ENABLING THE PRODUCTION OF BLACK SOLDIER FLY LARVAL PROTEIN FROM LIGNOCELLULOSIC BIOMASS THROUGH PRE-TREATMENT AND ENZYMATIC HYDROLYSIS.

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Abstract

Due to the rapid increase in the world's population, alternative food resources are crucial to sustain the human diet. Insects can produce valuable proteins and nutrients that can be used as high-quality animal feedstuff and help to reduce organic waste. *Hermetia illucens,* also known as the Black soldier fly, has attracted the attention of researchers due to the veracious nature of the fly larvae (BSFL). These insects can be reared on any organic waste, consuming large quantities of raw organic material more efficiently and faster compared to other known fly species. While consuming organic waste, BSFL build up their body composition to approximately 30% fat and 40% protein, which can be a substitute for nutritionally comparable fishmeal in pet foods and animal feed. Although BSFL are mainly used for animal feed, they represent a promising source of nutrients for human consumption, although limited knowledge regarding the use of BSFL for human consumption is available. Lignocellulose could serve as an alternative, "clean" feedstock consisting of carbohydrates, that can be fed to Black soldier fly larvae. However, due the recalcitrant nature of this biomass, pre-treatment is needed to expose the constituent fibres, making them accessible for rapid degradation with the use of enzymes and microorganisms. This project demonstrated that BSFL, assisted by enzymes and probiotic and/or cellulolytic bacteria, were capable of consuming pre-treated lignocellulosic biomass to produce proteins fit for human consumption.

The first part of the project investigated the use of enzymes to hydrolyse steam pre-treated sugarcane bagasse (SB) as lignocellulosic biomass source, before BSF larval rearing. The use of enzymes ranging from 2.5 FPU/gDW to 7.5FPU/gDW showed an increase in the larval biomass as the enzyme dosage increased. BSFL that were fed untreated SB had a mean dry weight of 0.033 g compared to BSFL that were fed on SB treated with enzymes that a mean dry weight ranging from 0.037 g to 0.043 g. We also investigated the use of a mixed culture of microorganisms to aid with the digestion of sugarcane bagasse, without addition of exogenous enzymes. No difference in the larval biomass was observed when steam pre-treated SB was compared to microbially treated SB. BSFL that were fed on steam pre-treated SB had a mean dry weight of 0.035 g compared to BSFL fed on microbially treated SB which had a mean dry weight ranging from 0.033 g - 0.036 g. Finally, we investigated rearing BSFL on SB exposed to a combination treatment of enzymes and microorganisms. This combination

produced the best overall results as BSFL reached a mean dry weight of 0.055 g (DW), the highest dry weight recorded for the three different experimental trials. A significant difference was observed between the BSFL reared on the steam pre-treated sugarcane bagasse and the BSFL reared on the co-treated sugarcane bagasse. These samples also had the highest protein and fat content, which suggested a correlation between the BSFL biomass and the protein and fat content. These results are a promising indication that BSFL could be reared on a clean feed which could be fit for human consumption. However, there is a need for further optimisation of the processes to ensure that the maximum amount of protein production is achieved for human consumption.

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List of Abbreviation

ADF	Acid detergent fibre
ADL	Acid detergent lignin
BSF	Black soldier fly
BSFL	Black soldier fly larvae
CMC	Carboxy-methylcellulose
DBR	Dry bioconversion rate
DM	Dry matter
DMR	Dry matter reduction
GIT	Gastrointestinal tract
GHG	Greenhouse gas
HMF	Hydroxymethylfurfural
LCB	Lignocellulosic biomass
NDF	Neutral detergent fibre
PVC	Polyvinyl chloride
SHF	Separate hydrolysis and fermentation
SSF	Simultaneous saccharification and fermentation
SoSF	Solid state fermentation
SB	Sugarcane bagasse
A_w	Water activity
WIS	Water insoluble solids

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CHAPTER 1: INTRODUCTION

1.1. Background

The world population has increased rapidly and is expected to reach 9 billion by 2050 (Jansen, 2018), with the African population is predicted to increase at least a 3.5-fold (Onsongo *et al*., 2018). Accordingly, more resources will be consumed at a faster rate to sustain this growing population. However, according to Liu *et al*., (2017) agricultural resources available such as soybeans, maize and rice are expected to decline by approximately 55%, 67% and 42% respectively, due to this rapid increase in the human population. During recent years, insects have gained popularity for their potential as a food source (Manyara, 2018). Literature revealed that over 2000 edible insect species has been documented globally and consumed worldwide for many years (Yu *et al*., 2009). According to Van Huis (2013), insects have numerous advantages as a protein source compared to plants and animals, such as (i) lowering greenhouse gas emissions during large scale production, (ii) low water demand, (iii) conversion of organic waste into edible biomass, while (iv) producing a high protein content and crude fat, making these insects an excellent replacement feed source for human consumption. Insect species that have been evaluated as feed resources included grasshopper, silkworm, locust, cricket, and *Hermetia illucens*, also known as the Black soldier fly (BSF) (Jayanegara *et al*., 2015).

Black soldier fly larvae (BSFL) are a potentially low-cost, nutrient-rich alternative protein source. It is similar or even superior in protein content compared to fishmeal (Sumbule *et al*., 2021) and one of the most efficient insects for bioconversion of organic waste, producing a feedstock which is high in nutrition and suitable for livestock. To date, substantial research was invested in optimising BSFL biomass yield and quality in response to different substrates and rearing strategies as this affects the final value of the biomass and nutrient content of the larvae (Sumbule *et al*., 2021). The biomass of healthy larvae contains about 40%-45% protein and 24%-30% fat. BSFL has been extensively researched for animal feed, however, it may also provide a great source of nutrients for human food, although the research into BSFL for human consumption is still in its infancy and extensive further research is needed (Bessa *et al*., 2020). Most current BSFL rearing methodologies use food waste that may lead to by-products that are not fit for human consumption.

One option to create a clean alternative bioconversion process is the use of lignocellulosic biomass (LCB) as the feedstock for rearing BSFL. Lignocellulosic biomass is an affordable and abundant material, which can be converted to sugar monomers by several microorganisms containing cellulolytic and hemicellulolytic enzymes under aerobic and anaerobic conditions (Amin *et al*., 2017). However, this treatment is very energy dependent. Bacterial and fungal organisms are the most abundant biological organisms present in nature, with the ability to break down both manmade and natural polymers (Chukwuma *et al*., 2021). Enzyme treatment, in particular high-solids enzymatic hydrolysis, offers higher sugar yields at lower rates of water consumption and lower operational cost compared to other methods of saccharification (Pino *et al*., 2019). Sugarcane bagasse (SB) is a well-known agricultural LCB residue produced by sugar mills. Sugarcane is a widely planted sugar crop and approximately 260–280 kg of wet SB is produced per ton of sugarcane, with total production of more than 279 million metric tons (MMT) annually worldwide (Xu *et al*., 2019). South Africa is known for its high production of sugarcane, making SB readily available without competition for arable land (Armah *et al*., 2020).

This research study focused on using lignocellulosic biomass, specifically sugarcane bagasse, for the cultivation of BSFL. The purpose of this project was to determine suitable substrate pre-treatment conditions to allow Black soldier fly larvae to thrive on LCB using steam pre-treated substrate in combination with bacterial treatment and the addition of hydrolytic enzymes. Pre-treated substrates were then fed to BSFL to determine whether they could consume the available carbohydrates, thus improving larval biomass. Three different methods of treating the steam pre-treated sugarcane bagasse before feeding it to BSFL were conducted to achieve this namely: (i) steam pre-treated SB treated with enzymes, (ii) steam pre-treated SB treated with microorganisms and (iii) steam pre-treated SB treated with enzymes and microorganisms.

1.2. Research approach

1.2.1. Research questions

a) To what extent will BSF larvae be capable of utilising lignocellulose derived sugars and/or solids present in the feed, or do they subsist on the microorganisms present in the feed?

b) To what extent will the addition of microorganisms in the feed support BSFL growth as a source of both nutrition and hydrolytic enzymes?

c) What is the minimum amount of exogenously added commercial enzyme required that would allow the larvae to consume the available sugars?

1.2.2. Aim and objectives

The main aims and objectives of this study are summarised as follows:

1 To test the production of Black soldier fly larvae protein from partially hydrolysed lignocellulosic feedstocks.

   a) Evaluate the effects of different enzyme loadings on steam pre-treated LCB to determine the optimal enzyme loading for partial enzymatic hydrolysis.

2 Identify bacterial strains that are able to grow on pre-treated and partially digested LCB.

   a) Screen a variety of probiotic bacteria on pre-treated LCB substrate to determine if they can grow in the presence of inhibitors.

   b) Test whether selected bacterial strains produce cellulolytic enzymes, thus hydrolysing available cellulose and hemicellulose.

3 Rear BSFL on treated substrates and determine their protein and fat content.

   a) Determine the protein and fat content of BSFL.

   b) Evaluate which treatment (enzymes, microbial or SSF) accumulated the highest protein and fat content.

4 Determine if cellulose/hemicellulose was digested by BSFL or the microorganisms present in the feed.

   a) Evaluate fibre content before and after BSFL rearing.

CHAPTER 2: LITERATURE REVIEW

2.1. Black Soldier fly

2.1.1. Description

Hermetia illucens, also known as the Black soldier fly (BSF), is a true fly belonging to the *Stratiomyidae* family, normally found in tropical and temperate regions such as Australia (Park & Kim, 2012). Black soldier fly larvae are very resilient, being able to live in harsh environmental conditions including nutrient shortage and drought (Jansen, 2018). Adult BSFs do not feed on organic material. Instead, they rely on fat reserved from the larval stages for sustenance (Wynants *et al*., 2018). BSFs are not considered pests because they do not possess stingers and they do not approach humans as compared to other flies such as *Muscu domestica* (house fly). Adults range from 15-20 mm in length and tend to live and breed in areas that are suitable for their larvae. Adult BSFs are known to have short life spans of about five to eight days wherein females lay approximately 500 eggs (Bessa *et al*., 2020). The hatched larvae are capable of digesting various types of organic matter. The biomass of a healthy larvae contains approximately 40% protein as well as 30% fat which is a potential feedstock high in nutrition and suitable for livestock. (Newton *et al.,* 2005).

2.1.2. Life cycle

The BSF undergoes complete metamorphosis within 40-44 days (Mutafela, 2015). The larval and pupa stages are the longest stages in the cycle with the egg and adult stage being shorter. Once the eggs are deposited, they take approximately 3-4 days to hatch into larvae (Manyara, 2018). Once hatched, they will find available waste material to consume immediately. In the right environmental conditions, the larvae will then mature into prepupa within two weeks. During this stage, their colour changes from white to dark brown, and they will find a dark medium to burrow in (Barry, 2004). The last stage before emerging as an adult is the pupation stage. As with the larval stage, depending on the condition of the environment, this might take up to two weeks, subsequently turning into adults, completing their life cycle as seen in Figure 2.1.

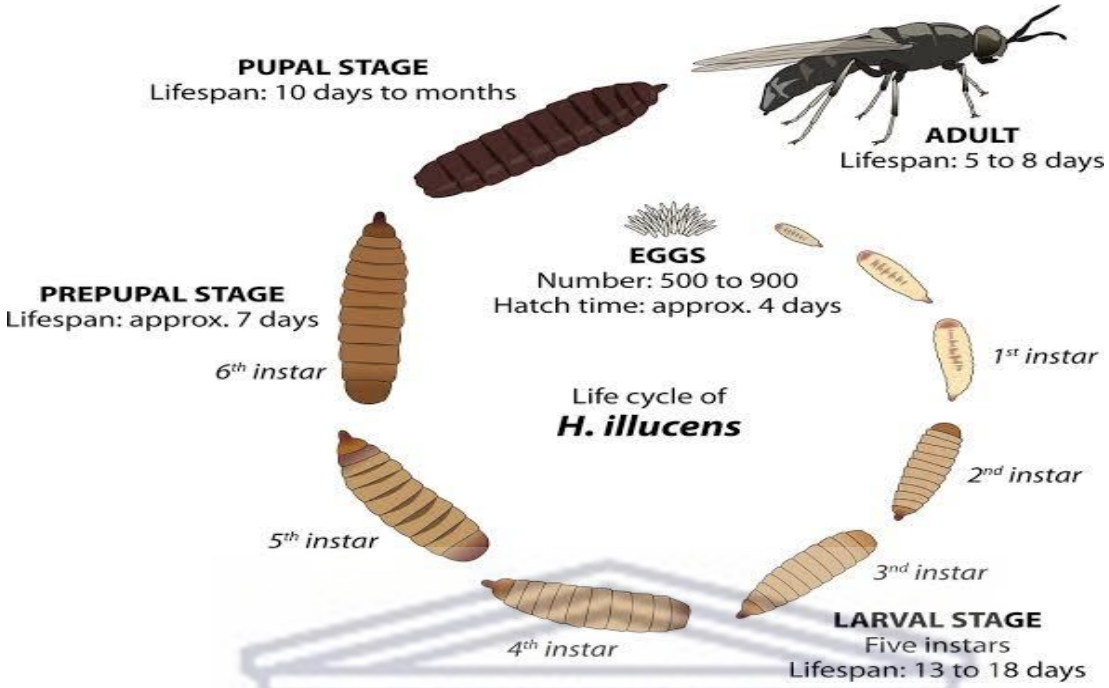

Figure 2.1: The life cycle of a Black soldier fly. The different development stages are shown along with the average duration of each stage (De Smet *et al.,* 2018*).*

2.1.3. Rearing and dietary conditions

BSF environmental requirements will vary depending on the different stages of the life cycle (Newton *et al.*, 2005). Numerous studies have reported on the sensitivity of the Black soldier fly to abiotic factors including temperature, humidity, and light density to ensure successful development and breeding requirements (Wang & Shelomi, 2017). BSFL are voracious and capable of thriving on a variety of organic materials including kitchen waste, animal manure, pig liver as well as agriculture waste, due to special microbiota in their gut. (Wang & Shelomi, 2017). Although these larvae can transform waste material into valuable, nutritive biomass rich in proteins, lipids, ash (11- 28% of DM) and other valuable nutrients such as calcium (5-8% DM) and phosphorus, they were previously mainly used for waste management rather than animal feed products (Newton *et al.*, 2005, van Huis, 2013). According to Yu *et al.*, (2009) BSF larvae biomass composition favourably compares with that of fishmeal and soya, which combined currently supply over 90% of the protein requirements of animal feeds. This shows that BSFL biomass, reared on selected organic wastes, can serve as a suitable animal protein source. One challenge to more widespread application of BSFL for protein production is that abundant agricultural waste such as rice straw is difficult to consume due to its high lignocellulosic content. However, Li *et al*., (2015) concluded that BSFL were able to convert

LCB into a clean feedstock that can subsequently be used for several applications. They also proved that the larvae could consume glucose and xylose, thereby producing protein and lipids. For efficient utilization of the LCB, co-digestion of BSFL with microorganisms can improve the conversion efficiency, development time as well as protein and oil content (Zheng *et al*., 2012). For example, Xiao and co-workers (2018), reported increases in the BSFL weight (15.9%), waste reduction rate (13.4%) and bioconversion rate (12.7%) when *Bacillus subtilis* was inoculated into chicken manure as compared to BSFL without the bacterial supplement. A recent study showed a slight increase in protein and fat percentage (55.3 ± 0.6% and 30.0 ± 0.6% respectively) of BSFL when soybean curd residues were inoculated with *Lactobacillus buchneri* compared to soybean curd residues only (Somroo *et al*., 2019). These studies confirm that a symbiotic relationship between the BSFL and bacteria could contribute to successful growth and reproduction and improved product yields. Recently, studies have also shown that symbiotic relationships of microorganisms in different environmental habitats and life stages of insects play an important role in the insects' growth and digestive enzyme production (Ur Rehman *et al*., 2017b).

2.1.4. Microbiome of BSF

A microbiome refers to all the microorganisms in a certain environment (Wynants *et al*., 2018). Regarding the microbiomes of most insect species, it is not always clear which organisms are native to the host and which are transient organisms picked up from their environment or through their diet (Dillon & Dillon, 2004). Intestinal microbiota is crucial to the host as these microorganisms often have functions that the insect's metabolism might lack. They assist the insects with the digestion of certain feedstocks as well as with the detoxification of certain allelochemicals such as alkaloids and tannins produced by plants to protect them against herbivores. Extensive research has shown that the microbiota of traditional farm animals such as pigs and ruminants contribute significantly to the performance of the animal (Bruno *et al*., 2019). In traditional farm animals, the gastrointestinal tract (GIT) contains a complex microbiome that is composed of a range of organisms such as bacteria, fungi, protozoa and even viruses, all contributing to the survival, growth, and health of the animal (De Smet *et al*., 2018). Although the gut of insects also contains bacteria that play a vital role in the functioning of the insect, so far, little is known about the microbial aspect of the BSF (Bruno *et al*., 2019).

The GIT of the BSFL can be categorised as the foregut, midgut, and hindgut, all of which contain different microbial communities (De Smet *et al*., 2018). It was also suggested that alterations in the BSFL farming environment play a vital role in the combination and the type of bacteria present in the gut microbiome. Furthermore, BSFL can digest various organic substrates during various stages of decay, which raises questions with regards to the larvae's microbiome and the roles of theses microorganisms. Although few studies have been done on the microbial aspect of BSF larvae, the feeding diet of the larvae will likely have a significant impact on the gut microbial community. Microorganisms present in the organic feed may also have potential to optimise the growth performance of larvae (Wynants *et al*., 2018).

