Investigating Various Types of Pre-treatments of Whiskey Distillery and Brewery Wastes for Anaerobic Digestion

A Thesis Submitted in Fulfilment of the Requirements for the Degree of

Doctor of Philosophy (PhD)

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DECLARATION

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Doctor of Philosophy is entirely my own work, and that I have exercised reasonable care to ensure that the work is original, and does not to the best of my knowledge breach any law of copyright, and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

Signed: ……

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Date: 12/09/2019

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List of Abbreviations 1

Symbol	Description
A Dose	Alkaline dose
ABR	Anaerobic baffled reactor
AD	Anaerobic digestion
Adeq. Precision	Adequate Precision
Adj. R^2	Adjusted R^2
AF	Anaerobic filter reactor
AnMBR	Anaerobic membrane reactor
ANOVA	Analysis of variance
Amp%	Amplitude ratio
ASBR	Anaerobic sequential batch reactor
BBD	Box-Behnken design
BOD	Biological oxygen demand
BOD_0	Initial biological oxygen demand
BT	Beating time
CAPEX	Capital expenditure
CHP	Combined heat and power
COD	Chemical oxygen demand
COD_0	Initial chemical oxygen demand
Cor. Total	Total sum of squares corrected for the mean
CSTR	Continuous stirred tank reactor
C:N	Carbon nitrogen ratio
d	Desirability
DD	Degree of disintegration
df	Degree of freedom
DOE	Design of experiment
EGSB	Expanded granular sludge blanket
ET	Exposure time
EU	European Union
GBR	Granular bed reactor
GRABBR	Granular bed anaerobic baffled reactor
HPWS	High pressure water scrubbing
HRT	Hydraulic retention time
I/S	Inoculum substrate ratio
k	Hydrolysis rate constant
MS	Mean square
MW Power	Microwave power
OLR	Organic loading rate
ORP	Oxygen reduction potential
OPEX	Operational expenditure
Pred. R^2	Predicted R^2
pH_0	Initial pH
PSA	Pressure swing adsorption
RSM	Response surface methodology
SS	Sum of squares
SS-AD	Solid state anaerobic digestion

List of Abbreviations 2

Symbol	Description
S%	Sludge percentage
T	Temperature
TS	Total solids
UASB	Upflow anaerobic sludge blanket
VS	Volatile solids
$(SO_4)_0$	Initial sulphate value

Greek Notations

Symbol	Description
α	Significance level
Ɛ	Random error

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Abstract

Waste to energy conversion potential of whiskey distilleries and breweries is attracting more and more interest due to the high energy demands of manufacturing processes as well as environmental concerns regarding typical waste disposal methods. Whiskey distillery/brewery waste streams, commonly known as pot ale and spent grain, are classified as lignocellulosic material. A pre-treatment step prior to anaerobic digestion is therefore necessary to obtain sufficient lignin degradation and to achieve an increased hydrolysis rate. In this research project, a combination of alkaline pre-treatment with beating, microwave and ultrasonic pre-treatments were introduced to the literature as novel pre-treatment methods for whiskey distillery/brewery waste streams. Alkaline-beating hybrid pre-treatment was applied and optimised for both pot ale alone and a pot ale–spent grain mixture. The biogas generation of non-treated pot ale and pot ale-spent grain with a 50% inoculum on a wet basis was found to be 205 ± 21.4 ml biogas/g VS with 19.3% CH_4 and 239 ± 3 ml biogas/g VS with 49.1± 2% CH_4 respectively. A significant enhancement was seen after the implementation of a 1M NaOH and 7.5 min beating pre-treatment, and the biogas yields increased to 550±6 ml/g VS with a CH_4 content of 54.3% and to 360±10 ml/g VS with 49.1% CH_4 respectively. Response Surface Modelling was employed for modelling, statistical analysis and optimisation of the process. The most powerful design factor was identified as inoculum amount. Therefore, seeding ratio was scanned from 50 – 95% on wet basis with application of thermochemical pre-treatment. A 3-fold increase was achieved in biogas yield by reaching 2768±234 ml/g VS with combined 1 M NaOH alkaline and 240 W microwave pre-treatment prior to anaerobic digestion of pot ale with a 95% inoculum. On the other hand, no significant difference was seen in biogas yield after implementation of alkaline-ultrasonic pre-treatment. However, combined 3 M NaOH alkaline and 1 amplitude ultrasonic pre-treatment for 2 hours on pot ale offered a reduction in COD of 57±6.1% and a reduction of 60±3.3% in BOD. The mineral quality of the digestate (P, K, Ca, Mg, Fe, Mn, Zn and Cu) was analysed to assess it's potential for agricultural use as a fertilizer; this compared favourably with an industrial digestate. A full-scale AD plant for industrial application was modelled based on the experimental results; CAPEX/OPEX estimations were obtained from published resources with data from 7 AD plant. It was determined that CSTR with an inoculum ratio of 65 – 95% in combination with CHP.

CHAPTER 1: INTRODUCTION

Widespread usage of non-renewable fuels (in particular fossil fuels) for energy production has been implicated as the cause of many ecological and environmental concerns having a direct impact on human migration and climate conditions, primarily due to the continuous emission of greenhouse gases (Bundhoo et al. 2015). In order to address this problem, the European Union aims to reduce the total greenhouse gas emission in developed countries to 20% (or possibly as low as 5%) of 1990 levels by 2050 (European Commission 2018). This, along with increasing energy demand as well as economic and environmental concerns, have led to a search for alternative energy sources. Biogas (a methane rich gas produced by biological means) is considered to be one of the most environmental friendly fuels owing to its non-toxic characteristics and potential for ease of use as an alternative to traditional fossil fuels (Ghanavati et al. 2015).

Beer and whiskey are the most consumed alcoholic beverages in Ireland. They are made from very few raw materials; barley, water and yeast are common ingredients for beer and whiskey production (Hamill 2015) while beer manufacturing involves some flavour additives such as hops. The manufacturing processes of beer and whiskey also have similarities: malting, mashing and fermentation are the common production steps (Uzal et al. 2003; Murunga et al. 2016). In beer production fermentation finalizes the alcohol content, followed by addition of hops and sometimes other ingredients (Ferreira et al. 2010). In whiskey production, however, fermentation is followed by a number of distillation steps, to significantly increase the alcohol levels. Traditionally whiskey is distilled twice in copper stills (Graham et al. 2012). Stills are shaped like a wide bowl base rising up to a thin neck, with slight differences in the shape having an effect on the taste of the final product. Whiskey and beer manufacturing processes are known to be highly energy intensive with a huge thermal energy demand. In beer production, mashing and boiling steps have high heat demand while whiskey production demands further heating for distillation steps. Therefore whiskey manufacturing in particular is considered to be more energy intensive, with 96% of the total energy demand corresponding to thermal energy demand (Duguid & Strachan 2016). In order to reduce heat demand in breweries and distilleries, distilleries are investing in development and implementation of new

technologies such as dynamic boiling with an internal boiler, or use of waste heat recovery, to help to reduce the energy expenditure on the manufacturing step (Meadows 2015). Whiskey and beer manufacturing processes also generate large amounts of high strength co-products which contain high levels of chemical oxygen demand (COD), biological oxygen demand (BOD), phosphorus, ammonia, heavy metals (including copper, iron and magnesium), as well as complex organic materials such as lignin (Dionisi et al. 2014). Due to the characteristics of the waste streams, the alcoholic beverage industry is potentially a highly polluting industry (Saha et al. 2005), with approximately 3.4 million tonnes of solid wastes including spent yeast and spent grain produced per year, in addition to the production of approximately 8 – 15 L aqueous waste generated per litre of malt whiskey and 3 – 10 L/L of beer (Saha et al. 2005; Dionisi et al. 2014; Aliyu & Bala 2013; Acharya et al. 2008; Chen et al. 2016). Disposal of brewery and distillery wastes has been legislated for in most countries for more than 20 years (Pant & Adholeya 2007). In countries such as Ireland and the UK there has been a massive increase in the occurrence of small "craft" breweries and distilleries. These small or micro-breweries/distilleries in particular would benefit from potential methods for reducing costs associated with waste treatment. Figure 1 provides the number of micro scale breweries in Ireland since 2012, with data referring to the number of breweries in production at approximately mid-year (Feeney 2018).

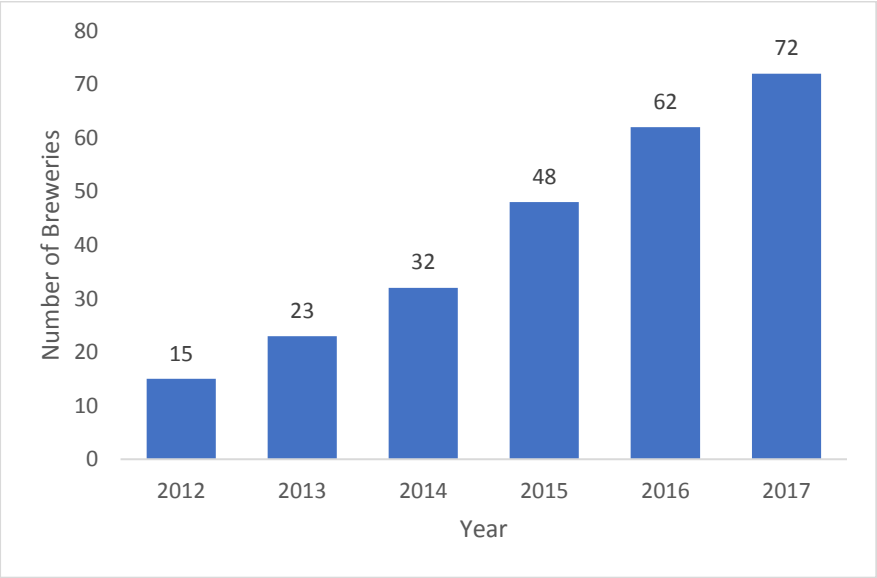


Figure 1. Number of Irish microbreweries in production

According to Irish Whiskey Association data, the number of whiskey distilleries in operation increased from 4 to 18 between 2013 and 2017, with a further 16 planned (ABFI 2018).

Anaerobic digestion (AD) is becoming more widely accepted as an efficient method to convert organic matter into biogas, which can significantly improve the energy balance and economics of industry (Liao et al. 2016; Cesaro & Belgiorno 2014). AD has proven to be more efficient than conventional methods in terms of the treatment of high strength wastes, such as the highly recalcitrant waste streams of distilleries/breweries (Akunna & Clark 2000; Handous et al. 2017). The establishment of anaerobic digestion plants for the treatment of high organic content wastes has undergone major development amongst wastewater treatment facilities in Europe (Cesaro & Belgiorno 2014), and application of AD in the treatment of distillery/brewery wastes is increasing. In addition, the potential for sustainable substrate supply throughout the year for AD renders this technology more appealing for waste streams of distilleries and breweries than other energy reduction approaches from environmental and energy recovery standpoints. However, whiskey distillery/brewery waste streams are highly lignocellulosic, which makes them resistant to degradation by biological means (Raud et al. 2015). Distillery/brewery wastes have a complex heterogeneous structure, and primarily due to the high lignin content, implementation of pre-treatments is considered to have great potential in order to obtain a higher biogas yield from AD (Weiß et al. 2010). The key role of the pre-treatments is modifying the structure of the substrates to make cellulose and hemicellulose more easily degradable. Different types of pre-treatments are discussed in detail in Chapter 2 Section 2.8.

The experimental planning and data analysis technique Design of Experiment (DOE) was introduced to the scientific community in the 1920s to determine the influence of various fertilisers on land. Since then, usage of DOE has been applied for many applications in different scientific disciplines (Benyounis & Olabi 2006). DOE has an important role in planning experiments as well as conducting, analysing and interpreting the experimental data. When a certain quality of a product is affected by many independent variables, the aim is then to design a set of experiments to find a valid, reliable solution in an effective and efficient manner. It should be noted that some variables might have a stronger effect

than others on the response of interest, also an interaction between the variables can sometimes be seen. The aim of a well-designed set of experiments is therefore to identify which factors affect the overall performance of the process as well as the best level of these factors in order to reach the desired quality of the response (Tedesco et al. 2014; Fisher 1936). Response surface methodology (RSM) is the most common design type of DOE design (Benyounis & Olabi 2006). It is a combination of mathematical and statistical techniques, which are used for modelling, interpreting, predicting and optimising a process of several input variables (Fisher 1936; Athijayamani et al. 2016). RSM is also capable of indicating the relationship between the measured response(s) and controllable process variables (Pavani et al. 2016). The surface response can be expressed as a function of the factors given in Eq 1 when the factors are measurable and reproducible with an insignificant error.

$$\mathbf{y = f(X_1, X_2 \dots X_k)} \qquad \text{Eq 1}$$

Where; y is the response of interest, X_i are the design factors, and k is the number of factors.

There are various methods in use for application of RSM design, including the Box-Behnken method applied in this research, which minimizes the number of experiments required to generate a reliable mathematical model, particularly in comparison to a standard factorial design matrix (Benyounis & Olabi 2006; Rakić et al. 2014).

1.1 Research Objectives

Considering that the whiskey manufacturing industry generates large volumes of high strength wastewater, AD technology provides a sustainable waste management opportunity in addition to a great energy recovery potential. The aim of this work was to investigate and optimise the energy recovery potential of distillery waste streams through anaerobic digestion by evaluating the impacts of different types of pre-treatments on the lignocellulosic structure of the waste stream.

The main parts of the investigation process were;

1. Evaluating the enhancements in biogas yield and quality (CH_4 content) from anaerobic digestion of distillery/brewery waste streams after implementation of various pre-treatments.

2. Modelling and optimisation of process and pre-treatment parameters for anaerobic digestion of pot ale.

3. Creating a scalable anaerobic digestion model for industrial application.

To assess biogas enhancements due to applied pre-treatment on pot ale and spent grain, first of all, anaerobic digestion experiments were conducted at low sludge inoculum ratios (5 – 50% on wet basis) to minimise the costs for full scale implementations. Pot ale was selected as the substrate of interest; subsequently application of thermal pre-treatment (microwave) on pot ale was investigated with an inoculum ratio range of 65 – 90% on wet basis. The impact of ultrasonic pre-treatment on the early stages of AD (first 2 days) was investigated with an inoculum ratio of 90% on wet basis. Finally, a theoretical study of the use of the end products (digestate and biogas) was carried out, and the design of an industrial application model based on inoculum ratios was outlined.

Modelling and optimisation processes were carried out throughout the experimental research by implementing the Design of Experiment (DOE) software to investigate the;

1. Parameters involved in the AD process
2. Parameters involved in the pre-treatment steps

The aim of these optimisation steps was to identify the optimum conditions to maximise the methane content of the biogas while aiming to minimise the other impurity components.

A discussion on a scaling up aspect of the project was addressed as it was acknowledged that the major challenge was how to relate the bench scale findings to industrial scale.

1.2 Thesis Outline

Chapter 1 provides an overview of the project and the approaches taken. It also highlights the key research areas.

Chapter 2 aims to provide the reader with a comprehensive literature review including fundamental knowledge about anaerobic digestion, whiskey/beer manufacturing processes and the characteristics of the waste streams. It also describes the current laboratory scale to full scale research in AD technology for distillery/brewery wastes, along with the various applied pre-treatment types. The principles of Design of Experiment software for modelling and optimisation tools are also included as part of the literature review.

Chapter 3 reports the detailed methods of the analytical techniques used as well as the experimental justifications for the parameters under investigation for each of the AD experiments.

Chapter 4 gives detailed characterisation of pot ale along with statistical analysis of the results of anaerobic digestion and co-digestion with draff, modelling and optimisation studies. This chapter compares the feasibility of two different substrates for anaerobic digestion after combined alkaline and beating pre-treatments at low inoculum ratios (5 – 90% on wet basis).

Chapter 5 presents the anaerobic digestion of combined alkaline-microwave, alkaline-ultrasonic pre-treated pot ale; the statistical analysis of each with generated mathematical models is outlined. The significance of the achieved results in comparison with the literature are explored here.

Chapter 6 focuses on the potential use of digestate as a biofertilizer by investigating the inorganic contents of the lab scale experimental samples. Comparisons between the experimental results, an industrial reference digestate, and the literature were conducted along with the relevant regulations on agricultural application limits for the EU.

Chapter 7 describes a theoretical scaling up study for industrial implementation of anaerobic digestion technology with a major focus on the contribution of applied pre-treatments to the cumulative biogas yield. This chapter presents a model that was

designed based on different inoculum ratios (50 – 90%) to address the unique needs/constraints of the individual needs of the distillery.

Chapter 8 presents the main conclusions and contributions of this work to the field of study as well as future research suggestions.

CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

This chapter aims to provide a comprehensive description of anaerobic digestion and a critical literature review on the use of whiskey distillery/brewery waste streams for purpose of biogas generation.

This chapter is divided into 8 main sections. The graphical summary of the literature review is given in Figure 2.

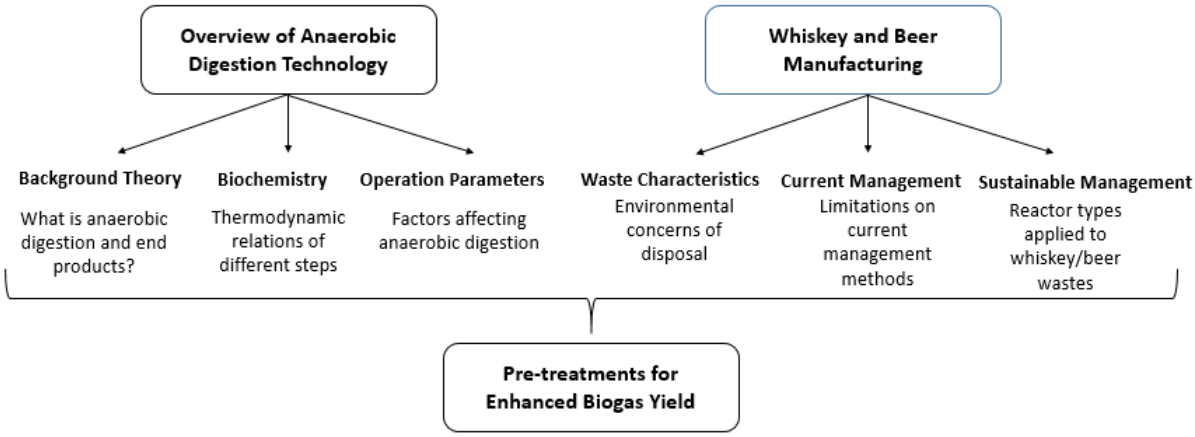


Figure 2. Graphical summary of literature review

2.2 Background Theory

Anaerobic digestion (AD) is considered a widely accepted and well-studied technology for the treatment of organic wastes (Kamalinasab et al. 2016). Among the known current technologies, it has been shown to be very appropriate for stabilizing high organic content matters not only due to its limited environmental impact, but also for its high energy recovery potential (Ariunbaatar et al. 2014; Boe 2006). It can be possible to convert a significant amount of COD (> 50-75%) to biogas, which might be used as an in-plant fuel (energy self-sufficient distilleries/breweries) depending on further purification. This would be required due to the existence of impurities such as hydrogen sulphide, nitrogen and most importantly carbon dioxide (Mallick et al. 2009; Petersson & Wellinger 2009).

An industrial AD process consists of four main stages which are; pre-treatment, digestion, gas upgrading or combined heat and power (CHP) unit, and digestate use. Figure 3 illustrates the overview of the AD process and the usage of the digestate as an end product of the process. Each stage will be discussed in detail individually.

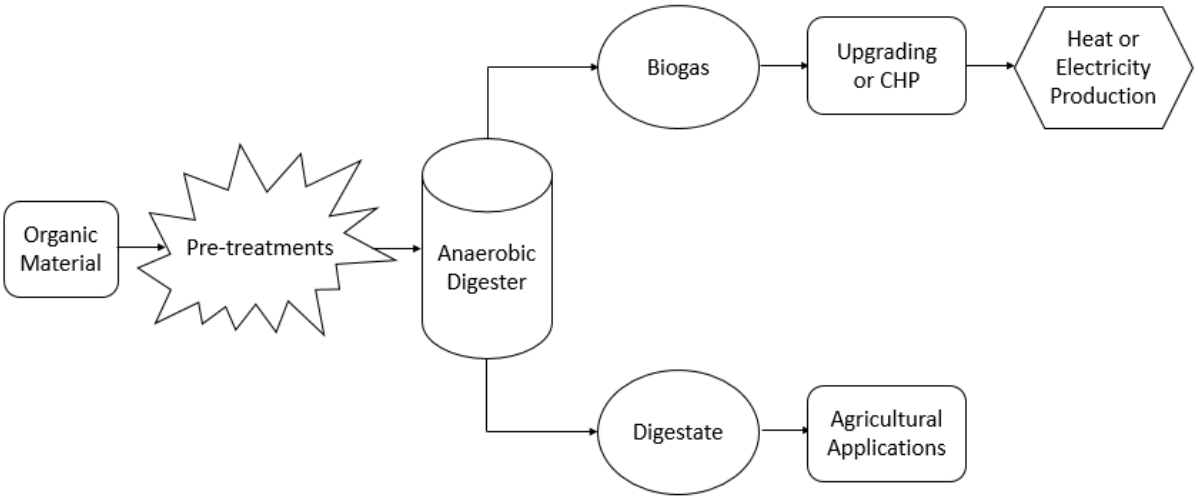


Figure 3. The overview of AD and the potential usage of the final products (adapted from Aboerheeba, 2013)

AD has some advantages over conventional aerobic wastewater treatment technologies; for example it has less sludge production, low energy consumption, potential for the destruction of the pathogens in the sludge, reduction in odour problems arising from the existence of putrescible matter, and a higher ability to cope with recalcitrant distillery and brewery wastes (Jáuregui-Jáuregui et al. 2014; H. Li et al. 2012). Considering these benefits, anaerobic digestion processes should be well suited to the treatment of distillery and brewery wastes.

2.2.1 Pre-treatment

Research on pre-treatments for AD, over the past 30 years, has focused on chemical, biological, mechanical and thermal processes; with the aim of enhancing organic compound solubilisation and biodegradability of the feed stream, to obtain a higher methane yield and improve the rate of hydrolysis. Those pre-treatments offer a deep modification, by weakening the molecular bonds between lignin and carbohydrates thereby reducing the degree of polymerisation. Thus, an increased surface area is obtained for bacterial attack (Lafitte-Trouqué & Forster 2002; Cesaro & Belgiorno 2014; Carlsson et al. 2012; Nayono 2009a; Fdez.-Güelfo et al. 2011).

To investigate the influence of pre-treatments, the biomethane potential test (a standard method developed based upon DIN 2006; ISO 1995), is commonly used. This method provides information about the cumulative amount of biogas generated as well as the rate of its production (Montgomery 2016; Sosa-Hernandez et al. 2016). Figure 4 compares

three different situations under three different time frames. It can be easily seen that a pre-treatment method can increase the rate of anaerobic digestion (case b) or can increase the methane yield (case c) in comparison to the nontreated substrate (case a).

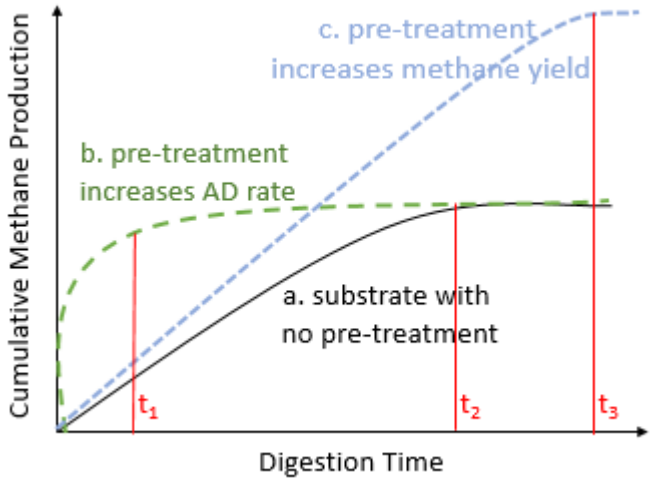


Figure 4. Effects of pre-treatments on the rate of anaerobic digestion and total methane production (adapted from Montgomery, 2014).

2.2.2 Digestion

Digestion takes place in the reactor where the bacteria break down the substrate in an oxygen depleted environment. There are many different types of reactors being used in industry, and their overall yield depends on a variety of operating parameters, such as temperature, pH, carbon-nitrogen ratio of the feedstock (C:N), hydraulic retention time (HRT), organic loading rate (OLR), and the existence of inhibitory compounds like volatile fatty acids and ammonia (Aboerheeba 2013; Ostrem 2004). Process parameters are discussed in Section 2.4.

2.2.3 End Product Use

AD typically results in the production of biogas with 50-75% CH_4 and 30-45% CO_2. Upgrading is necessary for direct use of biogas due to the existence of impurities such as hydrogen sulphide, oxygen, nitrogen, water vapour, ammonia, siloxanes and particulates, in order to avoid corrosion of the equipment as well as to maximise the energy density per volume of biogas (Yu 2016). The upgraded biogas, which reaches higher levels of CH_4 (75-82%), is chemically identical to natural gas (Montingelli 2015). Primary removal of hydrogen sulphide is essential for most of the upgrading systems. This is commonly accomplished by adding iron hydroxide to the digesters; when the biogas contains high

concentration of H_2S (< 20000 ppm), the employment of a H_2S bio-scrubber might be required before CO_2 removal. The three most cost effective applied upgrading techniques are; high pressure water scrubbing (HPWS), pressure swing adsorption (PSA) and chemical (amine) scrubbing (Santos et al. 2011; Cozma & Wukovits 2015). HPWS upgrading systems are currently regarded as being the most attractive systems for industry due to their simplicity of operation (Browne 2014). CHP, on the other hand, provides direct use of generated biogas through AD as an alternative to biogas upgrading. A typical CHP unit consists of internal combustion engines, microturbines, gas combustion and fuel cells (Shen et al. 2015). As a result of biogas ignition in a CHP unit, approximately 55% of the energy present in biogas can be converted to heat while approximately 30% can be recovered as electricity; meaning that the overall process efficiency is 85% (Nguyen 2014; Pöschl et al. 2010). In order to prevent the corrosion risk in a CHP system, removal of H_2S should be considered by either adding iron hydroxide or alkaline lubricant oil to the digester (Montingelli 2015). Combining an AD plant with a CHP unit is the most preferred method for industrial scale wastewater treatment plants (Duguid & Strachan 2016; Shen et al. 2015; Nally et al. 1982; Pöschl et al. 2010).

Digestate is the main solid by-product of the anaerobic digestion process. The inorganic nutrients of the raw input material remain in the digestate and are readily accessible for soil and crops. Therefore it is considered to have great potential in terms of substitution of mineral fertilizer (Nasir et al. 2012). Digestate is also an easy product to handle and apply. However, quality of digestate mainly depends on the nutrient present in feedstock of anaerobic digestion process (Faisal-Cury & Menezes 2006). The main nutrients of digestate can be categorised as macro nutrients such as phosphorus (P), potassium (K), nitrogen (N), calcium (Ca) and magnesium (Mg), and micro nutrients like iron (Fe), manganese (Mn), Zinc (Zn) and copper (Cu) (Kuusik et al. 2017; Kumar 2004; Barik & Sahin 2006; Santiveri et al. 2008). Although trace amounts of heavy metals like Cu and Zn are required by plants, animals and humans, excess amounts and presence of other heavy metals such as arsenic (As), cadmium (Cd), cobalt (Co), molybdenum (Mo) and mercury (Hg) are toxic to them (Kuusik et al. 2017; Selling et al. 2008).

The agronomic value of the digestate is measured using the levels of the three major plant nutrients, N, P and K (Holm&Jensen 2010). The total nitrogen concentration of the raw

material is considered to be a fundamental factor affecting the concentration of plant nutrient in digestate. The inorganic form of nitrogen (NO_3^-) is the most important for agricultural crop growth (Makádi 2012). In terms of P and K reserve, digestate has a higher concentration than most composts. As with nitrogen, some of the phosphorous content of the raw materials is converted to the inorganic form during anaerobic digestion, as such it is then is easily absorbable by crops (Kuusik et al. 2017), however all phosphorous forms in digestate are known to be readily available (Makádi 2012). In addition, using digestate as a substitute for compost is attractive as it limits the emissions of unpleasant odours by degradation of volatile organic compounds mainly iso-butonic acid, butonic acid, iso-valeric acid and valeric acid in the digestion step (Faisal-Cury & Menezes 2006). It is therefore considered to be a superior supplementation method of these missing macronutrients in soils. Unlike N and P, the forms of the other macro nutrients (K, Ca, Mg) do not change as these elements particularly do not get affected by the process of anaerobic digestion. They are present in dissolved form which is readily accessible for plants (Kuusik et al. 2017).

Despite the sufficient fertilizer value of digestates, the presence of significant levels of heavy metals such as Cu, Zn, Co, Ar, Pb and Cd suggests the potential for environmental contamination. The possibility of environmental pollution of heavy metal release into soils, water and plants brings the risk of public health though the food chain though uncontrolled agricultural use of digestate (Bonetta et al. 2014). The raw material governs the potential presence or absence of organic pollutants, heavy metals, pesticides and pathogenic bacteria in the digestate. Agricultural applications of digestate might trigger the risk of introducing those to soil ecosystem. The presence of heavy metals in digestates can be seen where wastewater treatment plant sewage sludge substrate is used for biogas generation (Bonetta et al. 2014). Nondegradable pollutants like heavy metals are not altered during anaerobic digestion therefore they might be more concentrated in the digestate than in the substrate because of the mass reduction effect of digestion (Selling et al. 2008; Kuusik et al. 2017).

Some bacteria such as *Bacillus, Pseudomonas, Penicillium, Aspergillus* and *Clostridium spp.* are known to boost the biofertilizer efficiency by increasing phosphorus solubility and nitrogen fixation in soil (Alfa et al. 2014). However, the presence of pathogenic bacteria

such as *Salmonella* and *Klebsiella* cause a potential health risk (Owamah et al. 2014). The presence of pathogenic bacteria in digestates are predominantly associated with the source of the raw material fed to the digester. In particular, substrates of animal and human origin are known to contain various pathogenic bacteria, parasites, virus, fungi and moulds (Mouat et al. 2010; Bonetta et al. 2014). There are several studies in the literature that cover digestate utilisation originating from animal and human raw input materials such as municipal organic solid waste, cattle manure (Kuusik et al. 2017), pig slurry (Abubaker et al. 2015), kitchen waste and human excreta (Owamah et al. 2014), cow dung and chicken droppings (Alfa et al. 2014), fish farm waste and slaughterhouse waste (Kuusik et al. 2016), kitchen waste (Iqbal et al. 2014) horse manure and maize (Selling et al. 2008). Although a few days digestion time under mesophilic conditions is capable of destroying up to 90% of bacteria causing diseases, further treatment of the digestate is required as per the EU derivates EC Regulation No 1774/ 2002 when human and animal by-products are used as raw materials (Alfa et al. 2014; Owamah et al. 2014; Faisal-Cury & Menezes 2006; Kuusik et al. 2017). According to EC Regulation No 1774/ 2002, it is required to pasteurise digestate of human and animal origin at 70 °C for at least 1 hour (uninterrupted) for elimination of infectious agents, after reduction of the particle size to below 12 mm. Alternatively, sterilisation at 113°C for a minimum of 20 minutes without interruption with an absolute pressure of 3 bar provided by saturated steam must be applied before any agricultural implementation (EUROPEAN COMMUNITIES 2002). Due to the risk of spreading pathogens on animal feed crops and introducing them to the food chain, the use of digestate from human and animal origin on grassland is banned in Scotland. As such, mineral fertilizer and non-animal/human by-product digestate use is predominantly preferred as these are considered to have lower risk (Mouat et al. 2010).

Agricultural application of digestate should be managed according to the seasonal plant uptake to prevent nutrient leaching, in particular N and P, into ground and surface waters. Phosphate overload potentially causes eutrophication due to its accumulation in coastal and inland waters especially in environmentally sensitive areas (of which there are many across Ireland, Northern Ireland and the UK) (A. Mouat, A. Barclay 2010). The main nitrogen source of digestate, NO_3^-, does not bind to negatively charged soil particles, consequently travelling through the soil with a risk of accumulation in underground water

reserves and breaking the natural N cycle. The EU Nitrate Directive 91/676/EEC protects the water reserves from agricultural nitrate leaching by setting the limit at 50 mg/L (Tsachidou et al. 2019). The nutrient leaching risk is considered to be higher on sandy soil due to poor water retention capacity. This problem can be managed by avoiding the application of digestate in autumn and winter when low plant uptake or high rainfall occur. Storing digestate, on the other hand, might cause a risk of volatilization of ammonia and methane gases. In order to minimise the risk of gas releases, using flexible storage bags or covering the storage tanks with airtight membranes were reported to be efficient approaches to reduce the gas emission to less than 1% (Faisal-Cury & Menezes 2006; Kuusik et al. 2017). The required storage capacity is determined as 4 months for the UK to reach less than 1% gas emission. Furthermore, the legal limit for maximum nutrient load using digestate as fertilizer is 170 kg N/ha/year along with limitation of the spreading season to 1st February – 14th October (Faisal-Cury & Menezes 2006). The legal limit for amount of copper that can be added to agricultural land is determined as 12 kg ha^{-1} year^{-1} by the EU (Santiveri et al. 2008), with the maximum Cu concentration in the animal diet stated as being 25 to 30 mg/kg to prevent adverse health outcomes in animals (Council 1980).

2.3 Biochemical Reactions in Anaerobic Digestion

AD is a complex and sequential process, which provides the degradation of organic materials by microorganism activity in an oxygen depleted environment (oxidation reduction potential (ORP) <-200 mV); resulting in the production of biogas rich in methane (Nayono 2009a; Appels et al. 2008). The type of bacteria involved in the sequential steps are known as hydrolytic, acidogenic (or fermentative), acetogenic (or syntrophic), and methanogenic bacteria (Moraes et al. 2015). The presence of sulphate, sulphite, or thiosulfate in the reaction mixture results in the reduction of oxidized sulphur matter to different forms of dissolved sulphide (HS^-, S^{-2}, H_2S) in the digestate, and to hydrogen sulphide (H_2S) in the generated biogas (O'Flaherty et al. 2006). The types of bacteria commonly involved in each stage of AD are given in Table 1 and Figure 5 illustrates the process of AD of complex organic materials with the group of bacterial activity in each step.

Table 1. Type of Bacteria for each stage of AD adopted from (Frischmann 2012)

Degradation stage	**Bacterial group**	**Type of conversion**	**Type of bacteria**
Hydrolysis	Hydrolytic bacteria	Proteins to soluble peptides and amino acids	*Clostridium, Proteus vulgaris, Peptococcus, Bacteriodes, Bacillus, Vibrio*
		Carbohydrates to soluble sugars	*Clostridium, Acetovibirio celluliticus, Staphylococcus, Bacteriodes*
		Lipids to higher fatty acids or alcohols and glycerol	*Clostridium, Micrococcus, Staphylococcus*
Fermentation (Acidogenesis)	Acidogenic bacteria	Amino acids to fatty acids, acetate and NH_3	*Lactobacillus, Escherichia, Staphylococcus, Bacillus, Pseudomonas, Desulfovibrio, Selenomonas, Sarcina, Veillonella, Streptococcus, Desulfobacter, Desulforomonas*
		Sugars to intermediary fermentation products	*Clostridium, Eubacterium limonsum, Streptococcus*
Acetogenesis	Acetogenic bacteria	Higher fatty acids or alcohols to hydrogen and acetate	*Clostridium, Syntrophomonas wolfeii*
		Volatile fatty acids and alcohols to acetate or hydrogen	*Sytrophomonas wolfeii, Sytrophomonas wolinii*
Methanogenesis	Carbon dioxide reducing methanogens	Hydrogen and carbon dioxide to methane	*Methanobacterium, Methanobrevibacerium, Methanoplanus, Methanospirillum*
	Acetoclastic methanogens	Acetate to methane and carbon dioxide	*Methanosaeta, Methanosarcina*

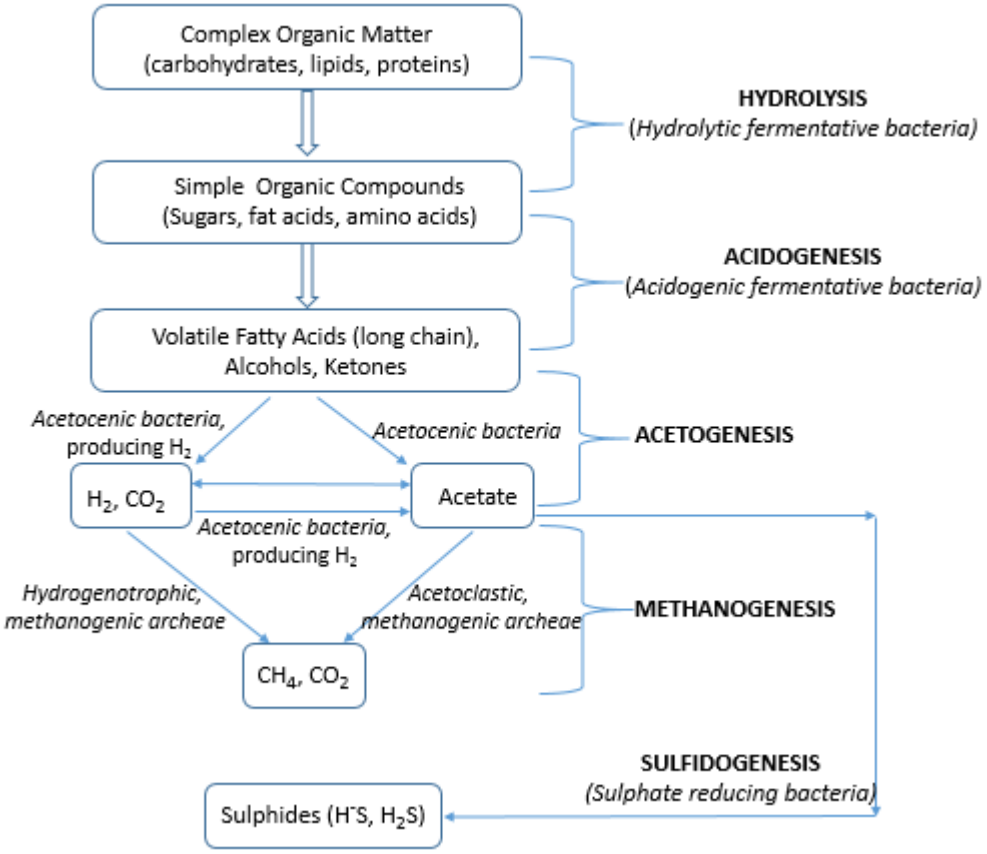


Figure 5. Stages of anaerobic digestion process adapted from (Moraes et al. 2015)

The thermodynamic relation with Gibbs free energy of the biochemical reactions that take place in AD is given in Table 2. Acetogenesis reactions are thermodynamically unfavourable; nevertheless, they occur naturally during AD as a result of interaction of the activity of methanogenetic and acetogenetic bacteria (Moraes et al. 2015), while all other reactions are thermodynamically favourable (Table 2). On the other hand, the sulfidogenesis stage also limits AD of distillery/brewery wastes due to favourable bacterial competition to sulphate reducing bacteria based upon the thermodynamics of the reactions (Collins et al. 2003). To prevent thermodynamic impediments, H_2 produced by acetogenic bacteria should be continuously purged. This ensures that the production of acetate is not blocked as it is the key intermediate product. Such biological reactions are favourable under low hydrogen partial pressure (Madsen et al. 2011; Wirth et al. 2012; Leitão et al. 2006; O'Flaherty et al. 2006).

Table 2. Common reactions in anaerobic digestion with Gibbs free energy in standard conditions (adapted from Collins et al. 2003; Moraes et al. 2015; A.J.M Stam et al. 2005)

Reaction Type		ΔG° (kJ/reaction)
Acidogenesis	$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COO^- + 2CO_2 + 2H^+ 4H_2$	-206
	$C_6H_{12}O_6 + 2H_2 \rightarrow 2CH_3CH_2COO^- + 2H_2O + 2H^+$	-358
	$C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COO^- + 2CO_2 + H^+ + 2H_2$	-255
Acetogenesis	$CH_3CH_2COO^- + 3\ H_2O \rightarrow CH_3COO^- + HCO_3^- + H^+ + 3H_2$	+76
	$CH_3CH_2CH_2COO^- + 2H_2O \rightarrow 2CH_3COO^- + H^+ + 2H_2$	+48.1
	$CH_3CH_2OH + H_2O \rightarrow CH_3COO^- + H^+ + 2H_2$	+9.6
Methanogenesis	$CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^- + 2H_2$	-31.0
	$H_2 + 1/4\ HCO_3^- + 1/4\ H^+ \rightarrow 1/4\ CH_4 + 4/3\ H_2O$	-33.9
	$HCOO^- + 1/4\ H_2O + 1/4\ H^+ \rightarrow 1/4\ CH_4 + 3/4\ HCO_3$	-32.6
Sulfidogenesis	$CH_3CH_2COO^- + 3/4\ SO_4^{2-} \rightarrow HS^- + 4H_2O$	-37.7
	$CH_3CH_2COO^- + 1/2\ SO_4^{2-} \rightarrow 2CH_3COO^- + 1/2\ HS^- + 1/2\ H^+$	-27.8
	$CH_3CH_2OH + 1/2\ SO_4^{2-} \rightarrow CH_3COO^- + 1/2\ HS^- + 1/2\ H^+ + H_2O$	-66.4

2.3.1. Hydrolysis

Complex organic materials are first decomposed to their component monomers as a result of hydrolytic enzymatic attack within the hydrolysis (the first) step. Degradation of complex molecules into their monomers has a significant importance prior to the acidogenesis step as acidogens cannot absorb complex organic compounds directly into their cells (Boe 2006). Acidogenic fermentative bacteria convert the end product of the hydrolysis step, soluble monomers of complex feedstocks, into simple organic matters. These are predominantly short-chain (volatile) organic acids such as acetic, propionic, butyric, isobutyric acid, valeric acid, isovaleric acid, pentanoic acid; alcohols, for instance, methanol and ethanol; and aldehydes, such as carbon dioxide and hydrogen. Acetate is the most important organic acid as it can be directly used as a substrate by methanogenic bacteria (Ziemiński & Frąc 2014).

Hydrolytic bacteria provide hydrolytic enzymes to convert biopolymers into simple soluble monomers. However, different biopolymers require specific enzymes with the corresponding molecular conversion (Figure 6).

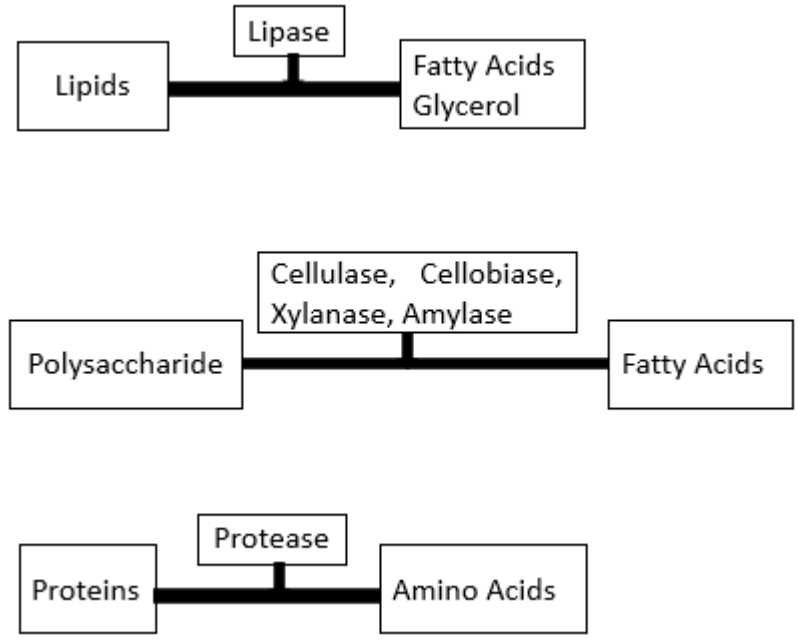


Figure 6. Enzymatic hydrolysis in AD

The hydrolysis process itself includes five different steps namely, enzyme production, diffusion, adsorption, reaction, and enzyme deactivation (Boe 2006). The rate of the hydrolysis process can be affected by parameters such as: particle size of substrate, pH, enzyme production rate, and the rate of diffusion and adsorption of enzymes on the particles of wastes subjected to the AD process (Montingelli 2015). On this basis, hydrolysis is a function of both biomass and substrate concentration. It is also commonly considered a rate limiting step if the feedstock is a complex cellulolytic waste, which contains lignin, due to the relatively slow biodegradability of this kind of substrate (Aboerheeba 2013; Alfarjani 2012). High lignin content feedstock, therefore, needs to be pre-treated prior to AD in order to achieve higher process yields (Zhang et al. 2015).

2.3.2 Acidogenesis

The second stage of the overall process is acidogenesis, also known as fermentation due to the oxidation of the organic molecule by acting as terminal electron acceptor in an environment depleted of exogenous electron acceptors (nitrate, sulphate) (Burchall 2016). Acidogenic reactions are carried out by acid forming (fermentative) bacteria known as acidogens. The intermediate products, most importantly acetate which can be used by acetoclastic methanogens directly, are produced as a result of acidogenic activity (Ziemiński & Frąc 2014). The concentration of the end products of this step varies

depending on the type of bacteria in conjunction with the physical conditions, e.g. temperature and pH of the culture. Glucose fermentative bacteria are able to metabolise the feedstock in different pathways which produce different intermediate products (Boe 2006). While glucose can be easily degraded by acidogens, VFAs must be oxidised by an external electron acceptor. Amino acids can be degraded through Stickland reactions where one amino acid acts as an electron acceptor while another acts as an electron donor (Ramsay & Pullammanappallil 2001). Acidogenic bacteria can have ten to twenty-fold higher growth rate than methanogens as well as five-fold higher bacterial yields and conversion rates. Anaerobic reactors therefore can be subjected to a sharp drop in pH as a result of non-dissociated VFAs, especially when the reactors are overloaded with VFAs (Nguyen 2014).

2.3.3. Acetogenesis

During acetogenesis, fatty acids longer than two carbon atoms, alcohols longer than one carbon atom, and branched-chain and aromatic fatty acids are oxidised to acetate and H_2 by proton reducing bacteria, as they cannot be used by methanogens (Schink 1997; Stam et al. 2005). VFAs are considered one of the main intermediates, so in this step, acetogenic bacteria break down the low molecular weight VFAs such as propionate, i-butyrate, butyrate and i-valerate to acetate, hydrogen gas and carbon dioxide; for this reason acetogenesis is often considered to be part of a single acid forming stage (Zhang et al. 2015). Acetogenesis and methanogesis steps usually run in parallel due to the symbiosis of the two groups of microorganisms.

2.3.4 Methanogenesis

In the methanogenesis stage, methane is produced through two different pathways by two different groups of bacteria. Approximately 70% of the methane is produced by division of acetate molecules into carbon dioxide and methane by acetoclastic methanogens. The remaining part is produced by the reduction of carbon dioxide with hydrogen by hydrogen-oxidizing methanogens (Aboerheeba 2013). Figure 7 illustrates the reactions that occur in the methanogenesis phase.

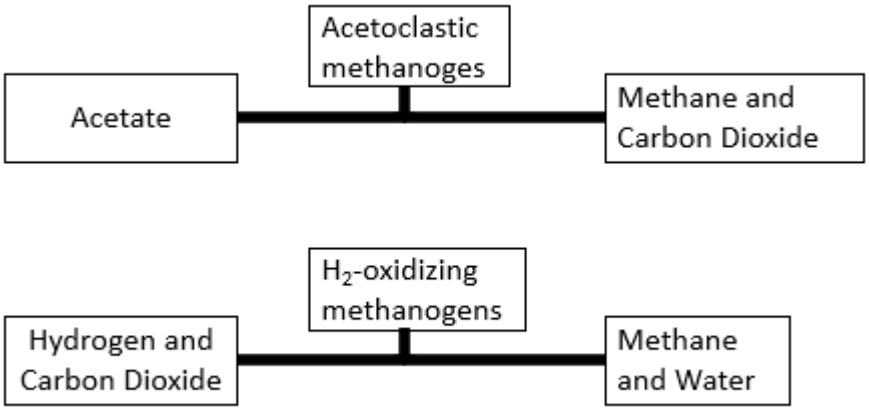


Figure 7. Methanogenesis biochemical reactions

The methanogenic bacteria are severely affected by the chosen process parameters. Therefore, it is a critical stage for the entire process. Operating temperature and pH, feedstock composition, organic loading rate and hydraulic retention time are the main examples of the factors affecting methanogen activity. Digester overloading, temperature and pH changes can potentially result in termination of methane production (Kim & Park 2014). Furthermore, the optimum conditions for the microorganisms involved in acidogenesis and methanogenesis steps vary widely in terms of desired environmental conditions, nutrients and growth kinetics. Hence, an inability to maintain the balance between these two groups of microorganisms can lead to failure of the AD (Montingelli 2015).

2.4 Operating Parameters of Anaerobic Digesters

The stability of AD is severely affected by the operating parameters, thus providing the most suitable conditions for the microorganisms is critical for the overall yield of AD. To ensure the stability of the AD systems, equilibrium must be achieved between the different bacterial groups which are involved in the complex interactions of the different stages of AD. The potential changes which might occur in the reaction environment could impair the equilibrium and might eventually inhibit the AD process (Ostrem 2004). For instance, if the methanogenic bacterial activity is slowed due to a sudden environmental change, it will result in acid accumulation in the digester and the process will not complete. In order to increase bacterial activity and methane production yield, the necessity for monitoring the physicochemical conditions such as pH, temperature, C:N ratio, hydraulic retention time, organic loading rate, volatile fatty acid content and composition, bacterial competition, nutrient content, the presence of toxicants and moisture content must be taken into account. It is important to ascertain the optimum ranges for all parameters. This section provides an overview of the major process and operating parameters.

2.4.1 pH

The pH is the measurement of the acidity/alkalinity of the solution. If the overall process is considered, the optimum pH value would be between 5.5 and 8.5 (Aboerheeba 2013). However, methanogens i.e. *Methanospirillum hungatei, Methanosarcina barkeri* and *Methanobacterium formicicum* are extremely sensitive to pH and require a neutral pH. Most methanogens favour a pH range of between 7.0 and 7.2 although the range between 6.8 and 7.2 is acceptable (Moraes et al. 2015), whereas hydrolysis and acidogenesis occur at pH 5.5 and 6.5, respectively (Migliore et al. 2012). pH levels below 6 and above 8.3 reduce methanogen activity significantly (Ostrem 2004). Many designers would prefer separating the hydrolysis/acidogenesis and the acetogenesis /methanogenesis into a two stage process in order to prevent the impairment between the different kinds of bacterial community (Weiland & Weiland 2013).

Ideally, most of the metabolic products of a well-balanced AD process continue to undergo catalytic process without accumulation (Nayono 2009a). However, acidogens produce a high volume of organic acids during the acidogenesis step and this leads to a

sharp pH drop below 5 which is fatal for the methanogens. Therefore, external action is required such as increasing the amount of the recycling water. On the other hand, the pH might show an upward trend (up to pH 8) in digesters as a result of the degradation of proteins due to ammonia release. Increasing pH (over 8) impedes the reactions which are carried out by acidogens. Adding larger amount of fresh feedstock can solve that problem by spurring acid forming bacteria growth (Ostrem 2004). A neutral initial pH is also crucial to achieve a well-balanced AD process (Zhou et al. 2016; Mallick et al. 2009). The initial pH adjustment can be performed by using sodium (bi-) carbonate, potassium (bi-) carbonate, calcium carbonate (lime), calcium hydroxide (quick lime) or sodium nitrate. Bicarbonate alkalinity is better for the function of methane forming bacteria (Nayono 2009a).

2.4.2 Temperature

Temperature is one of the major parameters of AD process, as different types of microorganisms favour different temperatures. AD can occur under psychrophilic (10 to 20°C), mesophilic (20 to 40°C), and thermophilic (40 to 60°C) conditions (Caye et al. 2008). Optimum temperatures are defined according to different methane-forming bacteria strains (Ogejo et al. 2009). Early research indicated that higher methane yield is achieved under thermophilic conditions; however, increased energy output per unit volume of reactor at lower digestion temperatures has been found more recently (Saady & Massé 2013). Moreover, psychrophilic and mesophilic digesters have advantages in comparison with thermophilic digesters such as lower energy demand for heating and ease of control, as cold adapted bacteria are not as sensitive to unexpected temperature fluctuations as mesophilic and thermophilic bacteria (Aboerheeba 2013). A small temperature change (± 1°C) has a negative impact on biogas production under thermophilic conditions while mesophilic bacteria can tolerate fluctuations up to ± 3°C without a significant reduction of biogas generation (Kim & Park 2014). Thus, reactors under psychrophilic and mesophilic conditions have higher process stability (Ferrer et al. 2008). Furthermore, thermophilic conditions show a suppressive effect on methanogens which results in a lower biogas yield due to the formation of volatile gases such as ammonia (Trent et al. 2012). Hence, operation under mesophilic condition (35-37°C) is generally preferred by most biogas plants as the process is more stable, has a better biogas yield and is less energy intensive.

2.4.3 Organic Loading Rate (OLR) and Hydraulic Retention Time (HRT)

The organic loading rate (OLR) and the hydraulic retention time (HRT) are considered the most important operating parameters of AD since they both affect biogas yields and plant economy. The OLR is the amount of organic dry matter that can be fed into the digester per time unit and per volume, according to (Eq 2)

$$\mathbf{OLR} = \frac{\mathbf{m} \times \mathbf{c}}{\mathbf{V}} \qquad \text{Eq 2}$$

Where OLR represents organic loading rate [kg day^{-1} m^{-3}], m is the mass of feedstock fed per unit time [kg day^{-1}], c is the concentration of organic matter [%] and V is the digester volume [m^3].

The HRT is the average required time to obtain complete degradation of organic substrates in a digester. The hydraulic retention time is linked to the digester volume and the volume of substrate fed per unit time, according to (Eq 3)

$$\mathbf{HRT} = \frac{\mathbf{V}}{\mathbf{Q}} = \frac{\mathbf{m} \times \mathbf{c}}{\mathbf{Q} \times \mathbf{OLR}} \qquad \text{Eq 3}$$

Where HRT refers to hydraulic retention time [day] and V is the volume of substrate fed per time unit [m^3 day^{-1}].

Variation in the organic concentration of the feedstock directly influences the HRT and it causes changes in the value of OLR (Nayono 2009a). As long as the feedstock of the process has stability in concentration of the organic fraction, it would be possible to be run with shorter HRT and higher OLR. Typical HRT ranges have been identified as 15 to 30 days and 12-14 days for digesters run under mesophilic and thermophilic conditions, respectively (Monnet 2003; Verma 2002). Many system failures have been reported by industrial plants because of overloading. The reason of the failure would be the effect of increasing OLR on the acidogens; thereby increasing acid production rate. Methanogens require a relatively longer time to grow, therefore methanogenic bacteria would not be able to deplete fatty acids simultaneously. Accumulation of the organic fatty acids in the reaction environment results in an abrupt pH drop which is lethal for the methanogenic bacteria (Harun et al. 2010; F. Alfarjani 2012). The minimum HRT must be defined depending on the anaerobic bacteria which grow at the lowest rate. There is a linear relationship between HRT and reactor size. A lower volume is generally desired in terms

of cost savings. Moreover, shorter residence time results in higher methane production per unit volume of digester as long as the required preventative measures for stability of the reactor have been taken into account (Nayono 2009a).

2.4.4 Nutrients

Macronutrients, for instance carbon (C), nitrogen (N), phosphorous (P), and sulphur (S), are essential for the growth of anaerobic microorganisms. Unbalanced nutrients are considered a critical limiting factor for AD (Fricke et al. 2007). The C:N ratio is generally subcategorized within AD nutrients as the relative amount of carbon and nitrogen that has a direct effect on the methane production due to its direct relation to potential inhibitions (Li et al. 2011). The known optimum C:N ratio is between 20:1 – 30:1 for any type of anaerobic digester to supply adequate nitrogen for bacterial growth (Wang et al. 2014). A high C:N (> 35:1) ratio (Mao et al. 2015) is not suitable for bacterial growth, in particular methane forming bacteria, because of the inadequate level of nitrogen. It can result in lower methane production. The substrate with low C:N ratio (< 15:1) (Wu et al. 2010), on the other hand, can lead to ammonia accumulation by means of methanogenic activity; as a result of this, the pH could reach a value above 8, which is toxic for acidogens (Alfarjani 2012; Ostrem 2004).

2.4.5 Volatile Fatty Acids (VFA)

The stability of AD systems is determined by the concentration of the intermediate products in particular VFAs, which include carbon chains of up to 6 atoms such as acetate, propionate, butyrate and lactate, produced via the acidogenesis step (Searmsirimongkol et al. 2011). Under the condition of overloading, methanogens are not able to remove the hydrogen and volatile organic acids as rapidly as they are produced (Siegert & Banks 2005). Correspondingly, a sharp pH drop is observed in the bioreactor, resulting in an increase in the fraction of undissociated VFAs. This then becomes more toxic by flowing through the cell membrane where dissociation can occur, which in turn lowers the internal pH of the cells, disrupting homeostasis (Montingelli 2015; Appels et al. 2008). VFA accumulation has different effects on batch reactors such as, microbiologically distinct hydrolysis, acidogenesis biogas production and a reduction in overall system pH. Fermentative bacteria (acidogens) have shown less sensitivity to VFA inhibition (Siegert & Banks 2005).

2.4.6 Ammonia

Ammonia is generated as a result of biological degradation of nitrogenous compounds such as proteins and urea within the digester (Chen et al. 2008). Ammonia concentration below 200 mg/L are widely accepted as beneficial for anaerobic processes, since nitrogen is an essential nutrient for anaerobic bacteria (Boe 2006). Although ammonia has a significant role in the growth of microorganisms, it may reduce the activity of methanogens, which are extremely sensitive; ammonia present in the digester can be regarded as a potential inhibitor (Kim & Park 2014). Higher digestion temperatures increase free ammonia accumulation, therefore the risk of ammonia inhibition is greater under thermophilic conditions (Yenigün & Demirel 2013; Boe 2006).

It is possible to eliminate ammonia inhibition by lowering the pH and temperature, adjusting the substrate concentration, C:N ratio and external compound addition (Yenigün & Demirel 2013; Chen et al. 2008).

2.4.7 Moisture Content

Anaerobic digesters can be classified as “dry” or “wet” based on the amount of total solids (TS) in the feedstock. Dry bioreactors have TS of 22-40%, whereas wet bioreactors contain 16%, or less, TS (Montingelli 2015). High moisture content catalyses AD, and, correspondingly, it has been reported that the highest methane generation kinetics occur at 40-20% TS, evidenced by increased specific methanogenic activity (Khalid et al. 2011).

2.5 Process Description of Whiskey Distilleries and Breweries

The whiskey manufacturing process, outlined in Table 3 and Figure 8, can be divided into six main steps: malting, mashing, fermentation, distillation and maturation (Graham et al. 2012; Goodwin et al. 2001).

Table 3. Whiskey production steps

Process Step	Purpose
Malting	The grain is steeped in water and dried to give characteristic malt flavour to the grain
Milling	Reduction of barley grain size; removal of husks
Mashing	Long chain starch molecules broken down to soluble sugars by enzymatic action at high temperature; Wort
Fermentation	Wort is fermented using yeast to obtain 6-7% ethanol
Distillation	Alcohol is separated as a top product with 20% ethanol concentration; Pot Ale produced as bottoms
Maturation; Bottling	Flavour establishment typically in wooden casks

The manufacture of craft beer has many similarities with the initial stages of the whiskey production process. It also starts with malting and mashing steps of barley or other grains. Hops are also added to give the characteristic bitterness flavour of beer and avoid bacterial spoilage. The product of the fermentation step is then subjected to filtration and stabilization, maturated, and bottled/kegged (Ferreira et al. 2010).

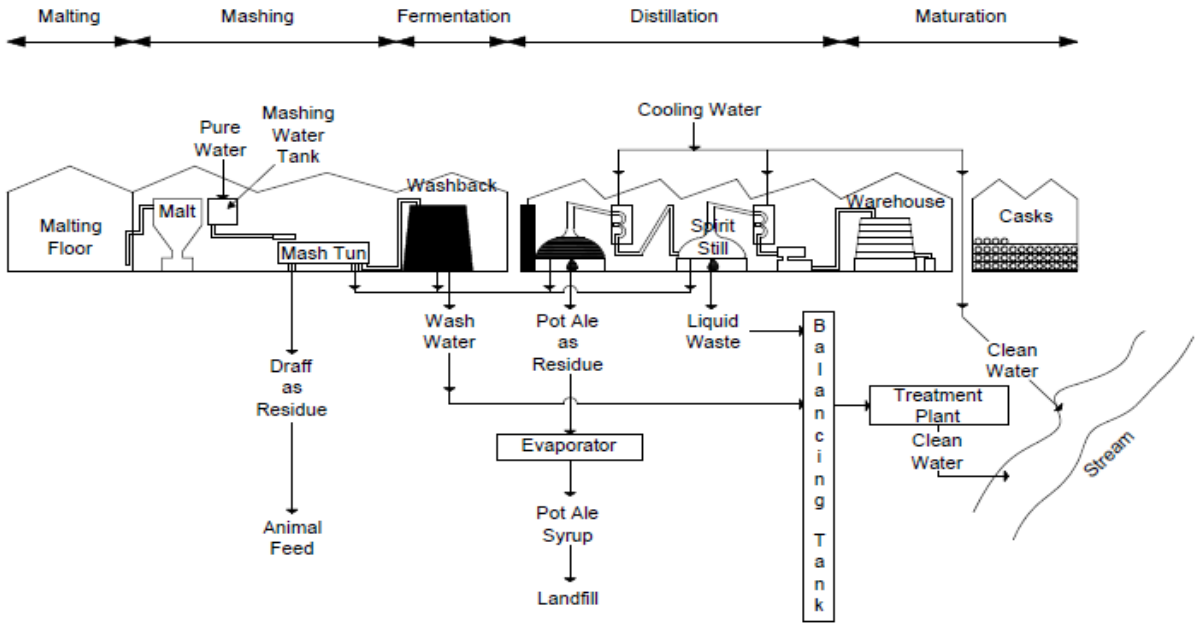


Figure 8. Main steps of whiskey distillery process adapted from (Graham et al. 2012)

Due to the similarities of these two processes, solid waste fractions, spent barley and spent yeast, are not much different; however, distilleries also generate massive amounts of pot ale (8.5 – 11.5 L per litre of malt whiskey) as a co-product of the distillation steps. Spent wash is also a significant liquid waste (e.g. 16-21 L per litre of grain whiskey (Mallick et al. 2009)).

2.5.1. Characteristics and Toxicity Profile of the Wastes

In a typical whiskey distillery, liquid residues left in the wash and spirit still after the distillation steps comprise the majority of the waste stream, known as pot ale and spent lees, respectively. In terms of solid waste, spent grain (also called draff) and spent yeast arise from the mash tun and fermenter of both distilleries and breweries (Goodwin et al. 2000).

Pot ale is a highly turbid, concentrated, caramelised and cumbersome liquid effluent (Graham et al. 2012); with millions of m^3 produced globally per year (Tokuda et al. 1998). Hence disposing of liquid waste is a major concern for distilleries; typical pot ale characteristics are summarized in Table 4. Pot ale has a high COD and BOD, and significant levels of phosphorus and ammonia (Goodwin et al. 2001; Mallick et al. 2009; Tokuda et al. 1998; Pant & Adholeya 2007). As copper stills are typically used in the distillation step, copper, which is toxic to micro and macroorganisms, is commonly seen in pot ale due to mass transfer between refluxing liquid and hot stills (Graham et al. 2012; Dionisi et al. 2014). Pot ale is harmful especially for aquatic life because of the high level of COD/BOD leading to decreases in the level of solubilised oxygen and eutrophication (Ansa-Asare et al. 2000). Due to its dark coloured nature it can block the penetration of sunlight into the receiving water, reducing the level of dissolved oxygen by restricting photosynthesis (Ravikumar et al. 2010). Spent lees have lower COD/BOD and contain volatile organic acids such as formic, acetic, propionic, butyric and pentanoic, which are the intermediate products of AD (Goodwin et al. 2001; Aboerheeba 2013).

The polluting strength of these liquid waste streams is significantly high, due to the large amounts of biodegradable organic material (sugars, lignins, hemicelluloses, dextrins, resins and organic acids) and fertilizers such as potassium, phosphorus and nitrogen (Pant & Adholeya 2007; Sangave & Pandit 2004; Mallick et al. 2009). They can cause odour

problems as a result of the decaying organic matters by releasing volatiles such as skatole, indole and other sulphur containing compounds (Pant & Adholeya 2007; Sangave & Pandit 2004; Mallick et al. 2009). Furthermore, uncontrolled land discharge of distillery and brewery waste water causes high levels of acidification. It has been shown that land discharge of distillery liquid wastes can impair seed germination (Ramana et al. 2002); potentially due to a decline in soil pH, leading to inhibition of agricultural crops (Mohana et al. 2009).

Table 4. Characteristics of distillery liquid residues

Parameter	Pot ale	Spent lees	Reference
Total solids	23	17	(Mallick et al. 2009)
Total suspended solids	9.6	4.5 – 7	(Sankaran et al. 2014)
Volatile suspended solids	9.4	8.1	(Mallick et al. 2009)
Total nitrogen	37	5 – 7	(Acharya et al. 2008; Mallick et al. 2009)
COD	30 – 50	85 – 110	(Goodwin et al. 2001; Sankaran et al. 2014)
BOD	25 – 35	25 – 35	(Goodwin & Stuart 1994; Tokuda et al. 1998)
pH	3.5 – 4.5	4.0 – 4.2	(Mallick et al. 2009)

* Units are in g/L except pH.

Environmental regulations force distilleries to enhance existing treatment technologies as well as adopt new and more efficient methods for waste management (Tokuda et al. 1999). In addition, it is mandated that the amount of landfilled biowaste is decreased to less than 10% of waste generated (Directive (EU) 2018/850 of the European Parliament and of the Council of 30 May 2018 amending Directive 1999/31/EC on the landfill of waste, (Capros et al. 2016). Thus, recovery of organic waste streams has become a major focus of waste management policies, with biological processes, predominantly anaerobic digestion, being seen as the main solution for high organic content wastes (Cesaro & Belgiorno 2014).

The yeast commonly used in the alcoholic beverage industry is generally divided into two classes: namely top fermenting and bottom fermenting yeast. Top fermenting yeast has significantly high usage (more than 90%) globally. Although similar surface ultrastructure is seen in top and bottom brewing, the flexibility of the cell wall is different due to the different levels of polysaccharides and hydrophobic compounds (Ferreira et al. 2010).

Yeast cells are covered by a thick cell wall, which is a complex matrix of phosphomannans, glucans, chitin and protein; thus they are not easily biodegraded (Mallick et al. 2009).

Spent grain, draff, such as spent barley, spent yeast and spent hops (breweries only), are generated in relatively large amounts, with more than 3.4 million tonnes being produced in the UK every year (Mussatto et al. 2006; Aliyu & Bala 2013). Spent grain basically consists of kernel husk, pericarp and seed coat, which have high levels of cellulose (16.8–25.4%), hemicellulose (mostly arabinoxylans) (21.8–28.4%), lignin (11.9–27.8%), proteins and fibres. Hence, it is considered a lignocellulosic material (Panji et al. 2015; Sežun et al. 2000). Spent grain is being used for animal feed , mainly for cattle, due to its both highly nutritious content and low/no cost, either in wet form or as dried conventionally (Öztürk et al. 2002). However, this might be poisonous when pot ale and spent grain mixture are used depending upon copper level as many animals cannot metabolise copper, particularly sheep (Graham et al. 2012). Lignin limits the degradation of lignocellulosic material due to its high level of recalcitrance (Neves et al. 2006).

2.5.2 Current Methods of Waste Disposal

Pot ale is the major problem of disposal for whiskey distilleries due to the large amount of production, millions of m^3 per year, as well as high organic content. Apart from high COD and BOD levels, the presence of copper limits the disposal alternatives. Current methods to dispose of or treat pot ale include: spreading on land as fertiliser, release to water bodies after treatment and evaporation to reduce the amount of effluent into pot ale syrup for use in animal nutrition (Jack et al. 2014; Graham et al. 2012). However, all of these disposal or treatment methods have been limited by waste management policies (Cesaro & Belgiorno 2014). Uncontrolled land spreading has potential toxicity effects for the environment due to the contained pollutants in pot ale. Releasing to water bodies, which is limited due to the risk of eutrophication, is only possible if the location of the distillery allows. Producing pot ale syrup to be used as animal feed is expensive due to the high energy demand for evaporation. Moreover, the use of pot ale syrup in the animal feed industry is limited because of its copper content (Graham et al. 2012; Jack et al. 2014; Dionisi et al. 2014). Although spent lees and wash water have relatively low organic content and are generated in lower amounts in comparison with pot ale, the same

limitations apply to disposal or treatment of these waste streams (Mallick et al. 2009; Satyawali & Balakrishnan 2008; Prakash et al. 2014).

Spent grain, which is the common solid effluent for whiskey distilleries and breweries, is the extracted residue of malted barley. It has therefore a lignocellulosic nature as well as a high protein and fibre content (Spinelli et al. 2016; Moreira et al. 2012). It is currently being used in animal nutrition as well as in human food for instance flour, bread, cookies and meat due to its both highly nutritious content and low/no cost either in wet form or as dried (Öztürk et al. 2002).

Spent yeast, on the other hand, is the secondary waste stream of breweries after spent grain. It is considered a great source of protein as well as nucleic acids, minerals and vitamins (particularly B-complexes). Brewery spent yeast is predominantly used in the food industry after heat inactivation to produce yeast protein concentrates while retaining its nutritive values and functional quality. Those products are generally found in the form of tablets, powders or in liquid form (Mallick et al. 2009; Ferreira et al. 2010). Usage of spent yeast as animal feed is also popular worldwide (Ferreira et al. 2010).

Although both whiskey and beer are among the most consumed alcoholic beverages globally, anaerobic digestion of the waste streams has received little attention in the literature to date (Goodwin et al. 2001; Ferreira et al. 2010). It is attractive for the treatment of distillery/brewery waste, with advantages over conventional wastewater treatment processes such as less sludge production, lower energy consumption, destruction of pathogens, odour limitation and a higher ability to cope with high organic content (Gonçalves et al. 2015; H. Li et al. 2012; Baloch & Akunna 2003). Accordingly, research into the development of the usage of anaerobic digestion has become worthy of attention in order to solve environmental concerns and the treatment of the beverage industry's waste streams by converting the organic fraction of the wastes into biogas and thus generate renewable energy, thereby reducing non-renewable energy sources currently in use (Rajeshwari et al. 2000; Mallick et al. 2009). Prior to AD of the effluents of whiskey distilling and brewing industries, pre-treatments are necessary in order to overcome their recalcitrant nature to obtain higher biogas yield, modifying the structure

of the substrates to make them more easily degradable (Neves et al. 2006; Raud et al. 2015; Weiß et al. 2010). Different types of pre-treatments are discussed in Section 2.8.

2.6 AD Reactor Configurations for Distillery/Brewery Waste Streams

A variety of reactor configurations have been used for anaerobic digestion of whiskey distillery/brewery wastes at different scales (Table 5). Reactors can be categorized based on the design (vertical, horizontal, inclined), feedstock (single, co-digestion), mode of operation (batch, continuous, semi-continuous) and operating temperature (psychrophilic, mesophilic, thermophilic). Batch reactors provide better process control than continuous mode reactors (Tauseef et al. 2013; Nayono 2009b). Reactors for AD can also be run as single, two stage or multi stage, which can be advantageous due to the different pH requirements of microbes involved in the different stages of the process (Massé et al. 2011; Mottet et al. 2009; Jang et al. 2014). In single stage systems all biochemical reactions occurs simultaneously in one reactor whereas in a two/multi stage AD system the hydrolytic-acidogenic stage is spatially or temporally separated from the methanogenic stage; in this way acidogenic microbes could be stimulated to produce more enzymes, so resulting in more expanded degradation (Parawira et al. 2005; Ariunbaatar et al. 2014). Both single and multi-stage reactors have advantages and drawbacks. For example, single stage digesters are required to operate under the same system conditions despite different microbial growth rates and optimal pH for each step of AD. This results in sudden pH changes within the reactor and consequently inhibits methanogenic activity. To overcome this problem in multi stage digesters, first reactor parameters are designed to maximise breaking down biopolymers and releasing fatty acids (hydrolysis/acidogenesis). Methanogenesis then occurs in the second reaction stage with the product of the first reactor; however a decline in biogas potential is sometimes observed due to the loss of solid particles from the feedstock to the further stage(s) where distinct reactor vessels are employed rather than sequencing batch reactors (Nayono 2009b).

