Cape Peninsula University of Technology

NEPENTHES MIRABILIS PLANT DIGESTIVE ENZYMES FOR SEMI-DELIGNI-HOLOCELLULOLYSIS OF MIXED AGRO-WASTE

By

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DECLARATION

I, **Justine Oma Angadam**, 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 or the National Research Foundation of South Africa.

All intellectual concepts, theories, methodologies, and material derivations and model developments used in this thesis and published in various scientific journals (except those that the candidate is not the first author in) were derived solely by the candidate and first author of the published manuscripts. Where appropriate, the intellectual property of others was acknowledged by using appropriate references. The contributions of co-authors for the conference and the published manuscript were provided in a training or supervisory capacity, or for research assistance.

Signed: Justine Oma Angadam **Date:** 09/03/2023

ABSTRACT

Environmental concerns regarding the dumping of agro-waste by agro-food processing industries in the environment are a major concern for human health in developing countries. Several of these industries are found in the Western Cape Province, South Africa, and the disposal of these agro-wastes is a challenge, as most of the agro-wastes are disposed of in landfills. A new approach is being proposed, where repurposing of the waste can be implemented cost-effectively, using appropriate technologies such as pretreatment of the waste for the extraction of value-added products. This approach has become a new promising strategy and a sustainable solution for the effective utilisation of this waste. Initially, this study started by developing a proof concept for semi-deligni-holocellulolysis, a term developed to describe agro-waste pretreatment as a partial delignification and holocellulolysis process. The current understanding is that ligninolysis and subsequent holocellulolysis occur. However, this study proposes that agro-waste pretreatment is a simultaneous semi-lignino-holocellulolysis, particularly for milled lignocellulosic waste. The study further proposes that digestive enzyme cocktails from the monkey cups of *Nepenthes mirabilis* and some plant exudates can be used entirely to perform agro-waste pretreatment for the extraction of fermentable hydrolysates focusing on total reducible sugars (TRSs) while minimising total phenolic compounds (TPCs). Generally, plant exudates and enzyme cocktails can be used, as they contain very useful constituents that can be harnessed for bio-waste and lignocellulosic waste pretreatment.

Nepenthes mirabilis (*N. mirabilis*) pitcher fluids were identified as suitable to pretreat mixed agro-waste while reducing phenolic compounds in the hydrolysate. In this instance, different particle sizes, i.e., 106µm, >75µm *x*<106µm were pretreated with *N. mirabilis* pitcher fluid, revealing the efficacy of the pitcher fluid to pretreat the agro-waste, imparting a green chemistry approach for the pretreatment of agro-waste.

For the pretreatment of the mixed agro-waste using *N. mirabilis* pitcher fluid, the pitcher fluid was fractionated (<3 kDa, >3 kDa, <10 kDa, >10 kDa) and slurried with the mixed agro-waste, i.e., 20% (w/w) for each wasteorange peels, apple peels, maize cobs, grape pomace, and oak plant leaf litter of various particle sizes, i.e., >75 µm *x*< 106 µm and >106 µm. The process produced a high concentration of total reducible sugars (TRSs) with the lowest production of total phenolic compounds (TPCs), with a particle size of >106 µm, pretreatment for 72 h, and an enzyme fraction of <10 kDa being identified as the best pretreatment conditions, whereby 97 g/L of TRSs were produced with a significantly lower TPCs load (1 g/L). Furthermore, the <10 kDa showed preferable physico-chemical properties, with the highest reduction-oxidation potential including acidity. Several enzymes, i.e., β-1,3-glucanase, putative peroxidase 27, and thaumatin-like protein, among others, were identified in the <10 kDa fraction, i.e.,

enzymes known to perform various functions in plant-based waste. Furthermore, when assessing the pretreatment of the agro-waste using – under different pH, temperature, and co-factor (trace element) solution conditions – a pH of 2 and lower than ambient temperature conditions was determined to be suitable. The supplementation of the pretreatment slurry showed a minimal effect. Additionally, the correlation between the milled mixed agro-waste porosity and <10 kDa fraction pitcher fluid efficacy was determined. With an average pore size of 2.84 nm (28.4 Å) for the > 106 µm particle size of the agro-waste, the <10 kDa fraction pitcher fluid containing the identified enzymes can be embedded into the pores of the agro-waste. To further provide researchers with a platform to develop the study further, an artificial neural network assessment was done, which also provided a way to simulate the data obtained in this thesis. Overall, the ANN structure used was 1-5-2-2 (0.54 at epoch 3) with experimental run 12 (> 106 µm x> 3 kDa) being identified as having a potential to perform similarly to experimental run 14 (> 106 µm x< 10 kDa), i.e. an experimental run with the highest production of TRS's.

This study supported and demonstrated that agro-waste pretreatment can be facilitated using an environmentally friendly approach solely using biological means, i.e., using fractionated *N. mirabilis*pitcher fluids. For such a process to be understood and applied on an industrial scale, an interdisciplinary approach encompassing environmental health, process engineering, and microbiology is required.

DEDICATION

I dedicate this thesis to my late father,

Mr. Angadam Joseph who wanted to see me become an independent strong woman.

To my sweet mother,

Mrs. Angadam Christina Eyasa, my backbone.

To my siblings and my family,

and

To my friends and well-wishers.

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RESEARCH OUTPUTS

The following research outputs represent the contributions of the candidate to scientific knowledge and development during the doctoral candidacy (2019-2022):

DHET accredited manuscripts published/accepted for publicationforming part of this thesis

Angadam, J.O., Ntwampe, S.K.O., Chidi, B.S., and Okudoh, V.I. 2023. *Nepenthes mirabilis* pitcher fluid functionality for agro-waste pre-treatment: Effect of pH, temperature, trace element solution and the pore size of the waste. *Sustainability*, 15, 3906. https://doi.org/10.3390/su15053906. (Impact Factor: 3.889)

Angadam, J.O., Njoya, M., Ntwampe, S.K.O., Chidi, B.S., Lim, J-W. Okudoh, V.I. and Hewitt. P.L, 2022. *Nepenthes mirabilis* fractionated pitcher fluid use for mixed agro-waste pretreatment: Advocacy for non-chemical use in biorefineries.*Catalysts,* 12(7): 726. https://doi.org/10.3390/catal12070726. (Impact Factor: 4.501)

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Other submittedor published manuscripts

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LAYOUT OF THESIS

This research study was conducted at the Bioresource Engineering Research Group (*BioERG*), Department of Biotechnology and Consumer Science, Cape Peninsula University of Technology, South Africa. The references listed in the thesis were listed in accordance with the Harvard method of referencing.The thesis was divided into the following chapters:

Chapter 1covers the background of the research study, the problem statement, the motivation for the study, the hypothesis and research questions and the overall aim and the objectives, as welll as delimitations of the study.

Chapter 2 reports on the concept of the simultaneous lignino-holocellulolysis process, identifying research gaps and niches based on this concept. This chapter has a supplementary section describing the suitability of plant exudates and enzymes cocktails for semi-delignino-holocellulolysis for agro-waste pretreatment. Both Chapter 2 and the supplementary section were published.

Chapter 3 reports on proofing the concept of the pitcher fluids to be effectively used for reducible sugar production while reducing the concentration of phenolics in the hydrolysates. Like Chapter 2, Chapter 3 also does have a supplementary section.

Chapter 4reports on the effect of agro-waste pretreatment conditions by varying pH, temperature, co-factor (trace element) solution and the porosity of the best agro-waste particle size, linking themto the best performing pitcher fluid fraction.

Chapter 5 discusses the development of an artificial neural network using the generated experimental data such that the process agro-waste*N. mirabilis* pretreatment process can be simulated.

Chapter 6enlists overall observations from the study (conclusion), highlights, and recommendations for future studies.

References: These are listed according to the Harvard reference style and are listed at the end of each chapter.

Appendices: Theselist proteomic information used to identify the enzymes, chemical solution preparation, TRS and TPC calibration, statistical analysis for actual TRS concentration for Chapter 5, preliminary results for TRS, and experimental runs for chapter 3, including ANN training tool and train.

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GLOSSARY

Abbreviations/Symbols	Definition (units)
CO	Conductivity
DNS	Dinitrosalicylic acid
LC-MS	Liquid chromatography with tandem mass spectrometry
ORP (Eh)	Redox potential (mV)
SG	Specific gravity
PDF	Probability density function
TES	Trace element (co-factor) solution
TPCs	Total phenolic compounds
TRS	Total reducing sugar
Terms	**Description**
Agro-waste	Residual biomass from processing agricultural produce.
Cellulases	Enzymes capable of degrading cellulose to simple sugars/carbohydrates.
Cellulose	Polysaccharides present in plant biomass cellular walls.
Delignification	Removal of a structural polymer called lignin from plant materials.
Digestion	Mechanical and chemical breakdown of food into small organic fragments.
Feedstock	Agricultural residues, dedicated energy crops, wood residues, and paper waste.
Holocelluloses	This term describes a combination of hemicelluloses and celluloses.
Lignocellulosic biomass	Plant biomass made of three components: cellulose, hemicellulose, and lignin.
Concepts	**Definition**
Holocellulolysis	This refers to a simultaneous breaking down of hemicellulose and cellulose (used extensively as a term in this thesis).
Semi-deligni-holocellulolysis	Partial breakdown of lignin and holocelluloses (the concept developed in this study).

CHAPTER 1

GENERAL INTRODUCTION

CHAPTER 1

INTRODUCTION

1.1. Introduction

Discarding agro-waste poses several environmental challenges such as water, air, and soil pollution. In the agricultural industry, agro-waste from agro-processing is abundant in the Western Cape Province, South Africa. Due to the production of low-quality produce, landfilling is the preferred method of disposal, which produces large quantities of greenhouse gases (GHG) which contribute greatly to global warming, and thus environmental deterioration. However, agricultural waste (agro-waste) does have prospective applications as a primary biorefinery feedstock to produce value-added products that complement petroleum energy products. Bioethanol is one of the most common primary bio-products being produced in biorefineries (Demirbas 2009), and is used as a fuel supplement in countries with socio-economic characteristics similar to those of South Africa, such as Brazil. Although bioethanol manufactured using the biorefinery concept does raise concerns associated with its production using agricultural produce intended for human consumption, other alternatives can be used, i.e., agro-processing wastes, which might reduce and mitigate food security concerns. Owing to concerns associated with the production of bioethanol and biodiesel from edible agronomic foodstuffs, there is a need for the development of second and in particular third-generation bio-refineries for developing countries, such as South Africa.

A bio-refinery can be considered as a method used in the sustainable processing of biomass into a range of value-added products and energy. There are three main classifications of biorefineries: first-, second-, and third-generation biorefineries. First- and second-generation biorefineries use feedstock to generate a limited number of bioproducts compared to conventional petroleum-based and/ or refinery processes.However, the products have limited market acceptability due to cost-income inconsistency and high operational costs (Lennartsson et al., 2014). The quantities, types of bio-alcohols, and value-added products generated are the limitations of biorefineries (Oke et al., 2016; Lennartsson et al., 2014). Another way to relieve food security challenges is by using waste biomass comprising mixed agro-waste which can provide a workable, renewable bioresource to produce fermentable hydrolysates to ease downstream separation and process intensity. The reuse of agro-waste for the sustainable creation of materials provides a solution that requires less natural resource usage while generating energy sources (Salehi Jouzani and Taherzadeh 2015; Madurwar et al., 2013).

Usually, first-generation biorefineries use a single feedstock to yield one product such as bioethanol. Examples of feedstock used in this type of biorefinery are corn starch or sugarcane bagasse which are

used independently as the only carbon source because their components are easy to ferment; nonetheless, these feedstocks are also used in food and animal feed production (Lennartsson et al., 2014; Lan and Liao 2013). Thus, food concerns in developing countries abound because manufacturers and/or suppliers of this kind of feedstock prefer to sell their produce to these biorefineries instead of for human consumption, as they make higher profits from it. Although it is advantageous to trade produce for the highest profits, human needsshould supersede the profit motive. There is therefore a need for an increased quantity of food that cannot be diverted for higher monetary gain (Hughes et al., 2013).

Second-generation biorefineries also use a single feedstock, including exhaustive pretreatment processes to produce a range of different bio-alcohols and other profitable co-products (de Jong et al., 2009). To expand the first and second-generation biorefineries, the concept of using plant digestive enzymes solely for the semi-deligni-holocellulolysis of agro-waste and extraction of value-added products and easily fermentable hydrolysatesis proposed here. This has become a new promising strategy and a sustainable solution towards the effective utilisation of agro-waste to upgrade traditional techniques, especially if the digestive enzymes are available in adequate quantities. The new strategy is aimed at easing the impact of converting agricultural products for biorefineries using physico-chemo-based methods while exploiting social and economic benefits from such a strategy. This strategy reduces environmental health-related deterioration challenges which is a focus of this study, i.e., to develop integrated processes for plant digestive enzyme usage for the concept termed herein *semi-deligni-holocellulolysis* of agro-waste, an environmentally benign process suitable for a green chemistry approach in biorefineries.

1.2. Problem statement

Landfilling agro-waste into the environment by the food and agro-processing industries is a major source of concern for environmentalists and human health in developing countries. There are numerous wastes, and they require appropriate disposal methods, which involve cumulative capital costs. In South Africa, there are over 1800 food manufacturing companies. The majority of these companies are found in the Western Cape Province, South Africa, while the disposal of the produced waste is becoming a serious concern, as most of the agro-waste is disposed of in landfills (Igumbor et al., 2012). Several types of agro-waste can be reprocessed and used in the manufacturing of fertilizers, while large quantities of unmanaged waste still contribute to environmental pollution.

Presently, numerous research studies have shown that agricultural waste can be repurposed for the production of biofuels and many high-value-added chemicals, specifically lignocellulosic waste materials in which a variety of readily reducible sugars in the form of holocelluloses are available (Cheng et al., 2012; Dinita et al., 2011; Cardona and Sánchez 2007; Dürre 2007). Cellulosic feedstocks are abundantly

available from agricultural produce residue, and they pose a challenge during their pretreatment to release fermentable sugars, e.g., glucose, mannose, etc. (Cheng et al., 2012).

Furthermore, research has proven that pretreatment processes such as hot water and dilute sulphuric acid as well as cellulase pretreatment for the agro-waste is being encouraged and considered to be inexpensive, suitable, and effective. The use of harsh delignification methods such as the dilute acid method does affect subsequent cellulases hydrolysis to produce fermentable sugars (Cheng et al., 2012) and can be categorized as inappropriate for a green chemistry approach for bioprocess development due to the production of residual environmental contaminants such as inhibitory by-products (e.g., furfural, phenolic compounds, etc.). These residual environmental contaminants can affect enzymatic hydrolysis and microbial action towards hydrolysate conversion during fermentation due to their toxicity (Panagiotou and Olsson 2007). Thus, there is a need to implement cost-effective, naturally suitable technologies such as plant digestive enzymes for the semi-deligni-holocellulolysis of agro-waste and extraction of value-added products. This approach has become a promising new strategy and a sustainable solution towards effectively utilising this waste and upgrading traditional techniques using available local biological materials (Madurwar et al., 2013), which is a major motivation/aim of this study.

1.3. Hypothesis

It is hypothesised that plant digestive enzymes can be used as semi-deligni-holocellulolysis agents for agro-waste to produce hydrolysates, which are easily fermentable with minimised inhibitory by-products under optimum conditions. It is important to note that this study focused on regionally available agro-waste.

1.4. Objectives of the study

The specific objectives were:

- To present a perspective on the semi-deligno-holocellulolysis concept, i.e., that pretreatment of milled agro-waste is a partial process, whereby the delignification and holocellulolysis occur simultaneously instead of being a sequential process, e.g., delignification > holocellulolysis.
- To identify a pitcher plant and apply pitcher fluids from the plant to assess their efficacy in pretreating mixed agro-waste of different particle sizes, and to:
    - Identify the fraction in the pitcher fluid which has high efficacy in pretreating the mixed agro-waste.
    - From the best performing pitcher fluid fraction, identify the enzymes contained therein.

- Identify the particle size which is best suited to be used with the best-performing pitcher fluid fraction.
- Determine the porosity of the best agro-waste size and evaluate the correlation with the best performing pitcher fluid.

- To assess the influence of different pH, temperature, and co-factor (trace element) solution on the agro-waste pretreatment process using the pitcher fluids.
- To develop a platform that describes the pretreatment process developed from which the study can be continued using available software for future research.

1.5. Significance of the research

A suitable biotechnological/environmental i.e. bioprocess strategy, for plant digestive enzymes usage for the semi-deligni-holocellulolysis concept/mechanism for agro-waste and the subsequent extraction of value-added products, has become a promising new strategy and a sustainable solution towards the effective utilisation of agro-waste and upgrading traditional techniques which use heat, dilute inorganic acids, with readily available bio-based materials, for a process which must therefore be developed for the benefit of society from which the agro-waste is produced.

1.6. Delineation of the study

In this study, the use of plant digestive enzymes for semi-deligni-holocellulolysis of agro-waste were its primary focus, concentrating only on the pretreatment technology to produce suitable hydrolysates. Digestive fluids from *Nepenthes mirabilis* was the only pitcher plant fluid used in this study to hydrolyse agro-waste as it was determined to be suitable for the concept proposed herein. Economic evaluation/feasibility can be a subject of other research studies.

1.7. References

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CHAPTER 2

LITERATURE REVIEW

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CHAPTER 2

LITERATURE REVIEW

2.1. Introduction

Environmental pollution by lignocellulosic waste dumped into the environment by various processing industries has been acknowledged to be toxic (Rex et al., 2016), thus negatively affecting the earth's ecology and human health. Therefore, the excessive landfilling of this type of waste is discouraged. Lignocellulosic waste generates acidic leachate with phenols being one of the major toxicants that poison water bodies (Svensson 2014). Such leachate has been determined to harm several aquatic organisms (Libralato et al., 2007, Taylor et al., 1996). Although there are other industries, i.e., petroleum refineries, gas and coking industries, pharmaceutical manufacturers, explosives/ munition manufactures, phenol–formaldehyde resin manufacturers, plastic, and varnish industries among others (Hussain et al., 2015), producing phenol-containing waste, the focus of this perspective was on lignocellulosic waste.

Generally, 1.77 – 2.4 Gt of waste from cereals, roots and tubers, oilseeds, and fruit and vegetables are produced globally annually (Santos, 2013) and most, can be classified as lignocellulosic waste with significant cellulose, hemicellulose, and lignin content (Szymańska-Chargot et al., 2017). Cellulose, hemicellulose, and lignin content in lignocellulosic waste vary in most lignocellulosic materials. Research has successfully demonstrated that lignocellulosic waste can be treated using several techniques (Barana et al., 2016; Pielhop et al., 2016; Bensah and Mensah, 2013), with landfilling being one of the oldest disposal methods; however, it produces greenhouse gasses in the form of carbon which is released as CH_4 which further pollutes the environment (O'Dwyer et al., 2018). To further understand lignocellulosic waste, and to mitigate its influence on the environment for repurposing and cleaner disposal, understanding its composition and currently available pretreatment methods, i.e., pretreatment strategies such as physical, chemical, biological, and physico-chemical (Ravindran and Jaiswal 2016), must be further developed.

Overall, it is difficult for enzymatic hydrolysis to decouple the lignin in lignocellulosic waste into its primary components due to the bond structure of lignin-cellulose-hemicellulose. This limits the delignification efficiency via natural ligninolysis of the lignocellulosic waste (Zabed et al., 2016); however, lignin can be degraded using different physico-chemical methods. These methods cannot be classified as environmentally benign. However, they boost the enzymatic hydrolysis of residual holocelluloses, i.e., cellulose and hemicellulose, in a sequential process whereby the lignocellulosic waste is initially physico-chemically pretreated and subsequently hydrolysed using cellulases from different

species, i.e., *Aspergillus*, *Penicillium*, and *Trichoderma* spp. (Passos et al., 2018). Some of these pretreatment processes are ineffective in delignifying the firm organization of lignin.

However, peroxidases from fungi such as *Phanerochaete chrysosporium* have been proven to facilitate ligninolysis and thus biodegrade the rigid structure of lignin (Leonowicz, et al., 1999). This provides for biological ligninolysis, exposing the holocellulosic matrix of the biomass to hydrolysis, and reducing the need for chemical solution usage in lignocellulosic waste pretreatment (Angadam, 2018). These peroxidases have been produced in high volumes using membrane bioreactor technology (Ntwampe, 2005). It is, therefore, feasible to introduce a biological pretreatment of lignin-containing lignocellulosic waste, in a process optimized for ligninolysis to expose holocellulosic components to hydrolysis, i.e., holocellulolysis. Lignocellulosic waste is predominantly made up of irregular β-1.4 glycosidic bonds. These bonds can be lysed by cellulases and β-1.3-β-1.4 glucanases from different microorganisms, e.g., *Chaetomium* sp. (Al-Kharousi et al., 2015) and some *Nepenthes* species enzymes, i.e., enzymes produced by *N. alata* (Jaeger, 2016) and*N. ventrata* (Zakaria et al., 2018). Furthermore, it was previously reported that there is evidence of leaf (Raza et al., 2019) decomposition and mineralization in the cups of *N. ampullaria* (Moran et al., 2003). The use of pitcher monkey cup extracts for lignocellulose waste pretreatment has also been reported elsewhere (Dlangamandla et al., 2019b). Rottloff et al. (2016)reported that *Nepenthes* sp. has evolved an arsenal of enzymes and the digestive fluids that are composed of proteins, including hydrolytic enzymes, some of which can be useful in lignocellulose waste pretreatment. The availability and cultivation of species such as those of *Nepenthes* sp. can be achieved using a hydroponic growth method (Anonymous, 2016) to mitigate against regional unavailability and for large-scale digestive juice production.

Consequently, the absolute hydrolysis of lignocellulosic waste into fermentable sugars requires a cocktail of oxidative and hydrolytic enzymes. Although researchers have used cellulases for years to ease the pretreatment process post-physico-chemical treatment, cellulases are not adequate to complete ligninolysis and holocellulolysis simultaneously. Moreover, cellulose is also usually intricated in hemicellulose, i.e., cellulose is entrapped in hemicellulose, a significantly abundant natural polymer found in lignocellulosic waste. Furthermore, hemicellulose is constituted by xylan with further ester bonds bound to lignin (Tayeb et al., 2018). Its hydrolysis involves diverse conditions and numerous methods to competently hydrolyse it into fermentable monomers (Wang et al., 2013). The primary constituent of hemicellulose, xylan, can be hydrolysed by a cocktail of enzymes (Manavalan et al., 2017; Zabed et al., 2016) to decouple any residual bonds. These are enzymes produced as exudates or extracellular bioproducts from a variety of microorganisms (see Table 1), which can be used to lyse lignocellulosic waste. This can be affected via beneficiation and further sustainable utilization of obtained hydrolysates for the production of added-value products. Therefore, the simultaneous partial biological delignification (ligninolysis) and holocellulose decoupling (holocellulolysis), herein referred to as semi-

lignino-holocellulolysis, can describe mechanisms associated with enzymatic ligninolysis, hemicelluloses and celluloses lysis in lignocellulosic waste without the use of chemical pretreatment. Overall, in lignocellulosic waste pretreatment, some lignin and holocellulose remain intact (see Table 2, highlighting other pretreatment methods and their products) even when an alkali pretreatment is used; a method previously determined to increase cellulose digestibility by enhancing lignin solubilisation (Yang et al., 2014). Thus, the notion of semi-ligninolysis and holocellulolysis is valid.

Overall, for the progress of an environmentally friendly set-up, delignification (ligninolysis) is warranted using appropriate plant and microbial oxidative/digestive enzymes. The latter can be used as one of the key processes that are appropriate to limit environmental contamination by the lignocellulose waste, while efficiently ligninolysing the recalcitrant lignin structure to uncover holocelluloses (Balat, 2011) for simultaneous ligninolysis and holocellulolysis. This was initially reported in Dlangamandla et al. (2019b) and Angadam et al. (2018) with some of the digestive enzymes reported in Hasan et al. (2006)being shown to support semi-lignino-holocellulolysis; however, feasibility studies for implementation on an industrial scale are ongoing. Even the assessment of oxidation-reduction potential and acidic strength evaluations of symbiont extracts from the *Nepenthes* sp. pods indicates a high potential to facilitate oxi-reductive reactions which are very compatible with even those of the commonly used dilute (1% v/v) sulphuric acid solution (see Table 3);this is preferred to increased cellulose accessibility by weakening the hemicellulose bonds. Overall, the use of plant and microbial enzymes including extracts will require a physical pretreatment step such as milling the lignocellulose waste to increase the overall biocatalysis area and to condition the lignocellulosic waste for enhanced lysis.

Overall, research has proved that commonly used lignocellulosic waste pretreatment processes such as hot water, dilute sulphuric acid and alkali methods including cellulases are being encouraged and considered to be inexpensive, suitable, and effective. The use of harsh delignification methods, such as chemical treatment methods, does affect subsequent cellulase/enzyme facilitated hydrolysis to produce fermentable sugars (Cheng et al., 2012). This can be categorized as inappropriate for a green chemistry approach for biorefinery development due to the production of residual toxicants such as furfural, phenolic compounds, etc., all of which affect fermentation. These residual toxicants can affect enzymatic hydrolysis and microbial action towards hydrolysate conversion during fermentation due to their toxicity (Panagiotou and Olsson, 2007). Therefore, in summary, this paper contributes to a new understanding that lignocellulosic waste pretreatment is largely a partial and simultaneous lignino-holocellulolysis process even when chemical pretreatments are used. This process can be supported solely by a cocktail of enzymes from a variety of plant and microbial sources. Previously, Dlangamandla et al. (2019b) also demonstrated total phenolic compound reduction, i.e., < 3 g/Kg, in hydrolysates solely pretreated with digestive juices of *N. mirabilis*, albeit with 2.6 g/Kg reducible sugars from mixed lignocellulose waste

using non-optimum conditions. The hydrolysates from this pretreatment showed higher alcohol production and reduced phenolics when *N. mirabilis* pretreatment was used (Dlangamandla et al., 2019a).

Table 1. Components in lignocellulosic waste, processes and a few examples of enzymes and their source associated with the waste component lysis.

Components in lignocellulosic waste	Process		Enzymes	Source of enzymes in plants and/or organisms	Reference
Lignin		Delignification (ligninolysis)	Lignin peroxidase, Manganese peroxidase, Laccase	*Phanerochaete chrysosporium*, *Phaseolus vulgaris*, *Ganoderma lucidum* IBL-05, *Trametes villosa*	(Angadam, 2018, de Oliveira Carneiro et al., 2017, Shaheen et al., 2017, Wesenberg et al., 2003)
Cellulose	Cellulolysis	(holocellulolysis)	β-glucosidases Cellulases Endo-glucanase Exo β-1.3-β-1.4-glucanase Acetyl xylan esterase Cellulase-free xylanase Arabinofuranosidase α-arabinofuranosidase, feruloyl esterase	*Bacillus sonorensis* BD92 *N. mirabilis* *Bacillus subtilis* CBS31 *Thielavia terrestris* Co3Bag1 *Alkalibacillus favidus* *Paenibacillus* sp. N1 *Streptomyces lividus* *Aspergillus horta*i CRM1919, *Lactobacillus crispatus*	(Olajide et al., 2020, Terrone et al., 2020, Regmi et al., 2020, Ali and Mahmood, 2019, Raza et al., 2019, Rodríguez-Mendoza et al., 2019, Xu et al., 2019,Pathania et al., 2012, Zhang and Lynd 2004, Danna, 1998)
Hemicellulose	Hemicellulolysis				

Table 2. Evidence of partial lignin-hemicellulose-cellulose lysis during lignocellulose waste pretreatment using alkaline pretreatments (basis: 35 g of waste feedstock) (Park and Kim, 2012). *Other by-product constituents in lignocellulose waste from different pretreatment methods (Jönsson and Martín, 2016).

