Alkalic salt-based pretreatment strategies for enhancing sugar recovery from corn cobs and process development for simultaneous saccharification and bioethanol production

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By

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MSc. Microbiology (*cum laude*)

Submitted in fulfilment of the academic requirements for the degree of

Doctor of Philosophy

In Microbiology

School of Life Sciences

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Pietermaritzburg Campus

South Africa

13 March 2018

PREFACE

The research contained in this dissertation/thesis was completed by **Yeshona Sewsynker-Sukai (210550313)** while based in the Discipline of Microbiology, School of Life Sciences of the College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Pietermaritzburg Campus, South Africa. The research was financially supported by the National Research Foundation (Grant number: 101316).

The contents of this work have not been submitted in any form to another university and, except where the work of others is acknowledged in the text, the results reported are due to investigations by the candidate.

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Signed: Professor Evariste Bosco Gueguim Kana

Date: 13 March 2018

DECLARATION 1: PLAGIARISM

I, **Yeshona Sewsynker-Sukai**, declare that:

(i) the research reported in this dissertation, except where otherwise indicated or acknowledged, is my original work;

(ii) this dissertation has not been submitted in full or in part for any degree or examination to any other university;

(iii) this dissertation does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons;

(iv) this dissertation does not contain other persons' writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:

a) their words have been re-written but the general information attributed to them has been referenced;

b) where their exact words have been used, their writing has been placed inside quotation marks, and referenced;

(v) where I have used material for which publications followed, I have indicated in detail my role in the work;

(vi) this dissertation is primarily a collection of material, prepared by myself, published as journal articles or presented as a poster and oral presentations at conferences. In some cases, additional material has been included;

(vii) this dissertation does not contain text, graphics or tables copied and pasted from the Internet, unless specifically acknowledged, and the source being detailed in the dissertation and in the References sections.

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Signed: Yeshona Sewsynker-Sukai

Date: 13 March 2018

DECLARATION 2: PUBLICATIONS AND MANUSCRIPTS

This thesis consists of three published papers and one manuscript (under review). The first author (student) contributed towards experimental work, data collection and manuscript preparation and was guided by Professor E.B. Gueguim Kana (supervisor).

1. Sewsynker-Sukai, Y., Gueguim Kana, E.B., 2017. Optimization of a novel sequential alkalic and metal salt pretreatment for enhanced delignification and enzymatic saccharification of corn cobs. *Bioresource Technology* 243, 785-792. (Chapter 3).

2. Sewsynker-Sukai, Y., Suinyuy, T.N., Gueguim Kana E.B., 2018. Development of a sequential alkalic salt and dilute acid pretreatment for enhanced sugar recovery from corn cobs. *Energy Conversion and Management* 160, 22-30. (Chapter 4).

3. Sewsynker-Sukai, Y., Gueguim Kana, E.B., 2018. Simultaneous saccharification and bioethanol production from corn cobs: Process optimization and kinetic studies. *Bioresource Technology* 262, 32-41. (Chapter 6).

4. Sewsynker-Sukai, Y., Moodley, P., Gueguim Kana, E.B. Progress in the development of alkalic and metal salt catalysed lignocellulosic pretreatment: Potential for bioethanol production. Submitted to *Energy Conversion and Management*. (Chapter 2).

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Signed: Yeshona Sewsynker-Sukai

Date: 13 March 2018

CONFERENCE CONTRIBUTIONS

1. Sewsynker-Sukai, Y. A two-stage alkalic salt and dilute acid pretreatment for enhanced enzymatic saccharification of corn cobs. 13th Biotechnology Congress. 28-30 November 2016. San Fransisco, United States of America. Oral Presentation.

2. Sewsynker-Sukai, Y. Optimization of a novel sequential alkalic and metal salt pretreatment for enhanced delignification and enzymatic saccharification of corn cobs. School of Life Science Research day. 23 May 2017. University Of KwaZulu-Natal (Westville campus), Durban, South Africa. Oral Presentation.

ABSTRACT

Fossil fuel depletion combined with environmental pollution from its combustion are stimulating the development of renewable and sustainable fuel carriers from lignocellulosic biomass. The identification of bottlenecks that limit industrial lignocellulosic bioethanol production with subsequent development of high-ethanol-performance processes is crucial for scale up. These include cost-effective lignocellulosic pretreatment regimes and fermentation processes that result in high fermentable sugar and ethanol yields. To achieve this, a review of literature on the development of alkalic and metal salt catalysed lignocellulosic pretreatments and their potential for bioethanol production was carried out. Then, two sequential alkalic salt-based pretreatment strategies for enhancing sugar recovery from corn cobs were developed and optimized using the Response Surface Methodology (RSM). These pretreatments were thereafter comparatively assessed on their potential suitability for microbial production of ethanol fuels and value-added products. Following the comparison of these pretreatments, simultaneous saccharification and fermentation (SSF) processes with prehydrolysis (PSSF) and without prehydrolysis (OSSF) were modelled and optimized. Subsequently, the kinetics of microbial cell growth and bioethanol production for optimized PSSF and OSSF processes were assessed under microaerophilic and anaerobic conditions using *Saccharomyces cerevisiae* BY4743.

Two lignocellulosic pretreatment techniques consisting of: (a) a sequential alkalic salt and metal salt (SAMS) and (b) a sequential alkalic salt and dilute acid (SASA) were modelled and optimized. The SAMS pretreatment inputs included alkalic salt concentration (5-15%), metal salt concentration (1-5%) and solid to liquid ratio (5-15%). For the SASA pretreatment, the process inputs consisted of alkalic salt concentration (5-15%), acid concentration (1-3%) and solid to liquid ratio (5-15%). The developed pretreatment models gave high coefficient of determination (R^2) values >0.90. The optimized SAMS pretreatment (14.02% $Na_3PO_4.12H_2O$, 3.65% $ZnCl_2$ and 5% solid to liquid ratio) gave a reducing sugar yield of 1.10 g/g compared to 0.99 g/g for the SASA pretreatment (12.70% $Na_3PO_4.12H_2O$, 1.04% H_2SO_4 and 14.49% solid to liquid ratio). These techniques gave higher reducing sugar yields (>8-fold) compared to previous corn cob pretreatment reports. Corn cob structural compositional analysis displayed comparable cellulose (59.98 and 58.89%), hemicellulose (28.33 and 29.01%) and lignin (2.30 and 2.77%) fractions for the SAMS and SASA

pretreatments, respectively. Similarly, the SAMS and SASA pretreatments gave high glucose (0.71 and 0.69 g/g) yields respectively, with low fermentation inhibitor concentrations (<1 g/L). Slight variations were observed between the SAMS and SASA experimental data and these were considered negligible. Although the SAMS pretreatment was shown to be effective for high reducing sugar production, the SASA pretreatment yielded a higher quantity of pretreated substrate (2.9-fold) with a lower alkalic salt concentration. Thus, the SASA pretreatment could potentially enhance the techno-economics of biofuel production processes such as bioethanol.

After comparing the sequential pretreatments, the SASA regime was selected for the RSM optimization of the SSF processes. The PSSF and OSSF inputs consisted of yeast titre (1-5 times the base level), solid loading (10-30%) and enzyme loading (10-30 FPU/g) with bioethanol concentration and bioethanol conversion as the model responses. Both the PSSF and OSSF RSM models gave R^2 values >0.90, thus indicating their significance. The optimized PSSF conditions (yeast titre of 2 times, 17.50% solid loading and enzyme loading of 30 FPU/g) gave a high bioethanol concentration (36.92±1.34 g/L) and bioethanol conversion (62.36±2.27%). Similarly, the optimized OSSF conditions (yeast titre of 1 time, 17.82% solid loading and enzyme loading of 30 FPU/g) resulted in a bioethanol concentration and bioethanol conversion of 35.04±0.170 g/L and 58.13±0.283%, respectively. Thus, negligible variations in the bioethanol concentration and conversion were observed between the PSSF and OSSF processes.

The logistic and modified Gompertz models were thereafter used to study the kinetics of microbial cell growth and bioethanol formation under microaerophilic and anaerobic process conditions. The kinetic data showed that *S. cerevisiae* growth in the $OSSF_{microaerophilic}$ process gave a higher maximum specific growth rate (μ_{max}) of 0.274 h^{-1} compared to 0.186 h^{-1} for the $PSSF_{anaerobic}$ process. The $PSSF_{microaerophilic}$ condition gave the highest potential maximum bioethanol concentration (P_m) of 42.24 g/L compared to 27.62 g/L for the $OSSF_{anaerobic}$ process. Experimental data from the kinetic study showed that the microaerophilic process conditions resulted in optimal cell growth and bioethanol concentration. This was further elucidated by the high P_m value and short process lag time (t_L) obtained for the $OSSF_{microaerophilic}$ (37.87 g/L) and $PSSF_{microaerophilic}$ (1.98 h) processes, respectively. Additionally, maximum bioethanol production rate ($r_{p,m}$) was shown to be highest for the

$PSSF_{anaerobic}$ (3.25 g/l/h) process and was attributed to metabolic shifts toward ethanol formation under anaerobic conditions.

The developed sequential alkalic salt-based pretreatment regimes significantly enhanced sugar recovery and demonstrated high efficiency for microbial production of fuels and high value commodities. These pretreatments could be considered as cost-effective alternatives to commonly used expensive treatment catalysts such as sodium hydroxide. Optimization of the SSF processes indicated that prehydrolysis stages do not significantly impact on the bioethanol concentration and conversion. This eliminates energy intensive prehydrolysis stages and helps improve the SSF process design for large scale bioethanol production. Furthermore, the kinetic study demonstrated that microaerophilic rather than anaerobic culture conditions enhanced *S. cerevisiae* cell growth and bioethanol production, thus circumventing costly anaerobic environments for industrial scale production processes.

Keywords: Corn cobs, Lignocellulosic pretreatment, Alkalic salt, Bioethanol production, Kinetic models, *Saccharomyces cerevisiae*

ACKNOWLEDGEMENTS

First and foremost, I would like to thank God Almighty for the courage, wisdom and strength that allowed me to successfully complete this research.

I would also like to extend my appreciation to a number of people who have contributed towards this research:

My husband and soulmate, Avinash, words cannot do justice in thanking you for everything that you have done. You are my pillar of strength and you encouraged me to pursue this PhD without hesitation. You epitomise what a perfect life partner should be.

My supervisor, Prof. Kana, thank you for your guidance and pushing me beyond all limits. You have moulded me into a strong, successful researcher and I will never forget all that I have learnt during this time.

Ms. Fowlds, without you, this research would never have been complete in the short period of time that it has and I cannot thank you enough for being so kind and constantly lending me chemicals and equipment. Celeste, you always assisted when I needed something even at short notice and I am grateful for all your efforts.

Prof. Coetzer, thank you for going out of your way and assisting me with lab equipment and apparatus. Your success and achievements inspire me as a female researcher and your demeanour is the ultimate definition of the word humble.

Prof. Schmidt, I enjoy picking your brain and your broad research knowledge never seizes to amaze me, thank you for always entertaining my constant inquiries. Dr. Hunter, I am thankful for all the academic advice and opportunities that you have allowed me during this time.

To my family, thank you for all the well wishes and support. You all will definitely be seeing a lot more of me now that this research has been completed.

Preshanthan and Daneal, I will always cherish our fun lunch dates on the grass and I will forever be a proud member of the prestigious ‘BT group’. To the rest of our Lab 117 research group: Zane, Funmi, Isaac, Reisha, Caitlyn, Mariam, Kimberley and Gabriel, I appreciate all the great times we shared.

I am also thankful to all my department colleagues and the administrative staff. Matt and Heather, you guys are ever so willing to help and I am grateful for all your assistance. Natalie, Tanya and Pat, thank you for being so wonderful and making our lives simpler.

Last but not least, I acknowledge the National Research Foundation (NRF) of South Africa (Grant number: 101316) for financially supporting this work. Opinions expressed and conclusions arrived at, are those of the author and are not necessarily attributed to the NRF.

This thesis is dedicated to all my loved ones in heaven:

"In life I loved you dearly

In death I love you still

In my heart you hold a place

No one else could ever fill."

-Author unknown

TABLE OF CONTENTS

Note: This thesis consists of a compilation of publications and manuscripts (under review) whereby each chapter is presented as an individual entity according to the respective journal's rules and regulations. Therefore, some repetition between chapters was unavoidable.

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CHAPTER 1
General Introduction

1. Background

1.1.The need for renewable and sustainable fuel sources

Rapid depletion of fossil fuel-derived sources combined with environmental pollution from its combustion has threatened global energy security (Aguilar-Reynosa et al., 2017). Crude oil reserves are the most exploited fossil fuels with the Middle East being the major global oil contributor (47.7%) that can only sustain about 50.6 years of global production (Figure 1) (BP, 2017). The exhaustion of these fossil fuels as well as its negative environmental impact has accelerated research towards renewable, sustainable and cost efficient energy alternatives.

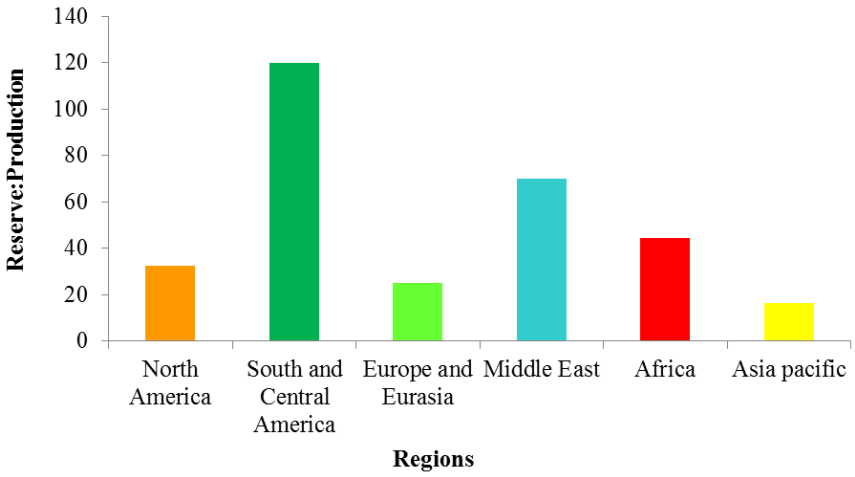

Figure 1. The regional oil reserves to production ratio for 2016 (BP, 2017) .

1.2.Bioethanol as a fuel alternative

Microbial biofuels such as hydrogen, methane, ethanol and biodiesel have shown to be valuable alternative energy sources (Naik et al., 2010). In the recent time, ethanol has received significant attention as a potential replacement fuel for gasoline (Aguilar-Reynosa et al., 2017). The advantages of ethanol over fossil fuels include its renewable and sustainable nature, ease of storage, higher oxygen content and higher octane number, among others (Putra et al., 2015). Large scale bioethanol production has been impeded by the lack of an

abundant and cost-effective feedstock for long term use. Lignocellulosic bioethanol production is emerging as a suitable replacement fuel to curb food security concerns (Aguilar-Reynosa et al., 2017; Zabed et al., 2016). Various countries around the world including the United States of America (USA), Brazil, China, Canada and several European Union (EU) member states have indicated their allegiance to bioethanol development programs in an attempt to lessen the dependence on conventional fossil fuels (RFA, 2016). Their contributions are depicted by the gradual increase in the annual bioethanol production from the year 2007 to 2016 as shown in Figure 2. In the same vein, African countries such as South Africa have committed themselves to strategic greenhouse gas mitigation actions that will result in a 42% reduction below its emission growth trajectory by the year 2025. South Africa has also displayed renewed interest in the development and improvement of the renewable energy market (DoE, 2015).

Figure 2. Global ethanol production by country from 2007 to 2016 (RFA, 2016).

1.3.Lignocellulosic biomass for bioethanol production

Globally, lignocellulosic biomass is produced at approximately 200 billion ton/year, whereby 8 to 20 billion tons can be used for biofuel production (Saini et al., 2014). Agricultural waste residues are mainly derived from corn, sugarcane, rice and wheat. Currently, several potential biofuel lignocellulosic feedstocks are being examined and these include sugarcane bagasse (Ramadoss and Muthukumar, 2015), corn stover (Liu et al., 2009) and corn cobs (Mao et al., 2012), among others. Corn production exceeds 1.03 billion metric tons annually, about 50%

of which makes up the leaves, stalks, husks and cobs that are usually disposed as wastes (USDA, 2017). Corn cobs consist of 32-45% cellulose, 39% hemicellulose and 6-14% lignin (Foley, 1978). It has a relatively high energy density that is between 4960-5210 MJ/kg and is approximately two-fold higher than other lignocellulosic substrates such as corn stover (2550 MJ/kg) and switchgrass (2500 MJ/kg). Furthermore, corn cobs has a low lignin content compared to corn stover and switchgrass , which makes it a superior competitor for microbial biofuel production processes (Potumarthi et al., 2012). Nevertheless, the major drawback of using lignocellulosic material such as corn cobs is attributed to their resistant structure that prevent enzymatic attack of the glucose rich polymer cellulose. Biomass pretreatment techniques are used to degrade recalcitrant lignocellulosic structures for the improvement of both enzymatic and microbial accessibility as illustrated in Figure 3.

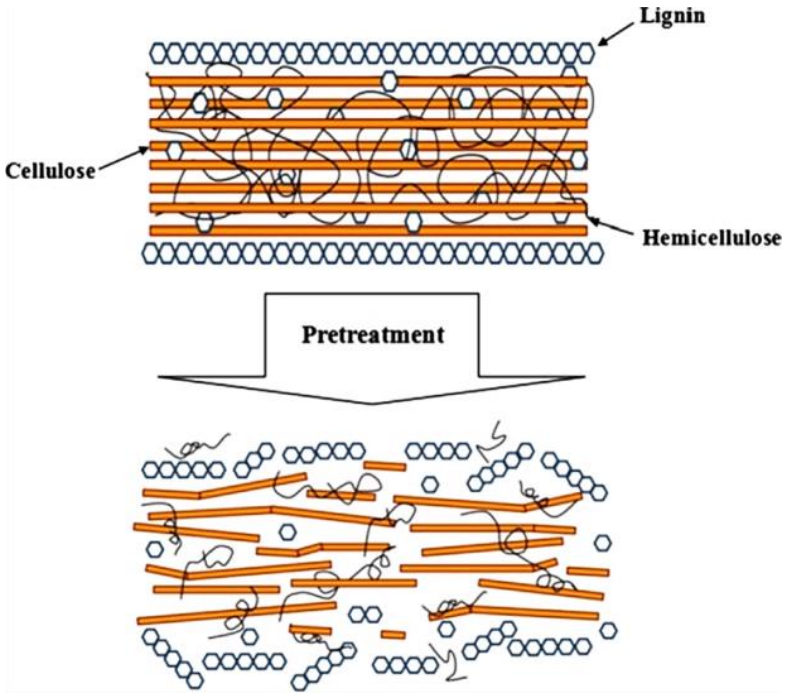

Figure 3. The effect of pretreatment on lignocellulosic material (Adapted from Mood et al., 2013).

1.4.Current lignocellulosic biomass pretreatments and their limitations

Several lignocellulosic pretreatment strategies have previously been investigated and these include acid, alkaline, ionic liquid and organosolvent, among others (Aguilar-Reynosa et al., 2017). However, these have been plagued with very high cost and energy demand. Acid hydrolysis is often utilized at toxic concentrations and has shown to result in the corrosion of

reactors or may require costly specialised equipment. In addition, acid pretreatments produce a high level of fermentation inhibitor compounds that are detrimental to enzymatic saccharification and fermentation processes. On the other hand, the main disadvantage of alkaline pretreatment is the high cost. Recently, the development of novel pretreatment regimes that are energy efficient, environmental friendly, cost-effective and produce high sugar yield has become a prime focus.

Pretreatment regimes with inorganic salts are attracting significant attention due to their low-cost, environmentally benign nature and reusability compared to inorganic acids (Liu et al., 2009). Inorganic salts may be grouped as either alkalic salts (Qing et al., 2016a) or metal salts (Li et al., 2009). Generally, the reaction mechanisms of alkalic salts and metal salts differ and may have variable impacts on the chemical composition and biomass structure (Yu et al., 2011). Alkalic salts act as weak bases and have been described as effective replacement catalysts for expensive alkali-based pretreatments such as NaOH (Qing et al., 2016a). Examples of some alkalic salts include $Na_3PO_4.12H_2O$, Na_2S and $Na_2CO_3 \cdot 1.5H_2O_2$ (Qing et al., 2016a; Qing et al., 2016b; Nakashima et al., 2016; Kim et al., 2014). Alkalic salts have shown to result in the dissolution of lignin and hemicellulose structures, de-esterification of intermolecular ester bonds (Kim et al., 2016), rearrangement and alteration of lignin and modification of the crystalline state of cellulose (Geng et al., 2014). On the other hand, metal salts lead to the formation of metal cations that function as Lewis acids in aqueous state and cleave glycosidic bonds present within the lignocellulosic structures (Loow et al., 2015; Kamireddy et al., 2013). Metal salts include NaCl, KCl, $CaCl_2$, $MgCl_2$, $FeCl_2$, $FeSO_4$, $FeCl_3$, and $Fe_2(SO_4)_3$, among others.

Alkalic and metal salt pretreatments have garnered significant attention as effective treatment catalysts but have been limited to very few reports that have assessed their efficiency individually or combined with other chemicals in single stage systems (Qing et al., 2016a; Qing et al., 2016b; Kamireddy et al., 2013). Combined salt and acid pretreatments have raised concerns about double replacement reactions, which render the chemical pretreatment inefficient (Helmenstine, 2016). Double replacement reactions or salt metathesis involves a biomolecular process in which chemical molecules containing counter ions are interchanged. H_2SO_4 and $FeCl_2$ are the most commonly used inorganic salt combined with acid pretreatment. These chemical species (H_2SO_4 and $FeCl_2$) react to form HCl and $FeSO_4$ in the presence of water, which implies that HCl causes the net chemical pretreatment effect

(IUPAC, 1997). Other limitations of common pretreatment catalysts such as acids include the partial degradation of the lignocellulosic matrix, low sugar recovery, high cost and energy related issues. An additional major challenge encountered during lignocellulosic biomass pretreatment is the formation of fermentation inhibitor compounds such as acetic acid, furfural and 5-Hydroxymethyl furfural (HMF). Fermentation inhibitor compounds have negative influence on enzymatic saccharification as well as on the microbial fermentation process (Harmsen et al., 2010). These inhibitor compounds released from lignocellulosic pretreatments have shown to: (1) result in intracellular anion accumulation causing a lower cell pH, which inhibits microbial cell activity, and (2) cause damage to the cell membranes and negatively impacts on the microbial cell activity, growth and sugar assimilation (Harmsen et al., 2010).

1.5. Bioethanol production and bioprocess kinetic studies

Bioethanol can be produced using three major processes, each with its own advantages and drawbacks: (1) separate hydrolysis and fermentation (SHF), (2) simultaneous saccharification and fermentation (SSF) without prehydrolysis (OSSF) and, (3) prehydrolysis followed by simultaneous saccharification and fermentation (PSSF) (Carrillo-Nieves et al., 2017). From the aforementioned process types, simultaneous saccharification and fermentation without prehydrolysis (OSSF) are being investigated as effective operational strategies to reduce the production costs, increase ethanol concentration and ethanol conversion with shorter times due to the elimination of separate, long saccharification steps. OSSF processes are performed in a single reactor with the same working temperature and the glucose produced is simultaneously metabolized by the bioethanol producing microorganism. Moreover, carbohydrate feedback inhibitory effects caused by high glucose yields during the enzymatic hydrolysis step are significantly reduced (Koppram et al., 2013). Previous studies have indicated that SSF processes are influenced by several input parameters that include solid loading, enzyme loading and yeast titre (Aguilar-Reynosa et al., 2017; Zhao et al., 2015; Zhu et al., 2015). Zhu et al. (2015) recorded a 38% higher bioethanol concentration when the solid loading was increased from 15 to 25%. Similarly, a 9% enhancement in the bioethanol conversion was observed by Aguilar-Reynosa et al. (2017) using a solid loading of 10% compared to 12.5%. Likewise, Zhao et al. (2015) achieved an 18% higher ethanol yield when

the yeast titre was raised from 1 time (8.0×10^7 cells/mL) to four times (3.2×10^8 cells/mL) the base level.

Additionally, kinetic modelling is considered fundamental for bioprocess scale up. The kinetic models define the production process under different conditions, which can improve the product yield, productivity and reduce undesirable by-products thus, reducing costs and increasing product quality. Some kinetic models that have previously been used for bioethanol processes include the logistic and modified Gompertz models (Phukoetphim et al., 2017; Dodic et al., 2012; Yan et al., 2013). Logistic models describe the changes in microbial cell growth as a function of growth rate, initial and maximum biomass concentration, and time (Phukoetphim et al., 2017) whereas the modified Gompertz model determines production lag time, maximum production rate, and maximum product concentration on a given substrate (Dodic et al., 2012).

2. Problem statement

Dwindling fossil fuels are stimulating the development of renewable and sustainable fuel carriers such as lignocellulosic bioethanol production. However, industrial scale lignocellulosic bioethanol production has been impeded by ineffective pretreatment regimes and fermentation processes resulting in low concentration of fermentable sugar and ethanol (Aguilar-Reynosa et al., 2017; Zhao et al., 2015). The major drawbacks of current acid and inorganic salt lignocellulosic pretreatments may include salt metathesis, low fermentable sugar yields, high inhibitor concentrations and high cost. Therefore, the development of sequential pretreatments, which incorporate alkalic salt with metal salt or dilute acid solutions that: (1) release low inhibitor concentrations, (2) generate high fermentable sugar yields and (3) are cost-effective has gained renewed interest.

On the other hand, ethanol production from SSF processes can be enhanced by optimizing the key input parameters. There has been a dearth of knowledge on the individual and interactive effects of yeast titre, solid loading and enzyme loading on the bioethanol concentration and bioethanol conversion in SSF processes. Likewise, there is a lack of consensus on the effect of prehydrolysis stages consisting in SSF processes (Zhu et al., 2015; He et al., 2016). Prehydrolysis steps improve the ethanol concentration and conversion but incur additional process time and energy input, which reduces its economic feasibility at large scale (He et al.,

2016; Zhu et al., 2015; Liu et al., 2014). Combining the enzymatic hydrolysis and fermentation steps reduces the capital investment by more than 20% (Wingren et al., 2003). Therefore, modelling and optimization of SSF processes with and without prehydrolysis stages on inputs of yeast titre, solid loading and enzyme loading are necessary to enhance the bioethanol concentration and conversion.

Furthermore, a knowledge gap exists on the kinetics of *Saccharomyces cerevisiae* cell growth and ethanol formation under microaerophilic and anaerobic conditions in SSF processes. Aerobic and microaerophilic process conditions promote *S. cerevisiae* cell growth whereas anaerobic environments enhance bioethanol formation (Lin et al., 2012). However, a high cost is associated with maintaining anaerobic conditions at large scale thus decreasing its economic viability (Podkaminer et al., 2012; Azhar et al., 2017). Kinetic knowledge on *S. cerevisiae* cell growth and bioethanol production under microaerophilic and anaerobic process conditions could significantly influence the bioethanol process design for large scale application.

3. Aims and objectives

This research aimed to develop efficient sequential alkalic salt-based pretreatment regimes for enhanced sugar recovery from corn cobs. Additionally, bioethanol production using simultaneous saccharification and fermentation strategies on the pretreated corn cobs were modelled and optimized. Furthermore, kinetic studies on microbial cell growth and bioethanol production in microaerophilic and anaerobic environments on the optimized SSF processes were investigated using *Saccharomyces cerevisiae* BY4743.

In order to achieve this aim, the following specific objectives were undertaken:

(i) A literature review on the development of alkalic and metal salt catalysed lignocellulosic pretreatments and their potential for bioethanol production.

(ii) The development of two different sequential alkalic salt-based lignocellulosic pretreatments consisting of: (1) a sequential alkalic salt and metal salt (SAMS) and (2) a sequential alkalic salt and dilute acid (SASA) for enhanced sugar recovery from corn cobs.

(iii) Comparisons of the SAMS and SASA pretreatments on their suitability for microbial production of ethanol fuels and value-added products.

(iv) Modelling and optimization of simultaneous saccharification and fermentation (SSF) processes with prehydrolysis (PSSF) and without prehydrolysis (OSSF) for maximum bioethanol concentration and bioethanol conversion.

(v) Then, the kinetics of microbial cell growth and bioethanol production for the optimized PSSF and OSSF processes were assessed under microaerophilic and anaerobic conditions.

4. Outline of thesis structure

This thesis includes seven chapters and conforms to the "research paper format" as outlined in the thesis template by the College of Agriculture, Engineering and Science (AES) of the University of KwaZulu-Natal, South Africa.

Chapter 1 provides the basis of this research and states the aims and objectives.

Chapter 2 reviews the available literature on alkalic and metal salt catalysed lignocellulosic pretreatments and the potential for bioethanol production.

Chapter 3 and Chapter 4 describe the development of: (1) a sequential alkalic salt and metal salt (SAMS) pretreatment and (2) a sequential alkalic salt and dilute acid (SASA) pretreatment for enhanced sugar recovery from corn cobs. Input parameters that were considered for the SAMS pretreatment included alkalic salt concentration, metal salt concentration and solid to liquid ratio. The SASA pretreatment inputs consisted of alkalic salt concentration, acid concentration and solid to liquid ratio.

Chapter 5 comparatively evaluates the previously developed SAMS and SASA pretreatment types on their suitability for microbial production of ethanol fuels and value-added products.

Chapter 6 models and optimizes the simultaneous saccharification and fermentation (SSF) processes with (PSSF) and without prehydrolysis (OSSF) using the SASA pretreated corn

cobs. Input parameters that were considered for the PSSF and OSSF processes included yeast titre, solid loading and enzyme loading with the bioethanol concentration and bioethanol conversion as the responses. Subsequently, the logistic and modified Gompertz models were used to assess the kinetics of microbial cell growth and bioethanol production on the optimized PSSF and OSSF processes under microaerophilic and anaerobic environments.

Chapter 7 states major conclusions derived from this study and provides recommendations for future research.

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CHAPTER 2

Progress in the development of alkalic and metal salt catalysed lignocellulosic pretreatment: Potential for bioethanol production

This chapter has been submitted to *Energy Conversion and Management* with the title: Progress in the development of alkalic and metal salt catalysed lignocellulosic pretreatment: Potential for bioethanol production.

The manuscript is presented in the following pages.

Progress in the development of alkalic and metal salt catalysed lignocellulosic pretreatment: Potential for bioethanol production

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Abstract

Lignocellulosic biomass is well suited to address present day energy and environmental concerns since it is an abundant, environmentally benign and sustainable feedstock. However, its commercial application has been limited by its recalcitrant structure. To date, several biomass pretreatment systems have been developed to address this major bottleneck but they are toxic and costly. Alkalic and metal salt pretreatment regimes have emerged as promising non-toxic and low-cost treatments. This paper examines the progress made in lignocellulosic biomass pretreatment with alkalic and metal salts. The alkalic and metal salt reaction mechanism and their effect on lignin removal, hemicellulose solubilization, cellulose crystallinity, physical structural changes, inhibitor profiles and enzymatic digestibility are discussed. Additionally, the potential of salt pretreatment for bioethanol production is evaluated with a focus on ethanol process type and kinetics. Furthermore, the challenges and future prospects on lignocellulosic pretreatment and bioethanol production are highlighted.

Keywords: Alkalic salt, Metal salt, Pretreatment, Lignocellulosic biomass, Bioethanol

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1. Introduction

Rapid depletion of fossil fuels coupled with its negative environmental effects has driven research towards renewable and sustainable fuel sources such as bioethanol (Qing et al., 2016a). Lignocellulosic biomass has shown to be an excellent feedstock for bioethanol production processes due to its abundance, renewable-nature and cost-effectiveness. Its fractional components consist of 30-50% cellulose, 20-40% hemicellulose and 10-30% lignin (McKendry, 2002; Binod and Pandey, 2015; Zamani, 2015). Lignocellulosic waste material includes sugarcane leaf wastes (Moodley and Gueguim Kana, 2015), corn stover (Qing et al., 2016a), corn cobs (Guo et al., 2016), bamboo shoot shell (Qing et al., 2016b), sorghum leaf wastes (Rorke and Gueguim Kana, 2017) and rice straw (Lü and Zhou, 2011), among several others.

Despite its advantages, lignocellulosic waste poses numerous challenges at a large scale owing to its complex and recalcitrant nature. Biofuel producing microorganisms cannot directly metabolize lignocellulosic biomass since the lignin layer makes the glucose rich cellulose polymer inaccessible. Commonly used species such as *Saccharomyces cerevisiae* are only able to convert simple carbohydrates such as glucose to bioethanol and are unable to utilize xylose (Rorke and Gueguim Kana, 2017). Few microbial strains such as *Pichia stipitis*, *Candida shehatae*, and *Fusarium oxysporum* metabolize xylose (Sánchez et al., 2002; Paschos et al., 2015) but are still unable to degrade resistant lignocellulosic structures. Consequently, the use of lignocellulosic waste for bioethanol production requires effective chemical pretreatment systems that will disrupt the resistant structures. These pretreatment regimes will improve enzymatic saccharification, thus yielding high fermentable sugar for microbial cell growth and bioethanol production (Kang et al., 2013).

A number of pretreatment techniques have been investigated and include acid, alkaline, microwave, ionic liquid, organosolvent, thermal and inorganic salts, among many others (Aguilar-Reynosa et al., 2017). These reported pretreatment techniques are challenged by high cost, toxicity and energy demand. Therefore, recent efforts focus on alternative pretreatment strategies with the aim of improving process cost, toxicity and energy reduction. Compared with other chemical pretreatments, inorganic salts have only recently been reported as an effective pretreatment strategy. Inorganic salts encompass alkalic and metal salts and have shown to be less corrosive, low-cost and recyclable compared to inorganic acids (Qing et al., 2016a). Limited studies have focused on the application of alkalic and metal salt pretreatments for lignocellulosic bioethanol production (Qing et al., 2016b; Ramadoss and Muthukumar, 2015; Ramadoss and Muthukumar, 2016). Inorganic salts are therefore emerging as an efficient biomass pretreatment strategy for enhancing sugar yields and bioethanol production. This paper examines the recent advancements in alkalic and metal

salt biomass pretreatments and their effects on the lignocellulosic structure, enzymatic digestibility and inhibitor profiles. In addition, the potential application of alkalic and metal salt pretreatment for bioethanol production processes are presented. Furthermore, existing challenges and future prospects for alkalic and metal salt catalysed pretreatments are outlined.

2. Lignocellulosic biomass

Lignocellulosic biomass (LB) are naturally designed complex composites from plant dry matter. Approximately 200 billion tons are produced annually, accounting for nearly 50% of the global biomass production, with a major fraction considered waste (Kabir et al., 2015). There is a general consensus on the replacement of fossil-derived fuels and products with LB due to its high abundance, renewability and low cost (Zamani, 2015). LB is a heterogeneous matrix containing the carbohydrate polymers cellulose and hemicellulose bound together by lignin. Generally, the fraction of these components range from 30-50% cellulose, 20-40% hemicellulose and 10-30% lignin, depending on the plant type (McKendry, 2002; Binod and Pandey, 2015; Zamani, 2015). Cellulose is an unbranched glucose polysaccharide held together by a β-1,4-glycosidic bond. Hemicellulose is an amorphous, single-chain branched polysaccharide containing both pentose and hexose sugars such as arabinose, mannose, glucose, galactose and xylose. Lignin is an amorphous phenolic polymer that contains guaiacyl, sinapyl and *p*-hydroxyphenyl units linked by ether and carbon bonds. Lignin provides the impermeable and recalcitrant characteristic to plant cell walls, thereby preventing microbial and chemical attack (Loow et al., 2015).

