Bioconversion of steam-pretreated sugarcane bagasse to single-cell protein

By

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ABSTRACT

Identifying affordable, environmentally friendly and sustainably produced proteins with high nutritional value is crucial to meet the increasing strain placed on global food production, driven by rapid population growth. Consequently, there is much opportunity in the bioconversion of low-value plant materials, such as sugarcane bagasse, into single-cell proteins (SCPs).

This project investigated the bioconversion of steam-exploded sugarcane bagasse to produce SCPs using generally regarded as safe (GRAS) microbial strains. The sugarcane bagasse was steam-exploded at 185 °C for 10 minutes to produce a highly digestible product with low concentrations of inhibitors, such as furfural (0.25 ± 0.01 g/L), 5-hydroxymethylfurfural (5-HMF; 0.004 ± 0.002 g/L) and acetic acid (3.79 ± 0.22 g/L). The resulting steam-exploded sugarcane bagasse underwent separate hydrolysis and fermentation (SHF) for SCP production. Enzymatic hydrolysis was performed at a solid loading of 15% (w/v) pressed steam-exploded sugarcane bagasse and a Cellic® CTec3 enzyme dosage of 7.5 FPU/g dry weight (DW) substrate for 72 h, which resulted in a final glucose concentration of 42.26 ± 0.80 g/L, representing a 77.6% ± 0.4% conversion of cellulose to glucose. The resulting enzymatic hydrolysis product contained negligible concentrations of furfural and 5-HMF, together with 4.44 ± 0.56 g/L of acetic acid.

Six GRAS microbial strains, *Bacillus subtilis CAB1111*, *Lactobacillus delbrueckii*, *Streptococcus thermophilus* (bacterial strains), *Fusarium venenatum*, *Pleaurotus ostreatus* CAB13 and *Saccharomyces cerevisiae* CAB79 (fungal strains), were screened as SCP candidates in undiluted enzymatic hydrolysate to determine which microbes were able to tolerate the inhibitor concentrations, such as acetic acid. *S. cerevisiae*, used as a control, tolerated the inhibitors best as it consumed all of the glucose and achieved a final biomass concentration of 20.37 ± 11.81 g/L after 48 h. Of the five alternative strains, *S. thermophilus* tolerated the inhibitors present best, as it consumed 47.48% ± 3.75% of the glucose and achieved a final biomass concentration of 1.28 ± 0.17 g/L after 48 h. Therefore, *S. thermophilus* was selected for comparison to *S. cerevisiae* in pulse fed-batch fermentations to maximise biomass formation by exposing the strain at lower initial inhibitor concentrations.

Pulse fed-batch fermentations, with an initial and final hydrolysate concentration of 20% (v/v) and 80% (v/v), respectively, substantially increased the final biomass concentrations of both *S. cerevisiae* and *S. thermophilus*, as compared to batch fermentation in undiluted hydrolysate. *S. cerevisiae* achieved a higher final biomass concentration (52.65 ± 0.80 g/L) than *S. thermophilus* (6.57 ± 0.09 g/L).

The protein contents of *S. cerevisiae* and *S. thermophilus* were 46.9% ± 2.6% (DW) and 66.1% ± 1.3% (DW), respectively. *S. cerevisiae* produced 24.71 ± 1.44 g/L of protein, while *S. thermophilus* produced 4.34 ± 0.10 g/L of protein. Therefore, *S. cerevisiae* is a promising strain to convert steam-exploded sugarcane bagasse to SCPs as it resulted in higher protein and biomass concentrations than *S. thermophilus*. Additionally, the maximum ethanol concentration produced by *S. cerevisiae* during pulse fed-batch fermentation was 8.13 ± 0.27 g/L after 36 h; therefore, the co-production thereof with SCP may further enhance the economic attractiveness of this process. Although, it is likely to be at the expense of the latter.

The technical and economic feasibility of producing SCPs from steam-exploded sugarcane bagasse on an industrial scale using *S. cerevisiae* and *S. thermophilus* were determined and compared. The minimum selling price (MSP) was used as the determining factor as it takes into consideration the biomass yield, the total capital investment (CAPEX) and the operating costs (OPEX) and was used to obtain the pre-specified internal rate of return (IRR) of 20%. *S. cerevisiae* achieved the lowest MSP of 2 319 US$/tonne biomass (ZAR 41/kg), which was about ten times less than the MSP achieved by the production of *S. thermophilus* (20 436 US$/tonne biomass; ZAR 353/kg). Therefore, on an industrial scale, *S. cerevisiae* is the most promising strain for the bioconversion of steam-exploded sugarcane bagasse to SCPs.

OPSOMMING

Om bekostigbare, omgewingsvriendelike en volhoubaar geproduseerde proteïene met hoë voedingswaarde te identifiseer, is krities om aan die verhoogde druk wat op globale kosproduksie, gedryf deur vinnige populasiegroei, te voldoen. Vervolgens, is daar baie geleentheid in die bio-omsetting van lae-waarde plantmateriale, soos suikerrietbagasse, na enkelselproteïene (SCPs).

Hierdie projek het die bio-omsetting van stoomontplofde suikerrietbagasse om SCPs te produseer ondersoek deur algemeen beskou as veilige (GRAS) mikrobiese lyne. Die suikerrietbagasse is gestoomontplof by 185 °C vir 10 minute om ’n hoogs verteerbare produk met lae konsentrasies van inhibeerders te produseer, soos furforaal (0.25 ± 0.01 g/L), 5-hidroksielmetielfurfuraal (5-HMF; 0.004 ± 0.002 g/L) en asynsuur (3.79 ± 0.22 g/L). Die resulterende stoomontplofde suikerrietbagasse het aparte hidrolise en fermentasie (SHF) vir SCP-produksie ondergaan. Ensimatiese hidrolise is uitgevoer by ’n soliede lading van 15% (w/v) gedrukte stoomontplofde suikerrietbagasse en ’n Cellic® CTec3-ensiemdosering van 7.5 FPU/g droë gewig (DW) substraat vir 72 h, wat ’n finale glukosekonsentrasie van 42.26 ± 0.80 g/L tot gevolg gehad het, wat ’n 77.6% ± 0.4% omsetting van sellulose na glukose verteenwoordig. Die resulterende ensimatiese hidrouliese produk het weglaatbare konsentrasie furfuraal en 5-HMF bevat, saam met 4.44 ± 0.56 g/L asynsuur.

Ses GRAS-mikrobiese lyne, *Bacillus subtilis CAB1111*, *Lactobacillus delbrueckii*, *Streptococcus thermophilus* (bakteriese lyne), *Fusarium venenatum*, *Pleaurotus ostreatus* CAB13 en *Saccharomyces cerevisiae* CAB79 (funguslyne), is gekeur as SCP-kandidate in onverdunde ensimatiese hidrolisaat om te bepaal watter mikrobes die inhibeerderkonsentrasies soos asynsuur, kon verdra. *S. cerevisiae*, gebruik as kontrole, het die inhibeerders die beste verdra omdat dit al die glukose verbruik het en ’n finale biomassakonsentrasie van 20.37 ± 11.81 g/L na 48 h bereik het. Van die vyf alternatiewe lyne het *S. thermophilus* die teenwoordige inhibeerders die beste verdra, omdat dit 47.48% ± 3.75% van die glukose verbruik het en ’n finale biomassakonsentrasie van 1.28 ± 0.17 g/L na 48 h bereik het. Daarom is *S. thermophilus* gekies vir vergelyking met *S. cerevisiae* in pulsvoerlotfermentasies om biomassaformasie te maksimeer deur die lyn bloot te stel aan laer aanvanklike inhibeerderkonsentrasies.

Pulsvoerlotfermentasies, met ’n aanvanklike en finale hidrolisaatkonsentrasie van 20% (v/v) en 80% (v/v), onderskeidelik, het die finale biomassakonsentrasie van beide *S. cerevisiae* en *S. thermophilus* beduidend verhoog, in vergelyking met lotfermentasie in onverdunde hidrolisaat. *S. cerevisiae* het ’n hoër finale biomassakonsentrasie (52.65 ± 0.80 g/L) as *S. thermophilus* (6.57 ± 0.09 g/L) bereik.

Die proteïeninhoud van *S. cerevisiae* en *S. thermophilus* was 46.9% ± 2.6% (DW) en 66.1% ± 1.3% (DW), onderskeidelik. *S. cerevisiae* het 24.71 ± 1.44 g/L proteïene produseer, terwyl *S. thermophilus* 4.34 ± 0.10 g/L proteïene geproduseer het. Daarom is *S. cerevisiae* ’n belowende lyn om stoomontplofde suikerrietbagasse na SCPs om te sit omdat dit hoër proteïen- en biomassakonsentrasie tot gevolg het as *S. thermophilus*. Daarby, die maksimum etanolkonsentrasie deur *S. cerevisiae* geproduseer word gedurende pulsvoerlotfermentasie was 8.13 ± 0.27 g/L na 36 h; daarom, die ko-produksie daarvan met SCP kan die ekonomiese aanloklikheid van hierdie proses verder vergroot. Dit is egter moontlik dat dit ten koste van die laasgenoemde sal wees.

Die tegniese en ekonomiese uitvoerbaarheid van produsering van SCPs uit stoomontplofde suikerrietbagasse op ’n industriële skaal deur *S. cerevisiae* en *S. thermophilus* is bepaal en vergelyk. Die minimum verkoopsprys (MSP) is gebruik as die bepalende faktor omdat dit die biomassa-opbrengs, die totale kapitale belegging (CAPEX) en die bedryfskostes (OPEX) in ag neem. *S. cerevisiae* het die laagste MSP van 2 319 US$/ton biomassa (ZAR 41/kg) bereik, wat omtrent tien keer minder was as die MSP bereik met die produksie van *S. thermophilus* (20 436 US$/ton biomassa; ZAR 353/kg). Daarom, op ’n industriële skaal is *S. cerevisiae* die mees belowende lyn vir die bio-omsetting van stoomontplofde suikerrietbagasse na SCPs.

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NOMENCLATURE

Symbols		
$\log(R_0)$	Severity factor	-
R_0	Reaction ordinate	-
s	Substrate concentration	g/L
T	Temperature	°C
t	Time	h
x	Biomass concentration	g/L
Y	Biomass yield coefficient	g/g
Greek symbols		
μ	Specific growth rate	h^{-1}
Subscripts and superscripts		
$\frac{x}{s}$	Biomass concentration vs substrate concentration	
max	Maximum	
p	productivity	
r	Reaction	
0	Initial	
Acronyms		
AFEX™	Ammonia fibre expansion	
ANOVA	Analysis of variance	
AOAC	Association of Official Agricultural Chemists	
BCA	Bicinchoninic acid	
CAF	Central Analytical Facility	
CAPEX	Capital expenditure cost	
CBP	Consolidated bioprocessing	
CCD	Central composite design	
CEPCI	Chemical engineering plant cost index	
COD	Chemical oxygen demand	
COP	Coefficient of performance	
CSL	Corn steep liquor	
DCF	Discounted cash flow	
DM	Dry mass	
DW	Dry weight	
EPA	Environmental Protection Agency	

FAO	Food and Agricultural Organisation
FCI	Fixed capital investment
FDA	Food and Drug Administration
FPA	Filter paper assay
FPU	Filter paper units
GHG	Greenhouse gases
GRAS	Generally regarded as safe
HMF	5-hydroxymethylfurfural
HPLC	High-performance liquid chromatography
IRR	Internal rate of return
IUPAC	International Union of Pure and Applied Chemistry
MEAA	Modified essential amino acid
MIC	Minimum inhibitory concentration
MSP	Minimum selling price
NREL	National Renewable Energy Laboratory
PM	Particulate matter
RDA	Recommended dietary allowance
RO	Reverse osmosis
SCP	Single-cell protein
SF	Submerged fermentation
SHF	Separate hydrolysis and fermentation
SSF	Simultaneous saccharification and fermentation
S-SF	Solid-state fermentation
STEX	Steam explosion
TCI	Total capital investment
UNU	United Nations University
US	United States
UV/VIS	Ultraviolet-visible
WHO	World Health Organisation
WIS	Water-insoluble solids
WSS	Water-soluble solids
YPD	Yeast extract, peptone & glucose

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1:

INTRODUCTION

1.1 Background

Population growth, climate change and changing consumption patterns are straining global food production. These factors are key drivers of growth in the agricultural sector, which has an increasingly large environmental footprint. Consequently, agricultural intensification and growth are progressively contributing to greenhouse gas (GHG) emissions, eutrophication and the loss of biodiversity through land degradation and deforestation (Laurance, Sayer and Cassman, 2014). The low efficiency associated with the conventional conversion of feed to primarily animal-based protein for human consumption (e.g. 6kg of feed to produce 1 kg of animal-based protein) means that the agricultural sector is not sustainable (Ritala *et al.*, 2017). Alternative protein sources have recently garnered much attention as an alternative, more efficient means of converting feed to protein, thus alleviating the pressure that is currently placed on the environment (Linder, 2019). These alternative protein sources that are presently being utilised for human consumption include; insects, in-vitro meat (artificial meat grown in laboratories), plant-based proteins and single-cell proteins (SCPs) from microbial biomass (Linder, 2019). However, the use of microorganisms as an alternative protein source has received less attention, even though microbes have been utilised as food sources throughout history. For example, Germany replaced nearly half of their imported protein sources during World War I with fodder yeast (Ugalde and Castrillo, 2002). Additionally, Brewer's yeast is an extract in many readily available sandwich spreads and microbial cultures are the main constituent of yoghurt and cheese (Linder, 2019). An additional problem resulting from population growth is an increase in ecological implications. One of which arises due to the accumulation of agricultural and agro-industrial plant waste in the form of by-products from crops used for food or feed (Petre, 2017). However, there is much opportunity in the conversion process of agro-processing residues, specifically lignocellulosic biomass, to a high-value product in the form SCPs, in a more sustainable and environmentally friendly way than that of conventional protein sources, such as that of beef, chicken and pork (Anupama and Ravindra, 2000).

Using a low-value substrate to produce SCPs provides additional benefits, including reducing costs of the SCP production process (Johnston, Fanzo and Cogill, 2014). The provision of affordable and high-quality proteins is crucial, as proteins are an essential constituent in human diets required for growth and development, where the recommended dietary allowance (RDA) is 0.83 g/kg body weight per day (WHO, FAO and UNU, 2002). Additionally, protein deficiency results in malnutrition and other health disorders, such as kwashiorkor and marasmus (Yousufi, 2012).

Various microbial strains, such as white-rot fungi, are capable of utilising lignocellulosic biomass. However, with no prior pretreatment, using microbial strains directly is ineffective and a timeous process. Pretreatment of the low-value lignocellulosic biomass increases the accessibility and availability of carbohydrates, which is crucial for the timeous bioconversion of lignocellulosic biomass to SCP (Moo-

Young, Chisti and Vlach, 1993; Sánchez, 2009; Nasseri *et al.*, 2011; Fatemeh, Reihani and Khosravi-Darani, 2019). There are various methods used to pretreat lignocellulosic biomass. Some involve using hazardous chemicals, such as sulphuric acid (H_2SO_4), hydrochloric acid (HCl) and sodium hydroxide (NaOH), that could be harmful for human consumption, resulting in additional costs to remove and recover these chemicals from the final product (Nasseri *et al.*, 2011). Autocatalysed steam explosion is a preferred method of pretreatment because it does not use hazardous chemicals and requires only high-pressure steam. Therefore, it does not require downstream processing, which would increase the operating costs of the process (Mosier *et al.*, 2005). Steam explosion modifies and partially removes the lignin and hemicellulose fractions, therefore improving cellulose conversion to glucose (Philippoussis, 2009; Wallace, 2013). Additionally, steam explosion has a low environmental impact and a low capital investment (Duque *et al.*, 2016).

Steam explosion results in a highly digestible product that would be suitable for subsequent enzymatic hydrolysis to produce fermentable sugars for bioconversion to protein. Separate hydrolysis and fermentation (SHF) is one of the process configurations that can be used for such lignocellulose conversion, allowing for the optimal enzymatic hydrolysis conditions required by the enzyme to be used (Merino and Cherry, 2007). The high yields of glucose that can be achieved in such enzymatic hydrolysis may lead to end-product inhibition. However, the commercial enzyme Cellic® CTec3 is known to have a higher end-product tolerance (Mokomele, 2019). The resulting hydrolysate is then used in the fermentation process.

Submerged fermentation (SF) is the desired fermentation process as compared to solid-state fermentation (S-SF) as it allows for ease of handling of the final product and better monitoring capabilities (Singhania *et al.*, 2010). SF is ideal for the growth of bacterial strains as they require a high water activity compared to fungal strains (Pandey, 2003; Rani *et al.*, 2009). SF is also known to achieve higher protein yields than S-SF, as *Pleurotus ostreatus* achieved a protein content of 29.76% when grown under SF as compared to 24.69% when grown under S-SF (Hatakka and Pirhonen, 1985; Mumpuni *et al.*, 2017). *Pleurotus sajor-caju* achieved a crude protein concentration of between 40% and 49% in SF and between 27% and 35% in S-SF (Hatakka and Pirhonen, 1985; Tanaka and Matsuno, 1985; Chahal, 1989; Gupta *et al.*, 2013).

Previous reports on SCP production from lignocellulose have focused on the bioconversion of acid- or alkali-pretreated lignocellulosic biomass under S-SF (Soccol and Vandenberghe, 2003; Rani *et al.*, 2009; Suman *et al.*, 2015; Mumpuni *et al.*, 2017; Letti *et al.*, 2018). Therefore, there is limited information on the bioconversion of enzymatically pretreated, steam-exploded sugarcane bagasse to SCPs via batch and pulse fed-batch fermentations under submerged fermentation conditions. Additionally, the economics of the production of SCPs from steam-exploded sugarcane bagasse has received little attention.

1.2 Thesis outline

CHAPTER 2 is the literature review, which provides an overview of alternative protein sources for human consumption, specifically focusing on the production of SCP from pretreated lignocellulosic biomass using various microorganisms that have GRAS status. Various pretreatment methods and different

saccharification and fermentation processes are discussed and compared. The effects of various process conditions and processing steps on the protein yield of the microorganisms are identified and discussed. **CHAPTER 3** outlines the research aims and objectives to be achieved in the current investigation. **CHAPTER 4** describes the methods (experimental and analytical), the equipment and the chemicals used in obtaining the experimental results. **CHAPTER 4** also outlines the experimental results from the enzymatic hydrolysis of the steam-pretreated sugarcane bagasse and the submerged fermentation of the resulting enzymatic hydrolysate by the preferred microbial strain. Furthermore, a detailed discussion of the findings is provided. The economics of the process of producing SCP on an industrial scale is discussed in **CHAPTER 5**. Additionally, the method used to develop the economic model and the estimation of the various costs are discussed in detail. The main conclusions and recommendations of the investigation are summarised in **CHAPTER 6**.

CHAPTER 2:

LITERATURE REVIEW

2.1 Conventional protein sources versus single-cell proteins

The protein content of various microbial strains is comparable to conventional protein sources. Meat, milk and soybean consist of about 45%, 25% and 35% protein on a dry weight basis, respectively. In contrast, microorganisms, such as algae, bacteria and fungi, consist of between 30% to 60% protein on a dry weight basis (Ghasemi, Rasoul-amini and Morowvat, 2011; Ritala *et al.*, 2017). Thus, microbial-based proteins could, arguably, be more suitable for human consumption. Another consideration would be the amino acid composition of the microbial biomass, as the composition thereof determines the nutritional value of the biomass (Ritala *et al.*, 2017). The amino acid composition of various microbial strains compares favourably with the Food and Agricultural Organization (FAO) standards for human nutrition (Appendix A, Table A.1 to Table A.4). Therefore, the higher protein content combined with a lower environmental footprint as well as a promising amino acid composition could make microbial protein a suitable protein alternative.

Furthermore, the agricultural sector is responsible for about 70% to 85% of the water footprint and about 30% of greenhouse gases (GHG) emitted worldwide (Smetana *et al.*, 2015). Meat production is responsible for approximately 18% of GHG emitted from the agricultural sector (Smetana *et al.*, 2015). Additionally, livestock rearing contributes a large portion of methane emissions and is the largest land resource user, thus contributing to deforestation and land degradation (Lipper *et al.*, 2010). Plant-based proteins are nutritional and healthy protein sources but require large quantities of water and land for cultivation. Therefore, the production of plant-based proteins results in land degradation and loss of biodiversity (Ritala *et al.*, 2017). Eutrophication is also a problem with the large-scale production of plant-based protein sources, as it leads to nutrient pollution due to the presence of Nitrogen and Phosphorus in the fertilisers that are used (Smetana *et al.*, 2018).

With the aforementioned issues in mind, there is a need to investigate a more sustainable and environmentally friendly protein source for human consumption. A promising alternative would be the production of single-cell proteins (SCPs). However, the current methods and substrates used to produce mycoprotein (SCP from fungi) have a significant GHG emission impact on the environment. Table 2.1 compares the global warming potential and the land requirements needed to produce various protein sources, specifically to produce mycoprotein, chicken and pork products.

Table 2.1: Comparison of the global warming potential and land requirements of mycoprotein, chicken and pork production, per 1 kg of product (Smetana *et al.*, 2018).

Protein Source	Carbon dioxide equivalent (kg $CO_{2,\ eq}$)	Land requirement (m^2a/kg)
Mycoprotein	5.6 to 6.2	2.0
Chicken	2.0 to 4.0	5.0 to 7.0
Pork	4.0 to 6.0	7.0 to 8.0

According to Table 2.1, the CO_2 emissions released during mycoprotein production is greater than the emissions released during the production of pork and chicken. However, the utilisation of lignocellulosic biomass as a substrate to produce SCPs is expected to reduce the carbon emissions to between 2 kg CO_{2eq} and 4 kg CO_{2eq}, similar to chicken and lower than pork, as determined from a Life Cycle Assessment (LCA) performed by Smetana *et al.*, 2018 (Smetana *et al.*, 2018, Table 2.1). Smetana et al. (2018) also suggested that the land usage requirement for mycoprotein production will decrease from 2 m^2a/kg to 0.5 m^2a/kg and the energy requirements will decrease from between 15 kWh/kg and 20 kWh/kg to about 10 kWh/kg when lignocellulosic biomass is used as a substrate. The increase in the production of SCPs could decrease the demand for animal- and plant-based proteins if SCPs are viewed as equivalent to conventional proteins, which would further reduce emissions and lead to a decrease in land degradation and deforestation (Nasseri *et al.*, 2011). The use of steam-pretreated lignocellulosic biomass as a substrate to produce SCPs is promising and may alleviate the environmental impact associated with the production of conventional protein sources.

The production of SCPs allows for the rapid production of highly nutritious food due to their short doubling time, which is defined as the time required to double the mass of the protein source (Kurcz *et al.*, 2018). Microbial biomass requires a few minutes to several hours, while alternative protein sources, such as plant- and animal-based proteins, require a doubling time of at least one week to several months (Table 2.2). The production of SCPs will allow for the rapid production of a more sustainable and environmentally friendly alternative protein source.

Table 2.2: The time required to double the mass of various protein sources (Nigam and Singh, 2014).

Organism	Mass doubling time
Bacteria and yeasts	20 to 120 minutes
Moulds and algae	2 to 6 hours
Grass and plants	1 to 2 weeks
Chickens	2 to 4 weeks
Pigs	4 to 6 weeks
Young cattle	1 to 2 months

Another advantage that the production of SCPs has over the production of conventional protein sources is that SCPs have a greater efficiency concerning the conversion of substrate to protein (Nigam and Singh, 2014). The resulting lower substrate requirement should decrease the operating costs of the production

process, which would reduce the cost to the end consumer. However, the lower operating costs depend on the type and amount of substrate used and the pretreatment process the substrate will have to undergo. In terms of mass, *Fusarium venenatum* (Table 2.3) can convert 1 kg of feed to three times more protein than conventional methods (Anupama and Ravindra, 2000). Due to the inefficiency of the conversion of feed to animal-based protein in terms of mass, it would be more advantageous to convert steam-pretreated lignocellulosic biomass to SCP for human consumption than use the pretreated substrate as animal feed (Anupama and Ravindra, 2000).

According to Nigam and Singh (2014), the protein production rate obtained from a 250 kg cow and 250 g of microbial biomass are comparable, as both will produce approximately 200 g of protein per day. In comparison, the microbial strain will, theoretically, produce approximately 25 tonnes of protein in the same time frame as one 250 kg cow, with the potential for application in the mass production of SCPs as an alternative protein source for human consumption.

Table 2.3: Protein and total mass achieved through the conversion of 1 kg of a substrate by different protein producers (Beech, Melvin and Taggart, 1985; Anupama and Ravindra, 2000; Nigam and Singh, 2014).

Protein producer	Substrate (1 kg)	Product	
		Protein (g)	Total mass of product (g)
Chicken	Feed	49	240 †
Cow	Feed	14	68 †
Pig	Feed	41	200 †
Mycoprotein *Fusarium venenatum*	Carbohydrate and inorganic nitrogen	136	1080 □

† Meat

□ Wet mass

2.2 Single-cell protein

Single-cell protein (SCP) refers to crude or refined protein that is produced via various microbial strains, such as bacteria, fungi, or algae (García-Garibay *et al.*, 2003). The dried cells of the microorganisms are typically used as protein supplements in animal feed and human food (Munawar *et al.*, 2010; Nasseri *et al.*, 2011). SCPs would be beneficial as an alternative protein source due to their high protein, vitamin, essential amino acid and lipid content, specifically that of long-chain polyunsaturated fatty acids (García-Garibay *et al.*, 2003; Nasseri *et al.*, 2011). The approximate protein content of microbial biomass is between 30% and 60% of its dry weight (Table 2.5) and can increase to 70% (w/w) when grown under optimal conditions, which depends on the strain used. In comparison, meat, milk and soybean contain about 45% (w/w), 25% (w/w) and 35% (w/w) protein, respectively, on a dry mass basis (Ghasemi, Rasoul-amini and Morowvat, 2011; Ritala *et al.*, 2017; Linder, 2019).

The amino acid composition of the SCPs determines the nutritional value of the microbial biomass, as essential amino acids cannot be produced naturally by the human body (Ritala *et al.*, 2017). The human body requires a specific daily intake of various essential amino acids (Table 2.4), as recommended by the

FAO, the World Health Organization (WHO) and the United Nations (UN) (WHO, FAO and UNU, 2002). Essential amino acids are the building blocks of proteins, hormones and various neurotransmitters, hence the importance of meeting the daily intake required by the human body (Table 2.4).

Table 2.4: WHO and FAO recommended essential amino acid requirements for adults (WHO, FAO and UNU, 2002).

Amino acid protein	**Concentration**	
	mg/kg per day	**mg/g protein**
Histidine	10	15
Isoleucine	20	30
Leucine	39	59
Lysine	30	45
Methionine + cysteine	15	22
Methionine	10	16
Cysteine	4	6
Phenylalanine + tyrosine	25	38
Threonine	15	23
Tryptophan	4	6
Valine	26	39
Total essential amino acids	184	277

The amino acid composition of most SCPs studied meets the requirements set by the FAO and WHO (Appendix A) regarding the essential amino acid scoring pattern for human nutrition (Matassa *et al.*, 2016). Thus, the human population can benefit from using SCPs as a food source, which is further substantiated by the fact that SCPs have a better amino acid composition than plant-based proteins (Filho *et al.*, 2019). However, the nucleic acid content of microbial biomass is too high for human consumption. Therefore, additional processing steps are required to reduce the nucleic acid content of the SCPs (Table 2.5). The nucleic acid content of SCPs and their side effects are discussed in further detail in Section 2.2.2.

Table 2.5: Average composition of the main types of microorganisms (Nasseri *et al.*, 2011).

Compound	**Composition (% DW)**			
	Bacteria	**Fungi**	**Yeast**	**Algae**
Protein	50 – 65	30 - 45	45 – 55	40 - 60
Fat	1 – 3	2 – 8	2 – 6	7 – 20
Ash	3 – 7	9 – 14	5 – 10	8 – 10
Nucleic acid	8 – 12	7 – 10	6 – 12	3 – 8

2.2.1 SCP Market

The current SCP market primarily consists of people that are aiming to reduce their meat intake, as SCPs can resemble the texture and quality of meat but do not have the associated health risks, such as an increased risk of developing cardiovascular diseases (McIlveen, Abraham and Armstrong, 1999; Sadler,

2004). Furthermore, the market for the sale of SCPs would be environmentally conscious consumers if the GHG benefits of producing SCPs outweigh those of animal-based products. Mycoprotein, a tasty and convenient SCP with high protein content, is one such meat alternative (Wiebe, 2002; Nigam and Singh, 2014). However, as microbial protein has a nucleic acid content that is too high for human consumption, various methods have been adapted to reduce the concentration thereof. Quorn® makes use of thermal treatment to break down the nucleic acids present in the mycoprotein produced, as discussed in more detail in Section 2.2.1 (Trinci, 1992; Wiebe, 2002)

Mycoprotein can be used in stews, casseroles and other dishes when sold in chunks, similar to meat portions. The production of mycoprotein-based foods focuses on the health aspect, aims to provide food to promote a healthier diet and has GHG benefits if improvements in production methods and/or substrate can be achieved (Wiebe, 2002; Smetana *et al.*, 2018). SCPs are currently used worldwide as a health food, for example, Quorn® products and spirulina supplements, for their benefits of controlling obesity and reducing cholesterol levels. SCPs are also utilised by athletes for instant energy in the form of snack and energy bars (Mensah and Twumasi, 2016). The incorporation of freeze-dried mycoprotein into other food products has also been considered, whether it was used as a fat replacer or to boost the nutritional value of the foods to which it was added (Turnbull, Leeds and Edwards, 1992; Wiebe, 2002).

According to a study, Quorn® mycoprotein is similar to chicken in terms of texture, flavour, appearance and aroma. Moreover, Quorn® ready-made meals had better scores in all four departments than ready-made chicken meals and tofu-based products (McIlveen, Abraham and Armstrong, 1999).

Mycoprotein is the most extensively studied SCP, while bacterial strains have received less attention due to their negative connotations, as they are associated with various diseases. Hence, this negatively affects the sale and marketing of bacteria-based products as an alternative protein source (Anupama and Ravindra, 2000; Nasseri *et al.*, 2011; Suman *et al.*, 2015). However, there is a pre-existing market for the sale of bacteria-based products. A few examples include probiotics, yoghurt, cheese and kombucha (Anupama and Ravindra, 2000). Therefore, the challenge is positioning bacteria-based SCP products to gain similar acceptance as other naturally fermented products.

2.2.2 Safety evaluation of SCPs

The main concerns with the production of SCPs include the nucleic acid content of the microbial biomass, the possibility of the microorganisms producing toxins, allergies that may arise and the presence of harmful substances such as heavy metals derived from the substrate used (Ritala *et al.*, 2017). Various fungal species produce mycotoxins which cause allergic reactions and, in more extreme cases, diseases and liver cancer (Anupama and Ravindra, 2000). Therefore, selecting microorganisms with GRAS status is essential to avoid the presence of toxins in alternative protein products (Ritala *et al.*, 2017).

The high nucleic acid content in single-cell proteins is problematic for human consumption as it causes an increase in uric acid within the blood plasma (Ritala *et al.*, 2017). Humans should not consume more than 2 g of nucleic acid per day to avoid gout and the formation of kidney stones (Moo-Young, Chisti and Vlach, 1993). When comparing the nucleic acid content of various food types (Table 2.6), there is no

nutritional hazard in consuming the muscle portion of beef and pork. However, Baker's yeast and oyster mushrooms have a high nucleic acid concentration and pose a nutritional hazard. Therefore, additional processing is required to decrease the nucleic acid content of SCPs to ensure that they are safe for human consumption and considered an alternative protein source.

Heat shock treatment is a processing step that can reduce the biomass's RNA content. Heat shock treatment is advantageous as it minimises the degradation or loss of protein and the fibre structure of the microbial biomass. Heat shock treatment is the process used to reduce the RNA content during the manufacturing of Quorn® products and has, therefore, been widely studied (Trinci, 1992; Wiebe, 2004).

Table 2.6: Average RNA and DNA contents of various foods. Adapted from Eenennaam and Young (2017).

Organism	Food	RNA (g/kg DM)	DNA (g/kg DM)	Total (g/kg DM)
Yeast	Baker's yeast	66.2	6.0	72.2
Mushrooms	Oyster	24.1	1.4	25.5
Cattle/ beef	Kidney	13.5	16.1	29.6
	Liver	22.1	19.5	41.6
	Muscle	3.2	1.7	4.9
	Pancreas	87.9	16.2	104.1
Pigs/ pork	Kidney	15.5	17.6	33.1
	Liver	32.1	14.8	46.9
	Muscle	4.1	1.9	6.0
	Pancreas	71.4	21.2	92.6

2.3 Lignocellulosic biomass

Lignocellulose is produced by plants during photosynthesis and is the main constituent of agricultural residues. Additionally, lignocellulosic biomass is one of the most abundant renewable carbon sources. Lignocellulose consists of three main components: lignin, hemicellulose and cellulose (Anupama and Ravindra, 2000; Howard *et al.*, 2003). Different substrates have different compositions of these three components (Table 2.7). Therefore, feedstock and conditions should be carefully considered to maximise the available sugar for microbial growth (Patil *et al.*, 2010).

Table 2.7: Chemical composition of different agricultural residues (Sun and Cheng, 2002; Howard *et al.*, 2003; Haghdan, Renneckar and Smith, 2016; Baruah *et al.*, 2018; Alokika *et al.*, 2021).

Lignocellulosic material	Cellulose (%, w/w)	Hemicellulose (%, w/w)	Lignin (%, w/w)
Corn cobs	45	35	15
Nut shells	25 – 30	25 – 30	30 – 40
Rice straw	32	24	18
Sugarcane bagasse	32 – 45	19 – 32	17 – 32
Wheat straw	30 – 40	20 – 50	15 – 20

A pretreatment step is required to decrease the lignocellulosic biomass recalcitrance, i.e., degree of crystallinity and lignification, among others, to allow for the upcycling of the biomass to be successful. Lignin acts as a physical barrier and provides the plant with rigidity. Lignin is bonded to the cellulose microfibrils and hemicellulose through hydrogen and covalent bonds to protect the cellulose and hemicellulose from microbial degradation. Therefore, lignin decreases the saccharification performance of cellulose and hemicellulose into sugars of the untreated material, while the hemicellulose fraction also prevents cellulolytic hydrolysis (Pengilly, 2013; Spalvins, Zihare and Blumberga, 2018; Hamann, 2020). Hemicellulose achieves this by acting as a physical barrier between the enzymes and cellulose. The cellulose fraction comprises of many glucose units, which is the preferred carbon source for microorganisms (Pengilly, 2013; Mokomele, 2019). Cellulose is present in fibrils linked together by hydrogen bonds, thus, forming a crystalline structure that provides the plant with strength and rigidity (Alokika *et al.*, 2021).

The conversion of the biomass can be achieved through the steam explosion (STEX) of the residues, followed by enzymatic hydrolysis to yield sugars that many GRAS microbes would be able to utilise to yield SCPs. The conversion of the lignocellulosic biomass would lead to a decrease in agricultural residues and an increase in value-added products, specifically SCPs (Moo-Young *et al.*, 1977; Moo-Young, Chisti and Vlach, 1993; Kahar, 2013). The bioconversion of lignocellulosic biomass into SCPs can be achieved in five general stages (Figure 2.1). These five stages are discussed in more detail in Sections 2.4, 2.5, 2.6 and 2.7.

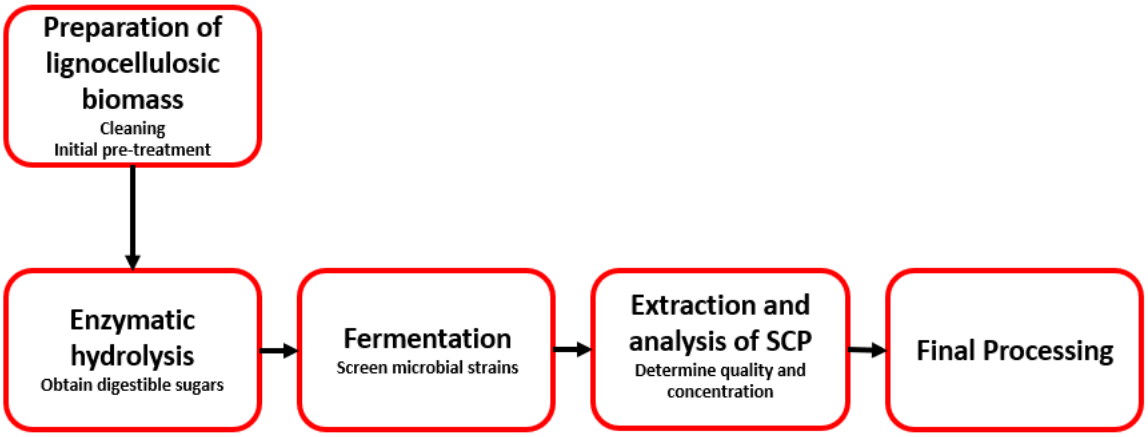


Figure 2.1: Five general stages followed for the bioconversion of lignocellulosic biomass to SCPs (Nasseri *et al.*, 2011; Ritala *et al.*, 2017).

The potential substrates for the growth of SCPs must meet specific criteria: the substrate must be non-toxic, inexpensive, abundant and renewable (Fatemeh, Reihani and Khosravi-Darani, 2019). Lignocellulosic biomass, especially sugarcane bagasse, meets the specified criteria, as discussed in Section 2.3.1.

2.3.1 Sugarcane bagasse as a potential substrate for the production of SCP

Sugarcane is a grass that belongs to the genus *Saccharum*, family *Poaceae* and is widely cultivated in South America, Asia and Africa for its high sucrose content (Chambon *et al.*, 2018; Khattab and Watanabe, 2019). Sugarcane bagasse is the lignocellulosic waste product from the sugar industry, which is left over after the extraction of sugarcane juice from the sugarcane stems (Das and Singh, 2004; Chambon *et al.*, 2018).

Sugarcane bagasse accounts for approximately 27% to 28% of the sugarcane crop produced worldwide, on a dry weight basis. Therefore, about 540 million metric tonnes of sugarcane bagasse are produced annually (Bian *et al.*, 2012; Bezerra, 2016; Alokika *et al.*, 2021). Approximately half of the sugarcane bagasse produced is incinerated in boilers to produce steam to power the sugar mills and the remainder is stockpiled or burnt (Chambon *et al.*, 2018). The incineration of the bagasse to power the mills is an inefficient process as it is designed to ensure that there is no surplus of bagasse left (Bizzo *et al.*, 2014). The burning of the sugarcane bagasse produces large concentrations of particulate matter (PM), which results in air pollution. The high concentration of PM results in the sugar mills not meeting regulations set out by the Environmental Protection Agency (EPA) (Alokika *et al.*, 2021).

There is much opportunity for the upcycling thereof, especially since sugarcane bagasse is an abundant, renewable and inexpensive feedstock (Tyagi *et al.*, 2019). An additional advantage of sugarcane bagasse as a substrate is its low ash content (1% to 9%), which is advantageous for microbial bioconversion. This is because ash has inhibitory effects, specifically on cellulase, during enzymatic hydrolysis (Bin and Hongzhang, 2010; Alokika *et al.*, 2021). Furthermore, sugarcane bagasse has been extensively studied for

bioethanol production, with less attention on the bioconversion thereof to SCPs (Mokomele *et al.*, 2018; Khattab and Watanabe, 2019; Tyagi *et al.*, 2019).

2.4 Pretreatment of sugarcane bagasse

Pretreatment of lignocellulosic biomass is required to increase the enzymatic digestibility of the biomass, which increases the accessibility and availability of carbohydrates for microbial cultivation. Therefore, pretreatment of the bagasse prior to fermentation improves the protein and biomass yields (Moo-Young, Chisti and Vlach, 1993; Nasseri *et al.*, 2011). There are microorganisms, specifically white-rot fungi, capable of utilising raw and unprocessed lignocellulose, but the process is slow and the protein and biomass yields are low compared to using pretreated lignocellulose (Tomás-Pejó *et al.*, 2011). Hatakka and Pirhonen (1985) compared the protein yield of wood-rotting fungi on alkali-pretreated and untreated wheat straw, where the pretreated wheat straw resulted in a higher protein yield than the untreated wheat straw. Therefore, motivating the benefit of pretreating lignocellulosic biomass.

The various pretreatment methods available can be separated according to their mode of action, which includes physical, chemical and biological or a combination thereof. Physical pretreatment includes mechanical methods such as ball milling and disc refining, whilst chemical pretreatment involves chemical reactions. STEX is a combination of both a physical and chemical pretreatment process, as it physically disrupts the lignocellulosic structure and cleaves the chemical bonds in the structure (Jacquet *et al.*, 2015). Biological pretreatment makes use of enzymes and, in some processes, fungi as they produce the required enzymes to break down the biomass (Fatemeh, Reihani and Khosravi-Darani, 2019). However, physical pretreatment is required before biological pretreatment as it allows for the cellulose to be more accessible in the biological pretreatment process (Tanaka and Matsuno, 1985). Physical pretreatment reduces the particle size, which increases the specific surface area and decreases the degree of polymerisation and crystallinity. Thus, promoting the enzymatic digestibility of the biomass (Rajendran *et al.*, 2018; Alokika *et al.*, 2021).

Pretreatment yields a feedstock that is more accessible for enzymatic digestion, which produces free sugars. The accessibility of the feedstock and the sugar yield depends on the pretreatment method and conditions used (Trinci, 1992; Nasseri *et al.*, 2011). The pretreatment method selected should be cost-effective, yield soluble sugars and result in few inhibitory by-products. A highly digestible feedstock, with few degradation products present, would lower the enzyme dosage required to achieve an intended conversion of cellulose to glucose (Tomás-Pejó *et al.*, 2011). The decrease of enzymes required would ultimately reduce the operating costs of the process. Therefore, a process with low energy demands and low capital and operating costs is desirable. Additionally, a pretreatment process is advantageous and profitable if it allows for a high solid loading and results in a high concentration of liberated sugars in the liquid fraction (Galbe and Zacchi, 2007). Therefore, the advantages and disadvantages of various pretreatment methods should be compared to ensure an effective method is selected (Table 2.8).

Table 2.8: Advantages and disadvantages of various pretreatment methods of lignocellulosic materials. Adapted from Maurya, Singla and Negi (2015).

