Assessment of Enzyme Recycling Schemes for Paper Sludge Fermentation using Kinetic Modelling

by

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DECLARATION

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Abstract

Ethanol for road transport is well established, but it also presents a “gateway molecule” in the bigger circular economy setting, where it is used for jet fuels, bioplastics, organic solvents, etc. These high-value products make ethanol production an attractive route compared to other biomass valorisation methods such as incineration, anaerobic digestion, and composting. Cheaper raw materials such as agricultural and industrial wastes have been found as suitable feedstock for bioethanol production. Paper sludge (PS) is one major waste stream from the pulp and paper industry that is mainly landfilled. Due to the extensive pulping processes, PS has substantial accessible cellulose content which makes it appropriate for bioethanol production. However, the cost of enzymes required during the process is one of the major bottlenecks to the commercialization of bioethanol from PS.

This work sought to achieve significant savings on the enzyme dosage required in a simultaneous saccharification and fermentation (SSF) process by recycling enzymes in the clarified supernatant or whole fermentation broth, consisting of solids and liquid. To achieve this goal, kinetic models that predict the loss of cellulase activity and ethanol concentrations during the fermentation process were developed.

The loss of cellulase activity was assumed to follow a first-order reaction for batch enzyme feeding in the reactor system. This model was used to predict the level of cellulase activity at any time in the fermentation. It was based on the overall decay of residual activities from the preceding reactor and fresh enzyme supplementations at the beginning of each round of fermentation. The model that describes the enzymatic hydrolysis of PS used the Avrami-Kolmogorov-Erofeev (AKE) equation to determine the kinetic conversion rates. Assuming a constant ethanol yield from sugars, the AKE equation was used to predict ethanol concentrations.

Model predictions were validated experimentally by evaluating the performance of the recycling schemes over multiple recycling rounds. Approximately 48% and 40% of the initial activity of Cellic® CTec 3 remained in the supernatant and broth respectively after 72 h of SSF in shake flask. Although

the recycling schemes showed no significant variation, contamination with lactic acid-producing bacteria decreased ethanol yields during successive recycling steps.

During the scale-up of enzyme recycling in 5L bioreactors, close to 35% of the initial enzyme activity was measured in the supernatant after 168 h of fed-batch fermentation. With only 65% fresh enzyme supplementation, the final ethanol concentration in the fermentation broth increased from 40 to 70 g/L. However, the experimental data deviated from model predictions as the ethanol yields decreased from 280 to 200 kg ethanol/ton dry PS for the first and last fermentations respectively. The observed reduction in the overall enzymatic hydrolysis rate was possibly due to the effect of time-wise loss of enzyme potency/synergy.

The kinetic models were able to predict the performance of PS fermentation with some reasonable level of accuracy. In spite of the inherent factors that affected PS fermentation, the advantages of high ethanol concentrations and significant reduction in enzyme dosages achieved can be very beneficial to the economic viability of the process.

Opsomming

Etanol vir padvervoer is reeds goed gevestig, maar dit bied ook ’n “toegangsmolekule” in die groter sirkulêre ekonomiese omgewing, waar dit gebruik word vir stralerbrandstof, bioplastiek, organiese oplosmiddels, ens. Hierdie hoë-waarde produkte maak etanolproduksie ’n aantreklike roete in vergelyking met ander biomassa valorisasiemetodes soos verbranding, anaerobiesevertering, en komposvorming. Goedkoper roumateriale soos landbou- en industriële afval is gevind om gepaste voermateriaal vir bio-etanolproduksie te wees. Papierslyk (PS) is een groot afvalstroom van die pulp-en-papierindustrie wat hoofsaaklik op die vullisterrein eindig. As gevolg van die omvattende verpulpingsprosesse, het PS aansienlike toeganklike selluloseinhoud wat dit gepas maak vir bio-etanolproduksie. Die koste van ensiemes wat nodig is gedurende hierdie proses, is egter een van die groot knelpunte tot die kommersialisering van bio-etanol uit PS.

Hierdie werk het beoog om beduidende besparings te bereik op die ensiemdosis wat vereis word in ’n gelyktydige sakkarifikasie en fermentasie (SSF) -proses deur ensieme in die verhelderde supernatant of hele fermentasiesop, wat uit vastestowwe en vloeistowwe bestaan, te herwin. Om hierdie doelwit te bereik, is kinetiese modelle wat etanolkonsentrasies en die verlies van sellulase-aktiwiteit gedurende die fermentasieproses voorspel, ontwikkel.

Dis is aangeneem dat die verlies van sellulase-aktiwiteit ’n eerste-orde reaksie volg vir lotensiemvoer in die reaktorsisteem. Hierdie model is gebruik om die vlak van sellulase-aktiwiteit op enige tydstip in die fermentasie te voorspel. Dit was gebaseer op die algehele afbreking van residuele aktiwiteite uit die voorafgaande reaktor en vars ensiemaanvullings aan die begin van elke rondte van fermentasie. Die model wat die ensiemhidroliese van PS beskryf, het die Ayrami-Kolmogorov-Erofeev (AKE) -vergelyking gebruik om die kinetiese omsettingstempo’s te bepaal. Met die aanname dat daar ’n konstante etanolopbrengs uit suikers is, is die AKE-vergelyking gebruik om etanolkonsentrasies te voorspel.

Vir modelvoorspellings om gevalideer te word, is die doeltreffendheid van die herwinningskemas eksperimenteel verrig oor verskeie herwinningsrondtes. Ongeveer 48% en 40% van die aanvanklike aktiwiteit van Cellic® CTec3 het in die supernatant en sop na 72 uur van SSF in skudfles, onderskeidelik, gebly. Al het die herwinningskemas nie beduidende variasies getoon nie, het kontaminasie met melksuur-produserende bakterieë etanolopbrengste laat afneem gedurende opeenvolgende herwinnigstappe. Die herwinningskemas het ’n tweevoudige toename in ensiemproduktiwiteit (g Etanol/FPU) toegelaat toe ensiemdosis verminder is met 50% van die aanvanklike lading; 10 FPU/gds, wat ’n 38%-vermindering in die algehele ensiemlading tot gevolg gehad het.

Gedurende die opskaal van ensiemherwinning in 5 L-bioreaktors is na aan 35% van die aanvanklike ensiemaktiwiteit gemeet in die supernatant na 168 uur van voerlotfermentasie. Met slegs 65% vars ensiemaanvulling, het die herwinning van die verhelderde supernatant se eksperimentele data afgewyk van modelvoorspellings soos die etanolopbrengste afgeneem het van 280 na 200 kg etanol/ton droë PS vir die eerste en laaste fermentasies, onderskeidelik. Die waargenome afname in die algehele ensiematiese hidrolisetempo is moontlik as gevolg van die effek van tydsgewyse verlies van ensiemkrag/sinergie.

Die kinetiese modelle kon die doeltreffendheid van PS-fermentasie voorspel met ’n redelike vlak van akkuraatheid. Ten spyte van die inherente faktore wat PS-fermentasie affekteer, kan die beduidende afname in ensiemdosisse bereik, baie voordelig wees vir die ekonomiese lewensvatbaarheid van die proses.

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BGL	β-glucosidase
CBH	Cellobiohydrolase
CBM	Carbohydrate binding module
CBP	Consolidated bioprocessing
COD	Chemical oxygen demand
CR-PS	Corrugated recycle paper sludge
EG	Endoglucanase
FPU	Filter paper unit
GDP	Gross domestic product
gds	Gram dry solid
HPLC	High-performance liquid chromatography
IUPAC	International Union of Pure and Applied Chemistry
LAB	Lactic acid bacteria
LPMO	Lytic polysaccharide mono-oxidase
NREL	National Renewable Energy Laboratory
PAMSA	Paper Making Association of South Africa
PEG	Polyethylene glycol
PS	Paper sludge
SHF	Separate hydrolysis and fermentation
SSCF	Simultaneous saccharification and co-fermentation
SSF	Simultaneous saccharification and fermentation
TPR-PS	Tissue and printed recycle paper sludge
VP-PS	Virgin pulp paper sludge
WHC	Water holding capacity

Chapter 1 : Introduction

1.1 Background and Motivation

Bioethanol production from waste materials has gained international recognition due to the increasing global energy demand coupled with the environmental impact of waste disposal. Bioethanol is considered to be a renewable energy source that has the potential to replace fossil fuels as transportation fuel and as a key intermediate for other high-value products (Khanal 2009). Commercial bioethanol, for example, can be produced by fermentation of glucose – the sugar platform derived from starch-based crops and other lignocellulose materials and industrial waste feedstocks (Vertes et al. 2010).

South Africa's legislation prohibits landfilling of industrial organic waste streams with moisture contents above 40%. Waste disposal results in negative environmental effects such as the emission of greenhouse gases and groundwater pollution and therefore, many landfills/cities are actively discouraging the landfilling of such organic waste materials. Paper sludge, a wastepaper stream emanating from pulp and paper processing, is one of the several sugar-rich solid, organic wastes presently being landfilled. Boshoff (2015) estimated that 500,000 wet tons of paper sludge is produced annually by the members of the Paper Making Association of South Africa (PAMSA). and therefore, considering PS for ethanol production would be a suitable route for valorizing this waste material. Paper sludge has high levels of accessible cellulose and previous fermentation studies, for instance, those by Boshoff et al. (2016), Kang et al. (2010), and Robus et al. (2016) have demonstrated its suitability for efficient bioethanol production using industrial yeast, with further bio-energy production from the solid and liquid residues after fermentation (Donkor 2019).

Regardless of this suitable feedstock, ethanol yields are often reduced at high-solid loadings due to inefficient mixing and mass transfer limitations. High enzyme dosages are used to overcome this, as high solids are essential for the final ethanol concentration threshold (40 g/L), as required for distillation (Boshoff et al. 2016). The cost of enzymes has, however, been reported to be an expensive

component (up to 60% of the annual production cost) that affects the commercial production of bioethanol from lignocellulose biomass (Humbird et al. 2011; Klein-Marcuschamer et al. 2012).

Enzyme recycling is one of the promising strategies for minimizing the dosage of enzymes required during the hydrolysis step (Gomes et al. 2015; Jørgensen & Pinelo, 2017). The possible strategies for recovering enzymes include ultrafiltration (Baral et al. 2020; Gomes et al. 2016), re-adsorption of enzyme onto fresh substrate (Lindedam et al. 2013), alkaline elution (pH adjustment) (Rodrigues et al. 2012), the addition of desorbents (Tu et al. 2009) and partial recycling of whole fermentation broth (Østergaard et al. 2015).

Previously, Gomes et al. (2016; 2018a) demonstrated the feasibility of recycling enzymes in PS fermentation via ultrafiltration. Although 53% savings on enzyme dosage was achieved, the cost associated with the ultrafiltration membranes affected the economic viability of the recycling process (Gomes et al. 2018b). In this regard, the core aim of this study was to determine if recycling enzymes in the supernatant or whole fermentation broth can significantly reduce the enzyme dosage required for the simultaneous saccharification and fermentation (SSF) of PS. This was achieved by developing kinetic models that predict the loss of cellulase activity and ethanol concentrations during the fermentation process. These models were then used to make predictions about the recycling schemes and were validated based on the performance of the recycle schemes completed experimentally at low (6% (w/w), shake flask), and high-solid loadings (18% (w/w), 5L bioreactors).

To our knowledge, the enzyme recycling schemes with clarified supernatant or whole fermentation broth during SSF fermentation of PS have not been reported in literature. These recycle schemes indicate a simpler and potentially cheaper approach for minimizing enzyme dosage and improving the economic viability of bioethanol production from PS. The mathematical models developed for enzyme kinetics and ethanol production would be a valuable tool for predicting the preferred recycling scheme for future process development and optimizations.

1.2 Thesis Layout

Chapter 1: Introduction. This chapter presents the background and motivation for this study.

Chapter 2: Literature review. This chapter provides a literature review on paper sludge production from pulping industries in South Africa. It discusses the fermentation process used in paper sludge bioconversion and highlights the effect of key parameters such as solids loading and enzyme dosage on the process. Also, the impact of enzyme recycling on bioethanol production is reviewed.

Chapter 3: Evaluation of Cellulase Recycling Schemes for Paper Sludge Fermentation using Kinetic Modelling. This chapter presents the experimental approach, methodology, materials, and equipment used for generating data for the study. It presents and discusses the results obtained from all experimental work. The results are discussed in relation to the study's research aims and objectives and is written as a manuscript submitted to the Journal of Environmental Chemical Engineering.

Chapter 4: Conclusions and recommendations. This chapter presents the general findings and conclusions to the study, with recommendations for future work.

Chapter 2 : Literature Review

2.1 The Pulp and Paper Industry in South Africa

South Africa has a pulp and paper industry that contributes approximately R29 billion to the country's economy and serves as a source of employment for 150,000 people (PAMSA, 2016). South Africa's pulp and paper industry is recognized worldwide as the 15th major pulp producer and 24th in paper production (PAMSA, 2016).

The industry has suffered a decline in production as a result of the surge towards electronic media against hard copies over the last five years (Figure 2-1). Despite the significant decrease, the industry is responsible for contributing to 0.44% of the total South African Gross Domestic Product (GDP) (PAMSA, 2016). Regardless of the advances in technology, the pulp and paper industry is still making several products that cannot be substituted with information technology. These include pulps used in textile and cloth making, security paper used for printing currencies, and tissue paper; an indispensable necessity for daily personal hygiene (Boshoff 2015). Most importantly, pulps for product packaging and storage has been on the increase due to the advent of online shopping such as Amazon, Takealot, etc. This has kept the economic viability of local manufacturing industries in most developing countries.

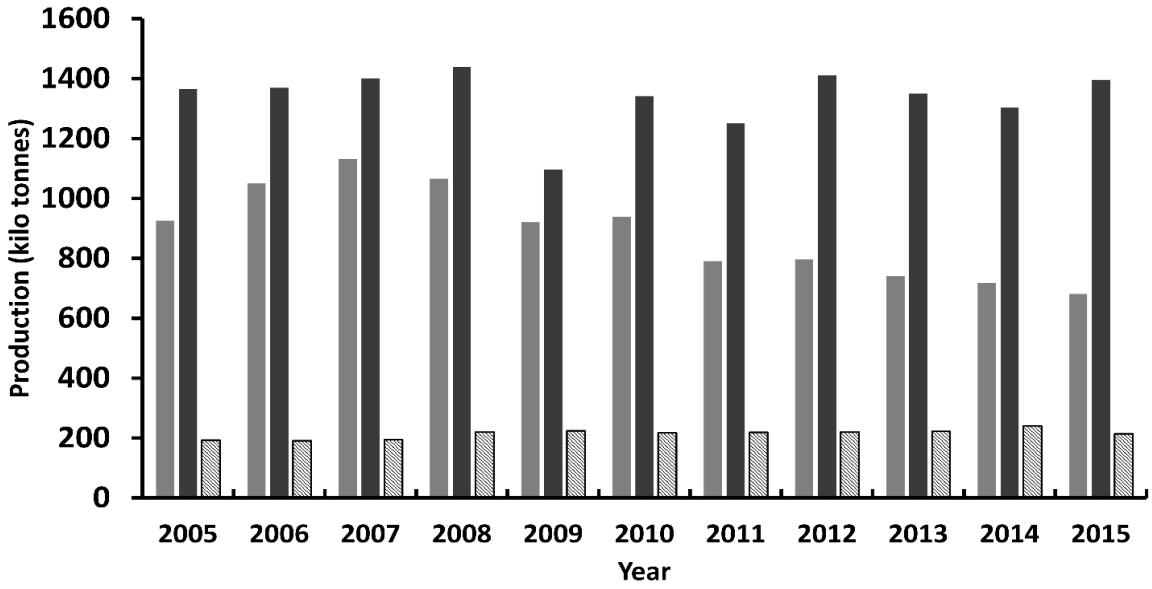


Figure 2-1: South Africa's annual pulp and paper production (■ Printing and writing paper ■ Packaging paper ■ Tissue paper) (PAMSA 2016).

2.2 Pulp and Paper Manufacturing Process

Paper production can be categorized into two main stages namely, pulping of fibrous raw material (virgin wood or recycled paper), and the papermaking process (Bajpai 2018). First, harvested raw virgin wood is prepared to remove unwanted materials such as dirt and bark, and comminuted into wood chips for further processing (Figure 2-2). Pulping begins when raw wood chips are separated into individual cellulosic fibres by the removal of lignin, a typical component of wood responsible for its rigidity, and most of the hemicelluloses (Bajpai 2013). The pulping process can be categorized into three groups namely; chemical pulping, mechanical pulping, and a combination of chemical and mechanical processes, depending on the specific desired product (Bajpai 2018). In chemical pulping, pure cellulose fibres are separated from lignin and hemicellulose by cooking the raw wood materials in either sodium hydroxide (Kraft pulping) or sodium sulfide (sulfite pulping) at high temperature and pressure, dissolving all non-wood fractions (Figure 2-2). These pulps are mostly used for newsprints, fine papers, and specialty papers (Gottumukkala et al. 2016). On the other hand, mechanical pulping including waste paper recycling uses mechanical energy (grinding) to gradually break bonds between the fibres, separating fibres from each other (Bajpai 2018). Although mechanical pulp retains more lignin, the fibres are stronger compared to chemical pulp and therefore makes it suitable for printing papers (Bajpai 2013).

After the pulping process, the dry pulp mixture is dissolved in water and the slurry is continuously passed through several refiners to enhance its bonding ability (Bajpai 2013). The finished stock is then treated with several chemical additives (resins, fillers, dyes, etc.) to impart specific properties such as color, texture, etc. The paper is then pressed, dried, and calendared to create a very smooth and glossy surface as well as a uniform thickness of the paper (Bajpai 2018).

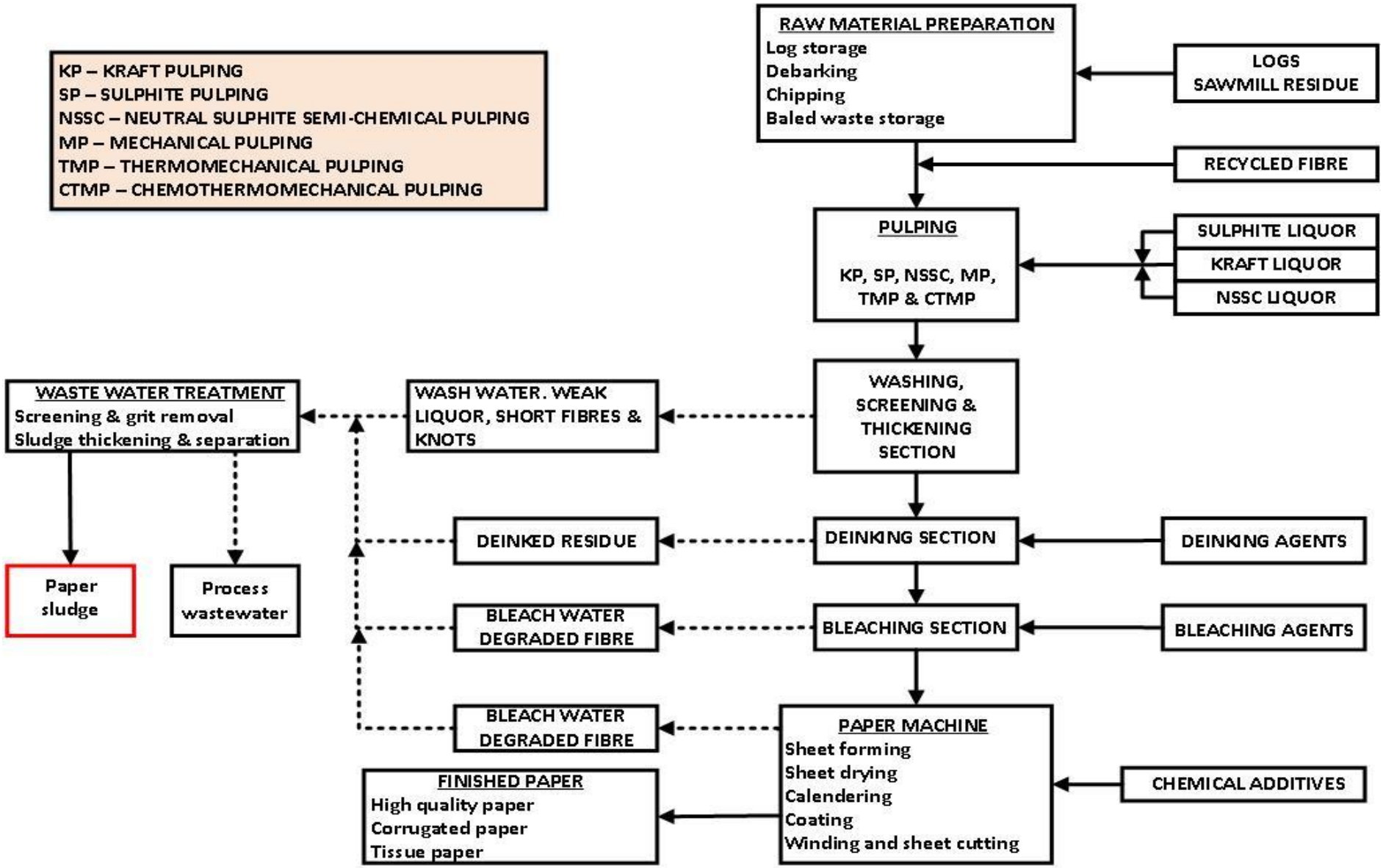


Figure 2-2: A Simplified Diagram of the Pulp and Paper Mill (Adapted from GAPS Guidelines, 2015; Williams, 2016).

2.3 Composition of Paper Sludge

Paper waste sludge (PS), also known as primary sludge, clarifier sludge, or paper waste is a solid waste residue emanating from the various stages of the aforementioned pulp and paper operations (Figure 2-2). It is composed of shortened lignocellulose fibres along with process waste water, and ash. Ash is composed of dyes, clay, and other chemicals that are not useful in the papermaking process (Prasetyo & Park 2013). Typically, the composition of paper sludge varies because it is a mixture of waste streams originating from various processes in the mill such as washing, bleaching, and papermaking units, etc. (Figure 2-2). Two main types of paper sludges are produced in pulp and paper processing, namely primary and secondary sludge. Primary sludge is the residue generated in the primary clarifier as a result of gravity settling and mostly contains short fibres and fillers. Secondary sludge is an auxiliary product of biological wastewater treatment and due to the high microbial content and very low sugar content, it is not suitable for bioethanol production (Gottumukkala et al. 2016).

Boshoff et al. (2016) grouped primary or clarifier paper sludges into three major classifications based on the feedstock utilized and the chemical composition of the sludge. Paper sludge was grouped as either:

1. Tissue and Printed Recycle Paper Sludge (TPR-PS)
2. Corrugated Recycle Paper Sludge (CR-PS)
3. Virgin Pulp Paper Sludge (VP-PS).

TPR-PS is produced from recycling newsprints, writing, and printing paper. It can be characterized by the lowest average cellulose content of about 20% w/w and a predominantly high ash content of approximately 60% w/w (Donkor 2019). CR-PS is found in mills that produce containerboards, liner board, and coated boards and are attributed with significantly high ethanol yields of 15 g/100 g PS on average due to the presence of high cellulose content (~ 40% w/w) in the sludge (Boshoff 2015). VP-PS is known to have the highest cellulose content and the least ash content of about 55% (w/w) and 10% (w/w) respectively. It is produced from mills that generate dissolved, mechanical and

chemical pulps from virgin wood (Boshoff 2015). The section below summarizes the typical composition of paper sludge and its impact on the fermentation process in general.

2.3.1 Cellulose

Cellulose is the fundamental component of the plant cell wall. It comprises simple and linear glucose chains that are linked by β-1, 4-glycosidic bonds. However, the physical properties of cellulose are complex depending on the degree of polymerization within a single cellulose fibril as well as the hydrogen bonding and Van der Waals interactions between the cellulose chains (Frei et al. 2014). The typical degree of polymerization of cellulose chains usually ranges from 10,000 to 15,000 units (Ramesh et al. 2017).

2.3.2 Hemicelluloses

Hemicelluloses are the next predominant fraction within the cellulose cell walls and function as a binder – to keep the microfibrils of cellulose together. This polymer is made up of several short, branched monosaccharides including pentose (xylose, arabinose, and rhamnose) and hexose (glucose, galactose, and mannose) sugars. It is quite amorphous and hence, breaks down easily due to branches in its structure (Frei et al. 2014; Ramesh et al. 2017). Hemicelluloses are usually of a much lesser molecular weight than cellulose (Pettersen 1983); their typical contents in paper sludges range between 5 and 16% (Table 2-1).

2.3.3 Lignin

Lignin is the third most abundant polymer in the plant cell wall. It is made of a complex, macrostructure of cross-linked polymers of phenolic monomers. Its presence offers strong and impervious resistance to microbial damage and oxidative stress as it fills up spaces within the cell walls. Paper sludge has a typical lignin content ranging from 5 to 20% (Table 2-1). Lignin is usually known to be a limiting factor in the enzymatic and microbial saccharification process due to its association with cellulose microfibrils (Ramesh et al. 2017).

2.3.4 Extraneous components

Extraneous components are substances (extractives and ash) that do not contribute to cellular structures such as cellulose, hemicellulose, and lignin. Extractives are a range of organic compounds such as fats, waxes, gums, and resins. Though present in very small quantities, organic extractives are responsible for certain properties of biomass including color and smell (Vertes et al. 2010).

Usually, TPR-PS is noted to have ash contents higher than 50% w/w. This is as a result of chemicals such as fillers and clay used during pulping as well as inks used in the printing process (Boshoff 2015). These additives shield the cellulose fibres and reduce the accessibility of enzymes to the cellulose substrate during hydrolysis prior to fermentation (Kang et al. 2011). Given that ash has a strong buffer action, paper sludge with high ash content can increase the pH of the fermentation broth to between pH 7 and 10, reduce sugar content by mass and increase capital cost due to increased equipment size (Chen et al. 2014).

Paper sludge with high cellulose content is theoretically considered to be a suitable feedstock for bioethanol production due to the potential for high ethanol yields per dry weight paper sludge (Boshoff et al. 2016). Virgin pulp PS with 55.70 g/100g of glucose was reported to produce 19.01 g/100g ethanol as compared to 13.33 g/100g ethanol produced from TPR-PS with only 20.80 g/100g glucose (Table 2-1).

Table 2-1: Chemical composition and ethanol yields from different paper sludge samples (Adapted from Boshoff 2015; Donkor 2019).

Sample	Glucose (g/100g)	Xylose (g/100g)	Lignin (g/100g)	Extractives (g/100g)	Ash (g/100g)	Ethanol (g/100g)
VP[1]	55.70 ± 0.84	16.70 ± 0.69	20.95 ± 0.88	3.58 ± 0.83	2.94 ± 0.42	19.01 ± 0.02
CR[1]	42.24 ± 0.21	13.76 ± 0.41	10.05 ± 0.36	6.09 ± 0.84	26.98 ± 0.18	16.85 ± 0.44
VP[2]	58.20 ±0.40	12.20 ± 0.10	4.10 ± 0.10	5.40 ± 0.10	20.80 ± 0.10	18.88 ± 0.50
CR[2]	37.50 ± 0.40	13.10 ± 1.10	13.10 ± 0.10	10.40 ± 0.10	25.90 ± 0.30	14.55 ± 0.51
TPR[2]	20.80 ± 0.10	4.90 ± 0.20	6.40 ± 0.10	5.10 ± 0.10	62.90 ± 0.40	13.33 ± 0.58

[1] VP=Virgin pulp (Sappi Ngodwana), CR=Corrugated recycle (Mpact Springs); Boshoff, (2015)

[2] VP=Virgin pulp (Mondi Richards Bay), CR=Corrugated recycle (Mpact Felixton), TPR= Tissue printed recycle (Nampak Bellville); Donkor, (2019)

2.4 Paper Sludge as Substrate for Bioethanol Production

Bioethanol production from paper sludge has been studied at bench and pilot-scale (Gottumukkala et al. 2016). Below are the advantages and disadvantages of using PS as a substrate for bioethanol production.

Advantages:

No pretreatment required: The pulp and paper making process subjects the feedstock (pulpwood or recycle paper) to several process conditions that ensure the removal of lignin, making the fibres easily accessible to enzymes and the microbial community during bioethanol production.

According to Kang et al. (2010), 15 FPU/gds of Spezyme CP applied to Kraft mill paper sludges yielded glucose and xylose quantities that were equivalent to 94.6% and 66.3% of the theoretical maxima, respectively.

Reduction of industrial waste: To minimize the environmental impacts of wastewater disposal, the new South African regulations have legislated the sustainable reduction, recycling, or re-use of wastewater products instead of the traditional method of landfilling (Mokonyama et al. 2017). Also, the outright ban on landfilling of solids that contain more than 40% moisture, came into effect in August 2019 (Personal communications). As such, several landfills/cities have also banned the landfilling of organics altogether. The re-use of paper sludge significantly reduces the operation cost of sludge disposal and also the emission of greenhouse gases by incineration of paper sludge (Williams 2016).

Negative cost feedstock: Raw material cost is considered to be the main contributor to the operating cost of paper sludge bioconversion to ethanol (Fan & Lynd 2007a). Paper sludge is a preferred feedstock for bioethanol production over several raw materials such as wood sources or agricultural residue because it is available at a permanent production location. This nullifies the cost of harvesting and transportation assuming the ethanol plant is annexed to the mill. This also creates a reliable and stable supply all year round (Chen et al. 2014). Secondly, pulp and paper mills pay for paper sludge to be collected. These current disposal costs will become part of the revenue of future ethanol plants.