Agricultural wastes are considered the major contributor to annual LB production, and include many different types of crop residues such as corn cobs and stover, sugarcane leaves and baggase, sorghum leaves, wheat straw and rice straw among others (Loow et al., 2015;

Zamani, 2015, Zabed et al., 2016). Several types of fuels and bioproducts have been produced from LB as shown in Table 1. Corn and sugarcane wastes are among the most promising feedstock candidates owing to their high annual global production of 1.03 billion and 1.91 billion tonnes, respectively (Loow et al., 2015; USDA, 2017). Furthermore, sugarcane has a high biomass yield and residues are considered a good source for second generation bioethanol while corn is an energy dense biomass with established technologies (Zabed et al., 2017; Potumarthi et al., 2012). Cellulose, hemicellulose and lignin content in sugarcane leaves are 44, 28 and 10%, respectively; whereas corn cobs contains 32-45% cellulose, 40% hemicelluloses and 6-14% lignin, further highlighting their feedstock potential (Moodley and Gueguim Kana, 2015; Foley, 1978).

Sugarcane leaves constitute 40% of the total plant dry weight and is usually burnt prior to harvest or dumped in landfill sites, posing serious health and environmental concerns (Smithers, 2014). The carbohydrate polymers found in the cell wall of the leaves and culm accounts for two thirds of the total energy content in sugarcane (de Souza et al., 2013). Furthermore, the recoverable dry leaves possess the energy equivalent to ten tons of coal per hectare (Smithers, 2014). Few studies have reported bioethanol production from sugarcane leaves. Krishna et al. (1998) reported 2% bioethanol using *Trichoderma reesei* QM9414 and *S. cerevisiae* NRRL-Y-132 in a simultaneous saccharification and fermentation (SSF) system. Another study employing acid pretreated sugarcane leaves observed an ethanol yield of 4.71 g/L (Jutakanoke et al., 2012).

Likewise, about 50% of corn harvest consists of the leaves, stems, husks and cobs and are discarded as waste material (USDA, 2017). A recent report by Li et al. (2016) investigated the effect of acid pretreatment on different parts of corn wastes (stem, leaf, flower, husk and cob) for bioethanol production and revealed that corn cobs gave the highest glucose yield and

bioethanol concentration of 94.2% and 24 g/L, respectively. Additionally, Kreith and Krumdieck (2013) reported that approximately 510 L of ethanol could be produced per ton of corn cobs compared to 450 L/t using corn stover.

Table 1. Bioproducts from various lignocellulosic residues.

Lignocellulosic biomass	Bio-product	Reference
Sugarcane leaves	Xylose and glucose; biohydrogen	Moodley and Gueguim Kana (2015)
Corn cobs	Glucose; bioethanol	Li et al. (2016)
Sugar beet	Vanillin	Aarabi et al. (2017)
Wheat straw	Glucose; bioethanol	Ruiz et al. (2012)
Corn residues	Xylitol	Irmak et al. (2017)
Sugarcane baggase	Xylitol	Vallejos et al. (2016)
Corn stover	Biobutanol	Cai et al. (2017)
Cotton	Acetic, formic and lactic acid	Gao et al. (2013)
Pine	Biogas	Brown et al. (2012)

3. Overview of chemical pretreatment regimes

Biomass pretreatment strategies are crucial for degradation of complex, resistant lignocellulosic structures (Loow et al., 2015). Pretreatment results in various effects on these structures that include an increase in the surface area and porosity, alteration of the lignin structure, lignin removal, partial break down of hemicellulosic components, and reduction of cellulose crystallinity. These effects enhance the enzymatic saccharification stage, thus releasing higher fermentable sugars that can be recovered for fermentation processes (Harmsen et al., 2010; Yang and Wyman, 2008). A previous study reported that only about 20% of fermentable sugar can be recovered without chemical pretreatment compared to approximately 80% when pretreatment is applied (Singhvi et al., 2014). Pretreatment may be

classified into three main groups that include mechanical, chemical and biological. Chemical pretreatment causes the disruption of recalcitrant biomass structures and may include dilute acid, alkaline, organosolvent, and ionic liquids (Harmsen et al., 2010). Alkaline-based pretreatments has been presented as one of the most effective chemical pretreatment regimes due to its low polluting, non-corrosive nature that involves less intensive chemical conditions compared to other technologies. The most commonly employed alkali-based pretreatment is sodium hydroxide (NaOH) which effectively removes lignin with low release of sugar degradation compounds and furan derivatives (Qing et al., 2016b). On the other hand, acid pretreatment techniques have been shown to solubilize cellulose and hemicellulose components (Zheng et al., 2013). Some examples of acid-based catalysts include hydrochloric (HCl), sulfuric (H_2SO_4) and phosphoric acid (H_3PO_4). Pretreatment with H_2SO_4 is most often used due to its high catabolic activity and has therefore been studied on a wide range of lignocellulosic wastes. Low acid concentrations are typically used since higher concentrations resulted in the corrosion of pretreatment reactors (Zhu et al., 2016). In addition, sugar molecules may be degraded to form furan derivatives such as furfural and 5-Hydroxymethyl furfural (HMF) and becomes inhibitory to fermentation processes (Jönsson and Martín, 2016). Microwave-assisted pretreatment has also attracted significant interest owing to its low cost, short reaction times, low energy requirements and high efficiency (Aguilar-Reynosa et al., 2017). Microwave irradiation employs an electromagnetic field to accelerate the molecules, creating rapid rotations and collisions resulting in friction and causing a rapid increase in temperature (Zhu et al., 2016). Lu et al. (2011) observed a 56% improvement in glucose yield from rape straw after microwave irradiation. Similarly, microwave-assisted alkali pretreatment of oil palm trunk was found to reduce lignin by 15% and enhance glucose yield by 79% (Lai and Idris, 2016). Despite the high volume of literature on the various pretreatment regimes, industrial scale application has significantly

been impeded by high cost, toxicity and energy related issues. Advantages and disadvantages of some common biomass pretreatment types are listed in Table 2.

Table 2. Commonly employed pretreatment technologies

Pretreatment	**Mode of action**	**Advantage (s)**	**Disadvantage (s)**	**Reference**
Irradiation	Cellulose is degraded into fragile fibres and oligosaccharides	Improves enzymatic hydrolysis	High cost Challenges with scale-up	Akhtar et al. (2015)
Alkaline	Cleaves linkages in lignin and glycosidic bonds of polysaccharides	Requires low temperature and pressure Low inhibitors generated Produces highly digestible substrate	High cost Generation of irrecoverable salts	Sindhu et al. (2015)
Acid	Hydrolyzes hemicellulose to xylose Modifies lignin structure	Simple method. Thermal energy not required	High cost Produces toxic inhibitor compounds	Jung and Kim (2015)
Microwave-chemical	Dipolar polarization achieves heating Rapid oscillation causes molecules to vibrate	Uniform heating Improves pretreatment speed Decreased energy input	Dependent on properties of the material Formation of hot spots Challenges with scale-up	Xu (2015)

Table 2. Continued…

Pretreatment	Mode of action	Advantage (s)	Disadvantage (s)	Reference
Alkalic salt	Cleavage of ester bonds and glycosidic linkages in the cell wall matrix	Low cost Low toxicity Recyclable Low inhibitors generated	Requires thermal energy Partial degradation of cellulose	Qing et al. (2016a)
Metal salt	Act as Lewis acids Dissociate into complex ions and rupture glycosidic linkages	Low cost Low toxicity Low inhibitors generated	Partial degradation of lignocellulosic matrix	Kang et al. (2013)
Ozonolysis	Degrades lignin	Low inhibitors generated Operates at ambient temperature	Highly reactive High energy demand	Zabed et al. (2016)
Organosolv	Cleavage of ether and glycosidic bonds	Fractionates biomass with high purity Easily recovered and reused	High cost Requirement for removal of solvent	Zhang et al. (2016)
Ionic liquids	Depolymerizes lignin by cleavage of β-*O*-4 linkage	No toxic or odour emissions Mild temperatures required Recyclable	High cost Requires washing for reuse	Zabed et al. (2016); Yoo et al. (2017)

4. Reaction mechanism of inorganic salt pretreatments

Fewer studies have previously reported on the use of inorganic salt pretreatment with its increasing importance (Liu et al., 2009a). Inorganic salts are commonly coupled with steam heating (Qing et al., 2016a) whereas limited studies are reported with microwave irradiation (Lu and Zhou, 2011). Similarly, these salts have been combined with a range of other chemicals such as acids (Mao et al., 2012), organosolvents (Park et al., 2010), ionic liquids (Li et al., 2009), and other inorganic salts (Qing et al., 2016a). Inorganic salts may be classified as alkalic (Qing et al., 2016a; Qing et al., 2016b) or metal type salts (Liu et al., 2009; Kamireddy et al., 2013; Kang et al., 2013; Ramadoss and Muthukumar, 2015; Ramadoss and Muthukumar, 2016). The mechanism of these salt types may differ substantially and are briefly discussed below.

4.1 Alkalic salt

Alkalic salts behave like weak bases and have been described as potential alternatives to expensive alkali-based pretreatments (Qing et al., 2016a). Some examples of these include $Na_3PO_4.12H_2O$, Na_2CO_3, Na_2S (Qing et al., 2016a; Qing et al., 2016b). Alkalic salt-based catalysts result in the dissolution of lignin and hemicellulose structures, de-esterification of intermolecular ester bonds (Kim et al., 2016), restructuring and conversion of lignin and the alteration of the crystalline state of cellulose (Geng et al., 2014). In addition, alkalic salts result in effective removal of acetyl groups from xylan polymers, which have shown to ameliorate cellulose digestibility, thus leading to higher fermentable sugar release (Kim et al., 2014a). Furthermore, strong nucleophilic species present in alkalic salts (PO_4^{3-}, HPO_4^{2-} and HS^-) would augment the cleavage of phenolic β-aryl ether bonds of lignin, thus enhancing delignification with reduced attack on carbohydrate molecules (Gu et al., 2013).

4.2 Metal salts

Several metal salts have been used for biomass pretreatment studies and include sulfates, phosphates and chlorides (Kamireddy et al., 2013; Kang et al., 2013; Yu et al., 2011). Various reaction mechanisms have been suggested for metal salts. Metal type salts result in the formation of metal cations that act as a Lewis acid when it is in its aqueous state and essentially cleaves glycosidic linkages within lignocellulosic structures (Loow et al., 2015; Kamireddy et al., 2013). A Lewis acid is described as a molecular body that functions as an electron pair acceptor that can react with a Lewis base to form what is referred to as a Lewis adduct (Zhang et al., 2011). Consequently, coordinate covalent bonds containing six water molecules as monodentate ligands are formed around the central metal cation. Metal chlorides such as Al^{3+} and Fe^{3+} are believed to follow this reaction mechanism to form six coordinate covalent bonds with water molecules. On the other hand, Cu^{2+} obtains a stable complex ion by coordinating as a tetradentate ligand (Loow et al., 2015). The formation of these metal cations eventually acts as Lewis acids that result in the cleavage of glycosidic linkages present within hemicellulosic moieties (Kamireddy et al., 2013).

Alternatively, metal ions undergo hydrolysis when they are combined with water to produce a hydronium ion (H_3O^+). This would result in a Brønsted acid character, which is similar to hydrochloric acid (HCl) since it depolymerizes hemicelluloses to monosaccharide type sugars. Chemical species such as $FeSO_4$ have been suggested to enhance the degradation of glycosidic linkages. This is attributable to the adsorption of Fe^{2+} to hydroxyl oxygen atoms and the oxygen of the cellulose pyran ring, which produces a carbohydrate complex (Marcotullio et al., 2011; Zhang et al., 2013). Furthermore, the pretreatment activity of metal chlorides increases with the valence of the metal cation since higher valence molecules such

as Fe^{3+} are able to form strong cations and complex with lignin more effectively than weaker cations such as Na^{+} (Kamireddy et al., 2013; Kang et al., 2013).

5. Effect of inorganic salt pretreatment on lignocellulosic biomass

5.1 Structural composition

The primary objective of pretreatment is to disrupt the lignocellulosic matrix. Ideally, the biomass should undergo efficient delignification and hemicellulose solubilization to enhance enzymatic saccharification and microbial fermentation. Therefore, the quantification of cellulose, hemicellulose and lignin of native and pretreated samples are key in establishing the pretreatment efficiency (Sluiter et al., 2010). Since metal chloride salts act as Lewis acids, their main activity involves hemicellulose solubilization (Loow et al., 2015). Liu et al. (2009a) reported up to 100% hemicellulose removal from corn stover with 0.1 M $FeCl_3$ at 140-200 °C for 5-30 min. Similarly, the hemicellulose fraction in sugarcane baggase was decreased from 19.4 to 3.33% after $CrCl_3$ pretreatment (Chen et al., 2014). The combination of metal chlorides and chemical catalysts has also been investigated to enhance lignocellulosic degradation. Barley straw pretreated with acidified $ZnCl_2$ resulted in hemicellulose and lignin removal of 80 and 30%, respectively (Kim et al., 2014b). Raghavi et al. (2016) reported a novel sequential pretreatment for sugarcane trash using $FeCl_3$, crude glycerol and NaOH. These authors reported a significant decrease in lignin (from 27.11 to 5.71%) and hemicellulose (19.41 to 9%). By contrast, alkalic salts have been shown to aid in lignin dissolution, owing to its ability to act as a weak base, with enhancement in cellulose content and minimal effects on hemicellulose. For instance, Kim et al. (2014a) optimized a sodium carbonate (Na_2CO_3) pretreatment and reported a 63% delignification. Likewise, high delignification (75%) and cellulose improvement (72%) with low hemicellulose removal

(17.6%) was reported from bamboo shoot shell pretreated with $Na_3PO_4 \cdot 12H_2O$ (Qing et al., 2016b). However, a higher hemicellulose solubilization was reported when alkali salt was combined with Na_2S (Qing et al., 2016a). Qing et al. (2016a) reported a maximum delignification of 62.2%, cellulose improvement of 56.31% and hemicellulose removal of 36.24% from corn stover using a combined Na_3PO_4 and Na_2S pretreatment regime. Therefore, the combination of inorganic salt and either an acid or base ultimately enhances the overall pretreatment efficiency of lignocellulosic biomass.

Fourier Transform Infrared (FTIR) spectroscopy is another method routinely employed in determining changes in the lignocellulosic structure. The β-glycosidic linkage in cellulose is usually assigned to the band at ~900 cm^{-1} whereas bands at ~1045 cm^{-1} and ~3420 cm^{-1} represent the pyranose ring vibration and OH stretching vibration of intramolecular hydrogen, respectively, in cellulose (Qing et al., 2016b). Increases in intensity at these band positions characteristically indicate the recovery of cellulose in the solid residue after pretreatment. Mustard stalk and straw pretreated with NaCl was shown to somewhat increase the relative absorbance of band 898 cm^{-1} from 1.02 to 1.11 while bands at 1056 cm^{-1} and 3435 cm^{-1} increased from 2.13 to 2.43 and 1.64 to 1.92, respectively (Banerjee et al., 2016), signifying high recovery of cellulose. The combination of 10% sodium sulfide and 4% sodium phosphate on corn stover had a lesser effect on cellulose after pretreatment (Qing et al., 2016b). Bands at 900 cm^{-1}, 1045 cm^{-1} and 3420 cm^{-1} increased from 0.086 to 0.099, 0.162 to 0.192 and 0.153 to 0.176, respectively. Bands depicted at 1215 cm^{-1} and ~1500 - 1602 cm^{-1} represent the C–C + C–O stretching and the aromatic skeletal C=C stretching vibration, respectively, in lignin (Xu and Wang, 2016). The relative peak intensities for bands at 1511 and 1602 cm^{-1} were shown to increase after sugarcane bagasse was pretreated with H_2O_2, $MnSO_4 \cdot H_2O$ and ZnO (Ramadoss and Muthukumar, 2015). Similar banding patterns were observed with NaCl pretreatment by Banerjee et al. (2016). More specifically, the relative

absorbance of peaks at 1248 cm^{-1} and 1630 cm^{-1} increased from 1.06 to 1.18 and 0.93 to 1.05, respectively thereby indicating a change in the lignin structure. However, Qing et al. (2016b) reported slight decreases in absorbance for bands at 1245 cm^{-1}, 1510 cm^{-1} and 1627 cm^{-1} from 0.119 to 0.117, 0.095 to 0.084 and 0.113 to 0.107, respectively.

Changes in the crystallinity of lignocellulosic biomass is often measured using X-ray diffraction (XRD) (Wikandari et al., 2016). In addition to providing data on the crystalline and amorphous fractions of cellulose, XRD also measures the crystallinity of the lignin-based material in its entirety (Karimi and Taherzadeh, 2016; Wikandari et al., 2016). Intermolecular hydrogen bonds between chains in lignocellulose make crystalline cellulose highly recalcitrant thereby hampering degradation (Sun et al., 2010). The ratio of crystalline cellulose to the amorphous region is expressed by the crystallinity index (CrI) using a calculation developed by Segel et al. (1959). A high CrI indicates a low crystalline structure whereas a high crystalline structure is represented by a low CrI (Jin et al., 2016, Lai and Idris, 2016). However, XRD is not routinely employed in pretreatment studies and its use is often confirmatory to other structural analysis. Some studies have examined the effect of various metal and alkalic salt pretreatments on the crystallinity of cellulose. Zhang et al. (2017) explored the effects of $FeCl_3$ with additives such as Tween 80 and biosurfactant (BSA) on the enzymatic digestibility of sugarcane bagasse. These authors reported a 15.6% increase in CrI with 0.1 M $FeCl_3$ and 150 mg/g BSA at 160 ^{o}C for 10 min. The increase in CrI was attributed to the solubilization of amorphous hemicellulose and cellulose whilst retaining crystalline cellulose. The effect of NaCl on enhancing the enzymatic digestibility of mustard stalk and straw has also been reported (Banerjee et al., 2016). Surprisingly, this monovalent salt significantly increased the CrI from 36.84 to 62.68% with 1 M NaCl. Another study investigating the effect of ultrasonic enhancement of cellulose hydrolysis with HCl-$FeCl_3$ reported a 20.1% increase in CrI of cellulose using 2.5 M HCl, 0.3 M $FeCl_3$ at 80 $^{\circ}$C for 70

min with 300 W ultrasonic treatment (Li et al., 2015). Alkalic salts have also been reported to increase the CrI. For instance, Qing et al. (2016b) examined the effect of alkalic salt and hydrogen peroxide on the enzymatic saccharification of bamboo shoot shell. The combination of 0.3 g/g H_2O_2 with 9% $Na_3PO_4.12H_2O$ was found to increase the CrI by 5.1%, compared to the native sample (Qing et al., 2016b). Similarly, Kim et al. (2014a) reported a 23% increase in the CrI when pretreated under moderate conditions of 4.1% Na_2CO_3 at 142.6°C for 18 min.

Physical changes in lignocellulosic biomass can be observed using scanning electron microscopy (SEM). SEM allows changes in morphology, surface structure and microstructure to be discerned (Amiri and Karimi, 2015). Untreated corn stover was shown to have a smooth and contiguous surface compared to the reduced particle size and cell structure damaged observed after pretreatment with $FeCl_3$ (Liu et al., 2009a). Similar observations were reported by Kang et al. (2013) for inorganic salt pretreatment of Miscanthus straw. These authors observed a smooth and intact surface with the native untreated samples compared to the degraded straw with cell structure damage exposing the cells inner contents. SEM micrographs have also been reported to show the delignification process by the formation of pores and lignin droplets on the plant surface. Pretreatment of corn stover with acidic ferrous ions showed the appearance of lignin droplets with the removal of a large percentage of matrixing material (Wei et al., 2011). Likewise, lignin droplets were observed on the surface of sweet sorghum baggase pretreated with $CuCl_2$ (Yu et al., 2011). Donohoe et al. (2008) proposed that pretreatment temperatures beyond the lignin phase transition causes lignin to coalesce into larger molten bodies that redeposit on the surface of plant cell walls. Alkalic salts such as sodium phosphate combined with sodium sulfide was shown to significantly increase porosity and fragmentation of corn stover (Qing et al., 2016a). These same authors investigated the effects of sodium phosphate and hydrogen peroxide on bamboo shoot shell,

and observed partial fibre disruption with a rough surface compared to the highly ordered surface of the native sample (Qing et al., 2016b).

5.2 Enhancing enzymatic digestibility

Inorganic salts have been shown to improve the enzymatic hydrolysis of lignocellulosic biomass either in combination with other pretreatments or alone (Table 3). Metal salts such as alkali metals (Li, Na, K); alkaline earth metals (Ca, Mg); and transition metals (Cr, Fe, Cu, Mn, Co, Zn) are often employed as chloride salts (Romero et al., 2016). These metal salts can dissociate into complex ions owing to their Lewis acid activity, and solubilize hemicellulose (Mamman et al., 2008). Several studies have reported the effects of metal salts on enzymatic hydrolysis of lignocelluloses. The saccharification efficiency of mustard stalk and straw increased from 16 to 82% with 1 M NaCl pretreatment (Banerjee et al., 2016). In another study exploring the effects of KCl, NaCl, $ZnCl_2$, $CaCl_2$ and $FeCl_3$ on Miscanthus pretreatment, Kang et al. (2013) reported 100% xylan removal and 71.6% enzymatic hydrolysis using 0.5% $FeCl_3$ at 200°C for 15 min. NaCl was shown to be the least effective salt while $ZnCl_2$ had a positive effect on the glucan recovery compared to $FeCl_3$. Microwave-assisted inorganic salt pretreatment has been shown to achieve an improvement in enzymatic digestibility due to the field-induced motion of salt ions resulting in a higher heating efficiency compared to steam pretreatment. Liu et al. (2009b) reported that microwave-assisted $FeCl_3$ pretreatment on corn stover effectively solubilized the hemicellulose fraction into simpler sugars and caused major disruptions between the ether and ester linkages in the bonding matrix. Microwave-assisted $FeCl_3$ pretreatment of rice straw has also been reported (Lu and Zhou, 2011). Under optimal conditions of 0.14 M $FeCl_3$, 160°C, 19 min and 109 g/l substrate concentration, enzymatic digestibility was improved, yielding 6.62 g/l of reducing

sugar compared to 2.3 g/l from the untreated substrate. On the other hand, alkalic salts have been effective in the removal of acetyl groups from xylan polymers, which ameliorate enzymatic saccharification and cellulose digestibility (Kim et al., 2014a). Yang et al. (2012) observed a 71.7% total sugar recovery from Na_2CO_3 pretreated rice straw under moderate conditions of 8% Na_2CO_3 at 140 °C. Likewise, Qing et al. (2016b) reported enhanced enzymatic digestibility of bamboo shoot shell, yielding 50.6% more reducing sugar using 9% $Na_3PO_4.12H_2O$ and 0.3 g/g H_2O_2 at 80 °C for 2 h. These same authors also observed a 91% reducing sugar yield and 64% glucose yield from corn stover pretreated with Na_3PO_4 and Na_2S (Qing et al., 2016a).

Table 3. Inorganic salt pretreatment of lignocellulosic biomass for enhanced enzymatic digestibility

Substrate	**Pretreatment**	**Key findings**	**Reference**
Rice straw	0.1 M $FeCl_3$ at 170 ^oC for 30 min	Increased enzymatic digestibility to 95.1%	Chen et al. (2015)
Corn stover	0.1 M $FeCl_3$ at 140 ^oC for 20 min	91% hemicellulose removed 89% recovered sugars	Liu et al. (2009)
Miscanthus straw	5% $ZnCl_2$ at 200 ^oC for 25 min	Increased enzymatic digestibility to 62.2%	Kang et al. (2013)
Mustard stalk and straw	2 M NaCl at 121 ^oC for 60 min	Increased enzymatic digestibility to 72%	Banerjee et al. (2015)
Barley straw	7.3% $ZnCl_2$ (acidified) at 67.9 ^oC for 10.5 min	Increased enzymatic digestibility to 69.3%	Kim et al. (2014)
Rice straw	0.14 M $FeCl_3$ at 800 W for 19 min	58.3% increase in sugar yield	Lu and Zhou (2015)
Corn cobs	2% $NaHCO_3$ with *EBI at 180 kGy for 600 min	34.7% delignification 67.6% glucose recovery	Guo et al. (2016)
Rice straw	8% Na_2CO_3 at 120 °C for 50 min	71.7% total sugar recovery	Yang et al. (2012)
Bamboo shoot shell	9% $Na_3PO_4.12H_2O$ and 0.3 g/g H_2O_2 at 80 ^oC for 2 h	87.7% delignification 97.1% reducing sugar yield	Qing et al. (2016b)
Corn stover	4% Na_3PO_4 and 10% Na_2S at 120 °C for 40 min	62.2% delignification 91.1% reducing sugar yield	Qing et al. (2016a)

Footnote: *EBI- electron beam irradiation

5.3 Inhibitor profile of hydrolysate

Hydrolysis of lignocellulosic biomass under varying pretreatment severities generates inhibitory by-products such as acetic acid, formic acid, 5-hydroxymethyl furfural (HMF), furfural and other phenolic-based compounds (Jung and Kim, 2015). Mussatto and Roberto (2004) have arranged the relative toxicity of these inhibitor compounds on the bioethanol fermentation process in decreasing order: phenolic compounds>furfural>HMF>acetic acid>extractives. These compounds are inhibitory to both cellulosic enzymes and fermenting microorganisms (Cavka and Johnson, 2013). Threshold values >1 g/L of furfural and HMF concentrations have shown to negatively impact the bioethanol production process. Likewise, acetic acid concentrations that exceed 1.5 g/L have shown to be inhibitory for bioethanol production (Wikandari et al., 2010). Phenolic compounds also inhibit bioethanol fermentation process above >1 g/L (Liu et al., 2016). Formation of acetic acid occurs when ester and acetyl linkages within lignocellulosic structures are degraded (Kamireddy et al., 2013). Unlike acetic acid, which is released when acetyl linkages within hemicellulose are disrupted, phenolic compounds are produced when ether bonds in lignin macromolecules are disintegrated (Harmsen et al., 2010). Alternatively, furan derivatives (furfural and HMF) are generated during decomposition of sugar molecules (Ravindran and Jaiswal, 2016), which generally occur at a higher exposure time to stronger chemical conditions or temperatures (Harmsen et al., 2010). Alkalic and metal salt pretreatment produced low concentrations of inhibitors compared to acid pretreatment, which is known to produce high amounts of acetic acid, HMF and furfural (Loow et al., 2015). Alkalic salt pretreatments release phenolic compounds due to the degradation of lignin cross-links or from extractives. In addition, alkalic salts may result in the formation of acidic compounds including organic acids from lignin as well as acetic acid from hemicellulose (Kim et al., 2014a; Qing et al., 2016a; Qing

et al., 2016b). Qing et al. (2016a) observed an acetic acid concentration of 2.04 g/L using a combined Na_3PO_4 and Na_2S pretreatment on corn stover. The same authors observed a lower acetic acid concentration (0.95 g/L) when bamboo shoot shell was pretreated using a combined $Na_3PO_4.12H_2O$ and H_2O_2 treatment (Qing et al. 2016b). Alternatively, metal salt pretreatments majorly release acetic acid owing to the breakdown of the hemicellulosic acetyl groups. In addition, trivalent cations may result in furfural production since they remain active in the presence of acids such as acetic acid (Kamireddy et al., 2013). For instance, corn stover pretreated with 0.125 M $CuCl_2$ at 150 $^{\circ}$C generated no furfural with 0.24 g/L HMF compared to 1.85 g/L furfural and 0.90 g/L HMF with 0.125 M H_2SO_4 at 150 $^{\circ}$C (Kamireddy et al., 2013). Low inhibitor concentrations (0.01 g/L furfural and 0.148 g/L HMF) were also reported with a combination of organosolv and $FeCl_3$ for barley straw pretreatment (Kim et al., 2010).

Table 4. Inhibitor profile from alkalic and metal chloride salt pretreatment

Substrate	Pretreatment conditions	Inhibitors (g/L)			Reference
		Acetic acid	Furfural	HMF	
Bamboo shoot shell	9% $Na_3PO_4.12H_2O$, 0.3 g/g H_2O_2, 1% S:L, 80°C, 120 min	0.95	ND	ND	Qing et al. (2016b)
Sugarcane bagasse	0.1 M $ZnCl_2$, 10% S:L, 170°C, 30 min	ND	3.46	2.52	Chen et al. (2014)
Sugarcane bagasse	0.1 M $FeCl_3$, 10% S:L, 170°C, 30 min	ND	5.11	0.75	Chen et al. (2014)
Corn stover	4% Na_3PO_4, 10% Na_2S, 1% S:L, 120°C, 40 min	2.04	ND	ND	Qing et al. (2016a)
Corn stover	0.125 M $FeCl_3$, 160°C, 10 min	3.30	1.19	0.52	Kamireddy et al. (2013)

Footnote: ND- Not determined; HMF- 5-Hydroxymethyl furfural.

6. Potential of inorganic salt pretreatment for lignocellulosic bioethanol production

6.*1 Process type*

Cellulosic bioethanol production consists of three main steps and includes lignocellulosic biomass pretreatment, enzymatic hydrolysis and fermentation. Enzymatic hydrolysis is an integral step in the bioethanol production process since it releases the fermentable sugars that will ultimately be metabolised into ethanol. Therefore, the selection of an appropriate enzyme hydrolysis and fermentation approach is essential. Microbial bioethanol can be produced using three process types, each with their own advantages and drawbacks: (1) separate hydrolysis and fermentation (SHF), (2) simultaneous saccharification and fermentation without prehydrolysis (OSSF) and, (3) prehydrolysis followed by simultaneous saccharification and fermentation (PSSF) (Carrillo-Nieves et al., 2017). The main feature of the SHF strategy is it allows the independent optimization of the saccharification and fermentation stages thus allowing enhanced product recovery from each stage. This however, leads to the drawback of requiring two reactors for enzymatic hydrolysis and fermentation. Additionally, carbohydrate feedback inhibition effects on cellulolytic enzymes can occur when sugar molecules accumulate (Koppram et al., 2013). Furthermore, the separation of the solid residues from the enzymatic hydrolysate requires a filtering or centrifugation stage, hampering process economics and productivity at a large scale (Aden and Foust, 2009). On the contrary, the OSSF does not require separate reactors for saccharification and fermentation, and it minimizes cellulase enzyme inhibition through simultaneous fermentation by the microorganism. The drawback of this system is mass and heat transfer problems at high solid loading. In addition, the main shortcoming of SSF is the

difference in optimum temperature for the enzyme and fermenting microorganism, usually 50°C and 30°C, respectively (Olofsson et al., 2008). Alternatively, the prehydrolysis strategy in SSF processes has shown to improve the bioethanol concentration and bioethanol conversion. This is mainly due to enhanced saccharification efficiency at high temperatures that are usually required for optimal enzymatic activity (Carrillo-Nieves et al., 2017; Zhu et al., 2015) and reduced initial viscosity at the beginning of fermentation (He et al., 2016). Despite these advantages, prehydrolysis stages require additional time and energy input, thus reducing its economic feasibility. Combination of enzymatic hydrolysis and fermentation steps reduces the number of vessels needed. This would decrease the investment cost by more than 20% when SSF processes without prehydrolysis have been used (Wingren et al., 2003).

6.2 Process kinetics

Kinetic models are useful tools in predicting the behaviour of microorganisms and product formation in various fermentation processes. Several kinetic models have been developed that describe growth and product formation (Phukoetphim et al., 2017). These models include Monod, logistic and modified Gompertz, among others (Dodic et al., 2012; Rorke and Gueguim Kana, 2017). The Monod model is a simplistic unstructured kinetic model that describes the growth kinetics of a microorganism in relation to a limiting substrate (Comelli et al., 2016). Several studies have examined the Monod growth kinetics of bioethanol production using glucose (Sing and Sharma, 2015), oil palm frond juice (Srimachai et al., 2015) and sweet sorghum juice (Thangprompan et al., 2013). The logistic model also describes the change in microbial

cells as a function of growth rate, initial and maximum biomass concentration and time. This model assumes sufficient substrate is present and ignores substrate inhibition (Phukoetphim et al., 2017). Studies using sugar beet raw juice (Dodić et al., 2012) and sweet sorghum juice (Phukoetphim et al., 2017) have employed the logistic model for bioethanol production processes. The modified Gompertz model was initially used to describe human populations and was later modified to describe microbial growth as a function of biomass concentration and productivity. It was then modified further to describe the production potential and maximum production rate of bioethanol and biohydrogen processes (Phukoetphim et al., 2017). This model is routinely employed in bioethanol production and has been reported using food waste (Yan et al., 2013), oil palm frond juice (Srimachai et al., 2015) and sugar beet raw juice (Dodic et al., 2012).

7. Challenges and Future prospects

7.1 Current alkalic or metal salt pretreatment strategies

Alkalic and metal salt pretreatment regimes have recently emerged as efficient pretreatment catalysts. Nevertheless, they have been limited by few studies that have briefly examined their efficacy in single stage systems either individually or in combination with other chemical strategies. Combined pretreatments with salts and other chemicals have illustrated significant improvements compared to individual treatments. Despite the reported improvements using combined systems, various challenges may hinder its advancement. One major limitation of salt and acid combined systems is the formation of double-replacement reactions, which render chemical pretreatments inefficient. Other challenges that have plagued these pretreatment

catalysts include the partial degradation of the lignocellulosic matrix, low sugar recovery, high fermentation inhibitor production, high cost and energy related issues.

Alkalic and metal salt pretreatment methods have several advantages over commonly employed acid and alkali pretreatment technologies. Acid hydrolysis is often employed in toxic concentrations and thus causes corrosion of reactors or requires costly specialised equipment. Moreover, acid hydrolysis generates a high amount of fermentation inhibitors. The main drawback with alkali pretreatment is the high cost associated with high concentrations. On the contrary, alkalic and metal salts are considered environmentally friendly, low-cost and does not require specialised reactors to minimize corrosion. Additionally, alkalic and metal salts generate a low concentration of inhibitors compared to commonly used pretreatments and is, therefore, considered more favourable for bioethanol production and other fermentation processes. There is little research on the combination of alkalic or metal salt with other chemical catalysts. For instance, sequential pretreatment systems that incorporate salts with dilute acid or alkaline could enhance enzymatic digestibility as well as reduce the cost of lignocellulosic biomass pretreatment. The application of dilute acid and alkaline solutions combined with alkalic or metal salts could enhance the sugar recovery from lignocellulosic biomass and at the same time reduce the negative impacts that include reactor corrosion and high costs. Furthermore, knowledge on the implementation of intelligent models such as Artificial Neural Networks (ANN) to extract functional relationships between alkalic or metal salt pretreatment inputs and the sugar recovery is scanty. Future studies on alkalic or metal salt pretreatment regimes could apply ANN models to determine functional relationships and gain an in depth understanding of the treatment inputs on the corresponding sugar yield.

