EFFECT OF TEMPERATURE AND CARBON TO NITROGEN RATIO ON THE PERFORMANCE OF AN UPFLOW ANAEROBIC SLUDGE BLANKET REACTOR TREATING SUGARCANE MOLASSES

by

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DECLARATION

I, Afika Ndobeni, declare that the contents of this thesis represent my own unaided work, and the thesis has not been previously submitted for any academic exemption towards any qualification. Furthermore it represents my own opinions and not necessarly those of the Cape Peninsula University of Technology.

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Signed **Date**

ABSTRACT

The sugar industry contributes to the development of the economy in many countries, including South Africa. The wastewater generated by this industry has a high pollution load, and therefore requires treatment before discharge to the environment. The primary aims of this study were to determine the performance of an upflow anaerobic sludge blanket (UASB) reactor treating sugarcane molasses and to develop an empirical model to predict the behaviour of the UASB in terms of chemical oxygen demand (COD) removal and biogas production.

A UASB (46 L working volume) was inoculated with granular sludge from the brewery industry and was used to investigate the treatment of synthetic sugar industry wastewater with an average COD of 4101 mg/L. The experiments were designed using Design-Expert® Software Version 10. The analysis of variance for the models and the optimisation of reactor temperature and feed carbon to nitrogen (C/N ratio) were carried out using response surface methodology. The UASB was operated at constant hydraulic retention time and organic loading rate of 2.04 days and 2.01 kg/m^3.d, respectively. A start-up period of 22 days was required to reach steady-state.

The developed empirical models for total COD removal efficiency and biogas production rate were found to be statistically significant with Prob > F values of 0.0747 and 0.0495 and the determination coefficients (R^2) were found to be 0.80 and 0.65, respectively. The optimal conditions were found to be at a temperature of 38^{o}C and C/N ratio of 22 mgTOC/mgTN. The corresponding removal efficiencies in terms of total COD, five day biological oxygen demand, total nitrogen, total phosphorus, and sulphate was 77.7, 85.9, 99.2, 44.4 and 57.2%, respectively. Biogas was produced at a rate of 0.832 L/L.d with a methane, carbon dioxide and molecular oxygen content of 65.2, 32.8 and 0.6%.

Results suggest that UASBs may offer a feasible option for reducing the organic strength of sugar industry wastewater, while simultaneously generating methane-rich biogas.

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DEDICATIONS

To my loving family

GLOSSARY

Term	Definition
Bagasse	Sugarcane lignocellulosic (fibre) by-product remaining from crushing cane and extraction of sugar juice in the milling stage.
Biological oxygen demand	Quantity of oxygen consumed by microorganisms that degrade organic matter in wastewater.
Chemical oxygen demand	Measure of the oxygen equivalent to the amount of organic matter that is susceptible to oxidation by a strong oxidising agent.
Degrees Brix (^{o}Bx)	Percentage weight of sugar content (sucrose) in a solution. 1^{o}Bx is equivalent to 1 g of sucrose in 100 g of solution.
Filter mud	Solids and soil precipitated from the clarification of sugar juice in the milling process.
Imbibition water	Water used to wash the sugar out of the fragmented cane (bagasse) remaining after crushing in the milling stage.
Massecuite	Mixture of crystalline sugar and a liquor produced from evaporation of water from sugar syrup in an evaporator.
Molasses	Dark viscous liquid obtained when sugar crystals are separated from sugar syrup in crystallisation and centrifugation processes.
Total Kjeldahl nitrogen	Total concentration of organic nitrogen plus ammonia.

LIST OF ACRONYMS

Acronym	Full name
AAE	Acetic acid equivalent
ABF	Anaerobic baffled filter
AF	Anaerobic filter
AgMRC	Agricultural marketing resource center
AHR	Anaerobic hybrid reactor
APHA	American public health association
ASBR	Anaerobic sequencing batch reactor
ASBR	Anaerobic sequencing batch reactor
AWWA	American water works association
CAS	Conventional activated sludge
CCD	Central composite design
CS	Cassava factory sludge
CSTR	Constant stared tank reactor
DWS	Department of water and sanitation
EGSB	Expanded granular sludge bed
EPA	Environment protection agency
GLSS	Gas liquid solids separator
H-EGSB	Hybrid-expanded granular sludge bed
HPLC	High pressure liquid chromatograph
HRT	Hydraulic retention time
HYB-AF	Hybrid digester and anaerobic filter
IHT	Inter-species hydrogen transfer
LCFAs	Long chain fatty acids
NAMC	National agricultural marketing council
NESC	National environmental services center
OLR	Organic loading rate
PS	Palm oil mill sludge
PU	pH unit
RBC	Rotating biological contactor

RSM	Response surface methodology
SACU	South African customs union
SASA	South african sugar association
SEM	Scanning electron microscopy
SFS	Seafood factory sludge
SIWW	Sugar industry wastewater
SRB	Sulphate reducing bacteria
SRT	Silids retention time
UAF	Upflow anaerobic filter
UAFB	Upflow anaerobic fixed bed
UAPB	Upflow anaerobic packed bed
UASB	Upflow anaerobic sludge blanket
UASB-AF	Upflow anaerobic sludge blanket-anaerobic filter
UBF	Upflow blanket filter
UNEP	United nations environmental programme
USDA	United States department of agriculture
USEPA	United states environmental protection agency
VFAs	Volatile fatty acids
WEF	Water environment federation
WWF	World wildlife fund

NOMENCLATURE

Abbreviation	Term	Unit of measurement
Y_{CH_4}	Methane yield rate	L CH_4/gCOD_{re}
μ_m	Maximum specific bacterial growth rate	g VSS/g VSS.d
k_s	Half-velocity constant	g/m^3
r_{su}	Substrate utilisation rate	g bsCOD/m^3.d
BOD	Biological oxygen demand	mg/L
BOD_5	Biological oxygen demand 5 day test	mg/L
bsCOD	biodegradable soluble chemical oxygen demand	mg/L
CD	Current density	A/m^2
COD	Chemical oxygen demand	mg/L
COD_{in}	Influent chemical oxygen demand	mg/L
COD_{out}	product stream chemical oxygen demand	mg/L
COD_{re}	Removed chemical oxygen demand	mg/L
G_{pr}	gas production	L/L.d
HRT	Hydraulic retention time	hrs
OLR	Organic loading rate	g COD/L.d
sCOD	Soluble chemical oxygen demand	mg/L
SRT	Solids retention time	hrs
SS	Suspended solids	mg/L
TDS	Total dissolved solids	mg/L
TKN	Total kjeldahl nitrogen	mg/L
TOC	Total organic carbon	mg/L
TP	Total phosphorus	mg/L
TS	Total solids	mg/L
TSS	Total suspended solids	mg/L
VSS	Volatile suspended solids	mg/L

CHAPTER ONE
INTRODUCTION

1.1 Background

Like many agri-industries, the sugar industry has arguably had a negative effect on the environment. The conversion of natural habitats for cane cultivation in coastal areas and tropical islands has led to critical environmental damage, loss of biodiversity and ecosystem services at landscape levels (World Wild Fund, 2015; Rein *et al*., 2011; Corcoran *et al*., 2007; World Wild Fund, 2004). In addition, the operations of sugar mills produce effluents that may contain massive quantities of organic matter and sludge washed from the sugar mills, which can degrade fresh water resources and deplete dissolved oxygen resulting in mortality of aquatic species (World Wild Fund, 2015; Sahu *et al*., 2015; Saranraj & Stella, 2014; Ahmad & Mahmoud, 1982). The industry generates waste from both milling and refining processes. Wastewater is generated from cleaning process pipes and equipment, washing of floors, and leakages (Rein *et al*., 2011; Sanjay & Solomon, 2005; Steffen *et al.*, 1990). In some countries with poor environmental regulations and laws, the annual cleaning of sugar mills results in large quantities of toxic matter which is usually discharged directly into surface waters (Saranraj & Stella, 2014; Levin *et al*., 2012). In order to alleviate environmental damage, sugar industry wastewater should be treated before discharge (Qureshi *et al.*, 2015). Therefore, further research is required to develop feasible and effective wastewater treatment technology to handle effluent volumes and reduce the pollutants in sugar industry wastewater.

Currently, there are 14 sugar mills in the Republic of South Africa, with an average production rate of 2.3 million tons of sugar per season [South African Sugar Association (SASA), 2015]. The South African sugar industry is concentrated on the east coast of KwaZulu Natal province with recognisable operations in the Limpopo and Eastern Cape provinces (SASA, 2015). Significant quantities of water are used by the industry: the water consumption of the industry is approximately 565 kL per ton of sugarcane processed and an average of approximately 2184 kL of wastewater per ton of sugarcane processed is discharged (Illovo Sugar Limited, 2015). Wastewater produced from the sugar industry contains high concentrations of organic nitrogen, oil and grease, and sugars (Sajani & Muthukkaruppan, 2011 Guray *et al*., 2008). The chemical oxygen demand (COD) concentration of sugar industry wastewater typically ranges between 3000 and 6000 mg/L, but values as low as 35 mg/L and as high as 7432 mg/L have been reported (Table 1) (Muhammad & Ghulam, 2015; Sahu *et al*., 2015; Saranraj & Stella, 2014; Shivayogimath & Rashmi, 2013; Siddiqui & Wassem, 2012; Sajani & Muthukkaruppan, 2011; Anand *et al*.,

2009). The range, as reported by various researchers is significantly higher than the general authorisations for irrigation of biodegradable industrial wastewater in terms of the South African National Water Act (Act No. 36 of 1998) which stipulates a COD limit of 5000 mg/L for irrigation of up to 50 kL/day and COD < 400 mg/L for irrigation of up to 500 kL/day (Government Gazette number 19182 of 2013). In addition to high COD concentrations, water from washing raw sugarcane contains crop pests, pesticide residues, and pathogens. Disposal of sugar factory effluents without effective treatment may cause environmental problems such as eutrophication of water bodies (Sahu *et al*., 2015; Sahu & Chaudhari, 2014).

Table 1: Typical sugar mill effluent values and Department of Water and Sanitation limits for discharge of wastewater into a water resource in South Africa.

Parameter	Typical effluent values*	Discharge limit	Reference
pH	7.1-9.1	5.5-9.5	Kaur *et al*., 2010
COD (mg/L)	35-7432	75	Saranraj & Stella, 2014; Siddiqui & Wassem, 2012
Chlorides (mg/L)	50-1894	0.25	Saranraj & Stella, 2014; Sahu *et al*., 2015
EC (mS/m)	470-1009	70	Turinayo, 2017
Nitrates (mg/L)	2.9-4.1	15	Muhammad & Ghulam, 2015
Cadmium (mg/L)	0.004-0.040	0.005	Muhammad & Ghulam, 2015; Suresh *et al*., 2015
Zinc (mg/L)	0.0338-1.40	0.1	Muhammad & Ghulam, 2015; Siddiqui & Wassem, 2012
Iron (mg/L)	0.140-12.80	0.3	Muhammad & Ghulam, 2015; Podar & Sahu, 2015
Copper (mg/L)	0.042-0.135	0.01	Muhammad & Ghulam, 2015; Podar & Sahu, 2015
Mercury (mg/L)	0.024-0.105	0.005	Muhammad & Ghulam, 2015

***Typical effluent values taken from references provided**

Waste stabilisation ponds (WSPs) are often used to treat sugar industry wastewater in South Africa (Welz and Ndobeni, 2017). Other methods that have been described for treating sugar industry wastewater include chemical precipitation, coagulation and flocculation and conventional activated sludge (CAS). The latter entails bubbling air into wastewater in aeration tanks to cultivate aerobic bacteria (biomass) which degrade organic matter and some inorganic compounds. The biomass (sludge) is separated from the treated wastewater by gravity settling in settling tanks (Guray *et al*., 2008). The drawbacks of the CAS is that it produces large quantities of sludge for disposal, has high maintenance costs, as well as high capital and operational costs due to installation of large process equipment, and energy

requirements for aeration and pumps (Sahu *et al*., 2015; Sahu & Chaudhari, 2014; Shivayogimath & Rashmi, 2013; Andreoli & Von, 2007; Steffen *et al.*, 1990).

While aerobic treatment systems such as CAS are widely adopted for treating low strength wastewaters (COD < 1000 mg COD/L) like municipal wastewater (Mrowiec & Suschka, 2009; Barbosa & Sant`anna, 1989), anaerobic systems such as upflow anaerobic sludge blanket (UASB) reactors greatly reduce oxygen demand in sugar industry effluents and can treat industrial wastewaters of high organic strength (Mrowiec & Suschka; Bruijn, 1975). UASBs have the advantages of low sludge production, compact equipment space, low maintenance and operational costs, and the ability to recover energy through biogas production (Bruijn, 1975).

1.1.1 Research problem statement

Wastewater generated from the sugar industry contains high concentrations of organic pollutants, suspended solids, pathogens, soil contaminated with residual pesticides, and nitrates and phosphates from fertilizers. Discharge of untreated sugar industry wastewater into water courses creates environmental problems such as eutrophication and acidification of water bodies, rapidly depletes dissolved oxygen due to biological oxidation, and causes mortality of aquatic and terrestrial species.

Physicochemical and biological technologies are typically used to treat sugar industry effluents, e.g. coagulation with alum in the presence of lime, followed by biological oxidation. Both processes are generally not able to produce treated effluent up to the discharge standards established by environmental agencies in sugar producing countries across the globe. In South Africa, this non-compliance can result in financial strain from government fines.

1.1.2 Aims

To examine and improve the performance of a 46 L UASB reactor treating sugar industry effluent in terms of COD reduction and biogas production.

1.1.4 Objectives

In order to achieve the aims, the following objectives were set to:

- Determine the physicochemical characteristics of sugar industry wastewater via a comprehensive literature review
- Formulate a synthetic wastewater with a COD concentration based on literature values
- Operate the UASB reactor in a start-up phase until a steady-state is reached

- Operate the UASB reactor under a range of temperature and C/N ratios
- Develop a mathematical model to predict COD reduction and biogas production in the UASB reactor
- Establish optimum temperature and C/N ratios yielding maximum organics removal and biogas production
- To identify the dominant bacteria and archael communities in the granules

1.1.3 Research questions

The following research questions underpinned the study:

- What is the start-up period required for an experimental UASB inoculated with anaerobic granules from the brewery industry?
- What combination of C/N ratio and temperature achieve maximum COD reduction and biogas production from the treatment of synthetic sugar industry wastewater (SIWW) in the experimental UASB?
- Can an empirical model be developed to predict COD reduction and biogas production from the experimental UASB treating synthetic SIWW?
- What known methanogenic species are present in the granules obtained from the experimental UASB?
- What is the COD concentration of the treated effluent from an experimental UASB treating synthetic SIWW, and does it comply with applicable South African discharge limits?
- What is the quality and quantity of biogas produced by the experimental UASB in terms of potential energy production?

1.1.5 Significance of research

It is proposed that by treating sugar industry effluent using UASB reactors (i) the environmental problem (eutrophication and acidification of water bodies) caused by sugar industry effluent discharge into aquatic environments (rivers) and (ii) irrigation with contaminated effluents from sugar mills may be reduced. In addition, the anaerobic digestion of sugarcane molasses by UASB produces a methane rich gas (>65% CH_4) which presents a source of bio-engergy.

CHAPTER TWO
LITERATURE REVIEW

2.1 Sugar and the South African sugar industry

The South African sugar industry is world renowned for its cost-competitive, quality sugars [National Agricultural Marketing Council (NAMC, 2015)]. In terms of volume, it frequently rates in the top15 and more often in the top 10 of more than 80 global sugar-producing countries, accounting for about 1% of the global production (NAMC, 2015). Brazil, China and India are the largest sugar producers (NAMC, 2015; Chauhan *et al*., 2011). According to the United States Department of Agriculture (USDA) (2015), the global sugar production in the May 2015/16 season amounted to an estimated total of 1.733×10^8 tonnes. The local sugar industry manufactures an estimated total of 2.3×10^6 tonnes of sugar per season of which most of it is sold in the Southern African Customs Union (SACU) region and the remainder is marketed in Africa, Asia and international markets [South African Sugar Association (SASA, 2015)]. According to SASA (2015), a total annual income of R12 billion is generated by the industry, contributing to the national economy through foreign exchange earnings and job creation.

The sugar industry is a multi-functional industry that integrates the agricultural activity of growing cane with the industrial activity of producing sugar (NAMC, 2015). The industry produces a variety of sugar products ranging from raw sugar, syrups, specialised sugars, refined sugar, and valuable by-products. Additionally, it is a potential producer of renewable energy, bio-plastics and biofuels (NAMC, 2015; Department of energy, 2013; Siddiqui & Waseem, 2012; Wienese & Purchase, 2004; Valdes, 2000).

The sugar content (mainly sucrose) in the sugarcane degrades within a few hours after harvesting, and sugarcane is bulky and relatively expensive to transport so it is inappropriate to export sugarcane without prior processing [Agricultural Marketing Resource Center (AgMRC, 2016); Mohammad *et al,* 2013]. It is for this reason that mills and refineries are located close to cane fields (NAMC, 2015). The process of producing white crystalline sugar involves harvesting, milling and refining (SASA, 2015). Harvesting entails cutting cane at ground level, and stripping leaves and tops off the cane stalks (Tongaat Hullet SA, 2015). In the milling process, the cane is crushed in a series of mills to extract sugar juice (Sugar Association, 2016). Raw sugar is produced by crushing sugarcane, evaporating and crystallising the sugar juice (Antonio, 2001; Chauhan *et al*., 2011). The refineries purify raw sugar from the mills and produce white sugar for domestic use and food industries (AgMRC, 2016).

2.1.1 The sugar milling process

Harvested raw sugarcane stalks or sugar beet roots from the plantation are delivered to the sugar mill in bulk. The sugarcane is processed in a series of processes to produce raw sugar (Figure 1). Sugarcane is carried into a series of horizontal rotary mills by conveyor belts and the cane is squashed in the mills, releasing sugar juice concentrated with solids and suspended particles (Chauhan *et al*., 2011). Bagasse is the lignocellulosic by-product of the mills and is washed with water (imbibition water) to recover sugar and maximise extraction of the juice (Antonio & Carlos, 2001).

Emerging from the mills, the sugar juice goes into a clarifying tank where lime ($Ca(OH)_2$) is added to precipitate suspended solids. The precipitated solids from the clarifier emerge as a thick mud that is pressure filtered to release filter juice and filter cake (filter mud). Filter juice is recycled to the clarifying tank and the filter cake is withdrawn from the process. The clarified juice is concentrated in a multi-effect evaporator in which steam heats and boils the sugar juice and water is evaporated (Chauhan *et al*., 2011) (Figure 1). The concentrated sugar juice (containing approximately 35% water and 65% solids) is crystallised by boiling in large vacuum pans with boiling seed grains added to initiate growth of sugar crystals (James & Chung, 1993). This produces sugar syrup containing a mixture of sugar crystals and sugar liquor (massecuite) (Antonio & Carlos, 2001). The sugar crystals are recovered by centrifugation and a blackish, viscous liquor (molasses) emerges as a by-product (Antonio & Carlos, 2001) (Figure 1).

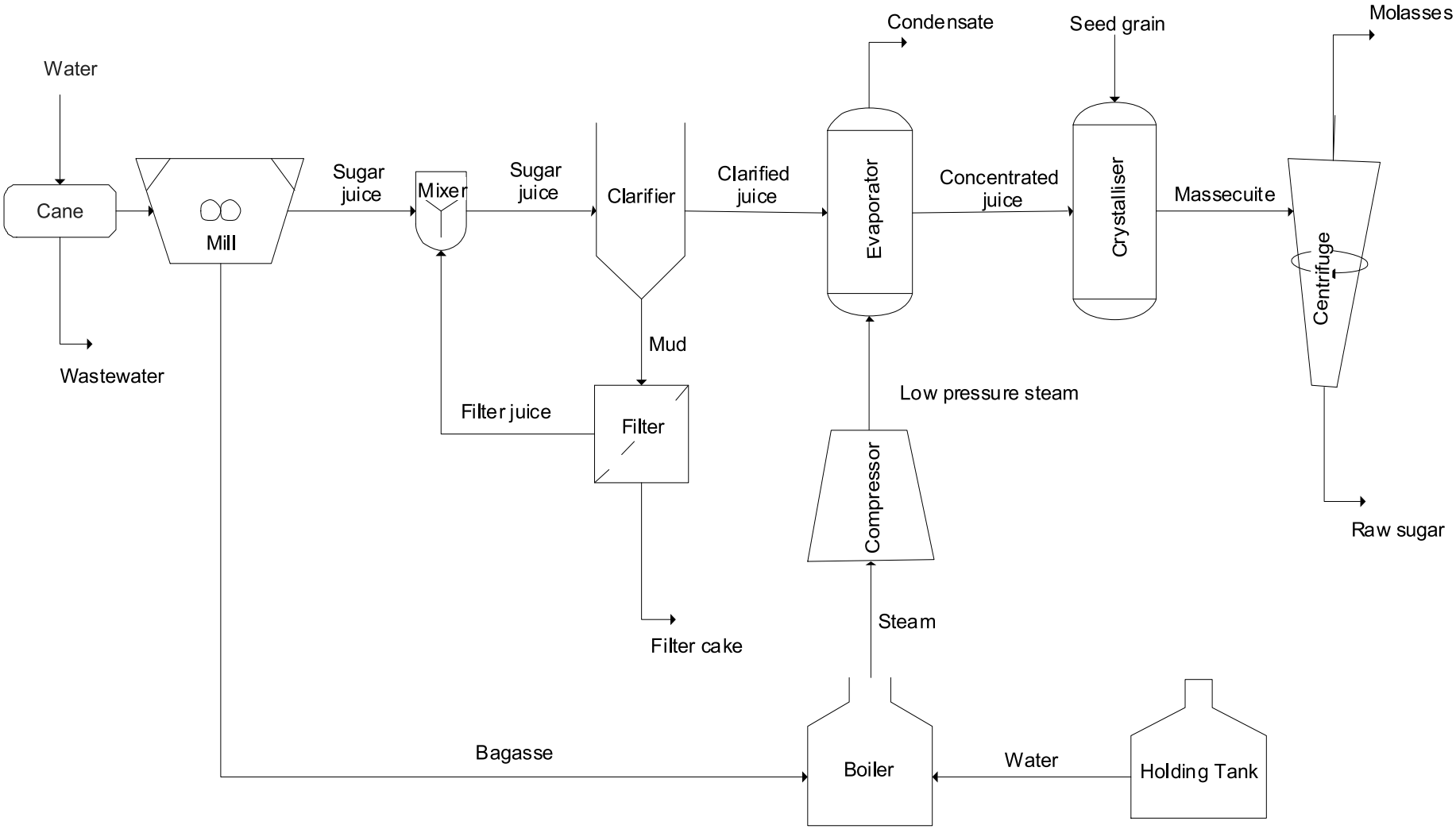


Figure 1: Flow diagram of a typical sugarcane milling process (Modified from Antonio & Carlos, 2001)

2.1.2 Sugar refining process

Raw sugar produced in the milling process has an undesirable colour and contains high ash content and non-sugar materials (Antonio & Carlos, 2001). Raw sugar is refined in a series of units (as indicated in Figure 2) to produce white sugar with high sucrose content (Antonio & Carlos, 2001).

The raw sugar crystals from the milling process are melted in high quality hot sweetwater to form a melt liquor with a sugar content ranging between 68°-72° Brix. Sweetwater is derived mainly from process water contaminated with sucrose and steam from the evaporators is used for heating the sweet water (Welz & Ndobeni, 2017).

Purification takes place by either phosphatation or cabonatation. In phosphatation, phosphoric acid (H_3PO_4) and hydrated lime ($Ca(OH)_2$) are added to the sugar liquor and heated to promote the formation of octacalcium phosphate ($Ca_8H_2(PO_4)_6$) which is a flocculent (Antonio & Carlos, 2001; Chou, 2000; James & Chung, 1993). The mixture is then sent to a clarifying tank in which non-sugar materials such as ash content and soil particles are settled along with ($Ca_8H_2(PO_4)_6$). A froth containing impurities such as starch, gum and suspended matter forms on the surface of the sugar juice and is removed by the rotating arms of the clarifier (Antonio & Carlos, 2001).

The carbonatation process is cheap and can achieve up to 30-50% colour removal and produces sugar of improved quality than that produced by phosphatation (Antonio & Carlos, 2001). The process entails bubbling carbon dioxide (CO_2) and adding $Ca(OH)_2$ in the melt liquor to form calcium carbonate ($CaCO_3$) as a flocculent which agglomerates and flocculates foreign residual matter such as pith (Antonio & Carlos, 2001).

The settled material (mud) in the clarifier is pressure filtered and produces filter mud and filter juice in which a fraction of the the latter is recycled. The clarified liquor is pumped into a fixed packed bed reactor packed with activated carbon or ion exchange resins where about 60% of the organic impurities and colloidal particles in the liquor are removed by adsorption (decolourisation) (Chou, 2000). The decolourised liquor is filtered then pumped into multi-effect evaporators where steam heats and boils the liquor, concentrating the sugar content by evaporation. The concentrated liquor from the evaporator is boiled and crystallised in large pans forming soft sugar syrup containing molasses and sugar crystals, and white sugar crystals are then recovered from the syrup by centrifugation (James & Chung, 1993).

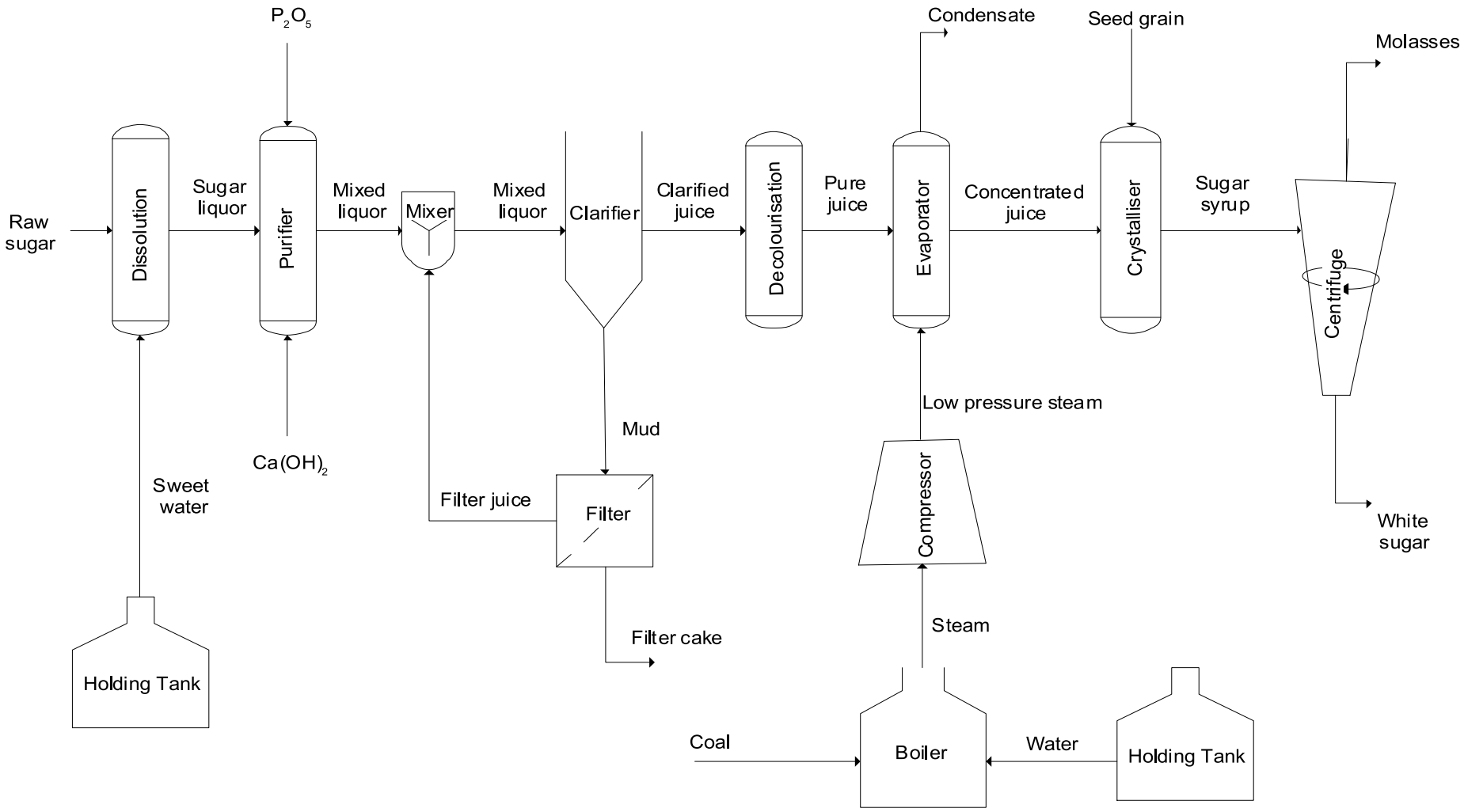


Figure 2: Flow diagram of a typical sugar refining process (Adapted from Antonio & Carlos, 2001)

2.1.3 Physicochemical characteristics of sugar industry effluent

Due to the use of re-circulated water from evaporators and vacuum pans, wastewater consists largely of surplus condensate, cooling water and boiler blow down (Podar & Sahu, 2015). Large amounts of wastewater also result from backwash water, water from decolourisation plants and general wash water (Reddy *et al*, 2014). Wastewater pollutant sources include entrained sugar and liquors such as syrup, molasses and sugar juice. Low pollution potential sources of wastewater are discharges from process units (boilers, vacuum pumps and condensers), washing and cleaning of floors and filters (Rein *et al*., 2011; Sanjay & Solomon, 2005).

Sugar industry wastewater (SIWW) is reddish in colour, and because of the presence of organics, is characterised by high COD and biological oxygen demand (BOD) values (Table 2). The wastewater from boiler blow down contains significant amounts of ash, suspended solids, and dissolved solids such as magnesium salts. These are periodically discharged with the water to prevent scale build up in the boiler (Reddy *et al.*, 2014). Hydrochloric acid is used to remove scale on the internal surfaces of heat exchangers and evaporator tubing. Acid washing contributes significant amount of chlorides to the resulting effluent (Sahu & Chaudhari, 2015).

The mill house wastewater is generated from water that is used for cleaning and cooling the mills, and is generally created from spills and sugar juice that is entrained from leakages (Kumar & Srikantaswamy, 2015). The wastewater contains organic matter such as sucrose, bagacillo, oil and grease from the bearing house of the mill (Kumar & Srikantaswamy, 2015; Kaur *et al*., 2010; Memon *et al*., 2006). The mill house wastewater is significant in volume and the BOD concentration ranges between 195 and 210 mg/L whilst the pH varies between 4.6 and 7.4.

Factories that use ion-exchange to decolourise sugar juice generate coloured wastewater. The coloured component of the wastewater is due to the presence of melanoidins which are brown polymers resulting from the Maillard amino-carbonyl reaction, and the presence of degradation products of sugars such as caramels (Zheng *et al*., 2013).

The pH of the wastewater is reduced in facilities that still use phosphoric acid and sulphur dioxide for clarifying the sugar juice (Siddiqui & Wassem, 2012; Akbar & Khwaja, 2006).

