Cape Peninsula University of Technology

TERTIARY BIOVALORISATION OF GRAPE POMACE

By

JUSTINE OMA ANGADAM

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Supervisor(s) : Prof. S.K.O. Ntwampe

Dr. E.F. Itoba-Tombo

Cape Town

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Supervisors/Advisors

Prof. Seteno Karabo Obed Ntwampe (EngD,HDHET*)
Asociate Professor; Biotechnology
HOD/Chair: Biotechnology department
HEAD/PI: Bioresource Engineering Research Group (BioERG)
Cape Peninsula University of Technology
Faculty of Applied Science
District 6 campus- Cape Town
South Africa

Dr. Elie F. Itoba Tombo (PhD, Cert. Nat. Scient.- SACNASP, IAIA)
Lecturer/ECP Coordinator: Environmental Management Programme
Department: Environmental and Occupational Studies
Cape Peninsula University of Technology (CPUT)
District Six Campus - Cape Town
South Africa

DECLARATION

I, **Angadam Justine Oma**, declare that the contents of this thesis represent my own unaided work, and that the thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology and the national research foundation of South Africa.

All intellectual concepts, theories and methodologies used in this Thesis and published in various scientific Journals and / or peer reviewed conference proceedings, here derived solely by the candidate and first author of the publised mannuscipts. Co-authors of manucripts were either in a supervisory capacity and / or training capacity.

15 December 2018

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ABSTRACT

In the Western Cape, South Africa and other regions globally, grape pomace (GP) is one of the abundant agro-waste from the winery industry. This study reports on the hyper-extraction of fermentable sugars from GP treated with white rot fungi (WRF) *Phanerochaete chrysosporium* BKMF 1767 to facilitate improved biovalorisation for total reducing sugars (TRS) extraction in conjunction with *Nepenthes mirabilis* digestive fluids. TRS were quantified using the 3,5-dinitrosalicylic acid (DNS) reagent method.

The free readily dissolvable sugars from the GP recorded for the bio-treated (BT) samples was 206.39 ± 0.06 mg/L and for the untreated (UT) samples was 271.05 ± 0.02 mg/L. Overall, the TRS yield for the Bio-treated (BT) and untreated (UT) samples was recorded as 205.68 ± 0.09 and 380.93 ± 0.14 mg/L, respectively, using hot water pretreatment (HWP) with 2266.00 ± 0.73 (BT) and 2850.68 ± 0.31 mg/L (UT), respectively, for dilute acid pretreatment (DAP); with 2068.49 ± 6.02 (BT) and 2969.61 ± 8.054 mg/L (UT) respectively, using the cellulase pretreatment (CP) method. Using the HWP as a reference, the relative increases imparted by the bio-treatment was higher (51%) for DAP and low (33%) for CP.

The combination of conventional used pre-treatment methods (hot water pre-treatment, dilute acid pre-treatment, and cellulase pre-treatment) in a single pot system was also done while monitoring the total residual phenolics (TRPCs) in the samples. Furthermore, powder X-ray diffraction (pXRD) were used to measure the crystallinity index (CrI) and functional groups of pre- and post-pretreated GP to ascertain the efficiency of the pre-treatment methods, with quantification of lignin, holocellulose, and ash. Overall, the TRS yield for *N. mirabilis* pre-treated agro-waste was 951 mg/L ± 4.666 mg/L, with biomass having a lower CrI of 33%, and 62% residual lignin content. Furthermore, reduced TRPCs were observed in hydrolysate, suggesting limited inhibitory by-product formation during *N. mirabilis* pre-treatment.

Keywords: Agro-waste, Grape pomace, *Nepenthes mirabilis*, Total reducing sugars, *Phanerochaete chrysosporium.*

DEDICATION

To

My sweet mother: Angadam Christina Eyasa

And

My husband: Anyik John Leo
My kids: Leoma and Neymar

Your love and support kept me going

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I wish to thank:

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LIST OF SYMBOLS AND ABBREVIATIONS

Nomenclature

Symbol	**: Description**	**Units**
T	: Temperature	°C
AD	: Anaerobic digestion	-
AIR	: Acid insoluble residue/lignin	-
ASL	: Acid soluble lignin	-
BOD	: Biochemical oxygen demand	(mg/L
BT	: Bio-treated	-
CCD	: Central Composite Design	-
CPPs	: Carnivorous pitcher plants	-
COD	: Chemical oxygen demand	(mg/L)
CrI	: Crystallinity index	-
C_2	: Acetic acid	(mEq)
CBHs	: Cellobiohydrolases	-
DAP	: Dilute acid pretreatment	-
DM	: Dry matter	-
EGs	: Endo-β-1, 4 glucanase	-
FW	: Food waste	-
FW_m	: Food waste coming from food manufacturing	-
FSCW	: Food supply chain waste	-
GP	: Grape pomace	-
GPE	: Grape pomace extract	-
GSF	: Grape skin flour	-
GHGs	: Greenhouse gases	-
ISW	: Industrial solid waste	-
ISWM	: Industrial solid waste management	-
LCC	: Lignin-carbohydrate complex	-
LCB	: Lignocellulosic biomass	-
LCM	: Lignocellulosic material	-
MC	: Moisture content	-
Mg	: Milligrams	-

Min	: Minutes	s
MSW	: Municipal solid waste	-
NA	: Nutrient agar	-
NIR	: Near-infrared spectroscopy	-
rdTRS	: Readily dissovable total reducing sugars	-
RSM	: Response surface methodology	-
SD	: Standard deviation	-
SRT	: Solid retention time	(d)
STP	: Standard temperature and pressure	(0 °C, 1 atm)
TS	: Total solids	(mg/L, %)
TRS	: Total reducible sugars	(mg/L)
TPCs	: Total phenolic compounds	(mg/L)
TROAs	: Total residual organic acids	(mg/L)
TOC	: Total organic carbon	(mg/L)
WRF	: White rot fungi	-
DNSA	: 3,5-Dinitrosalicylic Acid	-

Greek symbols

ε	: Extenction coefficients	$M^{-1}cm^{-1}$

GLOSSARY

GLOSSARY/BASIC TERMS AND CONCEPTS

Agrowaste : Residual materials from processing agricultural produce.

Antioxidant : Compounds which inhibit oxidation type reactions including the efficacy of oxidative radicals some of which are available in minute quantities in food waste.

Biomass : Carbon-based constituents which cannot be classified as edible materials for humans, which are mostly constituted by material having lignin, hemicellulose and celluloses i.e. holocelluloses.

Biovalorisation : This can be classified as a process whereby**,** synthetic chemicals are not utilized, focusing on an ambient temperature facilitated biological valorisation processes.

Food waste : Residual materials from processed edible materials as a result of manufacturing, home remains or leftovers meant for human consumption

Green chemistry : Environmentally benign approach which focuses on strategy of developing processes that uses natural products and microorganisms for beneficiating low value feedstock thus decreasing consequential environmental challenges which can be imparted by the use of refined and/or synthetic chemical compounds, which are considered harmful.

Holocelluloses **:** This is hemicelluloses plus celluloses

Lignocellulosic biomass : Plant based biomass composed of cellulose, hemicellulose, and lignin.

Micro-algal biomass : Refers to microalgae-derived biomass

Organic waste : Waste constituted by seeds, peels that are not fit for human consumption. This type of waste forms part of agrowaste.

Pyrolysis : High temperature valorisation of waste.

Relative increase : The absolute change as a percentage of the value of the reference in the initial treatment method used

Valorisation : The reprocessing of unwanted waste materials into more value-added products.

CHAPTER 1

INTRODUCTION

CHAPTER 1

INTRODUCTION

1.1 Background

Generally, all unusable organic constituents from agro-processing can be considered as waste, e.g., corn stover from maize (Zea mays), including lignin from woodchip pulping for paper making (Tuck *et al.*, 2012). The production or generation of unwanted materials some of which are agro-waste is an indisputable part of the human anthropogenic activity (Vandermeersch *et al.*, 2014; Taherzadeh and Karimi, 2008). Environmental pollution culminates as a result, due to agro-waste landfilling (Chan *et al.*, 2016; Mirabella *et al.*, 2014; Liguori *et al.*, 2013). Plant dry matter (lignocellulosic biomass) such as forest-based woody materials, agricultural remains, and public waste, are well-known feedstock for bioethanol and the production of value-added products because of its extraordinary accessibility and low cost; although, large-scale processes and manufacturing have not yet been established (Spyridon *et al.*, 2016).

Challenges associated with environmental pollution caused by dumping untreated agro waste in to landfills, has led to research in the production of value-added products from agro waste using environmentally benign processes in order to reduce ecological degradation waste can be of great economic and environmental interest because of its availability in large quantities and its composition; therefore, agro waste can be used as a low-cost material for the production of other useful products; thereby, reducing pollution and landfilling costs. The use of agro waste material limits its disposal as it contains phenolics and other toxic compounds post-processing which can end up in the environment. A portion of this agro waste can be repurposed to produce fertilisers; however, a large portion is left to decay; albeit this type of waste is inexpensive and can be applied on a large scale in various industries (Saini *et al.*, 2015; Bhatia *et al.*, 2014).

Current studies have shown countless processes used in treating lignocellulosic biomass including agro-waste, for biorefinery fuel and valued-added products

production by minimising the pretreatment parameters such as processing time and energy intensity, by using environmentally benign processes, thus eliminating the use of chemicals and reduce process cost (Shafiei *et al.*, 2015; Kudakasseril Kurian *et al.*, 2013). Some of this lignocellulosic biomass is in the form of pomace from the winery and juice industries.

Grape juice and wine production are produced from different species of grapes. Grape berries are constituted by 6–12% of the skin, 2–5% of the seeds and 85–92% of the pulp which is also the main part of the grapes. Grapes composition varies with the conditions used during their growth; including the make-up of the soil, application of fertilizers and also the use of herbicides (Botelho *et al.*, 2018). According to Ruberto et al. (2007), ~20% of grape processing culminates in pomace with an enormous quantity of such waste being released during winemaking; hence, the primary reason for selecting this kind of waste as it is an important regional feedstock in the Western Cape, South Africa. Furthermore, grape pomace can become a valuable source for monetary gains from beneficiation which can result in economic gains for wine manufacturers. The pomace can also be used for animal feed production and/or can be used in the production of fertilisers. Most importantly other useful chemical substances can be manufactured from grape pomace, using environmentally benign methods for the transformation of such waste. Additionally, it is feasible to reprocess the pomace into different constituents for use in biochemical production and/or bio-refineries for biofuel production (Mirabella *et al.*, 2014).

Some examples of value-added products extracted from grape pomace are; phenols, polyphenols, flavonoids, tannins and anthocyanins, antioxidants, amino acids, bio-surfactants, ethanol, lactic acid, resveratrol, tartaric acid, xylitol and other compounds (Botelho *et al.*, 2018; Ferri *et al.*, 2016; Ruberto *et al.*, 2007). Moreover, phenolic compounds and tartaric acid, found in grape pomace and seeds, can be purified and sold. Another essential value-added product which is an important class of phenolic compounds is flavan-3-ols in grape seeds. Therefore, the effect of agricultural residues on landfills can be reduced while developing novel methods that can generate an income (Devesa-Rey *et al.*, 2011), which is highly needed for the sustainability of grape processing industries.

Generally, landfilling of such agro-waste is also a universal challenge as a considerable quantity can be used as feedstock for other industries. This signifies human environmental and economic challenges associated with such waste. Concerns associated with foodstuff and agro-waste residue are a focus of the scientific community since the 1990's (Kosseva, 2017; Mirabella *et al.*, 2014). Grape pomace can be a vital source of bioactive complexes which can be extracted for use as antioxidants in food, pharmaceuticals, cosmetics, and as well as in bio-pesticides. Similarly, phenolic compounds have biological active characteristics, thus have been used as antimicrobial agents to extend the shelf-life of products by hindering lipid oxidation, improving color, flavor, and aroma of foods when used in minute quantities.

In humans, research has shown that regular intake of antioxidants can safeguard against several illnesses including cancers and cardiovascular diseases. Alternatively, grape pomace can be used as an additive in animal feed (Fontana *et al.*, 2013), or as feedstock for the biorefinery industry (Zheng *et al.*, 2012).

1.2 Research problem

The disposal of grape pomace and other agro-waste into the environment by vineyards and food processing industries has become a major source of concern for environmental and human health. As such, the development of valorisation methods for the pre-treatment and extraction of value-added products from grape pomace and mix agro-waste has become a new promising strategy and a sustainable solution towards the efficient utilisation of this waste (Nayak *et al.*, 2016; Chandrasekaran and Bahkali, 2013). As the Western Cape (South Africa), is classified as a regional hub for grape growers and processors, the production of grape pomace and its disposal challenges is becoming a concern, as most of the pomace is disposed-off in landfills.

1.3 Hypothesis

It is hypothesized that value-added products can be produced from *Nepenthes mirabilis* extract facilitated biovalorisation of grape pomace.

1.4 Research aims and objectives

The general aim of the study was to develop a suitable process for the biovalorisation of grape pomace. To achieve this aim, the research was divided into two phases with the following objectives:

Phase 1: Objective 1) to initially identify a suitable agro-waste for biovolarisation for TRS extraction, and

Objective 2) to ascertain the effect of bio-pretreatment on TRS extraction using *Phanerochaete chrysosporium* BKMF1767 in comparison with different commonly used pretreatments methods for hyper-extraction of TRS from GP (identified in objective 1) as the selected feedstock.

Phase 2: Objective 1) to develop a suitable process for the holocellulosic/TRS extraction of grape pomace (GP) using naturally available bioresources, i.e. *N. mirabilis* digestive fluids as pretreatment aliquots, in comparison to commonly used pre-treatment method, using a single reaction vessel strategy,

Objective 2) to quantify the activity of delignifying and cellulolytic enzymes in the proposed *Nepenthes mirabilis* extracts,

Objective 3) to quantify the percentage of residual lignin, cellulose, and hemicellulose (holocelluloses) in the grape pomace residue post pretreatment to determined which of the methods is better as a holocellulose/TRS extraction method, and

Objective 4) to identify organic compound residues such as organic acids and phenolics in the grape pomace hydrolysate and their fate during grape pomace pre-treatment using *Nepenthes mirabilis* extracts.

Overall, *N. mirabilis* extracts were proposed as an alternative source of biovalorisation extracts to minimise synthetic chemical usage and an external energy source for the treatment of grape pomace, due to their oxidative constituents.

1.5 Significance of the study

This study investigated and developed an eco-friendly alternative method of producing value-added products from waste grape pomace, using a green chemistry method involving plant-based oxidative extracts of *N. mirabilis* strategy which was never been reported before.

1.6 Delineation of study

The following was not considered in this study:

- Scale-up experiments to pilot or industrial scale,
- Economic evaluation/feasibility studies at a larger scales, and
- Microbial kinetics, of organisms used and/or identified during the study.

All these as listed above can be a subject of other research studies for further development of the tertiary biovalorisation concept initiated and reported in this thesis.

CHAPTER 2

LITERATURE REVIEW

CHAPTER 2

LITERATURE REVIEW

Environmental pollution by fruit waste has become a global challenge due to excessive landfilling of such waste. This chapter examined the challenges waste management and strategies involving valorisation, with a particular focus on food waste, including, grape pomace as a regional waste of concern and some mixed agro-waste. Discussion of different waste treatment techniques, their advantages and disadvantage and a proposed application of biological processes that can be used to pre-treat agro waste to derive more value-added products and subsequent reduction of environmental pollution, is elucidated.

2.1 Challenges with food waste

Dumping of food waste by landfilling and incineration is a deleterious ecological challenge. Currently, 15 million tonnes of wasted food is being dumped annually in the United Kingdom (Salemdeeb *et al.*, 2017). Mostly, dumping is being done by landfilling via composting or anaerobic digestion. These processes yield a huge amount of greenhouse gases. Furthermore, disease outbreak such as swine fever, foot-and-mouth disease, can be transmitted via meat waste. Thermal treatment can be performed with the waste being safe to be a composite in animal feed. In Japan and South Korea, 35.9% and 42.5% respectively, of food waste is reused as animal feed (Salemdeeb *et al.*, 2017). However, for developing countries, this is not the case, with landfilling being the primary method available and thus used extensively.