Second-generation bioethanol production: Besides paper sludge having easily accessible cellulose content and digestibility up to 70% and 94% respectively (Kang et al. 2010), PS is considered a desirable feedstock for second-generation bioethanol production because it makes a positive contribution to diversification from fossil fuels without threatening the global food supply (Brethauer & Wyman 2010). Lastly, the diversion of PS from landfills will decrease the land space required by such facilities, with benefits to communities.

Disadvantages:

Ash: One of the significant drawbacks to successful bioethanol production from paper sludge is the high content of ash (60% w/w) originating from recycling mills feeding mostly printed recycle material (Kang et al. 2010). The presence of ash in paper sludge, especially $CaCO_3$ is not only responsible for the buffering effect in the fermentation broth but also binds with cellulosic fibres, thereby reducing efficiency in both enzymatic hydrolysis and fermentation (Chen et al. 2014; Robus, 2013). Utilization of paper sludge with high ash content (56-65% w/w) increases the bulk density of the material, intensifies the capital cost due to increase in equipment sizes and intense energy inputs (Robus 2013).

Water Holding Capacity (WHC): The WHC is an indicator of the amount of water that a gram of paper sludge can saturate. This is estimated as the amount of water that will be landfilled when mechanically-pressed paper sludge is disposed. Boshoff et al. (2016) recorded the highest WHC (8.61 g water/g PS) for virgin pulp paper sludge in comparison to corrugated recycle paper sludge (6.62 g water/g PS). The difference in WHC is attributed to the fibre length of PS. Owing to the mild chemical treatment, virgin pulp paper sludge has higher fibre length as compared to recycled PS which has been subjected to multiple pulping operations, causing its fibres to be more degraded (Bester 2018). Boshoff et al. (2016) reported that WHC was directly linked to viscosity at high solid loadings and proposed intermittent feeding/fed-batch fermentation to alleviate the viscosity issues.

Background microbial contamination: The fact that PS is readily digestible, means that it will be easily susceptible to microbial contamination. Also, depending on the origin of the PS; for instance, those from recycled fibres, and pulping processes applied beforehand, PS can come with a lot of background microbial load. Although it is not necessary to pretreat the material for improved enzyme hydrolysis, it is important to sterilise it, using methods that can eliminate the background microbial load (Agalloco et al. 2004). However, sterilisation represents a substantial fraction of the cost of the process (Robus 2013).

2.5 Bioethanol Production from Paper Sludge

Generally, the type of feedstock considered for bioethanol production influences the process to be used (Khanal 2009). But in the case of paper sludge, which requires little or no pretreatment, the main biological processes involved are sterilization, enzymatic hydrolysis, yeast fermentation, and distillation as illustrated in Figure 2-3.

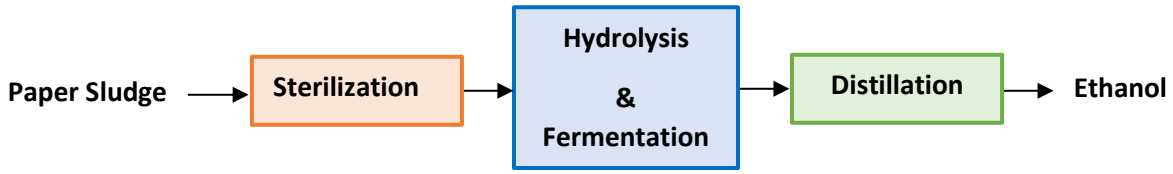


Figure 2-3: Schematic representation of ethanol production from paper sludge.

2.5.1 Sterilization

Before hydrolysis and fermentation, sterilization is considered to ensure a closed and sterile system that prevents the formation of undesired products. Therefore, efforts should be made to sterilize all process equipment and inputs (feedstock, reactors, chemicals, surroundings, etc.) (Agalloco et al. 2004). During hydrolysis and fermentation, significant losses in sugar and ethanol yields have been attributed to microbial contaminants such as lactic acid bacteria (LAB) (Serate et al. 2015). LAB depletes nutrients required for yeast growth and shifts the metabolism of sugars for the formation of lactic and acetic acids (Khatibi et al. 2014). Given that PS is not a heat-sensitive material, the most common method of sterilization is drying the PS before storage. This is a way of "long-term sterilisation," since most microbes cannot thrive in the absence of moisture. Other methods such as heat sterilization (autoclave); uses dry heat to penetrate the PS fibres (Robus et al. 2016).

2.5.2 Enzymatic Hydrolysis

Enzymatic hydrolysis involves the use of enzymes to catalyse the conversion of cellulose and hemicellulose into monomeric sugars (Suhag & Singh 2014). Davison et al., (2016) remarked that enzymatic hydrolysis of cellulose occurs in three stages: adsorption of cellulases to the cellulose surface, hydrolysis of cellulose, and desorption of cellulases. This biochemical process is very slow and is highly affected by the structure of the biomass substrate (Mittal & Decker 2013).

To date, the best available sources of commercial cellulases are those obtained from genetically altered strains of *Trichoderma reesei* (Rodrigues et al. 2012). The three types of cellulases secreted by production strains such as *T. reesei* and required for complete conversion of cellulose to fermentable sugars are endoglucanases (EG), cellobiohydrolases (CBH), and β-glucosidases (BGL). The synergistic action of these enzymes increases the hydrolysis efficiency of converting cellulose to monomeric sugars. EG spontaneously reduces the degree of polymerization by cleaving the internal bonds along the cellulose chain. New cello-oligosaccharides which are formed are further hydrolyzed by CBH to release either glucose or cellobiose. BGL then converts cellobiose to glucose monomers (Davison et al. 2016).

During enzymatic hydrolysis, the accumulation of liberated sugars can severely inhibit the enzymes and reduce their hydrolysis efficiency (Vertes et al. 2010). Cellobiose has been found to inhibit both EG and CBH, while BGL is inhibited by glucose. With the conversion of cellobiose to glucose being the major rate-limiting step in the hydrolysis of cellulose, BGL is usually added in excess to enzyme cocktails to prevent the inhibition of cellulases during the biomass conversion process (Van Dyk & Pletschke 2012).

2.5.3 Fermentation

In the fermentation process, microorganisms convert sugars released from carbohydrates by enzymatic hydrolysis, into ethanol. Three modes of fermentation exist namely:

Batch fermentation: It is a closed system where all the materials and nutrients necessary for the organisms' growth and product formation are added aseptically to a sterile medium at the beginning of the fermentation process (McNeil & Harvey 2008).

Fed-Batch fermentation: This process is characterized by the controlled addition of substrates, nutrients, or inducers to the bioreactor during the course of cultivation; this can be intermittent or continuous. This feeding approach allows for improved nutrient uptake, desired cell growth, minimizing unwanted by-products, and production of target metabolites (Cinar et al. 2003).

Continuous fermentation: It is an open system where fresh nutrients are continuously added to the system while products, spent medium and old cells are continuously withdrawn from the system. If both the chemical and physical process parameters are kept at constant values, then the system will eventually reach steady-state, which may have benefits for improved product yield (Wertz & Bédué 2013).

Generally, there are four popular configurations for bioethanol production, which can be applied in batch, fed-batch, or continuous processes, namely; Separate Hydrolysis and Fermentation (SHF), Simultaneous Saccharification and Fermentation (SSF), Simultaneous Saccharification and Co-Fermentation (SSCF), and Consolidated Bioprocessing (CBP), as illustrated in Figure 2-4.

Separate Hydrolysis and Fermentation (SHF): In the SHF process, enzymatic hydrolysis and fermentation are carried out in separate vessels, with every stage operated at optimum conditions (temperature, time, pH, etc.). Glucose released from the hydrolyzed substrate is converted to ethanol by microorganisms. There can be end-product inhibition during the hydrolysis step, as a result of the accumulation of sugars (glucose and cellobiose), which will limit cellulase activity (Harun et al. 2009). There is a high tendency for contamination of the sugar slurry since hydrolysis and fermentation are carried out in separate vessels.

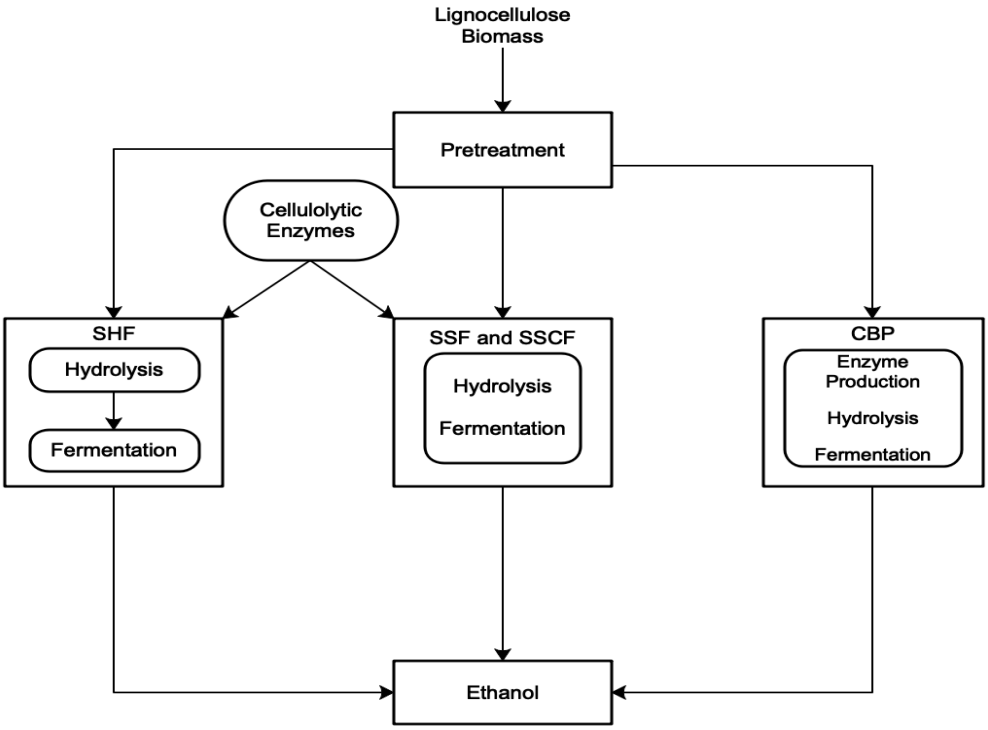


Figure 2-4: The four main process configurations for bioethanol production from lignocellulose biomass (Adapted from Waldron, 2010).

Simultaneous Saccharification and Fermentation (SSF): SSF is an important bioethanol production process strategy to eliminate the end-product inhibition of enzymatic hydrolysis by accumulated sugars (cellobiose and glucose) and thus, improving process performances. SSF incorporates both enzymatic hydrolysis and fermentation of sugars into a single reactor (Guan et al. 2015). In this process, the fermenting microorganism converts glucose hydrolyzed by the cellulases to ethanol thereby, reducing inhibitory effects from the broth, unlike SHF.

Lately, the general perception of SSF alleviating the inhibitory effect of sugars on hydrolysis has changed due to the addition of new auxiliary enzymes to modern cocktails such as the Cellic range of enzymes produced from the company Novozymes in Denmark. Cannella & Jørgensen, (2013) reported that the final ethanol yield for the SHF strategy was 20% higher than SSF when applying Cellic® CTec 2. Also, the typical temperature for SSF is approximately 37 °C and this represents a major drawback to enzymatic activities and fermentation as they occur at sub-optimal conditions (Wertz & Bédué 2013).

Simultaneous Saccharification and Co–Fermentation (SSCF): The SSCF process is an improved configuration of SSF because it utilizes mixed cultures or a single recombinant microorganism that ferments all the sugars released during hydrolysis (as opposed to just glucose) to ethanol in a single reactor (Harun et al. 2009). This process serves as an alternative to minimize bioethanol production cost by increasing the ethanol yield (Wertz & Bédué 2013).

Nevertheless, few industrial strains have demonstrated the ability to efficiently utilize the glucose and xylose sugars that are predominant in lignocellulosic hydrolysates. The commonly modified microorganisms (yeast and bacteria) for this function are *Saccharomyces cerevisiae* and *Zymomonas mobilis*. Genes capable of converting xylose have been successfully expressed in the aforementioned microorganisms (Faraco 2013; Waldron 2010).

Consolidated Bioprocessing (CBP): CBP is recognized as the largest cost reduction alternative compared to any of the bioethanol production configurations as it eliminates costs associated with dedicated cellulase production (Fan 2014). Consolidated bioprocessing uses one microbial strain or community that integrates cellulase production, biomass saccharification, and fermentation of released sugars into a single bioreactor (Abrego et al. 2017).

However, the major drawback is that an active CBP microorganism or consortia that exhibit all these features does not exist naturally and can only be achieved through genetic expression of cellulase genes into yeasts as illustrated in Table 2-2 (Fan 2014). *S. cerevisiae* has been identified as a suitable CBP organism due to its high ethanol yields and resistance to inhibitors compared to other microorganisms such as bacteria. *S. cerevisiae* has been proved to be easily genetically engineered and is generally regarded as safe due to its long association with the food industry (Hasunuma & Kondo 2012).

Also, another disadvantage to the practical feasibility of CBP is the contrast in optimal temperatures between enzymatic saccharification and fermentation. But the application of thermotolerant strains, hydrolysis and fermentation can even be achieved at elevated temperatures (Hasunuma & Kondo 2012).

Table 2-2: The comparison of potential CBP organisms (Adapted from Xu et al. 2009).

Characteristics	Naturally ethanologenic		Naturally cellulolytic	
	S. cerevisiae	***Z. Mobilis***	***C. thermocellum***	***T. reesei***
Cellulase genes	Some attempts to express heterologous genes for key cellulases have failed	Unknown	Naturally expresses cellulases	Naturally produces several cellulases
Cellulase production	Barely detectable activity for some enzymes from cloned genes	Unknown	Produces a few grams per liter	Produces more than 100 g/L
Ethanol production	Up to 160 g/L of ethanol	Up to 130 g/L of ethanol	Very slow rate and low yield	Very slow rate and low yield
Ethanol tolerance	Very high	High	Very low	Low
Multi – sugar usage in native strains	No	No	Do not utilize xylose	Yes
Resistance to inhibitors in biomass hydrolysates	High	High	Low	Very high
Amenability to genetic manipulation	Excellent	Good	Very poor	Good
Commercial acceptance	Very high	Acceptable	Unknown	Very high

2.5.4 Process Parameters in Paper Sludge Fermentation

To offset the cost-intensive distillation processes and maintaining economic feasibility in ethanol production, the minimum ethanol concentration in the product broth at the end of the fermentation run, should be 40 g/L. This achievement is mostly hampered by the expenditure on enzymes required to overcome the viscosity limitations during high solid fermentations (Robus et al. 2016). To achieve economic viability, the following modifications are applied to these process factors.

2.5.4.1 Temperature and pH

The typical optimal temperature and pH for hydrolysis are ~50 °C and pH 5.0-5.5 respectively, while that of fermentation are 28-37 °C and pH 6.0 depending on the microbe of choice (Hasunuma & Kondo 2012; Wertz & Bédué 2013). Given this, high-temperature fermentations (32-40 °C) can be achieved in SSF with thermotolerant strains. This is because, the enzymes have higher optimal temperatures (40-50 °C) than fermenting organisms, thus ensuring reductions in associated cooling costs for large-scale ethanol production processes (Abdel-banat & Hoshida 2010).

Maintaining the correct pH of the fermentation broth is essential for the enzymes and the yeasts. Owing to the presence of calcium carbonate in paper sludge, the pH during fermentation ranges between pH 4.8 and 5.5. This is a result of the buffering effect of the calcium carbonate and therefore, the pH levels for both saccharification and fermentation have to be optimized for the system since their optimum ranges from 5.0 to 5.5 (Boshoff et al. 2016; Robus et al. 2016).

2.5.4.2 Solids loading and agitation

To achieve a high final ethanol concentration that is vital for the economics of the fermentation-distillation process, higher substrate loadings (>8 % (w/w)) are required. However, the relation between increasing solid content and product yields is not linear (Modenbach & Nokes 2013). Xu et al. (2018) reported a 14% increase in final ethanol concentration when the substrate loading increased from 20% to 30% (w/w) although ethanol yield decreased by 13%.

As solids loading increases, one major potential problem that affects enzymatic hydrolysis is the reduction of the water content in the reactor. Water serves as the medium for mass transfer of products and also increases the lubricity of the slurry (Van Dyk & Pletschke 2012). During high-solids processes, the physical and chemical properties of the substrate determine the water retention capacity of the biomass. For example, the cellulose fibres in PS have a high WHC which causes viscous slurries in the fermenter (Boshoff et al. 2016). This leads to poor mixing of the broth, diminished enzyme adsorption to the substrate, and enzyme inhibition due to glucose build-up (Olofsson et al. 2008). Boshoff et al. (2016) observed an increase in viscosity for both corrugated recycle and virgin pulp PS when the solid loading was increased from 3% to 8 % (w/w) at all tested enzyme dosages (5, 15, and 25 FPU/gds). To improve mass transfer, high agitation rates (up to 1500 rpm) was employed. However, Boshoff (2015) stated that at 1500 rpm, a 15% reduction in the average activity of the enzyme Optiflow RC 2.0 was observed compared to a lower agitation rate (150 rpm). This observation was attributed to the mechanical damage of enzymes and fermenting organisms caused by shear stress.

The effect of lignin and ash enrichment during enzymatic hydrolysis can also fasten the slowdown of conversion during high-solids processes. Wallace et al. (2016) studied the recalcitrant sold accumulation effect on hydrolysis. It was reported that changes in substrate composition during the course of hydrolysis increased unproductive adsorption of enzymes to lignin, once the glucan content in the substrate was significantly reduced (Wallace et al. 2016). Although long reaction time did not improve the conversion, using a cost effective enzyme dosage and shorter reaction time could increase the hydrolytic potential of high-solid saccharification and fermentation (Modenbach & Nokes 2013).

2.5.4.3 Enzyme dosage

Previous studies on paper sludge fermentation have shown that ethanol yields can be improved as enzyme dosage increases. For instance, Kang et al. (2011) showed in a 120 h SSF of PS that, increasing enzyme dosage from 5 to 15 FPU/g-glucan results in a 25% increase in ethanol yields. Although adding more enzymes increases ethanol concentrations and yields, it is not practically feasible since their cost is a major drawback in attaining an economically viable process.

Enzymes could cost as high as 4 US$/kg cellulase (Gomes et al. 2018b) and as such, it is necessary to aim at minimizing enzyme dosage in any bioethanol process development. Optimization studies by Boshoff 2015 and Robus 2013 focusing on attaining the final ethanol concentration threshold (40 g/L), have been achieved at low enzyme dosages (11-15 FPU/gds) as summarized in Table 2-3. Even with such optimized enzyme loading, economic analyses still establishes enzyme cost as the overarching expense that would make the PS to ethanol production not viable in relation to commercial market ethanol (Robus 2013).

Table 2-3: Optimized enzyme dosages used in PS fermentation.

Paper sludge	Solids loading (% w/w)	Enzyme (FPU/mL)	Enzyme dosage (FPU/gds)	Ethanol concentration (g/L)	Reference
Sample 1[a]	20.79	Optiflow RC 2.0	15	47.38	
Sample 2[a]	21.75	145 FPU/mL	14.23	57.06	(Robus 2013)
VP-PS[b]	18.00	Optiflow RC 2.0	20	34.2	
CR-PS[c]	27.00	130 FPU/mL	11	45.5	(Boshoff 2015)

[a]: Recycle paper sludge from Nampak Tissue (Pty) Ltd.
[b]: Virgin pulp paper sludge from Sappi Ngodwana
[c]: Corrugated recycle paper sludge from Mpact Springs

2.5.4.4 Microorganisms used in bioethanol production: fermentation

The most well-known microorganisms used in bioethanol production from lignocellulose include *Escherichia coli*, *Z. mobilis*, *S. cerevisiae*, and *Pichia stipitis* (Faraco 2013). These microorganisms are essential for the conversion of hydrolysate produced after saccharification into ethanol. Table 2-4 summarizes the relevant characteristics of these microorganisms for bioethanol production.

S. cerevisiae is the widely used yeast for industrial ethanol production as it outperforms the other organisms due to its tolerance for high ethanol concentrations thus, making the process less susceptible to infection and inhibition (Azhar et al. 2017). However, *S. cerevisiae* can only ferment hexose sugars but not pentose sugars except for modified strains like *S. cerevisiae* strain D5A (Boshoff 2015; Robus 2013) and *S. cerevisiae* CelluX^{TM}4 (Basuyaux 2017).

A study on consolidated bioprocessing of lignocellulose to ethanol by Hasunuma & Kondo (2012) suggested that thermotolerant and ethanologenic CBP yeast strains would enhance the production of high-titre ethanol by facilitating enzyme production and saccharification, while consuming multiple sugars during high-temperature fermentations. However, such strains do not exist naturally, and genetic modifications have been limited to a few species.

Table 2-4: Features of most relevant microorganisms considered for ethanol production (Adapted from Gírio et al. 2010).

Characteristics	Microorganisms			
	E. coli	***Z. mobilis***	***S. cerevisiae***	***P. stipitis***
Glucose fermentation	+	+	+	+
Other hexose utilization	+	–	+	+
Pentose utilization	+	–	*	+
Anaerobic fermentation	+	+	+	–
Mixed – product formation	+	w	W	w
High ethanol productivity from glucose	–	+	+	w
Ethanol tolerance	w	w	+	w
Inhibitor tolerance	w	w	+	w
Osmotolerance	–	–	+	w
Acidic pH range	–	–	+	w

+: Positive; -: Negative; w: Weak; *: Modified strains of *S. cerevisiae* that utilizes C5 sugars

2.5.4.5 Product inhibition

The progression of enzymatic hydrolysis and bioethanol production is often affected by several obstacles. One of them is the feedback inhibition of lignocellulosic products. Although high product concentrations can improve the ultimate yield, they can severely affect the performance of enzymes and yeast cells, limiting subsequent production processes (Zhang et al. 2015). Inhibitors such as furans and phenolic derivatives are formed during the pretreatment step of biomass which uses physical, chemical, and biological methods to open up the lignocellulose structure to enable better hydrolysis. More specifically to paper sludge that requires no pretreatment due to the rigorous pulping process, end-product inhibition may arise from the biomass as short-chain organic acids, sugars, ethanol, etc. Below is a summary of several product inhibitors and their effect on the efficiency of microorganisms and enzymes:

Sugars (Cellobiose, Xylose, Glucose): Enzymatic hydrolysis of cellulose, due to its heterogeneous nature, is a multi-step reaction initiated by the adsorption of cellulases unto the substrate and the identification of a susceptible bond on substrates surface by CBH and EG (Bansal et al. 2009). The enzyme-substrate complex formation is established to initiate hydrolysis of glycosidic bonds to form cellobiose, which is converted to glucose by BGL (Levine et al. 2010). However, cellobiose, pentose sugars, and glucose can be inhibitory to cellulases during hydrolysis.

A recent paper determined for the first time the real-time effect of cellobiose on the adsorption of exo and endoglucanases on cellulose (Thielemans & He 2018). It was reported that a cellobiose concentration of 3 g/L restricted the adsorption of CBH1, indicating the inhibition of enzyme-cellulose complexes. CBH2 on the other hand, effectively hydrolysed cellulose until cellobiose concentrations reached 12 g/L (Thielemans & He 2018). A similar trend was reported by Du et al. (2010), who observed a strong inhibition of CBH1's activity by cellobiose while CBH2 displayed less inhibition by the intermediate product. However, Puri et al. (2013) reported that cellobiose accumulation and feedback inhibition can be avoided by increasing the BGL loading or using enzyme cocktails containing high BGL activity.

Similar to the case of cellobiose, increasing concentrations of glucose decreases hydrolysis efficiency but to a much lesser extent (Silva et al. 2020). Thielemans & He (2018) concluded that glucose was not a strong inhibitor of CBH activity as concentrations up to 12 g/L did not severely affect hydrolysis efficiency. However, the inhibitory effect by glucose is intensified in a high-solids process due to the reduction of enzymatic activity. The exogenous addition of glucose (up to 110 g/L) to the hydrolysis of empty fruit bunches (20% w/w, 40 FPU/g glucan CTec 3) resulted in a 40% decrease in glucose yield (Kim et al. 2019). The inhibition by glucose can therefore be controlled by incorporating an SSF setup that combines enzymatic hydrolysis of biomass and fermentation of sugars into a single reactor (Olofsson et al. 2008).

Also, intermediates of hemicellulose hydrolysis such as xylan, xylose, and xylooligomers have been identified to be strong inhibitors of enzymatic hydrolysis (Kumar & Wyman 2009). Qing et al. (2010) reported that the presence of 12 g/L of xylose reduced the initial hydrolysis rate of Avicel by 38%, while the final hydrolysis yield decreased by 20%. Although glucose and cellobiose are more inhibitory to the initial hydrolysis rate, xylooligomers were found to have a stronger long-term effect on enzymatic hydrolysis (Qing et al. 2010). These results support the importance of improving xylanase activity in enzyme formulations to boost the hydrolysis of xylooligomers into less inhibitory derivatives (Kumar & Wyman 2009).

Ethanol: One of the general requirements to reduce distillation costs and improve the economic benefit of ethanol fermentation is to maintain high ethanol concentrations (Vertes et al. 2010). But, high concentrations of ethanol present in high-solid fermentations strongly impede yeast performance, restricting cellulosic ethanol production (Zhang et al. 2015). In a previous study by Chen & Jin (2006), it was reported that ethanol concentrations between 1%-7% (v/v) also inhibited enzymatic hydrolysis efficiency. Zhang et al. (2015) reported ethanol to be the primary product that inhibits yeast growth and ethanol productivity in high-solid fermentation processes. They observed that at 70 g/L of ethanol, cell growth and fermentation activity was completely restricted.

Alternatively, acclimatization significantly improves the yeast density, cell morphology, and fermentation performance during high-solid fermentation. In response to ethanol-induced stress, yeast cells change their membrane composition to stabilize the plasma membrane against fluidization and oxidative damage to the inner cell structure (Ding et al. 2009). Zhang et al. (2015) reported that ethanol yields from *S. cerevisiae* improved by approximately 25% after five cycles of acclimatization.

Short-chain organic acids: The inhibitive effect of short-chain organic acids such as acetic and lactic acids has been of interest to ethanol manufacturers since both acids can synergistically inhibit yeast growth (Narendranath et al. 2001). Acetic acid mostly originates from the degeneration of acetyl-groups present in extractives, lignin, and hemicellulose of lignocellulose biomass (Sjulander & Kikas 2020). Acetic acid is also a minor by-product of fermentation by *S. cerevisiae*, with inhibitory concentrations produced by contaminating lactic acid bacteria and/or acetic acid bacteria (Narendranath et al. 2001). Lactic acid, on the other hand, is a primary metabolite of lactic acid bacteria: a non-motile and non-sporeforming homofermentative strain that is resistant to low pH, low oxygen concentrations, and high ethanol concentrations, which reduces the pH in the fermenter to suppress yeast performance (Brexó & Sant'Ana 2017). The pH change in the medium resulting from the accumulation of these organic acids causes stress and affects the viability of the cells. This change to the natural homeostasis of microorganisms causes a shift in their metabolism, as energy used for growth would be redirected to maintain their neutral intracellular matrix (Mira et al. 2010).

In addition, increasing concentrations of acetic and lactic acid inhibit nutrient uptake, glucose consumption, and ethanol production of *S. cerevisiae* (Sjulander & Kikas 2020). Early experiments by Narendranath et al. (2001) noted an exponential reduction in the growth rate of *S. cerevisiae* as a result of the strong synergistic inhibition by acetic and lactic acids. Although it was reported that the minimum inhibitory concentrations for yeast growth were 6 g/L and 25 g/L for acetic and lactic acid respectively, concentrations as low as 0.5 g/L and 2 g/L for acetic and lactic acid respectively severely reduced glucose consumption rates (Narendranath et al. 2001).

2.6 Enzyme Recycling in Bioethanol Production

The complex nature of lignocellulose substrates requires high dosages of enzymes to release sugars for fermentation. This remains one of the major process bottlenecks to the commercialization of this bioconversion technology. According to Johnson (2016), the cost of enzymes on ethanol production can represent up to 35% of the annual ethanol cash costs.

Enzyme recycling is one major process alternative aimed at minimizing the dosage of enzymes required for efficient hydrolysis (Østergaard et al. 2015). Recycling enzymes makes use of the enzymes for multiple stages of hydrolysis, thus decreasing the enzyme production cost (Jørgensen & Pinelo 2017). Gomes et al. (2016) previously reported that for an annual ethanol production of 30 million gallons, enzyme recycling could allow about $8 million saving on enzyme cost by decreasing enzyme supplementation to 30%.

2.6.1 Adsorption and Desorption of Enzymes during Biomass Hydrolysis

During the hydrolysis process, the adsorption of cellulases onto the cellulose fibres is an essential step in the initiation of hydrolysis. Most cellulases (EG and CBH) have a carbohydrate binding module (CBM), which ensures that the catalytic module of the enzyme is precisely positioned on the substrate (cellulose) (Liu et al. 2009). The presence of CBMs is, therefore, essential for attaining a fast hydrolysis equilibrium (Jørgensen et al. 2007).

