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Bioconversion of Waste Lignocellulosic Biomass (South African Corn cob) to Succinic Acid in Molten Hydrate Solvent System

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A thesis submitted to the Faculty of Engineering and the Built Environment, University of the Witwatersrand, Johannesburg in fulfilment of the requirement for the degree of Doctor of Philosophy in Engineering.

Supervisor: Prof. M. O. Daramola

Co-Supervisor: Prof. R. Adeleke

April, 2018

Declaration

I declare that this thesis is my own work. It is being submitted for the degree of Doctor of Philosophy in the University of Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.



Ayotunde Ayokunle Awosusi

6th day of April 2018

Ise oluwa awa maridi

Abstract

Lignocellulosic biomass (LCB) resources are central to modern, sustainable development of economies worldwide. They are abundant, cheap and do not contribute to net CO_2 emissions hence their refinement for energy and valuable industrial commodities production provides a necessary alternative to crude oil. LCB have been used as solid fuels since the paleolithic ages. Today, advances on bio-chemo catalysis and carbon chemistry have seen more efficient processes converting biomass to a wide range of valuable products e.g. chemicals, biofuels and other bio-based commodities. Likewise, increase in awareness of the dangers of the continuous use of non-renewable fossil have continued to drive and accentuate the potential of biomass as a sustainable source of organic carbon but the high cost of biomass processing, poses a major threat to proposals for further development and commercialisation. LCB is an abundant, cheap and sustainable resource but it is also naturally inert and recalcitrant to biocatalytic conversion. As such, biorefining processes generally require a feed activation step (pre-treatment) prior to efficient conversion. The high cost of pre-treatment have made the need for modern strategies targeting cheaper, effective, catalyst-compatible and sustainable pre-treatment techniques very imperative. Cheap, recyclable and sustainable non-derivatising zinc chloride molten hydrate salt systems was proposed in this work as a plausible alternative to acid and alkali pre-treatment by means of investigations probing a) pre-treatment efficiency at relatively mild conditions, and (b) influence on biocompatibility of fermentation medium.

This work sought to establish a holistic approach for improved techno-feasibility of large-scale biorefining by exploring the potentials of an intensified and integrated process. The proposed process involved mild pre-treatment by solvation of local waste LCB in inorganic $ZnCl_2.nH_2O$ and whole-cell microbial conversion of liberated sugar to value-added products (e.g. succinic acid). Succinic acid (BSA) is a valuable, bio-based commodity with a growing

demand and market value as a top-platform chemical. The biocommodity has a wide-range of application in several industries such as biopharmaceuticals, food and materials production. By targeting anaerobic fermentative synthesis of SA, a waste-to-value concept was explored, further contributing to the proposed strategy of cheaper, sustainable bioprocess. The efficiency and general potentials of the bio-chemocatalytic process for SA production was described by charting specific objectives such as, (a) feedstock analysis (organic and inorganic composition), (b) pre-treatment efficiency as described by biomass deconstruction in molten hydrate salt (MHS) (c) statistical optimisation of sugar yield, and (d) metagenomic analysis of media selectivity of microbial catalysts as correspondent to yield.

The suitability of locally-sourced corn cob wastes as feeds in a bioconversion system was assessed by means of the characteristic organic and inorganic composition of the materials. The high (82.5 %) percent composition of sugars in the biomass was promising for anaerobic fermentation process as explored in this study. Using zinc chloride tetrahydrate $ZnCl_2.nH_2O$ salt solvent system, this work demonstrated the efficient pre-treatment of locally sourced waste corn cobs at relatively mild conditions of 70 ^oC for 60 minutes. The solvation process resulted in microstructural modifications of the materials that were correlated for reduced crystallinity and improved fiber reactivity. In comparison to common techniques (such as dil. acid or alkali treatment at relatively harsher operating conditions) used in overcoming biomass recalcitrance, the effect of $ZnCl_2.nH_2O$ treatment on biomass recalcitrance was promising especially from cost (energy and solvent) and sustainability perspective. Implications for biomass susceptibility to biological digestion of inherent saccharose was also assuring as approximately 85 and 95.1 % of cellulosic and hemicellulosic fractions were recovered upon enzymatic saccharification of treated samples.

A predictive second-order polynomial model, developed using a $_2$3 Box-Behnken design was used to describe the effect (main and interactive) of pre-treatment process variables (time,

temperature, and solvent loading) on yield towards the parametric optimisation of the process. The experimental yield of reducing sugars (Y_E) was well-fitted into the resulting model according high f-value (> p-value) obtained from the regression analysis and ANOVA tests of the model. ANOVA tests also reiterated the significance of the response model (Reducing sugar yield) with a reliability of 92.5 %, (adjusted R^2= 88. %), suggesting an error margin of < 8%. The optimal parameters (90 minutes, 107 ^oC , 18 g/g liquid-to-solid ratio) for the recovery of a total of 90 % of total sugars (based on biomass compositional data) were obtained by multi-objective numerical optimisation and cross-validated experimentally.

The fermentability of the dissolved biomass sugars at varying concentrations of $ZnCl_2$ salt was characterised by the observed synthesis of organic intermediates and target product; Succinic acid, at select salt concentrations. Anaerobic fermentation set-up was a batch simultaneous saccharification and co-fermentation (SScoF) bioprocess with feed of pre-treatment slurry at varying solvent; $ZnCl_2.4H_2O$ loading, catalysed by a mixed-consortia of rumen bacteria at standard anaerobic fermentation conditions. The mixed acid- fermentation process of the unconditioned $ZnCl_2$ MHS pre-treatment slurry, resulted in the conversion (up to 2 %) of dissolved biomass sugars to SA (10.4 g L^{-1}). SA production declined with increasing salt concentration but the original findings were promising especially considering the uncontrolled carbon channelling resulting from the use of mixed-consortium of microbial biocatalysts. Analysis of SA yield results were reconciled with bioactivity; as measured by DNA quantification, at the different salt concentration while microbial inventory analysis was done at constant slurry salt concentration.

The choice of a mixed-consortia of rumen bacteria as to single-cell cultures was utilized to broaden the sampling range for SA-producing biocatalysts (using $ZnCl_2$ salt concentration as a singular factor to model their micro-environments). Selectivity of media for microbes was profiled by characterizing the inoculum (cow dung) and fermenter samples using advanced

next-generation sequencing (NGS) tools. The characteristic operational taxonomic units (OTUs) of the active species in raw cow dung were determined towards informing specific microbial activity in media. The microbiome of the cow-dung to consisted of approximately 1500 OTUs, with the detection of up to 265 genera (of which only 18 genera constituted over 1 % of relative abundance). The dominant phyla was *Firmicutes* and *Actinobacteria* both of which are notable for immense industrial applicability in organic acid and alcohol biosynthesis. The observed strains of phyla such as *thermileophilia, Acidimicrobiia* have potentials in metageneomic engineering for programmed cell-free catalysis and synthetic biomanufacturing designs which could find applicability in proposed process.

This thesis is a careful documentation of proof of concept of SA biosynthesis in an intensified bio-chemocatalytic process and experimental proof of efficient biomass deconstruction; characterised by ultra-hemicellulosic solubulization and increased bioreactivity (saccharification and digestion) of residual cellulose in $ZnCl_2.nH_2O$ solvent systems at relatively mild conditions. The findings as described in this thesis have the potentials to be fundamental to the design of advanced process for cheaper, efficient and integrated one-pot, bio-chemocatalytic pre-treatment and conversion of abundant waste LCB resources such as corn cob to valuable commodities such as bio-based succinic acid (BSA). Some of the findings as described in this dissertation have been communicated as scientific articles in reputable journals and as contributions to scientific conferences.

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Research Outputs from the study

Journals Publications

I) L. M. Mohlala, M. O. Bodunrin, **A. A. Awosusi**, M. O. Daramola, N. P. Cele, P. A. Olubambi (2016) Beneficiation of corncob and sugarcane bagasse for energy generation and materials development in Nigeria and South Africa: A short overview. *Alexandria Engineering Journal.*, 55(3), 3025-3036.

II) **A.A. Awosusi**, A.O. Ayeni, R. Adeleke and M. O, Daramola. (2017) Effect of water of crystallization on the dissolution efficiency of molten zinc chloride hydrate salts during the pre-treatment of corncob biomass. *Journal of Chemical. Technology and Biotechnology,* 92: 2468–2476. Doi:10.1002/jctb.5266

III) P.T. Sekoai, **A.A. Awosusi,** K.O. Yoro, M. Singo, O. Oloye, A.O. Ayeni, M. Bodunrin, M. O, Daramola. (2018) Microbial cell immobilization in biohydrogen production: A short overview. *Critical Reviews in Biotechnology,* 38(2):157-171. doi: 10.1080/07388551.2017.1312274.

IV) **A.A. Awosusi**, A.O. Ayeni, R. Adeleke and M. O, Daramola. (2017) Biocompositional and thermodecompositional analysis of South African agro-waste corncob and husk towards production of biocommodities. *Asia-Pacific. Journal of Chemical. Engineering.*, 12 (6): 960-968 doi: 10.1002/apj.2138.

Conference Proceedings/ Presentations

I. **A.A. Awosusi**, O. Oluwasina and M.O. Daramola. (2015), Dissolution of South African corncob in inorganic hydrate salts ($ZnCl_2.xH_2O$) towards efficient biocatalytic depolymerisation In: *Asia Pacific Confederation of Chemical Engineering Congress (APCChE 2015),* 27 September-1 October 2015, Melbourne, Australia, 232-243. ISBN: 9781922107473.

II. **A.A Awosusi,** Sekoai, P.T., Ayeni, A.O., Adeleke, R., M.O Daramola. (2017) Biocompatibility Profiling of Molten Inorganic Zinc Chloride Pre-Treatment Medium Using Corn Cob as Carbon Source. In. *European Biomass Conference and Exhibition Proceedings (EUBCE 2017)*, 12-15 June, 2017, Stockholm, Sweden., 1264-1270 ISBN: 978-88-89407-17-2

III. **A. A. Awosusi**, A.O. Ayeni, R. Adeleke, M. O. Daramola., Zinc chloride pre-treatment of corn cob: effect of Water of crystallization on efficiency.,*1st International Conference on Bioresource Technology for Bioenergy, Bioproducts & Environmental Sustainability in Sitges*, nr Barcelona, Spain from the 23-26 October 2016 **(Poster Presentation)**

IV. **A. A. Awosusi**, A.O. Ayeni, R. Adeleke, M. O Daramola., Statistical Optimisation molten zinc chloride tetra hydrate salt pre-treatment of South African corn cob. *International conference on Environment Materials and Green Technology* in Sebokeng, South Africa from the 24^{th}-25^{th} November, 2016 **(Oral Presentation)**

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Chapter 1

1.1 Background

1.1.1 Biorefinery Platform

Global challenges with greenhouse gas (GHG) emissions (as contributory to climate change), depleting crude oil reserves have triggered a strategic diversification towards more renewable and sustainable energy sources globally. Currently, fossil-sourced energy serves as the major driver of human activities globally but recent developments in renewable energy technologies and governmental policies have resulted in a gradual shift towards more sustainable processes. The conversion of biomass materials to energy and other valuable commodities is one of the leading, sustainable alternatives being proposed as potential sources of energy for modern industrial processes and human activities. Currently, up to 10% of global primary energy supply and 30% of the total output of the chemical industry have been forecasted to come from biomass by 2025 (Isikgor and Becer, 2015). Technically, biorefining platform, can yield all the intermediates currently recoverable from fossil resources (Kamm et al., 2000), hence, providing a viable, modern substitute for fossil refining across the entire spectrum of products.

Biomass, best described as naturally occurring materials derived from living organisms (Ladisch et al., 2013), are central to the overall structure of a bio-based economy. Biomass is undoubtedly the most abundant resource on earth for commodity and energy production, and as such is relatively cheap, uniformly distributed across the globe and stable in supply (Brenes, 2006). In addition to abundance, their use as raw materials in processing is encouraged because, unlike their fossil alternative, they do not contribute to greenhouse gas (GHG) emission; a leading cause of climate change globally (Brenes, 2006). Biomass resources are an immense repository of hydrocarbons (Ladisch et al., 2013), which have been

used since pre-historic times as a source of energy. The discovery of fossil fuels (in the early 20th century) and subsequent development as the primary energy of choice resulted in a decline in global interest and industrial relevance of biomass resources. The turn of the millennium has seen an active transition back to biomass-based economy, although, one which was met by a lapse in technological advancements and a well-defined system for efficient production of value-added products. This shift in global consciousness, backed by governmental reforms have since sought to contribute to the development of bio-based economy, thus, research has been targeted at frameworks for an integrated biorefinery platform (Cherubini, 2010). The proposed concept like similar outlooks in academia and industry involves the design of biorefinery processes for co-production of high-value-low-volume (HV-LV e.g. nutraceuticals) and low-volume, high value products (LV-HV e.g. biofuels) from low-cost, waste substrates (Fernando et al, 2006). The resulting flexibility in the process culminates to improve overall techno-feasibility, sustainability of biorefinery platforms, such that the resulting final products are commercializable and competitive with their respective fossil analogs (Cherubini, 2010).

A biorefinery is a complex system of sustainable processes converting biomass into a spectrum of marketable, value-added commodities (see Figure 1.1) (Kamm et al., 2008). A fully integrated biorefinery platform has been described as a potential sustainable analogue of the oil refinery. The efficiency of biorefining platforms is central to the marketability of bio-based commodities (food, materials, chemicals) and secondary energy carriers (e.g. fuels, heat (Werpy et al., 2004). The United States, Department of Energy (US-DOE) established via the NREL (Pacific Northwest National/National Renewable Energy Laboratory), 12 important high-volume-high-value (HV-HV) bio-based molecules (such as succinic, malic and fumaric acid) which are co-producible with secondary biorefinery products. The

proposed commodities, also widely referred to as platform chemicals, were regarded central to the strategic development of biorefining because they have considerable industrial relevance and are also convertible in two-steps or less, to several other HV-LV chemicals (Farmer and Mascal, 2015). However, the efficient and sustainable co-production of HV-HV and LV-HV in an efficient and cheap system from low-cost feeds is unarguably a promising strategy for increasing the potentials of the sustainable bio-based economy globally.

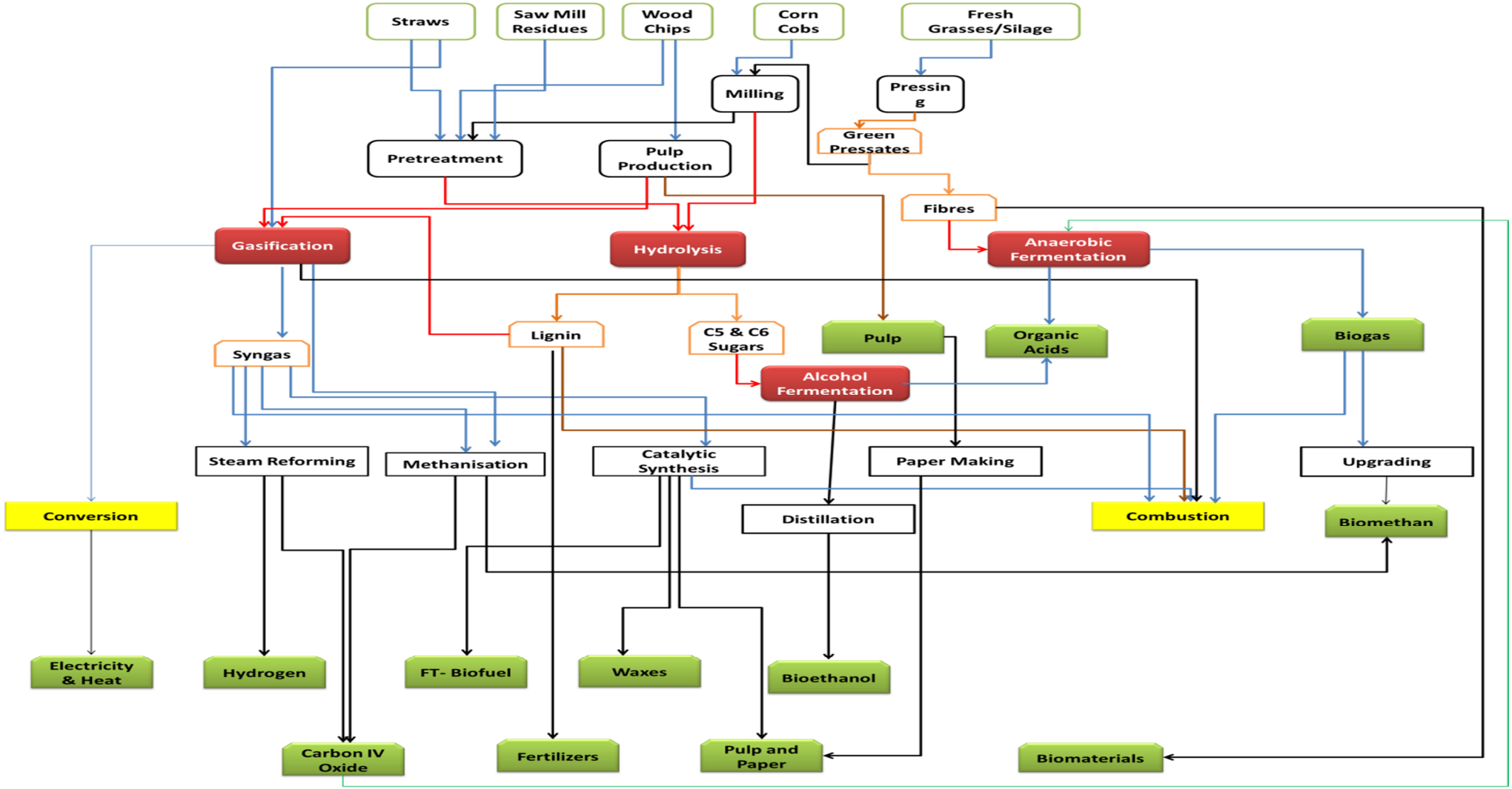


Figure 1. 1: Summary of conversion technologies and targeted end-products from lignocellulosic biomass processing in an integrated biorefinery platform (Mohlala et al., 2016).

1.1.2 Lignocellulosic biomass as a resource for sustainable production of bio-based products.

Biomass are generated from available atmospheric CO_2, water and sunlight through biological photosynthesis and as such considered to be the only sustainable source of organic carbon on earth (Brenes, 2006). Research into their use has shown them to be a viable, cheaper and more sustainable alternative to crude oil; as over 200 value-added chemicals and products currently produced from fossil resources can be derived from their processing using various conversion techniques (Werpy et al., 2004). Historically, biomass have played major roles as building blocks of economies worldwide as clothing, construction materials, dyes, medicines, industrial chemicals, and fuels were sourced majorly from plant and animals up until the early 19th century (Hale, 1939). Proposition to base global industrialization at the time on fuels and materials from biological resources was common, as pioneers such as Henry Ford believed the implementation of a bio-economy was a logical and necessary option for the growth of any civilization (Isikgor and Becer, 2015). The cost and challenges; especially as regard the threats associated to continuous dependence on a fossil economy, have since rekindled the global consciousness prioritizing the use of organic carbon in sustaining human development. David Morris in the 1980s coined the term carbohydrate economy, envisaging the shift of our global economy from dead carbons back to living carbons (Morris, 2006). Today, this theme is backed by coalitions and government policies which have sought to encourage the sustainable use of organic carbon in energy generation while inorganic carbon conversions incur sanctions. The development of a bio-based economy, especially as regard the sustainability and the socio-economic impact of the production of fuels and chemicals from biomass, has been intensely scrutinized in many academic and socio-political arena. For example, there are critical concerns regarding the production of first-generation biofuels as the process relies on edible, and widely-consumed starch and sugar crops. Current challenges with food security in several parts of the world

have led to the discouragement of this approach and similar approaches which may potentially pose competition to food production either directly or indirectly (Escobar et al., 2009). LCB utilization does not entirely contravene this perspective as they are not particularly edible but their production (e.g energy crops) can still compete with land resources important for food production (Huang et al., 2012). However, forestry, agricultural and agro-industrial LCB waste materials are an important class of beneficiable resources which are accumulated every year in large quantities (García et al., 2012).

Agricultural residues generated as waste during or after harvesting or processing of agricultural crops and forest products are very rich in cellulose and hemicellulose hence, their immense potentials for use as feedstock in biomass conversion technologies.

Waste LCB refining as a waste management strategy in the agro-sector has cost-saving and ecological implications; as current disposal of these wastes by burning or landfilling are expensive and poses environmental problems. From an economic point-of-view, the beneficiation of invaluable, low cost LCB waste materials in an integrated biorefinery system contributes in part to the potentials of low-cost production of value-added materials. A necessary outlook considering, the surge in marketability of bio-based products has not been matched by the market competitiveness, in terms of the cost of production and final cost of the products, when compared to the fossil alternatives. As such, strategies with potentials to contribute to the overall feasibility of biomass processing are being encouraged. LCB are naturally recalcitrant to degradation, possessing highly structural and defensive properties which make their processing a generally expensive procedure. In addition to logistical challenges such as storage and transport, this characteristic property of LCB impacts techno-feasibility of refining platforms due to high processing costs (Cherubini, 2010). Research

targeting cheaper ways to overcome LCB recalcitrance is currently being undertaken extensively globally.

In summary, LCB especially agro-industrial waste materials are fast becoming the ideal resources upon which modern industrialization can be centred and human development fostered sustainably. The development of efficient, consolidated processes capable of high selectivity and yield at economical costs is central to the establishment of a functional bio-based economy globally.

1.1.2.1 Lignocellulosic agro-waste resource: A South African outlook.

The dependence of South Africa on coal as the sole source of energy has prompted the need for more modern, sustainable and efficient renewable energy technologies. In addition to pressures from global sustainability agenda; especially concerning carbon emission, climate change-related challenges, food security and water scarcity, have continued to influence national industrial policies for sustainable renewable energy platforms like biorefining. Although, agro-waste beneficiation is seldom proposed, the strategy holds immense potentials for sustainable energy security and reduction in carbon emission in developing African countries (Maltitz et al., 2009).

In South Africa, residues generated across the entire value-chain of agriculture and forestry product processing accounts for over 6% of the waste generated in the country (Baloyi et al, 2015). Experts value the globally-recognized, secondary resource potentials of these waste to be upwards of ZAR15 billion annually (Baloyi et al., 2015). Yet, current practices involve burning and landfilling; both of which pose cost and environmental challenges. Agro-wastes such as corn cobs, wheat husks are very promising resources for beneficiation as they are

generated extensively in South Africa. South Africa as one of the top 10, corn producing countries globally, generates an ample amount of corn and corn wastes (see Figure 1.2).

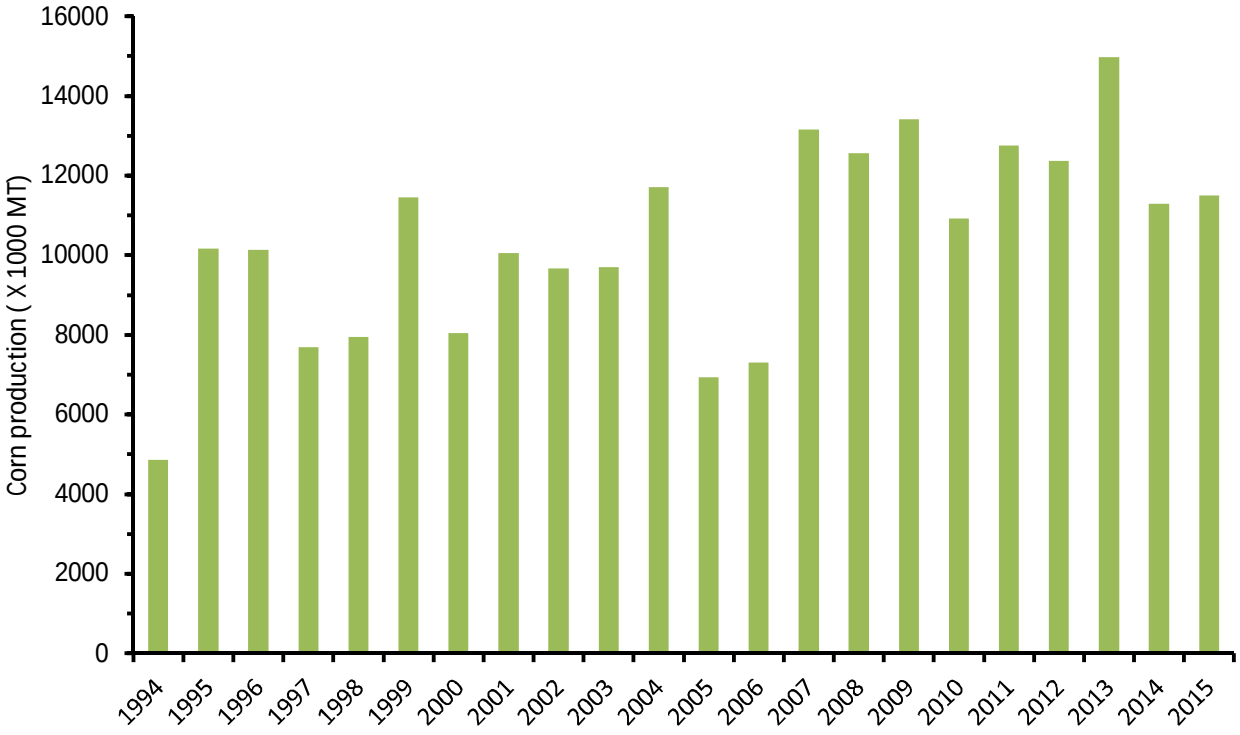


Figure 1. 2: Outlook of annual corn production in South Africa for 21 years (1994-2015) (FAOStat, 2017)

Over the lifecycle of maize (i.e. from harvesting to processing and consumption), beneficiable waste materials such as cobs, husks and stovers are generated at products to waste ratio as high as 1:1 (Zych, 2008). The abundance and availability of these resources for bio-based economy in Africa; South Africa and Nigeria as case studies was reviewed in-depth as part of this work (see Appendix P-1 for a copy of the review article that emanated from the study). Waste corn cobs are lignocellulosic biomass materials which have been subjects of biofuels and bio-based commodities production research, globally. They are proven sugar repositories which upon pre-treatment can be converted to a wide variety of

value-added products in several processes of the biorefinery platform (Jansen and Lübberstedt, 2012). In developing African countries, opportunities for socio-economic development presented by agro-industrial waste further amplify the well-reported merits such as energy security, waste management and process yields (Amigun et al., 2008). The development of modern, agro-waste-based sustainable systems for chemical and energy production explores these opportunities without compromising on limited resources; food, land and water (Janssen and Rutz, 2012).

1.1.3 Process Intensification

Available LCB feedstocks obtainable from plant resource have the energy equivalent of approximately two-thirds of the world's energy requirement. According to the DOE-USDA Billion-Ton Study, corn stover and perennial crops e.g switchgrass have the potentials for the generation of 1.3 billion tons of biomass resource for a bio-based economy by the mid-twenty-first century (Boe and Lee, 2007). Towards, the actualization of this potential, the development of cost-effective large-scale technologies with a billion ton of biomass/annum capacity is imperative.

Despite extensive studies, being carried out to meet the future challenges of bioenergy generation globally, there is yet to be a self-sufficient, feasible process or platform for the efficient conversion of lignocellulosic biomass to final products. The major reason for which is the naturally crystalline nature of LCB, which contributes to unreactivity and recalcitrance to catalytic hydrolysis of inherent polysaccharides (Klemm et al, 2005). Several chemical pre-treatment methods (e.g steam and acid/alkali-steam) have been evaluated for effective deconstruction and decrystallization of different types of LCB. However, the utilization of these pre-treatment methods has resulted in major drawbacks for the overall economy of LCB bioconversion.

The current biomass conversion process can be summarized into 5 major steps (a) comminution (b) pre-treatment (c) conditioning (d) hydrolysis (e) fermentation and as outlined by flow diagram illustrated in Figure 1.3.

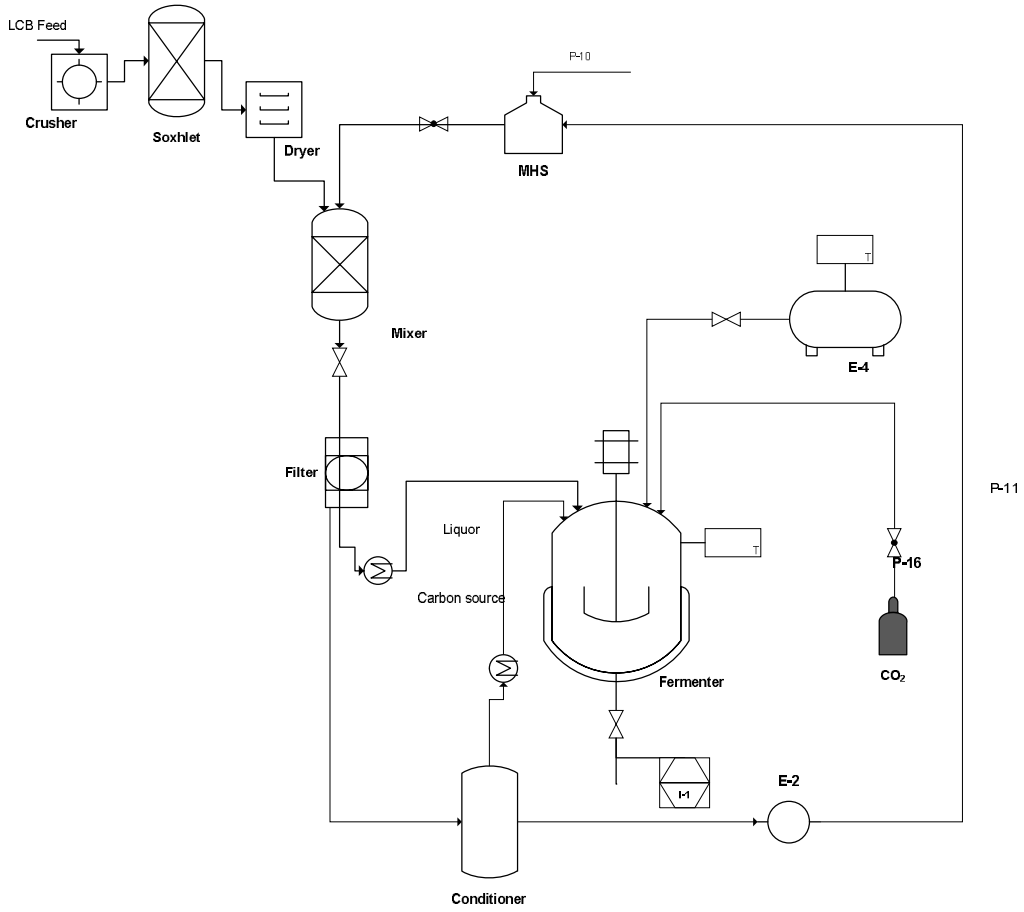


Figure 1. 3: Process flow diagram for biomass conversion process using conventional pre-treatment method such as dilute -acid treatment.

Conventional chemical pre-treatment methods usually include the use of corrosive, toxic media (e.g. sodium hydroxide, perchloric acid, peracetic acid, acid hydrolysis using sulfuric and formic acids, ammonia freeze explosion, and organic solvent) at harsh conditions. The processes are usually characterised by high costs (energy and infrastructure) and formation of sugar-degradation products which inhibits downstream fermentation process. To mitigate against the reduction in carbon throughput, different conditioning strategies such as filtration, neutralization and activated charcoal treatment have been researched but have further impacted the techno-feasibility of waste biomass beneficiation. The use of molten $ZnCl_2$

hydrate salt as a LCB pre-treatment medium at relatively mild conditions, as explored in this work is an alternative process. The inorganic media was investigated for efficient pre-treatment and towards use in an intensified bio/chemocatalytic conversion process (as illustrated in Figure 1.4) targeting a high-value product such as biosuccinic acid (BSA).

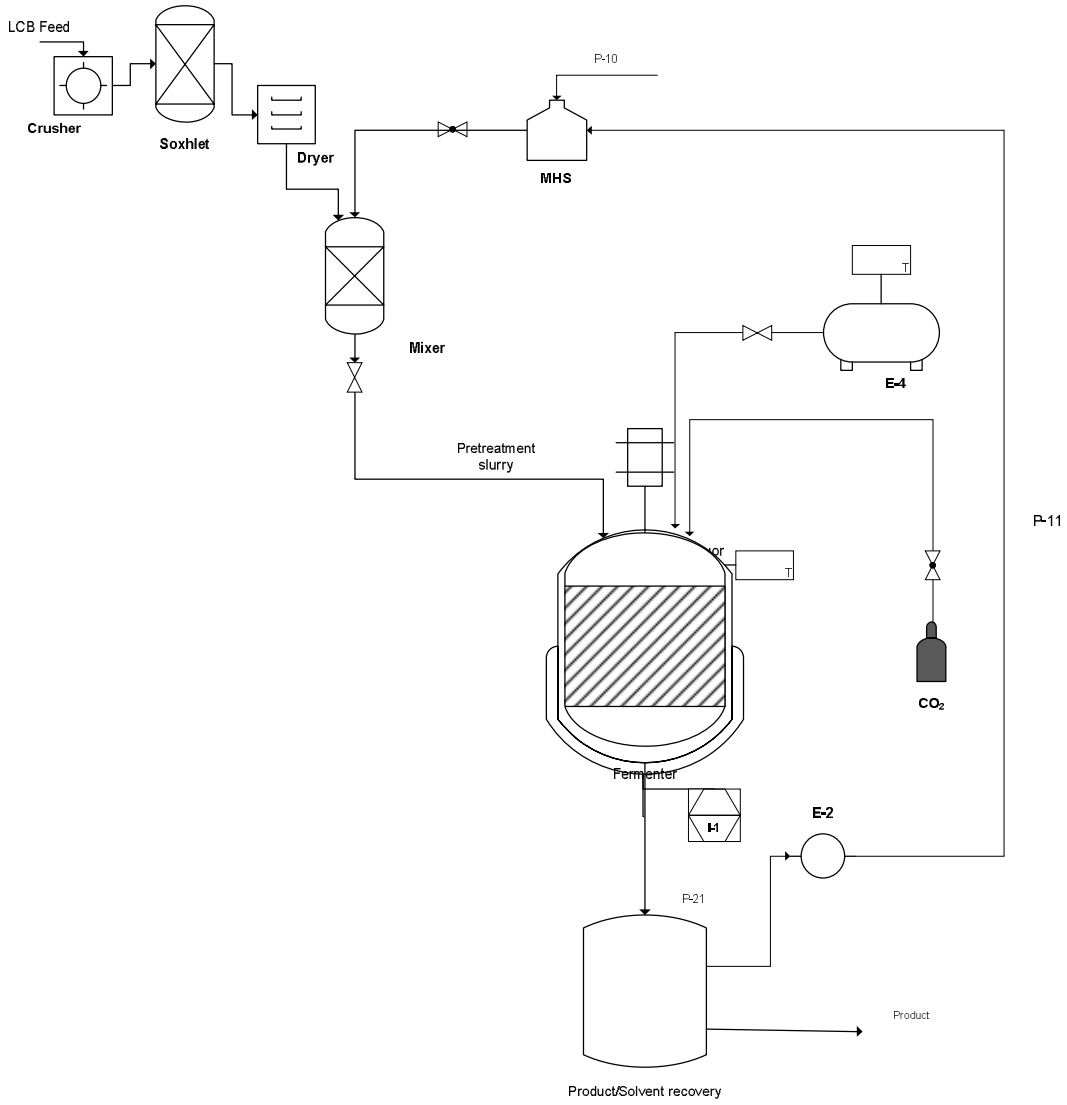


Figure 1. 4: Proposed intensified biomass conversion process based on mild $ZnCl_2$ MHS pre-treatment.

1.2 Research questions and Objectives

Against the background presented above, the aim of this research was to investigate the potentials of an intensified process combining biomass pre-treatment and conversion (via fermentation) of locally-sourced corn cob to BSA. In particular, this research study explored the development of a biochemo-catalytic platform, predesigned for the integration of the

three major biomass processing steps; pre-treatment, hydrolysis and whole-cell conversion of the corn cob to BSA. Consequently, three major concepts were identified to be fundamental to achieving the goal of a functionalized one-pot biosynthesis of BSA from lignocellulosic corn cob substrate.

These include; (a) a thorough understanding of the biomass resource and effects of pre-treatment in hydrate salt medium, (b) optimisation of pre-treatment for enhanced enzymatic saccharification, and (c) whole-cell fermentability of hydrate salt-treated LCB subject to outcome of (a) and (b) for BSA biosynthesis. Towards the provision of proof of the aforementioned concepts, the following research questions were posed and investigated:

(i) What are the cellulose, hemicellulose composition and general physicochemical properties of locally-sourced corn cob biomass?

(ii) What is the effect of $ZnCl_2.nH_2O$ treatment on surface morphology, crystallinity, fiber reactivity, surface chemistry and enzymatic saccharification of corn cob biomass?

(iii) What is the effect of pre-treatment operating conditions of $ZnCl_2.nH_2O$ pre-treatment process and enzymatic yield of sugar?

(iv) What is the effect of $ZnCl_2.nH_2O$ media on the anaerobic fermentation of corn cob by a host of rumen microbiome?

(v) What is the genomic composition of the inoculum employed in (iv) and how compatible is the medium for sustaining bioactivity?

Based on the research questions, the following research objectives were proposed:

(i) Assessment of the biocompositional and physiochemical properties of agro-industrial cob wastes of native South African maize crops (Zama white hybrid) towards establishment of resource credentials in a sustainable biocommodity producing process.

(ii) To investigate the pre-treatment of corn cobs and subsequent hydrolysis of the inherent polysaccharides in inorganic molten Zinc Chloride hydrate salt at varying conditions.

(iii) To optimize the solvation and hydrolysis process by investigating the relationship of pre-treatment process parameters such as time, temperature and solvent loading on (ii) using a response surface methodology approach.

(iv) To assess the bacterial diversity of inoculum and examine influence of $ZnCl_2$ media on microbial activity.

(v) Examine the effect of $ZnCl_2$-corn cob LCB feed on the anaerobic fermentation of MHS-treated corn cob to succinic acid using a mixed-consortia of rumen microbial catalysts.

1.3 Research approach, scope and thesis outline

The core of this research is a conceptual consolidated biomass process (CBP) designed to integrate the LCB pre-treatment step with the established bioconversion process. The conceptual design uses a cheap, recyclable and eco-friendly inorganic salt $ZnCl_2.nH_2O$ medium as a platform for the pre-treatment, hydrolysis, and anaerobic fermentation of locally-sourced waste corn cobs. To fully explain the potentials of the integrated conversion system, research efforts were dedicated to finding the empirical proof of a functional relationship between the severity of the hydrate salt medium and the bioactivity of a mixed consortium of bacteria in cow dung. The resulting information was used to develop a biocompatibility profile, which accounts for the physiological limitations of biocatalysts and informs the yield of value-added products; with emphasis on BSA, in the conceptual "one-pot" conversion system.

This thesis expounded the scope of this research by undertaking the following studies:

a) A review of existing literature on the development of lignocellulosic biomass pre-treatment technologies and the conversion of hydrolysates to platform chemicals is also documented in chapter 2 of this thesis.

b) *Characterizing the biomass:* A comprehensive biocompositional analysis of locally -generated corn cob was carried out and findings are documented in chapter 3 of this thesis. Using proximate, ultimate and other analytical techniques, the raw biomass was analysed for the organic and inorganic content. The beneficiability of the saccharide components, physicochemical characteristics of lignocellulosic material and elemental profile of the biomass were established. The findings served as a benchmark upon which other yields were based.

c) *Defining the MHS pre-treatment media:* This study (Chapter 4) was an in-depth investigation documenting the effect of zinc chloride hydrate salt pre-treatment on native corn cob. The study reported the correlations between physicochemical characteristics (such as hydration) of the salt solvent system and pre-treatment efficiency. In addition, variations in pre-treatment severity factor of $ZnCl_2$ pre-treatment on was also correlated to the observed effects such as biomass fractionation, richness of pre-treatment liquor, surface chemistry, and crystallinity, surface morphology of corn cob LCB.

d) *Relating $ZnCl_2.4H_2O$ pre-treatment to enzymatic saccharification:* The effect of $ZnCl_2.4H_2O$ pre-treatment on the enzymatic digestibility of the saccharide components of LCB was established. Response surface methodology (RSM) was used to empirically optimize the pre-treatment process parameters for optimised enzymatic digestion. The proposed regression model described the influence of the process conditions (main and interactive) on the yield of sugar monomers. The outcome of this investigation is presented in chapter 5 of this thesis.

e) *Biocompatibility profile studies:* Insights into the microbial selectivity and biocompatibility of the zinc chloride hydrate salt, as a medium constituent for anaerobic fermentation using mixed consortia of cow-dung bacteria was investigated and findings are documented in chapter 6 of this thesis. 16S-metagenomic analysis of DNA extracted from active microbial catalysts in conversion systems of varying pre-treatment severity is reported as well.

f) *Anaerobic fermentation study:* Finally, $ZnCl_2$ molten hydrate salt (MHS) treated biomass and pre-treatment liquor were used as a feedstock in a batch consolidated biomass processing (CBP) using a mixed-consortia of rumen bacteria from cow dung at under anaerobic conditions. The study (Chapter 6) was carried out in a 1litre Schott-abbott bottles and the yields of non-volatile organic acids; succinic, lactic, acetic acids and ethanol at standard conditions were confirmed using a high performance-liquid chromatography technique (HPLC).

1.4 Summary and contribution to knowledge.

Sustainable pre-treatment of lignocellulosic biomass (LCB) is a major challenge in current biomass processing technologies. The use of non-corrosive, environmentally benign biomass pre-treatment measures in an intensified bio/chemo catalysed process, at relatively mild conditions, does not only align with current ideas and ethos for green processes, it could also improve process techno-feasibility. Harnessing the merits of pre-treatment and subsequent optimisation for improved efficiency of subsequent biomass processing steps requires the need of cheaper and more sustainable pre-treatment systems. Hydrate salts such as $ZnCl_2$ salt–water binary system, is one of such systems with proven biomass deconstruction at relatively mild conditions (Sen et al., 2013). Recommendations for extensive use in biorefining is dependent on the following

1) Pre-treatment efficiency
2) A concise understanding of implications to downstream conversion process steps (fermentation).

These themes were explored in this work, but more importantly the potentials of the solvent system as a platform for simultaneous pre-treatment and bioconversion towards the possibility of an integrated bioprocess system that combines designed and optimised for one simple and solitary infrastructure. Knowledge of the behaviour of biological catalysts in electrochemical micro-environments is central to the development of bioconversion processes in an integrated biorefinery platform for production of platform and bulk commodities. Well-reported developments in protein engineering for applicability in non-aqueous solvents (Arnold, 1988, 1990, Gupta et al, 2009) have continued to exclude whole-cells; an important class of biological catalysts for biorefining processes. As such, this work correlates the interaction between $ZnCl_2$ MHS pre-treatment system and process feed (waste LCB) and biocatalysis (mixed microbial consortium) with yield of targeted product (succinic acid). The findings inform process integration strategies which are undoubtedly essential for the improvement of biorefining processes. This work exacerbates the waste-to value concept through the design of sustainable process and conversion systems while contributing to existing knowledge in bioprocessing by means of identification of whole-cells with potentials for applicability in sub-optimal electrochemical environment.

Chapter 2

Literature review

This chapter provides a comprehensive review of succinic acid bioproduction from waste lignocellulosic biomass. The dynamics of substrate-MHS interactions and reaction media biocompatibility are described towards contextualizing the potentials of an optimal bio/chemo catalytic system for the production of valuable bio-based commodities. Important process phenomenon such as biomass pre-treatment in hydrate salts, sugar liberation from matrix by means of enzyme hydrolysis of polysaccharides, mechanism of acidogenesis in biofermentation and whole-cell immobilization for media tolerance of biocatalysts were reviewed and documented in this chapter.

2.1 Biomass

LCB materials, by definition, are organic, carbon-rich living matter, which can be used as a fuel either directly or after a conversion process (EIA, 2017). Biomass resource have been utilized as fuel for thousands of years, and are still central to the functionality of the modern biorefinery platform. The global agendas for sustainability, energy security and accessibility to improved living are developed in-part around their prospects as important, eco-sustainable energy and manufacturing resources.

2.1.1 Biomass Classification

Biomass for use as feedstocks in biorefining are usually classified based on their chemical composition as:

a) ***Starch-based biomass:*** They mostly contain long complex chains of sugar molecules which can be easily converted to ethanol and drop-in fuels by fermentation. Starch-based biomass feedstocks are usually edible and could be staples in the most vulnerable, food insecure nations. As such, their use is widely discouraged for energy

generation and commodity production purposes. Examples of starch-based feedstocks include popular grains (such as corn or wheat) and tubers (such as potatoes and cassava).

b) ***Triglyceride- based biomass***: They have relatively high oil content hence are employed in production of biodiesel and proteins (Field et al., 2008). Examples of such biomass include vegetable oil, animal fat, waste cooking oil and algae.

c) ***Lignocellulosic biomass:*** Generally regarded as the most promising, natural, renewable, abundant feedstocks for a modern, sustainable and functional industrial system globally. This class of biomass are composed majorly of cellulose, hemicellulose and lignin (Ladisch et al., 2013). LCB is relatively cheap as they are not only abundant in nature and uniformly distributed globally, they are also major waste products of several industrial processes. This class of biomass are the ideal resource, considering their net-carbon emission and opportunities for socio-economic development. Cost-implications and challenges with the techno-feasibility of processing have been targeted at by intense research and process development strategies towards a more economical and efficient conversion of these resources to value-added products.

The leading sources of lignocellulosic biomass for use in processing are classified as energy crops, forestry waste and agricultural/agro-industrial waste are shown in Table 1.

Table 2. 1: Major sources of lignocellulosic biomass

Biomass Source	Cost (USD/ton)	Examples	Characteristics
Annual and perennial dry energy grasses	100-120	Switchgrass, Miscanthus, Canary grass, Giant reed, Napier grass	Requires extensive land resource but not necessarily water and nutrient demanding.
Forestry waste	60-80	Saw mill/dusts, Paper mill wastes, Wood chips, Poplar	Waste management options for the paper making industry
Agricultural waste	60-80	Corn cob, Corn stover, Sugar cane bagasse, Rice husks, Wheat straw	Positive contribution to the value chain of agroproducts.

Corn cob, sugar cane bagasse and rice straw are one of the most popular and applied lignocellulosic biomass in process today. Their prominence is due to factors such as high sugar content (as high as 80 % (Prasad et al., 2007)), 67 % (Kim and Day, 2011)) and 56 % (Prasad et al. 2007) for corn cob, sugar cane bagasse and rice straw respectively). Corn cob are low-cost by virtue of their waste credentials and the relative ease of handling, valuation, collection and storage (Jansen and Lübberstedt, 2012).

