A *Mucor circinelloides*-based integrated biorefinery

Submitted in complete fulfillment for the Degree of Doctor of Philosophy in Biotechnology in the Department of Biotechnology and Food Science, Durban University of Technology, Durban, South Africa

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November 2023

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REFERENCE DECLARATION

I, Mr. J.T. Zininga – 21143318 and Prof Kugen Permaul do hereby declare that in respect of the following dissertation:

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AUTHORS DECLARATION

This study presents original work by the author. It has not been submitted in any form to another academic institution. Where use was made of the work of others, it has been duly acknowledged in the text. The research described in this dissertation was carried out in the Department of Biotechnology and Food Science, Faculty of Applied Sciences, Durban University of Technology, South Africa, under the supervision of Prof Kugen Permaul and Prof Suren Singh.

Student's signature

DEDICATION

This work is dedicated to my dearest mother Enes Gonda Zininga who passed on the 4th of January 2019. All I have, I would give so she could just stand here with me on the finishing line. Though she may no longer be here physically, but I always feel her presence, whenever I remember her prayers for me, that provide such great comfort in the most difficult of times. Even in death I thank God for her life.

CONTENTS

ACKNOWLEDGEMENTS

First and foremost, l would like to thank my Lord Jesus Christ for His love, His kindness, His mercy, and His grace.

I would like to give special thanks to my supervisor, Prof Kugen Permaul for his guidance, insightful recommendations, correction and above all, mentoring. This would be incomplete if I dot mention my co-supervisor Prof Suren Singh as well and as Sir Isaac Newton once said, “If I have seen further, it is only by standing on the shoulders of giants”. I am grateful for your shoulders.

All the postgrad students and post docs in the Enzyme Technology Group (Dr Adarsh, Melvin, Nonhlanhla, Anele, Ayanda)

Ms. Siphi Mbamali for technical assistance and Ms. Kameshnee Mellem for your encouragement

Department secretaries Ms. Priscilla Phillips and Ms. Zinhle Mcoselli

Not forgetting Ms. Sohana Ranglal

My family for their tremendous love and support

My young brother Archford Zininga

I would also like to acknowledge the NRF for making this project possible through their funding.

ABSTRACT

The utilization of agro-based waste residue is a prominent example for establishing a circular bioeconomy. When agro-based waste is used to replace commercial growth media in microbial fermentation processes, it becomes expedient that the implementation of the biorefinery concept becomes integrated as the microbial biomass and their metabolites will add to the product spectrum. This study demonstrates an integrated approach towards valorization of sugarcane bagasse and microbial biomass comprising of *Mucor circinelloides* ZSKP. A maximum reducing sugar recovery of 80.67 g/l was achieved after combining lignocellulosic pretreatment with saccharification. A low temperature, glycerol and ammonium phosphate pretreatment method was established, where glycerol pretreatment conditions were reduced to 150°C and almost a third of the initial duration. This strategy will potentially result in lower energy consumption costs, decreased release of inhibitors and lower water consumption. The ammonium phosphate-containing hydrolysate yielded 12.89 g/l of fungal biomass after fermentation to add to 20.8 g lignin from the delignification step. Sequential fermentations were performed by addition of *Saccharomyces cerevisiae* as a co-culture to produce biomass and ethanol. The initial aerobic *M. circinelloides* fermentation was followed by mixed culture anaerobic fermentation. Fermentation of *M. circinelloides* (3 days aerobic growth) and *S. cerevisiae* (5 days of anaerobic growth) generated a balanced biomass yield of 17.9 g/l and ethanol yield of 18.69 g/l. Ethanol production demonstrated effective utilization of bagasse hydrolysate and offered the possibility of concurrent production with biodiesel in this biorefinery. The mixed biomass gave a lipid yield of (3.72 g/l) and a chitosan yield of (1.84 g/l), The lignin infused glycerol plasticized chitosan biocomposite plastic had a 100% improvement in thermogravimetric properties with almost 50% more energy needed to increase the temperature of the material when compared to glycerol-only-plasticized biocomposite. The deproteination step in chitosan extraction was modified and replaced with a protein recovery step, with protein yield being improved to a maximum of 187.89 mg/g. The amino acid composition of the protein showed that it has a relatively high content of lysine making it suitable as an animal feed supplement. This study presents strategies to address feasibility concerns for production of fungal chitosan, biodiesel and bioethanol from lignocellulosic waste and realizing the goal of a circular bioeconomy.

LIST OF ABBREVIATIONS

AIM	Alkaline Insoluble Material
MLC	Minimum Lethal Concentration
CoA	Coenzyme A
FTIR	Fourier Transform Infrared
GC-MS	Gas Chromatography-Mass Spectrometry
GlcNAc	D-Glucosamine
HPLC	High Performance Liquid Chromatography
MIC	Minimum Inhibitory Concentration
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
CPTL	Combined pretreatment and lignin extraction
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
TGA	Thermogravimetric Analysis
SDA	Sabouraud Dextrose Agar
TDS	Transdermal Drug Delivery System
UDP	Uridine Diphosphate

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1: INTRODUCTION

Countries have been focusing on economic growth to address the demands and aspirations of a bigger and modernized population, and this has seen a massive increase in industrialization as well as agricultural activities. These increased activities comes with new challenges to waste management systems. Agro-industrial wastes are now estimated to be one-third of the world's agricultural waste. The United Nations Environment Programme estimates this at 1.3 billion tons. Environmental concerns and a persistent population growth trajectory demands innovative and sustainable ways of economic activity. A circular economy based on reutilization and reuse of material becomes imperative (Yafetto *et al*.2023).

A circular economy allows for a closed resources loop based on renewable utilization of non-toxic material for energy and other products. The implementation of a circular economy from a biotechnological perspective must be sustainable, not just an endeavor to replace fossil resources with renewable ones but rather have a minimum net carbon effect, supply chains that are sustainable. There should also be innovatively disruptive conversion technologies of these renewable resources into bio-based products and fuels (Tan and Lamars 2021).

The principles that govern the circular bioeconomy concepts include agro-ecosystems by-products recycling and utilization and use of renewable energy whilst at the same time reducing overall energy demand. An effective utilization of sugarcane waste in South Africa to potentially produce biofuels and other by-products would be a perfect scenario of a circular bioeconomy (Muscat *et al*. 2021). Agricultural waste residue build-ups can be divided into two types: cycle build ups and field deposits. Field deposits are residues of the unutilized biomass in the production stage. The cycle build ups form in the field after the yield harvesting cycle. It is the cycle build ups that that would require effective management after harvesting. Burning them contributes to air pollution and global warming thus it becomes necessary to utilize them for deriving economic value. Stalks, cobs, leaves, molasses, and bagasse fall under this category. In KwaZulu Natal province in South Africa there is a lot of sugarcane production and with it comes the sugarcane related waste that need to be managed (Sivakumar *et al*. 2022).

Sugarcane bagasse is a fibrous lignocellulosic waste generated after sugarcane is crushed to extract the juice and is composed of cellulose (35-50%), hemicellulose (25-35%), lignin (20-30%) with the rest being ashes and extractives. It has had applications in the paper industry, as feed stock and as fibre in the textile technology industry among other applications. It contains a significant amount of cellulose, and this can be extracted. The major advantage of using bagasse

is that it is agro-based waste and after pretreatment, the applications spectrum of this material is even more enhanced. The biodegradability of any product from bagasse is a relevant solution to the current environmental challenges in waste management (Amin *et al.* 2020; Mahmud and Anannya 2021).

The bagasse constituents are chemically and physically bonded together by predominantly lignin and cell wall polysaccharides linkages. Pretreatment is therefore needed to breakdown the complex structure to allow access for enzymatic degradation of cellulose and hemicellulose into simple sugars. The target of a successful pretreatment process is to achieve a liberated cellulose component that can easily be hydrolyzed to give high yields of glucose. This now includes evaluating other components on the complex structure. The initial focus on lignocellulosic material pretreatment was necessitated by the need to maximize production of bioethanol from the material (Zoghlami and Paes 2019).

Current trends in biomass utilization point to a major shift towards maximizing the overall yield of as many products as possible from valorization of the material. A consideration of the constituents of lignocellulosic material like lignin and hemicellulose, should however inform the evolution of current strategies. Subsequently, pretreatment should not only be focused on extraction of cellulose, but the recovery of high value polymers like lignin as well. The possibility of meeting the high energy requirements through internal integration of high energy streams makes lignin attractive. It has been reported that even in an optimized mill where the lignin pulp is utilized for energy, 20-30% of the lignin is still available to be utilized for various industrial applications like wood panel products, biodispersants, polyurethane foams, automobile brakes material etc.

The integration of many biomass conversion pathways to produce a wider product spectrum with subsequent value addition is the essence of an integrated biorefinery. There are various pathways already available to produce a vast array of products from different biomasses and this has led to strategies with the objective of integrating processes and optimizing frameworks. There has been a drive for integrated biorefinery strategies to be thermal energy self-sufficient to remove or reduce any dependence to the national grid at a time when there is an energy deficit in most developing countries. Thus, value added products like biodiesel and fermentation products such as ethanol are necessary for the feasible culmination of the integrated process (Demirbus, 2019, Rodionova *et al*. 2022).

The combination of enzymatic hydrolysis after pretreatment of the cellulose and hemicellulose components with biochemical conversion will result in the production of biofuels. This is due to the fermentations of monomeric sugars to biofuels. The hydrolysate generated from pretreatment

and enzymatic saccharification contains reducing sugars with mostly hexoses (glucose) being dominant, in addition to pentoses mainly (xylose and arabinose). It becomes expedient that there is efficient utilization of all these sugars in fermentations. Production of ethanol from *Saccharomyces cerevisiae* utilizing bagasse hydrolysate has been the flagship process in the pursuit of unlocking the potential of sugarcane lignocellulosic material. To make sure that the bioethanol derived from this process is competitive in terms of pricing, it is important that all the reducing sugars present in the hydrolysate are converted into ethanol or valuable biomass. Some microbes used in fermentations can utilize 5-carbon sugars. The challenge is that this is usually suppressed when glucose is available (Baja *et al.2022;* Broda *et al*. 2022).

Microorganisms tend to prefer glucose in fermentation processes which they selectively utilize. In *S. cerevisiae* (wild type) production of ethanol using hydrolysate from lignocellulose, the xylose is largely unutilized. The recovery of xylose from a pretreatment process can be up to 80%-90%. *Mucor circinelloides* has been reported to be able to simultaneously utilize both glucose and xylose, even though glucose is assimilated at a faster rate. Carvalho *et al.* (2019) reported that both glucose and xylose were completely utilized after 120 h of *M. circinelloides* growth. *M. circinelloides* is a dimorphic fungus that changes into the yeast form under anaerobic conditions (Lee *et al.* 2013). This led to its investigation for ethanol production under both anaerobic and aerobic ethanol production (Lubbehusen *et al*. 2004). The biomass of this oleaginous zygomycete is a source for lipids, chitosan and just like *S. cerevisiae* is also a potential source of single cell proteins for fish feed.

The aim of this project is to effectively valorise *Mucor circinelloides* biomass and sugarcane bagasse through implementation of an integrated biorefinery process to produce high value biochemicals and second-generation biofuels. This aim was achieved using the following objectives:

- to optimize a pre-treatment strategy for sugarcane bagasse;
- to incorporate lignin extraction in bagasse pretreatment;.
- to enhance the conversion of cellulose and hemicellulose in sugarcane bagasse into fermentable sugars using hydrolytic enzymes by combining this process with the pre-treatment step;
- to co-ferment pre-treated bagasse for ethanol production;
- to replace the deproteination step in chitosan extraction with a protein extraction step;
- to develop a sequential process for the extraction of lipids, proteins, and chitosan from microbial biomass; and
- to quantify and characterise the bioproducts using analytical techniques and thereby propose potential applications.

CHAPTER 2: LITERATURE REVIEW

2.1. Plant-based biomass

A greener and energy secure economy are the major two objectives that humans seek to adopt for a future that is sustainable (Nanda *et al.*2015). There is a biomass resource that has a high energy content that can be used to address the energy crisis that the world faces, and this is in the form of lignocellulosic biomass such as sugarcane bagasse, wood residue, corn stalk, rice and wheat straw (Chum and Overend 2001; Long *et al.* 2013). An estimate of the global production of plant-based biomass is 11.9 B tons (Popp *et al.* 2021).

The offer of carbon sequestration benefits as well as harnessing bioenergy and bio-products has made the use of a renewable carbon source like plant-based biomass strategically vital. There is however an apparent obstacle to the utilization of such biomass to generate energy and other bio-products, which is the issue of supply as well as the technologies that would enable efficient conversion to get maximum yields (Hossain *et al.* 2017). This would enable a sustainable and feasible operation that would be a reliable alternative. Studies have shown further barriers to this process in the form of geographical distribution, seasonality and batch variations that would see a low calorific value (Zhu *et al.* 2021a). There is also the sensitive issue of competition for arable land at a time when food security is a major concern against an ever-growing population (Gavrilescu 2014).

2.1.1. Biomass and land usage

Biomass is strategic in lessening the dependence on fossil fuels which have a played a devastating role in climate change. Energy from biomass is unique in the sense that it is directly tied to the farming activities, ecosystems, and forests from which this biomass feedstock is obtained. Thus, utilization of biomass will have either negative or positive impact on the environment and society. This impact will extend to water sources, land, ecosystems, and biodiversity. This therefore means consideration on the sustainability of the utilization of this biomass is of paramount importance. Sustainability would address food production, forest stewardship, wildlife management etc. In fact, one of the contentious issues is the seeming conflict between production of biomass for renewable energy and food production. This would disqualify many crops that have massive potential as feedstock for biofuels like maize, vegetable oils, sugarcane etc. There is also the possibility that farmers might be tempted to prioritize growing biomass for biofuels which is potentially more lucrative and thereby leaving less arable

land for actual food production. There are other factors however, that have contributed to the potential sustainability of this pursuit, like the rising costs of fossil fuels.

2.2. Agro-based waste

Due to the ever-increasing human population, the need to increase agricultural production to feed the larger population is necessary. In the last 50 years alone, there has been a 300% increase in agricultural production (Lynch *et al.* 2021). This has been enabled by strategic technological advancement as well as expansion of arable land. This increased production represents 24 million tons of food. The enhanced agricultural activity as well as the huge yields present unprecedent environmental challenges. It has been reported that 21% of greenhouse gas emissions are coming from agricultural activities (Lynch *et al.* 2021). This has prompted the need to balance the required increase in food production whilst at the same time addressing environmental concerns. What is inescapable is the huge amounts of solid agricultural waste generated. Thus, there is a need to address this challenge in a way that is sustainable and economically beneficial. Recycling of agricultural waste offers the benefits of reducing greenhouse emissions as well as the creation of a green economy that will provide jobs bioenergy and other bio-chemicals.

Agricultural wastes are increasing annually at a rate of 7.5% and they can be classified as solid waste from animal production, solid waste from crop production, chemical waste from pesticides, waste from horticulture production, industrial agriculture solid waste (Sadh *et al.* 2018). There are different kinds of agricultural waste, and these include animal production wastes, on-farm medical wastes, horticulture products waste, agriculture industry waste and chemical wastes from the use of agro chemicals like pesticides. Most of the focus has been on crop production solid wastes as these have the largest contribution. These are associated with agricultural activities that include crop production. The examples of these would include husks, corn cobs, rice straw, sugarcane bagasse etc. Rice straw is one of the most abundant agrobased waste in Asia and some parts of Africa. Most of the waste has been directed to animal food consumption and the bigger remainder being disposed of using open field burning (Goodman 2020; Santolini *et al.* 2021).

2.2.1. Lignocellulosic material

Plant-based biomass has cell walls that have a high organic polysaccharides content. The biomass which comes from the plants is the most abundant source of renewable material. These polysaccharides which includes pectin, lignin, hemicellulose, and cellulose, give the cell wall its rigidity and complexity (Ning *et al.* 2021). It is critical to optimize the utilization of biomass by

incorporating processing of the wastes generated during the various conversion steps. These would then have to be converted to valuable bio-based product streams. This integration would give rise to the concept of a biorefinery. It is here where various conversion technologies be they biochemical, microbial fermentations, combustion, bio-catalysis etc. are integrated to ensure the much-desired efficient utilization of the biomass to produce a sustainable stream of bio-based products, bio-fuels and bioenergy (Sindhu *et al.* 2016).

2.2.2. Sugarcane bagasse

Sugarcane bagasse stands out as one of the residues that can potentially be used to address the issue of green energy even within the context of the energy crisis, environmental issues as well as concerns over food security (Scaramucci *et al*. 2006). There is about 300 million metric tons of sugarcane bagasse produced worldwide annually from the sugarcane yield of about 2.0 billion metric tons (Zhao and Li 2015). South Africa is one of the major sugarcane producers in Africa producing about 25 million tons every year with most of the product going towards food. A significant chunk of that production figure (30%) is lost as waste that is about 7.5 million tons of sugar bagasse are produced every year and these are rarely utilized (Mashoko *et al.* 2013).The production of sugarcane brings about the need to process such massive waste residue that comes with it and it becomes very important as this can also become a serious environmental issue if the waste is not properly handled (Amin *et al.* 2020).

2.3. Components of the plant cell wall

2.3.1. Cellulose

The major component in lignocellulosic biomass is the polymer cellulose (Fig.1). It is in the cell wall and constitutes between 40-50%. There are numerous reports of cellulose in the cell walls of bacteria, fungi and algae (Szymanska-Chargot *et al.* 2011; Garcia-Rubio *et al.* 2019). It is a homopolymer, whose β-D-glucopyranose subunits connect through a β-(1.4) glycosidic bond which allows for a linear structure. The cellulose fibres are combination of microfibrils (20-300) which interact through Van der Waals forces, covalent and hydrogen bonding (Kumar Gupta *et al.* 2019). It is the hydrogen bonds within the microfibrils that confers the linearity of the homopolymer chain. Hydrogen bond interactions between the chains however influence the crystallinity of the cellulose structure. Crystallinity influences the resistance to microbial or enzymatic attack that means crystallinity improves resistance to hydrolysis in contrast to the

amorphous form. Naturally, cellulose is hydrolyzed by extracellular enzymes, endoglucanases, b-glucosidases and cellobiohydrolases (Zoghlami and Paes 2019; Mankar *et al.* 2021).

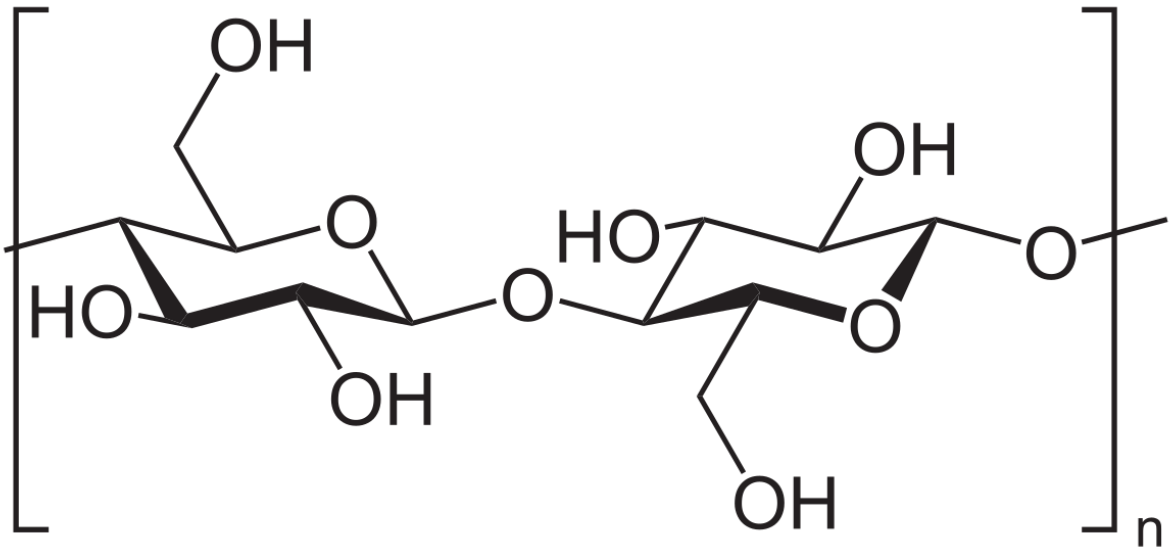


Figure 1:The structure of cellulose (Hu *et al*. 2020)

2.3.2. Hemicellulose

This is a heterogenous polymer which is branched and make up between 20-50% of lignocellulosic biomass (Zhou *et al.* 2016). The polymer is a mixture of hexoses, pentoses and acetylated sugars (Fig. 2). The difference with cellulose is that it is amorphous, with less polymerization. This means that it can be hydrolyzed much more easily when compared to cellulose and thus would not need harsh reaction conditions. The composition of hemicellulose is dependent on the type of plant, with hardwood hemicelluloses made up mostly of xylan, unlike softwood. Softwood contains the least amount of xylan of between 7-12%. Hard wood contains about 20-25% which is almost like annual plants (15-30%). Lignin bind xylan to cellulose. They are also, via arabinosyl subunits covalently bonded to phenolic residues (Berglund *et al.* 2020; Huang *et al.* 2021). Hemicelluloses are most prone to thermos-chemical treatments yet by virtue of their role of coating the cellulose-fibrils they are a hindrance to efficient cellulose hydrolysis. To hydrolyze the cellulose properly at least 50% of the hemicellulose should be eliminated. This can easily be done by application of higher temperatures, but this also presents a challenge of by products being formed which end up inhibiting the enzymatic saccharification process and microbial fermentation. Therefore, there is a need to balance the pretreatment parameters, to allow the removal of hemicellulose whilst at the same time not releasing furfurals and hydroxymethyl furfurals which are products of hemicellulose hydrolysis and have been shown

to inhibit microbial growth. This has the potential to affect the sugar recovery from cellulose hydrolysis. Endoxylanases and endomannases can be used to hydrolyse hemicellulose.(Nanda *et al.* 2015; Ning *et al.* 2021).