Table 2: Average wastewater characteristics from sugar factories

Wastewater source	Colour	pH	COD (mg/L)	BOD (mg/L)	BOD/COD (ratio)	TP (mg/L)	TSS (mg/L)	TDS (mg/L)	Chloride (mg/L)	Reference
Final untreated effluent	Dark yellow	5.5	3682	NG	NG	5.9	540	NG	50	Sahu *et al*., 2015
Not given	Black	4.2	3140	970	0.31	NG	NG	1480	NG	Sajani & Muthukkaruppan, 2011
Final untreated effluent	Greenish yellow	5.1	6400	2250	0.35	0.8	380	1008	NG	Shivayogimath & Rashmi, 2013
Not given	NG	6.6	290	NG	NG	3.9	ND	617	255	Muhammad & Ghulam, 2015
Final untreated effluent	Blackish grey	6.7	6820	2987	0.43	NG	447	NG	522	Anand *et al.*, 2009
Final untreated effluent	Dark yellow	5.5	3682	970	0.26	5.9	790	1480	250	Podar & Sahu, 2015
Cooling waters and washings	NG	6.2	3400	1820	0.53	NG	NG	NG	NG	Ramjeawon, 2000
Boiler blowdown	NG	10.1	300	130	0.43	NG	65	NG	NG	Ramjeawon, 2000
Final untreated effluent	Clear	8.1	7432	6856	0.92	NG	NG	2516	1894	Saranraj & Stella, 2014
Final untreated effluent	Dark brown	5	35	98	2.8	NG	110	2980	210	Siddiqui & Wassem, 2012
Settled effluent	Yellowish	9.0	2265	1635	0.72	NG	220	1268	1245	Kumar & Chopra, 2010

NG: not given; COD = chemical oxygen demand; BOD = biological oxygen demand; TP = total phosphate; TSS = total suspended solids; TDS = total dissolved solids

2.1.4 Disposal and treatment of sugar industry effluent in South Africa

A variety of treatment methods for handling SIWW exist. However the decision for selecting a treatment method depends on a number of factors including the characteristics of wastewater to be treated, land availability, economic and social constraints (capital, cost of operation and public health), climate and geography. In a recent survey, it was found that land application by irrigation, followed by discharge to river systems were the most applied methods for disposing of treated and/or untreated SIWW in South Africa (Welz & Ndobeni, 2017). In South Africa, the SIWW quality requirements for discharge are regulated by the Department of Water and Sanitation (DWS) and the integrated coastal management agency. The discharge guidelines/limits are issued as General Authorisations in terms of Section 39 of the South African National Water Act, Act No. 36 of 1998 (Government Gazette number 19182 of 2013) (Table 3 & 4). Some of the technologies commonly employed in the treatment of SIWW are discussed in Sections 2.2, 2.3 and 2.4.

2.1.5 Land application/irrigation

In many parts of the globe, various crops have been irrigated with treated and untreated effluents of sugar mills. Researchers recognise that this practice may be beneficial as it is a source of organic matter and plant nutrients, and may therefore serve as a fertiliser (Mane, 2015; Saranraj & Stella, 2014; Chaurasia & Tiwari, 2012; Usha & Vikram, 2012).

Various plants are capable of reducing the pollution load of effluents by taking up dissolved organic matter and micro and macro nutrients (Usha & Vikhim, 2012). Nutrients such as dihydrogen phosphate ($H_2PO_4^-$), ammonium nitrogen (NH_4-N) and nitrate nitrogen (NO_3-N) in mill effluents can provide essential growth nutrients. However, other effluent components such as metals may accumulate to toxic levels in plant tissues and may also pose a risk of toxicity on humans via ingestion of contaminated plants (Usha & Vikram, 2012). With respect to negative effects of metals on plants, different plants have different sensitivities to the same metal, and vice versa, and the extent of damage can therefore vary (Pramod *et al.*, 2015)

Irrigation with wastewater has been shown by some researchers to enhance yields of most crops and reduces the need for chemical fertilisers resulting in net cost savings for farmers (Kumar & Chopra, 2010). However, it has also been shown that irrigation of crops with untreated sugar industry effluents can reduce soil health, plant growth and crop yield (Pramod *et al*., 2015; Kumar & Chopra, 2010). In a study by Sajani and Muthukkaruppan (2011), the decline in paddy (*Oryza Sativa L*) seed germination rates was negatively correlated with the concentration of effluent used for irrigation. Similar results were obtained by Siddiqui and Wassem (2012) when they investigated the impact of irrigating cereal crops

with untreated sugar industry effluent on the percentage of seed germination. However, no impact of heavy metals on crop growth was determined, as the concentration was not high enough (Cu = 0.03 mg/L, Cr = 0.01 mg/L, Cd = 0.04 mg/L, Co = 0.08 mg/L, Zn = 1.40 mg/L and Ni = 0.09 mg/L). The reduction on seed germination with untreated effluent was attributed to increased osmotic pressure associated with highly concentrated sugar factory effluent (Siddiqui and Wassem, 2012; Sajani & Muthukkaruppan, 2011).

Table 3: General authorisations for irrigation of biodegradable industrial wastewater (South African National Water Act, Act No. 36 of 1998)

Irrigation volume (KL/day)	≥ 500 but ≤ 2000	≥ 50 but ≤ 500	≤ 50
pH	5.9-9.5	6-9	6-9
Chemical oxygen demand (mg/L)	≤ 75	≤ 400	≤ 5000
Electrical conductivity (mS/m)	70-150	≤ 200	≤ 200
Sodium adsorption ratio	-	≤ 5	≤ 5
Faecal coliforms (per 100 ml)	≤ 1000	≤ 1000	≤ 1000
Ammonia as N (mg/L)	≤ 3	-	-
Nitrates/nitrites as N (mg/L)	≤ 15	-	-
Free chlorine (mg/L)	≤ 0.25	-	-
Suspended solids (mg/L)	≤ 25	-	-
Ortho-phosphate (mg/L)	≤ 10	-	-
Fluoride (mg/L)	≤ 1	-	-
Fats, oil and grease (mg/L)	≤ 2.5	-	-

Table 4: General authorisations for discharge into a water resource with biodegradable industrial wastewater (South African National Water Act, Act No. 36 of 1998)

Discharge volume into a water resource (KL/day)	**≤ 2000**
pH	5.5-9.5
Chemical oxygen demand (mg/L)	≤ 75
Electrical conductivity (mS/m)	70-150
Sodium adsorption ratio	-
Faecal coliforms (per 100 ml)	≤ 1000
Ammonia as N (mg/L)	≤ 6
Nitrates/nitrites as N (mg/L)	≤ 15
Free chlorine (mg/L)	≤ 0.25
Suspended solids (mg/L)	≤ 25
Ortho-phosphate (mg/L)	≤ 10
Fluoride (mg/L)	≤ 1
Fats, oil and grease (mg/L)	≤ 2.5
Dissolved Arsenic (mg/L)	≤ 0.02
Dissolved Cadmium (mg/L)	≤ 0.005
Dissolved Chromium (VI) (mg/L)	0.05
Dissolved Copper (mg/L)	≤ 0.01
Dissolved Cyanide (mg/L)	≤ 0.02
Dissolved Iron (mg/L)	≤ 0.3
Dissolved Lead (mg/L)	≤ 0.01
Dissolved manganese (mg/L)	≤ 0.1
Mercury and its compounds (mg/L)	≤ 0.005
Dissolved Selenium (mg/L)	≤ 0.02
Dissolved Zinc	≤ 0.1
Boron (mg/L)	≤ 1

2.2 Introduction to biological wastewater treatment

Biological wastewater treatment systems depend largely on the metabolic activities of different microbial cultures/communities contained in the system to remove biodegradable organic matter from the waste stream (Viessman & Hammer, 1998). In most cases, biological treatment systems are used as integrated units of a wastewater treatment plant (Mittal, 2011). Biological treatment systems can treat municipal and industrial effluent that contains soluble biodegradable organic pollutants (Mittal, 2011; Sperling & Lemos, 2005), and operate by creating controlled environments with suitable substrates and growth nutrients for microorganisms to proliferate (Viessman & Hammer, 1998).

For a biological system to function successfully, the inorganic nutrients necessary for the growth of microorganisms must be available in adequate amounts (Ghasimi *et al*., 2009; Metcalf and Eddy, 2003). The nutritional requirements for microbial populations in biological treatment depends on the microbial species that constitute the treatment process (Lemos, 2007). Generally, nitrogen (N) and phosphorus (P) are the most important inorganic nutrients required to maintain a diverse microbial community and enhance bacterial growth in biological treatment (Annachhatre, 1996). The ratio of N and P to COD is typically used to determine whether the process will be nutrient limited or not. Examples of minimum nutrient requirements (COD:N:P ratio) reported in literature are 300:5:1, 250:5:1, and 100:2:0.5, (Ammary, 2004; Ragen, 2004; Annachhatre, 1996). If N and P are not available in ideal concentrations, the organic loading rate should be decreased, or the treatment efficiency will be unsatisfactory (Ghasimi *et al*., 2009).

2.2.1 Attached and suspended growth systems

Biological processes for the treatment of wastewater can be divided into two principal categories: suspended growth and attached growth systems (Metcalf & Eddy, 2003). Their application may vary depending on the type of wastewater and may include carbonaceous BOD removal, nitrification, denitrification and phosphorus removal (Metcalf & Eddy, 2003).

Fixed film systems (sometimes called attached growth systems or biofilms) are efficient biological treatment processes that use inert support materials such as rock, sand, stones peat or slag to provide a medium for the attachment and growth of microorganisms on the surface and/or within porous structures (USEPA, 2016; Zheng *et al*., 2013; Sasse, 1998). Extra-cellular polymeric substances produced by microorganisms and abiotic particles captured from the liquid medium form a hydrated biological structure on the filter media. This slime layer, known as biofilm, sloughs off from the surface of the filter media from time to time, and leaves with the treated wastewater [Tilley *et al*., 2014; United Nations Environmental Programme (UNEP), 2004; Mara, 2003; Gavrilescu & Macoveanu, 2000].

There are numerous attached growth processes, with trickling filters and rotating biological contactors being the most widely applied [National Environmental Services Center (NESC) 2016; Cheremisinoff, 2002]. These are discussed by way of examples of attached growth systems in Sections 2.2.2.1 and 2.2.2.2.

In contrast to attached growth systems, in suspended growth systems, the microbial populations remain suspended within the wastewater and need to be physically removed by settling and/or filtration prior to final effluent discharge (Metcalf & Eddy, 2003). The activated sludge process is the most commonly employed suspended growth system, and is described by way of an example of a suspended growth system in Section 2.2.2.3.

2.2.2.1 Trickling filters

Trickling filters have been successfully used to remove organics, and nitrify ammonium compounds in municipal and aquaculture wastewaters (Godoy-Olmos *et al*., 2016; Zhang *et al*., 2016; Tekerlekopoulou & Vayenas, 2007; Mara, 2003, Metcalf & Eddy, 2003; Gouzinis *et al*., 1998; Michalakos *et al*., 1997). Biological filters have also been used successfully to reduce BOD and COD content of sugar factory wastewater (Bruijn, 1975). Trickling filters can maintain high biomass concentrations and the biofilm structure provides resistance to organic shock loading and can tolerate high hydraulic loadings (Vayenas, 2015). The conventional design is the non-submerged trickling filter. The wastewater is spread over the surface of the media (for example a stationary bed of wooden chips or rocks) in the form of drops via spray nozzles and trickles down through the media in the direction of the under-drain where it collects and discharges (Figure 3) (Sperling, 2007; Weiner & Matthews, 2003; Cheremisinoff, 2002). The wastewater percolates through the biofilm, promoting contact between the microorganism and organic matter (Sperling & Lemos, 2005). Air supplied through vent pipes circulates in the empty spaces of the packing material and provides oxygen for the respiration of microorganisms. Trickling filters are simple, reliable bioprocesses and are compactable in areas in which land is limited and are less energy intensive than the conventional activated sludge process (Australian Meat Processor Cooperation Ltd, 2015; USEPA, 2000)

Disadvantages attributed to fixed film treatment processes include clogging of pore material which is caused by biomass growth, influent suspended solids and biosolids (NESC, 2016; Tilley *et al*., 2014; Ersahin *et al*., 2011)

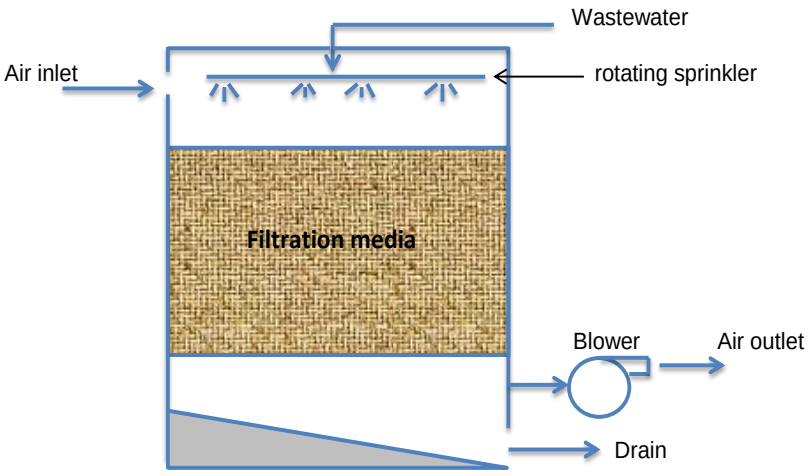


Figure 3: Diagrammatic representation of a non-submerged trickling filter

2.2.2.2 Rotating biological contactors

The rotating biological contactor (RBC) is another type of attached growth system (Spellman, 2014). Basic designs of an RBC consist of a series of closely packed circular disks attached to a rotating shaft in which each disk is partially submersed in a tank that contains the wastewater being treated (Figure 4) (Buyukgungor & Gurel, 2009; Cheremisinoff, 1996). As the disks rotate, the biofilm/support media is cycled between being submerged, where it comes into contact with the wastewater and emerged where it is exposed to atmospheric oxygen. When emerged, oxygen is used for aerobic organic decomposition and nitrification, while denitrification takes place during the submerged phase (Spellman, 2014; EPA, 1997). The RBC has been widely applied for secondary treatment of domestic and industrial wastewater for BOD removal, nitrification and denitrification (Ghawi & Kris, 2009; Metcalf & Eddy, 2003; Woordard, 2001; Liu & liptak, 1997). It has also been used for COD, phenol removal and decolourisation of sugar refinery wastewater (Guimaraes *et al*., 2005). The RBC system offers an outstanding option for wastewater treatment owing to the fact that it has low maintenance and power consumption, high activated sludge concentration, high specific area and process stability (Ghawi & Kris, 2009; Tawfik *et al*, 2006). Problems associated with RBC systems include physical failure of the shaft, and odour nuisance (Tawfik *et al*, 2006).

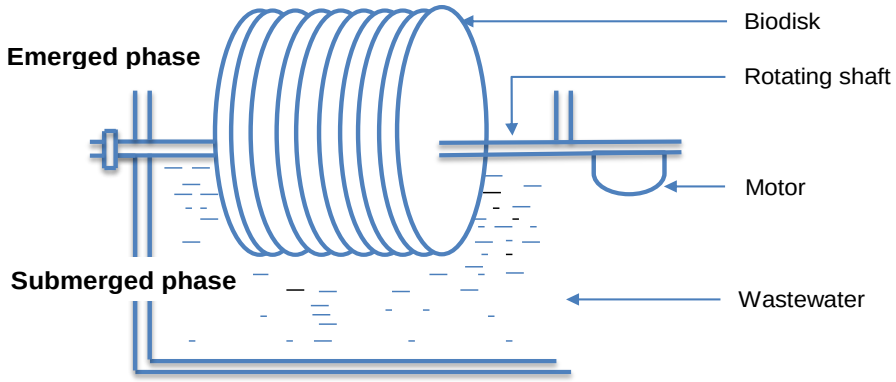


Figure 4: Diagrammatic representation of a rotating biological contactor (RBC)

2.2.2.3 The activated sludge process

The activated sludge process is characterised by the growth of microorganisms that are kept in suspension by mixing in the reactor, typically using mechanical agitation or pneumatic aeration (Dabi, 2015; Hung *et al*., 2012; Kuhn *et al*., 2010). Mechanical agitation (surface aeration) introduces oxygen by spraying the mixed liquor into the air using a forceful fountain-like effect. Pneumatic (fine bubble) aeration relies on diffusers to introduce bubbles into the bottom of the mixed liquor, which then rise to the surface (Grady *et al*., 2011; EPA, 1997). In the aeration (oxidation) vessel, aerobic and facultative microorganisms oxidise soluble organic matter and colloidal organics into new cells, carbon dioxide (and other gases), and water (Kolmetz, 2016). The organics and nutrients are therefore either mineralised, or incorporated in the biomass (EPA, 1997). The microorganisms agglomerate and form bioflocs which are joined together by proteins and polysaccharides (Kuhn *et al*., 2010). The flocs are then allowed to settle out in a clarifier, leaving a clear supernatant, which is the treated effluent.

To maintain the desired concentration of biomass in the aeration vessel, the wastewater solids and flocs (sludge) from the clarifier are recycled (also known as returned activated sludge) to the aeration vessel (Kuhn *et al*., 2010). The excess sludge is usually dewatered and may undergo further treatment before disposal to landfill (Haandel & Lubbe, 2007; EPA, 1997). In some instances, treated sludge is used as an organic fertiliser (Ahansazan *et al*., 2014; Arceivala & Asolekar, 2008; Haandel & Lubbe, 2007; EPA, 1997). The clarified water is typically disinfected prior to discharge or re-use.

The aerobic activated sludge system offers exceptional performance in removing organics, and depending on the configuration process, can also remove N and/or P from domestic wastewater (Chen *et al*., 2016; Davies, 2005). Other organic-rich effluents such as mixed municipal-textile wastewater have also been treated successfully (Lotito *et al*., 2014). Laboratory scale investigations on SIWW have been promising. For example, Li (1992) found that when SIWW (BOD = 770 mg/L) was treated in a 5.2 L aerobic activated sludge system, the BOD reductions at an HRT of 2.47 d and 0.41 d were 96.2 and 85.6 %, respectively.

Disadvantages attributed to activated sludge systems include copious sludge production that needs to be disposed of or treated, intensive energy requirements for aeration and pumps, high maintenance cost, and sizeable areas of land for installation of large process equipment, (Sahu *et al*., 2015; Sahu & Chaudhari, 2014; Shivayogimath & Rashmi, 2013; Steffen *et al.*, 1990). A schematic representation of the conventional activated sludge process is illustrated in Figure 5.

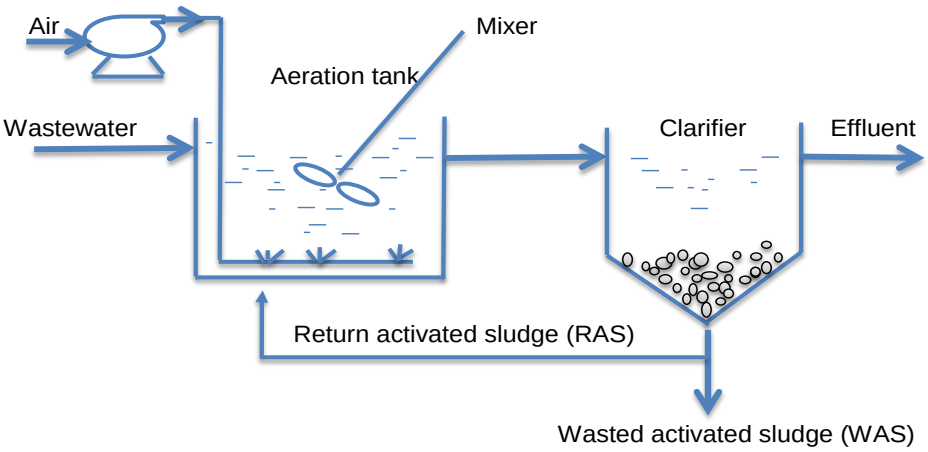


Figure 5: Schematic representation of a simple activated sludge process

2.3 Anaerobic digestion

Both solid and liquid waste can be 'digested', and most anaerobic digesters used for wastewater treatment are suspended growth systems (Section 2.2.1).

The first recognition of anaerobic digestion dates back to Italy in 1776 when Volta showed that anaerobic digestion processes present an effective way of converting organic waste material into methane (Verma, 2002). He showed the formation of combustible air-methane from streams, ponds and sediments in lakes (Fang, 2010; Verma, 2002). The first full scale installation of an anaerobic digester was the Mouras`s Automated Scavenger for the treatment of domestic wastewater in 1860 (Khanal, 2008). Then, in 1895, the first septic tank was built with the provision for retaining solids in sewage and recovery of biogas for heating

and lighting (Khanal, 2008). In 1904, a new two-stage anaerobic process, known as the Travis tank, was put into operation (Khanal, 2008). This was later in 1905 modified by Karl Imhoff to a continuous flow anaerobic reactor which enhanced settling and digestion of settled solids (Lier *et al*., 2016). The Imhoff tank was used for municipal wastewaters and is still functional in some parts of the world, especially in regions with warm climates (Lier *et al*., 2015).

A variety of industries, including the distillery and fermentation industry, pulp and paper industry, food industry, brewery and beverage industry, chemical and petrochemical industries are typically characterised by high organic matter in their wastewaters with COD values > 2000 mg/L (Mutombo, 2004). While aerobic treatment is adopted for treating low strength wastewaters (COD < 1000 mg COD/L), anaerobic treatment is preferable for wastewaters with a soluble organic fraction such as those found in the food industry, including SIWW. The biodegradable organic content in food industry substrates such as SIWW makes anaerobic treatment attractive in comparison to aerobic treatment (Rais & Sheoran, 2015; Lemos, 2007). Anaerobic digestion requires less energy, produces less sludge, and generates biogas that can be converted into energy (Tafdrup, 1995; Lettinga *et al*., 1984; McCarthy, 1964). In addition to waste removal, other environmental benefits include pathogen control, odour control, and reduction of greenhouse gas emissions (Cassie *et al*., 2010; Wilkie, 2005).

2.3.1 The anaerobic digestion process: biodegradation of organic compounds

Anaerobic digestion is a microbial process in which micro-organisms degrade organic matter in the absence of free molecular oxygen and produce new cell mass and biogas (methane, hydrogen, carbon dioxide, trace hydrogen sulphide) (Alkaya & Demirer, 2011; Metcalf & Eddy, 2003). Studies have shown that anaerobic digesters such as UASB reactors can achieve high COD reduction and can handle high strength industrial wastewaters (Mrowiec & Suschka, nd; Mehrdad *et al*., 2007). Anaerobic digestion takes place in four stages, namely, hydrolysis, acidogenesis, acetogenesis, and methanogenesis (Adekunle & Okolie, 2015; Yasar & Tabinda, 2010; Appels *et al*., 2008; Lemos, 2007) (Figure 6).

2.3.1.1 Hydrolysis: Step 1 of anaerobic digestion is the hydrolysis of polymeric particles: this is a surface-based process in which polymeric chemical compounds are degraded to monomeric chemical compounds [Lier *et al*., 2008; United States Environmental Protection Agency (USEPA), 2006]. During hydrolysis, proteins are hydrolysed to amino acids, polysaccharides (complex carbohydrates) to simple sugars, and lipids (fats and triglycerides) to long chain fatty acids (LCFAs) and glycerol (Yasar & Tabinda, 2010; Lier *et al*., 2008; Lemos, 2007).

Since complex organic molecules are too large to pass through the cell wall and membrane of microorganisms, some microorganisms secrete extracellular enzymes into the environment (Schnurer & Jarvis, 2009). These enzymes break down larger chemical precursors into smaller molecules that can diffuse through the cell wall and membrane of microorganisms and be used as a source of energy for growth and metabolic function (Schnurer & Jarvis, 2009). Enzymes are highly substrate specific and some microbial species secrete specialised enzymes (Schnurer & Jarvis, 2009). Table 5 lists some groups of hydrolytic enzymes and their functions.

Table 5: Some groups of hydrolytic enzymes and their functions (Adapted from Schnurer & Javis, 2009; Gerardi, 2013)

Enzyme	Organic substrate	Hydrolysis product/s
Lipase	Fats	Fatty acids and glycerol
Proteinase	Proteins	Amino acids
Amylase	Starch	Glucose
Cellulase	Cellulose	Cellubiose and glucose
Pectinase	Pectin	Sugars (galactose, arabinose, and polygalactic uronic acid)
Hemicellulase	Hemicellulose	Sugars (glucose, xylose, mannose and arabinose)

2.3.1.2 Acidogenesis: in the acidogenesis phase, the hydrolysed products (amino acids, simple sugars and LCFAs) diffuse through the cell wall and membrane of fermentative bacteria and are subsequently converted (fermented) to small organic compounds such as acetate (CH_3OO^-), carbonic acid (H_2CO_3), volatile fatty acids (VFAs), hydrogen gas (H_2), ammonia (NH_3), alcohols and carbon dioxide (CO_2) (Yasar & Tabinda, 2010; Appels *et al*., 2008; Lier *et al*., 2008). LCFAs are converted to H_2 and CO_2 by anaerobic oxidation, and propionate ($C_3H_6O_2$) and butyrate ($C_4H_8O_2$) are produced as intermediary products (Lier *et al*., 2008). This process takes place by anaerobic fermentation in which micro-organisms use electron acceptors from organic material and inorganic oxides contained in the wastewater (Lier *et al*., 2008). Exclusion of oxygen gas (O_2) in the process is achieved by containment. Equations 1-3 illustrate the conversion of sucrose to acetate, propionate and butyrate respectively (Lier *et al*., 2008).

$$C_{12}H_{22}O_{11} + 9H_2O \rightarrow 4CH_3COO^- + 4HCO_3^- + 8H^+ + 2H_2 \quad \text{Equation 1}$$

$$C_{12}H_{22}O_{11} + 5H_2O \rightarrow 2CH_3CH_2CH_2COO^- + 4HCO_3^- + 6H^+ + 4H_2 \quad \text{Equation 2}$$

$$C_{12}H_{22}O_{11} + 3H_2O \rightarrow 2CH_3COO^- + 2CH_3CH_2COO^- + 2HCO_3^- + 6H^+ + 2H_2 \quad \text{Equation 3}$$

2.3.1.3 Acetogenesis: The third stage of anaerobic digestion is known as acetogenesis. Compounds generated by acidogenesis, such as ethanol, and intermediate compounds ($C_3H_6O_2$) and ($C_4H_8O_2$) are further converted to CH_3COO^- and new cell material. Homoacetogenic bacteria produce enzymes that catalyse the conversion of H_2 and CO_2 to CH_3COO^-, as well as reverse reaction (Lier *et al*., 2008). Equations 4-5 demonstrate the conversion of propionate and butyrate to acetate (Ralph & Dong, 2010; Wei, 2007).

$$CH_3CH_2COO^- + 3H_2O \rightarrow CH_3COO^- + HCO_3^- + H^+ + 3H_2 \quad \text{Equation 4}$$

$$CH_3CH_2CH_2COO^- + 2H_2O \rightarrow 2CH_3COO^- + H^+ + 2H_2 \quad \text{Equation 5}$$

2.3.1.4 Methanogenesis: methanogenesis is the ultimate phase of anaerobic digestion, where methanogenic bacteria convert organic products from preceding stages to biogas [methane (CH_4), CO_2, H_2, trace hydrogen sulphide (H_2S)] (Yasar & Tabinda, 2010; Lier *et al*., 2008; USEPA, 2006). Acetoclastic and hydrogenotrophic methanogens are methanogenic bacteria that utilise specifically CH_3OO^- and H_2 as substrates from the acetogenesis stage to produce CH_4 (reaction 7-8) (Lier *et al*., 2008). These bacteria reduce CO_2 by using H_2 as an electron donor and decarboxylate CH_3OO^- to form CH_4 (Fig 4) (Yasar & Tabinda, 2010; Appels *et al*., 2008; Lier *et al*., 2008;). Methanogenesis is the rate limiting step in which the COD in wastewater is converted to a gaseous phase (CH_4) that eventually leaves the reactor (Lier *et al*., 2008). The energy initially contained in the biomass material is converted and produced as biogas, which can be used as an energy source. The acetoclastic and hydrogenotrophic methanogenesis reactions occur according to Equations 6-7 (Abdelgadir *et al*., 2014; Ralph & Dong, 2010; Chen, 1995).

Acetoclastic methanogenesis (acetate reduction)

$$CH_3COOH \rightarrow CH_4 + CO_2 \quad \text{Equation 6}$$

Hydrogenotrophic methanogenesis (carbon dioxide reduction)

$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O \quad \text{Equation 7}$$

In addition to biomethanation, there is a syntrophic relationship between microorganisms such as sulphate-reducing bacteria (SRB) or nitrate-reducing bacteria which consume hydrogen gas through a process known as inter-species hydrogen transfer (IHT) (Schnurer & Jarvis, 2010). Although digesters produce hydrogen in significant amounts, the effectivity of interspecies hydrogen transfer is such that the hydrogen that escapes to the environment is reduced to insignificant levels (Belaich *et al*., 1990).

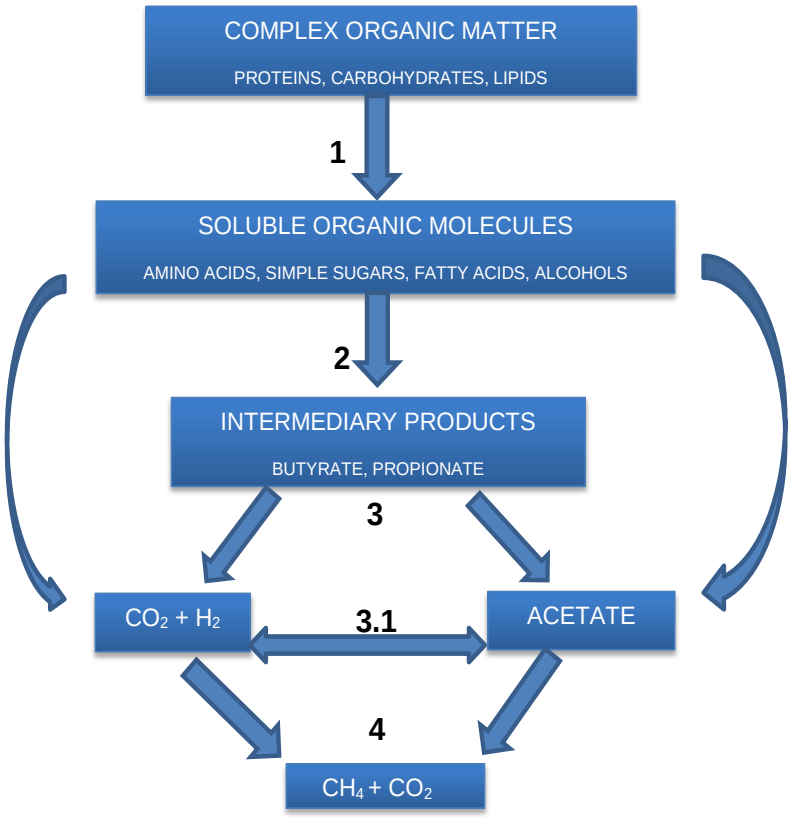


Figure 6: Schematic representation of the anaerobic digestion process (Modified from Yasar & Tabinda, 2010)

1 = hydrolysis; 2 = acidogenesis; 3 = acetogenesis; 3.1 = homoacetogenesis; 4 = methanogenesis

2.3.2 The effect of microbial substrate competition by sulphate reducing and nitrifying bacteria on methanogenesis

Certain microbial taxa proliferate in similar environments (e.g. temperature, presence/absence of oxygen and other electron donors, and pH), and compete with one another for growth factors.

Anaerobic digesters comprise of microbial communities in which various bacteria, including SRB and denitrifying bacteria, compete with methanogens for methanogenic substrates (Lier *et al*., 2008; Mizuno *et al*., 1998). Most SRB and denitrifying bacteria are heterotrophic bacteria that utilise sulphate (SO_4^{2-}) and nitrates/nitrites, respectively, as electron acceptors during the oxidation of organic compounds (Lier *et al*., 2008). Heterotrophic bacteria are fast-growing bacteria, also known as chemoorganotrophs, which rely on electrons obtained from the breakdown of organic molecules for energy.

SRB can utilise methanogenic organic substrates such as CH_3OOH and CO_2, and non-heterotrophic SRB can also utilise H_2 as electron donors for sulphate reduction (Figure 7)

(Lier et al., 2008; Mizuno *et al*., 1998; Dar *et al*., 2008; Stams *et al*., 2005). However, the utilisation of the fermentative products by SRB depends on the availability of sulphate as an electron acceptor (Lier *et al*., 2008; Dar *et al*., 2008).

The outcome of this competition for common substrates is determined by conversion kinetics (k_s and μ_{max}) of SRB, methanogens and acetogens (Lier *et al*., 2008; Stams *et al*., 2005). The COD/sulphate ratio is an important factor that is widely used to control biological sulphate removal in the treatment of sulphate-rich streams (Jeong *et al*., 2008). According to Lier *et al*. (2008), Jeong *et al*. (2008), and Vela *et al*. (2002), only wastewaters with a COD/sulphate ratio that is equivalent or greater than 0.67 contain sufficient sulphate for complete oxidation of the organic matter via sulphate reduction.