Method	Advantages	Disadvantages
Milling	- Decrease in crystallinity of cellulose - Decrease in polymerization of cellulose	- High energy requirements - Partially digestible product
Steam explosion (STEX)	- Cost-effective - Lower environmental impact - Improves enzymatic hydrolysis - Energy efficient - High sugar recovery - Transforms lignin - Solubilises hemicellulose	- Partial hemicellulose degradation - Formation of inhibitors
Liquid hot water	- No chemical requirements - No corrosion-resistant equipment required - Avoids formation of inhibitors	- High energy requirements - High water requirements
Concentrated acid	- High yield of glucose - Ambient temperatures - Solubilization of hemicellulose and lignin	- Expensive - Toxic and hazardous - Formation of inhibitors
Dilute acid	- High hydrolysis yields - Low yields of inhibitors	- High energy requirements
Alkali	- Decrease in crystallinity of cellulose - Decrease in polymerization of cellulose - Efficient removal of lignin - Moderate temperatures	- Expensive - Not used on a large-scale - High water requirements
Biological	- Low energy requirements - Low capital costs - Decrease in polymerization of cellulose - No chemical requirements - Mild conditions - Partial hydrolysis of hemicellulose - Eco-friendly	- Biomass requires pretreatment - Hydrolysis rate is low - Not very effective

There has been minimal research into autocatalysed steam-exploded lignocellulosic biomass as a substrate to produce SCP. The commonly used pretreatment methods are alkali and acid hydrolysis, which have many disadvantages due to the toxic nature of the processes, due to the high concentration of sulphuric acid and sodium hydroxide required.

Additonal to autocatalyzed steam explosion is acid-catalyzed steam explosion, where an externally-added acid catalyst is used, which can be viewed as an upgrade to the pretreatment process (Martín *et al.*, 2021). With dilute-acid steam explosion, the hemicellulosic fraction of the lignocellulosic biomass is completely hydrolysed. It was found that autocatalyzed steam explosion does not require the addition of sulfuric acid to yield good digestibility and achieve higher fermentability by *Saccharomyces cerevisiae*. Therefore, there is much opportunity to investigate the effects of autocatalyzed steam explosion pretreatment on the productivity and yield of SCP microbial strains.

2.4.1 Autocatalyzed steam explosion (STEX)

STEX pretreatment is cost-effective and is a commonly used method for pretreating lignocellulosic biomass (Talebnia, Karakashev and Angelidaki, 2010; Baruah *et al.*, 2018). The STEX process involves exposing the biomass to steam at high temperatures and pressure for between 0.5 minutes and 20 minutes (Rajendran *et al.*, 2018). The temperature range is between 160 °C and 260 °C, while the pressure range is between 0.6 MPa and 4.8 MPa (Talebnia, Karakashev and Angelidaki, 2010; Baruah *et al.*, 2018; Rajendran *et al.*, 2018). During the process, the saturated steam condenses on the surface and inside the lignocellulose as the steam transfers heat to the material. At high temperatures, water can act as an acid, which initiates an autohydrolysis reaction to occur due to the generation of organic acids from the acetyl groups found in the hemicellulose (Maurya, Singla and Negi, 2015; Duque *et al.*, 2016). The process ends with an explosive discharge during which the pressure is suddenly reduced, which causes the condensed water molecules to evaporate, resulting in split fibres (Baruah *et al.*, 2018).

The solid fraction that remains after STEX is made up of lignin, unhydrolysed hemicellulose and digestible cellulose, which is ideal for enzymatic saccharification. STEX modifies and partially removes the lignin, which is desirable as lignin decreases the saccharification performance (Wallace, 2013). Additionally, the use of STEX as a pretreatment method improves the extraction of hemicellulose and results in high yields of glucose (Cardona, Quintero and Paz, 2010; Maurya, Singla and Negi, 2015). The main disadvantage of STEX as a pretreatment method is the production of inhibitors such as furfural, which negatively affects the productivity and the growth of the microbial strains and enzymes (Martín *et al.*, 2018). The advantages of STEX are that it has moderate energy requirements and requires no chemicals, as water is used as the pretreatment liquid. Therefore, there are no chemical recovery costs involved in the process. The capital investment of STEX as a pretreatment step is relatively low and it has the ability to pretreat a variety of feedstocks.

2.4.1.1 Inhibitor formation

The formation of inhibitors depends on the substrate and the pretreatment conditions used (Du *et al.*, 2010). Various inhibitors are formed during STEX of sugarcane bagasse due to the degradation of hemicellulose, lignin and sugars, which occurs under severe pretreatment conditions (Vancov and Mcintosh, 2011). Therefore, one of the significant advantages of using mild pretreatment conditions is that sugar degradation is often avoided, which leads to a decrease in by-product formation (Vancov and Mcintosh, 2011). The formation of inhibitors leads to decreased yields and, if the concentrations are high enough, inhibit enzyme- and microorganism-saccharification productivity (Du *et al.*, 2010; Tomás-Pejó *et*

al., 2011; Pengilly, 2013). The effects of high inhibitor concentrations are essential to note, as a high solid concentration is required to achieve high product yields. Therefore, with a high solid loading, there is an increase in inhibitor concentrations (Pengilly, 2013; Mokomele, 2019).

Inhibitors can belong to three groups: furan derivatives, weak acids and phenolic compounds. All three of these groups can be produced during STEX of lignocellulosic biomass. The main inhibitors formed during STEX are furfural, hydroxymethylfurfural (HMF) and acetic acid, which are known as primary inhibitors (Tomás-Pejó *et al.*, 2011). The majority of the primary inhibitors are produced from the degradation of the hemicellulose fraction (Figure 2.2). Phenolic compounds at low concentrations, produced during the partial degradation of lignin, have been found to severely inhibit the fermentation of lignocellulosic hydrolysate (Palmqvist and Hahn-Hägerdal, 2000; Chandel, da Silva and Singh, 2013). However, an increase in the severity of the pretreatment conditions can lead to the formation of secondary inhibitors: formic acid and levulinic acid (Pengilly, 2013; Xiao, Song and Sun, 2017; Mokomele, 2019).

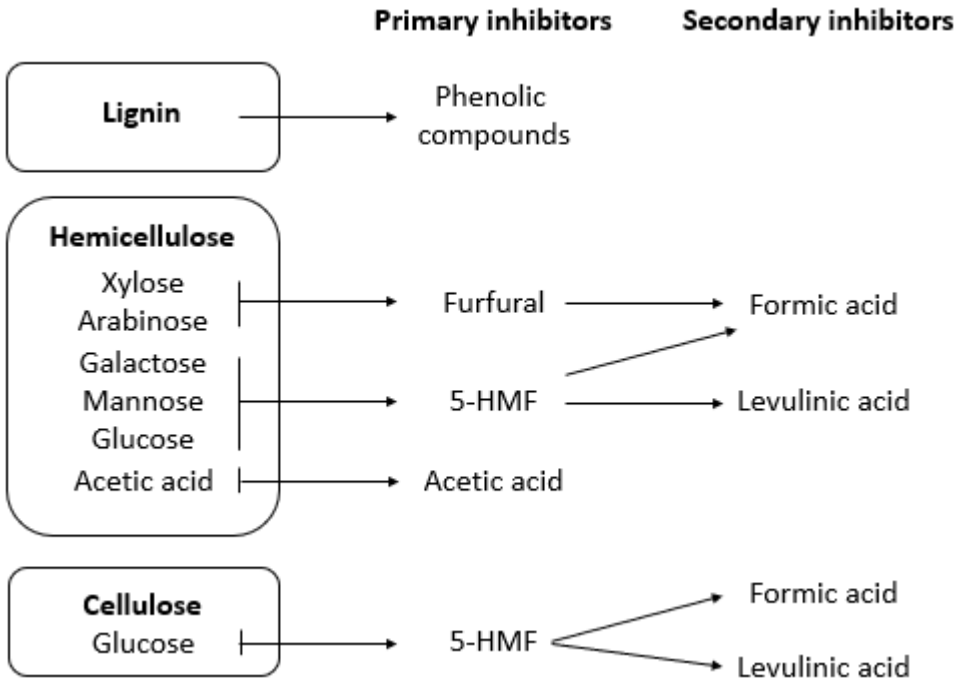


Figure 2.2: Inhibitory compounds produced during STEX (Palmqvist and Hahn-Hägerdal, 2000; Jing, Zhang and Bao, 2009; Pengilly, 2013; Kim, 2018; Mokomele, 2019).

The formation of oligomers from hemicellulose degradation during STEX has inhibitory effects. The oligomers inhibit various microorganisms as the microbes are unable to assimilate the oligomers for their metabolism (Silveira *et al.*, 2018). Acetic acid, aldehydes and other weak acids formed during STEX inhibit the growth of bacteria and yeast (Palmqvist and Hahn-Hägerdal, 2000; Kim *et al.*, 2011). However, a few microorganisms can withstand and even biodegrade some of these inhibitors. These microorganisms include *Bacillus subtilis* (bacteria), which can biodegrade furfural and *Pleurotus ostreatus* (fungi), which can metabolize 5-HMF (Feldman *et al.*, 2015; Zheng *et al.*, 2015). These two microbial strains have been selected for further investigation.

Furthermore, the inhibitors formed also affect the efficiency of cellulase enzymes, which leads to higher enzyme dosage requirements to achieve desirable conversions of cellulose (Jing, Zhang and Bao, 2009). However, the enzymes are unaffected if various inhibitors are below a specific concentration. Acetic acid formed during STEX does not affect cellulase enzymes up to a concentration of 2 g/L. However, the presence of acetic acid has a toxic effect on various microbial strains, hence the use of acetic acid as a preservative (Trček, Mira and Jarboe, 2015). Additionally, the presence of furfural and 5-HMF at concentrations between 0.5 g/L to 2 g/L does not affect the cellulase activity of the enzymes (Cantarella *et al.*, 2004b). Pentoses in the form of xylose and xylo-oligosaccharides inhibit cellulases, more so the xylo-oligosaccharides. Formic acid, at a concentration of 11.5 g/L and higher, has been found to inhibit the enzyme cellulase during enzymatic hydrolysis (Cantarella *et al.*, 2004b).

2.4.1.2 Reducing inhibitors and their effects during enzymatic saccharification and fermentation

Inhibitors have a negative impact on the yield and productivity of both the enzymatic hydrolysis and fermentation processes. Therefore, processes that mitigate the effects of inhibitors need to be investigated and are discussed below.

2.4.1.2.1 Enzymatic saccharification

One of the critical aspects in optimising any pretreatment process is ensuring a highly digestible product is produced and that the formation of inhibitors is kept to a minimum without increasing the capital and process costs (Teter, Sutton and Emme, 2014). Three methods can be utilised to reduce the effects of inhibitors on the enzyme and microbial strains selected. These three strategies are: (1) selecting less severe pretreatment conditions to reduce the generation of inhibitors, although this may negatively affect sugar yields from subsequent enzymatic hydrolysis, (2) engineering enzymes and/or microbes capable of resisting inhibitors and (3) making use of either physical or chemical separation processes to aid in the removal of the inhibitors (Merino and Cherry, 2007; Teter, Sutton and Emme, 2014; Mokomele, 2019).

A physical separation process that can be used to reduce the effects of inhibitors is the washing of the substrate after steam explosion or other pretreatment methods. Washing the substrate results in the removal of soluble components and allows for better cellulose conversion than unwashed substrate undergoing enzymatic hydrolysis under the same conditions (Merino and Cherry, 2007). The main drawbacks regarding washing the substrate are the dilution of sugars and the need to manage waste streams (Teter, Sutton and Emme, 2014). Managing waste streams will lead to a more complicated process, which will increase capital and operating costs. Additionally, if the substrate were to be washed, water utilisation would increase within the process; thus, water removal methods would need to be considered.

Research has been conducted into the use of various microbial strains, which can detoxify the resulting steam-exploded lignocellulosic biomass slurry. With the use of *in situ* microbial detoxification, there will be a decrease in inhibitors as well as the production of high-value products, depending on the microbial strains selected (Chandel, da Silva and Singh, 2013). However, the detoxification of the process stream

requires an additional and separate process step, which would lead to additional costs and thus would not be desirable (Alkasrawi *et al.*, 2006).

A simple process of solid-liquid separation would reduce the concentration of inhibitors and the recycling of catalysts would still be possible, if catalysts were used, specifically in the case of acid-catalyzed steam explosion (Teter, Sutton and Emme, 2014). Solid-liquid separation can be achieved by pressing the steam-exploded substrate to remove as much of the liquid fraction as possible. Most of the inhibitors, as well as the catalysts, would be present in the liquid fraction. Therefore, solid-liquid separation is the more desirable method to reduce the inhibitors' effects. However, management of the liquid waste stream would still be required at an additional cost. However, this liquid waste stream could be considered for biogas production if the waste stream's nutritional content meets the requirements thereof (Petersen, Okoro, *et al.*, 2021). The pressing of the steam-exploded substrate to reduce the inhibitor concentration was used in the present study.

2.4.1.2.2 Fermentation process

Mitigating the effects of the inhibitors in the fermentation of the hydrolysate is of utmost importance to ensure that high productivity and biomass yields are achieved. Therefore, it would not be acceptable to lower the sugar yield to achieve a low inhibitor concentration by making use of insufficient pretreatment conditions, as this would result in lower productivity and biomass yields (Jönsson, Alriksson and Nilvebrant, 2013). Therefore, various methods can be followed to reduce the effect of inhibitors on the fermentation process.

These methods include: (1) selecting microbial strains that exhibit resistance to the inhibitors present, (2) adapting the selected microbial strain to the inhibitors or (3) genetically modifying the desired microbial strain (Jönsson, Alriksson and Nilvebrant, 2013). Using a large inoculum could also counteract the effect of the inhibitors, as this will ensure that high cell viability is achieved (Cantarella *et al.*, 2004a; Bezuidenhout, 2021). However, the use of a large inoculum is not desirable in industry, as it would increase the required retention of microbial biomass for re-inoculation rather than processing towards final products (Jönsson, Alriksson and Nilvebrant, 2013).

Identifying microbial strains that are resistant to inhibitors would aid in mitigating the effect of the inhibitors. However, an investigation of the productivity of the selected microbial strain is important as their resistance to inhibitors does not mean that they will be suitable for industrial processes (Jönsson and Martín, 2016). Additionally, the adaptation of the microbes to the inhibitors would also be ideal as this would lead to inhibitor tolerance (Alkasrawi *et al.*, 2006). This can be achieved by exposing the microbial strain to the inhibitors, in small concentrations, before the fermentation process (Johansson, Brandberg and Larsson, 2011). This adaptation would positively impact both the productivity and yield of the microorganisms selected (Kim, 2018). The adaptation of the microorganisms is also an improvement compared to the detoxification of the feed stream, as it can increase both the fermentation rate and yield.

Adapting the microbial strain to high concentrations of inhibitors can be achieved using an acclimation culture or a fed-batch or continuous fermentation process. These fermentation processes are fed

continuously with small amounts of media/hydrolysate, which results in a low initial inhibitor concentration, whereas batch fermentation starts with a high inhibitor concentration (Taherzadeh and Karimi, 2011). Pulse fed-batch fermentation allows for better biomass concentrations as this fermentation type allows the microbial strains to metabolise the glucose present below lethal inhibitor concentrations (Nilsson, Taherzadeh and Lidén, 2001; Zhang *et al.*, 2014). Additionally, fed-batch has the ability to eliminate substrate inhibition, thus improving the productivity of the microorganism (Giridhar and Srivastava, 2000). The substrate inhibition is eliminated by pulse feeding the culture with small amounts of the high-concentration media, allowing the maximum specific growth rate to be obtained. Therefore, pulse fed-batch fermentation will be investigated with regard to the maximisation of the microbial biomass.

2.5 Enzymatic saccharification

Enzymatic saccharification or hydrolysis is a biological process that involves the use of enzymes to degrade the cellulose and hemicellulose components of the biomass to monomeric sugars (Sun and Cheng, 2002). Cellulase is a commonly used enzyme that is used to convert cellulose to reducing sugars, including glucose. The optimal operating conditions of cellulase are at a temperature ranging from 45 °C to 50 °C and a pH between pH 4 and pH 5 (Sun and Cheng, 2002; Merino and Cherry, 2007; Niju, Swathika and Balajii, 2019).

Enzymatic hydrolysis is one of the most effective methods used to obtain sugars from lignocellulosic biomass, as it allows for the hydrolysis of cellulose to occur. There are many advantages to the application of enzymatic hydrolysis. Some of these advantages are that the process has low energy requirements and does not use hazardous chemicals (Maurya, Singla and Negi, 2015). Additionally, the use of enzymatic hydrolysis also results in a lower inhibitor concentration compared to chemical pretreatment processes (Alkasrawi *et al.*, 2006). However, the biomass must undergo pretreatment before enzymatic hydrolysis to improve process efficiency, as lignin hinders the enzymatic activity (Mabee, McFarlane and Saddler, 2011).

Additionally, pretreatment allows the enzymes to perform optimally in degrading the lignocellulosic biomass (Cardona, Quintero and Paz, 2010; Talebnia, Karakashev and Angelidaki, 2010; Baruah *et al.*, 2018). However, the optimum conditions for maximum hydrolysis yield do not always result in the economic optimum. Therefore, the optimum depends on the cost, the feedstock used and the final product value (Pihlajaniemi *et al.*, 2020).

2.5.1 Enzymatic saccharification and fermentation

Three main process configurations can be used to convert lignocellulosic biomass into valuable products (Figure 2.3). The three main process configurations include: (1) separate hydrolysis and fermentation (SHF), (2) simultaneous saccharification and fermentation (SSF) and (3) consolidated bioprocessing (CBP).

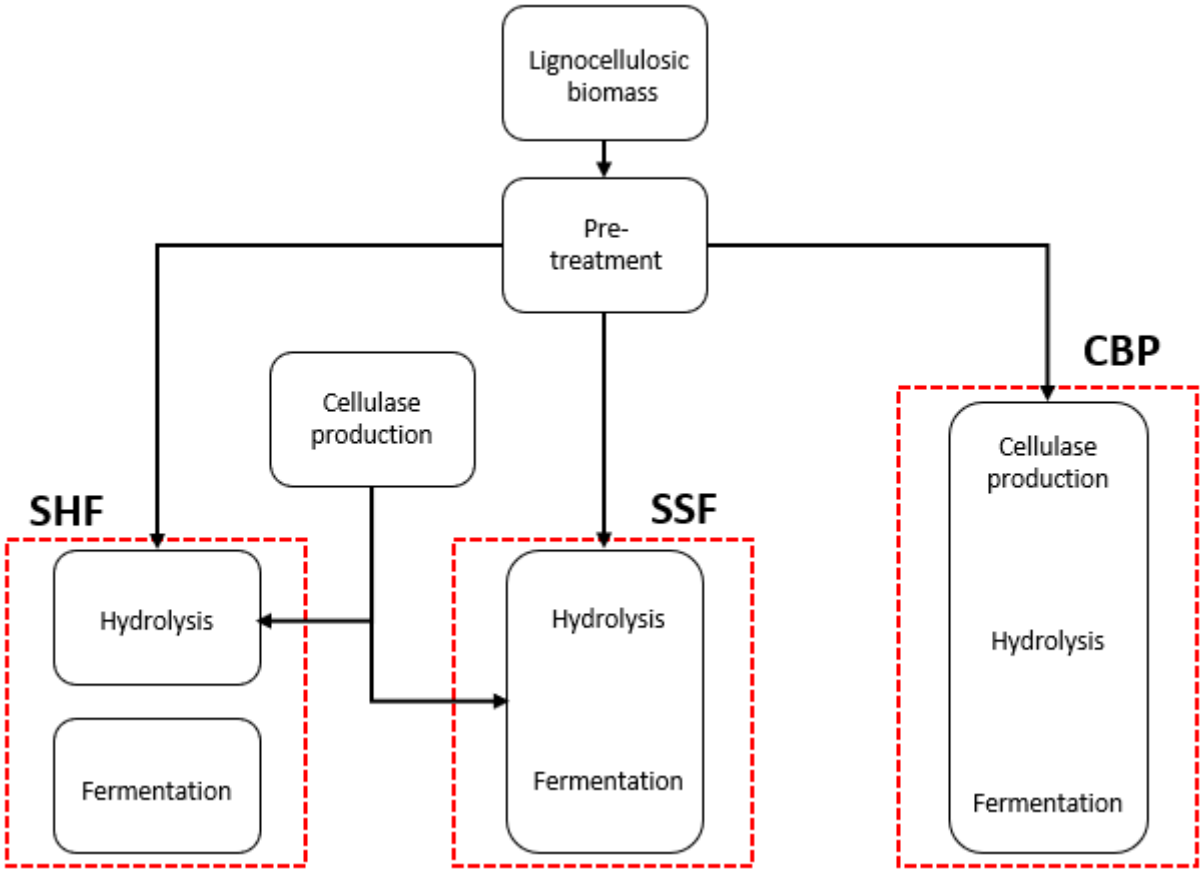


Figure 2.3: Schematic of the three main process configurations used to convert lignocellulosic biomass into valuable products. Adapted from Pengilly (2013).

2.5.1.1 Separate hydrolysis and fermentation

SHF occur when the enzymatic hydrolysis and the fermentation processes occur in two different reactors, with the hydrolysis process preceding the fermentation process (Mosier *et al.*, 2005). A significant advantage of SHF is that the enzymatic hydrolysis step is allowed to proceed to completion at the enzymes' optimal operating conditions. The main drawback of SHF is that product inhibition of the enzymes may result due to the released monomeric and oligomeric sugars, which results in a slower rate of hydrolysis (Merino and Cherry, 2007). However, Cellic® CTec3, a commercial enzyme, has a higher end-product tolerance; therefore, it is not greatly affected by product inhibition (Mokomele, 2019). Another drawback of SHF is the increased chance for contamination to occur and the overall productivity of the process is lower than that which can be achieved with SSF.

2.5.1.2 Simultaneous saccharification and fermentation

SSF is the process where the enzymatic hydrolysis and the fermentation steps occur simultaneously in a single reactor (Mosier *et al.*, 2005). Microbes, known to produce cellulase and other enzymes, can be used in conjunction with enzymatic hydrolysis in an SSF process. An advantage of SSF is the removal of the produced sugars, which helps prevent end-product inhibition. The main downfall of SSF is that the enzymes will not be operating at their optimal temperatures and pH levels, resulting in a reduced rate of

hydrolysis (Merino and Cherry, 2007). The optimal temperature range cannot be used to grow microbes as the microbial strains are unable to grow or grow very slowly at these high temperatures unless a thermophilic microbial strain is selected. Microbes also prefer pH levels higher than pH 5.5, depending on the chosen strain.

2.5.1.3 Consolidated bioprocessing

CBP is the combination of the enzymatic hydrolysis of pretreated lignocellulosic biomass and the fermentation process. The combination of the two is achieved through the use of a single organism or consortium to convert lignocellulosic biomass into valuable products (Nagarajan, Lee and Chang, 2019). This configuration will result in lower operational costs as no external enzymes would be required (Wallace, 2013; Nagarajan, Lee and Chang, 2019). Additionally, the process should yield higher hydrolysis rates, which will reduce reactor volume, thus resulting in a decrease in capital costs (Lynd *et al.*, 2005).

Microbial strains capable of hydrolysis and fermentation to produce high yields of protein are required for CBP to succeed. Currently, such microorganisms are not available. However, research into developing such microbes is being conducted (Lynd *et al.*, 2005). This research includes genetically modifying cellulolytic microbes to improve their enzyme and end-product yields, as the currently available microbes do not produce a sufficient amount of enzymes. Additionally, high enzyme activity from the microbes results in a decrease in protein yield, as more enzymes, in the form was cellulase would be produced (Erikkson and Larsson, 1975; Hatakka and Pirhonen, 1985).

2.6 Fermentation processes

The production of SCPs using fermentation processes has many advantages over conventional and non-conventional protein products, which rely on agriculture. A few of these advantages are that the process is not weather dependent and the product quantity and quality are more easily controlled. Therefore, better yields can be obtained (Moo-Young *et al.*, 1977; Suman *et al.*, 2015). Two main types of fermentation methods are used in the production of SCPs, solid-state and submerged, which are discussed below.

2.6.1 Submerged fermentation

Submerged fermentation (SF) is the method by which microbial strains are grown in a liquid media. SF is generally preferred over solid-state fermentation (S-SF) for ease of handling the final product and better monitoring capabilities (Singhania *et al.*, 2010). Additionally, SF processes have a shorter incubation period, allow for higher yields to be achieved with reduced risk of contamination and can be conducted in a smaller area (Zeltina *et al.*, 1987; Papaspyridi *et al.*, 2012; Mumpuni *et al.*, 2017).

SF is ideal for the growth of bacteria and yeast and appears to be a promising alternative for the efficient growth of edible mushrooms (Suman *et al.*, 2015). Various fungal strains can grow and result in high yields of mycelium biomass under SF conditions, thus revealing the opportunity of using SF to produce mycoprotein products commercially (Humfeld, 1948; Sugihara and Humfeld, 1954; Tellez-Tellez *et al.*, 2008). This was emphasised when ten wood-decaying fungi were grown under SF and S-SF conditions. SF

resulted in the fungal strains producing higher yields of protein than the S-SF process, i.e. 30% in SF and 25% in S-SF for *Pleurotus ostreatus* (Hatakka and Pirhonen, 1985; Mumpuni *et al.*, 2017). Additionally, the mycelium biomass produced under SF conditions has a high nutritional value as their amino acid composition compares favourably to other protein sources and they are a good source of vitamin B (Block *et al.*, 1953). Under SF conditions, microbial biomass can be easily harvested as the biomass is recovered through the use of simple filtration and centrifugation methods (Tanaka and Matsuno, 1985; Coradi *et al.*, 2012).

The main drawback of SF is the low productivity of the microorganisms, which can be avoided by increasing the substrate concentration to increase mass transfer. The disadvantage of this approach is that there will be an increase in viscosity, which will lead to oxygen limitation (Zeltina *et al.*, 1987). SF also requires a high capital investment and has high operating costs (Suman *et al.*, 2015; Upadhyaya *et al.*, 2016). Another drawback of SF is that the process requires agitation, which could damage the biomass and reduce the efficiency of the process (Hatakka and Pirhonen, 1985). An air-lift bioreactor would be an advantageous alternative.

Quorn® uses SF in an air-lift bioreactor to produce large quantities of mycoprotein with high protein yields. However, Quorn® uses glucose as a carbon source, not pretreated lignocellulosic biomass (Trinci, 1992; Wiebe, 2002, 2004). Therefore, there are opportunities to investigate the protein content of various microbial strains, especially those that grow on steam-pretreated lignocellulosic biomass under SF conditions, as there has been minimal research in this field, especially for human consumption.

2.6.2 Solid-state fermentation

Solid-state fermentation (S-SF) is the process where microbial biomass is grown on a solid substrate with moisture levels close to that of saturation, therefore, with no free water (Kavanagh, 2005; Rani *et al.*, 2009). However, the substrate's moisture content needs to support the growth and metabolism of various microbial strains (Rani *et al.*, 2009). S-SF has been extensively studied as it replicates the natural growing conditions of microbial biomass and, as such, it is used commercially to produce fungal fruiting bodies for human consumption (Soccol and Vandenberghe, 2003; Rani *et al.*, 2009; Suman *et al.*, 2015; Mumpuni *et al.*, 2017; Letti *et al.*, 2018).

S-SF does not require as much agitation as SF, which reduces operating costs (Hatakka and Pirhonen, 1985). The static nature of the system, however, leads to a decrease in heat and mass transfer, which results in the formation of undesirable temperature and concentration gradients and affects the microbes' productivity and yield (Kosseva, 2013).

The substrate used in S-SF does not need extensive pretreatment, which reduces operating costs. However, a lack of pretreatment requires microbes to secrete large amounts of cellulolytic enzymes, which is not ideal when wanting to produce high yields of protein as the high rate of enzyme production puts strain on the protein production mechanism of the microorganism (Erikkson and Larsson, 1975; Hatakka and Pirhonen, 1985). Therefore, the crude protein concentrations in S-SF are lower than in SF processes, i.e. between 40% and 49% in SF and between 27% and 35% in S-SF for *Pleurotus sajor-caju*

(Hatakka and Pirhonen, 1985; Tanaka and Matsuno, 1985; Chahal, 1989; Gupta *et al.*, 2013). It is also difficult to separate the microbial biomass from the substrate in S-SF. Therefore, if not used for producing fungal fruiting bodies, S-SF is used for producing protein-enriched feeds, antibiotics, enzymes and organic acids (Kosseva, 2013).

The major challenges involved with the use of S-SF are the scale-up of the process, biomass concentration estimation and purification of the end products (Rani *et al.*, 2009). Downstream processing of the products would be expensive especially because it is difficult to separate the microorganisms from the substrate. This separation would result in low protein yields (Tanaka and Matsuno, 1985). Additionally, the system is difficult to monitor and control due to the specific environmental conditions required as well as the high risk of contamination (Bravo *et al.*, 1994; Kavanagh, 2005).

Fungi and yeast are suitable microorganisms to be grown using S-SF; however, bacterial strains are unsuitable due to them requiring a high water activity (Pandey, 2003; Rani *et al.*, 2009). Therefore, SF would have to be used to compare the protein yield of the various microbial strains.

2.7 Bioconversion of pretreated lignocellulosic biomass into SCP for human consumption

The ability to convert agricultural residues to SCPs for human consumption reveals a biologically efficient method for upcycling lignocellulosic biomass. If a quarter of the annual cereal straw (about 2 325 million tonnes produced globally) were to be used to cultivate the growth of SCPs, about 377.8 million tonnes of protein-rich microbial biomass could be produced (Mane *et al.*, 2007). Thus, revealing the benefits of the upcycling of low-value lignocellulosic biomass to SCPs. However, a range of suitable microorganisms needs to be investigated to produce SCPs for human consumption from lignocellulosic biomass.

2.7.1 Bacterial protein

Lignocellulosic biomass, specifically steam-pretreated lignocellulosic biomass, has rarely been used as a substrate to produce bacterial SCPs. Bacterial strains that produce SCPs typically have a protein content between 50% and 80% (Anupama and Ravindra, 2000; Nasseri *et al.*, 2011; Ritala *et al.*, 2017). Bacteria have fast growth rates and would, therefore, be ideal for producing SCPs as they can produce high yields of protein in a short period (Suman *et al.*, 2015). The protein content of the investigated bacterial strains depends on the substrate used and differs between the various strains (Table 2.9).

Many bacterial strains investigated to produce SCPs have nutritional benefits, produce valuable by-products such as enzymes and are used in commercially produced foods. The SCPs from most bacterial strains investigated contain all essential amino acids at concentrations exceeding the FAO standard (Appendix A) (Ritala *et al.*, 2017). Some bacterial strains have a methionine content of between 2.2% and 3.0% on a dry weight basis, which is higher than that obtained from most algal and fungal SCP biomass (Schulz and Oslage, 1976; Anupama and Ravindra, 2000). Additionally, some bacterial strains produce important lipids and B-group vitamins and are probiotics, therefore, having many health benefits such as inhibiting intestinal pathogens (Stanton *et al.*, 2005; Sharma *et al.*, 2014; Ritala *et al.*, 2017). *Bacillus subtilis* has been found to have the ability to combat the influenza virus by inhibiting the replication of

the virus, while food products containing *Streptococcus thermophilus* may include anti-carcinogenic properties (Sharma *et al.*, 2014; Starosila *et al.*, 2017; Tarrah *et al.*, 2018).

It is expensive to produce SCPs from bacteria limiting the use thereof. The high cost is mainly due to the harvesting of the small cells, as the cells must be flocculated to achieve a slurry with a higher concentration of solids before the centrifugation process can occur (Anupama and Ravindra, 2000). Bacteria are also known to have a high nucleic acid content, between 15% and 16% (Anupama and Ravindra, 2000). Therefore, final processing steps are required to reduce the nucleic acid content.

The production of SCPs from bacteria is promising due to their fast growth rates and high protein content. However, further investigation on using steam-pretreated lignocellulosic biomass as a substrate for the growth of bacterial biomass is required.

Table 2.9: Summary of the protein content, substrate utilised and fermentation process of bacterial strains investigated as potential sources of SCP.

Microorganism	Substrate	Fermentation process	Protein content (%)	Substrate pretreatment method	GRAS status	Cellulolytic	Vitamins	Antioxidants	References
Bacillus subtilis	Ram horn	Submerged	71	Acid	Yes	Yes	B2, K2	Yes	(Kurbanoglu and Algur, 2002; He, Zhang and Lu, 2010; Lefevre *et al.*, 2017)
	Soybean hull	Submerged	12	-					
Cellulomonas biazotea	Kallar grass	Submerged	60	Alkali	Yes	Yes	-	-	(Anupama and Ravindra, 2000; Rajoka, 2005)
Lactobacillus acidophilus	*Stickwater	Submerged	68 - 71	-	Yes	Yes	B2, B11, B12	Yes	(LeBlanc *et al.*, 2011; Kam, Kenari and Younesi, 2012; Naghmouchi *et al.*, 2019; Baghbani-Arani, Asgary and Hashemi, 2020; Yonsei University, 2021)
Lactobacillus delbrueckii subsp. *bulgaricus*	Dried whey	Submerged	33	-	Yes	Unknown	B11, B12	Yes	(El-Sabaeny, 1996; Yin *et al.*, 2017; Baghbani-Arani, Asgary and Hashemi, 2020)
Lactobacillus fermentum	MRS broth	Submerged	60 - 80	-	Yes	Yes	B2, B11, B12	Yes	(Nielsen *et al.*, 2007; Melo *et al.*, 2017; Naghmouchi *et al.*, 2019; Kannan *et al.*, 2020; Yonsei University, 2021)
Lactococcus lactis	Sugarcane bagasse	Submerged	-	Steam explosion and acid hydrolysis	Yes	Yes	B11	Yes	(LeBlanc *et al.*, 2011; Song *et al.*, 2017; Yonsei University, 2021)

Rhodobacter sphaeroides	Soybean wastewater	Submerged	52	-	Yes	Yes	B12, E	Yes	(Noparatnaraporn and Nagai, 1986; He, Zhang and Lu, 2010; Yonsei University, 2021)
	Pineapple waste	Submerged	67						
Streptococcus thermophilus	Trypticase soy broth	Submerged	-	-	Yes	Yes	B1, B6, B11	Yes	(Erdman, Bergen and Adinarayana Reddy, 1977; LeBlanc *et al.*, 2011; Kanmani *et al.*, 2013; Umamaheswari *et al.*, 2014; Gu and Li, 2016; Tarrah *et al.*, 2018; Yonsei University, 2021)

* Liquid obtained from the processing of meat or organic waste

2.7.2 Fungal protein

Using fungi as a protein source would be beneficial as fungi are well-studied and have been characterised accordingly, leading to less resistance from consumers, as fungi are traditionally eaten (Trinci, 1992). Additionally, various fungal strains have been associated with meat for many years and have been described as having a meaty flavour (Trinci, 1992).

Fungal strains used for producing SCPs have a protein content of between 30% and 50% and a well-balanced amino acid composition that compares well with the FAO guidelines (Appendix A) (Anupama and Ravindra, 2000; Nasseri *et al.*, 2011; Ritala *et al.*, 2017). However, it has been noted that the content of threonine and lysine is generally high, while that of methionine is usually lower in fungi than in other SCP sources (Ritala *et al.*, 2017). The protein content of the investigated fungal strains depends on the substrate used and differs between the various strains (Table 2.10).

Mycoprotein can replace conventional protein sources that humans are currently consuming, as fungal fermentations are more efficient at converting carbohydrates into edible proteins and are more cost-effective than that of common livestock (Trinci, 1992). Mycoprotein can convert 1 kg of carbohydrates and inorganic nitrogen to 136 g of protein, while common livestock can only produce between 14 g to 49 g of protein from 1 kg of feed (Anupama and Ravindra, 2000).

Various fungal strains have been found to produce vitamins, specifically from the B-complex group (biotin, thiamine, folic acid and so forth) as well as provide a source of fibre, specifically chitin and glucan (Trinci, 1992; Sadler, 2003; Ritala *et al.*, 2017). Mycoproteins have a low sodium content and contain sufficient amounts of zinc and selenium for human consumption (Trinci, 1992; Denny, Aisbitt and Lunn, 2008). However, mycoproteins' iron and vitamin B12 contents are lower than in red meat (Denny, Aisbitt and Lunn, 2008). Additionally, the iron contained in mycoproteins is present in an inorganic form and is not as well absorbed as the haem iron that meat contains (Trinci, 1992). Mycoprotein offers a protein source that is low in calories, has no cholesterol and is low in saturated fats (Trinci, 1992). Fungi also produce a variety of lipids, including phospholipids, sterols, sterol esters, mono-, di- and tri-glycerides and free fatty acids (Sadler, 2003). The lipid content of fungi is generally minor. It, therefore, does not add to the nutritional value of the fungi other than the presence of ergosterol, which is the most important of the pro-vitamin D group. Additionally, the fatty acids that are present in fungi are predominantly unsaturated (Breene, 1990). Fungi have low to moderate nucleic acid content, between 7% to 10%, which is too high for human consumption (Anupama and Ravindra, 2000; Nasseri *et al.*, 2011; Ritala *et al.*, 2017). Therefore, additional processing steps are required to reduce the nucleic acid content of the fungal biomass.

Fungi are one of the only major organisms that have the capability to break down and modify lignin that is present in lignocellulosic biomass (Nasseri *et al.*, 2011). However, this is a slow process and pretreatment of the lignocellulosic biomass is required to increase the bioconversion thereof. Additionally, filamentous fungi are more easily harvested than bacterial and yeast biomass (Trinci, 1992; Nasseri *et al.*, 2011). However, their slower growth rate and possibly lower protein yield are disadvantages (Nasseri *et al.*, 2011).

2.7.2.1 Yeast protein

Yeast is an ideal source of SCPs as it has a high nutritional quality and produces high yields of protein. Yeast has a protein content of between 45% and 55% and nucleic acid content of between 6% and 12% (Nasseri *et al.*, 2011). The high nucleic acid content of yeast reveals that further processing of the yeast biomass is required to reduce the nucleic acid content to ensure that the yeast is safe for human consumption. The protein content of the investigated yeast strains depends on the substrate used and differs between the various strains (Table 2.10).

Yeast is easily harvested from fermented media due to its large cell size. Additionally, yeast can grow in acidic media, which is advantageous as it helps to reduce the risk of contamination from other microbes. Spent brewer's yeast (*S. cerevisiae*) has been used commercially as a yeast extract in Marmite®, Vegemite® and other spreads. This confirms yeast's acceptance as a food source for human consumption (Nasseri *et al.*, 2011). Additionally, yeast is a good source of five crucial group B-vitamins and is a rich source of glutamic acid (Ritala *et al.*, 2017).

There have been many studies on the use of yeast, specifically *S. cerevisiae*, to produce second-generation ethanol from sugarcane bagasse. However, little investigation has been conducted on the use of yeast as an alternative protein source.

Table 2.10: Summary of the protein content, substrate utilised and fermentation process of fungal strains investigated as potential sources of SCP.

Microorganism	Substrate	Fermentation process	Protein content (%)	Substrate pretreatment method	GRAS status	Cellulolytic	Vitamins	Antioxidants	References
Candida langeronii	Sugarcane bagasse	Submerged	48	Acid	Yes	Yes	-	-	(Nigam, 2000)
Candida tropicalis	Sugar beet bagasse	Submerged	51 – 54	Enzymes	No	Yes	-	-	(Pessoa, Mancilhab and Sao, 1996; Gao, Li and Liu, 2012; Patelski *et al.*, 2015)
	Sugarcane bagasse	Submerged	31	Acid					
	Soy molasses	Submerged	56	-					
Candida utilis	Potato wastewater	Submerged	28 – 49	-	Yes	Yes	B1, B2, B3, B5, B6, B7, B9, B12, β-carotene,	Yes	(Munawar *et al.*, 2010; Kurcz *et al.*, 2018; Mogmenga *et al.*, 2019)
	Fruit waste	Submerged	49	-					
Chaetomium cellulolyticum	Hardwood sawdust	Solid	13	Alkali	No	Yes	-	-	(Pamment *et al.*, 1978; Chavez, Touchburn and Moo-Young, 1988; Upadhyaya *et al.*, 2016)
	Molasses	Submerged	45	-					
Fusarium venenatum	Glucose	Submerged	42 – 44	-	Yes	Yes	A, B1 - B3, B5 - B7, B9	Yes	(Trinci, 1992; Denny, Aisbitt and Lunn, 2008; Hosseini and Khosravi-Darani, 2011; Nasseri *et al.*, 2011; Finnigan, Needham and Abbott, 2017; Filho *et al.*, 2019)
	Date juice	Submerged	46	-					
Paecilomyces variotii	Eucalyptus	Submerged	34	Acid	No	Yes	-	Yes	(Bajpai and Bajpai, 1987; Almeida e Silva *et al.*, 1995; Pereira *et al.*, 2010; Nasseri *et al.*, 2011)
	Rayon pulp mill waste	Submerged	45	Alkali					
Penicillium janthinellum	Sugarcane bagasse	Submerged	46	Steam	No	Yes	-	-	(Rao, Varma and Deshmukh, 2010; Ritala *et al.*, 2017)

Pleurotus florida	Corn straw	Solid	26 – 30	-	Yes	Yes	B1, B2, B3, B9	Yes	(Ahmed *et al.*, 2008; Khan *et al.*, 2008; Ahmadi *et al.*, 2010; Chanakya, Malayil and Vijayalakshmi, 2015; Fakruddin, Hossain and Ahmed, 2017; Salami, Bankole and Salako, 2017; Raman *et al.*, 2021)
	Paddy straw	Solid	23	Pasteurised					
Pleurotus ostreatus	Sawdust, rice bran & potato dextrose mix	Submerged	30	-	Yes	Yes	B1, B2, B3, B9	Yes	(Patil *et al.*, 2010; Mumpuni *et al.*, 2017; Raman *et al.*, 2021)
		Solid	25	-					
	Wheat straw	Solid	21	-					
Pleurotus sajor-caju	Corn stover	Submerged	40 – 49	Alkali	Yes	Yes	B1, B2, B3, B9	Yes	(Chahal, 1989; Gupta *et al.*, 2013; Kandasamy *et al.*, 2020; Raman *et al.*, 2021)
	Wheat straw and mahua cake	Solid	27 – 35	-					
S. cerevisiae	Cactus pear waste	Submerged	27	Acid	Yes	Yes	A, C, D2, E, B1 - B3, B5 - B7, B9, B12	Yes	(Mondal *et al.*, 2012; Haddish, 2015; Hezarjaribi, Ardestani and Ghorbani, 2016; Samadi, Mohammadi and Najafpour, 2016; Fakruddin, Hossain and Ahmed, 2017; Mogmenga *et al.*, 2019)
	Sugarcane bagasse	Solid	13	Alkali					
	Cucumber waste	Solid	53	-					
	Orange peels	Solid	31	-					
	Glucose	Submerged	45	-					

2.7.3 Strain Selection

Six microbial strains, consisting of bacterial and fungal strains, were investigated, namely *Bacillus subtilis, Lactobacillus delbrueckii, Streptococcus thermophilus* (bacterial strains), *Fusarium venenatum, Pleurotus ostreatus* (fungal strains) and *Saccharomyces cerevisiae* (yeast strain). The strains were selected based on their GRAS status, high nutritional value and their availability. The bacterial strains were of particular interest as there has been little investigation into the production of SCPs from bacterial strains, especially when grown on steam-exploded sugarcane bagasse. Additionally, the three bacterial strains are used in probiotic supplements, contain various vitamins and has antioxidant properties.

