Design of integrated processes for a second generation biorefinery using mixed agricultural waste

by

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DECLARATION

.

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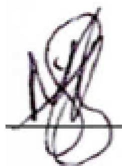

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ABSTRACT

Lignocellulosic biomass (agro-waste) has been recommended as the most promising feedstock for the production of bioalcohols, in the biofuel industry. Furthermore, agro-waste is well-known as the most abundant organic matter in the agricultural and forestry product processing industry. However, the challenge with utilizing agro-waste as a feedstock is its highly recalcitrant structure, which limits hydrolysis to convert the holocelluloses into fermentable sugars. Conventional pre-treatment methods such as dilute acid, alkaline, thermal, hot water and enzymatic, have been used in previous studies. The challenge with these conventional methods is the generation of residual toxicants during the pretreatment process, which inhibits a high bioalcohol yield, by reducing the microbial populations' (fermenter) ability to be metabolically proficient during fermentation. Numerous studies have been developed to improve the engineered strains, which have shown to have an ability to reduce the inhibition and toxicity of the bioalcohols produced or by-products produced during pre-treatment, while enhancing the bioalcohol production.

In the present study (chapter 5), evaluation of common conventional methods for the pretreatment of the mixed agro-waste, i.e. (>45µm to <100µm) constituted by *Citrus sinensis, Malus domestica* peels, corn cobs from *Zea mays* and *Quercus robur* (oak) yard waste without a pre-rinsing step at a ratio of 1:1 at 25% (w/w) for each waste material, was undertaken, focusing on hot water pre-treatment followed by dilute acid (H_2SO_4) pre-treatment. To further pretreat the mixed agro-waste residue, cellulases were used to further hydrolyse the pre-treated agro-waste in a single pot (batch) multi-reaction process. The TRS concentration of 0.12, 1.43 and 3.22 g/L was achieved with hot water, dilute acid and cellulases hydrolysis as sequential pretreatment steps, respectively, in a single pot multi-reaction system. Furthermore, a commercial strain was used to ascertain low (C_1 to C_3) and high carbon content (C_4^+) bioalcohol production under aerobic conditions. Multiple bioproducts were obtained within 48 to 72 h, including bioethanol and 1-Butanol, 3-methyl, which were major products for this study. However, undesirable bio-compounds such as phenolics, were detected post fermentation.

Since multiple process units characterised by chemical usage and high energy intensivity have been utilized to overcome delignification and cellulolysis, a sustainable, environmental benign pretreatment process was proposed using *N. mirabilis* "monkey cup" fluids (extracts) to also reduce fermenter inhibitors from the delignification of mixed agrowaste; a process with minimal thermo-physical chemical inputs for which a single pot multi-reaction system strategy was used. *Nepenthes mirabilis* extracts shown to have ligninolytic, cellulolytic and xylanolytic activities, were used as an enzyme cocktail to pretreat mixed agro-waste, subsequent to the furtherance of TRS production from the agro-waste, by further using cellulase for further hydrolysis. *N. mirabilis* pod extracts were determined to contained carboxylesterases (529.41±30.50 U/L), β-glucosidases (251.94±11.48 U/L)

and xylanases (36.09±18.04 U/L), constituting an enzymatic cocktail with a significant potential for the reduction in total residual phenolic compounds (TRPCs). Furthermore, the results indicated that maximum concentration of TRS obtainable was 310±5.19 mg/L within 168 h, while the TRPCs were reduced from 6.25±0.18 to 4.26 ±0.09 mg/L, which was lower than that observed when conventional methods were used. Overall *N. mirabilis* extracts were demonstrated to have an ability to support biocatalytic processes for the conversion of agro-waste to produce fermentable TRS in a single unit facilitating multiple reactions with minimised interference with cellulase hydrolysis. Therefore, the digestive enzymes in *N. mirabilis* pods can be used in an integrated system for a second generation biorefinery.

Additionally, the hydrolysates from *Nepenthes mirabilis* pod extracts including cellulases pre-treated mixed agro-waste (*N. mirabilis*/CP), were utilized for bioalcohols production using *Saccharomyces cerevisiae* under aerobic conditions in single spot batch system. The results obtained were compared with conventional biomass pre-treatment methods, i.e. hot water, dilute acid and cellulases (HWP/DAP/CP) hydrolysates fermentation. During fermentation a maximum cell concentration of 1.47 CFU/mL (x 10^{10}) was achieved with HWP/DAP/CP hydrolysates, with a relative difference of 21.1 % when compared to the *N. mirabilis*/CP cultures; whereby, the relative difference for product yield of biomass generation was achieved as 20.2% higher for *N. mirabilis*/CP cultures, albeit, the product yield based on biomass generation was relatively (20.2%) higher for *N. mirabilis*/CP culture. For the TRPCs generation, a relative difference (24.6%) between *N. mirabilis*/CP and HWP/DAP/CP pretreatment systems was observed, suggesting the *N. mirabilis*/CP pretreatment regime generates lower inhibition by-products. This was further evidenced by the lowest substrate utilization rate (3.3 x 10^{-4} g/L.h) for the *N. mirabilis*/CP cultures while achieving relatively similar product formation rates to those observed for the HWP/DAP/CP. Better correlation (R^2 0.94) was obtained when predicting substrate utilization for *N. mirabilis*/CP cultures. Generally, the pretreatment of mixed agro-waste using *N. mirabilis*/CP, seemed appropriate for producing hydrolysates which *Saccharomyces cerevisiae* can effectively use for bioalcohol production.

Therefore, this study showed the ability of *Nepenthes mirabilis* extracts to pre-treat lignocellulosic biomass (agro-waste) to enhance TRS extraction while reducing TRPCs for production of bioalcohols in a single pot multi-reaction system. The addition of *N. mirabilis* extract offers environmental benignity while reducing fermenter inhibitors from delignification.

Keywords: Agro-waste; Bioalcohol; Biorefinery; β-Glucosidases; Carboxylesterases; Cellulases; Holocelluloses; *Nepenthes mirabilis*; Total reducing sugars; Kinetic models; *Saccharomyces cerevisiae*; Xylanases

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- My whole family, for their support during my studies, they have been supportive in many different ways.

Nkosikho Dlangamandla

December 2018

DEDICATION

To my late Mother and Grandmother

Sylvia Dlangamandla and Nowayilesi Dlangamandla

To my family,

To my Wife Khwezikazi Stofile Dlangamandla,

My young ones

Lisa and Inakokonke Dlangamandla

And

To all the Dlangamandla family

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NOMENCLATURE

Symbols	Definition (Units)
Abs/A	Absorbance (cm^{-1})
ε	Extinction coefficient of lignin (g/L)
Ash (%)	Mass of ash by percentage (%)
B	cuvette path length (1 cm)
D_f	Dilution factor
I_{002}	Intensity for the crystalline phase (counts)
I_{am}	Amorphous (crystalline) phase portion (counts)
K_S	Substrate saturation constant ($g.\ L^{-1}$)
m_s	Cell maintenance parameter (h^{-1})
M_a	Mass of the dry sample (g)
M_c	Ash mass (g)
MIC	Minimum Inhibitory concentration ($g.L^{-1}$)
n	Leudeking–Piret constant (Area %.mL/CFU.h)
P	Product concentration (Area %)
P_o	Initial product concentration (Area %)
R^2	Correlation coefficient
r_P	Product formation rate (Area %/h)
r_s	Substrate utilisation rate ($g.\ L^{-1}\ h^{-1}$)
r_x	Cellular growth rate ($CFU.\ L^{-1}\ h^{-1}$)
S	Limiting substrate concentration ($g\ L^{-1}$)
S_o	Initial substrate concentration ($g\ L^{-1}$)
X	Cell concentration ($CFU.\ mL^{-1}$)
$X_{f/max}$	Final/maximum cell concentration ($CFU\ mL^{-1}$)

X_o	Initial cell concentration (CFU. mL $^{-1}$)
$Y_{p/x}$	Product yield coefficient (Area %.mL/CFU)
$Y_{x/s}$	Biomass yield coefficient (CFU/g substrate)

Abbreviations	**Meaning**
ABE	Acetone-Butanol-Ethanol fermentation
AFEX	Ammonia fibre expansion
AIR	Acid-insoluble residue lignin
ASL	Acid-soluble lignin
CBP	Consolidated bioprocessing (i.e. Utilisation of mono or co-cultures of microorganisms which ferment cellulose to biofuels)
CFU	Colony-Forming Units
Cr_I	Crystallinity index
CV	Coefficient of variance
DAP	Dilute acid pretreatment
DNA	Deoxyribonucleic acid
DNS	Dinitrosacylic acid
Eh	Redox potential (mV)
FTIR	Fourier-transform infrared spectroscopy
GC-MS	Gas Chromatography- Mass Spectrophotometry
HMF	5-Hydroxymethylfurfural
LCC	Lignin-carbohydrate complex
LHW	Liquid hot water
LODP	Level-off basic degree of polymerization
MDS	Mass selective detector
NCBI	National Center for Biotechnology Information
OD_{600nm}	Optical density (measured at 600nm)
PDA	Potato Dextrose Agar
PCR	Polymerase Chain Reaction
ρNPA	ρ-nitrophenyl acetate
pNPG	ρ-nitrophenyl-β-D-glucopyranoside
RPM	Revolutions per minute (rev/min)
SAA	Soaking in aqueous ammonia
SEC	Size Exclusion Chromatography

SEM	Standard error of mean
SEP	Steam explosion pretreatment
SHF	Separate hydrolysis and fermentation
SSF	Simultaneous saccharification and fermentation
SSFC	Simultaneous saccharification and co-fermentation
TLC	Thin-Layer Chromatography
TROAs	Total residual organic acids
TRPCs	Total residual phenolic compounds
TRS	Total reducing sugar
XRD	X-ray diffraction
YPD	Yeast/Peptone/Dextrose

Greek symbols

μ_{max}	Maximum growth rate (h^{-1})
μ	Specific growth rate (h^{-1})
ε	Extinction coefficient ($M^{-1}.cm^{-1}$)

PREFACE TO THE THESIS

The research presented in this thesis was conducted at the Chemical Engineering Department, the Bioresource Engineering Research group (*BioERG*) Laboratory, Department of Biotechnology; and the Instrumentation and Analytical Chemistry Laboratory, Department of Chemistry – all on the Cape Town campus of the Cape Peninsula University of Technology, South Africa.

This thesis is composed of ten chapters as follows:

Chapter 1 Enlists the background of the study, the potential and principles of alcohol based biofuel constituents and their production, including applications. The background of the research problem, problem statement, research questions/strategy of the research, aim and objectives of the study, also provides an introduction to the thesis. Study delineation, highlighting delimitations of the study are also listed,

Furthermore, the literature review is divided into **Chapter 2, and 3,** reporting on a comprehensive review of relevant literature on lignocellulosic biomass types, it's pre-treatment for biofuel production and the need to produce high calorific energy containing bioalcohols including their separation techniques from fermentation broth. Furthermore, these chapters illustrate the challenges of the biorefinery process at an industrial/commercial scale. Process integration for biorefinery is also advocated for, focusing on the pre-treatment of suitable biomass for reduced energy intensity (usage), refined organic chemical usage and a reduced plant foot print for the development of environmental benign processes, while

Chapter 4, reports on the highlights (methods and procedures) of experimental procedures that were used to complete the study and attain the objectives, with

Chapter 5, 6, and 7, reporting on the highlights (results) of experiments, with a suitable exposition required to explain the fundamental outcomes of the research including limitations thereof, in order to add to scientific knowledge as required for a doctoral candidacy.

Chapter 8, focused on the conclusions of the study and general recommendations for future studies for purposes related to the development of the concept of using *N. mirabilis* for the pretreatment of agro-waste to benefit the biorefinery industry.

Chapter 9, list all references used, and

Chapter 10, lists all appendices.

CHAPTER 1

INTRODUCTION

CHAPTER 1

1. INTRODUCTION

1.1 Background to the research problem

Recently, researchers have been focusing on the development of sustainable alternatives to fossil fuels to reduce greenhouse gas (GHG) emissions contributing to global warming, and thus environmental deterioration. Since the 1980s, biomass feedstock including agricultural waste (agro-waste) has been shown to have potential as a primary biorefinery feedstock to produce value added products that supplements and/or is added to petroleum products. Furthermore, bioethanol has been the primary and outmost bio-product being produced (Demirbas, 2009). However, bioethanol production using the biorefinery concept for biodiesel production has unintended consequences with a majority of challenges related to its production using agricultural produce destined for human consumption which raises food security concerns. Due to these concerns, and to negate perceived ethical challenges associated with the production of bioethanol and biodiesel from edible agricultural products, there is an urgent need for the development of second and in particular third generation bio-refineries for developing countries, such as South Africa.

A bio-refinery can be defined as a process used in the sustainable processing of biomass into a spectrum of marketable products and energy. Biorefineries are classified into three categories: first, advanced second and third generation bioprocesses. The first and second generation biorefineries have limited market acceptability due to cost-income disparities as limited feedstock is used to generated a limited quantity a bioproducts when compared to conventional petroleum based and/ or refinery processes (Naik *et al.*, 2010). These limitations include the quantity and types of bioalcohols including added value products produced, and downstream processes required for the recovery of the products from the residual liquid broth initial used, i.e. hydrolysates (Hughes *et al.*, 2013). An alternative required to allay food security concerns, is the use of waste biomass including mixed agro-waste which can provide a sustainable, renewable bioresource, to produce fermentable hydrolysates in order to facilitate a fermentation process in a biorefinery in order to replace a significant portion of edible agricultural produce currently being used, in particular for the production of bioalcohols (Tracy, 2012).

Generally, first generation biorefineries are classified by the utilisation of agricultural crops as the feedstock, while, using a single feedstock to produce a single product such as bioethanol and biodiesel(Moncada *et al.*, 2014). In this type of biorefinery, feedstock such as corn starch or sugar cane are used individually as the sole carbon source as their constituents are readily fermentable; however, these types of feedstock are also used in food production (Hughes *et al.*, 2013; Lan and Liao, 2013), raising concerns about food security, as producers and/or distributors of this type of

agricultural produce, derive higher monitory benefits if they batter their produce to these biorefineries in comparison to when they sell the produce for food production thus human consumption. Although it makes economic sense to divert the produce to a market which has the highest returns, the human population with its increase, requires an increased quantity of food which cannot be circumvented for monitory gain, particularly for African countries (Hughes *et al.*, 2013).

Similarly, second generation biorefineries are based on the utilisation of a single feedstock, using pre-treatment technologies such as thermo-chemical and biological hydrolysis of cellulose, hemicellulose and lignin conversion processes to produce a range of different bio-alcohols and marketable co-products (de Jong *et al.*, 2009). To improve the first and second generation biorefineries, the concept of a third generation biorefinery was then introduced, to reduce the impact of edible agriculture produce usage in biorefineries while maximising social and economic benefits, which includes minimisation and/or the reduction of environmental health related deterioration challenges. A third generation biorefinery is the most advanced type of a biorefinery which is based on the production of a variety of products using a diverse array of biomass feedstock (Dürre, 2007). To date, lignocellulose biomass and agrowaste have been identified as the least expensive and most abundant form of biomass for use in this type of biorefinery, thus a focus of this study, i.e. to develop integrated process for an advanced second generation biorefinery, while minimising environmental health challenges, an environmental benign –a green chemistry approach.

1.2 Problem statement

Agro-waste disposal is becoming a major concern for the agricultural industry and municipal authorities internationally, as a large quantity of this waste requires appropriate disposal methods, a situation requiring large-scale usage of landfills which in certain instances involves increasing capital costs associated with the disposal of such wastes. Some of the agro-waste is recyclable and is used in the production of fertilizers, while large quantities remain unused and thus in many instances contribute to environmental pollution. Currently, several research studies have shown that waste from the agricultural industry can be utilized in the production of bioenergy and several high added-value chemicals, in particular, waste containing lignocellulosic materials and a variety of free sugars (Cardona and Sánchez, 2007; Cheng *et al.*, 2012; Dürre, 2007).

The most sustainable and abundant cellulosic feedstock widely available is derived from agricultural produce residue. A critical challenge with the use of agricultural residue is its pre-treatment, to release fermentable growth promoting substrates using efficient and economically feasible processes (Cheng *et al.*, 2012). The pre-treatment of waste biomass is an important step for the

release of simple sugars such as glucose, xylose, mannose, etc., sugars which can be used as potential substrates for the production of various biological products through fermentation (Choi *et al.*, 2013). For the development of a biorefinery, a large proportion of research studies focuses on first and second generation biorefineries (Cardona and Sánchez, 2007). In general terms, there are limited research studies which focus on the development of third generation biorefineries, whereby mixed agro-waste is utilized in the production of multiple products, in particular those with high added value potential.

Additionally, research conducted on the pre-treatment processes for agro-waste, involve high-investment costs, and can be classified as unsuitable for a green chemistry approach for bioprocess development due to the production of residual persistent environmental contaminants as a results of refined chemical compound usage. In certain instances, biomass pre-treatment processes involve the use of dilute sulphuric acid which is used for biomass delignification and the conversion of the crystalline structure of cellulose, i.e. cellulolysis. However, the use of refined in -/organic chemicals, including high energy intensive systems, i.e. steam explosion, may generate inhibitory by-products that can affect enzymatic hydrolysis and microbial action towards hydrolysate conversion during fermentation (Gould and Freer, 1984). Generally, the utilization of chemical methods such as the dilute sulphuric acid pre-treatment is being advocated for; albeit it is perceived to be inexpensive, convenient and effective in the pre-treatment of biomass, it does affect subsequent cellulases hydrolysis to produce fermentable reducible sugars (Chandra *et al.*, 2012; Chen *et al.*, 2011; Diep *et al.*, 2012; Ranjan *et al.*, 2013; Zheng *et al.*, 2009). This necessitates the design, evaluation including the integration of suitable processes which have low capital cost inputs requirements thus sustainability, for medium to large scale biorefineries – a major proponent of this study.

Therefore, a multiple feedstock (mixed agrowaste), i.e. mixed agricultural waste consisting of citrus, starch, including yard (tree leaf) waste (Vancov and McIntosh, 2012), single-stage biological process to produce multiple high value products, thus an advance second generation biorefinery, is proposed for this study.

1.3 Research/Process development strategy

Therefore, a suitable biotechnological i.e. bioprocess engineering strategy, for a single pot facile delignification, cellulolysis and hydrolysis must therefore be developed, with the primary aim being two-fold 1) maximised total reducing sugar (TRS) extraction from the agro-waste used and 2) while reducing pre-treatment inhibitory by-products, to achieve an adequately successful fermentation.

1.4 General aim of the study

The primary aim of this study was to develop an advanced second generation biorefinery using mixed agricultural waste in a one-pot multiple reaction system to reduce plant foot print with minimal energy (heating) and refined chemical compound usage, including a potential to reduce pre-treatment toxicants, i.e. inhibitory by-products, which can have a negative impact on both environmental and downstream processes (from the pre-treatment stages).

1.5 Specific objectives

- To produce different bioalcohols such as low (C_1 to C_3) and high (C_4^+) carbon content alcohols in an integrated single pot system under aerobic conditions using a mixed agro-waste extracts as a sole carbon source, using a commercial *S. cerevisiae* strain for the production of the bioalcohols and other added value products for the biorefinery industry. The fermentations were analysed for different bioproducts to determine the potential of the single pot system to produce added value products for the biorefinery industry.
- To evaluate an integrated pre-treatment system of mixed agro-waste destined for a second generation biorefinery using *N. mirabilis* extracts. Such research is required to ascertain the applicability and biocatalytic efficacy of *N. mirabilis* extracts in an integrated process for biomass pre-treatment to maximise the extraction of TRS.
- To determine the microbial growth, substrate utilization and the product formation kinetic parameters during fermentation processes using hydrolysates of *N. mirabilis*/cellulase (*N. mirabilis*/CP) in comparison to those of hot water/dilute acid/cellulase (HWP/DAP/CP)-mixed agro-waste hydrolysis systems for fermentations facilitated by a commercial South African *S. cerevisiae* strain (VIN13).

1.6 Hypothesis

It is hypothesised that the second generation biorefinery utilising agro-waste can be designed to effectively convert agro-waste from various sources to produce multiple products such as bioethanol, biobutanol, including other products. However, this study focused on non-food crop biomass, i.e. regionally available agro-waste.

1.7 Delineation of the study

In this study, an advanced second generation biorefinery was the primary focus of the study, with a specialisation of pretreatment technology to produce suitable hydrolysates. The first and second generation biorefinery and bioalcohol recovery were not studied. However, a suitable bioalcohol recovery process will be recommended. The bioalcohols will be limited to low C_1 to C_3 and high carbon content C_4 (biobutanol) bioalcohols. *Nepenthes mirabilis* will be the only pitcher plant that will be used in this study to hydrolyse the agro-waste.

CHAPTER 2

LITERATURE REVIEW

CHAPTER 2

2. LITERATURE REVIEW: PART 1: Lignocellulosic biomass and its pre-treatment

2.1 Lignocellulosic biomass

Lignocellulosic biomass is an attractive feedstock for biorefineries and is the most abundant organic matter that is available on earth. Lignocellulosic biomass contains three components, primarily, lignin, cellulose, and hemicelluloses, with some, i.e. agro-waste, containing residual readily fermentable sugars. The cellulose and hemicellulose contain monomers such as C_5 and C_6 carbon-based constituents, which are bound together in a lignin matrix. Lignocellulosic biomass, has been used traditionally as a source of combustible fuel, for more than 2000 years, i.e. for cooking, heating and for light (Agbor *et al.*, 2011), with petroleum based products being an alternative source for these activities. The depletion of petroleum has ultimately led to research focusing on biomass as an alternative that can be beneficiated to produce add value products thus reduce the reliance on petroleum and associated fossil fuel products. Although, lignocellulosic biomass has been identified as a major renewable resource for production of value added products, as it is more abundantly available and cheap, suitable process must be designed to achieve this objective. Furthermore, bioalcohols produced from lignocellulosic biomass, are seen as being attractive as an alternative source of energy and an additives to fuels. During the process of bioalcohols production, lignocellulosic biomass is converted to alcohol and other products in a three step process whereby the biomass is converted to cellulose by pre-treatment, while the cellulose produced is converted to fermentable sugars, i.e. total reducing sugars (TRS) such as glucose by hydrolysis and finally with the TRS produced being fermented to produce the bioalcohols and other value added by products (Chiaramonti *et al.*, 2012; Taherzadeh and Karimi, 2008). However the complex structure of lignocellulosic biomass, has reduced its attractiveness for the commercialization of biomass-base processes (Kim and Han, 2012).

Lignocellulosic biomass composition depends on its source type of the material. Table 2.1 illustrates differential of the composition of lignocellulosic biomass, from different renewable sources.

2.1.1 Lignin

In plant material, cellulose and hemicellulose are covered and significantly integrated with the lignin polymeric structure. Lignin plays an important role in the protection of essential plant cells. Numerous polymeric compounds form the basis of the lignin structure, acting as a barrier for environmental, thus harsh conditions, for plant cells. Lignin, forms 15 to 40 wt% of dry matter for woody plants (Taherzadeh and Karimi, 2008; Timilsena *et al.*, 2013; Tong *et al.*, 2013; Wang *et al.*,

2013). A large quantity, i.e. 40 to 50 million tons of lignin is produced per annum globally as waste, with 60 to 90% being disposed to landfills (Varanasi *et al.*, 2013).

Current research studies have shown the re-purposefulness of lignin waste, i.e.to utilize it as a feedstock in the biorefinery industry (Timilsena *et al.*, 2013). During the beneficiation process, the lignin structure must first be degraded using different pre-treatment methods depending on the desired outcome in order to enhance enzymatic hydrolysis of residual hemicellulose. However, not all of these pretreatment processes are able to delignify the rigid structure of lignin, with peroxidase enzymes from species such as white-rot fungus demonstrating their ability to biodegrade the lignin matrix, exposing the cellulosic structure of the biomass to hydrolysis. This biodecomposition can be facilitated by extra-cellular enzyme from species such as *Phanerochaete chrysosporium*, *Trichoderma reesei* and *Aspergillus niger*, which are large producers of delignification enzymes, i.e. peroxidases. Therefore, by opting to use enzymes from the similar species, minimisation of chemical compound usage in pre-treatment can be achieved.

2.1.2 Cellulose

In lignocellulose biomass, cellulose is another major constituent forming plant cell walls. It consists of linear polysaccharide polymers which are linked by β – 1.4 glycosidic bonds. Cellulose is well known as a polymer of glucose, since it consist interlinked molecules of glucose. Generally, the properties of cellulose are directly proportional to the degree of polymerization, with its polymeric chains ranging from 10,000 to 15, 000 units (Agbor *et al.*, 2011). During pretreatment cellulose is extracted from lignocellulosic biomass and further hydrolysed using enzymes to breakdown it into fermentable sugars. The enzymatic degradation of cellulose involves enzymes that will subsequently decouple linkages and bonds of cellulose to fermentable sugars. Enzymatic hydrolysis is based on the decomplexation of bonds by β-1−4-endoglucanases and β-1−4-exoglucanases to yield cellobiose which is further converted to glucose by β-glucosidases (Mansfield *et al.*, 1999). The endoglucanases hydrolyse amorphous and soluble by-products of cellulose. This phase reaction is facilitated by the decleavage of β-1-4-glycosidic bonds and oxidative degradation of the polysaccharides which release fermentable sugars. While the next phase of reaction involves cellobiohydrolases (CBHs), which have an ability to degrade cellulose from both reducing and non-reducing ends. Whereby, terminal (final) reaction is facilitated by β-glucosidase to catalyse the hydrolysis of cellobiose residues to glucose. Therefore, to completely degrade biomass in to fermentable sugars, a cocktail of enzyme is required to complete the hydrolysis of lignocellulosic biomass in a single pot system. However, cellulases have been used for decades to facilitate such a hydrolysis process, since the cocktail contains a variety of enzymes; albeit cellulases are not sufficient to complete the whole process.

2.1.3 Hemicellulose

Hemicellulose is the second constituent prevalent in plant cell walls, consisting of heteropolymers such as pentoses (xylose, arabinose units), hexoses (glycosegyalactose, mannose units) and xylan (xylose, arabinose, glucuronic acid units), with their characteristics being associated with β-1,4 D-xylose polymers bonds linked by β - D - Xylopyranose units (Manavalan *et al.*, 2017). Therefore, xylan is the major constituent of hemicellulose. However, hemicellulose in biomass is differentiated in composition, e.g. straw and grasses, are mainly composed of xylose, while softwood contains a large quantity of glucomannan (Wang *et al.*, 2013). Hemicellulose has an added advantage, as it is hydrolysable through enzymatic hydrolysis than cellulose and lignin, due to its amorphous structure characteristics. Recent studies have shown that the hydrolysis of xylan to xylose is facilitated by xylanases, exo-xylanases, endo-xylanases, and β-xylosidases, which can be found in natural plants extracts (Manavalan *et al.*, 2017). For the complete hydrolysis of xylan, requires a combination of xylanases and β-xylosidases including α-larabinofuranosidases, carboxylesterases (acetyl xylan esterases), feruloyl esterases and α-glucuronidases. While the carboxylesterase catalyse the cleavage of acetyl substituents from acetylated xylan and α-L-arabinofuranosidases hydrolyze the glycosidic bonds (Yang *et al.*, 2017).

Table 2.1: Lignocellulosic biomass composition from different renewable resources [adapted from (Sun and Cheng, 2002) and (Szymańska-Chargot et al., 2017)]

Lignocellulosic biomass source	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Stem/Leaves			
Leaves (yard waste)	15–20	80–85	0
Hardwood stems	40–55	24–40	18–25
Softwood stems	45–50	25–35	25–35
Grasses			
Coastal Bermuda grass	25	35.7	6.4
Grasses	25–40	35–50	10–30
Agro-waste			
Wheat straw	30	50	15
Cotton seed hairs	80–95	5–20	0
Nut shells	25–30	25–30	30–40
Corn cobs	45	35	15
Sugarcane bagasse	30.2	56.7	13.4
Rice straws	39.2	23.5	36.1
Orange peels	14.46	11.93	2.17
Apple peels	8.81	5.44	2.98

2.2 Pre-treatment methods for lignocellulosic biomass

Pre-treatment of biomass is one of the essential steps required to achieve high hydrolysis of biorefinery feedstock for the production of value added products in biorefinery operations (Taherzadeh and Karimi, 2008). It enables the delignification and thus the exposure of cellulose and hemicellulose for effective hydrolysis with minimal energy consumption, to achieve maximum fermentable sugar recovery (Limayem and Ricke, 2012). Several methods have been used to remove the recalcitrant lignin in lignocellulosic biomass depending on the feedstock being used. Some of these methods have been determined to be unfeasible and uneconomical due to several technical challenges such as yield and the production of inhibitory by-products such as furfural from xylose and hydroxymethyl furfural (HMF) in addition to residual phenolics including organic week acids (Kumar *et al.*, 2009a).

These delignification methods include physical (milling), thermal (hot water), chemical (dilute acid, caustic) and microbial based processes (Mood *et al.*, 2013). Acid pretreatment is associated with the formation of toxic inhibitors during the delignification process (Agbor *et al.*, 2011; Taherzadeh and Karimi, 2008); although, it is still the preferred pretreatment method for industrial applications to date. Acid either as in- or or-ganic can be used in concentrated or diluted forms to disintegrate the rigid lignin structure of the biomass. Numerous acids are used for such a pre-treatment strategy, i.e. sulphuric acid (H_2SO_4), hydrochloric acid (HCl), phosphoric acid (H_3PO_4) and nitric acid (HNO_3) (Du *et al.*, 2010; Menon and Rao, 2012). However, dilute sulphuric acid is the most commonly used, due to its suitability for the pre-treatment of a wide variety of biomass (Maddox *et al.*, 2000; Menon and Rao, 2012). According to Menon and Rao (2012), an effective design for the pre-treatment of biomass can include both acid and sequential alkaline pre-treatment method to obtain, a semi-purified hemicellulose and cellulose final product.