2.2 Food waste: Characterisation, treatment and environmental impact

Industrial waste can be classified into two key groups depending on the source of the material. Food waste can be made from animal or plant material. Plant-based food waste is primarily produced during agricultural produce processing and secondly, during postharvest operations or storage, in developing countries (Galanakis, 2012). The food industry is rapidly growing due to the growing population, thus several food handling, treatment, and packaging procedures generate diversified waste and

quantities such that dumping becomes an unintended consequence with accumulative disposal challenges emerging and contributing to environmental degradation thus pollution. Moreover, if the pre-treatment processes used are inadequate to facilitate the processing of the waste, i.e. if such processes are inefficient, valuable biomass and nutrients from the waste can be discarded (Kroyer, 1995). Therefore, suitable and proficient methods are required, to repurpose such waste, for other industrial purposes to minimise landfilling.

2.2.1 Characteristics of food waste

Food processing industry produces waste characterised by the following quantifiable parameters in liquefied matrices (Kroyer, 1995).

- High organic content thus organic carbon (total organic carbon) from proteins, carbohydrates and lipids including incinerable constituents, some of which are classified as either macro and/or micronutrients,
- High suspended solids in liquefied matrices, such as wastewater,
- High biochemical oxygen demand (BOD) or chemical oxygen demand (COD), constituents of total organic carbon (TOC),
- High suspended solids including fats, oil or grease, and
- Variations in pH.

All these parameters, can be used to assess the extent of pollution, thus the development of suitable mitigation strategies.

2.2.2 Treatment of food waste

Many different methods of treating food waste have been established and some of these methods have shortcomings. These methods include physical and chemical methods with biological and biotechnological methods being considered favourable due to their environmental benignity. To treat food waste, TOC, BOD and COD reduction can be used to assess the efficacy of the methods being utilized (Kroyer, 1995). First generation bioconversion technologies for food waste treatment include:

❖ Aerobic processes: It involves slurrification procedures and trickling filters. Herein, layers of microorganisms are used as oxidizing catalysts that transform organic materials to carbon dioxide and microbial biomass.

❖ Anaerobic processes: Utilizes an anaerobic digester and organic materials are being transformed to biogases and biomass sludge (Kroyer, 1995). All these processes primary goal is to minimise organic constituents loading into the environment.

2.2.3 Environmental and human health impact of food waste

Food manufacturing, conveyance and packing which leads to food waste, have been identified as having several effects on the earth's ecology and human health due to dumping. Dumping of untreated food waste in the environment can cause serious environmental and human health problems such as environmental degradation and diseases to humans including animals (Marchante *et al.*, 2018; Salemdeeb *et al.*, 2017).

2.3 Fruit waste

2.3.1 Nutritional and biochemical composition of fruit waste

Most of the broadly examined substrates for value-added product production are fruits and vegetable waste using adaptable treatment technologies for the extraction of numerous product types such as antioxidants and nutritional fibre. Citrus type agricultural produce is one of the principal fruit crop types globally, with the peels from these fruits being used for pectin and flavonoids production. Apart from flavonoids, essential oils and carotenoids can also be extracted from some citrus fruit peel. Due to the functional diversity of products from such citrus waste, some, i.e. carotenoids, can be used as food or beverage preservatives, further enhancing the shelf-life of the primary products produced from such fruit (e.g., juicing operations), further delaying the formation of off-flavours and rancidity (Galanakis, 2012): (Ferrentino *et al.*, 2016).

Some food industries that use fruits as their feedstock for the manufacturing of fruit juices, concentrates, jams and dried fruits, produces wastes constituted by the peels from these fruits. As such, researchers are interested in studying the constituents and

composition of fruit waste, focussing on peels, seeds, stalks, etc., as some fruit peel contain natural antioxidants including reducible sugars which can be easily fermented into other high-value products including bioproduct such as alcohols which can be used in bioenergy generation. It has been proven that value-added by-products and other bioactive compounds from some fruit peels have clinical significant outcomes in human health (Ibrahim *et al.*, 2017; González-Centeno *et al.*, 2013).

2.3.2 Fruit waste management/challenges

Fruits waste similarly poses serious environmental challenges due to its high biodegradability. Additionally, plant based waste materials and nutritious substances in such waste, if discarded, can culminate into economic losses too. This is a primary reason to focus on developing policies and treatment methods for managing the fruit waste. Currently, the primary challenge is waste accumulation, it's handling, and repurposing as a raw material for other industries e.g Enzyme production industry. Hence, valorisation of such, can lead to increasing economic growth in other sectors. Generally, the collection, transportation, recovery and disposal of waste is known as waste management (Catana *et al.*; Plazzotta *et al.*, 2017), which must be implemented rigorously in the agro-processing industries.

2.3.3 Enzymes role in using fruit waste processing

Enzymes have been used for many years in fruit waste processing and the production of other useful enzymes for the benefit of the biotechnology industry. One of the largest industry is that of enzyme production through microorganism facilitated processes, some of which can be solely based on fruit waste as a carbon source. Fruit waste can be treated using enzymes such as cellulases and pectinases, ensuring elevated end-product extraction. Enzymes aid in lessening plant tissue bonding to extractable constituents thus liberating such high-value products subsequent to the enzymes being recovered for further use in other operations and/or for reuse in the same treatment technologies (Chandrasekaran and Bahkali, 2013).

A majority of enzyme requirements in South Africa are being met by importation mostly from the European Union (EU). These enzymes, are vital to numerous

industries because they can be used to reduce potable water usage, energy consumption and reduce gas emissions. For example, enzymes from *Aspergillus niger* and *A. oryzae* can be used to decompose starch-based waste into fermentable sugars which can be used to produce other high-value products (Khan *et al.*, 2015). Minimal research has been done concerning enzyme manufacturing from fruit waste in South Africa (Khan *et al.*, 2015).

2.3.4 Microorganisms associated with fruit waste beneficiation

Green chemistry methods have been proven to efficiently extract valuable products from fruit waste thus lessen the burden on the environment since numerous species of microorganisms has been demonstrated to alter fruit waste for the extraction of different biological products. Some of these organisms include Saccharomyces cerevisiae which has been added to fruit waste to produce proteins that can be used to prepare the animal feed. Similarly, Aspergillus sp. was acknowledged to facilitate the production of citric and lactic acids from fruit waste with Bacillus sp. being used to produce enzymes such as cellulase, amylase, and protease, using fruit waste feedstock (Panda *et al.*, 2016; Chandrasekaran and Bahkali, 2013). It is such beneficiation processes that can add economic value in the processing thus beneficiation of fruit waste.

2.3.5 Types of products manufactured from fermentable fruit waste

Handling of fruits result into two categories of waste which includes;

- Solid residue from peels, shells, etc., and
- Liquefied residue.

As such, commonly found constituents in liquefied fruit waste include papain which can be obtained from papaya, bromelain from pineapple and ficin from figs. These are proteins obtained via enzymatic biocatalysis and can be utilized in biodetergents and the brewing industry. Similarly, mango peel are used in the soap and the essential oil industry. Previously, it has been determined that mango peels are also a good source of manure for plants. For human consumption, pineapple peel has been used for the preparation of jam with a high content of pectin. The production of vinegar being an alternative outcome, a similar product outcome which can be

obtained using peach waste or some citrus based waste products. Manufacturers of cosmetics and deodorants have determined that such waste can benefit their industry, with fragrances being developed in alcohol-base mixtures. As observed in nature, some citrus fruits have pest repellent capabilities. Therefore, extracts from citrus fruits waste have been proposed for mosquito repellent. As grape pomace is of interest to this study, grape seeds and pomace can be used for extraction of oil, tartaric acid and tannins (Wazir *et al.*, 2005); (Ferri *et al.*, 2016; Ruberto *et al.*, 2007).

2.4 Valorisation of food and agrowaste

Valorisation is essentially a concept of recycling waste for the manufacturing of high-value chemicals (Pfaltzgraff *et al.*, 2013). The exploitation of food and agro-waste for value-added commodities production is an encouraging and systematic way of waste management (Lin *et al.*, 2013). A quantity of 1.3 billion tonnes of waste is misdirected globally (Luque and Clark, 2013; Pleissner and Lin, 2013). Such waste is generated at all production stages, i.e. from produce harvesting to household usage. Regardless of the site whereby waste is generated, landfilling becomes an easier disposal option with unintended ecological and environmental including human health-related complications (Pleissner and Lin, 2013), particularly in South Africa. Hence, valorisation is needed with numerous high-value product production stream being the intended goal as shown in Figure 2-1.

2.5 Types of valorisation

On the basis of developing a conceptual framework, it is proposed that valorisation is classified into primary, secondary and tertiary valorisation, analogous to the biorefinery concept.

2.5.1 Primary valorisation

This involves the physical pre-treatment of the waste with examples of pre-treatment processes being drying**,** irradiation and milling including pressing (Selvaraj and Vasan, 2018) to distort or extract a liquid fraction from the waste. Therefore, this type of process will require high physical energy.

2.5.2 Secondary valorisation

This can be categorized as the chemical processing of the waste with the application of either dilute organic (Selvaraj and Vasan, 2018; Bensah and Mensah, 2013) and inorganic acids being used to pre-treat the waste. Overall, it will involve synthetic chemicals which can add to operational costs and contribute to residual chemical constituents in the remainder of the waste, which can further contribute to environmental pollution, and stunted downstream secondary i.e, fermentation processes (Myat and Ryu, 2016)

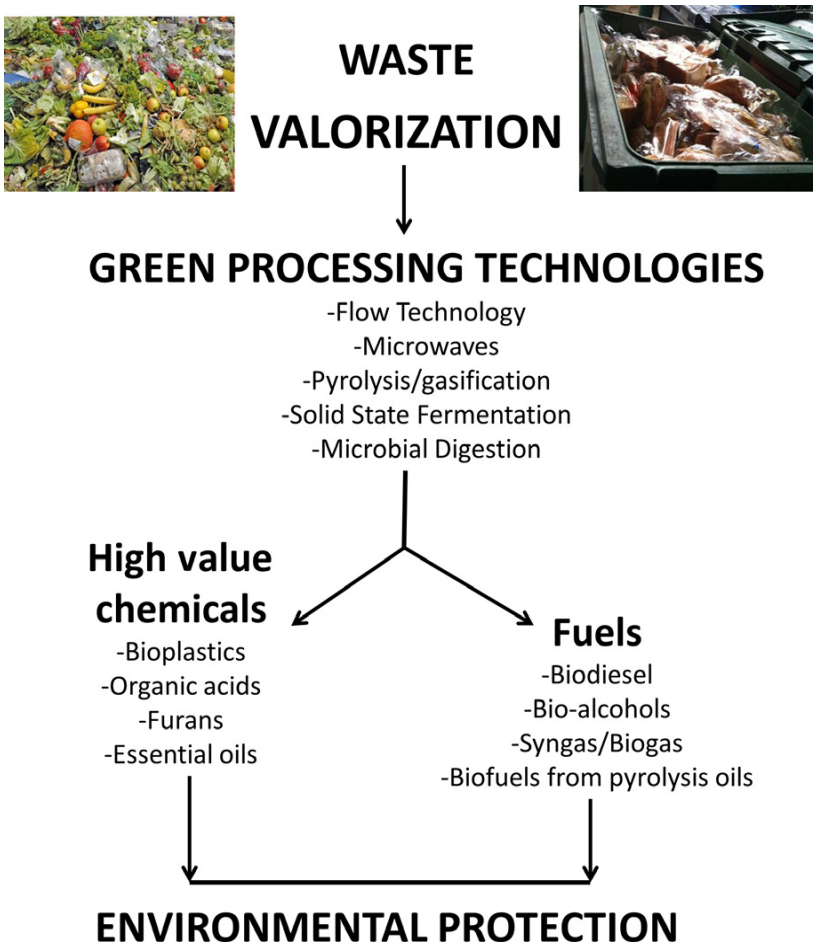

Figure 2-1: **Illustration of valorisation processes for waste into value-added products. These processes reduce landfilling challenges by applying a green chemistry approach (Adapted from (Arancon *et al.*, 2013)).**

2.5.3 Tertiary valorisation

This can be hypothetically classified as a biological pre-treatment process using microorganisms to pre-treat waste under low energy input requirements, imparting environmentally benignity to the processing and/or beneficiation of the waste feedstock. For this study, i.e. tertiary biovalorisation of grape pomace, would thus have to involve a process, whereby synthetic chemicals are not utilized, focusing on ambient temperature facilitated processes, for the production of numerous high valued added by-products. This would constitute low investment requirements for the repurposing of waste in developing countries, such as South Africa.

2.6 Processing of lignocellulosic biomass: A focus on grape pomace

The processing of any waste material constituted in the form of lignocellulosic biomass, is a crucial point to efficiently recover any valuable product within the waste in order to recover beneficial materials to produce bio-products (Negro *et al.*, 2003). Most pre-treatment methods aimed at exposing thus extraction of any high-value product from the waste biomass structure can be classified into different types of pre-treatments, categorized into acidic, biotic, alkaline, organosolvent, manual and physicochemical pre-treatments (Bhatia *et al.*, 2014; Sarkar *et al.*, 2012).

Biotic processes are preferable because of their environmental benignity attributes involving mild conditions; hence, minimal energy requirements are needed (Sebayang *et al.*, 2016). For such a process to be effective, the process is aimed at separating lignin, cellulose, hemicellulose and other constituents from the waste biomass.

In this research study, biological pre-treatment is proposed because it is a green chemistry approach (no chemical usage). Several studies proposed the use of white rot fungi to delignify the biomass to release hemicellulose and cellulose, i.e. holocelluloses. During such pre-treatment processes, quantities of holocellulose can be decomposed to monosaccharaides, i.e. mostly into D-glucose, D-xylose and D-arabinose. Thereafter, the pre-treated biomass is pressed to separate liquids and solids, with the extractant being fermented to produce more value-added products (Dinita *et al.*, 2011).

2.6.1 Types of carbohydrates present in grape waste pomace

Free glucose, galactose, mannose, xylose, arabinose, etc., are all readily available in grape pomace from grape berry pressing processes. Among these, polymeric constituent such as glucomannan and xylan can be found, some of which can be bio-catalytical converted to some reducible sugars that are fermentable to produce products such as bio-ethanol and other value-added products. However, prior to their availability, delignification must occur to expose the holocelluloses which can then be further biocatalytically converted into fermentable sugars. The Figure 2-2 shows cellulose being exposed as a result of pretreatment.

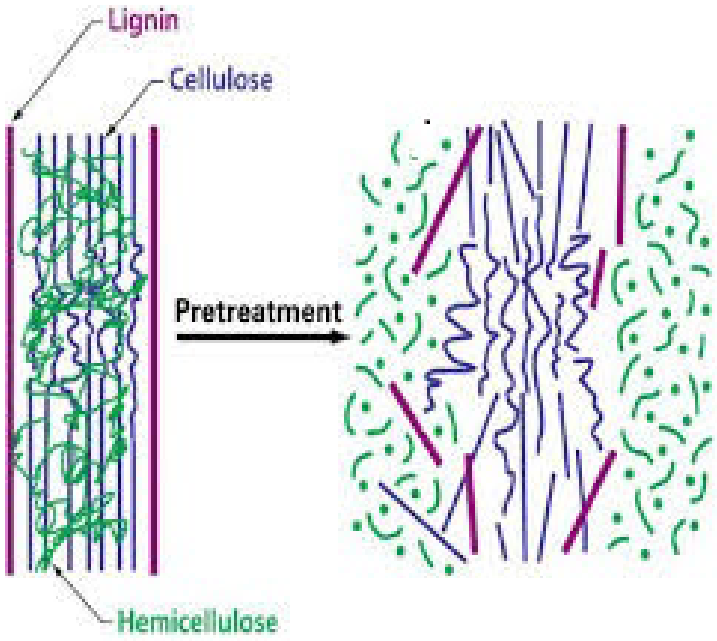

Figure 2-1: Simplified diagram showing the effect of pretreatment on lignocellulosic material as adapted from (Wu *et al.*, 2014; Bhatia *et al.*, 2012).