Other lignocellulosic biomasses that have been investigated for use as feedstocks in biorefinery processes includes waste newspapers, wheat straw, sponge gourd fibres (Guimares et al. 2009) nutshells (Howard et al. 2007), grasses; switch grass (Boe and Lee, 2007) and forest resources; hardwood, soft wood (da Silva et al., 2013).

2.1.2 Structure and composition of Lignocellulosic Biomass

The structure of lignocellulosic biomass is highly characterised by a sequestered, rigid, highly crystalline biomass matrix built on a network of inter and intra-polymer hydrogen bonds (Figure 2.1).

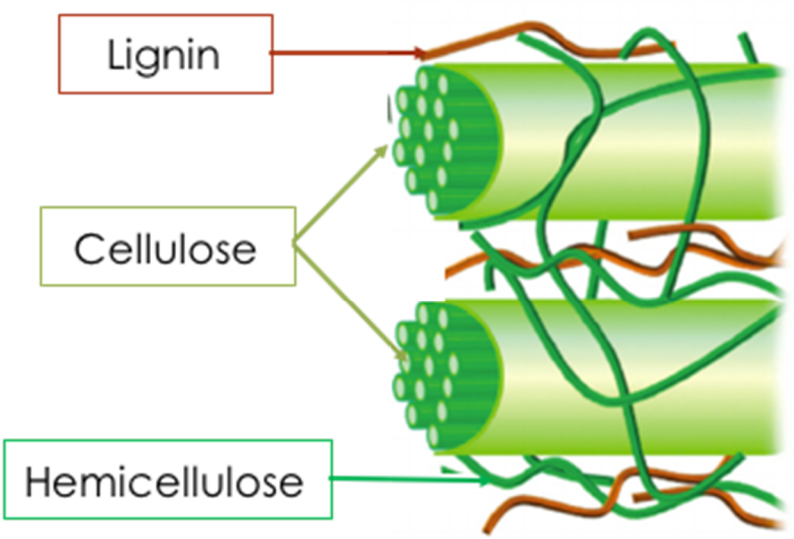


Figure 2. 1: Schematic illustration of the ordered structure of lignocellulosic biomass matrix (Adapted from Ladisch et al., 2013)

2.1.2.1 *Cellulose*

Cellulose is regarded an inexhaustible resource with an estimation of approximately 1.5 trillion tons producible yearly from biomass (Kumar et al, 2008). Cellulose is a regular, linear chain of 6-carbon D-anyhdroglucopyranose (AGU) monomeric units (degree of polymerization ranging from ~300 – 15000) which are connected by β-1,4-glycosidic linkages, as shown in Figure 2.2.

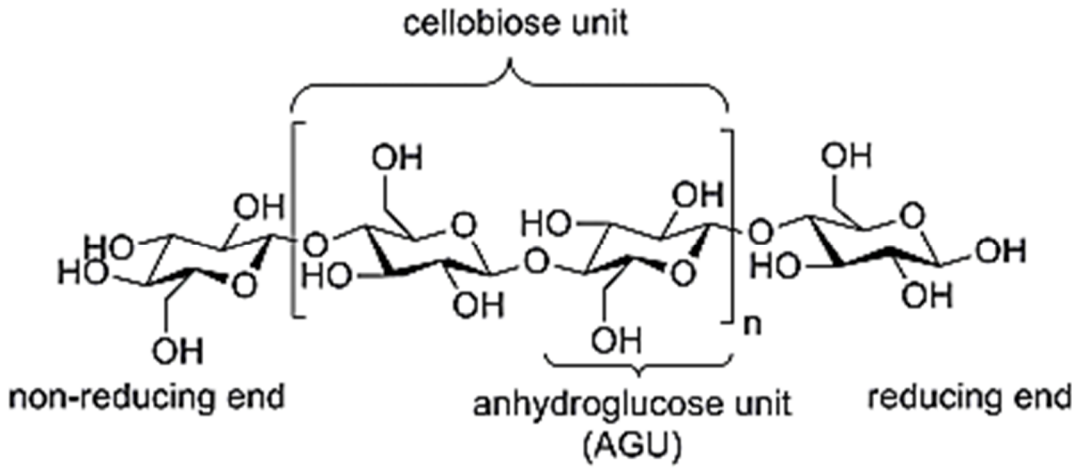


Figure 2. 2: Structure of cellulose showing cellobiose; the simplest unit of the polysaccharide. ((Olsson and Westman, 2013) Copied in terms of Fair Dealing – Section 12 of the SA Copyright Act No. 98 of 1978 (as amended)).

Cellulose consists of well oriented microfibrils which coalesce into longer, larger, crystalline and hydrophobic fibrils. The resulting structural material enables cellulose to play very important physicochemical and structural roles in plant cell walls (Gross and Chu, 2010). Since it was first isolated and identified by Anselme Payen in 1838, extensive work has been done on the biopolymer and knowledge of the intramolecular structure is not scarce in literature. Cellulose has a characteristic 2-fold screw axis along the chain direction that results in a 180^{o} rotation of every second AGU, such that the desired bond angles of the connecting β-(1-4)- glycosidic bonds is easily adjusted to achieve a flexible orientation (Klemm et al., 2005). The sets of two AGU units before each rotation are cellobioses; the simplest form of cellulose. Cellobioses (Figure 2.3) have eight free hydroxyl groups, one acetal linkage and one hemiacetal linkage all of which allows for very strong inter- and intra-molecular bonding opportunities with other AGUs and compounds. X-ray diffractions (XRD) and Nuclear magnetic resonance (NMR) readings have shown that AGUs have unique orientations assumed to be a $^{4}C_{1}$ chair conformation which allows for the hydroxyl groups of the monomers and their hydrogen atoms to be situated at the equatorial and axial planes

respectively (Klemm et al., 2005, O'sullivan, 1997). In other words, all three hydroxyl groups on a glucopyranose ring are situated on the equatorial position and the hydrogen atoms on the axial position. The resulting structure implies cellulose do have an intrinsic anisotropy characterised by major variations in polarity at respective sides of the molecule; while the equatorial direction of the glucopyranose ring is hydrophilic, the axial is hydrophobic. Hence, the higher the degree of polymerization of the AGU (100- 20,000), the more complex the inter- and intramolecular hydrogen bonding network and the more crystalline the biopolymer.

Cellulose crystallinity is an important factor in biomass processing, as it has been shown to be directly correlated with increased LCB recalcitrance to catalytic degradation (Klemm et al, 2005). Hence, strategies for the disruption of the naturally organized network of the cellulose polymer as in this study is essential for biomass activation for use as feeds in biorefining. Efforts on non-derivatizing solvation of cellulose in biomass pre-treatment will be discussed later in this review.

2.1.2.2 *Hemicellulose*

Hemicellulose is the second most abundant polymer on earth, after cellulose. It is an amorphous material made up of random branched chains of 5-carbon sugars, 6-carbon sugar monomeric units and occasionally organic acids. Hemicellulose has a relatively lesser degree of polymerization (~70-200) and functions as a non-covalent link between cellulose bundles (Khalil et al., 2007). The monomeric components of hemicellulose are 5-C sugars such as xylose, arabinose, and mannose, all of which also have found use as substrates for the biomanufacturing of bio-based products (e.g. xylitol from xylose).

2.1.2.3 *Lignin*

Lignin is a robust, hydrophobic, immensely cross-linked, poly-condensed, amorphous, phenolic macromolecule which forms a protective sheath across both cellulose and hemicellulose in the biomass matrix (Figure 2.1). The building blocks for lignin include three types of monolignol; guaiacyl (G), sinapyl (S) and *p*. Hydroxylphenyl (H), which are held together by aryl ethers or strong hydrogen bonds (Vanholme et al., 2010). Lignin is an extremely complex structural macromolecule which also functions as a conduit for nutrients and water transport in plant cells. It is an extensive biopolymer with degree of polymerization that may exceed 15000 (Vanholme et al, 2010). Their excellent physical properties contribute to the characteristic recalcitrance of LCB to degradation. The lignin sheath barrier in LCB matrix effectively forms anti-hydrolytic refractory effects via a selective adsorption mechanism that restricts access and uniformity in the distribution of chemical and microbial agents in the cell wall. As such, delignification is regarded as an efficient pre-treatment parameter and has been directly correlated in several reports to improved hydrolysis of the inherent sugar polymers in LCB (Park et al., 2015; Singh et al., 2014).

In addition to the afore-described structural components of biomass, non-structural components such as protein, pectin, ash and extractives are also found in LCBs at usually negligible quantities (Ladisch et al., 2013).

The general make-up and bonding characteristic of the polymeric constituents of LCB materials, both respectively and collectively necessitates the need for an activation step prior to conversion. Mosier et al, (2005) argued that no more than 20% of inherent sugar molecules in biomass can be recovered for conversion without an efficient pre-treatment process; bringing to fore, techno-feasibility challenge which discourages LCB utilization as

process feeds in their untreated state (Mosier et al., 2005). Thus, innovative engineering solutions tailored to enhance the accessibility of catalysts and substrate susceptibility to catalysis currently dominates the scope of LCB processing.

2.2 Lignocellulosic Biomass Processing

The current process of producing biocommodities from LCB can be categorized into three major steps; a) pre-treatment b) hydrolysis c) Bioconversion

2.2.1 Pre-treatment

Pre-treatment involves the use of physical, biological or thermochemical processes (Table 2.2) to increase the responsivity of the natural biomass to catalysis (bio or chemical) (Yang and Wyman, 2008). As illustrated in Figure 2.4 efficient pre-treatment is usually characterised by the disorganization of the ordered recalcitrant structure of the LCB matrix. In order to overturn the characteristic recalcitrance of biomass an efficient pre-treatment system would have achieved some or all of the following; (a) increased surface area and porosity of saccharides (b) reduced cellulose crystallinity (c) delignification (d) hemicellulose solubilisation, and (e) degree of polymer depolymerisation (Bensah and Mensah, 2013).

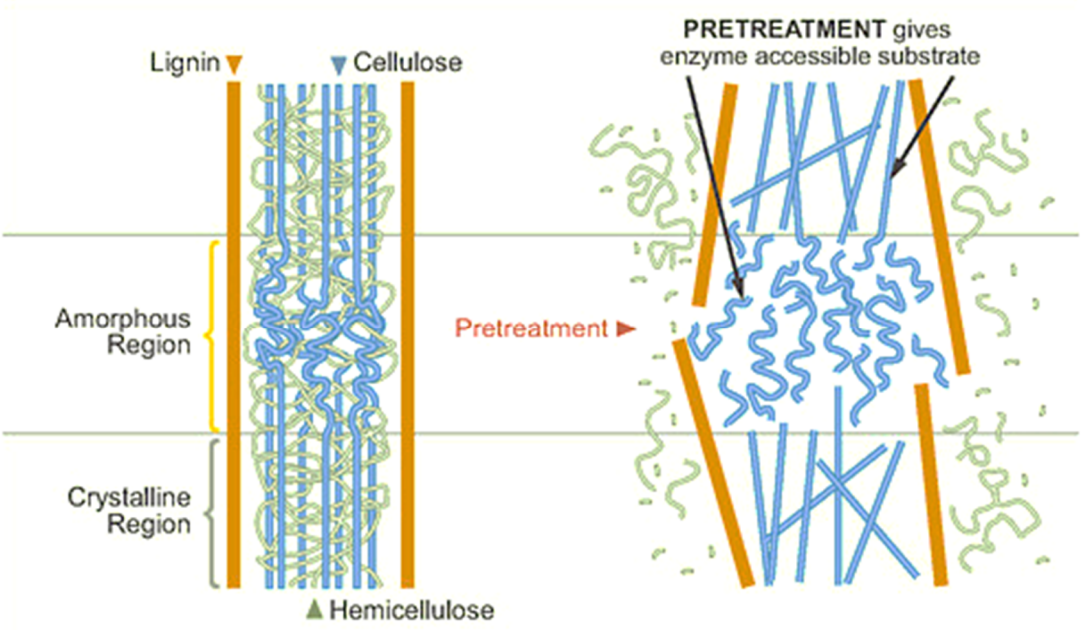


Figure 2. 3: Schematic illustration of the effect of pre-treatment on the integrity of biomass cell wall ((Mosier et al., 2005) Copied in terms of Fair Dealing – Section 12 of the SA Copyright Act No. 98 of 1978 (as amended)).

The aforementioned effects can be achieved by a variety of techniques designed to disorganize, de-lignify and de-crystallize the biomass matrix. For example, thermochemical and physical comminution methods disorganize the biomass matrix by increasing the surface area of the biomass while solvating methods such as dilute acid and lime treatment delignify LCB. Though effective, in terms of achieving the targeted effects on biomass, these methods are plagued by cost, sustainability and process challenges which are as summarized in Table 2.2. In general, the major challenges with current pre-treatment methodologies includes the following:

1) *Cost*: Exorbitant energy, reagent, maintenance and disposal costs especially for energy-intensive (such as hydrothermolysis) and corrosive processes (such as alkali and acid pre-treatment). Yang and Wyman carried out a techno-feasibility study of dilute acid pre-treatment in the production of bioethanol from waste LCB and the step accounted for approximately 25 % of the entire process cost (Yang and Wyman, 2008). Hence, recent developments for improved bioprocesses have seen a shift in research focus to efficient pre-

treatments at reduced costs and mild conditions in cheaper recyclable media.

2) *Eco-sustainability:* The use and disposal of non-environmentally friendly pre-treatment media such as acids, alkali and ionic liquids contravenes the core ethos of green processes (Prasad et al., 2016). The eco-toxicity of these materials requires the treatments of waste streams prior to disposal into natural environments.

3) *Process Efficiency:* The effects of current pre-treatment techniques on downstream fermentation and bioconversion efficiency have been a major challenge for bioprocesses. Current solvent techniques employed in biomass pre-treatment have been directly linked to the formation of degradation products such as hydroxyl methyl furfural (HMF) and pseudo-lignin (Ju et al., 2013). Degradation products are derivatives of monomeric sugar substrates for downstream processes which have been shown to effectively inhibit biocatalysts (microbial and other).

The formation of degradation products in pre-treatment media is correlated to the severity factor (R^1_o), calculated for using Equation 2.1.

$$\log R_0^1 = \log\left(t.e^{(T-100)/14.75}\right) - \mathrm{pH}, \qquad (2.1)$$

The severity factor summarizes the multiple effects of the different pretreatment factors on the susceptibility of the biomass matrix to saccharose depolymerization (Pedersen et al, 2010). Pre-treatment conditions such as media pH, time (t) and temperature (T), have directly correlated effects on the digestibility of polysaccharides (e.g. cellulose to glucose) and the simultaneous degradation of monosaccharides (e.g. simple sugars to furans). In addition to the aforementioned process parameters, other factors such as such as biomass particle size and liquid-to-solid ratio also contributes to the severity of pre-treatment media (Li, 2012).

Table 2. 2: Overview of pre-treatment methodologies for lignocellulosic biomass

	Media	Advantages	Disadvantages	References
Hydrothermolysis	High-pressure saturated steam at reduced pressure conditions	Simple operation, Low enzyme demand	Energy intensive process. High water input required. Pentose sugar are yielded in oligomeric forms, hence need for further processing Inhibitory and degradation products formed	(De Bari et al., 2007)
Alkali hydrolysis	NaOH, $Ca(OH)_2$, NH_3	Delignifies at relatively mild conditions. Relatively low production of inhibitory nor degradation products.	Lengthy pre-treatment High cost of solvent/chemical Requires complex recovery process.	(Alves et al., 2016)
Ammonia fiber expansion (Afex)	Liquid NH_3 at high T and P, 1-2g of NH_3 per g of dry biomass. 90 ^oC for 30 mins	Low levels of inhibitors and side-products generated. Characterised by efficient enzymatic hydrolysis which requires low enzyme.	Safety and environmental issues of Ammonia. Sugars are yielded as oligomers and as such require further processing pre-fermentation.	(Gao et al., 2014)
Acid hydrolysis	H_2SO_4 and HCl	Generally high yields of fermentable sugar Current analytical standard for biomass processing	Sugar recovery rate is low. High levels of inhibitory, side and by-products released. Very corrosive technique Acid recovery systems required for feasibility of technology. Neutralizations required for further processing.	(Wang et al., 2011)
Inorganic hydrate salts		Non-derivatising cellulose solvation. Cheap, non-toxic, easily recyclable	Relatively infant method	(Sen et al., 2013)
Biological	Brown-, white- and soft- rot Fungi	Low energy demand. Environmentally friendly	Process too slow to be feasible	(Sen et al., 2013)
Ionic Liquids		Dissolves cellulose Minimal environmental impact	High reagent costs Challenges with salt regeneration Toxicity and causticity issues with solvents	(Gao et al., 2014)

Modern efficient pre-treatment techniques are being tailored to increase fiber reactivity, maximise sugar yield and recovery from biomass, while producing negligible amounts of enzyme-inhibiting side products, at minimized energy demands (Bensah and Mensah, 2013). This study as such sought to circumvent the aforementioned challenges with biomass treatments by increasing cellulose fiber reactivity by solvation in non-derivatising zinc chloride molten hydrate salts system.

Non-derivatising solvation of cellulose occurs without any form of prior chemical modification to the biopolymer (Sen et al., 2013). In other words, solvation; accompanied by increase in surface area and decrystallization of the material, in aqueous media is achieved without prior functionalization. Cellulose structure is altered in-media as a result of hydrogen bond formations between the ionic components of the solvent system and the numerous hydroxyl groups of the biopolymer. The new bonding formations results in the disruption of the initial inter and intra-molecular hydrogen bonding thus resulting in the deconstruction of the biopolymer (see Figure 2.4). Non-derivatising dissolution limits the formation of degradation products as the reaction requires minimal hydrolysis for the breakage of the hydrogen bonds along the equatorial plane of the polymer.

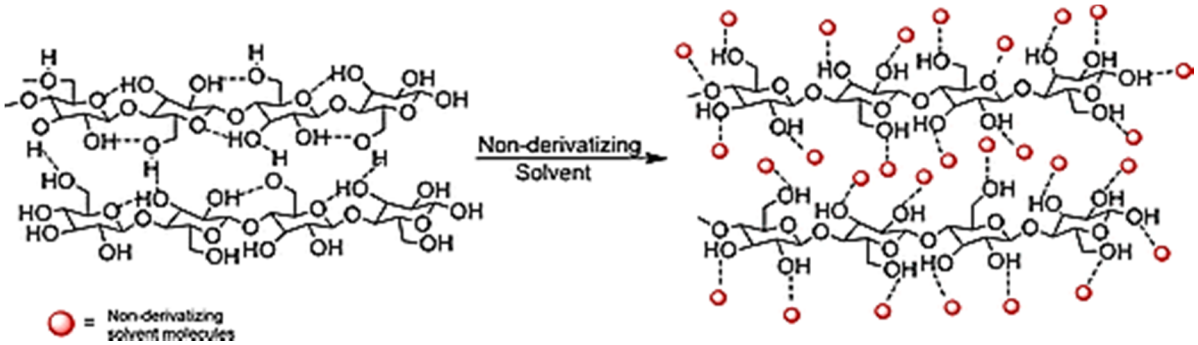


Figure 2. 4: Cellulose solvation mechanism of non-derivatising solvent systems ((Sen et al, 2013) Copied in terms of Fair Dealing – Section 12 of the SA Copyright Act No. 98 of 1978 (as amended)).

2.2.1.1.Molten hydrate salt pre-treatment of lignocellulosic biomass.

Molten hydrate salts (MHS) are an important class of non-derivatising cellulose solvent and a promising LCB pre-treatment technique. Molten hydrate salts are inexpensive, non-corrosive, green, easy-to-prepare and recyclable cellulose solvent systems. In addition, they are relatively non-toxic and environmentally benign, hence, the growing interest in them as alternatives to acid and conventional ionic liquids for sustainable LCB treatment and as reaction medium for sugar conversion (Li et al., 2013). They are generally described as aqueous salt systems with water to salt molar ratio close to the coordination number of the strongest hydrated cation (Leipner et al., 2000). MHS are simple salt-water systems with concentrations varying between aqueous salt solutions and solid hydrates.

Braunstein, in 1968, differentiated molten hydrate salts from aqueous salt solutions by classifying liquid cyclometallated salt mixtures into five classes based on the approximate ranges of water-mole ratio: (I) Debye−Hückel limiting (1 to >0.99); (II) extended Debye−Hückel theory (0.99−0.9); (III) hydrate melts (0.9−0.75); (IV) incomplete hydration sheaths (0.75−0.01); (V) gas solubility (<0.01) (Braunstein, 1968). 20 years later, Emon concluded that MHS are salt melts where only ion-water contacts occur (without any linear ion-ion or water–water contacts) (Emons, 1988). These assumptions factored the general tendencies of thermodynamic and transport properties as well as the co-ordination behaviour of the systems as related to specified concentration scales. The proposed ideal structure of MHS involves a single hydration sphere shielding the strongest hydrated cation from the anions as illustrated in Figure 2.5. Based on the proposed structure, the theoretical possibility of hydrate melt systems within a mole fraction range of salt from 0.1 to 0.25 is evident. To this end, Emon (1988) argued that the properties of hydrate salts are hinged on the stability and dominance of formed ion-water associations and the extent of competition from anion-

water coordination; which subsequently results in cation-anion, water-water contacting.

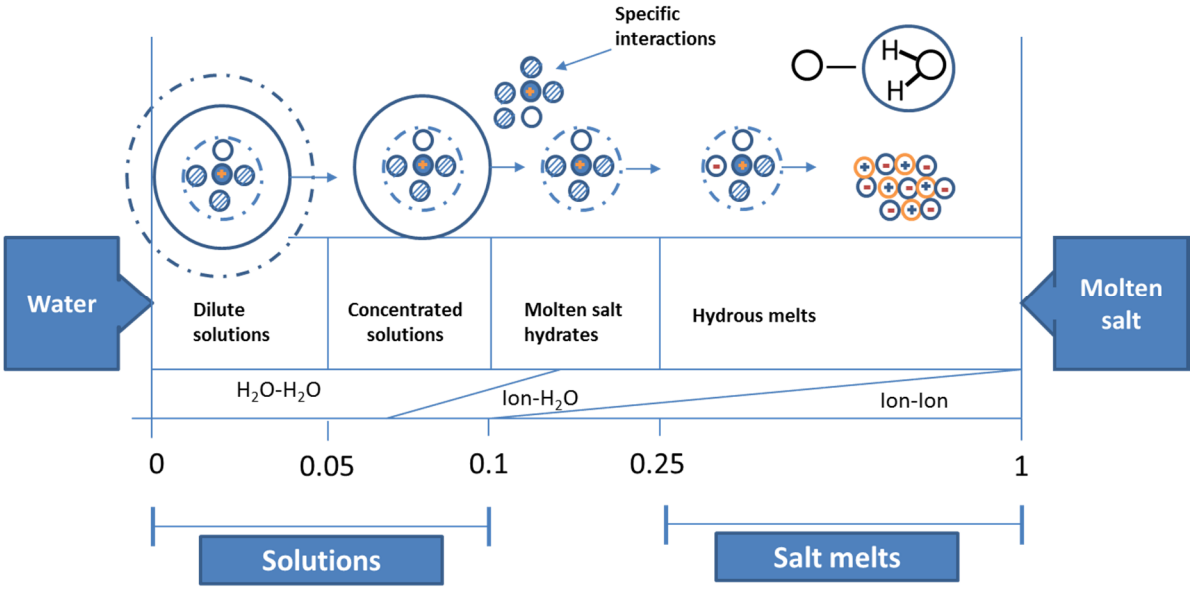


Figure 2.5: Proposed scheme for the demonstration of coordination and contact interaction for the differentiation of molten hydrate salts from molten salts and dilute solutions using the concentration scale. (Adapted from Emons, 1988))

More recent and crystallographic investigations into the molecular orientations of MHS have supported initial reports of the tight binding of water molecules to the inner coordination sphere of the metal cation (Wilcox et al., 2015). For example, Hennings et al, (2014) reported the crystallographic orientations and structure of $ZnCl_2.2.5H_2O$, showing the Zn^{2+} cation in i) a tetrahedral coordination with Cl^- and ii) an octahedral environment surrounded by five water molecules and one Cl^-, which was shared with $[ZnCl_4]^{2-}$ (Hennings et al., 2014) The trihydrate specie {(hexaaquazinc tetrachloridozinc) $[Zn(H_2O)_6][ZnCl_4]$} on the other hand, consisted of three different Zn^{2+} cations, two of which are octahedrally surrounded by water molecules.

The effects of MHS on biopolymers vary with different MHS properties, which according to Emon's model is affected by complex formation, thermal stability and lewis acidity. These properties are believed to differentiate between MHS in the context of cellulose processing. MHS can be classified, based on their effects on cellulose into the following groups:

a) No reaction
b) Strong swelling (e.g. $ZnCl_2 \cdot 2H_2O$, $ZnCl_2.4H_2O$, $LiCl_2.2\text{-}5H_2O$, $LiNO_3 \cdot 2H_2O$)
c) Dissolution (e.g. $Ca(NCS)_2 \cdot 3H_2O$, $LiClO_2.3H_2O$, $LiSCN \cdot 2H_2O$, $ZnCl_2.3H_2O$, $LiI_2 \cdot 2H_2O$).

The different types of MHS that have been investigated for potential applications in modern corn stover LCB processing are summarized in Table 2.3. The potentials of water-salt (binary and tenary) systems for major industrial cellulose treatment application have been a subject of intense investigation as far back as the early 20^{th} century. The solvating potentials of concentrated salt solutions was first reported by Weirman in 1912, two decades before Letters provided proof of increased polymer surface area (swelling) and dissolution of the polysaccharide in 55 and 63 % (w/w) aqueous zinc chloride. The observed structural changes in cellulose have been linked with polymer mercerization (Katz and Derksen, 1930) hence, propositions for salt solutions in cellulose functionalization, depolymerization and beneficiation. The opportunities for large-scale materials development have prompted numerous studies seeking to explain the effect of different salt melts on cellulose. For example, the proof of non-derivatization in MHS dissolution was substantiated by ^{13}C NMR studies of cellulose dissolved in $LiClO_4.3H_2O$ and $ZnCl_2.3H_2O$ (Fischer et al., 2003). Likewise, FT-IR spectra of regenerated cellulose from $ZnCl_2.3H_2O$ did not show any additional peak which was not recorded for the original material (Lu and Shen, 2011).

Table 2.3: Review of hydrate salts used for the treatment of corn stover.

MHS	Sugar recovery	Conditions	References
$FeCl_3$	89 % Xylose 94 % Xylose	160^oC, 20 min, 0.1 mol/ L$FeCl_3$ 160^oC, 20 min, 0.125 mol/ L$FeCl_3$	(Liu et al., 2009) (Kamireddy et al., 2013)
$Fe(NO3)_3$	91.8 % Xylose, 19 % Glucose, 96.74% Arabinose 81.66 % Xylose	150^oC, 10 min, S/L=1:9 (w/w), 0.05 mol/ L$FeCl_3$ 150^oC, 21.2 min, L/S= 9:1 (w/w), 0.05 mol/ L	Sun et al., 2011 Sun et al., 2011
$CuCl_2$	93% Xylose	160^oC, 10 min, 0.125 mol/ L $CuCl_2$	Kamireddy et al., 2013
$FeSO_4$	60.3 % Xylose	180^oC, 20 min, 10 % (w/v) $FeSO_4$	Jing et al., 2011
$ZnCl_2.4H_2O$	95% Xylose 25% Glucose	70 ^oC, 5 h, S/L = 1: 12	Awosusi et al, 2017

MHS hydrolyzes in water to form strong acidic solutions which dissolves cellulose. Details into the mechanism of interactions between the polysaccharide and aqueous salt systems have been the subject of numerous research attempts over the years. A concise understanding, of which, can provide the basis for the improvement of current process economics and development of modern, integrated approaches to beneficiating biomass.

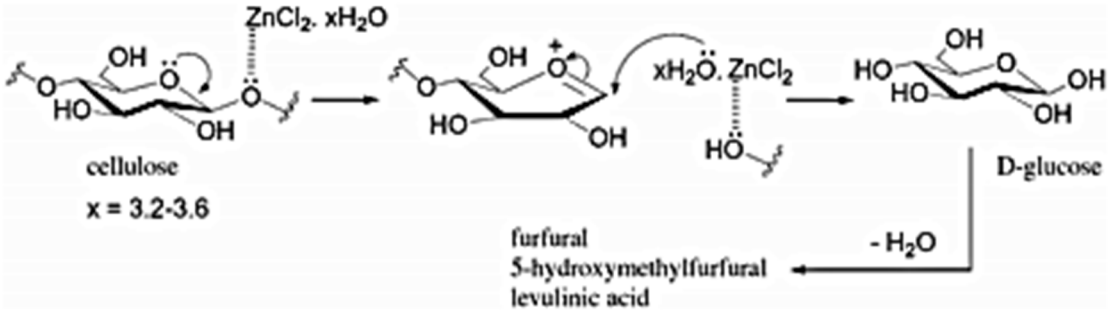


Figure 2.6: Lewis acid mechanism for cellulose dissolution in Zinc chloride at 200 ^{o}C ((Amarasekara et al, 2002) Copied in terms of Fair Dealing – Section 12 of the SA Copyright Act No. 98 of 1978 (as amended))

Richards et al, (1970) attempted to describe the behaviour of polysaccharides in aqueous zinc chloride using model glucopyranosides (Richards and Williams, 1970). In what is generally regarded as one of the earliest successes recorded, methyl β-D-glucopyranoside in aqueous $ZnCl_2$ was observed to undergo changes in their optical rotation. The rotation was attributed to the electrical environment that resulted from ionic reactions of the electrolyte. This standpoint was further buttressed by H-NMR studies which confirmed a deviation in the chemical shift of both the anomeric and ring protons of the sample thus implying direct interactions between the polysaccharide and MHS ionic constituents.

Further clarifications as regards the exact mechanism of dissolution came by means of ^{1}H-NMR analysis of the behaviour of glucopyranosides with varying levels of o-Methylation;

2,3,4,6-tetra-O-methyl-B-D- Glucopyranoside, hexa-O-methyl was myo-inositol, ethyl 3,4,6, tri-O methyly β-D- glucopyranoside and methyl-4,6-diO-methyl β-D glucopyranoside, in aqueous $ZnCL_2$. Of all four samples, only methyl-4,6-di-O-methyl β-D glucopyranoside reflected the earlier observed shift, which was reminiscent of the standard glucopyranoside $ZnCl_2$ interactions (Richards and Williams, 1970). The defining structural difference which the observed change of orientation in methyl-4,6-di-O-methyl β-D glucopyranoside could be attributed to was the presence of free proximate hydroxyl(OH) groups. Hence, the proposition of the possibility of complex formation involving the MHS cation and the hydroxyl groups of the sugar polymer.

Cellobiose; simplest form of cellulose, differ structurally from glucopyranosides but the findings of Richard et al, (1970) remains very significant as cellulose / cellobioses do have β 1-4, linkages characterised by pairs of hydroxyl groups (Sen et al, 2013). Thus, a rational assessment of cellulose solubilised in MHS infers a complex formation between the cation and the hydroxyl groups across the β- 1-4 glycosidic linkages (Figure 2.7). Xu and Chen, (1999), assumed the formation of this or similar complexes when the generated UV spectra for cellulose dissolved in 67 wt% $ZnCl_2$ (1:3) showed distinct peaks at 290nm (Xu and Chen, 1999). As such, the formation of complexes is deemed instrumental to the mechanism of hydrate salt solvation of highly crystalline polymers such as cellulose. Using $[Ca(NCS)_2 \cdot nH_2O]$, Hattori et al, described the mechanism of MHS solvation to be highly dependent on the efficiency of two molecular interactions between the salt and the biopolymers simultaneously (Hattori et al., 1998b). First, is an endothermic process; resulting from the strongly acidic solution formed by the rapid hydrolysis of salts in water that results in the breaking of the hydrogen bonds in cellulose and biomass. While the secondary event is an exothermic process, which as aforementioned is the formation of co-ordination bonds

(Emon et al, (1988) also argued, competes strongly with salt dissociation and plays a huge role in hypothesized characteristic of MHS) (Hattori et al., 1998a).

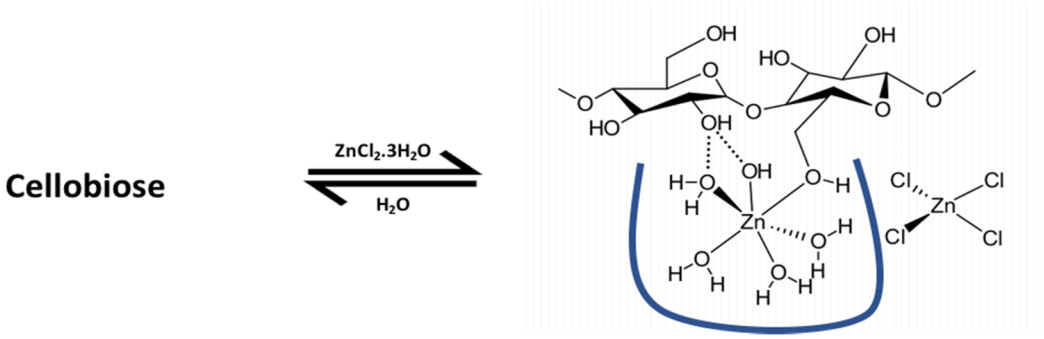


Figure 2.7: **S**chematic illustration of the proposed Zinc-cellobiose complex formation in cellulose treated in zinc chloride trihydrate (Awosusi et al, 2017).

Differential Scanning Calorimetry (DSC) studies by Hattori et al, differentiates between the two processes by calculating the amount of heat dissipated with increasing salt concentrations. The findings showed that an aqueous solution of > 48.5 wt% was required for cellulose dissolution at elevating temperatures (depending on cellulose concentration) in [$Ca(NCS)_2$. nH_2O]. Cellulose dissolution in MHS has been shown to keep the chemical composition of the biopolymer intact but then several effects on the regenerated cellulose have been reported. MHS dissolution has been observed to have considerable effects on cellulose crystallinity, molecular weight and thermal properties (Sen et al, 2013). Fischer and co-workers reported a reduction in the average molecular weight (M_w) of cellulose with a degree of polymerization (DP) of 1511 and Molecular weight 751,400 g/mol upon dissolution in $LiCl_2$. $3H_2O$ was to 284,000 g/mol (Fischer et al., 1999) In a similar study, reduction of molecular weight was linked with polymer chain length as the cellulose samples with the smallest chain length shows minimum reduction in DP, while the longer polymer chains show higher reduction in DP. The contributions of elevated temperature to this hypothesis

were suspected and in a similar study involving bacterial cellulose (initial DP = 4135) at elevated temperature (95 °C for 0.5 h) were found to facilitate the reduction in DP of cellulose (Final DP = 855) (Lu et al, 2011). Sen et al, (2013) although surmised that the inherent acidic nature of the inorganic salts hydrates is responsible for the polymer chain scission and thus reduction of the molecular weight.

X-ray scattering and Raman spectroscopy studies of regenerated cellulose sample showed a loss in-part of its crystallinity and in some cases the highly crystalline cellulose I was reported to have been converted to the less crystalline cellulose II (Leipner et al, 1999). Investigating the crystallinity of cellulose, Leipner et al, (1999) studied the solid state ^{13}C CP/MAS spectra of the polymer regenerated from $LiClO_4 \cdot 3H_2O$ and $ZnCl_2 \cdot 4H_2O$. This comparative study revealed cellulose regenerated from $ZnCl_2 \cdot 4H_2O$ shows higher crystallinity than the cellulose regenerated from $LiClO_4 \cdot 3H_2O$. Further buttressing initial reports of $ZnCl_2 \cdot 4H_2O$ being only a swelling agent only and revealing $LiClO_4 \cdot 3H_2O$ as a true cellulose solvent (Leipner et al, 1999). Both the observed decrease in the molecular weight and the degree of crystallinity was suspected to also have an effect on the thermal properties of cellulose (Lu et al, 2011). Like other morphological changes observed in the regenerated cellulose there are chances they are as a result of polymorph inter-conversion of cellulose I to cellulose II (Sen et al, 2013).

The mechanism of cellulose solvation in MHS isn't completely defined yet, but recent works have linked observed effects to the structure of the binary salt-water system. One of such is Wilcox's work, using advanced crystallographic approaches defined both crystal and liquid structures of zinc chloride hydrates across varying temperatures and hydration (2.5- 4 H_2O/

$ZnCl_2$ for crystals and up to 11.6 H_2O /$ZnCl_2$ for liquid structures). This work proved that $ZnCl_2$ dissociates in water, not into Zn^{2+} and Cl^{-1} ion but rather sees the complete hydration of one Zn ion $Zn(H_2O)_6^{2+}$ and complete chlorination of the other Zn; $ZnCl_4^{2-}$, to form hexa-aqua Zn^{2+} tetrachlorozincate (Wilcox, 2009). The work argued with empirical proof the thermodynamic feasibility of the occurrence of $ZnCl_4^{2-}$ in comparison to the hypothetical $ZnCl_2(H_2O)_2^{2-}$ (Wilcox, 2009). Vibrational modes in raman were detected for the $ZnCl_4^{2-}$ anion in the aqueous system at up to 98 % dilution with water; suggesting the molecular specie would not be hydrated (Wilcox, 2009). As shown in Figure 2.8, both ions were held together by a weak, non-chain, hydrogen bonding occurring between the respective chlorine and hydrogen.

The polar aprotic solvent; $[Zn(H_2O)_6^{2+}]$ $[ZnCl_4^{2-}]$ in further correspondences were described as possessing typical ionic liquid characteristics (Sen et al, 2016). Ionic liquids are substances which are liquid at room temperature and composed majorly of ions (van Rantwijk and Sheldon, 2007). They are green, high-performance reaction media which have been described in several reports as the ideal media for high-tech sustainable processing. ILs are characterised by high thermal stability, low vapor pressure and flexibility in terms of physical properties such as polarity, hydrophobicity and solvent miscibility behaviour (van Rantwijk and Sheldon, 2007). These properties have been the basis of the growing interests for their application for organic synthesis in biocatalysis. An exhaustive review focusing on the dynamics, development and optimisation of biocatalytic processing in ILs was published in 2007. The review like other similar overviews on scope tends to place emphasis on organic ILs as these are the most explored materials on scope while completely excluding major process challenges with synthesis cost, purity and ecotoxicity issues with disposal (Ozokwelu et al., 2017). The cheaper, easily recyclable and completely inorganic alternative

of $ZnCl_2$ hydrates; with properties reminiscent of the organic IL analogues, as recently described by Wilcox's work promises new opportunities for biocatalysis in industrial processing of the future. Been a relatively new scope, biocatalysis in the ionic, electrochemically-active, inorganic $ZnCl_2$ salt system, a concise understanding of the flexibility of the nucleophillicity of the $ZnCl_4^{2-}$, interactions with proteins and is imperative for efficient biotransformations.

2.2.2 Hydrolysis

Hydrolytic processes drive the depolymerization of both polysaccharides; cellulose and hemicellulose, yielding digestible sugar monomers. Hydrolysis can be achieved by means of a) Chemical; e.g acid, alkali (Li, 2012) and b) Biological; enzyme, microbial, treatments (Wang et al., 2012).

2.2.2.1 *Cellulose depolymerization*

The chemical procedure (acid hydrolysis) for cellulose monomerization entails the use of acids (ranging between 10 and 30 %w/w), such as hydrogen chloride (HCl), Nitric acid (HNO_3) and sulfuric acid (H_2SO_4); at reduced pressure and relatively high temperature conditions (Daruwalla and Nabar, 1956; Rinaldi and Schüth, 2009). Enzymatic hydrolysis of cellulose is generally better favoured in-process although due to lower energy requirements considering the mild conditions (<50 ^oC at atmospheric pressure), high sugar yields and less waste products. In addition, enzymatic hydrolysis contributes to the sustainability profiles of biomass processes (Mandels et al., 1974; Sun and Cheng, 2002). Enzymes involved in cellulose hydrolysis include the following:

a) *Cellulase*: is the main enzyme responsible for enzymatic hydrolytic cleavage of the β-1-4 glucoside bond the cellulose structure, this yields cellodextrins (short) and glucose (Sweeney, & Xu, 2012).

b) *Cellobiohydrolases:* Degrades crystalline cellulose; the sequential hydrolytic ability is accosted to the active site bearing a tunnel-like topology (Sweeney and Xu, 2012). The cellulose chain is threaded through the active site, where cellobiosyl component is cleaved off, it motions down the molecule, and proceeds with the subsequent hydrolytic step (Sweeney and Xu, 2012).

c) *β-Glucosidase*: Hydrolyses cellodextrins and cellobiose. They generally do not have carbohydrate binding molecules in their active site; however, they do have an active site which enables a catalytic attack on the on the non-reducing ends of glucose (Sweeney and Xu, 2012). They act on soluble substrates rather than insoluble ones (Sweeney and Xu, 2012).

d) β-*1-4-Endoglucanase:* Contributes to the breakage of glycoside bonds by creating free chain ends which allow for optimal cellobiohydrolase hydrolysis of cellulose. (Sweeney, & Xu, 2012). The active site of this enzyme assumes the shape of a groove, and may consist of carbohydrate binding molecules, which allows cellulose or cellodextrins to be dissolved efficiently (Sweeney and Xu, 2012).

The enzyme hydrolysis reaction is rate limiting, hence, the reaction velocity (V) is proportional to the concentration of uninhibited enzyme-substrate complex (Equation 2.2) (Holtzapple et al., 1994, 1984).

$$V = k[EG_x] \qquad (2.2)$$

Enzymatic hydrolysis of cellulose was described by a generalized theoretical HCH-1 model (Equation 2) proposed by Holzapple et al, 1984.

$$V = -\frac{dG_x}{dt} = \frac{kG_xEi}{\propto + \phi G_x + \epsilon E} \quad (2.3)$$

Where, G_x and E is the concentration of cellulose and enzyme respectively. κ, α, and ε are parameters describing the degree of substrate reactivity. ϕ represent the fraction of cellulose sites that are free, which is usually close to 1($\phi \cong 1$) at high substrate concentrations. *i* (inhibition parameter) can be described using Equation 2.4.

$$i = \frac{1}{1 + \beta_1 G_1 + \beta_2 G_2} \quad (2.4)$$

Assumptions by the model, that the binding-constants for cellobiose (β_2) is higher than for glucose (β_1) meant higher cellobiase activities would result in the further conversion of inhibitory cellobiose, thus implying a *i* (inhibition parameter) which is closer to one.

Generally, the model described a linear plot of the rate of conversion of cellulose against the logarithm of the enzyme loadings. Enzyme loading is an important factor in the processing of biomass as the high cost of enzymes impacts the feasibility of biomass processing negatively. Hence, assuming constant substrate reactivities, adsorption, and enzyme binding constants HCH-1 model can be used to relate rate of reaction, efficiency of conversion to the concentration of enzymes with known cellobiase activities.

2.2.2.2 *Hemicellulose degradation*

Hemicellulose, been amorphous by nature are easily degraded and removed by most pre-treatment steps. Dilute-acid treatment of biomass in solvents such as nitric acid (HNO_3), H_2SO_4, phosphoric acid (H_3PO_4), and Hydrochloric acid (HCl) have been shown to degrade and hydrolyse the hemicellulosic fraction of biomass. Hemicellulases such as polysaccharide lyases (cleavage of glycoside bonds), glycoside hydrolases (hydrolysis of glycoside bonds), and carbohydrate esterases (hydrolysis of ester bonds) liberates 5-carbon sugar monomers in the hemicellulose polysaccharide (Moraïs et al., 2010). Endo-hemicellulases such as mannases and xylanases cleaves the backbones of glycoside bonds facilitate the disruption of Manno/glucopyranosyl polymers and (galacto)(gluco)mannans, (or β (1→4)-D-mannosyl); which consists of an inconsistent linkage of seldom acetylated α (1→6)-D-Galactose side chain (Sweeney, & Xu, 2012).

Enzymatic hydrolysis and pre-treatment are mutually exclusive steps as the rate and efficiency of hemicellulose depolymerization is for the most part a consequence of the efficiency of pre-treatment (Moraïs et al., 2010).

2.2.3 Bioconversion

Bioconversion describes processes which utilizes biological agents (cells or enzymes) as catalysts (biocatalysts) in the production of valuable commodities (Menon and Rao, 2012). Under ideal conditions, biocatalysts (enzymes more especially) have high binding specificity for sugar substrates, converting them over biochemical steps to specific products (de Jong and Jungmeier, 2015; Ghisalba et al., 2010). Alcohols (such as Bioethanol, Xylitol), organic acids (such as succinic, lactic, citric etc.) are a few of the products which have been produced by means of bioconversion on a large-scale basis (Farmer and Mascal, 2015; Isikgor and Becer, 2015; Yuryev and Liese, 2010). In contrast to chemical catalysts, biocatalysed processes are generally characterised by excellent product quality and the mild conditions at which processing occurs resulting in reduced energy demand and costs. Biocatalyst-driven

transformation of sugar monomers plays an essential role in global strategies to fully integrate and standardize biorefinery platforms (Schmid et al., 2001). The concept of biotransformation in bio-manufacturing is quite advanced (Fermentation especially) but also constantly growing in terms of developments for large-scale feasibility.

Bioprocess development requires exceptional emphasis on a variety of scope which contributes to the overall economic feasibility of biocatalysis (Schmid *et al*., 2001). For example, several biocatalytic processes use immobilization techniques which allows for catalyst reuse and recovery (Schmid *et al*., 2001). When screening for enzymes or whole cell, the selected biocatalysts need to be able to facilitate the desired reaction (Schmid et al., 2001). Biocatalysis maximizes performance, when using enzymes; protein engineering is employed, for improved enzyme stability and substrate range; whereas in whole cells, substrate import, solvent resistance, product export and the elimination of by-products is improved (Schmid *et al*., 2001).

Biomass conversion processes differ based on how the aforementioned biological events (Figure 2.8) are integrated in-process. The resulting process-specific configurations like the eventual bio-based products are informed by the type of biocatalysts employed and classified into either of the following; a) Separate hydrolysis and fermentation (SHF) b) Separate hydrolysis and co-fermentation (SHcF) c) Simultaneous saccharification and fermentation (SSF) d) Simultaneous saccharification and co-fermentation (SScF) e) Consolidated biomass processing (CBP).