7.2 Lignocellulosic bioethanol production processes

Economical cellulosic bioethanol production is associated with several key technological issues. SSF processes with and without prehydrolysis are significantly challenged by low bioethanol concentration and bioethanol conversion due to ineffective operational strategies. Optimization of key operational strategies that define the interactive effects of key parameters for maximum bioethanol concentration and bioethanol conversion are necessary. Additionally, there is a lack of studies focusing on the kinetics of bioethanol production from alkalic or metal salt pretreated lignocellulosic waste. Future research on alkalic or metal salt pretreated waste that is centred on the kinetics of bioethanol production could potentially improve productivity and reduce costs. *S. cerevisiae*, an industrially-known bioethanol producing strain has shown to exhibit changes in growth behaviour under microaerophilic and anaerobic environments. For instance, microaerophilic conditions have shown to promote microbial biomass formation whereas anaerobic environments enhance bioethanol production by reducing the lag phase of microbial growth. Thus, knowledge on the kinetics of cell growth and bioethanol production under microaerophilic and anaerobic conditions are required for enhancement of SSF processes.

8. Conclusion

Pretreatment is a complex process exploiting lignocellulosic wastes as potential feedstocks for biofuel production combined with reducing waste materials. More specifically, alkalic and metal salt pretreatment regimes have gained significant interest as effective treatment catalysts. Screening and optimization of efficient alkalic or metal salt pretreatments is required to improve process economics, reduce fermentation inhibitors and enhance sugar recovery. This review highlighted recent progress in the

development of alkalic and metal salt catalysed pretreatment regimes for biomass conversion. In addition, the potential of bioethanol production from lignocellulosic wastes were evaluated. A better understanding of bioethanol production by studying kinetics in SSF processes will enhance the process performance and economics for large scale application.

Acknowledgements

The financial assistance of the National Research Foundation of South Africa (NRF) [grant numbers 101316 and 101275] towards this research is hereby acknowledged. Opinions expressed and conclusions arrived at, are those of the authors and are not necessarily attributed to the NRF.

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CHAPTER 3

Optimization of a novel sequential alkalic and metal salt pretreatment for enhanced delignification and enzymatic saccharification of corn cobs

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This chapter has been published in *Bioresource Technology* (243, 785-792) with the title: Optimization of a novel sequential alkalic and metal salt pretreatment for enhanced delignification and enzymatic saccharification of corn cobs.

The published paper and supplementary material are presented in the following pages.

Bioresource Technology 243 (2017) 785–792

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Optimization of a novel sequential alkalic and metal salt pretreatment for enhanced delignification and enzymatic saccharification of corn cobs

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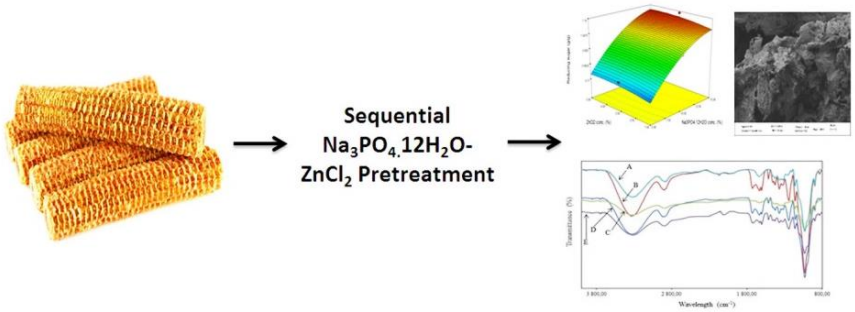
HIGHLIGHTS

- First report on a sequential alkalic and metal salt pretreatment of corn cobs.
- High delignification of 63.61% was observed.
- Maximum reducing sugar yield of 1.10 ± 0.01 g/g was obtained.
- This method showed a high yield improvement compared to previous reports.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 28 April 2017
Received in revised form 28 June 2017
Accepted 29 June 2017
Available online 1 July 2017

Keywords:
Sequential
Inorganic salt pretreatment
Corn cobs
Lignocellulosic biomass

ABSTRACT

This study presents a sequential sodium phosphate dodecahydrate ($Na_3PO_4{\cdot}12H_2O$) and zinc chloride ($ZnCl_2$) pretreatment to enhance delignification and enzymatic saccharification of corn cobs. The effects of process parameters of $Na_3PO_4{\cdot}12H_2O$ concentration (5–15%), $ZnCl_2$ concentration (1–5%) and solid to liquid ratio (5–15%) on reducing sugar yield from corn cobs were investigated. The sequential pretreatment model was developed and optimized with a high coefficient of determination value (0.94). Maximum reducing sugar yield of 1.10 ± 0.01 g/g was obtained with 14.02% $Na_3PO_4{\cdot}12H_2O$, 3.65% $ZnCl_2$ and 5% solid to liquid ratio. Scanning electron microscopy (SEM) and Fourier Transform Infrared analysis (FTIR) showed major lignocellulosic structural changes after the optimized sequential pretreatment with 63.61% delignification. In addition, a 10-fold increase in the sugar yield was observed compared to previous reports on the same substrate. This sequential pretreatment strategy was efficient for enhancing enzymatic saccharification of corn cobs.

1. Introduction

Fossil fuel depletion combined with environmental pollution has stimulated research towards renewable and sustainable fuel carriers using lignocellulosic biomass (Travaini et al., 2016). Lignocellulosic biomass has shown to be an attractive feedstock for alternative energy production, since it is abundant, renewable, and cost-effective. It is primarily composed of approximately 38–50% cellulose, 23–32% hemicellulose and 15–25% lignin (McKendry, 2002).

Currently, a variety of potential biofuel lignocellulosic substrates are being explored and include sugar cane leaf wastes (Moodley and Gueguim Kana, 2017), sorghum leaf wastes (Rorke et al., 2017), sugarcane bagasse (Ramadoss and Muthukumar, 2015), corn stover (Liu et al., 2009) and corn cob wastes (Mao et al., 2012), among others. The annual worldwide corn production exceeds 1.03 billion metric tons. Of this, nearly 50% makes up the

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http://dx.doi.org/10.1016/j.biortech.2017.06.175
0960-8524/

leaves, stalks, husks and cobs which are usually discarded as waste material (USDA, 2017). Corn cobs are primarily composed of 32–45% cellulose, 39% hemicellulose and 6–14% lignin (Foley, 1978). Its energy density is between 4960 and 5210 MJ/kg, which is approximately two-fold higher than commonly used biomasses such as corn stover (2550 MJ/kg) and switchgrass (2500 MJ/kg). In addition, the low lignin content of corn cobs makes it a superior contender for biofuel production processes compared to corn stover and switchgrass (Potumarthi et al., 2012).

Despite the aforementioned advantages, lignocellulosic biomass such as corn cobs presents several challenges as a result of its complex and recalcitrant nature. Overcoming these challenges for the improvement of both enzymatic and microbial accessibility necessitates the use of pretreatment methods to break down the resistant structures, thus allowing fermentable sugar recovery (Kang et al., 2013). In the last few years, several pretreatment strategies have been investigated and these include dilute acid, alkaline, microwave, ionic liquid, organosolvent, thermal and inorganic salts, among others (Qing et al., 2016 b). Pretreatment techniques that apply acid efficiently solubilize lignin and hydrolyse hemicellulose (Lopez-Arenas et al., 2010). Thermal pretreatment causes damage to lignin structures thus, reducing the rate of polymerization of biomass with simultaneous hydrolysis of hemicellulose to xylose (Eggeman and Elander, 2005). Recent studies have reported that hybrid pretreatment strategies release a higher fermentable sugar yield compared to single pretreatment techniques (Mao et al., 2012; Ramadoss and Muthukumar, 2015; Qing et al., 2016 b). Despite the abundance of literature on various pretreatment regimes, their implementation at a large scale has been significantly impeded by the high cost and energy related issues. Thus, current pretreatment methods are centred on the development of efficient, environmental friendly and cost-effective treatments.

The use of inorganic salt as a chemical pretreatment strategy has recently gained significant interest since they are less corrosive, cost-effective and recyclable compared to inorganic acids (Liu et al., 2009). Additionally, they have shown to enhance the degradation rates of cellulose, hemicellulose and lignin (Kamireddy et al., 2013; Qing et al., 2016a). Inorganic salts have most recently been coupled with several heating methods such as microwave irradiation (Moodley and Gueguim Kana, 2017), thermal heat (Qing et al., 2016b) and electron beam radiation (Guo et al., 2016). Moreover, these salts have been combined with various chemicals which include acids (Mao et al., 2012), organosolvents (Park et al., 2010), ionic liquids (Li et al., 2009), and other inorganic salts (Qing et al., 2016 b). Inorganic salts may be classified as either alkalic (Qing et al., 2016a,b) or metal salts (Li et al., 2009; Kamireddy et al., 2013; Kang et al., 2013; Ramadoss and Muthukumar, 2015). The mode of action of these different salt types may have a variable effect on the chemical composition and biomass structure (Yu et al., 2011).

Alkalic salts act as weak bases and have been reported as viable replacement catalysts for costly alkali-based pretreatments such as sodium hydroxide (NaOH) (Qing et al., 2016a). These include $Na_3PO_4{\cdot}12H_2O$, $NaCO_3$, Na_2S and $Na_2CO_3{\cdot}1.5H_2O_2$ (Qing et al., 2016a,b; Nakashima et al., 2016; Kim et al., 2014). This salt type results in the dissolution of lignin and hemicellulose structures, de-esterification of intermolecular ester bonds (Kim et al., 2016), restructuring and conversion of lignin and alteration of the crystalline state of cellulose (Geng et al., 2014). Qing et al. (2016b) investigated a combined $Na_3PO_4{\cdot}12H_2O$ and H_2O_2 pretreatment of bamboo shoot shell and reported a total reducing sugar yield of 97.1%. Likewise, Qing et al. (2016a) optimized a combined Na_3PO_4 and Na_2S pretreatment of corn stover and gave a total reducing sugar yield of 91.1% (Qing et al., 2016a). Similarly, Kim et al. (2014) reported on a Na_2CO_3 pretreatment and observed a maximum glucose yield of 0.27 g/g from corn stover.

Some metal salts reported in lignocellulosic pretreatment include the sulphates, phosphates or chlorides (Kamireddy et al., 2013; Kang et al., 2013; Yu et al., 2011). Metal salts result in the formation of metal cations that act as Lewis acids when in solution and essentially cleave glycosidic linkages within lignocellulosic structures (Loow et al., 2015; Kamireddy et al., 2013). Liu et al. (2009) studied the effects of NaCl, KCl, $CaCl_2$, $MgCl_2$, $FeCl_2$, $FeSO_4$, $FeCl_3$, and $Fe_2(SO_4)_3$ as catalysts and reported maximum hydrolysis of corn stover using $FeCl_3$ (98%). Similarly, Kang et al. (2013) studied the effects of NaCl, KCl, $CaCl_2$, $ZnCl_2$, and $FeCl_3$ on the pretreatment of *Miscanthus* straw and found that although $FeCl_3$ resulted in a 100% xylan removal, $ZnCl_2$ released a higher glucose content (90%) compared to $FeCl_3$ (55%). A microwave-assisted inorganic salt pretreatment by Moodley and Gueguim Kana (2017) reported a 1.7-fold and 2.3-fold increase in the reducing sugar yield when $FeCl_3$ was used compared to $ZnCl_2$ and NaCl, respectively.

Additional challenges in the chemical and thermal treatment of lignocellulosic biomass is the formation of various fermentation inhibitor compounds such as acetic acid, furfural and 5-Hydroxymethyl furfural (HMF). These have negative influence on the enzymatic hydrolysis step as well as the fermentation process (Rorke et al., 2017). Recent investigations are focusing on the development of pretreatment methods with reduced inhibitor concentrations. There is a dearth of knowledge on the impact of sequential pretreatment of lignocellulosic biomass with alkalic and metal salts. This study investigated the effect of a sequential sodium phosphate dodecahydrate ($Na_3PO_4{\cdot}12H_2O$) then zinc chloride ($ZnCl_2$) pretreatment for enhanced delignification, enzymatic saccharification and reduced inhibitor yields from waste corn cobs. The effect of this sequential inorganic salt pretreatment on the physical structure was further assessed using scanning electron microscopy (SEM) and Fourier Transform Infrared (FTIR) analysis.

2. Materials and methods

2.1. Materials

The corn cobs used in this study were obtained from the Ukulinga research farm (Pietermaritzburg, South Africa) (29° 67′ E, 30° 40′ S). These were subsequently oven dried at 60 °C for 24 h and thereafter milled to a particle size of less than 1–2 mm by a centrifugal miller (Retsch ZM-1, South Africa). Powdered corn cobs were then stored at room temperature. Compositional analysis of native (untreated) and pretreated biomass was determined using the NREL method (Sluiter et al., 2008). All chemicals were purchased from Merck, South Africa. Cellic CTec 2 enzyme was generously provided by Novozymes (Novozymes A/S, Denmark).

2.2. Preliminary screening

A preliminary screening was carried out to determine the influence of the alkalic salt ($Na_3PO_4{\cdot}12H_2O$) and metal salt ($ZnCl_2$) (both combined and sequential pretreatments) on the enzymatic saccharification of corn cobs. These initial experiments were performed using a total volume of 100 mL and a solid to liquid ratio of 10% (w/v). The $Na_3PO_4{\cdot}12H_2O$ and $ZnCl_2$ concentration ranges were selected based on the previous works by Qing et al. (2016b) and Kang et al. (2013), respectively. The combined pretreatment (COM–$Na_3PO_4{\cdot}12H_2O$–$ZnCl_2$) was carried out in a single stage process and consisted of 10% (w/v) $Na_3PO_4{\cdot}12H_2O$ and 3% (w/v) $ZnCl_2$. The sequential pretreatment ($Na_3PO_4{\cdot}12H_2O$–$ZnCl_2$) was carried

out using 10% (w/v) $Na_3PO_4{\cdot}12H_2O$ (first stage) and 3% (w/v) $ZnCl_2$ (second stage). The reverse sequential order was also assessed ($ZnCl_2$–$Na_3PO_4{\cdot}12H_2O$). Control experiments were carried out to determine the effect of single pretreatments and these included water alone, 10% (w/v) $Na_3PO_4{\cdot}12H_2O$ alone and 3% (w/v) $ZnCl_2$ alone. Pretreatments were carried out in a laboratory autoclave at setpoint conditions of 121 °C for 30 min (15 min per stage for the sequential pretreatment) according to a previous study on corn cobs (Gao and Rehmann, 2014). The pretreatment efficiency of each treatment was evaluated based on reducing sugar yields after enzymatic hydrolysis.

2.3. Modeling and optimization of the sequential pretreatment

The Box-Behnken design was used to generate a total of seventeen experimental runs for model development. Input parameters consisted of $Na_3PO_4{\cdot}12H_2O$ concentration (5–15%), $ZnCl_2$ concentration (1–5%) and Solid to liquid ratio (5–15%) (Table 1). The experimental reducing sugar yields obtained after enzymatic hydrolysis were used to fit a polynomial model equation, relating the input parameters to the reducing sugar yields using Design Expert software (Stat-Ease Inc., USA).

2.4. Sequential inorganic salt pretreatment process

Powdered corn cobs (g) were immersed in 100 mL of inorganic salt solution according to the experimental design (Table 1). $Na_3PO_4{\cdot}12H_2O$ and $ZnCl_2$ were used for the first and second pretreatment stages, respectively. The first pretreatment stage was performed using $Na_3PO_4{\cdot}12H_2O$ solution and was autoclaved at 121 °C for 15 min. Pretreated biomass was then filtered and washed three times with deionized water. The solid residue obtained after the first stage was oven dried at 70 °C overnight and thereafter used for the second pretreatment stage ($ZnCl_2$) which was also autoclaved at 121 °C for 15 min. After the second stage pretreatment, the treated biomass was filtered, washed and dried as previously specified and the solid residue obtained was used for the enzymatic hydrolysis stage.

2.5. Enzymatic saccharification

The pretreated corn cobs were mixed with citrate buffer (pH 4.8, 0.05M) in a 100 mL Erlenmeyer flask and the solid loading was kept at 10% (w/v). Cellic CTec2 (10 FPU/g substrate) was added to the pretreatment mixture. Enzymatic saccharification was carried out at 50 °C and 100 rpm for 72 h. After 72 h, the samples were centrifuged to remove any unhydrolyzed biomass. Reducing sugar and glucose were analysed using the 3.5-dintrosalicylic acid method (Miller, 1959) and Megazyme glucose kits (Megazyme, Ireland) respectively. All seventeen experiments were carried out in duplicate.

Table 1
Experimental runs for the sequential inorganic salt pretreatment.

Run	$Na_3PO_4{\cdot}12H_2O$ concentration (%)	$ZnCl_2$ concentration (%)	Solid to liquid ratio (%)	Reducing sugar (g/g)
1	10	5	15	0.79
2	5	3	15	0.72
3	10	3	10	0.92
4	10	3	10	0.99
5	5	3	5	0.82
6	15	3	5	1.13
7	10	1	5	0.98
8	10	3	10	0.95
9	5	5	10	0.76
10	15	3	15	0.86
11	5	1	10	0.78
12	15	5	10	0.98
13	15	1	10	0.99
14	10	5	5	0.97
15	10	1	15	0.97
16	10	3	10	0.93
17	10	3	10	0.94

2.6. Analytical methods

2.6.1. Scanning electron microscopy (SEM)

The corn cobs (native, controls and optimally pretreated) were examined under scanning electron microscopy (SEM). Samples were mounted on aluminium specimen mounts, gold sputter coated (Eiko IB-3 Ion Coater) and observed using conventional SEM at a magnification of 500×(ZEISS EVO LS 15).

2.6.2. Fourier Transform Infrared analysis (FTIR)

Functional group changes of native, control and optimally pretreated biomass were analysed by Fourier Transform Infrared spectroscopy (FTIR) using a Perkin Elmer 100 (Waltham, MA, USA). The samples were ground with spectroscopic grade KBr and pressed to produce diameter pellets. The FTIR spectra were recorded between 380 and 4000 cm^{-1}.

2.6.3. Inhibitor analysis

The hydrolysate obtained after the chemical pretreatment process (controls and optimally pretreated) was evaluated for inhibitors such as acetic acid, furfural and 5-hydroxymethyl furfural. The samples were analysed using coupled Varian 3800 gas chromatography (Varian Palo Alto, California, USA) and Varian 1200 mass spectrometry (GC–MS) as previously described by Rorke et al. (2017).

3. Results and discussion

3.1. Primary screening stage

The sequential pretreatments resulted in higher reducing sugar yields of 1.03 g/g ($Na_3PO_4{\cdot}12H_2O$–$ZnCl_2$) and 0.90 g/g ($ZnCl_2$–$Na_3PO_4{\cdot}12H_2O$) compared to 0.45 g/g observed for the combined pretreatment (COM–$Na_3PO_4{\cdot}12H_2O$–$ZnCl_2$). The $Na_3PO_4{\cdot}12H_2O$–$ZnCl_2$ sequential order was then further considered for optimization. Control pretreatment experiments performed with native, water pretreated, $ZnCl_2$ pretreated and $Na_3PO_4{\cdot}12H_2O$ pretreated biomass, gave reducing sugar yields of 0.29 g/g, 0.49 g/g, 0.58 g/g and 0.89 g/g, respectively.

3.2. Assessment of model significance

Assessment of the model fitness was performed using the Analysis of Variance (ANOVA) and the results are presented in Table 2. The model F-value of 13.05 and p-value of 0.0013 suggests that it was significant. Generally, a p-value <0.05 indicates model significance (Qing et al., 2016a). It was determined that $Na_3PO_4{\cdot}12H_2O$ concentration was the most significant parameter among all parameters (<0.0001) followed by solid to liquid ratio (0.0013) whereas the least significant parameter was $ZnCl_2$ concentration (0.0812). In addition, an R^2 value of 0.94 was obtained, indicating that the developed model could account for 94% of the variation in the observed data. These statistical indices show that the developed model was efficient for describing the effect of the inputs on the corresponding output. The model polynomial Eq. (1) is shown below:

Table 2
Analysis of Variance of the developed sequential inorganic salt pretreatment model.

Factor	Sum of squares	Degrees of freedom (*df*)	Standard error	*F* value	*p* value (probability > *F*)
Intercept or model	0.17	9	0.019	13.05	0.0013 significant
A- (%)	0.097	1	0.097	66.30	<0.0001
B- (%)	6.050E-003	1	6.050E-003	4.14	0.0812
C- (%)	0.039	1	0.039	26.85	0.0013
AB	2.500E-005	1	2.500E-005	0.017	0.8996
AC	7.225E-003	1	7.225E-003	4.95	0.0615
BC	7.225E-003	1	7.225E-003	4.95	0.0615
A^2	0.014	1	0.014	9.29	0.0186
B^2	5.813E-004	1	5.813E-004	0.40	0.5481
C^2	1.918E-004	1	1.918E-004	0.13	0.7277
Residual error	0.010	7	1.460E-003	–	
Lack of fit	7.300E-003	3	2.433E-003	3.33	0.1376 Not significant
Pure error	2.920E-003	4	7.300E-004	–	

$$\text{Reducing sugar yield}(g/g) = 0.34 + 0.084A + 0.044B + 0.021C + 0.00025AB - 0.0017AC - 0.0043BC - 0.0023A^2 - 0.0029B^2 - 0.00024C^2 \quad (1)$$

where, A, B and C are $Na_3PO_4{\cdot}12H_2O$ concentration, $ZnCl_2$ concentration and solid to liquid ratio, respectively.

3.3. Interactive effect of the input process parameters on the reducing sugar yield

The reducing sugar yields obtained from the experimental runs are shown in Table 1. Maximum reducing sugar yield (1.13 g/g) was obtained using pretreatment conditions of 15%, 3% and 5% for $Na_3PO_4{\cdot}12H_2O$ concentration, $ZnCl_2$ concentration and solid to liquid ratio, respectively. The lowest reducing sugar yield (0.72 g/g) was obtained under milder pretreatment conditions of 5%, 3% and 15% for $Na_3PO_4{\cdot}12H_2O$ concentration, $ZnCl_2$ concentration and solid to liquid ratio, respectively.

The combined effect of $ZnCl_2$ concentration and $Na_3PO_4{\cdot}12H_2O$ concentration on the reducing sugar yield when solid to liquid ratio is maintained at its midpoint value is shown in Fig. 1A. Low reducing sugar yields (<0.80 g/g) were obtained with pretreatment at low concentrations of $Na_3PO_4{\cdot}12H_2O$ (<7.5%) and $ZnCl_2$ concentration in the range of 1–5%. An increase in $Na_3PO_4{\cdot}12H_2O$ concentration from 5% to 15% resulted in an increase in the reducing sugar yield from 0.74 g/g to 1.01 g/g. These results further suggest that the $ZnCl_2$ concentration had a minimal effect on the reducing sugar yield compared to the $Na_3PO_4{\cdot}12H_2O$ Sharp increases in the reducing sugar yields observed at higher $Na_3PO_4{\cdot}12H_2O$ concentrations may be attributed to the degradation of cellulose into monomers and oligosaccharides (Qing et al., 2016a).

The interactive effect of solid to liquid ratio and $Na_3PO_4{\cdot}12H_2O$ concentration on the reducing sugar yield when $ZnCl_2$ concentration is kept at its centre point is shown in Fig. 1B. Higher solid to liquid ratios (7.5–15%) and low $Na_3PO_4{\cdot}12H_2O$ concentrations (5–7.5%) resulted in low reducing sugar yields (<0.83 g/g). On the other hand, lower solid to liquid ratios (<7.5%) and higher $Na_3PO_4{\cdot}12H_2O$ concentrations (5–15%) resulted in an increase in reducing sugar yield from 0.73 to 1.11 g/g. Similar results were reported by Qing et al. (2016 b) where a 69% increase in the reducing sugar yield was observed when the $Na_3PO_4{\cdot}12H_2O$ concentration was increased from 1% to 11%. The higher reducing sugar yields observed for the present study may be as a result of improved enzymatic saccharification which was enhanced by the accessibility of cellulose due to the sequential $Na_3PO_4{\cdot}12H_2O$ and $ZnCl_2$ pretreatment.

The interactive effect of solid to liquid ratio and $ZnCl_2$ concentration on the reducing sugar yield when the $Na_3PO_4{\cdot}12H_2O$ concentration is maintained at its median value is shown in Fig. 1C. When the solid to liquid ratio was kept at its minimum value (1%) and the $ZnCl_2$ concentration was varied from 1 to 5%, the reducing sugar yield increased from 0.98 to 1.01 g/g. On the other hand, an increase in the solid to liquid ratio from 5 to 15% while keeping the $ZnCl_2$ concentration at its lowest value (1%) led to a decrease in the reducing sugar yield from 0.98 to 0.93 g/g. This further illustrates that solid to liquid ratio has a marginal effect on the reducing sugar yield. Kang et al. (2013) reported a maximum enzymatic digestibility of 62.2% when 5% $ZnCl_2$ was used against 42.6% digestibility when a lower concentration of 0.5% $ZnCl_2$ was used. $ZnCl_2$ has shown to be an effective swelling chemical reagent which facilitates biomass fractionation, thus resulting in the degradation of glycosidic linkages within cellulosic structures (Alfred, 1964; Amarasekara and Ebede, 2009). Additionally, water molecules from the hydrated $ZnCl_2$ subsequently act as nucleophiles which yield D-glucose (Amarasekara and Ebede, 2009).

3.4. Validation of the optimized model

Model validation carried out using the optimized conditions of 14.02% $Na_3PO_4{\cdot}12H_2O$ concentration, 3.65% $ZnCl_2$ concentration and 5% solid to liquid ratio gave a reducing sugar yield of 1.10 ± 0.01 g/g against a predicted value of 1.09 g/g. Thus, the sequential pretreatment gave a 1.2-fold and 1.9-fold improvement in the reducing sugar yield compared with the $Na_3PO_4{\cdot}12H_2O$ control and $ZnCl_2$ control, respectively. The observed reducing sugar yield above 1 g/g using the present sequential pretreatment is explained by an 11.1% and 13.6% mass gain when a water molecule reacts with each sugar unit during cellulose and hemicellulose hydrolysis, respectively, as previously described by Wyman et al. (2005). In addition to this, hydrothermal pretreatment has been shown to affect the average molecular weight of cellulose (Fan et al., 2016). Moreso, aldehyde molecules with molecular weights smaller than glucose in the hydrolysate can react with the DNS reagent, resulting in a slight overestimation of reducing sugar yield (Rivers et al., 1984). The glucose yield was found to be 0.71 g/g for the optimized sequential sample compared to the native (0.16 g/g), water pretreated (0.22 g/g), $ZnCl_2$ pretreated (0.25 g/g) and $Na_3PO_4{\cdot}12H_2O$ pretreated sample (0.61 g/g). The high glucose yields further substantiate the observed reducing sugar yield.

3.5. Comparison of the sequential inorganic salt pretreatment with reported studies on corn cobs

Table 3 compares the sugar yield reported in previous pretreatment studies with the present optimized sequential inorganic salt

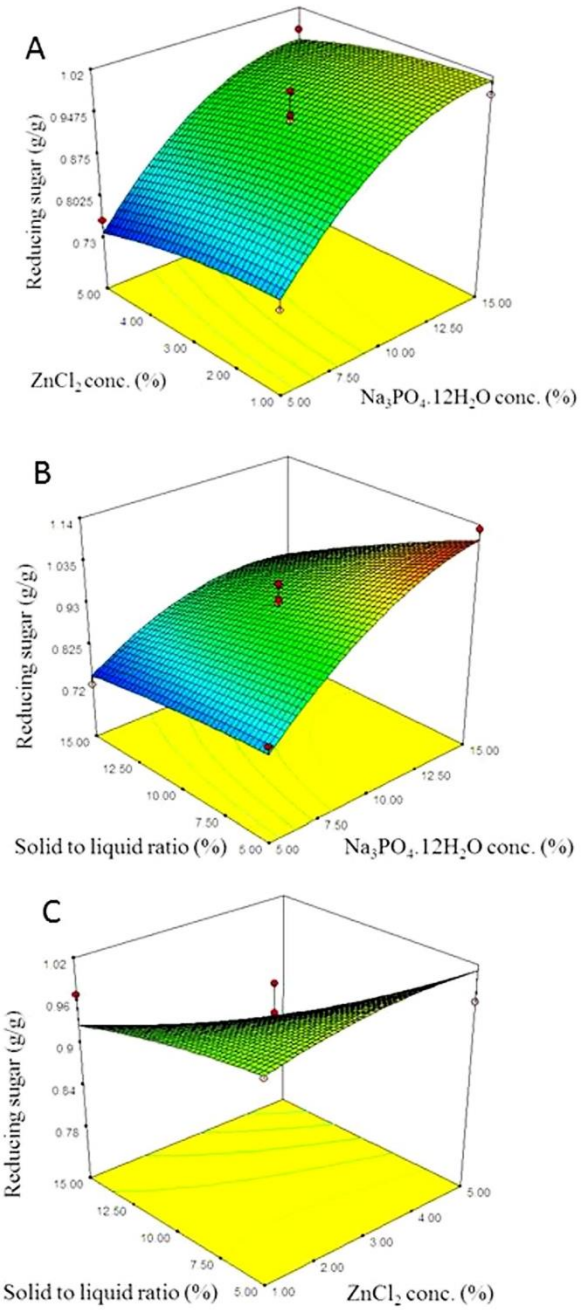

Fig. 1. Response surface plots illustrating the interactive effect of the various input parameters affecting the sequential pretreatment of corn cobs (A) interaction between $Na_3PO_4{\cdot}12H_2O$ and $ZnCl_2$ concentration, (B) $Na_3PO_4{\cdot}12H_2O$ concentration and Solid to liquid ratio, (C) $ZnCl_2$ concentration and Solid to liquid ratio.

pretreatment. The optimized sequential pretreatment was shown to be more effective for higher reducing sugar yields compared to previous studies using the same substrate. For instance, the present study gave a 1.3-fold increase in the sugar yield compared to a previous report by Satimanont et al. (2012) when corn cobs was treated using a dilute acid pretreatment method (1.75% H_3PO_4). Additionally, Potumarthi et al. (2012) pretreated corn cobs under moderate alkaline conditions (1M NaOH) and recorded a reducing sugar yield of 0.11 g/g which was shown to be 10-fold lower compared to the present study. Interestingly, a reported microwave-assisted alkaline pretreatment under mild conditions (2% KOH) gave a 5.5% lower reducing sugar yield compared to the present optimized sequential pretreatment (Wanitwattanarumlug et al., 2012).

3.6. Influence of the sequential $Na_3PO_4{\cdot}12H_2O$-$ZnCl_2$ pretreatment on the physical structure of corn cobs

3.6.1. Characterization of native and pretreated corn cobs

Within native lignocellulosic biomass, cellulose and hemicellulose structures are cemented together by layers of recalcitrant lignin moieties that prevent both chemical and biological degradation (Saha et al., 2013; Li et al., 2015). Therefore, increments in the cellulose content, hemicellulose removal and delignification are important indices for pretreatment efficiency (Loow et al., 2015). The native biomass in the present study consisted of 34.21% cellulose, 39.08% hemicellulose and 6.32% lignin (Table 4). The optimized sequential pretreated sample contained 59.98% cellulose, 28.33% hemicellulose and 2.30% lignin. Thus, a 75.33% increase in the cellulose content, 27.50% hemicellulose removal and 63.61% delignification was observed with the optimized sequential pretreatment. Strong nucleophilic species within alkalic salts (PO_4^{3-}, HPO_4^{2-} and HS^-) have shown to facilitate the cleavage of phenolic β-aryl ether bonds of lignin, resulting in effective delignification with limited attack on carbohydrates (Gu et al., 2013). Furthermore, alkalic salts act as weak bases and result in effective removal of acetyl groups from xylan polymers which ameliorate enzymatic saccharification and thus cellulose digestibility (Kim et al., 2014). On the other hand, metal salts such as $ZnCl_2$ act as Lewis acids that promote the degradation of glycosidic linkages within lignocellulosic structures (Amarasekara and Ebede, 2009). These properties may account for the observed high cellulose content, hemicellulose removal and delignification in the present optimized sequential pretreatment.

Table 4
Structural composition of control and optimized samples.

Sample	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Native	34.21	39.08	6.32
H_2O alone	37.94	38.95	6.24
$ZnCl_2$ alone	42.22	37.15	6.11
$Na_3PO_4{\cdot}12H_2O$ alone	55.97	29.75	3.99
Optimized sequential	59.98	28.33	2.30

Table 3
Comparison of the various pretreatment strategies on corn cobs.

Substrate	Pretreatment conditions	Reducing sugar yield (g/g)	References
Corn cobs	14.02% (w/v) $Na_3PO_4{\cdot}12H_2O$, 3.65% $ZnCl_2$, 5% S:L, 121 °C, 30 min	1.10	This study
Corn cobs	2% (w/v) KOH, 5% S:L, 120 °C, 25 min	1.04	Wanitwattanarumlug et al. (2012)
Corn cobs	1.75% (w/w) H_3PO_4, 3.33% S:L, 140 °C, 10 min	0.82	Satimanont et al. (2012)
Corn cobs	0.5M NaOH, 12.5% S:L, 121 °C, 30 min	0.92	Gao and Rehmann (2014)
Corn cobs	1 M NaOH, 20% S:L, 121 °C, 20 min	0.11	Potumarthi et al. (2012)

S:L – Solid to liquid ratio.

The control samples showed a lower cellulosic content, hemicellulose removal and delignification compared to the optimized sequential pretreatment. The water pretreated sample contained 37.94% cellulose, 38.95% hemicellulose and 6.24% lignin whereas the $ZnCl_2$ pretreated sample consisted of 42.22% cellulose, 37.15% hemicellulose and 6.11% lignin. In contrast, the $Na_3PO_4{\cdot}12H_2O$ pretreated sample had a high cellulose content (55.97%) and low hemicellulose (29.75%) and lignin content (3.99%). The optimized sequential pretreatment was the most effective treatment and resulted in a 1.8-fold and 2.7-fold increase in the cellulose content and delignification, respectively, compared to the native corn cob sample. A similar observation was reported by Qing et al. (2016a) where the cellulose content and delignification in corn stover increased by 56.3% and 20.5%, respectively, when pretreated with combined 4% Na_3PO_4 and 10% Na_2S at 120 °C for 40 min in contrast to the untreated sample. Likewise, Qing et al. (2016 b) reported a 1.7-fold and 3.3-fold increase in the cellulose content and delignification, respectively, from bamboo shoot shell when pretreated with combined 5% $Na_3PO_4{\cdot}12H_2O$ and 0.6 g/g H_2O_2 at 80 °C for 120 min compared with its native substrate sample. Similarly, Kang et al. (2013) and Chen et al. (2014) observed an 89.5% and 41.4% increase in the glucan recovery using 2M and 0.1M $ZnCl_2$, respectively.