A study by Jeon *et al*., (2011) demonstrated that the microbiome diversity found in the gut of the BSFL would differ depending on the diet and influence the gut microflora of the BSFL. BSFL were cultivated on three different diets, namely calf forage, cooked rice, and food waste. Pyrosequencing of the 16S rDNA was used to sequence and identify bacterial strains extracted from the gut of the BSFL. The dominance of the bacterial communities for the three groups showed that bacterial diversity was influenced by the diet (Figure 2.2). Cooked rice contained mainly carbohydrates and the relevant samples fed on this substrate were shown to have less diversity compared to calf forage and food waste. They also found thirty-six shared bacterial strains from the three diets concluding that BSFL gut microbiota was unique. Four aerobic bacterial strains selected based on their ability to degrade organic compounds were extracted from the gut of the BSFL and identified. These organisms were identified as *Bacillus amyloliquefaciens* ssp., *Bacillus stratosphericus* sp., Family *Paenibacillaceae* sp. and *Paenibacillaceae mirabilis* sp. all of which were shown to either have high amylase, cellulase, lipase or protease activity. Zhineng *et al*., (2021) showed that there were over 11,000 intestinal bacteria in the Black soldier fly larvae gut, indicating the presence of a high abundance of microorganisms in the gut. Their results proved that the taxonomic order was similar for different groups, thus suggesting that different feeds played a role in the overall microbial community of the BSF larvae.

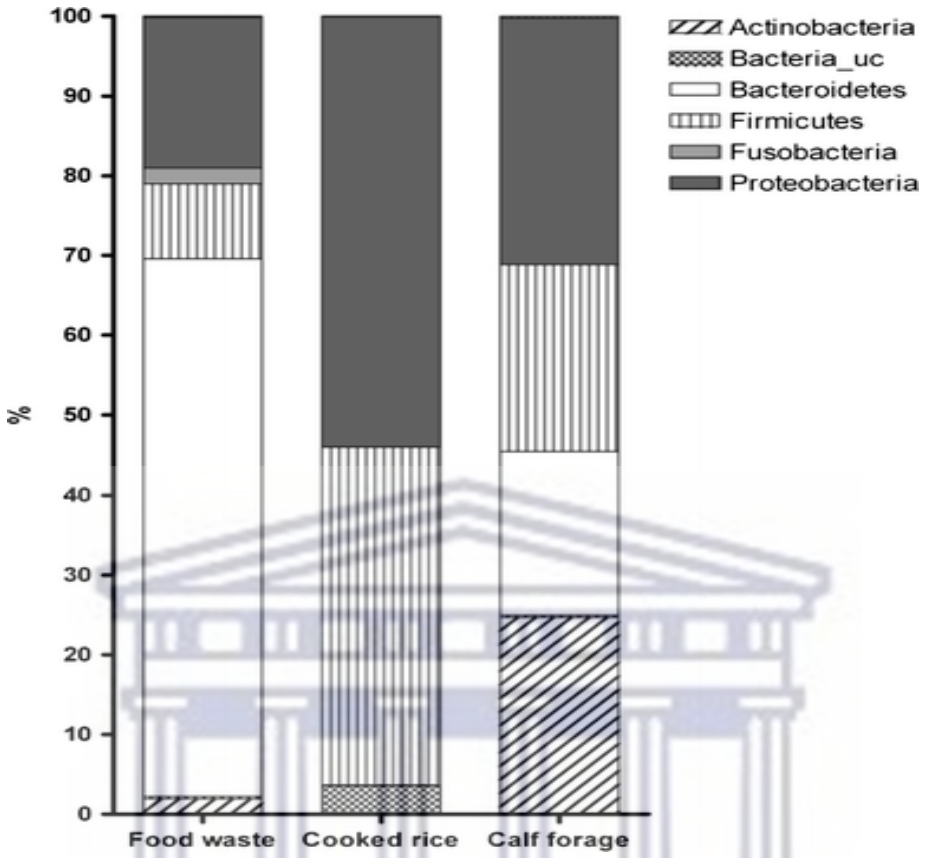

Figure 2.2: Bacterial communities in the gut of BSFL. Metagenomic DNA was extracted from the gut after feeding on 3 different diets: food waste, cooked rice, and calf forage. Major phyla were determined using pyrosequencing of 16s rRNA. (Jeon *et al.*, 2011)

2.1.5. Nutritional aspects

BSFL offer an interesting perspective on nutritional value, since the nutrients found in the BSFL are the same ones needed for human health (Bessa *et al*., 2020). Its diversity of micro- and macronutrients makes it an ideal feed source for a variety of animals including humans. The nutritional compositions of the larvae vary based on several factors such as environment, substrate consumed as well as the age of the larvae when harvested. This offers information which can be used to harvest larvae at different stages if a certain nutritional profile is needed. As the larvae ages, protein content seems to decrease as the fat content increases. Micronutrients such as iron and zinc are found in BSFL, ranging from 2.1 to 3 mg/100 g and 6.8 to 15 mg/100 g for iron and zinc respectively (Bessa *et al*., 2020). This could be essential in malnourished countries since these minerals are important for the human diet. Another important mineral found in BSFL is calcium with values ranging from 840 to 934 mg/ 100 g, similar to that of milk. The protein and fat content in BSFL are quite high, ranging from 30 to

53 g/ 100 g for protein content and 20 to 41 g/ 100 g for lipid content. This makes them comparable to other sources of protein and fat (Table 2.1). Studies found that BSFL have high saturated fatty acid content with the most abundant fatty acid being lauric acid (C12:0). Lauric acid is well known for its antimicrobial activity against gram positive bacteria. Other fatty acids found in BFSL are linoleic acid (C18:2n-6), palmitic acid (C16:0) and oleic acid (C18:1n-9) which are all present in high concentrations (Bessa *et al*., 2020).

Table 2.1: Nutritional content of beef, chicken, mealworm, and Black soldier fly larvae (g/100 g Dry Matter Basis)

Species	Crude protein	Total fat	Chitin	Reference
Beef	50.8 to 65.4	18.6 to 32.7	0.0	U.S. Department of Agriculture, Agricultural Research Service (2019)
Chicken	66.8 to 86.2	10.0 to 26.8	0.0	U.S. Department of Agriculture, Agricultural Research Service (2019)
Mealworm (*Tenebrio molitor*)	50.1 to 76.2	27.2 to 31.1	3.7 to 5.7	(Ghaly & Alkoaik, 2009)
Black soldier fly larvae (*Hermetia illucens*)	30.0 to 52.9	20.0 to 40.7	2.1 to 9.0	(Bessa *et al*., 2020)

2.2. Lignocellulosic biomass

2.2.1. Structure

Lignocellulosic biomass (LCB) is a renewable and natural resource abundantly available on earth (Amin *et al*., 2017). It is nonedible plant material that includes wood residues, dedicated crops, agricultural wastes, and forest residues (Verardi *et al*., 2012). LCB has a complex structure composited of lignin, an aromatic polymer, as well as the carbohydrate polymers cellulose and hemicellulose (Amin *et al*., 2017). LCB contains approximately 15-20% lignin, 50-60% cellulose and 20-30% hemicellulose (Sindhu *et al*., 2016), however the composition of the polymers varies depending on the type of lignocellulose material (Table 2.2). Within the plant cell wall, cellulose is a homopolysaccharide of glucose and the major structural component that is responsible for the mechanical strength of the plant (Anwar *et al*., 2014). Cellulose is composed of β-D-glucopyranose units, linked by β-1,4-glycosidic bonds with the smallest repetitive unit being cellobiose formed by two glucose monomers (Verardi *et al*., 2012).

Table 2.2: Composition of various lignocellulose materials

Lignocellulose material	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Source
Wheat straw	32.6	24.7	16	(Belal, 2013)
Sugarcane bagasse	40-45	30-35	20-30	(Sudiyani *et al*., 2016)
Rice straw	32-47	19-27	5-24	(Belal, 2013)
Corn stover	38	23	20	(Wan & Li, 2010)
Corn cob	30	32	13	(Syawala *et al*., 2013)
Barley straw	38	35	16	(Sun *et al*., 2005)
Sweet sorghum bagasse	34	15	21	(Sudiyani *et al*., 2016)

Long-chain cellulose polymers are packed together into microfibrils by hydrogen and van der Waal's bonds. Hemicelluloses are polysaccharides that are often repeated polymers of pentose and hexose sugars with a variety of side chains (Verardi *et al*., 2012). Lignin consists of three aromatic alcohols (coniferyl alcohol, sinapyl alcohol and *p*-coumaryl alcohol) and is the most complex of the three polymers, that adds strength to cell walls (Ghaffar & Fan, 2013). Hemicellulose together with lignin acts as a matrix for the cellulose microfibrils as shown in Figure 2.3 (Ghaffar & Fan, 2013). Lignin also provides the most resistance to biological attack, compared to cellulose and hemicellulose. Lignin is covalently connected to hemicellulose, surrounding the cellulose, thus preventing cellulolytic enzymes from attacking their substrate. Trace amounts of pectins, proteins and extractives such as nitrogenous material and ash are also found in LCB (Verardi *et al*., 2012).

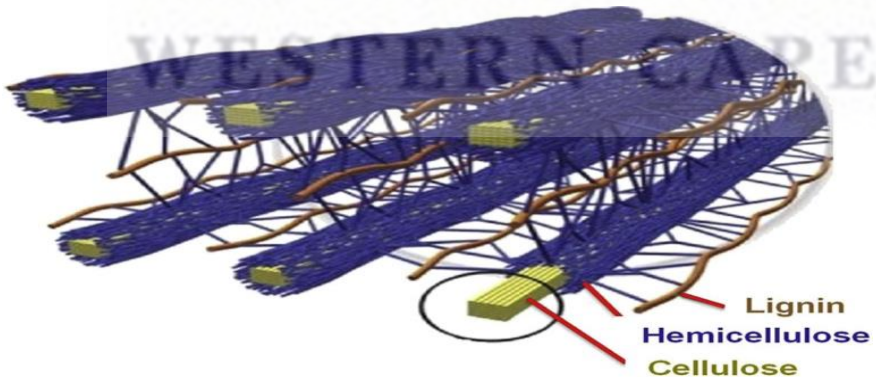

Figure 2.3: Schematic diagram of the lignocellulose structure. (Jensen *et al*., 2017)

2.2.2. Pre-treatment

Pre-treatment of lignocellulosic material prior to saccharification is essential, as various physical and chemical barriers block cellulolytic enzymes from entering the LCB structure, thus preventing efficient enzymatic hydrolysis and minimizing the recovery yield of

fermentable sugars (Yu *et al*., 2018). It is considered the most critical step of biomass to biofuel conversion processes and influences the outcomes of all subsequent steps. Therefore, research has focussed on finding the fastest and most efficient pre-treatment methods that enable rapid substrate hydrolysis with minimal production of toxic by-products (Galbe & Zacchi, 2012). The selected pre-treatment method should produce large quantities of carbohydrates, and high cellulose digestibility should be observed in subsequent enzymatic hydrolysis. In addition, it should also require low capital and operational cost while limiting the formation of inhibitors. Currently, researchers are working on improving several pre-treatment methods at laboratory and pilot scale (Sindhu *et al*., 2016). Based on the mechanism used, these methods are grouped into different categories namely physical, chemical, and biological or various combinations of these. Although numerous pre-treatment strategies have been developed, it seems only a few are promising at industrial scale (Galbe & Zacchi, 2012). Table 2.3 outlines some advantages and disadvantages of some popular pre-treatment methods that are often used.

Table 2.3: Summary of the advantages and disadvantages of different pre-treatment methods

Pre-treatment method	Category	Advantages	Disadvantages	Reference
Steam explosion	Physico-chemical	Low environmental effects, no chemical use.	Production of inhibitors influences subsequent hydrolysis process.	(Baruah *et al*., 2018)
Alkaline hydrolysis	Chemical	Less formation of inhibitors leading to less solubilization. Can be performed at lower temperatures.	Low digestibility in softwoods, requires large amount of water. Very costly.	(Jönsson & Martin, 2016)
Milling	Physical	Cost- effective	High energy consumption	(Aftab *et al*., 2019)
Microbial treatment	Biological	Degrades lignin, requires little energy, chemical-free, mild conditions	Biological treatment is very slow. A fraction of the carbohydrates is consumed by the microorganisms.	(Amin *et al*., 2017)

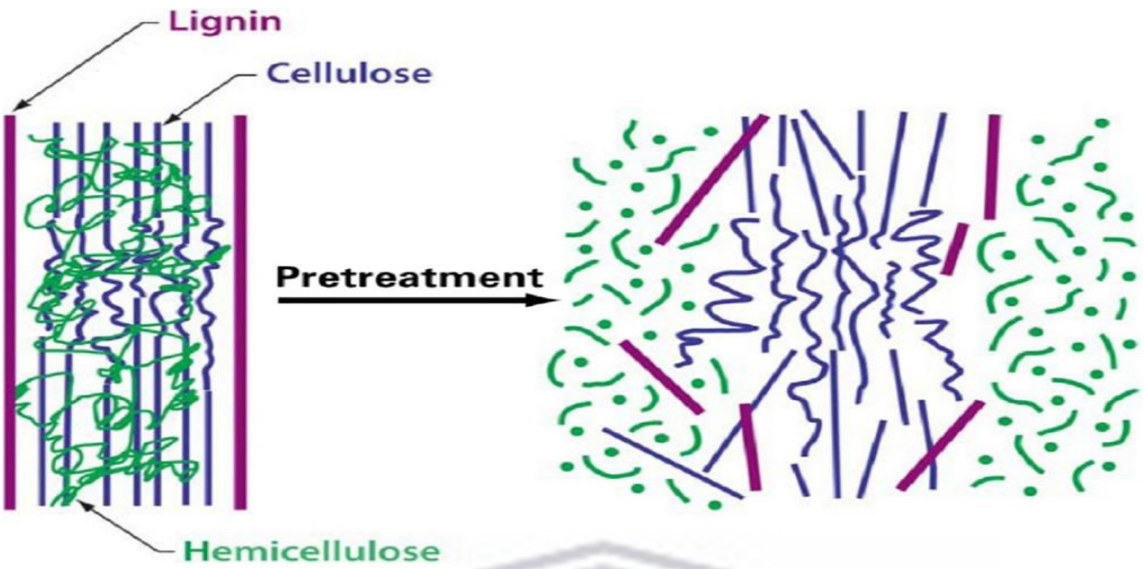

Figure 2.4: The effects of pre-treatment on lignocellulosic biomass (Mosier *et al*., 2005)

Currently, steam explosion is one of the most employed methods of pre-treating LCB (Baruah *et al*., 2018). During this physio-chemical process, the substrate is treated with high pressure and temperatures of about 180-220°C. This pressure is then released rapidly, and the steam causes the lignocellulosic matrix to expand, disrupting the cell wall structure (Figure 2.4) and resulting in a slurry. This disruption releases the fibres, thus increasing the digestibility of the LCB. (Singh *et al*., 2005). Steam explosion also removes a fraction of the lignin and redistributes it to the surface of the fibres (Behera *et al*., 2014). However, steam explosion will result in the release of inhibitory by-products which can have an impact on the downstream processes (Jönsson & Martin, 2016).

2.2.3. Inhibitor formation and detoxification

During the pre-treatment process, different severity conditions can produce inhibitory by-products at different concentrations, that can prevent subsequent processes such as enzymatic hydrolysis and microbial fermentation (Daehwan, 2018). Therefore, it is important to consider the pre-treatment methods as well as the type of LCB as these factors influence the concentration and toxicity of the different inhibitors produced. The most common inhibitors that are formed during this pre-treatment step include hydroxymethylfurfural (HMF), acetic acid, formic acid, furfural and levulinic acid (Jönsson & Martin, 2016). Depending on the origin, functionality and their effects on fermenting microorganisms, these

inhibitors are categorized into different groups. Furfural is easily formed at high temperatures and therefore usually found in high percentages (Weiss *et al*., 2013). It is also important to note that furfural is more toxic compared to HMF which has fewer inhibition effects. However, HMF is known to last longer in the medium thus causing a decrease in cell growth (Mhlongo *et al*., 2015). Lowering the severity of pre-treatment could prevent large amounts of inhibitors being formed. However, this might affect the subsequent enzymatic hydrolysis and fermentation processes. If high amounts of inhibitors are present in the hydrolysate, some form of detoxification method may be required to remove inhibitors selectively, but detoxification must be balanced against cost-effectiveness (Zabed *et al*., 2016).

There are several detoxification methods that can be employed to decrease the concentration of inhibitors, such as over-liming with calcium hydroxide (Lanka *et al*., 2011). Although over-liming reduces the concentration of various inhibitors, it also results in the loss of sugar. Other methods that can be employed include evaporation, which significantly removes volatile compounds such as HMF, furfural and acetic acid, but it may lead to an increase in concentrations of non-volatile compounds (Larsson *et al.*, 1999). Furthermore, enzymatic detoxification with peroxidase and laccase can be used to remove phenolic compounds (Chandel *et al*., 2012).

2.3. High-solids enzymatic hydrolysis

Enzymatic hydrolysis is an important step in lignocellulosic degradation. Not only does it determine the amount of sugars that are released for further downstream processes, but it also offers advantages over chemical hydrolysis such as, less formation for undesirable by-products and the potential for almost complete conversion (Fenila & Shastri, 2016). Compared to acid and alkaline hydrolysis, enzymatic hydrolysis is cost effective because it is performed at moderate conditions (pH 4.8 and temperature of 45-50°C). Enzymatic hydrolysis is a multi-step reaction (Yang *et al*., 2011). Cellulose hydrolysis is carried out using highly specific endoglucanases and cellobiohydrolases, followed by further catalytic cleaving of the cellobiose units through the action of β-glucosidase, with the resulting product being the reducing sugar glucose (Sun & Cheng, 2002) as shown in Figure 2.5. Novozymes Cellic® CTec3 is a highly efficient commercial cellulase and hemicellulase enzyme cocktail with proprietary enzyme activities (Fockink *et al*., 2017). It has been applied successfully in various processes

requiring pre-treatment, hydrolysis, and fermentation of LCB. It has been tested extensively at lab scale but has been applied at pilot and industrial scale as well. Cellic® CTec3 is comparatively cost effective when it comes to converting lignocellulosic materials to fermentable sugars. It offers process and substrate versatility for process optimization and higher conversion yields.