2.6.1 Conventional Anaerobic Reactor Configurations for Whiskey Distillery/Brewery Waste Treatment

2.6.1.1 Anaerobic Batch Reactor

Batch reactors are loaded with fresh feedstock with inoculum and sealed for the retention time, which ensures completion of biochemical reactions within the reactor. Once a batch reactor is opened, the residual is removed. Batch reactors are generally considered accelerated landfill boxes even though they show much higher biogas production rate then typical landfill areas (Nayono 2009a). Integration of a recirculating liquid phase, known as leachate, which requires little investment and maintenance, into traditional batch reactors enhances the AD yield. Upgraded batch reactors are known as leaching batch reactors (Degueurce et al. 2016) (Figure 9)

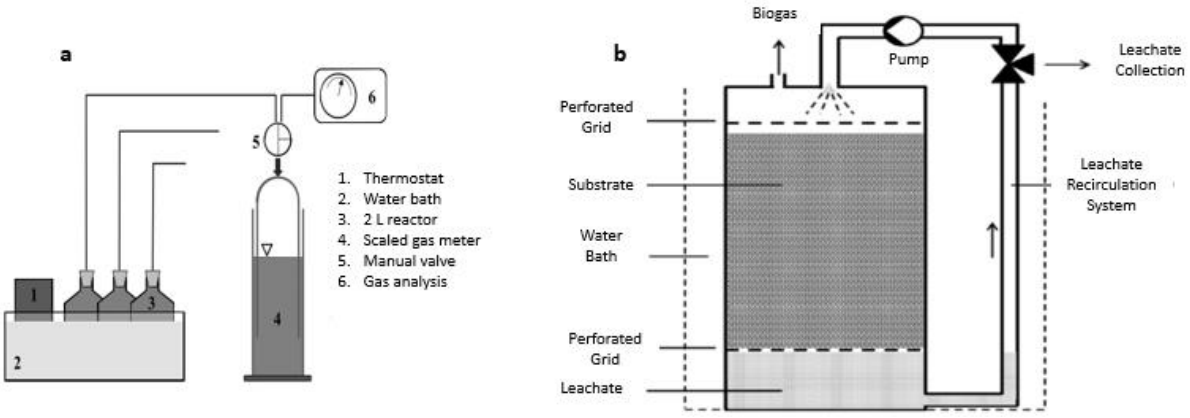


Figure 9. Schematic diagram of (a) batch and (b) leaching batch reactor (Degueurce et al. 2016)

Leachate has a key role on the biogas production rate as it does not only provide a better dispersion of microorganisms and feedstock, but also guarantees better mixing conditions (Degueurce et al. 2016). Therefore, leaching batch reactors, also known as leaching bed reactors, do not require complicated mixing or agitation equipment, or expensive high pressure vessels, which correspondingly reduces the capital costs (Nayono 2009a).

In terms of operational ease and low investment cost, single batch reactors have been used for full scale biogas production (Gonçalves et al. 2015). Despite better mixing conditions, the risk of blockage of the leaching process is the main shortcoming of leaching batch reactors. Using the mixture of bulk materials and feedstock could alleviate the compaction by controlling the thickness of the wastes (Nayono 2009a; Xie et al. 2012). The fundamental knowledge of new generation anaerobic digesters comes from batch

assays. Usage examples of batch reactors for whiskey distillery/brewery waste streams from lab to full scale are given in Table 5.

2.6.1.2 Solid State Anaerobic Reactor

Solid-state anaerobic digestion (SS-AD) has been gaining popularity with a sharp increase in Europe since the early 1990s for treatment of lignocellulosic biomass (Ge et al. 2016; Brown & Li 2013). SS-AD is capable of digesting high solid content feedstock, typically operating at 15-40% total solid content. Single stage SS-AD as well as the combination of SS-AD and granular bed reactor (GBR) for the treatment of brewery spent yeast has been employed previously (Panji et al. 2015) (Figure 10). The working principle of GBR is very similar to the upflow anaerobic sludge blanket (UASB) (Tauseef et al. 2013), which is discussed in detail in Section 2.6.2.3.

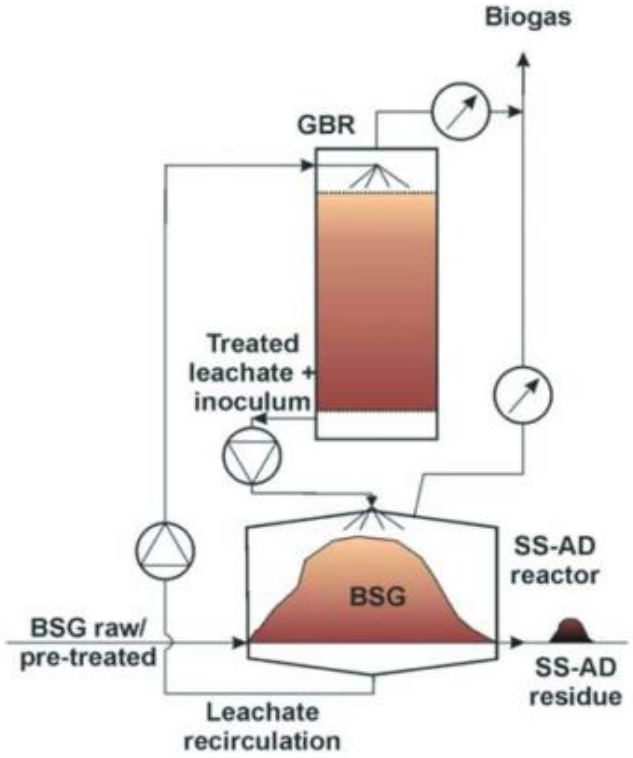


Figure 10. Experimental set up for SS-AD coupled with granular bed reactor (Panji et al. 2015)

The main advantages of SS-AD over liquid anaerobic digesters include; an ability to treat more material in the same size, lower energy demand for heating and process operation, and the creation of less effluent production. Furthermore, it has 2-7 times greater methane production capacity than liquid digesters (Wantanasak et al. 2015). It is therefore considered a more suitable configuration for lignocellulosic matter like whiskey

distillery/brewery wastes (Panji et al. 2015; Yang et al. 2015). There are also, however, potential operational problems; such as a longer retention time requirement, a relatively slower mass transfer rate between the biomass and the cell walls in comparison with liquid digesters due to inaccessibility of solid biomass (Li et al. 2011). The potential for process inhibition, such as accumulation of ammonia and VFAs arising from those problems (along with potential solutions) have been reported (Li et al. 2011; Yang et al. 2015; Ge et al. 2016). Premixing and leachate recirculation, treated or diluted with water in order to eliminate the risk of inhibition and usage of two stage reactor configuration, which separates the most sensitive methanogens, are common techniques to encourage optimum mass transfer (Li et al. 2011; Yang et al. 2015; Ge et al. 2016). SS-AD has received scant attention in the literature for AD of whiskey distillery/brewery wastes, although bench scale application on spent grain resulted in 74% methane yield under psychrophilic conditions (Table 5) (Panji et al. 2015).

2.6.1.3 Anaerobic Sequential Batch Reactor

Since the inception of the anaerobic sequential batch reactor (ASBR) in the 1930s, the ASBR has received increased attention in the literature recently due to its operational simplicity, efficient quality control of the effluent, flexibility of use as well as better process control advantage (Singh & Srivastava 2011; Tauseef et al. 2013; Massé et al. 2003). The ASBR works under fill-and-draw treatment cycle, which includes feed, reaction, settling and discharge stages. Once the tank is filled, it operates as a batch reactor for certain period of time, and after reaching the desired level of treatment, it is allowed to settle and the clarified supernatant is taken out of the reactor. The reactor operates under a series of periods of this cycle. There is a requirement for good mixing which can be performed by an agitator or a recycling stream, to ensure good mass transfer during the reaction time. The biomass settling determines the system performance. A sufficient self-immobilization to achieve good settleability is not seen in ASBR naturally. Therefore, usage of inert supports such as polyurethane foam is needed to perform high organic compound removal efficiency and high solids retention (Camargo et al. 2002; Mao et al. 2015; Tauseef et al. 2013; Rajagopal et al. 2013). ASBR has received little attention in the literature to date in particular for whiskey distillery/brewery wastes; however a gas yield

with 77 ± 5% of biomethane has been achieved for AD of malt whiskey pot ale (Table 5) (Uzal et al. 2003).

2.6.1.4 Continuous Stirred Tank Reactor

The 1950s saw the introduction of intense mechanical mixing within anaerobic reactors. This is considered a first-generation high rate anaerobic digestion. A suspended growth bacteria system is evidenced within the CSTR with intermittent or continuous agitation, facilitated by good contact between bacteria and substrate. However, slight mass transfer resistance is also seen. CSTRs have been shown to be suitable for treating high levels of suspended solids, with 2-3-fold improvement in performance over low rate digesters; unstirred or intermittently stirred reactors. Along with the effluent, the microbial population is washed out of the reactor in low-rate digesters. Prevention of microbial washout is thought to lead to microorganisms having a greater concentration in the reactor, therefore improving the efficiency of the digester (Tauseef et al. 2013; Hu 2013; Mao et al. 2015). Rapid acidification takes place due to mixing and continuous stirring as a result of large VFA production. To overcome this problem, the feedstock is diluted with recirculated digestate. CSTRs can operate as single and two stage as well as in plug flow or semi continuous modes (Tiwary et al. 2015; Mao et al. 2015) and have been shown to be effective at 30 L scale (Figure 11) for AD of whiskey distillery/brewery waste with spent grain and different applied pre-treatments (Table 5) (Sežun et al. 2000).

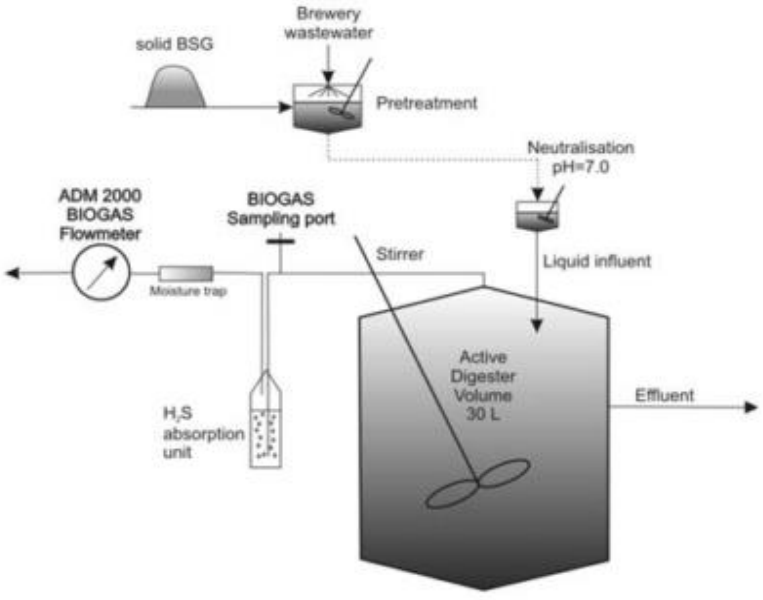


Figure 11. Experimental set up for semi- continuous stirred tank reactor (Sežun et al. 2000)

2.6.2 Second Generation Anaerobic Reactor Configurations for Whiskey Distillery/Brewery Waste Treatment

The concept of second-generation anaerobic digesters is based on the ability of retaining high viable biomass via a method of bacterial sludge immobilisation.

2.6.2.1 Anaerobic Filter Reactor

An anaerobic filter reactor (AF) has a packed bed biofilm configuration, which offers intimate interaction between influent and bacterial mass by attached support media. A schematic diagram of an upflow anaerobic filter reactor is given in Figure 12. A longer biomass retention time than the HRT is possible. As a characteristic of this configuration, a supporting biofilm is generated on the packing media that supports the biomass separated from the effluent (Young & McCarty 1969; Tauseef et al. 2013).

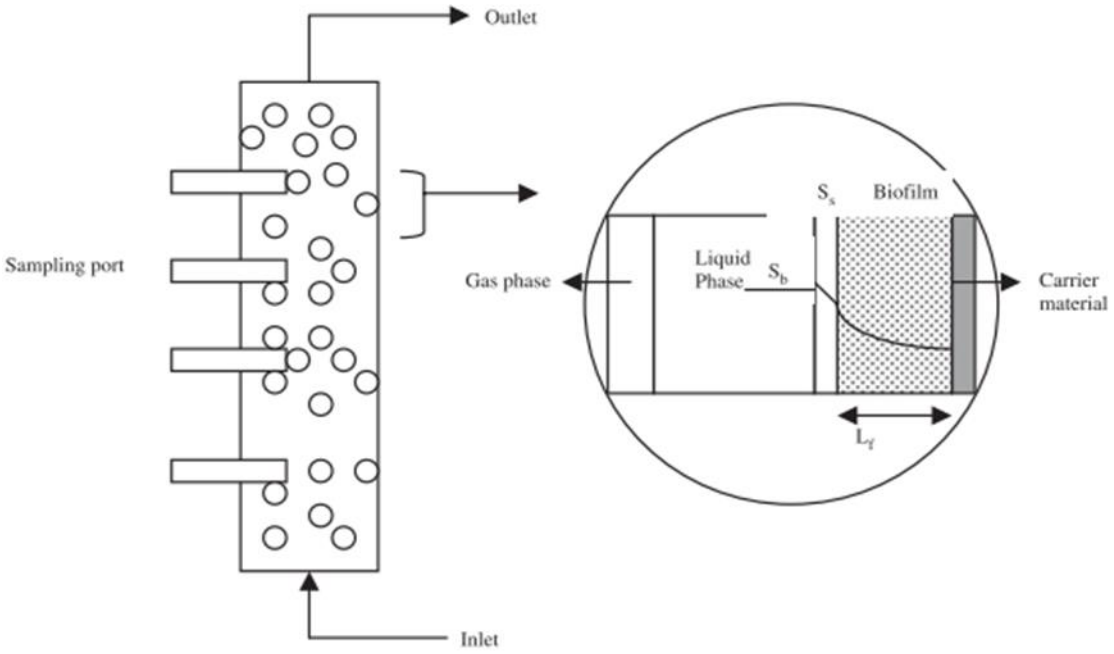


Figure 12. Schematic diagram of upflow anaerobic filter reactor (Saravanan & Sreekrishnan 2006).

AF can be run either though upflow or downflow pathway, but recycling and up flow operation is more common for treating highly recalcitrant wastes because it leads to the formation of a high concentration of suspended biomass in the structure of the fixed bed (Young & McCarty 1969; Mao et al. 2015; Saravanan & Sreekrishnan 2006). On the other hand, the risk of clogging of filter media during the treatment of high suspended solids containing waste is a potential risk of failure of the system (Mao et al. 2015). Nonetheless,

the success of AF reactors creates the fundamentals of novel high-rate anaerobic digesters (Tauseef et al. 2013; V. Blonskaja et al. 2003). The Single AF reactor has been used for AD of pot ale (Tokuda et al. 1998) and brewery wastewater (Leal et al. 1998) as well as in combination with UASB for anaerobic digestion of mix distillery waste (Blonskaja et al. 2003). AD of brewery wastewater showed a high methane yield, with 0.15 m^3 CH_4/ kg COD removed, along with 96% CH_4 (Table 5).

2.6.2.2 Anaerobic Membrane Bioreactor

The anaerobic membrane bioreactor (AnMBR) combines membrane filtration with an AD reactor, allowing removal of treated effluent with retention of sludge, offering high biomass operations (Padmasiri et al. 2007; Smith et al. 2012). Better retention of microorganisms eventually leads to a greater hydrolysis and decomposition as only a small amount of particulate matter is expelled from the system. Thus, AnMBR reactors are expected to be operated efficiently with short HRT and at low temperatures as nearly absolute biomass retention is obtained within the reactor in comparison to conventional digesters (Smith et al. 2013; Lew et al. 2009). AnMBR can be subcategorised based on the location of the membrane as external cross-flow, internal submerged, or external submerged. Fouling of the membrane, mainly as a result of organic matter adsorption, inorganic matter precipitation and microbial cell adhesion to the membrane surface, is considered the common challenge for all configurations. Reactor subcategories, effects of HRT and STR as well as fouling control have been thoroughly reviewed by (Smith et al. 2012). Although AnMBR is capable for both high and low strength waste treatment, high strength wastes have received scant attention in the literature, with little application to whiskey distillery/brewery wastes. It has however been shown that 0.53 m^3 CH_4/ kg $COD_{removal}$ biogas yield is achievable even under mesophilic conditions (Table 5) (Chen et al. 2016).

2.6.2.3 Up Flow Anaerobic Sludge Blanket Reactor

The up flow anaerobic sludge blanket (UASB) reactor (Figure 13), which is a high rate anaerobic digester, was invented in the 1970s by observing the development of sludge into granules which causes self-separation of active sludge from feedstock (Tauseef et al. 2013). Feed enters through the bottom of the reactor and flows upward, through a 'sludge blanket', composed of sludge and granular sludge. A gas–liquid–solid separator is

necessary to ensure that the solid granular sludge is retained in the system while gas and liquid effluent are removed (Ahn et al. 2001; Zupančič et al. 2012).

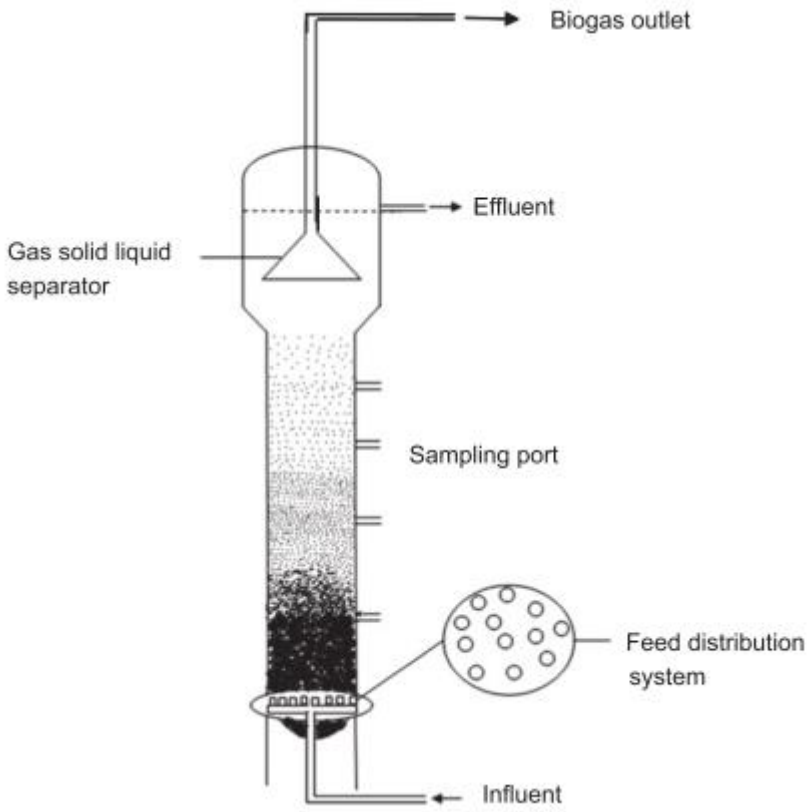


Figure 13. Schematic diagram of UASB (Tauseef et al. 2013)

The UASB is one of the most popular high rate anaerobic reactor configurations with many examples of plot and lab scale operation for treatment of high recalcitrant brewery and whiskey distillery pot ale (Mallick et al. 2009; Akarsubasi et al. 2006; Harada et al. 1996; Chen et al. 2016; Gao et al. 2007). The efficiency of the reactor mainly relies on the existence of settleable active granules, consisting of aggregated self-immobilised compacted anaerobic bacteria. A satisfactory level of methanogen retention in the system provides high digestion potential in terms of COD removal and methane yield as well as a better quality of effluent (Tauseef et al. 2013; Mallick et al. 2009; Gao et al. 2007; Mohana et al. 2009). OLR is another major parameter which can impair microbial ecology within the UASB and correspondingly, the performance of the reactor. Accumulation of VFAs and insufficient (too short) HRT, not giving enough time for the microbes to degrade the substrate, are considered the main reasons of this limitation (Aslanzadeh et al. 2014). In order to address this problem, two stage anaerobic digestion technology was suggested

based on flexibility of working with different OLR and HRT for each stage. The proximity of the syntrophic microorganisms, for instance hydrogen producing microorganisms (acidogens and acetogens) and hydrogen utilizing microorganisms (methanogens), has a significant role on both overall reactor performance and degree of granulation (McCarty & Smith 1986; Saravanan & Sreekrishnan 2006; V. Blonskaja et al. 2003). Investigation of AD process has been separated into two stages; first where hydrolysis, acidification, and liquefaction take place and next, where acetate, hydrogen, and carbon dioxide are converted into methane (Ahn et al. 2001; Aslanzadeh et al. 2014; V. Blonskaja et al. 2003). The main advantages of UASB are, good removal efficiency at low temperatures, lower energy consumption than CSTR, low sludge production, ability of long term preservation of inoculum, good mixing. In terms of disadvantages these include a requirement for strict temperature control, partial remove of pathogens, and working with relatively low OLR (Latif et al. 2011).

2.6.2.4 Expanded Granular Sludge Blanket

The UASB reactor was upgraded in terms of hydrodynamics by increasing (i) capacity of accommodating high organic and hydraulic loadings, (ii) treating wastewaters containing lipids and toxic/inhibitory compounds and (iii) feasibility of acidifying wastewaters under psychrophilic conditions (Tauseef et al. 2013). This new variation of UASB, called expanded granular sludge blanket (EGSB), provides more advantages over a conventional UASB through the special use of granular sludge, greater mixing due to the higher up-flow velocities and improved mass transfer, a slight bed expansion due to higher up-flow liquid velocity under standard operating conditions, and increased stable granules biofilms leading to better mass transfer between substrate and sludge aggregates (Lettinga 1987; Mao et al. 2015). A schematic diagram of the UASB is presented in Figure 14.

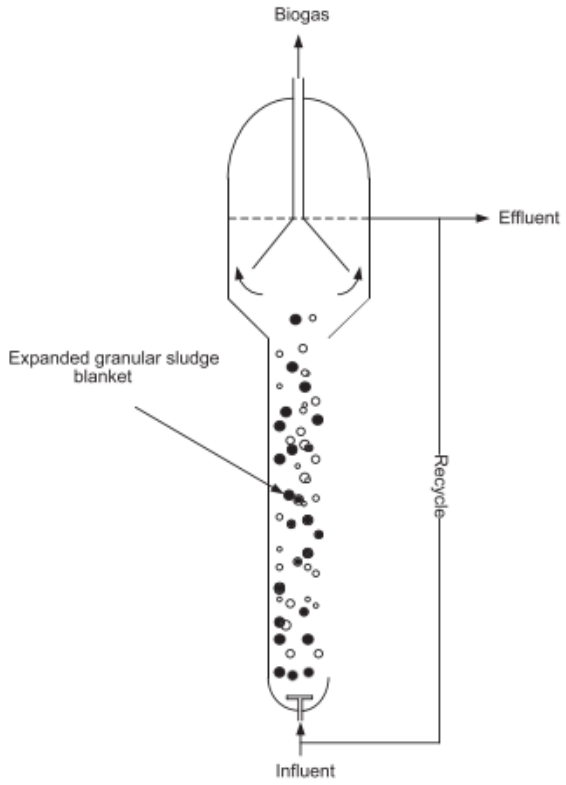


Figure 14. Schematic diagram expanded granular sludge blanket (EGSB) reactor (Tauseef et al. 2013).

A relatively higher superficial upflow velocity (4–10 m/h) is a distinctive feature for EGSB. This is obtained by changing the height/diameter ratio of the reactor or/and by the recycling of the effluent. It prevents the failure of the reactor because of the accumulation of inhibitory compounds at the influent portion of the reactor (Tauseef et al. 2013). Operation at a high upflow velocity provides a better hydraulic mixing than the levels that can be achieved by UASB, and also minimises the blind areas within the reactor. Thus, it enhances the diffusion of substrate from the bulk to the granule biofilms and results in improved biodegradation of substrate (Chou et al. 2008). Application of EGSB is commonly seen for large scale treatment of a wide range of strength (low – high) feedstocks (Tauseef et al. 2013; Zupančič et al. 2012; Mao et al. 2015; Chou et al. 2008). However, the removal of suspended solids may not be performed very well (Mao et al. 2015). The operation of a full scale EGSB at 5.15 ± 2.18 kg COD/m^3day OLR for AD of brewery waste water and spent yeast mix resulted in a biogas generation rate of 2.59 ± 1.12 m^3/m^3 day and 82.1 ± 3.9% methane conversion (Table 5) (Zupančič et al. 2012). However, other than that reference EGSB has received scant attention in the literature to the date for whiskey distillery/brewery wastes.

2.6.3 Anaerobic Reactors with Phase Separation

2.6.3.1 Granular Bed Anaerobic Baffled Reactor

The granular bed anaerobic baffled reactor (GRABBR) is a hybrid reactor, which combines the advantages of the anaerobic baffled reactor (ABR) and UASB by using anaerobic phase separation and granular biomass characteristics. It is therefore considered an upgraded version of UASB empowered with the ability of phase separation of the ABR, due to the existence of compartmentalization (Mohana et al. 2009; Akunna & Clark 2000). The GRABBR has shown a superior process stability to UASB at high OLR in a comparative study (Shanmugam & Akunna 2010). The reactor layout is given in Figure 15. UASB has already been discussed, but ABR has not been applied for whiskey distillery/brewery wastes so there is no section for it specifically. However, the typical characteristics of ABR in terms of capability of separating acidogenesis and methanogenesis stages longitudinally and handling organic shock loadings was summarised by (Barber & Stuckey 1999).

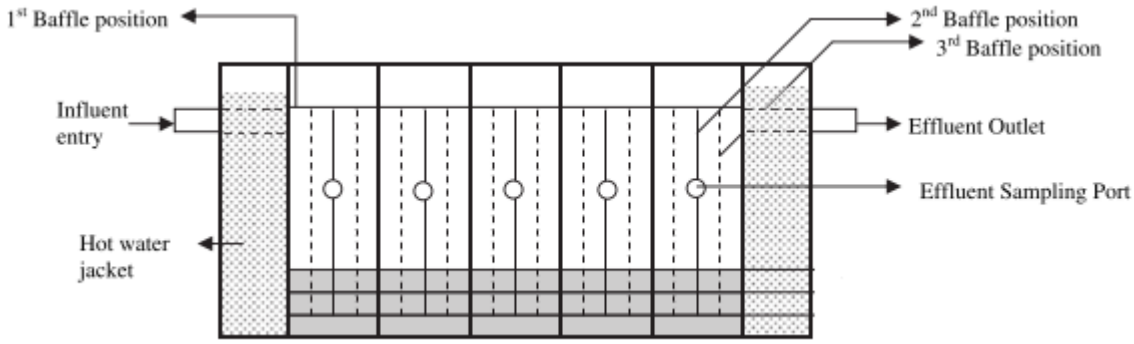


Figure 15. Schematic diagram of Granular bed anaerobic baffled reactor (Shanmugam & Akunna 2010)

In the structure of GRABBR, wastewater is forced up through the blanket sludge and this flow characteristic results in a horizontal movement (gently rising and settling with a slower rate) of the bacteria within the reactor. This movement enhances the phase separation within the GRABBR and allows the bacteria to develop under the most favourable conditions by separating acidogenesis and methanogenesis longitudinally down the reactor (Mao et al. 2015; Baloch & Akunna 2003; Tauseef et al. 2013). The division of different microbial communities is also possible, where front compartments were occupied by acidogens, and methanogens were dominant in the rear compartments (Akunna & Clark 2000). In the poor settling of GRABBR, acidogenic sludge occurs upstream of the granular methanogenic sludge zone, eventually, preventing the wash out of the former with the influent from the reactor and enhancing process stability (Tauseef et al.

2013). Accordingly, GRABBR is considered a solution to the high capital costs of two stage anaerobic reactors (Baloch & Akunna 2003). On the other hand, the major operational challenge of GRABBR is the occurrence of an excessive back pressure which impairs the characteristic flow pattern of the reactor and decreases the contact period between wastewater and microorganisms. The excessive back pressure can potentially cause overflow, leakages, and a reversed flow inside the reactor which may lead to reactor failure (Shanmugam & Akunna 2010).

Table 5. Studies of anaerobic digestion for whiskey distillery/brewery wastes in the literature

Substrate	Temp (˚C)	Reactor Configuration /Operating volume	Pre-treatment	[1]COD (feedstock) (mg L^{-1})	HRT (d)	[2]OLR	[3] ηCH_4	[4]εCOD (%)	Reference
Pot ale	Mesophilic, 35	UASB two stages, 113 ml	$NaHCO_3$ addition	37060 – 50700	29	30	0.019	55	(Uzal et al. 2003)
Pot ale	Mesophilic, 35	UASB, 1050 ml	$NaHCO_3$ addition	21050	2.1	10.2	N/A	93	(Goodwin et al. 2001)
Pot ale	Mesophilic, 37	Batch, 1000 ml	Enzymatic	61500	10	N/A	N/A	87	(Mallick et al. 2009)
Spent wash	Mesophilic, 37	Batch, 1000ml	Enzymatic	46300	10	N/A	N/A	45	(Mallick et al. 2009)
Spent wash	Mesophilic, 37	Full scale UASB, 143 m^3	-	25000 – 33000	N/A	6 – 11	0.25	90	(Akarsubasi et al. 2006)
Spent grain	Mesophilic, 35	EGSB, 3800 ml	Enzymatic	800 – 4000	45	1 – 10	[5]0.7	90	(Wang et al. 2015)
Spent grain	Mesophilic, 37	UASB, 8180 ml	-	16500 – 22520	3.4 – 0.4	33.3	0.318	80 – 97.3	(Gao et al. 2007)
Spent grain	Mesophilic, 37	Batch, 250 ml	Glucose addition	7614	28 – 30	N/A	0.516	90	(Gonçalves et al. 2015)
Spent grain	Thermophilic, 55	Batch, 250 ml	Glucose addition	7299	28 – 30	N/A	0.373	44	(Gonçalves et al. 2015)

[1] Influent chemical oxygen demand
[2] Organic loading rate in kg COD/ m^3day
[3] Methane yield coefficient in m^3 CH4/ kg $COD_{removal}$
[4] COD removal efficiency
[5] Unit in $gCOD_{CH4}$/ g COD_{fed} (The amount of methanised COD relative to the amount of COD fed to the reactor).

Substrate	**Temp (˚C)**	**Reactor Configuration /Operating volume**	**Pre-treatment**	**[6]COD (feedstock) (mg L^{-1})**	**HRT (d)**	**[7]OLR**	**[8] ηCH_4**	**[9]εCOD (%)**	**Reference**
Spent grain	Psychrophilic, 20	SS-AD, 3000 ml	Acid	N/A	1	N/A	[10]74	N/A	(Panji et al. 2015)
Spent Grain	Mesophilic, 37	CSTR, 30 000 ml	High shear homogenizer	107200	33.7	2.51	N/A	69.6	(Sežun et al. 2000)
Spent grain	Mesophilic, 37	CSTR, 30 000 ml	Alkali	101400	39.5	2.07	N/A	73.1	(Sežun et al. 2000)
Spent grain	Mesophilic, 37	CSTR, 30 000 ml	Thermo-chemical	101000	35.1	1.85	N/A	70.4	(Sežun et al. 2000)
Excess yeast (2.8%) + spent wash	Mesophilic, 32-35	UASB, 12 000 ml	Alkali addition for pH (6.5) adjustment	2240	N/A	12.6 ± 3.48 0.290	N/A	85.7 – 95.8	(Zupančič et al. 2012)
Excess yeast (0.7%) + spent wash	Mesophilic, 32-35	EGSB, 4000000 ml Full scale	Alkali addition for pH (6.5) adjustment	3522	0.6	5.15 ± 2.18	N/A	82.1 ± 3.9	(Zupančič et al. 2012)
Spent yeast	Mesophilic, 37	Batch, 180 ml	NH_4Cl addition	3800	45	N/A	0.042 2	24.2	(Sosa-Hernandez et al. 2016)

[6] Influent chemical oxygen demand
[7] Organic loading rate in kg COD/ m^3day
[8] Methane yield coefficient in m^3 CH4/ kg COD $_{removal}$
[9] COD removal efficiency
[10] Percentage of biomethane potential

Substrate	**Temp (˚C)**	**Reactor Configuration, volume**	**Pre-treatment**	**[11]COD (feedstock) (mg L^{-1})**	**HRT (d)**	**[12]OLR**	**[13] ηCH_4**	**[14]εCOD (%)**	**Reference**
Distillery wastewater	Mesophilic, 37	GRABBR, lab scale	Alkali, NaOH	16600 – 58000	4	4.75	N/A	80 – 92	(Shanmugam & Akunna 2010)
Mix of distillery waste	Mesophilic, 36	Two-Stages AF+ UASB, 1500ml	-	49000 – 53000	[15]10 – 19 [16]20 – 39	[7]2.5 – 5.1 [8]0.6 – 2.5	N/A	[14]54 [15]93	(V Blonskaja et al. 2003)
Brewery wastewater	Mesophilic, 35	AnMBR, 15000 ml	-	10200	1.8	3.5 – 11.5	0.53 ± 0.015	98	(Chen et al. 2016)
Brewery wastewater	Mesophilic, 35	Two-Stage UASB, 3000 ml	Acidic	1910	0.1 – 1.9	25	0.27 – 0.30	80	(Ahn et al. 2001)
Brewery wastewater	Mesophilic, 34-36	AF, 5 843 000 ml	-	2832	0.4	8	0.15	96	(Leal et al. 1998)
Pot ale	Mesophilic, 37	[17]UAFP,780 ml	Enzymatic	Given as TOC: 15380	18	[18]0.0108	N/A	N/A	(Tokuda et al. 1998)
Pot ale	Mesophilic, 37	Batch, 165 ml	-	57100	40	N/A	N/A	N/A	(Barrena et al. 2018)

[11] Influent chemical oxygen demand
[12] Organic loading rate in kg COD/ m^3day
[13] Methane yield coefficient in m^3 CH4/ kg $COD_{removal}$
[14] COD removal efficiency
[15] In the 1st stage
[16] In the 2nd stage
[17] Upflow anaerobic filter process
[18] Unit in kg TOC/L day

2.7 Current Applications of AD Technology at Industrial Scale for Distillery Wastes

Although anaerobic digestion technology is commonplace e.g. for municipal wastewater, industrial scale implementation for whiskey distilleries or breweries has not been widely utilised. Scotland is the most progressive, with several companies applying anaerobic digestion as a waste and energy management method. The Scottish Whiskey Association targets to deliver 20% of the primary energy requirements from sustainable energy sources by 2020, with a further aim of 80% by 2050 (Grant et al. 2013)

Diageo is the largest UK whiskey distiller with 28 malt distilleries and 1 grain distillery in Scotland. Diageo's Dailuaine Distillery has been using pot ale to generate biogas then used in a CHP to produce electricity and steam for use in their on-site distillery dark grains plant since 2010. The AD plant produces 0.5 MW of biogas, providing 40% of electrical demand for the site as well as reducing CO_2 emissions by 250 tonnes. The solid fraction of the digestate is used as biofertiliser whereas the liquid part can be discharged to river, meeting the regulatory requirements for discharge (SWA 2012). Diageo's Roseisle Distillery also recovers 8.6 MW of energy, which is equivalent to about 84% of its total steam load requirement, by a combination of biomass combustion and anaerobic digestion as well as reducing the potential CO_2 emission by approximately 13000 tonnes (Brinkerhoff 2012; J. Andrews et al. 2011). Meanwhile Cameronbridge Distillery in Fife is estimated to produce 30 MW of energy, recovering 95% of site electricity and 98% of the total steam demand through anaerobic digestion and CHP (J. Andrews et al. 2011; Duguid & Strachan 2016). Glendullan Distillery at Duffton treats approximately 1000 m^3 malt whiskey by-products per day with the capacity of producing 8000 MWh of thermal energy for the distillery, with about 1 million m^3 biogas generated annually (Clearfleau 2015; Duguid & Strachan 2016). William Grant & Sons Distillery has the capacity of producing renewable energy in the form of 25 MWh of heat and 60 MWh of electricity on a daily basis by burning the produced biogas in the turbines. A reduction in the level of COD in the plant effluent was achieved as a result of the implementation of anaerobic digestion (Brinkerhoff 2012). The North British Distillery also aims to generate up to 1 MW of renewable electrical energy and reduce CO emissions by 9000 tonnes per year which is equivalent of removing 3000 cars from street. Glenmorangie Tain distillery performed onsite feasibility studies to construct a membrane based AD plant in 2016 (Nation 2017).

Since then, 95% COD was removed from the aqueous waste stream. Over a 10 year period, it is predicted that 12 million tonnes of water will be treated to remove 45000 tonnes of COD from the discharge. Utilising the generated biogas will reduce the CO_2 emissions by 2.7 million kg CO_2 annually (Nation 2017). Slane Distillery, established in 2017, is the only distillery in Ireland utilising AD (an EGSB reactor) for biogas generation and sludge biofertilizer production (WEW 2018).

2.8 Pre-treatments for Anaerobic Digestion of Distillery/Brewery Wastes

Whiskey distillery and brewery waste streams are considered lignocellulosic biomass based on the hemicellulose (21.8–28.4%), cellulose (16.8–25.4%) and lignin (11.9–27.8%) fraction of their structure (Panji et al. 2015; Sežun et al. 2000). Cellulose is the most abundant polymer of the lignocellulosic material (Agbor et al. 2011). The straightness of the cellulose chains is determined by the existence of hydrogen bonds within the microfibril. However, interchain hydrogen bonds introduces order (crystalline) and disorder (amorph) to the structure which contribute to the resistance to biodegradation (Agbor et al. 2011; Gao et al. 2013; Kim et al. 2011; Ahring et al. 2014).Hemicellulose is the second main polymer of the lignocellulosic material which is different from cellulose with its heterogeneous branched structure (Raud et al. 2015). Hemicellulose is also considered a coat of cellulose-fibrils hence it contributes to the recalcitrant nature of crystalline cellulose (Agbor et al. 2011). Therefore removing hemicellulose from the structure increases cellulose degradation (Palmqvist 2000). Lignin is the least abundant constituent polymer in the structure of the lignocellulosic biomass. However it is the most recalcitrant part of the structure owing to its impermeable nature as well as resistance to oxidative stress and microbial attack (Hendriks & Zeeman 2009; Agbor et al. 2011). Lignin exists in the structure in the form of an amorphous heteropolymer consisting of three main phenyl propane units (p-coumaryl, coniferyl and sinapyl alcohol) held together by different linkages which act like glue, binding hemicellulose and cellulose (Gao et al. 2013; Kim et al. 2011). This is because cellulose and hemicellulose are physically protected from degradation thus rendering lignin resistant to the hydrolysis stage of AD process. Pre-treatments are therefore needed to modify or remove structural obstacles prior to the hydrolysis stage (Gao et al. 2013; Kim et al. 2011; Ahring et al. 2014; Raud et al. 2015;

Hendriks & Zeeman 2009; Aliyu & Bala 2013). Figure 16 illustrates the structure of the lignocellulosic materials before and after pre-treatment.

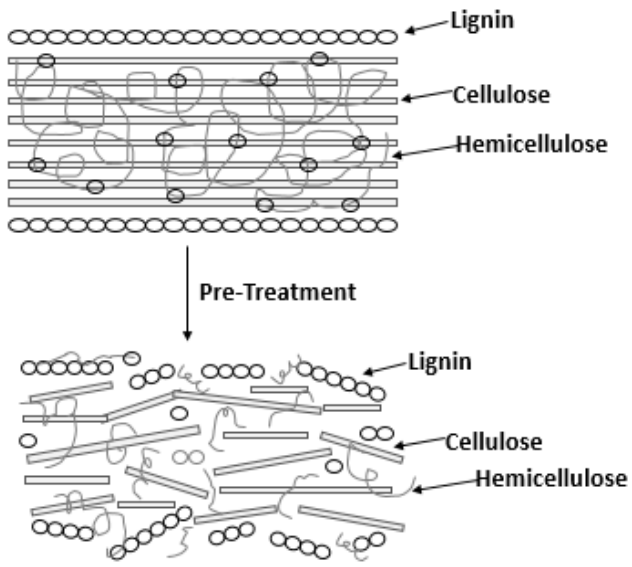


Figure 16. Effects of pre-treatment onto lignocellulosic materials

Cellulose and hemicellulose are inaccessible to enzymes in the structure of lignocellulosic materials, such as the pot ale and spent grain of whiskey distillery/brewery wastes. This results in the decrease of the rate of hydrolysis step of AD as discussed in Section 2.3.1. During the pre-treatment of the feedstock, reduction in the crystalline structure of cellulose molecules and degree of polymerisation is achieved in addition to the removal of lignin (Sun & Cheng 2002).

2.8.1 Effects of Chemical Pre-treatments of Whiskey Distillery/Brewery Wastes Prior to Anaerobic Digestion

Chemical pre-treatment, which is considered a cost effective method for maximising biodegradation of complex materials, is used to degrade organic compounds by means of strong acids, alkalis, bicarbonates or peroxide (Aboerheeba 2013; Of et al. 2012). Adjustment of pH is generally required for AD of whiskey distillery wastewater to balance their acidic nature , thus alkali pre-treatment is one of the most preferred chemical methods (H. Li et al. 2012). Acidic pre-treatments and oxidative methods such as ozonation are also used to improve the biogas production and enhance the hydrolysis rate (Ariunbaatar et al. 2014). The effects of chemical pre-treatment may change according to the type of method applied and the characteristics of the substrates. Chemical pre-

treatment is considered not to be appropriate for easily biodegradable substrates which contain high amounts of carbohydrates, due to their accelerated degradation and subsequent accumulation of VFA, which can lead to failure of the methanogenesis step. On the other hand, it has a positive effect on substrates rich in lignin (Ariunbaatar et al. 2014; Gillian 2011; Fernandes et al. 2009).

2.8.1.1 Ionic Liquid (IL) Pre-treatment

Ionic liquids (ILs) are organic salts, being in the liquid phase below 100˚C and contain organic cation and inorganic anion pairs. The most widely used IL types for delignification, dissolution of cellulose and precipitation of the cellulose by water, are imidazolium, tetra alkyl phosphonium, pyridinium, pyrrolidinium and quaternary ammonium (Kim et al. 2011; Bundhoo et al. 2015). ILs are considered "designer solvents" because of their adjustable characteristics regarding the combination of anions and cations. Moreover, IL pre-treatments are environmentally friendly owing to their high recyclability and thermal stability, low volatility, and non-flammable features (Gao et al. 2013). ILs are used as green solvents for pre-treatments of lignocellulosic biomass which contains cellulose, hemicellulose, and lignin. Many types of imidazolium-based ILs such as $[C_n mim]Cl$ (where n= 2-6) play an important role to break down the three-dimensional structure of lignocellulosic matter into its constituent substances (Kim et al. 2011; Gao et al. 2013). Furthermore, ILs are able to interact with the ionic, π–π, and hydrogen bonds in the biomass to disturb the three dimensional crystal structure of the lignocellulosic matter (Gao et al. 2013). Consequently, it causes a separation within the cellulose fraction by leading to swelling of amorphous structured cellulose to become more accessible for the enzymatic attack (Kim et al. 2011; Bundhoo et al. 2015). IL pre-treatment therefore provides an enhanced hydrolysis rate and higher biogas production yield (Bundhoo et al. 2015).

2.8.1.2 Alkaline Pre-treatment

Addition of a suitable amount of alkali in the forms of lime or sodium/potassium hydroxide to the digesters prior to anaerobic digestion is necessary in many cases in order to keep the pH within the neutral area (H. Li et al. 2012). Alkali pre-treatment is especially required for pH adjustment of distillery/brewery wastes due to their acidic character prior to AD (Mohana et al. 2009; Cesaro & Belgiorno 2014). Alkaline pre-treatment has

advantages over other chemical pre-treatment methods such as operating simplicity, usage of simple devices and high methane recovery efficiency. Alkali pre-treatments generally occur at ambient temperature and the most commonly used alkali agents are, in order of efficacy, NaOH > KOH > $Mg(OH)_2$ > $Ca(OH)_2$ (Kim et al. 2003; Carrère et al. 2010). Excessive concentration of Na^+ or K^+ ions, on the other hand, may lead to the formation of inhibitors of AD (Carrère et al. 2010). During the alkaline pre-treatment, the first reactions that take place, between free carboxylic groups and neutralization of diversified acids originating from degradation of organic materials, are solvation and saponification of uronic acids and acetyl esters, which improves the swelling of solids (Ariunbaatar et al. 2014; Kim et al. 2003). Consequently, an increased specific surface area is achieved and the substrates become more accessible to anaerobic microbes (Ariunbaatar et al. 2014). Implementation of alkali pre-treatment has received little attention in the literature to date for whiskey distillery/brewery wastes; however in one study the COD solubilization level increased from 17.6% to 86.5% by addition of NaOH (21 g/L at 0 ˚C), as a result of saponification of uronic acids and reaction of acetyl esters (Kim et al. 2003). The dose of alkali reagent, mainly NaOH, is critical, and is commonly kept at a low level of 0.08 – 0.25 g/g total sludge solids. Although there is a linear correlation between sludge disintegration and increasing dose of NaOH, usage of overdose alkali agents may cause the destruction of the bicarbonate buffer system in anaerobic digesters. Subsequently, it leads to an excessive increase in pH and potentially inactive anaerobic bacteria because of the presence of NaOH in the pre-treated feedstock (H. Li et al. 2012).

2.8.1.3 Acid Pre-treatment

Acid pre-treatment is desirable for protein rich lignocellulosic substrates as it breaks down the lignin content and prevents ammonia inhibition at the same time. It is not commonly preferred for distillery/brewery wastes because of their natural low pH (Ariunbaatar et al. 2014; Cesaro & Belgiorno 2014). The main reaction that takes place during acid pre-treatment is the hydrolysis of hemicellulose into monosaccharides, while the lignin condensates and precipitates (Hendriks & Zeeman 2009). Strong acid pre-treatment should be avoided because it may lead to the production of inhibitory by-products, such as furfural and hydroxymethylfurfural (HMF) due to excessive hemicellulose degradation. Thus pre-treatment with dilute acids might be matched with thermal methods (Agbor et

al. 2011; Modenbach & Nokes 2012). Other drawbacks associated with acid pre-treatment are; loss of fermentable sugar as a result of degradation of complex substrates, high cost of acids and the chemical extra cost for neutralizing the pH before the AD (Ariunbaatar et al. 2014).

2.8.1.4 Ozonation

Ozonation provides breakdown of recalcitrant compounds and pathogen removal as well as an increase in the biodegradability of organic matter by transforming complex molecules such as polysaccharides, proteins and lipids into smaller molecular weight compounds through lysis of the cell membrane. This also results in the release of intercellular components. Ozone acts as strong oxidant, which decomposes into radicals and reacts with organic substrates, resulting in the recalcitrant compounds becoming more biodegradable and accessible to anaerobic bacteria (Carballa et al. 2007). However this method is not considered economically feasible as it is extremely expensive if used with the intention of eliminating all of the contaminants in the waste (Ariunbaatar et al. 2014; Cesaro & Belgiorno 2014).

2.8.2 Effects of Mechanical Pre-treatments onto Whiskey Distillery/Brewery Wastes Prior to Anaerobic Digestion

Mechanical pre-treatments are generally achieved by special devices such as grinder, miller, high shear homogeniser, screw press. They operate by increasing the surface area of the lignocellulosic matter. By increasing the surface area, this allows better interaction between the anaerobic bacteria and the substrate which enhances the hydrolysis yield by 5-25% (Ariunbaatar et al. 2014), and subsequently the overall anaerobic digestion yield (Carrère et al. 2010). Mechanical pre-treatment methods are preferred as they are promising for maximising degradation of total suspended solids up to 90% (Ariunbaatar et al. 2014). Moreover, no odour is generated, it has easy application and offers greater dewaterability of the final anaerobic residue (Toreci et al. 2009). On the other hand, excessively small particle size can potentially increase the risk of VFA accumulation (Izumi et al. 2010).

2.8.2.1 Ultrasonication

Ultrasonic pre-treatment is the method in which sound waves are used for the destruction or inactivation of the biological cell. The release of the cytoplasm membrane from the cell

wall is achieved as a result of thinning the cell walls by the sound waves (Pilli et al. 2011). The two mechanisms occurring during ultrasonication are cavitation and chemical reactions. High frequency signals are used to create an empty space within the biomass which results in the formation of cavitation bubbles that were generated by the solid forces in the liquid molecules. The liquid that surrounds the bubbles undergoes powerful equilibrium forces, the most effective frequencies that are used are below 100 kHz. The collapse of the bubble causes the temperature to rise to around 5000 K and the pressure to rise several hundred atmospheres. The extreme conditions noted have the potential to generate chemical reactions in the form of reactive hydroxyl radicals ($OH^{\bullet}$, $HO_2^{\bullet}$, and $H^{\bullet}$) due to the thermal destruction of compounds in the cavitation bubbles. Once the intracellular components of the sludge cells are released they are exposed to further AD (Sharma et al. 2013; Carrère et al. 2010; Boni et al. 2016; Harris & McCabe 2015; Peces et al. 2015; Zhen et al. 2017; Carlsson et al. 2012).

2.8.2.2 Beating

The Reina model Hollander Beater is suitable equipment for treating lignocellulosic biomass. It was originally designed for the mechanical treatment of pulp and paper industry. The Hollander Beater (Figure 17) mainly consists of a beater tub, a 24 bladed drum and a crank handle which is used for the adjustment of the gap size between the beater tub and drum's blades. The beater can perform two actions concurrently which are a cutting action as a result of rotation of the bladed drum and a high-pressure beating action caused by an inclined plate located at the exit of the drum. This results in an increased specific surface area of feedstock in addition to releasing intercellular compounds (Montingelli 2015; Tedesco et al. 2013; Esposito et al. 2011). This makes the feed stock more accessible for the microbes by increasing the specific surface area and as well as resulting in intercellular compound release (Esposito et al. 2011).

Motor	1hp (746 watts)
	220v
	6.9 Amps
	1 Phase
	1450 rpm
Drum Speed	580 rpm
Tub Volume	Maximum Capacity =90 L Working capacity = 40 L
Drum diameter	200mm

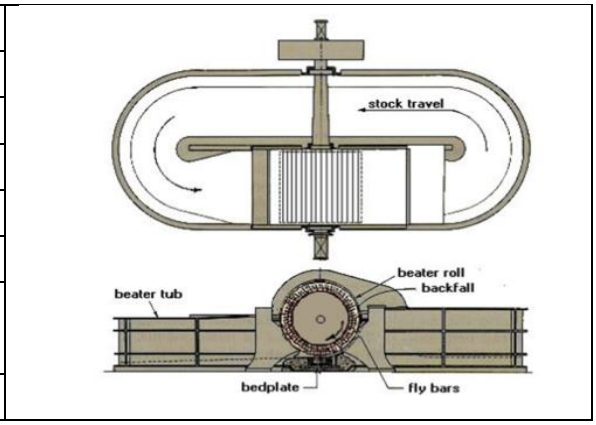


Figure 17. Hollander Beater Specification (adopted from Montingelli 2015)

2.8.2.3 High Pressure Homogeniser

Homogenisers were developed with the intended use for stabilisation of food and dairy emulsions. In 1990, the development of high pressure homogenisers enabled them to withstand higher pressures 10 to 15 times higher than classical homogenisers due to the different geometry in the reaction chamber. The pressures that can be applied from this technology are as high as 3000-5000 bar. High-pressure homogenisation is currently applied at large scale for the disruption of cells in complex microbial structures. The advantages associated with the use of high-pressure homogenisation over other pre-treatments are; chemical changes are absent, operation is easy, investment and operational cost are low and efficiency of cell lysis is high (Zhao et al. 2012; Paquin 1999).

2.8.3 Effects of Thermal Pre-treatments onto Whiskey Distillery/Brewery Wastes Prior to Anaerobic Digestion

Thermal treatment is one of the most studied pre-treatment methods and has been successfully applied as a conditioning process for the sludge at industrial scale due to its ability to improve the pathogen elimination and dewaterability of the wastes (Ariunbaatar et al. 2014; Cesaro & Belgiorno 2014). The main effect of thermal pre-treatment of feedstock is dispersion of cell membranes, therefore resulting in solubilisation of organic compounds. COD solubilisation and temperature have a direct correlation: higher solubilisation can be obtained at lower temperatures, but longer treatment time is necessary (Mottet et al. 2009; Ferrer et al. 2008). A wide range of thermal pre-treatments have been performed, from 60 to 270°C (Appels et al. 2010), although commonly applied pre-treatment temperatures across various different kinds of thermal pre-treatments

rarely go above 180˚C (Ferrer et al. 2008; Cesaro & Belgiorno 2014). The literature shows that thermal pre-treatment at under 100˚C does not result in complete degradation of complex compounds (Ariunbaatar et al. 2014). The most commonly accepted optimum range for high temperature pre-treatment is 160 - 180 ˚C. This is generally coupled with high pressure (between 600 - 2500 kPa) (Liao et al. 2016). Thermal pre-treatments at high temperatures provide gel structure degradation and cell wall lysis by increasing internal pressure of cell and intracellular water evaporation apart from the effects of the low temperature treatments. Hence, soluble organic compounds are released into the water (Carrère et al. 2010; Zhang et al. 2015). A combination of chemical and thermal pre-treatments is suggested to enhance the efficiency of thermal pre-treatment (Toreci et al. 2009; Carlsson et al. 2012). However, production of inhibitory matters, mainly in the form of phenolic compounds originating from lignin content, should be controlled (Sežun et al. 2000). No significant difference has been found between steam and electric heating, while microwave heating solubilized more biopolymers (Carrère et al. 2010).

2.8.3.1 Thermal Pre-treatment at Low Temperatures (<100 °C)

Pre-treatments implemented at temperatures below 100˚C are considered low temperature thermal pre-treatments (Appels et al. 2010; Carlsson et al. 2012). Application of low temperature thermal pre-treatments has been gaining importance in recent years due to the massive energy consumption and strict demands on devices of high temperature treatments (Liao et al. 2016). Thermal pre-treatment at 50˚C – 90˚C can break down lipids to volatile fatty acids, enhance removal of particulate carbohydrates, and increase soluble organic substance fraction and pathogen removal in sludge solids (Ferrer et al. 2008; Ariunbaatar et al. 2014; Skiadas et al. 2005), but it requires longer time treatment time (Liao et al. 2016). Consequently, low temperature thermal treatment is a potential method to accelerate sludge hydrolysis (Liao et al. 2016). In order to meet EU Regulation EC1772/2002 requirements, organic solid waste has to be treated 70˚C at least for 1h in order to reach adequate pathogen removal (Ariunbaatar et al. 2014)

2.8.3.2 Thermal Pre-treatment at High Temperature

High temperature treatment (160 – 180˚C at 600 – 2500 kPa) is frequently performed using steam injection or steam explosion with pressure being suddenly released in a flash tank (Liao et al. 2016). Thermal pre-treatment is largely accepted as a potential method in order to improve the AD process and it is currently being used in full-scale AD plants in many countries (Carlsson et al. 2012). High temperature thermal treatment time is significantly less than low temperature thermal treatment (Carrère et al. 2010). Even though optimum pre-treatment conditions and biogas production yield are predominantly dependant on the characteristic of the feedstock, recent research indicates that pre-treatment at 170 ˚C doubled the methane production and the existence of VFAs compared with AD of untreated sludge (Liao et al. 2016; Zhang et al. 2015). Furthermore, it has been shown that soluble proteins and carbohydrates exhibit a dramatic increase after being treated at 135˚C and it has a positive effect on the formation of VFAs during the acetogenesis stage of AD (Zhang et al. 2015). On the other hand, thermal pre-treatments at higher than 180˚C result in the formation of inhibitory intermediates which can be lethal for anaerobic bacteria and/or recalcitrant soluble organic matters, hence, sludge biodegradability decreases despite achieving higher solubilisation. This is usually associated with the Maillard reactions between amino acids and carbohydrates resulting in the generation of complex substrates, melanoidins (high-molecular-weight heterogeneous polymers), which are difficult to degrade (Mottet et al. 2009; Carrère et al. 2010; Appels et al. 2010; Liao et al. 2016; Zhen et al. 2017; Mata-Alvarez et al. 2014). These reactions can also take place during low temperature pre-treatment depending on a longer interaction time. In addition to Maillard reactions, higher temperature thermal pre-treatments might result in the decline in volatile fatty acids in sludge and correspondingly lower biomethane production from easily degradable organic matters (Ariunbaatar et al. 2014). High temperature thermal pre-treatment is not considered to be a cost efficient method due to the high energy demand (Appels et al. 2010).

2.8.3.3 Microwave Pre-treatment

The principles of microwave pre-treatment can be divided into two categories: thermal and athermal effects (Bundhoo et al. 2015). Thermal effects provide heat generation by means of microwaves and its absorption to polar materials in order to obtain temperature increase. It results in wall disruption due to the internal pressure (Zhang et al. 2015; Bundhoo et al. 2015). Athermal effects, non-thermal effects, are created by continuous alignment and realignment of macromolecules in polar liquid and result in breakage of hydrogen bonds, generation of frictional heat, modification of the hydration zone, increased solubilisation of sludge, improvements of volatile solids destruction and enhancement of biogas production (Bundhoo et al. 2015; Toreci et al. 2009; Zhang et al. 2015). Microwave pre-treatment has a significant effect on disruption of recalcitrant structure of cellulose, due to selective heating of the more polar part (Cesaro & Belgiorno 2014). Regarding athermal effects, the electromagnetic field enhances the rate of destruction of crystalline structure. As a result of both effects, organic matter in cells are transferred to the soluble phase and become accessible for biological attack (Bundhoo et al. 2015). The success of microwave pre-treatment depends on parameters such as, the size and shape of the vessel, the microwave power and intensity (penetration depth), the contact time, and the temperature (Wahidunnabi & Eskicioglu 2014; Zhang et al. 2015).

Considering conduction and convection heating, microwave has internal and direct interaction between the heated materials that can minimise heat loss through both mechanisms (Cesaro & Belgiorno 2014; Toreci et al. 2009). Compared to conventional heating methods microwave is not only more cost effective, but also a more environmentally friendly method (Zhang et al. 2015).

2.8.4 Effects of Biological Pre-treatment onto Whiskey Distillery/Brewery Wastes Prior to Anaerobic Digestion

Biological pre-treatment methods include both aerobic and anaerobic methods, besides the addition of specific enzymes such as peptidase, carbohydrolase, lipase or a certain type of bacteria such as hydrolytic bacteria to the AD system (Ariunbaatar et al. 2014). Among known biological pre-treatment methods, the addition of enzymes has been applied onto a range of whiskey distillery/brewery waste streams by different researchers.

2.8.4.1 Enzymatic Pre-treatment

Enzymatic pre-treatment is considered an efficient method for breaking the lignocellulosic materials into their component monomers in an environmentally friendly way (Pérez-Rodríguez et al. 2016). In addition, it provides solubilisation of phenolic compounds so avoiding their potential inhibitory effect on biogas yield (Schroyen et al. 2014). Therefore, enzymatic pre-treatment overcomes the drawbacks of chemical pre-treatments such as side product formation (furfurals, levulinic acid or formic acid) (Taherzadeh & Karimi 2008). On the other hand, it requires a much longer treatment time and a higher operation cost for maintaining constant temperature during the treatment (Kratky & Jirout 2011). Commercial enzymes such as lyticase, alpha amylase, cellulase, beta-glucosidase, beta-glucanase, lipase, protease, enzyme complex and papain have been used for AD of pot ale in order to support microorganisms. This results in higher yields of monosaccharides, and correspondingly better yields of biochemical reactions in the subsequent steps of AD (Quiñones et al. 2012; Mallick et al. 2009; Moraes et al. 2015). Lyticase and beta-glucanase combined with protease achieved approximately 90% cell lysis (the highest) and 80% cell digestion in pot ale at the optimum conditions of 37˚C and pH 7.5 (Mallick et al. 2009).

2.10 Conclusions of the Literature Review

Based on the literature review conducted, major research gaps were identified as; comprehensive organic and inorganic characterisation of pot ale, implementation of pre-treatment steps, influences of the pre-treatments on the lignocellulosic structure of pot ale and spent grain as well as implementation of AD technology at industrial scale. Therefore, the main objectives of this programme for research were set as follows;

1. Evaluation of the enhancements in anaerobic digestion yield of distillery/brewery waste streams after implementation of various pre-treatments
2. Modelling and optimisation of the process and pre-treatment parameters for anaerobic digestion of pot ale
3. Creating a scalable anaerobic digestion model for industrial applications

According to the literature investigation, the liquid waste stream of the distilleries, pot ale, has the major impact on the environment while solid waste (draff) of distilleries/breweries is typically utilized in animal nutrition. Therefore, the applicability of AD technology for pot ale treatment was evaluated first. Subsequently co-digestion of pot ale and draff was tested under different pre-treatment conditions. This research particularly focused on following the key findings which were considered crucial based upon the literature review for the investigation of AD of whiskey distillery/brewery waste streams.

For AD of whiskey distillery/brewery waste streams, investigation of spent grain was more commonly seen than AD of pot ale. However very few pre-treatments have been performed on the waste streams of distilleries/breweries although it shows a significant improvement on the biogas quality and the quantity. Mechanical and chemical pre-treatments have been commonly performed due to their simplicity and capability of modifying the resistance of the structure to achieve an effective treatment. Selecting the most suitable sort of pre-treatment strategy based on the literature was not possible since different reactor configurations and operating conditions do not allow comparison of the effects of pre-treatments on biogas yields. Therefore, the investigation was started with introducing a novel hybrid (chemical and mechanical) alkaline and beating pre-treatment

prior to AD of pot ale and spent grain. The experimental plan was initiated with single digestion of pot ale after the pre-treatment step. It was then followed with co-digestion of pot ale and spent grain mixture under the same experimental design in order to investigate a possibility of more sustainable management method for spent grain instead of using it as animal feed. The Design Expert software was used for the first time to assess the produced biogas quality and quantity by anaerobic digestion of whiskey distillery/brewery waste streams. Mathematical models were developed to have better understanding of the process and the process parameters. Based on the biogas yields of the initial screening experiments, pot ale was found to be a more suitable substrate for the characteristics of the batch reactor employed regarding pot ale and draff mixture.

Based on the literature review, microwave and ultrasonic pre-treatments were found to be highly capable in breaking down recalcitrant lignocellulosic structures, however a literature gap was detected in their application on distillery/brewery wastes. Therefore microwave and ultrasonic pre-treatments standalone and in combination with alkaline pre-treatment were introduced to AD of pot ale. Amongst the process parameters of anaerobic digestion that has a direct influence on methane production from distillery/brewery wastes, the inoculum ratio and the reaction pH were reported as one of the most critical. The experiments were designed to optimise the most crucial process parameters. Considering the acidic nature of the liquid waste as well as the pH sensitivity of the methanogenic bacteria, varying seeding ratios and initial reaction pH values were tested in order to achieve a balanced digestion.

Finally, a theoretical study was conducted on potential usage of the end products (biogas and digestate), and a scalable process design was developed for industrial implementation incorporating financial estimations of AD plants at different industrial scales. In addition to biogas use, the mineral quality of pot ale digestate was assessed for its potential agricultural application, which had not previously been addressed in the literature.

This project mainly aims to close the literature gaps in detailed characterisation of pot ale (particularly the lignocellulose structure), the evaluation of the influence of three different novel pre-treatments on lignocellulose fractions of pot ale, and enhanced biogas

quality and quantity. Furthermore, a link between the lab scale experiments and their industrial implementation was created including the both biogas and digestate use.

A detailed analysis of substrate characterisation and the experimental planning is reported in the next chapter.

CHAPTER 3: MATERIALS AND METHODS

3.1 Introduction

This chapter presents the analytical and experimental methodologies developed based upon the main findings of the literature review and used throughout the project. Detailed analytical methods for identifying the sample characteristics and the changes in the lignocellulosic composition of samples due to the applied pre-treatments are summarised. The configurational and the operational details of the lab-scale batch reactor are described. The objectives and the reasoning of all experiments are stated under separate headings in detail.

Box-Behnken Design, within Design Experiment Software, was defined as the experimental design methodology due to its broad statistical and mathematical data analysing capability with performing the least number of experiments. For the optimisation step of the modelling, numerical optimisation was followed by graphical optimisation in order to increase the analysis sensitivity in accordance with the literature. With the scope of this project, DOE technique was introduced to AD of distillery/brewery wastes.

3.2 Materials

In this project whiskey distillery waste streams, which are known as pot ale and draff, are the main substrate of anaerobic digestion. The distillery wastes were supplied by a small-scale Whiskey Distillery[19] in Dublin. Sludge, used as inoculum, was collected from a large-scale Irish mesophilic anaerobic digestion plant[20] processing industrial food waste.

3.3 Methods

3.3.1 Analytical Methods

Pot ale samples were initially characterized in terms of total solids, volatile solids, moisture content, sulphate, phosphate, nitrite, nitrate, chemical oxygen demand (COD), biological oxygen demand (BOD) and volatile fatty acids (VFAs). The supernatant of all samples (as a mixture of sludge and pot ale/draff) were vacuum filtered through a glass microfiber filter (1.5 µm pore size) after centrifuging at 10000 rpm for 30 minutes using a

[19] Production capacity is 500 m^3 pure alcohol annually.
[20] Treatment capacity is 6000 000 m^3 per day.

Sorvall RC 5B Plus centrifuge (Meixner et al. 2015; Tokuda et al. 1998) prior to and after anaerobic digestion, in order to minimise the risk of sludge masking on COD, BOD and SO_4 removals. Different dilution factors were needed for measurement of the individual chemical composition of the samples.

3.3.1.1 Moisture Content (MC), Total Solids (TS) and Volatile Solids (VS) Analysis

For determination of the moisture content, each sample (pot ale and draff) was dried at 105 ˚C for 4 hours (until a constant weight was achieved). All containers were dried at 105 ˚C before using to maximise the accuracy of the test in accordance with (Sluiter et al. 2008). The moisture content of each sample was calculated according to Eq 4.

$$\mathbf{MC}[\%] = [\mathbf{1} - \frac{\mathbf{D_c - C}}{\mathbf{W_c - C}}] \times \mathbf{100} \qquad \text{Eq 4}$$

Where; D_c [g] is the mass of the dry matter and container, W_c [g] is the mass of the wet matter and container, and C [g] is the weight of container.

The TS fraction was then calculated according to Eq 5.

$$\mathbf{TS}[\%] = \mathbf{100} - \mathbf{MC}[\%] \qquad \text{Eq 5}$$

The amount of VS was determined according to Eq 6 by igniting a known weight of dried sample at 550 ± 25°C for 4 hours with respect to standard methods (Verein Deutscher Ingenieure (VDI) 2006). A known amount of dry matter was ignited in a muffle furnace (WiseTherm, Model F-03, 0 - 1000˚C) for 4 hours until less than 5% weight change was observed.

$$\mathbf{VS}\ [\%\mathbf{TS}] = \left(\frac{\mathbf{D - A}}{\mathbf{D}}\right) \times \mathbf{100} \qquad \text{Eq 6}$$

Where; D [g] is the weight of dry matter at 105 ˚C and A [g] is the weight of ash (dry matter after ignition at 550 ˚C).

3.3.1.2 Chemical Oxygen Demand (COD) Analysis

The COD was measured using the Hach 8000 Method for water, wastewater and seawater (Hach Lange Company). The detailed method is given in Appendix 1. High range plus (0-15000 ppm Hach Lange, Düsseldorf, Germany) Hach standard kit was used to carry out the measurements and a Hach spectrometer version DR 2000 was used for the

measurements. The results in mg/L COD were defined as the mg of O_2 consumed per litre of sample after heating samples for 2 hours with sulfuric acid and a strong oxidizing agent, potassium dichromate.

3.3.1.3 Biochemical Oxygen Demand (BOD) Analysis

The biological oxygen demand measurement was performed using a Lennox BD system. The BOD measuring unit is a closed system, which has a gas compartment with a defined quantity of air in the test bottle. The simultaneously forming carbon dioxide is chemically bound by the potassium hydroxide in the seal cup of the test bottle. As a result, a pressure drop occurs in the system, which is measured by the BOD sensor and shown on the display as a BOD value in mg/L O_2. The detailed method is given in Appendix 2.

3.3.1.4 Sulphate Analysis

Sulphate concentrations were determined by following USEPA Sulfa-Ver 4 Hach Method 8051 provided by Hach Lange Company for water, wastewater and seawater. The detailed method is given in Appendix 3. In this method, the sulphate ions in a sample react with barium in the Sulfa-Ver 4 kit, which results in the precipitation of barium sulphate. The sulphate concentration is proportional to the amount of turbidity formed. The Sulfa-Ver 4 reagent kit also contains a stabilising agent to retain the precipitate in suspension for accuracy of analysis. A Hach spectrometer version DR 2000 was used for the measurements.

3.3.1.5 Nitrogen-Nitrate Analysis

The concentration of Nitrogen-Nitrate was measured using the Cadmium Reduction Hach Method 8039 provided by Hach Lange Company for water, wastewater and seawater. The detailed method is given in Appendix 4. Nitra-Ver 5 reagent powder pillow is the reagent of the test. Cadmium metal present in the reagent reduces nitrate present in the sample to nitrite. The nitrite ions react in an acidic medium with sulphanilic acid, an intermediate diazonium salt is then formed. This salt couples to gentisic acid which causes an amber coloured product. Results were then adjusted with the concentration of nitrite already present in the sample.

3.3.1.6 Nitrogen-Nitrite Analysis

Nitrogen-Nitrite was measured according to Ferrous Sulphate Hach Method 8153 provided by Hach Lange Company for water, wastewater and seawater. The detailed method is given in Appendix 5. Nitri-Ver 2 Reagent powder pillow kits were used and readings were obtained using a Hach spectrometer version DR 2000. This method uses ferrous sulphate in an acid medium to reduce nitrite to nitrous oxide. Ferrous ions combine with the nitrous oxide to form a greenish-brown complex in direct proportion to the nitrite present.

3.3.1.7 Nitrogen-Ammonia Analysis

Nitrogen-Ammonia was measured according to the Salicylate Hach Method 10031 provided by Hach Lange Company for water, wastewater and seawater. The detailed method is given in Appendix 6. Ammonia salicylate and ammonia cyanurate powder pillows were used as the reagent of the method and the measurements were obtained using a Hach spectrometer version DR 900.

3.3.1.8 Phosphorus Analysis

The concentration of Phosphorus was measured according to the Molybdovanate Hach Method 8114 (also known as the Orthophosphate Method) provided by Hach Lange Company for water, wastewater and seawater. The detailed method is given in Appendix 7. Standard Molybdovanate solution is used as the reagent of the method and a Hach spectrometer version DR 2000 was used for measurement.

3.3.1.9 Copper Analysis

Dissolved copper was determined using a Perkin Elmer 3100 Atomic Absorption Spectrophotometer according to (Korda et al. 2008). The detailed method is given in Appendix 8.

3.3.1.10 Lignocellulosic Content analysis

Hemicellulose, cellulose and lignin content was measured according to the Detergent Method developed by (Van Soest & Wine 1967; Van Soest 1963) prior to and after the implementation of each pre-treatment for evaluation of the amendment of lignocellulosic structure with regard to the applied pre-treatment before AD. The samples were subjected to sequential steps of hydrolysis and extraction using different reactants;

neutral detergent, acid detergent and 72% H_2SO_4 solution; in order to assess the lignocellulosic fractions of the sample with respect to their sensitivity to acid hydrolysis. The detailed method is given in Appendix 9.

3.3.1.11 Volatile Fatty Acids Analysis

The concentration of acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid and valeric acid was determined in order to calculate the total short chain volatile fatty acids in the substrate for sample characterisation, as well as substrate and inoculum mixture prior to and after AD with gas chromatography. The detailed method is given in Appendix 10.

3.3.1.12 Degree of Disintegration

The influence of ultrasonic pre-treatment on biomass was also associated with the degree of substrate disintegration which was assessed based on the changes in chemical properties by means of increase in soluble COD level (Pilli et al. 2011) over the treatment time according to Eq 7.

$$\mathbf{DD_{COD}}, \% = \left[\frac{\mathbf{COD_{us} - COD_0}}{\mathbf{COD_{NaOH} - COD_0}}\right] \times \mathbf{100} \qquad \text{Eq 7}$$

Where; COD_{us} [mg/L] is the COD in the supernatant of the ultrasonicated sample, COD_0 [mg/L] is the COD in the supernatant of the untreated sample, and COD_{NaOH} [mg/L] is the maximum COD release in the supernatant after NaOH disintegration by treating the sample with 1M NaOH, at a ratio of 1:2 for 10 min at 90 ˚C (Pilli et al. 2011).

