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The potential nutritional benefit of paper and cardboard fibre for ruminant feed.

by

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Dissertation

Submitted in fulfilment of the requirements for the degree

Master of Science

in

Chemistry

in the Faculty of Science, University of the Witwatersrand, Johannesburg, South Africa

Supervisor: Prof Dean Brady

Co-supervisor: Dr Lucia Steenkamp

September 2025

DECLARATION

I declare that this dissertation is my own, unaided work. It is being submitted for the degree of Master of Science at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.



(Signature of candidate)

September 2025

ABSTRACT

The increasing need for sustainable and cost-effective animal feed, particularly in dry regions, makes it necessary for the innovative approaches in lignocellulose feed formulation. Recycled wastepaper and cardboard fibre can be considered as good examples of lignocellulosic resources. However, through biodegradable and readily available. Quantitative data on their enzymatic digestibility, reducing sugar yield efficiency and toxicity level remain limited. The goal of this study is to investigate the potential of enzymatic hydrolysis of recycled wastepaper and cardboard fibre as ruminant feed additives.

The recycled lignocellulosic biomass was pretreated using different methods: milling, enzymatic conditioning with laccase, autoclaving and alkaline hydrolysis with sodium hydroxide, were applied to disrupt the lignin matrix and enhance cellulose accessibility. Enzymatic hydrolysis was carried out using commercial enzymes and buffer systems (sodium citrate and sodium acetate, pH 5). It was subsequently optimised for enzyme loads, buffer molarity, and reaction conditions (temperature, time and agitation rate), with resultant improvements in saccharification and reduced enzyme dosage.

Naturase® Penicillium Cellulase achieved 402.64 mg/g (citrate buffer, 24 hrs), while Acid Cellulase BSJD025 reached 361.28 mg/g (citrate) and 362.42 mg/g (acetate). The buffer strength was critical: 0.1 M acetate buffer with 1000 mg Acid Cellulase BSJD025 produced 643.49 mg/g glucose yield, as compared to 299.76 mg/g at 0.01 M. The Multizyme® Cellulase CEP yielded 663.49 mg/g (citrate, 1000 mg), and addition of Tween 80 surfactant increased 500 mg load yields from 576.23 mg/g to 670.98 mg/g.

Elemental analysis confirmed traces of metals in the wastepaper and cardboard fibre, and UPLC-QToF-MS profiling identified the bioactive compounds such as Heliamine, Guanosine and L-Tyrosine. However, bioactive compounds that were identified are not enzymatically hydrolysable, therefore, they will not be present in the reducing sugars. The majority of metals, predominantly in ionic form (as salts), found in the wastepaper and cardboard fibre are soluble in water, and they are within the permissible intake for the ruminant feed.

This study validates the potential of converting the recycled wastepaper and cardboard fibre into ruminant feed additives, offering both the enhancement of animal nutrition amidst feed shortages and contributing to the environmental sustainability through waste management. The study limitation includes the absence of *in vivo* digestibility trails and the classification of wastepaper and cardboard fibre being hydrolysed. The future studies should focus on classifying the wastepaper and cardboard used, rumen simulation as well as long-term feed safety evaluation.

DEDICATION

This dissertation is dedicated to my family and friends.

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NOMENCLATURE

µl - microliter

ADF – Acid detergent fibre

ADL – Acid detergent lignin

Ag – Silver

Al – Aluminum

ANFs – Anti-nutritive factors

As – Arsenic

B – Boron

Ba – Barium

Be – Beryllium

Bi – Bismuth

Ca – Calcium

Cd – Cadmium

Co - Cobalt

CO_2 – Carbon dioxide

Cr – Chromium

Cu^{2+} - Copper cation

DNS – Dinitrosalicylic acid

°C – Degrees Celsius

Eu – Europium

Fe - Iron

Fe^{3+} - Ferric cation

g - Gram

GDP – Gross domestic product

HCl – Hydrogen chloride

Hg – Mercury

Ho - Holmium

Hrs – Hours

ICP-OES - Inductively Coupled Plasma Optical Emission Spectrometry

K - Potassium

kDA – Kilodalton

kg – Kilogram

L – liter

La – Lanthanum

Lac – Laccase

LiP – Lignin peroxidase

M – Molar

Mg - Magnesium

mg - milligram

ml – Milliliter

Mn – Manganese

MnP – Manganese peroxidase

Mo – Molybdenum

Na - Sodium

NaOH – Sodium hydroxide

NDF – Neutral detergent fibre

Ni – Nickel

NIR – Near-Infrared Reflectance

nm – nanometer

P – Phosphorus

Pb – Lead

% - Percentage

pH – Potential of hydrogen

Re – Rhenium

Rh – Rhodium

S – Sulfur

Sb – Antimony

Sc – Scandium

SF – Submerge fermentation

Sr – Strontium

SSF – Solid state fermentation

Th – Thorium

Tl – Thallium

U – Uranium

UPLC-QToF-MS - ultra-performance liquid chromatography coupled to quadrupole time-of-flight mass spectrometry

V – Vanadium

VFA – Volatile fatty acid

VP – Versatile peroxidase

Yb – Ytterbium

Zn - Zinc

CHAPTER ONE – INTRODUCTION

1. General introduction

The livestock industry is a backbone of nations' agricultural economy. It provides for about 40% of world agricultural GDP, and it employs about 830 million to 1.3 billion people (Bathaei & Štreimikienė, 2023) (Steinfeld et al., 2006). The poor continue to eat inadequately as they lack access to sufficient quality protein and trace elements.

The demand for animal feed is on the rise with the growing number of humans and consumer preferences shifting to animal products like meat and milk (Delgado, 2003). Boland et al., (2013), anticipate that, by year 2050, the population growth and increasing living standards in the developing countries will be a massive increase in the need for protein derived from animals.

For animal productivity, supply of high-quality feed is vital, especially where nutrient availability is limited. Animal nutrition deteriorates in the absence of sufficient high-quality feed, resulting in low productivity and possible negative health issues. This factor is prominent in sub-Saharan Africa, where livestock productivity is significantly limited by poor access to high-quality-feed (Amole et al., 2022). From research, animals have also been able to offset nutritional deficiencies by consuming more feed when presented with poor-quality pasture (Imaz et al., 2019). This results in scrambling for improved feeds under adverse grazing conditions.

True determination of a diet's feeding value is required to be able to effectively use it for various classes of ruminants. According to Madsen et al., (1997), the nutritional aspect of the feed eaten helps to determine and evaluate the nutritional value as it moves through the gastrointestinal tract. Both optimal levels of animal performance and numerous animal feed outcomes are benefits of broad knowledge of the beneficial components in the feeds.

Farmers acknowledge that consumption rates of the feed have been documented to reduce quite substantially from the rainy season to the dry season, limiting animal husbandry (Lamega et al., 2021). Southern Africa has two diverse seasons that may be explained as the dry season (rarely rain) and the rainy season (frequent rain). The wet (rainy) season, is between October/November and March/April, and it is when over 95% of the yearly rainfall will occur, in the form of brief but frequent events. Dry

season is between mid-April and September/October, with extended durations of aridity and little or no precipitation (Cooke et al., 2025).

Interestingly, water limitation is perhaps the predominant constraint on grassland productivity. The herbaceous plants with soft-stemmed growth die, exposing the land surface. The intake and how well the feed delivers nutrients to meet the animal's needs (maintenance plus performance) determine if a feed will stimulate animal productivity (Cooke et al., 2025).

There is great potential to use cellulose and hemicellulose as the main components of ruminant drought feeding programs, especially when it comes to improving forage nutritional value during environmental stress conditions. Forage crops with high cellulose and hemicellulose content, such as alfalfa and enset, have been shown to improve animal nutrition, particularly in dry seasons when normal feed may be hard to find (Woyesa & Kumar, 2022).

Alfalfa has the potential to improve feed quality through changes in carbohydrate composition and an increase in the leaf-to-stem ratio under drought (Fiasconaro et al., 2012). Since stressed plants can have better nutritional content and water limitation tolerance, such adaptation results in a more acceptable forage profile (Ray et al., 2019).

One of the biggest challenges for farmers is the scarcity of high-quality feed for the dry season. This problem can be reduced by adding free sugars to ruminant diets, which contain key nutrients that tend to be lacking in feed that has been damaged by drought (Afolayan et al., 2017). The increased sugar content that is found in the leaves of drought-tolerant crops like moringa, for instance, increases the energy profile that is available to animals during times of serious dryness (Seradj et al., 2019).

To enable ruminants to digest food efficiently, rumen fermentation processes depend on free sugars. Volatile fatty acids (VFAs), the main energy end product of rumen fermentation, are more readily produced when sugars are present. Higher VFA concentrations enable better nutrient absorption and feed efficiency overall, which is especially critical under conditions of limited nutrients. This benefit is most applicable during dry seasons when high-fibre feeds typically do not have adequate energy content to maximise rumen fermentation (Murillo-Ortiz et al., 2013).

The ruminants can utilise free sugars as a source of readily energy rapidly to satisfy their metabolic demands. Traditional grasses and leguminous crops, which under normal conditions supply energy and nutrients, may be in limited supply during times of drought and, as it were, this may result in livestock experiencing energy deficiencies. By providing a quicker source of energy, free sugar consumption works to offset the nutritional deficiencies that happen at such times (Melo et al., 2021).

The sweet taste is preferred by the dairy cows instead of the usual taste of the feed, as stated by Nombekela et al., (1994). Previous studies also show that there is an increase in the consumption of dry matter by dairy cows when there is sugar supplementation in the diet (Ondarza et al., 2017). Inclusion of sugars in the diet has a potential of increasing the production of butyrate in the rumen, microbial protein synthesis as well as milk components (Oba, 2011). Production of butyrate is important for the growth and development of papillae and ruminal epithelium, which helps in pH regulation (Redmond et al., 1995).

To be more precise, when feed quality is lowered due to drought, palatability becomes an important factor in ruminant feed intake stimulation. According to Van Loon et al., (2014), free sugars can make different sources of feed more palatable and encourage increased levels of feed intake. When the animals are not inclined to eat poorer forages that are usually available at such a time, increased palatability may be of great value.

In addition, flexibility can be brought into ruminant feeding systems by tactically mixing free sugars with conventional feed sources. Livestock producers can formulate diets that meet both the nutritional needs under stress and also stimulate resilience to the effects of climate uncertainty by exploiting the characteristics of easily accessible free sugars, especially in semi-arid and arid ecosystems (Niaz et al., 2021).

For ruminant feed, there are numerous unconventional cellulose sources, including vegetable residues (Mahgoub et al., 2019), legume tree stems and leaves like those of the Australian adzuki bean, and leaves of particular trees like poplar and orchard trees (Johnson et al., 2023) (Temel & Pehluvan, 2015). They are high in fibre content, which is necessary for rumen function, and have variable nutritional value.

For instance, vegetable waste was demonstrated to be fed to ruminants, which contributes to the process of food waste conversion into nutritional food (Mahgoub et

al., 2019). Additionally, research on tree leaves shows the nutritional content may be similar or even better compared to conventional forages (Temel & Pehluvan, 2015).

Another alternative that would ease the load on the environment is the recycling of wastepaper and cardboard – inkjet-printed sheets, photocopy paper, newsprint are the three significant sources of primal material for recycling used sheets (Mohandass & Raghukumar, 2005). According to Zhang, et al., (2015), among one of the large-scale industrial sectors that produce an enormous quantity of lignocellulosic waste is the pulp and paper industry**.**

Recycled paper is a promising novel ruminant feed. Because recycled paper is usually readily available and cheap, it helps to mitigate fears over feed shortage and cost. Being high in fibre content, it can be used as a source of cellulose in ruminant feeds, helping to meet ruminant dietary fibre needs and aiding agricultural waste minimisation (Duncan et al., 2023).

Since fibre takes time to ferment in the rumen, it has the impact of decreasing voluntary feed intake (Allen, 1996). The use of enzymes, therefore, pre-digests the fibre (biomass) into reducing sugars, which are water-soluble carbohydrates, and their fermentation in the rumen takes place quickly because they are a partially available energy (Oba, 2011) (Ravelo et al., 2022).

Of specific interest to the present study is the lignocellulosic biomass that is found in wastepaper and cardboard fibre. The most crucial aspect will be the enzymatic hydrolysis of cellulose to produce glucose, as bioconversion is efficient and friendly to the environment in the handling of the wastepaper and cardboard products (Ojewumi et al., 2022). The bioconversion of cellulose has numerous uses that may be beneficial, for this project it was being screened for use in ruminants feed (Varotkar et al., 2016).

In the context of this research, bio-catalysis will substitute chemical catalysis. Bio-catalysis is defined as the use of enzymes or microbes in conducting chemical conversion (Wu, et al., 2021). At the time of writing, wastepaper and cardboard have not been used to produce products for ruminant feed. The enzymes that are to be used are readily available commercially, and they have been selected as they are able to hydrolyse cellulose and starch to produce glucose, which is the main target of the research. The production of glucose will help when feeding ruminants, as ruminant

feeding has become a problem during drought seasons, while also addressing the pulp and paper waste available and beneficiation and reduction thereof.

1.2 Literature review

Plants make up 82% of all biomass on earth (Bar-On et al., 2018), half of which is cellulose (Zhang et al., 2019). This represents a vast supply of carbohydrates for feeds, materials and energy. Locally a significant percentage of cultivated biomass is used by the pulp and paper industry, which currently contributes about R50 billion to the domestic economy through the production of pulp, paper, board and tissue (PAMSA, 2023).

1.2.1 Papermaking process

Approximately 850 million trees are currently growing on 676 000 hectares of South African land set aside for paper and pulp manufacturing (PAMSA, 2023). As the trees require a decade to mature, only a tenth of this area can be felled annually, the same area being used to plant new trees (Molony, 2021). Therefore, a maximum of 85 million trees are converted into paper and cardboard annually (PAMSA, 2023). The papermaking process is seen in **Figure 1**.

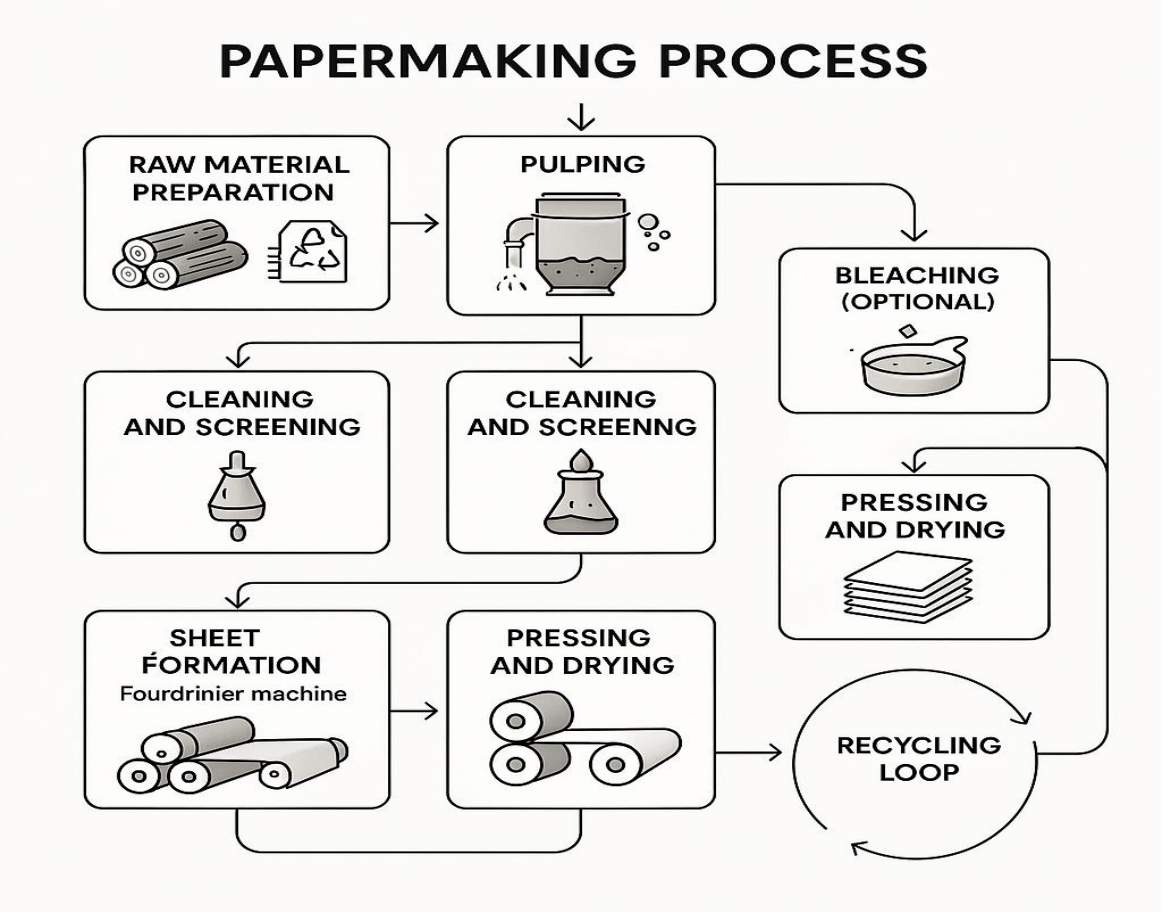


Figure 1: The process of papermaking (Smook Gary, 2016)

The paper-making process, as shown in the flowchart above starts with the preparation of raw materials: sorting and cleaning both virgin fibre derived from logs and recycled paper. This first step is crucial to contaminant screening, particularly in recycled fibre applications, pertinent to feed safety. The prepared materials are subjected to pulping, either mechanical or chemical, in which fibres are broken down into slurry. Beaching is optionally done using agents such as chlorine dioxide or hydrogen peroxide to remove lignin and brighten the pulp.

Cleaning and screening takes place twice (after pulping and before sheet formation) to eliminate non-fibrous materials and oversized particles. The phase of pressing and drying removes moisture mechanically and thermally. The sheet formation is achieved by means of a Fourdrinier machine, leading to continuous rolls of paper or cardboard. Lastly, recycling returns used paper into the stream of raw materials.

Inevitably the majority of the paper and cardboard is single use, and eventually goes into recycling.

1.2.2 Recycling wastepaper and cardboard

The paper recycling industry of South Africa is booming because of the efforts of waste management companies, collectors, and end-users. Paper recycling is the most commonly employed method of waste stewardship, and South Africa has been recycling since 1920. Three years prior to its target, South Africa achieved its 2020 goal of around 71% recycling of paper in 2017 when 1.3 million tons of cardboard and paper had been recycled. **Figure 2** shows a sample perspective of wastepaper and cardboard collection.

These astounding recycling rates are evidence of the success and growth of the industry. Then industry body, the Paper Manufacturers Association of South Africa (PAMSA), collaborates with producer responsibility organisations, namely: Fibre Circle, member mills, waste collectors, and small business to improve paper recycling in its paper recycling division RecyclePaperZa (Mackay, 2022). The primary factor that can drive the amounts of recycled paper, however, is still separation at the source. To substantiate these recycling rates, consumers and companies must continue to separate their paper waste from other types waste (Averda - South Africa, 2021).



Figure 2: Collection of used paper and cardboard (Mackay, 2022).

Paper and pulp recycled waste

A beneficial by-product is one that is valuable or can be reused, according to industry. The papermaking industry will always seek ways to use more of the tree, and this is a field of much research and development. The forestry-paper value chain has a variety of other types of “waste”.

Reuse of used paper gives the company a suitable substitute fibre for fresh products, particularly paper packaging and tissue. There are several types of paper found in South Africa, as shown in **Table 1.** Recycling paper not only keeps a useful product out of the landfill, but also keeps carbon out of the atmosphere for a long period (The Paper Story, 2023a).

Table 1: Different kinds of paper made in South Africa (The Paper Story, 2023b).

Printing and writing grades	Office paper, bond paper for long-run printing, scholastic grades & newsprint.
Packaging	Corrugated, container board, kraft paper, bag grades, packaging paper & moulded fibre.
Tissue	Toilet tissue, napkins/serviettes & wipes, paper/kitchen towel, industrial paper towel, facial tissue.
Special, security and Technical Papers	Passports, identity documents, label paper and wax paper.

The chemical make-up of paper and cardboard is primarily cellulose, with varying amounts of hemicellulose and lignin, depending on the product and intended use (**Table 2**). Recycled paper and cardboard products also include other constituents, including product additives and common contaminants (examples given in **Table 3**).

Table 2: Commonly used lignocellulosic materials' chemical make-up (Kumar & Sharma, 2017) (Sun & Cheng, 2002).

Lignocellulosic material	**Cellulose (%)**	**Hemicellulose (%)**	**Lignin (%)**	**Additives (fillers, coating or starch) (%)**
Paper	84 - 99	0	0 - 15	0
Cardboard	50 - 53	20 - 30	10 - 20	0 - 10

Table 3: The constituents usually found in recycled paper and cardboard (Wiley-Blackwell, 2007) (Elmas, 2017).

Additives	Colourants, fillers and coatings
Contaminants	Cadmium, chromium, lead and zinc
Per- and polyfluoroalkyl substances (PFSA)	Chemicals that are utilised for their resistance to water and grease
Plasticizers	Phthalates

The plastic contaminants that are usually found in recycled wastepaper and cardboard fibre can be seen in **Table 4**.

Table 4: Plastic contaminants found in recycled wastepaper and cardboard fibre (South African Plastics Pact, 2022) (WWF South Africa, 2021) (Wypych, 2019).

Contaminant	**Source**
Polyethylene	Plastic-lined paper and coated cartons
Polypropylene	Tapes, packaging films and labels
Polystyrene	Inserts and foam trays
Polyvinyl Chloride	Adhesive labels and synthetic papers
Polyethylene Terephthalate	Beverage labels and laminated paper
Pressure sensitive adhesives	Tapes and stickers

Wet strength resins	Food packaging and paper towels
Phthalates	Plasticizers, coatings and inks
N-butylbenzene sulfoamide	Plasticizer in laminates
Tris(2,4-di-tert-butylphenyl) phosphate	Flame retardants in coated paper

1.2.3 Composition of lignocellulosic biomass

The “lignocellulosic biomass” refers to plant material with a greater portion of lignified plant cell walls, which are composed of cellulose and hemicellulose along with lignin. Apart from that, lignocellulosic biomass may include traces of ash, proteins, and pectins (Grabber, 2005).