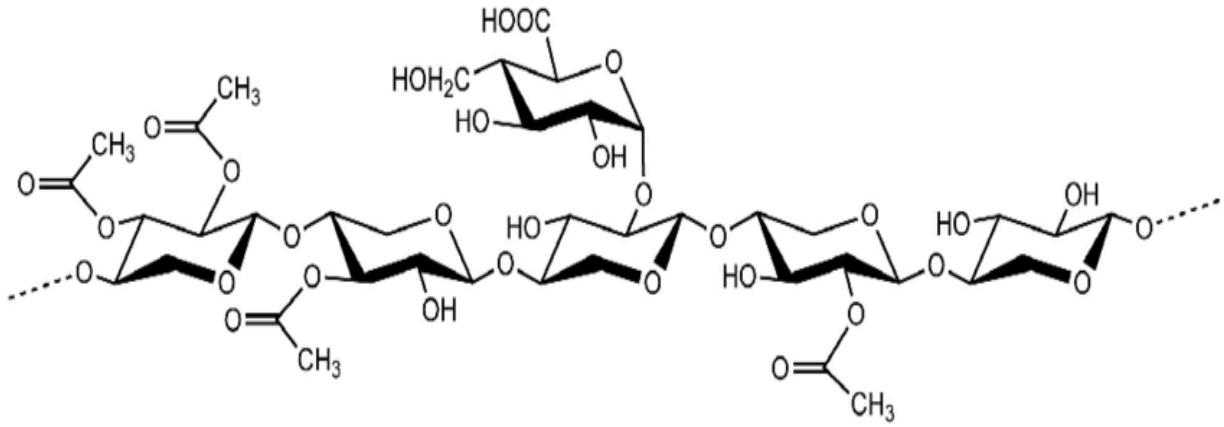


Figure 2: Structure of hemicellulose (Hu *et al*. 2020)

2.3.3. Pectin

Heteropolysaccharides that are made up of α-1,4-linked D-galacturonic acid monomers are known as pectin and they are found in most plant cell walls (Xiao and Anderson 2013). The polymer is made up of two regions: the smooth region, where the D-galacturonic acids monomers contain simple methylated or acetylated small side chains; and the 'hairy' region where the D-galacturonic acid monomers 1,4 glycosidic bond linked backbone is interrupted by α-1,2 glycosidic bond linked L-rhamnose residue side-chains (Fig. 3). The side chains can be also further linked to D-galactose and L-arabinose residues chains (Long *et al.* 2013; Ning *et al.* 2021).

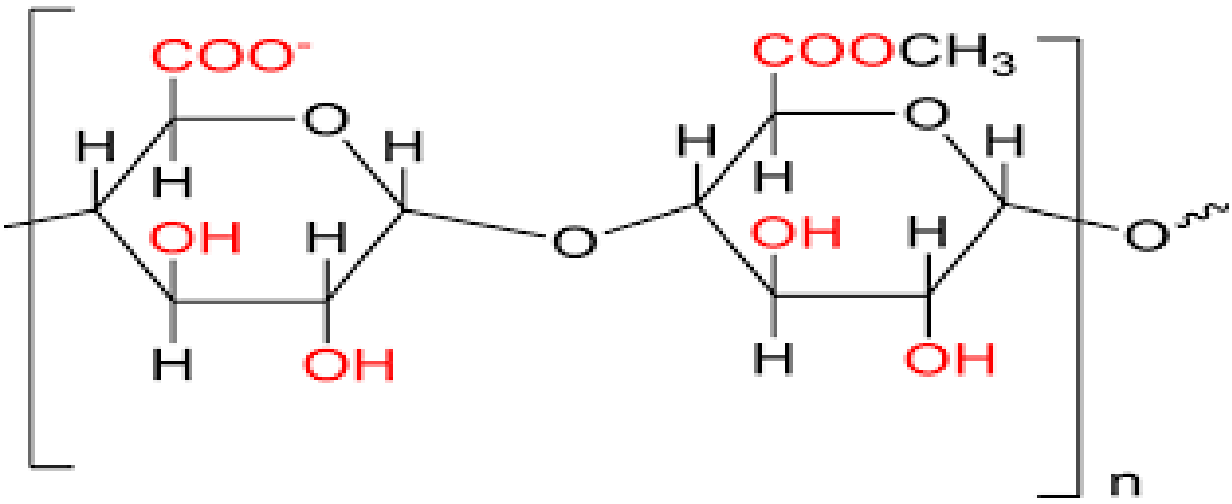


Figure 3: Structure of pectin (Maryana *et al.* 2014).

2.3.4. Lignin

About 15-30% of lignocellulosic biomass is lignin, which provides rigidity and impermeability to the plants, as well as protection against microbial attack. Three cinnamyl alcohol precursors, p-coumaryl, coniferyl and sinapyl alcohol amorphously make up this heteropolymer. They are linked to each other by ether linkages (Lu *et al.* 2017; Erfani Jazi *et al.* 2019). Lignin structure (Fig.4) has been elucidated using several models. It plays a role of gluing together the different components of lignocellulosic conferring the natural hydrophobicity of the structure. Lignin, a cross-linked aromatic polymer of about 100 kDa in molecular weight, also presents a challenge in the breakdown of lignocellulosic material. The composition of lignin precursors is dependent on the type of plant, as well as the location of the polymer in the plant cell wall. These precursors consist of an aromatic ring, up to two methoxy groups and a 3-carbon side chain. This polymer is energy rich with a carbon content of as high as 40%. Hydrolysis of lignin would need pretreatment (Arni *et al*. 2007; Yu *et al.* 2018).

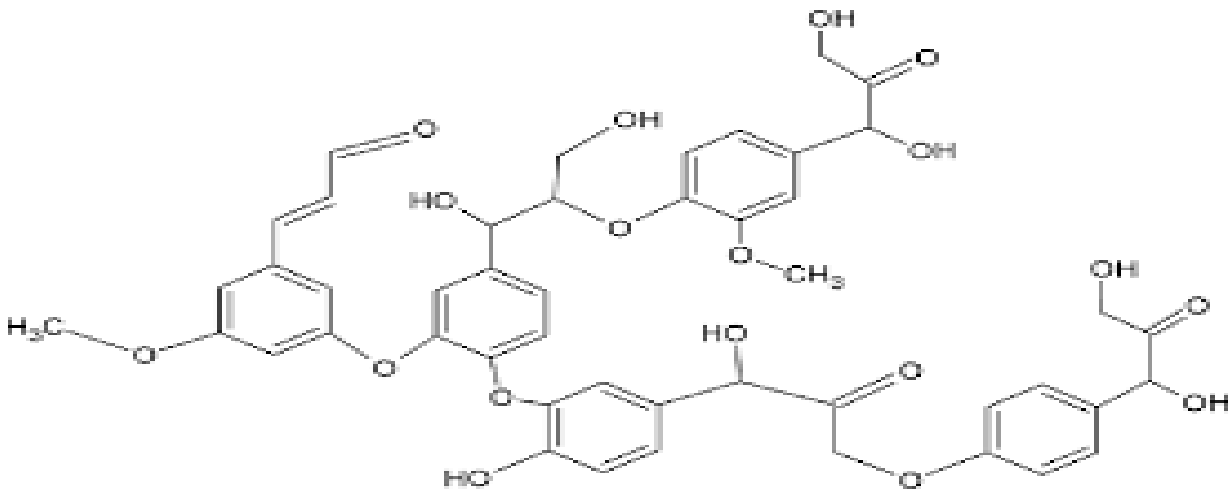


Figure 4: Structure of lignin (Maryana *et al.* 2014).

2.4. Pretreatment of lignocellulosic material

The need for second generation biofuels that can be produced from lignocellulosic material has encouraged attempts to find ways to break the recalcitrant and complex structure of these plant-based biomass. The complexity of the lignocellulosic structure prevents easy access to the reducing sugars monomers which are needed to make biofuel. Thus, efficient pretreatment methods to overcome the recalcitrant substrate are necessary (Kumar *et al.* 2009), to allow enzymatic access to the sugars present in the holocellulose component and break them down into fermentable sugars. There are three major components which contributes to the recalcitrant nature of lignocellulosic material are cellulose (35-50%), lignin (5-30%) and hemicellulose (20-35%) and the composition varies based on the source of the feedstock (Mankar *et al.* 2021).

2.4.1. Mechanical extrusion

Application of temperatures above 300˚C accompanied with thorough mixing has been commonly applied in pretreatment. The major disadvantage is the costs of the infrastructure required to implement these conditions, especially when considering scaling up at industrial level. The type of screw design, compression ratio, barrel temperature and screw speed influence the pretreatment of biomass (Zheng and Rehmann 2014; Duque *et al*. 2017).

2.4.2. Milling

Cellulose crystallinity can be significantly reduced by mechanical grinding, milling, and chipping. Pieces of 10-30 mm can be obtained through chipping but to get even smaller pieces (as small as 0.2 mm) there is need to employ milling and grinding. Mechanical pretreatment

prevents mass heat transfer loss, generates shear forces that disrupts cellulose crystallinity and the size of the biomass particles (Qu *et al.* 2017). The conditions needed for mechanical pretreatment are depended on the type of biomass and they create a larger surface area for enzymatic hydrolysis. There is no production of chemical inhibitors like furfurals and levulunic acid (da Silva *et al.* 2010; Patri *et al.* 2019).

2.4.3. Microwave

The use of low energy microwaves to generate heat in short durations can be employed to breakdown lignocellulosic biomass without generating inhibitors. The irradiation pretreatment can be done at selected power surges over specific time durations. Microwave irradiation was also applied together with the acid and alkali chemical pretreatment of Miscanthus. There was a 12-fold increase in the number of sugars released compared to conventional alkali and acid pretreatment over the same duration. The major drawback is that this is capital intensive (Choudhary *et al.* 2012; Aguilar-Reynosa *et al.* 2017).

2.4.4. Ultrasound

Ultrasound waves have both a chemical and physical effect on lignocellulosic biomass whenever they are used in pretreatment. The mechanism of action is due to small cavitation formation which cause the rupturing of hemicellulose and cellulose, thereby increasing the accessibility of cellulose-to-cellulose specific hydrolysis enzymes in-order to get simpler reducing sugars (Zhao *et al.* 2021). Various studies have employed ultrasound frequencies of ranges between 10-100 kHz when treating lignocellulosic biomass. Using sonication method with higher power presents its own set of challenges like costs and oxidation of cellulose (Xi *et al.* 2013; Hassan, Williams and Jaiswal 2018).

2.4.5. Pulsed electric field

Exploding lignocellulosic material at high voltage (5-20 kV/cm) over very short durations (nanoseconds to milliseconds) has been explored as a pretreatment alternative method. The mechanism is such that the pores in the cell membrane are opened thereby exposing cellulose to enzymatic attack. The short durations needed mean simple instruments that utilizes low energy can be designed making this method very attractive. There are reports of an increased total protein and polyphenol from rapeseed stem biomass exposed to this pretreatment. (Ade-Omowaye *et al.* 2001; Hassan *et al*. 2018).

2.4.6. Chemical pretreatment

The method that is most frequently used in pretreating lignocellulosic biomass is dilute acid hydrolysis. This was expediently adopted during the second world war in response to the need for utilization of agro-based waste as a source for reducing sugars. The major disadvantage is that use of dilute acid generates inhibitors in large quantities (phenolic acids, furfurals and aldehydes. This presents a challenge if the sugars generated need to be used in microbial fermentations (Bensah and Mensah 2013). Another issue is the corrosive nature of acids make it necessary to use material that are corrosive resistant. This complicates scale-up. Various studies have shown that sulphuric acid is mostly used, and it has been employed on different kinds of biomass that includes poplar, spruce, corn stover and switchgrass. A yield of 565 and 287 mg/g of simple sugars was produced from rice straw and wheat straw, respectively (Kim *et al.* 2013). There was less formation of toxic compounds as well. There have been efforts to pursue the use of much milder organic acids like maleic and oxalic acid as these would release less inhibitors. Oxalic acid was used on corncobs giving a yield of 13%5 sugar. Maleic acid favors cellulose breakdown into glucose (Badiei *et al.* 2014; Behera *et al.* 2014; Hassan *et al.* 2018).

2.4.7. Alkali pretreatment

To address some of the challenges faced with the dilute acid pretreatment method, alkali pretreatment methods have been employed (Jankovičová *et al.* 2022). These are less hazardous and demonstrates relatively lower amounts of inhibitors. Ambient parameters can generally apply to these methods. Ammonia, sodium hydroxide, potassium hydroxide and calcium hydroxide have mostly been the solutions applied. However, it seems like sodium hydroxide is mostly preferred. Alkali pretreatment changes the structure of lignin by breaking ester linked side chains and glycosides, breaks down hemicellulose to its monomers and reduces the crystallinity of cellulose. Ethanol production was carried out on rice straw that had been alkali pretreated. There is now a growing focus on alkaline hydrogen peroxide (AHP) biomass treatment. In the paper industry, this method is involved in pulping and bleach. Of greater interests is its ability to remove lignin and selectively retaining cellulose (Soares and Gouveia 2013; Maryana *et al.* 2014; Kim *et al.* 2016).

2.4.8. Ozonolysis

One pretreatment that has been used mainly for lignin removal is ozone treatment (Wang *et al.* 2022) . It breaks down the lignin component in the lignocellulosic biomass cell wall. It has been implemented on residues like bagasse, peanut, pine, wheat straw and sawdust. This treatment does not release inhibitory compounds and therefore allows for uninhibited enzymatic saccharification and microbial fermentation (de Barros Rda *et al.* 2013; Travaini *et al.* 2016)

2.4.9. Ionic liquids

The use of ionic solvents with a low melting point of below 100˚C, high polarity, high thermal stabilities, and low vapour pressure has been one of the pretreatments frequently used over the last two decades. The mechanism of action is that the ionic liquids untangle the lignocellulosic complex by attacking the hydrogen bonds present within the complex polymer structure (Reddy 2015). The amount of hemicellulose and lignin that can be broken down this way can be as high as 80% (Brandt *et al.* 2011; Li *et al.* 2011). They give high cellulose and lignin recoveries, but cost is the main constraint.

2.4.10. Physico-chemical methods

An example of the combination of physical and chemical pretreatment methods on lignocellulosic biomass is the steam explosion pretreatment. The physical treatment is mechanical in the form of pressure drop and the chemical the autohydrolysis of acetyl groups in the hemicellulose. High pressure (0.7-4.8 MPa) saturated steam is directed at the biomass at high temperatures (160–260˚C) for a few minutes' duration. Factors such as the size of the biomass sample, moisture content, temperature and the retention time determines the severity of the pretreatment regime. The entry of steam into the sample will result in the expansion of the cell wall and this partially degrade the biomass, thus providing a larger surface area for the enzymes to access and this has been done on wood chips, bagasse, rice straw and miscanthus. It is clean, cost effective and doesn't require a lot of energy input (Cruz *et al.* 2018; Hassan *et al.* 2018; Thite and Nerurkar 2019).

2.4.11. Liquid hot water pretreatment

Water at a high temperature (170–230˚C) and high pressure (5 MPa) instead of steam is also used in pretreatment of lignocellulosic biomass. This is also called hot compressed water

pretreatment. This use of liquid hot water dissolves hemicellulose and removes lignin. This makes the cellulose to be available for enzymatic hydrolysis without release of inhibitors, which would affect the subsequent fermentation. There are three ways in which this technique can be implemented. These are based on the flow of water within the reactor and the biomass. The first way would be to con-currently pretreat, that is, heat up the water and slurry together over a constantly fixed duration and temperature before allowing to cool. Secondly, counter-currently pretreat by flushing hot water at an optimum temperature into the biomass. Thirdly, to pump hot water into biomass and then have the hydrolysate drained from the reaction as a flow through (Brandt *et al.* 2011; Zhuang *et al.* 2016; Suriyachai *et al.* 2020).

2.4.12. Organosolv pretreatment process

The use of organic solvents commonly known as organosolv pretreatment to remove lignin and hemicellulose has also been pursued. This post solid-liquid separation would result in a fraction that is glucan rich with a high purity. This has led to intensive interests in this method with a wide range of organosolv solvents being tested. The different solvents include aliphatic alcohols like methanol, polyhydric alcohols like glycerol and organic acids. There has been comparative studies on the effect of various organosolv systems on lignocellulosic materials by Zhang *et al.* (2013b). The comparison is focused on the mechanism of action and the products of the pretreatment. What makes organosolv pre-treatment unique from the other pretreatment methods is that the catalyst is added during the pretreatment. This results in a high degree of hemicellulose hydrolysis and α- and β-aryl ether bonds cleavage in lignin (Zhang *et al.* 2016). Thus, this results in the fragmentation of hemicellulose and solubilizing the lignin. The protocol can be optimized to give a high purity stream using lower temperatures and shorter retention times. The lignin can be recovered from the glucan rich fraction by precipitation from the liquid, after solid-liquid separation of the stream. The precipitation can be done by diluting with water which would lower the solubility of the lignin leaving only the hydrolyzed components of hemicellulose in solution. Recovery and reusing the solvents is therefore critical when considering the economic feasibility and environmental concerns that would arise from the use of these solvents (Nath *et al.* 2021).

2.4.12.1. Combined severity factor in organosolv pretreatments

The combined severity factor considers the inclusion of the catalyst in the pretreatment, instead of only focusing on the parameters of stand-alone pretreatment in standard severity factor analysis. The severity factor is useful to predict lignocellulosic breakdown and to control the

process. It can also be used to predict the yield and purity after pretreatment. For the other pretreatment methods, the severity of the pretreatment protocol will directly influence the extent of the polymer breakdown and polymer recovery (like glucan) whilst at the same time resulting in the release of inhibitory chemicals. Thus, there must be a balance between hemicellulose breakdown; delignification on one hand and cellulose recovery on the other. The efficacy of organosolv, it has been hypothesized, depends on tailor making the system based on the sensitivity of the lignocellulosic biomass to hydrolysis. Rigorous conditions, with temperatures as high as 175-190 °C and 0.25-1% sulphuric acid concentrations would result in more inhibitors being released, despite the increased hydrolysis of hemicellulose. The conditions in organosolv pretreatment also affect the molecular weight of lignin which would ultimately determine its practical applications. A direct correlation between the extent of β-ether bonds cleavage the severity of the pulping process. The amount of phenolic compounds release as well as the potential to release antiradicals has also been directly linked to the applied temperature and catalyst loading (Hochegger *et al.* 2019). Increasing the process severity also lowers molecular weight and the presence of aliphatic hydroxyl groups. There are a few pretreatments methods that can hydrolyze lignocellulosic waste to fermentable sugars. These includes, alkaline pulping-based pretreatments, wet explosion, acid steam explosion and organosolv pretreatments. The concern, however is still on the costs, as they are still way too high to enable a feasible commercial application (Goh *et al.* 2011; Seibert-Ludwig *et al.* 2019; Vaidya *et al.* 2022).

2.5. Inhibitors released by pre-treatment processes

Pretreatment of lignocellulosic material breaks down the complex polymers by targeting intra and inter-hydrogen bonding as well as the glycosidic linkages. Breakdown of cellulose releases hexoses that can be dehydrated to 5-hydroxymethyl furfural. Other inhibitors that can be formed from the breakdown of hemicellulose include phenolic compounds, furan, alcohols and aldehydes. These affect the biological utilization of the lignocellulosic biomass. They slow down or inhibit the growth of fermentation microorganisms (Jonsson and Martin 2016; Yang *et al.* 2018; Kumar *et al.* 2020).

2.5.1. Detoxification of pretreated hydrolysates

It is important that inhibitors are to be eliminated for effective utilization of lignocellulosic biomass. There have been several approaches to achieve this, e.g., anion exchanger, treatment with reducing agents, addition of activated charcoal, overliming and allowing microorganism

adaptation to inhibitory conditions. These can be divided into three categories: physical; biological; and chemical methods (Chandel *et al.* 2007; Canilha *et al.* 2012; Cavka and Jonsson 2013).