The industrial practice of delignification of lignin from wood has been practised using the Kraft process for paper production. The Kraft process is used to convert wood to wood pulp that consists of cellulose fibres. It is also well known as the process that removes 95% of lignin from woody biomass. This type of process is based on the digestion of wood chips at high temperature and pressure in an alkaline solution. The delignification of the wood chip is normally conducted in digesters, categorised into either batch or continuous digesters. Some recent studies have shown positive results culminating from the delignification of lignocellulosic biomass in an alkaline solution, whereby cellulosic content above 70% from the woody feedstock was achieved, with increased delignification, while retaining the structural integrity of cellulose and hemicelluloses (Cheng *et al.*, 2012). This can improve enzymatic hydrolysis by cellulases (Taherzadeh and Karimi, 2008).

Since alkaline pre-treatment is limited to the biodegradation of lignin, acid hydrolysis has been found to be more effective for both delignification with partial distortion and destabilization of the cellulose and hemicellulose structure, culminating in improved TRS availability subsequent to enzymatic hydrolysis (Lan and Liao, 2013). Due to this, dilute acid hydrolysis is the most widely used pretreatment method of lignocellulosic biomass as a feedstock in the biorefinery industry (Guo *et al.*, 2012). In certain instances, this type of pretreatment requires high temperature, short thermal retention times, which has similar outcomes as long pretreatment retention times is required at low temperature (Taherzadeh and Karimi, 2008). Furthermore, dilute acid treatment although preferred, sometimes it can results in incomplete delignification of biomass, requiring, supplementary pretreatment methods.

For the development of an environmental benign operation, biological delignification using suitable enzymes, can be used as one of the key process selection variables which is suitable to limit environmental pollution due to its low process cost, while effectively delignifying the recalcitrant lignin structure to exposed cellulose and hemicelluloses (Balat, 2011).

2.2.1 Physical pretreatment methods

The advantage of this type of pretreatment methods is that it does not cause toxic by-product formation during the treatment process, though process cost can escalate depending on the method chosen.

2.2.1.1 Mechanical/Milling

Milling and size reduction of lignocellulosic biomass is one of the major steps used to increase the digestibility of lignocellulosic material, by enhancing the accessible surface area of the biomass while, reducing the degree of polymerization including cellulose crystallinity (Sun and Cheng, 2002), resulting in enhanced enzymatic hydrolysis. Most biomass or feedstock has to be milled prior to pretreatment; although, due to intensity energy requirements in milling, input costs must be minimised (Kim *et al.*, 2013a), as the energy consumption is directly dependent on the milling including size reduction equipment used. Milling equipment types include ball, two-roll, hammer, colloid, and vibro energy mills (Taherzadeh and Karimi, 2008). Since lignocellulosic biomass comes as either dry or wet material, different types of equipment can be used depending on the lignocellulosic biomass moisture content, with colloid mills, fibrillator and dissolver being among appropriate equipment used for wet biomass, while roller, cryogenic, hammer and extruder mills are usually used for dry materials. The ball mill is the only equipment that can be used for both dry and wet materials. Kim *et al*. (2013) have studied the

effect of three different milling modes (i.e. ball, attrition and planetary mills), reporting that a milling process has an ability to reduce toxic compounds thus inhibition by-products formation, which can add value to downstream processes such as saccharification and fermentation (Kim *et al.*, 2013a).

2.2.1.2 Irradiation

Irradiation pretreatment of biomass include the use of gamma rays, electron beam, ultrasounds and microwaves. This type of process has been determined to be unsuitable due to its energy intensivity and exposure of personnel to harmful rays, which can culminate in undesirable clinical outcomes, such as cancer. The use of an electromagnetic field produces microwaves which are transferred directly to the biomass to heat the biomass with a reduced thermal gradient, thus reduced processing time (Zheng *et al.*, 2014). The advantage of the irradiation processes is the rapidity to heat a large volume of biomass within fewer periods, which can reduce biorefinery input cost.

Moretti *et al.* (2016) have used microwave irradiation in aqueous system for the pretreatment of sugarcane bagasse and straw, culminating in a high yield of TRS, i.e. 250.9 mg/g from straw and 197.4 mg/g from bagasse (Moretti *et al.*, 2016).

2.2.2 Chemical pretreatment methods

Lignocellulosic biomass treatment methods include alkaline/caustic, acidic and autohydrolysis methods, with each having varying degrees of success; although some have disadvantages as highlighted in the subsequent sections.

2.2.2.1 Alkaline /Caustic

Since pretreatment of biomass is required to improve enzymatic hydrolysis (Kim and Han, 2012; Park and Kim, 2012), pretreatment using an alkaline/caustic solution to breakdown the lignin can be achieved using sodium hydroxide (NaOH), ammonia recycle percolation (ARP), ammonia fibre explosion (AFEX), aqueous ammonia soaking (AAS) and lime ($Ca(OH)_2$), i.e. chemicals globally seen as being readily available, inexpensive and reliable. This pretreatment method is effective at low temperature and pressure. Since alkaline pretreatment utilizes less water, less energy consumption as compared to dilute acid pretreatment during and post pretreatment, lower enzyme loading is therefore required for enzyme hydrolysis, which lowers the overall cost of the biorefinery process (Rawat *et al.*, 2013). Recent

studies have demonstrated the efficacy that is provided by lignocellulosic biomass pretreatment with alkaline solutions. However, the use of NaOH has been reported in significantly more research outputs than other caustic chemicals. Wang *et al.* (2010), has studied alkaline pretreatment of coastal Bermuda grass, focusing on the optimization of enzyme loading for the hydrolysis of pretreated biomass, analytical determining that 71% improvement in the yield of TRS can be achieved using NaOH; an indication of the effectiveness of alkaline pretreatment using NaOH (Wang *et al.*, 2010). An alternative to NaOH, is $Ca(OH)_2$, which when used under optimum conditions, it can achieved results similar to those observed by NaOH (Wang *et al.*, 2012b).

Since ammonia pretreatment processes are although categorised as ARP, AFEX and AAS the primary goal of these three processes is also to breakdown lignin, destabilize and swell hemicellulose to increase digestibility. In ARP process, lignocellulosic material is pretreated in a packed-bed flow-through type reactor, to obtain a high degree of delignification (Kim and Lee, 2005). The pretreatment is facilitated by the reaction of ammonia with lignin, whereby the ammonia cleave to the bonds between the lignin and hemicellulose, i.e. C-O and C-C bonds, respectively, as well as ester and ether groups in the lignin-based structure of the lignocellulosic biomass (Yoon *et al.*, 1995). Generally, ARP facilitate reactions that are influenced by temperature, ammonia concentration, reaction time and the volume of liquid drawn in the output stream of the system, a demonstration reported by Kim and Lee (2005) using corn stover in a flow-through column reactor, with the liquid throughput, including reaction temperature being determined to highly contribute to an increase in process costs; although, 70 to 85% of the lignin was delignified.

AFEX is another of numerous methods used in alkaline pretreatment processes of biomass, which are used on an industrial scale for producing TRS from lignocellulosic feedstock. The AFEX process operates using aqueous ammonia at the temperature range between 90 to 100°C, with pretreatment time ranging from 15 to 30 min under pressure (2 kPa), subsequent to a depressurisation procedure to vaporise the ammonia, some of which can be simply recovered and recycled for reuse in subsequent pretreatment cycles (runs) (Bals *et al.*, 2011), to reduce process input costs. During this type of pretreatment process, four major cofactors are classified as having an influence, i.e. ammonia loading, concentrated water loading, residence time and operating temperature (Taherzadeh and Karimi, 2008).

Similarly AAS, can be used, with its advantage being the ammonia without significant deterioration of carbohydrates in the biomass treated, while operating at ambient temperature and pressure (Kim *et al.*, 2009). AAS was determined to be effective when low lignin containing biomass is used.

2.2.2.2 Hydrogen peroxide (H_2O_2)

Hydrogen peroxide is also one of the widely used chemical used in pretreatment methods to release TRS from the lignocellulosic biomass. H_2O_2 has been historical used as a bleaching agent of pulp, paper and wood (Gould and Freer, 1984). Furthermore, the use of hydrogen peroxide has an added advantage, as its residue in biomass, decomposes into oxygen and water (Diaz *et al.*, 2013). Moreover, the use of hydrogen peroxide treatment culminates in the in-signification production of furfural or HMF (inhibitory biomass degradation products) after pre-treatment (Taherzadeh and Karimi, 2008). Gould and Freer (1984) demonstrated that by using this alkaline peroxidation pretreatment method, wheat straw and corn stover can be converted easily to TRS with an excellent yield close to 100%. These findings demonstrated that wheat straw hemicelluloses were nearly completely solubilised by the (H_2O_2); although, under restricted and well desired conditions. Furthermore, the overall efficiency of pretreated of other biomass was arranged at 50 to 90%, respectively in particular for corn cobs, stalks and husks, with an indication that the pre-treatment pH played a vital role during the process. Similarly, Cheng *et al*. (2012), reported on the suitability of using this method using agricultural residues, i.e. rice straw and sugarcane bagasse, subsequent to fermentation using the ABE process. For bioalcohol production, Diaz *et al*. (2013) also showed an elevated conversion of rice hulls to TRS, indicating desirable results of 77% improvement in sugar extraction using minute (7.5%) quantities of H_2O_2, under a pH of 11.5, 90°C with the maximum treatment time being 2 h. Comparative TRS extraction analysis was done between lime and H_2O_2 in the pretreatment of bagasse, with a higher TRS yield of 691 mg/g when H_2O_2 was used at 25 °C and 7.35% (v/v) of H_2O_2, than when lime was used (Rabelo *et al.*, 2011).

2.2.2.3 Ozonolysis

For ozonolysis pretreatment, lignocellulosic biomass is pretreated with a large quantity of ozone (O_3), which is also an oxidizing agent commonly used in the paper and pulp industry as a bleaching agent (Barros *et al.*, 2013). During this process, lignin and partial degradation of hemicellulose can be achieved (Taherzadeh and Karimi, 2008), at ambient temperature and atmospheric pressure (Barros *et al.*, 2013; García *et al.*, 2011; Karunanithy *et al.*, 2014a; Panneerselvam *et al.*, 2013a), which culminates in the destruction of the aromatic rings in lignin (Bensah and Mensah, 2013), with minimal production of inhibitory compounds (Panneerselvam *et al.*, 2013b). Due to its low energy intensitivity, makes the process attractive for industrial scale applications, some challenges still remain unresolved with one being the large quantity of ozone required; although some studies have indicated that ozone can be generated onsite (Panneerselvam *et al.*, 2013b).

2.2.2.4 Dilute In/Organic acids

As previously discussed, acid pretreatment can be completed with a mild or a strong acid, with sulphuric acid being the widely used acid for biomass pretreatment at both laboratory and industrial scale. Some studies have reported pretreatment using acids such as a HCl, phosphoric, nitric and acetic acid (Idrees *et al.*, 2013), with operating conditions for acid pretreatment being either at high temperature and low concentration or vice versa. High acid concentration pretreatment (e.g. 30 to70%) is undesirable, due to its corrosivity particularly that of inorganic acids, which effectively increase process designed input capital costs, since specific material of construction for equipment would be required (Taherzadeh and Karimi, 2008); an indication that dilute acid pretreatment is preferable at an industrial scale.

For some studies dilute acid hydrolysis was carried out at an acid concentration of 2 to 5% (v/v), at a 140 to 190°C (Agbor *et al.*, 2011). The advantage of this type of process is that it can be used as the pretreatment or direct treatment to hydrolysed lignocellulose to valuable TRS for fermentation (Rodrigues *et al.*, 2010), with a high yield associated with the conversion of xylan to xylose, which increases production of some desired products such as bioalcohols (Sun and Cheng, 2002). This is facilitated by the primary reaction that takes place during the acid hydrolysis process, i.e. conversion of hemicellulose (Hendriks and Zeeman, 2009); although, the presence of sulphurous species as residue from the use of H_2SO_4 can be disadvantageous (Taherzadeh and Karimi, 2008). Generally, hemicellulosic constituents can be readily hydrolysed by dilute acids under moderate conditions, but much more extreme conditions are needed for the hydrolysis of cellulose.

Idrees *et al.* (2013) have demonstrated that the optimization of pretreatment of lignocellulosic biomass into TRS, is achievable, even when other acids are used, i.e. phosphoric and maleic acid, have demonstrated a higher hydrolysis yield as comparable to when sulphuric acid was used; although, most research studies largely focused on sulphuric acid (El-Zawawy *et al.*, 2011; Idrees *et al.*, 2013; Ranjan *et al.*, 2013; Ranjan and Moholkar, 2012). By varying and selectively choosing an appropriate acid concentration, treatment times, including other process conditions, desirable results can be achieved, culminating in less toxic hydrolysates that can be obtained, a key parameter for a successful fermentation in biorefinery operations.

2.2.3 High temperature and pressure pretreatment methods

2.2.3.1 Steam explosion (autohydrolysis)

Steam explosion pretreatment of biomass has received some attention in the biorefinery industry. Steam explosion can remove most of the lignin and expose hemicellulose such that it is available for improved enzymatic hydrolysis. This process operates at a high temperature 160 to 260°C and a high pressure between 7 to 5 MPa; whereby, the biomass is exposed to such conditions momentarily, to decouple and weaken lignin bonds, which exposes hemicelluloses to enzymatic biocatalysis to TRS. The disadvantage of this type of pretreatment is its generation of inhibitory compounds such as furfural, acetic acid, formic acid and levulinic acid (Oliva *et al.*, 2003). The toxicants generated during the pretreatment process can have an effect on the hydrolysis during hemi- and cellulose conversion and the subsequent fermentation, due to the production of inhibitors; although, the effect of inhibitors can be reduced by diluting the hydrolysate containing TRS using a high volume of water, which effectively reduce the saccharification yield.

2.2.3.2 Carbon dioxide (CO_2) explosion

For CO_2 explosion pretreatment, supercritical carbon dioxide (SC–CO_2) is used under pressure (7 to 28 Mpa) to delignify lignocellulosic biomass (Agbor *et al.*, 2011) albeit at low temperature, i.e. 35 to 80°C (Zheng *et al.*, 1995). The main advantage of using supercritical carbon dioxide is that, it does not generate toxic by-products, while adding/increasing acidity during the pretreatment process. The effect of the SC–CO_2 is such that penetration into the pores of the lignocellulosic biomass is achievable, culminating in the significant exposure of hemicellulose and cellulose to other biocatalylic agents, thus, effective enzymatic hydrolysis for TRS extraction.

2.2.3.3 Liquid hot-water (LHW)

Heating of the lignocellulosic biomass using hot water forms part of hydrothermal processes, which are used for the pretreatment of biomass, a process that has been used in the pulp industry (Taherzadeh and Karimi, 2008). The experimental design of liquid hot water pretreatment process is similar to that of steam (expansion) pretreatment, the temperature for the LWH pretreatment being 160 to 240°C, without synthetic chemical supplementation. This method has attracted the biorefinery industry since it has an ability to recover pentose's, while achieving a high cellulose enzymatic digestibility (Gao *et al.*,

2013; Hongdan *et al.*, 2013; Wang *et al.*, 2012a; Yu *et al.*, 2013), with minimal corrosive /acidification and inhibitory compound production being observed, effectively rendering this process an environmentally benign process.

2.2.4 Hybridised pretreatment methods

2.2.4.1 Microwave-chemical

The microwave pretreatment biomass, although widely used for the treatment of lignocellulosic biomass to extract TRS for biofuel production, it has been found to have similar outcomes to that observed in conventional heating (high temperature) process (Barros *et al.*, 2013). The application of microwaves generates heat which increases disruption of the lignin structure with the additional use of chemicals, slightly improving the efficacy of the delignification process than the conventional heating method used (Karunanithy *et al.*, 2014b).

2.2.4.2 Organosolv

The Organosolv pretreatment process uses organic or aqueous organic solvent solutions to improve the delignification of lignin in the biomass being pretreatment. During the process the lignin structure and some of hemicellulose degrade, with the removal of some of lignin including destabilisation of hemicellulosic constituents to release TRS (Koo *et al.*, 2011), at 150 to 200°C in the absence or presence of a catalyst or without chemical compounds. Numerous chemicals such as sulphuric acid, sodium hydroxide, magnesium chloride including acetone have been used (Park *et al.*, 2010a).

2.2.4.3 Wet oxidation

For wet oxidation pretreatment of biomass, water and air or oxygen are used at both a high temperature (150 - 320°C) and pressure (5 - 20 MPa) using in an aqueous phase saturated with oxygen (Wang *et al.*, 2007). Furthermore, due to the type of reactions taking place, i.e. a high-temperature oxidative reaction, the generation of inhibitors occurs, which culminates in hydrolysate purification process requirements thereafter (Martin *et al.*, 2007).

2.2.5 Biological pretreatment of lignocellulosic biomass

Microorganisms can also be used for the pre-treatment of lignocellulosic biomass by degrading lignin, exposing the cellulose for effective hydrolysis; a process facilitated by organism such as white-rot, brown-rot, and soft-rot fungi including some bacterial species. Recent studies have shown that white-rot fungi are the most effective microorganisms for biomass delignification (Narayanaswamy *et al.*, 2013; Taherzadeh and Karimi, 2008), as the fungi has an ability to completely biodegrade the lignin, a process facilitated by several ligninolytic enzymes, such as extracellular enzymes, lignin peroxidases, manganese peroxidases, and laccases (Kamei *et al.*, 2012). These enzymes are produced by fungi such as *Phanerochaete chrysosporium, Trametes versicolor, Phlebia radiata* and *Pleurotus ostreatus*. However, lignin and manganese peroxidases are predominantly produced by *P. chrysosporium*. Similarly, brown-rot fungi also have an ability to degrade cellulose and hemicellulose, without degrading the lignin during the process (Rasmussen *et al.*, 2010); although recoverable TRS might be lower as the organisms utilize the bioconverted hemicellulose and cellulose for growth.

2.2.5.1 Digestive enzyme from organisms isolates from termites (Isoptera)

Many research studies have been conducted on the biological pretreatment of lignocellulosic biomass using digestive termites gut organisms to biodegrade the lignin in lignocellulosic biomass. Termites have digestive systems that biodegrade lignin in soft and hard woody biomass (Sethi *et al.*, 2013). As termites live in trees, they feed on the recalcitrant lignocellulosic biomass as a primary food source, utilizing gut symbionts to facilitate lignin biodelignification (Ohkuma, 2003), a process facilitated under anaerobic conditions, which is unimplemented, i.e. at an industrial scale. However, some recent studies are still focusing on the elucidation of processes facilitated by termites gut symbionts, in particular termites gut biodecomposition of lignin biomass, a process which is still less understood (Ni and Tokuda, 2013). Therefore, alternatives are required, in particular for aerobic process development.

2.2.5.2 Digestive enzymes from *Nepenthes mirabilis* (pitcher plant extract)

Pitcher plants are well known as carnivorous plants. These plants include the *Nepenthes* species. Most pitcher plant species produces an acidic fluid, with a pH ranging from 1.5 to 6 depending on the species, a fluid which facilitates the decomposition of living and dead organisms or materials which come into direct contact with the acidic fluid (Takeuchi *et al.*, 2011). Although numerous studies have been

conducted to profile the constituents of such acidic fluids produced by different species of pitcher plants; as there is minimal information on pitcher plants acidic fluid (extract) usability in novel biorefinery processes (Takeuchi *et al.*, 2015), and their ability to facilitate the biodegradation of lignin and holocelluloses in biomass. However, recent studies have clarified some previously unknown information, indicating that a diverse and complex enzymatic cocktail does exit with a high concentration of digestive / hydrolytic enzymes, from variety of microorganisms within the pods of *Nepenthes* sp. The type and activity of the digestive enzymes including whether they can be used in biodelignification process of biomass for a biorefinery, is still unclear (Takeuchi *et al.*, 2015). Table 2.2 enlist a summary of the classification of different biomass pretreatment process, including their limitations.

Table 2.2: Classification of lignocellulosic biomass pretreatment processes

Classification	Processes	Comments	Reference
Physical	Milling	The disadvantage of milling is the high energy required for operation	(Hideno *et al.*, 2009)
	Irradiation	Less pollution due to minimal or no solvent usage in the process	(Saini *et al.*, 2015)
Chemical	H_2SO_4	Degradation of biomass with less energy consumption, minimal processing period and lower temperature. Residual toxicity production observed	(Alvira *et al.*, 2010)
	HCL	This type of process requires high energy to operate, which is costly. Inhibitory by-products formation observed.	(Maurya *et al.*, 2015)
	HNO_3	This type of process has been extensively researched for decades and it has high operational cost, with toxicant production, which inhibits cellulolysis	(Kim and Lee, 2005)
	NaOH	This type of pretreatment has been significantly researched more than other chemicals	(Wang *et al.*, 2010)
	$Ca(OH)_2$	Similar outcomes to NaOH process, with the cost of the chemical and energy consumption being identified a draw back for the process	(Kaar and Holtzapple, 2000)
	Ammonia	The process is able to recover ammonia for reuse, which makes the process feasible for continuous industrial pretreatment process	(Agbor *et al.*, 2011)
	H_2O_2	Its disadvantage is the cost operation associated with H_2O_2 procurement	(Diaz *et al.*, 2013)
Physico - chemical	Microwave	Uses high pressure and temperature, which make the process not feasible for industrial scale operation, due to high cost.	(Li *et al.*, 2016)
	CO_2		
	Steam explosion	Minute chemical usage and it has high energy requirements	
	Wet oxidation	Oxidative delignification cost are the disadvantage of the process, which are higher than those used in alkaline pretreatment processes	(Sun *et al.*, 2016)
	Ionic liquid	The drawback of the process is the toxicity and the costs of the solvent.	(Clough *et al.*, 2015)
Biolog ical	plant extracts	More work has to be done on the organisms from termites, anaerobic conditions required therefore diffident to develop large scale process.	(Ni and Tokuda, 2013)
	Microbial extracts	Minimal significant research has been done until present	(Chan *et al.*, 2016)

CHAPTER 3

LITERATURE REVIEW

CHAPTER 3

3. LITERATURE REVIEW: PART 2: Kinetic parameter evaluation and value added bioproducts from total reducing sugars in biomass pretreatment hydrolysate fermentations

3.1 Biofuels as an energy source

Biomass is seen as the primary source of energy in developing countries. It is normally used as charcoal for cooking, wood, agro-residues (for fire), lighting, and heating. Due to disforestation, biofuels such as bioethanol, biobutanol, biodiesel and other semi-modified vegetable oils; although used sparingly, can play an insignificant role as a source of energy. Furthermore, due the energy security concerns, biofuels are considered as an alternative energy source globally. However, due to the energy security crisis, biobutanol global demand has increased to above 1.2 billion gallons per year from renewable biomass (Pereira *et al.*, 2018). The primary renewable biomass comes from waste lignocellulosic biomass form the processing of agricultural products such as corn, wheat, sugar cane, beet sugar and etc. For an example, Brazil has been utilizing sugar cane waste for more than 30 years as a primary feedstock for ethanol production in first generation biorefineries (Mariano *et al.*, 2013). Biofuels can be also be produced from biomass or animal fats through a variety of biological and thermochemical processes. The bioalcohols including other products obtained from these processes have similar attributes to those obtained from fossil fuels (i.e. petroleum). The characteristic similarity of these products, gives them an advantage over fossils fuels as they can be produced using readily available feedstock, infrastructure and equipment, which is currently available in the market. Since, these bioproducts, some of which are marketed as biofuels, can be used in numerous consumer goods, instead of petroleum products without equipment modification including the utilization of existing petroleum based distribution systems. Therefore, they are advocated for, as an acceptable alternative energy source, to meet energy needs of the populace globally. Table 3.1 list some of the common biofuels and /or their constituents, highlighting the physical characteristic and /or properties which make them suitable as an alternative energy source.

3.2. Production of bioproducts from agro-waste through process integration

Lignocellulose biomass contributes a large fraction of municipal solid waste (MSW). Its constituents include agricultural residue, animal manure, wood, and wood-based pulp process residue (Hughes *et al.*, 2013; Ziemiński *et al.*, 2012). Such biomass can be converted into biofuels through two different processes, i.e. biochemical and/or thermo-chemical conversion, though four major essential steps such as pre-treatment, hydrolysis and fermentation and recovery of the bioproducts must be performed

(Cheng *et al.*, 2012). In most instances, to reduce the cost of lignocellulosic biomass beneficiation, a co-culturing system is used which has the potential to be cost-effective, as the consolidation of bioprocesses can reduce both infrastructural (fixed) and indirect (variable) operational costs (Nakayama *et al.*, 2011). The production of bioproducts from cellulosic feedstock has been limited with single feedstock, which results in uneconomical process development thus an unfeasible biorefinery. Since, biofuels such as biodiesel, bioethanol and biogas can be produced from agro-waste, to mitigate against increases in the price of crude oil and increases associated with low petroleum availability and prospecting for new petroleum sources, renewal bioresources provides a suitable alternative (Naik *et al.*, 2010). Therefore, an integrated process in which the utilization and adequate processing of cellulosic feedstock for sustainable process development can be achieved would thus culminate in economic gains including cost savings, whereby mixed agro-waste can be utilized as useful feedstock for the production of multiple products.

3.2.1 First generation biorefinery

A first generation biorefinery use a single feedstock to produce single products such as bioethanol or biodiesel. In this type of biorefinery, feedstock such as corn starch or sugar cane are used as the primary constituents for cellulose and hemicelluloses, including readily fermentable sugars; however, these types of feedstock are also used in food production (Hughes *et al*., 2013). As previously mentioned, the challenge with first generation biorefineries is related to food security concerns.

3.2.2 Second generation biorefinery

Similar to first generation biorefinery, second generation biorefineries are also based on the utilisation of a single feedstock, using pre-treatment technologies such as thermo-chemical and biological conversion processes to produce a range of different bioproducts and marketable co-products. To improve the first and second generation biorefinery concept, the development of a third generation biorefinery was introduced to minimised the impact of using feedstock targeted for food production in particular focusing on the reduction of environmental pollution while benefiting from available, renewable bioresources in particular agro-waste. By definition, a third generation biorefinery is the most advanced type than the other two biorefineries. This is based on the production of a variety of products using a diverse array of biomass raw material (Timung *et al.*, 2015). Overall, lignocellulose biomass from agro-waste has been identified as the most suitable and least expensive abundant form of biomass, for use in this type of biorefinery. However, the selected of agro-waste, will be large dependent on

regionally available feedstock, depending on the largest agricultural and /or commercial active agro-processing facilities. Recent studies have illustrated the development of the second generation biorefinery not only with simulation, but with validated experimental data (Dias *et al.*, 2012).

3.2.3 Third generation biorefinery

This biorefinery concept attempts to apply the methods that have been applied to the refining of petroleum (Demirbas, 2009; Hughes *et al.*, 2013), with a focus on the production of multiple products such as bioalcohols, non-alcoholic, bio-based chemicals and heat from pretreated multiple and/or mixed feedstock using multiple processes; which provides for viable options in a bio-economy (Demirbas, 2009). Furthermore, the added benefits and/or possible of power generation thus energy harvesting or production, would allow this biorefinery to utilize the bioproducts to sustain heating requirements for other processes in the refinery, culminating in reduction of overall operation costs.

In general, the primary objective of a second generation biorefinery is to optimize the use of resources while minimizing waste generation; thereby, maximizing benefits and its overall profitability. Biomass waste, generated in large quantities from several agricultural sub-sectors, such as forestry, agriculture and by the municipality and many others, can be harvested for this type of biorefinery. To minimise landfill usage, as most are reaching their maximum capacity, necessitates the development of alternative disposal and diversion strategies including the use of such waste as the primary feedstock for a biorefinery. This would facilitate resource recovery, recycling or beneficiation of waste materials using appropriate processes with intended outcomes being, to commodify the products produced while achieving environment protection (Taherzadeh and Karimi, 2008). For this study, environmentally benign process design would be suitable, while proclaiming bioproducts such as biobutanol, due to its high calorific value, i.e. energy density.

Generally, bioethanol is the most wildly produced commodity from a biorefinery and it used as an additive in the production of biofuel for motor vehicles and has rapidly grown in the global market. However, the challenge associated with bioethanol is its lower energy content, which culminates in more interest being on similar bioalcohols such as biobutanol. Biobutanol has demonstrative abilities as a biofuel, with its production being favourable due to its high energy content, which can derive higher monitory value for the benefits of the biorefinery industry. Similarly to bioethanol, biobutanol is compatible with the current infrastructure used in the petroleum industry. Table 3.1 list some properties of some biofuels and bioalcohols which are used as additives to augment fossil fuels.

