

**STRATEGIES FOR ENHANCING SUGAR RECOVERY FROM SUGARCANE
LEAF WASTE AND KINETIC MODELLING FOR BIOETHANOL PRODUCTION
USING *SACCHAROMYCES CEREVISIAE* BY4743**

By

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PREFACE

The research contained in this thesis was completed by the candidate while based in the Discipline of Microbiology, School of Life Science, College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Pietermaritzburg, South Africa. The research was financially supported by the National Research Foundation.

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: Prof. E.B. Gueguim Kana (Supervisor)

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DECLARATION 1: PLAGIARISM

I, Preshanthan Moodley, declare that:

- (i) the research reported in this thesis, except where otherwise indicated or acknowledged, is my original work;
- (ii) this thesis has not been submitted in full or in part for any degree or examination to any other university;
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DECLARATION 2- PUBLICATIONS

This thesis represents a compilation of manuscripts/ published work where each chapter is an individual entity prepared as per the journals' specifications thus some repetition between chapters has been unavoidable. The first author (student) conducted all experimental work, data collection and manuscript preparation, under the guidance of the second and/or third author (supervisor).

1. Moodley, P., Sewsynker-Sukai, Y., Gueguim Kana, E.B., 2017. Progress in the development of alkalic and metal salt catalysed lignocellulosic pretreatment: Potential for bioethanol production. Submitted to *Energy Conversion and Management*. Under review. (Chapter 2)
2. Moodley, P., Gueguim Kana, E.B., 2017. Comparison of a two-stage and a combined single stage salt-acid based lignocellulosic pretreatment for enhancing enzymatic saccharification. *Industrial Crops and Products*. 108, 219-224. (Chapter 3).
3. Moodley, P., Gueguim Kana, E.B., 2017. Microwave-assisted inorganic salt pretreatment of sugarcane leaf waste: Effect on physiochemical structure and enzymatic saccharification. *Bioresource Technology*. 235, 35-42. (Chapter 4).
4. Moodley, P., Gueguim Kana, E.B., 2017. Development of a steam or microwave-assisted sequential salt-alkali pretreatment for lignocellulosic waste: Effect on delignification and enzymatic hydrolysis. *Energy Conversion and Management*. 148, 801-808 (Chapter 5).
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CONFERENCE PROCEEDINGS AND CONTRIBUTIONS

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4. Moodley, P., Gueguim Kana, E.B., Microwave-assisted inorganic salt pretreatment of sugarcane leaf waste. 19th International Conference on Chemical and Biological Engineering. 29 July 2017. Zurich, Switzerland.

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ABSTRACT

Depleting fossil fuel reserves and environmental concerns from its combustion have led to increasing interest in bio-based fuels and products. Lignocellulosic biomass is a potential feedstock for renewable biofuels such as bioethanol through microbial fermentation. However, this bioprocess is challenged by the recalcitrant nature of the lignocellulosic matrix which lowers the efficiency of enzymatic and microbial conversion. Current lignocellulosic pretreatment methods have significant drawbacks such as high cost, toxicity and energy requirements. This has further negative ramifications on bioethanol yield, and requirement for additional costly unit operations at upstream and downstream stages. In this study, three lignocellulosic pretreatment strategies were developed, optimized and assessed for enhancing enzymatic saccharification of sugarcane leaf waste (SLW). Experimental data from these studies were further used to develop two artificial neural network tools to predict sugar yields from inorganic salt-based pretreatments. The kinetics of *Saccharomyces cerevisiae* BY4743 growth and bioethanol production on pretreated SLW was also investigated.