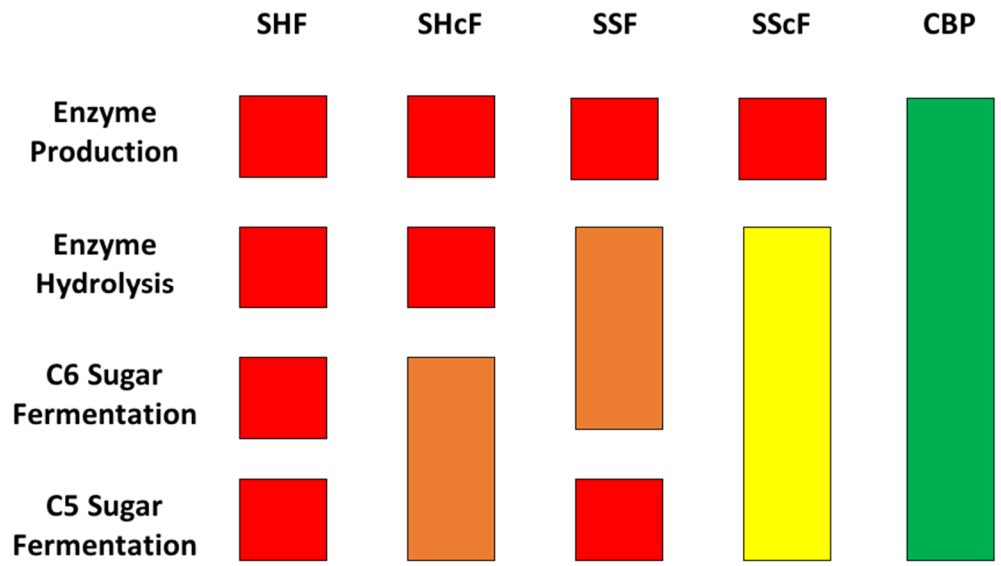


Figure 2.8: Process configurations for biomass conversion (Adapted from Lynd, 1996)

2.3 Succinic acid: The ideal platform bio-based product

Succinic acid (SA) ($C_4H_6O_4$), (IUPAC name: butanedioc acid) is a non-volatile, water soluble, dicarboxylic acid. SA, was first isolated in 1556 by Agricola, by distilling amber; a fossilized plant resin renowned for the decorative and preservatory functions. Since Agricola's work, it has been established that SA is a key intermediate of the tricarboxylic acid (TCA) cycle; an important intracellular metabolic pathway in most living organisms (Zeikus et al., 1999). Better understanding of the linear saturated dicarboxylic acid (Table 2.4) have since grown and overall properties have encouraged application in several sectors Today, succinate is used extensively in the food industry as a pH regulator and bacteriostatic in addition to its application as an important precursor in the production of a wide-range of products (such as biodegradable polymers, phthalate-free plasticizers, green solvents, resins, detergents, surfactants, coatings, pigments, perfume esters and lacquers) (Bechthold et al., 2008).

Table 2. 4: Physical properties of Succinic acid (Perry et al., 1997)

Molecular weight	118.09 g mol^{-1}
Melting point	235 ^{o}C
Boiling point	189 ^{o}C
Dissociation constant,	
$pK_{\alpha 1}$ at 25ºC	4.21
$pK_{\alpha 2}$ at 25ºC	5.6
Solubility at 20 ^{o}C	58 g L^{-1}
Density	1.56 gcm^{-3}

SA, like other linear saturated dicarboxylic acids is a platform chemical i.e., it can be converted in one-step to several bulk, value-added chemicals such as tetrahydrofuran, gamma-butyrolactone, adipic acid (Figure 2.9). In 2004, the US department of energy described bio-based succinic acid (BSA) as "a bio-commodity of utmost importance to the global sustainability agenda", due to its suitability for use as a building block for several valuable specialty chemicals produced conventionally from crude oil (Werpy et al., 2004).

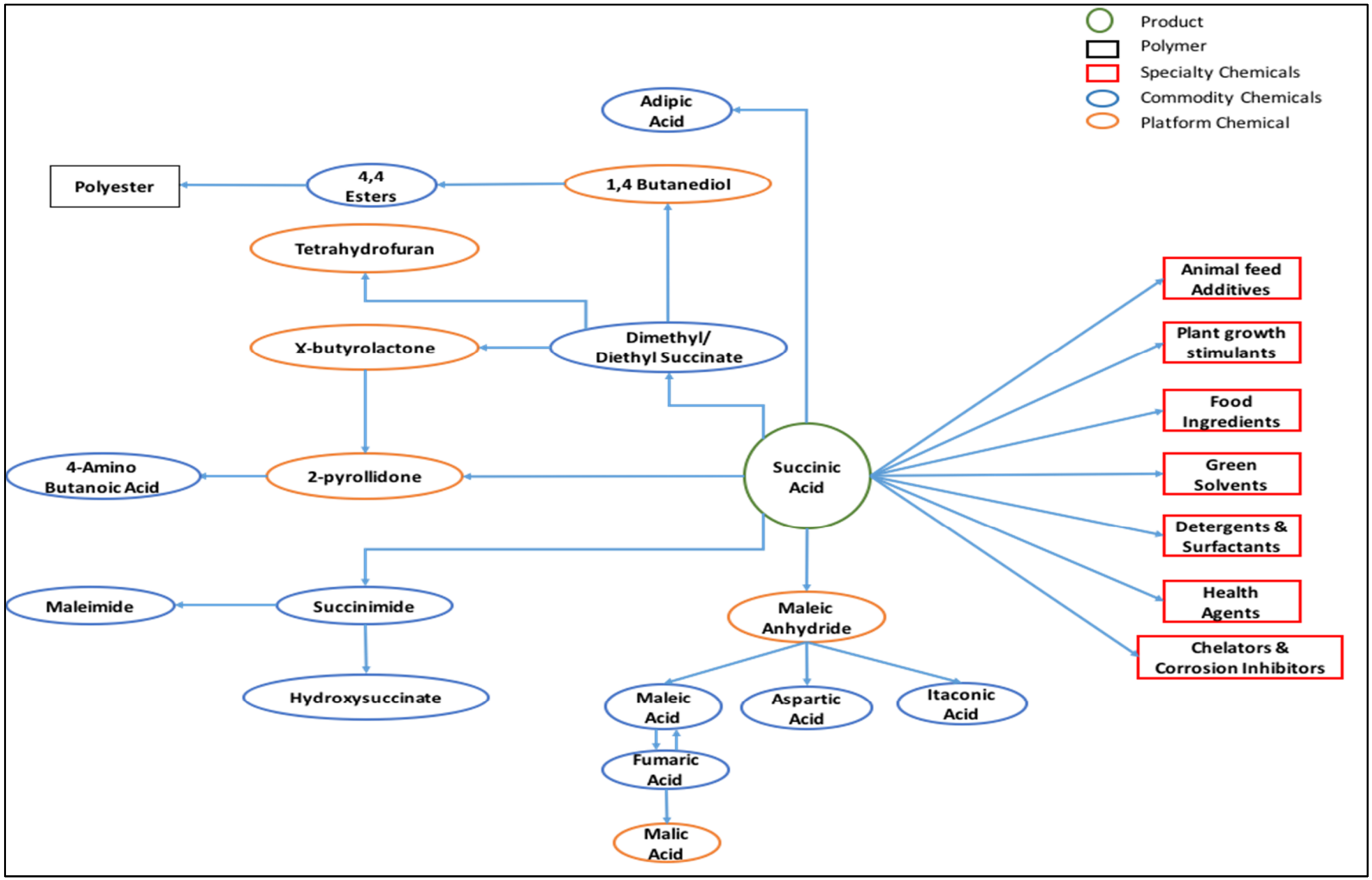


Figure 2. 9: Platform chemical credentials of Succinic acid (Adapted from Zeikus et al., 1999)

Given the opportunities presented for global market growth by the potential of BSA and of course, the current favourable climate, for sustainable practises, the market demand has been forecasted to increase by 800 % (30 to 270 kt/annum) by 2030 (McKinlay et al., 2007). Unlike the conventional method for succinic acid production; which involved the catalytic hydrogenation of petrochemicals such as maleic anyhydride and malic acid to succinic anhydride then succinic acid, post-hydrolysis (Bruk et al., 1998), bio-based production of SA, is produced by fermentative acidogenesis using proven biocatalysts (Beauprez et al., 2010). In comparison to the chemical process, fermentation processes usually occur at relatively mild conditions and as a result saves on energy costs and carbon footprint (Cok et al., 2014; Cukalovic and Stevens, 2008; Liu et al., 2013). The status of both chemical and bio-based routes of large scale succinic acid production is summarized in Table 2.5

Table 2. 5: General comparison of petrochemical and bio-based routes in the production of valuable commodities and materials.

	Bio-based routes	**Petrochemical Routes**
Origin	Biomass- The most abundant material on earth; Uniform distribution across the globe.	Fossil Sources- Deposits arguably in decline; Major cause of climate change due to extreme greenhouse gas emissions. Socio-political influenced fluctuations in supply
Technology	Young but efficient technologies. Developments for optimised yields still been explored.	Well-advanced, optimised, large-scale processes.
Processing Costs	Currently very expensive downstream processing	Relatively cheaper, in comparison to most renewable technologies
Productivity	Non-optimised yield and variations in products formed in systems.	Generally high yields of commodities.
Major disadvantages	Physiological constraints of biocatalysts such as sensitivities to fluctuations in operating conditions.	High energy demands; Disposal of spent catalysts; Environmental pollution
Public Perception	Increased interest and attention characterised by government subsidies, industrial investments and academic research.	Decreasing popularities; Reduced government commitments

BSA production is a developing scope in biomanufacturing currently characterized by limited commercial production worldwide (Table 2.6). The few existing large-scale projects, although yet advanced in terms of microbial technology employed, continue to define and push the frontiers of the underlying bioprocess technology in BSA production. The strategies of projects is dependent on different microbial catalysts and their technologies yet optimised for improved yields at minimal process costs. In general, better knowledge of metabolic pathways for BSA accumulation, metagenomics engineering for improved physiological tolerance of biocatalysts have informed ideas for more efficient, integrated and cheaper bioprocess designs. The resulting optimised BSA bioprocesses are essential for overall techno-feasibility and sustainable production.

Table 2.6: Large-scale plants producing succinic acid

Company	**Catalyst**	**Output (tons/annum)**	**Location**
Bioamber	*Saccharomyces cerevisiae*	2,000	Pomacle, France
DSM/ Roquette	*Actinobacillus succinogenes*	10- 50,000	Lestrem, France
Myriant	*Escherichia coli*	13,500	Louisiana, USA
BASF	*Basfia succiniproducens*	25,000	Spain

2.3.1 Succinic acid fermentation process

Biological cells undergo two major metabolic processes; catabolism and anabolism. Anabolic processes are fundamental to growth and development of the cells and consumes energy while catabolic processes generate energy by breaking down complex compounds; usually hydrocarbons to simple carbon compounds. For example, d-Glucose; a 6C hexose is catabolized in eukaryotic biological cells via the glycolytic cycle, producing a net amount of 2 adenosine triphosphate (ATPs), NADH and 2 molecules of Pyruvate (3C) (R2.1).

$$C_6H_{12}O_6 + 2NAD^+ + 2ADP + 2P \rightarrow 2CH_3(C=O)\,COOH + 2ATP + 2NADH + 2H^+. \quad (R2.1).$$

ATP is the energy currency of biological cells, as chemical energy generated is stored and transferred intracellularly via the nucleotide. Overall, the amount of ATPs generated can go up to 32 upon transfer of the pyruvate into the other phases of aerobic catabolism; tricarboxylic acid (TCA) cycle and electron transport chain (ETC). The ETC completes the process of cellular respiration via the oxidative phosphorylation of nicotinamide adenine dinucleotide (NADH) by means of electron transfer to an exogenous electron acceptor, such as oxygen (Villadsen et al., 2011). In contrast to respiration, fermentation involves the use of an endogeneous electron acceptor (which is usually an organic compound) as an alternative when there is no oxygen. In deprived oxygen conditions, cellular respiration is not activated hence oxidative phosphorylation does not occur and substrate builds up (Tracy et al., 2012). This leads to a phenomenon described as substrate phosphorylation where the substrate undergoes a balanced series of oxidative and reductive processes at which the reaction (R2.2) is in equilibrium:

$$NAD^+ + NADP^+ \longleftrightarrow NADH + NADPH \quad (R\ 2.2)$$

In other words, the substrate undergoes redox modifications intracellularly so that the oxidation of the electron carriers-NADH to form NAD^+ is achieved helping the latter resume glycolytic functions. Hence, the substrate is metabolised to various compounds such as ethanol, lactic acid, and a host of other organic intermediates (depending on cells), which upon build-up in the cytoplasm is excreted as waste (Shuler and Kargi, 2002).

Succinic acid is a fermentation end-product alongside other intermediates such as acetate, formate, lactate, pyruvate and ethanol in select bacterial strains which uses glucose and glycerol as carbon sources. Mixed-acid fermentation for BSA production is a carbon dioxide fixation process reaction, R2.3 shows.

$$C_6H_{12}O_6 + 2CO_2 \rightarrow C_4H_6O_4 + C_2H_4O_2 + CH_2O_2 \qquad \text{(R 2.3)}$$

The C4 metabolic pathway characteristic of native SA producing fermentation processes is the reductive branch of the TCA cycle (Figure 2.10).

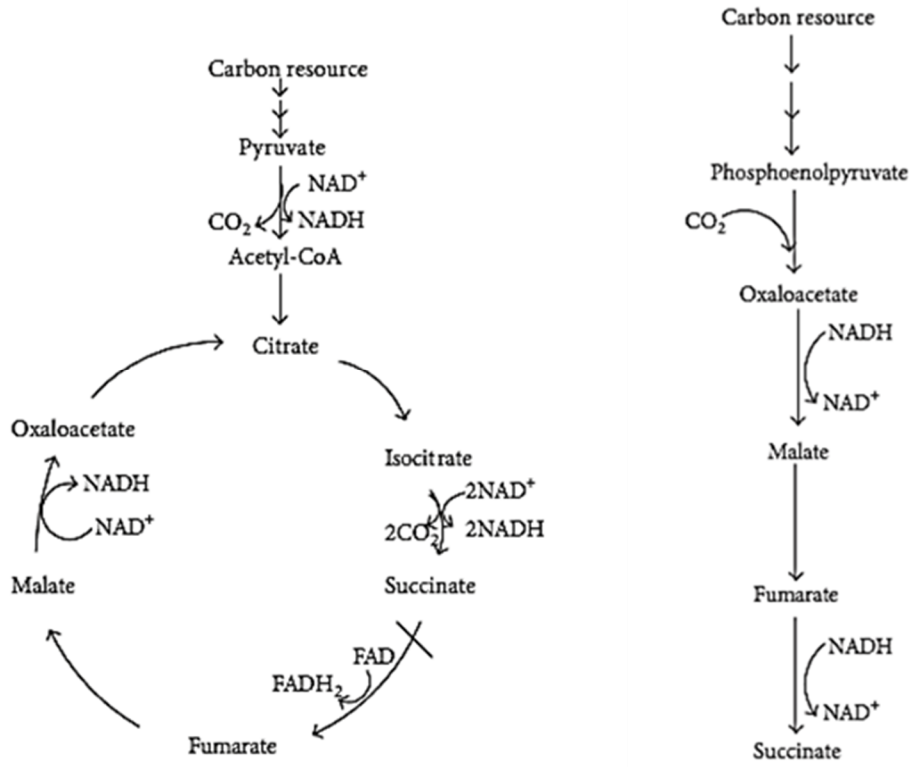


Figure 2. 10: Metabolic pathway for succinic acid biosynthesis in (a) genetically engineered *Escherichia coli* showing succinate dehydrogenase deletion as an alteration of the oxidative arm of the TCA cycle, and (b) C4 fermentation pathway (reductive arm of TCA cycle) for phosphoenolpyruvate conversion to succinic acid in *A. succinogenes* ((Li et al, 2014) Copied in terms of Fair Dealing – Section 12 of the SA Copyright Act No. 98 of 1978 (as amended)).

The active fixation of CO_2 by PEP carboxykinase allows for the conversion of phosphoenolpyruvate to oxaloacetate (Figure 2.11 (a)) thus driving the dynamics away from the C3 pathway to the desired C4 pathway for BSA synthesis.

$$PEP + CO_2 + 2NADH \rightarrow Succinate + 2NAD^+ \quad \text{(R2.4)}$$

It is important to note that both R2.3 and R2.4 are simplified stoichiometric assumptions, disregarding factors such as biomass formation, maintenance and in particular, minor side products such as ethanol (R2.5). The overall yield of the fermentative pathway varies significantly, depending on the assimilation of the carbon dioxide and hydrogen by the microbial systems and PEP carboxykinase activity. In theory, an optimal yield of 1.71 mol succinate per mole of d-glucose (1.2 g g^{-1}) is obtainable at high CO_2 concentrations (R2.5) (McKinlay et al., 2010).

$$7C_6H_{12}O_6 + 6CO_2 \rightarrow \quad 12HOOCCH_2CH_2COOH + 6H_2O \quad \text{(R2.5)}$$

In the presence of additional exogenous sources of electrons (e.g. H_2) the aforementioned yield of 1.71 mol mol^{-1} of glucose value can go up to 2 mol of succinate per mol glucose (1.31g.g^{-1}) (R2.6).

$$C_6H_{12}O_6 + 2CO_2 + 2H_2 \rightarrow 2HOOCCH_2CH_2COOH + 2H_2O \quad \text{(R2.6)}$$

Physiological challenges; such as poor cell growth and slow carbon throughput, which results from the limited catabolism of glucose and subsequent minimized energy generation due to the absence of oxygen also impacts yield in fermentation system. Attempts at aerobic synthesis of SA in microbial cells, although as characterised with faster production rates, haven't resulted in improving yield either. Lin et al, (2005) described SA accumulation under aerobic conditions by metabolic engineering of the oxidative arm of the TCA cycle such that the gene *sdha* which codes for succinate dehydrogenase; the enzyme which converts succinate to fumarate, was deleted in *Escherichia coli* (Figure 2.11 (a)) (Lin et al., 2005). Succinate biosynthesis was characterised by a much reduced optimal theoretical yield as carbon was lost to CO_2 production. The stoichiometric relation is as shown in R2.7:

$$2\text{Pyrurate} + 2H_2O + 3NAD^+ \rightarrow \text{Succinate} + 3\text{NADH} + 2CO_2 \qquad \text{(R2.7)}$$

Another SA biosynthetic routes under aerobic condition is the glyoxylate pathway. Glyoxylate pathway is a cascade of anaplerotic reaction necessary for filling up the molecule pool of the TCA cycle upon microbial adaptation to growth on acetate (Cheng et al., 2012). The pathway converts two moles of acetyl coA to 1 mol succinate *as per* the reaction R2.8:

$$2\text{Acetyl CoA} + 2H_2O + NAD^+ \rightarrow \text{Succinate} + 2\text{CoASH} + \text{NADH} + 2H^+ \text{ (R2.8)}$$

2.3.1.1 Biocatalysts

Extensive work has been carried out on the whole cell fermentation of SA and emphasis and focus have been placed especially on natural, highly efficient BSA producing microorganisms such as *Actinobacillus succinogenes, Anaerobiospirillum succiniciproducens, Mannheimia succiniciproducens,* and *Basfia succiniciproducens* (Song

and Lee, 2006). Advancements in molecular biology have also resulted in the use of genetically engineered *Corynebacterium glutamicum* and *E.coli* bacterial strains for SA biosynthesis (Wendisch et al., 2006). The microbial cells are all active agents for mixed acid fermentation using a wide range of simple sugars and glycerol as substrates. *A. succinogenes, F. succinogenes, A. succiniciproducens* and *M. succiniciproducens* have all been shown to produce SA and a variety of other organic acids from the most common reducing sugars; glucose, fructose, xylose and arabinose, sourced from a variety of raw materials. Proof of BSA production from LCB such as corn wastes (Wang et al., 2014; Yu et al., 2010), cane molasses (Liu et al., 2008b), cotton stalk, sake lees (Chen et al., 2010), wheat straws (Li et al., 2010), and whey (Wan et al., 2008) as carbon sources abound in literature.

Microbes that have been reported in literature to catalyse natural mixed-acid fermentation; although are members of different families, do have many similarities. The bacteria are gram-negative, facultative anaerobes (with the exception of *A. succiniciproducens* which is an obligate anaerobe) and are almost commonly found in rumen microbiomes (Martin, 1998). *M. succiniciproducens* (MBEL55E) (a capnophilic member of the *Pasteurellaceae* family) was first isolated from the rumen of a Korean cow and have been shown to possess similar genomic traits to *A. succinogenes* and *B. succiniciproducens* (also of the Pasteurellaceae family). Similarly, *E. coli* has been shown to grow in the lower intestine of warm-blooded animals (Wendisch et al., 2006). The link between their favourability of this environment and the digestion of animal feed which is majorly lignocellulosic in nature could not be far-fetched. Henderson et al, reiterated the influence of diet on dominance in rumen microbiomes while providing proof of bacterium such as the species of the *Fibrobacter* phylum forming the core of rumen microbiome (Henderson et al., 2015). *F. succinogenes* is a common,

ruminal cellulolytic organism which is also well sought after in biomass processing for SA fermentation. Their ability to degrade cellulose gives an added advantage to bioprocess as a multi-feedstock approach could then be employed with negligible effect on yield. Using *F. succinogenes*, a variety of substrates (glucose, cellobiose, microcrystalline cellulose, acid-treated cellulose, pulped paper, and steam-exploded yellow poplar) which ranged in degree of polymerization, were used as feed for SA fermentation (Gokarn et al., 1997). The study compared SA productivity using the aforementioned substrates with *Ruminococcous flaveciens* at the same culture conditions and found *F. succinogenes* to be a more efficient producer (Gokarn et al, 1997). Likewise, using agricultural waste such as orange peels and wheat straw as substrate, SA was produced by a one-step consolidated bioprocessing efficiently in *F. succinogenes* catalysed systems (Li et al., 2010). The bacteria were stable regardless of D-limonene (a well-known antibacterial agent) in the orange peels and with reported yields of 1.9 g. L^{-1}. At 1.9 g. L-1 h^{-1} productivity is relatively uncompetitive as biocatalysts such as yields as high as 2.2 $g.L^{-1}.h^{-1}$ have been documented for *A. succinicproducens*, using glycerol as carbon source in a continuous process (Lee et al., 2001)

Knowledge of the genomic sequence of other biocatalysts such as E. coli and *C. glutamicum* have informed molecular bioengineering strategies for the developments of more efficient biocatalysts in SA biosynthesis. Naturally *E. coli* undergoes heterolactate fermentation; producing SA, lactate, ethanol and acetate at deprived oxygen conditions. Metabolic engineering of the pathway for by-products formation have resulted in improved yield of succinate reported in literature (Lin et al, 2004). The potentials of SA biosynthesis under aerobic conditions have also been explored by means of sdHa deactivation, but as aforementioned, CO_2 production, impacted carbon throughput and SA yields (Lin et al,

2005). Likewise, Okino et al., 2005 successfully engineered a strain of *C. glutamicum* with a yield of 0.92 g g^{-1} in a fed-batch salt medium.

Another important factor with the use of these biocatalysts is their pathogenicity, as non-pathogenic biocatalysts can infer cost savings in effluent disposal into the environment. With the exception of *A. succinogenes* (which according to McKinlay et al. 2010) is most likely to be non-pathogenic), most members of the *Pasteurellaceae* family are infectious and as such need to have effluents sterilized prior to disposal (Shuler & Kargi, 2002).

Table 2.7 summarizes the different strains of the various bacteria which have been reported to give substantial yields of SA in literature. This work harnesses opportunities presented especially as regards cost, stable microbial associations and multi-microbial functionalities; cellulose degradation and SA fermentation, by using a mixed-consortia as inoculum for fermentation. Rumen bacteria were utilized, characterised in-process, towards an understanding of their roles in defining a flexible bioprocess at different salt and sugar concentrations in-media.

Table 2. 7: Overview of SA yields, rates and reported side products for microbial fermentation of glucose and lignocellulosic biomass.

Strain	SA Fermentation process						
	Substrate	Method	Concentration ($g\ l^{-1}$)	Productivity ($g\ l^{-1}\ h^{-1}$)	Yield ($g\ g^{-1}$)	By-products[b]	Reference
			A. Succinogenes				
FZ6	Corn fiber	Anaerobic. Batch	70.5	0.7	0.88	Fo, Py, Pr, Ac.	Yu et al, 2010
130Z	Glucose	Anaerobic. Batch	67.2	0.8	0.7	Fo Py Ac	Beauprez et al, 2010
CGMCC1716	Corn fibre hydrozylate	Anaerobic. Batch	35.4	0.98	0.73		Chen et al, 2010
			A. succiniciproducens				
ATCC53488	Glucose	Anaerobic. continuous culture	83	10.4	0.88	Fo, La, Ac	McKinlay et al, 2008
			M. succinoproducens				
MBEL55E	Glucose	Anaerobic. Batch	14	1.87	0.70	Fo, La, Ac.	Lee et al, 2002
			E. coli				
SD121	Corn stalk	Dual-phase	57.8	0.8	0.87		Wang et al., 2011
SBS550MG	Glucose	Anaerobic. Fed-Batch	40	0.42	1.06	Fo, Ac	Sánchez et al, 2005
HL27659k-	Glucose	Aerobic, Fed-Batch	58.3	0.72	0.62	Py, Ac	Lin et al, 2005
			C. glutamicum				
R$^{\Delta}$*Idh*A-pCRA717	Glucose	Micro- aerobic, fed batch with cell	146	3.17	0.92		Okino et al, 2005
			B. fragilis				
MTCC1045	Glucose	Anaerobic. Batch	20	0.83	0.57		Isar et al, 2006
			P. ruminocola				
ATCC 19188	Glucose	Anaerobic. Batch	18.9	0.52	-		Guettler et al, 1998
			F. succinogenes				
S85	Orange peel	Anaerobic. Batch	1.75	0.025	0.044		Li et al., 2010
			S. cerivisae				
AH22ura3	Glucose	Aerobic. Batch	3.62	0.022	0.072		Raab et al, 2010

2.3.1.2 Reaction media

Previous works on SA fermentation have established the relative flexibility of proven whole-cells in terms of preferred substrates, metabolic pathway and by-products formation. A rather encompassing factor is the physicochemical trait of the reaction media and the respective interactions of the microbial catalysts. Fermentation media are generally standardized and as such kept constant or at least within a specified range in most SA fermentation studies. The mesophilic nature and sensitivity of biocatalyst to their microenvironments have been a fundamental reason and as such efforts to recreate the optimal medium have been crucial to yield-centred investigations in literature (Liu et al, 2008). The implication of this tend to border on issues as regards the overall flexibility of SA producing systems and even worse their potentials for integration into the wider biomass refining platform. For example, the use of 6C sugars from pre-treated waste biomass hydrolyzates in SA fermentation is characterised by low efficiency that has been attributed to the presence of inhibitors such as furans and phenolic compounds (Li et al, 2007). In other words, derivative products formed from cellulose degradation during pre-treatment contaminates cultures thus having a negative effect on biocatalyst activity. Detoxification processes such as activated carbon adsorption and over-liming of feeds have been employed but observations of much lower fermentation parameters in comparison to synthetic medium still persists (Liu et al, 2008).

A promising strategy for the efficient use of LCB in optimised SA fermentation will be by efficient pre-treatment characterised by reduced formation of inhibitors. Non-derivatising solvation in $ZnCl_2$ hydrate salts have potentials for the execution of this strategy but of interest will be the effect of the salt on fermentation efficiency. (Liu et al., 2008a) reported the effects of varying concentrations of Na^+, Ca^{2+} and Mg^{2+} cations on *A. succinogenes* (CGMCC1593) growth and SA biosynthesis. Ca^{2+} inhibited cell growth at concentrations

over 0.05 mol L^{-1} while Na^{+} was toxic at concentrations above 0.1 mol L^{-1} and Mg^{2+} had no quantifiable effect on both cell growth and SA production (Liu et al, 2008a). Effect of Zn^{2+} is yet to be documented in literature for any of the proven biocatalysts but the same study reported Cl^{-} ions had negligible effect on either cell growth and SA biosynthesis. Other medium components that have been linked to optimal SA biosynthesis include yeast extract (YE) and corn steep liquor. (Guettler et al., 1996) reiterated the positive effects of yeast extract on cell growth and direct correlation between corn steep liquor and improved cell growth and SA yield in *A. succinogenes* (ATCC 55618).

Significant process parameters for the maintenance of the physiological integrity of SA producing biocatalysts, their sustained activity, growth and yield of SA includes the following: a) pH, b) Inorganic carbon source, c) electron donor (Cheng et al, 2011).

pH of media

The pH of fermentation media is an important factor in succinic acid biofermentation for a number of reasons. Firstly, BSA production is a CO_2 fixing process and the pH of media affects the solubility of CO_2 and availability of HCO^{-}_3 for sustained activity and growth of the succinic –acid producing anaerobes (Cheng et al, 2011). Secondly, cell membrane maintenance and general physiology of whole cells are affected by media pH (Liu et al, 2008)). Uncontrolled fermentation cultures catalysed by *Actinobacillus sp. 130Z* was linked to acidulation as decline in pH from 7.0 to 5.2 was observed and high quantities of inhibitory side products such as formic acid, lactic acid and acetic acid were reported (Van der Werf et al., 1997). Similar findings were documented for prominent SA producing bacterial strains such as *A. succinogenes* CGMCC1593, with limited SA accumulation at pH below 5.5 (Liu et al, 2008). The use of neutralizing agents such as $MgCO_3$, $CaCO_3$, Na_2CO_3, NaOH and NH_4OH as buffers is highly recommended for sustaining bioactivity in-process (Cheng et al, 2011). Studies have shown a positive response in terms of cell growth and sustained

bioactivity with time when $MgCO_3$ was used, while, Na_2CO_3 or NaOH had negative effects after 16 h of fermentation and NH_4OH was outright toxic to the biocatalysts without succinate accumulation in *A. succinogenes* (Cheng et al, 2011). Liu et al, 2008. Reported that *A. succiniciproducens* ATCC 53438 also followed the same pattern with optimal pH at 6 and substrate consumption and cell growth declining at pH above or below 6 (Nghiem et al., 1997). Bacteria with sustained activities at much wider range have been reported, for example *Enterococcus flavescens* produced succinate (with maximum yield of 0.92 g l^{-1} (at optimal pH of 6.5)) over a wide pH range of 4.0 to 9.0 (Anwar et al., 2014). Metabolic engineering of microbial catalysts such as *Yarrowia lipolytica Y-3314* have resulted in succinate yields of 17 g l^{-1} from glycerol at pH below 3.5 after 72h of fermentation (Yuzbashev et al., 2010).

Dissolved CO_2 in media

Carbonates dissolve in water to produce HCO_3^- and CO_3^-, with relative distribution and equilibrium with CO_2 being a function of the medium's pH. CO_2 availability in medium plays a major role in activation of metabolic pathways for the targeted synthesis of final products (or side-products) (Cheng et al, 2011). Using $MgCO_3$ as CO_2 source, Samuelov et al., 1991 reported a critical value of CO_2 above which, *A. succiniciproducens* would rather produce succinate instead of lactate (Samuelov et al., 1991). Analysis of intracellular enzyme activity further buttressed the claims of the study, considering PEP carboxykinase (enyme converting PEP to OA) activity is higher under CO_2-HCO_3^- conditions while lactate dehydrogenase and ethanol dehydrogenase activities were relatively low. At Increased pH conditions of 7.5 and low CO_2-HCO_3^- condition the inverse is the case; PEP carboxykinase activity is low while lactate dehydrogenase and alcohol dehydrogenase were active (Cheng et al, 2011). The effect of direct CO_2 availability was well correlated with the pH of the fermentation medium. These arguments have shown pH to affect the solubility of CO_2 in

medium hence influencing the utilizable CO_2 for microbial catalysts. The effect of CO_2 availability and partial pressure on the succinate yield and efficiency of *M. succiniciproducens* MBEL55E catalysed conversion systems at specific pH ranges (Cheng et al, 2011). Biocatalysts have varying optimal CO_2 concentrations and as such the need for empirical proof of specific CO_2 (flow, partial pressure and carbonate concentration) for yield respective conversion systems is essential.

H_2 in media

In addition to CO_2, studies have shown that hydrogen also plays an important role as an electron donor, influencing cellular metabolism for optimised BSA production. Hydrogen reduces cellular redox potential improving NADPH recycles in the process. H_2/CO_2 ratio of 1 to 19 was shown to promote glucose conversion to succinate in *A. succiniciproducens* catalysed conversion process by directly incorporating electrons into the metabolic pathways (Lee et al., 1999). In a different study, 5 % H_2 (95% CO_2) aeration in *Actinobacillus sp. 130Z* resulted in significant increase in succinate production and less acetate formed in comparison to 100 % CO_2 flow (Van der Werf et al., 1997). The fermentation of glucose for succinate production requires four electrons hence external supply of H_2 reduces the redox stress for better product accumulation (Cheng et al, 2011). Been a highly reduced fermentative product, succinate biosynthesis is preferably optimised with a good supply of electron donors or reduced substrates such as mannitol and arabitol (Cheng et al, 2011).

2.4 Zinc chloride MHS: aqueous medium for bio-catalysis.

The advantages of a conceptual integrated process of biomass pre-treatment and sugar conversion is numerous. Reduced operating costs; energy, material and equipment loads and improved process efficiency are but a few of the merits of process intensification (Sanders et al., 2012; Xu et al., 2016), which in the context of biorefining, have the potentials of improving the overall techno-feasibility of biomass conversion to value-added products.

Process integration of biomass refining systems is a largely unexplored scope, owning to the unestablished nature of bioprocesses and variations in documented yield. Rapid pre-treatment of biomass and biocatalytic conversion of released sugars are two well-researched, inter-dependent scopes with immense potentials for scalability. Biomass pre-treatment process designs are commonly characterised by harsh chemical reaction tailored for disorganization of biomass matrix and release of fermentable sugars while bioconversion processes are highly sensitive to physicochemical conditions and restrictive for optimal activities of the easily denatured biological catalysts. In fact, several pre-treatment methodologies require a post-pre-treatment step for efficient catalysis due to the production of bio-inhibitory intermediates (sugar degradation products). Building on existing knowledge of both processes this project sought to establish the potentials of process intensification as a strategy for increasing the market competitiveness of bio-based products by exploring pre-treatment of biomass at mild conditions in a "weak" electrolyte MHS media ($ZnCl_2.4H_2O$) and improved biocatalysts tolerance for efficient conversion to final products. As such, information relating the efficiency of the individual process units, final bio-based product yield (succinic acid) and the interactions of the proposed platform ($ZnCl_2.4H_2O$), dissolved substrates (reducing sugars and side-products) and biocatalysts (mixed-consortia of rumen bacteria) formed the scope of this research.

Zinc chloride salt, although in trace amounts, is important for biological processes (McCall et al., 2000). The reduction of biomass susceptibility to biocatalytic conversion in MHS systems requires the solvent media in more proportionate amount, hence need for a concise understanding of the interactions of the salt and resulting physicochemical conditions on bioactivity growth and growth-related yield of targeted bio-based products. One of the earliest and most profound works on salt interactions with biological macromolecules (protein, cellulose) was the hoffmeister series (HS). HS classified the ionic constituents of

salt molecules based on their effect on molecular water structure into kosmotrope (water structuring) and chaotropes (water structure disrupting). Figure 2.11 summarizes the effects of kosmotropic and chaotropic, anions and cations but in general the HS hypothesizes that kosmotropic anions; (before Cl^{-1} in Figure 2.12) tend to stabilize proteins in solutions while chaotropic (water structure-disrupting) anions (after Cl^{-1} in Figure 2.12), tend to "salt in" the peptide moiety of proteins thereby denaturing the macromolecule (Kunz et al., 2004).

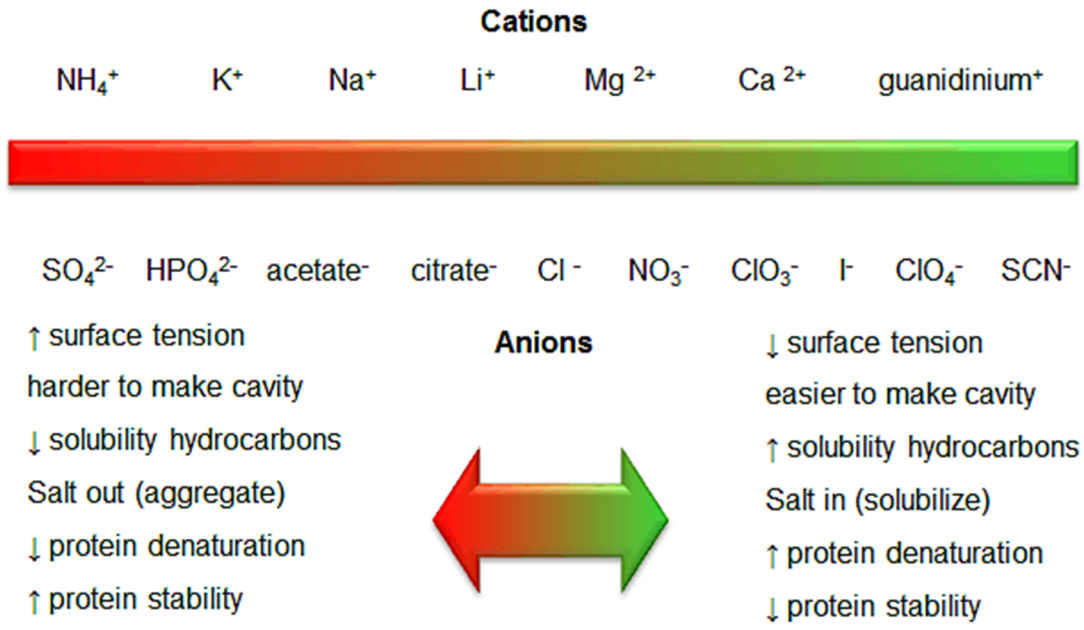


Figure 2. 11: Classification and properties of ions present in the Hofmeister series ((Kader, 2014), Copied in terms of Fair Dealing – Section 12 of the SA Copyright Act No. 98 of 1978 (as amended))

HS, although not unanimously agreed on by experts, have formed the bedrock of Coulombic forces-related investigations probing the interactions of ionic constituents of salts with phospholipids (membrane lipids). Binder et al, (2001) s work on scope focused on defining the interactions of a series of monovalent alkali metal cations; Li^{+}, Na^{+}, K^{+}, divalent alkaline earth; Be^{2+}, Mg^{2+}, Ca^{2+}, Sr^{2+}, Ba^{2+} and transition metal; Cu^{2+}, Zn^{2+}) ions in the presence of chlorides with anionic surfactant; 1,2-palmitoyl-oleoyl-phosphocholine (POPC) (Binder et

al., 2001; Binder and Zschörnig, 2002a, 2002b). Findings emphasised the exhibition of membanotropic effects of Zn^{2+}in model systems that resulted from its strong interactions which was in turn characterised by desolvation effects that was as proven with the lyotropic gel-liquid crystalline phase transition of the lipid (Binder et al, 2001). Zn^{2+}forms a covalently-linked complex with the negatively charged phosphate heads of the lipid such that it forms an adduct link between neighbouring lipid molecules which results in the stabilization of the gel phase. The complex formation results in increased hydrophobicity of the lipid heads resulting in increased fusogenic (membrane fusion) tendencies of membrane lipids (Ohki and Arnold, 2000). The behaviour of Zn^{2+}, consistent with HS been a kosmotrope (physiologically stabilizing) but yet also capable of physicochemical "de-activation" of the whole-cell catalysts.

The scope of whole-cell biocatalysis in ionic environments is not yet fully understood, and as such, it currently poses a major challenge to the integration and downstream-processing of MHS treatment of biomass. For example, Marcotuilo et al, (2011) reported the inhibitory effects of residual Fe^{2+} and Cl^{-} on iron chloride pretreated wheat straw during downstream cellulolysis and conversion (Marcotullio et al., 2011). The Fe cations were thought to bind with cellulose, reducing substrate affinity (K_m) of cellulase while metal cation of chromium was hypothesized to denature and deactivate cellulase. Other studies such as Kamireddy et al, (2013) suggested that the increased observed activity (K_{cat}) in their study was due to the formation of metal complexes with lignin, thus improving substrate accessibility and reducing inhibition in-process (Kamireddy et al., 2013). Kim et al, (2010) demonstrated dilution with deionized water till non-detection of ionic species was achieved but in order to contribute to process integration developments on scope, this project develops a buffer capacity profile for SA-producing whole-cell catalysts instead. An understanding of

physiological acclimatization as reflected in activity and product yield across a range of ionic strength (fermentation media to pre-treatment platform) can inform bioengineering and biomimetic strategies such as genetic mutation and physical immobilization for improved solvent tolerance.

2.5 Immobilization as a strategy for improved solvent tolerance in whole-cell biotransformation.

The physicochemical and molecular interactions of reaction media with substrates, catalysts and products at specified operating conditions are central to the efficiency of bioprocesses. The challenges posed by the negative effects of these interactions on suspended cells in homogenous media warrants the immobilization of biocatalysts for improved activity in media. By definition, immobilization involves techniques that confine a catalytically active biocatalyst (microbial cells or enzyme) to a matrix within a reactor system, preventing its entry into the bulk phase containing the substrate and product (Sekoai et al., 2017). The augmentation of the physical structure facilitates favourable micro-environment conditions for improved cell growth, activity and stability of protein structure (Qureshi et al., 2005; Sekoai et al., 2017; Tébéka et al., 2009). Immobilization is categorized into active and passive immobilization (Table 2.8). Passive immobilization involves the natural process of biofilm formation. Biofilms are a structured community of bacterial cells enclosed in a self-produced, protective, exopolysaccharide (EPS) matrix which also facilitates their adhesion to surfaces (Sekoai et al., 2017). The EPS provides a diffusive barrier to toxic compounds thus increasing solvent tolerance of the biocatalysts. The EPA barrier may also pose mass transfer limitations for nutrients and substrates getting to the cell in the inner parts of the matrix limiting cell growth and metabolic conversion respectively.

Table 2. 8: Overview of immobilization techniques in biocatalytic processing.

Immobilization technique	Advantages	Disadvantages
Active		
Membrane reactor	High productivities, high cell concentration can be achieved inside the reactor, clear permeates for further separation	Fouling with cells, cost prohibits their use in low cost large volume chemical production
Immobilized cell reactors Covalent bond formation	High cell concentration may be achieved, high productivity	Cell growth inside matrix may be restricted, cells leach out of the matrix and hence centrifugation of effluent may be required, chemical may affect the cells
Entrapment	High cell concentration may be achieved, high productivity	Matrix often starts disintegration with time, cells leach out of matrix, centrifugation of reactor effluents is required for further separation
Passive		
Biofilm	Comparatively high reactor productivities and high cell concentrations are achieved, reactors run longer and are economic to operate	Effluent centrifugation is required

Active immobilization involves the use of physical entrapment in microcapsules, adhesion to membrane, adsorption and chemical covalent bonding (for enzymes). A comprehensive review of the different types of active immobilization techniques and potential merits for anaerobic fermentation have been previously communicated in an academic journal (critical reviews in biotechnology) and is as attached to this thesis (Appendix P-2).

2.6 Summary

This chapter describes the state-of-the-art of LCB conversion to valuable commodities by providing a general overview of the fundamental concepts with focus on the feedstock, pre-treatment step, sugar release, bioconversion and potentials for general process intensification. The potentials of zinc chloride MHS as a pre-treatment additive and as a platform for cheaper LCB processing were the major highlights while possible strategies for improving catalysts stability was also explored. The development of integrated processes for the optimisation of valuable bio-based BSA; by means of efficient, low-cost activation of biomass and biocatalysis was described as a central strategy to improving the feasibility of biorefining processes and market competitiveness of the resulting bio-based products. This review of existing body of knowledge on scope informed the following conclusions:

- Waste LCB are abundant and have the potentials of contributing to an energy-secure South Africa.
- Zinc chloride hydrate salts pre-treatment media is proven for cellulose treatment and could also find immense use in cheap, sustainable biomass refining.
- A fully intensified, relatively mild pre-treatment step and solvent-tolerant biocatalytic process tailored to produce valuable bio-based product such as succinic acid will contribute immensely to the general techno-feasibility of biorefining platforms.
- Active immobilization techniques can be explored for improving solvent tolerance and stability of whole-cells.

The remaining chapters of this dissertation explored the aforementioned theme by investigating the efficiency of molten hydrate salt (zinc chloride hydrate) pre-treatment system and defining its biocompatibility for whole-cell SA producing microbes. Findings

from this study could pave the way for the development of a bioprocess for the sustainable, low-cost processing of LCB resources to valuable (bio)commodities.

Chapter 3

Biocompositional and physicochemical analysis of South African maize wastes

Cobs and husks are the major wastes generated from maize processing and this study assesses the benefication potentials of these lignocellulosic materials as low-cost feeds for sustainable biomass conversion processing. Results of organic and inorganic composition of these waste materials are presented in this chapter. The outcome of the compositional analysis of these waste materials serves as impetus for pre-treatment and bioconversion studies reported in subsequent chapters.

3.1 Introduction

This study placed emphasis on identifying the characteristics of locally-generated corn cob biomass with potentials for use as feedstocks in biorefining processes. Waste LCBs are promising resources upon which sustainable policies and socioeconomic developmental platforms can be centered, but their suitability as feedstocks in thermochemical and bioconversion processes depends on ease of conversion which in turn depends on the physicochemical property and organic composition of material. The physicochemical properties and organic composition of the raw biomass wastes were studied towards an assessment of biomass quality and potentials for beneficiability. LCBs are generally composed of sugar polymers cellulose (6C), hemicellulose (5C), and lignin. Cellulose and hemicellulose are the repositories of fermentable sugars; hence, their use as feedstocks in microbial conversion processes for the production of second-generation bioethanol and several value-added biocommodities (e.g. BSA). Using IR, thermogravimetric and qualitative methods, this chapter describes the organic characteristic of husks and cob wastes derived from maize processing. The outcomes of these investigations have been documented

in previous communication, as a publication in Asia-Pacific Journal of Chemical Engineering (see Appendix P-3 for a copy of the article).

3.2 Materials and Methods

Materials used in the investigation included sulphuric acid, nitric acid, corn cob (Zama star), acetone, ethanol, deionized water, acetonitrile.

Raw cobs of *Zea mays* Zama star (white (CCW) and red (CCR)) hybrid and husks (CCH) was obtained through the Agricultural Research council- (ARC-) Potchefstroom from a verified farmland in the province of Limpopo (23.89°S, 29.44° E). Corn cobs (weight w_i (g)) was cut to size using a band-saw (Mössner August, GmbH Co., Germany) and then shredded in a rotor mill (Retsch® SE-200, Germany) fitted with 640 μm - 4 mm sieve.