Table 3.1: Properties of some biofuels and alcohols which are used as additives to augment fossil fuels

Properties n-	Biodiesel	Diesel	Gasoline	Methanol	Ethanol	n-Butanol
Molecular formula	C_{12}-C_{22}	$C_{12} – C_{25}$	$C_4 – C_{12}$	CH_3OH	C_2H_5OH	C_4H_9OH
Boiling point (°C)	182-338	180-370	25-225	64.5	78.4	117.7
Density at 20°C (g/ml)	0.86-0.89	0.82-0.86	0.7-0.8	0.7966	0.7851	0.8098
Solubility in 100 g of water	Immiscible	immiscible	Immiscible	miscible	Miscible	Immiscible
Energy density (MJ/L^{-1})	32.6	35.86	32	16	19.6	27-29.2
Auto-ignition temperature (°C)	177	~210	~300	470	434	385
Heat of vaporization (MJ/kg)	-	0.27	0.36	1.2	0.92	0.43
Specific heat capacity Cp at 20°C (kJ/kg.K)	-	1.75	2.22	2.54	2.47	2.40
Flash point (°C) in closed cup	100-170	65-88	-45 to -38	12	8	35
Cetane number	48-65	40-55	0-10	3	8	25
Research octane number	136	129	91-99	-	-	96
Motor octane number	-	-	81-89	104	102	78
Octanol/Water partition Coefficient (as logPo/w)	-	~3.3	3.52±0.62	-0.77	-0.31	0.88
Stoichiometric air/Fuel ratio (wt./wt.)	13.8	14.3	14.7	6.49	9.02	11.21
Latent heating (kJ/kg) at 25°C	-	270	380-500	1109	904	
Flammability limits (%vol.)	-	1.5-7.6	0.6-8	6.0-36.5	4.3-19	
Saturation pressure (kPa) at 38°C	-	1.86	31.01 -	31.69	13.8	582 -
Viscosity (mm2/s) at 40° C	1.9-6.0	1.9-4.1	0.4-0.8 (20°C)	0.59	1.08	1.4-11.2

3.3 Motivation to produce high energy content bioalcohols

Biobutanol has been produced since the early 20th century, using acetone, butanol, and ethanol (ABE) fermentation process. During this time, the primary product was acetone from starch with the demand of acetone becoming high just after World War 1 (Weizmann, 1915). Industrial scale ABE process were performed in USA, Russia, and South Africa until the early 1980s. However, China has currently developed an advanced industrial scale ABE process that has the capacity to produce more that 1 million ton of ABE fermentation products and by-products annually (Ni and Sun, 2009). On the other hand bioethanol through fermentation from biomass has been conducted for centuries using the yeast, *Saccharomyces cerevisiae*. Additionally, recent studies reported that United States of America and Brazil are the first and second largest producers of bioethanol in the world respectively for the automotive industry (Sonego *et al.*, 2016).

Nowadays, biobutanol produced from lignocellulosic biomass has gained popularity over bioethanol from industry. The advantage of biobutanol over bioethanol is that the biobutanol has high energy content and a lower heat of evaporation, which makes it easier to blend with gasoline in higher ratios. Currently in the world the top producers (such as Cobalt Biofuels, Cathay Industrial Biotech, Gevo, Butamax, and Green Biologics) of bioethanol are conducting intensive research on the biobutanol, while retrofitting the existing bioethanol plant to suite the biobutanol production (Huang *et al.*, 2014). However, most existing biobutanol plants currently use corn as feedstock, which competes with human food and animal feed requirements. Therefore, many researches have been developed on the non-food lignocellulosic biomass as an alternative feedstock for biobutanol plants. Table 3.2 illustrate a comparative analysis for biobutanol and biobutanol, which illustrates that biobutanol, might be preferable, if it is added to other petroleum based products.

Table 3.2: Properties of Biobutanol and bioethanol

Properties	Biobutanol	Bioethanol
Molecular Weight (g/mol)	74.12	46.1
Density (kg/m^3)	809.8	789
Energy density (MJ/L	29.2	19.6
Air fuel ratio	11.2	9
Research octane number	96	129
Motor octane number	78	102
Boiling temperature (^oC)	117.7	78.37
Lower heating value (MJ/kg)	34.37	26.95
High heating value (MJ/kg)	37.33	29.85
Heat of evaporation (MJ/kg)	0.43	0.93

3.4 Fermentation for alcohols: History

3.4.1 Bioethanol production on an industry scale

Bioethanol has been a promising alternative fuel among other biofuels such as biobutanol and biodiesel. Currently, global production of bioethanol was approximately 100 billion cubic meters; whereby the majority was produced by the United States, Brazil and China (Lopes *et al.*, 2017). This rapid growth of bioethanol demand is influenced by the depletion of fossil fuels and the introduction of vehicles with dual fuel technology systems that can utilized bioethanol as a fuel (Zabed *et al.*, 2016). On an industrial scale, bioethanol has been produced from edible sources (sugars and starch) and nowadays lignocellulosic biomass, which has led to lessened attention (Gupta and Verma, 2015). Traditionally, bioethanol has been produced using *Saccharomyces cerevisiae* strains. Recent research reports, have developed alternative non-food feedstock for bioethanol production (Bayrakci and Koçar, 2014). Lignocellulosic biomass has been considered as a cheap renewable raw material that contains the cellulose and hemicellulose that are required for production of added value products. Although, recent studies have developed methods for utilization of agro-waste biomass, there is still less studies that are focusing on agricultural and forest waste in combination with a suitable biological method of pre-treatment. However, South Africa is one the leading countries in terms of agricultural produce

production such citrus fruit, corn and apple, while it has many forestry residues including trees that have an ability to generate waste for more than 500 years. However, abundant lignocellulosic biomass require prior steps of pretreatment to degrade the recalcitrant structure to release the fermentable sugars (Govumoni *et al.*, 2013). Numerous reports on pretreatment are available but the challenge with inhibition still prevails (Chaturvedi and Verma, 2013). Therefore, more alternative methods are required to overcome these challenges (Zabed *et al.*, 2016).

3.4.2 Biobutanol production under anaerobic conditions

Historically biobutanol production was facilitated by *Clostridium* sp. which has been used for fermenting reducible sugars under anaerobic conditions. In early 1912, a strain identified as *Clostridium acetobutylicum* was isolated for the production of acetone, using an ABE (Acetone-butanol-ethanol) fermentation process, a process developed by C. Weizmann at Manchester University (Jones and wood, 1986). During this time (i.e. 1912), acetone was in demand, with its intended use being the production of smokeless nitrocellulose explosives (cordite), while the biobutanol was being used as the primary solvent for the production of rubber. However, the challenge of producing biobutanol by an ABE fermentation process using *Clostridium* sp. is due to the alcohols toxicity and its inhibition of the fermenter used and requirements associated with anaerobic conditions requirements (Al-Shorgani *et al.*, 2015a). Generally, biobutanol becomes toxic to the fermenter, at a concentration of 13 to 20 g/L (Jones and Woods, 1986). The ABE fermentation process is sub-divided into two phases, i.e. acidogenic and solventogenic phases, with the acidogenic phase, facilitating the fermenters cell growth at an exponential growth, while the primary carbon source is fermented to acetate and butyrate, with the subsequent solventogenic phase facilitating the conversion of the acidic extracellular bio-products to acetone, biobutanol and bioethanol. To limit bioproduct inhibition, an in-situ biobutanol recovery system can be used. Other species know to produce biobutanol under anaerobic conditions include *Clostridium beijerinckii* (Huang *et al.*, 2014) and *Bacillus* sp. under aerobic conditions (Ng *et al.*, 2015).

3.4.3 Recent development on the biobutanol production under aerobic conditions

Recently, studies have developed new methods to produce biobutanol under aerobic conditions, and to improve the tolerance of the fermenter to high biobutanol concentration. Organisms such as *Escherichia coli*, *Saccharomyces cerevisiae* and *Bacillus subtilis* utilize glucose or lignocellulosic biomass hydrolysates as the carbon source. *Bacillus* sp. has been shown to have an ability to produce biobutanol and

bioethanol under aerobic conditions, with a 12.3 g/L titer being achieved (Ng *et al.*, 2015). This was achieved under a controlled pH. However, *Bacillus* sp. has shown also an ability to operate under both anaerobic and aerobic condition; however, under anaerobic conditions, lower biobutanol titers were achieved, which indicated that the strain (*Bacillus* sp.) preferred aerobic conditions.

3.5 Microbial growth, substrate consumption and product formation kinetics

3.5.1 Biomass growth kinetics

3.5.1.1 Monod equation

Various microbial growth rate kinetic models had been used to characterise the cell concentration in fermentation processes. Furthermore, the studies have shown that the growth rate of microbial biomass is directly proportional to the concentration of the microbial cells. Numerous mathematical models and theories have been developed for many decades to further explain the relationship between the specific growth rate of cells and substrate consumption and limiting substrate for biomass growth. The first model was firstly proposed by Malthus (1798) for a first-order growth model, which has been used to quantify the exponential growth phase of microbial cells. Therefore, the mathematically model can be expressed as:

$$dX/dt \propto X \tag{3.1}$$

Hence,

$$\frac{dX}{dt} = r_x = \mu, \tag{3.2}$$

Therefore,

$$\frac{dX}{dt} = \mu X \tag{3.3}$$

Where, X, describe the cell concentration in colony-forming units/mL (CFU/mL) and determined as an increase over specific period; dX/dt is the growth rate (CFU L^{-1} h^{-1}); μ is the specific growth rate (h^{-1}); while t is the time (h). In late 1940's, a model was developed to verify the relationship between the cell growth rate and substrate concentration, where Monod equation was discovered as followed (Monod, 1949):

$$\mu = \frac{\mu_{max} S}{K_S + S} \qquad 3.4$$

Where, μ_{max}, is the maximum growth rate (h^{-1}) for specified substrate and K_S is estimate half saturation constant (g/L) i.e., (when $\mu = ½\ \mu_m$), while S is the substrate concentration (g/L).

However, half saturation constant K_S illustrate how quick the specific growth rate (μ) can progress from minimum to its maximum specific growth rate (μ_{max}). Furthermore, the Monod model is well-known to be applicable, when is assumed that the toxic metabolic products has no functional role during the fermentation.

3.5.1.2 Logistic model (LM)

Unstructured kinetic models had been used for quantifying the cell growth concentration during fermentation, which considers the total biomass concentration as a single component. Therefore, in this study, an unstructured model that describes the microbial growth, i.e. the logistic kinetic model - Eq. 3.5, can be an alternative model to use.

$$\frac{dX}{dt} = \mu_{max} X \left(1 - \frac{X}{X_{max}}\right) \qquad 3.5$$

Where, X is the biomass concentration (CFU/L), X_m is the maximum biomass concentration (CFU/L), μ_{max} is maximum specific growth rate (h^{-1}). Biomass proliferation can further be expressed as a mathematical function by integrating Eq. 3.5 with initial conditions of t = 0, $X = X_0$ – see Eq. 3.6.

$$ln\frac{X}{(X-X_{max})} = \mu_{max}t + ln\frac{X_0}{(X_{max}-X)} \qquad 3.6$$

Therefore, after rearrangement the biomass concentration can be estimated as in Eq. 3.7.

$$X = \frac{X_0 e^{\mu_{max}t}}{\left(1-\left(\frac{X_0}{X_{max}}\right)\right)(1-e^{\mu_{max}t})} \qquad 3.7$$

3.5.1.3 Leudeking–Piret model (LP)

The Leudeking–Piret model (1959) specifically describes the relationship between cell growth and product formation rates- Eq. 3.8.

$$\frac{dP}{dt} = \alpha\frac{dX}{dt} + \beta.X \qquad 3.8$$

Where, α is the growth associated formation coefficient of the product while β is the non-growth associated formation coefficient of the product. The initial term of Eq. 3.8 (i.e. $\alpha\frac{dX}{dt}$) illustrates that the cells growing produce the product in proportion of their growth, while the second term (i.e. β) illustrate the cell growth is proportional to substrate concentration regardless of the growth phase (Dhavale *et al.*, 2016).

The mathematical representation of the product formation using Luedeking-Piret model was developed by substituting Eq. 3.8 into Eq. 3.7 followed by integration with initial conditions of t = 0: P = 0, X = 0, culminating into Eq. 3.9.

$$P = P_0 + \alpha X_0\left(\frac{exp(Kt)}{1-\left(\frac{X_0}{X_{max}}\right)(1-exp(Kt))} - 1\right) + \beta\frac{X_{max}}{K}ln\left(1-\left(\frac{X_0}{X_{max}}\right)(1-exp(Kt))\right) \qquad 3.9$$

3.5.1.4 Substrate consumption model

Theoretically, the substrate consumption is directly proportional to biomass generation and product formation. Therefore, in this study substrate consumption were evaluated using Luedeking–Piret model and can be describe as follows Eq. 3.10 (Dhavale *et al.*, 2016):

$$-\frac{dS}{dt} = p\frac{dX}{dt} + qX \qquad 3.10$$

Where, p = 1/ $Y_{x/s}$ (g substrate/g biomass), while q is the maintenance coefficient (1/h). Therefore, Eq. 3.11 can be rearranged into Eq. 3.11.

$$-dS = pdX + q\int X(t)\,dt \qquad 3.11$$

Therefore, the specified substrate was determined by substituting Eq. 3.3 into Eq. 3.6 and integrating the final equation with initial conditions of t = 0, S = S_0, resulting in Eq. 3.12.

$$S = S_0 - pX_0\left\{\frac{e^{\mu_{max}t}}{\left\{1-\left(\frac{X_0}{X_{max}}\right)(1-e^{\mu_{max}t})\right\}} - 1\right\} - q\frac{X_{max}}{\mu_{\max}}ln\left\{1-\left(\frac{X_{max}}{\mu_{max}}\right)(1-e^{\mu_{max}t})\right\} \qquad 3.12$$

3.6 Biobutanol recovery systems

The production of biological products such as bioethanol and biobutanol has culminated in the marketability of renewable biofuels. Recent studies have shown that biobutanol has beneficial attributes, thus its suitability in renewable biofuel production. Some microorganisms that produce biobutanol are susceptible to biobutanol toxicity, thus their inhibition during fermentation, a consequent resulting in low yields, requiring appropriate and innovative process designs, i.e. for in-situ biobutanol recovery. Historically, distillation has been traditionally used for the recovery of biobutanol, with the primary disadvantage being its energy usage; a recovery method unfavourable in low energy intensity biorefineries.

Recent studies have shown different processes (liquid-liquid extraction, gas striping, pervaporation, adsorption and vacuum separation), which can be used to recover biobutanol from fermentation broth

(Rohani *et al.*, 2015). All these methods are well known for their low input costs, due to minimal usage of chemicals and low energy requirements. The main focus of this study is to use a green and environmentally benign process system for recovery.

3.6.1 Liquid-liquid extraction (LLE)

LLE is a process that is used for separation of components in a liquid mixture using an immiscible solvent to recover a dissolved solute resulting in liquid-liquid phase separation. In the ABE fermentation liquid-liquid process, extraction is also used for separation of bioproducts from ferment broth (Ezeji *et al.*, 2004; Vane, 2008; Xue *et al.*, 2014; Xue *et al.*, 2013). LLE is more favourable for biobutanol recovery than for bioethanol, due to biobutanol being more hydrophobic; hence, less miscible with water than bioethanol. For the LLE process to be effective, the fermentation broth and extractant can be directly in contact through mixing or in packed columns, with the use of membrane separation technology to separate the two phases. The latter procedure is often referred to as perstraction (Ezeji *et al.*, 2007a; Vane, 2008). The employment of membranes in perstraction to separate the two phases is to minimise challenges usually associated with traditional liquid-liquid extraction system, including emulsion formation, loss of extractant, and the transfer of biomass from the broth to the extractant phase (Ezeji *et al.*, 2007b). Thereafter, the extractant is enriched with bioalcohols. These alcohols must be recovered in a regeneration unit in order to achieve the desired product quality and recovery of the extractant for reuse in subsequent recovery and recycle cycles. Common extractant regeneration methods include: distillation, vacuum evaporation, and pervaporation (Ezeji *et al.*, 2004; Vane, 2008). For this process to be effective, suitable solvents must be selected based on numerous factors that are applicable to the process.

3.6.2 Gas stripping

Gas striping is one of the most favourable methods that are used for the recovery of biobutanol. Gas stripping is a process whereby a gas is passed through a liquid phase to mobilize the product of interest. After the gas has passed through a liquefied medium, it is further passed through a condenser to recover the products in the gas phase into a liquid form. Packed or tray columns are normally used for gas stripping operation. The sustainability of this method is due to its low energy requirements and it is not affected by fouling and clogging (Ezeji *et al.*, 2003). The design of a gas stripping unit is such that it has a recycling stream to recover all of the solvent in the gas phase; although, single-pass systems are

also used. In this process fermentable off gases, CO_2 and H_2 and/or sparging using inert gasses such as nitrogen gas can be used as carries gases (Vane, 2008). This design can be used without using fermentation processes with a recycle.

The primary principle behind gas stripping is such that a counter current operation, of the carrier gas to fermentation broth would takes place; whereby, the alcohols ratio to inert gas is a strong function of the stripping temperature and partial pressure of the volatile compound to be recovered, i.e. alcohol or water, with the partial pressure of the gas being described as in Eq. 3.13:

$$P_i = y_i P_{Total} = x_i \gamma_i P_i^{Sat} \qquad 3.13$$

Where: P_i - is the partial pressure of the gas phase, yi_i and x_i - are the fractions of components in the gas and fermentation broth, respectively, γ_i - is the fermentation broth-phase activity coefficient, P_{Total} - is the total pressure of the gas phase, and P_i^{Sat} - is the saturated vapor pressure of bioproducts, i, at operating temperature.

Overall, saturation vapour pressure can be determined by the variation of temperature; whereby, an increase in temperature will increase the P_i^{Sat}, thus affect the P_i in the gas carrier phase (Vane, 2008). Foaming during gas stripping can affect the performance of a gas stripping unit, particularly because fermentation broth is known to foam, a problem which can be addressed using antifoams. Foams can affect the mass transfer, culminating in high gas flow rate requirement in the system. Table 3.3 list fermentation processes in which gas stripping operation was used.

Table 3.3: Removal of butanol using gas stripping

Fermentation process (conditions)	Strain used	Biomass	Yield (g/g)	ABE Product (g/L)	Butanol Production (g/L)	References
Batch (Anaerobic)	*C. Saccharoperbutylacetonicom N1-4*	Algae hydrolysate	0.270	16.0	10.4	(Tan *et al.*, 2013)
Batch (Anaerobic)	*C. beijerinckii P260*	Corn stove	0.28	16	10.4	(Qureshi *et al.*, 2010)
Fed-Batch(Anaerobic)	*C. Acetobutylicum*	Glucose	0.36	195.9	150	(Xue *et al.*, 2012)
Batch (Anaerobic)	*C. Acetobutylium JB200*	Glucose	0.25	532.3	420.3	(Xue *et al.*, 2013)
Batch (Anaerobic)	*C.Asscharobutylicum N1 – 4*	Pam oil	-	2.09	0.9	(Al-Shorgani *et al.*, 2015b)
Batch (Anaerobic)	*Acetobutylicum YM1*	Glucose	-	-	12.94	(Al-Shorgani *et al.*, 2015b)
Batch (Anaerobic)	*C. beijerinckii*	Sugar cane bagasse	0.16	11.9	6.4	(Su *et al.*, 2015)
Batch (Anaerobic)	*C. beijerinckii*	agro-industrial wastes	0.239		9.3	(Maiti *et al.*, 2016)
Batch (Anaerobic)	*C. Asscharobutylicum DSM 13864*	Corn stove	0.33	-	7.9	(Ding *et al.*, 2016)

3.5.3 Recover pervaporation

Pervaporation is a basic membrane technique used for the separation of alcohols (Richardson *et al.*, 2007), using membranes such as that made from polydimethylsiloxane (PDMS), with the solvents passing through the lumen side of the membrane, while the fermentation broth being interacted with membrane, facilitating the rejection of biomass (Ezeji *et al.*, 2007a). Since, the fermentation broth has different chemical and physical properties, the selectivity of the membrane material, is a determining factor (Rohani *et al.*, 2015).

In recent years pervaporation process has been intensively studied for bioethanol and biobutanol recovery from water and fermentation broths (Cai *et al.*, 2016). Samantha and Ray, (2015) studied the bioethanol recovery from water using pervaporation using a mixed matrix copolymer membrane, constituted with butyl acrylate and styrene and organophilic clay filler, retaining high flux, and bioethanol selective recovery (Samanta and Ray, 2015). Similarly, Shin et al. (2015) highlighted critical issues of biobutanol recovery from continuous ABE fermentations with high cell densities (Shin *et al.*, 2015), maintaining a flux of 1643 and 941 g/m^2h using polystyrene-b-polydimethylsiloxane-b-polystyrene (SDS) and polydimethylsiloxane (PDMS) membranes, respectively, corresponding to volumetric productivity between 0.94 and 0.66 g of ABE/L h. Similar results were obtained by Rozicka et al. (2014) with high separation efficiency. However, it must be included that the two membranes used, are selective towards biobutanol and non-selective to other constituents in water, i.e. acetone and water–bioethanol mixtures, which represent the complexity of the fermentation broth (Rozicka *et al.*, 2014).

3.5.4 Perstraction separation

From the literature review conducted, it is that membrane assisted solvent extraction, also referred to as membrane solvent extraction, can be used for bioproducts recovery. However, the extractant in this type of process does not get in contact directly with the fermentation broth (Abdehagh *et al.*, 2014). In the perstraction separation, the solvents diffuse through the membrane, while the extractant and fermentation broth are separated by another membrane (Huang *et al.*, 2014). Some of the extractant rejected by the membrane during the process are left in the fermentation broth. The challenge with this process is membrane fouling and clogging (Abdehagh *et al.*, 2014; Ezeji *et al.*, 2007b; Groot *et al.*, 1990; Zheng *et al.*, 2009), which leads to high membrane costs, since membrane must be periodically replaced. Qureshi and Maddox (2005) have investigated the application of perstraction to enhance butanol

production, while the oleyl alcohol as the perstraction solvent, achieving low flux values (Qureshi and Maddox, 2005).

3.6.5 Recovery using adsorption

Adsorption as an alternative process to distillation, absorption and liquid extraction, to recover desired components from a fermentation broth, as it is seen as efficient than other process in terms of needed energy requirements as seen in Table 3.4 during the process.

Table 3.4: Different energy required for different butanol recovery [Adapted from (Ruthven and Ching, 1989)]

Recovery System	Energy Requirements (MJ/kg) for Biobutanol Recovery
Distillation	24
Gas Stripping	22
Pervaporation	14
Extraction/Perstraction	9
Adsorption	8

In the adsorption process, molecules moves from the bulk fluid to the solid side of an adsorbent material used. Different types of adsorbents can be used for biobutanol recovery. However, the material must be hydrophobic and hence less miscible with water, during the fermentation process. Zeolite and silicate materials are much more preferable for adsorption processes, for biobutanol recovery, due to the zeolite structure with a very high SiO_2/Al_2O_3, that is also hydrophobic (Abdehagh *et al.*, 2014).

In 1982 Maddox studied biobutanol recovery using adsorption process, with silicates as adsorbents, whereby the concentration of butanol in fermentation broth was 11.7 to 16.8 g/L, obtaining 41 to 96 % of recovery of biobutanol from fermentation broth, with adsorption quantified between 64 to 85 biobutanol in mg/g of adsorbent.

Similarly, Faisal *et al.* (2014) has indicated that zeolite with high content of silica to alumina ratio, are effective in the adsorption of biobutanol and butyric acid, with biobutanol having a much higher affinity for zeolite than the adsorbent material. However, broth kinetic parameters must be known for rapid recovery, as this would determine loading rates (Abdehagh *et al.*, 2014).

3.7 Process integration of bioalcohol production

The primary objective of the integration process is to combine processes such as pretreatment, hydrolysis, and fermentation into single process, while using lignocellulosic biomass as a suitable feedstock to reduce the high costs related with the biorefinery system. In biorefinery processes, multiple enzymes that can do multiple functions in parallel in a one pot operation are required to facilitate the process (Hailes *et al.*, 2007). The advantage of the one pot operation will be the reduction of unit operation and cost of the operations. This can be only feasible, if the multiple enzymes can come from one source, meaning they were functioning in the same source even prior to utilization in another process. This means that the enzyme will function effectively with the similar conditions of the original source, which can add value on the functionality of the enzymes. The enzyme effectiveness in pretreatment can be examined in the one pot operation, assessing their ability to degrade the biomass and reduces toxic by-products.

CHAPTER 4

MATERIALS AND METHODS

CHAPTER 4

4. MATERIALS AND METHODS

4.1 Lignocellulosic biomass feedstock: mixed agro-waste

4.1.1 Collection and preparation

Feedstock, i.e. agro-waste constituted with *Citrus sinensis, Malus domestica* peels, cobs from *Zea mays* and *Quercus robur* (oak) yard waste, was collected from an agricultural produce market in the vicinity and the garden of the Cape peninsula University of Technology (CPUT), District 6 campus (Western Cape, Cape Town, South Africa) respectively, subsequent to drying at 80°C for 24 h and pulverization (>45µm to <100µm) without a pre-rinsing step. The *C. sinensis* peels were further re-dried for 48 h and subsequently re-pulverized. These agro-waste were used as mixed agro-waste feedstock, using a 1:1 ratio, i.e. 25% (w/w) for each; 1g *C. sinensis,* 1g *M. domestica*, 1g *Z. mays* cob and 1g *Q. robur,* since minimal is known about the biodelignification of such mixed agro-waste, with most research studies focusing on the use of a single feedstock. The dried agro-waste (2 g) was slurried in sterile distilled water (sdH_2O, 200 mL) using airtight multiport Erlenmeyer flasks (250 mL, triplicate) fitted with a sampling syringe, corresponding to a 1% (w/v) slurry (Cheng *et al.*, 2012), at ambient temperature (25 to 30°C) in a shaking (120 rpm) incubator (LABWIT- ZWY-240, Shangai Zhicheng Analytical, Shanghai-China), to ensure homogenization. The pH was measured using a pH meter (Lasec Pty Ltd, Ndabeni, SA), at different stages of the experiments.

4.2 Mixed agro-waste lignin and holocelluloses content

The lignin and holocelluloses content of the mixed agro-waste was determined for unpre-treated and *N mirabilis* pretreated samples with further pre-treatment using commercial cellulase in comparison to pretreated biomass. The solids samples from the pre-treatment steps were dried at 80°C in an oven for 24 h to reduce the moisture content (Dhavale *et al.*, 2016). Thereafter, the samples were cooled to ambient temperature and stored under dry conditions. The analyses were carried-out by slurring 300 mg of dried pretreated agrowaste in a 100 mL Schott bottles, with 1 mL of 72 % of H_2SO_4, subsequent to agitation with a glass rod until the agro-waste was homogenously mixed (Mansouri and Salvadó, 2006). Thereafter, the Schott bottles were place at 30±0.5°C using a water bath for 1 h. To further treat the samples, 28 mL of sterile distilled water (sdH_2O) was added subsequent to autoclavation at 121°C for 30 min. The solution was cooled to 80°C and filtered with the pre-weighed fibre glass filter (Garnier and Gaillet, 2015). The filtrates were transferred into 50 mL conical tubes and the acid-soluble lignin was

determined at 205 nm using a Jenway 7305 UV/Vis spectrophotometer (Cole-Parmer, UK), whereby the extinction coefficient of lignin (110 g/L.cm, according to TAPPI UM 250) was used to quantify the acid-soluble lignin using Eq. 4.1.

$$ASL = \frac{A.D.V}{a.b.M} \tag{4.1}$$

Where, A is the absorption at 205 nm, while D is the dilution factor, V is the volume of the filtrate (i.e. 0,029 L), a is the extinction coefficient of lignin (i.e. 110 g/L.cm, according to TAPPI UM 250), b is the cuvette path length, in cm (i.e. 1 cm) and M is the oven-dry weight of sample (i.e. as 100% dry matter) before acid digestion.

Furthermore, the residues were washed with hot water and a mixture of ethanol, benzene and ether until the residues were contaminant free (Dhavale *et al.*, 2016). The residue were dried at 105°C in the oven for 2 h, to quantify the total lignin content as the sum of both acid soluble and insoluble lignin. The residues on the fibre glass filter papers were transferred to crucibles and calcined at 700°C for 1 h in a furnace (Labofurn furnace, model EMF260, Cape Town) to quantify total ash determined by the mass difference of the ash contend of acid-insoluble lignin and ash content of the filters without the residues. This method was applied to all samples in triplicate. The total ash content was determined according to Eq. 4.2.

$$Ash(\%) = \frac{M_c}{M_a} \times 100 \tag{4.2}$$

Where, Ash (%) is the percentage by mass of ash, M_c is the ash mass (mass difference between crucible filter with residue, crucible with control filter and empty crucible), and M_a is the mass of the dry sample.

4.3 Inhibitory compound quantification: total residual phenolic compounds (TRPCs)

The analyses were conducted by transferring 5 mL of the slurried mixed agro-waste in to 15 mL conical tubes subsequent to centrifugation at 4000xg for 5 min with a volume (3 mL) of the recovered supernatant being diluted with an equivalent volume (3 mL) of sdH_2O in clean 15 mL conical tubes, while the remaining volume (1 mL, undiluted) was used for total reducing sugar analyses (section 4.5.4). Thereafter, the following analyses were periodically conducted during the pre-treatment process taking into account the dilution factor.

Phenolic compounds in agro-waste are known as inhibitors of β-glucosidase (Kim *et al.*, 2011), which is primarily responsible for facilitating the hydrolysis of oligosaccharides to fermentable sugars (Singhania *et al.*, 2013). Therefore, TRPCs were quantified using the Folin-Ciocalteu method (Makkar *et al.*, 1993), with a volume (100µL) of the diluted supernatants being added to an assay mixture containing sdH_2O (1.5 mL) and the Folin-Ciocalteu reagent (250 µL) subsequent to the addition of sodium carbonate (1 mL, 20% w/v) after 3 min; thereafter, the assay mixture was homogenised in darkness for 1 h. A volume (1 mL) of the assay mixture was then analysed using plastic cuvettes (1.5 mL) at 650 nm using a Jenway 7305 UV/Vis spectrophotometer (Cole-Parmer, UK), with the TRPCs concentration being determined using 2 to 10 mg/L of 1,2-dihydroxybenzene in sdH_2O for the calibration curve (Baba and Malik, 2015; Lavate *et al.*, 2013).