2.6.2 Lignin in grape pomace

Lignin is a non-toxic biopolymer (Wu *et al.*, 2014), which binds plants/biomass fibres together. As such, it is an abundant renewable aromatic compound, with its existence in woody biomass signifying a major challenge for the development of biomass conversion processes. Lignin if separated from waste biomass, it can be used for energy generation as it contains a high energy content thus produce all the heating and electricity requirements needed for a valorization plant. The main enzymes

involved in lignin degradation are lignin peroxidases, manganese peroxidases and laccases (Zabed *et al.*, 2016) some of which are constituents of cellulases. It is generally accepted that lignin obstructs cellulose hydrolysis into its monomeric constituents during biomass transformation. The fundamental structure that makes up the composition of lignin is constituted by coniferyl, synapyl, and p-coumaryl alcohols (see Figure 2-3) which are formed during photosynthesis (Achinas and Euverink, 2016).

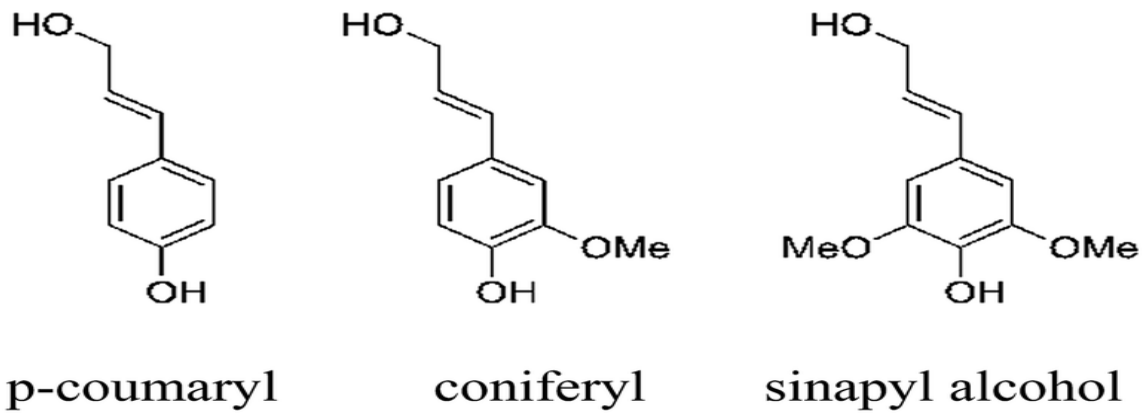

Figure 2-2: Different alcohols or structural units found in lignin (Xu *et al.*, 2014)

As such, the destallibition of the bond structure of these alcohols can be of benefit during delignification. Binding and inactivation of enzymes by the lignin component seems to be significant factors limiting the delignification efficiency of waste biomass. Figure 2-4 illustrates the enzymatic delignification mechanism of biomass with lignin components.

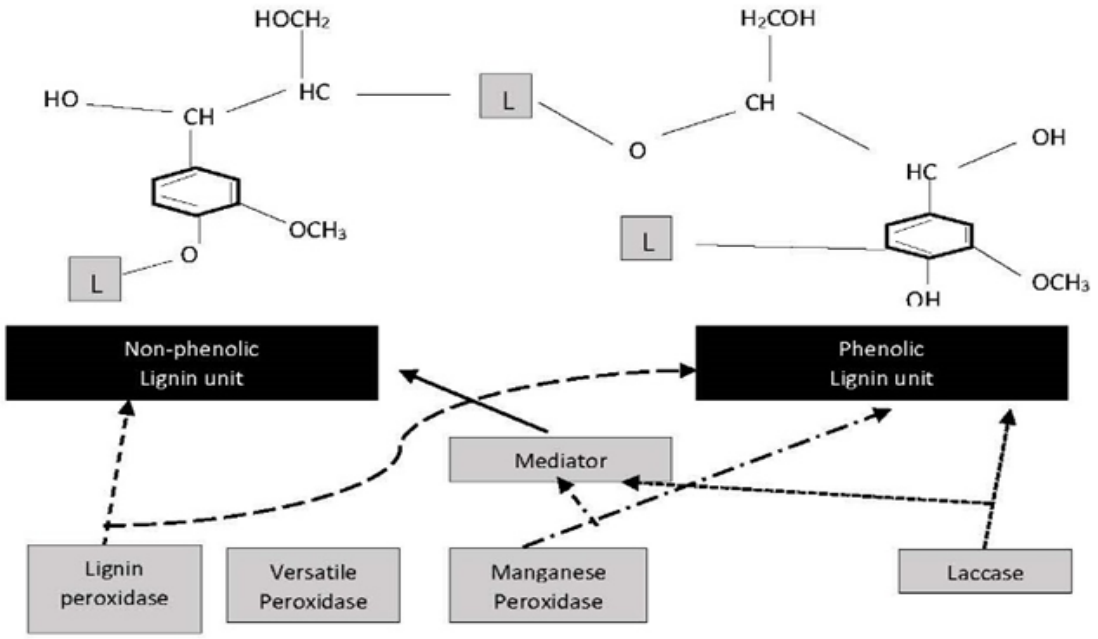

Figure 2-3: Enzymes involved in the enzymatic hydrolysis of lignin (Zabed *et al.*, 2016).

2.6.3 Hemicellulose in grape pomace

Agrowaste has been determined to have a large proportion of hemicellulose material than woody biomass. There exist some main heteropolymers of hemicellulose which include; xylan, xyloglucan, mannan, and arabinan (Vries *et al.*, 2018; Bastawde, 1992). Hemicelluloses are heterogeneous polymers of pentoses, hexoses and some organic chemicals some of which are inhibitory products (toxicants) during downstream secondary processes, especially, fermentation. Xylan is the most abundant constituent of hemicelluloses. The hemicellulosic chain contains the following; 90% xylose and 10% (m/m) arabinose which are derived from xylan, a primary constituent of hemicellulose. Hemicellulose is bonded with cellulose by hydrogen bonding and with ester linkages to lignin. Xylan composition varies in each waste material use; hence, its breakdown involves different conditions and several enzymes to efficiently hydrolyse the hemicellulose into fermentable monomers (Sarkar *et al.*, 2012; Bastawde, 1992).

Enzymes comprise in hemicellulose degradation include; Endo1,4-β-xylanase or endoxylanase, xylan 1,4-β-xylan esterases, ferulic and p-coumaric esterases, α-1-arabinofuranosidases, α-glucuronidase, α-arabinofuranosidase, acetylxylan esterase

and α-4-O-methyl glucuronosidases xylosidase. Endo–xylanases are responsible for the breaking down of the main chains of xylan and β–xylan esterases decouples xylooligosaccharides into xylose. Furthermore, the α–arabinofuranosidases and α–glucuronidases act on the xylan backbone and remove arabinose and 4–o–methyl glucuronic acid, respectively, where acetyl esterases debonds the acetyl substitutions on the xylose moieties, with feruloyl esterases hydrolyzing the ester bonds located between arabinose substitutions and ferulic acid (Zabed *et al.*, 2016).

2.6.4 Cellulose in grape pomace

Cellulose is another polymer found in plant tissue, and in most instance, it constitutes more than 30% of most plant biomass. Cellulases are the enzymes used to hydrolyzed cellulose. These enzymes are distributed into there main groups which are endoglucanases, cellobiohydrolases (exoglucanases), and β-glucosidases (Kumar *et al.*, 2008). It is characterised by its 1, 4-β-glucosidic bonds attaching a multitude of d-glucose units. The cellulose chains are filled up with hydrogen bonds held together by van der Waals forces (Bhatia *et al.*, 2012). These linkages give cellulose a compact structure. To decouple such a structure, cellulases constituting a cocktail with various enzymes, facilitates the decoupling process whereby endo-β-1,4 glucanases (EGs) decouples the internal hydrogen bonds of the cellulose releasing long polymers (glucans) while cellobiohydrolases (CBHs) facilitate the decoupling of the reduced ends the of hydrogen bonds of the cellulose, producing Oligosaccharides, disaccharides/tetrasaccharides, which are further reduced to monomers by β-glucosidases which are easily fermentable (Achinas and Euverink, 2016; Agbor *et al.*, 2011). CBHs comprise several types of enzymes, e.g. exoglucanase and rarely cellodextranase which is a CBH that hydrolyse cellulose from its non-reducing ends, which literally hydrolyse the glucan chain of cellulose fibers, randomly.

Celluloses are very fine microfibrils made up of two regions, namely; crystallinity and amorphous regions. Usually, these microfibrils are linked as bundles or macrofibrils which gives cellulose a complex arrangement that is impervious to both biological and chemical pretreatments (Bhatia *et al.*, 2012). This arrangement is shown in Figure 2-5.

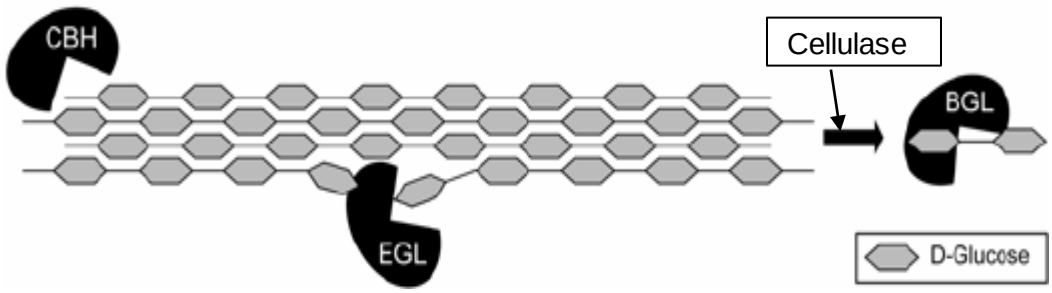

Figure 2-1: Shows a representation of cellulose, its decoupling D-glucose (Vries *et al.*, 2018).

A considerable portion of cellulose is made up of the crystalline form. Cellulases readily hydrolyses the amorphous portion of cellulose and if there is less accessibility to the crystalline part, less decoupling to fermentable monomers will ensure. (Taherzadeh and Karimi, 2008). Many varieties of bacteria, fungi, and wild yeasts which have been isolated, some of which are from the termite gut, have been shown to possess genes which facilitate the production of these cellulose bio-decoupling enzymes (Achinas and Euverink, 2016). Figure 2.6 illustrates the processes involved in the biocatalytic conversion of both the hemicellulose and cellulose.

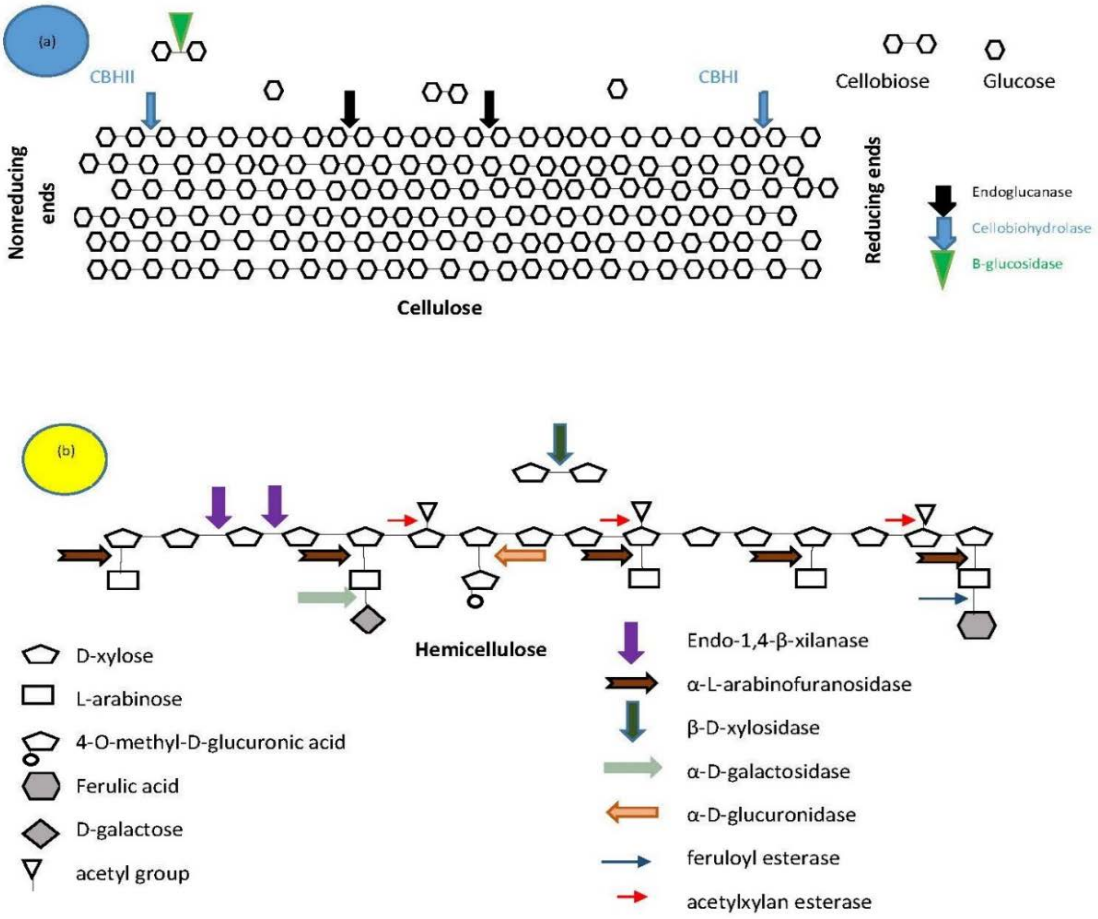

Figure 2-2: Shows enzymes involved in the biocatalytic decomposition of (a) cellulose and (b) hemicellulose (as adapted from (Zabed *et al.*, 2016)).

2.6.5 Cellulose, hemicellulose, lignin and ash content in biomass feedstocks from different sources as adopted from

The protective presence of lignin in unpretreated lignocellulosic biomass causes difficulties in accessing cellulose to produce biofuels or paper. Lignocellulosic biomass varies in its hemicelluloses content. Hardwood contains up to 35% hemicelluloses, with softwood averaging slightly less.Cellulose polymer molecules do not differ in chemical structure, having no side chains, but the proportion of cellulose in a given biomass does vary (Lynam, 2011). Table 2.1 highlight Cellulose, hemicellulose, lignin and ash content in biomass feedstocks from different sources.

Table 2-1: **Cellulose, hemicellulose, lignin and ash content in biomass feedstocks from different sources (Szymańska-Chargot *et al.*, 2017; Saini *et al.*, 2015; Juneja *et al.*, 2011; Sun *et al.*, 2005; Sun and Cheng, 2002).**

Lignocellulosic materials	Cellullose (%)	Hemicellulose (%)	Lignin (%)	Ash %
Biomass in general	35–50	20–35	10–30	6.33 ± 0.06
Different grass mixes	28.8 - 36.0	17.9 -24.7	13.4 -17.5	nd
Wheat straw	40.2	38.8	17.0	nd
Grasses	25–40	25–50	10–30	nd
Hardwoods	45 ± 2	30 ± 5	20 ± 4	0.6 ± 0.2
Softwoods	42 ± 2	27 ± 2	28 ± 3	0.5 ± 0.1
Cornstalk	39–47	26–31	3–5	12–16
Newspaper	40–55	18–30	18–30	nd
Sorghum stalks	27	25	11	nd
Corn stover	38–40	28	7–21	3.6–7.0
Coir	36-43	0.15-0.25	41-45	2.7-10.2
Bagasse	32-48	19-24	23-32	1.5-5
Rice straw	28-36	23-28	12-14	14-20
Sorghum straw	32	24	13	12
Sweet sorghum Bagasse	34-45	18-28	14-22	nd
Carrot	10.01	5.73	2.50	nd
Tomato)	8.60	5.33	5.85	nd
Cucumber	16.13	4.33	4.51	nd
Apple	8.81	5.44	2.98	nd
Sugar cane bagasse	42	25	20	nd
Banana waste	13.2	14.8	14	nd
Nut shells	25 - 30	25 - 30	30 - 40	nd

Table 2-1: *cont.*

Cotton, flax	80–95	5–20	-	nd
Leaves/yard waste	15-20	80-85	0	nd
Switch grass	45	31.4	12	nd

nd – not determined

2.7 Pre-treatment of biomass

In this study, three kinds of pretreatment, i.e. dilute acid, liquid hot water, and cellulases pretreatment were analsyed in comparison to a newly pretreatment method i.e. tertiary biovalorisation intended to impart process integration and minimal chemical usage, using extracts of *Nepenthes mirabilis* known to contain enzymes such as proteases, nucleases, peroxidases, chitinases, a phosphatase, and a glucanase (Lee *et al.*, 2016).These pretreatment methods were carried out at their optimum working conditions as per previous research (Bensah and Mensah, 2013) while the newly developed pre-treatment method was conducted at ambient temperature.