3.3.1.13 Mineral Analysis of Digestate

The digestate quality was investigated with and without pre-treatment prior to anaerobic digestion to compare with an industrial digestate sample which was collected from a full-scale biogas plant processing mixed food waste in Ireland. All samples were analysed in terms of total macro (P, K, Ca, Mg) and micro (Fe, Mn, Zn, Cu) nutrients as well as heavy metals such as As, Cd, Co and Mo, using a 5100 ICP-OES (Agilent). A detail SOP for the digestion and analysis steps are given in Appendix 11 and Appendix 12 respectively.

3.3.2 Bioreactor set-up

A lab scale batch system was used as an anaerobic digester. The bioreactors consist of 2 main parts which are 500 ml conical flasks with an active working volume of 400 ml, and the biogas compatible sampling bag. The connection in between those parts is provided

by plastic tubing, quick release tubing connectors and 3-way valves (Figure 18). In order to ensure anaerobic conditions, nitrogen was flushed twice for 5 minutes each time to the system to clear any residual trace of oxygen from the flasks as well as the tubing (Valero et al. 2016; Verein Deutscher Ingenieure (VDI) 2006). The flasks were immersed in water baths employed to ensure a constant mesophilic reaction temperature. A biogas analyser, BIOGASS 5000, was used for the qualitative and quantitative analysis of the generated biogas as well as validation of the anaerobic conditions which were created while setting the reactors up.

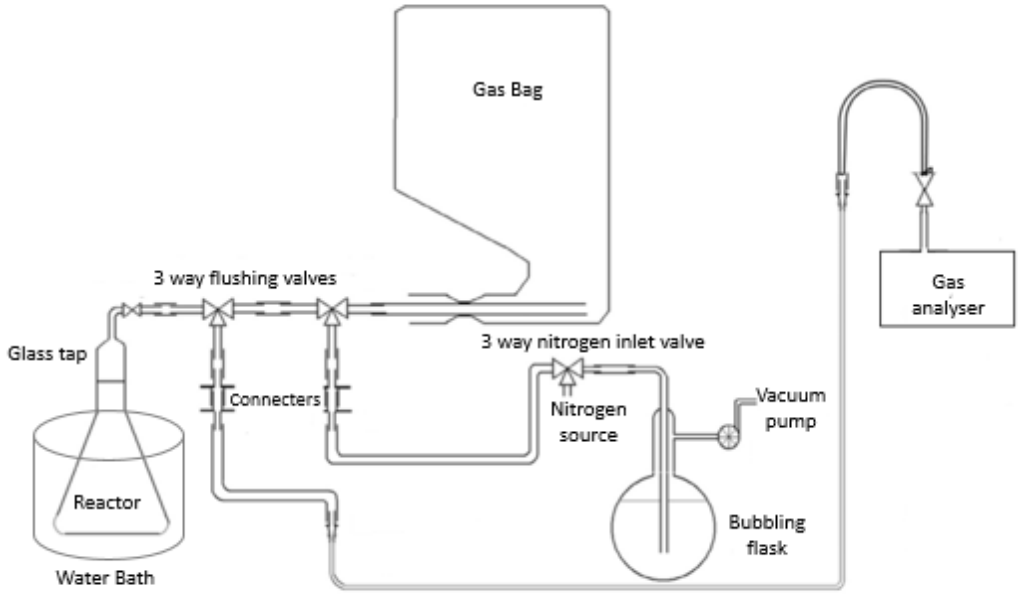


Figure 18. AD bioreactor set up

Samples were kept running in the batch system until the biogas production rate reduced by less than 1% of the most recent measured rate based on the cumulative biogas production (Tedesco et al. 2014).

3.3.3 Box-Behnken Design (BBD)

Box-Behnken design, which was developed by Box and Benken in 1960 (Benyounis & Olabi 2006), is based on evaluating the factors at 3 levels in order to find a reliable approximation for true functional relationship between the surface response and the independent process factors. BBD was selected as the modelling tool of the project in order to minimise the number of experiments to be performed, as each experimental run

has a retention time of 21 days. By using BBD, a sufficient amount of data is produced to develop a mathematical model of the process with less than the half of the possible combinations of operating parameters. It also provides statistical analysis of the generated data.

Usually the second order polynomial equations are used in RSM (Eq 8).

$$\mathbf{y} = \mathbf{b_0} + \sum \mathbf{b_i}\,\mathbf{X_i} + \sum \mathbf{b_{ij}}\mathbf{X_i}\mathbf{X_j} + \sum \mathbf{b_{ii}}\,\mathbf{X_{ii}^2} + \varepsilon \qquad \text{Eq 8}$$

BBD combines two level factorial design with incomplete block design after adding several centre points. For example, for investigation of three factors, the total number of the design points is equal to 12 and then 5 centre points are added, making 17 design points (Rakić et al. 2014). Even though 12 unique combinations of three factors at three levels represents less than one half of the possible combinations, it provides enough information to fit 10 coefficients of the equation in Eq 8. Figure 19 gives the schematic diagram for BBD for three factors (Ferreira et al. 2007).

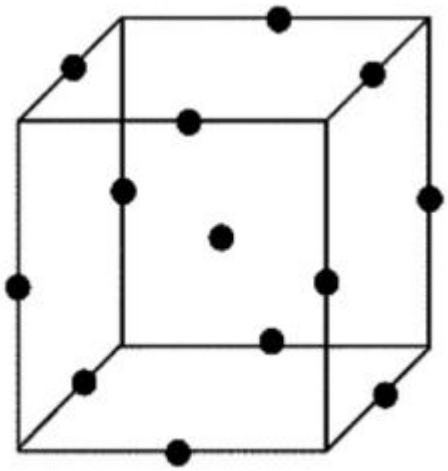


Figure 19. Schematic diagram for BBD for three factors (Ferreira et al. 2007).

3.3.3.1 Statistical Analysis for Box-Behnken Design

To determine the values of the 10 coefficients, namely b_0, b_i, b_{ii}, b_{ij}, in the polynomial equation given in Eq 8, the following Equations 9 – 12 can be used. A, B, C_1 and D_1 are constant and their values are 1/8, 1/4, -1/16 and 1/4 for three factor design.

$$\mathbf{b_0 = \bar{y}_0} \quad \text{Eq 9}$$

$$\mathbf{b_i = A \sum_{u=1}^{N} x_{iu} y_u}\text{, N: Number of the factors} \quad \text{Eq 10}$$

$$\mathbf{b_{ii} = B \sum_{u=1}^{N} x_{iu}^2 y_u + C_1 \sum_{i=1}^{N} x_{ii}^2 y - \left(\frac{\bar{y}_0}{S}\right)} \quad \text{Eq 11}$$

$$\mathbf{b_{ij} = D_1 \sum_{u=1}^{N} x_{iu} x_{ju} y_u} \quad \text{Eq 12}$$

The sum of squares for the developed model and each term of BBD with 3 factors can be calculated with the Equations 13 – 20 (Benyounis & Olabi 2006; Aboerheeba 2013).

$$\mathbf{SS_T = \sum_{i=1}^{N} (y_i - \bar{y})^2} \quad \text{Eq 13}$$

$$\mathbf{SS_M = \sum_{i=1}^{N} (\hat{y}_i - \bar{y})^2} \quad \text{Eq 14}$$

$$\mathbf{SS_R = \sum_{i=1}^{N} (y_i - \hat{y}_i)^2} \quad \text{Eq 15}$$

$$\mathbf{SS_{PE} = \sum_{i=1}^{N} (y_i - \hat{y}_i)^2}\text{, for center points only} \quad \text{Eq 16}$$

$$\mathbf{SS_{lof} = SS_R - SS_{PE}} \quad \text{Eq 17}$$

$$\mathbf{SS_{b_i} = A \sum_{i=1}^{N} (x_i y_i)^2}\text{, N: Number of factors} \quad \text{Eq 18}$$

$$\mathbf{SS_{b_{ij}} = D_1 \sum_{u=1}^{N} \left(x_{iu} x_{ju} y_u\right)^2} \quad \text{Eq 19}$$

$$\mathbf{SS_{b_{ii}} = b_0 \sum_{u=1}^{N} y_u + \sum_{u=1}^{N} b_{ii} x_{iu}^2 y_u - \sum_{u=1}^{N} \frac{(y_n)^2}{N}} \quad \text{Eq 20}$$

3.3.3.2 Development of Design Matrix

The design matrix in coded values for BBD is given in Table 6. The lowest and the highest limits of the factors are represented by -1 and 1 respectively, where 0 refers to the centre points. As stated earlier the number of experiments is 17 for investigating three factors at three levels. These experimental runs provide sufficient information to calculate the coefficients in the mathematical model given in Eq 8.

Table 6. Design matrix for BBD, in terms of coded factors

Std	Run	Factor 1	Factor 2	Factor 3
1	16	-1	-1	0
2	5	1	-1	0
3	8	-1	1	0
4	11	1	1	0
5	12	-1	0	-1
6	9	1	0	1
7	10	-1	0	1
8	4	1	0	1
9	6	0	-1	-1
10	2	0	1	-1
11	1	0	-1	1
12	13	0	1	1
13	15	0	0	0
14	14	0	0	0
15	7	0	0	0
16	17	0	0	0
17	3	0	0	0

3.3.3.3 Development of the Mathematical Models

The functional relationship between any response of interest and the associated design factors can be expressed as y=f (P, S, F), and Eq 8 can be expressed in the form of Eq 21. P, S, F notation was chosen to represent the design parameters in order to prevent confusion since these letters were not used for anything else.

$$\boldsymbol{Y = b_0 + b_1 P + b_2 S + b_3 F + b_{11} P^2 + b_{22} S^2 + b_{33} F^2 + b_{12} PS + b_{13} PF + b_{23} SF}$$

Eq 21

The values of the coefficients in Eq 21 are specified by applying regression analysis to the generated data Equations 9 – 12 are applied to ascertain the coefficients for the responses of the experiments.

3.3.3.4 Assessing the Adequacy of the Developed Models

Analysis of variance (ANOVA) is used to evaluate the adequacy of the developed mathematical models. Both the significance of the developed models and the significance of each term in the regression equation is assessed statistically by applying sequential F-test, lack of fit test, and other adequacy measures (such as R^2, Adjusted R^2, Predicted R^2 and Adequacy Precision ratio), using the DOE software to achieve the best fit. The aim is

to ensure adequacy measures close to 1, which therefore indicates that the model is adequate. An adequate model discrimination is measured with the value of adequate precision being greater than 4 (Montgomery 2016). The probability > F (also called as p-value) of the model can be computed using ANOVA. If the Prob. > F of the model and each term in the model is less than the level of significance (e.g. α=0.05), the model is then considered to be adequate within the interval of this confidence (1-α). In the case of a lack of fit test, it can only be considered when the Prob. >F of the lack of fit exceeds the level of significance (e.g. greater than 0.05). An adequate model suggests that the reduced model has successfully passed all of the required statistical tests and therefore can be used in prediction of process responses and in process optimisation (Eltawahni et al. 2011). The summary of the ANOVA table is given in Table 7 and the calculation of the other adequacy measures is given in Equations 22 – 26.

Table 7. ANOVA Table for a full model

Source	SS	df	MS	$F_{cal.}$ - Value	p-value or Prob.>F
Model	SS_M	p			
P	SS_1	1			
S	SS_2	1			
F	SS_3	1			
PS	SS_{12}	1	Each SS divided by its df	Each MS divided by MS_E	From table or software library
PF	SS_{13}	1			
SF	SS_{23}	1			
P^2	SS_{11}	1			
S^2	SS_{22}	1			
F^2	SS_{33}	1			
Residual	SS_R	N-p-1			-
Lack of Fit	SS_{lof}	N-p-n_0			From table
Pure Error	SS_{PE}	n_0-1			-
Cor Total	SS_T	N-1	-	-	-

Where; P is the number of coefficients in the model, N is the total number of runs, n_0 is the number of centre points, SS is the sum of squares, df is the degrees of freedom and MS is the mean square.

$$\boldsymbol{R^2 = 1 - \left[\frac{SS_R}{SS_R + SS_M}\right]} \quad \text{Eq 22}$$

$$\boldsymbol{Adj\ R^2 = 1 - \left[\left(\frac{SS_R}{df_R}\right) \times \left(\frac{SS_R + SS_M}{df_R + df_M}\right)^{-1}\right]} \quad \text{Eq 23}$$

$$\boldsymbol{R^2_{Pred} = 1 - \left[\frac{PRESS}{SS_R + SS_M}\right]} \quad \text{Eq 24}$$

$$\boldsymbol{PRESS = \sum_{i=1}^{n}\left(y_i - \hat{y}_{i,-1}\right)^2} \quad \text{Eq 25}$$

$$\boldsymbol{Adeq.precision = \left[\frac{Max(\hat{Y}) - Min(\hat{Y})}{\sqrt{\frac{p \times MS_R}{n}}}\right]} \quad \text{Eq 26}$$

Where p is the number of model parameters (including the intercept b_0) and n is the number of experiments.

3.3.3.5 Model Reduction by Stepwise Regression Method

The full model, which is given in Eq 21, usually contains insignificant terms along with the significant ones. Insignificant model terms (i.e. those model terms with p values greater than the level of significance α) should be eliminated manually or automatically. In this research, selection of the variables was performed by using a stepwise regression method as it combines the advantages of forward and backward regression (Aboerheeba 2013).

In a stepwise regression procedure, the model starts with the constant term only. The variable which has the highest correlation with y, the response of interest, is then added to the model to test the significance of the regression coefficient of this variable. If it is significant, according to a standard t-test, the variable remains in the model. After adjusting y for the effect of the first variable added, the same steps are applied for searching the other variables. However, it is also possible to delete a variable that might have been added at earlier stages. This method provides an advantage of assuming different or similar levels of significance for inclusion or exclusion of the variables from the final reduced equation which gives an opportunity to challenge the model (Benyounis & Olabi 2006).

3.3.3.6 Development of the Final Reduced Model and Post Analysis

The final reduced model is built through stepwise regression that contains only the significant terms along with the necessary terms to maintain the hierarchy. An ANOVA table is produced for the reduced quadratic model and all terms in the model (Aboerheeba 2013). When the final reduced model successfully passes all the required statistical tests, it can be used to predict process responses. Perturbation, contours and 3D graphs are plots that can then be generated post-analysis to examine the effect of the factors and their levels of contribution to the response.

3.3.4 Optimisation

The Design Expert software offers an important tool to optimise the interest of response where it is subjected to particular constraints of the independent parameters.

3.3.4.1 Desirability Approach

To optimise a multiple response process, all of the responses are combined into a dimensionless measure of performance which is known as the overall desirability function. The software also provides a flexibility weighting, giving more importance to individual responses. In this approach, each estimated response, Y_i, is defined within the boundary of $0< d_i < 1$, where a higher value of d_i indicates a better desirability of the response. In other words, $d_i = 1$ means a completely desired response or vice versa when $d_i = 0$ (Ferreira et al. 2007; Hosseini & Aziz 2011). In this work the desirability of each response (d_i) was calculated using Equations 27 – 30.

The desirability function can be created differently for each response by assigning a relative importance to each other (r) as well as setting different weight fields (w_{ti}) for each goal. Weights can be either used for adding emphasis to the upper and lower bounds or emphasising the target value of the interest of response. Importance can vary from the value of 1 (+), the least important, to the value of 5 (+++++), the most important.

For a goal of maximising, the desirability is calculated as:

$$d_i = \begin{cases} 0 & , Y_i \leq Low_i \\ \left(\frac{Y_i - Low_i}{High_i - Low_i}\right)^{wt_i} & , Low_i < Y_i < High_i \\ 1 & , Y_i \geq High_i \end{cases} \quad \text{Eq 27}$$

For a goal of minimising, the desirability is calculated as:

$$d_i = \begin{cases} 1 & , Y_i \leq Low_i \\ \left(\frac{High_i - Y_i}{High_i - Low_i}\right)^{wt_i} & , Low_i < Y_i < High_i \\ 0 & , Y_i \geq High_i \end{cases} \quad \text{Eq 28}$$

For a particular target *T* as the goal, the desirability is calculated as:

$$d_i = \begin{cases} \left(\frac{Y_i - Low_i}{T_i - Low_i}\right)^{wt_i} & , Low_i < Y_i < T_i \\ \left(\frac{Y_i - High_i}{T_i - High_i}\right)^{wt_i} & , T_i < Y_i < High_i \\ 0 & , \text{Otherwise} \end{cases} \quad \text{Eq 29}$$

For a goal within a range, the desirability is calculated as:

$$d_i = \begin{cases} 1 & , Low_i < Y_i < High_i \\ 0 & , \text{Otherwise} \end{cases} \quad \text{Eq 30}$$

$$\boldsymbol{D} = \left(\prod_{i=1}^{n} \boldsymbol{d_i^{r_i}}\right)^{\frac{1}{\sum r_i}} \quad \text{Eq 31}$$

In case of assessing different importance to individual responses, the overall desirability function D is calculated using Eq 31 (Benyounis & Olabi 2006).

3.3.4.2 Optimisation Approach in DOE Software

The DOE software provides numerical and graphical optimisation which both allow the user to set the desired goals for each factor and response (Tedesco et al. 2013). The numerical optimisation is used to maximise the objective function by finding a point(s) in the factor domains. In the graphical optimisation of multiple responses, the regions where the requirement meet the proposed criteria are highlighted by overlaying a response contour. A visual search for the best fit then becomes possible (Benyounis & Olabi 2006). Using numerical optimisation prior to graphical optimisation is advised where numerous responses are recorded. The regions of the feasible response values of individual factors are then displayed using graphical optimisation (Tedesco et al. 2014). The areas which do not fit the criteria of the optimisation are eliminated. The flowchart of the optimisation steps is given in Figure 20.

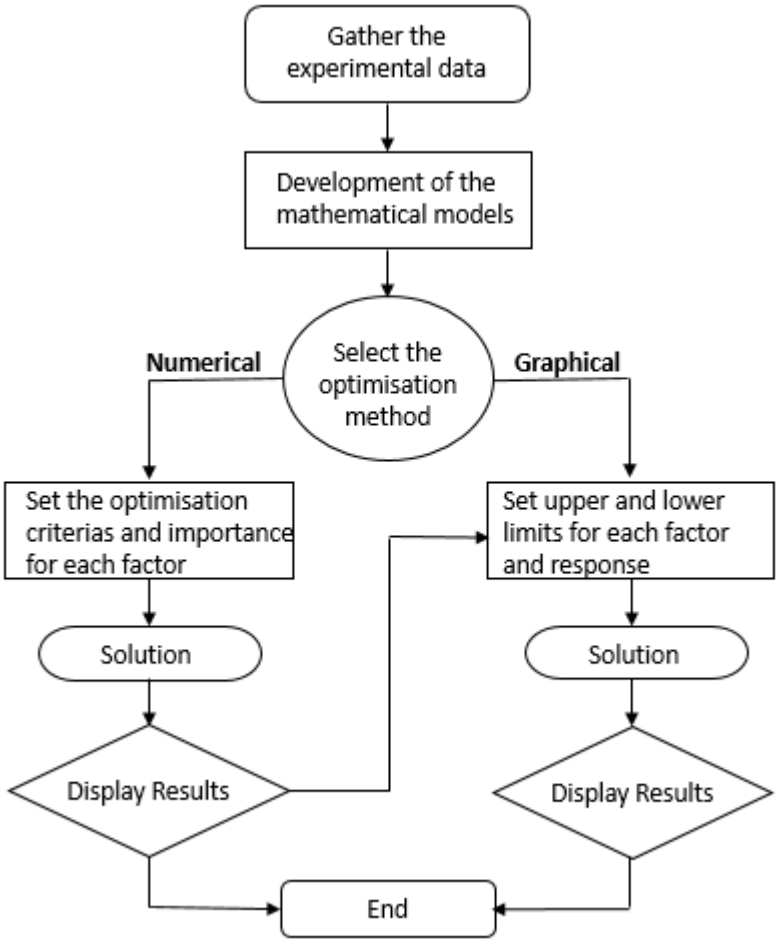


Figure 20. Optimisation flowchart

3.3.5 Experimental Methodologies

3.3.5.1 Application of the Alkaline and Beating Pre-treatments

Alkaline pre-treatment of pot ale is necessary prior to anaerobic digestion in order to balance its acidic nature (pH around 4.3). Therefore, initial screening experiments were performed to assess the combined impacts of alkaline with beating pre-treatment on AD of pot ale. Alkaline pre-treatment was performed in 400 ml batches in accordance with the literature (Kim et al. 2003; Carrère et al. 2010) by dropwise addition of 1 M NaOH into the pot ale sample until pH 10 was obtained, (Sežun et al. 2000) then the sample was stirred for 6 min at 500 rpm. For beating pre-treatment, the device was fed with 25 L of pot ale sample to have a full circulation in the beating tub. Beating pre-treatment was performed on pot ale prior to, after and without pH 10 NaOH treatment for 0, 5 and 7.5 minutes. All samples were seeded with 50% sludge (by volume, on wet basis).

For the alkaline pre-treatment of pot ale – draff mixture, the volume of pot ale was kept constant as 400 ml per batch (which was approximately 400 ± 2.5 g of pot ale due to the density of pot ale) and the required amount of draff was added with mixing ratios 1:1, 1:3 and 1:5 by wet weight, with alkaline pre-treatment as described previously (1 M NaOH addition until pH 10 with stirring at 500 rpm for 6 min). For the beating pre-treatment of the mixed compounds, 25 L alkali pre-treated pot ale and 12.5, 6.25 and 4.2 g alkali pre-treated draff was then fed to the beater in order to achieve 1:1, 1:3 and 1:5 mixing ratios by weight. The beating pre-treatment was performed for 7.5 and 15 minutes for each mixing ratio.

In order to evaluate the influence of alkaline pre-treatment on the lignocellulosic composition of pot ale and draff, the same pre-treatment method was followed for pot ale whereas in the case of treating draff alone, the liquid phase (pot ale) was replaced with pH 10 adjusted DI water (with a 1:5 draff water ratio by weight) in order to isolate the influence of alkaline pre-treatment on draff alone.

3.3.5.2 Effect of Different Sludge Percentages

Anaerobic digestion of both as received and pH 10 treated pot ale samples were performed with 0, 5, 10, 20, 50% of sludge, after the neutral pH adjustment. Inoculum with different sludge fractions was placed into 500 ml conical flasks with the total reaction volume of 400 ml. 10 different pot ale samples were observed in triplicate at two different digestion temperatures, which were 35 and 38˚C within the mesophilic range. All alkali treated pot ale was neutralised to pH 7 by 3% acetic acid due to its mild nature which could not cause an interference with the applied pre-treatment (Taherzadeh & Karimi 2008). A similar pH adjustment was performed on non-treated pot ale samples by 1M NaOH after the addition of the sludge prior to AD to ensure a consistent starting pH of 7.

3.3.5.3 DOE for AD of Pot Ale and Pot ale & Draff mix (5:1 by weight)

Pot ale samples were treated with 1M NaOH first, then beaten for 0, 7.5 and 15 min based on significant preliminary results. The required ratio of sample and sludge (10 – 50% sludge) for 400 ml of total volume were placed into 500 ml conical flasks and AD experiments were performed at three different temperatures under mesophilic conditions (35 ± 3 ˚C, as specified). All experiments were run in triplicate as indicated for

each parameter (beating time, sludge percentages and temperature). The design factors and responses are outlined in Table 8. The same design parameters were followed for AD of pot ale and draff mix by considering the potential dilution effects of draff addition on the copper concentration of pot ale.

Table 8. Summary of the design factors, factor levels and the responses for alkaline and beating pre-treatments

Parameters under investigation (Design factors)	**Levels**	**Responses**
Beating time (min)	0 7.5 15	Biogas Generation (ml/g VS) CH_4% CO_2%
Sludge seeding ratio (%)	10 30 50	
Digestion Temperature (˚C)	32 35 38	
Feedstock	Pot ale Pot ale draff mix (5:1 by wet weight)	

Recorded responses were used to develop mathematical models for both DOE designs. They were then optimised using a combination of numerical and graphical optimisation by employing the desirability function.

3.3.5.4 Evaluation of the Effects of Increased Solid Content on Anaerobic Digestion Performance

In order to evaluate the impacts of increased solid content on anaerobic digestion, draff and pot ale were seeded with 50% sludge of the total working volume of 400 ml with different mixing ratios (1:1, 1:3 and 1:5 by weight); the experiments were performed at 35 ˚C. Beating pre-treatments for 7.5 and 15 minutes and alkaline pre-treatment were performed according to the methods in Section 3.3.5.1.

3.3.5.5 Investigation of Initial Reaction pH on Anaerobic Digestion Yield

Pot ale samples were treated with 1M NaOH according the method given in the Section 3.3.5.1. The initial pH value (7 – 8 – 9) of the reaction was investigated. Following previous experiments, the reactor system was modified to allow online pH monitoring by inserting a probe from the side of the flask in order to investigate the pH profile in more detail. Each experiment was run in triplicate and one of each sample group was disconnected

over 9 days of AD to have a comparison on biogas generation within the first 9 days of AD and cumulative generation. The level of VFAs and organic matter removal percentages in terms of COD, BOD and SO_4 were measured for 3 experiments to define the time of inhibition. Sludge seeding ratio was selected as 50% by volume on a wet basis according to previous experiment results. Alkali pre-treated pot ale sample was seeded with 30 and 50% sludge to monitor the pH values over the reaction time in order to assess the effects of sludge seeding ratio on the pH fluctuations.

3.3.5.6 DOE of Pot Ale after Implementation of Combined Alkali and Microwave Pre-treatments

Alkaline pre-treatment was conducted using 1 M NaOH solution prior to microwave pre-treatment. Microwave pre-treatment was performed using a Sharp Compact microwave oven with an 800 W maximum power. In order to decide on the design limits of microwave power, a temperature profile (Table 9) was created based on the time of pre-treatment applied on 400 ml of alkali pre-treated pot ale at various power settings which were 10, 30 and 50% of the maximum power, as a longer period of time at lower power setting is advised in the literature in order to avoid potential inhibition arising from Maillard reactions as well as production of phenolic compounds (Sapci 2013). The temperature of the samples with 30% power reached the boiling point after 11 minutes of treatment, whereas the samples treated with 50% power setting reached boiling after 7 minutes. In the case of the 10% power setting, the sample did not reach the boiling point, in other words it was only subjected to low temperature thermal pre-treatment by using microwave.

Table 9. Temperature profile of microwave pre-treatment on alkali pre-treated pot ale

	Temperature (˚C)		
Time of Pre-treatment (min)	**10% Power (80 W)**	**30% Power (240 W)**	**50% Power (400 W)**
1	20	30	36
2	25	38	50
3	29	46	62
4	33	54	74
5	38	61	84
6	42	69	95
7	47	77	100
8	51	84	103
9	55	90	105
10	60	95	107
11	66	99	110

Along with the microwave power, increased sludge seeding ratio and initial pH were the parameters under investigation for this design. A range of 1 – 5 for inoculum substrate ratio (I/S) (sludge seeding ratio) was decided upon based on VS contents of inoculum (sludge) and pre-treated substrate (pot ale) according to (Vazifehkhoran et al. 2018) which corresponds to 65 – 90% on a wet volume basis. The experimental design is given in Table 10. Recorded responses were used to develop a mathematical model on cumulative methane production by using Box Behnken Design in RSM. It was then optimised by using a combination of numerical and graphical optimisation by employing desirability function.

Table 10. Summary of the design factors and the responses for alkaline and microwave pre-treatments

Parameters under investigation (Design factors)	**Levels**	**Responses**
I/S	1. 1 (65%) 2. 3 (85%) 3. 5 (90%)	1. Cumulative Methane Yield (ml/g VS) 2. Cumulative CO_2 Yield (ml/g VS) 3. Cumulative H_2S (ppm)
Initial pH	1. 7 2. 8 3. 9	
Microwave Power (%)	1. 10 2. 30 3. 50	

A kinetic study was conducted on microwave treated samples in order to evaluate the effects of pre-treatments by comparing the hydrolysis rate constants (Vazifehkhoran et al. 2018). The first order kinetic model is given in Eq 32. A t-test (2 tailed distribution with unequal variance) was used for statistical analysis.

$$\boldsymbol{B_t = B_0\,[\,1 - e^{(-\,k_h\,t)}]} \qquad \text{Eq 32}$$

Where; B_t [ml CH_4/g VS] is the cumulative CH_4 yield at time t, B_0 [ml CH_4/g VS] is the ultimate CH_4 yield, k_h [d^{-1}] is the first-order hydrolysis constant, and t is the time of digestion (days).

3.3.5.7 DOE of Pot Ale after Implementation of Combined Alkali and Ultrasonic Pre-treatments

The effects of ultrasound pre-treatment prior to anaerobic digestion of pot ale, which was performed using a Hielscher Ultrasonics UP 400S with a H22L2D horn, was assessed on a standalone basis as well as in combination with alkaline pre-treatment at different doses of NaOH 1.5 and 3 M. The amplitude ratio (40, 70 and 100%) and exposure time (1, 2 and 3h) were the investigated parameters of ultrasonic pre-treatment with the indicated levels. The experimental design is outlined in Table 11. I/S was decided as 5 on dry basis (which corresponds to a 90% sludge seeding ratio on by volume on wet basis) based on preliminary experimental results. The initial pH value was left at the pH of inoculum (8) with no adjustment since it was found as an insignificant parameter in the previous experiments with increased sludge seeding. The process factors were investigated at three levels with Box Behnken Design within Surface Response Methodology.

Table 11. Summary of the design factors and the responses for alkaline and ultrasonic pre-treatments

Parameters under investigation (Design factors)	**Levels**	**Responses**
Amplitude Ratio (%)	40	Methane Yield in 2 days (ml/g VS)
	70	CO_2 Yield in 2 days (ml/g VS)
	100	H_2S generation 2 days (ppm)
Exposure Time (h)	1	
	2	
	3	
NaOH Dose (M)	0	
	1.5	
	3	

The degree of disintegration on the feedstock was calculated according to Eq 7, while the kinetic study was performed according to Eq 32.

The recorded responses were used to develop a mathematical model for methane production within the first two days of AD. It was then optimised using a combination of numerical and graphical optimisation by employing the desirability function.

3.4 Chapter Summary

In this chapter, the materials, analytical and experimental methods were presented in detail along with the upper and lower limits of the chosen pre-treatments for performing lab-scale AD and subsequent mathematical modelling. A visual summary of the work sequence followed throughout this project is given in Figure 21. The following chapter presents the results and discussion on combined alkaline and beating pre-treatments in pot ale and pot ale draff mixture along with major outcomes of the performed experiments to carry on the further stages of the project.

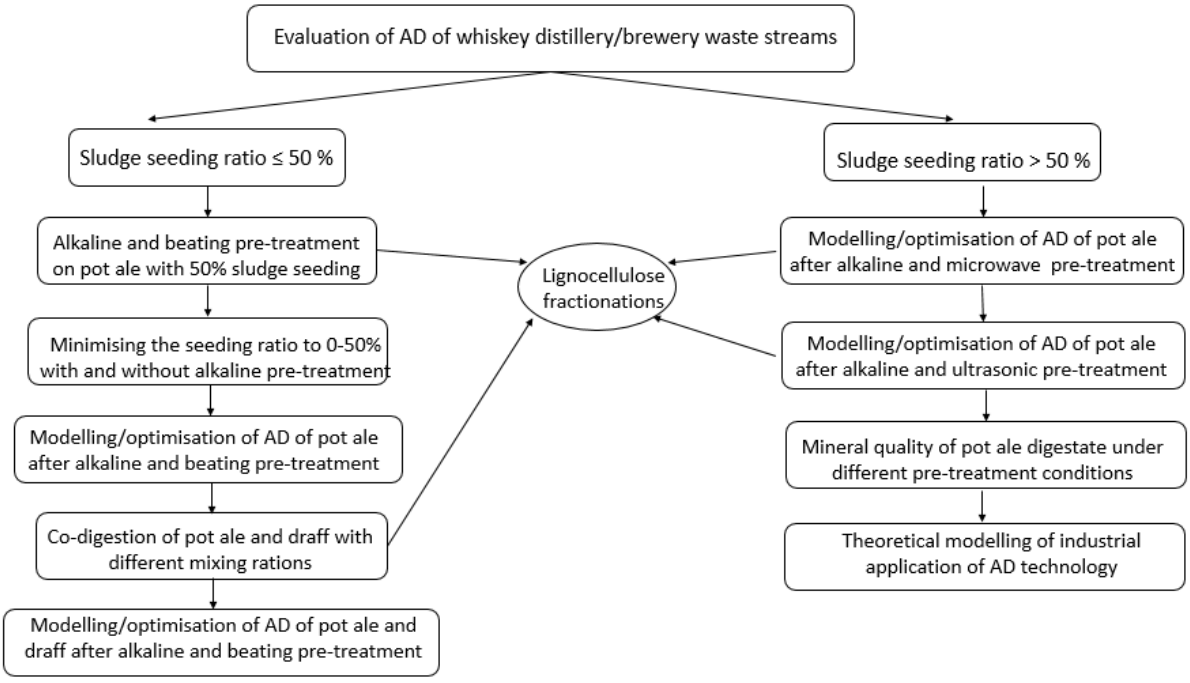


Figure 21. Summary of the work sequence

CHAPTER 4 RESULTS OF COMBINED ALKALINE-BEATING PRE-TREATMENT WITH LOW SLUDGE SEEDING RATIOS

4.1 Introduction

This chapter presents details of the physicochemical characteristics of pot ale, along with the effects of alkaline pre-treatment on the lignocellulosic structure of pot ale and draff. The modelling of anaerobic single digestion of pot ale and co-digestion of pot ale and draff is outlined with various sludge seeding ratios (0 – 50%) after implementation of a novel hybrid pre-treatment (combined alkaline and beating). The results of mathematical modelling and optimisation studies conducted in order to select the optimum conditions for AD are presented. A particular focus was placed on maximising the biogas yields with applied pre-treatments with the minimum use of sludge.

4.2 Results of Initial Screening

4.2.1 Sample Characterisation and Assessment of Pre-treatments

The pot ale was characterised as received in terms of in terms of TS, VS, moisture content, COD, BOD, sulphate, phosphate, nitrogen-nitrate, nitrogen-nitrite, nitrogen-ammonium, hemicellulose, cellulose, lignin, VFAs and dissolved copper. These characteristics and others are shown in Table 12. The volatile fatty acids (acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid and isovaleric acid) were determined from single replicate, whereas other characteristics are the average of triplicate analysis.

Table 12. Characterisation of pot ale as received

Compound	Value
TS (g/g sample)	0.089 ± 0.0004
VS (g/g sample)	0.077 ± 0.008
Moisture (%)	91.13 ± 0.042
COD (mg/L)	38867 ± 115
BOD (mg/L)	30965 ± 666
SO_4 (mg/L)	190 ± 31
VFAs (mM)	134.89 ± 1.5
Hemicellulose (%)	11.5 ± 0.3
Cellulose (%)	10.6 ± 1.8
Lignin (%)	26.9 ± 1.6
P-PO_4^{3-} (mg/L)	778 ± 7
N-NO_3 (mg/L)	111 ± 20
N-NH_3 (mg/L)	45 ± 7
N-NO_2 (mg/L)	33 ± 4
TOC	105 ± 7
Cu (mg/L)	14.7 ± 1.021
pH	4.3 ± 0.2

The summary of the applied pre-treatments on pot samples and the corresponding sample abbreviations are given in Table 13.

Table 13. Alkaline and beating pre-treatment summary

Sample abbreviation	Pre-treatment
NT	Nontreated (control)
A	Alkaline treated
B5	5 min beaten
B5A	5 min beaten + Alkaline treated
B7.5	7.5 min beaten pot ale
B7.5A	7.5 min beaten + Alkaline treated
AB5	Alkaline treated + 5 min beaten
AB7.5	Alkaline treated + 7.5 min beaten

The efficiency of alkaline pre-treatment was assessed in terms of modifying the lignocellulosic structure of pot ale. After implementation of alkaline pre-treatment (1 M NaOH) on pot ale, the hemicellulose fraction was increased, lignin was decreased and cellulose remained static (Figure 22). One-way ANOVA tests indicated that alkaline pre-treatment of pot ale resulted in a significant increase (5%) in the hemicellulose fraction and a significant decrease (9%) in the lignin fraction, with *p* values of 0.0320 and 0.0254

respectively. The cellulose content of the structure was not affected significantly by the pre-treatment. Effects of beating pre-treatment on the lignocellulosic structure could not be assessed because of the grinding requirement (particle size between 0.5 mm and 1 mm) of the method.

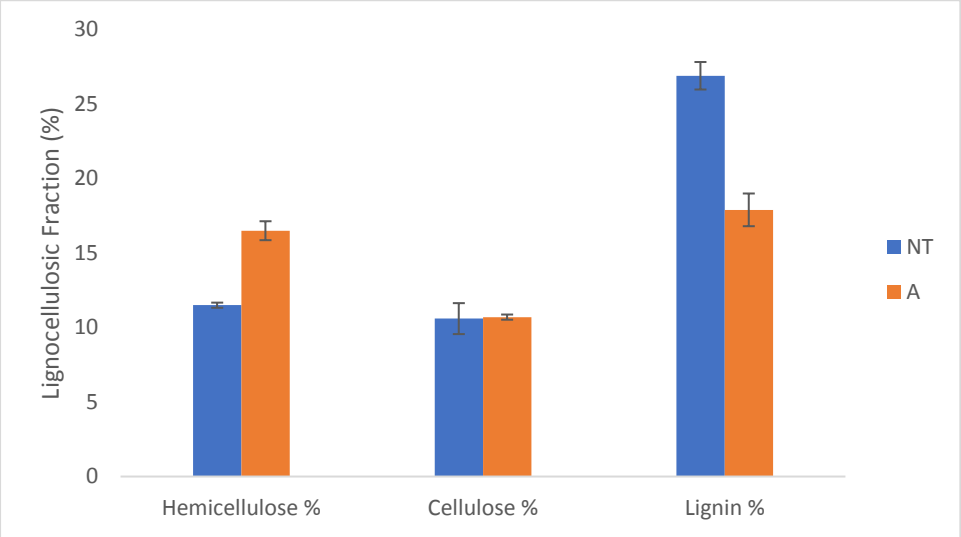


Figure 22. Lignocellulosic structure of pot ale before and after alkaline pre-treatment

The total solids (TS), volatile solids (VS) and moisture content of each sample and the biogas production results with regards to the applied pre-treatments are given in Table 14. The abbreviations of the sample names are given in Table 13. Mechanical pre-treatment alone did not significantly improve biogas generation and CH_4 percentages, while alkaline pre-treatment in isolation had a significant effect. The combination of alkaline pre-treatment followed by beating pre-treatment showed an improvement in the amount of biogas generated, in comparison with alkaline pre-treatment alone, whereas the biogas quality varied depending on the time of beating. Biogas generation, per gram VS, with combined alkaline/mechanical pre-treatment was improved 3-fold over the untreated samples, with biogas generation of 629 ± 8.5ml/g VS after 21 days digestion.

Table 14. Biogas yields preliminary experiments with untreated and pre-treated pot ale

Sample Name	TS (g/g sample)	VS (g/g sample)	Moisture %	Generated Biogas ± STD (ml/g VS)	CH_4 %	CO_2 %
NT	0.089	0.077	91.129	205 ± 21.4	19.3	65.1
A	0.088	0.070	91.207	523 ± 10.4	48.1	35.8
B5	0.087	0.072	91.266	209 ± 0.8	16.4	69.4
B7.5	0.087	0.071	91.303	188 ± 24.4	19.7	71.5
B5A	0.086	0.069	91.411	476 ± 6.2	37.1	47.3
AB5	0.095	0.077	91.517	541 ± 19.4	49.5	45.3
B7.5A	0.087	0.070	91.314	555 ± 5.3	43.0	52.3
AB7.5	0.090	0.070	90.977	629 ± 8.5	51.3	42.6

The highest cumulative biogas production over the 21 days of AD was achieved with alkaline pre-treatment following by beating for 7.5 min as 629 ± 8.5 ml/g VS, whereas the control could only reach 205 ± 21.4 ml/g VS – indicating that 1 M NaOH pre-treatment resulted in a 307% enhancement of overall biogas generation. A p-value of 0.0032 (<0.05) was found upon application of a one-way ANOVA (given in Table 15), indicating that the order in which the pre-treatments were performed had a significant effect on total biogas generation. As such, alkaline pre-treatment followed by beating pre-treatment was selected as the baseline hybrid pre-treatment for further experiments. This was attributed to greater mechanical pre-treatment after implementation of alkaline pre-treatment since it causes swelling of biomass.

Table 15. One-way ANOVA results on the order of the alkaline and beating pre-treatments

Groups	Count	Sum	Average	Variance
B5A	2	952.47	476.24	77.90
AB5	2	1082.53	541.27	749.79
B7.5A	2	1110.11	555.06	57.20
AB7.5	2	1257.81	628.91	144.79

ANOVA

Source of Variation	SS	df	MS	F	P-value	Fcrit
Between Groups	23538.36	3	7846.12	30.48	0.003247	6.59
Within Groups	1029.683	4	257.42			
Total	24568.04	7				

Due to the inability of the biogas analyser to measure both biogas quality and the quantity at the same time, 2 samples of each triplication were kept for measuring the biogas volume manually, the third sample was used for the measuring the biogas quality. Thus, standard deviations for biogas quality could not be reported in Table 14.

4.2.2 Effect of Lower Sludge Contents

In order to minimise the amount of sludge usage, non-treated and alkali treated samples were seeded with varying sludge range, 0 – 50 % of 400 ml, at the digestion temperatures of 35 and 38 ˚C. Extension outside of this rage of temperatures was not investigated due to the thermal sensitivity of the microbes within the mesophilic range. Total solids, volatile solids and the moisture content of nontreated (NT) and alkali treated (A) pot ale sample are given in Table 16.

Table 16. Total solids, volatile solids and the moisture content of pot ale sample

Sample Name	TS (g/g sample)	VS (g/g sample)	Moisture%
NT	0.087 ± 0.0025	0.080 ± 0.0022	91.21 ± 0.25
A	0.082 ± 0.0004	0.073 ± 0.0005	91.81 ± 0.04

The effects of the sludge content within the reactors on the biogas generation is given in Figure 23. Reactions at lower percentages of sludge seeding (<50%) resulted in considerably low biogas yields regardless of the digestion temperature or application of the alkali pre-treatment. However, when alkali pre-treated samples were seeded with 50% sludge, a significant enhancement was seen. Moreover, a significant difference (according to the one-way ANOVA tests) arose from the application of the alkali pre-treatment on pot ale within the 50% sludge seeded sample group, with the p values of 0.00056 and 0.000039 at 35 and 38 ˚C digestion temperatures, respectively.

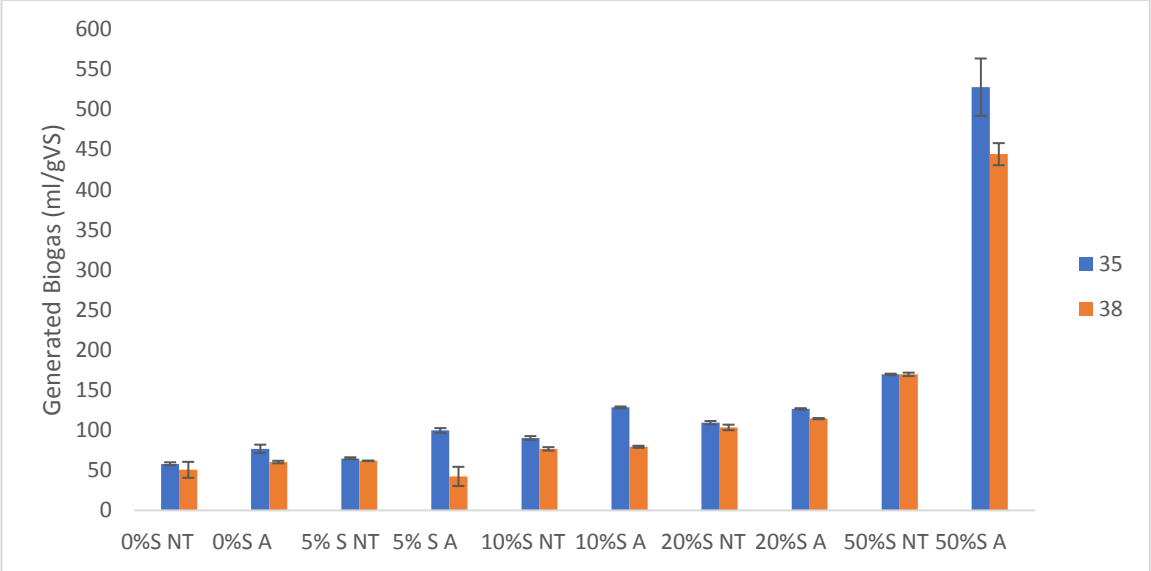


Figure 23. Effect of different sludge seeding percentages on biogas generation by AD of nontreated and alkali treated pot ale at different digestion temperatures

The biogas quality results and initial and final pH values of the samples based on 21 days of digestion time are given in Table 17. The lower percentages of sludge seeding ratios had a similar impact on biogas quality as biogas quantity. Increased sludge percentages (up to 20%) had no significant effect on the CH_4 content of generated biogas whereas 50% sludge seeded samples showed greater CH_4 percentages regardless of different digestion temperatures and implementation of alkaline pre-treatment. On the other hand, application of alkaline pre-treatment increased CH_4 percentages from 19.6 to 48.6 (p value 7.06×10^{-5}) and from 17.9 to 53.2 (the p value 0.00054) at the digestion temperatures of 35 and 38 ˚C, respectively.

The concentration of H_2S was higher than the measurement range (>10000 rpm) for the reactions that were carried out with less than 50% sludge. Alkali pre-treated samples showed a higher H_2S generation in comparison to nontreated sample at two different digestion temperatures. It should be noted that values of H_2S concentration are higher at 38 ˚C than at 35°C for both non-treated and alkali treated samples with 50% sludge seeding ratio. The high levels of H_2S and low CH_4 concentrations indicates the activity of the sulphate reducing bacteria through the sulfidogenesis path, which is the side reaction of methanogenesis.

Although all samples were neutralised prior to anaerobic digestion, pH drops (around 5) were seen in all of the samples except for alkali treated and 50% sludge seeded (50%S A) for both digestion temperatures.

Table 17. Effect of different sludge percentages on biogas quality at 35 and 38 ˚C digestion temperatures

Sample Name	Digestion Temperature(˚C)	CH_4%	CO_2 %	H_2S (ppm)	BAL %	Initial pH N/A	Final pH N/A
0%S NT	35	0.1	58.2	>>>	39.8	7.2	5.3
0%S A	35	0.2	55.6	>>>	41.9	7.1	5.3
5% S NT	35	2.4	65.8	>>>	28.1	7.3	5.4
5% S A	35	1.7	72.4	>>>	24.8	7.3	5.5
10%S NT	35	2.5	66.2	>>>	27.6	7.1	5.3
10%S A	35	1.5	64.3	>>>	32.7	7.1	5.5
20%S NT	35	1.8	68.3	>>>	27.8	7.2	5.4
20%S A	35	4.5	69.6	>>>	24.2	7.1	5.9
50%S NT	35	19.6	64.7	2429	14.2	7.1	5.9
50%S A	35	48.6	34.7	1139	9.9	7.1	8.1
0%S NT	35	0.0	56.7	>>>	40.2	7.2	5.7
0%S A	38	0.3	58.5	>>>	38.9	7.1	5.3
5% S NT	38	1.9	67.1	>>>	28.7	7.1	5.7
5% S A	38	2.7	57.1	>>>	35.1	7.1	5.3
10%S NT	38	3.1	68.7	>>>	25.7	7.1	5.4
10%S A	38	2.7	64.8	>>>	29.5	7.1	5.6
20%S NT	38	3.8	69.9	>>>	24.4	7.1	5.2
20%S A	38	5.4	70.1	>>>	22.5	7.1	5.7
50%S NT	38	17.9	68.5	4174	11.7	7.1	5.8
50%S A	38	53.2	35.8	1876	9.4	7.1	8.1

* All values are average of triplication

The organic matter removal percentages in terms of COD, BOD and SO_4 obtained with AD at 35 ˚C is given in Table 18 along with the initial values for both nontreated and alkali treated sample with the sludge seeding percentages varied within the range of 0 – 50. Mainly an increased trend was seen in COD and BOD removals with the increased sludge amounts with the highest values of 32 and 56% for nontreated (50% S NT), and 44, 62% for alkali treated samples (50% S A), respectively. In contrast, the same samples had the lowest SO_4 removal percentages along with the lowest H_2S and the highest CH_4 generations (Table 17). This was attributed to the bacterial competition between acetoclastic methanogens, which is the group of bacteria that produces around 70% of the CH_4 in anaerobic digestion, and sulphate reducing bacteria.

Table 18. Percentage removal of organic compounds in pot ale-sludge mixture after anaerobic digestion at 35 ˚C with different sludge percentages

Sample Name	*COD_0 (mg/L)	COD %	*BOD_0 (mg/L)	BOD %	*$(SO_4)_0$ (mg/L)	SO_4 %
0% S NT	16400	9 ± 4.3	13284	16 ± 3.9	854	72 ± 0.7
5% S NT	20452	10 ± 7.9	16361	21 ± 6.9	780	78 ± 1.8
10% S NT	18800	24 ± 2.3	14100	29 ± 2.2	1026	67 ± 1.7
20% S NT	18613	20 ± 6.3	14332	28 ± 5.7	767	73 ± 0.7
50% S NT	20523	32 ± 4.3	17445	44 ± 3.5	687	56 ± 1.4
0% S A	18307	10 ± 6.3	14645	21 ± 3.5	773	81 ± 0.8
5% S A	21420	23 ± 4.1	15851	28 ± 3.9	867	79 ± 0.6
10% S A	19667	16 ± 9.4	14553	21 ± 8.7	885	85 ± 0.3
20% S A	21773	15 ± 7.2	16112	20 ± 6.8	757	71 ± 1.5
50% S A	24083	56 ± 2.4	19267	62 ± 2.1	663	43 ± 5.0

*Initial values, average of triplicate runs

Only alkali pre-treated samples seeded with 50% sludge were not affected by the sharp pH drops regardless of the digestion temperature, thus produced biogas quality and quantity is significantly higher in comparison to the other samples (Table 18).

A novel hybrid pre-treatment, alkaline and beating, was introduced here to the body of knowledge. The order in which the pre-treatments were applied (alkaline first followed by beating) was found to be significant, based on the initial screening experimental results. Therefore, the combined pre-treatment with this order was applied in the further experimental designs.

4.3 Development of the Mathematical Models on Anaerobic Digestion of Pot Ale

The results of the solid analysis and the moisture content of each sample is given in Table 19.

Table 19. Total solids, volatile solids and moisture content of pot ale samples based on applied pre-treatment

Sample Name	TS (g/g sample)	VS (g/g sample)	Moisture%
A 0B	0.087 ± 0.001	0.070 ± 0.0009	91.336 ± 0.174
A 7.5B	0.087 ± 0.003	0.069 ± 0.0004	91.269 ± 0.290
A 15B	0.090 ± 0.002	0.072 ± 0.0006	91.000 ± 0.186

The Design of Experiments Box-Behnken design matrix, in a standard order, is given in Table 20 to present the experimental results. A variety of methane content was seen in the generated biogas. The maximum biogas quality and quantity were achieved with sample 12. Corresponding biogas production and CH_4 percentages were 550 ± 6 ml/g VS

and 54.3% CH_4 in respectively. Although all reactions were started at the neutral pH, mainly low final pH values were seen for all samples with a slight difference (higher) in the ones seeded with 50% sludge, however it was still lower than the optimum pH range for the methanogens (in the case of experiment numbers 7, 8, 11 and 12). These particular samples also had significantly higher CH_4 percentages than the rest.

Table 20. Design matrix for anaerobic digestion of alkali and beating pre-treated pot ale

Std	Run	Factor 1 A: BT min	Factor 2 B: T ˚C	Factor 3 C: Sludge %	Response 1 Biogas ml/g VS	Response 2 CH_4 %	Response 3 CO_2 %	Final pH
1	16	0	32	30	145 ± 9	3.4	42.1	5.24
2	5	15	32	30	177 ± 11	5.4	39	5.61
3	8	0	38	30	173 ± 7	3.8	33.8	5.47
4	11	15	38	30	214 ± 6	6.2	43.9	5.34
5	12	0	35	10	109 ± 5	1.5	16.7	5.41
6	9	15	35	10	134 ± 7	1.7	15.9	5.17
7	10	0	35	50	400 ± 19	46.8	22.4	6.12
8	4	15	35	50	510 ± 17	48.7	24.4	6.51
9	6	7.5	32	10	141 ± 3	1	35.4	5.42
10	2	7.5	38	10	124 ± 9	1.4	13.2	5.38
11	1	7.5	32	50	532 ± 12	43.6	26.7	6.45
12	13	7.5	38	50	550 ± 6	54.3	29.4	6.24
13	15	7.5	35	30	180 ± 20	3.1	41.2	5.71
14	14	7.5	35	30	223 ± 12	4.6	38.4	5.64
15	7	7.5	35	30	190 ± 9	2.5	37.5	5.24
16	17	7.5	35	30	211 ± 7	3.5	48.4	5.21
17	3	7.5	35	30	217 ± 8	2.9	41.9	5.43

The percentage removals of the organic compounds are given in Table 21 along with the initial concentration of COD, BOD and SO_4 which was organised based on sludge seeding percentage since it has the most powerful impact on all responses. The higher COD and BOD removals were achieved with the samples seeded with 50% sludge. Furthermore, relatively higher final pH values were seen with the same samples (number 7, 8, 11 and 12 in Table 20). This indicates that a higher sludge amount behaves like a buffer solution which delays the pH drops; thus, methanogens can survive longer to transform the organic matter into CH_4. The samples seeded with 10 and 30% sludge have lower COD and BOD removals in comparison with the samples seeded with 50% sludge (Table 21). Particularly sample number 4 and 10 showed the least COD and BOD removals. In terms of SO_4

removals, the opposite effect is observed. The highest SO_4 decomposition was seen with the samples which contain 30% sludge, it was followed with a slight difference by the ones seeded with 10%. However, SO_4 removal of 50% sludge seeded samples was only one third of the values obtained with 30% sludge. Due to bacterial competition, a sludge seeding ratio lower than 50% results in an unsuitable environment for methanogenic bacterial growth, and it triggers the activity of the sulphate reducing bacteria. Digestion under these conditions results in low CH_4 generation and high (> 10000 ppm) H_2S generation.

Table 21. Percentages removal of organic compounds in pot ale-sludge mixture after anaerobic digestion of pot ale

Exp no	COD_0 (mg/L)	COD (%)	BOD_0 (mg/L)	BOD (%)	$(SO_4)_0$ (mg/L)	SO_4 (%)
S% = 10						
5	13340	52 ± 8	6030	50 ± 9	285	56 ± 1
6	11780	48 ± 8	7860	43 ± 4	282	60 ± 5
9	19760	52 ± 7	11563	47 ± 9	276	65 ± 6
10	12760	16 ± 4	9214	22 ± 1	615	68 ± 5
S% = 30						
1	19360	43 ± 5	9489	44 ± 6	605	79 ± 3
2	18440	45 ± 4	10367	49 ± 4	556	79 ± 4
3	17020	39 ± 1	9014	37 ± 3	276	76 ± 4
4	11440	23 ± 3	7963	41 ± 5	615	81 ± 7
13-17	11940	28 ± 3	8145	60 ± 5	326	71 ± 2
S% = 50						
7	10160	58 ± 3	5905	55 ± 6	606	29 ± 2
8	10840	78 ± 5	5697	61 ± 4	628	25 ± 1
11	11020	64 ± 4	6982	49 ± 2	276	24 ± 1
12	12020	59 ± 2	9123	35 ± 4	615	36 ± 6

The VFA concentrations of the samples (in the form of pot ale and sludge mix) were analysed in terms of acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid and valeric acid prior to and after anaerobic digestion and the results are presented in Table 22 and Table 23 respectively.

Table 22. Volatile fatty acid concentration of the pot ale sludge mixture prior to AD

Std no	Sample Name	Acetic Acid	Propionic Acid	Isobutyric Acid	Butyric Acid	Isovaleric Acid	Valeric Acid	Total (mM)
1,3	30%S 0B	3.51	0	0	0	0	0	3.51
2,4	30%S 15B	1.62	0	0	0	0	0	1.62
5	10%S 0B	4.75	0.82	0	0	0	0	5.57
6	10%S 15B	8.76	0	0	0	0	0	8.76
7	50%S 0B	3.77	0	0	0	0	0	3.77
8	50%S 15B	7.64	0	0	0	0	0	7.64
9,10	10%S 7.5B	5.76	0	0	0	0	0	5.76
11,12	50%S 7.5B	6.01	0	0	0	0	0	6.01

A significant increase was seen in the final total volatile fatty acid concentration of all samples after AD. The samples which have 50% sludge (number 7,11 and 12 in Table 23) have lower total VFA concentrations. Moreover, those samples had a significantly higher biogas generation with a significantly higher methane content (Table 20), indicating that they are not affected by VFA inhibition as much as the ones containing a lower sludge amount. Sample number 12 in particular had the lowest final total VFA value and it has the highest biogas quality and the quantity (Table 20).

According to the level of total VFA concentrations after AD, samples with 30% sludge (number 1,2,3,4 and 13-17 in Table 23) are impaired by VFA inhibition more than the samples seeded with 10% sludge (number 5,6,9 and 10). This was attributed to the occurrence of potential bacterial competition due to the overloading of the substrate in the samples containing 10% sludge. As a result of imbalance in the reaction environment, either the activity of acidogenic bacteria was inhibited or the higher amount of VFAs were converted to H_2S.

Table 23. Volatile fatty acid concentration of pot ale sludge mixture after AD

Std no	Sample Name	Acetic Acid	Propionic Acid	Isobutyric Acid	Butyric Acid	Isovaleric Acid	Valeric Acid	Total (mM)
1	30%S 0B 32˚C	78.78	3.91	0	138.93	0	13.52	235.13
2	30%S 15B 32˚C	35.45	0.42	3.18	48.83	3.58	0	91.46
3	30%S 0B 38˚C	48.63	1.11	4.83	67.57	5.43	1.94	129.52
4	30%S 15B 38˚C	122.90	1.27	3.08	154.74	3.46	3.25	288.70
5	10%S 0B 35˚C	28.48	0	0.44	10.69	0.50	0	40.11
6	10%S 15B 35˚C	41.75	0	0	34.45	0	0	76.20
7	50%S 0B 35˚C	14.31	0	0	28.11	0	0	42.41
9	10%S 7.5B 32˚C	7.75	0	0	44.32	0	0	52.07
10	10%S 7.5B 38˚C	14.76	0	0	24.63	0	0	39.39
11	50%S 7.5B 32˚C	5.48	0	0	18.73	0	0	24.21
12	50%S 7.5B 38˚C	4.02	0	0	12.30	0	0	16.32
13-17	30%S 7.5B 35˚C	41.18	0.48	3.33	57.54	1.15	0	107.42

Modelling of the anaerobic digestion of pot ale with three factors and three responses was specified in accordance with the initial experimental results and the literature. The equipment and the procedures were explained in Section 3.3.3. The average of three responses in terms of biogas yield, CH_4% and CO_2% were recorded for 17 runs (Table 20). Samples which were seeded with low sludge percentages did not result in a high CH_4 generation, while significantly higher CH_4 production was achieved with the samples containing 50% sludge.

Design of Experiment Software, Version 10, was used to analyse the recorded responses. Experimental data was examined using a stepwise regression method, which excludes the insignificant model terms automatically, and the best fit was achieved by second order polynomials for identifying the relevant mathematical model terms. A sequential F-test and lack of fit test as well as adequacy measures were then implemented for obtaining the best models. The same statistical software was used to generate response plots. The statistical analysis of the suggested reduced quadratic models is given in ANOVA (Table 25 – 27) for each response along with R^2, adjusted R^2 and predicted R^2. All of the adequacy measures were close to 1 indicating a sufficient regression for the models. An adequate precision value greater than 4 indicates adequate model discrimination achieved here in all cases. The quadratic mathematical models for biogas production, CH_4 and CO_2 concentration which passed all statistical analysis steps are given in Eq 33, Eq 35, Eq 37

(coded factors) and Eq 34, Eq 36, Eq 38 (actual factors). The coding format of the variables is given in Table 24.

Table 24. Variable coded factors

	Coded Factors		
Variable	**-1**	**0**	**1**
A: Beating Time	0	7.5	15
B: Temperature	32	35	38
C: Sludge %	10	30	50

Coding parameters within the area of -1 to 1 reduces the influence of the magnitude of each parameter on the equations. It allows plotting of different design factors with different units in the same graph (perturbation graphs) to compare their individual effects on the response of interest. The coefficients of each factor in the equations in terms of coded factors were used to determine the influence strength of the parameters on the response of interest. On the other hand, prediction of experimental responses for validation of the models was carried out using the equations with the actual factors.

Analysis of variance (ANOVA) indicates (Table 25) that the percentage of sludge (C), the beating time (A) and their second orders (C^2, A^2) were the significant terms of the biogas generation model in a 95% confidence interval for AD of pot ale. According to the coefficients in the final equation in terms of coded factors (Eq 33), the impact of those terms on biogas generation is $C > C^2 > A^2 > A$.

Table 25. ANOVA table for biogas production quadratic model α=0.05

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	347790.58	4	86947.64	125.38	< 0.0001	significant
A-Beating Time, min	5408	1	5408	7.80	0.0162	
C-Sludge,%	275282	1	275282	396.95	< 0.0001	
A^2	5829.66	1	5829.66	8.41	0.0133	
C^2	63196.49	1	63196.49	91.13	< 0.0001	
Residual	8321.90	12	693.49			
Lack of Fit	6971.09	8	871.39	2.58	0.1879	not significant
Pure Error	1350.8	4	337.7			
Cor Total	356112.47	16				

R^2: 0.9766, Adj R^2: 0. 9688, Pred R^2: 0. 9435, Adeq Precision: 30.40

The final equation in terms of coded factors is given in Eq 33

$$\textbf{Biogas}\ (ml/gVS) = 208.74 + 26A + 185.5C - 37.16A^2 + 122.34C^2 \quad \text{Eq 33}$$

The final equation in terms of actual factors is given in Eq 34:

$$\textbf{Biogas}\ (\text{ml/gVS}) = 142.60 + 13.38\ BT - 9.08\ S\% - 0.6(BT)^2 + 0.31(S\%)^2 \quad \text{Eq 34}$$

ANOVA results for the model for CH_4 concentration are given in Table 26. The significant design parameters of this model were temperature (B), the percentage of sludge (C), the second order effect of percentage of sludge (C^2) as well as interaction between temperature and sludge percentage (BC), within a 95% confidence interval. The relative strength of influence of these parameters on the CH_4 concentration can be identified as C > C^2 > BC > B based upon the coefficients of the final equation in terms of coded factors (Eq 35).

Table 26. ANOVA table for CH4% quadratic model α=0.05

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	6311.44	4	1577.86	692.14	< 0.0001	significant
B-Temperature	18.91	1	18.91	8.30	0.0138	
C-Sludge,%	4408.61	1	4408.61	1933.86	< 0.0001	
BC	26.52	1	26.52	11.63	0.0052	
C^2	1857.40	1	1857.40	814.76	< 0.0001	
Residual	27.36	12	2.28			
Lack of Fit	24.79	8	3.10	4.83	0.0727	not significant
Pure Error	2.57	4	0.64			
Cor Total	6338.80	16				

R^2: 0.9956, Adj R^2: 0.9942, Pred R^2: 0.9869, Adeq Precision: 63.62

The final equation in terms of the coded factors is given in Eq 35:

$$\textbf{CH}_4\ (\%) = 3.93 + 1.54B + 23.48C + 2.57BC + 20.94 \times C^2 \quad \text{Eq 35}$$

The final equation in terms of the actual factors is given in Eq 36:

$$\textbf{CH}_4\ (\%) = 42.96 - 0.78T - 3.47S\% + 0.043\ T\ S\% + 0.05(S\%)^2 \quad \text{Eq 36}$$

For CO_2% analysis, ANOVA results are given in Table 27. Within a 95% confidence interval, the significant model terms were the second order effect of sludge% (C^2) and the

interaction between temperature and sludge percentage (BC). The first order effects of temperature (B) and sludge% (C) were added to the model by the software during stepwise regression to support model hierarchy. The strength of influence of the model terms on CO_2 content of the generated biogas can be defined based on the coefficient of the final equation in terms of coded factors (Eq 37), which was $C^2 > BC > B > C$.

Table 27. ANOVA table for CO2% quadratic model α=0.05

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	1602.75	4	400.69	18.73	< 0.0001	significant
B-Temperature	65.55	1	65.55	3.06	0.1055	
C-Sludge,%	58.86	1	58.86	2.75	0.123	
BC	155.00	1	155.00	7.25	0.0196	
C^2	1323.34	1	1323.34	61.87	< 0.0001	
Residual	256.66	12	21.39			
Lack of Fit	183.19	8	22.90	1.25	0.4433	not significant
Pure Error	73.47	4	18.37			
Cor Total	1859.42	16				

R^2: 0.8620, Adj R^2: 0.8160, Pred R^2: 0.6091, Adeq Precision: 12.89

Final Equation in terms of coded factors:

$$\mathbf{CO_2}\ (\%) = \mathbf{40.69 - 2.86\ B + 2.71\ C + 6.22\ BC - 17.68\ C^2} \quad \text{Eq 37}$$

Final Equation in terms of actual factors:

$$\mathbf{CO_2}\ (\%) = \mathbf{139.18 - 4.07\ T - 0.84\ S\% + 0.10\ TS\% - 0.044\ (S\%)^2} \quad \text{Eq 38}$$

4.3.1 Validation of the Developed Models

The diagnosis of the estimated models was performed by DOE software as part of the post statistical analysis. The diagnostic plots, the normal plot of residuals and the predicted vs actual plot for AD of pot ale, are given Figure 24 and Figure 25, respectively. In both graphs design points were coloured according to the value of biogas generation with the unit of mL/g VS. The normal distribution of residual data in Figure 24 proves that ANOVA can be applied to the dataset. Figure 25 gives the comparison between the actual data set and the predicted data set by developed model. It is seen that the residuals were minimal since all the design points tended to be close to the y=x line. The developed reduced

model was therefore adequate. The similar trend in both normal plot of residuals and actual vs predicted plot is seen for the built models on CH_4 and CO_2 percentages for AD alkaline and beating pre-treated pot ale as well as the models developed for AD of alkaline and beating pre-treated pot ale and pot ale draff mixture.

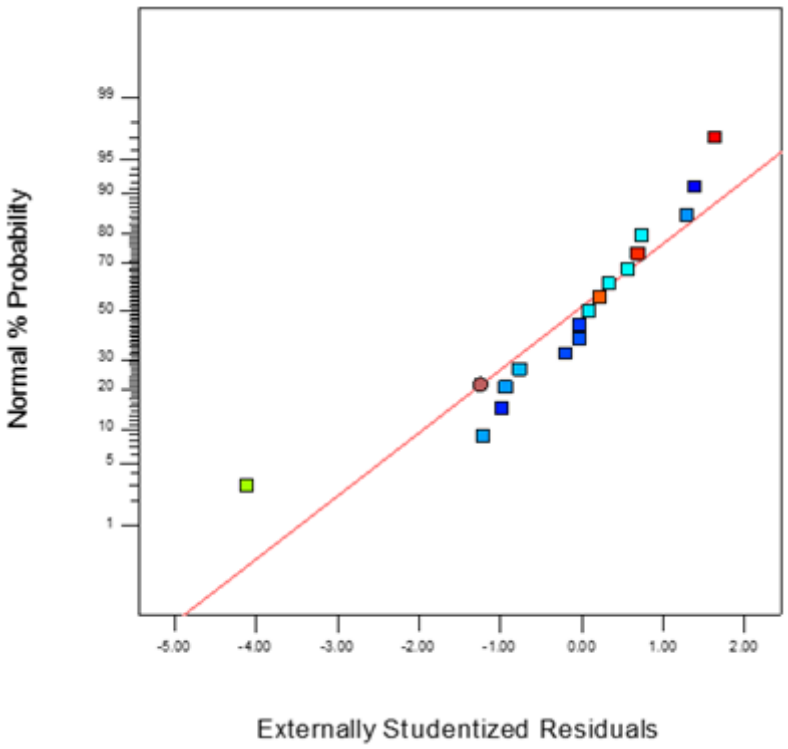


Figure 24. Normal plot of residuals on biogas generation (ml/g VS) for AD of pot ale

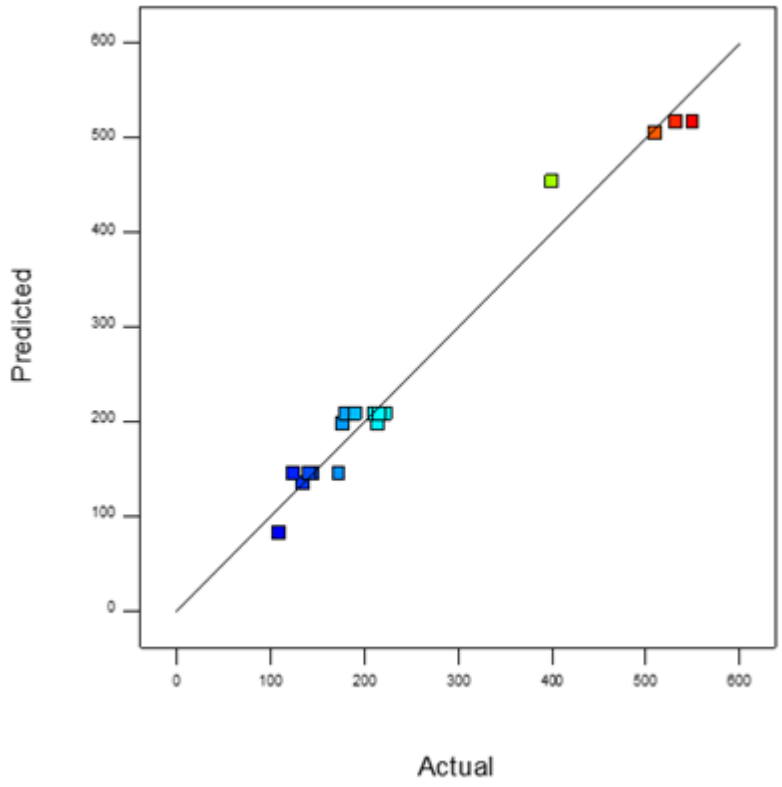


Figure 25. Scatter diagram biogas generation (ml/g VS) for AD of pot ale, design points coloured based on biogas yield

In addition to post analysis conducted by the software, the models were challenged using independent AD of pot ale experiments for a further validation step. Three validation points (which were not used for model development) were decided upon and the results of the experiments were compared with the values for each response of interest (biogas yield, CH_4% and CO_2%) predicted by the models given in Equations 34, 36 and 38. The differences between the predicted and the experimental results were statistically analysed by t-test (2 tailed distribution with unequal variance). The modelling of the biogas quality and the quantity was validated within a 95% confidence interval according to the *p* values (Table 28).

Table 28. Validation experiment results versus predicted results for AD of pot ale

Validation Points	**Biogas Yield (ml/g VS)**		**CH_4%**		**CO_2%**	
	Experimental	Predicted	Experimental	Predicted	Experimental	Predicted
0B 50%S 38˚C	464 ± 26	532	54.2 ± 9	46.5	33.1 ± 10	22.5
	p: 0.672		*p: 1.000*		*p: 0.226*	
0B 10%S 38˚C	91 ± 11	83	1.2 ± 0.2	0.4	33.3 ± 10.4	29.7
	p: 0.327		*p:0.084*		*p: 0.059*	
0B 30%S 35˚C	167 ± 14	149	9.0 ± 2.4	4.71	50.8 ± 6.9	36.9
	p: 0.155		*p: 0.089*		*p: 0.075*	

4.3.2 Model Graphs

The perturbation plots give the effect of all factors, which have different units, in the same graph since it is plotted in terms of coded factors. The lines show the individual behaviour of each factor while keeping the other at a constant ratio (their centre points by default), therefore these types of graphs do not show the effect of any possible interaction. Where more than one factor is displayed on a plot, it can be used to identify which factor affects the response of interest the most. Figure 26 (1, 2 and 3) presents the perturbation plots for biogas generation, CH_4 and CO_2 percentages respectively. The effect of sludge percentage (C) was a significant parameter for all responses and it was the strongest factor for all the responses. However, the beating time (A) was a significant parameter for biogas generation whereas temperature (B) was seen in CH_4 and CO_2% with a linear trend but reversed effect.