4.4 Pre-treatment of the mixed agro-water using hot water, dilute acid and cellulases

4.4.1 Hot water hydrolysis of the mixed agro-waste

Biomass (2g, mixed agro-waste) was treated with hot water at a high temperature (120°C) using an autoclave for 15 min in 250 mL Schott bottles, whereby 200 mL of distilled water was added, Most studies have shown hot water treatment to be effective at temperature between 120 to 200°C (Ko *et al.*, 2015). The challenge in pretreatment process for hot water hydrolysis is high energy consumption, which leads to high process cost implications. Therefore, the temperature (120°C) was selected since it was the minimum allowable and optimal temperature condition in aiming to reduce energy consumption. The mixture was cooled to ambient temperature. Aliquots (3 mL) were sampled into sterile 15 mL conical tubes, subsequent to centrifugation at 4000x g for 5 min to recover sedimented agro-waste biomass. A volume (1 mL) of the sample was diluted with 9 mL of sterile distilled water (sdH_2O) for TRS analysis. A volume (2 mL) of the withdrawn sample was returned to the Schott bottle. The recovered mixed agro-waste pellets were dried and kept at ambient temperature for further analyses for structural modification determination using Fourier Transform Infra-red Spectroscopy (FTIR) and powder X-ray diffraction (XRD) systems.

4.4.2 Dilute acid hydrolysis of the mixed agro-waste

The recovered hot water pre-treated biomass (agro-waste) was further treated with diluted sulphuric acid (1% v/v) in a batch system (Taherzadeh and Karimi, 2008), in triplicates at 121°C for 15 min (Ko *et al.*, 2015); the mixture was analysed for TRS before proceeding to enzymatic hydrolysis, using a

procedure as reported in section 4.4.1.

4.4.3 Enzymatic hydrolysis of the mixed agro-waste

Similarly after diluted sulphuric acid pre-treatment, the agro-waste was further treated with cellulases (24.67 U/mL). A volume of cellulases, 600 µL per g of mixed agro-waste, was added to the reaction mixture to further enhance pre-treatment outcomes at 55°C and pH 4.5 for 72 h, a pH was attained using sodium acetate buffer. Thereafter, the filtrate obtained was used for TRS analysis using proceedings in section 4.4.1.

4.4.4 Confirmatory identification of the commercial yeast used for fermentation

The genomic DNA (gDNA) extraction was performed according to the protocol analogous to that described in the Zymo Research, Catalogue No. D6005. DNA was extracted from the 24 h YPD pure yeast culture using the ZR DNA Kit (Zymo Research, Catalogue No. D6005, UK). The ITS target region was amplified using One Taq Quick-Load 2X Master Mix (NEB, Catalogue No. M0486), using primers ITS1-5'-TCCGTAGGTGAACCTGCGG-3' and ITS2-5'-TCCTCCGCTTATTGATATGC-3', with repeated sequencing using forward 27F-5'-AGAGTTTGATCMTGGCTCAG-3'and reverse 1492R-5'-GGTTACCTTGTTACGACTT-3' primers (Nimagen, Brilliant Dye Terminator Cycle Sequencing Kit V3.1, BRD3-100/1000) to ascertain correctness in the identification of the isolate (Zymo Research, Catalogue No. D4001, UK). The PCR products (i.e. extracted fragments) were run on a gel and a gel extraction with the Zymoclean™ Gel DNA Recovery Kit was performed thereafter. PCR was conducted in 100 µL reactions, while 100 ng of gDNA was used (Ng *et al.*, 2015). The PCR conditions were set-up as 98°C denaturation cycle for 30s, followed by 36 cycles of 98°C denaturation for 30s, primer annealing at 60°C for 20s, and elongation at 72°C for 60s. PCR products were further gel extracted (Zymo Research, Zymo Clean™ Gel DNA Recover kit), and purified (Zymo Research, ZR DNA sequencing clean-up kit catalogue D4050) while the resultant extracts were sequenced (forward/ reverse direction). Thereafter, analysis on the ABI PRISM 3500xl Genetic analyser ensued. The PCR products were further purified using Zymo Research, ZR-96 DNA Sequencing Clean-up kit (catalogue No D6006) and analysed using a CLC main workbench. Thereafter, the sequences generated were compared with available nucleotide sequences in NCBI Genbank database (http://blast.ncbi.nlm.nih.gov/Blast.cgi), for confirmatory identification of the *S. cerevisiae* strain used, with an accession number KT32652.1 being assigned (Altschul *et al.*, 1997).

4.4.5 Fermentation inoculum preparation and yeast cell counts

The yeast (10 mg) was grown in a medium containing 100mL of Yeast Extract-Peptone-Dextrose (YPD) broth (i.e. yeast extract, 10g/L; peptone, 20g/L; dextrose, 20g/L), incubated for 24 h at 30°C. The yeast was further cultured on a Potato Dextrose Agar at 30°C for 48 h. Colonies were further streaked out onto other PDA petri-dishes to ensure the purity of the yeast used for inoculum. The inoculum was prepared by pure freshly grown yeast cultures, with numerous colonies being picked to inoculate 5 mL YPD broth which incubated for 24 h at 30°C. To further prepare the fermentors, 100µL of the overnight (24 h) YPD-yeast cultures, were inoculated into individual flasks containing 50 mL of the hydrolysate from different hydrolysis methods and incubated for 72 h at 30°C in a shaking incubator (LABWIT- ZWY-240, Shangai Zhicheng Analytical, Shanghai-China) at 120 rpm. All fermentations were done in triplicate.

The quantification of yeast cell counts was performed using a MediXgraph CFU Scope v1.5 software (free trial version) used for the quantification of colony forming units (CFU/mL) on agar plates. The software is used as a mobile application for rapid and semi-automated CFU determination under a controlled environment (Sánchez-Femat *et al.*, 2016). Furthermore, the Optical Density (OD) of the yeast cultures during fermentation was determined using a Jenway 7305 UV/Vis spectrophotometer (Cole-Parmer, UK) at 600nm as a further method to ascertain kinetic parameter accuracy; albeit this method also detects biomass which is unculturable (dead). All cell counts and OD measurements were done in triplicate.

4.5 *Nepenthes mirabilis* extracts

The *Nepenthes* plants are carnivorous that use specialized pitfall trap, i.e. pitcher, which traps inserts and decompose organisms that are unbreakable by fibrous chitin and filled with protein (Lee *et al.*, 2016). Most pitcher plant species produces an acidic fluid, with a pH ranging from 1.5 to 6 depending on the species (Takeuchi *et al.*, 2011). Although numerous studies have been conducted to profile the constituents of such acidic fluids produced by different species of pitcher plants; there is minimal information on pitcher plants acidic fluid (extract) usability in novel processes (Takeuchi *et al.*, 2015), and their ability to facilitate the biodegradation of lignin in biomass. However, recent studies have addressed some previously unknown information, indicating that a diverse and complex enzymatic contain does exit with a high concentration of digestive / hydrolytic enzymes, from variety of microorganisms (Chan *et al.*, 2016).Therefore, Chan *et al*. 2016, reported that the biodegradation of the insert includes complex chitinolytic, proteolytic, amylolytic, and cellulolytic and xylanolytic activities.

Therefore, *N. mirabilis* plant was selected due to its availability in the Western Cape, South Africa, Southeast Asia and other tropical regions.

4.5.1 Collection and preparation

Pooled extracts from *N. mirabilis* plants grown in a greenhouse under controlled conditions, were collected from Pan's Carnivores Plant Nursery (21 Kirstenhof, Tokai, Cape Town, SA). The extracts were collected using sterile 50 mL conical tubes, and immediately stored on ice, prior to transportation to the laboratory, whereby they were centrifuge at 4000xg for 15 min and filter sterilised with 0.22µm millipore membranes (Isopore™, Massachusetts, USA) with subsequent storage at 4 °C prior to use, i.e. without dilution or the use of a buffer. About 15 to 35 mL of the extract was collected per "monkey cup" of the *N. mirabilis* plants, depending on the size of the cup.

4.5.2 *N. mirabilis* extracts characterisation

4.5.2.1 Physico-chemical characteristics of the *N. mirabilis* extracts

The physicochemical characteristics of the *N. mirabilis* extracts such as pH, conductivity, specific gravity and redox potential, were determined using a multi-parameter meter (Eutech Instruments Pte Ltd/ Thermo Fisher Scientific, Singapore) as highlighted in Table 4.1. The *N. mirabilis* plants are characterized by leaves modified into pitcher traps, which can attract, capture, and digest insect prey, while producing digestive enzymes (Bazile *et al.*, 2015). The common *Nepenthes* sp consist of three vital sections, i.e. a slippery upper rim, waxy inner wall and bottom pit for attracting and trapping prey and prevent prey from escaping, while the acidic fluid digest the trapped prey (Lee *et al.*, 2016)..

Table 4.1: Physico-chemical characteristics of the *N. mirabilis* extracts

Characteristic/Parameter	Values (units)
pH	1.80 – 2.2
Specific gravity	0.67 – 0.82
Redox potential*	510 - 526 mV
Conductivity	3.5 - 5.89 mS/cm

* A high redox potential is indication of the oxidative ability of the extracts

4.5.2.2 Microbial population identification in the *N. mirabilis* extracts

Prior to filtration, microbial population identification was initially done using a VITEK 2 systems V07:01 (BioMérieux, France) utilising Gram-negative cards (GN cards) and Gram-positive cards (GP card) as per the manufacturers' instructions (Pincus, 2005). Further identification of microbial strains in the extracts was performed using a DNA extraction method including sequencing. For DNA extraction, the cultures were cultivated by adding 1 μL of the *N. mirabilis* extracts into 15 mL glass test tubes that contained Luria broth (5 mL) subsequent to incubation (37°C) for 24 h. Thereafter, the cultures were inoculated on Luria bertani agar (LBA) plates at 30˚C for 24h, with single colonies being sub-cultured for specie purification and identification (Chan *et al.*, 2016). A staining procedure was also performed for each isolate for morphological assessments.

In this study, DNA extraction and Polymerase Chain Reaction (PCR) amplification of 16S ribosomal deoxyribonucleic acid (rDNA) was performed in an external laboratory (i.e. Inqaba biotech, Pretoria, South Africa) using the commercial genomic DNA purification kit (Zymo Research; Fungal/Bacterial DNA Kit, UK), as per the manufacturer's instructions. The genomic DNA of strain was extracted for PCR using universal bacterial primers targeting the 16S rDNA gene (Mekuto *et al.*, 2018). The DNA was assessed using 1) a 0.5% (v/v), i.e. 500 µl per 100mL of the genomic lysis buffer, while the cell disruptor were processed at maximum speed for 5 min, subsequently centrifuge at 10,000xg for 1 min in a lysis tube; 2) A volume of 400 µl supernatant of Zymo-Spin was transfer into spin filter in a collection tube and was also centrifuge at 7,000 rpm for 1 min; 3) A volume 1.2 ml of Fungal/Bacterial DNA binding buffer was added to the filtrate in the collection tube from Step 2; 4) A volume 800 µl of the mixture from Step 4 was transfer to a Zymo-Spin column in a collection tube and centrifuge at 10,000 x g for 1 min. The flow through was discard and the process was repeated; 5) A volume 200 µl DNA pre-wash buffer was added to the Zymo-Spin column and centrifuge at 10,000 x g for 1 min., and then wash w/ 500 µl

Fungal/Bacterial DNA Wash Buffer; 6). The mixture in the column was transfer to a clean 1.5 ml micro-centrifuge tube and a volume of 100 µl DNA elution buffer directly to the column matrix, subsequently to be centrifuge at 10,000 x g for 30s to elute the DNA. The PCR amplification was conducted using primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'), which were universal primes for 16S rDNA. PCR was conducted in 100 µL reaction, while 100 ng of genomic DNA was used (Ng *et al.*, 2015).The PCR conditions were set up as 98°C denaturation cycle for 30s, followed by 36 cycles of 98°C denaturation for 30s, primer annealing at 60°C for 20s, and elongation at 72° C for 60s. PCR products were further gel extracted (Zymo Research, Zymo Clean Tm Gel DNA Recover kit), while the forward and reverse sequenced primers on the ABI PRISM 3500xl Genetic analyser. The PCR products were further purified using Zymo Research, ZR-96 DNA Sequencing Clean-up kit and analysed using CLC main workbench. Thereafter, 16S rDNA were compared with available nucleotide sequences in NCBI Genbank databased (http://blast.ncbi.nlm.nih.gov/Blast.cgi), for identification of strain that are available in the *N. mirabilis* pod extracts with the following accession number KY249126.1, DQ513324.1 and KU948294.1 (Altschul *et al.*, 1997).Furthermore, the schematic diagram of PCR is shown in Fig. 4.1, with universal primers being used.

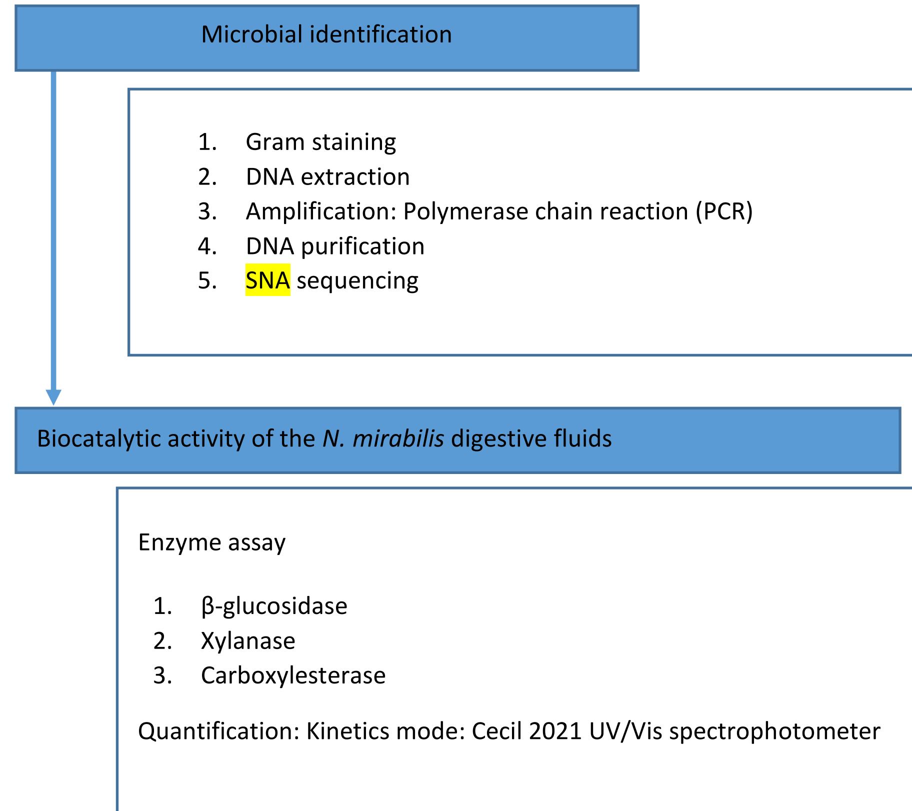

Figure 4.1: A schematic flow diagram of the microbial population identification and biocatalytic activity of the *Nepenthes mirabilis* digestive fluids

4.5.2.3 Biocatalytic activity of the *N. mirabilis* extracts

Specific enzymes were selected in order to quantify their activity in *N. mirabilis* extracts based on their efficacy to hydrolyse the agro-waste constituents including by-products formed. Enzymes of interest, 1) β-glucosidases, 2) xylanases and 3) carboxylesterases were previously identified as; 1) being an essential cellulose biodecomposing component facilitating the penultimate and a bottleneck for biocatalytic conversion of cellobiose, a reducing sugar, to glucose (Chan *et al.*, 2016), 2) having the potential to biodegrade thus solubilize hemicellulose (García-Huante *et al.*, 2017) and 3) having the potential as candidate phenolic acid esterases with a hydrolytic activity against carboxylesterase bonds between holocelluloses sugars and lignin (Manavalan *et al.*, 2017).

As such, β-glucosidase activity in the *N. mirabilis* extracts was quantified using ρ-nitrophenyl-β-D-glucopyranoside (*p*NPG) as a substrate (Marques *et al.*, 2003), with the rate of formation for xylose, a reducing sugar from xylan, also being determined from xylanase assays using xylan (Zabed *et al.*, 2016; Lopes *et al.*, 2017; Bazile *et al.*, 2015). Similarly, carboxylesterase activity was determined using ρ-nitrophenyl acetate (ρNPA) as the substrate (Ljungquist and Augustinsson, 1971; Manavalan *et al.*, 2017; Kannan *et al.*, 1998). All these assays were performed using a *N. mirabilis* extracts in an appropriately buffered mixture at ambient temperature, using a Cecil 2021 UV/Vis spectrophotometer (Lab Equipment, England) set in a kinetics mode – see Table 4.2 for enzymatic assay conditions. Overall, the activity quantified was based on the concentration of product formed per min (U/L), computed using Eq. 4.3.

Table 4.2: Enzyme activity assays for *N. mirabilis* extracts

Reagent/Parameter	1,4-β-glucosidase	endo-Xylanase	Carboxylesterase
Substrate concentration	0.35mM*p*NPG	54.2mM Xylan	0.5 mM ρNPA
Substrate volume	0.8 mL	1.8 mL	0.8 mL
Product formed	*p*NP	Xylose	*p*NP
Buffer(s) & volumes	50mMsodium acetate, pH 6, 600 µL	100mM McIlvaine, pH 5, 1600 µL	100 mM Tris-HCL, pH 7.8, 200 µL
Volume of enzyme	200 µL	200 µL	300 µL
Temperature	25°C	25°C	25°C
Wavelength	410 nm	586 nm	410 nm
Extinction coefficient (ε)	18100 $M^{-1}.cm^{-1}$	135 $M^{-1}.cm^{-1}$	17000 $M^{-1}.cm^{-1}$

$$\text{Enzyme activity (U/L)} = \left[\left(\frac{\frac{dA}{dt}.D_f}{\varepsilon}\right) \times 60 \times 10^6\right] \qquad 4.3$$

Where, dA/dt is the initial rate reaction, while the D_f is dilution factor and ε is the extinction coefficient.

4.5.3 Mixed agro-waste pre-treatment procedure using *N. mirabilis* extracts and sequential commercial cellulases hydrolysis

The pre-treatment of the mixed agro-waste followed a sequence whereby, the mixed agro-waste was slurried (see section 4.1.1) for 72 h to solubilise some of the constituents in the waste, prior to the direct supplementation of the pooled *N. mirabilis* extracts into each homogenized Erlenmeyer flasks subsequent to further (96 h) ambient temperature incubation for a total experimental time of 168 h. The furtherance of cellulolysis for the slurried agro-waste, cellulase (Sigma-Aldrich, Germany) supplementation was performed after 48 h post *N. mirabilis* supplementation for each Erlenmeyer flask i.e. sequential to the pre-treatment using *N. mirabilis* extracts, the mixed agro-waste was further treated with cellulases (Sigma-Aldrich, Germany) after the slurrification procedure and supplementation with *N. mirabilis* extracts, i.e. after an additional 48 h, at a stage whereby the experiment was at 120 h from its initiation. A volume (1200 µL) of cellulases (24.67 U/mL) was added to each flask, constituting

600 µL cellulases /g agro-waste (Shi *et al.*, 2011), without pH correction, with requisite analyses being conducted. This process was conducted in a single pot, multiple reaction system. After each intermediate pre-treatment stage, agro-waste free samples (5 mL), were collected for various analyses (TRPCs) by initially centrifuging (4000xg for 5 min) the supernatant containing agro-waste biomass using a preparatory strategy analogous to that reported in section 4.3. Thereafter, the agro-waste pellets were thoroughly rinsed with sdH_2O and air dried at room temperature, to reduce the moisture content prior to the assessment of structural modifications using Fourier Transform Infra-red Spectroscopy (FTIR) and powder X-ray diffraction (XRD) systems, for each of the agro-waste samples recovered. Control experiments with the untreated mixed agro-waste served as a reference (El-Zawawy *et al.*, 2011).

4.5.4 Determination of total reducing sugars (TRS)

The TRS produced during hydrolysis of the agro-waste were quantified by using a 3.5 dinitrosacylic acid (DNS) reagent, composed of DNS (10 g), phenol (2 g), sodium sulphite (0.5 g) and sodium hydroxide (10 g) made-up to 1 L; whereby the sample (1000 µL, prepared as highlighted in section 4.3) was diluted in 9 mL of sdH_2O. The assay mixture contained 1.5 mL of the diluted aliquots, 1500 µL DNS reagent in sterile 15 mL test tubes subsequent to heated up to 90^oC for 10 min. The assay mixture was cooled to ambient temperature, prior to the addition of a 0.5 mL of 40% (w/v) sodium potassium tartarate solution. The absorbance was determined using a Jenway 7305 UV/Vis spectrophotometer (Cole-Parmer, UK) at 575 nm. The reference was analysed using a similar procedure without the TRS containing samples, i.e. which was replaced with sdH_2O, with different glucose concentrations being used to generate a suitable calibration curve (Miller, 1959).

4.5.5 Fourier Transform Infrared Spectroscopy analyses

Functional group modifications in the mixed agro-waste was determined to determine the effectiveness of the pre-treatment method being studied, using an α-FTIR spectrometer (Bruker Pty Ltd, SA) and Smart iTR with a diamond crystal window; a mass of the un- and pre-treated mixed agro-waste samples were placed in the diamond crystal window of the Smart iTR. Initially, the measurements were taken against a background spectrum of the diamond window without the mixed agro-waste. The spectra scans were collected from a range of 400 to 4000 cm^{-1} with a spectral resolution of 4 cm^{-1} at 100 scans per min (Zeng *et al.*, 2011).

4.5.6 Powder X-ray Diffraction Analyses

To quantify crystallinity, the rinsed and air dried mixed agro-waste obtained prior and post pre-treatment, was analysed using a pXRD (Bruker Pty Ltd, SA) at 40kV and 40 mA with a D2 phaser with a Lynxeye, providing a suitable peak-to-background ratio (Qiu *et al.*, 2012). The scanning range (2θ) was 10 to 50° at a ramping scale of 0.017^{o}, using a zero background holder plates (50μm depth), with the crystallinity index (CrI) being determined using Eq. 4.4. For these analyses, 5 mg of agro-waste was used.

$$CrI(\%) = \frac{(\Delta I)}{I_{002}} \times 100 \qquad 4.4$$

Where, ΔI was $I_{002} - I_{am}$ .with I_{002} being the intensity for the crystalline portion of the agro-waste at 2θ between 21 to 22°, while I_{am} was the amorphous portion of the agro-waste at 2θ between 14 to 19° (Segal *et al.*, 1959).

4.6 Experimental data handling, computations and statistical analyses

The presence of bioalcohols was analysed using Jones and Lucas methods as described by Tojo and Fernàndez (2006). The Jones method was designed for primary and secondary alcohol determination, i.e. 1° and 2° alcohols, while the Lucas method, was developed for tertiary alcohols (2° and 3° alcohols). The analysis were conducted in 10 mL test tubes, with 10 µL of the fermented samples being added to 1 mL of acetone and 2 µL of Jones reagent, with a positive bioalcohol presence being represented by a slightly green colour. Lucas alcohols test was conducted by mixing 0.2 mL of fermented solution samples with 2 mL of the Lucas reagent in 10 mL test tubes at ambient temperature. Thereafter, the presence of bioalcohols was observed by an insoluble layer or emulsion (Tojo and Fernández, 2006). To implement the single pot multi-reaction pre-treatment process of the mixed agro-waste, a total experimental run time of 168h was implemented, with periodic sampling for various analyses at 24, 48, 72, 96, 120 and 168 h, prior to the supplementation of *N. mirabilis* extracts (72 h) and cellulases (120 h). The data generated were analysed to determine the mean value and standard error of the mean (SEM), for the raw data obtained. All experimental data were computed to take into account sample dilutions to quantify the actual concentrations for parameters monitored. Furthermore, all experimental analyses were conducted in triplicate, i.e. n = 3 samples per experiment and analyses with the SEM being determined using Eq. 4.5.

$$\boldsymbol{SEM} = \frac{\boldsymbol{Standard\ deviation}}{\sqrt{\boldsymbol{Number\ of\ sample\ tested}}} \qquad 4.5$$

For FTIR and XRD, the recovered air dried agro-waste, were pooled to attain a composite sample which was then used for analyses.

An additional quantification of the presence of bioalcohols production was determined using a modified GC-MS method developed by Rossouw and Bauer (2016). A gas chromatograph (6890N, Agilent technologies network) attached to a CTC Analytics PAL auto sampler and coupled to an Agilent technologies inert XL EI/CI Mass Selective Detector (MSD) (5975B, Agilent technologies Inc., Palo Alto, CA) operated in a full scan mode with the source and quad temperatures being at 230°C and 150°C, respectively, was used or to quantify the production of bioalcohols – i.e. bioethanol, biobutanol, phenylethyl alcohol, separation of the fermentation broth volatiles was performed on a polar STABILWAX (60 m, 0.25 mm ID, 0.25 µm film thickness) Zebron 7HG-G007-11 capillary column. Helium was used as the carrier gas at a flow rate of 2 mL/min with the injector temperature being maintained at 250°C. The sample was injected in a splitless mode. The oven temperature was maintained at 35°C for 10 min and ramped up to 240°C at a rate of 15°C/min. The transfer line temperature was maintained at 250°C with the mass spectrometer operated under electron impact mode at ionization energy of 70eV, at a scanning range 35 to 500 m/z (Rossouw and Bauer, 2016).

CHAPTER 5

Production of low (C_1 to C_3) and high carbon content (C_4^+) alcohols under aerobic conditions using total reducing sugar from mixed agro-waste

RESULTS: Part 1

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Published as: ***N. Dlangamandla***, S.K.O. Ntwampe*, J.O. Angadam, and E.F. Itoba-Tombo, B.S.Chidi, 2018. Production of Low (C_1 to C_3) and High Carbon Content (C_4+) Alcohols Under Aerobic Conditions Using Total Reducing Sugar from Mixed Agro-Waste. Paper presented at the 10th International Conference on Advances in Science, Engineering, Technology and Healthcare (ASETH-18). 19-20, November 2018, Cape Town, South Africa. Paper ID - EAP1118256.

CHAPTER 5

5. PRODUCTION OF LOW (C1 TO C3) AND HIGH CARBON CONTENT (C4+) ALCOHOLS UNDER AEROBIC CONDITIONS USING TOTAL REDUCING SUGAR FROM MIXED AGRO-WASTE

5.1 Introduction

Agro-waste has been recommended as the most promising and attractive feedstock for biorefineries and is the most abundant organic matter residue in agro-processing (Amiri *et al.*, 2015). In the biofuels industry, bioethanol and biobutanol are the most high value alcohols compared to other alcohols. In particular, biobutanol has a high energy content and is water resistant; it can be distributed through the existing petroleum pipeline system and can be used as a fuel, or supplement without modifying the current engines' system (Abdehagh *et al.*, 2016). Acetone–Butanol–Ethanol (ABE) fermentation has been the primary biological process used to produce biobutanol using agro-waste as a feedstock (Chua *et al.*, 2013). *Clostridia* species such as *Clostridium acetobutylicum* and *Clostridium beijerinckii* are the most used bacterial strains for biobutanol production under anaerobic conditions. However, the challenge with these strains is the lower biobutanol yield during fermentation, which increase the cost related with biobutanol recovery at the end of the fermentation process (Xu *et al.*, 2015). Therefore, these challenges need to be addressed in order for the biofuels industry to compete with fossil fuel processors. As a result, various engineered strains of *Clostridia* sp. have been developed to improve the biobutanol yield. Some separation methods have also been reported for the recovery of biobutanol from fermentation broth. Some studies have shown that *Escherichia coli* (E. coli) are the most suitable and easily engineered strain with *Clostridia* sp., for the improvement of biobutanol yields. More development imparted traits have been reported, such as the biobutanol production under aerobic conditions using different *Bacillus* sp. isolated from soil as reported by Ng *et al*. (2015). However, on an industry scale, commercial yeast (Saccharomyces cerevisiae) has been traditionally used for the production of alcohols (Lian *et al.*, 2014). Furthermore, specific strains are required to biodegrade the sugars from the pre-treatment of agro-waste bioalcohols, while reducing the effect of inhibitors such as acetic acid, furfural, hydroxymethyl furfural (HMF) and phenolics (Lopes *et al.*, 2017).

5.2 Objectives

This part of the study, focused on the production of different bioalcohols such as low (C_1 to C_3) and higher (C_4^+) alcohols in an integrated single pot system under aerobic conditions using a mixed agro-waste extracts as sole carbon sources, while using as commercial S. cerevisiae strain for the production of bioalcohols and other added value products for the biorefinery industry. The samples were analysed

for different bioproducts to determine the potential of the single pot system to produce added value products for the biorefinery industry.