Lignocellulose waste component	Concentration in untreated waste (%)	Concentration in pretreated waste (%)
Cellulose	43.4 – 35.9	42.8 – 32.4
Hemicellulose	29.1 – 18.7	23.1 – 10.7
Lignin	30.1 - 29.0	25.8 – 4.1

*Acid-based methods: aliphatic carboxylic acids, phenylic compounds, furans; Hydrothermal processing: acetic acid, furan aldehydes; Mild alkaline methods: acetic acid, hydroxy acids, dicarboxylic acids, phenolic compounds; Oxidative methods: aldonic and aldaric acids, furoic acid, phenolic acids, acetic acid (Jönsson and Martín, 2016).

Table 3. Direct comparison of dilute (1% v/v) sulphuric acid solution (Grauer, 1991) to *N. mirabilis* pod extracts (Dlangamandla et al., 2019b,Angadam, 2018).

Solution/Extract (characteristics)	Oxidation reduction potential (mV)	pH	Acid strength
N. mirabilis digestive pod extracts (contains symbionts and a cocktail of enzymes known for lignino-holocellulolysis)	510 - 526	1.8 - 2.2	Strong
Sulphuric acid (1% v/v, free of symbionts and enzymes)	354.2	0.7	Strong

2.2.Current beneficiation of lignocellulosic waste using different pretreatment techniques

Pretreating lignocellulosic waste is a mandatory phase to accomplish hydrolysis of biorefinery feedstock aimed at producing value-added products, i.e., fermentable sugar (highest release of reducing sugars, i.e., up to 10.70 ± 0.14 g/Kg biomass of glucose and 12.41 ± 0.34 g/Kg biomass of xylose from lignocellulose waste (Raza et al., 2019) in biorefinery processes (Taherzadeh and Karimi 2008). Pretreatment facilitates ligninolysis and consequently, uncovering holocelluloses for the subsequent successful hydrolysis of the lignocellulosic waste with negligible energy intake, to accomplish the outmost fermentable sugar extraction (Nitsos et al., 2019; Limayem and Ricke 2012). Numerous techniques such as hot water, dilute sulphuric, etc. have been utilized to eliminate the recalcitrant lignin in lignocellulosic waste focusing on the feedstock being beneficiated to mitigate environmental pollution. Several lignocellulosic waste pretreatment approaches have been impractical and wasteful due to some technical challenges, which include the low yield and the formation of inhibitory by-products, e.g., furfural (up to 34.5 g/Kg), hydroxymethyl furfural (up to 29.5 g/Kg), total phenolic compounds (up to 4.1 g/Kg) and weak organic acids (up to 114.9 g/Kg) including total furans (up to 34.5 g/Kg) (Jampatesh et al., 2019; van der Pol et al., 2014). The processes involved in bio-physico-chemical ligninolysis and holocellulolysis of lignocellulosic waste include physical (milling), thermal (hot water), chemical (dilute acid, caustic), and microbial-based processes (Balan and Sousa, 2019; Zhang et al., 2019). Formation of by-product toxicants is usually observed during dilute acid and alkaline pretreatment as lignin is being partially degraded (Jampatesh et al., 2019; Taherzadeh and Karimi 2008); however, it is still assumed to be the ideal pretreatment method for an industrialized approach to date. Some common types of acids used in pretreating lignocellulosic waste from agriculture and forestry include dilute sulphuric (H_2SO_4), phosphoric (H_3PO_4), hydrochloric (HCl), and nitric (HNO_3) acids (Dąbkowska et al., 2019). Nonetheless, it has been proven that dilute sulphuric acid is the most frequently used in chemo-ligninolysis, because of its appropriateness for degrading a widespread selection of lignocellulosic waste even in a mixed form (Maddox et al., 2000, Menon and Rao 2012).

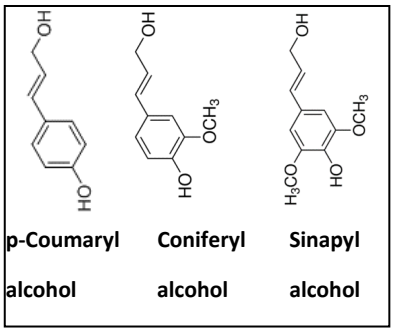
The alkaline (corrosive) pretreatment methods, including those using sodium hydroxide (NaOH) solutions, are commonly known for lignin lysis. Some other alkaline-based methods include ammonia fibre explosion (AFEX), ammonia recycle percolation (ARP), lime [$Ca(OH)_2$], and aqueous ammonia soaking (AAS), using chemicals generally observed to be readily available, cheap, and capable of consistent lignocellulosic waste pretreatment. Alkaline pretreatment techniques are dynamic at low heat and pressure; however, they require a large quantity of water, while using less energy when compared to dilute acid pretreatment, consequently lowering enzyme loading which is essential for hydrolysis, and hence they can lower general operational costs of a biorefinery (Rawat et al., 2013).

2.3. Ligninolysis of lignocellulosic waste: Physico-chemical and biological methods

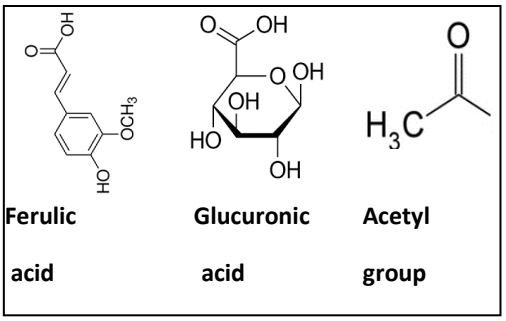
Ligninolysis describes the decoupling of lignin from lignocellulosic waste through ligninolysis achieved initially by milling/grinding to reduce the waste crystallinity (Amin et al., 2017). Chemical oxidation or acidification to reduce lignin-holocellulose bond strength by disrupting aryl-ether, carbon–carbon and xylosidic links and breaking acetyl ester linkages (Bensah and Mensah, 2013) is a process that can also be achieved solely by biological means. This includes the decomposition of ether bonds (Pielhop et al., 2016), after the decoupling of phenolic/non-phenolic structures within the lignocellulosic waste (Nadir et al., 2019). All these types of ligninolysis can be described as physico-chemi-ligninolysis, as they involve physical and chemical means of lignin lysis for which an alternative technological approach is needed for the process to be environmentally benign. Overall, this means a biological approach.

The act of enzymatic hydrolysis of lignin can be described as ligninolysis. Filamentous fungi and other microorganisms, including those in the family of Basidiomycetes, such as white rot fungi (WRF) for ligninolysis, and brown rot fungi (BRF) for holocellulolysis, have proven to be the most commonly known natural matter disintegrators for the breaking down of lignin (Barr and Aust, 1994), in particular lignocellulosic waste. WRF-facilitated ligninolysis is principally the hydrolysis of lignin with insignificant holocellulose breakdown (Narayanaswamy et al., 2013). The major enzymes involved in such ligninolysis include lignin peroxidases (LiPs), manganese peroxidases (MnPs), and laccase (Lac) produced by a variety of fungi (e.g.,*Phanerochaete chrysosporium*, *Pleurotus ostreatus* (Jacq.), *Pleurotus kumm* (MCC16) under nutrient-limited conditions; similarly for BRF, such as *Chaetomium* sp., *Ceratocystis* sp., and *Kretzschmaria deusta*, which produce enzymes such as cellulases, laccase, lignin peroxidases, all known to solubilise lignino-holocelluloses (Al-Kharousi et al., 2015). However, there are uncertainties as to whether these are the sole streamlined bio-delignifiers and bio-holocellulolisers of lignocellulosic waste. Although lignin is resistant because of its low porosity, and thus protects the energy-rich holocellulose of plants' cell walls, it can be solubilised using digestive extracts of plants such as those of *Nepenthes* sp. in combination with cellulases releasing coniferyl, synapyl, and p-coumaryl

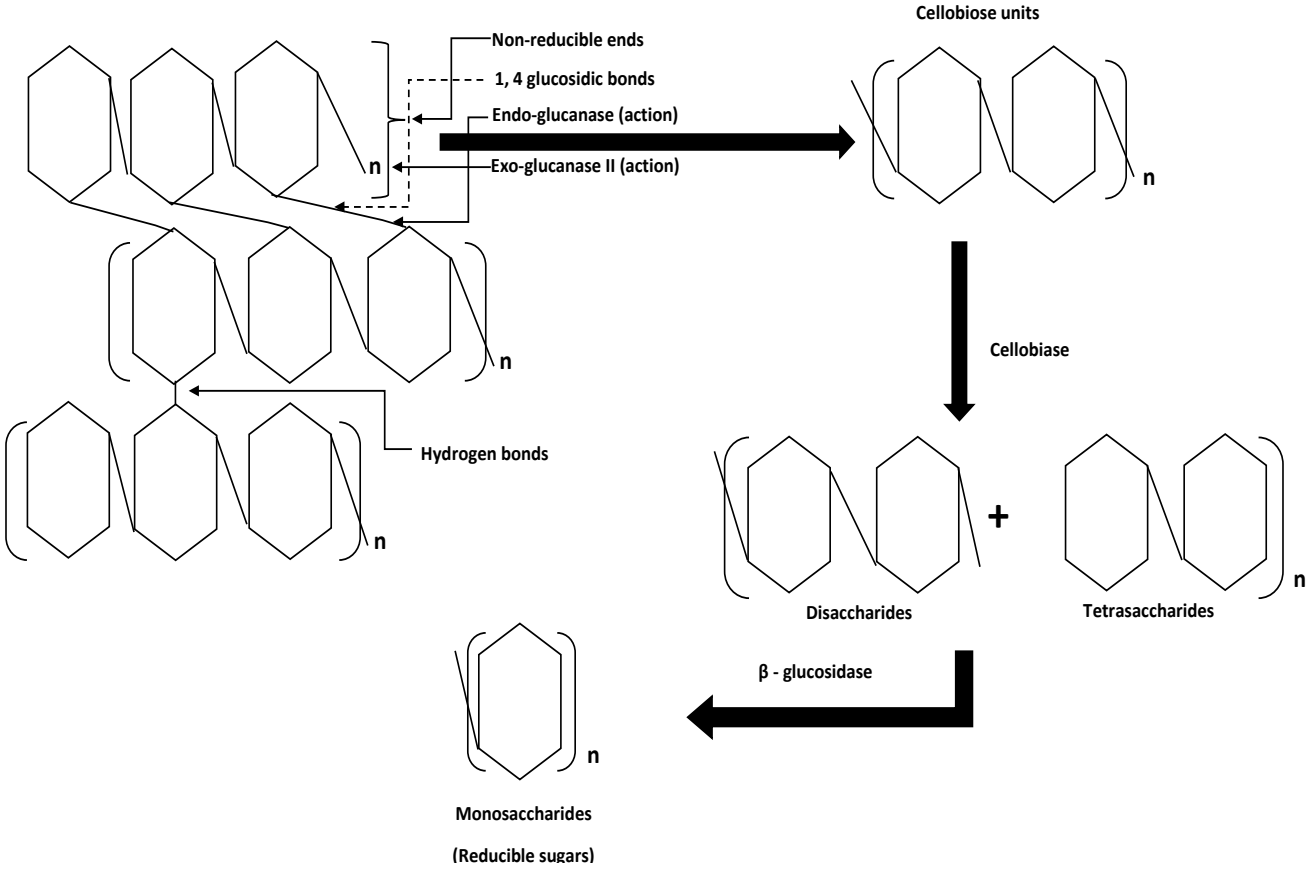
alcohols, including ferulic acid, glucuronic acid and acetyl group (Achinas and Euverink, 2016) (see Figure 1(A)1 and (A)2).


p-Coumaryl alcohol
Coniferyl alcohol
Sinapyl alcohol

(a)1

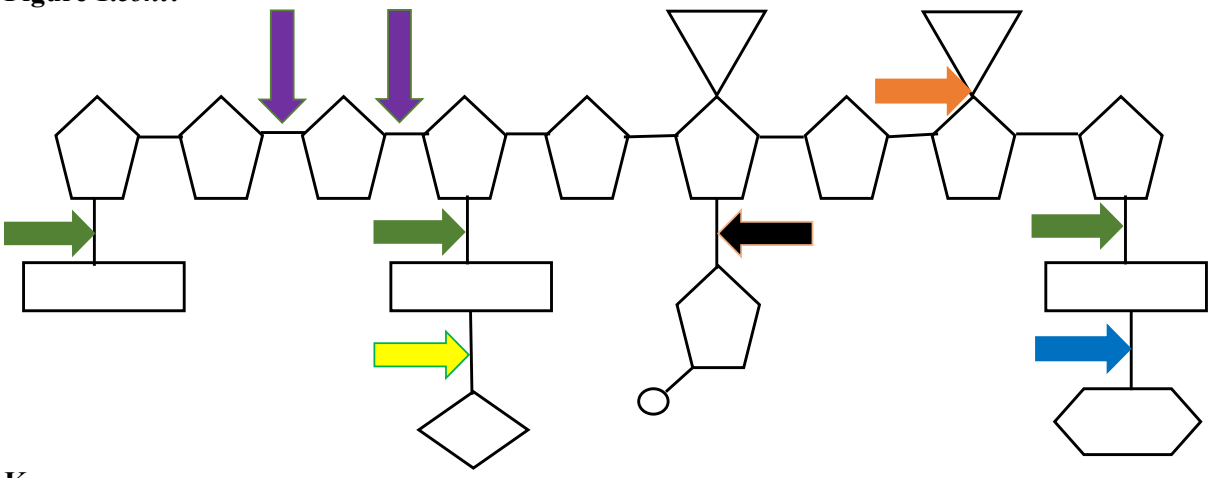

Ferulic acid
Glucuronic acid
Acetyl group

(a)2

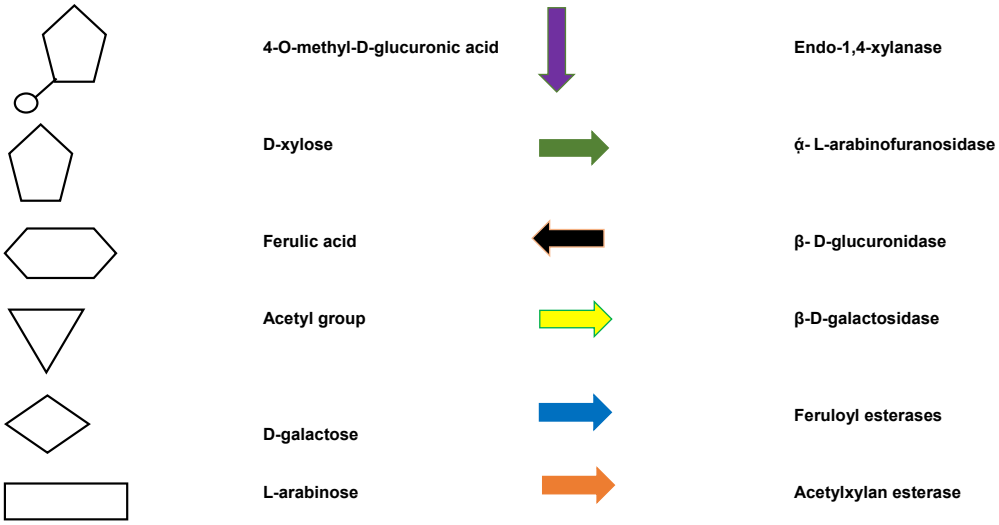

Non-reducible ends
1, 4 glucosidic bonds
Endo-glucanase (action)
Exo-glucanase II (action)
Cellobiose units
Cellobiase
Hydrogen bonds
Disaccharides
Tetrasaccharides
β - glucosidase
Monosaccharides
(Reducible sugars)

(b)

Figure 1.*cont*:



Key:



(c)

Figure 1. Molecular structures of products of **(a)1** ligninolysis and **(a)2** holocellulolysis; **(b)** process of cellulolysis including enzymes at each biocatalysis step, and **(c)** enzyme action site involvement in hemicellulolysis including end-products.

2.4. Holocellulolysis of lignocellulosic waste

Research has shown that when lignocellulosic waste is pretreated either by physical, chemical, or biological methods, lignin, and holocelluloses are partially lysed simultaneously. The main enzymes responsible for the breaking down of the crystalline and amorphous structure of cellulose are known as endo-glucanase (EGs) and exo-glucanase II (CBHs-II). The endo-glucanase decomposes the 1,4-β-glycosidic bonds while the CBHs decouple the non-reducible ends of the crystalline structure of the cellulose. When the EGs and CBHs act on cellulose, an amorphous structure is produced which is catalysed by cellubiase to produce certain products. These are cellubiose units that are further biotransformed into di- and tetra-saccharides. Finally, β-glycosidase reduces these sugars into monosaccharides. This scenario is diagrammatically represented in Figure 1(b). Unlike cellulose,

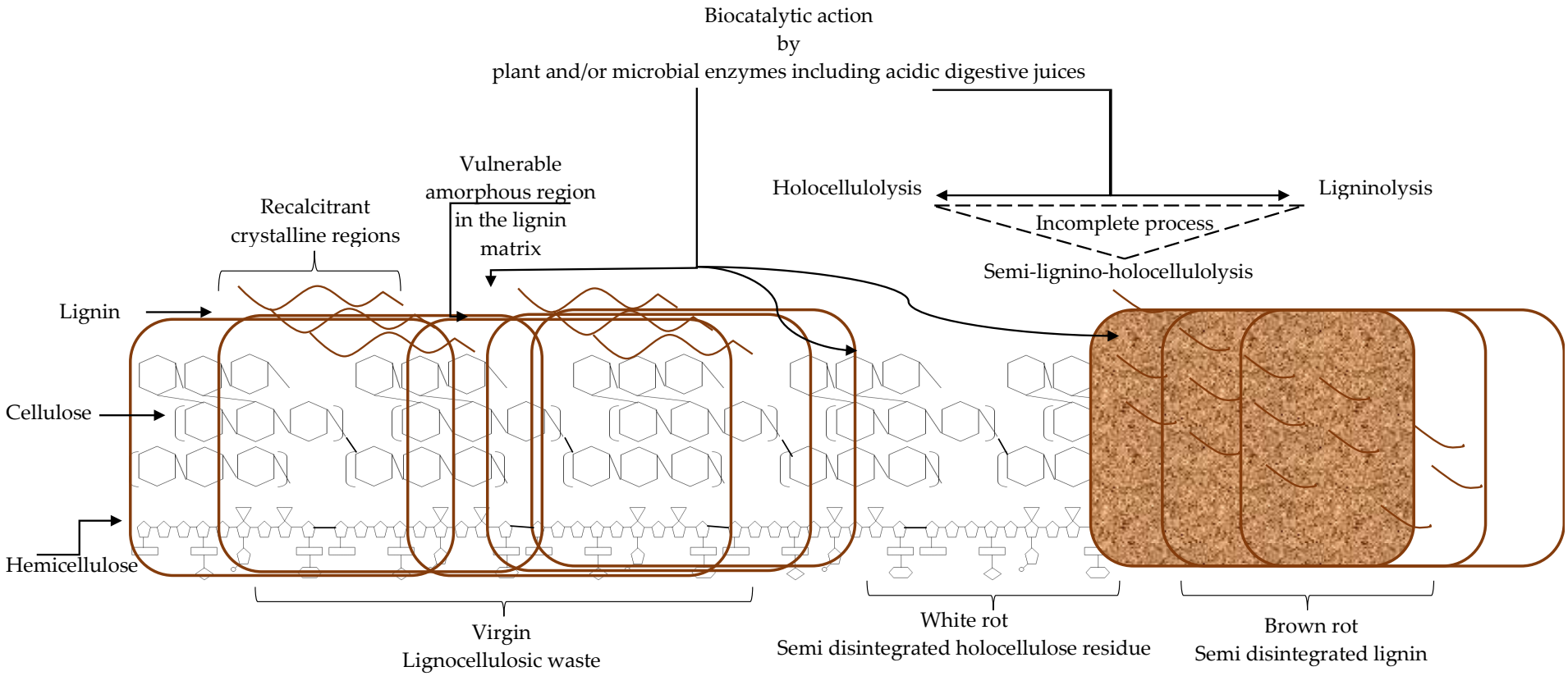
hemicellulose (xylan) is more complex, and its lysis requires more specific and multiple enzymes. Endo-xylanase hydrolyses the main chains of xylan, and β-xylan esterase reduces xylooligosaccharides into xylose, with α-arabinofuranosidase and α-glucuronidase acting on the xylan backbone for the removal of arabinose and 4-o-methyl glucuronic acid. The esterases thereafter reduce the acetyl substitutions on the xylose, while feruloyl esterases hydrolyse the ester bonds located between arabinose substitutions and ferulic acid. Further, feruloyl esterases also make it easier to decouple hemicellulose in lignocellulosic waste. Figure 1(c) illustrates the hemicellulose structure and individual enzyme types, including actions on individual bonds to release a variety of reducible sugars and other by-products. As illustrated in Table 1, a large number of these enzymes can be sourced from plant or microbial sources.

2.5. Simultaneous partial biological ligninolysis and holocellulolysis

2.5.1. Perspective on semi-delignino-holocellulolysis

Lignin is responsible for the rigidity and the nature of plants including lignocellulosic waste. The mash structure of the lignin contains surface pores, which are amorphous in nature (see Figure 2a) and consist of irregular based carbon molecules. This explains why some plants can secrete plant exudates externally to the bark of a tree, some of which are used as a defence mechanism. This enables organisms such as WRF, which produces lignin and manganese peroxidases, including laccase, to initially lyse this amorphous region to start the ligninolysis of the lignin barrier directly (Leonowicz et al., 1999). Similarly, BRF exploits the amorphous regions in lignocellulosic waste by initially producing oxidation reactive species to further weaken the amorphous regions of the lignocellulosic waste after holocellulolysis, via a cocktail of glycoside hydrolases, leaving the lignin residue (see Figure 2(a). Additionally, simultaneous (co-current) ligninolysis and holocellulolysis can take place during the decomposition of such lignocellulosic waste in a symbiotic environment, implying that the hydrolysate will be richer in both lignin products and holocellulose constituents such as mono- and tetra-saccharides. Therefore, the classification of this phenomenon, i.e., simultaneous ligninolysis and holocellulolysis, can be termed lignino-holocellulolysis. Overall, during the pretreatment of lignocellulosic waste, some residual lignin and holocellulose are present in the residue, which indicates partial, i.e., semi-pretreatment. It is therefore logical to have the classification ‘semi-lignino-holocellulolysis'. Lignin and holocellulose can consequently be partially degraded simultaneously because as lignin is recalcitrant, some acidic extracellular bio-products can directly act on the lignin structure. For example, the production of LiP and MnP from *P. chrysosporium* was shown to reduce the pH of the environment where the organism was grown. At the same time, the oxidative-reduction potential increased (Ntwampe, 2005). This would thereafter enable the hydrolases and other enzymes to biocatalytically lyse holocelluloses while ligninolysis ensues. This can be optimised and effectively used to pretreat even mixed lignocellulosic waste. Some plant digestion extracts, i.e., those of *N. mirabilis*, have high acidic strength and can therefore solubilise some lignin components (Moran et al., 2003), weakening their structure such that cellulases can easily penetrate the holocellulose (Dlangamandla et al., 2019b). This type of reaction is

irreversible and is assumed to follow for multiple bioreactions in parallel, as illustrated in Figure 2(c), and not in series as shown in Figure 2(b) (Barana et al., 2016).


Biocatalytic action
by
plant and/or microbial enzymes including acidic digestive juices
Vulnerable
amorphous region
in the lignin
matrix
Recalcitrant
crystalline regions
Holocellulolysis
Ligninolysis
Incomplete process
Semi-lignino-holocellulolysis
Lignin
Cellulose
Hemicellulose
Virgin
Lignocellulosic waste
White rot
Semi disintegrated holocellulose residue
Brown rot
Semi disintegrated lignin

(a)

Figure 2.*cont*:

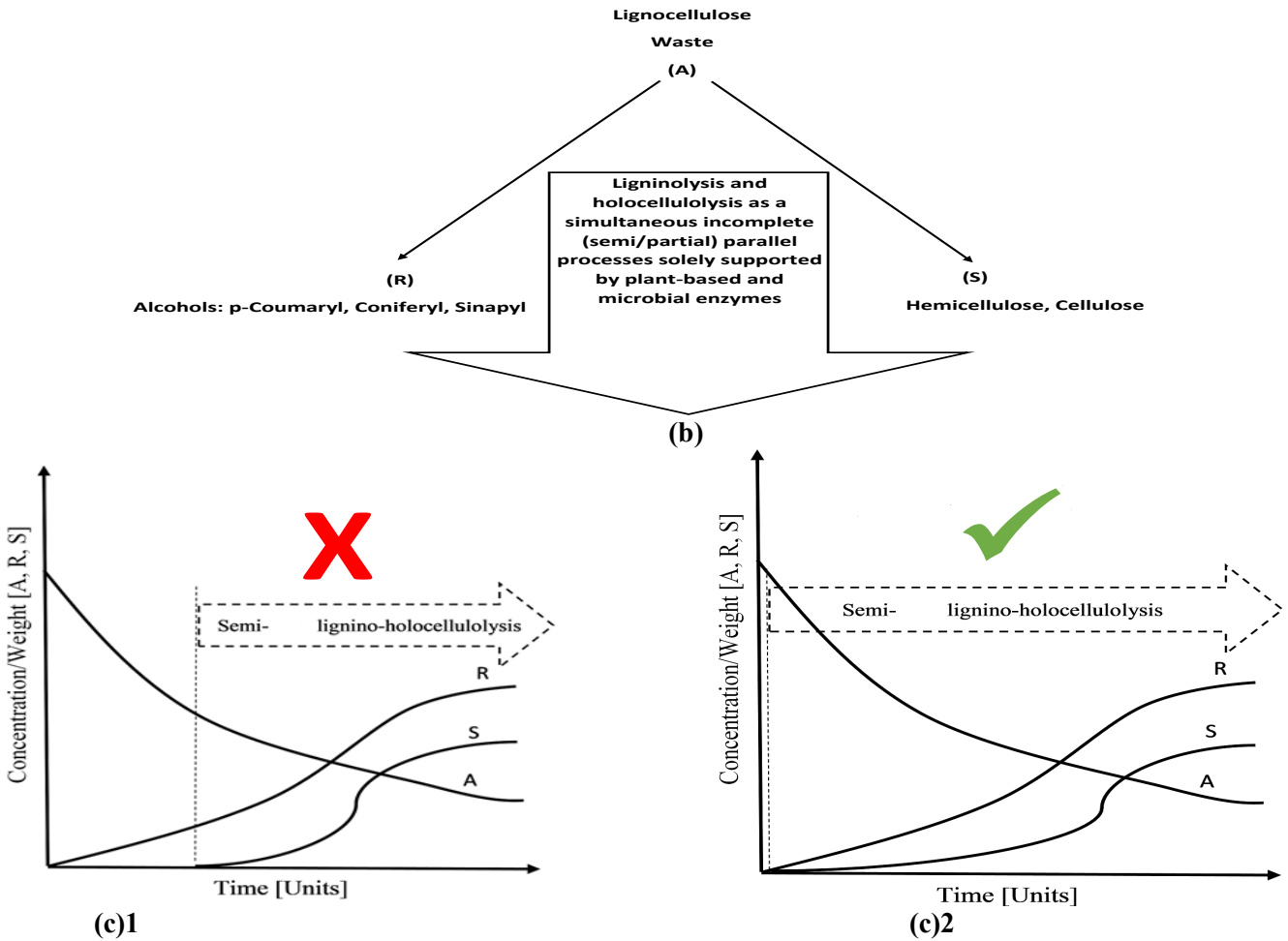


Figure 2. (a) Illustration of the rigid and amorphous regions of lignocellulosic waste and how plant-based or microbial enzymes will facilitate semi-lignino-holocellulolysis; (**b**) depiction of the hydrolysis of lignocellulose waste (A) into primary products, R (alcohols) – for ligninolysis and S (cellulose/hemicellulose) – for holocellulolysis simultaneously,**(c)1** demonstration of semi-lignino-holocellulolysis as irreversible reactions in series, and (**c)2** demonstration of simultaneous semi-lignino-holocellulolysis as irreversible first order reactions in parallel.