Cellulose

The foremost component of the lignocellulosic biomass, cellulose ($C_6H_{10}O_5$)n, is a polysaccharide made up of a linear chain of D-glucose bound by –(1,4)-glycosidic linkages, as seen in **Figure 3**. In the production of cellulose fibrils, the chains of cellulose are joined. There are various inter- and intramolecular hydrogen bonds that bind the cellulosic fibres together (Li et al., 2010).

Research has shown that drought-induced cellulose content alteration is a controlled, complex process determined by a wide range of internal and external factors. According to Liu et al., (2020), cell wall thickening is usually a consequence of overexpression of the cellulose synthase gene and has been linked to increased drought tolerance in a range of species.

The plants adapt better to the physical stresses caused by water limitation through reinforcing their structure with greater cellulose content. Cell wall property alteration of vital impact on mechanisms of drought response is accompanied by the implication that cellulose alteration can augment structural tolerance as well as functional capacity under conditions of stress (Sun et al., 2022).

Apart from maximising the rich, renewable, readily biodegradable, and biocompatible character of cellulose resources, cellulose-derived functional materials can introduce multifarious functionalities like optical, magnetic, electric, catalytic, and adsorptive capabilities through hybridization or reaction with other functional monomers. This

significantly expands the applications of cellulose (Xie et al., 2020) (Farooq et al., 2020) (Xing et al., 2018).

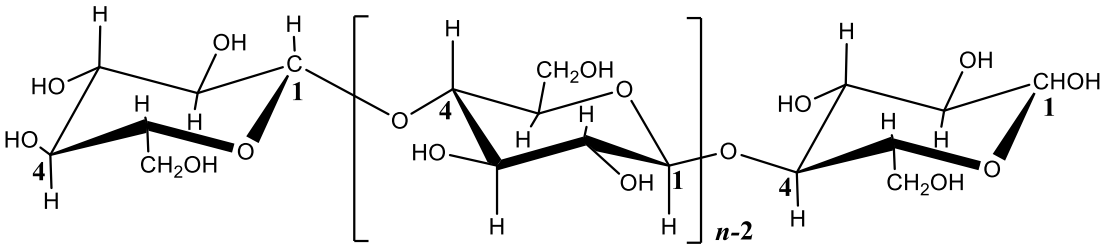


Figure 3: The structure of cellulose molecule drawn using ChemDraw (Singh & Satapathy, 2018).

Hemicellulose

Hexoses (D-galactose, D-mannose & D-glucose) and/or uronic acids (D-glucuronic, -D-4-O-methylgalacturonic and α-D-galacturonic acids) are present in heterogeneous branching biopolymers know as hemicelluloses $(C_5H_8O_4)_m$, shown in **Figure 4**, which are present in secondary cell walls (Gírio et al., 2010).

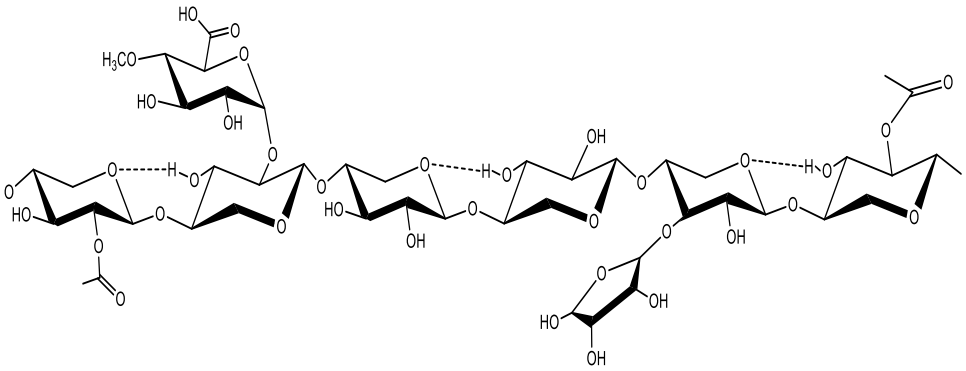
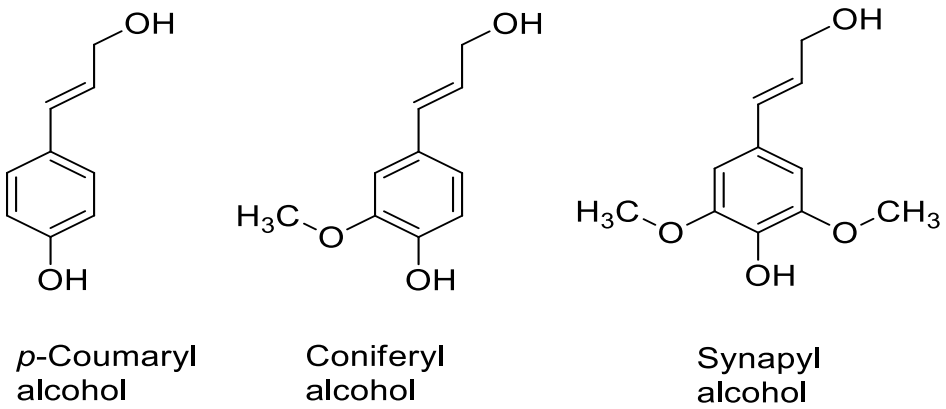


Figure 4: The structure of hemicellulose drawn using ChemDraw (Singh & Satapathy, 2018).

Lignin

According to its biochemistry, lignin, as shown in **Figure 5**, is made up of three phenylpropane units ($C_{31}H_{34}O_{11}$), known as guaiacyl units, from the former syringyl units of coniferyl alcohol (G) and precursor sinapyl alcohol (S) through the shickimate

pathway. The production of the fundamental lignin building unit requires the shickimate pathway (Higuchi, T., 1990).



p-Coumaryl alcohol | Coniferyl alcohol | Synapyl alcohol

Figure 5: Coniferyl-, *p*-coumaryl-, and sinapyl alcohol: the assembling blocks of 3D polymer lignin drawn using ChemDraw (Singh & Satapathy, 2018).

1.2.4 Agricultural by-products as feedstuff

Any material that, when ingested by the animal, is digestible, absorbable, and does not contain anti-nutritional factors and/or toxic compounds can be considered feedstuff (Tomar et al., 2022).

1.2.5 By-products

By-product feeds are commonly low-cost sources of nutrition. The high moisture content in certain by-product foods can deter intake, which would be injurious to animal performance and growth.

Sugar cane, both fresh or ensiled, utilised on farms, dried sugar cane pulp, and sugar cane molasses yielded in sugar mills are by-products of the sugar cane industry. They are sources of carbohydrates that are of improved taste and of high quality (Habeeb et al., 2017). By-products are used to produce concentrated feeds.

By-products classification

Numerous feedstuff by-product types exist, namely:

1. Cereal by-products: bran, distillers' grain and corn gluten feed.
2. Legume by-products: pea and lentil meal; and bean hulls.
3. Oilseed by-product: soybean meal, canola meal and sunflower meal.
4. Vegetable and fruit by-products; beet pulp and potato pulp; citrus pulp.

By-products may be classified in terms of their nutritional value, source, fermentable organic matter content and water content (Preston, 1981) (Ørskov, 1980) (Boucque & Fiems, 1988).

1.2.6 Effects that limit the by-products:

Anti-nutritive factors of the by-products:

Anti-nutritive factors (ANFs) are compounds, shown in **Table 5**, that either directly or indirectly, via their metabolites, interfere with the use of dietary nutrients by the body, promoting decreased growth and feed use and possible implications on animal health (Huisman & Tolman, 1992).

Table 5: The list of anti-nutritive factor compounds that are usually found in wastepaper and cardboard fibre (Nte et al., 2023):

Compounds	**Factors**
Carbohydrates	Non-starch polysaccharides Beta-glucans
Phenolic compounds	Tannins Lignin
Fibre compounds	Soluble and insoluble fibre
Fatty acids	Unsaturated fatty acids
Vitamins and minerals	Oxalates Phytates

Moisture

The moisture content of by-products can limit the quantity of dry matter that may be eaten, the distance it may be transported to , the economical price, and the length of time it may be stored prior to spoilage (rot). Because the dry matter content of the entire diet drops below 50%, ruminants tend to consume less total dry matter. Due to a higher market and longer storage times, dry by-products are more marketable than the equivalent by-product in wet fork. Dry by-products tend to be more expensive in general (Schingoethe, 1991). The dry by-products are more stable over time, they are easy to incorporate into dry feeds, they reduce microbial risk and are easier to ship, more especially in large quantities.

1.2.7 Compositional variety

In trying to properly design nutritionally balanced diets for their animals, farmers will be confronted by variations in the composition of feed materials, whether or not they are by-products. The product must then be analysed to determine chemical makeup, e.g., whether or not the protein level, fibre level, or other essential constituents vary greatly (Schingoethe, 1991). Costs are impacted by variations in the nutritional value of feedstuffs (Weiss & St-Pierre, 2009). A number of factors, such as the age of the specific ruminant, the desired production of the ruminant, and desired changes in the ruminant, affect the ideal nutritional contents (Kubkomawa et al., 2015).

1.2.8 Mineral Content

There are by-products which are not usable because of mineral content, but on the other hand, it is a cheap source of nutrients to be added as a replacement of a nutrient shortage in the remainder of the diet. Ruminants are prone to overconsumption of heavy metals like copper, zinc, and even lead. Excesses of these would make the products (feeds) unfit (Ørskov, 1977).

1.2.9 By-product feeds evaluation

The assessment of the feed should have values that show the potential carbohydrate structure, consumption of feeds, protein, mineral and vitamin, physical consumption, special anti-nutrition factor content, energy level and fat level. According to Van Soest., (1982), by using the detergent procedures; neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL), feeds are classified under the following:

- Cell contents soluble in neutral detergents include the following: organic acids, water-soluble matter, sugars, lipids, starch, pectin, and fluid protein and non-protein nitrogen.
- Cell wall pieces insoluble in neutral detergents include the following: cellulose, hemicellulose, and lignin

For practical purposes, detergent analysis in practice tends to be done using Van Soest's procedures, which were specifically designed for fodder measurements. By this repeated detergent extraction process, NDF is isolated through neutral detergent solutions and ADF and lignin through weaker acid detergent solutions (Costa et al., 2022). Verification of the detergent processes against good references, e.g., the

Kjeldahl method for the estimation of crude protein, which offers a good platform for nutrient evaluation, is required for sound appraisal (Massignani et al., 2021).

Assessment of forage quality has been improved by faster and more efficient methods brought about by technology, Near-Infrared Reflectance (NIR) spectroscopy, which enables multiple estimates of NDF, ADF, and other nutritional parameters at one time (Chen et al., 2021). For accuracy among varying types of forages and to improve efficiency of work in forage management, calibration of the NIRS model needs large datasets that relate spectral information with conventional chemical tests (Yamada et al., 2024).

They exist in different applications of NIRS (Ramirez et al., 2015) (Aenugu et al., 2011), namely:

1. Agricultural NIRS – used in feed analysis for the prediction of digestibility and nutrient concentration.
2. Industrial NIRS – used in textile, food, and pharmaceutical quality control.
3. Biomedical NIRS – used to monitor tissue oxygenation and blood perfusion, especially in the investigation of muscles and brain.

Summarily, detergent procedures play an important role in the classification of fodder as they reflect the fibre content, which has direct implications on cattle digestibility and nutrition availability. The efficacy of forage management is contingent on more precise and timely analysis with the aid of the increasing advancement of association of conventional procedures with new analytical procedures like NIRS (Kallida et al., 2022) (Vasileva et al., 2019).

1.2.10 Estimates of the energy content of feeds

As pointed out by Madsen et al., (1997), depending on the method of assessment being employed, the energy content of a meal is expressed as digestible energy, metabolisable energy, or net energy. In whichever system, the first step in determining the energy value of feedstuffs is measuring organic matter digestibility of the feed, and this also is by far the most important factor.

Several efforts have been made to relate various chemical analysis with digestibility determined in animals, but digestibility studies in cattle and sheep are cumbersome and extremely expensive. Because the standard deviations of sheep’s regressions on

digestibility against a series of feeds on a lignin, ADF, crude fibre and NDF basis will always be of the order of 9 – 11% units, none of these correlations can be considered universal (Van Soest, 1982).

1.2.11 Evaluating the feed digestibility

Digestibility of feeds is defined through a series of biological methods to establish the capacity of animals to use the nutrients in their diet.

Biological methods that are available include:

- Digestion by rumen micro-organisms, improved by two-stage in vitro systems, with digestion of fibres stressed as important by Foster et al. (2023), and enzymatic digestion assays improved by Zhang et al. (2025) to assess protein digestion rates or gas development techniques (GDT) (Magnani et al., 2024) (Rippstein et al.,2025).
- The *in situ* degradation (nylon bag technique) (Assunção et al., 2022) and cell-free enzymatic assays (Zhang et al., 2025).

Foster et al. (2023) confirmed that two-stage systems in vitro are pivotal for forage digestibility measurements, where anaerobic incubation in rumen fluid copies microbial digestion processes and subsequent treatment with acid-pepsin imitates an abomasal environment (acidic, digestive fourth stomach of the cow). Variations of this GDT have broadened this focus: thus, gas produced from feed sample fermentation processes in buffered ruminal fluids was assessed as a GHG source by gas evolution on a feed sample substrate (Magnani et al., 2024).

Rippstein et al. (2025) assessed ruminal compared with faecal inoculum on GPR system reproducibility and availability. Improvements for *in situ* degradation measurements of tropical feed materials are being reported on (Assunção et al., 2022), although Foster et al. (2023) pointed out that “more accurate and precise methods” are being explored and applied. More recently, advances in enzymatic incubation have shifted protein digestion studies from static end-point measurements to dynamic modelling approaches, allowing researchers to quantify nutrient availability in real time and better predict feed efficiency.

Cell-free fungal cellulase (enzymatic methods)

Since in part due to the growing availability of commercial enzymes, enzymatic digestibility techniques employing enzymes rather than microbes have been

increasingly refined. Foster et al. (2023) gave prominence to improvements in in vitro systems, mentioning their contribution toward mimicking ruminal fibre degradation in controlled conditions. Enzymatic analysis procedures, developed by Zhang et al. (2025), are able to estimate protein digestion kinetics. Moving from static endpoint values to provide dynamic insight into nitrogen solubilisation and hydrolysis, but have some disadvantages,
as seen in **Table 6**. The great benefit for the enzymatic system over the rumen fluid is that it does not need a fistulated animal to serve as an inoculum donor.

Table 6: Drawbacks of enzymatic procedures for feed digestibility assessment (Magnani et al., 2024) (Rippstein et al.,2025) (Assunção et al., 2022) (Zhang et al., 2025) (Foster et al., 2023):

Category	**Drawbacks**	**Mitigation techniques**
Sensitivity to environmental conditions	Temperature and pH dependence Inhibition by environment	Use buffered systems Employ thermostable enzymes Optimise assay conditions
Specificity and cross-reactivity	Specificity limitations Non-target interference	Combine with chromatographic or spectroscopic confirmation Validate with control assays
Cost and resource requirements	Reagent and equipment costs Preparation and time investment	Bulk procurement Integrate automation to reduce labour Use standardized kits
Limited space of application	Not universally applicable Degradation of analytes	Select appropriate enzyme cocktails Apply complementary microbial or chemical methods
Calibration and standardization issues	Need for calibration Variability in enzyme preparation	Source from certified suppliers

		Implement ISO/SANAS calibration protocols
Interference from sample matrix	Complex sample matrices Sample preparation	Pretreatment Matrix-matched standards Spike recovery tests
Data interpretation challenges	Complex data analysis Potential for misinterpretation	Integrate with LIMS for traceability Use statistical modelling Replicate assays

1.2.12 Compounds in cattle feed

An ingredient is a constituent, component or any combination or mixture that is added to and forms animal food (feed). Some examples of possible ingredients of animal food are grain, minerals, by-products of mills, supplementary vitamins, other diet and energy sources and fats/oils (Center for Veterinary Administration, 2023).

Grains (concentrates) and roughages are the two categories of feed. Roughages typically have a lot of fibre while concentrates typically have a lot of protein or carbohydrates. An animal must consume adequate energy and protein for maintenance, growth, and production to enable it to grow well and put on weight (Bath, 1981).

1.2.13 Concentrated feeds:

Concentrated feeds exist in two forms, namely carbonaceous and proteinaceous:

- Carbonaceous (energy) concentrates feed: the total digestibility of the nutrients is high, and the protein is below 11%
- Proteinaceous (protein) concentrates feed: the protein is above 15% (high) (Aminah & Nurfadilah, 2010)

1.2.14 Nutrients' importance in animal health and production

The level of nutrition and management practices employed in the animals determine the effectiveness with which animals are being produced. Nutrition has significant part to play in animal production and health (Tomar et al., 2022) because:

- It realises the potential genetic makeup of the animal and that would not be realised if animal is undernourished.
- Since 70 – 75 percent of the overall animal production cost is incurred in feeding animals, it makes an animal farming cost-effective.
- With the use of unconventional feeds in animal production it minimises competition between humans and animals for food.
- It changes feed ingredients in order to maximise the use of nutrients.

One needs to estimate the requirements for nutrients and then calculate appropriate and affordable diets that will meet the demands (Staff, 2020). Nutrients are the chemical components and substances in feed and fall into six classes: water, carbohydrates, proteins, lipids or fats, minerals, and vitamins. Based on the cattle's nutritional need, each composite component's quantity will differ (Harris, L. E., 1980).

In addition, the animal's age, production stages and goals, and season all impact the demand for nutrients. Beef cattle, for example, require more protein than dairy cattle which fattens the animal for beef production. The total protein level is estimated from the analysis of feed's nitrogen content (Mamphogoro et al., 2024).

1.2.15 Lignin impact

Due to its negative effect on the supply of nutrition within plant fibre, lignin is considered to be a counter-quality factor of feed. Lignin acts as a physical obstacle to microbial enzymes, preventing them from hydrolysis of the cell-wall polysaccharides (Moore & Jung, 2001). Lignin is consequently reducing the digestibility of feed by the ruminants (Susmel & Stefanon, 1993).

According to Jung et al., (2012) and Getachew et al., (2018), lignin is a significant structural part of the plant cell wall that restricts digestibility of forage by physically preventing microbial and enzymatic access to cellulose and hemicellulose. Other than concentration, its chemical structure and cross-linking with polysaccharides may affect its adverse effects; for instance, lignin's syringyl-to-guaiacyl (S/G) ratio (whether lignin is easy or hard to degrade) can completely change its inhibitory effect on cell-wall digestion (Getachew et al., 2018). Tissue maturity and immaturity experiments of forage crops support the idea that lignin accumulates and changes composition during plant growth, particularly in stems, further decreasing digestibility of the fodder. For

instance, reduced digestibility of fodder correlates with an increase in lignin at maturity (Wu et al., 2022).

To reduce the negative impacts of lignin on fodder quality, several approaches have been established. One major method is to decrease the production of lignin by the plant using genetic alteration. For example, *brown midrib* (*bmr*) mutants are extensively researched in alfalfa and sorghum forage. Downregulation of genes involved in lignin biosynthesis, for instance, some P450 enzymes, results in altered lignin composition that improves cell-wall degradability and lower lignin concentration (Reddy et al., 2005). As long as the decrease in lignin is balanced, such genetic alterations have been reported to improve the nutritional quality and digestibility of the forage without affecting overall agronomic performance (Jung et al., 2012).

Apart from genetic methods, post-harvest treatments have been investigated to eliminate or alter lignin to enhance the digestibility of fodder. Cell wall polysaccharides are more available to ruminal microbes when chemical pretreatment (such as alkaline treatments) and enzymatic treatment degrade lignin-carbohydrate complexes (Jung et al., 2012). While they are highly effective, such methods are more used during the processing phase of biofuel production than with direct feed, owing largely to logistics and economics. For optimal feed quality, however, studies have continued exploring combined approaches that merge plant breeding and intervention during processing.

In conclusion, lignin inhibits the microbial breakdown of cell wall components, which has detrimental effect on the digestibility of fodder. Both in planta approaches (approaches to plant transformation that bypass the need for conventional tissue culture steps, hence improving and making genetic modification more efficient and convenient) like physical and chemical pretreatments, and in planta approaches, like genetic modifications (e.g., *bmr* mutants and specific downregulation of lignin biosynthetic genes), can minimise its negative impacts. It is required to carefully adapt each method to preserve plant fitness as well as economic viability (Getachew et al., 2018; Grev et al., 2020; Jung et al., 2012; Oliver et al., 2005; Reddy et al., 2005; Wu, J. et al., 2022).

1.2.16 Heavy metals in animal feed

Heavy metal presence in animal feed, seen in **Table 7**, particularly for ruminant animals, is a concern due to their inherent toxicity and the risk of bioaccumulation in

food animal products such as milk and meat. The published reports have clearly established that heavy metal contamination of feeds such as lead, cadmium, arsenic and mercury can occur through a number of routes, namely contaminated raw material, soil and water contact during plant growth, and cross-contamination during handling and transport (Elliott et al., 2017).

Table 7: General regulatory limits of heavy metals in animal food (Department of Agriculture, Land Reform and Rural Development).

Heavy metal	**Maximum limit (mg/kg dry wt.)**
Lead	10.0
Cadmium	1.0
Mercury	0.1
Arsenic	2.0

These values are typically expressed on a dry matter basis and relate to total feed. The tolerance may vary depending on the feed type. In general, most of the heavy metals will be retained in solid residue or eliminated in the purification step. Trace levels may remain in the glucose solution if the raw material is very contaminated.

1.2.17 Hydrolysis of paper

The bioconversion process of converting paper and cardboard lignocellulosic biomass to fermentable sugars through enzymatic hydrolysis to final products (employing various enzymes) depends on the configuration of the raw materials (Brethauer & Studer, 2015), which will now be discussed in detail.

1.3 Biological Conversions of Lignocellulosic Biomass

1.3.1 Pretreatment Methods

Size reduction techniques

To improve the digestibility of lignocellulosic biomass, various mechanical size reduction techniques have been employed like cutting, slicing, milling, and crushing (Agbor et al., 2011).

Physical-chemical pretreatment

The most researched and utilized physical-chemical method of pretreatment of biomass is steam pretreatment, also known at times as steam-explosion. Because it was supposed that for the fibres to be hydrolysable there must be an “explosive” process. The milled biomass is usually placed in a high pressure (autoclave) from 100 to 200 °C (Agbor et al., 2011).

Ma et al., (2022), reports the “explosive” process:

1. Breaks lignin barrier, which prevents cellulose and hemicellulose from being easily hydrolysed by enzymes. Relaxation of the lignin barrier occurs due to steam explosions.
2. Increases the surface area: more cellulose becomes available to enzymatic action when fibres are physically broken due to the explosive release of pressure.
3. Improves enzymatic accessibility: the enzymes are able to hydrolyse cellulose to fermentable sugars once the fibre structure has been broken.
4. Reduces chemical dependency: steam explosion is a cleaner process.