5.3 Materials and methods

The feedstock material was collected and prepared as describe in chapter 4 (section 4.1.1), whereby *C. sinensis* peels were further re-dried for 48 h and subsequently re-pulverized. Mixed agro-waste feedstock, using a 1:1 ratio, i.e. 25% (w/w) was used with a mass (2 g) was slurried in sterile distilled water (sdH_2O, 200 mL) using airtight multiport Erlenmeyer flasks fitted with a sampling syringe, corresponding to a 1% (w/v) slurry (Cheng *et al.*, 2012), at ambient temperature in a shaking (120 rpm) incubator to ensure homogenization. The pH was measured using a pH meter, at different stages of the experiments. The hot water, diluted acid and cellulases pre-treatments were performed as discussed in chapter 4 (section 4.4). Moreover, the fermentation was conducted with hydrolysates from final pre-treatment stage (i.e. cellulases hydrolysis).

5.4 Results and Discussion

5.4.1 Effect of pre-treatment methods on TRS production

In this study, pre-selected mixed agro-waste was pre-treated with three different pre-treatment processes, i.e. hot water, dilute acid and cellulases hydrolysis. The results for TRS analyses are illustrated in Fig. 5.1, whereby the residual sugar concentration in solution for the mixed agro-waste (i.e. orange peel, apple peel, corn cob, and yard waste) is highlighted. The highest achievable actual TRS was 3.22 ± 0.38 g/L. The combined pre-treatment process in the single-pot led to the highest actual TRS conversion from the mixed agro-waste. The actual TRS for hot water pre-treatment was very low when compared to dilute acid pre-treatment and cellulases hydrolysis with 0.18 ± 0.01 g/L being considered freely dissolvable TRS from slurrification of the mixed agro-waste at the beginning of the experiments. The freely dissolvable sugars were determined by the quantity of TRS released within 1 h of slurrying the mixed agro-waste in sterile distilled water. Generally, the freely dissolvable sugars were determined to quantify the amount of the TRS released due to milling, as milling is also considered a biomass pre-treatment method. However, in most studies, the freely dissolvable sugars are simply ignored, but they have an impact on the overall TRS outcomes attributed by other pre-treatment procedures. A similar study was done by Guo et al. (2009), in which the accounting of the released sugars, as freely dissolvable sugars, was determined to be 1.45 g/L within in 1 h (Guo *et al.*, 2009). The results obtained at the end of

the experiments showed that the enzymatic hydrolysis was proficient in TRS extraction from the mixed agro-waste used in comparison to the hot water and mild acid pre-treatment methods, which showed a reduced conversion of the holocelluloses, with cellulases being observed to easily penetrate the complex structure of the mixed agro-waste culminating in further TRS generation.

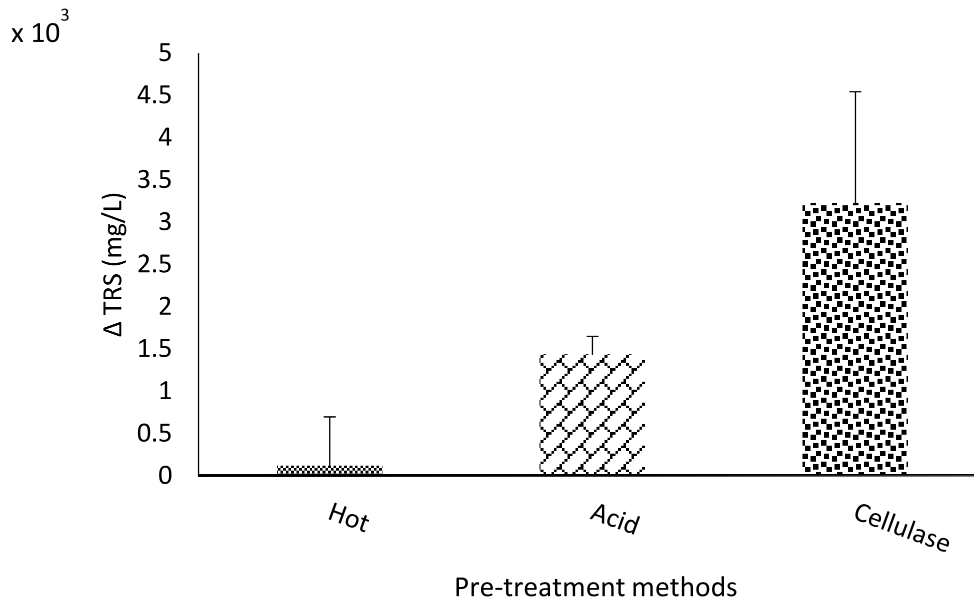

Figure 5.1: Total reducing sugar (TRS) from different agro-waste pre-treatment processes

The actual yield of the TRS is one of the important outcomes associated with the outcomes of pre-treatment procedures for agro-waste. Overall, the mixed agro-waste TRS yield had increased from 1.19 % to 32.25% using the three pre-treatment methods, as shown in Table 5.1. By sequentially pre-treating the agro-waste with hot water and dilute acid in a single pot system, compromised the integrity of the complex structure of the mixed agro-waste, leading to the high efficacy of the cellulases hydrolysis process. The higher yield of TRS illustrated the ability of these processes to produce fermentable sugars for the biorefinery industry, from mixed agro-waste.

Table 5.1: Actual total reducing sugar and yield of mixed agro-waste

Pre-treatment method	TRS (g/L) x10^3	Yield (%)
Hot water	0.12	1.19
Dilute acid	1.43	14.33
HWP/DAP/CP	3.22	32.25

5.4.2 FTIR analyses of agro-wastes

The effectiveness of the pre-treatment was further quantified using FTIR, to verify the change in structure of the mixed agro-waste. The results obtained for the FTIR are illustrated in Fig. 5.2, whereby the bands at 3329 to 3350 cm^{-1} (O-H) are more enhanced in the dilute acid pre-treatment and cellulases hydrolysis. The deformation of the hydrogen bonds of cellulose was observed at 2920 cm^{-1}, which illustrated the C-H stretching region of the mixed agro-waste for different pre-treatment methods. Furthermore, at 2359 and 1906 cm^{-1} significant peaks were observed, which are associated with carbohydrate peaks, assigned C=C, C=O, C–H, C–O–C and C–O, associated with the breakdown of the carbohydrates (Guo *et al.*, 2009). Absorbance associated with hydroxyl groups, phenolic hydroxyl group bands were observed at 1380 and 1330 cm^{-1}, respectively, while the absorbance related to primary hydroxyl and secondary hydroxyl groups present in the lignin were observed at 1035 and 1100 cm^{-1}. This illustrated the reduction of primary and secondary hydroxyl groups from agro-waste with the use of different pre-treatment methods, with all pre-treatment methods showing the degradation of the agro-waste. Other absorbance bands were observed at 1453, 2835 and 2942 cm^{-1}, which are related to the methoxy groups (–OCH_3) that are present in lignin (Guilherme *et al.*, 2015). In addition, the C-OH group band, absorption peaks at 1639, 1501 and 1410 cm^{-1} (C-H vibration of aromatic ring) and other absorption peaks between 1100 and 1330 cm^{-1} related to ester bonds (O=C–O–C) were observed.

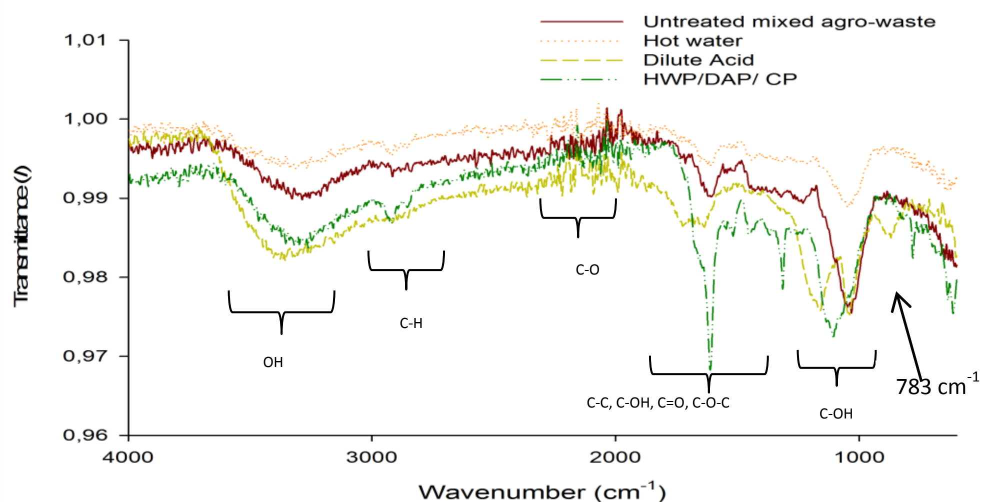

Figure 5.2: FTIR spectrum for different pre-treatment

These FTIR functional groups have tentatively illustrated the pre-treatment decomposition of the mixed agro-waste used with reference to previous studies of lignocellulosic biomass, illustrating the characteristic differentiation of the organic structure of the mixed agro-waste in relation to different pre-treatment methods.

5.4.3 X-ray diffraction analysis for mixed agro-waste

In this part of the study, XRD analyses were conducted to analyse the deformation of the structure of the agro-waste. Therefore, the XRD was also used to determine the effect of the pre-treatment methods (i.e. hot water, dilute acid and cellulase hydrolysis). Theoretically, any lignocellulosic biomass contains holocelluloses and lignin (Taherzadeh and Karimi, 2008); therefore, the purpose of the pre-treating the agro-waste was to degrade these structures that are bound together in the agro-waste. Therefore, in this part of the study, the CrI values of mixed agro-waste for different pre-treatment methods was calculated by measuring the relative amount of crystalline cellulose in untreated and pre-treated agro-waste samples (Xu *et al.*, 2010). Therefore, the Crl is determine from the height ratio between the intensity of the crystalline peaks (I_{002} - I_{AM}) and highest intensity (I_{002}) accounting for the background signal measured without the samples. Based on the results obtained (Fig. 5.3), the crystallinity values (30.05 % as shown in Table 5.2) for enzymatic hydrolysis was higher compared to hot water and dilute acid pre-treatment, which indicated that a more crystalline structure of the residual agro-waste remained after pre-treatment procedures. Overall, two typical diffraction peaks for characterizing crystallinity of cellulose were observed at around $15^{o}\pm$ 41 and

21°± 23 at 2θ, which relate to minimum peak (I_{101}) and highest peak (I_{002}) lattice planes of crystalline cellulose (Park *et al.*, 2010b). Furthermore, these three pre-treatment methods used in this study have shown an ability to enhance the hydrolysis of agro-waste to extract fermentable sugars, which is associated to an amorphous formation of the cellulose during pre-treatment, with the residual biomass having a higher crystallinity associated acid insoluble and enzyme hydrolysis resistant cellulose in the mixed agro-waste – see Table 5.2 and Table 5.3, which shows that after pre-treatment procedures, a larger proportion of the agro-waste was constituted by insoluble lignin.

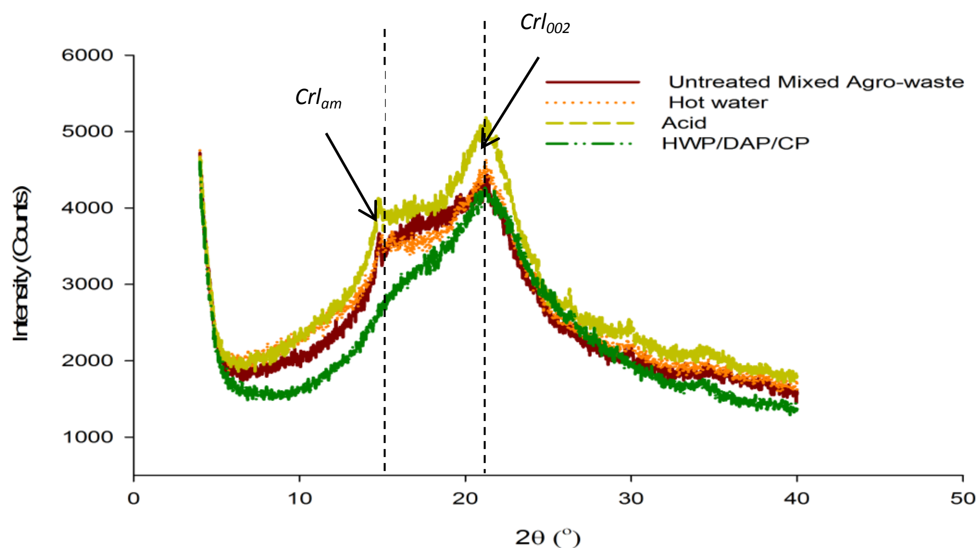

Figure 5.3: X-ray diffraction analysis for different pre-treated mixed

Table 5.2: Crystallinity of mixed Agro-waste residue after pre-treatment agro-waste

Pre-treatment method	Crystallinity index (*CrI* %)
Hot water	25.82±1.0
Dilute acid	24.50±0.8
Cellulases hydrolysis	30.05±0.3

5.4.4 Lignin and ash content

The acid-insoluble residue (AIR), acid-soluble lignin (ASL) and ash in the agro-waste determined by Klason lignin method and total lignin content is shown Table 5. 2. This method was applied for untreated and final residue samples (enzymatic hydrolysis) after hot water and diluted acid pre-treatment. The results obtained show a significant difference in ASL content of 1.32% and 3.34 %, for

untreated and Hot water/dilute acid/enzymatic hydrolysed agro-waste respectively. These results illustrated the extraction of some holocelluloses during the pre- treatment process, which culminated in AIR Overall, the pre-treated samples showed less recoverable and/or convertible lignin content compared to the untreated samples. Furthermore, the ash content was 0.132 and 0.340 % for untreated agro-waste and cellulases hydrolysis respectively

Table 5.3: Lignin and ash content of untreated and enzymatically hydrolysed agro-waste

Pre-treatment method	Acid-insoluble residue (%)	Acid-soluble Lignin (%)	Ash (%)
Untreated agro-waste	1.32	0.07	0.132
Cellulases hydrolysis	3.40*	0.01*	0.340

5.4.5 Enhancement of alcohol production

The fermentation of extracts from mixed agro-waste pre-treatment was performed using the commercial strain VIN13 (*Saccharomyces cerevisiae*). To further quantify the presents of bioalcohols, Jones and Lucas methods were used (Jones and Woods, 1986). Table 5.4, shows the preliminary test for the presents of C_1 to C_3 and C_4^+ bioalcohols, during fermentation. The results obtained showed positive results for bioalcohols, which led to further analyses of the samples using GC-MS.

Table 5.4: Bio-Alcohol preliminary tests agro-waste

Strain	C_1 to C_3	C_4^+
S. cerevisiae (VIN13)	++	+

+ Presence of alcohol//- Absents of alcohol

The GC-MS results obtained are listed in Table 5.5, which indicated that the bioalcohols were detected from 24 h of fermentation for C_1, whereby the highest (C_4), i.e. 1-Butanol, 3-methyl-, was obtained after 48 to 72 h. Furthermore, some unwanted biocompounds were also detected during the fermentation. These compounds included phenolic, Octanone and acetic acid which are not shown in Table 5.5.

The results obtained have shown the ability of the *S. cerevisiae* (VIN13) to utilise the pre-treated extract to produce bioalcohols in an integrated single pot system. The increase in bioalcohol production is necessary, with better yields required. In addition, other added value bio-products were also observed during fermentation, which can add value for the biorefinery industry. Further analyses and development of the pre-treatment of the agro-waste is required for this study.

Table 5.5: Bio-alcohols produced agro-waste

Bio-product	Max Area%
Ethyl alcohol/Ethanol grain	1.02
1-Butanol, 3-methyl-	0.53
Benzyl alcohol	0.06

5.5. Summary

In this part of the study, an integrated single pot system was developed for mixed agro-waste pre-treatment to attain hydrolysates which can be used as substrate (pre-treated extract) for bio-alcohol production. The results showed an efficient TRS production process using hot water and dilute sulphuric acid with an addition of cellulases further increasing TRS concentration. Moreover, using commercial fermenter strain, resulted in positive bioalcohol production; albeit with a higher production of C1 to C3 bioalcohols. Therefore, the single pot pretreatment system has shown potential to produce different bioalcohols and other multiple products, which can benefit the biorefinery industry. Bioalcohol production using a variety of microbial species has had challenges due to the toxicity and inhibition of bioalcohols produced on microbial populations used during fermentation, which generally results in low yields. This has resulted in research focusing on a variety of strategies to produce alcohols using suitable biocatalysts and efficient downstream processes for bioalcohol recovery. The next chapter focused on an investigation of new biocatalysts or a cocktail of enzymes to reduce inhibition by-products while maintaining or increasing TRS in hydrolysates from the pretreatment of mixed agrowaste.

CHAPTER 6

Integrated pre-treatment of mixed agro-waste for a second generation biorefinery using *Nepenthes mirabilis* pod extracts

RESULTS: PART 2

Article submitted and published

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CHAPTER 6

6. INTEGRATED PRE-TREATMENT OF MIXED AGRO-WASTE FOR A SECOND GENERATION BIOREFINERY USING *NEPENTHES MIRABILIS* POD EXTRACTS

6.1 Introduction

The challenge associated with environmental pollution and petroleum depletion has ensured an interest in the production of biofuels including value added products using environmentally benign processes. The production of such commodities has demonstrated the feasibility and applicability of biorefinery processes that are microbially mediated, using lignocellulosic biomass feedstock such as agro-waste. Moreover, the biorefinery processes designed, consists of several stages, i.e. processing units, which includes the preparation and pre-treatment of the feedstock to be used, i.e. lignocellulosic biomass including agro-waste, in the upstream processes subsequent to fermentation and recovery of bio-products in downstream processes. Recent studies, have investigated numerous processes to pre-treat the lignocellulosic biomass including agro-waste for biorefineries (Kudakasseril-Kurian *et al.*, 2013); whereby, the evaluation for the development of such processes, focused on reduced pre-treatment time for maximizing the extraction of fermentable carbohydrates, reduced energy intensity, environmental benignity by eradicating inorganic compound usage and minimisation of capital thus operational costs (Lee *et al.*, 2009). These processes, albeit achieving varying successes include, chemical, physical, biological and physicochemical pre-treatment technologies, either as individualised and/or amalgamated processes, with varying success (Cheng *et al.*, 2012; Chiaramonti *et al.*, 2012; Kumar *et al.*, 2016; Lee *et al.*, 2009; Martín and Thomsen, 2007; Narayanaswamy *et al.*, 2013; Procentese *et al.*, 2017; Rajan and Carrier, 2014).

Despite the recent successes in lignocellulosic biomass pre-treatment using the aforementioned processes, several challenges still prevail. Currently, biomass pre-treatment is conducted in two to four stages including enzyme hydrolysis, depending on the desired outcomes, which can increase the operational costs of a biorefinery. Furthermore, pre-treatment as it is used for the delignification of biomass to extract fermentable carbohydrates, culminates in the production of inhibitors, which may inhibit enzymatic hydrolysis and subsequently, downstream fermentation processes. This necessitates further improvement of pre-treatment methods that are currently in use, since the pre-treatment process is one of a few costly processes in a biorefinery. By maximizing extractable fermentable sugars production, herein referred to as total reducing sugars (TRS), while reducing inhibitors and minimizing operational costs, which could be vital to the success of a biorefinery even in developing countries.

Currently, a single stage and an environmentally benign process, has not been developed for the pre-treatment of feedstock for a second generation biorefinery, particularly focusing on the use of a

renewable bioresource such as mixed agro-waste for an effective and efficient way to resolve challenges associated with, 1) delignification, 2) cellulolysis and 3) the production of recalcitrance residual inhibiting by-products from the biomass pre-treatment process, all of which have an effect on other downstream processes (Jönsson and Martín, 2016). To achieve this, process integration is required to improve and reduce some of the stages in the pre-treatment of biomass, i.e. using a suitable single pot multi-reaction process for biodelignification, cellulolysis and the reduction of inhibitory by-products from the feedstock. For such a strategy to succeed, digestive enzymes, such as those produced by *Nepenthes* mirabilis, which have been previously associated with delignification and cellulolysis (Chan *et al.*, 2016), with the potential to biodegrade delignification inhibitors, are required.

Generally, *N. mirabilis* plant extracts, i.e. from the pitcher "monkey cup", have been reported to contain digestive enzymes, which are capable of biodegrading complex and polymeric molecules such as glycan, starch and elemental metallic species even under anaerobic conditions (Chan et al., 2016). The application of such digestive enzymes in a biorefinery can minimise energy requirements, plant footprint, the use of hazardous chemical compounds while reducing production of fermentation inhibitors, making such extracts suitable for a single pot multi-reaction biomass pre-treatment system, thus an integrated system which can advance process capacity in the biorefinery industry; a daunting assertion when mixed agro-waste is to be considered as a feedstock. Overall, *N. mirabilis* (pitcher plant) extracts, have not been explored for the pre-treatment of lignocellulosic biomass destined for biorefineries; although the extracts; 1) have the ability to decompose a variety of polymeric substances (Lee *et al.*, 2016), 2) are acidic (Siragusa *et al.*, 2007) thus can be used directly for the pre-treatment of biomass, a process analogous to dilute acid pre-treatment and 3) contain microbial populations of the *phyla Proteobacteria, Bacteroidetes, Actinobacteria, Verrucomicrobia, Planctomycetes*, which are associated with the production of numerous hydrolytic enzymes (Chan *et al.*, 2016).

6.2 Objectives

The primary objective of this part of the study was to evaluate integrated pre-treatment of mixed agro-waste destined for a second generation biorefinery using *N. mirabilis* extracts. Such research is required to ascertain the applicability and biocatalytic efficacy of *N. mirabilis* extracts in an integrated process for biomass pre-treatment of mixed agro-waste to maximise the extraction of TRS.

6.3 Material and methods

The final Feedstock, i.e. agro-waste (*C. sinensis*, *M. domestica*, cobs from *Z. mays* and *Q. robur* (oak) were selected and used based on the results in chapter 4 (section 4.1.1). Furthermore, to develop an environmental benign process, a natural digestive enzyme cocktail was identified and used to pretreat the mixed agro-waste, - see chapter 4 (section 4.5.3); while the lignin and holocelluloses content was quantify as explained in chapter 4 (section 4.2). Analyses associated with acid soluble and insoluble lignin, were carried-out by slurring 300 mg of dried pretreated agrowaste in a 100 mL Schott bottles, with 1 mL of 72 % of H_2SO_4, subsequent to agitation to homogenously mix the slurry. The *N. mirabilis* plants extracts used, were collected from Pan's Carnivores Plant Nursery. The phenolic compounds and physico-chemical characteristics of the *N mirabilis*, were also analysed as discussed in chapter 4 (section 4.3 and 4.5.2). The pre-treatment of the mixed agrowaste using *N. mirabilis* extract was conducted at ambient temperature - see chapter 4 (section 4.5.3). FITR and XRD were also used to quantify the morphological structural changes of the agro-waste post pre-treatment.

6.4 Results and discussion

6.4.1 Selection of agro-waste

The mixed agro-waste feedstock selected was based on its regional availability in South Africa, especially in Western Cape, South Africa. Overall citrus trees, and oak, are largely available, focusing on the specifications requirements of the feedstock to be used in a second generation biorefinery. Second generation biorefinery feedstock may include wood waste, non-food crops, waste cooking oil, forestry agricultural residues (Hughes *et al.*, 2013). These types of feed stock reduces the reliance on edible crops (Altschul *et al.*, 1997). Western Cape Province (SA) is the third largest province that produces large quantity of Citrus fruit in South Africa (Hunlun *et al.*, 2017), with 95% of Apples (*M. domestica*) being produced in this province. Furthermore, oak trees (*Q. robur*) generate a large quantity of yard waste with the plant being able to live for 300 – 600 years. Therefore, the agro-waste was selected to reduce landfilling, by convert it into fermentable sugars. In previous studies, agro-waste in particular, *C. sinensis, M. domestica,* corn cobs from *Z. mays* and *Q. robur* (oak), had been determined to contain sufficient quantities of extractable fermentable sugars (Mekuto *et al.*, 2018; Xu *et al.*, 2013; Su *et al.*, 2016; Taghizadeh-Alisaraei *et al.*, 2017); although, some produce inhibiting by-products such as ferulic, glucuronic, ρ-coumaric and acetic acids, phenolics including residual heavy metals during pre-treatment (Jönsson and Martín, 2016), some of which were hypothetically assumed to be biodegradable using *N. mirabilis* extracts. To further extract fermentable sugars from pre-treated agro-waste, further potential hydrolysis of the residual pre-

treated agro-waste using commercial cellulases must be feasible, even when a different cocktail of lignocellulolytic enzyme is used (Singhania *et al.*, 2013), in particular an enzyme cocktail having properties such as those observed in *N. mirabilis* plant extracts (Chan et al., 2016).

From the results (Table 6. 1), the increment in lignin content in residual pre-treated agro-waste in comparison to the reduction in holocellulose, was directly attributed to the pretreatment regime implemented; albeit, it was observed that the recalcitrant acid-insoluble lignin, was ineffectively decomposed by the enzymatic pretreatment regime used, an advantageous attribute as the system seemed to only decrease holocellulosic content of the biomass feedstock used. This suggests a reduction in the production of phenolic from the agro-waste.

Total residual lignin constituted the acid-insoluble residue (AIR), acid-soluble lignin (ASL) determined by the Klason lignin method, for untreated and *N. mirabilis* plant extracts pretreated mixed agro-waste, showed significant increases of residual lignin content in cellulases supplemented system. Overall, lower lignin concentration and its composition contribution was observed to be 27 to 39% when the agro-waste was pre-treated solely with *N. mirabilis* plant extracts, while the holocelluloses were 72.9 and 60.7%. These results illustrated a potential extraction of some holocelluloses during pretreatment processes, which was further reduced to 40.7%, when commercial cellulases were supplement in *N. mirabilis* pre-treatment system culminating with the residual mass being highly crystalline with a large proportion of unreactive ash. Therefore, the pre-treated samples showed recoverable and/or convertible holocelluloses content using the *N. mirabilis*/cellulase pre-pretreatment regime. Generally, with the *N. mirabilis* plant extracts pre-treatment, a larger fraction of the lignin content in agro-waste remains in the acid insoluble residue; while for conventional acid based pre-treatment methods used, acid soluble by-products were observed, which can lead to inhibitors generation. By using *N. mirabilis* plant extracts, the pre-treatment of the agro-waste was hypothesised to enhance fermenters performance with minimal inhibition. By sequentially using conventional methods, i.e. hot water, dilute acid and cellulase pre-treatment in a single reactor system, a large proportion of lignin seemed to have biodegraded into solution, leaving a higher quantity of holocelluloses (56.8%) intact in the residual biomass compared to *N. mirabilis* subsequent cellulases pretreated system.

Table 6.1: Composition of Lignin content and Holocellulose of Untreated and *N. mirabilis* plant extracts and cellulases pre-treated agro-waste

Pre-treatment methods	**Residual Lignin (%)**	**Residual Holocellulose (%)**	**Ash (%)**
Untreated mixed agro-waste	27	72.9	0.1
*N. mirabilis**	39	60.7	0.1
N. mirabilis / CP	59	40.7	0.3
HWP/ DAP/ CP	43	56.8	0.2

***** *Nepenthes* pitcher extract

6.4.2 Biophysico-chemical characteristics of the *N. mirabilis* pod extracts

The dominant bacteria in *Nepenthes* pitcher extracts were *Klebsiella oxytoca* (KF55591), *Bacillus thuringiensis* (KF557957); although, *Bacillus cereus* and *Bacillus anthracis* were not identified with the Vitek method as show in Table 6.2. The most abundant enzymes in the pitcher extracts were identified as proteases, nucleases, peroxidases, chitinases, a phosphatases and glucanases, including carboxypeptidases (Lee *et al.*, 2016). Lee *et al*. (2016) also identified these proteins, whereby the carboxypeptidases and prolyl endopeptidases were identified as novel proteins. The enzymes that were observed in the *Nepenthes* sp. extracts have been shown to be stable under acidic (i.e. low pH), oxidative and low nitrogen conditions (Lee *et al.*, 2016). Similarly, it is expected that bacteria producing these enzymes are able to live under such conditions, while sustaining an environment that facilitates the degradation of complex carbohydrates such as starch, xylan, hemicellulose and cellulose (Chan *et al.*, 2016). In previous studies, *Klebsiella oxytoca* was determined to be chitinolytic, with *Bacillus thuringiensis* (H1M), *Bacillus* H1a (*Bacillus* sp.) being shown to have proteolytic, amylolytic and cellulolytic activity; producing glycoside hydrolases, which are constituted by chitanase and glucanases (Kim *et al.*, 2007). For this study, there was evidence of microbial proliferation in the *N. mirabilis* pods extract, with the dominant microbial population being to *Bacillus* sp., i.e. *B. cereus*, *B. thuringiensis* and *B. anthracis* including *Klebsiella oxytoca* which was also identified. The evaluation of gastro intestinal bacterial population in termites, determined for the decomposition of holocelluloses included *B. cereus, B. thuringiensis* and *B. subtilis*, which have been determined to be cellulases and xylanases producers with varying biocatalylic activity (Asem *et al.*, 2017). However the functionality of these microorganisms at pH 2 and Eh + 500 mV is unknown.

The redox potential (Eh) of the extracts were measured to verify the ability of the *N. mirabilis* extracts to accept or lose electrons, i.e. facilitate oxidation reactions. For highly effective oxidative reactions, a positive Eh of up to +810 mV under ambient and/or mild conditions (pH 7.0, 30°C) is

required (Hoffman and Winston, 1987). In microbial systems the Eh might be directly related to the growth of microorganisms, with aerobes being able to grow and proliferate in Eh from +300 to +500 mV, while for anaerobes were determined to proliferate from -250 to +100 mV, with slower growth rate occurring at higher Eh due to the highly oxidative environment which can calumniate in oxidative species generation, which are known to be harmful to cellular membranes (Hoffman and Winston, 1987). Overall, the *N. mirabilis* pod extracts had an Eh averaging +510 mV, which was indicative of the oxidative properties of the extracts (Abreu *et al.*, 2002).