2.5.2. Plant exudates and enzyme cocktails for semi-lignino-holocellulolysis of lignocellulosic waste

Plant exudates contain bioactive compounds such as amylases, invertases, phosphatases, proteases, and polygalacturonases, which include certain amino acids, organic acids, reducible sugars, phenolics, flavonoids, etc. Furthermore, studies have shown that plant extracts from plants of the genus *Nepenthes* contains β-glucosidases, xylanases and carboxylesterases, proteases, ribonucleases, nucleases, phosphatase hydrolase, esterases, ribonucleases, and amylases, which are used to digest insects trapped in the fluid inside monkey cups (Mithöfer 2011). These fluids are acidic and have a pH between 1.5 to 6, depending on the species (Dlangamandla et al., 2019b). A lot still needs to be researched regarding the bioactive ingredients found in carnivorous plant cocktails (Takeuchi et al., 2015). There are some common enzymes found in both plant exudates and *Nepenthes* sp. that can be useful in the delignification of lignocellulosic waste and in particular, the lysis of holocellulose (Schulze et al., 2012; Stephenson and Hogan, 2006). Overall, alternatives for lignino-holocellulolysis in a process in which there is minimal use of chemicals or high temperature and pressure will provide for a better, environmentally benign process that will have minimal impact on the environment whilst providing for process sustainability and integration.

2.6. Future perspective, mitigation of limitations and economic impact

A future perspective: from the preceding studies, it appears that the various techniques that have been exploited in the quest to pretreat bio-waste for the extraction of value-added products are posing newer challenges. These include the production of inhibitory products such as phenolic compounds, furfural, organic weak acids, etc. (Kumar et al., 2009). Although, chemical pretreatments produce more inhibitors such as phenolic compounds, the presence of lipases and esterases in pitcher digestive juices (Higashi et al., 1993) for lignin-holocellulolysis is advantageous as these enzymes have been determined to hydrolyse some phenolic compounds (Bornscheuer et al., 2005). The use of acids to pretreat bio-waste such as lignocellulosic waste can therefore be expensive, and it is not eco-friendly. The future lies with the sole use of plant-based digestive extracts and microorganism-based enzymes under optimal conditions, as some plant digestive enzymes have been shown even to have similar characteristics to those of certain dilute strong acids, albeit with an added advantage of having active enzymes and symbionts within such extracts.

Mitigation of limitations: biological pretreatment of lignocellulosic waste, using for example *N. mirabilis* digestive extracts, can be effective, and the carboxylesterases that are available in the plant extracts can assist in reducing the inhibitory bio-products produced during pretreatment of bio-waste, such as lignocellulosic waste (Dlangamandla et al., 2019b). The use of such plant extracts in the pretreatment of lignocellulosic waste will limit environmental harm in any process developed. However, further development of this strategy is required.

Economic impact: In developed countries, the use of renewables is rising rapidly (Maroušek et al., 2020), and there lie opportunities for developing countries to use their locally available biomass for value-added product manufacturing. For lignocellulose waste beneficiation, industries can use a well-defined ecological criterion and bring savings in terms of emissions trading among many other economic benefits, as reported elsewhere (Maroušek et al., 2020). Recently, Angadam (2018) reported on the pretreatment, i.e., using *N. mirabilis* digestive juices, of lignocellulosic pomace as a waste for high reducible sugar production. This is just one of the examples which can be used for economic benefits while considering environmental safety. Furthermore, the exploitation of the knowledge and understanding of the idea of lignocellulosic waste biological pretreatment in which partial simultaneous lignino-holocellulolysis ensues will therefore affect reactor system prototype designs (Maroušek et al., 2015).

2.7. Summary

Lignocellulosic waste is made up of holocelluloses that are bonded together in a lignin matrix. This type of bio-waste can be used in biorefineries for the production of value-added products such as bio-alcohols by using bio-physico-chemical pretreatment methods and enzymes from plants and microorganisms. Holocellulose hydrolysis can be achieved solely by a cocktail of enzymes such as endoglucanases, cellobiohydrolases, and β-glucosidases enzymes. These can lyse the lignocellulosic waste via lysis of the hemicellulose by decoupling of β-1,4 D-xylose polymers bonds by endo 1,4-β-xylanase or endoxylanase, 1,4-β-xylan esterases, α-1-arabinofuranosidases, and α-glucuronidases, etc. This occurs in a process whereby there is a concurrent semi-biological deligninolysis and holocellulolysis using a process herein termed semi-lignino-holocellulolysis. To achieve a higher efficacy of semi-lignino-holocellulolysis, ligninolysis accomplished by milling/grinding to reduce the lignocellulosic waste crystallinity has to be the primary step as this will disrupt linkages within the waste matrix for effective hydrolysis. In general, there are some enzymes found both in plant exudates, e.g., *Nepenthes* sp., and which are microbially produced extracellularly, that can be used for semi-lignino-holocellulolysis of lignocellulosic waste to allow an environmentally benign process and to mitigate against the use of chemicals, heat, and pressurised systems, which this literature review contributes as a necessary discussion for biorefinery development.

2.8. References

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LITERATURE REVIEW: SUPPLEMENTARY

S2.1. Introduction

Plants have been used from the beginning of mankind as food, as a source of beverages as well as for medicinal purposes. To gain from all the benefits mentioned, it is necessary to know the chemical constituents present in the plant. This is essential because microbial toxins can sporadically enter the food chain (Puri and Hall, 1998). Plants, like human beings, face and respond to environmental challenges which include soil conditions and the availability of microorganisms that biodegrade organic matter such that nutrients become available to the plants. When injured, they respond by excreting polysaccharide gums known as exudates.

These exudates are produced from several parts of plants (examples include plant cell walls, seeds, tuber/roots, etc.). In food industries, these exudates are used as additives. The plant exudates comprise sugars, amino acids, peptides, enzymes, vitamins, organic acids, nucleotides, fungal stimulators, inhibitors, attractants, etc. These exudates can be stimulatory or inhibitory. Roots are the main source of water and nutrient uptake, i.e., from the roots to the leaves, and some exudates released from plant leaves also defend the plant efficiently against detrimental microorganisms (Mirhosseini and Amid, 2012).

As such, plants secrete a plethora of high and low molecular weight defence compounds due to pathogen stimulation. The low molecular weight antimicrobial compounds that are present on the plant as a response to stress and pathogen proliferation are known as phytoanticipins. Additionally, other low molecular weight antimicrobial complexes that are not measurable in healthy plants are known as phytoalexins. Furthermore, plant exudates contain numerous chemicals, including a class of phenolics and terpenoids that have antibacterial and antifungal abilities.

S2.2. Plant exudates: Overview

Research has also shown that molecules such as amino acids can function as stimulators for a certain group of microorganisms which can be suppressors for other soil bacteria. Phenylpropanoids are abundant plant phenolics that are largely found in root exudates including flavonoids, which are characterized as one of the major classes of phenylpropanoids and constitute a large proportion of plant root exudates which are used as a defence against pathogens (Baetz and Martinoia 2014, Wen, et al. 2007). Another class of chemicals that plants use as defence mechanisms both above the soil surface and underground are terpenoids, which also form part of the root exudates. Recently, volatile organic compounds that also serve as a defence mechanism were shown to be released from roots (Wen et al., 2007).

S2.2.1.The usefulness of plants exudates

The composition of plant exudates, if known, will be useful to a wide range of researchers, i.e., food scientists, nutritionists, chemical ecologists, pharmacologists, biologists, and phytochemists. This is largely because the exudates contain phenolics, alkaloids and essential oils, and triterpenoids (Puri and Hall,1998). The components of plants are diverse and are used in different areas of life. This research is focused specifically on the bioactive components of plant exudates in roots underground and tree stems, branches, and leaves above ground.

S2.2.2. Bioactive constituents in plant exudates

There exist some bioactive constituents in plant exudates, particularly enzymes, which include amylase, invertase, phosphatase, protease, and polygalacturonase, including amino acids, organic acids, reducing sugars, phenolics, and flavonoids, etc. Plants exudates can be used for medicinal purposes such as the treatment of bone fractures, arthritis, inflammation, obesity, cardiovascular disease, and lipid disorders. For example, the plant exudate from *Pycanthus angolenses* has been reported to be effective in the treatment of cancer, leprosy, and diabetes, and is used to waterproof boats and other structures (Pichersky and Raguso, 2018). Some plants' exudates are commercially important, e.g., rubber. Several terpenoids exist in exudates of several plants and herbs, and some are present in spices, contain a significant concentration of terpenoids that serves as flavourants in food, and alcoholic drinks, including wine specifically, which is due mostly to the presence of terpenes. They are also used as food preservatives because of their microbicidal and insecticidal properties.

S2.2.3.Co-factor influence and reasons for exudate production by plants

These plant exudates are influenced by some growth factors such as p-amino benzoic acid, auxins, biotin, choline, inositol, n-methyl nicotinic acid, niacin, pantothenate, pyridoxine, thiamine, etc. (Pichersky and Raguso, 2018; Okwu and Nnamdi, 2008; Flores and Ricalde, 1996). Plants produce exudates for several reasons, some of which are a defence against herbivores and pathogens. Figure 3 illustrates some exudates from different plant parts.

Figure 3. Plant stems and root releasing exudates.

S2.2.4. Bioactive constituents of the plant fluid of the genus *Nepenthes*

It has been found that plant cocktails from carnivorous pitcher plants of the genus *Nepenthes* also constitute several bioactive fluids, which are used to entrap insects and subsequently digest them in the pitcher fluid, as well as exudates produced by fly traps. Though pitcher fluid has been known for many decades, our awareness of the pitcher fluid composition is quite narrow (Angadam et al., 2019; Dlangamandla et al., 2019; Mithöfer, 2011). These *Nepenthes* species are insectivorous by nature, and they are characterised by their specialized monkey cup traps, thus the name "pitcher". These trap their prey and digest them, including the exoskeleton containing a chitin-type structure which was previously considered considered indestructible, thereby filling the monkey cups with a protein-rich pitcher fluid for the *Nepenthes* plant's nutrition (Lee et al., 2016).

Figure 4 illustrates different traps which produce digestive fluids or exudates. Some of the enzymes found in both plants' exudates and *Nepenthes* species, have been demonstrated to play a role in the delignification of agro-waste and also the breaking down of holocelluloses (Dlangamandla et al., 2019; Schulze et al., 2012; Gallie and Chang, 1997).

Figure 4. Prey entrapment plant parts for different *Nepenthes* plants.

S2.2.4.1. Quantifying biocatalytic activity of enzymes in *Nepenthes* species digestive fluids

Research was conducted on the activity of a few enzymes, namely carboxylesterases, β-glucosidases, and xylanases, based on their ability to decompose agro-waste components forming by-products such as total reducible sugars. Some of these enzymes are found in pitcher fluids and exudates. They were earlier acknowledged as being indispensable lignin-holocellulose biodegrading agents, aiding the transformation of holocellulose into cellobiose, which is a reducing sugar, and further to glucose. Similarly, they are known for their ability to also biodegrade hemicellulose (Manavalan et al., 2017; Chan et al., 2016), and again, they decouple the bonds between holocelluloses and lignin. For the research, test assays were conducted in mixing tubes; thereafter, the supernatants were transferred into glass cuvettes to read absorbance in a kinetic mode. All reactions were performed at 25°C, with some studies reporting the influence of fraction components in the fluids, to identify the most potent fraction in the pitcher fluid.

Enzyme fractionation is a separation technique that is used to disassociate a mixture or suspension into different fractions in which the composition varies in molecular weight or gradient. Different methods can be used, e.g., tangential flow filtration (TFF), which is a speedy and effective technique for the separation and purification of enzymes. This is done by connecting the TFF device to a pump and pressure gauge(s) with tubing and fittings. The process is easy, quick, and above all, it accomplishes greater concentrations in less time compared to techniques such as dialysis, centrifugal devices, or stirred cells (Schwartz and Seeley, 2002). However, recent methods include centrifugation tubes with membranes. Overall, different plant fluids or exudates from different parts of plants include enzymes that can be repurposed, with some being a source of several useful enzymes which can be exploited for lignin decomposition

(delignification) and cellulose, including hemicellulose decomposition (holocellulolysis), albeit, under limited conditions, and thus the concept of semi-delignino-holocellulolysis.

S2.3. References

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CHAPTER 3

NEPENTHES MIRABILIS FRACTIONATED PITCHER FLUID USE FOR MIXED AGRO-WASTE PRETREATMENT: ADVOCACY FOR NON-CHEMICAL USE IN BIOREFINERIES

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CHAPTER 3

NEPENTHES MIRABILIS FRACTIONATED PITCHER FLUID USE FOR MIXED AGRO-WASTE PRETREATMENT: ADVOCACY FOR NON-CHEMICAL USE IN BIOREFINERIES

3.1. Introduction

Nowadays, sustainable energy generation is paramount for energy security globally. Hence, the availability and utilization of bioenergy from waste materials have resulted in its generation receiving considerable attention from industry and researchers (Leong et al., 2021). This has become a focus area for researchers as there is a need to find feasible substitutes for fossil-based fuels, thus the development of biorefineries with a focus on co-manufacturing of biofuels, eco-clean energy, and chemicals from renewable biomass sources (Ghatak, 2011). Recent studies have proven that some processes used in green energy generation are not always eco-friendly. The practice involving biomass conversion to biofuels in biorefineries also results in a wide range of pollutants that damage the environment and cause humans harm via chemical use. These are known as biorefinery-derived pollutants (Heath, 2022; Lee et al., 2021).

Furthermore, biorefineries are water-intensive, and thus place substantial stress on available water supplies. For example, maize-based biofuel refineries produce 13 litres of wastewater for each litre of ethanol produced. Some experts have cautioned that biofuel production puts additional pressure on natural resources. The wastewater produced can cause pollution that extends throughout the ecosystem, resulting in the deterioration of rivers and land with silt and sediment contaminated mostly by chemical residues emanating from biomass/agro-waste pretreatment processes translocated by natural means to pristine areas (Levidow, 2015).

They are several agro-waste pretreatment methods, among which chemical pretreatment methods, i.e., alkaline/caustic, dilute acid/organic acid, steam explosion (autohydrolysis), carbon dioxide (CO_2) explosion, liquid hot water (LHW), microwave-chemical, organosolv and wet oxidation, are among the favourites. Some of these pretreatment methods generate hazardous waste that is detrimental to human health and the environment (soil, air,

water). For example, LHW pretreatment produces solid residue that needs disposal, while processes such as organosolv need solvents to be drained and recycled with solvent residues being in the solid waste (Dlangamandla, 2018; Capolupo and Faraco, 2016). These methods can be replaced by plant-based enzymes containing digestive fluids, such as those found in monkey cups of some *Nepenthes* sp.

Nepenthes sp., also known as pitcher plants, are a group of carnivorous plants that grow in tropical regions with about 130 species having been identified, and numerous species being discovered annually (Ellison, 2010). One of these species is *Nepenthes mirabilis* (*N. mirabilis*) which has monkey cups containing pitcher fluid in which numerous enzymes are embedded. Indeed, to ascertain large-scale availability, the hydroponic growth method in combination with vertical farming can be used to grow pitcher plants such that they produce the enzyme-containing pitcher fluid on a large scale (Jürkenbeck et al., 2019; Anonymous, 2016). The pitcher fluid has been known to completely digest both insects and leaf litter. Thus, it could potentially be used as a sole means of pretreating agro-waste. The pitcher fluid produced by most pitcher plant species is acidic with a pH range of 1.5 to 6. The acidity of the pitcher fluid aids in the plants' insect and leaf litter hydrolysing capabilities (Chan et al., 2016; Takeuchi et al., 2015). Enzymes found in the pitcher fluid include β-xylosidase, aspartic proteinase nepenthesin I, β-1,3-glucanase, β-1,3-glucanase, class III chitinase, oxidoreductase, class IV chitinase, acid chitinase, carboxylesterase, xylanase, and thaumatin-like protein, etc. (Hatano and Hamada, 2012; Takeuchi et al., 2011; Hatano and Hamada, 2008), with some having properties which can enhance delignification and holocellulolysis to produce fermentable hydrolysates from agro-waste. Since pretreatment is an important step involved in the production of bioethanol from lignocellulosic biomass, we envisage that the pitcher fluid can replace some of the current biomass/agro-waste pretreatment methods in which chemicals are used.

The selection of a specific agro-waste depends largely on regional accessibility including availability and thus this was the primary motivation to use a mixture of apple peels, oak yard (leaf) waste, grape pomace, and maize cobs. These agro-wastes are readily available in South Africa (SA), especially in the Western Cape Province where this study was conducted. Several researchers have been reporting on the use of a single agro-waste when performing biomass pretreatment studies. This is not ideal, as agro-waste in most instances consists of two or more different types of waste, especially in an industrial setting. The Western Cape Province is the third leading province in SA, producing a large quantity of citrus fruit, with 95% being apples (Hunlun et al., 2017). Moreover, there is a plethora of oak trees in the Western Cape Province, and they produce a large quantity of yard waste. These agro-wastes were selected with the

intention of reducing the reliance on landfilling; hence transforming such waste into value-added products or crude products, such as hydrolysates containing fermentable sugars, would be advantageous. This is because such hydrolysates can be used in the generation of bioenergy. *N. mirabilis* pitcher fluid was selected to pretreat the mixed agro-waste because other studies have proven that it is not necessary to have a pure form of enzyme cocktails for biomass pretreatment (Adsul et al., 2020). Furthermore, its monkey cup, on average, is larger compared to other *Nepenthes* sp. and can thus store a larger quantity of the pitcher fluid. As elucidated previously, *Nepenthes* sp. pitcher fluid contains an assortment of enzymes that might facilitate the hydrolysis of lignin-containing agro-waste while reducing phenolic reductants.

The purpose of this part of the study was therefore to assess the feasibility of using *N. mirabilis* pitcher fluid as a suitable agent to pretreat agro-waste for the extraction of total reducible sugars (TRSs) while reducing total phenolic compounds (TPCs) which are known to be toxicants for fermentation systems. Furthermore, it was imperative to also determine optimal conditions, namely the most suitable particle size of the agro-waste, the fraction in the pitcher fluid responsible for high TRSs production while reducing TPCs,as well as identifying the enzymes in the high-performing fraction.

3.2. Materials and methods

3.2.1. Collection and processing of the mixed agro-waste

Agro-waste such as *Malus domestica* (apple) peels, *Quercus robur* (oak) yard waste, *Citrus sinensis* (orange) peels, *Vitis vinifera* (grape) pomace, and cobs from *Zea mays* (maize) were obtained from a local fruit/vegetable store while yard waste was gathered from the garden of the Cape Peninsula University of Technology (CPUT), District 6 campus (Cape Town, Western Cape Province, SA). These agro-wastes were dried separately in an oven at 80°C for 24 h, except for *C. sinensis* peels, which were dried for 72 h. The different agro-wastes were pulverized and screened to > 75 µm x< 106 µm and > 106 µm particle sizes, eliminating a pre-cleansing step. A composite was made by accurately weighing 10 g of each of the milled waste by pooling in equal proportions of 20% (w/w), and subsequent homogenous mixing. The basis for using mixed agro-waste is that mixed lignocellulosic biomass (MLB) has been demonstrated to contribute to cost savings with bench scale experiments, indicating a better ethanol yield when MLB was used in comparison to single feedstocks (Oke et al., 2016).

3.2.2. Collection, physico-chemical characterization, and sample fractionation of the *N. mirabilis* pitcher fluid

N. mirabilis pitcher plants were cultivated hydroponically in a greenhouse under ambient conditions of 25–30 °C. These plants were grown at Pan's Carnivores Plant Nursery (21 Kirstenhof, Tokai, Cape Town, SA). Pitcher fluid samples (10 to 40 mL) were collected from both the opened and closed monkey cups depending on the size of the individual cups. This fluid was transferred from the monkey cups into sterile 50 mL conical tubes and instantly stored on ice before transportation to the laboratory. The plant's pitcher fluid was then centrifuged at 4000 x g for 15 min and filter sterilised with a 0.22 µm Millipore membrane filter (Merck, Burlington, MA, USA) followed by pooling to make a single batch. From the single batch, the pitcher fluid was passed through a 10 kDa filter via centrifugation at 4000 x g for 10 min. The flow through from the 10 kDa filter was then concentrated on a 3 kDa filter (Pall OD003C34) by centrifugation at 4000 x g for 10 mins. The different fractions, i.e., < 3 kDa, > 3 kDa, < 10 kDa, > 10 kDa, were stored at -20 °C before further processing, i.e., without dilution or use of a buffer. The selection for the 10 kDa fraction was based on a method used by Lee et al. (2016), whereby a 10 kDa Amicon ultramolecular weight cut-off centrifugal filter (Millipore) was used. Furthermore, since the nominal molecular weight limit (NMWL) below 10 kDa is 3 kDa, this fraction was also assessed. Overall, the characterization of the *N. mirabilis* pitcher fluid was carried out by using a multi-parameter meter (Eutech Instruments Pty Ltd, Thermo Fisher Scientific, Singapore) to determine the physico-chemical properties, i.e., pH, redox potential, and conductivity of the pooled samples and the individual fractions. The specific gravity of the pooled pitcher fluid was determined by weighing 1 mL of the fluid. Protein quantification was accomplished, using the QuantiPro BCA assay kit (Sigma QBCA) according to the manufacturer's instructions, to ascertain the presence of enzymes (proteins) within the fluid.

3.2.3. Pitcher fluid fraction in-solution digestion and proteome analysis via LC-MS/MS

An LC-MS/MS analysis was piloted with a Q-Exactive Quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, USA) coupled with a Dionex Ultimate 3000 nano UPLC system. Data were developed using Xcalibur v4.1.31.9, Chromelean v6.8 (SR13), Orbitrap MS v2.9 (build 2926), and Thermo Foundations 3.1 (SP4). The mobile phase was made by dissolving peptides in 0.1% formic acid (FA, Sigma 56302), 2% acetonitrile (ACN, Burdick & Jackson BJLC015CS) and loaded on a C18 trap column (PepMap100, 9027905000, 300 µm × 5 mm × 5 µm). The volume of the sample injected was approximately 400 ng of peptide for each sample. Samples were trapped in the analytical column and washed for 3 min before the valve was switched on and the peptides eluted. Chromatographic

separation was performed with a Waters nanoEase (Zenfit) M/Z Peptide CSH C18 column (186008810, 75 µm × 25 cm × 1.7 µm). The solvent system used was solvent A: LC water (Burdick and Jackson BJLC365), 0.1% FA, and solvent B: ACN, 0.1% FA. All data acquisition was obtained using Proxeon stainless steel emitters (Thermo Fisher TFES523). The multi-step gradient for peptide separation was generated at 300 nL/min with a time change of 5 min, gradient change: 2 – 5%; Solvent B, time change 40 min, gradient change 5 – 18%; Solvent B, time change 10 min, gradient change 18 – 30%; and Solvent B, time change 2 min, gradient change 30 – 80%. The gradient was then held at 80% Solvent B for 10 min before returning to 2% Solvent B for 15 min. The mass spectrometer was operated in a positive ion mode with a capillary temperature of 320°C. The applied electrospray voltage was 1.95 kV. Details of data acquisition conditions are shown in Table 16 in the Appendices. Furthermore, database interrogation was performed with a Byonic$_{TM}$ Software v3.8.13 (Protein Metrics, USA) using the *Nepenthes* sp. database of reviewed and unreviewed proteins containing 1790 proteins sourced from the NCBI (www.ncbi.nlm.nih.gov) dated 19/10/2021. Details of search parameters are displayed in Table 17 in the Appendices.

3.2.4. Trace element (co-factor) solution preparation for pitcher fluid supplementation

Enzyme performance can be enhanced by several factors, with a trace elements solution (TES) being one of the solutions that can provide enzyme co-factors, thus improving the digestive fluid performance. Trace elements are believed to work as co-factors and are essential in minute quantities to enhance the biological functioning of enzymes (Keskin et al., 2018). The oxidation-reduction reactions of biomass degradation can be enhanced by some metallic ions; examples include iron and copper (Šelih et al., 2007). The trace element solution used in this study was prepared by dissolving 1.5 g of nitrilotriacetate in 800 mL sterile distilled water. Subsequently, the pH was adjusted to 6.5 by using 1M KOH (8 g/500 mL). These compounds – i.e., $ZnSO_4.7H_2O$ (0.1 g), $FeSO_4.7H_2O$ (0.1 g), $MgSO_4$ (3 g), $MnSO_4$ (0.5 g), NaCl (1 g), $CuSO_4$ (0.1 g), $AlK(SO_2)_2.12H_2O$ (0.01 g), H_3BO_3 (0.01 g), $Na_2MnO_4.2H_2O$ (0.01 g), $MgSO_4.7H_2O$ (6.14 g), $MnSO_4.H_2O$ (0.56 g), $CoCl_2.6H_2O$ (0.187 g) and $CoCl_2$ (0.1 g) – were weighed as specified and added to the nitrilotriacetate solution and the solution was made up to 1000 mL. The solution was filter sterilised using a 0.22 µm filter and autoclaved. It was then stored at 4 °C before use – see Appendices A1 for the preparation of the solutions.