Alkalic salts such as $Na_3PO_4{\cdot}12H_2O$ have shown to cleave ester and glycosidic bonds in the cell wall matrix, thus causing lignin disruption, cellulose swelling and decrystallization (Qing et al., 2016a; Cheng et al., 2010). Metal salts such as $ZnCl_2$ play a substantial role in the breakage of ether bonds between xylan polymers consequently resulting in ample hemicellulose removal (Kang et al., 2013; Moodley and Gueguim Kana, 2017). The combined effect of these mechanisms may account for the observed high cellulose content, hemicellulose removal and delignification.

3.6.2. Scanning electron microscopy (SEM)

Morphological changes in biomass surface structures were analysed by scanning electron microscopy. The native biomass displayed a smooth, well-ordered and intact structure compared to the pretreated biomass. All pretreated biomass exhibited distorted, porous and disintegrated structures with many cracks on the surface of the biomass. More pronounced structural changes were observed in the optimized sequential pretreated sample which showed extreme cavitation and fissures of the surface structures compared to the native and control samples. Similar results were reported by Guo et al. (2016) when a $NaHCO_3$ pretreatment was used. The damaged surface area of the pretreated biomass is attributable to the weakening of the cell wall during delignification (Ramadoss and Muthukumar, 2015). The highly porous and disorganized surface appearance observed with the optimized sequential pretreated sample suggests that it was more efficient for the modification of the resistant lignocellulosic structure. This result was in line with the observed high cellulose content, hemicellulose removal and delignification. These findings showed that the sequential pretreatment caused major structural damage which allowed for improved enzymatic saccharification. The disruption to the corn cob surface structure is substantiated by the modifications in structural components shown by the FTIR analysis.

3.6.3. Fourier Transform Infrared (FTIR) spectra analysis

FTIR spectral data revealed the presence of lignin, hemicellulose and cellulose structures usually found within lignocellulosic biomass. Absorption enhancement of band peaks relating to cellulose, hemicellulose and lignin were shown to be stronger for the optimized sequential pretreated sample compared to the native and control samples. The band peak at 898 cm^{-1} corresponds to breakage of β-glycosidic linkages in cellulose whereas the peaks at 1031 cm^{-1} and 1162 cm^{-1} represents the vibrations of C–O–C associated with the pyranose ring skeleton and C–O–C asymmetric stretching in cellulose, respectively. In addition, the absorption spectra at 1371 cm^{-1} and 1426 cm^{-1} are linked with C–H bending vibrations and symmetric CH_2 bending and scissoring of cellulose, respectively (Chen et al., 2015). C–H stretching and –OH stretching of intramolecular hydrogen within cellulose were indicated by absorptive peaks 2895 cm^{-1} and 3312 cm^{-1}, respectively (Qing et al., 2016a). Strong absorption peaks at 1243 cm^{-1} and 1514 cm^{-1} relates to C=O stretching vibration and C=C stretching vibration of the aromatic ring in lignin, respectively (Qing et al., 2016a). C=O stretching vibrations of acetyl groups in hemicellulose and ester groups in lignin were represented by band position 1727 cm^{-1} (Ramadoss and Muthukumar, 2015). Previous studies on inorganic salt pretreatments revealed similar observations (Qing et al., 2016a,b; Ramadoss and Muthukumar, 2015). Spectral data from the FTIR analysis showed that the optimized sequential pretreatment was effective for the unravelling of cellulose, hemicellulose and lignin characteristic structures and thus enhanced the enzymatic saccharification of corn cobs.

3.7. Inhibitor profile of the pretreatment hydrolysate

Some major fermentation inhibitor compounds formed after chemical pretreatment include acetic acid, furfural and 5-hydroxymethyl furfural (HMF). These may inhibit microbial growth and metabolism and thus negatively impact on the fermentation process (Sindhu et al., 2016). Table 5 shows the concentrations of major inhibitors present in the control and optimized sequential pretreated samples. The $ZnCl_2$ control sample led to the highest concentration of acetic acid (2.4×10^{-2} μg/g) whereas the lowest concentration was observed in the optimized sequential pretreatment sample (7×10^{-3} μg/g). Acetic acid concentrations above 1.5 g/L have shown to be inhibitory for bioethanol production by *Saccharomyces cerevisiae* (Wikandari et al., 2010). Acetic acid formation is largely due to the disruption of ester and acetyl linkages within lignocellulosic structures (Kamireddy et al., 2013). The high acetic acid concentration produced by the $ZnCl_2$ control pretreatment may be due to its high Lewis acid activity (Leshkov and Davis, 2011). Metal salts such as $ZnCl_2$ cause disruption of acetyl groups in hemicellulose and thus may have led to the higher acetic acid concentration compared to the other samples. On the other hand, acetic acid formation observed for the $Na_3PO_4{\cdot}12H_2O$ control sample may be due the breakage of ester bonds in lignin and acetyl linkages in hemicellulose. Furthermore, $Na_3PO_4{\cdot}12H_2O$ possesses excellent buffering capacity which has previously shown to stimulate the production of acidic compounds such as acetic acid (Qing et al., 2016a,b).

Furfural concentration was found to be highest for the optimized sequential pretreated sample (3.7×10^{-2} μg/g) with the lowest concentration detected for the water control sample (1.4×10^{-3} μg/g). On the other hand, the $ZnCl_2$ pretreated sample (1.4×10^{-3} μg/g) showed a higher release of HMF compared to the other samples where no HMF was detected. Furan derivatives such as furfural and HMF typically form during pentose and hexose

Table 5
Inhibitor profile of control and optimized sequential inorganic salt pretreated corn cobs.

Sample	Inhibitor concentration (μg/g)		
	Acetic acid	Furfural	HMF
Water	1.5×10^{-2}	1.4×10^{-3}	ND
$ZnCl_2$	2.4×10^{-2}	2.1×10^{-2}	1.4×10^{-3}
$Na_3PO_4{\cdot}12H_2O$	1.4×10^{-2}	2.3×10^{-4}	ND
Optimized sequential	7×10^{-3}	3.7×10^{-2}	ND

HMF – 5-Hydroxymethyl furfural.
ND – Not detected.

Table 6
Comparison of inhibitor concentrations produced using inorganic salt pretreatment on different substrates.

Substrate	Pretreatment system	Pretreatment conditions	Inhibitors (μg/g)			References
			Acetic acid	Furfural	HMF	
Corn cobs	Sequential	14.02% (w/v) $Na_3PO_4{\cdot}12H_2O$, 3.65% $ZnCl_2$, 5% S:L, 121 °C, 30 min	7.4×10^{-3}	3.7×10^{-2}	ND	This study
Bamboo shoot shell	Single	9% Na_3PO_4, 0.3 g/g H_2O_2, 1% S:L, 80 °C, 120 min	9.5×10^{4}	–	–	Qing et al. (2016b)
Sugarcane bagasse	Single	0.1 M $ZnCl_2$, 10% S:L, 170 °C, 30 min	–	3.46×10^{4}	2.52×10^{3}	Chen et al. (2014)
Corn stover	Single	4% $Na_3PO_4.H_20$, 10% Na_2S, 1% S:L, 120 °C, 40 min	2.0×10^{5}	–	–	Qing et al. (2016a)
Sugarcane leaf waste	Single	2 M $ZnCl_2$, 10% S:L, 800 W, 6 min	7.8×10^{-3}	2.3×10^{-2}	2.1×10^{-3}	Moodley and Gueguim Kana (2017)

S:L – Solid to liquid ratio.
ND – Not detected.

degradation (Ravindran and Jaiswal, 2016). Furfural and HMF concentrations >1 g/L have shown to disrupt the bioethanol production process by affecting microbes such as *Saccharomyces cerevisiae* (Wikandari et al., 2010). Generally, the quantities of these compounds are elevated when the process conditions become more severe, either with a higher exposure time to stronger chemical conditions or temperatures (Harmsen et al., 2010). The high furfural concentration recorded for the optimized sequential pretreated sample may be due to the moderate severity of pretreatment conditions ($Na_3PO_4{\cdot}12H_2O$ and $ZnCl_2$) employed. On the other hand, the low HMF concentrations observed in this study may be attributed to the rehydration of this compound to levulinic acid (Yang et al., 2012).

The present optimized sequential pretreatment generated a low acetic acid concentration of 7.4×10^{-3} μg/g compared to single stage inorganic salt pretreatment studies on bamboo shoot shell (9.5×10^{4} μg/g) (Qing et al., 2016 b), corn stover (2.0×10^{5} μg/g) (Qing et al., 2016a) and sugarcane leaf waste (7.8×10^{-3} μg/g) (Moodley and Gueguim Kana, 2017) (Table 6). Similarly, a low furfural concentration (3.7×10^{-2} μg/g) was observed using the present optimized sequential pretreatment compared to an earlier pretreatment on sugarcane bagasse (3.46×10^{4} μg/g) (Chen et al., 2014). The concentrations of inhibitor compounds detected for the present sequential pretreatment are below the commonly reported inhibitory concentrations and therefore eliminate the use of expensive detoxification methods.

4. Conclusion

A novel sequential alkalic and metal salt pretreatment of corn cobs was evaluated in this study. Optimization of this pretreatment strategy gave a high R^2 value of 0.94. A maximum reducing sugar yield of 1.10 ± 0.01 g/g and delignification of 63.61% was obtained with 14.02% $Na_3PO_4{\cdot}12H_2O$, 3.65% $ZnCl_2$ and 5% solid to liquid ratio. SEM and FTIR analysis revealed major structural changes after pretreatment. Fermentation inhibitor compounds were present in low quantities (<1 μg/g) and thus circumvent costly detoxification methods. A 10-fold increase in the sugar yield was observed compared to previous reports on the same substrate.

Acknowledgements

This work was supported by the National Research Foundation (NRF) (Grant number: 101316) of South Africa. Opinions expressed and conclusions arrived at, are those of the author and are not necessarily attributed to the NRF.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2017.06.175.

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Chapter 3: Supplementary material

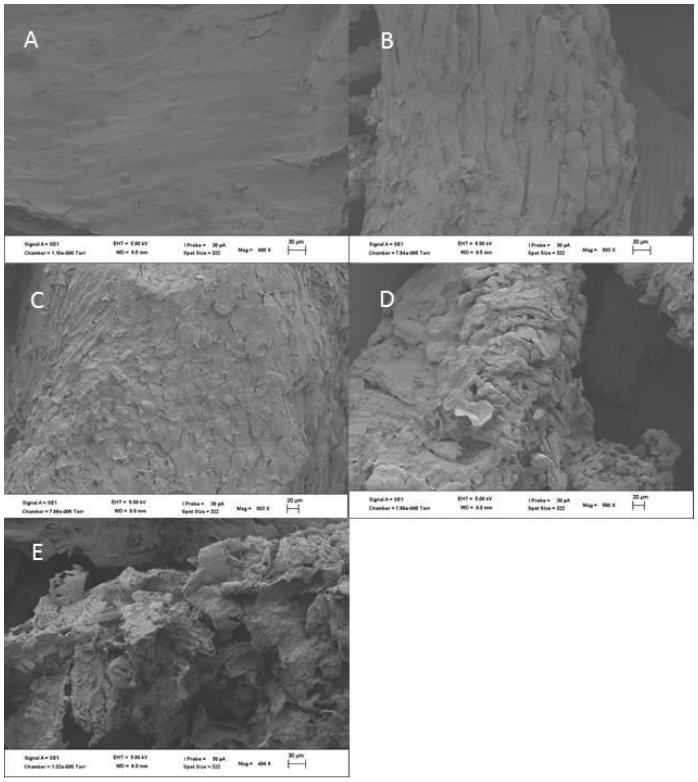

Fig. S1. SEM micrographs of corn cobs (A) native (B) water alone (C) $ZnCl_2$ alone (D) $Na_3PO_4.12H_2O$ alone (E) Optimized sequential.

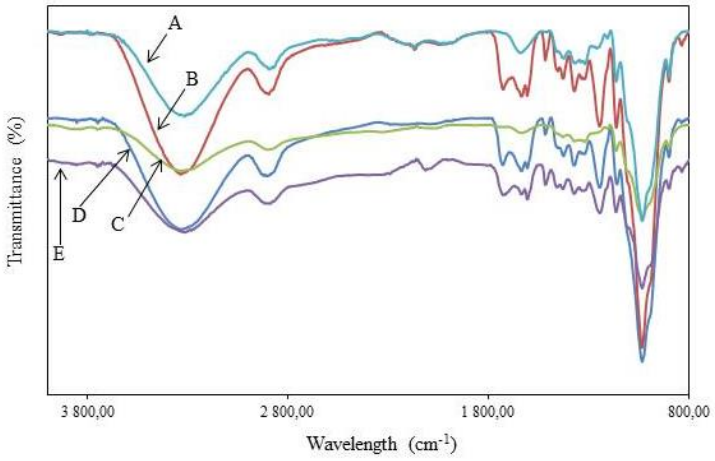

Fig. S2. FTIR spectra of corn cob samples: (A) native; (B) water alone; (C) $ZnCl_2$ alone; (D) $Na_3PO_4.12H_2O$ alone and (E) Optimized sequential.

Table S1. Characteristics and variations of absorption bands observed in the FTIR analysis of native and pretreated corn cobs.

Wave number (cm^{-1})	Functional group	Band assignment	Native	Water alone	$ZnCl_2$ alone	$Na_3PO_4.12H_2O$ alone	Optimized sequential
898	β-glycosidic bond	cellulose	0	0	0.029	0.034	0.051
1031	C–O–C associated with the pyranose ring skeletal vibration	cellulose	0.070	0.152	0.070	0.162	0.110
1162	C−O−C asymmetric stretching	cellulose	0.018	0.031	0.020	0.048	0.065
1243	C=O stretching vibration	lignin	0	0.018	0.020	0.051	0.066
1371	C−H bending vibrations	cellulose and hemicellulose	0	0	0.025	0.038	0.054
1426	symmetric CH_2 bending and scissoring	cellulose	0	0	0.023	0.033	0.052
1515	C=C stretching of the aromatic ring	lignin	0	0	0.017	0.022	0.049
1727	C=O stretching of acetyl or carboxylic acid	hemicellulose and lignin	0	0	0.018	0.038	0.055
2895	C−H stretching	cellulose	0	0.001	0.030	0.044	0.060
3312	−OH stretching intramolecular hydrogen	cellulose	0.012	0.042	0.041	0.075	0.077

Table S2. Observed and RSM predicted reducing sugar yields obtained for each run.

Run	Observed reducing sugar (g/g)	RSM predicted reducing sugar (g/g)
1	0.79	0.79
2	0.72	0.75
3	0.92	0.95
4	0.99	0.95
5	0.82	0.80
6	1.13	1.11
7	0.98	0.98
8	0.95	0.95
9	0.76	0.74
10	0.86	0.88
11	0.78	0.80
12	0.98	0.96
13	0.99	1.01
14	0.97	1.01
15	0.97	0.93
16	0.93	0.95
17	0.94	0.95

Footnote: RSM- Response Surface Methodology.

Table S3. Analysis of Variance of the developed model.

Factor	Sum of Squares	Degrees of freedom (*df*)	Mean Square	*F* value	*p* value (probability>*F*)
Intercept or model	0.17	9	0.019	13.05	0.0013 significant
A- (%)	0.097	1	0.097	66.30	< 0.0001
B- (%)	6.05×10^{-3}	1	6.05×10^{-3}	4.14	0.0812
C- (%)	0.039	1	0.039	26.85	0.0013
AB	2.50×10^{-5}	1	2.50×10^{-5}	0.017	0.8996
AC	7.23×10^{-3}	1	7.23×10^{-3}	4.95	0.0615
BC	7.23×10^{-3}	1	7.23×10^{-3}	4.95	0.0615
A^2	0.014	1	0.014	9.29	0.0186
B^2	5.81×10^{-4}	1	5.81×10^{-4}	0.40	0.5481
C^2	1.92×10^{-4}	1	1.92×10^{-4}	0.13	0.7277
Residual Error	0.010	7	1.46×10^{-3}	-	
Lack of fit	7.30×10^{-3}	3	2.43×10^{-3}	3.33	0.1376 not significant
Pure Error	2.92×10^{-3}	4	7.30×10^{-4}	-	

CHAPTER 4

Development of a sequential alkalic salt and dilute acid pretreatment for enhanced sugar recovery from corn cobs

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This chapter has been published in *Energy Conversion and Management* (160, 22-30) with the title: Development of a sequential alkalic salt and dilute acid pretreatment for enhanced sugar recovery from corn cobs.

The published paper and supplementary material are presented in the following pages.

Energy Conversion and Management 160 (2018) 22–30

Contents lists available at ScienceDirect

Energy Conversion and Management

journal homepage: www.elsevier.com/locate/enconman

Development of a sequential alkalic salt and dilute acid pretreatment for enhanced sugar recovery from corn cobs

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ARTICLE INFO

Keywords:
Alkalic salt
Acid
Pretreatment
Corn cobs
Lignocellulosic biomass

ABSTRACT

This study presents a sequential sodium phosphate dodecahydrate ($Na_3PO_4{\cdot}12H_2O$) and sulfuric acid (H_2SO_4) pretreatment to enhance sugar release from corn cobs. The effects of $Na_3PO_4{\cdot}12H_2O$ concentration, H_2SO_4 concentration and solid to liquid ratio on the reducing sugar yield were investigated. The developed model had a coefficient of determination value (R^2) of 0.98. Maximum reducing sugar yield of 0.99 ± 0.01 g/g was obtained with 12.70% $Na_3PO_4{\cdot}12H_2O$, 1.04% H_2SO_4 and 14.49% solid to liquid ratio. A 9-fold increase in the sugar yield was observed compared to previous reports on corn cobs. An intelligent model was developed to determine functional relationships between the input and output parameters. Reducing sugar yield was majorly dependent on $Na_3PO_4{\cdot}12H_2O$ concentration and fits a Weibull type of relationship. Sequential pretreatment of lignocellulosic material with alkalic salt and dilute acid significantly enhanced sugar recovery and demonstrated high efficiency for microbial production of biofuels and bioproducts.

1. Introduction

Lignocellulosic biomass provides an excellent feedstock for microbial production of various biofuel processes and value-added products since it is plentiful, cost-effective and does not compete with global food security [1–3]. It is majorly comprised of cellulose (38–50%), hemicellulose (23–32%) and lignin (15–25%) [4]. In the present time, various potential biofuel lignocellulosic feedstocks are being assessed and include bamboo shoot shell [5], sorghum leaf wastes [6], sugarcane bagasse [7], sugarcane leaves [8], corn stover [9] and corn cobs [10], among others. The annual global corn production exceeds 1.03 billion metric tons. About 50% of this comprises the leaves, stalks, husks and cobs and are commonly disposed of as waste material [11]. Corn cobs are predominantly composed of 32–45% cellulose, 39% hemicellulose and 6–14% lignin. Corn cobs have an energy density between 4960 and 5210 MJ/kg, which is about two times higher than frequently used biomasses such as corn stover (2550 MJ/kg) and switchgrass (2500 MJ/kg). Moreover, it possesses a relatively low lignin content and this makes it a competitive candidate for use in biofuel production processes compared with corn stover and switchgrass [12].

Nevertheless, lignocellulosic biomass such as corn cobs present several challenges owing mainly to resistant structural components. The inability of industrial microorganisms to directly metabolize lignocellulosic biomass represents a major limitation for biofuel production. For example, *Saccharomyces cerevisiae* converts glucose to bioethanol but cannot ferment xylose [13] whereas few microbial strains such as *Pichia stipitis*, *Candida shehatae*, and *Fusarium oxysporum* have been found capable of xylose fermentation [14,15]. Thus, the use of lignocellulosic waste for bioethanol production necessitates effective chemical pretreatments to degrade complex structures. This will enhance the enzymatic hydrolysis step leading to high fermentable sugar yields as carbon and energy sources for microbial growth and product formation [16].

Several chemical pretreatment strategies have been reported and include acid [17], alkaline [18,19], inorganic salts [16] and sequential dilute acid and alkali [20,21], among others. Despite the availability of studies on various pretreatment systems, their industrial application has been significantly hampered by the high energy demand. As a result, recent pretreatment studies focus on the development of efficient, environmentally friendly and low-cost treatments. Acid pretreatment types have shown to be effective for high solubilization of cellulose and hemicellulose components [22]. Nevertheless, acid-catalysed treatments at high concentrations results in the corrosion of pretreatment reactors in addition to the formation of fermentation inhibitory compounds such as acetic acid, furfural and 5-Hydroxymethyl furfural (HMF). Fermentation inhibitor compounds have shown to disrupt the bioethanol production process by affecting the microbial culture. Inhibitory effects of these compounds observed during microbial fermentation processes include a longer lag-phase, slower microbial growth, lower cell density, low substrate intake, reduced productivity

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https://doi.org/10.1016/j.enconman.2018.01.024
Received 14 November 2017; Received in revised form 10 January 2018; Accepted 11 January 2018

and fermentation efficiency [23]. Therefore, despite the lower sugar yields, dilute acid treatments are favoured over concentrated ones [17,24]. On the other hand, alkaline pretreatment has shown to be the most efficient pretreatment type since it results in the removal of acetyl groups from xylan polymers, decomposition and removal of lignin structures, decrease in cellulose crystallinity and enhanced porosity with low release of sugar degradation compounds and furan derivatives [1,25].

In recent times, alkalic salts that function as weak bases have been reported as potential alternatives to commonly used expensive alkali-based pretreatments such as sodium hydroxide (NaOH) [1]. Alkalic salts are categorized as inorganic salts along with metal type salts [1,16]. Inorganic salts have recently attracted considerable attention as an effective chemical pretreatment method due to their cost-effectiveness and recyclable nature [9]. The mechanisms of alkalic and metal type salts have different effects on the chemical composition and biomass structure [1,5,16]. Alkalic salts that have been investigated in lignocellulosic treatment include $Na_3PO_4{\cdot}12H_2O$ [1], $NaCO_3$ [5] and Na_2S [1]. These salts result in lignin and hemicellulose dissolution, de-esterification of intermolecular ester bonds [26], rearrangement and alteration of lignin and the crystalline state of cellulose [27]. Metal salts reported in lignocellulosic pretreatment include $ZnCl_2$ [16], $FeSO_4$ [28] and $FeCl_3$ [9]. They form metal cations that act as Lewis acids when in solution and cleave glycosidic linkages within lignocellulosic structures [29]. Some combinations of alkalic or metal salts with other chemicals for efficient lignocellulosic pretreatment have been reported [1,5,10,30]. For example, alkalic salts have been combined with other inorganic salts [1] as well as oxidative agents [5] while metal type salts have been combined with organic acids [10], inorganic acids [30], organosolvents [31] and oxidative agents [32]. Qing et al. [5] explored a combination of $Na_3PO_4{\cdot}12H_2O$ and H_2O_2 on bamboo shoot shell and observed a high fermentable sugar yield of 86%. In the same vein, Qing et al. [1] optimized a combined Na_3PO_4 and Na_2S pretreatment strategy and reported a 91.11% reducing sugar yield on corn stover. Similarly, reports on sequential and simultaneous combination of metal salts with other chemical treatments are limited. Park et al. [31] examined a combined $MgCl_2$ and C_2H_6O pretreatment type for pitch pine which gave a 75.8% glucose yield. Ramadoss and Muthukumar [32] investigated a combined TiO_2 and H_2O_2 treatment regime on sugarcane bagasse and reported a 78.72% delignification. A combined $FeCl_3$ and CH_3COOH of corn cobs gave a 54.79% delignification [10].

Few studies have reported on combined inorganic salt and inorganic acid lignocellulosic pretreatments [30,33–34], and have raised great concerns about the salt metathesis reactions which render the chemical pretreatment inefficient [35]. Salt metathesis also referred to as a double replacement reaction consists of a biomolecular process where chemical molecules containing counter ions are interchanged. Commonly used inorganic salt combined with acid treatments employ H_2SO_4 and $FeCl_2$. However, when these reactive species are in aqueous state, H_2SO_4 and $FeCl_2$ react to form HCl and $FeSO_4$, thus indicating that HCl results in the net chemical pretreatment effect [36]. Therefore, the application of sequential pretreatments avoids the occurrence of salt metathesis reactions. In addition, the application of dual chemical pretreatments effectively disrupts the resistant lignocellulosic matrix that usually prevents enzymatic attack of the glucose rich cellulose polymer. Alkalic salts such as sodium phosphate behave like strong alkaline catalysts such as NaOH and result in effective delignification and hemicellulose removal whereas dilute acid is known for high hemicellulose solubilization [24].

Despite the aforementioned advantages on the use of sequential chemical pretreatments, a significant knowledge gap exists on alkalic salts such as sodium phosphate with dilute acid for lignocellulosic biomass pretreatment. Modelling and optimization of pretreatment conditions is crucial to understand possible functional relationships between pretreatment inputs and fermentable sugar production to ultimately improve the yield. Artificial Neural Networks (ANNs) are mathematical tools that are highly efficient in modelling non-linear complex processes [6]. Therefore, this study modelled and optimized a novel sequential alkalic salt ($Na_3PO_4{\cdot}12H_2O$) and dilute acid (H_2SO_4) pretreatment strategy for the enhancement of sugar recovery from corn cobs. Functional relationships between these pretreatment inputs and the reducing sugar yield were extracted using a developed Artificial Neural Network (ANN) model. Additionally, physical structural properties of native and pretreated biomass were assessed using Scanning Electron Microscopy (SEM) and Fourier Transform Infrared (FTIR) analysis.

2. Materials and methods

2.1. Materials

Corn cobs were obtained from the Ukulinga research farm (Pietermaritzburg, South Africa) (29° 67′ E, 30° 40′ S). The corn cobs were oven dried at 60 °C for 24 h then milled to a particle size of less than 1–2 mm using a centrifugal miller (Retsch ZM-1, South Africa). The milled corn cobs were then stored at room temperature. The native (untreated), controls and optimally pretreated biomass composition was analysed using the National Renewable Energy Laboratory method [37]. Native corn cobs consisted of 34.21% cellulose, 39.08% hemicellulose and 6.32% lignin. All chemicals were purchased from Merck, South Africa. Cellic CTec 2, a cellulase-based enzyme was generously provided by Novozymes (Novozymes A/S, Denmark). The enzyme activity of Cellic CTec 2 was 160 FPU/ml and was determined according to the Laboratory Analytical Procedure (LAP) of the National Renewable Energy Laboratory [38].

2.2. Primary screening

A primary screening was performed to determine the effect of the alkalic salt ($Na_3PO_4{\cdot}12H_2O$) and dilute acid (H_2SO_4) (combined and sequential pretreatments) on the enzymatic hydrolysis of corn cobs. All preliminary experiments were carried out using a total volume of 100 mL and a solid to liquid ratio of 10% (w/v). The $Na_3PO_4{\cdot}12H_2O$ and H_2SO_4 concentration ranges were selected based on previous studies [5,39]. The combined pretreatment (COM–$Na_3PO_4{\cdot}12H_2O$–H_2SO_4) was performed in a single stage system and consisted of 10% (w/v) $Na_3PO_4{\cdot}12H_2O$ and 2% (v/v) H_2SO_4. The sequential pretreatment ($Na_3PO_4{\cdot}12H_2O$–H_2SO_4) was carried out using 10% (w/v) $Na_3PO_4{\cdot}12H_2O$ (first stage) and 2% (w/v) H_2SO_4 (second stage). The reverse sequential order was also evaluated (H_2SO_4–$Na_3PO_4{\cdot}12H_2O$). Control experiments were performed and these included water alone, 10% (w/v) $Na_3PO_4{\cdot}12H_2O$ alone and 2% (v/v) H_2SO_4 alone. The pretreatment process was carried out in a laboratory autoclave at 121 °C for 30 min (15 min per stage for the sequential pretreatment) according to a previous report on corn cobs [40]. The reducing sugar yield obtained after enzymatic hydrolysis was considered as the efficiency index for the pretreatment.

2.3. Experimental design for process modelling and optimization

The Box-Behnken design was used to generate a total of seventeen experimental runs for model development. The pretreatment input parameters consisted of $Na_3PO_4{\cdot}12H_2O$ concentration (5–15%), H_2SO_4 concentration (1–3%) and solid to liquid ratio (5–15%) (Table 1).

2.4. Pretreatment process

Milled corn cobs (g) were submerged in 100 mL of alkalic salt solution then dilute acid according to the experimental design (Table 1). $Na_3PO_4{\cdot}12H_2O$ and H_2SO_4 were used for the first and second pretreatment stages, respectively. Corn cobs were pretreated with $Na_3PO_4{\cdot}12H_2O$ for 15 min at 121 °C and were thereafter washed with

Table 1
Experimental runs for the sequential alkalic salt and dilute acid pretreatment.

Run	$Na_3PO_4{\cdot}H_2O$ concentration (%)	H_2SO_4 concentration (%)	Solid to liquid ratio (%)	Observed reducing sugar (g/g)	RSM predicted reducing sugar (g/g)	ANN predicted reducing sugar (g/g)
1	15.00	3.00	10.00	0.75	0.75	0.43
2	10.00	1.00	5.00	0.72	0.66	0.69
3	15.00	2.00	15.00	0.98	0.93	0.83
4	10.00	2.00	10.00	0.41	0.44	0.46
5	10.00	1.00	15.00	0.86	0.87	0.86
6	10.00	2.00	10.00	0.40	0.44	0.46
7	5.00	2.00	15.00	0.34	0.33	0.35
8	10.00	3.00	15.00	0.29	0.35	0.34
9	10.00	2.00	10.00	0.48	0.44	0.46
10	5.00	1.00	10.00	0.70	0.71	0.51
11	5.00	2.00	5.00	0.39	0.44	0.32
12	15.00	2.00	5.00	0.67	0.68	0.61
13	10.00	2.00	10.00	0.46	0.44	0.46
14	5.00	3.00	10.00	0.22	0.22	0.29
15	10.00	2.00	10.00	0.44	0.44	0.46
16	10.00	3.00	5.00	0.43	0.42	0.33
17	15.00	1.00	10.00	0.93	0.98	0.92

deionized water and dried at 70 °C overnight followed by H_2SO_4 pretreatment for 15 min at 121 °C. After the second stage pretreatment, the biomass was filtered, washed and dried as previously stated and the solid residue obtained was used for enzymatic saccharification.

2.5. Enzymatic saccharification

The chemically pretreated corn cobs were then added to 100 mL Erlenmeyer flasks containing 10 mL sodium citrate buffer (pH 4.8, 0.05 M) with a solid and enzyme loading of 10% (w/v) and 10 FPU/g respectively. The pretreatment flasks were incubated at 50 °C and 100 rpm for 72 h. Reducing sugar and glucose yields were determined by the 3,5-dinitrosalicylic acid method (DNS) [41] and Megazyme glucose kits (Megazyme, Ireland) respectively. All seventeen experiments were carried out in duplicate.

2.6. Analytical methods

2.6.1. Scanning electron microscopy (SEM)

Corn cobs samples (untreated, controls and optimally treated) were analysed using a ZEISS EVO LS 15 scanning electron microscope. The samples were mounted onto aluminium specimen mounts, gold sputter coated (Eiko IB-3 Ion Coater) and viewed at a magnification of 500×.

2.6.2. Fourier Transform Infrared (FTIR) analysis

Functional group changes of native and pretreated biomass were examined by Fourier Transform Infrared (FTIR) spectroscopy using a Perkin Elmer 100 (Waltham, MA, USA). Samples were milled with spectroscopic grade KBr and pressed to produce diameter pellets. FTIR spectra were recorded between 800 and 3800 cm^{-1}.

2.6.3. Inhibitor analysis

The chemical hydrolysate (controls and optimally pretreated) were assessed for major inhibitors such as acetic acid, furfural and 5-hydroxymethyl furfural (HMF). Samples were analysed using coupled Varian 3800 gas chromatography (Varian Palo Alto, California, USA) and Varian 1200 mass spectrometry (GC–MS) as previously outlined by Rorke et al. [6].

2.7. Modelling and optimization of the alkalic salt and dilute acid pretreatment

2.7.1. Response Surface Methodology (RSM) model development

The experimental reducing sugar yields obtained after enzymatic saccharification were used to fit the polynomial equation that relates the input parameters to the output using Design Expert software (Stat-Ease Inc., USA). The general form of the polynomial model is shown in Eq. (1):

$$Y = \alpha_0 + \alpha_1 x_1 + \alpha_2 x_2 + \alpha_3 x_3 + \alpha_{11} x_1^2 + \alpha_{22} x_2^2 + \alpha_{33} x_3^2 + \alpha_{12} x_1 x_2 + \alpha_{13} x_1 x_3 + \alpha_{23} x_2 x_3 \quad (1)$$

where Y represents the response output, α_0 is the intercept, $\alpha_1 x_1$, $\alpha_2 x_2$, $\alpha_3 x_3$ are the linear coefficients, $\alpha_{11} {x_1}^2$, $\alpha_{22} {x_2}^2$, $\alpha_{33} {x_3}^2$ are the quadratic coefficients and $\alpha_{12} x_1 x_2$, $\alpha_{13} x_1 x_3$, $\alpha_{23} x_2 x_3$ represent the interaction of coefficients.

2.7.2. Artificial intelligent model development to determine functional relationships

Artificial Neural Networks were used to develop an intelligent model to determine functional relationships between pretreatment inputs and the reducing sugar yield. The neural network had a topology of 3-2-1 which corresponds to the number of neurons of input, hidden and output layers. The inputs included $Na_3PO_4{\cdot}12H_2O$ concentration, H_2SO_4 concentration and solid to liquid ratio while the output was the reducing sugar yield. The experimental data were divided into two sets consisting of 75% (training) and 25% (validation). The logistic transfer function was employed for the hidden layer. The hidden layer had two main functions: (1) addition of weighted inputs and the linked bias and, (2) shift input data to a non-linear form as shown in the following Eqs. (2)–(4) [42]:

$$\text{sum} = \sum_{i}^{n} = 1^{xiwi} + \theta \quad (2)$$

where w_i ($i = 1, n$) are the connection weights, θ is the bias and x_i is the input variable

$$f(sum) = \frac{1}{1 + \exp(-sum)} \quad (3)$$

Experimental data were normalized according to the following equation:

$$\text{Normalized } (e_i) = \frac{e_i - E_{min}}{E_{max} - E_{min}} \quad (4)$$

where *ei* is the normalized data and E_{min} and E_{max} represent the minimum and maximum values.

The network was trained using a back propagation algorithm with the goal of achieving a minimum net error on the validation data set while preventing overtraining or memorization. The model accuracy was assessed using regression analysis on predicted and observed

process outputs.

2.7.3. Determination of functional relationships between process inputs and output

Mathematical equations were used to extract the functional relationships between treatment inputs and the reducing sugar yield from the developed model. The rate and direction of output change when each treatment input was varied from its minimum to maximum values, while the other two inputs were maintained at their median value were shown. These equations were derived using curve fitting [6].

3. Results and discussion

3.1. Primary screening

The sequential pretreatments resulted in higher reducing sugar yields of 0.52 g/g ($Na_3PO_4{\cdot}12H_2O$–H_2SO_4) and 0.50 g/g (H_2SO_4–$Na_3PO_4{\cdot}12H_2O$) compared to 0.40 g/g recorded for the combined pretreatment (COM–$Na_3PO_4{\cdot}12H_2O$–H_2SO_4). The $Na_3PO_4{\cdot}12H_2O$–H_2SO_4 sequential order was then further considered for optimization.