The stoichiometric reaction for the conversion of hydrogen and acetate by SRB occurs according to the following equations:

$$H_2 + 1/4\, SO_4^{2-} + 1/4\, H^+ \rightarrow 1/4 HS^- + H_2O \qquad \text{Equation 8}$$

$$CH_3OO^- + SO_4^{2-} \rightarrow HS^- + 2HCO_3^- \qquad \text{Equation 9}$$

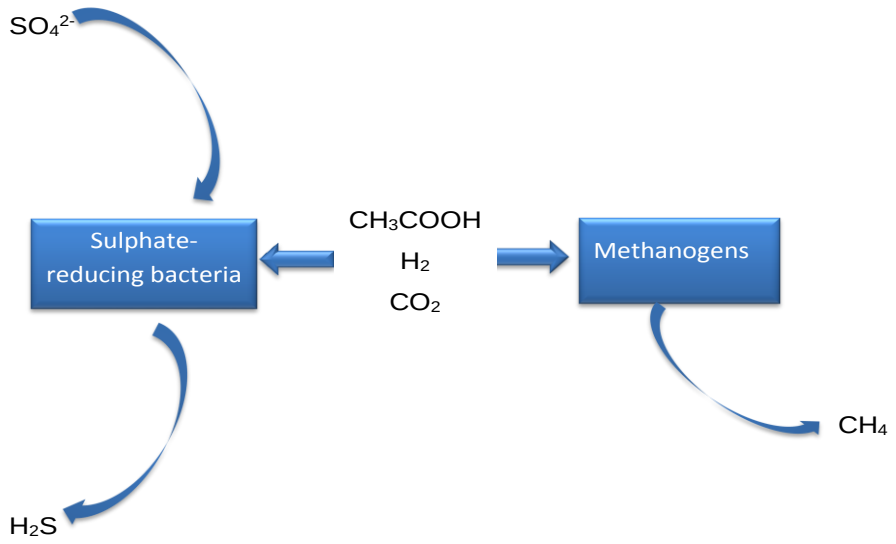


Figure 7: Schematic diagram representing the interspecies treanfer of hydrogen gas (Modified from Shah *et al*., 2014)

Denitrification is mediated by facultative heterotrophs or strictly anaerobic bacteria that convert nitrate and nitrite to molecular nitrogen (N_2) (Sousa *et al*., 2008; Akuna *et al*., 1992). Using glucose as an electron donor, the stoichiometric reactions for the conversion of nitrate to nitrite, nitrite to N_2, and nitrate directly to N_2 occur according to equations 10 to 12:

$$NO_3^- + 1/2\ C_6H_{12}O_6 \rightarrow +NO_2^- + 1/2\ CO_2 + 1/2\ H_2O$$ Equation 10

$$NO_{32}^- + 1/8 C_6H_{12}O_6 + H_2O \rightarrow 1/2\ N_2 + 3/4\ CO_2 + 5/4\ H_2O + OH^-$$ Equation 11

$$NO_3^- + 5/24 C_6H_{12}O_6 + H_2O \rightarrow 1/2\ N_2 + 5/4\ CO_2 + 7/4\ H_2O + OH^-$$ Equation 12

Denitrification can be expected to take place when the influent contains significant amounts of nitrate and nitrite (Lier *et al*., 2008). Not only do denitrifiers compete with methanogens for substrate, but N-oxides can inhibit the growth and activity of methanogens (Tugtas *et al*., 2009)

2.3.2 High rate anaerobic reactors

In 1955, high rate anaerobic reactors such as the anaerobic contact process were developed to handle soluble organics and dilute wastewaters (Khanal, 2008). High rate anaerobic reactors are characterised by three fundamental concepts: (i) enhanced contact between biomass and substrate which minimises problems of diffusion of substrates and products from the bulk liquid to biomass, (ii) accumulation of active biomass by means of settling or attachment on a medium, and (iii) ability to retain high concentrations of viable biomass (biofilms or granular aggregates) that allow enhanced activity rates and settling velocities, thereby reducing reactor volume requirements (Hassan *et al*., 2013; Bodik *et al*., 2000; Lier *et al*., 1997; Rebac *et al*., 1995). Widely used high rate anaerobic technologies include anaerobic sequencing batch reactors (ASBR), anaerobic filters (AF), expanded granular sludge bed reactors (EGSB), and UASB reactors (Lemos, 2007). The ASBR, AF and EGSB are described briefly in Sections 2.3.2.1 to 2.3.2.3. A separate Section has been dedicated to a detailed description of the reactor used in this study, the UASB (Section 2.4).

2.3.2.1 Anaerobic sequencing batch reactors

The anaerobic sequencing batch reactor (ASBR) is a single batch anaerobic digestion system developed by Dargue and co-authors at Iowa state University (Ames, Iowa, USA) (Sung & Dung, 1992; Sung & Dague, 1995). A scheme of an ASBR is shown in Figure 8. The sequencing batch reactor process operates through four main cyclic phases; fill, react, settle and decant (Steele, 2013; Rodrigues *et al*., 2004). With this kind of operation the ASBR decouples the solids retention time (SRT) from the hydraulic retention time (HRT) so that the solids have a prolonged residence time, while the biodegradable liquids spend a short time in the vessel (Ndegwa *et al*, 2005). The benefit of this is that the ASBR can handle more volume of substrate per unit time and therefore the required volume of the digester can be reduced (EPA, 1999; Ndegwa *et al*, 2005).

During the fill (feed) stage, the substrate concentration increases in the vessel and the food to microorganism (F/M) ratio and metabolic activity of anaerobic microorganisms increase to their highest value (Timur & Ozturk, 1999; Sung & Dung, 1992). In the react stage the organic matter in the substrate undergoes biodegradation. The time required for the reaction step may be driven by a number of parameters such as quality and strength of the substrate, reactor temperature, required effluent quality and biomass concentration (Sung & Dague, 1995). As a result of high substrate concentration (high F/M ratio) in the beginning of the react stage, the rate of substrate uptake is high, and continuous agitation ensures good contact of substrate and microorganisms. This provides the driving force for high rate conversion of organics to biogas (Wei, 2007). At the end of the react phase the substrate concentration is reduced, evidenced by a low F/M ratio (Ndegwa *et al*, 2005).

The low F/M ratio enhances the settling properties of the biomass in the settling phase, and the heavy flocs settle and entrain low weight flocs (Dague *et al*., 1992; Sung & Dung, 1992). The decant stage involves the removal of the treated effluent from the sludge blanket and is performed slowly to avoid agitation and disturbance of settled solids (Sung & Dung, 1992). The cycle is repeated in regulated process conditions.

The major benefits of the ASBR process is that biological treatment and solids capture is achieved in one reactor vessel, and the need for secondary clarification is eliminated. This results in capital savings (Al-Rekabi *et al*., 2007).

The technical applicability of this technology has been widely assessed on various types of effluents such as dairy wastewater (de Souza Santana *et al*., 2016) domestic sewage (Sarti *et al*., 2007), landfill leachate (Contrera *et al.*, 2014), and low strength wastewater (Rodrigues *et al*., 2004). Table 6 gives examples of results from some studies performed using anaerobic digesters, including ASBRs.

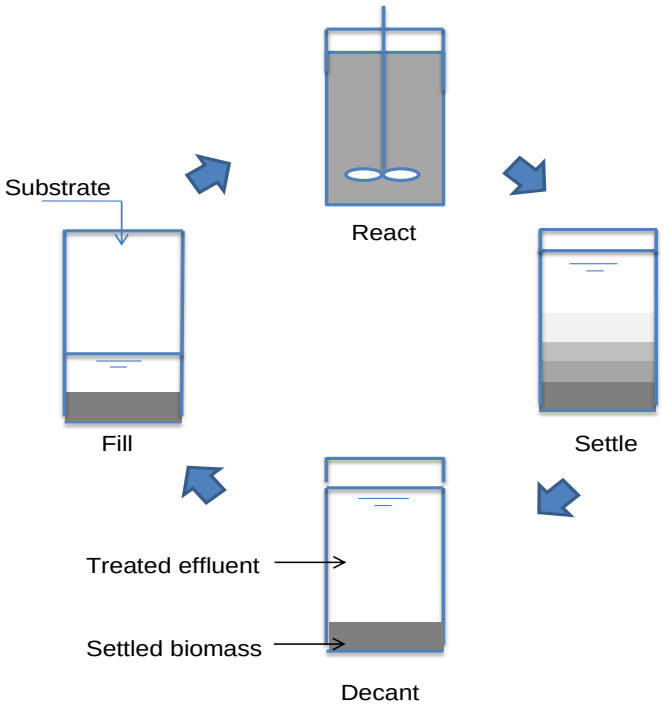


Figure 8: Diagrammatic representation of an anaerobic sequencing batch reactor

2.3.2.2 Anaerobic filter

The anaerobic filter (AF) is an attached growth system (Figure 9). The conventional AF is filled with a natural or synthetic packing media (rock or plastic) which provides a surface for microbial attachment and is similar to the aerobic trickling filter (Bodik *et al*., 2000). However, the wastewater is distributed in the bottom of the filter and flows in an upward direction through the filter medium so that the entire surface of the bed is completely submerged (Rose, 1999). Anaerobic microorganisms attach on the surface of the filter and occupy the void space of the filter. The increased surface area of the filter ensures good contact between the substrate and microorganisms and ensures successful degradation of organic matter (Gutterer *et al*., 2009; Young & McCarty, 1969).

The AF is widely used for the treatment of wastewaters with a low concentration of suspended solids and a narrow range of COD/BOD ratio, for example as a secondary unit receiving effluent from a septic tank (Gutterer *et al*., 2009). It has also been used in the treatment of SIWW (Mehrdad *et al*., 2007).The AF is ideal for treating organic wastewater because, (i) a high solid matter concentration is maintained in the filter and this allows the process to resist environmental changes (pH and temperature) and adapt easily to new operating conditions such as increased organic loading, (ii) very low volume of sludge is produced and the effluent is free of suspended solids, and (iii) no heating is required to achieve high treatment efficiencies (Kara, 2007; Harris, 1992; Dahab, 1982). On the other hand, a high concentration of suspended solids retained in the filter medium can cause long

term problems such as clogging, which may lead to short circuiting of the wastewater (Bodkhe, 2008; Henze, 2008)

In addition to widespread application in the treatment of agro-food processing industry wastewater, AFs have also been used extensively in the treatment of low strength domestic and municipal sewage wastewater (Ladu & Lu, 2014; Fia *et al*., 2012; Bodkhe, 2008; Manariotis & Grigoropoulos, 2006; Bodik *et al*., 2000; Kobayashi *et al*., 1983).

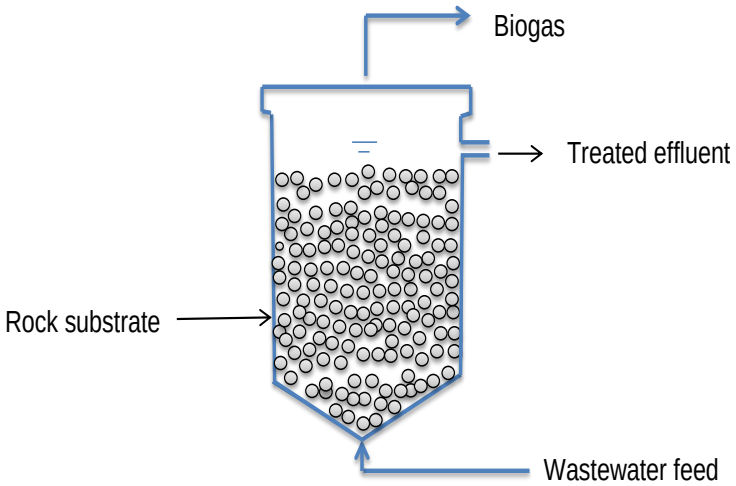


Figure 9 Schematic diagram of an upflow anaerobic filter (Adapted from Tonon *et al*., 2016)

2.3.2.3 Expanded granular sludge bed reactor

The expanded granular sludge bed (EGSB) reactor is a further modification of the UASB technology, described in detail in Section 2.4. Unlike the conventional UASB reactors with hydraulic velocities ranging between 0.5-2.5 m/h, the EGSB reactor operates at increased superficial hydraulic velocities (>4 m/h) and the height/diameter (H/D) ratio is increased (Liu *et al*., 2006; Jeison & Chamy, 1999). This significantly increases contact between the sludge and substrate (Liu *et al*., 2006; Jeison & Chamy, 1999).

Similar to the conventional UASB reactor, the EGSB is based on self-immobilisation of microorganisms and development of granular biomass (Liu *et al*., 2006). According to Yoochatchaval *et al*. (2008), the UASB process is usually applicable to high strength wastewaters at mesophilic (30-35° C) and thermophilic (55-65° C) temperatures and offers excellent process performance due to retainment of methanogenic biomass and prolonged SRT.

Many organic wastewaters (domestic and a variety of industrial) are discharged at low ambient temperatures (10-25^{o}C) with low COD concentrations of < 600 mg/L (Yoochatchaval

et al., 2008; Lettinga *et al*., 2001). It has been established that the application of the UASB process for such wastewaters is limited because the deficiency of substrate causes deterioration and disintegration of the physical characteristics of granules. This has been attributed to low gas production rate and ineffective mixing inside the UASB reactor which yields dead-zones in the sludge bed (Zheng *et al*., 2014; Yoochatchaval *et al*., 2008; Lettinga *et al*., 2001). The EGSB reactor was developed to improve substrate to biomass contact by expanding the sludge bed and intensifying hydraulic mixing to allow treatment of low strength wastewater (Zheng *et al*., 2014; Angenent *et al*., 2001). According to Zheng *et al*. (2014), Musee (2013) and Jeison and Chamy (1999), the system`s efficiency is significantly increased at superficial hydraulic velocity (7-10 m/h) which can be achieved by applying increased recirculation rates. This also facilitates hydraulic mixing which enhances mass transfer between substrate and biomass. The EGSB reactor is illustrated in Figure 10.

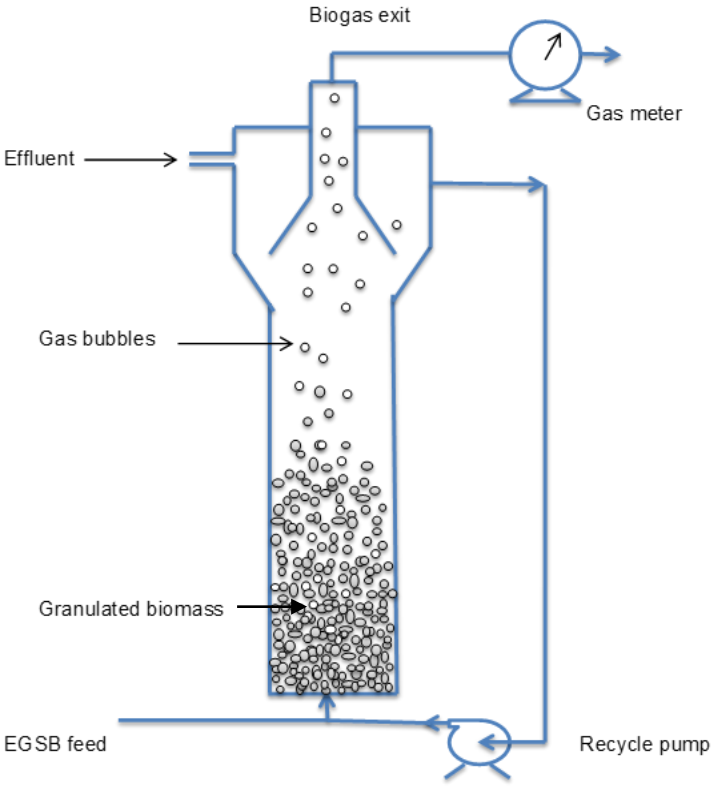


Figure 10: Diagramatic representation of an expanded granular sludge bed reactor (Adapted from Wang *et al*., 2009)

Table 6: Performance and methane yield of some high rate anaerobic reactors treating various wastewaters

Wastewater	COD_{IN} (mg/L)	Type of reactor	Volume (L)	Temperature (ºC)	HRT (days)	OLR ($kgCOD/m^3.d$)	% COD removal	MPR ($L.CH_4/g.COD_{re}$)	Methane content (%)	Reference
Brewery	2440	UBF	NM	20-29	0.17	14.64	82	0.29-0.33	71	Yu & Gu, 1996
Brewery	44400	AF	3.6	35	10	4.44	71	0.379	54.7	Lo & Liao, 1989
Brewery	5000	ASBR	45	33	2	1.5-5.0	90	0.48	68	Shao *et al*., 2008
Yeast	14400-25700	ASBR	0.7	35 ± 2	2.5-10	1.4-9.16	84	0.187	NM	Krapivina *et al*., 2007
Cheese whey	10 000000	UAPB	30	ND	5	83	94-96	NM	50-52	Patil *et al*., 2012
Slaughterhouse	3740-10410	UASB-AF	4.2	35	0.5-1.5	2.49-20.82	90.2-93.4	0.345	56-74	Borja *et al*., 1998
Slaughterhouse	4306	CSTR	2	30	27	0.16	75	0.45	NM	Padilla-Gasca *et al*.,2011
Poultry Slaughterhouse	1544-7700	UAF	5.4	29-35	0.5	10.05	78	0.24	46-56	Rajakumar *et al*., 2011
Baker`s yeast	11000-88000	HYB-AF	10	35	3	1.8-10	65-67	0.207-0.208	38-62	Van Der Merwe & Britz, 1993
Palm oil mill	24000-55000	AHR	5.8	55	10-20	1.2-5.5	80-90	0.32	65-73	Wanitanukul *et al*., 2013
Palm oil mill	4000-34725	UASFF	4.4	38	1.5-3	2.63-23.15	89-97	0.346	62-82	Najafpour *et al*., 2006
Palm oil mill	19700	AHR-ABF-ADF	40	36	0.7-2.4	0.91-23	93.5	0.171-0.269	59.5-78.2	Choi *et al*., 2013
Cold meat	3500	UAF	5	37	20	1.17-3.5	84-88	0.422	NM	Leon-Becerril *et al*., 2016
Rice winery	13000-16000	UAF	1.4	35	2.2-10	0.6-8.5	50-91	0.31-0.33	NM	Jo *et al*., 2015
Glucose 2,4 dichlorophenol	8000-12000	EGSB	5.4	30 ± 1	2	4-6	80	0.088	NM	Puyol *et al*., 2009

Table 6 continued

Wastewater	COD_{IN} (mg/L)	Type of reactor	Volume (L)	Temperature (°C)	HRT (days)	OLR ($kgCOD/m^3.d$)	% COD removal	MPR ($L.CH_4/g.COD_{re}$)	Methane content (%)	Reference
Palm oil mill	4331-35000	EGSB	20.5	35	2	1.45-17.5	91	NM	70	Yejian *et al*., 2008
Laundry	800-2665	EGSB	1.4	30	1.58	NM	90-92	NM	NM	Delforno *et al*., 2014
Winery	CODs/COD = 0.66	H-EGSB	135	37	1.08	2	96	0.31	NM	Petropoulos *et al*., 2016
Beet sugar molasses	2000- 8000	UAFB	180	32-34	0.83	2.4-9.6	75-93	NM	NM	Mehrdad *et al*., 2007
Olive mill & pig	20000-60000	UAF	2.5	35	6-7	3-10	70-80	0.348	65-75	Marques, 2001
Cheese whey	500-4000	ASBR	5	30±2	NM	0.6-4.8	>90	NM	NM	Mockaitis *et al*., 2006

NM = not mentioned; HRT = hydraulic retention time; OLR = organic loading rate; COD = chemical oxygen demand; MPR = methane production rate; AHR = anaerobic hybrid reactor; ABF = anaerobic baffled filter; UBF = upfflow blanket filter; AF = anaerobic filetr; UASF = upflow anaerobic sludge –fixed film; UAF = upflow anaerobic filter; HYB-AF = hybrid digester and anaerobic filter; H-EGSB = hybrid-expanded granular sludge bed; EGSB = expanded granular sludge bed; ASBR = anaerobic sequencing batch reactor; UAFB = upflow anaerobic fixed bed; UAPB = upflow anaerobic packed bed; CSTR = constant stared tank reactor; UASB-AF = upflow anaerobic sludge blanket reactor and anaerobic filter

2.4 The upflow anaerobic sludge blanket reactor

The basic design of an upflow anaerobic sludge blanket (UASB) reactor is a cylindrical or rectangular shaped tank that may be equipped with an inverted cone that serves as a gas-liquid-solids (GLS) separator (Figure 11). The wastewater flows in an upward direction through the sludge bed and the organic matter in the wastewater is converted to biogas and new cell mass by anaerobic microorganisms contained in the sludge (Tanksali, 2013). The biogas, which also provides mixing, bubbles up through the reactor carrying with it some residual solids that separate at the GLS and fall back to the sludge blanket (Govindaradjane & Sundararajan, 2013; Matangue & Campos, 2011; Lemos, 2007). The biogas flow rate out of the reactor can be measured with a gas meter and captured in a container. In North Africa, the UASB technology has been applied to domestic and industrial wastewater and has gained popularity in countries such as Indonesia and Angola. However, the long period of start-up required for the development of proper granules and the lack of understanding the granulation process limits its application (Khalil *et al*., 2008; Liu *et al*., 2003).

A variety of wastewaters have been treated successfully using the UASB technology. Table 7 provides a review of some of the wastewaters, together with operating parameters, and performance data. The COD removal percentage for these studies ranged between 39-96%. The volume, temperature, OLR and HRT varied between 0.5-500 L, 20-55^{o}C, 0.76-28 kgCOD/m^{3}.d and 0.071-8 days, respectively.

In addition to the volume and operating conditions, the source of inoculum and the size of granules also impacts on the performance of the reactor (Table 7). For example, cassava wastewater was treated in identical reactors inoculated with granules from different sources: cassava factory sludge (CS), a seafood factory sludge (SFS) and a palm oil mill sludge (PS), with a granule size range of 1.5-1.7 mm, 0.7-1.0 mm and 0.1-0.2 mm, respectively (Jijai *et al*., 2015). The reactors inoculated with mixed granules (CS+SFS and CS+PS) responded differently to the control reactor inoculated with granules from own source (CS). In addition, as the HRT of the reactors was reduced systematically from 5 days to 1 day, the COD removal efficiencies declined from 91.4 to 43.2%, 89.4 to 45.1% and 87.2 to 33.0% for reactor with CS, CS+SFS and CS+PS respectively (Jijai *et al*., 2015).

After performing a comprehensive search using Google Scholar, Scopus and Science Direct search engines, only six literature studies could be found that focus on the treatment of SIWW using UASB reactors. It was concluded that there is limited research available about the treatment of SIWW using UASB reactors.

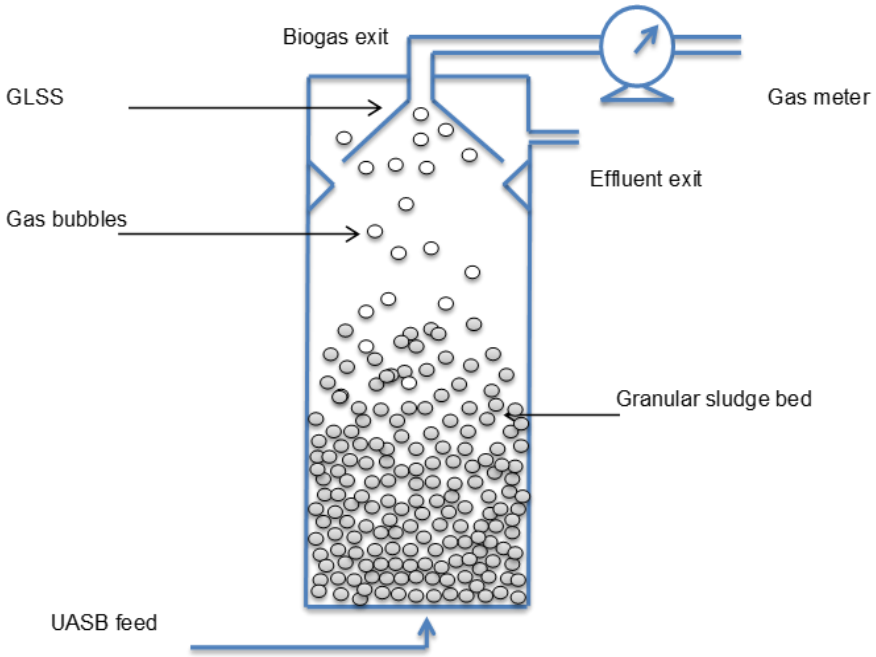


Figure 11: Diagramatic representation of an upflow anaerobic sludge blanket reactor (Adapted from Powar *et al*., 2013)

Table 7: Performance of the upflow anaerobic sludge blanket reactor in the treatment of various types of wastewaters

Wastewater	$COD_{influent}$ (mg/L)	Volume (L)	Temp. (°C)	Inoculum	HRT (hrs)	OLR ($kgCOD/m^3.d$)	pH	COD removal (%)	MPR ($L.CH_4/g.COD_r$)	Reference
Starch: synthetic	1000	6	35	Anaerobic granules	6-24	4	6.9-7.5	63.2-94.9	NM	Lu, 2016
Slaughterhouse	2000-6200	7.2	35	Anaerobic granules	14-22	2.7-10.8	7.5-8.5	80-85	NM	Caixeta *et al*., 2002
Winery	3000-5000	10.2	35	NM	48	6.1-18	7.0-7.33	>90	NM	Laubscher *et al*., 2001
Winery grain distillery	4300	2.3	35	Anaerobic granules	48	4.3	7.2	>90	NM	Gie, 2007
Sugarcane molasses (pilot)	3750	16.5	24-32	Digested activated sludge	39.6-12.5	2.3-7.14	7.0-7.3	59-91	0.33-0.39	Gonzalez *et al*., 1998
Sugar	1000-4340	7.95	29-37	Digested sewage sludge	6	16	NM	89.4	NM	Hampannavar & Shivayogimath, 2010
Cane molasses vinasse (pilot)	10000	140	55	NM	8.4-99.8	2.4-28	NM	39-67	0.29	Harada *et al*., 1996
Cane molasses (pilot)	800-2600	500	35-38	Anaerobic digested sludge	5-6	NM	7.0	90	NM	Atashi *et al*., 2010
Cane molasses	1000	10	NM	Acclimated sludge	10.1-2	6.7	NM	60-81	NM	Ragen *et al*., 2001
Synthetic sugar	2000	8.4	26-39	Septic tank sludge	48-12	1-6	NM	80-96	NM	Tanksali, 2013
Sewage	474	4.6	20	Cow dung	3-12	0.95-3.8	NM	72.6-80	NM	Rizvi *et al*., 2014
Pharmaceutical	10000	6	37 ± 1	Granular sludge	31.2	8	8.5-7.1	70		Weicheng *et al*., 2015
food waste (VFA & alcohol)	3104	40	37 ± 1	Partially granulated sludge	5.76	12.9	7.6-7.8	96	4.1	Han *et al*., 2005
Brewery	870-5065	30	35±1	Granular sludge	84	7	NM	95	0.3	Oktem & Tufekci, 2006

Table 7 continued

Wastewater	$COD_{influent}$ (mg/L)	Volume (L)	Temp. (ºC)	Inoculum	HRT (hrs)	OLR ($kgCOD/m^3.d$)	pH	COD removal (%)	MPR ($L.CH_4/g.COD_r$)	Reference
Cheese production	2050	4	35±1	Anaerobic sludge	1.7	31	NM	90	0.32	Gutierrez *et al*., 1991
Poultry manure	12100	15.7	32±2	Granular sludge	192	0.76	8.28	90.7	NM	Yetilmezsoy & Sakar, 2008
Cow manure	37034	9	Thermo-philic	NM	172.8	5.06	8.0-8.3	54.8	0.3	Castrillon *et al*., 2002
Fementation & distillation	24000	12	35±1	Thickener sludge	72	8	NM	84	0.52	Amin & Vriens, 2014
Potato processing	6800	0.5	37	Granular sludge	125-12.5	1.3-13.1	NM	71-90	0.3	Manhokwe *et al*., 2009
Cassava	18800	2.06	NM	CS	120-24	3.76-18.8	7.2-5.8	91-43	NM	Jijai *et al*., 2015
Cassava	18800	2.06	NM	CS+SS	120-24	3.76-18.8	7.2-5.8	89-45	NM	Jijai *et al*., 2015
Cassava	18800	2.06	NM	CS+PS	120-24	3.76-18.8	7.2-5.8	87-32	NM	Jijai *et al*., 2015
Dairy	1500-17600	31.7	35±1	Flocculent sludge	12	2.5	NM	80 COD_s	NM	Nadais *et al*., 2005
Combined industrial	514	15.5	NM	Activated sludge	3-12	1-4.1	NM	76-84	NM	Yasar *et al*., 2007

NM = not mentioned, HRT= hydraulic retention time, OLR = organic loading rate, COD = chemical oxygen demand, MPR = methane production rate, CS = cassava factory sludge, SS = seafood factory sludge, PS = palm oil mill sludge.

2.4.1 Parameters affecting the performance of the up-flow anaerobic sludge blanket reactors

There are several factors that affect the performance of a UASB reactor. Factors with significant impact include temperature, HRT, OLR, F/M ratio, pH, and the alkalinity of the influent (Gomez, 2011 Yasar & Tabinda, 2010; Lemos, 2007).

2.4.1.1 Temperature

Temperature has a significant impact on the physical and chemical properties of the wastewater in the reactor liquor, and also influences the growth rate and metabolism of microorganisms (Appels *et al*., 2008). For example, the viscosity of liquids increases as their temperature decreases. Therefore, at psychrophilic temperatures of <15°C, the diffusion and mass transfer rates of soluble organic compounds between the substrate and biomass drops and the sludge bed may become less easily mixed (Lettinga *et al*., 2001). This can result in ineffective contact between the substrate and biomass and can ultimately reduce the rate of substrate uptake.

The rate of degradation of organic matter is optimal under mesophilic conditions (between 25 and 40˚C) (Table 6) (Yasar & Tabinda, 2010; Schnurer & Jarvis, 2009). Hydrolysis is slower at temperatures below 20˚C, and in the thermophilic range (40-70˚C), mesophiles decay and only thermophiles reproduce rapidly (Schnurer & Jarvis, 2009; Lemos, 2007).

High temperatures (>70°C) can have some advantages and disadvantages. According to Appels *et al*. (2008) and Chen (1995), increasing temperatures can increase the solubility of the organic matter in the substrate, increase pathogen mortality, and enhance biological and chemical reaction rates. On the other hand, temperatures exceeding 70˚C are bactericidal to most bacteria and may reduce the microbial population numbers in the reactor, resulting in reduced degradation rates.

2.4.1.2 Hydraulic Retention Time

The HRT is a fundamental design parameter for anaerobic reactors. It is defined as the average time that a specific volume of wastewater spends in the reactor during treatment. The HRT can be determined by dividing the actual liquid volume in the reactor by the influent flow rate (Yasar & Tabinda, 2010; Lemos, 2007). In general, by lengthening the HRT, the contact time between the substrate and the functional bacteria is increased. Therefore, better COD removal efficiencies are expected at higher HRTs (0.5-1 day). However, very

long HRTs (> 7 days) have a negative effect on the process of sludge granulation, caused by ineffective mixing (Table 7).

As most of the methane is derived from methanogenic fermentation of acetic acid, shorter HRTs (< 1 hr) can cause washout of methanogens and accumulation of VFAs which can be followed by a severe drop in pH if the buffering capacity of the system is poor (Stamatelatou *et al*., 2003). However, the appropriate retention time depends on a number of factors, including the properties of the individual substrates, such as biodegradability, which varies from industry to industry, environmental conditions, and required effluent quality (Arsova, 2010; Yasar & Tabinda, 2010).

2.4.1.3 Organic Loading Rate

The OLR is the amount of organic material fed per unit volume of reactor per unit of time (Lemos, 2007). Microorganisms in anaerobic digesters depend on the organic content of the substrate for growth. At increased OLR, microorganisms grow rapidly, but if the OLR is increased excessively, microbial toxicity can occur (Gomez, 2011). Conversely, at low OLR, insufficient substrate may result in decreased microbial growth (Gomez, 2011).

Some researchers have reported that an increase in OLR in UASBs treating sugar and winery wastewater can be accommodated successfully without deterioration in effluent quality. They also found a positive correlation between COD removal efficiency and increasing OLR, up to a certain limit (Atashi *et al*., 2010; Laubscher *et al*., 2001; Ragen, 2001). Excessive increase in OLR leads to operational problems such as accumulation of organic acids, upliftment of the sludge bed, and loss of viable microorganisms (Abdelgardir *et al*., 2014).