3.2.5. Conditions for pitcher fluid facilitated mixed agro-waste pretreatment

A mass (0.5 g) of the mixed agro-waste was weighed into each 100 mL Schott bottle and a volume (10 mL) of the individual *N. mirabilis* pitcher fluid fractions was added to each Schott bottle, to constitute a 5% (w/v) slurry. Thereafter, the trace element solution (0.1 mL) was added as a supplement. The mixed agro-waste and the pitcher fluid were mixed by swirling in a shaking (120 rpm) incubator (LABWIT ZWY-240, Shanghai Zhicheng Analytical, Shanghai, China) to ensure uniformity at a temperature maintained at 25-30 °C, to mimic ambient temperature. Sampling (3 mL) was done at 24 h and 72 h, and the samples were centrifuged at 4000 x g for 10 min. The supernatant collected was used for further analyses, i.e., TRSs and TPCs quantification. All experiments were done in triplicate, and average values were used for data analysis.

3.2.6. Quantification of total reducible sugars and residual phenolic compounds in agro-waste pretreatment hydrolysates

TRSs were quantified from individual samples collected as supernatant. This test was performed using a dinitrosalicylic acid (DNS) assay protocol (Miller, 1959) and the concentration of TRSs was determined using a calibration curve ($R^2 = 0.95$ – see Figure 18 in the Appendices). Consequently, the Folin–Ciocalteu method was used to measure TPCs (Agbor et al., 2014), with a calibration curve ($R^2 = 0.91$ – see Figure 19 in the Appendices) being used to determine the actual concentration in individual samples. The assay protocols were done using a Jenway 7305 UV/Vis spectrophotometer (Cole–Parmer, Staffordshire, UK). All measurements were done in triplicate and the averages were used in data analysis.

3.2.7. Data analytics and rationale

The data collected from this experiment were analysed with Python using different libraries, i.e., Matplotlib, Pandas, Seaborn, and SciPy. The rationale was to determine which arrangement of parameters yielded the most TRSs while generating the least TPCs. To this end, the enzyme fraction, and the particle size were each divided into classes as observed in Table 4.Appendices Table 22 lists experimental run classifications.

Table 4. Classification of the enzyme fraction and particle size for data analytics.

Enzyme fraction (kDa)	Enzyme fraction class	Particle size class	Particle size (µm)
< 3	1	1	> 75 *x*< 106
> 3	2	2	> 106
< 10	3	-	-
> 10	4	-	-

This classification was geared towards creating a cluster of observations presenting the same characteristics and thus facilitating a categorical analysis meant to inform which arrangement was more effective.

To evaluate the correlation between these classes and the contact time on the production of both TPCs and TRSs, the Pearson correlation coefficient was used. The latter shows the correlation between the parameters assessed and the target variables (TRSs, TPCs) without providing causation. When assessing several key parameters, a correlation matrix can be used to show the Pearson correlation coefficient between each of these parameters. Although the Pearson correlation doesnot inform on the causation of observed experimental outputs, it can inform on the influence that each of these parameters has on the target variable by providing the degree of covariance between them.

Further data analysis and graphical evaluation were done, i.e., to evaluate the influence that each of these key parameters had on the production of TRSs, including a statistical summary, and 3-D plots of the effects of the variation of each class on the production of TRSs. Furthermore, a comparison of the probability density function (PDF) of the production of TRSs and TPCs for each class was also done. The statistical summary provided the mean, standard deviation, minimum, p25, p50, p75, and maximum values of the production of TRSs and TPCs concerning each class of the key parameters. The 3-D plots give a visual representation of this analysis, and the PDF illustrations compare the distribution of the TRSs and TPCs production for each class of the key parameters. The combination of these analyses contributes to understanding the variation of the production of TRSs, informs on the most relevant key parameters, and guides on what arrangement to select to minimize the generation of TPCs.

3.3. Results and discussion

3.3.1. Physico-chemical characteristics of the fresh and fractionated *N. mirabilis* pitcher fluid

The physico-chemical properties of *N. mirabilis* pitcher fluid were determined with a focus on the following characteristics: redox potential, specific gravity, conductivity, and pH - see Table 5. Even though *N. mirabilis* pitcher fluid comprises a cocktail of enzymes, the observation was that they must have acidity tolerant traits. Furthermore, the pitcher fluid characteristics were determined to be comparable to those of the 1% (v/v) dilute sulphuric acid solution usually used in biomass treatment, although the solution has a higher redox potential (Angadam et al., 2021). When a solution has a high redox potential, it is an indication that reduction-oxidation reactions can occur more rapidly.

Table 5. Physico-chemical properties of *N. mirabilis* pitcher fluid juice before fractionation.

Factors	Values (units)
pH	2.0-2.09
Specific gravity (SG)	0.73-0.81
Redox potential (ORP)	501-520 mV
Conductivity (CO)	3.86-4.93mS/cm

After fractionation, the < 10 kDa fraction was observed to have somewhat preferable physico-chemical properties as highlighted in Table 6, with less TPC formation including a slightly higher TRS formation. As such, this fraction was determined to be suitable for the pretreatment of mixed agro-waste.However, all fractions were assessed, and a confirmatory statistical analysis was needed.

Table 6. Averaged (n =3) physico-chemical properties of the different fractions of *N. mirabilis* pitcher fluid in comparison to dilute (1% v/v) sulphuric acid [a], and the initial assessment of the best performing fraction in terms of TRSs and TPCs formation [b].

Factors	< 3 kDa	> 3 kDa	< 10 kDa	> 10 kDa	[a]1% (v/v) H_2SO_4
			A		
pH	2.04	2.02	**2.00**	2.06	0.70
S.G.	0.73	0.73	**0.81**	0.80	1.08
ORP	503	501	**510**	511	354.2
CO	3.91	3.86	**4.93**	3.97	[c]n/d
			B		
[b]TRSs	31.31	33.50	**33.87**	30.82	
[b]TPCs	13.42	11.66	**11.45**	16.95	

[a]Angadam et al., [b]Conditions (initial evaluation) = 72 h, > 106 µm particle size, ambient temperature, without TES, [c]n/d – not determined

To hydrolyse lignin-containing agro-waste into fermentable sugars, both oxidative and hydrolytic enzymes are needed, as well as an acidity that can culminate in the dissolution of some constituents in the waste. Therefore, when there is a cocktail of enzymes that are acid tolerant within the pitcher fluid which is highly acidic, numerous advantages can be gained, among which are: 1) ease of bond decoupling, 2) lysis of lignin, 3) extraction of cellulose and hemicellulose (holocellulolysis), 4) the decomposition of toxicants produced as by-products, and 5) the deactivation of agro-waste decomposers, e.g., fungi and bacteria.

3.3.2. Identified enzymes in the *N. mirabilis* pitcher fluid

Several researchers have confirmed the existence of certain enzymes in numerous pitcher plant fluids, including the pitcher fluid of *N. mirabilis*. Examples include β-1,3-glucanase, class III chitinase, class IV chitinase, and a thaumatin-like protein. Class III acid endochitinase was also identified from the monkey cup of the carnivorous pitcher plant by other researchers (Rottloff et al., 2016). β-1,3-glucosidase, xylanase, and carboxylesterase were also determined to be in the pitcher fluid of *N. mirabilis* (Dlangamandla et al., 2019b). The presence of two or more different chitinases within the pitcher fluid was proved by Senevirathna et al. (2019). Another study also reported the prevalence of putative peroxidase, class III chitinase, glucanase, oxidoreductase, putative peroxidase, class IV chitinase, and acid chitinase in *Nepenthes* sp. pitcher fluid (Hatano and Hamada, 2012). In a study carried out by Hatano and Hamada (2008), thaumatin-like protein, aspartic proteinase nepenthesin I, chitinase precursor, and β-1,3-glucanase were observed (Athauda et al., 2004).

Consequently, in this study, using proteomic analyses performed on the < 10 kDa fraction, i.e., determined to produce a high concentration of TRSs with a low TPC load of pitcher fluid from both opened and closed pitchers, the following enzymes were identified; β-1,3-glucanase, purple acid phosphatases, putative peroxidase 27, class IV chitinase, aspartic protease, thaumatin-like protein, and class III chitinase. Some of these enzymes have functional attributes that can be advantageous when pretreating mixed agro-waste (see Table 7). In summary, putative peroxidase 27 can degrade lignin to water (H_2O), carbon dioxide (CO_2) and H_2O_2 to H_2O and oxygen (O_2) (Isroi et al., 2011). Overall, such peroxidases are an exceptional group of ligninolytic enzymes, and due to their high redox potential, they can oxidise lignin (Ravichandran and Sridhar, 2017). When combined with β-1,3-Glucanase, they can play a significant role whereby a lignin-glucan-rich substrate can be decoupled into simple saccharides (Michalko et al., 2013). Overall, β-1,3-glucanase can hydrolyse some constituents in the agro-waste by targeting 1,3-linked glucose polysaccharides using an inverting mechanism for glucan hydrolysis (McGrath and Wilson, 2006). A similar trait can also be attributed to thaumatin-like proteins, which have an ability to breakdown polymeric β-1,3-glucans via hydrolysis to oligosaccharides (Grenier et al., 1999), and they can also facilitate the degradation of p-hydroxybenzoic acid, which is a component of lignin (Fierascu et al., 2019). Class III and IV chitinases hydrolyse glycosidic bonds; specifically, those associated with chitin with a specificity of decoupling glycosidic bonds between two or more carbohydrates or between a carbohydrate and a non-carbohydrate moiety (Rottloff et al., 2016). Therefore, all of the

combined attributes of the individual enzymes identified can facilitate an effective way to pretreat agro-waste for the production of crude hydrolysates which can be further processed without the use of chemicals.

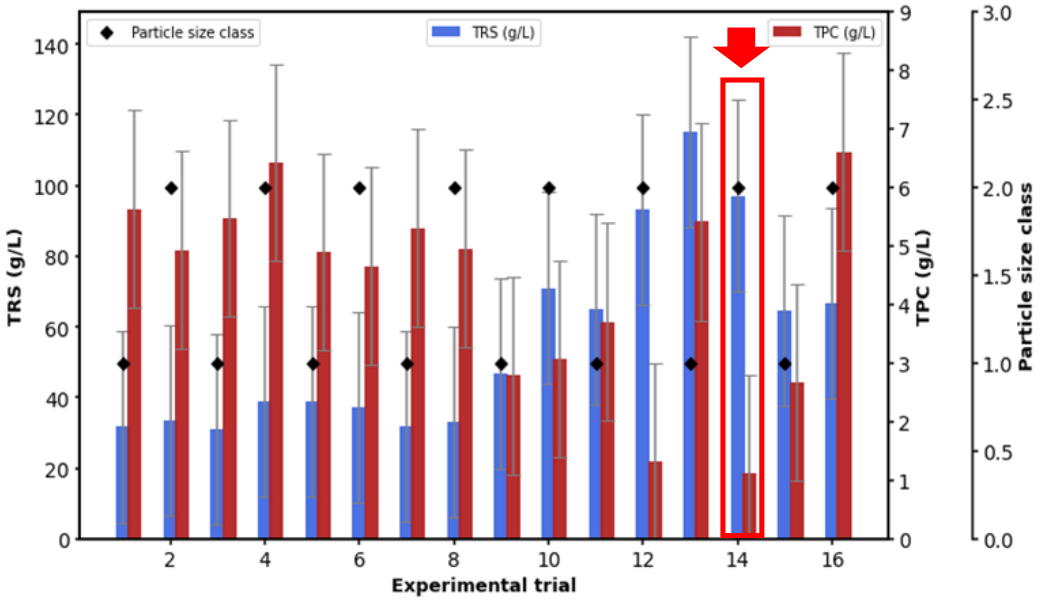
Table 7. Enzymes identified in the < 10 kDa *N. mirabilis* pitcher fluid via a Byonic™ software search and their rankings based on the best score.

Rank	Enzyme	Accession number	Functional attributes in relation to agro-waste pretreatment
1	β-1,3-Glucanase	BAM28611	Degrades glucan into oligosaccharides or reducing sugars (Jose et al., 2014)
2	Purple acid phosphatase	BAW35430	Degrades phosphate monoesters in plant tissue (Kong et al., 2018)
3	Class IV chitinase	QBC75407	Prevents agro-waste decomposers such as fungi to proliferate (Slavokhotova et al., 2014)
4	Putative peroxidase 27	AMN14864	Oxidation of toxic reductants and degradation of lignin (Anonymous, 2022b)
5	Aspartic protease nepenthesin I	AFV26024	Decouples proteins associated with some agro-products into amino acids (Anonymous, 2022a)
6	Thaumatin like protein	ACU31850	Degrades polyphenols into phenolic acids (Ma et al., 2020)
7	Class III chitinase	BAM28610	Has an ability to breakdown β-1-4 glycosidic bonds (Hamid et al., 2013)

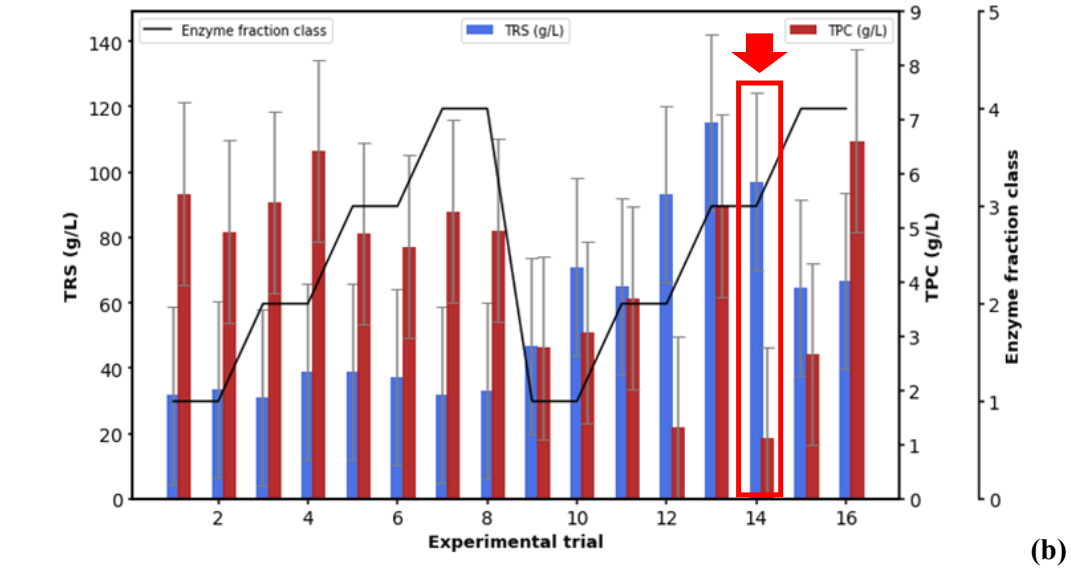
3.3.3. Analysis of reducible sugar production and residual phenolic compounds in hydrolysates from mixed agro-waste pretreatment

Table 8 provides a statistical summary of the influence of the assessed parameters on the production of TRSs and TPCs which are residual toxicants in pretreated agro-waste hydrolysates. Overall, the mean production of TRSs was higher when the agro-waste particle size was > 106 µm and > 75 µm *x*< 106 µm (Experiments 13 and 14 – see Figure 5); albeit, using the < 10 kDa enzyme fraction. The < 10 kDa enzyme fraction in combination with > 75 µm *x*< 106 µm particle size produced hydrolysates with a higher concentration of TPCs at 5 g/L (Experiment 13).

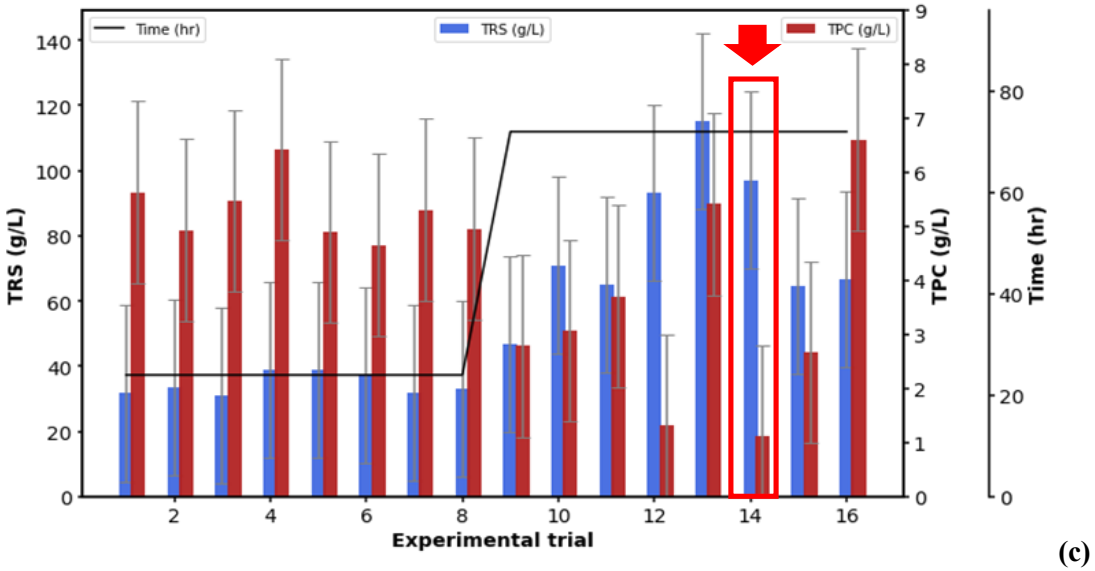
There was an indication that the mean generation of TPCs was higher when the agro-waste particle size was smaller and after 24 h than at 72 h (Figure 5A). This is an indication that the *N. mirabilis* pitcher fluid might have provided some enzymes that biodegrade TPCs as indicated elsewhere (Dlangamandla et al., 2019b). Furthermore, the mean production of the TRSs was higher after 72 hthan at 24 h(Figure 5C) using enzyme fraction class 3 (Figure 5B). Therefore, there was a need to ascertain which of the parameters are suited for better performance for downstream processes such as fermentation, i.e., with the least quantity of toxicants, i.e., TPCs. Refer to Table 8 for statistical validation of the distribution between TRS and TPC production.



(a)



(b)



(c)

Figure 5. Variation of TRS and TPC production with **(a)** particle size, **(b)** enzyme fraction, and **(c)** time. Refer to Table 22 in the Appendices for experimental run classifications.

Table 8. Statistical summary of the study with respect to the parameters assessed, providing standard deviations to inform the validity of the distribution to produce TRSs and TPCs for each experiment.

	Count	Mean	Std dev.	Min.	25%	50%	75%	Max.
TRS production (> 106 µm)	8	53.13	28.73	31.08	31.78	42.73	64.80	115.19
TRS production (>75 µm x < 106 µm)	8	58.88	26.80	33.08	36.41	52.79	76.59	97.13
TPC production (> 106 µm)	8	4.48	1.24	2.67	3.48	5.10	5.43	5.63
TPC production (>75 µm x < 106 µm)	8	4.13	2.11	1.11	2.64	4.79	5.32	6.61
TRS production (24 h)	4	33.35	3.68	31.08	31.50	31.74	33.58	38.84
TRS production (72 h)	4	72.91	29.48	46.62	60.17	64.91	77.64	115.19
TPC production (24 h)	4	5.33	0.32	4.89	5.20	5.39	5.51	5.63
TPC production (72 h)	4	3.64	1.27	2.67	2.76	3.25	4.13	5.41
TRS production (> 10 kDa)	2	39.14	10.59	31.65	35.39	39.14	42.88	46.62
TRS production (< 10 kDa)	2	48.10	24.08	31.08	39.59	48.10	56.61	65.13
TRS production (> 3 kDa)	2	77.01	53.99	38.84	57.93	77.01	96.10	115.19
TRS production (< 3 kDa)	2	48.26	23.24	31.83	40.04	48.26	56.47	64.69
TPC production (> 10 kDa)	2	4.21	2.01	2.79	3.50	4.21	4.92	5.63
TPC production (< 10 kDa)	2	4.59	1.25	3.71	4.15	4.59	5.03	5.48
TPC production (> 3 kDa)	2	5.15	0.37	4.89	5.02	5.15	5.28	5.41
TPC production (< 3 kDa)	2	3.99	1.86	2.67	3.33	3.99	4.65	5.31

To ascertain the veracity of the experimental data, a Pearson correlation matrix (Figure 6) between the amendable parameters was done as it provides the linear correlation between the parameters evaluated.

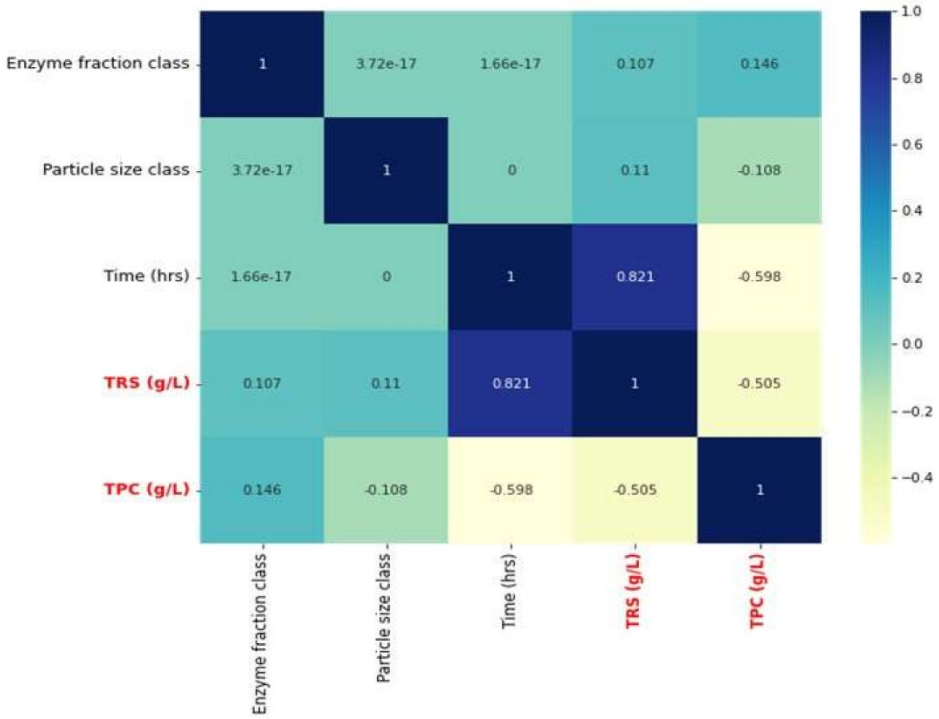


Figure 6. Correlation matrix between the amendable parameters for TRS and TPC production.

This correlation matrix was used as a diagnostic tool to attest to a correlation between variables (n = 2). It was observed that the contact time was the parameter most correlated to TRS production (Pearson Product Moment correlation of 0.82) while TPC production in the hydrolysates was unrelated to the contact time, with a coefficient of -0.6. Normally, it is expected that as the incubation period increases, the generation of TPCs must cumulatively increase in the hydrolysates. This suggested the hydrolysis of some phenolic components by the pitcher fluid, although further verification of this assertion is needed. Carboxylesterases with a potential to hydrolyse phenolic-type compounds present in *N. mirabilis* digestive fluids were suggested as candidate phenolic acid esterases, with TPC bioconversion potential (Manavalan et al., 2017). Furthermore, after evaluating the output of each experiment to the parameters that were modified, i.e. individually and then collectively, it was observed that a trade-off between the production of a high concentration of TRSs with the lowest quantity of TPCs was ideal.This was achieved under the following conditions: 1) particle size of > 106 µm, 2) contact time of 72 h, and 3) enzyme fraction < 10 kDa (Experiment trial 14, refer to Figure 5); although 1) particle size of > 75 µm *x*< 106 µm, 2) contact time of 72 h, and 3) enzyme fraction < 10 kDa produced hydrolysates with 115 g/L of TRSs while the concentration of TPCs was high.

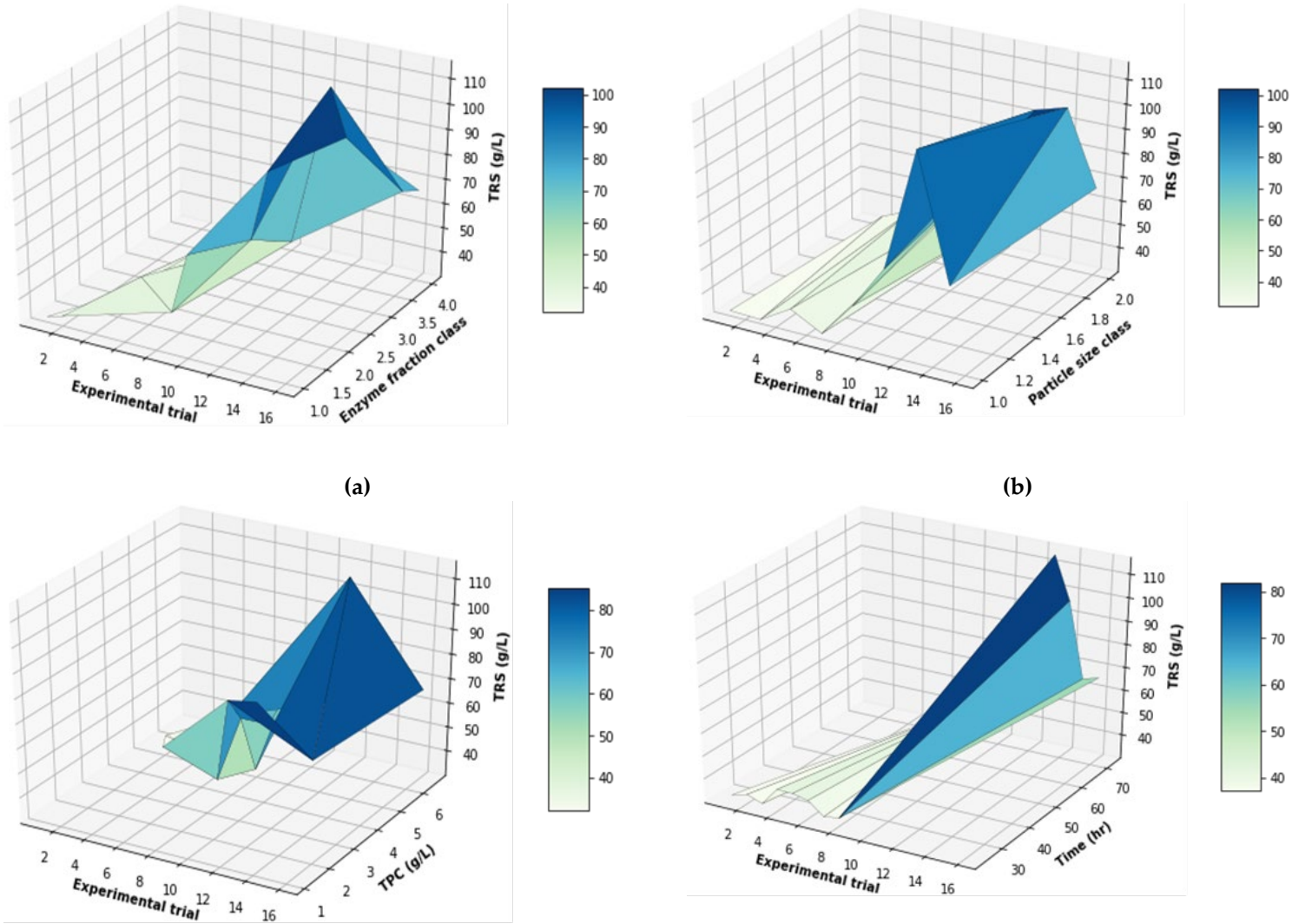


Figure 7. 3-D plots of the variation of TRS production with enzyme fraction class **(a)**, particle size class **(b)**, time **(c)** and TPC generation **(d)**. Refer to Table 4 for enzyme fraction and particle size classes.