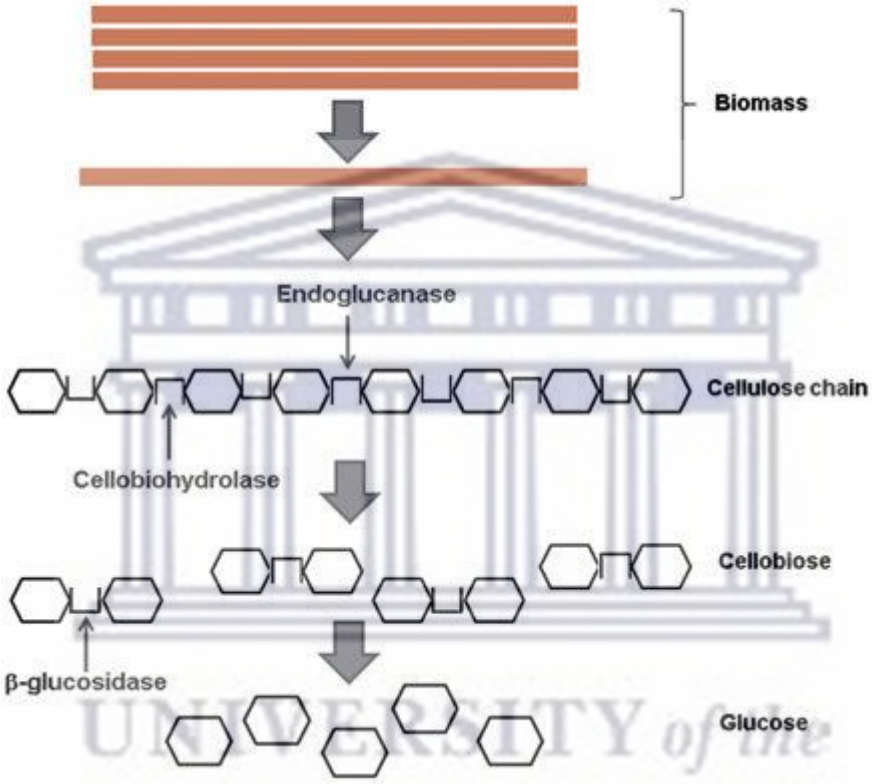

Figure 2.5: Cellulose hydrolysis by the three main types of cellulases: endoglucanase breaks the covalent bonds in the amorphous structures of cellulose. Cellobiohydolase cleaves two glucose units (cellobiose) from the ends of the chains and finally cellobiose is hydrolysed to glucose by β-glucosidase. (Gomez del Pulgar & Saadeddin, 2013)

The use of high-solids enzymatic hydrolysis (≥ 15 % solids w/w) offers certain advantages such as high final sugar concentration, low energy and water consumption and lower equipment cost (Xu *et al*., 2019). The disadvantage with high solids loading is that a high viscous slurry is formed that affects the substrate and enzyme interaction and reduces the mixing efficiency (Pino *et al*., 2019). The high sugar concentration that is released at the initial stage also inhibits the action of the enzymes, which limits its industrial application. Previous work has demonstrated the feasibility of achieving high sugar release with almost 30% (w/v) solids content or even higher substrate concentration in hydrolysis with various kinds of substrates (Liu *et al*., 2020). However, it was observed that hydrolysis yield decreased with

increasing slurry concentration for most LCB. Furthermore, this aspect becomes more pronounced at higher solids levels. The decrease in yield seems to be an intrinsic or generic effect of saccharification with increasing solids loads, which is undesirable for it offsets the advantages of high solids hydrolysis. The reason for the ‘‘solids effect’’ is so far unknown (Liu *et al*., 2020).

2.4. Nutritional enhancement of agro-industrial products through microorganisms

Symbiotic bacteria may constitute a critical factor in the enormous success of insects in adapting to new environments and food sources (Somroo *et al*., 2019). However, most agro-industrial by-products are poor in nutrients such as proteins and vitamins and are rich in fibres with low digestibility. The utilization of microorganisms, such as bacteria and fungi which can be cultivated on a large scale and be used to convert the agro-industrial products has been investigated. Using microorganisms to produce nutritionally enhanced raw materials for feed has certain advantages over the conventional method of making feeds (Villas-Boasa *et al*., 2002). Microorganisms grow quickly and create significant amounts of protein - about 600 g/Kg dry biomass. Various feed formulations have been using microbial by-products from traditional fermentation businesses for a while. The cattle feed sector greatly depends on these items. Compound feeds can also contain a variety of by-products from different fermentation industries as sources of nutrients (Villas-Boasa *et al*., 2002). A promising process would be the inoculation of one or more specific microorganisms in non-sterilized or lightly pasteurized lignocellulosic wastes, to create a microbial consortium that involves both microorganisms from the natural microflora and added microorganisms. This kind of process has not yet been properly evaluated. However, when working with endogenous microorganisms the risks related to possible toxins increase, needing a more careful evaluation of possible toxicological effects (Villas-Boasa *et al*., 2002). The microorganisms that were selected for this project was based on several factors such as their ability to grow in the presence of pre-treatment derived inhibitors, their ability to degrade hemicellulose and cellulose as well as possibly being beneficial (probiotic) to the BSFL.

2.5. Solid state fermentation

Solid state fermentation (SoSF) can be referred to as a process that replicates a natural environment, allowing for microbial fermentation to occur in the absence or near absence of free water (Soccol *et al*., 2017). However, enough moisture is required to ensure the growth

and metabolic activity of the microorganism. (Manan & Webb, 2017). SoSF has been used f around the world or centuries and has attracted the attention of researchers due to its applicability in industries such as pharmaceuticals, foods, and bioenergy (Lizardi-Jiménez & Hernández-Martínez, 2017). A well-known example of SoSF is the growth of *Aspergillus oryzea* on a bed of cooked rice, also known as koji. Compared to submerged fermentation where the substrate is inundated in liquid containing the necessary nutrients for microbial growth (Manan & Webb, 2017), SoSF offers a unique set of advantages such as, (i) inexpensive substrates (Manan & Webb, 2017), (ii) ease of operation, (iii) no foam formation, (iv) reduced capital and energy demands as well as (v) easy control of contamination (Kapilan, 2015). Although SoSF has distinctive advantages over submerged fermentation, there are certain disadvantages that must be overcome, such as (i) the uneven distribution of nutrients, cell mass, pH and moisture content, (ii) the difficulty in achieving steady agitation of the substrate as well as (iii) measuring the biomass for bacterial growth (Manan & Webb, 2017).

SoSF must be carried out under a controlled environment to achieve maximum performance and product yield when using selected microorganisms, all while constraining possible inhibitors as well as unwanted microbial activity (Manan & Webb, 2017). The advantages of SoSF comes from its simplicity and its relatedness to the natural habitat of the micro-organisms used. New initiatives have been developed to improve the productivity of SoSF. The performance of SoSF depends on factors such as solid substrate, bioreactor design and the microorganisms used (bacterial and fungal) that influence the outcome. These factors can be divided into three groups namely, biological factors, physio-chemical factors, and mechanical factors (Bhargav *et al*., 2008). Substantial research has been done to understand these aspects; hence new initiatives through modern biotechnology allows for the improvement of SoSF productivity.

2.5.1. Biological aspects

The selection of a suitable strain for the development of SoSF is an important factor to consider and depends on various aspects such as the environmental conditions and the substrate used (Kapilan, 2015). Currently filamentous fungi are still considered the most popular choice for SoSF since these methods simulates their natural environment. However, certain species of bacteria (e.g., *Bacillus subtilis* and *Lactobacillus*) have also been reported to synthesise enzymes and other metabolites in large quantities using SoSF (Soccol *et al*., 2017).

The resistance of the microorganism to catabolic repression in the presence of substrates such as glucose and other carbon sources is an important factor to consider (Lizardi-Jiménez & Hernández-Martínez, 2017). The inoculum is defined as the number of viable cells from pure bacterial culture needed to start a new culture which brings about desirable changes and influence the progression of fermentation (Manan & Webb, 2017). Therefore, the microorganism must be precultured in the correct medium and harvested at the correct age before inoculation. Using higher concentrations of inoculum decreases the chances of contamination that might occur during fermentation.

The solid substrate is of great importance during SoSF since it is the support structure for the growing microorganisms (Kapilan, 2015). Additionally, the substrate provides the carbon as well as other nutrients needed for the biosynthesis of the microorganisms. SoSF substrates can be divided in four categories: (i) starchy substrates which are rich in carbohydrates such as cassava, barley oats and corn meal, (ii) substrates packed with proteinaceous nutrients such as sunflower oil cake and canola oil cake, (iii) lignocellulosic substrates including wheat straw, sugarcane bagasse and barley straw that contain high levels of cellulose (Aftab *et al*., 2019) and (iv) substrates that contain substantial amounts of soluble sugars such as lemon peel, pineapple waste and coffee pulp. Various inert carriers such as clay granules, vermiculite and perlite can also be used as solid substrates during SoSF. These inert carriers offer a variety of advantages including better control over temperature control, reuse of the material, and direct biomass measurement. Another important aspect to consider with the substrate is its water capacity that will determine the moisture content.

2.5.2. Physio-chemical aspects

Water activity (A_w) is defined as the free water present in the solid substrate and is important for microbial activity (Manan & Webb, 2017). The availability of water also determines the type of microorganisms that can be used in the SoSF process. Because of limited water present in SoSF, growth and metabolism of the microorganism can be restricted (Bhargav *et al*., 2008). Bacteria can grow on substrates with A_w of about 0.9, whereas filamentous fungi can thrive on much lower A_w values ranging between 0.6 - 0.7. During SoSF, heat is generated due to microbial activity, thus increasing the temperature within the reactor. This heat influences the growth of the microorganisms and needs to be dissipated to alleviate this problem (Manan & Webb, 2017). A few strategies have been developed to address this such

as the control of the aeration rate that can simultaneously control the temperature and moisture in the reactor. An additional problem that occurs is that heat creates condensation. Large amounts of water then return to the substrate increasing the moisture content and making it difficult to maintain a constant temperature. A solution to this problem would be to install a cooling system or air can be blown into the system, while the generated heat escapes via a gas outlet (Bhargav *et al*., 2008).

Another challenging factor in SoSF is measuring and controlling pH due to the very low water content as well as nature of the substrate (Manan & Webb, 2017). Currently there is no reliable electrode that can measure pH in a solid medium. The use of microorganisms that can thrive on a wide pH range would thus be preferable in this case. Yeast and fungi generally prefer acidic pH ranges, whereas bacteria prefer to grow in pH ranges near neutrality. Aeration requirements also depend on the microorganisms used in SoSF. High oxygen levels are maintained during aeration and ensure that the carbon dioxide levels are low in the solid substrate (Bhargav *et al*., 2008). The quality and rate of the oxygen feed are important aspects to consider with aeration. Saturated air controls the moisture gradient and the temperature of the substrate and ensures that the substrate does not lose any moisture content. Alternatively, dry air has certain advantages ensuring heat removal, however, it also influences the moisture of the substrate (Manan & Webb, 2017). The particle size of the substrate is an important factor to consider during SoSF since it affects the surface area needed for full contact of the microorganism with the nutrients. Larger particle sizes provide a smaller surface area per volume, but the diffusion of oxygen is not good. Smaller particle size is good for full contact of microorganism with nutrients since it has a larger surface area per volume. The particle size of the substrate may change during SoSF, affecting the growth of the microorganisms, substrate consumption water content and heat conductivity.

2.5.3. Mechanical aspects

Agitation plays an important role in the SoSF process and may improve the conditions of microbial growth (Bhargav *et al*., 2008). Agitation is beneficial in that it provides even distribution of the substrate throughout the reactor. Additionally, it can also be used to solve heterogeneity problems that occur during SoSF (Bhargav *et al*., 2008). On the other hand, continuous mixing can lead to cell damage, especially when filamentous fungi are used. However, for bacteria agitation is preferred since the cells are not in close contact with the

substrate. SoSF bioreactors can be grouped based on their mixing system and aeration requirements. Common bioreactors are perforated trays and horizontal drums, although these bioreactors come with their own advantages and disadvantages (Wang & Yang, 2007). Therefore, it may be necessary to develop novel bioreactors using improved designs to commercialize SoSF to produce value-added products (Soccol *et al*., 2017). When designing the reactors, certain aspects must be considered such as the nature of the substrate that will be used as the growth medium, size and the strength of the reactor and the efficiency of mixing and aeration required (Soccol *et al*., 2017). Reactors with continuous mixing and aeration are believed to perform best. Another important aspect to consider is the microbial morphology which will influence the choice of agitation and the rate of aeration. This may increase the complexity of the bioreactor design. Rotating drums are usually a cylindrical drum mounted on its side onto rollers that support and rotate the vessel (Wang & Yang, 2007). Humified air is circulated through inlets and outlets, and the drums are often equipped with baffles for agitation of the substrate for enzyme and microbial production (Figure 2.6). Their main disadvantage is that the drum can only be filled to about 30% to ensure thorough mixing. Tray fermenters are used extensively to produce fermented oriental foods and enzymes (Singhania *et al*., 2009). The substrate layer is usually 2.5-5 cm deep and humified air is forced in to allow for aeration (Figure 2.7). The bed temperature must be monitored and is usually controlled by adjusting the recycled air flow. Column bioreactors mostly use glass or plastic columns that contain loosely packed solid substrate. A jacket surrounding the column is used to control the temperature. These systems are used for production of organic acids, ethanol, and biomass (Wang & Yang, 2007). The production of biomass for animal feed is also achieved by using fluidized bed reactors. Continuous agitation is achieved with forced air to prevent adhesion and aggregation in these systems (Figure 2.8). Damage to fungal mycelia can occur in this type of reactor, due to shear (Singhania *et al*., 2009).

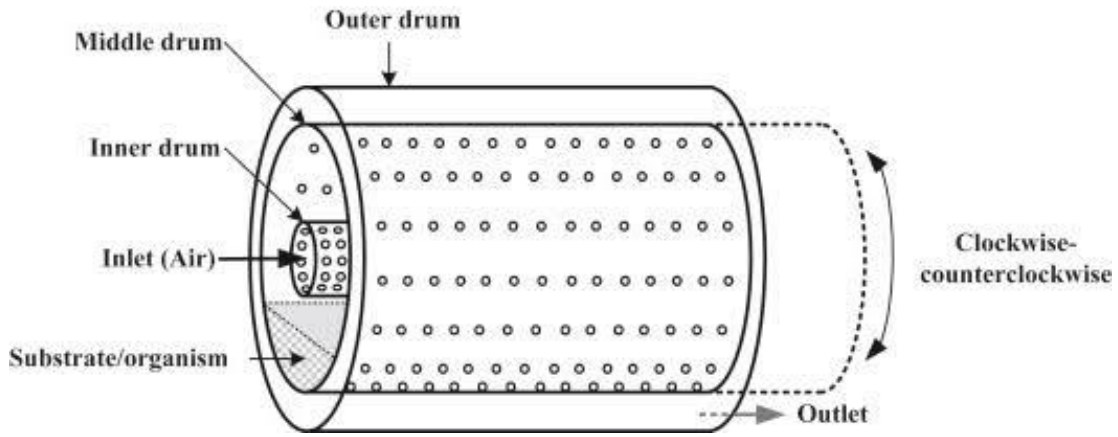

Figure 2.6: Schematic view of a rotating drum bioreactor. (Ge *et al*., 2017)

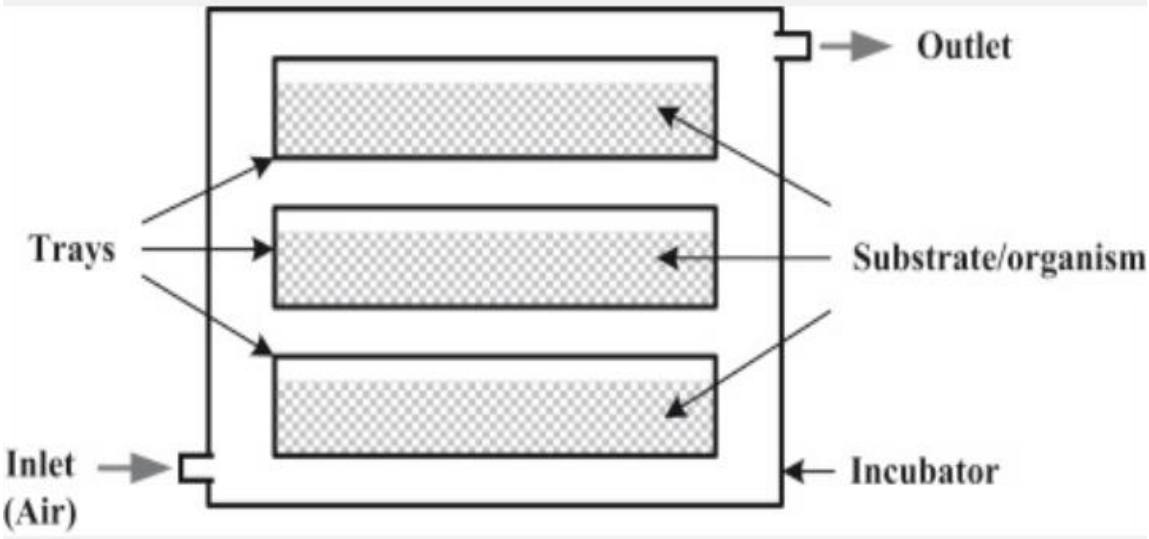

Figure 2.7: Schematic view of a tray fermenter (Ge *et al*., 2017)