The adsorption of cellulase to cellulose is considered reversible because most cellulases are released into the liquid phase once complete hydrolysis of cellulose is achieved (Gomes et al. 2015). However, it is reported that after hydrolysis 40%-50% of the cellulases (EG and CBH) remain adsorbed on the lignin-rich residue (Jørgensen & Pinelo 2017). This “unproductive” binding of cellulases and other enzymes (BGL) unto lignin is one of the challenges to effective enzyme recycling. Paper sludge samples are typically low in lignin, usually ranging from 5 to 20% (Table 2-1). However, in a study by Gomes et al. (2016), it was revealed that approximately 30% of the cellulase activity from Celluclast was lost from the liquid phase and was adsorbed onto the final solids (unconverted carbohydrates and lignin) during the hydrolysis and fermentation of PS.

2.6.2 Routes Considered for Enzyme Recovery and Re-use

Considering a generic biorefinery from biomass pretreatment, hydrolysis, fermentation, and finally to distillation, there are three typical points in the process where enzymes for recycling could be obtained. These routes include the hydrolysate (for SHF processes only) (1), the fermentation broth (2), and the stillage from the distillation column (3) (Figure 2-5).

The routes listed above provide complementary schemes for recovery and re-use of enzymes with/without alternative addition of enzymes (Gurram & Menkhaus 2014). To decide which process stream to consider for efficient recycling of the enzymes, they must fulfil these requirements:

1. The streams should not contain compounds that would inhibit enzymes and the fermenting microorganisms
2. The enzymes should not bind irreversibly to the biomass
3. The enzymes should be stable to chemical and physical stresses for extended periods

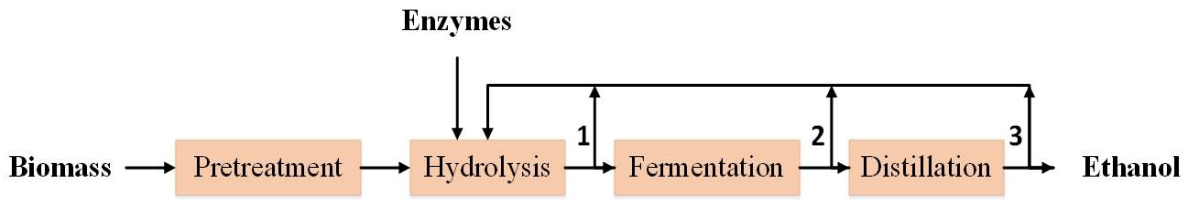


Figure 2-5: Potential routes for recycling enzyme activity in a generic bioethanol process (Adapted from Lindedam et al. (2013)).

The advantages and disadvantages of the routes listed above are:

1. Recycling enzymes from the hydrolysate has the benefit of recycling relatively fresh enzymes, while avoiding the build-up of unhydrolyzed solids. With this route, it is possible to remove water and minor inhibitors such as sugars (Du et al. 2010), while concentrating the enzymes through an ultrafiltration step (Gomes et al. 2018a). However, ultrafiltration reportedly suffers from fouling as a result of the enzymes and/or impurities in the liquid (Jørgensen & Pinelo 2017).

Two factors that affect the feasibility of this route is the degree of enzymatic hydrolysis and the lignin content of the biomass (Jørgensen & Pinelo 2017). At industrial process conditions (~25% dry matter content), the amount of enzymes that might be adsorbed to unhydrolyzed solids (lignin) can be substantial and might require a desorption step, since not all enzymes would be present in the liquid phase (Thoresen et al. 2020).

2. Recycling the whole broth slurry after fermentation (reusing enzymes from both liquid and solid phases) is known to be the simplest method as no separation method is required. This alternative can be used at industrial conditions since the recirculation of slurry increases the final ethanol concentration in the broth and reduces energy consumption during distillation (Lindedam et al. 2013). Also, product inhibition by glucose and cellobiose is nullified by fermenting sugars to ethanol. Nonetheless, Chen & Jin (2006) reported that ethanol concentrations between 1%-7% (v/v) in the process stream slightly inhibited enzymatic hydrolysis efficiency.

   Østergaard et al. (2015) recycled 20% of the fermentation broth at a demonstration-scale (~20% DM of wheat straw) and reported a 5% reduction in enzyme dosage. However, there was a 30% decrease in cellulose conversion, which was attributed to the final ethanol concentration, unfermented xylose, and the accumulation of lignin.

3. Enzymes in the stillage can be recycled if distillation is carried out under vacuum or during low-temperature stripping (below 60 °C) to avoid denaturing the enzymes. There is no introduction of inhibitors, as the stillage is a lean stream with no sugars or ethanol. However, the activities of the enzymes in the stillage will be severely reduced, since it would have been subjected to extended processing time and exposed to stress from pumps and passages in the distillation column (Jørgensen & Pinelo 2017). Lindedam et al. (2013) reported that only 25% of the initial enzyme activity was recovered after vacuum distillation.

2.6.3 Factors affecting Enzyme Stability during Hydrolysis of Biomass

The recalcitrant nature of lignocellulose materials particularly as a result of lignin, restrains hydrolysis efficiency, due to its high affinity to bond with cellulases (Van Dyk & Pletschke 2012). Therefore, most of the recent commercial enzymes used in the conversion of biomass polysaccharides into monomeric sugars consist of a cocktail of several enzymes (cellulases, hemicellulases, and auxiliary/accessory enzymes) that boost hydrolysis efficiency (Modenbach & Nokes 2013).

For enzymes to be successfully re-used, the enzyme mixture must maintain its characteristics and performance over the entire process time. Below are some of the well-known factors that can affect enzyme stability and the potential of recycling enzymes:

Temperature: Considering that optimum enzymatic hydrolysis occurs around 50 °C, enzymes with outstanding thermostability signify a great potential for enzyme re-utilization. Although, according to a study by Gomes et al. (2018a), Celluclast, Accellerase, and Cellic® CTec 2 recorded an increasing loss of hydrolysis efficiency during 72 h incubation of recycled paper sludge at 45, 50, and 55 °C. At 55 °C, the conversion was reduced to 59%, 74%, and 80% for Celluclast, Accellerase, and Cellic® CTec 2, respectively. Therefore, the thermal effect on enzyme stability and performance were observed to worsen at elevated temperatures (>50 °C).

Substrate-enzyme interaction: Enzymes degrade cellulose by binding to the cellulose chain surface. However, the major drawback lies with the binding of cellulases onto lignin, due to electrostatic and hydrogen bonding involved in the enzyme-lignin interaction (Haven & Jørgensen 2013). Alkaline elution and addition of surfactants have proven to increase desorption of enzymes from lignin but these procedures affect other sections of the process (Rodrigues et al. 2012). For example, the addition of surfactants improves cellulose conversion although such desorption agents can be inhibitory to enzymes and can dilute other process streams (Jørgensen & Pinelo 2017).

2.6.4 Enzyme Recovery

During the hydrolysis of lignocellulosic biomass, the mechanism of cellulase enzymes in the system exist in equilibrium such that, enzymes may either be free in solution (free enzymes) or remain bound to unhydrolyzed solids including lignin and unconverted carbohydrates (bound enzymes) (Pribowo et al. 2012; Tu et al. 2009). It has been observed that about 60% of the enzymes can remain in solution after the adsorption equilibrium is attained (Hu et al. 2018), whereas the remaining enzymes are bound to the residual substrate (cellulose and lignin) (Jørgensen & Pinelo 2017). Therefore, an efficient enzyme recycling system would require the recovery of both free and residual enzymes.

For instance, Gomes et al. (2016) demonstrated that free enzymes can be recovered by ultrafiltration and readsorption to fresh substrate, while other studies have applied specific surfactants and alkaline solutions to recover solid bound enzymes (Baral et al. 2020; Rodrigues et al. 2012). Below is a summary of some of the relevant enzyme recovery technologies applied to biofuel production.

Ultrafiltration: This is an efficient method used in cellulase recovery as well as continuously separating products that might inhibit the hydrolysis process. Cristina et al (2014) indicated that ultrafiltration allowed up to 80% recovery of soluble enzymes after fermentation of wheat straw. However, Gomes et al. (2016) and Rodrigues et al. (2015) reported about 29% activity loss during the ultrafiltration process.

Readsorption to fresh substrate: Free enzymes in solution may be recycled by readsorption of enzymes unto new biomass (Emert & Paul 1980). Hu et al. (2018) reported a 50% reduction in enzyme dosage when free/unadsorbed enzymes in solution were recycled, while maintaining cellulose hydrolysis yields greater than 70%. The major drawback of this system is that when the enzymes used have a high affinity for residual solids like lignin. A separate desorption step would sometimes be required before the enzymes can be reused (Jørgensen & Pinelo 2017).

Alkaline elution or pH adjustment: This is a popular technique used to desorb adsorbed cellulases from lignin. As described by Rodrigues et al. (2012), the process involves a mild change in pH from pH 4 to 9 or 10 using a sodium acetate buffer. It was reported that conformational changes to enzymes during the pH shift was completely reversible and did not result in any loss of activity at temperatures below 40 °C. However, only 37.5% of the initial activity remained when the pH was done at 50 °C. Hence, one should be mindful of the temperature effect if pH adjustment is to be considered during enzyme recycling (Rodrigues et al. 2012).

Addition of Surfactants: The addition of surfactants (Tween 20 and Tween 80) and surfactant precursors (Polyethylene glycol (PEG)) has been shown to improve enzymatic performance, by reducing unproductive binding of cellulases to unhydrolyzed biomass (lignin) and increasing the amount of enzymes free in solution (Baral et al. 2020; Jørgensen et al. 2007; Jørgensen & Pinelo 2017; Tu et al. 2009). These surfactants have a high affinity for lignin and would preferentially be adsorbed and form a coating on the lignin surface. This mechanism of preferential adsorption of surfactants to lignin improves enzyme stability by minimizing the adsorption, inactivation, and potential precipitation of enzymes. The addition of PEG 6000 to the hydrolysis of acid pretreated sugarcane bagasse using Cellic® CTec 2 did not only result in approximately 75% cellulose hydrolysis efficiency but also facilitated the desorption of about 55% of the enzymes from the lignin-rich biomass (Baral et al. 2020).

2.7 Enzyme Recycling in Paper Sludge Fermentations

The only enzyme recycling studies on paper sludge was by Gomes et al. (2016, 2018a), who focused on enzyme recycling from the solid and liquid phases by incorporating ultrafiltration and alkaline elution into the SHF setup. Although this strategy allowed for 53%–60 % enzyme savings, the additional separation processes employed were undesirable and adversely affected process economics (Gomes et al. 2018b).

In this study, Celluclast enzyme cocktail was more thermostable than Accellerase and Cellic® CTec 2. Its activity was not noticeably affected in the temperature ranges of 30-45 °C, although approximately 50% of the enzyme activity was lost at 50 °C (Gomes et al. 2016).

To determine the strategy to recover the enzymes, the distribution of cellulase activity during hydrolysis was monitored. At 35 °C (to avoid enzyme degradation), most of the final enzyme activity was present in the liquid fraction; although a significant amount (30%) of the enzymes remained adsorbed to the solid residues. By using the alkaline elution procedure as described by Rodrigues et al., (2012), close to 82% of the solid-bound enzymes were recovered without a substantial loss of activity.

To test the reusability of recovered enzymes, four sequential rounds of 72 h of hydrolysis and fermentation (SHF) were undertaken with only 30% fresh enzyme supplemented at each stage. Although glucan conversion gradually decreased from 94% in the first stage to 71% in the last stage (Table 2-5), close to 71% of the enzyme activity was preserved over the entire process. Although the ultrafiltration step resulted in about 29% loss in enzyme activity, this scheme of enzyme recycling resulted in a 53% reduction in the overall enzyme consumption for 8.8% w/w ethanol. This could translate to tremendous economic gains of about $8 million for a production rate of 30 million gallons per year (Gomes et al. 2016).

Table 2-5: Sequential rounds of PS fermentation (SHF) with enzyme recycling (20 FPU/g cellulose; 30% fresh enzymes) (Adapted from Gomes et al. (2016)).

Round	Ethanol (g/L)	Glucan conversion (%)	Glucans in residues (%)
1	7.70 ± 0.02	94.27 ± 0.16	1.78 ± 0.05
2 (Recycle 1)	7.59 ± 0.06	91.95 ± 0.80	2.50 ± 0.25
3 (Recycle 2)	6.63 ± 0.03	83.01 ± 0.30	5.27 ± 0.09
4 (Recycle 3)	5.65 ± 0.08	70.69 ± 0.91	9.08 ± 0.28

2.8 Kinetic Modelling of the Enzymatic Hydrolysis and Fermentation of Lignocellulosic Biomass

This section summarizes the key aspects, applications, and shortcomings of various models of enzymatic hydrolysis and fermentation. Kinetic modelling is an important tool for rapidly investigating the inherent biomass conversion mechanisms to understand the underlying kinetics, improve process design and optimization, large-scale production, and economic assessment (Wang et al. 2011). To fully identify the various bottlenecks that reduce the conversion rates of lignocellulose materials to ethanol, mathematical modelling should be applied to analyse the sequence of experimental results.

2.8.1 Enzymatic Hydrolysis Kinetic Modelling

The wildly proposed mechanism of the enzymatic hydrolysis of cellulose is complex and involves several steps. The process is initiated by the adsorption of cellulases unto the surface of the substrate by its carbohydrate binding module. The formation of enzyme-substrate complex leads to hydrolysis of glycosidic bonds that yields cellobiose. Lastly, there is desorption of enzymes from the substrate's surface and transfer of products (cellobiose and glucose) to the aqueous phase. (Bansal et al. 2009).

Due to the heterogeneity of cellulose hydrolysis, mechanistic models that simulate the hydrolysis time course can be categorized as either enzyme-based or substrate-based depending on the functional approach and methodology used. The enzyme-based kinetic models focus on elucidating the rate expressions of the enzyme-cellulose reaction whiles assuming the insoluble substrate as inert. Substrate-based kinetic models explicitly account for the continuous transformation of the insoluble cellulose substrate to identify rate-limiting properties during hydrolysis (Jeoh et al. 2017).

1. Enzyme-based kinetic models

Enzyme-based kinetic models focus on providing insight into the intermediate enzyme-cellulose interaction steps at the molecular level, mainly:adsorption/desorption, complexation/decomplexation, and hydrolysis (Bansal et al. 2009; Jeoh et al. 2017). For example, Maurer et al. (2013) used quartz crystal microbalance (QRM) to elaborate the kinetic behaviour of aqueous enzymes on a thin film of

insoluble cellulose. The kinetic rates for the enzyme mixture included competitive adsorption, irreversible binding, complexation, and cooperative enzyme activity, and were modelled as a first order with respect to the enzyme species considered: CBH1&2 (Table 2-6). Experimental data of this model shows that complexation is the rate-limiting step of CBH1's activity whiles CBH2 is limited by adsorption to the cellulose surface. Similarly, Shang et al. (2013) developed a spatially resolved kinetic model that incorporates the non-classical behaviour in enzyme kinetics to illuminate the effect of surface restriction or interfacial confinement on CBH activity (Table 2-6). It was reported that the primary bottlenecks to crystalline cellulose decomposition are over-crowding of enzymes at high surface densities and the slow complexation of CBH unto cellulose, while the catalytic rate constant did not affect the overall conversion rates (Jeoh et al. 2017). To accelerate the rate of cellulose hydrolysis, Shang et al. (2013) suggested the optimization of the complexation kinetics to overcome the enzyme interfacial confinement.

Alternatively, Praestgaard et al. (2011) expanded hydrolysis in multiple processive steps. The model bases on the burst-phase kinetic formalism to explain the initial burst in cellulose hydrolysis rate, usually followed by a rapid rate decline. More specifically, the model does not consider adsorption/desorption as limiting steps but includes the complexation of enzymes to the cellulose chain. In contrast to Maurer et al. (2013), Praestgaard et al. (2011) included an inactivation step due to irreversibly complexed enzymes. It was concluded that the decline in hydrolysis rate was due to the slow decomplexation rate (K_3, Table 2-6) and steric obstacles on the cellulose surface. Cruys-Bagger et al. (2016) also specifically elucidated if complexation or decomplexation may be the rate limiting step for the overall hydrolysis of crystalline cellulose. The model uses a complex processive mechanism that assumed the enzyme-cellulose interaction leads to a complex "threaded" enzyme: associated non-threaded and threaded enzyme states. Cruys-Bagger et al. (2016) analysed the complexation of associated enzymes (ES_{assoc}, Table 2-6) and concluded that decomplexation was the main determinant of enzymatic hydrolysis as compared to complexation and adsorption/desorption rates.

Table 2-6: Mechanisms of some enzyme-based kinetic models (Adapted from Jeoh et al. (2017))

Reference	Mechanism	Variables	Rate-limiting step
Maurer et al. (2013)	Reversible adsorption/desorption $CBH \underset{K_{D,CBH}}{\overset{K_{A,CBH}}{\leftrightarrow}} r_{CBH}$ Irreversible binding $r_{CBH} \xrightarrow{K_{I,CBH}} r_{CBH,I}$ Complexation of reversibly bound CBH $r_{CBH} \underset{K_{-1,CBH}}{\overset{K_{1,CBH}}{\leftrightarrow}} r_{CBH-s*}$ Complexation of irreversibly bound CBH $r_{CBH-1} \underset{K_{-1,CBH}}{\overset{K_{1,CBH}}{\leftrightarrow}} r_{CBH-s*,I}$	CBH: Free enzyme (ppm) r_{CBH}: Reversibly bound CBH (mg/m^2) r_{CBH-s*}: Reversibly bound, complexed CBH (mg/m^2) $r_{CBH,I}$: Irreversibly bound CBH (mg/m^2) $r_{CBH-s*,I}$: Irreversibly bound, complexed CBH (mg/m^2) $K_{A,CBH}$: Reversible adsorption ($ppm^{-1}h^{-1}$) $K_{D,CBH}$: Reversible desorption (h^{-1}) $K_{I,CBH}$: Complexation ($m^2mg^{-1}h^{-1}$) $K_{-1,CBH}$: Decomplexation (h^{-1})	Complexation
Shang et al. (2013)	Adsorption/desorption $E + S \underset{K_d}{\overset{K_a}{\leftrightarrow}} E_{uc}$ Surface diffusion $E_{uc} \overset{K_{diff}}{\leftrightarrow} E_{uc} + RE$ Complexation/decomplexation $E_{uc} + RE \underset{K_{dc}}{\overset{K_c}{\leftrightarrow}} E_{active}$ Hydrolysis $E_{active} \xrightarrow{K_h} P + E_{active}$ Unjamming by decomplexation $E_{blocked} \xrightarrow{K_{dc}} E_{uc}$	E: Free enzyme S: Surface cellobiose residue E_{uc}: Surface adsorbed enzyme RE: Cellulose reducing ends E_{active}: Active enzyme P: Soluble product $E_{blocked}$: Jammed enzyme K_a: Adsorption (s^{-1}) K_d: Desorption (s^{-1}) K_{diff}: Diffusion (s^{-1}) K_c: Complexation (s^{-1}) K_{dc}: Decomplexation (s^{-1}) K_h: Hydrolysis (s^{-1})	Complexation (with an optional decomplexation rate)

Praestgaard et al. (2011)	Complexation/decomplexation $E + C_n \underset{K_{-1}}{\overset{K_1}{\leftrightarrow}} EC_n$ Processive hydrolysis $EC_n \xrightarrow{K_2} EC_{n-i} + C \ldots \xrightarrow{K_2} EC_z$ Dissociation $EC_{n-i} + C \xrightarrow{K_3} E + C_{n-i}$ Inactivation $EC_{n-i} + C \xrightarrow{K_4} IC_{n-i}$	E: Enzyme (μM) C_n: Cellulose chain of length n (μM) EC_z: Enzyme-substrate complex (μM) n – i: Cellulose chain position i: counting index (from 0 to n-x) K_1: Complexation ($s^{-1}\mu M^{-1}$) K_{-1}: Decomplexation (s^{-1}) K_2: Hydrolysis (s^{-1}) K_3: Dissociation (s^{-1}) K_4: Inactivation (s^{-1})	Decomplexation
Cruys-Bagger et al. (2016)	Association of enzyme $E + S_\alpha \underset{K_{-a}}{\overset{K_a}{\leftrightarrow}} ES_{assoc}$ Threading of cellulose into active site $ES_{assoc} + S_\beta \underset{K_{-b}}{\overset{K_b}{\leftrightarrow}} ES_{thread} + S_\alpha$	E: Enzyme (μM) S_α: Unoccupied association site (μM) ES_{assoc}: Associated enzyme (μM) S_β: Unoccupied threading site (μM) ES_{thread}: Threaded, active enzyme (μM) K_a: Association ($\mu M^{-1}s^{-1}$) K_{-a}: Dissociation ($\mu M^{-1}s^{-1}$) K_b: Threading ($\mu M^{-1}s^{-1}$) K_{-b}: Unthreading ($\mu M^{-1}s^{-1}$)	Decomplexation

Enzyme-based models are solely focused on rate-limiting enzyme-cellulose interaction steps without considering substrate properties and its depletion during hydrolysis. As a result, enzyme-centric models mostly capture the initial hydrolysis course. In order to model a full-time hydrolysis course, substrate properties must be incorporated alongside the intrinsic enzyme properties to account for the overall rate of cellulose hydrolysis (Bansal et al. 2009; Jeoh et al. 2017).

2. Substrate-based kinetic models

The mechanism of enzymatic hydrolysis is hypothesized to be affected by the action of complex structural properties of heterogeneous insoluble substrates on enzymes (Bansal et al. 2009). Substrate-based kinetic models express the rate determining features of surface and structural heterogeneity of cellulose (geometry, dynamic substrate transformation, etc.) that impede enzyme-substrate interactions and reduce hydrolysis yields (Jeoh et al. 2017). For example, Seo et al. (2012) developed a simple cellulose conversion model to examine the role of substrate area (substrate derived factor) and adsorbed cellulase (enzyme derived factor) on the hydrolysis kinetics. It was shown that the retardation of hydrolysis was dependent on the initial available surface area (initial radius: R_0, Table 2-7), and that increasing the initial surface area improves hydrolysis yields. Similarly, Levine et al. (2010) modelled the role of surface area changes on hydrolysis rates. The model used a mechanistic description that captures enzyme adsorption, distinct complexation steps, and the dynamic interaction between enzymes and cellulose during hydrolysis. However, it was reported that surface area was only a rate limiting factor in the early stages of hydrolysis. Thus, the slowdown that follows the initial hydrolysis burst might be a result of product inhibition and/or thermal deactivation of enzymes. In a similar modelling framework to Levine et al. (2010), Huron et al. (2016) modelled the impact of substrate morphology and its evolution with time on hydrolysis It was reported that an additional first-order deactivation term was included to capture the slowdown phase during extended hydrolysis times. Therefore, the addition of such “compensation terms” indicate that enzyme and substrate related properties only are unlikely to successfully model the cellulose hydrolysis profile.

Table 2-7: Summary of some substrate-based kinetic models (Adapted from Jeoh et al. (2017))

	Seo et al. (2012)	Levine et al. (2010)	Huron et al. (2016)
Enzyme-substrate interaction step	Not explicitly modeled; enzyme-substrate interaction steps are not considered to be rate limiting	-Adsorption/desorption -Complexation/decomplexation -Hydrolysis	Not explicitly modeled; enzyme-substrate interaction steps are not considered to be rate limiting
Substrate geometry	Cylinders	Mono and polydisperse spheres	Monodisperse cylinders
Insoluble substrate depletion	Rate of conversion as a function of initial radius, adsorbed enzyme, exponential decay and product inhibition effects: $\frac{d\alpha}{dt} = \frac{k_1}{R_0^2}\frac{Q}{S_0}\left(\exp\left(\frac{-k_2}{q_m}\frac{\alpha}{1-\alpha}\right) + k_3\right)$	Rate of change of cellulose particle radius: $\frac{dR}{dt} = R\frac{SA}{3} MW_1 \sum_{i=1} 6ir_i^1$	Rate of surface area change as a function of glucose and cellobiose production rates: $\frac{dS}{dt} = \frac{S}{S_{ext}} 2\Pi N_p L \frac{dR}{dt}$ $= \frac{-S}{S_{ext}} \frac{V_{water}}{R}\left(\frac{d[G_1]}{dt} V_{G_1} + \frac{d[G_2]}{dt} V_{G_2}\right)$
Variables	α: Cellulose conversion ratio t: Time (s) k_1: Reaction rate constant (m^2/s) R_0: Initial cellulose fibril radius (m) Q: Total adsorbed cellulase (kg) S_0: Initial weight of cellulose (kg) k_2: Rate constant q_m: Maximum adsorbed cellulase in monolayer (kg/kg cellulose) k_3: Rate constant	R: Cellulose particle radius (dm) t: Time (s) SA: Cellulose surface area (dm^2/g) MW_1: Cellulose monomer molecular weight (g/mmol) i: Cellulose chain index r_i^1: Rate of formation of a length i soluble sugar ($\frac{mmol}{dm^2.s}$)	S: Cellulose particle surface (m^2) t: Time (s) S_{ext}: External cellulose surface accessible to enzymes (m^2) N_p: Number of cellulose particles L: Cellulose particle length R: Cellulose particle radius (m) V_{water}: Volume of water (m^3) $[G_1]$: Concentration of glucose (mol/m^3) V_{G_1}: Volume of one mole of glucose (m^3/mol) $[G_2]$: Concentration of cellobiose (mol/m^3) V_{G_2}: Volume of one mole of cellobiose (m^3/mol)
Initial rate limiting cellulose property	Initial surface area	Cellulase accessible surface area	Initially accessible cellulose surface

On a different approach, empirical models have been widely used to correlate hydrolysis with time without resorting to complex equations whiles providing other pertinent information (Sarkar & Etters 2004). Although empirical models do not provide any mechanistic details of the enzyme-substrate interaction, they can provide insight about other properties that affect cellulose hydrolysis rates (Bansal et al. 2009). For example, mass transfer effects: diffusion of enzymes into the insoluble substrate, diffusion of products from the substrate into the aqueous phase and/or the presence of a diffusional boundary layer, are crucial in the hydrolysis kinetics (Wang et al. 2011).

Several researchers have used the Avrami-Kolmogorov-Erofeev (AKE) equation shown below to accurately study the diffusional effect of enzyme-cellulose systems that were not incorporated in other models (Etters 1980; Ioelovich 2015; Knezevic et al. 1998; Sarkar & Etters 2004).

$$\ln (1 - \alpha) = - Kt^n$$

Where α refers to the degree of cellulose conversion; K is the effective rate constant; t is time (h) and n is the effective order of the process that indicates the adsorption mechanism.

The physical meaning of the Avrami parameters (k and n) provide a phenomenological description of where adsorption kinetics occur without providing any detailed molecular information (Etters 1980; Knezevic et al. 1998). Sarkar and Etters (2004) used the empirical model to describe the hydrolysis of cotton fibres. It was reported that for n values greater than 1, it suggests that the process is limited by surface properties (i.e. enzyme transfer to the substrate is not a rate limiting step). If the value of n ranges between 0.5 and 1, it indicates that the hydrolysis reaction is limited by the transfer of enzymes to the substrate (Ioelovich 2015).

2.8.2 Fermentation Kinetic Modelling

The fundamental framework for modelling lignocellulose fermentations comprises of rate equations for sugar uptake or product formation and microorganisms (Nosrati-Ghods et al. 2020). Sugar uptake rate for cell growth and maintenance, product synthesis, and competitive inhibition of sugars are represented by adsorption models such as Langmuir isotherm and Michaelis-Menten kinetics (Unrean 2016). The growth of microorganisms is modelled using the Monod model for microbial growth kinetics (Monod 1949).

Shao et al. (2007, 2009) developed a fermentation model by modifying the mechanistic rate equation proposed by South et al. (1995). The latter used an equilibrium model to describe the rate of substrate conversion as a function of enzymes adsorbed on cellulose surface. The modification by Shao et al. (2007, 2009) used a dynamic enzyme adsorption model to incorporate cellulose conversion whiles accommodating intermittent feeding of substrate which reduces enzyme loading required to achieve a given conversion rate. However, one of the major drawbacks of the predictability of the model developed by Shao et al. (2007, 2009) is that it does not incorporate co-fermentation.

Zhang et al. (2009) developed a kinetic model for co-fermentation by combining glucose and xylose hydrolysis and the competitive uptake of the sugars by a modified *S. cerevisiae* strain for xylose utilization. It was reported that co-fermentation alleviated xylose inhibition during hydrolysis (Qing et al. 2010). Although it was shown that glucose and xylose uptake rates were competitively inhibitory, ethanol was the primary rate-limiting factor among the fermentation related parameters.

In contrast to the above models, Andrade et al. (2009) developed a dynamic flux balance model for bioethanol production by including cell, substrate, and product inhibition terms (Table 2-8). Ethanol production from sugarcane using *S. cerevisiae* was simulated with the model and a good predictability was found in validation to experimental data. Andrade et al. (2009) concluded that the most influential parameters in the system were maximum specific growth rate (μ_{max}), maximum ethanol concentration for cell growth (P_{max}) and product yield based on cell growth ($Y_{P/X}$.); Table 2-8.