The developed lignocellulosic pretreatments consisted of: (a) a two-stage salt-acid pretreatment, (b) a microwave assisted inorganic salt (MAIS) pretreatment, and (c) a steam or microwave-assisted two stage salt-alkali pretreatment (MSA and SSA respectively). All developed pretreatment models showed strong correlation to experimental data ($R^2 > 0.84$). The two stage salt-acid pretreatment showed a 90% hemicellulose solubilization and a sugar yield of 0.293 g/g under optimal pretreatment conditions of 3.32 M $ZnCl_2$, 1.84% (v/v) H_2SO_4 , and 9.26% (w/v) solid loading. This method exhibited a 1.9 fold yield improvement compared to previously reported pretreatments based on acid and sequential acid-alkali regimes. The microwave-assisted inorganic salt pretreatment gave a maximum hemicellulose removal and sugar yield of 71.5% and 0.406 g/g respectively with 2 M $FeCl_3$ at 700 W for 3.5 min. The MAIS regime gave a 3.1 fold improvement in sugar yield compared to previous reports using a sequential acid-alkali or peroxide-based pretreatments. The most effective pretreatments were the developed sequential SSA and MSA techniques. The SSA regime gave a sugar yield of 1.21 g/g with 1.73 M $ZnCl_2$, 1.36 M NaOH and 9.69% solid loading whereas the MSA method gave 1.17 g/g using 1.67 M $ZnCl_2$, 1.52 M NaOH at 400 W for 10 min. These pretreatment strategies showed an improvement of up to 2.7 fold compared to previous reports. These pretreatments were further used to develop predictive models.

To implement the artificial neural network-based predictive models, data from 90 experimental runs under varied pretreatment conditions were used to develop the microwave- and steam-based models. The input parameters for the microwave model consisted of salt concentration, alkali concentration, power intensity and heating time whereas the input parameters for steam model were salt concentration, acid concentration, alkali concentration, solid loading and stage (single or two-stage). The topology of the steam-based model comprised one input layer of five neurons, two hidden layers of thirteen neurons each and one output layer with one neuron (5-13-13-1) while the microwave model had an ANN topology consisting of one input layer of four neurons, two hidden layers of thirteen neurons each and one output layer with one neuron (4-13-13-1). These models gave high coefficients of determination (R^2) of 0.97 and showed high accuracy when predicting sugar yields. Knowledge extraction revealed that the reducing sugar yield for both models were highly dependent on salt and alkali concentration, exhibiting a sigmoidal and dose response relationship respectively for the steam model, and a regression and sigmoidal relationship respectively for the microwave model.

The recovered fermentable sugars from the SSA and MSA pretreated SLW were then used as a carbon and energy source for bioethanol production using *Saccharomyces cerevisiae* BY4743. The effect of the SSA and MSA pretreatment as well as filtered (F) and unfiltered (U) enzymatic hydrolysate on kinetic models were investigated. Fermentation data from the bioethanol production process were used to fit the empirical Monod, Logistic and modified Gompertz models with coefficients of determination $R^2 > 0.98$. The maximum specific growth rates (μ_{\max}) were 0.24, 0.26, 0.28 and 0.29 h^{-1} for *S. cerevisiae* BY4743 grown on SSA-F, SSA-U, MSA-F and MSA-U respectively. Potential maximum bioethanol concentration (P_m) values of 30.49, 31.06, 23.26 and 21.79 g/L were obtained for SSA-F, SSA-U, MSA-F and MSA-U respectively. Unfiltered enzymatic hydrolysate had a negligible effect on μ_{\max} and P_m , thus suggesting a possible reduction in the number of unit operations at large scale.

This study developed novel lignocellulosic pretreatment strategies to provide cost-efficient, low-energy alternatives to enhance enzymatic hydrolysis. In addition, the developed intelligent models may be useful as initial screening tools to identify suitable pretreatment conditions prior to optimization thus shortening pretreatment development time. Furthermore, kinetics data revealed that SSA pretreated SLW is a suitable feedstock for bioethanol production thereby providing a low cost alternative to other considered agricultural waste.

Keywords: Bioethanol, Lignocellulose, Pretreatment, Sugarcane leaf waste, Inorganic salt

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“Never doubt that a small group of thoughtful, committed citizens can change the world; indeed, it's the only thing that ever has.”