The biomass was treated for extractives by means of solvent extraction in a soxhlet apparatus (Sluiter et al., 2005). Raffinate biomass was recovered, oven-dried (50 ^{o}C, 48 h) until all residual solvent had evaporated and then re-weighed (w_o (g)). Raffinate biomass (1 g)) in triplicates per batch) was analysed for cellulose, hemicellulose by means high performance liquid chromatographic HP-LC (Bruker, Germany) of monosacccharides upon dilute-acid treatment as outlined in the standard NREL procedure for determination of carbohydrates in biomass (Sluiter et al., 2011). The Klason lignin and ash compositions were analysed for via a thermogravimetric approach while acid soluble lignin (ASL) was determined by means of spectrophotometric analysis.

The inorganic composite of the biomass was analysed by combusting the raw corn cob biomass in a muffle furnace set at 700 ^{o}C. The recovered ash was digested in 70 % HNO_3

prior to Inductive coupled plasma- mass spectrometry (ICP-MS) (Bruker instruments, Germany) analysis.

Proximate analysis of the corn cob biomass was carried out in a simultaneous thermogravimetric analyser (Q600 SDT thermal analyser (TA Instruments, USA). Ultimate analysis was done using a CHNS analyser and the resulting values were used in estimating the higher heating value (Equation 3.1) of the samples. The calorific values of the corn cob biomass were derived in a calorimeter (TA systems, USA).

$$\text{HHV (MJ/kg)} = \left(\frac{33.5 \times \text{wt.\% C}}{100}\right) + \left(\frac{142.3 \times \text{wt.\% H}}{100}\right) - \left(\frac{15.4 \times \text{wt.\% O}}{100}\right) \quad (3.1)$$

Crystallinity ratios; lateral order index (LOI) (α1437 cm^{-1}/α899 cm^{-1}), total crystallinity index (TCI) (α1378 cm^{-1}/α2900 cm^{-1}) and hydrogen bond intensity (HBI) (α3400 cm^{-1}/α1320 cm^{-1}) as hypothesized by Segal, et al, (1959) was determined by analysing raffinate biomass in a PerkinElmer Fourier transform infrared (FT-IR) spectrometer (PerkinElmer, USA) using the attenuated total reflectance (ATR) method.

3.3 Results and Discussion

The results of the characterization study of the raw LCB corn cob samples confirm the viability of the materials as a substrate for beneficiating bioconversion systems. Figure 3.1 shows dry corn cob (CCW) sample, prior to comminution.

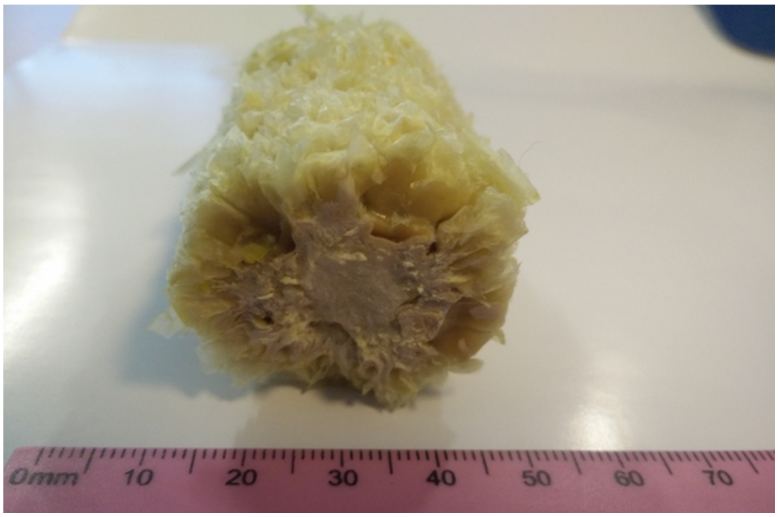


Figure 3. 1: Lignocellulosic corn cob biomass.

Waste LCBs are currently the most abundant and promising sources of organic hydrocarbons for the production of value-added commodities in an integrated biorefinery platform. The general composition and structural traits of LCB directly impact digestibility and conversion of LCB to final products in an efficient high-yield bioconversion system. Previous studies have established the variations in these parameters are linked to numerous factors; such as geography, genetics, environment, etc., which are contributory to the syngenesis and development of plant matter (Datta et al, 2012). Hence, the quantitative and qualitative study of the organic carbon potentials of waste LCB feedstock is important for the evaluation and standardized assessment of biocatalytic activity for efficient substrate conversion (Schimdt et al, 2001).

Standard qualitative approaches for investigation of the applicability of materials as solid fuels or carbon resources were employed in a more holistic framework towards a concise assessment of the feedstock credentials of agro-waste samples. Results of the physicochemical study; proximate and ultimate analyses of the materials are as summarised in Table 3.1. Ultimate analysis of biomass samples described the atomic makeup of the

organic matters showing relatively high carbon composition (44.5 % for CCR, CCW and CCH respectively) in the biomass samples. The results (Table 3.1) were interpreted to establish the empirical formula of the samples (CCW, CCR and CCH: $CH_{1.51}O_{0.83}$, $CH_{1.49}O_{0.84}$, and $CH_{1.62}O_{0.94}N_{0.001}$, respectively).

Table 3.1: General physicochemical properties of untreated locally sourced corn cob biomass.

	CCR	**CCW**	**CCH**
Proximate (wt.%)			
Ash	2.09±1.8	2.1±1.1	1.7±0.9
Volatile matter	75.25±1.4	77.93±2.6	77.57±1.9
Fixed Carbon	22.66 ± 0.7	19.97 ± 0.4	20.73
Moisture[a]	7.53±0.4	7.37±0.3	13.1±1.7
Heat of Combustion (MJ/kg)			
Experimental	20.09± 2.8	19.39±1.7	16.61±1.4
Theoretical, HHV	15.18	15.24	14.04
Ultimate			
Carbon	44.5 ± 0.9	44.70 ±1.2	41.94±0.9
Hydrogen	5.58 ± 1.2	5.69 ±1.5	5.72 ±0.6
Nitrogen	0.17 ± 0.6	> 0.1	0.5 ±1.2
Sulfur	-	-	>0.01
Oxygen (by diff)	49.75 ± 1.1	49.62 ± 1.1	52.29±0.7
Atomic ratio			
O/C	0.84	0.83	0.94
H/C	1.49	1.51	1.62
Empirical formula	$CH_{1.49}O_{0.84}$	$CH_{1.51}O_{0.83}$	$CH_{1.62}O_{0.94}N_{0.001}$
Infrared crystallinity ratios			
TCI (α1437 cm^{-1}/ α899cm^{-1})	0.96	0.97	0.97
LOI (α1378 cm^{-1}/ α2900cm^{-1})	1.07	1.11	1.09
HBI (α3400 cm^{-1}/ α1320cm^{-1})	1.00	0.91	1.00
Structural components (wt.%)			
Cellulose (Y_G)	40.29 ± 3.8	41.34 ± 2.6	28.06±4.2
Hemicellulose (Y_x)	45.34 ± 7.1	39.35 ± 4.5	30.89±3.2
Acid insoluble lignin	4.65 ± 2.0	7.5 ± 1.2	10.9±1.5
Acid soluble lignin	2.06 ± 0.7	2.40 ± 0.2	3.05±0.2
Extractives	7.60 ± 1.2	9.38 ± 0.7	27.11±0.1

[a]- percent weight was calculated using raw biomass.
ANOVA test: null hypothesis was rejected as F > F (crit.), and P-value (0.002) < α = 0.05 for both sets of data reported for 6-C and 5-C polymeric sugars.

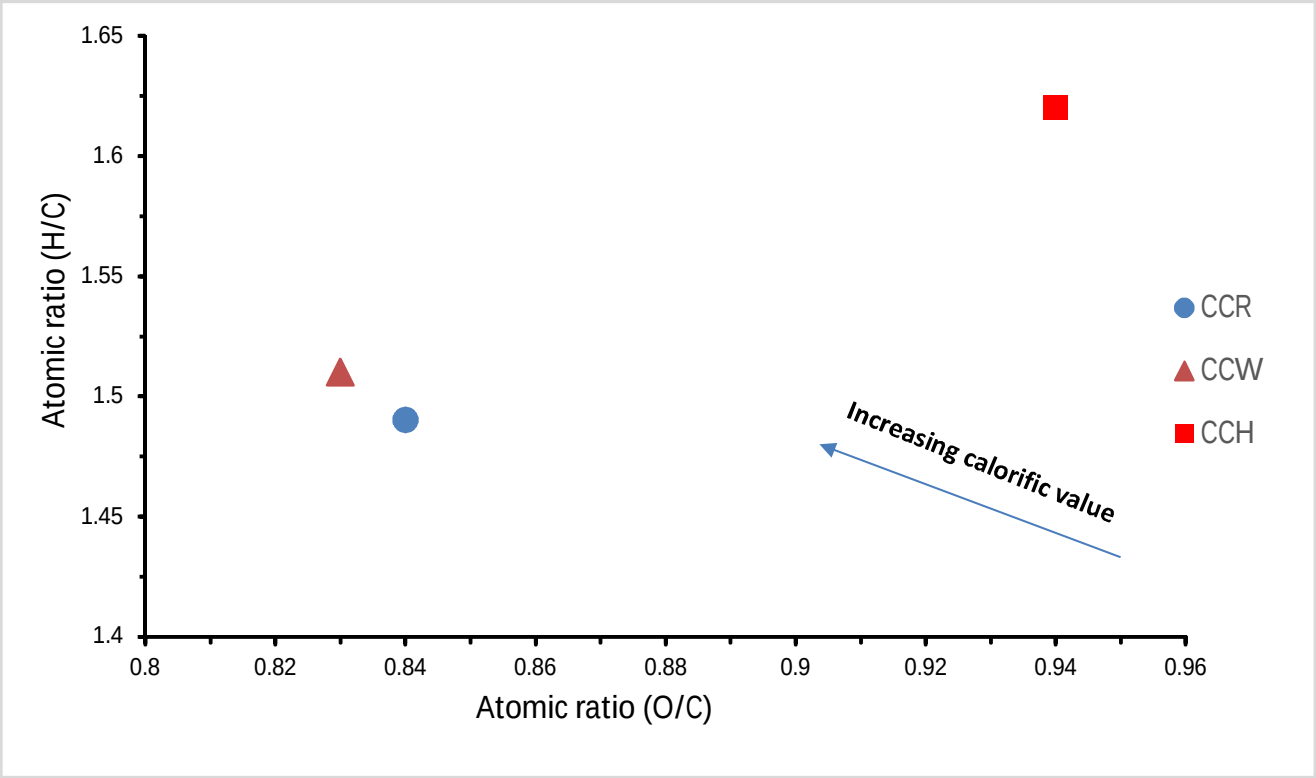


Figure 3. 2: van Krevelen plot describing the energy potential of maize wastes.

The observed variation in elemental distribution of the biomass wastes was reflected in the energy potential (as estimated by van Krevelens plot (Figure 3.2) and DuLong equation (Equation 3.1). The cob samples; CCW and CCR, had considerably higher C contents (44.6 and 44.1 wt.%, respectively) relative to CCH (41.94 wt.%) and as such resulting in the observed differences in the energy potentials of the materials as illustrated with the van Krevelen plot (Figure 3.2). Likewise, the HHVs of the materials as deduced from DuLong's, reiterated the inherent chemical energy in the materials to be in the order of CCH< CCR< CCW (although with only a negligible difference between the cobs). The trend was substantiated with empirical proof of the calorific value of the materials (Table 3.1), which was closely aligned with the estimated HHV, thus giving credence to the viability of the data obtained for the atomic framework of the biomass samples. The observed CV for the cob

samples; (CCR (20.09 MJ/kg), CCW (19.31 MJ/kg) was within the range of earlier reported values in literature for corn cob (sourced from Eastern Cape, South Africa (18.02 MJ/kg) (Anukam et al, 2017) and in other geographic zones such as Eastern Canada (18.75 MJ/ kg) (Lizotte et al, 2015) and Southern Asia (18.36 MJ/kg) (Danish et al, 2015). In general, the amount of obtainable energy was a reflection of the carbon content of the materials (CCH was characterised with relatively low CV (16.61 MJ/kg)). The calorific value of biomass increases considerably with the carbon content as there is more energy inherent in C–C bonds than in C–O and C–H bonds (Nanda et al, 2013).

Proximate analyses of the biomass samples (as summarized in Table 3.1) was described by means of thermogravimetry (Figure 3.3A). Differences in the physicochemical traits: ash, volatile matter (VM), and fixed carbon (FC) as interpreted for by the thermodecompositional behavior of the biomaterials was negligible across samples. The only exception to which was the characteristically high moisture content (13.1%) in CCH; an undesirable feedstock credential due to the increased susceptibility of biomass to microbial degradation during storage and the energy cost of drying.

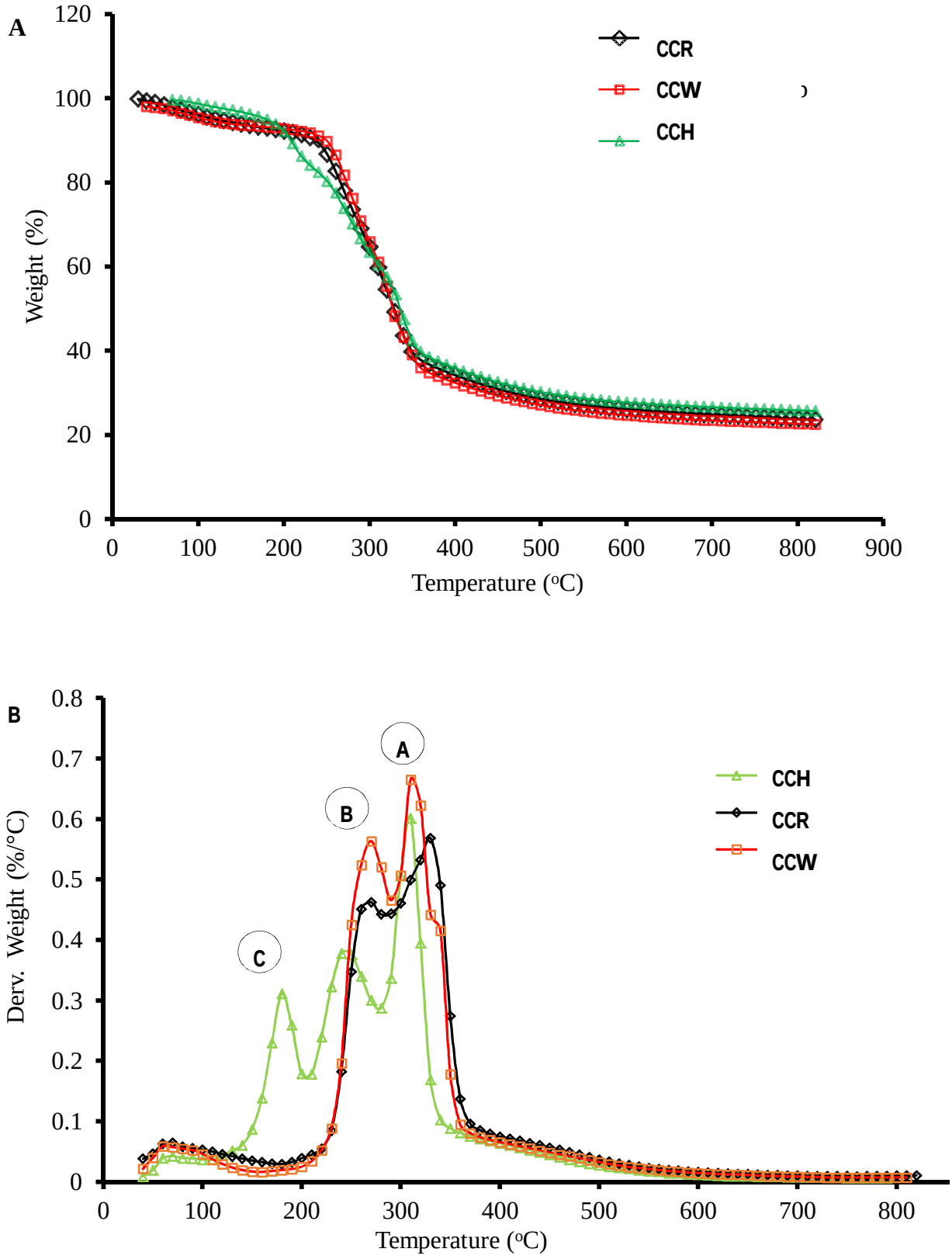


Figure 3.3: *A*) Residual mass ratio thermograms of corn cob samples: Red corn cob (CCR), white corn cob (CCW) and corn cob husks (CCH). *(B*) DTG thermograms of corn cob samples: Red corn cob (CCR), white corn cob (CCW) and corn cob husks (CCH). Where points (A)- Hemicellulose; (B)- Cellulose (C)- Extractives; decomposition peaks respectively.

The decomposition behaviour of the corn cob (Figure 3.3B) samples suggested the main composition of the biomass as moisture, extractives, cellulose, hemicellulose and lignin. As such, raffinate biomass (w_o) used throughout this work was as described in Equation (3.1).

$$w_o = \sum Y_G Y_X \, L \, A \qquad (3.1)$$

Where, w_o is the weight of raffinate biomass in (g), Y_G is inherent glucan in raw corn cob biomass, Y_x, inherent xylan in corn cob, L, weight of lignin and A, ash composition in (g).

The general optimisation of fermentation processes for sugar uptake accentuates the importance of high sugar composition in feeds. The weight percent distribution of cellulose and hemicellulose in the three biomass samples (in relation to lignin) is as shown in Table 3.1. In both local corn cob samples analysed, sugar-to-lignin ratio of 12.67 and 8.15 were promising and in agreement with report for corn cobs in other geographical regions such as Southern Asia (12.4) (Danish et al, 2015). Using conversion factors (Equation 4.4) for the inherent sugar polymers, it was established via this characterization study, the sugar content of the biomass was in the range of 800 - 960 mg (515 mg: xylan content and 445 mg: glucan) per gram of corn cob LCB. The high percent composition of saccharose in biomass was a major indication of the suitability as feeds for high yields of bio-based products. Saccharide to lignin ratio was significantly low (4.3) in the husk samples with relatively low amount of cellulosic composite. The cellulosic (6C) content of biomass is central to the yield of bio-based processes for biofuels and biocommodities (such as BSA) due to the selectivity of whole-cell catalysis for 6C monomers. Also, in addition to improved yield, higher saccharose-lignin ration may imply improve processability of the feeds; as biomass

recalcitrance is contributed to in-part by lignin content. The relatively low amount of lignin in corn cob samples analysed suggested the susceptibility of samples to pre-treatment processes, although the crystallinity of the biomass material (especially cellulosic content) also reduces substrate reactivity. FT-IR analysis of the biomass wastes (Figure 3.4) was used to determine their respective crystallinity ratios (Table 3.1) towards informing the structural traits of the samples. Relatively high amounts of cellulose especially in comparison to the more amorphous hemicellulose explains the higher crystallinity ratios in the cob samples, although CCW was characterised by a more organized inter-molecular hydrogen bonding network.

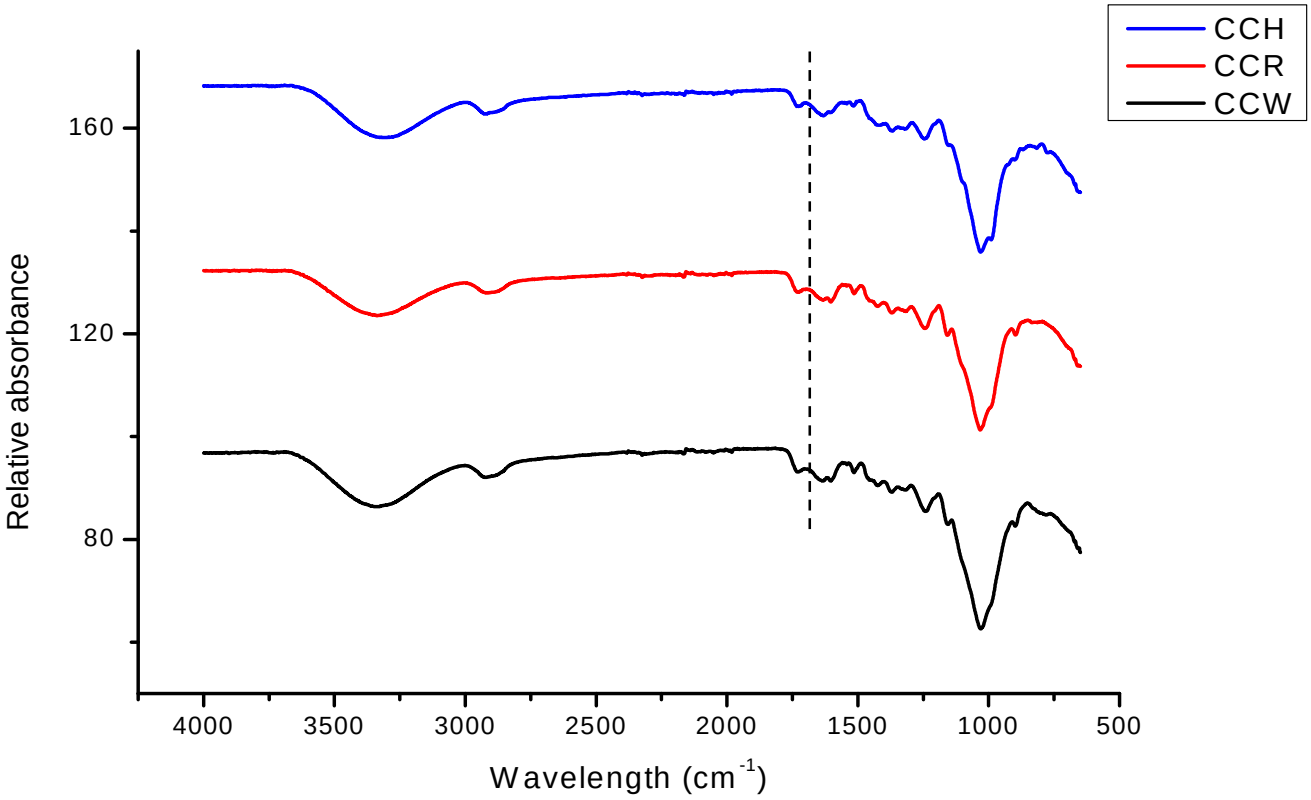


Figure 3. 4: FT-IR spectra of maize agro-wastes (Cobs; CCR & CCW) and husks (CCH).

Finally, analysis of the mineral contents (Table 3.2) gave important insights into the inorganic elemental composites in biomass samples: such as alkali metals; K, Mg, Zn, Fe and

Ca, Heavy metals; Th and U, in the corn cobs. The presence of these elements could be attributed to the land, farming practises and geographic elements that defined the syngenesis of the plants.

Table 3.2: Elemental composition (ppb) by ICP-MS analyses of waste untreated corn cob biomass.

Element	CCW	CCR	CCH
Li	13.20	4.85	12.12
Na	14.58	2.04	14.03
Mg	103464.8	7638.34	124659.7
Al	3570.44	4744.19	4216.26
K	572315.00	<0.00	422763.00
Ca	5307.22	239.89	3811.72
Cr	1342.07	90.58	983.22
Mn	1253.49	131.27	1455.17
Fe	6836.66	2379.13	7695.77
Co	30.75	53.95	33.88
Ni	556.88	57.60	617.65
Cu	744.29	77.33	843.58
Zn	13386.26	1010.95	13091.02
As	3.29	0.17	3.15
Sr	399.61	32.36	474.11
Ag	2.96	0.17	4.91
Cd	0.52	0.00	0.49
Hg	1.46	1.07	1.13
Pb	111.55	7.58	121.93
Th	0.97	0.37	0.85
U	0.16	0.05	0.15

3.4 Summary

In general, corn cob samples are promising for use in bioconversion process due to the characteristic calorific values and saccharide content. High saccharide compositions implies that analysed local maize waste materials are promising resources as feedstocks with immense potentials for very high yields of valuable bio-based products. CCH is recommendable for use as feedstock in large-scale processes in an augmenting role for the cob samples, although cost of storage may influence techno-feasibility. Finally, observed

crystallinity suggests the need for efficient biomass pre-treatment processes for improved saccharose accessibility and optimised carbon throughput for an efficient downstream conversion process.

Chapter 4

Molten zinc chloride hydrate salt pre-treatment of corn cob: Effect of salt hydration, process conditions on biomass digestibility.

This chapter describes the pre-treatment of corn cob waste in molten hydrate zinc chloride salt. The effect of pre-treatment on the structural and susceptibility of the LCB matrix to biodecomposition was investigated at different solvent hydrations and process conditions. The findings from this chapter have been documented as an article published in the Journal of Chemical Technology and Biotechnology (see Appendix P3) and an article in the proceedings of the Asia-Pacific Conference of Chemical Engineers (see Appendix P4).

4.1 Introduction

The underlying mechanism of non-derivatising MHS solvation of cellulose in zinc chloride MHS systems is related to the unique molecular structure of the salt systems. Molten zinc chloride hydrate salt media are characterised by metal cations (Zn^{2+}) which are embedded within hydrant spheres in a thermodynamically unstable state. The nature (charge and radius) of the cationic species influences the reorganization of water structure (Wilcox, 2009) thus modifying the hydrophobic interactions of media with macromolecular orientations. Crystallographic proof has shown the structure and physicochemical properties of the salt-water system to be unaltered for select $ZnCl_2.nH_2O$ (n=3, 4, 7, 21) but the effect of the respective media on cellulose solvation and derivatization varies. The respective MHS studies have maintained in contrast to molecular structural posits, the influence of hydration on the lewis acidity of systems and the consequential effects of the ionic media on cellulose biopolymers (Loow et al., 2015). The effect of the select $ZnCl_2.nH_2O$ (n= 3, 4) salt hydrates on cellulose; especially as pertaining to increasing saccharose surface area and reduced crystallinity is well documented in literature. The findings of this study have maintained that

proven MHS systems have unique hydrations levels at which the optimal α-cellulose solvation occurs. $ZnCl_2.nH_2O$ (n= 3-4) has been subsequently recommended in literature for efficient cellulose processing. Studies of MHS solvation of cellulose have been directly correlated to lignocellulosic biomass treatment, although with little to null empirical proof, despite the obvious differences in the compositional and structural make-up of both materials. Hence, this investigation focused on the effect of $ZnCl_2.nH_2O$ MHS hydration on lignocellulosic corn cob using parameters such as surface morphology, decrystallinity and sugar extraction to define the efficiency of pre-treatment in the respective media.

Residual polysaccharides in treated biomass are hydrolysed into reducing sugars for further processing by means of enzymatic hydrolysis post biomass pre-treatment. Pre-treatment plays a very important role in the efficiency of enzymatic hydrolysis. Efficient pre-treatment on LCB is generally linked to an increase in polymer surface area and subsequent amplification of the accessibility of cellulolytic activities. Reducing sugars are the most common substrates for fermentation products, and an increase in their release from biomass matrixes may imply considerable improvement of the efficiency of fermentation system. Against the aforementioned, this study also investigated the efficiency of the enzymatic hydrolysis step of the untreated and $ZnCl_2.4H_2O$ treated corn cobs.

4.2 Materials and Methods

Materials used include zinc chloride (anhydrous (Sigma-aldrich)), deionized water, corn cob, and acetonitrile

4.2.1 Effect of salt hydration on pre-treatment efficiency

Anhydrous zinc chloride was stored in an oven set at 100 ^{o}C over-night to ensure there is no residual moisture content in salts. The different salt hydrates were made by dissolving salt at the respective molecular weight ratio as shown in table 4.1

Table 4. 1: Preparation of hydrate salts by salt dissolution in water.

MHS	Percent weight (% w/w)
$ZnCl_2.3H_2O$	71.6
$ZnCl_2.4H_2O$	65.4
$ZnCl_2.5H_2O$	60.2
$ZnCl_2.7H_2O$	52.1

To study the effect of hydration, 300 mg of corn cob was added to 3.6 g of the respective zinc chloride hydrates in 50 mL autoclave vials. The vials were transferred to a shaking incubator at 70 ^{o}C for 60 min. Upon cooling, the respective slurries were separated by means of vacuum filtration. The solid fraction was washed prior to drying and analysis while the liquid fraction was stored at -4 ^{o}C.

4.2.2 Effect of severity on $ZnCl_2$ tetrahydrate pre-treatment.

To study the effect of process condition; time (t) and temperature (T), on the pre-treatment efficiency of $ZnCl_2.4H_2O$, 3 g of milled corn cobs (Approximate particle size of 2.4 mm) were dissolved in $ZnCl_2.4H_2O$ at a ratio of 1:12 in 50 ml autoclave vials. The vials were transferred into an autoclave set at temperatures (70 ^{o}C and 105 ^{o}C respectively) for varying pre-treatment time ranging from (0 - 60 min). The recovered samples were vacuum filtered and both the liquid and solid fractions were stored for analysis.

4.2.3 Analysis of $ZnCl_2$ MHS treated LCB

4.2.3.1 Analysis of pre-treatment liquor

The pre-treatment liquor was analysed for reducing sugar content and degradation products by means of HPLC analysis as described elsewhere (Agblevor et al., 2007). Prior to analysis

a weak acid post-hydrolysis treatment of the pre-treatment liquor samples was carried out. Pre-treatment liquor (1 ml) was treated with 4 %w/w H_2SO_4 (121 °C, 10 min). $Ba(OH)_2$ was added to the treated liquor towards precipitating the sulphates which was then separated by centrifugation. Glucose and xylose were quantified using a pre-calibrated HPLC (Agilent tech., USA) equipped with a refractive index (RI) detector. A Zorbax carbohydrate column (4.6 x 250 mm) was fitted for use on the HPLC and 70 %wt/wt of acetonitrile solution was used as the mobile phase. Column temperature was set at 60 °C and flow rate was 1.4 mL sec^{-1}. The injection volume was 20 µL with a run time of 45 min. Dissolved lignin was analysed with the use of a spectrophotometer. A separate aliquot of the liquor was used to analyse for degradation products such as acetic, formic, malic, succinic and glycolic acids were also quantified using the aforementioned HP-LC conditions.

4.2.3.2 Biocompositional analysis of solid fraction

The biomass residue was washed, dried (in a convection oven set at 40 ^oC for 72 h) and weighed (w_g) for mass loss. The sugar, lignin and ash content of the pre-treatment residues was determined by diluting the components in a two-step sulphuric acid hydrolytic process described by Sluiter et al, (2011). The polymeric sources (i.e. cellulose and/or starch) of the glucose monomers quantified via HPLC could not be differentiated; hence, it was classified in general, as glucan. The quantity of the components was expressed on an extractives free basis.

4.2.3.3 Characterization of pretreated and untreated lignocellulosic biomass.

Physicochemical properties of the recovered solid fraction was analysed for using a variety of techniques such as fourier-transform infrared spectroscopy (FT-IR), scanning electron microscopy (SEM-EDX) and X-ray diffraction (XRD). In addition, thermogravimetric analysis (TGA) was carried out on raw and pre-treated samples of corn cob on a simultaneous Q600 SDT thermal analyser (TA Instruments, USA). Approximately 11.5 mg of the oven dried (105 ^{o}C for 6 h) samples were loaded and heated under nitrogen flow (20 mL min-1) from room temperature 30 ^{o}C to 800 °C at a ramping rate of 10 ^{o}C/min. The temperature was held at the final temperature for 20 min. The weight loss and decomposition rate were recorded for all samples.

Variations in surface morphology of biomass resulting from pre-treatment was analysed by means of scanning electron microscopy equipped (SEM). Both raw and MHS-treated corn cob biomass were washed with deionized water after which they were left to dry in a conventional oven at 40 ^{o}C for 72 h. The dried residues were then stored in capped 20 mL glass sample vials. The dried samples were mounted on aluminium stubs by means of a carbon tape. The residues were examined using a field emission scanning electron microscope (FE-SEM) (Carl-Zeiss Sigma, Germany) equipped with energy dispersive x-ray (EDX) functions.

The raw and pre-treated samples were evaluated for the variations in surface chemistry of their constituents with a PerkinElmer Fourier transform infrared (FT-IR) spectrometer (PerkinElmer, USA) using the attenuated total reflectance (ATR) method. The crystallinity of the polymeric components of the biomass samples was investigated using infrared ratios as hypothesized by Segal, et al, (1959). The ratios of lateral order index (LOI) (α1437 cm^{-1}/α899 cm^{-1}), total crystallinity index (TCI) (α1378 cm^{-1}/α2900 cm^{-1}) and hydrogen bond intensity

(HBI) (α3400 cm^{-1}/α1320 cm^{-1}) were used in calculating the crystalline structure of the cellulosic content in terms of the inter-relationship with the other polymeric components. The spectra acquired using 32 scans were in the range of 4000 to 400 cm^{-1}, at a resolution of 4 cm^{-1}.

Crystallinity and phase changes of the untreated and zinc chloride MHS treated biomass samples were obtained by means of a continuous scan on a D2 advance diffractometer (Bruker, Germany) using cobalt as anode material at 30 kv and 10 mA. XRD analysis was at room temperature and the monochromatic CoKα1 radiation was at a wavelength of 1.79 nm in the 2θ region between 10 ^{o} and 70 ^{o}. Raw data was processed using the EVA DIFFRAC software. Crystallinity index (CrI) was calculated as described by Segal et al., 1959 and shown in Equation (4.1)

$$CrI = \frac{I_{002} - I_{am}}{I_{002}} \times 100 \qquad (4.1)$$

where, I_{002} is intensity of the diffraction from the 002 plane at 2theta = 22.6^{O}, and I_{am} is intensity measured at 2theta = 18.7^{O}

4.2.3.4 *Rheological characterization of pre-treatment slurries*

Rheological properties of MHS-corn cob slurries from the solvent systems $ZnCl_2.3H_2O$ and $ZnCl_2.4H_2O$ slurries were determined using a Rheometer (Anton-Paar, Germany).

4.2.4 Enzymatic saccharification

Materials used for enzymatic hydrolysis study include: zinc chloride (anhydrous), cellulase (*Trichoderma reesei*), dinitrosalicylic acid, sodium hydroxide, citrate monohydrate, sodium azide, deionized water and acetonitrile.

Enzymatic digestion of the raw and $ZnCl_2.4H_2O$ pre-treated corn cob biomass samples was carried out in 50 ml stoppered conical flasks in an air-bath shaker set at 50 ^oC. *Trichoderma reesei* cellulase (1.2 mg protein/ml, 74 FPU) was added at a 2 % w/w of media to corn cob samples in citrate buffer (5 ml, 0.1M, pH 4.8). 0.5 ml of sodium azide solution (1 %w/w) was added to inhibit microbial contamination. The activity of the cellulase was expressed in filter paper units (FPU); 1 FPU defined as the amount of enzyme producing 1 mol of reducing sugars per minute. The FPU of the cellulase employed was determined by the dinitrosalicylic acid (DNS) assay according to the NREL procedure (Adney and Baker, 1996).

The untreated and pretreated corn cob samples were hydrolyzed at varying enzyme loadings for 3 days and samples were collected at 24 h intervals.

The collected samples were boiled for 15 min; towards deactivating the enzyme, before centrifugation (10 000 rpm for 5 min) and supernatant recovery. The supernatant was analysed for reducing sugars with a HP-LC and the obtained results based on sugar concentration media which is released per treated biomass using Equation 4.2.

$$Y = \frac{[reducing\ sugar] \times D \times V}{W_o} \qquad (4.2)$$

Where, D is dilution factor, V is working volume in ml, W_o weight of biomass and Y is weight (mg) of reducing sugars released per gram of corn cob biomass. Reducing sugar concentration was quantified (in mg/ml) by means of HPLC analysis (see Appendix D1 for calibration curves) .

Hydrolysis yield of glucose (Y_g) from cellulose was expressed based on the stoichiometry of hydrolysis (R 4.1) using Equation (4.4), as gramme of reducing sugar per gramme of saccharide in corn cob biomass.

$$\underset{\text{Cellulose (Mw 162.2)}}{[C_6H_{10}O_5]n} + n{\cdot}H2O \rightarrow \underset{\text{Glucose (Mw 180.2)}}{n{\cdot}C_6H_{12}O_6} \qquad \text{(R 4.1)}$$

$$Y_g = \frac{g\ of\ cellulose\ hydrolysed}{g\ of\ cellulose\ in\ pretreated\ biomass} \qquad (4.3)$$

$$Y_g = \left[\frac{g\ of\ glucose\ (quantified)}{g\ of\ cellulose}\right] \times \left[\frac{162.2}{180.2}\right] \qquad (4.4)$$

4.3 Results and Discussion

4.3.1 Effect of salt hydration on biomass deconstruction

The effect of pre-treatment on corn cob using varying hydrates species of zinc chloride salt were evaluated based on their respective effect as regard sugar yield and the physicochemical integrity of the biomass structure. Increased surface area and disarrangement of the microstructures of dissolved biomass gave credible insights into the efficiency of the respective pre-treatment media. Figure 4.1 shows the effect of respective salt systems on the surface morphology of lignocellulosic biomass indicating a disorganization of the biomass matrix in the salt solvent systems. The degree of disorganization reduced with increasing zinc chloride water of crystallization. $ZnCl_2.xH_2O$ (where x= 3 and 4, Figure 4.1B and 4.1C) completely altered the surface morphology of the biomass while the higher hydrated (x= 5 and 7, Figure. 4.1D and 4.1E) only slightly affected biomass organization. Structural organization of the biomass matrix is directly correlated to biomass recalcitrance to catalytic hydrolysis of the polysaccharides (Ladisch et al., 2013). Increased surface area as observed by microscopic analysis of pre-treated biomass infers improved accessibility of saccharifying biochemical agents.

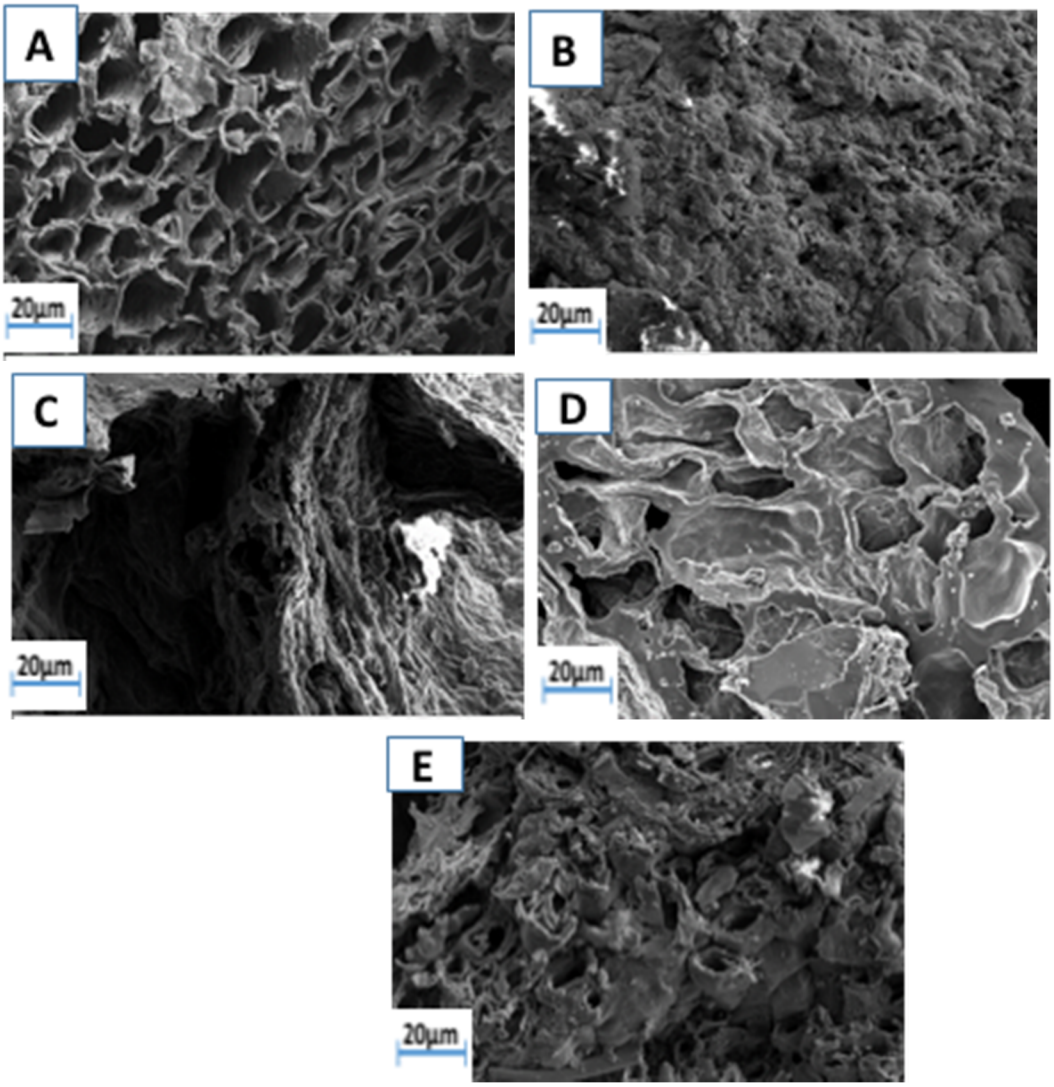


Figure 4. 1: SEM images of corn cob biomass. untreated corn cob (A), $ZnCl_2.3H_2O$ residue (B), $ZnCl_2.4H_2O$ residue (C), $ZnCl_2.5H_2O$ residue (D), $ZnCl_2.7H_2O$ residue (E).

Biomass deconstruction was accompanied by sugar yields as observed by quantitative analysis of reducing sugars in the pre-treatment liquor of $ZnCl_2.3H_2O$ and $ZnCl_2.4H_2O$ systems (Table 4.2). Hemicellulose content is an amorphous composite of biomass and is as such susceptible to dissolution and depolymerisation in pre-treatment media. Hemicellulose content in biomass reduced immensely in the solid fraction of biomass dissolved in $ZnCl_2.3H_2O$ (up to 44.26 % w/w) and $ZnCl_2.4H_2O$ (up to 33 %w/w) at the stipulated process conditions. In the higher hydrated salt systems, hemicellulose removal reduced significantly

in $ZnCl_2.5H_2O$ (11 %w/w) and $ZnCl_2.7H_2O$ (6.24 %w/w). Xylose concentration in the pre-treatment liquor was not reflective of observed hemicellulose solvation and a likely reason for which is the presence of other 5-carbon sugars such as mannan, galactan and arabinan in hemicellulose chains. Mannose was quantified to be present in the liquor samples being highest in the $ZnCl_2.4H_2O$ liquor (Table 4.2). The trend of biomass cellulose solvation in respective media correlated with results reported for cellulose as a stand-alone process feed, although solvation efficiency was higher in the latter. Cellulose I (degree of polymerization 2060) was completely dissolved and transformed to the less crystalline cellulose II in $ZnCl_2.3H_2O$ at 80 ^oC for 135 minutes (Lu and Shen, 2011). Cellulose solvation was negligible in the higher hydrated $ZnCl_2.xH_2O$ solvents (x= 5 and 7), while up to 28 %w/w of cellulose was removed from the untreated biomass. Cellulose solvation was negligible in $ZnCl_2.3H_2O$ media (2.6 %w/w) and it has been argued that the viscoelastic properties of the slurry and resulting mixing challenges may have played a role in the efficient solvation of cellulose (Awosusi et al, 2017).

Table 4. 2: Effect of salt hydration on composition of $ZnCl_2$ pre-treated biomass residues at conditions of 70 ^oC for 60 mins, using a biomass to solvent ratio of 1:12 wt/wt.

	Corn Cob (Untreated)	$ZnCl_2.3H_2O$	$ZnCl_2.4H_2O$	$ZnCl_2.5H_2O$	$ZnCl_2.7H_2O$
SOLID FRACTION (% w/w)[a]					
Dry Solid residue	100	78	88.5	95.5	96.9
Extractives	13.0 ± 0.8	-	-	-	-
Cellulose[a]	40.04 ± 3.2	39.09 ±1.2	28.46 ± 1.6	40.83 ±3.2	40.42 ± 2.5
Hemicellulose[a]	45.66 ±2.4	25.45 ± 2.7	30.9 ± 0.5	39.80 ± 2.1	42.81 ± 3.1
Lignin[a, b]	6.34 ± 4.7	6.9 ± 2.1	7.5 ±1.7	7.37 ± 1.4	6.77 ± 2.0
LIQUID FRACTION (g L^{-1})					
Cellobiose	-	-	-	-	-
Glucose	-	6.96 ±1.5	7.34 ± 3.9	5.10±1.4	0
Xylose	-	3.39 ± 0.3	4.33 ± 0.2	3.10 ±0.5	0.69 ± 0.3
Mannose	-	-	1.35 ±0.5	-	-
pH	-	0.29	0.44	0.60	1.40
CS	-	0.6	0.45	0.29	-0.51

- [a]Reported on extractive free basis
- [b]- sum of Klason and acid-soluble lignin content
- (values are expressed as mean and standard deviation)

Analysis of the liquid fraction showed $ZnCl_2.3H_2O$ had a considerable amount of recovered glucan content although $ZnCl_2.4H_2O$ had a higher amount of the reducing sugar. $ZnCl_2.4H_2O$ pre-treatment liquor was rich in both xylose and glucose, hence the perceived potentials of the solvent for applicability in biofermentation system for second-generation biofuel and biocommodities production. With the exception of the observed anomalies in $ZnCl_2.3H_2O$ media, pre-treatment efficiency in runs was inversely proportional to the physicochemical characteristics (pH) as summarized in the severity constant CS (Table 4.2).