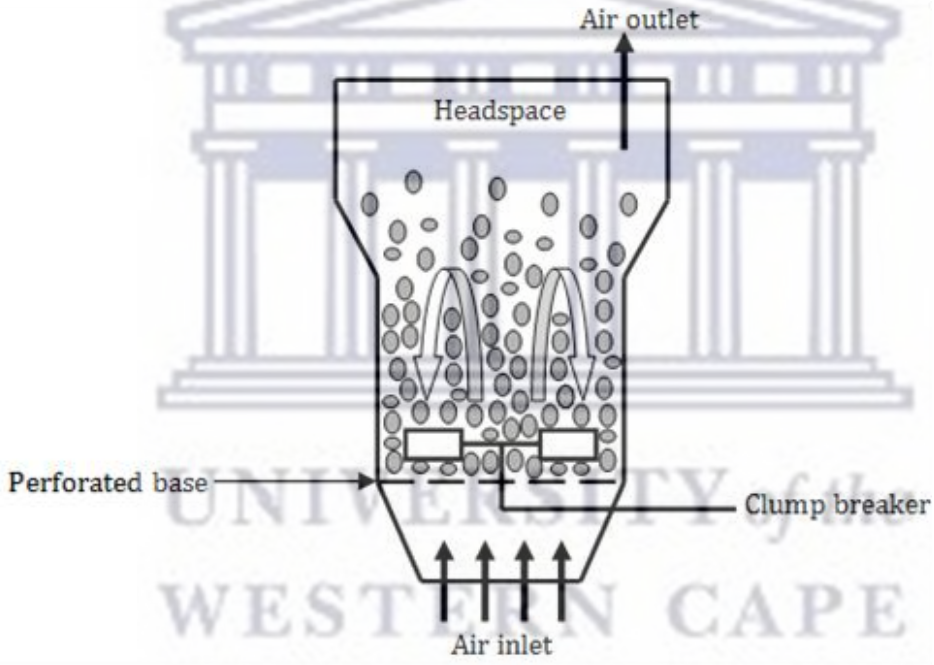

Figure 2.8: Schematic view of a fluidized bioreactor (Manan & Webb, 2017)

2.6. Other hydrolysis and fermentation processes

Conventional lignocellulose conversion to ethanol involves four biological processes, (i) enzyme production, (ii) hydrolysis of the substrate followed by the fermentation of (iii) hexose and (iv) pentose sugars (den Haan *et al*., 2013). In separate hydrolysis and fermentation (SHF), the material is hydrolysed to glucose following fermentation which takes place in a separate unit. This process can be very time consuming and may even result in microbial contamination. One possibility for the contamination could be due to microorganisms in the enzymes used (Taherzadeh & Karimi, 2007). Practically it is challenging to sterilise large

amounts of cellulase since it must be filtered. The main disadvantage of SHF is the inhibition of the cellulase activity by the released sugars. Simultaneous saccharification and fermentation (SSF) describes a process where hydrolysis and fermentation are combined so that hydrolysed glucose is immediately consumed by the fermenting organism, eliminating the inhibition effects of cellobiose and glucose since these sugar concentrations are kept low (Nielsen *et al*., 2019). The capital cost is also reduced in SSF since both processes take place in one reactor. It has also been reported that lower amounts of enzymes were required for this process and compared to SHF, the risk of contamination was lower due to the presence of the alcohol in the reactor (Nielsen *et al*., 2019). One drawback of this process is that the hydrolysis and the fermentation processes are optimally conducted at different environmental conditions. Fermentative microorganisms usually grow at a temperature between 30°C and 37°C whereas optimal enzymatic hydrolysis temperature occurs around 45°C and 50°C, therefore it is not possible to attain optimal conditions for both processes. These processes are performed as submerged fermentations. In the present study, the goal was not to produce large amounts of ethanol, but to ensure that most carbohydrates in LCB are available for larval feeding. Furthermore, we used SoSF to cultivate bacterial strains that could benefit the conversion process either by releasing sugars or acting as probiotics for the BSFL.

CHAPTER 3: MATERIALS AND METHODS

3.1. Feedstock preparation

Sugarcane bagasse (SB) was sourced from TSB Sugar (Malelane, Mpumalanga, South Africa) and pre-treated at 185°C for 10 minutes using a steam gun supplied by the Department of Process Engineering at Stellenbosch University (Theron, 2022). This condition was selected based on preliminary BSF larval rearing trials performed by a local BSF rearing facility in the Western Cape that showed SB steam pre-treated at 185°C for 10 minutes to be the best condition to support larval growth (Theron, 2022). The slurry produced after steam pre-treated consisted of water insoluble solids (WIS) and the liquid hydrolysate containing soluble sugars and most of the inhibitors, with a moisture content of approximately 74%. The chemical composition of the pre-treated substrate was determined according to the NREL procedure (Sluiter *et al*., 2008). The substrates were divided into 700g per bag, based on the quantity needed for one batch of experiments and placed in vacuum seal bags for sterilization by irradiation. The substrates were sterilized by using a cobalt-60 radiation source (HEPRO, Cape Town, South Africa) at a dosage of 20kGy (kilogray) and subsequently stored at -20°C.

3.2. Experimental setup for substrate treatment

High-solids enzymatic hydrolysis and solid-state fermentation experiments were carried out in a closed rig manufactured by the Department of Process Engineering, Stellenbosch University. Figure 3.1 shows a cross-sectional view of the reactor used. Horizontal reactors 600 mm in length and 115 mm in diameter with a capacity of 6 L were designed using polyvinyl chloride (PVC) pipes. A stainless-steel rod and scraper were placed inside the reactor and supported by the caps attached to mimic gravimetric mixing (Figure 3.2). A heater was added to the rig to apply heat connected to a thermostat controller to monitor the temperature (Figure 3.3). The reactors were placed in the rig and rotated at 12.5 rpm.

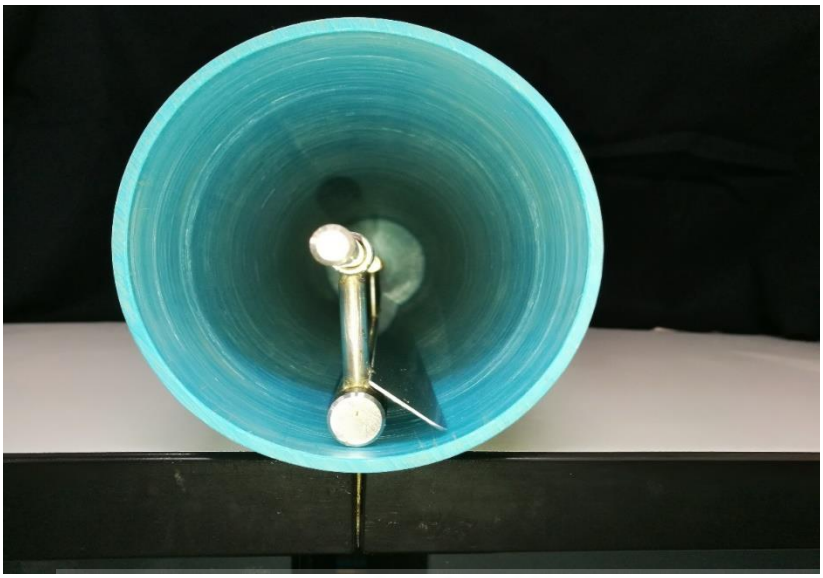

Figure 3.1: Cross sectional view of the reactor used for high solids experiments.

Figure 3.2: Stainless scraper used for mixing.

Figure 3.3: High-solids reactor placed on a rig. Heater was placed inside the rig.

3.3. Microorganisms

The bacterial strains used in this study were selected based on their ability to produce cellulase, their resistance to pre-treatment derived by-products, and their ability to provide probiotic effects. *Bacillus amyloliquefaciens* DSM 7, *Lactobacillus pentosus* DSM 20314, *Pediocccus pentosaceus* DSM 20283 were purchased from the Leibniz Institute DSMZ-German Collection of microorganisms and Cell Culture GmbH (Germany). *Lactobacillus plantarum* and *Pediococcus acidilactici* was obtained from culture collections at the Department of Microbiology at Stellenbosch University. *Lactobacillus brevis* (ATCC® 367™) and *Lactococcus lactis subsp. lactis* (ATCC® 15577™ renamed *Lactobacillus xylosus*) were obtained from the culture collection at the Department of Process Engineering at Stellenbosch University. *Streptomyces* spp. was obtained from the Institute for Microbial Biotechnology and Metagenomics at the University of the Western Cape. Strains were stored in 1 mL aliquots at -85 °C using 30% (v/v) glycerol.

3.4. Growth media

Bacillus amyloliquefaciens DSM 7 was maintained on Nutrient broth (1 g/L beef extract, 2 g/L yeast extract, 5 g/L sodium chloride (NaCl), 5 g/L peptone) (Sigma-Aldrich SA, Kempton Park, South Africa). *Lactobacillus plantarum*, *Pediococcus acidilactici*, *Lactobacillus pentosus* DSM 20314, *Pediococcus pentosaceus* DSM 20283, *Lactobacillus brevis* (ATCC® 367™) and *Lactobacillus xylosus* were maintained on MRS broth (5 g/L yeast extract, 10 g/L peptone, 5 g/L sodium acetate, 2 g/L sodium citrate, 2 g/L K_2HPO_4, 0.58 g/L $MgSO_4 \cdot 7H_2O$, 0.12 g/L $MnSO_4 \cdot 7H_2O$ and 0.05 g/L $FeSO_4 \cdot 7H_2O$) (Sigma-Aldrich SA, Kempton Park, South Africa). *Streptomyces* was maintained on YEME (4 g/L yeast extract, 10 g/L malt extract, 4 g/L glucose) (Sigma-Aldrich SA, Kempton Park, South Africa). All bacterial strains were cultivated at 37°C overnight.

3.5. Identification for cellulolytic bacteria

3.5.1. Primary screening for cellulolytic activity

To determine the presence of endoglucanase activity, bacterial cultures were spotted on its respective media supplemented with 1% carboxy-methylcellulose (CMC) and incubated at 37°C overnight. Following incubation, plates were stained with 0.1% Congo red solution for

30 minutes, followed by de-staining with 1.2 M $NaCl_2$. The presence of a clear zone around the colony was an indication of cellulolytic activity.

3.5.2. Enzymatic assay

Bacterial cultures were inoculated in triplicates in 100 ml Erlenmeyer flasks containing 10 ml of their respective media and cultivated overnight at 37°C on a rotary shaker at 150 rpm. Determination of the endoglucanase activity was performed as described by La Grange *et al.,* (2001) using 1% CMC as a substrate. Endoglucanase activity was determined at 50°C for 5 minutes including a buffer blank (50mM NaOAc pH5). The absorbance was measured at 540 nm on a SPECTROstar (BMG LABTECH) plate reader. A standard curve with a range of 0.5 g/L to 10 g/L D-glucose was used to determine the enzymatic activity.

A 1:20 dilution was performed on all samples and OD_{600} values were determined to calculate the dry cell weight (DWC) which was used to normalize the volumetric values of the bacterial cultures. Enzyme activities were determined as units/g DCW in which one unit (IU) was defined as the amount of enzyme needed to release one µmol of reducing sugar equivalent per minute.

3.6. Determination of inhibitor tolerance

Cultivations in a 250 mL Erlenmeyer flask with a working volume of 50 mL were used to determine the inhibitor tolerance of the strains under aerobic conditions. Colonies grown on agar plates were transferred to their respective cultivation media and incubated overnight at 37°C, prior to culturing into flasks containing 75% SB hydrolysate. The remaining 25% volume represents fermentation media. Each strain in the cultivation media was transferred to the 75% SB hydrolysate flasks yielding an initial OD_{600nm} of 1 and then incubated for 3 days at 37°C. Samples were taken after incubation and bacterial cells in the media were counted in duplicates on a Petroff-Hausser Counting Chamber. All bacterial cultures were tested in triplicate and the average results along with the standard deviation were calculated.

3.7. Identification of contaminants

Because solid-state fermentation was conducted under non-sterile conditions, contamination within the reactors was expected. Two predominant contaminants were routinely isolated, and their extracted DNA was used to amplify 16s rRNA (bacterial contaminant) and 18s rRNA (fungal contaminant) using primers, E9-F and U1510-R (5'-GAGTTTGATCCTGGCTCAG-3' and

5’-GGTTACCTTGTTACGACTT-3’) and EukA and EukB (5’-AACCTGGTTGATCCTGCCAGT-3’ and 5’-TGATCCTTCTGCAGGTTCACCTAC-3’) respectively. Reactions of 50µl were set up using 2X Red Taq PCR master mix (AMPLIQON) according to the manufacturer’s instructions. Table 3.1 below outlines the polymerase chain reaction (PCR) parameters used for 30 cycles. The amplicons were purified and sent for Sanger sequencing at CAF (Stellenbosch University). The sequence was analysed in BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Table 3.1: PCR steps and conditions used for amplification

	Bacterial strain		Fungal strain	
Reaction step	**Temperature °C**	**Time**	**Temperature °C**	**Time (min)**
Initial step	96	2 min	95	3 min
Denaturation	96	45 sec	95	45 sec
Annealing	54	30 sec	55	45 sec
Primer extension	72	2 min	72	3 min
Final extension	72	5 min	72	10 min

3.8. High-solids enzymatic hydrolysis and Solid-state fermentation

3.8.1. High-solids enzymatic hydrolysis

The commercial enzyme preparation Novozymes Cellic CTec 3 (Novozymes A/S, Denmark) was used for high-solids enzymatic hydrolysis of SB. The enzyme preparation had an activity of 148 FPU/ml as measured by the filter paper assay (Pengilly *et al.*, 2015). Partial hydrolysis was carried out using 700 g steam pre-treated SB slurry (wet weight) mixed with 1 M sodium citrate buffer to a final concentration of 0.05 M (pH 4.8) to ensure a moisture content of approximately 74%. Enzyme dosages of 2.5, 5 and 7.5 FPU/g (per dry weight) were added, and the reaction mixture was incubated at 40°C for 21h using the set-up detailed in Section 3.2, prior to BSF larval trials. All enzymatic hydrolysis experiments were performed in triplicate and the average result along with standard deviations were calculated.

3.8.2. Solid-state fermentation

Solid-state fermentation was conducted in the reactors containing 700 g steam pre-treated SB slurry (wet weight) supplemented with casein hydrolysate (10 g/L) as a nitrogen source and inoculated with 1×10^9 CFU/ml of each of the selected bacterial cultures. The reactors were incubated at 37°C for 3, 6 and 10 days using the set-up detailed in Section 3.2, prior to

BSF larval trials. All experiments were performed in triplicate and the average result along with standard deviations were calculated.

3.8.3. Simultaneous saccharification and fermentation

Simultaneous saccharification and fermentation (SSF) was conducted in the reactors containing 700 g steam pre-treated SB slurry (wet weight) substrate supplemented with casein hydrolysate (10 g/L) as a nitrogen source and inoculated with 1×10^9 CFU/ml of each of the selected bacterial cultures as well as 7.5 FPU/g (per dry weight) Cellic CTec 3 enzyme. The reactors were incubated at 37°C for 3, 6 and 10 days using the set-up detailed in Section 3.2, prior to BSF larval trials. All experiments were performed in triplicate and the average result along with the standard deviations were calculated.

3.9. BSF Larval rearing

BSF larvae, as well as the formulated standard feed which contained a high wheat bran content used in this study, were collected from BSF Breeding (Goodwood, Cape Town). Nursey feed was prepared according to the specifications described by van ser Vyver, 2022. Treated SB substrate was mixed with a formulated standard feed at an inclusion ratio of 50%. Fifty 4-day old larvae were maintained on 75 g (wet weight) feed at a moisture content of 73% during the grower stage until 11-days old. All experiments were performed in replicates of eight and the average result along with the standard deviations were calculated. Harvested larvae was sacrificed by freezing at -20 °C for 24 hours. Larvae were removed from the freezer and allowed to defrost at room temperature before drying in a ventilated oven at 65°C for 24 hours. After drying, the larvae were milled through a 3 mm sieve using a Christy and Norris junior laboratory mill. Milled samples were stored in a cold room until laboratory analyses.

3.10. Analytical methods

Analytical methodologies were performed at the Department of Animal Science (Stellenbosch University) except for HPLC analysis that was done at the Department of Process Engineering (Stellenbosch University).

3.10.1. Dry matter determination

The dry matter (DM) of the larvae and residue was determined according to the Association of Official Analytical Chemists International (2002), Official Method 934.01. Two grams of

each sample were placed in a crucible and allowed to dry for 24 hours at 100°C. Thereafter the dry sample was weighed, and the DM content was calculated using Equation 1:

Equation 1

$$\% \text{ Moisture} = \frac{(A + B) - C}{\text{Sample mass (g)} \times \text{dry matter*}} \times \frac{100}{1}$$

Dry Matter (%) = 100 - Moisture (%)

Where:

A = Weight of empty and dry crucible

B = Weight of test sample

C = Weight of crucible and moisture free test sample

3.10.2. Neutral detergent fibre determination

The neutral detergent fibre (NDF) determination was performed according to Raffrenato & Van Amburgh (2011). NDF is the residue or insoluble fraction left after boiling a feed material in a neutral detergent solution. Duplicate samples weighing 1 g were placed into a glass crucible and into the Fibertec extrusion apparatus. To each sample 100 ml cold NDF solution was added. Upon boiling, 0.1 ml heat stable α-Amylase (Sigma #A3306) was added and the samples were left to boil for 60 minutes where after which it was washed three times with 100 ml distilled water. Samples were washed with 15 ml acetone to remove water. After the completion of this procedure the samples were dried at 100°C for 24 hours and then placed in a furnace for 6 hours at 500°C. The NDF content was then calculated by using Equation 2:

Equation 2

$$\% \text{ NDF} = \frac{A - B}{\text{Sample mass (g)} \times \text{dry matter*}} \times \frac{100}{1}$$

Where:

A = Sample and crucible after drying, in g

B = Sample and crucible after ashing, in g

* = Dry material of sample (100 – Moisture content)

3.10.3. Acid detergent fibre determination

The acid detergent fibre (ADF) determination was performed according to Raffrenato & Van Amburgh (2011). ADF is a measure of the plant components in forages that are the least digestible by livestock, including cellulose and lignin. Duplicate samples weighing 1 g were placed into a glass crucible and into the Fibertec extrusion apparatus. To each sample 100 ml cold ADF solution was added, and the samples were left to boil for 60 minutes where after it was washed three times with 100 ml distilled water. Samples were washed with 15 ml acetone to remove any trace of water. After the completion of this procedure the samples were dried at 100°C for 24 hours and then placed in a furnace for 6 hours at 500°C. The ADF content was then calculated by using equation 3. The NDF content along with the ADF content was used to determine the hemicellulose content using equation 4.