3.2. Assessment of RSM model significance

The model fitness was assessed using Analysis of Variance (ANOVA) and these results are shown in Table 2. The model F-value of 30.81 and p-value of < .0001 suggests that it was significant. Generally, a p-value < .05 indicates model significance [5]. It was determined that $Na_3PO_4{\cdot}12H_2O$ concentration was the most significant input among all parameters (< 0.0001) followed by the H_2SO_4 concentration (< 0.0013). A coefficient of determination (R^2) value of 0.98 was obtained, indicating that the developed model could account for 98% of the variation in the observed data. The model polynomial Eq. (5) is shown below:

$$\text{Reducing sugar yield (g/g)} = 1.73 - 0.117A - 0.58B - 0.034\,C + 0.015AB + 0.0036AC - 0.014BC + 0.0046A^2 + 0.096B^2 + 0.0016C^2 \quad (5)$$

where A, B and C are $Na_3PO_4{\cdot}12H_2O$ concentration, H_2SO_4 concentration and solid to liquid ratio, respectively.

3.3. Assessment of the developed ANN model

The developed ANN model displayed a high correlation between the experimental and predicted data with a coefficient of determination (R^2) value of 0.81. The high R^2 value obtained suggests a high accuracy and reproducibility in the prediction of the reducing sugar yield when subjected to novel pretreatment conditions.

3.4. Effect of pretreatment input parameters on reducing sugar yield

The observed reducing sugar yields obtained for each experimental run are shown in Table 1. The highest reducing sugar yield (0.98 g/g) was obtained using pretreatment conditions of 15%, 2% and 15% for $Na_3PO_4{\cdot}12H_2O$ concentration, H_2SO_4 concentration and solid to liquid ratio respectively. The lowest reducing sugar yield (0.22 g/g) was recorded when milder pretreatment conditions (5% $Na_3PO_4{\cdot}12H_2O$ concentration, 3% H_2SO_4 concentration and 10% solid to liquid ratio) were used. This large variation indicates the high sensitivity of sugar recovery on the investigated pretreatment input conditions. The effects of varying input parameter values within the ranges used, on reducing sugar yield is shown in Fig. 1A–C.

The impact of $Na_3PO_4{\cdot}12H_2O$ concentration on reducing sugar production when other pretreatment parameters are kept at their median value showed a gradual increase in reducing sugar yield from 0.37 to 0.71 g/g when $Na_3PO_4{\cdot}12H_2O$ concentration was varied from 7 to 15% (Fig. 1A). This trend fits a Weibull type of relationship as shown by the ANN model. The high sensitivity of reducing sugar yield to the $Na_3PO_4{\cdot}12H_2O$ concentration may be accounted for by its ability to act like a weak base, thus removing lignin and enhancing cellulose and hemicellulose digestibility [5]. The ANN model indicated that an increase in the H_2SO_4 concentration from 1 to 3% resulted in a drastic decrease in reducing sugar yield from approximately 0.80–0.33 g/g, illustrating a high sensitivity within this region (Fig. 1B). The direction and rate of change of reducing sugar yield as a function of acid concentration could be illustrated by a dosage-response type of relationship with a threshold concentration at 2% H_2SO_4. Higher acid concentrations have shown to result in lower sugar yields due to the degradation of these carbohydrate molecules to furan derivatives such as furfural and 5-Hydroxymethylfurfural [6,43]. Baek et al. [43] investigated the effect of H_2SO_4 concentration on the pretreatment of cellulose and illustrated that H_2SO_4 concentrations > 1% led to increased furfural production. Decreases in reducing sugar yield above 2% H_2SO_4 observed in the present study may be attributed to the degradation of sugar compounds to furan derivatives. The influence of solid to liquid ratio on the reducing sugar yield showed that an increase in this input from 5 to 15% led to a linear increase in the reducing sugar yield from 0.40 to 0.56 g/g (Fig. 1C). This relationship was best illustrated by using a dosage-response type equation. Sindhu et al. [17] reported an optimum solid to liquid ratio of 10% for maximum reducing sugar yield (0.43 g/g) from acid pretreated chili post-harvest. Studies have reported that higher solid loadings (> 8%) led to lower inhibitor compounds release with high sugar yields [6,44]. This may account for the observed simultaneous increase in reducing sugar yield with incremental variation in the solid to liquid ratio parameter. The developed Artificial Neural Network model has been deposited into the Repository of Intelligent Models [45] with an accession number (PRZF001725). To compare the relative sensitivity of ANN model on input parameters, the

Table 2
Analysis of variance of the developed sequential pretreatment model.

Factor	Sum of squares	Degrees of freedom (*df*)	Mean Square	*F* value	*p* value (probability > *F*)
Intercept or model	0.84	9	0.093	30.81	< 0.0001 significant
A-$Na_3PO_4{\cdot}12H_2O$ conc.	0.35	1	0.35	116.88	< 0.0001
B-H_2SO_4 conc.	0.29	1	0.29	95.67	< 0.0001
C-solid to liquid ratio	8.450×10^{-3}	1	8.450×10^{-3}	2.80	0.1382
AB	0.023	1	0.023	7.45	0.0293
AC	0.032	1	0.032	10.73	0.0136
BC	0.020	1	0.020	6.49	0.0382
A^2	0.057	1	0.057	18.77	0.0034
B^2	0.039	1	0.039	12.86	0.0089
C^2	7.078×10^{-3}	1	7.078×10^{-3}	2.34	0.1696
Residual error	0.021	7	3.019×10^{-3}	–	
Lack of fit	0.017	3	5.550×10^{-3}	4.96	0.0781 not significant
Pure error	4.480×10^{-3}	4	1.120×10^{-3}	–	

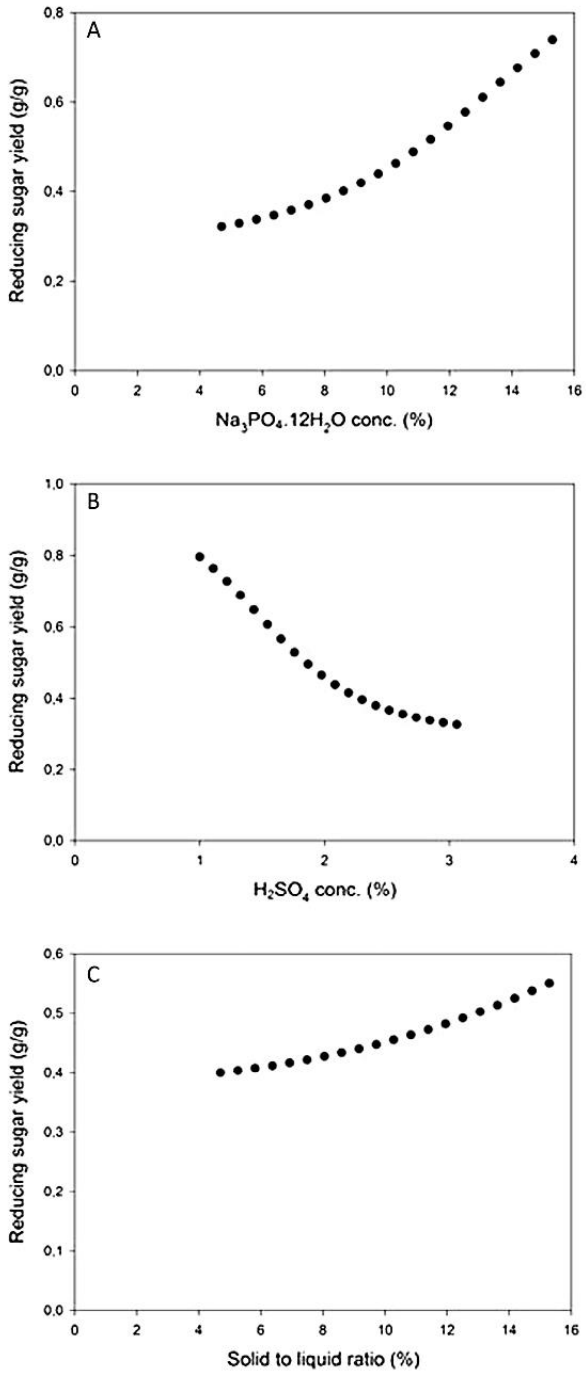

Fig. 1. Impact of linear variations in input parameters (A) $Na_3PO_4{\cdot}12H_2O$ concentration, (B) H_2SO_4 concentration and (C) solid to liquid ratio on the reducing sugar yield.

slope of each series (Fig. 1A–C) was obtained. Assessment of these gradients indicated that $Na_3PO_4{\cdot}12H_2O$ concentration (0.04) had the most significant influence on the reducing sugar yield followed by solid to liquid ratio (0.01) and H_2SO_4 (−0.24) concentration in decreasing order. The high slope value observed for $Na_3PO_4{\cdot}12H_2O$ concentration illustrated that slight changes in this treatment input would significantly impact on the reducing sugar yield. Generally, higher $Na_3PO_4{\cdot}12H_2O$ concentrations create an alkaline environment by effectively removing acetyl groups and enhancing cellulose digestibility

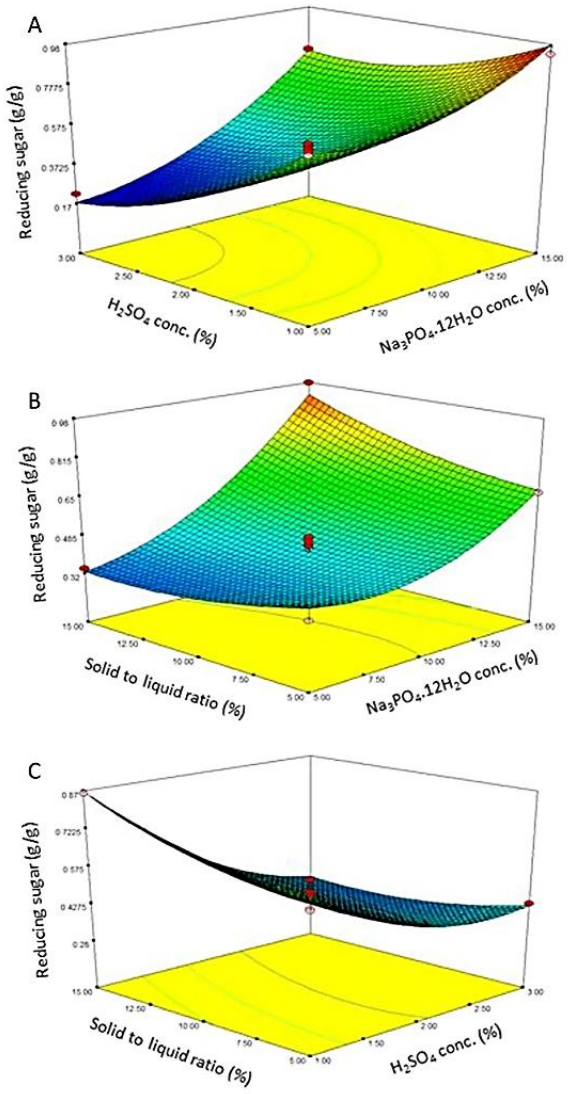

Fig. 2. Response surface plots showing the interactive effect of the various input parameters affecting the pretreatment of corn cobs (A) interaction between $Na_3PO_4{\cdot}12H_2O$ concentration and H_2SO_4 concentration, (B) $Na_3PO_4{\cdot}12H_2O$ concentration and solid to liquid ratio, (C) H_2SO_4 concentration and solid to liquid ratio.

[1,5,26]. On the other hand, the negative gradient observed for H_2SO_4 concentration is due to the fact that concentration beyond the threshold (1%) negatively impacted the sugar yield. High acid concentrations (> 1%) would result in low reducing sugar yields due to rapid conversion of monomeric sugars to furan derivatives, whereas low acid concentrations result in high sugar yields. The relatively high slope value for solid to liquid ratio indicated that this parameter significantly impacted the reducing sugar yield. Previous reports on solid to liquid ratio have indicated that optimum reducing sugar yield is obtained between 5 and 15% [17,46–47].

3.4.1. Pairwise interactive effects of the treatment inputs on reducing sugar yield

The interactive effect of H_2SO_4 and $Na_3PO_4{\cdot}12H_2O$ concentrations on the reducing sugar yield when the solid to liquid ratio is maintained at its median value is shown in Fig. 2A. Low reducing sugar yields (0.17–0.37 g/g) were obtained with pretreatment at low concentrations of $Na_3PO_4{\cdot}12H_2O$ (5–7.5%) and high H_2SO_4 concentrations in the range

Table 3
Reducing sugar yields from corn cobs under various pretreatment strategies.

Pretreatment conditions	Reducing sugar yield (g/g)	Reference
12.70% $Na_3PO_4{\cdot}12H_2O$, 1.04%, 14.49% S:L, 121 °C, 30 min	0.99	This study
2% NaOH, 6.7% S:L, 100 °C, 30 min	0.68	Boonsombuti et al. [18]
1.75% H_3PO_4, 3.33% S:L, 140 °C, 10 min	0.82	Satimanont et al. [47]
0.5 M NaOH, 12.5% S:L, 121 °C, 30 min	0.92	Gao and Rehmann [40]
1 M NaOH, 20% S:L, 121 °C, 20 min	0.11	Potumarthi et al. [12]
1% H_2SO_4, 10% S:L, 121 °C, 60 min	0.52	Boonsombuti et al. [39]
1% H_2SO_4, 20% S:L, 108 °C, 180 min	0.58	Chen et al. [51]

S:L – Solid to liquid ratio.

of 2–3%. On the other hand, when the H_2SO_4 concentration was maintained below 2% and the $Na_3PO_4{\cdot}12H_2O$ concentration was increased from 5 to 15%, a simultaneous increase in the reducing sugar yield (0.50–0.98 g/g) was observed. Steep increases in the reducing sugar yields observed under stronger alkalic salt ($Na_3PO_4{\cdot}12H_2O$) conditions may be ascribed to enhanced accessibility to cellulose due to effective lignin removal, thus resulting in the formation of monomeric and oligosaccharide type sugars [1]. The combined effect of solid to liquid ratio and $Na_3PO_4{\cdot}12H_2O$ concentration on the reducing sugar yield when H_2SO_4 concentration is kept at its midpoint value is shown in Fig. 2B. Low reducing sugar yields of 0.32–0.45 g/g were obtained with pretreatment at low concentrations of $Na_3PO_4{\cdot}12H_2O$ (5–7.5%) and solid to liquid ratios in the range of 5–10% respectively. When the solid to liquid ratio was varied (5–15%) and the $Na_3PO_4{\cdot}12H_2O$ concentration was simultaneously increased from 5 to 15%, the reducing sugar yield drastically increased from 0.34 to 0.96 g/g. This high saccharification efficiency is attributed to effective degradation rates of hemicellulose and lignin at high concentrations of alkalic salts, thus exposing the cellulose moieties to enzymatic attack [1]. Previous reports on alkalic salt pretreatment have only utilized this chemical in combined pretreatment regimes as opposed to sequential treatments. For instance, Qing et al. [1] observed a 20% increase in the reducing sugar yield when the Na_3PO_4 was increased from 1 to 4%. Likewise, Qing et al. [5] reported a simultaneous increase in the sugar yield from 35 to 75% when the $Na_3PO_4{\cdot}12H_2O$ was increased from 1 to 11% and the H_2O_2 was maintained at 0.3 g/g.

Pairwise effects of solid to liquid ratio and H_2SO_4 concentration on the reducing sugar yield when the $Na_3PO_4{\cdot}12H_2O$ concentration is maintained at its middle value is shown in Fig. 2C. A simultaneous increase in the solid to liquid ratio from 5 to 15% and H_2SO_4 concentration from 1 to 3% resulted in a decrease in reducing sugar from 0.42 to 0.28 g/g. On the other hand, high reducing sugar yields of 0.50–0.87 g/g were obtained when the solid to liquid ratio was increased from 5 to 15% and the H_2SO_4 concentration was maintained below 2%. Further increases in acid concentration beyond the threshold values (2%) showed an antagonistic effect on the reducing sugar yield. This may be attributed to the degradation of sugar molecules to volatile inhibitor compounds at higher acid concentrations. Inhibitor compounds have been shown to negatively impact the enzymatic hydrolysis stage thus leading to low saccharification and sugar yield [17,43]. Zheng et al. [22] observed a 13% higher reducing sugar yield when the H_2SO_4 concentration was reduced from 1 to 0.66%. Alkalic salt and dilute acid catalysed pretreatment types have been shown to effectively enhance hemicellulose and lignin removal, thus optimization of their sequential effect positively enhanced enzymatic accessibility for the conversion of cellulose to glucose [1,17,47].

3.5. Experimental validation of the optimized model

The developed model predicted a reducing sugar yield of 0.98 g/g for the sequential pretreatment under optimal conditions of 12.70% $Na_3PO_4{\cdot}12H_2O$, 1.04% H_2SO_4 and 14.49% solid to liquid ratio. Experimental validation carried out in duplicate yielded 0.99 ± 0.01 g/g for the sequential pretreatment. Control experiments with native, water pretreated, H_2SO_4 pretreated and $Na_3PO_4{\cdot}12H_2O$ pretreated biomass gave reducing sugar yields of 0.29, 0.49 g/g, 0.50 g/g and 0.89 g/g, respectively. Compared to the control treatments, the sequential pretreatment showed improvements of 10%, 45%, and 51% for the $Na_3PO_4{\cdot}12H_2O$, H_2SO_4 and H_2O control samples respectively. High reducing sugar yields (0.99 ± 0.01 g/g) observed using the optimized sequential alkalic salt and dilute acid pretreatment can be explained by an 11.1% and 13.6% mass gain when a water molecule reacts with each sugar unit during cellulose and hemicellulose hydrolysis, respectively [48]. Furthermore, aldehyde molecules with molecular weights lower than glucose can react with the DNS reagent, which may cause a slight overestimation of reducing sugar [49]. A high glucose yield of 0.69 g/g was observed for the optimized sequential sample compared to the native (0.16 g/g), water pretreated (0.22 g/g), H_2SO_4 pretreated (0.32 g/g) and $Na_3PO_4{\cdot}12H_2O$ pretreated sample (0.61 g/g). The high glucose yields obtained further validate the observed high reducing sugar yield.

3.6. A comparative assessment of the novel sequential pretreatment with reported pretreatment studies on corn cobs

The reducing sugar yields reported in previous pretreatment studies on corn cobs were compared with the present optimized sequential pretreatment and data are shown in Table 3. The present study gave a 1.2-fold increase in the sugar yield compared to a study reported by Satimanont et al. [50] using a single stage dilute acidic pretreatment (1.75% H_3PO_4) on corn cobs. Interestingly, the present optimized sequential pretreatment gave a 7.1% higher reducing sugar yield compared to a previous alkaline-based treatment regime (0.5 M NaOH) [40]. Similarly, an 89% lower reducing sugar yield was observed using an alkaline pretreatment strategy (1 M NaOH) on corn cobs [12]. Likewise, Chen et al. [51] and Boonsombuti et al. [39] recorded a 41% and 47% lower sugar yield using acid-based pretreatments (1% H_2SO_4). A microwave-hydrothermal pretreatment on corn cobs gave low glucose recovery (4.7%) with high xylose yield (64.7%) [52]. Higher reducing sugar yields observed in the present study compared to previous reports on the same substrate is accounted for by the dual effect of sequential pretreatment with alkalic salt, followed by dilute acid treatment which enhanced the breakdown of lignocellulosic structures thus easing the enzymatic saccharification process and improving the sugar yield.

3.7. Effect of the sequential $Na_3PO_4{\cdot}12H_2O$–H_2SO_4 pretreatment on the physical structure of corn cobs

3.7.1. Characterization of native and pretreated corn cobs

Native lignocellulosic biomass consists of cellulose and hemicellulosic structures that are tightly held together by layers of resistant lignin that prevent both chemical and biological degradation [53]. The lignin, cellulose and hemicellulose content in the native corn cob biomass were 6.32, 34.80 and 39.08% respectively. The optimized sequential pretreated sample contained 58.59% cellulose, 29.01%

hemicellulose and 2.77% lignin. Therefore, the optimized sequential pretreatment resulted in a 71.27% increase in the cellulose content, 25.77% hemicellulose removal and 56.17% delignification. Characteristics such as the strong nucleophilic species within alkalic salts (PO_4^{3-}, HPO_4^{2-} and HS^-) have shown to enhance the cleavage of phenolic β-aryl ether bonds of lignin, leading to effective delignification with reduced antagonistic effects on carbohydrates [54]. In addition to this, alkalic salts behave like weak alkaline treatments that cleave ester and glycosidic bonds within the cell wall matrix, leading to modification of the lignin structure and the partial degradation of cellulose [1,25]. On the other hand, acidic compounds such as H_2SO_4 aid biomass fractionation, resulting in effective hydrolysis and solubilization of cellulose and hemicellulose respectively [22]. These chemical characteristics may therefore account for the observed increase in cellulose content, hemicellulose removal and delignification using the sequential pretreatment. Control samples displayed lower cellulosic content, hemicellulose removal and delignification compared to the sequential optimized pretreatment. For example, water pretreated sample gave cellulose, hemicellulose and lignin content of 37.94%, 38.98% and 6.24% respectively.

Acid (H_2SO_4) pretreated sample resulted in cellulose and hemicellulose content of 40% and 38.83% respectively. An increase in the lignin content from 6.32% (native) to 6.61% (H_2SO_4) was noted for the acid pretreatment control. Lignin in the solid residue gradually increases with the severity of chemical (e.g. acid) treatment as a result of the degradation or solubilization of carbohydrates or due to the generation of pseudo-lignin from sugar-degradation products that results in 'false' high values for lignin [55]. These phenomena may account for the higher lignin content observed in the H_2SO_4 control compared to the native biomass. The alkalic salt ($Na_3PO_4{\cdot}12H_2O$) pretreated sample gave cellulose, hemicellulose and lignin contents of 55.97%, 29.75% and 3.99%, respectively. Nonetheless, the optimized sequential pretreatment was found to be the most efficient treatment and resulted in a 1.7-fold and 2.3-fold increase in the cellulose and delignification respectively when compared with the native corn cob sample. The effectiveness of alkalic salts is mainly due to the cleavage of ester and glycosidic bonds within the cell wall matrix, thus causing alteration of lignin structure, cellulose swelling, and the partial decrystallization of cellulose [1,5,25]. Conversely, acidic compounds such as H_2SO_4 have shown to cause damage to glycosidic linkages within cellulose and ether bonds between xylan polymers [56]. The dual effect of these chemical pretreatment processes may account for the improved cellulose content, hemicellulose removal and delignification observed in the present study. Qing et al. [1] observed a 56.3% increase in the cellulose content and 20.5% increase in the delignification when pretreated under mild alkalic salt conditions (4% Na_3PO_4 and 10% Na_2S at 120 °C for 40 min) compared to the untreated sample. A combined alkalic salt and oxidative agent pretreatment under moderate conditions (5% $Na_3PO_4{\cdot}12H_2O$ and 0.6 g/g H_2O_2 at 80 °C for 120 min) gave a 1.7-fold and 3.3-fold increase in the cellulose content and delignification, respectively, compared with its native substrate sample [5]. Likewise, a 1.2-fold and 2.3-fold increase in the cellulose content and delignification respectively, were recorded by Sindhu et al. [17] using a sono-assisted acid pretreatment (4% H_2SO_4, 20% solid to liquid ratio, 4 min sonication time and 60 min incubation time) compared to its native sample. An increase in the cellulose content (42%) and delignification (13.8%) has recently been reported by Yu et al. [52] using a microwave-hydrothermal pretreatment on corn cobs.

3.7.2. Scanning electron microscopy (SEM)

Structural and surface morphological changes of the native, controls and optimized sequential pretreated biomass were recorded using Scanning Electron Microscopy (SEM). The native sample exhibited a well-ordered and intact structure that was smooth and non-porously compressed compared to the pretreated biomass. Pretreated samples demonstrated fragmental and porous surface structures that were rough with several perforations. A more distinct structural change was observed for the optimized sequential pretreated sample which displayed extreme cavitation and fissures of the surface structures compared to the native and control samples. Similar results were reported by Guo et al. [57] and Sindhu et al. [17] when electron beam-assisted $NaHCO_3$ and sono-assisted H_2SO_4 pretreatments were used respectively. The disintegrated porous surface area of the pretreated biomass combined with the increased surface area may be attributed to the weakening of the cell wall during delignification [7]. The highly perforated and disorganized surface appearance observed for the sequential pretreated sample implies that it was more effective for the alteration of recalcitrant lignocellulosic structures. These observations were in line with the high cellulose content, hemicellulose removal and delignification. Findings from the present study revealed that the sequential pretreatment resulted in major structural damage which ameliorated enzymatic saccharification and cellulose digestibility. The fragmented corn cob surface structure is corroborated by the modifications in the structural components shown by the FTIR analysis.

3.7.3. Fourier Transform Infrared (FTIR) spectra analysis

FTIR spectral data showed the presence of lignin, hemicellulose and cellulose moieties commonly found within lignocellulosic biomass. Significant differences in the banding patterns were observed between the pretreated sample and the native biomass, thus indicating structural changes after pretreatment. FTIR spectra did not show the formation of any new peaks. Absorptive peaks associated with cellulose, hemicellulose and lignin were shown to be stronger for the optimized sequential pretreated sample compared to the native and control samples. Cellulose consists of β-D-glucopyranose units linked with (1 → 4) glycosidic bonds [58]. A strong band peak at 898 cm^{-1} depicts the cellulose spectrum corresponding to the β-glycosidic bond usually found between sugar units in cellulose structures [7]. The H_2SO_4 pretreated sample displayed the most intense peak at 898 cm^{-1} compared to the other samples. H_2SO_4 has shown to disrupt glycosidic linkages within cellulose and ether bonds between xylan polymers [54]. The more severe acid concentration used for the acid control (2%) compared to the sequential pretreatment (1.04%) may account for the strong absorption peak at 898 cm^{-1}. Sharp peaks observed at 1031 cm^{-1} and 1162 cm^{-1} indicates vibrations of C—O—C related to the pyranose ring skeleton and C—O—C asymmetric stretching in cellulose, respectively.

Additionally, the absorptive peaks at 1371 cm^{-1} and 1426 cm^{-1} were associated with C—H bending vibrations and symmetric CH_2 bending and scissoring at the C(6) region in cellulose, respectively [59]. High intensity peaks at 2895 cm^{-1} and 3312 cm^{-1} are linked with C—H stretching and —OH stretching of intramolecular hydrogen within cellulose, respectively [5]. The band peak at 1243 cm^{-1} belongs to C=O stretching vibration of the aromatic ring in lignin, xylan and ester groups. The absorbance observed at band peak 1514 cm^{-1} indicates the presence of the aromatic skeletal vibrations in C=C stretching of lignin [5,7]. Absorption peak at 1727 cm^{-1} was attributed to C=O stretching vibrations of acetyl and ester groups in hemicellulose and lignin, respectively [7]. Previous reports on inorganic salt pretreatment have recorded similar banding patterns [1,5,7]. FTIR analysis suggests that the optimized sequential pretreatment was effective for the unwinding of cellulose, hemicellulose and lignin characteristic structures and thus improved the enzymatic hydrolysis of corn cobs.

3.8. Inhibitor profile of the pretreatment hydrolysate

Key fermentation inhibitor compounds present after chemical pretreatment include acetic acid, furfural and 5-hydroxymethyl furfural (HMF). They are inhibitory to microbial growth and metabolism at specific concentration ranges [17]. The sequential optimized sample resulted in the highest concentration of acetic acid (1.83×10^{-2} μg/g) whereas the H_2SO_4 pretreated sample gave the lowest yield of acetic acid (1.7×10^{-4} μg/g).

Table 4
Comparison of inhibitor concentrations produced using alkalic salt or acid pretreatment on different substrates.

Substrate	Pretreatment system	Pretreatment conditions	Inhibitors (μg/g)			Reference
			Acetic acid	Furfural	HMF	
Corn cobs	Sequential	12.70% $Na_3PO_4{\cdot}12H_2O$, 1.04% H_2SO_4, 14.49% S:L, 121 °C, 30 min	1.83×10^{-2}	9.4×10^{-2}	3.7×10^{-4}	This study
Bamboo shoot shell	Single	9% Na_3PO_4, 0.3 g/g H_2O_2, 1% S:L, 80 °C, 120 min	9.5×10^{4}	–	–	Qing et al. [5]
Corn stover	Single	4% $Na_3PO_4{\cdot}12H_2O$, 10% Na_2S, 1% S:L, 120 °C, 40 min	2×10^{5}	–	–	Qing et al. [1]
Chili post harvest	Single	4% H_2SO_4, 20% S:L, 4 min (sonication), 60 min (incubation)	1.1×10^{4}	ND	ND	Sindhu et al. [17]
Sugar beet pulp	Single	0.5% H_2SO_4, 2% S:L, 140 °C, 30 min	2.5×10^{4}	ND	2.9×10^{3}	Zheng et al. [22]

S:L – Solid to liquid ratio.
ND – Not detected.

Acetic acid becomes inhibitory to *Saccharomyces cerevisiae* during bioethanol production when its concentration exceeds 1.5 g/L [13]. Formation of acetic acid may be attributed to the disruption of ester and acetyl linkages within lignocellulosic structures [29]. High acetic acid concentration observed for the sequential optimized sample can be accounted for by the buffering capacity of $Na_3PO_4{\cdot}12H_2O$ which stimulates the production of acidic compounds [1,5]. Highest furfural concentration was observed for the H_2SO_4 pretreated sample (0.14 μg/g) with the lowest concentration detected for the $Na_3PO_4{\cdot}12H_2O$ pretreated sample (2.3×10^{-4} μg/g). Similarly, the highest HMF yield was observed for the H_2SO_4 pretreated sample (2.8×10^{-3} μg/g) with no HMF detected for the other control samples. The sequential optimized pretreated sample displayed a low HMF concentration of (3.7×10^{-4} μg/g). Concentrations of furfural and HMF > 1 g/L have shown to inhibit the bioethanol production process [13]. Furfural and HMF are furan derivatives and are usually produced during pentose and hexose degradation. Quantities of furan derivatives become elevated when the pretreatment process conditions become more severe, either by a higher exposure time to stronger chemical conditions or temperatures [60]. The high furfural and HMF concentrations observed for the H_2SO_4 pretreated sample may be as a result of the severe pretreatment conditions (2% H_2SO_4) employed. Relatively low furfural and HMF yields observed for the other samples may be ascribed to the rehydration of this compound to levulinic acid in the presence of a water solvent [61]. The sequential alkalic salt and acid pretreatment generated a low acetic acid concentration of 1.83×10^{-2} μg/g compared with single stage pretreatment studies on bamboo shoot shell (9.5×10^{4} μg/g) [1], corn stover (2.0×10^{5} μg/g) [5], chili post harvest (1.1×10^{4} μg/g) [17] and sugar beet pulp (2.5×10^{4} μg/g) [22] (Table 4).

Likewise, low HMF concentration (3.7×10^{-4} μg/g) was recorded using the sequential optimized pretreatment compared to an earlier study on the acidic pretreatment of sugar beet pulp which reported a high HMF concentration (2.9×10^{3} μg/g) [22]. A low furfural concentration (9.4×10^{-2} μg/g) was observed using the sequential optimized pretreatment which was comparable to the acidic pretreatment studies by Sindhu et al. [17] and Zheng et al. [22] where no furfural was detected. Yu et al. [52] observed a furfural concentration of 1.8 g/L using a microwave-hydrothermal pretreatment of corn cobs. Furfural concentrations in the range of (0.12–13.71 g/L) have been reported for corn cob pretreatment using acid and alkali [39,47]. The observed low inhibitor concentrations detected in this study were shown to be below the reported inhibitory levels (< 1 g/L) and therefore eradicate the use of costly detoxification methods [13].

4. Conclusion

A sequential $Na_3PO_4{\cdot}12H_2O$ and H_2SO_4 pretreatment of corn cobs was assessed in this study. Optimization gave a high R^2 of 0.98. Maximum sugar yield of 0.99 ± 0.01 g/g was achieved with 12.70% $Na_3PO_4{\cdot}12H_2O$, 1.04% H_2SO_4 and 14.49% solid to liquid ratio.

A 9-fold increase in the reducing sugar yield was observed compared to previous reports. An intelligent model was developed to elucidate functional relationships and revealed that reducing sugar yield exhibited high sensitivity to $Na_3PO_4{\cdot}12H_2O$ concentration. The developed sequential pretreatment demonstrated high efficiency at enhancing sugar yields from lignocellulosic wastes that could be harnessed for microbial biofuel production and value-added products.

Acknowledgements

The work was financially supported by the National Research Foundation (NRF) (Grant number: 101316) of South Africa. Opinions expressed and conclusions arrived at, are those of the author and are not necessarily attributed to the NRF.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.enconman.2018.01.024.

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Chapter 4: Supplementary material

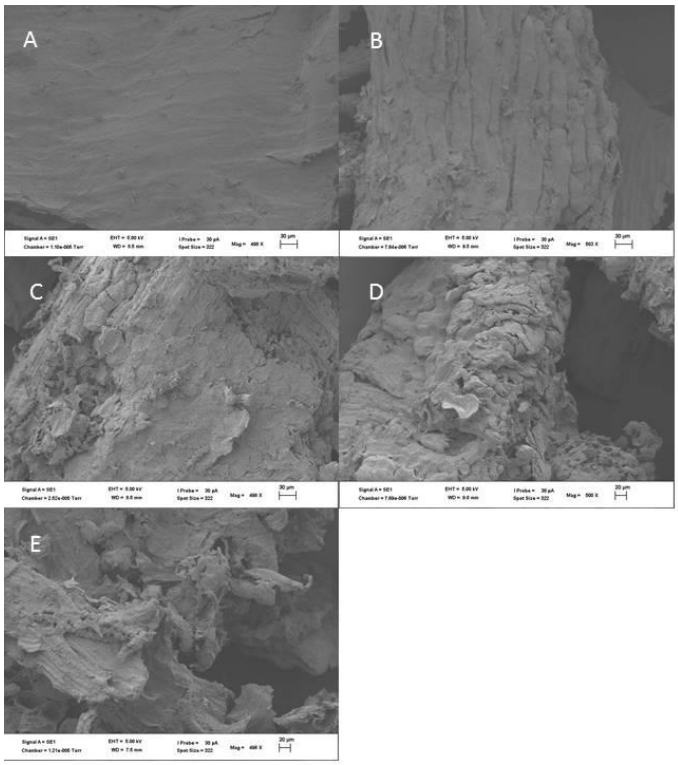

Fig. S1. Scanning electron microscopy images of corn cobs (A) native (B) water alone (C) H_2SO_4 alone (D) $Na_3PO_4.12H_2O$ alone (E) optimized sequential.