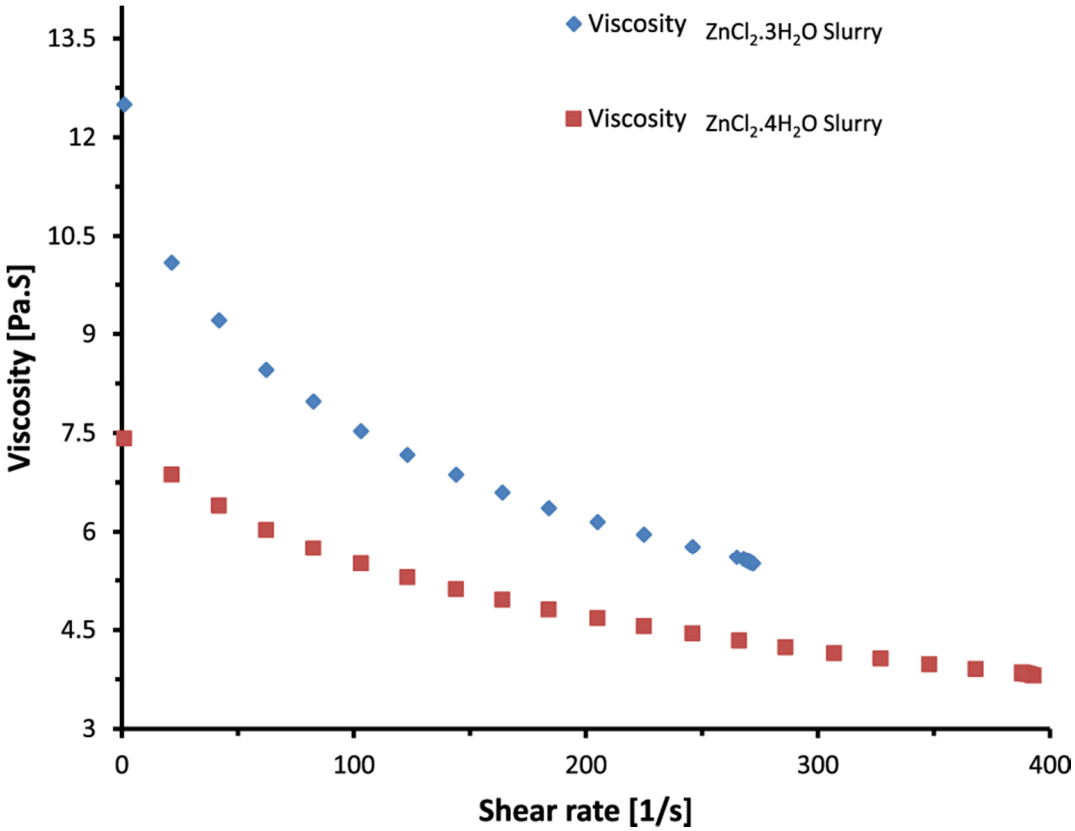


Figure 4. 2: Rheological profile of $ZnCl_2$ MHS-treated corn cob slurries at 70 ^{o}C.

Several studies have emphasised the efficiency of $ZnCl_2.3H_2O$ for cellulose solvation especially over $ZnCl_2.4H_2O$ (Fischer et al., 1999; Li, 2012), with the latter regarded as swelling agent and not necessarily a solvent of the macromolecule. Physicochemical observation of the resulting slurries from both solvent systems presented a widely overlooked process challenge with mass-transfer and optimal mixing, which might impact overall suitability for biomass pre-treatment (Figure 4.2). The apparent higher viscosity of the $ZnCl_2.3H_2O$ slurry, meant higher temperature is required for efficient mixing thus, undermining the idea of mild pre-treatment for energy cost-savings especially for large-scale applications. As such, efficient biomass pre-treatment is recommended at x= 3-4 (70.1 – 64.5 % (w/w)) range, and was conducted throughout the project at a 65.4 % (w/w) $ZnCl_2.xH_2O$ salt.

4.3.2 Zinc Chloride tetra-hydrate salt pre-treatment of corn cob

$ZnCl_2.4H_2O$ MHS pre-treatment of corn cob biomass catalysed the breakdown of the corn cob biomass matrix by solubilizing major components. The efficiency of the pre-treatment step has been related to process parameters such as pH, temperature and reaction time as summarized by the severity CS constant (Equation 2.1). The resulting variations in the solvating credentials of the pre-treatment process at varying CS was determined and documented here. Figure 4.3 showed the mass profiles of corn cob treated in $ZnCl_2.4H_2O$ media at varying temperature and reaction time. The relative mass loss of the composites is an excellent indication of compositional changes and morphology alterations. A significant amount of the biomass composites were lost in the $ZnCl_2.4H_2O$ solvent systems at varying temperatures (70 and 110 ^oC). After an hour of pre-treatment, the mass of residual solid fraction (w_p, g) accounted for up to 88.5 % (w/w) of initial biomass weight (w_o, g). In comparison to hydrothermal pre-treatment (H_2O, 180 ^oC) the mass loss was not very significant at relatively mild process condition of 70 ^oC but increased with increasing temperature (110 ^oC). The variations in observed mass losses however was negligible across batches of runs, thus validating the efficiency of the pre-treatment reactor and the handling procedures for filtering and washing of the biomass slurry.

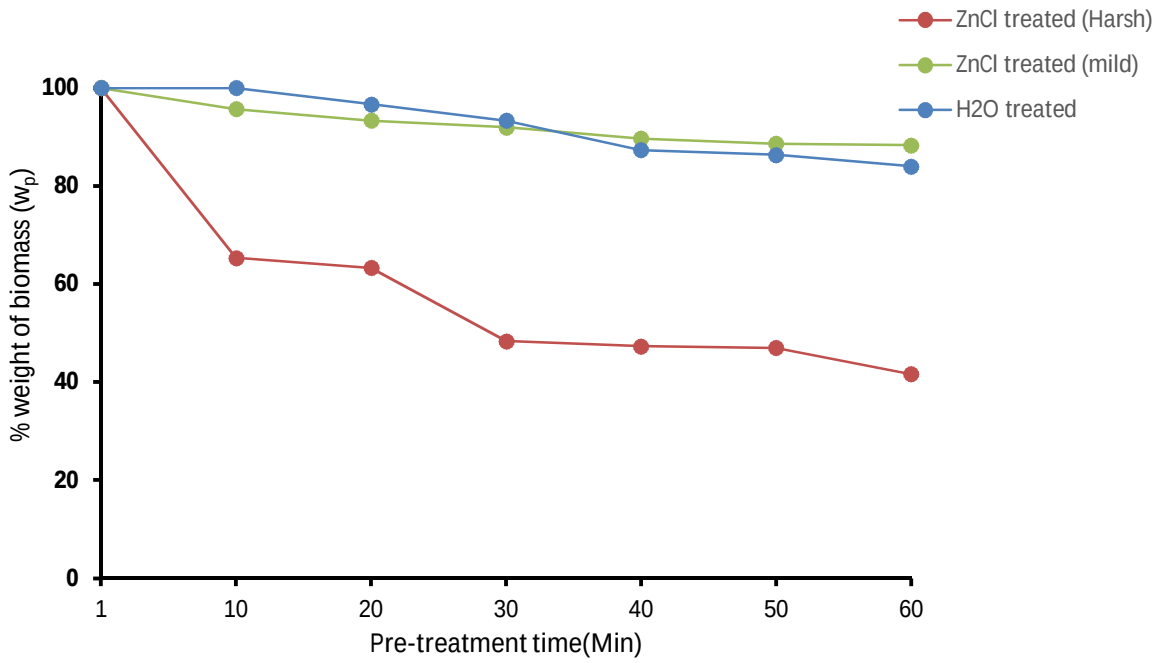


Figure 4. 3: Weight loss of corn cob biomass in $ZnCl_2.4H_2O$ hydrate salt at different temperatures.

The general outlook of mass loss suggested major compositional changes which may have resulted from MHS pre-treatment. The implications of $ZnCl_2.4H_2O$ MHS pre-treatment for the holocellulose in biomass was of immense interest to study, hence, the findings as illustrated in Figure 4.4. Ultra-dissolution (99 %) of the xylan content of corn cob biomass after 50 minutes in the 110 ^oC runs was a profound observation (Figure 4.4(a)). Several reports in literature have obtained extremely high yields of hemicellulosic content especially at harsh conditions such as high temperatures or lengthy reaction times. Chen et al, (2010) reported the severe degradation (93.7 %) of xylan content after non-oxidative treatment of corn stover in lime for 16 weeks at 55 ^oC while (Marcotullio et al., 2011) reported 94 % xylan removal using Iron chloride salts at 160^oC for 20 minutes. Approximately 39.6 % of the initial cellulosic fraction in raffinate biomass was lost, with a negligible difference (± 2.4 % w/w) at both pre-treatment conditions. The stability of the glucan at varying pre-treatment condition suggested that the lag phase of the cellulose-$ZnCl_2.4H_2O$ dissolution reaction upon

the assumption that dissolution of the crystalline component is the rate-determining step. The rate of glucan extraction from biomass matrix was high in the first 10 minutes, an observation that could be due to the easily degraded amorphous cellulose in the biomass matrix. Xiao et al. (2013) reported a similar occurrence in the hydrothermal treatment of bamboo biomass at 140 °C for different time (10-120 min) and arrived at the same conclusion although with proof of increased crystallinity index (CrI) in the residual biomass (Xiao et al., 2013). It was hypothesized that the residual glucan fraction are only accessible by catalytic depolymerization by acids e.g HCl (Marcotullio et al., 2011) or enzymatic saccharification.

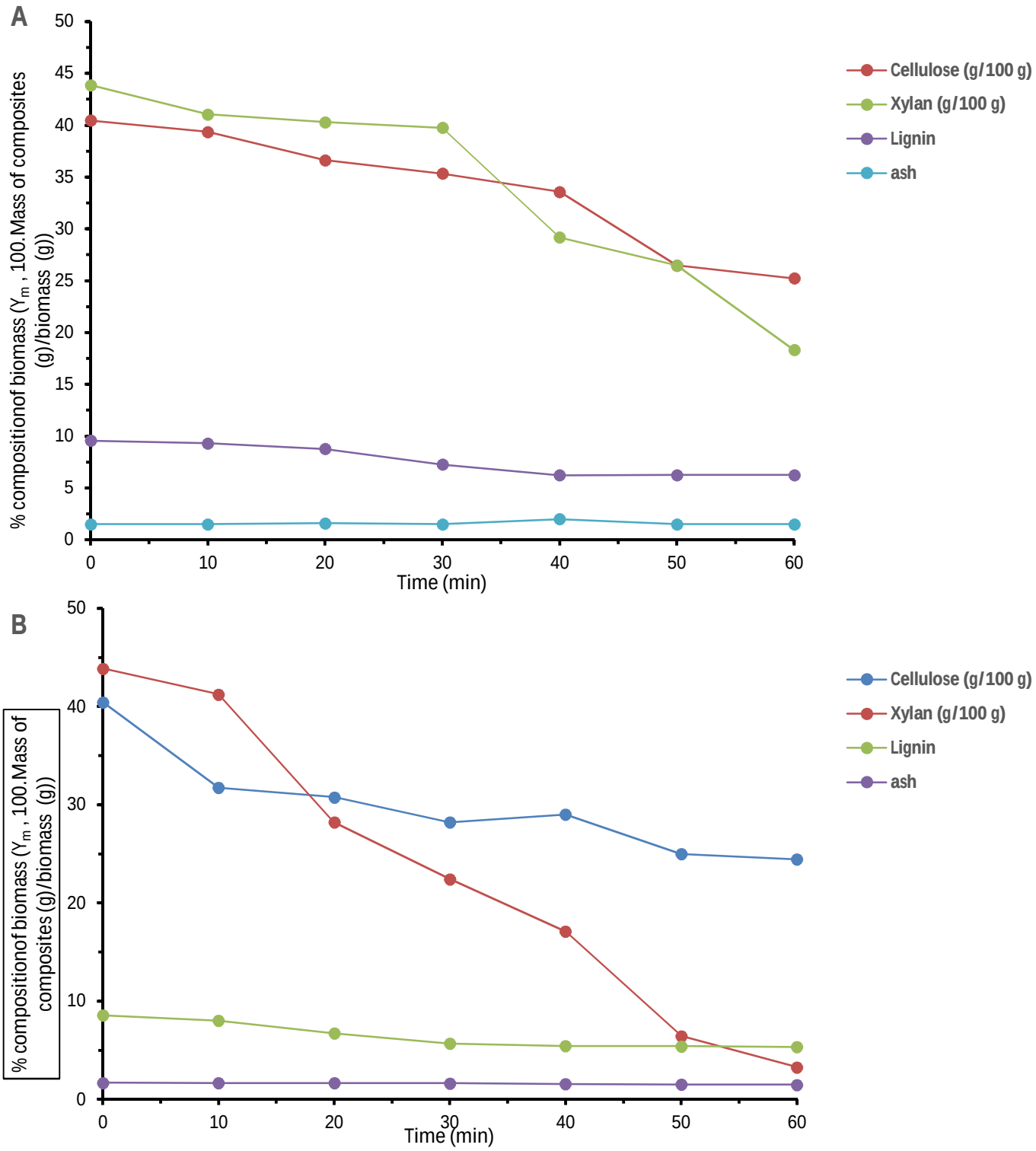


Figure 4. 4: Mass profile of total solids, holocellulose, lignin, ash of corn cob biomass in $ZnCl_2.4H_2O$ at 70 ^oC (a) and 105 ^oC (b)

In addition to hemicellulose removal, biomass delignification also directly influences the efficiency of enzymatic saccharification of cellulose as it inhibits the biochemical physiological processes of biocatalysts. Approximately 70 % of inherent lignin remained in the corn cob with only a negligible difference between both process conditions. The effect of inorganic salt pre-treatment on the modification of lignin composites of biomass is not yet established, unlike other pre-treatment technologies such as non-oxidative alkaline treatment and acid hydrolysis (Ayeni et al, 2012). For example, acetic sodium cholorite treatment, (Wise et al., 1946) is a standard approach used in the pulp industry, with up to 95 % efficiency in removing lignin. Chang and Holzapple, (2000) suggested lignin content removal of up to 90 % for an effective enzymatic hydrolysis (Chang and Holtzapple, 2000). Even though the required cost (both process and substrate-throughput) have not made the aforementioned methods justifiable in literature. More recent opinions on scope have highlighted the importance of accentuating the resulting effect (i.e increasing cellulase accessibility and enzyme-substrate affinity, (K_m)) of delignification in biomass treatment processes instead of simply quantifying lignin removal (Rollin et al., 2011). Reported efforts taretting the delignification of LCB dissolved in inorganic salts have employed synergetic strategies involving techniques such as electron beam irradiation (Guo et al., 2016).

4.3.2.1 Effects of pre-treatment on structural features of corn cob biomass

SEM imaging of pre-treated biomass samples (Figure 4.1) suggested $ZnCl_2$ treatment have a pronounced effect on biomass structure. It is common knowledge that the bio-digestibility of LCB is a function of characteristics such as the degree of crystallization, lignification and acetylation of hemicellulose (Chang and Holtzapple, 2000). In addition to lignin content, Chang and Holzapple's work, also identified acetyl content as a major barrier to cellulase reactivity and as such suggested the complete removal of acetyl groups for optimal digestibility of LCB. The degree of acetylation is directly correlated to the hemicellulosic

component of biomass because aliphatic *O*-acetyl groups bind to 5-carbon sugars such as xylose and glucomannnans at a maximum ratio of 2 to 1 (Mitchell et al., 1990). Acetyl group (unlike phenolic hydroxyl groups found in lignin) actively reduces hydrophobic interactions thus grossly negating enzyme adsorption capacities of substrates (Guo et al., 2014). Infrared analysis of the acetyl group (C=O), which was detected at wavelengths (ν(C=O)a, cm^{-1}) of 1738-1750 cm^{-1} (Filippov et al., 1978) informed the investigations into the effect of Zinc chloride MHS pre-treatment on the acetyl content of biomass in this study.

The peak at the corresponding wavelength (1740 cm^{-1}) as shown in the FT-IR spectra (Figure 4.5) showed a reduction in intensity for the pre-treated samples in comparison to the raw corn cob. the absorbance at 1740 cm^{-1} for the acetyl functional group of the raw samples was identical and comparable to the standard cellulose. The influence of pre-treatment condition on the intensity for acetyl group was negligible as such reiterating the potentials of biomass deacetylation in $ZnCl_2.4H_2O$ treatment at relatively mild conditions.

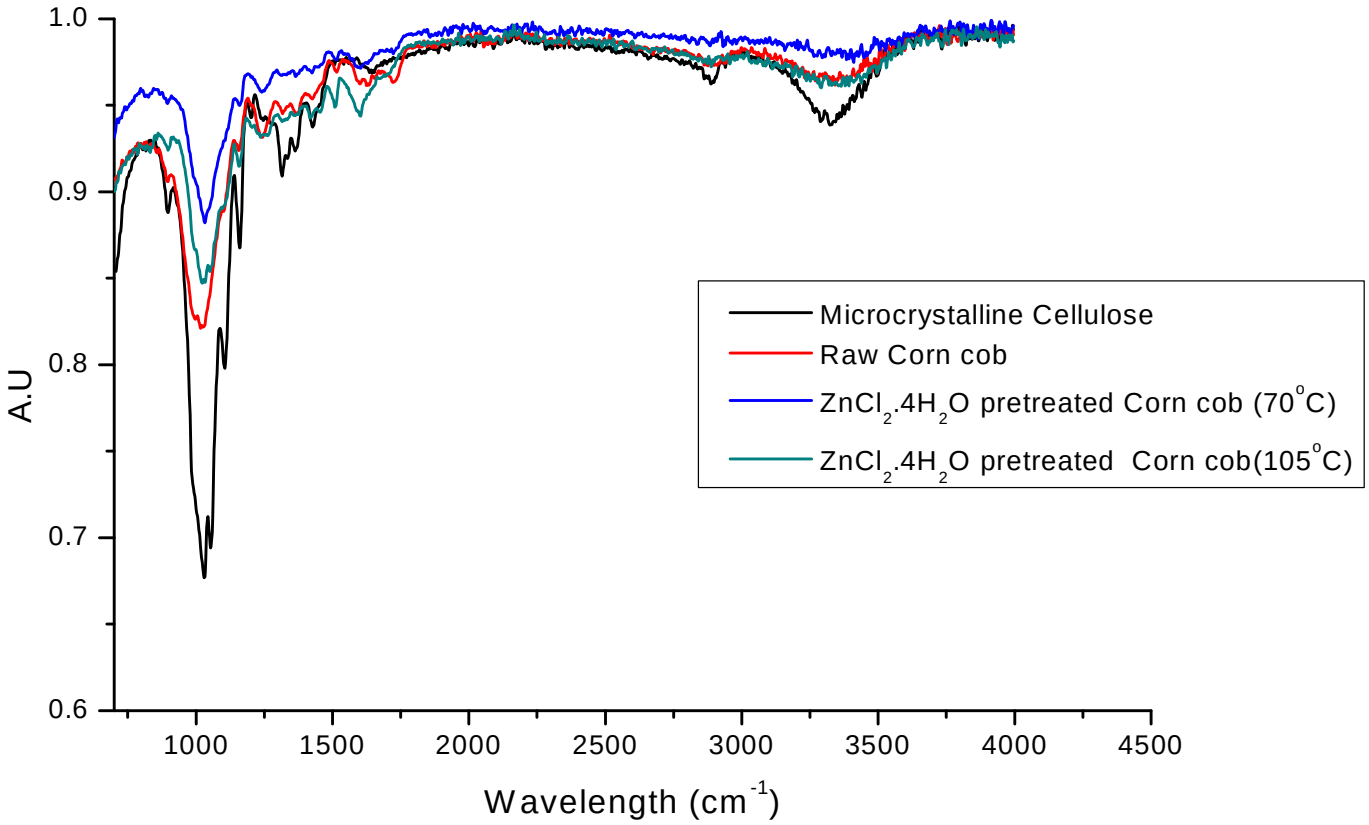
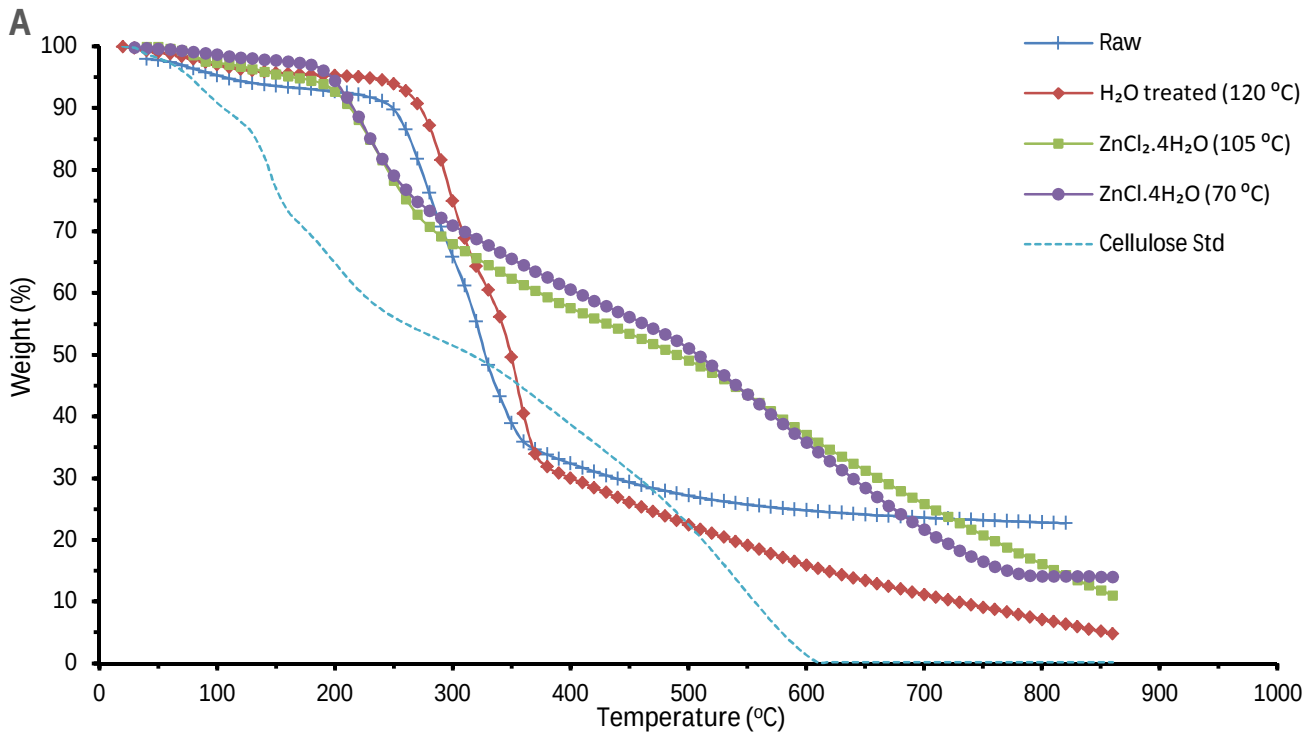


Figure 4. 5: FT-IR spectra of $ZnCl_2.4H_2O$ treated and untreated corn cob biomass

Significant differences in the intensity of peaks for other functional groups (C-O, C-OH: 1050-1150 cm^{-1}; C=C: 1400-1600 cm^{-1}, C-H: 2850-3000 cm^{-1}, O-H: 3200-3600 cm^{-1}) was observed for $ZnCl_2.4H_2O$ treated biomass at different conditions. In comparison to earlier IR studies of biomass materials the spectra was definitive of the distribution of the respective composites of the biomaterial. higher absorbance was expected for the OH in all composites and C-O, C=O specifically for the cellulosic and hemicellulosic composites respectively (Yang et al., 2007).

Characteristic similarities in the absorbance behaviour of lignin and cellulose although (with the exception of a sharp peak at 1025 cm^{-1}) meant insights into organic make-up with the

exception of acetylation couldn't be drawn. Hence, a thermogravimetric approach was used to describe the compositional behaviour of the biomass constituents towards an assessment of the structure and potential digestibility. Figure 4.6 describes the thermodecompositional behaviour of treated biomass in comparison to standard cellulose and untreated corn cob. The behaviours exhibited by the different samples were based on the distribution of their respective components. Previous data on biomass decomposition have established the respective decomposition ranges of cellulose (315 – 400 ^{o}C), hemicellulose (220 -315 ^{o}C) and lignin (0 -900 ^{o}C). The trend of $ZnCl_2.4H_2O$ treated biomass differed significantly from the decomposition pattern of both raw and hydrothermal treated biomass suggesting major variations in distribution of biomass. At 500 ^{o}C, only 50 % of the treated corn cob have been lost compared to approximately 75 % and 80 % of hydrothermal treated corn cob and untreated biomass respectively (Figure 4.6(A)). At this temperature, 80 % of standard cellulose was also lost, suggesting a higher quantity of carbohydrates in both the untreated and water-treated biomass.



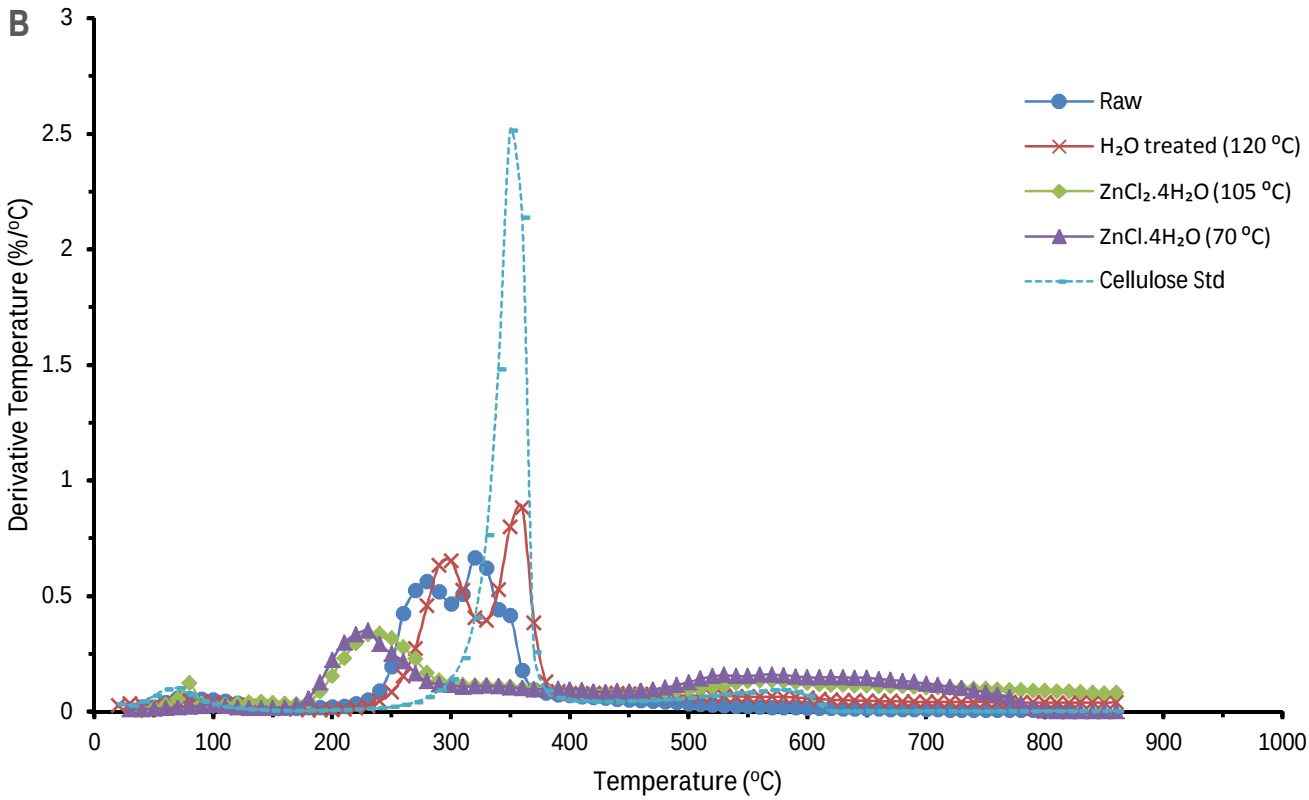


Figure 4. 6: Thermal decomposition analysis of $ZnCl_2.4H_2O$ treated corn cob biomass showing A) Percent weight loss B) Derivative temperature DTG spectra of weight loss

The DTG spectra (Figure 4.6(B)) showed the intensity of treated biomass peaked at 170-250 ^oC after which it normalized till 900 ^oC without major activities unlike the other analysed materials. It was believed that the treated samples had a relatively higher lignin to saccharide ratio but overall less lignin than the untreated biomass as the final residue was slightly less in the treated samples.

Reduction in lignin content and deacetylation led to investigations into the crystallinity of the pretreated biomass. Crystallinity have been shown to have a significant effect on the rate of enzyme hydrolysis. Figure 4.7 shows the overlayed x-ray diffraction pattern of treated and untreated corn cob. Table 3.5 Summarizes the crystallinity indexes of the samples.

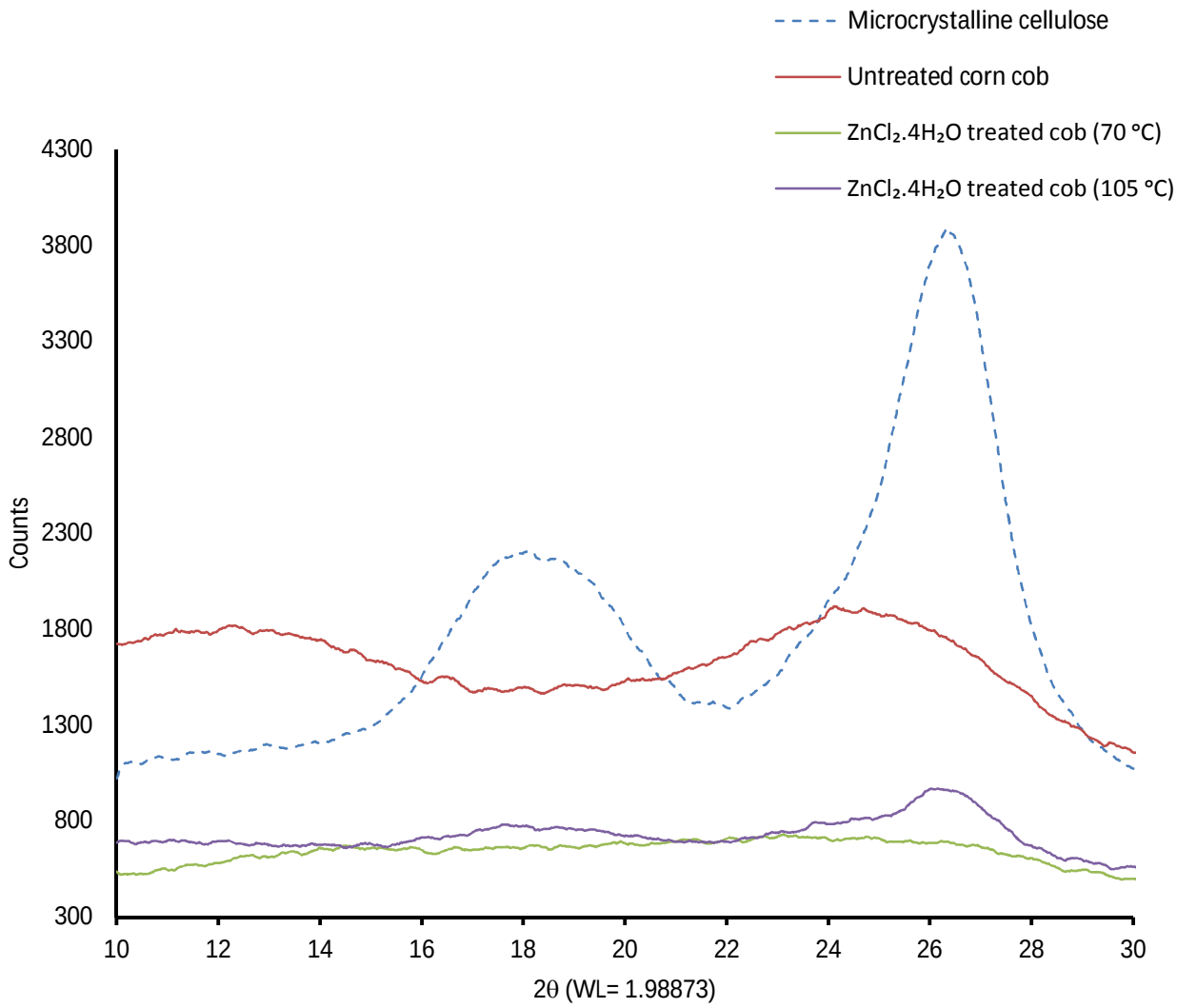


Figure 4. 7: XRD spectra of untreated and pre-treated corn cob biomass.

Table 4.3 showed the significant decrease in the crystallinity index (CrI) between the untreated and treated materials. CrI in pretreated biomass have been directly linked with the ratio of residual glucan in several texts. For example, Kim 2004, reported a slight increase in crystallinity to the high glucan to lignin and hemicellulose ratio resulting from an efficient lime pre-treatment of LCB. It was implied that the pre-treatment had no effect on the microstructural orientation of the cellulosic component, thus substrate reactivity might still be impaired due to the micro-fibrillated arrangement of the cellulose fraction. The effect of hydrate salt solvation on cellulose include conversion of the highly crystalline cellulose Iβ to

the swollen, more amorphous, cellulose II hydrate; which is characterised by faster enzymatic hydrolytic rates. Reduction in crystallinity as observed for the pretreated materials resulted from the swelling of the cellulose micro-fibrils which causes an increase in the polymer surface area and degree of amorphous nature observed. It was suggested that the pretreated biomass would as such be more susceptible to enzymatic hydrolysis as reduced crystallinity could infer increased accessibility and improved substrate reactivity.

Table 4. 3: Crystallinity index (CrI) of raw and pre-treated corn cob biomass.

	Crystallinity index (CrI, %)
Standard Cellulose	45.16
Untreated Corn cob	14.23
$ZnCl_2.4H_2O$ pretreated corn cob (70 ^oC)	6.4
$ZnCl_2.4H_2O$ pretreated corn cob (105 ^oC)	6.6

4.3.2.2 Inhibitory products formation

The pre-treatment liquor was analysed for common degradation products by means of HP-LC. Hydroxycarboxylic acids are formed by end-wise depolymerization of oligo- and polysaccharides by β-elimination reactions which is usually catalysed by alkali and acidic environments (Bensah & Mensah, 2014). The rate of the β-elimination reaction increases with pre-treatment severity forming monomeric phenols (e.g. vanillin) and furan derivatives (e.g. 2-furfural) derived from cellulose and hemicellulose degradation respectively. Degradation products presents a carbon-through challenge for bioprocessing but the formation of phenols and furfurals have been linked to immense inhibition of biological conversion downstream processes.

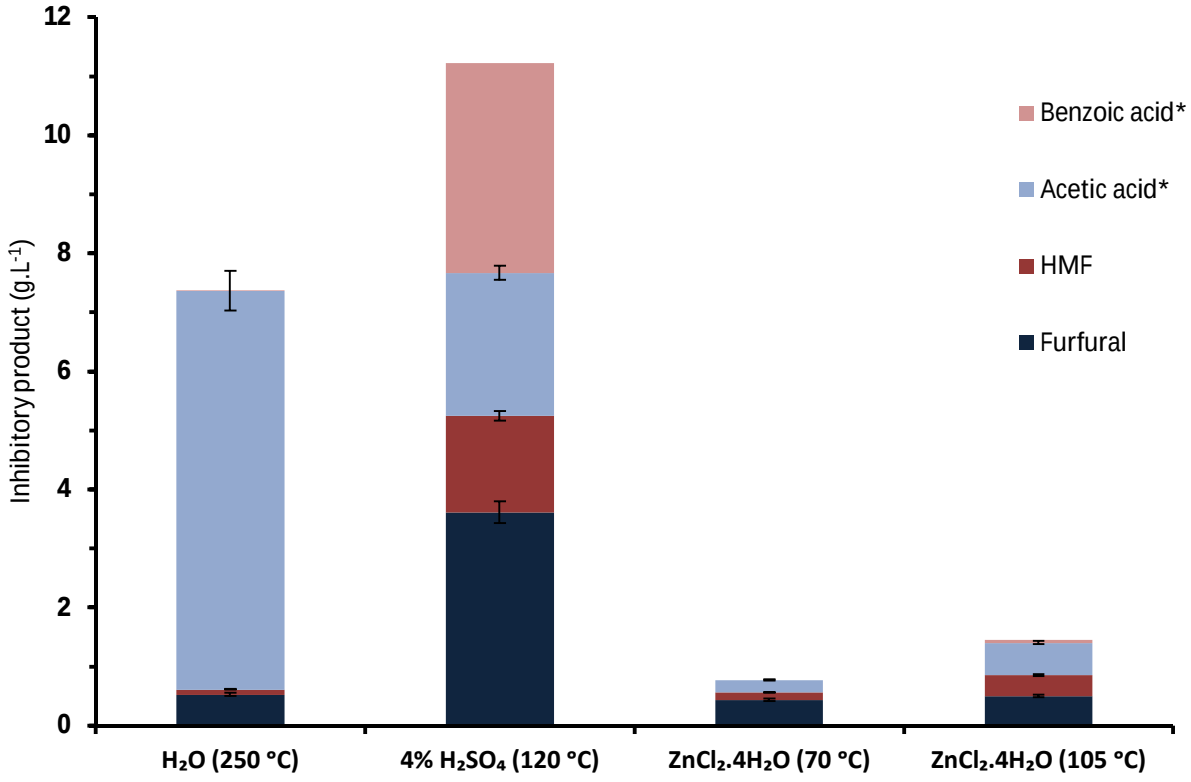


Figure 4. 8: Degradation products yield in $ZnCl_2.4H_2O$ at 70 ^oC (a) and 105 ^oC (b) *- quantified as the salt acetate and benzoate.

Figure 4.8 shows the analysed fragmentation products which could have an inhibitory effect on biocatalyst activity in downstream bioprocessing. In comparison to current standard techniques, the formation of inhibitory products in MHS media at both conditions was close to negligible. Formation of inhibitory products for pre-treatment methods such as alkali treatment and dilute acid pre-treatment is an uncontrolled and difficult to monitor phenomenon. Several potent inhibitory side-products of pre-treatment with varying degrees of inhibitory effects on biocatalytic conversion (cell-free and microbial) are been continuously discovered and reported in literature. For example, 26 oligophenols which are formed during hydrothermal treatment of biomass have only recently been reported to deactivate cellulase (*Trichoderma reesei* complex) media (Rasmussen et al., 2017). The authors concluded that post-pre-treatment conditioning of the pre-treatment liquor was

necessary for optimised carbon throughput and efficient conversion of recovered sugars. Molten hydrate salts pre-treatment at mild conditions were observed to limit the formation of sugar degradation products in-media.

4.3.3 Enzymatic hydrolysis of $ZnCl_2.4H_2O$ pre-treated corn cob biomass

Liberation of glucose was significantly improved by $ZnCl_2$ MHS pre-treatment of corn cob biomass as illustrated in Figure 4.10. The digestibility of raw corn cob biomass was as expected low relative to the treated biomass samples. It was hypothesized that the kinetics of cellulase activity at different enzyme concentrations was favoured until the amorphous cellulosic content was exhausted, after which, reaction velocity (Equation 2.3) slows as a result of increase in inhibition parameter (*i:* as contributed to by reduces substrate reactivity of crystalline cellulose) (Equation 2.4). Figure 4.9 provides a linear function (Equation 4.5) relating enzyme concentration (as derived experimentally from the untreated cobs) to sugar yields.

$$Y = A.\ln(X) + B \qquad (4.5)$$

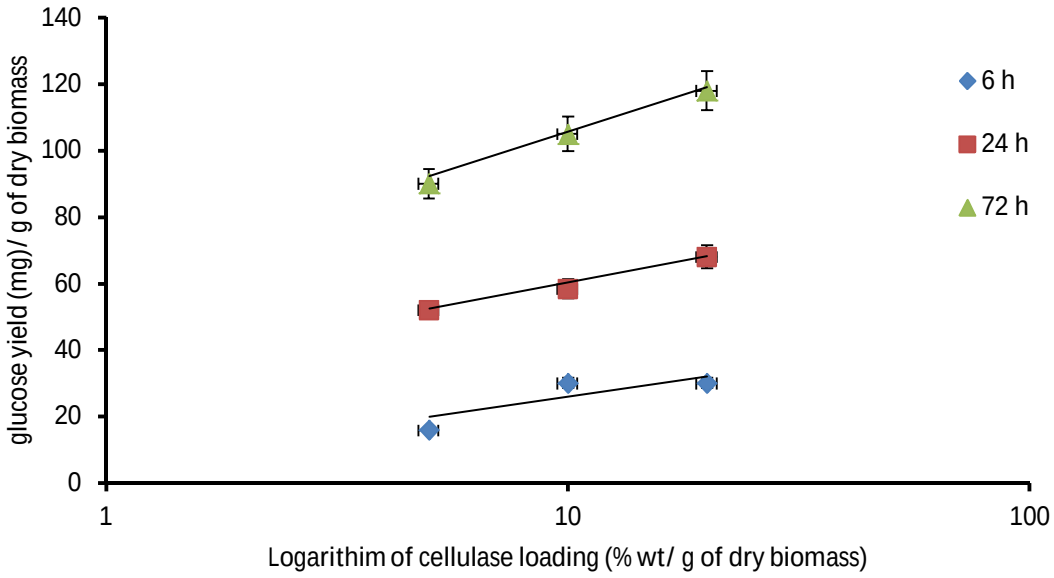
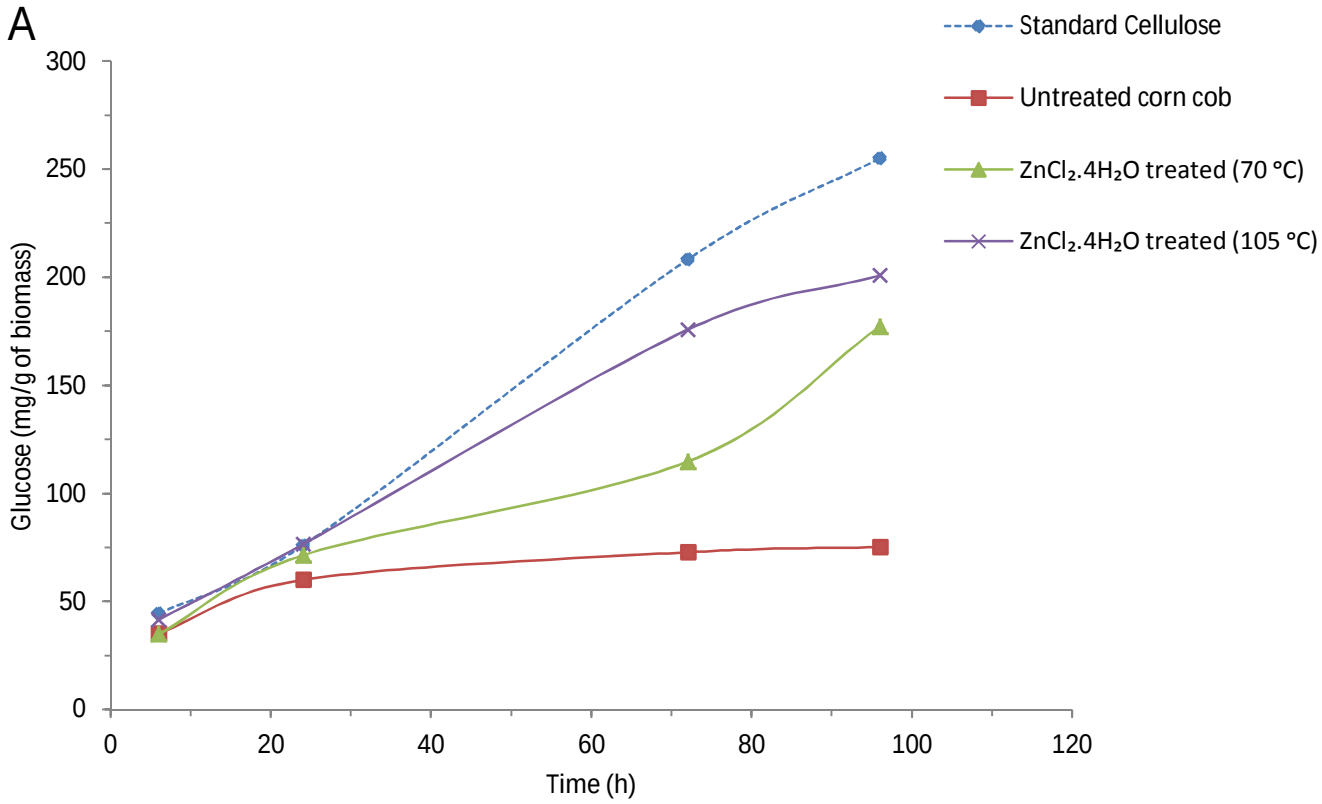


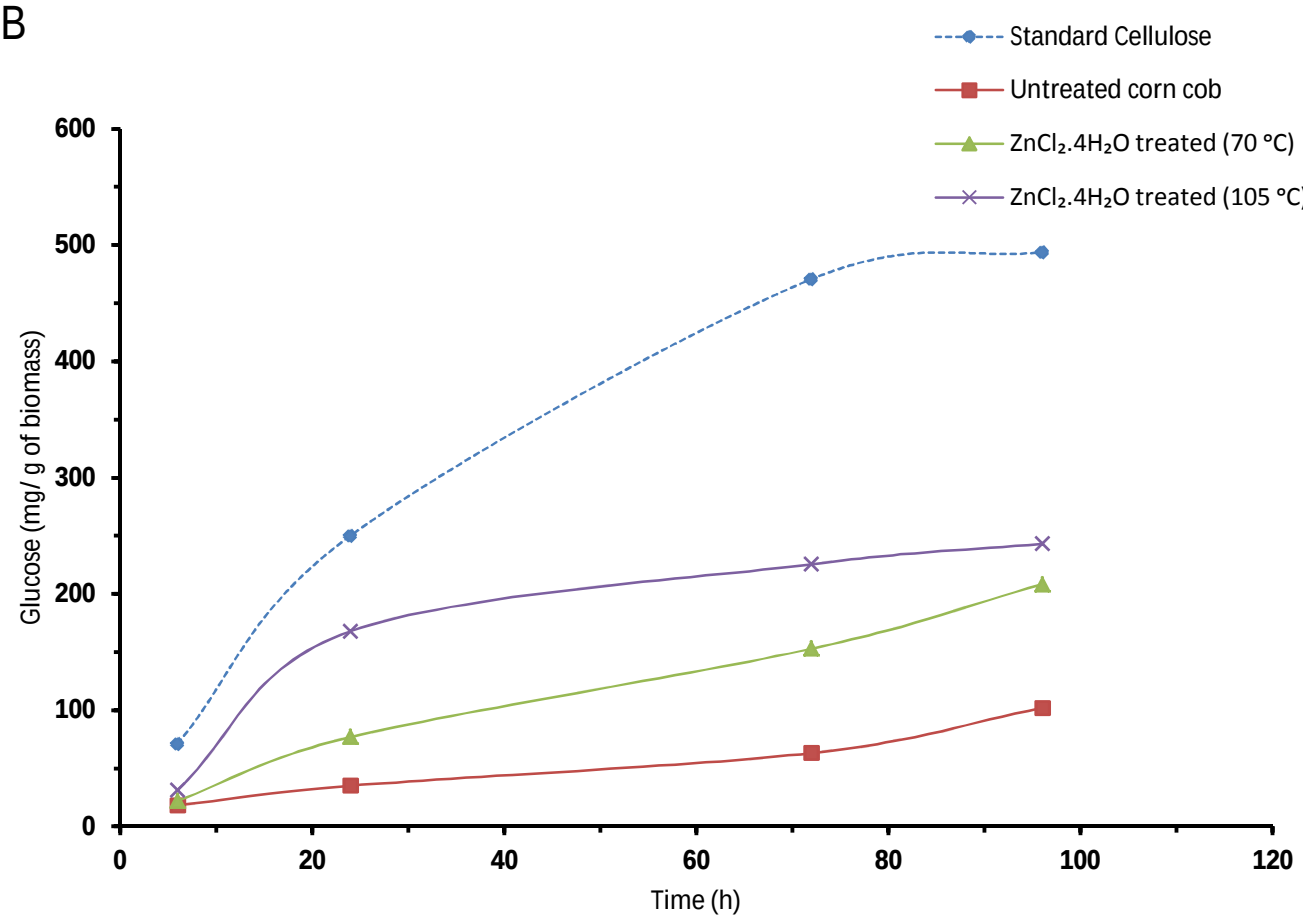
Figure 4. 9: Effect of cellulase loading on glucose yield at 6, 24 and 72 h reaction times.