Equation 3

$$\% \text{ ADF} = \frac{A - B}{\text{Sample mass (g)} \times \text{dry matter}^*} \times \frac{100}{1}$$

Where:

A = Sample and crucible after drying, in g

B = Sample and crucible after ashing, in g

* = Dry material of sample (100 – Moisture content)

Equation 4

% Hemicellulose = % NDF – % ADF

3.10.4. Acid detergent lignin determination

The acid detergent lignin (ADL) determination was performed according to Goering & van Soest (1970). Duplicate samples weighing 1 g were placed into a glass crucible and into the Fibertec extrusion apparatus. To each sample 100 ml cold ADF solution was added, and the samples were left to boil for 60 minutes. After boiling, samples were washed three times with 100 ml distilled water. Samples were washed with 15 ml acetone to remove any trace of water. After the completion of this procedure the sample were dried at 100°C for 24 hours. After drying 72% sulphuric acid was added to each crucible and allowed to extract for 3 hours stirring every 30 minutes. Crucibles were then placed back into the vacuum system and rinsed three times with 150 ml hot water. Samples were washed with 15 ml acetone and dried at

100°C for 24 hours then placed in a furnace for 6 hours at 500°C. The ADL content was then calculated by using equation 5. The ADF content along with the ADL content was used to determine the cellulose content using equation 6.

Equation 5

$$\% \text{ ADL} = \frac{A - B}{\text{Sample mass (g)} \times \text{dry matter*}} \times \frac{100}{1}$$

Where:

A = "Hot Weighing" Mass of residue in crucible after drying, in g

B = "Hot Weighing" Mass of residue in crucible after ashing, in g

* = Dry material of sample (100 – Moisture content)

Equation 6

% Cellulose = % ADF - % ADL

3.10.5. Crude protein determination

The crude protein content of the larvae and residue samples were determined by measuring the total nitrogen (N) content according to the method described by the Association of Official Analytical Chemists International (2002), Official Method 4.2.07, in the LECO FP828 apparatus. Two subsamples each weighing 0.1 g were placed in a tin foil cup and then placed into the LECO FP828. Thereafter the N content was directly taken from the LECO FP828, and the Crude Protein content was calculated by using Equation 7:

Equation 7

Crude Protein (%) = Nitrogen (%) × 6.25

3.10.6. Crude fat determination

Crude fat was determined through an acid hydrolysis, method 954.02 (AOAC 2002). Duplicate samples with a weight of 2 g each were added to a test tube, followed by 2 ml of ethanol and 10 ml HCL solution (3%). Test tubes were boiled for 30 minutes in a water bath. Once cooled, the mixture was poured into a separating funnel. The tube was rinsed with 10 ml ethanol and added to the funnel. A volume of 25 mL of diethyl ether was added to the funnel and shaken for one minute. Afterwards, 25 mL of petroleum ether was added, and the mixture was shaken again for one minute. The transparent upper portion of liquid was transferred into a

fat beaker. This process was repeated two more times using 15 mL diethyl ether and petroleum ether. Fat beakers were placed in a warm sand bath until all the ether evaporated and weighed to determine crude fat content using equation 8.

Equation 8

$$\% \text{ Crude fat} = \frac{(\text{Mass of fat cup+ Fat}) - (\text{Mass of fat cup})}{\text{Sample mass (g)} \times \text{dry matter*}} \times \frac{100}{1}$$

* = Dry material of sample (100 – Moisture content)

3.10.7. HPLC analysis

The concentrations of sugar monomers (glucose and xylose) as well as acetic acid and the pre-treatment by-products formic acid, furfural and 5-hydroxymethyl-2-furaldehyde (5-HMF) were determined using HPLC. The Aminex HPx-87 column used was equipped with a cation-H Micro Guard Cartridge and an AS3000 AutoSampler (Bio-Rad, Johannesburg, South Africa). The column temperature was operated at 65 °C with 5 mM H_2SO_4 as a mobile phase at the flowrate of 0.6 mL/min. Sugar concentrations were measured with an RI detector (Shodex, RI-101, Munich, Germany) operated at 45°C. 5-HMF and furfural were analysed on a Phenomenex Luna C18(2) reversed phase column equipped with a Phenomenex Luna C18(2) precolumn (Separations, Johannesburg, South Africa) with column temperature set to 25°C and a flow rate of 0.7 mL/min.

3.11. Statistical analysis

The mean comparison between the different groups within the same feeding regime was determined on Microsoft Excel 2016, using a student t-test, assuming unequal variances and unpaired samples with a significant level of $P \leq 0.05$.

CHAPTER 4: RESULTS AND DISCUSSION

4.1. Chemical composition of sugarcane bagasse

Sugarcane bagasse (SB) is a fibrous residue of sugarcane stalks left over after crushing and extraction of the juice (Pandey *et al.*, 2000). It is one of the largest agro-industrial by-products and inefficient sugar mills burn most of their bagasse in boilers for heating energy (Baruah *et al*., 2018). Generally, 280 kg of bagasse (dry basis) is generated from 1 ton of sugarcane (Soccol *et al.*, 2010). SB is thus a widely available potential feedstock for BSFL cultivation if an appropriate conversion process can be devised. Steam explosion is a common pre-treatment method used for SB. Steam explosion (SE) conditions were selected based on preliminary BSF larval rearing trials performed by a local BSF rearing facility in the Western Cape, which showed that SB steam pre-treated at 185°C for 10 minutes was the best condition to support larval growth (Theron, 2022). The chemical composition of SB and steam pre-treated SB are shown in Table 4.1. The cellulose content of SB and steam pre-treated SB were 38.2 g/100 g DM and 50.3 g/100 g DM, respectively. Hemicellulose content was 14.7 g/100 g DM and 8.6 g/100 g DM, respectively. SB has high glucan content; thus, more sugars can be made available which will make it advantageous for BSFL larvae.

Table 4.1: Chemical composition of sugarcane bagasse and steam pre-treated sugarcane bagasse (% (w/w) per dry mass)

Components	Untreated Sugarcane Bagasse	Steam pre-treated Sugarcane Bagasse
Cellulose	38.2±0.3	50.3±0.2
Hemicellulose	14.7±0.2	8.6±0.2
Lignin	29.5±0	26.3±0.1
Extractives	5.8±0	13.6±0
Ash	2.6±0.1	3.6±0
Total Mass Closure	90.7±0.6	102.3±0.4

The chemical composition of the SB was similar to what was previously reported in literature (Table 4.2). The chemical composition of SB depends on various factors, including breeding, variety, geographical location, and time of harvest (Benjamin, 2014). This explains the difference in cellulose (32.8 g/100 g to 44.9 g/ 100 g DM), hemicellulose (14.7 g/ 100 g to 24.8 g/ 100 g) and lignin (18.9 to 29.5 g/100 g DM). SB is high in cellulose content, thus making it

a suitable feedstock for the bioconversion processes. However, untreated cellulose is relatively indigestible due to its recalcitrant nature and lignin promotes unproductive binding of hydrolytic enzymes (Diedericks, 2013). NREL analysis showed that after SE, the substrate contained sufficient polysaccharides to sustain the growth of the BSF larvae if the sugars could be liberated by the actions of microbial enzymes.

Table 4.2: Comparison of the chemical composition of sugarcane bagasse (% (w/w) per dry mass). Chemical composition was determined using the NREL method

Cellulose	Hemicellulose	Lignin	Extractives	Ash	Reference
38.6	15.8	27.4	6.9	2.7	(Koekemoer, 2018)
39.1	24.8	18.9	6	4	(Diedericks, 2013)
39.6	20.8	22.4	5	1.3	(Benjamin, 2014)
40.2	23.4	23.0	5.9	3.2	(Pius, 2017)
38.2	14.7	29.5	5.8	2.6	This work

4.2. Screening and selection of microorganisms: Screening for cellulolytic activity

Several insects, including BSFL, depend heavily on their symbiotic bacteria for successful growth and reproduction (Somroo *et al*., 2019). These nutrient interactions have attracted considerable attention, and for most insect–bacteria symbiotic systems, insects benefit from nutrient availability. Selection of bacterial strains from previously published work for this project was based on three criteria, namely (i) probiotic strains that were shown to have health benefits when consumed, (ii) ability to grow in the presence of inhibitors such as HFM, furfurals and acetic acids and/or (iii) ability to secrete cellulolytic enzymes capable of digesting cellulose and hemicellulose. Table 4.3 shows the bacterial strains that were selected and screened for cellulolytic activity.

Table 4.3 Criteria for bacterial strain selection

Bacterial Strains	Benefits	Reference
***Bacillus amyloliquefaciens* DSM 7**	Cellulolytic activity + Probiotic	(Manhar *et al*., 2015)
Lactobacillus pentosus	Probiotic + Inhibitor tolerant	(Boguta *et al*., 2014), (Unban *et al*.,2021)
Pediocccus pentosaceus	Probiotic + Inhibitor tolerant	(Bagad *et al*., 2017), (Boguta *et al*., 2014)
Lactobacillus plantarum	Probiotic + Inhibitor intolerant	(Tu *et al*., 2019), (Lee *et al*., 2019)
Pediococcus acidilactici	Cellulolytic activity + Inhibitor tolerant	(Liu *et al*., 2013)
Lactobacillus brevis	Probiotic + Inhibitor tolerant	(Koekemoer, 2018), (Hacıoglu *et al*., 2021)
Lactobacillus xylosus	Probiotic + Inhibitor tolerant	(Koekemoer, 2018), (Rahman *et al*.,2020)
Streptomyces	Cellulolytic activity	(Unban *et al*.,2021)

A standard CMC plate screen (Figure 4.1) was performed to determine whether the bacterial cultures were able to produce cellulolytic (endoglucanase) activity. All the bacterial cultures except *L. plantarum* and *P. acidilactici* produced clear zones on the CMC plate assay. The amount of endoglucanase activity was subsequently quantified for each of the cultures (Figure 4.2). All bacterial cultures showed endoglucanase production with *P. pentosaceus* having the highest activity (6165,88 IU/gDWC) despite showing the smallest clearing zone (data not shown). These results differed from the plate screening results, where *L. plantarum* and *P. acidilactici* displayed cellulolytic activity of 1081.15 and 446.01 IU/gDWC respectively. This may be due to cell-associated endoglucanase activity as opposed to enzymes that are secreted into the growth medium. According to (Oke *et al*., 2016) factors such as amount of agar, the molecular weight of the enzyme and the size of the enzyme can also influence the migration on the enzyme on the agar plate, explaining the disparity in zone size versus quantitative activity data. The selected bacterial strains thus showed promise in

bioaugmentation of BSF cultivation. However, they also need to be able to withstand inhibitors present in the SB released during SE.

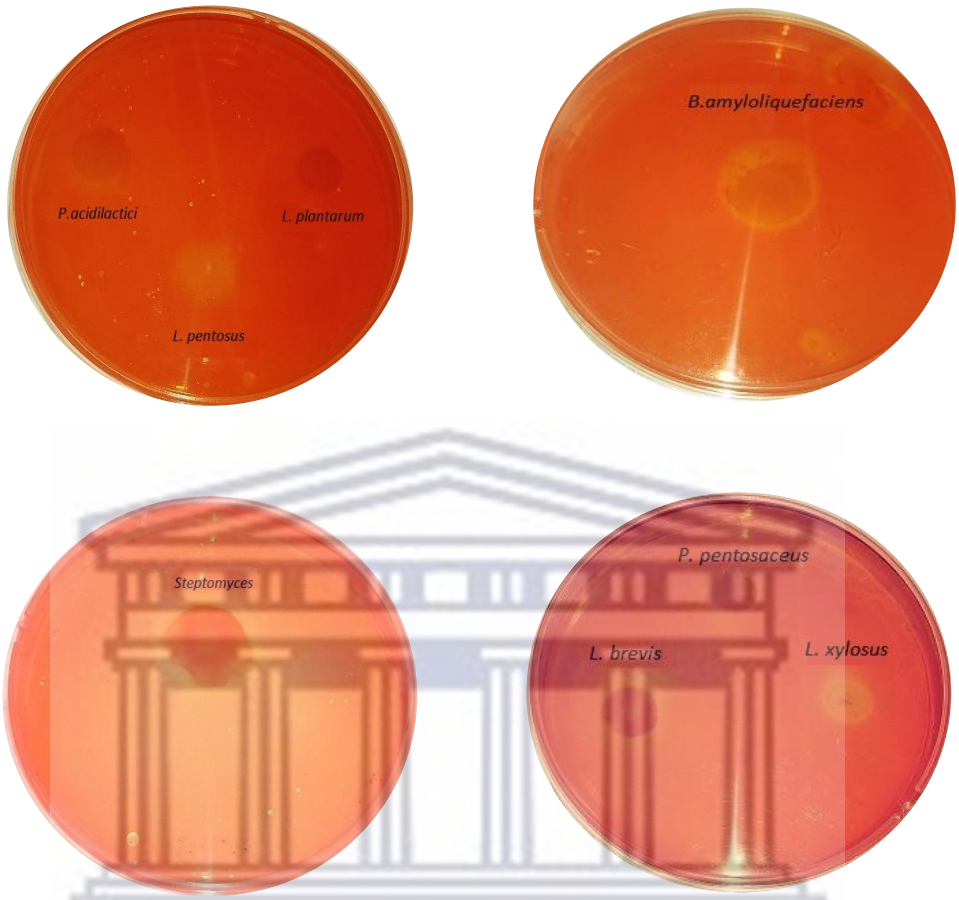

Figure 4.1: Plate screening of bacteria for cellulolytic activity. A clear zone indicated the presence of cellulase. A *Streptomyces* strain, previously shown to have cellulolytic activity, was used as positive control.

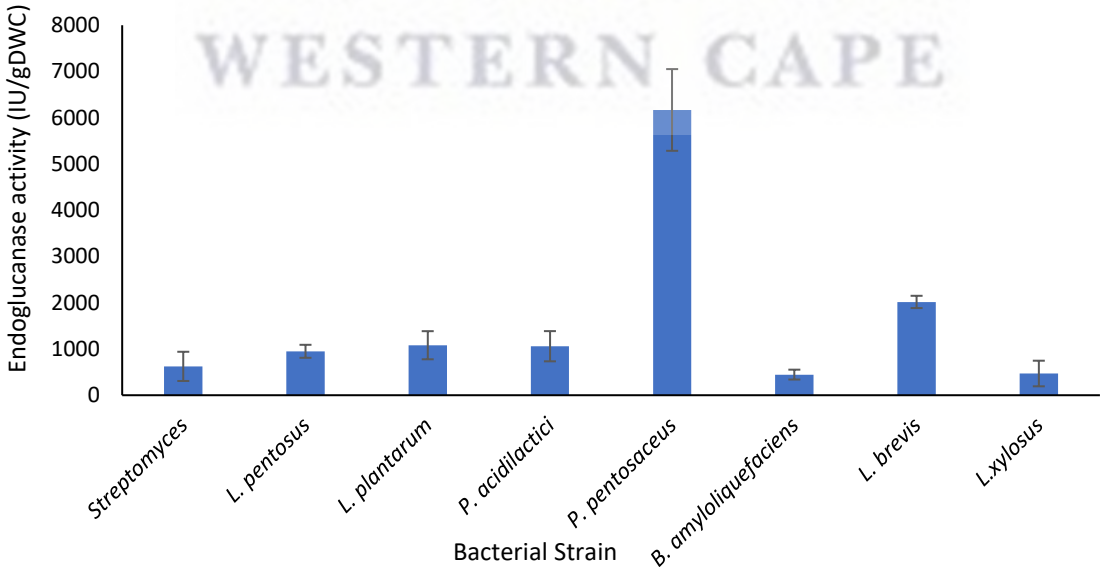

Figure 4.2: Liquid endoglucanase (CMCase) assay for the different bacterial strains. A *Streptomyces* strain, previously shown to have cellulolytic activity, was used as positive control. All values represent mean values of assays done in triplicate with error bars indicating standard deviation. Control organisms with no cellulases displayed activities close to zero in an assay (not shown).

4.3. Screening and selection of microorganisms: Tolerance to inhibitors in the hydrolysate of steam pre-treated sugarcane bagasse

The ability to survive the conditions presented by pre-treated SB due to the presence of inhibitors (Table 4.4) was tested to identify potential strains for solid state fermentation. An initial screen was performed using 75% hydrolysate where the remaining volume represents fermentation media. All bacterial strains were inoculated at OD 1 and after 3 days of incubation the cell count was recorded (Figure 4.3). The best performing strains were *L. brevis, L. plantarum* and *L. xylosus* with cell counts of 1.57×10^{8}, 7.05×10^{7}, 2.6×10^{7} cells/ml, respectively, with *L. brevis* showing a 4.5-fold increase in cell count over the incubation period. These results were comparable with previously published reports. According to Boguta *et al.* (2014) strains of *L. brevis* exhibited robust growth on up to 7 g/L furfural, 2.7g/L acetic acid and 5.9 g/L HMF. Although the amount of inhibitors present in the SB used for this project was lower, *L. brevis* displayed the highest growth after 3 days of cultivation. Another study by (Tu *et al*., 2019) reported that a *L. plantarum* isolate was able to tolerate 8 g/L furfural and 6 g/L HMF inhibitors. Van der Pol *et al*. (2016) investigated the effects of different inhibitors on the growth of *Lactococcus lactis* DSM 20481 among other strains. At 2.5 g/L HMF, *L. lactis* showed a 39% increase in its growth, however a reduction of the growth performance was detected at 2.5 g/L furfural. In contrast to that, they also showed that the growth performance increased despite a low concentration of acetic acid (122% at 5 g/L).

Table 4.4: Concentration of pre-treatment derived inhibitors in steam pre-treated sugarcane bagasse at 75% hydrolysate, prior to bacterial inoculation

Inhibitory Compound	Steam pre-treated (g/L)	75% hydrolysate (g/L)
Formic acid	1.23 ± 0.24	0.38 ± 0.043
Acetic acid	5.86 ± 0.47	4.32 ± 0.379
5-Hydroxymethylfurfural (HMF)	n.d	n.d
Furfurals	0.28 ± 0.009	n.d

n.d. – 5-HMF and furfural concentration was below HPLC detection limit.