2.7.3.1 Bacillus subtilis

B. subtilis has GRAS status, is a commercially produced probiotic and has been extensively studied and used in the fermentation of soybeans (He, Zhang and Lu, 2010; Lefevre *et al.*, 2017). When grown under SF conditions on hydrolysate derived from rams' horns, *B. subtilis* was found to have a total protein content of 71% and achieved a biomass concentration of 5.2 g/L (Kurbanoglu and Algur, 2002). Zheng et al. (2015) found that *B.* subtilis is capable of degrading furfural (an inhibitor produced during STEX), as it achieved 31.2% furfural degradation. Furthermore, *B. subtilis* is known to have a well-balanced amino acid composition (Appendix A), which is ideal for human consumption. However, its ability to produce SCP from steam-exploded sugarcane bagasse has received little attention.

2.7.3.2 Saccharomyces cerevisiae

S. cerevisiae was used as a control as it is a well-studied strain, specifically for producing bioethanol from steam-exploded sugarcane bagasse and is well known for its high tolerance to inhibitors (Rudolf *et al.*, 2008). Cavka and Jornsson (2014) compared the inhibitor tolerance of five microorganisms, namely, *Aspergillus niger, Trichoderma reesei* (fungal strains), *Yarrowia lipolytica*, *Pichia pastoris* and *S. cerevisiae* (yeast strains), and found that *S. cerevisiae* had the highest inhibitor tolerance as it was able to grow in high concentrations of hydrolysate, unlike the other four strains. The authors further suggested that *S. cerevisiae* be used in consolidated bioprocessing as it can be used for both the fermentation process and the production of enzymes (Van Zyl *et al.*, 2007; Cavka and Jornsson, 2014). Additionally, *S. cerevisiae* is capable of converting furfural and 5-HMF present in fermentation media to 5-HMF alcohol and 5-hydroxymethyl furan carboxylic acid, respectively (Taherzadeh *et al.*, 2000). Therefore, explaining *S. cerevisiae's* tolerance to various inhibitors.

2.7.3.3 Lactobacillus delbrueckii

L. delbrueckii is a well-known probiotic that has been found to have the ability to maintain the immune system of the elderly (Moro-garcía *et al.*, 2013). This bacterial strain is already present in many food sources and thus is safe for human consumption (Duggal, 2020). There has been limited research into the production of SCPs from this strain.

2.7.3.4 Streptococcus thermophilus

S. thermophilus is a thermophilic bacterial strain and is produced industrially for the production of cheese and yoghurt, and as such, has GRAS status (Tarrah *et al.*, 2018). *S. thermophilus* is a probiotic, aids in inhibiting some intestinal pathogens and has an amino acid composition that is comparable to that of the FAO standard (Appendix A). Moreover, it aids in the prevention of side effects caused by antibiotics. *S. thermophilus* may have the ability to utilise lignocellulosic biomass (Umamaheswari *et al.*, 2014). However, its ability to produce SCP has not been extensively studied and reveals a gap in research.

2.7.3.5 Fusarium venenatum

F. venenatum is a filamentous fungus that is commercially grown to produce mycoprotein for human consumption by Quorn® and has a minimum protein content of 42% (w/w) (Trinci, 1992). When grown commercially by Quorn®, *F. venenatum* is grown on a glucose-ammonia-biotin-mineral salts media and is grown under submerged fed-batch fermentation conditions in an airlift bioreactor at a temperature of 30 °C (Trinci, 1992; Denny, Aisbitt and Lunn, 2008). Furthermore, it was found to have a maximum growth rate (μ_{max}) of 0.28 h^{-1} and a doubling time of 2.5 h (Trinci, 1992). However, minimal research has been conducted on the bioconversion of lignocellulosic biomass by *F. venenatum*. Furthermore, the use of lignocellulosic biomass as a substrate instead of glucose could reduce production costs.

2.7.3.6 Pleurotus ostreatus

P. ostreatus is a GRAS microorganism whose fruiting body is consumed worldwide. *P. ostreatus* produces hydrolytic and oxidative extracellular enzymes that aid in the degradation of lignocellulosic biomass (Philippoussis, 2009; Nasseri *et al.*, 2011). Therefore, it would be beneficial to investigate *P. ostreatus* as a candidate for SCP production from steam-exploded sugarcane bagasse. Furthermore, *P. ostreatus* achieved a higher protein content when grown under SF conditions (29.76% (w/w)) as compared to S-SF conditions (24.69% (w/w)) when grown on a mixture of sawdust, rice bran and potato dextrose (Mumpuni *et al.*, 2017). *P. ostreatus* has a well-balanced mineral and amino acid composition (Appendix A) and is a good source of vitamin B and folic acid (Patil *et al.*, 2010).

2.8 Factors influencing the production of SCPs

The variables that affect the volumetric productivity (g/(L·h)) and the concentration (g/L) of microbial strains used for the production of SCPs are the environmental conditions (pH and temperature) and the composition of the media (Fatemeh, Reihani and Khosravi-Darani, 2019). The method of substrate pretreatment used and the type of substrate utilised also affect the productivity and yield of the microorganisms (Anupama and Ravindra, 2000). The subsequent sections discuss these factors and their influences in more detail.

2.8.1 Temperature and pH

Temperature is one of the main factors that influence microbial productivity and yield (Fatemeh, Reihani and Khosravi-Darani, 2019). The optimal operating temperature varies for various microbial strains. Therefore, it is frequently used as a variable in factorial designs to determine the optimal growth

conditions of microbes. The optimal operating temperature range for the growth of most microorganisms for SCPs is between 25 °C and 35 °C (Table 2.11). It is ideal to work at a higher operating temperature as it would reduce the risk of contamination by other microorganisms (Nigam, 2000).

The pH of the growth media also affects the growth of microorganisms (Basu *et al.*, 2015). All microorganisms have an optimal pH for growth (Table 2.11). Microorganisms can either be acidophilic (from pH 0 to pH 5.5), neutrophilic (from pH 5.5 to pH 8.0) or alkalophilic (from pH 8.5 to pH 11.5). Most bacterial strains are classified as neutrophiles as they prefer a more neutral pH.

The metabolism of fungi requires a more acidic environment due to the transmembrane proton motive force (the force that aids proton movement across membranes), which is vital for fungal nutrition and for regulating the cytoplasmic pH (Slayman, 1985; Kavanagh, 2005; Fatemeh, Reihani and Khosravi-Darani, 2019). A more acidic media reduces the risk of contamination as few microorganisms can grow optimally under acidic conditions (Nigam, 2000). As discussed in the subsequent section, the effect of pH on various microorganisms is crucial when nitrogen supplementation is required, as the nitrogen source selected changes the pH.

Table 2.11: Optimal temperature and pH ranges for various microbial strains.

Microorganisms	Optimal operating conditions		References
	Temperature (°C)	pH	
Bacterial strains			
Bacillus subtilis	30	7.0	(Kurbanoglu and Algur, 2002)
Bifidobacterium animalis	36 – 38	6.5 – 7.0	(Shah, 2011; Biavati and Matterelli, 2015)
Bifidobacterium longum	36 – 38	6.5 – 7.0	(Shah, 2011; Biavati and Matterelli, 2015)
Lactobacillus acidophilus	30 – 37	5.5 – 6.8	(Erdman, Bergen and Adinarayana Reddy, 1977; Kam, Kenari and Younesi, 2012)
Lactobacillus bulgaricus	30 – 47	5.5 – 6.8	(Erdman, Bergen and Adinarayana Reddy, 1977; Radke-Mitchell and Sandine, 1986)
Lactobacillus fermentum	30 – 37	5.0 – 7.0	(Erdman, Bergen and Adinarayana Reddy, 1977)
Lactococcus lactis	30	6.3 – 6.9	(Rault, Bouix and Béal, 2009; Chen *et al.*, 2015)
Rhodopseudomonas palustris	25 – 40	5.5 – 8.5	(Çetinkaya Dönmez, Öztürk and Çakmakçi, 1999; Fang *et al.*, 2012)
Streptococcus thermophilus	30 – 45	6.5 – 7.0	(Rault, Bouix and Béal, 2009; Tarrah *et al.*, 2018)
Fungal strains			
Candida langeronii	40 – 42	6.0	(Nigam, 2000)
Candida tropicalis	30	6.0	(Patelski *et al.*, 2015)
Candida utilis	30 – 35	5.0 – 6.0	(Munawar *et al.*, 2010; Akanni *et al.*, 2015)
Fusarium venenatum	30	5.8	(Trinci, 1992; Prakash, Namashivyam and Narendrakumar, 2014)
Lentinula edodes	26 – 28	3.0 – 4.0	(Quaicoe *et al.*, 2014; Matjuškova *et al.*, 2017; Krupodorova *et al.*, 2019)
Neurospora sitophila	35 – 37	5.5	(Moo-Young, Chisti and Vlach, 1993)
Paecilomyces variotii	30	6.0	(Bajpai and Bajpai, 1987)
Penicillium janthinellum	28		(Rao, Varma and Deshmukh, 2010)
Pleurotus florida	21 – 25	6.5 – 7.0	(Chanakya, Malayil and Vijayalakshmi, 2015)(Bellettini *et al.*, 2019)
Pleurotus ostreatus	20 – 35	6.5 – 7.0	(Chahal, 1989; Bellettini *et al.*, 2019)
Pleurotus sajor-caju	30	6.0	(Chahal, 1989)
Saccharomyces cerevisiae	25 - 37	5.5	(Haddish, 2015; Kumar, Gautam and Dutt, 2016; Mensah and Twumasi, 2016; Samadi, Mohammadi and Najafpour, 2016)

2.8.2 Nitrogen source and concentration

Nitrogen supplementation of the growth media is needed as lignocellulosic biomass has a low nitrogen content and is required to produce important amino acids, which is necessary to produce a high-quality protein (Lee, 1997; Kavanagh, 2005). The nitrogen source and concentration directly affect the cost of production and the biomass yield achieved by the microorganisms (Fatemeh, Reihani and Khosravi-Darani, 2019). Therefore, a low-cost nitrogen source would be ideal to ensure that the final product is affordable.

The most commonly used nitrogen sources for the growth of microorganisms are ammonia, ammonium salts, urea, nitrate and organic nitrogen (Fatemeh, Reihani and Khosravi-Darani, 2019). The selected nitrogen source depends on the microorganisms' preferred pH. Nitrate addition causes an increase in the pH, while ammonium causes a decrease in the pH (Clarke, 2013). Therefore, fungi, which prefer a lower pH, favour supplementation with an ammonium salt, while bacteria prefer a neutral to a higher pH and favour a nitrate salt. Ammonium sulphate, an inorganic nitrogen source, is commonly used in fungal growth media as it provides a source of utilisable sulphur (Kavanagh, 2005).

Corn steep liquor (CSL) is an organic nitrogen source and a by-product of the starch industry. CSL is a low-cost source of nitrogen and, if used instead of ammonia and yeast extract, should reduce operating costs (Fatemeh, Reihani and Khosravi-Darani, 2019). CSL is used as a replacement for yeast extract as it is a cheaper alternative that contains a mixture of amino acids, vitamins, minerals and trace elements (Tan *et al.*, 2016; Taiwo, Madzimbamuto and Ojumu, 2018). The suspended solids in CSL are rich in organic nitrogen (Loy and Lundy, 2018). The effect of different nitrogen sources on the cell mass production of *Candida utilis* was investigated and it was found that the use of CSL increased the cell mass production by about 47.1%, while urea resulted in adverse effects (Zhao, Zhang and Zhang, 2010). Additionally, CSL resulted in the best growth rate and protein content of *C. utilis*. The other nitrogen sources investigated were ammonium sulphate, ammonium nitrate, sodium nitrate and urea (Rajoka *et al.*, 2006).

2.8.3 Inoculum size and age

The optimum microbial growth depends on the size and age of the seed culture, as they influence the biomass yield. Different inoculum sizes influence various microbial strains' final biomass concentrations (Fatemeh, Reihani and Khosravi-Darani, 2019). The larger the inoculum size, the higher the cell density. The higher cell density ensures that high cell viability is achieved for a longer time (Bezuidenhout, 2021). Therefore, in a growth media that contains a high concentration of inhibitors, a higher cell density is desired.

Additionally, the size and age of the seed culture affect the cost of the process. An increase in the size of the inoculum leads to an increase in biomass productivity, which in turn could lead to an increase in protein yields (El-Nawwi and El-Kader, 1996; Zhang, Wu and He, 2002; Hosseini and Khosravi-Darani, 2011; Yunus, Nadeem and Rashid, 2015). However, a larger inoculum size poses higher operating costs, as larger equipment would be required.

2.9 Economic assessment

There has been a significant focus on the economics involved in the bioconversion of lignocellulosic biomass to bioethanol. However, there has been less attention on the economics associated with the bioconversion of steam-exploded sugarcane bagasse to SCPs. Much insight can be gained from research into the economics of the bioconversion of steam and the enzymatic pretreated sugarcane bagasse process.

High product yields, such as high protein yields and a high final biomass concentration, are necessary to reduce production costs. Additionally, improved pretreatment methods are required to reduce production costs (Galbe *et al.*, 2007). However, the main costs of the process are incurred through the pretreatment process, specifically thermochemical pretreatment and the enzymatic hydrolysis of the proposed lignocellulosic feedstock (Lynd *et al.*, 2017). It is noted that the cost of the final product is determined by the substrate used, the yield and the nutritive value of the final product (Anupama and Ravindra, 2000).

Enzymatic hydrolysis is a key cost factor when producing high-value products from pretreated lignocellulosic biomass, as the maximum hydrolysis yield is not necessarily the economic optimum (Pihlajaniemi *et al.*, 2020). Therefore, it is important to perform an economic assessment of the process being investigated to produce SCPs on an industrial scale. The economic evaluation will provide insight into which microbial strain investigated would be more economically feasible on an industrial scale. The profitability of the two strains' production processes, the selected strain and *S. cerevisiae* (control), will be compared to determine which is more financially viable.

The profitability of the SCP production process will be determined by calculating the minimum selling price (MSP) of the final biomass product. The MSP is determined through various iterations of the selling price until the net present value (NPV) is zero. The NPV is an important economic indicator and is the difference between the present value of the annual cash flows of the project and the initial investment that was required to start the project of interest (Peters and Timmerhaus, 1991). The stipulated internal rate of return (IRR) is used to determine the profitability of potential investments and is the discount rate that ensures that the NPV is equal to zero for cash flows in a discounted cash flow (DCF) analysis (Annoh-Quarshie, 2018; Fernando, 2022).

2.10 Literature conclusions

SCPs produced from lignocellulosic biomass are generally used for feed in the agricultural sector and have not received much attention as a protein alternative for human consumption. Furthermore, sugarcane bagasse is a commonly used feedstock to produce bioethanol and, in some cases, SCPs. The commonly used pretreatment process used on bagasse is alkali or acid pretreatment. There is minimal research into the use of steam-explode sugarcane bagasse followed by enzymatic hydrolysis for producing SCPs.

The production of fungal mycelium under SF with lignocellulosic biomass as a substrate has received less attention than the production of fungal fruiting bodies under S-SF. Furthermore, bacterial strains have received the least attention for the bioconversion of lignocellulosic biomass to SCP for human

consumption. This is mainly due to the high production costs associated with harvesting the small cells and that bacteria are associated with disease. However, there is much opportunity in bacterial SCP if bacterial strains are found to have a high protein content as they have a fast growth rate and high nutritional value. Additionally, there has been minimal research on the production of SCPs from bacterial strains when grown using steam-exploded sugarcane bagasse. Therefore, there is much insight to gain from the present study.

The use of pulse fed-batch fermentation for producing SCPs grown on steam-exploded and hydrolysed sugarcane bagasse has not been thoroughly investigated to minimise the inhibitors' toxic effects. Therefore, further investigation of the effects of pulse fed-batch fermentation on biomass concentration and protein content of the microbial strains will be insightful.

There has been extensive research into the profitability of the bioconversion of lignocellulosic biomass to bioethanol. However, minimal research has been conducted into the profitability of the bioconversion of steam-exploded sugarcane bagasse to SCPs. Therefore, there is much opportunity to further investigate the economics of this particular production process.

CHAPTER 3:

RESEARCH AIMS AND OBJECTIVES

This investigation aimed to determine whether single-cell protein (SCP) from a generally regarded as safe (GRAS) microbial strain would be a suitable alternative protein source for human consumption when grown on steam- and enzymatically-pretreated sugarcane bagasse. Consequently, the microbial strain is required to produce high yields of protein and be economically feasible.

The aim of this investigation was achieved through the fulfilment of the following objectives:

1. Investigate different enzymatic hydrolysis conditions, hydrolysis times, solid loadings and enzyme dosages, to achieve an 80% conversion of cellulose to glucose as well as produce a hydrolysate with a high glucose concentration at a low enzyme dosage.
2. Perform a screening experiment to identify which microbial strains tolerate the inhibitors present in undiluted enzymatic hydrolysate best by comparing various growth parameters, specifically the proportion of glucose consumed and final biomass concentration, to *S. cerevisiae*, which was used as a control.
3. Make use of a pulse fed-batch process to increase the glucose consumed and to maximise the biomass concentration of the selected strain. The selected strain's protein content, biomass concentration and final glucose concentration are to be compared to that achieved by *S. cerevisiae* to determine the feasibility of the process.
4. Use a pre-existing Aspen Plus® model to determine and compare the technical and economic feasibility of the process when using the selected strain against *S. cerevisiae*.

CHAPTER 4:

SUBMERGED FERMENTATION OF STEAM-PRETREATED SUGARCANE BAGASSE TO PRODUCE SINGLE-CELL PROTEINS

Preface

The change in the composition of raw sugarcane bagasse after steam explosion under a preferred set of conditions, as defined in Theron's unpublished work (2022), was investigated to determine the effects of the selected steam explosion conditions. The change was observed by comparing the composition of raw sugarcane bagasse to that of steam-exploded sugarcane bagasse. The concentration of inhibitors formed, the glucose yield and the concentration of oligosaccharides formed throughout the pretreatment process of the sugarcane bagasse were measured. The steam-exploded sugarcane bagasse was then enzymatically pretreated to convert the cellulose present in the sugarcane bagasse to glucose.

Three different enzymatic hydrolysis conditions, solid loadings, times and enzyme dosages, were investigated to determine the conditions required to obtain a close-to 80% cellulose conversion to glucose of the steam-exploded sugarcane bagasse. An ≈ 80% conversion was selected as this is the goal for bioethanol biorefineries to be commercially viable (Mokomele, 2019). Additionally, the selected enzymatic hydrolysis conditions were to result in high glucose yields while ensuring that the lowest possible enzyme dosage was used.

Furthermore, this chapter investigated the ability of six generally regarded as safe (GRAS) microbial strains to tolerate inhibitors present in the enzymatic hydrolysate. The six microbial strains selected were *Bacillus subtilis CAB1111*, *Lactobacillus delbrueckii*, *Streptococcus thermophilus* (bacterial strains), *Fusarium venenatum*, *Pleaurotus ostreatus* CAB13 and *Saccharomyces cerevisiae* CAB79 (fungal strains). The GRAS strains used for this investigation were selected based on their availability, nutritional value and high protein contents, as established from previous studies. The bacterial strains were selected as they are commercially produced probiotics and little research has been conducted on these strains' capability of converting lignocellulosic biomass to SCP.

The microbial strains' ability to tolerate the inhibitors present was achieved through submerged fermentation (SF) of the undiluted enzymatic hydrolysate. The enzymatic hydrolysate was supplemented with corn steep liquor (CSL) as the nitrogen source and other salts. Various growth parameters of the different microbial strains were calculated and compared with *S. cerevisiae* as a control, as it is a well-studied strain and is known to have a high tolerance for inhibitors when compared to other microbial strains. The strain that tolerated the inhibitors best was selected for further pulse fed-batch fermentations.

The pulse fed-batch fermentation runs allowed for the maximisation of the microbial biomass to be investigated for comparison of the preferred microbe from the selection above to the *S. cerevisiae* control. Additionally, the protein content of the microbial strains after pulse fed-batch fermentation was

determined and the concentration of protein produced from both microbial strains was compared to determine which strain would be more industrially feasible. The experimental results obtained were then used in CHAPTER 5 to determine the economic feasibility of the process.

ABSTRACT

This study evaluated enzymatic hydrolysis conditions to achieve an approximate 80% conversion of cellulose to glucose and a high glucose concentration from sugarcane bagasse pretreated under preferred steam explosion conditions. A final glucose concentration of 42.26 ± 0.80 g/L and a 77.6% ± 0.4% conversion of cellulose to glucose were achieved by enzymatically hydrolysing steam-exploded sugarcane bagasse with a solid loading of 15% (w/v) and a Cellic® Ctec3 enzyme dosage of 7.5 FPU/g DW substrate for 72 h. The resulting enzymatic hydrolysate was used in submerged batch fermentations to determine the inhibitor tolerance of six GRAS microbial strains when grown in the undiluted hydrolysate. *S. cerevisiae* CAB79 was used as a control.

S. cerevisiae outperformed the alternative five strains that underwent screening in undiluted hydrolysate, as it consumed all of the glucose present and achieved a final biomass concentration of 20.37 ± 11.81 g/L after 48 h. Of the five alternative strains, *S. thermophilus* tolerated the inhibitors produced during steam explosion best and consumed 47.48% ± 3.75% of the glucose in the hydrolysate. *S. thermophilus* achieved a final biomass concentration of 1.28 ± 0.17 g/L after 48 h.

The pulse fed-batch fermentations substantially increased the final biomass concentrations of *S. cerevisiae* and *S. thermophilus* by allowing the strains to acclimate to a lower initial inhibitor concentration. *S. cerevisiae* achieved a final biomass concentration of 52.65 ± 0.80 g/L, which was significantly higher than the final biomass concentration of 6.57 ± 0.09 g/L achieved by *S. thermophilus*. The protein concentrations achieved by *S. thermophilus* and *S. cerevisiae* were 4.34 ± 0.10 g/L protein and 24.71 ± 1.44 g/L protein, respectively.

Converting steam-exploded sugarcane bagasse to SCP would be more beneficial using *S. cerevisiae* as it resulted in higher protein and biomass concentrations than *S. thermophilus*. Additionally, the co-production of ethanol with SCP may further enhance the economic attractiveness of this process as the maximum ethanol concentration produced by *S. cerevisiae* during pulse fed-batch fermentation was 8.13 ± 0.27 g/L after 36 h. Although, the co-production thereof is likely to be at the expense of the latter and further investigation is required.

4.1 Introduction

The demand for protein products increases with a continuously growing global population, which places considerable strain on the agricultural sector to keep up with the demands. As plant protein is inefficiently converted to animal-based protein (≈ 6kg of feed to produce ≈ 1kg of animal-based protein), the agricultural sector will be unable to continue meeting the increasing demands for protein, alluding to the unsustainability of the agricultural sector (Ritala *et al.*, 2017). Alternative protein sources that are environmentally friendly, sustainable and provide the required nutrients, especially the correct amino acid composition, have recently received much attention. The production of single-cell proteins (SCPs) from microbial biomass is one such alternative, as many microbial strains are able to convert agro-processing residues, in the form of lignocellulosic biomass, to SCPs. The ability to convert agricultural residues to SCPs for human consumption reveals a biologically efficient method for upcycling lignocellulosic biomass.

Lignocellulosic biomass is a waste material that is produced from various agricultural processes, such as the production of feed and food crops, as well as various forestry practices. Sugarcane bagasse is lignocellulosic biomass produced as a by-product from the sugarcane industry. On an annual basis, approximately 540 million metric tonnes of sugarcane bagasse are produced (Bian *et al.*, 2012; Bezerra, 2016; Alokika *et al.*, 2021). A large portion of the bagasse is incinerated as fuel to fire boilers to produce steam to power the sugar mills. However, burning the bagasse as fuel is inefficient and contributes to air pollution, as large concentrations of particulate matter are released upon incineration (Bizzo *et al.*, 2014; Alokika *et al.*, 2021).

An alternative use for the sugarcane bagasse would be the upcycling thereof to produce SCPs for human consumption. The upcycling of the bagasse will unlock many benefits, especially since sugarcane bagasse is an abundant, renewable and inexpensive feedstock (Tyagi *et al.*, 2019). The benefits that would result include a decrease in the cost of the SCP production process, providing a more affordable protein source to poorer communities and alleviating food and nutrition insecurity (Johnston, Fanzo and Cogill, 2014).

Sugarcane bagasse has a high cellulose composition (32% to 45%), which, once pretreated, would result in high glucose yields. Glucose is the preferred carbon source for microorganisms and therefore, high glucose yields would result in high microbial biomass (Pengilly, 2013; Mokomele, 2019). Pretreatment of the sugarcane bagasse is required to increase the accessibility and availability of the cellulose present and to decrease the time required to bioconvert the bagasse to SCPs (Moo-Young, Chisti and Vlach, 1993; Sánchez, 2009; Nasseri *et al.*, 2011; Fatemeh, Reihani and Khosravi-Darani, 2019).

Steam explosion is the preferred method of pretreatment prior to enzymatic hydrolysis as it does not require downstream processing to remove harmful chemicals, as high-pressure steam is all that is required in the process. Therefore, steam explosion has a low environmental impact and lower operating costs than alternative pretreatment processes that require harmful chemicals (Duque *et al.*, 2016). Steam explosion is a standard pretreatment method as it modifies and partially removes the lignin and hemicellulosic fractions of the sugarcane bagasse. Thus, improving the enzymatic hydrolysis process that

is used to convert the cellulose to glucose and reducing the enzyme dosage required to achieve high yields of glucose (Philippoussis, 2009; Wallace, 2013).

Steam-exploded sugarcane bagasse is a widely studied substrate for the production of bioethanol. However, there is limited research on the bioconversion of enzymatically pretreated steam-exploded sugarcane bagasse to SCPs via batch and pulse fed-batch fermentations under submerged fermentation conditions. In this study, the bioconversion of enzymatically digested steam-exploded sugarcane bagasse to SCP under submerged fermentation using *B. subtilis*, *L. delbrueckii*, *S. thermophilus* (bacterial strains), *F. venenatum*, *P. ostreatus* and *S. cerevisiae* (fungal strains) was investigated to determine which of the six strains is able to tolerate the inhibitors produced during steam explosion best. The bacterial strains were of particular interest as there has been little investigation into the production of SCPs from bacterial strains, especially when grown on steam-exploded lignocellulosic biomass, specifically sugarcane bagasse. Additionally, the three bacterial strains are used in probiotic supplements, contain various vitamins and are known antioxidants. *S. cerevisiae* was used as a control, as it is well-studied and known for its resistance to the inhibitors produced during steam explosion.

4.2 Materials and methods

4.2.1 Approach

The experimental approach that was followed for this investigation is depicted in Figure 4.1 and Figure 4.2. Sugarcane bagasse was dried, sieved and milled before being steam-exploded under preferred pretreatment conditions (185 °C for 10 minutes). After the autocatalyzed steam explosion (STEX), the substrate was pressed, through the use of a hydraulic bench press, to separate the liquid fraction, with a high inhibitor concentration, from the solid fraction. The resulting solid fraction, which still contained approximately 40% to 45% moisture, underwent enzymatic hydrolysis using Cellic® Ctec 3. The enzymatic hydrolysis was conducted at various solid loadings and enzyme dosages to determine which conditions achieved an approximately 80% conversion of cellulose to glucose and a high glucose concentration. An ≈ 80% conversion was selected as this is the goal for bioethanol biorefineries to be commercially viable (Mokomele, 2019). Therefore, as the production of bioethanol from lignocellulosic biomass is a well-studied and established process, it is desirable to ensure the same conversion is achieved in the SCP production process. The hydrolysate of the enzymatic hydrolysis obtained at the desired conditions was filtered and supplemented with corn steep liquor (CSL) and other salts. The resulting hydrolysate was used in the submerged batch fermentations.

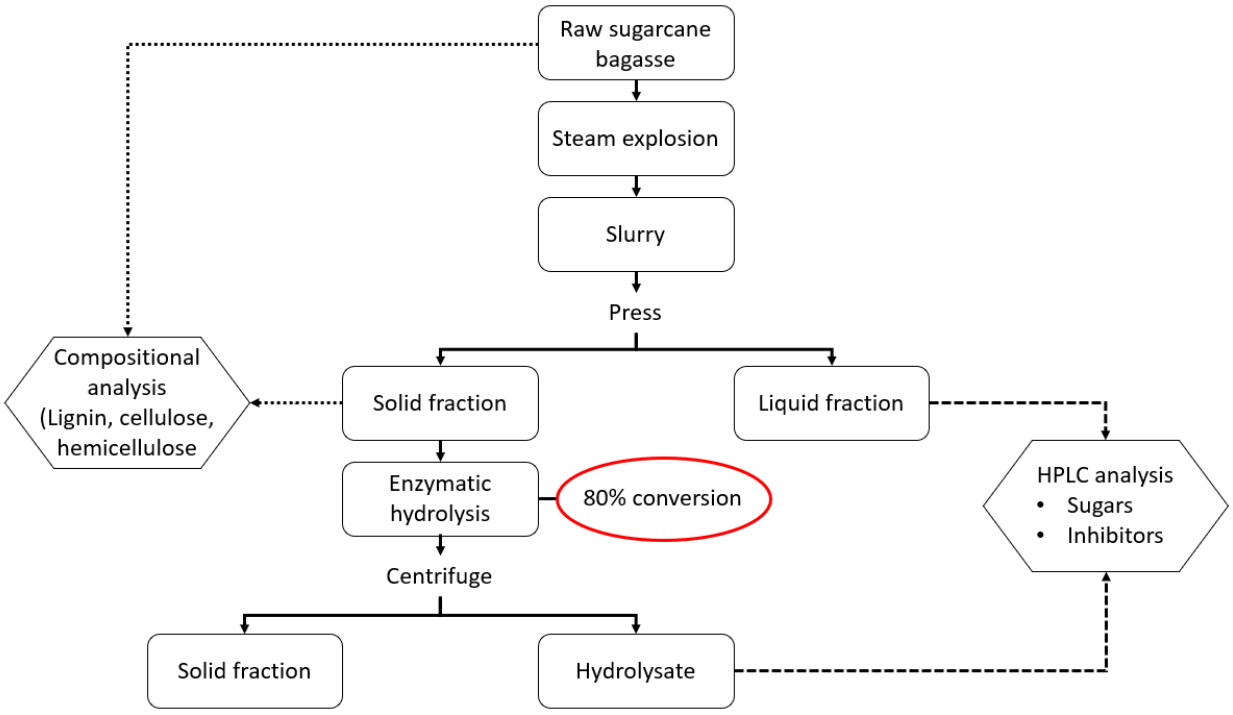


Figure 4.1: Experimental approach followed to pretreat the sugarcane bagasse.

A screening experiment was conducted to determine which microbial strains selected were able to tolerate the inhibitors present in the undiluted hydrolysate. The strains selected all have GRAS status. The various microbial strains' growth parameters were compared to the growth parameters obtained for *S. cerevisiae,* as it is known to have a high inhibitor tolerance. The microbial strain that was identified as being able to tolerate the inhibitors was further investigated in a pulse fed-batch fermentation to

investigate the effect of pulse fed-batch fermentation on the microbe's final biomass concentration and determine the microbe's protein content (Figure 4.2). Once again, *S. cerevisiae* was used as a control.

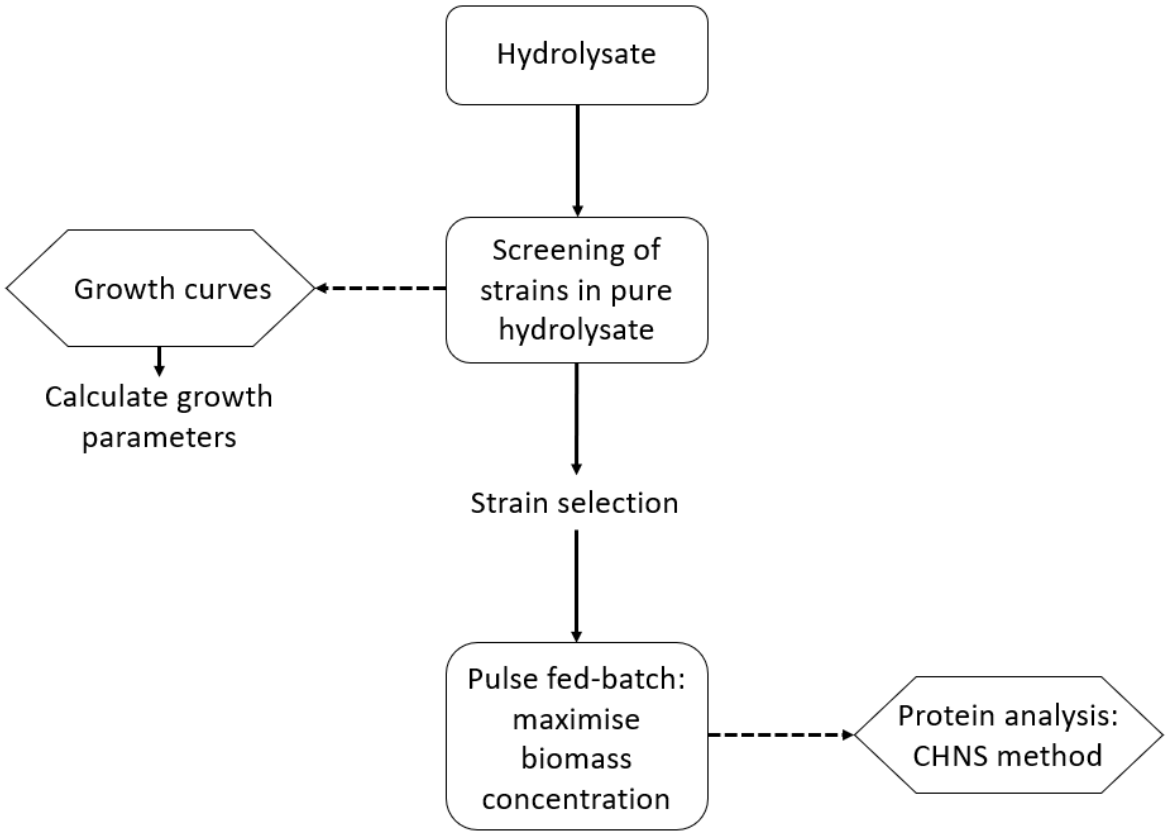


Figure 4.2: Experimental approach followed for selecting the microbial strain and maximising the final biomass concentration of the selected microbial strain.

4.2.2 Materials

Sugarcane bagasse was kindly donated by RCL Sugar, Malelane, Mpumalanga Province, South Africa. The enzyme used for enzymatic saccharification, Cellic® CTec3, was obtained from Novozymes, Denmark.

In order to complete this investigation, a variety of chemicals were required (Table 4.1). The same chemicals from the same suppliers were used throughout to remove variability between the various experimental runs. The procedures in the subsequent sections mention the specific use of the chemicals.

Table 4.1: Chemicals and their properties used in this investigation.

Chemical name	Chemical formulae	Molar mass (g/mol)	Purity (%)
Agar	-	-	-
Bacteriological peptone	-	-	-
Calcium chloride	$CaCl_2$	110.98	≥97
Citric acid anhydrous	$H_3C_6H_5O$	210.14	99.9
Corn steep liquor	-	-	-
D-(+)-Glucose	$C_6H_{12}O_6$	198.17	99.6
D-(+)-Xylose	$C_5H_{10}O_5$	150.13	≥99
Magnesium sulphate heptahydrate	$MgSO_4 \cdot 7H_2O$	120.37	98
Potassium dihydrogen phosphate	KH_2PO_4	136.09	99
Potassium hydroxide	KOH	56.11	85
Tri-sodium citrate	$Na_3C_6H_5O_7 \cdot 2H_2O$	294.10	99
Yeast extract powder	-	-	-

4.2.3 Analytical and experimental methods

4.2.3.1 Substrate preparation

The sugarcane bagasse was dried to achieve a moisture content of approximately 12%. The moisture content was determined using a moisture analyser (AND ML-50). Once the desired moisture content was achieved, the bagasse was quarter-sampled and sieved. Quarter-sampling involves quartering the bagasse, taking one quarter at a time, and re-mixing the four individual quarters. This method ensured that the bagasse was well-mixed and that each bag was representative of all the bagasse in terms of quality and makeup. The sieving allowed for various impurities, such as sand, bagasse pith and fines, to be removed. The well-mixed bagasse was milled using a knife-mill and placed in zip-tied plastic bags before undergoing STEX.

4.2.3.2 Steam explosion

STEX of the milled bagasse was achieved using an automated batch pilot-scale unit (IAP GmBH, Graz, Austria) equipped with a 19 L stainless steel reactor vessel and a 100 L expansion vessel (Figure 4.3). The reactor vessel was fitted with a 40-bar steam boiler, which makes use of saturated steam to reach the desired temperatures.

The STEX reaction vessel was top loaded with about 600 g milled bagasse per batch and was directly heated with 30 bar (abs) saturated steam. The bagasse was heated to a temperature of 185 °C and a pressure of 10 bar. After a holding time of 10 minutes, the bagasse was discharged into an expansion vessel, which was maintained at atmospheric pressure. This allowed for immediate depressurisation of the pretreated substrate to occur. The conditions selected were based on the findings of Hamann (2020) and the unpublished data of Theron (2022) and Bothma (2022).

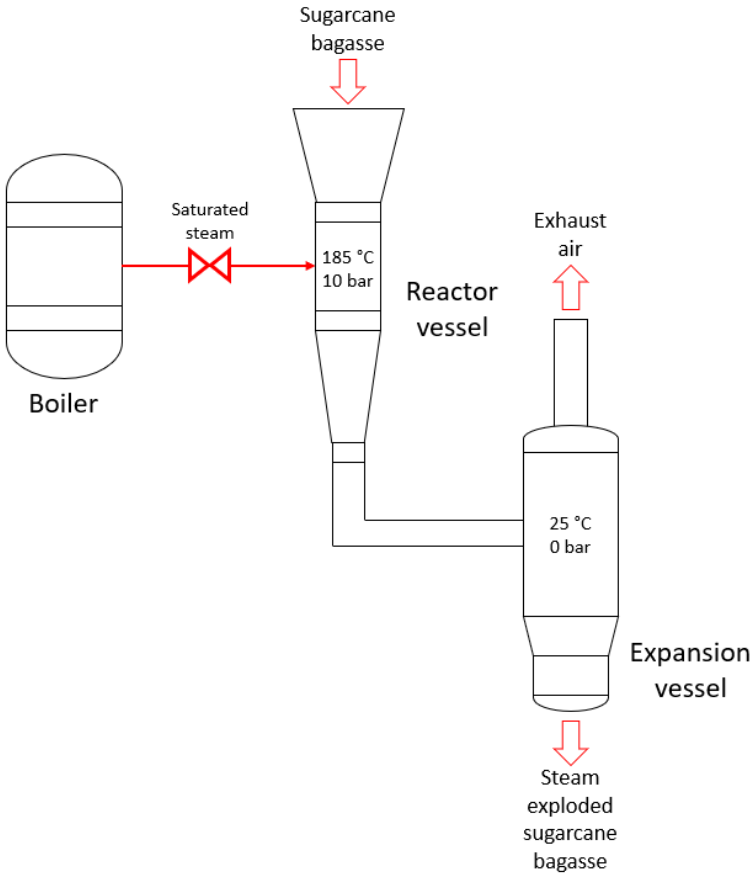


Figure 4.3: Simplified flow diagram of the automated batch pilot-scale unit used for the steam explosion of the sugarcane bagasse.

The severity of the steam explosion pre-treatment process was estimated through the calculation of a severity factor, which considers the effects of both temperature and retention time. The lower the severity of the steam explosion process, the lower the inhibitor concentration and the lower the digestibility of the product. Therefore, selecting pretreatment conditions that maximise the digestibility of the product to ensure a lower enzyme dosage is required as well as ensuring a low inhibitor concentration, is essential.

The severity factor of the STEX conditions was calculated by taking the logarithm of the reaction ordinate (Equation C.2). The reaction ordinate was determined through the use of Equation C.3 below (Overend, Chornet and Gascoigne, 1987; Martín *et al.*, 2021).

$$SF = log(R_o) \quad 4.1$$

$$R_0 = t \cdot exp\left(\frac{T_r - 100}{14.75}\right) \quad 4.2$$

Where t is the holding time and T_r is the STEX process's temperature.

The resulting steam-pretreated bagasse (slurry) was collected from the expansion vessel and weighed. The slurry had a moisture content of between 72% and 75%. The slurry was separated into liquid and

solid fractions by making use of a hydraulic bench press. The pressed solids had an approximate moisture content of between 40% and 45%. The moisture was determined using a moisture analyser (AND ML-50).

Triplicates of the pretreated solids were taken to determine the composition of the substrate after steam explosion. These triplicates were pressed and their liquor was analysed with high-performance liquid chromatography (HPLC) to determine the sugar and inhibitor concentrations. The liquor was also subjected to post-acid-hydrolysis (described further in Section 4.2.3.4) to determine the concentration of oligosaccharides that were present. The solid fraction was washed to determine the water-soluble solids (WSS), water-insoluble solids (WIS) and, thus, the total solids.

4.2.3.3 Composition analysis

The cellulose, hemicellulose and lignin composition of the milled and steam-exploded sugarcane bagasse was determined according to National Renewable Energy Laboratory's (NREL) standard laboratory analytical procedures (Sluiter *et al.*, 2012).

4.2.3.4 Post-acid hydrolysis

Post-acid hydrolysis using dilute sulphuric acid (H_2SO_4) allows for the recovery of oligomeric sugars, present in the liquid fraction of the steam-exploded sugarcane bagasse, in monomeric form (Mokomele, 2019; Pihlajaniemi *et al.*, 2020). The post-acid hydrolysis was performed by using 4% H_2SO_4 at 121 °C for 1 h on the liquid fraction of the steam-exploded sugarcane bagasse (Pihlajaniemi *et al.*, 2020). The procedure followed was according to the NREL protocols (Sluiter *et al.*, 2008). The concentration of oligosaccharides was determined through the use of a mass balance by taking the difference in the monomeric sugar concentration before and after post-acid hydrolysis as well as considering the additional formation of inhibitors (Swart, 2021). The sugar and inhibitor concentrations were determined using HPLC (Bio-Rad Aminex HPX-87H column, 5 mM H_2SO_4 eluent at 0.6 mL/min, 65 °C).

4.2.3.5 Enzyme properties

A commercial enzyme, Cellic® CTec3, obtained from Novozymes, Denmark, was used for the enzymatic hydrolysis. The enzyme cellulase activity was found to be 148 FPU/mL and the step-by-step method followed is provided in APPENDIX B.

The cellulase activity of the Cellic® CTec3 enzyme was determined through the use of the standardised filter paper assay (FPA), recommended by the International Union of Pure and Applied Chemistry (IUPAC) (Wood and Bhat, 1988). The protocols by Zhang et al. (2009) and Wood and Bhat (1988) were used with the exception that HPLC was used to determine the glucose released instead of the DNS method.