Table 6.2: Identity of bacteria isolated from *N. mirabilis* extracts

Identity	Vitek 2	16S rDNA	Accession number
Bacillus sp.	+	-	-
Klebsiella oxytoca	+	-	-
Bacillus cereus	-	+	KY249126.1
Bacillus thuringiensis	-	+	DQ513324.1
Bacillus anthracis	-	+	KU948294.1

6.4.2.1 Carboxylesterase, β-glucosidase, xylanase and commercial cellulases activity

Carboxylesterases are well known as acetylxylan esterases, which facilitates the hydrolysis of xylan. They facilitate the removal of ferulic acids from xylan including the bioconversion of holocelluloses (Mewa-Ngongang *et al.*, 2017). In general, these esterase's catalyses the hydrolysis of numerous acetyl groups in polymeric such as xylan, acetylated xylose and acetylated glucose. The carboxylesterase activity was assessed as a hydrolysis based reaction for the formation of 1 µmol of p-nitrophenol per min, corresponded to an activity of 529.41±30.57 U/L. Recent studies have shown carboxylesterase activity in *Penicillium chrysogenum*, whereby the activity was found to be 5.4 U/L (Yang *et al.*, 2017), with *Aspergillus* and *Trichoderma* sp. being other organisms that were reported for carboxylesterase activity (Komiya *et al.*, 2017; Manavalan *et al.*, 2017). This illustrated the capability of the *N. mirabilis* pods extracts to hydrolyse acetyl groups including those associated with xylan. Furthermore, *Bacillus* sp., in particular *B. vallismortis* was confirmed to have the ability to produce short chained acetyl xylan esterases (Turner Jr *et al.*, 1969). The decoupling of the xylan backbone is highly dependent on the endo/β-xylosidases; albeit acetyl xylan esterases including xylanases are largely involved (Komiya *et al.*, 2017). By reducing the acetyl groups, the endo-xylanases will thus be effective during the pre-treatment of mixed agro-waste (Manavalan *et al.*, 2017), furthering the decomposing of xylan to xylose.

Furthermore, β-glucosidase activity (251.94±11.48 U/L), was measured using the formation of pNPG per minute resulting in similar results to those observed in the study by Kim et al. (2007); albeit in this study, pNPG has been used as a substrate to quantify the activity of β-glucosidase using pitcher plant extracts as active enzymes biocatalisers instead of cellobiases. Hydrolytic enzyme activities including β-glucosidase, were positively quantify in numerous *Nepenthes* spp., with the activity of β-glucosidase, disassociated with the influence of pH. Pitcher fluid in *Nepenthes* sp. are determined to contain a higher activity of digestive enzymes, with high concentration of natural antibacterial agents, and the bacteria from these fluids, were considered to be robustly adaptive to even produce β-glucosidase, including xylanase.

Xylanase activity has been previous studied by Khan (1995) using *Trichoderrna reesei* extracellular extracts using a method for which the enzymes aliquots were substituted with *N. mirabilis* digestive fluids, whereby the degradation of xylan to xylose was detected. This indicated the presence of xylanase in the *N. mirabilis* extracts with an activity of 36.09±18.04 U/L. Therefore, *N. mirabilis* extracts can be used to degrade a component of the holocellulose in particular, hemicellulose embedded in the mixed agro-waste to produce fermentable reducible sugars. Furthermore, xylanase, has the ability to degrade hemicellulose in agro-waste, in particular β-1,4-xylan, with the B. cereus being determined to facilitate the decoupling of beta-bonds in xylan due to a xylanase mechanisms (Moser, 1958).

6.4.3 Integrated pretreatment single one pot process

6.4.3.1 Total residual phenolic compounds (TRPCs)

The total residual phenolic compounds determined using the Folin-Ciocalteau reagent, as phenolics compounds inhibit the efficacy of β-glucosidase, thus depletes their functionality due their reactive species scavenging properties, which can reduce the efficacy of these enzymes in an oxidative environment (Baba and Malik, 2015). The highest TRPCs (6.25 ±0.1 mg/L) were observed in slurrified mixed agro-waste supplemented with *N. mirabilis* extract, as shown in Table 6.3, with decrease in TRPCs being observed subsequently to the addition of cellulases. The TRPCs concentration in untreated mixed agro-waste remained at 3.95±0.12 mg/L throughout the 168 h of experimentation period, at attribute imparted by insignificant reactions that were taking place in the untreated sample. A concentration of 5.65 ±0.44 mg/L for TRPCs was also observed at the end of experiments conducted using conventional methods. The results obtained indicated that the presents of the *N. mirabilis* extract in the pretreatment system designed has an ability to reduce phenolic content generated; albeit to a minimised extend. A similar study, was conducted and reported (Bader, 1978);

whereby, the TRPCs were observed vary from 26 to 34% of the total lignin loads in pre-treatment system used. This further confirmed that the mixed agro-waste has a large phenolic content, which can negatively impact downstream process. Therefore, these results must be taken into account for optimization of feedstock preparation to minimise high phenolic content production.

Table 6.3: Total residual phenolic content at initial and after enzymatic hydrolysis

Processes	Sampling time (h)	Total phenolic** content (mg/L)	SEM
Untreated mixed agro-waste	168	3.95±0.12	0.07
N. mirabilis	72	6.25±0.18	0.11
N. mirabilis /CP	168	4.26±0.09	0.05
HWP/DAP/ CP	168	5.65±0.44	0.25

** Average value ± SD (n=3)

6.4.3.2 Performance of the single pot multi-reaction pre-treatment process

The performance of the single pot process designed for ambient temperature operations, using *N. mirabilis* extracts, included the initial slurrification of the biomass, subsequent to *N. mirabilis* supplementation at 72 h, with the further of holocellulolysis using commercial cellulases being implemented at 120 h. The effect of the pitcher plant extracts on biodegradation of the mixed agro-waste was distinctively observed after 48 h of the *N. mirabilis* extract supplementation. The results indicated that the concentration of the pitcher plant extract had a significant effect on the degradation of holocelluloses with the maximum (max) TRS concentration measured being 310±5.19 mg/L. The average TRS concentration (Table 6.4) illustrates the specific impact of the addition of *N. mirabilis* extract and further cellulases supplementation in a single pot system, with the maximum TRS being achieved after 168 h. The cellulases effect on the pre-treatment process step was significant as illustrated by the increase of the TRS after 120 h. For conventional methods an adequate increase from 1.4±0.578 to 3.22±0.219 g/L of TRS was observed in 72 h, which illustrated the impact of the cellulases hydrolysis. Furthermore, comparing the conventional methods and the proposed *N. mirabilis* supplementation extracts, similar results have been obtained for both methods. However, the *N. mirabilis* supplementation has further shown a reduction of inhibitor concentration during the process, while the conventional methods produced a higher concentration of inhibitors, i.e. TRPCs.

6.4.3.3 Furtherance of agro-waste hydrolysis using commercial cellulases

To further evaluate the effect of the pre-treatment and to quantify the biodegradation suitability of the agro-waste using *N. mirabilis* extract, cellulases hydrolysis was performed. The process was completed after 72 h subsequent to the addition of cellulases, with a sharp TRS increase after 24 h when cellulases were added to the pre-treatment mixture. The maximum concentration of 310 mg/L was obtained. However, similar results were obtained by Chang *et al*. (2012), with acid pretreatment, which was 8.11 ± 0.02 g/L TRS from rice straw. Furthermore, the maximum total reducing sugar yield was obtained as 27.97% results which were similar to those obtained by Chen, (2007), whereby the TRS yield of untreated agro-waste was 5.46% as shown in Table 6.4. This illustrates an ability of *N. mirabilis* extract to extract TRS at ambient conditions without the use of chemicals. The secondary function of *N. mirabilis* extracts was to reduce the TRPCs, while increasing TRS extraction in a single pot system. This further biodegradation of the mixed agro-waste and reduction of inhibitors can be provided for by suitable cocktail of suitable enzyme. Generally, the acid based pretreatment method has been associated with high energy consumption and the production of inhibitors (Rajan and Carrier, 2014) and chemical usage which are not environmental benign (Taherzadeh and Karimi, 2008).

Table 6.4: Average TRS concentration of *N mirabilis* extract from 72, 120 & 168 h

Processes	Sampling time (h)	Average TRS concentration**(mg/L)	SEM	Yield %
Untreated mixed agro-waste	168	60.69±1.7	1.02	5.46
N. mirabilis	72	244.91±20.55	11.9	22.04
N. mirabilis	120	269.164±18.94	10.9	24.22
N. mirabilis / CP	168	310.55±5.19	3.00	27.95

** Different values represents the mean ±SD (n=3)

6.4.3.4 X-ray diffraction analysis

The XRD analyses were conducted to analyse the deformation of the crystalline structure of the mixed agro-waste. Any biomass is constituted by cellulose, hemicellulose (i.e. holocellulose) and both are embedded in lignin (Taherzadeh and Karimi, 2008). Theoretically, the aim of pre-treating agro-waste was to decouple these structures that are bound together in the agro-waste for ease of hydrolysis. Therefore, the CrI values were determined to vary depending on the effect of pre-

treatment processes used and in their ability to disintegrate the structure of the agro-waste thus reduce its crystallinity (Kumar *et al.*, 2009b). However, the crystalline determination for entire lignocellulosic biomass can be difficult, since pooled samples were used as a representative of the crystalline structure of the biomass such that true crystallinity of holocellulosic material (mixed agro-waste) being evaluated, can be comprehensively assessed. This is due to X-ray diffraction methods measuring total crystallinity of lignocellulosic biomass, which includes the combined holocellulose and lignin. Therefore, in this study the CrI values of agro-waste were measured by the relative crystallinity of the holocelluloses for untreated and pre-treated agro-waste (Xu *et al.*, 2010). As such, the CrI were determined using the ratio between the intensity of the crystalline peaks (I_{002} - I_{AM}) and the highest the intensity (I_{002}), while accounting for the background signal measured without the samples.

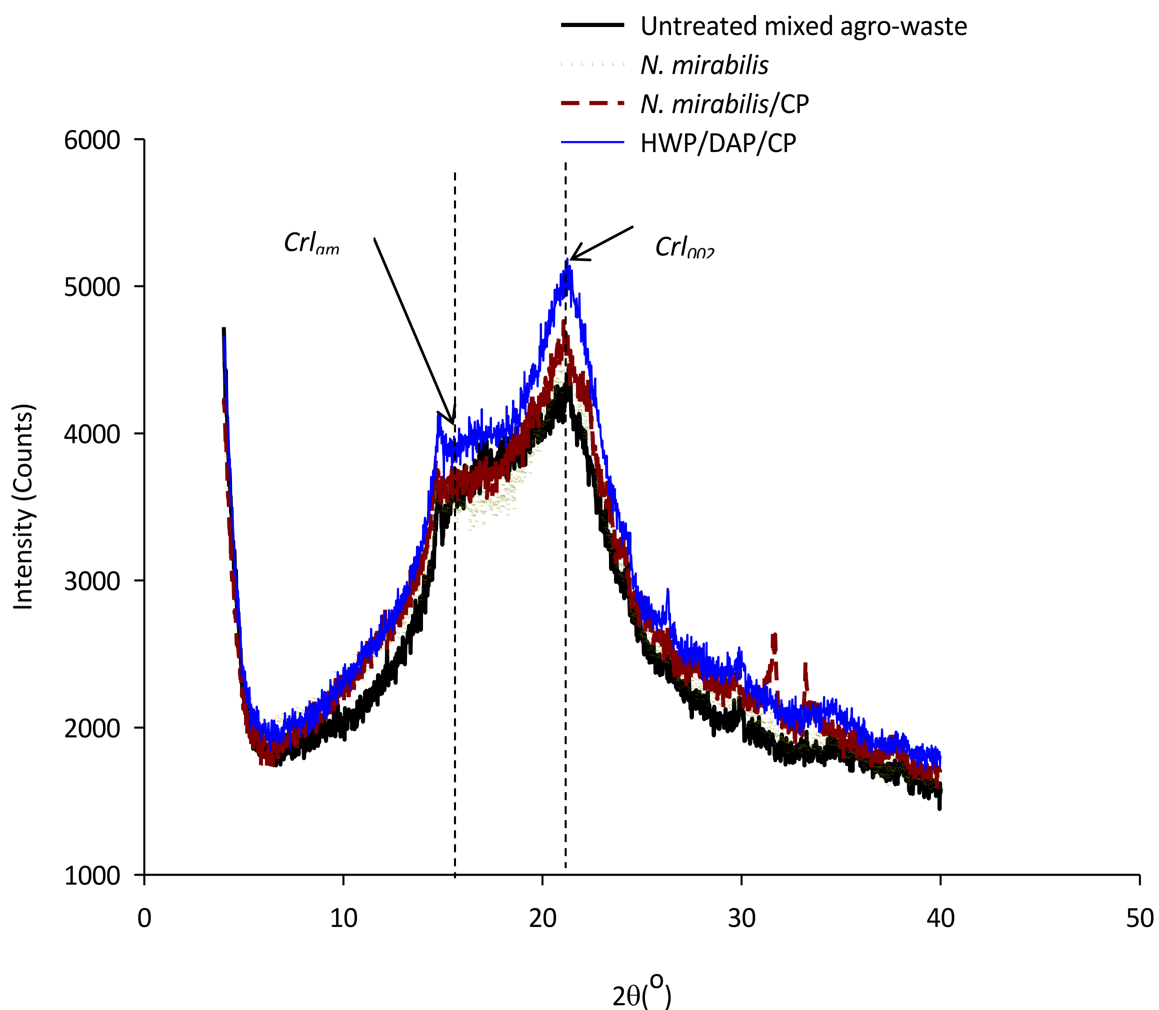

Figure 6.1: X-ray diffraction spectrum for un-and pre-treated mixed agro-waste

Based on the results (Fig. 6.1), crystallinity values for untreated and mixed agro-waste were lower compared to the pre-treated biomass, with *N. mirabilis* which showing a higher Crl value. This illustrated the significant effectiveness of the plant extracts used to pre-treat the lignocellulosic biomass. The agro-waste pre-treated with *N. mirabilis* extract, had shown higher Crl values (see Table 6.4), which confirmed the deformation of the biomass structure. There are two typical diffraction peaks for characterizing crystallinity of ligno-holocellulosic biomass waste were observed at around 18°.41 and 22°.01 at 2θ, which relate to the minimum peak (101) and highest peak (002) in the lattice planes of the biomass (Park *et al.*, 2010b) . However, the peak for the cellulose are much broader compared to others chemical compounds (Qiu *et al.*, 2012). For biomass, the amorphous peaks normally occur at around 2θ of 18,7^{o}, which was also observed in a study by Park *et al*. (2010). The crystalline values are as shown in Table 6.1, which are calculated based on the peak height method.

Table 6.1: Crystallinity index values of mixed agro-waste after 168 h of pretreatment in a single pot system

Pre-treatment	**Crystallinity index ($Crl\%$)**
Untreated mixed agro-waste	15.64
*N. mirabilis**	23.14
N. mirabilis /CP	30.05
HWP/DAP/CP	25.82

*Pitcher plant extracts = *Nepenthes* mirabilis extracts

6.4.3.5 FTIR Analyses for mixed agro-waste

To further quantify the effectiveness of the pre-treatment method using *N. mirabilis* extracts, FTIR was also used to quantify the change in the structure and functional group distortion of the agro-waste. The main of using the FTIR spectra was indicating the ability of *N. mirabilis* extract to deconstruct the biomass similarly to traditional methods, i.e. thermochemical methods, in order to observe the structure of mixed agro-waste constituents and chemical changes taking place in agro-waste due to integrated treatments process (Chundawat *et al.*, 2011). The results obtained from the FTIR (Fig. 6.2), included a broad spectral peak at around 3324 to 3350 cm^{-1} for all pre-treated agro-waste, which is associated with the O-H stretching region enhanced for the *N. mirabilis* extract pre-treated biomass. The deformations of hydrogen bonds of holocelluloses were observed at 2913 cm^{-1}, which illustrated the asymmetric C-H stretching region of the mixed agro-waste for the *N. mirabilis* extract pre-treated agro-waste. Similar results were observed, whereby the same region, i.e. 2920 cm^{-1}, was indicative of the methyl and aliphatic methylene group in holocelluloses (Poletto *et al.*,

2014). Furthermore, from 1035 to 1722 cm^{-1} significant peaks were observed, which are associated with carbohydrate peaks, assigned C=C, C=O, C–H, C–O–C and C–O associated with crystalline cellulose and xylan stretching bonds, with the reduced peak prominence being attributed to the breakdown of some lattice structure of the agro-waste (Guo *et al.*, 2009). The absorbance related with hydroxyl groups, phenolic hydroxyl group bands were observed at 1380 and 1330 cm^{-1}, respectively, while the absorbance related to primary hydroxyl and secondary hydroxyl groups present in lignin was observed at 1035 and 1100 cm^{-1} (Zeng *et al.*, 2011). Furthermore, this indicated the reduction of primary and secondary hydroxyl groups from the mixed agro-waste with the use of *N. mirabilis* extract for pre-treatment process, showing the degradation of functional groups, thus biodegradation of the agro-waste during pre-treatment. Furthermore, other absorbance bands were observed at 1453, 2835 and 2942 cm^{-1}, which are related to the methoxy groups ($–OCH_3$) that are normally present in lignin (Guilherme *et al.*, 2015). In addition, absorption peaks at peaks at 1639 cm^{-1} associated with aromatic C=C vibration and C=O stretching, 1501 cm^{-1} aromatic ring associated with lignin and 1410 cm^{-1} (C-H vibration deformation of aromatic ring in lignin), while other absorption peaks between 1100 and 1330 cm^{-1} are related to ester bonds (O=C–O–C) of crystalline cellulose (Barnette *et al.*, 2012).

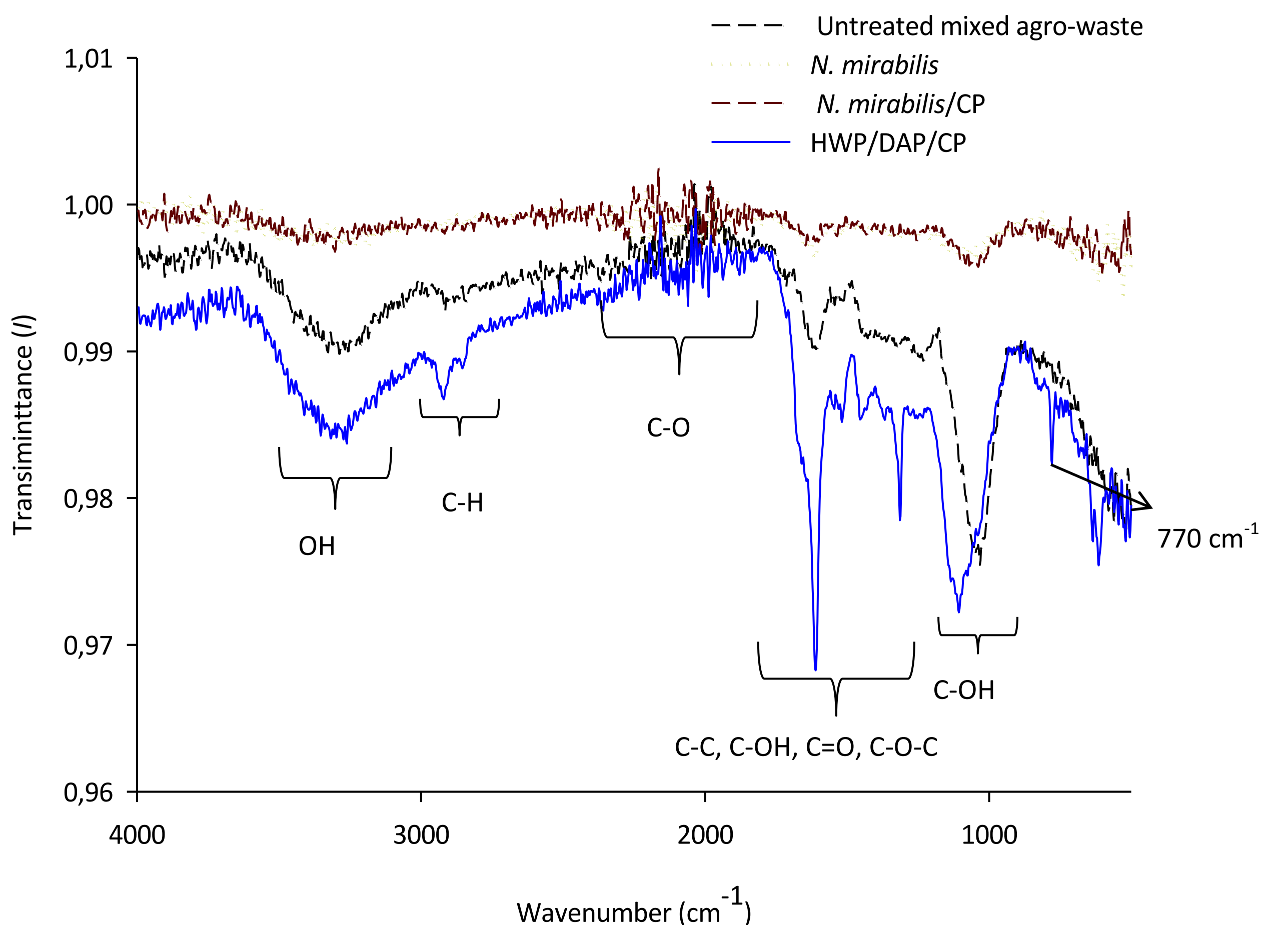

Figure 6.2*:* FTIR spectrum of pre-and un-treated mixed agro-waste

Furthermore, the results obtained showed a decrease on transmittance (*I*) from I_{2850}, I_{1603} and I_{1035}, with a high value of the transmittance being indicative of high crystalline structure in the residual agro-waste. Similar studies had previous confirmed that the FTIR spectral peaks can be utilized to analyse some aspects of crystallinity for different samples that contained holocellulose with amorphous holocellulose structure being easily observable when other components of the pre-treated agro-waste are decoupled from the lignified backbone of the waste (Sudfeldt, 1990). Primarily the pre-treatment of lignocellulosic biomass release TRS from solid side to liquid side of broth (i.e. insoluble to soluble). Hence, the results obtained shown lower C-C type linkages that are associated with holocellulose of the hot water/ dilute acid/cellulase when compared with *N. mirabilis*/cellulase. Thus, the holocellulose are degraded and are being released to the broth, whereby the less content of the holocellulose was left on the solids (insoluble biomass). This has been also indicated by high lignin content with *N. mirabilis*/ cellulase, while high holocellulose was observed (i.e. see Table 6.4).

6.5 Summary

In this part of the study, agro-waste was pre-treated with *N. mirabilis* extracts in single pot multi-reactions at ambient temperature. The results obtained demonstrated an ability of digestive enzymes to biodegrade lignocellulosic biomass (agro-waste), while increases the TRS extraction and reduction of phenolic compound based on the analyses conducted. The results observed have proven that single pot multi-reaction processes can be used an alternative pre-treatment method for the biorefinery industry. Furthermore, cellulases hydrolysis has shown an ability to further increases in TRS in a single pot system in the presents of *N. mirabilis* extracts as a suitable cocktail of enzymes. The analytical methods, i.e. Vitek and 16 rDNA that were used indicated different microorganisms that are present in the *N. mirabilis* extracts, including enzymes, confirmed by positive enzyme activity of carboxylesterases, xylanases and β-glucosidases. The pXRD and FTIR spectroscopy analyses demonstrated diffraction peaks related to cellulose crystallinity in residual pre-treated agrowaste, with a significant degradation profile from *N. mirabilis* pre-treated agro-waste. Therefore, the next chapter focused on the microbial growth rate, product formation and substrate consumption; using hydrolysates from the *N. mirabilis* pre-treatment in comparison to the combined hot water, dilute acid, cellulases pretreatment discussed in chapter 5. This was done using appropriate kinetic models for fermentations using a commercial strain of *S. cerevisiae*.

CHAPTER 7

Kinetic parameters of *Saccharomyces cerevisiae* bioalcohol production using *Nepenthes mirabilis* pod extracts - mixed agro-waste hydrolysates

RESULTS: PART 3

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CHAPTER 7

7. KINETIC PARAMETERS OF *SACCHAROMYCES CEREVISIAE* BIOALCOHOL PRODUCTION USING *NEPENTHES MIRABILIS* POD EXTRACTS - MIXED AGRO-WASTE HYDROLYSATES

7.1 Introduction

Fermentation is the well-established process for production of bioproducts (i.e. bioethanol, biobutanol, isobutanol, lactic acid, citric acid and etc.) from glucose and/or lignocellulosic biomass hydrolysates (Meintjes, 2011). However, the utilization of lignocellulosic biomass (agro-waste) hydrolysates as a sole carbon source largely relies on extractable and fermentable constituents, i.e. holocelluloses, in the biomass which can be extracted by pretreatment technologies involving physical, chemical and enzymatic hydrolysis, subsequent to fermentation to products, including bioalcohols using commercial strains of *Saccharomyces cerevisiae* (Bailey, 1992; Jung *et al.*, 2011; Khamaiseh *et al.*, 2014; Kumar *et al.*, 2009c). Comparatively, *S. cerevisiae* is the most commonly used yeast for production of bioethanol at an industrial scale (Bailey, 1992); albeit, using easily fermentable constituents in a broth.

The challenges associated with fermenter performance for bioalcohols production is largely attributed to product hydrolysates inhibition during fermentation (Jönsson and Martín, 2016). Inhibitors can also be directly linked and associated with hydrolysis methods used to extract fermentable total reducible sugars (TRS) from lignocellulosic biomass (Jönsson and Martín, 2016), resulting in stunted cell growth of fermenters further leading to low bio-product concentration and fermenter productivity. Common inhibitory compounds are classified into three groups: 1) phenolic compounds (determined in this study), 2) furan derivatives, and 3) weak organic acids (Baba and Malik, 2015; Kim *et al.*, 2011; Parmar and Rupasinghe, 2012), largely produced during lignocellulosic biomass hydrolysis among fermentable holocellulose constituents galactose, mannose and xylose (Jin *et al.*, 2011), with cellulose predominantly producing glucose (Alvira *et al.*, 2010; Diaz *et al.*, 2013; Gao and Rehmann, 2014; Procentese *et al.*, 2017; Qing and Wyman, 2011). Overall, the suitability of pretreatment/hydrolysis methods used, i.e. biological, physical, and chemical , have not been developed to reduce the toxicity of constituents in the resultant pretreatment hydrolysate.

Recent studies have shown that biological hydrolysis has an ability to reduce the inhibitory by-products mostly from chemical hydrolysis, positively influencing productivity and biomass concentration generation during bioalcohol production (Narayanaswamy *et al.*, 2013). For most studies, hydrolysis is solely performed using cellulases; albeit, there are other enzyme cocktails that can be effectively used to perform both the delignification and holocellulolysis of renewal resources such as lignocellulosic biomass, including agro-waste, without the use of synthetic chemical and high energy processes. These enzyme cocktails include those found in the pods of *Nepenthes mirabilis,*

which were found to be suitable for holocellulolysis as they contain β-glucosidase, xylanases and carboxylesterase (Chan *et al.*, 2016). However, the fermenter performance in hydrolysate recovered from such *N. mirabilis* pod extract hydrolysis must be compared to hydrolysates of combined conventional hydrolysis methods, i.e. hot water, dilute acid and cellulases - an evaluation which can be understood using kinetic model parameter evaluations.

For an effective performance parameter determination thus evaluation, suitable mathematical kinetic models and experimental design to assess the impact of fermenter conditions including hydrolysates, is required (Ali *et al.*, 2017). The output of the kinetic models can lead to the assessment of optimal conditions and system control thus efficiency including media (hydrolysate) selection (Dhavale *et al.*, 2016). Previously, Monod, Moser, Tessier, Logistic, and Leudeking-Piret models have been used to describe the microbial growth, substrate consumption and product formation rates (Ali *et al.*, 2017; Dhavale *et al.*, 2016; Garnier and Gaillet, 2015). Therefore, they can be used to comparatively analyse hydrolysate suitability. However, the selection of these models depends on the required purpose of individual studies.

7.2 Objectives

The purpose for this part of the study was to determine the microbial growth, substrate utilization and the product formation kinetic parameters during fermentation processes using hydrolysates of *N. mirabilis*/cellulase (*N. mirabilis*/CP) in comparison to those of hot water/dilute acid/cellulase (HWP/DAP/CP)-mixed agro-waste hydrolysis systems for fermentations facilitated by a commercial South African *S. cerevisiae* strain (VIN13).

7.3 Materials and methods

The mixed agro-waste was prepared as discussed in Chapter 4 (section 4.1.1). The *S. cerevisiae* was grown in a medium containing 100mL of (YPD) broth (section 4.1 to 4.3), incubated for 24 h at 30°C. The broth aliquots were further cultured in a PDA medium at 30°C for 48 h until growth was observed. The colonies were further streaked out onto new PDA media plates to obtain pure cultures as reported in chapter 4. The inoculum size for fermentation was prepared by inoculating pure yeast isolates from the freshly grown cultures subsequent to inoculation in were into 5 mL YPD broth and incubated for 24 h at 30°C. To further prepare the inoculum, 100μL of the overnight culture was inoculated in 50 mL sterile YPD broth (pH 4.5) and incubated for 24 h at 30°C in a shaking incubator at 120 rpm, as reported in chapter 4 (section 4.4). The S. cerevisiae was then inoculated into hydrolysates from *N. mirabilis*/CP and HWP/DAP/CP pre-treatments.