Table 2-8: Summary of fermentation kinetic models considering limiting effects for biomass growth and ethanol production

Reference	Biomass growth	Ethanol production	Conditions	Variables
Shao et al. (2007, 2009)	$r_{Xc} = \frac{[X_C]\ X\ \mu_{max}\ X\ [G]}{[G]+K_G}\ x\ (1 - \frac{[Eth]}{K_{X/Eth}})$	$r_{Eth} = r_{Xc}\ x\ \frac{Y_{Eth/G}}{Y_{X/G}}$	Temp: 37 °C pH: 5.8 150 – 200 rpm	r_{Xc} : Rate of reaction for yeast cells (g/(L.h)) $[X_C]$: Concentration of yeast cells (g/L) μ_{max} : Maximum cell growth rate (h^{-1}) [G]: Concentration of glucose (g/L) K_G: Monod constant (g/L) [Eth]: Concentration of ethanol (g/L) $K_{X/Eth}$: Inhibition of cell growth by ethanol (g/L) r_{Eth}: Rate of reaction for ethanol (g/(L.h)) $Y_{Eth/G}$: Ethanol yield per substrate consumed (g/g) $Y_{X/G}$: Cell yield per substrate (g/g)
Zhang et al. (2009)	$r_X = (\mu_G + \mu_X - K_d - K_{de})\ x\ [X]$	$r_{Eth} = \left(\frac{\mu_G}{Y_{x/G}^{max}} + m_G\right) x[X]xY_{Eth/G} + \left(\frac{\mu_X}{Y_{x/x}^{max}} + m_x\right) x[X]xY_{Eth/x}$	Temp: 37 °C pH: 5.5 250 rpm	r_X: Cell formation rate (g/L h) μ_G: Maximum specific growth rate on glucose (h^{-1}) μ_X: Maximum specific growth rate on xylose (h^{-1}) K_d: Natural cell death rate (h^{-1}) K_{de}: Cell death rate by ethanol toxicity (g/L) [X]: Cell mass concentration (g/L) r_{Eth}: Ethanol formation rate (/L h) $Y_{x/G}^{max}$: Maximum cell yield on glucose (g/g) $Y_{x/x}^{max}$: Maximum cell yield on xylose (g/g) m_G: Maintenance factor for growth on glucose (g/L g cell) m_x: Maintenance factor for growth on glucose (g/L g cell) $Y_{Eth/G.X}$: Ethanol yield on glucose and xylose (g/g)
Andrade et al. (2009)	$r_X = \mu_{max}\ \frac{S}{K_s + S}\ \exp(-K_i S)(1 - \frac{X}{X_{max}})^m (1 - \frac{P}{P_{max}})^n$	$r_p = Y_{P/X} r_X + \frac{\beta_{mp} S}{K_{\beta S1} + S} X$	28-34 °C 200 rpm High solids fermentation	r_X: Cell growth rate equation μ_{max}: Maximum specific growth rate (h^{-1}) S: Substrate (kg/m^3) K_s: Substrate saturation constant K_i: Substrate inhibition parameter (m^3/kg) X: Total cell mass (kg/m^3) X_{max}: Maximum substrate concentration for cell growth (kg/m^3) P: Ethanol (kg/m^3) P_{max}: Maximum ethanol concentration for cell growth (kg/m^3) r_p: Ethanol formation rate $Y_{P/X}$: Product yield based on cell growth (kg/kg) β_{mp}: Cell maintenance parameter (kg/kg h) $K_{\beta S1}$: Saturation parameter (kg/m^3) m,n: empirical parameters

2.9 Gap in the literature

The suitability of recycling enzymes in PS fermentation has been well-reported in literature (Gomes et al. 2016, 2018a; 2018b), but these experimental successes were carried out at conditions that are not industrially feasible (e.g. alkaline elution, ultrafiltration, etc.). Therefore, the performance of other potentially viable recycling schemes such as supernatant and broth recycling needs to be explored. Consequently, models capable of predicting enzyme activity decay and ethanol concentrations at high solid loadings need to be developed since it would be a useful tool to understand the enzyme recycling process and to facilitate further process optimizations.

2.10 Aim and Objectives

This study aims to propose theoretical kinetic models that predict process performance when recycling enzymes in the supernatant or fermentation broth. To accomplish this aim, the following objectives were outlined:

1. To evaluate different enzyme cocktails and yeast strains for efficient hydrolysis and fermentation of paper sludge.
2. To experimentally determine the retention of enzyme activity after paper sludge fermentation.
3. To develop mathematical models for enzyme activity decay and enzymatic hydrolysis of paper sludge to predict the performance of recycling either the clarified supernatant or fermentation broth, while investigating the effect of enzyme supplementations.
4. Validation of the proposed models for the selected PS sample, enzyme, and yeast strain by developing a sequential 5 L fed-batch SSF process with the selected recycling route (either supernatant or fermentation broth) to produce ethanol yields that are comparable to a non-recycle fermentation using decreased enzyme dosages.

The following section focuses on the outcomes and insights gained from these recycling schemes. In order to highlight the novelty of these recycling schemes, special references will be made by comparing results to literature findings on enzyme recycling in PS fermentation.

Chapter 3 : Kinetic Modelling of Paper Sludge Fermentation for Evaluating Cellulase Recycling Schemes

Abstract

Kinetic models were developed to predict the feasibility of enzyme recycling during paper sludge bioconversion by simultaneous saccharification and fermentation. The models accounted for the loss of enzyme activity with time, and the effect of fresh enzyme supplementation on ethanol yields over multiple recycle rounds. The activity of Cellic® CTec 3 followed a logarithmic decline with approximately 48% and 40% of the initial activity remaining in the supernatant and broth, respectively, after one round of SSF. Ethanol yields from recycling either the enzyme-containing supernatant or the whole fermentation broth showed no significant variation during successive recycle steps. Although contamination had a negative effect on the ethanol yields, the recycle schemes proved to be an effective strategy to reduce enzyme dosage used in paper sludge fermentation. With only 50% fresh enzyme supplementation for every round of fermentation, about 38% savings on enzyme dosage, and a two-fold increase in enzyme productivity were achieved using shake flasks (6% w/w PS). Based on model predictions on shake flask experiments, recycling of the clarified supernatant was tested at high solids loading (18% w/w) over three fed-batch fermentations in 5L bioreactors. With only 65% fresh enzyme supplementation, the experimental data deviated from model predictions as the ethanol yields reduced from 280 to 200 kg ethanol/ton dry PS for the first and last fermentations respectively. The observed reduction in the overall enzymatic hydrolysis rate was possibly due to the effective loss of the overall enzyme potency during the course of the fermentation. Despite the decline in ethanol yields, recycling the clarified supernatant allowed up to 23% savings on the overall enzyme loading in the bioreactor. The observed decrease in enzyme dosage can be beneficial to the economic viability of paper sludge fermentation.

3.1 Introduction

Bioethanol is considered to be a renewable energy source for large scale production either as a pure fuel or blended with gasoline. It has the potential to significantly reduce the overdependence on fossil fuels, which have been associated with accelerating the global climate change crisis (Vertes et al. 2010). This has inspired research into bioethanol production from cheaper and abundant non-food sources such as agricultural and industrial waste feedstocks.

Paper sludge (PS), one of the major waste streams from the pulp and paper industry, has demonstrated its suitability for efficient bioethanol production by yeast fermentation (Fan & Lynd 2007b; Kang et al. 2011; Kang et al. 2010) owing to its substantial quantities of cellulose fibres (Boshoff et al. 2016; Robus et al. 2016). However, large scale implementation of bioethanol production from paper sludge fermentation is yet to be achieved. One of the main reasons is the cost and high dosages of enzymes required to achieve a commercially viable ethanol titre (~40 g/L) (Robus et al. 2016). According to Klein-Marcuschamer et al. (2012), the cost of enzymes on ethanol production is about $ 0.68 per gallon and can represent up to 35% of the annual ethanol cash costs (Johnson 2016).

Enzyme recycling has been identified as a promising concept for reducing the amount of enzyme required and thus, the overall cost of the process (Gomes et al. 2015; Jørgensen & Pinelo 2017). The concept of enzyme recycling is based on the assumption that by recovering and reusing the active residual enzymes at the end of one process, it would be possible to reduce the dosage of new enzymes required for subsequent hydrolysis and hence, increasing the enzyme productivity (Weiss et al. 2013). Previous work has shown that after enzymatic hydrolysis of lignocellulosic biomass, the enzymes may either be free in the liquid phase (supernatant) or remain bound to the residual substrate (Pribowo et al. 2012). Possible strategies for recovering enzymes include ultrafiltration (Baral et al. 2020; Gomes et al. 2016), alkaline elution (pH adjustment) (Rodrigues et al. 2012), the addition of desorbents (Tu et al. 2009), recycling of enzyme present in the supernatant onto fresh substrate (Lindedam et al. 2013) and partial recycling of whole fermentation broth (Østergaard et al. 2015). Gomes et al. (2018b) estimated the economic viability of cellulase recycling by ultrafiltration-

recovery, for bioethanol production from PS using SHF. It was shown that although 88% of the final cellulase activity could be recycled, enabling a 50% reduction in enzyme dosage, the cost associated with enzyme recovery (ultrafiltration) negatively affected the viability of the recycling process.

Despite the notable efforts on enzyme recycling in PS fermentation in the SHF configuration (Gomes et al. 2018a, 2018b, 2016), there is no reported study on the recycling of enzymes from an SSF process. Enzyme recovery methods like ultrafiltration and pH adjustment are impracticable in an SSF setup, where the enzymes are mixed with yeasts and unconverted PS solids. The (partial) recycling of the clarified supernatant or whole fermentation broth could be simpler and potentially cheaper because it eliminates these complex methods of enzyme isolation and recovery. Also, more detailed insight is required about the kinetic behaviour of cellulase activities during the hydrolysis and fermentation, where the loss of activity and residual substrate build-up might severely reduce enzyme recycling potential. A mathematical model that describes enzyme kinetics and the performance of paper sludge fermentation with enzyme recycling would be a useful tool for future process optimizations.

This work aimed to determine if, by recycling enzyme in the supernatant or whole fermentation broth from an SSF process, a significant decrease in the fresh enzyme dosage required to reach a similar level of ethanol yield could be achieved. The two strategies evaluated for recycling enzymes after PS bioconversion by SSF included recycling enzymes in the clear supernatant (Supernatant recycle) and recycling the whole fermentation broth (Broth recycle).

Data on enzyme activity and enzymatic hydrolysis were used to develop kinetic models that predict the loss of cellulase activity and ethanol concentrations during the fermentation process. Model predictions were validated experimentally based on the performance of these recycle schemes at low (shake flask) and high-solid loadings (5L bioreactors), while varying the supplementation of fresh enzymes. The resulting kinetic model will be used subsequently in process optimisation.

3.2 Materials and Methods

3.2.1 Experimental Approach

The experimental work began with the collection of two paper sludge samples (virgin pulp and recycled PS) and their corresponding clarifier wastewaters from their respective mills in South Africa (Figure 3-1). Preparation of feedstock and characterization of paper sludge and process wastewater samples were conducted respectively. The use of clarifier wastewater as make-up water rather than clean water for fermentation was justified by Donkor (2019) after successful ethanol production was achieved with clarifier wastewater. This modification was necessitated due to the recent water crisis in South Africa.

Fermentation screening was conducted to select the suitable microorganism (yeast) and enzyme cocktail for the recycling experiments. Based on experimental data, *S. cerevisiae* CelluX^{TM}4 was selected due to its strong co-fermentation ability. Cellic$^{®}$ CTec 3 was the preferred enzyme cocktail based on hydrolysis efficiency, thermostability, and retention of enzyme activity after the fermentation process.

The effect of recycling enzymes in the clear supernatant or whole fermentation broth was conducted in shake flasks with the selected PS sample (Batch SSF), while investigating the effect of enzyme supplementation on ethanol production. Two models were developed by considering the functional enzyme decay over time and the hydrolysis of the paper sludge fibres. Model parameters were obtained from enzyme activity assays and hydrolysis data. The models were validated by comparing model predictions to data from the recycling experiments.

Recycling enzymes in the supernatant were tested at high solids loading (18% w/w) by conducting SSF fed-batch experiments in 5L bioreactors. The enzyme activity model was used for enzyme supplementation during the recycle experiments in the bioreactors, whereas model predictions on ethanol production were validated at the optimized enzyme dosage. Chemical analysis on solids after fermentation was performed to determine the extent of hydrolysis and perform mass balances on the recycling process in the bioreactor.

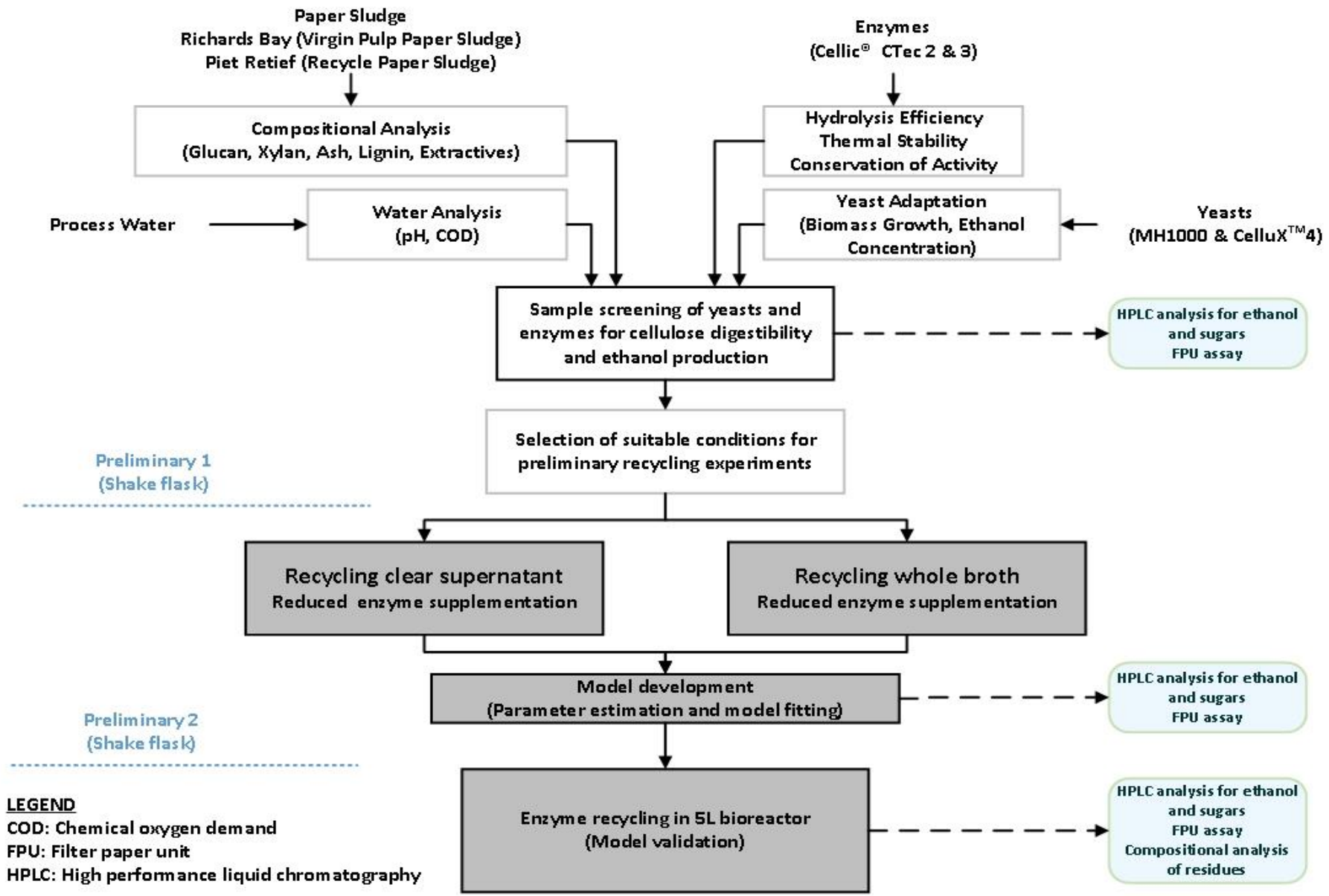


Figure 3-1: Experimental plan used in this study. The shaded sections indicate the novelty of this study.

3.2.2 Materials and Methods

3.2.2.1 Paper sludge, enzyme cocktails, and yeast strain

The paper sludge samples were collected from Mondi South Africa Ltd. in Richard's Bay (Kraft pulp mill) and Mpact Ltd. in Piet Retief (Recycle pulp mill). The samples were dried in a greenhouse at 40-45 °C to a 10% (w/w) moisture content and milled with a hammer mill (Drotsky S1) fitted with a 2 mm screen. The PS samples were pelletized (MPEL200, ABC Hansen Africa) to form compacted pellets (~6 mm diameter), dried again at 75 °C to a moisture content of 4% (w/w), and sealed in plastic bags at room temperature for storage. The chemical composition of the PS was determined according to the National Renewable Energy Laboratory (NREL) standard procedures (Sluiter et al. 2008, 2012).

Enzymatic hydrolysis was conducted with two enzyme cocktails: Cellic® CTec 2 and 3 (Novozymes, Denmark). The total cellulase activities were determined to be 129 and 148 FPU/mL respectively, according to the standardized filter paper assay published by IUPAC in 1987 (Ghose 1987).

The recombinant strains of *S. cerevisiae* MH1000 (Stellenbosch University culture collection) and CelluX™4 (provided by Leaf by Lesaffre, France), were stored as glycerol stock cultures at -85 °C in an ultralow freezer (NU-966BE, Lasec, South Africa). Cultures for fermentation experiments were grown in a medium containing: 10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose (all Merck, South Africa) for 18 h at 30 °C in an orbital shaker (LM-575D, Lasec – South Africa) at 150 rpm.

3.2.2.2 Enzyme stability assays and retention of cellulase activity

The stability of the enzyme cocktail was determined by measuring the residual cellulase activities during a 72 h incubation of 6% (w/w) paper sludge at different temperatures (30, 37, and 50 °C). After the incubation period, the hydrolysis performance of the enzymes was assessed by quantifying the glucose yields from the PS sample. The retention of cellulase activity between the clear supernatant and broth was also evaluated by measuring the total cellulase activity (in terms of filter paper units, FPUs) in both fractions after the 72 h fermentation.

3.2.2.3 Batch and fed-batch fermentation

Fermentation of the PS sample in batch culture experiments was conducted in 250 mL rubber capped baffled flasks. The medium consisted of 3.0 g/L corn steep liquor (Sigma-Aldrich, South Africa) and 0.62 g/L $MgSO_4.H_2O$ (Merck). Paper sludge at a solid loading of 6% (w/w) was added to the medium in the flasks and sterilized at 121 °C for 15 min in an autoclave. The medium was supplemented with the enzyme cocktail at a dosage of 10 FPU/gds and inoculated with the YPD-grown yeast culture (5% of the total reaction volume) and incubated at 37 °C and 150 rpm for 72 h.

Fed-batch experiments were performed in jacketed BIOSTAT® Bplus-5L CC twin bioreactors (Sartorius BBI Systems GmbH, Switzerland) fitted with Rushton impellers and an Easyferm plus K8 pH probe (Mecosa, South Africa). The total working volume and solids loading were 2.5 L and 18% (w/w), respectively. The initial solid loading upon inoculation was 6% (w/w) with subsequent feedings of 6% (w/w) every 24 h. The bioreactors were inoculated with 125 mL (5% v/v) of CelluX^{TM}4 seed culture together with 5 FPU/gds of Cellic® CTec 3 and cultivations were allowed to continue for 7 days at 37 °C. Samples were collected at regular intervals and the ethanol yields were calculated using eq.(1) and (2) respectively.

$$\text{Theoretical ethanol concentration (g/L)} = \text{Solids fed (g/L)} \times \text{Glucose fraction in PS} \times 0.511 \quad (1)$$

$$\text{Ethanol yield (\%)} = \frac{\text{Experimental ethanol concentration (g/L)}}{\text{Theoretical ethanol concentration (g/L)}} \times 100 \quad (2)$$

3.2.2.4 Multiple rounds of fermentation with enzyme recycling

For the recycling experiments, the first round of fermentation was conducted as described for a single batch and fed-batch experiment for shake flask and bioreactor respectively. At the end of each round of fermentation, portions of the supernatant were aseptically removed after the solid fractions had settled out, and then added to freshly sterilized paper sludge. A portion of the whole broth containing enzymes, unhydrolyzed solids, and yeast were transferred to freshly sterilized paper sludge in shake flask only. The volume of enzymes recycled depended on the amount of enzymes supplemented to prevent the dilution effect in the next fermentation; 25% volume transfer for 75% fresh enzymes etc.

The new paper sludge suspensions, including recycled enzymes, were supplemented with different dosages of fresh enzymes to replenish enzyme activities at the beginning of each new round of fermentation. The ethanol concentration was measured in the new PS mixture at time zero to account for ethanol carried over from the previous fermentation. These recycle experiments were subjected to the same fermentation conditions and completed over 4 batch PS fermentations in shake flasks (Figure 3-2) and 3 fed-batch fermentations in the 5L bioreactors (Figure 3-3).

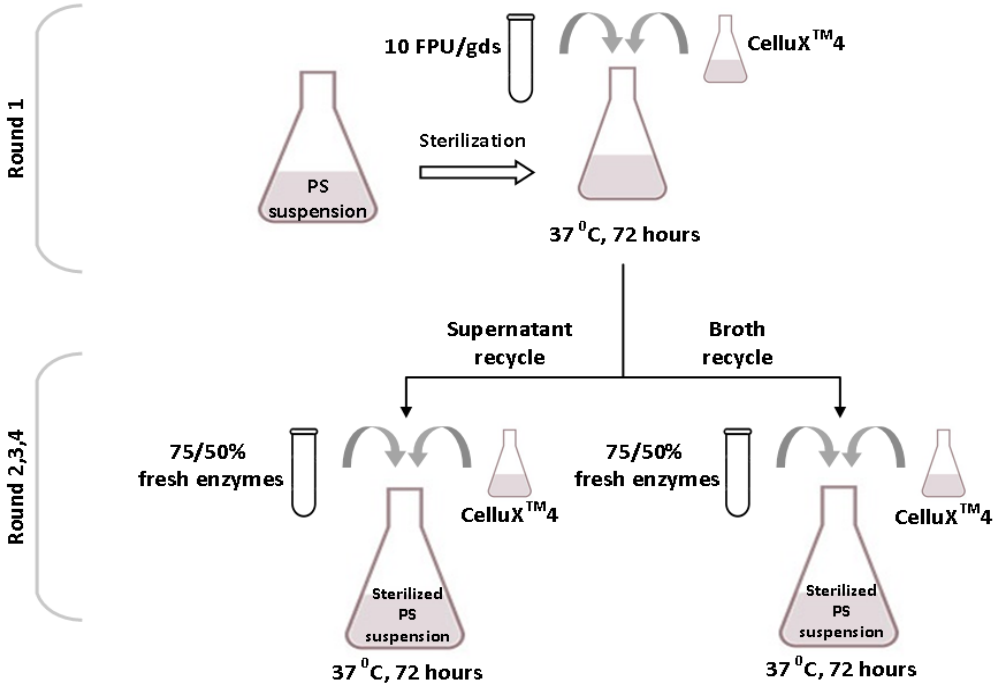


Figure 3-2: Schematic of paper sludge fermentation with enzyme recycling

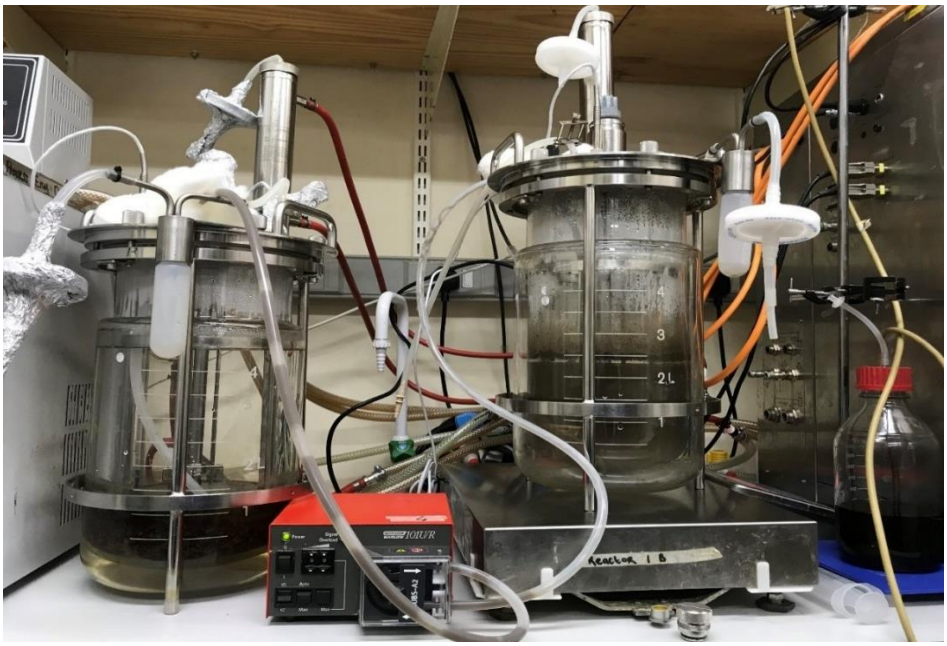


Figure 3-3: Transferring enzymes in the supernatant from one 5Lbioreactor (right) to the other (left).

3.2.2.5 Chemical analysis for sugars and fermentation products

The concentrations of glucose, xylose, organic acids, and ethanol in the samples collected during hydrolysis and fermentation were measured by high-performance liquid chromatography (HPLC) fitted with an Aminex HPx-87 column, a cation-H Micro Guard Cartridge, R1-101 detector, pump, and an AS3000 AutoSampler (all Thermo Scientific Products, Bio-rad, South Africa). The HPLC column temperature was maintained at 65 °C with 5mM sulfuric acid as a mobile phase at a flow rate of 0.6 mL/min.

3.2.2.6 Measurement of enzyme activities

The residual cellulase activities were expressed in FPU where one unit of filter paper activity (FPU) was determined as the amount of enzyme that releases 2.0 mg of reducing sugar (glucose) from 50 mg of Whatman No. 1 filter paper within 1 h (Adney & Baker 2008). The experiment was carried out in a reaction mixture containing 0.5 mL of diluted enzyme solution, 1.0 mL of 0.1 M citrate buffer (pH 4.8), and 50 mg of a 1× 6 cm strip of filter paper and incubated at 50 °C for 1 h in a conical tube (Adney & Baker 2008).

3.2.3 Kinetic Model Development

3.2.3.1 Enzyme decay

To obtain a correlation between enzyme activity and hydrolysis performance, it is required to know the variation of cellulase activity during the hydrolysis process. In this study, the loss of enzyme activity for batch enzyme feeding in a reactor system (Figure 3-4) can be assumed to follow a single-step scheme where active enzymes are transformed into its inactive form. Following a first-order irreversible reaction, a mass balance can be performed as shown below:

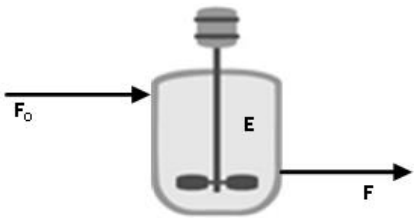


Figure 3-4: Schematic of Enzyme *E* in a reactor system.

Accumulation = Input – Output + Generation – Consumption

$$\frac{dE}{dt} = F_o - F + r_g - r_c \quad (3)$$

Where; E = Concentration of enzyme (FPU/mL)

F_o = Flowrate of enzyme into reactor (FPU/mL.hr)

F = Flowrate of enzyme out of reactor (FPU/mL.hr)

r_g = Rate of generation (FPU/mL.hr)

r_c = Rate of enzyme decay (FPU/mL.hr)

For batch enzyme feeding; $F_0 = F = 0$; $r_g = 0$

$$\frac{dE}{dt} = -r_c \quad (4)$$

$$\frac{dE}{dt} = -K_{det}[E] \quad (5)$$

Where [E] is the concentration of active enzymes (FPU/mL) and K_{det} (h^{-1}) is the first-order rate of enzyme deactivation. Integrating eq (5) with initial conditions ([E] = [E_0] at t = 0) yields:

$$E = [E_0]\exp(-K_{det}\, t) \quad (6)$$

With regards to multiple fermentation experiments incorporating batch enzyme feeding and recycling (Figure 3-5), eq (6) can be used to predict the residual activity (E). This is defined as the concentration of active enzymes (cellulase activity) at any time in the fermentation. It can be applied to every subsequent fermentation by taking into account both the recycled enzyme activities and fresh enzyme supplementation as follows:

For reactor 1, the residual active enzymes after fermentation ($E_{1, end}$) can be deduced as:

$$E_{(1, end)} = E_{0\,(1, initial)} \exp(-K_{det}\, t) \quad (7)$$

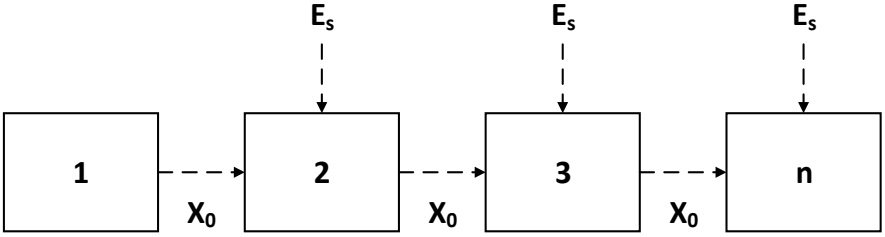


Figure 3-5: Diagrammatic representation of batch reactor operation with enzyme recycling.