Margaret Mead

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CHAPTER 1

General Introduction

1.1 Research rationale

The global energy consumption is projected to increase by 48%, from 549 quadrillion British thermal units (Btu) in 2012 to 815 quadrillion Btu in 2040 as a result of population growth which is estimated to exceed 9.7 billion by 2050 (International Energy Outlook, 2016). The current energy demands are primarily met by conventional fossil fuels, accounting for 80% of the total energy market (Zabed et al., 2016). However, coal and oil reserves are rapidly depleting and are estimated to meet demand for the next few decades at current extraction rates (Day and Day, 2017). In addition, the exponential increase in greenhouse gas (GHG) emissions from fossil fuel combustion is causing a rise in global temperatures, creating severe environmental concerns (Aditiya et al., 2016). Therefore, it is essential to transition towards a more sustainable carbon-neutral bio-economy. The South African Department of Energy has mandated that clean renewable energy should comprise 30% of the total market by 2025, and 42% by 2030 (DoE Strategic Plan 2015-2020). Biofuels such as bioethanol will play a key role in shifting towards this bio-economy (De Bhowmick et al., 2017).

Bioethanol is a renewable and sustainable fuel that has been earmarked as a potential alternative to gasoline. In addition, the combustion of bioethanol is relatively cleaner and results in lower toxic emissions, owing to its high oxygen content (Aditiya et al., 2016). First generation bioethanol is produced from crop oils and sugars whereas second generation is produced from lignocellulosic biomass. First generation fuels are severely constrained by using limited food crops thus contributing to the food versus fuel debate while second generation fuels utilize non-food lignocellulosic biomass. Thus, it is vital to develop a sustainable approach to produce second generation bioethanol where lignocellulosic biomass plays a central role.

Lignocellulosic biomass (LB) has an annual production of 200 billion tons, the majority of which is considered waste (Kabir et al., 2015). The annual growth rate of LB per hectare of land is equivalent to 30-240 Barrels of oil (Huber et al., 2006), thus LB is an abundant, sustainable, low-cost and energy dense feedstock for biofuel production. Agricultural waste residues make up a large fraction of LB. Sugarcane is an important agricultural crop worldwide

with an annual production of 328 teragrams (Tg), owing to its economic importance (Sindhu et al., 2016). Sugarcane leaf waste (SLW) constitutes 40% of the plants total biomass. They are burned or dumped in landfill sites, constituting 131 Tg of an underutilized bio-resource (Smithers, 2014).

There are various bioprocessing routes for LB such as SLW for the production of biofuels and biomaterials. A typical biorefinery for lignocellulosic biomass to produce biofuels and bioproducts, as illustrated in Fig 1.1, entails three sequential operations: (1) pretreatment of LB, (2) enzymatic hydrolysis of pretreated LB and (3) fermentation (Raghavi et al., 2016).