4.2.3.5.1 Digestibility of steam-exploded sugarcane bagasse

The digestibility of the steam-pretreated bagasse was determined by enzymatically hydrolysing 2% (w/v) water insoluble solids (WIS) in 250 mL baffled Erlenmeyer flasks with a working volume of 100 mL using a 0.05 M sodium citrate buffer adjusted to pH 5. An enzyme dosage of 15 FPU/g DW substrate of Cellic® CTec3 was added to the flasks. The flasks were incubated at a temperature of 50 °C for a period of 72 h in an orbital shaker at 150 rpm. Samples were taken at 0 h and 72 h and were prepared for sugar analysis

via HPLC (Pengilly, 2013; Koekemoer, 2018; Hamann, 2020). The enzymatic hydrolysis was completed in triplicates for statistical purposes.

The procedure that was followed was according to the standard laboratory analytical procedures stipulated by the NREL procedure (Resch, Baker and Decker, 2015). WIS was prepared according to the procedure described by Hamann (2020), where the pressed solids were washed with excess reverse osmosis (RO) water to remove the residual soluble solids. The mass ratio that was used was 1:10 solids to water. After washing, the excess water was removed and the resulting solids had a moisture content of between 77% and 80%. The washed solids were then subjected to enzymatic hydrolysis as described above.

4.2.3.5.2 Solid loading, enzyme dosage and hydrolysis duration

Solid loadings of 10% (w/v) and 15% (w/v), on a dry basis, of pressed sugarcane bagasse were used in 250 mL baffled Erlenmeyer flasks, with a reaction volume of 100 mL 0.05 M sodium citrate buffer at a pH 5.0. A solid loading of 20% (w/v) was also investigated. The flasks were incubated in an orbital shaker at a temperature of 50 °C and 150 rpm for 72 h. Different enzyme dosages were investigated: 5 FPU/g DW substrate, 7.5 FPU/g DW substrate, 10 FPU/g DW substrate and 15 FPU/g DW substrate. Samples were taken at 0 h, 24 h, 48 h and 72 h and these samples were analysed via HPLC to determine the conversion achieved by the various enzymatic hydrolysis conditions. The lowest enzyme dosage and the solid loading that yielded a conversion of cellulose to glucose of ≈ 80% and a high glucose concentration were selected for further investigation. Each condition combination was completed in triplicates for statistical analysis.

4.2.4 Separate hydrolysis and fermentation screening experiment

4.2.4.1 Enzymatic hydrolysate preparation

The hydrolysate obtained after enzymatic hydrolysis with a solid loading of 15% (w/v) dry bagasse and an enzyme dosage of 7.5 FPU/g DW substrate was centrifuged at 4 000 rpm for 10 minutes (Hermle Labortechnik GmbH ZK 496). Centrifugation allowed for the separation of unhydrolyzed solids from the hydrolysate. The hydrolysate underwent filtration through 0.45 μm nylon membrane filters using a glass Buchner funnel connected to a vacuum line.

Additionally, various minerals were required for the growth of the microbial strains; thus, the hydrolysate required supplementation (Table 4.2). The hydrolysate specifically needed to be supplemented with a nitrogen source as lignocellulosic biomass has a low nitrogen content (Lee, 1997). The selected nitrogen source was CSL. A buffer salt was also needed as the pH of the media was not controlled. Potassium dihydrogen phosphate (KH_2PO_4) was the selected buffer salt. The pH of the enzymatic hydrolysate was adjusted through the addition of KOH pellets to obtain the desired pH for the microbial strains. The hydrolysate with all required supplementation was autoclaved at 121 °C for 15 minutes to sterilise the growth media.

Table 4.2: Concentration of minerals required for supplementation of the hydrolysate (Pamment *et al.*, 1978; Hatakka and Pirhonen, 1985; Mokomele, 2019).

Chemical name	Chemical formulae	Concentration (g/L)
Calcium chloride	$CaCl_2$	0.02
Corn steep liquor	-	5% (v/v)
Magnesium sulphate heptahydrate	$MgSO_4 \cdot 7H_2O$	0.3
Potassium dihydrogen phosphate	KH_2PO_4	2.0

4.2.4.2 Fermentation

The conditions that were selected for the various microbial strains were obtained based on literature (Table 4.3). These values were selected based on Table 2.11.

Table 4.3: Selected operating conditions, temperature and pH for selected microbial strains.

Microorganisms	Temperature (°C)	pH
Bacterial strains		
Bacillus subtilis	30	6.5
Lactobacillus bulgaricus	37	6.5
Streptococcus thermophilus	37	6.5
Fungal strains		
Fusarium venenatum	30	5.5
Pleurotus ostreatus	30	6.5
Saccharomyces cerevisiae	30	5.5

All microbial strains were preserved in 1 mL aliquots at -80 °C using 10% (v/v), 40% (v/v) and 60% (v/v) glycerol for fungal, yeast and bacterial strains, respectively, as a cryoprotectant. During inoculation, one vial of stock culture was thawed and transferred aseptically to a cotton-stoppered 250 mL baffled Erlenmeyer flasks that contained 100 mL YPD broth, which was sterilised in an autoclave for 15 minutes at 121 °C. The bacterial strains and *S. cerevisiae* were incubated in the orbital shaker for 24 h at 150 rpm. In comparison, the fungal strains were incubated in the orbital shaker for 48 h at 150 rpm, as the fungal strains have a slower growth rate than the bacterial and yeast strains. The incubator's temperature was set according to the selected operating temperature for each strain, as specified in Table 4.3. The initial growth of the microbial strains in YPD was designated as the starter culture.

When the desired time elapsed, 5% (v/v) of the starter culture was transferred into sterilised cotton-stoppered 250 mL baffled Erlenmeyer flasks that contained sterilised 25% (v/v) hydrolysate in YPD of 95 mL to ensure a final volume of 100 mL. The 25% (v/v) hydrolysate allowed the strains to acclimate to the presence of inhibitors and thus was designated as the pre-conditioned inoculum culture. The inoculum cultures were incubated in the orbital shakers until the late exponential phase of the strains was obtained (Table 4.4).

Table 4.4: Identified times of the start of the late exponential growth phase of the microbial strains.

Microbial strain	Time (h)
Bacillus subtilis CAB1111	7 – 10
Lactobacillus bulgaricus	8 – 12
Fusarium venenatum	18 – 24
Pleurotus ostreatus CAB13	18 – 24
Saccharomyces cerevisiae CAB79	13 – 16
Streptococcus thermophilus	8 – 14

The desired volume of the inoculum cultures was then aseptically transferred to the prepared growth media flasks containing hydrolysate (Figure 4.4).

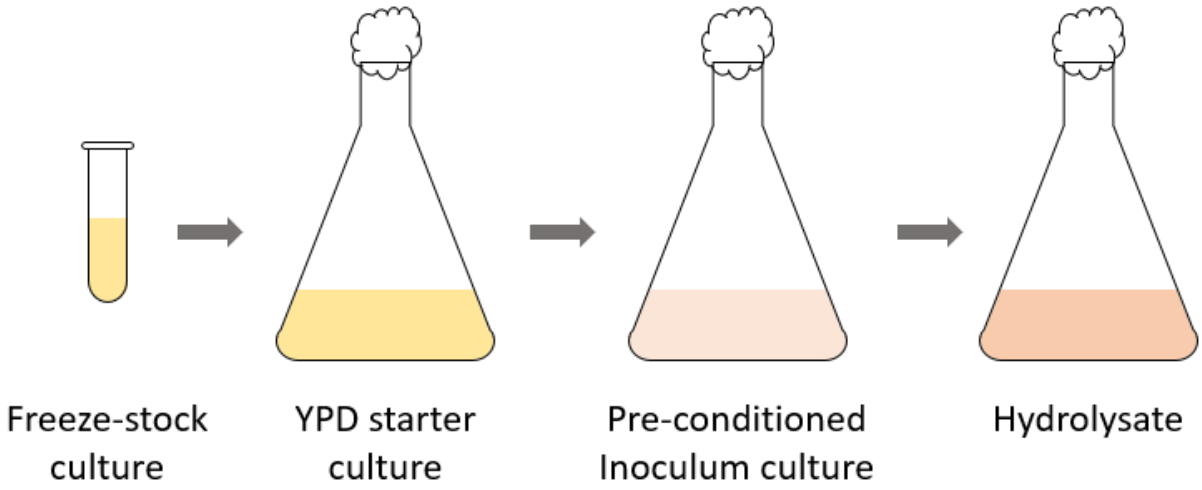


Figure 4.4: Schematic of inoculation sequence followed.

The fermentation of the undiluted hydrolysate was performed in 250 mL baffled Erlenmeyer flasks with a working volume of 100 mL. The cultures were incubated in the orbital shaker at 150 rpm at the specified conditions for each of the strains (Table 4.3).

Samples were taken at regular intervals. The gravimetric method was used to determine the biomass concentration of the yeast and bacterial strains. Samples were taken from the flasks at various time intervals and were centrifuged at 3 200 rpm for 10 minutes. The liquid fraction was removed for HPLC analysis and the cell pellet was washed twice with 0.9% saline solution. The pellet was then resuspended in 0.9% saline solution and was vacuum filtered through a pre-weighed 0.22 μm nylon membrane filter. The filter with the biomass was dried at 105 °C overnight. Once dried, the filter was weighed and the biomass concentration was determined. The biomass concentration was determined by making use of Equation 4.3 below.

$$C_{biomass} = \frac{\left(mass_{filter\,+\,biomass}(g) - mass_{clean\ filter}\,(g)\right)}{Volume\ of\ sample\ taken\ (mL)} \cdot 1000\,\frac{mL}{L} \qquad 4.3$$

Similarly, the sacrificial-gravimetric method was used for the fungal strains to obtain the biomass concentration at various time intervals, specifically 0 h, 3 h, 6 h, 9 h, 12 h, 24 h, 38 h and 48 h, as the fungal strains grew in clumps. Therefore, the entire volume of the flask was used to determine the biomass concentration at the selected time intervals instead of analysing samples taken from the same flask throughout. Large error is associated with this method as the biological makeup will differ from flask to flask. The biomass concentration was also determined by making use of Equation 4.3.

4.2.4.2.1 Growth parameters

Samples of the hydrolysate used for the growth curve runs were analysed using HPLC to determine the change in the composition of the growth media at the selected time intervals. The change in the glucose concentrations, in conjunction with the growth curves, was used to determine the maximum specific growth rate (μ_{max}), the minimum generation time (t_d) and the biomass yield in terms of the substrate consumed ($Y_{x/s}$). The glucose uptake rate and percentage of glucose consumed were also determined.

The minimum generation time (t_d) was calculated using Equation 4.4, while the biomass yield in terms of the substrate consumed ($Y_{x/s}$) was calculated using Equation 4.5. The maximum specific growth rate (μ_{max}) was determined from the fitted gradient of the growth curves (Maier, 2009).

$$t_d = \frac{\ln(2)}{\mu_{max}} \tag{4.4}$$

$$Y_{x/s} = \frac{\frac{dx}{dt}}{-\frac{ds}{dt}} \approx \frac{x - x_0}{s_0 - s} \tag{4.5}$$

Where x represents the biomass concentration at the start of the stationary phase, x_0 represents the initial biomass concentration, s represents the substrate concentration at the start of the stationary phase and s_0 represents the initial substrate concentration. The substrate, in this case, would be glucose.

The glucose uptake rate was determined using Equation 4.6, where the time that was used was the total duration of the experiment or when the final glucose concentration was zero.

$$Glucose\ uptake\ rate = \frac{Glucose\ consumed\ \left(\frac{g}{L}\right)}{Time\ (h)} \tag{4.6}$$

While the percentage of glucose consumed was determined using Equation 4.7.

$$\%\ Glucose\ consumed = 100\% \cdot \frac{Initial\ glucose\ concentration\left(\frac{g}{L}\right) - Final\ glucose\ concentration\ \left(\frac{g}{L}\right)}{Initial\ glucose\ concentration\ \left(\frac{g}{L}\right)} \tag{4.7}$$

Batch culture kinetic curves were fitted to the growth curves of the microbial strains to calculate the growth parameters. This allowed for the coefficient of determination (R^2) between the actual data obtained and the fitted data to be compared to assess the accuracy of the kinetic curves.

4.2.5 Pulse fed-batch fermentations

A method similar to that used by Bezuidenout (2021) was followed for the pulse fed-batch fermentation runs. The pulse fed-batch fermentations were conducted by making use of 250 mL baffled Erlenmeyer flasks. The selected microbial strain was *S. thermophilus*, which was incubated in an orbital shaker at

37 °C and 150 rpm. The hydrolysate, with an adjusted pH, was filter-sterilized through 0.22 μm nylon membrane filters before being added to the sterilised RO water containing the required salts and CSL. The RO water that contained the required salts and CSL was autoclaved at 121 °C for 15 minutes. The pulse fed-batch runs that were conducted were compared to a batch fermentation run with a hydrolysate concentration of 80% (v/v).

4.2.5.1 Initial pulse fed-batch runs

The pulse fed-batch fermentations used a six-pulse feeding scheme of undiluted hydrolysate to increase the initial hydrolysate concentration to a final concentration of 80% (v/v). Three different initial hydrolysate concentrations were investigated, 20% (v/v), 40% (v/v) and 60% (v/v), each with an initial working volume of 50 mL. Therefore, the three initial concentrations' medium composition and pulse volumes differed (Table 4.5).

Table 4.5: Medium composition for the three different initial hydrolysate concentrations.

Media requirements	**Initial hydrolysate concentration (% v/v)**		
	20	**40**	**60**
Calcium chloride (g)	0.004	0.003	0.002
Corn steep liquor (mL)	15	11.2	7.5
Magnesium sulphate heptahydrate (g)	0.06	0.045	0.03
Potassium dihydrogen phosphate (g)	0.4	0.3	0.2
Initial volume of hydrolysate (mL)	10	20	30
Volume of media (mL)	37.5	27.5	17.5
Pulse volume (mL)	25	16.7	8.3
Volume inoculum (mL)	2.5	2.5	2.5

The feeding scheme that was followed was a six-pulse feeding scheme, where the pulses occurred after 12 h intervals. However, the time of the initial pulse for each of the three initial concentrations differed, due to the different initial glucose concentrations. It was assumed that the initial glucose present in the different runs would be depleted at varying times. Hence the different times for the initial pulse. After the initial pulse, the three different initial concentrations' pulse times occurred simultaneously (Table 4.6). The three different pulse fed-batch runs were completed in duplicate.

Table 4.6: Pulse feeding scheme for the three different initial hydrolysate concentrations and the change in the hydrolysate concentration after each pulse with undiluted hydrolysate.

Time (h)	Initial hydrolysate concentration (% v/v)					
	20		40		60	
	Pulse volume (mL)	% Hydrolysate (v/v)	Pulse volume (mL)	% Hydrolysate (v/v)	Pulse volume (mL)	% Hydrolysate (v/v)
0	0	20	0	40	0	60
6	25	46.7	0	40	0	60
12	0	46.7	16.7	55	0	60
24	25	60	16.7	64	8.3	65.7
36	25	68	16.7	70	8.3	70
48	25	73.3	16.7	74.3	8.3	73.3
60	25	77.1	16.7	77.5	8.3	76
72	25	80	16.7	80	8.3	78.2
84	0	80	0	80	8.3	80

4.2.5.2 Final pulse fed-batch run

An initial hydrolysate of 20% (v/v) was selected for the final pulse fed-batch runs and *S. cerevisiae* was used as a control. A six-pulse feeding scheme was used where the pulses occurred every 24 h (Table 4.7) and the same media makeup was used (Table 4.5). The final pulse fed-batch run was conducted in triplicate for statistical purposes.

Table 4.7: Pulse feeding scheme for an initial hydrolysate concentration of 20% (v/v) and the change in the hydrolysate concentration after each pulse with undiluted hydrolysate.

Time (h)	Pulse volume (mL)	% Hydrolysate (v/v)
0	0	20
6	25	46.7
24	25	60
48	25	68
72	25	73.3
96	25	77.1
120	25	80

4.2.5.3 Protein determination

The biomass obtained from the final pulse fed-batch runs of *S. cerevisiae* and *S. thermophilus* were analysed for CHNS elemental composition at the Central Analytical Facility (CAF), Stellenbosch University. The biomass obtained from the batch fermentation run for *S. thermophilus* grown in 80% (v/v) hydrolysate concentration was also analysed for CHNS elemental composition. CHNS elemental composition analysis determines the nitrogen content of the samples. Thus, the conversion factor of 6.25

was used to convert the nitrogen content to protein content, which is the same conversion factor used for the Kjeldahl protein analysis method (Pihlajaniemi *et al.*, 2020).

4.2.6 Statistical analysis

For statistical purposes, all experiments were conducted in triplicates, unless otherwise stated, and the averages, with the respective standard deviations, were provided. Microsoft Excel was used to calculate all relevant averages and standard deviations and perform a one-way analysis of variance (ANOVA) to determine the statistical differences and significance between the various results obtained for the variables investigated. The significant differences were determined at a confidence level of 95%, which means that there were significant differences when $p < 0.05$.

4.3 Results and discussion

4.3.1 Composition of untreated and pretreated sugarcane bagasse

The chemical compositions of the sugarcane bagasse before and after steam explosion were analysed using NREL procedures for the three main components found in lignocellulosic biomass, namely, lignin, cellulose and hemicellulose, as well as the ash content (Table 4.8).

The chemical composition of the untreated sugarcane bagasse used in this study was comparable to the values reported previously, with a cellulose content of 29.94% ± 0.16% DW, a hemicellulose content of 25.42% ± 0.25% DW, a lignin content of 25.86% ± 0.07% DW and an ash content of 3.14% ± 0.01% DW (Table 4.8). Reported values have ranged between 32% to 45% cellulose, 20% to 32% hemicellulose, 17% to 32% lignin and 1.0% to 9.0% ash, all on a dry weight basis (Haghdan, Renneckar and Smith, 2016; Alokika *et al.*, 2021). The slight decrease in the cellulose content of the sugarcane bagasse was attributed to the variety and cultivation of the sugarcane, which influences the composition and the analytical methods used to determine the composition thereof (Canilha *et al.*, 2011). Sugarcane bagasse is also a by-product of the sugarcane industry; thus, the composition will differ from process to process (Hames *et al.*, 2003).

Table 4.8: Chemical composition of untreated and steam-pretreated sugarcane bagasse at STEX conditions of 185 °C for 10 minutes.

Component	Untreated sugarcane bagasse (% DW)	Steam-pretreated sugarcane bagasse (% DW)
Cellulose	29.94 ± 0.16	36.49 ± 1.46
Hemicellulose	25.42 ± 0.25	18.74 ± 0.13
Lignin	25.86 ± 0.07	24.36 ± 0.29
Ash	3.14 ± 0.01	3.25 ± 0.02

The change in the composition of the sugarcane bagasse is a result of the high temperatures and the sudden pressure change that occurs during the discharge into the expansion vessel (Table 4.8). As expected, the hemicellulose fraction of the bagasse decreased from 25.42% ± 0.25% to 18.74% ± 0.13% after steam explosion. This decrease can be attributed to the formation of inhibitors, such as furfural and 5-hydroxymethylfurfural (5-HMF), and pentoses in the form of xylose and xylo-oligosaccharides (Palmqvist and Hahn-Hägerdal, 2000; Cantarella *et al.*, 2004b). It should be noted that the NREL method used to determine the composition of the steam-exploded sugarcane bagasse washes the liquid fraction out. Therefore, the composition is only for the water-insoluble solids (WIS) in the pretreated material, on a dry basis. On the other hand, the decrease in the hemicellulose fraction causes enrichment of the cellulose component after pretreatment, as evident from a 6.55% increase in cellulose content (Table 4.8).

The concentrations of furfural and 5-HMF in the liquid fraction of the pretreated bagasse (Table 4.9) were found to be 0.25 ± 0.01 g/L and 0.004 ± 0.002 g/L, respectively. These concentrations were in agreement with the selected steam explosion conditions of 185 °C for 10 minutes, having a severity factor of 3.5 that

falls within the intermediate range, resulting in a lower concentration of inhibitors (Du *et al.*, 2010; Tomás-Pejó *et al.*, 2011; Martín *et al.*, 2021).

The concentrations of furfural and 5-HMF in the liquid fraction were negligible (Table 4.9) and, therefore, whatever liquid is left in the pretreated solids after pressing is unlikely to significantly affect microbial productivity and growth, due to furfural and 5-HMF alone. Similarly, the formic acid present at a concentration of 1.08 ± 0.05g/L is below the toxic concentration for microbial growth of 11.5 g/L (Cantarella *et al.*, 2004b), and minimal adverse effects from formic acid are expected. However, the concentration of acetic acid in the liquid fraction was 3.79 ± 0.22 g/L, which is almost double the 2 g/L lethal threshold for most microbial strains (Cantarella *et al.*, 2004b; Trček, Mira and Jarboe, 2015). The minimum inhibitory concentration (MIC) of acetic acid for *S. cerevisiae* was found to be 9 g/L at pH 4.0 (Stratford *et al.*, 2013), showing particularly high resistance to this inhibitor (Trček, Mira and Jarboe, 2015). However, the pressing of the pretreated materials will remove the majority of the inhibitors prior to enzymatic hydrolysis and bioconversion, while further dilution of these inhibitors will occur by the addition of sodium citrate buffer during enzymatic hydrolysis. The presence of acetic acid is not expected to have an adverse effect on the cellulase activity, considering both these benefits of pressing and water-dilution of the pretreated solids, as well as the robustness of Cellic® Ctec3 enzymes to inhibitors, compared to other enzymes (Sun *et al.*, 2015). Oligosaccharides (Table 4.8), specifically xylo-oligosaccharides, inhibit cellulases (Merino and Cherry, 2007). The steam-exploded sugarcane bagasse was pressed to remove most of the liquid fraction as it contained the oligosaccharides with a total concentration of 30.38 ± 0.56 g/L.

Table 4.9: Concentration of sugars and inhibitors present within the liquid fraction of the steam-exploded sugarcane bagasse.

Compound	**Concentration (g/L)**
Monomeric glucose	0.27 ± 0.02
Oligomeric glucose	2.32 ± 0.02
Monomeric xylose	2.60 ± 0.19
Oligomeric xylose	28.06 ± 0.02
Acetic acid	3.79 ± 0.22
Formic acid	1.08 ± 0.05
Furfural	0.25 ± 0.01
5-HMF	0.004 ± 0.002

4.3.2 Enzymatic hydrolysis

4.3.2.1 Digestibility of steam-exploded sugarcane bagasse

The digestibility of the steam-exploded sugarcane bagasse was determined by enzymatically hydrolysing 2% (w/v) WIS with 15 FPU Cellic® CTec3/g DW WIS, as per the NREL procedure described in Section 4.2.3.5.1. The digestibility of the steam-exploded sugarcane bagasse was evident from the glucose yield of 98% ± 0.06% of the theoretical maximum, which was equivalent to 38.7 g glucose/100 g DW WIS. The digestibility achieved support that the mild pretreatment conditions selected are ideal as a high sugar yield can be achieved during enzymatic hydrolysis and a lower enzyme dosage could be accommodated, which may have an economic benefit (Mokomele, 2019).

The glucose yield or digestibility achieved by the steam-exploded sugarcane bagasse is more than that achieved by Koekemoer (2018). Koekemoer (2018) showed a glucose yield of 35.3 g/ 100 g DM at steam pretreatment conditions of 190 °C for 15 minutes. However, the cellulose content of the sugarcane bagasse used was 38.6% (w/w), per dry basis (Koekemoer, 2018), as compared to the 29.9% (w/w), per dry basis used in the present study. The sugarcane bagasse composition after steam explosion was not provided by Koekemoer (2018); therefore, a comparison between the two digestibilities cannot be made. Koekemoer (2018) used the Cellic® CTec2 enzyme, which is not as potent as the Cellic® CTec3 enzyme (used in the present study) for the conversion of lignocellulosic material, as Cellic® Ctec2 is not as resistant to the presence of inhibitors as Cellic® Ctec3 and Cellic® Ctec2 is affected more by end-product inhibition.

Mokomele (2019) investigated the impact of three different steam explosion conditions on the digestibility of sugarcane bagasse. After steam explosion at 185 °C for 15 minutes, the cellulose component of the sugarcane bagasse increased from 39.50% ± 0.41% to 68.9% ± 1.92%. Mokomele (2019) achieved a glucose yield or digestibility of 57% of the theoretical maximum. The yield achieved is significantly lower than that achieved in this investigation. Mokomele (2019) made use of a combination of Cellic® CTec2 and HTec2.

4.3.2.2 Selection of process conditions

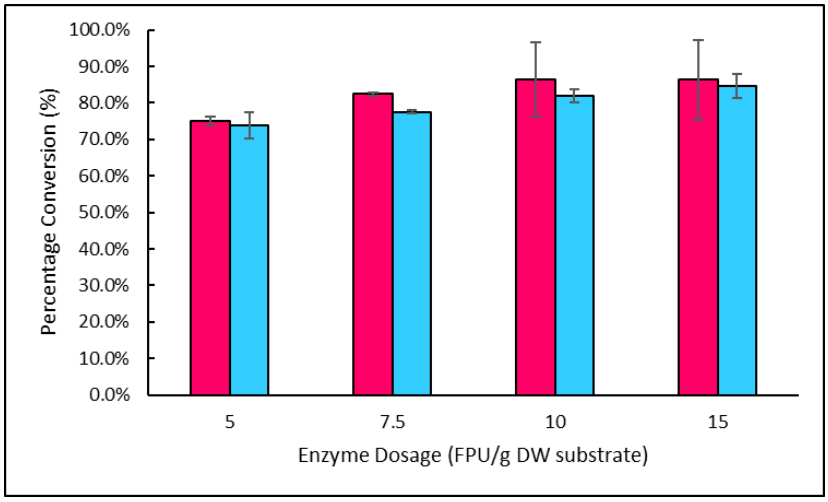
As previously mentioned, an approximate 80% conversion of cellulose to glucose is the conversion goal of biorefineries to achieve during enzymatic hydrolysis to ensure that they are commercially viable (Mokomele, 2019). Therefore, selecting the enzymatic hydrolysis process conditions is essential as it determines the solid loading, enzyme dosage and hydrolysis duration required to achieve this goal and ensure high glucose concentrations. The enzyme dosage has a large impact on the economics of the process, as the cost of enzymes (6.00 US$/kg solid cellulase protein) is one of the main contributors to the operating costs of the process (Pihlajaniemi *et al.*, 2020; Petersen, Okoro, *et al.*, 2021). Additionally, the optimum conditions for maximum hydrolysis yield do not always result in the economic optimum.

The steam-exploded sugarcane bagasse was pressed to remove the majority of the inhibitors present in the liquid fraction of the slurry, specifically to reduce the oligomeric sugars concentration of 30.38 ± 0.56 g/L, which also avoids some of its inhibitory effects on enzymatic hydrolysis (Merino and Cherry, 2007; Mokomele, 2019).

A combination of two solid loadings, 10% (w/v) and 15% (w/v), and four enzyme dosages of Cellic® CTec3, 5 FPU/g DW substrate, 7.5 FPU/g DW substrate, 10 FPU/g DW substrate and 15 FPU/g DW substrate, were investigated to determine which combination would yield an approximate 80% conversion of cellulose to glucose and a high concentration of glucose after 72 h (Figure 4.5). A solid loading of 20% (w/v) was investigated; however, inadequate mixing in the 250 mL baffled flasks was experienced. A one-way analysis of variance (ANOVA) showed that enzyme dosage and solid loadings had significant effects on the conversion efficiency ($p \leq 0.05$). There was a general increase in conversion efficiency at higher enzyme dosages (Figure 4.5). For the 15% (w/v) solid loading, the conversion achieved at an enzyme dosage of 5 FPU/g DW substrate was 73.8% ± 3.6%, while at an enzyme dosage of 15 FPU/g DW substrate, the conversion achieved was 84.6% ± 3.2% (Figure 4.5). The same trend was observed in Mokomele's (2019) work, where higher enzyme dosages resulted in higher glucose yields. Mokomele (2019) found that an enzyme dosage of 25 mg/g glucan resulted in a glucose conversion of 77% from ammonia fibre expansion (AFEX™)-treated bagasse, while an enzyme dosage of 15 mg/g glucan resulted in a glucose conversion of 65%. The high conversions achieved in the present study could, in part, be attributed to the effectiveness of the steam explosion conditions of 185 °C for 10 minutes.

A solid loading of 15% (w/v) pressed steam-exploded sugarcane bagasse resulted in a higher concentration of free monomeric sugars, specifically glucose, as compared to a solid loading of 10% (w/v) (Figure 4.5), confirming that there is a positive relationship between free monomeric sugars and solid loadings (Cara *et al.*, 2007). The glucose concentration achieved with a solid loading of 15% (w/v) and an enzyme dosage of 15 FPU/g DW substrate was 50.3 ± 1.1 g/L. In comparison, a glucose concentration of 34.2 ± 1.3 g/L was achieved at a 10% (w/v) solid loading at the same enzyme dosage of 15 FPU/g DW substrate (Figure 4.5).

A)



B)

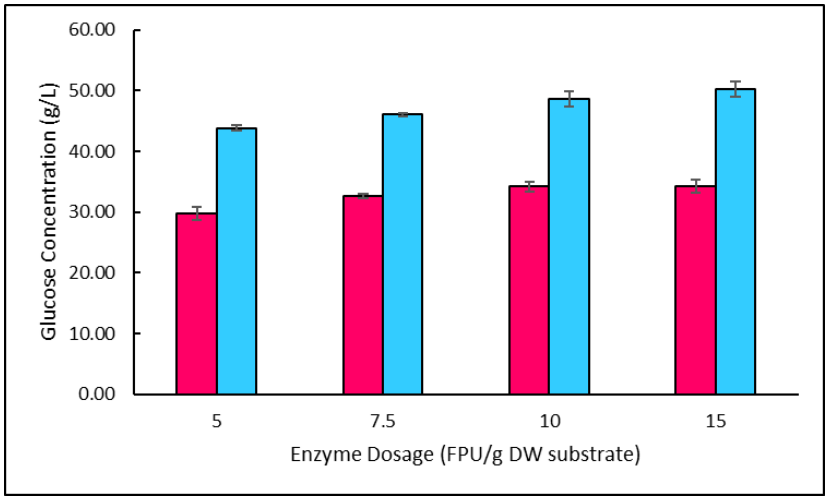


Figure 4.5: Bar graphs showing the **(A)** percentage conversion of cellulose to glucose and **(B)** glucose concentration after 72 h of incubation at 50 °C and pH 5.0 at solid loadings of 10% (w/v) (pink) and 15% (w/v) (blue).

Additionally, the conversion of cellulose to glucose at a solid loading of 15% (w/v) at four enzyme dosages was investigated over 72 h, where samples were taken every twenty-four hours (Figure 4.6). An approximate 80% conversion of cellulose to glucose was achieved after 72 h with an enzyme dosage of 7.5 FPU/g DW substrate, 10 FPU/g DW substrate and 15 FPU/g DW substrate, resulting in 77.6% ± 0.4%, 81.8% ± 1.8% and 84.6% ± 3.23% conversion of cellulose to glucose, respectively (Figure 4.6). The lowest enzyme dosage, 7.5 FPU/g DW substrate, was thus used for the remainder of the investigation. The lowest enzyme dosage was selected as minimising the required enzyme dosage allows for the STEX-based biorefineries to be influenced less by the fluctuations in the enzyme cost, thus minimising the changes in the production costs that would occur (Lynd *et al.*, 2017).

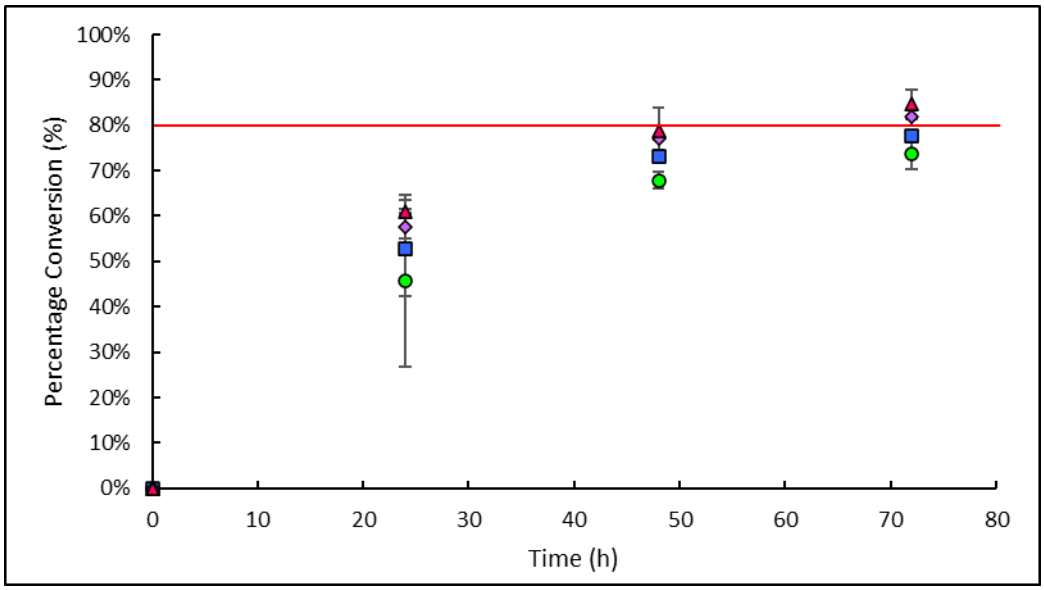


Figure 4.6: Scatter plot showing the percentage conversion of cellulose to glucose after 72 h incubation at 50 °C and pH 5.0 at a solid loading of 15% (w/v) and enzyme dosages of 5 FPU/g DW substrate (green circles), 7.5 FPU/g DW substrate (blue squares), 10 FPU/g DW substrate (purple diamonds) and 15 FPU/g DW substrate (pink triangles).

4.3.3 Submerged fermentation using hydrolysate as a carbon source

The six generally regarded as safe (GRAS) microbial strains that were considered as candidates for single-cell protein (SCP) production from a lignocellulosic hydrolysate, namely, *B. subtilis*, *L. delbrueckii*, *S. thermophilus* (bacterial strains), *F. venenatum*, *P. ostreatus* and *S. cerevisiae* (fungal strains), were compared in terms of biomass formation and tolerance to inhibitors by considering the proportion of glucose in the undiluted hydrolysate that was consumed, the biomass yield and the final biomass concentration (Figure 4.7 and Table 4.11). The submerged batch fermentations were completed with undiluted sugarcane bagasse hydrolysate in 250 mL baffled shake-flasks, with the strains first preconditioned by cultivation in YPD medium that contained 25% (v/v) hydrolysate.

The fungal strains, *F. venenatum* and *P. ostreatus*, appeared to tolerate the inhibitors in the hydrolysate the least, as no glucose was consumed (Figure 4.7 and Table 4.11). The poor performance was likely due to the high concentration of acetic acid (Figure 4.8), which was present between 4.0 and 5.5 g/L (Table 4.10), which is more than double the lethal concentration of 2 g/L (Trček, Mira and Jarboe, 2015). The acetic acid concentration increased during enzymatic hydrolysis as the acetyl groups in the hemicellulose were hydrolysed (Balat, 2011; Jönsson and Martín, 2016). 5-HMF and furfural are known to be more potent inhibitors that are generated via steam pretreatment. However, the 5-HMF and furfural concentrations in the undiluted hydrolysate were below the detection limits (Table 4.10), as they were diluted out during enzymatic hydrolysis of the steam-exploded sugarcane bagasse. It must be noted that phenolic compounds from lignin degradation are also toxic to microbial strains in small concentrations (Palmqvist and Hahn-Hägerdal, 2000); however, they were not detected via HPLC.

Table 4.10: Concentration of inhibitors present in the enzymatic hydrolysate.

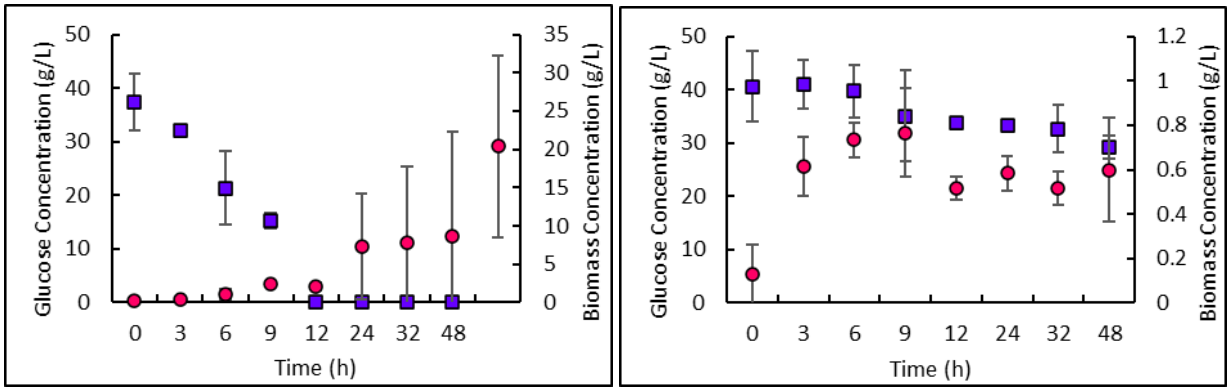
Compound	Concentration (g/L)
Acetic acid	4.0 – 5.5
Formic acid	-
Furfural	-
5-HMF	-

* '-' as the concentrations fell below the detection limits

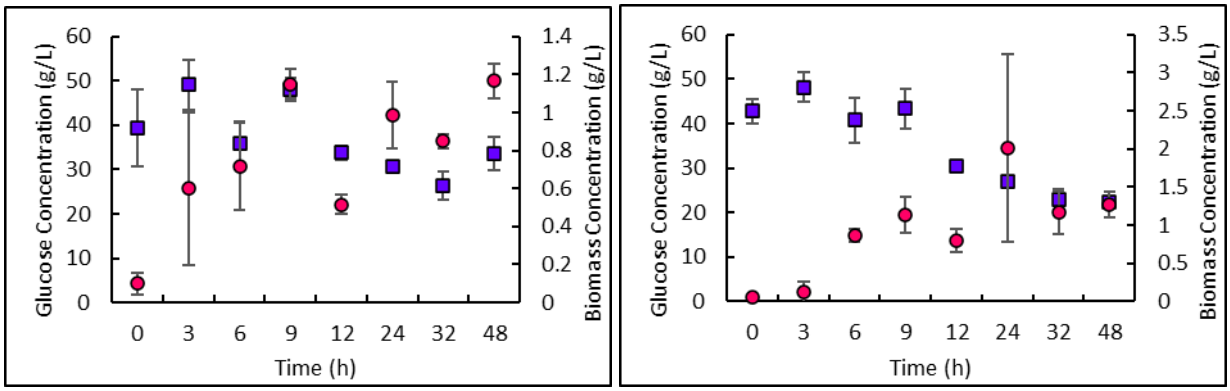
The bacterial strains, *L. delbrueckii* and *S. thermophilus,* appeared to tolerate the inhibitors reasonably well, as these two strains achieved the most glucose consumption of 29.56% ± 21.08% and 47.48% ± 3.75%, respectively, compared to *B. subtilis, F. venenatum* and *P. ostreatus*, where 27.24% ± 9.18%, 0% and 0% glucose was consumed, respectively (Figure 4.7 and Table 4.11).

S. cerevisiae achieved 100% glucose consumption when grown in undiluted enzymatic hydrolysate. Therefore, it reveals its high inhibitor tolerance compared to the other five microbial strains investigated (Figure 4.7 and Table 4.11). *S. cerevisiae* has a particularly high resistance to acetic acid (Trček, Mira and Jarboe, 2015), as its minimum inhibitory concentration (MIC) is 9 g/L at pH 4.0 (Stratford *et al.*, 2013). Additionally, *S. cerevisiae* has a known capability of converting furfural and 5-HMF present in hydrolysates into 5-HMF alcohol and 5-hydroxymethyl furan carboxylic acid, respectively, which have substantially lower inhibitory effects (Taherzadeh *et al.*, 2000). Therefore, it explains the improved *S. cerevisiae* tolerance to various inhibitors.

A) *S. cerevisiae* **B) *B. subtilis***



C) *L. delbrueckii* **D) *S. thermophilus***



E) *F. venenatum* **F) *P. ostreatus***

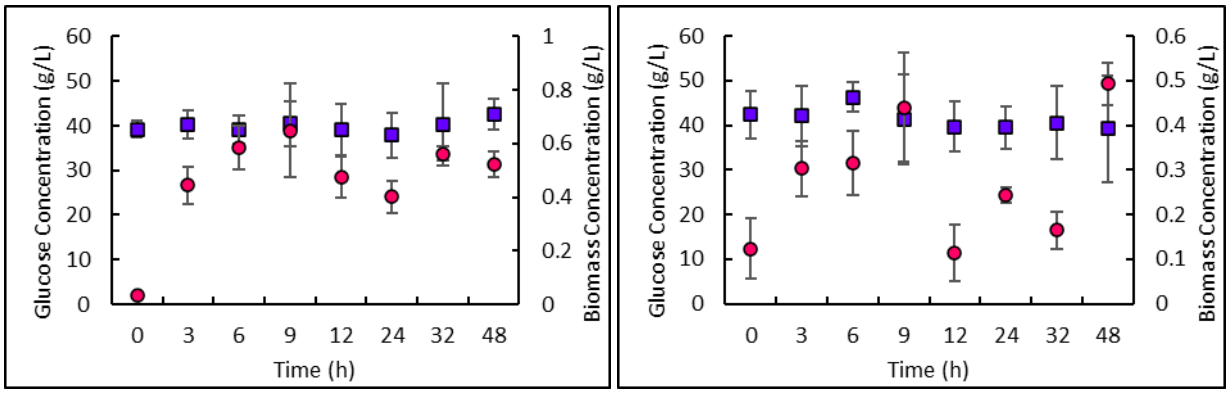
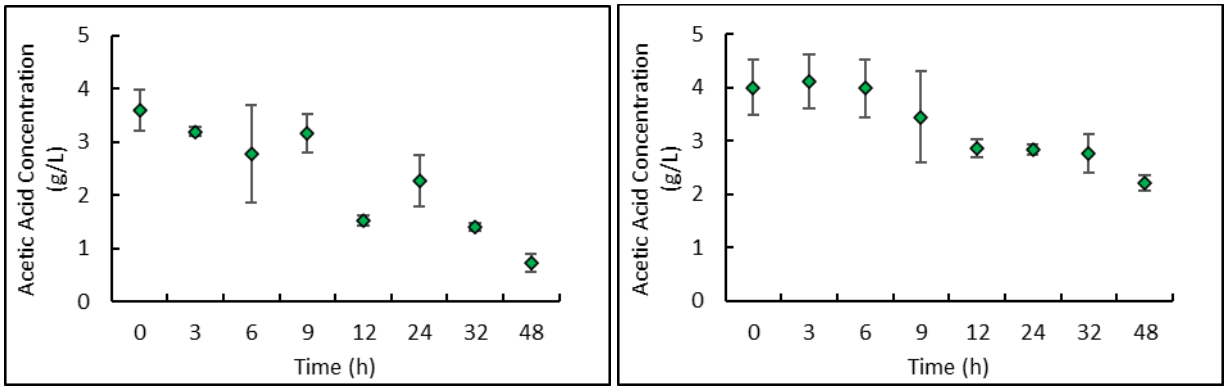


Figure 4.7: Glucose (purple squares) and microbial biomass (pink circles) concentrations over 48 h for **(A)** *S. cerevisiae*, **(B)** *B. subtilis*, **(C)** *L. delbrueckii*, **(D)** *S. thermophilus*, **(E)** *F. venenatum* and **(F)** *P. ostreatus* grown in undiluted enzymatic hydrolysate.