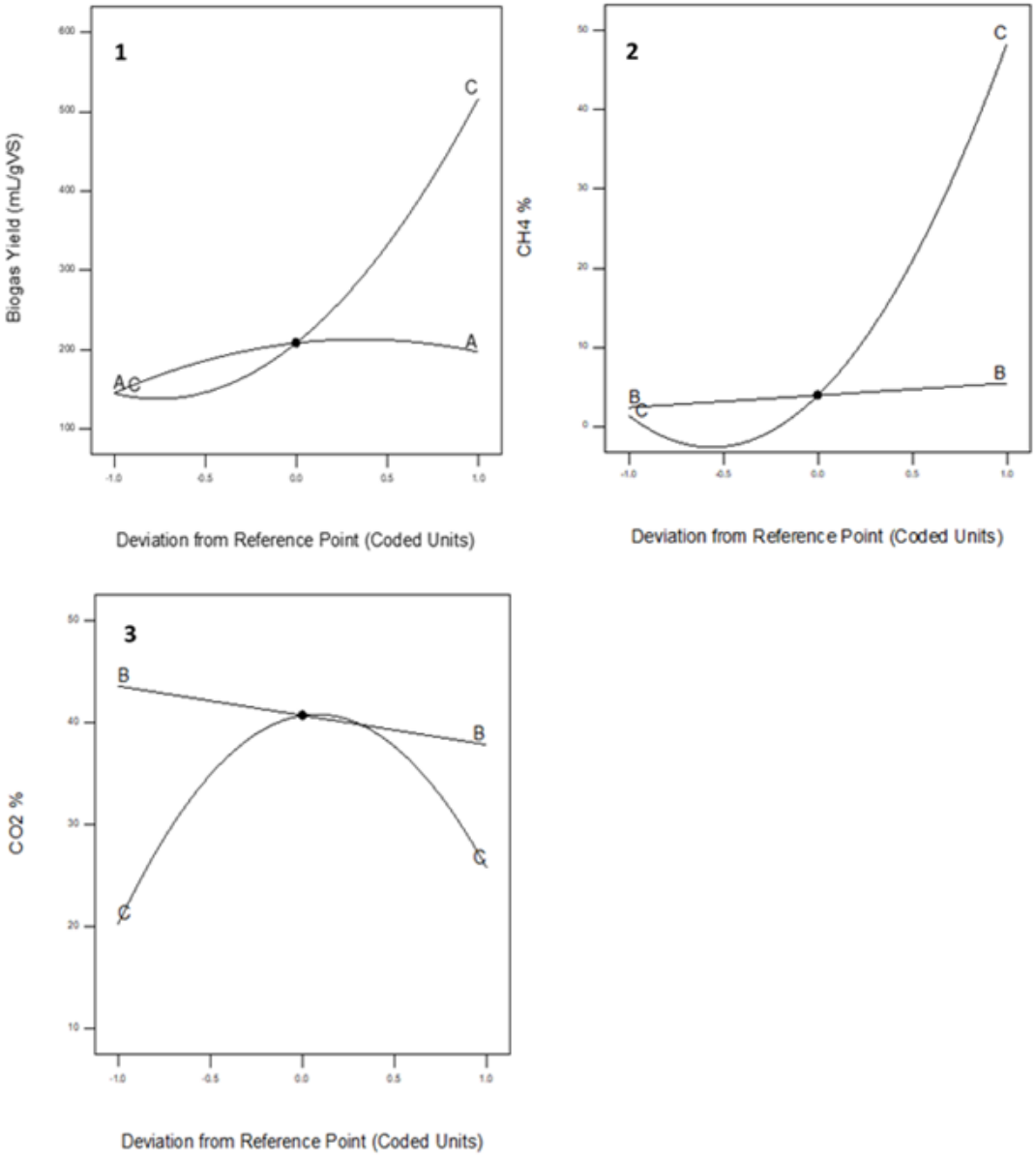


Figure 26. Perturbation graphs of the developed models on 1. Biogas generation, 2. CH_4%, 3. CO_2%

There was no interaction between the factors in biogas generation model. The interaction between the sludge percentages and the digestion temperatures for CH_4 and CO_2 percentages are given in Figure 27 (1 and 2).

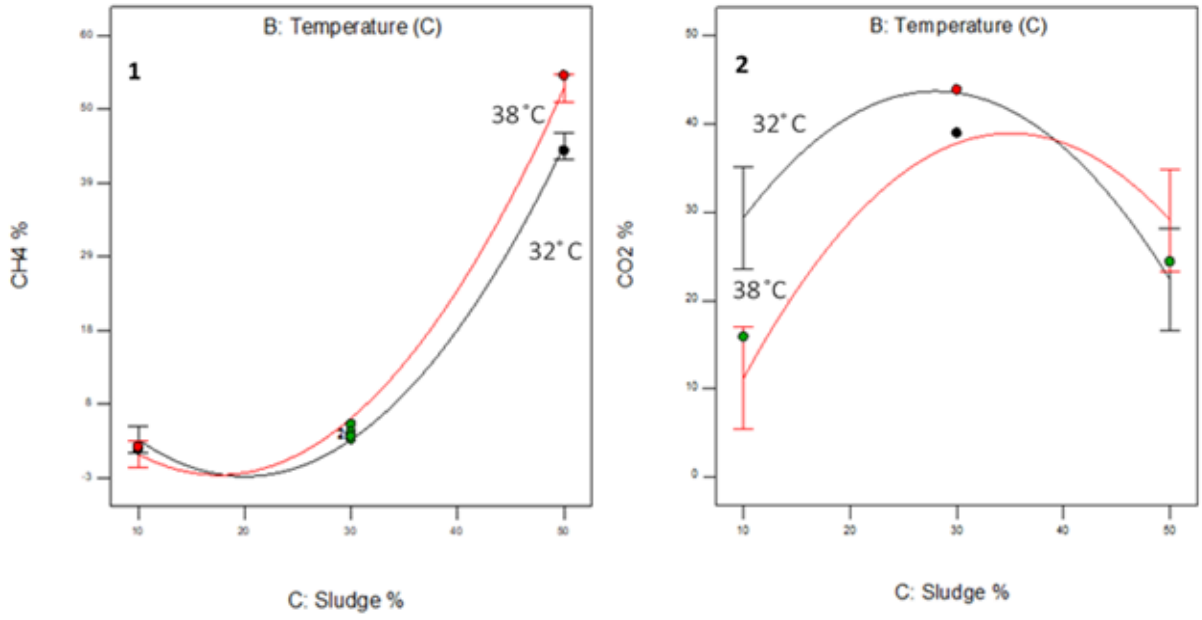


Figure 27. Interaction graphs between the digestion temperatures and sludge percentages for 1. CH_4 2. CO_2 percentages

According to Figure 27 (1), the digestion temperature had no significant impact on the CH_4 content at low sludge seeding ratios (<20%). However, when the sludge percentage increased, an increase in the digestion temperature to 38 ˚C led to a significant improvement in the biogas quality. Digestion temperatures did not have a significant effect on CO_2% at high sludge seeding ratios (35 – 50%), whereas at lower sludge seeding ratios digestion at 38 ˚C results in a significantly lower CO_2 % than digestion at 32 ˚C (Figure 27). With regards to the interaction graphs, the desired AD conditions are seeding with the higher sludge percentages at 38 ˚C digestion temperature. Figure 28 (1, 2 and 3) show the contour graphs for biogas yield, CH_4 and CO_2 percentages respectively. Mainly straight lines were seen in the biogas production contour. The influences of sludge percentage and temperature on the CH_4 content of the produced biogas (Figure 28 (2)) showed only a linear trend, with the highest CH_4 content achieved with high sludge percentage and digestion temperature (the area marked red on the contour). The effects of the sludge percentage and temperature on the CO_2 content (Figure 28 (3)) had the

highest value in the area centred around 30% sludge with a slight effect of temperature. However, lower CO_2 percentages were seen with the experiments performed with high temperature and low sludge as well as high sludge and low temperature conditions. Only higher sludge seeding ratio and digestion temperatures were considered desired conditions for generated biogas quality, because it provides higher CH_4 and lower CO_2 content at the same time (Figure 28 (2, 3)).

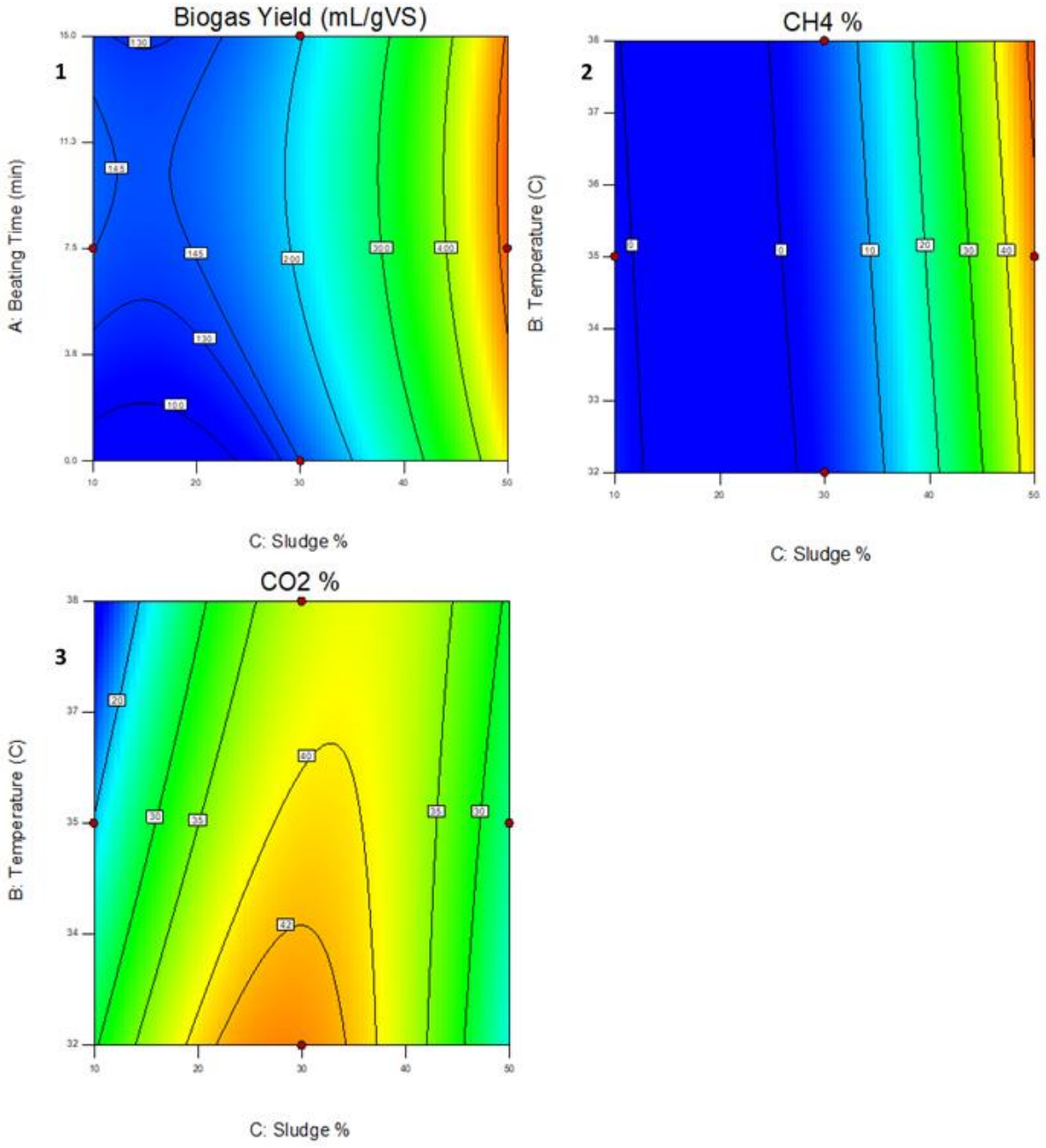


Figure 28. Contour graphs for 1. biogas yield, 2. CH_4% and 3. CO_2%

4.3.3 Optimisation

An optimisation tool is offered by DOE software in order to predict the best levels of each factor to maximise the biogas production and the CH_4 content while minimising the CO_2 content. The desirability function outlined in Section 3.3.4.2 was applied to the RSM dataset by implementing numerical optimisation and graphical optimisations. In the numerical optimisation, design factors were kept in range, however biogas yield and CH_4% were maximised with the importance of 5 whereas CO_2% was minimised with the same importance level. The optimisation conditions are outlined in Table 29. Suggested solutions are given in Table 30.

Table 29. Optimisation conditions for AD pot ale

Variable	Goal	Lower Limit	Upper Limit	Importance
A: Beating Time (min)	is in range	0	15	3
B: Temperature (˚C)	is in range	32	38	3
C: Sludge (%)	is in range	10	50	3
Biogas Yield (ml/g VS)	maximise	109	550	5
CH_4%	maximise	1	54.3	5
CO_2%	minimise	13.2	48.4	5

Table 30. Numerical optimisation solutions for AD of pot ale

No	A:Beating Time (min)	B:Temperature (˚C)	C:Sludge (%)	Biogas Yield (ml/g VS)	CH_4 (%)	CO_2 (%)	Desirability N/A
1	10.14	32.00	50.00	521.13	44.24	22.36	0.82
2	7.65	32.00	50.00	517.08	44.24	22.36	0.81
3	9.96	35.01	50.00	521.11	48.37	25.74	0.80
4	11.76	34.94	50.00	519.37	48.27	25.66	0.80
5	7.81	36.15	50.00	517.59	49.92	27.01	0.79
6	8.92	36.40	50.00	520.17	50.27	27.29	0.79
7	8.81	37.44	50.00	519.99	51.69	28.46	0.79
8	6.96	37.32	50.00	514.49	51.52	28.32	0.78
9	0.63	32.00	50.00	461.53	44.24	22.36	0.78
10	10.06	32.00	10.00	150.12	2.44	29.37	0.11

The graphical optimisation result is given in Figure 29. The target area coloured with yellow was delimited by constraints set according to the numerical optimisation solutions (Table 30). All solutions with a high desirability (>0.78) suggested that sludge percentage be set at 50. Lower and upper limits of the optimum area is 520 and 521 ml/g VS for biogas

generation with 22 and 24% for CO_2 content. In case of CH_4% only the lower limit (48.3%) is visible on the chart because the lower limit does not overlap with the other criterions.

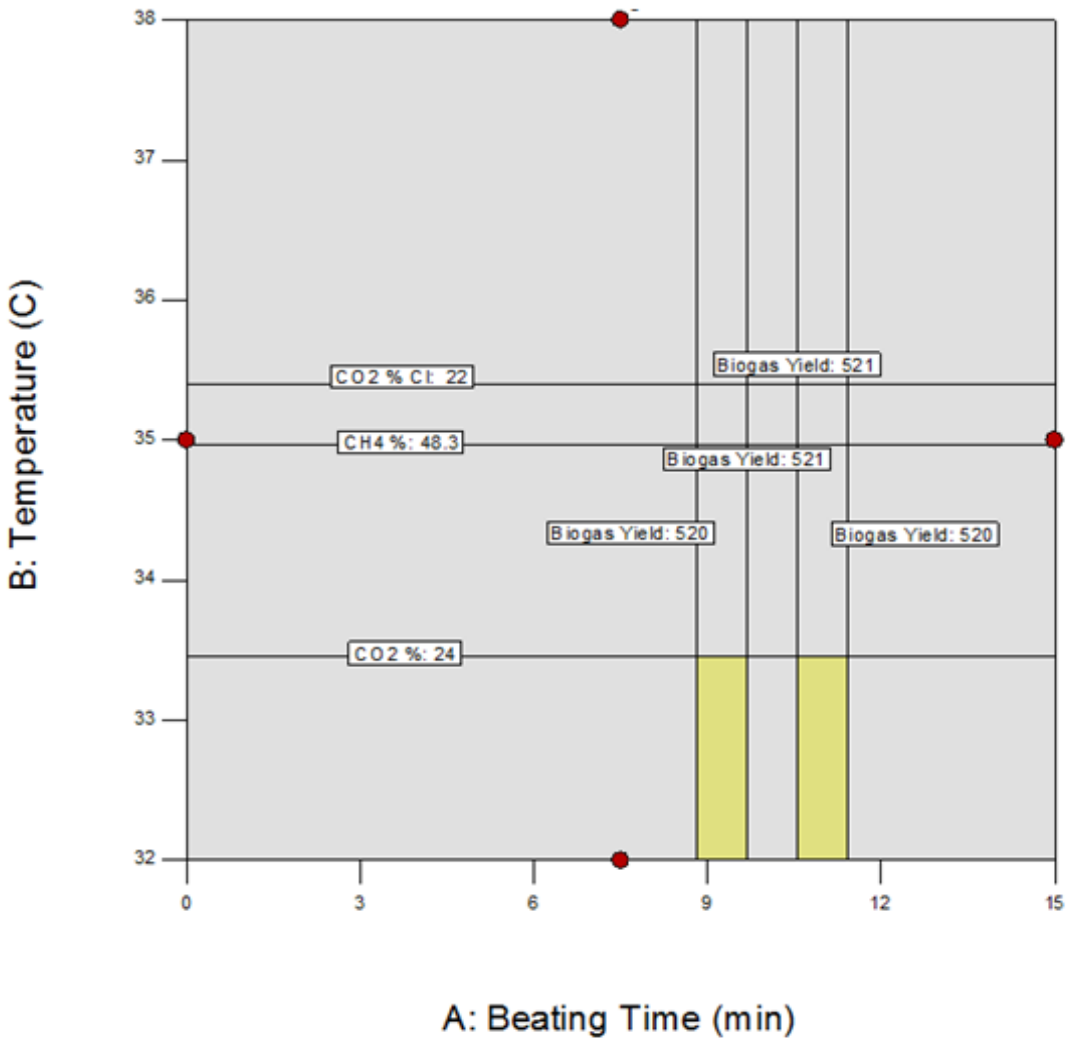


Figure 29. Graphical optimisation of AD of pot ale at sludge ratio of 50%.

Lignocellulose fractionation of pot ale before and after application of alkaline pre-treatment were introduced to the literature. The sludge ratio was found to be most powerful design parameter on both biogas quality and quantity according to the data analysis by DOE. The highest biogas generation was found to be 550±6 ml/g VS with a CH_4 content of 54.3% with AD at 38˚C with a 50% sludge seeding ratio after pre-treatment with 7.5 min beating after 1M NaOH pre-treatment. The significant model parameters for biogas yield were found to be the beating time and sludge percentage, whereas the

significant design parameters for CH_4 content was found to be the temperature and sludge seeding ratio. Digestion at higher temperature (38˚C) led to a significantly higher CH_4 and CO_2 yield at high sludge seeding ratios (40 – 50% on wet basis). Therefore, the optimum conditions were identified as 50% sludge seeding ratio, 33˚C digestion time and 8 – 11 min beating time.

4.4 Effects of Increased Draff Content on Anaerobic Digestion.

The total solids, volatile solids, moisture content of spent grain and pot ale mixed in ratios of 1:1, 1:3, 1:5 by wet weight are given in Table 31.

Table 31. TS, VS, and Moisture % of the non and pre-treated pot ale and draff mix

Sample Names	TS g/g sample	VS g/g sample	Moisture %
1:1 NT	0.181 ± 0.018	0.161 ± 0.004	81.86 ± 1.8
1:1 A 0B	0.183 ± 0.004	0.163 ± 0.007	81.70 ± 0.4
1:1 A 7.5B	0.195 ± 0.19	0.178 ± 0.001	80.53 ± 1.9
1:1 A 15B	0.197 ± 0.012	0.174 ± 0.002	80.34 ± 1.2
1:3 NT	0.143 ± 0.003	0.134 ± 0.011	85.70 ± 0.3
1:3 A 0B	0.147 ± 0.010	0.141 ± 0.002	85.35 ± 1.0
1:3 A 7.5B	0.153 ± 0.002	0.145 ± 0.005	84.67 ± 0.2
1:3 A 15B	0.137 ± 0.015	0.115 ± 0.008	86.26 ± 1.5
1:5 NT	0.112 ± 0.007	0.114 ± 0.009	88.77 ± 0.7
1:5 A 0B	0.121 ± 0.004	0.100 ± 0.007	87.85 ± 0.4
1:5 A 7.5B	0.115 ± 0.007	0.089 ± 0.008	88.55 ± 0.7
1:5 A 15B	0.117 ± 0.014	0.110 ± 0.005	88.27 ± 1.4

The impact of alkaline 1M NaOH pre-treatment on the lignocellulosic structure of draff (spent barley) is given in Figure 30. Alkaline pre-treatment achieved a significant increase in hemicellulose fraction (16%) and a significant decrease (10%) in lignin fraction with *p* values of 0.00032 and 0.000032 respectively. The cellulose content of the structures was not affected significantly by the pre-treatment. Effects of beating pre-treatment on the lignocellulosic structure could not be assessed because of the grinding requirement (particle size between 0.5 mm and 1 mm) of the method.

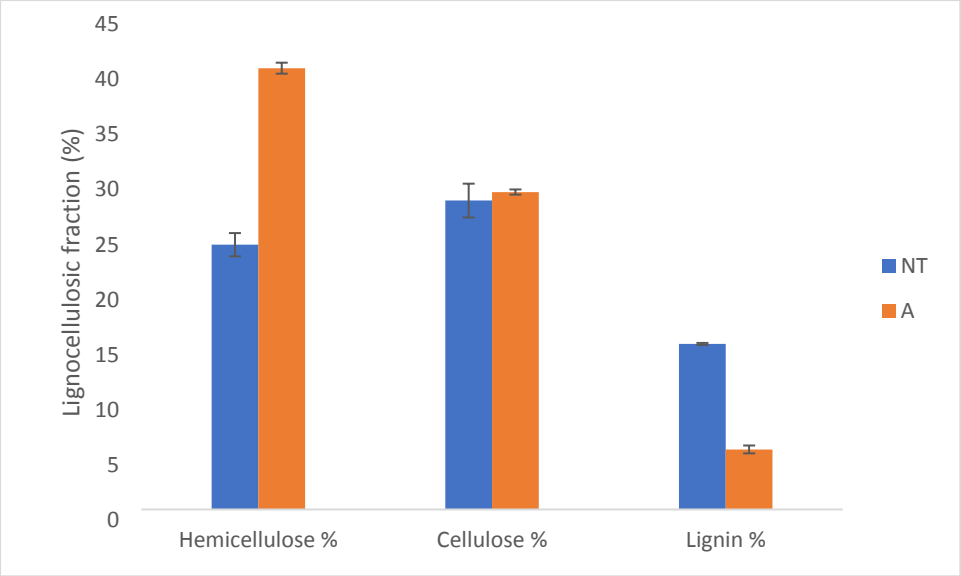


Figure 30. Lignocellulosic structure of draff before and after alkaline pre-treatment

The biogas quantity and quality for AD of pot ale-draff mixture is given in Figure 31 and Table 32, respectively. Combined alkaline and beating pre-treatments enhanced biogas quantity significantly over the control for all mixing ratios (Figure 31). However, biogas generation with 1:1 and 1:3 draff to pot ale mixing ratios was found to be significantly lower than the mixing ratio of 1:5, regardless of the application of pre-treatments. Application of 7.5 minutes beating pre-treatment after alkaline pre-treatment had the highest biogas generation and CH_4 percentage with the values of 469 ± 7 ml/g VS and 53.3% respectively in a mixing ratio of 1:5. The pre-treatment conditions for this sample had a significant impact on biogas generation in comparison with alkaline pre-treatment alone, and followed by 15 minutes beating, with *p* values of 0.0033 and 0.00016. On the other hand, single digestion of pre-treated pot ale had higher biogas yields with the *p* values of 0.0291 and 0.0169 for alkaline pre-treatment in combination with 7.5 and 15 minutes beating pre-treatments respectively.

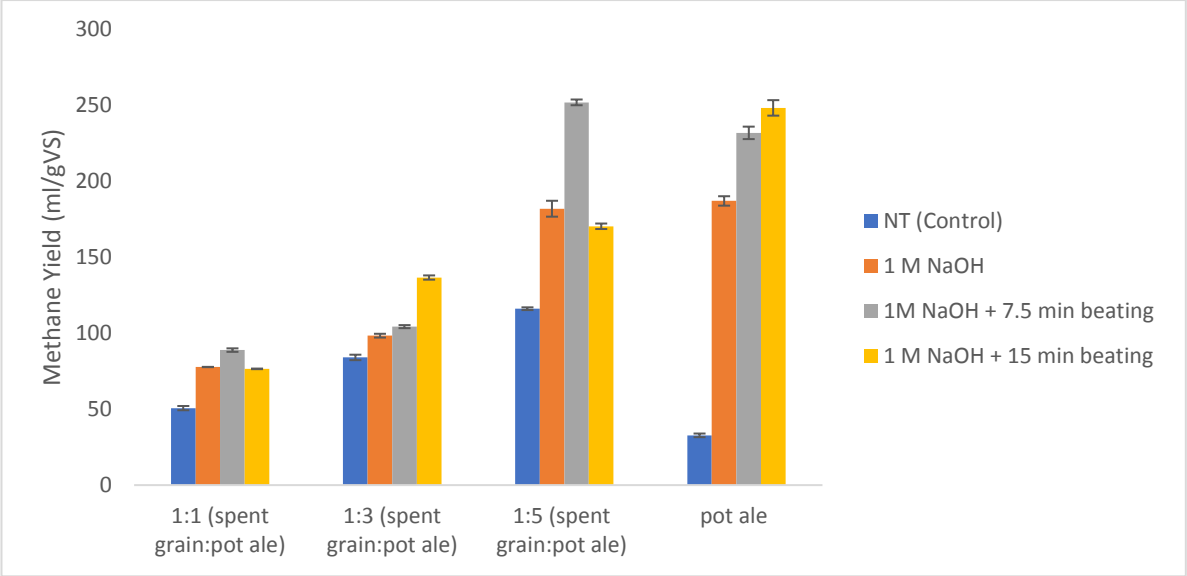


Figure 31. Cumulative biogas generation of draff and pot ale mixtures after alkaline and beating pre-treatments with different mixing ratios

Table 32. The components of generated biogas by anaerobic digestion of pot ale and draff mixtures

Sample Name	CH_4 (%)	CO_2 (%)	BAL (%)	*H_2S (ppm)	*Initial pH -	*Final pH -
1:1 Control	42.3 ± 9	44.0 ± 8	9.6	1572	7.20	5.33
1:1 A0B	50.0 ± 3	39.1 ± 2	9.3	1273	7.15	5.47
1:1 A7.5B	50.5 ± 5	34.4 ± 7	13.3	632	7.12	5.57
1:1 A15B	45.3 ± 2	40.1 ± 2	10.6	896	7.21	5.48
1:3 Control	50.5 ± 3	39.9 ± 3	8.4	1628	7.18	5.37
1:3 A0B	52.4 ± 6	42.9 ± 4	9.3	1354	7.09	5.54
1:3 A7.5B	48.5 ± 3	41.2 ± 3	10.6	1692	7.08	5.34
1:3 A15B	48.4 ± 3	40.7 ± 1	9.0	1120	7.12	5.78
1:5 Control	49.1 ± 2	34.0 ± 1	7.9	800	7.19	5.44
1:5 A0B	51.4 ± 3	34.5 ± 4	12.1	834	7.11	5.67
1:5 A7.5B	53.3 ± 1	35.2 ± 6	17.3	913	7.14	5.78
1:5 A15B	50.9 ± 3	37.9 ± 1	9.4	757	7.18	5.75

* Average of triplicate results.

The biogas quality (Table 32) was improved over the control after applying combined alkaline and beating pre-treatments for each mixing ratio except for 1:3, however, this small decrease in the biogas quality was not found to be statistically significant (p<0.05). The concentration of H_2S is within the range of measurement and it shows a higher trend with the 1:3 mixing ratio sample group. Correspondingly, the sulphate removal percentages had the greatest values for these samples (Table 33). In contrast, the COD

and BOD removal percentages had a positive linear correlation with increased amount of draff within the reactor.

Table 33. Percentage removal of organic compounds in pot ale-sludge mixture after anaerobic digestion of pot ale and draff mix, 1:1, 1:3 and 1:5 by weight

Sample Name	COD_0 (mg/L)	COD %	BOD_0 (mg/L)	BOD %	$(SO_4)_0$ (mg/L)	SO_4 %
1:1 Control	22215	31 ± 5	9495	22 ± 1	780	33 ± 4
1:1 AOB	21135	38 ± 3	8325	21 ± 1	924	18 ± 1
1:1 A7.5B	18600	35 ± 1	8400	33 ± 3	728	12 ± 4
1:1 A15B	21435	34 ± 4	9620	30 ± 2	632	35 ± 1
1:3 Control	23310	25 ± 1	11610	46 ± 6	623	47 ± 2
1:3 AOB	23760	34 ± 3	13755	43 ± 4	609	35 ± 5
1:3 A7.5B	22935	43 ± 3	11295	44 ± 2	621	39 ± 3
1:3 A15B	22560	45 ± 6	14160	42 ± 3	643	37 ± 1
1:5 Control	23160	41 ± 4	12540	47 ± 3	576	32 ± 1
1:5 AOB	16935	59 ± 3	12840	48 ± 4	642	30 ± 3
1:5 A7.5B	23580	60 ± 5	16870	51 ± 2	638	26 ± 2
1:5 A15B	20100	56 ± 6	14874	55 ± 1	642	32 ± 5

The assessment of anaerobic co-digestion of pot ale and spent grain with and without pre-treatment was a gap that had been identified in the literature. The highest methane yield was achieved as 252±1.9 ml/g VS with a mixing ratio of 1:5 (spent grain : pot ale based on wet weight) with a 50% sludge seeding ratio. In addition, significantly higher COD, BOD and lower SO_4 were seen with the mixing ratio of 1:5 indicating a greater methanogenesis activity than with mixing ratios of 1:1 and 1:3. Therefore 1:5 mixing ratio was selected for the DOE experiments to provide a comparison with the single digestion potential of pot ale.

4.5 Development of the Mathematical Models for Anaerobic Digestion of Pot Ale & Draff Mixture (5:1)

The analysis of total solids, volatile solids and the moisture content of pot ale draff mix after applied pre-treatment, with a mixing ratio of 5:1 by wet weight, is given in Table 34.

Table 34. TS, VS and Moisture% for draff and pot ale mix, 1:5 ratio by weight

Sample Name	TS (g/g sample)	VS (g/g sample)	Moisture %
A 0B	0.120 ± 0.007	0.115 ± 0.004	88.0 ± 0.7
A 7.5B	0.124 ± 0.010	0.112 ± 0.011	87.6 ± 1.0
A 15B	0.134 ± 0.011	0.111 ± 0.009	86.6 ± 1.1

The same design was followed in order to evaluate the impact of increased solid content after adding draff into pot ale (1:5, by wet weight) on anaerobic digestion as well as to ascertain the influence of the operating parameters on AD of the mixed substrate. The design matrix with the corresponding responses is given in Table 35. A similar range of CH_4 generation was seen the previous design for anaerobic digestion of pot ale. The greatest biogas quality and quantity are obtained from the case of sample number 12 with 55 ± 0.4% CH_4 in 360 ± 10 ml/g VS biogas. It should be noted that the final pH value for this particular sample is maintained within the neutral area whereas it dropped to approximately 5.5 for the other samples.

Table 35. Design matrix for anaerobic co-digestion of pot ale and draff (5:1, by weight)

Std	Run	Factor 1 A: BT min	Factor 2 B: T ˚C	Factor 3 C: Sludge %	Response 1 Biogas mL/g VS	Response 2 CH_4 %	Response 3 CO_2 %	H_2S* ppm	Final *pH
1	16	0	32	30	49 ± 2	3.9 ± 1.1	56.8 ± 1.8	>>>	5.5
2	5	15	32	30	55 ± 8	10.0 ± 0.8	55.2 ± 0.8	>>>	5.4
3	8	0	38	30	67 ± 13	8.5 ± 0.7	55.9 ± 0.7	>>>	5.7
4	11	15	38	30	84 ± 11	16.1 ± 0.2	56.2 ± 1.5	>>>	5.7
5	12	0	35	10	42 ± 2	2.2 ± 0.3	50.7 ± 0.2	>>>	5.4
6	9	15	35	10	50 ± 5	10.8 ± 0.2	55.1 ± 0.8	>>>	5.6
7	10	0	35	50	207 ± 19	28.0 ± 2.1	39.2 ± 0.8	1782	5.6
8	4	15	35	50	279 ± 11	48.5 ± 2.3	35.0 ± 1.7	782	5.5
9	6	7.5	32	10	57 ± 3	2.2 ± 0.5	52.8 ± 1.6	>>>	5.4
10	2	7.5	38	10	48 ± 7	4.9 ± 1.4	52.8 ± 1.6	>>>	5.3
11	1	7.5	32	50	258 ± 8	29.3 ± 2.1	48.2 ± 8.3	1472	5.6
12	13	7.5	38	50	360 ± 10	55.0 ± 0.4	36.0 ± 0.3	1375	7.1
13	15	7.5	35	30	63 ± 11	14.0 ± 1.4	51.8 ± 2.7	>>>	5.5
14	14	7.5	35	30	69 ± 9	13.0 ± 0.9	5.70 ± 1.8	>>>	5.5
15	7	7.5	35	30	76 ± 8	13.8 ± 0.7	57.5 ± 2.1	>>>	5.4
16	17	7.5	35	30	46 ± 4	15.0 ± 0.5	59.1 ± 3.4	>>>	5.3
17	3	7.5	35	30	61 ± 7	14.5 ± 0.7	53.6 ± 3.7	>>>	5.3

* Average of triplicate runs

Pre-treatment, however, had a significant impact on biogas quality for the same samples i.e. in the experiment numbers 1, 2 and 5, 6 in (Table 36), CH_4% increased 2 and 5-fold over due to the effect of 15 minutes beating pre-treatment, respectively. The extended active surface area on solid material in the reactors stimulated microbial activity especially in early stage of AD which resulted in higher CH_4 generation. However, the availability of the large amounts of fresh feedstock caused imbalanced reaction rates, with sequentially sharp pH drops occurring that does not allow the bacteria, in particular the acetoclastic methanogens, to survive to carry out the reactions.

All samples were characterised in terms of COD, BOD and SO_4 before and after anaerobic digestion; the percentage removal is given in Table 36 along with the initial values. The experiments performed with 10% and 30% sludge resulted in very low COD and BOD removal percentage levels regardless of the application of the pre-treatments; correspondingly low levels of biogas yields were seen in those samples. The data gathered on the percentage of sulphate removal, which is significantly higher for the samples

seeded with 10 and 30% sludge, support the statement made above because of the over-activation of sulphate reducing bacteria. Moreover, the concentration of the H_2S were higher than the limit of the measurement (>10000ppm) for the same samples.

Table 36. Percentage removal of organic compounds in pot ale-sludge mixture after anaerobic digestion of pot ale and draff (5:1, by weight)

Exp no	*COD_0 (mg/L)	COD (%)	*BOD_0 (mg/L)	BOD (%)	*$(SO_4)_0$ (mg/L)	SO_4 (%)
S% = 10						
5	28450	12 ± 2	21622	14 ± 1	614	95 ± 2
6	29060	23 ± 3	23539	17 ± 1	603	74 ± 4
9	23610	11 ± 1	14880	22 ± 3	475	87 ± 1
10	19180	9 ± 1	14880	16 ± 4	381	69 ± 7
S% = 30						
1	28070	15 ± 3	16170	25 ± 2	629	71 ± 1
2	24160	23 ± 1	14870	14 ± 1	407	88 ± 1
3	28070	29 ± 2	16170	29 ± 6	629	40 ± 2
4	24160	37 ± 2	14870	32 ± 4	407	56 ± 6
13-17	19920	39 ± 5	16135	33 ± 7	441	49 ±7
S% = 50						
7	23890	58 ± 7	18156	40 ± 3	499	32 ± 5
8	19510	66 ± 3	14633	46 ± 2	307	23 ± 3
11	19180	49 ± 4	10320	48 ± 5	381	38 ± 1
12	23610	73 ± 6	10320	63 ± 7	475	31 ± 7

* Average of triplicate results.

The highest COD and BOD removals were achieved with the samples seeded with 50% sludge along with the lowest SO_4 (Table 36) in comparison to the lower sludge seeding ratios. Application of 15 minutes beating pre-treatment has a significant impact on both biogas quantity and quality as seen from comparison of experiments 7&8 (Table 35) with a rise from 207 ± 19 to 279 ± 11 ml/g VS in biogas quantity and a rise from 28 to 48.5 % CH_4. Correspondingly greater COD/BOD removals are shown in Table 36, with less H_2S production due to the 9% less SO_4 reduction (Table 35).

Different digestion temperatures were seen to significantly influence biogas production and CH_4 content (experiment numbers 11 & 12, Table 35). The digestion at 38 ˚C rather than 35 ˚C increased the biogas yield from 258 ± 8 to 360 ± 10 ml/g VS and increased the CH_4 % from 29.3 ± 2.1 to 55.0 ± 0.4 because of higher COD/BOD removals (Table 36). Lower H_2S production was achieved with experiment number 12 along with lower SO_4 removal percentages (Table 36).

The results of the VFA analysis of the sample before and after anaerobic digestion is given in Table 37 and Table 38 respectively.

Table 37. VFA concentration of pot, draff and sludge mix before AD of pot ale draff mix

Std no	Sample Name	Acetic Acid	Propionic Acid	Isobutyric Acid	Butyric Acid	Isovaleric Acid	Valeric Acid	Total
1,3	30%S 0B	1.20	0	0	0	0	0	1.20
2,4	30%S 15B	59.83	0	0	36.13	0	0	95.96
5	10%S 0B	71.15	0	0	27.24	0	17.71	116.10
6	10%S 15B	43.38	0	0	46.93	0	15.15	105.46
7	50%S 0B	9.10	1.57	1.40	25.30	1.58	3.57	42.52
8	50%S15B	30.87	0	0	10.73	0	10.28	51.88
9,10	10%S 7.5B	101.66	7.73	0	93.18	0	38.80	241.37
11,12	50%S 7.5B	57.14	2.02	0	43.50	0	39.84	142.50
13-17	30%S 7.5B	53.48	0.79	0	42.02	0	22.09	118.38

Although total VFA concentration of all samples after AD was significantly higher ($p< 0.05$) than the initial corresponding values, samples which are seeded with 50% sludge (sample number 7, 8 and 12 in Table 38) had the lowest final total VFAs values indicating that these particular samples were not affected by the VFA inhibition as much as the others. Therefore, their biogas yields as well as methane percentage were significantly higher than the other samples (Table 35).

AD of pot ale draff mix had much higher final VFA concentration than pot alone ale even though the same design factors are applied for both feedstock types. This was attributed to higher impact of the lack of internal agitation within the system for AD of the mixed compounds since it has higher solid content. In other words, microbes are physically blocked to reach the draff which leads to competition for the limited amount of pot ale as the feedstock.

Table 38. VFA concentrations of pot ale, draff and sludge mix after AD of pot ale draff mix

Std no	Sample Name	Acetic Acid	Propionic Acid	Isobutyric Acid	Butyric Acid	Isovaleric Acid	Valeric Acid	Total
1	30%S 0B 32˚C	416.07	63.29	110.97	1101.55	124.79	389.87	2206.54
2	30%S 15B32˚C	527.92	37.28	71.58	1237.56	80.49	54.29	2009.13
3	30%S 0B 38˚C	304.67	20.21	40.77	815.88	45.85	32.82	1260.20
4	30%S 15B 38˚C	286.35	34.40	78.23	589.32	87.98	198.64	1274.91
5	10%S 0B 35˚C	393.32	30.30	78.46	1118.17	88.24	36.36	1744.85
6	10%S 15B 35˚C	653.05	43.39	97.35	1768.60	109.47	97.96	2769.82
7	50%S 0B 35˚C	40.28	77.70	0	0	0	30.04	148.01
8	50%S 15B 35˚C	0.99	14.77	0	9.11	0	0	24.89
9	10%S 7.5B 32˚C	8.15	136.90	0	47.80	0	33.66	226.51
10	10%S 7.5B 38˚C	510.34	41.47	111.60	2044.33	125.50	229.17	3062.40
12	50%S 7.5B 38 ˚C	62.07	16.11	16.12	92.46	9.38	0	196.15

ANOVA for the suggested reduced models for biogas generation, CH_4 and CO_2 percentages are given in Table 39, Table 40 and Table 41. The quadratic equations built based upon statistical analysis in terms of coded and actual factors are given in Eq 39, Eq 41 and Eq 43 and Eq 40, Eq 42 and Eq 44 for three of the responses of interest respectively. The format of coding was stated in Table 24.

The model developed for biogas generation by anaerobic digestion of pot ale and draff mixture was statistically analysed by ANOVA (Table 39) at an alpha value of 0.1 instead of 0.05 for this particular case due to present significant lack of fit within the confidence level of 95%. To address this problem adjusting the alpha value was necessary**.** The coefficient of the final model in terms of coded factors (Eq 39) indicates the strength of impact of the individual factors on biogas generation as being $C > C^2 > BC > B$.

Table 39. ANOVA Table for biogas production quadratic model α=0.01

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	1.50E+05	4	37568.12	56.84	< 0.0001	significant
B-Temperature	2455.95	1	2455.95	*3.72*	0.0779	
C-Sludge,%	1.03E+05	1	1.03E+05	*155.55*	< 0.0001	
BC	3039.32	1	3039.32	*4.6*	0.0532	
C^2	41961.97	1	41961.97	*63.48*	< 0.0001	
Residual	7931.94	12	661			
Lack of Fit	7446.01	8	930.75	*7.66*	0.0331	not significant
Pure Error	485.93	4	121.48			
Cor Total	1.58E+05	16				

R^2: 0.9499, Adj R^2: 0. 9332, Pred R^2: 0. 8345, Adeq Precision: 20.22

Final equation in terms of coded factors:

$$\mathbf{Biogas\ (ml/gVS)} = \mathbf{63.26 + 17.52\ B + 113.37\ C + 27.57\ BC + 99.54\ C^2} \quad \text{Eq 39}$$

Final Equation in Terms of Actual Factors:

$$\mathbf{Biogas(ml/gVS)} = \mathbf{395.14 - 7.94\ T - 25.34\ S\% + 0.46\ T\ S\% + 0.25\ S\%^2} \quad \text{Eq 40}$$

The dataset for CH_4% values were analysed by ANOVA (Table 40). The significant model terms were the beating time (A), first and the second order effects of temperature (B) and sludge% (C), as well as the interaction effect of temperature and sludge percentage (BC). The coefficients of the final equation for CH_4 gives the strength of the influence on CH_4 percentage which was C > C^2 > BC > B > A > -B^2. The final equation on CH_4% in terms of actual factors are given in Eq 41.

Table 40. ANOVA Table for CH_4% quadratic model α=0.05

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	3980.98	6	663.50	116.82	< 0.0001	significant
A-Beating Time	129.82	1	129.82	22.86	0.0007	
B-Temperature	189.33	1	189.33	33.34	0.0002	
C-Sludge	2857.14	1	2857.14	503.06	< 0.0001	
BC	132.08	1	132.08	23.26	0.0007	
B^2	36.74	1	36.74	6.47	0.0292	
C^2	650.96	1	650.96	114.62	< 0.0001	
Residual	56.80	10	5.68			
Lack of Fit	46.32	6	7.72	2.95	0.1575	not significant
Pure Error	10.48	4	2.62			
Cor Total	4037.77	16				

R^2: 0.9859, Adj R^2: 0. 9774, Pred R^2: 0.9255, Adeq Precision: 33.23

Final Equation in terms of coded factors:

$$\mathbf{CH_4}(\%) = \mathbf{12.98 + 4.03A + 4.86B + 18.9C + 5.75\ BC - 2.95\ B^2 + 12.42\ C^2} \quad \text{Eq 41}$$

Final Equation in terms of actual factors:

$$\mathbf{CH_4}\ (\%) = \mathbf{-349.18 + 0.54\ BT + 21.69\ T - 4.27\ S\% + 0.10\ T\ S\% - 0.33\ T^2 + 0.031\ S\%^2} \quad \text{Eq 42}$$

ANOVA results for CO_2% are presented in Table 41. The first and second order effects of sludge percentages (C, C^2) were shown to have the greatest influence on the response of interest. However, temperature (B) and the interaction effect of temperature and sludge % (BC) were also added to the model after the stepwise regression to improve the model by the software. According to the final equation built in terms of coded factors (Eq 43), the relative impact of the parameters on CO_2 content was found as C^2 > C > BC > B. The final equation for CO_2% in terms of actual factors is given in Eq 44.

Table 41. ANOVA Table for C02% quadratic model α=0.05

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	803.08	4	200.77	24.11	< 0.0001	significant
B-Temperature	18.30	1	18.30	2.10	0.1640	
C-Sludge	351.13	1	351.13	42.17	< 0.0001	
BC	37.21	1	37.21	4.47	0.0561	
C^2	396.45	1	396.45	47.61	< 0.0001	
Residual	99.92	12	8.33			
Lack of Fit	63.86	8	7.98	0.89	0.5923	not significant
Pure Error	36.06	4	9.02			
Cor Total	903.00	16				

R^2: 0.8893, Adj R^2: 0.8524, Pred R^2: 0.6959, Adeq Precision: 14.29

Final Equation in terms of coded factors:

$$\mathbf{CO_2}\ (\%) = \mathbf{55.90 - 1.52\ B - 6.64\ C - 3.03\ BC - 9.67C^2} \quad \text{Eq 43}$$

Final Equation in terms of actual factors:

$$\mathbf{CO_2}\ (\%) = \mathbf{8.70 + 1.01\ T + 2.89\ S\% - 0.05\ T\ S\% - 0.02\ S\%^2} \quad \text{Eq 44}$$

4.5.1 Validation of the Developed Models

The models were used as part of the post statistical analysis to produce the normal plot of residuals and the predicted vs actual plot. The same trend was seen in the plots of the models for AD of pot ale and draff mix as for AD of pot ale (Figure 24 and Figure 25). Normal distribution of the residuals confirms that ANOVA can be used to analyse the dataset. Adequacy of the model was validated with actual vs predicted graph, with the design points tending to be on the diagonal line.

The models were then tested with further independent validation experiments and the corresponding results are given in Table 42. The final equations in terms of actual factors (Eqs 40, 42 and 44) were used to predict the results for biogas yield, CH_4 and CO_2 percentages respectively. Subsequently, a t-test (2 tailed distribution with unequal variance) was used to analyse the differences between experimental result and the predicted results. According to the *p* values the developed models are statistically significant within a 95% confidence interval.

Table 42. Validation experiment results versus predicted results for AD of pot ale and draff mix

Validation Points	**Biogas Yield (ml/g VS)**		**CH_4%**		**CO_2%**	
	Experimental	Predicted	Experimental	Predicted	Experimental	Predicted
0B 50%S 38˚C	378.2 ± 84	325.4	44.2 ± 7.3	52.4	40.1 ± 12	46.5
	p: 0.391		*p: 0.207*		*p: 0.479*	
0B 10%S 38˚C	78.4 ± 35	40.1	2.2 ± 0.8	1.1	62.7± 12.6	54.9
	p: 0.202		*p: 0.161*		*p: 0.399*	

4.5.2 Model Graphs

The perturbation plots are given in Figure 32 (1, 2 and 3) for biogas generation, CH_4 and CO_2 percentages after anaerobic digestion of pot ale and draff mixture. The sludge % (C) had the greatest impact on all of the responses with the same trend for biogas yield and CH_4% , whereas a reversed effect was seen for CO_2%. The content of CH_4 was influenced by all factors whereas biogas production and CO_2% were not significantly affected by beating time (A).

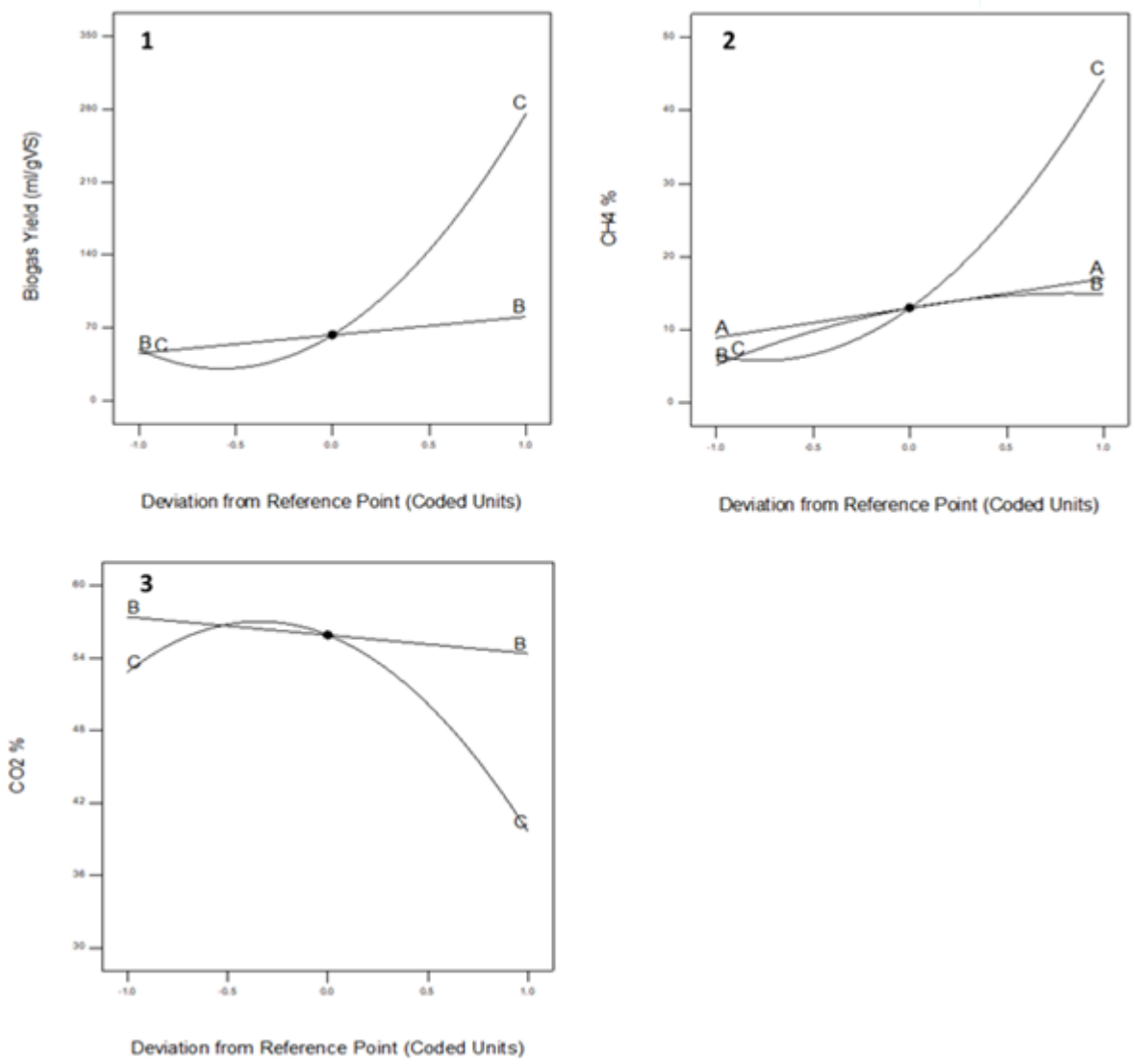


Figure 32. Perturbation graphs of the developed models on 1. biogas generation, 2. CH_4%, 3. CO_2%

An interaction between the digestion temperature and sludge percentages was seen in biogas production, CH_4 and CO_2 percentages which are given in Figure 33 (1, 2 and 3) respectively. The different digestion temperature did not have a significant effect on any of the responses with lower sludge seeding ratios (<30%). However, once the sludge seeding ratio was increased, increase in digestion temperature to 38˚C led to a significantly enhanced biogas yield and CH_4% (Figure 33 (1, 2)). In contrast, the CO_2% was reduced (Figure 32 (3)) which is considered to be desired condition to achieve higher biogas quality and quantity at the same time.

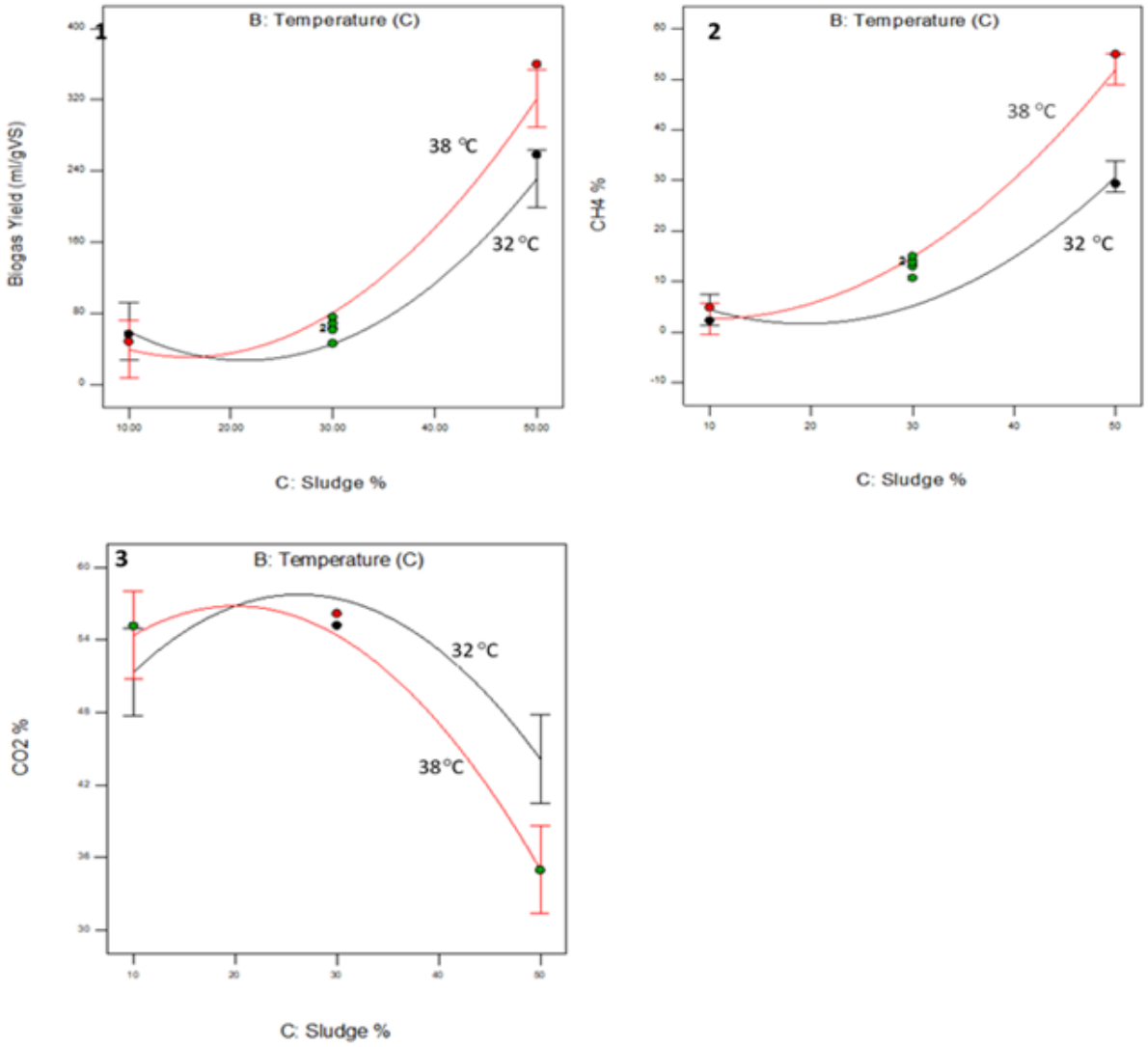


Figure 33. Interaction graphs between the digestion temperatures and sludge percentages for 1. Biogas generation, 2. CH_4, 3. CO_2 percentages

Unlike the design for anaerobic digestion of pot ale, an interaction was seen between sludge percentage and the digestion temperature with the design for anaerobic digestion of pot ale and draff mixture for biogas generation (Figure 33 1). On the other hand, the same interaction trend is obtained for the biogas quality for both experimental designs. A greater biogas production and CH_4% was achieved with higher sludge seeding ratios and higher digestion temperatures.

The contour graphs were plotted and coloured in accordance with the value of the generated biogas, CH_4% and CO_2% and given in Figure 34 (1, 2 and 3), respectively. The effects of temperature and sludge percentages mainly have straight lines on all responses. However, the trend of the lines changed into peaks for the area around 20-30% sludge

which gives a lower yield for biogas generation and methane content with the greatest CO_2% values. The greatest biogas generation and the highest CH_4 percentage were achieved under the condition of higher sludge seeding ratio and digestion temperature.

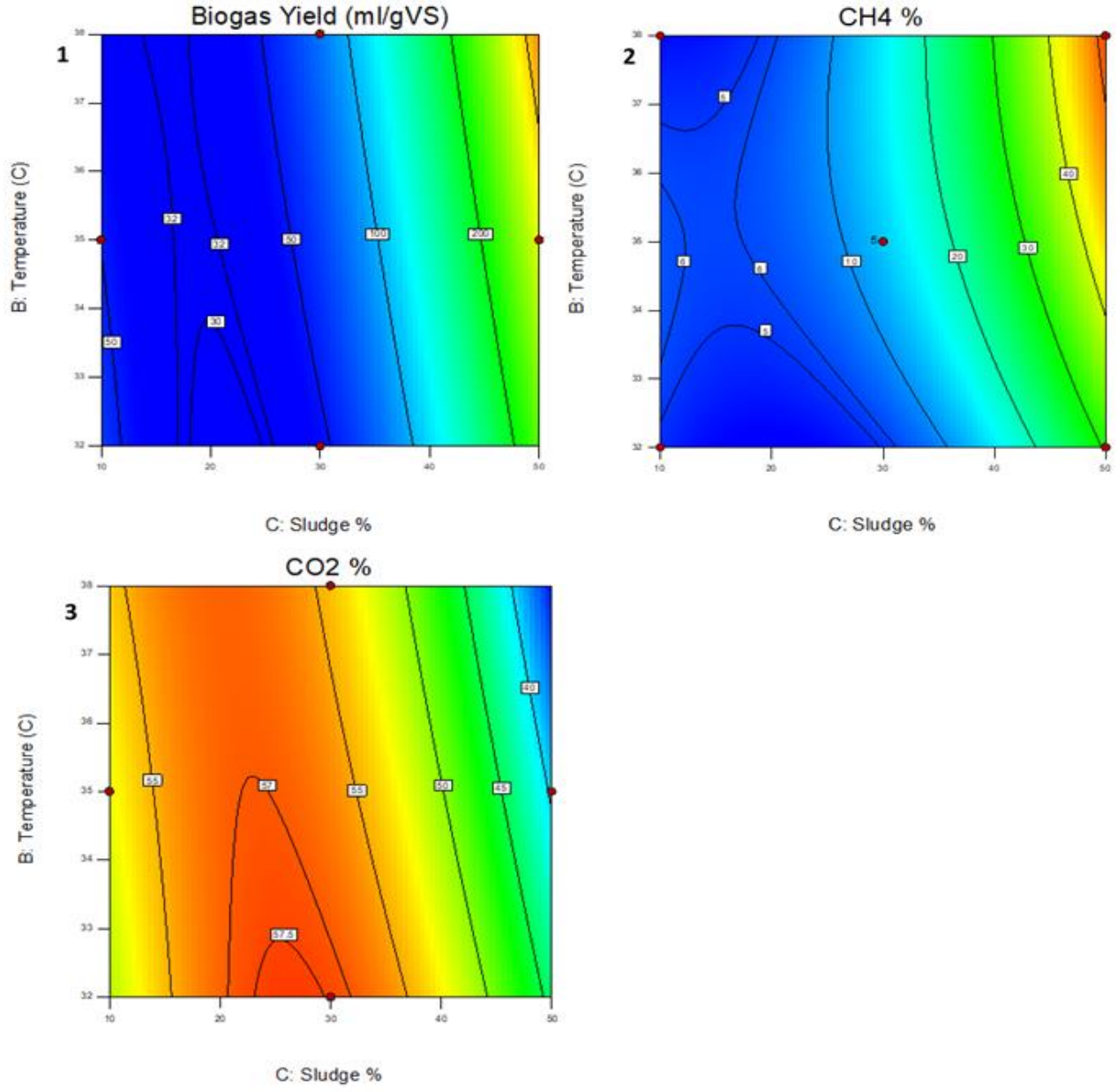


Figure 34. Contour graphs for 1. biogas generation, 2. CH4% and 3. CO2%

4.5.3 Optimisation

The desirability function, in conjunction with numerical and graphical optimisations, was applied to the RSM dataset in order to maximise the biogas yield and CH_4% with an importance level of 5 while minimising the CO_2% with the same importance (Table 43) which is the same optimisation approach as DOE design for AD of pot ale. Suggested solutions are given in Table 44. The optimum sludge percentage was found to be 50 according to the numerical optimisation solutions.

Table 43. Optimisation conditions for AD pot ale draff mix.

Variable	Goal	Lower Limit	Upper Limit	Importance
A: Beating Time (min)	is in range	0	15	3
B: Temperature (˚C)	is in range	32	38	3
C: Sludge (%)	is in range	10	50	3
Biogas Yield (ml/g VS)	maximise	42.5	359.9	5
CH_4%	maximise	2.2	55.0	5
CO_2%	minimise	35.0	59.1	5

Table 44. Numerical optimisation solutions for AD of pot ale and draff mix

No	A: BT (min)	B:T (˚C)	C:Sludge %	Biogas Yield (ml/g VS)	CH_4 %	CO_2 %	Desirability N/A
1	13.09	38.00	50.00	321.21	54.95	35.05	0.96
2	11.06	38.00	50.00	321.25	53.87	35.04	0.96
3	8.69	38.00	50.00	321.25	52.59	35.04	0.95
4	15.00	37.09	50.00	307.64	54.30	36.42	0.93
5	15.00	35.66	49.93	285.02	50.36	38.68	0.84
6	15.00	35.11	50.00	277.87	48.72	39.42	0.82
7	0.00	35.52	50.00	284.01	42.03	38.80	0.79
8	14.99	33.48	50.00	253.37	42.20	41.90	0.71
9	0.00	38.00	45.76	253.66	38.00	40.76	0.70
10	15.00	32.23	10.00	58.71	8.82	51.46	0.13

The result of the graphical optimisation is given in Figure 35. The optimum area marked with yellow is defined by the constraints set according to the numerical optimisation solutions (Table 44). In this design, defining lines for the target area are 317.03 ml/g VS biogas production, 53.2 CH_4 and 36% CO_2 percentages.

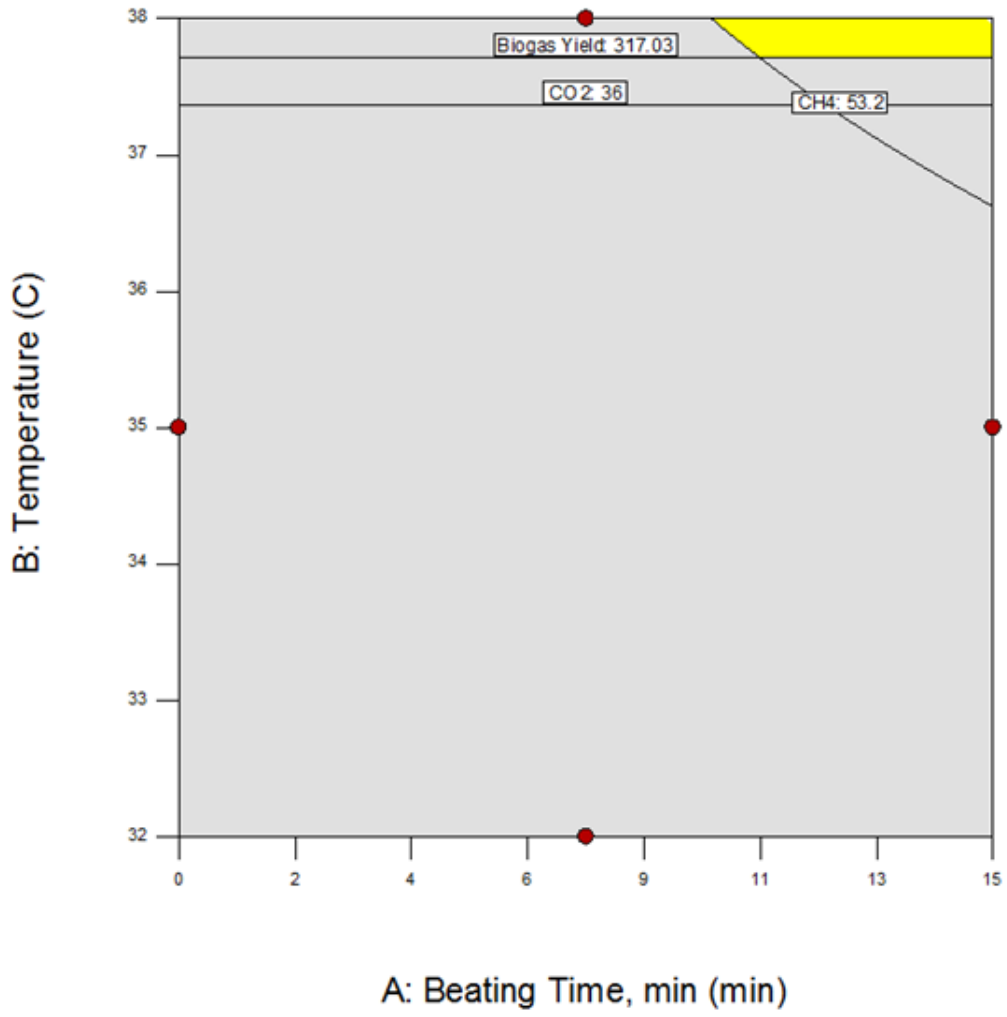


Figure 35. Graphical optimisation of AD of pot ale and draff mix at 50% sludge

The lignocellulose fractions of spent grain before and after application of alkaline pre-treatment were introduced to literature. Similar to the single digestion of pot ale, the most powerful design parameter was identified as the sludge seeding ratio according to the data analysis with DOE. The highest biogas generation was found to be 360±10 ml/g VS with a CH_4 content of 55.0±0.4% for AD at 38˚C with a 50% sludge seeding ratio after application of 7.5 min beating after 1M NaOH pre-treatment. The significant model parameters for biogas yield were found to be sludge seeding ratio and its interaction with temperature while beating time and temperature were also found to be significantly impact the CH_4 content of the produced biogas. The higher digestion temperature 38˚C led the higher and lower CH_4 and CO_2 yields respectively at high sludge seeding ratios (40 – 50% on wet basis). Therefore, the optimum reaction conditions were identified as 50%

sludge seeding ratio, 38˚C digestion time and 10 – 15 min beating time. On the other hand, significantly higher total VFA concentrations were seen in all experiments which caused low pH values in the reactors and inhibition of methanogenic activity.

A comparative summary of the mathematical models developed for biogas yield, CH_4 and CO_2 percentage models with anaerobic single digestion of pot ale and pot ale draff mixture is given in Table 45. Equations in terms of actual factors were used for data prediction for the validation step of the models as well as the optimisation step. The design parameters beating time, temperature and sludge seeding ratio were coded as A, B and C respectively. It brings the effect of different parameters in different units to a dimensionless area in order to assess their strength of influence on the responses.

Table 45. A summary of the developed models of AD of pot ale and pot ale draff mixture after combined alkaline and beating pre-treatment

Pot ale	$* \mathbf{Biogas}\ (\boldsymbol{ml/gVS}) = \mathbf{208.74 + 26A + 185.5C - 37.16A^2 + 122.34C^2}$
	$\mathbf{Biogas}\ (\mathbf{ml/gVS}) = \mathbf{142.60 + 13.38\ BT - 9.08\ S\% - 0.6(BT)^2 + 0.31(S\%)^2}$
Pot ale & draff	$* \mathbf{Biogas}\ (\boldsymbol{ml/gVS}) = \mathbf{63.26 + 17.52\ B + 113.37\ C + 27.57\ BC + 99.54\ C^2}$
	$\mathbf{Biogas\ (ml/gVS) = 395.14 - 7.94\ T - 25.34\ S\% + 0.46\ T\ S\% + 0.25\ S\%^2}$
Pot ale	$* \mathbf{CH_4}\ (\%) = \mathbf{3.93 + 1.54B + 23.48C + 2.57BC + 20.94 \times C^2}$
	$\mathbf{CH_4}\ (\%) = \mathbf{42.96 - 0.78T - 3.47S\% + 0.043\ T\ S\% + 0.05(S\%)^2}$
Pot ale & draff	$* \mathbf{CH_4}(\%) = \mathbf{12.98 + 4.03A + 4.86B + 18.9C + 5.75\ BC - 2.95\ B^2 + 12.42\ C^2}$
	$\mathbf{CH_4}\ (\%) = \mathbf{-349.18 + 0.54BT + 21.69T - 4.27S\% + 0.10T\ S\% - 0.33(T)^2 + 0.031\ (S\%)^2}$
Pot ale	$* \mathbf{CO_2}\ (\%) = \mathbf{40.69 - 2.86\ B + 2.71\ C + 6.22\ BC - 17.68\ C^2}$
	$\mathbf{CO_2}\ (\%) = \mathbf{139.18 - 4.07\ T - 0.84\ S\% + 0.10\ TS\% - 0.044\ (S\%)^2}$
Pot ale & draff	$* \mathbf{CO_2}\ (\%) = \mathbf{55.90 - 1.52\ B - 6.64\ C - 3.03\ BC - 9.67C^2}$
	$\mathbf{CO_2}\ (\%) = \mathbf{8.70 + 1.01\ T + 2.89\ S\% - 0.05\ T\ S\% - 0.02\ (S\%)^2}$

* Equations in terms of coded factors.

4.6. Influence of Initial Reaction pH on AD Yield

The results of TS, VS and moisture content of 1 M NaOH pre-treated pot ale was determined as 0.0949±0.0059 g TS/g sample, 0.0825±0.0011 g VS/g sample and 91.1±0.1% respectively. Cumulative biogas and methane yields for AD of alkaline pre-treated pot ale with 50% seeding ratio and different initial reaction pH values are given in Table 46.

Table 46. Cumulative biogas and methane yields

Sample	Initial pH	Biogas Yield ml/g VS	Methane Yield ml/g VS	*H_2S ppm	*Final pH
1 M NaOH pre-treated pot ale	7	165 ± 2	21 ± 3.7	573	5.44
	8	141 ± 2	34 ± 1.3	542	5.56
	9	203 ± 8	43 ± 2.5	497	5.86

* Average of triplicate runs

The initial reaction pH was found to be a significant parameter with the *p* values of 0.0003 and 0.0000014 for biogas and biomethane yields respectively. On the other hand, the final pH values of all design samples were found to be lower than the neutral range due to VFA accumulation which was not ideal for the methanogenic bacteria to grow and carry out balanced reactions in the final step of AD. In order to determine the time of pH drops, sample with an initial pH value of 8 was monitored at 30 and 50% sludge seedings on wet basis. The results are given in Figure 36. The decrease in the pH values was much sharper for the 30% seeding ratio by reaching below 5 within the first day of AD whereas the pH never went below 5 for the 50% seeding ratio. In the 2nd and the 3rd days of digestion acetogenic activity increased in the sample contains 30% sludge than the 1st day which increased the VFA consumption to recover the pH level. After the 3rd day of AD a similar pattern is seen regardless of sludge content.

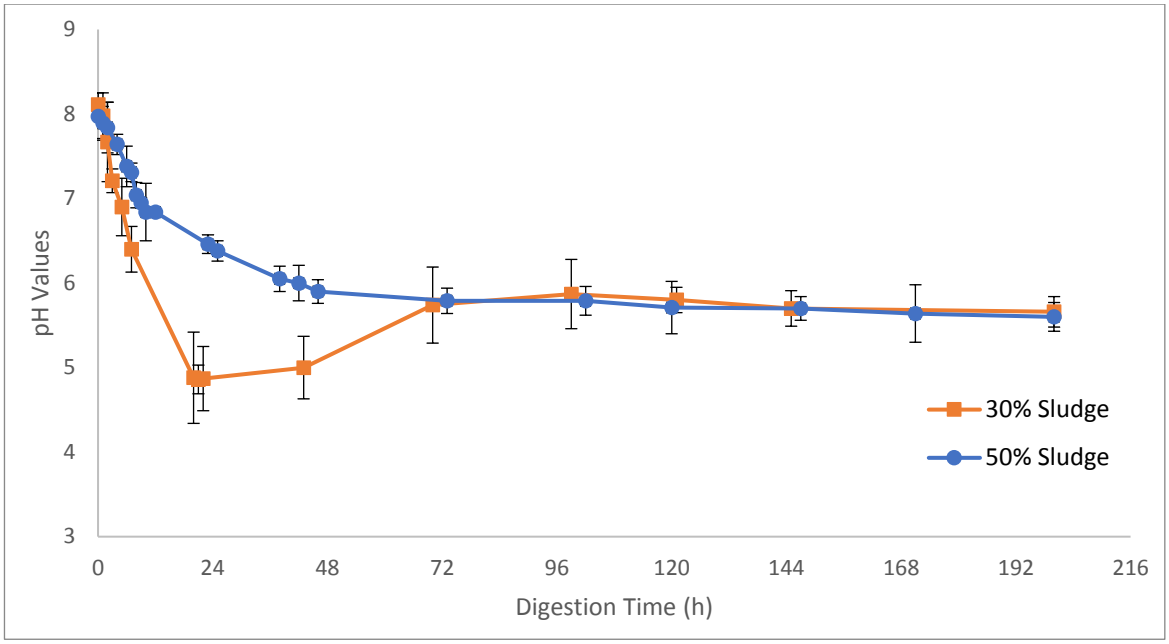


Figure 36. pH profile of the 1 M NaOH pre-treated sample with 30 and 50% seeding ratios

The total volatile fatty acids (acetic acid, propionic acid, butyric acid, iso butyric acid, valeric acid and isovaleric acid) were analysed before and after AD as well as the 9th day of the digestion. The results of VFA analysis are given in Figure 37. A huge increase in the level of total VFA was seen after the first 9 days of AD for all samples. Based on the pH profiles and the VFA concentrations, the major part of total VFA was produced within the first 1 and 2 days of AD with the sludge seeding ratios of 30 and 50% respectively.