For subsequent reactors (2, 3 …., n), the residual active enzymes during the fermentation would depend on the residual enzymes from the preceding reactor (E_{n-1}), the amount of enzymes supplemented at the beginning of that round (E_s), and the decay in the enzyme activity during the course of the fermentation. These enzyme activities are not simply lumped together. Instead, the model accounts for the fact that some enzymes are older than others and hence, that older enzymes would be intrinsically different from the freshly added enzymes. The amount of enzymes recycled and supplemented for each round would depend on the fractional volume of transfer (X_o) from the preceding reactor, to balance off the dilution effect in the next reactor, and the contributions of each to the overall activity present in the SSF culture at any time was modelled separately:

$$E_{(2,\,end)} = E_{0\,(2,\,initial)}\exp(-K_{det}\,t)\;;\; E_{0\,(2,\,initial)} = E_{(1,\,end)}(X_0) + E_s(1 - X_0) \quad (8)$$

$$E_{(n,\,end)} = E_{0\,(n,\,initial)}\exp(-K_{det}\,t)\;;\; E_{0\,(n,\,initial)} = [\textstyle\sum_{n=1}^{n=n-1} E_{(n,\,end)}(X_0)] + E_s(1 - X_0) \quad (9)$$

3.2.3.2 Paper Sludge Hydrolysis and Fermentation

Several kinetic models have been proposed in literature to determine the effective rate of enzymatic hydrolysis of a wide range of substrates (Wang et al. 2011). As a basis of this work, the kinetic model for cellulose hydrolysis as proposed by Ioelovich (2015) was considered. This model used the Avrami-Kolmogorov-Erofeev (AKE) equation to accurately describe experimental kinetic curves for several cellulose substrates (eq. (10)).

$$\text{In}\,(1 - \alpha) = -Kt^n \quad (10)$$

Where α refers to the degree of cellulose conversion (%), the ratio of sugars released to the total cellulose content, which is the ratio of sugars released to the actual cellulose content of the substrate; K is the effective rate constant; t is time (h) and n is the effective order of the process that indicates the kinetic mechanism. To obtain the kinetic parameters of the experimental kinetic curves, eq. (10) was linearized into a logarithmic form:

$$\text{In}\,F = \text{In}\,K + n\,\text{Int} \quad (11)$$

Where; $F = -\text{In}\,(1 - \alpha)$

After parameter estimation, the validity of the model was tested by comparing model predictions to experimental results from the recycle scenarios. It was assumed that the solid loading and ethanol yield from sugars (0.511 g ethanol /g sugars) were constant during the recycling experiments. Model sensitivity was not evaluated, but the validity of the models was determined by analysis of variance between the data from validation experiments and model predictions.

3.2.4 Statistical Analysis

Experimental data obtained from measuring enzyme activities, cellulose conversion, and ethanol concentrations were used to validate the mathematical models. Model constants were determined by using regression analysis in Microsoft Excel. The coefficient of determination (R^2) between experimental data and predicted values were used to judge the accuracy of the models.

3.3 Results and Discussion

3.3.1 Paper Sludge Composition

The paper sludge samples used in this study were analysed to determine the carbohydrate, lignin, and ash contents. The highest glucan and xylan fractions of 59.2% and 11.5% (w/w), respectively, were recorded for Richards Bay PS. This was at least 43% (w/w) higher than that of Piet Retief PS. Richards Bay PS also had the lowest ash content (15.2% w/w) as compared to Piet Retief PS, for which an ash content of 22.6% (w/w) was recorded (Table 3-1). The substantial carbohydrate content and low ash content in Richards Bay PS were similar to previous reports on virgin pulp PS and, therefore, suggested that high ethanol yields could be attainable with this substrate (Boshoff et al. 2016; Donkor 2019; Williams 2016). Although virgin pulp PS originates from mills that use virgin wood as their starting material, seasonal variations in feedstock and pulping processes are expected to slightly affect the composition and fermentation performance of the same category of PS from one production batch to the other (Chen et al. 2014).

Table 3-1: Chemical composition of paper sludge samples.

Component (w/w)	Piet Retief PS*	Richards Bay PS*	Boshoff et al. (2016)	Williams (2016)	Donkor (2019)
Glucan	22.9 ± 0.20	59.2 ± 0.34	35.5	52.0 ± 0.4	58.2 ± 0.4
Xylan	4.8 ± 0.01	11.5 ± 0.20	17.7	10.6 ± 0.1	12.2 ± 0.1
Extractives	10.7 ± 0.12	5.2 ± 0.06	7.1	7.4 ± 0.1	5.4 ± 0.1
Lignin	39.0 ± 0.54	8.9 ± 0.62	19.3	5.1 ± 0.1	4.1 ± 0.1
Ash	22.6 ± 0.17	15.2 ± 0.12	20.4	24.8 ± 0.1	20.8 ± 0.1

* The values are the average of triplicates ± standard error

Both paper sludge samples were compared in an SSF batch culture in shake flasks with *S. cerevisiae* strain MH1000 and the final ethanol concentration obtained in batch culture at 5 FPU/gds of Cellic® CTec 2 showed significant variation (Figure 3-6). The carbohydrate content in the Richards Bay sample (~70% w/w) enabled its ethanol concentration to reach values (~9 g/L), almost three times higher than that obtained for Piet Retief PS (3.2 g/L). This observation indicated the Richards Bay PS sample as a preferred feedstock for bioethanol production and was selected for further optimization.

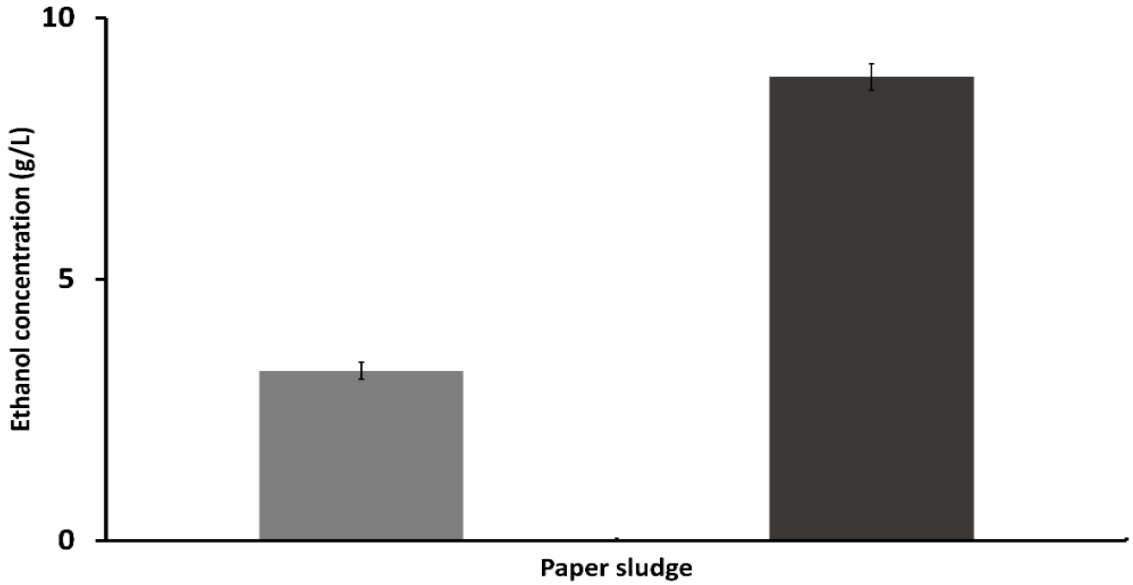


Figure 3-6: Ethanol concentration (g/L) obtained from 6% (w/w) paper sludge (■Piet Retief PS ■Richards Bay PS) after 72 h fermentation with Cellic® CTec 2 and *S. cerevisiae* MH1000 in shake flask batch cultures. The ethanol concentrations shown represent the average of triplicate experiments with standard error bars.

3.3.2 Yeast and Enzyme screening

3.3.2.1 Effect of clarifier overflow water on yeast growth and ethanol production

The chemical oxygen demand (COD) measured was 5130 mg/L and 2280 mg/L for Piet Retief and Richards Bay water samples respectively. This wastewater stream is a mixture of several effluent streams from different sections of the pulp and paper mill and is known to contain several pollutants such as fatty acids, phenolic and sulphur compounds as well as heavy metals (Rintala & Puhakka 1994).

The final biomass concentrations measured for strains cultured in clarified overflow water did not show statistical significance (p-value (0.176) > 0.05) as shown in Figure 3-7a. In comparison to strains cultured in clean water, the final biomass concentration measured for *S. cerevisiae* CelluX^{TM}4 was approximately 16% greater than that of clarified overflow water ($p < 0.05$), however, the opposite was observed for *S. cerevisiae* MH1000 (p-value (0.072) > 0.05).

Similarly, ethanol concentrations for strains cultured in clean water were significantly higher than those in clarified overflow water ($p < 0.05$). The level of toxicity of the Piet Retief water sample (COD = 5130 mg/L) was possibly the reason for its lower ethanol production compared to that of the Richards Bay water sample (p-value (0.022) < 0.05). Although clarified overflow water had some negative impact on yeast performance, it is worth noting that, the final ethanol concentrations ranged between 19 g/L - 23 g/L for both clean and process water; which is relatively close to the maximum theoretical ethanol concentration of 25 g/L as shown in Figure 3-7b. These observations are similar to that of Donkor (2019) who demonstrated the application of clarified overflow water as make-up water for the fermentation process.

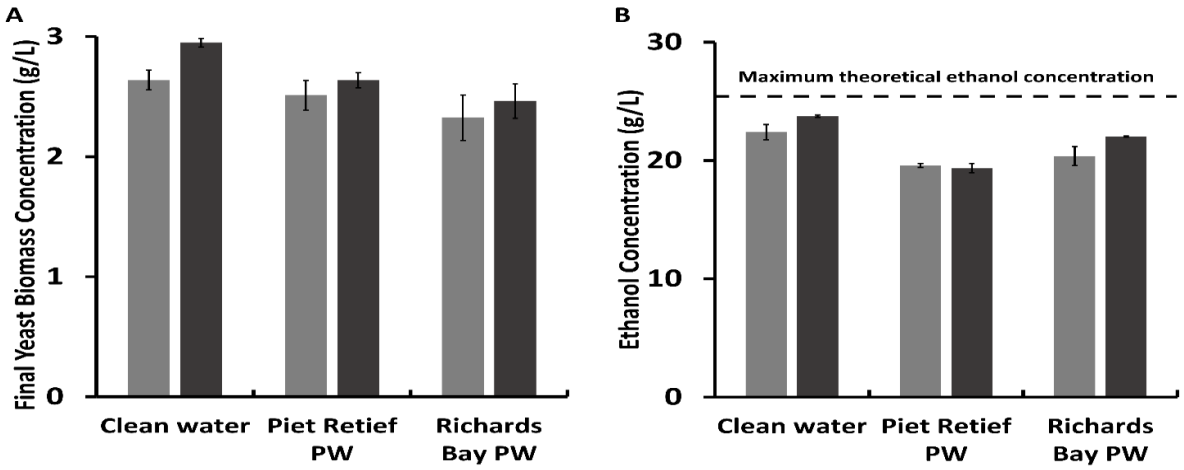


Figure 3-7: Effect of process water on yeast performance (■MH1000 ■CelluX^{TM}4): Final biomass concentration (g/L) (A); Ethanol concentration (g/L) (B). Data represent the average of triplicate experiments after 144 h shown with standard error bars.

3.3.2.2 Screening of yeast strains

The final ethanol concentrations produced by *S. cerevisiae* CelluX^{TM}4 was averagely 17% and 32% higher than *S. cerevisiae* MH1000 for both enzyme cocktails tested; Cellic® CTec 2 and 3 respectively (p-value (0.013) < 0.05) (Figure 3-8). Similar to Basuyaux (2017), the successful expression of a xylose isomerase into CelluX^{TM}4 yeast, allowed the utilization of glucose and xylose in the paper sludge. With the hemicellulose content in Richard Bay PS being only 12%, the higher ethanol production of CelluX^{TM}4 yeast observed (17% and 32%) shows that *S. cerevisiae* CelluX^{TM}4 is also more efficient at converting glucose compared to *S. cerevisiae* MH1000 (Figure A-2, Appendix). CelluX^{TM}4 was therefore selected for further optimization since co-fermentation is beneficial for ethanol production (Vertes et al. 2010).

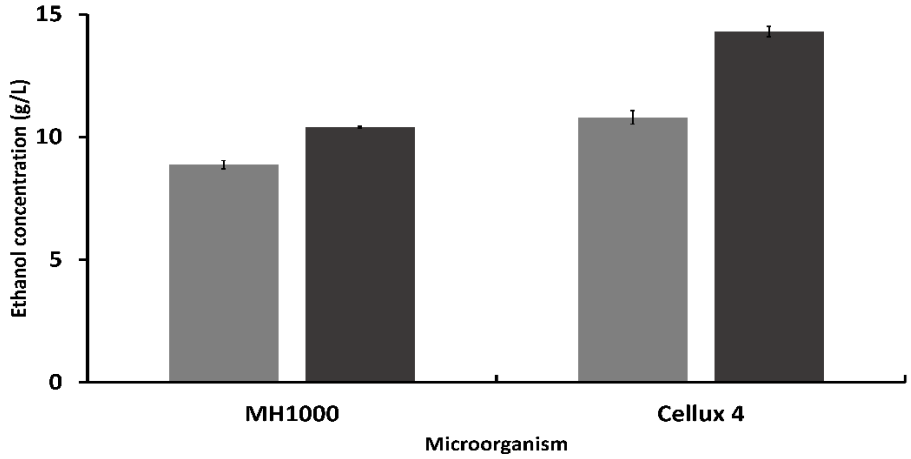


Figure 3-8: Ethanol concentration (g/L) obtained for Richards Bay (RB) sample during yeast strain screening. Both yeast Strains were tested with 5 FPU/gds of Cellic® CTec 2 (■) & 3 (■). The ethanol concentrations reported are the highest values measured after 72 h with standard error bars for the deviation of triplicate experiments.

3.3.2.3 Thermal stability and hydrolysis efficiency of Cellic® CTec 2 & 3

A progressive loss of activity was observed when each enzyme cocktail was incubated for 72 h at different temperatures; 30, 37, and 50 °C (Figure 3-9). For hydrolysis at 37 °C, a minimum of 73% and 81% of the initial enzyme activity was present after 72 h of incubation for Cellic® CTec 2 and 3 respectively. However, at 50 °C, the residual enzyme activity was reduced to 60% and 75% for Cellic® CTec 2 and 3 respectively. Minor thermal deactivation of enzymes was observed during the earlier stages of incubation (up to 48 h) at 30 °C and 37 °C (p-value (0.158) > 0.05). For incubation at 50 °C, significant enzyme loss was found at the earlier stages ($p < 0.05$). For incubation periods equal or higher than 48 h, Cellic® CTec 3 proved to be more stable particularly at low to moderate temperatures, 30 °C and 37 °C compared to incubation at 50 °C (p-value (0.037) < 0.05).

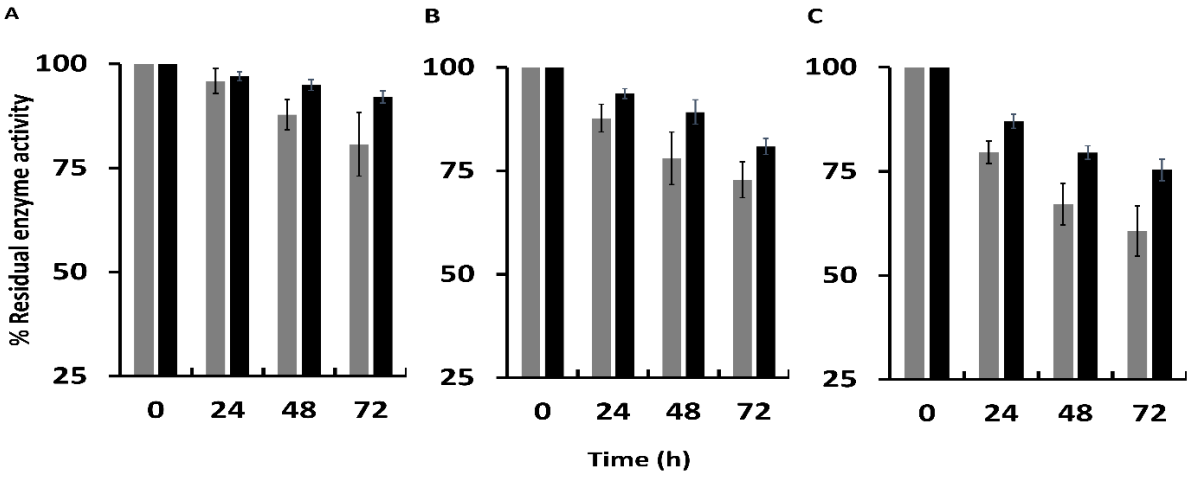


Figure 3-9: Variation of enzymatic activity by Cellic® CTec 2 (■)and 3 (■) during the hydrolysis of paper sludge at different temperatures: (A) 30 °C, (B) 37 °C, (C) 50 °C. 10 FPU/gds was added initially and the values reported are the average of triplicates, error bars represent ± standard error.

It was observed that using Cellic® CTec 3 produced significantly higher final glucose yields ($p < 0.05$) compared to Cellic® CTec 2 after 72 h of hydrolysis for all incubation temperatures (Figure 3-10). Also, 37 °C was selected as a suitable temperature for hydrolysis and fermentation. This is due to the marginal loss of enzyme activity observed (Figure 3-9b) as well as comparable glucose yields with hydrolysis at 50 °C (Figure 3-10). Previous reports have shown 37 °C to be a suitable temperature for enzyme recovery (Gomes et al. 2015) and ethanol production with several *S. cerevisiae* strains (Kang et al. 2010; Robus et al. 2016).

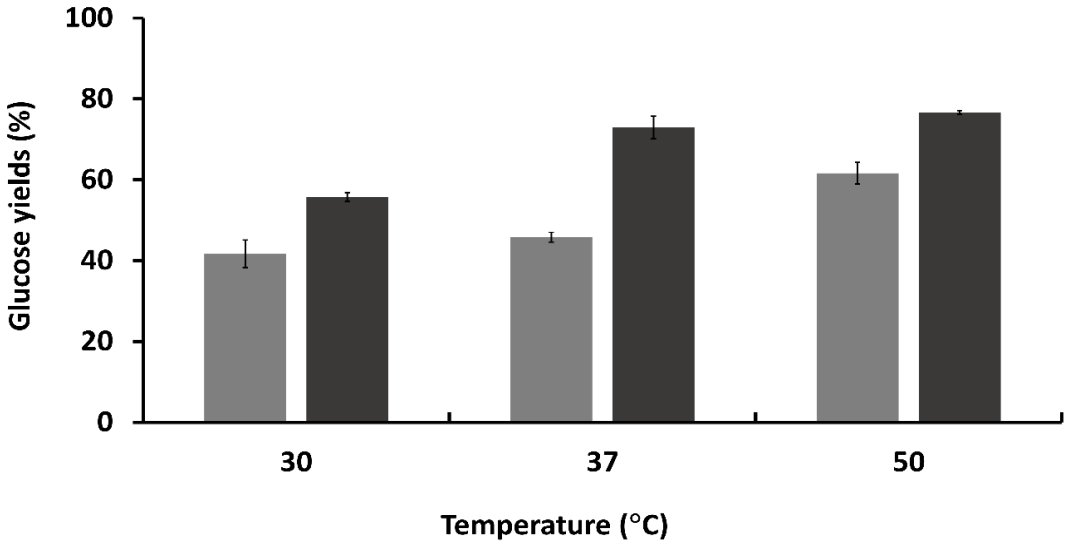


Figure 3-10: Glucose (g/L) released from 6% (w/w) PS by CTec 2 (■)and 3 (■) during the hydrolysis-only of Richards Bay PS at different temperatures. The error bars represent the standard error of triplicate experiments.

3.3.3 PS fermentation with enzyme recycling in shake flasks

In this section, the retention of enzyme activity after 72 h of batch fermentation was measured for the supernatant and broth in comparison to the initial enzyme dosage. Enzyme recycling is conducted in shake flasks at 6% (w/w) solids, over 4 batch fermentations for different enzyme supplementations (50% and 75% fresh enzymes). The yields from recycling either the broth or supernatant were measured. Some limitations to the recycling schemes were identified and discussed below.

3.3.3.1 Determination of enzyme activity after fermentation: Clarified supernatant vs whole broth

The enzyme activities measured in the supernatants were higher ($p < 0.05$) than those observed in the whole broth, for both enzyme cocktails (Figure 3-11a and b). This observation was attributed to the reversible mechanism of cellulase adsorption during cellulose hydrolysis, where most cellulases are released to the liquid phase upon completion of hydrolysis (Gomes et al. 2015). The slightly lower residual activities reported for the broth may be due to the adsorption of β-glucosidase to residual solids (mostly lignin) in the broth (Haven & Jørgensen 2013; Kim et al. 2019). However, the absence of cellobiose accumulation in the fermentation broth is in agreement with results reported by Haven et al. (2013), who showed that the adsorption of β-glucosidase to residual solids is not a major concern as these enzymes remained catalytically active and could be recycled (Weiss et al. 2013).

Generally, the enzyme activities that remained after fermentation was higher in the case of Cellic® CTec 3 (Figure 3-11a and b). This trend has already been observed by several researchers due to the addition of improved supplementary enzymes which boosts the performance of Cellic® CTec 3 over CTec 2 (Kim et al. 2019; Novozymes 2017). Considering the lowest enzyme dosage (10 FPU/gds), it presents a scenario where about 48 and 40% of the initial enzyme activity of Cellic® CTec 3 was located in the supernatant and broth respectively (Figure 3-11b). This, therefore, suggests the possibility of minimizing fresh enzyme dosage in the subsequent stages of hydrolysis.

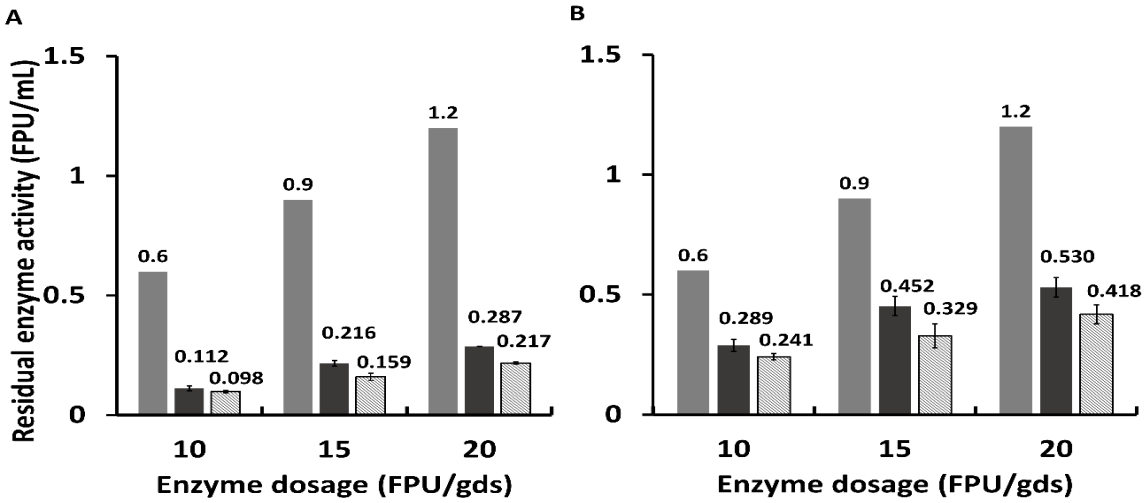


Figure 3-11: Enzyme activities measured after 72 h fermentation (■initial ■Supernatant ▣Broth) with Cellic® CTec 2 (A); Cellic® CTec 3 (B). The activities are relative to controls without substrate added and the average of triplicate experiments are shown with standard error bars.

3.3.3.2 Comparison between enzyme recycling schemes: Supernatant vs broth recycling

Higher enzyme supplementations slightly favoured ethanol yields in the first recycle round (R1) compared to the initial round (R0). The 75% enzyme supplementation increased ethanol yields by 11 ± 0.27% and 13 ± 0.05% whereas the 50% enzyme supplementation increased the yields by only 5±0.37% and 9 ± 0.55% for the recycled supernatant and broth, respectively, in comparison to the initial batch fermentation, R0 (Figure 3-12a and b). For recycle R2 and R3, the ethanol yields obtained with the 75% enzyme supplementation were significantly higher (p-value (0.004) < 0.05) than when enzyme supplementation of 50% was used. This observation suggests that there could be an accumulation of enzyme activity when 75% fresh enzyme supplementation was used (Xue et al. 2012). Thus, the lower enzyme supplementation; 50% enzymes was only sufficient to achieve similar ethanol yields in the first round of recycling (R1) compared to the R0.

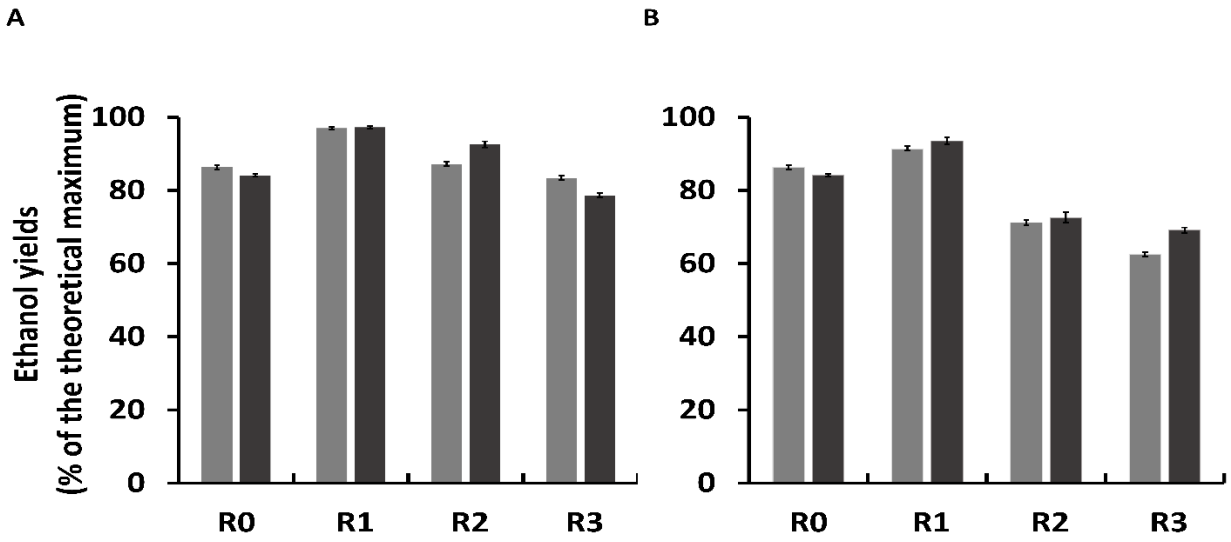


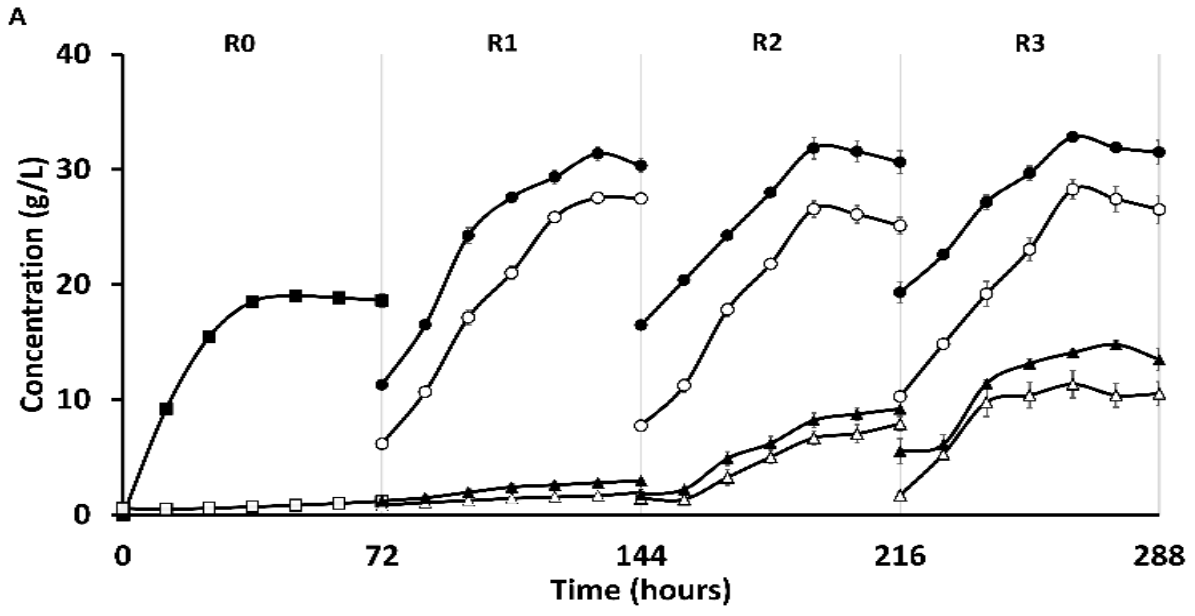
Figure 3-12: Final ethanol yields obtained from recycling either the supernatant (■) or broth (■). 10 FPU/gds of Cellic® CTec 3 was applied to the initial fermentation (R0) followed by 75% (A) and 50% (B) fresh enzyme supplementations to the recycle rounds; R1, R2, R3. Experimental data are the average of triplicate experiments shown with standard error bars.