2.7.1 Dilute acid pretreatment (DAP)

There are two types of acid pretreatment which include strong and mild acid pretreatment. Dilute sulphuric acid is a common pretreatment agent used for biomass at both laboratory and industrial scale (Idrees *et al.*, 2013; Alvira *et al.*, 2010). During acid pretreatment, conditions such as temperature, sample concentration and reaction time play an important role to maximize the yield. At 170°C, sulphuric acid pretreatment produces less free xylose than at 150°C, because about 23% of the xylose would degrade to furfural which is fermentation toxicant, while at a reduced temperature, less toxicants are produced (Kootstra *et al.*, 2009).

The percentage of inhibitors produced when a weak acid such as organic acids, i.e. maleic acid or fumaric acid, are used at the same temperature, minimal toxicant formation is observed. Optimal conditions for dilute acid pretreatment was reported for sulphuric acid to be 0.6mol/L for 15 min at 121°C (Rocha *et al.*, 2014), with minimal detectable quantities of furfural being produced. It is beneficial to use mild

acid pretreatment due to its associated high yield, in terms of cost, and method cruelty. The sulphurus residue associated with sulphuric acid can be disadvantageous for downstream processes (Taherzadeh and Karimi, 2008) particularly for fermentations in which commercial yeast are used.

2.7.2 Hot water pretreatment (HWP)

This type of pretreatment makes use of hydrothermal apparatus which is another effective method of pretreating biomass. It involves no chemical or catalyst usage but it utilises high pressure to sustain water at high temperatures(160–240°C) which can aggravate changes in the organization of the lignin in biomass. To prevent the formation of inhibitors, the pH should be mild (4-7) and reaction time minimal, 30 min (Georgiev *et al.*, 2014; Yu *et al.*, 2013; Alvira *et al.*, 2010; Taherzadeh and Karimi, 2008). Hot water makes it easy for the accessibility of holocellulose by loosening the lignin while minimising the creation of fermentation inhibitors. (Kim *et al.*, 2011), reported that HWP perform best if heated at 200°C for 10min under pressure. This pretreatment method has been reported to recover high yield of fermentable sugars, hence; it is suitable for large-scale industrial processes (Gao *et al.*, 2013).

2.7.3 Cellulase pretreatment (CP)

Agrowaste can also be pre-treated with microorganisms by decomposing the lignin and exposing the holocellulose structure for effective hydrolysis. The biological pretreatment involves microorganisms such as white-rot and soft-rot fungi. Literature review has shown that white-rot and soft-rot fungi predominantly breakdown the plant lignin with negligible holocellulose degradation (Narayanaswamy *et al.*, 2013). White rot fungi (WRF) falls under members of the Enmycota a group of basidiomycetes characterized by their distinctive, unlimited degradative structure that utilitize the extracellular habitat (Barr and Aust, 1994). White rod fungi such as *Phanerochaete chrysosporium* expresses lignin peroxidase (LiP), manganese peroxidase (MnP) and lactases in nutrient-limited conditions, enzymes which solubilities lignin nutrient-limited conditions are often required for the activation of the lignin biodegradation exposing the holocelluloses. In this study, white rot fungi and *Nepenthes mirabilis* plant extracts made up of a cocktail of enzymes were designated to be suitable for application in a pretreatment, a method termed tertiary biovolarisation.

Table 2-2: Some common methods of biomass pre-treatment/hydrolysis.

Pretreament/hydrolysis method	Working conditions	Advantages	Disadvantages
Dilute inorganic acids	140-190^{o}C	Can pre-treat a variety of biomass, Short reaction time, High yields, Direct pre-treatment to fermentable sugars	High cost, High temperature, Corrosion potential, residual sulphurous species
Ammonia oxidation	60–90 °C	High degree of delignification	High process costs
Ammonia fiber expansion (AFEX)	90-100^{o}C, 15-30mins, (2 kPa), the process is controlled by four main co-factors Ammonia loading, water loading, residence time and temperature	Rapid reaction time, Increases reaction surface area, Increases reaction surface area, Less inhibitors formation	High cost of Ammonia,
Aqueous ammonia soaking (AAS)	20-30^{o}C	Can be operate at ambient temperature and pressure	Residual Ammonium by-products, cost of Ammonia
Organosolvent	150-200°C, catalyst required	Catalyst recycling possible	High temperatures, organic chemicals usage, by-product

Table 2-2: ***Cont.***

Steam explosion (autohydrolysis)	160-260^{o}C, 7-5Mpa	Less chemicals usage	High temperatures, generates inhibitory by-prodcuts which affect fermentation
Carbon dioxide explosion	35-80°C, 7-28Mpa	Minimal toxic compounds generated	Hydrlysate acidity increases during this process.
Liquid hot water (LHW)	160-240°C	High yields, minimal corrosiveness, environmentally benign, No chemicals usage, size reduction of the biomass is not needed	High temperatures, high water requirements, toxicant generation
Microwave chemical	35 °C	Generate high heat for effective delignification than chemo-thermal processes	Exposure to irradiation, costly equipment, similar outcomes to that of conventional heating
Hydrothermal	121 - 270^{o}C	Rapid reaction time	Generate inhibiting by-products

Table 2-2: ***Cont.***

Wet oxidation	150-320°C, 5-20 MPa	Minimal inhibitor generation	High temperatures and pressure, high cost
Ozonolysis	20-30^{o}C, 101.3 KPa	Minimal inhibitor generation, low energy intensity	Residual oxidation reactive species residue
Biological	Ambient to 55 ^{o}C, atmospheric pressure	Low energy input, low temperature, environmentally benign, low cost	Optimal conditions required, sufficient quantities of biological catalyst, slow
Alkaline caustic	Low temperatures and pressure	Low energy consumption, low cost	Caustic-based residue generation
Milling	Ambient and up to 80°C, a few minutes to 1-3 days	Reduction of particle size, increase surface area, increased biomass porosity, minimal toxic by-products formed	High cost, energy intensive
Hydrogen peroxide	90°C, pH 11.5, up to 2 hrs	Less inhibitory products produced, high yield	Oxidation reactive species residue

2.7.4 Tertiary biovalorisation of grape pomace

This can be defined as the usage of microorganisms in the repurposing of grape pomace without the utilization of either high energy or synthetic chemicals with the sole assistance of a natural product for the delignification thus biocatalytic decomposition of complex polymeric carbohydrates containing structures in the agro-waste for the release of fermentable monosaccharides for the production of other value-added products. This concept was developed for use in this research study and has not been reported elsewhere!

2.8 Green chemistry approach: Biovalorisation using *Nepenthes mirabilis* extracts (pitcher plant)

Some plants produce oxidative extracts with a very low pH (<2), high redox potential (510mv), with a density lower than that of water (1 g/cm^3). Such plants include *Nepenthes mirabilis,* see Figure 2-7.

a) closed *Nepenthes mirabilis pod* b) open *Nepenthes mirabilis* pod

Figure 2-3: *Nepenthes mirabilis* pods (a) open and (b) closed

N. mirabilis and other associated plants, produces natural enzymatic extracts with an ability to valorise a multitude of materials. By utilising such extracts, an environmentally benign method which focuses on the strategy of process development using renewable resources, in order to decrease the environmental

burden associated with harmful synthetic substances can be implemented. In green chemistry applications, there is minimal chemical usage, with some reaction being free of synthetic chemicals with minimal energy intensity.

2.8.1 Proposed biovalorisation strategy for producing value-added products from grape pomace

Some essential nutrients can be confined within agro-waste and can be directly removed or be transformed with the assistance of microorganisms into wanted bio-products, e.g. functional foods used in nutriceuticals, chemicals and monomers for bio-plastics production, etc (Lin and Sze, 2012). Therefore, the proposed strategy for biovalorisation of grape pomace will follow the following sequence.

- Slurrification to dissolve freely available materials (compounds),
- Treatment with a suitable mixture of enzymes such as those found in *N. mirabilis* pods, and
- Further treatment with cellulases, for the furtherance of the pre-treatment processes, thus increased product yield, without the use of an external heat source nor synthetic chemicals.

2.8.2 Other methods which can be used to extract value-added products from the grape pomace

Leading green technologies used for pre-treatment or during the extraction processes to recover high-value products from agrifood and/or agro-waste are enzyme-aided extraction, ultrasound-aided extraction, microwave-aided extraction, electrically aided extraction, liquid hot water extraction, supercritical-liquid extraction, and instant controlled pressure drop extraction (Carciochi *et al.*, 2017). All these methods can be used to augment the proposed tertiary biovalorisation pre-treatment method to extract valuable constituents from the grape pomace.

2.9 Summary

Repurposing of grape pomace has been researched by many researchers to produce valued-added products by using chemicals and high energy input which are not environmentally benign processes. In this research study, grape pomace will be pretreated with *N. mirabilis* (green chemistry approach) to release fermentable

monosaccharides for the production of other valued-added products. This has not been reported elsewhere.

CHAPTER 3

MATERIALS AND METHODS

CHAPTER 3

MATERIALS AND METHODS

3.1 Introduction

This chapter registers, the materials used, experimental procedures and the logic for each procedure used for this research study. The chapter also deliberates on some of the analytical tools which were used to present and /or evaluate the results achieved. All the experiments and analyses were conducted in triplicates to ensure that there were reproducible.

3.1.1 Collection and preparation: Grape pomace as a holocellulosic biomass feedstock

GP (*Vitis vinifera*) waste, was collected from ARC's Nietvoorbij experimental cellar farm (with permission), Stellenbosch, Cape Town (Western Cape, South Africa). The GP was immediately stored in a plastic bag and placed on ice prior to transportation, and subsequent to storage at -20 ºC. These samples were dried in an oven at 80°C for 3days. The samples were milled to a powder form (>45µm to<100µm) using a ball mill without a pre-washing step. A mass (2g) of the milled GP was weighed and transferred into Scott bottles, (200mL) of sterile distilled water in a slurrification process (6h). Additionally, a mass (10g) of milled GP was weighed and mixed with *Phanerochaete chrysosporium* BKMF1767 inoculum (10% v/v) grown in agar plates as described in Ntwampe (2005) and placed in an incubator (37°C) for 7days. Therefore, 2g of the *P. chrysosporium* bio-treated samples were slurrified in 200mL sterile distilled water. Overall, different (n=2) samples were prepared; i.e. 1) untreated GP (UGP) and 2) *P. chrysosporium* bio-treated GP samples.

3.1.2 Grape pomace lignin, holocellulose and ash content analyses

The analyses were done using the Biorefinery Test Method L2 (2016). The total lignin content, i.e. the acid soluble lignin (ASL) and the acid insoluble residue/lignin (AIR), were quantified as residual lignin content of the un- and pre-treated GP. For this, a volume (1mL) of 72% sulphuric acid was added to beakers containing the homogenised milled GP (300mg) samples. The samples were stirred using a glass rod until the test samples began to dissolve. A volume (28mL) of sterile distilled water

were added to the samples subsequent to placement in a water-bath (30°C) for 1h. Thereafter, autoclavation (120°C) for 1h ensued subsequent to cooling to 80°C. The samples were each filtered using a pre-weighed compacted glass fibre filters. The ASL was then quantified by measuring the filtrate absorbance (205nm) using the Jenway 7305 UV/Vis spectrophotometer (Cole-Parmer Instrument Co. Ltd, Cambridgeshire, UK). The extinction coefficient of lignin to determine ASL in this method, was as per TAPPI UM 250 method (TAPPI, 1991) using Eq. 3.1. The residual biomass on the filters was oven dried (105°C) for 2h subsequent to reweighing. The AIR was then determined. Lastly the samples were ashed at 800°C for 2h using a furnace.

$$ASL = \frac{A.V}{\varepsilon.l.M} D_f.C_{GP} \quad (3\text{-}1)$$

A is absorption at 205 nm, D_f is dilution factor, *V* is volume of the filtrate, in Litres (0,029 L), ε is extinction coefficient of lignin, in g/L.cm (110 g/L.cm, according to TAPPI UM 250), *l* is cuvette path length, in cm (1 cm), *M* is weight of sample (as 100% dry matter) before acid hydrolysis/suspension (g), *CGP* is the concentration of grape pomace used.

3.1.3 Hot water pretreatment (HWP)

The samples were then autoclaved at 121ºC for 15mins. The samples were cooled and samples (6mL) from a homogenous mixture were centrifuged at 4000xg for 5mins. A volume sample (1mL) was then added to distilled water (9mL) for dilution. The essay mixture was composed of equivalent aliquots of the diluted sample and DNS (1,5mL) in sterile test tubes, with a 7305 UV/Vis spectrometer (Cole-Parmer, UK) being used to measure the absorbance at 575nm; hence, TRS quantification.

3.1.4 Dilute acid pretreatment (DAP)

DAP commenced immediately after HWP. A volume (1mL) of 1% (v/v) dilute sulphuric acid was added to the Schott bottles containing the samples. It was heated for 30mins at 121ºC. The samples were cooled subsequent to centrifuging at 4000xg for 5mins. A

volume of samples (1mL) was then added to distilled water (9mL) for dilution. Thereafter, TRS was quantified.

3.1.5 Cellulase pretreatment (CP)

After the DAP process, CP commenced. The pH of the samples was adjusted to 4.5 using sodium acetate buffer (pH 5.6). A volume 600 µL/g waste of commercial cellulase (24.67 U/L) was pipetted into the Schott bottles containing the samples. It was heated to 55ºC for 72h. Thereafter, TRS was quantified and some of the filtrate was stored at -20 ºC for future use.

3.1.6 Grape pomace pretreatment using conventional methods

For comparative analysis of the new pretreatment method being developed, conventional methods i.e. Hot Water, Dilute Acid and Cellulase pretreatment (HWP/DAP/CP) methods were sequentially used in a single reaction vessel, i.e. using Schott bottles a process analogous to the proposed *N. mirabilis* pre-treatment process being developed herein. During HWP, samples were autoclaved at 120ºC for 15 min (Selvaraj and Vasan, 2018; Taherzadeh and Karimi, 2008), subsequent to sample cooling to ambient temperature. Subsequently, dilute sulphuric acid for DAP was added to the Schott bottles containing the samples to constitute a 1% (v/v) concentration, with heating being instituted for 30 min at 121ºC (Maurya *et al.*, 2015; Idrees *et al.*, 2013; Alvira *et al.*, 2010; Kootstra *et al.*, 2009). Thereafter, samples were also cooled to ambient temperature prior to the commencement of the CP. Prior to CP, the samples were (pH=4.5) adjusted by using a sodium acetate buffer (pH 5.6) with a volume (600µL) of commercial cellulases (25 U/mL) being added into the Schott bottles containing the samples, with heating (55ºC) ensuing thereafter for 72h (Zeng *et al.*, 2011). After this, the samples were cooled, subsequent to centrifugation at 4000xg for 5mins, with total reducible sugars (TRS), total residual phenolic compounds (TRPCs), powder X-ray diffraction (pXRD), Fourier transform infrared Spectroscopy (FTIR) analyses being conducted thereafter, using liquid samples and the recovered residual GP biomass. This process was denoted the classification HWP/DAP/CP.