2.5.2. Biological methods

Microorganisms or enzymes that are able to breakdown or utilize the inhibitors can be used for detoxification (Guo *et al.* 2022). Enzymes would be more advantageous because of their specificity, and they will not utilize the desired reducing sugars. If microorganisms are used, then this would have to be done by co-culturing as has been reported for the sequential co-culturing of *Thermoanaerobacter pentosaceus* and *Saccharomyces cerevisiae*. The *T. pentosaceus* utilized the furfural inhibitors in this fermentation. *Trametes versicolor* has been reported to be able to breakdown phenolic compounds in co-culturing with *S. cerevisiae* (Maurya *et al*. 2015; Sindhu *et al.* 2016).

2.5.3. Chemical detoxification

There are various examples of chemical detoxification that includes, ion exchange, overliming, solvent extraction and activated charcoal. There have been extensive reports on the use of activated charcoal after saccharification with enhanced fermentation results. Activated charcoal removes inhibitors through adsorption. Reducing agents and lime can also be used but the issue is they are very costly especially when you want to scale up industrial production. Reducing agents like sulphite and dithionite have been reported to enhance ethanol production as high as 5-fold on bagasse hydrolysate. The mechanism of action on how overliming detoxify is not clear but it seems there is a combination of precipitation and chemical conversion at high pH. Precipitation is effective against on levulinic and acetic acid inhibitors as they would be neutralized (Jönsson *et al.* 2013; Kim 2018).

2.5.4. Physical detoxification

The most common methods for physical detoxification are membrane separation and evaporation of the inhibitors in the hydrolysate. Some of these inhibitors like acetic acid, furfural, and some lignin degradation chemicals are volatile and can be reduced by evaporation. While evaporation is a simple procedure that does not cost much in terms of infrastructure to implement, it creates high energy demand. However, it can play a dual role of removing inhibitors and regulating the concentration of sugars in the hydrolysate (Luo *et al.* 2021). Removing the inhibitors using

organic solvents like ethyl acetate and trialkylamine can be applied. The major challenges to this approach are scaling up, emulsifying obstacles and flooding. Thus, membrane extraction becomes more attractive. The advantage is that there is no need to have two phases. The membrane can be modified, as has been reported where the polypropylene membrane base were modified via deposition of polyelectrolytes enabling the removal of different kinds of inhibitors. What would give solvent extraction a small advantage though would be the possibility of recycling which would enhance feasibility. These methods can be combined synergistically, like over-liming followed by charcoal method or over-liming followed by adsorption using ion-exchange column (Marton *et al.* 2006; Kim 2018; Kumar *et al.* 2020).

2.6. The use of fungal biomass

Filamentous fungal microorganisms generate interest due to their ability to grow on a wide range of substrates, including agro-based and lignocellulosic waste. These can then be subsequently scaled-up in bioreactors. Zygomycota, Ascomycota, Chytridiomycota, Basidiomycota and Deuteromycota make up the fungal kingdom. Two fungal classes have been extensively used in various fermentation processes: zygomycetes and ascomycetes (Ferreira *et al*. 2013). Zygomycetes have been reported to be the highest chitosan accumulators (Nyman *et al*. 2013; Vaingankar and Juvekar 2014). Some species which belong to the zygomycetes like *Mucor sp*. have also been reported to be oleaginous. Zygomycetes are mainly saprophytes which are fungal strains growing on dead organic matter. Some have also shown pathogenicity in plants and animals; however, suitably selected strains have been used in the food industry. They can grow on different kinds of carbon sources from glucose to complex polymers like cellulose. Zygomycetes biomass contains chitosan, chitin, amino acids, lipids, and proteins which can be present in substantial quantities. This makes it suitable as a feed for both humans and animals. They demonstrate versatility by growing in different cultures, media and conditions. It is important to have inexpensive unspecific substrate for biomass production to ensure that the process is economically viable. The range of carbon and nitrogen sources in which they can grow is wide, including complex sugars and lignocellulosic material. The ascomycetes are a bigger group when compared to zygomycetes. Consequently, they have a wider range of applications. The production of antibiotics by *Penicillium chrysogenum* has made this species one of the most studied industrial strains together with Aspergillus griseus. Aspergillus sp have been widely used in fermentations to produce organic acids (Ferreira *et al.* 2016). They produce enzymes that break down recalcitrant industrial residues and lignocellulosic wastes. Filamentous fungal growth allows easier biomass harvesting as opposed to single cell cultures. Non-filamentous and

unicellular members of the phylum ascomycota have also been found to be producers of fermentation products like organic acids, amino acids, fatty acids, lipids etc. Therefore, the use of fungal biomass in biorefineries is a critical, relevant, and strategic development to the endeavor of establishing feasible biorefineries, in that, fungal biomass offers a different and ever-growing spectrum of products. The production of organic acids, amino acids, enzymes and antibiotics has already been reported using different fungal fermentation techniques (Pietrzak *et al.* 2016; Ahmed *et al.* 2017).

2.7. Co-culturing of microbes

In the natural environment microorganisms do not exist in isolation but rather in complex microbial communities where there is competition and cooperation. Thus, co-culturing is one way to mimic the natural environment to address challenges and concerns arising from microbial fermentations. Whenever lignocellulosic biomass hydrolysate is being used for ethanol production by *S. cerevisiae*, there are two fermentation challenges that arise and these are, the potential presence of inhibitors in the hydrolysate which would lengthen the lag phase and lower the ethanol output. The other challenge is that *S. cerevisiae* cannot utilize the five-carbon sugars that are readily available in the hydrolysate. Therefore, there is a need to pursue the idea of co-culturing to address this. The *S. cerevisiae* can be co-cultured with other microorganisms that removes inhibitors and utilize the five-carbon sugars (Chen 2011; Duarte *et al*. 2013).

2.8. Microbial degradation of plant cell wall polysaccharides

Microorganisms have developed various physiological mechanisms to adapt to the environment and to thrive. The most critical activity for microorganisms is nutritional uptake, especially when considering that the nutrients are outside the cell. Competition in the natural environment means that the simple and readily available nutrients are not easy to come by. Thus saprophytism, is common amongst microorganisms. This involves the release of enzymes by the microorganism on dead complex organic matter, breaking it down extracellularly and then absorbing the nutrients. These enzymes can breakdown the complex polymers in the plant biomass releasing simple sugars that the microorganism can utilize. This is important for the ecosystem as it completes the carbon cycle. Focus has been set on these extracellular enzymes for industrial applications. Most of the microorganisms that have these saprophytic properties are fungi and bacteria. A select genus of bacteria such as, *Ruminococcus, Caldicellulosiruptor, Cellulomonas, Erwinia, Yersinia, Fibrobacter, Cytophaga, Acetivibrio* to name but a few. However much

shifted on fungal cellulose utilization due to the potential of high enzyme titres. The saprophytic cellulose utilization is across the entire fungal kingdom (de Souza 2013; Sindhu *et al*. 2016).

2.9. Fungal enzyme sets for lignocellulosic degradation

Fungi produce a cocktail of lignocellulosic degradative enzymes which carry out saprophytic breakdown of the polysaccharides. The composition of the cocktail is unique to different fungal species. Thus, it is important to comprehend fungal diversity which will enable better access to the different cocktails available in the natural breakdown of lignocellulosic matter with the view to adopt a biological protocol for industrial applications (Andlar *et al.* 2018).

Enzymes that breakdown carbohydrates are usually categorized based on the catalytic centre amino acid sequence. In fungi, these carbohydrate-active enzymes mostly have a carbohydrate binding module, which allows for the interactions between the substrate and the enzyme. There are at least 35 glycoside hydrolase, 3 carbohydrate esterase and 6 polysaccharide lyase families amongst these fungal derived enzymes which act on plant biomass degradation. Classification allows for focus on specific enzymes, however there are still some challenges because some families may have more than one enzymatic activity. The complex nature of the polysaccharide matrices in the biomass points towards synergistic cooperation by these carbohydrates degrading enzymes (Breen and Singleton 1999).

Lignin is a polymer that is worth more in its intact form than when its degraded. There are fungal classes like Actinomycetes and Basidiomycetes which produce enzymes that degrade lignin. Extracellular peroxidases have been reportedly produced by *Phanerochaete chrysosporium* and *Phlebia radiate,* with *Coriolus tersicolor* reported to produce haem peroxidase intracellularly (Barber-Zucker *et al.* 2022). A synergistic combination of laccase and manganese peroxidase have been shown to solubilize lignin (Hatakka 1994).

2.9.1. Cellulose degradation

Three classes of enzymes are needed to breakdown the β-1,4-linked D-glucopyranose monomers during cellulose breakdown. These are β-1,4-endoglucanases, exo-glucanases or cello-biohydrolases and β-glucosidase. The internal cleaving of cellulose chains in the amorphous region is carried out by endo-gluconases which release derivatives to be further hydrolyzed by cellobiohydrolases and β-glucosidases. Cellobiohydrolases work from the end of the polysaccharide chain by cleaving the disaccharides derived from cellulose also known as

cellobiose. The cellobiose is then broken down by β-glucosidases to glucose monomers which can be utilized by the microorganisms (Romsaiyud *et al.* 2009).

For complete degradation of the cellulose complex there must be synergistic and sequential cooperation between the three cellulose degrading enzymes. This involves the endo cleaving by β-1,4-endoglucanases, the exo-cleaving by cellobiohydrolases and the breaking down of the released oligosaccharides to glucose by β-glucosidases. *Trichoderma reesei* has been reported to be the most efficient when it comes to breaking down cellulose because of the synergistic cooperation of the hydrolysis enzymes even though it is not known to have the widest spectrum of cellulose degrading enzymes. The β-glucosidases from this strain are prone to negative feedback product inhibition. Thus, in industrial applications the cellulose degrading enzymes from *Trichoderma reesei* would have to be supplemented with β-glucosidases from other fungal strains like Aspergilli (Beguin and Aubert 1994; Eibinger *et al.* 2014).

2.9.2. Hemicellulose degradation

Hemicellulose is a composition of mannan, xylan and xyloglucan which make up three backbones that are entangled into a complex polymer. The complex nature of the bio structure would require synergistic and concerted enzyme cooperation to break it down. This involves endo-enzymes attacking the main chain internally, exo-enzymes cleaving from the end to release oligomers and accessory enzymes dealing with the side chains as well as the oligosaccharides released until monosaccharides are released (Lopez-Mondejar *et al.* 2016).

The degradation of xylan is done by ß-1,4-endoxylanase and which breaks down the ß-1,4-linked D-xylose units backbone into oligosaccharides and β-1,4-xylosidase which then breaks down the oligosaccharides into xylose. There is a variation in fungal ß-1,4-endoxylanases because of a difference in substrate specificity. Those that have a broader substrate specificity have been shown to be effective in breaking down highly branched and substituted xylan. They are also effective in breaking down smaller xylo-oligosaccharides after degrading the linear chains of the 1.4-linked D-xylose residues. To complete the breakdown of the xylan, ß-xylosidases which are specific for unsubstituted oligomers must be incorporated. There has also been reports of ß-xylosidases being involved in the making of specific oligosaccharides through a process of transxylosylation (Broeker *et al.* 2018). Xyloglucan is made up of D-glucose monomers linked with a ß-1,4 glycosidic bond substituted with mainly D-xylose. It therefore requires a combination of ß-glucosidases and (endo) xyloglucanases. There has been reports of some endoglucanases which only cleaves specifically the xyloglucan substituted backbone, without being able to breakdown cellulose. Xyloglucanase from *T. reesi* reportedly cleaved at branched

glucose residues, while xyloglucanase from *A. niger* was more specific on xylogluco-oligosaccharides six glucose residues and above, with at least one of the glucose residues not being branched.

Mannans are made up of a ß-1,4-linked D-mannose backbone. There are also D-galactose side chained with D-glucose residues. ß-endomannanases (ß-mannanases) and ß-mannosidases are needed to breakdown this hemicellulose. ß-mannanases breaks down the galactomannans backbone to give off mannooligosaccharides. There are factors that affect the efficacy of ß-mannanases in breaking down the mannan backbone. These include mannose to glucose ratio and the degree of substitutions in the backbone. ß-mannanases activity is deterred by galactose residues attached to the mannan backbone. Mannobiose and mannotriose are the products mainly released from ß-mannanase breakdown of mannan. ß-Mannosidases then releases mannose by attacking the non-reducing ends of the mannooligomers. Terminal mannose units can be released if the adjacent subunits are not substituted (Houfani *et al.* 2020). The main extent of substitutions in the hemicellulose structure is such that there is need for different accessory enzymes to complement each other if all the substitutions are going to be removed. This complete breakdown of hemicellulose can only be realized after the substitutions are removed from the backbone first.

Another common sugar in hemicellulolse which exists is xyloglucan a xylan substitute. The enzymes α-arabino furanosidases and arabinoxylan arabino furanohydrolases breakdown the polymer to release arabinose. There are differences in the substrate specificity amongst these enzymes as highlighted by a report on two arabino furanosidase enzymes from *A. niger* ,one was able to specifically breakdown arabin and sugar beet pulp and release L-arabinose whilst the other specifically broke down xylan to release L-arabinose (Patel *et al.* 2015). The α-1,3- and α-1,2-linkages that binds the L-arabinose units in arabinoxylan are attacked by arabinoxylan arabino furanohydrolases, however, the enzyme is sensitive to the presence of D-xylose substitutions as reported on the arabinoxylan arabino furanohydrolases from *A. niger* failed to cleave arabinobiose from xylan because the D-xylose residues were adjacent to D-glucuronic acid residues. The enzymes responsible for the removal of the D-xylose residues are α-xylosidases, with the differences among this set of enzymes being based on the type of glycoside they hydrolyze (Thygesen *et al.* 2003; Bomble *et al.* 2017).

2.9.3. Pectin degradation

Pectins are made up of an α-1,4-linked D-galacturonic acid backbone. There are two distinct regions, the smooth region consisting of methylated or acetylated D-galacturonic acid residues and the hairy region, which has the same backbone of D-galacturonic acid with α-1,2-linked L-

rhamnose residues interruptions. The L-rhamnose side chains can also have side chains of D-galactose and L-arabinose if they are longer. The breakdown of pectins, just like that of hemicellulose and cellulose requires synergistic enzyme action. Polysaccharide lyases and glycoside hydrolases are the two sets of enzymes involved in breaking down the pectin backbone (Benoit *et al.* 2012).

Endo-polygalacturonase and exo-polygalacturonase are the glycoside hydrolases involved in breaking down of the backbone in the smooth regions. Xylogalacturonases, exo-rhamnogalacturonases, endo-rhamnogalacturonase, α-rhamnosidases, glucuronyl hydrolases (unsaturated) and rhamnogalacturonan hydrolases are involved in further hydrolysis of the hairy regions. The α-1,4-glycosidic bonds in the galacturonic acid backbone are broken-down by a combination of exo and endo-polygalacturonases. The α-1,2-glycosidic bonds that attaches L-rhamnose branch to the pectin backbone hairy region are broken down by rhamnogalacturonases. There hasn't been much characterization of enzymes like glucuronyl hydrolases, α-rhamnosidases and rhamnogalacturonan hydrolases and this is necessary for the breakdown of the main pectin chain (Abbott and Boraston 2008).

Pectin lyases and pectate lyases from fungi have been studied as well as their involvement in the breakdown of α-1,4-linked D-galacturonic acid residues backbone found in the smooth regions. A greater extent of methylesterification is preferred by pectin lyases whilst lower extent of esterification is preferred by pectate lyase. Calcium cations are needed by pectate lyases to catalyze and this is not necessary for pectin lyases (Ali *et al.* 2015). Rhamnogalacturonan and xylogalacturonan pectin structures are such that they require various enzymes, whose action range from removing the side chains and opening up the backbone to pectinolytic enzymes attack (Garg *et al.* 2016).

2.9.4. Lignin degradation

As stated earlier, lignin plays the role of strengthening the plant cell wall and giving its recalcitrant properties. It is insoluble due to its branched nature, with phenylpropane substitutes attached via ether and carbon-carbon linkages. This provides a network that is cross linked throughout the cell wall. Breaking down the lignin structure is complicated, even for microbes because of three major structural challenges which are; the size of the lignin polymer (which would require that all the enzymes involved are extracellularly produced; the carbon-carbon and ether bonds (which requires the enzymes' mode of action has to be oxidative instead of hydrolytic),the irregularity of the stereochemistry of the lignin (which would require enzymes characteristics of less specificity compared to hydrolytic enzymes). There are various enzymes

that are able to do this and these are peroxidases (lignin, manganese) laccase, aryl alcohol oxidase and glyoxal oxidase (Janusz *et al.* 2017).

Catalysis by peroxidases involves a series of oxidative steps dependent on hydrogen peroxide. The non-phenolic elements of lignin are oxidized by lignin peroxidase. This is done by removing electrons to oxidize cationic radicals which then go through chemical decomposition. Manganese peroxidases differ in that they do not directly oxidize to create the non-phenolic radicals but rather uses Mn^{3+}, which is a product of the Mn^{2+} oxidation reaction, a reaction that manganese peroxidases catalyze. The Mn^{3+} then attacks the lignin structure from the inside (Hatakka 1994; Arni *et al.* 2007).

Phenolic compounds are oxidized by laccase which results in the reduction of molecular oxygen to water. Laccase catalyzes the oxidative genesis of phenoxyl radicals as well as their spontaneous reactions. This would see the conversion of the phenolic group to ketone, cleavage of alkyl-aryl and phenolic substructure Cα-Cβ bonds. The versatile peroxidases are even broader in their specificity from, phenolic to aromatic compounds without the phenol group and even Mn^{2+} oxidation (Brown and Chang 2014).

Microorganisms would need an extracellular supply of hydrogen peroxides to be able to breakdown lignin using peroxidases. The hydrogen peroxide in nature which would be accessible to these microbes would arise because of the reduction of the oxygen molecule to hydrogen peroxide by extracellular oxidases. Enzymes like glyoxal oxidase and alcohol oxidase are involved in this generation of hydrogen peroxide.

Pure cultures of white rot fungi have been reported to effectively mineralize lignin to carbon dioxide and water, hence the extensive studies on these particular fungi (Hou *et al.* 2020). *Pleurotus ostreatus*, *Ceriporiopsis subvermispora*, *Phanerochaete chrysosporium* and *Phlebia subserialis* have been reported to breakdown lignin in lignocellulosic biomass utilization. The brown-rot fungus, *Postia placenta,* has also been shown to metabolise lignin (Riley *et al.* 2014). There are also some bacterial species that have demonstrated lignin-metabolising ability such as *Marinomonas mediterranea* and *Azospirillium lipoferum. Pycnoporus cinnabarinus* is regarded as having very high lignocellulosic biomass utilization potential because it overproduces a laccase with high redox potential. Subsequent studies focused on producing different laccases for various industrial applications using this saprotrophic homobasidiomycete (Del Cerro *et al.* 2021). This would include using an *A. niger* host in heterologous expression. *Cyanthus bulleri* and *Cyanthus cinnabarinus* also part of the white rot fungi family demonstrated an ability to degrade lignin (Bomble *et al.* 2017).

The composition of the biomass to be utilized will determine the enzyme complex necessary for its breakdown. What is clear is that on top of the arrangement of the various enzymes in the complex, the degradation is reliant on a concerted action of the enzymes. The process of gene expression up to secretion from the microbial cell is a resource intensive process hence it is tightly regulated. The induction of the gene expression of various enzymes which breakdown lignocellulosic waste is an important tool in the production of these enzymes in a fermentation process (Janusz *et al.* 2017; Lee *et al.* 2019).

2.10. Utilisation of lignin

The concept of the cellulosic biorefinery has been commercially pursued in Brazil, USA and the EU, however, the full potential of the concept has not been realized yet. One way to get closer to that is to look at lignin, which is a major constituent in the lignocellulosic biomass, as a potential product of value. Lignin from bagasse is of better quality when compared to lignin from other sources. Lignin can be used to generate energy which can then subsequently be used to power the onsite biorefinery operations (Obydenkova *et al.* 2019). Another option would be to convert the lignin to other high value products.

Lignin is the best source for natural aromatic compounds and has various chemical applications (Obydenkova *et al.* 2017). Phenols, phenolic resins, carbon fibres and polyurethanes are some of the chemicals derived from lignin. For this wide range of products to be manufactured commercially, there must be viable lignin production in terms of the costs involved and the quantities produced. The initial prudent step would be to look at lignin as an energy source for the biorefinery. Prioritizing its conversion to other chemical products of value can only be based on a proven sustainable energy strategy for the biorefinery put in place first, or a sufficient energy source if a surplus is attained. It has been reported that, in order to achieve energy self sufficiency for a biorefinery producing ethanol from lignocellulosic material, a 40% yield of dry lignin was needed (Sassner *et al.* 2008) also suggested that for ethanol production levels of around 34-40% would suffice. It has also been reported that an energy output surplus of 10-13 MW can emerge from utilizing lignin and biogas. It is important to evaluate how much lignin can be extracted from the process that produces the biorefinery target (Bu *et al.* 2014; Obydenkova *et al.* 2019; Zhou *et al.* 2022).