Recycling solids in the whole broth did not affect enzymatic hydrolysis during the first round of recycling (R1) since the ethanol yields recorded when recycling the supernatant (94 ± 0.55%) and broth (95 ± 0.63%) were very similar for both enzyme dosages (Figure 3-12a and b). This result contradicted earlier findings where the supernatant was reported to contain most of the enzyme activities after fermentation (Figure 3-11b), and thus, was expected to produce higher ethanol titers compared to the broth. Previously, Kim et al. (2019) and Weiss et al. (2013) successfully demonstrated that enzymes bound to residual solids could be recycled without impacting sugar yields. However, the buildup of unhydrolyzed solids during broth recycling could have affected ethanol concentrations as recycling progressed (R2 and R3). With 75% fresh enzyme supplementation, the final ethanol concentration decreased from 21.3 g/L after the first recycle to 17.9 g/L at the end of the experiment, which represents a decrease of approximately 16% (Figure 3-12a). The accumulation of unhydrolyzed solids including ash, unconverted cellulose, and lignin has been reported to have an inhibitory effect on cellulose hydrolysis and fermentation (Wallace et al. 2016). The accumulation of solids further indicates that recycling the fermentation broth would not only lead to decreased ethanol yields but also increase the capital cost for larger process equipment and energy demands for mixing and pumping (Jørgensen & Pinelo 2017).

Although data on the ethanol yields from these recycling schemes could not prove a significant difference between recycling enzymes in the supernatant or broth, it suggests that the level of enzyme activity in each round of fermentation was similar. The best trade-off for process intensification, i.e. high solid loadings and low enzyme dosage, however, would be to recycle the enzymes in the supernatant as it eliminates solids accumulation and produces satisfactory ethanol yields.

3.3.3.3 Effect of contamination on enzyme recycling

Contamination by lactic acid-producing microbes negatively affected ethanol yields during enzyme recycling, despite the sterilisation of the PS solids prior to fermentation, by storage of dried samples in airtight bags and autoclaving these before fermentation. The average lactic concentration increased for both recycle schemes from the onset of round 2 (R2) (Figure 3-13a and b). For example, the concentrations of lactic acid increased to approximately 8 ± 0.62 g/L and 12 ± 1.31 g/L for rounds 2 and 3, respectively, for both recycling schemes (Figure 3-13a and b). This observation could be the reason for the plateauing of ethanol production as contaminants outperform yeasts in competition for the same carbon source. Lactic acid concentrations of 2 g/L have been reported to inhibit *S. cerevisiae* growth rate, glucose consumption, and ethanol formation (Narendranath et al. 2001). Although these recycling experiments were conducted under aseptic conditions, the lactic acid production possibly originated from contaminants already present in the paper sludge (Donkor 2019).



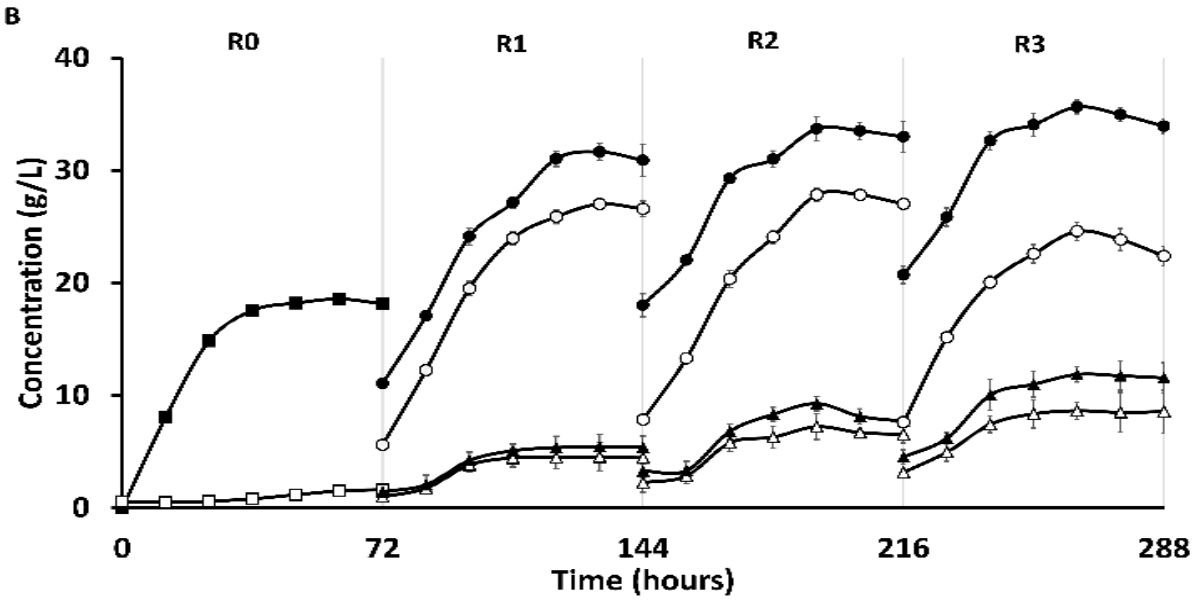


Figure 3-13: Concentrations of ethanol (■,○,●) and lactic acid (□,△,▲) obtained with 100%, 75%, and 50% enzymes respectively with supernatant (A) and broth recycling (B). R0, R1, R2, R3 refers to the initial process and the first, second, and last rounds of recycling. Data shown are the average of triplicate experiments with standard error bars.

3.3.4 Modelling of enzyme recycling

The section uses experimental data obtained during recycling (section 3.3.3) for model development. Model predictions were validated based on actual recycling data obtained during the experiments.

3.3.4.1 Analysis of cellulase activity decay

A model was developed to successfully predict the residual cellulase activities of Cellic® CTec 3 during the fermentation process. This model is in contrast to that developed by South et al. (1995), whom assumed a constant enzyme activity during fermentation. The relationship between the overall decline of measured residual cellulase activity during the fermentation was expressed as the following equation:

$$\text{In}\ \frac{[E]}{[E_0]} = -\ 0.009t \qquad (12)$$

The value of the characteristic rate constant (K_{det}= 0.009 h^{-1}) determined from the fitting shown in Figure 3-14, was specific to the enzyme (Cellic® CTec 3) and substrate considered in this study. This is due to the unique enzyme-substrate behaviour during hydrolysis and fermentation (Ioelovich 2015). The predicted data from the first-order decay kinetics showed a close correlation to the experimental data as seen by the coefficient of determination, R^2 = 0.8255 (Figure 3-14).

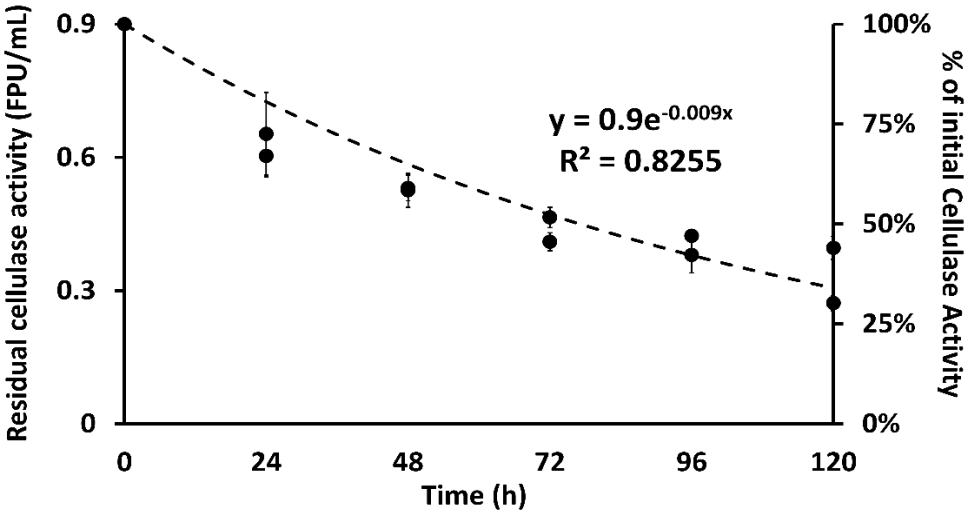


Figure 3-14: An exponential decay of cellulase activity during paper sludge fermentation; Experiment (solid dot), Model (broken line). Figure shows residual activity as well as the % of original activity on the secondary axis

Data presented in Figure 3-14 shows that at approximately 77 h, 50% of the initial cellulase activity was present and decreased to nearly 30% at the end of the fermentation (120 h). The half-life of cellulases estimated by Levine et al. (2010) (*T. reesei* cellulase mixture: 42.5h) is lower than what was estimated in this study (~77 h). This difference could possibly be attributed to Cellic® CTec 3 being a more recently developed cellulase and thus, could have higher stability compared to older generations of cellulase cocktails.

3.3.4.2 Enzymatic hydrolysis of paper sludge

A high enzyme dosage contributed to a greater degree of cellulose conversion of PS; the ratio of sugars released to the actual cellulose content of the PS. The maximum cellulose conversions were found to be about 60% and 90% with 5 and 10 FPU/gds of Cellic® CTec 3, respectively (Figure 3-15). The effective rate constant and order of the hydrolysis process were determined by fitting eq. (10) to experimental conversion data from the batch fermentation. The model parameters were estimated by performing a regression analysis on the experimental data. The accuracy of the model fit was examined by estimating the R^2 between the predicted data and experimental data. The R^2 values (0.9891 and 0.9921 for hydrolysis at 5 and 10 FPU/gds, respectively) suggest the kinetic model is accurate at predicting the course of PS hydrolysis (Table 3-2) and is in line with previous studies where hydrolysis yields increased with increasing enzyme dosage (Fan & Lynd 2007b).

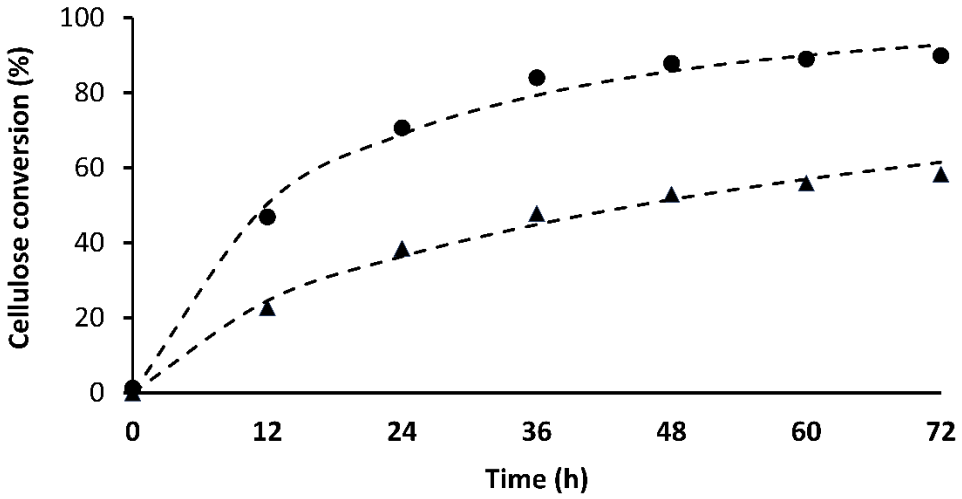


Figure 3-15: Model (broken line) and experimental (solid objects) hydrolysis profiles of 6% (w/w) PS with 5 (▲) and 10 (●) FPU/gds Cellic® CTec 3.

Table 3-2: Model parameters for enzymatic hydrolysis determined from the regression analysis of experimental data using eq. (10).

Parameters	Enzyme dosage	
	5 FPU/gds	10 FPU/gds
n	0.6809	0.7386
K	0.0519	0.1118
R^2	0.9891	0.9921

Ethanol concentrations were predicted using the time-course conversion data (eq. (10)) and assuming a constant ethanol yield on sugars (0.511 g ethanol /g sugars) during the fermentation experiment. According to Figure 3-16, the model accurately described the experimental data (R^2 = 0.9698) for the batch fermentation conditions used (6% solids loading, 10 FPU/gds Cellic® CTec 3, 72 h). The final ethanol concentration predicted by the model is approximately 20.1 g/L; which amounts to 91% of the theoretical maximum ethanol concentration and 8% higher than actual experimental data (Figure 3-16).

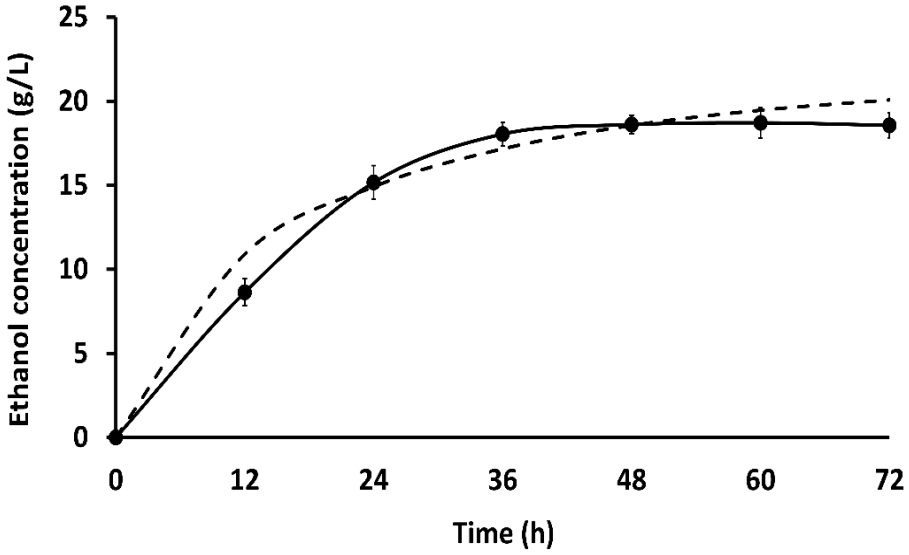


Figure 3-16: Ethanol concentration profile predicted by the model using parameters obtained from batch fermentation data versus experimental values (C); Experiment (solid object), Model (broken line)

3.3.4.3 Model validation - Cellulase recycling over multiple rounds of fermentation

The model accurately predicted ethanol concentrations in the early stages of the process (R0 and R1) but differed as recycling progressed (R2 and R3). As shown in Figure 3-17, there was no substantial difference between predicted and experimental ethanol concentrations for R0 and R1 (p-value (0.077) > 0.05). However, the opposite was observed for R2 and R3, apparently due to a substantial accumulation of lactic acid, which was not accounted for in the kinetic model, possibly resulting in a decrease in the overall ethanol yield for the recycle experiments. The ethanol yields predicted by the model for the overall recycling experiments were approximately 12% and 20% higher than experimental yields at 75% and 50% enzyme supplementations, respectively (Table 3-3). Despite these differences, the model prediction and actual ethanol yields were substantially higher (>40%) than what some authors have reported on PS fermentation (Boshoff et al. 2016; Donkor 2019; Gomes et al. 2018, 2016; Williams 2016) (Table 3-3). This improvement could be attributed to the high carbohydrate content (~70% w/w) and co-fermentation of sugars by the yeast used in this study (CelluX^{TM}4), which enabled the production of more ethanol per unit substrate compared to saccharification and fermentation with the yeast strains and substrates used in those studies.

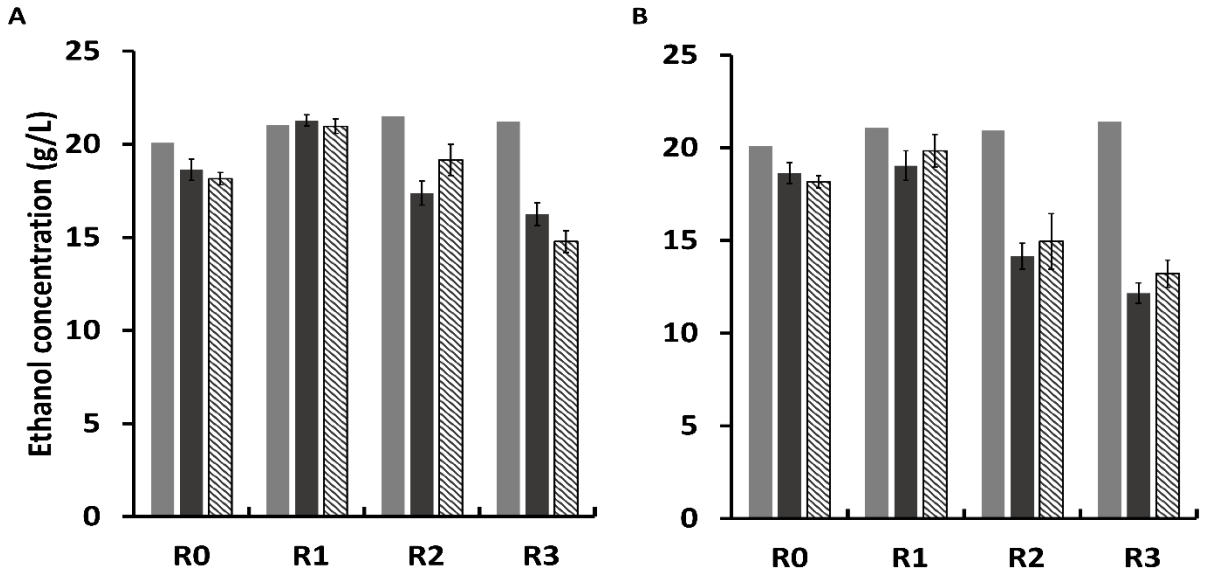


Figure 3-17: Final ethanol concentration over the entire recycle rounds based on model predictions and experimental scenarios (Model Supernatant Broth): 75% fresh enzymes (A) and 50% fresh enzymes (B). R0, R1, R2, R3 refers to the initial process and the first, second, and last rounds of recycling.

Model predictions on residual active enzymes revealed that enzyme activity was not the limiting factor to the degree of hydrolysis as there were substantial residual cellulase activities at the start and end of each round of fermentation (Table 3-4). Mass balance analysis also showed that final cellulose conversions reached approximately 93% and 95% at the end of fermentation when recycling supernatant and broth, respectively (Table 3-5). However, the lower ethanol concentrations reported for these latter rounds (R2 and R3) shows that, although the enzymes hydrolysed the paper sludge, the sugars released were not completely utilized by the yeasts for ethanol production but were also used by the LAB for lactic acid production.

In this study, the diversion of sugars towards the formation of products other than ethanol, i.e. lactic acid, as well as the antimicrobial activities of these organic acids, negatively affected the ethanol yields of the experimental scenarios considered (especially R2 and R3). Data presented in Table 3-5 shows that experimental ethanol yields would have improved if contamination was avoided (Albers et al. 2011), partly due to the fact that sugars lost to contamination would have increased the final ethanol concentrations for all recycling scenarios considered. Thus, the developed mathematical model would be more accurate at predicting ethanol concentrations for all the recycling rounds and scenarios if contamination can be controlled.

Table 3-3: Comparison of fermentation yield markers in this study to some reported literature on paper sludge fermentation

Non-recycle fermentation systems					
Glucan content (%)	**Solids loading (g/L)**	**Enzyme dosage (FPU/gds)**	**Ethanol conc. (g/L)**	**Ethanol yield (g eth. / g PS)**	**Reference**
58.2	180	20 (Viscamyl Flow-84.7FPU/mL)	49.6	0.276	(Donkor 2019)
55.7		20 (Optiflow RC 2.0-130FPU/mL)	34.2	0.190	(Boshoff et al. 2016)
58.2		20 (Viscamyl Flow-140FPU/mL)	46.8	0.260	(Williams 2016)
Recycle fermentation systems					
Glucan content (%)	**Solids loading (g/L)**	**Enzyme dosage (FPU/gds)**	**Total ethanol conc. (g/L)**	**Ethanol. yield (g eth. / g PS)**	**Reference**
30.98	50	20 (Celluclast-45FPU/mL, +20% fresh enzymes)	23.59[i]	0.118	(Gomes et al. 2016)
		20 (Celluclast-45FPU/mL, +30% fresh enzymes)	27.57[i]	0.138	
27.1	220	20 (Celluclast-45FPU/mL, +50% fresh enzymes)	88.47[i]	0.101	(Gomes et al. 2018a)
59.17	60	10 (Cellic® CTec 3-148FPU/mL, +75% fresh enzymes)	73.53[1]	0.306[a]	This study
			73.06[2]	0.304[b]	
			83.85[3]	0.349[c]	
		10 (Cellic® CTec 3-148FPU/mL, +50% fresh enzymes)	63.98[1]	0.267[a]	
			66.16[2]	0.276[b]	
			83.46[3]	0.348[c]	

eth refers to ethanol
conc refers to concentration
[i] Total ethanol produced from 4 batches of PS fermentations with enzyme recycling
[1, 2, 3] Total ethanol produced for the entire recycle rounds (Supernatant, Broth, and model predictions respectively)
[a, b, c] Overall ethanol yield (g total ethanol/ g paper sludge fed) for the entire recycle rounds (Supernatant, Broth, and model predictions respectively)

Table 3-4: Model predictions of residual activities for 75% and 50% enzyme supplementation scenarios

Time (hrs)	**Residual active enzymes (FPU/mL)**							
	75% enzymes				**50% enzymes**			
	Round 0	**Round 1**	**Round 2**	**Round 3**	**Round 0**	**Round 1**	**Round 2**	**Round 3**
0	0.6000	0.5285	0.5191	0.5179	0.6000	0.4569	0.4195	0.4097
24	0.4834	0.4258	0.4183	0.4173	0.4834	0.3682	0.3380	0.3301
48	0.3895	0.3431	0.3370	0.3362	0.3895	0.2966	0.2723	0.2660
72	0.3139	0.2764	0.2715	0.2709	0.3139	0.2390	0.2194	0.2143

Table 3-5: Mass balance for PS fermentation with enzyme recycling in 250mL flasks

Operating conditions					
Cellic® CTec 3 (FPU/gds)			10		
Mass of PS fed each round (g)			6		
Glucan fraction (% w/w)			59.17		
Xylan fraction (% w/w)			11.53		
Total glucose fed each round (g)			3.55		
Total xylose fed each round (g)			0.69		
			Round 0		
	Supernatant recycle		**Broth recycle**		**Model prediction**
Lactic acid (g/L)	1.19		1.64		-
Acetic acid (g/L)	3.04		3.08		-
Ethanol concentration (g/L)	18.63[1] (21.68)[2]		18.15[1] (21.47)[2]		20.08
Theoretical ethanol yield (%)	85.9[1]		83.7[1]		92.6
Ethanol yield (g ethanol/g sugars)	0.394[1]		0.384[1]		0.425
Productivity (g/(L.hr))	0.259[1]		0.252[1]		0.279
Cellulose conversion (%)	89.9		90.1		-
			Round 1		
	Supernatant +75%	**Supernatant +50%**	**Broth +75%**	**Broth +50%**	**Model prediction**
Lactic acid (g/L)	0.57	2.97	0.96	2.75	-
Acetic acid (g/L)	0.28	1.39	0.40	0.60	-
Ethanol concentration (g/L)	21.28[1] (21.80)[2]	19.04[1] (21.70)[2]	20.97[1] (21.79)[2]	19.84[1] (21.76)[2]	21.03[3] (21.07)[4]
Theoretical ethanol yield (%)	98.2[1]	87.8[1]	96.7[1]	91.5[1]	97.0[3] (97.2)[4]
Ethanol yield (g ethanol/g sugars)	0.450[1]	0.403[1]	0.444[1]	0.420[1]	0.445[3] (0.446)[4]
Productivity (g/(L.hr))	0.296[1]	0.264[1]	0.291[1]	0.276[1]	0.292[3] (0.293)[4]
Cellulose conversion (%)	94.6	94.7	98.5	98.2	-
			Round 2		
Lactic acid (g/L)	5.25	9.22	3.41	7.72	-
Acetic acid (g/L)	2.06	3.18	1.10	3.14	-
Ethanol concentration (g/L)	17.38[1] (21.78)[2]	14.15[1] (21.53)[2]	19.16[1] (21.83)[2]	14.96[1] (21.51)[2]	21.52[3] (20.92)[4]
Theoretical ethanol yield (%)	80.2[1]	65.3[1]	88.4[1]	69.0[1]	99.3[3] (96.5)[4]
Ethanol yield (g ethanol/g sugars)	0.368[1]	0.299[1]	0.405[1]	0.317[1]	0.455[3] (0.443)[4]
Productivity (g/(L.hr))	0.241[1]	0.197[1]	0.266[1]	0.208[1]	0.299[3] (0.291)[4]
Cellulose conversion (%)	96.5	93.7	99.5	92.8	-
			Round 3		
Lactic acid (g/L)	7.82	13.50	8.50	11.57	-
Acetic acid (g/L)	1.34	3.09	2.82	3.14	-
Ethanol concentration (g/L)	16.24[1] (21.42)[2]	12.16[1] (21.72)[2]	14.78[1] (21.50)[2]	13.21[1] (21.79)[2]	21.22[3] (21.39)[4]
Theoretical ethanol yield (%)	74.9[1]	56.1[1]	68.2[1]	60.9[1]	97.9[3] (98.7)[4]
Ethanol yield (g ethanol/g sugars)	0.344[1]	0.257[1]	0.313[1]	0.279[1]	0.449[3] (0.453)[4]
Productivity (g/(L.hr))	0.226[1]	0.169[1]	0.205[1]	0.183[1]	0.295[3] (0.297)[4]
Cellulose conversion (%)	95.4	92.6	92.3	94.7	-

[1] Determined from fermentation broth with HPLC
[2] [Ethanol produced by sugars fed + Ethanol lost due to contamination]
[3] Model predictions for 75% enzyme supplementation scenario
[4] Model predictions for 50% enzyme supplementation scenario

3.3.4.4 Effect of recycling on enzyme productivity

Enzyme recycling increased the total enzyme productivity. Enzyme productivity is defined as the total amount of ethanol produced over the course of the fermentation per unit enzyme dosage added to the experiment. For all experimental scenarios, enzyme productivities ranged from 0.03-0.048 and 0.03-0.071 g ethanol/ FPU for 75% and 50% enzyme supplementation respectively (Figure 3-18).

In comparison to the enzyme productivity corresponding to the maximum theoretical ethanol concentration (22 g/L), enzyme productivity almost doubled (0.03 to 0.071 g ethanol/ FPU) when the amount of fresh enzymes added was reduced to 50% (Figure 3-18b); allowing up to 38% saving on total enzyme dosage for the entire recycle experiment. As shown in Figure 3-18a, slight improvement (~30%) in enzyme productivities were observed when the enzyme dosage for the subsequent rounds increased (75% enzyme supplementation).

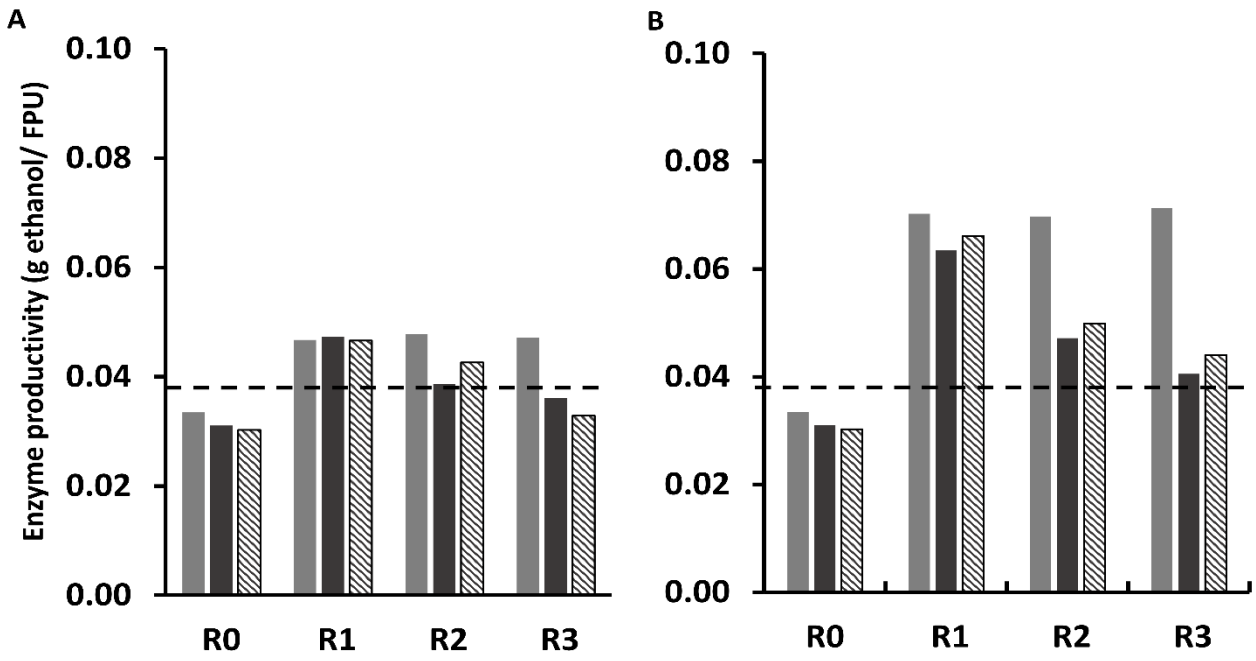


Figure 3-18: Enzyme productivities over the entire recycle rounds based on model predictions and experimental scenarios (Model Supernatant Broth): (A) 75% fresh enzymes and (B) 50% fresh enzymes. R0, R1, R2, R3 refers to the initial process and the first, second, and last rounds of recycling. The broken line represents the enzyme productivity for the maximum theoretical ethanol concentration (22 g/L) and an initial dosage of 10 FPU/gds with no recycling.

3.3.5 Scaled-up fermentation with enzyme recycling in 5L bioreactors

This section summarizes the performance of recycling the clarified supernatant at high solids loading (18% w/w). Experimental results were compared to model predictions and some limitations to the recycling scheme were identified and discussed below.