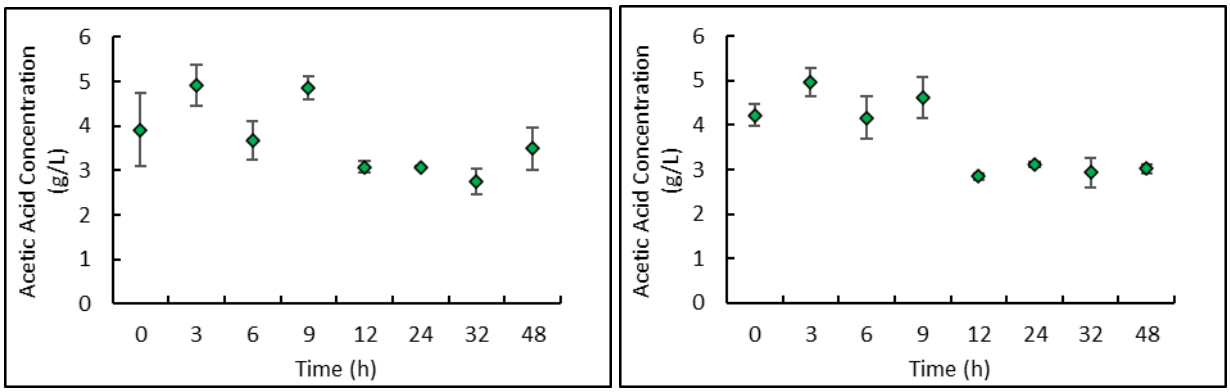
An interesting observation was that the bacterial and yeast strains appeared to consume the acetic acid present in the medium (Figure 4.8). However, the acetic acid concentrations of the two fungal strains remained constant. The decrease in acetic acid concentrations is unusual as acetic acid is a known microbial growth inhibitor that is used in the food industry (Palma, Guerreiro and Sá-Correia, 2018). Furthermore, Taherzadeh et al. (2000) found that when microorganisms consume inhibitors present, a

decrease in carbon source consumption is experienced. As such, it is possible that the decrease in glucose consumption by the bacterial strains was because of the consumption of acetic acid (Piper *et al.*, 2001).

A) *S. cerevisiae* **B) *B. subtilis***



C) *L. delbrueckii* **D) *S. thermophilus***



E) *F. venenatum* **F) *P. ostreatus***

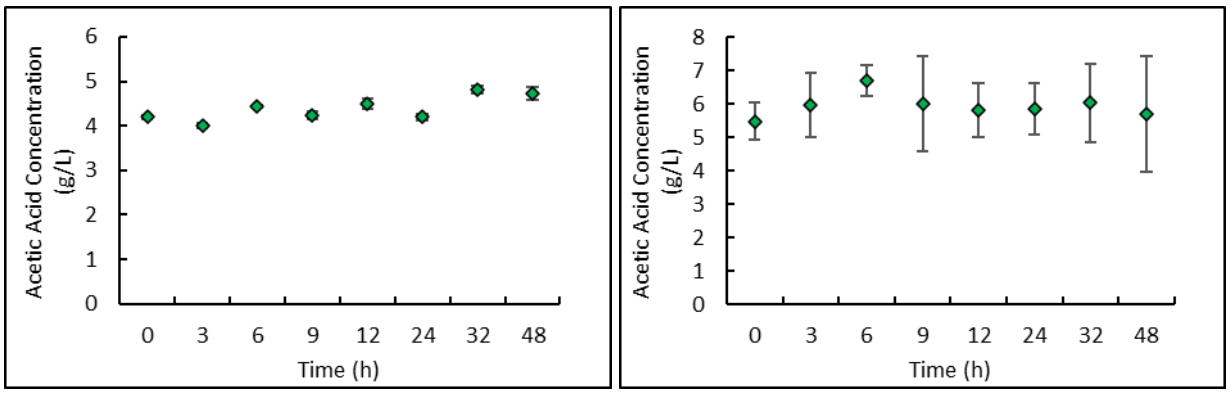


Figure 4.8: Acetic acid concentrations over 48 h for **(A)** *S. cerevisiae*, **(B)** *B. subtilis*, **(C)** *L. delbrueckii*, **(D)** *S. thermophilus*, **(E)** *F. venenatum* and **(F)** *P. ostreatus* grown in undiluted enzymatic hydrolysate.

The calculated growth parameters, specifically the yield and the final biomass concentration, provide better insight into the performance of the microbial strains in the undiluted hydrolysate in the presence of inhibitors (Table 4.11). *S. cerevisiae* was the only strain that was able to consume all the glucose in the hydrolysate, resulting in a final biomass concentration of 20.37 ± 11.81 g/L. The large error associated with the biomass concentration value was due to the *S. cerevisiae* strain forming clumps, which affected

the accuracy of the biomass determination via the gravimetric method. As a consequence, the measured biomass yield ($Y_{x/s}$) of 0.73 ± 0.21 g/g for *S. cerevisiae* measured with the gravimetric method was above the well-known theoretical limit of 0.51 g/g for this yeast. Therefore, a more accurate yield should be determined through the use of the sacrificial gravimetric method.

S. thermophilus achieved the highest yield ($Y_{x/s}$) of 0.061 ± 0.01 g/g compared to the other four strains screened, indicating a low tolerance to the inhibitors present. Additionally, its μ_{max} of 0.38 ± 0.07 h^{-1} was higher than the μ_{max} achieved by *S. cerevisiae* (0.35 ± 0.05 h^{-1}; Table 4.11). The improved growth parameters of *S. thermophilus*, compared to the other four strains, were attributed to a better tolerance for the inhibitors present and it was selected for further investigation.

Table 4.11: Summary of the calculated growth parameters of the six microbial strains grown, under submerged fermentation conditions, on undiluted steam-exploded sugarcane bagasse enzymatic hydrolysate after 48 h.

Microbial strain	μ_{max} (h^{-1})	Final biomass concentration (g/L)	Generation time (t_d) (h)	$Y_{x/s}$ (g biomass/g glucose)	Glucose uptake rate (g/L·h)	Glucose consumed (%)
Bacillus subtilis	0.14 ± 0.07	0.60 ± 0.23	4.79 ± 2.48	0.01 ± 0.004	0.24 ± 0.11	27.24 ± 9.18
Fusarium venentum	0.30 ± 0.02	0.52 ± 0.05	2.31 ± 0.15	NA	NA	NA
Lactobacillus delbrueckii	0.26 ± 0.07	1.17 ± 0.09	2.74 ± 0.72	0.023 ± 0.002	0.27 ± 0.25	29.56 ± 21.08
Pleuroutus ostreatus	0.17 ± 0.07	0.49 ± 0.05	4.17 ± 1.68	NA	NA	NA
Saccharomyces cerevisiae	0.35 ± 0.05	20.37 ± 11.81	2.00 ± 0.28	0.73 ± 0.21	1.55 ± 0.22	100.00 ± 0.00
Streptococcus thermophilus	0.38 ± 0.07	1.28 ± 0.17	1.83 ± 0.34	0.061 ± 0.01	0.42 ± 0.06	47.48 ± 3.75

4.3.4 Pulse fed-batch fermentation for the maximisation of microbial biomass

Pulse fed-batch fermentation provides a way to gradually expose the microbial strain to the inhibitors instead of 'shocking' the strain with a high concentration of toxic compounds upon inoculation of the culture medium (Bezuidenhout, 2021). A pulse fed-batch system allows the culture to acclimate to the inhibitors at a low concentration, thus maximising the biomass concentration of the microbial strain. The pulse fed-batch fermentations were used to decrease the inhibitor concentrations that microbes were exposed to during cultivation.

4.3.4.1 Pulse fed-batch cultivation of S. thermophilus with 6-pulses every 12 hours and different initial hydrolysate concentrations

The initial pulse fed-batch cultivation of *S. thermophilus* followed a six-pulse feeding scheme using undiluted hydrolysate to increase the hydrolysate concentration from three different initial concentrations, 20% (v/v), 40% (v/v) and 60% (v/v), to a final hydrolysate concentration of 80% (v/v). Additionally, a batch fermentation with a hydrolysate concentration of 80% (v/v) was performed to determine whether the pulse fed-batch fermentation increased the final biomass concentration (Figure 4.9 and Table 4.12). The initial pulse fed-batch runs were used to establish the best feeding strategy.

The 20% (v/v) initial hydrolysate concentration pulse fed-batch fermentation run performed better than the 40% (v/v) and 60% (v/v) initial hydrolysate concentrations with regard to the final biomass concentration achieved by *S. thermophilus* (Table 4.12). The 20% (v/v) pulse fed-batch run achieved a final biomass concentration of 3.68 ± 0.67 g/L as compared to the 40% (v/v) and 60% (v/v) pulse fed-batch runs, which achieved final biomass concentrations of 2.43 ± 0.07 g/L and 2.30 ± 0.35 g/L, respectively. It was expected that the 20% (v/v) hydrolysate pulse fed-batch fermentation run should outperform the 40% (v/v) and 60% (v/v) hydrolysate pulse fed-batch fermentation runs, as the cultures were introduced to a lower initial inhibitor concentration. It is suggested that the lower initial concentration of inhibitors allowed the culture opportunity to adapt to the inhibitors present in the hydrolysate before the gradual addition of more sugars and inhibitors. This gradual exposure to inhibitors has previously been shown to improve the conditioning and performance of microbes for the conversion of lignocellulosic hydrolysates (Bezuidenhout, 2021). Additionally, the pulse fed-batch fermentations resulted in higher final biomass concentrations than achieved previously in the batch culture of *S. thermophilus* with undiluted hydrolysate, i.e., 1.28 ± 0.17 g/L (Table 4.11). Modifications to the timing of pulses in fed-batch cultivation may further improve this performance.

Table 4.12: The final glucose and biomass concentrations achieved by *S. thermophilus* after 144 h of fermentation with the three different initial hydrolysate concentrations of 20% (v/v), 40% (v/v) and 60% (v/v) pulse fed-batch runs and the 80% (v/v) batch fermentation run.

Initial hydrolysate concentration (% (v/v))	Concentration after 144 h (g/L)	
	Glucose	Biomass
20	20.98 ± 0.09	3.68 ± 0.67
40	24.44 ± 0.73	2.43 ± 0.07
60	32.75 ± 0.92	2.30 ± 0.35
80*	13.59 ± 2.29	5.73 ± 2.51

* Batch fermentation run only

The proportion of glucose consumed by *S. thermophilus* in the 80% (v/v) batch fermentation (65.55% ± 0.72% after 144 h; Table 4.12 and Figure 4.9) was higher than that achieved in the undiluted hydrolysate batch fermentation run (47.48% ± 3.75% after 48 h; Table 4.11), while a higher final biomass concentration of 5.73 ± 2.51 g/L (after 144 h; Table 4.12) was also achieved as compared to the 1.28 ± 0.17 g/L (after 48 h; Table 4.11). While the increase in fermentation time from 48 h to 144 h would have benefitted the 80% (v/v) batch fermentation compared to the undiluted hydrolysate fermentation after 48 h, these results do demonstrate a positive effect of hydrolysate dilution on *S. thermophilus* cultivation and that the microbe remains particularly sensitive to inhibitor concentrations. The increase in CSL concentrations from 5% (v/v) for the undiluted hydrolysate batch fermentation to 7.5% (v/v) for the pulse fed-batch and 80% (v/v) hydrolysate batch runs alone, as a result of potential nitrogen limitations, do not fully explain the increases in biomass formation.

Table 4.13: The estimated mass of glucose achieved after the six pulses for the three different initial hydrolysate concentrations of 20% (v/v), 40% (v/v) and 60% (v/v) pulse fed-batch runs and the 80% (v/v) batch fermentation run before being consumed by *S. thermophilus*, the mass left after 144 h of fermentation and the percentage of glucose consumed.

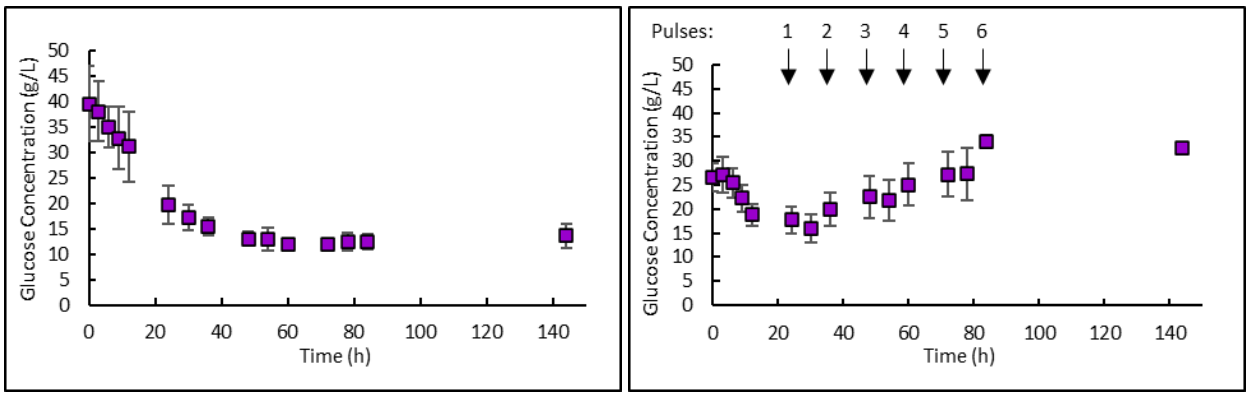
Initial hydrolysate concentration (% (v/v))	Total volume (mL)	Estimated mass of Glucose after six pulses (g)		Glucose consumed (%)
		Total left	Total before being consumed	
20	200	4.2	7.2	41.7
40	150	3.7	5.4	31.5
60	100	3.3	3.6	8.3
80*	100	1.4	3.6	61.1

* Batch fermentation run only

More glucose was consumed in the 80% (v/v) hydrolysate batch fermentation as compared to the pulse fed-batch runs (Table 4.12, Table 4.13 and Figure 4.9). The 80% (v/v) hydrolysate batch fermentation run achieved a final glucose concentration of 13.59 ± 2.29 g/L and resulted in 61.1% glucose consumption, while the 20% (v/v), 40% (v/v) and 60% (v/v) pulse fed-batch runs achieved final glucose concentrations of 20.98 ± 0.09 g/L, 24.44 ± 0.73 g/L and 32.75 ± 0.92 g/L, respectively (Table 4.12 and Figure 4.9) and

41.7%, 31.5% and 8.3% glucose consumption, respectively (Table 4.13). This indicated that further increases in the time between pulses in the fed-batch culture might be required to increase the time available for the adaptation of the microbial culture to inhibitors. Therefore, the microbial strain is 'shocked' by the sudden increases in inhibitor concentration. In contrast, for the batch fermentation run, the microbial strain is given an extended period of time to acclimate to the unchanging inhibitor concentration.

A) Batch Culture **B) Fed-batch with batch culture in 60% hydrolysate**



C) Fed-batch with batch culture in 40% hydrolysate **D) Fed-batch with batch culture in 20% hydrolysate**

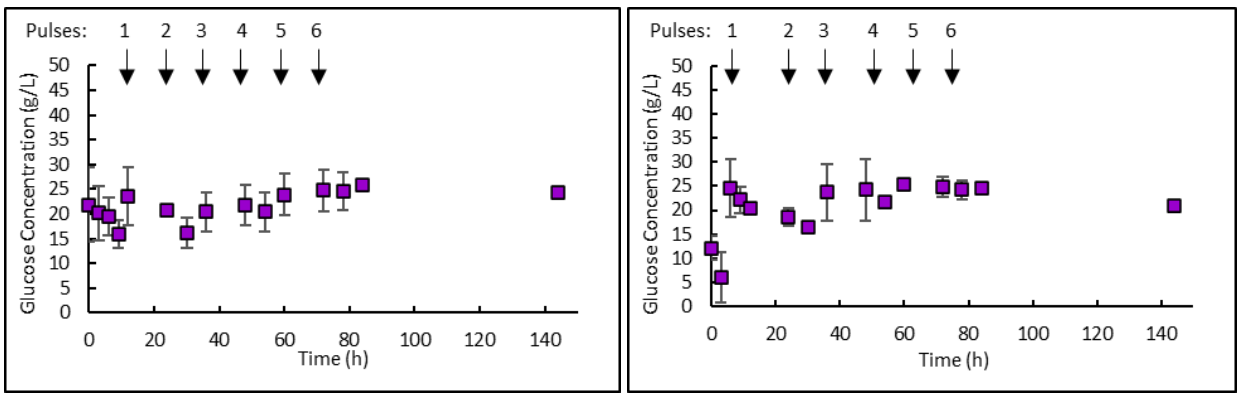


Figure 4.9: Glucose concentrations during the cultivation of *S. thermophilus* over 144 h in either **(A)** 80% (v/v) hydrolysate for batch fermentation, or fed-batch configurations for six-pulse fermentations with initial hydrolysate concentrations of **(B)** 60% (v/v), **(C)** 40% (v/v) and **(D)** 20% (v/v). Pulses contained undiluted hydrolysate and the feeding times are indicated by the arrows.

4.3.4.2 Pulse fed-batch fermentation of S. thermophilus and S. cerevisiae with 20% (v/v) initial hydrolysate concentration and 6-pulses every 24 h

The pulse fed-batch fermentation with an initial hydrolysate concentration of 20% (v/v) and feeding in pulses with undiluted hydrolysate was further investigated. The pulse-feeding scheme was adjusted to six pulses every 24 h rather than every 12 h to allow more time for the culture to acclimate to the changes in inhibitor concentrations. The pulse fed-batch fermentation run of *S. thermophilus* (Figure 4.10) was compared to the pulse fed-batch fermentation run of *S. cerevisiae* (Figure 4.11). The same hydrolysate compositions and pulse-feeding schemes were used for both pulse fed-batch runs, with the only cultivation differences in terms of the incubation temperatures and the pH of the hydrolysate.

Pulse fed-batch fermentations allowed for better biomass concentrations, as this fermentation type allows the microbial strains to metabolise the glucose present below lethal inhibitor concentrations (Nilsson, Taherzadeh and Lidén, 2001; Zhang *et al.*, 2014). As they can metabolise the sugars below lethal concentrations, the yield and productivity of the microbial strains are improved. The growth of both strains was improved in the pulse fed-batch fermentation runs compared to the batch fermentation runs. This was seen by the increase in the final biomass concentrations achieved by *S. thermophilus* (6.57 ± 0.09 g/L; Table 4.14) and *S. cerevisiae* (52.65 ± 0.80 g/L; Table 4.14) in the pulse fed-batch runs as compared to the final biomass concentrations achieved by the strains in the batch fermentation runs (1.28 ± 0.17 g/L and 20.37 ± 11.81 g/L, respectively; Table 4.11), thus further emphasising the benefits of pulse fed-batch fermentations.

Table 4.14: The final glucose and biomass concentrations achieved by *S. thermophilus* and *S. cerevisiae* after 168 h of fermentation with an initial hydrolysate concentration of 20% (v/v).

Microorganism	**Concentration after 168 h (g/L)**	
	Glucose	**Biomass**
S. thermophilus	22.75 ± 0.19	6.57 ± 0.09
S. cerevisiae	0	52.65 ± 0.80

S. cerevisiae consumed all the glucose between pulses, which was not achieved by the *S. thermophilus* strain (Figure 4.10 and Figure 4.11). Additionally, the glucose concentration for the *S. thermophilus* strain fermentation run plateaued near the end (Figure 4.10), which may have been caused by the increase in the concentration of inhibitors, such as acetic acid. Thereby, the *S. thermophilus* strain remains sensitive to the inhibitors present in the hydrolysate, even at an increased time between pulses. Furthermore, the decrease and plateauing of the glucose concentration could also be a sign of a secondary effect of nitrogen limitation, indicating that the bacterial strain could have a higher nitrogen demand than the yeast strain.

The final biomass concentration of *S. thermophilus* achieved for the 24 h pulse-feeding scheme (6.57 ± 0.09 g/L; Table 4.14) is almost double that achieved by the *S. thermophilus* (3.68 ± 0.67 g/L; Table 4.12) with an initial hydrolysate concentration of 20% (v/v) and a 12 h pulse-feeding scheme. The increase in the biomass concentration reveals that the strain required an extended period to acclimate to the increase in the inhibitor concentrations.

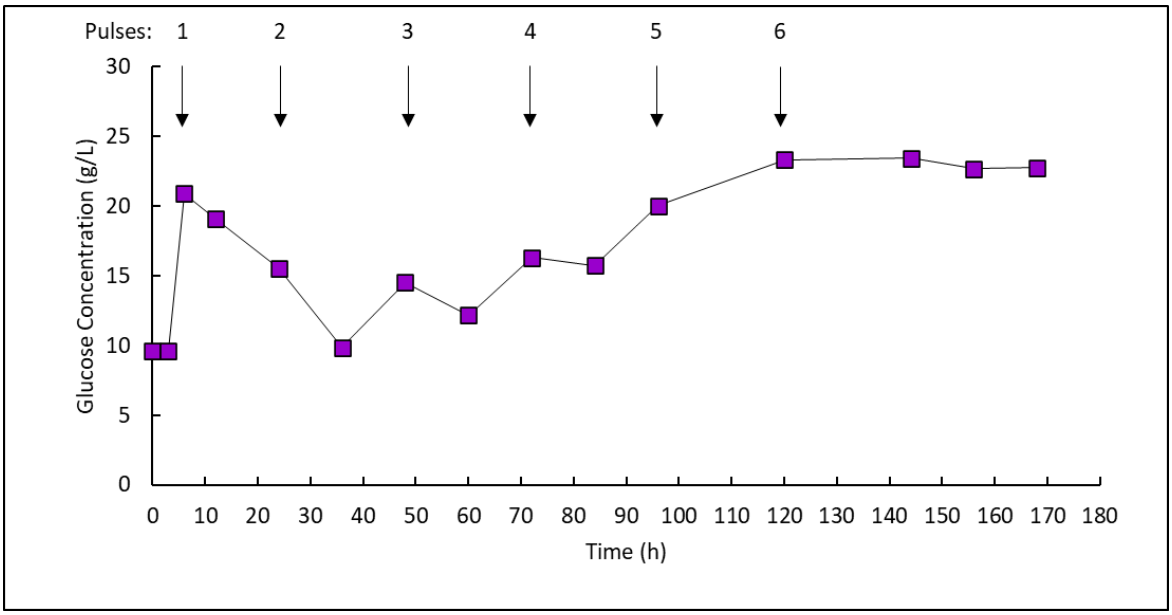


Figure 4.10: Fermentation profile for the six-pulse fed-batch fermentation of *S. thermophilus* with an initial and final hydrolysate concentration of 20% (v/v) and 80% (v/v), respectively. Pulses contained undiluted hydrolysate and the feeding times are indicated by the arrows. The black line is used to indicate the change in the glucose concentration over time.

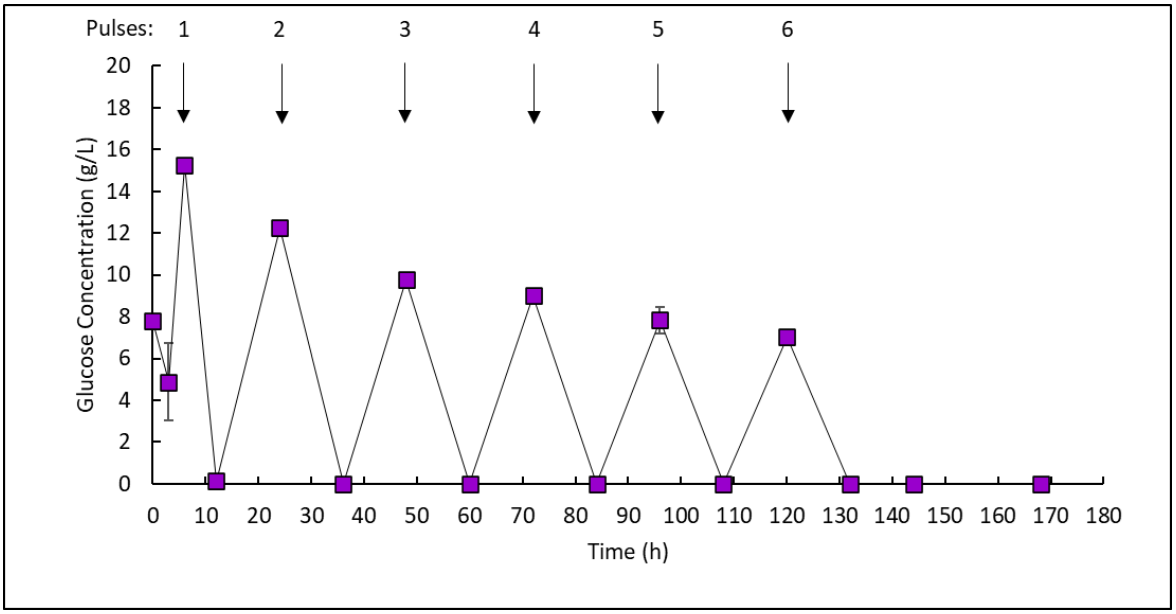


Figure 4.11: Fermentation profile for the six-pulse fed-batch fermentation of *S. cerevisiae* with an initial hydrolysate and final hydrolysate concentration of 20% (v/v) and 80% (v/v), respectively. Pulses contained undiluted hydrolysate and the feeding times are indicated by the arrows. The black line is used as a visual aid to indicate the change in the glucose concentration over time.

S. cerevisiae achieved a final biomass concentration of 52.65 ± 0.80 g/L (Table 4.14), which was substantially higher than the 6.57 ± 0.09 g/L (Table 4.14) achieved by *S. thermophilus*. Therefore, further investigation into these two strains' protein contents and concentrations needs to occur.

In addition, *S. cerevisiae* also produced bioethanol during the pulse fed-batch fermentations (Figure 4.12). The final ethanol concentration achieved by the pulse fed-batch fermentation was 5.62 ± 1.43 g/L, with a slight decrease from 132 h to 168 h (Figure 4.12), which corresponded with the depletion of glucose in the culture (Figure 4.11). The decrease in the ethanol concentration occurs as the yeast is capable of consuming the accumulated ethanol once all sugars in the culture have been depleted and require the presence of oxygen (Thomson *et al.*, 2005). The maximum ethanol concentration (8.13 ± 0.27 g/L after 36 h; Figure 4.12) was similar to the maximum achieved when a recombinant *S. cerevisiae* strain was grown on spent sulphite liquor (SSL) using pulse fed-batch fermentation (7.8 ± 0.1 g/L), albeit on xylose as the primary carbon source (Bezuidenhout, 2021). However, the ethanol concentrations achieved in the present study were substantially lower than the ethanol concentrations of 16.8 g/L and 19.4 g/L that were achieved after 120 h and 144 h, respectively, when *S. cerevisiae* was grown under SSF and SHF conditions using steam-pretreated corn stover as feedstock (Öhgren *et al.*, 2007). This indicated the potential to further increase ethanol co-production with SCP, although this is likely to be at the expense of the latter. The interplay between SCP and ethanol yields from available sugars should be further considered from an economic point of view.

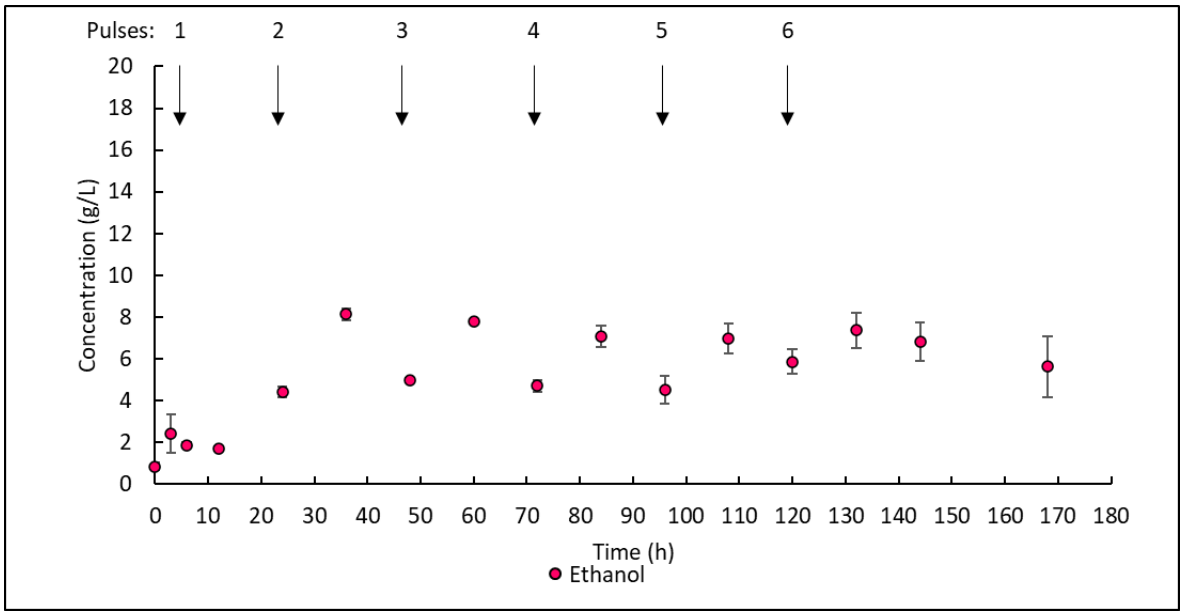


Figure 4.12: Ethanol concentration for the six-pulse fed-batch fermentation of *S. cerevisiae* with an initial hydrolysate concentration of 20% (v/v). Pulses contained undiluted hydrolysate and the feeding times are indicated by the arrows.

4.3.4.2.1 Protein contents of *S. thermophilus* and *S. cerevisiae*

The protein contents of *S. cerevisiae* and *S. thermophilus* biomass harvested at the end of the pulse fed-batch runs were determined from the elemental composition of the biomass and multiplying the elemental nitrogen content by a factor of 6.25 (Pihlajaniemi *et al.*, 2020). This approach is also used when making use of the Kjeldahl method.

Table 4.15: The protein content and concentrations of *S. thermophilus* and *S. cerevisiae* after 168 h of pulse fed-batch fermentation with an initial hydrolysate concentration of 20% (v/v).

Microorganism	Protein Content (% DW)	Protein Concentration (g/L)
S. thermophilus	66.10 ± 1.26	4.34 ± 0.10
S. cerevisiae	46.92 ± 2.64	24.71 ± 1.44

On a dry weight basis, the protein content of *S. thermophilus* was 66.1% ± 1.3% (DW) after the final fed-batch run (Table 4.15), which was similar to the 67.1% ± 2.5% (DW) in biomass grown under batch-fermentation conditions, with an initial hydrolysate concentration of 80% (v/v). The increased biomass yields in pulse fed-batch cultivations directly translated into an increased production of SCP.

The protein contents of *S. cerevisiae* after pulse fed-batch fermentation with an initial hydrolysate concentration of 20% (v/v) was 46.9% ± 2.6% (DW) (Table 4.15). The protein content achieved is similar to the protein content of 44.6% (DW) for *S. cerevisiae* when grown under submerged fermentation conditions using glucose as the carbon source (Hezarjaribi, Ardestani and Ghorbani, 2016).

Although the protein content of *S. cerevisiae* was lower than that of the *S. thermophilus* strain, the increase in the final biomass concentration of the former in pulse fed-batch fermentation (52.65 ± 0.80 g/L; Table 4.14) resulted in more SCP production per unit volume of bioreactor, compared to the protein present in the much lower final biomass concentration of *S. thermophilus* (6.57 ± 0.09 g/L; Table 4.14). The SCP produced per unit volume of bioreactor by *S. cerevisiae* and *S. thermophilus* was 24.71 ± 1.44 g/L and 4.34 ± 0.10 g/L (Table 4.15), respectively. *S. cerevisiae* was therefore preferred for SCP production from lignocellulose hydrolysate, primarily due to the severely negative effect of inhibitors on the growth performance of *S. thermophilus*. Increasing the availability of nitrogen in *S. thermophilus* cultures may improve biomass production, in addition to the implementation of detoxification methods to remove inhibitors from lignocellulose hydrolysates.

4.4 Conclusions

This study aimed to identify a generally regarded as safe (GRAS) status microbial strain that would be a suitable candidate for the bioconversion of steam-exploded and enzymatically pretreated sugarcane bagasse to single-cell protein (SCP). The objective was to identify a GRAS status microbial strain that could tolerate the presence of the inhibitors from steam explosion, such as furfural, 5-HMF and acetic acid, and maximise the biomass produced through the use of pulse fed-batch fermentation using *S. cerevisiae* as a control.

The steam explosion conditions of 185 °C for 10 minutes resulted in highly digestible sugarcane bagasse with a glucose yield of 98% ± 0.06% of the theoretical maximum of cellulose present, which is equivalent to 38.7 g glucose/100 g DW WIS. The product's high digestibility was achieved through the robust and potent enzyme Cellic® CTec3. Low concentrations of furfural, 0.25 ± 0.01 g/L and 5-HMF, 0.004 ± 0.002 g/L, were produced during steam explosion, where the acetic acid concentration of 3.79 ± 0.22 g/L exceeded the lethal dosage of 2.0 g/L. Therefore, the concentration of acetic acid was expected to have a toxic effect on the microbial strains. As a digestible product was produced, it is assumed that a lower enzyme dosage would be required to achieve high glucose concentrations during enzymatic hydrolysis.

The enzymatic hydrolysis conditions that resulted in an approximate 80% conversion of cellulose to glucose was a solid loading of 15% (w/v) of steam-exploded sugarcane bagasse and a Cellic® CTec3 enzyme dosage of 7.5 FPU/g DW substrate for 72 h, which resulted in a final glucose concentration of 42.26 ± 0.80 g/L and a 77.6% ± 0.4% conversion of cellulose to glucose. The 77.6%± 0.4% conversion achieved is close to the desirable 80% conversion that is required for bioethanol biorefineries to be commercially viable. Therefore, these enzymatic hydrolysis conditions were used to produce the hydrolysate used in the screening and pulse fed-batch fermentations.

During the screening experiment that was used to determine which of the GRAS status microbial strains were able to tolerate the presence of inhibitors best, *S. cerevisiae*, which was used as the control, outperformed the other five strains. *S. cerevisiae* consumed all of the glucose in the undiluted enzymatic hydrolysate and achieved a final biomass concentration of 20.37 ± 11.81 g/L after 48 h. In comparison, out of the five strains, namely, *B. subtilis* CAB1111 (bacteria), *F. venenatum* (fungus), *L. delbrueckii* (bacteria), *P. ostreatus* CAB13 (fungus) and *S. thermophilus* (bacteria), *S. thermophilus* achieved the highest consumption of available glucose (47.48% ± 3.75%) and a final biomass concentration of 1.28 ± 0.17 g/L, after 48 h. These findings suggest that the five screened strains did not exhibit a high tolerance to the inhibitors, such as acetic acid, present in the hydrolysate.

Pulse fed-batch fermentation with an initial hydrolysate concentration of 20% (v/v), fed with undiluted hydrolysate to achieve a final concentration of 80% (v/v), substantially increased the final biomass concentrations of both *S. cerevisiae* and *S. thermophilus*, compared to batch cultures. *S. cerevisiae* achieved a higher final biomass concentration (52.65 ± 0.80 g/L) than *S. thermophilus* (6.57 ± 0.09 g/L). The protein concentration produced by *S. cerevisiae* was 24.71 ± 1.44 g/L, while *S. thermophilus* produced 4.34 ± 0.10 g/L. Therefore, *S. cerevisiae* would be the best strain to convert steam-exploded

sugarcane bagasse to single-cell proteins as it results in the best final biomass concentrations, resulting in the highest mass of protein. Co-production of ethanol with SCP may further enhance the economic attractiveness of this process as the maximum ethanol concentration produced by *S. cerevisiae* during pulse fed-batch fermentation was 8.13 ± 0.27 g/L after 36 h. However, this is likely to be at the expense of the latter.

CHAPTER 5:

TECHNO-ECONOMIC ANALYSIS

Preface

This chapter aims to determine whether *S. thermophilus* or *S. cerevisiae* from CHAPTER 4 would be more technically and economically feasible to produce single-cell protein from steam-exploded sugarcane bagasse for human consumption on an industrial scale. An Aspen Plus® software model that was developed in previous studies was used, where the experimental data obtained from CHAPTER 4 was used as inputs to obtain realistic industrial-scale costs to assess the processes' economic and technical feasibility. The Aspen Plus® model integrated a sugar mill and the SCP production process. Furthermore, the necessity of the additional washing stage of the developed Aspen Plus® model after the bagasse underwent steam explosion was investigated for the *S. cerevisiae* strain.

ABSTRACT

The technical and economic feasibility of producing single-cell protein (SCP) from a hydrolysate obtained through the steam explosion and enzymatic hydrolysis of sugarcane bagasse was investigated. The SCP production process was integrated into a sugar mill, where the bagasse feedstock could be available, processed and converted into SCP through the microbial culture of *S. cerevisiae* or *S. thermophilus*. The technical feasibility of the processes was assessed by determining and comparing the biomass yields per kilogram of sugarcane bagasse of the two strains and the utility requirements. Additionally, the overall energy balance of the integrated sugar mill and SCP plant that was required to ensure that sufficient bagasse-based process energy remained available for the complex to be energy self-sufficient was compared. These factors were determined through mass and energy balances simulated in Aspen Plus® software. The economics of the two processes were compared on the basis of their total capital investment (CAPEX), operating costs (OPEX) and minimum selling price (MSP). The higher specific yield (0.11 kg biomass/kg bagasse) achieved by *S. cerevisiae* resulted in the lowest MSP of 2 319 US$/tonne biomass (ZAR 41/kg) for SCP, which was an order of magnitude lower than the MSP of 20 436 US$/tonne biomass (ZAR 353/kg) for SCP from *S. thermophilus*, due to the lower specific yield of the latter (0.01 kg biomass/kg bagasse). Furthermore, the necessity of the washing stage for the steam-exploded bagasse in the Aspen Plus® model was investigated for the *S. cerevisiae* strain and it was found that the washing stage marginally increases the product purity by 3% and, therefore, is not required. Consequently, it would be recommended that the model be adjusted to improve the efficiency of the washing stage to achieve a higher product purity.

5.1 Introduction

Sugarcane bagasse is a by-product of the sugarcane industry, where at least half of the bagasse produced is combusted in boilers for steam generation to power the sugar mill (Chambon *et al.*, 2018). The other half of the bagasse is either stockpiled or burnt. Therefore, there is much opportunity in the upcycling of the surplus sugarcane bagasse.

The bioconversion of steam-exploded sugarcane bagasse to single-cell proteins (SCPs) for human consumption reveals a biologically efficient method for upcycling the bagasse. There has been much research into the economics of the production of bioethanol from sugarcane bagasse. However, minimal research has been conducted on the technical and economic feasibility of the production of SCPs from sugarcane bagasse. Therefore, using the experimental research in CHAPTER 4, the two SCP production processes from steam-exploded sugarcane bagasse using *S. thermophilus* and *S. cerevisiae* were modelled.

The industrial process that will be modelled will be that of a biorefinery integrated with a sugar mill so as to allow the excess steam provided by the mill's boiler to be used in the biorefinery process. Therefore, the technical feasibility of the process will be determined by ensuring that the bagasse produced is (1) enough to feed the biorefinery process for the production of the desired product, (2) can be used to provide the steam and electrical requirements of the biorefinery and (3) provide all of the steam and electrical demands of the sugar mill itself.

The biorefinery process will include additional processing steps above the steam pretreatment and separate hydrolysis and fermentation (SHF) that were investigated during the experimental research. These additional process steps include a washing step after the steam explosion of the bagasse, isolating and drying the high-content microbial biomass, biogas production and water purification, for which process descriptions were obtained from literature.

The processing costs include operational costs, such as energy, biocatalyst and chemical costs, as well as capital costs. The main operating cost contribution is the cost of the conversion of the sugarcane bagasse, as the feedstock has a low value and hence would not incur too much cost to the process (Lynd *et al.*, 2017). Furthermore, the main operating costs are associated with the pretreatment of the sugarcane bagasse (Lynd *et al.*, 2017). The pretreatment process includes the thermochemical pretreatment and the enzymatic hydrolysis of the sugarcane bagasse.

The main purpose of this work is to determine whether *S. thermophilus* or *S. cerevisiae* would be more profitable on an industrial scale and how the final SCP product's required minimum selling price (MSP) compares to existing protein products for human and animal consumption. The experimental work showed that *S. cerevisiae* achieved higher biomass yields and utilised all of the glucose available in the enzymatic hydrolysate during submerged batch fermentation (CHAPTER 4). However, the technical and economic performances of producing SCPs from *S. cerevisiae* on an industrial scale need to be investigated and compared to producing SCPs from *S. thermophilus*.

An Aspen Plus® simulation model, developed by Abdul Petersen (Petersen *et al.*, 2022), was adapted to simulate the two different processes for *S. thermophilus* and *S. cerevisiae*, to obtain the necessary mass

and energy balances for the integrated sugar mill with annexed SCP plant. Inputs from the experimental work provided in CHAPTER 4 were used in the simulation and various utilities required in the process were specified to determine the capital and operating costs of the process. The costs of the process were determined through the use of an economic model, also developed by Abdul Petersen, which was linked to the Aspen Plus® simulation and was therefore updated by the inclusion of experimental results from CHAPTER 4 into the Aspen Plus® simulation. The economic model was used to perform a discounted cash flow (DCF) analysis to determine the estimated MSP to produce a tonne of SCPs from the two strains. The MSP was compared to determine which of the two would result in a more economically feasible production process and how these MSPs compare to existing protein products in the market, such as beef, pork and chicken meat for human consumption and animal feed such as soybean oilcake.

A washing stage was incorporated into the Aspen Plus® model after the steam pretreatment stage, which was an additional step as compared to the experimental method followed. Therefore, the necessity of the washing stage of the model was investigated for the *S. cerevisiae* process by comparing the final product purity for a model that contained a washing step and for one that followed the experimental method and did not contain a washing step.

5.2 Methodology

5.2.1 Process model

Aspen Plus® is a software that is used to model processes and can also be used for process monitoring, optimisation and conceptual design. It is primarily used by chemical process industries (Saha, 2022) and allows one to create a process model by first developing a flowsheet. Once a flowsheet has been developed, the chemical components and operating conditions are specified for the specific process being modelled. The process simulation then performs calculations needed to determine various outcomes of the process, specifically mass and energy balances. These calculations are performed by making use of the inputs provided to Aspen for the specific process being modelled. Thus, the model predicts the behaviour of the process based on the inputs provided (Fogler and Gurmen, 2008).