In the treatment of domestic wastewater using a fluidised bed UASB reactor, the decline in methane yield from 0.285 to 0.0988 LCH_4/gCOD was negatively correlated to the decrease in HRT from 6 to 2.5 hr. The decrease in methane yield was attributed to the corresponding increase in OLR from 7.76 to 10 kgCOD/m^3.d, respectively (Moharram *et al*., 2016).

2.4.1.4 Food to microorganism ratio and source of inoculum

The F/M ratio is the ratio of organic matter present to the amount of bacterial available, and is an important factor to consider in the control and operation of anaerobic digesters (Dennis & Burke, 2001). The concentration of volatile suspended solids (VSS) is typically used as a proxy for bacterial concentrations. Individual bacteria can only degrade a limited amount of substrate per unit time. Therefore, to achieve the desired effluent chemical concentrations, sufficient numbers of microorganisms need to be present in the reactor (Kamthunzi, 2008;

Dennis & Burke, 2001). At reduced F/M ratios, the lack of substrate will result in a reduced microbial metabolic rate, and hence reduced methane yield. On the other hand, with excessively increased F/M ratios, the microbial substrate requirements may be exceeded, which may be toxic and bactericidal (Hadiyarto *et al*., 2015).

Inocula from different sources contain microorganisms with distinctive metabolic functions. Therefore, the F/M ratio required for optimal degradation of a particular substrate may vary according to the inoculum source (Shah *et al*., 2014). For example, in the biomethanation of SIWW and beet-pulp in batch reactors inoculated with anaerobic seed sludge, a maximum methane yield of 0.311 L CH_4/gCOD was observed at an F/M ratio of 0.77 gCOD/gVSS (Alkaya & Demirer, 2011). In the batch methanogenesis of fish offal waste and microbial sludge obtained from the curing of fish and river mud discharges, Hadiyarto *et al*. (2015) reported a maximum biochemical methane potential of 0.164 L CH_4/gCOD with sewage sludge at an F/M ratio of 0.2 gCOD/gVSS.

2.4.1.5 pH and alkalinity

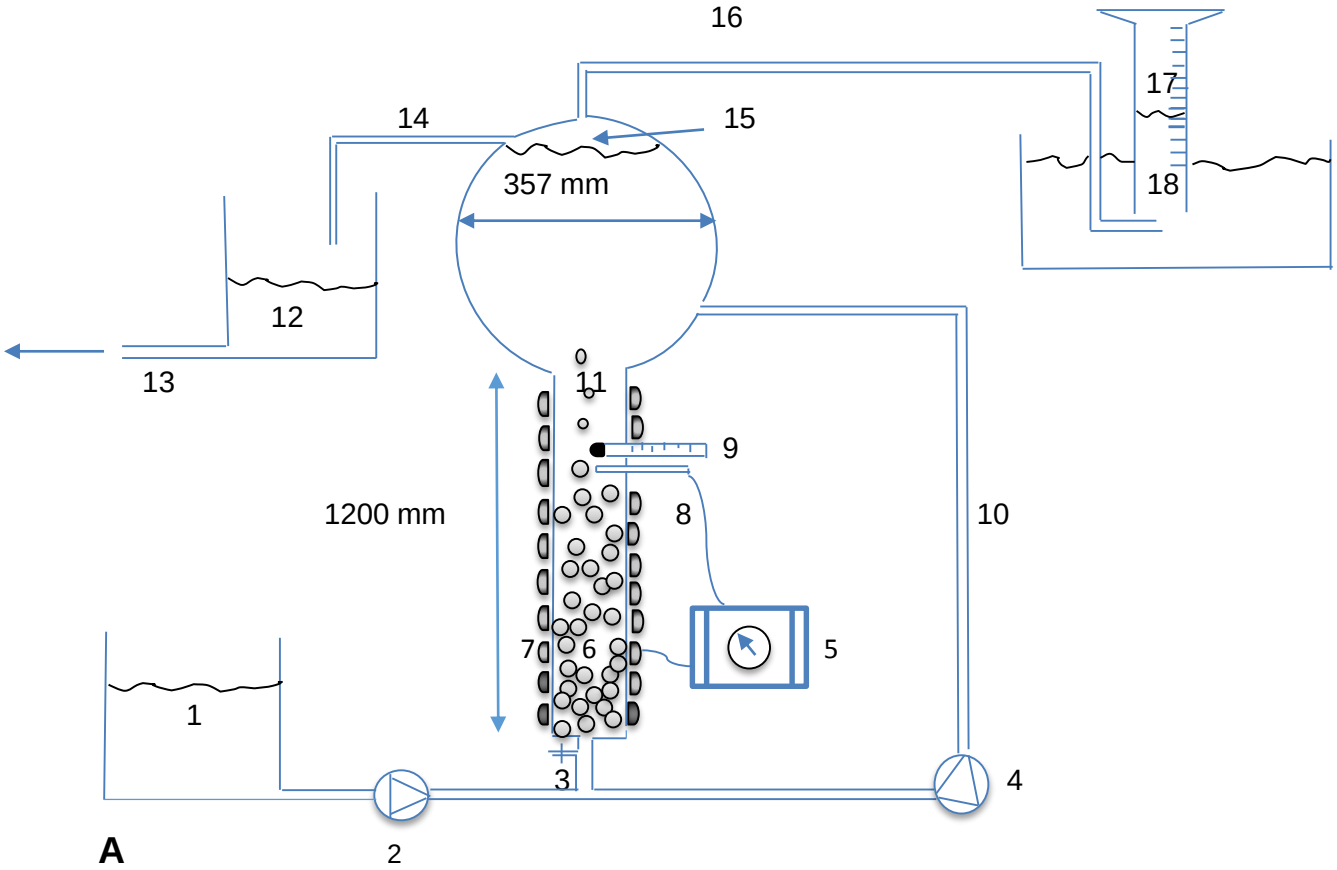
According to Gomez (2011), there are three functional bacterial groups that participate in biogas production: hydrolysing bacteria, acid-forming bacteria, and methane-forming bacteria. Methanogenesis of acetate is the rate limiting step in anaerobic digestion. At pH values lower than 6 and greater than 8 the rate of methanogenesis is suppressed (Lemos, 2007). Conversely, the acid-forming bacteria are less sensitive to low and high pH ranges. Therefore, acid fermentation will prevail over methanogenic fermentation, which may consequently cause souring of the reactor contents and accumulation of organic acids (Yasar & Tabinda, 2010; Kavitha, 2009; Lemos, 2007). The accumulation of organic acids may decrease the pH of the wastewater to values less than 6, and this may ultimately inhibit the activity and growth of methanogenic bacteria and subsequently decrease the performance of the reactor.

Alkalinity in the range of 1500 - 3000 mg $CaCO_3$/L is required for effective digestion and is essential to serve as a buffer to resist sudden pH variations (Kavitha, 2009; Marti, 2008; Gerardi, 2003). A reduction in alkalinity below optimal values may result in considerable pH fluctuations, and may lead to process failure. Hence, chemicals should be added or the operating conditions should be adjusted to maintain desired alkalinity (Gie, 2007). For a stable process, the VFA/alkalinity ratio should be maintained between 0.14-0.3 (Tanksali, 2013).

CHAPTER THREE
MATERIALS AND METHODS

3.1 Experimental setup

A single-stage biotreatment process consisted of a 46 L glass UASB reactor with a 22.3 L column (internal ø = 154 mm, *h* = 1200 mm) and a 23.8 L bulb (internal ø = 357 mm) (Figure 12). A head space was left at the top of the bulb to allow for separation of liquids, solids and biogas. The influent reservoir consisted of six 5 L Schott Duran glass bottles, and the waste effluent was collected into a 40 L polypropylene container. The influent was fed into the bottom of the reactor via 8 mm silicon tubing using a Watson-Marlow peristaltic pump (Spirax-Sargo Engineering plc, Cheltenham, England) controlled by a Yaskawa Varispeed controller (Illinois, USA) (work rate of 1.2 KW, 2.4 mm clamp size). A portion (2.52 L/hr) of the treated effluent was recycled via the influent stream. The recycle stream apparatus consisted of 8 mm silicon tubing and a dedicated Watson-Marlow peristaltic pump (Spirax-Sargo Engineering plc) controlled by a Yaskawa Varispeed controller. The temperature was controlled by a self-regulating heating cable (ELSR-H-BOT up to 210°C) connected to an electronic temperature controller (ELTC-14) and a temperature probe (Pt100) supplied by Eltherm South Africa (Pty) Ltd. The temperature probe and thermometer were submerged into the sludge bed. The gas delivery tube emerging from the overhead port of the UASB reactor was inserted into the inverted cylinder of the water displacement system. The water displacement system consisted of a 10 L square bucket with a graduated inverted cylinder filled with tap water. The Biogas analyser consisted of a suction tube that was connected to a delivery tube that carried the biogas that was produced from the UASB reactor and an exit tube which discharged the analysed biogas from the analyser to the atmosphere.



B

1) Influent reservoir
2) Peristaltic pump
3) Sludge withdrawal valve
4) Peristaltic pump
5) Temperature controller
6) Sludge Bed
7) Self regulating heating cable
8) Temperature probe
9) Thermometer
10) Recycle stream
11) Sludge blanket
12) Effluent container
13) Drainage tube
14) Treated effluentstream
15) Overhead space
16) Gas delivery tube
17) Measured gas
18) Tap water

Figure 12: A) Schematic diagram, and B) Photograph of the experimental set-up of the upflow anaerobic sludge blanket reactor

3.1.2 Inoculation of the upflow anaerobic sludge blanket reactor

In order to reduce the start-up period and acclimatisation time, the UASB reactor was inoculated with anaerobic granular sludge and mixed liquor volatile suspended solids (MLVSS). The granular sludge was sourced from a full-scale UASB reactor treating brewery wastewater [South African Brewery (SAB), Newlands brewery, South Africa]. The MLVSS was obtained from a full-scale activated sludge wastewater treatment plant treating domestic wastewater (Bellville wastewater works, Bellville, South Africa). The MLVSS and granular sludge were mixed and the UASB reactor was filled with the mixed inoculum up to a volume of 20 L and made up to 46 L with synthetic SIWW with a COD concentration of 1880 mg/L. Prior to start up the UASB reactor was put into a recycle mode for 36 hours at a recirculation rate of 2.5 L/hr.

3.1.3 Formulation of synthetic substrate

Due to (i) the scarcity of sugar factories in the Western Cape and hence inavailability of fresh SIWW, and (ii) the need for consistent influent for long-term comparative testing, synthetic SIWW was made using sugarcane molasses. Molasses is a by-product obtained in the manufacturing process of raw sugar and white crystalline sugar. It has a content of carbohydrates and cysteine which makes it a suitable substrate for anaerobic digestion.

Fresh batches of SIWW were made up each day and were used immediately. Standing batches were stored at 4°C for 12 hours. Each batch consisted of 187 g of molasses diluted with 30 L of tap water. 75 g of sodium bicarbonate ($NaHCO_3$) and 17.5 g of sodium hydroxide (NaOH) was added to each as a buffer. Macro and micro-nutrutrients were added in final concentrations recommended by Appels *et al*. (2008) and Show *et al*. (2004) for the activation of enzymes and co-enzymes in anaerobic digesters: $MgSO_4$ = 36.7 mg/L; $FeSO_4 7H_2O$ = 9.50 mg/L, $CaCl_2 2H_2O$ = 30 mg/L; H_3BO_3 = 2 mg/L; $MnSO_4$ H_2O = 2 mg/L; $CoCl_2 6H_2O$ = 2 mg/L. 3 g of urea (46% N) and 1.5 g of sodium dipotasium phosphate (22.7% P) were added as sources of nitrogen and phosphorus to give a COD:N:P ratio of 250:3.4:1. The COD:N:P ratio of the substrate indicates that there was adequate amount of nutrients for bacterial growth compared to the minimum requirement of 100:2:0.5 (Ragen *et al*., 2001). The average COD concentration of the fresh substrate was 4101 mg/L (Table 8). Each batch of the substrate was sterilised in a vertical steam steriliser at a temperature of 120°C and a pressure of 1.5 kg/cm^2 for 20 minutes to prevent *in-situ* degradation prior to feeding. Prior to feeding, the batch was cooled to ambient temperature. Each batch was characterised using the methods provided in Section 3.1.7.

Table 8: Characteristics of synthetic sugar industry wastewater used in the study

Parameter	Range	Average
pH	10.09-10.48	10.25
COD (mg/L)	4075-4150	4101
TN (mg/L)	35-77	55.9
TP (mg/L)	16.02-16.5	16.38
SO_4^{2-} (mg/L)	140-132	138
Alkalinity (mg$CaCO_3$/L)	3903-4233	4123
BOD_5 (mg/L)	1061-1038	1055
TOC (mg/L)	1730-1790	1760

ND = not determined; COD = Chemical oxygen demand; TN = Total Nitrogen; TP = Total phosphorus; SO_4^{2-} = Sulfate; BOD = Boilogical oxygen demand; TOC = Total organic carbon

3.1.4 Operation and monitoring of the upflow anaerobic sludge blanket reactor

Table 9 shows the operating conditions of the UASB reactor during the study. Temperature and C/N ratio were varied to optimise the treatment conditions. The temperature of the reactor was varied between 25 and 38°C using the electronic temperature controller. The C/N ratio was varied between 22 and 50 by changing the amount of urea added into the substrate. TOC and HRT were maintained constant at 1760 mg/L and 48 hrs. A pH meter and pH 7000 probe (Eutech Instruments, Stanger, South Africa) was used to measure the pH of the feed and outlet stream. The UASB feed flow rate and outlet flow rate were monitored daily using a stop watch and a 40 L container.

3.1.5 Experimental design and statistical analysis

The experiments were carried out based on central composite design (CCD) and the experimental data was analysed with response surface methodology (RSM) using Design-Expert® Software Version 10 (Stat-Ease, Inc., Minneapolis, USA). RSM uses a group of statistical techniques to develop an adequate empirical model which explains the relationship between the response (output variables) and the associated independent variable (input variable). The principal objective is to optimise the response which depends mainly on the input variable. In this study, temperature and C/N ratio were chosen as the independent operational variables.

Table 9: Randomised two level factorial design

Standard order of runs	Run No.	Factor 1 (A): Temperature (°C)	Factor 2 (B): C/N ratio (mg TOC/mg N)
5	1	25	36
3	2	25	50
9	3	31.5	36
7	4	31.5	22
11	5	31.5	36
1	6	25	22
6	7	38	36
8	8	31.5	50
10	9	31.5	36
2	10	38	22
4	11	38	50

Table 10 shows factors in coded and actual units with experimental ranges. Each independent variable was varied over three levels (-1 to +1). In order to determine the experimental error and validate the reproducibility of the polynomial model fitted using linear regression analysis the centre point (31.5°C, 36 mg TOC/mg N) represented by zero was repeated three times (Beszedes *et al*., 2011).

Table 10: Range of factors

Factors	Range and levels		
	-1	0	+1
(A) Temperature (°C)	25	31.5	38
(B) C/N ratio (mg TOC/mg N)	22	36	50

C/N = carbon to nitrogen TOC = total organic carbon

The coefficients of the second-order polynomial model (Equation 13) proposed by Rastegar *et al*. (2011) and Montgomery (1997) were determined by calculating the process`s responses (COD removal efficiency and biogas production rate)

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i,1}^{k} \sum_{i=1}^{k} \beta_{ij} x_i x_j + \varepsilon \qquad \text{Equation 13}$$

In which β_o is the constant term, k is the number of independent variables, β_i , β_{ii} and β_{ij} are the linear, quadratic and interaction effect coefficients of the terms. x_i and x_j are the coded independent variables. ε is the residual of the experiment or random error (Nair & Ahmmed, 2015; Sathish & Vivekananda, 2015; Bashir *et al*., 2012 Yetilmezsoy *et al*., 2009). The significance and reliability of the independent variables and their interactions were evaluated by analysis of variance (ANOVA) and regression analysis processed by Design-Expert® Software Version 10 (Stat-Ease). The quality of fit of the second-order polynomial model was determined using the F-test, Prob > F test, determination coefficient (R^2) and lack of fit (LOF) test. A Prob > F of 0.05 was used to determine the statistical significance of the model and model terms.

3.1.6 Measurement of biogas flow rate and composition

Prior to measuring the gas flow rate, biogas was bubbled through the water in the bucket of the water-displacement system to saturate it with CO_2 gas. This eliminated any discrepancies in displacement volume that may have arisen due to absorption of CO_2 into the water during biogas measurement. The flow rate of the biogas produced from the UASB reactor was measured by accounting for the volume of water displaced in the inverted cylinder over the period of 1 hour. All measurements were performed in triplicate for each run when the process was at steady state. In general, the biogas produced by anaerobic digesters consists mainly of methane with the balance being mostly carbon dioxide (Bothi, 2007; Rasi *et al*., 2007). Biogas poses a fire hazard in a confined environment and the UASB reactor was therefore mounted outdoors and the space was well ventilated. The biogas produced from the reactor was released into the atmosphere via the delivery tube. At each operational condition the gas produced was analysed using a Geotech Biogas 5000 instrument (Goodspeed environmental services, Everton, South Africa) (Figure 13) according to the manufacturers' instructions. The Biogas 5000 analyser was programmed to analyse the % of methane, carbon dioxide and molecular oxygen in the biogas.

Figure 13: Biogas analyser used to determine the composition of the biogas

3.1.7 Sampling and analysis of influent and effluent

The UASB was run for a period of 160 days for the start-up period, 48 days for the experimental runs and 9 days for the validation experiments. During start-up, fresh samples (~30 ml) were collected from the effluent tubing over a cycle period of 3-10 days for 6 months. The operational phase commenced once the COD concentration had stabilised. During the operation phase when each run had stabilised, samples (~30 ml) were taken in triplicate. In addition, 28 ml of each batch of SIWW was taken for analysis during the operation phase (Table 11).

An Agilent (Agilent Technologies, Santa Clara, USA) 1100 series HPLC instrument and an Agilent 1200 series refractive index detector were used to determine the concentrations of sugars in the molasses. An Agilent Zorbax carbohydrate column and acetonitrile/water (80/20) solution as a mobile phase were used for separation of the sugars. The sample retention time, flow rate and temperature were set at 30 min, 0.80 mL/min and 30°C, respectively. The sugar concentrations were calculated automatically by the instrument from standard concentrations of sugars run under the same conditions (see Appendix B for standard graphs).

A Merck Hitachi LaChrom high performance liquid chromatography (HPLC) instrument with a D-7400 refractive index detector set at 210 nm was used for the detection and quantification of selected sugars, alcohols and acids in the final effluent. A phenomenex Rezex RHM-Monosaccharide H+ column and a 1 mM sulfuric acid (H_2SO_4) solution as a mobile phase were used for the separation of the components. The sample retention time and flow rate were set at 60 min and 0.550 mL/min. Concentrations of the components were calculated manually from standard graphs prepared using analytical standards (Appendix B).

Merck cell tests and kits (Table 11) were used to determine the COD, VFA, TN, SO_4^{2-}, Alk, TP, TOC and BOD_5 according to the manufacturers' instructions (Appendix C). A pH 700 probe and instrument (Eutech Instruments, Stanger, South Africa) were used to determine the pH of the influent and effluent.

Table 11: Methods used to analyse the influent and effluent samples: Range of factors

Analysis	Influent	Method	Reference or kit
COD (mg/L)	4101	Chromosulfuric acid oxidation, chromium (III) determination	Merk COD cell test cat nos: 14541, 14555
VFA (mg/L)	ND	Esterification	Merk VOA cell test cat no: 01763
Sucrose (mg/L)	X	HPLC (RI Detector)	Welz *et al*., 2011
Glucose (mg/L)	X	HPLC (RI Detector)	Welz *et al*., 2011
Xylose (mg/L)	ND	HPLC (RI Detector)	Welz *et al*., 2011
Glycerol (mg/L)	ND	HPLC (RI Detector)	Welz *et al*., 2011
Acetate (mg AAE/L)	ND	HPLC (RI Detector)	Welz *et al*., 2011
TN (mg/L)	35-77	Peroxodisulfate oxidation, nitrospectral	Merk Nitrogen cell test cat no: 14537
SO_4^{2-} (mg/L)	138	Bariumsulfate, turbidimetric	Merk Sulphate cell test cat no: 14548
Alk (mg/L)	4123	Indicator reaction	Merk Acid capacity cell test cat no: 01758
TP (mg/L)	16.38	Peroxodisulfate oxidation, phosphormolybdenum blue	Merk Phosphate cell test cat no: 14543
TOC (mg/L)	1760	Peroxodisulfate oxidation, indicator	Merk TOC cell test cat no: 14878
BOD_5 (mg/L)	1055	Modification of Winkler method	Merk BOD cell test cat no: 00687
TS (mg/L)	ND	Drying method	Viessman & Hammer (1998)
pH	8-9.9	pH meter	Eutech instrument, pH 700

ND: not determined; COD = chemical oxygen demand; VFA = volatile fatty acids; TN = Total Nitrogen; TP = Total phosphorus; SO_4^{2-} = Sulfate; BOD = Boilogical oxygen demand; TOC = Total organic carbon; HPLC = high performance liquid chromatography

3.1.8 Imaging of granule structure by scanning electron microscopy

On the last day of UASB operation, samples of the biomass were withdrawn from the bottom of the reactor via the desludging valve. The samples were stored in a refrigerator in 50 ml tubes until analysis. The defrosted samples were fixed in glutaraldehyde ($C_5H_8O_2$), then washed in a buffer solution and taken through a dehydration series of varying percentages of ethanol (25 – 100%). A small amount of the sample was then dropped into a nucleopore membrane and dried using hexamethyldisilazane (HMDS). Once the samples were dry, they were coated with gold/palladium alloy ready to be viewed in the scanning electron microscope (SEM). The SEM was a FEI NanoSEM 230 with a field emission gun and the samples were viewed at 5.00 KeV.

3.1.9 Determination of bacterial community composition of granules using next generation sequencing

PCR products were quantified, and library quality control was performed using an Agilent 2100 Bioanalyzer (Santa Clara, USA). Amplicons were indexed using the Nextera XT dual indexing kit (Illumina, San Diego, USA), and was run on the Illumina MiSeq platform MCS version 2.5.0.5, according to the manufacturers' instructions. 20 Mb of 2x300 base pairs paired end reads were produced for each amplicon.

The V3 and V4 variable regions were amplified in a 25 ul reaction using Q5 ® Hot start High Fidelity 2x Master Mix (New England Biolabs, USA). Amplicon library PCR was performed on all replicate extractions separately. The DNA primers used were Truseq Tailed 341F and 785R (5'TGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCCTACGGGNGGCWGCAG-3') and (5'ACACTCTTTCCCCACACGACGCTCTTCCGATCTGACTACHVGGGTATCTAATCC-3), respectively.

The thermocycler settings for PCR amplification were as follows: (1) initial denaturation at 95°C for 2 min (2) 30 cycles of 95°C for 20 s (3) 55°C for 30 s (4) 72°C for 30 s and final elongation at 72°C for 5 min. The amplicon libraries were purified using the Agencourt® Ampure® XP bead protocol (Beckman Coulter, USA). Library concentration was measured using Nebnext Library quant kit (New England Biolabs, USA) and quality validated using Agilent 2100 Bioanalyser (Agilent Technologies, USA). The samples were pooled in equimolar concentrations and diluted to 4n M based on library concentrations and calculated amplicon sizes. The library pool was sequenced on a MiSeq™ (Illumina, USA) using a MiSeq™ Reagent kit V3 600 cycles PE (Illumina, USA). The final pooled library was at 12 pM with 15% PhiX as control.

CHAPTER FOUR
RESULTS AND DISCUSSION

4.1 Start-up of the UASB reactor

The start-up of the UASB reactor took place in four phases. In each phase, adjustments were made to attempt to improve the efficiency of the system. The operating conditions for the UASB during the different phases are given in Table 12. Figure 15 shows the inlet and outlet COD and the COD removal efficiency of the UASB reactor during start-up.

Table 12: Operating conditions applied to the UASB during the start-up priod

Phase	Period (days)	HRT (hr)	OLR (kgCOD/m^3d)	COD removal (%)	Temp. (^oC)
1	7-65	30.72-40.35	4.88-2.45	20.1-81.2	25-30
2	65-124	47.52-48.9	1.83-1.86	61.5-84.0	25-30
3	124-138	48.9-14.05	1.86-6.49	78.3-22.8	25-30
4	139-160	48.9	0.92-1.91	60.4-73.6	30-33

HRT: hydraulic retention time, OLR: organic loading rate

4.1.1 Phase 1

During phase 1 the holding tank was open to the atmosphere and spontaneous pre-fermentation of the organic material took place because of bacterial contamination from the container, by flies, and by atmospheric deposition. As a result, the inlet COD concentration and the OLR were unstable and fluctuated between 3815 mg/L and 4202 mg/L, and 4.88 kgCOD/m^3.d and 2.45 kgCOD/m^3.d, respectively. The molasses used in this study had a high content of readily biodegradable sugars [total sugars = 40.9% (wt/wt) of which sucrose = 12.0% (wt/wt), fructose = 13.3% (wt/wt), glucose = 15.6% (wt/wt)] (Figure 14). The spontaneous pre-fermentation process of the feed yielded organic acids which depleted the alkalinity of the feed from 2472 mg$CaCO_3$/L to 1286 mg$CaCO_3$/L and ultimately caused the pH of the feed to drop from 7.2 to 6.1 (Table 18). Similar observation was reported by Mehrdad *et al.* (2007) when fermentation of the feed (beet sugar molasses) caused a change in the pH of the effluent. The buffering capacity of the feed was unstable, and in some instances the alkalinity and pH of the feed decreased markedly from 2472 mg$CaCO_3$/L to 358 mg$CaCO_3$/L and 7.2 to 5.1, respectively (Table18).

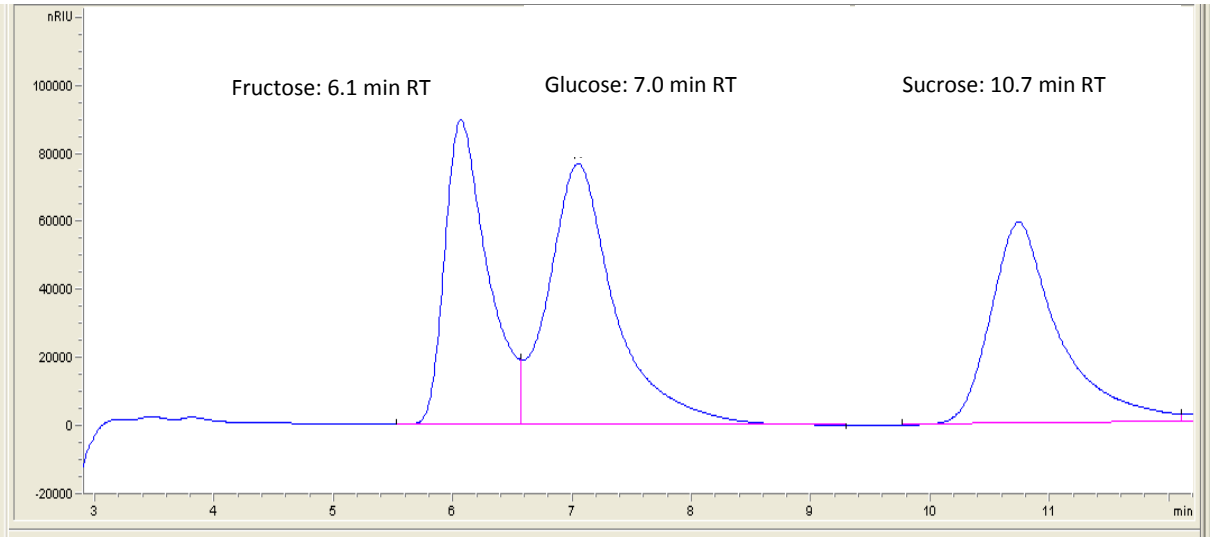


Figure 14: Chromatogram showing peaks and retention times of sugars detected in the molasses using high performance liquid chromatography. RT = retention time

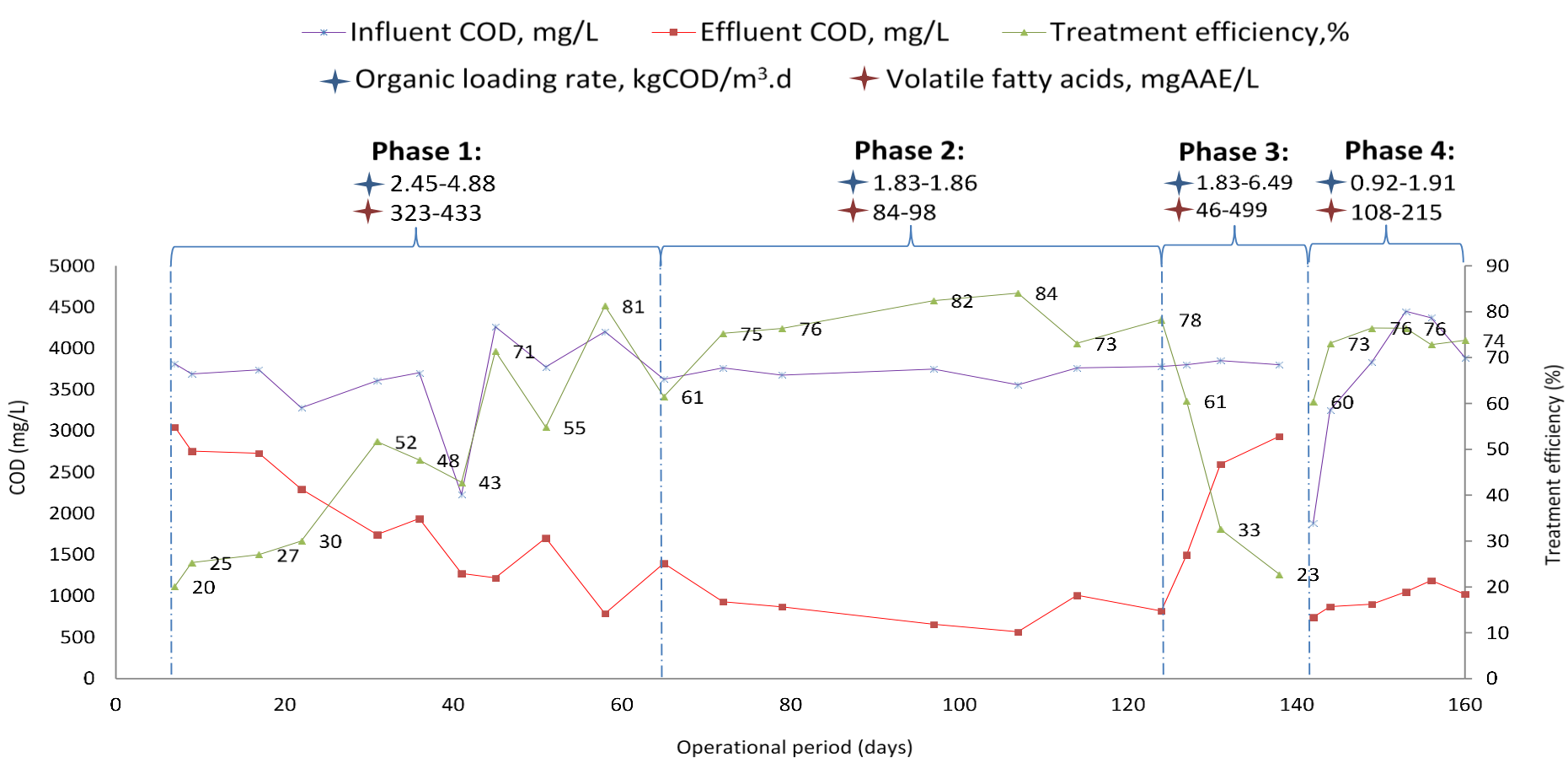


Figure 15: Influent and effluent COD and removal (treatment) efficiency, and the applied organic loading rates and effluent volatile fatty acid concentration during the four phases of the start-up of the UASB. The removal efficiency at each sampling point is shown on the graph

The concentration of VFAs in the effluent accumulated to 323 mgAAE/L and 433 mgAAE/L, respectively (Figure 15). The COD removal efficiency showed an increasing trend from 20.1 % to 81.2%, with some fluctuations between 71.4% and 61.5% (Figure 15). Biogas production varied between 0.56 L/L.d and 0.65 L/L.d and reached a maximum of 1.04 L/L.d (Table 18). During the start-up of a UASB treating synthetic SIWW, Tanksali (2013) obtained a 95% COD removal and 1.85 L/L.d biogas production.The low treatment efficiency in this study during phase 1 may be attributed to the fact that the microbial population of the inoculum had to acclimatise to the substrate and flow regime, and the performance of the reactor was affected by pre-fermentation caused by contamination in the holding tank. In addition, reactor scale can play a significant role in the performance of AD reactor (Ruffino *et al*., 2015). The reactor used by Tanksali *et al*. (2013) was small-scale (8.4 L) while that employed in the current study was pilot-scale (46 L).