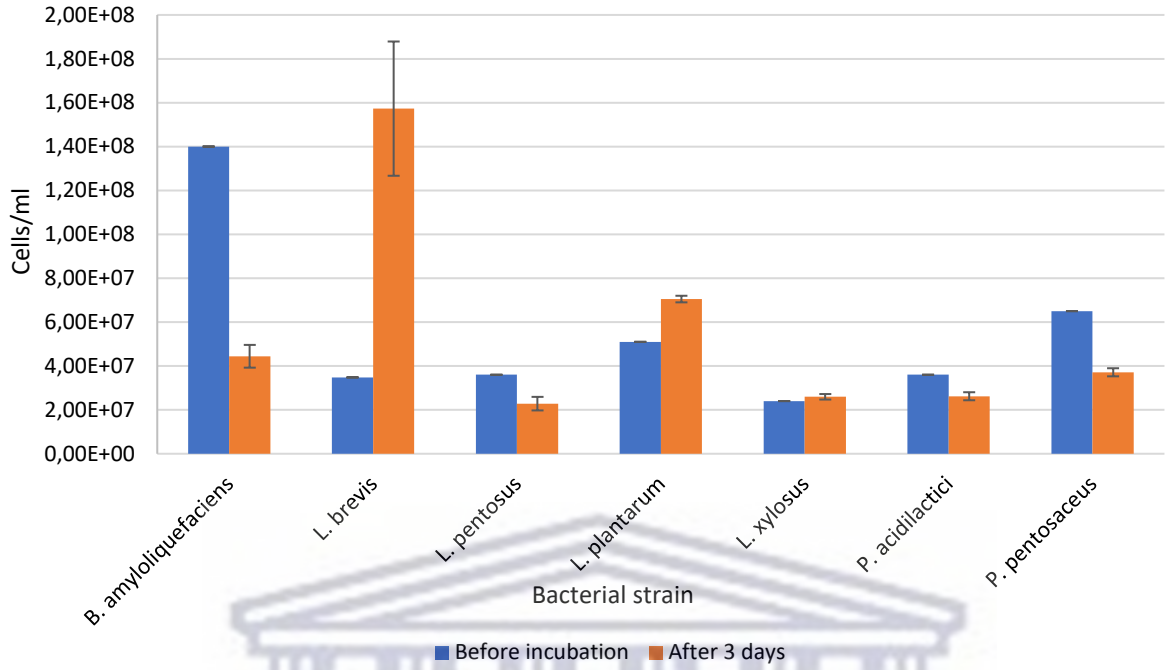

Figure 4.3: Cell count of micro-organisms in 75% SB hydrolysate. Cells were counted before incubation and after 3 days of incubation. All values represent the mean of cell counts with error bars indicating the standard deviation of triplicate experiments.

L. pentosus, P. acidilactici, P. pentosaceus and *B. amyloliquefaciens* all showed a decrease in cells, with *B. amyloliquefaciens* showing a significant decrease after 72 hours of cultivation. Boguta *et al*. (2014) reported that after 48 hours identified isolates of *L. pentosus*, *P. pentosaceus* and *P. acidilactici* were not only highly resistant to the different inhibitors at high concentrations but were also able to consume the xylose and arabinose present. In our study the strains failed to perform well possibly due to a lack of available free sugars (1.14mg/g glucose and 5.74 mg/g xylose) or the combined effect of inhibitors including phenolic inhibitors which have been shown to have a synergistic effect in combinations. These results together with that of published literature, shows that *L. plantarum, L. brevis* and *L. xylose* were good candidates for microbial treatment of pre-treated SB. They are all known to be probiotic strains with cellulolytic activity (Boguta *et al*., 2014).

4.4. Identification of SoSF contaminants

The selected bacterial strains were subsequently tested on steam pre-treated SB using SoSF. The roller system used for SoSF was designed using PVC pipes placed in a closed rig, with a heater place inside to allow for temperature regulation. However, because SoSF was conducted in a non-sterile environment, as would be applicable to an industrial process, this

led to contamination even after the SB was gamma radiated (Figure 4.4). Two persistent contaminants were isolated, a bacterial and a fungal species. DNA sequencing methodologies were used to identify the strains as a *Pseudomonas* strain (*Pseudomonas salomonii*) and a *Byssochlamys* strain (*Byssochlamys spectabilis*) (Figure 4.5). These strains thus became part of the mixed microbial culture that was produced during the SSF on pre-treated SB.

P. salomonii is a gram-negative, non-spore forming, motile bacterial strain that affects garlic (Gardan *et al*., 2002). It is identified by white-cream, circular, smooth colonies, 3–4 mm in diameter after 48h growth at 28°C. Growth of *P. salomonii* was observed on both D-xylose, D-glucose. *B. spectabilis* is a fungal species belonging to the *Trichocomacae* family. *B. spectabilis* is thermophilic and is commonly associated with the contamination of heat-treated foods and juices. De Morais *et al*., (2018) recently isolated two fungal strains, one of which was *B. spectabilis*, with the potential for β-glucosidase production in low-cost media. They concluded that 51 U/g dry substrate β-glucosidase was produced when *B. spectabilis* was cultivated on wheat bran and 1.7 ± 0.1 U/g dry substrate of β-glucosidase was produced when *B. spectabilis* was cultivated on sugarcane bagasse.

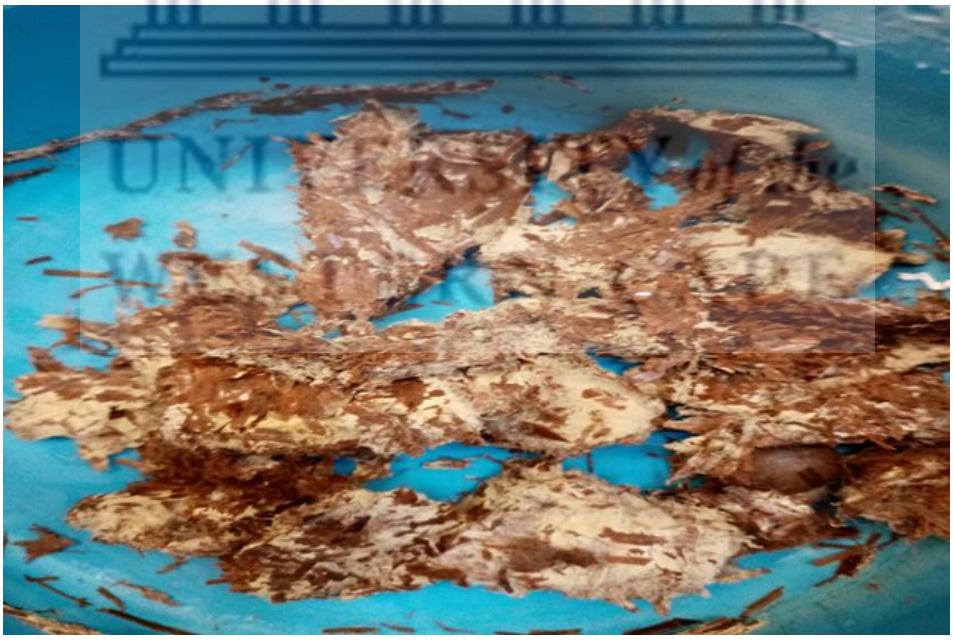

Figure 4.4: Sugarcane bagasse contaminated with *Pseudomonas* and *Byssochlamys* strains after solid-state fermentation.

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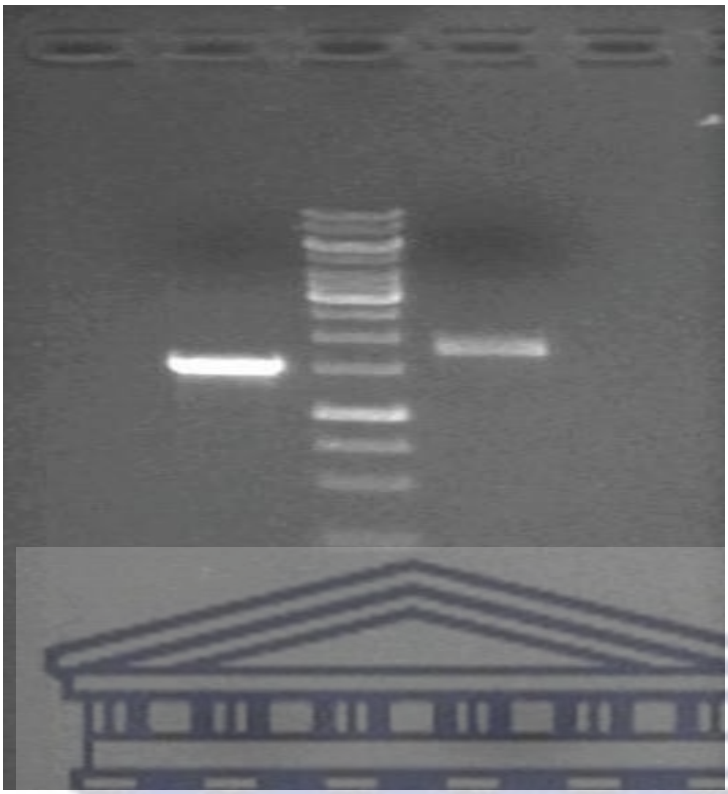

Figure 4.5: **1% agarose gel electrophoresis of 16s rRNA (1: bacterial contaminant) and 18s rRNA (2: fungal contaminant) PCR amplification using extracted genomic DNA as template. Sanger sequencing was performed on the amplicons and sequences were searched on the NCBI nucleotide database using BLAST. Alignment identified *Pseudomonas* (*Pseudomonas salomonii*) (1) and *Byssochlamys* (*Byssochlamys spectabilis*) (2) as the contaminant species. The middle lane represents the ThermoScientific 1Kb marker.**

4.5. BSF larval rearing trials

4.5.1. Biomass conversion

In this study, sugarcane bagasse was evaluated as a potential diet for protein and fat accumulation in BSF larvae. Because SB lacks nitrogen, casein hydrolysate (10 g/L) was added in as a nitrogen source. The experimental work in this study focused on (i) steam pre-treated SB treated with enzyme, (ii) steam pre-treated SB treated with microorganisms and (iii) steam pre-treated SB treated with enzymes and microorganisms. The treated sugarcane bagasse was then mixed with a standard formulated feed at a 50% inclusion rate. Biomass conversion is explained by BSFL dry mean weights (the actual dry weight of the larvae after feeding), dry bioconversion rate (DBR) (the quantity of the diet converted to pre-pupae biomass expressed as a percentage) and dry matter reduction (DMR) (the percentage of the diet consumed on a dry matter basis). Tables 4.4 to 4.6 show the amount of feed given to larvae, the amount consumed and the residues in each case. At the end of each trial, all eight tubes were pooled together.

4.5.1.1. Enzyme treatment

Trial 1 consisted of steam exploded SB treated with three different enzymes doses, 2.5 FPU, 5.0 FPU and 7.5 FPU per gram dry weight for 24 h in the roller system, to liberate monomeric sugars from the feedstock for larval consumption. This partially hydrolysed feedstock was then mixed with the standard formulated feed and the BSFL growth trial was initiated. To compare the larval weights of all the treatments, the larvae were collected and weighed after the rearing process and the mean BSFL weight were determined. As shown in Table 4.5 below for Trial 1, we observed a slight increase in the dry weight of the larvae as the enzyme dose increased. The t-test showed a significant difference between the steam pre-treated SB and the pre-treated SB also treated with different enzymes doses, however there was no significant difference between the SB treated with different enzymes doses.

Table 4.5: BSF Larval Trial 1: Steam pre-treated SB was treated with different enzyme dosages (per g DW) for 24 h, then mixed with a formulated standard feed at an inclusion ratio of 50%. Larval dry weight, dry bioconversion, and dry matter reduction were recorded. All values represent mean values of assays done in triplicate. Calculated standard errors are displayed with the data

Treatment	Feed Added (g)	Residue (g)	Feed Consumed (g)	Mean BSFL Dry Weight (g)	DBR (%)	DMR (%)
Formulated feed	162	78.7 ± 7.8	83.3 ± 1.1	0.055 ± 0.01	12.1 ± 3.2	51.4 ± 4.8
Steam pre-treated	162	99.2 ± 7.1	62.8 ± 7.1	0.033 ± 0.00	7.2 ± 1.5	34.4 ± 1.2
2.5 FPU	162	98.5 ± 6.3	63.5 ± 6.3	0.037 ± 0.00	8.7 ± 0,3	39.2 ± 3.9
5.0 FPU	162	113.0 ± 4.4	49.0 ± 4.4	0.038 ± 0.00	9.6 ± 0.7	30.3 ± 2.7
7.5 FPU	162	106.3 ± 7.7	55.7 ± 7.7	0.043 ± 0.00	8.67 ± 0.7	34.4 ± 4.7

Formulated feed: 100% formulated standard feed (no sugarcane bagasse), Steam pre-treated: No enzymes, DBR: Dry bioconversion rate, DMR: Dry matter reduction. Enzyme dosage was added based on a per dry weight basis.

The dry bioconversion rate was lower when the treated substrate was compared to the formulated feed (100% formulated standard feed - no sugarcane bagasse), thus meaning that the treated SB was, as expected, less efficient in terms of feed conversion into larvae biomass compared to the formulated feed. The addition of enzymes resulted in higher bioconversion rates. SB treated with 5.0 FPU enzyme yielded the highest bioconversion rate of 9.6%, with the lowest being the SB treated with no enzyme with a bioconversion rate of 7.21%. In the enzyme treated samples, enzymes were expected to hydrolyse complex macromolecules such as cellulose and hemicellulose into readily available carbohydrates. It was found that enzyme-

treatment at 45 °C increased the carbohydrate concentration significantly compared with the control (Figure 4.6). This resulted in a gradual increase in the dry larval weight that was observed as the proportion of glucose and xylose increased.

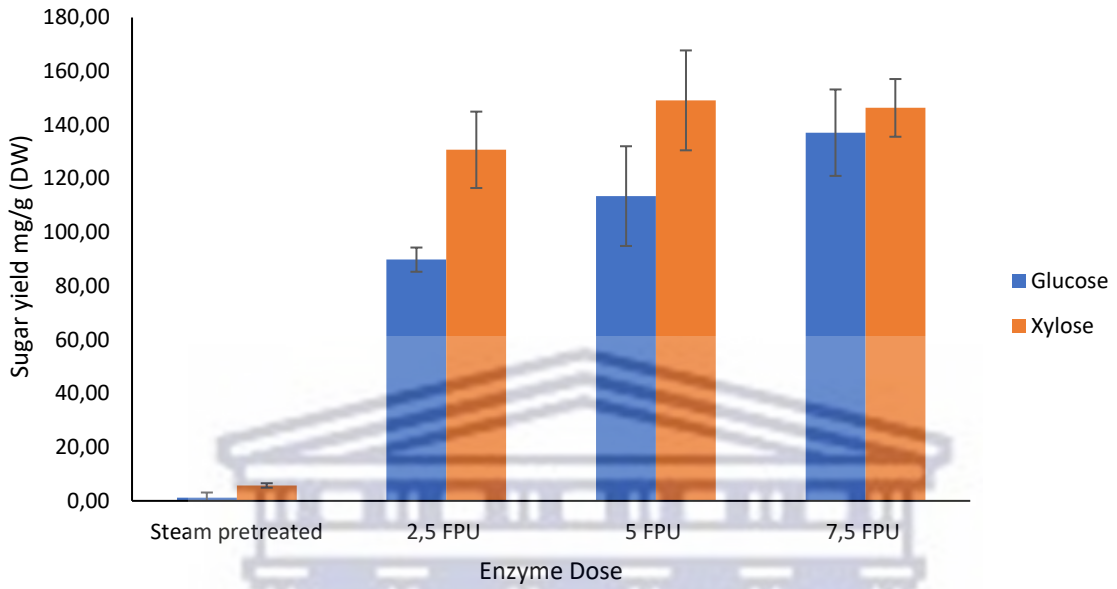

Figure 4.6: Glucose and xylose yields from steam pre-treated sugarcane bagasse after 24h enzymatic hydrolysis with Cellic CTec 3. All values represent mean values of assays done in triplicate with error bars indicating standard deviation.

The structure of the substrate is important for BSFL rearing, as it enables aeration so that the larvae can actively move through the substrate without respiration through their spiracles being hindered (Barros *et al*., 2019). Higher enzyme doses led to visibly reduced structure in the substrate in the present study, which could explain why DBR did not improve. However, it does not explain why DBR, and matter reduction were similar in the treatment with directly added enzymes compared with the steam pretreated samples. According to Ur Rehman *et al*, (2017a) although the energy content is high in manure, it consisted to a large extent of lignin, which the larvae cannot degrade. This could be a reason for the low larvae weight when reared on steam pre-treated bagasse in our study.

4.5.1.2. Microbial Treatment

Rearing of BSF larvae on SB with and without the addition of microorganisms were studied in trial 2. Steam pre-treated SB samples were inoculated with equal concentrations of *L. brevis, L. plantarum* and *L. xylosus* then placed in the roller- system for 3, 6 and 10 days cultivation. After the SoSF, samples were taken to determine if the microorganisms consumed the available cellulose and hemicellulose in the SB. Figure 4.7 shows the degradation of cellulose

and hemicellulose after fermentation. Because lignin is highly recalcitrant and the selected strains are not known to have ligninolytic activity, the process was standardised by assuming that the weight of lignin would remain the same throughout the fermentation process as previously described (Ruiz-Dueñas & Martínez, 2009). Significant amounts of cellulose and hemicellulose were consumed by the mixed culture; however, this took place within the first three days after inoculation, with no further reduction in cellulose and hemicellulose observed on days 6 or 10. It was, therefore, concluded that the microbial consortium was capable of utilising both cellulose and hemicellulose to a limited extent.