These results were further confirmed with the 3-D plots (Figures 7a – d) showing the variation of TRS production given the variation of the individual parameter in each experimental trial. From the observed literature, there are no studies associating the production of TRSs with a particular enzyme fraction, that is < 10 kDa, with no further association with the waste particle size as observed in Figure 7a, whereby Experiment 9 indicated the lowest TRSs production when compared to Experiment 13 with the highest TRSs production. However, there is a linkage between TRS production and the particle size of the milled agro-waste (Figure 7b), whereby in some studies it was reported that the crystallinity of cellulose can be reduced by milling, with the accessibility of the hemicellulose by hydrolysing enzymes increasing.

Mechanical agro-waste reduction can result in reduced energy consumption with an increase in the accessibility of the enzymes to the biomass, thus the digestibility of lignocellulosic waste, a result that can impart moderate chemical or physicochemical co-treatments (Barakat et al., 2014). However, observations have been made that when using ionic liquids as a green chemistry approach to biomass, there is a likelihood that reducing the waste to much smaller < 0.150 µm might reduce glucose yields; however, this can vary with the type of pretreatment liquid (Bahcegul et al., 2012). It was observed that when particle sizes range from 38 to 105 µm, similar sugar yields can be attained using commercial microcrystalline cellulose (Peters et al., 1991). Overall, the hydrolysis is time-dependent (Figure 7d), with the observation being that: 1) there are lower hydrolysis rates with smaller particle sizes, including those in excess of 300 µm, with 2) longer hydrolysis time being preferable at near ambient temperature conditions (Li et al., 2012). While a longer hydrolysis period might be preferable, there is an increasing chance that TPCs might accumulate as seen in Experimental run 17 (Figure 7c) even when using a >106 µm mixed agro-waste; albeit with a > 10 kDa enzyme fraction, unless mitigation mechanisms or enzymes such as laccases are supplemented to reduce the phenolics (De La Torre et al., 2017). An assertion was made by Dlangamandla et al. (2019b) that the *N. mirabilis* pitcher fluid has enzymes that reduce TPCs.

By using a probability density function (Figure 8), i.e., normal distribution (Gaussian), of the TRS and TPC production, further confirmation was ascertained of the observations achieved, i.e., the variation of several individual experimental conditions for the agro-waste pretreatment using the pitcher fluid of *N. mirabilis*. At 72 h, both the TRSs and TPCs had a symmetrical probability density function PDF (Figure 8c& 8d), with attributes of a platykurtic (kurtosis < 3) profile, which further suggested the lack of outliers. Previously, Lai et al. (2014) indicated that a 72-hr process for an enzyme hydrolysis scheme was appropriate, with hydrolysis of hard- and softwoods being assessed. In another process, i.e., organosolv-enzyme hydrolysis, whereby lignocellulose of feedstock was used to produce butanol and ethanol, a similar enzymatic hydrolysis incubation period was used (Nanda et al., 2014). Therefore, the 72-hr enzyme incubation period was used as a baseline for probability analysis. The 72-hr incubation period was also confirmed in this study as being suitable and adequate for agro-waste pretreatment using pitcher fluids.

Since TPCs are a challenge downstream in fermentation processes, the minimum and maximum threshold, or a range, of the total phenolics in the hydrolysates, needed to be verified, i.e., its probability assessment using the PDF. Although the quantification of the type of phenolics was not done in this study, it is imperative to assess TPCs formation probability, i.e., their presence in the hydrolysates, as this influences fermentation outcomes, especially when using the popular ethanol-producing strain, *Saccharomyces cerevisiae* (*S. cerevisiae*). Although different classes of by-products might be present in the hydrolysates of pretreated agro-waste, phenolics were the focus of this study, for which 4-Hydroxy-3-methoxycinnamaldehyde, coniferyl alcohol, and p-coumaric acid were prevalent among others (Sharma et al., 2022). It was determined that 4-Hydroxy-3-methoxycinnamaldehyde (coniferyl aldehyde) inhibited *S. cerevisiae* at 0.32 g/L (Adeboye et al., 2014), a very low threshold. Furthermore, Klinke et al. (2003), mentioned that by using alkaline wet oxidation treating wheat straw, 0.27 g/L of phenols were produced. An IC_{50} of 0.46 g/L (50% inhibition) for *S. cerevisiae* was also reported elsewhere (Hrenovic et al., 2005). As such, two values, i.e., 0.3 (min) and 1 g/L (max) as observed in this study, were used to assess the probability of TPC formation using these values at 72 h for different particle sizes, and including the enzyme fractions used.

For TPC formation using > 106 µm agro-waste particles, PDF had a slight negative skewness, with a semi-bimodal profile (Figure 8b), further suggesting a near normal distribution. However, for TRSs (Figure 8a), a positive skewness was observed, suggesting that the mean was slightly greater than the median, with the > 75 µm x< 106 µm agro-waste particles having a higher mode than the > 106 µm particle size with a near log normal profile.

For the > 106 µm particle size, the probability of their concentration being above 0.3 g/L is almost certain (100%), with standard deviations being 3.37 below the mean (z-score). This is reduced minutely (4%) when considering the > 75 µm x< 106 µm agro-waste particles. A similar trend was also observed for TPCs at a concentration of 1 g/L for both particle sizes assessed. Additionally, TRSs (< 3 kDa/> 3 kDa) and TPCs (> 10 kDa/< 3 kDa) in Figures 8e& 8f are centred around the mean with higher modes, suggesting a likelihood that there is an equivalent frequency when the fractions are used, thus a similar outcome will ensue, e.g., higher TPC production at a lower TRS production when compared to the < 10 kDa fraction. Since toxicant by-products from agro-waste pretreatment such as phenolics are known to inhibit fermentation processes whereby the inhibition of fermenters such as *S. cerevisiae* including enzymic conversion occurs, the use of inhibitor-resistant strains might provide a prudent outcome in such downstream systems, i.e., develop strains via tolerance engineering (Wang et al., 2018).

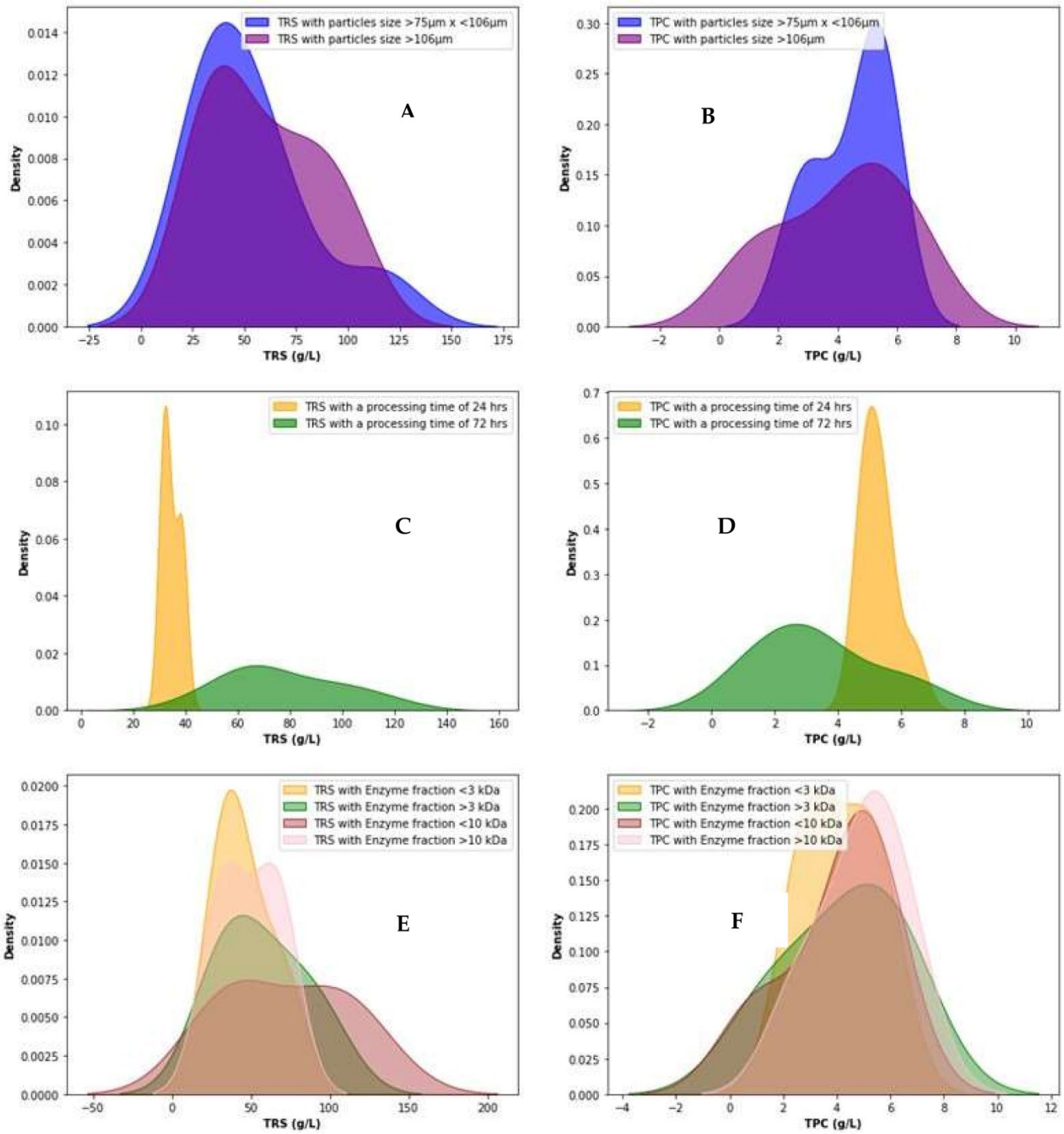


Figure 8. Probability density function of the TRS and TPC production with respect to various parametric conditions.

3.3.4. Chemical compounds and the use of pitcher fluids for agro-waste pretreatment in biorefineries

Traditional lignocellulosic biomass pretreatment methods such as chemical pretreatment methods are not environmentally friendly and can thus be classified as being unsuitable for biorefineries, which take into

cognizance the environmental burden imparted by the use of chemicals. Therefore, a green chemistry approach will be warranted in such cases, whereby a strategy can be implemented while achieving similar results in terms of producing similar hydrolysates to those obtained using chemicals, thus using a less harmful approach. Generally, the ultimate aim of the pretreatment stage in agro-waste repurposing requires that a high concentration of TRSs be produced at a low cost while preventing the loss of fermentable carbohydrates, using minimal chemical reagents and energy requirements. When using a high temperature, there is a risk of TRS decomposition, which leads to the formation of Levoglucosan, a six-carbon ring compound generated when carbohydrates are pyrolysed (Harris et al., 1985).

Acid solution hydrolysis of agro-waste targets hemicellulose, whereby the hemicelluloses are more readily hydrolysed than cellulose and lignin. As such, maize cobs containing up to 30% xylan can be easily hydrolysed to produce xylose. Others, including Hassan et al. (2016), have also demonstrated that 2.9 g/L of TRSs can be obtained using an *Aspergillus niger* facilitated decomposition of maize cobs, with up to 35 g/L TRSs production when 5.5 % (v/v) dilute sulphuric acid is used (Ayeni et al., 2020); however, this can lead to lower enzymatic hydrolysis yields downstream of the pretreatment process, suggesting the generation of toxicants (Dziekońska-Kubczak et al., 2018). Likewise, 10.26 g/L were generated from a 5% (w/v) sulphuric acid, and (3 % w/v) grape pomace. However, when the acid solution concentration was increased to 5% (w/v), the TRS content in the grape pomace hydrolysate was reduced to 8 g/L (Kurt and Cekmecelioglu, 2021), suggesting reducing sugar decomposition when high acid concentration solutions are used for grape pomace pretreatment. Furthermore, since most yard waste in the form of leaves contains a higher concentration of hemicellulose with minimal lignin, glucose and fructose will be the dominant TRSs when such waste is hydrolysed using dilute acid solutions, with traces of xylose and sucrose being observed (Nykvist, 1963). This will not be the case for orange and apple peels which have a slightly higher cellulose content than hemicellulose (Dlangamandla, 2018). For orange peels, the main reducing sugars are glucose > fructose > sucrose, albeit with a TPC content between 0.6 – 7.3 % (dry biomass wt), which can contribute significantly to the souring of a fermentation process (Tsouko et al., 2020). Additionally, apple peels generally have higher TPCs, irrespective of the source cultivar (Ergün, 2021).

Acid hydrolysis uses a high temperature exceeding 80 ˚C, albeit with a shorter pretreatment period of 30 min (see Table 9). Some of the research studies also use a single feedstock, i.e., sugarcane bagasse, rice hulls, corn stover, etc., which in terms of their management and repurposing are much simpler in composition compared to mixed waste. Some processes also use secondary pretreatment processes such as sonication and enzyme hydrolysis (Rehman et al., 2014; Chen et al., 2012). This can be mitigated by using *N. mirabilis* pitcher fluid, which is highly acidic and contains some enzymes with hydrolysis capabilities. Furthermore, reducible sugars from dilute sulphuric acid pretreatment of different wastes, includes xylose from hemicellulose (Jacobsen and Wyman, 2002). By increasing the acid concentration, the quantity of galactose in the hydrolysates can increase (Sun and Cheng, 2002). Thus, the observation

was made that 5.5 g/L galactose was formed from a 3.3% (v/v) dilute sulphuric acid pretreatment of mixed hardwoods (Park and Um, 2015). Although the individual reducible sugars had not been identified, in our previous study, it was determined that a similar mixed agro-waste hydrolysate as that obtained in this study, *S. cerevisiae*can be used for fermentation to produce ethanol. Further observations were that when *N. mirabilis* pitcher fluid was used instead of dilute sulphuric acid solution, a higher ethanolyield can be obtained (Dlangamandla et al., 2019a).

Table 9. Dilute sulphuric acid pretreatment of different lignocellulose waste under different conditions.

Pretreatment method (Dilute sulphuric acid)	Waste type	Secondary treatment method	Temp. (˚C)	Time (min)	TRSs (type/conc.)	TPCs (type/conc.)	Reference
Acid hydrolysis (2% v/v)	Sugarcane bagasse	-	122	60	Xylose (19.1 g/L) Arabinose (2.2 g/L)	-	Martín et al. (2007)
	Rice hulls	-	122		Glucose (33, 5 g/L)	-	
Acid hydrolysis (5.5 % v/v)	Corn stover	-	100	300	Xylose (18.73 g/L) Glucose (6.64 g/L)	Furfural (0.63 g/L)	Lu et al. (2008)
Acid hydrolysis (0.75% v/v)	Corn stover	Cellulases	150	30	Glucose and xylose (0.50 g/g)	n/d	Chen et al. (2012)
Acid hydrolysis (10% v/v)	Rice straw	Sonication	80	50	TRS (0.32 g/g)	-	Rehman et al. (2014)
Acid hydrolysis (3.3% v/v)	Mixed hardwoods	-	130	50.2	Galactose (5.5 g/L)	-	Park and Um (2015)
Acid hydrolysis (1% v/v)	Sorghum	-	121	120	Xylobiose (18.02 mg/g) Xylose (225 mg/g)	Furfural (4.6 mg/g)	Deshavath et al. (2017)
Acid hydrolysis (4% v/v)	Teft straw	-	120	55	TRS (26.65 mg/g)	-	Tesfaw and Tizazu (2021)
Acid/enzyme hydrolysis (100% pitcher fluid) > 10 kDA fraction	**Mixed agro-waste**	-	**25 – 30 (Ambient)**	**4320**	**TRSs (97 g/L)**	**TPCs (1 g/L)**	**This study**

Overall, the *N. mirabilis* pitcher fluid with its cocktail of enzymes has demonstrated an ability to decompose the components of mixed agro-waste. For biorefineries, and other similar sustainable energy process systems, it is imperative that the use of pitcher fluids be assessed to ascertain whether applicability on a large scale is feasible. Furthermore, a trade-off between using ambient conditions and high temperature must be further investigated to determine which of the processes might be financially beneficial while taking into cognizance the environmental impact of chemical use for agro-waste pretreatment.

3.4. Summary

In this study, the pretreatment of mixed agro-waste was performed using *N. mirabilis* pitcher fluid, whereby the agro-waste was initially milled, screened into sizes > 75 µm x < 106 µm and > 106 µm, and subsequently pretreated with fractionated (< 3 kDa, > 3 kDa, < 10 kDa, > 10 kDa) pitcher fluid. The best performing fraction was < 10 kDa, with hydrolysates pretreated with this fraction having a higher TRS load, with significantly fewer TPCs. This fraction was further analysed using LC-MS/MS to identify the enzymes contained therein, as it also had significantly better physico-chemical characteristics than the other fractions studied. Putative peroxidase 27, β-1,3-glucanase, class III and IV chitinases, thaumatin like protein, aspartic protease nepenthesin I, and purple acid phosphatase were identified as being in the < 10 kDa fraction. From the investigation, it was concluded that *N. mirabilis* digestive fluid is indeed made up of a cocktail of digestive/hydrolytic enzymes that are capable of pretreating milled agro-waste into a significant quantity of fermentable sugars. It is therefore prudent to suggest that the pitcher fluids were able to decouple some carbohydrates into TRS under ambient conditions, limiting the accumulation of TPCs within the hydrolysates. The maximum formed was 97 g/L (TRSs) and 1 g/L (TPCs).

This study is relevant to the bioenergy industry as it provides an alternative biomass pretreatment method. This green chemistry method of agro-waste pretreatment can be considered to be eco-friendly and cost-effective. It is recommended that 1) further feasibility studies be undertaken, to assess implementation at a large scale; 2) optimisation to increase TRSs while reducing TPCs;and 3) identifying and quantifying the individual reducible sugars and the type of toxicants in the hydrolysates from *N. mirabilis* pretreated agro-waste.

3.5. Recommendations

It is recommended that the following be considered in future studies:

- To determine the different enzymes contained within the individual pitcher fluid fractions assessed to ascertain the reasons the < 10 kDa fraction performed better than other fractions.
- Since TRSs reported herein constitute a measure of all reducing sugars within the hydrolysates from pretreated agro-waste, the concentration of the individual reducing sugars could be quantified.

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CHAPTER 3: SUPPLEMENTARY

S3.1. Introduction

Enzymes are life's great facilitators. They are protein constituents synthesized by living organisms, facilitating indefinite metabolic and biochemical reactions. Enzymes have been used for centuries to produce food and beverages and even in waste treatment. Further, in the last century, the biocatalytic activity of enzymes has been exploited to include usage in biorefineries for biofuel production, therefore renewable energy constituents are used in fuels. Some enzymes from various bacteria are known to degrade recalcitrant polymeric substances by hydrolytic action. The main end-products in most cases are fermentable hydrolysates, i.e., disaccharides and monosaccharides, which can be fermented to produce fuel additives for energy generation. In general, enzyme engineering also can enhance enzyme efficacy and increase the number of biotransformation products available.

The enzymes found in the acidic fluid of pitcher plants can therefore be utilized. Recent studies have clarified some previously unknown information, indicating that a diverse and complex enzymatic cocktail does exit with a high concentration of digestive/hydrolytic enzymes from within the pods (monkey cups) of *Nepenthes* species. The type and activity of the digestive enzymes – including whether they can be used in the bio-delignification process of lignocellulosic biomass, and agro-waste for a biorefinery –are still unclear (Afolalu et al., 2021). Note,however, thatfractionation of such a cocktail of hydrolytic enzymes might be necessary.

Fractionation is a separation procedure used for the division of mixtures into several smaller quantities (fractions) based on differences in specific properties of the individual fractions. Fractionation makes it possible to isolate more than two components in a mixture in a single run (Bazile et al., 2015; Johnson and Bock, 1974). It has been proven that enzymes in the pod juice of the *Nepenthes* species are capable of degrading plant litter in the environment in which they grow; however, it is not clear which fraction within the digestive pod juice is responsible for this.

There is abundant agro-waste that is continuously being dumped into the environment by various industries. Agro-processing industries are among the top commercial and significant agro-economic conglomerates globally, producing a diverse quantity of lignin-holocellulose-containing waste. After waste generation, the inappropriate disposal practices of agricultural-based wastes that contain mostly peels, seeds, and stems, which are the main constituents of holocellulose, can lead to environmental pollution with landfilling being potentially harmful to human and animal health. These agro-waste residues are considered the cheapest and most copious organic waste that can be easily transformed to value-added products as they are mostly made up of holocellulosic material which is considered a fundamental source of fermentable sugars for the manufacturing of value-added products (Kim et al.,

2011), and fuel additives for energy generation. The deficiency of traditional sources of energy to meet the ever-increasing needs of people has led to researchers discovering other potentials for harnessing energy from numerous other renewable resources, including agro-waste.

Some research has been done on the effect of biological pretreatment of milled agro-waste to fermentable sugar and then into fuel or additives in biorefineries. This research focused on the effect of biological pretreatment of a defined particle size milled mixed agro-waste using plant digestive enzymes found in *N. mirabilis* pods for the extraction of fermentable sugars with minimal phenolic compound formation, or conversion as reported elsewhere (Dlangamandla et al., 2019), which are known to be inhibitory for fermenters in fermentation systems.

S3.2. Materials and methods: Overview

S3.2.1. Collection and preparation of the mixed agro-waste

Agro-waste comprised of pomace from *Vitis vinifera* (grape) fruit, cobs from *Zea mays*, *Malus domestica* (apple) peels, *Quercus robur* (oak) yard waste, and *Citrus sinensis* (orange) peels were sourced from marketplaces around the neighbourhood and the garden of the Cape Peninsula University of Technology (CPUT), District 6 campus (Western Cape Province, Cape Town, SA). After drying all the waste at 80°C for 24 h, *C. sinensis* peels were re-dried for a further 48 h. The individual agro-waste was milled and screened to a >75 µm x< 106 µm size without a preliminary rinsing step. These agro-waste were mixed in equal proportions of a 1:1 ratio for each: 1 g orange peels, 1 g apple peels, 1 g maize cobs, 1 g grape pomace, and 1 g oak plant yard litter.

S3.2.2. Collection and characterization of the *N. mirabilis* juice

N. mirabilis plants can grow in a greenhouse under regulated conditions. The plants selected were grown at Pan's Carnivores Plant Nursery (21 Kirstenhof, Tokai, Cape Town, SA). The plants' digestive juice from individual pods was collected into sterile 50 mL conical tubes and immediately stored in ice, before transportation to the laboratory. In the laboratory, the plants' digestive juice was centrifuged at 4000 x g for 15 min and filter sterilized with a 0.22 µm Millipore membrane filter with subsequent pooling to make a single batch and subsequently stored at -20 °C before use, i.e., without dilution or the use of a buffer. Approximately 10 to 40 mL of the digestive juice was collected per pod, i.e., monkey cup, of the *N. mirabilis* plants, a volume which was dependent on the size of the individual cups. The specific gravity, pH, redox potential, and conductivity which are the physico-chemical properties of the *N. mirabilis* digestive pod juices, were measured by making use of a multi-parameter instrument (Eutech Instruments Pty Ltd, Thermo Fisher Scientific, Singapore).

Some factors can promote the functionality of enzymes even when in a cocktail, with trace elements being one of the solutions which can facilitate enzyme co-factor availability, thus enhancing the digestive juices' performance.

S3.2.3. Trace element stock solution preparation

Trace elements are essential in small amounts for the biological functioning of enzymes as co-factors. Some metallic ions such as iron and copper contribute to oxidation-reduction reactions in biomass breakdown (Šelih et al., 2007). The trace element solution used was prepared by dissolving 1.5 g of nitrilotriacetate in 800 mL sterile distilled water. Thereafter, the pH was adjusted to 6.5 by using 1M KOH (8 g/500 mL). The following compounds, i.e., MgSO4 (3 g), MnSO4 (0.5 g), NaCl (1 g), FeSO4.7H2O (0.1 g), CoCl2 (0.1 g), $ZnSO_4.7H_2O$ (0.1 g), $CuSO_4$ (0.1 g), $AlK(SO_2)_2.12H_2O$ (0.01 g), H_3BO_3 (0.01 g), $Na_2MnO_4.2H_2O$ (0.01 g), $MgSO_4.7H_2O$ (6.14 g), $MnSO_4.H_2O$ (0.56 g), $CoCl_2.6H_2O$ (0.187 g), were added to the solution and the solution was made up to 1000 mL. The solution was filter sterilized using a 0.22 µm filter and autoclaved. It was then stored at 4 °C before use. The colour of the solution was light yellow.

S3.2.4. Enzyme-facilitated hydrolysis of the mixed agro-waste

It has been found that plant cocktails from carnivorous pitcher plants of the genus Nepenthes also constitute several bioactive substances as a result of catching insects which are digested in the pitcher fluid with the help of the special features of the pitcher traps. Though pitcher fluid has been known for many decades, our awareness of the pitcher fluid components is quite narrow (Mithöfer 2011; Angadam, Dlangamandla, et al. 2019;Ntwampe, et al. 2019). These *Nepenthes* sp. are insectivorous by nature, and they are characterised by their specialized traps;hence the name pitcher, which traps prey and breaks down insects that have a perceived indestructible exoskeleton, thus the monkey cups are filled with protein (Lee et al., 2016).

A mass (0.5 g) of the mixed agro-waste was weighed into each 100 mL Schott bottle and a volume (10 mL) of the individual fractions was added to each Schott bottle. Thereafter, the trace element solution (0.1 mL) was added as a supplement. The mixed agro-waste and the digestive juices were mixed by swirling in a shaking incubator at a temperature maintained at 25-30 °C, to mimic ambient temperature. Sampling was done at 24 h intervals and the samples were centrifuged at 4000 x g for 10 min. The supernatant collected was analyzed for total reducible sugars (TRSs) using a dinitrosalicylic acid (DNS) assay protocol. Phenolic content, which is known as an inhibitory compound to enzymes such as β-glucosidase in the degradation of polysaccharides to simpler sugars, was quantified using the Folin-Ciocalteu assay protocol.The depiction of the experimental process is highlighted in Figure 9.