2.11. Ethanol production from lignocellulosic biomass

Most bioethanol production is done using starch or sucrose as the substrate. These substrates are derived from agricultural crops like sugarcane, corn, and wheat. Brazil is the world's largest producer of ethanol from yeast fermenting sugarcane substrate. The processes involved are already established. The need to obtain ethanol as a biofuel means production must be enhanced which would optimize the economies of scale, thus the use of lower substrates like lignocellulosic biomass is currently being pursued. The processing of sugarcane produce involves milling the sugarcane to get sucrose and this produces large quantities of sugarcane bagasse which is a rich source for lignocellulosic polymers. Bagasse offers the advantage that it's a waste and addresses food security concerns; production of ethanol from lignocellulosic material is dependent on the efficacy of the following biological processes (Alabi 2022). These are access to free cellulose and hemicellulose through delignification, the breakdown of the cellulose and hemicellulose to release free sugars and the utilization of the mixture of 5-carbon and 6-carbon sugars in a fermentation process to produce ethanol. Most of the free sugars released during breakdown of lignocellulosic material is glucose which can be as high as 60%. *Saccharomyces cerevisiae* has been proven to be the best ethanol producing yeast. The challenge, however, is its inability to utilize the 5-carbon sugars like xylose and arabinose which are significantly present in the hydrolysate.

Microorganisms that are able to utilize 5-carbon sugars to produce ethanol are *Pichia stipitis*, *Pachysolen tannophilus* and *Candida shehatae* with *P. stipitis* being the best producer under very low aeration conditions (Busic *et al.* 2018). This ethanol production from 5-carbon xylose, however, cannot be compared to ethanol production by *S. cerevisiae* glucose fermentation. Genetic engineering tools have been used to try and enable *S. cerevisiae* to utilize 5-carbon sugars as well as adopt a high ethanol tolerance level to boost the ethanol yield. Genetic engineering to enable utilization of 5-carbon sugars has been expanded to *Escherichia coli*, *Zymomonas mobilis* and *Candida utilis.* Candidate genes to be added to the yeast strains to be engineered can always be obtained from studying fungal degradation of lignocellulosic material (Canilha *et al.* 2010; Deesuth *et al.* 2012; Maryana *et al.* 2014; Pietrzak *et al.* 2016; Hossain *et al*. 2017).

2.12. Supplementation of external nitrogen sources and growth factors

To enhance utilization of sugars in ethanol production and subsequently increase ethanol production, it is important to supplement the medium with an exogenous nitrogen sources like

peptone, ammonium nitrate, ammonium sulphate, yeast extract, malt extract etc. This works best in the presence of vital cofactors like riboflavin and biotin (Sato *et al.* 1992). There has been reports on *S. cerevisiae* YS preferring corn steep liquor over yeast extract and peptone. This was attributed to the presents of other nutrients in corn steep liquor like water soluble vitamins, minerals, and amino acids. Bloom algae powder also enhanced ethanol production under high gravity technology. The use of distillers dried grains, yeast extract and ammonium sulphate were reported not to improve ethanol production when using a thermotolerant *S. cerevisiae* DQ1 on solid state fermentation using corn stover, thus it becomes imperative to ensure that the supplementation is balanced according to the needs of the microorganism together with other physico-chemical parameters that influences growth. (Deesuth *et al.* 2012; Li, Wang and Shi 2017; Almeida *et al.* 2020)

2.13. Fish feed

Concomitant with the ever-growing human population are concerns about food security. Proteins are a key consideration because their deficiency is a serious threat to living organisms. The challenge now is that the protein food sources are either in short supply, that is, are unable to meet the growing demand and even when they are available, they are beyond the economic reach of most. This brings into perspective the rapid growth in aquaculture. The Food and Agriculture Organization (FAO) has reported in their 2022 State of World Fisheries and Aquaculture report that fish production is to record a 14% growth by 2030. On the contrary, wild fish capture fisheries have not seen the same level of growth and marine capture which makes up the bulk component has been on a steady decline. As of now, of the 177.8 million tons of the total fish produced, 49.2% is coming from fish aquaculture.

Fish farming is heavily dependent on a protein rich feed supplement. The major ingredient in this feed supplement is fishmeal. Fishmeal is a protein supplement from fish that is not suitable for human consumption, and this is in most cases wild fish. This means the decline in the capture of wild fish will affect the availability of fish meal. This shortage *vis a vis* the increase in fish aquaculture production, allows the economic law of supply and demand which has seen a sharp rise in the price of fishmeal. Meeting this demand is only possible by finding an alternative. This alternative must be a protein source that has nutritional values that are compatible to the aquaculture requirements. This compatibility is in terms of protein content, amino acid profile and amino acid balance. Various sources which include rapeseed, lupine, soybean meal, cotton seed meal, corn gluten amongst others, have been investigated. However, the challenge encountered has been their low digestibility, which would mean lower intake of the feed. There

is also an issue of their potential adverse effect to the intestinal microbiota of the fish. Then there are also issues of the presence of antinutrients like phytic acid, protease inhibitors, tannins, saponins and antivitamins which would impair the immune system and reduce growth. Whilst animal-based protein supplements have the obvious advantage over the plant-based protein supplements in that they don't present the mentioned challenges, but the issue of high costs is the biggest impediment, after all this is a business and cost-effective inputs would enhance profitability hence viability. The use of material that can potentially be used for human consumption is always frowned upon and is subject to ethical criticism, there is therefore a need for biotechnological solutions to address these challenges. (Zhang *et al.* 2013a; Ritala *et al.* 2017; Glencross *et al.* 2020; Jones *et al.* 2020)

2.14. Single cell biomass proteins

Microorganisms like fungi, bacteria and microalgae are known as single cell protein sources. These can have the potential to be utilized as a component of fish feed. The versatility of filamentous fungi which allows them to grow on broad spectrum of organic matter, makes them very attractive (Derbyshire and Delange 2021). Fungi have a high protein content; they have fatty acids and other nutrients which would stimulate growth. Filamentous fungal fermentations are already being applied in various industries like food, pharmaceuticals, agriculture etc. Their ability to produce cocktails of enzymes that can breakdown complex inexpensive polymers has seen the increased use of these polymers as substrates in various fermentations (Bratosin *et al.* 2021).

This nutritional assimilation results in the formation of products which are of economic value as well as biomass. A lot of fungal species are categorized as generally regarded as safe paving the way for their use in the food industry. *Neurospora sp* and *Aspergillus sp* have been reported to be extensively used traditionally in some East Asian indigenous dishes and beverages. *Fusarium venenatum* was reported in the production of a mycoprotein called quorn. The amount of protein in filamentous fungus can be as high as 50% dry weight, depending on the culture conditions and type of fungal strain. The major obstacle, however, is the costs of the cultivation medium. Even when replacing the much more expensive inorganic components, still the organic dominant cultivation medium must be of low costs for the product to be of competitive pricing. This therefore directs focus to the use of waste biomass as a culture medium (Ritala *et al.* 2017; Bratosin *et al.* 2021).

2.15. Biorefineries

A biorefinery is the culmination of the sustainable processing of biomass into a spectrum of marketable products and energy. The application of petroleum refinery methods to biomass production brings about the biorefinery concept. This normally entails producing bio-based chemicals as well as biofuels. Bio-based heat and power can be generated too (Bu *et al.* 2014). It has been suggested that the ideal biorefinery scenario is one where a bio-fuel is produced along with a bio-based chemical bioproduct (Galbe and Wallberg 2019). The efficiency of these biorefineries can be enhanced when the heat and energy released during the generation of one product is used for another co-product as well as the further development of the co-product (Ferreira *et al.* 2016). The setting up of biorefinery industries are likely to result in further development, especially to rural areas. These biorefineries have the advantage of flexibility in terms of size unlike petroleum-based refineries which are always large. The different sizes also mean different biorefinery installations can combine in terms of material flow, thus completely utilizing the biomaterial which may be locally available in most rural settings. The production of biofuels for the transportation sectors is expected to drive increased investment in biorefineries. This will be due to the high demand for fuel. That means the success of biorefineries is hinged on the feasible production of biofuels, which is still a major issue of concern as the feasibility of numerous bio-based chemicals has already been proven (Ferreira *et al.* 2013).

2.15.1. Biorefinery platforms

There are four platforms that make up a biorefinery and these are mechanical, chemical, thermochemical and biological conversions (Takkellapati *et al*. 2018). These result in the actual conversion of the biomass into products of value, be it primary or secondary products. Primary products are those directly derived from the biomass, and they are still raw, whilst the secondary products are the refined primary products. Secondary products arise because of value-addition. It is important to have a higher yield of products and at the same time it should be easy to separate these products if successful biomass valorization is to be achieved (Takkellapati *et al.* 2018; Tong *et al.* 2022).

2.15.2. Process integration and optimization

A sustainable biorefinery concept would have to incorporate the integration of processes with aim to optimize production and minimize costs (Walmsley *et al.* 2018). It is the conversion

processes that must be integrated holistically. This process integration is meant to achieve optimal production requirements, at the same time reducing the raw materials and energy used. It is also key to the objectives of making the entire process greener by reducing wastes and emissions (Klemeš and Kravanja 2013; Al-Aboosi and El-Halwagi 2018).

2.15.3. Lignocellulosic biorefineries

Lignocellulosic waste is a cheap and rich source of carbohydrates. Thus, they are an important raw material in any large scale biorefinery implementation as they offer the obvious advantages of being sustainable, greener, and not affecting food security. These are regarded as second-generation biomass feedstock, and they are a direct response to the concerns raised with the use of first-generation feedstock that competes for arable land with food crops. The wide spectrum of plant biomass from which the lignocellulosic waste is derived, makes them a very practical solution especially in tropical climates. Annually, about 1.3 billion tons of lignocellulosic waste are generated with only a paltry 3% being utilized in any way (Baruah *et al.* 2018; Usmani *et al.* 2021). Various sources of lignocellulosic waste include wheat, sugarcane, bagasse. sorghum stalks, wood. rice, coconut husk, barley straw.

2.15.3.1. Biomass valorization

This involves value addition to the biomass. A biorefinery, with all its conversion technologies, provides a platform for biomass valorization. Before selecting the biomass to use in a biorefinery, there has to be a thorough assessment of the availability of the biomass both in terms of quantity and quality. Thus, an estimate of what is potentially available quantitatively in a particular geographical region and a characterization of the available material to determine potential use is critical (Ubando *et al.* 2020; Okolie *et al.* 2022). There is a possibility that the energy stored in biomass can be released and used as renewable heat or electricity. Dry biomass cand go through combustion and gasification to generate heat energy. The gasification process would involve anaerobic microbial digestion. The heat can then be transformed to electric power which has been one of the most efficient uses of the biomass.

2.16. Gaps in literature

Current trends in bagasse pretreatment are focused on alkaline pretreatment. This strategy, as stated, involves dissolving lignin thereby exposing the cellulose and hemicellulose to enzymatic hydrolysis. The main challenges to this are the release of fermentation inhibitors which makes the hydrolysate application as microbial fermentation processes media, problematic. The

hydrolysate would have to go through another complex process of detoxification to be able to be utilized by microbes. Using alkaline solvents would need numerous washing steps of the substrate to neutrality before setting the conditions necessary for enzymatic hydrolysis. This consumes a large quantity of water. Thus, an ideal pretreatment method has not yet been found. Even though pretreatment is now more inclined towards alkaline solvents there hasn't been deliberate efforts to integrate pretreatment with lignin recovery. These initiatives have been pursued separately.

There is currently no commercial production of microbial fungal chitosan. There is no commercial production of biodiesel from microbial oils. There is also limited commercial production of bioethanol from lignocellulosic material. The major issue in all the scenarios is the extremely high cost of production. One way of addressing costs is to increase the product(s), quantitatively and qualitatively. In addressing this, the first successful step which was done in previous work, was to concomitantly produce chitosan with lipids from *M. circinelloides*. The second step would be to replace the expensive defined media with hydrolysate from agro-based residue and even this hydrolysate would have to be produced at minimum cost. The third step would be enhanced utilization of the hydrolysate in ethanol production. There have been previous attempts to use co-cultures in ethanol fermentations, but non within the concept of a biorefinery and under pulsed aerobic and anaerobic conditions. The fourth step would be enhanced valorisation of the microbial biomass (*M. circinelloides* and *S. cerevisiae*). The chitosan and lipids concomitant production from fungal biomass strategy could be further modified to add more valuable products as this has not been done. The integration of sugarcane bagasse and microbial biomass valorisation in full implementation of the biorefinery concept has also not been done. An integrated approach to value addition of biorefinery products is another area that has not been exhaustively pursued.

CHAPTER 3: INTEGRATED BIOREFINERY OF *Mucor circinelloides* BIOMASS AND SUGARCANE BAGASSE FOR APPLICATION OF HIGH-VALUE BIOPOLYMERS

3.1. Introduction

According to the International Energy Agency (IEA) Bioenergy Task 42, a biorefinery is the sustainable processing of biomass into a spectrum of marketable products and energy. The global biorefineries market is expected to reach USD 52 680 million by 2027, at a compound annual growth rate of 2.2%. The efficiency of these biorefineries can be enhanced by producing value-added co-products during the process. Sustainable and economical bioconversion process is a prerequisite for the biorefinery concept. Sugarcane based biorefineries have been a leading approach in the production of cellulosic biofuels (Galbe and Wallberg 2019; Zetterholm *et al.* 2020).

With an annual production of about 25 million tons, South Africa is the largest producer of sugarcane in Africa. However, the country also produces about 7.5 million tons of bagasse annually during sugarcane processing, which largely remains underutilized (Mashoko *et al.* 2013). Therefore, efficient utilization of this waste biomass becomes expedient. This can be achieved by incorporating processing steps for the wastes generated after various conversion steps. The utilization of biomass such as bagasse, which contains complex polymers like cellulose, hemicellulose and lignin is performed by a combination of physico-chemical pretreatment methods and enzymatic breakdown of the free cellulose and hemicellulose after pretreatment. Despite active research on the use of sugarcane bagasse (SCB) to bioethanol and other biochemicals, low saccharification due to recalcitrance of biomass remains a global challenge (Guna *et al.* 2019; Patel *et al.* 2019).

Pretreatment is a crucial step in biorefinery process that facilitates enzymatic access to the trapped cellulose within the crystalline structure of lignocellulosic and agro-based residue like SCB, for subsequent hydrolysis. Pretreatment enhances the access of saccharification enzymes to the carbon polymers. During the last few years various chemical, physical, and biological pretreatment methods have been developed to expand the surface area, improve the overall porosity, dissolve the hemicellulose and lignin and reduce the particle size of the recalcitrant lignocellulosic biomass. However, there is an immense variation in composition and structure of lignocellulosic biomass, and therefore no single universal pretreatment method can be applied to all types of lignocellulosic biomass. There is a need to combine the pretreatment processes to achieve a balanced strategy with higher efficacy. Additionally, removal of sturdy lignin, which

is highly resistant to solubilization, is a major technical and economic challenge (Sindhu *et al*. 2016; Marin-Batista *et al.* 2021).

Although dilute acid pretreatment is most used, several disadvantages are associated with this method. Alkali pretreatment has emerged as a promising alternative. An attractive feature of this method is its ability to remove lignin with selective retention of cellulose. This mode of action shares some similarities with the organosolv pretreatment. However, the organosolv process is targeted towards delignification with solubilization of hemicellulose to varying degrees. Glycerol has been reported to be effective as an organosolv. The use of glycerol raises interesting prospects since it is a byproduct of the biodiesel production process (Sun *et al.* 2016; Meighana *et al.* 2017).The combination of alkaline and organosolv pretreatment methods may result in lignin extraction under milder processing conditions. High energy costs and high costs of robust infrastructure to withstand extreme pretreatment conditions are always deleterious to biorefinery projects. The combination of pretreatment methods may result in the amelioration of harsh and expensive pretreatment conditions without compromising on the desired yield. An ideal pretreatment method should be cost effective, minimize the release of inhibitors, minimize water consumption and safe to the environment without compromising the yield of fermentable sugars.

Previously, *Mucor circinelloides* ZSKP was used for concurrent production of chitosan and lipids, with the lipids being used for biodiesel production (Zininga *et al.* 2019). The oleaginous zygomycete, *M. circinelloides* is of special interest due to its ability to produce lipids, chitosan, pigments, ethanol and many other industrially important biomolecules (Rodrigues Reis *et al.* 2019). Interestingly, this dimorphic fungus is reported to grow on several lignocellulosic biomass including SCB (Carvalho *et al.* 2019), corn straw (Zhang and Song 2021), wheat straw, pomegranate, and tangerine peels (Al Mousa *et al.* 2022). This study was focused on developing a biorefinery approach by valorization of *M. circinelloides* ZSKP and SCB biomass through a new pretreatment strategy using alkalic salts and glycerol to generate supplemented bagasse hydrolysate as an alternative cultivation medium. An attempt was made to implement a multi-step valorization approach to produce a wide array of bio-products. Additionally, produced lignin and chitosan were utilized for value-addition.

3.2. Methodology

3.2.1. Fungal cultivation

Mucor circinelloides ZSKP was isolated previously with growth, chitosan and lipid accumulation conditions being established (Zininga *et al.* 2019). The strain was sub-cultured on potato dextrose agar plates by incubation at 27°C for 5 days. Spores of *M. circinelloides* ZSKP were collected from agar plates using sterile distilled water and used as seed cultures for fermentation.

3.2.2. Characterization of the bagasse

Sugarcane bagasse (SCB) was also obtained from SMRI, Durban, South Africa. The bagasse was washed and then air-dried. The dry bagasse was milled into fine biomass and sieved into a storage container using a 300 µm sieve. Cellulose, hemicellulose, and lignin were quantified according to methods described by Sluiter *et al.* (2012) and their compositions (Table 1).

Table 1: Cellulose, hemicellulose, and lignin composition of the sugarcane bagasse

Sugarcane bagasse component	Composition (%)
Cellulose	44.12
Hemicellulose	23.89
Lignin	25.78
Others*	6.21

*Other components include ashes, proteins, and extractives

3.2.3. Pretreatment of the bagasse

Five grams of bagasse was sieved using through 2 mm up to 0.075 mm sieves. This was then pretreated using 50 ml of 1% sulphuric acid, 1.5% sodium hydroxide and 80% glycerol at 120˚C, 160˚C and 250˚C respectively, at durations of 1 h for the sulphuric acid and sodium hydroxide pretreatments and 2 h for the glycerol pretreatment. The pretreatment procedure was then modified to combine the glycerol and dilute alkaline sodium hydroxide. Another combination treatment involved the use of glycerol and 5% alkalic salts such as sodium phosphate, sodium carbonate, ammonium phosphate and ammonium carbonate, as reported by Qing *et al.* (2016) with a 1.5% sodium hydroxide lignin extraction being the final recovery step after saccharification. The pretreatment was done in 1 l Schott bottles which were sealed and

immersed in an oil bath (Schutzart DIN 40050-1P20) preheated to the desired temperature. The effect of different pretreatment parameters such as different temperatures of the glycerol pretreatment, different pretreatment duration times and for the ammonium phosphate pretreatment such as different temperatures and concentrations on total reducing sugar yield was investigated in triplicate. The mean difference between different pretreatments was analyzed by ANOVA. Glycerol was procured from Sigma Aldrich.

3.2.4. Saccharification

Three commercial enzyme cocktail containing cellulase (500000 U/g), xylanase (290000 U/g) and β-glucosidase (50000 U/g) purchased from Shandong Longda Bio-Products Company Limited (China) was loaded at a ratio of 10 mg of enzyme per g of dried pretreated bagasse substrate (Osipov *et al.* 2020). The enzyme cocktail was dissolved in 50 ml 0.05 M acetate buffer, pH 5.5 (Sukumaran *et al.* 2009). The saccharification was carried out in 250 ml Erlenmeyer flasks for 48 h at 50°C. The final sugar concentrations were analyzed using high performance liquid chromatography (HPLC) and 3,5-dinitrosalicyclic acid method. The reducing sugars yield was calculated using the formulae reported by Qing *et al.* (2016).

3.2.5. HPLC analysis

Concentration of different sugars in liquid samples after enzymatic hydrolysis was analysed by HPLC (Shimadzu LCMS-2020). The HPLC system was equipped with an ELSD detector and Aminex HPX-87P column (Shimadzu). Degassed Milli-Q water was used as the mobile phase at a flow rate of 0.6 ml/min at 50°C. Analytical standards of sugars (Sigma Aldrich) were used for determination of unknown concentrations.