Two separate process flowsheets for *S. thermophilus* and *S. cerevisiae* were developed by Abdul Petersen using Aspen Plus® version 11 and the experimental results obtained from this investigation were used as inputs for the flowsheets. The process flowsheets were identical except for the inputs into the models, such as the temperatures of the reactor, biomass yield and so forth.

An economic model, developed previously in Microsoft Excel (Petersen, Okoro, *et al.*, 2021), was linked to the process flowsheet developed in Aspen Plus®. The integration of the two allowed various inputs specified in the Excel spreadsheet to be automatically linked to the process flowsheet and vice versa. This integration allowed for the mass and energy balances developed in the process simulation to be used directly in the equipment sizing and the economic evaluation of the process.

5.2.2 Development of the process flowsheet

The process flowsheet was developed in several parts. The first part was developed for the steam explosion of the sugarcane bagasse. The second part simulated the enzymatic hydrolysis of the washed solid fraction obtained from the steam explosion and was followed by the fermentation of the resulting hydrolysate. The remainder of the flowsheet consists of wastewater treatment, biogas production to produce energy and utilisation of the waste bagasse to generate steam for the production process (Figure 5.1).

The same production process was simulated and used for *S. cerevisiae* and *S. thermophilus* to ensure that the economic evaluation of both simulations was comparable. The differences between the two simulations resulted from the inputs that were used, such as the temperature of the fermentation process, the protein and biomass yields and the glucose consumption achieved by the two strains, which were obtained from the pulse fed-batch experiments reported in CHAPTER 4.

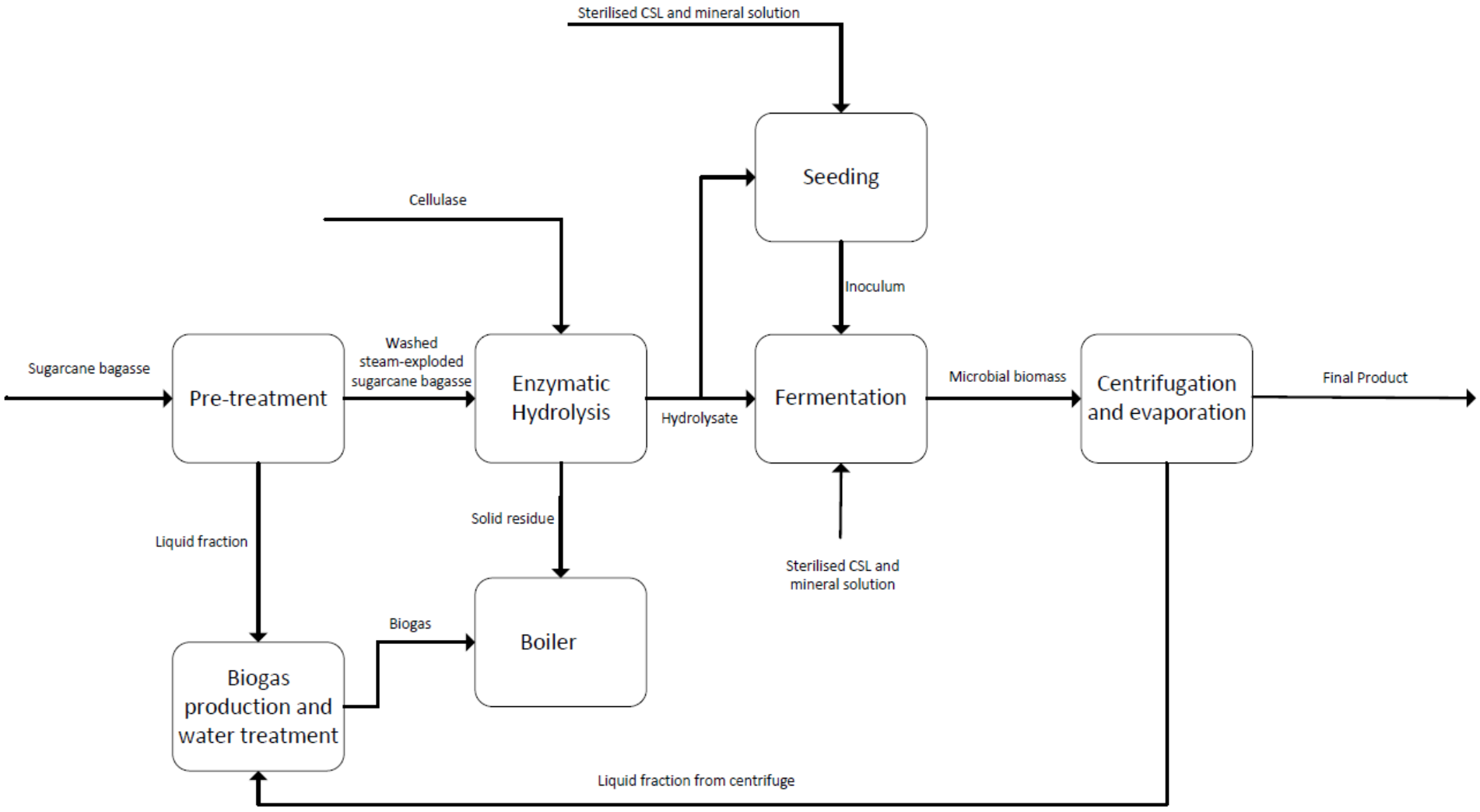


Figure 5.1: Block flow diagram of the single-cell protein production process.

5.2.2.1 Steam explosion of the sugarcane bagasse

The sugarcane bagasse was steam-exploded to ensure that the resulting product was more digestible. The experimental results were used as inputs to determine the by-products produced, the sugars produced and the water content of the resulting steam-exploded sugarcane bagasse (Table 4.9).

The steam explosion of the sugarcane bagasse was simulated through the use of an RStoic reactor. The reactor's temperature was specified through the use of a calculator block, which calculated the required amount of steam, at a pressure of 10 bar, to achieve the desired temperature. The resulting steam-exploded sugarcane bagasse undergoes a two-stage counter-current washing and filtration process to split the solids and to wash the residual by-products present in the liquid fraction from the solid fraction. The water used to wash the sugarcane bagasse was recycled from the wastewater treatment section of the flowsheet.

5.2.2.2 Separate enzymatic hydrolysis and fermentation

The washed solid fraction of the steam-exploded sugarcane bagasse enters the enzymatic hydrolysis phase. Again, an RStoic reactor was used for the enzymatic hydrolysis pretreatment step. However, before entering the reactor, a calculator block was used to determine the volume of water required to dilute the solid fraction to obtain the desired solid loading of 15% (w/v). Additionally, the required enzyme dosage was calculated to ensure that a 7.5 FPU/g DW substrate was achieved, based on experimental results in CHAPTER 4. The resulting enzymatic hydrolysate underwent filtration to ensure that only the liquid fraction of the hydrolysate was used during the fermentation step.

For the fermentation step, a 2% split of hydrolysate was diverted to the seeding train, which was placed prior to the fermentation. The seeding train allowed for the growth of the microbial inoculum and allowed for the acclimation of this strain to a small dosage of the inhibitors present in the hydrolysate. The corn-steep liquor (CSL) and the mineral solution dosage required in the seeding train were also calculated using a calculator block (Table 5.1). The mineral solution was added at an equal mass ratio as the CSL. The CSL calculated value was multiplied by 1.5 to ensure that the CSL was present in excess. The seeding train also included a sterilisation step at 121 °C for the mineral solution and CSL. As this is an aerobic process, a calculator block was used to determine the required air input based on the amount of glucose and xylose present in the inlet stream to the reactor.

Table 5.1: A summary of the chemical dosing requirements per kilogram of sugarcane bagasse.

Chemicals	**Dosing requirements (g/kg bagasse)**
Calcium Chloride ($CaCl_2$)	0.012
Cellulase solution (10% solid cellulase protein)	55.14
Corn steep liquor	587.40
Magnesium sulphate ($MgSO_4$)	0.086
Potassium dihydrogen phosphate (KH_2PO_4)	1.18

The resulting microbial inoculum generated in the seeding train is introduced to the fermentation process. The fermentation process was simulated through the use of an RStoich reactor, which was set at the pre-determined temperature for the microbial strain being used in the process, which was 37 °C for *S. thermophilus* and 30 °C for *S. cerevisiae*. Once again, the required mineral solution, which comprised of $CaCl_2$, $MgSO_4$ and KH_2PO_4, and CSL dosages were calculated and sterilised before being added to the reactor. The CSL was calculated to ensure that a 9.3 mass% of CSL was achieved for the entire volume in the reactor. Once again, the mineral solution was added at an equal mass ratio as the CSL.

5.2.2.3 Preparation of the product

The biomass produced from the liquid hydrolysate and microbial inoculum during the fermentation process subsequently entered a separation process. Before the separation process occurred, the biomass entered a heater that was set at 65 °C, which was used to inactivate the material and degrade the RNA (Trinci, 1992; Wiebe, 2002). The degradation of the RNA is essential as it ensures that the biomass produced is safe for human consumption as there is a limit on the amount of nucleic acid one can consume, which is not more than 2 g of nucleic acid per day (Moo-Young, Chisti and Vlach, 1993). A temperature of 65 °C was selected as this is the temperature used in the Quorn® production process. Additionally, a temperature above 65 °C results in the denaturing of the protein produced, which is undesirable, as the goal of the production process is to produce a high-quality protein product (Annoh-Quarshie, 2018).

Subsequent to thermal treatment, the resulting biomass entered a centrifuge to remove the excess liquid from the fermentation process, resulting in a cake with a moisture content of 95%. The remaining water was sent for biogas production. The resulting solids entered an evaporation step to concentrate the slurry to about 50% solids before the cells were dried in a spray dryer. Thus, the final product would be ready for packaging and sales.

5.2.2.4 Water Treatment

The water that was removed during the centrifugation of the biomass is treated in a biodigester, which removes 80% of the chemical oxygen demand (COD) and produces biogas (Dias *et al.*, 2012). The water from the biodigester is then treated in an aerobic reactor, where a water balance is performed to

determine the water requirements of the process. The water balance determines how much water from the aerobic effluent requires treatment through sterilisation, ultrafiltration and reverse osmosis (RO) (Petersen, Okoro, *et al.*, 2021).

5.2.2.5 Integrated Energy Supply

To ensure that the energy and steam demands of the process are met, the solid hydrolysate residue is used to feed the combined heat and power (CHP) of the sugar mill and the utility boiler to generate sufficient electricity and steam for both the sugar mill and the SCP plant (Petersen, Okoro, *et al.*, 2021). The utility boiler is installed to ensure that the steam demands that are in excess of the CHP process are met. The biogas that is produced in the water treatment step is used as additional fuel for the boilers. Therefore, the process is energy self-sufficient, where no additional external sources are required for the production of SCPs.

5.2.3 Determining the necessity of the washing stage in the model

The washing of the steam-exploded sugarcane bagasse was an additional stage that was incorporated into the model; however, it was not incorporated into the experimental method. Therefore, it is essential to investigate the effect that the washing stage of the model has on product purity by comparing the mass flow rates of xylose and acetic acid in the product stream with and without the washing stage. The washing stage in the *S. cerevisiae* process model was removed and the simulation was re-run to determine the effect thereof on product purity.

5.3 Technical evaluation of the process

The technical evaluation of the production of SCPs from steam-exploded sugarcane bagasse was evaluated by investigating three factors: whether the bagasse produced (1) is enough to feed the biorefinery process for the production of SCPs, (2) it can be used to provide the steam and electrical requirements of the biorefinery process and (3) provide all of the steam and electrical demands of the sugar mill itself.

The mass of bagasse required for the two biorefinery processes was determined iteratively to ensure technical efficiency. The iterative solving of the required mass of bagasse was determined by the energy requirements of the two different process models. An energy balance was used to determine the energy requirements of the process.

5.4 Economic evaluation of the process

The SCP production process's economic success depended on the yields of microbial biomass in the process and their protein contents, with the latter aimed primarily at protein sources for human consumption.

The economic performance of the SCP production process will be determined by the final product's MSP, where the final product is the SCP. The MSP will be determined through the use of a discounted cash flow (DCF) analysis to meet the desired internal rate of return (IRR) (Petersen, Brown, *et al.*, 2021)

The assumptions that will be used to perform the processes' economics are as follows:

1. Start-up of the plant will take 2 years, including construction, preparing equipment and so forth.
2. Plant life will be 25 years.
3. The desired IRR is set to 20% (real).
4. Taxation rate of 28%.
5. It is assumed that the plant runs for 5 000 hours a year, which is equivalent to 208 days, based on the harvest season of sugarcane bagasse in South Africa.
6. No salvage value at the end of the project's life.
7. 50% of the potential revenue is obtained in the first year after start-up and 100% thereafter.
8. Cash flows are discounted at a rate of 20%.
9. Depreciation is calculated using the straight-line method to zero value over the financial period.

5.4.1 Capital expenditure cost (CAPEX)

The capital cost estimate that will be used will take the form of a first order estimate. This estimate is used as a screening and feasibility estimate and will allow for a rough comparison to occur between the two microbial strains used in the production of SCP (Turton *et al.*, 2018). The resulting error range of this particular cost estimation is ± 30% (Peters and Timmerhaus, 1991).

The majority of the capital costs were calculated using an in-house tool that was developed and described in previous publications (Petersen, Franco and Görgens, 2018; Petersen, Brown, *et al.*, 2021). The in-house tool makes use of numerous simulated variables and uses these variables to size and estimate the cost of the equipment required as well as the cost of the various utilities. The CAPEX considers

installation, sundry equipment and indirect and working capital costs. The working capital was calculated as 5% of the total capital investments (TCI).

5.4.2 Operating cost estimations (OPEX)

The operating cost of the two biorefinery processes was calculated by taking the sum of the raw material, disposal and other fixed costs. The steam and electricity requirements of the process were determined by the mass and energy balances that were simulated in the Aspen Plus® model. The cold utilities were also simulated and converted to electrical equivalents using a coefficient of performance (COP) of 12%. A summary of the operating costs can be found in Table 5.2 below.

Table 5.2: Summary of operating costs.

Item	Unit Cost (US$)	Reference
Electricity (US$/kWh)	0.052	Sirius Engineering
Steam (US$/tonne)	7.67	Sirius Engineering
Glucose for seeding train*	2.35	www.alibaba.com
Solid cellulase protein (US$/kg)	6.00	(Petersen, Okoro, *et al.*, 2021)
Enzyme solution (9% concentration) (US$)	0.54	
Bagasse (US$/kg)	0.12	(Diederichs *et al.*, 2016)
Magnesium sulphate ($MgSO_4$)	1.07	www.futurama.co.za
Calcium Chloride ($CaCl_2$)	1.55	www.labequipsupply.co.za
Potassium dihydrogen phosphate (KH_2PO_4)	1.83	www.gardengoods.co.za
Microorganisms	0.01	(Petersen, Okoro, *et al.*, 2021)
Corn steep liquor	0.09	(Humbird *et al.*, 2011)
Operating labour costs ($/h)	164.8	(Petersen, Okoro, *et al.*, 2021)

* Estimated based on molasses

5.4.3 Cash flow analysis and project profitability

A DCF analysis was used to determine the NPV and the MSP. The MSP was determined through various iterations of the selling price until the NPV was zero. The stipulated IRR is used to determine the profitability of potential investments and is the discount rate that ensures that the NPV is equal to zero for cash flows in a DCF analysis at the end of the project (Annoh-Quarshie, 2018; Fernando, 2022).

5.5 Results and discussion

5.5.1 Technical Assessment

A summary of the technical outcomes regarding the biomass produced, sugarcane bagasse required, utility requirements and electricity requirements are summarised in Table 5.3 below. The technical requirements were based on the simulated mass and energy balances from the Aspen Plus® model, with the experimental data from CHAPTER 4 as inputs.

S. cerevisiae achieved the highest specific biomass yield of 0.11 kg biomass/kg bagasse (Table 5.3). In comparison, *S. thermophilus* achieved a specific biomass yield of 0.01 kg biomass/kg bagasse (Table 5.3). Therefore, *S. cerevisiae*, from a yield perspective, would be the preferred strain to produce SCP on an industrial scale.

The purity of the microbial biomass produced by *S. cerevisiae* was 47%, while that of *S. thermophilus* was 41% (Table 5.3). The purity of the cells from the production of SCP is an important aspect to consider, as the product will contain entrained sugars and proteins that were obtained from the corn steep liquor (CSL). The entrained proteins result from CSL containing a mixture of amino acids, vitamins, minerals and trace elements (Tan *et al.*, 2016; Taiwo, Madzimbamuto and Ojumu, 2018). All proteins were modelled as glutamine to develop reactions for protein production by the microbial strains (Table 5.3). Additionally, the entrained proteins will contribute to the overall protein content of the microbial biomass.

The heating utility requirements of *S. cerevisiae* (64 455 MJ/h; Table 5.3) were higher than that of *S. thermophilus* (2 029 MJ/h; Table 5.3), as more bagasse required steam explosion (30 721 kg/h compared to 29 210 kg/h; Table 5.3), which requires a large portion of the heating utility requirements of the process. More bagasse was required by *S. cerevisiae* as it achieved a higher yield per bagasse fed (0.11 kg biomass/kg bagasse; Table 5.3) as compared to *S. thermophilus* (0.01 kg biomass/kg bagasse Table 5.3). The *S. cerevisiae* production process required more bagasse to ensure that both the mill and the SCP production process were energy self-sufficient as well as being able to produce large quantities of SCP. Furthermore, as *S. cerevisiae* produced more biomass (3 395 kg/h; Table 5.3) than *S. thermophilus* (387 kg/h; Table 5.3), *S. cerevisiae* required higher cooling demands due to cellular respiration (3 988 kW compared to 972 kW; Table 5.3).

The bagasse requirements of the two processes were determined iteratively based on the energy requirements of the two processes. The iterations were required to ensure that the processes were technically efficient, resulting in the processes' energy self-sufficiency. For the *S. thermophilus* process to be self-sufficient, 29 tonne/h of bagasse is required, while 31 tonne/hr of bagasse is required for the *S. cerevisiae* process (Table 5.3). As aforementioned, *S. cerevisiae* requires more bagasse due to its higher utility demands and higher specific biomass yield.

Table 5.3: Summary of technical outcomes from the production process of *S. thermophilus* and *S. cerevisiae*.

	Units	*Streptococcus thermophilus*	*Saccharomyces cerevisiae*
Inputs			
Sugarcane bagasse	kg/hr	29 210	30 721
Outputs			
Biomass: mixed fraction	kg/h	387	3 395
Yield ($Y_{x/s}$) per bagasse fed	kg biomass/kg bagasse	0.01	0.11
Cell Purity	%	41	47
Glutamine	kg/h	110	1 255
Electricity availability for biorefinery	kW	3 948	3 901
Requirements			
Electricity	kW	2 188	5 228
Process energy integration			
Hot utilities			
Total	MJ/h	2 029	64 455
Cold utilities			
Total	kW	972	3 988

5.5.2 Economic Evaluation

The CAPEX required for the *S. cerevisiae* process was 140 million US dollars compared to 125 million US dollars required for the *S. thermophilus* process (Figure 5.2). The *S. cerevisiae* process required a higher capital investment as more biomass was produced; hence larger equipment capacity and sizing were required in downstream processing to produce a desirable final product. The OPEX of the *S. cerevisiae* process (2.50 million US$/annum; Figure 5.2) was higher than that of the *S. thermophilus* process (1.11 million US$/annum; Figure 5.2), as the *S. cerevisiae* process had higher bagasse demands and thus higher chemical requirements.

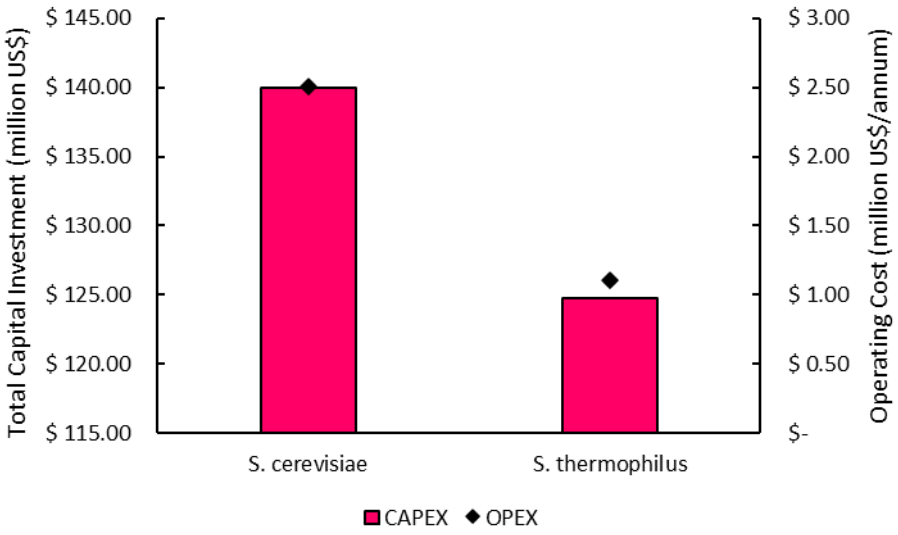


Figure 5.2: CAPEX (bars) and OPEX (diamond points) costs for the production of SCP from *S. cerevisiae* and *S. thermophilus*.

The two microbial strains' MSPs differs significantly (Table 5.4). The MSP of *S. cerevisiae* is 2 309 US$/tonne biomass (ZAR 41/kg; Table 5.4), whereas the MSP of *S. thermophilus* is 19 705 US$/tonne biomass (ZAR 353/kg; Table 5.4). The MSP for SCP from *S. cerevisiae* were below the wholesale prices for chicken meat (ZAR 59; Table 5.4) and beef meat (ZAR 60; Table 5.4) in South Africa, although above the wholesale price for pork meat (ZAR 27/kg; Table 5.4)and the effective protein price for animal feed, based on the current market prices for soybean oilcake (ZAR 17/kg; Table 5.4). This implied that the process would not be economically feasible if it were sold as animal feed (Pihlajaniemi *et al.*, 2020). The significant difference in MSP can be attributed to *S. cerevisiae* producing about ten times more biomass than *S. thermophilus* (Table 5.3) while incurring similar CAPEX and OPEX costs in the SCP facility. Also, it must be noted that the economics of adding a step for extraction and purification of SCP from the isolated microbial biomass has not been considered here.

Table 5.4: MSPs of *S. cerevisiae* and *S. thermophilus* per tonne of biomass compared to wholesale market prices of chicken, pork and beef meat and animal feed in the form of soya bean oilcake (Morning Star Feeds, 2022; NAMC, 2022; Western Cape Government, 2022a, 2022b).

	Units	*S. thermophilus*	*S. cerevisiae*	Whole Chicken	Pork	Beef	Soya bean oilcake
MSP	US$/tonne	19 705	2 309	-	-	-	
	ZAR/kg	353	41	59[a]	27[b]	60[c]	17

[a] 2021
[b] Apr 2022
[c] A-grade in Apr 2022

5.5.3 Necessity of the washing stage in the process model

A washing stage was incorporated into the Aspen Plus® for the *S. cerevisiae* and *S. thermophilus* models, which was a deviation from the experimental method followed. An investigation into the necessity of the washing stage was conducted for only the *S. cerevisiae* process.

When the washing stage is implemented, the product purity is 47% (Table 5.5), while with no washing stage, the product purity is 44% (Table 5.5), which results from a slight increase in the xylose mass flow rate in the product (1 622 kg/h to 1 948 kg/h; Table 5.5). Therefore, from these results, a washing stage is not required, as only a 3% increase in product purity was achieved. However, improvements to the washing stage in the pre-existing Aspen Plus® model are recommended to improve the purity of the final product.

Table 5.5: Mass flow rates of glucose, xylose and acetic acid in the product stream for SCP production from *S. cerevisiae* with and without a washing stage.

	Units	**Washing stage**	**No washing stage**
Glucose	kg/h	366	376
Xylose	kg/h	1 622	1 948
Acetic acid	kg/h	0.3	0.5
Product purity	%	47	44

5.6 Conclusions

The technical and economic feasibility of the production of SCPs from the bioconversion of steam-exploded sugarcane bagasse was evaluated for *S. cerevisiae* and *S. thermophilus.* The biorefinery process was modelled to be integrated with sugar mill operations to secure a supply of bagasse and process energy for SCP production. The technical feasibility of the process was determined by ensuring that the bagasse produced allowed for the processes to be energy self-sufficient and was enough to feed the biorefinery process for the production of the desired product. The economics of the two processes were compared based on their total CAPEX, OPEX and MSP.

S. cerevisiae resulted in a higher specific biomass yield of 0.11 kg biomass/kg bagasse, while *S. thermophilus* achieved a specific biomass yield of 0.01 kg biomass/kg bagasse. Therefore, based on these specific biomass yields alone, *S. cerevisiae* is the preferred strain to produce SCP on an industrial scale. Additionally, *S. cerevisiae*'s higher biomass yield resulted in an MSP of 2 319 US$/tonne biomass (ZAR 41/kg) compared to the MSP of *S. thermophilus* of 20 436 US$/tonne biomass (ZAR 353/kg), as the resulting CAPEX and OPEX would be less per unit of product produced. Furthermore, the MSP of *S. cerevisiae* was below the wholesale prices for chicken (ZAR 59) and beef (ZAR 60) meat in South Africa.

Therefore, to produce a more affordable alternative protein product, *S. cerevisiae* would be a beneficial candidate as it resulted in the lowest MSP, produced higher biomass yields and, thus, produced more protein on a mass basis as compared to *S. thermophilus*.

The necessity of the washing stage in the Aspen Plus® model was investigated for the *S. cerevisiae* process. The washing stage was implemented in the pre-existing Aspen model to improve the product purity. However, upon investigation, it was found that without the washing stage, a product purity of 44% was achieved, while a product purity of 47% was achieved with a washing stage. Therefore, as the washing stage marginally increases the product purity by 3%, the washing stage is not required in this process unless improvements to the washing stage are made so as to increase the product purity substantially.

CHAPTER 6:

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

The bioconversion of steam-exploded and enzymatically hydrolysed sugarcane bagasse to single-cell protein (SCP) using generally regarded as safe (GRAS) microbial strains was investigated. Various GRAS microbial strains were identified and screened to determine which could tolerate the presence of various inhibitors, such as furfural, 5-HMF and acetic acid. The biomass production by the strain that tolerated the inhibitors best, compared to *S. cerevisiae*, was maximised through pulse fed-batch fermentation, where *S. cerevisiae* was once again used as a control.

Highly digestible steam-exploded sugarcane bagasse was produced under the steam explosion conditions of 185 °C for 10 minutes, where a glucose yield of 98% ± 0.06% of the theoretical maximum of cellulose present was achieved through the application of a high dosage of Cellic® CTec3. The steam explosion conditions resulted in negligible concentrations of furfural and 5-HMF of 0.25 ± 0.01 g/L and 0.004 ± 0.002 g/L, respectively, while the acetic acid concentration, 3.79 ± 0.22 g/L, was above the lethal dose of 2 g/L and is thus expected to have a toxic effect on the microbes. Furthermore, due to the high digestibility of the bagasse, lower enzyme dosages of 7.5 FPU/g DW substrate could be applied to achieve the desired ≈ 80% conversion required for the biorefinery to be commercially viable.

Enzymatic hydrolysis conditions that resulted in an approximate 80% conversion of cellulose to glucose were a solid loading of 15% (w/v) of steam-exploded bagasse and a Cellic® CTec3 enzyme dosage of 7.5 FPU/g DW substrate for 72 h. A 77.6% ± 0.4% conversion was achieved and a glucose concentration of 42.26 ± 0.80 g/L. These conditions were used to produce hydrolysate for the submerged batch fermentations and pulse fed-batch fermentations.

The submerged batch fermentation was used during the screening experiment to identify SCP candidates with a high tolerance for the inhibitors produced during steam explosion from six GRAS microbes. *S. cerevisiae* (yeast), used as the control, outperformed the alternative five strains, namely, *B. subtilis* CAB1111 (bacteria), *F. venenatum* (fungus), *L. delbrueckii* (bacteria), *P. ostreatus* CAB13 (fungus) and *S. thermophilus* (bacteria). *S. cerevisiae* consumed all of the glucose and achieved a final biomass concentration of 20.37 ± 11.81 g/L after 48 h. *S. cerevisiae* is a well-studied strain and is well-known for its high tolerance to inhibitors; therefore, its performance is expected. In contrast, *S. thermophilus* tolerated the inhibitors, such as acetic acid, the best out of the other strains investigated, where it consumed 47.48% ± 3.75% of the glucose and achieved a final biomass concentration of 1.28 ± 0.17 g/L after 48 h. This revealed that the five strains screened, excluding *S. cerevisiae*, cannot tolerate the concentrations of inhibitors, such as acetic acid, present in the hydrolysate.

The maximisation of the final biomass concentration of *S. thermophilus* using pulse fed-batch fermentation was investigated, with *S. cerevisiae* as a control. An initial hydrolysate concentration of

20% (v/v) was used and the system was pulsed with undiluted hydrolysate until a final hydrolysate concentration of 80% (v/v) was achieved. Pulse fed-batch fermentation substantially increased the final biomass concentrations of *S. thermophilus* and *S. cerevisiae* compared to the aforementioned batch cultures. *S. cerevisiae* outperformed *S. thermophilus* and achieved a higher final biomass concentration of 52.65 ± 0.80 g/L, while *S. thermophilus* achieved a final biomass concentration of 6.57 ± 0.09 g/L. Additionally, the protein concentration produced by *S. cerevisiae* was 24.71 ± 1.44 g/L, while *S. thermophilus* produced 4.34 ± 0.10 g/L. For the production of SCP from steam-exploded sugarcane bagasse, *S. cerevisiae* would be the best candidate as it would be able to efficiently convert the substrate to protein and achieve high biomass concentrations. Additionally, the co-production of ethanol with SCP may further enhance the economic attractiveness of this process as the maximum ethanol concentration produced by *S. cerevisiae* during pulse fed-batch fermentation was 8.13 ± 0.27 g/L after 36 h. Although, the co-production thereof is likely to be at the expense of the latter and further investigation is required.

The technical and economic feasibilities of the two strains were investigated and compared. Once again, *S. cerevisiae* performed better than *S. thermophilus* and is the preferred strain to produce SCP on an industrial scale from steam-exploded sugarcane bagasse. *S. cerevisiae*'s higher yield resulted in a minimum selling price (MSP) of 2 319 US$/tonne biomass (ZAR 41/kg) compared to the MSP of *S. thermophilus* of 20 436 US$/tonne biomass (ZAR 353/kg), as the resulting CAPEX and OPEX would be less per unit of product produced. Furthermore, the MSP of *S. cerevisiae* was below the wholesale prices for chicken meat (ZAR 59/kg) and beef meat (ZAR 60/kg) in South Africa. Therefore, *S. cerevisiae* would be a beneficial SCP candidate to produce a more affordable protein alternative.

A washing stage was implemented in the Aspen Plus® model, which was a deviation from the experimental method. Therefore, the necessity of the additional stage was investigated. The washing stage was implemented to increase the product purity; however, it marginally increased the product purity by 3% and, therefore, is not required in this process. It is suggested that improvements to the washing stage are made to ensure a higher product purity is achieved.

6.2 Recommendations

6.2.1 Higher solid loading

Higher solid loadings should be investigated for the enzymatic hydrolysis of the steam-exploded sugarcane bagasse. A high solid loading reactor should be used to ensure that adequate mixing of the substrate is achieved. The higher solid loading should result in higher glucose yields and, with adequate mixing, may result in better cellulose-to-glucose conversions.

6.2.2 Consolidated bioprocessing

The use of consolidated bioprocessing (CBP) should be investigated as an alternative method to produce SCPs from steam-exploded sugarcane bagasse. CBP should result in lower production costs, as there should be no need for the addition of cellulase enzymes.

6.2.3 Co-production of SCP and bioethanol

The interplay between SCP and ethanol yields from available sugars should be further considered from an economic point of view. The effect of the co-production would be of interest to determine the effects thereof on the biomass and ethanol yields obtained. Therefore, an experimental and an economic investigation should be considered.

6.2.4 Economic evaluation of a protein extraction and purification step

Further investigation into the economics of adding a protein extraction and purification step to isolate the SCP product from the microbial biomass should be investigated experimentally and included in the Aspen Plus® simulation.

6.2.5 Improvement of the washing stage

An improvement to the washing stage should be implemented in the pre-existing Aspen Plus® model to increase the product purity achieved. Additionally, the economics of such an improvement should be investigated. Further, the optimisation between product purity and the economics of the improved washing stage should be compared to determine to what extent the washing stage can be improved without compromising the cost of the process.

6.2.6 Investigate the growth of the microbial strains without inhibitors

Grow the microbial strains in a synthetic growth media replicating the sugars, minerals and CSL concentrations of the undiluted hydrolysate media. However, the media will not have the inhibitors present. This experiment will act as a benchmark to determine how much the inhibitors affect the growth of the microbial strains.

REFERENCES

Ahmadi, A.R. *et al.* (2010) 'Determination of the amount of protein and amino acids extracted from the microbial protein (SCP) of lignocellulosic wastes', *Biological Sciences*, 13(8), pp. 355–361.

Ahmed, S.A. *et al.* (2008) 'Biological Efficiency And Nutritional Contents Of Pleurotus florida (Mont.) Singer Cultivated On Different Agro-wastes', *Nature and Science*, 7(1), pp. 44–48.

Akanni, G.B. *et al.* (2015) 'Protein enrichment of an Opuntia ficus-indica cladode hydrolysate by cultivation of Candida utilis and Kluyveromyces marxianus', *Journal of the Science of Food and Agriculture*, 95(5), pp. 1094–1102. doi:10.1002/jsfa.6985.

Alkasrawi, M. *et al.* (2006) 'Influence of strain and cultivation procedure on the performance of simultaneous saccharification and fermentation of steam pretreated spruce', *Enzyme and Microbial Technology*, 38, pp. 279–286. doi:10.1016/j.enzmictec.2005.08.024.

Almeida e Silva, J.B. *et al.* (1995) 'Microbial protein production by Paecilomyces variotii cultivated in eucalyptus hemicellulosic hydrolyzate', *Bioresource Technology*, 52(2), pp. 197–200. doi:10.1016/0960-8524(95)00029-E.

Alokika *et al.* (2021) 'Cellulosic and hemicellulosic fractions of sugarcane bagasse: Potential, challenges and future perspective', *International Journal of Biological Macromolecules*, 169, pp. 564–582. doi:10.1016/j.ijbiomac.2020.12.175.

Annoh-Quarshie, J. (2018) *Development and comparison of processes for the extraction of dietary protein from yellow peas*. Stellenbosch University.

Anupama and Ravindra, P. (2000) 'Value-added food: Single cell protein', *Biotechnology Advances*, 18(6), pp. 459–479. doi:10.1016/S0734-9750(00)00045-8.

Baghbani-Arani, F., Asgary, V. and Hashemi, A. (2020) 'Cell-free extracts of Lactobacillus acidophilus and Lactobacillus delbrueckii display antiproliferative and antioxidant activities against HT-29 cell line', *Nutrition and cancer*, 72(8), pp. 1390–1399.

Bajpai, P. and Bajpai, P.K. (1987) 'Single Cell Protein Production from Rayon Pulp Mill Waste by Paecilomyces variotii', *Fermentation Technology*, 65(3), pp. 349–351.

Balat, M. (2011) 'Production of bioethanol from lignocellulosic materials via the biochemical pathway: A review', *Energy Conversion and Management*, 52(2), pp. 858–875. doi:10.1016/j.enconman.2010.08.013.

Baruah, J. *et al.* (2018) 'Recent trends in the pretreatment of lignocellulosic biomass for value-added products', *Frontiers in Energy Research*, 6(141), pp. 1–19. doi:10.3389/fenrg.2018.00141.

Basu, S. *et al.* (2015) 'Evolution of bacterial and fungal growth media', *Bioinformation*, 11(4), pp. 182–184. Available at: www.bioinformation.net.

Beech, G.A., Melvin, M.A. and Taggart, J. (1985) 'Food, drink and biotechnology', *Biotechnology: principles and applications*, pp. 73–110.

Bellettini, M.B. *et al.* (2019) 'Factors affecting mushroom Pleurotus spp.', *Saudi Journal of Biological Sciences*, 26(4), pp. 633–646. doi:10.1016/j.sjbs.2016.12.005.

Bezerra, T.L. (2016) 'A review of sugarcane bagasse for second-generation bioethanol and biopower production', *Biofuels, bioproducts and biorefining*, 10(5), pp. 634–647. doi:10.1002/bbb.

Bezuidenhout, N. (2021) *Development of a fed batch fermentation strategy to produce bioethanol from non-detoxified hardwood spent sulphite liquor*. Stellenbosch.

Bian, J. *et al.* (2012) 'Isolation of hemicelluloses from sugarcane bagasse at different temperatures: Structure and properties', *Carbohydrate Polymers*, 88(2), pp. 638–645. doi:10.1016/j.carbpol.2012.01.010.

Biavati, B. and Matterelli, P. (2015) 'Bifidobacterium', *Bergey's Manual of Systematics of Archaea and Bacteria* [Preprint].

Bin, Y. and Hongzhang, C. (2010) 'Effect of the ash on enzymatic hydrolysis of steam-exploded rice straw', *Bioresource Technology*, 101(23), pp. 9114–9119. doi:10.1016/j.biortech.2010.07.033.

Bizzo, W.A. *et al.* (2014) 'The generation of residual biomass during the production of bio-ethanol from sugarcane, its characterization and its use in energy production', *Renewable and Sustainable Energy Reviews*, 29, pp. 589–603. doi:10.1016/j.rser.2013.08.056.

Block, S.S. *et al.* (1953) 'Experiments with Submerged Culture', *Agricultural and Food Chemistry*, 1(14), pp. 890–893. doi:10.1021/jf60014a006.

Bravo, O. *et al.* (1994) 'Growth of Chaetomium cellulolyticum in solid-state fermentation of sugar cane bagasse treated with water and alkali at several liquid/ solid ratios', *Biotechnology letters*, 16(8), pp. 865–870. doi:10.1007/BF00133969.

Breene, W.M. (1990) 'Nutritional and medicinal value of specialty mushrooms', *Journal of Food Protection*, 53(10), pp. 883–894. doi:10.4315/0362-028x-53.10.883.

Canilha, L. *et al.* (2011) 'A study on the pretreatment of a sugarcane bagasse sample with dilute sulfuric acid', *Journal of Industrial Microbiology and Biotechnology*, 38(9), pp. 1467–1475. doi:10.1007/s10295-010-0931-2.

Cantarella, M. *et al.* (2004a) 'Comparison of different detoxification methods for steam-exploded poplar wood as a substrate for the bioproduction of ethanol in SHF and SSF', *Process Biochemistry*, 39(11), pp. 1533–1542. doi:10.1016/S0032-9592(03)00285-1.

Cantarella, M. *et al.* (2004b) 'Effect of Inhibitors Released during Steam-Explosion Treatment of Poplar Wood on Subsequent Enzymatic Hydrolysis and SSF', *Biotechnology Progress*, 20(1), pp. 200–206. doi:10.1021/bp0257978.

Cara, C. *et al.* (2007) 'Influence of solid loading on enzymatic hydrolysis of steam exploded or liquid hot water pretreated olive tree biomass', *Process Biochemistry*, 42(6), pp. 1003–1009. doi:10.1016/j.procbio.2007.03.012.

Cardona, C.A., Quintero, J.A. and Paz, I.C. (2010) 'Production of bioethanol from sugarcane bagasse: Status and perspectives', *Bioresource Technology*, 101(13), pp. 4754–4766. doi:10.1016/j.biortech.2009.10.097.

Cavka, A. and Jornsson, L.J. (2014) 'Comparison of the growth of filamentous fungi and yeasts in lignocellulose-derived media', *Biocatalysis and Agricultural Biotechnology*, 3(4), pp. 197–204. doi:10.1016/j.bcab.2014.04.003.

Çetinkaya Dönmez, G., Öztürk, A. and Çakmakçi, L. (1999) 'Properties of the Rhodopseudomonas palustris Strains Isolated From an Alkaline Lake in Turkey', *Tr. J. of Biology*, 23, pp. 457–463.

Chahal, D.S. (1989) 'Production of protein-rich mycelial biomass of a mushroom, Pleurotus sajor-caju, on corn stover', *Journal of Fermentation and Bioengineering*, 68(5), pp. 334–338. doi:10.1016/0922-338X(89)90008-1.

Chambon, C.L. *et al.* (2018) 'Pretreatment of South African sugarcane bagasse using a low - cost protic ionic liquid: a comparison of whole, depithed, fibrous and pith bagasse fractions', *Biotechnology for Biofuels*, 11(247), pp. 1–16. doi:10.1186/s13068-018-1247-0.

Chanakya, H.N., Malayil, S. and Vijayalakshmi, C. (2015) 'Cultivation of Pleurotus spp. on a combination of anaerobically digested plant material and various agro-residues', *Energy for Sustainable Development*, 27, pp. 84–92. doi:10.1016/j.esd.2015.04.007.

Chandel, A.K., da Silva, S.S. and Singh, O. V. (2013) 'Detoxification of Lignocellulose Hydrolysates:

Biochemical and Metabolic Engineering Toward White Biotechnology', *Bioenergy Research*, 6(1), pp. 388–401. doi:10.1007/s12155-012-9241-z.

Chavez, E.R., Touchburn, S.P. and Moo-Young, M. (1988) 'Microbial biomass protein from the fungus Chaetomium cellulolyticum. I. Composition and nutritive evaluation', *Animal Feed Science and Technology*, 22(1–2), pp. 1–11. doi:10.1016/0377-8401(88)90069-7.

Chen, J. *et al.* (2015) 'Adaptation of Lactococcus lactis to high growth temperature leads to a dramatic increase in acidification rate', *Scientific Report*, 5(14199), pp. 1–15. doi:10.1038/srep14199.

Clarke, K.G. (2013) 'Microbial kinetics during batch, continuous and fed-batch processes', in *Bioprocess Engineering - An Introductory Engineering and Life Science Approach*. Woodhead Publishing, pp. 97–146. Available at: https://app.knovel.com/web/toc.v/cid:kpBEAIELS6/viewerType:toc/root_slug:bioprocess-engineering-an?hierarchy=kt00C5KSPJ.

Coradi, G.V. *et al.* (2012) 'Comparing submerged and solid-state fermentation of agro-industrial residues for the production and characterization of lipase by Trichoderma harzianum', *Annals of Microbiology*, 62(2). doi:10.1007/s13213-012-0500-1.

Das, H. and Singh, S.K. (2004) 'Useful Byproducts from Cellulosic Wastes of Agriculture and Food Industry — A Critical Appraisal', *Critical Reviews in Food Science and Nutrition*, 44(2), pp. 77–89. doi:10.1080/10408690490424630.

Denny, A., Aisbitt, B. and Lunn, J. (2008) 'Mycoprotein and health', *Nutrition Bulletin*, 33(4), pp. 298–310. doi:10.1111/j.1467-3010.2008.00730.x.

Dias, M.O.S. *et al.* (2012) 'Integrated versus stand-alone second generation ethanol production from sugarcane bagasse and trash', *Bioresource Technology*, 103(1), pp. 152–161. doi:10.1016/j.biortech.2011.09.120.