1.3.2 Enzyme degradation of lignocellulose

Biological pretreatment

The biological pretreatment has been interesting as an aftermath of the fact that it has an upper hand over the physical and chemical pretreatment (Kirk & Chang, 1981) namely:

a) Significant reaction and substrate specificity,

b) Low energy demands

c) Low pollution creation

d) Significant production of desired results.

The application of microorganisms’ enzymatic activity to depolymerize lignin and breakdown lignocellulosic structures is included in the biological pretreatment (Isroi et al., 2011). According to Hatakka, (1983) and Lundquist et al., (1977), white rot fungi are considered the best suitable microorganisms for biological pretreatment, with lignin-degrading capability to water and CO_2 under pure culture qualities.

One of the white rot fungi such as *Phlebia subserialis*, *Phanerochaete chrysosporium*, *Pleurotus ostratus* and *Ceriporiopsis subvermispora* has the capability in metabolising lignin in a broad spectrum of lignocellulosic biomass (Isroi et al., 2011).

According to Deepa & Dhepe., (2015), different forms of lignin are depolymerized into solid acid catalyst monomers with aromatic properties, producing yields of about 60% with a substantial mass balance. The discovery suggests that the improvement of catalyst properties including acidity and surface area is essential to enhance the overall efficiency of lignin depolymerisation processes. The contribution of β-O-4 bonds within lignin structure toward selective depolymerisation results is addressed such as the role of reactor design and practices that can improve lignin solubility to attain maximum value under treatment (Choi et al., 2023).

White-Rot Fungi

White rot fungi can decay all wood components, like lignin, and whiten and render wood fibrous in nature. White rot fungi grow mainly on hardwood e.g., birch and aspen. Blanchette, (1995) states that certain species of *Phlebia genus*, *Phellinus pini* and *Heterobasidion annonsum* radiate grow on softwoods like spruce and pine. White rot fungi decay lignin in two types of activities, e.g., selective and non-selective decays (Blanchette, 1995).

White-rot fungi lignin degradation depends on the genotype of lignocellulose, incubation time, and other factors. Non-selective degradation is the breakdown of more or less equal weights of all fractions of lignocellulose. Selective decay involves the breakdown of hemicellulose and lignin fractions while the cellulose fraction is more or less intact (Isroi et al., 2011).

The fungi have also been researched in relation to a variety of ligninolytic enzymes, such as versatile peroxide (VP), lignin peroxidase (LiP) and manganese peroxidase (MnP) (Higuchi, Takayoshi, 2004) (Wong, 2009). Biological pretreatment by means of white-rot fungi has predominantly been conducted using solid-state fermentation (SSF).

It has been discovered that ligninolytic enzyme production is greater in SSF than it is during submerged fermentation (SF) (Fujian et al., 2001). Enzyme activity and ligninolysis are affected by a number of factors including nutrient composition

(nitrogen, Cu^{2+} and Mn^{2+}), and water, aeration composition, fungal strain, temperature, and pH (Isroi et al., 2011).

Furthermore, extensive uses have been witnessed in the biodegradation of crop residues' lignin by white rot fungi. For instance, the use of white rot fungus in biodegradation tobacco stalks registered a 37.60% degradation rate of lignin after a week, which indicates their efficiency in breaking down lignin-containing material into products that can be potentially used (Su et al., 2016). This is especially applicable where waste management is involved since waste in agriculture tends to have a high lignin content, making it difficult to use waste. Recycling agricultural waste also reduces environmental contamination while promoting the utilization of biomass in a sustainable manner using white rot fungi (Qi et al., 2023).

Oxidases: Laccase

Laccases (Lac, EC 1.10.3.2, benzenediol:oxygen oxidoreductase) are a blue copper oxidases capable of catalysing one-electron oxidation of amines and phenolics like lignin phenolic substructures laccase is secreted by the majority of white rot fungi, with enormous diversity in the amounts (Wong, 2009). Insects, plants, and microorganisms are among the living things that have high concentrations of laccase, the only variation being that fungi-based laccases have a more significant redox potential compared to bacteria-based laccases (Kunamneni et al., 2007).

Laccase production in some of the fungi such as *Ganoderma lucidum* and *C. subvermispora* (Fukushima & Kirk, 1995), is enhanced on incubation with lignocellulosic material. According to Rodriguez-Couto et al., (2001) and Leonowicz et al., (2001), laccase production in white rot fungi is induced by aromatic compounds such as veratryl alcohol (Rodriguez-Couto et al., 2001) or Cu^{2+} (Palmieri et al., 2000) and 2,5–xylidine (Eggert et al., 1996). Fungal laccase has been implicated in fungal plant pathogen interaction, morphogenesis, detoxification and stress defence against by-products of lignin degradation.

Hydrolases:

Cellulases

A diverse range of microorganisms, including *actinomycetes*, other than bacteria, and fungi, secrete a complex group of enzymes known as cellulases (Jayasekara, 2019).

Cellulases are a collection of enzymes that catalyse the hydrolysis of cellulose, a polysaccharide made up of β-1,4 linked glucose units. This collection includes enzymes from classes' endo-1,4-glucanase (EC 3.2.1.4), β-D-glucosidase (EC 3.2.1.21) and exo-1,4-glucanase (EC 3.2.1.91) (Deng & Tabatabai, 1994).

Cellulase components

Endo-β-1,4-glucanases, break the internal β-1,4-glucosidic linkages in cellulose chains to produces shorter oligosaccharides. They have a primary role in the generation of new chain ends that are further hydrolysed by other cellulases (Huang et al., 2017) (Zhang et al., 2019). Cellulose is a linear polymer of β-1,4-linked D-glucose units containing repeating disaccharide units, called cellobiose. Cellobiose is cleaved from the reducing or non-reducing ends of cellulose chains by complementary enzymes referred to as β-1,4-glucanases (Zhang et al., 2019) (Huang et al., 2017).

Cellobiose is also hydrolysed to glucose by enzymes β-glucosidases. B-glucosidases are crucial to the cellulase system as they prevent the accumulation of cellobiose, which would otherwise inhibit the other cellulases (Liu et al., 2016) (Zhang et al., 2019).

Cellulase importance in biomass conversions

The capability of various cellulases' to work synergistically to convert lignocellulosic biomass to glucose is one of the most important factors in their effectiveness. Studies have determined that when these enzymes are present in an optimal ratio, saccharification process work more efficiently (Bhatt et al., 2023)

Recent research has identified that certain fungi, like *Trichoderma reesei* and *Aspergillus awamori*, are capable of producing cellulase. *T. reesei* is a model organism for industrial enzyme production and is well noted for its high cellulase production (Naher et al., 2021). Cellulase production is further increased through genetic engineering of fungi like *T. reesei*, where focus is given to certain gene networks that are related to cellulase expression (Matsu-ura et al., 2018).

Factors and mechanisms affecting cellulase activity

Substrate type, pH, temperature, and the presence of inhibitory substances such as lignin degradation products are some of the parameters that affect the activity of cellulase. Cellulases and lignin can interact hydrophobically, thus possibly reducing

the efficiency of the enzyme and causing non-productive binding (Ilić et al., 2021). One of the methods to mitigate these effects is the use of nonionic surfactants such as Tween 80, which can enhance enzyme access to cellulose by inhibiting non-specific adsorption onto lignin (Marsola et al., 2022).

1.4 Aim

The aim of the present study was to extract sugars from wastepaper and cardboard fibre to be utilised in supplementing ruminant feed by bio-catalysis.

1.5 Objectives:

The objectives of the current study are to:

- Analyse the processed wastepaper for:
    - Fibre organic constituents/compositions (e.g., cellulose, hemicellulose, and lignin)
    - Metal content
    - Possible toxic compounds
- Screen for enzymes that will be viable and beneficial in the breakdown of the fibre to valuable products,
- Optimise the bio-catalysis conditions to produce a valuable product for use in ruminant feed,
- Apply all analysis methods to produce the carbohydrates used in ruminant feed,
- Formulate the product and production of a sample for testing,
- Beneficiation of the ruminant feed with the developed product.

1.6 Hypothesis and Questions

1.6.1 Hypothesis:

Wastepaper and cardboard fibre are rich in nutritional value that could be of beneficial in ruminant feeds; particularly during drought seasons when feed shortages lead to increased costs.

1.6.2 Research questions to guide the study:

1. What enzymes can be used in the breakdown of wastepaper and cardboard fibre into high sugar ruminant feed additives?
2. What are the nutritional values/benefits as determined chemically?
3. How will the wastepaper and cardboard fibre pulp influence the price of a formulated product once it is processed into feed?

CHAPTER TWO – Methods and Materials

2.1 Materials and Equipment

- A Hidex Sense PlateReader, Turku, Finland
- Inductively coupled plasma sector-field mass spectrometer (ICP-SFMS): ELEMENT2, Thermo Fisher, Germany, equipped with a self-aspirating microflow nebulizer of 100 l/min, a PFA spray chamber and auto-sampler (CETAC Technologies, USA).
- Microwave digestion system: ETHOS UP with 15 position SK (high pressure) rotor and reference temperature vessel in position1 (Milestone, Italy)
- Semi-micro analytical balance: Cubis II, Sartorius, Germany.
- Paper and cardboard fibre pulp.
- 96 well plate, Lasec.
- ALP autoclave, Lasec.
- Overhead Stirrer ES, Velp Scientifica
- Waterbath
- Excella E24 Incubator Shaker Series
- Ultra-Performance Liquid Chromatography-Quadrupole-Time of Flight Mass Spectrometry (UPLC-Q-TOF MS) (Framingham, MA, USA)
- Tween 80
- D-glucose
- High-performance liquid chromatography (HPLC)
- Buchner funnel
- Muslin cloth
- Rotary evaporator

2.2 Sample material

The pulp (wastepaper and cardboard fibre mixture) shown in **Figure 6**, was received at the CSIR, based in Pretoria, from Khepri biosciences, based in Mogale City.



Figure 6: Wastepaper and cardboard fibre mixture received from Khepri biosciences.

2.3 Pulp preparation

Of the pulp that was received, 1 kg was soaked in a 5 L bucket of deionised water. The mixture was agitated by using an overhead stirrer for 30 minutes, then the liquid was removed by filtration. A repeat 5 L wash with deionised water was performed. The pH from the second wash was recorded at 7.24 as compared to after the first wash which was 8.34.

From the washed pulp (**Figure 7**), 20 g was sampled and placed in an oven (120 °C) for 2 hours. Once the oven drying was complete, the pulp was removed, and it was allowed to cool down to room temperature and weighed. The yield was 5.26 g dry mass.



Figure 7: The paper pulp sample after washing.

2.4 Enzymes

The enzyme preparations were chosen on basis that they are commercially available in bulk and that they are able to hydrolyse cellulose (**Table 8)**. The exception is laccase, which is an oxidative enzyme that has activity against lignin.

Table 8: Commercial enzymes for cellulose hydrolysis.

Enzyme	**Supplier**
Naturase® *Penicillium* Cellulase	OenoBiotech
Acid Cellulase (CB111523)	Creative Biogene
Laccase	Sigma-Aldrich
Enzeco® Cellulase CEP Multizyme® Cellulase CEP	Enzyme Development Corporation
Acid Cellulase BSJD025 Bescell VRE Compound Cellulase BL3.0	Bestzyme
Cellic Ctec 3 HS	Novozymes A/S.

2.5 Buffer solutions

Buffers were prepared using edible compounds according to AAT Bioquest (2006). Preparation of 0.1 M sodium citrate buffer pH 5 or pH 6 in 1 L: sodium citrate dehydrate (24.269 g), and citric acid (3.358 g) were dissolved in 800 ml deionised water: adjusted to pH 5 or pH 6 with HCl or NaOH, then topped up to volume with distilled water to 1 L and stored at 4 °C.

Preparation of 0.1 M sodium acetate buffer pH 5 or pH 6 in 1 L: sodium acetate (7.721 g), and acetic acid (352.5 mg), were dissolved in 800 ml deionised water: adjusted to pH 5 or pH 6 with HCl or NaOH, then topped up to volume with deionised water to reach 1 L and stored at 4 °C.

Preparation of 0.01 M sodium citrate pH 5 in 1 L: sodium citrate dehydrate (1.71 g), and citric acid (804.2 mg), were dissolved in 800 ml deionised water: adjusted to pH 5 with HCl or NaOH, then topped up to volume with deionised water to reach 1 L and stored at 4 °C.

Preparation of 0.01 M sodium acetate buffer pH 5 in 1 L: sodium acetate (0.136 g), and acetic acid (0.06 mg), were dissolved in 800 ml of deionised water: adjusted to pH 5 with acetic acid; then topped up to volume with deionised water to reach 1 L and stored at 4 °C.

2.6 Enzyme solution

The enzyme solutions were prepared as follows:

The enzyme dilutions were made by dissolving the required amount of enzyme (from 10 mg to 1000 mg) in 100 ml of (0.1 M and 0.01 M) sodium citrate buffer or sodium acetate buffer at pH 5.

2.7 Biomass Analysis

2.7.1 High-performance liquid chromatography

This process was done in the Biorefinery Industrial Development Facility (BIDF) department in Durban, by Pule Seemela.

Biomass sample was carefully weighed (0.20 g) and dissolved in sulphuric acid in the test tubes. At the same time, the moisture content of the sample was determined by using the moisture analyser. The contents of the test tube were heated in a constant temperature water bath for 60 minutes with regular stirring, using a glass rod. The

resulting blend was rinsed into Schott bottles using ultrapure water and autoclaved for 1 hour. The samples were then cooled to room temperature and filtered through a Buchner filtration setup with weighed nylon filter paper and ready for HPLC analysis.

The sample was screened using HPLC to determine the concentration of carbohydrates present in unknown samples. Monosaccharides were analysed utilizing a Carbopac PA1 analytical column along with a pulsed amperometric detection on a Thermo ICS5000+ HPLC system. ICS5000+ HPLC system which includes autosampler, quaternary LC pump, column oven, pulsed amperometric detector, isocratic LC post column pump and Dionex CarboPac PA1 analytical column (in conjunction with Dionex borate trap and Phenomenex SAX guard cartridge).

2.7.2 Klason/Insoluble lignin analysis

The klason lignin (insoluble lignin content) was determined gravimetrically according to the Technical Association of the Pulp and Paper Industry (TAPPI) 222om-88 after acid hydrolysis of the sample.

2.7.3 Soluble lignin analysis

The hydrolysate was read by Genesys 150 spectrometer. The ultraviolet-visible spectroscopy (UV-Vis) utilizes light to quantify the absorbance of a chemical species in solid-state or aqueous solution. The sample was placed in a quartz cuvette (1 cm path length) containing 3% sulphuric acid as a solvent.

2.8 Colorimetric analysis

2.8.1 Pretreatment of pulp material

The pulp was washed twice with deionised water, then the excess liquid was removed and then the pulp was oven dried at 120 °C for two hours. After the pulp was cooled to room temperature as shown in **Figure 8**, it was milled, to increase the surface area for the enzymatic hydrolysis, Bansod et al., (2024) using a blender for approximately 3 minutes as shown in **Figure 9**.

For large quantities, the wet pulp can be placed in an open room for drying (low cost). This process took approximately 5 days to be dried and milled at 4.5 mm particle size sieve.



Figure 8: The oven dried pulp sample.



Figure 9: The milled pulp after being placed in a blender

The milled pulp (2 g) was placed in a Schott bottle and then 20 ml of deionised water was added. The mixture was then placed in an autoclave at 121 °C for one hour. Once the mixture had cooled down, the excess liquid was removed, and the pulp was allowed to dry up and await enzymatic hydrolysis.

Pretreatment with NaOH

The milled pulp (100 g) was placed in a 2 L volumetric flask that contains 1 L of 5% NaOH (Jung et al, 2020). The mixture was then autoclaved (121 °C) for 20 minutes. Once autoclaved, the pH of the mixture was 12.52.

Lignin separation after NaOH autoclaving

After the pretreatment, the mixture was allowed to cool to room temperature. The filtration took place using a Buchner funnel under vacuum to separate the solid residue (cellulose and hemicellulose pulp) and black liquor (NaOH solubilized solution).

The black liquor was acidified by slowly adding sulphuric acid, while gently stirring, to pH 2. The acidified solution was then filtered using the Buchner funnel under vacuum. The filtered cake was then washed with acidified deionized water (pH 2) to remove residual NaOH. The lignin cake was the oven-dried for 10 minutes at 120 °C. Once it was dried, it was allowed to cool to room temperature and then weighed.

The solid residue (cellulose and hemicellulose) was then brought to pH 5 using 2.5% sulphuric acid (Xu et al, 2014). Once the pH was corrected, the liquid and solids were further separated by filtration, and the solids went through enzymatic hydrolysis seen in **Figure 10**.



Figure 10: The enzymatic hydrolysis of 100 g wastepaper and cardboard fibre pulp.

Pretreatment with Laccase

The milled pulp (2 g) was placed in a Schott bottle and then 20 ml of laccase-citrate enzyme solution was added. The mixture was then incubated at 50 °C, 200 rpm overnight. The liquid and solids were separated by filtration, and the solids went through enzymatic hydrolysis.

Enzymatic hydrolysis

The procedure of the enzymatic hydrolysis, 1 g of pulp mixed with 10 ml of enzyme solution as shown in **Figure 11**, was placed in a shaker. The conditions of the shaker were 50 °C and 200 rpm. The reactions gave good results after multiple testing with different conditions and enzyme solutions.

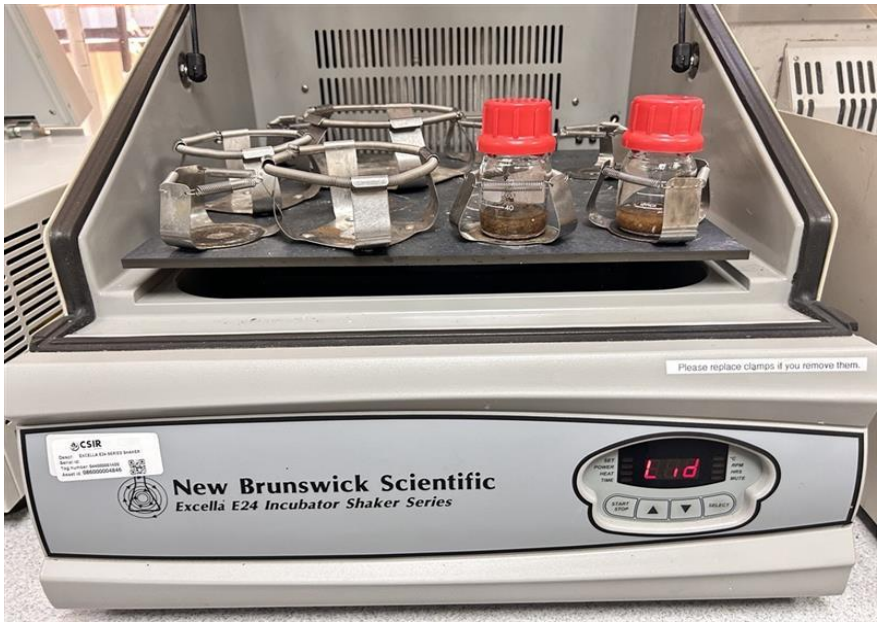


Figure 11: The pulp, buffer and enzyme mixture in Schott bottles placed in a shaker.

To accurately assess the impact that the enzyme has on the pulp, the experiment was repeated at a larger scale using 80 g of wet pulp was placed in an Erlenmeyer flask in 200 ml of buffer, the mixture was covered and then autoclaved for an hour. Once it had cooled, the mixture was added to the reactor as can be seen in **Figure 12,** with an addition of 200 mg of enzyme. The reaction conditions were set to 50 °C and 500 rpm.

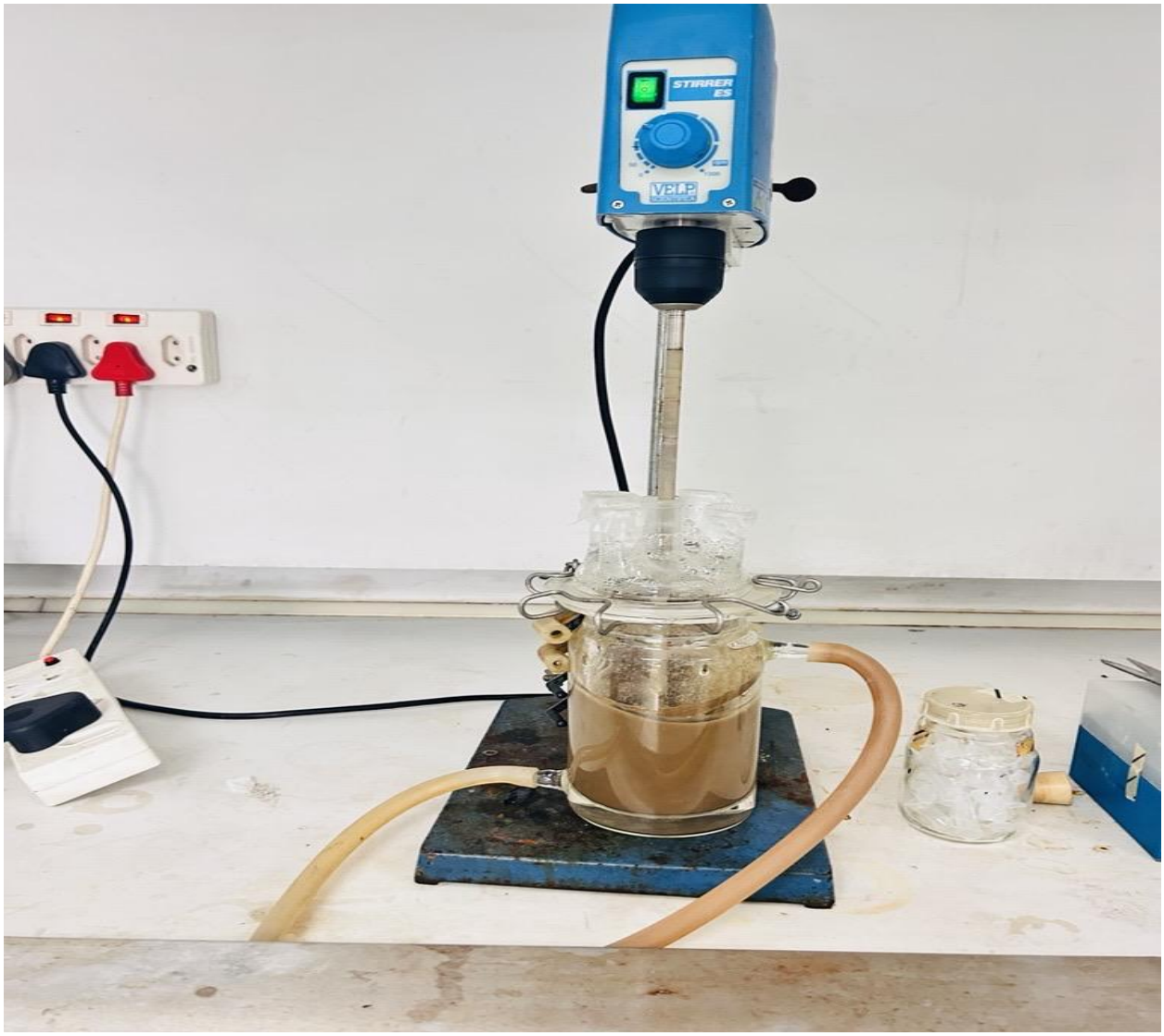


Figure 12: The pulp, buffer and enzyme mixture placed in a reactor and an overhead stirrer used for mixing.