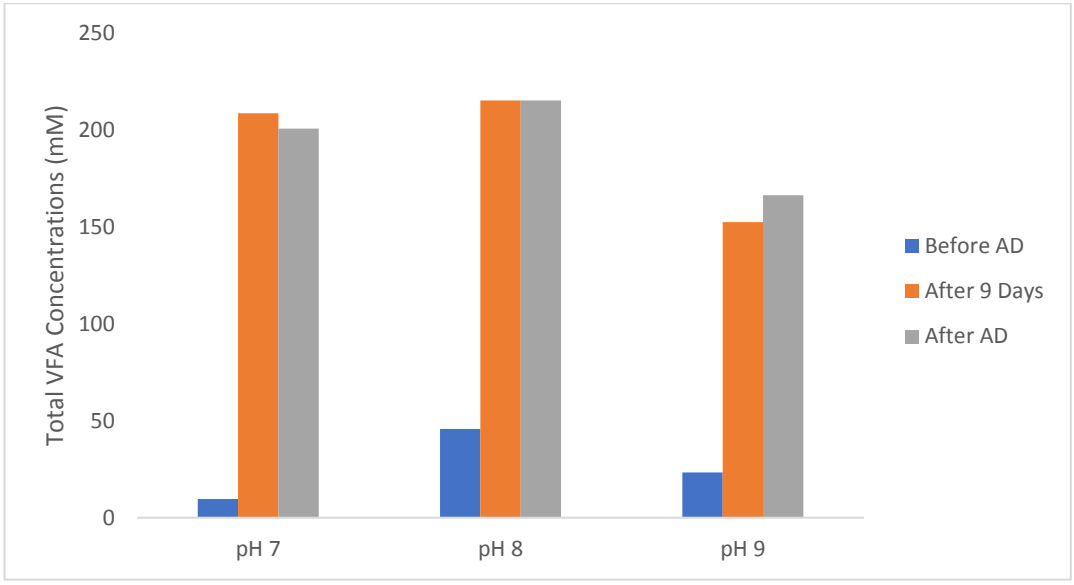


Figure 37. Total VFA concentrations before, after 9 days and after AD

Comparative methane yields and organic removal percentages of first 9 days and 21 days of the samples are given in Figure 38 and Table 47 respectively. The highest methane generations were achieved by the samples with the initial pH value of 9, while the lowest methane production was obtained with the sample which had the initial pH value set to 7. However, no significant difference was found between the cumulative methane production results and after the first 9 days results indicating that methanogenic activity was severely inhibited in the early stages of the digestion for all cases.

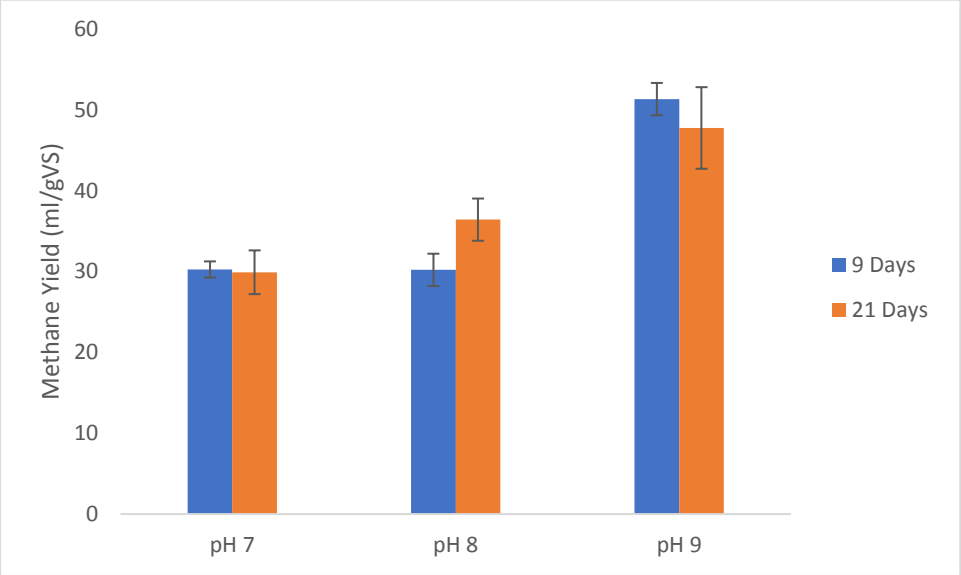


Figure 38. Methane yields after 9 and 21 days of AD

A similar pattern was seen in the organic removal percentages. Approximately 90% of overall COD and BOD degradations were obtained within the first 9 days of AD for all samples (Table 47). However, a significant rise (ranging from 14.7% to 18.1%) was seen in the cumulative SO_4 degradation in comparison with first 9 days degradations for all samples. Based upon this organic matter removal and methane yields methanogenic bacteria activity was impaired within the first 9 days of AD which led the sulphate reducing bacteria to carry out the reactions mainly through the sulfidogenesis path, which resulted in H_2S production instead of CH_4. Cumulative H_2S production is given in Table 46.

Table 47. Organic matter removal within first 9 days and after 21 days AD

		COD (%)		BOD (%)		SO_4 (%)	
Sample	***pH_0**	**9 Days**	**Cumulative**	**9 Days**	**Cumulative**	**9 Days**	**Cumulative**
1 M NaOH	7	70 ± 1	71 ± 1	64 ± 1	68 ± 2	39 ± 3	56 ± 2
pre-treated	8	66 ± 2	70 ± 2	59 ± 3	67 ± 3	40 ± 8	58 ± 5
pot ale	9	76 ± 1	78 ± 1	71 ± 2	75 ± 1	57 ± 9	71 ± 4

* Initial pH value

An approximate time of VFA generation/accumulation was determined to be 1 and 2 days with AD of 1 M NaOH pre-treated pot ale for the sludge seeding ratios of 30 and 50% of overall active reactor volume of 400 ml. In addition, no significant decrease in the total VFA concentration and methane production was detected after the 9^{th} day of AD indicating an inhibition in the reactor. On the other hand, initial pH level of the reaction was determined as a significant parameter as it potentially delayed the pH drops. Therefore, higher inoculum ratios and different initial pH values were investigated in the next experiments.

4.7 Discussion of Key Findings

The characteristics of non-treated pot ale was mainly found to be in agreement with the literature in terms of the concentrations of COD (38867 ± 115 mg/L), BOD (30965 ± 666 mg/L), SO_4 (190 ± 31 mg/L) within the reported ranges 30000 – 50000 mg/L for COD (Goodwin et al. 2001; Sankaran et al. 2014; Uzal et al. 2003; Graham et al. 2012), 25000 – 35000 mg/L for BOD (Goodwin & Stuart 1994; Tokuda et al. 1998; Graham et al. 2012), 223 – 284.8 mg/L for SO_4 (Kida et al. 1999). The pH (3.7) was also within the reported range of 3.5 – 4.5 (Mallick et al. 2009; Dionisi et al. 2014). A slight difference was found between the concentrations of PO_4^{3-} (778 ± 7 mg/L), N-NH_3 (45 ± 7 mg/L), N-NO_3 (111 ± 20 mg/L), N-NO_2 (33 ± 4 mg/L) and Cu (14.7 ± 1 mg/L) and the corresponding reported concentrations: 714mg/L (Ansa-Asare et al. 2000; Uzal et al. 2003) for PO_4^{3-}, 0.12 – 7.9 mg/L for N-NH_3 (Barrena et al. 2018), 8.3 – 53 mg/L for N-NO_3, 16 – 20 mg/L for N-NO_2 and 2 – 6 mg/L for Cu (Graham et al. 2012). These differences were attributed to use of different source of raw materials and process parameters of the sampling distillery. In addition to the chemical characterisation, the correlation between TS and VS (VS were 86.5% of TS w/w) was within the reported range of 85 – 92.9% (Barrena et al. 2018).

The lignocellulosic structure of pot ale has not previously been reported in the literature, therefore there is no comparison in terms of hemicellulose, cellulose and lignin fractions of pot ale as received and after 1M NaOH pre-treatment. On the other hand, the lignocellulosic structure of spent grains was reported to contain about 21.8–28.4% hemicellulose, 16.8–25.4% cellulose and 11.9–27.8% of lignin depending on the seasonality and conditions of fermentation (Panji et al. 2015; Sežun et al. 2000). The hemicellulose (25±1.1%) and lignin (16±0.1%) content of draff used in this study is in an agreement with the reported data whereas the cellulose content (29±1.5%) was about 4% higher than the upper limit for cellulose and lignin. This can be attributed to the potential slight differences between the fermentation conditions of breweries and whiskey distilleries. Since pot ale was a spent grain extracted waste stream of whiskey manufacturing, lower fractions of hemicellulose (11.5±0.2%), cellulose (10.6±1.0%) and lignin (26.9±0.9%) than draff was seen. 90% lignin removal was reported following 20% NaOH pre-treatment at pH10 on brewery spent grain (Sežun et al. 2000) whereas 40% lignin degradation was achieved in this study which was attributed to the weaker alkali agent used in this study.

A methane yield of 554 ± 67 ml/g VS was reported by Barrena et al. 2018 with AD of non-treated pot ale with a substrate inoculum ratio of 1:2 (on wet basis) at lab scale batch experiments, while in this study the highest methane yield was found as 322 ± 4 ml/g VS (Table 14) by AD of alkaline and 7.5 minutes beating pre-treated pot ale with a substrate to inoculum ratio of 1:1. The difference between the two yields was attributed to the lower inoculum ratio in the experiments as the reactor configurations and alkali conditions were similar in both works. Consequently increased solid content of the reactor by means of adding draff to be co-digested with pot ale (with a mixing ratio of 1:5) after alkaline and 7.5 minutes beating pre-treatment with a substrate inoculum ratio of 1:1 had biogas yield of 469 ± 7 ml/g VS (Figure 31) with 53.3 ± 1% CH_4 (Table 32) content. However, the biogas yield from AD of pot ale alone was found to be 629 ± 8.5 ml/g VS (Table 14) with 51.3% CH_4 content (Table 14). Considerably lower biogas generation in AD of draff and pot ale mix suggests an unsuitable reaction environment because of potential inhibitions such as the presence of VFAs (because of the low final pH values shown in Table 32) (Searmsirimongkol et al. 2011; Siegert & Banks 2005; Appels et al. 2008) as well

as lack of internal agitation within the system which led to a phase separation during the digestion time resulting in the influence of the pre-treatments on the biogas production being masked with lack of active contact between the feedstock and the microbes.

The efficiency of AD was evaluated based on the removal of organic matter by the treatment and various COD and BOD degradations have been reported for different reactor configurations and pre-treatment conditions for AD of pot ale. During the first 140 days of digestion a typical COD removal was reported as 70 – 90% by AD of alkaline ($NaHCO_3$) pre-treated pot ale using a UASB (Goodwin et al. 2001). Enhanced levels of COD (96%) and BOD (99%) removals were obtained by (Uzal et al. 2003) for AD of pot ale with addition of $NaHCO_3$ and nutrient supplement for accelerated growth of culture using two stage UASB. On the other hand, 13 and 50% COD reductions were obtained by AD of pot ale without and with enzymatic pre-treatment using 10% papain in a lab scale batch reactor (Mallick et al. 2009). In this work, an improvement of 75% (from 32±4.3 to 56±2.4) and 41% (from 44±3.5 to 62±2.1) was seen in the COD and BOD degradations respectively for AD of pot ale with a substrate inoculum ratio of 1:1 at 35˚C (Table 18) due to the application of alkaline pre-treatment. However, the highest COD and BOD degradations were achieved as 78±5% and 61±4% (std no 8 in Table 21) respectively due to combined effects of alkaline and 15 minutes of beating pre-treatment on pot ale under the same digestion conditions. Although the highest levels of organic matter removals (73±6% for COD and 63±7% for BOD) were seen in AD of pot ale and draff with a mixing ratio of 5:1 (std no 12 in Table 36) by wet weight among the digestion of the mixed substrates, it was slightly lower than digestion of pot ale alone. Organic degradation rates were found to be in an agreement with published works (Goodwin et al. 2001; Mallick et al. 2009). Although considerably higher organic matter removals were achieved by AD of draff and pot ale, biomethane generation per gram VS was 33.5% lower than AD of pot ale under the same digestion conditions (298 ± 3 CH_4 /g VS with std no 12 in Table 20 and 198 ± 5.5 ml CH_4 /g VS with std no 12 in (Table 35)). This was attributed to the VFA inhibition occurring in the case of co-digestion of pot-ale and draff due to the significantly higher final VFA value which was found as 1474.69 mM (std no 12 in Table 38), whereas it was found to be 142.50 mM for AD of pot ale (std no 12 in Table 37). Co-digesting draff with pot ale caused overloading of the reactor which consequently resulted in excessive VFA generation in the

acidogenesis step and imbalanced pH levels which impaired the methanogenic bacteria activity for further breakdown of the VFAs. A second potential reason was lack of internal agitation within the system which led to a phase separation during the digestion time resulting in a lack of contact between bacteria and spent grain. Consequently, higher COD and BOD degradation were the indicator of a more complete digestion on pot ale in the mixture since draff was physically blocked. Samples which have less than 1:1 inoculum substrate ratio (< 50% sludge) were affected by VFA inhibition even more because of the significant differences between the total VFA concentration before and after AD of pot ale (Table 22, Table 23) and AD of spent grain and pot ale (Table 37, Table 38). For example, std no 5 (alkaline pre-treated sample seeded with 10% S) had a total VFA concentrations of 5.57 mM (in case of AD of pot ale Table 22) prior to AD and a 7-fold increase was seen in total VFA concentration (40.11 mM in Table 23) after AD. Similarly, a 15-fold increase was seen in total VFA concentrations of std number 5 (in case of AD of pot ale and draff) before and after AD from 116.10 mM (Table 37) to 1744.85 mM (Table 38). Application of mechanical pre-treatment (beating in this study) has been reported to increase the risk of VFA accumulation with the reactors (Izumi et al. 2010). For example, std no 5 and 6 have exactly the same conditions apart from the beating time for both experimental designs. For AD of pot ale final VFA value increased from 40.11 mM (std no 5 in Table 22) to 76.20 mM (std no 6 in Table 37) due the implementation of 15 minutes of beating. In case of AD of draff and pot ale the difference was even clearer with an increase in the final VFA concentration from 1744.85 mM (std no 5 in Table 38) to 2769.82 mM (std no 6 in Table 38).

AD of pot ale and AD pot ale and draff were modelled using DOE software and the developed models were subsequently challenged by independent experiments. The difference between the predicted results by the models and experimental results were found to be insignificant according to applied t-test (Table 28, Table 42). The most important and powerful design factor was found to be the sludge seeding ratio for both systems. Therefore, the optimum conditions for both designs were predicted sludge seeding ratio as 50% by the numerical optimisation tool with desirability approach. The beating time and the digestion temperature were predicted as 10 minutes and 32˚C for AD of pot ale and 13 min and 38˚C for AD of draff and pot ale in order to maximise the

biogas yield and the CH_4% while minimising the CO_2% with an equal importance (Table 29, Table 43).

The pH profiles of AD pot ale after 1M NaOH pre-treatment were created for 30 and 50 % sludge seeding ratios of total volume of 400 ml. Although the starting pH value of the reactions were set to 8, sharp pH drops reaching 4.9 and 6.4 were seen in the first 24 h of AD for the sludge seeding ratios of 30 and 50 % respectively. The change in the pH profile was found to be in agreement with previously published results on AD of pot ale, where an approximate pH range of 5.6 – 6.2 were reported within the first 24 h AD of pot ale with combined enzymatic pre-treatment when the initial pH value was 7.5 for operation of 1 L batch reactor (Mallick et al. 2009). In the same study, non-treated pot ale also had a pH of 5.9 approximately at the end of the first day of the digestion. The sudden pH changes in the early stages of AD were attributed to accumulation of VFAs in the acidogenesis step, which consequently impairs methanogenic activity (Tedesco et al. 2014; Ferrer et al. 2008; Wang et al. 2014; Wei et al. 2014; Mumme et al. 2010; Aramrueang et al. 2016; M. E. Montingelli et al. 2016; Mao et al. 2015; Guo et al. 2017; Rathaur et al. 2017; Izumi et al. 2010; Yenigün & Demirel 2013; Madsen et al. 2011). In previously published research, when the methanogenic activity was not impaired severely by low pH values, the level of total VFAs concentration showed a declining trend after the 4^{th} and the 9^{th} days of AD in batch mode (Izumi et al. 2010 and Wang et al. 2014). However, in this work, no decrease was seen in the VFA concentration after 9 days of AD of alkali pre-treated pot ale with a 50% seeding ratio (Figure 37). The final VFA levels of the samples which had initial pH of 7, 8 and 9 were determined to be 208.54, 215.14 and 152.68 mM respectively after the first 9 days of AD, indicating that they reached the inhibitory VFA levels (100 – 200 mM) for methane production in batch mode (Izumi et al. 2010). Consequently, no significant difference was found between the first 9 days and overall methane yields regardless of the initial pH value of 7, 8 and 9 (Figure 38) according to the *p* values of 0.6077, 0.2936 and 0.5151 respectively, proving the VFA inhibition in methanogenic activity. Due to the inhibition in the methanogenesis step, sulphate reducing bacteria carried out the side reactions (sulfidogenesis). Therefore, a significant increase in SO_4 removals were seen in the samples with initial pH value of 7, 8 and 9 after 9 days of AD with the *p* values of 0.047, 0.0462 and 0.0488 respectively. Necessity of pH

control by circulating an alkali reagent for AD of non/pre-treated pot ale has been suggested for different continuous reactor configurations (Tokuda et al. 1999; Jang et al. 2014; Kida et al. 1999; Harada et al. 1996; Uzal et al. 2003; Gao et al. 2007). When it is not possible such as in operation of batch reactors, increased amount of inoculum with an inoculum substrate ratio of 2:1 based on volatile solids (corresponding to approximately 70 % sludge of overall reactor volume) has been shown to prevent pH drops in batch mode (Barrena et al. 2018). In addition to pH drops in AD of pot ale, a slight pH drop (from 7 to 6.5) when paper waste (Rathaur et al. 2017) and (from 7.5 to 6.71±0.04) when brown seaweed (Montingelli et al. 2016) was used as substrate for lab scale batch mode reactor with a sludge seeding ratio from 60 – 80% on wet basis.

CHAPTER 5 RESULTS OF MICROVAWE AND ULTRASONIC PRE-TREATMENTS WITH HIGHER SLUDGE SEEDING RATIOS

5.1 Introduction

This chapter presents the results of the anaerobic single digestion potential of pot ale and co-digestion of pot ale and draff was tested in various sludge seeding ratios (65 – 90% of total working volume of 400 ml on wet basis) after implementation of a novel hybrid pre-treatment (combined alkaline-microwave and ultrasonic) . Then the results of mathematical modelling and optimisation studies on were conducted in order to selection of optimum substrate for AD.

5.2 Modelling of AD of Pot Ale with Combined Alkaline and Microwave Pre-treatments

Moisture content and the solid analysis of each sample for this experimental design is given in Table 48.

Table 48. TS, VS, moisture content of inoculum, non-treated, alkaline and microwave pre-treated pot ale

Sample Name	TS (g/g sample)	VS (g/g sample)	Moisture %
Inoculum	0.065 ± 0.001	0.039 ± 0.0004	93.5± 0.1
Non-treated pot ale	0.110 ± 0.002	0.105 ± 0.0010	89.0 ± 0.2
Alkaline + microwave (10% Power)	0.059 ± 0.001	0.048 ± 0.0020	94.1 ± 0.1
Alkaline + microwave (30% Power)	0.060 ± 0.001	0.050 ± 0.0002	94.0 ± 0.1
Alkaline + microwave (50% Power)	0.068 ± 0.002	0.055 ± 0.0050	93.2 ± 0.2

The lignocellulosic composition of non-treated, alkaline (1M NaOH) and combined alkaline and microwave (with 10, 30 and 50% of 800 W power settings) pre-treated pot ale are given in Figure 39. Alkaline standalone and in combination with microwave pre-treatment on pot ale had a significant increase in the hemicellulose fraction as well as a significant decrease in removal of lignin from the structure with regard to non-treated (NT) pot ale regardless the power setting of the microwave pre-treatment according to Figure 39. Moreover, alkaline pre-treatment followed by microwave pre-treatment at lower power settings (AP10 and AP30) enhanced the hemicellulose fraction significantly in comparison with alkaline (A) pre-treatment alone with the *p* values of 0.0140 and 0.0005 respectively. Alkaline pre-treatment followed by 30% microwave power (AP30)

significantly influenced cellulose fractions over alkaline pre-treatment alone with a *p* value of 0.0053. The highest hemicellulose release and the cellulose and lignin degradations were seen in AP30.

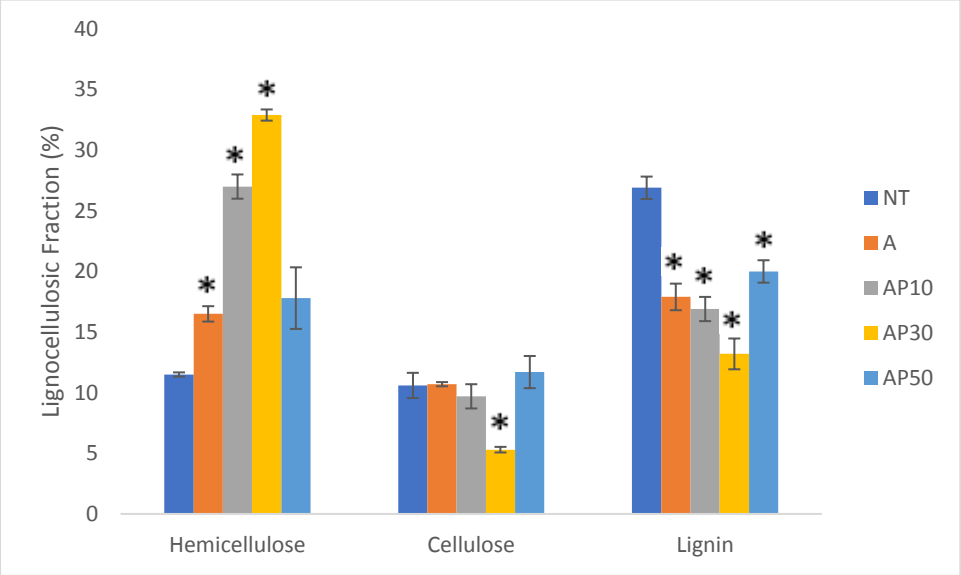


Figure 39. Lignocellulosic structure of pot ale before and after alkaline, combined alkali-microwave pre-treatments.

Box Behnken design matrix for modelling combined alkaline and microwave pre-treatments and recorded responses are given in Table 49. In addition to the design points, biogas results of the non-treated pot ale sample as control group (with 3 different sludge seeding ratios) are given in Table 49 along with corresponding responses. Significant enhancements in CH_4 yields were seen due to applied alkaline and microwave pre-treatments in comparison with the relevant control for all inoculum substrate ratios (I/S). The highest methane yield was achieved when alkaline pre-treatment followed by microwave pre-treatment at 30% power setting (AP30) on pot ale and with an I/S of 5 on dry basis (std no 4 in Table 49). The highest methane yield was found to be 1614 ± 168 ml CH_4/g VS corresponding to a more than 3-fold increase over the control (Control 3 in Table 49). On the other hand, the highest H_2S concentrations (> 1000 ppm) were seen in the samples (std no 1, 3, 5, 7 and Control 1) which had the lowest I/S. The pH values of the all samples remained within the neutral range (6.8 – 7.4) for the entire digestion period for all samples.

Table 49. Design matrix for combined alkaline and microwave pre-treatments

		Factor 1	Factor 2	Factor 3	Response 1	Response 2	Response 3	Response 4	Response 5
Std	Run	A: I/S	B: pH	C: Microwave Power	CH_4 Yield	CO_2 Yield	Biogas Yield	H_2S Generation	CH_4
		-	-	%	ml/g VS	ml/g VS	ml/g VS	ppm	%
Control 1		1	N/A	N/A	227 ± 6	191 ± 2	509 ± 2.4	1049 ± 31	54 ± 1.5
Control 2		3	N/A	N/A	391 ± 23	235 ± 13	709 ± 7.7	734 ± 143	55 ± 2.7
Control 3		5	N/A	N/A	518 ± 1	286 ± 10	925 ± 10.4	701 ± 210	56 ± 0.6
1	2	1	7	30	728 ± 30	554 ± 22	1341 ± 40	1117 ± 231	54 ± 0.7
2	8	5	7	30	1430 ± 50	948 ± 12	2548 ± 69	680 ± 100	56 ± 6.4
3	12	1	9	30	525 ± 115	307 ± 47	854 ± 151	1028 ± 409	56 ± 4.9
4	16	5	9	30	1614 ± 168	867 ± 75	2768 ± 234	727 ± 517	60 ± 0.9
5	10	1	8	10	557 ± 7	480 ± 2	1205 ± 4	1300 ± 81	55 ± 1.7
6	15	5	8	10	764 ± 11	677 ± 15	1714 ± 38	570 ± 66	53 ± 0.7
7	13	1	8	50	685 ± 13	509 ± 15	1255 ± 29	1207 ± 138	55 ± 0.4
8	17	5	8	50	1019 ± 51	632 ± 39	1915 ± 89	576 ± 20	53 ± 6.4
9	9	3	7	10	547 ± 178	445 ± 90	1106 ± 255	624 ± 98	51 ± 2.5
10	4	3	9	10	640 ± 11	459 ± 17	1279 ± 35	645 ± 88	57 ± 2.2
11	6	3	7	50	879 ± 111	630 ± 66	1621 ± 157	839 ± 92	54 ± 1.6
12	11	3	9	50	861 ± 18	462 ± 26	1474 ± 6	948 ± 36	58 ± 0.9
13	14	3	8	30	918 ± 84	712 ± 60	1696 ± 142	795 ± 202	54 ± 0.5
14	3	3	8	30	1058 ± 115	612 ± 37	1838 ± 95	444 ± 102	52 ± 1.5
15	1	3	8	30	1121 ± 72	637 ± 52	1891 ± 87	455 ± 35	53 ± 1.2
16	7	3	8	30	1108 ± 106	747 ± 54	2037 ± 145	766 ± 99	54 ± 0.6
17	5	3	8	30	916 ± 85	621 ± 23	1689 ± 160	958 ± 67	54 ± 2.1

The hydrolysis rate constant as outlined in Eq 32 in Section 3.3.5.6 was then calculated according to the first order kinetic model for the first two and the six days of biomethane yields to assess the influence of the pre-treatments. The results are given in comparison to the corresponding controls in Table 50. Initial reaction pH was found a statistically insignificant parameter therefore, it was disregarded for the experiments performed with the controls.

The reaction rates were found to be significantly lower within first 2 days of digestion over the 2 – 6 days for all experiment (except control 2, 3, std no 3, 6 and 13-17 in Table 50) according to applied paired t-test on the hydrolysis constants. In case of control 2, 3 and std no 3, 4, 6 there was no significant increase in the reaction rates between the first 2 and 6 days of digestion. On the other hand, std no 13-17 (centre point of the design) have shown a significant decrease (*p:*0.0413) in the reaction rates for the same time period. Std 3 the reaction rate was significantly higher than the control for the first 2 days of AD with the hydrolysis constants of 0.1706 ± 0.034 and 0.1204 ± 0.004 day^{-1} (p =0.0328) respectively due to substrate modification by the pre-treatment. A similar situation was seen with the std no 13-17 as std 3 with the hydrolysis constants of 0.3417 ± 0.015 and 0.2165 ± 0.012 day^{-1} (p=0.0373). For these particular samples the conditions of the pre-treatments, alkaline followed by microwave with 30% power setting, had the highest hemicellulose release and cellulose and lignin degradation (Figure 39). Therefore, a higher I/S ratio (in std no 13-17) has accelerated the reaction in the early stage AD more than in std no 3 due to higher fresh organic loading. Therefore, the reaction rate decreased for the std o 13-17 whereas it has shown a slower increase for the std no 3. In terms of AD of non-treated pot ale (controls), increased I/S ratios (3 and 5) reached higher reaction rates within 2 days of digestion than what lower I/S 1 could reach by the end of 6 days. Therefore, no further increase was seen in the reaction rates in Control 2 and 3 (in Table 50)

Table 50. Hydrolysis constants of each sample for the first 2 and the 2nd- 6th days of digestion for modelling microwave pre-treatment

Std No	Sample Name	k_h (day^{-1}) (Day 2)	k_h (day^{-1}) (Day 6)	*p* values
Control 1	I/S 1 NT	0.1204 ± 0.004	0.1995 ± 0.001	0.0223
1	I/S 1 7 P30	0.1173 ± 0.004	0.1898 ± 0.008	0.0017
3	I/S 1 9 P30	0.1706 ± 0.034	0.1952 ± 0.022	0.2451
5	I/S 1 8 P10	0.1190 ± 0.008	0.1898 ± 0.005	0.0006
7	I/S 1 8 P50	0.1236 ± 0.017	0.1944 ± 0.027	0.0086
Control 2	I/S 3 NT	0.2165 ± 0.012	0.2898 ± 0.025	0.0799
9	I/S 3 7 P10	0.2075 ± 0.038	0.2614 ± 0.079	0.0499
10	I/S 3 9 P10	0.1981 ± 0.012	0.2817 ± 0.011	0.0002
11	I/S 3 7 P50	0.2271 ± 0.032	0.3275 ± 0.033	0.0014
12	I/S 3 9 P50	0.1749 ± 0.015	0.2763 ± 0.014	0.0016
13-17	I/S 3 8 P30	0.3417 ± 0.015	0.3208 ± 0.023	0.0413
Control 3	I/S 5 NT	0.2268 ± 0.007	0.2617 ± 0.003	0.5908
2	I/S 5 7 P30	0.2531 ± 0.006	0.3287 ± 0.024	0.0251
4	I/S 5 9 P30	0.2265 ± 0.043	0.3041 ± 0.041	0.0325
6	I/S 5 8 P10	0.2104 ± 0.064	0.2889 ± 0.018	0.0638
8	I/S 5 8 P50	0.2402 ± 0.015	0.3138 ± 0.041	0.0394

Initial VFA concentrations of the supernatant of all samples in form of sludge mixture are given in Table 51. Final VFA concentrations of the same samples were analysed after AD and no VFA was seen in any of the samples indicating that it was fully broken down in the acetogenesis step.

Table 51. VFA concentration prior to AD for modelling alkaline and microwave pre-treatments

Std no	Sample Name	Acetic Acid	Propionic Acid	Isobutyric Acid	Butyric Acid	Isovaleric Acid	Valeric Acid	Total (mM)
1	I/S 1 7 P30	91.29	0.96	0.06	2.23	0.07	0	94.61
2	I/S 5 7 P30	0	0	0	0	0	0	0
3	I/S 1 9 P30	18.89	0	4.08	3.14	4.59	0	30.70
4	I/S 5 9 P30	11.71	0.27	0	0	0	0	11.98
5	I/S 1 8 P10	20.17	0	0	0	0	0	20.17
6	I/S 5 8 P10	10.32	0.14	0	1.11	0	0	11.57
7	I/S 1 8 P50	38.31	0	0	0	0	0	38.3
8	I/S 5 8 P50	49.43	0	0	0	0	0	49.43
9	I/S 3 7 P10	0.44	0	1.19	0	0	0	21.04
10	I/S 3 9 P10	10.02	0.13	0	1.08	0	0	11.23
11	I/S 3 7 P50	42.02	0	0	0	0	0	42.02
12	I/S 3 9 P50	39.57	0	1.23	0	0	0	40.80
13-17	I/S 3 8 P30	139.99	0	0	0	0	0	139.99

Organic matter removals in terms of COD, BOD and SO_4 are given in Table 52. The table is organised by inoculum substrate ratios as it is the major factor of biodegradation. The highest COD and BOD removal were achieved with the std no 4 with a corresponding 69 ± 1.4% and 58 ± 5.0% (Table 52) degradations. Furthermore, the SO_4 removal of std no 4 was found to be the minimum, 40 ± 8.3%, among the samples seeded with an I/S of 5. Std no 4 has also shown the highest CH_4 yield (Table 49) indicating that organic matter was broken down through the methanogenesis path predominantly. Higher COD and BOD removals were seen with microwave pre-treatment with higher power settings (P30 and P50) for the I/S of 1 and 5. For example COD and BOD degradations of std no 7 were found to be significantly higher than std no 5 according to the applied 2 tailed t-test with the *p* values of 0.001 and 0.010 respectively.

In case of the I/S of 5, COD and BOD removal percentages of std 8 were found to be significantly higher than std no 6 with regard to the *p* values of 0.037 and 0.023 respectively. On the other hand, in case of I/S ratio of 3, no significant difference was found in the COD and BOD removals of std no 10 and 12 arising from the pre-treatment at different microwave power. However, std no 9, which was pre-treated at 10% microwave power, had a significantly (*p:* 0.006) higher COD removal than std no 11 (with 50% microwave power pre-treatment setting). In terms of SO_4 removals, various levels ranging from 40 ± 8.3 to 78 ± 0.9% were seen.

Table 52. Organic matter removal percentages of each sample with alkaline and microwave pre-treatment

Std no	Sample Name	*COD_0 (mg/L)	COD (%)	*BOD_0 (mg/L)	BOD (%)	*$(SO_4)_0$ (mg/L)	SO_4 (%)
I/S = 1							
1	I/S 1 7 P30	17093	62 ± 1.8	13760	66 ± 1.7	497	59 ± 8.2
3	I/S 1 9 P30	27520	51 ± 6.0	20960	50 ± 4.1	475	57 ± 4.7
5	I/S 1 8 P10	19840	34 ± 1.7	14027	34 ± 6.0	517	51 ± 1.6
7	I/S 1 8 P50	30667	65 ± 0.2	22507	65 ± 1.3	539	61 ± 2.9
I/S = 3							
9	I/S 3 7 P10	19627	55 ± 1.2	13280	51 ± 3.8	853	78 ± 0.9
10	I/S 3 9 P10	16000	37 ± 6.4	13493	36 ± 5.1	704	52 ± 2.4
11	I/S 3 7 P50	21547	48 ± 1.1	15147	48 ± 5.0	891	60 ± 2.8
12	I/S 3 9 P50	20053	44 ± 4.5	14293	44 ± 6.3	944	60 ± 2.1
13-17	I/S 3 8 P30	19488	38 ± 5.0	14144	33 ± 5.0	880	69 ± 2.6
I/S = 5							
2	I/S 5 7 P30	20133	50 ± 4.5	14240	53 ± 3.9	565	62 ± 3.9
4	I/S 5 9 P30	24747	69 ± 1.4	18293	58 ± 5.0	853	40 ± 8.3
6	I/S 5 8 P10	20213	46 ± 2.8	16053	52 ± 4.3	843	81 ± 0.5
8	I/S 5 8 P50	18293	37 ± 3.5	13483	40 ± 2.6	800	58 ± 2.6

*Average of triplicate results

Experimental results for methane generation were used for modelling and optimisation of the design parameter by Design Expert Software version 10. The experimental data were analysed with stepwise regression to eliminate the insignificant model terms. The best fit was achieved with a quadratic model for identifying the influences of the relevant model terms on response of interest. The developed model was then tested by sequential F-test, lack of fit test and adequacy measures. The statistical analysis of the developed polynomial models for biomethane yield and CO_2 are given in ANOVA Table 53 and Table 55 respectively along with R^2, adjusted R^2 and predicted R^2 values. All adequacy measures were found to be close to 1 indicating a sufficient regression for model development. Moreover, adequate precision was found to be greater than 4 which defines adequate model discrimination. The same software was used to plot the model graphs and to optimise the design parameters. The final mathematical models on CH_4 and CO_2 generation for applied alkaline and microwave pre-treatments are given in Eq 45 and Eq 47, Eq 46 and Eq 48 in terms of coded and actual factors respectively.

Table 53. ANOVA table for methane production quadratic model α= 0.05

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	1.18E+06	3	3.92E+05	17.45	< 0.0001	significant
A- I/S	6.80E+05	1	6.80E+05	30.24	0.0001	
C- Microwave Power (%)	1.10E+05	1	1.10E+05	4.87	0.0459	
C^2	3.87E+05	1	3.87E+05	17.23	0.0011	
Residual	2.92E+05	13	22481.86			
Lack of Fit	2.52E+05	9	27971.49	2.76	0.1704	not significant
Pure Error	40520.8	4	10130.2	17.45	< 0.0001	
Cor Total	1.47E+06	16				

R^2: 0.8010, Adj R^2: 0.7551, Pred R^2: 0.6116, Adeq Precision: 13.783

The ANOVA table indicates that I/S (A), first and second order effect of microwave power (C, C^2) were the significant model terms while initial pH value was insignificant in a 95% confidence interval for AD of pot ale. Moreover, a significant model fit was achieved in accordance with the *p* value (0.1704,) of the applied lack of fit test.

The coding format of the variables are given in Table 54. The coded variables were used to for data prediction and for presentation of different design factors on a same plot in a unitless scale.

Table 54. Variables' coded factors for modelling combined alkaline and microwave pre-treatment

	Coded Factors		
Variable	**-1**	**0**	**1**
A: I/S	1	3	5
B: Initial pH	7	8	9
C: Microwave Power (%)	10	30	50

Final Equation in terms of coded factors:

$$\mathbf{CH_4(ml/gVS) = 1046.44 + 291.5\ A + 117\ C - 302.44\ C^2} \quad \text{Eq 45}$$

Final Equation in terms of actual factors:

$$\mathbf{CH_4(ml/gVS) = -246.8 + 145.6\ I/S + 51.22 \times MW\ Power - 0.76 \times MW\ Power^2} \quad \text{Eq 46}$$

Furthermore, the coefficients of the design factors in the final equation in terms of coded factors (Eq 45) gives the influence strength of the parameters on methane yield. For this design, the second order effect of microwave power (C^2) had the most powerful influence (negative) on methane yield. It was then followed by first order effects of I/S (A) and microwave power (C).

According to the ANOVA for analysing CO_2 production model (Table 55), the significant model terms were I/S (A), second order effect of microwave power (C^2) as well as initial pH values (B). The first order effect of microwave power was included to the model to support the hierarchy. The model significance was tested with F-test (*p:* 0.0002) and sequential lack of fit test (*p:* 0.1926) to ensure developed model was significant with an insignificant lack of fit.

Table 55. ANOVA table for CO_2 production quadratic model α= 0.05

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	303782.4	4	75945.59	12.62	0.0002	significant
A-I/S	202884.5	1	202884.5	33.71	8.39E-05	
B-Initial pH	58140.5	1	58140.5	9.66	0.0090	
C-Microwave Power (%)	98	1	98	0.02	0.9005	
C^2	42659.38	1	42659.38	7.09	0.0207	
Residual	72213.39	12	6017.782			
Lack of Fit	60312.19	8	7539.024	2.53387	0.1926	not significant
Pure Error	11901.2	4	2975.3			
Cor Total	375995.8	16				

R^2: 0.8079, Adj R^2: 0.7439, Pred R^2: 0.5592, Adeq Precision: 12.0657

The final CO_2 production model in terms of coded factors (Eq 47) identifies the influence strength of the design parameters on the response of interest which was A > -C2 > -B > -C.

Final equation in terms of coded factors:

$$\boldsymbol{CO_2(ml/gVS) = 662.11 + 159.25\,A - 85.25\,B - 3.5\,C - 100.36\,C^2} \quad \text{Eq 47}$$

Final equation in terms of actual factors:

$$\mathbf{CO_2(ml/gVS) = 884.7 + 78.6I/S - 85\,pH_0 + 14.9 \times MW\ Power - 0.3MW\ Power^2} \quad \text{Eq 48}$$

The final equations in terms of actual factors for CH_4 (Eq 46) and CO_2 (Eq 48) generated were used for data prediction for validation.

5.2.1 Validation of Developed Models

Developed models were diagnosed by the software as part of the post statistical analysis. Normal plot of the residuals and the predicted vs actual plots for methane generation are given in Figure 40 and Figure 41. The normal distribution of the residuals shows that ANOVA can be used for statistical analysis of the models. Comparison between the actual experimental data and the predicted data by the developed model for methane generation is given in Figure 41. A homogeneous distribution of the design points close to the diagonal line was seen indicating that model is adequate. A similar trend in both normal plot of residuals and actual vs predicted plot is seen in CO_2 generation as well as the developed CH_4 and CO_2 models with combined alkaline and ultrasonic pre-treatments.

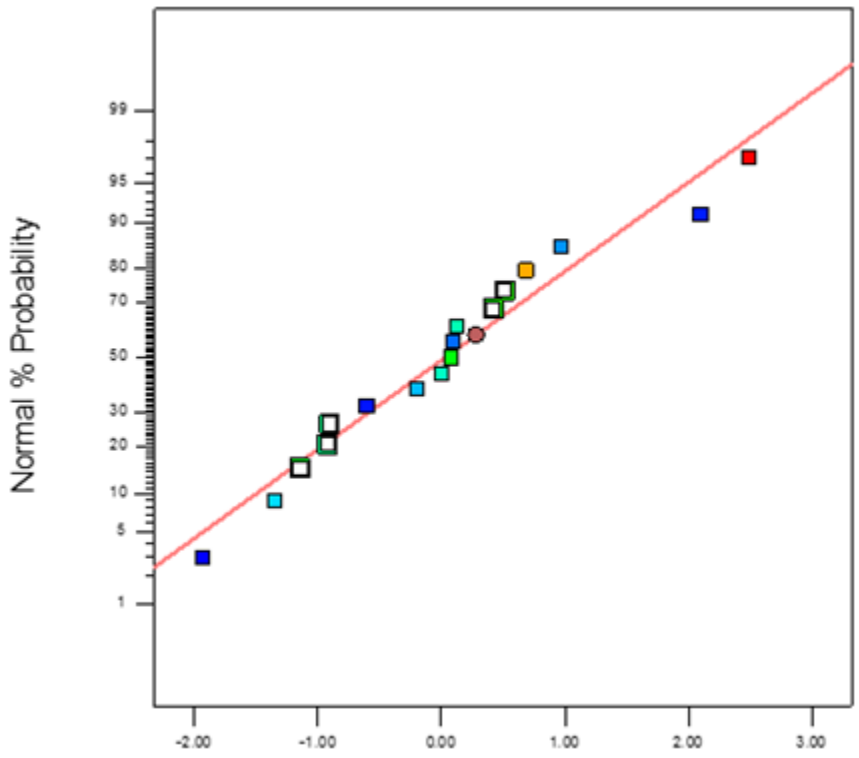


Figure 40. Normal plot of the residuals on methane generation (ml/g VS)

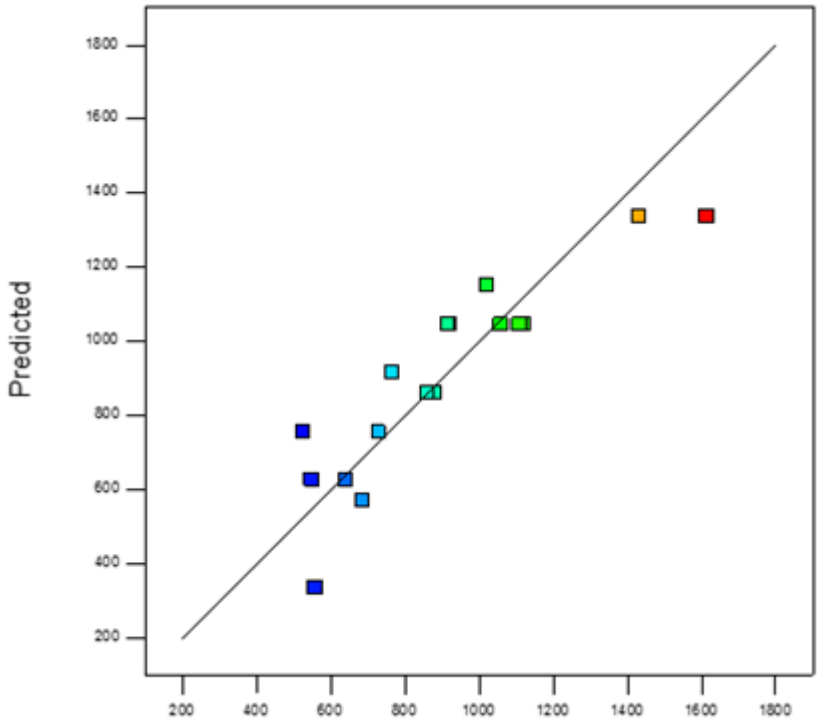


Figure 41. Scatter diagram for methane generation with combined alkaline and microwave pre-treatment, design points coloured bases on methane yield

In addition to the post analysis provided by the software, mathematical models were challenged by independent experiments. Three validation points (which were not used for model development) were chosen and the results of the experiments are given in Table 56 along with the predicted values by the models for CH_4 and CO_2 generations. The difference between the predicted and the experimental results are then statistically analysed by t-test (2 tailed distribution with unequal variance). There was no significant difference between the experimental data and the predicted data in any case and the developed models were validated within the 95% level of confidence.

Table 56. Results of validation experiments for alkaline and microwave pre-treatment

Validation Points	CH_4 Yield (ml/g VS)		CO_2 Yield (ml/g VS)	
	Experimental	**Predicted**	**Experimental**	**Predicted**
I/S2 8 P30	825 ± 14	897	557 ± 61	537
	p: 0.0878		*p:* 0.1498	
I/S4 8 P30	1067 ± 22	1189	525 ± 36	694
	p: 0.0816		*p:* 0.0605	
I/S4 8 P50	980 ± 16	997	453 ± 26	512
	p: 0.3633		*p:* 0.0735	

5.2.2 Model Graphs

The perturbation plots of modelling AD of pot ale with combined alkaline and microwave pre-treatments are given for methane and CO_2 generations in Figure 42 1 and 2 respectively. The significant design parameters I/S (A) and microwave power (C) were plotted in terms of coded factors by keeping the other factor constant (at the centre point by default) at a time. I/S (factor A) has shown a linear relationship with methane yield. However, microwave power (factor C) has a polynomial relationship with methane generation with a peak in the centre area corresponding to around 30% microwave power setting. Factor A and C have shown similar effects on CO_2 generation as CH_4. In addition to that, initial pH of the reaction (factor B) was a significant parameter on CO_2 generation. It had a reverse effect compared with factor A by achieving lower CO_2 production at higher pH values.

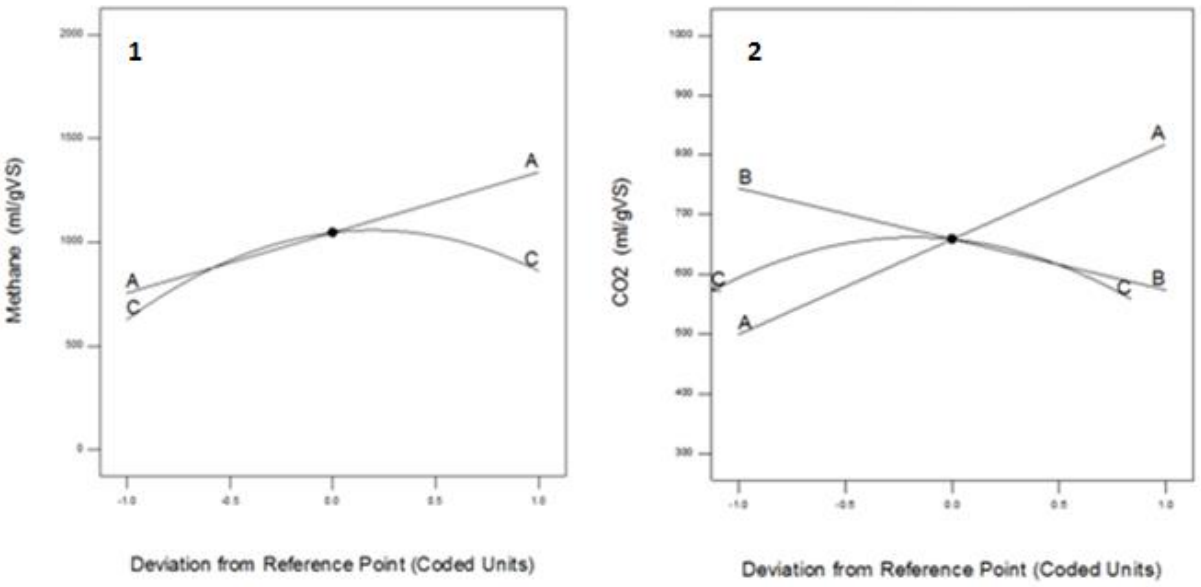


Figure 42. Perturbation graphs of the 1. CH_4 and 2. CO_2 yields for combined alkaline-microwave pre-treatment

The contour graphs of CH_4 and CO_2 generations are given in Figure 43 1 and 2 respectively. Mainly polynomial curves were seen in both graphs. The higher CH_4 and CO_2 productions were achieved with the conditions of higher I/S ratios and mid-range (approximately 35% of 800W) microwave power.

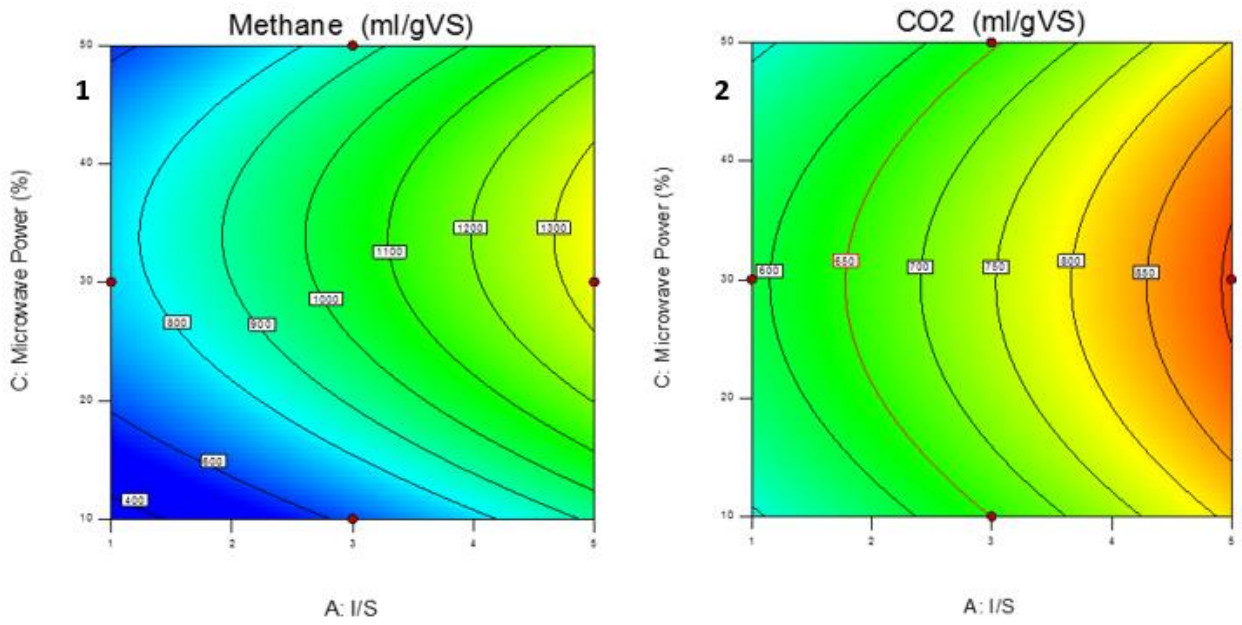


Figure 43. Contour graph for 1. CH_4 and 2. CO_2 yields for combined alkaline-microwave pre-treatment

5.2.3 Optimisation

A combination of numerical and graphical optimisation was performed under two different constraints on design factors to identify the optimum digestion conditions as well as minimum energy consumption in the pre-treatment step. In the first optimisation approach all design factors were kept in range while methane generation was aimed to maximise with an importance level of 5. Carbon dioxide and H_2S generations on the other hand was minimised with the importance levels of 3 and 5 respectively. The conditions of the first optimisation approach are outlined in Table 57.

Table 57 Numerical optimisation conditions for approach 1 for combined alkaline-microwave pre-treatment

Variable	Goal	Lower Limit	Upper Limit	Importance
A: I/S	in range	1	5	3
B: Initial pH	in range	7	9	3
C: Microwave Power (%)	in range	10	50	3
CH_4 (ml/g VS)	maximise	525	1614	5
CO_2 (ml/g VS)	minimise	307	948	3
H_2S (ppm)	minimise	444	1300	5

The solutions of the numerical optimisation approach as a function of desirability is given in Table 58. The upper and lower limits of all responses of interest in Table 58 were used to set the highest and the lowest yields for graphical optimisation.

Table 58. Numerical optimisation solutions for approach 1 for combined alkaline and microwave pre-treatment

No	I/S	Initial pH	Microwave Power (%)	CH_4 (ml/g VS)	CO_2 (ml/g VS)	H_2S (ppm)	Desirability
1	4.6	8.9	39	1274	684	630	0.6424
2	4.6	8.9	39	1273	683	630	0.6424
3	4.6	8.9	39	1267	680	629	0.6423
4	4.7	8.9	38	1291	694	630	0.6419
5	4.8	8.9	40	1297	696	632	0.6417
6	4.9	8.9	41	1294	695	634	0.6410
7	4.8	8.9	46	1211	654	632	0.6359

The result of the graphical optimisation for approach 1 is given in Figure 44. The optimum area (marked in yellow) was identified by overlapped limits of each interest of response (1211 – 1274 ml/g VS for methane, 653 – 694 ml/g VS for CO_2 and 630 – 634 ppm for H_2S). In order to achieve maximum CH_4 with AD, the optimum microwave power setting was

found to be approximately 40% of 800W overall power, while the most the most desired inoculum substrate ratio range was determined as 4.7 – 4.9 dry basis when there were no constraints applied on design parameters. The pH was found to be optimum at 8.9.

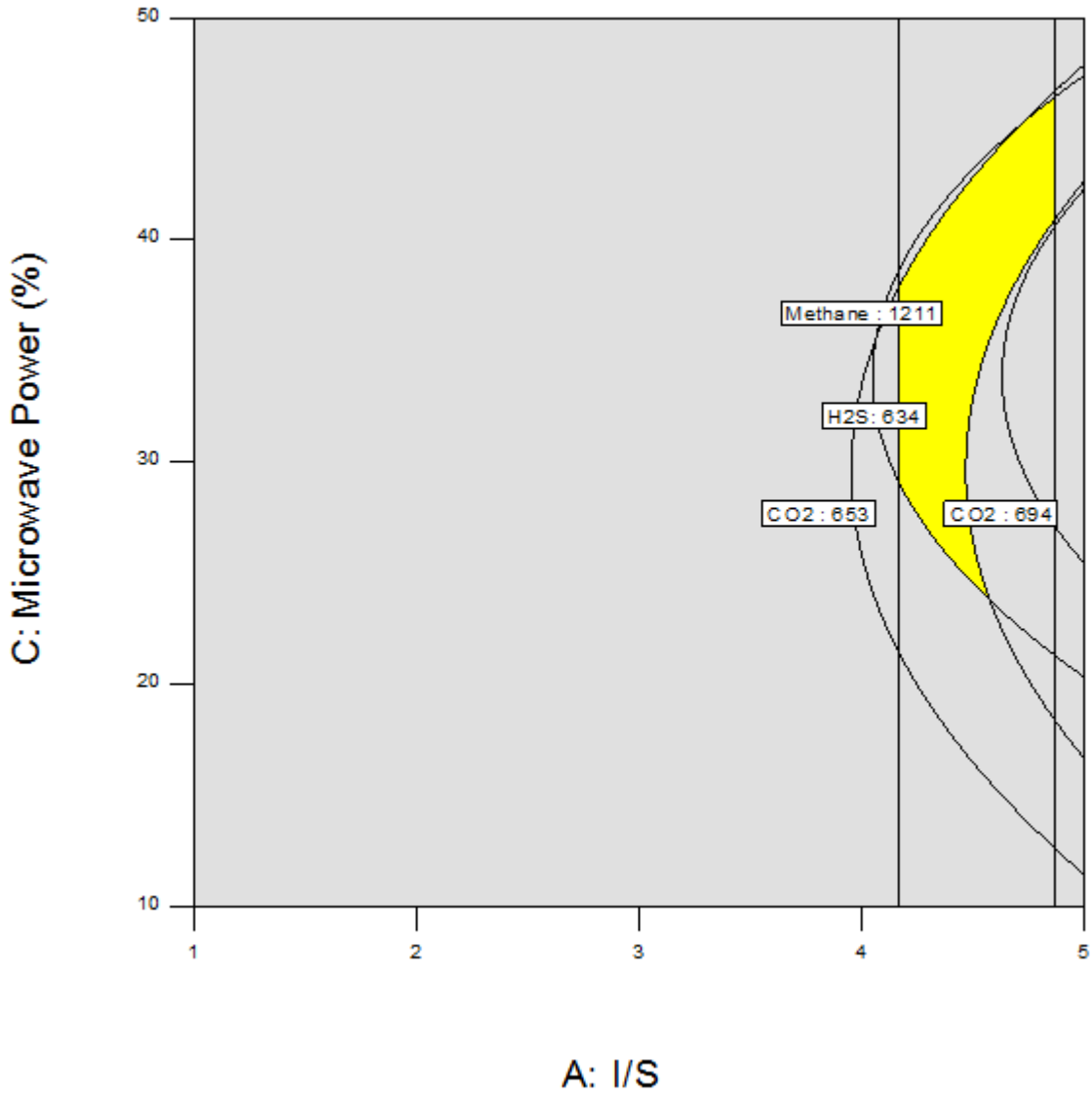


Figure 44. Graphical optimisation for optimisation approach 1 for combined alkaline and microwave pre-treatment at pH 8.9

The second numerical optimisation with the constraints in process parameters is outlined in Table 59. As opposed to the first optimisation plan, the design factors A (inoculum substrate ratio) and C (microwave power) were aimed to minimise with an importance 5 whereas the initial pH value was kept in range since it was not a significant parameter for modelling of CH_4 yield. The goals for the interest responses were kept the same as approach 1.

Table 59. Numerical optimisation for approach 2 combined alkaline and microwave pre-treatment

Variable	Goal	Lower Limit	Upper Limit	Importance
A: I/S	minimise	1	5	5
B: Initial pH	in range	7	9	3
C: Microwave Power (%)	minimise	10	50	5
CH_4 (ml/g VS)	maximise	525	1614	5
CO_2 (ml/g VS)	minimise	307	948	3
H_2S (ppm)	minimise	444	1300	5

The numerical optimisation solutions for the second optimisation criteria are given in Table 60. The upper and lower limits of the CH_4, CO_2 and H_2S were used to create graphical optimisation. The result of the graphical optimisation is given in Figure 45.

Table 60 Numerical optimisation solutions for approach 2 combined alkaline and microwave pre-treatment

No	I/S	Initial pH	Microwave Power (%)	CH_4 (ml/g VS)	CO_2 (ml/g VS)	H_2S (ppm)	Desirability
1	2.8	8.9	22	937	552	738	0.5591
2	2.8	8.9	23	939	552	739	0.5591
3	2.9	8.9	22	939	553	736	0.5591
4	2.8	8.9	23	950	554	740	0.5588
5	2.7	8.9	23	939	547	753	0.5585
6	2.8	8.9	24	950	551	748	0.5584
7	2.7	8.9	21	892	537	753	0.5572

The boundaries of the graphical optimisation (891 – 950 ml/g VS for methane, 536 – 553 ml/g VS for CO_2 and 735 – 950 ppm for H_2S) were initially determined by using the solutions of the numerical optimisation. With the applied constraints, the optimum condition for microwave pre-treatment was found to be approximately 20% of overall power of 800 W. In addition, the optimum inoculum substrate ratio was determined as 2.8 ± 0.1 on dry basis which corresponds to approximately 80% sludge use on wet basis.

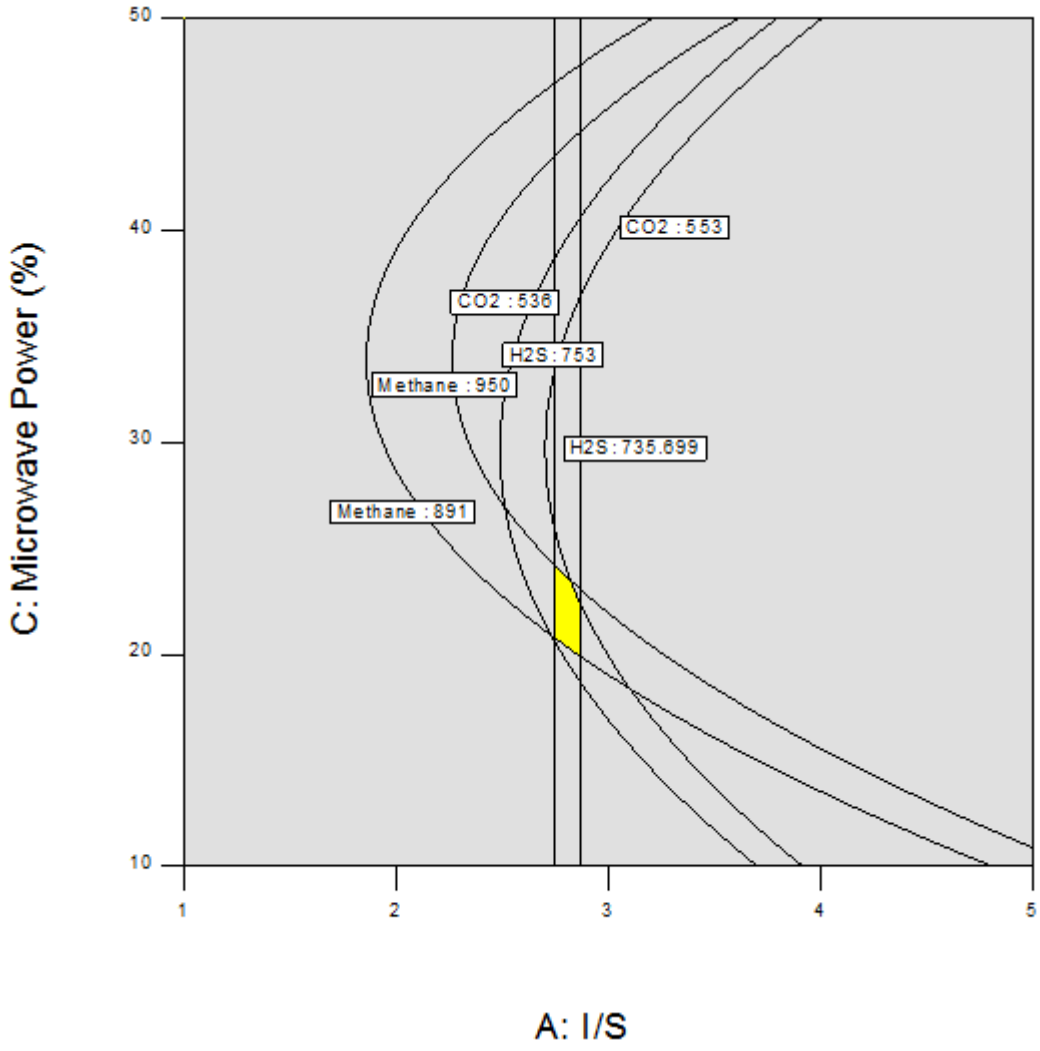


Figure 45. Graphical optimisation for approach 2 combined alkaline and microwave pre-treatment at pH 8.9

The impact of combined alkaline and microwave pre-treatment was assessed on lignocellulosic structure of pot ale for the first time. A significant lignin degradation was observed up to 14% removal from the structure. The most powerful design parameters on methane yield were determined as I/S and microwave power. The highest methane yield was found to be 1614±168 ml/g VS (more than 8-fold increase over the relevant control) with an I/S ratio at 5 on dry basis (90% on wet basis) and 30% microwave power setting. The increase was attributed to the impact on pre-treatment on the lignocellulosic structure as well as the addition of acetic acid for pH adjustment. The optimum process parameters were identified as I/S of 4.5 and microwave power 39 – 46% of overall 800W.

Developed models for AD of pot ale after implementation of alkaline and microwave pre-treatment were summarised in Table 61. Equations in terms of actual factors were used

for data prediction for the validation step of the models as well as the optimisation step. The design parameters, I/S, initial reaction pH and microwave power were coded as A, B and C respectively which enables comparing power of influence on the responses.

Table 61 Summary of developed model of AD of pot ale after combined alkaline-microwave pre-treatment

$* \mathbf{CH_4}(\boldsymbol{ml/gVS}) = \mathbf{1046.44 + 291.5\ A + 117\ C - 302.44\ C^2}$
$\mathbf{CH_4}(\boldsymbol{ml/gVS}) = \mathbf{-246.8 + 145.6\ I/S + 51.22 \times MW\ Power - 0.76 \times MW\ Power^2}$
$* \mathbf{CO_2}(\boldsymbol{ml/gVS}) = \mathbf{662.11 + 159.25\ A - 85.25\ B - 3.5\ C - 100.36\ C^2}$
$\mathbf{CO_2}(\boldsymbol{ml/gVS}) = \mathbf{884.7 + 78.6I/S - 85\ pH_0 + 14.9 \times MW\ Power - 0.3MW\ Power^2}$

* Equations in terms of coded factors

5.3 Modelling of first 2 Days of AD of Pot Ale with Combined Alkaline and Ultrasonic Pre-treatments

The results of the solid analysis and the moisture content of each sample within the design matrix as well as non-alkaline (NA), 1.5 M and 3 M NaOH treated pot ale and sludge are given in Table 62.

Table 62. Total solids, volatile solids and moisture content of non-treated and pre-treated pot ale

Exp No	Sample Name	TS (g/g sample)	VS (g/g sample)	Moisture %
Inoculum	Sludge	0.062 ± 0.002	0.046 ± 0.0002	93.8 ± 0.010
Control 1	NA	0.072 ± 0.003	0.069 ± 0.0001	92.8 ± 0.002
Control 2	1.5M	0.071 ± 0.001	0.057 ± 0.0011	93.1 ± 0.093
Control 3	3M	0.074 ± 0.002	0.055 ± 0.0033	92.6 ± 0.007
1	1.5M 0.4A 1h	0.076 ± 0.001	0.055 ± 0.0000	92.4 ± 0.000
2	1.5M 1A 1h	0.076 ± 0.000	0.056 ± 0.0005	92.4 ± 0.029
3	1.5M 0.4A 3h	0.081 ± 0.001	0.057 ± 0.0008	91.9 ± 0.030
4	1.5M 1A 3h	0.083 ± 0.000	0.066 ± 0.0014	91.7 ± 0.015
5	NA 0.4A 2h	0.084 ± 0.004	0.080 ± 0.0004	91.6 ± 0.012
6	NA 1A 2h	0.084 ± 0.001	0.080 ± 0.0001	91.6 ± 0.005
7	3M 0.4A 2h	0.087 ± 0.001	0.065 ± 0.0002	91.3 ± 0.007
8	3M 1A 2h	0.089 ± 0.001	0.061 ± 0.0099	91.1 ± 0.001
9	NA 0.7A 1h	0.081 ± 0.001	0.078 ± 0.0009	91.9 ± 0.054
10	NA 0.7A 3h	0.081 ± 0.002	0.062 ± 0.0045	91.9 ± 0.177
11	3M 0.7A 1h	0.085 ± 0.001	0.061 ± 0.0029	91.5 ± 0.052
12	3M 0.7A 3h	0.090 ± 0.002	0.065 ± 0.0012	91.0 ± 0.073
13-17	1.5M 0.7A 1.5h	0.077 ± 0.002	0.055 ± 0.0004	92.3 ± 0.056

Modifications in the lignocellulosic structure of pot ale due to implementations of 3M NaOH pre-treatment (3M) and 70% amplitude ultrasonic pre-treatment for 3 hours (NA 0.7A 3h std no 10 in Table 62) standalone as well as combination with 3M NaOH pre-treatment (3M 0.7A 3h std no 12 in Table 62) in comparison with non-alkalised (NA) pot ale are given in Figure 46. Neither ultrasonic pre-treatment alone (NA 0.7A 3h) nor in conjunction with 3M NaOH pre-treatment (3M 0.7A 3h) had a significant impact on lignin and cellulose removals from the structure. In addition, no significant increase was seen in the hemicellulose content regarding to these pre-treatments. 3M alkaline pre-treatment in isolation on the other hand, had a significant increase in the hemicellulose content (with a *p* value of 0.0043) and a significant decrease in the cellulose fraction (with a *p* value of 0.0402) in comparison with non-alkalised pot ale sample (NA). A 7.7 ± 0.6% decrease in

the hemicellulose content of pot sample when 3M alkaline pre-treatment followed by 0.7A ultrasonic pre-treatment for 3h.

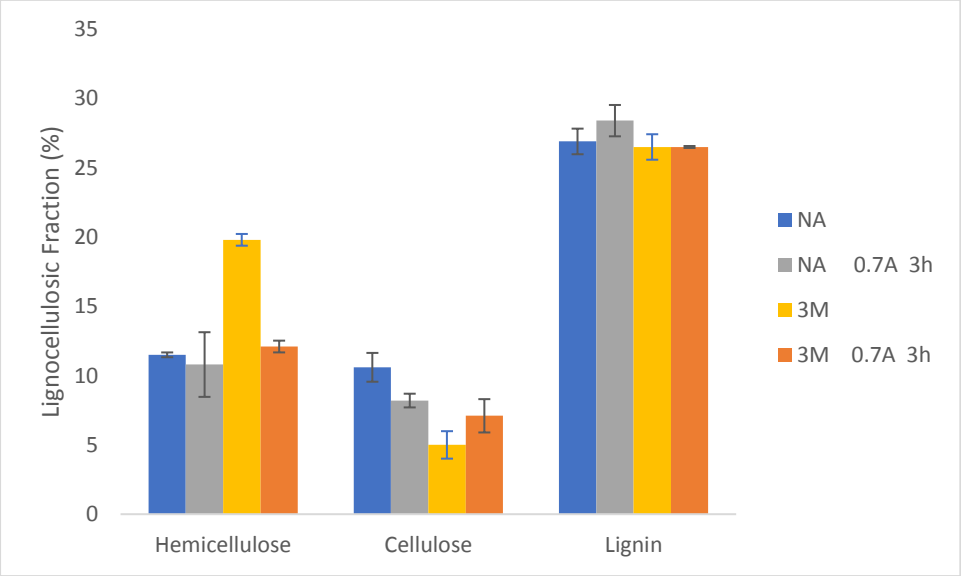


Figure 46. Lignocellulosic structure of non-treated, US treated and combined alkaline US treated pot ale

Box Behnken design matrix and controls, on non-alkalised (Control 1), 1.5 M (Control 2) and 3 M NaOH (Control 3) pre-treated pot ale with recorded responses are given in Table 63. The effects of diverse alkaline and ultrasonic pre-treatment conditions on the CH_4, CO_2 yields and H_2S production for the first 2 days of AD was modelled and statistically analysed using Design Expert version 10. Moreover, cumulative biogas, CH_4, CO_2 yields and H_2S generation are given in Table 63.

No significant difference was found in the cumulative methane and biogas yields between the design points (std no 1 – 17) and their individual comparison with the corresponding controls regarding the applied pre-treatments. However, the conditions of the ultrasonic pre-treatment had a significant negative impact reaction kinetics the first 2 days of AD, given in Table 64. For example, when 1.5 M NaOH pre-treatment was applied prior to ultrasonic pre-treatment, in case of std no 1, 2, 3, 4 methane yield for the first 2 days of AD was found to be significantly lower than 1.5 M NaOH pre-treatment standing alone (Control 2) regardless of the different conditions of the ultrasonic pre-treatment in Table 63. On the other hand, ultrasonic pre-treatment on std no 13 – 17 did not have a significant impact on methane yield in comparison to Control 2 (p: 0.4825).