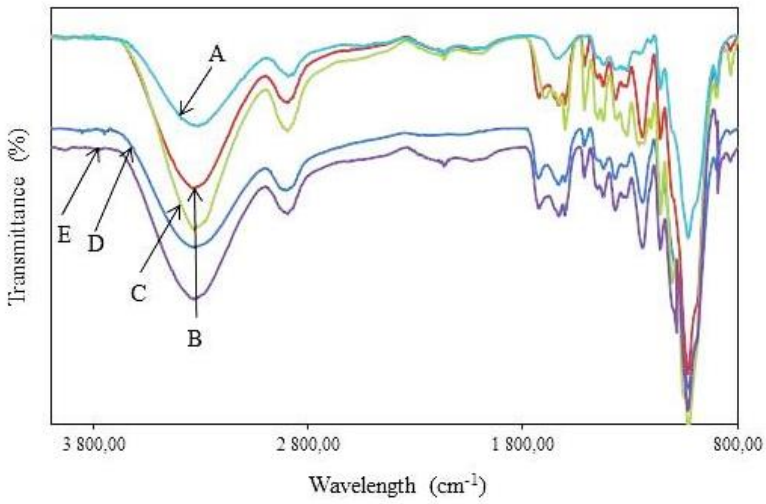

Fig. S2. Fourier transform infrared spectroscopy of corn cobs (A) native (B) water alone (C) H_2SO_4 alone (D) $Na_3PO_4.12H_2O$ alone (E) optimized sequential.

Table S1. Model equations illustrating the functional relationships between the treatment inputs and the reducing sugar yield when input parameters were varied within their boundaries.

Eq.	Process input/output	Model equation form	Equation type	Fitted model	R^2 value
(A)	$Na_3PO_4.12H_2O$ concentration: Reducing sugar yield	$y = a - b^{-cx^d}$	Weibull	$y = 1.05 - 0.74^{-0.000094x^{3.35}}$	0.99
(B)	H_2SO_4 concentration: Reducing sugar yield	$y = \alpha + \frac{\theta x^\eta}{\kappa^\eta + x^\eta}$	DR-Hill	$y = 0.86 + \frac{-0.58x^{4.14}}{1.63^{4.14} + x^{4.14}}$	0.99
(C)	Solid to liquid ratio: Reducing sugar yield	$y = \alpha + \frac{\theta x^\eta}{\kappa^\eta + x^\eta}$	DR-Hill	$y = 0.39 + \frac{20.42x^{2.25}}{131.69^{2.25} + x^{2.25}}$	0.99

Footnote: DR- Dosage response.

Table S2. Structural composition of control and optimized pretreated corn cobs samples.

Sample	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Native	34.21	39.08	6.32
H_2O alone	37.94	38.95	6.24
$Na_3PO_4.12H_2O$ alone	55.97	29.75	3.99
H_2SO_4 alone	40	38.83	6.61
Optimized sequential	58.59	29.01	2.77

Table S3. Inhibitor profile of controls and optimized sequential alkalic salt and dilute acid pretreatment of corn cobs.

Sample	Inhibitor concentration (μg/g)		
	Acetic acid	Furfural	HMF
Water	1.5×10^{-2}	1.4×10^{-3}	ND
H_2SO_4	1.7×10^{-4}	0.14	2.8×10^{-3}
$Na_3PO_4.12H_2O$	1.4×10^{-2}	2.3×10^{-4}	ND
Optimized sequential	1.83×10^{-2}	9.4×10^{-2}	3.7×10^{-4}

Footnote: HMF- 5-Hydroxymethyl furfural; ND- Not detected.

CHAPTER 5

Comparing the sequential alkalic salt and metal salt/dilute acid lignocellulosic pretreatment strategies for microbial production of ethanol fuels and value-added products

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This chapter compares the previously developed optimized sequential alkalic salt pretreatment regimes (Chapter 3 and 4) on their suitability for microbial production of ethanol fuels and value-added products.

The short write-up is presented in the following pages.

Comparing the sequential alkalic salt and metal salt/dilute acid lignocellulosic pretreatment strategies for microbial production of ethanol fuels and value-added products

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Abstract

This chapter comparatively evaluates two previously developed sequential alkalic salt-based lignocellulosic pretreatment strategies on their suitability for microbial production of ethanol fuels and value-added products. These pretreatment techniques included: (1) alkalic salt and metal salt (SAMS) pretreatment and (2) alkalic salt and dilute acid (SASA) pretreatment. These pretreatments were compared based on their impact on the lignocellulosic structural composition, sugar yield and inhibitor profiles. Pretreated corn cobs showed similar structural composition for cellulose (59.98 and 58.89%), hemicellulose (28.33 and 29.01%) and lignin (2.30 and 2.77%) for the SAMS and SASA, respectively. The SAMS and SASA pretreatments gave high reducing sugar (1.10 and 0.99 g/g) and glucose (0.71 and 0.69 g/g) yields, respectively. Inhibitor profile analysis displayed low concentrations (<1 g/L) for both pretreatments. Experimental data obtained for the structural composition, glucose yield and inhibitor profile showed negligible variations between the SAMS and SASA pretreatments. The SAMS pretreatment was shown to be effective for high reducing sugar production whereas the SASA pretreatment yielded a higher quantity of pretreated substrate (2.9-fold). Thus, the SASA pretreatment could potentially enhance the techno-economics of biofuel production processes such as bioethanol.

Keywords: Lignocellulosic biomass, Alkalic salt, Sequential, Pretreatment, Biofuel production

Contents:

1. Introduction

Alkaline pretreatments have emerged as one of the most promising approaches due to effective degradation of the lignocellulosic structure, high sugar yields and low release of fermentation inhibitor compounds compared to acid treatments. However, alkaline pretreatments have been limited by the high cost at industrial scale (Qing et al., 2016). Recently, alkalic salts such as sodium phosphate have garnered significant interest as effective replacement catalysts for expensive alkaline treatments such as sodium hydroxide (NaOH). Two sequential alkalic salt catalysed lignocellulosic pretreatments on corn cobs have been described in our previous studies and included: (1) alkalic salt and metal salt (SAMS) pretreatment (Sewsynker-Sukai and Gueguim Kana, 2017) and (2) alkalic salt and dilute acid (SASA) pretreatment (Sewsynker-Sukai et al., 2018). The optimized pretreatment conditions for the SAMS (14.02% $Na_3PO_4.12H_2O$, 3.65% $ZnCl_2$ and 5% solid to liquid ratio) and SASA (12.70% $Na_3PO_4.12H_2O$, 1.04% H_2SO_4 and 14.49% solid to liquid ratio) pretreatments gave high reducing sugar yields (>8-fold) compared to previous pretreatment reports on corn cobs.

Despite the development of these regimes, there has been a paucity of knowledge on the selection of appropriate pretreatment types for microbial fuels and value added-products. The selection of suitable pretreatment processes necessitates extensive knowledge on the lignocellulosic structure, sugar yield and inhibitor profile. This is mainly because microbial fermentation processes require lignocellulosic substrates that release sufficient sugar for cell

growth, metabolic functioning and product formation. Additionally, inhibitory compounds produced during lignocellulosic pretreatments impact on microbial growth and product formation. The pretreatment type ultimately determines the bioproduct that will be produced (Gao and Rehmann, 2014; Nasr et al., 2014; Pan-in et al., 2017). Product formation by various fermenting microorganisms is considerably influenced by the fermentable sugar concentration available during the bioprocess. Pretreatments that target high fermentable sugar usually apply high chemical concentration combined with high temperatures (Harmsen et al., 2010). In addition, the solid to liquid ratio (SLR) parameter has been shown to significantly impact on the pretreatment process. For example, pretreatments that employ high SLR cause less damage to the lignocellulosic matrix and may lead to moderate fermentable sugar yields. On the other hand, pretreatments with low SLR effectively disrupt lignocellulosic structures leading to high fermentable sugar yield. Even though high fermentable sugar yields are preferable for bioprocesses, pretreatments with low SLR can lead to the formation of elevated inhibitor concentrations, which negatively impact on microbial fermentations (Jönsson et al., 2013). Furthermore, lignocellulosic pretreatment types that employ low SLR drastically escalate the process cost by permitting low substrate quantities per pretreatment cycle (Harmsen et al., 2010).

Therefore, the selection of high efficiency pretreatment processes is necessary to enhance the economic feasibility of bioproduct formation at large scale. There is a lack of consensus for the most suitable lignocellulosic pretreatment type for microbial production of ethanol fuels and value-added products (Qing et al., 2016; Li et al., 2016). The selection of an effective pretreatment type is influenced by several factors that include: (1) degradation of the lignocellulosic structure, (2) fermentable sugar yield and (3) inhibitor compound profile. Efficient selection of appropriate lignocellulosic pretreatments for specific microbial fermentations could improve the techno-economic feasibility for large scale operations. A comparative assessment of the previously developed SAMS and SASA pretreatment regimes will provide knowledge on their relative effectiveness for microbial production of fuels and chemicals. The present study comparatively evaluated the impact of SAMS and SASA pretreatments on their potential suitability for microbial production of high value commodities. Comparisons were made on: (1) degradation of the lignocellulosic structure, (2) reducing sugar and glucose yields and (3) fermentation inhibitor concentrations. The prospect of using SAMS and SASA pretreatments for microbial production of ethanol fuels and value-added products were highlighted.

2. Comparative assessment of SAMS and SASA pretreatments

2.1. Degradation of the lignocellulosic structure

The compositions of the native and pretreated corn cobs are shown in Figure 1. The native corn cobs consisted of 34.21% cellulose, 39.08% hemicellulose and 6.32% lignin. The SAMS optimized sequential pretreated sample contained 59.98% cellulose, which was relatively similar to the SASA pretreatment (58.59%). A similar trend was observed for the hemicellulose and lignin fractions. For instance, the SAMS pretreatment gave a hemicellulose content of 28.33% compared to 29.01% by the SASA pretreatment. In the same way, lignin fractions were low for both the SAMS (2.30%) and SASA (2.77%) pretreatments. During enzymatic saccharification of lignocellulosic biomass, commercial cellulase-based enzymes target the glucose rich cellulose polymer. Cellulose and hemicellulose molecules are bound together by resistant lignin structures. Thus, break down of recalcitrant lignin moieties is crucial to release cellulosic components. Slight variations were observed in the cellulosic contents for the SAMS (59.98%) and SASA (58.59%) pretreatments and were considered negligible. Similar observations were noted for the hemicellulose and lignin composition. Studies on corn cob pretreatment have reported cellulose, hemicellulose and lignin fractions in the range of 50-59%, 10-32% and 7-23% ,respectively (Sahare et al., 2012; Chen et al., 2009; Yu et al., 2017).

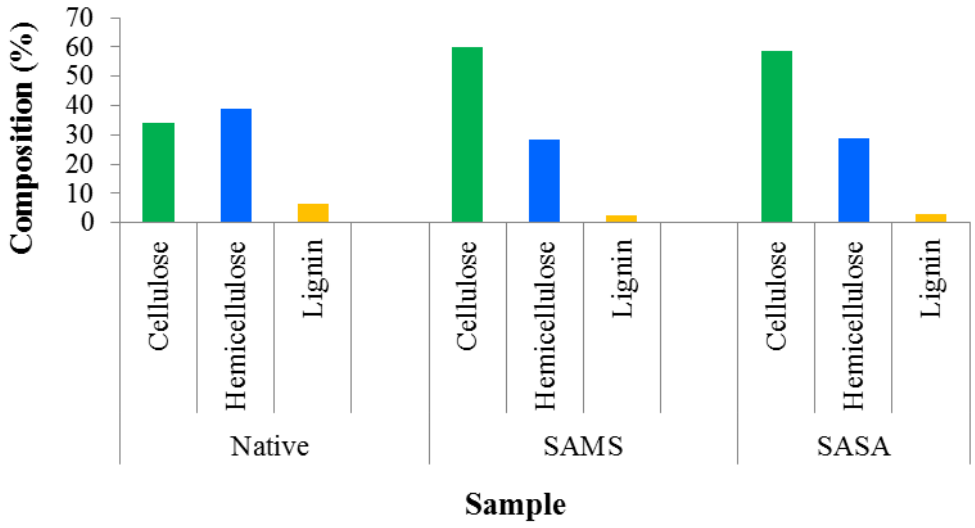

Figure 1. Composition of the lignocellulosic structure for the native and optimized samples.

Topographical changes in the lignocellulosic structure were visualized using scanning electron microscopy (SEM) (Figure 2). The SEM micrographs depicted major structural differences between the native (untreated) and pretreated corn cob biomass. The native sample exhibited a smooth and compact surface with minimal aberrations to the lignocellulosic structure. Both the SAMS and SASA pretreatments disrupted the structural integrity of the corn cobs and showed an increase in surface fractionation and roughness. The SAMS and SASA pretreatments displayed a high degree of structural damage with fragmentation and perforations. No significant structural differences were observed between the SAMS and SASA pretreated samples. This indicated that both sequential pretreatments were equally effective in the disruption of the lignocellulosic matrix. Similar observations in the corn cob surface structure were previously reported in different pretreatment studies using KOH (Wanitwattanarumlug et al., 2012), H_3PO_4 (Boonsombuti et al., 2015) and NaOH (Boonsombuti et al., 2013). The damaged lignocellulosic structure after pretreatment allows enzymatic attack of the cellulose polymer to produce glucose molecules that can be channeled towards microbial fermentative processes.

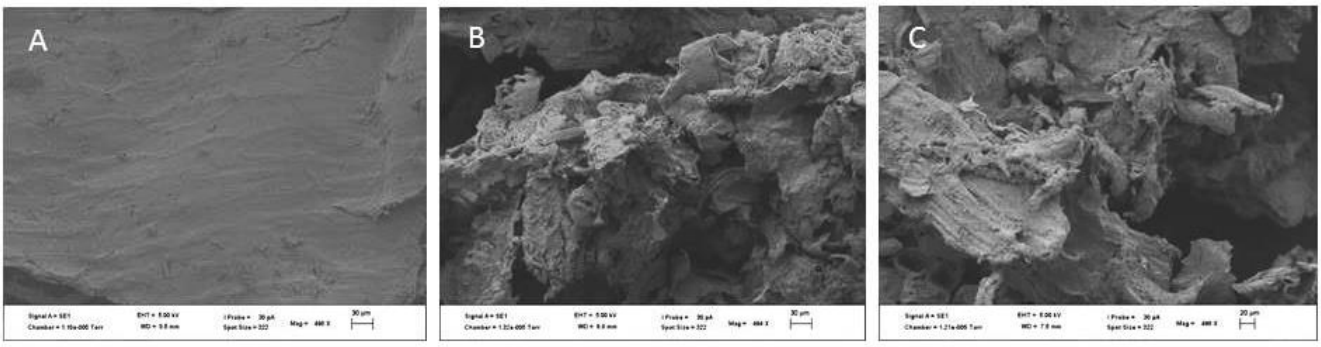

Figure 2. SEM micrographs of corn cobs (A) Native, (B) SAMS pretreated and (C) SASA pretreated.

2.2. Release of fermentation inhibitor compounds

Chemical pretreatment of lignocellulosic biomass releases various fermentation inhibitor compounds. Inhibitor profiles are highly dependent on the nature of the pretreatment employed. Major inhibitor compounds include acetic acid and furan derivatives (furfural, 5-Hydroxymethyl furfural). These volatile compounds inhibit microbial growth and metabolism, thus negatively impacting on fermentation processes (Mussatto and Roberto,

2004). Acetic acid is released when ester and acetyl linkages present within hemicellulose are disrupted (Kamireddy et al., 2013; Harmsen et al., 2010). Likewise, the formation of furfural and 5-Hydroxymethyl furfural (HMF) occur during pentose and hexose degradation (Ravindran and Jaiswal, 2016). Lignocellulosic pretreatment reports on inorganic salts have displayed low concentration of inhibitor compounds while acid treatments resulted in high furfural and HMF concentrations (Loow et al., 2015; Wikandari et al., 2010). Additionally, concentrated acids release significantly higher inhibitor concentrations compared to dilute acid solutions. As shown in Table 1, the SAMS pretreatment produced slightly lower concentrations of inhibitors compared to the SASA pretreatment. The SAMS optimized sample gave a low acetic acid concentration (7×10^{-3} µg/g) and furfural concentration (3.7×10^{-2} µg/g) with no HMF detected, whereas acetic acid, furfural and HMF concentrations of 1.83×10^{-2} µg/g, 9.4×10^{-2} µg/g and 3.7×10^{-4} µg/g, respectively, were obtained using the SASA optimized pretreatment (Table 1). The SASA pretreatment gave low concentrations of furfural and HMF, which was attributed to the use of dilute H_2SO_4 (1.04%) which reduces pentose and hexose degradation. Previous reports on corn cob pretreatment gave high acetic acid, furfural and HMF concentrations in the range of 1-15 g/L, 0.20-7.5 g/L and 0.40-1.5 g/L respectively (Van Eylen et al., 2011; Wang et al., 2011). Generally, acetic acid concentrations above 1.5 g/L and HMF concentrations >1 g/L have been shown to inhibit microbial growth and product formation (Wikandari et al., 2010). The SAMS and SASA pretreatments resulted in low fermentation inhibitor concentrations (<1 g/L) thus, significantly below the concentrations reported in previous studies as well as the inhibitory concentration. The low concentration of inhibitor compounds observed for the SAMS and SASA pretreatments further highlights their efficiency for industrial scale bioprocesses.

Table 1. Inhibitor profiles after the SAMS and SASA pretreatments.

Pretreatment	Acetic acid (µg/g)	Furfural (µg/g)	HMF (µg/g)	Reference
SAMS	7×10^{-3}	3.7×10^{-2}	ND	Sewsynker-Sukai and Gueguim Kana (2017)
SASA	1.83×10^{-2}	9.4×10^{-2}	3.7×10^{-4}	Sewsynker-Sukai et al. (2018)

Footnote: HMF- 5-hydroxymethylfurfural, ND- Not determined.

2.3. Reducing sugar and glucose yields

Chemically pretreated substrates are hydrolysed to produce glucose as the major end product. However, hydrolytic enzymes such as Cellic CTec 2 have been described as a blend of aggressive cellulases, β-glucosidases and hemicellulases (Novozymes A/S, 2010) that released both glucose and xylose (Aguilar-Reynosa et al., 2017; Zhao et al., 2015; Zhu et al., 2015). Enzymatic hydrolysis of the pretreated biomass gave high reducing sugar and glucose yields (Figure 3). The SAMS pretreatment gave a reducing sugar (RS) yield of 1.10 g/g compared to the SASA pretreatment (0.99 g/g). Thus, a 10% higher reducing sugar yield was observed for the SAMS pretreatment. Variations in the alkalic salt concentration and solid to liquid ratio accounted for the higher reducing sugar yield obtained for the SAMS pretreatment. For the SAMS optimized pretreatment, a low solid to liquid ratio (5%) was treated with a high alkalic salt concentration (14.02%) compared to 14.49% (solid to liquid ratio) and 12.70% ($Na_3PO_4.12H_2O$ concentration) for the SASA pretreatment. The higher alkalic salt concentration combined with a lower solid to liquid ratio enhanced the pretreatment efficiency by disrupting the lignocellulosic structures. Additionally, high glucose yields of 0.69 g/g (SASA) and 0.71 g/g (SAMS) were obtained under the optimal pretreatment conditions. The variation observed in the glucose yields can be attributed to the slightly higher cellulose and lower hemicellulose contents obtained for the SAMS pretreatment (Figure 1). Earlier reports on corn cobs gave reducing sugar and glucose yields in the range of 0.11-0.92 g/g and 4-54 g/L (Chen et al., 2009; Satimanont et al., 2012; Li et al., 2016; Potumarthi et al., 2012), respectively. Glucose is a versatile C6 monomeric sugar that is metabolised by several microbes (Loow et al., 2015). On the other hand, xylose is a C5 monosaccharide that can be converted to xylitol, which is of industrial significance as a natural sweetener (Swain and Krishnan, 2015). In addition, xylose can be channelled towards fuels such as biohydrogen and biogas through microbial fermentation processes. The SAMS and SASA pretreatments gave higher reducing sugar and glucose yields compared to previous reports on the same substrate thus, increasing the application of lignocellulosic substrates for industrial scale microbial fermentative processes.

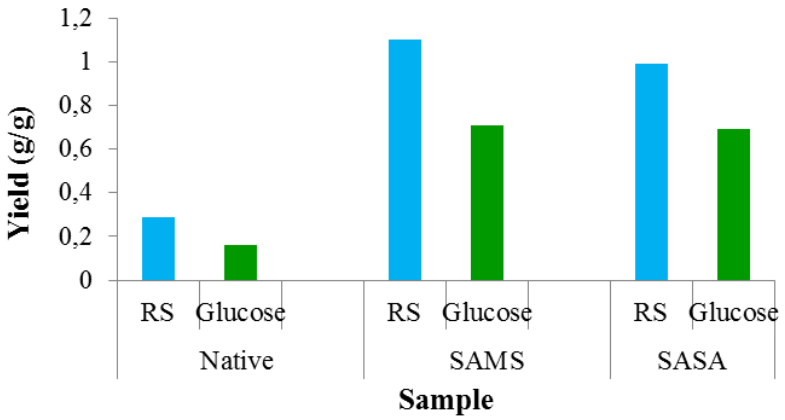

Figure 3. Reducing sugar and glucose yields from the native and optimized samples.

3. Prospect of using the SAMS and SASA pretreatments for microbial production of ethanol fuels and value-added products

Previous reports on microbial ethanol fuels and value-added products generated from corn cobs under various chemical pretreatments are depicted in Table 2. Different lignocellulosic pretreatment types produce different profiles of fermentable sugars and inhibitor compounds. Fermentable sugars produced may consist of glucose, xylose and arabinose while inhibitor compounds include acetic acid, furfural and HMF. While high fermentable sugars are desired for microbial fermentative processes, inhibitor compounds should be minimized. Inhibitor compounds exhibit a high toxicity and can lead to precipitation and irreversible inhibition of hydrolytic enzymes during the saccharification steps for sugar production (Jönsson et al., 2013; Harmsen et al., 2010). Additionally, these compounds impact on the microbial metabolic fluxes and may: (1) cause intracellular anion accumulation resulting in acid dissociation and thus a lower cell pH, which inhibits microbial cell activity, and (2) trigger partition and loss of integrity of cell membranes, thus, reducing microbial cell activity, growth and sugar assimilation (Harmsen et al., 2010).

The relative toxicities of acetic acid, furfural and HMF may vary from one microbial fermentation process to another. For example, low concentration of inhibitor compounds can be detrimental to microbial fermentations that employ pure cultures such as bioethanol production as opposed to biohydrogen or biomethane generation, which utilizes a mixed consortium. The metabolic machinery within the single pure species is drastically influenced by slight changes in the environmental factors (Harmsen et al., 2010). On the other hand,

methane and hydrogen-producing mixed microbial consortia consist of a range of microbes that are able to withstand a high level of inhibitors. In addition, pure cultures may be limited by a single metabolic pathway and can only ferment glucose whereas mixed microbial cultures can utilize glucose, xylose and arabinose (Nasr et al., 2014; Pan-in et al., 2017; Harmsen et al., 2010). Different lignocellulosic pretreatment types can be used to target specific bioproducts. For example, the SAMS pretreatment could prove valuable if the target product was reducing sugar. Reducing sugars may consist of several monosaccharides such as glucose, xylose and arabinose that can be metabolized by several microbial cultures, which facilitates its application in various microbial fermentative processes. Moreover, the SAMS pretreatment does not include acid and could prove beneficial when acid pretreatment processes are prohibited under some countries government legislations due to environmental concerns (Kumar and Sharma, 2017). The developed sequential pretreatment regimes showed negligible deviations in the lignocellulosic structural composition, glucose yields and fermentation inhibitor concentrations. Despite this, a large variation was observed for the optimal solid to liquid ratio parameter. Optimization gave a high solid to liquid ratio of 14.49% for the SASA pretreatment (Figure 4A) compared to 5% for the SAMS pretreatment (Figure 4B). The SASA pretreatment yielded a 2.9-fold higher solid to liquid ratio compared to the SAMS pretreatment. Lignocellulosic biofuel production processes require energy efficient and inexpensive pretreatments, which release sufficient fermentable sugar that can be used as carbon and energy sources for microbial growth and product formation. In addition, pretreatments that produce very low concentration of inhibitors are desirable (Jönsson et al., 2013). Although both the SAMS and SASA pretreatment strategies align with the aforementioned microbial necessities, the SASA pretreatment displayed a higher efficiency. For instance, the higher quantity of substrate (2.9-fold) achieved per pretreatment cycle using the SASA pretreatment compared to the SAMS strategy may potentially enhance the economics for large scale biofuel production processes such as bioethanol.

Table 2. Microbial ethanol fuels and value-added products generated from previous corn cob studies under different pretreatment regimes

Pretreatment conditions	Microorganism	Fuel/Value-added product	Reference
2% H_2SO_4 ,121°C, 60 min, 10% SLR	*Saccharomyces cerevisiae*	Ethanol	Li et al. (2016)
2.5% NaOH ,121°C, 30 min, 10% SLR	*Aspergillus niger*	Cellulases and Hemicellulases	Irfan et al. (2010)
1% H_2SO_4 ,121°C, 60 min, 10% SLR	*Clostridium beijerinckii*	Butanol	Boonsombuti et al. (2015)
0.5M NaOH, 121°C, 30 min, 12.5% SLR	*Clostridium saccharobutylicum*	Acetone, Butanol and Ethanol	Gao and Rehmann (2014)
2% NaOH, 25°C, 2880 min, 3% SLR	Mixed culture[a]	Methane	Pan-in et al. (2017)
Autohydrolysis with dilute acid[*]	Mixed culture[b]	Hydrogen	Nasr et al. (2014)
0.5% H_2SO_4 for 60 min, 121°C, 20 min, 14.3% SLR	*Clostridium hydrogeniproducens*	Hydrogen	Tang et al. (2013)
2% NaOH, 121°C, 30 min, 20% SLR	*S. cerevisiae* and *Candida tropicalis*	Xylitol and Ethanol	Latif and Rajoka (2001)

Footnote: SLR- Solid to liquid ratio; [*]- pretreatment conditions not stated; [a]- animal dung (pig, cow and goat); [b]- anaerobic digested sludge.

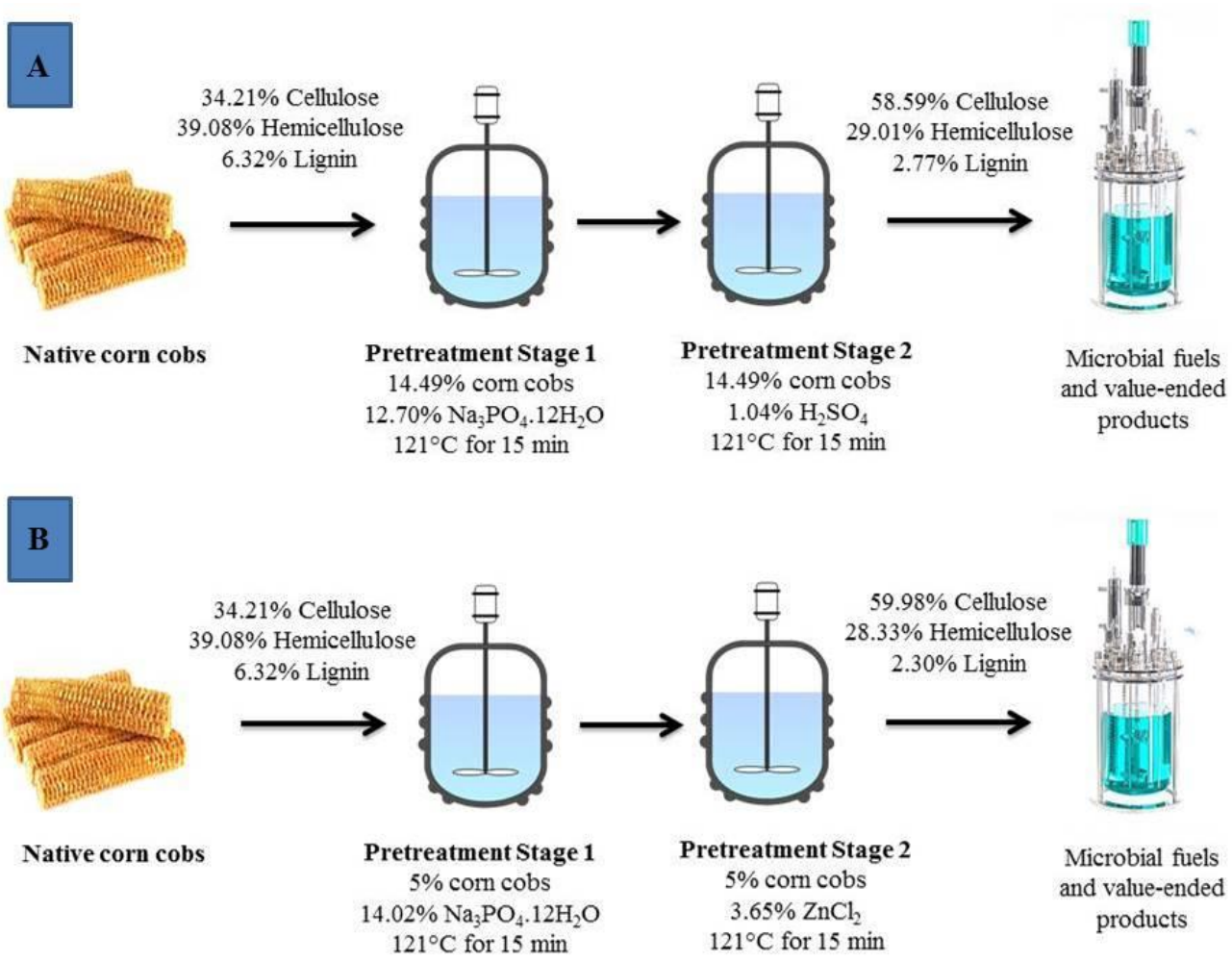

Figure 4. Flow diagram of the (A) SASA pretreatment and (B) SAMS pretreatment.

4. Conclusion

This chapter comparatively assessed two previously developed sequential alkalic salt catalysed pretreatments on their suitability for microbial production of ethanol fuels and value-added products. These pretreatments were compared based on the corn cob structural composition, sugar yield and inhibitor profile. Compositional analysis gave comparable cellulose (59.98 and 58.89%), hemicellulose (28.33 and 29.01%) and lignin (2.30 and 2.77%) fractions for the SAMS and SASA pretreatments, respectively. Likewise, the SAMS and SASA pretreatments displayed high reducing sugar (1.10 and 0.99 g/g) and glucose (0.71 and 0.69 g/g) yields respectively, with low inhibitor concentrations (<1 g/L). The SAMS

pretreatment was more effective for high reducing sugar production and could be used for several microbial bioprocesses. However, the SASA pretreatment yielded a higher substrate quantity (2.9-fold) compared to the SAMS pretreatment and may potentially improve the techno-economics of microbial biofuel production processes such as bioethanol.

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CHAPTER 6

Simultaneous saccharification and bioethanol production from corn cobs: Process optimization and kinetic studies

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This chapter has been published in *Bioresource Technology* (262, 32-41) with the title: Simultaneous saccharification and bioethanol production from corn cobs: Process optimization and kinetic studies.

The published paper and supplementary material are presented in the following pages.

Bioresource Technology 262 (2018) 32–41

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Simultaneous saccharification and bioethanol production from corn cobs: Process optimization and kinetic studies

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GRAPHICAL ABSTRACT

ARTICLE INFO

Keywords:
Bioethanol
Prehydrolysis
Simultaneous saccharification and fermentation
Corn cobs

ABSTRACT

This study investigates the simultaneous saccharification and fermentation (SSF) process for bioethanol production from corn cobs with prehydrolysis (PSSF) and without prehydrolysis (OSSF). Two response surface models were developed with high coefficients of determination (> 0.90). Process optimization gave high bioethanol concentrations and bioethanol conversions for the PSSF (36.92 ± 1.34 g/L and 62.36 ± 2.27%) and OSSF (35.04 ± 0.170 g/L and 58.13 ± 0.283%) models respectively. Additionally, the logistic and modified Gompertz models were used to study the kinetics of microbial cell growth and ethanol formation under microaerophilic and anaerobic conditions. Cell growth in the $OSSF_{microaerophilic}$ process gave the highest maximum specific growth rate (μ_{max}) of 0.274 h^{-1}. The $PSSF_{microaerophilic}$ bioprocess gave the highest potential maximum bioethanol concentration (P_m) (42.24 g/L). This study demonstrated that microaerophilic rather than anaerobic culture conditions enhanced cell growth and bioethanol production, and that additional prehydrolysis steps do not significantly impact on the bioethanol concentration and conversion in SSF process.

1. Introduction

Second generation biofuels such as lignocellulosic bioethanol production has gained significant interest as a potential replacement for fossil fuel-derived sources (Aguilar-Reynosa et al., 2017). Bioethanol exhibits several advantages over conventional fossil fuels which include its renewable and sustainable nature, ease of storage, higher oxygen content and higher octane number, among others (Putra et al., 2015). Lignocellulosic biomass sources such as corn cobs have emerged as suitable feedstocks for bioethanol production processes. However, economical cellulosic bioethanol production is associated with several key technological issues. Identification of the bottlenecks that limit

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https://doi.org/10.1016/j.biortech.2018.04.056
Received 27 February 2018; Received in revised form 11 April 2018; Accepted 13 April 2018
Available online 17 April 2018
0960-8524/

industrial ethanol fermentation with subsequent development of attractive high-ethanol-performance processes is considered fundamental for scale up. These include the need for cost-effective lignocellulosic pretreatment regimes that result in high sugar yields and low fermentation inhibitor compounds, efficient utilization of feedstock, fermentation processes that result in high ethanol yields and shorter fermentation times (Aguilar-Reynosa et al., 2017; Zhao et al., 2015).

Simultaneous saccharification and fermentation (SSF) processes are being considered as effective operational strategies to reduce production costs, increase ethanol concentration and ethanol conversion with shorter times due to the elimination of separate, long saccharification steps. SSF processes are performed in a single reactor with the same working temperature and the glucose that is produced is simultaneously metabolized by the bioethanol producing microorganism. Moreover, the inhibitory effects caused by high glucose yields during the enzymatic hydrolysis stage are significantly reduced. Nevertheless, differences in the optimal temperature for enzymatic activity (50 °C) and yeast growth (30 to 37 °C) have limited the implementation of SSF processes (Gonçalves et al., 2016; Ruiz et al., 2012).

Several studies have focussed on the enhancement of SSF processes (Zhao et al., 2015; Aguilar-Reynosa et al., 2017; Zhu et al., 2015). Zhu et al. (2015) observed a 1.4-fold higher bioethanol concentration when the solid loading was increased from 15 to 25%. Likewise, Aguilar-Reynosa et al. (2017) recorded a 9% improvement in the bioethanol conversion using a 10% solid loading compared to 12.5%. Similarly, Zhao et al. (2015) observed an 18% increase in the ethanol yield when the yeast titre was increased up to four times its base level. SSF processes are affected by several process parameters that include solid loading, enzyme loading and yeast titre. An optimum combination of these process inputs may overcome challenges associated with mass and heat transfer in addition to improving the overall ethanol concentration and conversion. Therefore, optimization of key parameters that influence the SSF process is crucial for achieving maximum bioethanol concentration and conversion. Bioprocess optimization is a complex stage that is necessary to improve product yield and maintain a level of consistency during scale up (Cheng et al., 2017). Statistical models such as the response surface methodology (RSM) can be used to identify the individual and interactive effects of process variables on the responses and to determine the optimum conditions during SSF processes.