4.1.2 Phase 2

The accumulation of VFAs caused by poor buffering capacity and acid fermentation in the feed causes the pH of anaerobic digesters to decrease and pH values lower than 6 may inhibit the growth of functional bacteria (Yasar & Tabinda, 2010; Kavitha, 2009). In order to minimise the effect of uncontrollable pH variations, on the 65th day after start-up, the OLR was reduced from 2.45 kgCOD/m^3.d to 1.86 kgCOD/m^3.d, and the buffering capacity of the system was increased by adding sodium bicarbonate ($NaHCO_3$) and sodium hydroxide (NaOH) such that the feed had a COD/alkalinity ratio in the range of 0.9<1 (Table 18). This was in accordance with the methodology applied by Gonzalez *et al*. (1998) for the treatment of sugarcane molasses by a UASB. Subsequently, the effluent VFA concentration dropped to between 84 mgAAE/L and 98 mgAAE/L and COD removal efficiency increased, to reach a maximum of 84% on day 107. Biogas production increased to between 1.05 and 1.15 L/L.d (Table 18).

4.1.3 Phase 3 (stress period)

From the 124th to the 138th day after start-up, the HRT of the reactor was gradually reduced from 48.90 hr to 14.05 hr, and the OLR increased from 1.86 kgCOD/m^3.d to 6.49 kgCOD/m^3.d (Table 12). During this period, the effluent COD concentration increased from 821 mg/L to 2935 mg/L, and the COD removal efficiency dropped from 78.3% to 22.8%. At an HRT of 14.05 hr and corresponding upflow velocity of 0.176 m/h, the reactor contents became ‘sour’ and VFAs in the effluent stream accumulated to 499 mgAAE/L (Table 18). There was also total upliftment of the sludge bed and excessive biomass washout. Biogas formation and pH declined to 0.31 L/L.d and 5.7 (Table 18). It was deduced that the reactor was shocked as a result of high OLR of 6.49 kgCOD/m^3.d. In the treatment of synthetic

SIWW, Atashi *et al*. (2010), Ragen *et al*. (2001), and Hampannavar and Shivayogimath (2010) managed to operate their UASBs at low HRT of 5,6 and 10 without excesive biomass washout. No information about sludge volume index (SVI) on the afforementioned studies could be found in literature, but their ability to operate at lower HRTs without operational falure could be attributed to good settling properties of the inocula used.

4.1.4 Phase 4

On day 139, a second start-up was initiated. The UASB reactor was re-inoculated with granular sludge and MLVSS, and was set in a 24 hr recirculation mode. To prevent pre-fermentation and acidification, all influent was sterilised by autoclaving in 5 L Schott bottles. The influent was fed to the UASB from the Schott bottles via autoclaved tubing. In order to allow the microorganisms to acclimatise to the substrate, a low concentration of substrate of 1880 mgCOD/L was applied and gradually increased stepwise to 3890 mgCOD/L. The corresponding OLR increased from 0.92 kgCOD/m^3.d to 1.91 kgCOD/m^3.d (Figure 15). The alkalinity of the feed was increased to approximately 4100 mg$CaCO_3$/L by adding sodium carbonate and sodium hydroxide (COD/alkalinity 0.9<1) and the problem of pH shock and souring caused by rapid acidification was mitigated. The second start-up was rapid, and the reactor stabilised quickly. The COD removal efficiency increased from 60.4% to 73.6% and did not change by >5% for two consecutive weeks, indicating that the reactor was at steady state (Figure 15). The concentration of VFAs in the effluent remained between 228 mgAAE/L and 250 mgAAE/L, and biogas production was stable between 0.91 L/L.d and 0.84 L/L.d. After day 160 it was assumed that the reactor was at steady state and optimisation experiments were initiated (Section 4.2, 4.5).

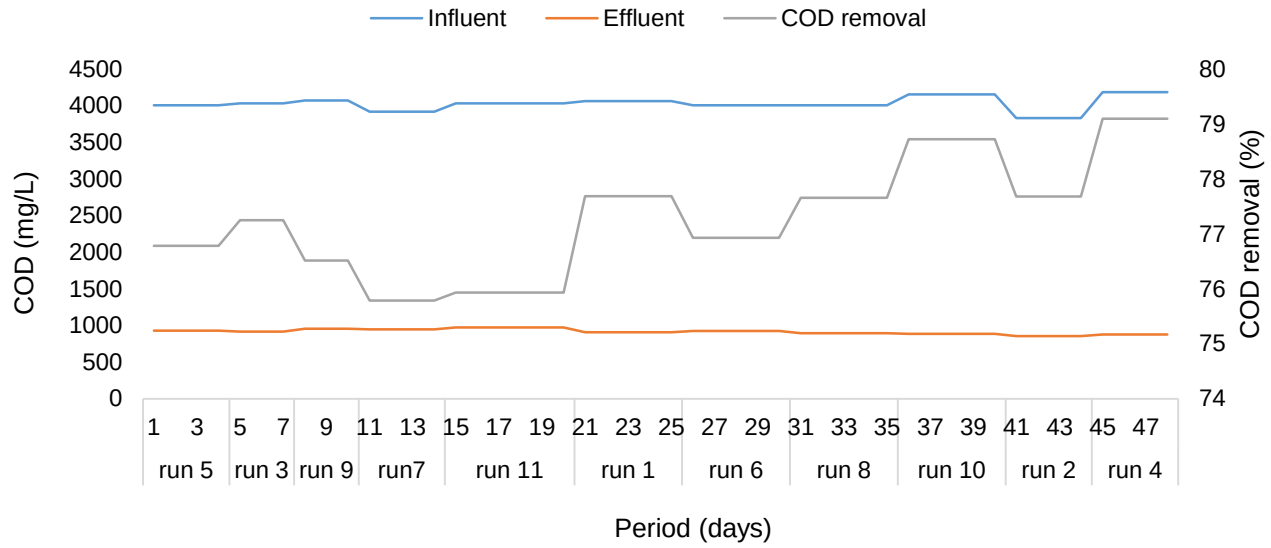
4.2 Performance of the UASB during the operational period

The COD concentration, COD removal efficiency, effluent VFA concentration, alkalinity, pH and biogas production were used to assess the performance of the UASB during the investigation (Figure 16). The UASB was operated for 48 days and the temperature and C/N ratio was varied between 25-38°C and 22-50 mgTOC/mgTN at constant HRT of 48.9 hr (Table 13).

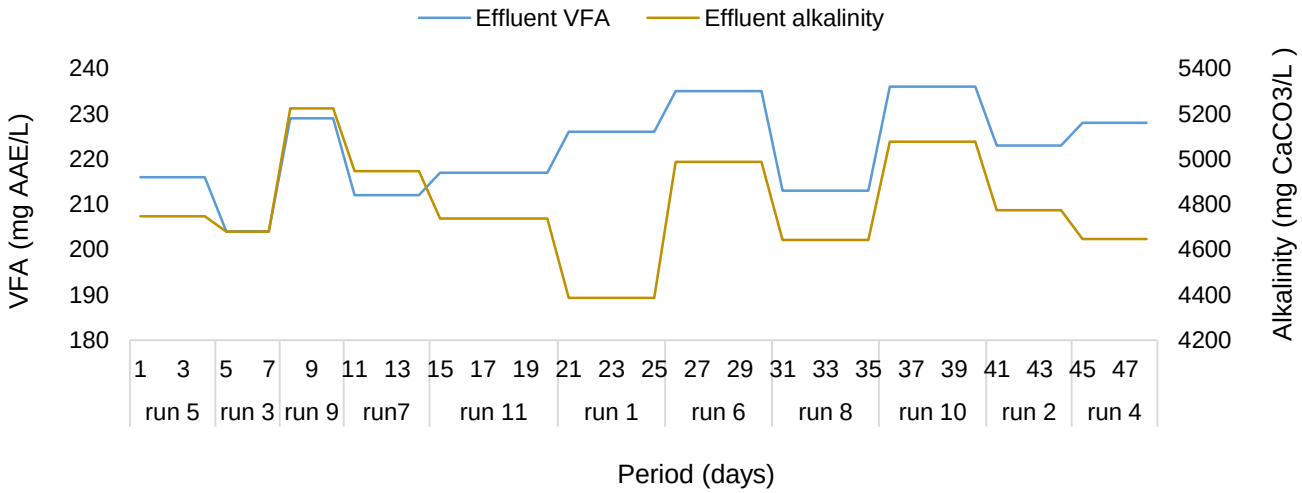
Table 13: Temperature and C/N ratios applied during runs when sugars and organic acids were detected in the final effluent of the UASB

Run No	Temperature	C/N ratio
1	25	36
2	25	50
3	31.5	32
4	31.5	22
5	31.5	33
6	25	22
7	38	36
8	31.5	50
9	31.5	36
10	38	22
11	38	50

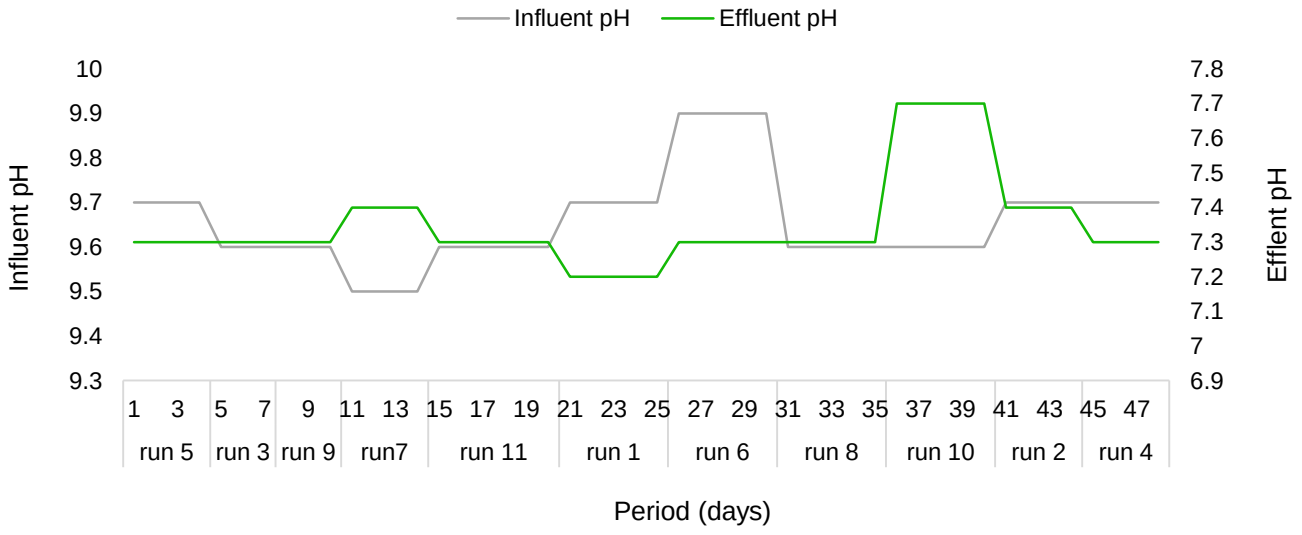
The COD concentration of the feed and OLR varied between 4005 and 4185 mg/L and 1.96 and 2.05 kgCOD/m^3.d respectively. The lowest effluent COD concentration obtained was 855 mg/L at a temperature of 25°C and C/N ratio of 50 mgTOC/mgTN (Figure 16A). The corresponding COD removal efficiency was 77.6% which falls within the range of 72.6-80% and 76-84% obtained in previous studies using sewage and combined industrial wastewater (Rizvi *et al*., 2014; Yasar *et al*., 2007). In terms of COD removal the system performed fairly well when compared to studies with molasses wastewater (Gonzalez *et al*., 1998; Harada *et al*., 1996) (Table 7). In terms of effluent COD, the treated effluent did not comply with the discharge limit of 75 mg/L applicable for most mills in SA (Welz and Ndobeni, 2017). In addition, the HRT of 48.9 hr of the system was high compared to 12, 6 and 2 hr HRT of other studies which investigated the treatment of SIWW by UASBs (Tanksali, 2013**;** Hampannavar & Shivayogimath, 2010; Ragen *et al*., 2001) (Table 7), but these investigations were conducted in small-scale (reactors<10 L).


Influent
Effluent
COD removal
COD (mg/L)
COD removal (%)
4500
4000
3500
3000
2500
2000
1500
1000
500
0
80
79
78
77
76
75
74
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39 41 43 45 47
run 5 run 3 run 9 run7 run 11 run 1 run 6 run 8 run 10 run 2 run 4
Period (days)

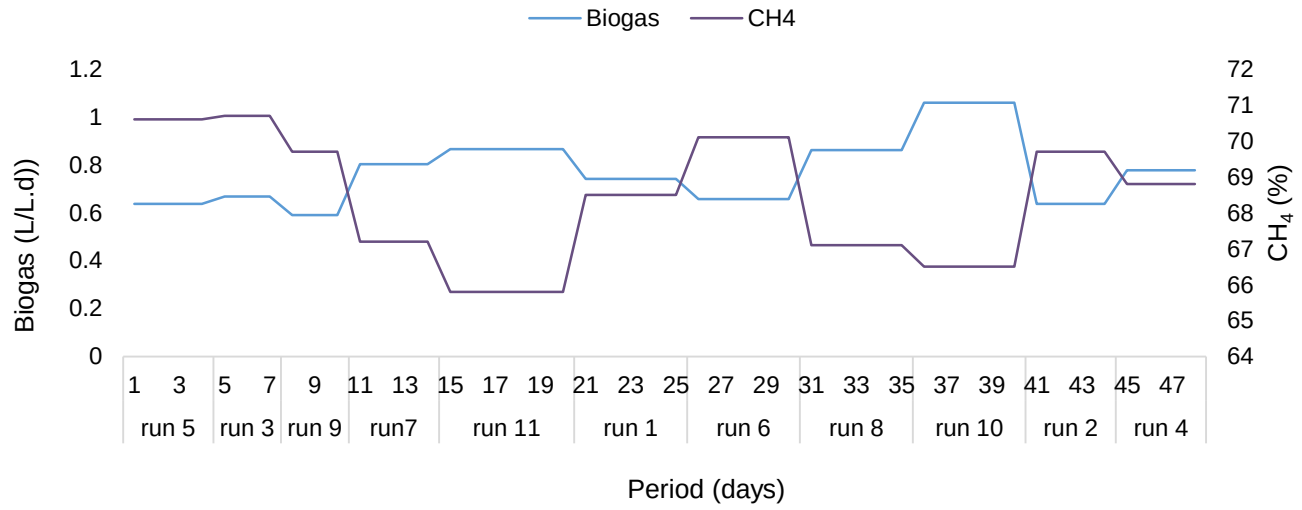
(A)


Effluent VFA
Effluent alkalinity
VFA (mg AAE/L)
Alkalinity (mg CaCO3/L)
240
230
220
210
200
190
180
5400
5200
5000
4800
4600
4400
4200
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39 41 43 45 47
run 5 run 3 run 9 run7 run 11 run 1 run 6 run 8 run 10 run 2 run 4
Period (days)

(B)


Influent pH
Effluent pH
Influent pH
Effluent pH
10
9.9
9.8
9.7
9.6
9.5
9.4
9.3
7.8
7.7
7.6
7.5
7.4
7.3
7.2
7.1
7
6.9
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39 41 43 45 47
run 5 run 3 run 9 run7 run 11 run 1 run 6 run 8 run 10 run 2 run 4
Period (days)

(C)



(D)

Figure 16: Influent and effluent parameters during the UASB operational period: chemical oxygen demand concentration and removal efficiency (A), volatile fatty acid concentration and alkalinity (B), pH (C), and biogas production (D)

The VFA concentration and alkalinity are the best early warning indicators for process stability in anaerobic reactors and under unstable conditions accumulation of VFAs may deplete total alkalinity and cause souring of reactor contents (Yasar & Tabinda, 2010; Kavitha, 2009; Lemos, 2007). The ratio of VFA/alkalinity has been used to monitor the metabolism of anaerobic digesters and detect signs of process deterioration (Li *et al*., 2014). A VFA/alkalinity ratio Less than 0.4 indicates process stability, a ratio between 0.4 and 0.8 indicates a shift toward process instability and a ratio greater than 0.8 indicates a suppression of methanogens and a potential process failure (Hampannavar & Shivayogimath, 2010; Callaghan *et al*., 2002). In this study, the concentration of VFAs and alkalinity of the treated effluent during the operational period ranged between 204 mgAAE/L and 236 mgAAE/L and 4387 mg$CaCO_3$/L and 5223 mg$CaCO_3$/L (Figure 16B). The corresponding VFA/alkalinity ratio was between 0.043 and 0.046, indicating process stability and compares well with the study of Ragen *et al*. (2001) (Table 7). Furthermore, the pH of the effluent remained between 7.2 and 7.7 (Figure16C) and was comparable with previous studies treating synthetic starch, winery grain distillery, food waste, slaughterhouse and sugarcane molasses wastewater (Lu, 2016; Gie, 2007; Han *et al*., 2005; Caixeta *et al*., 2002; Gonzalez *et al*., 1998) (Table 7). A maximum biogas production rate of 1.09 was obtained at a C/N ratio 22.8 mgTOC/mgN and temperature of 38°C. The methane content of the biogas ranged between 65.8% and 70.7%. In the treatment of SIWW by a small-scale (7.95 L) UASB, Hampannavar and Shivayogmath (2010) reported a maximum biogas production of 4.66 L/L.d, with a methane content ranging between 73 and 82%. Tanksali

(2013) reported an increase of biogas production to a maximum of 1.6 L/L.d with a methane content of 71% for the treatment of synthetic SIWW, also in a small-scale (8.4 L) reactor. In addition to different reactors and influent, the dissimilarity of the biogas production rate to that obtained by Hampannavar and Shivayogmath (2010) and Tanksali (2013) may be, among other factors, attributed to the fact that the UASBs were inoculated with inocula from different sources. It is likely that each source would contain distinctive microbial populations with distinctive metabolic functions (Shah *et al*., 2014). This would translate into different systems having different biogas production rates, specific methanogenic activities (SMA) and biomethane potentials (BMP).

4.2.1 The effect of reactor scale and substrate on the performance of UASBs treating sugar industry wastewater

Apart from differences in the inoculum, the variations in the performance of UASBs treating synthetic or real SIWW could be attributed (wholly or partially) to the differences in the substrate and/or the mixing properties with respect to reactor size (Ruffino *et al*., 2015; Ragen *et al*., 2001). For example, Tanksali. (2013) used a readily biodegradable sucrose-based solution, and Gonzalez *et al*. (1998) used a molasses-based substrate with 72% total sugar content. The molasses used in this study had a total sugar concentration of only 40.9%, with the remainder of the solids being unidentified. It is possible that the remaining fraction was more recalcitrant, as described by Ruffino *et al*. (2015).

The contents of small scale reactors are easily mixed compared to pilot and full-scale reactors. The dead-zones in the sludge bed of a pilot scale reactor caused by ineffective mixing causes poor mass transfer and diffusion of the substrate from the bulk liquid to the biomass and therefore reduces substrate uptake as compared to easily mixed contents of small scale reactors (Metcalf & Eddy, 2003; Lettinga *et al*., 2001).

4.2.2 Conversion of organic compounds

Figure 17 depicts the concentration of organic compounds of the treated effluent at varying operational conditions. The temperature of the UASB at runs 3, 4, 5 and 8 was maintained at a constant temperature of 31.5°C (Table 13). The C/N ratio of the feed at run 3, 4 and 5 varied between 22 and 38 and the concentration of glucose in the treated effluent remained low between 25 mg/L and 38 mg/L. The glucose concentration increased substantially to a peak of 143 mg/L with increasing C/N ratio of 50 mgTOC/mgN at run 8. This may be explained by the fact that acidogenic bacteria which utilise glucose as a source of carbon may decrease due to the nitrogen deficient environment caused by an increase in C/N ratio (Shah *et al*., 2014). In comparison to the other runs, a high concentration of sucrose (841

mg/L) was detected at run 4 when temperature and C/N ratio was 31.5°C and 22 mgTOC/mgN, respectively. The accumulation of sucrose can be attributed to poor hydrolysis of sucrose due to undernurishment of sucrose hydrolysing bacteria at 31.5°C (Schnurer & Jarvis, 2009; Lettinga *et al*., 2001)

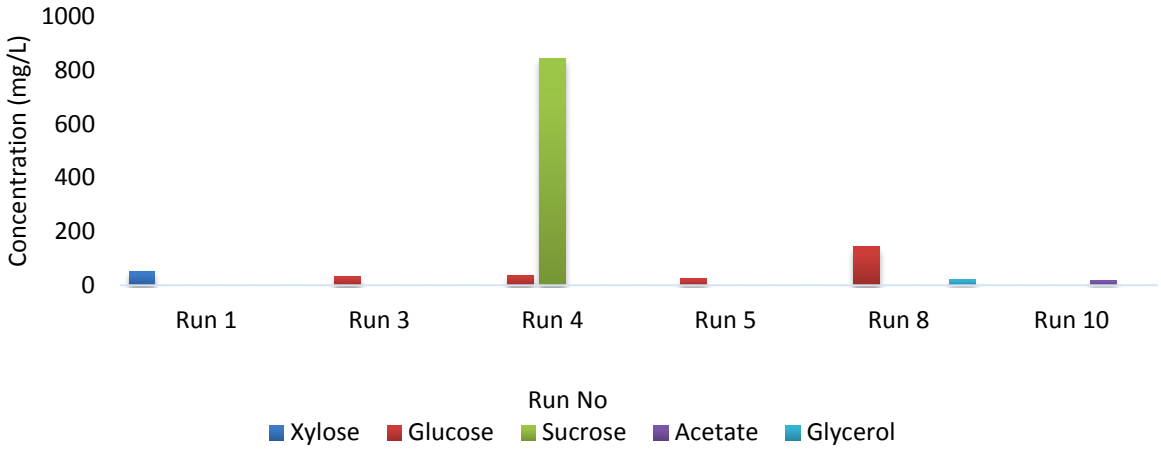


Figure 17: Concentration of some organic compounds in the effluent of the UASB during operation period.

Acetate, xylose and glycerol were present in the effluent at low concentrations of 19.5, 51.2 and 21 mg/L, respectively during runs 10, 1 and 8 (Table 13). During runs 2, 6, 9 and 11, no sugars or organic acids were detected in the final effluent. Other short chain VFAs (butyric and propionic acid) were not detected in the treated effluent of the UASB during any of the runs, indicating a good uptake of these VFAs (if present) by functional bacteria.

4.3 Microstructure and morphology of granules

During the last day of treatment (217th day), a sample of granules was extracted from the base of the UASB for analysis of the bacterial community structure and granule structure. The granule structure was visualised using scanning electron microscopy (SEM) (Figure 18). The surface of the granules was covered in slime, and exhibited micropores. It has been shown that abiotic and heterogeneous polymers such as melanoidins attach on the surface and form a hydrated slime-layer on UASB granules (Lu *et al*., 2015). The organic matter adsorbs onto the micropores, where it is biodegraded and biomethane is produced. Acetate-utilising methanogens (e.g. *Methanosaeta*) and volatile organic acid (butyrate and propionate) users (e.g. *Methanosarcina* spp.) are thick rod-shaped bacteria and cotton-like filamentous bacteria which could resemble those shown in Figure 18A and 18B (Narihiro *et al*., 2009; Angenent *et al*., 2004; Hulsoff Pol *et al*., 2004).

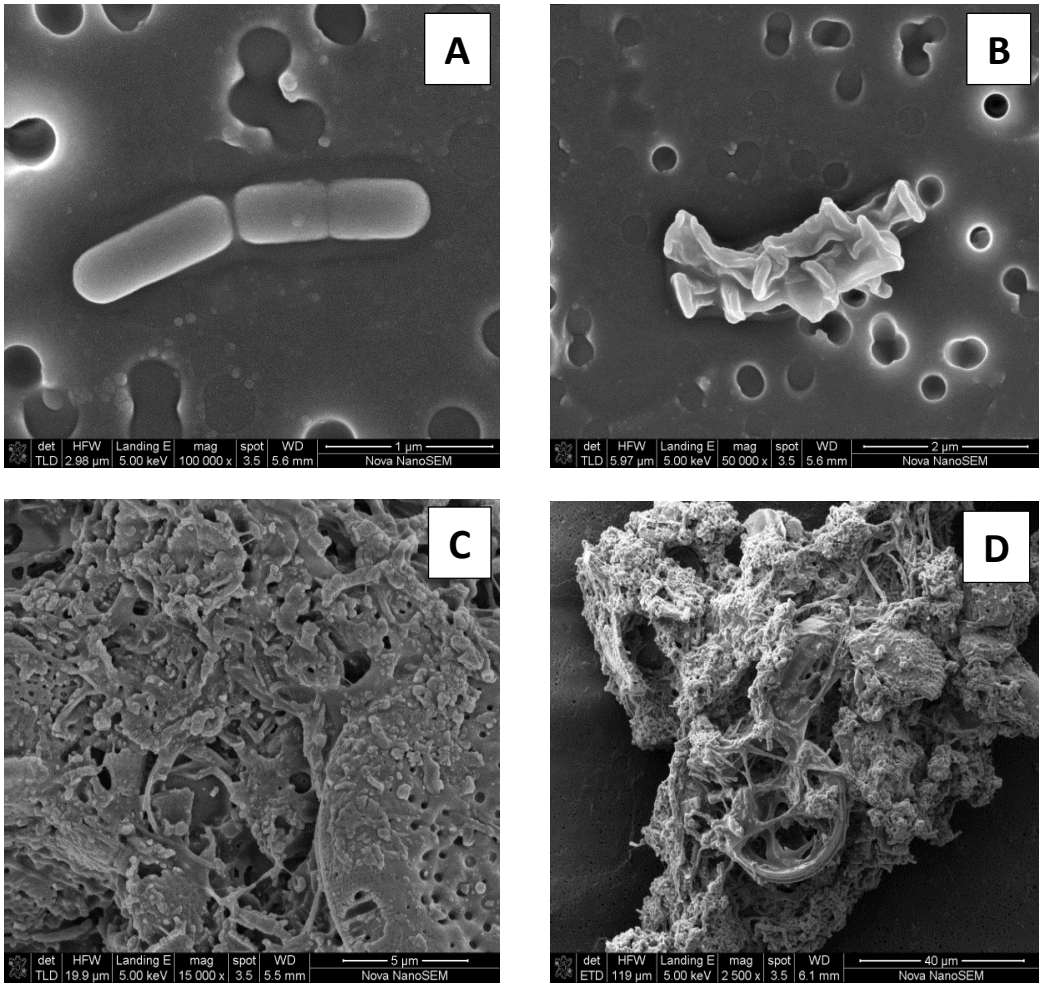


Figure 18: Scanning electron micrographs of UASB granules under 100 000x magnification (A), and 50 000x magnification showing slime, rod-shaped bateria and micropores (B), 15 000x magnification (C), and 2 500x magnification (D).

4.4 Bacterial and archaeal community structure in UASB granules

Figure 32 and Table 22 in Appendix D show the distribution of all the bacteria/archaea in the UASB granules detected using NGS. The population was highly diverse, with the most abundant genera being *Kosmotoga* spp. (18.3%), uncultured species E6 (17.5%) and OPB95 (16.6%). *Kosmotoga* spp. are thermophiles, growing over a temperature range of 20-80^{o}C. They are phylogenetically similar to the mesophilic *Thermotogales* order because of their ability to reproduce at high rates at 37^{o}C. In this study, the UASB was operated at mesophilic conditions (25-40^{o}C), ideal for the growth of *Kosmotoga* spp. The uncultured species E6, related to the genus *Synergistales* spp. has also been found to dominate in granular sludges of EGSB treating ionic components of laundry wastewater and UASBs treating potato processing wastewater and saline phenolic wastewater (Antwi *et al*., 2017; Wang *et al*., 2017; Delforno *et al*., 2014). The uncultured species of OPB95 was found to be

supported by hydrogen metabolism in obsidian pool (OP) sediment rich in reduced iron, sulphide, CO_2 and H_2 (Hugenholtz *et al*., 1998). OPB95 was also found to be a major population in phenol-degrading sludge used for the anaerobic treatment of phenolic wastewater (Fang *et al*., 2006).

In the class Methanobacteria and order *Methanosarcinales*, *Methanosaeta* spp. was the dominant genus, with an abundance of 3.1%. *Methanosaeta* spp. have previously been reported in granular sludge of a UASB reactor treating brewery wastewater (Diaz *et al*., 2006; Liu *et al*., 2002), and phenol (Na *et al*., 2016).

Other uncultured species related to the class *Actinobacteria* exhibited an abundance of 5.8%. These bacteria have been shown to play an important metabolic role in reducing carbohydrates in anaerobic digesters, and *Clostridium* spp. (2%) have been found in syntrophic association with hydrogenotrophic methanogens (Vanwonterghem *et al*., 2014). In the mesophilic anaerobic digestion of beet silage, *Clostridium* spp. have been found to exhibit a variety of metabolic functions, including homoacetogenesis, syntrophic acetate oxidation and production of fermentation products (VFAs and alcohols) from cellulosic substrates (Krakat *et al*., 2011). In a full scale anaerobic reactor digesting activated sludge, Guo *et al*. (2015) found *Clostridium* spp. to be the main acid fementers producing VFA, CO_2 and H_2. The authors also found that the unclassified *Treponema* genera associated with the *Clostridia* play an impotant role in acetate production in the acetogenesis stage. Other dominant families detected in the granules of the UASB are shown in Figure 18.