3.2 Quantification of total reducible sugars (TRS)

The TRS that were extracted during the pretreatment of the GP was measured using a DNS based method, with the assay mixture being composed of DNS (10g), phenol (2g), sodium sulphite (0.5g) and sodium hydroxide (10g), made-up to 1000mL using sterile distilled water (Miller, 1959). Aliquots of the diluted sample and DNS (1,5mL) was transferred into sterile test tubes and heated in a water bath for 10mins at 90ºC, subsequent to cooling to ambient temperature followed by the addition of a 40%(v/v) sodium potassium tartrate (0.5mL) solution into test tubes for TRS concentration analyses using a spectrophotometer (575nm). Different glucose concentration standards (0-1000mg/L) were used to produce an appropriate calibration curve. The control was distilled water and DNS solution, without the diluted samples. Figure 1A to 1C, highlight the results of TRS concentration obtained using different pre-treatment methods.

TRS during the pretreatment of GP was measured using dinitrosalicylic (DNS) based method, with 2g/200mL GP samples. The assay mixture being made up of DNS (10g), phenol (2g), sodium sulphite (0.5g) and sodium hydroxide (10g), in 1000mL of sterile distilled water (Miller, 1959). Equivalent volumes (1.5mL) of the diluted samples (1:9) and the DNS mixture, were transferred into sterile test tubes and heated in a water bath for 10min at 90ºC, subsequent to cooling to ambient temperature. Thereafter, a 40 %(v/v) sodium potassium tartrate (0.5mL) solution was added into the test tubes. Subsequently, the TRS concentration was analysed using a Jenway 7305 UV/Vis spectrophotometer (575nm Cole-Parmer, UK) using a calibration curve developed by different glucose concentration standards (0-1000 mg/L), to quantify TRS at different sampling intervals. The control used was distilled water and DNS mixture without the diluted pretreatment samples.

3.2.1 Data handling: Effectiveness of *P. chrysosporium* bio-treatment on TRS extraction using dilute acid and cellulases

In order to assess the effectiveness of biologically pre-treating the GP, comparative absolute relative increases between mild acid and cellulase pre-treated GP was assessed using the HWP processes as a reference, as it had a minor or insignificant contribution to the TRS extracted from the GP. This was computed using Eq. 3.2:

$$\% \text{ Relative Increase} = \left(\frac{\Delta TRS_{CP}}{\Delta TRS_{DAP}} - 1\right) \text{ X } 100 \quad (3\text{-}2)$$

Whereby ΔTRS for both cellulases (ΔTRS_{CP}) and mild acid (ΔTRS_{DAP}) pretreatment can be estimated using Eq. 3.3.

$$\Delta TRS_{CP/DAP} = \left(\Delta TRS^{*\backslash x}_{CP/DAP} - \Delta TRS^{*\backslash x}_{HWP}\right)/\Delta TRS^{*\backslash x}_{HWP} \quad (3\text{-}3)$$

With **/x* being ΔTRS for *P. chrysosporium* treated (*) or untreated GP (*x*). Standard deviation from a triplicate set of experiments was used in this study to account for variations in datasets.

3.2.2 Quantification of total residual phenolic compounds (TRPCs)

Agro-waste contains inhibitors known as phenolic compounds which are predominantly accountable for inhibiting the functionality of β-glucosidase (Kim *et al.*, 2011), a key enzymatic agent which facilitates the breaking down of oligosaccharides to simpler sugars (Singhania *et al.*, 2013). The Folin-Ciocalteu assay, composed of 15 g of lithium sulphate in 5 mL of water, and a drop of bromine, was used to quantify TRPCs in the samples (1:9) (Blainski *et al.*, 2013; Ainsworth and Gillespie, 2007; Singleton *et al.*, 1999). A volume (100µL) of the diluted samples was transferred into test tubes whereby a volume (250µL) of the Folin-Ciocalteu reagent and a volume (1.5mL) of sterile distilled water were added to each sample test tube allowing for a 3 min reaction time to lapse, subsequent to addition of 20% (w/v) Sodium carbonate (1mL). The assay mixture was kept in the dark for 1h prior to absorbance (650nm) analyses, a Jenway 7305 UV/Vis spectrophotometer (Cole-Parmer, UK). Thereafter, the TRPCs were quantified, using a calibration curve generated with 2 to 10mg/L of 1, 2-dihydroxybenzene, a hydroxylation by-product of phenol.

3.2.3 Quantification of total residual organic acids (TROAs)

Organic acids such as tartaric, fumaric, benzoic, formic, citric, shikimic, succinic and malic acids are commonly found in agro-waste in varying quantities. For the quantification of TROAs, a volume (3mL) of the centrifuged sample was measured and

the pH was recorded. Equivalent (3mL) aliquots of distilled water were used to dilute the sample and the pH was recorded after dilution. The method used was a titration method using phenolphthalein as an indicator as described by (Sinclair *et al.*, 1945). A volume (10 µL) of phenolphthalein was added to the sample and titrated with NaOH drop-wise until a colour change was observed. Hence, TROAs was measured. The volume of NaOH used is an indication that there are TROAs in the sample.

3.3 *Nepenthes mirabilis* digestive fluids

3.3.1 *Nepenthes mirabilis*: Collection and preparation

N. mirabilis digestive fluids were collected from Pan's Carnivores Plant Nursery (Tokai, Cape Town, South Africa). The pitcher plants were grown in a greenhouse under controlled environmental conditions. The digestive fluids were collected from both open (with prey) and closed (without prey) pitcher plants and pooled to form a composite sample. The digestive fluids were carefully collected into sterile 50mL conical tubes and were immediately stored in ice prior to transportation to the laboratory. Thereafter, the digestive fluids were filtered (0.2µm) and stored at 4°C. Upto 15 to 35mL of *N. mirabilis* digestive fluids were collected from each monkey cup depending on the sizes of the cups (Lee *et al.*, 2016; Adlassnig *et al.*, 2011).

3.3.2 *N. mirabilis* digestive fluid physico-chemical characteristics

Instruments used to measure the different physio-chemical characteristics of the digestive fluids from *N. mirabilis* cups were, a pH meter to measure both pH and redox potential, with the specific gravity being measured by using a hydrometer, while an electrical conductivity meter was used to measure the conductivity.

3.3.3 *N. mirabilis* digestive fluid microbial population

Prior to digestive fluid filtration, the fluid were analysed for microbial population proliferation using a VITEK 2 systems VO7:01 (BioMérieux, France), utilising gram-negative and positive cards (GN cards) (Pincus, 2005), as per the manufacturer instructions. DNA sequencing was further utilised to identify strains in the digestive fluid which were not identified by the Vitek system. Cultures were activated by adding a volume (1µL) of the *N. mirabilis* digestive fluids into 15mL glass test tubes that contained Luria broth (5mL) subsequent to incubation at 37°C for 24h. The cultures broth were then inoculated onto Luria Bertani agar (LBA) plates at 30°C for 24h, with pure cultures sub-cultured for specie purification and identification (Chan *et al.*, 2016). DNA was isolated from cultures using ZR Bacterial DNA kit™ (Zymo Research). PCR was performed by using DreamTaq™ DNA polymerase (Thermo Scientific™) and primers (16S-27F 5' – AGAGTTTGATCMTGGCTCAG – 3' and 16S-1492R 5' – CGGTTACCTTGTTACGACTT – 3') were used to amplify the 16S rDNA target region. Zymoclean™ Gel DNA Recovery Kit (Zymo Research) was used to

extract the PCR products (amplicons) from agarose gel, which was sequenced on the ABI PRISM™ 3500xl Genetic Analyser (Thermo Fisher Scientific, Massachusetts, USA) in both forward and reverse direction. The sequenced products were purified with ZR-96 DNA Sequencing Clean-up Kit™ (Zymo Research) and analysed with CLC Main Workbench 7 (QIAGEN Bioinformatics, Aarhus, Denmark). A BLAST search was also done on the NCBI website.

3.3.4 Biocatalytic activities of *N. mirabilis* digestive fluid

Biocatalytic activity of enzymes of interest was undertaken based on their ability to degrade the agro-waste constituents including by-products formed, with the enzymes of interest being.β-glucosidases, xylanases, and carboxylesterases determined earlier for their importance, and identified as; 1) having an essential cellulose bio-decomposing component for the biocatalytic conversion of cellobiose, which is a reducing sugar (Chan *et al.*, 2016); 2) having the capacity to biodegrade holocelluloses (García-Huante *et al.*, 2017) and 3) have the potential for hydrolytic activity against carboxylester bonds between holocellulose bound sugars and lignin monomers (Manavalan *et al.*, 2017). All assay mixtures were mixed in 3mL Eppendorf tubes, prior to assay mixture transfer in to glass cuvettes for absorbance reading, in a kinetic mode.

Since the *N. mirabilis* pre-treatment was conducted at ambient temperature (25°C), therefore the enzyme activity quantification was also conducted at ambient temperature, p-nitrophenyl-β-D-glucopyranoside (pNPG) was used as a substrate to measure the activity of β-glucosidase in the *N. mirabilis* digestive fluids (Kim *et al.*, 2012; Bailey *et al.*, 1992), with the rate of formation of xylose from endo-xylan being determined as an indication of xylanase (Sudffldt *et al.*, 1990). Additionally, p-nitrophenyl acetate (pNPA) was used as a substrate to determined carboxylesterase activity. Overall, the *N. mirabilis* digestive fluids were used to perform the above assays in a suitable buffer at ambient temperature, using a Jenway 6405 UV/Vis spectrophotometer (Cole-Parmer, UK), with a temperature controlled cuvette holder, set in a kinetics mode (Gilham and Lehner, 2005; Wheelock *et al.*, 2001; Ljungquist and Augustinsson, 1971; BRENDA). For β-glucosidase, the reaction mixture contained 600µL 50mM sodium acetate (pH 6) containing 0.35mM pNPG, enzyme

(200µL) with the reaction being performed at 420nm, using extinction coefficient of 18100$M^{-1}.cm^{-1}$ to monitor the product produced, i.e. ρ-nitrophenol (pNP) (Kim *et al.*, 2012; Bailey *et al.*, 1992). Similarly, the activity of xylanase was determined using a reaction mixture constituted by 100mM McIlvaine's citrate phosphate buffer (pH 5) in which 54.2mM endo-xylan was dissolved, at 25°C and 586nm using an extinction coefficient of 135 $M^{-1}.cm^{-1}$ (Sudffldt *et al.*, 1990) to quantify the product formed, i.e. xylose. To quantify the activity of carboxylesterases, the reaction mixture contained 200mM Tris-HCl buffer (pH 7.8) containing 0.5mM pNPA as a substrate, to monitor the reaction at 410nm, using extinction coefficient of 17000 $M^{-1}.cm^{-1}$ for the product formed, i.e. pNP (Gilham and Lehner, 2005; Wheelock *et al.*, 2001; Ljungquist and Augustinsson, 1971; BRENDA). The enzyme activity was quantified using Eq. 3-4.

$$Enzyme\ activity\ (U/L) = \frac{dA/dt}{\varepsilon}.Df \rightharpoonup .600 \qquad (3\text{-}4)$$

Where *dA/dt* is the rate of absorbance change, D_f is the dilution factor, ε is extinction coefficient of lignin, *U/L* is the µmol substrate converted per minute per litre.

3.3.5 Grape pomace pre-treatment using *N. mirabilis* digestive fluids

The agro-waste was allowed to initially undergo a slurrification process, for 72h with the first sample being conducted at 6h to quantify readily dissolvable sugars in the form of TRS from the unwashed GP prior to the direct supplementation of the *N. mirabilis* digestive fluids (1%) into each of the Erlenmeyer flasks used; a process analogous to when a 1% DAP is implemented. The flasks were incubated in a shaking incubator 120rpm at 25°C, with sampling being done at after 6, 72, 96, and 120 h during experimentation. The TRPCs, were measured alongside the TRS; afterward, the residual GP biomass recovered was recovered by centrifugation (4000xg, 5min, for 5days) and oven dried (80°C) to reduce the moisture content prior to the evaluation of structural deformation using Fourier transform infrared Spectroscopy (FTIR) and powder X-ray diffraction (XRD) systems. The HWP/DAP/CP samples were used as a reference to compare the efficacy of the *N. mirabilis* (NmGP) and *Phanerochaete chrysosporium/N. mirabilis* [NmBT (GP)] treated samples. All biomass samples were mixed to form a composite sample for XRD and FTIR analyses.

3.4 Powder X-ray diffraction (pXRD) and Fourier Transform Infrared Spectroscopy (FTIR) analysis

The pooled residual GP biomass obtained pre- and post- pre-treatment were oven dried (80°C for 24h) and the crystallinity index (*CrI* %) was quantified. This was done using a pXRD (Bruker Pty Ltd, SA) at 40kV and 40Ma with a D2 phaser with a Lynxeye, (Bruker South Africa (Pty) Ltd., Sandton, South Africa), which provided a suitable peak-to-background ratio (Qiu *et al.*, 2012). The scanning range (2θ) was 10 to 50° at a ramping scale 0.017°, using a zero background holder plate (50µm depth), with the crystallinity index (CrI%) being determined using Eq. 3-5.

$$CrI(\%) = \frac{\Delta I}{I002}.100 \qquad (3\text{-}5)$$

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

Furthermore, to ascertain the efficiency of the pre-treatment method developed, an α-FTIR spectrometer (Bruker Pty Ltd, SA) and smart iTR with a diamond crystal window was used to measure the organic, polymeric, and in some cases, inorganic materials including functional groups in the untreated and pre-treated GP. Firstly, the measurement were taken against a background spectrum of the diamond window without the agro-waste sample. A scan range of 400-4000 cm^{-1} at a spectral resolution of 4cm^{-1}, at 100 scans per min was used (Zeng *et al.*, 2011).

3.5 Experimental data handling, computations and statistical evaluation

The pretreatment of GP was done with the total experimental time being 120h with recurrent sampling being at 6, 72, and 120h to perform different analyses. The supplementation of *N. mirabilis* digestive fluids was at 52h after determining that readily dissolvable TRS concentration solubilised in the sterile distilled water stabilised at 52 to 72h, and the experiment was terminated at 120 h. The mean value, and standard error of the mean (SEM) was determined (Eq. 3-6) from the data produced from the various analyses. Experimental data were all computed in order to

take into account sample dilutions which were used to measure the actual concentrations for parameters monitored. All analyses where performed in triplicates.

$$SEM = \frac{\text{Standard Deviation}}{\sqrt{\text{Number of samples tested}}} \quad (3\text{-}6)$$

CHAPTER 4

RESULTS AND DISCUSSION

CHAPTER 4

RESULTS AND DISCUSSION

This chapter is divided into two phases:

Phase 1: Objective 1) to initially identify a suitable agro-waste for biovolarisation for TRS extraction,
Objective 2) to ascertain the effect of bio-pretreatment on TRS extraction using *Phanerochaete chrysosporium* BKMF1767 in comparison with different commonly used pretreatments methods for hyper-extraction of TRS from GP (identified in objective 1) as the selected feedstock.

Phase 2: Objective 1) to develop a suitable process for the holocellulosic/TRS extraction of grape pomace (GP) using naturally available bioresources, i.e. *N. mirabilis* digestive fluids as pretreatment aliquots, in comparison to commonly used pre-treatment method, using a single reaction vessel strategy.
Objective 2) to quantify the activity of delignifying and cellulolytic enzymes in the proposed *Nepenthes mirabilis* extracts,
Objective 3) to quantify the percentage of residual lignin, cellulose, and hemicellulose (holocelluloses) in the grape pomace residue post pretreatment to determined which of the methods is better as a holocellulose/TRS extraction method, and
Objective 4) to identify organic compound residues such as organic acids and phenolics in the grape pomace hydrolysate and their fate during grape pomace pre-treatment using *Nepenthes mirabilis* extracts.