Based on the 1-HCH model (Equation 2.2), the linear plot of Equation 3.5 is generally expected to increase with reduced inhibitory characteristics of biomass and increased cellobiase activity (both of which are summed up as increasing inhibition parameter). It is important to note that 1-HCH model was proposed on the assumptions of high enzyme loadings (Holtzapple et al., 1984); an uncommon recommendation in industrial processes. High costs of enzymes and related technologies impacts the techno-feasibility and as such biocatalytic applications especially on a large scale; which often requires an optimised balance between activity (K_{cat}) and weight-based total turnover number (TTN_w) (Rollin et al., 2013). Numerous efforts which are committed to the optimisation of saccharification reaction velocities (*V*) at much reduced enzyme concentrations (*E*) have been tailored for the increment of the inhibitory parameter ($i \rightarrow 1$) by costly, ultra-reduction of inhibitory elements (e.g. lignin) in process medium (Kim, 2005). Observed findings, on the other hand, showed potentials for optimisation by $ZnCl_2$ MHS treatment towards increasing the degree of substrate reactivity, by increasing free cellulose sites (ϕ) per specified concentrations (G*x*) of cellulosic fraction of LCB. The effect of MHS pre-treatment, although not delignifying in nature, have the potentials of increasing the surface area and decrystallizing the highly crystalline cellulosic fraction of biomass such that the binding constants for cellobiase activities (β_2) is increased. Rate of hydrolysis of sugar yield in pre-treated biomass mirrored the rate observed for standard cellulose at all enzyme concentrations (Figure 4.10). Sugar yield (in both standard cellulose and pretreated cob samples) peaked at approximately 85, 55 and 50 h time ranges for the 5, 10 and 20 %w/w concentration of cellulase enzyme (with original 74 FPU activity).

A


Standard Cellulose
Untreated corn cob
ZnCl₂.4H₂O treated (70 °C)
ZnCl₂.4H₂O treated (105 °C)
Glucose (mg/g of biomass)
Time (h)
0
50
100
150
200
250
300
0
20
40
60
80
100
120

B


Standard Cellulose
Untreated corn cob
ZnCl₂.4H₂O treated (70 °C)
ZnCl₂.4H₂O treated (105 °C)
Glucose (mg/ g of biomass)
Time (h)
0
100
200
300
400
500
600
0
20
40
60
80
100
120

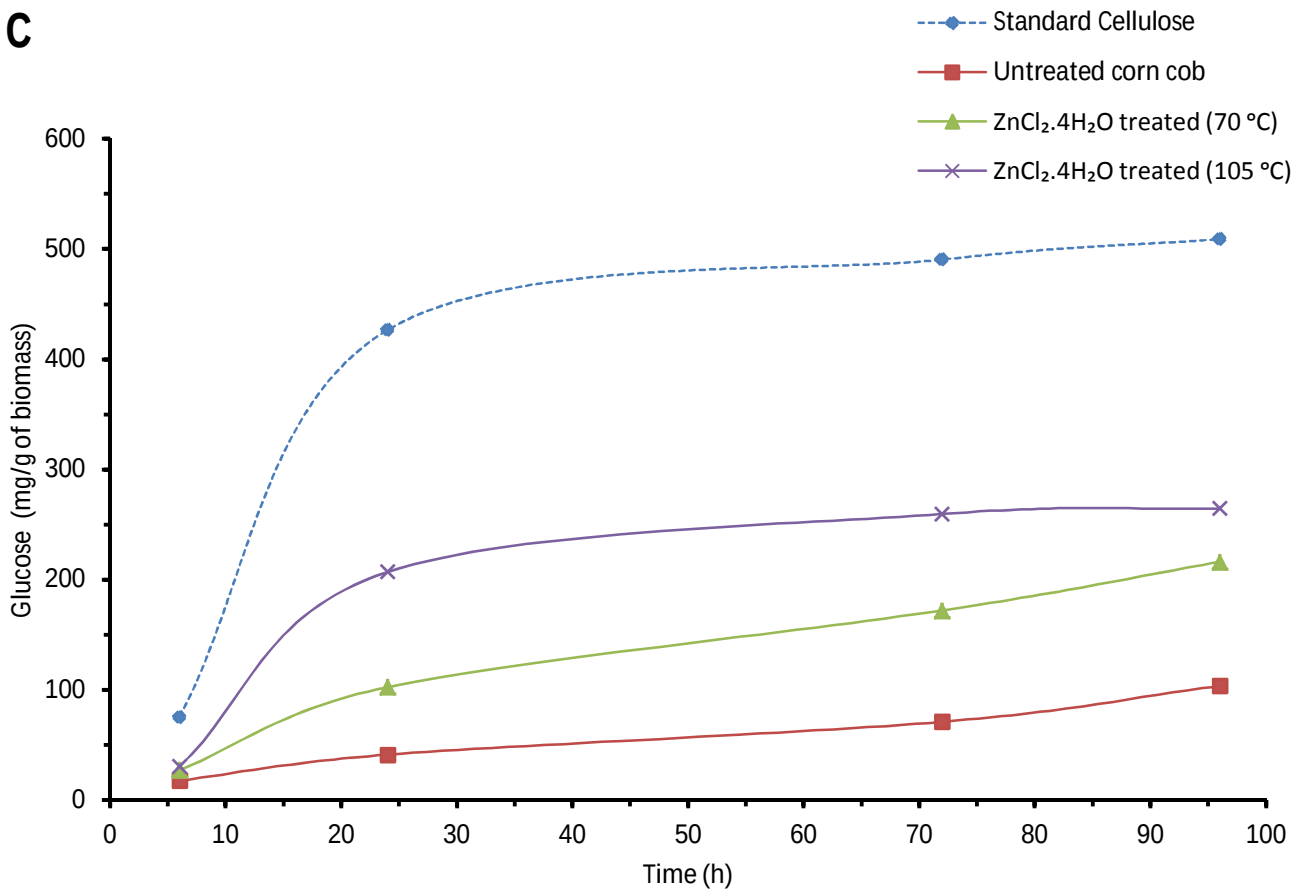
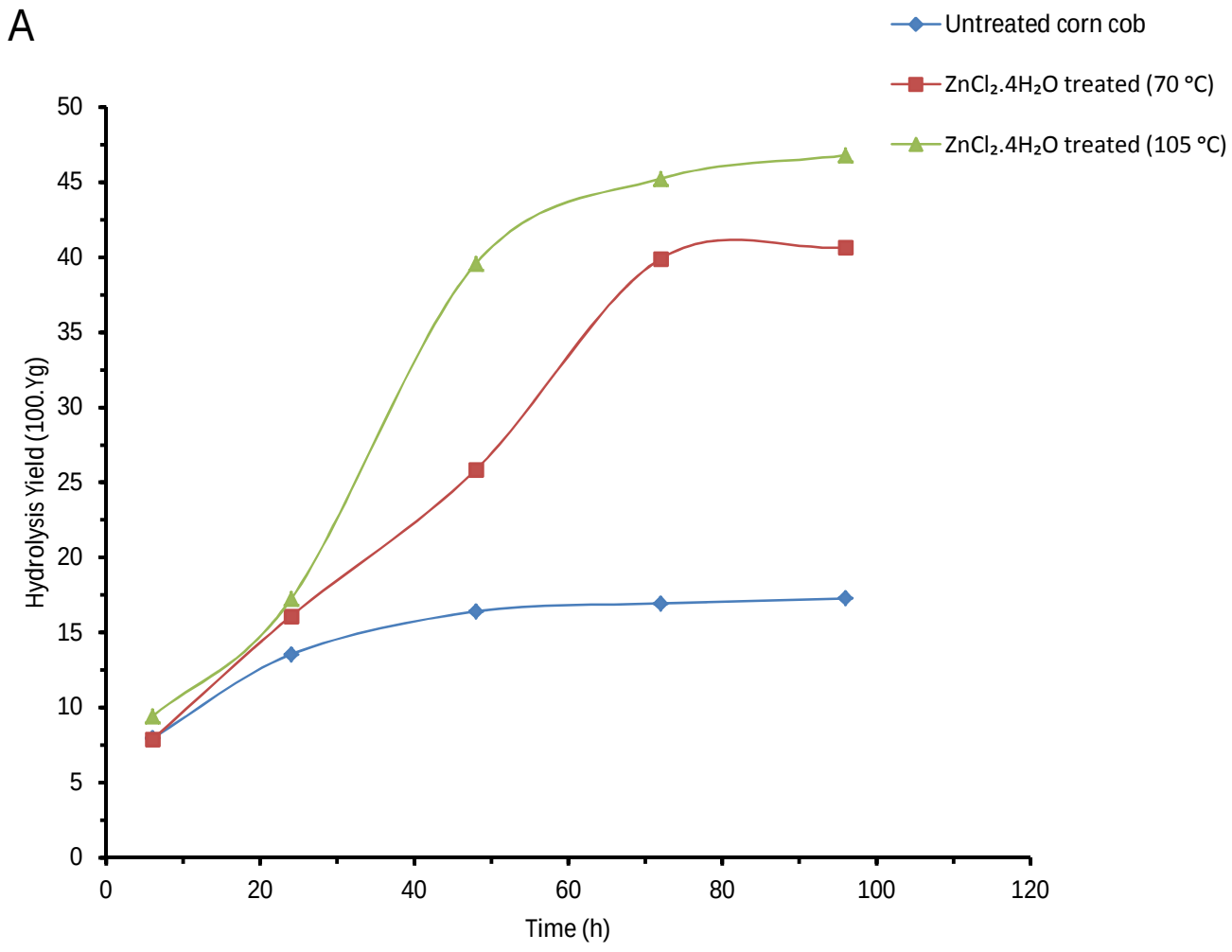


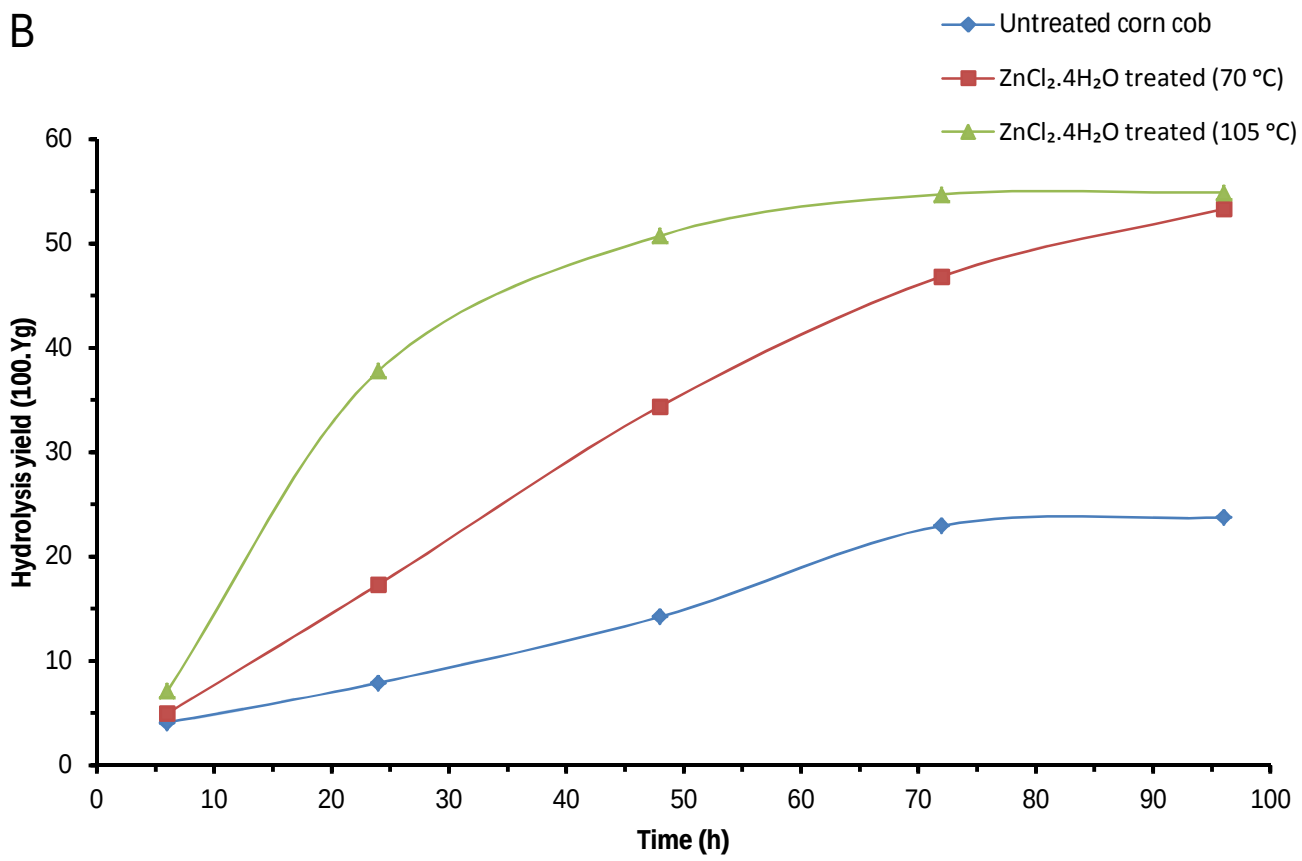
Figure 4. 10: Enzymatic hydrolysis of untreated and $ZnCl_2.4H_2O$ treated corn cob biomass at (A) 5% enzyme loading (B) 10 % enzyme loading and (C) 15 % (w/w) loading of cellulase enzyme.

The observed finding for enzymatic yield of glucose reiterates the reliability of the cellulose modification strategy and overall yield (≅ 332.3 mg/g of biomass, 85 % yield of cellulose at $ZnCl_2.4H_2O$ pretreated biomass at 70 ^oC for 60 mins and 10 %wt enzyme loading) showed the potency of $ZnCl_2.4H_2O$ MHS for cellulose modification. Hydrolysis yield (Y_g) of glucose as illustrated in Figure 4.11, is described as the percent cellulose hydrolysis (as corrected for residual cellulose in pre-treated biomass (estimated as ≅ 60 % (267 mg of glucose)) of original cellulose in raffinate corn cob (w_o)) and initial weight of cellulose in untreated cob samples). Up to 75 % (glucose equivalent of 200.2 mg/g) of residual cellulose was recovered at 10 % enzyme loading for cob samples treated at 70 ^oC for 1 h. As such, the combined

process of pre-treatment and saccharification released up to ≅84 % of inherent cellulose. As shown in Table 4.4, HPLC data suggested the solubulization of approximately 93.1 % of overall hemicellulosic fraction. The influence of pre-treatment conditions on sugar yield was within range for the varying pre-treatment conditions but varied across runs immensely (P_{70}: n= 12, mean= 415 ± 65 mg/g; P_{105}: n= 15, mean= 385 ± 75 mg/g). A likely reason for which is the high variability in biomass composition and sampling.



B


Untreated corn cob
ZnCl₂.4H₂O treated (70 °C)
ZnCl₂.4H₂O treated (105 °C)
Hydrolysis yield (100.Yg)
Time (h)
0
10
20
30
40
50
60
70
80
90
100

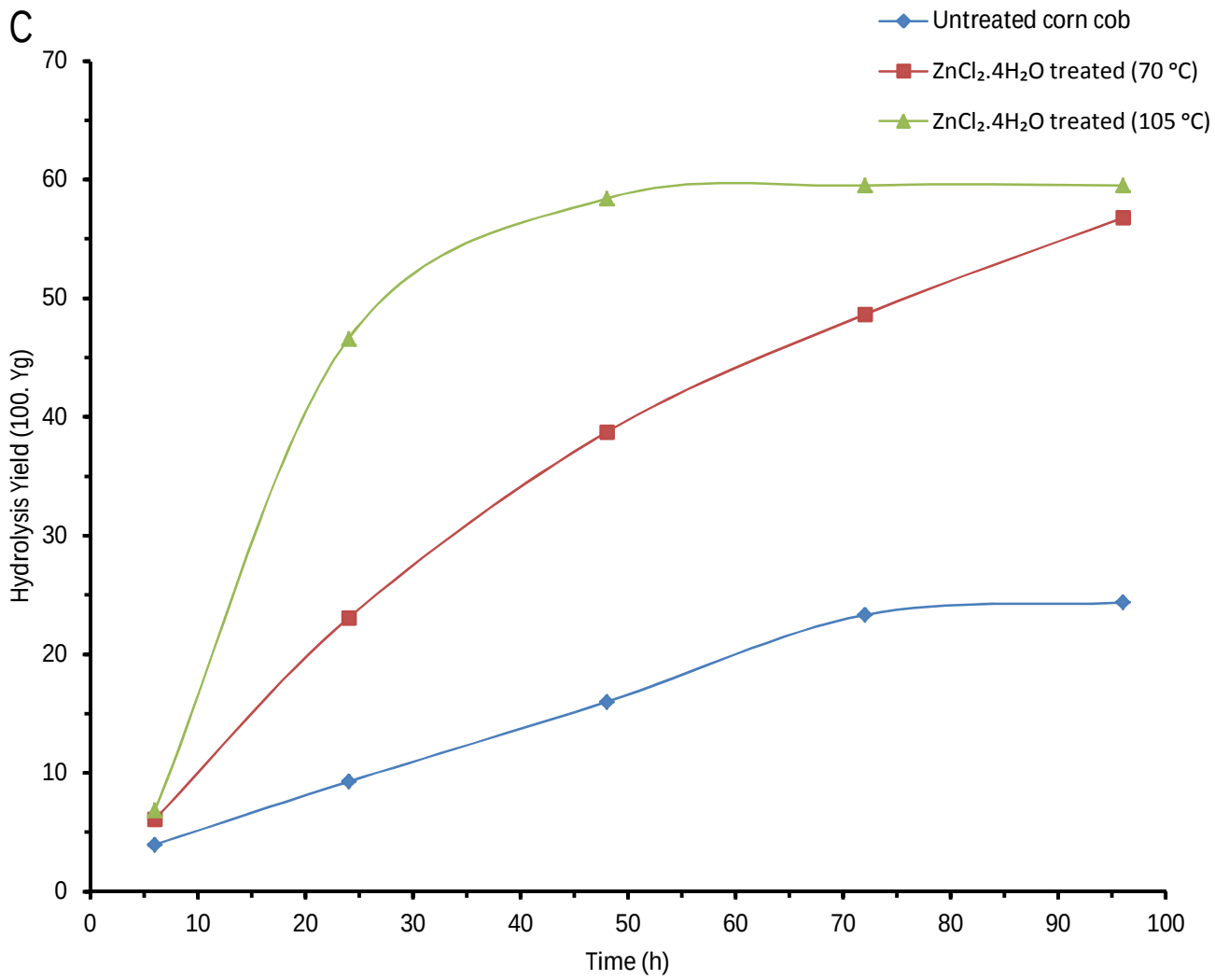


Figure 4. 11: Hydrolysis yield of glucose per g of residual cellulose in corn cob biomass at 5 % A), 10 % w/w B) 20 %w/w (C) enzyme loading.

Overall, Table 4.4 summarizes the overall yield of sugars from the pre-treatment and digestion processes.

Table 4.4: Summary of sugar yield from corn cob during pre-treatment and enzymatic digestion process.

	Raw Corn cob	$ZnCl_2.4H_2O$ Pre-treated corn cob	
		Mild[a]	Harsh [b]
Pre-treatment yield			
Y_g (mg / g of dry biomass) [c]	-	178.51 ± 6.25	182.26 ± 4.52
Y_x (mg / g of biomass) [c]	-	298.23 ± 3.51	514.76 ± 2.94
4*d*-enzymatic hydrolysis yield (10 %w/w enzyme loading)			
Y_G (mg/ g of biomass)[c, d]	102.95 ± 5.33	152.88	225.55
Y_X (mg / g of biomass) [c, d]	125.54 ± 3.21	127.20	25
Residual biomass			
Y_{RG}	343.1 ± 4.12	89 ± 5.03	15.01 ± 2.1
Y_{RX}	365 ± 3.87	65 ± 2.95	2.5± 1.05
Saccharide recovery (% biomass, [100.(1-(Y_{RG} +Y_{RX}) mg/sum of sugar in biomass). mg])	26.24	83.95	98.23

a- Process conditions of 70 ^oC, 60 mins
b- Process conditions of 105 ^oC, 60 mins
c- Values calculated on dry and extractives-free weight.
d- Relevant mass loss due to pre-treatment as corrected for.

Figure 4.12 compares the hydrolysis yield of glucose from $ZnCl_2.4H_2O$ MHS pre-treatment slurry to the recovered washed solid fraction at different pre-treatment process conditions after 4d- enzymatic digestion. The rate of digestion and yield differed significantly at higher pre-treatment temperatures. As earlier defined in Equation 2.1, temperature contributes immensely to pre-treatment severity factor, hence it was proposed that yield (as defined by quantifiable sugar) was affected significantly by factors such as sugar degradation (as earlier

observed (Figure 3.8) and ion-protein interactions in media. The observed differences in the rate of digestion (as represented by the difference in empirical constants for the rate of hydrolysis (Equation 3.5) for the same substrates concentration and enzyme loading) was also attributed to the inhibitory effect of either the ionic salt, substrate and side products on enzyme activity. Milder pre-treatment (70 ^{o}C) exhibited a similar degradation trend to the washed solids, thus reiterating the minimized formation of sugar degradative products and suggesting zinc chloride has a non-inhibitory effect on cellulase activity.

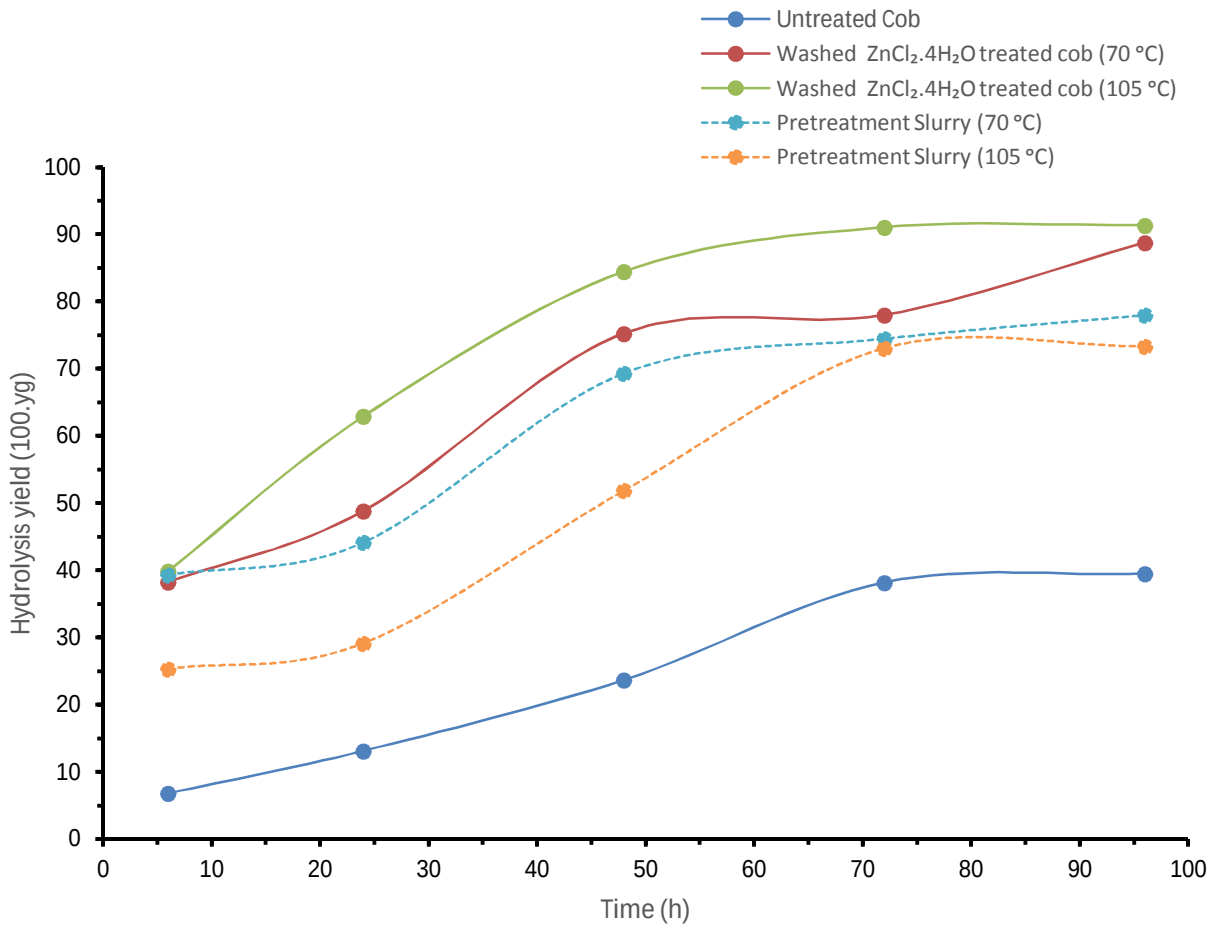


Figure 4. 12: Hydrolysis yield of glucose from corn cob pretreated in $ZnCl_2.4H_2O$.

4.4 Summary

The organic constitution and make-up of biomass is fundamental to their characteristic recalcitrance to biocatalysis. Lignin content, acetyl groups on hemicellulose and crystallinity

of cellulose represented the major barriers to efficient enzymatic digestibility of biomass. $ZnCl_2.4H_2O$ significantly reduced cellulose crystallinity, demonstrated ultra-solubulization of hemicellulose and subsequent deacetylation. Zinc chloride MHS pre-treatment of corn cob at relatively mild conditions, regardless of increased lignin to saccharose ratio, achieved high biomass digestibility upon treatment with cellulase at improved reaction velocities (V).

- Ultra-solubulization and removal of hemicellulosic component was in direct correlation with FT-IR proof of degree of deacetylation.
- MHS pre-treatment dissolved the amorphous cellulose fraction at relatively mild conditions and the residual cellulose was found to be very susceptible to enzymatic saccharification.
- The highest overall yield of fermentable hexose sugars was achieved by means of $ZnCl_2.4H_2O$ pre-treatment of the cob samples at conditions of 70 ^oC for 1 h and 4-d enzymatic digestion using cellulase in a standard medium.
- The overall yield of cellulose, hemicellulose and lignin over the two processes of pre-treatment and saccharification was 84.37, 95. 1 and 35.1 % of respective initial compositions
- Pre-treatment temperature had significant secondary effect on pre-treatment efficiency but had negligible effect on residual sugar yield during saccharification.
- Hydrolysis was in direct correlation with the reduction in crystallinity of the zinc chloride salt treated sample.
- Application of $ZnCl_2.4H_2O$ in currently standard isolated process and high enzyme loadings is viable, although techno-feasibility of production may still be challenging.

Chapter 5

Parametric optimisation of zinc chloride tetra-hydrate salt pre-treatment of corn for improved enzymatic saccharification yield of reducing sugars

This chapter presents a predictive response model relating the effect of LCB biomass $ZnCl_2.4H_2O$ pre-treatment process variables; time, temperature and solvent loading on enzymatic yields of reducing sugars; glucose and xylose.

5.1 Introduction

The proof of efficient $ZnCl_2$ pre-treatment of cob biomass at mild conditions was characterised by improved rate and yield of enzymatic saccharification of the cellulosic fraction. The overall removal of 80.83 % (glucose: 332.85 mg and Xylose: 425 mg) of inherent sugars (Table 4.4) was observed in the last chapter. Significant reduction in the aforementioned yield of reducing sugars was observed in the one-pot saccharification of pre-treated corn cob slurry.

Efficient depolymerization of biomass polysaccharides is an important preliminary step in bioconversion platforms; as reducing sugars are the preferred substrates in the production of biofuels and commodities. Hence, optimisation of sugar liberation from biomass for an improved carbon-throughput for downstream processes is strongly correlated with the final yield of the targeted product. Previous work on MHS pre-treatment have suggested the potentials of underlying factors such as deacetylation and decrystallization of cellulose to the observed efficiency of saccharification in the washed corn cob, while, the reduction in the rate of enzymatic digestion in the one-pot system could be linked to reduced substrate reactivity and inhibition parameters.

Previous studies in literature have either directly or indirectly suggested the potential effects of 1) ionic interactions (Hoffmeister series)(Binder et al., 2001; Binder and Zschörnig, 2002a; Marcotullio et al., 2011)

2) Pre-treatment mechanism (e.g cation-hydroxyl complexation) (Kamireddy et al., 2013; Loow et al., 2015)

3) pre-treatment severity (especially as contributory to inhibitory products formation) as characteristic of inorganic salt media on biocatalytic activity of enzymes and cellulolytic whole microbial cells (Kumar and Murthy, 2011).

Knowledge of the mechanism of biomass modification in hydrate salt and interactions of ionic constituents is still yet widely debated but the influence of pre-treatment parameters on yield of fermentable sugars is as summarized in the combined severity factor (CS). The effect of pre-treatment conditions; time, temperature and solvent loading are pronounced on sugar yield and as such are prominent variables for enzymatic sugar yield from biomass. This work explored the optimisation of overall reducing sugar yield from MHS corn cob pre-treatment slurry by means of a predictive mathematical modelling of operating conditions that are contributory to enzymatic digestion. Using response surface methodology (RSM), based on a 2^3- Box-Behnken design (BBD) of experiments, the multifactorial optimisation of sugar yield was described by a regression model, which was cross-validated experimentally. In addition to optimisation, the model was used to define the sugar substrate present for further conversion in downstream fermentation process.

5.2 Materials and Methods

5.2.1 Experimental design, model development and analysis

Enzymatic hydrolysis yield of reducing sugars (glucose and xylose) was used as response, while pre-treatment process conditions such as temperature, time and solvent loading were

used as variables. Using a $_2$3 Box-Behnken design, the effect of pre-treatment process conditions was investigated towards the proposal of a mathematical model for the optimisation of the response. Fifteen replicated runs (3 centre points) were formulated with MiniTab statistical software (Minitab Inc., PA, USA) and the decoded values are as shown in Table (5.1). The statistical significance (R^2 and adjusted coefficient R^2) of the resulting second-order polynomial regression model was evaluated by conducting one-way ANOVA test of the developed model. In addition, the main and interaction effects of the variables on the response were described using 3-D response surfaces and 2-D contour plots.

5.2.2 Biomass pre-treatment

$ZnCl_2.4H_2O$ MHS used for optimisation studies was prepared as earlier described (Section 3.1.2.1). Corn cob biomass (described in Table 3.1 of chapter 3) was pre-treated as earlier described in Section 3.1.2.1 of Chapter 3 at the stipulated operating conditions stated in Table 5.1. The resulting slurry from the pre-treatment was transferred to 100 ml Schott-Abbott flask containing citrate buffer (30 ml, 0.1M, pH 4.8). The mixture was shaken at a speedof 150 rpm in a shaking incubator at 50 ^oC for 1 h.

5.2.3 Enzymatic hydrolysis

4-d Enzymatic digestion method was according to the procedure described in section 3.3.1of Chapter 3. Cellulase enzyme diluted up to (10 %w/w dilution) was used at standard saccharification conditions. Samples were collected (in triplicates) after 72 h of incubation and analysed for reducing sugars using a pre-calibrated HP-LC technique (see Section 3.1 of Chapter 3 for conclusion). Equation (5.1) was used to calculate yield of sugars relative to biomass yield.

$$Y_E = \frac{Y_g \times (G/0.90) + Y_x \times (X/0.88)}{(G/0.9) + (X/0.88)} \qquad (5.1)$$

5.3 Results and Discussion

5.3.1 Response surface model and effects of pre-treatment conditions

Response surface methodology is a statistical tool for modelling and optimisation of multi-variable functions by quadratic interpolations of empirical proof as informed by sequential testing procedure (factorial DoE). The interactive effects of the main pre-treatment process variables was investigated using a Box-Benhken design matrix (as seen in Table 5.1) from which a second-order polynomial equation (Equation 5.1) relating, time (A), temperature (B) and $ZnCl_2.4H_2O$ loading per gram of biomass (C) to sugar yield was generated using empirical data obtained experimentation. Using 46 datasets, the mean amount of reducing sugar solubilised after *4d*- enzymatic hydrolysis of $ZnCl_2$ pre-treated biomass slurries (at the respective process variables) is summarized in Table (5.1).

$$\text{Amount } (Y_E, \text{mg } g^{-1}) = -324 + 4.15A + 9.08B - 8.9C - 0.0095A^2 - 0.0317B^2 + 0.553C^2 + 0.0197AB - 0.024AC + 0.0394\,BC \quad (5.1)$$

Where, A is pre-treatment time, B is Temperature in degree Celsius, and C is the solvent loading (solid to liquid ratio) of $ZnCl_2.4H_2O$ hydrate salt media.

Table 5.1: 2^3 Box-Behnken design matrix showing the decoded values of independent variables, hydrolysis yield (Y_E) and the model-predicted (Y_p) yield of reducing sugars.

Run	Time (A, minutes)	Temperature (B) (^oC)	Solvent loading (C) (g/g)	Experimental yield (Y_E) (mg/g)	Model -predicted (Y_P)(mg/g)
1	30	30	12	120.450	32.306
2	90	30	12	174.120	231.230
3	30	110	12	534.270	488.405
4	90	110	12	695.330	781.844
5	30	70	6	221.140	292.455
6	90	70	6	598.240	547.206
7	30	70	18	320.120	369.504
8	90	70	18	682.450	607.115
9	60	30	6	132.450	135.427
10	60	110	6	630.745	619.890
11	60	30	18	145.200	185.013
12	60	110	18	702.900	707.263
13	60	70	12	478.250	442.739
14	60	70	12	440.000	442.739
15	60	70	12	450.210	442.739
16	30	30	12	111.200	32.481
17	90	30	12	188.020	231.405
18	30	110	12	543.210	488.580
19	90	110	12	701.670	782.019
20	30	70	6	200.450	292.630
21	90	70	6	600.210	547.381
22	30	70	18	315.200	369.679
23	90	70	18	695.450	607.290
24	60	30	6	135.210	135.602
25	60	110	6	672.210	620.065
26	60	30	18	162.000	185.187
27	60	110	18	715.170	707.438
28	60	70	12	451.250	442.914
29	60	70	12	412.250	442.914
30	60	70	12	425.000	442.914

The predicted yield of reducing sugar (Y_p (Table 5.1)) was derived by substituting for the independent variables A, B, C from the model equation as described by Myers and Montgomery (1995). Figure 5.1 shows the predicted responses (Y_P) is in good agreement with experimental sugar yield (Y_E), thereby suggesting the model is in good measure, in terms of validity.

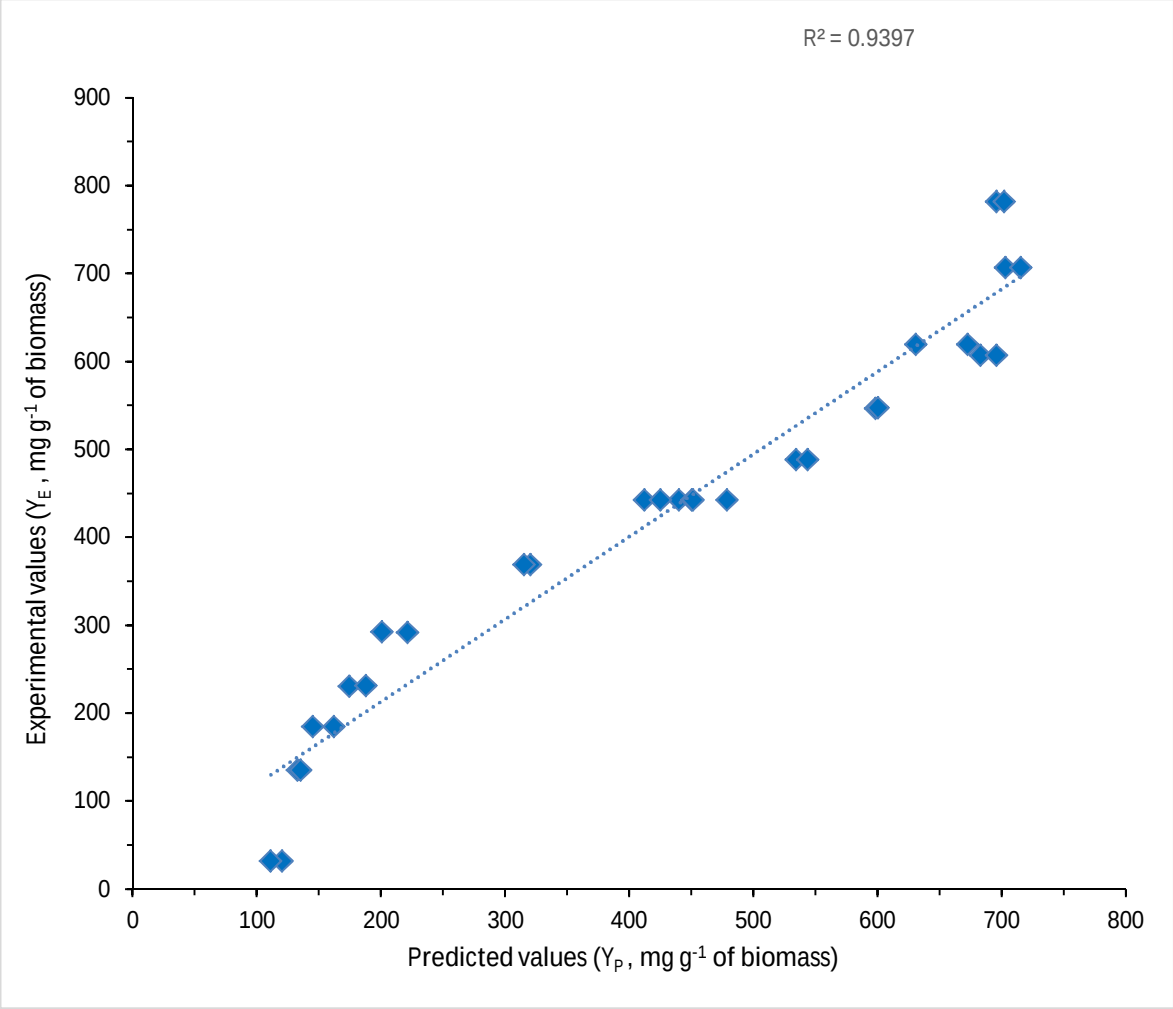


Figure 5. 1: Experimental versus predicted reducing sugar yield values.

One-way ANOVA (analysis of variance) for the quadratic polynomial model is given in Table 5.2. The statistical significance of the model as summarized by the multiple correlation coefficient; R^2 = 93.97 % (adjusted R^2 = 90.79 %) gives credence to the observed similarities

in the predicted (Y_p) and experimental (Y_E) yield of sugars. ANOVA test informed the adequacy and reliability of response fits for linear, dual-function interaction and quadratic functions of the process variables.

Table 5. 2: One-way ANOVA test for surface response quadratic polynomial model of sugar yield.

Term	**Effect**	**Coefficient**	**Standard Error**	**t-Value**	**p-Value**
Constant		442.8	27.1	16.35	0.000
A (Time (minutes))	246.2	123.1	16.6	7.42	0.000
B (Temperature ^oC)	503.4	251.7	16.6	15.17	0.000
C (Solvent loading (g / g))	68.5	34.2	16.6	2.06	0.053
A^2	-17.1	-8.6	24.4	-0.35	0.730
B^2	-101.5	-50.7	24.4	-2.08	0.052
C^2	39.8	19.9	24.4	0.81	0.425
AB	47.3	23.6	23.5	1.01	0.327
AC	-8.6	-4.3	23.5	-0.18	0.857
BC	18.9	9.4	23.5	0.4	0.692

Model summary: S= 66.3616, R^2= 93.97 %, R^2(adj)= 90.79 %, R^2 (pred)= 83.02%

The non-significance of the lack-of-fit test (0.007) (Table 5.3) (which compares the residual and pure errors at replicated points) implied the resulting fits were well defined and fitted by the model. Large *f*-values (25.04) implies that most of the variations in the sugar yield (response) can be explained by the regression model equation. The *f*-test for analysis of variance (ANOVA), showed that the '$P > F$' value for the model was <0.0001, thus indicating the regression was statistically significant with a confidence interval of 99.99%. The *f*-value (29.60) implied that the model was significant and there was only a 0.01 % chance that a 'Model *f*-value' could occur due to noise.

Table 5. 3: Regression analysis of the Box-Behnken design for reducing sugar yield

Source	Degree of Freedom	Sum of Squares	Mean Square	f-Value	P-Value
Model	10	1303493	130349	29.60	0.000
Linear	3	1274651	424884	96.48	0.053
Square	3	23514	7838	1.78	0.185
2-Way Interaction	3	5327	1776	0.40	0.752
Error	19	83673	4404		
Lack-of-Fit	15	82098	5473	13.90	0.010
Pure Error	4	1575	394		
Total	29	1387166			

Statistical tests showed the model could be used to predict the yield of reducing sugar during the pre-treatment process under the investigated range of the operating variables. Optimal value of each variable was determined using multi-objective numerical approach as described elsewhere (Ayeni et al., 2013). The response optimizer objective was to target 830 mg/g (84 % saccharification yield) without constraints on the independent variables. The derived optimal variables were defined as the relative process conditions at which the percent saccharification yield of the pre-treatment slurry equals the washed cob substrate. Using, 10 %w/w of 74 FPU/g *Trichoderma reesei* Cellulase at standard pH and temperature of 40 ^{o}C, the multiple response prediction of the optimal independent variables was; 89.15 minutes, 107.27 ^{o}C and 11.96 g/g of $ZnCl_2.4H_2O$ with an overall desirability function (*D*=0.988122). The optimal values for the operating conditions were cross-validated experimentally with a total yield of 810 mg/g which was in the same range as the yield observed for highest determined for RSM.

RSM is a common statistical technique used for optimisation of enzymatic digestion studies of a several of low-cost lignocellulosic substrates. Mahat et al., (2004) and Tanyildizi et al. (2006) reported an increase in sugar yield up to 34 % and 15 %, respectively, using RSM.

Similar studies have resulted in percent yield as high as; 96 %, 90 %, and 88 % from delignified bagasse (Adsul et al., 2005), alkali-H_2O_2 treated rice hull (Saha and Cotta, 2007) and NaOH treated-rice husk (Jeya et al., 2009) using cellulase from a variety of sources. In this particular study, RSM; using pre-treatment process conditions as factors, helped model the substrate reactivity of pretreated corn cob at the investigated enzyme concentration. The optimised sugar yield was reminiscent of the derived summation of yield from the different steps; pre-treatment and digestion, of pre-treated, washed and conditioned biomass substrate.

5.3.2 Interactive effects of pre-treatment conditions on sugar yield.

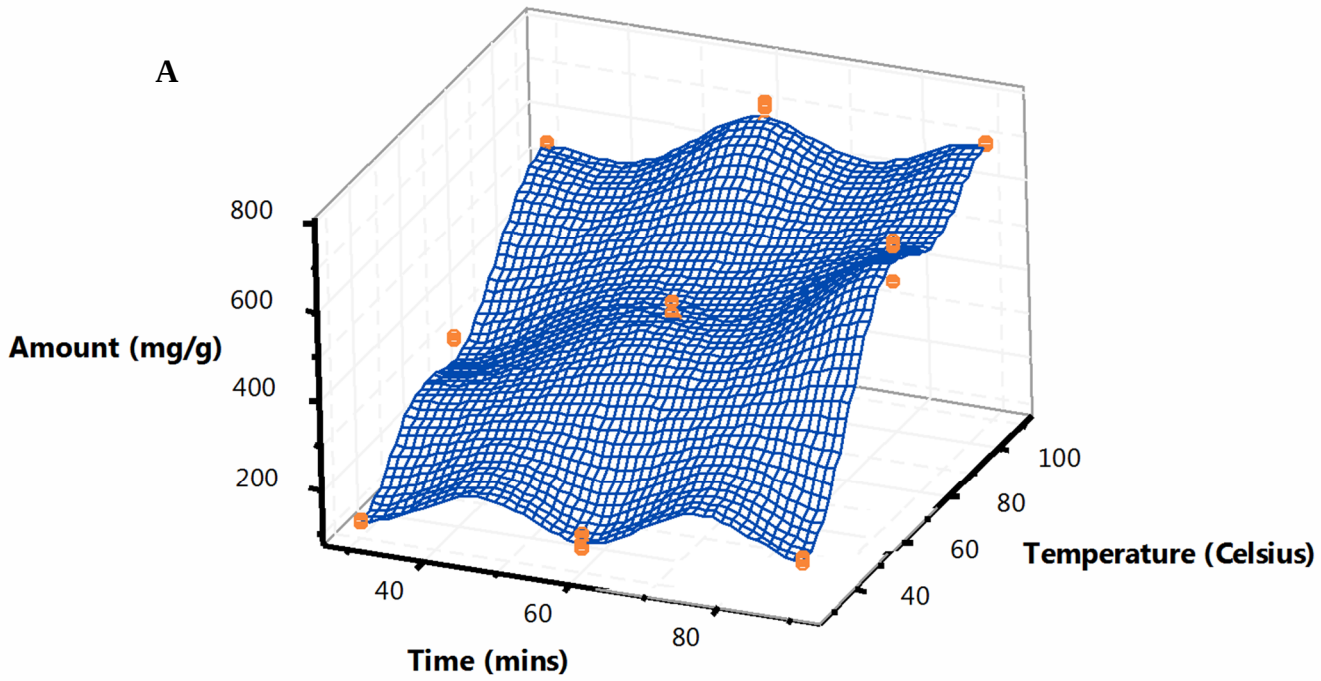
Response surface plots showed the main and linear interactive effects of two variables on the yield of sugars (z-axis) (Figure 5.2). Temperature had a pronounced effect with time on biomass susceptibility to digestion and overall release of sugar from the biomass. The highest amount of sugar (715 mg/g) that was observed from the BBD runs was at 1h, but at the highest temperature (110 ^{o}C) and solvent loading (g/g). The results were in agreement with previous studies on the effect of pre-treatment severity on sugar yield as the values reduced at high temperatures (110 ^{o}C) for longer time (90 minutes) due to the conversion of sugars to degradation products at the relatively harsher conditions. Optimal studies resulted in yields in the range of observed sugar yield in the divergent processes (including enzymatic hydrolysis of washed cobs) at high temperatures, time and solvent loading. Recent RSM attempts by Ayeni et al, (2016) have supported the role of solvent-loading in effective solvation of annual plant lignocellulosic residues in batch aqueous treatment systems. An important posit although contrasting reports such as by Viamajala et al., (2009), emphasises ultra-comminution; a costly, energy-intensive step, in the processing of biomass.