2.8.2 Determination of reducing sugars

In this method, the DNS reagent, which contains 3,5-dinitrosalicylic acid in an alkaline medium, reacts with free aldehyde or ketone groups of reducing sugars to form 3-amino-5-nitrosalicylic acid. This reaction produces a coloured complex whose intensity, measured spectrophotometrically, is directly proportional to the concentration of reducing sugars present in the sample (Gonçalves et al., 2010) (Wood et al., 2012).

In order to connect absorbance with concentration, the experimental setup should additionally involve creating a calibration curve using standard reducing sugars, often D-glucose. Furthermore, it is advised to validate the method for the particular sample

type because the presence of other reducing chemicals in the sample matrix may interfere with the assay (Gonçalves et al., 2010).

The standard curve of the glucose was generated using D-glucose. The stock solution was prepared as 15 mg of D-glucose in 3 ml of deionised water. The working solution was prepared as seen in **Table 9**.

Table 9: Working solutions of D-glucose for standard curve.

Concentration (mg/ml)	Stock solution (µl)	Deionised water (µl)
0	0	1000
0.2	40	960
0.5	100	900
0.8	160	840
1.2	240	760
1.5	300	700
2.0	400	600
2.5	500	500
3.0	600	400
3.5	700	300
4.0	800	200
4.5	900	100
5.0	1000	0

The preparation of the DNS and potassium sodium tartrate solution was as follows:

DNS reagent (light sensitive):

- Dinitrosalicylic acid: 10 g,
- Sodium sulfite: 0.5 g,
- Sodium hydroxide: 10 g,
- Dissolved in deionised water (1 L).

The mixture was covered with aluminium foil to block-out the light and then stored at room temperature.

Potassium sodium tartrate solution (Rochelle salt) (40%): 40 g dissolved in 100 ml of deionised water.

The water bath was heated to 90 °C. The glass tubes were placed in a metal rack and 1 ml of the sample (supernatant) was added to the tube followed by 1 ml of DNS as seen in **Figure 13**. The tube was then covered to avoid evaporation and contamination. The mixture was heated in the water bath for 10 minutes.

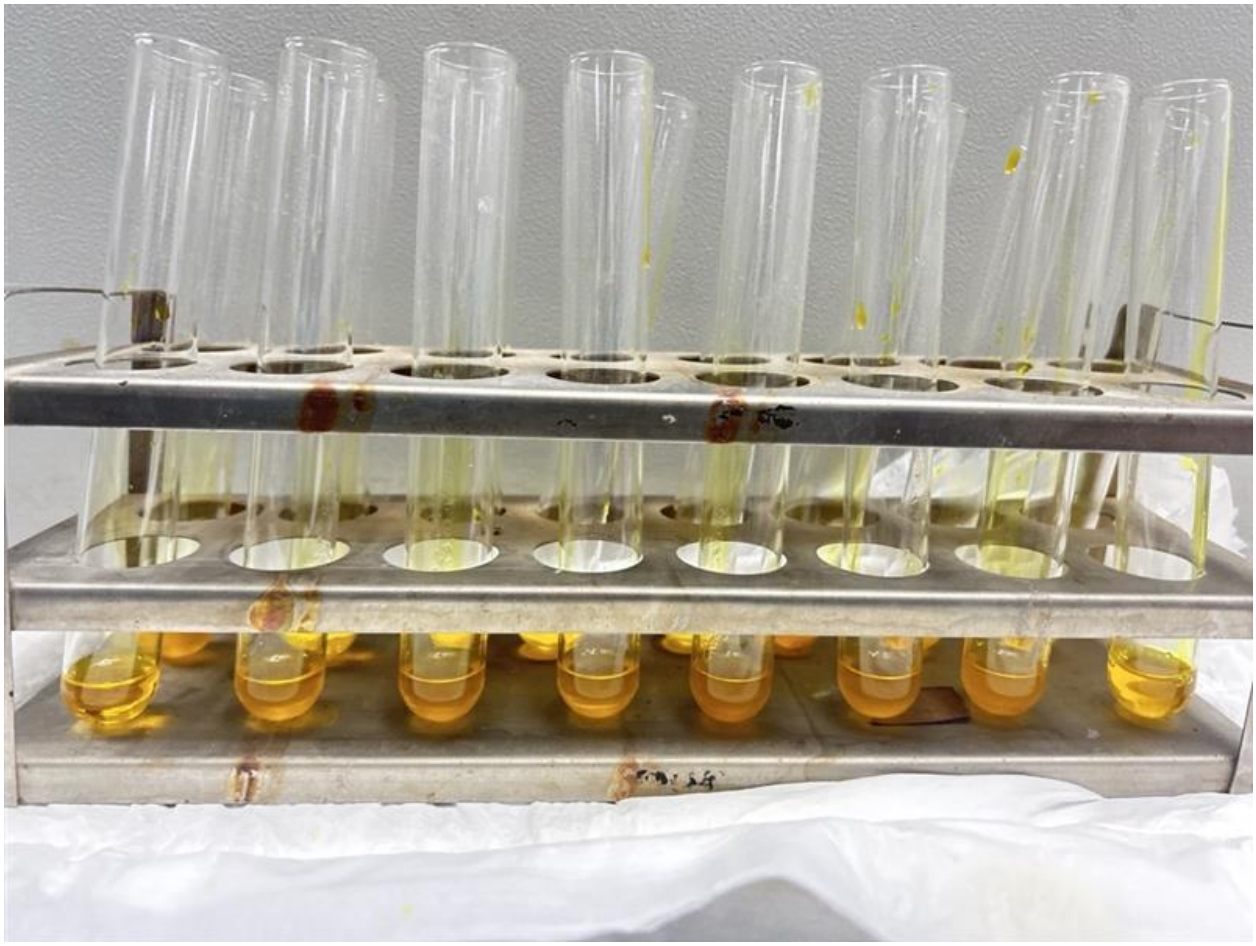


Figure 13: The sample and DNS mixture before heating up in a water bath.

After heating, the rack was removed from the water bath and 340 µl of the Rochelle salt was added to each of the tubes and the mixture was allowed to cool to room temperature, resulting in a colour change as seen in **Figure 14**. Thereafter 100 µl of the mixture (done in triplicates) was transferred to a 96 well microplate, to read the absorbance at 575 nm using a Hidex Sense PlateReader (**Figure 15)**.

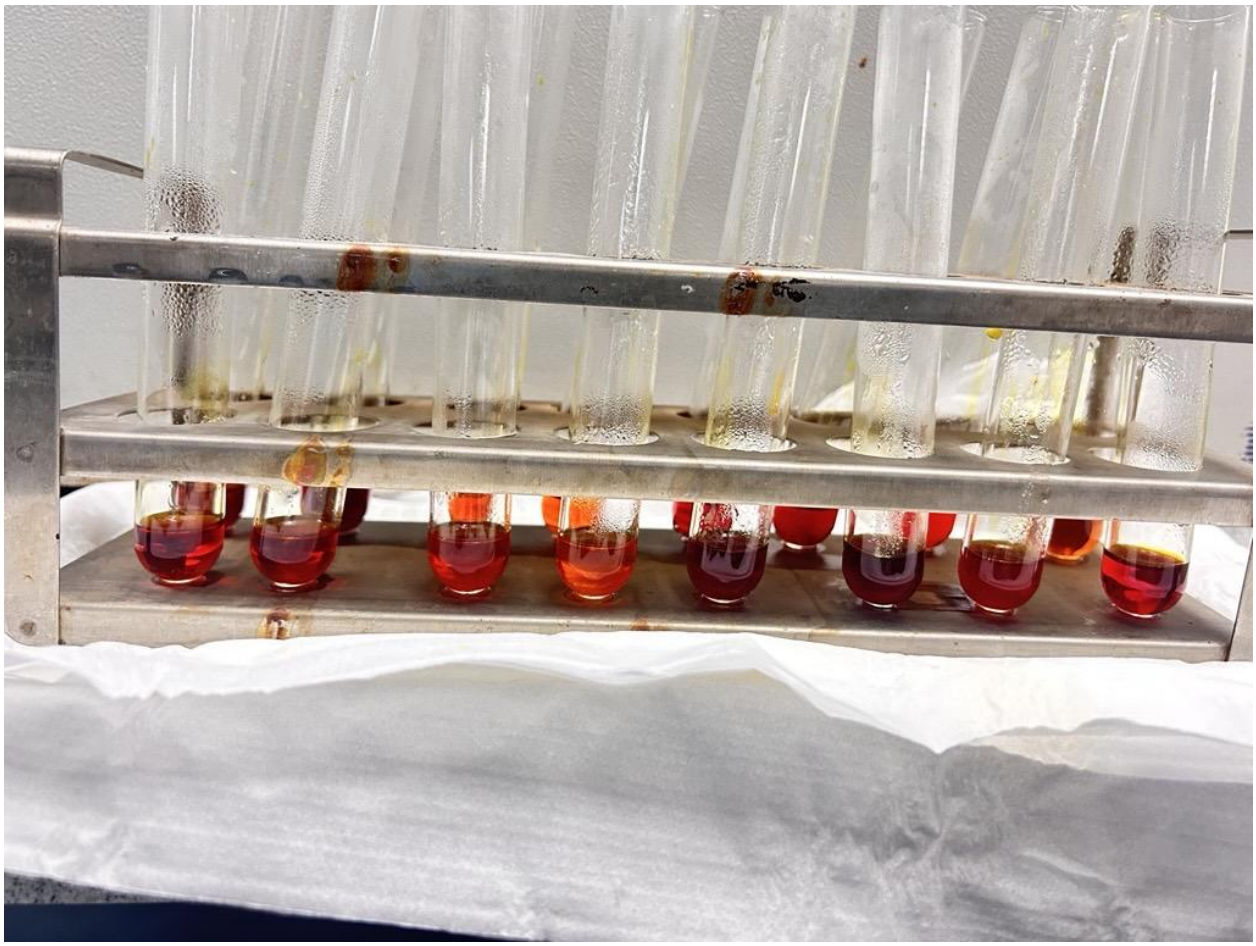


Figure 14: The sample and DNS reaction mixture after being placed in a water bath for 10 minutes.

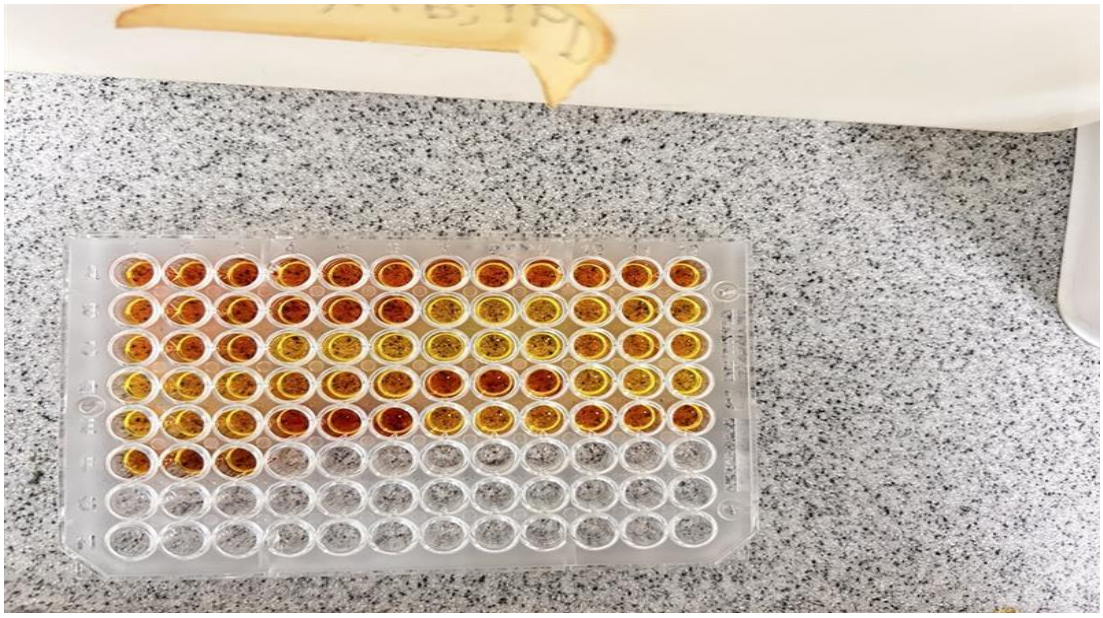


Figure 15: The DNS reaction sample in a 96 well plate before reading the absorbance.

2.9 Recovered glucose product

The mixture from the reactor was passed through the muslin cloth, then it was filtered by vacuum filtration two times. The rotary evaporation, at 60 °C, was used to remove the excess water that was found in the reaction.

2.10 Element analysis (major, minor and trace)

The process was done by the National Metrology Institute of South Africa, in Pretoria.

The simultaneous identification of major, minor, and trace elements in a variety of sample matrices is made possible by the reliable and adaptable analytical method known as elemental analysis using Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES). In order to guarantee full analyte dissolution and reduce any possible matrix interferences, the sample is usually prepared by acid digestion before being added to an aqueous solution (Ruiz-de-Cenzano et al., 2015).

Nebulized sample solution is placed into an argon plasma in the ICP-OES system, where the high temperature (often above 8000 K) effectively atomizes and excites the constituent elements. Each element's own wavelengths of light are released by the relaxed stimulated atoms, providing an element specific spectrum. A spectrometer is then used to gather and measure the optical emission signals, and multi-element standard solutions are used to calibrate the intensity. In order to guarantee accuracy and reduce biases caused by inter-element effects, these standards must be carefully

prepared, including precise matrix matching the sample (Lee et al., 2016). Because it enables the simultaneous measurement of elements present in concentrations spanning multiple orders of magnitude, this calibration method is especially crucial for evaluating complicated matrices (Leikin & Phillips, 2023).

The test material was stored at room temperature before preparation and analysis was prepared in a temperature-controlled, clean laboratory environment.

The sub-sample amounts of approximately 0.5 g were dissolved in a combination of double distilled, high purity nitric acid (HNO_3) and concentrated hydrofluoric acid (HF). Following microwave digestion, samples were diluted with deionised water to 50 ml in high-purity polypropylene vials. Following further dilution, samples were analysed by ICP-SFMS. The analysis was carried out at a temperature of 20 ± 3 ˚C.

For the ICP-SFMS, the multi-element quality control tune solution was used as a standard in the semi-quantitative method set-up.

2.11 UPLC- QToF-Mass Spec analysis

The process was done by Prof Paul Steenkamp at the CSIR, South Africa.

A sophisticated analytical method that has gained popularity because of its exceptional sensitivity, resolution, and speed is ultra-performance liquid chromatography coupled to quadrupole time-of-flight mass spectrometry (UPLC-QToF-MS). This method is especially well-suited for the in-depth qualitative and quantitative analysis of complex biological samples and natural products. By using smaller particle sizes in the stationary phase, UPLC produces better peak shape and resolution and enables faster separations than conventional high-performance liquid chromatography (HPLC) (Sun et al., 2015) (Asamenew et al., 2019).

The detection capabilities are greatly improved by the incorporation of QToF mass spectrometry. High-resolution mass measurements are made possible by the QToF system, which increases the precision of molecular structure determination and makes it easier to identify substances with comparable m/z ratios. UPLC-QToF-MS has been widely used to characterize phytochemical contents in biological matrices and complicated herbal formulations, enabling the simultaneous profiling of many compounds (Liu et al., 2017).

2.11.1 UPLC analysis

A Waters Premier UPLC coupled in series to a Waters SYNAPT XS HDMS mass spectrometer was used to generate accurate mass data. Optimisation of the chromatographic separation was done utilising a Waters HSS T3 C18 column (150 mm x 2.1 mm, 1.8 µm) and the column temperature controlled at 60 °C. A binary solvent mixture was used consisting of water (Eluent A) containing 10 mM formic acid (pH 2.4) and acetonitrile (Eluent B) containing 10 mM formic acid. The initial conditions were 100%A at a flow rate of 0.4 ml/min and were maintained for 1 minute, followed by a linear gradient to 10%A at 16 minutes. The conditions were kept constant for 2 minutes and then changed to the initial conditions. The runtime was 20 minutes, and the injection volume varied between 3 µl and 5 µl. Samples were kept cool at 8 °C in the Waters Sample Manager during the analysis.

2.11.2 TOF Mass Spec analysis

The SYNAPT XS mass spectrometer was used in sensitivity mode and operated with an electrospray probe to enable detection of all ESI-compatible compounds. Leucine enkephalin (100 pg/ml) was used as reference calibrant (Lock Mass) to obtain typical mass accuracies between 0.1 and 2 mDalton (mDa). The mass spectrometer was operated in both ESI positive and negative modes with a capillary voltage of 0.6 kV, the sampling cone at 30 V and the extraction cone at 4.0 V. The scan time was 0.1 seconds covering the 50 to 1500 Dalton mass range with an interscan time of 0.02 seconds. The source temperature was 120 °C and the desolvation temperature was set at 450 °C. Nitrogen gas was used as the nebulisation gas at a flow rate of 700 L/h and cone gas was added at 50 L/h. The software used to control the hyphenated system and do all data manipulation was MassLynx 4.2 (SCN 1028).

CHAPTER THREE – Results and Discussion

3.1 Analysis of biomass sample

The analysis of the biomass sample (after pulp preparation) was identified and quantified into monosaccharides (with HPLC) and lignin (insoluble lignin and soluble lignin analysis respectively), done in duplicate (R1 and R2) as seen in **Tables 10, 11 and 12**.

Table 10: Monosaccharides composition of a pulp sample.

Sample No.	Arabinose %	Galactose %	Glucose %	Xylose %	Mannose %	Total %
Biomass R1	0.28	0.39	55.57	1.90	2.15	60.29
Biomass R2	0.28	0.54	55.44	2.03	2.35	60.65

Table 11: Klason/Insoluble lignin of a pulp sample.

Sample No.	Lignin (%)
Biomass R1	38.27
Biomass R2	38.24

Table 12: Soluble lignin of pulp sample.

Sample No.	Lignin (%)
Biomass R1	0.32
Biomass R2	0.36

The results obtained above were used as a reference to compare with the results obtained after enzymatic hydrolysis.

The total % mass of biomass R1 = 98.88% and R2 = 99.25%

The elevated lignin content of the wastepaper and cardboard fibre arises from the removal of long cellulose fibres during paper recycling, which leaves behind a residue

of short cellulose fibres containing high levels of lignin (Celignis, 2025) (Green Agrochem, 2025). The residual fraction, which is not taken up into new paper, serves to concentrate lignin from the initial waste stream. This enrichment is consistent with the findings of pulp and wastewater analysis, which report high levels of lignin in residues of recycled fibre (Vikman et al., 2024)

The lignin that was recovered after 100 g fibre-NaOH autoclaving and lignin separation was 16.74 g. The 5% NaOH autoclaving pretreatment had a positive impact in removing lignin from the wastepaper and cardboard fibre pulp, ultimately exposing the cellulose to be enzymatically hydrolysed to glucose.

3.2 Glucose standard curve

The production of a standard curve for glucose, seen in **Figure 16**, has been used to great success in numerous studies. For instance, Ejekwu et al., (2021) and Miller (1959) described the production of standard curves for analysing total reducing sugars, and they measured the absorbance at 575 nm to get their quantitative assays.

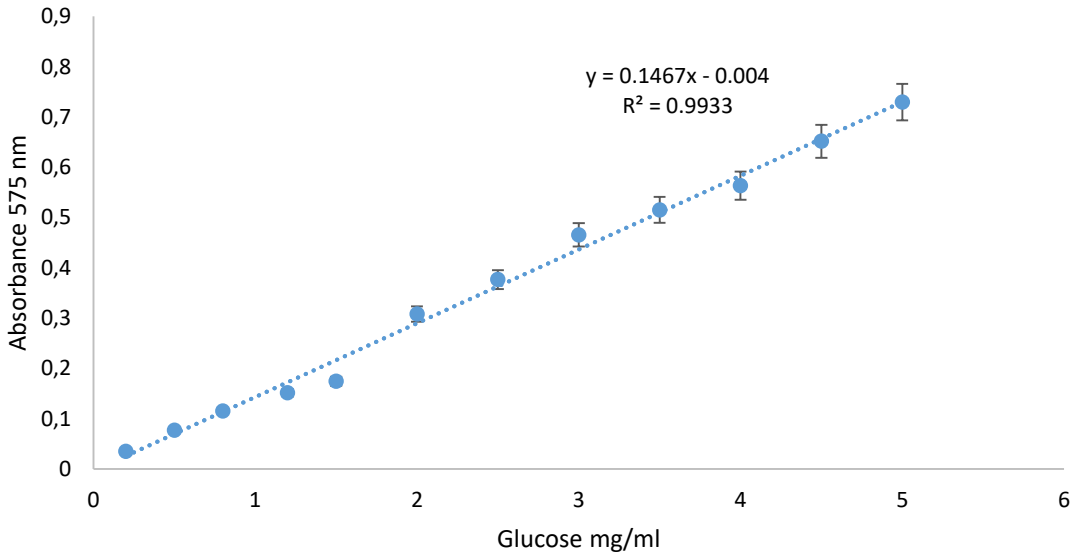


Figure 16: The glucose standard curve at different concentrations.