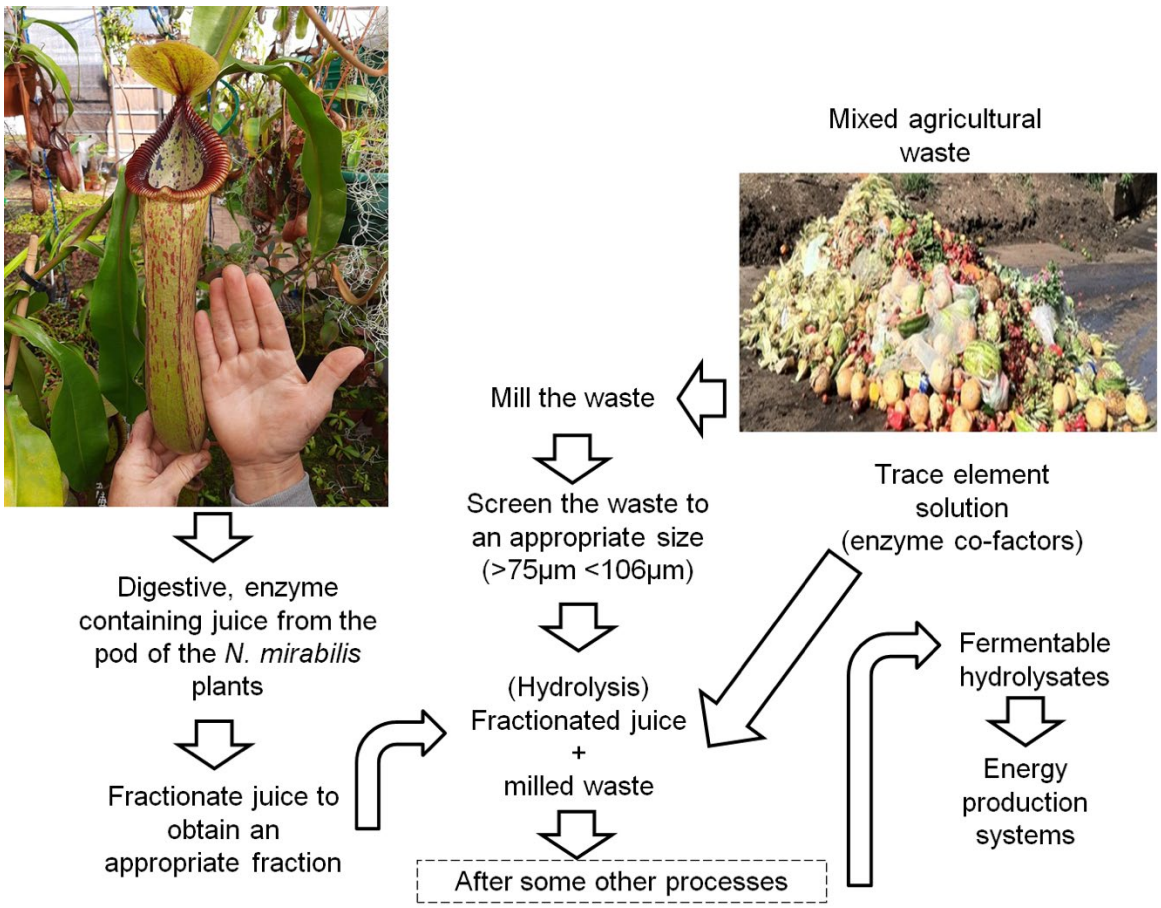

Figure 9. Experimental process undertaken for the study.

S3.3. Results and discussion: Initial observations

The specific gravity, pH, redox potential, and conductivity, i.e., the physico-chemical properties of the fresh *N. mirabilis* digestive juice collected, are listed in Table 10. Although *N. mirabilis* digestive juice contains a cocktail of enzymes, its properties are similar to those of 1% (v/v) dilute sulphuric acid solution, although with a higher redox potential (Angadam et al., 2021). This is a trait associated with the ability of a mixture to facilitate reduction-oxidation reactions. Overall, *N. mirabilis* plants are categorized by their monkey cups which can attract and trap insects and break down their exoskeletons, via a digestive, acidic enzyme-facilitated process. This is due to the acidic nature of the *N. mirabilis* pod juices, which also facilitate leaf litter digestion for the plant's nutrition (Moran et al., 2003). Hence the notion that such enzyme-containing acidic juices can be used to pretreat mixed agro-waste for TRS production from a variety of lignocellulose-containing biomass is valid.

Table 10. Physico-chemical properties of *N. mirabilis* digestive juice without fractionation.

Factors	Values (units)
pH	2.0-2.09
Specific gravity (S.g)	0.73-0.81
Redox potential (ORP)	501-520 mV
Conductivity (CO)	3.86-4.93mS/cm

Overall, the <10 kDa fraction was observed to have the best physico-chemical properties as highlighted in Table 11, which led to it performing better in terms of agro-waste pretreatment for TRS production and total residual phenolic compound (TRPC) reduction in preliminary studies.

Table 11. Physico-chemical properties of the different fractions of *N. mirabilis* extract.

Factors	< 3 kDa	> 3 kDa	<10kDa	>10kDa
pH	2.04	2.02	**2.00**	2.06
SG	0.73	0.73	**0.81**	0.80
ORP	503	501	**510**	511
CO	3.91	3.86	**4.93**	3.97

Overall, the suitable conditions were such that, a particle size of >106 µm, using a contact time of 72 h and an enzyme fraction of < 10 kDa produced the highest amount of TRSs with minimized TRPCs, animprovement over other agro-waste pretreatment methods, i.e., without the use of inorganic acids. Note though that the mixed agro-waste was milled. Others have observed a higher yield of TRS production using dilute inorganic acids, albeit with a higher TRPC load in the hydrolysates (Raza et al., 2019). This was a confirmation of the hypothesis that during pretreatment of agro-waste, there can be a reduction of inhibitory compounds by pre-treating the agro-waste with plant-based digestive juices from pitcher plants.

It has been reported that harsh delignification methods, based on chemical pretreatment methods, are inhibitory to subsequent cellulase-/enzyme-facilitated hydrolysis and fermentation to produce value-added products destined for renewable energy generation (Cheng et al., 2012). Chemical pretreatment

methods can be categorized as inappropriate for a less energy-intensive biorefinery, and the use of a green chemistry approach will lessen the burden on the environment and thus environmental impact for bio-refineries. Overall, a well-developed green pretreatment system will facilitate ligninolysis, and consequently show holocelluloses to be the most successful hydrolysis of agro-waste with negligible energy intake (Nitsos et al., 2019; Limayem and Ricke, 2012). For biorefineries, assessment of environmental impact based on final products achieved from known mass and energy input is important. For example, the use of steam in a biorefinery was also recently reported to harm human health (Hosseinzadeh-Bandbafha et al., 2022), and is largely recommended for biomass delignification on a large scale.

Biodegradation activity of enzymes, namely carboxylesterases, β-glucosidases, and xylanases was conducted, based on their ability to decompose the agro-waste components forming by-products. It was earlier acknowledged as being an indispensable cellulose biodegrading component aiding the transformation of cellobiose, which is a reducing sugar, to glucose; additionally, they are known for their abilities to biodegrade hemicellulose (Manavalan et al., 2017; Liuet al. 2017;Hong et al. 2016) and again, they hydrolyse the bonds between holocellulose sugars and lignin. All test assays were mixed in tubes; thereafter, they were transferred into glass cuvettes to read absorbance in a kinetic mode. All reaction was performed at 25°C, with some studies reporting the influence of fraction components in the fluids, to identify the most potent fraction.

Enzyme fractionation is a separation technique that is used to disassociate a mixture or suspension, etc., into smaller fractions in which the composition varies in weight or gradient. For example, tangential flow filtration (TFF) is a speedy and effective technique for the separation and purification of enzymes. This is done by basically connecting the TFF device to a pump and pressure gauge(s) with tubing and fittings. The process is easy, quick, and above all, it accomplishes greater concentrations in less time as compared to techniques such as dialysis, centrifugal devices, or stirred cells (Schwartz and Seeley, 2002). However, recent methods include centrifugation tubes with membranes. Overall, different plant exudates from different parts of plants including enzyme cocktails used in prey traps can be a source of several useful enzymes which can be exploited for lignin decomposition (delignification) and cellulose, including hemicellulose decomposition (holocellulolysis); albeit, under limited conditions, thus the concept of semi-delignino-holocellulolysis.

S3.4. Summary

Exploration for novel scientifically viable plant enzymes is never-ending, and carnivorous plants have shown to be a valuable resource for ongoing research in various fields. In this study, a fractionated portion of *N. mirabilis* extract were used to pre-treat mixed agrowaste of a particular particle size for the breaking down of holocelluloses to produce fermentable sugars and reduction of phenolic compounds thereof. The technique of enzymes fractionation helps to separate enzymes in solution according to their

molecular weight. The findings revealed a significant production of fermentable sugars and a substantial reduction in the inhibitory compound produced, i.e., phenolics. Consequently, using different fractions of *N. mirabilis* digestive juices to pre-treat mixed agrowaste of a particular particle size, has the potential to produce fermentable sugars and subsequently other value-added products, a strategy that can be profitable in biorefineries.

S3.5. References

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CHAPTER 4

NEPENTHESMIRABILIS PITCHER FLUID FUNCTIONALITY FOR AGRO-WASTE PRETREATMENT: EFFECT OF PH, TEMPERATURE, TRACE ELEMENT SOLUTION AND THE PORE SIZE OF THE WASTE

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CHAPTER 4
NEPENTHES MIRABILIS PITCHER FLUID FUNCTIONALITY FOR AGRO-WASTE PRETREATMENT: EFFECT OF PH, TEMPERATURE, TRACE ELEMENT SOLUTION AND THE PORE SIZE OF THE WASTE

4.1. Introduction

Nepenthes spp. have monkey cups which are filled with an acidic enzyme containing fluid, henceforth referred to as pitcher fluid, capable of degrading leaf litter and insects into useable nutrients which then serve as a source of energy for the plants' well-being and reproduction. Pitcher fluids produced by *Nepenthes* spp. have distinctive characteristics suited for agro-waste pretreatment. Since the pitcher fluid is highly acidic, i.e., with a pH range of 1.8 – 2 (Takeuchi, et al, 2011) it can be responsible for the delignification and decoupling of cellulose and hemicellulose chains into fermentable sugars. A handful of researchers have reported on the constituents of the fluids from a variety of *Nepenthes* spp. However, there is still insignificant evidence to suggest that such acidic fluid can be applied for biomass pretreatment at a large scale in biorefineries (Dlangamandla, et al., 2019; Ravee, et al., 2018; Lee, et al. 2016). Additionally, current investigations have proven that the pitcher fluid does have lignin-holocellulose hydrolyzing enzymes (Angadam, et al., 2021); however, their functionality outside of the monkey cups can be enhanced by varying environmental conditions to suit large-scale usage and efficacy. These environmental conditions or factors include but are not limited to pH, temperature, and a co-factor (trace element) solution.

Pretreatment of lignocellulose waste is generally influenced by pH, temperature, and holding times (Pedersen and Meyer, 2010). Generally, there is a direct impact on hydrolysate quality if the conditions are unsuitable. In general, preparation of fermentable hydrolysates, pretreatment of waste feedstock, pH, temperature, and to some extent trace elements in varying proportions, can produce hydrolysates that enhance a fermenter's performance and thus efficacy. Some researchers suggest that when there is lignocellulosic waste, a pH of 2 and a temperature of 33°C provide adequate conditions for pretreatment. Using *Nepenthes mirabilis* pitcher fluid with a pH range of 2 to 2.9 under ambient temperature conditions (15 to 25°C or up to 30°C) can satisfy these requirements. When pretreatment of agro-waste is performed using pitcher fluids which contain a cocktail of enzymes such as those from *Nepenthes* sp., it can be

advantageous for processes that focus on eco-friendliness, without the use of chemicals. Pretreating agro-waste at lower pH improves the solubilization of hemicellulose (Carvalheiro, et al., 2008).

Since it was determined that *N. mirabilis* pitcher fluids contain enzymes, such as β-1,3-glucanase, putative peroxidase 27, and thaumatin-like protein among others, their functionality must be determined by focusing on pH, temperature, and co-factors in the form of trace elements. Some enzymes were determined to be stimulated by Ca^{2+}, Mg^{2+}, and Mn^{2+} (Oumer and Abate, 2017). There is a suggestion that metal salts, including compounds such as EDTA, can improve the activity of some enzymes, among which endoglucanase, exoglucanase, and β-glucosidase, which are some of the important enzymes in agro-waste pretreatment. Those associated with *N. mirabilis*, such as purple acid phosphate, do have improved functionality under acidic pH while having no specific co-factor preference. However, supplementing a minute concentration of Mg^{2+}, Ca^{2+}, and Mn^{2+} to class IV chitinase, does increase the enzyme's activity (Cheba, et al., 2016). These considerations motivated the exploration of using a trace element solution. Therefore, the interlinkages between pH, temperature, and a trace element solution during milled agro-waste pretreatment as used in this study, using *N. mirabilis* pitcher fluid, must be assessed.

Another aspect that needs to be assessed is the relation between the porosity of the mixed agro-waste and the enzyme fraction (< 10 kDa) determined to be suitable for the pretreatment of the milled mixed agro-waste used in this study. This is because, if the pore size of the milled agro-waste is large enough in comparison to the size of the enzymes, the enzymes in the pitcher fluid can adequately be embedded in the pores of the agro-waste, thereby improving the yield of targeted products, in particular, total reducible sugars, thus improving the pretreatment process. This linkage has not been studied before.

Therefore, the aim of this study was to 1) assess the influence of pH, temperature, and trace element solution on the pretreatment of the milled agro-waste using *N. mirabilis* pitcher fluids, and 2) to assess the link between the porosity of the used agro-waste to the fraction of the pitcher fluid enzymes. Previously, it was determined that 72h holding time, a particle size of > 106 µm, and an enzyme fraction of < 10 kDa resulted in the highest production of total reducible sugars while total phenolic compounds were low. Therefore, these conditions were adopted for part of this study, specifically assessing total reducible sugars.

4.2. Materials and methods

4.2.1. Collection and processing of the mixed agro-waste

Mixed agro-waste containing *Malus domestica* (apple) peels, *Quercus robur* (oak) yard waste, *Citrus sinensis* (orange) peels, *Vitis vinifera* (grape) pomace, and cobs from *Zea mays* (maize), as previously

used, were collected from a fruit and vegetable store in Zonnebloem, Cape Town, South Africa, with *Quercus robur* (oak) yard waste (leaves) being collected from the campus of the Cape Peninsula University of Technology (CPUT), District 6 campus (Cape Town, Western Cape Province, SA). Following mixing with a pre-rinsing step, the agro-wastes were initially dried at 80°C for 24 h. This was extended to 72 h due to the adhesive nature of the *C. sinensis* peels. Following this, pulverisation ensued, followed by screening to obtain a > 106 µm particle size. It was demonstrated that mixing the agro-waste is cost-effective and produces better quality hydrolysates than single feedstocks (Oke, et al., 2016). As in previous experiments, 20% (w/w), i.e., 10 g per agro-waste, was used.

4.2.2. Collection, and sample fractionation of the *N. mirabilis* pitcher fluid

The *N. mirabilis* pitcher fluids were collected from plants cultivated at Pan's Carnivores Plant Nursery (21 Kirstenhof, Tokai, Cape Town, SA) and stored in ice. To remove debris, a centrifugation regime was applied whereby conditions were 4000 x g for 15 min with subsequent filtration at 0.22 µm using Millipore membrane filters (Merck, Burlington, MA, USA). To obtain the < 10 kDa, a filter with size <10 kDa was used, with the filtrate being used for the experiments. This was done using a centrifuge at 4000 x g for 10 min. Thereafter the filtrate was stored at -20 °C which was determined to be an adequate temperature before use in the experiments. After thawing, the filtrate was used as is.

4.2.3. Trace element solution preparation for pretreatment supplementation

The trace element solution used in this study was prepared as previously reported using analytical grade chemicals, i.e., by dissolving 1.5 g of nitrilotriacetate in 800 mL sterile distilled water with the pH being adjusted to 6.5 using 1M KOH (8 g/500 mL). Thereafter, the following compounds were added to the nitrilotriacetate: $ZnSO_4.7H_2O$ (0.1 g), $FeSO_4.7H_2O$ (0.1 g), $MgSO_4$ (3 g), $MnSO_4$ (0.5 g), NaCl (1 g), $CuSO_4$ (0.1 g), $AlK(SO_2)_2.12H_2O$ (0.01 g), H_3BO_3 (0.01 g), $Na_2MnO_4.2H_2O$ (0.01 g), $MgSO_4.7H_2O$ (6.14 g), $MnSO_4.H_2O$ (0.56 g), $CoCl_2.6H_2O$ (0.187 g) and $CoCl_2$ (0.1 g), with the solution being made up to 1000 mL (Tien and Kirk, 1988). The solution was filter-sterilised using a 0.22 µm filter and autoclaved. It was then stored at 4 °C before use, as previously elucidated.

4.2.4. Conditions for pitcher fluid facilitated mixed agro-waste pretreatment

The agro-waste was slurried at 5% (w/v) in 100 mL Schott bottles with 10 mL of the < 10 kDa pitcher fluid of the slurry being used. Subsequently, the pH was adjusted using either HCl or KOH to attain the

required pH of the slurry. The variation in the volume of the trace element solution used is shown in Table 12. Furthermore, a shaking (120 rpm) incubator (LABWIT ZWY-240, Shanghai Zhicheng Analytical, Shanghai, China) was used to vary the temperature. Sampling (3 mL) was done only at 72 h as this was the incubation time previously determined to be suitable to produce the highest total reducible sugars. All samples collected were centrifuged at 4000 x g for 10 min and the debris-free supernatant was used to quantify the total reducible sugars. Table 12 indicates the experimental conditions used per run (n = 7). The values for the individual parameters were analogous to those generated by the response surface methodology. All the runs were done in triplicate.

Table 12. Experimental runs used for this part of the study.

Runs	pH	Temperature (°C)	Trace element solution (µL)
1	12	25	50
2	10	16	-
3	2	16	-
4	2	16	100
5	6	25	50
6	2	33	-
7	10	16	100

4.2.5. Quantification of total reducible sugars from agro-waste pretreatment hydrolysates

Miller's (1959) dinitrosalicylic acid (DNS) assay protocol was used to determine the concentration of total reducible sugars. The basis of the method is such that DNS is reduced to 3-amino-5-nitrosalicylic acid, although different sugars yield different colours. Since the measurements in this study are for total reducible sugars in a mixture, it was expected the colorimetric measurements would be consistent. A Jenway 7305 UV/Vis spectrophotometer (Cole–Parmer, Staffordshire, UK) was used for this assay. The calibration curve correlation coefficient (R^2) was 0.95. All measurements were done in triplicate and the averages were used in data analysis.

4.2.6. Determination of the milled agro-waste porosity using the Brunauer-Emmett-Teller (BET) method

The surface area and pore properties of the samples were evaluated on a Micromeritics 3Flex adsorption analyser (supplied by Poretech, Roodepoort, South Africa), using N_2 as an adsorbate, with a bath temperature of -196.9 ºC, with a sample mass of 1.70g of the agro-waste at equilibrium intervals of 15 s.

The analysis free and ambient free space was 48 and 15 cm^3 respectively. Before sample analysis, all the samples were dried in a vacuum oven at 105 ºC for 3 h, before a representative of the samples was loaded into the sample tubes. Degassing of the samples was then conducted following the method described elsewhere. Briefly, the samples were degassed at 300 ºC for 12 h and further at 45 ºC for another 12 h before switching to low-pressure gas adsorption (LPGA) analysis, using both N_2 and CO_2 as adsorptive gases. The LPGA was conducted at –196 ºC in liquid N_2 for N_2 adsorption, and 0 ºC in an ice–water bath for CO_2 adsorption. Adsorption isotherm data were captured automatically from the equipment and analyzed on the Micrometrics Microactive v5.02 platform. Details of the data processing and surface area and pore property evaluations are given in Okolo et al. (2015).

4.3. Results and Discussion

4.3.1. Pretreatment of the agro-waste using *N. mirabilis* under different pH, temperature, and trace element solution conditions

Alkaline pretreatment has been said to be the most efficient pretreatment strategy as it rapidly solubilises lignin. However, this type of pretreatment strategy might be suited to pretreat agro-waste with high lignin content. Dlangamandla et al. (2019) have previously determined that the agro-waste used in this study has a lignin content of 27%, while holocellulose accounted for 73%. This distribution in constituents between both lignin and holocellulose suggests that alkaline pretreatment might be unsuitable. Additionally, the impression that corn cob has a higher lignin content because it is hardy is misleading. Orange and apple peels and grape pomace have a higher lignin content (Ayala, et al.,2021; Guardia, et al., 2019), which is greater by 5 to 6% when compared to that of corn cob. Similarly, the cellulose content of orange peel and grape pomace is higher than that of corn cob and apple peel. In this study, the observation was that a pH of 2 at below ambient temperature produces a higher concentration of total reducible sugars than when the temperature is higher (> 25 ºC), and particularly at 33 ºC. It was expected that a lower pH and a high temperature (33 ºC) would improve reactivity between the *N. mirabilis* pitcher fluid and the agro-waste. A higher temperature would likely have deactivated the constituents in the pitcher fluid. Even though alkaline (with a high pH) pretreatment is reportedly an efficient pretreatment method, low total reducible yields were at pH 10 to 12. Table 13 further indicates that the supplementation of the pretreatment regime with a trace element solution had minimal influence on the overall yield of total reducible sugars.

Table 13. Production of total reducible sugars under varying pH, temperature, and trace element solution.

Runs	pH	Temperature (ºC)	Trace element solution (µL)	Total reducible sugars (g/L)
1	12	25	50	65.00
2	10	16	-	231.25
3	**2**	**16**	**-**	**407.5**
4	**2**	**16**	**100**	**407.02**
5	6	25	50	148.75
6	**2**	**33**	**-**	**141.88**
7	10	16	100	202.50

This might suggest that the *N. mirabilis* pitcher fluid might contain sufficient metallic ions, thus supplementing the pitcher fluid with additional metallic ions, might have somehow inhibited the functioning of the cocktail of enzymes contained therein. Therefore, further exploratory studies are needed to ascertain the metal ion content of the pitcher fluid.

4.3.2. Correlation between the milled mixed agrowaste porosity and pitcher fluid efficacy

Researchers report that preparatory methods increase the porosity of the agro-waste (Peguero, et al., 2022), which then enhances enzyme accessibility to some extent. This is achieved by using numerous techniques, some of which are physical, e.g., milling as used in this study. Increasing the porosity of the agro-waste, in turn, increases the specific surface area. As such, agro-waste morphology including roughness, if not managed properly, can influence the hydrolysis of the waste and contribute to impediments to total reducible sugars and usable hydrolysate production. It is suggested that if a highly porous agro-waste is used as feedstock, low enzyme loading or quantities can be utilised (Kumar, et al., 2011). There is also a correlation between the size (kDa) and shape of the enzyme and the average pore size (nm) to which the enzyme can attach itself in the agro-waste. Such a correlation is hardly ever reported. It is estimated that the following enzyme sizes are associated with a minimum pore diameter, i.e., 5 kDa (2.2 nm), 10 kDa (2.84 nm), 20 kDa (3.56 nm), 50 kDa (4.8 nm), 100 kDa (6.1 nm), 200 kDa (7.68 nm), 500 kDa (10.42 nm) (Erickson, 2009) with 10 angstroms (Å), a unit measurement from BET, being equivalent to 1 nm.

Since < 10 kDa (associate size of 2.84 nm) pitcher fluid fraction was identified as being suitable to pretreat the milled agro-waste (> 106 µm) used in this study, its average pore size of 2.84 nm (28.4 Å) would thus also explain why the fraction performed satisfactorily, as it also contains a cocktail of enzymes associated with agro-waste decomposition. Table 14 lists pore diameter values, while Figure 10

represents the isotherm plot from the BET measurements. Additionally, the BET surface area (see Figure 11) for the >106 µm agro-waste was determined to be 0,6458 m^2/g.

Table 14. Adsorption and desorption pore diameter.

Parameter	Value (Å)	Value (nm)	Estimated kDa
Adsorption average pore diameter	79.90 - 76.91	7.99 - 7.68	200
Desorption average pore diameter	71.77 - 71.46	7.18 - 7.15	≤ 200

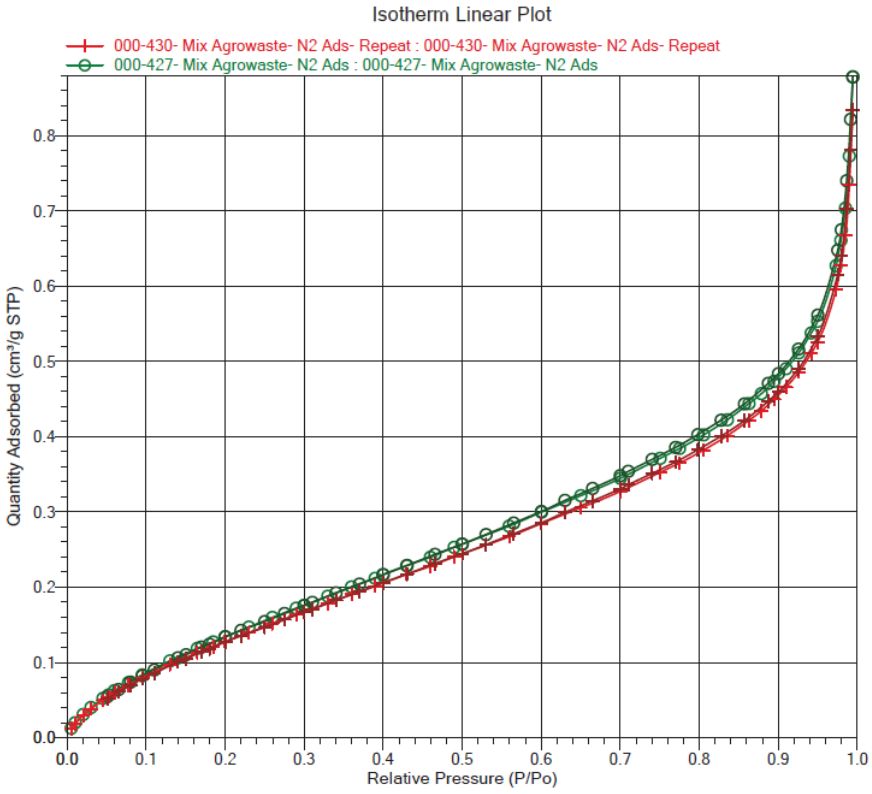

Figure 10. Isotherm linear plot for the milled agro-waste.