Diederichs, G.W. *et al.* (2016) 'Techno-economic comparison of biojet fuel production from lignocellulose, vegetable oil and sugar cane juice', *Bioresource Technology*, 216, pp. 331–339. doi:10.1016/j.biortech.2016.05.090.

Du, B. *et al.* (2010) 'Effect of varying feedstock-pretreatment chemistry combinations on the formation and accumulation of potentially inhibitory degradation products in biomass hydrolysates', *Biotechnology and Bioengineering*, 107(3), pp. 430–440. doi:10.1002/bit.22829.

Duggal, N. (2020) *How do you use Lactobacillus bulgaricus*, *Healthline*. Available at: https://www.healthline.com/health/digestive-health/lactobacillus-bulgaricus.

Duque, A. *et al.* (2016) 'Steam explosion as lignocellulosic biomass pretreatment', in Mussatto, S.I. (ed.) *Biomass Fractionation Technologies for a Lignocellulosic Feedstock Based Biorefinery*, pp. 349–368. doi:10.1016/b978-0-12-804296-0.00003-8.

El-Nawwi, S.A. and El-Kader, A.A. (1996) 'Production of single-cell protein and cellulase from sugarcane bagasse: Effect of culture factors', *Biomass and Bioenergy*, 11(4), pp. 361–364. doi:10.1016/0961-9534(96)00021-9.

El-Sabaeny, A.. (1996) 'Influence of medium composition on lactic acid production from dried whey by Lactobacillus delbrueckii', *Microbiologia*, 12(3), p. 411.

Erdman, M.D., Bergen, W.G. and Adinarayana Reddy, C. (1977) 'Amino acid profiles and presumptive nutritional assessment of single cell protein from certain lactobacilli', *Applied and Environmental Microbiology*, 33(4), pp. 901–905. doi:10.1128/aem.33.4.901-905.1977.

Erikkson, K.-E. and Larsson, K. (1975) 'Fermentation of waste mechanical fibers from a newsprint mill by the rot fungus Sporotrichum pulverulentum', *Biotechnology and Bioengineering*, 17(3), pp. 327–348.

Fakruddin, M., Hossain, M.N. and Ahmed, M.M. (2017) 'Antimicrobial and antioxidant activities of

Saccharomyces cerevisiae IFST062013 , a potential probiotic', *BMC Complementary and Alternative Medicine*, 17(64), pp. 1–11. doi:10.1186/s12906-017-1591-9.

Fang, L.C. *et al.* (2012) 'Characterization of Rhodopseudomonas palustris strain 2C as a potential probiotic', *Acta Pathologica, Microbiologica Immunologica Scandinavica*, 120(4), pp. 743–749. doi:10.1111/j.1600-0463.2012.02902.x.

Fatemeh, S., Reihani, S. and Khosravi-Darani, K. (2019) 'Influencing factors on single-cell protein production by submerged fermentation : A review', *Electronic Journal of Biotechnology*, 37, pp. 34–40. doi:10.1016/j.ejbt.2018.11.005.

Feldman, D. *et al.* (2015) 'Detoxification of 5-hydroxymethylfurfural by the Pleurotus ostreatus lignolytic enzymes aryl alcohol oxidase and dehydrogenase', *Biotechnology for Biofuels*, 8(1), pp. 1–11. doi:10.1186/s13068-015-0244-9.

Fernando, J. (2022) *Internal Rate of Return (IRR)*, *Investopedia*. Available at: https://www.investopedia.com/terms/i/irr.asp#:~:text=The internal rate of return,same formula as NPV does.

Filho, P.F.S. *et al.* (2019) 'Mycoprotein: Environmental impact and health aspects', *World Journal of Microbiology and Biotechnology*, 35(147), pp. 1–8. doi:10.1007/s11274-019-2723-9.

Finnigan, T., Needham, L. and Abbott, C. (2017) 'Mycoprotein: A Healthy New Protein With a Low Environmental Impact', in *Sustainable Protein Sources*. Elsevier Inc., pp. 305–325. doi:10.1016/B978-0-12-802778-3.00019-6.

Fogler and Gurmen (2008) *Introduction to Aspen Plus 12.1*. Available at: http://websites.umich.edu/~elements/help/software/html/aspen/intro.htm (Accessed: 27 July 2022).

Galbe, M. *et al.* (2007) 'Process engineering economics of bioethanol production', *Advances in Biochemical Engineering/Biotechnology*, 108(June), pp. 303–327. doi:10.1007/10_2007_063.

Galbe, M. and Zacchi, G. (2007) 'Pretreatment of lignocellulosic materials for efficient bioethanol production', *Advances in Biochemical Engineering/ Biotechnology*, 108, pp. 41–65. doi:10.3233/978-1-61499-566-1-3.

Gao, Y., Li, D. and Liu, Y. (2012) 'Production of single cell protein from soy molasses using Candida tropicalis', *Annals of Microbiology*, 62(3), pp. 1165–1172. doi:10.1007/s13213-011-0356-9.

García-Garibay, M. *et al.* (2003) 'Single-Cell protein| Algae', in *Encyclopedia of Food Sciences and Nutrition*. 2nd edn. Elsevier, pp. 5269–5276.

Ghasemi, Y., Rasoul-amini, S. and Morowvat, M.H. (2011) 'Algae for the production of SCP', in Liong, M.-T. (ed.) *Bioprocess Sciences and Technology*. Nova Science Publishers, Inc, pp. 163–184.

Giridhar, R. and Srivastava, A.K. (2000) 'Fed-Batch Sorbose Fermentation Using Pulse and Multiple Feeding Strategies for Productivity Improvement', *Biotechnology and Bioprocess Engineering*, 5, pp. 340–344.

Gu, Q. and Li, P. (2016) 'Biosynthesis of Vitamins by Probiotic Bacteria', in *Probiotics and prebiotics in human nutrition and health*, pp. 135–148.

Gupta, A. *et al.* (2013) 'Yield and nutritional content of Pleurotus sajor caju on wheat straw supplemented with raw and detoxified mahua cake', *Food Chemistry*, 141(4), pp. 4231–4239. doi:10.1016/j.foodchem.2013.06.126.

Haddish, K. (2015) 'Production of Single Cell Protein from Fruit of Beles (Opuntia Ficus-Indica L.) Peels Using Saccharomyces cerevisiae', *Journal of Microbiology & Experimentation*, 2(7). doi:10.15406/jmen.2015.02.00073.

Haghdan, S., Renneckar, S. and Smith, G.D. (2016) 'Sources of Lignin', *Lignin in Polymer Composites*, (1),

pp. 1–11. doi:10.1016/B978-0-323-35565-0.00001-1.

Hamann, M.L. (2020) *Uncatalysed steam pretreatment regimes for bagasse and harvest residues in a sugarcane biorefinery*. Stellenbosch. Available at: http://hdl.handle.net/10019.1/109744.

Hames, B.R. *et al.* (2003) 'Rapid Biomass Analysis', *Applied Biochemistry and Biotechnology*, 105, pp. 5–16. doi:10.1007/978-1-4612-0057-4_1.

Hatakka, A.I. and Pirhonen, T.I. (1985) 'Cultivation of wood-rotting fungi on agricultural lignocellulosic materials for the production of crude protein', *Agricultural Wastes*, 12(2), pp. 81–97. doi:10.1016/0141-4607(85)90001-0.

He, J., Zhang, G. and Lu, H. (2010) 'Treatment of soybean wastewater by a wild strain Rhodobacter sphaeroides and to produce protein under natural conditions', *Frontiers of Environmental Science and Engineering in China*, 4(3), pp. 334–339. doi:10.1007/s11783-010-0239-5.

Hezarjaribi, M., Ardestani, F. and Ghorbani, H.R. (2016) 'Single Cell Protein Production by Saccharomyces cerevisiae Using an Optimized Culture Medium Composition in a Batch Submerged Bioprocess', *Applied Biochemistry and Biotechnology*, 179(8), pp. 1336–1345. doi:10.1007/s12010-016-2069-9.

Hosseini, S.M. and Khosravi-Darani, K. (2011) 'Response Surface Methodology for Mycoprotein Production by Fusarium Venenatum ATCC 20334', *Journal of Bioprocessing & Biotechniques*, 1(1), pp. 1–5. doi:10.4172/2155-9821.1000102.

Howard, R.L. *et al.* (2003) 'Lignocellulose biotechnology : issues of bioconversion and enzyme production', *African Journal of Biotechnology*, 2(12), pp. 602–619.

Humbird, D. *et al.* (2011) *Process design and economics for biochemical conversion of lignocellulosic biomass to ethanol: dilute-acid pretreatment and enzymatic hydrolysis of corn stover*, *National Renewable Energy Laboratory*. doi:10.4324/9780429290602-8.

Humfeld, H. (1948) 'The Production of Mushroom Mycelium (Agaricus campestris) in Submerged Culture', *Science*, 107(2780), p. 373.

Jacquet, N. *et al.* (2015) 'Application of Steam Explosion as Pretreatment on Lignocellulosic Material: A Review', *Industrial and Engineering Chemistry Research*, 54(10), pp. 2593–2598. doi:10.1021/ie503151g.

Jing, X., Zhang, X. and Bao, J. (2009) 'Inhibition performance of lignocellulose degradation products on industrial cellulase enzymes during cellulose hydrolysis', *Applied Biochemistry and Biotechnology*, 159(3), pp. 696–707. doi:10.1007/s12010-009-8525-z.

Johansson, E., Brandberg, T. and Larsson, C. (2011) 'Influence of cultivation procedure for Saccharomyces cerevisiae used as pitching agent in industrial spent sulphite liquor fermentations', *Journal of Industrial Microbiology and Biotechnology*, 38, pp. 1787–1792. doi:10.1007/s10295-011-0965-0.

Johnston, J.L., Fanzo, J.C. and Cogill, B. (2014) 'Understanding Sustainable Diets: A Descriptive Analysis of the Determinants and Processes That Influence Diets and Their Impact on Health, Food Security, and Environmental Sustainability', *Advances in Nutrition*, 5(4), pp. 418–429. doi:10.3945/an.113.005553.

Jönsson, L.J., Alriksson, B. and Nilvebrant, N.-O. (2013) 'Bioconversion of lignocellulose: inhibitors and detoxification', *Biotechnology for Biofuels*, 6(16).

Jönsson, L.J. and Martín, C. (2016) 'Pretreatment of lignocellulose: Formation of inhibitory by-products and strategies for minimizing their effects', *Bioresource Technologys*, 199, pp. 103–112. doi:10.1016/j.biortech.2015.10.009.

Kahar, P. (2013) 'Synergistic Effects of Pretreatment Process on Enzymatic Digestion of Rice Straw for Efficient Ethanol Fermentation', in Petre, M. (ed.) *Environmental Biotechnology - New Approaches and Prospective Applications*. InTech, pp. 65–87. doi:10.5772/54949.

Kam, S., Kenari, A.A. and Younesi, H. (2012) 'Production of single cell protein in stickwater by lactobacillus

acidophilus and aspergillus niger', *Journal of Aquatic Food Product Technology*, 21(5), pp. 403–417. doi:10.1080/10498850.2011.605539.

Kandasamy, S. *et al.* (2020) 'Assessment of Antioxidant , Antibacterial Activities and Bioactive Compounds of the Wild Edible Mushroom Pleurotus sajor - caju', *International Journal of Peptide Research and Therapeutics*, 26, pp. 1575–1581. doi:10.1007/s10989-019-09969-2.

Kanmani, P. *et al.* (2013) 'Probiotics and Its Functionally Valuable Products — A Review', *Critical Reviews in Food Science and Nutrition*, 53, pp. 641–658. doi:10.1080/10408398.2011.553752.

Kannan, B. *et al.* (2020) 'Single Cell Protein (SCP) production from marine Lactobacillus spp.', *Indian Journal of Animal Research*, 54, pp. 1115–1119.

Kavanagh, K. (2005) 'Fungal Fermentation Systems and Products', in Kavanagh, K. (ed.) *Fungi: Biology and Applications*. 2nd edn. Wiley-Blackwell, pp. 89–112. doi:10.1002/0470015330.ch4.

Khan, A. *et al.* (2008) 'Comparative Study of the Nutritional Composition of Oyster Mushrooms Cultivated in Bangladesh', *Bangladesh J. Mushroom*, 2(1), pp. 9–14.

Khattab, S.M.R. and Watanabe, T. (2019) 'Bioethanol From Sugarcane Bagasse: Status and Perspectives', in Ray, R.C. (ed.) *Bioethanol Production from Food Crops*. Elsevier Inc., pp. 187–212. doi:10.1016/B978-0-12-813766-6/00010-2.

Kim, D. (2018) 'Physico-Chemical Conversion of Lignocellulose: Inhibitor Effects and Detoxification Strategies: A Mini Review', *Molecules*, 23(2), p. 309. doi:10.3390/molecules23020309.

Kim, Y. *et al.* (2011) 'Soluble inhibitors/deactivators of cellulase enzymes from lignocellulosic biomass', *Enzyme and Microbial Technology*, 48(4–5), pp. 408–415. doi:10.1016/j.enzmictec.2011.01.007.

Koekemoer, T. (2018) *Lactic acid production from sugarcane bagasse and harvesting residues*. Stellenbosch.

Kosseva, M.R. (2013) 'Recovery of Commodities from Food Wastes Using Solid-State Fermentation', in Kosseva, M.R. and Webb, C. (eds) *Food Industry Wastes: Assessment and recuperation of commodities*. 1st edn. Elsevier Inc., pp. 77–102. doi:10.1016/B978-0-12-391921-2.00005-6.

Krupodorova, T.A. *et al.* (2019) 'Effect of cultivation conditions on mycelial growth and antibacterial activity of Lentinula edodes and Fomitopsis betulina', *Czech Mycology*, 7(12), pp. 167–186. doi:10.33585/cmy.71204.

Kumar, A., Gautam, A. and Dutt, D. (2016) 'Biotechnological Transformation of Lignocellulosic Biomass in to Industrial Products: An Overview', *Advances in Bioscience and Biotechnology*, 7(3), pp. 149–168. doi:10.4236/abb.2016.73014.

Kurbanoglu, E.B. and Algur, O.F. (2002) 'Single-cell protein production from ram horn hydrolysate by bacteria', *Bioresource Technology*, 85, pp. 125–129.

Kurcz, A. *et al.* (2018) 'Application of Industrial Wastes for the Production of Microbial Single-Cell Protein by Fodder Yeast Candida utilis', *Waste Biomass Valorisation*, 9, pp. 57–64. doi:10.1007/s12649-016-9782-z.

Laurance, W.F., Sayer, J. and Cassman, K.G. (2014) 'Agricultural expansion and its impacts on tropical nature', *Trends in Ecology and Evolution*, 29(2), pp. 107–116. doi:10.1016/j.tree.2013.12.001.

LeBlanc, J.G. *et al.* (2011) 'B-Group vitamin production by lactic acid bacteria – current knowledge and potential applications', *Journal of Applied Microbiology*, 111, pp. 1297–1309. doi:10.1111/j.1365-2672.2011.05157.x.

Lee, J. (1997) 'Biological conversion of lignocellulosic biomass to ethanol', *Journal of Biotechnology*, 56(1), pp. 1–24. doi:10.1016/S0168-1656(97)00073-4.

Lefevre, M. *et al.* (2017) 'Safety assessment of Bacillus subtilis CU1 for use as a probiotic in humans', *Regulatory Toxicology and Pharmacology*, 83, pp. 54–65. doi:10.1016/j.yrtph.2016.11.010.

Letti, L.A.J. *et al.* (2018) 'Solid-State Fermentation for the Production of Mushrooms', in *Current Developments in Biotechnology and Bioengineering*, pp. 285–318. doi:10.1016/b978-0-444-63990-5.00014-1.

Linder, T. (2019) 'Making the case for edible microorganisms as an integral part of a more sustainable and resilient food production system', *Food security*, (11), pp. 265–278.

Lipper, L. *et al.* (2010) *'Climate-smart' Agriculture: Policies, practices and financing for food security, adaptation and mitigation*, *Food and agricultural organization of the United Nations*. doi:10.1111/j.1467-6346.2009.02662.x.

Loy, D.D. and Lundy, E.L. (2018) 'Nutritional properties and feeding value of corn and its coproducts', in *Corn: Chemistry and Technology, 3rd Edition*. 3rd edn. Elsevier Inc., pp. 633–659. doi:10.1016/B978-0-12-811971-6.00023-1.

Lynd, L.R. *et al.* (2005) 'Consolidated bioprocessing of cellulosic biomass: an update', *Current Opinion in Biotechnology*, 16, pp. 577–583. doi:10.1016/j.copbio.2005.08.009.

Lynd, L.R. *et al.* (2017) 'Cellulosic ethanol: status and innovation', *Current Opinion in Biotechnology*, 45, pp. 202–211. doi:10.1016/j.copbio.2017.03.008.

Mabee, W.E., McFarlane, P.N. and Saddler, J.N. (2011) 'Biomass availability for lignocellulosic ethanol production', *Biomass and Bioenergy*, 35(11), pp. 4519–4529. doi:10.1016/j.biombioe.2011.06.026.

Maier, R.M. (2009) 'Bacterial Growth', in *Environmental Microbiology*, pp. 37–54. doi:10.1016/B978-0-12-370519-8.00003-1.

Mane, V.P. *et al.* (2007) 'Bioconversion of low quality lignocellulosic agricultural waste into edible protein by Pleurotus sajor-caju (Fr.) Singer.', *Journal of Zhejiang University. Science. B.*, 8(10), pp. 745–751. doi:10.1631/jzus.2007.B0745.

Martín, C. *et al.* (2018) 'Formation of microbial inhibitors in steam-explosion pretreatment of softwood impregnated with sulfuric acid and sulfur dioxide', *Bioresource Technology*, 262(April), pp. 242–250. doi:10.1016/j.biortech.2018.04.074.

Martín, C. *et al.* (2021) 'Effects of operational conditions on auto-catalyzed and sulfuric-acid-catalyzed hydrothermal pretreatment of sugarcane bagasse at different severity factor', *Industrial crops and products*, 159. doi:10.1016/j.indcrop.2020.113077.

Matassa, S. *et al.* (2016) 'Microbial protein: Future sustainable food supply route with low environmental footprint', *Microbial Biotechnology* [Preprint]. doi:10.1111/1751-7915.12369.

Matjuškova, N. *et al.* (2017) 'Effect of lignin-containing media on growth of medicinal mushroom lentinula edodes', *Proceedings of the Latvian Academy of Sciences, Section B: Natural, Exact, and Applied Sciences*, 71(1), pp. 38–42. doi:10.1515/prolas-2017-0007.

Maurya, D.P., Singla, A. and Negi, S. (2015) 'An overview of key pretreatment processes for biological conversion of lignocellulosic biomass to bioethanol', *3 Biotechnology*, 5, pp. 597–609. doi:10.1007/s13205-015-0279-4.

McIlveen, H., Abraham, C. and Armstrong, G. (1999) 'Meat avoidance and the role of replacers', *Nutrition & Food Science*, 99(1), pp. 29–36. doi:10.1108/00346659910247653.

Melo, T.A. *et al.* (2017) 'Functional Profile Evaluation of Lactobacillus fermentum TCUESC01 : A New Potential Probiotic Strain Isolated during Cocoa Fermentation', *BioMed Research International*, 2017, pp. 1–7.

Mensah, J.K.M. and Twumasi, P. (2016) 'Use of pineapple waste for single cell protein (SCP) production

and the effect of substrate concentration on the yield', *Food Process Engineering*, pp. 1–9. doi:10.1111/jfpe.12478.

Merino, S.T. and Cherry, J. (2007) 'Progress and challenges in enzyme development for biomass utilization', *Advances in Biochemical Engineering/Biotechnology*, 108, pp. 95–120. doi:10.1007/10_2007_066.

Mogmenga, I. *et al.* (2019) 'Yeasts Biotechnologies Application and Their Involving in African Traditional Fermented Foods and Beverages.', *International Journal of Advanced Research*, 7(2), pp. 44–62. doi:10.21474/ijar01/8462.

Mokomele, T. *et al.* (2018) 'Ethanol production potential from AFEX^{TM} and steam-exploded sugarcane residues for sugarcane biorefineries', *Biotechnology for Biofuels*, 11(1), pp. 1–21. doi:10.1186/s13068-018-1130-z.

Mokomele, T. (2019) *Integrated bioenergy and animal feed production from AFEX and steam exploded sugarcane residues*. Stellenbosch.

Mondal, A.K. *et al.* (2012) 'Utilization of Fruit Wastes in Producing Single Cell Protein', *International Journal of Science, Environment and Technology*, 1(5), pp. 430–438.

Moo-Young, M. *et al.* (1977) 'SCP Production by Chaetomium cellulolyticum, a New Thermotolerant Cellulolytic Fungus', *Biotechnology and Bioengineering*, XIX, pp. 527–538.

Moo-Young, M., Chisti, Y. and Vlach, D. (1993) 'Fermentation of cellulosic materials to mycoprotein foods', *Biotechnology Advances*, 11(3), pp. 469–479. doi:10.1016/0734-9750(93)90015-F.

Morning Star Feeds (2022) *Afresh brands soya oilcake 50kg*. Available at: http://www.msfeeds.co.za/other-feeds/132-afresh-brands-soya-oil-cake-50kg.html.

Moro-garcía, M.A. *et al.* (2013) 'Oral supplementation with Lactobacillus delbrueckii subsp . bulgaricus 8481 enhances systemic immunity in elderly subjects', *Age*, 35(4), pp. 1311–1326. doi:10.1007/s11357-012-9434-6.

Mosier, N. *et al.* (2005) 'Features of promising technologies for pretreatment of lignocellulosic biomass', *Bioresource Technology*, 96(6), pp. 673–686. doi:10.1016/j.biortech.2004.06.025.

Mumpuni, A. *et al.* (2017) 'Growth and protein content establishment of Pleurotus ostreatus on liquid and solid medium', *Biosaintifika: Biology and Biology Education*, 9(3), pp. 572–578. doi:10.15294/biosaintifika.v9i3.11660.

Munawar, R.A. *et al.* (2010) 'Biosynthesis of Single Cell Biomass of Candida Utilis By Submerged Fermentation', *Science*, 62(1), pp. 1–5.

Nagarajan, D., Lee, D.J. and Chang, J.S. (2019) 'Recent insights into consolidated bioprocessing for lignocellulosic biohydrogen production', *International Journal of Hydrogen Energy*, 44(28), pp. 14362–14379. doi:10.1016/j.ijhydene.2019.03.066.

Naghmouchi, K. *et al.* (2019) 'Lactobacillus fermentum: a bacterial species with potential for food preservation and biomedical applications', *Critical Reviews in Food Science and Nutrition*, pp. 1–13. doi:10.1080/10408398.2019.1688250.

NAMC (2022) *South African Poultry Products Prices Monitor*. Available at: https://www.namc.co.za/wp-content/uploads/2022/04/Poultry-Products-Price-Monitor-March-2022.pdf.

Nasseri, A.T. *et al.* (2011) 'Single cell protein: Production and process', *American Journal of Food Technology*, 6(2), pp. 103–116. doi:10.3923/ajft.2011.103.116.

Nielsen, D.S. *et al.* (2007) 'The microbiology of Ghanaian cocoa fermentations analysed using culture-dependent and culture-independent methods', *International Journal of Food Microbiology*, 114, pp. 168–186. doi:10.1016/j.ijfoodmicro.2006.09.010.

Nigam, J.N. (2000) 'Cultivation of Candida langeronii in sugar cane bagasse hemicellulosic hydrolyzate for the production of single cell protein', *Microbiology and Biotechnology*, 16, pp. 367–372.

Nigam, P.S. and Singh, A. (2014) 'Single Cell Protein: Mycelial Fungi', *Encyclopedia of Food Microbiology*. Second Edi, 3, pp. 415–424. doi:10.1016/B978-0-12-384730-0.00311-6.

Niju, S., Swathika, M. and Balajii, M. (2019) *Pretreatment of lignocellulosic sugarcane leaves and tops for bioethanol production*, *Lignocellulosic Biomass to Liquid Biofuels*. INC. doi:10.1016/B978-0-12-815936-1.00010-1.

Nilsson, A., Taherzadeh, M.J. and Lidén, G. (2001) 'Use of dynamic step response for control of fed-batch conversion of lignocellulosic hydrolyzates to ethanol', *Journal of Biotechnology*, 89(1), pp. 41–53. doi:10.1016/S0168-1656(01)00283-8.

Noparatnaraporn, N. and Nagai, S. (1986) 'Selection of Rhodobacter Sphaeroides P47 as a useful source of single cell protein', *Journal of General and Applied Microbiology*, 32, pp. 351–359.

Öhgren, K. *et al.* (2007) 'A comparison between simultaneous saccharification and fermentation and separate hydrolysis and fermentation using steam-pretreated corn stover', *Process Biochemistry*, 42, pp. 834–839. doi:10.1016/j.procbio.2007.02.003.

Overend, R.P., Chornet, E. and Gascoigne, J.A. (1987) 'Fractionation of Lignocellulosics by Steam-Aqueous Pretreatments [and Discussion]', *Philosophical transactions of the Royal Society of London*, 321, pp. 523–536. doi:10.1098/rsta.1987.0029.

Palma, M., Guerreiro, J.F. and Sá-Correia, I. (2018) 'Adaptive response and tolerance to acetic acid in Saccharomyces cerevisiae and Zygosaccharomyces bailii: A physiological genomics perspective', *Frontiers in Microbiology*, 9(FEB), pp. 1–16. doi:10.3389/fmicb.2018.00274.

Palmqvist, E. and Hahn-Hägerdal, B. (2000) 'Fermentation of lignocellulosic hydrolysates. II: Inhibitors and mechanisms of inhibition', *Bioresource Technology*, 74(1), pp. 25–33. doi:10.1016/S0960-8524(99)00161-3.

Pamment, N. *et al.* (1978) 'Growth of Chaetomium cellulolyticum on Alkali-Pretreated Hardwood Sawdust Solids and Pretreatment Liquor', *Applied and Environmental Microbiology*, 36(2), pp. 284–290.

Pandey, A. (2003) 'Solid-state fermentation', *Biochemical Engineering Journal*, 13(2–3), pp. 81–84. doi:10.1016/S1369-703X(02)00121-3.

Papaspyridi, L.M. *et al.* (2012) 'Submerged fermentation of the edible mushroom Pleurotus ostreatus in a batch stirred tank bioreactor as a promising alternative for the effective production of bioactive metabolites', *Molecules*, 17(3), pp. 2714–2724. doi:10.3390/molecules17032714.

Patelski, P. *et al.* (2015) 'Utilisation of sugar beet bagasse for the biosynthesis of yeast SCP', *Journal of Food Engineering*, 167, pp. 32–37. doi:10.1016/j.jfoodeng.2015.03.031.

Patil, S.S. *et al.* (2010) 'The nutritional value of pleurotus ostreatus (JACQ.:FR.) kumm cultivated on different lignocellulosic agro-wastes', *Innovative Romanian Food Biotechnology*, 7, pp. 66–76.

Pengilly, C. (2013) *Study of Enzymatic Hydrolysis to Improve Sugar Production from Steam-Pretreated Sweet Sorghum Bagasse and Triticale Straw*. Stellenbosch.

Pereira, S.R. *et al.* (2010) 'Single cell protein production by Paecilomyces variotii from spent sulphite liquor', *Biotechnology*, (150S), p. 509. doi:10.1016/j.jbiotec.2010.09.801.

Pessoa, A., Mancilhab, I.M. and Sao, S. (1996) 'Cultivation of Candida tropicalis in sugar cane hemicellulosic hydrolyzate for microbial protein production', *Biotechnology*, 51, pp. 83–88.

Peters, M.S. and Timmerhaus, K.D. (1991) *Plant design and economics for chemical engineers*. 4th edn. McGraw-Hill Book Co.

Petersen, A.M., Brown, L.J., *et al.* (2021) 'Flowsheet Analysis of Valourising Mixed Lignocellulose and Plastic Wastes via Fast Pyrolysis at a Paper Mill', *Waste and Biomass Valorization*, 12(2), pp. 1025–1038. doi:10.1007/s12649-020-01033-1.

Petersen, A.M., Okoro, O. V., *et al.* (2021) 'Systematic cost evaluations of biological and thermochemical processes for ethanol production from biomass residues and industrial off-gases', *Energy Conversion and Management*, 243. doi:10.1016/j.enconman.2021.114398.

Petersen, A.M. *et al.* (2022) 'Optimizing the processes of extracting proteins from yellow peas and ethanol production from spent pea residues', *Biomass Conversion and Biorefinery*, 12(7), pp. 2913–2924. doi:10.1007/s13399-020-00851-6.

Petersen, A.M., Franco, T. and Görgens, J.F. (2018) 'Comparison of recovery of volatile fatty acids and mixed ketones as alternative downstream processes for acetogenisis fermentation', *Biofuels, Bioproducts and Biorefining*, 12(5), pp. 882–898. doi:10.1002/bbb.1901.

Petre, M. (2017) 'The semi-solid state cultivation of edible mushrooms on agricultural organic wastes', (January 2012).

Philippoussis, A.N. (2009) 'Production of mushrooms using agro-industrial residues as substrates', in Nigam, P.S. and Pandey, A. (eds) *Biotechnology for agro-industrial residues utilisation: Utilisation of agro-residues*. Springer Science and Business Media, pp. 163–196.

Pihlajaniemi, V. *et al.* (2020) 'Comparison of pretreatments and cost-optimization of enzymatic hydrolysis for production of single cell protein from grass silage fibre', *Bioresource Technology Reports*, 9, pp. 1–8. doi:10.1016/j.biteb.2019.100357.

Piper, P. *et al.* (2001) 'Weak acid adaptation: The stress response that confers yeasts with resistance to organic acid food preservatives', *Microbiology*, 147(10), pp. 2635–2642. doi:10.1099/00221287-147-10-2635.

Prakash, P., Namashivyam, S.K.R. and Narendrakumar, G. (2014) 'Optimization of Growth Parameters for Elevated Production of Mycoprotein - Fusarium venenatum Using Response Surface Methodology', *Journal of Pure and Applied Microbiology*, 8(6).

Quaicoe, E.H. *et al.* (2014) 'Nutrient Requirements And Environmental Conditions For The Cultivation Of The Medicinal Mushroom (Lentinula Edodes) (Berk.) In Ghana', *International Journal of Scientific & Technology Research*, 3(12), pp. 44–50.

Radke-Mitchell, L.C. and Sandine, W.E. (1986) 'Influence of Temperature on Associative Growth of Streptococcus thermophilus and Lactobacillus bulgaricus', *Dairy Science*, 69(10), pp. 2558–2568. doi:10.3168/jds.S0022-0302(86)80701-9.

Rajendran, K. *et al.* (2018) 'Updates on the pretreatment of lignocellulosic feedstocks for bioenergy production – a review', *Biomass Conversion and Biorefinery*, 8, pp. 471–483. doi:10.1007/s13399-017-0269-3.

Rajoka, M.I. (2005) 'Production of single cell protein through fermentation of a perennial grass grown on saline lands with Cellulomonas biazotea', *World Journal of Microbiology and Biotechnology*, 21(3), pp. 207–211. doi:10.1007/s11274-004-2889-6.

Rajoka, M.I. *et al.* (2006) 'Kinetics of batch single cell protein production from rice polishings with Candida utilis in continuously aerated tank reactors', *Bioresource Technology*, 97(15), pp. 1934–1941. doi:10.1016/j.biortech.2005.08.019.

Raman, J. *et al.* (2021) 'Cultivation and Nutritional Value of Prominent Pleurotus spp .: An Overview', *Mycobiology*, 49(1), pp. 1–14. doi:10.1080/12298093.2020.1835142.

Rani, R. *et al.* (2009) 'Recent advances in solid-state fermentation', *Biochemical Engineering Journal*, 44, pp. 13–18. doi:10.1016/j.bej.2008.10.019.

Rao, M., Varma, A.J. and Deshmukh, S.S. (2010) 'Production of Single cell protein, essential amino acids, and xylanase by Penicillium janthinellum', *BioResources*, 5(4), pp. 2470–2477. doi:10.15376/biores.5.4.2470-2477.

Rault, A., Bouix, M. and Béal, C. (2009) 'Fermentation pH influences the physiological-state dynamics of Lactobacillus bulgaricus CFL1 during pH-controlled culture', *Applied and Environmental Microbiology*, 75(13), pp. 4374–4381. doi:10.1128/AEM.02725-08.

Resch, M.G., Baker, J.O. and Decker, S.R. (2015) *Low Solids Enzymatic Saccharification of Lignocellulosic Biomass Laboratory Analytical Procedure (LAP) NREL/TP-5100-63351*.

Ritala, A. *et al.* (2017) 'Single cell protein-state-of-the-art, industrial landscape and patents 2001-2016', *Frontiers in Microbiology*, 8(OCT). doi:10.3389/fmicb.2017.02009.

Rudolf, A. *et al.* (2008) 'Simultaneous Saccharification and Fermentation of Steam-Pretreated Bagasse using Saccharomyces cerevisiae TMB3400 and Pichia stipitis CBS6054', *Biotechnology and Bioengineering*, 99(4), pp. 783–790. doi:10.1002/bit.

Sadler, M.J. (2003) 'Nutritional properties of edible fungi', *Nutrition Bulletin*, 28(3), pp. 305–308. doi:10.1046/j.1467-3010.2003.00354.x.

Sadler, M.J. (2004) 'Meat alternatives - Market developments and health benefits', *Trends in Food Science and Technology*, 15(5), pp. 250–260. doi:10.1016/j.tifs.2003.09.003.

Saejung, C. and Thammaratana, T. (2016) 'Biomass recovery during municipal wastewater treatment using photosynthetic bacteria and prospect of production of single cell protein for feedstuff', *Environmental Technology (United Kingdom)*, 37(23), pp. 3055–3061. doi:10.1080/09593330.2016.1175512.

Saha, P. (2022) *Aspen Plus simulation software - a basic course for beginners*. Available at: https://onlinecourses.nptel.ac.in/noc21_ch44/preview.

Salami, A.O., Bankole, F.A. and Salako, Y.A. (2017) 'Nutrient and Mineral Content of Oyster Mushroom (Pleurotus florida) Grown on Selected Lignocellulosic Substrates', *Journal of Advances in Biology and Biotechnology*, 15(1), pp. 1–7. doi:10.9734/JABB/2017/35876.

Samadi, S., Mohammadi, M. and Najafpour, G. (2016) 'Production of Single Cell Protein from Sugarcane Bagasse by Saccharomyces cerevisiae in Tray Bioreactor', *International Journal of Engineering*, 29(8). doi:10.5829/idosi.ije.2016.29.08b.01.

Sánchez, C. (2009) 'Lignocellulosic residues: Biodegradation and bioconversion by fungi', *Biotechnology Advances*, 27(2), pp. 185–194. doi:10.1016/j.biotechadv.2008.11.001.

Schulz, E. and Oslage, H.. (1976) 'Composition and nutritive value of single-cell protein (SCP)', *Animal Feed Science and Technology*, 1, pp. 9–24.

Shah, N.P. (2011) 'Bacteria, Beneficial: Bifidobacterium spp.: Morphology and Physiology', *Encyclopedia of Dairy Sciences: Second Edition*, pp. 381–387. doi:10.1016/B978-0-12-374407-4.00043-1.

Sharma, R. *et al.* (2014) 'Probiotic Efficacy and Potential of Streptococcus thermophilus modulating human health : A synoptic review', *Journal of Pharmacy and Biological Sciences*, 9(3), pp. 52–58. doi:10.9790/3008-09325258.

Shipman, R.H., Kao, I.C. and Fan, L.T. (1975) 'Single-Cell Protein Production by Photosynthetic Bacteria Cultivation in Agricultural By-products', *Biotechnology and Bioengineering*, 17(11), pp. 1561–1570.

Silveira, M.H.L. *et al.* (2018) 'Production of hemicellulosic sugars from sugarcane bagasse via steam explosion employing industrially feasible conditions: Pilot scale study', *Bioresource Technology Reports*, 3(August), pp. 138–146. doi:10.1016/j.biteb.2018.07.011.

Singhania, R.R. *et al.* (2010) 'Advancement and comparative profiles in the production technologies using

solid-state and submerged fermentation for microbial cellulases', *Enzyme and Microbial Technology*, 46(7), pp. 541–549. doi:10.1016/j.enzmictec.2010.03.010.

Slayman, C.L. (1985) 'Plasma membrane proton pumps in plants and fungi', *BioScience*, 35(1), pp. 34–37. Available at: https://academic.oup.com/bioscience/article-abstract/35/1/34/219748?redirectedFrom=fulltext.

Sluiter, A. *et al.* (2008) *Determination of Sugars, Byproducts, and Degradation Products in Liquid Fraction Process Samples: Laboratory Analytical Procedure (LAP) NREL/TP-510-42623*.

Sluiter, A. *et al.* (2012) *Determination of Structural Carbohydrates and Lignin in Biomass: Laboratory Analytical Procedure (LAP)*.

Smetana, S. *et al.* (2015) 'Meat alternatives: life cycle assessment of most known meat substitutes', *International Journal of Life Cycle Assessment*, 20(9), pp. 1254–1267. doi:10.1007/s11367-015-0931-6.

Smetana, S. *et al.* (2018) 'Agri-food waste stream utilization for development of more sustainable food substitutes', in Benetto, E., Gericke, K., and Guiton, M. (eds) *Designing Sustainable Technologies, Products and Policies*, pp. 145–155. doi:10.1007/978-3-319-66981-6_52.

Soccol, C.R. and Vandenberghe, L.P.S. (2003) 'Overview of applied solid-state fermentation in Brazil', *Biochemical Engineering Journal*, 13(2–3), pp. 205–218. doi:10.1016/S1369-703X(02)00133-X.

Song, A.A.-L. *et al.* (2017) 'A review on Lactococcus lactis: from food to factory', *Microbial Cell Factories*, pp. 1–15. doi:10.1186/s12934-017-0669-x.

Spalvins, K., Zihare, L. and Blumberga, D. (2018) 'Single cell protein production from waste biomass: comparison of various industrial by-products', *Energy Procedia*, 147, pp. 409–418. doi:10.1016/j.egypro.2018.07.111.

Stanton, C. *et al.* (2005) 'Fermented functional foods based on probiotics and their biogenic metabolites', *Current Opinion in Biotechnology*, 16, pp. 198–203. doi:10.1016/j.copbio.2005.02.008.

Starosila, D. *et al.* (2017) 'Anti-influenza Activity of a Bacillus subtilis probiotic strain', *Antimicrobial Agents and Chemotherapy*, 61(7), pp. 1–11.

Stratford, M. *et al.* (2013) 'Weak-acid preservatives: PH and proton movements in the yeast Saccharomyces cerevisiae', *International Journal of Food Microbiology*, 161(3), pp. 164–171. doi:10.1016/j.ijfoodmicro.2012.12.013.

Sugihara, T.F. and Humfeld, H. (1954) 'Submerged Culture of the Mycelium of Various Species of Mushroom', *Applied Microbiology*, 2(3), pp. 170–172.

Suman, G. *et al.* (2015) 'Single Cell Protein Production: A Review', *International Journal of Current Microbiology and Applied Sciences*, 4(9), pp. 251–262. doi:10.1016/S0167-8760(01)00179-9.

Sun, F.F. *et al.* (2015) 'Accessory enzymes influence cellulase hydrolysis of the model substrate and the realistic lignocellulosic biomass', *Enzyme and Microbial Technology*, 79–80, pp. 42–48. doi:10.1016/j.enzmictec.2015.06.020.

Sun, Y. and Cheng, J. (2002) 'Hydrolysis of lignocellulosic materials for ethanol production: A review', *Bioresource Technology*, 83(1), pp. 1–11. doi:10.1016/S0960-8524(01)00212-7.

Swart, L.J. (2021) *Techno-economic analysis of the valorization of brewers spent grains: production of xylitol and xylo-oligosaccharides*, *Journal of Chemical Technology and Biotechnology*. Stellenbosch. doi:10.1002/jctb.6683.

Taherzadeh, M.J. *et al.* (2000) 'Physiological effects of 5-hydroxymethylfurfural on Saccharomyces cerevisiae', *Applied Microbiology and Biotechnology*, 53(6), pp. 701–708. doi:10.1007/s002530000328.

Taherzadeh, M.J. and Karimi, K. (2011) *Fermentation Inhibitors in Ethanol Processes and Different*

Strategies to Reduce Their Effects. 1st edn, *Biofuels: Alternative Feedstocks and Conversion Processes*. 1st edn. Elsevier Inc. doi:10.1016/B978-0-12-385099-7.00012-7.

Taiwo, A.E., Madzimbamuto, T.N. and Ojumu, T.V. (2018) 'Optimization of Corn Steep Liquor Dosage and Other Fermentation Parameters for Ethanol Production by Saccharomyces cerevisiae Type 1 and Anchor Instant Yeast', *Energies*, 11(1740), pp. 1–20. doi:10.3390/en11071740.

Talebnia, F., Karakashev, D. and Angelidaki, I. (2010) 'Production of bioethanol from wheat straw: An overview on pretreatment, hydrolysis and fermentation', *Bioresource Technology*, 101(13), pp. 4744–4753. doi:10.1016/j.biortech.2009.11.080.

Tan, J.P. *et al.* (2016) 'Use of corn steep liquor as an economical nitrogen source for biosuccinic acid production by Actinobacillus succinogenes', *IOP Conference Series: Earth and Environmental Science*, 36(1). doi:10.1088/1755-1315/36/1/012058.

Tanaka, M. and Matsuno, R. (1985) 'Conversion of lignocellulosic materials to single-cell protein (SCP): recent developments and problems', *Enzyme and Microbial Technology*, 7, pp. 197–206.

Tarrah, A. *et al.* (2018) 'In vitro Probiotic Potential and Anti-cancer Activity of Newly Isolated Folate-Producing Streptococcus thermophilus Strains Bacterial Strains', *Frontiers in Microbiology*, 9(September), pp. 1–11. doi:10.3389/fmicb.2018.02214.

Tellez-Tellez, M. *et al.* (2008) 'Growth and laccase production of Pleurotus ostreatus in submerged and solid-state fermentation', *Applied Microbiology and Biotechnology*, 81, pp. 675–679.

Teter, S.A., Sutton, K.B. and Emme, B. (2014) 'Enzymatic processes and enzyme development in biorefining', in *Advances in Biorefineries: Biomass and Waste Supply Chain Exploitation*, pp. 199–233. doi:10.1533/9780857097385.1.199.

Thomson, J.M. *et al.* (2005) 'Resurrecting ancestral alcohol dehydrogenases from yeast', *Nature Genetics*, 37(6), pp. 630–635. doi:10.1038/ng1553.

Tomás-Pejó, E. *et al.* (2011) 'Pretreatment technologies for lignocellulose-to-bioethanol conversion', *Biofuels*, pp. 149–176. doi:10.1016/B978-0-12-385099-7.00007-3.

Trček, J., Mira, N.P. and Jarboe, L.R. (2015) 'Adaptation and tolerance of bacteria against acetic acid', *Applied Microbiology and Biotechnology*, 99(15), pp. 6215–6229. doi:10.1007/s00253-015-6762-3.