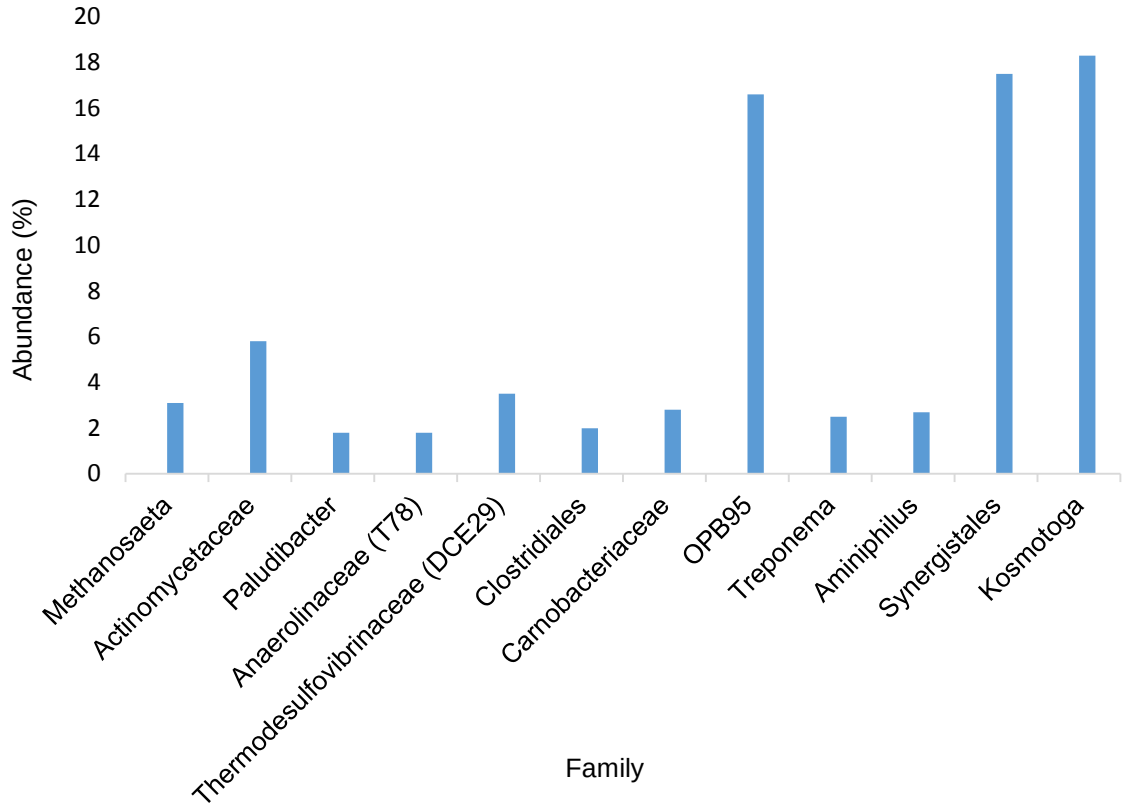


Figure 19: The most abundant bacterial and archaeal families found in the UASB granules using next generation sequencing of the 16S rRNA metagenome

4.5 Statistical analysis and diagnostic assessment of the model

Table 14 shows the experimental parameters applied and the measured responses. According to the factorial design protocol, a total of 11 runs, inclusive of 3 centre points, were carried out. Each run was continued until the COD removal efficiency did not change by > 5% from day to day. This was within 3-6 days for each run. Based on the COD removal efficiency and biogas production rate, the empirical models represented by Equations 14 and 15 were developed by applying regression analysis using response surface methodology. The empirical models for predicting COD removal efficiency and biogas production rate in terms of coded factors are as follows:

$$COD\ removal\ (\%) = 77.05 - 0.32A - 0.90AB - 0.62A^2 + 1.03B^2 \qquad \text{Equation 14}$$

$$Biogas\ production\ (L/Ld) = 0.69 + 0.12A - 0.022B + 0.12B^2 \qquad \text{Equation 15}$$

Where A is temperature (˚C) and B is C/N (mgTOC/mgN). The statistical significance of the model terms and their interaction effects was examined using the Prob > F and the quality of fit of the models is accepted at a Prob > F less than 0.05 that is, at 95% confidence interval (Wang *et al*., 2013; Asadi & Ziantizadeh, 2011).

Model terms and interaction terms with Prob > F greater than 0.1 are statistically insignificant and were eliminated from the quadratic models (Sathish & Vivekanandam, 2016). The results for analysis of variance (ANOVA) for the quadratic equations are summarised in Table 15.

Table 14: Design matrix of central composite design and corresponding responses

Run order	Run No	Factors		Responses	
		(A) Temperature	(B) C/N ratio	COD removal	Biogas production
		(^oC)	(mg TOC/mg N)	(%)	(L/L.d)
5	1	25	36	77.7	0.74
3	2	25	50	77.7	0.64
9	3	31.5	36	77.2	0.67
7	4	31.5	22	79.1	0.78
11	5	31.5	36	76.8	0.64
1	6	25	22	76.9	0.66
6	7	38	36	75.8	0.80
8	8	31.5	50	77.7	0.86
10	9	31.5	36	76.5	0.59
2	10	38	22	78.7	1.06
4	11	38	50	75.9	0.87

After eliminating terms and interactions which were statistically insignificant (Prob > F greater than 0.1), Equations 14 and 15 were obtained. The linear and quadratic terms of temperature (A & A^2) in Equation 14 and C/N ratio (B & B^2) in Equation 15 were insignificant with Prob > F value of 0.2906, 0.1942, 0.6022 and 0.0739. The linear interaction term of temperature and C/N ratio (AB) shown in Equation 14 was significant with a Prob > F value of 0.0407, indicating that they had a significant effect on COD removal efficiency. In Equation 14 the effect of B^2>A>B>A^2>AB with coefficient values of 1.03, -0.32, -0.57, -0.60 and -0.9, respectively. In Equation 15 the effect B^2 is equal to the effect of A but greater than the effect of B with coefficient values of 0.12 and -0.022. According to the ANOVA, Equation 14 was found to be insignificant with a Prob > F value of 0.0747. A lack of fit (LOF) test, as shown in Table 15, was also used to compare the residual error to pure error from the design points. Ideally a model should not lack in fitting the experimental data and should have insignificant LOF (i.e. Prob > F greater than 0.1) (Mourabet *et al*., 2017; Cheng *et al*., 2012). The LOF for Equations 14 and 15 was insignificant, with Prob > F values of 0.166 and

0.118, indicating that there was 16.6% and 11.8% chance that LOF could occur due to noise.

The determination coefficient (R^2) explains the overall efficiency of the model, and a model with a R^2 value nearer to 1 represents and fits the experimental data adequately (Cheng *et al*., 2012). Predicted R^2 (R^2_{pred}) is an indicator of how precise the model is in predicting a response value, and the adjusted R^2 (R^2_{Adj}) is the proportion of variance (difference) in the response variable explained by the estimate in the experimental data (Everitt & Howell, 2005). For a model, the R^2_{pred} and R^2_{Adj} should be within 0.2 of each other to be in good agreement (Mourabet *et al*., 2017). The predictions for COD removal efficiency and biogas production rate as shown in Figure 20 were relatively close to the diagonal lines and were randomly distributed along the line, suggesting a good correlation between the predicted and experimental values of the models. However, there were also outliers, which indicate the presence of residual errors. In this case the R^2 value was 0.8 for Equation 14 and 0.65 for Equation 15, meaning that 80% of the variations in COD removal efficiency could be explained by Equation 14 and 65% of the variations in biogas production rate could be explained by Equation 15. A high R^2 value of 0.8 and 0.65 signifies a good correlation of the models between predicted responses and actual experimental data. The difference of R^2_{pred} and R^2_{Adj} for both models was >0.2. This indicates a block effect or potential problems with the models and the negative predicted R^2 of Equation 14 as shown in Table 15 implies that the overall mean may be a better predictor of the response than the model itself. For biogas production rate and COD removal Rastegar *et al*. (2011) obtained a R^2 value of 0.95 and 0.96 which differs quite significantly to the findings of this study. The low determination coefficient value for biogas production may be attributed to noise encountered in the experiment due to deviation of the actual temperature from the set point (Appendix A).

Table 15: Analysis of variance (ANOVA) for response surface quadratic models

Response	Factor	Sum of squares	DF	Mean square	F-value	*p*-value Prob > F	
COD removal (%)	Model	8.79	5	1.76	4.07	0.0747	not significant
	A	0.60	1	0.60	1.40	0.2906	
	B	1.93	1	1.93	4.47	0.0882	
	AB	3.24	1	3.24	7.51	0.0407	
	A^2	0.97	1	0.97	2.25	0.1942	
	B^2	2.70	1	2.70	6.25	0.0545	
	Residual	2.16	5	0.43			
	Lack of Fit	1.91	3	0.64	5.16	0.1666	not significant
	Pure Error	0.25	2	0.12			
	R^2= 0.80						
	R^2_{Adj}= 0.61						
	R^2_{Prej}= -0.66						
Biogas production (L/L.d)	Model	0.12	3	0.041	4.37	0.0495	significant
	A	0.079	1	0.079	8.39	0.0231	
	B	2.817E-003	1	2.817E-003	0.30	0.6022	
	B^2	0.042	1	0.042	4.41	0.0739	
	Residual	0.066	7	9.457E-003			
	Lack of Fit	0.063	5	0.013	7.71	0.1188	not significant
	Pure Error	3.267E-003	2	1.633E-003			
	R^2= 0.65						
	R^2_{Adj}= 0.50						
	R^2_{Prej}= 0.06						

DF = degrees of freedom

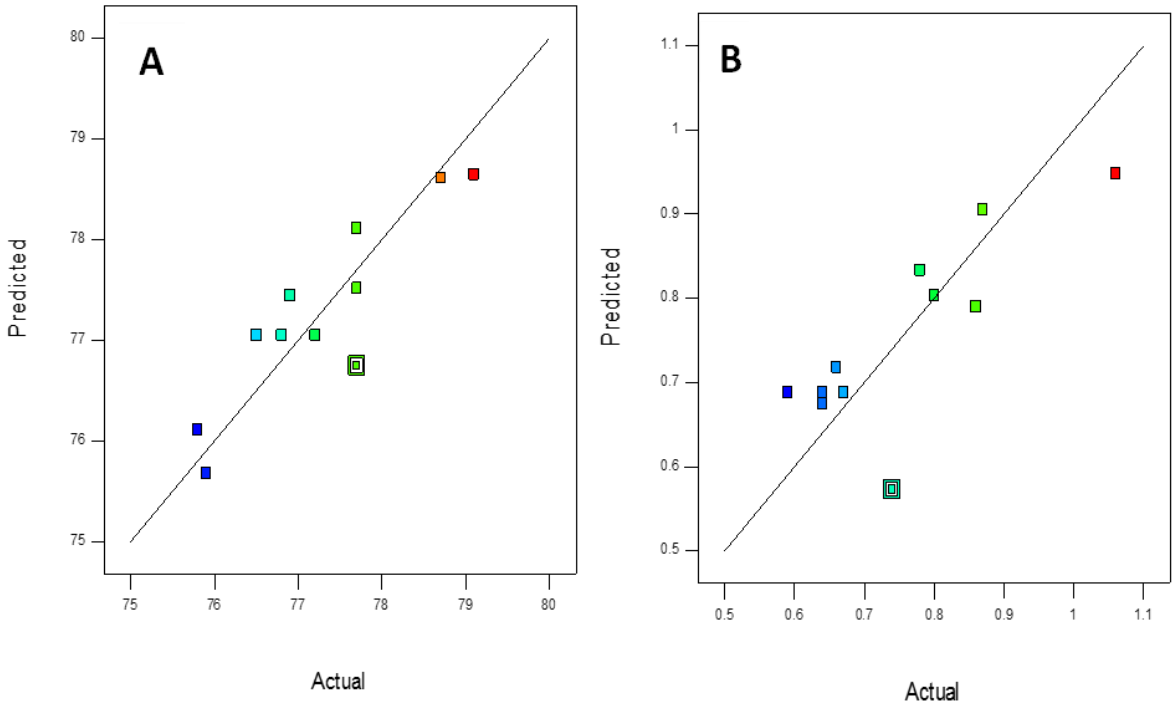


Figure 20: Actual versus predicted responses for COD removal efficiency (A) and biogas production rate (B)

4.5.1 Interaction of temperature and C/N ratio on COD removal efficiency

The contour plot and three-dimensional (3D) response surface plot was constructed using Equation 14 (Figure 21), and depicts the COD removal efficiency of the UASB reactor as a function of temperature and C/N ratio. As shown by the colour coding, the contour and the surface becomes hot (red) at high COD removal efficiencies and cold (blue) at low COD removal efficiencies. As shown in Figure 21 there was clearly a combined effect of temperature and C/N ratio on COD removal at constant HRT (48.9 hrs). As the C/N ratio decreased from 36 mgTOC/mgN to 22 mgTOC/mgN and the temperature increased from 25°C to 38°C, the COD removal efficiency increased from 76% to 78.7%. A negative trend is observed when C/N ratio increase from 36 mgTOC/mgN to 50 mgTOC/mgN at constant temperature of 38°C. The highest COD removal efficiency of 79.1% was obtained when the C/N ratio was 22 mgTOC/N at a temperature of 31.5°C. Increasing temperature enhances hydrolysis and increases diffusion and mass transfer of soluble organics from the substrate to the biomass therefore resulting in rapid substrate uptake (Appels *et al*., 2008 and Chen, 1995). The decrease in COD removal with increasing C/N ratio can be attributed to poor substrate degradation due to low microbial growth rate in the digester caused by nitrotrogen deficient environment at high C/N ratio of 50 mgTOC/mgN (Adriamanohiarisoamanana *et*

al.,2017; Zeshan *et al*., 2012; Yen & Brune, 2007). Most studies agree on an optimal C/N ratio to range between 20 and 35 (Dioha *et al*., 2013; Wang *et al*., 2013; Wang *et al*., 2012) which is in agreement with this study. C/N ratio as low as 15 has been reported (Wang *et al*., 2014; Siddiqui *et al*., 2011). Such C/N ratio (15) may result in a toxic environment for functional bacterial growth due to ammonia accumulation and may give poor performance in anaerobic reactors (Wang *et al*., 2014; Dioha *et al*., 2013; Akunna *et al*., 1992). For example, in the anaerobic digestion of sludge with different C/N ratio (8, 12 and 15) at 35°C the degradation efficiency obtained was only 57.1, 51.9 and 51.0%, respectively (Caijie *et al*., 2015)

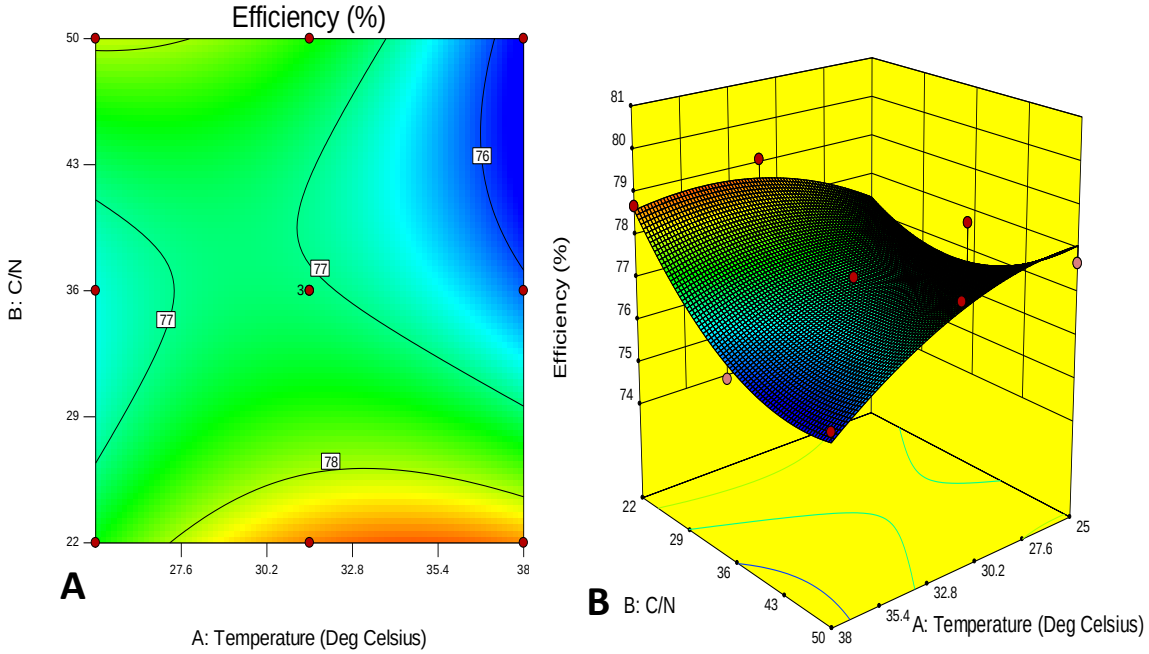


Figure 21: Contour plot (A) and response surface plot (B) of COD removal efficiency as a function of temperature and C/N ratio

4.5.2 Interaction of temperature and C/N ratio on biogas production rate

Figure 22 depicts the contour and response surface plots for biogas production with respect to temperature and C/N ratio. As expected, the biogas production rate increased with a decrease in C/N ratio and increase in temperature. Biogas production increased up to a maximum of 0.94 L/L.d when temperature rose from 25°C to 38ºC and from C/N ratio of 36 mgTOC/mgN to 22 mgTOC/mgN, respectively. As with COD removal, a negative effect on biogas production is also observed as temperature decreases from 38°C to 25°C. In the co-digestion of dairy manure, chicken manure and rice straw, improved methane potential was associated with an increase in temperature of up to 35°C and ammonia inhibition was attributed to a low C/N ratio of <15 (Wang *et al*., 2014). Increasing temperature to the margin of the mesophilic range (38-40°C) enhances hydraulic turbulence by reducing the viscosity

of the reactor contents. Microbial metabolic rates increase due to increased absorption of substrate and more biogas is produced (Abdelgadir *et al*., 2014). Lower C/N ratio increases nitrogen concentration and accumulation of ammonium-nitrogen which elevates pH. At pH levels exceeding 8.5, toxic environments that may inhibit methane formers prevail. Conversely, low concentration of nitrogen resulting from high C/N ratio of feed limits the availability of proteins for functional bacterial growth and biogas production decreases due to low microbial populations in the digester (Matheri *et al*., 2017; Abdalla & Hammam, 2014; Zeshan *et al*., 2012; Yen & Brune, 2007). Some studies agree on an optimal temperature of between 25 °C and 38°C and an optimal C/N ratio between 20 and 35, which is in agreement with this study (Pavi *et al*., 2017; Tanimu *et al*., 2014).

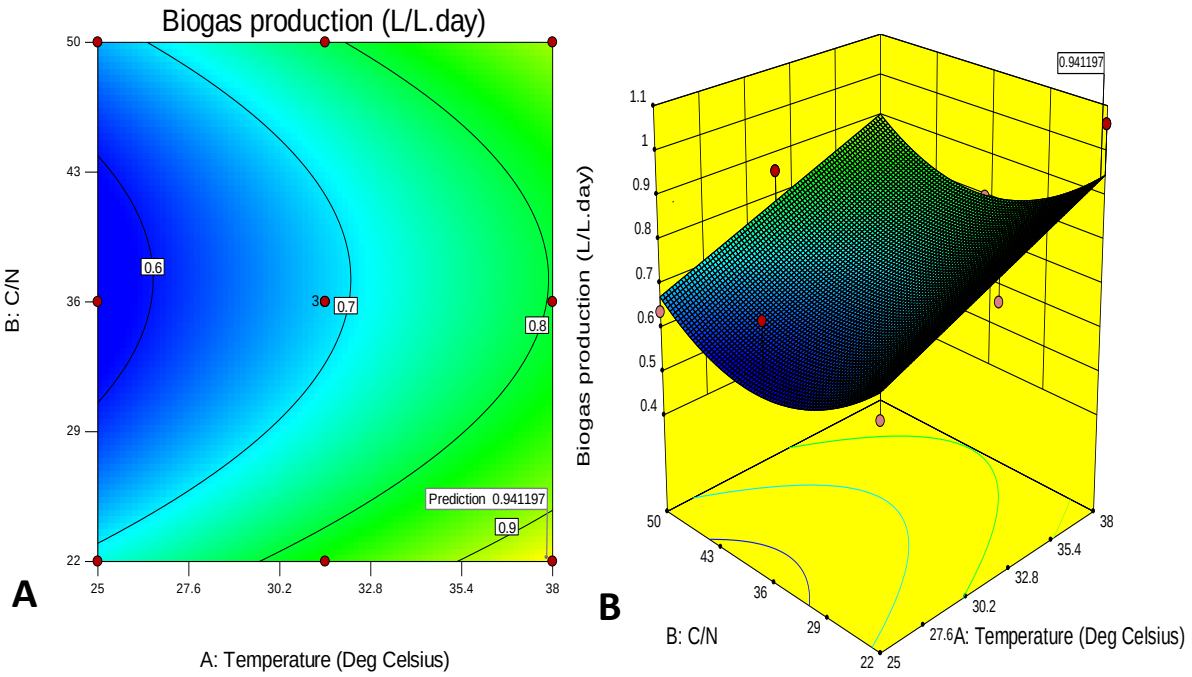


Figure 22: Contour plot (A) and response surface plot (B) of the biogas production rate as function of temperature and C/N ratio

4.5.3 Optimisation of UASB operating conditions and model validation

Graphical optimisation was used to fine-tune the region in which the C/N ratio and temperature in the ranges used in this study allowed optimal operation of the UASB in terms of COD removal and biogas production (Figure 23). The yellow region (enclosed by five contours on the overlay plot) displays the area which is most feasible for operation of the UASB. The COD removal efficiency and the biogas production rate represent the substrate metabolism rate and methanogenic activity of the digester, respectively. The aim of this study was to determine the optimal combination of the two parameters. Two optimal combinations of 38°C and 22 mgTOC/mgN, and 30°C and 50 mgTOC/mgN, were suggested by the model (Table 16). The latter displayed the highest total desirability (0.806). The

desirability scale ranges from zero, being the least desirable and one being the most desirable (Mourabet *et al*., 2015). The corresponding COD removal efficiency and biogas production rate under the optimal temperature and C/N ratio was 78.6% and 0.948 L/L.d, respectively. The quadratic models were validated by running the predicted optimal conditions shown in Table 16. The experimental values did not differ significantly from the predicted values (Table 16), therefore the quadratic models for COD removal efficiency and biogas production rate were confirmed to be adequate.

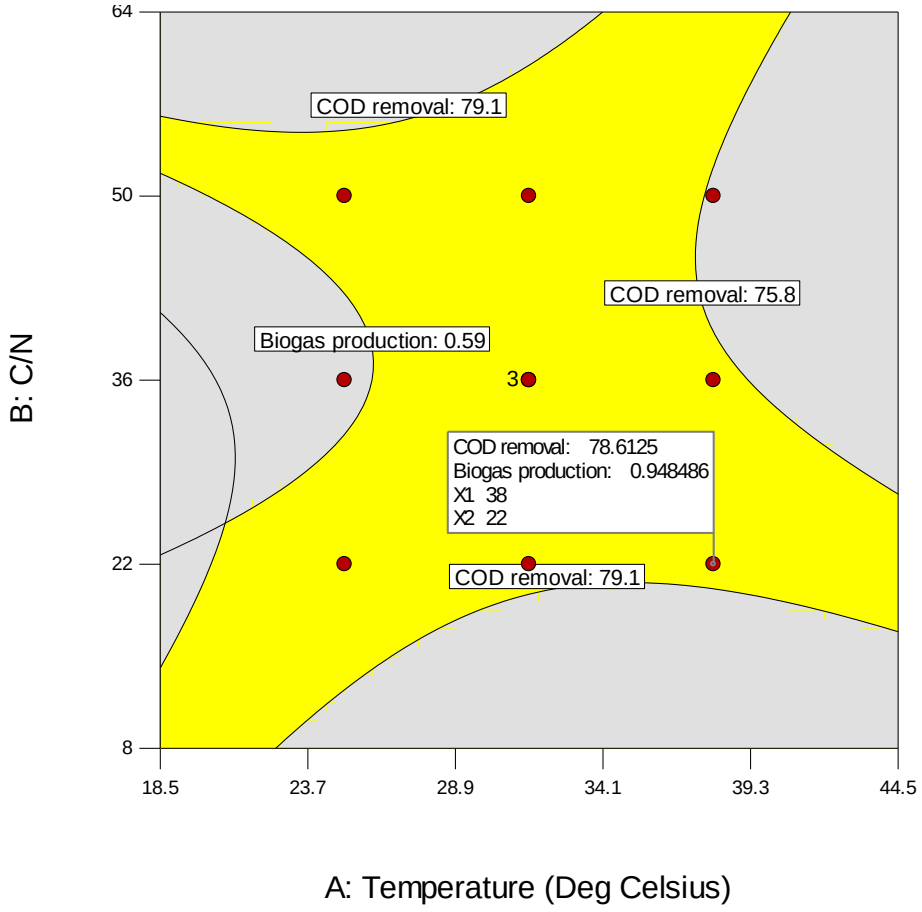


Figure 23: Overlay plot showing optimal operating conditions of the UASB within the range of parameters tested

Table 16: Predicted and actual responses

Solution	Temp. (ºC)	C/N (mgTOC/ mgTN)	COD removal (%) (model)	COD removal (%) (experiment)	Biogas (L/L.d) (model)	Biogas (L/L.d) (experiment)	Desirability
1	30.90	50	77.6	75.4	0.779	0.691	0.471
2	38.00	22	78.6	77.7	0.948	0.832	0.806

CHAPTER FIVE
CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

The aim of this study was to examine the performance of a UASB reactor treating sugar industry effluent and optimise COD reduction and biogas production. Temperature and feed C/N ratio were investigated as the main parameters.

The results showed that an empirical model for predicting COD removal and biogas production could be developed using Design-Expert® Software Version 10. A half factorial CCD design with a total of 11 runs inclusive of 3 center points was applied for the optimisation of reactor temperature and feed C/N ratio. Regression analysis of the results yielded second order quadratic models. The quadratic models were found to be statistically significant with Prob > F values of 0.0747 and 0.0495 and the determination coefficients (R^2) were found to be 0.80 and 0.65 indicating a good correlation between predictions and actual values. The results obtained using RSM indicate that interaction terms had significant effect on COD removal and biogas production. A combination of C/N ratio and temperature of 22 mgTOC/mgN and 38°C was required for optimal COD reduction and biogas production of 77.7% and 0.832 L/L.d, respectively within the range of parmaters tested.

The UASB successfully reduced the COD of synthetic SIWW from from a high COD concentration of 4155 mg/L to 884 mg/L.The average COD concentration of the treated effluent from the UASB did not comply with the applicable South African discharge limits. An additional polishing step would therefore be required before discharge.

In terms of biogas production, HRT, methane content and organic removal in the treatment of authentic SIWW, the current system exhibited a distinct performance when compared to other pilot and small scale UASBs treating authentic and synthetic SIWW (Table 17). This may have been related to different inocula, influent substrates, and reactor scales (less effective mixing of substrate and granules in larger reactors). No results about biogas production rates in pilot or full-scale UASBs could be found in literature, but the small scale reactors used by Tanksali (2013) and Hampannavar and Shivayogimath (2010) produced biogas at a maximum of 1.6 L/L.d and 4.66 L/L.d which is greater than 1.09 L/L.d reported in this study. Gonzalez *et al*. (1998), and Hampannavar and Shivayogimath (2010) operating smaller UASBs, also under mesophilic conditions, but with shorter retention times, obtained biogas with higher methane content (Table 17). Although the methane content obtained in this study and the study conducted by Tanksali (2013) was lower, the COD removal rate was more stable (Table 17). The organic removal rate obtained in this study compared favourably to other studies focussing on the treatment of molasses, with the exception of a pilot-scale

study conducted by Atashi *et al*. (2010), where 90% removal was achieved with a considerably shorter HRT. However, these authors did not report on biogas production rates (Table 17). During this study, when the HRT was reduced, biomass washout occurred with resultant operational failure. However, Atashi *et al*. (2010), Hampannavar and Shivayogimath (2010), and Ragen *et al*. (2001) managed to operate UASBs at low HRT without excessive biomass washout (Table 17), this could be due to good settling characteristics of the inocula used in the afforementioned studies as compared to the current study.

Table 17: Performance of small-scale and pilot-scale UASB reactors treating SIWW

Scale	Reactor capacity (L)	HRT (hrs)	Temp. (range)	Biogas (L/L.d)	CH_4 (%)	Organic removal (%)	Reference
Pilot	16.5	39-13	Mesophilic		79-94	59-91	Gonzalez *et al*., 1998*
Small	8.4	48-12	Mesophilic	1.6	71	80-96	Tanksali, 2013**
Pilot	140	8-100	Thermophilic	NM	NM	39-67	Harada *et al*., 1996*
Pilot	500	5-6	Mesophilic	NM	NM	90	Atashi *et al*., 2010*
Small	7.95	6	Mesophilic	4.7	73-82	89	Hampannavar & Shivayogimath, 2010***
Small	10	10	NM	NM	NM	60-81	Ragen *et al*., 2001*
Pilot	46	49	Mesophilic	1.1	66-71	75-79	This study*

Mesophilic = 25 - 40°C, Thermophilic = 40 - 70°C

*** Molasses based substrate**

**** Sucrose based substrate**

***** Actual sugar industry wastewater**

This study confirmed previous studies which showed that UASB technology can be successfully applied to reduce the organic load of SIWW and reduce the environmental problem caused by discharge of sugar industry effluents into aquatic environments. The UASB exhibited the potential to generate energy through biogas formation, being produced at a maximum of 1.05 L/L.d with a rich methane content of 66.5%. This biogas could potentially be used for heating, cooking or be converted to electrical power before or after scrubbing.

5.2 Recommendations for future work

- In order to understand the biomethane potential (BMP) of the inoculum, the specific methanogenic activity (SMA) of the granular sludge should be determined prior to inoculating UASBs.
- In order to improve the COD removal rates, additional research should be conducted to address challenges such as pH shock and souring of reactor contents in UASBs treating SIWW.
- A cost-effective polishing step should be incorporated in order to further reduce the COD concentration of the treated effluent to comply with the South African discharge limit.
- Further research should be conducted to evaluate the environmental impact, economic viability and technical feasibility of the technology for full-scale implementation for the South African sugar industry.
- Additional experiments should be conducted to look at the microbial composition and function in the sludge throughout the sludge bed and relate this to the treatment efficiency in that particular spatial position.
- The produced biogas should be scrubbed with a solution of sodium hydroxide to increase the methane content by removing CO_2.
- The mass transfer rate between the soluble organic compounds and the biomass could be improved by incorporating a mixing mechanism or baffles in the UASB.