3.2.6. Supplemented bagasse hydrolysate as a medium for fungal growth

The hydrolysate obtained after saccharification was supplemented with 5% corn steep solids from Sigma Aldrich, and inoculated with 1×10^5 *M. circinelloides* spores in 250 mL Erlenmeyer flasks containing 50 mL medium (pH 5.5) at 250 rpm, 30°C for 120 h. A 180 µm sieve and filter cloth were used to separate the media and biomass. The biomass was freeze-dried to obtain the dry biomass weight.

3.2.7. Lignin, lipids, and chitosan extraction

Lignin was initially extracted using different concentrations of commercial glycerol (Sigma) and 1.5% sodium hydroxide at 120˚C, with both extractions being carried out just before saccharification. The best lignin recovery (80%) was eventually achieved by glycerol pretreatment at 150˚C, followed by saccharification and a second 1.5% sodium hydroxide extraction being implemented at 120˚C on the residue after saccharification. The lignin residue was precipitated from the supernatant by adjusting the pH to 2.0 using 1 M HCl and oven drying at 60˚C. The lipids and chitosan were concomitantly extracted according to a previous method stated by (Zininga *et al.* 2019).

3.2.8. Preparation of the plastic biocomposites

A 1% (m/v) chitosan sample in 1% acetic acid was prepared with constant stirring at 23˚C (Xu *et al*., 2005). This was kept overnight with constant agitation with centrifugation being carried out thereafter to remove particulates. Glycerol and glycerol with dissolved lignin (from the lignin extraction process) were added as plasticizers. Each plasticizer was added to give a final plasticizer to chitosan weight ratio of 0.04 (Alvarez *et al.* 2013). The emulsions were then poured into petri plates after removal of bubbles and dried in an oven (35˚C, 24 h). The casted plastic films were recovered by peeling off the plate.

3.2.9. The effect of glycerol plasticizer on the tensile properties of the chitosan bio-composite plastic

Mechanical parameters (tensile strength, young's modulus or stiffness, breaking elongation) were measured for the lignin infused and non-lignin infused chitosan bio-composite plastic samples in the dry states. The mechanical properties of the dry samples were determined with the aid of an Instron 5943 universal testing machine, the base length of the composite film was 20 mm, and the height was 60 mm, and extension rate was 1 mm/sec. The thickness of the plastic was measured using a ruler in mm.

3.2.10. Thermogravimetric analysis

Thermogravimetric analysis was used to evaluate the degradation of the chitosan bioplastic with glycerol and lignin-infused glycerol plasticizers. An SDT Q600 V20.9 Build 20 thermal analyzer (TA instruments) was used over a temperature range of 25–590 °C at 10 °C/min heating rate. The tested samples weighed 5 and 10 mg each. Nitrogen was used over the samples. The weight loss was assessed using the associated software and expressed as a percentage (Abugoch *et al.* 2011).

3.2.11. FTIR analysis

The samples of chitosan produced were characterized in KBr pellets by Fourier-transform infrared (FTIR) spectroscopy in the range of 400 to 4000 cm^{-1}. This involved 0.8 mg of each sample being mixed with 200 mg of pure KBr (Merck) powder and the mix was compacted into thin pellets of 13 mm diameter. The transmission spectra of acetic and sulphuric acid-extracted chitosan were then compared to the spectrum produced by commercial chitosan extracted from shrimp shells (Sigma).

3.3. Results and Discussion

3.3.1. Comparison of pretreatment methods

The effect of sulphuric acid, glycerol, and sodium hydroxide pretreatment of bagasse on reducing sugar yield was determined after enzymatic saccharification (Table 2). The bagasse sample pretreated with sodium hydroxide gave the highest reducing sugars yield of 89.9 g/l. The lowest yield was recorded from the sulphuric acid pretreated sample at 34.6 g/l. The high temperature (250˚C) glycerol pretreatment gave a relatively high yield of 79.4 g/l

Table 2: Reducing sugars yield from 100 g of dry pretreated substrate using three pretreatment methods

Pretreatment method	Total reducing sugars (g/l)
Sulphuric acid	34.6±1.1
Glycerol	79.4±2.1
Sodium hydroxide	89.9±1.7

Pretreatment is the most important step in the valorization of agro-based residue like sugarcane bagasse. It is meant to enhance access of the saccharification enzymes to the carbon polymers and not necessarily to release fermentable sugars. It has been noted in literature that both alkaline (sodium hydroxide) and dilute acid (sulphuric acid) pretreatment methods release fermentation and saccharification inhibitors with the resultant hydrolysates needing detoxification steps. There is need to balance between a higher effective pretreatment and a lower release of saccharification and fermentation inhibitors. Therefor it may be possible to combine the pretreatment processes to strike a balance between a high yield of fermentable sugars after saccharification and a low or no release of inhibitors. Other factors such as high energy costs and high costs of robust infrastructure to withstand extreme pretreatment condition are always considered as well, hence, the combination should result in the moderation of the harsh and expensive pretreatment conditions without compromising on the desired yield.

Acid pretreatment is not efficient in dissolving lignin, which acts as a barrier to impede saccharification (Mankar *et al.* 2021) and this may be the reason for the lower reducing sugars yield (34.6 g/l) from the sulphuric acid method when compared to the sodium hydroxide and glycerol pretreatment methods. The current pretreatment strategies for sugarcane bagasse have focused on delignification, hence, the current trend towards mostly alkaline pretreatment methods and, in some instances, organosolv pretreatment methods. Alkaline pretreatment also

cleaves the uronic acid and acetyl groups on hemicellulose. This has an overall positive reactivity effect on the polysaccharides (Chen *et al.* 2013) whilst the organosolv pretreatment partially breaks down lignin bonds very efficiently. This results in a pulp that is rich in cellulose as it will also solubilize most of the hemicellulose sugars. The lignin selectivity of the solvent can be improved by adding a catalyst (Sun and Cheng 2002; Mesa *et al.* 2011). Unlike the aqueous process of alkaline delignification, the organosolv pretreatment offers the advantages of easier lignin recovery and concurrent recycling of the solvent through distillation (Mesa *et al.* 2011; Novo *et al.* 2011).

3.3.2. Combined pretreatment and lignin extraction (CPTL)

Lignin was concomitantly extracted during pretreatment using a combination of different strategies (L1 and L2, Table 3). Using both L1 and L2 strategies, maximum amount of total lignin was produced (0.4 g and 0.65 g, respectively) using a 80:20 (80% glycerol) glycerol to water ratio (Table 3). However, the lowest amount of reducing sugars (69.38 g/l) was produced using 80% glycerol. The pretreatment, in which 1.5% sodium hydroxide was used without glycerol, showed highest reducing sugar (90.6 g/l), but with lowest amount of total lignin (0.06 g and 0.4 g, respectively).

Table 3: The effect of different concentrations of glycerol on lignin extraction

Glycerol (%)	Lignin yield (g) first extraction (L1) *	Lignin yield (g) second extraction (L2) #	Total reducing sugar (g/l)†
0	0.06±0.00	0.40±0.09	90.6±0.9
20	0.26±0.01	0.49±0.1	80.9±1.1
40	0.27±0.02	0.58±0.06	76.9±1.6
50	0.33±0.01	0.60±0.04	78.9±0.6
60	0.36±0.03	0.60±0.03	77.12±0.7
80	0.40±0.01	0.65±0.05	69.38±1.8

* Using glycerol from an initial 5 g of bagasse sample at 120˚C for 15 min

using sodium hydroxide at 120˚C for 15 min

† from 100 g of dry pretreated substrate

The lignin yields obtained are consistent with a previous report (Novo *et al.* 2011), where a mixture of glycerol and water resulting in a pulp with less than 8% residual lignin. At least 80% delignification was achieved. However, the conditions that gave the highest lignin extraction yield did not correspond to the highest yield of reducing sugars, as expected, but rather a low yield of 69.38 g/l. This suggests the presence of saccharification inhibitors. This is exacerbated by the combination of high temperatures as well as the high degree of alkaline lignin solubilization which releases chemicals like furans and phenolic compounds (Luo *et al.* 2021; Yuan *et al.* 2021). Phenolics have been reported to be able to suppress hydrolysis of hemicellulose and cellulose by deactivating β-xylosidase and β-glucosidase parts of hemicellulose and cellulase enzymes thereby affecting the reducing sugars yield in saccharification.

Glycerol pretreatment as an organosolv process can be used for treatment of lignocellulosic materials like bagasse. The lignin was recovered easily with a possibility of recycling the solvents used. It is, however, important to note the comparatively milder conditions employed in this study. High temperature of 250˚C needed to apply the glycerol pretreatment means high energy costs and the need for costly infrastructure. Combination of solvents like glycerol and sodium hydroxide can prevent reprecipitation of the dissolved lignin, thereby enhancing lignin recovery.

3.3.3. The efficacy of hydrolysate generated from the pretreatment methods on *M. circinelloides* ZSKP growth

The hydrolysate from the bagasse samples pretreated with sodium hydroxide and glycerol was used as fermentation medium for *M. circinelloides* ZSKP growth and the biomass measured. There was growth in the glycerol pretreatment derived hydrolysate which gave a biomass yield of 2.93 g/l and no growth in the sodium hydroxide pretreatment derived hydrolysate (Table 4). A combination of glycerol and sodium hydroxide pretreatment also yielded no growth.

Table 4: Effect of fermentation inhibitors on growth of *M. circinelloides* ZSKP

Pretreatment method	Biomass yield (g) of *M. circinelloides* ZSKP after 5 days (g/l)
Sodium hydroxide	0.00±0.00
Glycerol and sodium hydroxide	0.00±0.00
Glycerol	2.93±0.08

Sodium hydroxide used in the alkaline pretreatment process breaks ester bonds between the lignin and hemicelluloses due to saponification reactions. This allows enhanced access of saccharifying enzymes to cellulose for subsequent hydrolysis. However, several fermentation inhibitors may be released when the lignin is broken down at high temperatures. The non-aqueous nature of glycerol lignin extraction (Demirba 1998) seems to reduce the release of fermentation inhibitors like hydroxy methyl furfural (HMF), phenolic compounds and solubilized organic acids like formic acid despite the high temperatures. This can be indirectly corroborated by the presence or absence of fungal growth.

3.3.4. Alkalic salt pretreatment methods and growth of *M. circinelloides* ZSKP

The possibility of an alkaline pretreatment method that is less rigorous like the use of weaker alkali salts as reported by Qing *et al.* (2016) was explored. Sodium phosphate pretreatment gave the highest reducing sugars yield of 38.9 g/l followed by ammonium phosphate at 21.7 g/l (Fig 5). However, both sodium phosphate and ammonium phosphate pretreatments gave reducing sugar yields much lower than sodium hydroxide pretreatment.

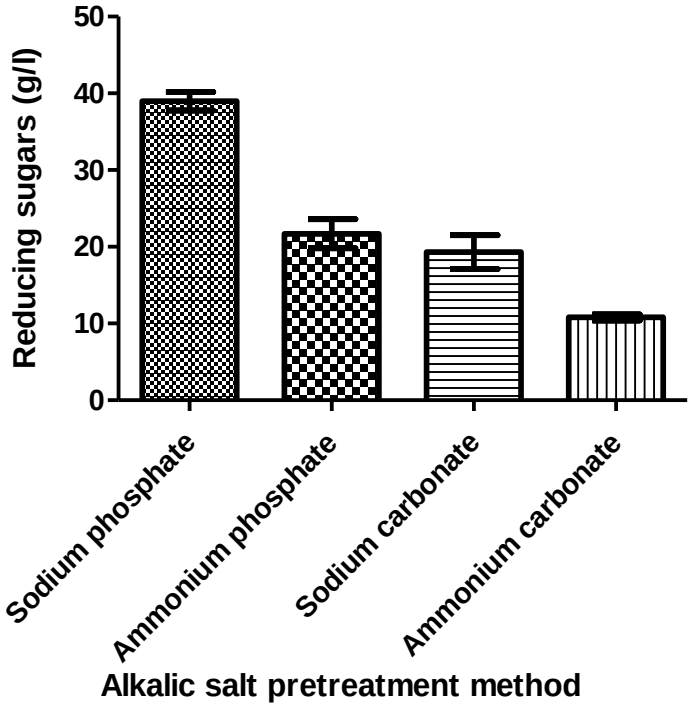


Figure 5: The effect of alkalic salt pretreatment on reducing sugar yield.

Alkaline pretreatment of biomass results in delignification involving condensation and redistribution of lignin, solubilization of the lignin and changes to cellulose crystalline state. The extent of the lignin targeting is influenced by the type of alkaline solution. The high temperatures applied and a high degree of lignin solubilization increases the chances of fermentation and saccharification inhibitors (Thoma *et al.* 2020; Troncoso-Ortega *et al.* 2021). A consideration of these factors would explain ammonium phosphate's potential suitability as a pretreatment solvent that releases minimum amount of inhibitors as demonstrated by the hydrolysate's ability

to support *M. circinelloides* growth without the need for detoxification and extensive washing of the pretreated substrate before saccharification.

The bagasse hydrolysate from the alkalic salts pretreatment was used as a medium for *M. circinelloides* ZSKP fermentation with biomass yield quantified. There was no growth in the sodium phosphate hydrolysate, however, there was growth on the ammonium phosphate pretreated hydrolysate (1.69 g/l) as well as the combined treatment of glycerol and ammonium phosphate (2.56 g/l) (Table 5)

Table 5: Alkalic salts pretreatment hydrolysate for the growth of *M. circinelloides* ZSKP

Pretreatment method	*M. circinelloides* ZSKP yield after 5 days (g/l)
Sodium phosphate	0.00±0.0
Ammonium phosphate	1.69±0.1
Glycerol and ammonium phosphate	2.56±0.2

The liquor obtained from the sodium phosphate pretreatment was black, pointing towards a high degree of delignification and solubilization of lignin, unlike the liquor obtained from the ammonium phosphate pretreatment, which was faint brown in colour. This suggests that ammonium phosphate, whilst enhancing accessibility of the cellulose to the enzymes, does not completely solubilize the lignin, hence lower amounts or no inhibitors were released.

3.3.5. Modification of the combined pretreatment and lignin extraction (mCPTL)

The pretreatment and lignin extraction strategy was modified to achieve a dual effect of high lignin yield and high reducing sugars in the hydrolysate with minimum microbial growth inhibitors. This was done by substituting the sodium hydroxide pretreatment stage with the weaker alkalic salt ammonium phosphate.

3.3.5.1. Combination of glycerol and ammonium phosphate pretreatment methods

The glycerol pretreatment was combined with ammonium phosphate pretreatment. Glycerol pretreatment was maintained at 120˚C, followed by ammonium phosphate pretreatment at a reduced temperature of 80˚C. The reducing sugar yield was improved from 18.9 g/l to 29.8 g/l (Fig 6).

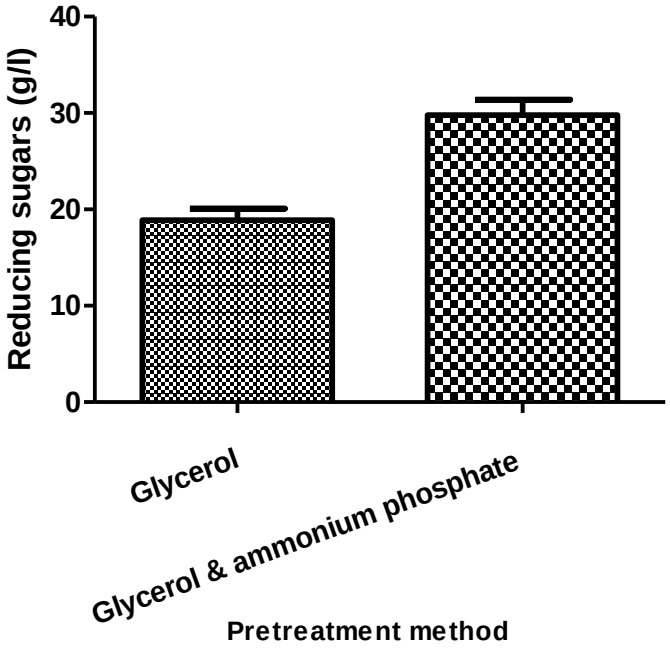


Figure 6: Combination of glycerol and ammonium phosphate (AP) pretreatment of sugarcane bagasse.

3.3.6. The effect of different parameters on the combined glycerol and ammonium phosphate pretreatment method

While studying the effect of different temperatures on the release of reducing sugars during ammonium phosphate pretreatment, it was observed that temperatures beyond 70˚C did not resulted a significant increase in the yield (Fig. 7). It should be noted that an initial glycerol pretreatment at 120˚C preceded this step. Therefore, 70˚C was selected as the most viable temperature for this pretreatment. Although maximum release of reducing sugars was observed at 100˚C, it could not be selected for further studies due to increased energy costs.

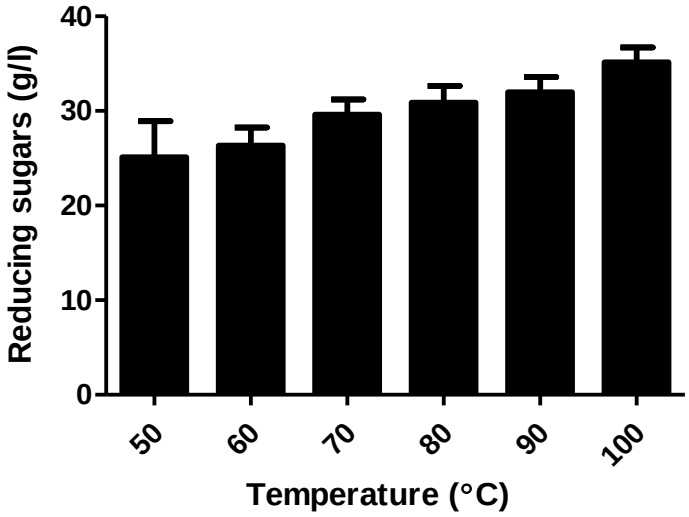


Figure 7: Effect of different ammonium phosphate pretreatment temperatures on total reducing sugars yield combined with glycerol pretreatment.

Increasing the pretreatment temperature for the glycerol lignin extraction resulted in an increase in the reducing sugars yield. The largest increase was observed between 120˚C and 150˚C, but there was no substantial increase between 150˚C and 180˚C (Fig. 8). The second step, ammonium phosphate pretreatment, was performed at 70˚C for 2 h. Therefore, 150˚C was selected due to high yield of reducing sugars and possibly lower energy costs.

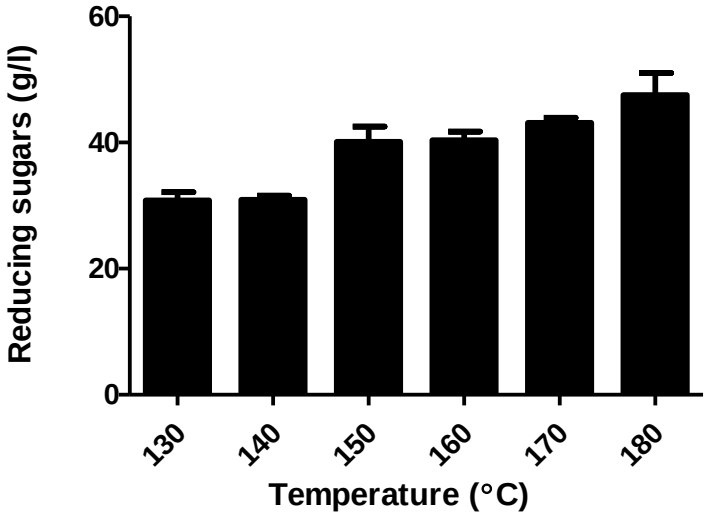


Figure 8: Effect of different glycerol extraction temperatures combined with ammonium phosphate extraction on reducing sugars yield after saccharification (g/l).

The impact of different glycerol pretreatment times on the release of reducing sugars was also studied. After 45 minutes of pretreatment, the reducing sugars yield increased to 60.96 g/l. There was a steady increase in reducing sugars yield from 15-45 min with lower increases after the 45 min. Therefore, 45 minutes was selected as further increases in pretreatment time did not result in a proportional increase in reducing sugars yield, making it potentially economically unfeasible in terms of cost of energy considerations against mimimal output increase (Fig. 9).

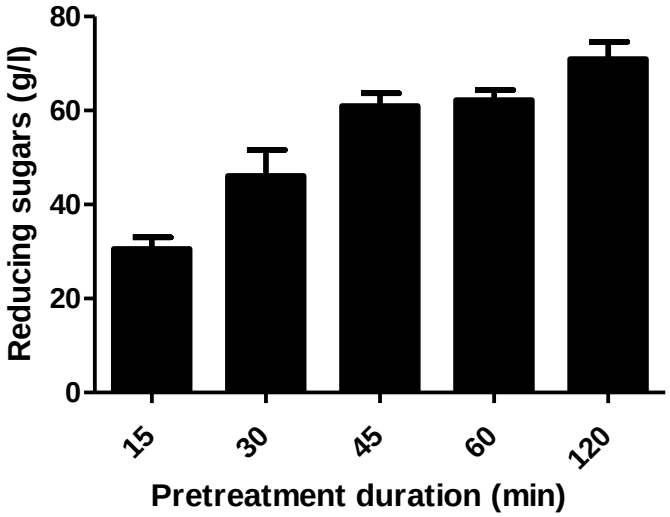


Figure 9: Effect of duration of glycerol pretreatment on reducing sugars yield at 150˚C.