4.1 *Phanerochaete chrysosporium* supported biovalorisation of grape pomace for hyper reducible sugar extraction

4.1.1 Introduction

There are bountiful, inexpensive and renewable lignocellulosic biomass being landfilled continuously by the winery industry. The winery and juicing industries are among the profitable and substantial agro-economic operations worldwide,

processing a variety of grape berries. After the extraction of the juice from grapes, approximately 20% end-up as grape pomace (GP) containing skins, seeds, and stems-major components of holocellulose, which are landfilled, culminating in growing environmental pollution concerns (Gama, 2013). Instead of land filling, this GP can be used as animal feed or as a source of fermentable sugars (Figueira, 2017; Korkie *et al.*, 2017). If the GP is not treated or handled accordingly, it can result in deleterious environmental pollution challenges including release of extracts which will seep into ground-water bodies; this being among the challenges identified (Devesa-Rey *et al.*, 2011). Thus, the repurposing of GP, will serve as a means to remove waste containing phenolics and other potential toxicants from the environment and also as an alternative nutrient source for the production of value-added products (Zheng *et al.*, 2014).

GP is composed mostly of holocellulosic material which is categorized as a key source of fermentable sugars for the production of value-added products (Kumar *et al.*, 2008); albeit, it is challenging to degrade because of the presence of lignin (Bhatia *et al.*, 2012). The effect of milling and biological pre-treatment (bio-treatment) on fermentable sugar extraction from such GP has not been studied effectively. Some pretreatment methods, can be used for fermentable sugar extraction, with hot water, dilute sulphuric acid and cellulase pretreatment methods being preferred (Shafiei *et al.*, 2015; Gao *et al.*, 2013; Idrees *et al.*, 2013; Alvira *et al.*, 2010). Recent studies have shown that, pre-treatment techniques used to pre-treat lignocellulosic biomass encompassing agro-waste, are making emphases on the reduced pre-treatment time, exploiting the removal of fermentable sugars while reducing energy intensity usage, including the utilization of environmentally benign processes by eliminating the use of chemicals and at reduced operating cost (Hyun *et al.*, 2009). These processes can be utilized either as independent or as combined processes (Procentese *et al.*, 2017; Kumar *et al.*, 2016; Rajan and Carrier, 2014; Narayanaswamy *et al.*, 2013; Cheng *et al.*, 2012; Chiaramonti *et al.*, 2012; Lee *et al.*, 2009).

4.1.2 Aim and objectives

Therefore, the objective of this part of the study was to initially identify a suitable agro-waste for biovolarisation for TRS extraction and to ascertain the effect of bio-pretreatment on TRS extraction using *Phanerochaete chrysosporium* BKMF1767 in comparison with different commonly used pretreatments methods for hyper-extraction of TRS from GP as the selected feedstock, since they are deemed to be easily fermentable into value added products.

4.1.3 Selection of agro-waste

Studies have identified holocellulosic materials as one of the major source of fermentable sugars for the production of bio-ethanol and other value-added products (Kumar *et al.*, 2008). GP is a regionally available and inexpensive feedstock in the Western Cape; albeit, its availability is seasonal. Furthermore, although GP can be used to produce fermentable sugars, it does contain some inhibitory compounds such as ρ-coumaric, ferulic, acetic, glucuronic acids including furfural and phenolics, which are released during pre-treatment processes (Jönsson and Martín, 2016). Alternatively, cellulases can reduce some of these fermentation inhibiting by-products, while catalyzing fermentable sugar extraction from different agro-waste.

4.1.4 Total readily dissolvable sugars

The freely dissolvable sugar obtained during GP slurrification for the untreated samples was 271.05 ± 0.02 mg/L and 206.39 ± 0.06 mg/L for the *P. chrysosporium* treated samples, respectively. This revealed that *P. chrysosporium* used some of the freely available sugars present in the slurrified samples thus the reduced quantity of the TRS at the initiation of the experiments with differentiation being determined to be miniscule in comparison to the TRS present in the untreated samples.

4.1.5 Hot water pretreatment (HWP)

This is a commonly used pretreatment method, with its function being to delignify/or loosen the holocellulose in order, to improve its penetrability during hydrolysis. Thermal methods broadens the penetrable and vulnerable surface area of densely, lignified biomass, for improved accessibility by hydrolytic enzymes (Selvaraj and Vasan, 2018; Taherzadeh and Karimi, 2008). For HWP, the TRS concentration for

the untreated sample was 380.93 ± 0.14 mg/L, with that observed for bio-treated samples being 205.68 ± 0.09 mg/L. Although this pretreatment method can be classified as advantageous due to non-use of chemicals including independence of the agro-waste particle size, it is however, energy intensive with a higher water requirement than some pretreatment methods and it produces some toxicants such as furfural and phenolics which can sour downstream processes.

4.1.6 Dilute acid pretreatment (DAP)

One of the effective pretreatment methods used to delignify and solubilised agro-waste components to fermentable sugars is the dilute sulphuric acid pretreatment method. Its function is to solubilize hemicellulose to monosaccharides. At a higher temperature, sulphuric acid will also degrade xylose and some waste components into inhibitory compounds such as soluble lignin (Jönsson and Martín, 2016; Maurya *et al.*, 2015; Idrees *et al.*, 2013; Alvira *et al.*, 2010; Kootstra *et al.*, 2009). The yield for TRS was 2850.68 ± 0.31 mg/L for the untreated sample, with 2266.00 ± 0.73 mg/L being the concentration of TRS for the bio-treated samples. The acid pretreatment in combination with bio-treated samples released a higher concentration of fermentable sugars as compared to the HWP. This is probably due to the loosened hemicellulose and cellulose (structure) which culminated in the ease of the holocellulose decoupling. Although acid pretreatment is effective and produces a high yield of fermentable sugars, it has some limitations which include corrosiveness and the degradation of xylose and glucose units at high temperatures (Selvaraj and Vasan, 2018; Maurya *et al.*, 2015).

4.1.7 Cellulase pretreatment (CP)

Cellulases are a cocktail of enzymes used to hydrolyze, hemicellulose and cellulose, and are constituted by endoglucanases, cellobiohydrolases (exoglucanases), and β-glucosidases (Kumar *et al.*, 2008). By using cellulases, the delignified biomass structure, can be easily converted to disaccharides and further into fermentable monosaccharides. As there is a need for environmental benign, efficient, and inexpensive processes, cellulase for agro-waste pretreatment is deemed suitable. Numerous pretreatment practices are required to alter the physical and chemical composition of the lignocellulosic biomass, with enhanced hydrolysis rates being

observed for biomass pre-treated with dilute acid prior to cellulase pre-treatment (Zeng *et al.*, 2011). Although, this can culminate into residual toxicants when compared to sole cellulase pre-treatment which produces less harmful by-products. By pretreating the agrowaste with hot water and dilute acid can facilitate efficient cellulase treatment to slacken hemicellulose and celluloses for efficient debonding by the cellulases. After hot water and acid pretreatment, cellulase pretreatment effectively produced 2969.61 ± 8.05 mg/L (untreated samples) and 2068.49 ± 6.02 mg/L TRS for the bio-treated samples. The cumulative TRS obtainable is represented by Eq. 4-1 (untreated) and Eq. 4-2 (bio-treated).

$$\Delta TRS_T = 6201.22 \pm 8.50 \text{ mg/L} \quad (4\text{-}1)$$

$$\Delta TRS_T = 4540.17 \pm 6.84 \text{ mg/L} \quad (4\text{-}2)$$

4.1.8 Relative increases of TRS by bio-pretreatment

Table 4-1: Higher relative increases of TRS by bio-treatment

Pre-treatment methods	Untreated (%)	Treated (%)	Relative increases (%)
Mild acid	650	980	51
Cellulase	682	886	33

Table 4-I, highlights relative increases of TRS. Relative increases are the difference in the change between the different pre-treatment processes for the untreated samples and bio-treated samples, using HWP as a reference.

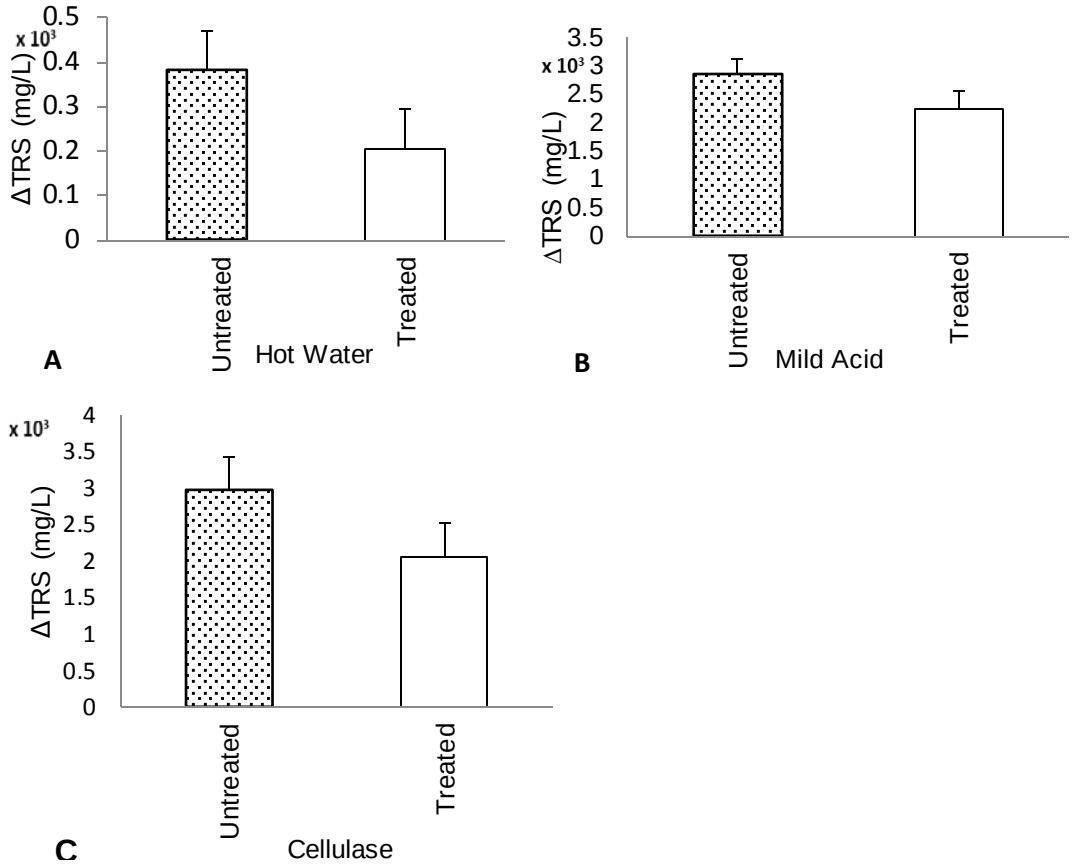

Figure 4-2: TRS results recorded for hot water (A), mild acid (B) and cellulase (C) pretreatments for the untreated and *P. chrysosporium* treated GP.

Overall, the relative increases for the bio-treated samples were higher than those of the untreated samples. This can be attributed to the fact that, the WRF helped in delignifying the samples; hence, making it easy for the pre-treatment procedures to perform optimally.

4.1.9 Summary

From the results obtained in this study, the cellulase pretreatment was determine to be an effective technique for the extraction of fermentable sugars from GP. This was seen in the TRS produced as 2969.61 ± 8.05 mg/L recorded for the untreated samples and 2068.49 ± 6.02 mg/L for the *Phanerochaete chrysosporium* treated samples. Overall, cumulative TRS was 6201.22± 8.50 mg/L for untreated samples and 4540.17 ± 6.84 mg/L for treated sample. Thus, from the results of this research,

it can be deduced that *Phanerochaete chrysosporium* used some of the readily available sugars in the samples for growth purposes. Although it contributed to higher relative increases for TRS extraction.

4.2 Sustainable *Nepenthes mirabilis* facilitated holocellulosic extraction from grape pomace

4.2.1 Introduction

The generation of waste is an indisputable part of human anthropogenic activity. As such, environmental pollution culminates as a result of waste landfilling, generating pollutant containing leachate (Mirabella *et al.*, 2014; Chandrasekaran and Bahkali, 2013; Liguori *et al.*, 2013). Similarly, the dumping and landfilling of agro-waste in pristine environments, is concerning. Overall, the repurposing and utilization of agro-waste is desirable and can limit its disposal, as such waste contains phenolics and other toxicants, which can pollute the environment.

Agro-waste contains micro- and macro-nutrients that can be used as a feedstock in the production of other value-added products by using environmentally benign processes. This can be achieved using numerous approaches which can include process integration, pre-treating the agro-waste appropriately for nutritional component extraction, exposing holocellulosic materials for further biodegradation and use in downstream processes (fermentation) for high value bio-products production (Shafiei *et al.*, 2015).

Current studies have shown that, many pretreatment techniques used to pre-treat holocelluloses embedded in agro-waste, emphasize the need to reduce the pre-treatment time to exploit the extraction of fermentable sugars as total reducible sugars (TRS), reduced energy intensity input, eliminating the use of synthetic chemicals thus reduced residual toxicants and reduced operating cost (Hyun *et al.*, 2009). Despite the fact that, currently utilised pre-treatment processes are successful, the utilisation of chemicals, mechanised processes, high energy input systems, in combination with biological pre-treatment techniques, is preposterous at an industrial scale even if they are either used as independent or inter mixed processes (Procentese *et al.*, 2017; Kumar *et al.*, 2016; Narayanaswamy *et al.*, 2013;

Cheng *et al.*, 2012; Chiaramonti *et al.*, 2012; Hyun *et al.*, 2009) with numerous challenges being encountered.

Presently, agro-waste pretreatment is carried-out in a number of processes, in which deligno-cellulolysis of the waste to fermentable sugars is facilitated; albeit producing inhibitors associated with the souring of downstream fermentations including, enzymatic hydrolysis. This requires the development of alternative and environmental benign holocelluloses valorisation methods for the pretreatment of agro-waste, for the production of value-added products, while limiting the production of toxicants from the lignin component of the waste which is a new promising alternative strategy towards the sustainable and efficient processing of numerous waste types (Nayak *et al.*, 2016; Chandrasekaran and Bahkali, 2013). Presently, a single stage or a pretreatment reactor system and an ecologically benign method free of chemical use and high temperature, have not been developed for the pretreatment of agro-waste, to efficiently address challenges associated with delignification, cellulolysis and production of inhibitors which in turn affects the downstream process (Jönsson and Martín, 2016).

In this research study, *Nepenthes mirabilis* plant digestive fluids were proven to be effective in targeting holocelluloses extraction, with a significant proportion of residual lignin and ash being left behind. The plant digestive fluids contained digestive aliquots which have the potential to biodegrade complex and polymeric molecules (Chan *et al.*, 2016). This pretreatment method proposed, requires less energy as it was operated at ambient temperature, and it eliminates the use of hazardous chemicals such as dilute inorganic acids; albeit the digestive fluid is acidic, with an added advantage of reducing the production of inhibitory compounds such as phenolics (Siragusa *et al.*, 2007).

4.2.2 Aim and objectives

Therefore, the objective of this part of the study was to develop a suitable process for the holocellulosic extraction of grape pomace (GP) in a single reaction vessel, using *N. mirabilis* digestive fluids as pretreatment aliquots, to exploit the extraction of the TRS. This process was compared to commonly use pre-treatment method, using a single reaction vessel strategy.

4.2.3 Results and discussion

From an availability perspective, GP is a regionally available feedstock, as in South Africa, more than 80% of table grapes are produced in the Western Cape region of South Africa (DAFF, 2012).