7.4 Kinect prediction models

7.4.1 Monod and microbial growth kinetic parameters

The Monod model (Eq. 7.1.) is the well-known model used to describe the proliferation of organisms under nutrients rich conditions (Monod, 1949). In this study, the Monod model was used to investigate microbial growth kinetic parameters for *S. cerevisiae* using agro-waste hydrolysates from a single pot pre-treatment system, as the sole carbon source.

$$\mu = \frac{\mu_{max} S}{K_S + S} \tag{7.1}$$

Where, μ_{max}, is the maximum growth rate (h^{-1}) for unspecified reducible sugars and K_S is estimated half saturation constant (g/L), while S is the residual TRS concentration (g/L).

The saturation constant reported herein, i.e. K_s, illustrated the rapidity of microbial proliferation and it ability to attain a maximum specific growth rate (μ_{max}), with the reducible sugars being utilised; albeit the Monod's model is well-known to be applicable when there is minimal presents of inhibitors, i.e. in the hydrolysates used as fermentation medium and as metabolic by-products produced during the fermentation (Khalseh, 2016). Similarly, Eq. 7.2 was used for quantifying the yeast growth rate during fermentation, and does consider the total biomass concentration as a single component, it is based on the modified Malthus equation (Mewa-Ngongang *et al.*, 2017). Therefore, to further quantify the microbial growth, the Malthus equation was used as follows - Eq. 7.2.

$$\frac{dX}{dt} = uX \tag{7.2}$$

Where, fermenter concentration (X) and its maximum (X_{max}), is described in colony-forming units (CFU/mL) taking into consideration the inoculum size (X_0).

7.4.2 Modelling TRS consumption for simultaneous biomass and product formation

Theoretically, the TRS consumption in fermentations is primarily and directly proportional to the biomass generation and product formed, with $p\frac{dX}{dt} \gg qX$ (Eq. 3) Therefore, in this study, TRS consumption was evaluated using the Luedeking–Piret model, Eq. 7.3 (Dhavale *et al.*, 2016); assuming that product formation is directly liked to biomass generation.

$$-\frac{dS}{dt} = p\frac{dX}{dt} + qX \qquad 7.3$$

Whereby, $p = 1/Y_{x/s}$ (g/CFU), while q is the product formation coefficient (1/h) Therefore, Eq. 7.3 can be rearranged as shown in Eq. 7.4.

$$-dS = pdX + q\int X(t)\,dt \qquad 7.4$$

For overall TRS consumption and to determine residual TRS concentration, with the substitution of Eq. 7.2 in Eq. 7.4 followed by integration with the initial conditions of $t = 0$ and $S = S_0$, Eq. 7.5 was developed.

$$S = S_0 - pX_0\left\{\frac{e^{\mu_{max}t}}{\left\{1-\left(\frac{X_0}{X_{max}}\right)(1-e^{\mu_{max}t})\right\}} - 1\right\} - q\frac{X_{max}}{\mu_{max}}ln\left\{1-\left(\frac{X_{max}}{\mu_{max}}\right)(1-e^{\mu_{max}t})\right\} \qquad 7.5$$

7.4.3 Production formation kinetic parameter determination

The productivity of a fermentation system can be quantified using a modified Luedeking–Piret model (Eq. 7.6), whereby the parameters can be directly evaluated in relation to fermentation data generated in particular bio-products of interest formed (Mewa-Ngongang *et al.*, 2017).

$$\mathrm{P(t)} - \mathrm{P_0} - \mathrm{n}\left(\frac{\mathrm{X_{max}}}{\mu_{\mathrm{max}}}\right)\ln\left[1-\left(\frac{\mathrm{X_0}}{\mathrm{X_{max}}}\right)(1.0 - \mathrm{e}^{\mu_{\mathrm{max}}\mathrm{t}})\right] = \mathrm{m}[\mathrm{X(t)} - \mathrm{X_0}] \qquad 7.6$$

With P being the product concentration (Area %), while n (Area %.mL/CFU.h) and m (Area %.mL/CFU) are associated the Luedeking–Piret model constants.

7.4.4 Data handling, relative differences and other kinetic parameters

The experimental data and kinetic models were computed and analysed using Microsoft Excel 2013, while for other models certain build-in functions such Solver® were used. Furthermore, the relative differences were determined (Eq. 7.7 and 7. 8) to illustrate the significance of the differences observed for the performance of the hydrolysates of the *N. mirabilis*/CP and the HWP/DAP/CP agro-waste pretreatment systems. For reporting, the minimal microbial concentration detectable limit used was $\log_{10}$ (CFU/mL) = 2. Other evaluated kinetic parameters are listed in Eq. 7.9 to 7.12, with the reference amount, for both absolute and relative differences being the *S. cerevisiae* (VIN13) fermentations using hydrolysates from HWP/DAP/CP-agro waste pretreatment systems.

$$\text{Absolute difference} = \left| New\ amount^{N.mirabilis/CP} - Reference^{HWP/DAP/CP} \right| \quad 7.7$$

$$\text{Relative difference} = \frac{\text{Absolute difference}}{Reference^{HWP/DAP/CP}} \text{ x } 100 \quad 7.8$$

$$Y_{x/s} = \frac{dX}{dS} \quad \text{(Biomass yield based on substrate consumption)} \quad 7.9$$

$$Y_{p/x} = \frac{dP}{dX} \quad \text{(Product yield based on biomass generated)} \quad 7.10$$

$$r_s = \frac{dS}{dt} \quad \text{(Substrate utilization rate)} \quad 7.11$$

$$r_p = \frac{dP}{dt} \quad \text{(Product formation rate)} \quad 7.12$$

7.5 Results and discussion

7.5.1 Microbial growth parameters using mixed agro-waste pre-treatment hydrolysates

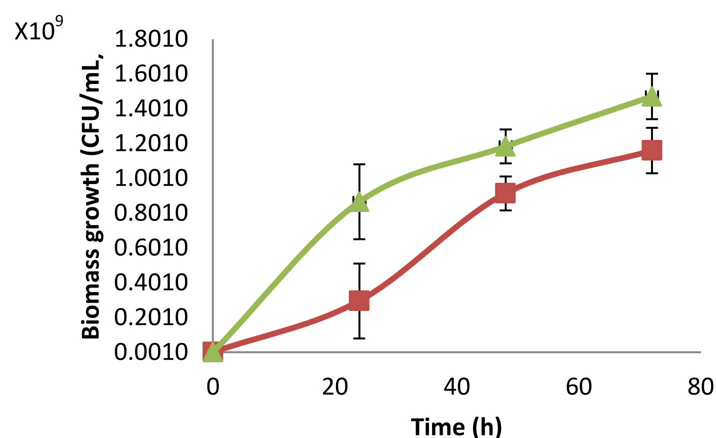
In this study, kinetics of cellular growth, substrate utilization and bioalcohols production were determined, using a commercial *S. cerevisiae* strain in hydrolysates obtained from the pre-treatment of mixed agro-waste constituted using peels of *C. sinensis* and *M. domestica*, including cobs of Z. mays and yard waste, i.e. from *Q. robur*. A source of the hydrolysates is the newly proposed *N. mirabilis*/cellulases pre-treatment method, which was compared to conventional HWP/DAP/CP

methods for the pre-treatment of lignocellulosic biomass. The maximum *S. cerevisiae* strain (VIN13) growth was determined using cellular counts; see Fig. 7.1 and Table 7.1, indicating a maximum cellular concentration of 1.47 x10^{10} CFU/mL for the HWP/DAP/CP hydrolysates compared to 1.16 x10^{10} CFU/mL attained for *N. mirabilis*/CP, with a differentiation quantified as a relative difference being 21.1 %. This was attributed to the highest TRS concentration attained during the pre-treatment using the HWP/DAP/CP; albeit this method is not specific to holocellulose extraction but does degrade the lignin, than the more holocellulose targeted *N. mirabilis*-based pretreatment method. This suggested that the *S. cerevisiae* strain would have had an adequate substrate supply during the fermentation process. However, maximum bioalcohols production was obtained using *N. mirabilis*/CP hydrolysates, which illustrated the limited inhibition characteristic of the hydrolysates as compared to those of the HWP/DAP/CP system, an observation which was confirmed by highest total residual phenolic content (TRPCs), i.e. 4.26 and 5.65 mg/L, for *N. mirabilis*/CP and HWP/DAP/CP hydrolysates, respectively, with the highest inhibition effects observed for the HWP/DAP/CP as only 1.31 Area % mL/CFU (x 10^{-10}), at a relative difference of 20.2% to that observed for *N. mirabilis*/CP. Overall, the impact of the TRPCs has been observed with stunted product formation in HWP/DAP/CP, whereby the formed product was lesser than that in which the hydrolysates of *N. mirabilis*/CP were used as the sole nutrient media source. Furthermore, the TRS consumption has been observed with an increase in the progression of the fermentation cycle. This showed that the metabolism of the *S. cerevisiae* strain used remain intact, thus continued product formation during the fermentation process. Theoretically, a high concentration in TRS results in a high volume of bioalcohols being produced (Sánchez-Femat *et al.*, 2016), unless if there are inhibitory compounds in the hydrolysates, in which case the fermenter will use most of the available TRS to counteract the effects of the inhibitors (Kim *et al.*, 2013b). The initial TRS concentration (S_o) was 0.311 and 3.22 g/L, with the residual TRS concentration (S) of 0.075, and 0.439 g/L at the end of *N. mirabilis*/CP and HWP/DAP/CP fermentations, respectively. This further confirmed of the suitability of using HWP/DAP/CP methods for delignification-cellulolysis operations as reported elsewhere. The consumption of TRS during the fermentation has been previously studied using lignocellulosic biomass extracts as primary substrates, while *S. cerevisiae* was used as the fermenter (Lian *et al.*, 2014; Ali *et al.*, 2017; Sánchez-Femat *et al.*, 2016), which successes attributed to the effectiveness of the pre-treatment methods used. By proposing a new method of pre-treatment, hydrolysates achieved, must perform similarly to conventional methods with added beneficial attributes, or should outperform the relatively established methods as is the case with *N. mirabilis/* CP pre-treatment proposed being proposed herein this study.

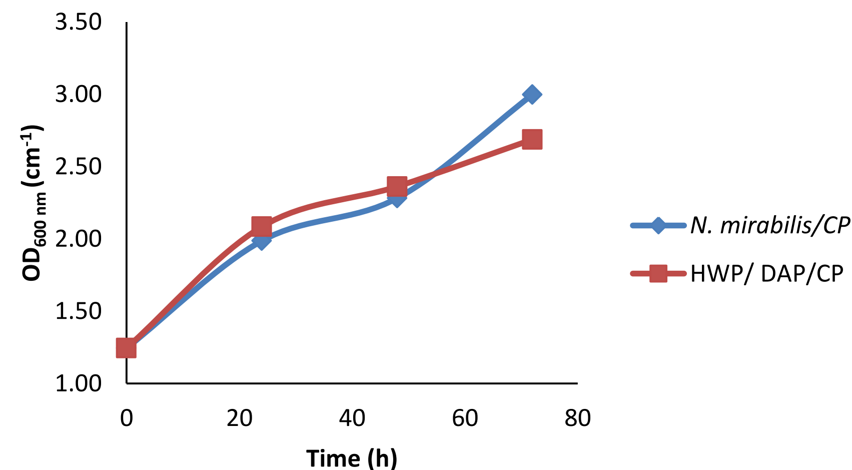
Table 7.1: Resultant kinetic parameters for *S. cerevisiae* (VIN13) fermentations using hydrolysates from *N. mirabilis* /CP- and HWP/ DAP/CP-agro waste pretreatment

Hydrolysates	X_{max} (x 10^{10} CFU/mL)	S_o/S (g/L)	P (%Area) (ethanol; butanol;phenylethyl)	$Y_{x/s}$ (CFU/g x 10^{-13})	$Y_{p/x}$ (Area %.mL/CFU x 10^{-10})	μ_{max}/μ (h^{-1})	K_s (g/L)	TRPCs (mg/L)
N. mirabilis /CP	1.16	0.311/0.075	1.83 (1.23;0.23;0.38)	4.92	1.58	1.76/0.095	1.32	4.26
HWP/DAP/CP	1.47	3.22/0.439	1.93 (1.02;0.53;0.38)	0.53	1.31	1.58/0.088	7.46	5.65
Absolute difference	0.31	n/d	0.1	4.39	0.3	0.2/0.01	6.1	1.4
Relative difference (%)	21.1	n/d	5.2	829.9	20.2	12/9.1	82.3	24.6

Xmax – maximum cell concentration (x 109 CFU/mL), So – initial substrate (TRS) concentration (g/L), S – residual substrate (TRS) concentration (g/L), P – bioalcohol production (% area GC-MS), TRPCs – total residual phenolic compounds (mg/L), Yx/s [Xmax/(So-S)] – biomass yield based on substrate consumption (CFU/g x 10-3), Bioalcohols – bioethanol, biobutanol, phenylethyl alcohol,



a)



b)

Figure 7.1: *S. cerevisiae* (VIN13) growth in *N. mirabilis*/CP and HWP/DAP/CP hydrolysates at 30°C a) biomass growth rate, b) Optical density

Furthermore, the alcohol concentration were obtained as 1.83 and 1.93 %Area for *N. mirabilis/*CP and HWP/DAP/CP respectively, with a relative difference of 5.2% (Table7.1). However, the higher TRS concentration, i.e. 3.22 g/L did not show significant difference in terms of the alcohol produced. Therefore, alcohol production was better when the *N. mirabilis*/CP hydrolysate was used as a carbon source as compared to HWP/DAP/CP, this was confirmed by the lower initial TRS concentration of *N. mirabilis*/CP hydrolysate, while obtaining similar alcohol production using hydrolysates from the HWP/DAP/CP system. A similar study has been done by Khalseh (2015), whereby 22.12 g/L (2.23 w/w %) of ethanol was obtained with 50 g/L of glucose. Furthermore, severally by-products (see Appendix 6, Table 10.9) were obtained, which can add value in the biorefinery industry.

The biomass formation yield ($Y_{X/S}$) based on the TRS consumption and product formation yield ($Y_{P/x}$) based on biomass generated were observed to be 4.92 and 0.53 (CFU/g x 10^{-13}), and 1.58 and 1.31 Area %.mL/CFU x 10^{-10} for *N. mirabilis*/CP and HWP/DAP/CP respectively, with the HWP/DAP/CP hydrolysates showing a relatively rudimentary biomass yield, as shown in Table. 7.1, which translated into a relative difference of 830 %. To overcome such a momentous challenge, the results obtained in this study showed that the higher bioalcohol production and reduction of inhibition by-products reported as TRPCs can be achieved when *N. mirabilis*/CP pre-treatment is used with the resultant hydrolysates being suitable as a media source for fermentation in the biorefinery industry. Moreover, and as an alternative, the cell density (OD_{600}) was also analyzed as showed in Fig. 7.1b, whereby the optical density was similar for both conventional and *N. mirabilis/*CP hydrolysates fermentation, albeit this methods quantifies the turbidity of the total biomass in the samples irrespective of its activity and/or cultivability-which ultimately can include dead biomass.

7.5.2 Kinetic data and model fitting

Kinetic rates of the *S. Cerevisiae* facilitated fermentation were investigated during bioalcohol production. Malthus model was used to determined and describe the microbial growth of the fermenter. The specific and maximum specific growth rates (Table 1) were, 1.76, 1.58 h^{-1} (i.e. μ_{max}) for *N. mirabilis*/CP and HWP/DAP/CP respectively, with the highest μ_{max} being observed for the *N. mirabilis*/CP hydrolysates, with a significant relative difference of 12 % and 9.1% for the specific growth rate. The μ_{max} value obtained in this study is relative similar to reported in recent studies, whereby the *S. cerevisiae* was used as a fermenter; albeit with commonly used (refined) media (Ali *et al.*, 2017). However, in some studies the μ_{max} related to *S. cerevisiae* in the batch system under acidic conditions was only 0.5717 h^{-1} (Bailey, 1992). Furthermore, Table 2 illustrate additional kinetic parameter deemed important in this study, whereby a biomass formation rate was observed as 1.61 and 2.04 x10^{8}CFU/mL.h, with an absolute difference of 21.1% for *N. mirabilis*/CP and HWP/DAP/CP

cultures, respectively; while the product formation rate for both *N. mirabilis*/CP and HWP/DAP/CP was very low, i.e. 0.025 and 0.027 Area %/h –with a minute 5.26% relative difference considered to be insignificant; albeit at laboratory scale with significant product margin being suggested to be large at an industrial scale. Similar, substrate utilization rates of 0.0033 and 0.0387 g/L.h for *N. mirabilis*/CP and HWP/DAP/CP respectively were indicative that a lot of the energy source, i.e. 91.1 % relative difference, was used for other metabolic biomass maintenance function that for product generation, an anomaly previously attributed to high TRPCs in hydrolysates of the HWP/DAP/CP pretreatment regime used.

Table 7.2: Additional kinetic rates for S. cerevisiae (VIN13) fermentations using hydrolysates from *N. mirabilis* /CP and HWP/ DAP/CP-agro waste pretreatment

Parameter	Description (units)	*N. mirabilis*/CP	HWP/DAP/CP	Relative difference (%)
r_x	Biomass formation rate ($x10^8$ CFU/mL.h)	1.61	2.04	21.1
r_p	Product formation rate (Area %/h)	0.025	0.027	5.26
r_s	Substrate utilization rate (g/L.h)	0.0033	0.0387	91.5

7.5.3 Product formation kinetics and substrate consumption rate and modeling

Additional parameters related to product formation were quantified using the modified Luedeking–Piret model, which integrates TRS biomass concentration and product formation using S. cerevisiae in *N. mirabilis*/CP and HWP/DAP/CP hydrolysates. By further modifying the Luedeking–Piret model to fit the experimental data, Eq. 7.7 was used by discarding the n constant function as in Eq. 7.8, since is non-growth associated product formation.

$$\mathrm{P(t) - P_0 = m[X(t) - X_0]} \qquad 7.8$$

Whereby, a plot of $P(t) - P_0$ versus $[X(t) - X_0]$ would generate a linear trend-line with a slope *m* (Area %. mL /CFU), which illustrate the achievable rate of bioalcohol formation.

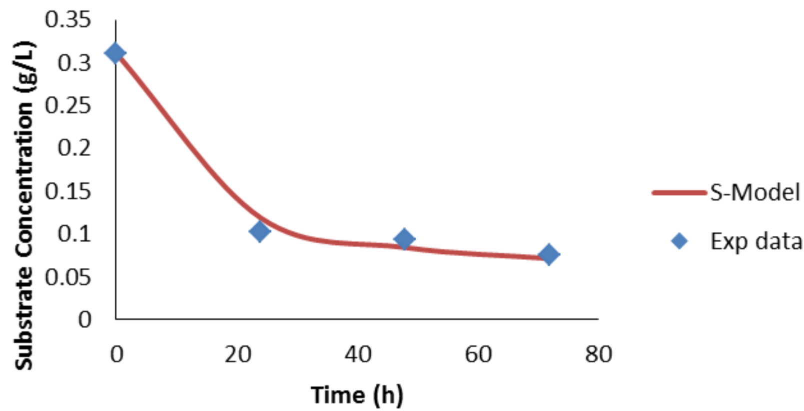
Table 7.3, illustrates the linkages of the product concentration with cell density and Luedeking–Piret models constants. The achievable bioalcohol formation rate was observed as 1.0035 and 0.4848 Area %.mL/CFU in relation with the fermenter concentration. The correlation coefficient (R^2) of 0.941 and 0.4981were obtained using modified Luedeking–Piret models for *N. mirabilis*/CP and HWP/DAP/CP hydrolysate cultures, respectively. However, the product formation for HWP/DAP/CP hydrolysates indicated a perhaps non-related production formation to the biomass generated as observed with a lower correlation coefficient (R^2) of 0.481, which can be associated with inhibitors. Furthermore, the results obtained showed that the cell growth rate and bioalcohol production are inter related with similar results have being by Ali *et al.* (2017), whereby the concentration of biomass increases resulted with the increase with bioalcohols production, in particular for the *N. mirabilis*/CP hydrolysate cultures. Therefore, with these results obtained, it was illustrated that the modified Luedeking–Piret model predictably demonstrated the product formation of the *N. mirabilis*/CP pre-treatment systems.

Table 7.3: Interlinkages of product concentration with cell maximum density and substrate utilization as eluded by the Luedeking–Piret model for N. mirabilis /CP and HWP/DAP/CP pretreatment extracts

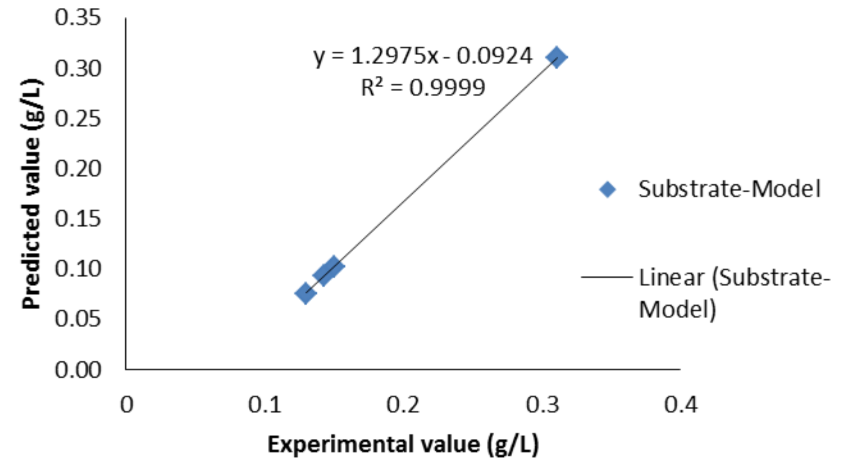
Parameter	Description (units)	*N. mirabilis*/CP	HWP/DAP/CP
R^2	Correlation coefficient	0.941	0.4981
m	Slope (Area %.mL/CFU)	1.0035	0.4848
**p*	g/CFU x10^{12}	2.0	18.9
**q*	1/h x 10^{-5}	7.84	0.185

* Luedeking–Piret model constant value generated using Microsoft Excel Solver

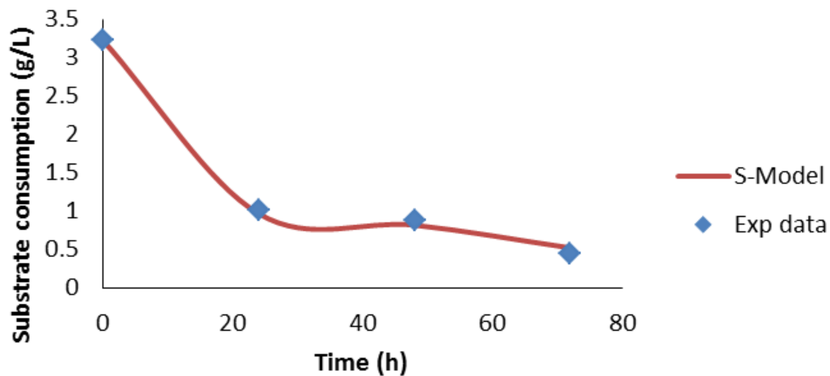
As the substrate was prepared by pretreating mixed agro-waste to produce fermentable sugars, i.e. total reducing sugar (TRS),to produce bioalcohols using *S. cerevisiae,* Microsoft Excel solver was used to predict the rates constant (i.e. p and q) of the Luedeking–Piret model – see Table. 7.3. In Fig. 7.3 a, and c , the response of the Luedeking–Piret model for substrate consumption was demonstrated, while Fig 7.3 b, and d shows a comparison between actual experimental and model predicted data for substrate consumption. Substrate consumption theoretically illustrates the propensity of microorganism to utilize the substrate for the purpose of producing the bioalcohols of interest, irrespective of the presence of inhibitory by-products from the pre-treatment of biomass. When the Luedeking–Piret model was used for predicting the substrate utilization kinetic parameters, coefficients determined, i.e. for *p* was 2.18 x10^{-8} and 1.75 x10^{-7} (g/CFU), while for *q* values attained were 7.84 x10^{-5} and 1.85 x10^{-4} (1/h), for *N. mirabilis*/CP and HWP/DAP/CP fermentations, respectively – see Table. 3. These parameters values are related to the fermentations substrate consumption rate and the microorganism's ability to proliferate – which in turn is assumed to be influential in product formation. The results obtained showed an excellent correlation with experimental data (i.e. R^2 of 0.9999 and 0.9999) for *N. mirabilis*/CP and HWP/DAP/CP, respectively, which confirmed a high significance of the model (see Fig. 7.3). Similar results have been obtained by Ali *et al.* (2017), whereby the Luedeking–Piret model was used to describe the batch fermentation with an adequacy correlation coefficient (R^2) of 0.984, between the model and the experimental data.



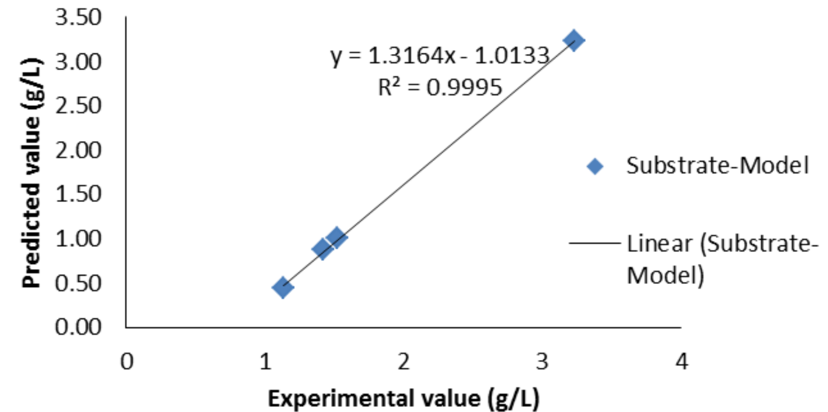
a) *N. mirabilis*/CP



b) Experimental and Model Predicted values of *N. mirabilis*/CP hydrolysates fermentations



c) HWP/DAP/CP



d) Experimental and Model predicted values of HWP/DAP/CP hydrolysates fermentations

Figure 7.2: Comparison of substrate consumption experimental data and kinetic model by Luedeking–Piret, a) Experimental data (dotted line)and model prediction (solid line) using N .mirabilis/CP , b) Comparison of predicted value versus experimental data using *N. mirabilis*/CP filtrate as a substrate, c) Experimental data (dotted line)and model prediction (solid line) using hot water/dilute acid/cellulases, and d) Comparison of predicted value versus experimental data using HWP/DAP/CP filtrate as a substrate**.**

7.6 Summary

In this study, the proposed kinetic models were useful for the evaluations of the cell growth rate, substrate consumption and bioalcohol production for a single pot system under aerobic conditions in which *N. mirabilis*/CP and HWP/DAP/CP pre-treatments hydrolysate were tested. The suitability of single pot multi-reaction system for applications in biorefinery industry has also been demonstrated, hence, the insignificant differences in the maximum cell biomass between *N. mirabilis* /CP and HWP/DAP/CP (conventional methods) pre-treatment hydrolysate. Additionally, the direct association between bioalcohols production and cell growth rates was demonstrated using a modified Luedeking–Piret model. Furthermore, a better correlation coefficient (R2) of predicted substrate utilization was observed for *N. mirabilis*/CP compared to HWP/DAP/CP pre-treatments hydrolysate fermentations. Based on the results obtained, the pretreatment of mixed agro-waste using *N. mirabilis*/CP proved, useful in bioalcohols fermentation using *S. cerevisiae*, and also for applications in the biorefinery industry.

CHAPTER 8

CONCLUSION AND RECOMMENDATIONS

CHAPTER 8

8. SUMMARY, CONCLUSION AND RECOMMENDATIONS

8.1 Summary and Conclusion

Generally, lignocellulosic biomass is the most abundant organic matter residue with the waste from the agricultural and forestry processing, being the most suitable for an advanced biorefinery. However, the recalcitrant structure of the biomass has led to new developments and processes being developed for the pretreatment of such biomass. The literature reviewed, has proven that acid pretreatment has been the recommended method for biomass pretreatment; albeit, the method still has challenges associated with inhibitor generation, which affects fermentation to produce added value products. Therefore, in this study, common conventional pre-treatment methods (i.e. hot water, dilute acid and cellulases) and *N. mirabilis* "monkey cup" extracts, were comparatively evaluated, using kinetic models to assess the *S. cerevisiae* performance and to determine the feasibility of the *N. mirabilis* pretreatment method proposed.

In this study, studies were conducted by developing an integrated single pot system with multi-reactions using common conventional methods to pre-treatment mixed agro-waste to produce fermentable sugars (i.e. hydrolysates as substrate) for bio-alcohol production. A high concentration of TRS was observed with significant increases, i.e. 3.22 g/L when a commercial cellulase was supplemented. To further quantify the suitability of this type of system, the FTIR and pXRD were analysed, whereby results demonstrate diffractions peaks associated with cellulose crystallinity in pretreated agro-waste. The commercial *S. cerevisiae* strain was able to produce low (C_1 to C_3) and high carbon content (C_4^+) bioalcohols during fermentation processes. For an integrated single pot system hydrolysates, demonstrated to have potential to produce different bioalcohols and multiple products, which can benefit the biorefinery industry. However, the common convention methods are associated with inhibitors during fermentation. Due to this, *N. mirabilis* extracts were evaluated, due to the diverse digestive enzymes that are available in it. The microbial community in the *N. mirabilis* extracts revealed *Klebsiella oxytoca* (KF55591), *Bacillus thuringiensis* (KF557957), *Bacillus cereus* and *Bacillus anthracis* using 16S rDNA and vitek system, as the dominant species, some of which have been associated with the production of enzymes responsible in cellulolysis. The enzyme activity for carboxylesterases, xylanases and β-glucosidases was confirmed, which was performed to determine the suitability of enzymatic cocktail of the pitcher juice to facilitate the pre-treatment of lignocellulosic biomass, which resulted in higher non-soluble lignin residue, than holocelluloses, which were extracted. Therefore, the results obtained from the pre-treatment of mixed agro-wastes with *N. mirabilis* extracts have demonstrated their ability to be effective in TRS extraction and phenolic compound reduction during pre-treatment.