Additionally, reports on SSF processes have indicated that a prehydrolysis step could significantly enhance the fermentation process and ethanol concentration and conversion (He et al., 2016; Zhu et al., 2015; Liu et al., 2014). This is due to the higher saccharification efficiency at elevated temperatures which are usually required for optimal enzymatic activity and reduced initial viscosity at the beginning of fermentation (He et al., 2016; Zhu et al., 2015). Even with these advantages, prehydrolysis stages require a longer process time and a higher energy input, thus reducing its economic feasibility. Combining the enzymatic hydrolysis and fermentation steps reduces the number of unit operations. A decrease in capital investment has been estimated to be more than 20% when SSF processes without prehydrolysis have been used (Wingren et al., 2003). This reduction is substantial since lignocellulosic bioethanol production is already limited by its high cost. There has been a lack of consensus on the effect of prehydrolysis stages in SSF processes (Zhu et al., 2015; He et al., 2016). Thus, modelling and optimization of SSF processes with and without prehydrolysis is imperative to determine its effect on the bioethanol concentration and conversion.

Kinetic modelling is considered as one of the most crucial steps in developing fermentation processes for large scale application. These process models define the production process under different input conditions which can help improve the product yield, productivity and reduce undesirable by-products. This will reduce costs and increase the product quality. Logistic models are employed to describe the changes in microbial cell growth as a function of growth rate, initial and maximum biomass concentration, and time (Phukoetphim et al., 2017). The modified Gompertz model has been used to determine production lag time, maximum production rate, and maximum product concentration on a given substrate (Dodic et al., 2012).

There is a dearth of knowledge on the kinetics of *Saccharomyces cerevisiae* growth and ethanol formation from corn cob wastes under microaerophilic and anaerobic conditions in SSF processes. A high cost is associated with maintaining anaerobic conditions at large scale thus reducing its economic viability (Podkaminer et al., 2012; Azhar et al., 2017). *S. cerevisiae* is one of the few yeasts that are able to grow under aerobic, microaerophilic and anaerobic environments however, the former two favours microbial cell growth and replication while the latter proceeds directly towards bioethanol fermentation (Lin et al., 2012). *S. cerevisiae* shifts to a mixed respiro-fermentative metabolism which produces ethanol when sufficient glucose concentration is available (0.8 mM) (Verduyn et al., 1984). Generally, aerobic ethanol production by *S. cerevisiae* depends on the relative capacities of the fermentative and respiratory pathways. This microbe does not generate ethanol under aerobic conditions when low glucose concentrations are present (Kappeli, 1986). Glucose uptake in *S. cerevisiae* is controlled by multiple hexose transporters (Ozcan and Johnston, 1999) which have demonstrated different substrate specificity and affinity when expressed under different, overlapping conditions (Reifenberger et al., 1997). Some studies have indicated that the presence of oxygen stimulates high levels of pyruvate decarboxylase in *S. cerevisiae* whereas yeasts such as *Candida utilis* and *Kluyveromyces lactis* display high levels of this enzyme under oxygen-limited conditions (Snoek and Steensma, 2007; Kiers et al., 1998; Weusthuis et al., 1994). Oxygen is required for lipid biosynthesis in *S. cerevisiae* and is essential for cell growth, plasma membrane integrity, and the maintenance of high glycolytic and ethanol production rates (Rosenfeld et al., 2003). Therefore, knowledge on the kinetics of microbial cell growth and bioethanol production under microaerophilic and anaerobic process conditions will significantly impact on bioethanol process design for large scale application.

The specific objectives of this work was to: (1) optimize the simultaneous saccharification and fermentation (SSF) process of bioethanol production from corn cobs with prehydrolysis (PSSF) and without prehydrolysis (OSSF), (2) determine the individual and interactive effects of yeast titre, solid loading and enzyme loading on the bioethanol concentration and bioethanol conversion, and (3) study the kinetics of microbial cell growth and bioethanol formation under microaerophilic and anaerobic process conditions using the logistic and modified Gompertz models.

2. Materials and methods

2.1. Materials

The corn cobs used in this study were obtained from the Ukulinga research farm (Pietermaritzburg, South Africa) (29° 67′ E, 30° 40′ S). These were then oven dried at 60 °C for 24 h and thereafter milled to a particle size of less than 1–2 mm by a centrifugal miller (Retsch ZM-1, South Africa). The powdered corn cobs were stored at room temperature. All chemicals used in this study were purchased from Merck, South Africa.

2.2. Pretreatment of corn cobs

Milled corn cobs were pretreated using an optimized sequential alkalic salt and dilute acid pretreatment as described in our previous study (Sewsynker-Sukai et al., 2018). During the first stage, the milled corn cobs with a solid loading of 14.49% (w/v) was treated with 12.70% (w/v) $Na_3PO_4{\cdot}12H_2O$ at 121 °C for 15 min then washed and dried at 60 °C and was thereafter treated with 1.04% (v/v) H_2SO_4 at 121 °C for 15 min. The treated biomass was filtered, washed and dried as previously described and the solid residue obtained was used for the

Table 1
Bioethanol concentration and conversion observed for PSSF and OSSF experiments.

Run	Yeast titre (times)	Solid loading (%)	Enzyme loading (FPU/g)	PSSF bioethanol concentration (g/L)	PSSF bioethanol conversion (%)	OSSF bioethanol concentration (g/L)	OSSF bioethanol conversion (%)
1	3.00	30.00	10.00	26.92	26.53	25.36	24.99
2	3.00	20.00	20.00	41.31	61.07	35.33	52.22
3	3.00	20.00	20.00	42.45	62.75	34.90	51.59
4	1.00	30.00	20.00	23.93	23.58	30.63	30.18
5	5.00	20.00	30.00	41.74	61.70	34.76	51.38
6	5.00	30.00	20.00	28.21	27.79	28.63	28.21
7	1.00	20.00	10.00	33.90	50.12	32.19	47.59
8	3.00	20.00	20.00	39.17	57.91	36.47	53.91
9	5.00	10.00	20.00	31.20	92.22	29.49	87.16
10	1.00	20.00	30.00	42.45	62.75	41.17	60.85
11	3.00	30.00	30.00	29.91	29.48	30.63	30.18
12	3.00	10.00	10.00	30.34	89.69	27.78	82.11
13	3.00	10.00	30.00	31.62	93.48	29.34	86.74
14	3.00	20.00	20.00	39.88	58.96	34.76	51.38
15	5.00	20.00	10.00	33.04	48.85	33.76	49.90
16	1.00	10.00	20.00	30.05	88.85	30.34	89.69
17	3.00	20.00	20.00	43.16	63.80	36.75	54.33

Footnote: 1 time (base level) = 2.70×10^6 cells/mL, 3 times the base level = 8.10×10^6 cells/mL, 5 times the base level = 1.35×10^7 cells/mL.

SSF processes. The composition of the sequentially pretreated corn cobs was determined following the National Renewable Energy Laboratory (Sluiter et al., 2008). The pretreated corn cobs in this study consisted of 59.72% cellulose, 28.21% hemicellulose and 2.41% lignin.

2.3. RSM modelling and optimization of the simultaneous saccharification and fermentation (SSF) process

The Box-Behnken design was used for the development of the SSF with prehydrolysis (PSSF) and the SSF without prehydrolysis (OSSF) models. The input parameters consisted of yeast titre (1–5 times the base level which corresponded to 2.70×10^6–1.35×10^7 cells/mL), solid loading (10–30%, w/v) and enzyme loading (10–30 FPU/g) with bioethanol concentration and bioethanol conversion as the response outputs (Table 1). The input ranges were selected based on the previous works by Zhao et al. (2015), Zhu et al. (2015) and Aguilar-Reynosa et al. (2017). Thus, four process models were investigated and designated as follows: (1) SSF with prehydrolysis for bioethanol concentration ($PSSF_{concentration}$), (2) SSF with prehydrolysis for bioethanol conversion ($PSSF_{conversion}$), (3) SSF without prehydrolysis for bioethanol concentration ($OSSF_{concentration}$), (4) SSF without prehydrolysis for bioethanol conversion ($OSSF_{conversion}$). A total of 17 experiments were evaluated for each SSF process. All experiments were carried out in duplicate. The experimental data were used to fit the polynomial model equations, relating the input parameters to the bioethanol concentration and bioethanol conversion using Design Expert software (Stat-Ease Inc., USA).

2.4. Enzyme and enzymatic activity

Cellic CTec 2, a cellulase-based enzyme was generously provided by Novozymes (Novozymes A/S, Denmark). The enzyme activity of Cellic CTec 2 was 160 FPU/ml and was determined according to the Laboratory Analytical Procedure (LAP) of the National Renewable Energy Laboratory (NREL, 2008).

2.5. Yeast inoculum

Saccharomyces cerevisiae strain BY4743 was obtained from the Department of Genetics, University of KwaZulu-Natal, Pietermaritzburg, South Africa. The cells were grown for 18 h at 30 °C in an orbital shaker (120 rpm) and yeast peptone dextrose (YPD) medium containing 20 g/L of glucose, 20 g/L of bacteriological peptone and 10 g/L of yeast extract. The standard yeast culture contained 2.70×10^6 cells/mL of *S. cerevisiae* and was determined using a cell count chamber (Thoma, Germany).

2.6. Simultaneous saccharification and fermentation (SSF) processes

2.6.1. Prehydrolysis stage

A 24 h prehydrolysis stage was performed for all PSSF experiments only. The reaction solution (22.5 mL) contained pretreated corn cobs with solid loading (10–30%), enzyme loading (10–30 FPU/g), citrate buffer (pH 4.8, 0.05 M) and nutrients (10 g/L peptone and 5 g/L yeast extract) and were incubated at 50 °C and 120 rpm for 24 h. The enzyme and substrate loading were fed based on the final working volume of 25 mL.

2.6.2. SSF process

For the PSSF experiments, the enzymatic hydrolysate from the prehydrolysis stage was used. The OSSF process (25 mL) contained: pretreated corn cobs with solid loading (10–30%), enzyme loading (10–30 FPU/g), citrate buffer (pH 4.8, 0.05 M) and nutrients (10 g/L peptone and 5 g/L yeast extract). The *S. cerevisiae* BY4743 cells were harvested by centrifugation for 8 min at 4 °C, 1800g and the cell pellet was re-suspended in 0.9% (w/v) NaCl solution. Inoculation was carried out at 10% (v/v) (yeast titre of 1 time = 2.70×10^6, 3 times = 8.10×10^6 cells/mL, 5 times = 1.35×10^7 cells/mL suspended in 0.9% NaCl) for the PSSF and OSSF experiments. 0.9% NaCl solution was proportionally added to ensure a 10% (v/v) inoculation of the final fermentation solution. After inoculation, both OSSF and PSSF experiments were incubated at 35 °C and 120 rpm over 48 h. These experiments were characterized as microaerophilic processes attributable to the initial exposure to molecular oxygen without subsequent aeration (Phukoetphim et al., 2017). For sample analysis, 0.5 mL aliquots were extracted after 0, 12, 24 and 48 h.

2.7. Analytical methods

2.7.1. Determination of ethanol, glucose and biomass concentration

The ethanol concentration was determined using Megazyme K-ETOH assay kits according to the manufacturer's instructions (Megazyme, Ireland) and VERSAmax tunable microplate reader (Molecular Devices, California, USA). Hydrolysed and unfermented glucose were detected spectrophotometrically using Megazyme glucose kits (Megazyme, Ireland). The bioethanol conversion yield was calculated using the following Eq. (1):

$$\text{Bioethanol conversion (\%)} = \frac{[EtOH]f}{0.51(f\,[biomass]1.111)} \times 100 \quad (1)$$

where $[EtOH]_f$ is the highest ethanol concentration generated during the fermentation (g/L), the denominator expression represents the theoretical ethanol concentration, where [biomass] is the dry lignocellulosic biomass concentration at the beginning of the fermentation (g/L), f is the cellulose fraction of dry biomass (0.597 g/g in this study), 1.111 is the conversion factor of cellulose to equivalent glucose and 0.51 is a conversion factor for glucose to ethanol based on the reaction stoichiometry (NREL, 2001; Aguilar-Reynosa et al., 2017).

Cell biomass was quantified by using the cell count as a function of the concentration of yeast cells. A 24 h *S. cerevisiae* culture grown in YPD broth was used. Samples were centrifuged at 10 000 rpm for 5 min and the pellets were used to determine the cell dry weight. Dry cell weights were determined by oven drying at 70 °C until a constant mass was obtained.

2.8. Validation on optimized OSSF and PSSF processes under microaerophilic and anaerobic conditions

Validation of the optimized PSSF and OSSF processes were carried out under microaerophilic and anaerobic conditions. These processes were designated as follows: (1) optimized SSF with prehydrolysis under microaerophilic conditions ($PSSF_{microaerophilic}$), (2) optimized SSF with prehydrolysis under anaerobic conditions ($PSSF_{anaerobic}$), (3) optimized SSF without prehydrolysis under microaerophilic conditions ($OSSF_{microaerophilic}$), (4) optimized SSF without prehydrolysis under anaerobic conditions ($OSSF_{anaerobic}$). Anaerobic processes were performed in modified screw cap Erlenmeyer flasks. After inoculation, the anaerobic experimental processes were flushed with N_2 gas for 1 min to create anaerobiosis. Experiments that were not flushed with nitrogen (N_2) gas were characterized as microaerophilic processes due to the initial exposure to molecular oxygen without subsequent aeration. Both OSSF and PSSF experiments under microaerophilic and anaerobic conditions were incubated at 35 °C and 120 rpm for 48 h. Sample analysis for PSSF and OSSF experiments (microaerophilic and anaerobic) were performed every 2 h. Ethanol and glucose analysis were determined as previously stated. All experiments were performed in duplicate. Control experiments (not inoculated) were performed under microaerophilic and anaerobic conditions to estimate the initial concentration of glucose released. This was thereafter subtracted from the test experimental samples to compute the glucose utilisation (%). Glucose utilisation was calculated using Eq. (2) (Srimachai et al., 2015):

$$\text{Glucose utilisation (\%)} = \frac{\text{Initial glucose concentration (g/L)} - \text{Final glucose concentration (g/L)}}{\text{Initial glucose concentration (g/L)}} \times 100 \quad (2)$$

2.9. Kinetic models and calculation of kinetic parameters

2.9.1. The logistic model

The logistic model in the differential form of Eq. (3) was integrated to form Eq. (4). Eq. (3) represents the exponential and stationary phases of growth. This logistic model illustrates the relationship of biomass (X) to initial cell concentration (X_0), maximum cell concentration (X_{max}) and maximum specific growth rate (μ_{max}) at specific times (t) during the exponential and stationary phases of yeast growth. Nevertheless, it does not predict the death phase of microorganisms after the stationary phase (Zajšek and Goršek, 2010).

$$\frac{dX}{dt} = \mu_{max}\left(1 - \frac{X}{X_{max}}\right)X \quad (3)$$

$$X = \frac{X_0 \exp(\mu_{max} t)}{1 - \left[\left(\frac{X_0}{X_{max}}\right)(1 - exp(\mu_{max} t))\right]} \quad (4)$$

2.9.2. The modified Gompertz model

The bioethanol production data derived from the microaerophilic and anaerobic conditions were used to fit the modified Gompertz model by using the least squares method (CurveExpert V1.5.5). This model revealed the lag time (t_L), maximum bioethanol production rate ($r_{p,m}$), and the maximum potential bioethanol concentration (P_m) as shown in Eq. (5).

$$P = P_m \cdot exp\left\{-exp\left[\frac{r_{p,m} \cdot exp(1)}{P_m}\right] \cdot (t_L - t) + 1\right\} \quad (5)$$

where P is bioethanol concentration (g/L), P_m is potential maximum bioethanol concentration (g/L), $r_{p,m}$ is maximum bioethanol production rate (g/L/h) and t_L is the time from the beginning of fermentation to exponential bioethanol production (h).

3. Results and discussion

3.1. RSM model development

Bioethanol concentrations and conversions obtained from the PSSF and OSSF experiments after 48 h (Table 1) were used to generate the polynomial Eqs. (6)–(9) (Table 2). The suitability of the developed models was assessed by Analysis of Variance (ANOVA). High F-values of 11.51, 80.56, 37.79 and 385.17 were observed for the $PSSF_{concentration}$, $PSSF_{conversion}$, $OSSF_{concentration}$ and $OSSF_{conversion}$ models respectively.

In addition, $PSSF_{concentration}$, $PSSF_{conversion}$, $OSSF_{concentration}$ and $OSSF_{conversion}$ models gave low p-values of 0.0020, < 0.0001, < 0.0001 and < 0.0001 respectively. The high F-values and low p-values illustrate the models significance. Significant input parameters are illustrated by having p-values < 0.05 (Qing et al., 2016). Among all investigated variables, the effect of enzyme loading (0.0158) was shown to be significant for the $PSSF_{concentration}$ model whereas yeast titre (0.0155) and enzyme loading (0.0002) were found to be significant for the $OSSF_{concentration}$ model. The enzyme loading largely impacts on the glucose recovery. This has shown to be more significant in SSF processes with a prehydrolysis stage since optimal cellulase enzymatic activity is achieved at 50 °C. The high temperature (50 °C) enhances saccharification and bioethanol production due to the availability of high glucose for yeast consumption. Conversely, the yeast titre affects the glucose consumption rates in SSF processes. Variations in this parameter may increase the glucose consumption rate while

Table 2
RSM polynomial model equations relating the input parameters to the ethanol concentration and conversion for the PSSF and OSSF processes.

Model	Equation	Equation number
$PSSF_{concentration}$	$-8.52 + 2.99A + 3.81B + 0.590C + 0.0391AB + 0.00188AC + 0.00428BC - 0.595A^2 - 0.105B^2 - 0.0103C^2$	6
$PSSF_{conversion}$	$101.05 + 5.11A - 3.43B + 1.11C + 0.0105AB + 0.00275AC - 0.0021BC - 0.841A^2 + 0.00576B^2 - 0.0168C^2$	7
$OSSF_{concentration}$	$2.20 + 0.809A + 2.45B + 0.6576C - 0.0143AB - 0.0998AC + 0.00928BC + 0.165A^2 - 0.0653B^2 - 0.00834C^2$	8
$OSSF_{conversion}$	$115.06 + 0.165A - 4.89B + 1.33C + 0.0070AB - 0.147AC + 0.00140BC + 0.319A^2 + 0.0450B^2 - 0.0153C^2$	9

Footnote: A = yeast titre, B = solid loading, C = enzyme loading.

minimizing the deleterious effects of fermentation inhibitor compounds in addition to mass and heat transfer limitations (Zhao et al., 2015).

The solid loading (< 0.0001) and enzyme loading (0.0125) were found to be significant for the $PSSF_{conversion}$ model whereas solid loading (< 0.0001), enzyme loading (0.0005) and yeast titre (0.0228) were significant for the $OSSF_{conversion}$ model. The solid loading parameter influences the mass and heat transfer mechanisms in microbial fermentation processes. Generally, a high viscosity of the lignocellulosic material observed at high solid loading has shown to reduce enzyme diffusion which negatively affects sugar release for yeast cell growth and bioethanol formation (Aguilar-Reynosa et al., 2017).

The $PSSF_{concentration}$, $PSSF_{conversion}$, $OSSF_{concentration}$ and $OSSF_{conversion}$ models showed coefficient of determination (R^2) values of 0.936, 0.990, 0.979 and 0.998 respectively. Thus, these models can account for 93.6% ($PSSF_{concentration}$), 99.0% ($PSSF_{conversion}$), 97.9% ($OSSF_{concentration}$) and 99.8% ($OSSF_{conversion}$) of variations in the observed data respectively. These statistical data showed that the developed models had a good fit in terms of relating the input parameters to the responses.

3.2. Interactive effect of the input process parameters on bioethanol concentration and bioethanol conversion

The bioethanol concentration and bioethanol conversion obtained for each experimental run is shown in Table 1. Bioethanol concentration ranged from 23.93 to 43.16 g/L (PSSF experiments) and 25.36 to 41.17 g/L (OSSF experiments). Bioethanol conversion ranged from 23.58 to 93.48% and 24.99 to 89.69% for the PSSF and OSSF experiments respectively. The PSSF process gave up to 18% higher bioethanol concentration and conversion compared to the OSSF process when all the input variables were maintained at their median values (run 3). Low yeast titre and enzyme loading (run 7) resulted in slightly higher bioethanol concentration and conversion values (5%) for the PSSF process compared to the OSSF process under similar conditions.

The interactive effect of the process inputs on bioethanol concentration and conversion is shown in Fig. 1(A–H) for the PSSF and OSSF models. A simultaneous increase in enzyme loading and yeast titre from 10 to 30 FPU/g and 1 to 3 times the base level respectively led to an increase in the bioethanol concentration from 29 to 43 g/L (Fig. 1A). A further increase in yeast titre from 3 to 5 times the base level resulted in a decrease in the bioethanol concentration from 43 to 35 g/L ($PSSF_{concentration}$ model). Conversely, the $OSSF_{concentration}$ model indicated that when the yeast titre was maintained at its minimum value (1 time the base level) and the enzyme loading was varied from 10 to 30 FPU/g, the bioethanol concentration increased from 31 to 41 g/L (Fig. 1B). Sharp increments in the bioethanol concentration observed at higher enzyme loadings may be attributed to the rapid conversion of cellulosic content to monomeric sugars such as glucose that may be available for utilization by the yeast culture. Dahnum et al. (2015) and Aguilar-Reynosa et al. (2017) reported a 33% and 17% higher bioethanol yield when the enzyme loading was increased from 10 to 40 FPU/g and 5 to 15 FPU/g respectively. Conversely, Zhao et al. (2015) observed that increasing the yeast titre from 1 to 4 times the base level (8.0×10^7 cells/mL to 3.2×10^8 cells/mL) led to a 14% improvement in the bioethanol yield.

The interactive effects of enzyme loading and solid loading on the bioethanol concentration when the yeast titre is kept at its median value are shown in Fig. 1C and D for the $PSSF_{concentration}$ and $OSSF_{concentration}$ models respectively. The $PSSF_{concentration}$ model showed that an increase in enzyme loading and solid loading from 10 to 30 FPU/g and 10 to 20% respectively resulted in an increase in the bioethanol concentration from 25 to 43 g/L (Fig. 1C). Further increases in solid loading (20 to 30%) showed a drastic reduction in bioethanol concentration (43 to 25 g/L) ($PSSF_{concentration}$ model). The $OSSF_{concentration}$ model showed that simultaneous increments in the enzyme loading (10 to 30 FPU/g) and solid loading (10 to 20%) led to an increase in the bioethanol concentration from 25 to 37 g/L (Fig. 1D). Further increases in the solid loading (20 to 30%) negatively impacted the bioethanol concentration and resulted in a decrease from 37 to 26 g/L. High bioethanol concentrations observed at low solid loadings may be ascribed to the effective mass and heat transfer that enhanced saccharification efficiency. The enhancement of the enzymatic hydrolysis step led to the production of high glucose concentrations. A high glucose concentration has been shown to promote glycolytic rates towards bioethanol formation (Kappeli, 1986). The estimated threshold of mass transfer limitations for the PSSF and OSSF processes ranged between 10 and 20% solid loading. Despite these limitations, high performing Cellic CTec2 enzymes show remarkable saccharification efficiencies at high solid loads (> 10%) (Aguilar-Reynosa et al., 2017). Advantages of Cellic CTec2 include its enhanced endo/exo activity ratio, lower end-product inhibition, higher tolerance to non-specific binding and the presence of auxiliary proteins such as oxireductases (Ramos et al., 2015). These oxireductases facilitate the accessibility of lignocellulosic biomass to cellulolytic enzymes (McQueen-Mason et al. (1992)). Ramos et al. (2015) reported a high glucan conversion (69.2%) at elevated solid loadings (20%) and low enzyme loadings (4.54 FPU/g) using Cellic CTec2. A similar result was recorded by Zhu et al. (2015).

The combined effects of solid loading and yeast titre on the bioethanol conversion when the enzyme loading is maintained at its median value is shown in Fig. 1E and F. Incremental variation in the solid loading from 10 to 30% and yeast titre from 1 to 5 times the base level resulted in low bioethanol conversion (< 30%) for the $PSSF_{conversion}$ model (Fig. 1E).

High bioethanol conversions in the range of 50–92% were observed when the solid loading was maintained between 10 and 15% and the yeast titre was increased from 1 to 5 times the base value. Likewise, the $OSSF_{conversion}$ model displayed high bioethanol conversions in the range of 60 to 89% when the solid loading was maintained at 10% and the yeast titre was increased from 1 to 5 times the base value (Fig. 1F). Simultaneous increases in the solid loading from 10 to 30% and yeast titre from 1 to 5 times its base value led to a decrease in the bioethanol conversion (89–59%). Low bioethanol conversion observed at high solid loadings may be due to (a) high levels of fermentation inhibitors and (b) high viscosity of the lignocellulosic material and traditional poor mixing observed in flask reactor systems. Fermentation inhibitor compounds have shown to reduce the yeast performance whereas high viscosity could reduce diffusion of enzymes and products of hydrolysis and fermentation (Zhao et al., 2015). Liu and Chen (2016) demonstrated that increasing the solid loading from 5 to 25% reduced the bioethanol conversion from 84.6 to 62.9%. Similarly, Aguilar-Reynosa et al. (2017) illustrated that increasing the solid loading (10–12.5%) decreased the bioethanol conversion from 92 to 83%.

Influences of enzyme loading and solid loading on the bioethanol conversion when the yeast titre is kept at its median value are shown in Fig. 1G and H for the $PSSF_{conversion}$ and $OSSF_{conversion}$ models respectively. High bioethanol conversions of 60 to 97% were noted when the enzyme loading was increased from 10 to 30 FPU/g and the solid loading was maintained at 10% (Fig. 1G). Further increases in solid loading above this threshold, from 11 to 30%, decreased the bioethanol conversion from 97 to 25%. Bioethanol conversion values ranged between 65 and 88% when the enzyme loading was increased from 10 FPU/g to 30 FPU/g and the solid loading was kept at its minimum value of 10% (Fig. 1H). Further incremental changes in the solid loading beyond this threshold (11–30%) resulted in a rapid decline in the bioethanol conversion from 88 to 23%. Experimental results from the present study indicated that although bioethanol concentration may be increased by raising the solid loading, the bioethanol conversion is reduced at higher solid loadings. This was in accordance with Liu and Chen (2016), Aguilar-Reynosa et al. (2017) and Zhu et al. (2015).

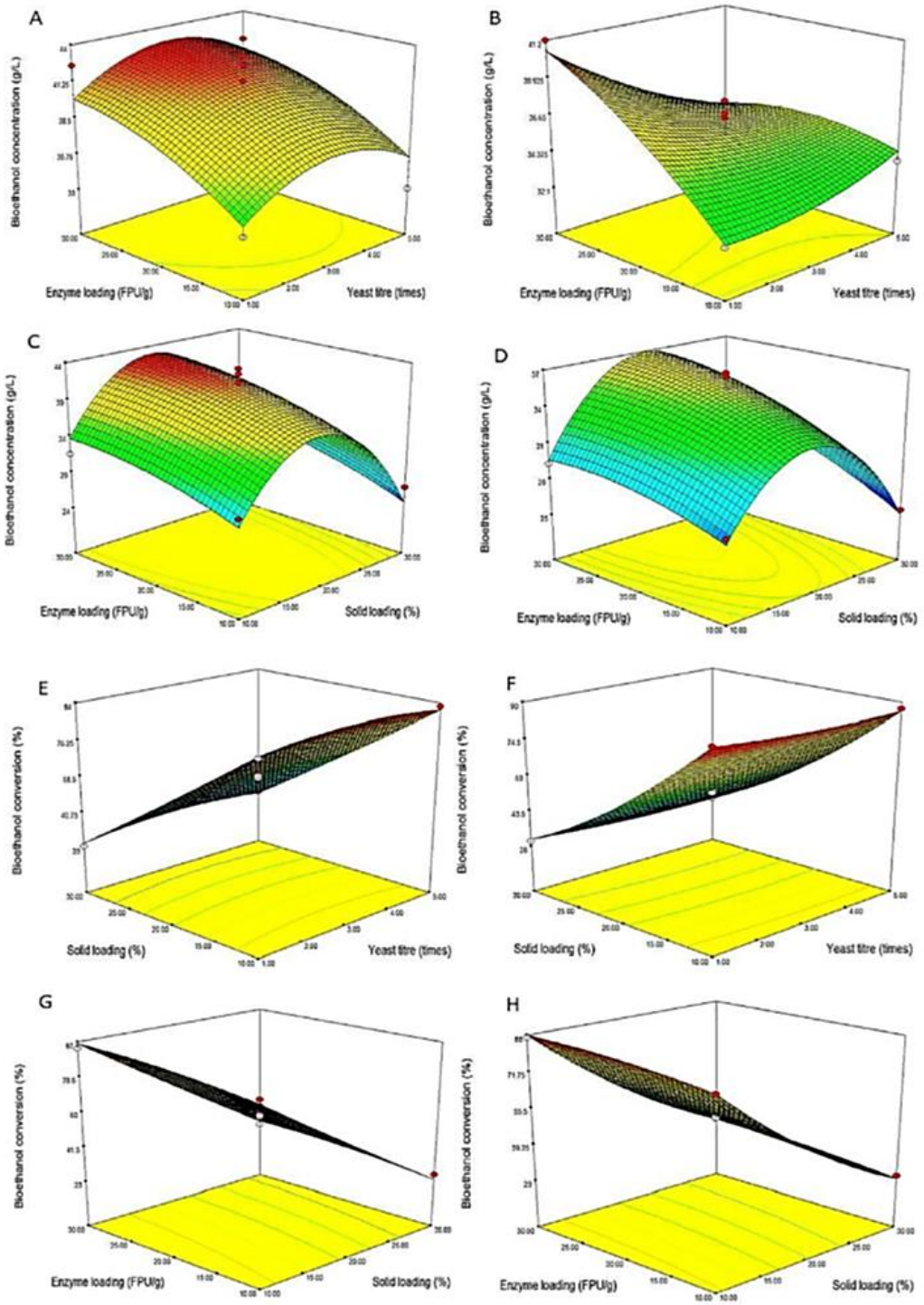

Fig. 1. Response surface plots showing the interactive effect of : (A) enzyme loading and yeast titre ($PSSF_{concentration}$); (B) enzyme loading and yeast titre ($OSSF_{concentration}$); (C) enzyme loading and solid loading ($PSSF_{concentration}$); (D) enzyme loading and solid loading ($OSSF_{concentration}$); (E) solid loading and yeast titre ($PSSF_{conversion}$); (F) solid loading and yeast titre ($OSSF_{conversion}$); (G) enzyme loading and solid loading ($PSSF_{conversion}$) and (H) enzyme loading and solid loading ($OSSF_{conversion}$).

Table 3
Validation of the optimized conditions for the PSSF and OSSF processes.

Run	Yeast titre (times)	Solid loading (%)	Enzyme loading (FPU/g)	Bioethanol concentration (g/L)		Bioethanol conversion (%)	
				Predicted	Observed	Predicted	Observed
PSSF	2	17.50	30	41.78	36.92 ± 1.34	70.52	62.36 ± 2.27
OSSF	1	17.82	30	39.99	35.04 ± 0.170	66.46	58.13 ± 0.283

Footnote: Yeast titre of 1 time the base level = 2.70×10^6 cells/mL, Yeast titre of 2 times the base level = 5.40×10^6 cells/mL.

3.3. Experimental validation of the developed PSSF and OSSF models

The models predicted bioethanol concentration and conversion values of 41.78 g/L and 70.52% for the PSSF models and 39.99 g/L and 66.46% for the OSSF models respectively (Table 3). Experimental validation carried out in duplicate yielded 36.92 ± 1.34 g/L and 62.36 ± 2.27% for the $PSSF_{concentration}$ and $PSSF_{conversion}$ models respectively. Similarly, bioethanol concentration and conversion of 35.04 ± 0.170 g/L and 58.13 ± 0.283% were achieved for the $OSSF_{concentration}$ and $OSSF_{conversion}$ models respectively. The PSSF process showed a 3% and 5% improvement in the bioethanol concentration and conversion compared to the OSSF process. The slightly higher bioethanol concentration and conversion obtained for the PSSF process (36.92 ± 1.34 g/L and 62.36 ± 2.27%) in this study could be negligible considering that prehydrolysis stages are cost and energy intensive.

3.4. Comparison of the bioethanol concentration and conversion with previous SSF reports on corn wastes

Higher bioethanol concentration and conversion were achieved with the optimized PSSF and OSSF processes compared to recent studies using corn cobs (Table 4). For instance, the optimized bioethanol concentration (PSSF experiments) gave a 1.9-fold increase in the bioethanol concentration compared to an SSF study on corn cobs that consisted of a 4 h prehydrolysis (He et al., 2016). The higher bioethanol concentration in the present study may be attributed to the optimized conditions employed. Similarly, OSSF experiments gave a 1.8-fold higher bioethanol concentration compared to the study by He et al. (2016). Liu et al. (2014) evaluated an SSF process with a 24 prehydrolysis stage and observed a 31% and 27% lower bioethanol concentration compared to the $PSSF_{concentration}$ and $OSSF_{concentration}$ models respectively. Likewise, the present study gave a 1.7-fold ($PSSF_{concentration}$) and 1.6-fold ($OSSF_{concentration}$) higher bioethanol concentration compared to a previous study by Koppram et al. (2013). Slight variations in the bioethanol concentration and bioethanol conversion observed for the PSSF and OSSF processes indicates that prehydrolysis stages did not significantly impact the process. SSF processes without prehydrolysis have shown to be preferable from an industrial perspective due to their lower operating costs (Koppram et al., 2013).

3.5. Kinetics of S. cerevisiae cell growth on the PSSF and OSSF processes under microaerophilic and anaerobic conditions

Changes in *S. cerevisiae* cell growth under the optimized PSSF (Fig. 2A) and OSSF (Fig. 2B) processes in microaerophilic and anaerobic environments showed a similar lag phase that lasted for 7 h and an exponential phase that occurred between 7 and 14 h for all four processes.

Despite the comparable growth patterns observed in the lag and exponential phases for the PSSF and OSSF processes under microaerophilic and anaerobic conditions, the anaerobic processes gave a slightly lower biomass concentration (3.12–3.13 g/L). Higher biomass accumulation in the microaerophilic processes (3.15–3.16 g/L) can be ascribed to oxygen availability which is crucial for *S. cerevisiae* cell growth and replication (Lin et al., 2012). Biomass production reached a plateau after 12 and 14 h for the PSSF and OSSF experiments respectively which corresponded with the exhaustion of fermentable sugars.