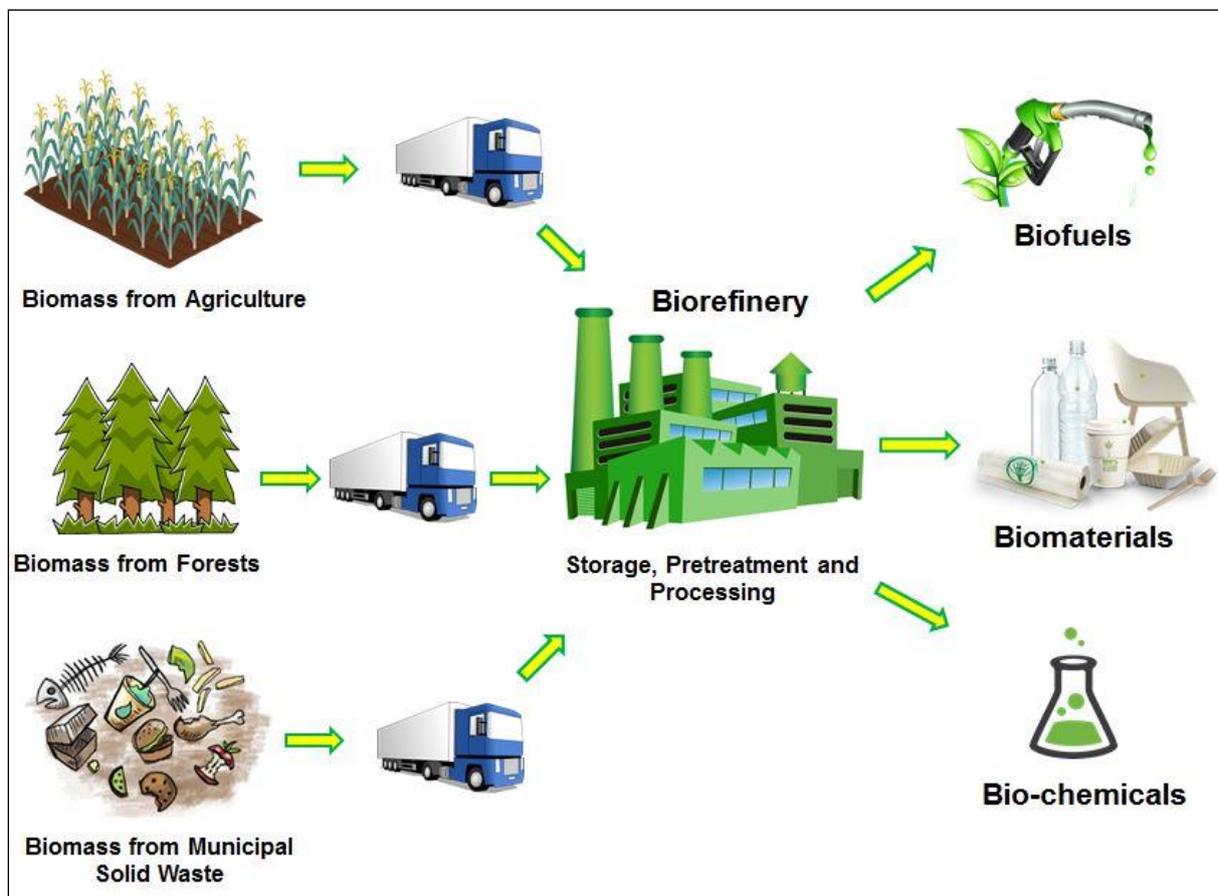


Figure 1.1. Lignocellulosic processing in a biorefinery system (Adapted from Kurian et al. 2013).

A major limiting step in the biorefinery of lignocellulosic biomass for biofuels and bioproducts is the pretreatment stage. The complex recalcitrant nature of SLW and other lignocellulosic materials hinders its bioconversion enzymatically or via microbial fermentation for bioethanol production. Microorganisms commonly employed in bioethanol fermentation such as *Saccharomyces cerevisiae* and *Pichia stipitis* are unable to access or metabolize the polymeric sugar molecules in cellulose or hemicellulose. Consequently, a pretreatment step is required to disrupt and degrade the complex lignocellulosic structure. A number of pretreatment methods have been developed, and include acid, alkali, steam explosion, ionic liquids, organosolv and inorganic salt among many others, with each having advantages and disadvantages (Kamireddy et al., 2013). These pretreatment methods are commonly challenged by high process costs, high concentration of fermentation inhibitors, high energy demands, high toxicity and partial degradation of the lignocellulosic matrix (Zabed et al., 2016; Jung and Kim, 2015; Kang et al., 2013). The development of alternative pretreatment strategies with enhanced sugar recovery, low concentration of fermentation inhibitory compounds, lowered cost and lesser energy input will significantly enhance the economic viability of lignocellulosic biofuels.

Inorganic salts have emerged as a promising pretreatment candidate, owing to their low cost, low toxicity and low generation of inhibitors (Kang et al., 2013). Previous studies have typically focused on conventional steam heating (Banerjee et al., 2016; Chen et al., 2014; Kang et al., 2013; Liu et al., 2009), which has resulted in average sugar yields. In addition, the combined pretreatment effect of inorganic salt and acid or other catalysts has only been explored in a combined single stage (Kim et al., 2014; Qing et al., 2016) rather than a sequential two stage system. Therefore, the development of novel inorganic salt-based strategies could improve sugar yields.