In terms of H_2S production, a significant model fit was seen for the first time with the data gathered within the first 2 days of AD. Ultrasonic pre-treatment standalone as well as in combination with alkaline pre-treatment led significantly lower levels of H_2S generation for the first 2 days of AD on comparison with the controls. For instance, applied ultrasonic pre-treatment under different conditions after 1.5 M NaOH had no H_2S generation for std no 1, 2, 3, 4 and the std no 13 – 17 (in Table 63) had a significantly lower H_2S production (p: 0.0467) than Control 2. Similarly, std no 5, 6, 9 and 10 had shown significantly lower H_2S generation due to the application of ultrasonic pre-treatment in comparison with Control 1 with the *p* values of 0.025, 0.029, 0.029 and 0.021 respectively within first 2 days of AD. However, implementation of ultrasonic pre-treatment after 3 M NaOH pre-treatment (std no 7, 8, 11 and 12 in Table 63) did not have a significant impact on reduction of H_2S generation with regards to 3 M NaOH pre-treatment in isolation (Control 3 in Table 63). Treatment at a higher amplitude ratio (in case of std no 8) caused almost a 2-fold rise in the H_2S formation in comparison with std no 7 (*p*: 0.048).

Table 63. Design for modelling ultrasonic pre-treatment with recorded responses

		Factor 1	Factor 2	Factor 3	Response 1	Response 2	Response 3	Response 4	Response 5	Response 6	Response 7	Response 8
Std	**Run**	**A: Amplitude Ratio**	**B: Exposure Time**	**C: Alkaline Dose**	**CH_4 Yield 2 days**	**CO_2 Yield 2 days**	**2 days H_2S production**	Cumulative Biogas Yield	Cumulative CH_4 Yield	Cumulative CO_2 yield	Cumulative H_2S Production	CH_4
		-	h	M	ml/g VS	ml/g VS	ppm	ml/g VS	ml/g VS	ml/g VS	ppm	%
Control 1		N/A	N/A	0	297 ± 21	265 ± 23	200 ± 14	1340 ± 42	724 ± 46	440 ± 11	738 ± 161	54 ± 1.7
Control 2		N/A	N/A	1.5	296 ± 50	232 ± 21	169 ± 13	1356 ± 3	625 ± 2	392 ± 4	354 ± 11	47 ± 0.1
Control 3		N/A	N/A	3	324 ± 27	294 ± 43	175 ± 17	1468 ± 35	806 ± 43	482 ± 36	377 ± 28	55 ± 1.7
1	3	0.4	1	1.5	44 ± 3	28 ± 1	0 ± 0	1356 ± 26	722 ± 22	401 ± 11	189 ± 47	53 ± 1.6
2	14	1	1	1.5	34 ± 8	21 ± 6	0 ± 0	1333 ± 28	705 ± 45	408 ± 3	228 ± 86	53 ± 3.6
3	10	0.4	3	1.5	42 ± 10	27 ± 10	0 ± 0	1346 ± 18	691 ± 78	404 ± 22	195 ± 94	51 ± 5.1
4	13	1	3	1.5	22 ± 2	14 ± 3	0 ± 0	1174 ± 21	642 ± 12	366 ± 8	241 ± 64	55 ± 2.0
5	7	0.4	2	0	228 ± 14	239 ± 19	72 ± 8	1097 ± 56	567 ± 60	375 ± 30	307 ± 97	52 ± 3.0
6	12	1	2	0	238 ± 19	255 ± 15	73 ± 14	1145 ± 98	590 ± 126	397 ± 48	289 ± 116	51 ± 5.1
7	16	0.4	2	3	240 ± 16	227 ± 5	77 ± 9	1573 ± 108	816 ± 4	462 ± 12	268 ± 99	52 ± 3.5
8	17	1	2	3	273 ± 3	244 ± 7	135 ± 26	1443 ± 17	810 ± 21	455 ± 10	404 ± 132	56 ± 1.1
9	11	0.7	1	0	252 ± 3	260 ± 10	88 ± 19	1225 ± 12	639 ± 7	417 ± 5	326 ± 63	52 ± 1.0
10	8	0.7	3	0	333 ± 5	404 ± 8	61 ± 9	1728 ± 18	876 ± 26	628 ± 6	315 ± 68	51 ± 1.6
11	4	0.7	1	3	255 ± 39	233 ± 25	72 ± 45	1241 ± 102	630 ± 157	392 ± 67	212 ± 160	50 ± 5.3
12	1	0.7	3	3	256 ± 30	234 ± 21	103 ± 11	1226 ± 93	605 ± 70	373 ± 29	265 ± 33	50 ± 5.6
13	6	0.7	2	1.5	252 ± 17	238 ± 10	80 ± 9	1526 ± 39	801 ± 104	468 ± 45	282 ± 118	53 ± 6.0
14	15	0.7	2	1.5	276 ± 9	250 ± 5	82 ± 7	1561 ± 65	894 ± 55	496 ± 14	386 ± 24	57 ± 2.5
15	9	0.7	2	1.5	250 ± 15	234 ± 17	94 ± 6	1482 ± 27	627 ± 74	410 ± 31	204 ± 34	42 ± 1.2
16	5	0.7	2	1.5	259 ± 17	247 ± 8	75 ± 11	1540 ± 15	818 ± 14	477 ± 27	134 ± 17	53 ± 0.7
17	2	0.7	2	1.5	230 ± 4	239 ± 3	73 ± 15	1532 ± 17	847 ± 94	485 ± 16	412 ± 21	55 ± 1.7

The reaction rates within the first 2 days of digestion for non-alkalised (NA) and 3M NaOH pre-treated prior to ultrasonic pre-treatment was found to be significantly higher according to applied 2 tailed t-test (*p* values are given in Table 64) than the reaction rates within 2^{nd} – 6^{th} days of AD. 1.5M NaOH pre-treatment followed by ultrasonic pre-treatment under different conditions showed a significantly slower reaction rates for the first 2 days of AD in comparison with digestion rate of 2^{nd} – 6^{th} days of AD except for control 2 (only 1.5M NaOH pre-treated) and std no 13-17 (1.5M NaOH pre-treatment in combination with ultrasonic pre-treatment at 70% amplitude for 1.5h). On the other hand, the first 2 days reaction rate for std no 13-17 (0.1916 ± 0.038) was found to be significantly lower than Control 2 (0.2819 ± 0.026) with a *p* value of 0.0491 indicating that implemented ultrasonic pre-treatment after the alkaline pre-treatment triggered inhibitions in the reactor (Table 64). However, the second day methane yield of std 13-17 was not lower than Control 2 (Table 63).

There was no significant difference in the first 2 days reaction kinetics between ultrasonic pre-treated samples (std no 5, 6, 9, 10) with Control 1 as well as between std no 7, 8, 11, 12 and Control 3 (Table 64) indicating no significant modifications were achieved with applied pre-treatment in lignocellulosic structure. Both ultrasonic pre-treatment in isolation NA 0.7A 3h (std no 10) and in combination with alkaline pre-treatment 3M 0.7A 3h (std no 12) did not show significant delignification (Figure 46). Similarly, when ultrasonic pre-treatment combined with 1.5 M NaOH pre-treatment, the first 2 days hydrolysis rate constants of std no 1, 2, 3, 4 and 13 – 17 were found to be significantly lower than Control 2 (Table 64). However, the first 2 days methane yield of std no 1, 2, 3, 4 were found to be significantly lower than Control 2 whereas there was no significant difference in the methane yield for the std no 13 – 17. It was related to the conditions of the ultrasonic pre-treatment. According to Figure 47, the specific energy delivered to only the std no 13 – 17 (63000 kJ/kg VS) (among ultrasonic 1.5 M NaOH pre-treatment sample group) was found to be within the range of 55000 – 85000 which was identified as a range for significantly higher intercellular compound release due to application of combined alkaline and ultrasonic pre-treatments.

Table 64. Hydrolysis constant of each sample for first 2 and 2nd – 6th days of digestion for ultrasonic pre-treatment.

Std No	Sample Name	k_h (day^{-1}) (Day 2)	k_h (day^{-1}) (Day 6)	*p* values
Control 1	NA Pot ale	0.2492 ± 0.018	0.1689 ± 0.016	0.0432
5	NA 0.4A 2h	0.2593 ± 0.025	0.0936 ± 0.004	0.0065
6	NA 1A 2h	0.2689 ± 0.065	0.0983 ± 0.002	0.0453
9	NA 0.7A 1h	0.2495 ± 0.001	0.0933 ± 0.001	0.0000
10	NA 0.7A 3h	0.2394 ± 0.005	0.1024 ± 0.002	0.0001
Control 2	1.5M Pot ale	0.2819 ± 0.026	0.0851 ± 0.006	0.0493
1	1.5M 0.4A 1h	0.0312 ± 0.002	0.1483 ± 0.005	0.0001
2	1.5M 1A 1h	0.0246 ± 0.005	0.1748 ± 0.008	0.0000
3	1.5M 0.4A 3h	0.0313 ± 0.006	0.1730 ± 0.017	0.0020
4	1.5M 1A 3h	0.0178 ± 0.002	0.1576 ± 0.006	0.0003
13-17	1.5M 0.7A 1.5h	0.1916 ± 0.038	0.1192 ± 0.007	0.0115
Control 3	3M Pot ale	0.2571 ± 0.010	0.0886 ± 0.006	0.0053
7	3M 0.4A 2h	0.1746 ± 0.014	0.0915 ± 0.010	0.0015
8	3M 1A 2h	0.2061 ± 0.008	0.1084 ± 0.002	0.0016
11	3M 0.7A 1h	0.2667 ± 0.041	0.1040 ± 0.005	0.0197
12	3M 0.7A 3h	0.2774 ± 0.048	0.1008 ± 0.007	0.0215

The specific energy delivered by ultrasonic pre-treatment to each sample is given in Table 65 along with ultrasound dose, density and intensity. Moreover, the degree of disintegration levels in std no 10 and 12 (representing ultrasonic pre-treatment alone and in combination with 3M NaOH pre-treatment) were monitored by measuring the increase in COD concentrations due to the intercellular compound release based on specific energy delivered per gram volatile solids.

Table 65. Details of ultrasound pre-treatment for each sample

Std no	Sample Name	Specific Energy (kJ/kg VS)	Ultrasound Dose (J/L)	Ultrasound Density (W/L)	Ultrasound Intensity (W/cm^2)
1	1.5M 0.4A 1h	18000	1440	0.4	0.042
2	1.5M 1A 1h	45000	3600	1	0.105
3	1.5M 0.4A 3h	54000	4320	0.4	0.042
4	1.5M 1A 3h	135000	10800	1	0.105
5	NA 0.4A 2h	36000	2880	0.4	0.042
6	NA 1A 2h	90000	7200	1	0.105
7	3M 0.4A 2h	36000	2880	0.4	0.042
8	3M 1A 2h	90000	7200	1	0.105
9	NA 0.7A 1h	31500	2520	0.7	0.074
10	**NA 0.7A 3h**	**94500**	**7560**	**0.7**	**0.074**
11	3M 0.7A 1h	31500	2520	0.7	0.074
12	**3M 0.7A 3h**	**94500**	**7560**	**0.7**	**0.074**
13-17	1.5M 0.7A 1.5h	63000	5040	0.7	0.074

The degree of disintegration was scanned for std no 10 and 12 based on specified energy delivered (from 10000 to 95000 kJ/kg VS) in order to evaluate intercellular compound release regarding application of ultrasonic pre-treatment both in isolation and in combination with 3 M NaOH pre-treatment and the result is given in Figure 47. There was no significant difference arising from the alkaline pre-treatment in the degree of disintegration with specific energy delivered up until 55000 kJ/kg VS. Combined 3 M NaOH and 70% amplitude ultrasonic pre-treatment for 3 h has showed a significantly higher disintegration levels than ultrasonic pre-treatment standalone within the specific energy range of 55000 to 85000 kJ/kg VS. Subsequently, no significant difference was seen within the range of 85000 – 95000 kJ/kg VS with regards to 3 M NaOH pre-treatment.

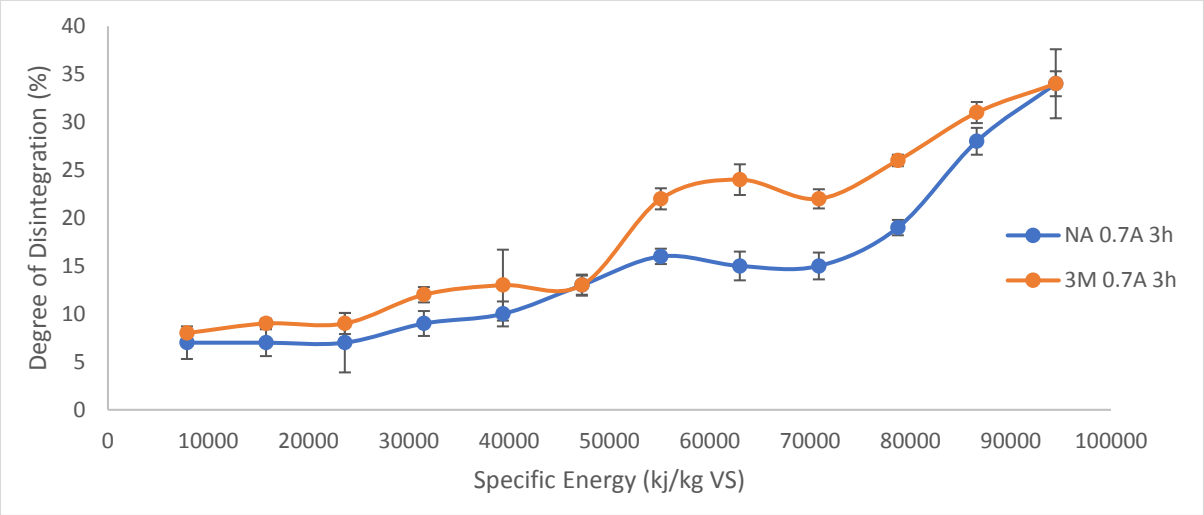


Figure 47. Degree of disintegration based on specific energy delivered during ultrasonic pre-treatment

The VFA concentration of the supernatant of all samples in form of pot ale sludge mixture prior to AD is given in Table 66. The supernatant of the same samples was also analysed after AD and no VFA was detected indicating that intermediate products were fully broken down.

Table 66. VFA concentrations of all samples and controls before AD

Std no	Sample Name	Acetic Acid	Propionic Acid	Isobutyric Acid	Butyric Acid	Isovaleric Acid	Valeric Acid	Total (mM)
C 1	NT	38.01	0	0	0	0	0	38.01
C 2	1.5M A	16.96	0	0	0	0	0	16.96
C 3	3M A	12.08	0	0	0	0	0	12.08
1	1.5M 0.4A 1h	27.40	1.02	0.86	8.38	0.97	0	38.64
2	1.5M 1A 1h	9.77	0.36	8.58	2.72	9.64	0	31.08
3	1.5M 0.4A 3h	26.30	0.42	0	5.56	0	0	32.28
4	1.5M 1A 3h	15.79	0.71	0	6.79	0	0	23.30
5	NA 0.4A 2h	26.09	0	0	4.71	0	0	30.80
6	NA 1A 2h	31.96	0.34	0	4.20	0	0	36.50
7	3M 0.4A 2h	14.87	0	0	0	0	0	14.87
8	3M 1A 2h	15.44	0	3.65	0	4.10	0	23.18
9	NA 0.7A 1h	9.84	1.95	0	0	0	0	11.79
10	NA 0.7A 3h	15.24	1.33	0	0	0	0	16.57
11	3M 0.7A 1h	11.18	0	0	0	0	0	11.18
12	3M 0.7A 3h	16.30	0	0	0	0	0	16.30
13	1.5M 0.7A 1.5h	18.01	0	0	15.95	0	0	33.97

Average of triplicate runs. C1, C2, C3 are controls.

In order to evaluate the efficiency of AD, the level of organic matter in the supernatant of pot ale and inoculum mix were measured prior to and after the reactions. Overall organic matter removals in terms of COD, BOD and SO_4 are given in Table 67. Mainly increased initial organic matter concentrations (COD_0 and BOD_0) were achieved with combined alkaline and ultrasonic pre-treatments with regards to non-alkalised pot ale (Control 1, NA) as well as alkaline pre-treatment standalone (Control 2, 1.5M and Control 3, 3M)

For only ultrasonic pre-treated samples (std no 5, 6, 9, 10), std no 10 had the highest COD, BOD and SO_4 removals as 48 ± 4.4 and 56 ± 3.5% (Table 67) respectively. Furthermore, significantly higher COD (*p:* 0.0356) and BOD (0.0435) removals were only achieved with std no 10 in comparison to Control. On the other hand, std no 5, 6, 9 and 10 had significantly lower H_2S degradations than Control 1 with the p values of 0.0027, 0.019, 0.0069 and 0.0034 respectively.

In case of 1.5 M NaOH pre-treatment combined with ultrasonic pre-treatments under different conditions (std no 1, 2, 3, 4 and 13 – 17), no significant difference was found in COD, BOD and SO_4 removals in comparison to Control 2 according to Table 67. Similarly, overall CH_4 yields and H_2S generation of std no 1, 2, 3, 4 and 13 – 17 were not found to be significantly different than Control 2 (Table 63).

Only std no 8 achieved a significantly more COD and BOD removal than Control 3 with p values of 0.0147 and 0.0106 respectively. This indicates that ultrasonic pre-treatment at 100% amplitude for 2 h after 3 M NaOH pre-treatment increased intercellular compound release based on initial COD value of 26663 mg/L and subsequently provided a better substrate quality than std no 12 based on the cumulative CH_4 yields of 810 ± 21 and 605 ± 70 ml/g VS (Table 63) respectively. On the other hand, ultrasonic pre-treatment on std no 7, 11 and 12 had no significant enhancement on COD and BOD degradation with regards to 3 M NaOH pre-treatment standalone (Control 3) in Table 67. Furthermore, no significant difference was found in SO_4 removals with std no 7, 8, 11, 12 in comparison to Control 3.

Table 67. Organic matter removal with alkaline and ultrasonic pre-treatment

Exp no		*COD_0 (mg/L)	COD Removal (%)	*BOD_0 (mg/L)	BOD Removal (%)	*$(SO_4)_0$ (mg/L)	SO_4 Removal (%)
Ultrasonic pre-treatment only							
Control 1 (NA)		20583	38 ± 2.5	12667	43 ± 2.3	745	52 ± 2.6
5	0.4A 2h	20900	37 ± 5.0	13173	52 ± 4.6	686	25 ± 2.1
6	1.0A 2h	25460	44 ± 2.5	12223	34 ± 2.3	662	35 ± 3.0
9	0.7A 1h	24953	46 ± 4.3	13490	48 ± 1.0	870	47 ± 2.1
10	0.7A 3h	25080	48 ± 4.4	12920	56 ± 3.5	886	38 ± 0.7
Control 2 (1.5 M)		20773	43 ± 5.0	11843	48 ± 4.6	640	33 ± 0.4
1	0.4A 1h	26080	51 ± 2.5	11780	55 ± 2.2	744	43 ± 4.6
2	1.0A 1h	25587	43 ± 5.0	13300	47 ± 4.0	841	28 ± 2.6
3	0.4A 3h	23940	39 ± 3.3	17100	44 ± 2.9	612	35 ± 3.7
4	1.0A 3h	25143	50 ± 2.7	12413	54 ± 3.7	699	35 ± 1.8
13-17	0.7A 1.5h	21090	34 ± 4.4	13933	50 ± 4.0	799	36 ± 1.9
Control 3 (3 M)		22293	44 ± 3.3	12477	48 ± 3.0	776	38 ± 0.1
7	0.4A 2h	22673	44 ± 4.1	12793	48 ± 3.7	891	40 ± 3.1
8	1.0A 2h	26663	57 ± 6.1	13463	60 ± 3.3	757	31 ± 4.8
11	0.7A 1h	25650	49 ± 4.2	13047	53 ± 3.9	861	41 ± 3.1
12	0.7A 3h	26600	51 ± 4.3	13173	55 ± 4.4	693	37 ± 2.1

*Average of triplicates

The influences of ultrasonic pre-treatment alone and in combination with alkaline pre-treatment on response of interests within the first 2 days AD, the coding format of the variables are given in Table 68.

Table 68. Coding format of the variables for ultrasonic pre-treatment

	Coded Factors		
Variable	**-1**	**0**	**1**
A: Amplitude Ratio (%)	0.4	0.7	1
B: Exposure Time (h)	1	2	3
C: NaOH Dose (M)	0	1.5	3

Statistical analysis of the design parameters and first 2 days CH_4, CO_2 and H_2S production models are summarised ANOVA tables given in Table 69, Table 70 and Table 71 respectively. A significant quadratic model fit with an insignificant lack of fit was achieved for all cases within a confidence level of 95%. The models passed the sequential adequacy tests by means of having R^2 close to 1 and adequate precision greater than 4.

For the first 2 days CH_4 generation, the second order effects of amplitude ratio (A^2), exposure time (B^2) and alkaline dose (C^2) were found to be significant model terms according to the *p* values lower than 0.0001 (Table 69). Furthermore, the first order effect of the parameters (A, B, C) as well as the interaction between the exposure time and alkaline dose (BC) were added to the model to support hierarchy.

Table 69. ANOVA table for methane production model α= 0.0.5

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	1.54E+05	7	22041.18	40.41	< 0.0001	significant
A-Amplitude (%)	21.13	1	21.13	0.039	0.8484	
B-Exposure Time (h)	578	1	578	1.06	0.3301	
C-Alkaline Dose (M)	91.13	1	91.13	0.17	0.6923	
BC	1600	1	1600	2.93	0.1209	
A^2	64298.02	1	64298.02	117.89	< 0.0001	
B^2	37461.92	1	37461.92	68.69	< 0.0001	
C^2	55611.6	1	55611.6	101.96	< 0.0001	
Residual	4908.7	9	545.41			
Lack of Fit	3805.5	5	761.1	2.76	0.1735	not significant
Pure Error	1103.2	4	275.8			
Cor Total	1.59E+05	16				

R^2: 0.9692, Adj R^2: 0.9452, Pred R^2: 0.7812, Adeq Precision: 17.509

The final model for CH_4 generation in the early stages of AD (first 2 days) is given in Eq 49 and Eq 50 in terms of coded and actual factors respectively. The impact strength of the parameters on CH_4 yield was found to be as $-A^2 > C^2 > -B^2 > -BC > B > -C > A$ according to the coefficient of model in terms of coded factors.

Final Equation in terms of coded factors:

$$\mathbf{CH_4(ml/gVS) = 253.4 + 1.62\ A + 8.5\ B - 3.37\ C - 20\ BC - 123.57A^2 - 94.33B^2 + 119.93C^2} \quad \text{Eq 49}$$

Final Equation in terms of actual factors:

$$\mathbf{CH_4(ml/VS) = -431.04 + 1927.69\ Amp\% + 425\ ET - 423.08\ A\ Dose - 20ET\ A\ Dose - 1373.06\ Amp\%^2 - 94.33\ ET^2 + 114.93\ A\ Dose^2} \quad \text{Eq 50}$$

In CO_2 generation, first and second order effects of alkaline dose (C, C^2), second order effects of amplitude ratio (A^2), exposure time (B^2) and interaction between exposure time and alkaline dose (BC) were found to be significant model terms according to the *p* values (Table 70). Subsequently, the first order effect amplitude ratio (A) and exposure time (B) were added to the model as part of the stepwise regression to support the hierarchy. CO_2 production model was developed with α set to 0.01 due to existence of significant lack of fit when it was set to 0.05.

Table 70. ANOVA table for CO_2 production model α= 0.01

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	181254.7	7	25893.52	31.16	< 0.0001	significant
A-Amplitude (%)	21.13	1	21.13	0.03	0.8768	
B-Exposure Time (h)	2346.13	1	2346.13	2.82	0.1271	
C-Alkaline Dose (M)	6050	1	6050	7.28	0.0244	
BC	5112.25	1	5112.25	6.15	0.0349	
A^2	69958.78	1	69958.78	84.20	< 0.0001	
B^2	32163.2	1	32163.2	38.71	0.0002	
C^2	72643.46	1	72643.46	87.43	< 0.0001	
Residual	7477.8	9	830.87			
Lack of Fit	7071	5	1414.2	13.91	0.0122	not significant
Pure Error	406.8	4	101.7			
Cor Total	188732.5	16				

R^2: 0.9604, Adj R^2: 0.9296, Pred R^2: 0.6334, Adeq Precision: 18.1745

The final models in terms of coded and actual factors are given in Eq 51 and Eq 52 respectively. The coefficients of the Eq 51 was used to determine the influence strength of the parameters on CO_2 production which was $C^2 > -A^2 > -B^2 > -BC > -C > B > A$

Final equation in terms of coded factors;

$$\mathbf{CO_2(ml/gVS) = 238.8 + 1.63\,A + 17.13\,B - 27.5\,C - 35.75\,BC - 128.9A^2 - 87.4B^2 + 131.35C^2} \quad \text{Eq 51}$$

Final equation in terms of actual factors;

$$\mathbf{CO_2(ml/gVS) = -763.28 + 2010.53\,Amp\% + 402.48\,ET - 145.8\,A\,Dose - 23.83\,ET\,A\,Dose - 1432.22\,Amp\%^2 - 87.4\,ET^2 + 58.38\,A\,Dose^2} \quad \text{Eq 52}$$

A significant model could be developed for H_2S for the first time with the data gathered within the first 2 days of AD with combined alkaline and ultrasonic pre-treatments on pot ale. According to ANOVA analysis (Table 71), significant model terms were found to be the first and the second order effects of alkaline dose (C,C^2), second order effect of exposure time (B^2) and amplitude ratio (A^2), the interactions between amplitude ratio and alkaline dose (AC) and exposure time and alkaline dose (BC). The first order impacts of amplitude ratio (A) and exposure time (B) on the other hand was added to the model to support the model hierarchy by the software.

Table 71. ANOVA table for H_2S production model α= 0.0.5

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	24672.42	8	3084.05	26.87	< 0.0001	significant
A-Amplitude (%)	435.13	1	435.13	3.79	0.0874	
B-Exposure Time (h)	2	1	2	0.02	0.8982	
C-Alkaline Dose (M)	1081.13	1	1081.13	9.42	0.0153	
AC	812.25	1	812.25	7.08	0.0287	
BC	841	1	841	7.33	0.0268	
A^2	5540.53	1	5540.53	48.28	0.0001	
B^2	8347.27	1	8347.27	72.74	< 0.0001	
C^2	8422.42	1	8422.43	73.39	< 0.0001	
Residual	918.05	8	114.76			
Lack of Fit	647.25	4	161.81	2.39	0.2097	not significant
Pure Error	270.8	4	67.7			
Cor Total	25590.47	16				

R^2: 0.9641, Adj R^2: 0.9283, Pred R^2: 0.6813, Adeq Precision: 16.7266

The final H_2S production models in terms of coded and actual factors are given in Eq 53 and Eq 54 respectively. The impact strength of the design factors on H_2S production was identified as C^2 > -B^2 > -A^2 > BC > AC > C > A > B according to the coefficient of Eq 53.

Final equation in terms of coded factors;

$$\mathbf{H_2S(ppm) = 80.8 + 7.36\,A + 0.5\,B + 11.63\,C + 14.25\,AC + 14.5\,BC - 36.28\,A^2 - 44.53\,B^2 + 44.73\,C^2} \quad \text{Eq 53}$$

Final equations in terms of actual factors;

$$\mathbf{H_2S(ppm) = -32.86 + 493.86 \times Amp\% + 149.6\ ET - 229.53\ A\ Dose + 47.5\ Amp\%\ A\ Dose + 14.5\ ET\ A\ Dose - 403.06\ Amp\%^2 - 44.53 \times ET^2 + 44.73\ A\ Dose^2} \quad \text{Eq 54}$$

5.3.1 Validation of the Developed Models

The developed models have been validated by using the same software as a part of the post analysis step. A similar trend was obtained in the normal plot of the residuals and predicted vs actual scatter diagram for all response of interests with the previous design given in Figure 40 and Figure 41 indicating the achievement of an adequate model development.

In addition to the post statistical analysis offered by the software, the developed models were challenged by independent experiments. Three validation points were selected as combination of varying alkaline conditions; non-alkalinised (NA), 1 M and 2.5 M NaOH alkalinised, with ultrasonic pre-treated at 70% amplitude for 1 h. First 2 days of CH_4, CO_2 yields as well as the H_2S production data was then predicted according to the individual conditions of the validations point by using the corresponding final equations in terms of actual factors Eq 50, Eq 52 and Eq 54). The comparative summary of the experimental and the predicted data is given in Table 72 along with the *p* values of the applied 2 tailed t-test with an unequal variance. No significant difference was found between the predicted and the experimental data according to the *p* values.

Table 72. Results of validation experiments for alkaline and ultrasonic pre-treatment

Validation Points	**CH_4 Yield (ml/g VS)**		**CO_2 Yield (ml/g VS)**		**H_2S production (ppm)**	
	Experimental	**Predicted**	**Experimental**	**Predicted**	**Experimental**	**Predicted**
NA 0.7A 1h	489 ± 48	577	282 ± 40	257	194 ± 16	220
	p: 0.0736		*p:* 0.5447		*p:* 0.2529	
1M 0.7A 1h	194 ± 10	248	194 ± 10	146	201 ± 25	149
	p: 0.0824		*p:* 0.0943		*p:* 0.2088	
2.5M 0.7A 1h	193 ± 7	187	193 ± 7	198	186 ± 33	111
	p: 0.4582		*p:* 0.4721		*p:* 0.1376	

5.3.2 Model Graphs

The perturbation plots of CH_4, CO_2 yields and H_2S generation are given in Figure 48 1, 2 and 3 respectively. Theses graphs were created in terms of coded factors which enables to observe the impacts of the parameters in different units in a same plot. All the parameters had a similar effect on the responses of interest. For example, amplitude ratio (A) and the exposure time (B) of the ultrasonic pre-treatment resulted in the highest CH_4, CO_2 yields and H_2S generation in the centre point of the experimental design which was 70% for the amplitude ratio and 2h for the exposure time. Alkaline dose (C), on the other hand, led the lowest values of the all responses when it was set to the centre point of the design (1.5 M NaOH).

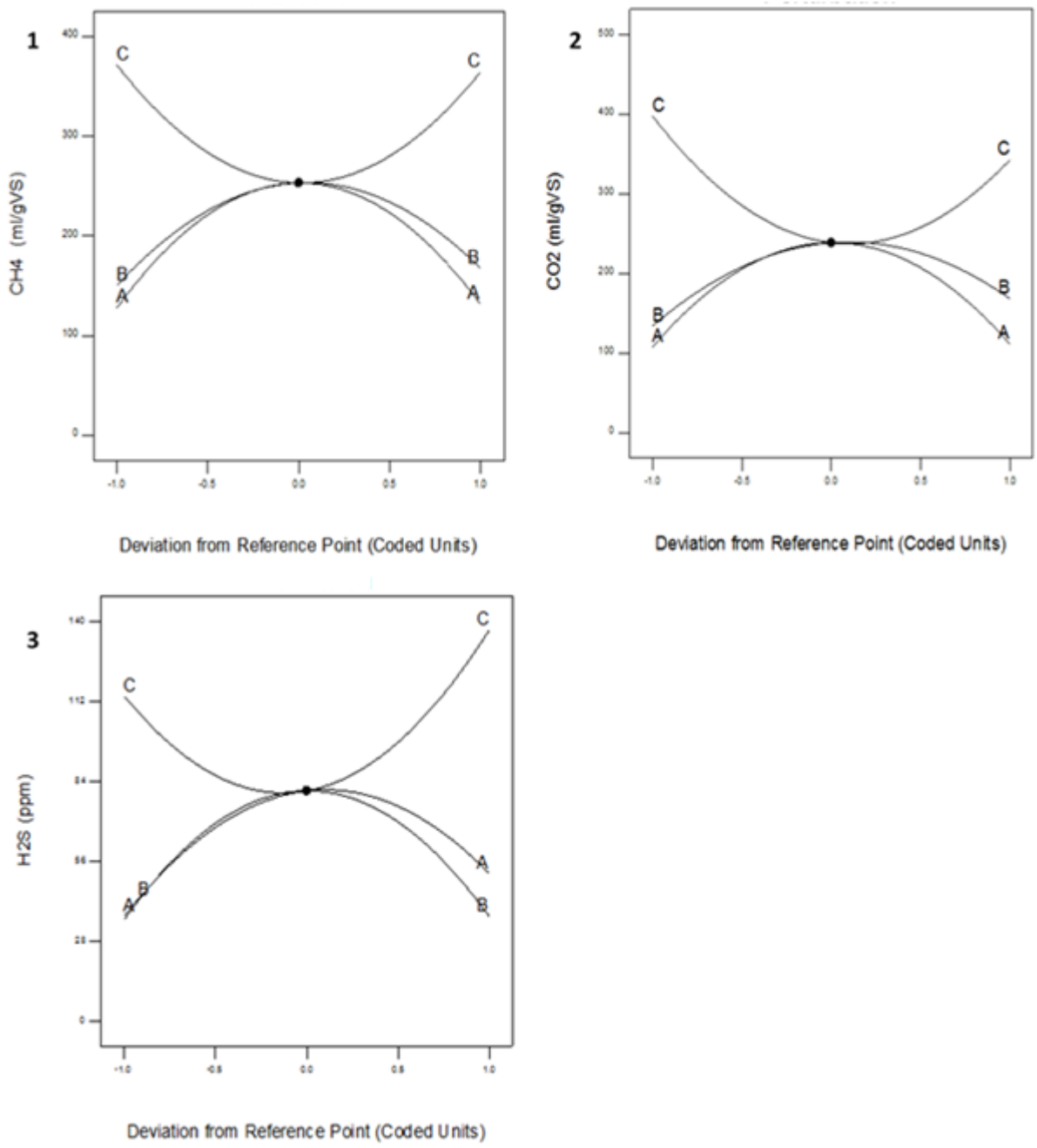


Figure 48. Perturbation graphs of the 1. CH_4, 2. CO_2 yields and 3. H_2S generation for combined alkaline-ultrasonic pre-treatment

A significant interaction was found between the exposure time (B) of the ultrasonic pre-treatment and alkaline dose (C) in CO_2 yield and H_2S production. The interaction plots are given in Figure 49. No significant difference was found in CO_2 and H_2S yields arising from the implementation of 3 M NaOH pre-treatment prior to AD in comparison with non-alkalised pot ale when the time of the ultrasonic pre-treatment was short. In contrast, when the exposure time was increased to 3 h, 3 M NaOH pre-treatment resulted in

significantly lower CO_2 yield (Figure 49, 1) while it caused a significant increase in H_2S production (Figure 49, 2) with regard to AD of non-alkalised pot ale.

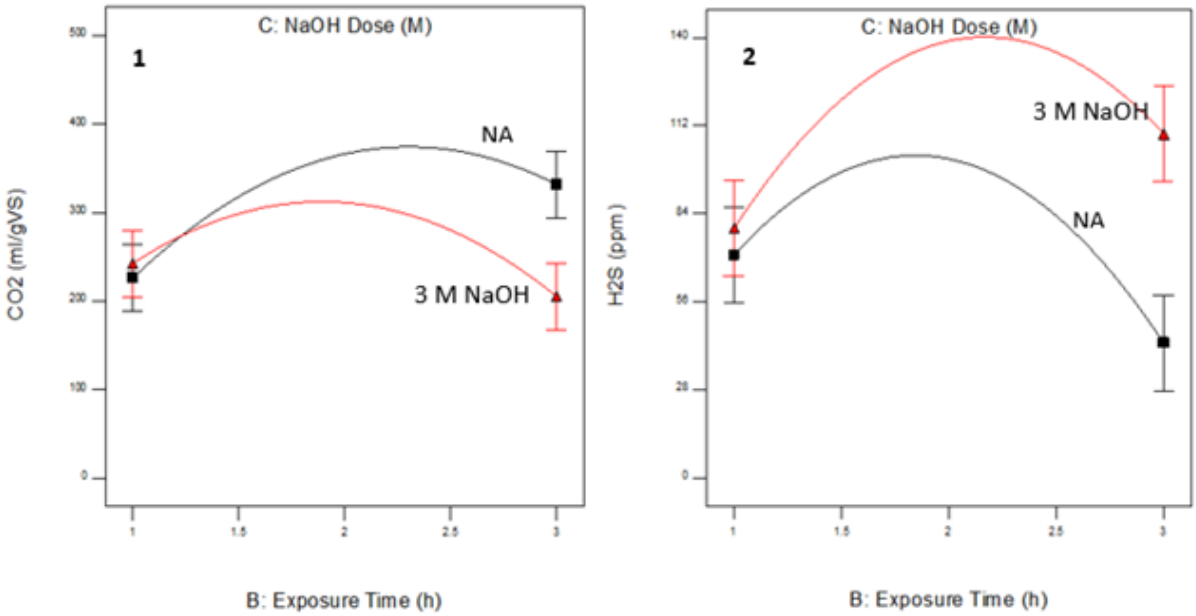


Figure 49. Interaction graphs of the 1. CO_2 yields and 2. H_2S generation for combined alkaline-ultrasonic pre-treatment

The contour graphs for CH_4, CO_2 yields and HS_2 generation are given in Figure 50 1, 2 and 3 respectively. The highest CH_4 yields were achieved when ultrasonic pre-treatment applied at 70% amplitude for 2h on both 3M NaOH pre-treated ad non-alkalised pot ale (the areas marked in red in Figure 50 1). Furthermore, the lowest CO_2 and H_2S yields were obtained when the alkali dose was higher than 0.5 M (Figure 50 2) and lower than 2.5 M (Figure 50 3) respectively regardless of the exposure time of the ultrasonic pre-treatment.

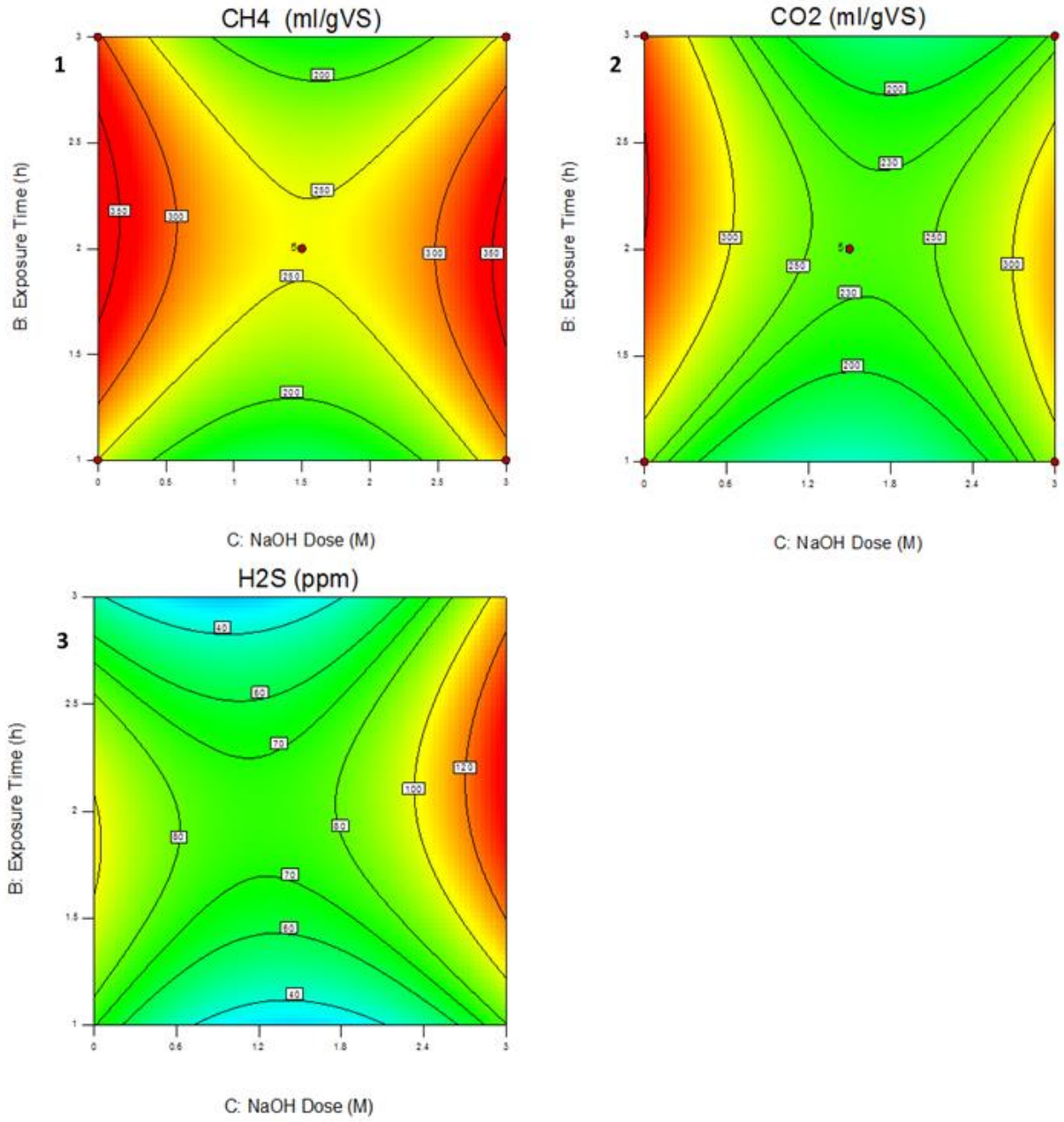


Figure 50. Contour graphs of the 1. CH_4, 2. CO_2 yields and 3. H_2S generation for combined alkaline-ultrasonic pre-treatment

5.3.3 Optimisation

A combined numerical and graphical optimisation approach was conducted under two different conditions on the design factors in order to optimise pre-treatment conditions and minimum energy consumption in the pre-treatment step. First of all, all design factors were kept in range with no restrictions while methane yield was aimed to maximise with an importance of 5. CO_2 and H_2S were set to be minimised with the importance level of 5 and 3 respectively. The first optimisation approach conditions are outlined in

Table 73.

Table 73. Optimisation approach 1 for combined alkaline-ultrasonic pre-treatment

Variable	Goal	Lower Limit	Upper Limit	Importance
A: Amplitude (%)	is in range	0.4	1	3
B: Exposure Time (h)	is in range	1	3	3
C: Alkaline Dose (M)	is in range	1	3	3
CH_4 (ml/g VS)	maximise	22	333	5
CO_2 (ml/g VS)	minimise	22	333	3
H_2S (ml/g VS)	minimise	0	135	5

The numerical optimisation solutions for the first approach as a function of the desirability are given in Table 74. The upper and lower limits of responses for graphical optimisation step were decided based on the numerical optimisation solutions in order to application of the graphical optimisation.

Table 74. Numerical optimisation solutions for approach 1 for combined alkaline-ultrasonic pre-treatment

No	Amplitude (%)	Exposure Time (h)	Alkaline Dose (M)	CH_4 (ml/g VS)	CO_2 (ml/g VS)	H_2S (ppm)	Desirability
1	0.4	1.0	2.9	176	162	28	0.62
2	0.5	1.0	3.0	199	187	36	0.62
3	0.4	1.0	3.0	188	175	33	0.62
4	1.0	3.0	0.0	184	236	11	0.62
5	0.6	3.0	0.8	188	212	29	0.61
6	0.7	3.0	0.9	198	219	34	0.61
7	0.6	1.0	2.1	171	153	38	0.60

The graphical optimisation results for the optimisation approach 1 are given in Figure 51. The optimum area, marked in yellow was identified based on the overlapped upper and lower limits of each interest of response which were obtained with the numerical optimisation solutions (Table 74). The boundaries of the optimum area were set as 171 – 198 ml/g VS for CH_4, 153 – 236 ml/g VS for CO_2 and 11 – 33 ppm for H_2S. The optimum pre-treatment conditions were found to be either ultrasonic pre-treatment (at 40% amplitude) for 3 h with no alkaline pre-treatment or 3 M NaOH pre-treatment followed by much shorter (1h) ultrasonic pe-treatment time at 40% amplitude.

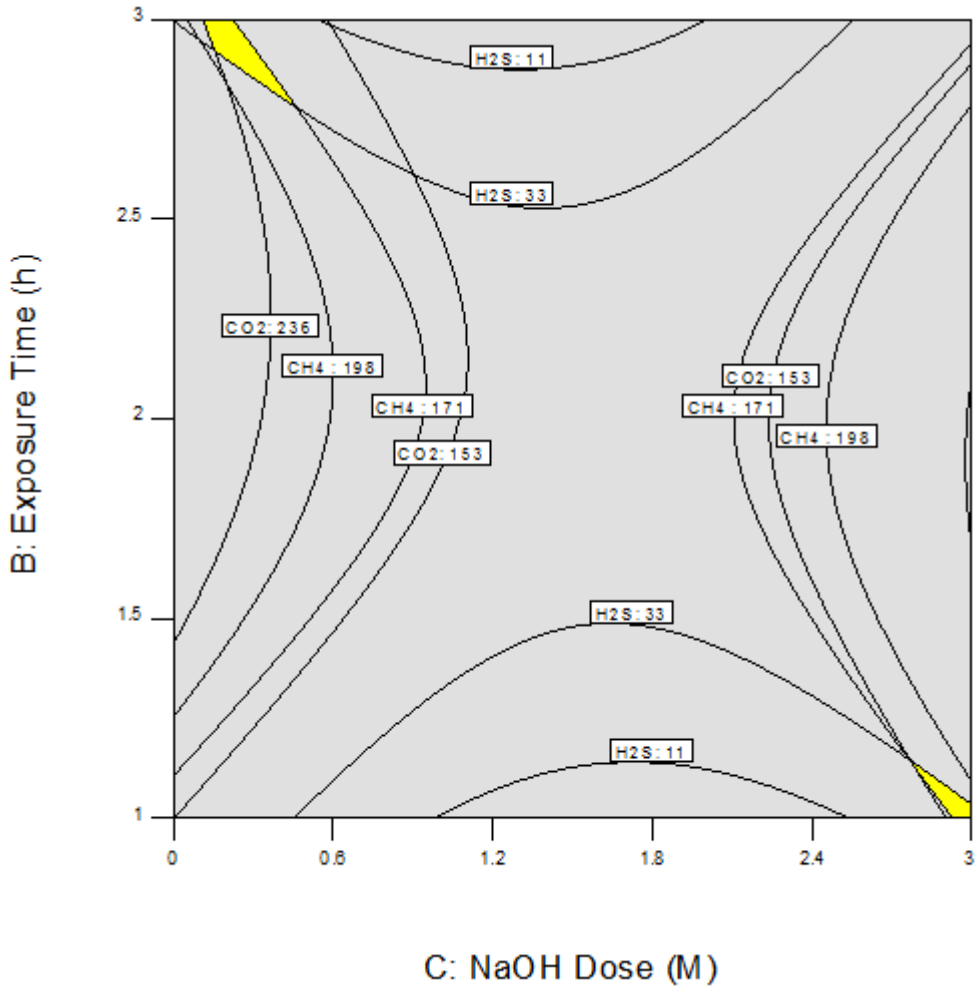


Figure 51. Graphical optimisation for optimisation approach 1 for combined alkaline-ultrasonic pre-treatment

In the second optimisation approach all design factors initially were aimed to minimised with an importance level of 5 to increase the application simplicity. However, it resulted in only 3 suggestions which had in significantly lower CH_4 yields in comparison with the results of the numerical optimisation approach one. Therefore, the alkaline dose was set to be in range. The goals for the responses were kept as same with the approach 1. The details of the second optimisation approach is given in Table 75.

Table 75. Optimisation approach 2 for combined alkaline-ultrasonic pre-treatment

Variable	Goal	Lower Limit	Upper Limit	Importance
A: Amplitude (%)	minimise	0.4	1	5
B: Exposure Time (h)	minimise	1	3	5
C: Alkaline Dose (M)	in range	1	3	3
CH_4 (ml/g VS)	maximise	22	333	5
CO_2 (ml/g VS)	minimise	22	333	3
H_2S (ml/g VS)	minimise	0	135	5

Suggested numerical optimisation solutions for the second approach is give in Table 76. The upper and the lower limits of each interest of response were decided according to the suggested solutions by numerical optimisation to complete a graphical optimisation.

Table 76. Numerical optimisation for approach 2 combined alkaline-ultrasonic pre-treatment

No	Amplitude (%)	Exposure Time (h)	Alkaline Dose (M)	CH_4 (ml/g VS)	CO_2 (ml/g VS)	H_2S (ppm)	Desirability
1	0.4	1.0	3.0	157	143	20	0.76
2	0.5	1.0	3.0	207	195	39	0.74
3	0.4	1.0	2.8	122	105	10	0.74
4	0.5	1.0	0.4	139	136	47	0.68
5	0.5	1.1	1.0	131	117	35	0.68
6	0.4	1.2	0.0	159	164	65	0.67
7	0.7	1.0	0.9	159	148	42	0.64

The result of the graphical optimisation is given in Figure 52. The optimum area, marked in yellow was determined according to the numerical optimisation solutions. The limits of CH_4, CO_2 and H_2S generations were set as 122 – 207 ml/g VS, 105 – 195 ml/g VS and 20 – 65 ppm respectively. The optimum exposure time was found to be 1 h based on the numerical optimisation solutions.

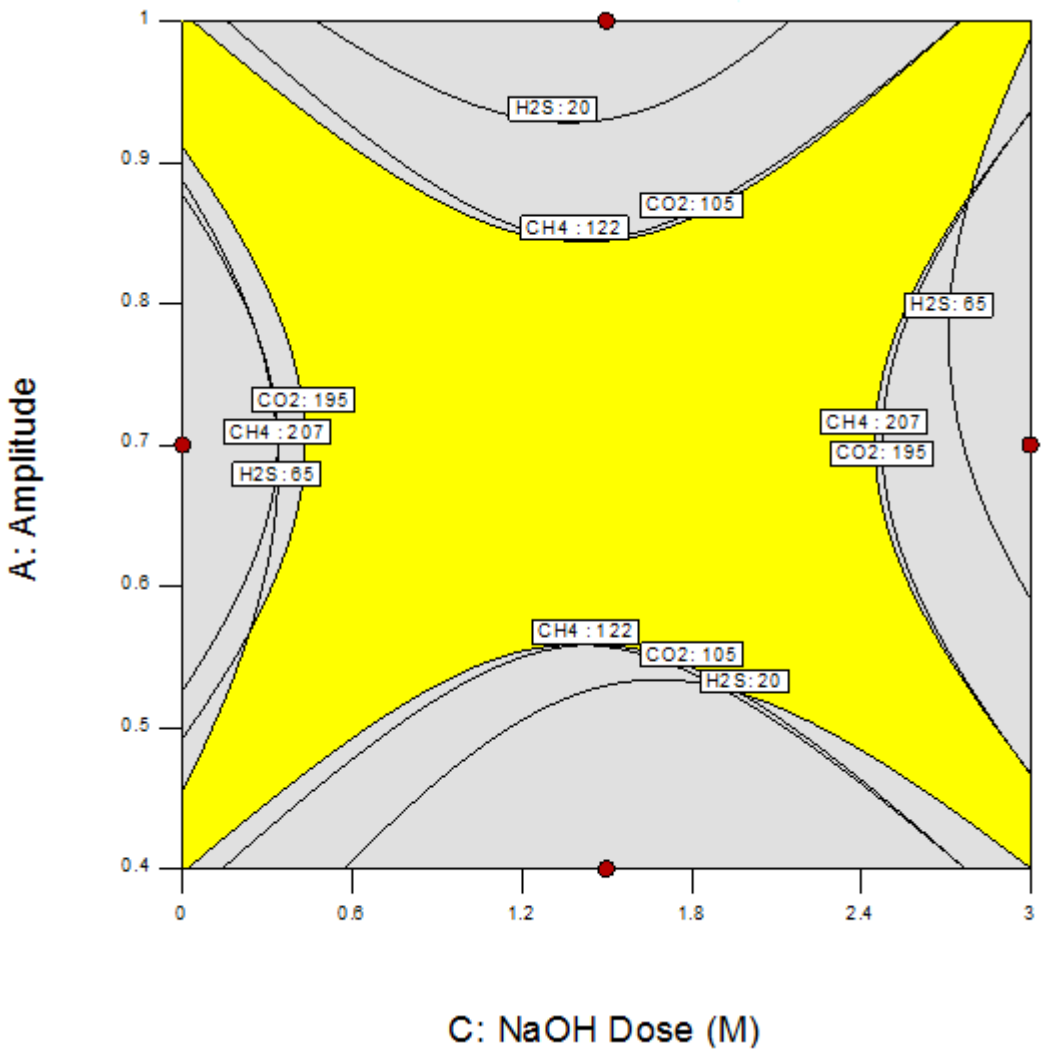


Figure 52. Graphical optimisation for optimisation approach 2 for combined alkaline-ultrasonic pre-treatment at exposure time of 1h

An increased I/S (5 on dry basis) was used in this experiment plan which lead to no significant difference between the combined alkaline pre-treated samples and their controls. However, alkaline pre-treatment (1.5 M NaOH) in combination with ultrasonic pre-treatment caused a significant delay on methane generations for the first 2 days of AD. There influences of the design parameters in the early stages of AD was modelled. The second order effect of the amplitude ratio and the exposure time of the ultrasonic pre-treatment had a descending effect on the first 2 days of methane yield, while alkaline dose had an ascending effect. The optimum pre-treatment conditions for the early stages of AD was identified as application of 3h ultrasonic pre-treatment at 70% amplitude in isolation and 1h ultrasonic pre-treatment at 70% amplitude in combination with 3M NaOH pre-treatment.

Developed models for the first 2 days pf AD of pot ale after implementation of alkaline and ultrasonic pre-treatment were summarised in Table 77 Equations in terms of actual factors were used for data prediction for the validation step of the models as well as the optimisation step. The design parameters amplitude ratio, exposure time of the ultrasonic pre-treatment and alkaline dose were coded as A, B and C respectively which enables comparing power of influence on the responses.

Table 77. Summary of developed models of first 2 days of AD of pot ale after alkaline and ultrasonic pre-treatment

$\mathbf{CH_4(ml/gVS)} = \mathbf{253.4 + 1.62A + 8.5B - 3.37C - 20BC - 123.57A^2 - 94.33B^2 + 119.93C^2}$
$\mathbf{CH_4(ml/gVS)} = \mathbf{-431.04 + 1927.69\ Amp\% + 425\ ET - 423.08\ A\ Dose - 20ET\ A\ Dose - 1373.06\ Amp\%^2 - 94.33\ ET^2 + 114.93\ A\ Dose^2}$
$\mathbf{CO_2(ml/gVS)} = \mathbf{238.8 + 1.63\ A + 17.13\ B - 27.5\ C - 35.75\ BC - 128.9A^2 - 87.4B^2 + 131.35C^2}$
$\mathbf{CO_2(ml/gVS)} = \mathbf{-763.28 + 2010.53\ Amp\% + 402.48\ ET - 145.8\ A\ Dose - 23.83\ ET\ A\ Dose - 1432.22\ Amp\%^2 - 87.4\ ET^2 + 58.38\ A\ Dose^2}$
$\mathbf{H_2S(ppm)} = \mathbf{80.8 + 7.36\ A + 0.5\ B + 11.63\ C + 14.25\ AC + 14.5\ BC - 36.28\ A^2 - 44.53\ B^2 + 44.73\ C^2}$
$\mathbf{H_2S(ppm)} = \mathbf{-32.86 + 493.86 \times Amp\% + 149.6\ ET - 229.53\ A\ Dose + 47.5\ Amp\%\ A\ Dose + 14.5\ ET\ A\ Dose - 403.06\ Amp\%^2 - 44.53 \times ET^2 + 44.73\ A\ Dose^2}$

5.4 Discussion of Key Findings

Microwave pre-treatment has not been studied on pot ale before therefore, the modifications on lignocellulosic fractions were compared with other types of lignocellulosic matter. The lignin fraction of non-treated pot ale was determined as 26.9 ± 0.9% (Figure 39). Significant reductions in lignin levels were seen as 9.1 ± 2 (p: 0.028), 12.8 ± 1.7 (p: 0.014) and 5.9 ± 1.8% (p: 0.037) due to the application of combined 1 M NaOH pre-treatment with 80, 240 and 400 W microwave treatment for 11 minutes respectively (AP10, AP30 and AP50 in Figure 39). Similar results were published in the previous studies on pre-treatment of different lignocellulosic substrates. As a result of combined 1% NaOH and 600W microwave pre-treatment for 3 minutes 13.1% delignification (from 18 to 4.9%) in sugarcane bagasse structure was published (Binod et al. 2012). Also 9% lignin removal (from 14.8 to 5.8%) was reported in wheat straw after implementation of 2.75% (w/v) NaOH and 800 W microwave pre-treatment (at 100˚C) (Singh & Bishnoi 2012). Applying 1% NaOH and 30 min 700 W microwave pre-treated rice straw had a 8.7% (from 13.6 to 4.9%) lignin degradation according to a published study (Zhu et al. 2005). Hemicellulose content of non-treated pot ale was found to be 11.5 ± 0.5%. A 15.5 ± 3.2% (*p:* 0.002) and 21.4 ± 0.4% (*p:* 0.000002) rise was seen in the hemicellulose fraction was observed after applying 1 M NaOH pre-treatment in combination with 80 and 240 W microwave pre-treatments respectively (AP10 and AP30 in Figure 39). 400 W microwave pre-treatment had only 8.4 ± 2.9% increase which was statistically insignificant. The cellulose content of the non-treated pot ale was found to be 10.6 ± 1.8%. The only significant difference was seen in 1M NaOH pre-treatment coupled with 240W microwave pre-treatment (AP30 in Figure 39) in with a 6.3 ± 1.5% decrease (*p:* 0.0163). Other pre-treatment conditions had no significant effect on cellulose fraction. Literature shows varying results on cellulose content of lignocellulosic biomass due to combined alkaline and microwave pre-treatment. Significant rises in cellulose content of lignocellulosic biomass such as rice straw (from 38.6 to 69.2%) (Zhu et al. 2006), oil palm fronds (from 41.9 to 68.9%) and oil palm trunks (from 50.8 to 71.69%) (Lai, L., Idris 2013) ,wheat straw (from 45 to 51%) (Singh & Bishnoi 2012) and (from 41.2 to 79.6%) (Zhu et al. 2006). Cellulose fraction, on the other hand, was not affected significantly according to the literature. For example, 1% NaOH and 700 W microwave pre-treatment for 1 h had

only 2.2% rise in the cellulose content of rice straw to 23.3% which was not reported to be significant (F. Li et al. 2012). After combined 1.4 M NaOH glycerol and 1300 W microwave pre-treatment for 2 min, cellulose fraction of corn straw and rise husk was reduced from 27.9% to 23.1% and from 56.7% to 44.4% respectively (Belen et al. 2015). Furthermore, cellulose content of herbal-extraction process residue has shown a slight reduction (insignificant) of 0.2% from 21.2% due to combined NaOH and 700 W microwave pre-treatment for 15 minutes (Cheng & Liu 2010). Similarly, 1.4 M NaOH glycerol and 1300 W microwave pre-treatment for 2 min (Belen et al. 2015) on corn straw did not affect cellulose fraction of 22.5%. The lignin and cellulose degradations were mainly found to be in an agreement with the previously published studies. The differences in cellulose fractions were attributed to the different conditions of pre-treatments and different substrate compositions such as varying crystallinity level of cellulose which gives a rigidness to structure (Taherzadeh & Karimi 2008; Agbor et al. 2011; Kratky & Jirout 2011) in substrates.

The highest delignification was observed with the combination of 1M NaOH and 240 W (30% of overall power) microwave pre-treatment (AP30 in Figure 39). The highest methane cumulative methane yield was achieved as 1614 ± 168 ml/g VS with std no 4 (Table 49) with an I/S of 5 on VS basis (90% sludge on wet basis) after 1 M NaOH and 240 W microwave (AP30) pre-treatment. While the control for I/S of 5 (Control 3 in Table 49) had only 518 ± 1 ml CH_4 per g VS. Microwave power and I/S were found to be significant model terms of CH_4 yield with the *p* values of 0.0459 and 0.0001 respectively according to ANOVA (Table 53) while starting pH of the digestion was insignificant. The negative effect combinations of 1M NaOH pre-treatment with 80 (AP10) and 400 W (AP50) microwave pre-treatment on cumulative methane yield is attributed to the lower levels of lignin degradation with AP50 than AP10 given in Figure 39.

In addition to alternations in the lignocellulosic structure, the efficiency of pre-treatments were also assessed by increased soluble organic matter namely COD (C. Bougrier, C. Albasi, J.P. Delgenes 2006; Ferrer et al. 2008; Mallick et al. 2009). The COD concentrations of supernatant of pre-treated pot ale and sludge mixtures before AD (Table 52) for combined alkaline and microwave pre-treatment, Table 67 for combined alkaline and

ultrasonic pre-treatment) were found to be lower than pot ale as received which was 38867 ± 115 (Table 1, Chapter 4). The difference was attributed to the dilution effect of sludge on pot ale. On the other hand, the initial concentration of SO_4 was found to be higher than the fresh pot ale which was 190 ± 31 mg/L (Table 1, Chapter 4) which was attributed to the potential sulphate existing in sludge. Combined alkaline and ultrasonic pre-treatment led to the largest increase in both COD and BOD (25.5% and 44.4% respectively, std 1 and std 3 in Table 52) while ultrasonic pre-treatment alone led to a maximum increase of just 23.6 and 6.5% respectively (std 9 in Table 52).

Among the sample group which had an I/S of 5, the highest initial COD value was determined as 24747 mg/L after combined 1 M NaOH and microwave pre-treatments at 30% (std no 4 in Table 52). It was found to be 35% higher than combined 1 M NaOH and microwave pre-treatment at 50% (std no 8 in in Table 52). Increased COD concentration also had a significant effect in biogas yields for std no 4 (1614 ± 168 in Table 49) and 8 (1019 ± 51 in Table 49) with a *p* value of 0.025. In addition, the initial COD concentrations of non-treated, 1.5 and 3 M NaOH pre-treated pot ale and sludge mixture were determined as 20583, 20773 and 22293 mg/ L respectively (control 1, 2 and 3 in Table 67). After application of ultrasonic pre-treatment on these controls, 24 (std no 1 in Table 67), 26 (std no 1 in Table 67) and 19 (std no 8 in Table 67)% of rise was seen in COD concentrations respectively. Enhancements in COD concentrations of diluted pot ale was also studied by (Mallick et al. 2009) arising from combined beta-glucanase plus protease and papain pre-treatments. The COD concentration of the supernatant of was reported as 5500 mg/ L and 24 and 63% increase was seen due to application of beta-glucanase plus protease and papain pre-treatments respectively prior to AD.

In this study methane yields of non-treated pot ale was found to be 227 ± 6, 391 ± 23 and 518± 1 ml/g VS (control 1, 2 and 3 in Table 49) for the I/S of 1, 3 and 5 on dry basis respectively. After implementation of combined 1 M NaOH and microwave (30% power) pre-treatment the highest methane yields were determined as 728 ± 30, 1108 ± 108 and 1614 ± 168 ml/g VS for the I/S of 1, 3 and 5 with the std no 1, 16 and 4 in Table 49. It indicates a significant enhancement in methane yields with the p values of 0.019, 0.004 and 0.013 over the Control 1, 2 and 3. On the other hand, when 1.5 M NaOH pre-

treatment in isolation applied on pot ale, the cumulative methane yield was found as 625 ± 2 ml/g VS (Control 2 in Table 63) with an I/S of 5, indicating a 20% increase (*p:* 0.0005) over AD of non-treated pot ale (Control 3 in Table 49). Furthermore, when 1.5 M NaOH pre-treatment combined with ultrasonic pre-treatment at 70% amplitude for 2 hours the methane yield increased 43% from 625 ± 2 9 (Control 2 in Table 63) to 894 ± 55 (std no 14 in Table 63) ml/g VS and the *p* value was found the be 0.003. The highest methane yield achieved with combined alkaline ultrasonic pre-treatment was 894 ± 55 ml/g VS (std no 14 in Table 63), lower than the highest methane yield achieved with combined alkaline and microwave pre-treatments (std no 4 in Table 49, 1614 ± 168 ml/g VS). This was attributed to lower delignification potential of combined alkaline ultrasonic pre-treatment as has already been discussed.

These results were found to be in an agreement with literature in terms of biogas yields. Effects of 2 different inoculum sources, namely whole digestate "sludge" from a mesophilic AD plant treating food waste, which is a similar source of this study, and "granular sludge" from a UASB treating whiskey distillery wastewater has been studied. The methane yields of AD of non-treated, filtered and deproteinated pot ale in lab scale batch mode with a continuous mixing at 110 rpm and an I/S ratio of 2 based on VS was published as 482 ± 10, 586 ± 19 and 483 ± 27 ml/g VS. Moreover, the methane yields were reported as 551 ± 31, 571 ± 10 and 520 ± 38 with the I/S of 4. In addition, when granular sludge was used as inoculum (with a I/S of 2) significantly enhanced methane yields were obtained as 630 ± 22 and 602 ± 21 for AD of non-treated and filtered pot ale (Barrena et al. 2018) suggesting inhibitions on AD when non-treated pot ale seeded with non-adopted inoculum. There was no data reported in organic matter removals. Moreover, a 780 ml seed sludge loaded to a lab scale UAF reactor for AD of pH 7 adjusted pot ale supernatant by using 1 M NaOH with a total organic carbon volumetric OLR of 2 g/ L day in a study published by (Kida et al. 1999). The biogas yield was calculated based on total organic carbon instead of COD concentration therefore direct comparison of the biogas yield was not possible. However, methane content of the produced biogas was reported as 56% (Kida et al. 1999) which is in an agreement with experimental results with upper and lower limits of alkaline-microwave (51 ± 2.5 - 60 ± 0.9% std no 4,6 in Table 63) and alkaline-ultrasonic (47 ± 0.1 – 57 ± 2.5, control 2 and std 14 in Table 63) pre-treatments. In addition,

AD of enzymatically pre-treated (saccharifying enzymes) pot ale at a lab scale (2 L) batch reactor with 50% of acclimatised sludge use on wet basis was published based with the same units by (Tokuda et al. 1998). A daily biogas generation was reported as 3000 – 4000 ml with an average methane content of 57% when the HRT was 18 h (Tokuda et al. 1998). These results are considered to be in an alignment with AD of non-treated pot ale (control 1 on Table 59) in 500 ml batch reactor which had a 2530 ± 26 ml biogas generation with a 32.2 ± 0.1% methane content after 2 days of AD. the differences in biogas generation and the methane content of it was attributed to use of acclimatised sludge as inoculum as well as 4 times bigger capacity of treatment. However, when combined 1M NaOH and microwave pre-treatment applied, the biogas quantity and quality was found to be 3636 ± 76 ml 27 ± 0.5% CH_4, 3193 ± 76 ml, 28.7 ± 0.6% CH_4, 3636 ±163 ml 29.3 ±2.4% CH_4 and 3032 ± 258 ml 44 ± 1% CH_4 for the std no 1, 5, 7, 9 and 13 – 17 in Table 49, respectively. Whereas, the highest biogas generation was only 2157 ± 39 ml with 39.2% CH_4 (std no 10 in Table 63) after 2 days of AD after 70% amplitude ultrasonic pre-treatment for 3 h. Seeing lower biogas quality than the literature can be explained by using non-adopted sludge as inoculum as inoculum source is considered to be one of the major factors on biogas yields according to (Neves et al. 2008). In another study ,AD of pot ale supernatant with a major focus on disintegration of intact yeast cells was observed by (Mallick et al. 2009) at lab scale batch (1 L) experiments. Seed inoculum from an AD plant treated domestic wastewater was used but no information was given for I/S as well as for biogas generation. After AD 87 and 50% of COD removals were seen in beta-glucanase plus protease and papain pre-treated samples from the initial concentration of 5500 and 6800 mg/L respectively. In this study COD of pre-treated pot ale was analysed in form pot ale sludge mix with the minimum concentrations of 17093 and 20773 mg/L for combined alkaline microwave and ultrasonic pre-treatments. The level of COD removals agrees with the ones found in this study with combined alkaline microwave and ultrasonic pre-treatments which are given as ranges of 34 ± 1.7 – 69 ± 1.4% (Table 52) and 34 ± 4.4 – 57 ± 6.1% (Table 67) respectively.

A lab scale (1.1 L) UASB was used for AD no-treated pot ale by Goodwin et al. 2001. Granular sludge obtained from an AD plant treating whiskey distillery wastewater was fed to the reactor with a ratio of 55% on wet basis. Only sodium hydrogen carbonate was

added to adjust alkalinity of the substrate. The biogas production rate raged from 0.5 L to 5 L per day depending on the OLR of 6.52 and 3.14 kg COD/m^3day respectively with a constant HRT of 2.1 day. The COD removals of 70 – 90% the during digestion time. It shows an agreement as in this study, biogas generation rate of non-treated pot ale digested with 60% sludge was found to be 2.5 ± 0.03 L (Control 1 in Table 49) within the first 2 days of digestion. A further significant enhancement in biogas production was also achieved as 3.6 ± 0.2 L (*p:* 0.017) after combined 1 M NaOH and 400 W microwave pre-treatment (std no 7 in Table 49). The COD removal percentage was slightly lower than literature since the it was determined as 65 ± 0.2%. However, the efficiency of the removal is approximately the same since the COD concentration of the influent was varied in the range of 5340 – 32860 mg/L in the publication (Goodwin et al. 2001) whereas in this study it was constant at 30667 mg/L.

AD of non-treated pot ale was studied (Tokuda et al. 1999) at a pilot scale UAF reactor (2 m^3) for 100 days at varying HRTs up to 10 h. The reactor was loaded with 400 L seed sludge and supernatant of pot ale with an OLR of 80 L per day indicating an 80% sludge use on wet basis. 48% NaOH solution was to maintain the pH at 7 throughout the reaction time. An average daily biogas yield was reported as 0.75 m^3/kg COD containing 65 – 75% CH_4 and 200 ppm H_2S which corresponds to 14460 ml biogas/ g suspended solids yield and a range of 6109 – 10845 ml CH_4/g suspended solids (Tokuda et al. 1999). An average COD removal was also reported as 76% from a 46900 mg/L initial value. However, in this study the highest cumulative biogas yield of non-treated pot ale was found as 925 ± 10.4 ml/g VS (Control 3 in Table 49) with an approximately 90% sludge seeding on wet basis. Although significant enhancements were achieved with combined alkaline microwave and ultrasonic pre-treatments increased the methane yield to 2768 ± 10.4 ml/g VS with a 60 ± 0.9% CH_4 and 1728 ± 18 with a 51 ± 1.6% CH_4 content respectively, they were still lower than yields reported by (Tokuda et al. 1999). The differences in biogas yields were mainly attributed to higher technology of UAF than enabling a better contact between the microbes and substrate while the batch reactors did not have even an internal agitation.

Two-stage AD of distillery wastewater was studied by employing a lab scale AF and UASB reactors with the influent COD concentration of 8510 – 16800 mg/L and 13600 mg/L for

the first and the second stage of the digestion (V. Blonskaja et al. 2003). Sludge content was reported constant as 30% of the reactor (1.5 L) while the wastewater dose was increased 5% every week from 10 to 30% indicating an I/S varying from 3 to 1 on wet basis. The methane production of the second stage, where methanogenesis occurred, was calculated as 10.1 L after the 20 days of HRT with an OLR of 2 kg COD/m^3day influent (13600 mg COD/L). However, in this study the methane generation after 21 days of digestion of non-treated pot ale with 65% sludge of total volume 400 ml was found as 3.8 ± 0.1 (control 1 in Table 49). When combined alkaline microwave pre-treated applied 4.2 ± 0.05 and 4.8 ± 0.09 cumulative methane productions were obtained with the std no 5 and 7 (Table 49) at 65% sludge seeding ratio. While combined alkaline and ultrasonic pre-treatment had only 2.1 ± 0.001 and 2.2 ± 0.007 L methane production with the std no 7 and on respectively with a 90% sludge seeding. The difference attributed to advantages of UASB over batch reactor arising from the high-speed reactor configuration providing a better contact between the wastewater and the microbes. As well as 3.75-fold higher capacity of treatment.

A granular bed anaerobic baffled reactor with a 35 L of effective capacity was employed by Akunna & Clark 2000 for AD of diluted malt whiskey wastewater. Daily biogas generations of 10 and 22 L were published by treating the influent had 9500 mg/L COD concentration with the HRT of 10 and 2 days respectively. The CH_4 content of the biogas was reported to be in a range of 60 – 70%. Moreover, COD and BOD degradations of the wastewater were reported to be 80 – 92 and 90 – 96%, respectively for the HRT 10 and 2 day. In other words 7600 – 8740 mg/L and 8550 – 9120 mg/L COD was removed for HTR 10 and 2 day in the study conducted by Akunna & Clark 2000. High biogas production rate and organic matter removal results were attributed to the combined effects of high treatment capacity and high technology of reactor which combines benefit of ABR and UASB by using anaerobic phase separation. In this study, COD removal ranges of 34 ± 1.7 – 69 ± 1.4% (for combined alkaline microwave pre-treatment in Table 52) and 34 ± 4.4 – 57 ± 6.1% (for combined alkaline ultrasonic pre-treatments in Table 67) was found. The COD removal ranges indicate that 6745 – 17075 mg/L and 7170 – 15198 mg/L COD removals respectively. Therefore, the efficiency of COD removals is considered to be in an agreement with literature.