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Appendix A: Experimental results of UASB during operation period

Table 18: **COD removal efficiency, biogas production, effluent pH and alkalinity and effluent VFA concentration of UASB reactor during start-up**

Period, days	Influent COD, mg O_2/L	HRT, (hr)	OLR, KgCOD/m^3.d	Effluent COD, mg O_2/L	COD removal efficiency,%	Biogas production, L/L.d	Influent-Effluent pH	Influent alkalinity, mg $CaCO_3$/L	Effluent alkalinity, mg $CaCO_3$/L	VFA, mg/L
7	3815	30.7	4.88	3048	20.1	ND	ND-6.4	ND	ND	ND
9	3691	30.7	4.72	2757	25.3	ND	ND-6.8	ND	ND	ND
17	3741	30.7	4.79	2730	27	ND	ND_7.0	ND	ND	ND
22	3280	30.7	4.20	2296	30	ND	ND-7.1	ND	ND	ND
31	3610	30.7	4.62	1745	51	ND	ND-7.1	ND	ND	ND
36	3705	30.7	2.89	1940	47.6	ND	ND-6.5	ND	ND	ND
41	2230	30.7	2.85	1275	42.8	0.56	ND-7.8	ND	3853	433
45	4263	40.3	2.53	1220	71.3	1.17	4.28-6.9	2472.12	2839	ND
51	3777	40.3	2.24	1705	54.9	1.04	6.10-7.0	1286.1	2066	322
58	4202	40.3	2.45	788	81.2	0.99	4.96-7.4	358.3	3298	172
65	3627	47.5	1.83	1397	61.4	0.65	8.19-7.2	3425.44	2809	306
72	3763	51.1	1.77	930	75.3	1.15	9.47-7.3	3169.67	1711	83
79	3676	50.4	1.75	870	76.3	ND	8-7.5	2870.74	4313	92
97	3750	48.9	1.84	660	82.4	ND	9.1-73	3721	4690	ND
107	3560	48.9	1.74	568	84.0	1.05	ND	ND	ND	95
114	3763	48.9	1.85	1011	73.1	ND	ND	ND	ND	ND
124	3781	48.9	1.86	821	78.3	0.95	9.2-7.3	3755	4598	97
127	3802	30.35	3.00	1500	60,5	0.71	ND	ND	ND	279
131	3852	18.23	5.07	2598	32,5	ND	ND	ND	ND	ND
138	3801	14.05	6.49	2935	22,7	0.31	9.5-7.6	3890	4632	499
142	1880	48.9	0.92	745	60.3	ND	ND	ND	ND	107
144	3250	48.9	1.60	873	73.1	ND	ND	ND	ND	ND
149	3837	48.9	1.88	903	76.4	0.91	9,7-7.4	4120	4785	198
153	4450	48.9	2.18	1050	76.4	0.86	ND	ND	ND	ND
156	4370	48.9	2.14	1188	72.8	ND	9,6-7.3	4023	4647	250
160	3890	48.9	1.91	1021	73.7	0.84	ND	ND	ND	228

Table 19: Experimental results of UASB treatment of synthetic SIWW

Run No	days	Temperature, (ºC)	C/N ratio	ExpTemp (ºC)	Exp TN, mg N/L	Measured C/N ratio	inlet pH	outlet pH	Inlet COD mg O_2/L	Outlet COD mg O_2/L	CODs mg O_2/L	BOD mg/L	TN mg/L	TP mg/L	TS g/L	SO_4^{2-} mg/L
5	4	31,5	36	29-33	53	33.2	9.7	7.3	4005	930	823	238	<0.5	10	ND	67
3	3	31,5	36	30-33	55	32.0	9.6	7.3	4030	917	827	238	<0.5	11	ND	64
9	3	31,5	36	30-33	58	30.3	9.6	7.3	4070	956	822	158	<0.5	13	4.2	67
7	4	38	36	36-39	53	33.2	9.5	7.4	3915	948	799	158	<0.5	18	4	74
11	6	38	50	36-39	38	46.3	9.6	7,3	4030	970	809	98	<0.5	11	3.8	65
1	5	25	36	23-27	57	30.9	9.7	7.2	4060	906	834	138,5	<0.5	10	ND	61
6	5	25	22	23-26	77	23	9.9	7.3	4005	924	824	ND	<0.5	14	4.6	52
8	5	31.5	50	33-34	35	50	9.6	7.3	4005	895	809	39	2.5	11	4.4	57
10	5	38	22	37-40	77	22.8	9.6	7.7	4155	884	795	59	<0.5	14	3,8	59
2	4	25	50	23-27	35	50	9.7	7.4	3830	855	809	159	0.3	9	ND	46
4	4	31.5	22	29-33	77	22.8	9.7	7.3	4185	875	804	180	0.4	9	4.2	52
Confirm	4	30.9	50	30-31	35	50	9.5	7.4	4020	987	830	162	0.7	10	ND	61
Confirm	5	38.0	22	37-39	77	22.5	9.6	7.3	4050	901	785	149	0.6	9	ND	59

Table 19 continued

Run No	days	VFA, mg/L	Alkalinity, mg $CaCO_3$/L	Biogas L/L.d	$\%CH_4$	$\%CO_2$	$\%O_2$	%COD removal
5	4	216	4747	0.637	70.6	28.9	0,3	76.7
3	3	204	4679	0.668	70.7	29.0	0,2	77.2
9	3	229	5223	0.590	69.7	29.5	0,5	76.5
7	4	212	4947	0.803	67.2	32.1	0,5	75.7
11	6	217	4737	0.866	65.8	33.2	0,6	75.9
1	5	226	4387	0.741	68.5	30	0,4	77.7
6	5	235	4987	0.657	70.1	28,9	0,5	76.9
8	5	213	4643	0.861	67.1	32	0,5	77.6
10	5	236	5077	1.059	66.5	31.9	0.7	78.7
2	4	223	4774	0.637	69.7	30.2	0.4	77.8
4	4	228	4647	0.777	68.8	29.6	0.8	79.1
Confirm	4	252	4690	0.691	70.5	28.4	0.7	75.4
Confirm	5	231	4869	0.832	65.2	32.8	0.6	77.7

Table 20: Deviation of operating conditions observed during operation of the UASB

Factor	Design value in CCD	Measured value in experiment	Standard deviation
Temperature, ^{o}C	31.5	30	2.69
	25	24	1.09
	38	37.5	1.48
C/N ratio, mg TOC/mgTN	36	33.2	1.31
	22	22.85	0.05
	50	51.7	1.35

Calculation of standard deviation of actual C/N ratio at run 1

The standard deviation in table 18 was computed using the following equation:

$$\sigma = \sqrt{\frac{\sum\left(X - \overline{X}\right)^2}{n-1}}$$

X	$\overline{X}$	$\left(X - \overline{X}\right)$	$\left(X - \overline{X}\right)^2$
33.20	31.92	1.28	1.63
32.00	31.92	0.08	0.00
33.20	31.92	1.28	1.63
30.30	31.92	-1.62	2.62
30.90	31.92	-1.02	1.04
			$\sum\left(X - \overline{X}\right)^2$ = 6.92

$\sum\left(X - \overline{X}\right)^2$ = 6.92

$n = 5$

$$\sigma = \sqrt{\frac{6.92}{5-1}}$$

$$\sigma = 1.31$$

Calculation of COD removal efficiency at run 1

$$\%COD = \left(\frac{COD_{in} - COD_{out}}{COD_{in}} \right) \times 100 = \left(\frac{4060 - 906}{4060} \right) \times 100 = 77.68$$

Calculation of methane yield at optimal conditions

$$CH_4 = \left(\frac{\%COD_t removal \times G_{pr} \times V_r}{100 \times COD_{re}} \right) \times 1000$$

$$= \left(\frac{77.75 \times 0.832 \times 46}{100 \times 3149} \right) \times 1000$$

$$= 9.45 LCH_4 / gCOD_{re}$$

Appendix B: HPLC results determined using refractive index detector

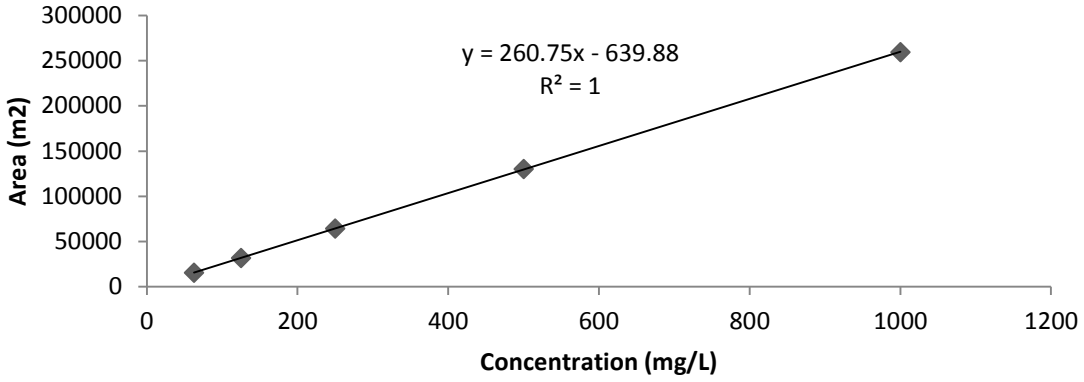

Figure 24: Standard graph for determining concentration of D-(+) xylose

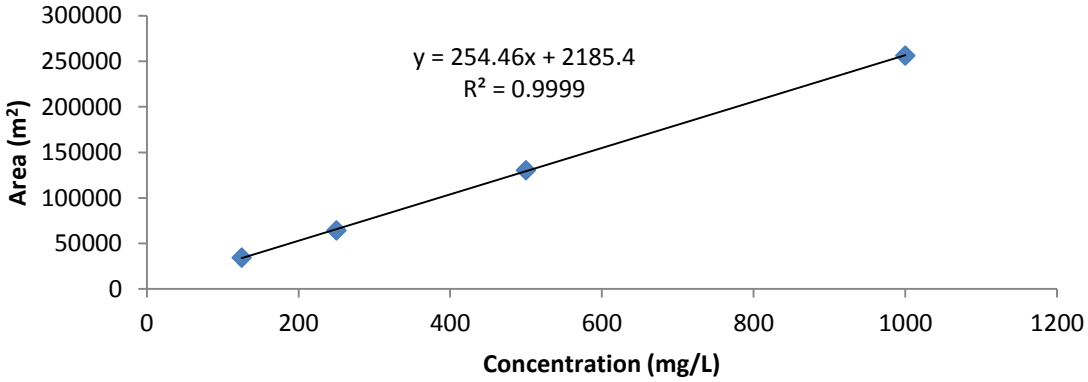

Figure 25: Standard graph for determining concentration of glucose

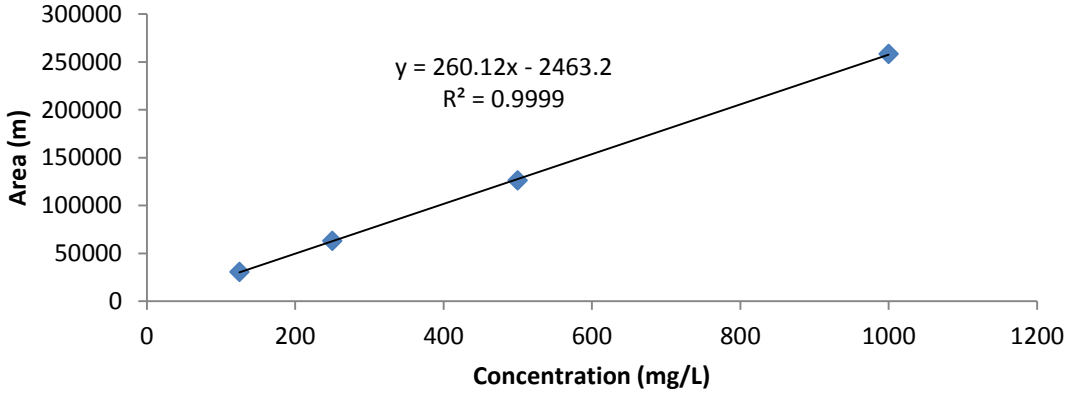

Figure 26: Standard graph for determining concentration of sucrose

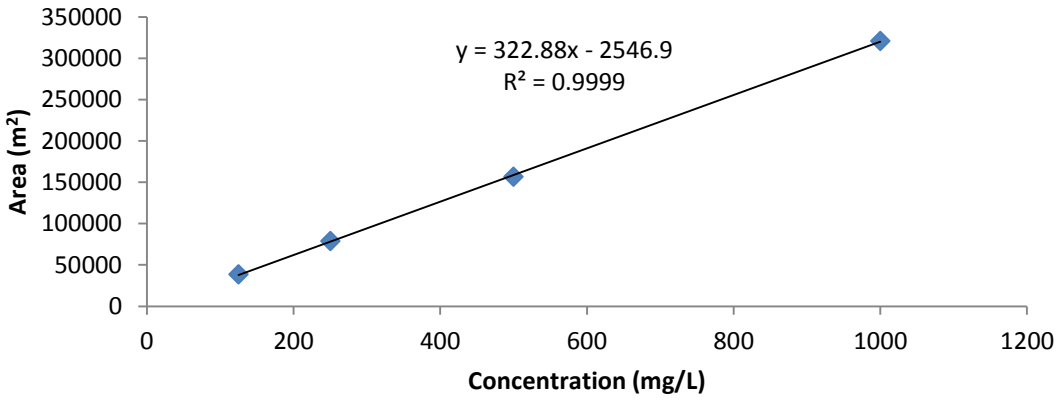

Figure 27: Standard graph for determining concentration of glycerol

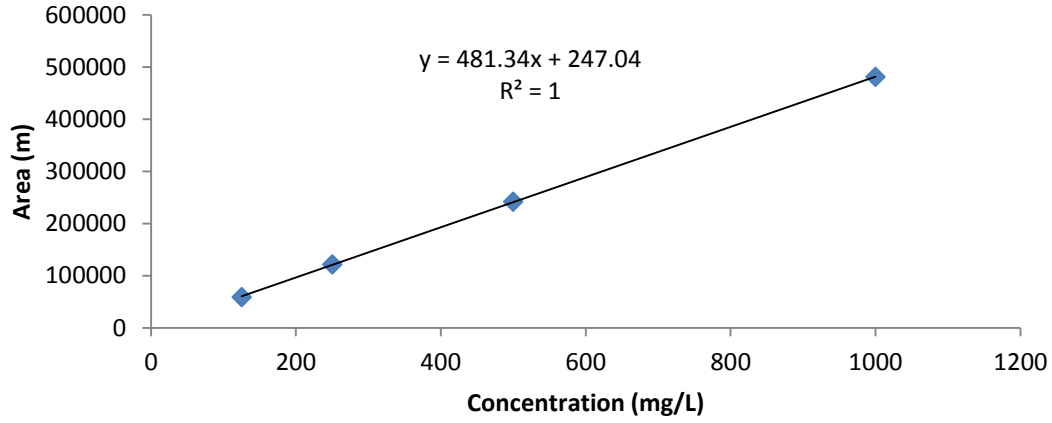

Figure 28: Standard graph for determining concentration of acetate

Table 21: Retention time and concentration of some organic compounds during operation of UASB

Run No	Peak number	Retention time (min)	Component	Area (unit^2)	Concentration (mg/L)
1	1	10.4		49723	
	2	12.2	Xylose	10856	51
	3				
2	1	10.0		48669	
	2	11.9200		3781	
	3				
3	1	9.9		34221	
	2	11.7	glucose	6245	33
	3	14.0		1858	
4	1	9.8	Sucrose	216298	841
	2	11.6	glucose	7569	38
	3				
5	1	9.7		32265	
	2	11.6	glucose	4243	25
	3	13.8		1487	
6	1	10.7		39368	
	2	13.0		5404	
	3	15.4		1251	
7	1	11.0		31859	
	2	13.0		5063	
	3	15.4		1462	
8	1	11.5	glucose	34897	143
	2	13.6		3417	
	3	16.3	Glycerol	3198	21
9	1	12.2		41216	
	2	14.4		5026	
	3	17.0		3095	
10	1	12.9		38120	
	2	18.2	Acetate	2604	19
	3				
Run 11	1	13.6		45732	
	2	19.3		3163	
	3				
Run 12 (confirmation)	1	14.8		45646	
	2	17.4		7366	
	3	20.8		3773	

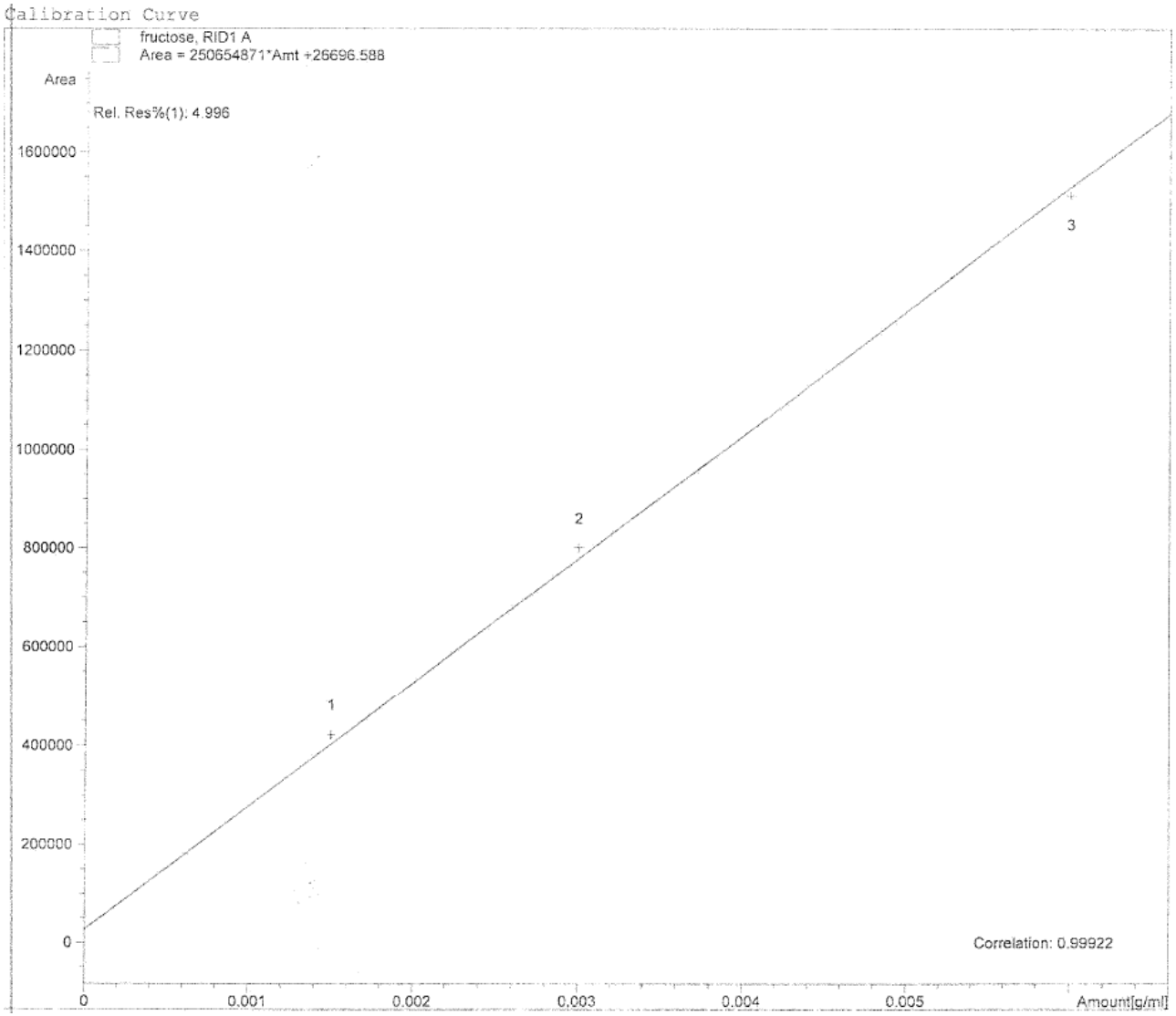

Figure 29: Standard graph used for determining concentration of fructose in the molasses

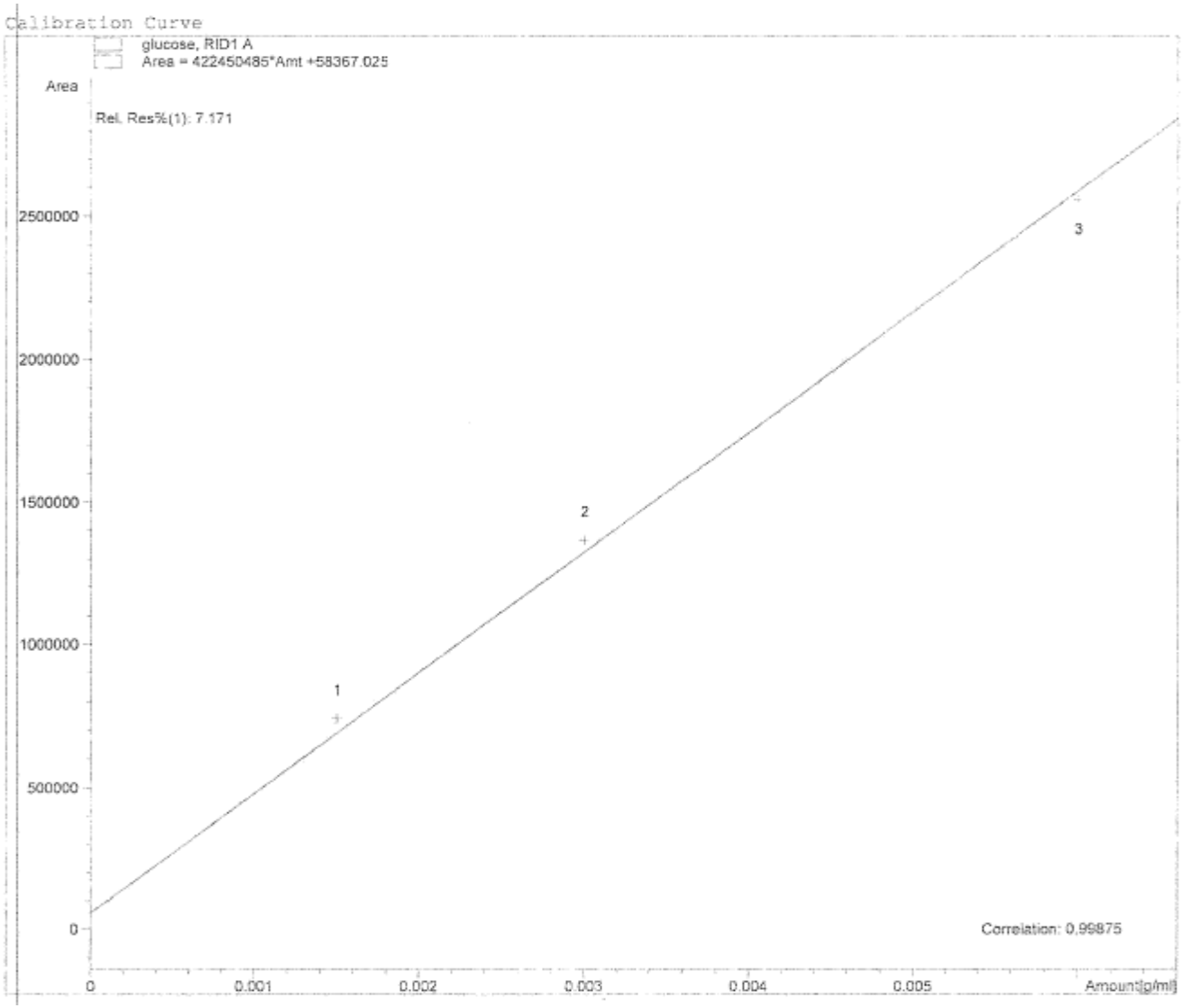

Figure 30: Standard graph used for determining concentration of glucose in the molasses

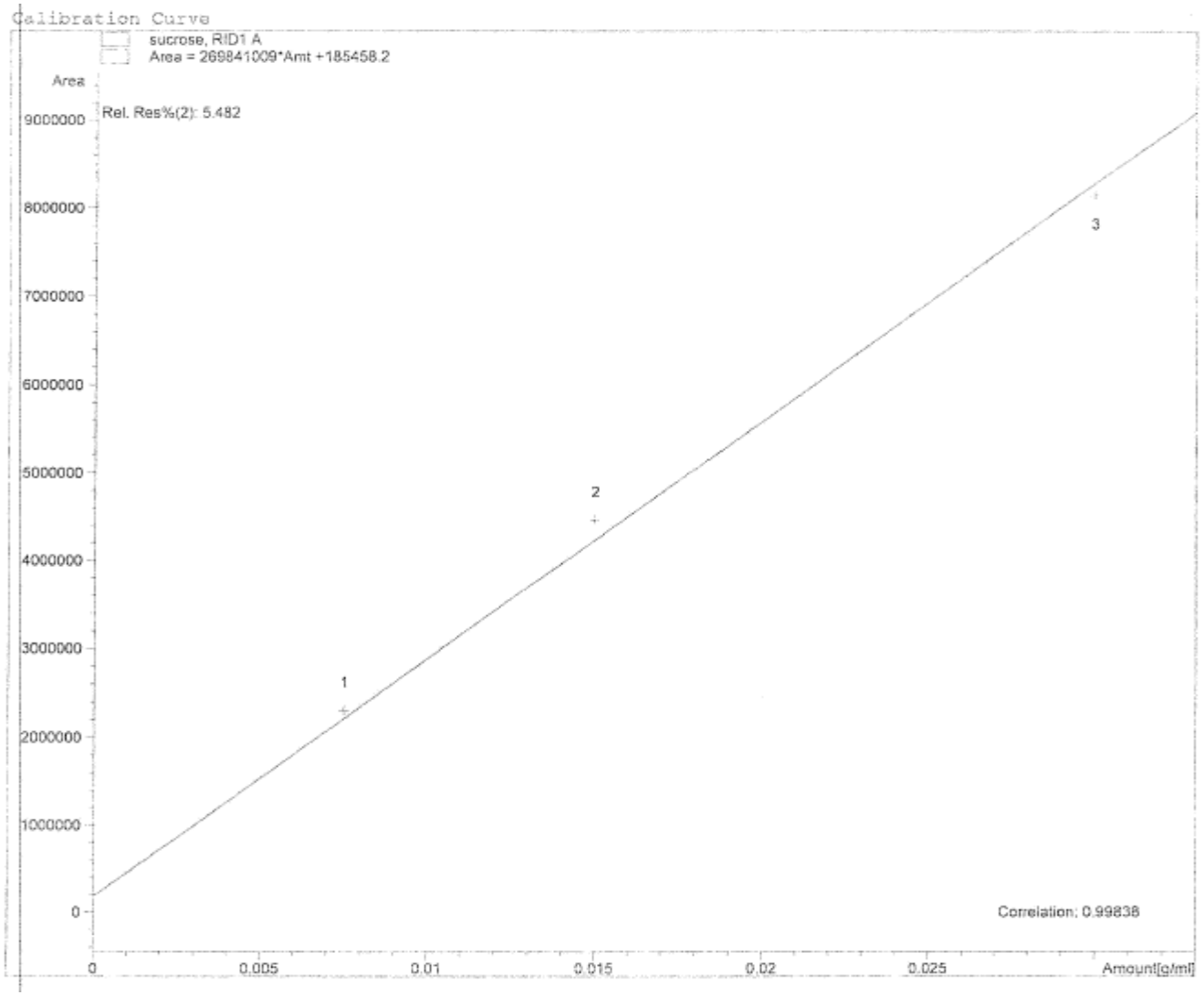

Figure 31: Standard graph used for determining concentration of sucrose in the molasses

Appendix C: analytical methods used to determine wastewater parameters

Determination of total COD (COD) concentration of the feed and product stream using 500-10000 and 25-1500 mg/L range. Cat No. 14555 and 14541. Chromosulfuric acid oxidation method.

1) The thermoreactor (LSE digital dry bath) was switched on and the temperature was set at 148^{o}C.
2) While the thermoreactor was heating up the reaction cell was swirled to suspend the bottom sediments of the reaction cell.
3) 1.0 mL and 3.0 mL of the sample was pipette out into the 14555 and 14541 reaction cells, immediately closed tightly with the screw cap and mixed vigorously.
4) The reaction cells were heated in the thermoreactor for 120 minutes and cooled in a test tube rack at room temperature.
5) Immediately after 10 minutes of cooling the cells were swirled and left to cool to room temperature in the test tube rack.
6) While the reaction cells were cooling the lamp of the spectrophotometer was warmed up by switching on the photometer for approximately 15 minutes.
7) The cells were placed into the cell compartment of the photometer and aligned to the line of the cell compartment.

Determination of soluble COD (COD_s) concentration of the product stream using 25-1500 mg/L range. Cat No. 14541. Chromosulfuric acid oxidation method.

1) The measurement sample was filled into a graduated pCR tube and spun into a centrifuge (Neofuge *13R*) at 10 000 rpm and 2^{o}C for 15 minutes.
2) 3.0 mL of the supernatant was pipette out into the reaction cell, immediately closed tightly with the screw cap and mixed vigorously.
3) The reaction cell was heated in the thermoreactor for 120 minutes and cooled in a test tube rack at room temperature.
4) Immediately after 10 minutes of cooling the cell was swirled and left to cool to room temperature in the test tube rack.
5) While the reaction cell was cooling the lamp of the spectrophotometer was warmed up by switching on the photometer for approximately 15 minutes.
6) The cell was placed into the cell compartment of the photometer and aligned to the line of the cell compartment.

Determination of total Nitrogen (TN) concentration of the feed and product stream using 0.5-15 mg/L measuring range. Cat No. 14537.Peroxidisulfate oxidation, nitrospectral.

1) The thermoreactor is switched on and the temperature is set to 120^{o}C.
2) While the thermoreactor is heating up to the desired temperature 10 mL of the sample is pipette into a clean empty cell and one level of blue microspoon (N-1K) and 6 drops of N-2K,are added.
3) The cell is closed tightly with the screw cap and the contents of the cell are mixed by shaking the cell vigorously.
4) The cell is heated in the thermoreactor at 120^{o}C for 60 minutes and allowed to cool to room temperature by placing it in a test tube rack.
5) After 10 minutes of cooling the cell is swirled briefly and replaced in the test tube rack to cool completely (pretreated sample).
6) One level of blue micro-spoon (N-3k) is added into a nitrogen reaction cell, and shaked for 1 minute to completely dissolve the solid substance.
7) 1.5 mL of the pretreated sample is slowly pipetted into the reaction cell, closed tightly with the screw cap and mixed briefly (cell becomes hot should be held by the screw cap)
8) For measurement of TN the nitrogen reaction cell is left out to cool for 10 minutes (reaction time) in a test tube rack then inserted into the cell compartment of the photometer.

Determination of biochemical oxygen demand (BOD_5) concentration of the feed and product stream using 0.5-3000 mg/L measuring range. Cat No. 00687.Modification of Winkler method.

1) 1 ml of the measurement sample and 1 ml of nutrient-salt solution were diluted separately with 199 ml of distilled water to make a 1:200 dilution ratio.
2) The pH of the fresh batch was in the alkaline range (9-10) and was dropped to a range of 6 and 8 with sulphuric acid.
3) Two oxygen reaction bottles were filled with the diluted measurement sample and two glass beads to overflow. The reaction bottles were closed with the slanted ground-glass stoppers and mixed thoroughly.
4) Two oxygen reaction bottles were filled with the diluted nutrient-salt sample and 2 glass beads to overflow and closed with the slanted ground-glass stoppers mixed thoroughly.
5) One oxygen reaction bottle of the measurement sample (result 2) and one oxygen reaction bottle of the nutrient-salt sample (result 2) were incubated at 20±1^{o}C for 5 days.
6) Five drops of BSB-1K and then 10 drops of BSB-2K were added into one oxygen reaction bottle filled with the measurement sample and one filled with nutrient-salt sample and was mixed thoroughly.

7) After 1 minute of reaction, 10 drops of BSB-3K were added into both reaction bottles, reclosed and mixed thoroughly.
8) Both samples were separately transferred into round cells and measured in a photometer (result 1).
9) After 5 days of incubation step 4 to 8 were performed to the incubated measurement sample (result 2) and nutrient-salt sample (result 2)

BOD of measurement sample = result 1-result 2 (measurement sample) = A mg/L

BOD of blank sample= result 1-result 2 (inoculated nutrient-salt sample) = B mg/L

*BOD of original sample (mg/L) = A*dilution factor-B*

Determination of total phosphorus (TP) concentration of the feed and product stream using 0.5-3000 mg/L measuring range. Cat No. 14543.Phophormolybdenum blue method.

1) 5 mL of the sample was pippeted into the phosphorus reaction cell, closed tightly and mixed.
2) One dose of P-1K was added to the reaction cell using the green-dose metering cap. The cell was closed tightly with the screw cap and heated in the thermoreactor for 30 minutes.
3) After heating the cell was placed in the test tube rack to cool to room temperature.
4) 5 drops of P-2K were added and mixed followed by 1 dose of P-3K using the blue dose-metering cap and closed tightly.
5) The reaction cell was shaked vigorously to dissolve the solid substance completely.
6) After five minutes of reaction, the cell was placed into the cell compartment of the photometer and the mark on the cell was aligned with that on the photometer.

Determination of sulphate (SO_4^{2-}) concentration of the feed and product stream using 5-250 mg/L measuring range. Cat No. 14548. Bariumsulfate turbidimetric method.

1) 5 mL of the measurement sample was pippeted into the sulphate reaction cell, closed tightly and mixed.
2) One level of green microspoon of SO_4-1K was added into the reaction cell, closed tightly with the screw cap and mixed vigorously to completely dissolved the solid substance.
3) To measure the sample the cell was placed into the cell compartment of the photometer and the mark on the cell was aligned with that on the photometer.

Determination of total solids (TS)

1) An empty aluminium dish was washed with distilled water and dried in the oven at 105^{o}C for 30 minutes.
2) The dried dish was allowed to cool to room temperature in the desiccator and weighed (M^1).
3) 28 mL of the sludge sample was poured into the aluminium dish and the moisture content of the sludge sample was evaporated in a fume cupboard overnight.
4) The evaporated sludge sample was dried in the oven at 105^{o}C until constant mass (M^2).

TS of sludge sample = Final mass of dish (M^2) – initial mass of dish (M^1)

Determination of volatile fatty acids (VFA) concentration of the product stream using 50-3000 mg/L measuring range. Cat No. 01763. Esterification method.

1) 0.75 mL of OA-1 was pipetted into the reaction cell and 2 drops of OA-2 were added.
2) 0.50 mL of the sample was pipetted into the reaction cell, the cell was closed thightly with the screw cap, mixed and heated in the thermoreactor for 10 minutes.
3) After heating, the cell was cooled to room temperature under running water and 5 drops of OA-3 were added followed by 0.50 mL of OA-4, closed with the srew cap and mixed.
4) After a reaction time of 3 minutes 0.50 mL of OA-5 was pipetted into the cell, closed with the screw cap and shaken vigorously.
5) The contents of the cell were allowed to react for 10 minutes. After reaction time the cell was placed into the cell compartment and the mark on the cell was alined with the one on the photometer.

Determination of acid capacity (total alkalinity) of the feed and product stream using 0.4-8.0 mmol/L measuring range. Cat No. 01758. Indicator reaction method.

1) 4.0 mL of AC-1 was pipetted into the reaction cell and 1.0 mL of the sample was pipetted into the cell, closed with the screw cap and mixed.
2) 0.5 mL of AC-2 was pipetted into the cell, closed with the screw cap and mixed.

1) After mixing the cell is placed into the cell compartment and the mark on the cell is aligned with that on the photometer.

NB: The reading is displayed in units of mmol CaCO3/L and can be converted to mg CaCO3/L by multiplying with the molecular weight of CaCO3.