Furthermore, the lignocellulosic pretreatment conditions have a significant impact on fermentation process for bioethanol production. Factors such as cell biomass yield, ethanol yield, productivity and ethanol production rate, among others are affected by the substrate- and pretreatment-type (Dodic et al., 2012; Ariyajaroenwong et al., 2016). Kinetic models have been employed in predicting the behaviour of microorganisms and product formation in different bioprocesses. Many models have been developed and include Monod, Logistic and modified Gompertz (Dodic et al., 2012). These models describe cell growth and product formation thereby providing insights towards scale up. The kinetic studies of cell growth and product formation on pretreated lignocellulosic biomass provide data on the behaviour of microorganisms in response to changes in fermentation conditions (Manikandan et al., 2008;

Phukoetphim et al., 2017). Knowledge of kinetic behaviour plays a significant role in process optimization and scale up. There is a paucity of kinetic studies of bioethanol production from pretreated lignocellulosic biomass (Birol et al., 1998; Dodic et al., 2012; Ariyajaroenwong et al., 2016). To the best of our knowledge, there are no reports on inorganic salt pretreated SLW. Furthermore, it is common practice to carry out bioethanol fermentation on the filtered enzymatic hydrolysate. This additional unit operation impacts on productivity and process economics (Aden and Foust, 2009). There is a dearth of knowledge on the kinetics of filtered and unfiltered enzymatic hydrolysate in bioethanol production. These knowledge gaps have hindered the development and potential scale up of bioethanol production from inorganic salt pretreated SLW.

1.2 Aims and objectives

The aim of this research was the development of effective inorganic salt-based pretreatment strategies for enhancing enzymatic saccharification of sugarcane leaf waste. Experimental data from these strategies were then used to develop artificial neural network tools to predict sugar yields. The kinetics of *Saccharomyces cerevisiae* BY4743 growth and bioethanol production on pretreated SLW were investigated.

Therefore, the following specific objectives were undertaken-

- i. Improvement of enzymatic hydrolysis using a two-stage salt-acid pretreatment for sugarcane leaf waste.
- ii. Enhancement of sugar recovery from sugarcane leaf waste using microwave-assisted inorganic salt pretreatment.
- iii. Development of a steam salt-alkali and microwave salt-alkali pretreatment to enhance enzymatic saccharification of sugarcane leaf waste.
- iv. Development of Artificial Neural Network based models to predict sugar yields from inorganic salt pretreated sugarcane leaf waste using experimental data from the above studies

- v. Investigating the kinetics of *Saccharomyces cerevisiae* BY4743 growth and bioethanol production under different pretreatment-types and using filtered and unfiltered enzymatic hydrolysate

1.3 Outline of thesis structure

This thesis comprises eight chapters presented in research paper format. Chapters' three to five deal with the development, screening and assessment of novel pretreatment strategies. The experimental data from these studies are used in chapter six for the development of intelligent predictive tools using Artificial Neural network. The kinetics of *S. cerevisiae* BY4743 growth and bioethanol production on SLW using the optimized pretreatment strategy is studied in Chapter 7. Each chapter is self-contained, with a literature review, materials and methods, results and discussion, and conclusion. The detailed outline is as follow:

Chapter two presents an overview of recent advances in inorganic salt pretreatment of lignocellulosic waste. In addition, the potential of inorganic salt pretreatment of sugarcane leaf waste for bioethanol production is detailed. Furthermore, the challenges and future prospects are discussed.

Chapter three compares the pretreatment efficiency of a single combined salt-acid regime and a two-stage sequential salt-acid regime. These pretreatments are modelled and optimized using Response Surface Methodology.

In Chapter four, three microwave-assisted inorganic salt-based pretreatment models are developed and optimized using Response Surface Methodology. The efficiency of each pretreatment is evaluated based on the sugar yield and changes in the lignocellulosic structure.