AD of ten-fold diluted non-treated malt whiskey wastewater to adjust influent COD strength to approximately 10000 mg/L while BOD was 2490 mg/L by using an UASB with an active volume of 140 L for a period of 430 days under thermophilic conditions was published by Harada et al. 1996 studied. An adopted granular culture was obtained from an AD plant processing whiskey wastewater was used as inoculum. The sludge seeding ratio was reported to be approximately 60% with an HRT of 0.53 day. An average daily biogas production rate was found to be 6.4 L with a methane content of 55%. COD and BOD removals were 39 – 60% and 80% respectively. I this study, after 2 days of AD of non-treated pot ale 2.5 ± 0.03 L (Control 1 in Table 49) with an I/S of 1 on dry basis which corresponds to approximately 65% indicating that biogas production rate was 2.5 times lower than the study published by Harada et al. 1996. After implementation of combined 1 M NaOH and microwave pre-treatment at 10, 30 and 50% power settings, the biogas generation was found as 3.2 ± 0.07 L with 26.7 ± 0.5% CH_4, 3.6 ± 0.07 L with 28.7 ± 0.6% CH_4 and 3.6 ± 0.2 L with 29.3 ± 2.4% CH_4 (std no 5, 1 and 7 in Table 49) respectively with 65% sludge content. It is important to note that when sludge content increased to 90% on wet basis significant rises in biogas were seen as 41.4 ± 0.4% (*p:* 0.004), 43.4 ± 2.1% (*p:* 0.0001) and 41.3 ± 1.2 (*p:* 0.005) respectively, However, biogas quantity was found to be significantly lower as follows 1.7 ± 0.002 L (*p:* 0.001), 2.3 ± 0.02 L (*p:* 0.006) and 1.8 ± 0.06 L (*p:* 0.04) in the same order of pre-treatment. The lower biogas quality and quantities are mainly attributed to 400 times less pot ale treatment capacity and use on non-adopted sludge in the lab scale batch experiments. As well as advantages of UASB reactor.

As opposed to higher biogas yields available in literature, lower biogas yields at lab scale batch experiments were published by Uzal et al. 2003. Two different inoculum sources, mixed anaerobic culture obtained from domestic wastewater treatment plant (filtered 1 mm a screen of 1 mm mesh size before use) and acetate enriched Methanosarcina culture from a fully established CSTR, were used collectively as inoculum with a volumetric ratio of 1:1 for lab scale (50 ml active volume) batch experiments published by Uzal et al. 2003. The highest biogas production of 260.5 ml (16.8 ml biogas/g VSS) and 332.6 ml (21.5 ml/g VSS) achieved with 15210 mg/L initial COD concentration with and without nutrient additives respectively under mesophilic conditions. It is also reported that nutrient additive does not increase the cumulative biogas production, but shortens the acclimation

time for the microbes. These results are considerably lower than the experimental results of non-treated pot ale seeded with an I/S of 1, 3 and 5 given in Table 49 (Control 1: 509 ± 2.4, Control 2: 709 ± 7.7 and 925 ± 10.4). It is explained by lower organic content of the influent of the reactor as well as 4 times smaller reactor volume than this study. No data for COD, BOD removals. 113 ml mixed granular culture was fed to a lab scale 2 stage UASB (250 ml) to treat 6.1 ± 0.2 g (approximately 6 ml) non-treated pot ale indicating a 95% sludge content. The HRT was reported as 25.8 h. An average of 69 and 99% of COD and BOD removals for the for the influent COD concentration of 33866 mg/L (Uzal et al. 2003). In this study when the sludge seeding ratio was 90% (I/S of 5 on dry basis), non-treated pot ale (control 1 in Table 67) had 38 ± 25 and 43 ± 2.3% COD and BOD removals from the influent concentration of 20583 and 12667 mg/L. Furthermore, 69 ± 1.4 (influent 24747 mg/L) and 57 ± 6.1% (influent 26663 mg/L) COD removals were achieved with the std no 4 in Table 52 and std no 8 in Table 67 respectively when combined alkaline microwave and ultrasonic pre-treatments implemented prior to AD. However, BOD removals could only reach 58 ± 5.0 and 60 ± 3.3% respectively. No biogas data was reported for the experiments performed with UASB.

Hydrolysis constant was calculated for the first 2 and 2^{nd} – 4^{th} days of AD of pot ale after alkaline-microwave pre-treatment for I/S ratios of 1, 3 and 5 and after alkaline ultrasonic pre-treatment of I/S of 5 according to the 1^{st} order kinetic model which is considered one of the most for especially for batch experiments according to previously published studies (Kafle et al. 2014; Khan et al. 2016; Rathaur et al. 2017; Deepanraj et al. 2015; Vazifehkhoran et al. 2018; Barrena et al. 2018; Xie et al. 2011; Yu et al. 2018; Astals et al. 2014)

The hydrolysis rate constant of non-treated pot ale (controls for combined alkaline microwave pre-treatment) was determined as 0.1204 ± 0.004, 0.2165 ± 0.012 and 0.2268 ± 0.007 day^{-1} for I/S ratios of 1, 3 and 5 on dry basis respectively for the first 2 days of AD (Control 1, 2 and 3 in Table 50). Similarly, the hydrolysis constant for no-treated pot ale with an I/S ratio of 5 (control for combined alkaline ultrasonic pre-treatment) calculated as 0.2492 ± 0.018 day^{-1} for the first 2 days of AD (Control 1 in Table 64). Increasing I/S ratio from 1 to 3 resulted in a significantly faster hydrolysis step with an increasing k constant

(0.1204 ± 0.004 to 0.2165 ± 0.012; *p* =0.0326) within the first 2 days of AD while no significant difference was found in the hydrolysis rates for I/S of 3 and 5. Dependency of hydrolysis rate on I/S was also published by Raposo et al. 2011 with AD of mung bean. When I/S was increased from 1 to 2 on dry basis consequently hydrolysis rate rose from 0.21 ± 0.13 to 0.30 ± 0.17 day^{-1}. Furthermore, source of inoculum was also reported as a crucial parameter on kinetics of the hydrolysis step of AD of pot ale at lab scale batch mode by Barrena et al. 2018. When granular sludge was used as inoculum at an I/S of 2 the hydrolysis rate constant was calculated as 0.38 ± 0.01, 0.40 ± 0.04 and 0.57 ± 0.04 day^{-1} for non-treated, filtered and deproteinated pot ale respectively. Only the reaction rate for deproteinated pot ale was reported to be significantly higher indicating that protein removal had a pre-treatment effect on AD. On the other hand, when sludge originating from a food processing AD plant, used with an I/S of 4, the hydrolysis rates were reported to be only 0.18, 0.17 and 0.19 day^{-1} respectively. Since there were only slight differences in the k values of AD of non-treated pot ale with an I/S of 1, 3 and 5 (0.1204 ± 0.004, 0.2165 ± 0.012 and 0.2268 ± 0.007 day^{-1}), this study could be considered in an agreement with literature. Although the highest hydrolysis constant of this study was calculated as 0.3417 ± 0.015 day^{-1} (std no 13-17 in Table 50) after 1M NaOH and 240 W microwave pre-treatment at an I/S of 3, it could not reach the values reported (0.38 ± 0.01, 0.40 ± 0.04 and 0.57 ± 0.04 day^{-1} for non-treated, filtered and deproteinated pot ale respectively) with granular sludge at an I/S of 2. It suggests that the sludge acclimatization has a bigger importance than increased I/S ratios.

No significant difference (*p:* 0.303) was found between k values obtained from two different experimentations of non-treated pot ale for first 2 days (Control 3 in Table 50 0.2268 ± 0.007 day^{-1} and Control 1 in Table 64 0.2492 ± 0.018 day^{-1}) and for 2^{nd} – 6^{th} days (0.2430 ± 0.0235 day^{-1} in Table 50 and 0.869 ± 0.0009 day^{-1} in Table 64) with an I/S 5. Thus, k values are considered to be comparable to assess the efficiency of the pre-treatments. The hydrolysis constants were found to be within a range of 0.2104 ± 0.064 – 0.2402 ± 0.015 day^{-1} for combined 1 M NaOH microwave pre-treatments at I/S of 5 (std no 2, 4, 6, 8 in Table 50) within first 2 days of AD. While the range was found as 0.1746 ± 0.014 – 0.2774 ± 0.048 day^{-1} for ultrasonic pre-treatment in isolation (std no 5, 6, 9, 10 in bb and in combination with 3 M NaOH pre-treatment (std no 7, 8, 11, 12 in Table 64). Based on

this data, no significant difference was found arising from the type of pre-treatment. However, kinetic data for 2nd – 6th days of AD was significantly higher in alkaline microwave pre-treated samples (0.2889 ± 0.018 – 0.3287 ±0.024 day^{-1}) than alkaline ultrasonic per-treated samples (0.0915 ± 0.010 – 0.1084 ± 0.002 day^{-1}) with *p* values 0.026 and 0.004 of lower upper limits respectively. It suggests that methane generation rate reduced significantly within the 2nd and the 6th days of AD due to the implementation of ultrasonic pre-treatment in isolation as well as in combination with 3 M NaOH pre-treatment. Hence almost 2-fold increase was seen in the cumulative methane yield with combined 1 M NaOH 240W microwave pre-treated sample (1614 ± 168 ml/g VS with std no 4 in Table 49) in comparison with combined 3 M NaOH 40% amplitude ultrasonic pre-treatment for 2 h (816 ± 4 ml/g VS std no 7 in Table 63). On the other hand, combined 1.5 M NaOH ultrasonic pre-treatment had substantially lower reaction kinetics ranging from 0.01178 ± 0.002 – 0.0313 ± 0.006 day^{-1} (Table 64) for the first 2 days of AD. Despite having an increased kinetics for the 2nd – 4th days AD, no difference was found in the overall methane yields. The lower biogas quality and quality results for alkaline ultrasonic hybrid were associated with the formation of the potential inhibitory compound such as hydroxymethylfurfural, furfurals and soluble phenolic compounds due to excessive local temperatures during ultrasonic pre-treatment of lignocellulosic biomass with regard to previously published studies (Ariunbaatar et al. 2014; Hendriks & Zeeman 2009; Zhen et al. 2017; Chen et al. 2014).

In the biogas modelling with alkaline microwave hybrid pre-treatment no significant interaction was found between the design factors for both CH4 and CO2 generations with combined alkaline and microwave pre-treatment. For the process optimisation, a higher importance was given to minimising H_2S (level 5) than CO_2 (level 3) because of its toxic nature as well as high corrosion risk in the end product use equipment such as biogas upgrader and CHP according to the study published (Yu 2016; Jeníček et al. 2017; Weiland & Weiland 2013)

The optimum I/S was determined within a range of 4.6 – 4.9 (Table 58) when there were no constraints which corresponds around 90% sludge use for a total 400 ml of pot ale sludge mixture. In order to ease the larger scale applications, minimising the amount of

sludge use was required. Therefore, in the second optimisation approach in addition to the microwave power, I/S were aimed to be minimised (Table 59). As a result, a slight drop in the desirability from 64% (Table 58) to 55% (Table 60) was seen. Dependency on microwave energy was reduced from approximately by 60% from 400 (50% Table 58) to 160W (20% Table 60) “When the design factors were constrained a significantly smaller optimum are was found with regard to the first optimisation approach with no constraints on the design factors.

A good model fit was achieved with the biogas data gathered within the first 2 days of AD after implementation of hybrid alkaline ultrasonic pre-treatment. H_2S had a significant model fit for the first time for this experimental plan. The interactions between alkaline dose and time for ultrasonic pre-treatment in 2 days of CO_2 yield and H_2S production (Figure 49). The same optimisation approaches for the responses of interest were followed as in combined alkaline and microwave pre-treatments. A 62% desirability (Table 74) was seen when the design factors had no constraints (Table 74). However, when time and amplitude ratio of the ultrasonic pre-treatment was aimed to minimised with an importance level of 5 to mitigate application conditions, the desirability increased to a range of 64 – 76% (Table 76). Consequently, a larger optimum area was obtained with graphical optimisation due to the broader upper and lower limits of suggested numerical solutions (Table 76) with regard to the solutions approach 1 (Table 74).

CHAPTER 6: Quality of Digestate for Agricultural Use

6.1 Introduction

The anaerobic digestion process has 2 end products (biogas and digestate) as stated in the literature review in Section 2.2.3. Previously in Chapter 4 and 5 AD digestion potentials of whiskey distillery/brewery waste streams has been discussed. Significant enhancements in the biogas quality and quantity has been achieved by implementation of various pre-treatments such as alkaline pre-treatment standalone an in combination with beating and microwave pre-treatments. This chapter covers the mineral quality of pot ale digestate representing different AD plans in order to have a complete research project on AD and its end products. Fertilization studies have received a scant attention while the physical and chemical properties of digestate have been investigated widely in the literature (Bonetta et al. 2014; Makádi 2012). Therefore, this chapter focuses the digestate quality of whiskey distillery waste in terms of macro (P, K, Ca, Mg) and micro nutrients (Fe, Mn, Zn, Cu) and its comparison with different types of digestate originating from different raw materials. Moreover, the feasibility of pot ale digestate in soil applications for industrial implementations of AD technology will be covered.

6.3 Mineral Analysis Results

Digestate samples were selected representing different experimental design with different pre-treatment conditions. The abbreviation of the samples is given in Table 78.

Table 78. Digestate sample abbreviations

Abbreviation	Digestion Conditions
90% S NT	90% S Non-treated pot ale
90%S 3M A	90%S 3 M NaOH pre-treated pot ale
80%S 1M AP30	80%S 1M NaOH + 30% microwave pre-treated pot ale
75%S 1M AP30	70%S 1M NaOH + 30% microwave pre-treated pot ale
90%S 0.7Amp 3h	90%S 0.7 Amplitude, 3h ultrasonic pre-treated pot ale
90%S 3M A 0.7Amp3h	90%S 3M NaOH +0.7 Amplitude, 3h ultrasonic pre-treated pot ale

In addition to the digestate samples originating from the lab scale experiments and industry, pot ale and inoculum in isolation were analysed to observe the changes in the mineral concentrations due to applied pre-treatments and digestion.

The results of mineral analysis of pot ale and inoculum in isolation as well as seven different digestate samples are given in Table 79. No heavy metals (As, Cd, Co and Mo) were detected in any of the samples. Volatile fatty acids also were not present in the digestate samples obtained from lab experiments.

Table 79. Total mineral analyses of non-treated pot ale, inoculum and digestate samples

Sample	P	K	Mg	Zn	Mn	Cu	Fe
NT Pot Ale	476.5 ± 11.8	954.2 ± 11.3	107.3 ± 0.4	0.49 ± 0.01	0.30 ± 0.01	53.04 ± 3.2	2.55 ± 0.2
Inoculum	1620.5 ± 18.2	1225.3 ± 3.7	209.6 ± 11.4	16.87 ± 0.4	18.47 ± 0.1	3.40 ± 0.1	349.0 ± 3.8
Ind. Sample	1082.6 ± 6.8	1090.0 ± 13.4	104.8 ± 2.3	12.52 ± 0.3	12.54 ± 0.2	2.70 ± 0.4	224.3 ± 3.5
90% S NT	1496.5 ± 27.2	1139.5 ± 2.2	156.2 ± 23.9	14.95 ± 0.3	17.47 ± 0.3	24.51 ± 0.3	291.9 ± 8.5
90%S 3M A	1466.3 ± 18.9	1127.9 ± 8.6	160.5 ± 10.4	14.12 ± 0.27	16.76 ± 0.12	61.05 ± 0.81	281.7 ± 9.0
80%S 1M AP30	1391.6 ± 17.7	1174.4 ± 6.1	151.9 ± 13.3	14.00 ± 0.21	15.13 ± 0.13	13.15 ± 0.14	279.8 ± 3.9
75%S 1M AP30	1267.0 ± 22.9	1106.4 ± 4.1	137.1 ± 13.2	12.17 ± 0.15	13.28 ± 0.14	18.01 ± 0.01	241.6 ± 3.6
90%S NA 0.7Amp 3h	1397.4 ± 17.9	1175.4 ± 6.7	153.5 ± 7.6	13.42 ± 0.70	15.46 ± 0.22	85.99 ± 0.7	266.6 ± 11.4
90%S 3M A 0.7Amp 3h	1347.0 ± 66.1	1140.8 ± 8.6	124.6 ± 34.2	13.07 ± 0.33	15.31 ± 0.50	62.76 ± 0.4	255.8 ± 17.3

* All units are in mg/L

Although the inorganic content of digestate is directly linked to raw material source the compositional analysis of samples of various pre-treatment conditions representing each experiment design was provided in order to have a detail analysis for the potential industrial implementations of each experiment plan.

Industrial digestate sample, which is already used as fertiliser in Ireland, was taken as a reference sample to evaluate the quality of the digestate to be used for agricultural purposes. The fertilizer value of the digestate is assessed with the concentration of P and K as these are essential for crop growth. The P and K concentrations of the reference samples was determined to be 1082.6±6.8 and 1090±13.4 mg/L respectively. All digestate samples regardless of the seeding ratio and pre-treatment had higher P and K concentration than the industrial sample within ranges of 1267±22.9 – 1496.5±27.2 and 1106.4±4.4 – 1175.4±6.7 respectively. In particular the P content was found to be remarkably higher than the industrial sample in all cases. As part of the macro nutrients, Mg concentration was determined to be higher in the lab scale digestate samples than the reference sample. 3 M NaOH pre-treated sample seeded with 90% inoculum had the highest Mg concentration of 160.5±10.4 mg/L which was approximately 50% higher than the reference sample (104.8 ± 2.3 mg Mg/L).

In terms of micro nutrients, similar figures were seen in the concentrations of Zn and Mn with digestate of the lab scale experiments with the reference sample. A maximum increase of 2.43 and 4.93 mg/L was seen the lab scale experiments digestates in the Zn and Mn concentrations respectively with regards to the reference sample. Slightly higher concentrations of Fe were seen in the digestates of the lab scale experiments with a maximum value of 291.9±8.5 mg/L (30% higher than the reference digestate). No big differences were seen in the Zn, Mn and Fe concentrations arising from the pre-treatment types/conditions as well as the different seeding ratios. However, various Cu concentrations ranging from 13.15±0.14 to 85.99±0.7 mg/L were observed depending on the pre-treatment types while the reference sample had only 2.70±0.4 mg Cu/L. In the presence of strong alkaline agents (3M NaOH) and ultrasonic pre-treatment prior to AD, 3-fold more Cu concentrations were determined in comparison to the digestate of the non-treated pot ale (24.51±0.3 mg/L) when the sludge seeding ratio was 90% on wet

basis. On the other hand, combined 1 M NaOH and microwave pre-treatment at 30% power resulted in 13.15±0.14 and 18.01±0.01 mg/L when the sludge seeding ratio was 80 and 75% respectively. Sludge and non-treated pot ale in isolation had only 3.40±0.1 and 53.04 ± 3.2 mg/L Cu concentration respectively.

6.4 Discussion of Key Findings

Very few studies in inorganic characteristics of pot ale have been published previously. A typical range of 990 – 1200 mg/L K was stated by Barrena et al. 2018 in a study conducted with pot ales from 4 different distillery, while a wider range of P concentrations (150 – 600 mg/L (Akunna & Clark 2000) and 740 mg/L (Tokuda et al. 1999)) was reported. In this study the concentration of K and P was determined as 954.2 ± 11.3 and 476.5 ± 11.8 mg/L which are in an alignment with the previous studies since they all fall into the relevant reported ranges. Moreover, the concentration of Cu and Zn were seen as 40 – 80 mg/L (Akunna & Clark 2000) and 0.368 – 1.8 mg/L (Barrena et al. 2018; Harada 1996) in the literature respectively indicating that determined concentrations of 53.04±3.2 mg Cu/L and 0.49±0.01 mg Zn/L agree with the previously published research. The concentration of Mg (107.3±0.4 mg/L) was found to be slightly lower than the published range of 120 – 270 mg/L (Tokuda et al. 1999; Barrena et al. 2018).The differences were attributed to the variety in the source of raw materials used in whiskey manufacturing.

The mineral quality of the whiskey distillery digestate with or without application of pre-treatment was not available in literature since it has not been investigated previously. Therefore, direct comparison of the results with a similar raw material source of a digestate was not possible. In order to evaluate the agricultural applications of the digestate, a food waste based digestate, which is already used as biofertilizer in Ireland, was selected as reference sample for comparison. The nutrient content of the digestates in terms of P, K, Mg, Zn, Mn and Fe was found to be in an agreement with the reference sample regardless of applied pre-treatments and different sludge seeding ratios. An average increase of 413.9, 85.4 and 55.7 mg/L in the concentration of P, K and Mg respectively was achieved with pot ale digestate at the lab scale experiment with respect to corresponding values of the reference industrial sample (1082.6±6.8 mg P/L, 1090.0±13.4 mg K/L and 104.8±2.3 mg Mg/L).

On the other hand, the copper concentration of pot ale was determined to be 52.14 ± 1.9 mg/kg where the legal limit was 300 mg/kg for organic biofertiliser (European Parliament 2019). The main reason for the higher Cu concentrations were associated with the occurrence of the mass transfer due to the employment of copper stills for the distillation (Graham et al. 2012). In addition, a variety range of Cu concentrations were determined in the digestates of the lab scale experiments. The minimum Cu concentration of 13.15±0.14 mg/L was achieved with the digestate of 1M NaOH and 240W microwave pre-treated pot ale which was still 4.8-fold higher than the industrial sample. Cu is one of the essential micro nutrients plant growth and it is transferred to animals and humans through the food chain. Therefore, excessive copper release to the soil might cause a risk for animal and human health (Laczi et al. 2017). The two stage AD was previously reported to an efficient method for metal mobilisation (due low treatment with low pH for hydrolysis and acidogenesis steps) and transferring to leachate after the 1st stage consequently achieving lower Cu concentrations in the final form of the digestate (Selling et al. 2008).

From environmental and public health standpoints, using digestate of whiskey distillery wastes as biofertilizer is considered to be a promising replacement material to animal by product digestates due to their high risk of containing of parthenogenic bacteria such as *Salmonella* and *Klebsiella spp. Klebsiella* were previously reported to be implicated in human infections. *Salmonella* are known to be transmittable to human and animals though contaminated food and water (Owamah et al. 2014). The presence of this pathogen has been reported in digestate originating from cattle manure, pig slurry (Kuusik et al. 2017), cow dung and chicken dropping (Alfa et al. 2014), food waste and human excreta (Owamah et al. 2014). Therefore pasteurisation step before applying digestate to farm land is required by the EU Regulation No 1774/ 2002 (EUROPEAN COMMUNITIES 2002).

Digestate characteristics are known to be specific to each reactor as it is a result of a living process. Therefore, its nutritious quality might vary for the different batches of the same digester and even within the same digestate batch (Faisal-Cury & Menezes 2006). Therefore, mineral analysis of digestate from different sampling points is advised for each

batch. Although presence of pathogenic bacteria is mainly associated with animal and human based digestate testing microbiological characteristics of pot ale digestate is recommended for increased public safety.

Chapter 7: Industrial Application Modelling of Pre-treatments

7.1 Introduction

This chapter aims to identify the potential energy recovery enhancements can be achieved though implementation of pre-treatment at full scale applications. The interpretation of lab scale experiment results is provided for creating an industrial implantation model to whiskey distilleries with different manufacturing capacities.

7.2 Research Approach

Pot ale was considered the main substrate of the AD since it causes the major concerns for the distilleries as well as high discharge rates even for small scale distilleries. Moreover, low solid content of pot ale (<8% TS) eases the industrial implementations in terms of pumping simplicity and smaller reactor volume requirements. In this theoretical modelling investigation, the following factors were considered;

1. Pre-treatment contributions in biogas generation and quality of the lab scale experiments
2. Analysis of different end product use technologies
3. Energy outputs and financial viability aspects of the model

7.3 Pre-treatment Contributions in Biogas Yield at Lab-Scale Experiments

The I/S ratio was determined to be the most powerful design parameter on the biogas quality and quantity with DOE software in Chapter 4 and 5 for alkaline and combined alkaline/thermal pre-treatments respectively. It also has a key role in defining the reactor size for industrial applications for diverse size of distilleries. Therefore, the sludge seeding ratio range of 0 – 90% (on wet basis) was scanned with non-treated (NT) as well as 1 M NaOH pre-treated pot ale. The biogas yield results for NT and 1M NaOH pre-treated pot ale is given in Figure 53 based on sludge seeding ratio.

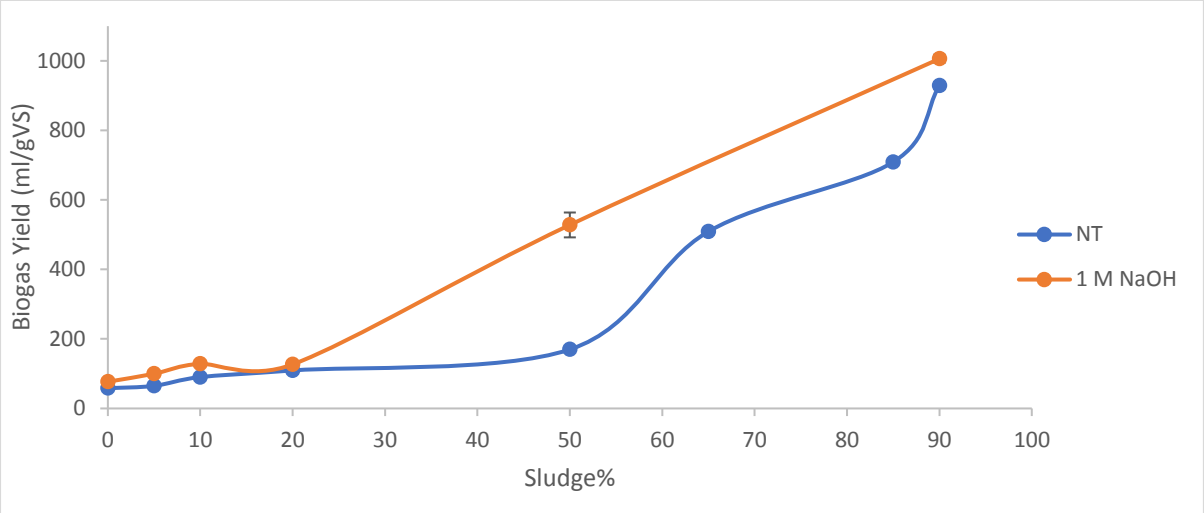


Figure 53. Biogas yields of NT and alkali pre-treated pot ale

In addition to low biogas yields, biogas quality (CH_4) was also found to be below 10% for the seeding ratios of 0 – 20% of total volume of 400 ml, whereas it increases to almost 20% with 50% seeding. Moreover, implementation of 1 M NaOH pre-treatment increased the biogas quality to 48.6 ± 2.9% CH_4 for the 50% sludge content. In addition to low CH_4 content, excessive H_2S productions, which was above the upper measurement limit of 10000 ppm, were seen when the sludge seeding ratio was lower than 50%, whereas with 50% the H_2S concentrations were determined as 1139 ± 27 and 2429 ± 697 ppm for non-treated and 1 M NaOH pre-treated pot ale respectively with 50% sludge seeding. Further biogas yield comparisons were therefore focused on higher sludge contents (≥50%). Biogas yields of NT pot ale as well as combination of 1 M NaOH pre-treatment with 80 and 240 W microwave pre-treatments were determined for the seeding ratios of 65, 85 and 90% on wet basis. 1 M NaOH pre-treatment without microwave was only performed for 50% and 90% sludge seeding due to space constraints. The results are given in Figure 54. The combined 1 M NaOH and 80 W microwave pre-treatment resulted in a significant increase in the biogas yield for 65 (*p:*0.0010), 85 (*p:* 0.0221) and 90% (*p:* 0.0421) sludge seeding ratios in comparison to the non-treated situation. For the sludge seeding ratios of 85 and 90%, coupled 1M NaOH and 240W microwave pre-treatment caused further enhancements in the biogas generation with the *p* values of 0.0155 and 0.0135 respectively with regards to non-treated pot ale. The only significant difference arising from the microwave power was seen between 80 and 400 W for the 90% sludge seeding

with a p value of 0.0051. Therefore, two lower power settings were considered to be more suitable for larger scale applications from energy consumption point of view.

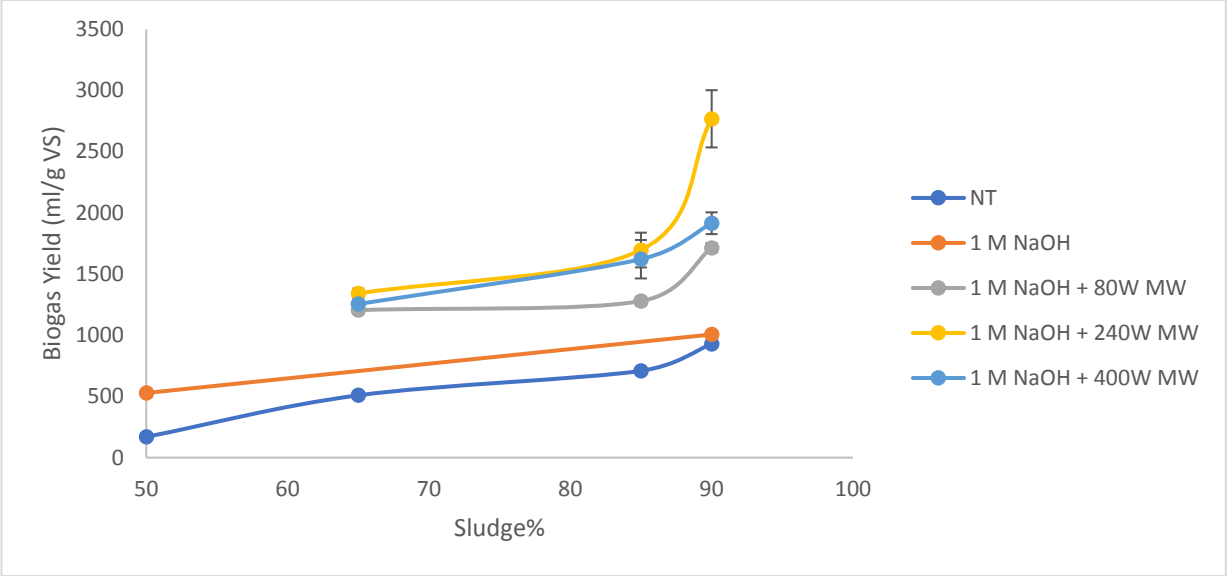


Figure 54. Biogas yields of NT, alkali and combined alkali thermal pre-treated pot ale

In addition to the biogas quantity, biogas quality results are also given in Figure 55 and Figure 56 in terms of CH_4 and H_2S respectively. The CH_4 content of NT pot ale increased to 54.4 ± 1.5% from 19.6 ± 0.6% (*p:*0.0068) when the seeding ratio was increased from 50 to 65%. No significant increase was seen in the CH_4 content arising from the different microwave power settings.

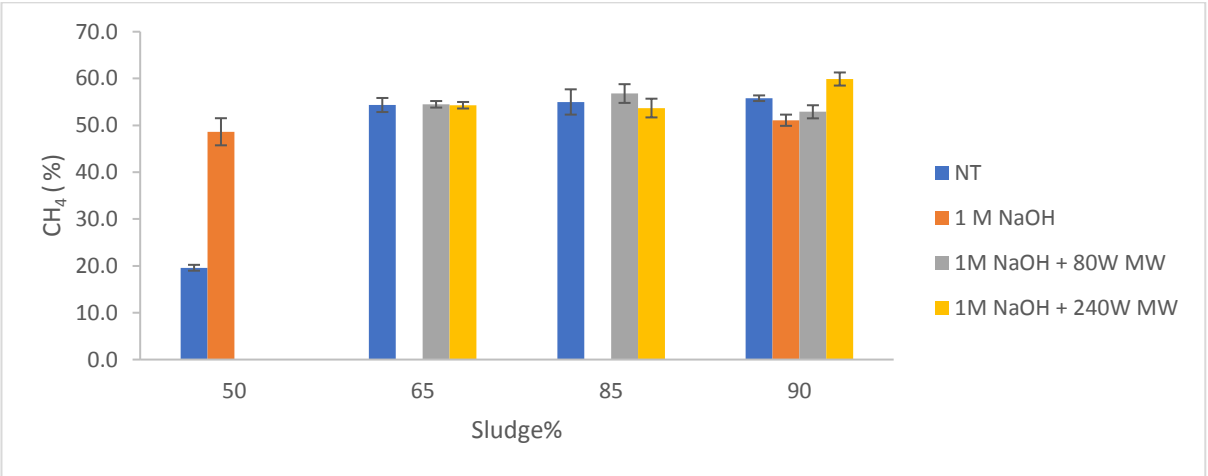


Figure 55. Biogas quality of NT, alkali and combined alkali thermal pre-treated pot ale

The 1 M NaOH pre-treatment led a significantly higher H_2S generation (*p:* 0.0018) for the sludge seeding ratio of 50% (Figure 56). On the other hand, increased amount of sludge use (90%) resulted in lower H_2S productions in comparison with the digestion of the non-treated pot ale.

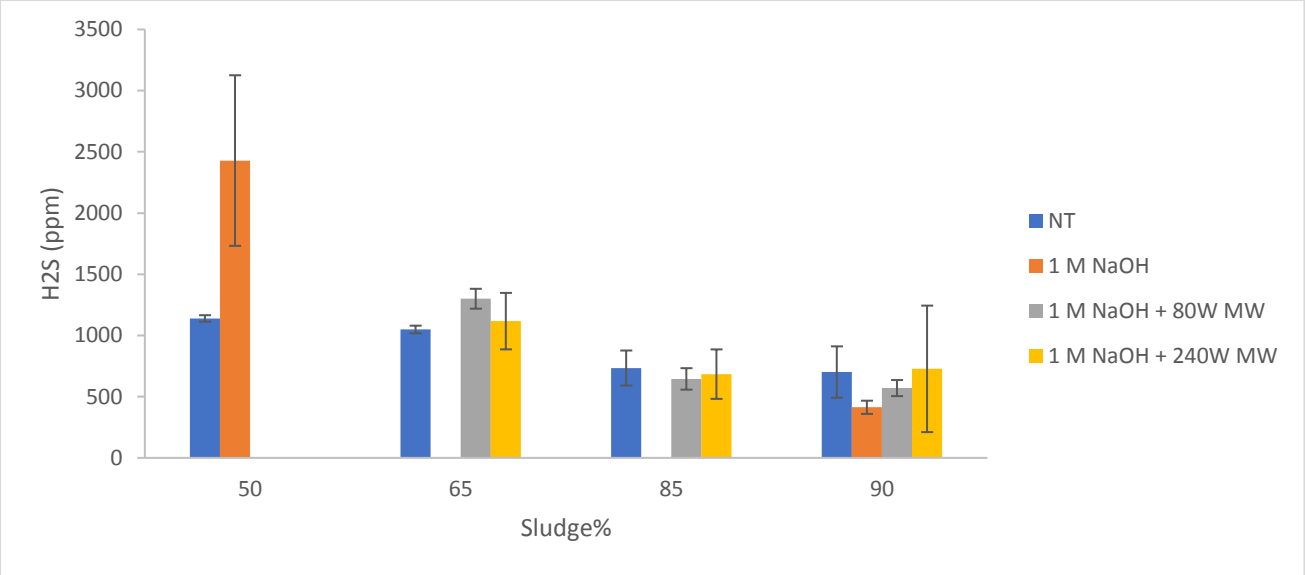


Figure 56. H_2S generation of NT, alkali and combined alkali thermal pre-treated pot ale

According to the lab-scale experiment results, four different scenarios were created based on the pre-treatment types applied on pot ale prior to AD in the lab scale experiment. The energy recovery yields were calculated for non-treated, 1M NaOH pre-treated alone and in combination with 240 and 80W microwave pre-treated as well as non-treated pot ale to assess the potential enhancements in bioenergy conversion yield based on different sludge seeding ratios. The summary of different scenarios is given in

Table 80. The implementation of the microwave pre-treatment would not be feasible for full-scale applications due to high volume of pot ale discharge. Therefore, it was considered as low temperature thermal pre-treatment (≤100˚C) based on the created temperature profile in Section 3.3.5.6 (Table 9) which allows a use for excess heat potential of the distilleries in pre-treatment step.

Table 80. Summary of different scenarios

Scenarios	Pre-treatment conditions
Scenario 1	Non-treated
Scenario 2	1 M NaOH pre-treated
Scenario 3	1 M NaOH + 65 ˚C Thermal pre-treated (1 M NaOH + 80W MW)
Scenario 4	1 M NaOH + 100 ˚C Thermal pre-treated (1 M NaOH + 240W MW)

7.4 Mass Balance and Energy Demand of Typical Malt Whiskey Distilleries

Mass balance of whiskey manufacturing process does not vary remarkably for individual plants depending on manufacturing scale, raw material selection etc (Pyke 1965; Goodwin et al. 2001), and distillery energy demand is directly related to the operation size. A typical mass balance for malt whiskey distillery producing 1 million litres of alcohol was reported by Hamill 2015. The operational flowchart of the distillery given in Figure 57 was used as the baseline for the assumptions of the theoretical calculations.

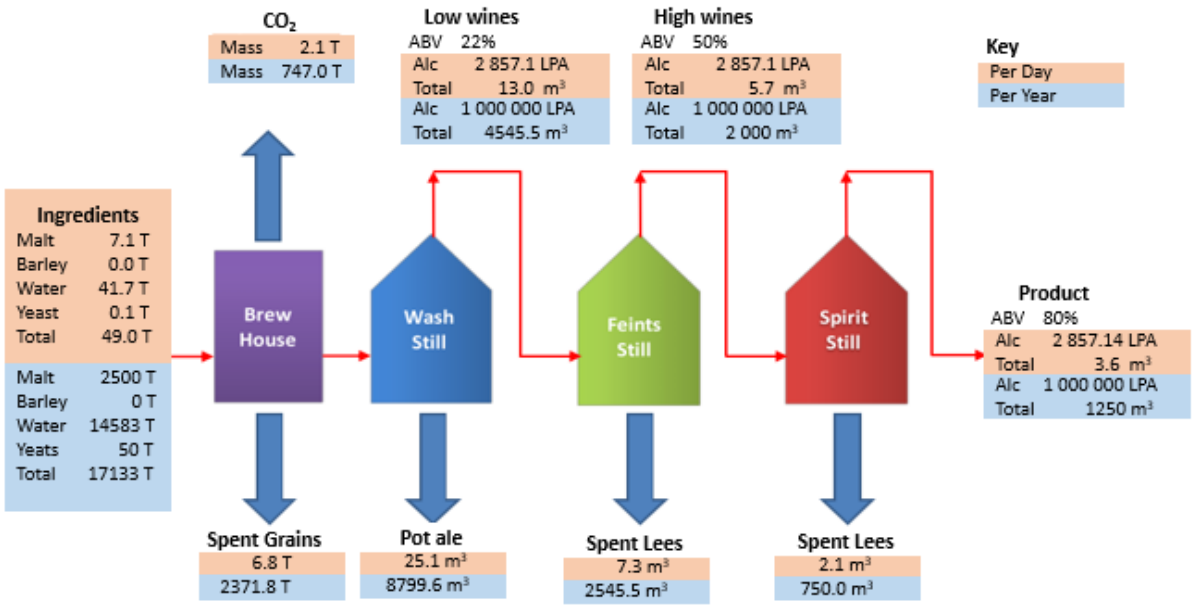


Figure 57. Mass balance for a typical malt whiskey distillery

In order to estimate the electrical and the thermal energy demands of a distillery producing 1 million L whiskey per year, a correlation was created based on a previous research conducted by (Meadows 2015) including the data from 7 different distilleries in the UK. The correlations for the electrical and the thermal demands are given in Figure 58 and Figure 59, respectively.

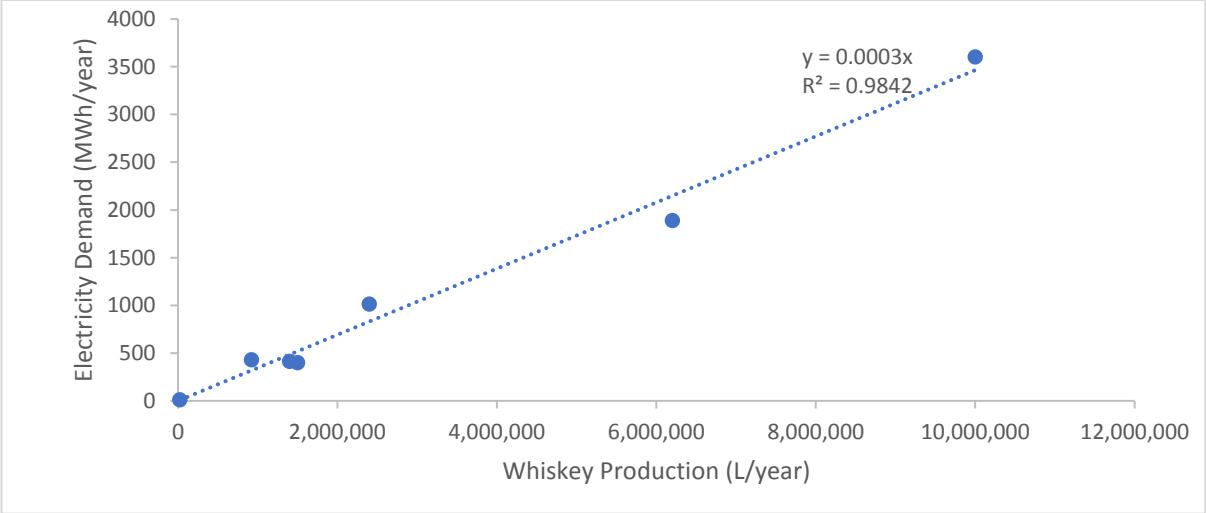


Figure 58. Electricity demand vs annual whiskey production

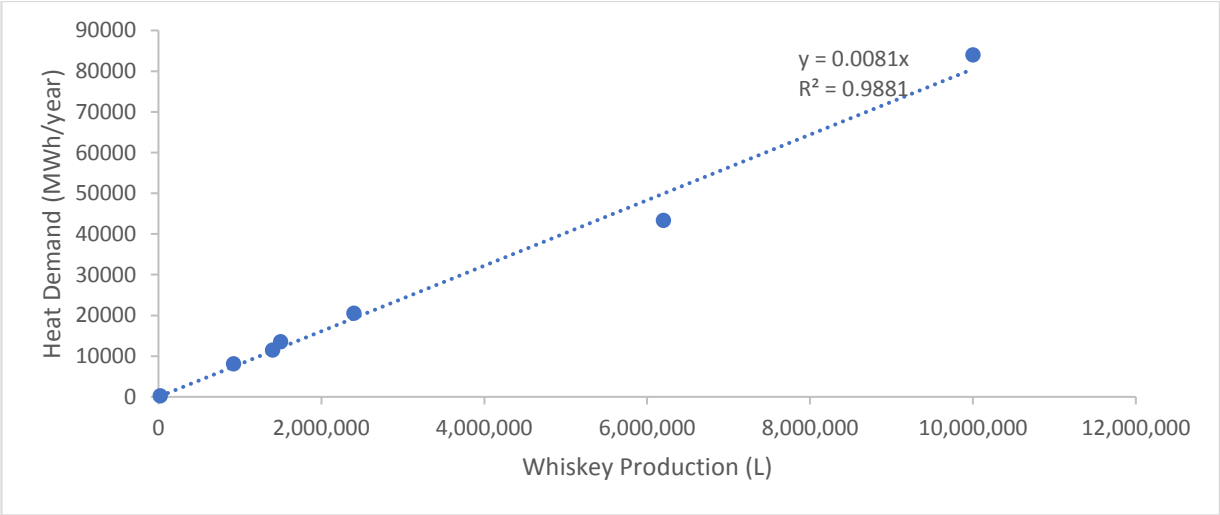


Figure 59. Thermal demand vs annual whiskey production

According to the standard curves, electricity and the heat demand of the distillery was estimated as 300 MWh/year and 8000 MWh/year respectively with a total energy demand estimation of 8300 MWh/year.

7.5 Generic Approach to AD Plant Design

The continuous stirred tank reactor (CSTR) is one of the most preferred configurations for industrial applications due to its operational simplicity in comparison with 3rd generation high rate reactors (Duguid & Strachan 2016) for biogas production. For the end product (biogas) usage of an AD, employing a CHP and a biogas upgrader are the most common technologies (SWA 2012; Brinkerhoff 2012). Main advantages of the CHP are that biogas

can be used with no further purification step, whereas biogas upgrading units demand further post-treatments to reach 97% methane within the produced biogas to be used as fuel. Moreover, the infrastructure required to develop the CHP is less complex and expensive than upgrading units since it does not require a connection to the grid (Goulding & Power 2013). The CHP is therefore considered a more feasible technique and is the most common application for whiskey distilleries at industrial scale for end product usage (Clegg 2017; Meadows 2015). Therefore, combining a CSTR with a CHP unit was selected for biogas yield estimations (Figure 60). Typical biogas losses in the CHP units were estimated to be 0.5%, with electrical and thermal efficiencies of 38 and 39% respectively (Goulding & Power 2013). Internal electrical and thermal energy demand was reported to be proportional to the production size. Typically, 6.9% of electricity produced by CHP is utilised to operate electrical components such as pumps, mixing units etc. as well as computer system of the plant (Pfiel 2007). Similarly, heat demand of the plant was published to be approximately 13.5% of thermal energy production of an AD plant (Goulding & Power 2013)

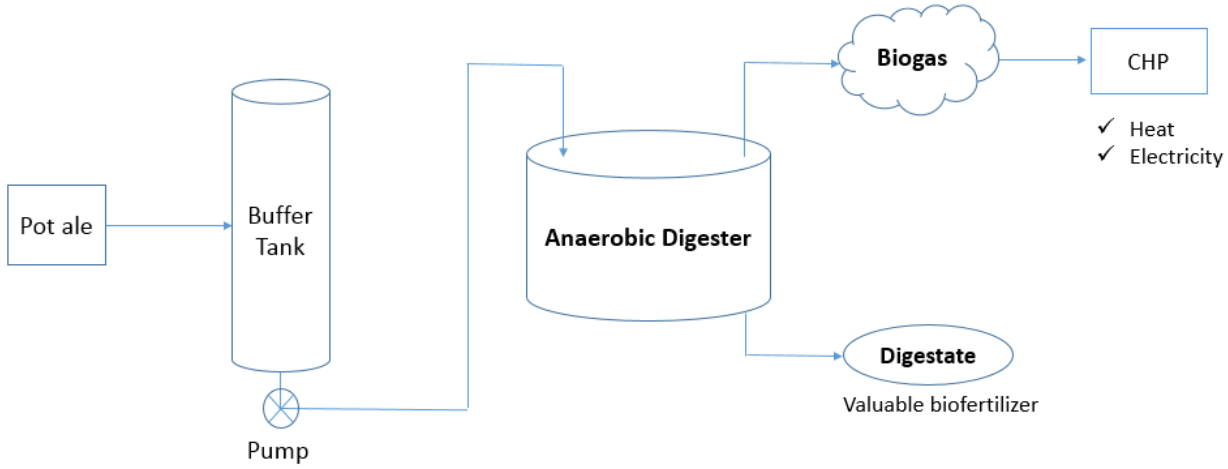


Figure 60. AD plant design

According to the mass balance of the distillery (Figure 57), approximately 8.8M L of pot ale is discharged per annum to produce 1M L of whiskey as the baseline of this study.

Assumptions:

1. The biogas yield of full-scale application was assumed to be the same as the lab scale experiments.

2. The OLR of the reactor was assumed to be 38.8 ± 0.12 kg/m^3 (which is the COD level of pot ale)
3. The HRT was assumed as 6 days since approximately 70 – 80% of the digestion was completed within first 6 days of AD in the lab scale experiments for all cases
4. Operation under mesophilic conditions (35 ˚C).

7.6 Energy Recovery Potential Results

The biogas yield estimations of full-scale applications were conducted based on the mass balance given in Section 7.4 for the 4 different scenarios given in Table 80 . Sample calculations for energy analysis of generated biogas and the required CHP capacity and energy output are given in Appendix 13 and Appendix 14 respectively. The biogas yields of the first 6 days of AD at lab scale were assumed to be the overall yield of the full-scale applications. The estimated results for the biogas annual biogas generation, energy potential of the produced biogas and energy demand of the plant is given in Table 81. The increased sludge seeding ratio significantly increased the biogas estimation per annum for AD of non-treated pot ale (scenario 1 in Table 81) with a *p* value of 0.0134, for 65% sludge (on wet basis) in comparison to 50%. Moreover, annual biogas generation of non-treated pot ale with 85% sludge seeding ratio was significantly (*p:* 0.0180) higher than 65% sludge. Similarly, only 5% increase in sludge content from 85% resulted in rising estimated annual biogas production from 561646 ± 2652 m^3 to 710075 ± 3249 m^3 (Table 81) with a *p* value of 0.0005. The implementation of 1 M NaOH pre-treatment (scenario 2 in Table 81) also had a significant rise (*p:* 0.0022) in annual biogas estimation from 90965 ± 2043 m^3 to 216883 ± 12189 m^3 for 50% sludge content. On the other hand, when the seeding ratio was increased to 90%, no significant difference was seen in annual biogas estimations regarding the application of the alkaline pre-treatment with regards to the non-treated case (Scenario 1). Similarly combined 1 M NaOH and 65 ˚C thermal pre-treatment (scenario 3 in Table 81) had no enhancements in estimated biogas production for any sludge seeding ratio in comparison with the non-treated pot ale (scenario 1). However, annual biogas generation estimations after applied 1 M NaOH and 100 ˚C thermal pre-treatment (scenario 4 in Table 81) was found to be significantly higher for the sludge ratios of 65 (*p:* 0.0285), 85 (*p:* 0.0009) and 90 (*p:* 0.0191)% than the non-treated pot ale. In addition, the maximum biogas generation was estimated as 970695 ± 64430 m^3/year.

Table 81. Biogas to CHP performance based on the sludge content for each scenario

	S%	Scenario 1	Scenario 2	Scenario 3	Scenario 4
Lab-scale biogas yields (m^3/tonne VS)	50	136 ± 4	356 ± 19	N/A	N/A
	65	376 ± 12	N/A	908 ± 15	1017 ± 23
	85	597 ± 3	N/A	1233 ± 31	1493 ± 22
	90	755 ± 3	689 ± 1	1058 ± 37	2208 ± 94
*Biogas production (m^3/year)	50	90965	216883	N/A	N/A
	65	360054	N/A	388590	447179
	85	561646	N/A	527872	656263
	90	710075	518107	452951	970695
*Biogas losses on CHP (m^3/year)	50	466	1134	N/A	N/A
	65	1760	N/A	1943	2236
	85	2799	N/A	2639	3281
	90	3539	2395	2265	4853
Energy input to CHP (MWh/year)	50	183 ± 9	1109 ± 126	N/A	N/A
	65	1774 ± 19	N/A	2131 ± 53	2437 ± 76
	85	3026 ± 86	N/A	2998 ± 62	3488 ± 75
	90	3858 ± 17	2854 ± 229	2408 ± 99	5447 ± 520
*Internal electricity demand (MWh/year)	50	5 ± 0.2	29 ± 3.3	N/A	N/A
	65	47 ± 0.5	N/A	56 ± 1.4	64 ± 2.0
	85	79 ± 2.2	N/A	79 ± 1.6	91 ± 2.0
	90	101 ± 0.5	75 ± 8.6	63 ± 2.6	150 ± 13.6
*Internal heat demand (MWh/year)	50	10 ± 0.5	58 ± 6.6	N/A	N/A
	65	93 ± 1.0	N/A	112 ± 2.8	128 ± 4.0
	85	159 ± 4.5	N/A	158 ± 3.2	184 ± 3.9
	90	203 ± 0.9	150 ± 17.3	127 ± 5.2	300 ± 27.4
CHP Capacity Estimation (kW)	50	15 ± 1	89 ± 10	N/A	N/A
	65	143 ± 2	N/A	172 ± 4	196 ± 6
	85	244 ± 7	N/A	242 ± 5	281 ± 6
	90	311 ± 1	230 ± 27	194 ± 8	439 ± 42

* Average of triplicate runs.

Annual net electrical and thermal energy production estimations of each scenario is given based on sludge seeding ratio in Figure 61 and Figure 62 respectively. The stepwise increase in sludge content, resulted in significant increases ($p < 0.05$) in both estimated electrical and thermal energy production per annum for scenario 1, 2 and 4. In case of scenario 3, increasing sludge content from 65 to 85% had a significant increase in electrical and thermal energy generation with the *p* values lower 0.0001. A further increase of 5% in the sludge content caused a significant reduction in both electricity (*p:* 0.0020) and heat (*p:* 0.0021) generations.

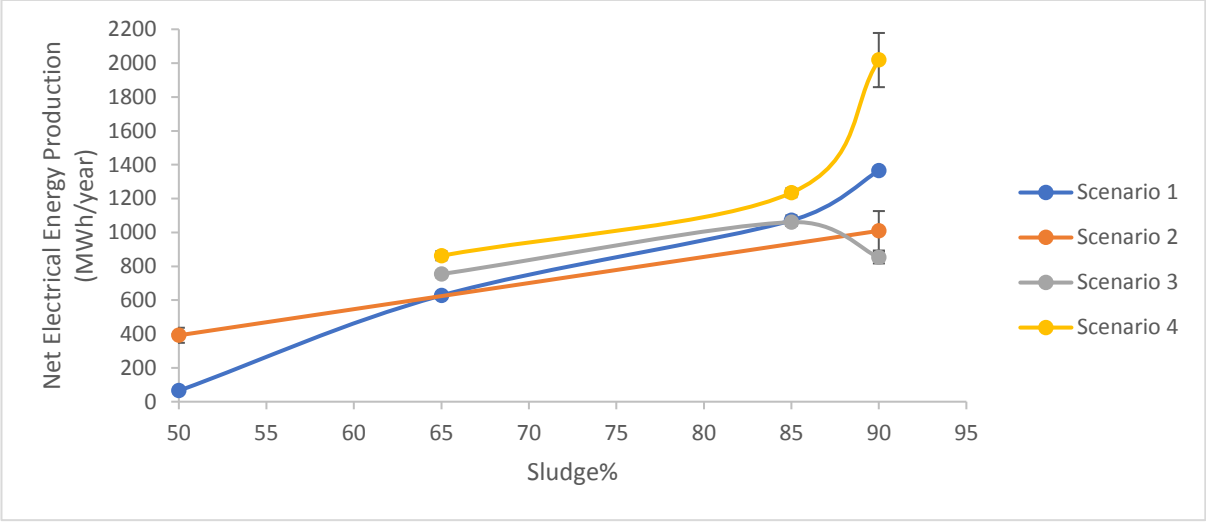


Figure 61. Net electrical energy production for each scenario

When the sludge seeding ratio was 65%, combined alkaline and thermal pre-treatments (scenario 3 and 4) had increased electricity (*p:* 0.0201 and 0.0224) and heat production significantly (*p:* 0.0201 and 0.0228) with regard to the Scenario 1 in Figure 61 and Figure 62 respectively. Furthermore, annual electricity and heat production with combined 1 M NaOH and 100 ˚C thermal pre-treatment (Scenario 3) were found to be significantly higher than non-treated pot ale in Scenario 1.

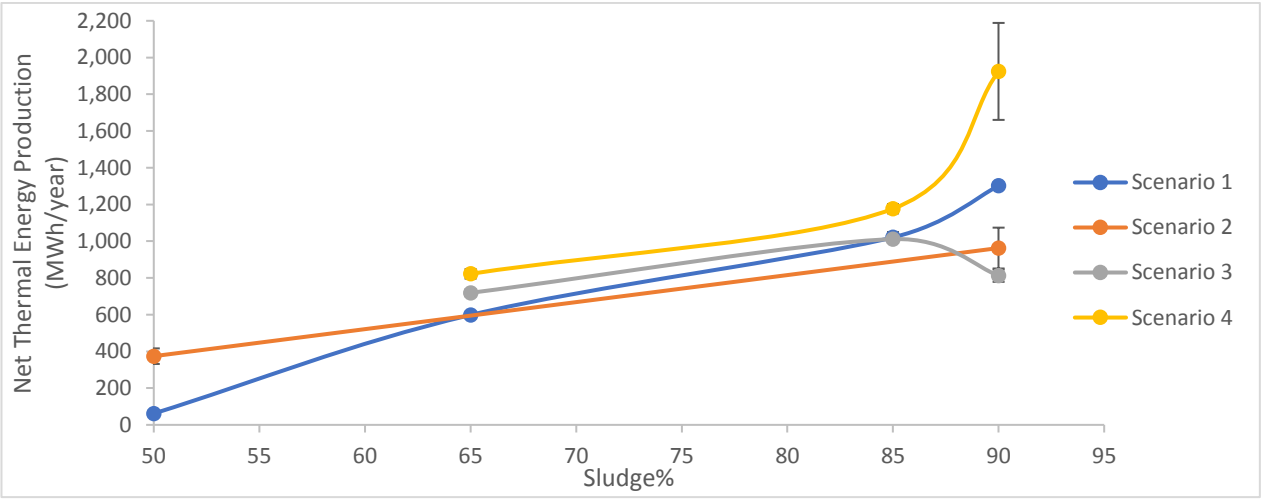


Figure 62. Net thermal energy production for each scenario

The electrical energy demand of the example distillery (Figure 57) which was estimated as 300 MWh/year was achieved in all cases except for 50% sludge seeding ratio of the

Scenario 1. It could only reach the 22 ± 1% of the electricity demand. The excess electricity production for all cases is given in Table 82.

Table 82. Excess electricity production of each scenario

	S%	Scenario 1	Scenario 2	Scenario 3	Scenario 4
Excess Electricity (MWh/year)	50	-	99 ± 45	N/A	N/A
	65	335 ± 7	N/A	461 ± 19	569 ± 27
	85	778 ± 30	N/A	768 ± 22	941 ± 26
	90	1078 ± 6	717 ± 117	559 ± 35	1725 ± 154

The potential heat recovery level of each scenario is given in Figure 63. Significant increases were seen in the potential heat recovery percentages due to the stepwise increased amount of sludge ratios in the Scenario 1,2 and 4. In case of Scenario 3, despite obtaining a significant increase (*p:* 0.0001) in the heat recovery percentage from 9 ± 0.2% to 12 ± 0.3% when the sludge ratio was increased from 65 to 85%, there was no significant difference in comparison to Scenario 1 at 85% sludge ratio. Moreover, a 5% further increase in the sludge percentage had a significant negative impact (*p:* 0.0228) on potential heat recovery. When the sludge ratio was 50% Scenario 2 enhanced the potential heat recovery significantly due to 1M NaOH pre-treatment with regards to Scenario 1. However, at 90% sludge seeding no significant difference was found between the Scenario 1 and 2 (Figure 63).

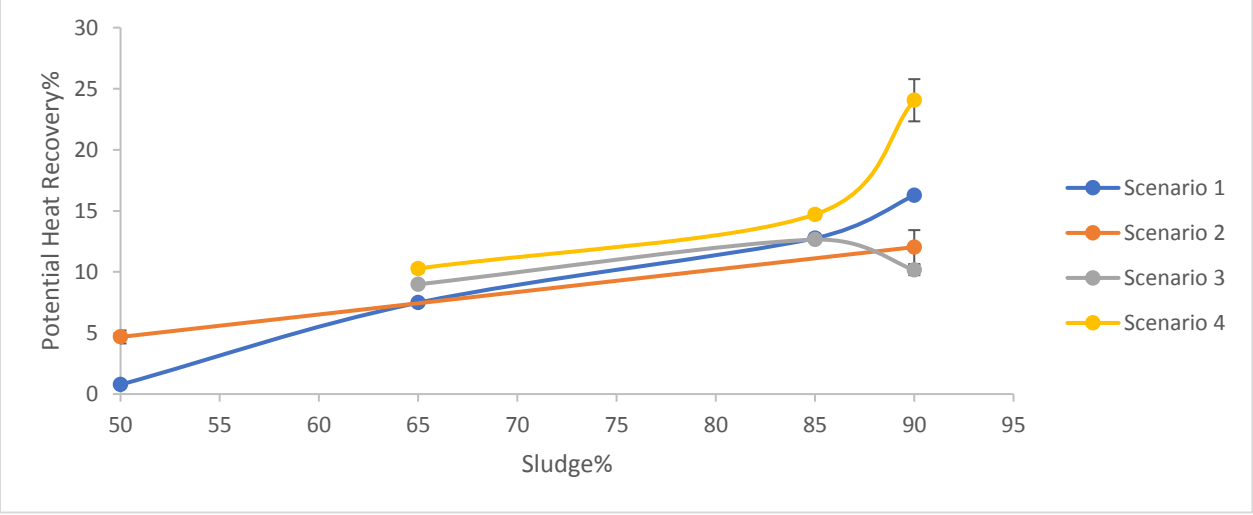


Figure 63. Potential heat recovery levels of each scenario

The maximum heat recoveries were seen in the Scenario 4 due to implementation of combined thermo-chemical pre-treatment regardless of the different sludge seeding ratios. Implementation of the combined 1M NaOH and 100˚C thermal pre-treatment (Scenario 4) increased the heat recoveries significantly in comparison to non-treated case (Scenario 1) with the *p* values of 0.0207, 0.02346 and 0.0276 for 65, 85 and 90% sludge seeding ratios reaching 10±0.3, 14±0.3 and 24±1.7% of total heat demand. Scenario 2 also caused a 2% increase (*p:* 0.0201) in the heat recovery with regards to Scenario 1 for the sludge seeding ratio of 65% whereas no significant difference arising from the application of thermo-chemical pre-treatment for sludge seeding ratio of 85%. Moreover, Scenario 1 (non-treated case) was found to be 6% (*p:* 0.0228) higher than Scenario 3 when the sludge seeding ratio was increased to 90%.

Energy recovery potentials have been presented based on varying sludge seeding ratios which adds flexibility to the model in terms of addressing the individual requirements of the distilleries (e.g different waste discharge capacity and space limitation on site) in real-life applications. Annual biogas production estimations and required CHP capacities were calculated (in Table 61) based on varying sludge seed rations within a range of 50 – 95 for a distillery with an annual whiskey production of 1 000 m^3 which can be scalable for other cases. According the experiment results, seeding ratios higher than 65% of active volume is advised to achieve high methane yields. Once the annual production capacity and seeding ratio was identified mass balance should be tailored accordingly in order to define the capacity of the AD plant and CHP unit required for finalising the financial aspect of the model. It is important to note that during start-up phase of the AD plant it will take some time for the microbial load in the reactor to reach the steady state mode before starting continuous operation as it's a growing mix culture and different types of bacteria have different growth rate. Once the bacteria reach steady state mode, pot ale should be fed to the reactor continuously.

7.7 Factors Affecting Feasibility of AD

7.5.1 CAPEX/OPEX Estimations of AD Plant and CHP Unit

It is impossible to obtain the exact price of the machinery as it differs based on the vendors for each plant as well as time dependency of pricing. Another aspect to take into consideration is that capital cost varies depending on scale. For example, a larger scale AD plant would have a lower capital investment per tonne of waste than a smaller identical technology (WRAP 2013). However, a range of capital and operation expenditures of 7 AD plant with various sizes (500 – 7400 MWh/year) was reported by (Duguid & Strachan 2016) for estimations. The CAPEX and OPEX values of AD plants located in Kilchoman, Ardbeg, Bowmore, Bruichladdich, Bunnahabhiain, Laphroaig and Lagavulin are given in Figure 64 based on the total energy production capacity of the plant. In both cases, relatively linear relationships were achieved therefore it can be used for estimations. Moreover, a typical cost range for grid connection was also reported to be £400 000 – £750 000 as part of CAPEX (Clegg 2017).

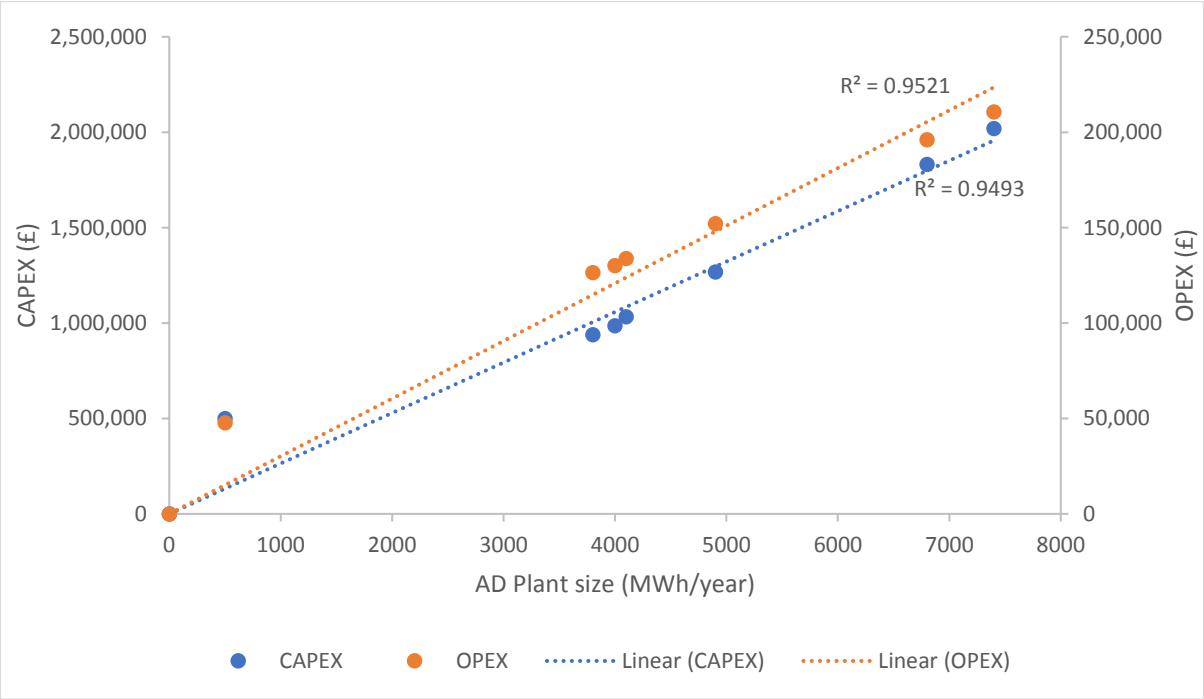


Figure 64. Relation between capacity of AD plants and its capital and operation costs

In this assumption capital costs include, storage/pre-treatment equipment for feedstock, full set of AD plant equipment (feedstock feeding pump, digestion tank and gas holder) as

well as feasibility studies, civil works and land costs. The operational costs include, staff costs in terms of training and salaries, plant maintenance and insurance.

In addition to the costs of AD plant, typical CAPEX and OPEX of CHP system, which was obtained from the plant owners, was also previously reported based on the capacity of unit for 4 different conditions. The correlation is given in Figure 65 Similar to the AD plant cost, it has shown a linear relationship with the CHP capacity.

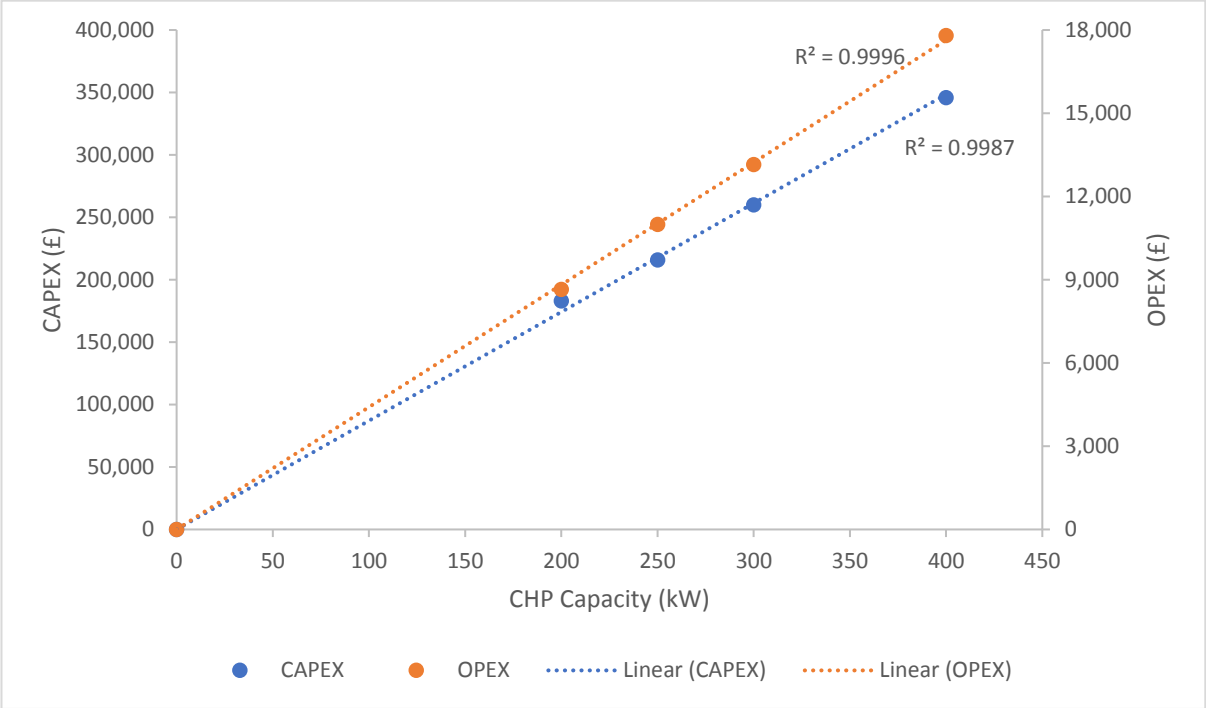


Figure 65. Relation between capacity of CHP unit and its capital and operation costs

In order to estimate the most accurate CAPEX and OPEX values, first of all sludge seeding ratio should be chosen depending on the annual pot ale discharge volume which is indirectly linked to the manufacturing size of the distilleries. For example, small scale distilleries have a smaller volume of pot ale generation thus higher sludge seeding ratios can be considered to be more feasible than large scale ones, and vice versa. Subsequently, the size of the digester should be calculated based on the volume of feed and the sludge seeding ratio as well as the HRT (set to 6 days in this design) allowing the substrate sufficient time in the digester to be degraded. Once the digestion parameters are fixed, annual energy production capacity of the AD plant and required CHP unit capacity is estimated according to the example calculation given in Appendix 11 and Appendix 12 the estimations of capital and operational costs of AD plant and CHP unit based on Figure 64 and Figure 65 respectively.

7.5.2 Revenue Assumptions

The main potential income of an AD plant for a distillery is minimising the energy demand by using the end product (biogas) of AD through CHP unit to produce electrical and thermal energy to be used in the manufacturing process. According to the theoretical scaling-up study, most of the scenarios could meet and even exceed the electricity demand of the example distillery. In case of thermal energy demand, up to 24±1.7% heat recovery was achieved though combined an AD and a CHP unit. Therefore, the energy savings are considered to be the main source of income for the distilleries. Besides energy savings, excess amount of electricity can be sold to grid for €65 per MWh (Enerpower 2018). Selling co-product of AD (digestate) as biofertilizer for a suggested price of £5/tonne is also considered an additional income source (A. Mouat, A. Barclay 2010).

7.8 Challenges of Scaling-up

Although anaerobic digestion is a promising sustainable technology for the concept of whiskey distillery waste management, there are several obstacles that challenge scaling up to industrial implementations. The major challenge is considered to be the predominance of empirical methodologies in the fundamental studies of AD of whiskey distillery wastes. Moreover, the pre-treatment aspect of the process has received a very scant attention in the literature to the date. The link between applicability of the pre-treatment at micro and macro scale is non-existent with respect to its impacts on the whole system. Although commonly used batch tests establish the methane production under specific pre-treatment and digestion conditions, it may fail to give accurate predictions for full scale continuous AD performance due its dependency on inoculum type, on the ratio of inoculum to substrate used, and on the different reactor configuration (Carlsson et al. 2012). Simply because it is impossible to achieve identical mixing conditions and nutrient management in the full-scale applications. Achieving a sufficient level of mixing in the full-scale applications is a challenge due to large operation volumes. However it is essential since it increases the mass transfer and reactor hydrodynamics (Yang et al. 2015). The diverse range of input nutrients in inoculum, maintain bacterial growth for the continuation of AD. For the full-scale application, it can be challenging to maintain long term operation stability during biogas production as well as during subsequent re-use of inoculum (Tiwary et al. 2015) whereas it is be replaced

with a fresh supply for lab/bench scale applications. In order to increase the accuracy of the prediction for scaling up purpose, an enhanced experimental should be followed evaluating mass transfer fluxes, actual kinetics involved in microbial growth and organic material conversion, the hydrodynamic behaviour of the selected reactor configuration as well as a deep investigation into the biochemistry and microbiology of the AD process (Moraes et al. 2015).