3.3.7. Concurrent pretreatment and saccharification by mCPTL

Ammonium phosphate pretreatment was combined with the saccharification process, and the effect on reducing sugars yield was measured. Treatment 6 which combined glycerol-ammonium sulphate pretreatment with saccharification, gave the highest yield of reducing sugars of 80.67 g/l as shown in Table 6. The hydrolysate was then used directly as media for the growth of *M. circinelloides* ZSKP biomass with Treatment 6 giving the highest biomass yield of 12.89 g/l, which is twice the yield of the second-best treatment at 6.5 g/l.

Table 6: The effect of a two-step ammonium phosphate pretreatment and saccharification on reducing sugar and *M. circinelloides* ZSKP biomass yield

Pretreatment	1st Pretreatment conditions	2nd Pretreatment conditions	Saccharification Conditions	Reducing sugars (g/l)	Biomass (g/l)
1	Glycerol, 150˚C, 45 min	5% ammonium phosphate, 70˚C, 2 h	sodium acetate buffer, pH 5.50	60.70±1.11	6.50±0.52
2	Glycerol, 150˚C, 45 min	5% ammonium phosphate, 70˚C, 2 h	5% ammonium phosphate directly from 2nd pretreatment, initial pH 5.50	36.61±0.80	3.13±0.12
3	Glycerol, 150˚C, 45 min	5% ammonium phosphate, 70˚C, 2 h	Washed and replenished 5% ammonium phosphate, initial pH 5.50	35.73±0.32	5.91±0.23
4	Glycerol, 150˚C, 45 min	-	5% ammonium phosphate dissolved in sodium acetate buffer, pH 5.50	47.17±0.74	5.89±0.10
5	Glycerol, 150˚C, 45 min	-	sodium acetate buffer, pH 5.50	33.64±1.12	2.98±0.11
6	Glycerol, 150˚C, 45 min	5% ammonium phosphate, 70˚C, 2 h	5% ammonium phosphate dissolved in sodium acetate buffer, pH 5.50	80.67±1.31	12.89±0.62

The ammonium phosphate plays a dual role of enhancing the accessibility of cellulose to the saccharification enzymes, as well as being a nitrogen source to supplement the hydrolysate during fermentation. This can save costs in establishing a sustainable biorefinery process. This also allows for the moderation of the glycerol pretreatment process to levels that reduces energy costs.

3.3.8. The replacement of commercial medium with the generated and supplemented bagasse hydrolysate

The bagasse hydrolysate generated from the two-step glycerol and ammonium phosphate pretreatment and saccharification process was used as fermentation medium and this was supplemented with 5% corn steep solids. With some adjustments to pH, inoculum size and aeration, based on previous growth optimization work on the fungal strain. A 100 g sample of bagasse gave a lignin yield of 20.8 g and a *M. circinelloides* ZSKP biomass yield of 17.69 g. The fungal biomass produced was used to extract chitosan and lipids giving yields of 2.36 g and 4.9 g respectively.

The lipids produced from *M. circinelloides* ZSKP have been used to produce biodiesel as reported in previous work (Zininga *et al.* 2019). The need for a sustainable and economical bioconversion process has brought about the emergency of the biorefinery concept. Sugarcane based biorefineries have been one of the leading concepts in cellulosic biofuels production. Biomass valorization is at the center of addressing feasibility concerns when it comes to the commercialization of these biorefineries (Ning *et al.* 2021). There is therefore an attempt to implement a multi-step valorization approach to produce a wide array of bio-products. Lignin and chitosan are high value products that could off-set the costs of producing biofuels in the same process. Lignin, lipids, and chitosan where successfully produced from the sugarcane bagasse and *M. circinelloides* biomass with value addition of these products being the next focus.

3.3.9. Characterization of the extracted lignin using FTIR analysis

The lignin extracted from the bagasse was compared to a commercial lignin standard using FTIR analysis (Fig.10). Functional groups associated with lignin including hydroxyl, carboxyl, carbonyl, methoxy with peaks at 3400-3600, 2920, 1700-1750 and 2650-2890, respectively, were detected.

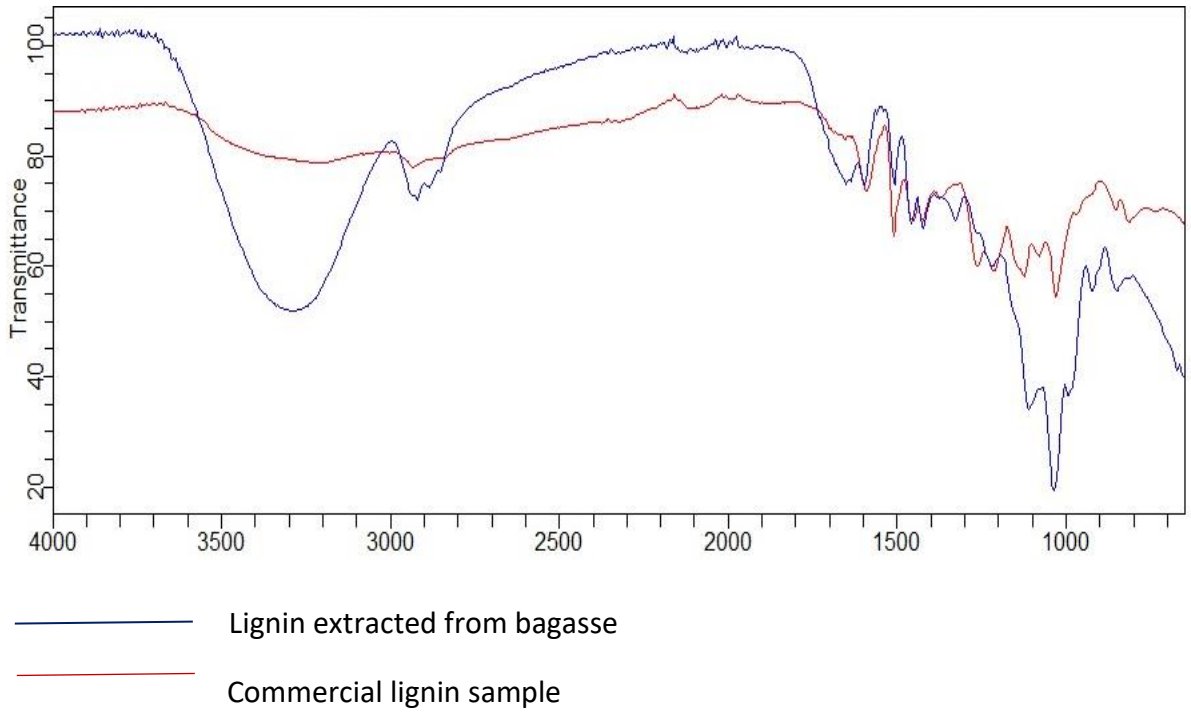


Figure 10: FTIR spectrum of the lignin extracted from bagasse against a commercial standard from wood.

Lignin can be classified according to sources which are hardwood, softwood, and grass. The chemical structure of the polymer is dependent on the biomass source. This would explain the differences in the width of some FTIR broad peaks as the commercial lignin sample is from hardwood and the lignin from bagasse is from grass. The extraction strategies can also influence the structure as the bagasse lignin was extracted using both alkaline and organoslv strategies.

3.3.10. The effect of glycerol plasticizer on the tensile properties of the chitosan biocomposite plastic

Additives are necessary in bioplastics to improve the mechanical properties of the plastic and make it more resilient, strong and soft. Glycerol has been shown to be effective as a plasticizer. Glycerol was used as a plasticizer on its own, as well as with dissolved lignin taken directly from the lignin extraction process. These plasticizers were added to the chitosan bioplastics to create different plasticized samples. The bioplastics with the added plasticizers had clearly improved tensile strength and thickness as shown in Table 7. However, addition of a plasticizer was shown to negatively affect the thermal properties of the bioplastic (Fig 11a).

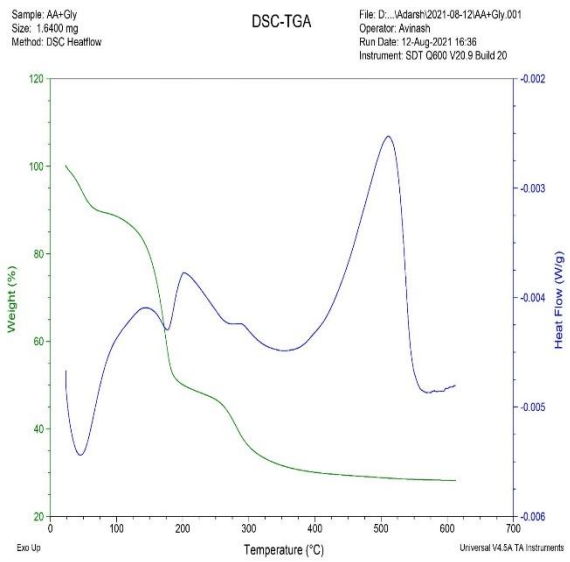
Table 7: Effect of plasticizer on thickness and tensile strength of the chitosan bioplastic

Film plasticizer composition	Thickness (mm)	Tensile strength (MPa)
Chitosan only	0.050	0.170
Chitosan and glycerol	0.380	7.757
Chitosan and lignified-glycerol from the lignin extraction process	0.383	7.412

3.3.11. Thermogravimetric and differential scanning calorimetry analysis of the extracted lignin incorporated with chitosan bioplastics

The glycerol containing the extracted lignin was directly used as a plasticizer in chitosan-based bio composite films. The glycerol with the dissolved lignin conferred clear improvements in the thermostability of the plastic having the least biomass loss as shown (Fig 11b). There was also more heat needed to increase the temperature of the same chitosan biocomposite plastic with lignin infused plasticizer. The chitosan biocomposite plastic with glycerol only plasticizer had the most inferior thermal properties (Fig 11a).

a



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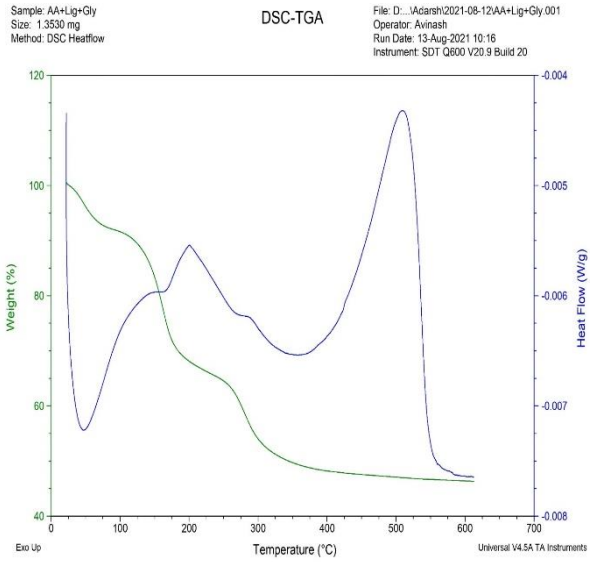

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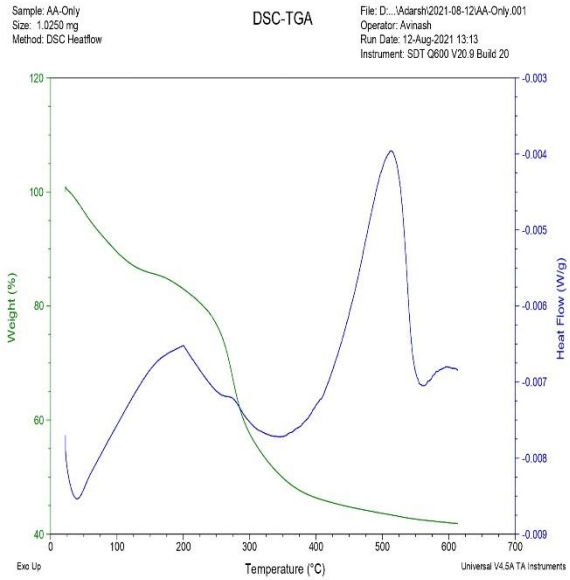


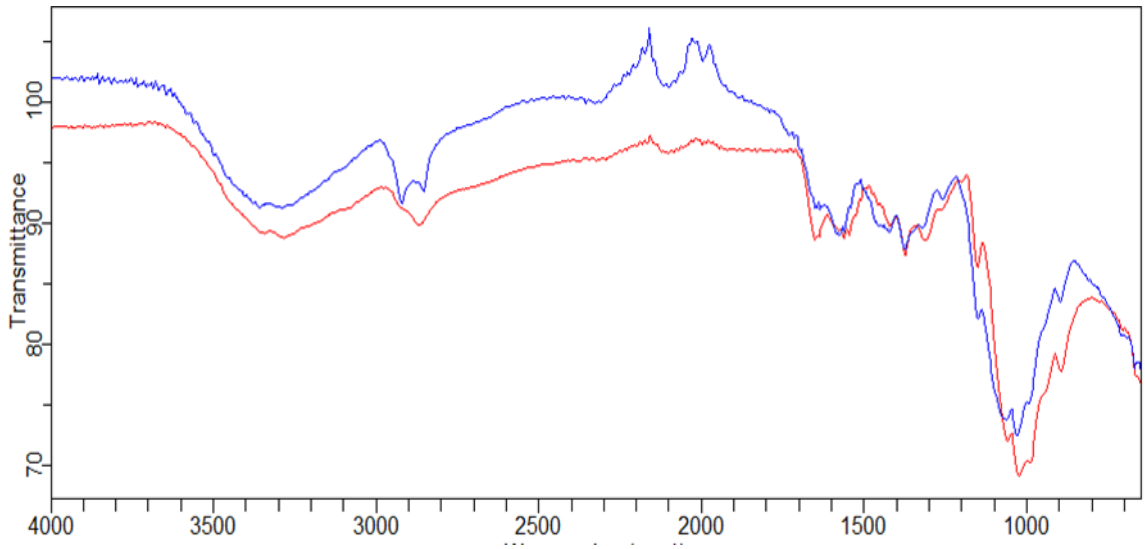
Figure 11: Thermogravimetric and differential scanning calorimetry analysis of the bio composite plastics a) chitosan biocomposite plastic with glycerol plasticizer b) chitosan biocomposite plastic with lignin infused glycerol plasticizer and c) chitosan biocomposite plastic without plasticizer.

Plastic has numerous different applications, and this can determine which mechanical properties are much more desirable. Glycerol has been reported to be a very good plasticizer for biocomposite plastics for polymers like starch and chitosan. The presence of glycerol in the

biocomposite plastic matrix increases elasticity, decreases material resistance as well as the glass transition temperature. However, this comes at the cost of thermal properties of the biocomposite plastic (Basiak *et al.* 2018; Tarique *et al*. 2021). Glycerol, a polyol, has hydrophilic groups whose hydrogen bonds interaction with water molecules allows for moisture absorption. Packaging is one area in which the potential application of chitosan plastic can be pursued. Thermostability properties are critical in plastic used in the food industry where heat is applied for preservation and extension of shelf life. In fact a lack of thermostability has been one of the major limiting factor to industrial application of plastic (Jeong *et al.* 2020). In the food industry, temperatures are important as high temperatures can be used to prolong the shelf life of the food, thus a plasticized chitosan plastic with enhanced thermos-properties due to the addition of lignin would open more possibilities.

3.3.12. Characterization of chitosan by FTIR analysis

To confirm the production of the chitosan as well as calculate the degree of deacetylation, FTIR analysis was carried out (Fig.12). This was done for both the acetic acid and sulphuric acid-derived chitosan with commercial shrimp shell chitosan as a reference. The peaks at 3417 cm^{-1} corresponds to the OH functional group and the absorption band at 3417 cm^{-1} is characteristic of amino groups vibrations. The methylene stretching CH is identified by the presence of the 2877 cm^{-1}peak. The deformation of the NH amino group is highlighted by the peak at 1600 cm^{-1}. Peaks at 1631 and 1747 cm^{-1} represents amide carbonyl and ester carbonyl group stretch vibrations respectively. The glucose amine ring is shown by the highlighting of the COC stretch vibrations through the presence of the 1072 cm^{-1} peak. All these functional groups are characteristic of chitosan.



M. circinelloides ZSKP chitosan

Commercial shrimp shell chitosan standard

Figure 12: FTIR spectrum of the fungal chitosan produced against that of commercial shrimp shell chitosan standard.

The chitosan produced showed antimicrobial properties against four microbial strains *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis* and *Aspergillus niger*. It was also effective as a 1% spray to preserve fresh fruit produce (Zininga *et al.* 2023). The possibility of fungal chitosan being commercialized is enhanced if chitosan is produced together with multiple products as demonstrated in this study. The next step would be to add value to the chitosan produced instead of selling it in its raw form. Value can also be added by purification and functionalization. Highly pure medical grade chitosan can fetch a price as high as US$25000/kg

3.4. Conclusions

An integrated process of lower temperature glycerol-ammonium phosphate bagasse pretreatment and concurrent saccharification, combined with lignin extraction was established. There was a significant moderation of the high temperatures normally applied in the glycerol organosolv processes as well as modification of the inhibitors inducing highly alkaline sodium hydroxide pretreatment. The process gave a relatively high yield of reducing sugars comparable to the sodium hydroxide and the high temperature glycerol pretreatment. Ammonium phosphate proved to be effective both in pretreatment as well as supplementing the hydrolysate as a nitrogen source as evidenced by the highest biomass yield. Addition of lignin to the product spectrum and its application as a value addition step, together with chitosan further enhances implementation of a biorefinery utilizing *M. circinelloides.*

CHAPTER 4: ETHANOL PRODUCTION TO SUPPLEMENT CONCURRENT SUGARCANE BAGASSE AND MICROBIAL BIOMASS VALORIZATION

4.1. Introduction

The efficacy of a circular bioeconomy as a sustainable tool to address environmental concerns and economic development is premised on the principle of waste biomass valorization and value addition. This is effectively realized through the implementation of the biorefinery concept. Lignocellulosic waste is a cheaper and rich source for carbohydrates and represents an important raw material in any large scale biorefinery implementation. As a second generation feedstock they offer the obvious advantages of being sustainable, greener, not used for food, and does not complete for arable land with food crops (Ning *et al.* 2021; Okolie *et al.* 2022).

Most of the bioethanol production has been done using starch or sucrose as the substrate. The processes involved are already established. Brazil is the world's largest producer of ethanol from yeast fermentation of sugarcane molasses. There is an increased demand for ethanol as a greener alternative to fossil fuels. This means production must be increased substantially, to meet the growing demand as well as leverage on the increased economies of scale. The use of lower cost substrates like lignocellulosic biomass therefore becomes necessary (Tesfaw and Assefa 2014; Busic *et al.* 2018).

Ethanol production from lignocellulosic material is dependent on pretreatment objectives that successfully allows saccharification enzymes access to cellulose and hemicellulose. This can be done through delignification or breakdown of hemicellulose. The breakdown of hemicellulose results in the release of 5-carbon and 6-carbon sugars which become substrates in a fermentation process to produce ethanol. In fact, most of the monosaccharides released during breakdown of lignocellulosic material is comprised of glucose, which can be as high as 60%. *Saccharomyces. cerevisiae* has been proven to be the best ethanol-producing yeast. The challenge, however, is its inherent inability to utilize the 5-carbon sugars like xylose and arabinose which are present in the hydrolysate (Saha and Cotta 2012; Tesfaw and Assefa 2014).

To achieve effective utilization of bagasse hydrolysate because of its characteristic 5-carbon and 6-carbon reducing sugars constituents, co-culturing may be a suitable strategy. In the natural environment, microorganisms do not exist in isolation but rather in complex microbial communities, where competition, cooperation and other interactions exist. These conditions mimicked in a co-culturing strategy may enable effective utilization of bagasse hydrolysate in microbial fermentations. The *S. cerevisiae* can be co-cultured with other microorganisms like

Mucor circinelloides that is able to utilize the Crabtree sugars and can grow as a yeast in anaerobic conditions due to its dimorphic nature. The five carbon Crabtree-positive nature of *M. circinelloides* and its relatively high tolerance to ethanol are potentially critical traits in mixed ethanol producing cultures (Lubbehusen *et al*. 2004; Tantipaibulvut *et al.* 2015; Carvalho *et al.* 2017).