Trinci, A.P.J. (1992) 'Myco-protein: A twenty-year overnight success story', *Mycological Research*, 96(1), pp. 1–13. doi:10.1016/S0953-7562(09)80989-1.

Turnbull, W.H., Leeds, A.R. and Edwards, D.G. (1992) 'Mycoprotein reduces blood lipids in free-living subjects', *American Journal of Clinical Nutrition*, 55(2), pp. 415–419. doi:10.1093/ajcn/55.2.415.

Turton, R. *et al.* (2018) *Analysis, synthesis and design of chemical processes*. 5th edn. Pearson Education Inc.

Tyagi, S. *et al.* (2019) 'Production of Bioethanol From Sugarcane Bagasse: Current Approaches and Perspectives', in Shukla, P. (ed.) *Applied Microbiology and Bioengineering*. Elsevier Inc., pp. 21–42. doi:10.1016/B978-0-12-815407-6.00002-2.

Ugalde, U.O. and Castrillo, J.I. (2002) 'Single cell proteins from fungi and yeasts', *Applied Mycology and Biotechnology*, 2, pp. 123–149. doi:10.1016/S1874-5334(02)80008-9.

Umamaheswari, T. *et al.* (2014) 'Streptococcus thermophilus strains of plant origin as dairy starters: Isolation and characterisation', *International Journal of Dairy Technology*, 67(1), pp. 117–122. doi:10.1111/1471-0307.12098.

Upadhyaya, S. *et al.* (2016) 'Microbial Protein: A Valuable Component for Future Food Security', *Microbes and Environmental Management*, (January), pp. 260–279. doi:10.13140/RG.2.1.1775.8801.

Vancov, T. and Mcintosh, S. (2011) 'Effects of dilute acid pretreatment on enzyme saccharification of wheat stubble', *Journal of Chemical Technology and Biotechnology*, 86(6), pp. 818–825. doi:10.1002/jctb.2594.

Wallace, J. (2013) *Enzymatic hydrolysis of steam pretreated bagasse: Enzyme preparations for efficient cellulose conversion and evaluation of physicochemical changes during hydrolysis*. Stellenbosch.

Wang, D., Sakoda, A. and Suzuki, M. (2001) 'Biological efficiency and nutritional value of Pleurotus ostreatus cultivated on spent beer grain', *Bioresource Technology*, 78, pp. 293–300.

Western Cape Government (2022a) *Beef Monthly Report*. Available at: https://www.elsenburg.com/wp-content/uploads/2022/06/LIVESTOCK-REPORT_APRIL-BEEF-2022.pdf.

Western Cape Government (2022b) *Pork Monthly Report*. Available at: https://www.elsenburg.com/wp-content/uploads/2022/06/LIVESTOCK-REPORT_APRIL-PORK.pdf.

WHO, FAO and UNU (2002) *Protein and amino acid requirements in human nutrition*.

Wiebe, M. (2002) 'Myco-protein from fusarium venenatum: A well-established product for human consumption', *Applied Microbiology and Biotechnology*, 58(4), pp. 421–427. doi:10.1007/s00253-002-0931-x.

Wiebe, M.G. (2004) 'Quorn™ myco-protein - Overview of a successful fungal product', *Mycologist*, 18(1), pp. 17–20. doi:10.1017/S0269915X04001089.

Wood, T.M. and Bhat, M.K. (1988) 'Methods for measuring cellulase activities', *Methods in Enzymology*, 160, pp. 87–112.

Xiao, L.-P., Song, G.-Y. and Sun, R.-C. (2017) 'Effect of hydrothermal processing on hemicellulose structure', in *Hydrothermal Processing in Biorefineries: Production of Bioethanol and High Added-Value Compounds of Second and Third Generation Biomass*, pp. 45–94. doi:10.1007/978-3-319-56457-9.

Yin, X. *et al.* (2017) 'Proteomes of Lactobacillus delbrueckii subsp. bulgaricus LBB.B5 incubated in milk at optimal and low temperatures', *Applied and Environmental Science*, 2(5), pp. 1–13.

Yonsei University (2021) *Cellulolytic Enzyme Database*. Available at: http://www.microbiome.re.kr/db/celldb/re_result_view.php?celldb_id=CelDB_1000&keyword=.

Yousufi, M.K. (2012) 'To Determine Protein Content of Single Cell Protein Produced By Using Various Combinations of Fruit Wastes and Two Standard Food Fungi', *International Journal of Advanced Biotechnology and Research*, 3(1), pp. 533–536.

Yunus, F. un N., Nadeem, M. and Rashid, F. (2015) 'Single-cell protein production through microbial conversion of lignocellulosic residue (wheat bran) for animal feed', *Journal of the Institute of Brewing*, 121(4), pp. 553–557. doi:10.1002/jib.251.

Zeltina, Mo *et al.* (1987) 'Submerged fermentation of cellolignin materials (compared with solid-state fermentation and other alternatives).', *Acta Biotechnologica*. Edited by MO Zeltina, 7(2), pp. 157–166. doi:10.1002/abio.370070214.

Zhang, C.H., Wu, J.Y. and He, G.Y. (2002) 'Effects of inoculum size and age on biomass growth and paclitaxel production of elicitor-treated Taxus yunnanensis cell cultures', *Applied Microbiology and Biotechnology*, 60(4), pp. 396–402. doi:10.1007/s00253-002-1130-5.

Zhang, J. *et al.* (2014) 'Modified method combining in situ detoxification with simultaneous saccharification and cofermentation (SSCF) as a single step for converting exploded rice straw into ethanol', *Journal of Agricultural and Food Chemistry*, 62(30), pp. 7486–7495. doi:10.1021/jf501703j.

Zhao, G., Zhang, W. and Zhang, G. (2010) 'Production of single cell protein using waste capsicum powder produced during capsanthin extraction', *Letters in Applied Microbiology*, 50(2), pp. 187–191. doi:10.1111/j.1472-765X.2009.02773.x.

Zheng, D. *et al.* (2015) 'Biodegradation of furfural by Bacillus subtilis strain DS3', *Journal of Environmental Biology*, 36(4), pp. 727–732.

Van Zyl, W.H. *et al.* (2007) 'Consolidated bioprocessing for bioethanol production using saccharomyces cerevisiae', *Advances in Biochemical Engineering/Biotechnology*, 108, pp. 205–235. doi:10.1007/10_2007_061.

APPENDIX A: AMINO ACID COMPOSITIONS OF MICROBIAL STRAINS

Table A.1: Amino acid composition of edible bacteria compared to FAO standards. Adapted from (Erdman, Bergen and Adinarayana Reddy, 1977; Moo-Young *et al.*, 1977; Kurbanoglu and Algur, 2002)

Amino acid (%)	***Bacillus subtilis***	***Lactobacillus acidophilus***	***Lactobacillus delbrueickii* subsp. *bulgaricus***	***Lactobacillus fermentum***	***Streptococcus thermophilus***	**Beef protein**	**Soya**	**FAO reference**
Threonine	5.2	4.0	3.8 to 4.3	3.6	3.7	4.4	4.0	2.8
Valine	6.1	4.9	4.9 to 5.8	4.9	4.8	5.5	5.0	4.2
Cystine	0.7	-	-	-	-	-	1.4	2.0
Methionine	2.6	1.9	1.9 to 2.2	2.0	1.9	2.5	1.4	2.2
Isoleucine	4.6	4.3	4.2 to 4.5	4.4	4.1	5.3	5.4	4.2
Leucine	8.7	7.4	6.1 to 6.5	6.3	6.4	8.2	7.7	4.8
Tyrosine	5.0	2.5	2.7 to 3.3	2.4	2.6	3.4	2.7	2.8
Phenylalanine	4.9	3.3	3.2	3.1	3.2	4.1	5.1	2.8
Lysine	4.5	10.4	7.9 to 9.3	7.1	9.0	8.6	6.5	4.2
Alanine	9.0	9.0	6.0 to 7.2	8.9	8.4	-	-	-
Arginine	7.9	5.1	4.0 to 4.5	5.1	4.3	-	-	-
Aspartic acid	10.6	9.7	10.0 to 10.5	7.7	10.6	-	-	-
Glutamic acid	12.6	11.1	9.1 to 9.8	10.3	9.7	-	-	-
Glycine	6.6	4.2	3.5 to 4.0	4.2	4.2	-	-	-
Histidine	2.3	2.4	1.9 to 2.2	2.2	2.2	-	-	-
Proline	4.4	3.5	3.0 to 3.6	2.6	2.4	-	-	-
Serine	4.7	2.5	2.3 to 2.6	2.7	2.3	-	-	-

Note: '-' states that no data was provided

Table A.2: Amino acid composition of edible bacteria compared to FAO standards. Adapted from (Shipman, Kao and Fan, 1975; Moo-Young *et al.*, 1977; Noparatnaraporn and Nagai, 1986; Rajoka, 2005; Saejung and Thammaratana, 2016)

Amino acid (%)	***Cellulomonas biazotea***	***Rhodobacter sphaeroides***	**Beef protein**	**Soya**	**FAO reference**
Threonine	2.5	2.9	4.4	4.0	2.8
Valine	4.5	2.7	5.5	5.0	4.2
Cystine	5.5	-	-	1.4	2.0
Methionine	1.9	1.5	2.5	1.4	2.2
Isoleucine	3.2	1.8	5.3	5.4	4.2
Leucine	5.9	3.9	8.2	7.7	4.8
Tyrosine	1.5	-	3.4	2.7	2.8
Phenylalanine	3.6	2.4	4.1	5.1	2.8
Lysine	3.7	2.6	8.6	6.5	4.2
Alanine	2.1	-	-	-	-
Arginine	1.5	-	-	-	-
Aspartic acid	3.4	-	-	-	-
Glutamic acid	5.5	-	-	-	-
Glycine	-	-	-	-	-
Histidine	1.6	1.0	-	-	-
Proline	4.8	-	-	-	-
Serine	1.6	-	-	-	-

Note: '-' states that no data was provided

Table A.3: Amino acid composition of edible fungi compared to FAO standards. Adapted from (Erdman, Bergen and Adinarayana Reddy, 1977; Moo-Young *et al.*, 1977; Chahal, 1989; Pessoa, Mancilhab and Sao, 1996; Anupama and Ravindra, 2000; Nigam, 2000; Wang, Sakoda and Suzuki, 2001; Ahmadi *et al.*, 2010)

Amino acid (%)	***Chaetomium cellulolyticum***	***Trichoderma viride***	***Pleurotus ostreatus***	***Pleurotus sajor-caju***	***Pleurotus florida***	***Candida tropicalis***	***Candida utilis***	***Candida langeronii***	**Beef protein**	**Soya**	**FAO reference**
Threonine	6.14	4.90	1.71	2.91	0.64	4.98	2.50	5.10	4.40	4.00	2.80
Valine	5.76	4.40	2.1	3.47	6.68	4.76	4.80	4.50	5.50	5.00	4.20
Cystine	0.31	1.45	0.38	0.70	-	-	0.70	0.70	-	1.40	2.00
Methionine	2.33	1.35	0.38	1.14	2.11	1.60	0.80	0.75	2.50	1.40	2.20
Isoleucine	4.70	3.50	1.62	2.88	7.32	3.99	3.90	4.10	5.30	5.40	4.20
Leucine	7.54	5.80	2.57	4.39	6.82	6.45	6.10	6.50	8.20	7.70	4.80
Tyrosine	3.26	3.30	1.33	2.09	-	3.29	-	-	3.40	2.70	2.80
Phenylalanine	3.77	3.70	1.52	2.04	4.37	3.55	3.50	3.50	4.10	5.10	2.80
Lysine	6.80	4.40	2.29	3.99	9.55	7.22	6.80	7.80	8.60	6.50	4.20
Alanine	-	-	2.86	5.08	-	-	-	-	-	-	-
Arginine	-	-	2.76	8.84	8.30	-	-	-	-	-	-
Aspartic acid	-	-	3.14	5.74	-	-	-	-	-	-	-
Glutamic acid	-	-	5.33	8.84	-	-	-	-	-	-	-
Glycine	-	-	1.71	-	-	-	-	-	-	-	-
Histidine	-	-	1.24	1.83	19.88	-	-	-	-	-	-
Proline	-	-	1.52	-	-	-	-	-	-	-	-
Serine	-	-	1.81	5.66	-	-	-	-	-	-	-

Note: '-' states that no data was provided

Table A.4: Amino acid composition of edible fungi compared to FAO standards. Adapted from (Moo-Young *et al.*, 1977; Almeida e Silva *et al.*, 1995; Rao, Varma and Deshmukh, 2010; Kam, Kenari and Younesi, 2012)

Amino acid (%)	***Penicillium janthinellum***	***Paecilomyces variotii***	***Saccharomyces cerevisiae***	**Beef protein**	**Soya**	**FAO reference**
Threonine	3.30	4.65	4.80	4.40	4.00	2.80
Valine	9.00	6.39	5.30	5.50	5.00	4.20
Cystine	1.30	1.17	-	-	1.40	2.00
Methionine	0.30	1.74	1.70	2.50	1.40	2.20
Isoleucine	-	5.08	4.60	5.30	5.40	4.20
Leucine	2.40	14.38	7.00	8.20	7.70	4.80
Tyrosine	4.60	5.86	-	3.40	2.70	2.80
Phenylalanine	2.70	4.31	4.10	4.10	5.10	2.80
Lysine	14.0	7.34	7.70	8.60	6.50	4.20
Alanine	-	7.19	-	-	-	-
Arginine	-	4.31	2.40	-	-	-
Aspartic acid	-	7.03	-	-	-	-
Glutamic acid	-	11.41	-	-	-	-
Glycine	-	4.99	-	-	-	-
Histidine	-	2.18	2.70	-	-	-
Proline	-	5.98	-	-	-	-
Serine	-	3.39	-	-	-	-
Tryptophan	-	-	1.00	-	-	-

Note: '-' states that no data was provided

APPENDIX B: STEP-BY-STEP PROCEDURES

B.1 Enzyme activity determination – modified filter paper assay:

Different enzyme dilutions were added to test tubes with 1 mL 0.05 M citrate buffer with a pH of 5.0. Whatman No.1 filter paper, 50 mg, was added to each test tube, and these test tubes were then incubated at a temperature of 50°C for 1 hour. After an hour, the samples were placed on ice and were prepared for HPLC analysis to determine which enzyme dilution resulted in the release of 2 mg of glucose. Thus, the activity of the enzyme was calculated.

The concentration of enzyme used to release 2 mg of glucose was determined by translating the dilutions into concentrations (Equation B.1).

$$Concentration = \frac{1}{dilution}\left(= \frac{volume\ of\ enzyme}{total\ volume}\right) \quad \text{B.1}$$

The filter paper unit (FPU) was then calculated (Equation B.2).

$$FPU = \frac{0.37}{enzyme\ concentration\ to\ release\ 2.0\ mg\ glucose}\left(\frac{FPU}{mL}\right) \quad \text{B.2}$$

The determination of the enzyme activity is important as it stipulates the required enzyme dosage that is required and allows for a comparison with other studies to occur. The enzyme required per dry weight of substrate was calculated using Equations B.3 and B.4.

$$Desired\ activity\left(\frac{FPU}{g\ substrate}\right) \times dry\ mass\ substrate\ (g) = Activity\ (FPU) \quad \text{B.3}$$

$$\frac{Calculated\ activity}{Enzyme\ activity} = volume\ of\ enzyme\ required\ (mL) \quad \text{B.4}$$

1. Add 1.0 mL 0.05 M, pH 5 sodium citrate buffer to test tubes
2. Cut Whatman No. 1 filter paper to the mass of 50 mg and add to the citrate buffer
3. Add different dilutions of enzymes to find what dosage releases 2.0 mg of glucose
4. Add test tubes to a water bath, pre-heated to 50 °C for an hour
5. Remove test tubes and prepare samples for HPLC analysis

APPENDIX C: SAMPLE CALCULATIONS

C.1 Conversion factors

The conversion factors were calculated through the use of equation C.1.

$$Conversion\ factor = \frac{MW_{product}}{MW_{reactant}} \quad \text{C.1}$$

Where $MW_{product}$ is the molecular weight of the product of the hydrolysis reactions in g/mol and $MW_{reactant}$ is the molecular weight of the molecule that reacts with water to produce the product, also in g/mol.

C.1.1 Cellulose to glucose

Cellulose has a repeating unit that has a molecular mass of 324.28 g/mol. Cellulose reacts with water in a hydrolysis reaction to produce 2 glucose molecules that each have a molecular mass of 180.16 g/mol. Therefore, the total molecular mass of the two glucose molecules is 360.32 g/mol. The conversion factor on cellulose to glucose can be calculated as follows:

$$Conversion\ factor = \frac{360.32\ g/mol}{324.28\ g/mol}$$

$$Conversion\ factor = 1.11$$

C.1.2 Hemicellulose to xylose

Hemicellulose is comprised of a repeating unit that has a molecular mass of 264.16 g/mol. Hemicellulose reacts with water in a hydrolysis reaction to produce 2 xylose molecules that each have a molecular mass of 150.13 g/mol. Therefore, the total molecular mass of the two xylose molecules is 300.26 g/mol. The conversion factor on hemicellulose to xylose can be calculated as follows:

$$Conversion\ factor = \frac{300.26\ g/mol}{264.16\ g/mol}$$

$$Conversion\ factor = 1.14$$

C.1.3 Xylose to furfural

Xylose reacts with water in a hydrolysis reaction to produce furfural, with a molecular mass of 96.08 g/mol. The conversion factor on xylose to furfural can be calculated as follows.

$$Conversion\ factor = \frac{96.08\ g/mol}{150.13\ g/mol}$$

$$Conversion\ factor = 0.64$$

C.1.4 Glucose to 5-HMF

Glucose reacts with water in a hydrolysis reaction to produce 5-HMF, with a molecular mass of 126.11 g/mol. The conversion factor on glucose to 5-HMF can be calculated as follows:

$$Conversion\ factor = \frac{126.11\ g/mol}{180.16\ g/mol}$$

$$Conversion\ factor = 0.7$$

C.2 Severity Factor

The severity factor of the STEX conditions was calculated by taking the logarithm of the reaction ordinate (Equation C.2). The reaction ordinate was determined through the use of Equation C.3 below (Overend, Chornet and Gascoigne, 1987; Martín *et al.*, 2021).

$$SF = log(R_o) \quad \text{C.2}$$

$$R_0 = t \cdot exp\left(\frac{T_r - 100}{14.75}\right) \quad \text{C.3}$$

Where t is the holding time and T_r is the STEX process's temperature.

Therefore, the severity factor calculated for steam explosion at 185 °C after a holding time of 10 minutes was calculated as follows:

$$R_0 = 10 \cdot exp\left(\frac{185 - 100}{14.75}\right)$$

$$R_0 = 3\,182.101$$

$$SF = \log(3\,182.101)$$

$$SF = 3.5$$

CHAPTER 7: ASPEN PLUS® MODEL FLOWSHEETS

7.1 Overall Flowsheet

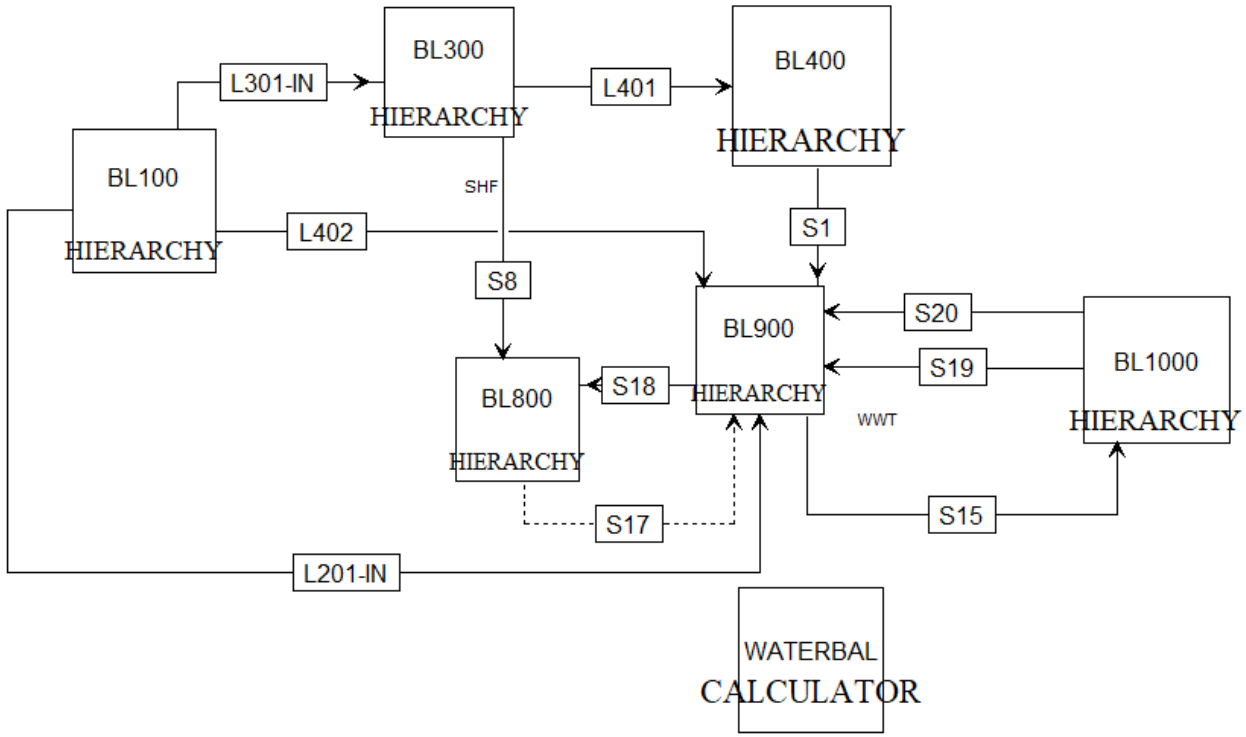


Figure 7.1: The overall Aspen Plus® Flowsheet indicating where each block fits into the flowsheet.

7.2 Steam Explosion and Washing (BL100)

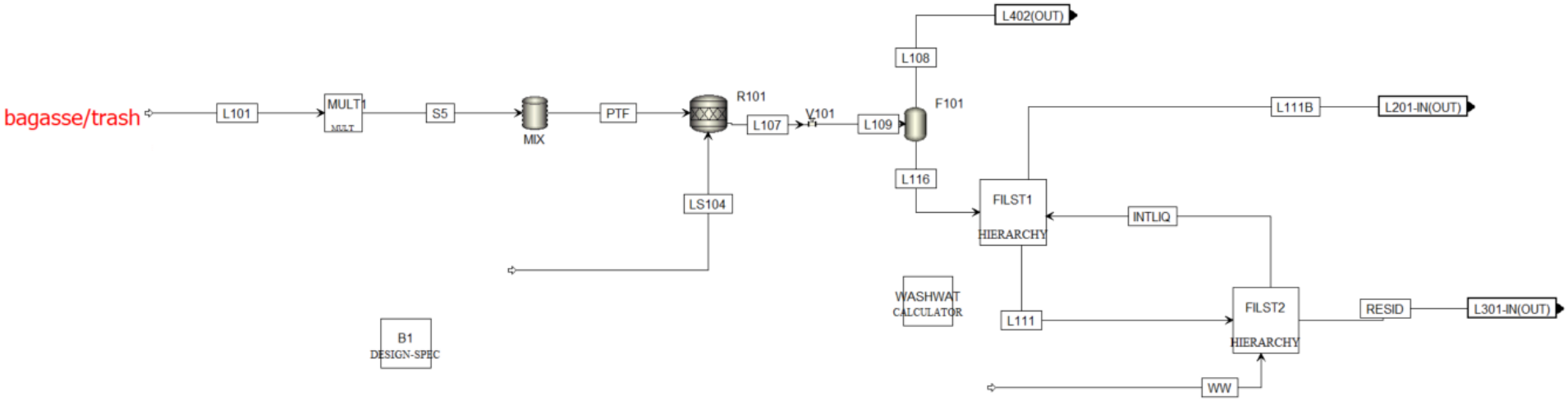


Figure 7.2: Steam explosion and the two-stage counter-current washing of the steam exploded bagasse, followed by filtration to separate the solid fraction from the liquid fraction.

7.3 Separate Hydrolysis and Fermentation (BL300)

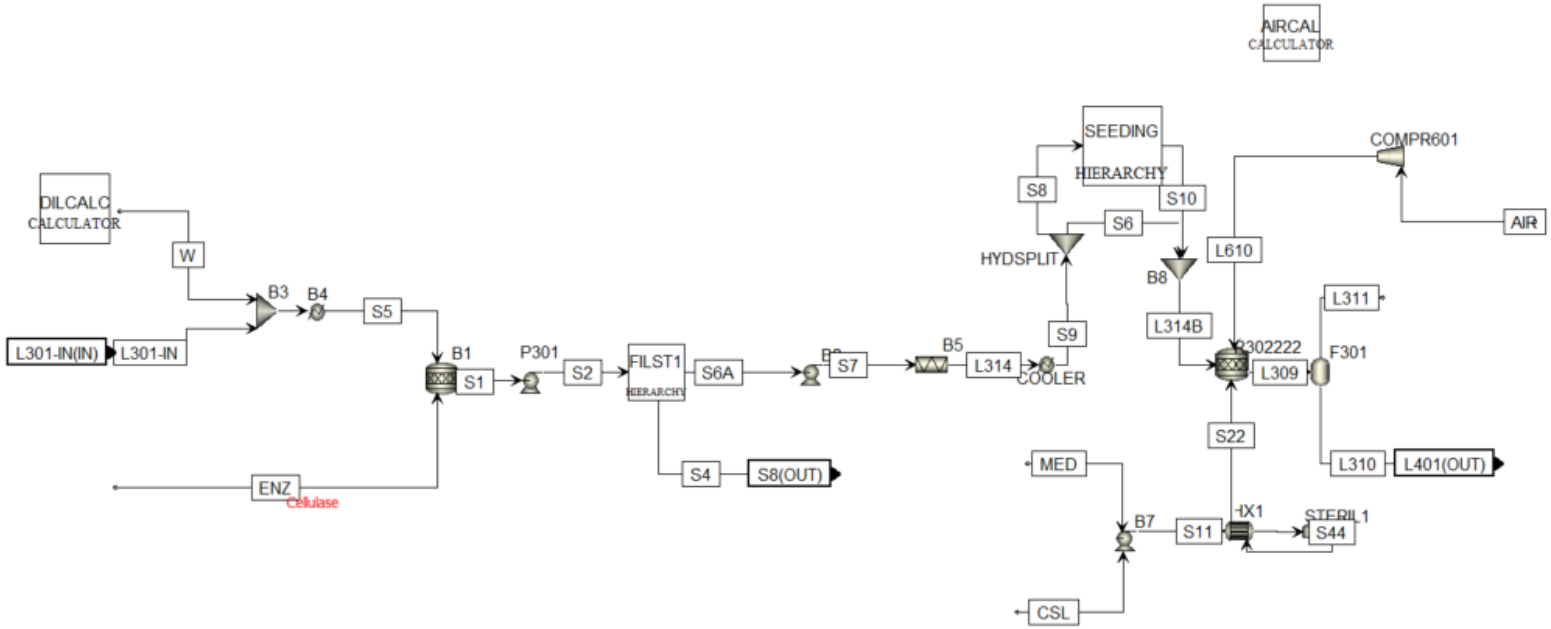


Figure 7.3: Separate hydrolysis and fermentation stage of the flowsheet, which includes a seeding train before the fermentation step occurs.

7.4 Centrifugation and Evaporation (BL400)

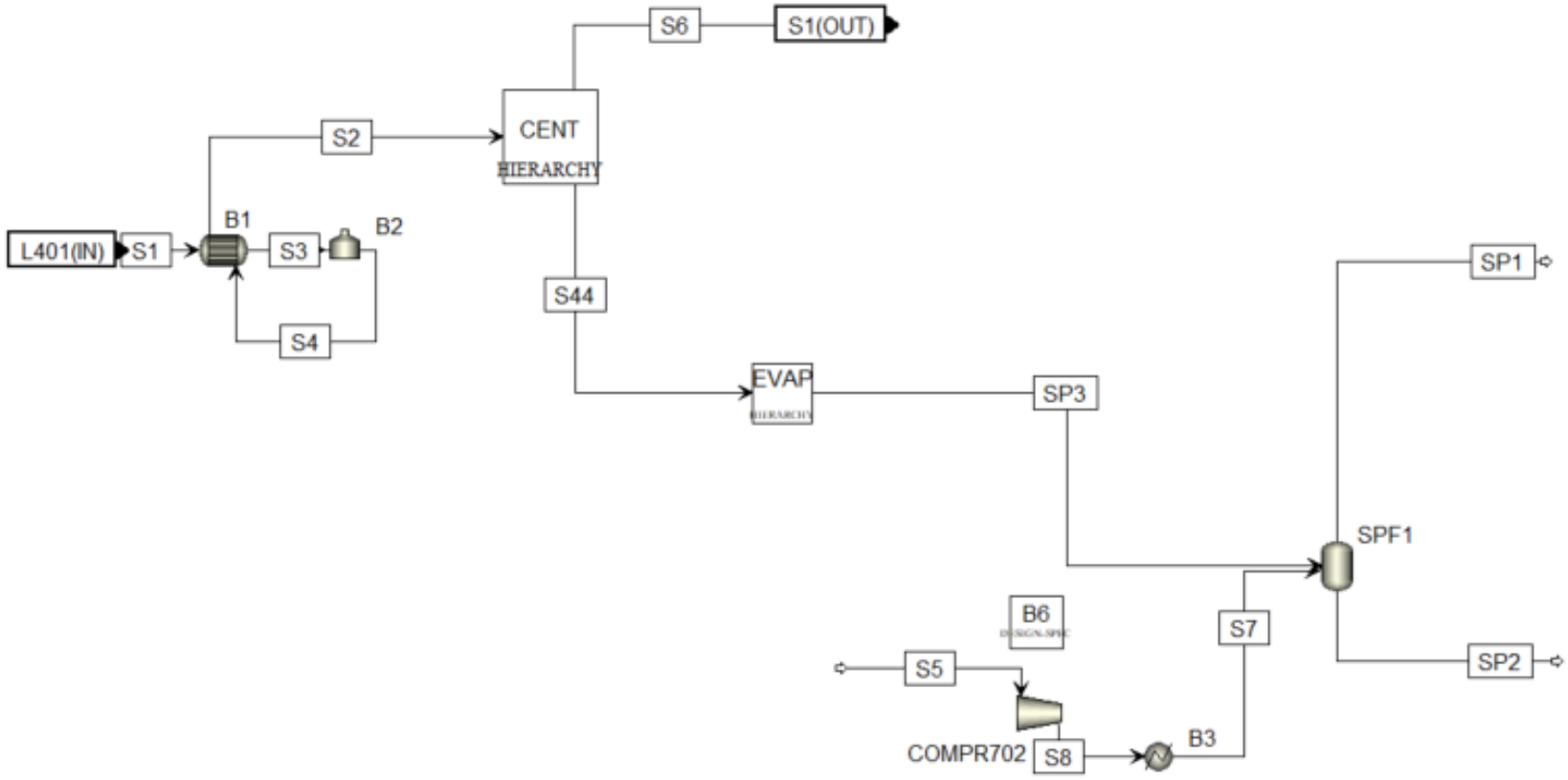


Figure 7.4: Centrifugation and evaporation of the microbial biomass product.

7.5 Boiler and Steam Generation (BL800)

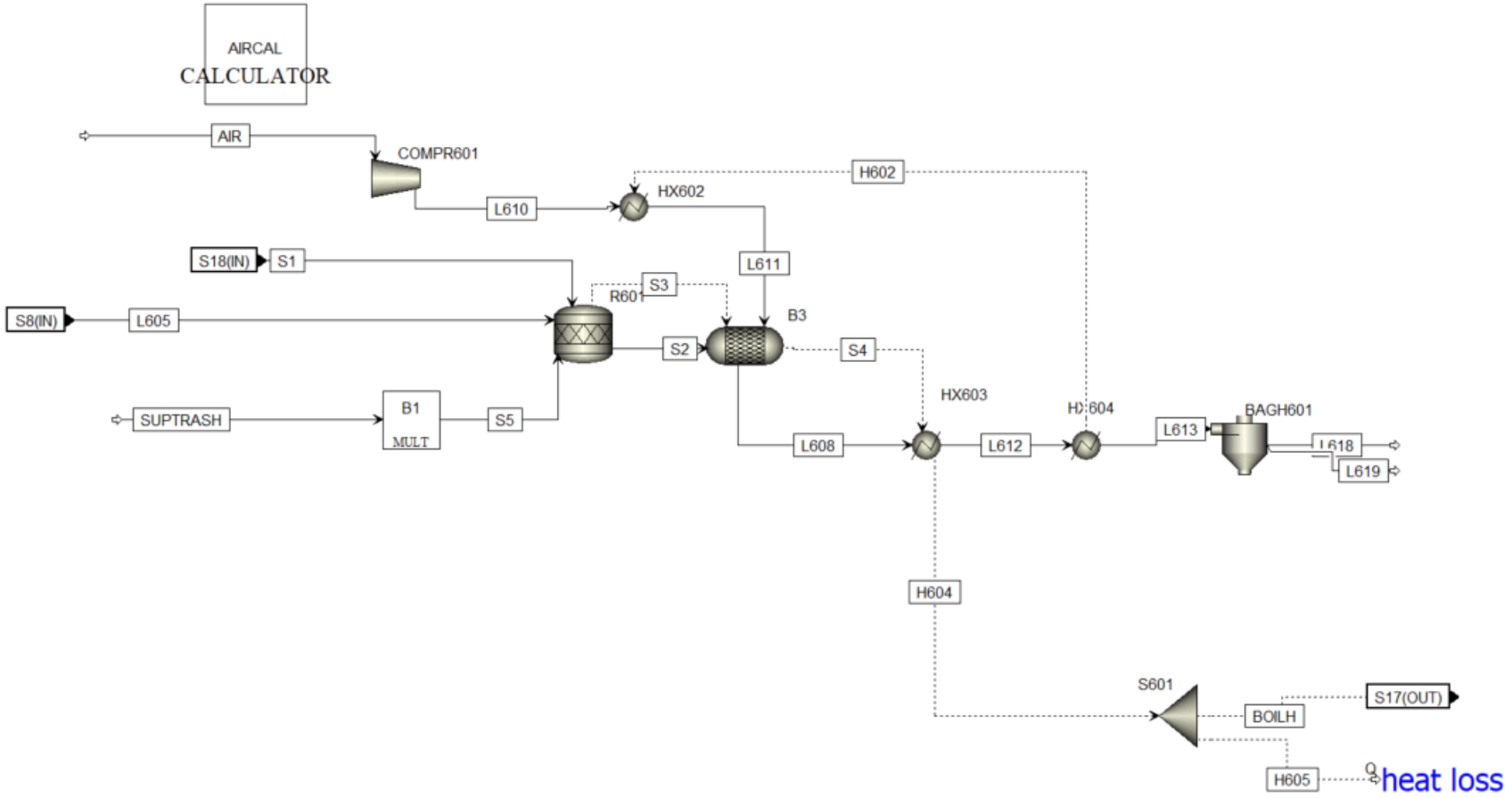


Figure 7.5: Boiler section of the flowsheet, where steam for the process is produced.

7.6 Water Treatment (BL900)

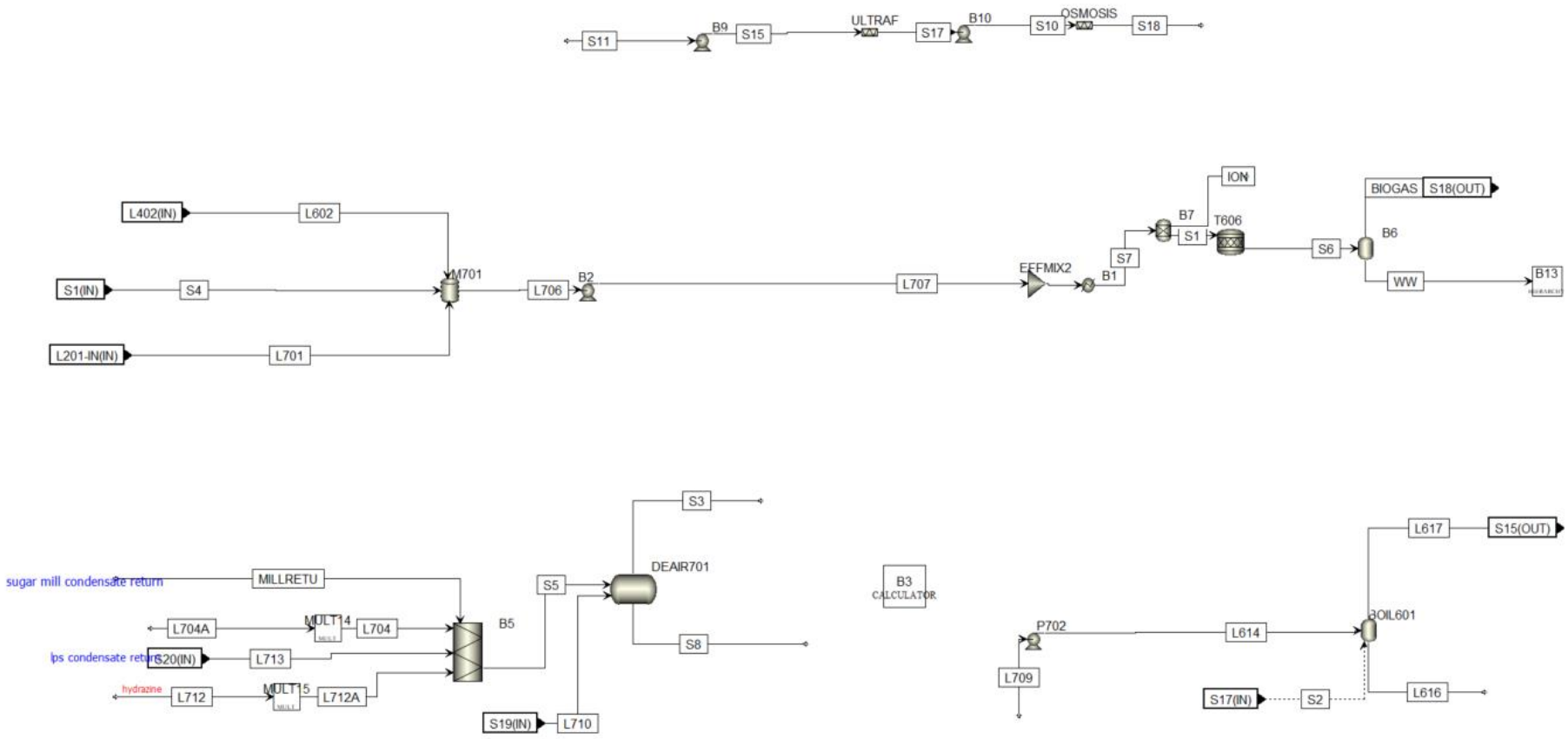


Figure 7.6: The wastewater treatment section of the flowsheet, where the water used for the washing of the steam-exploded bagasse is treated and recycled.

7.7 Energy Balance (BL1000)

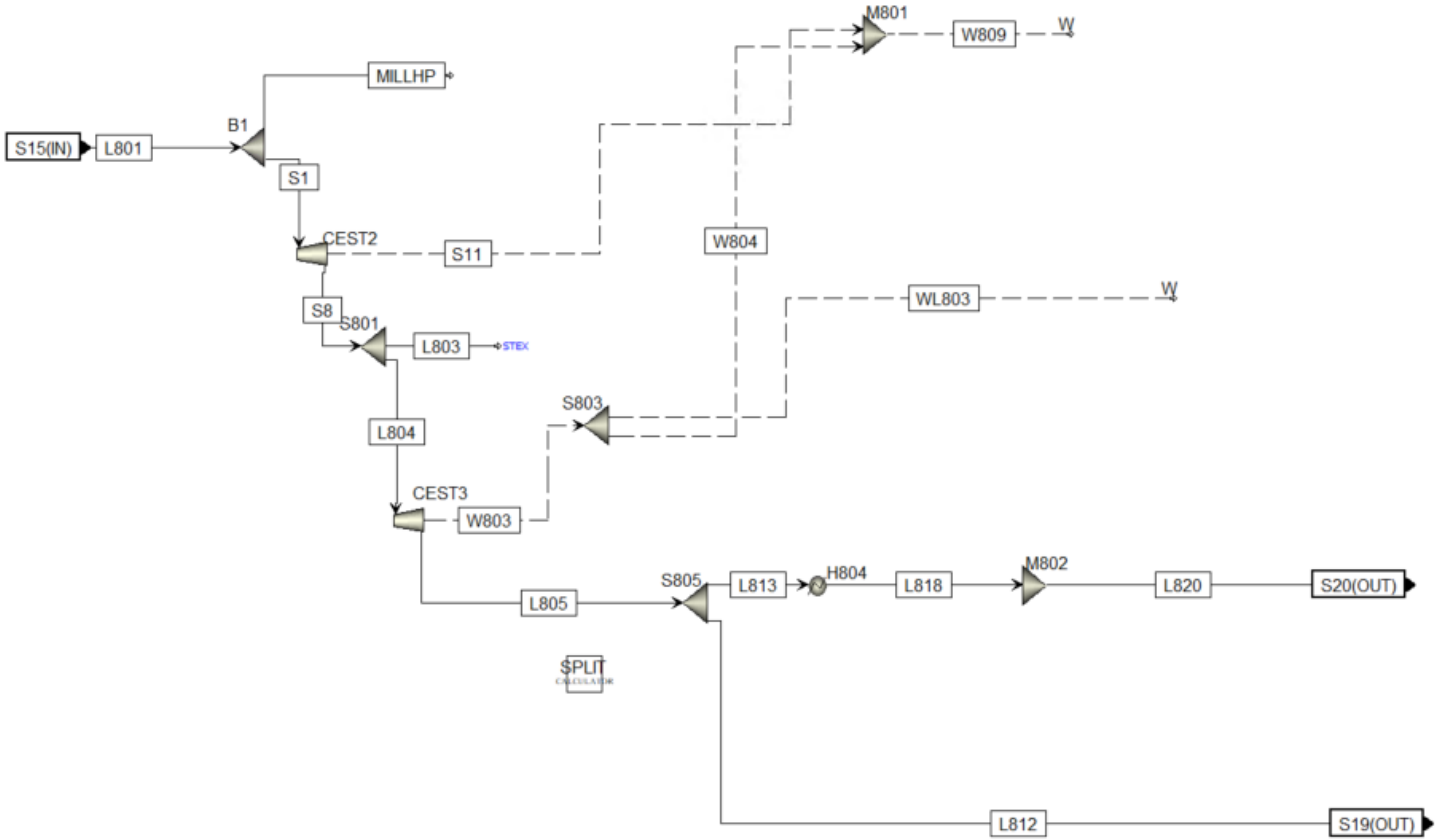


Figure 7.7: Energy balance to determine the bagasse requirements to ensure the process is self-sufficient.