3.3.5.1 Effect of enzyme dosage on ethanol yields

The effect of enzyme dosage on the ethanol yields was investigated at higher solid loadings (18% w/w) using an appropriate bioreactor system, where continuous mixing allowed fermentation to proceed at solids loadings higher than the 6% w/w applied in shake flask fermentations. After 7 days (168 h) of fermentation, 5 and 10 FPU/gds of Cellic® CTec 3 enzymes produced 56.6 ± 0.38 and 61 ± 0.84 g/L of ethanol corresponding to 88% and 95% of the theoretical ethanol yields, respectively (Figure 3-19). In comparison to previous fermentations on a similar feedstock and solids loading, the ethanol concentration obtained for 5 FPU/gds of Cellic® CTec 3 was at least 14% higher than those reported by Boshoff 2015, Donkor 2019, and Williams 2016 (Table 3-3). The lower enzyme dosage (5 FPU/gds) was therefore selected to evaluate cellulase recycling at high-solids fermentation, since it could produce comparable ethanol yields and could be beneficial to the economic viability of the process.

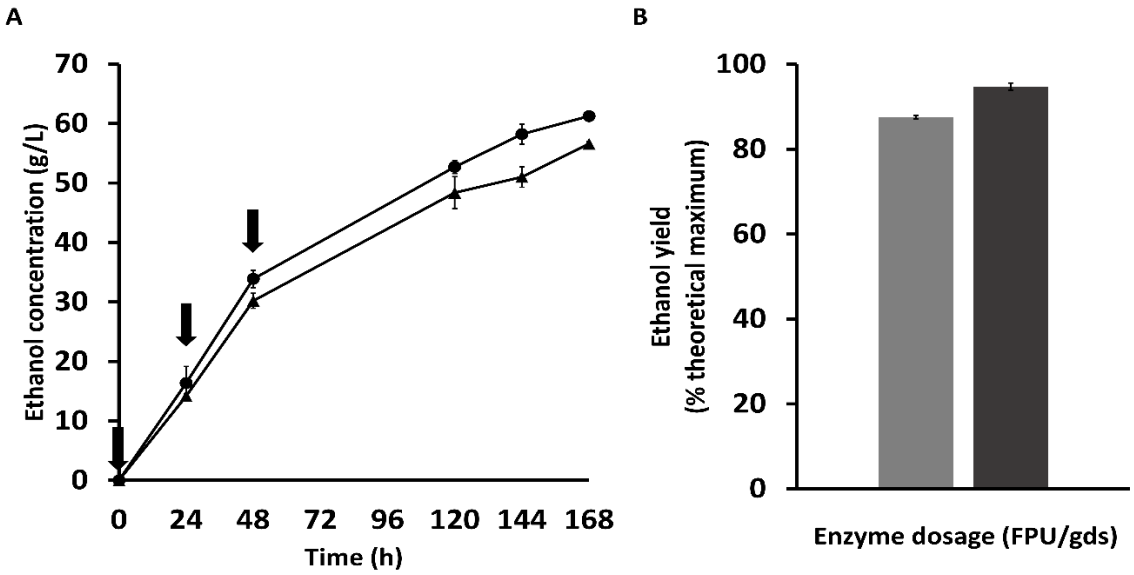


Figure 3-19: Ethanol concentration profiles at varying enzyme dosages (A) (▲5 FPU/gds ● 10 FPU/gds), arrows represent feeding points of solids (6% w/w); Final theoretical ethanol yields obtained after 168h of fermentation (B) (■5 FPU/gds ■10 FPU/gds). Experimental data are expressed as mean ± standard error of two independent experiments.

3.3.5.2 Recycling cellulases in the Supernatant

The residual activities measured in the supernatant during the first round of fermentation (R0) decreased from 88.3 ± 4.8% after 24 h to 34.8 ± 1.7% at the end of the experiment (168 h) (Figure 3-20a). To test the feasibility of achieving similar ethanol production by enzyme recycling, the supernatant was removed aseptically at the end of the first round of fermentation (R0), added to the same amount of substrate, and supplemented with different enzyme loadings (0%, 50%, and 65% of the initial dosage, 5 FPU/gds) for the next round of fermentation (R1) (Figure 3-20b).

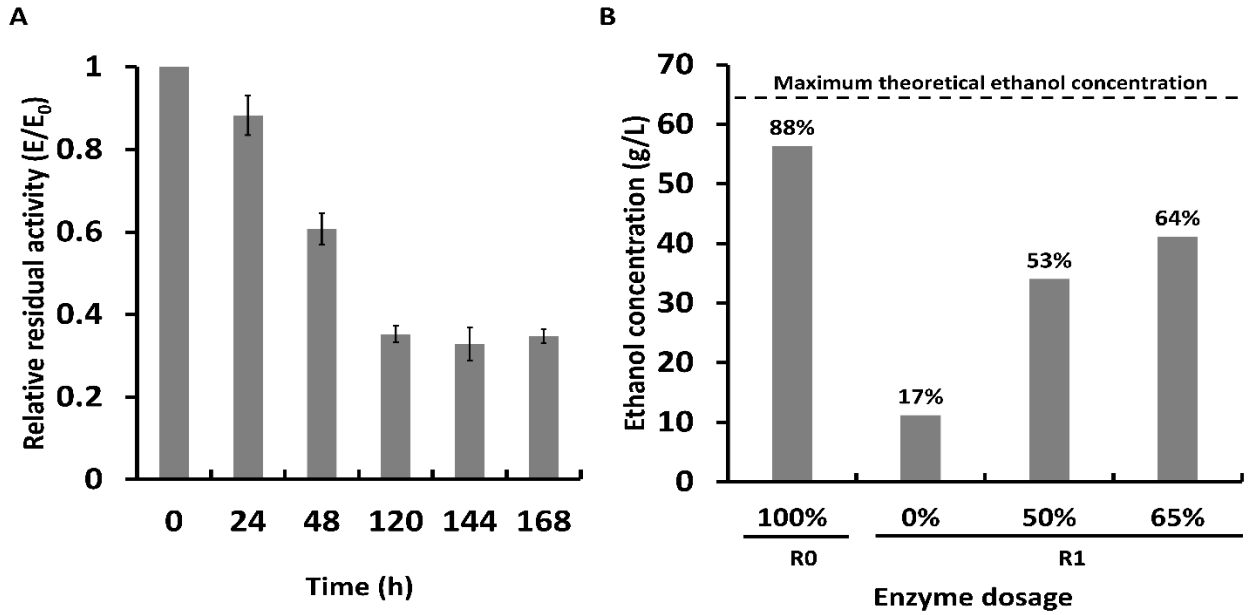


Figure 3-20: Residual cellulase activity during high-solids fermentation, data points represent the means of duplicate values with standard error bars (A); Effect of fresh enzyme dosages on ethanol production in the first round of recycling (R1) (B).

Ethanol concentration in the first round of recycling (R1) increased with increasing enzyme dosage (Figure 3-20b). Without enzyme supplementation (0%) in R1, the ethanol concentration reached a maximum of 11.21 ± 0.89 g/L (approximately 17% of the theoretical maximum). Although this ethanol concentration was very low compared to the other enzyme dosages (50% and 65%), this is still important, as it proves that the residual enzymes in the supernatant are still viable and can be reused. With approximately 35% of the initial enzyme activity being retained in the supernatant after fermentation (R0) (Figure 3-20a), it was assumed that supplementing 65% fresh enzyme would be able to achieve similar levels of ethanol as R0 (100% enzyme). However, this was not observed as ethanol concentration decreased by 24% relative to R0 (Figure 3-20b).

Also, the production of ethanol in each recycle step (R1 and R2) did not reach similar levels achieved in the first round of fermentation (R0) for both enzyme dosages (Figure 3-21). This observation contradicts the findings of Xue et al. (2012), who reported that the accumulation of enzymes during recycling improves enzymatic hydrolysis. It is also worth pointing out that in these experiments, the concentrations of inorganic acids (lactic and acetic acids) were below inhibitory levels (< 2 g/L), and therefore, the effect of sugar diversion was negligible.

Altogether, the observed decrease in the amount of ethanol produced per unit of recycled enzyme activity shows that, although significant enzyme activity (as measured with the FPU assay) remains in the supernatant, the enzyme must have lost much of its potency. The FPU assay used for measuring the enzyme activities did not accurately represent the true catalytical ability of the enzyme to liberate sugars from paper sludge for fermentation. This is because the assay uses an ideal substrate i.e. filter paper and therefore, its degree of digestibility could not be obtained with a more complex substrate like paper sludge.

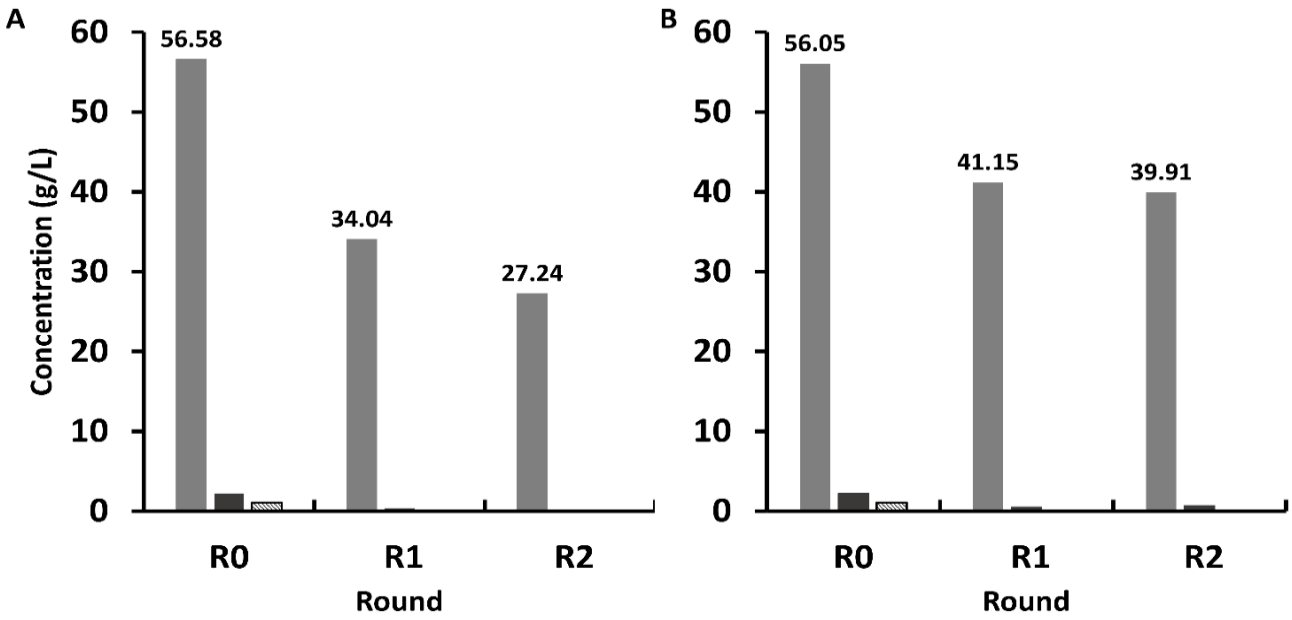


Figure 3-21: Concentration of products (■ Ethanol ■ Lactic acid ■ Acetic acid) during supernatant recycle with 50% (A) and 65% (B) enzyme supplementation. R0, R1, R2 refers to the initial process and the first and last rounds of recycling.

High-solids fermentation coupled with enzyme recycling resulted in the build-up of ethanol in the fermentation broth (60-70 g/L) (Figure 3-22). Aside from saving on enzyme consumption, high ethanol concentrations in the broth can make positive contributions to the economic viability of the process by minimizes the energy consumption for distillation (Kang et al. 2011). However, this concentration of ethanol could have possibly affected enzymatic hydrolysis during subsequent fermentations (R1 and R2) and thus, limited ethanol yields in the recycling rounds as previously stated. Chen & Jin (2006) reported a similar trend where ethanol (up to 70 g/L) inhibited both the hydrolysis efficiency and cellulase activity during SSF of crystalline cellulose. Therefore, the decline in enzyme performance and ethanol production in the recycling rounds could suggest the possibility of enzyme inhibition by ethanol during the multiple fermentations (Østergaard et al. 2015).

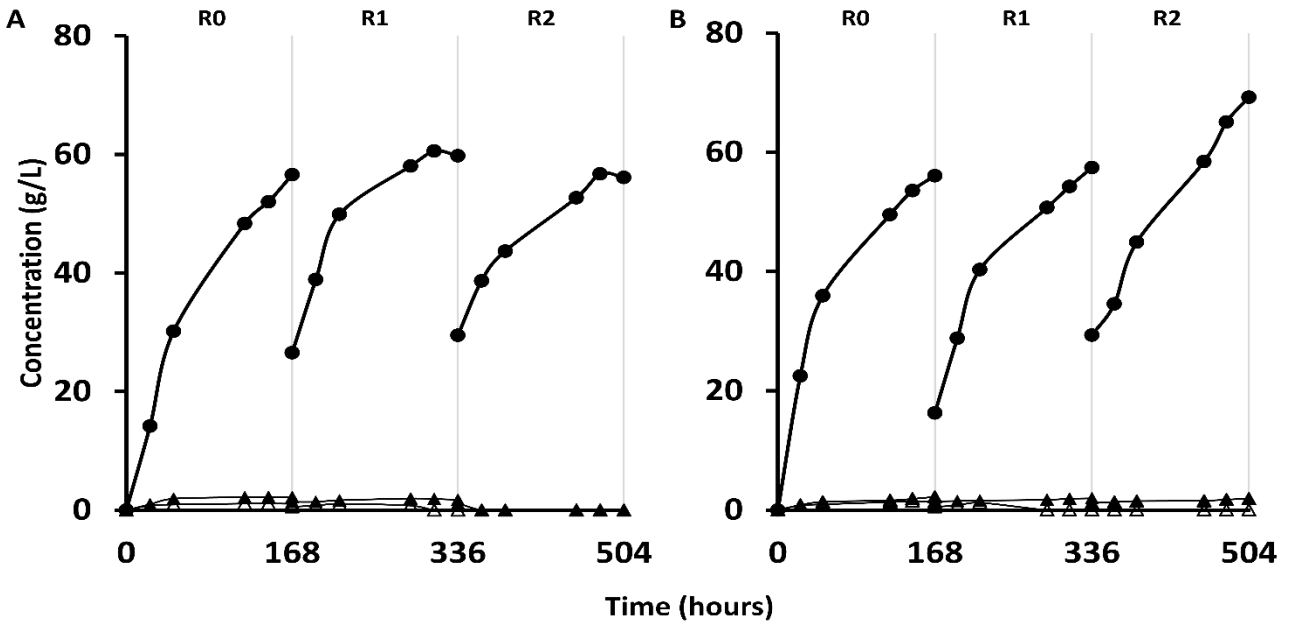


Figure 3-22: Concentration of Ethanol (●), Lactic acid (▲), and Acetic acid (△) during supernatant recycle with 50% (A) and 65% (B) enzyme supplementation.

The technique for transferring supernatant in the bioreactor (sterile tube and a pump, Figure 3-3) did not work perfectly because some solids, i.e. mainly unhydrolyzed carbohydrates and lignin, were transferred along with the supernatant. This carryover of solids reduced the free water content in the reactor, increased the viscosity of the slurry, and enhanced the slowdown of enzymatic hydrolysis (Modenbach & Nokes 2013).

The effect of the introduction of such lignin-rich residues (Figure 3-23) could not be alleviated by the increase in the hydrolysis time (multiple hydrolysis steps). This is because these recalcitrant substrates increase the unproductive adsorption of cellulases which contributes to a reduction in the glucose conversion rate (Wallace et al. 2016) for ethanol production as observed in the recycling experiments (R1 and R2).

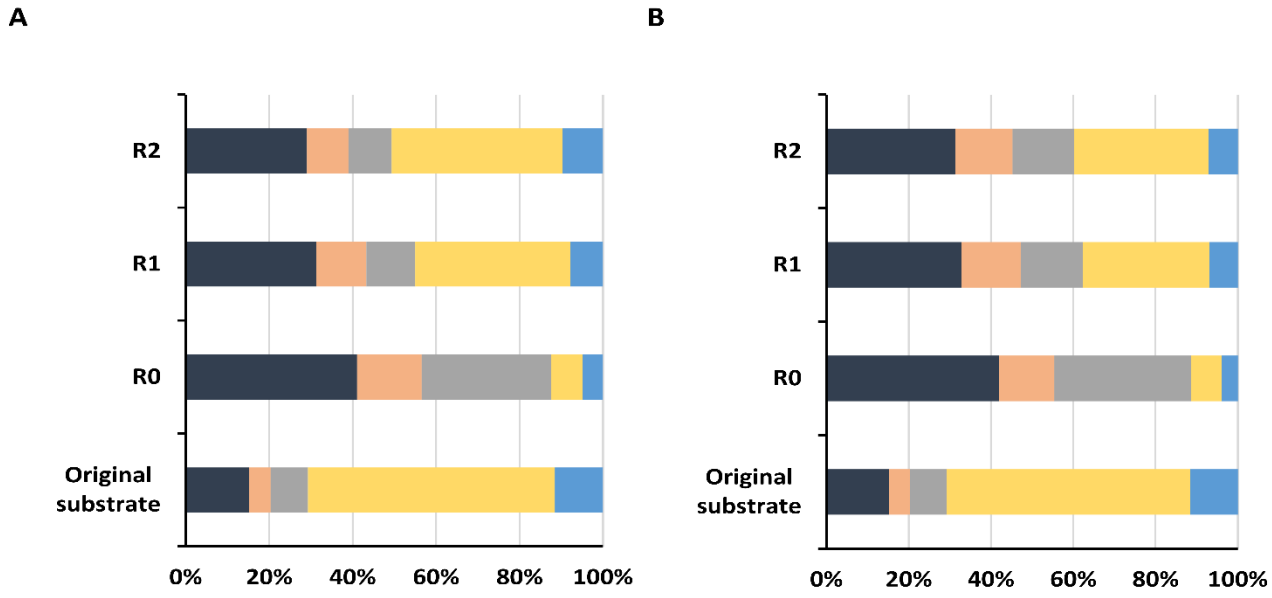


Figure 3-23: Compositional analysis (Ash, Extractives, Lignin, Cellulose, Hemicellulose) of dried residues from recycling in 5L bioreactors

3.3.5.3 Kinetic model validation

To determine the validity of the models proposed in section 3.2.3, experimental data obtained in the 5L bioreactors were compared to the kinetic model predictions. The results obtained for the model are based on regression analysis for both enzyme decay and PS hydrolysis.

There was no substantial variation between the model predictions and the first fermentation experiment (R0). However, the yields obtained during the recycling stages (R1 and R2 respectively) were approximately 37% and 43% lower than from the model's prediction (Figure 3-24). This can be attributed to the inefficient enzymatic hydrolysis performance discussed earlier.

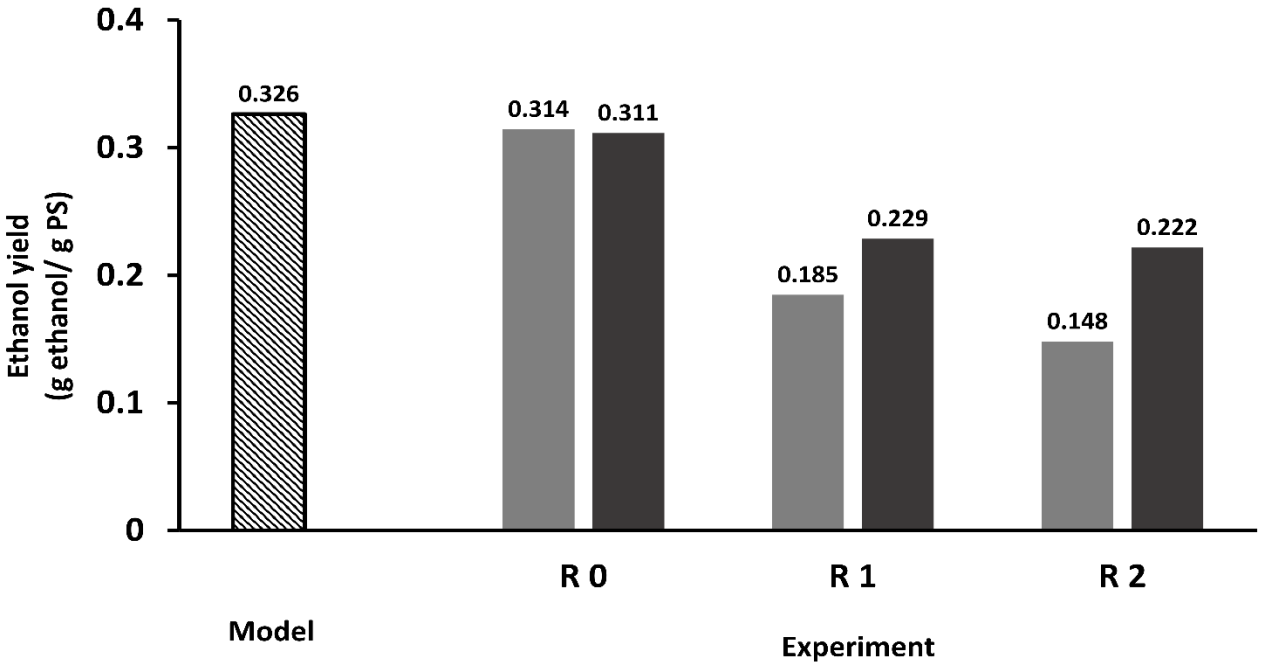


Figure 3-24: Comparison of the final ethanol yields based on model predictions (▨)and experimental values over the entire recycle rounds (■ +50% fresh enzyme ■+65% fresh enzyme). R0, R1, R2 refers to the initial process and the first, and last rounds of recycling.

Model predictions of residual cellulase activity show a strong fit with the experimental data (R^2 = 0.973 and R^2 = 0.944) for both enzyme dosages respectively (Figure 3-25). However, the retention of cellulase activity of Cellic® CTec 3 as determined with the assay, did not correlate with the conversion of total cellulose during the recycling experiments (Table 3-7). This is an indication that other enzyme components may have been depleted/deactivated during the fermentation thus, resulting in the reduction of enzyme synergism and glucose production (Modenbach & Nokes 2013).

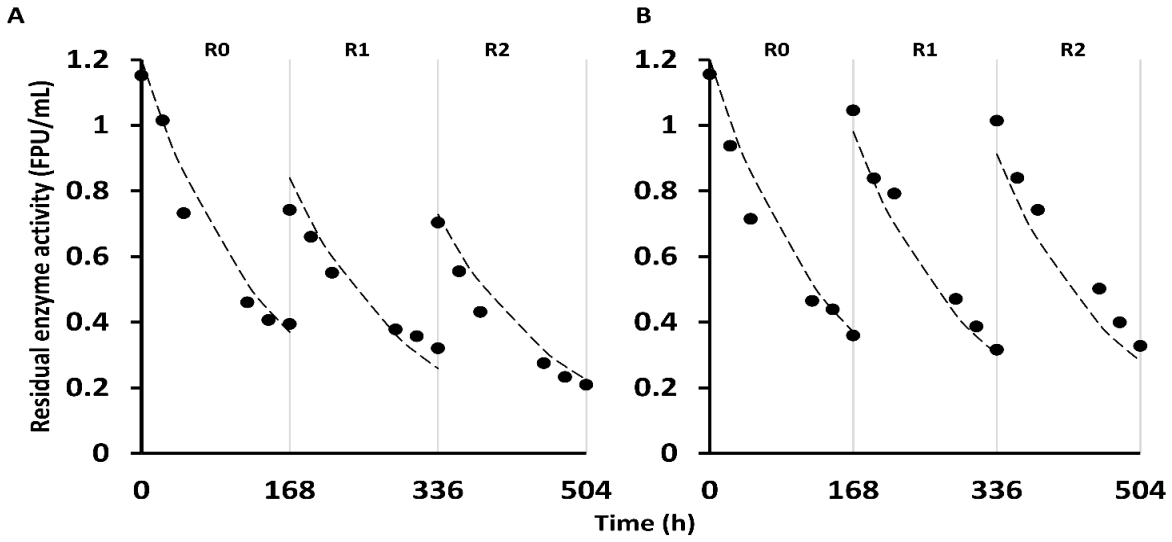


Figure 3-25: Residual enzyme activities during paper sludge fermentation with 50% fresh enzymes (A) and 65% fresh enzymes (B); Experiment (solid object). Model (broken line)

Cellulase cocktails depend to a large extent on the synergism between different enzymes present in the cocktails. This means, for example, that cellulase (CBH and EG) enzymes cannot hydrolyse lignocellulose efficiently without hemicellulases (xylanases) and other accessory enzymes such as CBMs and lytic polysaccharide mono-oxidases (LPMOs), etc (Modenbach & Nokes 2013). The FPU assay only measured cellulase activity and did not account for the activities of the other supplementary and accessory enzymes. Hence, the loss of these enzymes to the point where enzyme synergism disappears was not known. The deactivation of accessory enzymes is more likely as a mechanism of the enzymes and could be the cause of the reduced enzyme performance, even though the cellulase activity remains in the culture.

Hu et al. (2018) and Zhai et al. (2018) recently showed that the exoglucanase and xylanase activities of Cellic® CTec 3 were easily less stable and susceptible to depletion during cellulose hydrolysis. It is worth mentioning that, the retention of xylose in the paper sludge residue after fermentation could indicate the loss of xylanases in the enzyme cocktail (Table 3-6). Adding more enzyme would have increased the hydrolysis yields, but it tends to reduce the total amount of ethanol produced over the course of the fermentation per total enzyme dosage added (Figure 3-18) and defeats the primary objective of enzyme recycling, which is to minimize enzyme dosage in PS fermentation. However, enzyme recycling can be further improved by optimizing individual enzyme activities and supplementation of specific depleted enzyme components.

Table 3-6: Solid residues from recycling experiments in 5L bioreactors

Recycle round	+50% fresh enzymes				+65% fresh enzymes			
	Glucans in residue (w/w)	Xylans in residue (w/w)	Glucan conversion (%)	Xylan conversion (%)	Glucans in residue (w/w)	Xylans in residue (w/w)	Glucan conversion (%)	Xylan conversion (%)
0	7.48	4.94	93.6	78.1	7.35	3.93	95.9	88.9
1	37.12	7.89	58.66	55.6	30.67	6.93	73.41	69.2
2	40.88	9.74	45.9	33.9	32.59	7.18	71.3	67.5

The decline of enzyme performance also resulted in a reduction of the overall ethanol yield and productivity as recycling progressed (Table 3-7). Overall ethanol yields for the highest enzyme supplementation (65% fresh enzymes) decreased from 280 to 200 kg ethanol/ton dry PS for R0 and R2 respectively. However, a 23% saving on the overall enzyme dosage was achieved by recycling the clarified supernatant and reducing the fresh enzyme dosages to 65% in the 5L bioreactors, Although a much lesser enzyme dosage (5 FPU/gds) was applied, the ethanol yields obtained for supplementing 65% fresh enzymes, 0.222 g ethanol/g PS - 0.311 g ethanol/g PS (Table 3-7) were higher than previous recycling studies by Gomes et al. (2016; 2018a), 0.101 g ethanol/g PS - 0.138 g ethanol/g PS (Table 3-3).

Table 3-7: Mass balance for PS fermentation with supernatant recycling in 5L bioreactors

Operating conditions						
Enzyme dosage (FPU/gds)			5			
Percentage dry PS fed (% w/w)			18			
Mass of dry PS fed (g)			450			
Glucan fraction (%)			59.17			
Xylan fraction (%)			11.53			
Total glucose fed (g)			266.27			
Total xylose fed (g)			51.89			
	+50% fresh enzymes			**+65% fresh enzymes**		
	Round 0	**Round 1**	**Round 2**	**Round 0**	**Round 1**	**Round 2**
Glucose in residue (g)	17.16	110.20	144.01	10.96	70.74	76.48
Soluble residual glucose (g)	-	-	-	-	-	-
Total glucose consumed (g)	249.11	156.07	122.26	255.30	195.52	189.79
Conversion of total cellulose (%)	93.6	58.6	45.9	95.9	73.4	71.3
Xylose in residue (g)	11.34	23.04	34.32	5.76	15.99	16.84
Soluble residual xylose (g)	-	-	-	-	-	-
Total xylose consumed (g)	40.54	28.85	17.57	46.13	35.90	35.05
Conversion of total xylose (%)	78.1	55.6	33.9	88.9	69.2	67.5
Lactic acid (g/L)	2.19	0.36	-	2.22	0.51	-
Acetic acid (g/L)	1.09	-	-	1.08	-	
Carryover ethanol (g/L)	-	26.55	29.48	-	16.29	29.31
Final ethanol concentration (g/L)	56.58	59.79	56.13	56.05	57.44	69.22
Actual ethanol produced (g/L)	56.58	33.24	26.66	56.05	41.15	39.91
Theoretical ethanol yield (%)	87.0	51.1	40.9	86.2	63.3	61.4
Productivity (g/L.hr)	0.337	0.198	0.159	0.334	0.245	0.238
Ethanol yield (g ethanol/g glucose consumed)	0.512	0.480	0.491	0.495	0.474	0.474
Ethanol yield (g ethanol/g glucose fed)	0.479	0.281	0.225	0.474	0.348	0.338
Ethanol yield (g ethanol/g sugars fed)	0.399	0.234	0.188	0.395	0.290	0.281
Ethanol yield (g ethanol/g PS)	0.314	0.185	0.148	0.311	0.229	0.222
Overall ethanol yield (kg ethanol/ton dry PS)	283.24	166.38	133.43	280.57	205.99	199.77

3.3.6 The limiting effect of residual ethanol on cellulase recycling

Although SSF eliminates feedback inhibition of glucose and cellobiose on enzymatic hydrolysis, residual ethanol has been reported to significantly reduce hydrolysis yields (Philippidis et al. 1993; Podkaminer et al. 2011). For example, the concentration of ethanol before the first recycle (R0) was approximately 20 g/L even at low-solid loading (6% w/w), therefore the decrease in ethanol yields could be a result of the feedback inhibition of ethanol (Figure 3-13). Hence, the inhibition of cellulases by ethanol was investigated by adding various concentrations of ethanol to the enzymatic hydrolysis of PS.