Throughout human history, fish has been an important source for proteins. The increasing human population has necessitated an increase in fish production. This means natural supplies through wild fish capture fisheries must be supported by commercial production in the form of aquaculture which has seen a growth trajectory. Fish farming is heavily dependent on protein-rich feed supplement. The major ingredient in these feed supplements is fishmeal. Fishmeal is a protein supplement from fish that is not suitable for human consumption, and this is in most cases wild fish. This means the decline in the capture of wild fish will affect the availability of fish meal hence an alternative is required (Olsen and Hasan 2012; Shannon and Waller 2021). This alternative must be a protein source that has nutritional values that are compatible to the aquaculture requirements. This compatibility can be satisfied by comparing protein content, amino acid profile and amino acid balance. Various plant-based sources have been investigated. However, the challenges encountered has been their low digestibility, which would require higher intake of the feed to meet nutritional requirements. There is also an issue of their potential adverse effect to the intestinal microbiota of the fish. The presence of antinutrients like phytic acid, protease inhibitors, tannins, saponins and antivitamins can also impair the immune systems and reduce growth (Ritala *et al.* 2017; Junaid, Khawaja and Ali 2020; Nasrabadi *et al*. 2021).

This leads to the focus on microorganisms being a source for proteins, which have been dubbed single cell proteins as they have a high protein content, fatty acids and other nutrients which would stimulate fish growth. The protein content in microorganisms can be as high as 50% of dry weight, depending on the culture conditions and type of microbial strain. These organisms have the potential to be utilized as a component of fish feed. The metabolic versatility of these microorganisms which allows them to grow on broad spectrum of organic matter, makes them very attractive as sources of biomass. This chapter focuses on the effective utilization of bagasse hydrolysate through co-culturing to produce ethanol and a protein extract from the valorized microbial biomass.

4.2. Methodology

4.2.1. *M. circinelloides* growth and xylose utilization

Potato dextrose agar (PDA) plates were inoculated with *M. circinelloides* to generate a spore inoculum for fermentations. The fermentations were performed in 250 ml Erlenmeyer flasks. A total of 50 mL of Vogel's medium contained different ratios of xylose and glucose concentrations, to give a total concentration of 40 g/l. The cultivation conditions were pH 5.5, 250 rpm, 30°C for 120 h. The samples were collected in triplicate after 12-hour intervals for xylose and glucose utilisation analyses, as well as for biomass measurements. A 180 µm sieve and filter cloth were used to filter and separate the media and biomass from the broth. The broth was discarded, and the biomass dried in a freeze drier (Alpha 2-4 LDplus, Martin CHRIST).

4.2.2. Sugarcane bagasse hydrolysate generated medium

The sugarcane bagasse hydrolysate was produced through a series of pretreatment and saccharification steps according to the procedure described in section 3.2.3. The pretreatment conditions were 150˚C glycerol pretreatment for 45 minutes and 80˚C ammonium sulphate pretreatment for 1 h. The saccharification conditions were: 5% ammonium phosphate dissolved in sodium acetate buffer; pH 5.5; and a temperature of 50˚C. The hydrolysate was supplemented with 5% corn steep solids.

4.2.3. HPLC analysis

The concentrations of the different monomeric sugars in liquid samples after enzymatic hydrolysis were analyzed by High Performance Liquid Chromatography (Shimadzu LCMS-2020). The HPLC system was equipped with an ELSD detector and Aminex HPX-87P column (Shimadzu). Samples were analyzed using degassed Milli-Q water as the mobile phase at a flow rate of 0.6 mL/min at 50°C. Analytical standards from Sigma Aldrich were used to determine the concentration of sugars in the samples.

4.2.4. Effect of *S. cerevisiae* inoculum size on ethanol yield

Three yeast inoculum sizes (1%, 3%, and 5%) were used to determine their effect on ethanol yield. The medium used was 20 g $C_6H_{12}O_6$, 0.23 g $CaCl_2 \cdot 2H_2O$, 4 g $(NH_4)_2SO_4$, 1 g $MgSO_4 \cdot 7H_2O$. The fermentation was then carried out under anaerobic conditions at 30°C.

4.2.5. Co-culturing of *M. circinelloides* and *S. cerevisiae*

The co-culturing of *M. circinelloides* and *S. cerevisiae* was done in 250 ml Erlenmeyer flasks, using sugarcane bagasse hydrolysate supplemented with corn steep solids and the same conditions described in section 3.2.6. *M. circinelloides* (1×10^5 spores/ml) aerobic fermentation preceded the addition of *S. cerevisiae* (4×10^8 cells/ml) and the implementation of anaerobic co-culturing conditions for durations ranging from 1 to 5 days. The initial aerobic *M. circinelloides* fermentation was carried out at durations ranging from 1 to 5 days before anaerobic co-culturing. Ethanol quantification as well as biomass harvesting was done at the end of the fermentation runs.

4.2.6. Proximate analysis of the microbial biomass

The proximate analysis of the mixed *M. circinelloides* and *S. cerevisiae* biomass was done using the AOAC (2016) method. Moisture content was measured by drying the biomass in an oven at 105˚C for 24 h. The final weight was then subtracted from the initial weight to give the moisture content. The protein content was measured by Kjeldahl method, which involved sulphuric acid digestion and multiplying by the factor 6.5. The fibre content was determined by treatment with sodium hydroxide and sulphuric acid in a Fibretech apparatus (Labcono Corporation). The treated samples were heated in a muffle furnace at 600˚C (MF-1/02, PCSIR). The ash content was measured by charring and incinerating the sample at 600˚C for 5 h. The carbohydrate content (%) was estimated by subtracting the values of the other nutrients from 100.

4.2.7. Quantification of ethanol

Gas chromatography was used to determine the concentration of ethanol after fermentation. Distillates were filtered using a 0.2 µm filters prior to GC analysis. Ethanol was used as reference standard to determine the concentration of the analyzed samples. A Shimadzu Nexis GC-2030 was used to carry out the analysis of ethanol produced. The GC was equipped with a flame ionization detector and a flow rate of 30ml/min using an InertCap column and N_2 as the carrier gas.

4.2.8. Lignin, lipid, protein, and chitosan extraction

The lignin, the lipids and chitosan were extracted according to the method described in section 3.2.7. The proteins were extracted through modification of the deproteinization step during the chitosan extraction. The protein extraction was done on the pellet from lipid extraction. This was resuspended in 50 ml of 1 M NaOH and autoclaved at 121°C for 15 min (Hu *et al.* 1999). The mixture was then cooled and centrifuged at 5000 × *g* for 15 min. The pellet was stored 4°C for later use and the supernatant also stored for protein quantification.

4.2.9. Quantification of protein yield

A standard curve was derived using the Bradford assay that was then used to quantify protein recovery. The supernatants were used to quantify the protein recovery according to the Bradford assay. The data obtained was used to compare the parameters for the highest extraction of proteins. The protein yield was calculated using the formula:

Percentage yield= $\frac{protein\ recovered}{biomass\ used} X\ 100$

4.2.10. Effects of different solvents on the protein yield

The effect of distilled water and sodium chloride, in combination with sodium hydroxide as solvents in the protein extraction was investigated. The samples and the different solvent combinations were exposed to different temperatures (25˚C to 121˚C) and pH values (7 to 11). Different time durations (10-60 min) for the extraction were also compared. The supernatants were used for protein quantification and the pellets stored at 4°C for chitosan extraction.

4.2.11. Protein precipitation

A method described by Burgess (2009) was used to precipitate the proteins recovered from the stored supernatants. Ammonium sulphate was crushed and powdered using a pestle and mortar to easily dissolve in the protein solutions (supernatants). Different protein samples were decanted into a 100 ml beaker and 10 ml of the solution were added into five different beakers and used to determine the ammonium sulphate saturation point having the largest precipitate. The saturation points selected were 30, 50, 60, 70 and 80%. This was done by dissolving 1.64, 2.91, 3.61, 4.36 and 5.16 g of ammonium sulphate, respectively. The process of precipitation was carried out at 4°C while using a magnetic stirrer to dissolve the ammonium sulphate. When all the ammonium sulphate had dissolved, the solutions were decanted into separate 15 ml

centrifuge tubes and incubated in ice for 30 minutes then centrifuged at 2500 × *g* for 20 min. The pellet was dried and weighed to determine the saturation with the highest precipitate (protein) recovery. This saturation percentage was then used to precipitate all the protein from the protein solutions (Burgess 2009).

4.2.12. Desalting by dialysis

The precipitated protein solutions were centrifuged at 5000 × *g* for 20 min at 4°C. The supernatants were pooled and stored at 4°C and the pellets also combined. The combined proteins were resuspended in 100 ml deionised water and vortexed to homogenise the solution. A 30 cm piece of dialysis tubing was soaked in deionised water for 5 min before being used. The soaked tubing was knotted at one end and 5 ml of the homogenised protein solution was added through the open end using a pipette. The dialysis tubing was sealed by knotting. The dialysis tubing was then placed into a glass beaker filled with deionised water stirred by a magnetic stirrer to ensure circulation. The procedure was carried out at 4°C, for 4 h. The buffer was then changed, and dialysis proceeded for a further 8 h. The purified proteins were centrifuged at 5000 × *g* for 15 min then stored at -80°C to prevent degradation.

4.2.13. Amino acid analysis

The determination of the total amino acid content was done by Ultra Performance Liquid Chromatography using UV or fluorescence detection. This followed the derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC). The measurement of the amino acids was done after the 6 M HCl protein digestion and hydrolysis. It was also done on the free amino acids in solution. A photodiode array detector was used to detect amino acids separation. An amount of 1 µL carrying the derivatized amino acids from the digested sample was injected into the mobile phase using a C18 column (2.1 mm × 50 mm × 1.7 µm) at 60˚C. MassLynx software was used to analyze the data. The total amino acid content was reported in grammes per 100 g of the sample. Above analysis was carried out by the Central Analytical Facility, University of Stellenbosch.

4.3. Results and Discussion

4.3.1. Simultaneous xylose and glucose utilization by *M. circinelloides*

When the initial concentration of both glucose and xylose was equal in the medium (Fig.13), both glucose and xylose were utilized simultaneously by the fungi. The rate of glucose utilization was higher than that of xylose. However, after 60 h, the rate of xylose utilization increased as the concentration of glucose decreased. The medium yielded 7.9 g of biomass after 120 h of fermentation.

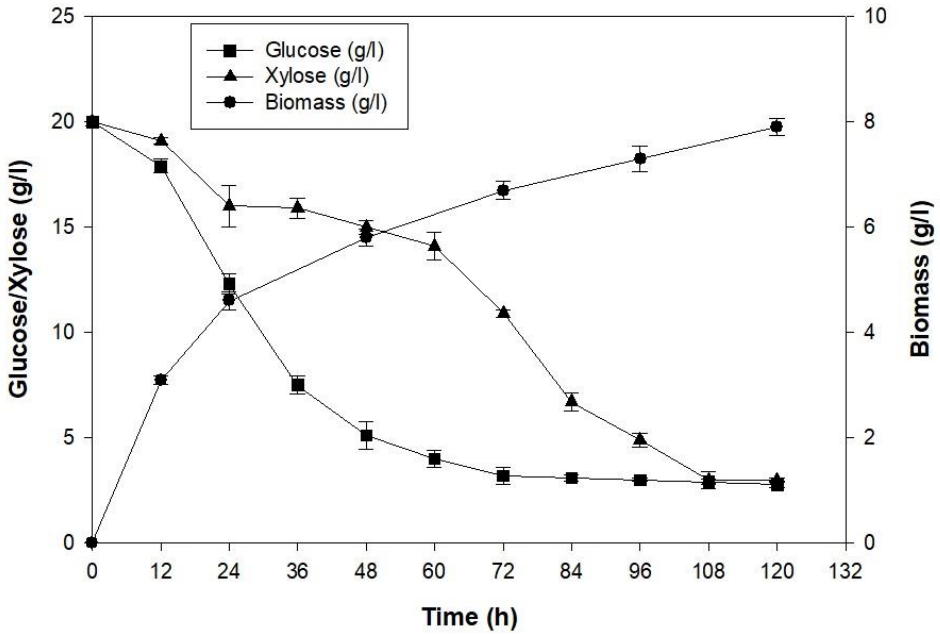


Figure 13: Utilization of xylose and glucose by *M. circinelloides* and biomass accumulation at equal concentrations of the two reducing sugars.

When the initial concentration of xylose was two times higher than that of glucose (Fig.14) both xylose and glucose were utilized at the same time. However, the rate of xylose utilization was faster than when xylose and glucose ratio were at equal concentrations. Again, the rate of xylose utilization accelerated after 60 h as the glucose concentration became depleted. The rate of xylose utilization was faster throughout the fermentation until 108 h when both sugars were completely utilized. In both media, the fungal biomass production rate was the highest in the first 24 hours and reached maximum levels at 120 h. The second medium gave a similar biomass yield of 7.7 g.

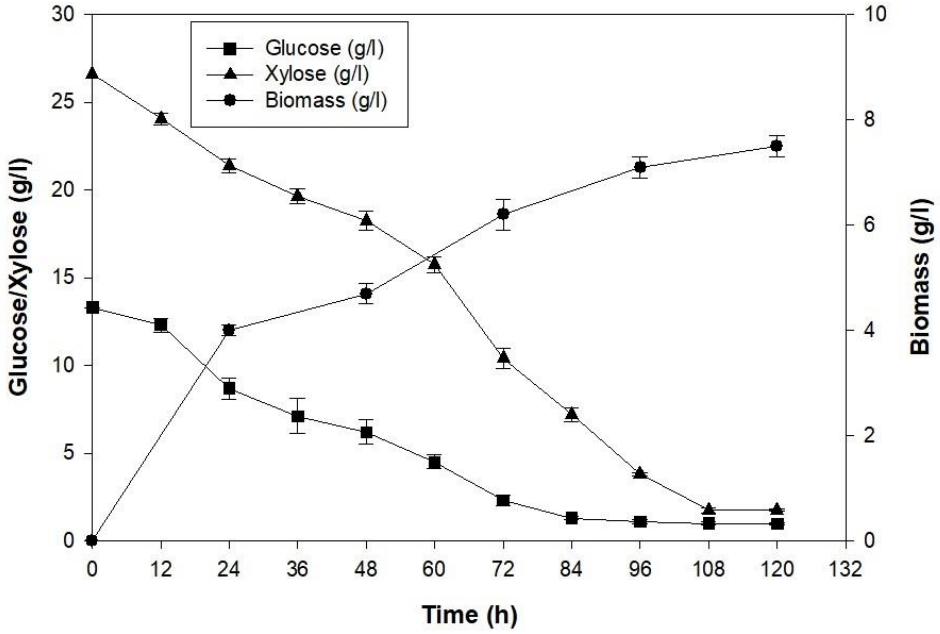


Figure 14: Utilization of xylose and glucose by *M. circinelloides* and biomass accumulation at 2:1 concentration of xylose: glucose.

Xylose is metabolized through the pentose phosphate pathway which is found in all microorganisms as it allows for the provision of D-ribose for the synthesis of nucleic acids, aromatic amino acids, and NADPH, needed for anabolic processes. The pentose phosphate pathway (PPP) has two phases. The hexose, D-glucose-6-phosphate is converted into a pentose D-ribulose-5-phosphate, with carbon dioxide and NADPH being produced. The D-ribulose-5-phosphate then undergoes several isomerization steps to eventually produce D-erythrose as one of the intermediates which combines with D-xylulose to yield glyceraldehyde-3-phosphate and fructose-6-phosphate, which are both directed back to glycolysis. In bacteria, xylose enters the PPP as D-xylulose after conversion by the enzyme xylose isomerase. In yeast and filamentous fungi, xylose goes through reduction and oxidation steps in the presence of the enzymes xylose reductase and xylitol dehydrogenase, respectively. This affects the demand for oxygen (Huntley and Patience 2018; Carvalho *et al.* 2019; Zhao *et al.* 2020).

Yeasts and bacteria are important for the utilization of xylose. Yeasts have an advantage over bacteria in commercial fermentations in that they are bigger in size, have a thicker cell wall and can grow well in acidic and nutritionally stressful conditions. *S. cerevisiae* does not utilize xylose naturally and has to be genetically engineered to be able to do so (Zhu *et al.* 2021b). Genetic engineering has also been used to modify xylose-utilizing yeasts like *Pichia stipitis* (Agbogbo

and Coward-Kelly 2008*).* Even though there has been successful attempts to genetically modify *S. cerevisiae*, the challenges of redox imbalances, high xylitol production, erratic gene expression and effective utilization of xylose still arise (Moyses *et al.* 2016). Conversion of xylose and glucose into value-added products is crucial for economic viability of bioprocess. There has been progress in metabolic engineering that has resulted in strains with unique improvements in glucose and xylose fermentations. There is however a major hindrance in that detailed knowledge of the impact of such changes on cellular metabolism is limited. The conversion of waste biomass like bagasse cannot be economical unless the hemicellulose is also utilized together with the cellulose especially considering that xylose is one of the most abundant carbohydrates, second only to cellulose (Moyses *et al.* 2016; Cunha *et al.* 2019; Zha *et al.* 2021).

The ability of *M. circinelloides* to accumulate biomass whilst concurrently utilizing glucose and xylose is important when evaluating use of lignocellulosic material. Lignocellulosic biomass is attractive as a second-generation source for sustainable production of chemicals and biofuels. Breaking down of lignocellulosic material results in the release of xylose and glucose as the main reducing sugars. The challenge is that most microorganism have a system of catabolic repression such that glucose and xylose cannot be utilized at the same time. Glucose is utilized first before xylose is utilized. The glucose and xylose utilization established in this study was that the rate of xylose utilization was dependent on the ratio of glucose and xylose in the medium. Various strategies, have been used to achieve efficient utilization of xylose which includes flux analysis, modelling and alteration and deletion of targeted genes (Hua *et al.* 2019; Lopez *et al.* 2020). *M. circinelloides* demonstrates natural co-utilization of glucose and xylose that can be useful.

4.3.2. The effect of inoculum size on ethanol production from *S. cerevisiae*

Varying amounts of yeast inoculum are used in fermentation reactors. The best inoculum size would be the one that gives the highest conversion of sugar to ethanol with a minimum production of undesirables (Mukhtar *et al.* 2010). Different amounts of *S. cerevisiae* were used as inoculum and the effect on the ethanol yield measured. There was an increase in ethanol yield when the inoculum was increased from 300-400 x 10^6 cells/ml. The inoculum with the cell count of 400 x 10^6 cells/ml produced the best yield 13.69 g/l (Table 8). Further increase of the inoculum to 450 x 10^6 cells/ml reduced the ethanol yield by 21%.

Table 8: The effect of inoculum size on ethanol yield

Inoculum (10^6 cells /ml)	Ethanol yield (g/l)
300	6.5
350	11.27
400	13.69
450	10.8

4.3.3. Co-culturing of *M. circinelloides* and *S. cerevisiae* for biomass and ethanol fermentation

Several fermentations with either *M. circinelloides* or S. cerevisiae alone or as co-fermentations. and aerobic then followed by anaerobic fermentation after the addition of *S. cerevisiae* was carried out and the biomass and ethanol yields were measured (Table 9). The anaerobic production of ethanol by *S. cerevisiae* yielded the highest amount of ethanol (25.78 g/l) but had the lowest biomass yield (2.22 g/l). The 10-day sequential aerobic and anaerobic fermentation with *M. circinelloides* and *S. cerevisiae* gave the highest biomass yield (21.3 g/l) but with the second-lowest ethanol production (4.45 g/l). Fermentation 8 which was the sequential fermentation of *M. circinelloides* (3 days aerobic growth) and *S. cerevisiae* (5 days of anaerobic growth) gave a biomass yield of 16.91 g/l and ethanol yield of 18.69 g/l.

Table 9: The effect of co-culturing *M. circinelloides* and *S. cerevisiae* on ethanol and biomass yields

Fermentation	Organism(s)	Conditions	Biomass (g/l)	Ethanol yield (g/l)
1	*S. cerevisiae*	Aerobic (5 days)	5.63±0.32	8.13±0.45
2	*S. cerevisiae*	Anaerobic (5 days)	2.22±0.12	25.78±1.98
3	*M. circinelloides*	Aerobic (5 days)	16.6±0.98	10.45±1.67
4	*M. circinelloides*	Anaerobic (5 days)	4.84±0.39	15.34±2.12
5	*M. circinelloides* and *S. cerevisiae*	Aerobic (5 days)	16.21±0.95	1.45±0.11
6	*M. circinelloides* and *S. cerevisiae*	Anaerobic (5 days)	4.65±0.23	20.04±1.89
7	*M. circinelloides* and *S. cerevisiae*	Aerobic (2 days) and Anaerobic (5 days)	13.87±1.22	19.69±1.78
8	*M. circinelloides* and *S. cerevisiae*	Aerobic (3 days) and Anaerobic (5 days)	16.91±0.96	18.69±1.23
9	*M. circinelloides* and *S. cerevisiae*	Aerobic (5 days) and Anaerobic (5 days)	21.32±1.43	4.45±0.32

Glucose has a higher affinity for hexose transporters in the cell wall and this slows down xylose utilization in co-metabolism. There has been attempts for simultaneous saccharification and fermentation, with the glucose being steadily released from low concentrations which would allow for enhanced uptake of xylose as well as enhanced overall fermentation (Öhgren *et al.*, 2006; Olofsson *et al.*, 2008b). The fermentations in samples 3-9 (Table 9) take advantage of an effective *M. circinelloides* aerobic glucose-xylose utilization matrix to favor biomass accumulation before the introduction of *S. cerevisiae* under anaerobic conditions to favour ethanol production. It was therefore useful to have excess reducing sugars in the hydrolysate and this strategy obviated the need to dilute the hydrolysate initially.