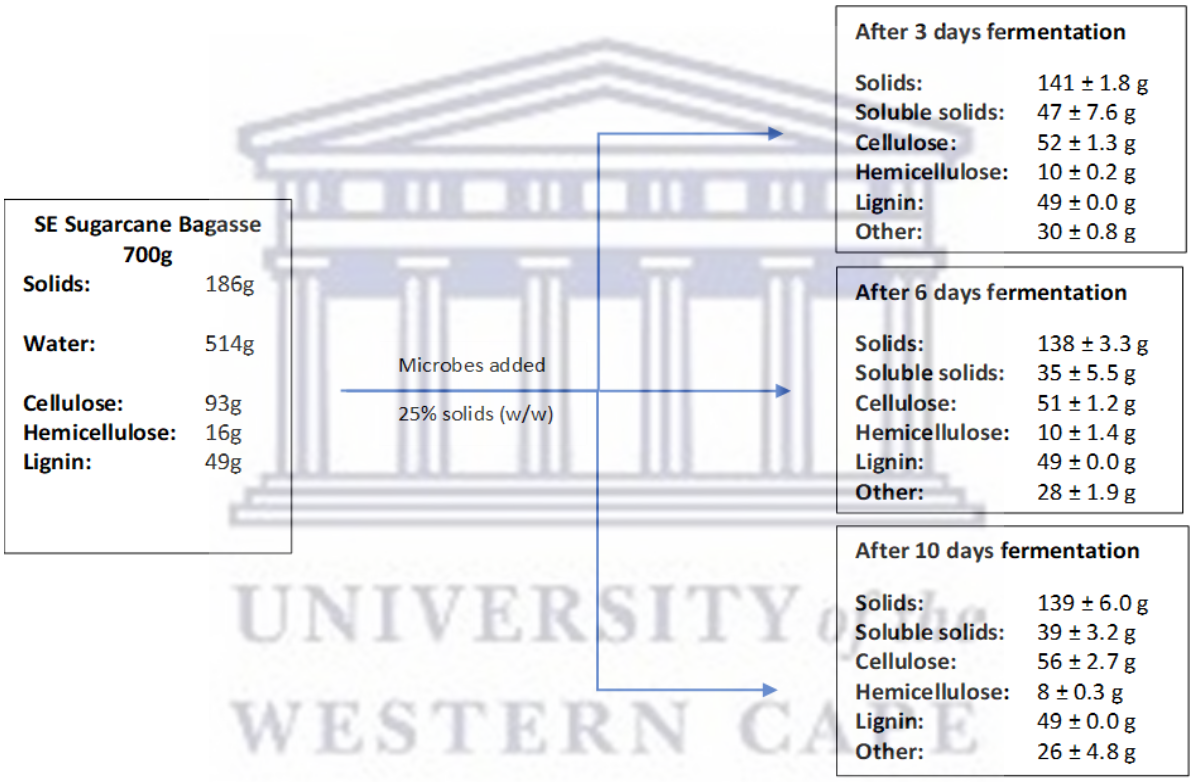

Figure 4.7: Flow diagram showing cellulose and hemicellulose degradation after fermentation. All values represent mean values of NREL assays done in triplicate. Calculated standard errors are displayed with the data.

Table 4.6: BSF Larval Trial 2: Steam pre-treated SB treated with a mixed microbial culture was mixed with a formulated standard feed at an inclusion ratio of 50%. Larval weight, dry bioconversion, and dry matter reduction were recorded. All values represent mean values of assays done in triplicate. Calculated standard errors are displayed with the data

Treatment	Feed Added (g)	Residue (g)	Feed Consumed (g)	Mean BSFL Dry Weight (g)	DBR (%)	DMR (%)
Formulated feed	162	71.6 ± 6.0	90.4 ± 6.0	0.060 ± 0.00	14.6 ± 0.9	55.8 ± 3.7
Steam pre-treated	162	92.7 ± 6.5	69.3 ± 6.5	0.035 ± 0.00	8.4 ± 0.7	42.8 ± 4.0
BT 1	162	90.2 ± 2.3	71.8 ± 2.3	0.036 ± 0.00	8.7 ± 0,1	44.3 ± 1.4
BT 2	162	89.8 ± 0.5	72.2 ± 0.5	0.035 ± 0.00	8.5 ± 0.5	44.6 ± 0.3
BT 3	162	89.8 ± 3.7	72.2 ± 3.7	0.033 ± 0.00	8.3 ± 0.34	44.6 ± 2.3

Formulated feed: 100% formulated standard feed, Steam pre-treated: No microbes added, BT1: BSFL trial after SB was treated with microbes for 3 days, BT2: BSFL trial after SB was treated with microbes for 6 days, BT3: BSFL trial after SB was treated with microbes for 10 days. DBR: Dry bioconversion rate, DMR: Dry matter reduction.

Mean BSFL dry weight results from Trial 2 are shown in Table 4.6. The use of the mixed microbial culture alone did not seem to have any impact on the growth of the larvae. The dry weight of the larvae from the treated and steam pre-treated (no microorganisms) substrates all averaged around 0.035 g. These results were not consistent from what was expected based on literature. Xiao *et al*., (2018) showed that the co-conversion by BSFL and synergistic bacteria rapidly decreased accumulated chicken manure. These differences are likely due to the notably different substrate sources and climatic conditions. *B. subtilis* inoculums accelerated BSFL growth and reduced chicken manure accumulation, while more larvae were harvested at the same conditions. The inoculated symbiotic bacteria maybe help insects to digest non-digestible nutrients or provide nutritional molecules that the Black soldier fly could not synthesize. They may also protect larvae from predators, and pathogens as demonstrated by the fact that inoculating poultry manure with bacteria isolated from Black soldier fly larvae influenced the growth and development of conspecific larvae feeding on the manure (Yu *et al*., 2011). However, in the present study the addition of microorganisms did not have any effect on the biomass conversion of the BSF larvae. Somroo *et al*. (2019) reported that BSFL cultivated with *L. buchneri* on soybean curd residue showed a significantly higher survival rate, and a significantly lower development time compared to BSFL fed on soybean curd residue without the addition of *L. bucherni* and the artificial feed. They also showed that BSFL with *L. bucherni* fed on soybean curd residue had significantly higher wet and dry larval

biomass weights. This shows that the addition of microorganisms could provide economic benefits to produce food and feeds with BSFL, such as increasing the feed conversion rate, shortening the development time, and increasing the survival rate.

Dry weight and bioconversion of the larvae in larval Trial 2 was the same as for the steam pre-treated substrate (Table 4.6). Possible reasons for our observations could be that the selected strains did not possess sufficient cellulolytic activity needed to break down enough of the cellulose and hemicellulose in the conditions provided. Lignocellulose is recalcitrant to biodegradation because lignin provides protection against microbial attack and oxidation. The lignocellulose was therefore largely intact after fermentation, and the nutrients were therefore unavailable for the microorganisms as well as for the larvae to grow. Furthermore, the moisture content was significantly lower than what would be expected for manure feedstocks and pre-treatment derived inhibitors may also have impeded the conversion process. The bioconversion rate for the formulated feed was 14.6%. The bioconversion rate was similar for all other treatments in this trial, including the steam pre-treated sample averaging just over 8%. There was no significant difference observed between any of the treatments for the parameters tested. The bioconversion rate results from larval Trial 1 (SB treated with different enzyme dosages for 24 h) was not significantly better than the results from Trial 2 (SB treated with a mixed microbial culture). Trial 2 showed the overall highest DMR, averaging around 44% (Table 4.6). There was no significant difference in the results when the trials were compared to each other.

4.5.1.3. Enzyme & Microbial Co-Treatment

Trial 3 comprised a simultaneous saccharification and fermentation process. SSF is a process that combines enzymatic hydrolysis with fermentation to obtain value-added products in a single step (Li *et al*., 2015). SSF was set up by adding steam pre-treated SB with 7.5 FPU/g per DW Cellic CTec 3 and the mixed culture described above into the reactors and incubating for 3 (SSF1), 6 (SSF2) and 10 (SSF3) days. The cellulose of the steam pre-treated SB was converted into glucose by enzymes while simultaneously the available glucose was available to be converted into microbial biomass (Wahono *et al*., 2015). The mean BSFL dry weight results from Trial 3 are shown in Table 4.7. Trial 3 yielded the best overall results, recording almost twice the amount of dry weight compared to the enzyme treatment and microbial treatment (Trials 1 & 2) results. There was a significant difference ($p < 0.05$) between the steam pre-

treated and the SSF treated SB. Again, most of the changes took place within three days after inoculation with little to no change up to days 6 and 10. In Table 4.7 below, the bioconversion rate for the formulated feed was 14.5% and that of the steam pre-treated control was 9.8%. The SSF treated substrate displayed improved bioconversion rates over the steam pre-treated control, with SSF 2 showing the highest bioconversion rate of 13.4%. The higher bioconversion rate reflects the effectiveness of the larvae when converting SB to larvae biomass. Dry matter reduction was also higher in the treated substrate (40% - 44.3%) when compared to the steam pre-treated control, with SSF2 showing the highest percentage at 44.3% (Table 4.7). These results are in line with previous reports, as dry mass reduction from soybean curd residue and artificial feed was 44–56% (Somroo *et al*., 2019), for chicken feed it was 33–40% (Diener *et al*., 2009) and 39% for pig manure (Newton *et al*., 2005).

Table 4.7: BSF Larval Trial 3: Steam pre-treated SB treated with a mixed microbial culture and 7.5 FPU/g per DW Cellic CTec3 enzyme, then mixed with a formulated standard feed at an inclusion ratio of 50%. Larval weight, dry bioconversion, and dry matter reduction were recorded. All values represent mean values of assays done in triplicate. Calculated standard errors are displayed with the data

Treatment	Feed Added (g)	Residue (g)	Feed Consumed (g)	Mean BSFL Dry Weight (g)	DBR (%)	DMR (%)
Formulated feed	162	73.2 ± 2.1	88.8 ± 2.1	0.061 ± 0.005	14.5 ± 1.9	54.8 ± 1.3
Steam pre-treated	162	110.6 ± 0.7	51.4 ± 0.7	0.039 ± 0.001	9.8 ± 0.2	31.9 ± 0.4
SSF 1	162	93.0 ± 7.7	69.0 ± 7.7	0.049 ± 0.004	12.0 ± 0.6	42.6 ± 4.7
SSF 2	162	90.2 ± 3.5	71.8 ± 3.5	0.055 ± 0.003	13.4 ± 0.7	44.3 ± 2.2
SSF 3	162	97.2 ± 2.8	64.8 ± 2.8	0.051 ± 0.004	12.6 ± 0.9	40.0 ± 1.7

Formulated feed: 100% formulated standard feed, Steam pre-treated: no microorganisms or enzymes added, SSF1: BSFL trial after SB was treated with microorganisms and enzymes for 3 days, SSF2: BSFL trial after SB was treated with microorganisms and enzymes for 6 days, SSF3: BSFL trial after SB was treated with microorganisms and enzymes for 10 days, DBR: Dry bioconversion rate, DMR: Dry matter reduction.

4.6. Black Soldier Fly Larvae Protein and Fat Content

The nutritional value of Black soldier fly larvae is strongly influenced by its feed. If the feed is high in protein, the larvae will contain high protein levels and the same goes for fat. (Aristi *et al*., 2020). Despite the protein content of the larvae not varying by much, the size of the larvae varied considerably. Therefore, for the purpose of comparisons, calculations in Table 4.8 were based on the protein and fat content presented in Appendix A-4. The BSFL trials showed a consistency with the literature values of about 40% larval protein (Table 4.8). Based on Trial

1 (Enzyme treatment) a similar pattern was observed to that of the larvae dry weight, where the protein values increased with increasing amounts of carbohydrates in the larval diets. No significant difference ($p>0.05$) was observed for the protein levels between the increasing enzyme dosages.

Table 4.8: The average amount of protein and fat produced per dry BSFL weight per Kg of feed of each treatment. All values represent mean values of assays done in triplicate. Calculated standard errors are displayed with the data

Treatment	Feed Added (Kg)	BSFL Dry Weight (g/Kg)	Crude Protein (g/Kg)	Crude Fat (g/Kg)
Trial 1				
Formulated feed	0.162	135.2 ± 4.0	59.9 ± 2.1	27.2 ± 1.1
Steam pre-treated	0.162	80.9 ± 0.5	37.7 ± 0.3	7.4 ± 0.3
2.5 FPU	0.162	90.7 ± 0.3	36.4± 0.1	14.2 ± 0.2
5.0 FPU	0.162	95.1 ± 0.4	39.5± 0.3	20.37 ± 0.3
7.5 FPU	0.162	105.6 ± 1.2	44.4 ± 1.2	24.1 ± 0.1
Trial 2				
Formulated feed	0.162	147.5 ± 1.8	66.0 ± 0.6	39.5 ± 1.1
Steam pre-treated	0.162	85.8 ± 1.1	40.7 ± 1.1	19.1 ± 0.2
BT 1	0.162	89.5 ± 0.4	42.6± 0.1	25.9 ± 0.1
BT 2	0.162	85.2 ± 0.8	42.6 ± 0.3	17.2 ± 0.4
BT 3	0.162	81.5 ± 0.7	37.0 ± 0.6	16.6 ± 0.3
Trial 3				
Formulated feed	0.162	151.2 ± 1.9	48.4 ± 1.1	45.7 ± 1.1
Steam pre-treated	0.162	97.5 ± 0.4	38.8 ± 0.2	28.4 ± 0.1
SSF 1	0.162	121 ± 1.4	51.2 ± 0.3	36.4 ± 1.0
SSF 2	0.162	135.2 ± 1.1	46.3 ± 0.8	47.5 ± 0.7
SSF 3	0.162	126.5 ± 1.5	38.8 ± 0.5	37.7 ± 0.6

Formulated feed: 100% formulated standard feed, Steam pre-treated: No microorganisms or enzymes added, BT1: BSFL trial after SB was treated with microorganisms for 3 days, BT2: BSFL trial after SB was treated with microorganisms for 6 days, BT3: BSFL trial after SB was treated with microorganisms for 10 days, SSF1: BSFL trial after SB was treated with microorganisms and enzymes for 3 days, SSF2: BSFL trial after SB was treated with microorganisms and enzymes for 6 days, SSF3: BSFL trial after SB was treated with microorganisms and enzymes for 10 days. Average BSFL dry weight is based on 400 BSF larvae.

For Trial 2, The amount of protein produced from the different treatments was all similar at about 37 – 42.6 g protein per Kg feed (g/Kg) and no significant difference was observed between the treatments. This was shown to have a similar trend to the dry weight of the larvae. Trial 3 showed the best overall protein production results with SSF1 yielding 51.2 g/Kg,

significantly higher than that of the formulated feed as seen in Table 4.8. BSFL were found to have high protein content when reared on the treated sugarcane bagasse, confirming that BSFL can be used for upcycling protein from lignocellulosic biomass into BSFL biomass suitable to be used as feed for human consumption. The larval protein content in this study is in line with what observed by (Lalander *et al*., 2019) whose larval protein ranged from 39.1 % and 44.2 % for larvae reared in different substrates.

While the fat content for SB was not determined in this study, according to literature SB consist of <3 % fat (Suryaningrum *et al*., 2021). Feed type is likely to have an impact on the fat content and composition for the BSFL since they need to accumulate energy in the form of fat to prepare for their adult phase (Lalander *et al*., 2019). BSFL fat is a promising source of food, feed, and biodiesel feedstock. BSFL fat is composed of saturated FAs, such as palmitic acid (C16:0), stearic acid (C18:0), and lauric acid (C12:0) and has desirable physical and chemical properties which facilitate further conversion into biodiesel. In Trial 1, the observed crude fat values of BSF larvae increased with increasing amounts of carbohydrates (Appendix A-1) ranging from 14.2 to 24.1 g/Kg. A t-test revealed that there was a significant difference between the steam pre-treated substrate and the 5 FPU and 7.5 FPU treatments, however no significant difference was found between 5 FPU and 7.5 FPU treatments. Aita & Moon, (2020) observed the same trend when they increased the amount of carbohydrate added to the BSF larvae diets. Moreover, Li *et al*. (2015) found increasing amounts of lipids in BSFL upon adding increasing levels of glucose and xylose to their diet. In Trial 2, the crude fat content varied strongly with BT1 having the highest level of crude fat at 25.9 g/Kg. BT2 and BT3 had the lowest crude fat at 17.2 and 16.6 g/Kg respectively. The results of this study showed that 6 and 10 days of cultivating mixed microorganisms on steam pre-treated SB had no significant effect on the crude fat content. Somroo *et al*. (2019) showed that the addition of *L. buchneri* to soybean curd residue increased fat content by 3.9%. It should be noted that *L. buchneri* was added at the start of the rearing trial to the soybean curd residue, unlike in this case where the microorganisms were cultivated for 3, 6 and 10 days before the rearing trial. BT1 was significantly different when compared to the other treatments. Trial 3 showed the highest fat content overall, ranging from 36.4 to 47.5 g/Kg. A t-test revealed that there was a significant difference between the steam pre-treated substrate when compared to SSF2 and SSF3 treatments, however no significant difference was found between SSF2 and SSF3. This

shows a similar pattern to the dry weight production of the larvae. These results indicated that the diet had a larger influence on the crude fat content of the BSL larvae since more nutrients in the substrate were available to utilize and convert to larval biomass with the aid of enzymes degrading the cellulose and hemicellulose, thus more sugars were available for microbial consumption resulting in greater fat yields.