Research has shown that GP (*Vitis vinifera* waste) encompasses adequate quantities of readily dissolvable and fermentable sugars. Readily dissolvable sugars (rdTRS) are free soluble sugars that dissolve into solution during the slurrification process without any pretreatment method being implemented on the GP. The results seen after 6h shows that, the UGP released some quantity of free sugars than the *Phanerochaete chrysosporium* (BT) pre-treated GP. A stabilised concentration in the free sugars was seen after 48h culminating in the supplementation of *N. mirabilis* digestive fluids, after 52h. After 72h, the fermentable sugars concentration was further observed to increase.

The residual lignin, holocellulose and ash content of different pre-treated GP samples are shown in Table 4-2 with UGP being determined to have one of the highest content of holocelluloses (63.8%) in comparison to other agro-waste, e.g. rice strew (62.7%), (Montusiewicz *et al.*, 2017). Generally, softwood lignin content varies between 30–60% with an ash content being up to 0.50 % (Kang *et al.*, 2014), results which are similar to those observed for the UGP.

Table 4-3: Residual lignin, holocellulose and ash content in different GP samples

	Residual Lignin (%)	**Residual Holocellulose (%)**	**Ash (%)**
UGP	36	63.8	0.2
NmBT (GP)	61	38.9	0.1
NmGP	62	38	0.02
HWP/DAP/CP	75	24.6	0.4

For the single reaction vessel HWP/DAP/CP pre-treatment system, significant changes in the organization of the holocelluloses in the residual GP biomass

occurred, with a high proportion residual lignin being observed in comparison to NmBT (GP) and NmGP samples. In reference materials, a high content of ash is observed in lignin dominant samples than in sample with high hemicellulose and cellulose. This suggest that for ash content, lignin > hemicellulose/cellulose (Stefanidis *et al.*, 2014).

4.2.4 *N. mirabilis* digestive fluid characteristics and microbial population

In order to evaluate the ability of *N. mirabilis* digestive fluids to degrade the GP, several parameters were evaluated to determine the reduction and the oxidative reaction potential of the *N. mirabilis* digestive fluids. High redox potential means the solution has a high electron accepting tendency. Physico-chemical characteristics indicated that the *N. mirabilis* digestive fluids had a pH of 2 (±0.12), with a specific gravity in the range of 0.745 (±0.04). Furthermore, the redox potential was found to be 519 (±4.04 mV) indicating the oxidative potential of the digestive fluids, at a conductivity averaging 4.695 (±0.69) mS/m which was relatively high when compare to that of water, i.e. 1.75 (±0.72) mS/cm. Similarly, a 1% (v/v) dilute sulphuric acid, has a pH of 1.65, a redox potential of 681.6 mV and has a conductivity 18.23 mS/cm. Generally, these physico-chemical characteristics of the dilute acid used in the DAP, are analogous to those observed for the digestive fluids of *N. mirabilis*.

Microbial identification and isolation including DNA analysis was done on the *N. mirabilis* digestive fluids to identified different species proliferating in the fluids which included *Bacillus spp*. and *Klebsiella oxytoca* identified via16S rDNA sequencing. A comparative analysis between NmBT (GP) and NmGP, indicated that both methods were similar; albeit the NmBT (GP) had a slightly higher residual lignin than the NmGP, which had the lowest ash with the HWP/DAP/CP process showing a higher ash content. The *Bacillus spp.* identified were: *Bacillus cereus, Bacillus thuringiensis* and *Bacillus anthracis* with accession numbers KY249126.1, DQ513324.1 and KU948294.1, respectively. All the species were identified as being prevalent in *N. mirabilis* digestive fluids (Chan *et al.*, 2016).

Furthermore, the enzyme activity assays, thus, the *N. mirabilis* digestive fluids contained Carboxylesterase, (1260 ±6.63 U/L), β-glucosidase (2645 ±17.647 U/L)

and Xylanase (360 ±10.418 U/L). These enzymes all have the biocatalytic ability to decompose agro-waste (Zabed *et al.*, 2016; Kim *et al.*, 2007).

4.2.5 Direct comparative analysis of TRS produced using conventional and *N. mirabilis* pre-treatment process in a single reaction vessel

The agro-waste used was milled, which is another form of pretreatment which alters the structure of the GP, thus the holocelluloses crystallinity index (Maurya *et al.*, 2015; Taherzadeh and Karimi, 2008). However, the pretreatment of agro-waste into fermentable sugars using the HWP/DAP/CP, has been widely researched (Rocha *et al.*, 2014; Idrees *et al.*, 2013; Alvira *et al.*, 2010); albeit in a single reaction vessel. The comparison of combined common pretreatment, i.e. HWP/DAP/CP used sequentially to pre-treat GP, were directly compared to the NmGP.

HWP assists in the delignifying of biomass and the partial break-down of the holocelluloses making it easier for improved holocellulolysis efficiency, broadening the penetrability and vulnerability of the biomass surface area (Selvaraj and Vasan, 2018; Taherzadeh and Karimi, 2008). One of the successful methods used to delignify agro-waste components to fermentable sugars is using dilute sulphuric acid (DAP) with 1% (v/v) being determined to be amicable by researchers (Idrees *et al.*, 2013; Alvira *et al.*, 2010). Similarly to the HWP, the function of the DAP in biomass pretreatment is also to delignify and partially solubilize holocelluloses using a high temperature. At a higher temperature, dilute acid can degrade lignin into furfural and other components (Rocha *et al.*, 2014; Idrees *et al.*, 2013; Alvira *et al.*, 2010; Kootstra *et al.*, 2009). Previously, it was determined that the DAP releases more fermentable sugars as compared to the HWP when used as stand-alone processes, a phenomena evidenced in this study probably because of the corrosiveness of the acid, which would lessen the integrated bondage holocelluloses and lignin which in turn making it easier for the hydrolysis of the polymers resulting in more fermentable sugars extraction. This type of pretreatment have some limitations which include production of phenolic compounds and causes corrosion to equipment. By subsequently applying cellulases post DAP, ensured further holocellulolysis facilitated by a cocktail of enzymes constituted primarily by endoglucanases, cellobiohydrolases (exoglucanases) and β-glucosidases. The HWP/DAP/CP have

disadvantages as individual processes and when they are used sequentially in single reaction vessels. Consequently, there is a need for more environmental benign and efficient processes such as the NmGP method evaluated. Numerous pretreatment practices are designed at altering the physical and chemical composition of the lignocellulosic biomass albeit producing toxicants which reduce hydrolysis rates and other downstream fermentation processes (Zeng *et al.*, 2011). The results reported herein, indicated that the combination of HWP and DAP, indeed made it easier for the CP to effectively hydrolyse the holocellulose; hence a better TRS production result; albeit with a higher TRPCs -see Table1. For the combined HWP/DAP/CP single reaction system, 3269 ±8.054 mg/L of TRS was recorded for the UGP. On the other hand, total reducible sugars recorded for the *N. mirabilis* digestive fluids treated GP (NmGP) was found to be 951 ±4.666.mg/L; albeit with: 1) reduced TRPCs and 2) without energy usage, i.e. at ambient temperature-see Figure 4-2. From the results obtained, it can be deduced that, HWP/DAP/CP system performed better than the *N. mirabilis* pretreatment but, the NmGP can be an alternative green method for pre-treating agrowaste for TRS extraction, specifically for targeted holocellulose biovalorisation.

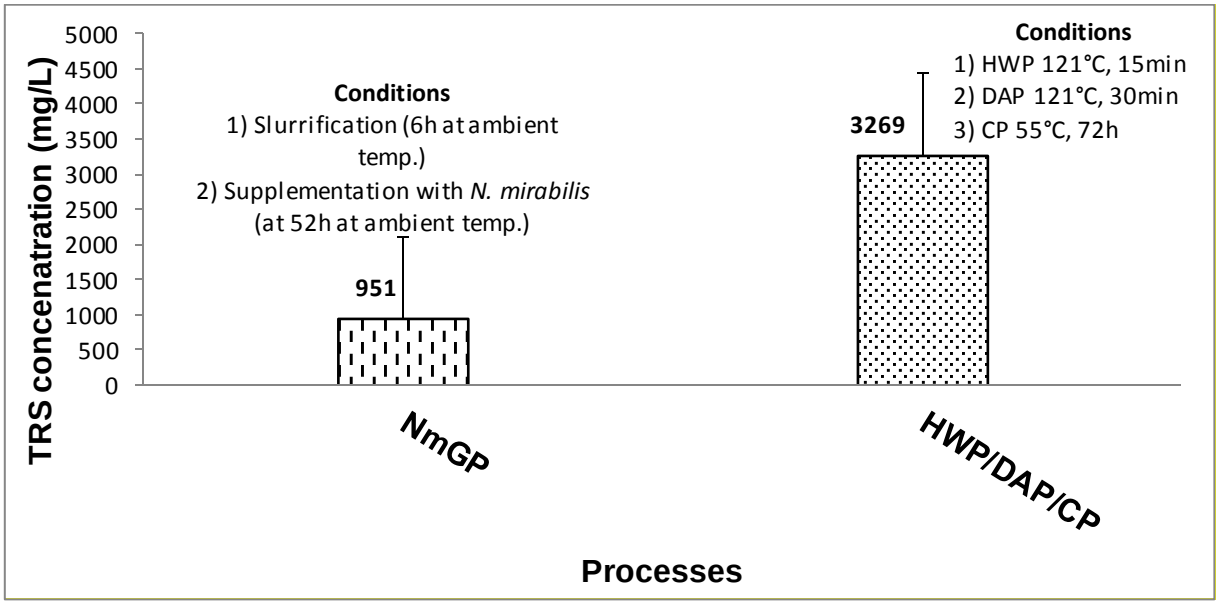

Figure 4-1. Direct comparison of pre-treatment processes in a single reaction vessel. TRS produced using conventional (HWP/DAP/CP) and *N. mirabilis* (NmGP) methods

4.2.6 Determination of total residual phenolic compounds (TRPCs)

Phenolics are one of the known inhibitory compounds in delignification of biomass for TRS extraction, with the inhibition of β-glucosidase being among the most detrimental in biorefineries hence the TRPCs quantification. TRPCs were measured at 6, 72, and 120h, 1) UGP (control), 2) NmBT (GP) - *Phanerochaete chrysosporium*/*N. mirabilis*, 3) *N. mirabilis* and 4) HWP/DAP/CP (used as reference) in a single reaction vessel system. Observations indicated the TRPCs were higher in the NmBT (GP) sample in comparison to the HWP/DAP/CP and UGP samples with less being observed for NmBT after operating the reaction vessels for 120h. There was an indication that the *N. mirabilis* digestive fluids had the capacity to reduce inhibitants such as phenolics which were lower at the end of the experimentation method, while that of NmBT (GP) were hypothetically assumed to have been increased by the *P*. *chrysosporium* decomposition of poly-phenols from the GP. The use of *N. mirabilis* digestive fluids for biological pretreatment of agro-waste was presumed to have the ability to reduce TRPCs formation at ambient temperature- Table 4-3

Table 4-4: TRPCs during conventional (HWP/DAP/CP), *N. mirabilis* (NmGP) and *Phanerochaete chrysosporium/ N. mirabilis* NmBT (GP) pretreatment methods

		TRPCs (mg/L)±SEM	
Process/Sample type	**Time (h)**	**NmBT (GP)**	**NmGP**
Slurrification	6	22.7±0.02	2.42 ±0.04
N. mirabilis	72	24.3±0.01	2.57±0.10
End	120	15.8±0.09	1.54±0.05
Control Experiments After 120 h TRPCs (mg/L)±SEM			
UGP		**HWP/DAP/CP**	
2.16±0.02		2.24 ±0.33	

4.2.7 Total residual organic acids (TROAs)

TROAs were quantified in the samples to actually show that they are organic acids in the samples by using the titration methods. This was done before and after the introduction of the *N. mirabilis.* The volume of NaOH titrated is directly proportional to the organic acids present in the samples and inversely proportional to the pH. The results shows the minimum and maximum TROAs and TRS at 72h. Quantification of TROAs using titration method with GP has not been extensively studied- see Table 4-4 and 4-5.

Table 4-5: Shows the TROAs for UGP at 72h

72 h				
	Min	**Max**	**Mean**	**SD**
Control	955	3042	1998.5	18
pH	4.01	4.32	4.17	0.214
TROAS (mg/L)	0.38	0.40	0.39	0.015
TRS (mg/L)	963.57	3076.47	2020.02	10.09

Table 4-6: Shows the TROAs for BT (GP) at 72h

72 h				
	Min	**Max**	**Mean**	**SD**
Control	478	1638	1058	5
pH	3.65	3.72	3.69	0.01
TROAS (mg/L)	0.42	0.45	0.44	0.003
TRS (mg/L)	506.06	1720.25	1113.16	3.78

4.2.8 Powder X-ray and FTIR evaluation of grape pomace

The effect of the pretreatment were further quantified by performing pXRD analysis prior and post pretreatment, to quantify the crystallinity index of the agro-waste samples. The deformation of the crystalline structures can be seen in the reduced crystallinity of biomass structures, into an amorphous phase. The untreated sample

(UGP) showed a crystallinity index (CrI) of 35% while other pre-treated samples had the following CrI (%) order: NmBT (GP) - 35% > HWP/DAP/CP - 34% > NmGP - 33%. The increase in CrI values as the pretreatment progresses indicated the removal of more holocelluloses leaving the crystalline lignin and ash fraction intact in the pre-treated solid residues (Timung *et al.*, 2016). Additionally, the high *CrI* for NmBT (GP) can be attributed to the fact that Phanerochaete chrysosporium contributed in the semi-delignification of the sample prior to ensuring N. mirabilis facilitated holocellulolysis pretreatment-refer to Figure 4-3.

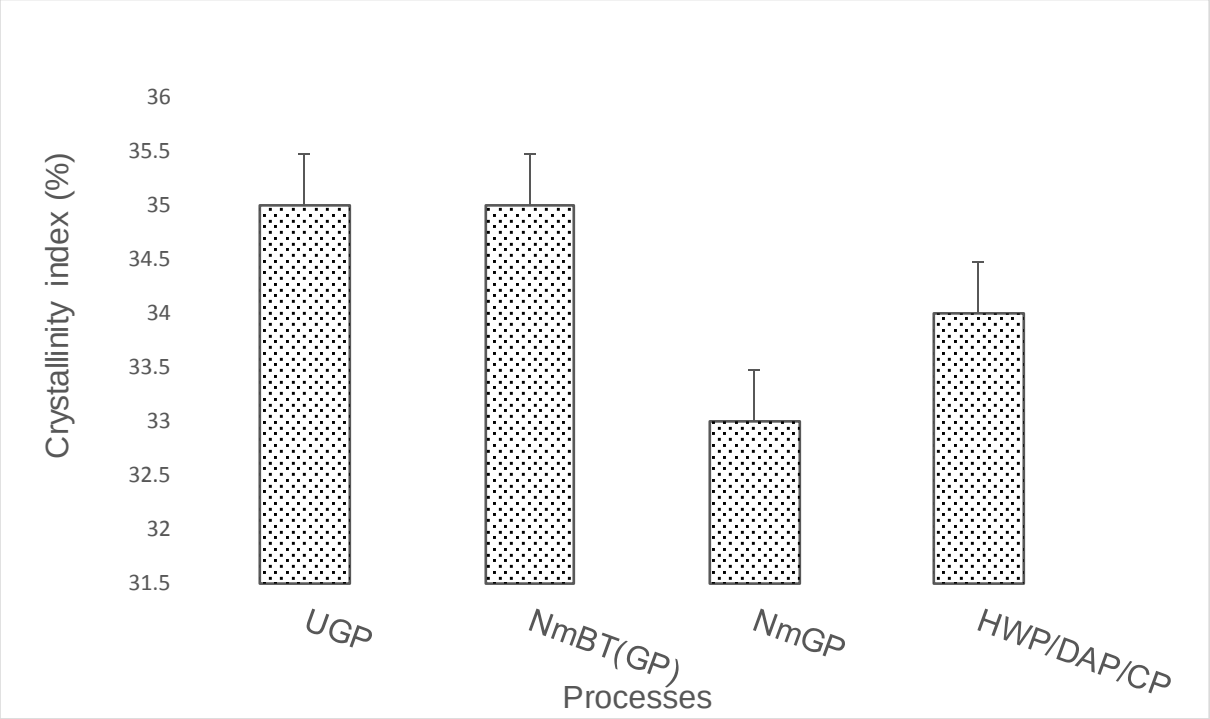

Figure 4-2: Graphical representation of the crystallinity index of the different pretreatment methods

Similarly, FTIR (Figure 4-4) was used to quantify the structural changes pre- and post-pretreatment. A broad peak was observed in the region of 3312cm^{-1} for all the pretreatment processes. These peaks are associated to the O-H group region of the FTIR spectrum. Also peaks such as; 2927 cm^{-1} (C-H stretching), 1745 cm^{-1}, (CH2/-SH/cellulose, C-H stretch, S-H stretch, C-H stretch associated with cellulose), 1605cm-1 (N-H, CH2, carbonyl stretching with aromatic rings, O-H stretch, C-H

stretch all associated with aromatic lignin), 1440 cm^{-1} (C-H, xylan C-O-C contribution) and 1030 cm-1 (C-O stretching vibration) were identified (Pavia *et al.*, 2014). The prominent peak at 950 to 1182 cm^{-1} is assigned to C-O, C-C, and C-OH bends in xylan; albeit overlapping with respect to C-O stretching at C-3 and C-O stretching at C-6 associated with cellulose. The FTIR spectra of cellulose and hemicellulose as constituents of holocellulosic material is very similar (Xu *et al.*, 2013; Rodd's Brudenell River Resort and Island, 2009). In previous studies, the bands formed at 895-897 cm^{-1} have been allocated as β-glucosidic bonds (Kunusa *et al.*, 2018; Liu and Kim, 2017), which were observed at wavenumber 896 cm^{-1} in this study; albeit there were not more pronounced in the HWP/DAP/CP, NmBT (GP) and NmGP samples. Both NmBT (GP) and NmGP samples, had very high transmittance which was indicative of a clustering of unexposed bonds, with pronounced inverted peaks at 1500, 1700, 3000cm^{-1}, related to emissions and lattice-bond strains.