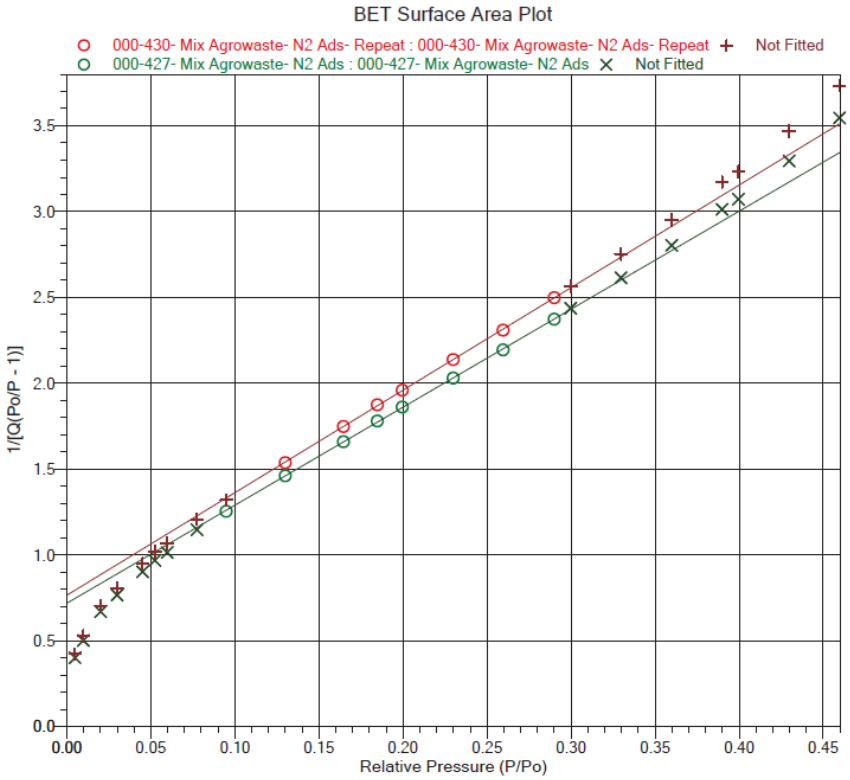

Figure 11. The BET surface area plot for the milled agro-waste.

In most instances, the preparation of agro-waste when using milling as in this study increases its porosity and surface area (Meng, et al., 2013). It was also mentioned that different preparation techniques for the agro-waste can result in variations in its structure, which can then affect hydrolysis with pore volume being strongly influential in hydrolysis (Zhang, et al., 2021), thus the production of hydrolysates with a high content of total reducible sugars. In this study, the single-point adsorption total pore volume was 0,001242 cm^3/g. Figure 12 illustrates the cumulative pore volume of the milled agro-waste used in this study. In general, terms, milling the agro-waste will result in the reduction of the mixed waste, i.e., particle size, thus increasing the pore volume as indicated elsewhere (Zoghlami and Paës, 2019). Barakat et al. (2014) have criticised the use of methods such as BET, indicating that they are not precise, especially when there are macropores in the samples. However, the BET method used herein was deemed sufficient. Although it is understood that milling the agro-waste before pretreatment with *N. mirabilis* pitcher fluid can assist in the effectiveness of hydrolysis, others advocate for wet milling (Nitsos, et al., 2019); however, this generates another waste stream.

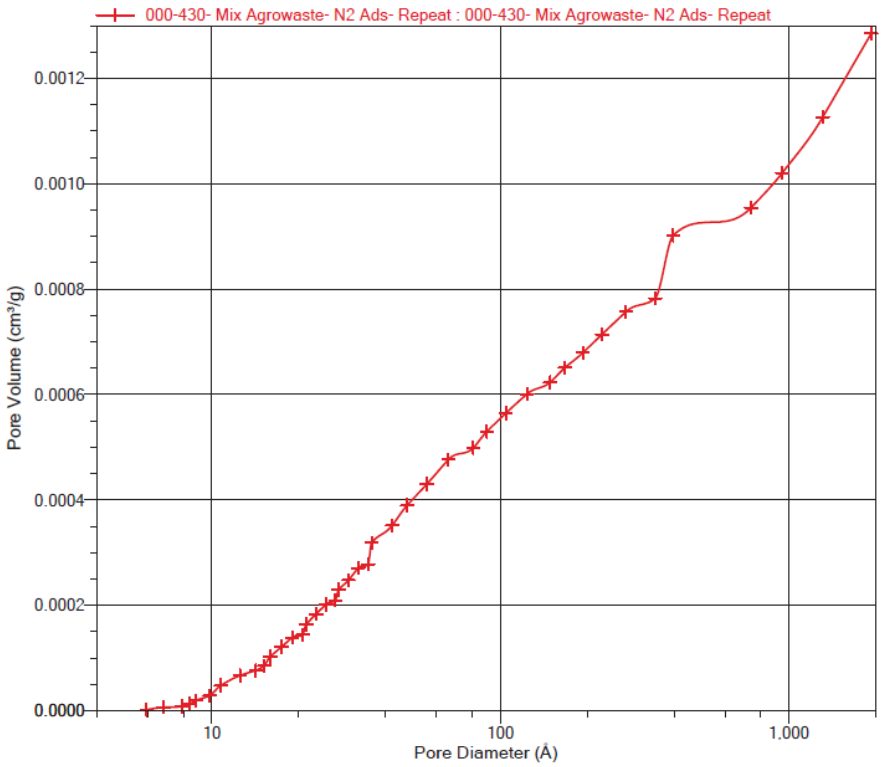

Figure 12. Cumulative pore volume of the milled agro-waste.

Agro-waste pre-treatment is presently 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. The development of alternative and environmental benign holocellulose valorisation methods for the pre-treatment of agro-waste, for the production of value-added products, while limiting the production of toxicants from the lignin component of the waste, is necessary. This will provide a new promising alternative strategy towards the sustainable and efficient processing of numerous agro-waste types (Nayak, et al., 2016; Chandrasekaran and Bahkali, 2013). In the present study, the *N. mirabilis* plant's digestive fluid was proven to be effective in targeting holocellulose extraction under optimal conditions of low pH, ambient temperature, trace element solution and porosity of agro-waste. The plant digestive fluids contained digestive enzymes which have the potential to biodegrade complex and polymeric molecules (Chan, et al., 2016). This pre-treatment method requires less energy as it was operated at ambient temperature, and it eliminates the use of hazardous chemicals such as dilute inorganic acids; however, the digestive fluid is acidic, with an added advantage of reducing the production of inhibitory compounds such as phenolics (Siragusa, et al., 2007). When a high temperature is used for agro-waste pre-treatment, there is a risk of TRSs decomposition, which leads to the formation of Levoglucosan, a six-carbon-ring compound generated when carbohydrates are pyrolysed (Harris et al., 1985). The acidic nature of the *N. mirabilis* pod juices, which also facilitate leaf litter digestion for the plant's nutrition (Moran, et al., 2003), can be used to pre-treat mixed agro-waste for TRS production from a variety of lignocellulose-containing biomass.

4.4. Summary

In this part of the study, we further demonstrated that it was prudent to utilise *N. mirabilis* pitcher fluid with a pH range of 2 – 2.09 to pretreat the milled mixed agro-waste at ambient temperature, as the highest concentration of total reducible sugars was determined to be at pH 2, albeit below ambient temperature. The < 10 kDa enzyme fraction was determined to be suitable as the pore size. In this instance, the pore diameter of the agro-waste was estimated to be 7.15 to 7.99 nm for which an enzyme size of 200 kDa could penetrate the pore of the agro-waste. The observed BET surface area of 0.6458 m^2/g was hypothesised to be suitable for effective enzyme-facilitated hydrolysis. The role of the trace element solution was non-evident, with a recommendation that metallic species in the pitcher fluid be investigated in future studies.

4.5. References

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CHAPTER 5

DATA: SUPPORTINGTHE RESEARCH THESIS OBSERVATIONS VIA ARTIFICIAL NEURAL NETWORK

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DATA: SUPPORTING THE RESEARCH THESIS OBSERVATIONS VIA ARTIFICIAL NEURAL NETWORK

5.1. Introduction

The purpose of this part of the study was to use neural networks to assess the veracity of the data generated, and perhaps for process modelling for the upscaling to pilot and large scale. The focus in this part of the study was total reducible sugars produced.

5.2. Specification information

The subject area of this part of the study is agro-waste (biomass) pretreatment, environmental engineering, health, and biotechnology. The data was acquired using bench-scale experiments. The data presented in this thesis was processed and obtained from numerous analytical methods. Experimental agents included *N. mirabilis* pitcher fluids which were sampled from Pan's Carnivores Plant Nursery (21 Kirstenhof, Tokai, Cape Town, SA). The data was generated at the Bioresource Engineering Research Group laboratory and Centre for Proteomic and Genomic Research (Cape Town, South Africa).

5.3. Value of the data

- This data provides information on using an artificial neural network (ANN) to predict the outcome of the experiments performed in this study.
- It further provides a platform for other researchers from which they can start to perform comparative studies using other pitcher fluids and optimization using other optimization software.

5.4. Data

Signifiantly, the data contains ANN prediction in comparison to experimental data – ANN topology is a function fit for the output, training, and validation, including test and actual output predictions. In this case, the output was the total reducible sugars. The ANN analysis was done using MATLAB

software neural network toolbox. Refer to Tables 20 and 21 in the appendices for statistical analysis of the data used for total reducible sugars determined at 24 and 72h.

5.5. Materials and methods

Experimental conditions are similar to those listed in section 3.2.5. Briefly, 5% (w/v) agro-waste/*N. mirabilis* slurry was prepared in Schott bottles (100 mL), whereby a trace element solution was supplemented without varying the pH and ambient temperature conditions used. The Schott bottles were shaken (120 rpm) in an incubator. Total reducible sugar (as output) was quantified as per the standard DNS method, with sampling at 24 and 72 h.

5.6. Graphical outputs and ANN predictions

An ANN topology (Figure 13) interlinks the input variables (particle size/enzyme fraction) and the outputs (May et al., 2011), i.e., total reducible sugars, produced in the hydrolysates at 24 and 72 h. Weights are randomly assigned to each input variable, adjusting the input variables to the most effective range. Subsequently, in the hidden layer, the number of neurons (nodes) within the hidden layer is assigned. The neurons determine the computational operations to be performed (Alaloul and Qureshi, 2020).

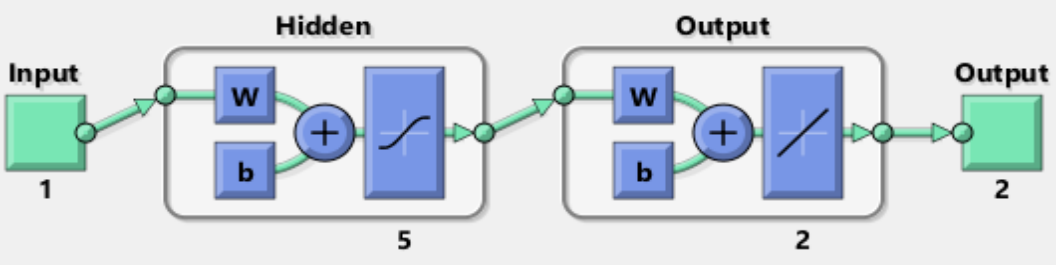

Figure 13.ANN topology (1 input, i.e., agro-waste/enzyme fraction, 5 hidden neurons, and 2 outputs, i.e., total reducible sugar concentration after 24 h and 72 h).

Since an ANN requires a learning procedure through training (Figure 14a: n = 6 samples; Figure 25: ANN train showing mean squared error and regression values and with n = 7 epoch iterations; Figure 24: the neural network training tool), which is a procedure whereby the assigned weights to individual inputs are modified (Knerr et al., 1990). In other words, the assigned weights to the inputs can either be positive (relevant), negative (ineffectual), or irrelevant. As such, the hidden layer neurons are modified, largely taking into consideration relevant weights to generate the desired output after validation (Figure 14b), i.e., such that the desired output is achieved. Epoch 3 at 0.54 was deemed optimal (Figure 15), which describes the number of cycles the training data was processed through the hidden layer (Sinha et al., 2010), with weights assigned to each input variable being varied for optimal

ANN output prediction. Thereafter, a test run was performed (Figure 14c) before generating an output (total reducible sugars, Figure 14d) at 24 and 72h in this study. Correlation coefficients were used to verify the validity of the process at individual stages (Figure 14). The training and validation had a mean square error of 4 x 10^{-2} and 5.4 x 10^{-1} including 1.2 x 10^{0} for testing (Figure 25 in the Appendices). This simply suggests how the ANN estimated expected values.

Table 15 lists particle size/enzyme fraction assigned codes (-1, -2. 1, 2, -3. -4, 3, and 4), including actual total reducible sugars in comparison with ANN predicted values (statistical analyses of the total reducible sugars are listed in Table 20 and 21, for 24 h and 72 h respectively);Figure 15 shows the validation performance with a network structure 1-5-2-2 (0.54 at epoch),including Figure 16 (a function fit for total reducible sugars) indicating errors between the ANN targeted values and the actual output values, i.e., experimental total reducible sugars. Errors for the following coded inputs were observed: 1, -1, -2, -3, and -4, meaning the ANN could not effectively predict the concentration of the total reducible sugars in the hydrolysates produced. Additionally, experimental runs coded 2, 3, and 4 had minimal errors, meaning that the ANN effectively predicted the outcomes. However, experimental runs coded 2 and 3 (experimental runs 7 and 8 – Table 22) had higher concentrations of total phenolic compounds (Figure 17, demarcated with a black arrow). Previously, experimental run 14 was deemed suitable (demarcated with a red arrow) for the pretreatment conditions of the milled agro-waste used, as observed in Chapter 3. Therefore, experimental run 12 (code 2, > 106 µm/> 3 kDa) can be further assessed, as the > 3 kDa is a purified fraction of the < 10 kDa fraction, and the conditions of the experimental run are similar resulting in a similar profile of total reducible sugar production, with a low total phenolic content (see Table 6 (Chapter 3) and Figure 17).

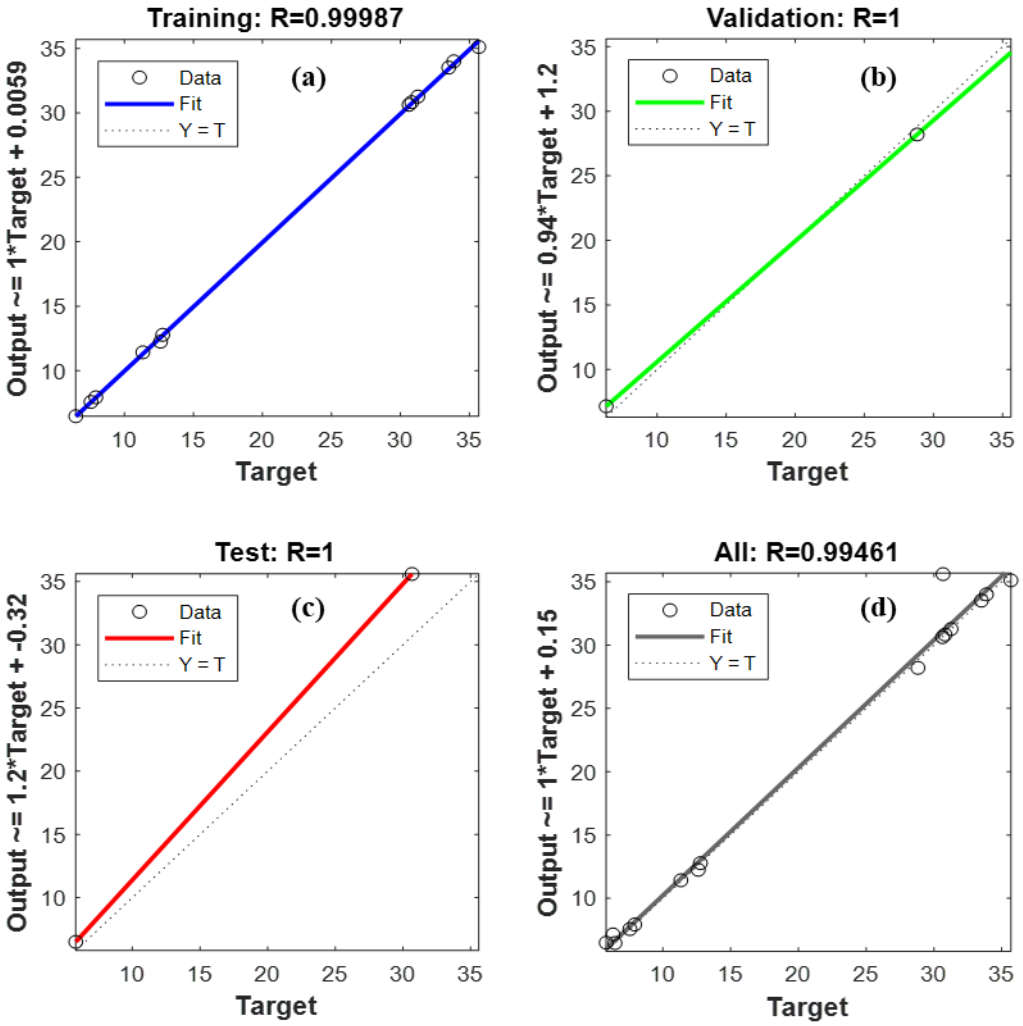

Figure 14. Training**(a)**, validation **(b)**, test run**(c)** and actual output **(d)**profiles.

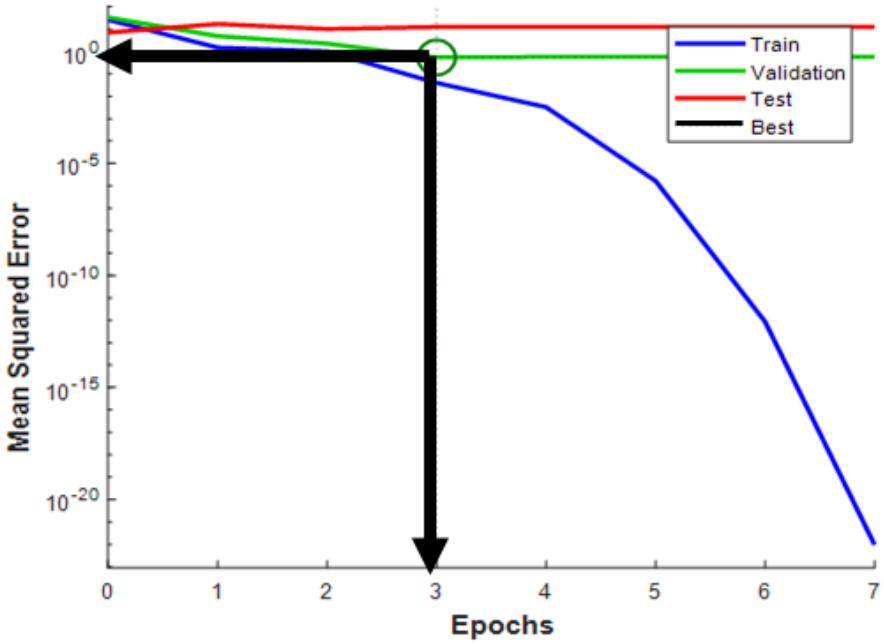

Figure 15. Validation performance with a network structure 1-5-2-2 (0.54 at epoch 3).

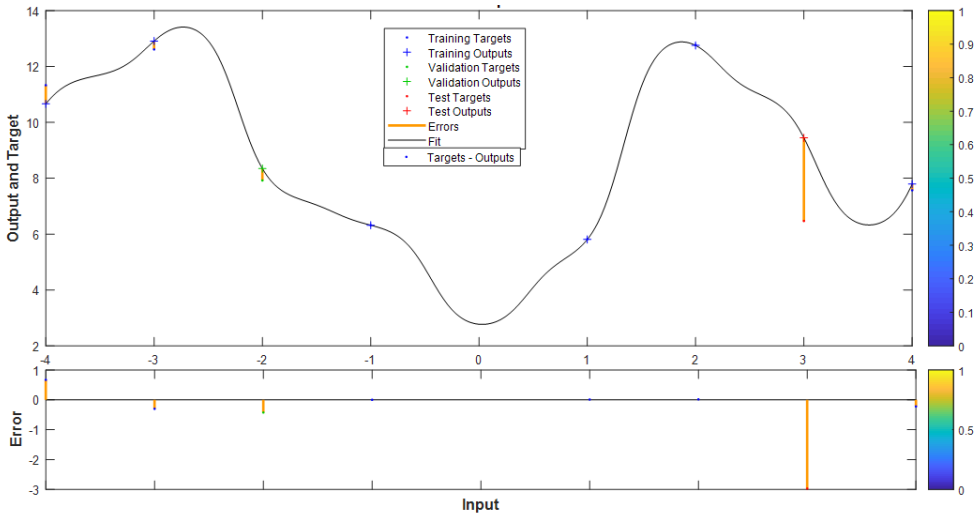

Figure 16. Function fit for output (total reducible sugars).

Table 15. Actual experimental values and ANN predicted values.

Particle size (enzyme fraction)	Mix agrowaste specific pitcher fluid fraction code	TRS (g/L) 24h actual	TRS (g/L) 72h actual	TRS (g/L) 24h ANN	TRS (g/L) 72h ANN
>75µm <106µm (<3kDa)[a]	-1	6,32	28,82	2,05	41,22
>106µm (<3kDa)[a]	-2	7,92	31,26	4,94	41,18
>75µm <106µm (>3kDa)[a]	1	5,82	30,67	2,86	35,71
>106µm (>3kDa)[b]	2	12,77	33,50	4,55	34,77
>75µm <106µm (<10kDa)[a]	-3	12,61	35,68	7,74	40,82
>106µm (<10kDa)[a]	-4	11,33	33,87	6,09	38,01
>75µm <106µm (>10kDa)[c]	3	6,47	30,63	6,29	33,89
>106µm (>10kDa)[c]	4	7,57	30,82	7,79	32,18

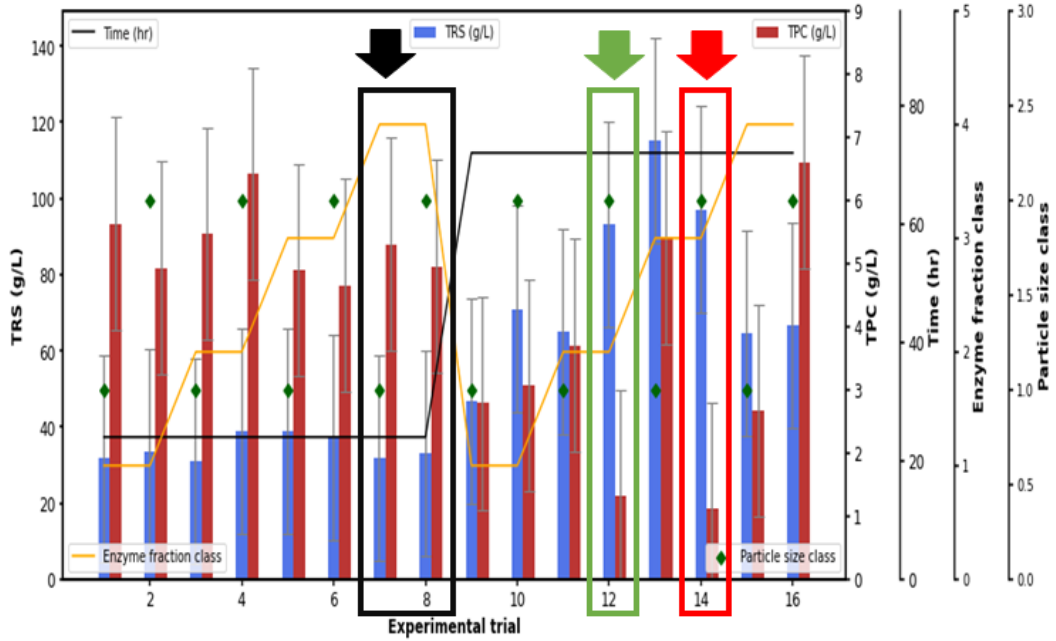

Figure 17. Experimental runs (Table 22) with current suitable conditions ⬇ (run 14),those which must be studied in future⬇(run 12)and undesired conditions ⬇ (run 7 and 8).

5.7. Summary

The feed-forward topology set-up, taking into consideration the inputs in relation to outputs, is an important step in ANN. Furthermore, determining the neurons (nodes) effectively determines the effectiveness of predicted outputs, which in this part of the study was the concentration of total reducible sugars in the hydrolysates of the pretreated milled mixed agro-waste. Three epochs were determined to be suitable during the validation process, with a 1-5-2-2 network structure, with particle size/enzyme fraction (1, input layer), 5 hidden neurons, and output layers (n = 2). In this stance, the topology might have to be redeveloped as it is suggested that the number of hidden layer neurons be in the range between inputs and output. Overall, the > 106 µm particle size seemed to be suitable for the number of coded inputs (2, 3, and 4), although coded inputs 3 and 4 (with the minimal error between the ANN and experimental values) were previously determined to have a high concentration of phenolics. Experiment run 12 (> 106 µm/> 3 kDa, input code 2, showed promising results with adequate predictability of the output in comparison to experimental data – results similar to those of > 106 µm (< 10 kDa).

5.8. References

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CHAPTER 6

CONCLUSION, HIGHLIGHTS AND RECOMMENDATIONS

CHAPTER 6

CONCLUSION, HIGHLIGHTSAND RECOMMENDATIONS

6.1. Conclusion

In this study, a proposal of a new concept termed 'semi-deligni-holocellulolysis' as a simultaneous process, particularly for milled agro-waste was proposed and motivated. It means that during the pretreatment of milled agro-waste, the solubilization of the whole agro-waste is largely not achieved, and for such milled waste, delignification and decoupling of cellulose, including hemicellulose, occurs as a simultaneous process due to the milling. Additionally, the study proposes that the pretreatment of the milled agro-waste (whether mixed or as a single feedstock) can be achieved by using non-chemical means. Therefore, an eco-friendly pretreatment had to be identified.

In this regard, plant-based constituents which can be used for agro-waste pretreatment were an alternative, as plant constituents are perceived to be eco-friendly. Therefore, a cocktail of acidic digestive pitcher fluids was identified as an alternative. *N. mirabilis* pitcher fluids were identified as being suitable and were assessed for agro-waste pretreatment to produce total reducible sugars while reducing toxicants, i.e., total phenolic compounds. *N. mirabilis* has larger monkey cups compared to other *Nepenthes* spp. The *N. mirabilis* pitcher fluids were determined to have a similar profile (acidic) and certain other characteristics associated with 1% (v/v) sulphuric acid, although the pitcher fluid had a higher redox potential, i.e., an indication that it can adequately facilitate redox type reactions.

From the *N. mirabilis* pitcher fluid, several enzymes were identified using proteomics. These enzymes are largely associated with agro-waste decomposition in one way or the other, i.e., they can decouple numerous constituents within the agro-waste to produce hydrolysates with a high total reducible sugar content.

The pitcher fluid was fractionated, with the < 10 kDa fraction, i.e., a fraction where the aforementioned enzymes were found, being identified as suitable to effectively pretreat > 106 µm particle size (identified as the prime particle size), with the highest total reducible sugar content and low total phenolic compounds in the hydrolysates. It proves that the application of the pitcher fluids can be an alternative to pretreating milled agro-waste effectively.

It was then necessary to assess the variation in pH, temperature, and co-factor solution (trace element solution) to determine their influence on the pretreatment process, particularly the production of a high concentration of total reducible sugars. Initially (Chapter 3), it was thought that supplementing the pitcher

fluids with a trace element solution would enhance the activity of the enzymes within the pitcher fluid, thus improving the pretreatment process. However, this was not evident when evaluating the association between the trace element solution, pH, and temperature. The observation was that a pH of 2 at a temperature below ambient (in this study we initially used ambient temperature) was effective in producing hydrolysates with a high total reducible sugar concentration. This was followed by determining the pore size of the > 106 µm agro-waste and linked to the fraction (< 10 kDa) used to pretreat it. The porosity values determined using the BET method indicated that pores with the agro-waste could accommodate < 10 kDa enzymes, i.e., the enzymes could be adequately embedded within the pores of the agro-waste used.