A standard curve was prepared using known glucose concentrations. Although the DNS assay measures total reducing sugars, the regression equation derived from the glucose standard curve was applied to the absorbance values of the hydrolysed wastepaper and cardboard fibre samples to estimate their reducing sugar content, expressed as glucose equivalents.

3.3 Enzymatic hydrolyses of paper and cardboard fibre

Since wastepaper and cardboard fibres are important renewable resources, the enzymatic hydrolysis of lignocellulosic biomass—including these materials—is an essential step in the reducing sugars production process. Effective biomass pretreatment and the choice of suitable enzyme systems are the two main factors that determine the effectiveness of enzymatic hydrolysis, which is crucial. Processing lignocellulosic materials into fermentable sugars typically involves two steps: enzymatic hydrolysis and a pretreatment phase to improve cellulose accessibility (Horn et al., 2012) (Gatt et al., 2019). The intrinsic recalcitrance of wastepaper and cardboard fibre pulp, which are composed of cellulose, hemicelluloses, and lignin, must be addressed by this two-phase method in order to increase the bioavailability of the enzymes that catalyze the hydrolysis reaction (Harris et al., 2010) (Horn et al., 2012).

The enzyme choice was based on commercial availability, activity spectrum and cost efficiency; buffer choice was guided by maintaining optimal pH for cellulase activity while testing ionic strength effects; optimization was structured as a stepwise process, beginning with enzyme screening, followed by systematic variation of buffer molarity, enzyme load and agitation

3.3.1 Cellulosic enzyme analysis

The paper and cardboard fibre pulp underwent enzymatic hydrolyses using various enzymes and the glucose yield release was measured by the DNS method as shown in **Figure 17**. The enzymes were tested using two safe and edible buffers (sodium citrate buffer and sodium acetate buffer) at 0.1 M, pH 5. The products that are expected are reducing sugars, the most abundant one being glucose, the rest being arabinose, galactose, xylose and mannose. The enzyme solutions were 1 g of enzyme in 100 ml of buffer.

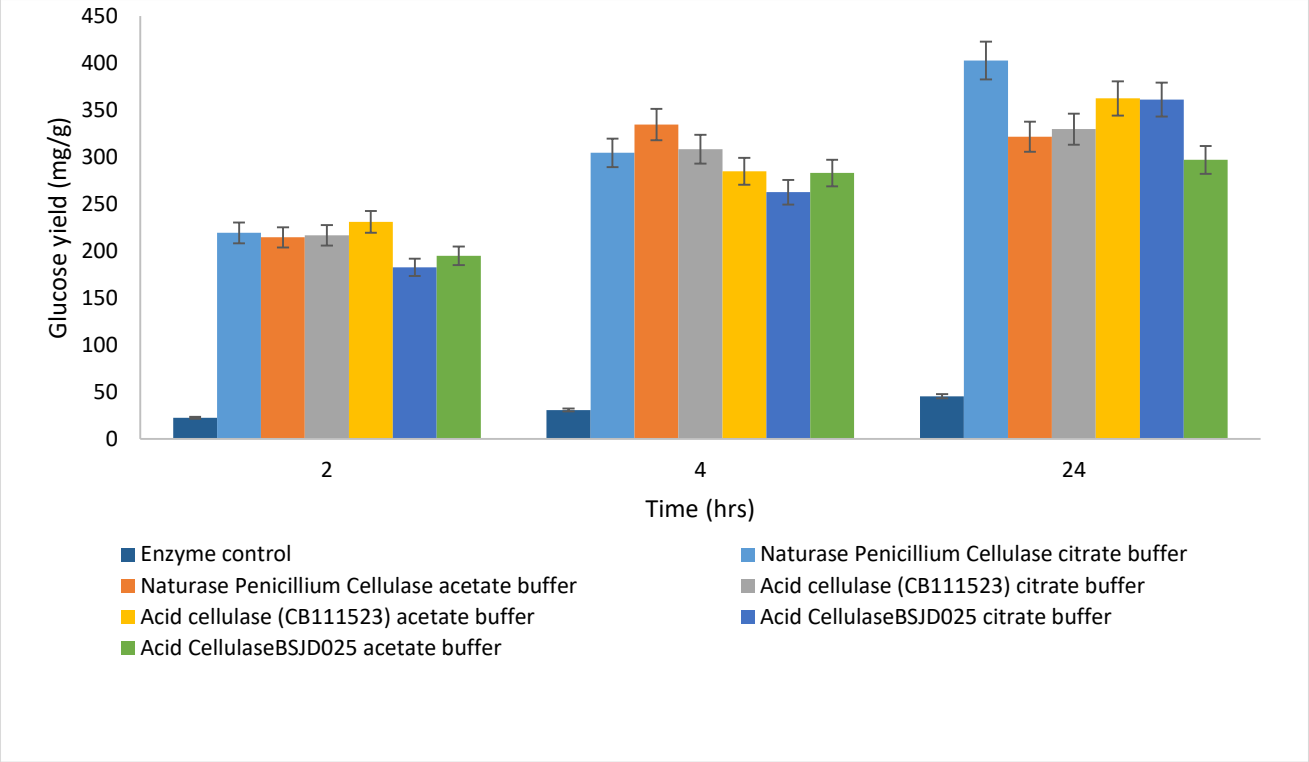


Figure 17: The paper and cardboard fibre enzymatic hydrolyses with different buffers and enzymes were performed at 50 °C, 200 rpm for 24 hours for glucose yield per gram. The DNS method was used to analyse the reducing sugar throughout the process.

Over time, enzymatic activity continues to break down wastepaper and cardboard fibre to create reducing sugars, evidenced by the increasing glucose yield for all treatments. More reducing sugar is always obtained with treatments that include citrate buffer and acetate buffer because they support enzymatic activity. Minimum reducing sugar yield is with the enzyme control (deionised water) treatment, less than 50 mg/g for the duration of the experiment, and it is suggested that there is not a lot of enzymatic activity when there is not specific buffer present.

Table 13: Glucose equivalent yield of different conditions and buffers after 2 and 24 hrs.

Conditions	**Buffer type**	**Glucose yield (mg/g) after 2 hrs**	**Glucose yield (mg/g) after 24 hrs**	**Average glucose release rate**

				µmol/g fibre /min
Enzyme control	Not applicable	22.52	45.32	0.17
Acid Cellulase BSJD025	Citrate	182.69	361.28	1.39
Acid Cellulase BSJD025	Acetate	194.86	296.98	1.14
Acid Cellulase (CB111523)	Citrate	216.77	329.70	1.27
Acid Cellulase (CB111523)	Acetate	231.08	362.42	1.40
Naturase® *Penicillium* Cellulase	Citrate	219.27	402.64	1.55
Naturase® *Penicillium* Cellulase	Acetate	214.50	321.75	1.24

The highest yield was achieved by the Naturase® *Penicillium* Cellulase at 402.64 mg/g glucose per biomass yield in citrate buffer after 24 hours. The contrast between acetate and citrate buffers is instructive regarding the buffer's function in enzyme activity. Naturase® *Penicillium* Cellulase provided a 402.64 mg/g glucose yield in citrate buffer, but only 75% of this yield was achieved in acetate buffer (321.75 mg/g glucose yield).

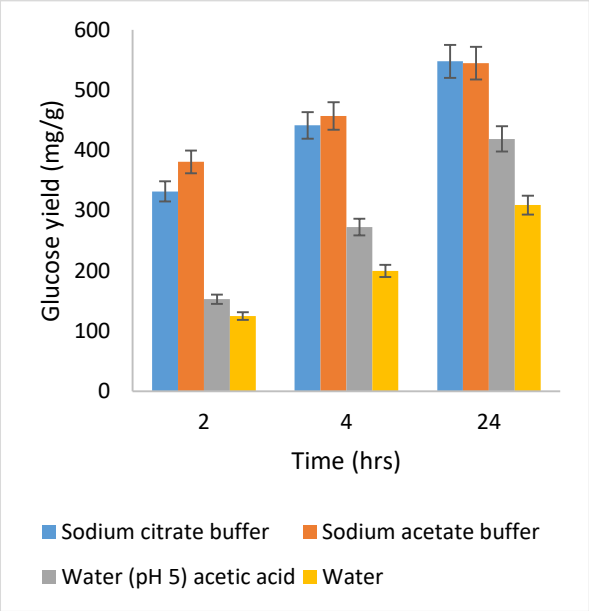
Acid Cellulase (CB111523) performs better with acetate buffer (362.42 mg/g glucose yield) than citrate buffer (329.70 mg/g glucose yield); while Acid Cellulase BSJD025 performs better in citrate buffer (361.28 mg/g glucose yield) as compared to acetate buffer (296.98 mg/g glucose yield).

In the long term, no significant release of glucose was observed in the enzyme control (deionised water), which indicates that enzymatic catalysis needs to be present for efficient degradation of cellulose to glucose (i.e. there is only a small amount of soluble glucose present in the biomass).

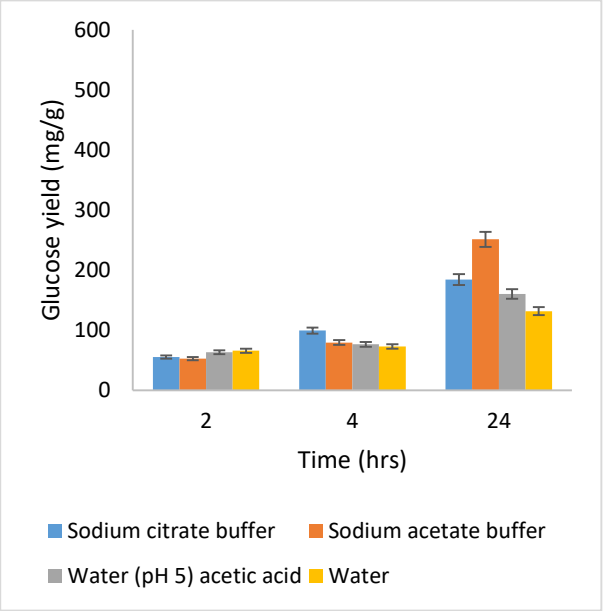
3.3.2 Impact of buffers

The two buffer conditions were used to see the impact they have in maximizing the various enzymes in breaking down the cellulose and hemicellulose into reducing sugars as shown in **Figure 18**. The buffer solutions that were used were sodium citrate buffer (0.1 M, pH 5), sodium acetate buffer (0.1 M, pH 5), deionised water and deionised water which the pH was adjusted to 5 using acetic acid. The enzyme solutions were 1 g of enzyme in 100 ml of buffer.

A



B



C D

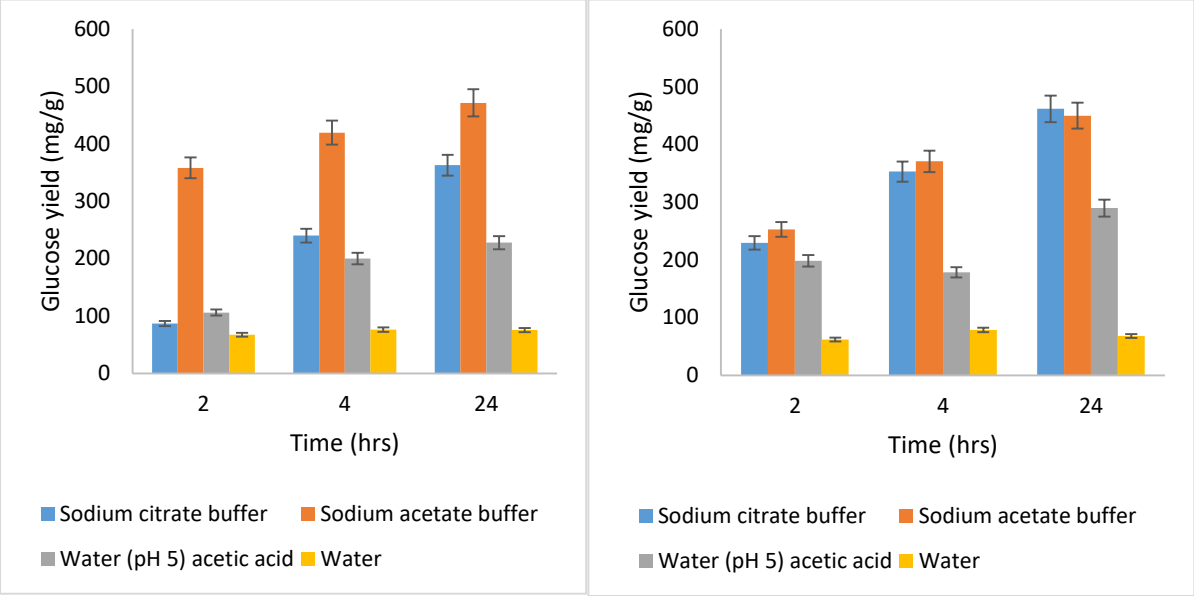


Figure 18: The different enzymes, namely Acid Cellulase BSJD025 (A), Bescell VRE (B), Naturase® *Penicillium* Cellulase (C) and Compound Cellulase BL3.0 (D), in different buffer solutions breaks down the carbohydrates differently. The conditions of the reactions were 50 °C, 200 rpm.

Table 14: Comparison of cellulose preparation activities.

Buffer type	**Glucose yield (mg/g) after 24 hrs**					
	Acid cellulase BSJD025	Bescell VRE	Naturase® *Penicillium* Cellulase	Cellulase BL 3.0	Average	Normalised (%)
Sodium acetate buffer	544.65	251.31	471.26	449.68	429.22	100%
Sodium citrate buffer	547.83	184.28	362.65	461.71	389.11	91%
Water (pH 5) acetic acid	419.00	160.19	227.68	289.71	274.14	64%
Deionised water	309.02	131.56	75.44	67.94	145.99	34%

The enzymatic hydrolysis of cellulose was evaluated under four treatment conditions, namely, sodium citrate buffer, sodium acetate buffer, deionised water (pH 5, acetic acid adjusted), and deionised water, for three periods of time: 2, 4 and 24 hours. The yield of glucose (mg/g) was considered as the primary parameter to estimate the efficiency of each of the treatments.

During the process of enzymatic hydrolysis, the sodium acetate buffer and, to a slightly lesser extent sodium citrate buffer, facilitated high yields of glucose after 24 hrs. When pH adjusted water (using acetic acid) was used as a solvent the glucose produced was on average 36% lower, while unadjusted water gave an average of 64% lower reducing sugar. The results therefore prove that buffering is a requirement to maximise the enzymatic hydrolysis of paper and cardboard fibre.

The Bescell VRE has phosphatase and α-galactosidase as key enzymes. The enzymatic solution produced the lowest glucose, 251.307 mg/g, after enzymatic hydrolysis as compared to the other tested enzymes after 24 hrs.

The Cellulase BL 3.0 enzyme in citrate and acetate buffers, respectively, both provided reasonable high glucose output, with citrate being slightly better.

The results indicate that buffered systems far outperform minimally buffered or no-buffered systems for enzyme-catalyzed hydrolysis. The sodium citrate buffer generated the highest glucose levels at every measurement time point most reliably, likely due to:

- Optimal pH maintenance
- Improved enzyme stability
- Potential chelation actions that can reduce inhibitory ions (Huang, W. et al., 2022).

3.3.3 Enzyme and buffer concentration

Since the impact of buffers has been established, the obvious questions concern whether the degree of buffering, as a function of buffer salt concentration, is sufficient, and whether increasing the biocatalyst load would enhance activity?

Solutions, of 10 mg, 100 mg and 1000 mg of enzyme were prepared in the sodium citrate and sodium acetate buffers (100 ml of 0.01 M at pH 5) respectively (**Figure 19**).

Then 10 ml of enzyme solution was added to the 1 g of pulp and the mixture was incubated. The glucose levels were checked at intervals, the last being 24 hours.

A B

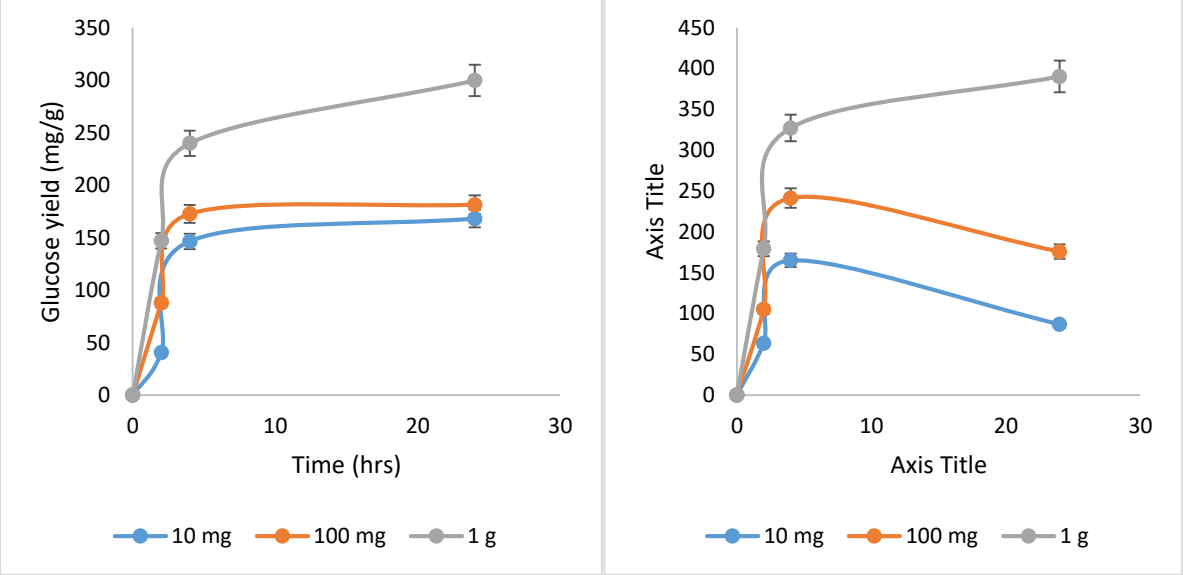


Figure 19: Acid Cellulase BSJD025 in acetate buffer (A) and citrate buffer (B) The paper and cardboard fibre enzymatic hydrolysis with different buffers and enzymes were performed at 50 °C, 200 rpm. The DNS method was used to analyse the conditions throughout the process.

Table 15: Glucose equivalent yield after 2 and 24 hrs at 0.01 M buffer concentration using Acid Cellulase BSJD025.

Enzyme concentrations (mg)	Glucose yield (mg) after 2 hrs in acetate buffer	Glucose yield (mg) after 24 hrs in acetate buffer	Glucose yield (mg) after 2 hrs in citrate buffer	Glucose yield (mg) after 24 hrs in citrate buffer
10	40.45	168.17	63.62	86.80
100	87.93	181.33	104.98	175.87
1000	147.01	299.76	179.28	390.37

All three curves in the acetate buffer exhibit an initial steep increase in glucose yield followed by a plateau phase, indicating that;

- The initial phase (0 – 10 hours) is the period of active hydrolysis where the interaction of enzymes with the substrate is most effective.

- The phase (after 10 hours) reflects that substrate either becomes limiting or enzyme activity declining via product inhibition, enzyme denaturation, or substrate recalcitrance.

The 1 g dose provides the highest glucose concentration, which means that it overcomes some of the restrictions at lower concentrations, such as crowding of enzymes or insufficiency of catalytic turnover, as compared to 10 mg and 100 mg (Völler, 2019).

For the citrate buffer, despite the decline, 1 g dose indicated the maximum glucose yield, while the 10 mg and 100 mg decreased.

The results indicate a diminishing effect of the enzyme on pulp hydrolysis, as its ability to generate reducing sugars decreases over time. The reason for that is either the enzyme could be inhibited (or denatured), microbial consumption or secondary reactions (oxidation) could have started reducing free glucose (Olkiewicz et al., 2022). The buffer strength, 0.01 M, also played a role as it can be see that the glucose produced is less than 50%.

The enzyme solutions, seen in **Figure 20**, of 10 mg, 100 mg and 1 g of enzyme were prepared in the sodium citrate and sodium acetate buffers (100 ml of 0.1 M at pH 5) respectively. The same information is shown in the graphs.

A B

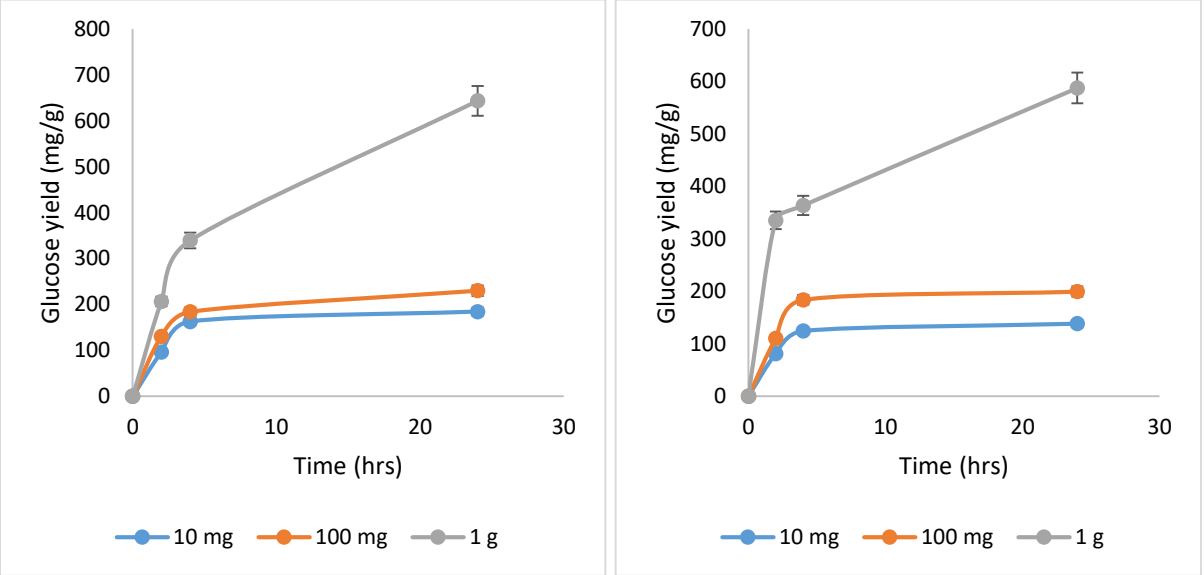


Figure 20: Acid Cellulase BSJD025 in acetate buffer (A) and citrate buffer (B) The paper and cardboard fibre enzymatic hydrolysis with different buffers and enzymes were performed at 50 °C, 200 rpm. The DNS method was used to analyse the conditions throughout the process.

Table 16: Acid Cellulase BSJD025 in acetate and citrate buffer (0.1 M).