7.9 Discussion of Key Findings

There is not an ideal waste management route for every distillery as it is strictly related to the scale of operation and the space constraints due to the geographical location. The theoretical scale up study was conducted based upon the biogas quality and the quantity results at lab scale with the major factor of sludge seeding ratio. The ratios lower than 50%, on wet basis, resulted in insufficient degradations therefore, higher ratios should be considered (from 50%) up until 90%). In addition, AD plant designed based on varying sludge seeding ratios increases applicability of the design to many different distilleries with different production capacity. For example, high sludge seeding ratios (>80-90) would be more suitable for the small/medium scale distilleries whereas it could be limited for the large scale distilleries due to higher daily waste disposal rates. Operating two digesters simultaneously at high sludge seeding rate would address this problem for the large scale distilleries. Once the seeding ratio is decided for continuous reactor (CSTR) operation based on unique need of the distillery, required amount of sludge, which can be obtained from wastewater treatment plant, is place in the reactor and the fresh feedstock is fed. During operation small amount of sludge (digestate) is taken out of the reactor to be sold as biofertiliser as well as to maintain the fresh feed percentage constant in the reactor all times. In this study, pre-treatment contribution on biogas yield was examined with the predictions of 4 different scenarios for the industrial scale predictions. To create different scenarios for combined thermochemical pre-treatment, alkali agent and dose were kept constant (1M NaOH), while the temperature of the thermal pre-treatment was set to 65 and 100 ˚C by changing the microwave power for the treatment time of 11 minutes.

A total annual energy demand of 8 300 MWh which corresponds 0.96 MW for the distillery producing 1 million litre whiskey annually by operating 7 days a week, 24 hours per day and 365 days per year (Hamill 2015). The thermal energy demand had a biggest part (~95%) of the total energy demand therefore it was the challenging part of the design to recover. AD of non-treated pot ale (Scenario 1), could only recover 0.8±0.03% of overall heat demand of the distillery when the seeding ratio was 50% on wet basis. Implementation of 1M NaOH pre-treatment (Scenario 2) could recover 4.6±1% of the total demand (*p:* 0.0059) at 50% sludge ratio. On the other hand, no significant differences were seen arising from the alkaline pre-treatment in case of sludge seeding increase to 90%. When 1 M NaOH pre-treatment was followed by 100˚C thermal pre-treatment (Scenario 4), higher heat recoveries were seen than its combination with 65 ˚C thermal pre-treatment (Scenario 3) for all mixing ratios (Figure 63). This was attributed to the higher lignin degradation capacity of the higher temperature treatment which was presented in Chapter 5. Moreover, increased AD capacity with higher temperature thermal pre-treatment due to higher level of cell membrane dispersion was also previously published (Carrère et al. 2010; Kim et al. 2003). The highest thermal energy recovery was achieved with Scenario 4 as 10.1±0.3, 14.4±0.3 and 23.6±1.7% for the sludge seeding ratios of 65, 85 and 90%. In terms of electrical energy recovery, the demand of the distillery was achieved all cases except for 50% sludge seeding ratio of non-treated pot ale which only reach 22±1% of the demand due to the low CH_4 content of generated biogas.

Diageo's Dailuaine facility with the capacity of 3 300 000 L alcohol production (Media GmbH & Co. KG 2018) operates a combined high rate anaerobic digester and a CHP unit to treat liquid residue. The AD plant produces 0.5 MW of biogas which meets 40% of the energy demand of the distillery (Organics et al. 2014). The capital cost of the plant was reported as £6 000 0000 (Scotch Whisky Association 2009). Another Diageo distillery, Roseisle, with an alcohol capacity of 10 000 000 – 12 000 000 L whiskey per annum (Wood 2015), was published to produce 8.6 MW biogas though treating pot ale with a combined AD and CHP unit, recovering 84% of the overall energy demand of the distillery. The capital cost of the plant was reported to be £17 000 000 (J. M. H. Andrews et al. 2011). Cameronbridge Distillery producing 120 000 000 L grain whiskey (Difford 2019) was

reported to have the highest capital cost of £65 000 000 for combined AD and CHP plant as well as membrane filtration unit for water recovery (J. M. H. Andrews et al. 2011). The plant was previously reported to produce up to 30 MW of energy per annum which recovery of approximately 95% of the total energy demand (Duguid & Strachan 2016). The total energy demand estimation of a distillery producing 1 000 000 L whiskey (0.96MW) was found to be in alignment with the industrial figures. For example, Dailuaine Distillery had an energy demand of 1.25 MW, despite its triple manufacturing capacity of the example distillery given in Figure 57. Based up on three AD plants of Diageo distilleries with similar technologies at different scale, the capital investment cost per litre whiskey production capacity was found as 1.81, 1.54 and 0.54 M GBP for Dailuaine, Roseisle and Cameronbridge distilleries respectively. Therefore, joint AD plant applications for small scale distilleries was seen in literature where the location allows the designers to integrate more cost-efficient treatment technologies. For example, Glendullan Distillery AD plant was reported to convert 1 000 m^3 distillery co-products into 1 MW of thermal energy per day by feeding the biogas into a boiler, reducing dependency on the fossil fuels by 25% for alcohol manufacturing (Gueterbock & Sangosanya 2017; Beverage 2016). On the other hand, it was previously published that the AD plant receives distillery wastes from Dufftown area and annually produces 2 million m^3 of biogas and 800 MWh thermal energy for the distillery (Duguid & Strachan 2016). The capital investment of the AD plants mainly relies on the production size. However, time dependency of the cost also should be considered for the comparisons.

The North British Distillery producing 60 000 000 L grain whiskey operates an AD to treat liquid residues and produce 24 000 MWh biogas to be used in CHP unit for heat and electricity recovery (Duguid & Strachan 2016). The plant was reported to be producing 7.2 MW of total energy (Brinkerhoff 2012). Moreover, Girvan distillery, owned by William Grant & Sons, was reported to produce 60 and 15 MWh electricity and heat per day by using a combined AD and CHP unit (Hamill 2015; Scotch Whisky Association 2009). The distillery meets its annual electrical energy demand of 8.4 MW with a further 2 MW to be exported to the electricity gird while it reaches only a 10% of the heat demand (Duguid & Strachan 2016).

A variety of energy recovery figures were seen in literature such as Dailuaine, Roseisle and Cameronbridge distilleries had 40, 84 and 95% of total energy recovery. The main challenge was seen in the thermal energy recovery levels. For example, North British Distillery could recover only 10% its heat demand while it had excess electricity generation. A similar situation was observed in this theoretical study with a main focus of enhancements due to the implementation of pre-treatments. The application of thermochemical pre-treatments (Scenario 3 and 4) was enhance the heat recovery significantly for the sludge ratio of 65, 85% on wet basis (Figure 63) in comparison to non-treated situation (Scenario 1). The maximum enhancement of 8±0.2% was seen with Scenario 4 with regards to Scenario 1 when the sludge ratio was 90% reaching a total heat recovery of 24±1.7%. While alkaline pre-treatment standalone only had a significant enhancement of 4±0.3% in heat recovery when the sludge mixing ratio was 50%.

In order to assess the routes available for implementation of AD technology to the distilleries it is advised that the existing distilleries that to create a scenario for treating annual pot ale discharge through AD. Calculation of the cost/benefit and energy recovery and savings potential of this scenario is then compared with the current waste management method. Subsequently, assessing advantages and disadvantages of applying AD technology to whiskey distillery is suggested from both economical and environmental standpoints to assess return of investment. In terms of planned distilleries, it is beneficial to obtain the following information;

1. How much pot ale is discharged for annum?
2. What are the possible waste management methods?
3. How much would AD plant cost? What are the benefits of AD?
4. What is the cost/revenue of AD per annum?
5. How much would other waste treatment methods cost?
6. Are there any costumers for selling digestate? Would they be available throughout the year on a regular basis (i.e. any seasonality limitations)
7. What is the practicality/ease of application treatment options?

Establishing a baseline scenario to investigate potential energy usage and cost of AD and other waste management methods from environmental and economical standpoints is

advised to planned distilleries. Alternatively, investigation of potential joint AD plants where the location allows for small/medium scale distilleries can reduce the cost of AD while increase the biogas yield due to balanced feedstock in terms of carbon and nitrogen sources.

To conclude, the outcome of this project is in favour of both existing and planned distilleries in both environmental and energy point of views as the biogas/methane yield enhancement due to applied pre-treatments has been proven.

Chapter 8: Conclusions and Future Work

8.1 Discussion

In this research project, a detailed characterisation of pot ale in terms of organic and inorganic constituents is provided. Application of anaerobic digestion process on pot ale and spent grain was tested at lab scale, in batch mode. The level of organic matter in the substrate in terms of COD, BOD and SO_4 was measured to assess the effectiveness of AD and the potential for VFA inhibition of the AD process. Enhanced biogas quality and quantity was achieved by implementation of a variety of pre-treatment steps, including alkaline pre-treatment alone and in combination with beating, microwave and ultrasonic pre-treatment, prior to anaerobic digestion with different sludge seeding ratios. A biogas yield of 205 ± 21.4 with ml/g VS a CH_4 content of 19.3% was achieved with AD of non-treated pot ale with 50% sludge seeding ratio. Enhanced biogas yields and quality (523±10.4 ml/g VS with 48.1% CH4 and 555±5.3 m/g VS with 43.0% CH_4) were achieved with implementation of 1M NaOH pre-treatment alone and in combination with 7.5 minutes beating respectively under the same digestion conditions. In addition to single digestion of pot ale, co-digestion with spent grain was investigated under the same pre-treatment and digestion conditions. Mathematical models of each process were developed to enhance understanding of the process and pre-treatment parameters as well as their interaction with each other. The amount of inoculum was found to be the most critical design parameter for each case. Models were then used for the optimisation of AD of pot ale and its co-digestion with spent grain. The optimum digestion conditions for single digestion of pot ale were identified as 50% sludge seeding ratio, 33 ˚C digestion time and 8 – 11 min beating time. In the case of co-digestion with spent grain, the optimum conditions were found to be 50% sludge seeding ratio, 38 ˚C digestion time and 10 – 15 min beating time. It is important to note that no significant improvement was seen with co-digestion of pot ale and spent grain. In addition, significantly higher levels of VFAs were seen after digestion, indicating the VFA accumulation in the reactor and inhibition of the methanogenic activity. In order to address this, single digestion of pot ale with higher sludge seeding ratio (65 – 95% on wet basis) was assessed after implementation of 1M NaOH pre-treatment in combination with microwave and ultrasonic pre-treatments. A methane yield of 1614 ± 168 ml/g VS, which is the highest methane yield achieved in

this study, was achieved with AD of pot ale with 95% sludge seeding on a wet basis, after implementation of 1 M NaOH pre-treatment followed by 240 W microwave pre-treatment for 11 minutes. It was attributed to the high buffer capacity of the inoculum, maintaining a more balanced reaction environment for the microbes to carry out the reactions completely. No VFA was determined after AD with high inoculum use indicating that the organic matter was fully broken down. The process and pre-treatment parameters were optimised (I/S of 4.5 on dry basis and microwave power 39 – 46% of overall 800 W) using the developed mathematical models. I/S of 5 on a dry basis was followed to evaluate the effects of alkaline dose and conditions of ultrasonic pre-treatment. The highest methane yield was obtained as 876 ± 26 ml/g VS after implementation of ultrasonic pre-treatment at 70% amplitude for 3 h. However, no significant difference was found arising from the different pre-treatment conditions.

In addition to biogas yields, the mineral concentration of the digestates of different experimental conditions were assessed to evaluate the potential for agricultural use. A comparison was provided with an industrial digestate sample obtained from a full-scale AD plant processing mixed food waste. No significant differences were seen in the compositional analysis of the pot ale digestate samples arising from different pre-treatment conditions since the inorganic content of digestate is directly linked to the raw material of the process. Significantly higher P and Cu concentrations were seen in the pot ale digestates than in the industrial digestate sample.

As the final part of this research, a scalable AD plant model was designed based on the lab scale experiment results. Annual biogas production estimations and required CHP capacities were calculated based on varying sludge seed ratios within a range of 50 – 95% for a distillery with an annual whiskey production of 1 000 m^3 which can be scalable for other cases. Energy recovery potentials of non-treated and thermo-chemically pre-treated pot ale was provided based on different I/S ratios and selecting the optimum I/S ratio was left to individual industrial implementations. CAPEX and OPEX estimations for the AD plant and CHP unit were provided for an annual capacity of 0 – 700 MWh/year and 0 – 400 kW respectively which enabled financial feasibility studies of individual industrial applications to be completed.

8.2 Conclusions

In this study various pre-treatment types were introduced to anaerobic digestion of whiskey distillery and brewery waste streams. In addition, RSM was employed to investigate the impact of the process parameters on biogas yield as well as for process modelling and optimisation. The main conclusions of this research project are outlined in this chapter as follows:

1. Among the whiskey distillery/brewery waste streams, pot ale and draff, the highest biogas yields were obtained with single digestion of pot ale after combined alkaline and beating pre-treatments. Pot ale proved to be a more suitable substrate for AD.
2. Among the applied pre-treatments on pot ale, (alkaline alone and alkaline in combination with beating, microwave and ultrasonic), the highest biogas yields were achieved with combined alkaline and microwave pre-treatment. Thus, for AD of pot ale, alkaline coupled with microwave pre-treatment represents a valuable method for biogas conversion.
3. From the digestate investigation, it can be concluded that pot ale digestate is a valuable biofertiliser due to its rich mineral content in terms of macro (P, K, N, Ca and Mg) and micro (Fe, Mn, Zn and Cu) nutrients of the soil.
4. For full scale implementation of the AD technology, a scalable AD plant was designed based on the inoculum to substrate ratio to address the individual needs and constraints of whiskey distilleries, with regard given to their waste disposal capacity and location.

8.3 Research Contribution

The research contributions are presented as follows.

1. This research project provided knowledge and understanding about the use of three novel pre-treatment methods for the scope of AD of whiskey distillery and brewery waste streams. This was the first study conducted for investigating the influences of alkaline pre-treatment in combination with beating, microwave and ultrasonic pre-treatment on the lignocellulosic structures of pot ale for biogas production.
2. Despite it being a very sustainable, commercially and environmentally important substrate for AD, very few studies were seen in the literature on pot ale. None of these dealt with introducing pre-treatments to process modelling and optimisation for full scale implementation. As such, pre-treatment steps prior to AD is not seen in industrial applications. Therefore, the current research produced novel data on the employment of pot ale for biogas generation while optimising pre-treatments.
3. This research project was able to provide new knowledge about the quality of pot ale digestate and its potential for use as a biofertiliser.
4. Datasets were produced and used for a scalable AD plant design which can be applicable to many distilleries regardless of their capacity, to create an environmentally friendly circular economy for the whiskey distilling industry.

8.4 Perspectives

To conclude; new research is needed in order to exploit the full bioenergy conversion potential of whiskey distillery and brewery waste streams in Ireland and beyond. Future research suggestions based on results of this research and the literature gaps are as follows:

1. This research aimed to address the major challenges for the whiskey distilleries in terms of waste management therefore pot ale was the main focus of the project. Also preliminary data was produced on pot ale draff co-digestion to find an alternative use for draff. A further optimisation of sludge seeding ratio for a range of 60-90 on wet basis is recommended. Moreover, adding nitrogen rich industrial food waste or fish farm waste to be digested with pot ale or pot ale draff mixture is recommended for achieving further enhancements in biogas yields.
2. Aim of theoretical scale-up study of this work was to provide the preliminary knowledge on techno-economic analysis. A progressive scale-up of the process could be achieved with the aid of computer simulation tools. The technology-integrated experimental approach could increase the accuracy of biogas yield predictions for full scale applications to obtain a more reliable AD plant design. A well-established simulation process (i.e. ADM1 model) can enable the investigation of various scenarios in a short period of time. It is therefore advised as the next step to take for the industrial modelling of various types of pre-treatments prior to anaerobic digestion.

In the research project application of the process of anaerobic digestion on distillery/brewery waste streams has been outlined in detail. The enhanced biogas yields were achieved by implementation of pre-treatment steps prior to anaerobic digestion. An industrial implementation was modelled based on the lab scale experiment results.

Appendix 1: COD Measurement SOP

SOP No: 1

Brewery/Distillery Co-Product Characterisation Procedures Manual	Issue No: 1
Chemical Oxygen Demand USEPA Reactor Digestion Hach Method 8000 0 – 15000 mg/L COD	Issue Date: 21/08/16 Issued by: Burcu Gunes

1.0 Sampling and Storage:

1.1 Collect samples in clean glass bottles. Use plastic bottles only if they are known to be free of organic contamination.

1.2 Samples may be stored up to 28 days by treating with sulphuric acid to a pH of less than 2 and cooling 4˚C or lower.

1.3 For better accuracy samples must be warmed to room temperature (~20˚C) before analysis.

2.0. Sample Pre-treatment

Dilution is required before analysis, 1:30 dilution rate is advised due to high COD levels.

3.0 Analysis Procedure

The mg/L COD results are defined as the mg of O_2 consumed per litre of sample under conditions of this procedure. In this procedure, the sample is heated for two hours with a strong oxidising agent, potassium dichromate. Oxidisable organic compounds react, reducing the dichromate ion ($Cr_2O_7^{2-}$) to green chromic ion (Cr^{3+}). When the 0 - 15000 mg/L colorimetric method is used, the amount of Cr^{3+} produced is determined. The COD reagents also contains silver and mercury ions. Silver is a catalyst, and mercury is used to complex chloride interferences

3.1 Homogenize 100 mL of sample in a blender and poor the homogenized sample into a 250mL beaker and stir with a magnetic stirrer.

3.2 Turn on the COD reactor. Preheat to 150 ˚C.

3.3 Remove the cap of a COD Digestion Reagent Vial.

Note: The reagent mixture is light-sensitive. Keep unused vials in an opaque shipping container, in a refrigerator if possible. The amount of light striking the vials during the test will not affect results.

3.4 Hold the vial at a 45-degree angle. Pipette 0.2 mL of sample into the vial. ***Note:*** For proof of accuracy, use COD standard solutions (preparation is given in section 4)

3.5 Replace the vial cap tightly.

3.6 Hold the vial by the cap and over a sink. Invert gently several times to mix the contents. Place the vial in the preheated COD reactor.

Note: The vial will become very hot during mixing.

3.7 Prepare a blank by repeating Steps 3 to 6, substituting 0.2 mL demineralised water for the sample.

Note: One blank must be run with each set of samples. All tests (samples and blanks) should be run with the same lot of vials. The lot number appears on the container label.

3.8 Heat vials for 2 hours.

Note: If desired, measure the concentration (while the vial is still hot) at 15 minutes intervals until it remains unchanged. At this point, the sample is completely digested. Cool the vials to room temperature for final measurement.

3.9 Turn the heating block off. Wait about 20 minutes for the vials to cool to 120 ˚C.

3.10 Invert each vials several times while still warm. Place the vials into a rack. Wait until the vials have reached to room temperature 20 ˚C

Note: If a pure green colour appears in the reacted sample, the reagent capacity may have been exceeded. Measure the COD and, if necessary, repeat the test with a diluted sample.

3.11 Enter stored programme number for chemical oxygen demand, high range plus by pressing 435 ENTER/READ.

3.12 Rotate the wavelength dial until the small display shows 620nm. When 620 nm is seen press READ/ENTER.

3.13 Place the COD Vial Adapter into the cell holder with the marker to the right.

3.14 Clean the outside of the blank with a tissue to remove fingerprints or other marks.

3.15 Place the blank into the adapter with the Hach logo facing the front the instrument. Place the cover of the adapter.

Note: The blank is stable when stored in dark.

3.16 Press ZERO The display will show 0 mg/L COD H.

3.17 Clean outside of the sample vial with a tissue.

3.18 Place the sample vial in the adapter with the Hach logo facing the front the instrument. Place the cover on the adapter.

3.19 Press READ/ENTER. The display will show the result in mg/L COD

Note: The displayed value multiplied by ten when the High Range Plus COD Digestion Reagent Vials (0 to 15000 mg/L) are used.

Note: In the constant-on mode, pressing READ/ENTER is not required. Wait until the display stabilizes, read the result.

4.0 <u>Accuracy Check:</u>

Accuracy of the test is checked by using 10000 mg/L COD Standard Solution. For greater accuracy standard should be performed in each test.

Preparation: Dissolve 8.500 grams of dried (120 ˚C, overnight) potassium phthalate and dilute to 1000mL with demineralised water. Use 0.2 mL of this solution as the sample volume; expected result will be 10000 mg/LCOD. Prepare this solution daily.

Appendix 2: BOD Measurement SOP

SOP No: 2

Brewery/Distillery Co-Product Characterisation Procedures Manual	Issue No: 1
Biological Oxygen Demand BOD System BD600 0 – 4000 mg/L O_2	Issue Date: 20/08/16
	Issued by: Burcu Gunes

1.0 Sampling and Storage:

1.1 Collect samples in clean glass bottles. Use plastic bottles only if they are known to be free of organic contamination.

1.2 Samples may be stored up to 28 days by treating with sulphuric acid to a pH of less than 2 and cooling 4˚C or lower.

1.3 For better accuracy samples must be warmed to room temperature (~20 ˚C) before analysis.

2.0. Sample Pre-treatment

Dilution is required before analysis, 1:30 dilution rate is advised due to high BOD levels.

3.0 Analysis Procedure

The BOD measuring unit, consist of 6 test bottles and BOD sensors, is a closed system. With the filled sample quantity, there is a gas compartment with a defined quantity of air in the test bottle. The bacteria in the waste water filled in the bottle consume the oxygen dissolved in the sample over the course of the BOD measurement. It is replaced by air oxygen from the gas compartment of the test bottle. The simultaneously developing carbon dioxide is chemically bound by the potassium hydroxide in the seal cup of the test bottle. As a result, a pressure drop occurs in the system, which is measured by the BOD sensor and shown in the directly in the display as a BOD value in mg/l O_2.

3.1 Estimate the measurement range of the sample to be tested and the sample volume as indicated in Table Appendix 2-1. The BOD value to be expected corresponds to approximately 80% of the COD value.

Table 1. Sample preparation for BOD measurement

BOD range in (mg/L)	Sample volume in (ml)	Nitrification inhibitor ATH dosage (drops)
0 – 40	428	10
0 – 80	360	20
0 – 200	244	5
0 – 400	157	5
0 – 800	94	3
0 – 2000	56	3
0 – 4000	21.7	1

3.2 Test the pH value of the waste water sample. The ideal pH value lies between pH 6.5 and 7.5 any greater deviation provides a lower BOD value. If a pH value is too high, it can be neutralised with diluted hydrochloric acid (1 M) or diluted sulphuric acid (1 M), and if the pH value is too low, it can be neutralised with a sodium hydroxide solution (1 M).

3.3 Measure the sample volume precisely with a volumetric flask and pour into the BOD bottle (use a funnel, if necessary).

3.4 Add the corresponding amount of nitrification inhibitor to prevent oxygen consumption by Nitrifying bacteria as indicated in Table 1.

3.5 Place the magnetic stir bar in the BOD bottles.

3.6 Fill the seal cup with 3 drops of KOH solution and place the seal cup in the test bottle

3.7 Screw the BOD sensors on the test bottles tightly.

3.8 Place the sample in the bottle rack

3.9 Turn on BD 600 Tintometer on.

3.10 Incubate the samples at 25°C for 5 days.

3.11 After the incubation time the results will be displayed in the unit of mg/L O_2.

Appendix 3: Sulphate Measurement SOP

SOP No: 3

Brewery/Distillery Co-Product Characterisation Procedures Manual	Issue No: 1
Sulphate USEPA SulfaVer 4 Hach Method 8051 2 – 70 mg/L SO_4^{2-}	Issue Date: 19/08/16
	Issued by: Burcu Gunes

1.0 Sampling and Storage:

1.1 Collect samples in clean plastic or glass bottle.

1.2 Samples may be stored up to 7 days by cooling 4˚C or lower.

1.3 For better accuracy samples must be warmed to room temperature before analysis

2.0. Sample Pre-treatment

Dilution is required before analysis 1:20 dilution rate is advised due to high sulphate concentration.

3.0 Analysis Procedure

Sulphate ions in sample react with barium in the Sulfa Ver 4 and form a precipitate of barium sulphate. The amount of turbidity formed is proportional to the sulphate concentration. The Sulfa Ver 4 also contains a stabilising agent to hold the precipitate in suspension.

3.1 Turn on Hach DR 2000 Spectrophotometer wait until the end of self-testing

3.2 Enter stored programme for sulphate (SO_4^{2-}) by pressing 680 ENTER/READ

3.3 Rotate the wavelength dial until the small display shows 450nm. When 450 nm is seen press READ/ENTER.

Note: For greater accuracy prepare an instrument calibration for each new lot of SulfaVer4 Reagent Power Pillows; see Calibration Section 4.

3.4 Fill a clean sample cell with 25 mL sample (prepared sample).

3.5 Add the content of one Sulfa-Ver 4 Reagent Powder Pillow to the sample cell. Swirl to dissolve.

Note: For proof of accuracy, use a 50 mg/L sulphate standard solution (see Accuracy Check) in place of the sample.

3.6 A 5-minute reaction period will begin. Allow the cell to stand undisturbed in a fume hood.

3.7 Fill a second sample cell with DI water (blank).

3.8 Place the blank into cell holder. Close the light shield.

3.9 Press ZERO. The display will show 0 mg/L SO_4^{2-}

3.10 After the reaction period finished, place the prepared sample into the cell holder. Close the light shield.

3.11 Press READ/ENTER. The display will show the results in mg/L SO_4^{2-} .

4.0 <u>Calibration</u>

4.1 A new calibration should be performed for each new lot of Sulfa-Ver 4 Sulphate Reagent Powder Pillows as follows.

4.2 Prepare standards of 0, 10, 20, 30, 40 and 50 mg/ L sulphate by pipetting 10, 20, 30, 40 and 50 mL of a 50 mg/L sulphate standard solution into 50 mL volumetric flasks.

4.3 Dilute to volume and mix well. Transfer 25 mL to each test cell.

4.4 Add reagents to standard solutions as described in steps 3.4 to 3.6, using demineralised water blank to perform the zero calibration.

4.5 After blanked zero calibration, enter the sulphate concentration of the first standard (10 mg/L) and measure the absorbance as directed as Hach DR 2000 instrument manual. React with the Sulfa-Ver 4 Reagent Powder Pillow and measure remaining standards.

4.6 Prepare a new calibration for each new lot of reagent, using the same stored programme number.

5.0 Accuracy Check:

5.1 Accuracy of the test is checked by using 50 mg/L Sulphate Standard Solution. For greater accuracy standard should be performed in each test.

Appendix 4: Nitrate-Nitrogen Measurement SOP

SOP No: 4

Brewery/Distillery Co-Product Characterisation Procedures Manual	Issue No: 1
Nitrate-Nitrogen Cadmium Reduction Methods Hach Method 8039 0 – 30 mg/L NO_3^--N	Issue Date: 20/08/16 Issued by: Burcu Gunes

1.0 Sampling and Storage:

1.1 Collect samples in clean plastic or glass bottle.

1.2 Samples may be stored up to 48 hours by cooling 4˚C or lower. For longer storage periods, add 4.0 ml of Mercuric Chloride Solution for each litre of sample taken and mix. Sample refrigeration is required. Avoid using acid preservatives.

1.3 For better accuracy samples must be warmed to room temperature (~20^{o}C) before analysis.

2.0. Sample Pre-treatment

Dilution is required before analysis, 1:5 and 1:10 dilution rates are advised to be tried.

3.0 Analysis Procedure

Cadmium metal reduces nitrate present in the sample to nitrite. The nitrite ion then reacts in an acidic medium with sulphanilic acid to form an intermediate diazonium salt. This salt couples to gentisic acid to form an amber coloured product.

3.1 Turn on Hach DR 2000 Spectrophotometer wait until the end of self-testing.

3.2 Enter stored programme number for high range nitrogen-nitrite (NO_3^-) by pressing 355 keys ENTER/READ

3.3 Rotate the wavelength dial until the small display shows 500 nm. When the 500 nm is seen press READ/ENTER.

3.4 Fill a clean sample cell with 25 ml sample (prepared sample).

3.5 Add the contents of one Nitra-Ver 5 Reagent Powder Pillow, shake to dissolve. ***Note:*** A deposit of unoxidized metal will remain after powder pillow dissolves. This deposit does not affect the test results.

Note: An amber colour will develop if nitrate nitrogen is present.

3.6 A 5-minute reaction period will begin.

3.7 Fill a second sample cell with 25 ml of demineralised water (blank).

3.8 Place the blank into cell holder. Close the light shield.

3.9 Press ZERO. The display will show 0 mg/L NO_2^- H

3.10 After the 5-minute reaction period finished, place the prepared sample into the cell holder. Close the light shield.

3.11 Press READ/ENTER. The display will show the results in mg/L NO_3^-.

4.0 <u>Calibration</u>

4.1 A new calibration should be performed for each new lot of Nitra-Ver 5 Reagent Powder Pillows as follows.

4.2 Prepare standards of 0 (for blank), 5, 10, 20 and 30 mg/ L Nitrate-Nitrogen standard solution by pipetting 2.5, 5, 10 and 15 ml of a 100 mg/L Nitrate-Nitrogen standard solution into 50 ml volumetric flasks.

4.3 Dilute to volume and mix well. Transfer 25 ml to each test cell.

4.4 Add reagents to standard solutions as described in steps 3.4 to 3.6, using demineralised water blank to perform the zero calibration.

4.5 After blanked zero calibration, enter the nitrite concentration of the first standard (5 mg/L) into the cell holder and measure the absorbance as directed as Hach DR 2000 instrument manual. React with Nitra-Ver 5 Reagent Powder Pillow and measure remaining standards.

4.6 Prepare a new calibration for each new lot of reagent, using the same stored - programme number for nitrite.

5.0 Accuracy Check:

5.1 Accuracy of the test is checked by using 10 mg/L Nitrate Nitrogen Standard Solution. For greater accuracy standard should be performed in each test.

Appendix 5: Nitrite Nitrogen Measurement SOP

SOP No: 5

Brewery/Distillery Co-Product Characterisation Procedures Manual	Issue No: 1
Nitrite Ferrous Sulphate Hach Method 5183 0 – 150 mg/L N-NO_2^-	Issue Date: 20/08/16 Issued by: Burcu Gunes

1.0 Sampling and Storage:

1.1 Collect samples in clean plastic or glass bottle.

1.2 Samples may be stored up to 48 hours by cooling 4˚C or lower. For longer storage periods, add 4.0 mL of Mercuric Chloride Solution for each litre of sample taken and mix. Sample refrigeration is required. Avoid using acid preservatives.

1.3 For better accuracy samples must be warmed to room temperature (~20^oC) before analysis.

2.0. Sample Pre-treatment

Dilution is required before analysis 1:10 dilution rate is advised due to the high Nitrite levels.

3.0 Analysis Procedure

The method uses ferrous sulphate in an acid medium to reduce nitrite to nitrous oxide. Ferrous ions combine with the nitrous oxide to form a greenish-brown complex in direct proportion to nitrite present.

3.1 Turn on Hach DR 2000 Spectrophotometer wait until the end of self-testing

3.2 Enter stored programme number for high range nitrogen-nitrite (NO_2^-) by pressing 373 keys ENTER/READ

3.3 Rotate the wavelength dial until the small display shows 585nm. When 585 nm is seen press READ/ENTER.

3.4 Fill a clean sample cell with 25 mL sample (prepared sample).

3.5 Add the contents of one Nitri-Ver 2 Reagent Powder Pillow, shake to dissolve. ***Note:*** A greenish-brown colour will develop if nitrite is present.

3.6 A 10-minute reaction period will begin.

3.7 Fill a second sample cell with 25 mL of demineralised water (blank).

3.8 Place the blank into cell holder. Close the light shield.

3.9 Press ZERO. The display will show 0 mg/L NO_2^- H

3.10 After the 10 minutes reaction period finished, place the prepared sample into the cell holder. Close the light shield.

3.11 Press READ/ENTER. The display will show the results in mg/L NO_2^-.

4.0 <u>Calibration</u>

4.1 A new calibration should be performed for each new lot of Nitri-Ver 2 Reagent Powder Pillows as follows.

4.2 Prepare standards of 0 (for blank), 20, 40, 60, 80 and 100 mg/ L nitrite by pipetting 10, 20, 30, 40 and 50 mL of a 100 mg/L nitrite standard solution into 50 mL volumetric flasks.

4.3 Dilute to volume and mix well. Transfer 25 mL to each test cell.

4.4 Add reagents to standard solutions as described in steps 3.4 to 3.6, using demineralised water blank to perform the zero calibration.

4.5 After blanked zero calibration, enter the nitrite concentration of the first standard (10 mg/L) into the cell holder and measure the absorbance as directed as Hach DR 2000 instrument manual. React with Nitri-Ver 2 Reagent Powder Pillow and measure remaining standards.

4.6 Prepare a new calibration for each new lot of reagent, using the same stored-programme number for nitrite.

5.0 Accuracy Check:

5.1 Accuracy of the test is checked by using 100 mg/L Nitrite Standard Solution. For greater accuracy standard should be performed in each test.

Preparation: Dissolve 0.150 grams of fresh sodium nitrite and dilute to 1000mL with demineralised water. Use this solution in place of sample, expected result is 100 mg/L. Note: Prepare this solution daily.

Appendix 6: Ammonia-Nitrogen Measurement SOP

SOP No: 6

Brewery/Distillery Co-Product Characterisation Procedures Manual	Issue No: 1
N-Ammonia, Test N Tube Salicylate Method 10031 0 – 50 mg/L N-NH_3	Issue Date: 20/08/16 Issued by: Burcu Gunes

1.0 Sampling and Storage:

1.1 Collect samples in clean glass bottles. Use plastic bottles only if they are known to be free of organic contamination.

1.2 Samples may be stored up to 28 days by treating with sulphuric acid to a pH of less than 2 and cooling 4˚C or lower.

1.3 For better accuracy samples must be warmed to room temperature (~20 ˚C) before analysis.

2.0. Sample Pre-treatment

Dilution is required before analysis, 1:40, 1:80 and 1:160 dilution rates should be tried.

3.0 Analysis Procedure

Ammonia compounds react with chlorine to form monochloramine. Produced monochloramine is then react with salicylate to form 5-aminosalicylate. The 5-aminosalicylate is oxidized due to the presence of a sodium nitroprusside catalyst to generate a blue coloured compound which is masked by yellow colour from the presence of excess reagent to give a green coloured solution.

3.1 Turn on Hach DR 900 Spectrophotometer wait until the end of self-testing.

3.2 Enter stored programme number for nitrogen, ammonia, test and tube by pressing RPGM key and pressing 67 keys for the program code and press ENTER.

3.3 The display will show mg/L, N-NH_3 and the ZERO icon.

3.4 Insert the TNT adaptor into sample holder by turning it until it clicks in the place then push it down to ensure that it is installed fully.

3.5 Remove the cap from 2 AmVer Diluent Reagent high range vials. Add 0.1 ml of DI water and sample into the first vial (blank) and the second vial (sample) respectively.

3.6 Add 1 Ammonia Salicylate Reagent Powder Pillow for 5 ml into each vial.

3.7 Add 1 Ammonia Cyanurate Reagent Powder Pillow for 5 ml into each vial.

3.8 Place the cap tightly and shake the vial thoroughly to dissolve the powder.

Note: A green colour will develop if ammonia is present.

3.9 A 20-minute reaction period will begin.

3.10 Clean the outside of the first vial with a tissue paper after 20 minutes, place it into the sample adapter and close the light shield.

3.11 Press ZERO. The display will show 0 mg/L NH_3-N

3.12 Place the prepared sample and close the light shield.

3.13 Press READ. The result in mg/L NH_3-N will be displayed.

<u>4.0 Accuracy Check</u>

4.1 Nitrogen ammonia solutions (10 - 50 mg/L) or Nitrogen Ammonia Volute Ampule Standard (50 mg/L) can be used to check accuracy. For greater accuracy standard should be performed in each test.

Appendix 7: Phosphorus Measurement SOP

SOP No: 7

Brewery/Distillery Co-Product Characterisation Procedures Manual	Issue No: 1
Phosphorus Molybdovanate (Orthophosphate) Method Hach Method 8114 0 – 45 mg/L P-PO_4^{3-}	Issue Date: 20/08/16 Issued by: Burcu Gunes

1.0 Sampling and Storage:

1.1 Collect samples in clean plastic or glass bottle.

1.2 Samples may be stored up to 48 hours by cooling 4˚C or lower. For longer storage periods, add 4.0 ml of Mercuric Chloride Solution for each litre of sample taken and mix. Sample refrigeration is required. Avoid using acid preservatives.

1.3 For better accuracy samples must be warmed to room temperature (~20 ^{o}C) before analysis.

2.0. Sample Pre-treatment

Dilution is required before analysis, 1:80 and 1:160 dilution rates should be tried.

3.0 Analysis Procedure

In molybdovanate method, phosphomolybdate is produced in acid medium due to the reactions of orthophosphate and molybdate. Yellow coloured vanadomolybdophosphoric acid is formed in presence of vanadium. Intensity of the yellow colour changes proportionally depending on the phosphate concentration.

3.1 Turn on Hach DR 2000 Spectrophotometer wait until the end of self-testing

3.2 Enter stored programme number for reactive phosphorus molybdovanate method by pressing 480 keys ENTER/READ

3.3 Rotate the wavelength dial until the small display shows 430 nm. When the 430 nm is seen press READ/ENTER. `

3.4 Fill a clean sample cell with 25 ml of DI water (blank) and 25 ml sample (prepared sample).

3.5 Add 1 ml of Molybdovanadate Reagent to each sample cell, swirl to mix ***Note:*** A yellow colour will form if phosphate is present. Presence of a small amount of yellow colour in the blank because of the reagent does not interfere the result.

3.6 A 3-minute reaction period will begin.

3.7 Place the blank into cell holder after the reaction time. Close the light shield.

3.8 Press ZERO. The display will show 0 mg/L PO_4^{3-} MoV

3.10 Place the prepared sample into the cell holder. Close the light shield.

3.11 Press READ/ENTER. The display will show the results in mg/L PO_4^{3-} .

4.0 <u>Calibration</u>

4.1 A new calibration should be performed for each new lot of Molybdovanadate Reagent as follows.

4.2 Prepare standards of 0 (for blank), 5, 15, 25, 35 and 45 mg/ L Phosphate standard solution by pipetting 5, 15, 25, 35 and 45 ml of a 50 mg/L Phosphate standard solution into 50 ml volumetric flasks.

4.3 Dilute to volume and mix well. Transfer 25 ml to each test cell.

4.4 Add reagents to standard solutions as described in steps 3.4 to 3.8, using DI water blank to perform the zero calibration.

4.5 After blanked zero calibration, enter the first standard solution (5 mg/L) into the cell holder and measure the absorbance as directed in the Hach DR 2000 instrument manual. React with Molybdovanadate Reagent and measure remaining standards.

4.6 Prepare a new calibration for each new lot of reagent, using the same stored-programme number for nitrite.

5.0 Accuracy Check:

5.1 Accuracy of the test is checked by using 10 mg/L Phosphate Standard Solution. For greater accuracy standard should be performed in each test.

Appendix 8: Copper Measurement SOP

SOP No: 8

Brewery/Distillery Co-Product Characterisation Procedures Manual	Issue No: 1
Copper Perkin Elmer 3100 Atomic Absorption Spectrophotometer	Issue Date: 19/09/16
	Issued by: Burcu Gunes

1.0 Sampling and Storage:

1.1 Collect samples in clean plastic or glass bottle.

1.2 Samples may be stored up to 30 days by cooling 4˚C or lower.

1.3 For better accuracy samples must be warmed to room temperature before analysis

2.0. Sample Pre-treatment

2.1 Sample need to be filtered with a 0.45 µm filter paper to prevent clogging risk of sample inlet.

3.0 Analysis Procedure

3.1 Installing the Hollow Cathode Lamp

3.1.1 inset the Hollow Cathode Lamp into the right-hand side of the instrument, taking the note of the minimum and maximum current values.

3.1.2 Set the wavelength to 63.54 ηm by adjusting the dial on the left had side of the instrument.

3.1.3. Turn on the machine.

3.1.4 When the model name (Perkin-Elmer Model 3100) appears press the Param Entry key.

3.1.5 Press 15 and ENT to measure Copper.

3.1.6 Enter the INT Time Value which is 0.2 for copper then press ENT

3.1.7 Press the ENERGY button. The value should read above 50. If not, adjust the position of the lamp by adjusting the knobs above the lamp and by slowly adjusting the wavelength dial. When the value increases press the GAIN button and continue until the energy value will no longer increase.

3.2 Adjusting the Burner Head Position

3.2.1 The Burner Head can be adjusted by using the Horizontal and Vertical knobs located at the base of the burner head.

3.2.2 Lower the Burner Head below the beam of light.

3.2.3 Press the Cont. button to observe absorbance readings.

3.2.4 Press the A/Z (Auto Zero) button to give a reading of 0.000

3.2.5 Slowly raise the burner head until a positive absorbance is obtained (i.e. 0.001 or 0.002)

3.2.6 Bring the burner head back down until a zero reading is obtained.

3.2.7 Give the vertical knob a further quarter turn to clockwise

3.4 Lighting the Flame

3.4.1 Turn on the Extractor Fan

3.4.2 Close the screen in front of the flame.

3.4.3 Turn on the Compressed Air tap.

3.4.4 Turn on the Acetylene Tap

3.4.5 Turn the Oxidant Knob to the AIR position and hold the Red Button until the flame ignites.

3.5 Standard Solution Preparation

3.5.1 Prepare 100 ppm Copper Stock Solution by mixing 0.393 g $CuSO_4.5H_2O$ with DI water up to the final volume of 1000 ml

3.5.2 Prepare 5, 10, 20, 30 and 40 ppm standard solution by diluting the stock solution.

3.5.3 Blank the instrument using distilled water. Adjust sample intake by adjusting the nozzle.

3.5.4 Run standard solutions starting from the most diluted one and record the corresponding absorbance to create standard curve absorbance versus concentration.

3.5.5 Run unknown sample, record the absorbance.

3.5.6 Calculate the concentration of the unknown by using the standard curve.

Appendix 9: Lignocellulosic Content Measurement SOP

SOP No: 9

Brewery/Distillery Co-Product Characterisation Procedures Manual	Issue No: 1
Neutral Detergent Fibre 1st Step of Van Soest Method	Issue Date: 20/8/17 Issued by: Burcu Gunes

1.0 Sampling and Storage:

1.1 Collect samples in clean glass bottles. Use plastic bottles only if they are known to be free of organic contamination.

1.2 Samples may be stored up to 5 days at 4˚C.

2.0. Sample Pre-treatment

2.1 The sample (pot ale, draff) is ait dried at 100 ˚C. At least 5 g of dried sample is necessary, therefore it is necessary to dry approximately 400 ml of pot ale sample due to its high water content.

2.2 The dried sample is ground by using manual grinder.

2.3 The particle size between 0.5 mm to 1 mm is chosen by using “Retsch AS 200 Shaker”

3.0 Analysis Procedure

The neutral-detergent fibre (NDF) procedure constituents is a rapid method for analysing the total fibre in organic matter while the soluble fraction is extracted with neutral detergent solution. Neutral detergent fibre is the sum of hemicellulose, cellulose and lignin (Goering & Van Soest 1970).

3.1 Weigh 0.5 g (± 1 mg) ground sample into a 250 ml round bottom flask.

3.2 Add 100 ml of neutral detergent solution at room temperature into the flask and insert a condenser.

3.3 Heat to boiling and reflux for 60 min starting from onset of boiling. Use some octanol if foam is produced.

Note: A special care needs to be taken to the level of boiling. If foam is over produced it might take the sample out of the solution.

3.4 Tare a crucible.

3.5 Place previously tared crucible on a vacuum filter. Do not apply vacuum until the crucible is filled.

3.6 Filter and wash the sample in the crucible with boiling DI water 3-5 times.

3.7 Place the crucible into a 50 ml beaker and wash it in 20 ml of acetone 3 times for 5 minutes.

3.8 Dry for 8 h at 100 ˚C.

3.9 Weigh.

3.10 Calculate neutral detergent fibre (NDF) and neutral detergent soluble (NDS);

$$NDF\ \% = \frac{(Weight\ of\ crucible + Weight\ of\ residue) - Weight\ o\ crucible}{Weight\ of\ original\ sample} \times 100$$

$$NDS\ \% = 100 - NDF\ \%$$

3.11 Ash the crucible with dried sample in it at 525 ˚C for 8 h in Wise Therm muffle furnace.

3.12 Weigh.

3.13 Calculate ash insoluble in neutral detergent;

$$Ash\ insoluble\ \% = \frac{Loss\ on\ ashing}{weight\ of\ sample}$$

4.0 Chemicals

4.1 Neutral detergent solution 5 L;

28.6 g Disodium hydrogen phosphate, (Na_2PO_4-$2H_2O$)

93.0 g Disodium ethylenediaminetetraacetate, (EDTA, $C_{10}H_{14}N_2Na_20_8$)

34.1 g Disodium tetraborate decahydrate, ($Na_2B_40_7$-$10H_2O$)

150 g Sodium lauryl sulphate neutral, ($C_{12}H_{25}NaO_4S$)

50 ml Ethylene glycol diethyl ether, ($C_4H_{10}O_2$)

Pour disodium hydrogen phosphate, disodium ethylenediaminetetraacetate and disodium tetraborate decahydrate in a 5 L flask and dissolve in 2500 ml of DI water.

Add sodium lauryl sulphate and mix until dissolved.

Add ethylene glycol diethyl ether and the remaining of DI water.

Control the pH which must be between 6.9 and 7.1

4.2 n-octanol ($C_8H_{18}O$)

4.3 Acetone

1.0 Sampling and Storage and 2.0. Sample Pre-treatment are the same with the 1st step of the method.

3.0 Analysis Procedure

In the acid detergent fibre (ADF) procedure, water soluble and hemicellulose fraction is extracted by using acid detergent solution while cellulose and lignin remain in the biomass. ADF is sum of cellulose and hemicellulose. Hemicellulose fraction can be calculated by subtracting ADF from NDF (Goering & Van Soest 1970).

3.1 Weigh 0.5 g (± 1 mg) ground sample into a 250 ml round bottom flask.

3.2 Add 100 ml of acid detergent solution at room temperature into the flask and insert a condenser.

3.3 Heat to boiling and reflux for 60 min starting from onset of boiling. Use some octanol if foam is produced.

Note: A special care needs to be taken to the level of boiling. If foam is over produced it might take the sample out of the solution.

3.4 Tare a crucible.

3.5 Place previously tared crucible on a vacuum filter. Do not apply vacuum until the crucible is filled.

3.6 Filter and wash the sample in the crucible with boiling DI water 3-5 times.

3.7 Place the crucible into a 50 ml beaker and wash it in 20 ml of acetone 3 times for 3 minutes.

3.8 Dry for 8 h at 100 ˚C.

3.9 Weigh.

3.10 Calculate acid detergent fibre (ADF)

$$ADF\ \% = \frac{(Weight\ of\ crucible + Weight\ of\ residue) - Weight\ o\ crucible}{Weight\ of\ original\ sample} \times 100$$

3.11 Ash the crucible with dried sample in it at 525 ˚C for 8 h in muffle furnace

3.12 Weigh.

3.13 Calculate ash insoluble in acid detergent;

$$Ash\ insoluble\ \% = \frac{Loss\ on\ ashing}{weight\ of\ sample}$$

4.0 Chemicals

4.1 Acid detergent solution 5 L;

100 g Cetyltrimethylammonium bromide technical grade, ($C_{19}H_{42}BrN$)

136 ml Sulfuric acid 96%, (H_2SO_4)

Place cetyltrimethylammonium bromide in a 5 L flask.

Add 3 L DI water.

Add slowly and carefully 136 ml H_2SO_4 while stirring to promote dissolution.

Add DI water up to 5 L

4.2 n-octanol ($C_8H_{18}O$)

4.3 Acetone

1.0 Sampling and Storage and 2.0. Sample Pre-treatment are the same with the 1st step of the method.

3.0 Analysis Procedure

In the acid-detergent lignin procedure, the acid-detergent fibre (ADF) procedure is a preparatory step to remove the protein and other acid-soluble material that would interfere with the lignin determination. The ADF residue consists of cellulose, lignin and acid-insoluble ash (mainly silica). Treatment with 72 percent sulfuric acid dissolves cellulose. Ashing of the residue will determine the crude lignin fraction. Cellulose fraction can be calculated by subtracting ADL from ADF (Goering & Van Soest 1970).

3.1 Weigh 0.5 g (± 1 mg) ground sample into a 250 ml round bottom flask.

3.2 Add 100 ml of acid detergent solution at room temperature into the flask and insert a condenser.

3.3 Heat to boiling and reflux for 60 min starting from onset of boiling. Use some octanol if foam is produced.

Note: A special care needs to be taken to the level of boiling. If foam is over produced it might take the sample out of the solution.

3.4 Tare a crucible.

3.5 Place previously tared crucible on a vacuum filter. Do not apply vacuum until the crucible is filled.

3.6 Filter and wash the sample in the crucible with boiling DI water 3-5 times.

3.7 Move the crucible to a 50 ml glass beaker (must be acid resistant).

3.8 Pour 72% H2SO4 in the crucible and into the beaker. The crucible must stay ¾ filled with acid all the time for 48 hours, use a clock glass to cover.

3.9 Use a glass spatula in each crucible to mix well at least twice a day.

3.10 After 48 hours wash outside the crucible carefully to avoid any potential damage to the filtration sealing.

3.11 Place crucible to vacuum filter and wash it thoroughly with warm DI water until acid is cleaned off. Confirm by pH of the wash water.

3.12 Place the crucible into a 50 ml beaker and wash it in 20 ml of acetone 3 times for 3 minutes.

3.13 Dry for 8 h at 100 ˚C.

3.14 Weigh.

3.15 Ash the crucible with dried sample in it at 525 ˚C for 8 h in muffle furnace

3.12 Weigh.

3.13 Calculate acid detergent lignin;

$$ADL\ \% = \frac{(Weight\ of\ crucible + residue\ after\ acid\ soak) - Weight\ of\ crucible\ after\ ignition}{Weight\ of\ original\ sample} \times 100$$

Appendix 10: VFA Measurement SOP

SOP No: 10

Brewery/Distillery Co-Product Characterisation Procedures Manual	Sheet 1 of 5
	Issue No: 1
Short Chain Volatile Fatty Analysis (VFA) Gas Chromatography	Issue Date: 20/8/17
	Issued by: Burcu Gunes

1.0 Sampling and Storage:

1.1 Collect samples in clean glass bottles. Use plastic bottles only if they are known to be free of organic contamination.

1.2 Samples may be stored up to 5 days at 4˚C.

1.3 For better accuracy samples must be warmed to room temperature (~20 ˚C) before analysis.

2.0. Sample Pre-treatment

2.1 VFAs are analysed in mixture of sample and sludge, therefore centrifuging at 10000 rpm for 20 min is required to ensure clear liquid phase.

3.0 Analysis Procedure

This procedure details the preparation of distillery/brewery waste and activated sludge mixture for short chain volatile fatty acid analysis using FID gas chromatography.

3.1 Gas Chromatography Conditions and Preparation of Samples for Short Chain VFA Analysis

3.1.1 Chromatography Conditions:

Chromatograph: Agilent 7890 gas chromatograph with split inlet and 7639A Auto injector
Column Specifications: Agilent CP 7686; Length: 25m, Diameter: 0.15 mm, Film: 0.25 µl

Temperature:
Initial Oven: 115˚C
Injector: 260˚C
Detector: 280˚C

Injection size: 0.5 µl
Injector pressure: 44.55 psi
Injector total flow: 41.25ml/min

Flow rates:
Hydrogen: 30 ml/min
Compressed air: 300 ml/min
Makeup (Helium): 30 ml/min
Column + Makeup flow: 30 ml/min

Run Time: 9 min

3.1.2 Analysis Procedure:

3.1.2.1 Label microcentrifuge tubes for VFA standards (25, 50, 75 and 100%) and samples

3.1.2.2 Pipette 1.5 ml of standards and samples into the corresponding micro-centrifuge tube.

3.1.2.3 Centrifuge them at 3000 rpm for 10 minutes at 4˚C

3.1.2.4 Place tubes into ice water bath after centrifuging.

3.1.2.5 Prepare a 2nd set of labelled microcentrifuge tubes.

3.1.2.6 Pipette 200 µl of 25% meta-phosphoric acid/60mM Crotonic acid into each microcentrifuge tube of the 2nd set.

Note: Special care needs to be taken with the meta-phosphoric acid/60mM Crotonic acid mixture as it is the internal standard.

3.1.2.7 Pipette 1 ml of supernatant from the 1st set of centrifuged tubes to the 2nd on to mix with the internal standard.

Note: Keep watch for separation while pipetting

3.1.2.8 Vortex the microcentrifuge tubes to mix completely.

3.1.2.9 Place the microcentrifuge tubes into freezer at least for 3-4 hours until fully frozen.

3.1.2.10 Allow to thaw in a refrigerator or bench top.

3.1.2.11 Once thawed, centrifuge it at 12000 rpm at 4˚C for 15 minutes.

3.1.2.12 Label GC vials for standards and samples and pipette 1 ml of the supernatant of standards and samples into corresponding vial.

3.1.2.13 Place vials onto GC tray and run. Every 5th vial should be DI water.

3.3 Preparation of VFA Standard Solutions

3.3.1 To make the stock solution add the amounts acid indicated below to a 250 ml volumetric flask then make it up to 250 ml with DI water

1.435 ml Acetic acid

0.656 ml Propionic acid

0.082 ml Isobutyric acid

0.462 ml Butyric acid

0.111 ml Isovaleric acid

0.124 ml Valeric acid

Note: Example of the volume according to the target molarity is given in Appendix 10 Table 1.

Table 1. Target molarity of each standard in stock solution along with required volumes

	Acetic acid	**Propionic acid**	**Iso-butyric acid**	**Butyric Acid**	**Iso-valeric acid**	**Valeric acid**
Density, g/ml	1.049	0.993	0.950	0.964	0.925	0.936
Molecular weight, g/mol	60.1	74.1	88.11	88.11	102.1	102.1
Molarity, M	17.42	13.39	10.67	10.83	8.97	9.08
Target, mM	**100**	**35**	**3.5**	**20**	**4**	**4.4**
ml per 250 ml	1.435	0.653	0.082	0.462	0.112	0.124

$$Molarity, M\ (\frac{mol}{L}) = \frac{\left(Density\ \left(\frac{g}{ml}\right) \times 1000\right) g/L}{Molecular\ weight\ (\frac{g}{mol})}$$

$$Required\ acid\ volume, ml = \frac{TargetMolarity(mM) \times StockSolutionVolume(ml)}{Molarity\ of\ acid, ml}$$

Example calculation for acetic acid;

$$M = \frac{\left(1.049\ \left(\frac{g}{ml}\right) \times 1000\right) g/L}{60.1\ g/mol} = 17.42\ M = 17420\ mM$$

$$V = \frac{100\ mM \times 250\ ml}{1740\ mM} = 1.435\ ml\ acetic\ acid\ is\ required$$

3.3.2 Bring the flaks to the volume with DI water.

3.3.3 Allow to stir for at least 1 hour.

3.3.4 Pipette the appropriate amount of ID water and stock solution into each vial by using Table Appendix 10-2.

Table 2. Content of standard solutions

Standard (%)	DI Water (ml)	Stock Solution (ml)
25	11.25	3.75
50	7.5	7.5
75	3.75	11.25
100	0	15

3.3.5 Label each vial and store it in the fridge for up to 2 months.

3.4 Preparation of 25% Meta-phosphoric Acid w. 60mM Crotonic Acid as Internal Standard

3.4.1 Weigh 25 g of metha-phosphoric acid into a 100 ml of volumetric flask.

3.4.2 Add about 75 ml of DI water.

3.4.3 Place ono a stir plate and stir for 3 hours.

3.4.4 Prepare 500 mM crotonic acid solution by dissolving 4.3 g crotonic acid into a second 100 ml of volumetric flask.

3.4.5 Add 12 ml of 500 mM crotonic acid into metha-phosphoric acid solution and stir until the solution is clear.

3.4.6 Bring the volume with DI water in 100 ml volumetric flask to finish preparing 25% Meta-phosphoric acid w. 60mM Crotonic acid solution.

3.5.7 Run one GC vial with 1 ml DI water and 200 µl metha-phosphoric acid/crotonic acid mixture (MPhos) to quantify the area of the internal standard which is Crotonic acid in the mixture.

Note: Due to mixing 200 µl internal standard with 1 ml sample, the concentration of the internal is diluted. Example calculation is given below;

$$Diluted\ Concentration\ of\ MPhos = \frac{Crotonic\ acid\ concentration\ in\ MPhos \times MPhos\ Volume}{Volume\ of\ MPhos + Volume\ of\ sample}$$

$$Diluted\ Concentration\ of\ MPhos = \frac{60\ mM\ \times 200\ \mu l}{1200\ \mu l} = 10\ mM$$

Appendix 11: ICP-OES Sample Preparation

SOP No: 11

Brewery/Distillery Co-Product Characterisation Procedures Manual	Issue No: 1
Sample Preparation Total Mineral Analysis Using 5100 ICP-OES Agilent Technologies	Issue Date: 17/03/19
	Issued by: Burcu Gunes

1.0 Sampling and Storage:

1.1 Collect samples in clean glass bottles. Use plastic bottles only if they are known to be free of organic contamination.

1.2 Samples may be stored up to 28 days by treating with sulphuric acid to a pH of less than 2 and cooling 4˚C or lower.

1.3 For better accuracy samples must be warmed to room temperature (~20 ˚C) before analysis.

2.0 Analysis Procedure

2.1 Weigh out required amount of sample into a 35 ml microwave vessel and an appropriate control,

- For **Se** analysis weight out ≈0.2 g,
- For **Mineral/Bioplex** analysis weigh out ≈0.1 g,
- For **Feed + Premix** weigh out ≈0.3 g
- Plant samples weight out ≈0.4 g

2.2 Add small magnet

2.3 Add 1 ml of H_2O and 10 ml of conc. HNO_3, slowly, (1 mL conc. HCl for Fe analysis),

2.4 Put cap on

2.5 Stir on magnetic plate until homogenous

2.6 Turn both fans on wall (red light) and both digesters (right side)

2.7 Put the microwave vessels into a desired location (A, B, C or D)

2.8 Chance the attenuator to a large one (35 ml)

2.9 In CEM software, select Yeast & Bioplex program and then click and drag to the position on autosampler

2.10 Double check if everything is correct

2.11 Click Play for digestion to begin

After Digestion

2.12 Take caps off, carefully

2.13 Wash/rinse the inside of cap with DI water at least 3-4 times into a 15 or 50 ml sterilin

2.14 Transfer the sample solution to the sterilin holding the magnetic wand so that the small magnet does not fall into the sterilin

2.15 Rinse the sterilin with DI water to make sure that all sample is contained

2.16 Make up to the mark and invert

2.17 Dilute if required

Standard Preparation

2.18 From 1000 ppm make 10 ppm stock (500 µL in 50 ml sterilin)

2.19 Make 0.1, 0.25, 0.5, 1, 2 and 5 ppm standards by making up to 50 ml with appropriate conc. of HNO_3 to matrix match the samples.

Appendix 12: ICP-OES Operating Procedure

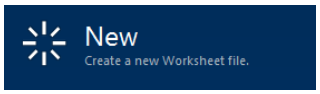
SOP No: 12

Brewery/Distillery Co-Product Characterisation Procedures Manual	Issue No: 1
ICP-OES Standard Operating Procedure	Issue Date: 17/03/19
	Issued by: Burcu Gunes

1.0 Procedure

1.1 Double click on **ICP-Expert** icon on the computer desktop.

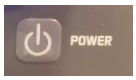
1.2 Create a new Worksheet file by clicking on '**New**'.



1.3 Save the file as YYYYMMDD and name format.

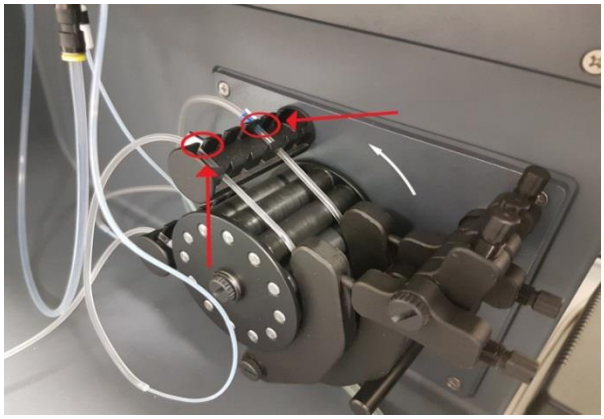
For example, 20190410 Total mineral analysis – digestate

1.4 Turn on the chiller (located on the floor, beside the autosampler) by pressing '**Power**' button.



1.5 Turn the '**ICP FAN**' on. The switch is located on a wall above the chiller (red light must be on).

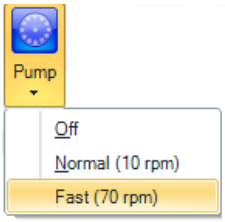
1.6 The sample introduction peri pump tubing must be replaced (depending on its use) and clamped. Make sure that the tubing is straight and is in appropriate section (sample introduction tube in section one and waste tubing in section four) as indicated below.



1.7 Visually check the torch for any cracks/dirt (if any cracks are present, report to lab curator)

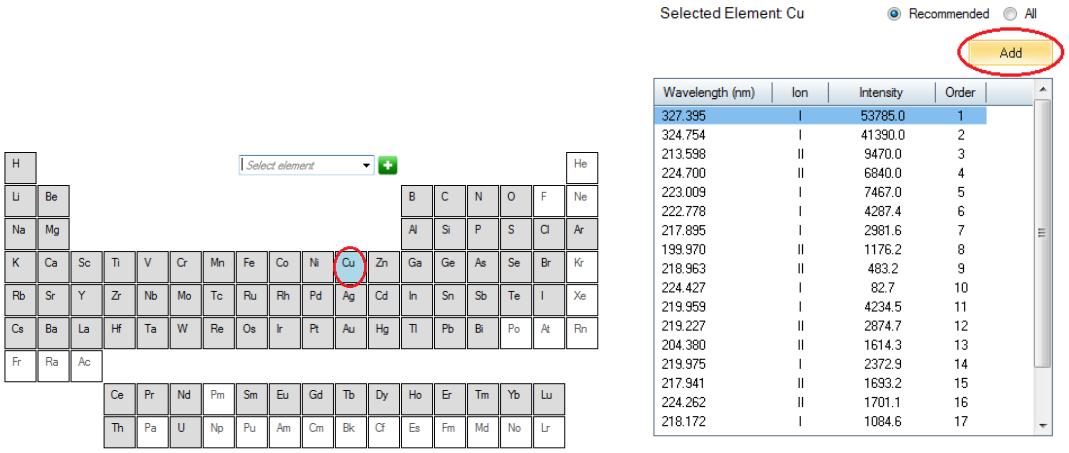
1.8 Turn on the plasma by clicking '**Plasma**' at the top of the worksheet. Let the instrument warm up for 20 minutes.

1.9 Go to the **Autosampler** tab and send the probe to rinse by double clicking on the rinse station icon. Check that the mist spraying from the nebulizer is constant and not pulsing (change pump speed to fast).



1.10 In the **Elements** tab select the required elements by clicking on them in the periodic table. Confirm selection by clicking '**Add**' on the right with highest intensity wavelength (nm).

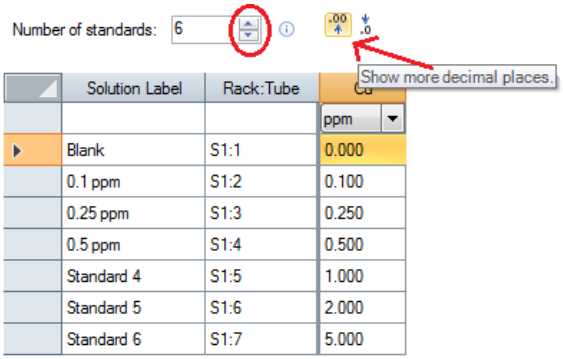
Example adding Cu below:



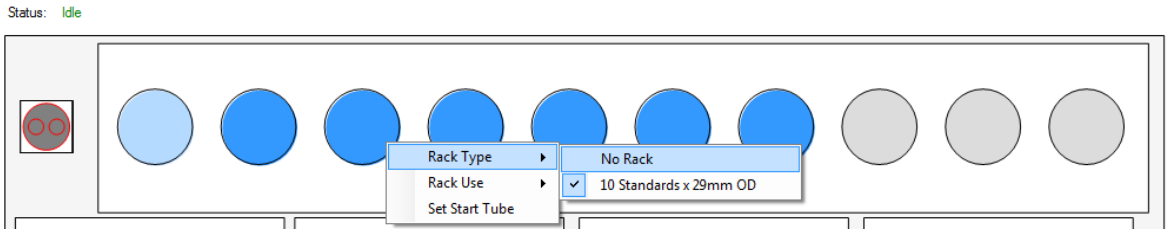
Some elements may interfere with one another (these will appear in red bands). Multiple wavelengths can be added by repeating point 10. Add wavelengths that don't have interferences (not red bands) while keeping the intensities as high as possible.

1.11 The **Conditions** tab has all the parameters already set, however sometimes depending on the sample type and concentrations some parameters such as 'Number of Replicates', 'Uptake Delay', 'Rinse Time' or 'Read Time' can be increased.

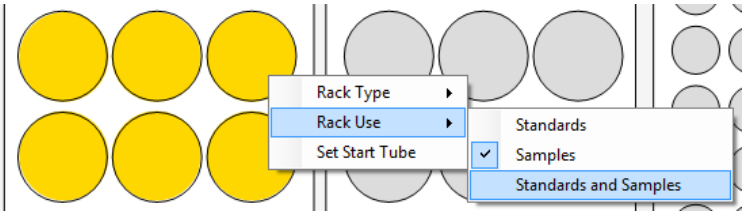
1.12 The **Standards** tab is used to select the 'Number of Standards' by using the arrows (circled in red). Enter standard names and their concentrations respectively. Click on show more decimal places and set the standards to three decimal places.



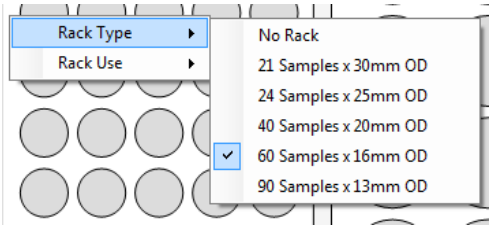
1.13 Next go to the **Autosampler** tab and right click the top rack -> Rack Type -> No Rack.



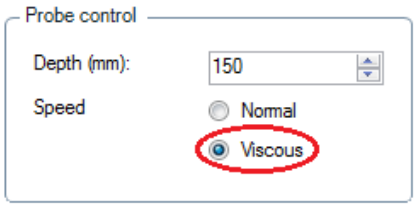
The first rack from the left should have ‘Rack Use’ selected as ‘Standards and Samples’, unless specified otherwise.



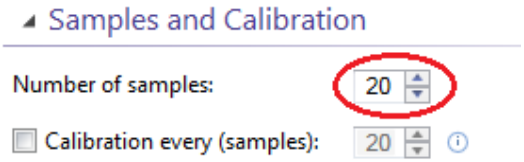
Then right click on the other racks going from the left to right and select the appropriate ‘Rack Types’ (Either 21 or 60 samples per rack).



Set the probe control speed to viscous.

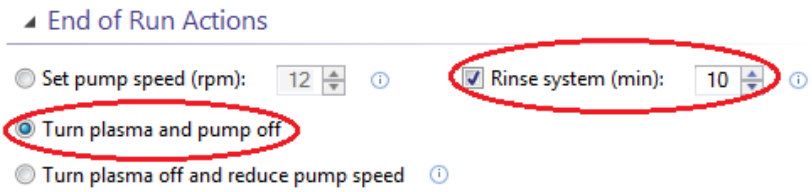


1.14 The **Sequence** tab allows selection of the ‘Number of Samples’. Click on the arrows (circled) or enter the number in the box.

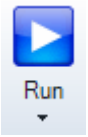


After placing the samples in the racks, check that ‘Rack: Tube’ numbers correspond to the positions of the samples. Type all the known information into the ‘Solution Labels’, ‘Weights’, ‘Volume’ and ‘Dilutions’.

Make sure to click on ‘Plasma and Pump off’ (unless there is another set of samples to analyse next) and also ‘Rinse system’ for 10 mins.



1.15 Double check that everything is correct and click ‘**Run**’ at the top of the worksheet.



1.16 Go to the **Analysis** tab to monitor the analysis.

1.17 After the plasma is turned off -> undo the peri pump clamp, loosen the peri pump tubing and turn off the fan and chiller.

Appendix 13: Energy Analysis Calculations for AD plant

Pot ale Discharge:

Annual pot ale discharge volume: 8 800 m^3

Density of pot ale: 1000 kg/m^3

Annual pot ale discharge mass: 8 800 tone

Experiment Results:

Biogas yield of lab scale experiment result of non-treated pot ale with a sludge seeding ratio of 65%:

Biogas yield per g VS after 2 days ($Biogas_2$): 184 ± 1 ml/g VS, 32.2 ± 0.1% CH_4

Biogas yield per g VS after 6 days ($Biogas_6$): 376 ± 12 ml/g VS, 50.3 ± 1.1% CH_4

Biogas yield per VS after 21 days ($Biogas_{21}$): 509 ± 2 ml/g VS, 54.4 ± 1.5% CH_4

VS: 0.107 g/g sample, 10.7% Dry Matter

74% of overall digestion yield was achieved within the first 6 days of AD, therefore $Biogas_6$ yield was chosen.

Assumption: Biogas yield will be the same for the full-scale implementation as lab scale.

Annual Biogas Production Potential at Full-Scale:

$$376\,\frac{m^3 Biogas}{tone\ VS} \times 8\,800\ \frac{tone\ pot\ ale}{year} \times 0.107\,\frac{tone\ VS}{tone\ pot\ ale} = 352\,084\ \frac{m^3 Biogas}{year}$$

Biogas loss estimation in CHP: 0.5%

Biogas loss in CHP;

$$354\,041\ \frac{m^3 Biogas}{year} \times \frac{0.5}{100}\ =\ 1760\ m^3/year$$

Net biogas input to CHP;

$$352\ 084\ \frac{m^3 Biogas}{year} - 1\ 760\ \frac{m^3 Biogas}{year} = 350\ 323\ \frac{m^3 Biogas}{year}$$

Available CH_4 within generated biogas for combustion;

$$350\ 323\ \frac{m^3 Biogas}{year} \times \frac{50.3\ CH_4}{100\ Biogas} = 175\ 978\ \frac{m^3 CH4}{year}$$

Appendix 14: Energy Analysis Calculations for CHP

Combustion Energy Output Potential:

Energy density of methane: 36 MJ/m^3, $1\text{MJ} = 0.00028\ \text{MWh}$

$$175\ 978 \frac{m^3 CH_4}{year} \times 36 \frac{MJ}{m^3 CH_4} \times 0.00028 \frac{MWh}{MJ} = 1\ 774\ \frac{MWh}{year}$$

Required Power Capacity of CHP:

$$1\ 774\ \frac{MWh}{year} \times \frac{1\ year}{365\ day} \times \frac{1\ day}{24\ h} = 0.2025\ MW, 202.5\ kW$$

Electrical efficiency estimation: 38%

Electrical energy output potential of CHP:

$$1\ 774 \frac{MWh}{year} \times 0.38 = 674\ \frac{MWh}{year}$$

Internal electrical energy demand estimation of the plant: 6.9%

Internal electricity consumption:

$$674 \frac{MWh}{year} \times 0.069 = 47 \frac{Mwh}{year}$$

Net electricity production potential:

$$674\ \frac{MWh}{year} - 47 \frac{MWh}{year} = 628 \frac{MWh}{year}\ , 627\ 556 \frac{kWh}{year}$$

Thermal efficiency estimation: 39%

Thermal energy output potential of CHP:

$$1\ 774 \frac{MWh}{year} \times 0.39 = 692\ \frac{MWh}{year}$$

Internal thermal energy demand estimation of the plant: 13.5%

$$692\ \frac{MWh}{year} \times 0.135 = 93\ \frac{MWh}{year}$$

Net heat production potential:

$$692\ \frac{MWh}{year} - 93\ \frac{MWh}{year} = 598\ \frac{MWh}{year}\ ,598\ 411\ \frac{kWh}{year}$$

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