The ANN structure used was 1-5-2-2 (0.54 at epoch 3), with 106 µm (> 3 kDa), 75 µm < 106 µm (> 10 kDa) and > 106 µm (10 kDa), i.e., with experimental runs coded 2, 3, 4, being deemed predictable by the ANN model. However, 75 µm < 106 µm (> 10 kDa) and > 106 µm (10 kDa) were determined to have high total phenolic compound concentration and therefore were unsuitable for further studies.

6.2. Highlights

- The thesis puts forward an argument that for milled agro-waste, the process of hydrolysis is a partial, simultaneous (parallel) process of lignin-holocellulose constituents in the agro-waste instead of it being a sequential process of ligninolysis > holocellulolysis (hydrolysis in a series of reactions).

- This study indicates that *Nepenthes mirabilis* pitcher fluids can hydrolyse mixed-agro waste under acidic conditions at below ambient temperature to produce hydrolysates with a high concentration of total reducible sugars.

- The use of a particular agro-waste in combination with a particular pitcher fluid fraction enzyme size is crucial in the performance of the sole use of *N. mirabilis* pitcher fluids.

- Proteomics confirmatory tests identified β-1,3-glucanase, purple acid phosphatase, class IV chitinase, putative peroxidase 27, aspartic protease nepenthesin I, thaumatin-like protein, and class III chitinase in the < 10 kDa pitcher fluid fraction – all of which can be useful in agro-waste pretreatment.

- Lastly, agro-waste porosity was associated with enzyme molecular weight, which subsequent elucidated the justification of < 10 kDa fraction efficacy.

6.3. Recommendations

As some recommendations have been made in the individual chapters, they will not be repeated here. It is therefore additionally recommended that:

- The metallic content of the *N. mirabilis* pitcher fluids be quantified (this has never been done before),
- Vertical farming be assessed for the *N. mirabilis* as there is uncertainty as to the availability of the pitcher fluid for medium to large-scale plants,
- The constituents besides total reducible sugars and total phenolic compounds, i.e., metallic species, in the hydrolysates produced by the agro-waste used in this study be determined, and
- The > 106 µm (> 3 kDa) (experimental run 12 – see Table 22 in theAppendices), which is a purified filtrate of the < 10 kDa fraction, must be investigated for the pretreatment of the milled mixed agro-waste as it produces hydrolysates with a similar total reducible sugar and phenolic compound profile as the < 10 kDa (-see Table 6, Chapter 3, and Figure 17).
- Other environmental factors associated with the pretreatment process suggested in this thesis must be assessed.
- Since a feed-forward was used in the study, a feedback topology might indicate what ranges of input variables, i.e., particle size including enzyme, to use. Additionally, the ANN topology can be optimized. One of the options is to increase the number of layers in the ANN, i.e., depth.

6.4. Declaration: as per external examiners recommendations

In this thesis, all the statistical analyses performed and the raw data (including appropriate controls) obtained from the experiments were submitted electronically for archiving as part of the thesis. Appropriate justification of the use of *N. mirabilis*, the agro-wastes chosen, and the restriction to assess factors such as pH, temperature, and trace element solutions are highlighted in Chapter 3, section 3.1 and Chapter 4, section 4.1.

APPENDICES

APPENDICES

Table 16. Mass spectrometry data acquisition parameters

Full scan	
Resolution	70.000 (@m/z 200)
AGC target value	3e6
Scan range	350 - 2000 m/z
Maximal injection time (ms)	100
Data-dependent MS/MS	
Inclusion	off
Resolution	17.000 (@m/z 200)
AGC target value	1e5
Maximal injection time (ms)	50
Loop count	10
Isolation Window width (Da)	3
NCE (%)	27
Data-dependent settings	
Underfill rate (%)	1
Charge exclusion	Unassigned 1, 7, 8, >8
Peptide match	Preferred
Exclusion isotopes	on
Dynamics exclusion (s)	60

Table 17. Byonic™ search parameters

Recorded protein search configuration		
Num	**Rule**	**Value**
0	Protein database	Nepenthes_NCBI_1790 proteins_191021.fasta
1	Spectrum-level FDR	Auto cut
2	Cleavage residues	RK
3	Digest cutter	C-terminal cutter
4	Peptides termini	Fully specific
5	Maximum number of missed cleavages	2
6	Precursor's tolerance	10.0ppm

7	Fragment tolerance	Frag:qtof_hcd 20.0ppm
8	Fragment tolerance version	2
9	Charges applied to charge unassigned spectra:	1,2,3
10	Precursor mass max	10000.0
11	N-glycan search	None
12	O-glycan search	None
13	Off by x isotopes	-2, -1, 0, +1, +2
14	Contaminants added	True
15	Decoy added	True
16	**Additional parameters:**	
17	Disulphide enables	False
18	Trisulphide enable	False
19	DSS crosslink enable	False
20	Custom crosslink enable	False
21	Wildcat enable	0
22	Combyne cut off score	Auto
23	Protein FDR cut off	1%
24	Focused DB created	False
25	Export mzldentML	True
26	Score version	2
27	Precursor assignment flags	2
28	Po_NumberMONOsReturn	1
29	Lock mass list	None
30	% Modification searches:	
31	Common_Modification_max	1
32	Rare_modifications_max	1

A1. Chemical solutions preparation

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

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

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

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

Trace element (co-factor) solution preparation for pitcher fluid supplementation

The trace element solution used in this study was prepared by:

- Dissolving 1.5 g of Nitrilotriacetate in 800 mL sterile distilled water;
- Adjusting the pH to 6.5 by using 1M KOH (8 g/500 mL).
- The following compounds, i.e., $ZnSO_4.7H_2O$ (0.1 g), $FeSO_4.7H_2O$ (0.1 g), $MgSO_4$ (3 g), $MnSO_4$ (0.5 g), NaCl (1 g), $CuSO_4$ (0.1 g), $AlK(SO_2)_2.12H_2O$ (0.01 g), H_3BO_3 (0.01 g), $Na_2MnO_4.2H_2O$ (0.01 g), $MgSO_4.7H_2O$ (6.14 g), $MnSO_4.H_2O$ (0.56 g), $CoCl_2.6H_2O$ (0.187 g) and $CoCl_2$ (0.1 g), were weighed as specified and added to the Nitrilotriacetate solution.
- The volume was made up to 1000
- The filter was sterilized using a 0.22 µm filter and autoclaved
- Stored at 4 °C prior to use.

Table 18. Standard calibration data of TRS.

Concentration (mg/L)	Final absorbance
2,5	0,016
5	0,021
10	0,036
20	0,037
40	0,04
80	0,067
160	0,248

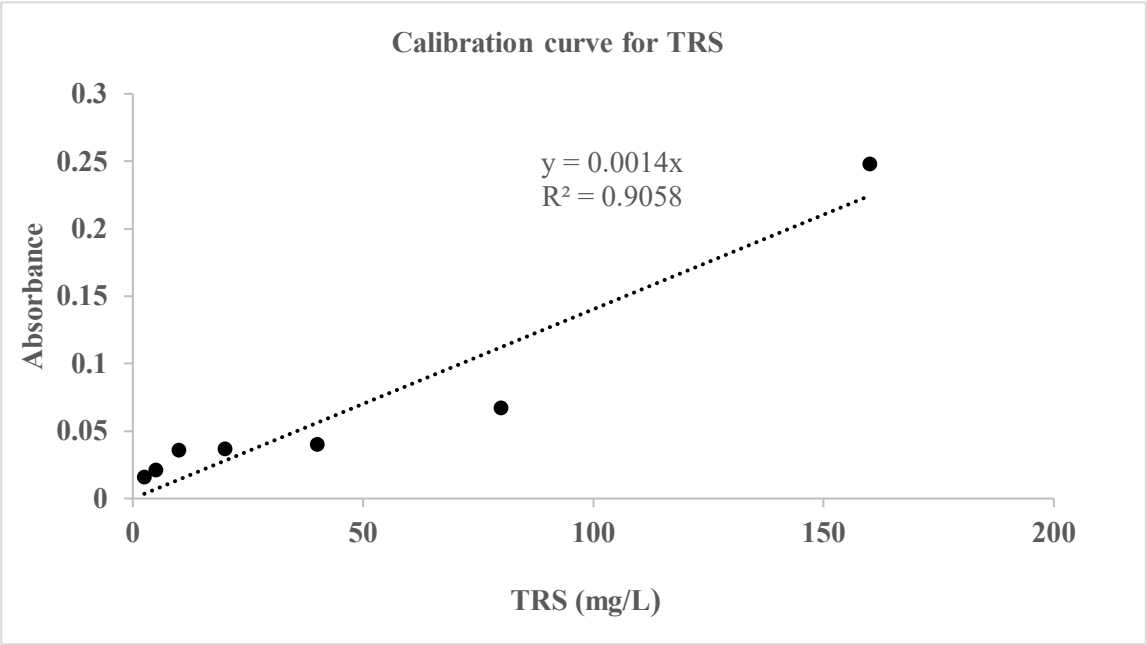

Figure 18. Standard calibration curve of TRS.

Table 19. Standard calibration data of TPC.

Concentration (mg/L)	Final absorbance
10	0,372
20	0,502
40	0,61
80	0,877
160	1,031

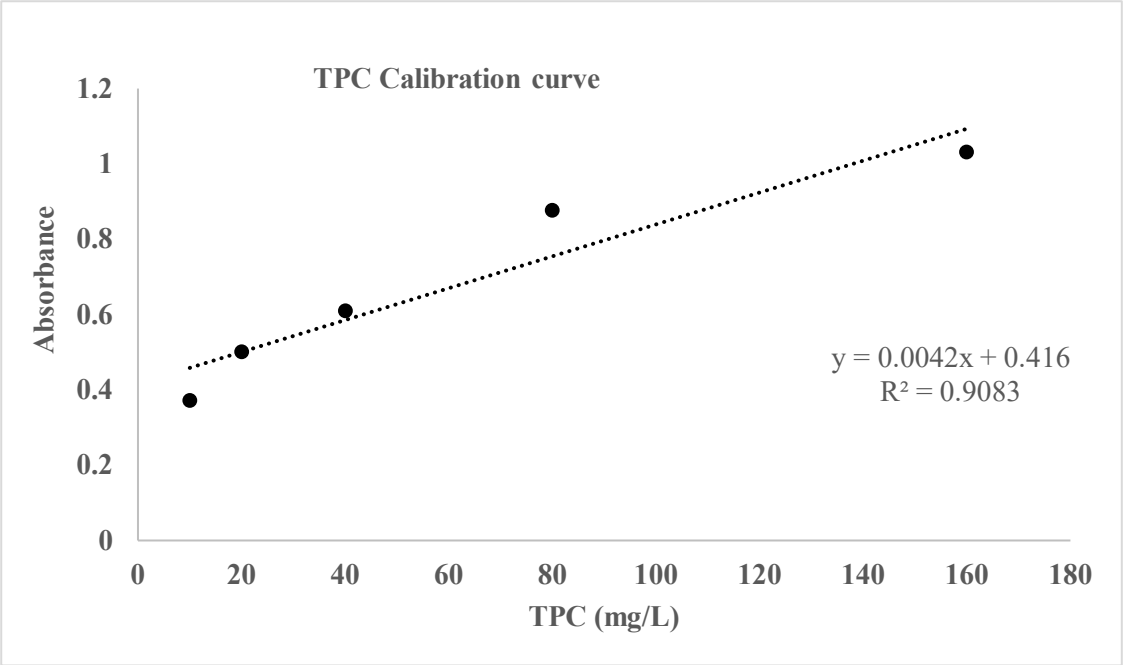

Figure 19. Standard calibration curve of TPC.

Table 20. Statistical analysis for TRS at 24 h.

Mix agrowaste/Specific juice fractions	P-value TRS	By conventional criteria, it is considered to be	t-value TRS	df TRS	Std error of difference TRS
>75µm <106µm (<3kDa)	0.0005	Extremely statistically significant	10.377	4	0.032
	0.0004	Extremely statistically significant	10.7117	4	0.035
>75µm <106µm (>3kDa)	0.0002	Extremely statistically significant	13.2273	4	0.023
	0.0001	Extremely statistically significant	17.2638	4	0.02
>75µm <106µm (<10kDa)	0.0001	Extremely statistically significant	151.3556	4	0.004
	0.0001	Extremely statistically significant	27.2210	4	0.01
>75µm <106µm (>10kDa)	0.0003	Extremely statistically significant	11.8907	4	0.029
	0.0065	Very statistically significant	5.2002	4	0.063

Table 21. Statistical analysis for TRS at 72 h.

Mix agrowaste/Specific juice fractions	P-value TRS	By conventional criteria,it is considered to be	t-value TRS	df TRS	Std error of difference TRS
>75µm <106µm (<3kDa)	0.0012	Very statistically significant	8.173	4	0.005
	0.0001	Extremely statistically significant	16.6495	4	0.019
>75µm <106µm (>3kDa)	0.0001	Extremely statistically significant	20.2282	4	0.028
	0.018	Very statistically significant	0.3716	4	0.036
>75µm <106µm (<10kDa)	0.0001	Extremely statistically significant	24.9325	4	0.027
	0.1564	Not statistically significant	1.7424	4	0.037
>75µm <106µm (>10kDa)	0.0048	Very statistically significant	0.078	4	0.078
	0.2482	Not statistically significant	1.3504	4	0.166

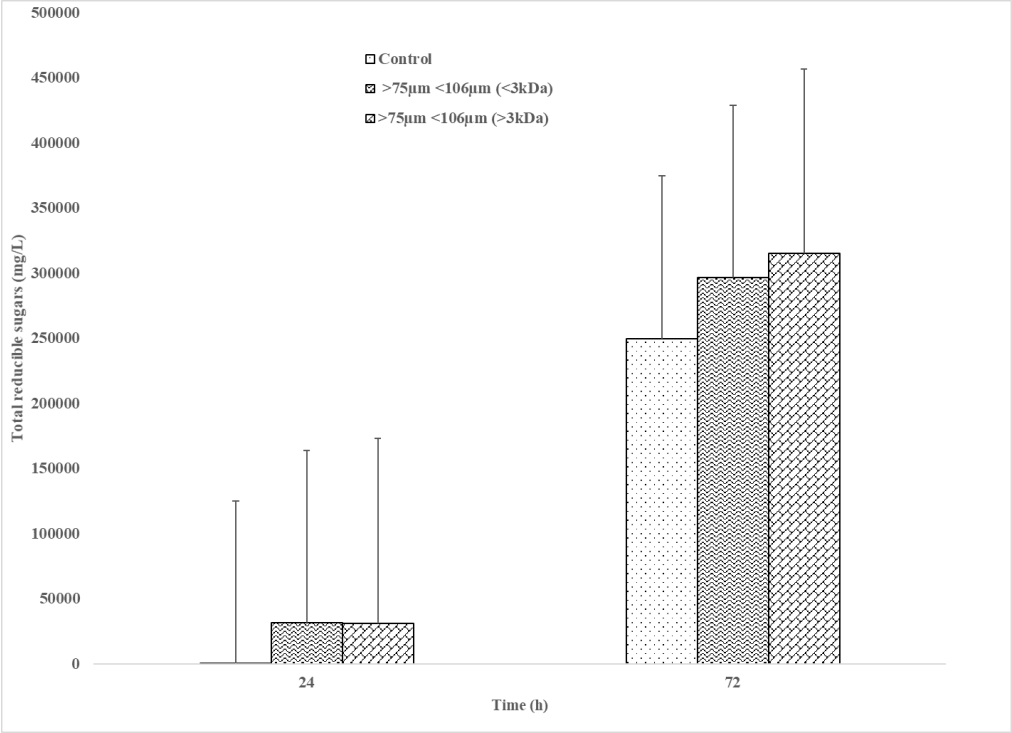

Figure 20. Preliminary results of the retentate and filtrate using the 3 kDa filter for TRS.

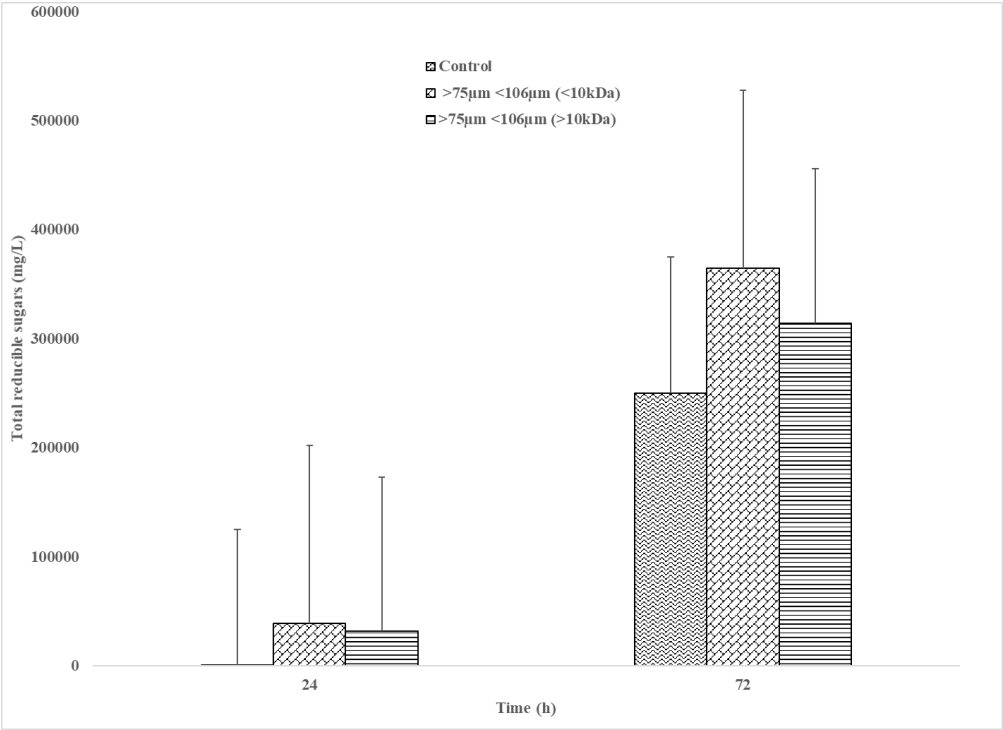

Figure 21. Preliminary results of the retentate and filtrate using the 10 kDa filter for TRS.

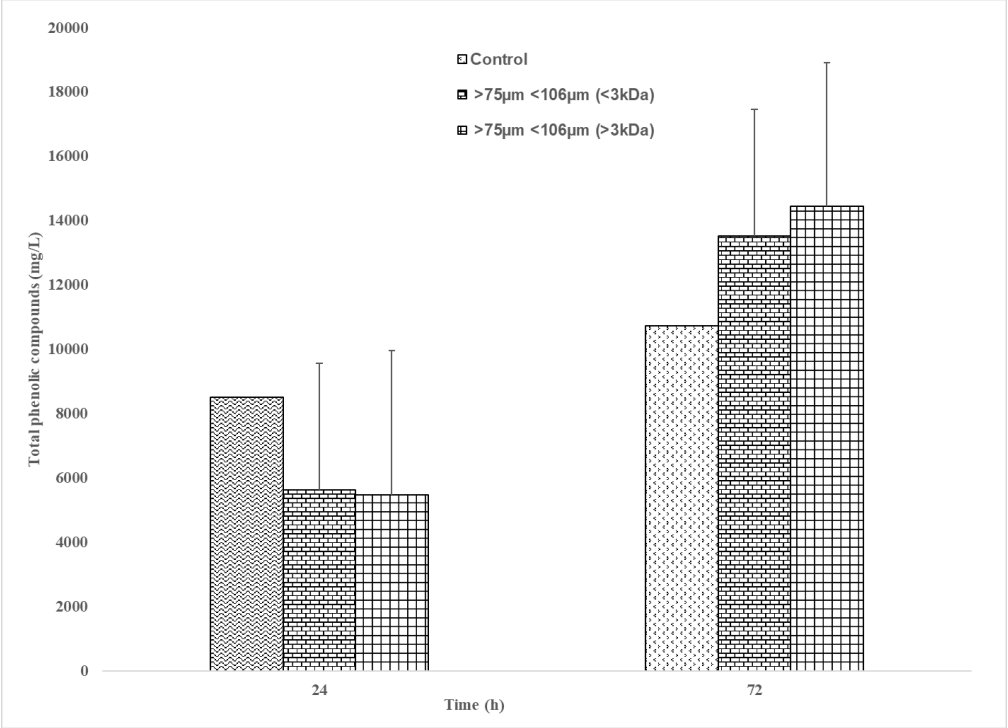

Figure 22. Preliminary results of the retentate and filtrate using the 3 kDa filter for TPC.

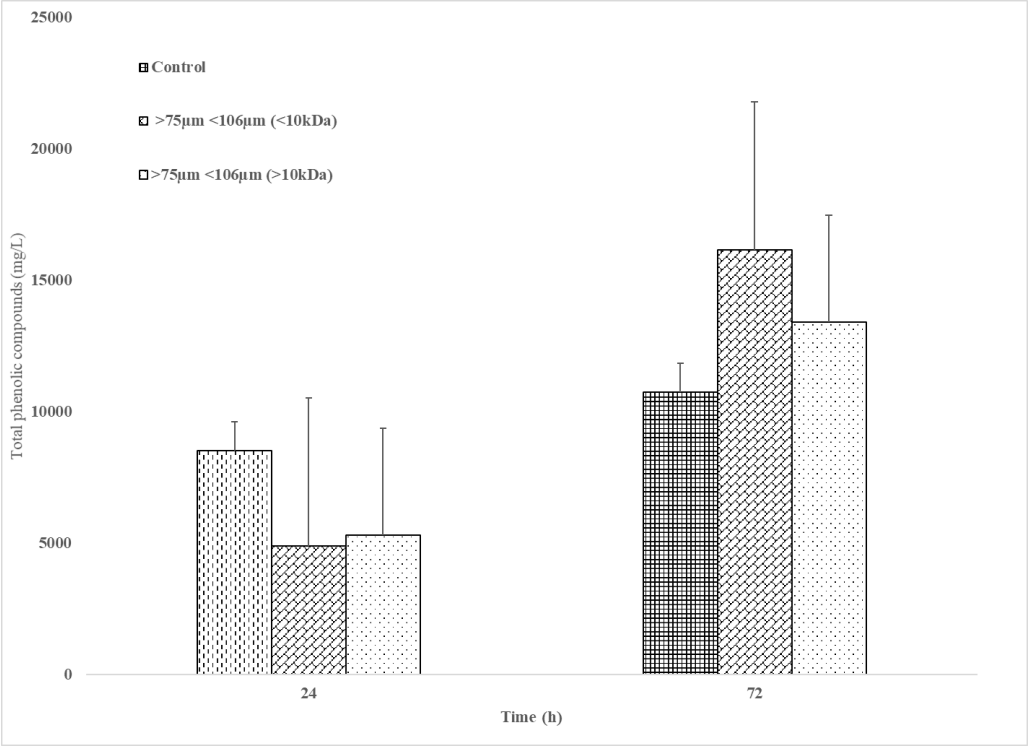

Figure 23. Preliminary results of the retentate and filtrate using the 10 kDa filter for TPC.

Table 22. Experimental run classifications.

Time (24 h)	
Agro-waste size/Pitcher fluid fractions	**Run**
>75µm *x* <106µm (<3kDa)	1
>106µm (<3kDa)	2
>75µm *x*<106µm (>3kDa)	3
>106µm (>3kDa)	4
>75µm *x* <106µm (<10kDa)	5
>106µm (<10kDa)	6
>75µm *x* <106µm (>10kDa)	7
>106µm (>10kDa)	8
Time (72 h)	
Agro-waste size/Pitcher fluid fractions	**Run**
>75µm *x* <106µm (<3kDa)	9
>106µm (<3kDa)	10
>75µm *x* <106µm (>3kDa)	11
>106µm (>3kDa)	12
>75µm *x* <106µm (<10kDa)	13
>106µm (<10kDa)	14
>75µm *x* <106µm (>10kDa)	16

Figure 24: Neural network training tool.

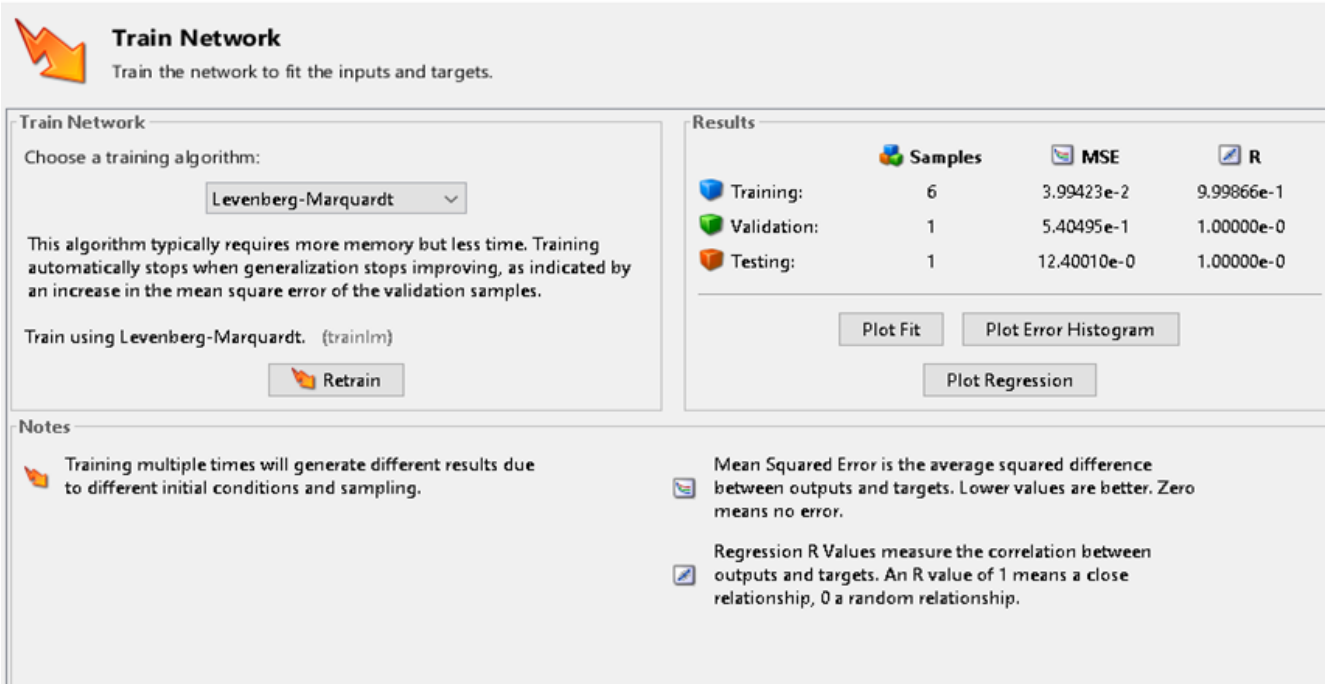

Figure 25. Neural network trainindicating mean squared error and the regression values.