M. circinelloides has demonstrated ability to grow effectively on hydrolysate containing both glucose and xylose, accumulating biomass as well as producing ethanol through its dimorphic nature. It has also been able to grow on medium containing only xylose. However, when comparing ethanol production, *S. cerevisiae* is the superior organism. *S. cerevisiae* is preferred for ethanol fermentations because it offers clear advantages over other microorganisms that can be used for fermentation, and these include high yields of conversion (up to 90%); higher productivity; and high tolerance to ethanol levels as high as 10% (v/v). It is generally regarded as safe microorganism. It has been shown previously that, *M. circinelloides* biomass serves as a source of lipids, chitosan and potentially single cell proteins (Zininga *et al.* 2019; Fazili *et al.* 2022). *S. cerevisiae* biomass has also been reported to be a reliable source for single cell proteins. Thus, the desired co-culturing outcome would be an *M. circinelloides*-dominated biomass mixture and an improved ethanol yield due to the presence of *S. cerevisiae.* The proximate analysis of the mixed microbial biomass was carried out showing significant lipid and protein content (Table 10).

Table 10: Proximate analysis of the mixed microbial biomass

Constituent	Composition (%)
Carbohydrate	21.6
Protein	33.5
Total Fat	26.3
Fibre	11.6
Ash content	4.9
Moisture content	2.1

A primary requirement of a co-culture is that there must be compatibility of the two strains, allowing them to grow together. *S. cerevisiae* has been reported to inhibit the growth of many yeast strains,yet it is the preferred organism for fermentation of glucose to ethanol. There are other yeast strains like *C. shehatae* and *P. stipitis* that have also been reported to inhibit *S. cerevisiae*. *M. circinelloides* and *S. cerevisiae* seem to be compatible and the fermentation conditions of the two fungi are similar. The interactions between the co-cultures can be direct via cell to cell or indirectly through utilization of the substrate. Thus, metabolic interactions can be used to stabilize co-cultures. The interactions can be negative, positive or have no effect. Positive effects can be attained through the utilization and exhaustion of oxygen by aerobic cultures to create anaerobic conditions perfect for an anaerobic strain. The two co-cultures may also negatively compete for a substrate. To determine the co-culture preference might not be just a case of deciding if the interactions are negative or not but the most important consideration is the output (Chen 2011). In the current study, the output was ethanol and biomass, products whose biochemical genesis are such that the optimal conditions favorable for one may not be the same for the other. *M. circinelloides* biomass is more useful for valorization than *S. cerevisiae*. Therefore, an initial aerobic fermentation was carried out before inducing ethanol production conditions together with the addition of *S. cerevisiae*.

4.3.4. Modification of the protein extraction method

The deproteination step in chitosan extraction was modified and replaced with a more comprehensive protein extraction method. Three solvents were used for protein extraction and the effect of temperature, pH and extraction duration on the protein yield was investigated.

Increased temperatures resulted in an improved yield of total proteins, yielding as much as 120.29 mg/g (Table 11).

Table 11: The effect of temperature on protein recovery

Temperature (˚C)	Protein Recovery			
	H_2O (mg/g)	0.5 N NaCl (mg/g)	1 M NaOH (mg/g)	Total (mg/g)
25	13.26±0.83	6.02±0.61	37.83±2.1	57.09
30	14.53±1.21	6.17±0.56	38.03±2.34	58.73
60	16.91±1.26	7.63±0.41	49.23± 3.21	73.77
80	20.12±1.87	11.53±0.89	73.03±6.89	104.68
100	20.73±1.98	13.51±1.12	86.01±7.92	110.25
120	20.13±1.98	21.83±2.13	86.43±8.24	118.39

The increase in protein yield because of an increase in temperature might be due to increased solubility of the protein. The high temperatures can also cause the improvement in the extraction of cell membrane associated proteins like glycoproteins (Baurin *et al.* 2022). However high temperatures can be unfavorable in terms of the quality of the protein due to denaturation, hydrolysis, and coagulation.

There was a further increase in protein yield when duration of the extraction procedure was increased from 20 to 45 min to give an improved protein yield of 157.9 mg/g (Table 12). A further increase in duration by 15 min to 60 min only resulted in a 0.75% increase in protein yield (158.76 mg/g)

Table 12: The effect of extraction duration on protein recovery

Time (min)	Protein Recovery			
	H_2O (mg/g)	0.5 N NaCl (mg/g)	1 M NaOH (mg/g)	Total (mg/g)
10	18.53±1.34	19.73±1.53	64.41±5.79	102.67
15	19.81±1.86	19.52±1.79	67.13±6.23	106.46
20	21.91±0.79	21.53±1.98	70.83±6.96	114.27
30	20.13±0.91	22.01±1.89	81.11±6.89	123.25
45	36.73±2.89	37.42±3.13	83.43±6.89	157.58
60	37.41±2.56	33.02±1.99	88.33±7.23	158.76

The improvement in the protein yield with increased duration can also be attributed to removal of cell wall associated recalcitrant proteins with longer durations necessary to remove the proteins. There is no significant increase after 45 minutes, due to possible denaturation of the proteins.

There was an increase in protein yield as the pH became more alkaline giving an improved protein yield as high as 187.89 mg/g (Table 13).

Table 13: The effect of alkaline pH on protein recovery

pH	Protein Recovery			
	H_2O (mg/g)	0.5 N NaCl (mg/g)	1 M NaOH (mg/g)	Total (mg/g)
7	31.22±2.34	21.62±1.97	39.63±2.13	92.47
8	63.91±5.89	18.92±1.23	37.21±2.45	120.04
9	81.93±7.12	28.83±1.79	35.13±2.92	145.89
10	77.76±7.34	20.71±1.93	70.52±6.88	168.99
11	81.61±7.22	70.82±6.54	35.43±2.33	187.86

Alkali-assisted extraction of proteins is the most preferred extraction method when compared to the enzyme and acid assisted extractions (Baurin *et al.* 2022). Acid-assisted extraction has the lowest efficiency, whilst enzyme-assisted extraction has the highest efficiency and does not cause corrosion of equipment as with alkali and acid assisted extraction. However, the cost of the enzyme assisted extraction is high. The efficacy of the alkali extraction can be attributed to increased protein solubility. It also does not need expensive and sophisticated infrastructure to carry out (Lu *et al.* 2019). The effect of pH on the recovery of proteins has been investigated and a similar increase of pH (8-13) resulted in a gradual increase in protein yield (Omana *et al.* 2010). However, when a pH of 14 was used especially when using higher concentrations of NaOH, there was a significant decrease in protein yield. There could be due to amino acids side chains reacting at the high temperatures and high pH. The issue of protein quality is also important, considering factors like amino acid isomerization and formation of lysinoalanine as this influences the nutritional value of the protein (Omana *et al.* 2010; Momen *et al* 2021).

Valorization of the bagasse and microbial biomass yielded lignin, lipids, proteins, chitosan and ethanol (Table 14). The conditions for fermentation 8 (Table 9) were implemented in these experiments. A 300 g sample of bagasse produced a lignin yield of 59.86 lignin and a mixed *M. circinelloides* and *S. cerevisiae* biomass yield of 17.91 g. The microbial biomass was used to extract 3.72 g lipid, 2.97 g proteins,1.84 g of chitosan and 28 ml of ethanol (based on an ethanol density of 789 kg/$m^{3)}$.

Table 14: Valorization of bagasse and microbial biomass

Lignin yield (g/100 g bagasse)	Microbial biomass yield (g/l) *	Lipids yield (g/l)	Protein yield (g/l)	Chitosan yield (g/l)	Ethanol yield (ml)
20.8±0.8	16.91±0.96	3.72±0.32	2.97±0.22	1.84±0.17	28.00±4.00

*From 100 g of dried pretreated substrate

The valorization of the microbial biomass presents an opportunity for a biorefinery whereby biodiesel and ethanol can be produced sequentially. We have previously demonstrated concomitant production of chitosan and biodiesel from *M. circinelloides* biomass after chitosan extraction and transesterification of extracted lipids (Zininga *et al.* 2019). This would allow a process that is more energy self-sufficient. The suitability of the protein and the biomass residue that is left after the initial valorization process as a fish feed was explored. The biomass residue can provide a suitable carbon source for the fish as they require 10-30% of carbohydrates. The exposure to high chitosan extraction temperatures, may be beneficial as digestibility has been reported to be remarkably improved when the feed is cooked (Royes and Chapman 2015).

4.3.5. Amino acid composition of the protein extract

The microbial biomass contained high amount of essential amino acids with leucine being the most abundant (Table 15). There was also a relatively high amount of lysine. Most plant sources of proteins have a low content of lysine. Sulphur containing amino acids like methionine are also in high quantities. Glutamine was the most abundant non-essential amino acids.

Table 15: The amino acid composition of the extracted proteins and residual microbial biomass collected after chitosan extraction

Amino acid	Extracted proteins	Residual biomass after chitosan extraction
	Amino acid quantity (% g/100g)	Amino acids quantity (% g/100g)
Histidine	0.43	0.09
Arginine	0.83	0.12
Serine	0.65	0.17
Glycine	0.72	0.23
Aspartic acid	1.22	0.33
Glutamine	1.61	0.44
Threonine	0.91	0.20
Alanine	0.86	0.28
Proline	0.73	0.23
Lysine	1.13	0.21
Tryptophane	0.70	0.19
Methionine	0.38	0.15
Valine	0.54	0.30
Isoleusine	0.70	0.28
Leusine	1.36	0.51
Phenylalanine	0.87	0.31

The proteins extracted from the mixed *M. circinelloides* and *S. cerevisiae* mixed biomass have an amino acid profile that that is suitable as fish feed according to FAO guidelines (Mohanty *et al.* 2014). They have a high content of lysine and threonine, but lower content of methionine even though it is still within the FAO guidelines. Methionine content can even be lower in some fungi thereby necessitating the need to supplement the cultivation medium to enhance sulphur rich amino acids. There is glucan in the cell walls of fungi which can add fibre to the diet.

Consumption of myco-protein from *Fusarium venenatum* has been shown to improve glucose and insulin regulation (Cherta-Murillo *et al.* 2020). The major challenge is the high content of nucleic acids as high as 10% and this would require processing (Zhang *et al.* 2013a) but high heat has been used to degrade nucleic acids especially RNA (Bitskinashvili *et al.* 2019).

New sources of protein are needed due to the increased demand for meat and dairy protein. Plant protein has the disadvantage of conversion inefficiency whereby 6 kg of plant protein is used to produce 1 kg of meat protein. Plant-based protein sources like soyabean which can contain as high as 35% protein content, need arable land to grow. An ideal protein source would have the desired amino acids and low-fat content. The amino acid composition of a protein determines the nutritional value of a protein (Mirmiran *et al.* 2017; Kumar *et al.* 2022). There are 20 amino acids that must be supplied through dietary means and these includes (valine, threonine, tryptophan, leucine, lysine, histidine, proline, glutamine, cysteine, arginine, tyrosine, isoleucine, methionine, phenylalinine, glycine, cysteine, glycine).

The pressure on the environment has triggered efforts to replace fish meal. Single cell protein at appropriate dietary levels have shown to be suitable. The composition of the nutrients however is dependent on the source of the protein as well as the substrate used to support its growth. Microbial proteins can be an important source of feed proteins. These are commonly referred to as single cell proteins. They are derived from bacteria and fungi. It is preferred if the biomass contains at least 30% protein. Unlike plant protein there is no need for conversion of microbial proteins. Microbes have been used to improve the protein content of fermented foods. The advantages that microbes have as a protein source are: high growth rate; ability to grow on cheaper substrate resulting in much more sustainable conversion than traditional agriculture (Matassa *et al.* 2016). The use of single cell proteins is much more prevalent in animal feed than in human consumption and this is expanding (Patthawaro and Saejung 2019).

4.4. Conclusions

The bagasse hydolysate was utilized to produce ethanol and microbial biomass. The deproteination step in chitosan extraction was modified to serve as protein recovery step. This allowed for the sequential extraction of lipids, proteins, and chitosan to add to ethanol in the product spectrum. The amino acid profile of the proteins extracted show that they are suitable as a protein source for fish feed. The residual biomass cooked through the high temperatures applied during protein and chitosan extraction can also be potentially a carbon and nitrogen source for fish.

CHAPTER 5: SUMMARY

Biofuels offer some attractive benefits, and these include: enhanced energy security; potential to create a diversified rural economy; saving foreign-currency; and notably all their contribution as a greener alternative source of energy to petroleum-based fuels. However, the greatest hindrance to biofuels replacing petroleum fuels is the high cost of production making the final product uncompetitive on the market. This necessitates the adoption of the biorefinery approach to the production of biofuels (Chum and Overend 2001; Bu *et al.* 2014; Galbe and Wallberg 2019).

The biorefinery approach has gone through various phases, with the first phase being a simple conversion of a single raw material to a single product. The second phase saw the product range being broadened. It is in the current integrated phase where a wide range of raw materials, technologies and processes are utilized sequentially or simultaneously to produce a broadened range of products. The main products are usually biofuel with other co-products like chemicals, power, and heat. The integrated biorefinery approach is premised on facilitating the conversion of biomass into energy and other value-added products in a manner that is sustainable (Galbe and Wallberg 2019; Okolie *et al.* 2022).

In this project, sugarcane bagasse biomass was used as a source of reducing sugars used in biomass fermentations by *M. circinelloides.* Bagasse itself is an inexpensive agrobased waste that is readily available in South Africa. The costs involved in the utilization of bagasse arises from the pretreatment process. There has been a variety of pretreatment processes established previously. In this work, the focus was to incorporate lignin extraction into the pretreatment process and use a weaker alkalic salt like ammonium phosphate instead of sodium hydroxide, a corrosive compound which poses an environmental hazard. Ammonium phosphate results in less water being required in the process as water is used to wash the substrate to neutrality. Another direct benefit of the ammonium phosphate pretreatment is the reduced amount of inhibitors released. The detoxification step would be a source of additional costs from the raw materials (Cavka and Jonsson 2013).

There has been an attempt to lower the energy consumption costs by moderating the high temperatures usually associated with the glycerol pretreatment process. The glycerol pretreatment is quite strategic in that glycerol itself is a waste product of the biodiesel production process. In future studies, it would be interesting to compare partially purified waste glycerol in the pretreatment process developed in this study. Biodiesel has been successfully produced from the lipids extracted from *M. circinelloides*. This means the glycerol waste would find immediate utilization in the pretreatment process, as well as for lignin extraction.

Lignin is a valuable polymer. It can be used as an unmodified and unchanged macromolecule and there are various applications for this. In this project, the unmodified lignin was used whilst still soluble in the extraction glycerol to enhance the plasticizing properties of glycerol (Ľudmila *et al.* 2015). Lignin modification can be in three ways: modification of the hydroxyl group, depolymerization and modification of the functional groups. Low-purity lignin can fetch between $50-280 /MT and it can go as high as $750 /MT for high-purity lignin. Organosolv lignin has been reported to fetch higher prices than the lignin from the Kraft process and lignosulphonates.

Riofrio *et al.* (2021) showed that a two-year investment in a chitosan processing plant of 5000 ton capacity can result in quick investment recovery, resulting in a value of $100 million being reached in the projected lifespan of the plant. The probability of a high return rate is 67%. The costs of production of chitosan are critical to the feasibility of this enterprise. Scaling-up allows for a reduction in the production costs even though the return rates might not differ. A return rate that is positive is an indication that an investment is viable. An investment of $750 000 in chitosan production, at a return rate of 75% can yield a net value of $5 million by the fifth year of production. The location and type of raw materials has a direct impact on the profit for the investor, in a market that has shown great potential. This leads to the focus on alternatives to crustacean-based chitosan. Fungal based chitosan can address the key issues of location, seasonal availability of the raw materials. Fungal-based chitosan has been widely reported in academic literature, however, there has not been commensurate actualization of this in commercial endeavors and this can be attributed to the economic viability of the process. To produce fungal biomass is more costly than harvesting crustacean waste and this will have a direct impact on production costs and subsequently the profit margins. The focus of this work was to expand the product spectrum, as well as add value to the products and offset the higher production costs. A waste product was used for fungal fermentations.

Ethanol production was the biofuel in the product spectrum. There has been a previous attempt to produce ethanol from bagasse with xylitol being the co-product (Damiao Xavier *et al.* 2018). The bagasse was pretreated using acidic and enzymatic processes. Hemicellulose sugars were used as the substrate to produce xylitol whilst cellulose was used as the substrate for ethanol production using *S. cerevisiae* as the fermenting microorganism. There was a 2.3-fold increase in profitability for the integrated process when compared to the stand-alone ethanol production from the bagasse hydrolysate process. The lipid extractants from *M. circinelloides* biomass were used to make biodiesel (Zininga *et al.* 2019) which gives this proposed biorefinery approach the

uniqueness of producing both ethanol and biodiesel at the same time. Single cell proteins (SCP) were also added into the production matrix. Single cell proteins can fetch as high as $270 per 10 g of sample according to the sigma website. According to global market insights, single cell protein market is to surpass $18.5 billion by 2030. This because it has gone beyond just the utilization of SCP in animal feed, but as a suitable supplement in vegan diets as a meatless substrate. SCP utilization can be commercially categorized into animal feed, food, beverages, and dietary supplements (Ritala *et al.* 2017; Bratosin *et al.* 2021).

There are various factors that increase the overall costs, and these include import tariffs, reagents and equipment. The concept of sustainable recycling can be implemented to lower the costs of reagents. In this project, glycerol being used from pretreatment and lignin extraction can potentially be recovered from the biodiesel transesterification process as glycerol is a by-product of the process (Zininga *et al.* 2019). The production of both biodiesel and bioethanol in this integrated biorefinery process can strategically place such an enterprise in a rural setting, as it can be potentially energy self-sufficient. High costs of production are the major challenges to the use of biofuels and bioenergy. The current endeavor has focused on addressing this challenge.

It is imperative that any strategy to feasibly produce biofuels is accompanied by economic analysis. This would need to consider possible alternative production technologies and feedstock types. The dependent variable would be the total investment costs which can include the capital investment cost and the operating cost. It is in the operating costs that expenses related to utilities, labour, repairs, and raw materials are encountered. Raw materials and utilities has been reported to be as high as 86% of the operating costs (Gebremariam and Marchetti 2018) and could get even higher with higher production capacity. Feedstock cost has been identified as one of the important factors to consider in addressing the total costs of the production process. There is also a risk of making this the sole criterion which makes the selection of alternative feedstock important. This can eventually be problematic as there are logistical issues as well in terms of availability. Thus, there has been attention on agro-based waste. This project focused on sugarcane bagasse because it goes beyond cost issues, as it is an abundant agro-based waste product in KwaZulu Natal, where the potential integrated biorefinery would have to be implemented.

An organosolv-alkalic salt bagasse pretreatment method was established using glycerol and ammonium phosphate. The method used relatively lower temperatures compared to those that are normally applied to glycerol pretreatment without compromising the reducing sugars yield. This initial glycerol pretreatment step was also used to extract lignin, followed by sodium

hydroxide lignin extraction of the same biomass after the pretreatment and saccharification process. The use of an alkalic salt (ammonium phosphate) instead of a strong alkali like sodium hydroxide resulted in minimum release of fermentation inhibitors and the hydrolysate could support the growth of *M. circinelloides* without the need for a detoxification process. The use of ammonium phosphate allowed for a combined pretreatment and saccharification process, without the extensive use of water for washing to neutrality, as required in the alkaline pretreatment process. The ammonium phosphate residue also potentially supplemented the hydrolysate in its use as a fermentation medium. Lignin recovery was incorporated into the pretreatment process. The lignin improved the thermogravimetric properties of chitosan bioplastics. The utilization of the generated bagasse hydrolysate was enhanced by sequential aerobic-anaerobic fermentation to produce ethanol and biomass. A modified chitosan extraction process was used to recover proteins. In conclusion, an integrated biorefinery approach was used to concurrently produce multiple biofuel and biochemical products (lignin, lipids, proteins, chitosan, and ethanol) from sugarcane bagasse and fungal biomass.

Future work would focus on optimization of the integrated process based on identifying priority products. This would have to be informed by various factors which would differ in terms of economic policies. Further work on the key aspect of value-addition to the multiple products with emphasis on derivatives of the highest value to optimize the value of the output. For example, potential biomedical applications for fungal chitosan will have to be investigated. Lignin is also another chemical with vast value-addition potential. This would enhance the economic viability of a process that has been demonstrated to be greener and sustainable. All future work would have to focus on scaling-up both the downstream and upstream processes from the benchtop to bigger bioreactors.

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