4.7. Fibre Content

For comparison purposes, the cellulose and hemicellulose content were calculated based on the proximate composition results obtained from the neutral detergent fibre, acid detergent fibre and acid detergent lignin (Appendix A1-3) and expressed as (g/Kg). Figure 4.8 shows the cellulose and hemicellulose hydrolysis of each treated substrate before and after BSF larval trials, with the highest cellulose and hemicellulose reduction values observed in the SSF treatment. With the aid of cellulase and the activity of microorganisms, about 44% to 49.7% of cellulose and 51.9% to 56.4% of hemicellulose could be consumed when the two agents worked together. The data suggests that the sugar and microbial products were then absorbed by BSFL for development. The lowest cellulose conversion values observed was in the microbial treated feed ranging from 15-26% (C) and the lowest hemicellulose conversion values was observed in the enzyme treated feed ranging from 35.7-40.3% (B). The hemicellulose reduction was similar between the bacterial treated and SSF feeds. All treatments including the controls showed significant reductions in both cellulose and hemicellulose after larval rearing trials. These cellulose and hemicellulose reduction percentage values were comparable those observed by Ur Rehman *et al*. (2017a). They showed that the highest cellulose reduction was in chicken manure at 67.7%, while the lowest were found in dairy manure at 49.9%. They also observed that the hemicellulose content of the dairy manure was reduced by 49.8%. From figure 4.8 it was clear that, in the absence of enzymes and microorganisms, about 21% to 32.1% cellulose and 33.9% to 46% hemicellulose was removed.

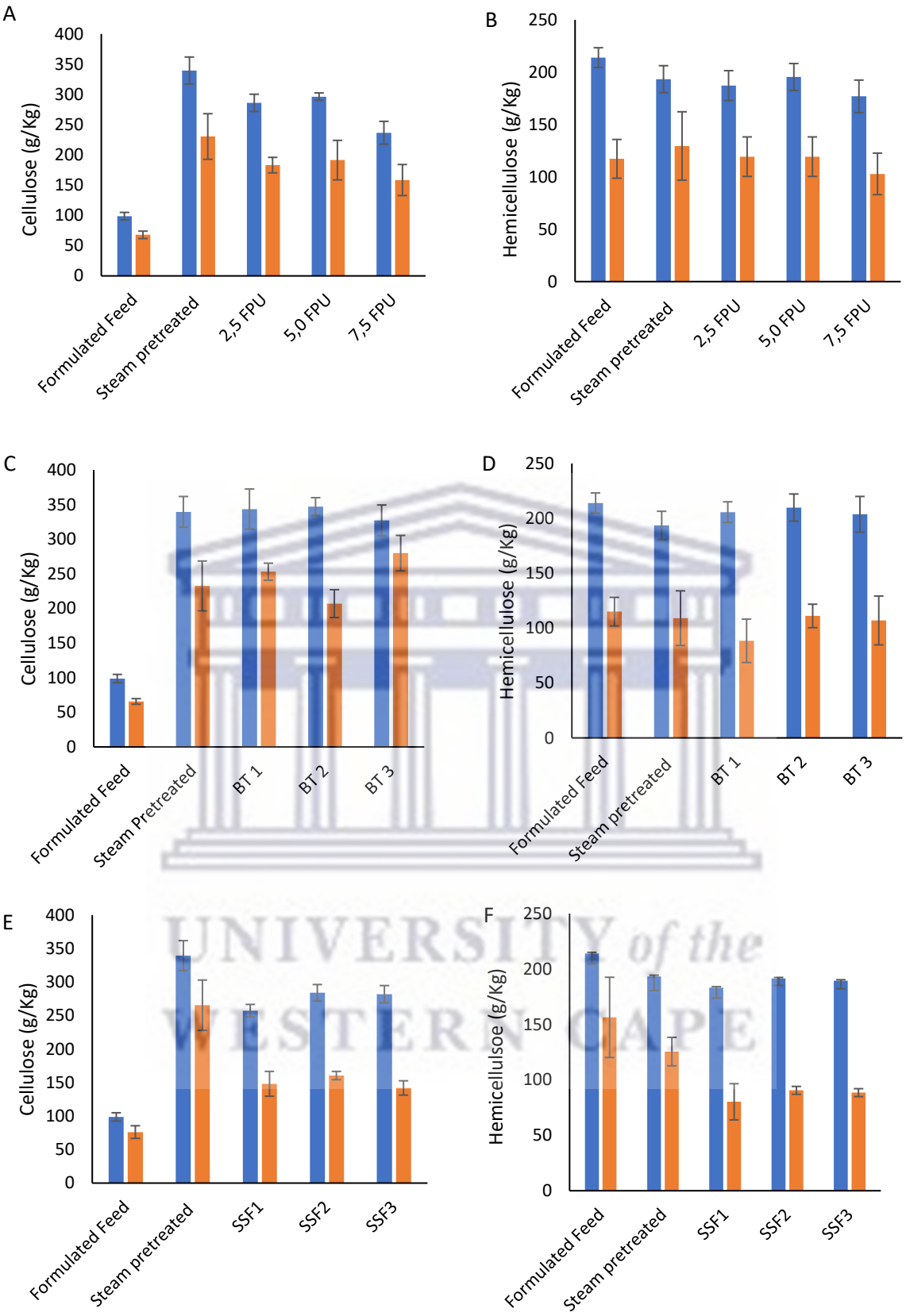

Figure 4.8: Total cellulose and hemicellulose content of feed (blue bars) and residues (orange bars) prior to and after larval rearing trial. Total cellulose content (g/Kg) before and after BSF larval rearing for Trial 1 (A) Trial 2 (C) and Trial 3 (E) on a dry matter basis. Total hemicellulose content (g/Kg) before and after BSF larval rearing for Trial 1 (B) Trial 2 (D) and Trial 3 (F) on a dry matter basis. Values represent averages of assays performed in triplicate and error bars show standard deviation.

These results were also comparable to with Zheng et al., (2012) who showed that in the absence of Rid-X (containing natural active bacteria that facilitated breakdown of solid organic wastes, and cellulase, lipase, protease, and amylase) about 27.9% cellulose and 32.6% hemicellulose were degraded by BSF larvae. They also reported that higher reduction percentages were achieved with the addition Rid-X. It is difficult to conclude whether this reduction was the result of the BSFL, or microorganisms present in the feed due to the trials being performed under non-sterile conditions. However, studies by Watanabe & Tokuda, (2010) and Li *et al*. (2011) have confirmed that larvae and their microbial symbionts were capable of digesting lignocellulosic components in their gut. The ash content before and after larval rearing trials ranged between 2.1% and 3.9% (Appendix A1-A3). This was in line with the results obtained from the chemical composition. The protein in the feed was similar amongst the different treatments. The protein content in SB was usually about 1-3% (Suryaningrum *et al*., 2021), however the protein content in the feed was found to be about 9.1-12.3%. The higher protein content could be because the steam pre-treated and treated SB substrate was mixed with the formulated standard feed at an inclusion ratio of 50%. In summary, the results demonstrate that BSFL has the potential to degrade cellulose and hemicellulose. The addition of cellulolytic enzyme and cellulolytic probiotic bacteria also improved the performance of the BSFL.

Chapter 5: CONCLUSION & RECOMMENDATIONS

BSF larvae have a wide range of potential applications ranging from waste management to food security benefits, making them one of the most important insects in the future bioeconomy. To benefit from these larvae, successful and sustainable development on a large industrial scale is required. This is essential considering the rising need for food security. Sugarcane bagasse contains large amounts of unutilised cellulose and hemicellulose. The recovery of glucose and xylose by hydrolysis of these polymers represent an attractive way to provide low-cost sugar for BSF larval rearing, to produce protein and fat that could supplement human diets.

Our fist aim was to evaluate biomass conversion by the larvae after rearing them on steam pre-treated SB, treated with different enzyme dosages for 24 h. It was established that as the total sugar in the feed increased, there was a concomitant increase in the mean BSFL weight. While a significant difference was observed between the steam pre-treated and the treated SB, no significant difference was observed between the three enzyme treatments. This suggested that any one of the three enzyme dosages chosen could sufficiently hydrolyse cellulose and hemicellulose. Because the use of enzymes on an industrial scale would be expensive, using a smaller enzyme dosage cold contribute to making the process more cost effective.

Our second aim was to evaluate what effect a mixed culture of microorganisms, cultivated on steam pre-treated SB for a period of 3, 6 and 10 days would have on BSFL rearing. It was clear that the mixed culture was capable of cellulose and hemicellulose degradation, however, there was no difference in the mean BSFL weights after rearing them on this substrate. Thus, the addition of the microorganisms alone did not have an impact on the biomass conversion. When the mixed culture was added to the SB with the addition of enzymes, it was observed that higher BSFL mean weights, ranging between 0.039-0.055 g were obtained compared to only microbial and enzyme treatment. A significant difference was also observed for the mean larvae weight between the steam pre-treated and the treated SB.

Our third aim, focused on the analysing the protein and fat content of the larvae produced in the study. The protein and fat contents were consistent with that reported in literature. Protein content for each trial were in line with that of the mean BSFL weight, with trial 3 (SB

treated with microorganisms and enzymes) displaying the highest protein and fat content overall.

The objective of aim four was to determine whether are not the BSFL were inherently capable of cellulose and hemicellulose degradation. This study showed that significant differences were observed for both cellulose and hemicellulose content in the feed before and after BSF larval rearing for all treatments as well as for the control. This suggested that either the larvae themselves, or organisms in their microbiome, had the necessary enzymes for cellulose and hemicellulose degradation.

Finally, taking our findings into consideration with the facts that (i) SB is a clean and abundant feedstock, (ii) BSFL can convert low quality substrates into high quality feed sources cost effectively and (iii) protein derived from BSF larvae is comparable to commercially accepted protein resources such as soyabean meal. It may be concluded that BSF larvae grown on enzyme and microbially treated SB is a viable source of protein for human consumption. However, substantial additional research is needed to determine the economic viability of rearing BSF larvae on a plant-based substrates and the safety of the product.

Recommendations for future studies:

1. Further studies into screening of non-pathogenic microorganisms with a tolerance for inhibitors formed during steam pre-treatment, that also have high cellulase and hemicellulase activities, thus being able to grow on the substrate without the need for exogenous enzymes.
2. Intensive research to determine if BSFL are capable of digesting lignocellulosic components in their gut via native enzymes, or if they rely on their microbiome. In the latter case, natural BSFL symbionts may be excellent prebiotics to aid the conversion process.
3. A study to determine the biomass conversion and nutritional value of BSFL reared on different lignocellulosic biomass sources to facilitate in extracting the maximum amount of protein and fat from BSFL.
4. Research on how to safely use BSFL for human consumption.
5. A techno-economic study to determine the cost of BSFL protein and fat production compared to alternative products with similar protein and fat content.

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Appendices

Appendix A-1: A comparison of the average (with standard errors) proximate composition of Trial 1 feed before and after BSF Larval rearing on a dry matter basis (%DM).

Composition (%)	Formulated Feed		Steam pre-treated		2.5 FPU		5.0 FPU		7.5 FPU	
	Feed	Residue	Feed	Residue	Feed	Residue	Feed	Residue	Feed	Residue
Dry Matter (g)	162.0±0	78.7±7.8	162.0±0	88.8±1.6	162.0±0	101.7±8.4	162.0±0	113.6±4.9	162.0±0	107.7±8.2
Ash	5.7±0.2	5.0±2.1	2.6±0.2	3.1±0.3	2.8±0.4	3.0±0.4	2.5±0.3	2.5±0.1	2.7±0.2	2.6±0.1
NDF	35.0±1.1	62.7±3.6	64.5±2.1	79.8±2.8	56.7±1.4	73.2±1.1	57.7±1.8	77.1±1.6	53.6±1.2	72.4±3.6
ADF	13.4±0.7	29.0±1.5	45.1±1.4	56.3±0.5	37.9±2.1	49.3±2.1	38.0±1.1	53.0±0.9	36.1±2.8	48.7±2.5
ADL	3.5±0.3	9.2±1.7	11.1±1.9	13.9±2.1	9.2±0.9	13.3±1.8	8.5±0.5	14.6±1.1	12.5±2.0	12.3±1.6
Hemicellulose	21.6±1.0	33.7±2.1	19.5±1.2	23.5±2.8	18.8±1.6	23.8±3.1	19.8±1.3	24.1±2.0	17.5±1.6	23.7±1.9
Cellulose	9.9±0.5	19.8±1	33.9±2.3	42.4±1.6	28.7±1.3	36.1±3.7	29.5±0.7	38.4±0.3	23.6±1.9	36.4±1.1
Protein	15.5±0	13.3±1.5	9.1±0.4	6.5±0.7	10.4±0.4	8.1±0.4	10.5±0.4	7.1±0.3	10.8±0.3	8.7±0.5

Appendix A- 2: A comparison of the average (with standard errors) proximate composition of Trial 2 feed before and after BSF Larval rearing on a dry matter basis (%DM).

Composition (%)	Formulated Feed		Steam pre-treated		BT1		BT2		BT3	
	Feed	Residue	Feed	Residue	Feed	Residue	Feed	Residue	Feed	Residue
Dry Matter (g)	162.0±0	72.1±5.5	162.0±0	93.1±6.3	162.0±0	90.8±2.4	162.0±0	90.1±0.5	162.0±0	89.8±3.7
Ash	5.7±0.2	5.4±0.4	2.6±0.2	2.7±0.2	2.7±0.3	3.8±0.1	3.0±0.2	3.9±0.4	3.3±0.4	3.7±0.3
NDF	35.0±1.1	65.6±0.8	64.5±2.1	76.5±1.4	62.8±2.8	69.8±5.2	64.5±1.3	74.2±0.3	65.0±1.4	75.0±1.0
ADF	13.4±0.7	30.5±0.6	45.1±1.4	56.2±1.7	42.1±2.8	54.5±3.8	43.4±1.1	55.5±1.5	44.6±1.3	58.5±1.3
ADL	3.5±0.3	10.5±0.9	11.1±1.2	12.9±0.8	7.9±0.3	11.3±1.4	8.8±0.3	12.4±1.1	11.9±1.5	15.2±0.6
Hemicellulose	21.6±0.5	35.1±0.7	19.5±1.2	20.3±1.5	20.6±1.2	15.2±3.3	21±1.4	18.7±1.2	20.4±1.6	16.5±1.1
Cellulose	9.9±0.5	20±1.4	33.9±2.3	43.3±1.6	34.2±3.1	43.2±2.4	34.7±1.3	43.1±2.4	32.8±2.5	43.3±1.7
Protein	15.5±0	13±1.6	9.1±0.4	7.0±1.3	10.1±0.7	7.2±0.9	10.1±0.5	7.1±0.2	10.3±0.6	7.0±0.2

Appendix A-3: A comparison of average (with standard errors) proximate composition of Trial 3 feed before and after BSF Larval rearing on a dry matter basis (%DM).

Composition (%)	Formulated Feed		Steam pre-treated		SSF1		SSF2		SSF3	
	Feed	Residue	Feed	Residue	Feed	Residue	Feed	Residue	Feed	Residue
Dry Matter (g)	162.0±0	73.8±2.1	162.0±0	111.0±0,9	162.0±0	93.7±7.7	162.0±0	91.7±5.3	162.0±0	99.3±2.9
Ash	5.7±0.2	5.5±0.4	2.6±0.2	3.2±0.4	2.1±0.1	3.3±0.4	2.4±0.1	3.5±0.3	2.1±0.1	2.7±0.1
NDF	35.0±1.1	66.4±1.2	64.5±2.1	74.3±4.2	54.1±0.8	65.0±4.5	57.0±1.1	73.5±2.3	55.9±0.7	72.3±2.5
ADF	13.4±0.7	30.4±1.7	45.0±1.4	55.0±4.0	35.7±0.8	46.0±2.2	38.1±0.6	51.2±1.6	36.9±0.9	50.0±2.2
ADL	3.5±0.3	10.0±0.7	11.1±1.2	14.0±0.2	9.9±1.6	11.0±0.5	9.5±0.6	12.2±0.6	8.6±0.3	14.2±1.8
Hemicellulose	21.6±0.5	36.0±1.9	19.5±1.2	19.3±0.4	18.3±1.0	19.0±2.3	22.2±1.0	19.0±0.5	19.0±0.4	22.2±0.4
Cellulose	9.9±0.5	20.4±1.1	33.9±2.2	41.0±3.9	25.8±0.8	35.0±1.7	28.6±1.2	39.0±1.9	28.3±1.2	35.8±0.8
Protein	15.5±0	11.8±0.5	9.1±0.4	5.4±0.3	12.3±0.4	8.3±0.6	11.7±0.5	7.3±1.0	11.9±0	7.2±0.6

Appendix A-4: Average protein and fat content for each treatment. All values represent mean values of assays done in triplicate with error bars indicating standard deviation.

Treatment	Protein (%)	Fat (%)
Trial 1		
Formulated feed	44.3 ± 2.0	24.5 ± 1.7
Steam pre-treated	46.2 ± 0.1	12.6 ± 2.8
2.5 FPU	40.4 ± 0.5	16.2 ± 1.2
5.0 FPU	41.3 ± 1.1	21.4 ± 3.1
7.5 FPU	42.2 ± 1.5	22.8 ± 1.1
Trial 2		
Formulated feed	44.7 ± 0.9	27.0 ± 3.8
Steam pre-treated	47.3 ± 1.2	22.7 ± 1.0
BT 1	47.8 ± 0.7	28.8 ± 0.6
BT 2	50.0 ± 1.0	20.3 ± 1.4
BT 3	45.5 ± 2.8	20.3 ± 2.0
Trial 3		
Formulated feed	32.8 ± 6.5	30.2 ± 2.1
Steam pre-treated	39.8 ± 1.8	29.1 ± 0.9
SSF 1	42.5 ± 2.2	30.0 ± 3.0
SSF 2	34.4 ± 2.5	35.1 ± 4.9
SSF 3	30.7 ± 2.0	29.8 ± 0.7

Formulated feed: 100% formulated standard feed, Steam pre-treated: No microorganisms &/or enzymes added, BT1: BSFL trial after SB was treated with microorganisms for 3 days, BT2: BSFL trial after SB was treated with microorganisms for 6 days, BT3: BSFL trial after SB was treated with microorganisms for 10 days, SSF1: BSFL trial after SB was treated with microorganisms and enzymes for 3 days, SSF2: BSFL trial after SB was treated with microorganisms and enzymes for 6 days, SSF3: BSFL trial after SB was treated with microorganisms and enzymes for 10 days.