Enzyme concentrations (mg)	Glucose yield (mg) after 2 hrs in acetate buffer	Glucose yield (mg) after 24 hrs in acetate buffer	Glucose yield (mg) after 2 hrs in citrate buffer	Glucose yield (mg) after 24 hrs in citrate buffer
10	96.34	184.52	81.12	138.61
100	130.88	230.05	110.43	199.69
1000	206.77	643.49	335.38	587.59

The 1000 mg enzyme dose treatment did much better than the others, and this is most likely due to one or more of the following reasons:

- Higher enzyme concentration, with more active sites for hydrolysis of cellulose.
- Greater substrate accessibility, possibly because effective pretreatment had reduced lignin content or crystallinity.

The 10 mg of enzyme was able to produce 184.52 mg and 138.61 mg of glucose from 1 g of pulp with acetate and citrate buffers respectively. When 1000 mg of enzyme

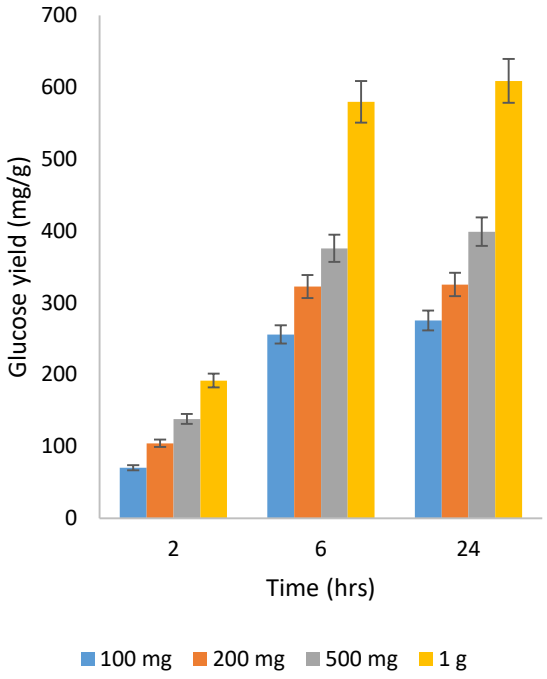
was used to breakdown 1 g of pulp, the glucose produced was 643.49 mg and 587.59 mg with acetate and citrate buffers respectively. In conclusion, it can be seen that 0.1M performed better that than 0.01M as it produced double the glucose yield.

Having determined that the enzymatic hydrolysis reaction worked, we obtained additional enzyme preparation types from various suppliers to determine which had superior properties.

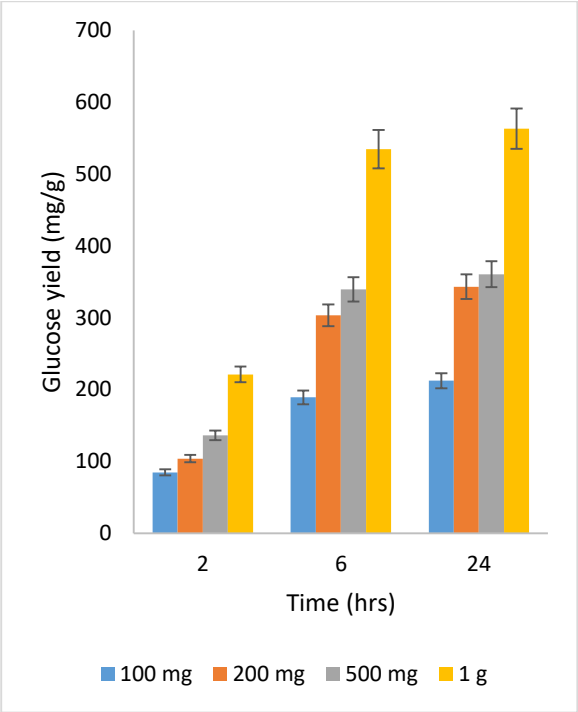
The enzyme solutions, seen in **Figure 21** of 100 mg, 200 mg, 500 mg and 1000 mg of enzyme were prepared in the sodium citrate and sodium acetate buffers (100 ml of 0.1 M at pH 5) respectively. The same information is shown in the graphs.

Then 10 ml of enzyme solution was added to the 1 g pulp.

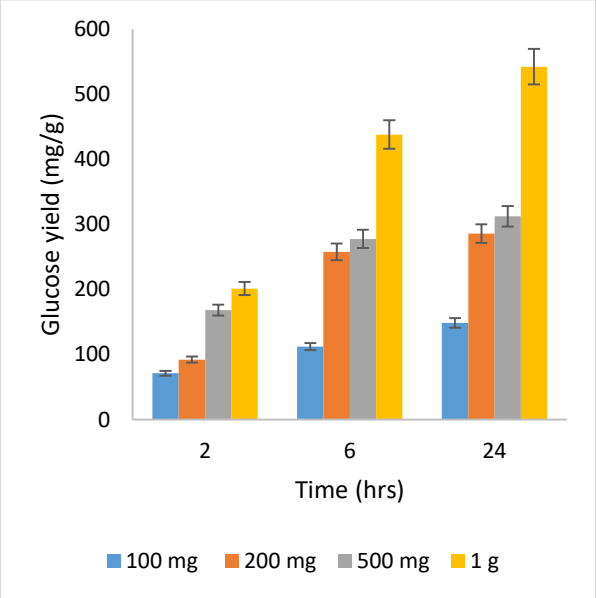
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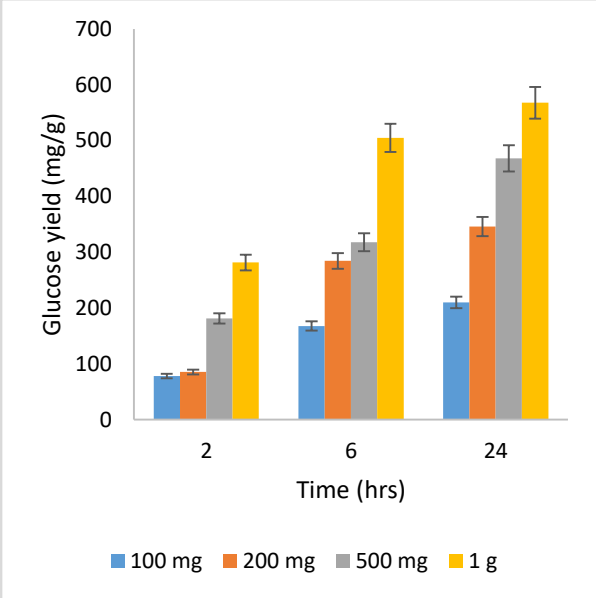
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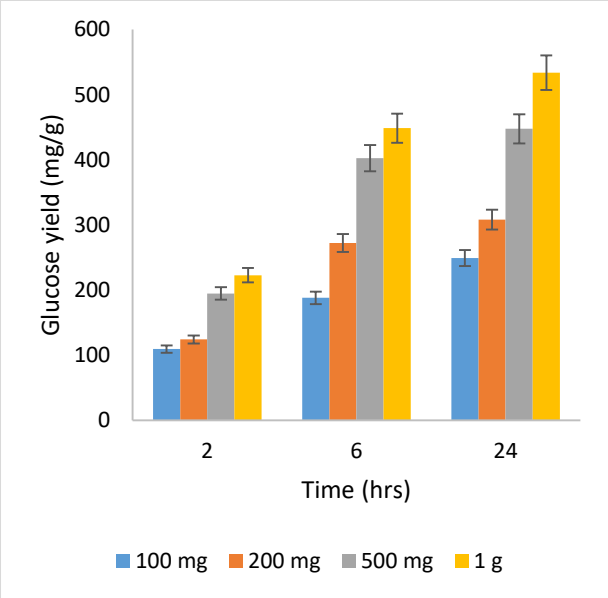
C


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Glucose yield (mg/g)
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Time (hrs)
100 mg
200 mg
500 mg
1 g

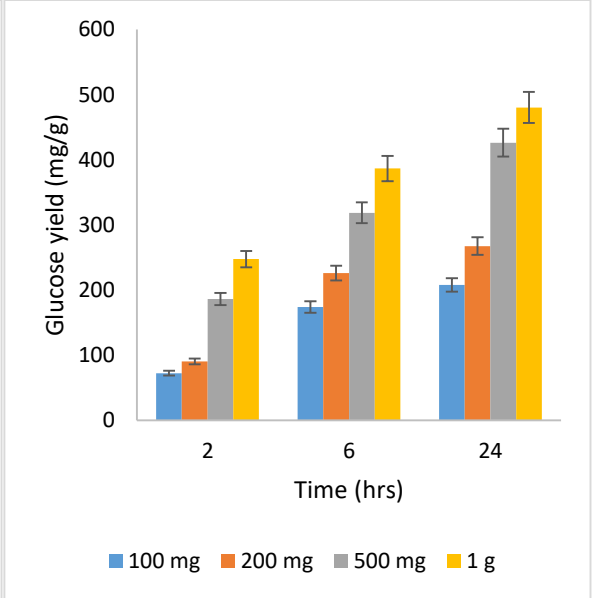
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Glucose yield (mg/g)
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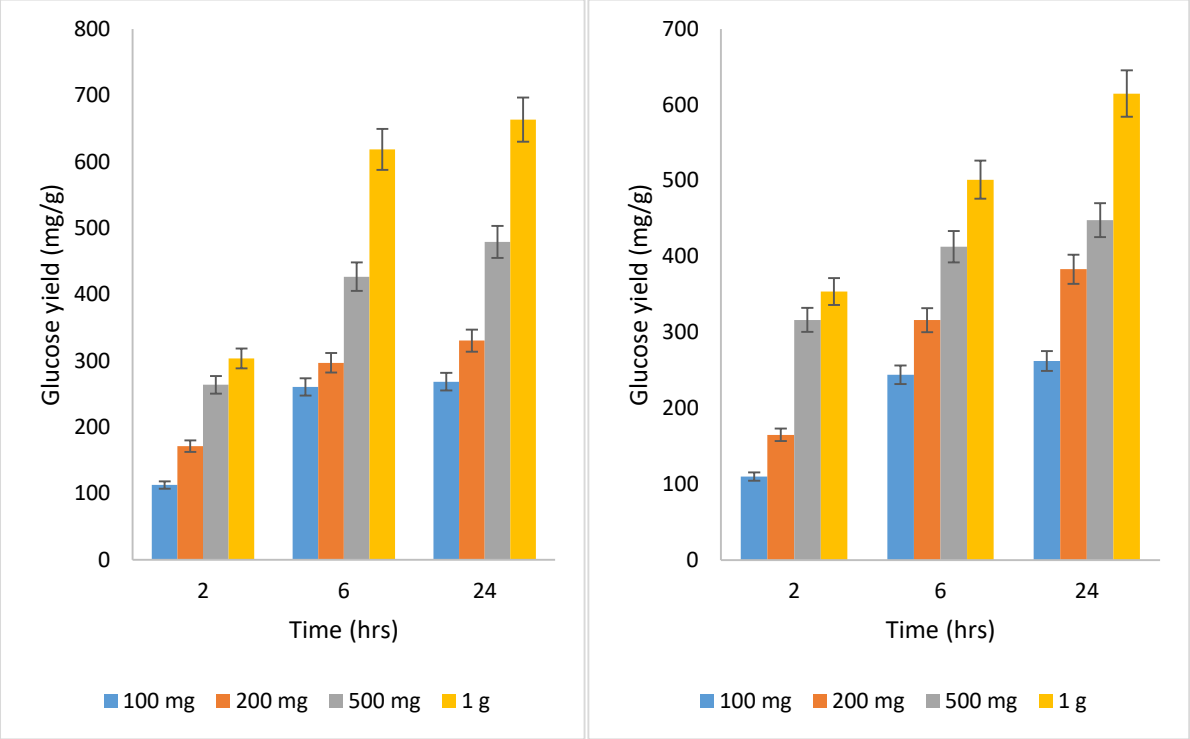
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Glucose yield (mg/g)
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Time (hrs)
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500 mg
1 g

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Glucose yield (mg/g)
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24
Time (hrs)
100 mg
200 mg
500 mg
1 g

G H



b

Figure 21: Acid Cellulase BSJD025 (A), Acid Cellulase (CB111523) (C), Enzeco® Cellulase CEP (E), and Multizyme® Cellulase CEP (G), respectively in sodium citrate buffer. Graphs B, D, F and H represent the same enzymes in sodium acetate buffer, respectively. The wastepaper and cardboard fibre enzymatic hydrolysis with different buffers was performed at 50 °C, 200 rpm for 24 hours. The DNS method was used to analyse the glucose yield throughout the process.

Table 17: Specific glucose release per mass of enzyme.

Enzyme Preparation		**Average specific rate of glucose release per enzyme load (mg glucose /g fibre / g enzyme/ hrs)**	
	Enzyme load (mg)	**Acetate buffer pH 5**	**Citrate buffer pH 5**
Acid cellulase BSJD025	100	88	115
	200	71	68
	500	30	33
	1000	23	25
Acid Cellulase (CB111523	100	88	62
	200	72	60
	500	**39**	26

	1000	24	23
Enzeco Cellulase	100	87	104
	200	56	64
	500	36	37
	1000	20	22
Multizyme® Cellulase CEP	100	109	112
	200	80	69
	500	37	40
	1000	26	28

The experimental findings show a clear inverse relationship between enzyme load and specific glucose release efficiency. At the lowest level of enzyme concentration (100 mg), maximum specific activity was realised for both acetate and citrate buffer systems, between 87 and 115 mg/g/g/h. At the higher enzyme load (1000 mg), specific activity declined drastically, indicating substrate saturation and diminishing returns due to excess enzyme. The average specific rate of glucose release per enzyme load between the two buffers show that on average, the activity is similar in spite of the differences in buffer salts.

These results have implications on the experiment in maximising enzymatic hydrolysis of recycled wastepaper and cardboard fibre for use in feed, so that enzyme loading and selection of buffer should be optimised co-ordinately to provide maximal efficiency at minimal cost.

The 1 g enzyme in 100 ml buffer solution produced over 50% glucose yield from the 1 g of paper and cardboard fibre pulp as to other enzyme concentrations. The Multizyme Cellulase performed better than the other enzymes in both the buffer types, as it produced over 60% of the glucose yield from the wastepaper and cardboard fibre.

Since the Multizyme® Cellulase CEP performed better in the previous reactions, it was further evaluated, but with smaller intervals in enzyme mass additions in order to better assess the impact of enzyme load (**Figure 22**). The enzyme solutions, of 500 mg, 600 mg, 700 mg, 800 mg, 900 mg and 1 g of enzyme were prepared in in sodium citrate and sodium acetate buffers (100 ml of 0.1M at pH 5) respectively.

A B

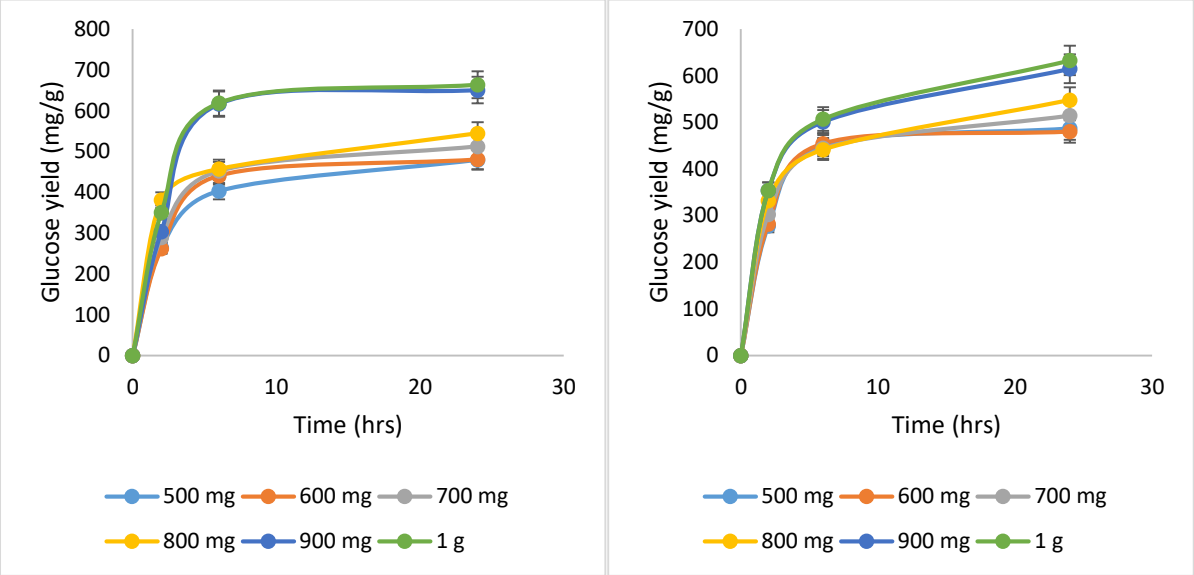


Figure 22: Multizyme® cellulase CEP in citrate buffer (A) and acetate buffer (B) The paper and cardboard fibre enzymatic hydrolysis with different buffers and enzyme concentrations were performed at 50 °C, 200 rpm. The DNS method was used to analyse the glucose yield throughout the process.

Table 18: Different buffer type and enzyme (Multizyme® cellulase CEP) concentrations.

	Glucose yield (mg/g) after 2 hrs		**Glucose yield (mg/g) after 24 hrs**	
Enzyme load (mg)	**Acetate buffer pH 5**	**Citrate buffer pH 5**	**Acetate buffer pH 5**	**Citrate buffer pH 5**
500	277.66	263.80	487.16	479.21
600	282.21	262.21	480.12	480.57
700	302.89	289.48	514.65	512.38
800	331.74	380.82	547.83	544.65

900	353.78	303.57	614.86	650.31
1000	353.78	350.37	632.58	663.49

The results demonstrate the effects of enzyme load and buffer composition on glucose production following the enzymatic hydrolysis of the wastepaper and cardboard fibre, determined between 2 and 24 hours. Glucose release at 2 hours is moderate in all enzyme loads, showing the initiation phase of converting the cellulose into glucose. The glucose yield improves with higher concentration of enzyme from 500 to 1000 mg, with both buffer systems showing an improvement.

From 500 to 700 mg, the sodium acetate buffer was producing slightly more glucose than the citrate, but from 800 to 1000 mg, the citrate was showing a high rise. The glucose yields are significantly higher by 24 hours, indicating a continued hydrolysis and full conversion of substrate

This biphasic trend is typical enzymatic saccharification processes and reflects the transition from active hydrolysis to a state of equilibrium or inhibition (Lischeske & Stickel, 2019).

3.3.4 Hydrolysis with a mixture of two enzymes

In this section, two enzymes were mixed in single buffer to see if it provided any synergy in hydrolysing the fibre into reducing sugars (**Figure 23**).

The 1% enzyme solutions, of enzyme (consisting of 500 mg each of Naturase® Penicillium cellulase and Compound cellulase BL3.0) were prepared in both sodium citrate and sodium acetate buffers (1 g enzyme powder, 100 ml in 0.1M buffer at pH 5) respectively.

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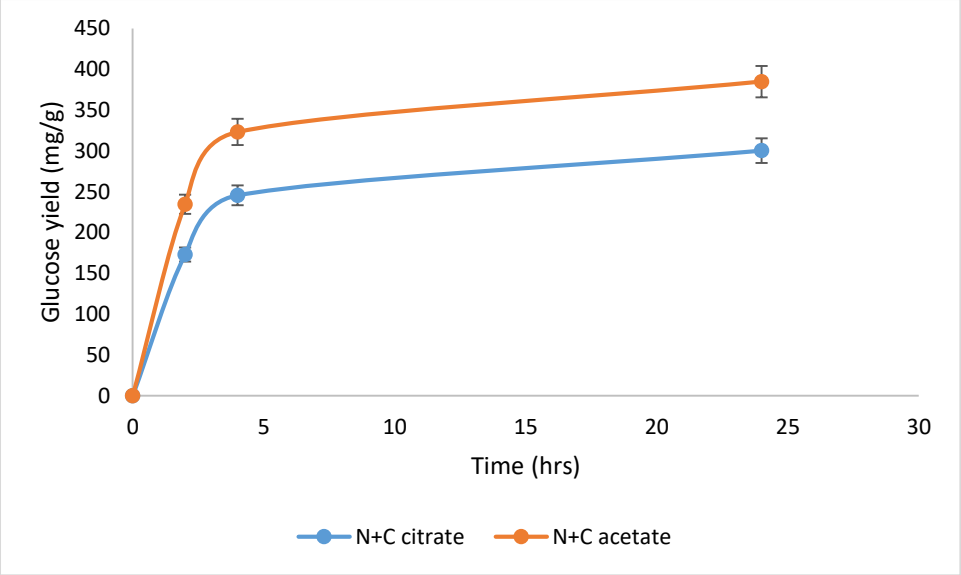


Figure 23: The Naturase and Cellulase BL enzymes mixture was used. The paper and cardboard fibre enzymatic hydrolysis with enzyme mixture with the different buffers (sodium acetate in orange and sodium citrate in blue) at pH 5 were performed at 50 °C, 200 rpm. The DNS method was used to analyse the conditions throughout the process.

The enzyme mixture produced low glucose yield from both buffer solutions. The glucose yield produced from the sodium citrate buffer was 300.39 mg/g (in blue) and 384.94 mg/g from the sodium acetate buffer (in orange). The enzyme mixture is the lowest glucose yield producer, indicating that there was no catalytic synergy.

3.3.5 Hydrolysis in the presence of nonionic surfactant

Non-ionic surfactants reduce the non-productive binding of cellulase enzymes to lignin, therefore improving the sugar hydrolysis yield (Min et al., 2015). For this study, Tween 80 was used. For this process, 500 mg of enzyme was added into 100 ml of sodium citrate buffer pH 5 to prepare the enzyme solution. Then 1 g of the pulp was added into 10 ml of the enzyme solution along with a 20 µl or 40 µl aliquot of Tween 80 (**Figure 24**). Then the mixture was incubated in a shaker.