As the addition of ethanol (residual ethanol concentration) increased from 0 to 30 g/L, hydrolysis yields reduced from 92% to 48% respectively (Figure 3-26a). On the contrary, the increasing residual ethanol concentration had a marginal effect on the loss of overall cellulase activity at 37 °C. Adding 30 g/L only heightened the loss of cellulase activity within the first 24 h. The final residual enzyme activity obtained for adding 30 g/L of ethanol was 0.48 ± 0.02, only 4% lower than that of the control (0 g/L); 0.52 ± 0.02 (Figure 3-26b). High-solids fermentation with enzyme recycling increased the concentration of ethanol to approximately 70 g/L in the fermentation broth (Figure 3-22) and could have possibly influenced the slowdown of hydrolysis efficiency during SSF (Chen & Jin 2006). This bottleneck in enzyme recycling can be overcome with the application of *in-situ* vacuum distillation (Zhang et al. 2017) to reduce residual ethanol concentration during SSF of PS.

However, the marginal loss of overall cellulase activity (FPUs) observed at low- solids SSF suggests that the decrease in enzymatic hydrolysis efficiency could have been predominated by the time-wise loss of enzyme synergism, as compared to the inhibitory effect of ethanol concentration (Malgas et al. 2017). The exact pattern for the time-effect of enzyme synergy during the hydrolysis of complex lignocellulosic material remains contradictory as several factors (substrate type, choice of enzyme, protein interaction, pretreatment, etc.) have been identified to affect the optimum synergistic interaction between enzymes (Van Dyk & Pletschke 2012).

For instance, Andersen et al. (2008) argued that a high level of enzyme cooperation exists in the early stages of hydrolysis but decreases towards the end of the process. The reason for this pattern is that a high degree of enzyme synergy is required to unravel the lignocellulose material. But as more active sites become accessible, the degree of cooperation between enzymes reduces (Van Dyk & Pletschke 2012). Contrarily, Jung et al. (2008) reported a positive correlation between hydrolysis time and enzyme synergy. They argued that there are limited binding sites for enzymes during the early stages of hydrolysis. Therefore, as hydrolysis progresses and more cleavage sites become accessible, the degree of synergistic effect increases as enzymes that were initially shielded gets access to the substrate (Malgas et al. 2017).

However, it is noteworthy to mention that the exact mechanism of the effect of time on the synergistic association between enzymes during PS hydrolysis was not investigated in this study. The exact nature of loss of enzyme potency or synergy needs to be evaluated to improve enzymatic hydrolysis and further expand the benefit of enzyme recycling in PS fermentation

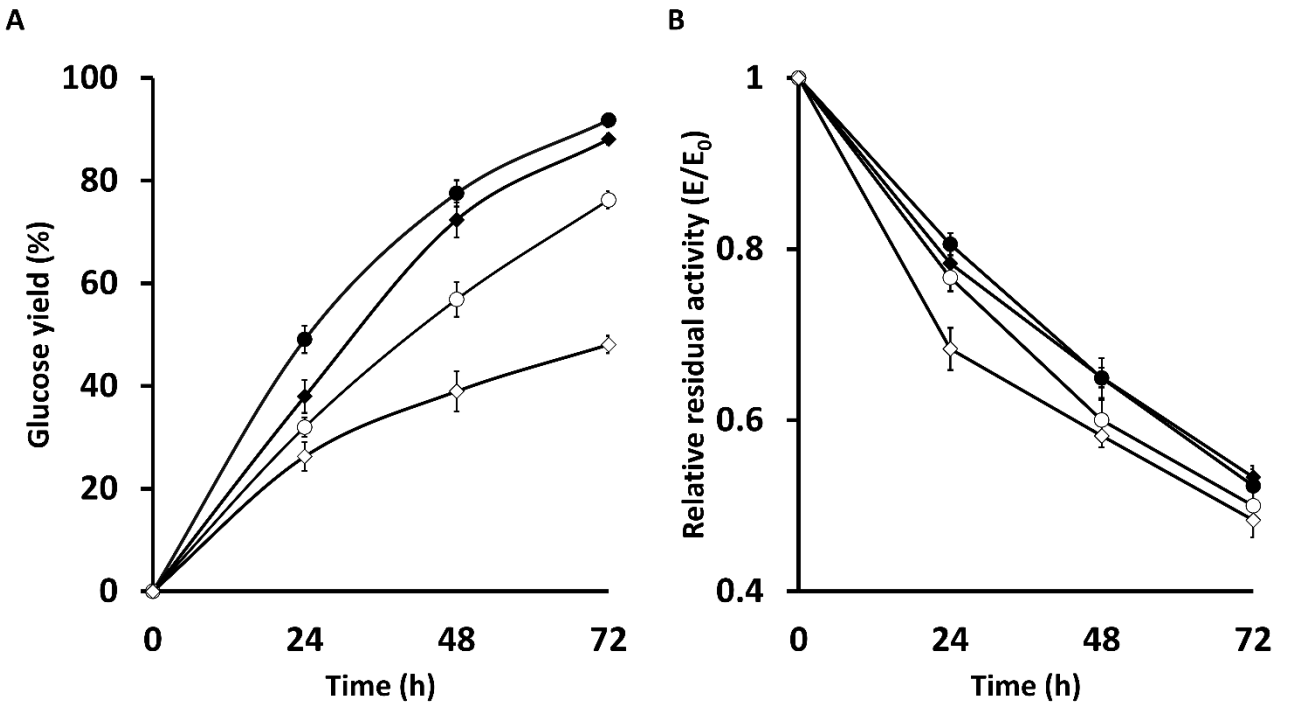


Figure 3-26: Effect of residual ethanol on the glucose yields from paper sludge hydrolysis at 37 °C (A); Effect of residual ethanol on cellulase activity (B): 0 g/L (●), 10 g/L (◆), 20 g/L (○), 30 g/L (◇). Experimental data is expressed as the mean of two independent experiments with standard error bars.

Chapter 4 Conclusions and Recommendations

The main aim of this study was to determine if significant savings on enzyme dosage required in the simultaneous saccharification and fermentation (SSF) of paper sludge can be achieved by recycling enzymes in the supernatant or whole fermentation broth. This was achieved by developing kinetic models that predict ethanol concentrations and the loss of cellulase activity during the process. Model predictions were validated experimentally based on the performance of the recycling schemes (supernatant and broth recycling) over multiple rounds while varying fresh enzyme supplementation.

4.1 Conclusions

The conclusions from this study are summarized below relating to the Aims and objectives outlined in Section 2.10.

1. *PS fermentation with enzyme recycling: Clarified supernatant vs whole broth*

From the available enzyme cocktails, the retention of enzyme activities was significantly higher with Cellic® CTec 3 after PS fermentation. After 72 h of fermentation, approximately 48 and 40% of the initial enzyme activity of Cellic® CTec 3 was located in the supernatant and broth respectively, signifying the possibility of recycling residual enzymes and minimizing fresh enzyme dosage in the subsequent rounds. Although a considerably high residual active enzyme concentration was measured in the supernatant, recycling either supernatant or broth did not prove any significant difference, evident from their respective ethanol yields. Whole broth recycle can make reductions to the inoculum required for fermentation as it contains yeast cells and enzymes. However, the inevitable buildup of solids (ash, unconverted cellulose, and lignin) during broth recycling could have an inhibitory effect on cellulose hydrolysis and fermentation. The accumulation of solids also indicates that broth recycling would not only lead to decreased ethanol yields but also increase the capital cost for larger process equipment and energy demands for mixing and pumping. The best trade-off for process intensification, i.e. high solid loadings and low enzyme dosage, however, was to recycle the enzymes in the supernatant as it eliminates solids accumulation and produces satisfactory ethanol yields.

Irrespective of the dosage of fresh enzymes added lactic acid contamination affected ethanol yields for the recycle schemes tested. Sterilisation of the PS solids before fermentation, by storage of dried samples in airtight bags and heat sterilization before fermentation, could not eliminate the background microbial load in the substrate. Since the trends in ethanol yields were similar for all the enzyme supplementations, it indicates that lactic acid bacteria (LAB) outperformed the yeast cells for the carbon sources that were released during enzymatic hydrolysis. Lactic acid concentrations of 2 g/L have been reported to inhibit *S. cerevisiae* growth rate and ethanol formation. Therefore, 8 g/L – 12 g/L of lactic acid measured during the recycling experiments could have inhibited the yeast performance, resulting in the decrease in ethanol yields.

2. *Fed-batch SSF with supernatant recycling*

The optimized process for high-solids fermentation (18% (w/w) solid loading and 5 FPU/gds enzyme dosage) with enzyme recycling resulted in the build-up of ethanol in the fermentation broth (~70 g/L), which is crucial to reaching an economically viable fermentation process. However, there was a reduction in the overall ethanol yields as recycling progressed; from 280 to 200 kg ethanol/ton dry PS for R0 and R2 respectively. The observed decrease in ethanol production per unit of recycled enzyme activity despite the retention of cellulase activity (FPUs) signifies that, the enzymes (cellulases, hemicellulases, accessory enzymes, etc.) must have lost their synergy. This mechanism of the enzymes affected the overall enzyme potency during the course of the fermentation.

3. *Kinetic model development for enzyme recycling in PS fermentation*

The kinetics of enzyme activity decay and enzymatic hydrolysis of PS were modeled separately. The loss of enzyme activity during multiple fermentations was proposed to follow a single-step first-order irreversible reaction in which the residual active enzymes during the fermentation would depend on the residual enzymes from the preceding reactor and the enzyme activities supplemented at the beginning of the fermentation. The model proposed for PS hydrolysis considered the rate of cellulose conversion and was used for generating data on ethanol production by assuming a constant yield of ethanol from sugars, an approach that has not yet been considered for PS fermentations. In comparison to other models found in literature; Levine et al. (2010) and Shao et al. (2007, 2009), our approach eliminates complex mathematical optimizations used for generating kinetic parameters.

The model was tested for supernatant and broth recycling at different enzyme supplementations over multiple recycling rounds. The conversion rate and overall cellulase activity (FPUs) as a function of time was measured during PS fermentation. The R^2 values determined suggested the kinetic models were accurate at predicting the course of the fermentation. However, the validity of the model reduced with time as experimental values from the recycling schemes deviated from predicted values. This significant reduction in the ethanol yields for the latter recycling stages was due to the decrease in enzymatic hydrolysis and the increase in lactic acid production. This is because, the model assumed a constant ethanol yield from sugars during the fermentation and thus, any reaction that deviated sugars from the yeasts (contamination) would directly affect the validity of the model. Although there was no significant variation between the recycling schemes, recycling the supernatant eliminated the build-up of solids during recycling and was considered for further process optimizations.

Summary

The models described in this study represent a reliable approach for predicting the process performance during simultaneous saccharification and fermentation of PS with enzyme recycling. Considerable savings on enzymes was observed with recycling either supernatant or broth and suggests improvements in the economic feasibility of PS fermentation.

4.2 General recommendations

1. *Removal of enzyme inhibitors*

The in-situ removal of ethanol from the fermentation broth could significantly improve the ethanol yields during enzyme recycling. Zhang et al. (2017) recently used vacuum distillation to remove ethanol after SSF of corncob residues. It was reported that ethanol removal allowed enzymes and yeast cells to be recycled up to five times. This configuration achieved up to a 24% reduction of the ethanol production cost as compared to conventional SSF without enzyme recycling.

2. *Enzyme synergy*

Studies have established the importance of a strong interaction between cellulases and xylanases in the hydrolysis process (Malgas et al. 2017). Therefore, understanding the synergy of such enzymes during PS hydrolysis would be useful in identifying the factors responsible for the inhibition/ deactivation of individual enzymes and thus, improving the conversion of this biomass.

3. *Application of consolidated bioprocessing (CBP) microorganisms to PS fermentation*

Consolidated bioprocessing uses a single microorganism that merges cellulase production, enzymatic hydrolysis, and co-fermentation in one reactor (Vertes et al. 2010). The combination of CBP and enzyme recycling in paper sludge fermentation would improve process economics by eliminating the cost associated with dedicated cellulase production which remains a major bottleneck to the commercial application of paper sludge fermentation (Robus et al. 2016).

4. *Lactic acid production from PS*

Given the recalcitrant nature of the background microbial load in PS, the coproduction of bioethanol and lactic acid can be considered in the biorefinery context (Santos et al. 2008). Lactic acid is an expanding precursor to biodegradable polylactate polymers used for commodity packaging and the development of drug delivery systems in biomedical sciences.

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Appendix

Appendix A: Additional Experimental Results

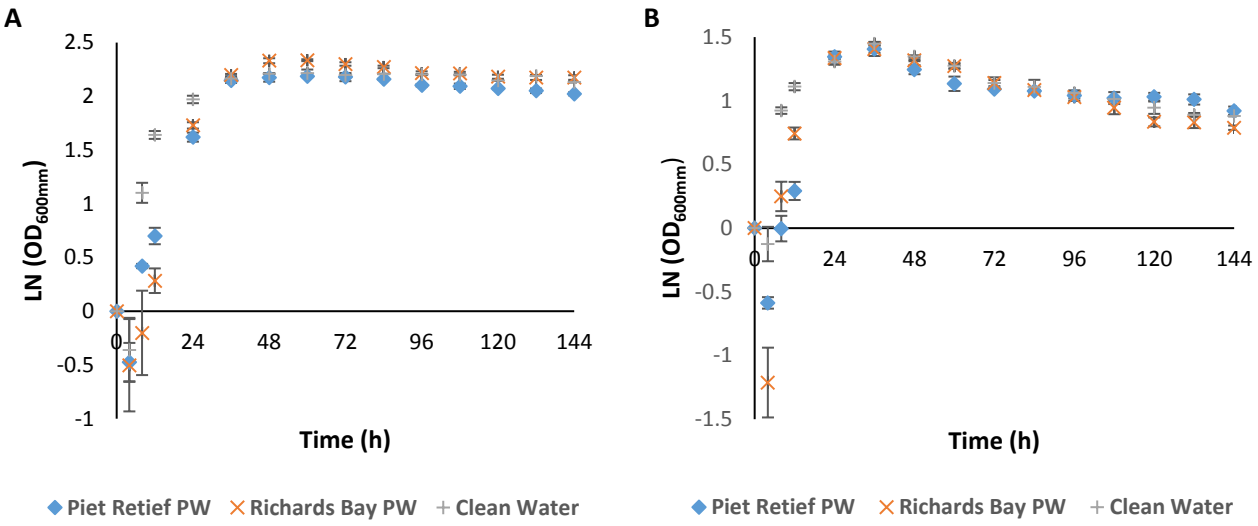

Figure A-1: Comparison of yeast growth in clarifier overflow wastewater/process water and clean water: MH1000 (A); CelluX™4 (B).

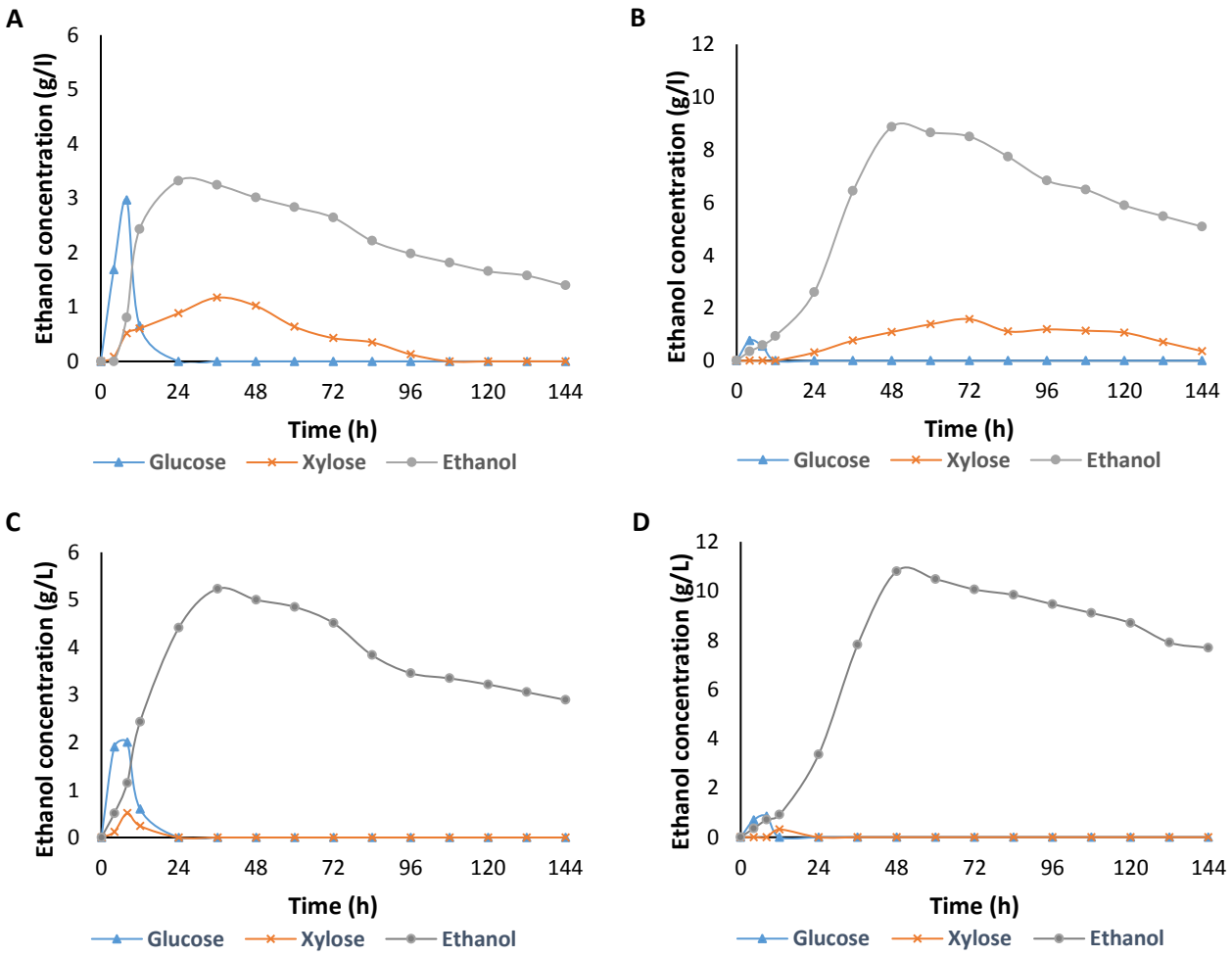

Figure A-2: Product concentration profiles from Piet Retief (A) and Richards Bay (B) paper sludge samples by MH1000; co-fermentation of glucose and xylose from Piet Retief (C) and Richards Bay (D) paper sludge samples by CelluX™4.

Appendix B: Model Equations

Enzyme balances:

$$\frac{dE}{dt} = -K_{det}[E] \tag{13}$$

$$E_{(1,\,end)} = E_{0\,(1,\,initial)}\exp(-K_{det}\,t) \tag{14}$$

For subsequent fermentations, the residual active enzyme concentration was accounted for by the cumulative effect of fresh enzyme supplementation and the recycled enzyme activities; though inherently of lower potency.

For n=3, 4, etc:

$$E_{(3,\,end)} = E_{0\,(3,\,initial)}\exp(-K_{det}\,t)$$

$$E_{0\,(3,\,initial)} = E_{(2,\,end)}(X_0) + E_s(1 - X_0) \tag{A-1}$$

$$E_{(n,\,end)} = E_{0\,(n,\,initial)}\exp(-K_{det}\,t)$$

$$E_{0\,(n,\,initial)} = E_{(n-1,\,end)}(X_0) + E_s(1 - X_0)$$

$$E_{(n,\,end)} = [\sum_{n=1}^{n=n-1} E_{(n,\,end)}\,(X_0)] + E_s(1 - X_0) \tag{A-2}$$

Cellulose mass balance:

$$\ln(1 - \alpha) = -Kt^n \tag{15}$$

$$\alpha = [1 - \exp(-Kt^n)]$$

$$\alpha = \frac{P_i\ (\text{Concentration of sugars at time t, g/L})}{S_0\ (\text{Initial concentration of cellulose, g/L})}$$

$$\alpha = \frac{\frac{1}{0.51}[\text{Ethanol}] + \frac{1}{0.97}[\text{Lactic Acid}] + \frac{1}{0.64}[\text{Acetic Acid}] + \frac{1}{0.95}[\text{Cellobiose}] + [\text{Glucose}]}{S_0} \tag{A-3}$$

Appendix C: Calculated Results

Determining the parameters of AKE's empirical equation

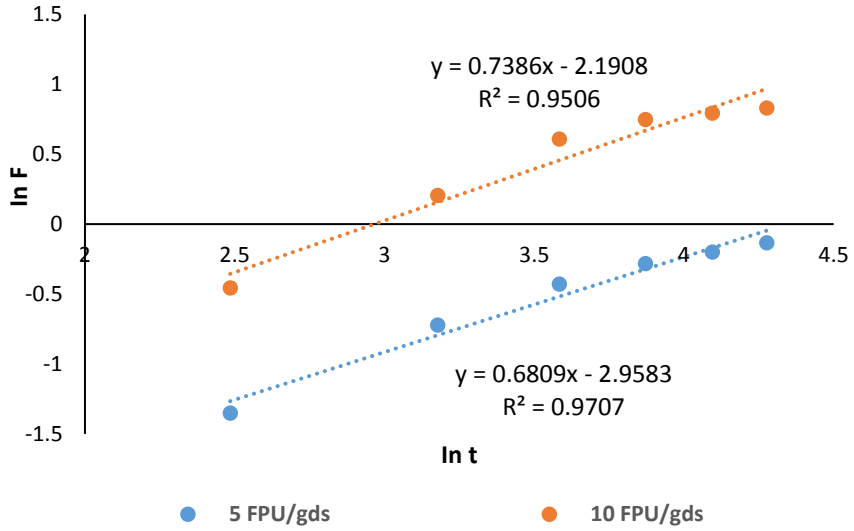

Figure A-3: Linearized kinetics of the hydrolysis of Richards bay PS in shake flask (6% w/w)

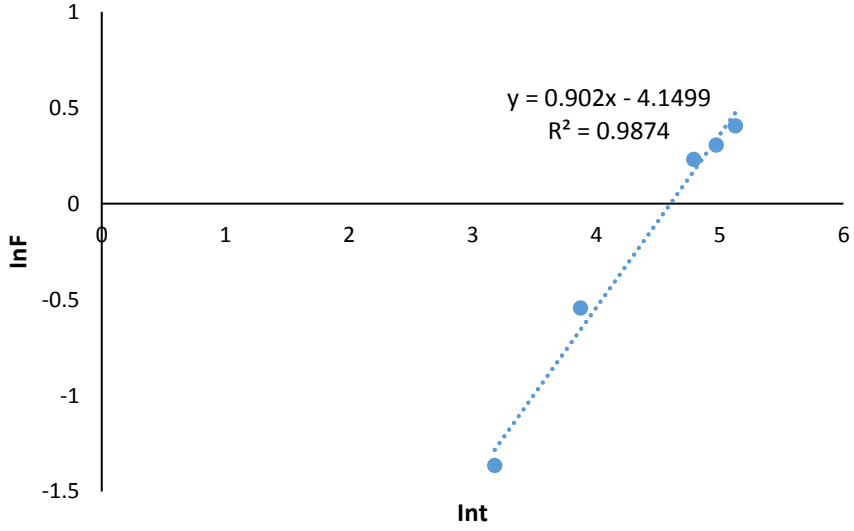

Figure A-4: Linearized kinetics of the enzymatic hydrolysis of Richards Bay PS in 5L bioreactor (18%w/w, 5PFU/gds Cellic® CTec 3)

Table A-1: Kinetic parameters of the enzymatic hydrolysis of Richards Bay PS

Parameters	Shake flask (6% w/w)		5L Bioreactor (18% w/w)
	5 FPU/gds	10 FPU/gds	5 FPU/gds
n	0.6809	0.7386	0.902
K	0.0519	0.1118	0.0158
R^2	0.9891	0.9921	0.9874

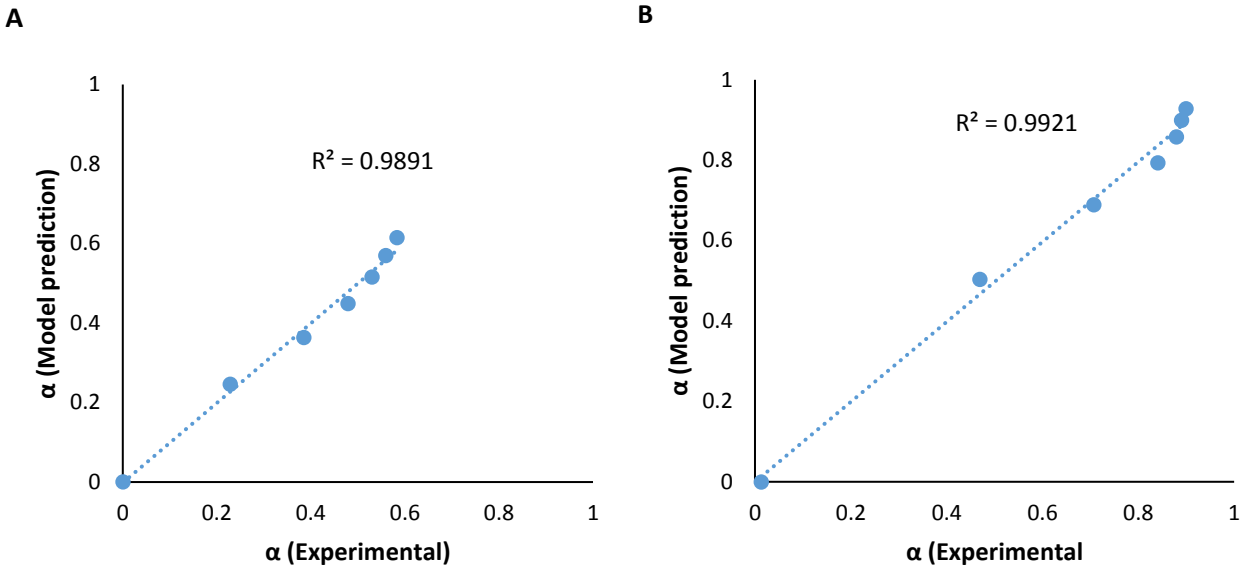

Figure A-5: Relation between the experimental and model predicted values for the cellulose conversion factor (α); (A) 5 FPU/gds and (B) 10 FPU/gds

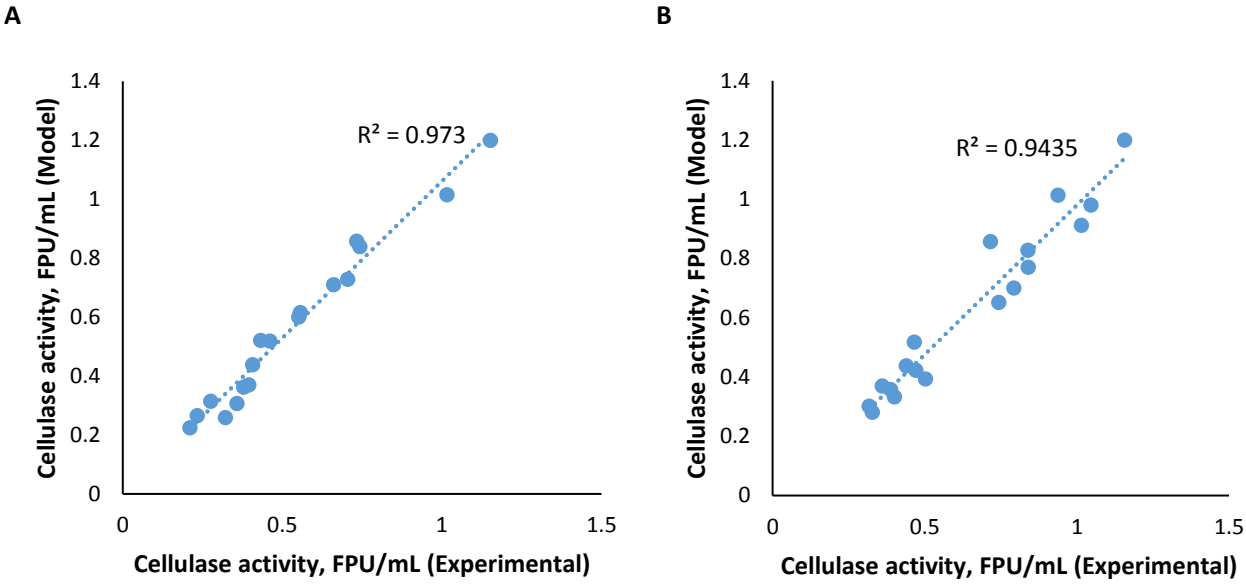

Figure A-6: Experimental and predicted values for the filter paper activity during recycling in bioreactors; (A) 50% and (B) 65% enzyme supplementation