Appendix D: Microbial composition of the sludge extracted from the base of the UASB

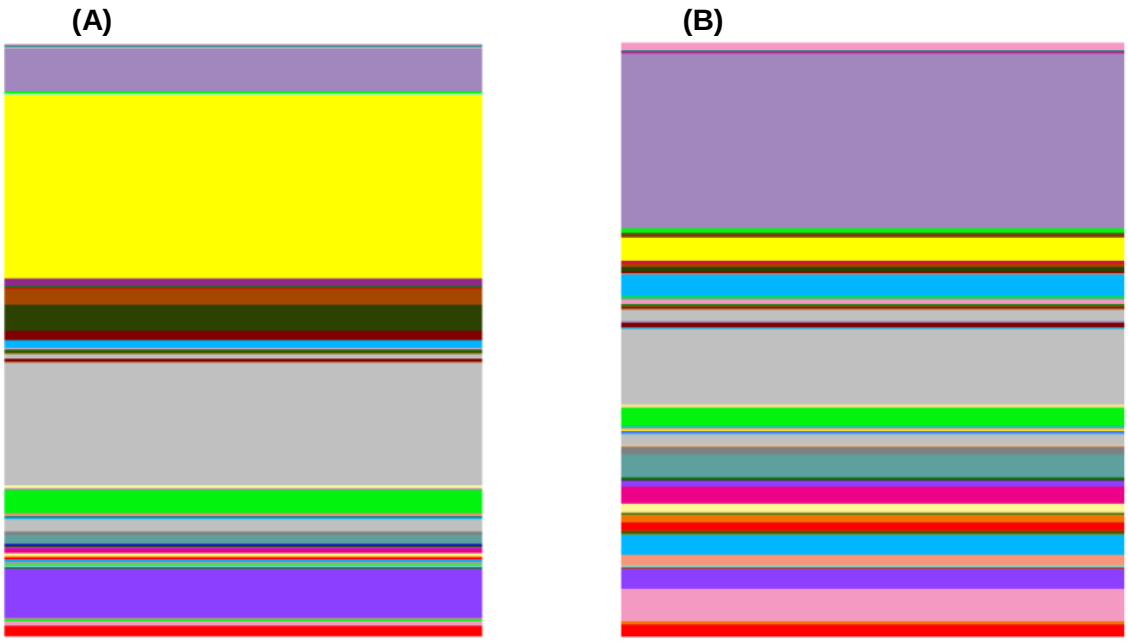

Figure 32: Taxanomy bar chart showing distribution of microorganisms in the UASB sludge

Table 22: Phylogenetic order of bacteria from granular sludge samples obtained at the base of UASB treating sugarcane molasses

Legend		Phylum	Class	Order	Family	Genus	A, %	B, %	Total, %
	Unassigned	Other	Other	Other	Other	Other	1.7	2.2	2
	Archaea	Crenarchaeota	MCG	pGrfC26			0.0	0.0	0.0
	Archaea	Euryarchaeota	Methanobacteria	methanobacteriales	Methanobacteriaceae	Methanobacterium	0.0	0.3	0.2
	Archaea	Euryarchaeota	Methanobacteria	Methanobacteriales	WSA2	NG	0.0	0.0	0.0
	Archaea	Euryarchaeota	Methanobacteria	Methanobacteriales	Methanoregulaceae	Candidatus Methanoregula	0.0	0.0	0.0
	Archaea	Euryarchaeota	Methanobacteria	Methanobacteriales	Methanoregulaceae	Methanolinea	0.0	0.0	0.0
	Archaea	Euryarchaeota	Methanobacteria	Methanobacteriales	Methanospirillaceae	Methanospirillum	0.0	0.0	0.0
	Archaea	Euryarchaeota	Methanobacteria	Methanosarcinales	Methanosaetaceae	Methanosaeta	0.9	5.4	3.1
	Archaea	Euryarchaeota	Methanobacteria	Methanosarcinales	Methanosarcinaceae	methanomethylovorans	0.0	0.0	0.0
	Archaea	Euryarchaeota	Thermoplasmata	E2	Methanomassiliicoccaceae	NG	0.0	0.0	0.0
	Archaea	Euryarchaeota	Thermoplasmata	E2	Methanomassiliicoccaceae	Methanomassiliicoccus	0.0	0.0	0.0
	Bacteria	NG	NG	NG	NG	NG	0.2	0.1	0.1
	Bacteria	Acidobacteria	Acidobacteriia	Acidobacteriales	Acidobacteriaceae	NG	0.0	0.0	0.0
	Bacteria	Acidobacteria	BPC102	MVS-40	NG	NG	0.0	0.0	0.0
	Bacteria	Acidobacteria	DAO52	Ellin6513	NG	NG	0.0	0.0	0.0
	Bacteria	Acidobacteria	GAL08	NG	NG	NG	0.0	0.0	0.0
	Bacteria	Acidobacteria	Solibateres	Solibacterales	NG	NG	0.0	0.0	0.0
	Bacteria	Acidobacteria	Solibacteres	Solibacterales	AKIW659	NG	0.0	0.0	0.0
	Bacteria	Actinobacteria	NG	NG	NG	NG	0.0	0.0	0.0
	Bacteria	Actinobacteria	Acidimicrobiia	Acidimicrobiales	NG	NG	0.0	0.0	0.0
	Bacteria	Actinobacteria	Actenobacteria	Actinomycetales	Other	Other	0.0	0.0	0.0
	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	NG	NG	0.0	0.0	0.0
	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	NG	NG	0.0	0.0	0.0
	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Actinomycetaceae	Other	0.0	0.0	0.0
	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Actinomycetaceae	NG	8.4	3.2	5.8
	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Actinomycetaceae	Actinobacterium	0.0	0.0	0.0
	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Beutenbergiaceae	Other	0.0	0.0	0.0

Table 22
continued

Legend		Phylum	Class	Order	Family	Genus	A, %	B, %	Total, %
	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Micromonosporaceae	Other	0.0	0.0	0.0
	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Nocardioidaceae	Propionicimonas	0.0	0.0	0.0
	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	propionibacteriacaea	NG	0.1	0.3	0.2
	Bacteria	Actinobacteria	Corobacteriia	Corobacteriales	Corobacteriaceae	NG	0.0	0.0	0.0
	Bacteria	Actinobacteria	Coriobacteriia	Corobacteriales	Corobacteriaceae	Atopobium	0.0	0.0	0.0
	Bacteria	Actinobacteria	Thermoleophilia	NG	NG	NG	0.0	0.0	0.0
	Bacteria	Actinobacteria	Thermoleophilia	Gaiellales	NG	NG	0.2	0.1	0.1
	Bacteria	Actinobacteria	Thermoleophilia	Solirubrobacterales	NG	NG	0.0	0.0	0.0
	Bacteria	Actinobacteria	Thermoleophilia	Solirubrobacterales	Conexibacteraceae	Conexibacter	0.0	0.0	0.0
	Bacteria	Armatimonadeteas	NG	NG	NG	NG	0.1	0.1	0.1
	Bacteria	Armatimonadetes	SHA-37	NG	NG	NG	0.0	0.0	0.0
	Bacteria	Armatimonadetes	SJA-176	RB046	NG	NG	0.0	0.0	0.0
	Bacteria	Armatimonadetes	SJA-176	TP122	NG	NG	0.1	0.0	0.1
	Bacteria	BRC1	NPL-UPA2	NG	NG	NG	0.0	0.0	0.0
	Bacteria	BRC1	PRR-11	NG	NG	NG	0.0	0.0	0.0
	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	NG	NG	0.4	1.7	1.1
	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	BA008	NG	0.0	0.0	0.0
	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	NG	0.0	0.0	0.0
	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	NG	0.0	0.0	0.0
	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Marinilabiaceae	NG	0.0	0.0	0.0
	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Other	0.0	0.0	0.0
	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	NG	0.0	0.1	0.1
	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Dysgonomanas	0.0	0.0	0.0
	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Paludibacter	0.4	3.2	1.8
	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	NG	0.0	0.0	0.0
	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	Blvii28	0.1	0.4	0.3
	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	SB-1	NG	0.0	0.0	0.0
	Bacteria	Bacteroidetes	Cytophagia	Cytophagales	Cyclobacteriaceae	NG	0.0	0.0	0.0
	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	NG	NG	0.0	0.0	0.0

Table 22 continued

Legend		Phylum	Class	Order	Family	Genus	A, %	B, %	Total, %
	Bacteria	Caldiserica	Caldisericia	Caldisericales	Other	Other	0.0	0.0	0.0
	Bacteria	Caldiserica	Caldisericia	Caldisericales	Caldisericaceae	Other	0.0	0.1	0.1
	Bacteria	Caldiserica	Caldisericia	Caldisericales	Caldisericaceae	NG	0.0	0.2	0.1
	Bacteria	Caldiserica	Caldisericia	Cadisericales	Caldisericaceae	Caldisericum	0.2	1.3	0.8
	Bacteria	Caldiserica	OP5	WCHB1-02	SHBZ1169	NG	0.0	0.0	0.0
	Bacteria	Caldiserica	TTA-B1	NG	NG	NG	0.1	1.4	0.8
	Bacteria	Caldiserica	WCHB1-03	NG	NG	NG	0.0	0.1	0.1
	Bacteria	Chlorobi	NG	NG	NG	NG	0.0	0.0	0.0
	Bacteria	Chlorobi	BSV26	C20	NG	NG	0.0	0.0	0.0
	Bacteria	Chlorobi	Ignavibacteria	Ignavibacteriales	Other	Other	0.0	0.0	0.0
	Bacteria	Chlorobi	Ignavibacteria	Ignavibacteriales	Ignavibacteriaceae	NG	0.0	0.0	0.0
	Bacteria	Chlorobi	Ignavibacteria	Ignavibacteriales	IheB3-7	NG	0.0	0.0	0.0
	Bacteria	Chlorobi	OPB56	NG	NG	NG	0.0	0.0	0.0
	Bacteria	Chlorobi	SJA-28	NG	NG	NG	0.0	0.0	0.0
	Bacteria	Chloroflexi	Anaerolineae	Other	Other	Other	0.0	0.0	0.0
	Bacteria	Chloroflexi	Anaerolineae	NG	NG	NG	0.0	0.0	0.0
	Bacteria	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolinaceae	Other	0.0	0.0	0.0
	Bacteria	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolinaceae	NG	0.0	0.2	0.1
	Bacteria	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolinaceae	Anaerolinia	0.0	0.0	0.0
	Bacteria	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolinaceae	C1 B004	0.0	0.1	0.0
	Bacteria	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolinaceae	Longilinea	0.3	1.2	0.8
	Bacteria	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolinaceae	SHD-231	0.0	0.1	0.0
	Bacteria	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolinaceae	T78	0.8	2.8	1.8
	Bacteria	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolinaceae	WCHB1-05	0.0	0.0	0.0
	Bacteria	Chloroflexi	Anaerolineae	Caldilineales	Caldilineaceae	NG	0.0	0.0	0.0
	Bacteria	Chloroflexi	Anaerolineae	GCA004	NG	NG	0.0	0.1	0.0
	Bacteria	Chloroflexi	Anaerolineae	OPH11	NG	NG	0.3	1.1	0.7
	Bacteria	Chloroflexi	Anaerolineae	S0208	NG	NG	0.0	0.0	0.0
	Bacteria	Chloroflexi	Anaerolineae	SBR1031	NG	NG	0.0	0.0	0.0
	Bacteria	Chloroflexi	Anaerolineae	SBR1031	SHA-31	NG	0.0	0.0	0.0

Table 22
continued

Legend		Phylum	Class	Order	Family	Genus	A, %	B, %	Total, %
	Bacteria	Chloroflexi	Anaerolineae	SHA-20	NG	NG	0.1	0.1	0.1
	Bacteria	Chloroflexi	Anaerolineae	SJA-15	NG	NG	0.2	0.1	0.1
	Bacteria	Chloroflexi	Anaerolineae	envOPS12	NG	NG	0.0	0.0	0.0
	Bacteria	Chloroflexi	Dehalococcoidetes	Dehalococcoidales	Dehalococcoidaceae	NG	0.1	0.1	0.1
	Bacteria	Elusimicrobia	Elusimicrobia	Elusimicrobiales	NG	NG	0.0	0.0	0.0
	Bacteria	Elusimicrobia	Endomicrobia	NG	NG	NG	0.0	0.1	0.1
	Bacteria	FCPU426	NG	NG	NG	NG	0.0	0.0	0.0
	Bacteria	Firmicutes	Bacilli	Bacillales	Planococcaceae	Other	0.0	0.0	0.0
	Bacteria	Firmicutes	Bacilli	Lactobacillales	Other	Other	0.0	0.0	0.0
	Bacteria	Firmicutes	Bacilli	Lactobacillales	Carnobacteriaceae	Other	0.0	0.1	0.1
	Bacteria	Firmicutes	Bacilli	Lactobacillales	Carnobacteriaceae	NG	1.6	3.9	2.8
	Bacteria	Firmicutes	Bacilli	Lactobacillales	Carnobacteriaceae	Carnobacterium	0.0	0.0	0.0
	Bacteria	Firmicutes	Bacilli	Lactobacillales	Carnobacteriaceae	Trihococcus	0.5	1.3	0.9
	Bacteria	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	Othetr	0.0	0.0	0.0
	Bacteria	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	Enterococcus	0.0	0.0	0.0
	Bacteria	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	Vagococcus	0.0	0.0	0.0
	Bacteria	Firmicutes	Bacilli	Lactobacillales	Streptoococcaceae	Lactococcus	0.0	0.0	0.0
	Bacteria	Firmicutes	Bacilli	Lactobacillales	Streptoococcaceae	Streptococcus	0.0	0.0	0.0
	Bacteria	Firmicutes	Clostridia	Other	Other	Other	0.0	0.0	0.0
	Bacteria	Firmicutes	Clostridia	Clostridiales	Other	Other	0.0	0.0	0.0
	Bacteria	Firmicutes	Clostridia	Clostridiales	NG	NG	2.1	2.0	2.0
	Bacteria	Firmicutes	Clostridia	Clostridiales	Caldicoprobacteraceae	Caldicoprobacter	0.0	0.0	0.0
	Bacteria	Firmicutes	Clostridia	Clostridiales	Christensenellaceae	NG	0.1	0.1	0.1
	Bacteria	Firmicutes	Clostridia	Clostridiales	Christensenellaceae	Christensenella	0.0	0.0	0.0
	Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	NG	0.0	0.0	0.0
	Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Clostridium	0.1	0.2	0.1
	Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Oxobacter	0.0	0.0	0.0
	Bacteria	Firmicutes	Clostridia	Clostridiales	Dehalobacteriaceae	Dehalobacterium	0.0	0.0	0.0
	Bacteria	Firmicutes	Clostridia	Clostridiales	EtOH8	NG	0.0	0.0	0.0
	Bacteria	Firmicutes	Clostridia	Clostridiales	Eubacteriaceae	NG	0.0	0.0	0.0

Table 22 continued

Legend		Phylum	Class	Order	Family	Genus	A, %	B, %	Total, %
	Bacteria	Firmicutes	Clostridia	Clostridiales	Eubacteriaceae	Acetobacterium	0.0	0.0	0.0
	Bacteria	Firmicutes	Clostridia	Clostridiales	Eubacteriaceae	Anaerofustis	0.0	0.0	0.0
	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	NG	0.0	0.0	0.0
	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Blautia	0.0	0.0	0.0
	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Coprococcus	0.0	0.1	0.1
	Bacteria	Firmicutes	Clostridia	Clostridiales	Peptococcaceae	NG	0.0	0.0	0.0
	Bacteria	Firmicutes	Clostridia	Clostridiales	Peptococcaceae	Dehalobacter	0.0	0.1	0.0
	Bacteria	Firmicutes	Clostridia	Clostridiales	Peptococcaceae	Syntrophobotus Desulfosporosinus	0.0	0.0	0.0
	Bacteria	Firmicutes	Clostridia	Clostridiales	Peptococcaceae	Desulfotomaculum	0.0	0.0	0.0
	Bacteria	Firmicutes	Clostridia	Clostridiales	Peptococcaceae	Desulfotomaculun	0.0	0.0	0.0
	Bacteria	Firmicutes	Clostridia	Clostridiales	Peptococcaceae	Desulfovirgula Desulfurispora	0.0	0.0	0.0
	Bacteria	Firmicutes	Clostridia	Clostridiales	Peptococcaceae	Pelotomaculum	0.0	0.0	0.0
	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Other	0.0	0.0	0.0
	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	NG	0.2	0.3	0.3
	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Ethanoligenens	0.0	0.0	0.0
	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Oscilospira	0.0	0.0	0.0
	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Ruminococcus	0.0	0.0	0.0
	Bacteria	Firmicutes	Clostridia	Clostridiales	Syntrophomonadaceae	Syntrophomonas	0.1	0.1	0.1
	Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Other	0.0	0.0	0.0
	Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	NG	0.0	0.0	0.0
	Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Acidaminococcus	0.0	0.0	0.0
	Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Anaeromusa	0.0	0.0	0.0
	Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Phascolarctobacterium	0.0	0.1	0.1
	Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Veilonella	0.0	0.0	0.0
	Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	VadinHB04	0.0	0.0	0.0
	Bacteria	Firmicutes	Clostridia	Clostridiales	[Mogibacteriaceae]	Other	0.0	0.0	0.0
	Bacteria	Firmicutes	Clostridia	Clostridiales	[Mogibacteriaceae]	NG	0.0	0.0	0.0
	Bacteria	Firmicutes	Clostridia	Clostridiales	[Mogibacteriaceae]	Anaerovorax	0.0	0.0	0.0
	Bacteria	Firmicutes	Clostridia	MBA08	NG	NG	0.0	0.0	0.0

Table 22 continued

Legend		Phylum	Class	Order	Family	Genus	A, %	B, %	Total, %
	Bacteria	Firmicutes	Clostridia	OPB54	NG	NG	0.0	0.0	0.0
	Bacteria	Firmicutes	Clostridia	SHA-98	D2	NG	0.0	0.0	0.0
	Bacteria	Fusobacteria	Fusobacteriia	Fusobacteriales	NG	NG	0.0	0.0	0.0
	Bacteria	GN04	GN15	NG	NG	NG	0.0	0.0	0.0
	Bacteria	Gemmatimonadetes	Gemm-2	NG	NG	NG	0.0	0.0	0.0
	Bacteria	Gemmatimonadetes	JL-ETNP-Z39	NG	NG	NG	0.0	0.0	0.0
	Bacteria	LD1	NG	NG	NG	NG	0.0	0.0	0.0
	Bacteria	Lentisphaerae	[Lentsphaeria]	Victivallales	victivallaceae	NG	0.0	0.0	0.0
	Bacteria	Lentsiphaerae	[Lentsphaeria]	Z20	R4-45B	NG	0.0	0.0	0.0
	Bacteria	NKB19	NG	NG	NG	NG	0.0	0.0	0.0
	Bacteria	NKB19	TSBW08	NG	NG	NG	0.0	0.0	0.0
	Bacteria	NKB19	noFP H4	NG	NG	NG	0.0	0.0	0.0
	Bacteria	Nitrospirae	Nitrospira	Nitrospirales	[Thermodesulfovibrionaceae]	DCE29	3.8	3.1	3.5
	Bacteria	OC31	NG	NG	NG	NG	0.0	0.0	0.0
	Bacteria	OD1	NG	NG	NG	NG	0.0	0.0	0.0
	Bacteria	OD1	ABY1	NG	NG	NG	0.0	0.1	0.1
	Bacteria	OP1	OPB14	NG	NG	NG	0.0	0.0	0.0
	Bacteria	OP11	OP11-3	NG	NG	NG	0.0	0.0	0.0
	Bacteria	OP8	OP8 1	Other	Other	Other	0.4	0.1	0.3
	Bacteria	OP8	OP8 1	NG	NG	NG	0.4	0.3	0.4
	Bacteria	OP8	OP8 1	OPB95	NG	NG	20.6	12.7	16.6
	Bacteria	OP9	JS1	BA02	NG	NG	0.0	0.0	0.0
	Bacteria	OP9	OPB46	OPB72	TIBD11		0.0	0.0	0.0
	Bacteria	OP9	OPB46	SHA-1	NG	NG	0.1	0.2	0.1
	Bacteria	Planctomycetes	Phycisphaerae	MSBL9	NG	NG	0.0	0.0	0.0
	Bacteria	Planctomycetes	Phycisphaerae	Pla1	NG	NG	0.0	0.0	0.0
	Bacteria	Planctomycetes	Phycisphaerae	S-70	NG	NG	0.0	0.0	0.0
	Bacteria	Planctomycetes	Phycisphaerae	mle1-8	NG	NG	0.5	0.6	0.5
	Bacteria	Planctomycetes	Planctomycetia	Pyrellulales	Pyrellulaceae	NG	0.0	0.0	0.0
	Bacteria	Planctomycetes	vadinHA49	PeHg47	NG	NG	0.0	0.0	0.0

Table 22 continued

Legend		Phylum	Class	Order	Family	Genus	A, %	B, %	Total, %
	Bacteria	Proteobacteria	Alphaproteobacteria	NG	NG	NG	0.0	0.0	0.0
	Bacteria	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	NG	0.0	0.0	0.0
	Bacteria	Proteobacteria	Alpharoteobacteria	Ellin329	NG	NG	0.0	0.0	0.0
	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Beijerinckiaceae	Other	0.0	0.0	0.0
	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Beijerinckiaceae	NG	0.0	0.0	0.0
	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Brydyrhizobiaceae	Other	0.0	0.0	0.0
	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Brydyrhizobiaceae	NG	0.0	0.0	0.0
	Bacteria	Proteobacteria	Alpharoteobacteria	Rhizobiales	Methylocystaceae	Other	0.0	0.0	0.0
	Bacteria	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	NG	0.0	0.0	0.0
	Bacteria	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Rhodospirillaceae	NG	0.0	0.0	0.0
	Bacteria	Proteobacteria	Betaproteobacteria	Other	Other	Other	0.0	0.1	0.0
	Bacteria	Proteobacteria	Betaproteobacteria	NG	NG	NG	0.0	0.0	0.0
	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Other	Other	0.0	0.0	0.0
	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Hylemonella	0.0	0.0	0.0
	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Other	0.0	0.0	0.0
	Bacteria	Proteobacteria	Betaproteobacteria	Gallionellales	Gallionellaceae	Gallionella	0.0	0.0	0.0
	Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclalales	Rhodocyclalaceae	Other	0.2	0.2	0.2
	Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclalales	Rhodocyclalaceae	Azospira	0.0	0.0	0.0
	Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclalales	Rhodocyclalacea	Propionivibro	0.8	1.8	1.3
	Bacteria	Proteobacteria	Betaproteobacteria	NG	NG	NG	0.0	0.0	0.0
	Bacteria	Proteobacteria	Betaproteobacteria	BPC076	NG	NG	0.0	0.0	0.0
	Bacteria	Proteobacteria	Betaproteobacteria	Bdellovibrionales	Bacteriovoracaceae	NG	0.0	0.0	0.0
	Bacteria	Proteobacteria	Betaproteobacteria	Desulfarculales	Desulfarculacea	Desulfarculus	0.0	0.0	0.0
	Bacteria	Proteobacteria	Betaproteobacteria	Desulfovibrionales	Desulfovibrionaceae	NG	0.0	0.0	0.0
	Bacteria	Proteobacteria	Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae	Desulfovibrio	0.1	0.1	0.1
	Bacteria	Proteobacteria	Deltaproteobacteria	Desulfuromonadales	Geobacteraceae	Geobacter	0.0	0.0	0.0
	Bacteria	Proteobacteria	Deltaproteobacteria	GW-28	NG	NG	0.1	0.2	0.1
	Bacteria	Proteobacteria	Deltaproteobacteria	MBNT15	NG	NG	0.1	0.1	0.1
	Bacteria	Proteobacteria	Deltaproteobacteria	Myxococcales	NG	NG	0.0	0.0	0.0
	Bacteria	Proteobacteria	Deltaproteobacteria	Myxococcales	0319-6G20	NG	0.0	0.1	0.1

Table 22
continued

Legend		Phylum	Class	Order	Family	Genus	A, %	B, %	Total, %
	Bacteria	Proteobacteria	Deltaproteobacteria	Myxococcales	Cystobacterineae	NG	0.0	0.0	0.0
	Bacteria	Proteobacteria	Deltaproteobacteria	Myxococcales	Myxococcaceae	Anaeromyxobacter	0.0	0.0	0.0
	Bacteria	Proteobacteria	Deltaproteobacteria	Syntrophobacterales	Syntrophaceae	NG	0.2	0.3	0.3
	Bacteria	Proteobacteria	Deltaproteobacteria	Syntrophobacterales	Syntrophaceae	Desulfomonile	0.0	0.0	0.0
	Bacteria	Proteobacteria	Deltaproteobacteria	Syntrophobacterales	Syntrophaceae	Syntrophus	0.0	0.0	0.0
	Bacteria	Proteobacteria	Deltaproteobacteria	Syntrophobacterales	Syntrophobacteraceae	Other	0.0	0.0	0.0
	Bacteria	Proteobacteria	Deltaproteobacteria	Syntrophobacterales	Syntrophobacteraceae	Syntrophobacter	0.2	0.8	0.5
	Bacteria	Proteobacteria	Deltaproteobacteria	Syntrophobacterales	Syntrophorabdaceae	NG	0.1	0.3	0.2
	Bacteria	Proteobacteria	Gammaproteobacteria	Chromatiales	Ectothiorhodospiraceae	NG	0.0	0.0	0.0
	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriacea	Other	0.0	0.0	0.0
	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriacea	NG	0.0	0.0	0.0
	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriacea	Citrobacter	0.0	0.0	0.0
	Bacteria	Proteobacteria	TA18	CV90	NG	NG	0.0	0.0	0.0
	Bacteria	SAR406	AB16	noFP H7	NG	NG	0.0	0.0	0.0
	Bacteria	Spirochaetes	MVP-15	Other	Other	Other	0.0	0.0	0.0
	Bacteria	Spirochaetes	MVP-15	PL-11B10	NG	NG	0.0	0.0	0.0
	Bacteria	Spirochaetes	Spirochaetes	M2PT2-76	NG	NG	0.0	0.0	0.0
	Bacteria	Spirochaetes	Spirochaetes	Sphaerochaetales	Sphaerochaetacea	Sphaerochaeta	0.0	0.0	0.0
	Bacteria	Spirochaetes	Spirochaetes	Spirochaetales	Spirochaetacea	NG	0.0	0.0	0.0
	Bacteria	Spirochaetes	Spirochaetes	Spirochaetales	Spirochaetacea	Treponema	1.2	3.8	2.5
	Bacteria	Spirochaetes	Spirochaetes	Spirochaetales	Spirochaetacea	Za29	0.0	0.0	0.0
	Bacteria	Spirochaetes	Brachyspirae	Brachyspirales	Brachyspiracea	NG	0.0	0.1	0.1
	Bacteria	Spirochaetes	Brevinematae	Brevinematales	NG	NG	0.0	0.0	0.0
	Bacteria	Spirochaetes	[Leptospiriae]	[Leptospiriales]	Sediment-4	NG	0.0	0.0	0.0
	Bacteria	Spirochaetes	[Leptospiriae]	[Leptospiriales]	Sediment-4	SJA-88	0.0	0.0	0.0
	Bacteria	Synergistetes	Synergistia	Synergistales	Other	Other	1.5	0.2	0.8
	Bacteria	Synergistetes	Synergistia	Synergistales	Aminiphilaceae	Aminiphilus	4.5	0.9	2.7
	Bacteria	Synergistetes	Synergistia	Synergistales	Anaerobaculaceae	NG	2.5	0.6	1.5
	Bacteria	Synergistetes	Synergistia	Synergistales	Dethiosulfovibrionaceae	HA73	0.3	0.2	0.2
	Bacteria	Synergistetes	Synergistia	Synergistales	Dethiosulfovibrionaceae	PD-UASB-13	0.0	0.0	0.0

Table 22 continued

Legend		Phylum	Class	Order	Family	Genus	A, %	B, %	Total, %
	Bacteria	Synergistetes	Synergistia	Synergistales	Synergistaceae	Thermoanaerovibrio	0.0	0.0	0.0
	Bacteria	Synergistetes	Synergistia	Synergistales	Synergistaceae	vadinCA02	0.2	0.1	0.1
	Bacteria	Synergistetes	Synergistia	Synergistales	TTA B6	Other	1.2	0.2	0.7
	Bacteria	Synergistetes	Synergistia	Synergistales	TTA B6	E6	31.2	3.9	17.5
	Bacteria	Synergistetes	Synergistia	Synergistales	Thermovirgaceae	NG	0.1	0.0	0.1
	Bacteria	TM6	SJA-4	NG	NG	NG	0.0	0.0	0.0
	Bacteria	TM7	TM7-3	Other	Other	Other	0.0	0.0	0.0
	Bacteria	TM7	TM7-3	NG	NG	NG	0.1	0.7	0.4
	Bacteria	TM7	TM7-3	EW055	NG	NG	0.0	0.0	0.0
	Bacteria	TPD-58	NG	NG	NG	NG	0.2	0.8	0.5
	Bacteria	Tenericutes	Mollicutes	NG	NG	NG	0.0	0.0	0.0
	Bacteria	Tenericutes	Mollicutes	Acholeplasmatales	Acholeplasmatacea	Acholeplasma	0.0	0.0	0.0
	Bacteria	Tenericutes	Mollicutes	RF39	NG	NG	0.0	0.0	0.0
	Bacteria	Thermotogae	Thermotogae	Thermotogales	Thermotogaceae	Fervidobacterium	0.0	0.0	0.0
	Bacteria	Thermotogae	Thermotogae	Thermotogales	Thermotogaceae	Kosmotoga	7.3	29.3	18.3
	Bacteria	Thermotogae	Thermotogae	Thermotogales	Thermotogaceae	S1	0.0	0.0	0.0
	Bacteria	Verrucomicrobia	Verruco-5	LD1-PB3	NG	NG	0.2	0.2	0.2
	Bacteria	Verrucromicrobia	Verruco-5	WCHB1-41	NG	NG	0.0	0.0	0.0
	Bacteria	Verrucomicrobia	Verruco-5	WCHB1-41	RFP12	NG	0.0	0.1	0.1
	Bacteria	Verrucomicrobia	Verruco-5	WCHB1-41	WCHB1-25	NG	0.0	0.0	0.0
	Bacteria	Verrucomicrobia	[Pedosphaerae]	[Pedosphaerales]	NG	NG	0.0	0.0	0.0
	Bacteria	Verrucomicrobia	[Pedosphaerae]	[Pedosphaerales]	Ellin515	NG	0.0	0.0	0.0
	Bacteria	Verrucomicrobia	[Pedosphaerae]	[Pedosphaerales]	R4-41B	NG	0.1	0.0	0.1
	Bacteria	WPS-2	NG	NG	NG	NG	0.2	0.3	0.3
	Bacteria	WS3	PRR-12	GN03	KSB4	NG	0.0	0.0	0.0
	Bacteria	WS3	PRR-12	PSB-III-9	NG	NG	0.0	0.0	0.0
	Bacteria	WS6	SC72	NG	NG	NG	0.0	0.0	0.0
	Bacteria	WS6	SC72	A-2AF	NG	NG	0.0	0.0	0.0
	Bacteria	WS6	SC72	WCHB1-15	NG	NG	0.0	0.0	0.0
	Bacteria	WWE1	[CLoacamonae]	[CLoacamonales]	SHA-116	NG	0.0	0.0	0.0

Table 22
continued

Legend		Phylum	Class	Order	Family	Genus	A, %	B, %	Total, %
	Bacteria	WWE1	[CLoacamonae]	[CLoacamonales]	[CLoacamonaceae]	Other	0.0	0.0	0.0
	Bacteria	WWE1	[CLoacamonae]	[CLoacamonales]	[CLoacamonaceae]	NG	0.0	0.0	0.0
	Bacteria	WWE1	[CLoacamonae]	[CLoacamonales]	[CLoacamonaceae]	Candidatus Cloacamonas	0.0	0.0	0.0
	Bacteria	WWE1	[CLoacamonae]	[CLoacamonales]	[CLoacamonaceae]	W22	1.2	0.2	0.7
	Bacteria	WWE1	[CLoacamonae]	[CLoacamonales]	[CLoacamonaceae]	W5	0.0	0.0	0.0