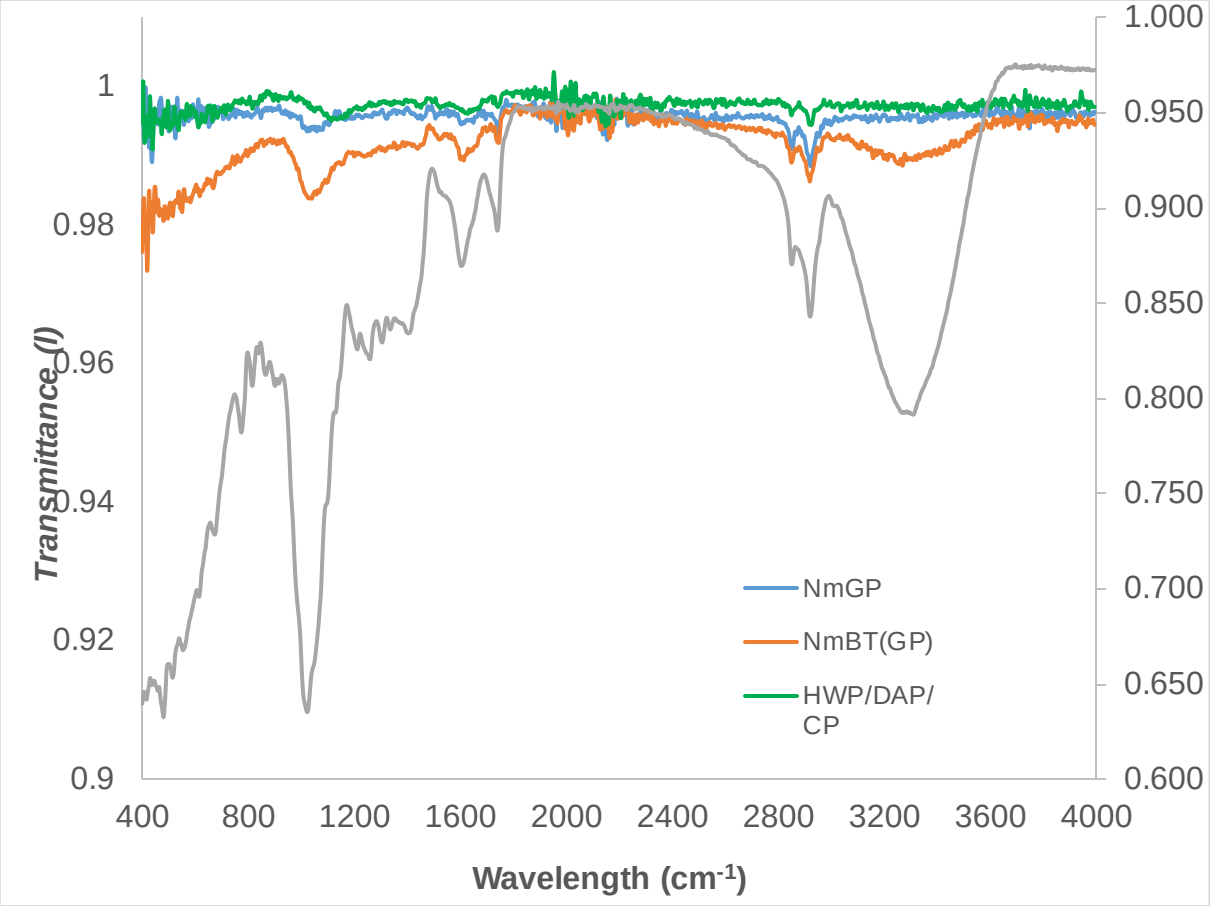

Figure 4-3: Represent FTIR for UGP, NmBT (GP), NmGP and HWP/DAP/CP

4.2.8 Summary

The results of this study has shown that *N. mirabilis* digestive fluids contains enzymes that have an ability to biodegrade holocellulose in agro-waste without heating. It was seen that, TRS produced by the conventional methods (HWP/DAP/CP) in a single reaction vessel was higher than that produced by *N. mirabilis* digestive fluids at ambient temperature. Results which were further confirm by pXRD, FTIR and lignin, holocelluloses content analyses in residual biomass. It can be assumed that, *N. mirabilis* digestive fluids can be used as an alternative environmental benign pretreatment method and still produce significant quantities of TRS as the conventional pretreatment methods.

Powder XRD diffraction and FTIR were also performed prior and post pretreatment methods to further quantify the efficiency of *N. mirabilis* digestive fluids. High crystallinity index was seen with the samples treated with *Phanerochaete chrysosporium*. FTIR also confirmed the distortion of lignin during the various pretreatment methods. Therefore, optimisation of the process conditions should be done in future studies including fermentations to ascertain whether the fermentation media constituted with *N. mirabilis* pre-treated agro-waste extractants, will perform similarly to those produced using conventional methods (HWP/DAP/CP).

CHAPTER 5

CONCLUSIONS

AND

RECOMMENDATIONS

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

Grape pomace (GP) is produced mainly by the winery industries and is being landfilled. These GP if not approriately diverted for other purposes, can cause enviromentalm pollution which can culminate in unfavourable human health outcomes and their extractants can leach into underground water bodies, further polluting the environment. Due to the landfilling of GP in the Western Cape, South Africa, there is the need for a suitable pretreatment method to further benefitiate this waste product of the winery industry. This study aimed to develop a suitable environmentally benign process for the biovalorisation of GP to produce total reducable sugars (TRS), which can be used in fermentation to produce value added products.

Several research works have proposed different methods for the pretreatment of GP, which are not eco-friendly due to either the use of chemicals and/or the application of high energy pretreatment systems which are costly. Thus, it was necessary to develop an environmentally benign and cheaper process for the biovalorisation of GP.

Therefore, this study began by measuring TRS produced by using *Nepenthes mirabilis* digestive fluids facilitated biovalorisation of GP, which is an environmentally benign method unlike the conventional pretreatment methods (HWP/DAP/CP) which involves the use of high energy input and chemicals. The TRS produced with *Nepenthes mirabilis* digestive fluids pretreatment were recorded as 951 ±4.666.mg/L, while that for HWP/DAP/CP were 3269 ±8.054 mg/L. The percentage residual lignin, residual holocellulose and ash in the GP were quantified using the Biorefinery Test Method (L 2:2016) with the result indicating that they were; 62, 38 and 0.02% respectively for NmGP and 75, 24 and 0.4% for HWP/DAP/CP, respectively, which indicated that if optimised, the application of *Nepenthes mirabilis* digestive fluids can be used for recovery of holocelluloses from agro-waste.

Phenolic compound residues in the GP were identified by using Folin-Ciocalteu method with reduced TPCs being observed in GP samples pretreated with *Nepenthes mirabilis* digestive fluids. Such results were determined to be attributed to enzymatic activity of numerous enzymes, i.e. carboxylesterase, glucosidase and xylanase, quantified to be present in the *Nepenthes mirabilis* digestive fluids. The results confirmed that, TRS production increases with further cellulases treatment of the GP. Furthermore, some microorganisms were identified in the *N. mirabilis* digestive fluids; i.e. *Bacillus cereus, Bacillus thuringiensis*, and *Bacillus anthracis* with accession numbers KY249126.1, DQ513324.1, and KU948294.1, organisms which were identical to those found by other researchers in *N. mirabilis*.

The scientific development that were accomplished from this study was:

- The successful application of *N. mirabilis* digestive fluids as a biovalorisation agent of GP, an agro-waste.

5.2 Recommendations

The following are recommended for future research:

- A study on the optimization of the process conditions for biovalorisation,
- Assessment of fermentations using the hydrolysates containing the TRS should be done in order to compare the product yield of hydrolysates from different pretreatment methods, i.e. to compare the *N. mirabilis* digestive fluids to conventional pretreatment methods hydrolysates, and
- Additional pre-treatment methods which will convert 100% of the holocelluloses in the GP into fermentable sugars.

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APPENDICES

Figure A-1: Wet grape pomace

Figure A-2: Dry grape pomace

Figure A-3: Wet orange peels

Figure A-4: Dry orange peels

Figure A-5: Wet apple peels

Figure A-6: Dry apple peels

Figure A-7: Wet corn cop

Figure A-8: Dry corn cop

Figure A-9: Wet oak leaves

Figure A-10: Dry oak leaves

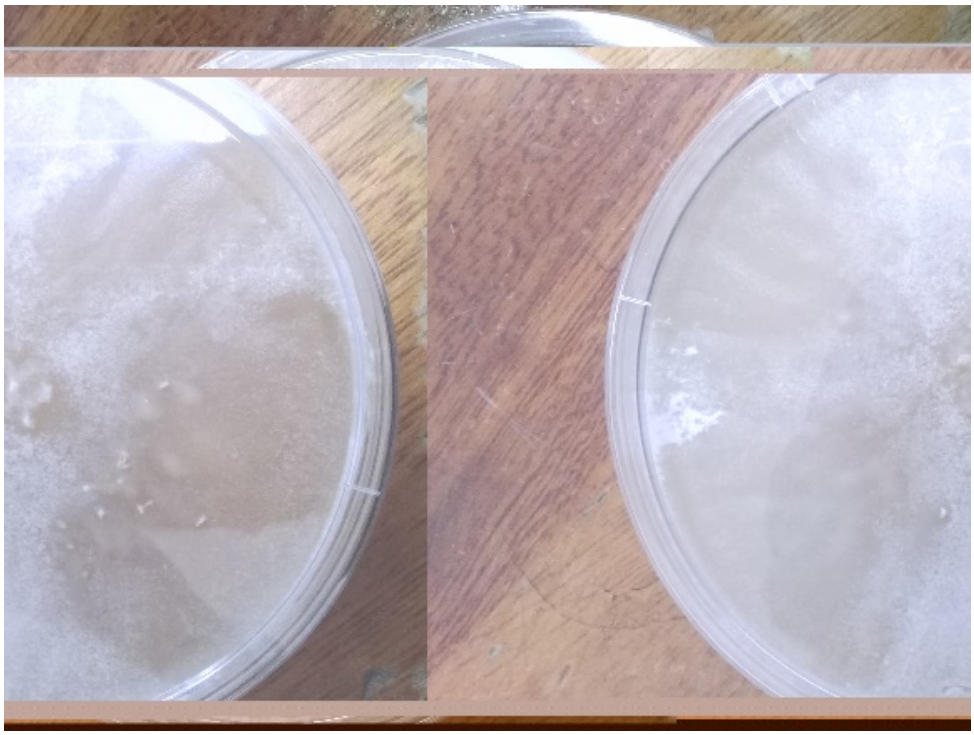

Figure A-11: White rot fungi growing on agar plate.

Figure A-12: Closed and opened *N.mirabilis*

Figure A-13: *Nepenthes mirabilis* pitcher extracts with prey

Calculations of enzymes activity

The calculation of enzyme activity was done in order to convert the reading units per litre; thus, 1 unit = µmol substrate which is converted in minutes.

- While, the readings of the absorbance is plotted in a graph in which there is time versus absorbance.
- $\frac{dA}{dt}$ is an initial slope gradient which determines the value of the reaction's initial rate.
- While, the above ratio is converted into $\frac{dc}{dt}$ by dividing it with the extension coefficient (17000 M^{-1}. Cm^{-1} as suggested by Lambert-Beer. Thus, the value will be converted into mol.min. $^{-1}L^{-1}$.
- Multiple by 10^6 in order to convert moles into micromoles. Finally, the 1 unit will be micro-moles. min. $^{-1}L^{-1}$ with the final answer unit expressed as units per litre.

The table 1 below shows the approximate regions where various common types of bonds absorb including vibrations such as stretching, bending, twisting and other types of bonds as adopted from (Pavia *et al.*, 2014) with modifications.

Table 1: A simplified corolation chat

FUNCTIONAL GROUPS		FREQUENCY (cm^{-1})	INTENSITY
Alkanes (stretch) $-CH_3$ (bend) $-CH_2-$ (bend) Alkenes (stretch) Aromatics (stretch) Alkyne (stretch) Aldehyde Ketone Carbonxylic acid Ester Amide Anhydride Alcohols, ethers, esters, carbonxylic acid, anhydrides	C-C C=C RCHO C=O RCOOH RCOOR' C-O	3000-2850 1450 and 1375 1465 3100-3000 3150-3050 Ca. 3300 2900-2800 1725-1700 1750-1730 1680-1630 1810 and 1760 1800 1300-1000	S m m m s s w s s s s s s
Alcohols, phenols Free H-bonded Carbonxylic acids	 O-H RCOOH	 3650-3600 3400-3200 3400-2400	 m m m
Primary and Secondary amines and amides (stretch) (bend)	N-H	 3500-3100 1640-1550	 m m-s
Amine	C-N	1350-1000	m-s
Imines and oximes	C=N	1690-1640	w-s
Nitriles	RCN	2260-2240	m
Allenes, Ketenes, isocyanates, isothiocyanates	X=C=Y	2270-1940	m-s

Culturing of Phanerochaete chrysosporium (strain BKMF 1767).

Phanerochaete chrysosporium were cultured on a growth media made up of the following chemicals:

Glucose = 10,03g,

Malt extract = 10g,

Peptone = 2g,

Yeast extract = 2g,

Asparagine = 1g,

KH_2PO_4 = 2g,

$MgSO_4.7H_2O$ = 1g,

Agar-Agar (nutrient agar) = 20g

Thiamin-HCL = 1mg.

The media was prepared in 1000ml and autoclave for an hour at 121°C. It was allowed to cool down. Thereafter, it was poured into petric dishes and test tubes for slants under laminar flow. The fungi was sub cultured on the dishes and slants and incubated for at 37°C for 7days. After 7days, there were mix growth on the plates, some were white, reddish and blackish colours. The white spores were re-cultured to obtain pure pores.

Discussion: The mixed growth of different colours was probably due to contamination and also yeast growth.