Experimental data from the biomass concentration over time (Fig. 2A and B) were used to fit the logistic models with high correlation coefficients (R^2) for the $PSSF_{microaerophilic}$ (0.893), $PSSF_{anaerobic}$ (0.892), $OSSF_{microaerophilic}$ (0.927) and $OSSF_{anaerobic}$ (0.932) processes. *S. cerevisiae* growth in the $OSSF_{microaerophilic}$ process gave the highest maximum specific growth rate (μ_{max}) of 0.274 h^{-1} whereas the lowest μ_{max} value (0.186 h^{-1}) was obtained for the $PSSF_{anaerobic}$ process (Table 5). Lower μ_{max} values were observed under the $PSSF_{microaerophilic}$ (0.216 h^{-1}) and $PSSF_{anaerobic}$ (0.186 h^{-1}) conditions compared to the $OSSF_{microaerophilic}$ (0.274 h^{-1}) and $OSSF_{anaerobic}$ (0.267 h^{-1}) processes. Oxygen is required for several biosynthetic pathways such as haem, sterols, unsaturated fatty acids, pyrimidines and deoxyribonucleotides (Anderasen and Stier, 1953; Chabes et al., 2000; Nagy et al., 1992). Moreover, *S. cerevisiae* growth under anaerobic conditions triggers transcriptional and metabolic changes within the cell (Snoek and Steensma, 2007) which impact on cell growth and metabolic processes. Anaerobic conditions diverted the cells metabolic activities toward ethanol formation and was evidenced by the low μ_{max} values obtained for the $PSSF_{anaerobic}$ (0.186 h^{-1}) and $OSSF_{anaerobic}$ (0.267 h^{-1}) processes. Furthermore, the inclusion of prehydrolysis stages in SSF processes resulted in the production of glucose that is immediately available for utilization by the yeast culture. High glucose levels increased glycolytic rates and stimulated ethanol production instead of cell

Table 4
Bioethanol concentration and conversion from SSF processes using corn wastes under microaerophilic conditions.

Microorganism	Substrate	SSF process conditions	PSSF/OSSF process	Bioethanol concentration (g/L)	Bioethanol conversion (%)	References
S. cerevisiae BY4743	Corn cobs	5.40×10^6 cells/mL[a], 17.50%[b], 30 FPU/g [c]	PSSF	36.92	62.36	This study
S. cerevisiae BY4743	Corn cobs	2.70×10^6 cells/mL[a], 17.82%[b], 30 FPU/g[c]	OSSF	35.04	58.13	This study
S. cerevisiae KE6-12	Corn cobs	5 g/L[a], 7.5%[b], 5 FPU/g[c]	OSSF	22	ND	Koppram et al. (2013)
S. cerevisiae	Corn stover	7.5×10^9 CFU/mL[a], 20%[b], 30 FPU/g[c]	PSSF	25.5	ND	Liu et al. (2014)
S. cerevisiae	Corn cobs	5 g/L[a], 10%[b], 15 FPU/g[c]	PSSF	19.3	76.5	He et al. (2016)

Footnote: [a] = yeast cell concentration, [b] = solid loading, [c] = enzyme loading, PSSF = simultaneous saccharification and fermentation with prehydrolysis, OSSF = simultaneous saccharification and fermentation without prehydrolysis, ND = Not determined.

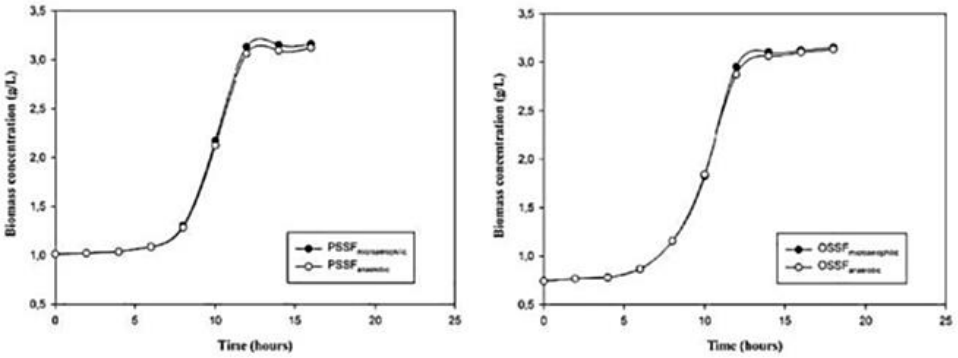

Fig. 2. *S. cerevisiae* cell growth during bioethanol production for the PSSF (A) and OSSF (B) processes.

Table 5
Kinetic parameters from the logistic models under microaerophilic and anaerobic conditions for the PSSF and OSSF processes.

Kinetic parameter	PSSF and OSSF kinetic process			
	$PSSF_{microaerophilic}$	$PSSF_{anaerobic}$	$OSSF_{microaerophilic}$	$OSSF_{anaerobic}$
μ_{max} (h^{-1})	0.216	0.186	0.274	0.267
X_o (g/L)	0.556	0.609	0.287	0.300
X_{max} (g/L)	3.65	4.05	3.52	3.51

Footnote: μ_{max} = maximum specific growth rate, X_o = initial cell concentration, X_{max} = maximum cell concentration.

development which elucidates the lower μ_{max} values observed for the $PSSF_{microaerophilic}$ (0.216 h^{-1}) and $PSSF_{anaerobic}$ (0.186 h^{-1}).

Maximum cell concentration (X_{max}) values achieved for the $PSSF_{microaerophilic}$ (3.65 g/L) and $PSSF_{anaerobic}$ (4.05 g/L) processes were higher than the $OSSF_{microaerophilic}$ (3.52 g/L) and $OSSF_{anaerobic}$ (3.51 g/L) processes. The higher X_{max} values obtained for the PSSF processes compared to the OSSF experiments may be accounted for by the higher yeast titre employed at the beginning of the process (two times the base level compared to one time for the OSSF process). Studies have indicated that increasing the yeast biomass concentration at the beginning of the SSF process could potentially enhance cell growth and ethanol formation (Wang et al., 2013; Zhao et al., 2015). Previous kinetic studies on bioethanol production gave comparable μ_{max} and X_{max} values under microaerophilic conditions. For instance, Phukoetphim et al. (2017) studied the kinetics of *S. cerevisiae* on sweet sorghum juice under high sugar concentration (160 g/L) and reported a μ_{max} of 0.154 h^{-1} and X_{max} of 5.15 g/L. Likewise, Dodic et al. (2012) recorded a μ_{max} value of 0.194 h^{-1} and X_{max} value of 8.38 g/L using *S. cerevisiae* from sugar beet juice containing 136 g/L of total sugar. In the same way, Echegaray et al. (2000) studied the kinetics of *S. cerevisiae* under anaerobic process conditions on diluted sugarcane molasses with a high glucose concentration (170 g/L) and recorded μ_{max} values between 0.019 and 0.24 h^{-1}. Although the aforementioned studies observed lower μ_{max} values compared to the present study (0.274 h^{-1}), they achieved significantly higher X_{max} values. These variations may be due to other factors such as substrate type and concentration, yeast strain and process operating conditions.

3.6. Kinetics of bioethanol production on the PSSF and OSSF processes under microaerophilic and anaerobic conditions

Bioethanol evolution under the optimized PSSF (Fig. 3A) and OSSF (Fig. 3B) process conditions revealed a short lag phase (4 h) for all four SSF processes. A sharp increase in the ethanol concentration up to 36.92 g/L ($PSSF_{microaerophilic}$) and 31.45 g/L ($PSSF_{anaerobic}$) was observed from 4 to 18 h. The $OSSF_{microaerophilic}$ bioprocess showed sharp increases in the bioethanol concentration up to 35.04 g/L from 4 to 20 h compared to 25.13 g/L from 4 to 16 h for the $OSSF_{anaerobic}$ experiment. Maximum bioethanol concentration occurred during the exponential growth phase in all four bioprocesses and was associated with a rapid conversion of glucose. No further increment in the bioethanol concentration was observed after the exponential phase (> 18 h) and this was attributed to sugar depletion (Mazzoleni et al., 2015). The production of fermentable sugars in SSF processes is drastically influenced by the enzyme loading (Wang et al., 2013). The high glucose concentration available within a short process time (< 18 h) was due to increased hydrolysis rates at high enzyme loading (30 FPU/g).

Owing to the complex nature of SSF processes in which the simultaneous release and degradation of glucose occurs, the glucose utilisation was estimated from the control (initial glucose concentration) and test (final glucose concentration) experiments. Maximum glucose utilisation of 98, 97, 92 and 94% were observed for the $PSSF_{microaerophilic}$, $PSSF_{anaerobic}$, $OSSF_{microaerophilic}$ and $OSSF_{anaerobic}$ processes respectively and occurred during the exponential phase for all four processes.

Experimental data from bioethanol production over time (Fig. 3A and B) were used to fit the modified Gompertz model with high R^2 values for the $PSSF_{microaerophilic}$ (0.958), $PSSF_{anaerobic}$ (0.985), $OSSF_{microaerophilic}$ (0.965) and $OSSF_{anaerobic}$ (0.985) processes. The obtained model parameters were compared to other bioethanol kinetic studies in Table 6. Lag times of 1.98, 2.68, 2.66 and 3.12 h were obtained for the $PSSF_{microaerophilic}$, $PSSF_{anaerobic}$, $OSSF_{microaerophilic}$, $OSSF_{anaerobic}$ experiments respectively. Unfavourable growth conditions such as low nutrient content and the lack of oxygen accounts for the high t_L value observed for the $OSSF_{anaerobic}$ experiment (3.12 h) compared to the $PSSF_{microaerophilic}$ process (1.98 h). The prehydrolysis stage may account for the shorter lag time observed for the $PSSF_{microaerophilic}$ process. However, the $OSSF_{microaerophilic}$ and $PSSF_{anaerobic}$ bioprocesses displayed relatively similar t_L values of 2.66 and 2.68 h respectively. This implied that bioethanol production was influenced by both the absence of oxygen and glucose. Previous kinetic studies on oil palm frond juice (Srimachai et al., 2015), sugar beet raw juice (Dodic et al., 2012) and sweet sorghum juice (Phukoetphim et al., 2017) observed lag times of 0.77 h, 1.04 h and 2.98 h respectively. The observed lag duration (1.98 h) in the present kinetic study is within the range reported in previous studies.

Bioprocesses under microaerophilic and anaerobic conditions gave high maximum ethanol production rates ($r_{p,m}$) of 2.39, 3.25, 2.14 and 2.33 g/L/h for the $PSSF_{microaerophilic}$, $PSSF_{anaerobic}$, $OSSF_{microaerophilic}$, $OSSF_{anaerobic}$ experiments respectively (Table 6). Processes carried out under anaerobic conditions exhibited higher $r_{p,m}$ values compared to the microaerophilic processes. During anaerobic bioethanol fermentation, high concentration of fermentable sugars are converted to ethanol while a lower quantity is utilized for yeast growth and metabolic maintenance (Ozmihci and Kargi, 2007; Rosenfeld et al., 2003). The

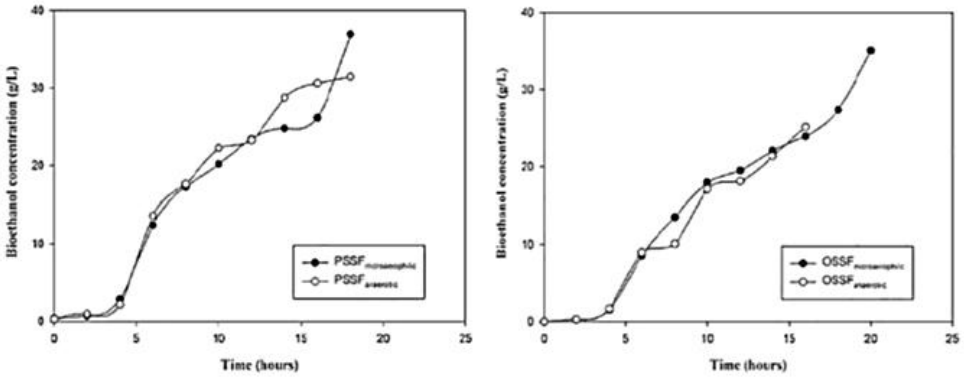

Fig. 3. Bioethanol formation using *S. cerevisiae* for the PSSF (A) and OSSF (B) processes.

absence of oxygen caused metabolic shifts within the cell towards ethanol formation which explains the higher $r_{p,m}$ values achieved under anaerobic process conditions.

Additionally, high glucose levels promote increased glycolytic rates that exceed the pyruvate dehydrogenase reaction which generates an overflow towards pyruvate decarboxylase, thus producing ethanol. Under anaerobic conditions, pyruvate decarboxylase and alcohol dehydrogenase convert pyruvate into ethanol and carbon dioxide to re-oxidize two molecules of NADH that was produced from the glycolytic pathway. This could account for the higher $r_{p,m}$ value achieved for the $PSSF_{microaerophilic}$ (2.39 g/L/h) process compared to the $OSSF_{anaerobic}$ (2.33 g/L/h). Phukoetphim et al. (2017) recorded a maximum ethanol production rate of 2.17 g/L/h from sweet sorghum juice which was slightly lower than the present $PSSF_{microaerophilic}$, $PSSF_{anaerobic}$, $OSSF_{anaerobic}$ processes. Similarly, Srimachai et al. (2015) observed a lower maximum ethanol production rate compared to the present PSSF and OSSF optimized experiments. On the contrary, Dodic et al. (2012) obtained a higher $r_{p,m}$ value of 4.39 g/L/h compared to 3.25 g/L/h in the current study.

SSF processes showed high maximum potential bioethanol concentration (P_m) values of 42.24, 32.09, 37.87 and 27.62 g/L for the $PSSF_{microaerophilic}$, $PSSF_{anaerobic}$, $OSSF_{microaerophilic}$, $OSSF_{anaerobic}$ processes respectively. Microaerophilic processes (PSSF and OSSF bioprocesses) gave higher maximum potential bioethanol concentrations compared to their equivalent anaerobic experiments. The observed higher P_m values in the $PSSF_{microaerophilic}$ and $OSSF_{microaerophilic}$ processes could be attributed to the presence of oxygen that supports lipid biosynthesis and enhances ethanol catalysing enzymes within *S. cerevisiae* (Rosenfeld et al., 2003; Snoek and Steensma 2007).

Thus, a microaerophilic environment was more favourable for bioethanol production using *S. cerevisiae* BY4743 compared to anaerobic conditions. Previous kinetic studies on oil palm frond juice (Srimachai et al., 2015), sugar beet raw juice (Dodic et al., 2012) and sweet sorghum juice (Phukoetphim et al., 2017) in microaerophilic environments displayed P_m values of 3.790, 73.31 and 88.48 g/L respectively. The high ethanol formation observed in the present study coincided with optimal cell growth thus higher P_m values were obtained under microaerophilic conditions compared to the anaerobic processes. Kinetic data suggested that bioethanol production showed a mixed growth associated pattern as evidenced by the high μ_{max} and P_m values obtained under microaerophilic conditions whereas high $r_{p,m}$ values were achieved for the anaerobic bioprocesses. This study highlighted the increased potentiality of corn cob wastes for lignocellulosic bioethanol production from SSF processes.

4. Conclusion

This study optimized two RSM models ($R^2 > 0.90$) on SSF processes with prehydrolysis (PSSF) and without prehydrolysis (OSSF). Experimental validation gave high bioethanol concentrations and conversions for the PSSF (36.92 ± 1.34 g/L and 62.36 ± 2.27%) and OSSF (35.04 ± 0.170 g/L and 58.13 ± 0.283%) models respectively. The highest μ_{max} was achieved for the $OSSF_{microaerophilic}$ (0.274 h^{-1}). Conversely, both the $PSSF_{microaerophilic}$ (42.24 g/L) and $OSSF_{microaerophilic}$ (37.87 g/L) processes gave high P_m values. Microaerophilic process conditions favoured *S. cerevisiae* cell growth and ethanol formation. The present study gave major implications for SSF bioethanol process design at large scale by eliminating energy intensive prehydrolysis steps and costly anaerobic conditions.

Acknowledgements

The authors thankfully acknowledge the National Research Foundation, South Africa (NRF) (Grant number: 101316) of South Africa for financially supporting this research. Opinions expressed and conclusions arrived at, are those of the author and are not necessarily attributed to the NRF.

Table 6
Kinetic values from the modified Gompertz models under microaerophilic and anaerobic conditions for the PSSF and OSSF processes compared to previous reports.

Substrate	Oxygen environment	P_m (g/L)	$r_{p,m}$ (g/l/h)	t_L (h)	Reference
Corn cobs	Microaerophilic	42.24	2.39	1.98	This study ($PSSF_{microaerophilic}$)
Corn cobs	Anaerobic	32.09	3.25	2.68	This study ($PSSF_{anaerobic}$)
Corn cobs	Microaerophilic	37.87	2.14	2.66	This study ($OSSF_{microaerophilic}$)
Corn cobs	Anaerobic	27.62	2.33	3.12	This study ($OSSF_{anaerobic}$)
Oil palm frond juice	Microaerophilic	3.790	0.08	0.77	Srimachai et al. (2015)
Sugar beet raw juice	Microaerophilic	73.31	4.39	1.04	Dodic et al. (2012)
Sweet sorghum Juice	Microaerophilic	88.48	2.17	2.98	Phukoetphim et al. (2017)

Footnote: P_m = maximum potential bioethanol concentration, $r_{p,m}$ = maximum bioethanol production rate, t_L = lag time.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.biortech.2018.04.056.

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Chapter 6: Supplementary material

Table S1. Analysis of Variance of the developed $PSSF_{concentration}$ model.

Factor	Sum of Squares	Degrees of freedom (*df*)	Mean square	*F* value	*p* value (probability>*F*)
Intercept or model	598.65	9	66.52	11.51	0.0020 significant
A- Yeast titre	1.86	1	1.86	0.32	0.5879
B- Solid loading	25.35	1	25.35	4.39	0.0745
C- Enzyme loading	57.89	1	57.89	10.02	0.0158
AB	2.45	1	2.45	0.42	0.5358
AC	0.00563	1	0.00563	0.000974	0.9760
BC	0.73	1	0.73	0.13	0.7325
A^2	23.87	1	23.87	4.13	0.0816
B^2	461.19	1	461.19	79.82	<0.0001
C^2	4.47	1	4.47	0.77	0.4081
Residual Error	40.44	7	5.78	-	-
Lack of fit	29.17	3	9.72	3.45	0.1315 not significant
Pure Error	11.28	4	2.82	-	

Table S2. Analysis of Variance of the developed $PSSF_{conversion}$ model.

Factor	Sum of Squares	Degrees of freedom (*df*)	Mean square	*F* value	*p* value (probability>*F*)
Intercept or model	8443.29	9	938.14	80.56	<0.0001 significant
A- Yeast titre	3.46	1	3.46	0.30	0.6027
B- Solid loading	8247.13	1	8247.13	708.16	<0.0001
C- Enzyme loading	129.77	1	129.77	11.14	0.0125
AB	0.18	1	0.18	0.015	0.9055
AC	0.012	1	0.012	0.00104	0.9752
BC	0.18	1	0.18	0.015	0.9055
A^2	47.65	1	47.65	4.09	0.0828
B^2	1.40	1	1.40	0.12	0.7393
C^2	11.87	1	11.87	1.02	0.3463
Residual Error	81.52	7	11.65	-	-
Lack of fit	56.96	3	18.99	3.09	0.1520 not significant
Pure Error	24.57	4	6.14	-	-

Table S3. Analysis of Variance of the developed $OSSF_{concentration}$ model.

Factor	Sum of Squares	Degrees of freedom (*df*)	Mean square	*F* value	*p* value (probability>*F*)
Intercept or model	248.48	9	27.61	37.79	<0.0001 significant
A- Yeast titre	7.39	1	7.39	10.12	0.0155
B- Solid loading	0.36	1	0.36	0.49	0.5046
C- Enzyme loading	35.32	1	35.32	48.35	0.0002
AB	0.33	1	0.33	0.45	0.5227
AC	15.92	1	15.92	21.79	0.0023
BC	3.44	1	3.44	4.71	0.0666
A^2	1.84	1	1.84	2.52	0.1563
B^2	179.60	1	179.60	245.85	<0.0001
C^2	2.93	1	2.93	4.00	0.0855
Residual Error	5.11	7	0.73	-	-
Lack of fit	1.77	3	0.59	0.71	0.5953 not significant
Pure Error	3.34	4	0.83	-	-

Table S4. Analysis of Variance of the developed $OSSF_{conversion}$ model.

Factor	Sum of Squares	Degrees of freedom (*df*)	Mean square	*F* value	*p* value (probability>*F*)
Intercept or model	6978.27	9	775.36	385.17	<0.0001 significant
A- Yeast titre	16.99	1	16.99	8.44	0.0228
B- Solid loading	6736.12	1	6736.12	3346.26	<0.0001
C- Enzyme loading	75.40	1	75.40	37.46	0.0005
AB	0.078	1	0.078	0.039	0.8492
AC	34.69	1	34.69	17.23	0.0043
BC	0.078	1	0.078	0.039	0.8492
A^2	6.84	1	6.84	3.40	0.1078
B^2	99.02	1	99.02	49.19	0.0002
C^2	9.86	1	9.86	4.90	0.0625
Residual Error	14.09	7	2.01	-	-
Lack of fit	6.77	3	2.26	1.23	0.4078 not significant
Pure Error	7.32	4	1.83	-	-

Table S5. Observed bioethanol concentration and conversion compared to the RSM predicted values for the PSSF and OSSF processes.

Run	PSSF observed bioethanol concentration (g/L)	PSSF RSM predicted bioethanol concentration (g/L)	PSSF observed bioethanol conversion (%)	PSSF RSM predicted bioethanol conversion (%)	OSSF observed bioethanol concentration (g/L)	OSSF RSM predicted bioethanol concentration (g/L)	OSSF bioethanol conversion (%)	OSSF RSM predicted bioethanol conversion (%)
1	26.92±0.85	25.45	26.53±0.84	23.58	25.36±0.36	25.88	24.99±0.35	24.99
2	41.31±0.78	41.19	61.07±1.16	58.80	35.33±0.14	35.64	52.22±0.21	53.69
3	42.45±0.07	41.19	62.75±0.11	58.80	34.90±0.28	35.64	51.59±0.42	52.69
4	23.93±0.43	25.30	23.58±0.42	26.03	30.63±0.64	30.81	30.18±0.63	31.11
5	41.74±0.28	40.99	61.70±0.42	63.48	34.76±0.71	34.62	51.38±1.05	51.10
6	28.21±0.28	27.83	27.79±0.28	27.35	28.63±0.43	28.31	28.21±0.42	28.48
7	33.90±0.71	34.65	50.12±1.05	54.11	32.19±0.85	32.34	47.59±1.26	47.87
8	39.17±1.21	41.19	57.91±1.79	58.80	36.47±1.21	35.64	53.91±1.79	52.69
9	31.20±0.36	29.83	92.22±1.05	91.56	29.49±0.50	29.31	87.16±1.47	86.23
10	42.45±0.85	39.95	62.75±1.26	62.17	41.17±0.57	40.53	60.85±1.26	59.90
11	29.91±0.43	31.04	29.48±0.42	30.72	30.63±0.57	31.09	30.18±0.56	30.20
12	30.34±0.28	29.22	89.69±0.84	86.88	27.78±0.43	27.32	82.11±1.26	82.09
13	31.62±0.14	34.60	93.48±0.42	93.48	29.34±0.21	29.66	86.74±0.63	87.95
14	39.88±0.57	41.19	58.96±0.84	58.80	34.76±0.14	35.64	51.38±0.21	52.69
15	33.04±0.14	35.54	48.85±0.21	55.43	33.76±0.71	34.40	49.90±1.05	50.85
16	30.05±0.28	30.43	88.85±0.84	90.25	30.34±0.36	30.66	89.69±1.05	89.43
17	43.16±0.64	41.19	63.80±0.95	58.80	36.75±0.78	35.64	54.33±1.16	52.69

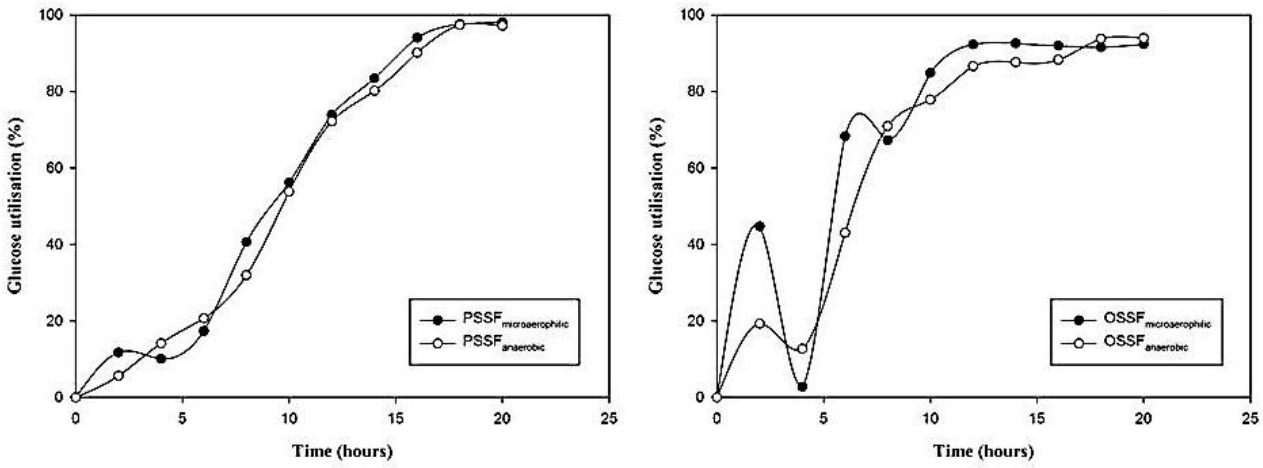

Fig. S1. Glucose utilisation during bioethanol production using *S. cerevisiae* for PSSF (A) and OSSF (B) processes.

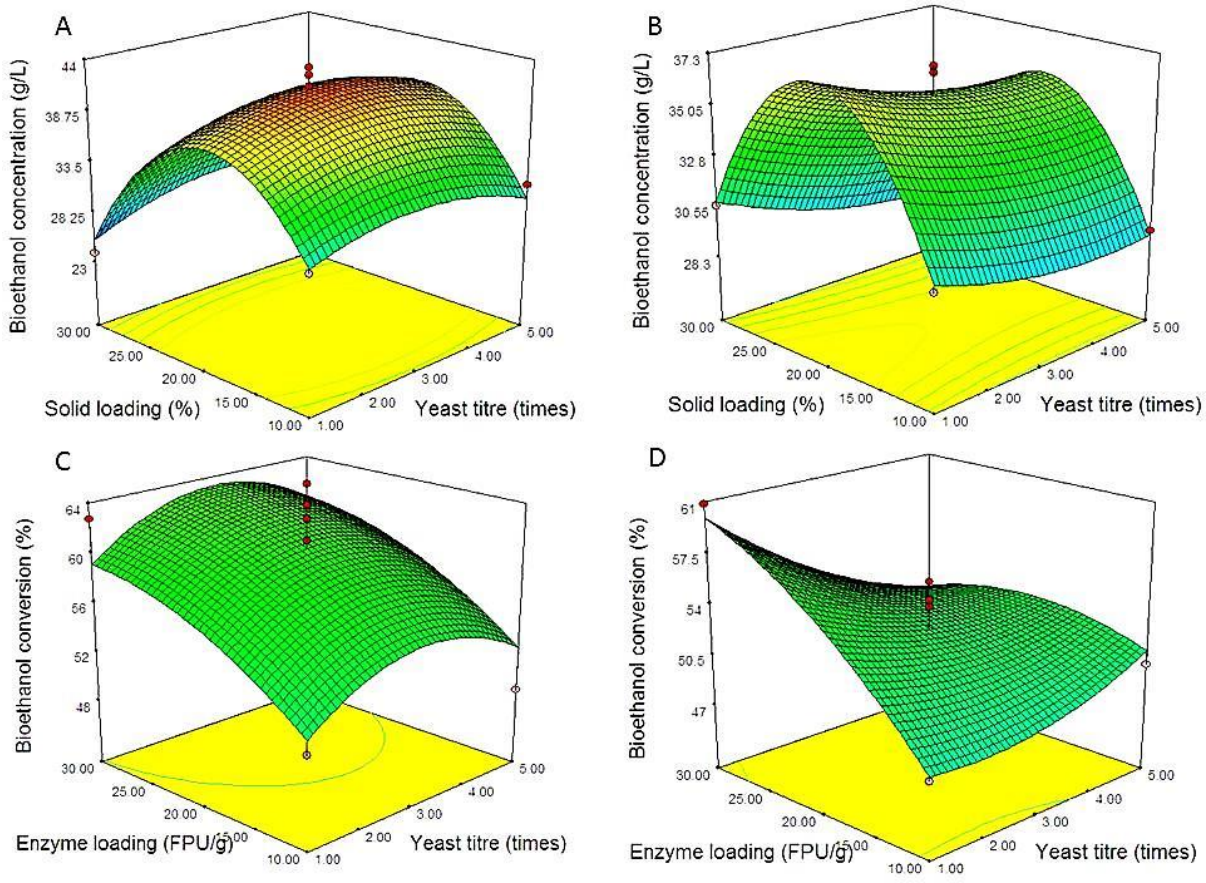

Figure S2. Response surface plots showing the interactive effect of: (A) solid loading and yeast titre ($PSSF_{concentration}$); (B) solid loading and yeast titre ($OSSF_{concentration}$); (C) enzyme loading and yeast titre ($PSSF_{conversion}$) and (D) enzyme loading and yeast titre ($OSSF_{conversion}$).

Supplementary material 2:

1. The standard method as previously reported by Van Soest (1973) was adopted for the compositional analysis. Neutral detergent fiber (NDF) analysis was determined by boiling the sample in a detergent solution (pH 7.0). The NDF contained cellulose, hemicellulose and lignin. Acid detergent fiber (ADF) was determined by boiling the sample in an acid detergent solution to remove the soluble portion. The ADF that consisted of cellulose and lignin. The resulting ADF components were treated with 72% H_2SO_4 to yield acid detergent lignin (ADL) and contained lignin.

References

1. Van Soest, P.J., McQueen, R.W. (1973). The chemistry and estimation of fibre, Proc. Nutr. SOC. 32, 123-130.

CHAPTER 7

Conclusions and Recommendations

7.1. Conclusions

The development of high-ethanol-performance processes from lignocellulosic wastes will enhance the global economy by facilitating sustainable fuel carriers. Bottlenecks that currently limit lignocellulosic bioethanol processes include the high cost and energy input coupled with the low fermentable sugar and ethanol yields. This research was aimed at addressing these limitations to potentially improve the industrial feasibility of bioethanol production from corn cob waste. Major findings derived from this study are summarized as follows:

7.1.1. Two different sequential alkalic salt-based pretreatment regimes were developed for enhanced sugar recovery from corn cobs and consisted of: (a) a sequential alkalic salt and metal salt (SAMS) and (b) a sequential alkalic salt and dilute acid (SASA). The sequential optimized SAMS pretreatment (14.02% $Na_3PO_4.12H_2O$, 3.65% $ZnCl_2$ and 5% solid to liquid ratio) gave a reducing sugar yield of 1.10 g/g compared to 0.99 g/g for the SASA pretreatment (12.70% $Na_3PO_4.12H_2O$, 1.04% H_2SO_4 and 14.49% solid to liquid ratio). Structural compositional analysis revealed similar cellulose (59.98 and 58.89%), hemicellulose (28.33 and 29.01%) and lignin (2.30 and 2.77%) fractions for the SAMS and SASA pretreatments, respectively. Likewise, the SAMS and SASA pretreatments resulted in high glucose (0.71 and 0.69 g/g) yields, respectively, and low fermentation inhibitor concentrations (<1 g/L). Thus, the developed sequential pretreatment strategies demonstrated high sugar yields (>8-fold) compared to previous reports on corn cobs. The high content of fermentable sugars and reduced concentration of inhibitor compounds observed with the SAMS and SASA pretreatments make these procedures highly suitable for the microbial production of ethanol fuels and value-added products. Moreso, the SASA regime gave a higher quantity of pretreated biomass (2.9-fold) compared to the SAMS method, and thus was subsequently selected for the simultaneous saccharification and fermentation (SSF) processes.

7.1.2. SSF processes with prehydrolysis (PSSF) and without prehydrolysis (OSSF) were optimized for maximum bioethanol concentration and bioethanol conversion using *Saccharomyces cerevisiae* BY4743. Process optimization gave bioethanol concentrations and conversions of 36.92±1.34g/L and 62.36±2.27% for the PSSF model (yeast titre of 2 times, 17.50% solid loading and enzyme loading of 30 FPU/g) compared to 35.04±0.170g/L and 58.13±0.283% for the OSSF model (yeast titre of 1 time, 17.82% solid loading and enzyme loading of 30 FPU/g), respectively. A negligible variation between the PSSF and OSSF processes was observed for ethanol concentration and conversion. The logistic and modified Gompertz models were used to study the kinetics of microbial cell growth and bioethanol formation under microaerophilic and anaerobic process conditions using the optimized PSSF and OSSF conditions. *S. cerevisiae* cell growth in the $OSSF_{microaerophilic}$ process gave a higher maximum specific growth rate (μ_{max}) of 0.274 h^{-1} compared to 0.186 h^{-1} for the $PSSF_{anaerobic}$ process. Bioprocess carried out under $PSSF_{microaerophilic}$ conditions gave the highest potential maximum bioethanol concentration (P_m) of 42.24 g/L while the lowest P_m (27.62 g/L) was observed for the $OSSF_{anaerobic}$ process. Kinetic data revealed that microaerophilic environments resulted in optimal cell growth and bioethanol concentration. This was substantiated by the high P_m value and short process lag time (t_L) obtained for the $OSSF_{microaerophilic}$ (37.87 g/L) and $PSSF_{microaerophilic}$ (1.98 h) processes, respectively. However, the maximum bioethanol production rate ($r_{p,m}$) was highest during the $PSSF_{anaerobic}$ (3.25 g/l/h) process and was ascribed to metabolic shifts toward ethanol formation under anaerobic environments.

7.1.3. In this study, the developed SAMS and SASA pretreatment regimes significantly enhanced sugar recovery from corn cobs and provided cost-effective alternatives to the commonly employed sodium hydroxide. The SAMS pretreatment demonstrated high efficiency for reducing sugar production and the SASA pretreatment resulted in a higher quantity of pretreated substrate. Thus, both developed pretreatment regimes enhance the techno-economics of microbial production of fuels and high value commodities. Additionally, SSF process optimization showed that additional prehydrolysis stages did not significantly impact on the bioethanol concentration and conversion thus reducing a unit operation. Furthermore, kinetic data revealed that microaerophilic instead of anaerobic process conditions resulted in optimal cell growth and bioethanol production. These findings will

significantly impact on the lignocellulosic bioethanol process design and improve the techno-economic output.

7.2. Recommendations

Based on the results derived from this study, the following recommendations can be made for future research on lignocellulosic bioethanol production:

7.2.1. The lignin fraction obtained from the developed pretreatments should be assessed for electricity generation to achieve a higher substrate conversion and energy efficiency.

7.2.2. The potentiality of recycling and reusing spent liquid after lignocellulosic pretreatment should be explored to reduce disposal and remediation costs and promote eco-friendly methods for lignocellulosic biofuel production processes.

7.2.3. The development of a biorefinery concept that integrates bioethanol production with other fuel processing technologies such as biodiesel, biogas and biohydrogen should be investigated to enhance substrate conversion, reduce costs and improve the energy efficiency using lignocellulosic wastes.

7.2.4. The improvement in the capability of the bioethanol-producing microorganisms for higher ethanol yields and the utilization of a wide range of carbohydrates using metabolic engineering could improve the industrial feasibility of bioethanol production.