contains glycerol that will make your DNA denser so that it will sink into the wells. It also contains dye molecules that are smaller and travel faster through the gel than the DNA molecules. The dye molecules provide a visual tracking method so you know how far the DNA has travelled through the gel.

- Pipette 5 µl (2 µl loading dye and 3 µl DNA ladder) as reference sample into the first well. Keep the tip of the pipette ABOVE the well. The DNA will sink into the well because it has been mixed with loading dye. If you puncture the bottom of the well your DNA run out through the bottom of the gel into the buffer tank.

    - Molecular biologists often use a size standard marker called a l kb DNA ladder. The DNA ladder produces several different sized fragments and can be used to estimate the size of an unknown DNA fragment.

- You are now ready to load the next sample into the next well. Repeat step 4 until all of the samples and controls, both positive and negative (blank without DNA), have been loaded into the gel.

- Close the top of the box. Plug the leads into the gel box. The black lead is the negative lead and should be plugged in closest to the wells. The red lead is the positive lead and should be plugged-in furthest from the wells.

- Plug the other end of the leads into the power source and turn it on. Electrophoresis at 80-170 volts until the loading dye has travelled ½ to ¾ of the way down the gel (about 0.5-1 hour).
- Turn off the power supply. Unplug the leads and power supply before opening the gel box.
- Visualize the DNA on a long wave UV light box and photograph with Polaroid camera lens aperture set at f/11 and exposure time of 30 milliseconds. Pull out the picture and allow developing for one minute.
- Photodocument the picture.

Calculations:

Calculate reagent's concentration according to manufacturer's specifications.

Interpretation and reporting of results

Read the gel picture for the efficient amplification of target sequence. The separated macromolecules in each lane can be seen in a series of bands spread from one end of the gel to the other. Target gene can be read against the standard molecular weight markers (DNA ladder).

The picture can then be documented in a file or record book.

Appendix 5: Algorithm for Gompertz and Logistic models

```
Murunga<-read.table("D:\\msc NOtes\\my work\\project\\project R code\\bacteriadata1.txt",header=T) # Reading data in from an external disk
attach(Muranga)
murunga1=data.frame(time,L7)# creating a data frame that holds time and values of isolate 7
plot(time,L7)
locator(1)
d=diff(L7)/diff(time)
mu=max(d) # obtaining the maximum gradient/slope
########################Fitting Gompertz model on isolate 7
murunga2=L7 ~ Y0 + A-Y0 * exp(-exp(mu * exp(1) *
    (lag - time)/((A -Y0) ) + 1))
data(growthcurve1)
nls2 <- nlsLM(murunga2,data=murunga1,list(lag = 4.068, mumax =mu, LOG10N0 = min(L7),LOG10Nmax = 9.896))# Fitting  the Gompertz regression model
#using the levenberg Marquardt algorithm
summary(nls2)
boot=nlsBoot(nls2, niter = 200) # obtaining the bootstrap confidence intervals of the estimates
summary(boot)
confint(boot,0.95)
AIC(nls2,nlsLog) # obtaining the AIC for both the Logistic and Gompertz models for comparisons
BIC(nls2,nlsLog)
plotfit(nls2,smooth=T,pch.obs = 16,lty = 1, lwd = 2, col.fit = "blue",xlab="Time",ylab="LOG(Y)",main="Gompertz curve for isolate 7")
# plotting the gompertz model
############################## Fitting logistic model on isolate 7
 Logi=L7~Y0+(A-Y0)/(1+exp(((4*u)/(A-Y0))*(L-time)+2))
nlsLog <- nlsLM(Logi,data=murunga1,list(L = 4.068, u = mu,Y0=min(L6),A=9.896)))# Fitting  the Logistic regression model
#using the levenberg Marquardt algorithm
summary(nlsLog)
boot1=nlsBoot(nlsLog, niter = 200)# obtaining the bootstrap confidence intervals of the estimates
summary(boot1)
t=seq(0,28,1)
l81 <- predict(nlsLog,list(time=t))
pdf(file = "isolate 7.pdf", bg = "transparent")# saving the plot on the external file
plotfit(nls2,smooth=T,pch.obs = 16,lty = 1, lwd = 1, col.fit = "black",xlab="Time(h)",ylab="LOG(N)")# plotting both the Gompertz and the logistic model on the same curve
lines(t,l81,col="black",lty="dashed",lwd=1) # superimposing the logistic curve on the same plot of Gompertz curve
legend("bottomright",c("Gompertz","Logistic"),lty=c("solid","dashed"),lwd=c(2,2))
rect(1, 5, 3, 7, col = "white")
dev.off()
```

Appendix 6: A section of aligned sequences by Clustal W in Mega Software version 5

Appendix 7: Definition of terms

Anaerobic digestion	It is the degradation of organic materials by microorganisms in the absence of oxygen to CO_2 and CH_4.
Biochemical techniques	Refers to a set of methods, assays and procedures to analyse the substances found in living organisms and the chemical reactions underlying life processes
Biodegradability	It is the ability of materials to be capable of being disintegration by bacteria, fungi, or other biological means.
BOD_5	It is the amount of dissolved oxygen needed by aerobic biological organisms to break down organic material present in a given water sample at certain temperature over a five day period.
COD	Is a measure of the capacity of water to consume oxygen during the decomposition of organic matter and the oxidation of inorganic chemicals such as Ammonia and nitrite.
Deoxyribonucleic acid (DNA)	A self-replicating material present in nearly all living organisms as the main constituent of chromosomes. It is the carrier of genetic information.
DNA Sequencing	Is the process of determining the sequences of nucleotide bases (As, Ts, Cs and Gs)

Environment The surroundings or conditions in which a person, animal, or a plant lives or operates.

Methanogens Microorganisms that produce methane as a metabolic byproduct in anioxic conditions.

Molecular techniques Methods involving manipulation of DNA, RNA, protein, and lipids

Morphological techniques Analysis of the organisms with respect to the form, structure, and their specific features.

Phylogenetic analysis Is the means of inferring or estimating relationships. The evolutionary history inferred from phylogenetic analysis is usually depicted as branching, tree like diagrams that represent an estimated pedigree of the inherited relationships among molecules, organisms or both.

Polymerase Chain Reaction (PCR) Is a method of oligonucleotide primer directed enzymatic amplification of a specific DNA sequence of interest. It amplifies a single copy or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence.

Primers Is a strand of DNA bases that enables DNA to be replicated. They are used primarily in Polymerase Chain Reaction and each primer is single stranded DNA and is designed to match a specific piece of template DNA. The specificity arises from the fact that each DNA

base can only pair with one other DNA base that is adenine (A) pairs only with thymine (T) in DNA and uracil (U) in RNA, and guanine (G) pairs only with cytosine (C). In order for copies to be made, the primer must bind to the right piece of DNA and the bases must match. If the matching occurs, then DNA polymerase (the enzyme that copies the DNA) can bind and amplify the DNA. If the primer does not match the DNA sequence, then the DNA polymerase cannot bind and no copies will be made.