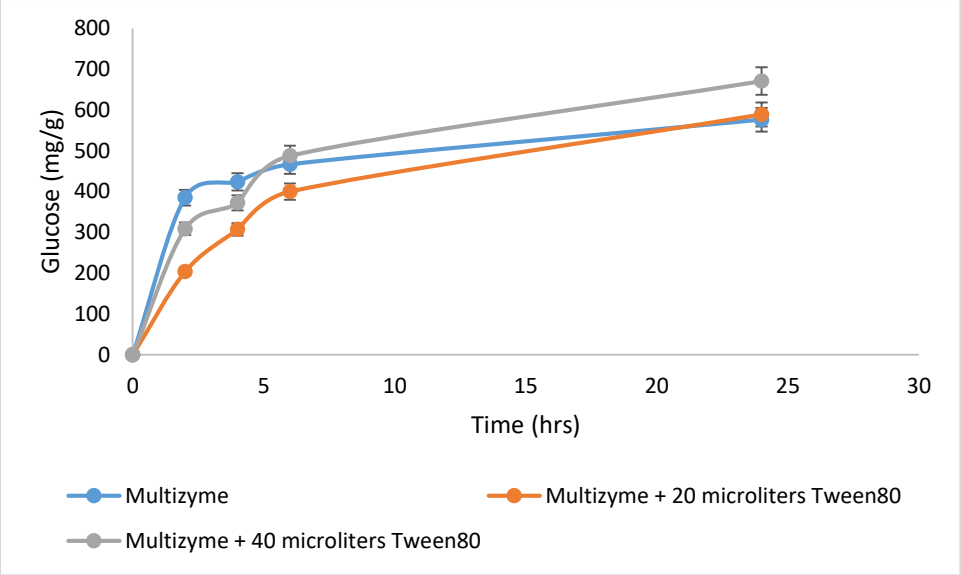


Figure 24: Multizyme cellulase CEP enzyme (500 mg) with 0, 20 or 40 µl of Tween 80, respectively. The paper and cardboard fibre enzymatic hydrolyses with different buffers and enzymes were performed at 50 °C, 200 rpm. The DNS method was used to analyse the reducing sugars throughout the process.

When the Multizyme cellulase CEP enzyme (500 mg load) was used without surfactant, the glucose that was produced is 576.23 mg/g. The addition of 40 µl Tween 80 surfactant increased this slightly when it was added in the reaction mixture, up to 670.98 mg/g (a 16% increase), making it comparable to the yields achieved with 1 g of enzyme.

3.3.6 Pulp Pretreatment

Lignin removal is one of the biggest challenges for enzymatic hydrolysis. Therefore, pretreatment is necessary to breakdown the lignin and expose the cellulose for enzymatic hydrolysis. The laccase enzyme was chosen because it oxidises lignin's phenolic groups, breakdown its complex structures and reduces interferences with enzymatic hydrolysis (Bajpai, 2016). While with NaOH, the cellulose fibres swell, the surface area increases and the enzyme accessibility improves (Zhao et al., 2012).

Pretreatment with Laccase

For the pretreatment with Laccase, 1 g of the Laccase enzyme was added into 100 ml of sodium citrate buffer pH 5 to create enzyme solution. Then 2 g of wastepaper and cardboard fibre (milled and not autoclaved) was added into 20 ml of the enzyme solution. The mixture was then incubated overnight at 50 °C, 200 rpm. The excess

liquid was then removed by filtration, then 1 g of the wastepaper and cardboard fibre was added into 10 ml of the Enzeco® Cellulase CEP enzyme solution, seen in **Figure 25**.

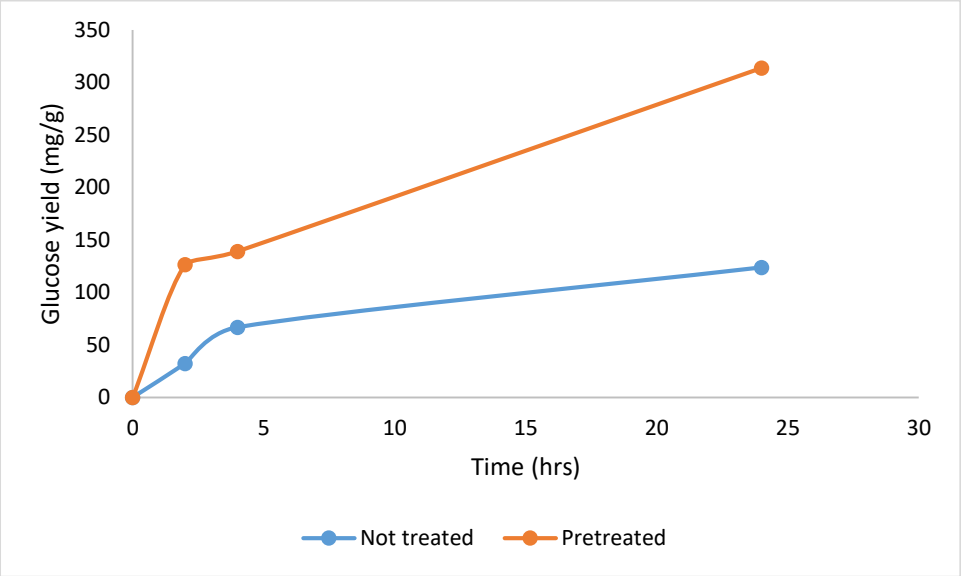


Figure 25: The Enzeco® Cellulase CEP hydrolysis of paper and cardboard fibre pre-treated by laccase. Reaction conditions were sodium citrate at pH 5 were performed at 50 °C, 200 rpm. The DNS method was used to analyse glucose release throughout the process.

The preteated fibre produced almost three times the glucose that was produced from the untreated at 2, 4 and 24 hours. This suggests that the lignin degradation took place during the overnight incubation with Laccase enzyme, thus making the cellulose accessible for hydrolysis. This is an indication that lignin is a big hindrance in the enzymatic hydrolysis of wastepaper and cardboard fibre into reducing sugars. The laccase enzyme has properties that breakdown the lignin in the wastepaper and fibre pulp, ultimately exposing the cellulose to be hydrolysed into glucose.

Pretreatment with NaOH

NaOH pretreatment has been shown to perform particularly well. Structural analysis (XRD, FTIR, SEM) has shown that NaOH pretreatment disintegrated biomass structure and exposes cellulose to enzymes. This leads to glucose production to be many times higher than that for untreated or acid-treated samples (Wang, Z. et al., 2021).

For this reaction, 100 g of NaOH pretreated pulp (after acid neutralization) was added into 1 L of buffer solution (0.1 M at pH 5), 1 g of enzyme added, and the mixture was placed in a reactor in **Figure 10** above. The glucose yield can be seen in **Figure 26**.

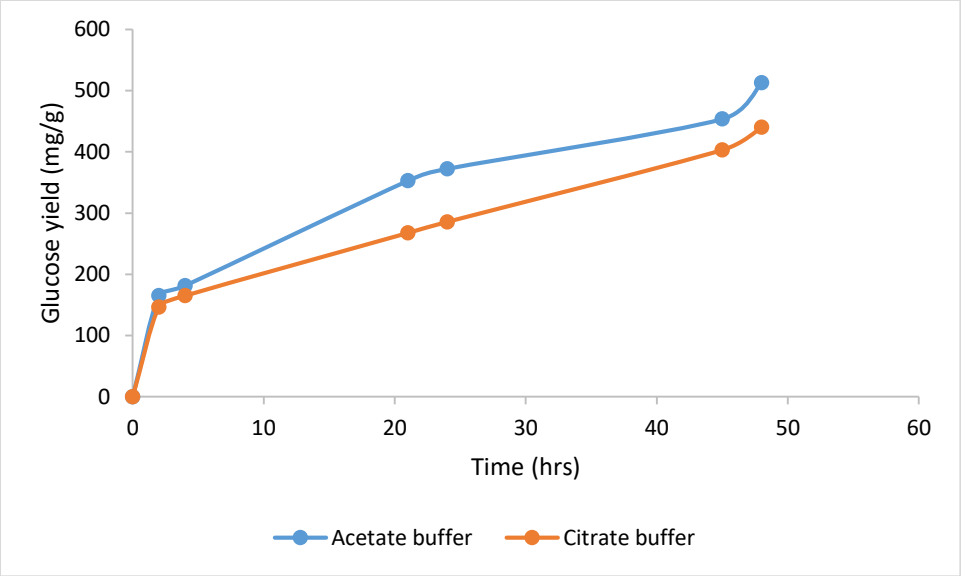


Figure 26: Cellic CTec 3 HS in citrate buffer (blue) and acetate buffer (orange) was used for enzymatic hydrolysis of alkali pre-treated paper and cardboard pulp to produce glucose, under 50 °C and 200 rpm. The DNS method was used to analyse the glucose concentration throughout the reaction.

The enzyme solution mixture made of acetate buffer produced more glucose (513.1 mg/g) as compared to the mixture made of citrate buffer (440.4 mg/g). The reaction was allowed to take place for 48 hours and the glucose production kept on increasing from after 2 hours till the reaction was stopped at 48 hours. The trend suggests that the acetate buffer provides a more favourable environment for enzymatic hydrolysis, resulting in higher glucose release over time.

Both buffers show a linear rise in glucose yield throughout the 48-hour period, with no clear plateau being reached until the final time point. This is suggestive of:

- Maintained enzymatic activity over the period.
- Gradual substrate breakdown, possibly due to the complexity of the structure of the lignocellulosic substrate.
- Minimum inhibition or deactivation of the product enzyme during the time frame.

Absence of an early plateau indicates that the reaction conditions were effective at maintaining enzyme activity and substrate accessibility.

The proper mixing of the solution assisted in further optimisation of the enzyme: fibre pulp (1:100) ratio. As compared to the reactions above where the enzyme and fibre pulp ratio was 1:1 and the highest glucose yield produced was 60%. The reactions were placed in a reactor. The solution mixture required higher enzyme concentration to breakdown the cellulose into glucose as the mixing was poor. With proper mixing, 1 g of enzyme was able to break down 100 g of fibre pulp into 513.10 mg/g (acetate buffer) and 440.40 mg/g (citrate buffer) glucose yield.

Conclusion on pulp hydrolysis studies

The pretreatment with the autoclave, 5% NaOH and with Laccase suggested the disruption of the lignocellulosic biomass and exposed the cellulose to the cellulase enzymes, as the reducing sugar was higher after undergoing the pretreatment processes. The cellulase enzymes in different concentrations and in different buffers were able to hydrolyse the wastepaper and cardboard fibre into reducing sugars. The buffer concentration has an impact in the enzyme activity as it could be seen that 0.1 M of the acetate and citrate buffer outperformed the 0.01 M in producing reducing sugars.

The Multizyme Cellulase CEP and Cellic Ctec 3 HS were the most productive enzymes in both the sodium citrate and sodium acetate buffers at pH 5, 0.1 M. The sodium acetate buffer produced more glucose than the sodium citrate buffer. In reactors with efficient mixing (stirring), smaller amounts of enzyme were required as compared to reactions conducted in a shaker, where mixing was less effective.

The sodium acetate buffer is also ideal because it presents an appealing profile for ruminant feed supplementation, particularly in cost-limited and fermentation-optimised feeds (Chen et al., 2022). If it is sourced in bulk or as a byproduct, it is often less expensive than citrate salts on a per-unit metabolisable energy basis, an advantage that aligns well with feed cost modelling and procurement conventions. Its safety record is also good because acetic acid and sodium acetate are endogenously produced and metabolically familiar to ruminants, which translates to high tolerance and lack of established maximum inclusion limits (European Food Safety Authority, 2012).

Functionally, sodium acetate promotes microbial activity by causing an increase in the levels of concentrations of ammonia nitrogen and microbial crude protein, and therefore supports microbial growth and protein synthesis. It also improves volatile fatty acid (VFA) profile by rising total VFA levels which consist of acetic, isobutyric, isovaleric and propionic acids without altering their molar proportions, indicating steady rumen fermentation (Chen et al., 2022).

The glucose which was the key focus in this study was analysed using the DNS method, and the yield was calculated referencing from the standard curve of the glucose that was created.

3.4 Techno-economic evaluation of the hydrolysate product

Recovered glucose product

The image in **Figure 27** is the glucose that was enzymatically hydrolysed from the wastepaper and cardboard fibre pulp. The pulp was dried, milled and then autoclaved with 5% NaOH for 20 minutes. When the temperature of the mixture had cooled to room temperature, filtration took place using muslin cloth. The wastepaper and cardboard fibre was then washed with tap water twice. After the second wash, the water was not removed and then 2.5% sulfuric acid was used to neutralise the pH to 5.

Once the pH was at 5, the water was filtered using the muslin cloth. The pretreated wastepaper and cardboard fibre was then added into the reactor (containing 0.1M acetate buffer, pH 5). The reactor was at 50 °C; rpm could go up to 600 to maintain thorough mixing.



Figure 27: Recovered glucose product.

Following the enzymatic hydrolysis, the mixture was filtered and the liquid fraction was concentrated by rotary evaporation to remove excess water. Because rotary evaporation selectively removes volatile components, the process not only concentrates the glucose, but buffer salts as well. Therefore, post evaporation residues are a mixture, and the salts may influence gravimetric estimates of yields as well as the validity of subsequent glucose assays since they may interfere with pH and ionic strength (Jones & Patel, 2020).

In animal nutrition, these salts are metabolically active in ruminants, acetate is a beneficial volatile fatty acid of the rumen and may be utilized as an energy source (Bergman, 1990). While moderate addition will not be detrimental, high sodium load

may disrupt electrolyte balance, affect water intake and modify the type of fermentation (NRC, 2001).

Glucose in its glutinous state is the final product, which is obtained from the enzymatic hydrolysis of wastepaper and cardboard fibre.

Beneficiation of product to ruminant feed

Ruminants (cattle, sheep and goats) do not absorb much glucose from feed directly because most of the carbohydrates are fermented in the rumen into volatile fatty acids. However:

1. Glucose is needed for milk lactose synthesis, fetal growth and brain function (Zhao, 2014).
2. Rumen-bypassed glucose supplementation can be directly supplemented to provide energy-demanding functions, especially early in lactation (Yu et al., 2024).

Glucose beneficiation strategies

Rumen bypass glucose

- Formulation: encapsulated glucose or glucose polymers using fat-coated or pH-sensitive-coated encapsulation,
- Function: sustain glucose from rumen fermentation so that it reaches the small intestine to be absorbed,
- Benefits: increases levels of blood glucose, peak milk yield and prevents subclinical ketosis (Yapin et al., 2021).

Glucose-enriched feed additives

- Application: glucose syrup added or spray-dried glucose added to silage.
- Uses: dairy cow transitions on diets, recovery diets for sick or stressed animals
- Starter feeds for young ruminants.

The enzyme (Cellic CTec 3 HS) used in the enzymatic hydrolysis was purchased for $20 (R360)/kg. 1 g of the enzyme produced 51 g of glucose yield from 100 g wastepaper and cardboard fibre. Glacial acetic acid (1 L) was purchased for R1 316.40, from that, 6.9118 ml was used. Sodium acetate (25 kg) was purchased for R2 346.45, from that, 151.392 g was used. The wastepaper and cardboard fibre can cost at R2.50 per kilogram, depending on the quality of it.

To produce 1 kg of glucose, the cost breakdown of materials used are seen in **Table 20**.

Table 19: Feed costs in store.

Feed	**Value (R/kg)**
Molasses (in store)	7.8
Hay	3.0
Glucose (in store)	56.0

Table 20: Glucose from reaction

Material	**Value (R/kg)**
Enzyme	7.06
Sodium acetate	14.20
Glacial acetic acid	9.02
waste	4.90
Glucose plus buffer solution	35.19

From the table above, it can be observed that the glucose yield produced from the wastepaper and cardboard fibre could have a competitive impact in feeds that are already in the market. The glucose and molasses price are from the AGRI store. This observation answers the aim of this study, the glucose was extracted from the wastepaper and cardboard fibre and it can be utilized in supplementing the ruminant feed. This outcome further show that wastepaper and cardboard fibre can play an essential role in the agricultural and animal feed sector.

3.5 Contaminants from wastepaper and cardboard fibre

Wastepaper and cardboard fibre used as feed input within drought-resistant livestock production system, can contain a wide diversity of chemical pollutants from its previous consumer and industrial applications. These chemical pollutants include alkaloids, aromatic hydrocarbons, nitrogenous metabolites, heavy metals and microbial residues (ARC, 2023) (SANAS, 2023).

Although the fibre has structural and nutritional benefits, the contaminants pose risks to animal health, food safety and regulatory compliance. In South Africa, feed ingredients are controlled by Farm Feeds, Fertilizers, Stock and Agricultural Remedies Act (Act 36 of 1974), managed by the Department of Agriculture, Land Reform and Rural Development (DALRRD), which demands rigorous screening for chemical biological impurities (DALRRD, 2021).

3.5.1 Element analysis ICP-OES

Primary contributors: Printing inks

There exists a close link between metal contamination and printing inks, some which contain inorganic pigments whose chromophores are heavy metals. Copper, for instance, is used in phthalocyanine blues and greens, while chromium, cadmium and lead appear in numerous yellow and red pigments (Chen et al., 2020).

Although changes in regulations in some sectors have reduced the occurrence of heavy metals in printing inks, same-type formulations continue to be utilized, especially on packages and imported products. Black inks will also contain trace amounts of nickel, manganese and mercury (Kumar & Sharma, 2021).

Surface Coatings and Additives

Paper and cardboard products tend to have additives for enhanced printability, strength, water resistance, or appearance. With the exception of some wax and oils, most of these chemicals will be sources of trace metal residues. Examples are:

1. Flame retardants add antimony or organometallic brominates;
2. Optical brighteners and bleach agents may include zinc or titanium dioxide;
3. Barrier coatings, particularly on food packaging, can introduce aluminum, iron, or composite polymers metallic oxide embedded (Moyo et al., 2023).

These materials, while effective in first application, contribute to the unwanted metals and compounds when the paper and cardboard are recycled.

Adhesives and Binding Agents

The adhesives used in book production, cartons, labels, and tapes also include metal compounds. Titanium and zinc compounds are common cross-linkers in adhesives, while tin-based catalysts can be employed in hot-melt glues (Ahmed et al., 2020). After pulping, such material is extracted only partially by means of routine flotation or

screening operations, and persists adsorbed on cellulose fibres or in the process sludge.

South African recycled fibre stream is generally heterogeneous, encompassing commercial, domestic and industrial wastepaper and cardboard. These include office paper, magazines, newspaper, corrugated containers, and mixed-paper bales imported from overseas. Each source specifically contributes to the overall contaminant load. Imported wastepaper and cardboard waste, however, especially from Asia, Europe and North America, brings in variability due to varying regulatory regimes governing printing ink content and material additives (Smith & Jones, 2019).

The results for the trace and ultra-trace elemental content as determined in the paper and cardboard fibre pulp samples (**Table 21**) performed by NMISA are presented in **Table 22**. The concentration values are presented in mass fraction units, milligram per kilogram (mg/kg).

Table 21: Samples tested.

Sample	**Description**
1	Pulp sample: 50 g wet pulp, wash with 200 ml dH_2O. Filter and dry pulp.
2	Pulp sample: 50 g wet pulp, wash with boiling 200 ml dH_2O. Filter and dry pulp.
3	Pulp sample: 50 g wet pulp, wash with 200 ml 0.1M EDTA and an additional 200 ml dH_2O. Filter and dry pulp.
4	10 g pulp after 5% NaOH autoclaving
5	10 g pulp after 72 hrs enzymatic hydrolysis reaction
6	Residual sugars post cellulase digestion/DSP

Table 22: Semi-qualitative results for trace and ultra-trace elements in paper and cardboard fibre pulp samples.

element	Average concentration (mg/kg)						Permissible intake (mg/kg)
	1	2	3	4	5	6	
Ag	0.075	0.063	0.115	0.024	0.010	0.130	0.1
Al	365	463	380	140	68.9	48.1	200
As	0.221	0.228	0.232	0.108	0.333	0.550	2
B	6.52	5.54	4.24	1.86	0.67	39.5	30
Ba	14.2	22.1	14.7	6.09	2.06	9.01	10
Be	0.019	0.025	0.021	0.008	0.005	0.005	0.1
Bi	0.057	0.069	0.183	0.024	0.014	0.006	0.1
Ca	2781	2969	814	969	327	8224	600 – 12000
Cd	0.201	0.036	0.028	0.112	0.071	0.017	0.5
Co	0.51	0.576	0.539	0.224	0.165	0.289	10
Cr	5.08	9.9	5.52	3.64	2.92	0.79	10
Cu	7.210	7.350	5.950	3.800	2.360	3.040	50
Eu	0.024	0.027	0.019	0.007	0.003	0.005	Not regulated
Fe	737	803	609	458	199	83	1000
Hg	0.007	0.009	0.023	0.024	0.002	0.002	0.1
Ho	0.009	0.009	0.007	0.003	0.001	0.001	Not regulated
K	556	576	423	179	71.7	3163	8000 (nutritional)
La	0.767	0.759	0.612	0.216	0.122	0.072	0.1
Li	0.94	0.92	0.86	0..33	0.14	0.86	0.5
Mg	350	939	766	712	317	306	3000 (nutritional)
Mn	34.6	36.4	14.9	13.1	5.37	51	100
Mo	0.197	0.210	0.187	0.339	0.177	0.430	5
Na	58.2	56.3	1073	23.5	222	Not reported	8000 (nutritional)
Ni	2.72	2.19	3.32	0.92	2.53	28.7	50

P	2.61	1.99	1.85	1.52	0.91	2.61	8000 (nutritional)
Pb	4.25	3.54	1.84	0.94	1.43	0.29	10
S	1.4	1.21	1.27	1.06	0.64	2.21	4000 (nutritional)
Sb	0.433	0.423	1.084	0.11	0.061	0.16	1
Se	14.2	14.7	14.2	11.4	8.8	27.5	Not regulated
Sr	10.2	10.7	6.28	3.62	1.23	25.8	50
Th	0.169	0.173	0.162	0.047	0.025	Not reported	Not specified
Tl	0.004	0.004	0.003	0.001	0	0.001	0.1
U	0.054	0.056	0.052	0.017	0.009	0.014	Not specified
V	1.257	1.349	1.112	0.421	0.251	0.383	10
(Yb)	0.005	0.006	0.005	0.001	0.001	0.001	Not regulated
Zn	11	9.61	5.11	5.48	2.36	2.57	120

The analyses were carried out at an ambient temperature of 20 °C ± 3 °C.

The permissible limits intake is stated by the Department of Agriculture, Land Reform and Rural Development.

Lanthanum, Cobalt, Europium, Ytterbium and Thorium are present in the wastepaper and cardboard fibre because of specialized industrial use or contamination.

The rare earth metals and trace metals are not chemically bound to the polysaccharides, that would therefore make it rare for them to be present in the sugar molecules. The metals will either be entrapped in the lignin matrix, solubilized during pretreated or remain in the solid fractions (da Silva et al., 2020).