Overlaid contour plots (Figure 5.3) reiterated the effects of the different process parameters

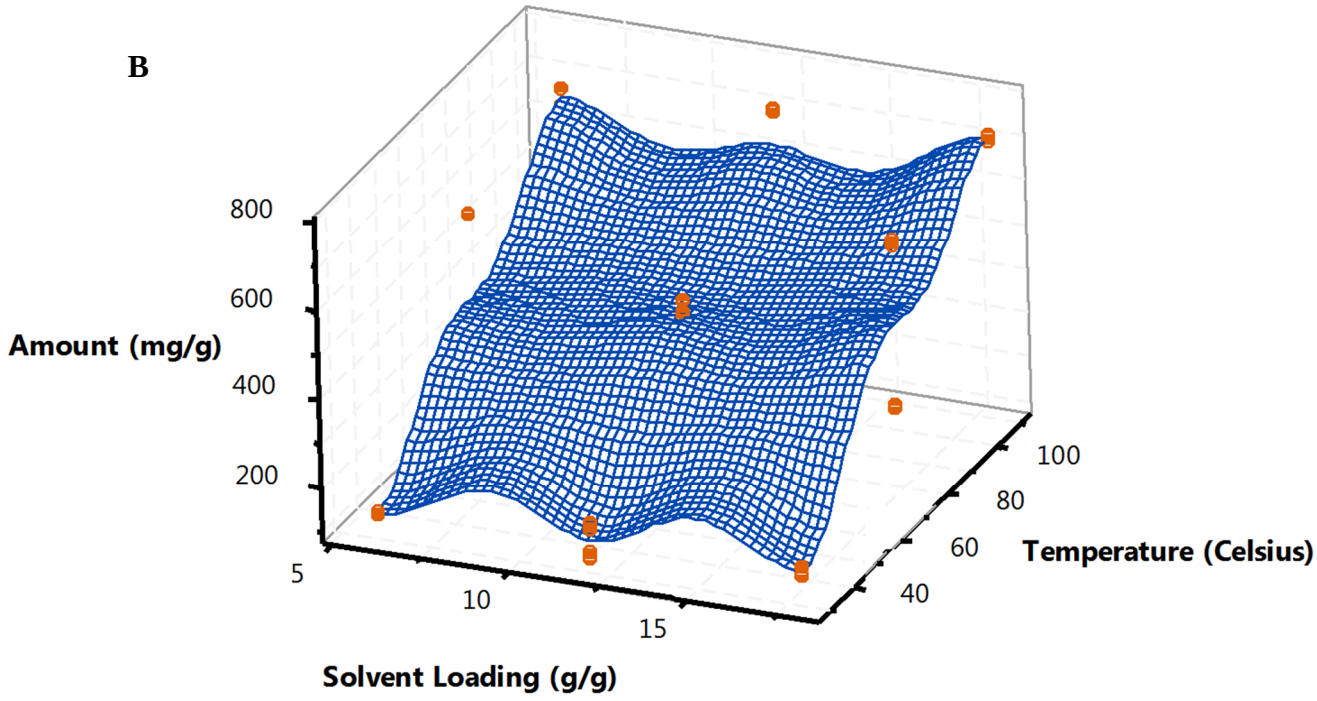
by defining their specific interactions in relation to yield of sugar. The effect of solvent loading at constant time and increasing temperature and vice versa is as illustrated in Figure (5.3A& B). Solvent loading had a visibly significantly lesser effect on sugar yield but at the optimal ranges of the different parameters, influence becomes more pronounced. At 18 $g.g^{-1}$ of $ZnCl_2.4H_2O$ solvent media, the amount of sugar produced increased at the 90 minutes and 110 ^oC respectively. The influence of solvent concentration on downstream biocatalysis will be addressed in future studies, but optimal sugar yield at milder conditions and process costs remain essential to process development, As such, an optimised amount of solvent loadings for specific biomass sizes as relatable to enhancement of sugar yield is a key recommendation for pre-treatment.

The synergetic effect of time and temperature on yield was also explored and illustrated in Figure 5.3C. It is common knowledge in pre-treatment studies that both factors contribute immensely on severity factor and as such have the potentials of affecting carbon-throughput. Temperature especially drove the yield of reducing sugars with time. The effect of time as a factor was important for applicability of the proposed solvent platform in a continuous process which have been show in various texts to be characterised with higher productivities of the sugar monomers as product inhibition is immensely reduced. The yield of reducing sugars increased with time and temperature as illustrated by contour plot. Similar observation is widely reported although for harsher pre-treatment techniques, yield of sugar decreases at higher conditions (Chum et al., 1990; Jing et al., 2011; Wang et al., 2011). Amarasekara et al (2002) described sugar yield from cellulose reached an asymptote without noticeable decline in yield at temperature as high as 200 ^{o} C.

A


800
600
400
200
Amount (mg/g)
40
60
80
Time (mins)
40
60
80
100
Temperature (Celsius)

B


800
600
400
200
Amount (mg/g)
5
10
15
Solvent Loading (g/g)
40
60
80
100
Temperature (Celsius)

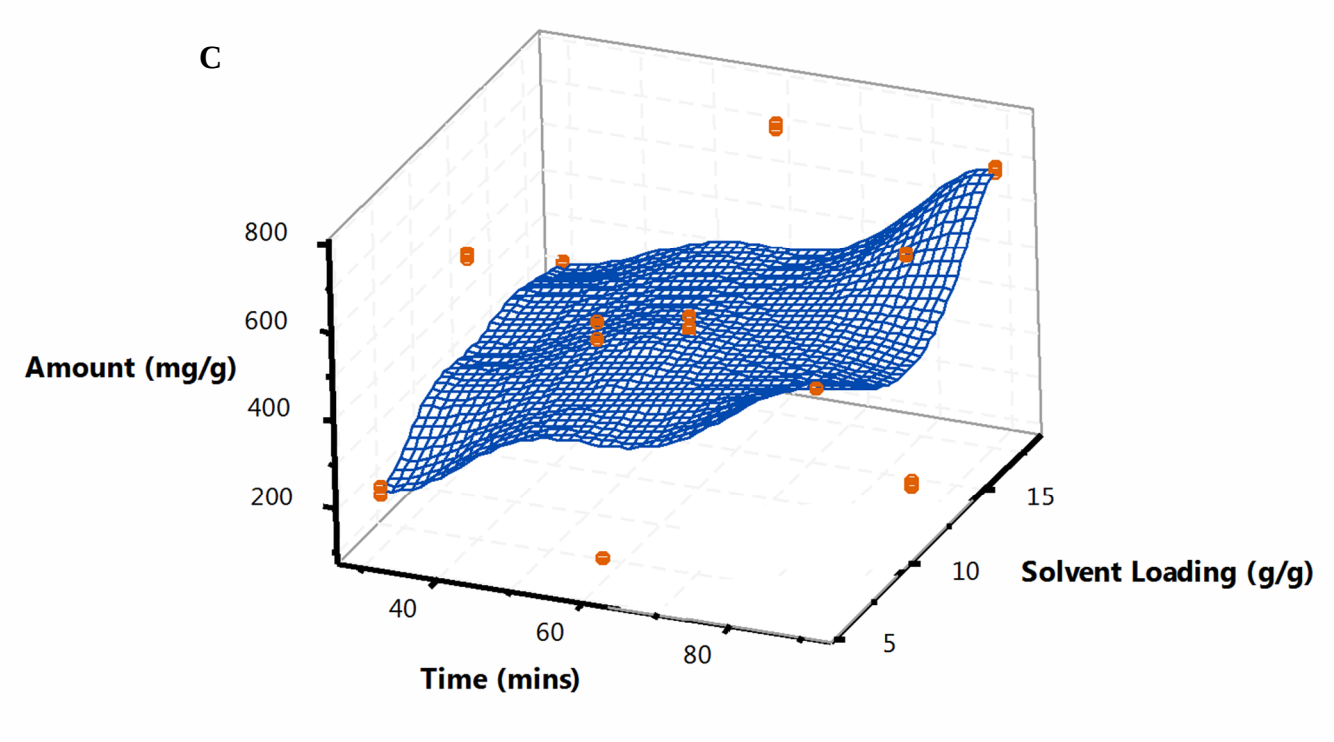
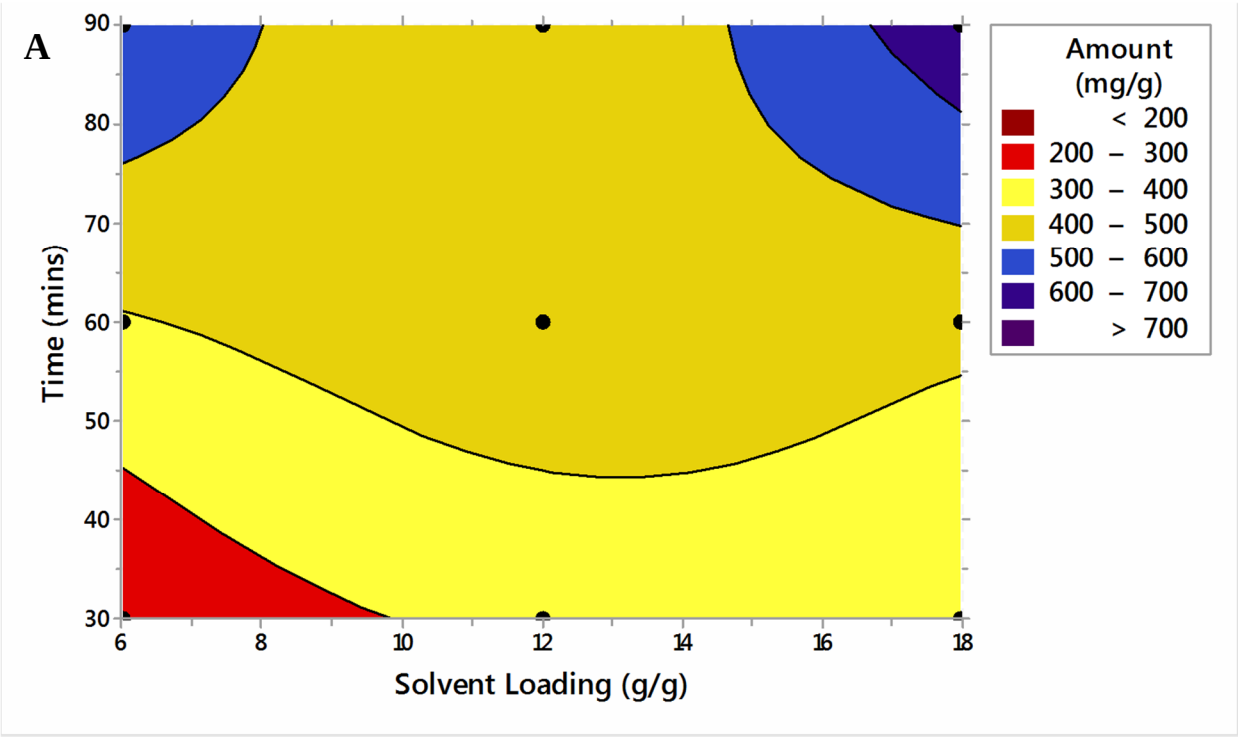


Figure 5. 2: Response surface plots illustrating the interactions of $ZnCl_2.4H_2O$ biomass pre-treatment process variables on the overall yield of reducing sugars. The interactions of (A) time (minutes) and Temperature (^oC); (B) Solvent loading (g/ g) and Temperature (^oC), and (C) pre-treatment time (minutes) and Solvent loading (g/ g) with corresponding sugar yield.



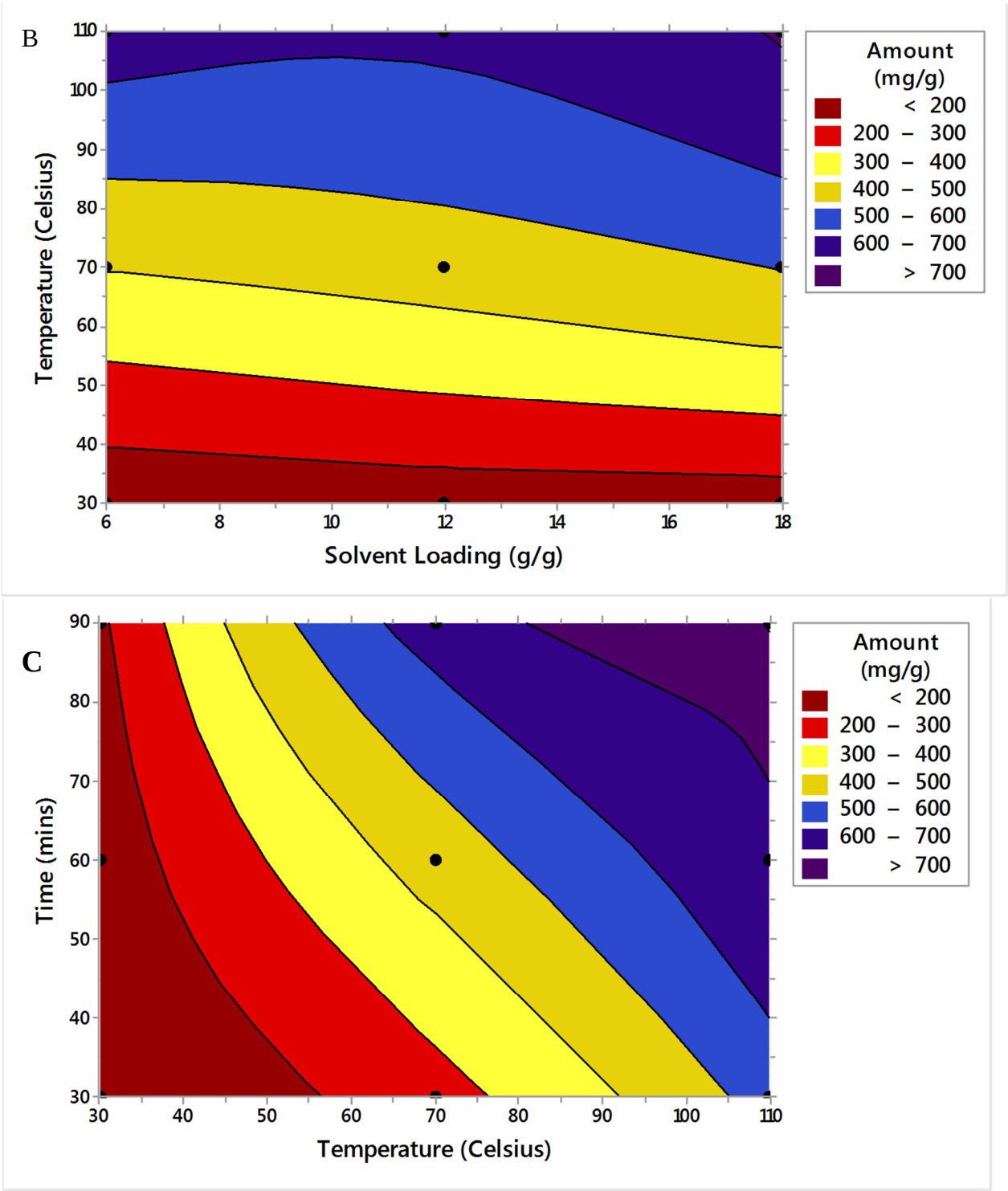


Figure 5. 3: Contour plot indicating the interactions of process variables Temperature and solvent loading A), Time and Temperature (B) Time and Solvent loading, and (C) Temperature and solvent loading, and the effect on sugar yield during ZnCl2.4H2O biomass pre-treatment process.

5.4 Summary

In summary, optimisation studies supports the applicability of $ZnCl_2.4H_2O$ as a pre-treatment solvent for corn cob biomass at mild conditions. The following points were established by means of RSM modelling of pre-treatment process conditions for enhancement of reducing sugar yield from corn cob biomass:

- A polynomial regression model was developed to describe the effect of operating variables on the pre-treatment of corn cob using $ZnCl_2.4H_2O$ solvent system for the release of sugar for downstream bioconversion to value-added bio-based molecules (e.g. Biosuccinic acid)
- Statistical tests show the validity of model (R^2: 93.9 %), as characterised with a non-significant lack-of-fit and large f-value thus, indicating the model fitted the data very well and as such suitable for the description of process within the investigated ranges of variables.
- The optimum operating conditions of 89.15 minutes, 107.27 ^oC and 11.96 g/g of $ZnCl_2.4H_2O$ with an overall desirability function (D=0.988122), for an enhanced yield of 810 mg/g of extractives-free corn cob. was described by the model and substantiated experimentally.

In general, the use of RSM approach enhanced sugar yield (within the indicated ranges of pre-treatment process variables) from dissolved corn cob biomass in $ZnCl_2.4H_2O$ MHS. The yield of reducing sugar (810 mg/g) is comparable to the recovered sugar from $ZnCl_2.4H_2O$ pre-treated biomass feed in a non-optimised process.

Chapter 6

One-Pot bio-conversion of zinc chloride pre-treated corn cob to succinic acid

This chapter presents the fermentation of inorganic $ZnCl_2.nH_2O$ (n = 4) MHS treated corn cob as characterised by yields of BSA and other valuable biobased products. Media selectivity of the wild bacterial strains was established by means of metagenomic analysis of inoculum and subsequent variations in the biodiversity of effluents were the focus of the investigation documented in this chapter.

6.1 Introduction

Succinic acid (BSA) is a top platform chemical and high-value product of LCB bioconversion. Low-cost and high yield production of BSA from waste LCB materials have been recommended as a key strategy for a competitive bio-based economy. BSA is a common co-product of microbial metabolism of reducing sugars in anaerobic mixed-acid fermentation processes; usually alongside other valuable bio-based molecules such as bioethanol (biofuel), lactate, acetate and biohydrogen (biogas). The underlying mechanism of biocatalytic conversion is well-understood with numerous reports of biocatalyst engineering solutions targeting improvement in yield, rate and product recovery. Feasibility of overall process yet remains a challenge hence this proposal of a design which explores (a) the utilization of low-cost feeds (waste LCB; corn cob), and (b) intensified pre-treatment and conversion process using biocompatible $ZnCl_2$. $4H_2O$ MHS medium. Process intensification as a design approach, explored throughout this work, for the development of cleaner, cheaper and energy-efficient biomass refining technology. The proposed strategy is a modification of the conventional LCB refining process (refer to Section 1.1.3), which utilizes $ZnCl_2.4H_2O$ MHS as an efficient medium for LCB sustainable pre-treatment and metabolic processing of sugar substrates to value-added final products.

Prior to this study, the efficiency of $ZnCl_2.4H_2O$ as a pre-treatment media have been established and sugar recovery in medium parametrically optimized. Towards a comprehensive description of the proposed energy-efficient bio/chemo-catalytic process, it was imperative to investigate the potentials of biocatalytic formulations and activities which have potentials for the yield of final product (BSA). As such, here, the media selectivity of the media was studied towards proposal of suitable whole-cells for bioconversion of the treated biomass to value-added products such as BSA. The microbial diversity analyses inform the general knowledge of the uptake of $ZnCl_2$ MHS treated feeds and the effects of the metal halide on summative and specific bioactivity. Using high-throughput microbial screening technique, the biocompatibility of the modified fermentation media was directly correlated with the quantified metabolites such as BSA. It is believed that the findings of the study have the potentials to pave the way for stable, novel biocatalyst formulations customized for the "one pot" conversion of MHS-treated LCB to valuable products in constitutive media.

The description of rumen microbiome activity in modified media, could imply that the simplicity of the proposed $ZnCl_2.nH_2O$ (n=4) LCB pre-treatment solvent system (as characterised by reduced capital and operating costs, optimised carbon throughput (due to negligible formation of inhibitory products), process sustainability, and CO_2 beneficiation (of anaerobic fermentation process)) can be translated to market competitiveness of waste LCB biorefining. Finally, knowledge of the effects of MHS on microbial activity and bioconversion of $ZnCl_2$ treated biomass to BSA will prove the conceptualisation of a MHS medium for "one-pot" biomass beneficiation bioprocessing.

6.2 Materials and Methods

Materials used for the anaerobic fermentation process runs include: Raw cow dung (inoculum), sodium alginate, zinc chloride, corn cob, peptone, yeast extract, magnesium carbonate.

6.2.1 Inoculum preparation

The inoculum used in this study was digested (anaerobic) cow dung collected from a dairy farm in Soweto (Johannesburg, South Africa) while the carbon source was from the cobs (as earlier described in Table 3.1 in Chapter 3). The pH of cow dung used was 7.1 ± 2.45 and total solids (TS), fatty acid, protein constituents are as summarized in Table 6.1.

Table 6. 1: Physicochemical characteristics of cow dung inoculum

Parameters	Value (g. L^{-1})
Total solids (TS)	35± 1.1
Volatile solids (VS)	21.5 ± 3.7
Protein	19.7 ± 2.3
Carbohydrates	6.1 ± 0.4
Lipids	3.1 ± 0.3

Prior to fermentation experiments, the inoculum was heat-treated at 70 ^{o}C for 5 minutes in an autoclave towards reducing the activity of methanogens (methane producers) and sustenance of the activity of targeted spore-formers for bioprocess (Soto et al., 1993). After the, heat treatment, the inoculum was centrifuged (10, 000rpm, 5 mins) and the pellets containing the microbes were recovered and stored at -4 ^{o}C.

6.2.2 Inoculum Immobilization

2 g of sodium alginate powder was dissolved in 100 mL of deionized water in an Erlenmeyer flask. The flask containing the mixture was then placed on a magnetic stirrer (set at 150 rpm) for 2 h. The resulting homogenous mixture was mixed with 10 g of inoculum such that the bacterial cells are entrapped into the alginate matrix. The gel beads were formed by dropping the inoculated alginate solution into 2 % $CaCl_2$ solution using a

peristaltic pump. The diameter of the beads was 3–4 mm and stored in 0.05% $CaCl_2$ solution at room temperature prior to use.

6.2.3 Bioreactor

Fermentation was conducted in 1 litre Schott-abott glass bottles with modified lids for sampling. The reactor was a batch setup in an anaerobic shaking incubator (150 rpm, 40 ^oC). The flasks were sparged with N_2 gas and immediately sealed to create anaerobic conditions.

6.2.4 Anaerobic fermentation

Pre-treatment slurry from 5 g of corn cob (particle size < 2.4 mm) dissolved in $ZnCl_2.4H_2O$ (S: L = 1: 12) for 1 h at 70 ^oC was fed into a batch desktop anaerobic fermenter (set at 45 ^oC and 150 rpm) (Datta et al., 1992). The fermentation medium (50 ml, pH 7.2) consisted of peptone (5 g L^{-1}), yeast extract (5 g L^{-1}), $MgCO_3$ (25 gL^{-1}) and distilled water. Fermentation media was sterilized in an autoclave at 121 ^oC for 45 mins. Inoculum was cultured for 24 h in the fermentation medium and thereafter immobilized in sodium alginate matrixes, before addition (1 g) to the respective setups in an anaerobic shaking incubator. Fermentation lasted for 96 h and samples were collected at 6 h intervals, stored at -20 ^oC for analysis. All experiments and sampling were conducted in triplicates. Control runs used standard glucose and xylose as carbon source, while the runs used the pre-treatment slurry (see Table 6.2).

Table 6. 2: Description of fermentation set-ups

Run		Carbon source (Concentration ($g.L^{-1}$))	Medium (1 L)
1	Control	Glucose (55 mg), xylose (45mg)	Citrate buffer, Yeast extract, Peptone, $MgCO_3$
2	Raw cob	Untreated corn cob (5g)	Citrate buffer, Yeast extract, Peptone, $MgCO_3$
3	Pt Slurry[a]	Pretreated biomass	Citrate buffer, Yeast extract, Peptone, $MgCO_3$
4	Pt Slurry[a] (5)	Pretreated biomass slurry	Citrate buffer, Yeast extract, Peptone, $MgCO_3$, $ZnCl_2.4H_2O$ (aq) (5 g)
5	Pt slurry[a] (25)	Pretreated biomass slurry	Citrate buffer, Yeast extract, Peptone, $MgCO_3$, $ZnCl_2.4H_2O$ (aq) (25 g)
6	Pt Slurry[a] (50)	Pretreated biomass slurry	Citrate buffer, Yeast extract, Peptone, $MgCO_3$, $ZnCl_2.4H_2O$ (aq) (50 g)

a- 5 g of biomass, 1: 12 solid-to-liquid ratio

6.2.5 High performance liquid chromatography analysis

Succinic acid, Lactate, Ethanol concentration was as determined by means of high performance liquid chromatography analysis as described in section 4.2.2.1.

6.2.6 Analysis of microbial community in process media

6.2.6.1 DNA extraction

DNA was extracted from inoculum and process effluent using the ZR Soil Microbe DNA Kit™ (Zymo research company, USA) *as per* manufacturer's instructions (Zymo Research, 2016). The integrity of DNA extracted from samples were verified by agarose gel electrophoresis, while DNA quantification was done using the Qubit 2.0 fluorometer (Invitrogen, California, USA).

6.2.6.2 PCR and DGGE profiling

The primer sets 968F (AACGCGAAGAACCTTAC) and 1401R (5´-CGGTGTGTACAAGACCC-3´) (Felske et al., 1996) targeting the V6-V8 region of the bacterial 16S rRNA gene was used with a 40 bp GC-clamp which was attached to the latter (Muyzer et al., 1993). PCR was carried out in a thermal cycler (SimpliAmp, Applied Biosystems, USA). The reaction mixtures containing 50 ng DNA template, 10 µl of Phusion Flash master mix (ThermoFischer Scientific, USA), 0.2 µM of each primer and nuclease-free water to make 20 µl in total. The PCR conditions was at 98 °C for 10 s hold, 30 cycles of 98 °C for 5 s, 58 °C for 1 s and 72 °C for 15 s, with a final extension of 72 °C for 1 min..

6.2.6.3 Next-generation sequencing of extracted biomaterials.

The first step was the preparation of partial 16S rRNA gene library as described in the Illumina MiSeq 16S library preparation guide. The sequencing method for the V5-V6 region (~336 bp) of the 16S rRNA gene was amplified using forward and reverse primers; 799F (5´-AACMGGATTAGATACCCKG-3´) and 1115R (5´- AGGGTTGCGCTCGTTG-3´) (Redford et al., 2010). Both primers used contained Illumina forward and reverse overhangs, respectively (Illumina Inc., California, USA). The PCR mixture contained12.5 µl of 2x KAPA HiFi HotStart ReadyMix (KAPA Biosystems, Massachusetts, USA), 5 µM of both forward and reverse primer, 12.5 ng template DNA and nuclease-free water to make a final volume of 25 µl. The PCR was run at 95°C for 3 min, followed by 25 subsequent cycles at 95, 60 and 72 °C for 30 s each and a final extension of 72 °C for 5 min. The major aim of the study was to obtain a snapshot of the active microbial elements in the respective process media, hence, the PCR amplicons were further processed according to a downstream library preparation procedure; involving purification and indexing steps, using Nextera XT primers (Illumina Inc., CA, USA). After indexing, the amplicon library was then quantified, normalised and pooled prior to loading on the MiSeq flow cell for a 2x 300 paired-end sequencing.

Upon completion of the sequencing in the MiSeq, reads were de-multiplexed using the on-system MiSeq reporter software. Analysis of the quality of reads was done using the FastQC software (v 0.11.5, Babraham Bioinformatics, UK) before the assembling of both forward and reverse reads using PANDAseq (Masella et al., 2012). The assembled reads were clustered into operational taxonomic units (OTUs) by using the "pick_open.reference_otus.py" script in QIIME (Caporaso et al., 2010) while aligning data against the Silva rRNA database (release 128) (Quast et al., 2013). The OTU table generated from the clustering step data was first normalized, prior to summarizing taxa, and computing alpha and Beta diversity in R software.

6.3 Results and Discussion

The integration of biocatalysts into various manufacturing processes; such as in the production of commodity and platform chemicals is a fast-developing scope, both in industry and academia. Well-reported advancements in molecular biology, high-throughput screening techniques, advanced instrumentation and bioreactor design, and metabolic engineering technologies have helped improve the efficiency of bioconversion processes by customizing the selectivity, stability and activity of biocatalysts to reaction media and process conditions (Zaks, 2001). Tolerance of biological catalysts to their microenvironment is integral to the conceptualization of bioconversion process through directed evolution. Numerous approaches are utilized in the identification of the customized catalytic activities but high-throughput microbial screening and selective enrichment technique are two of the most reliable. Hence this study, using the former technique, sought to propose whole-cell catalysts for the conversion of $ZnCl_2$ treated corn cob biomass to BSA from a mixed-consortia of rumen microbiome.

6.3.1 Effect of zinc chloride MHS on succinic acid production in batch fermentation process.

The fermentability of corn cob biomass $ZnCl_2 \cdot 4H_2O$ slurry was investigated in this study at different zinc chloride concentrations. Anaerobic fermentation involves the catabolism of organic substrates by anaerobes in microspheres characterised by low dissolved oxygen, producing value-added short-chain acids (SCFA) (e.g lactate, malic and succinic acid), volatile fatty acid (VFA) (e.g., propanoate, butyrate and acetate) and gases (methane, biohydrogen) (Guerrant et al, 1982). Dicarboxylic acids such as SA and Lactate are common SCFA produced in fermentative systems; such as human and animal guts (Cummings et al., 2004), and anaerobic fermenters. Using glucose and xylose Table 6.3, shows the production of intermediate products such as ethanol and notable SCFAs in a standard anaerobic digester. Yield of the different intermediates was highly varied as expected, considering the use of a mixed-consortia biocatalysed system means a trade-off of control of the molecular channelling and microbial metabolism of substrates accordingly. The biosynthesis of BSA (as shown in Figure 6.2 and 6.3) from corn cob-$ZnCl_2.4H_2O$ pre-treatment slurry, aligned standard and proposed fermentation process design by highlighting the potentials of “select” colonies from the rumen microbiome for BSA fermentation.

Table 6. 3: Alcohol and SCFA yield in in control run of 96 h batch fermentation medium.

Intermediate	Yield ($g.L^{-1}$)
Ethanol	29.4 ± 3.5
Acetic acid	6.5 ± 1.43
Formic acid	0.27 ± 4.23
Lactate	65.17 ± 1.67
Propionic acid	1.2 ± 0.5
Succinic acid	14.78 ± 2.78

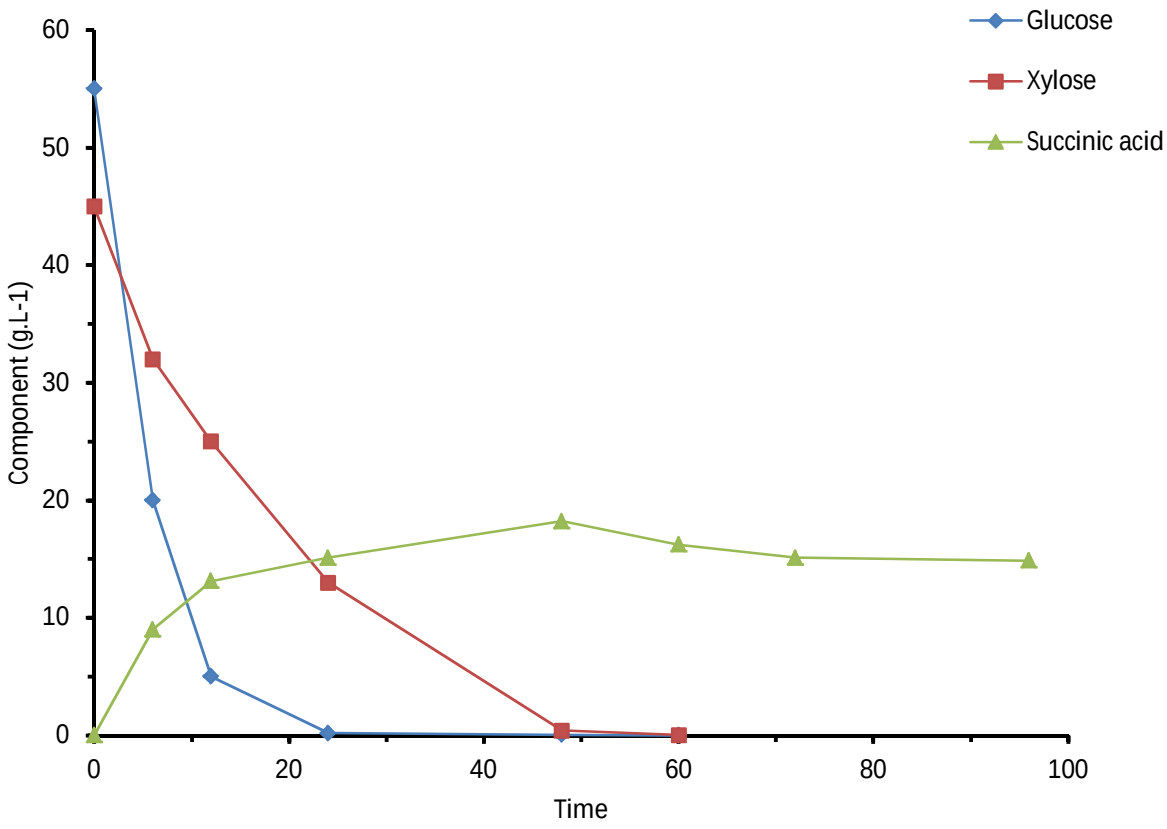


Figure 6. 1: Reducing sugars; glucose and xylose consumption and SA biosynthesis in anaerobic fermentation media.

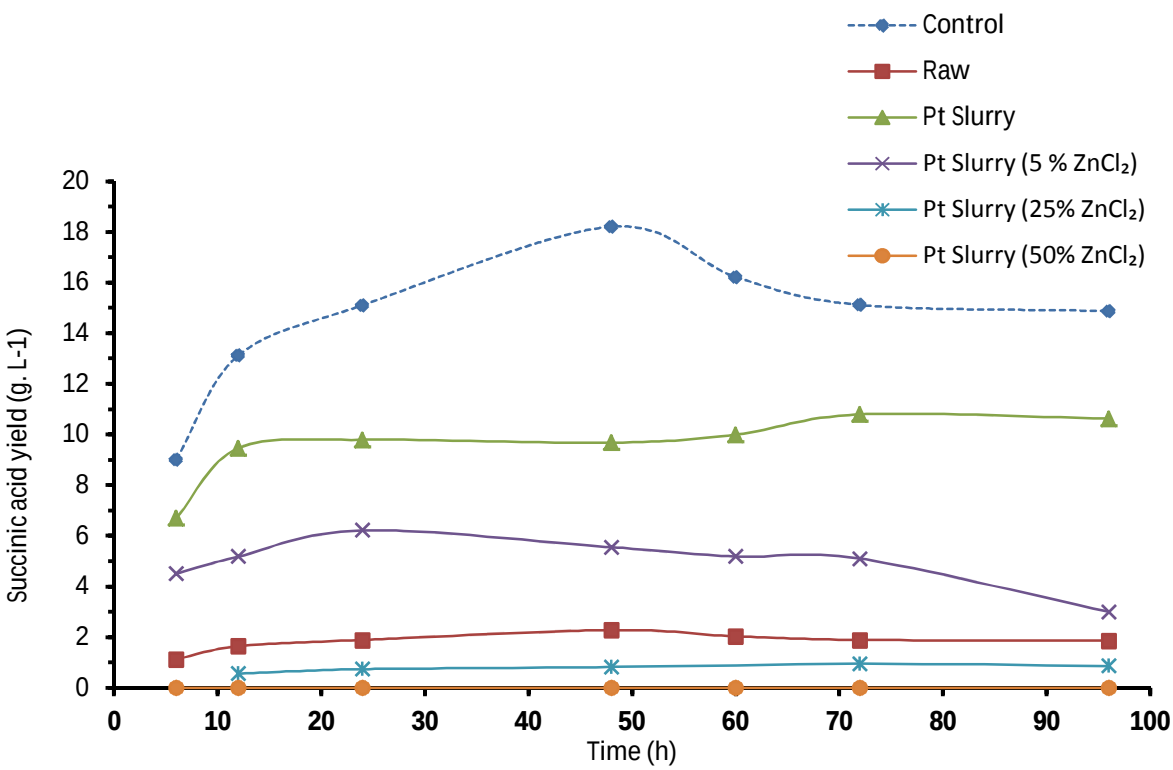


Figure 6. 2: Succinic acid biosynthesis catalysed by a mixed consortia of rumen bacteria at varying concentrations of the inorganic $ZnCl_2$ MHS media

Figure 6.2 shows the yield of the intermediate product using the biomass/MHS slurry at varying amounts of the active ionic constituents of the inorganic media; $ZnCl_2$. Biosynthesis of SA was observed in fermentation runs which uses pre-treatment slurry as carbon source up to 25 %w/w of fermentation media. In comparison to the raw corn cob, a 82 % increase yield over raw corn cob was observed in the fermentation runs suggesting $ZnCl_2$ pretreated corn cob were better substrates for SA bioproduction than untreated LCB. Higher concentration of the $ZnCl_2$ constituent implied increased ionic interactions affected the catalytic activity of the process as yield of intermediates was close to negligible throughout the duration of run. This finding was correlated with the concentration of reducing sugar which was stable during fermentation. The results agreed with previous reports, such as by Kamireddy et al, (2013), on the negative effects of metal halides on the activity of anaerobic biological conversion. Zinc cation is a strongly hydrated cation with high charge densities with kosmotropic tendencies and as such is theoretically, a more biocompatible media for

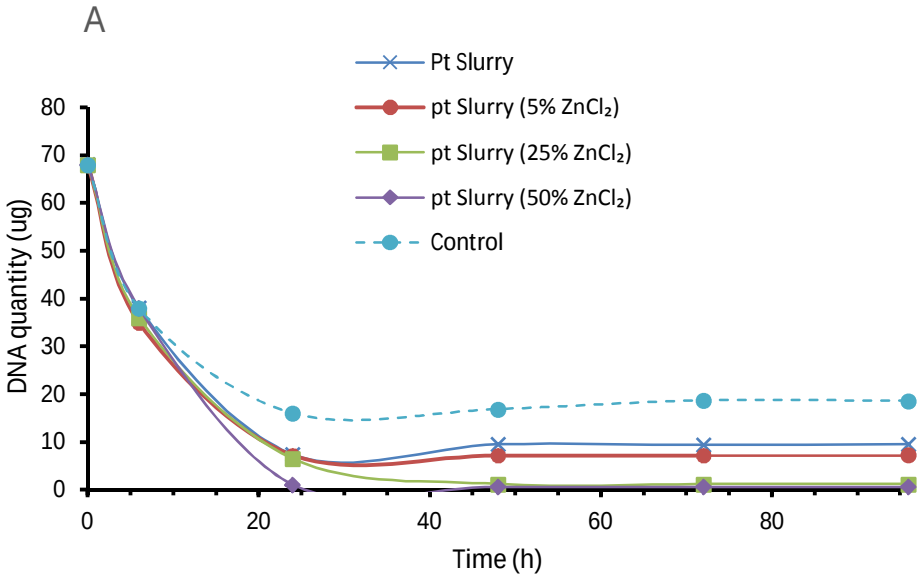
biocatalysis than other metal halides and solvent systems commonly used for pre-treatment e.g. ammonia (Collins, 2004). The effect of chaotropic chloride constituent of the fermentation media was evident in the observed limitation of the metabolic and overall activity of the microbial catalyst at increased salt concentration.

The pre-treatment slurry as a sole carbon source gave the optimum yield of BSA (0.02) in comparison to other runs at increasing salt concentration hence reiterating the effects of the increasing halophicity of the media on bioprocess. The effect of ionic media on the biomacromolecules of (a) microbial membrane, and (b) intracellular proteins and subsequent enzymatic activity were proposed to have reflected in the yield of SA in the fermentation system. In comparison to reported single-cell cultures of SA producing catalysts cultured on standard glucose, productivity was very low at 1.9 g g-1 of corn cob biomass (0.02 g g^{-1} of Y_g) in comparison to 1.2 g g^{-1} of glucose reported by studies such as McKinlay et al., (2010). Proven biosynthesis of BSA although, was a proof of conceptual design, with apparent potentials for optimisation upon identification of the active SA-producing biocatalyst in the reaction media.

Towards development of the microbial catalytic system, for the conversion of $ZnCl_2.4H_2O$ treated biomass to BSA in a large-scale process, the assessment of the active biocatalysts and characteristic selectivity in media specifically is imperative. DNA quantification and high-throughput microbial screening were used to understand the biocatalytic process.

The effect of concentration on the biological activity using different carbon sources as quantified in terms of DNA in the fermentation effluents supported the observed BSA and intermediate quantification results; as DNA reduced significantly with increasing salt concentration. Findings showed DNA reduced by 70 % in the first 24 h of fermentation

process, with both biomass and sugar carbon sources (Figure 6.3A-B). The detection of biology activity at highly saturated media was negligible (<2.5 µg/ml). At reduced MHS concentrations, DNA extracted varied significantly with difference of up to 10.6 µg/ml after 96h.Tolerance to inorganic media as illustrated in Figure 6.4B reduced significantly with increasing salt concentration. Reports from literature have long debated the role of electrostatic and physicochemical factors with several perspectives on scope supporting acidic to slightly acidic conditions as efficient conditions for ruminal fermentation. Unlike rumen bacteria, anaerobic halophilic (sourced from high salt environments e.g. sea water) bacterial, although characterised with low yield of organic acids, have seldom been used in SCFA fermentation but the metagenomic engineering of their mechanism of solvent tolerance could be engineered into the genome of proven SA-producing microbial cells towards improved MHS dissolved-sugar metabolism to final products.



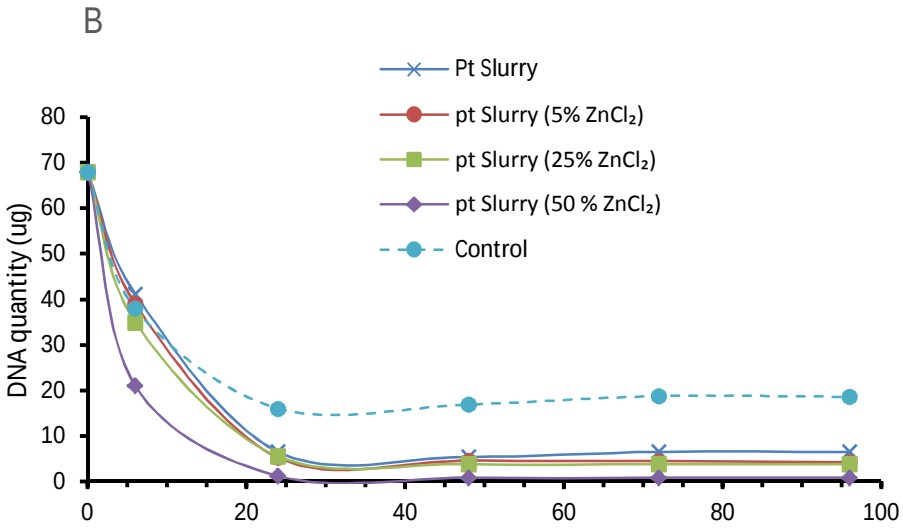


Figure 6. 3: Quantification of DNA extracted from fermentation process (anaerobic set-up, 50 ^{o}C) at varying $ZnCl_2$ salt concentrations using different carbon sources (A) corn cob (B) Glucose. (Pt implies pre-treatment)

6.3.2 Metagenomic characterization of cow dung inoculum

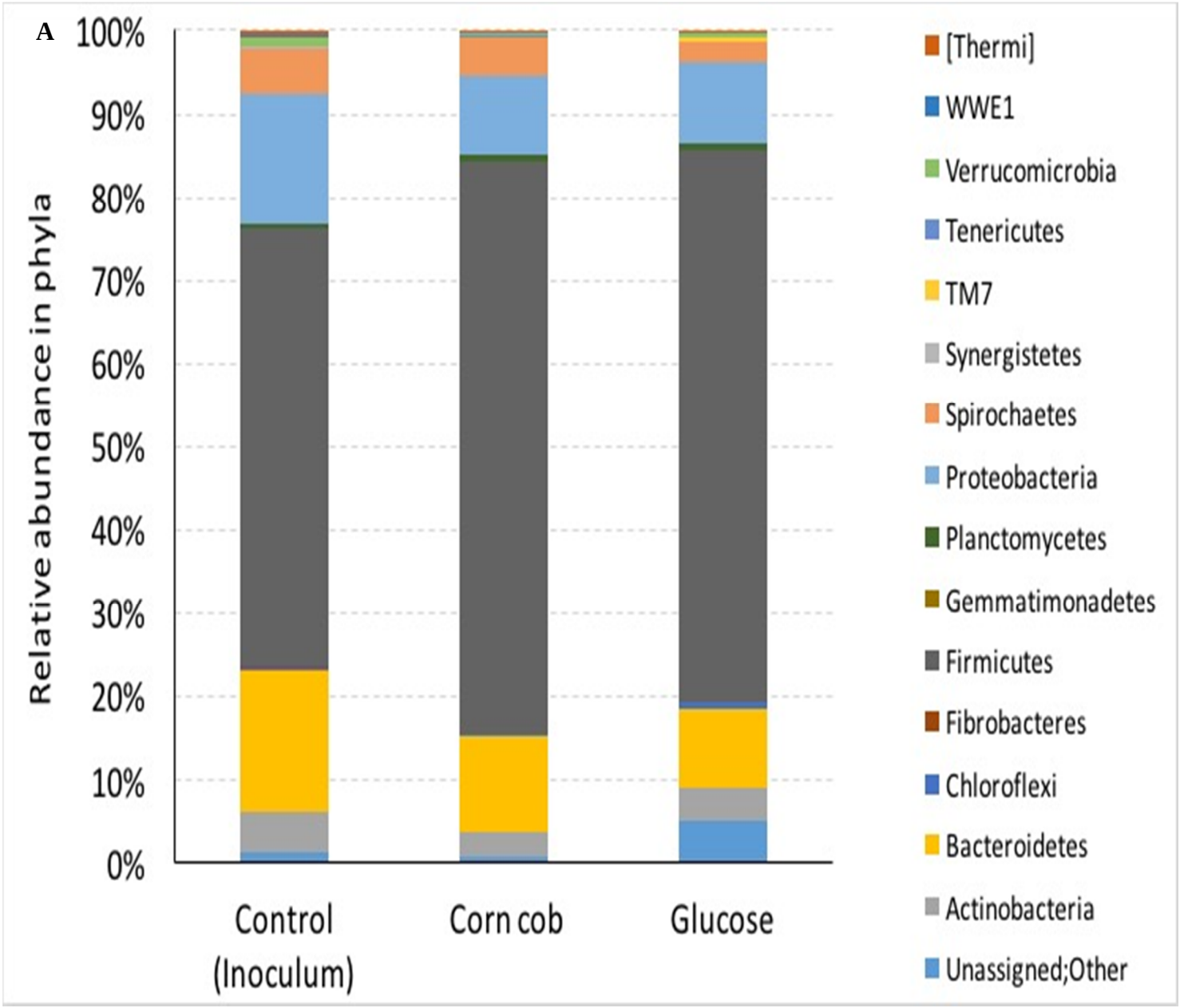
High-throughput microbial screening was achieved by means of next-generation sequencing (NGS) assessment of digester biodiversity. The results of the microbial diversity analysis of the inoculum and effluent of the most productive SA (run 3) was used as a basis for the evaluation of the biocompatibility of the substrate (pre-treated corn cob biomass in $ZnCl_2.4H_2O$). The constituting microbiota were observed based on their choice of carbon sources (glucose and corn cob biomass) in standard fermentation medium. Metagenomic sequence reads spanning the V5-V6 region of bacterial 16S rRNA gene were recovered from inoculum and process streams fed with both glucose and corn cob-MHS slurry. Upon trimming, high-quality reads of the sequences were processed by rarefactioning at a depth of 1500 reads per sample, corresponding to a total of 1765 operational taxonomic units (OTUs) in the process samples. The respective diversity indices in comparison to the

inocula are as shown in Table 6.4.