Therefore, the *N. mirabilis* supplementary extracts can be utilized as the alternative pretreatment method for the biorefinery industry. Moreover, an alternative single pot multi-reaction process can be advantageous in the pre-treatment stages of agro-wastes to completely biovalorise a mixture of different wastes. Furthermore, by further pre-treating the mixed ago-waste with commercial cellulases, further increases in TRS and reduction in TRPCs, i.e. from 6.25 ±0.18 to 4.26 ±0.09 can be obtained, when the single pot multi-reaction system is used. The pXRD diffraction peaks related to cellulose crystallinity in residual pre-treated agrowaste were demonstrated, with a significant degradation profile from *N. mirabilis* pre-treated agro-waste. FTIR analysis indicated the detection of amorphous and crystalline structure of cellulose, which was similar with other studies that have been done to quantify the crystalline of cellulose. Based on the results obtained, the FTIR analyses indicates also losses in functional groups associated with holocelluloses, i.e. the enzymatic hydrolysis analyses also confirmed that the pre-treatment with *N. mirabilis* extract has an ability to decompose the agro-waste, with functional group prominently associated with xylan/hemicellulose and cellulose being reduced; indicative of structural deformation, thus better holocellulose extraction.

To further quantify the feasibility of the *N. mirabilis*/CP pre-treatment methods, kinetic models were used to evaluate the microbial growth rate, product formation rate and substrate consumption using hydrolysates from the pretreatment methods. The results obtained showed a suitability of the single pot multi-reaction hydrolysate as an alternative method for mixed agro-waste pre-treatment for the biorefinery industry. The maximum cell biomass, i.e. 1.47 $x10^{10}$CFU/mL was observed with HWP/DAP/CP pre-treatment hydrolysate. The Luedeking–Piret model demonstrated that bioalcohols formation is related to cell growth rate, whereby the relative difference was insignificant between the two treatment methods. Better correlation coefficient (R^2) of predicted substrate utilization was observed for *N. mirabilis/*CP compared to HWP/DAP/CP pre-treatments hydrolysates. Based on the results obtained, the pretreatment of mixed agro-waste using *N. mirabilis/CP*, have demonstrated to be an alternative for producing hydrolysates for bioalcohols fermentation using *S. cerevisiae* for the biorefinery industry.

8.2 Recommendations

Several studies have been developed to reduce the inhibitory by-product formation during fermentable sugar extraction. The primary aim of this study was to develop a novel and integrated single pot multi-reaction system using renewal and natural bioresource, to reduce fermenter inhibitors that are associated with pre-treatment methods. To overcome these challenges a cocktail of enzyme from *N. mirabilis* pods was used, which demonstrated an ability to reduce phenolics while increasing the TRS concentration yield. Based on the literature reviewed there is less known about the ability of *N. mirabilis* extract to pre-treat lignocellulosic biomass. Therefore it is recommended that:

- More investigations are required on the biodegradation of individual substrates (e.g. corn cobs), when a *N. mirabilis* extract are used as sole supplements for pretreatmentprior to the evaluation of the effect of mixed agro-waste in comparison with single substrates.
- Investigation of individual enzymes (i.e. in the *N. mirabilis* cocktail) and the optimum conditions in which that are able to function effectively.
- More studies are required to further quantify the generation of other inhibitors including furfural, when a *N. mirabilis* extracts are used.
- To undertake comparative studies of energy and cost analysis including savings by forgoing multi process units, by further developing the integrated single pot multi-reaction system proposed in this study.

CHAPTER 9

REFERENCES

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CHAPTER 10

APPENDICES

CHAPTER 10

10. APPENDICES

Appendix 1: Feedstock availability in South Africa

Citrus fruit in South Africa is main produces in Limpopo, Eastern Cape, Mpumalanga, Western Cape and KwaZulu-Natal. Citrus fruit is among the top fruit that are produced in the world, e.g. in year season 2016/2017, 49.6 million metric tonnes were forecasted (Hunlun *et al.*, 2017). However the citrus pees from processing industry are ending up in the landfills. However for apples Western Cape and Eastern Cape are main producers of this type of fruit.

Table 10.1: Production areas of Citrus fruit (ha)

	Orange	Soft Citrus	Lemon & Lime	Grape fruit
Easter Cape	4226	2915	3795	208
KwaZulu-Natal	382	-	373	706
Limpopo	16008	1866	2569	4242
Mpumalanga	1966	381	213	1566
North West	49	-	34	-
Northern Cape	285	-	175	420
Western Cape	2339	3892	807	39

Appendix 2: Enzymes activities

A. β-glucosidase activity

Table 10.2: *β-glucosidase* assay reagents

	Blank (μL)	**Sample (μL)**
Substrate with buffer	500	500
Sterile distilled water	200	-
Enzyme (extract) solution	-	200
Na_2CO_3	-	-
Total	**800 μL**	**800 μL**

Spectrophotometer settings: β-glucosidase activity assay

The JENWAY 6405 UV/Vis spectrophotometer (Cole-Parmer, UK) settings used were as follows:

- Kinetics setting, with
- UV/Vis light switched on, for a
- Reading at 410 nm for 2 min at 10 sec. intervals, while the

- Temperature was 25 °C, using an
- Extinction coefficient of 18100 $M^{-1}.cm^{-1}$.

Preparation of 50 mM sodium acetate (pH 6)

- 800 mL of sterile distilled water was prepared in a 1000 mL Schott bottle.
- Add 3.859 g of Sodium Acetate (anhydrous Mw: 82 g/mol) to the 800 mL of sterile distilled water.
- Add 0.176 g of Acetic Acid (Mw: 60.05 g/mol) to the 800 mL of sterile distilled water.
- Adjust pH to 6 using 1 M NaOH and add distilled water until volume is 1 L.
- Store at 4°C

B. Xylanase activity

Table 10.3: Xylanase activity assay reagents

	Blank (μL)	**Sample (μL)**
Substrate & McIlvaine's buffer (1% w/v)	1000	1800
Sterile distilled water	500	-
Enzyme (extract) solution	-	200
DNS Reagent	-	-
Total	**2000μL**	**2000 μL**

Spectrophotometer settings: Xylanase activity assay
The JENWAY 6405 UV/Vis spectrophotometer (Cole-Parmer, UK) settings used were as follows:

- Kinetics setting, with
- UV/Vis light switched on, for a
- Reading at 575 nm for 2 min at 10 sec. intervals, while the
- Temperature was 25 °C, using an
- Extinction coefficient of 33000 $M^{-1}.cm^{-1}$.

Preparation of Buffer McIlvaine's (pH 5)

- 100 mM McIlvaine's (pH 5) was prepared by adding 10.30 mL of disodium phosphate (0.2 M, Mw: 178 g/mol) and 09.70 mL of citric acid (0.1M, Mw: 192.1 g/mol) in to a 50 mL Schott bottle to make up a volume of 20 mL.
- Store at 4°C

C. Carboxylesterase activity

Table 10.4: Carboxylesterase activity assay reagents

	Blank (μL)	**Sample (μL)**
0.1M Tris-HCL buffer (pH = 7.8)	200	200
0.6mM pNPA in acetone	300	300
Sterile distilled water	300	
Enzyme (extract) solution	-	300
Total	**800 μL**	**800 μL**

Spectrophotometer settings: carboxylesterase activity assay
The JENWAY 6405 UV/Vis spectrophotometer (Cole-Parmer, UK) settings used were as follows:

- Kinetics setting, with

- UV/Vis light switched on, for a
- Reading at 410 nm for 2 min at 10 sec. intervals, while the
- Temperature was 25 °C, using an
- Extinction coefficient of 17000 $M^{-1}.cm^{-1}$.

Preparation of 100 mM Tris -HCL (pH 7.8)

- ➢ The buffer was prepared by dissolving 27.15 g/mol of Tris – Hydroxymethyl amino methane [$C_4H_{11}NO_3$ (Mw: 121.14 g/ml)] in a 800 mL sterile distilled water.
- ➢ Adjust pH to 7.8 using
- ➢ Adjust the volume to 1000 mL using sterile distilled water.
- ➢ Autoclave at 121 for 15 minutes
- ➢ Store at °C

D. Cellulase activity

Procedure

Cellulases activity was measured by using filter paper as a substrate, with the assay being prepared using a suspension 100 to 600 µL enzyme/mL of a sodium citrate buffer (50 mM, pH 4.8) using an appropriate dilution (dilution factor) for computation.

Table 10.5: Cellulases activity assay reagents

	Blank (µL)	Sample (µL)	Sample (mg)
Sodium citrate buffer (pH = 4.8)	1000	1000	
Sterile distilled water	16600	16000	
Commercial Cellulase	-	600	
substrate			50
DNS	3000	3000	
Total	**20600 µL**	**20600 µL**	**50 mg**

Preparation of substrate

- The substrate by shredding Whatman No. 1 filter paper (i.e. cut into 1 × 6 cm strips)

Preparation of 50 mM sodium citrate buffer (pH 4.8)

- 800 mL of sterile distilled water was prepared in a 1000 mL Schott bottle.
- Add 15.54 g of Sodium Citrate dihydrate (mw: 294 g/mol) to the 800 mL of sterile distilled water.
- Add 9.059 g of Citric Acid (mw: 192.1 g/mol) to the 800 mL of sterile distilled water.
- Adjust pH to 4.8 using 1 M NaOH and add distilled water until volume is 1000 mL.
- Store at 4°C

Preparation of 3,5-dinitrosalicylic acid (DNS) Reagent

- The DNS reagent was prepared by adding 10 g of 3, 5-dinitrosalicylic acid (DNS), 2g of phenol (2 g), 0.5g sodium sulphite and 10 g sodium hydroxide was made-up to 1000 mL.

Preparation 40% (w/v) of sodium potassium tartarate

- Adding 40g of sodium potassium tartrate into 80 mL of sterile distilled water
- Add sterile distilled water until volume is 100 L
- store at 4°C

Procedure for cellulases activity

The assay was performed by adding cellulases (500 µL), in 1 mL of sodium citrate buffer (50 mM, pH 4.8) in test tubes (20 mL) followed by the addition of shredded Whatman No. 1 filter paper (50 mg, cut into 1 × 6 cm strips), subsequent to vortexing (CENCO, UK) for 1 min and incubation (1 h) at 50°C. The reaction was terminated by adding 3 mL of a 3,5-dinitrosalicylic acid (DNS) solution (Miller, 1959) to the reaction test tubes, subsequent to boiling (100°C) for 5 min. The assay mixture was cooled by adding a volume (16 mL) of sdH_2O prior to absorbance reading at 540 nm using a Jenway 7305 UV/Vis spectrophotometer (Cole-Parmer, UK). The blank was done using a similar procedure without the addition of the cellulases mixture (Adney and Baker, 1996; Eveleigh *et al.*, 2009). The cellulases activity quantification procedure was analogous to that developed by Yu et al. (2016), whereby the activity, i.e. as filter paper activity,

was calculated based on the quantity of 2 mg TRS being formed and/or released by a defined cellulases concentration used (Yu *et al.*, 2016). Eq.10.1 illustrates the quantification of activity of cellulases.

$$\text{Activity (U/L)} = \frac{0.37}{\text{Cellulase concentration to release 2 mg TRS}} \quad 10.1$$

Table 10.6: **FPU for cellulases at different concentration**

Cellulases concentration (mg/ml)	FPU (IU/ml
0.015	24.67
0.00875	42.29
0.00780	47.46
0.0075	49.33
0.005	74.00
0.00375	98.67

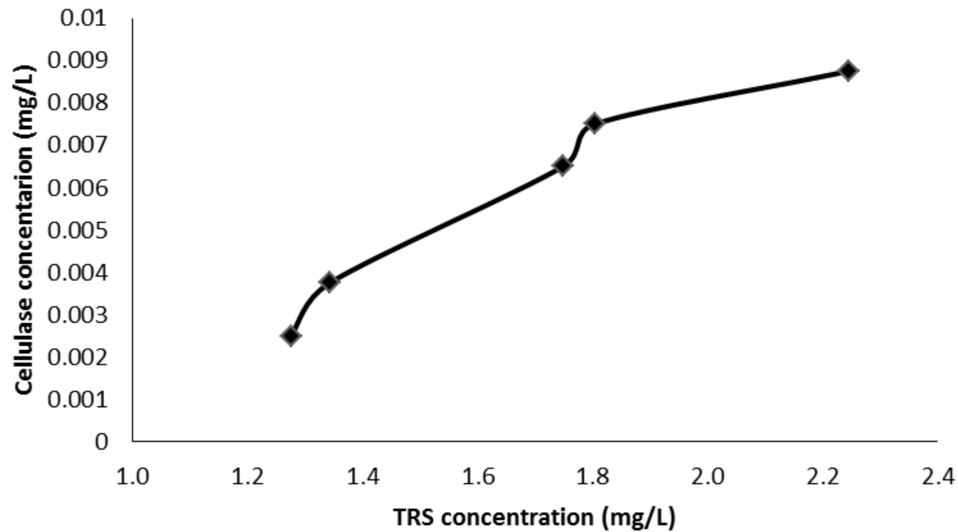

Figure 10. 1. : TRS concentration measure at different cellulase concentration

Appendix 3 Used Primmer for *N. mirabilis* and *S. cerevisiae*

A) **N. mirabilis**

Primers (reverse and forward) used:

27F (5’-AGAGTTTGATCMTGGCTCAG-3’)

1492R (5’-GGTTACCTTGTTACGACTT-3’)

N. mirabilis pod extracts accession number KY249126.1, DQ513324.1 and KU948294.1

Nucleotide sequences submitted on Genbank

Query 1 GCGTTAACTTCAGCACTAAAGGGCGGAAACCCTCTAACACTTAGCACTCATCGTTTACGG 60
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Subject 870 GCGTTAACTTCAGCACTAAAGGGCGGAAACCCTCTAACACTTAGCACTCATCGTTTACGG 811
Query 61 CGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCGCCTCAGTGTCAG 120
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Subject 810 CGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCGCCTCAGTGTCAG 751
Query 121 TTACAGACCAGAAAGTCGCCTTCGCCACTGGTGTTCCTCCATATCTCTACGCATTTCACC 180
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Subject 750 TTACAGACCAGAAAGTCGCCTTCGCCACTGGTGTTCCTCCATATCTCTACGCATTTCACC 691
Query 181 GCTACACATGGAATTCCACTTTCCTCTTCTGCACTCAAGTCTCCCAGTTTCCAATGACCC 240
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Subject 690 GCTACACATGGAATTCCACTTTCCTCTTCTGCACTCAAGTCTCCCAGTTTCCAATGACCC 631
Query 241 TCCACGGTTGAGCCGTGGGCTTTCACATCAGACTTAAGAAACCACCTGCGCGCGCTTTAC 300
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Subject 630 TCCACGGTTGAGCCGTGGGCTTTCACATCAGACTTAAGAAACCACCTGCGCGCGCTTTAC 571
Query 301 GCCCAATAATTCCGGATAACGCTTGCCACCTACGTATTACCGCGGCTGCTGGCACGTAGT 360
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Subject 570 GCCCAATAATTCCGGATAACGCTTGCCACCTACGTATTACCGCGGCTGCTGGCACGTAGT 511
Query 361 TAGCCGTGGCTTTCTGGTTAGGTACCGTCAAGGTGCCAGCTTATTCAACTAGCACTTGTT 420
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Subject 510 TAGCCGTGGCTTTCTGGTTAGGTACCGTCAAGGTGCCAGCTTATTCAACTAGCACTTGTT 451
Query 421 CTTCCCTAACAACAGAGTTTTACGACCCGAAAGCCTTCATCACTCACGCGGCGTTGCTCC 480
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Subject 450 CTTCCCTAACAACAGAGTTTTACGACCCGAAAGCCTTCATCACTCACGCGGCGTTGCTCC 391
Query 481 GTCAGACTTTCGTCCATTGCGGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTCTGGGCC 540
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Subject 390 GTCAGACTTTCGTCCATTGCGGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTCTGGGCC 331
Query 541 GTGTCTCAGTCCCAGTGTGGCCGATCACCCTCTCAGGTCGGCTACGCATCGTTGCCTTGG 600
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Subject 330 GTGTCTCAGTCCCAGTGTGGCCGATCACCCTCTCAGGTCGGCTACGCATCGTTGCCTTGG 271
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Subject 270 TGAGCCGTTACCTCACCAACTAGCTAATGCGACGCGGGTCCATCCATAAGTGACAGCCGA 211
Query 661 AGCCGCCTTTCAATTTCGAACCATGCGGTTCAAAATGTTATCCGGTATTAGCCCCGGTTT 720
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Subject 210 AGCCGCCTTTCAATTTCGAACCATGCGGTTCAAAATGTTATCCGGTATTAGCCCCGGTTT 151
Query 721 CCCGGAGTTATCCCAGTCTTATGGGCAGGTTACCCACGTGTTACTCACCCGTCCGCCGCT 780
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Subject 150 CCCGGAGTTATCCCAGTCTTATGGGCAGGTTACCCACGTGTTACTCACCCGTCCGCCGCT 91
Query 781 AACTTCATAAGAGCAAGCTCTTAATCCATTCGCTCGACTTGCATGTATTAGGCACGCCGC 840
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Subject 90 AACTTCATAAGAGCAAGCTCTTAATCCATTCGCTCGACTTGCATGTATTAGGCACGCCGC 31
Query 841 CAGCGTTCATCCTGAGCCATG 861
|||||||||||||||||||||
Subject 30 CAGCGTTCATCCTGAGCCATG 10

B) S. cerevisiae

Primers (reverse and forward) used:

ITS1: 5'-TCCGTAGGTGAACCTGCGG-3'

ITS2: 5'-TCCTCCGCTTATTGATATGC-3'

Saccharomyces cerevisiae with the following accession number UW5FPV9U015

Nucleotide sequences submitted on Genbank

Query 1 TTTTGAAATGGATTTTTTTGTTTTGGCAAGAGCATGAGAGCTTTTACTGGGCAAGAAGAC 60
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Subject 5 TTTTGAAATGGATTTTTTTGTTTTGGCAAGAGCATGAGAGCTTTTACTGGGCAAGAAGAC 64
Query 61 AAGAGATGGAGAGTCCAGCCGGGCCTGCGCTTAAGTGCGCGGTCTTGCTAGGCTTGTAAG 120
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Subject 65 AAGAGATGGAGAGTCCAGCCGGGCCTGCGCTTAAGTGCGCGGTCTTGCTAGGCTTGTAAG 124
7
Query 121 TTTCTTTCTTGCTATTCCAAACGGTGAGAGATTTCTGTGCTTTTGTTATAGGACAATTAA 180
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Subject 125 TTTCTTTCTTGCTATTCCAAACGGTGAGAGATTTCTGTGCTTTTGTTATAGGACAATTAA 184
Query 181 AACCGTTTCAATACAACACACTGTGGAGTTTTCATATCTTTGCAACTTTTTCTTTGGGCA 240
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Query 241 TTCGAGCAATCGGGGCCCAGAGGTAACAAACACAAACAATTTTATCTATTCATTAAATTT 300
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Subject 245 TTCGAGCAATCGGGGCCCAGAGGTAACAAACACAAACAATTTTATCTATTCATTAAATTT 304
Query 301 TTGTCAAAAACAAGAATTTTCGTAACTGGAAATTTTAAAATATTAAAAACTTTCAACAAC 360
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Subject 305 TTGTCAAAAACAAGAATTTTCGTAACTGGAAATTTTAAAATATTAAAAACTTTCAACAAC 364
Query 361 GGATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCGAAATGCGATACGTAATGTGAATT 420
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Subject 365 GGATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCGAAATGCGATACGTAATGTGAATT 424
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Subject 425 GCAGAATTCCGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCTTGGTATTCCAGGG 484
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Subject 485 GGCATGCCTGTTTGAGCGTCATTTCCTTCTCAAACATTCTGTTTGGTAGTGAGTGATACT 544
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Query 601 TTTCTCTGCGTGCTTGAGGTATAATGCAAA 630
||||||||||||||||||||||||||||||
Subject 605 TTTCTCTGCGTGCTTGAGGTATAATGCAAA 634

Appendix 4: Calibration standard

A) Standard Curve of p-Nitrophenol

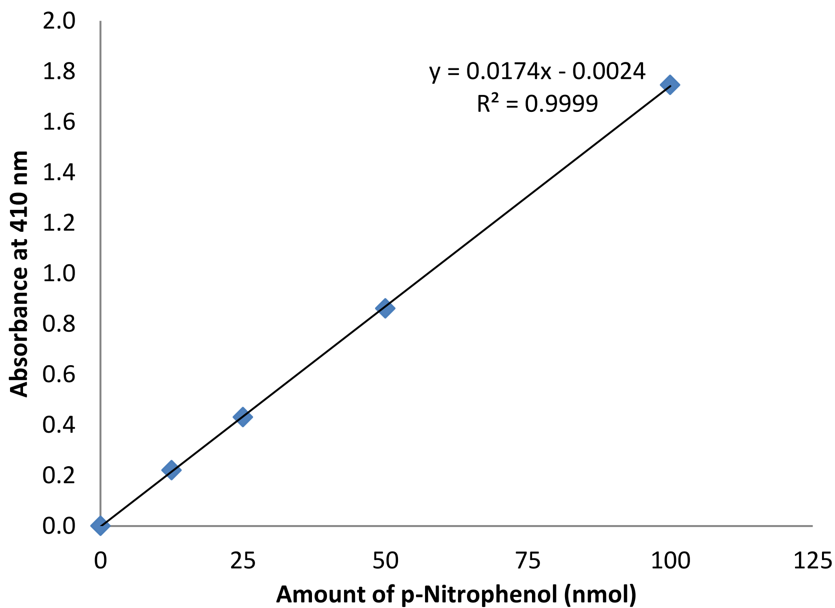

Figure 10. 2: Standard Curve of p-Nitrophenol

B) Standard Curve of Xylose

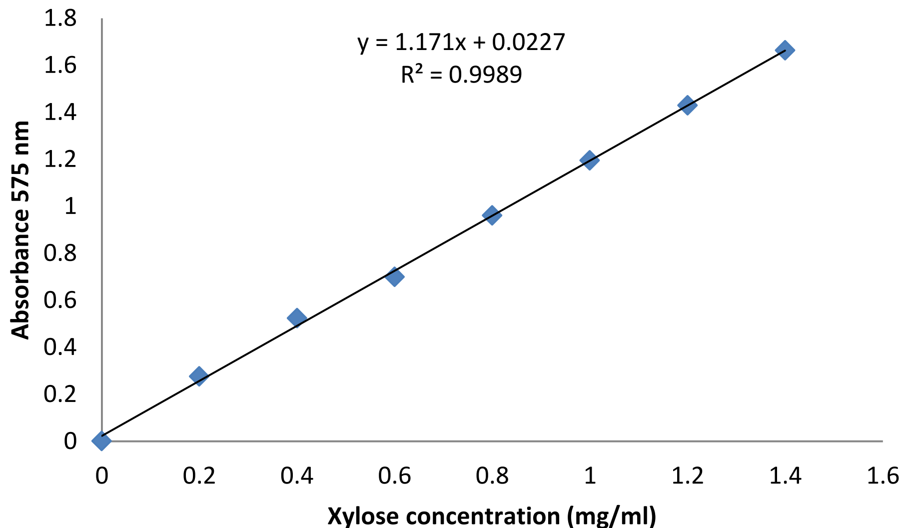

Figure 10. 3: Standard Curve of Xylose

C) Standard Curve of Glucose

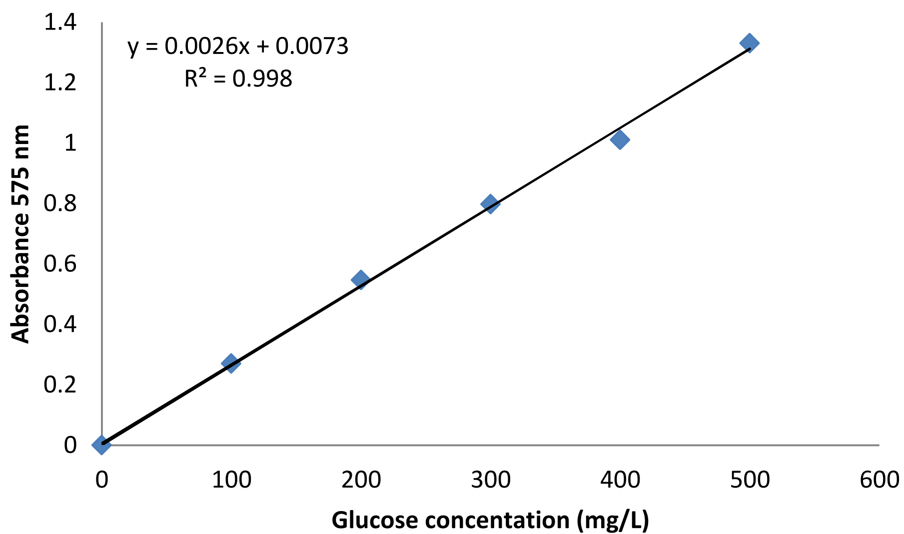

Figure 10. 4: Standard Curve of Glucose

D) Standard curve of 1, 2-dihydroxybenzene

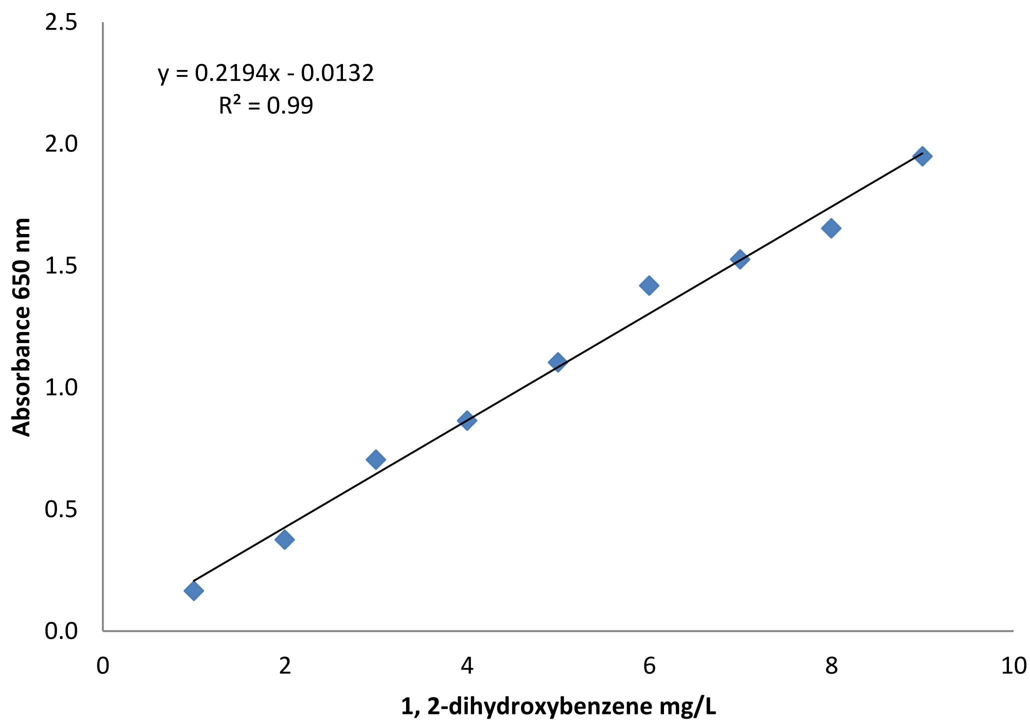

Figure 10. 5: Standard curve of 1, 2-dihydroxybenzene

Appendix 5: Microbial growth Kinetics

Table 10.7: **Common unstructured Kinetic models**

Model	**Kinetic mathematical representation**	**References**
Tessie (1942)	$\mu = \mu_{max}\left(1 - e^{-C_s/K_s}\right)$	(Şeker *et al.*, 1997)
Blackman (1905)	$\mu = \frac{\mu_{max}S}{K}$ if S<K $\mu = \mu_{max}$ if S≥K	(Bader, 1978)
Monod (1942)	$\mu = \frac{\mu_{max}S}{K_S + S}$	(Monod, 1949)
Haldane (1930)	$\mu = \frac{\mu_{max}}{K_s + S + S^2/K_i}$	(Garcıa-Ochoa and Casas, 1999)
Moser (1958)	$\mu = \frac{\mu_{max}S^n}{K_s + S^n}$	(Moser, 1958)
and Contois (1959)	$\mu = \frac{\mu_{max}S}{K_sX + S}$	
Logistic law (1969)	$\mu = \mu_{max}\left(1 - \frac{X}{K_s}\right)$	(Turner Jr *et al.*, 1969)

Appendix 6: Alcohols by-products

Table 10.9: By-products

Solvent	**% Area**
Furan, 3-methyl-	0.7264
3-Octanone	0.6576
Benzyl Alcohol	0.0359
Phenylethyl Alcohol	0.2285
Acetic acid	0.0726