3.5.2 UPLC- QToF-Mass Spec analysis

One of the principal methods in contemporary metabolomics and biochemical profiling, Ultraperformance Liquid Chromatography coupled with Quadrupole Time-of-Flight Mass Spectrometry (UPLC-QToF-MS) is a sophisticated analytical platform that offers high chromatographic and mass spectral resolution, sensitivity, and molecular characterization capability. Because the UPLC technology utilizes small particle diameters (less than 2 μm), the method can quickly separate complicated

mixtures, making the chromatographic process more efficient. When compared to traditional HPLC, studies have shown that UPLC significantly improves the method performance characteristics like chromatographic resolution, peak shape, limit of detection (LOD) and limit of quantitation (LOQ), leading to improved analytical efficiency and quicker analysis times. For example, erythropoietin analysis time was cut from 20 minutes to as low as 4 minutes by using UPLC (Gumułka et al., 2022).

The technique's strengths are improved when the UPLC is combined with QToF-MS detector because it gives detailed information regarding the ion mass-to-charge ratio, which is important in the identification of unknown substances within biological matrices. UPLC-QToF-MS is a crucial structural elucidation technique in the investigation of complex biological fluids and traditional medications because it can deliver high-resolution data which can translate isotopic abundances and elemental compositions of fragment ions (Zhao et al., 2019). Progress in UPLC in recent years has shown that it is significant in metabolomics, enabling untargeted analyses which allow the simultaneous generation of many metabolic profiles. The application of UPLC-MS in serum metabolomics, for example, proved the sensitivity of the method as well as the reliability of candidate metabolite quantification over prolonged time frames (Chan et al., 2019).

The bioactive compounds that were detected in the wastepaper and cardboard fibre before the pretreatment and enzymatic hydrolysis are listed in **Table 23**.

Table 23: Bioactive compounds in wastepaper and cardboard fibre (GreenSky Bio, 2024) (International Labour Organization, 2011) (Mofokeng et al., 2025) (Pereira et al., 2023) (Shimadzu, 2016) (Studocu, 2024).

Tentative compound identification	Predicted molecular formula	Detected m/z	Sources in wastepaper and cardboard fibre
Heliamine	$C_{11}H_{16}NO_2$	194.1179	Residual plant material, microbial degradation of biomass or ink additives.

L-Tyrosine	$C_9H_{11}NO_3$	182.0814	Microbial degradation of biomass, food packaging or amino acid coatings.
Guanosine	$C_{10}H_{14}N_5O_5$	284.0991	Enzymatic additives used in paper processing, RNA/DNA fragments from biological residues or microbial biomass.
Dibutyl phthalate	$C_{16}H_{23}O_4$	279.1596	Mineral oil hydrocarbons of coatings, adhesives of packages or printing inks.
Syringaldehyde	$C_9H_{11}O_4$	183.0286	Synthesized from vanillin or gallic acid
Vanillic acid	$C_8H_8O_4$	167.0332	Biotransformation of ferulic; vanillin oxidation

Compound identification was done by comparing the individual spectra to the NIST 2014 mass spectral library. The bioactive compounds spectra can be seen in **APPENDIX A**. The suggested compound identities must be considered as tentative and should be verified in future studies with reference standards or targeted analysis procedure. The presence of bioactive compounds in **Table 23** presents a formidable challenge to commerciality and regulatory compliance in Southern Africa. These challenges are especially critical under South African National Accreditation System (SANAS) accredited testing, regional livestock health protocols and feed safety regulations (SANAS, 2023) (Department of Agriculture, Land Reform and Rural Development, 2021).

Safety and regulatory concerns

In the regional countries and South Africa, feed inputs are regulated by the Fertilizers, Farm Feeds, Agricultural Remedies and Stock Remedies Act (Act 36 of 1947) which

mandates the safety screening of chemical contaminants (Department of Agriculture, Land Reform and Rural Development, 2021). Alkaloids and aromatic hydrocarbons may:

- Express neurotoxic or hepatotoxic effects in animals (AFMA, 2023)
- Bio-accumulate in body tissues, affecting milk and meat safety
- Implement non-compliance with DALRRD or SANAS-accredited laboratory limits (SGS, 2023).

Impact on marketability

The Southern Africa commercial feed buyers, especially feedlots, cooperatives and farmers with an export focus require:

- Certificate of analysis (CoA) from SANAS-accredited laboratories (SANAS, 2023)
- Evidence of compliance with SANS 10386 (AFMA, 2023).

The identification of toxic substances can lead to downgrading or rejection of the feed batch, undercut buyer confidence in recycled fibre inputs or entail costly remediation (e.g. enzymatic detoxification) (AFMA, 2023).

Perceived nutritional and quality value

Even if it is not acute poisons, synthetic or non-nutritive residues can;

- Reflect poor fibre sourcing or inefficient processing
- Impact digestibility assays
- Raise concerns over the chronic exposure risks in ruminants and monogastrics (Farmers Mag, 2024)

The free trails carried out in Limpopo and KwaZulu Natal have led to decreased palatability and intake where recycled fibre contains residual printing esters (Farmers Mag, 2024).

Mitigation measures

Conduct pre-screening using SANAS-accredited methods (e.g. GC-MS, LC-MS/MS) (ARC, 2023), then apply enzymatic hydrolyses to decompose esters and adsorption of aromatic compounds by activated carbon (AFMA, 2023).

CHAPTER FOUR: CONCLUSION

The objectives of this study were to determine wastepaper and cardboard fibre can provide nutritional benefit to ruminant feed when subjected to enzymatic hydrolysis rather than conventional chemical hydrolysis. The findings confirm that enzymatically hydrolysed wastepaper and cardboard fibre pulp does have nutritional benefit: it produces glucose that can be used directly as an additive in ruminant diets. Glucose yields consistently exceeded 500 – 600 mg/g, with efficiency strongly dependent on buffer type, enzyme formulation and enzyme dosage.

Optimisation of reaction conditions plays a vital role in producing maximum glucose yields. The conditions include, the type of stirring and speed used, the enzyme as well as paper and pulp concentration, the pretreatment of the pulp and paper, the temperature of incubation as well as the time the mixture is placed in the incubator. The milled dried pulp, pretreated with sodium hydroxide and steam or with Laccase exposes the cellulose for increased bioconversion.

From the enzymes that were assessed, the Multizyme® cellulase CEP and Cellic Ctec 3 HS proved to be the most efficient under the optimized conditions of 50 °C, and 200 – 600 rpm stirring depending on the amount of pulp fibre being hydrolysed. The consistency of the pulp and enzyme solution used was 1:10 (pulp: enzyme solution). The aim was to use as little enzyme as possible in order to reduce the expenses. Both the enzymes produced over 500 mg/g of glucose after 24 to 48 hours of hydrolysis. During initial scale up using an overhead stirrer, the enzyme concentration could be reduced 10 fold resulting in a ratio of 1: 100 enzyme: wastepaper and cardboard fibre.

The wastepaper and cardboard fibre pulp was also checked for minerals as well as compounds to evaluate the toxicity of the pulp. The sodium hydroxide pretreatment also dissolved the lignin, which could be removed from the reaction mixture and may have additional applications. Lignin has been shown to be detrimental to ruminants and needs to be removed before final formulations for feed. The soluble fraction will be taken and dried to remove water. The remaining fraction will then be used as a feed additive.

The study has shown that paper and pulp waste can be beneficial, the significant amounts of paper and pulp waste reduced thus benefitting the environment. The wastepaper and cardboard fibre has shown to be a very good source of cellulose and hemicellulose which can be bioconverted to reducing sugars.

In conclusion the objectives of this study were achieved. It demonstrates enzymatically hydrolysed wastepaper and cardboard fibre is a viable low-cost and sustainable nutritional feed supplement for ruminant nutrition. The pretreatment of the wastepaper and cardboard fibre pulp allowed the cellulose to be exposed to the cellulase and become hydrolyse to glucose.

Although this study has shown the viability of using recycled paper and cardboard fibre as a raw material for the production of fermentable sugars for use in ruminant nutrition, there are some limitations that should be considered. Using an alkaline treatment process such as sodium hydroxide autoclaving might not be environmentally friendly.

Also, the variation in concentration levels for trace metals and bioactive compounds among the different types of wastepaper used creates doubts about their long-term hazard variability. Future studies should therefore evaluate these raw materials for their whole toxicological spectrum, alongside the development of raw material treatment processes that avoid using large amounts of chemicals while maximising glucose yield.

Further studies should be conducted regarding the integration of enzymatic hydrolysis under continuous processing systems and should concentrate on prepreatment technologies like steam explosion, biological conditioning treatments, or laccase pretreatments. Dose optimisation efficiencies should be perfected among different enzyme formulations and buffer solutions.

Finally, feeding trails in ruminants on a pilot scale must be conducted to verify the nutritional value associated with the *in-vitro* responses and ensure that the liberation of glucose translates to better performances under real farm conditions. These deficiencies must be corrected to enhance the role of recycled fibre as a green feeding additive and work towards its acceptance as a legal additive in the international community and the Southern African region.

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APPENDIX A



Figure 28: Analysis of the Compound's Elemental Composition at 182.0814 m/z and Retention Time 2.010 min.



Figure 29: Analysis of the Compound's Elemental Composition at 194.1179 m/z and Retention Time 7.736 min.

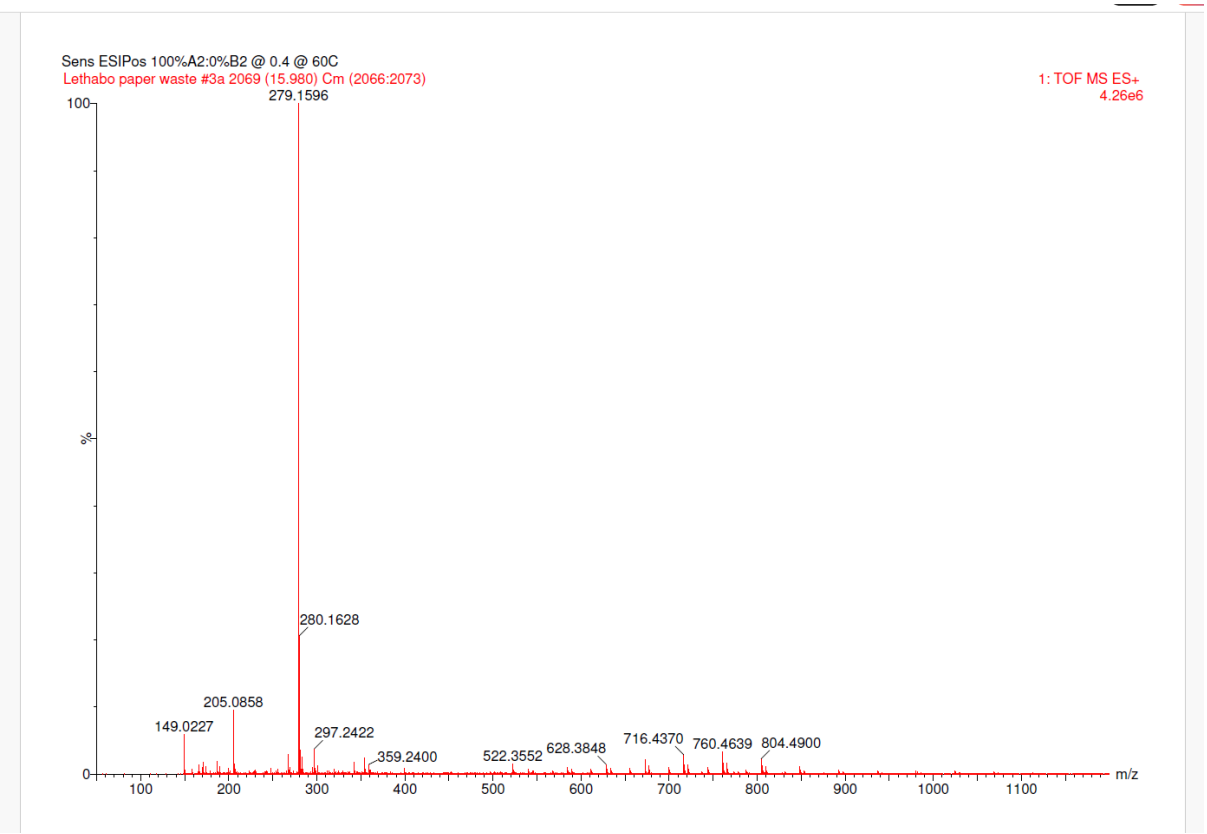


Figure 30: Analysis of the Compound's Elemental Composition at 279.1595 m/z and Retention Time 15.980 min.



Figure 31: Analysis of the Compound's Elemental Composition at 191.0175 m/z and Retention Time 1.419 min.

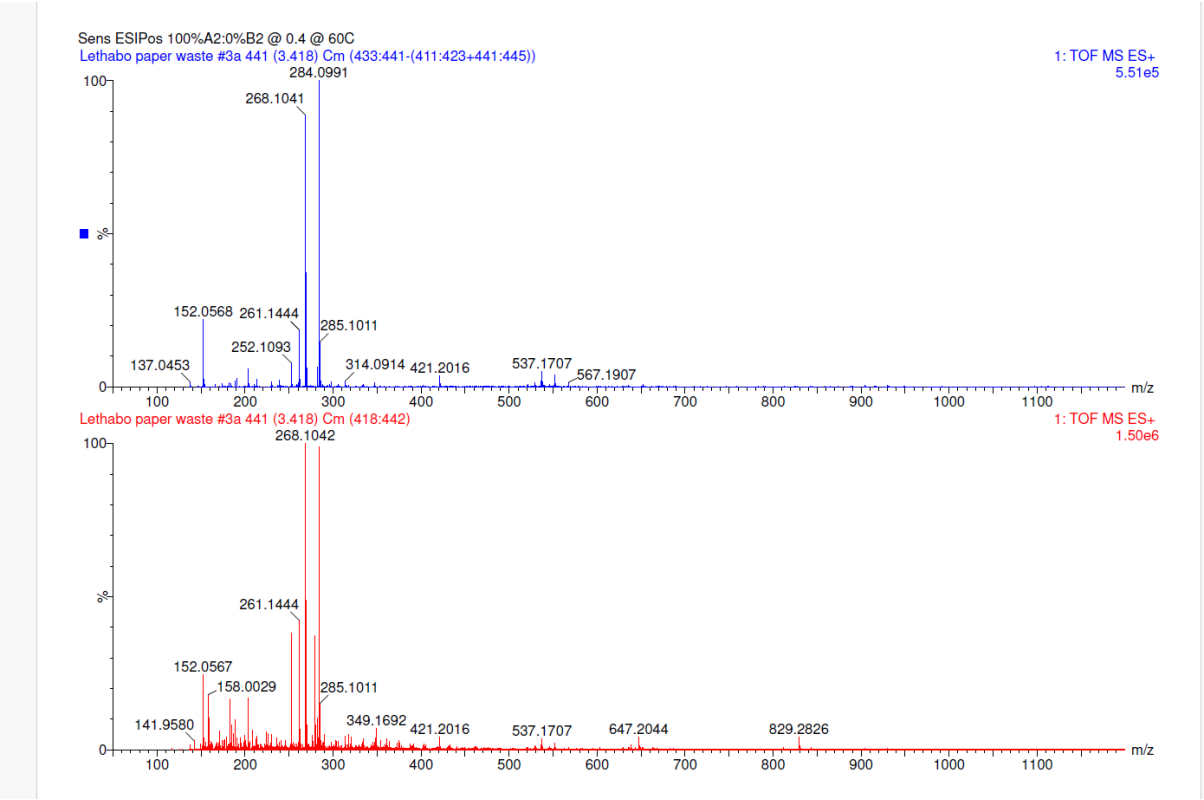


Figure 32: Analysis of the Compound's Elemental composition at 284.0991 m/z and Retention Time 1.419 min.

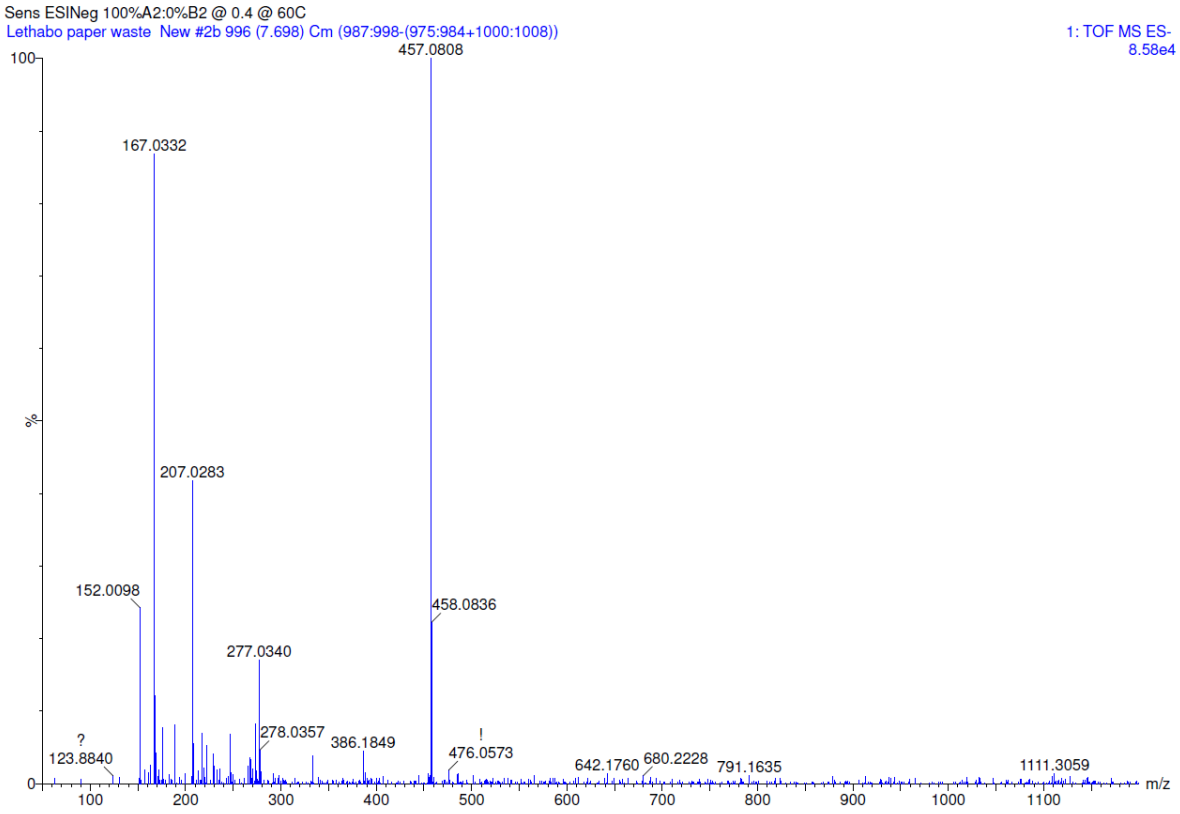


Figure 33: Analysis of the Compound's Elemental composition at 167.0332 m/z and Retention Time 7.698 min.

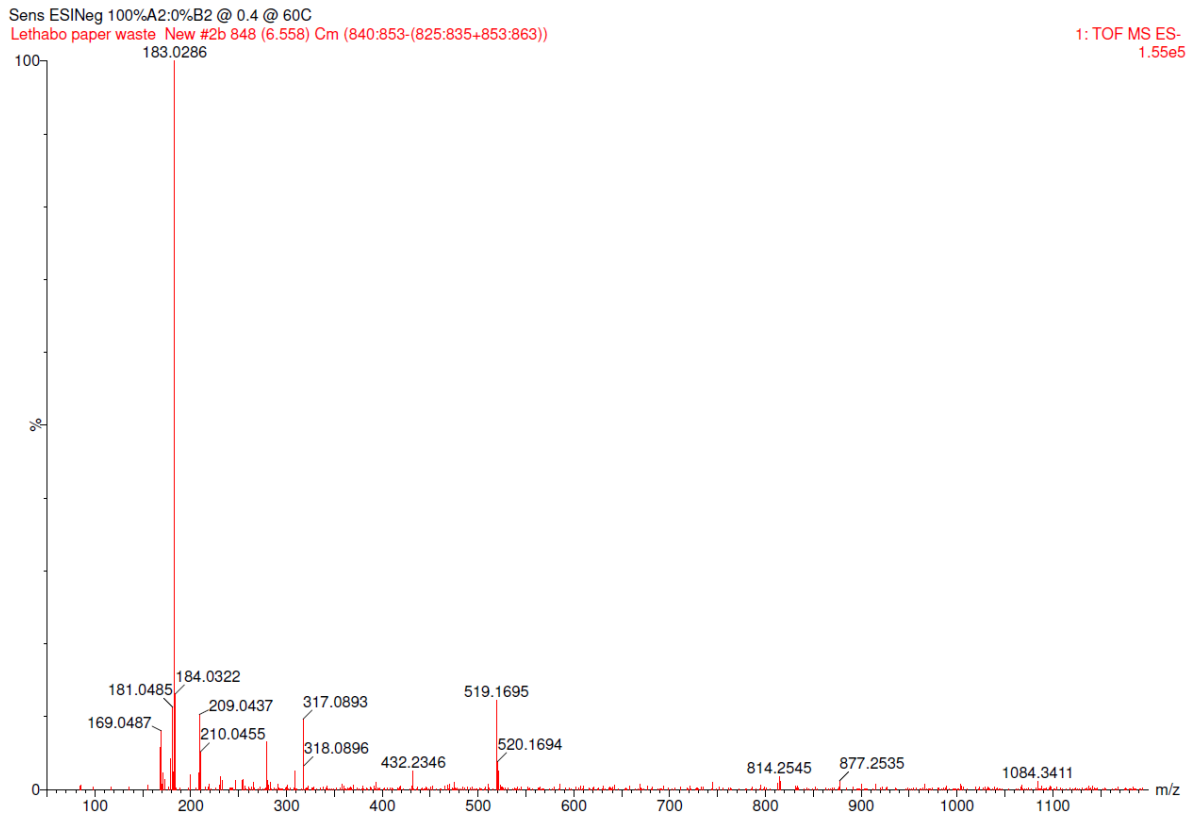


Figure 34: Analysis of the Compound's Elemental composition at 183.0286 m/z and Retention Time 6.558 min.

The washing with deionised water and pretreatment methods removes the compounds that are present in the raw material. The remaining bits remains behind after enzymatic hydrolysis when the soluble fraction is filtered from the insoluble fraction. Therefore, the compounds will not be present in the reducing sugars, since they were not hydrolysed.