Table 6. 4: Diversity indices of OTUs in process samples.

	Inoculum	$ZnCl_2.4H_2O$ slurry	
Diversity Indices		Glucose	Corn cob
OTUs	676	535	554
Shannon index (H')	7.88	7.13	7.38
Simpson index (D')	86.86	34.69	59.96
Chao1	1939.71	784.03	1740.06

The inoculum was found to have the highest amount of OTUs while the process sample with a single source of organic carbon had the least. Likewise, the alpha diversity indices, showed that at the same salt concentration, the treatment with the corn cob slurry had higher Shannon and Simpson indexes than glucose feeds. This suggested the selectivity of substrate was contributive to the activity of biocatalysts in the slightly ionic media. The chao1 index is instrumental in the estimation of the total number of species present in the reactor microbiota and as observed the cob-$ZnCl_2.4H_2O$ slurry feed had a higher true OTU richness than for the standard glucose feed. OTU richness emphasizes sustained bioactivity in-media; which have been argued to be directly correlated to fermentability under ideal conditions (but not necessarily to specific bio-based product yield). Identification of the specific biocatalysts with potentials for sustained activity and selectivity for the substrates (slurry) are important towards proposition for use in product-specific biosystem.


A
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Relative abundance in phyla
Control (Inoculum)
Corn cob
Glucose
[Thermi]
WWE1
Verrucomicrobia
Tenericutes
TM7
Synergistetes
Spirochaetes
Proteobacteria
Planctomycetes
Gemmatimonadetes
Firmicutes
Fibrobacteres
Chloroflexi
Bacteroidetes
Actinobacteria
Unassigned;Other

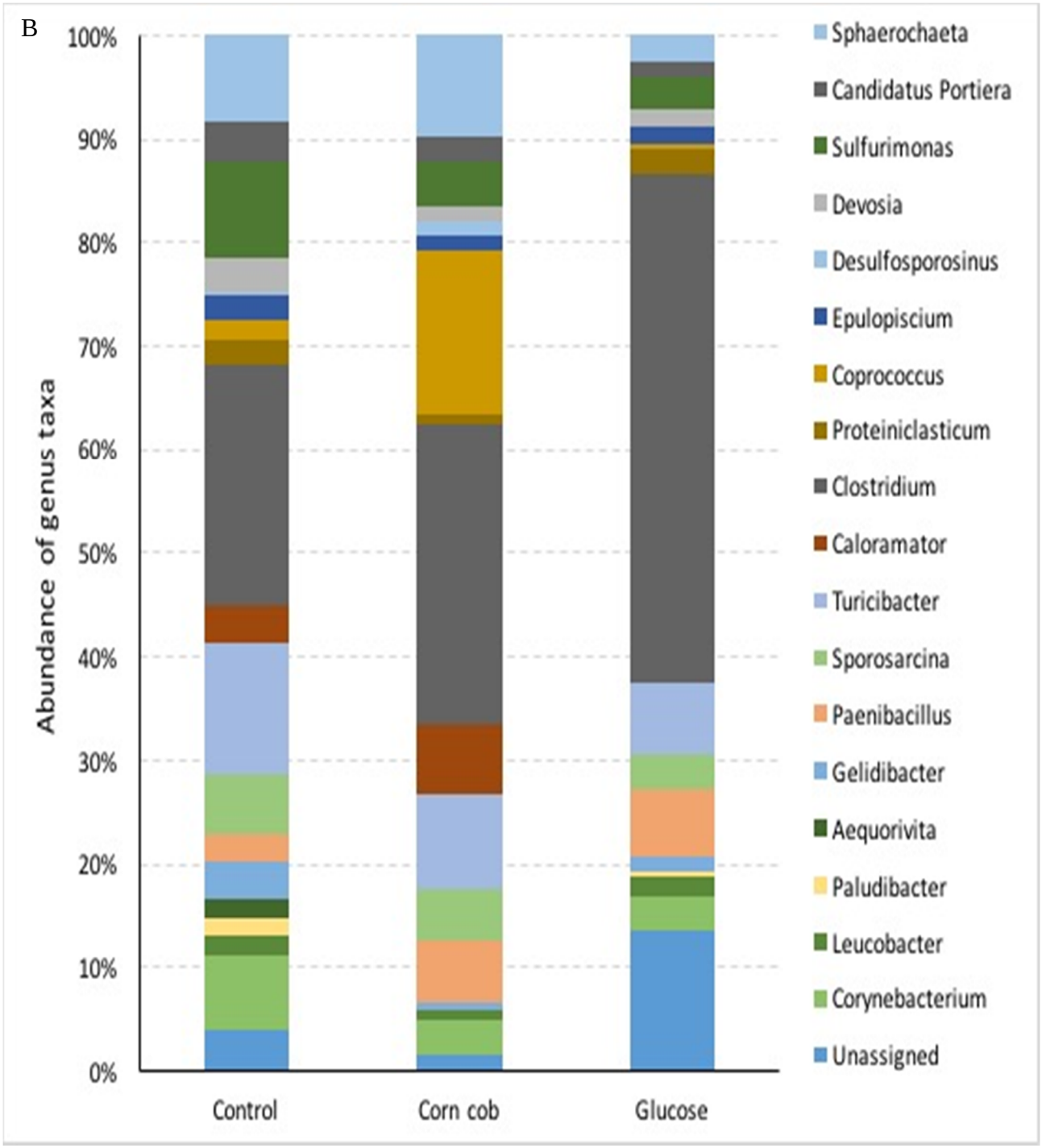
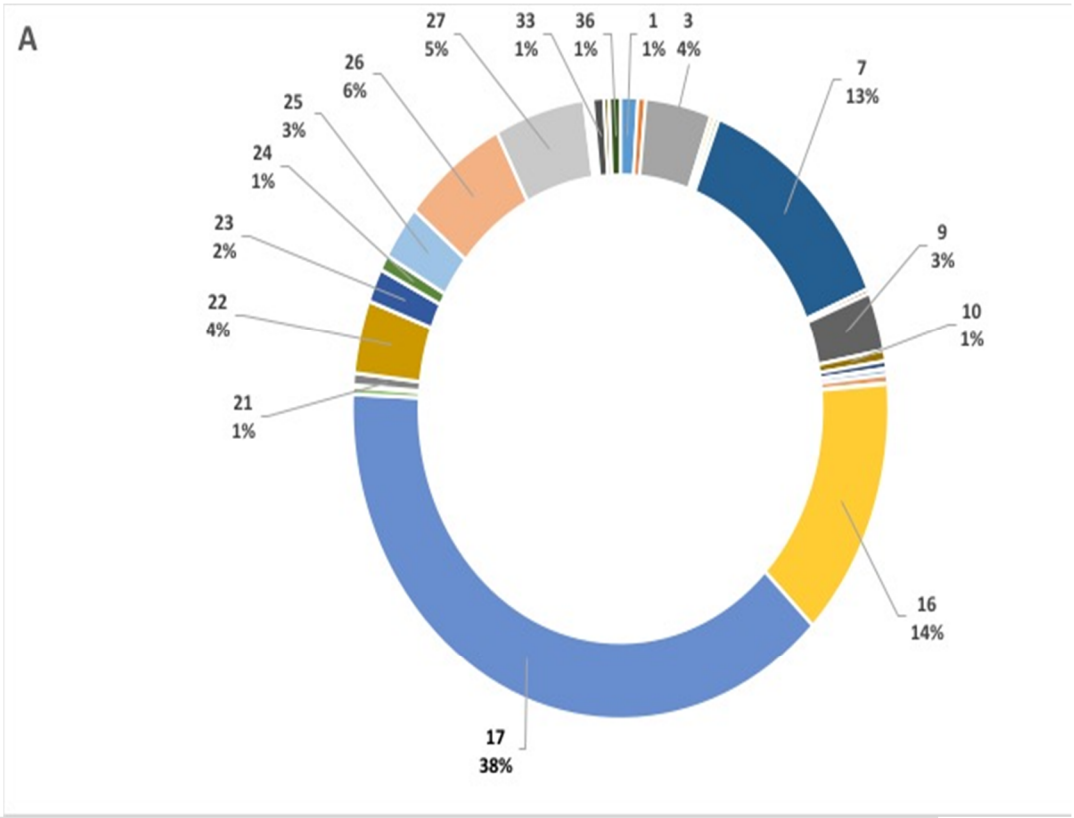
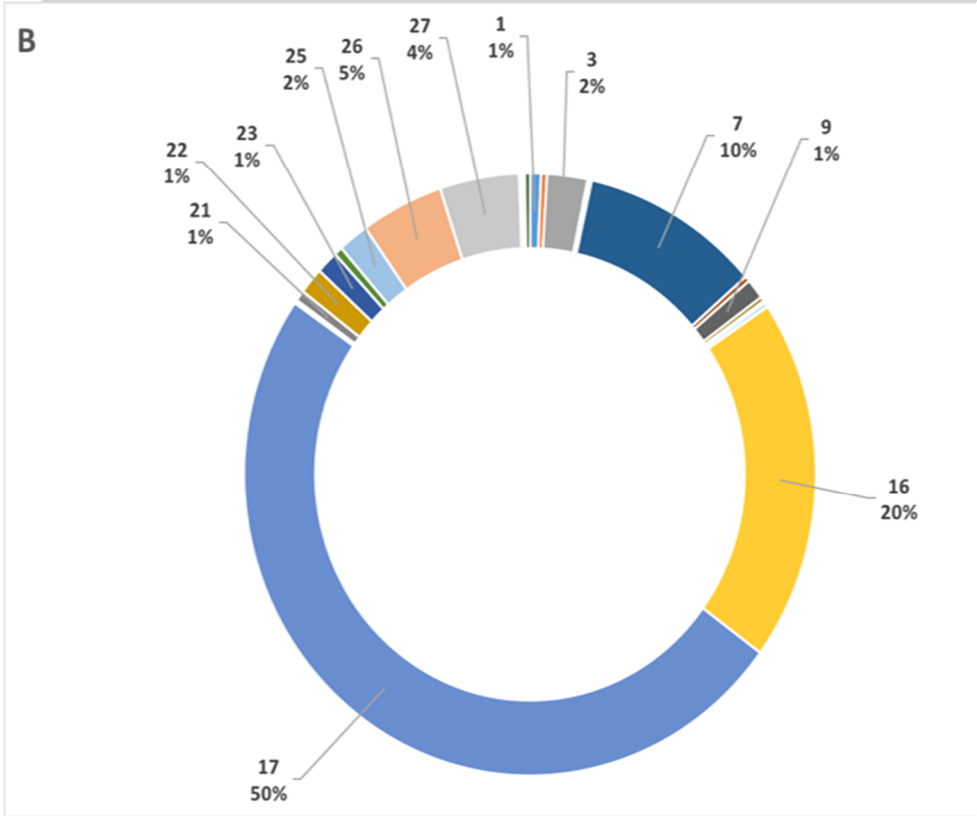


Figure 6. 4: Relative taxonomic abundance of A) phyla taxa B) genera taxa in inoculum with different carbon sources (unassigned refers to OTUs at phylum taxa level which was unclassifiable.)

Bacterial diversity assessment by means of OTU clustering analysis, taxonomically assigned the observed OTUs in all samples into 15 bacterial phyla, 43 classes, 80 orders and 266 genera. *Firmicutes* was the dominant phylum in the inoculum with increase in relative abundance regardless of carbon source. Other well represented phyla in the fermentation systems included; *Actinobacteria, Bacteriodetes, Proteobacteria and Plantomycetes.* The variation in salt concentration had a major effect on the abundance of the different phyla (except *Firmicutes*); grossly reducing their constitution or resulting in complete removal (e.g *Verrucomicroia).* Bacteria assigned to the phylum *Firmicutes* were found to be the most diverse and abundant group in the inoculum and process samples. The taxa class of *Firmicutes; Clostridia* (OTU 17) and *Bacilli* (OTU 16) comprises of 52 %, 66 % and 70 % of the microbiome in the control, glucose-feed and dissolved corn cob-feed sample process respectively (Figure 6.6). The *Firmicutes* phylum increased in the presence of the dissolved biomass and glucose, even though the phyla seem to slightly prefer the treated corn cob feed. This results suggested better metabolic affinity of the phylum to multi-sugar substrates than glucose. The observed effect of carbon sources was less distinct in comparison to salt concentration.


A
27
5%
33
1%
36
1%
1
1%
3
4%
26
6%
25
3%
24
1%
23
2%
22
4%
21
1%
7
13%
9
3%
10
1%
16
14%
17
38%


B
27
4%
1
1%
25
2%
26
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1%
16
20%
17
50%

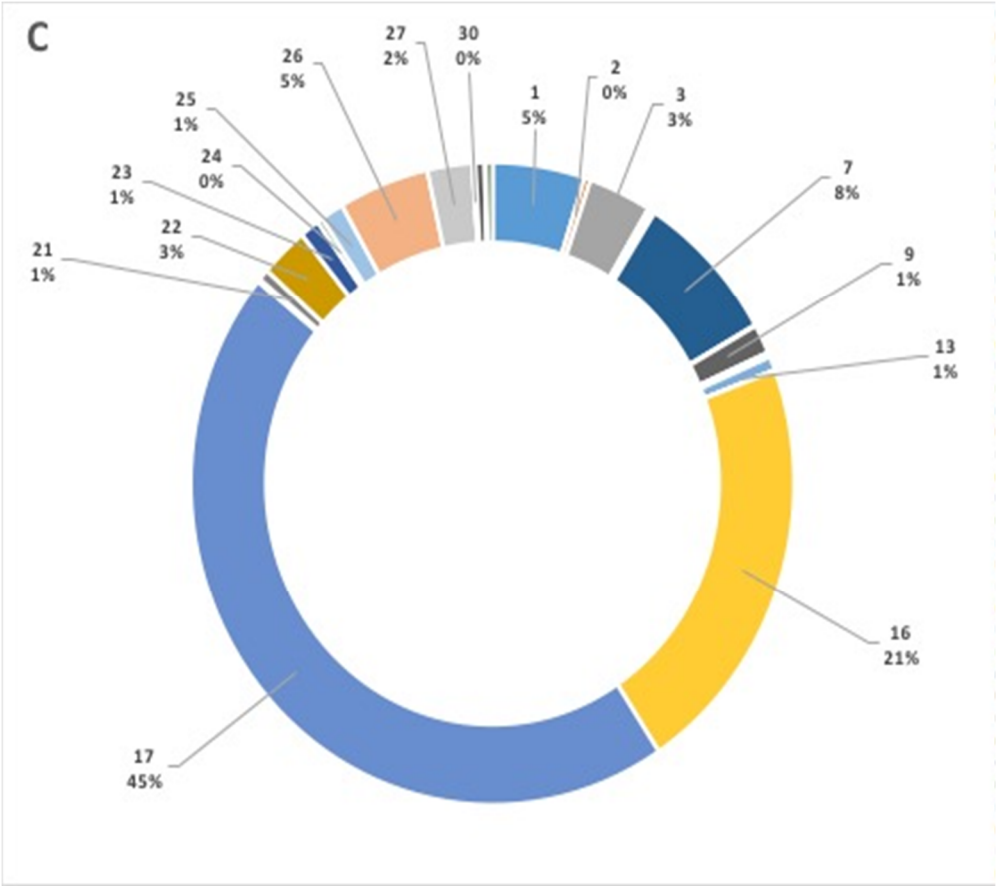


Figure 6. 5: Pie chart diagrams showing the OTU profiles of bacterial populations in A) Inoculum B) Process sample (Corn cob feed) C) Process sample (Glucose) The taxonomic assignments for specific OTUs (1-36) are shown in Table 6.5

Table 6. 5: Taxonomic assignment for select OTUs identified in anaerobic digesters.

OTU	Taxa (phylum-class)	OTU	Taxa (Phylum-class)
1	*Unassigned; Other*	19	*Planctomycetes-Phycisphaerae*
2	*Actinobacteria-Acidimicrobiia*	20	*Planctomycetes-Planctomycetia*
3	*Actinobacteria- Actinobacteria*	21	*Proteobacteria- Alphaproteobacteria*
4	*Actinobacteria- Coriobacteriia*	22	*Proteobacteria-Betaproteobacteria*
5	*Actinobacteria- Nitriliruptoria*	23	*Proteobacteria-Deltaproteobacteria*
6	*Actinobacteria-Thermoleophilia*	24	*Proteobacteria-Epsilonproteobacteria*
7	*Bacteroidetes-Bacteroidia*	25	*Proteobacteria-Gammaproteobacteria*
8	*Bacteroidetes-Cytophagia*	26	*Spirochaetes-Spirochaetes*
9	*Bacteroidetes-Flavobacteriia*	27	*Spirochaetes-Leptospirae]*
10	*Bacteroidetes-Sphingobacteriia*	28	*Synergistetes-Synergistia*
11	*Bacteroidetes-Saprospirae]*	29	*Planctomycetes-Phycisphaerae*
12	*Chloroflexi-Anaerolineae*	30	*Planctomycetes-Planctomycetia*
13	*Chloroflexi-Thermomicrobia*	31	*TM7-TM7-3*
14	*Fibrobacteres-Fibrobacteria*	32	*Tenericutes-RF3*
15	*Firmicutes-AHT28*	33	*Verrucomicrobia-Opitutae*
16	*Firmicutes-Bacilli*	34	*Verrucomicrobia-Verrucomicrobiae*
17	*Firmicutes-Clostridia*	35	*Verrucomicrobia-Spartobacteria]*
18	*Firmicutes-Erysipelotrichi*	36	*WWE1-Cloacamonae]*

The use of microbial species belonging to the most abundant classes of the *Firmicutes* phyla, have been widely explored in anaerobic fermentative processes with the most notable SA producers being *Clostridium thermosuccinogenes* (Sridhar et al., 2000). The detections of this wild strains in fermentation media were an highlight of the metagenomic studies due to the potentials of SA production at proposed pre-treatment conditions. *C. thermosuccinogenes* is a strictly anaerobic, thermophilic (up to 70 ^{o}C), spore-forming gram-positive bacterium which have been proven to ferment hexoses to succinate and acetate as major products and to lactate, ethanol, formate, hydrogen, and carbon dioxide as minor products (Drent et al., 1991). Fermentative studies of *C. thermosuccinogenes* have established the natural rechannelling of carbon metabolism from the acetate synthesizing pathways as acetate kinase activity reduces significantly with temperature (Sridhar and Eiteman, 2001). As such, SA is produced at higher temperatures of 65- 70 ^{o}C (Sridhar and Eiteman, 2001). Hence, synergistic SA fermentation systems as conceptualised in this study may find the biocatalyst applicable in an integrated system. In addition to SA production, *C. thermocellum* another member of the *Clostridium* genus, is a widely regarded high-utility catalyst, for use in consolidated biomass processing (CBP) applications (Akinosho et al., 2014). Similar to *C. thermosuccinogenes*, *C. thermocellum* is also an anaerobic, rod-shaped, gram-positive thermophile that has been shown to directly and efficiently saccharify cellulose and also capable of biosynthesizing ethanol (Kumar et al, 2008). The industrial relevance of this emerging biocatalyst was further reiterated by evidence provided in this work about the possibility of their metabolic dependence on zinc chloride-dissolved biomass polysaccharides. Hence, their potentials in bio-based fermentation, even though is not as explored in comparison to other natural producers of ethanol and BSA on a commercial scale, yet, do present an array of optimizable potentials and productivity.

Genera such as *Corynebacterium, Sulphurimonas, Candidatus poteiras, and Devosia* although characterised by reduced activity in the presence of both carbon sources could also find applicability in processing. Several *Corynebacterium* species are prominent in the production of organic acids with optimised activities in the presence of glucose (Blombach and Eikmanns, 2011). The stunted growth in diluted $ZnCl_2$ salt infers a high sensitivity to the ionic media, hence the observed decline in their relative abundance in media. Likewise, observed genera such as *Coprococcus* and *Sphaerochaeta* in the inoculum, showed a positive response in the salt media but only in the dissolved corn cob and not glucose. Choice of organic carbon played an important role in their sustained bioactivity, much like the *Proteinclasticum*, although in an inversed role. *Proteinclasticum* was observed to thrive better in the presence of glucose in comparison to dissolved biomass. Their preference for dissolved biomass at the process conditions and observed tolerance to salt concentration was as such understandable. *Paenibacillus* strains is another observed genus with potential applications in industrial process manufacturing especially in the production of saccharification enzymes such as cellulase, amylases and hemicellulases. The development of modern novel biocatalysts for optimal efficiency in inorganic media may require metabolic engineering, but direct evolution of microbial cells such as, as identified in this study can be explored for productivity and process modifications for techno-feasibility.

6.4 Summary

BSA biosynthesis was described in bio-chemo catalytic conversion of $ZnCl_2.4H_2O$ MHS-corn cob biomass pre-treatment slurry using a mixed-consortia of rumen bacteria. The wide range of biological catalysts as characteristic of the inoculum's microbiome provided insights for recommendations of single-cell culture, engineering and processing of corn cob biomass into the platform molecule in an efficient one-pot conversion system. Building on

previous knowledge of sugar liberation in inorganic hydrate salt media, this study investigated the fermentability of the sugar yield for SA acid production. BSA production by wild strains of naturally existing rumen bacteria in an anaerobic digester fed with pre-treatment slurry provided scientific basis for the proposed concept of an integrated refinery. In this study, 16S-rRNA genomic sequencing methods was used to establish the bacterial strains of potential catalysing agents and it was established that the *Clostridium* genus sustained activity and could as such find applicability in the proposed one-pot SA producing platform.

The fermentability of the substrate MHS-saccharose complexes is a profound and novel discovery which can be fundamental to the development of synergistic bio-chemo catalytic systems for lignocellulose biomass to targeted final products. In addition to a comprehensive microbial diversity analysis for the MHS solubilised carbon source the following highlights were proposed towards the development of a one-pot biorefinery media.

- BSA productivity, although relatively low (0.02 g g^{-1}) provided proof of the conceptual biocatalytic conversion of $ZnCl_2.4H_2O$ modified biomass to SA in an integrated process.
- Increase in the MHS concentration of fermentation media resulted in significant decline in collective bioactivity of the inoculating rumen microbiome, hence pre-treatment solvent loading have a pronounced effect on downstream bioprocessing.
- Cellulolytic and SA producing thermophiles (*C. thermocellum* and *C. thermosuccinogenes*) were central to SA yield, while *C. butyrium* and *C. propionicum* may have impacted SA yield.

Chapter 7

7.1 Conclusions

The development of a one-pot pre-treatment and bioconversion system have immense potentials for cost reduction in current biomass refining platforms. Process intensification as a strategy for further integration of biomass refining, although relatively unexplored may prove vital for a sustainable and feasible bio-based economy. Bio/chemo-catalytic set-ups for the simultaneous pre-treatment of low-cost lignocellulosic substrates and high-yield conversion of resulting sugar to valuable final products, have the potentials of reducing process costs; energy, material and infrastructure, without compromising on sustainability. The efficiency and viability of proposed intensified strategies although, is heavily reliant on the synergistic effect of chemical pre-treatment and biocatalytic conversion on bioproduction. It was as such imperative to obtain a yield-based assessment (intermediates; sugar monomers and final products; BSA) and empirical proof of the proposed concept; a bio/chemo-catalytic LCB processing unit. Using, an ionic, molten hydrate zinc chloride salt reaction media, locally-abundant corn cob wastes were used as substrates for the mixed-microbial consortia catalysed production of BSA; a platform chemical. The pre-treatment efficiency and biocompatibility of the reaction media at varying concentrations was investigated and directly correlated to bioactivity and yield of final products. In addition, process modulating factors such as feedstock quality; composition and physicochemical properties, were also investigated.

Findings established the potentials of $ZnCl_2.xH_2O$ (x=3-4) MHS reaction media as an efficnet platform for the deconstruction and digestibility of LCB and a selective biocompatible

medium, characterised by SA yield at lesser concentration. The following conclusions were deduced from the respective studies:

- *Lignocellulosic biomass substrate:* Low-cost and locally abundant corn cobs and husks are credible repositories of cellulose, hemicellulose and lignin; the three main components of LCB resources. Organic and inorganic composition of the agro-wastes were assessed and described towards their respective beneficiability in this thesis (Chapter 3). The cellulosic fraction is a stable, more refractory component (compared to hemicellulose) and more importantly, the source of C6 sugar monomer. The amount of inherent hexoses was quantified by means of acid hydrolysis as described in the standard NREL method, and approximately 447 mg of glucose was found per g of LCB. Glucose is a preferred carbon source for microbial catabolism and other forms of biocatalytic techniques for valuable end-products. Theoretically, one mole of glucose (C6) can be converted to 2 moles of succinate (C4), via a series of reaction which requires 2 moles of CO_2. As such, the obtainable amount of glucose from the agro-wastes accentuates the substrate credentials, in the context of microbial biosynthesis of BSA as targeted throughout this project. Implications of composition for calorific value was established in addition to numerous analytical techniques probing the holistic applicability of corn agro-wastes in a biorefinery platform. Findings were in agreement with earlier reported information for cobs in South Africa and other geographical region. Infrared spectroscopic results was interpreted towards defining the physicochemical property of the agro-wastes, especially crystallinity with the total crystallinity index (TCI) for the respective waste samples (cobs; CCW (0.97), CCR (0.96) and husks; CCH (0.97) samples) suggesting the recalcitrance of the materials to biocatalysis.

These findings represented the first objective of this work and provided a reference for subsequent yield analysis for pre-treatment, saccharification and fermentation steps.

- *MHS pre-treatment:* The recalcitrance of LCB materials to several forms of catalysis is a well reported phenomenon. Likewise, is the disadvantages of using conventional techniques involving highly corrosive solvents such as acids and alkali or/and energy intensive processes. Here, the potentials of a sustainable and cheap approach involving $ZnCl_2$ hydrate salt pre-treatment; by means of non-derivatising solvation of the cellulosic component of the biomass samples, at relatively mild conditions was explored. A large part of this research focussed on the efficiency of the solvent system in deconstructing corn cob LCB, towards improving its susceptibility to liberation of the inherent saccharosic composites. As such, solvent and process parameters such as effect of hydration and process variables, respectively were investigated towards a concise understanding of the effect of solvation on LCB. LCB pre-treatment were studied in batch set-ups at a temperature window of 70 – 110 ^{o}C. The work gave microscopic, spectroscopic and thermogravimetric proof of the effect of MHS on surface morphology, crystallinity and compositional of the cobs dissolved in $ZnCl_2.xH_2O$ MHS (where x = 3, 4, 5 and 7). Earlier reported observations for cellulose solvation in $ZnCl_2.xH_2O$ MHS were also established for multi-composite LCB material. Overall pre-treatment efficiency reduced with increasing water of crystallization; an observation which was in agreement with earlier documentation of the influence of hydration on the mechanism of complexation and solvation of cellulose in $ZnCl_2.xH_2O$ MHS solvent systems (Fischer et al, 1999). In order to elucidate the potential merit of biomass deconstruction as relatively mild conditions and compatibility for selectivity and physiological limitations of biocatalysts, pre-

treatment efficiency was described in relation to severity (CS) (Chapter 4). Pre-treatment efficiency was found to increase with increasing severity but yield of sugar was compromised by increased formation of degradation products; such as furfurals and organic acids, in medium. The occurrence of side-reactions in reaction media presents challenges with downstream fermentation as the amount of degradation products have been directly correlated with biocatalyst inhibition in process. As such, presenting major challenges for proposed integrated and simplified process, hence pre-treatment at mild conditions of 70 ^{o}C was central to a satisfactory process.

- *Enzymatic Saccharification:* The hydrolysis of residual sugar polymers in the pretreated biomass matrix is an important process step in biomass refining. Using cellulase *Trichoderma reesei* (74 FPU), the *4d-* enzymatic digestibility of $ZnCl_2.4H_2O$ treated cobs was studied in a controlled system. Liberation of glucose was significantly improved by $ZnCl_2$ MHS pre-treatment of corn cob biomass relative to the untreated corn cob biomass.

  The rate of hydrolysis of sugar yield in the pre-treated biomass mirrored the rate observed for standard cellulose and was in agreement with previously reported 1-HCH model (Holtzapple et al, 2005). Sugar yield (in both standard cellulose and pre-treated cob samples) peaked at approximately 85, 55 and 50 h time ranges for the 5, 10 and 20 %w/w concentration of cellulase enzyme. The washed MHS-treated LCB (at mild condition) displayed promising substrate potentials characterised by relatively high reaction velocities and high yield of residual sugars at the different enzyme loadings investigated, thus suggesting inhibition by residual lignin and degradation product was negligible. The effect of ionic media on the activity of the enzyme, was significant as established by reduced reaction velocity when the pre-treatment slurry were used directly as substrates. The inhibition parameter of the pre-

treatment slurry substrate was accentuated by the ionic media and as such yield of sugar monomers was reduced. Enzymatic yield of sugar is central to downstream fermentation process and in the context of this research and the proof of biodepolymerization in $ZnCl_2$ media concludes the second objective of this project.

- *Process Optimisation:* Having observed the effect of ionic media on the substrate parameters of the $ZnCl_2$ corn cob biomass in a bio-depolymerization (Chapter 4), it became paramount to optimize the yield of sugars from biomass slurry. Using Response surface methodology (RSM) statistical tool, based on a $_2$3 Box-Behnken design, this work described a second-order polynomial equation (Equation 5.1) relating the pre-treatment process variables; time, temperature and $ZnCl_2.4H_2O$ loading per gram of biomass to enzymatic sugar yield. Statistical tests (ANOVA (R^2= 93.7%) and regression analysis (f-value > p-value)) showed the model could be used adequately and reliably correlate sugar yield with pre-treatment conditions. Optimal yield of the response (approximately 84 % yield of reducing sugars) was derived by means of multi-objective numerical approach at conditions; 89.15 minutes, 107.27 ^oC and 11.96 g/g of $ZnCl_2.4H_2O$ with an overall desirability function (D = 0.988122). the values were cross-validated experimentally and the empirical proof (810 mg/g) was in agreement with the predicted value (830 mg/g).
- *Anaerobic fermentation:* Conventional downstream process configurations; SHF and SHcF, for bioconversion of biomass to final products is well optimised in terms of process control and yield but not for techno-feasibility and efficiency (Lynd, 1996). The characteristic challenges with process costs and carbon conversion are integral to the design (multi-unit) of the respective processes. Propositions for consolidation of the process units (pre-treatment, saccharification and fermentation) have resulted in integrated biorefining approaches such as SSF and CBP (see Figure 2.8). A one-pot

simultaneous chemical pre-treatment and bioconversion process is yet to be developed and as such was conceptualised and worked towards in this project. The development of a reaction media which effectively increases biomass susceptibility and also sustains biocatalytic activity was centred on the potentials of molten hydrate salts ($ZnCl_2.4H_2O$). Having demonstrated and optimised sugar yield by means of enzymatic saccharification of $ZnCl_2.4H_2O$ treated corn cobs (without conditioning steps), it became imperative to describe the selectivity of media for BSA producing biocatalytic activities. A mixed-consortia of rumen bacteria was employed in-process and results of specified bioactivity at different concentrations of the ionic $ZnCl_2.4H_2O$ MHS media was obtained by means of next-generation sequencing techniques. This study presented the scientific basis for a one-pot pre-treatment and biological conversion by proposing biological agents with proven activity for zinc chloride MHS slurry feed and a proven SA yield of 0.02 g g^{-1} of dissolved sugars. As expected the observed yield of BSA, although "a proof of concept", could not be directly translated to process efficiency because mixed-consortia inoculation meant the molecular channelling of carbon was uncontrolled; especially for dominant SA metabolism. Molecular characterization results of catalytic expressions in fermentation media suggested proven, thermophilic, SA producing microbes belonging to the *Clostridia* genus such as *C. thermocellum* and *C. thermosuccinogenes* may find applicability in an intensified bio-chemocatalytic biomass conversion process.

It is important to note that, although the simultaneous pre-treatment and bioconversion of LCB is as of this study presented, the fermentability of $ZnCl_2.4H_2O$ dissolved biomass polysaccharoses and observed affinity of the *Clostridia* and *Bacilli* class (for the MHS pre-treatment slurry) describes the bedrock of the proposed bio-chemocatalytic platforms for advanced industrial biocatalysis.

7.2 Recommendations for future work

Having described the potential of $ZnCl_2.4H_2O$ MHS for applicability as reaction media in the conversion of biomass to biocommodities and final products, it is important to note the required work for further scope development. Firstly, bacterial diversity assessment studies using rumen bacteria and other microbiomes (e.g. from saline sources such as sea water) at varying concentrations of MHS will build on current information and contribute towards the development of a robust microbiota base which will inform biocatalyst design strategies for the production of different intermediates in the proposed consolidated, bio-chemo catalytic fermentation systems.

In general, the optimisation of SA yield from waste corn cobs treated in $ZnCl_2.4H_2O$ hydrate media will continue to require interdisciplinary approaches tailored at the following scopes:

- *Catalyst engineering:* The bioengineering of natural SA producers with biological traits of halophilic strains with proven activity in inorganic $ZnCl_2$ hydrate salt media will be pursued towards the development of a one-pot biochemo-catalytic simultaneous pre-treatment and conversion platform for direct one-pot SA biosynthesis. The development of advanced industrial biocatalysts for pronounced and optimised activity is not rare in literature but application for SA production from low-cost lignocellulosic substrate in aqueous inorganic media is yet a fully developed concept. Bioinformatic frameworks for advanced bioengineering of whole-cells and cell-free biosynthetic approaches have been described in text with immense potentials for actualisation. Findings from study, in addition to available knowledge of the biological metabolisms and engineering techniques will be fundamental to the development of a novel innovative, low-cost system with immense potentials for large-scale applicability of this process concept.

- *Reactor design and modelling:* Efforts for biocatalysts immobilization, improved carbon throughput, mass transfer efficiency in a continuous process have been shown to result in improved BSA yield in conventional conversion processes. As such, bioreactor design strategies on a meso- and macro scale for the biochemo-catalytic system is central to scope development.
- *Product recovery:* SA is produced in media as Zinc (ii) succinate and as such might require recovery through a similar method to the dilute acid precipitation method described by Datta, et al, (1982). The addition of dilute acid may have an effect on the recyclability of the hydrate salts and reusability of the effluent catalysts. Other methods (e.g ion-exchange chromatography, membrane separation) need to be explored for product recovery in-media. (Datta et al., 1992)
- *Techno-economical assessment of process:* A comprehensive, standard, cost-benefit comparison of the economic quality of the proposed process, relative to conventional biomass conversion technologies is highly recommended prior upscaling.

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Appendix A: Copy of Publications from the Thesis

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REVIEW

Beneficiation of corncob and sugarcane bagasse for energy generation and materials development in Nigeria and South Africa: A short overview

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KEYWORDS

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Abstract The challenges of increasing energy demand and advanced materials for infrastructural development in developing countries have necessitated the search for sustainable sources of raw materials. The high amount of agricultural residues generated in Africa owing to vast availability of arable land has been an impetus for solving some of these challenges. Therefore, this review article provides information on beneficiation and challenges of the two largely generated agricultural residues, corncobs and sugarcane bagasse, in Nigeria and South Africa. The estimated quantities of corncob and sugarcane bagasse generated by these countries are reported. The potentials of beneficiating corncob and sugarcane bagasse in energy generation, in materials development and in other purposes such as production of platform chemicals are reviewed and discussed. Various technologies deployable in the beneficiation of these wastes are enumerated, and the benefits and challenges that are associated with beneficiating these wastes are briefly discussed.
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Research article

Biocompositional and thermodecompositional analysis of South African agro-waste corncob and husk towards production of biocommodities

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ABSTRACT: Biocompositional and thermodecompositional analyses of locally abundant agro-wastes of South African corn were evaluated for their potential utilization as biocommodities via thermochemical and bioconversion processing. Corncob of *Zea mays Zama star* (white) & *PAN 6Q-252* (yellow) hybrid as well as the ear husks of South African sweet corn, *Z. mays* var. Saccharata were utilized in the beneficiation process. Proximate and elemental analyses were conducted to check for the moisture content, the ash content, the volatile matter, and the elemental composition of the agro-waste samples. In addition, the thermal property of the waste, the surface chemistry and the calorific values were investigated using thermogravimetric analysis, Fourier-transform infrared (FTIR) spectroscopy and bomb calorimetric technique, respectively. Significant amounts of alkali metals (Li, Na, and K) were found in the corn cob samples, and heavy metals such as thorium and uranium were detected in the ear husks, indicating that the biomass might not be suitable for use in thermochemical process. However, a relatively high volatile matter content recorded for the waste samples supports their use in thermochemical process with improved yield per gram of feed. These and other findings such as the high calorific value of the cob samples and the relatively low lignin-to-sugar ratio of the waste confirm previous reports regarding their suitability as feedstocks in thermo-processing and bioprocessing. With the exception of the moisture content in ear husks, the minimal variation in the physicochemical property of the waste samples substantiates their suitability for co-processing without major upsets in process kinetics. Results of other investigations such as the thermodecompositional behavior of the waste samples and the surface chemistry further shed light on the physicochemical property of the locally sourced lignocellulosic biomass waste samples. © 2017 Curtin University and John Wiley & Sons, Ltd.

KEYWORDS: biocompositional analysis; thermodecomposition analysis; biocommodities; physicochemical property; lignocellulosic biomass

INTRODUCTION

Environmental challenges associated with the use of fossil materials for energy and commodities production have prompted a strategic shift towards the use of non-fossil materials such as biomass for green and sustainable processes.[1,2] In developing nations such as South Africa, the paradigm shift from the use of fossil materials to the use of non-fossil materials for energy and biocommodity production is instrumental to salvaging the currently stressed energy systems and revamping the declining manufacturing sector.

Biomass, a non-fossil material, is the most abundant source of organic carbon available for production of energy and biocommodities on earth.[2] In addition to abundance, the suitability of biomass as renewable energy resources of the future is centered on their (1) characteristic carbon neutrality, (2) noncompetition with food resources and related systems, and (3) feasibility of processing. As such, waste lignocellulosic biomass generated along the entire value-chain of common agro-products, such as maize, has amassed a lot of interest in academia and industry, as the ideal raw materials for the sustainable production of energy and biocommodities locally.

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Effect of water of crystallization on the dissolution efficiency of molten zinc chloride hydrate salts during the pre-treatment of corncob biomass

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Abstract

BACKGROUND: Agro-wastes albeit abundant and cheap, require pre-treatment steps tailored to overcome the characteristic recalcitrance of lignocellulosic biomass. In this study, zinc chloride hydrate salts were studied for their biomass deconstruction potential. The role of salts' hydration and to what degree it affects the dissolution efficiency was evaluated. Milled corn cobs from local farmlands were dissolved in the following solvent systems; $ZnCl_2.3H_2O$, $ZnCl_2.4H_2O$, $ZnCl_2.5H_2O$ and $ZnCl_2.7H_2O$ at 70 °C for 60 mins at a biomass to solvent ratio of 1:12.

RESULTS: The difference between the least and most hydrated solvent systems in terms of total released sugars as a percentage of recoverable sugar is 70.1%. The $ZnCl_2.4H_2O$ solvent system extracted up to 49.1% of glucose and 95% of xylose. There were significant differences in the crystallinity indices and surface morphologies between the untreated and treated biomass.

CONCLUSION: The findings as documented contribute to current insights on the mechanisms of disintegration of lignocellulosic biomass in molten hydrate salts. The extraction of sugar components of biomass by $ZnCl_2.4H_2O$ solvation was about 43% glucan and 99% xylan.
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Keywords: agro-waste beneficiation; biomass pre-treatment; molten hydrate salt

INTRODUCTION

Global dependence on fossil fuel sources for energy and commodity production has been associated with climate change and other common environmental challenges. A major holistic side-stepping approach involves the development of renewable energy technologies capable of competing with more conventional petrochemical energy sources.[1] Biomass processing by means of an integrated biorefining platform is one of such sustainable technologies currently being advanced globally.[2] The potential of bioenergy and biocommodities production are hinged on two major factors; the abundance of lignocellulosic biomass and the development (as with the continuous integration and standardization) of the biorefining scope.[3] Agro-industrial wastes such as corn cobs are important classes of bioenergy resources. In addition to their utilization as feed-stocks, they are also accepted for uses as energy resources in socio-economic spheres because they do not pose a direct threat to food security.[4,5] Agricultural wastes are generated in enormous amounts especially in agro-dependent nations of sub-Saharan Africa. In South Africa for example, waste biomass materials generated during the production of the most common agricultural products, maize and wheat, have been highlighted as significant resources capable of contributing to the advancement of sustainable industrial policy and socio-economic development agendas.[6]

Corn cob like several other agricultural waste products has been utilized as renewable feedstocks in the characteristic high-yield production of biofuels and chemicals.[7] Their applicability in the majority of these processes is often challenged by their characteristic recalcitrance to most forms of catalytic conversion. Lignocellulosic biomass in their natural state have highly organized and crystalline microstructures protected by lignin sheaths which make direct conversion slow and uneconomical.[8] Current practises in biomass processing and conversion generally involve thermochemical processes which are designed to

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Microbial cell immobilization in biohydrogen production: a short overview

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Dissolution of South African corncob in inorganic hydrate salts ($ZnCl_2.xH_2O$) towards efficient biocatalytic depolymerisation

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Abstract: A major challenge to agro-industrial waste management, beneficiation and applicability for productive processes is biomass recalcitrance. As a result, sustainable, cheap and effective pre-treatment techniques designed to make such waste biomass process-friendly (by enhancing their susceptibility to bio/chemical catalysis) have become an essential step in biomass conversion. Dissolution of lignocellulosic biomass in hydrated inorganic salt such as $ZnCl_2.3\text{-}4H_2O$ is a very promising technique with proven cellulose solvation in relatively mild process conditions. In this study, the dissolution of South African corncob in $ZnCl_2.xH_2O$ salts (x=3,4) is investigated. The dissolution mechanism and biocatalytic depolymerisation of this waste to the monomeric sugar are proposed as well. It was envisaged that the crystallinity of the sugar polymers, which constitute over 50% of the waste lignocellulosic biomass will be reduced upon dissolution in the hydrate salts solvating systems, thus an increase in biocatalysts' substrate-accessibility is expected. This relationship between efficient dissolution towards productive catalysis was shown via physico-chemical and gravimetric characterization of the dissolved polymers. The changes in the morphologies and crystallinity of the polysaccharides were also studied using the EDS-SEM and XRD analytical tools respectively. Using Raman spectroscopy (FT-IR), the mechanism of dissolution and the relationship between the metal cations of the hydrate $ZnCl_2.xH_2O$ (x=3,4) salts was shown. The outcome of this study would contribute to the design of sustainable sugar recovery systems from lignocellulose, aiding the integration of waste recycling platforms to the production of valuable biocommodities and energy.

Keywords: Agro-industrial waste, biomass pre-treatment, biocatalysis, depolymerisation, hydrate salts, lignocellulosic biomass, South African corn cob, waste beneficiation,

1) Introduction

Today, waste management and beneficiation both in-process and generally in the agro-industrial sector is a platform which is fast growing in importance. It is relevant in that it has the potentials to contribute immensely towards the complete development of an integrated biorefinery system; which is capable of producing biofuels, biocommodities (such as Xylitol, Lactic acid, Butanol) in a way that boosts the overall competitiveness of sustainable bioprocesses over their hazardous fossil alternatives.

South Africa produces millions of metric tonnes of agro-industrial waste annually a major percentage of which are corn cobs [1]. Corn cob is an abundant waste considering maize is a major staple in the republic and even though common practise encourages destruction by landfilling and burning, their role in building a sustainable green economy locally and globally cannot be overemphasized. Corn cob is a popular lignocellulosic biomass; a class of materials that have been widely acclaimed as promising feedstocks for sustainable processes due to their composition and abundance in nature. Lignocellulosic biomass consists majorly of three components; cellulose, hemicellulose and lignin, all of which are bond by a complex network of intra and inter hydrogen bonds in the cell wall to form a complex, rigid matrix that serves as a defence system against catalytic degradation of all forms in plants [2].

The necessity of bypassing the defence structures via pre-treatment of biomass have resulted in several thermal and solvating measures been designed and proven in the optimisation of their selective conversion to value-added products. The mechanism of pre-treatment employed in such processes been conceptualized hinges on its sustainable credentials for integration into a generally green biorefinery system and also an efficient defunctionalisation of the highly functionalized biopolymers. Balancing both strategies have posed several challenges for sustainable processes as