Chapter five focuses on the development of two salt-based pretreatment regimes namely steam salt-alkali (SSA) and microwave-assisted salt-alkali (MSA) to enhance sugar yields from enzymatic saccharification. These pretreatments are modelled and optimized using Response Surface Methodology.

In Chapter six, two Artificial Neural Network models are developed to predict sugar yields from inorganic salt-based pretreatments under varied novel conditions.

Chapter seven investigates the potential of bioethanol production from inorganic salt-based pretreated SLW. The effect of pretreatment-type and, filtered and unfiltered enzymatic hydrolysate on process kinetics are examined.

The final chapter, Chapter eight, integrates the various findings and provides conclusions and recommendations for future studies.

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CHAPTER 2

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

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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 have shown to be 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 reaction mechanism of alkalic and metal chloride salts on lignocellulosic biomass degradation are reviewed. The effect of salt pretreatment on lignin removal, hemicellulose solubilization, cellulose crystallinity, and physical structural changes are also presented. In addition, the enzymatic digestibility and inhibitor profile from salt pretreated lignocellulosic biomass are discussed. Furthermore, the potential of salt pretreatment of lignocellulosic biomass for bioethanol production is evaluated with a focus on system configuration and process kinetics. Finally, 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 (Lu 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). Lignocellulosic biomass 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., 2012). 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. Bio-products 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) and has shown to effectively remove lignin with low release of sugar degradation compounds and furan derivatives (Qing et al., 2016b). On the other hand, acid pretreatment techniques have shown to solubilize cellulose and hemicellulose components (Zheng et al., 2013). Some examples of acid-based catalysts include hydrochloric (HCl), sulfuric (H₂SO₄) and phosphoric acid (H₃PO₄). Pretreatment with H₂SO₄ 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 result 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. Advantages and disadvantages of 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
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., 2015
Ionic liquids	Depolymerizes lignin by cleavage of β - <i>O</i> -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

Few studies have previously reported on the use of inorganic salt pretreatment with its increasing importance most recently (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., 2009a; 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 $\text{Na}_3\text{PO}_4 \cdot 12\text{H}_2\text{O}$, Na_2CO_3 , Na_2S (Qing et al., 2016a; Qing et al., 2016b). Alkalic salt based catalysts have shown to 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^-) have shown to 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 reported in previous 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 have shown to 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 and Shahbazi, 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 $\text{Na}_3\text{PO}_4 \cdot 12\text{H}_2\text{O}$ (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 $\sim 900 \text{ cm}^{-1}$ whereas bands at $\sim 1045 \text{ cm}^{-1}$ and $\sim 3420 \text{ cm}^{-1}$ 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 $\sim 1500 - 1602 \text{ cm}^{-1}$ 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 , $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ 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 Segal 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).

Several 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 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 $160\text{ }^\circ\text{C}$ 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 $80\text{ }^\circ\text{C}$ 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 % $\text{Na}_3\text{PO}_4 \cdot 12\text{H}_2\text{O}$ 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 $142.6\text{ }^\circ\text{C}$ for 18 min. XRD is not routinely employed in pretreatment studies and its use is often confirmatory to other structural analysis.

Physical changes in lignocellulosic biomass can be observed using scanning electron microscopy (SEM). SEM allows the 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 was 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 ZnCl₂ had a positive effect on the glucan recovery compared to FeCl₃. 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 FeCl₃ 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 FeCl₃ pretreatment of rice straw has also been reported (Lu and Zhou, 2011). Under optimal conditions of 0.14 M FeCl₃, 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 shown to be effective for 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 Na₂CO₃ pretreated rice straw under moderate conditions of 8 % Na₂CO₃ at 140 °C. Likewise, Qing et al. (2016b) reported enhanced enzymatic digestibility of bamboo shoot shell, yielding 50.6 % more reducing sugar using 9 % Na₃PO₄·12H₂O and 0.3 g/g H₂O₂ at 80 °C for 2 h. These same authors also observed a 91.11 % reducing sugar yield and 64.01 % glucose yield from corn stover pretreated with Na₃PO₄ and Na₂S (Qing et al., 2016a).

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

Substrate	Pretreatment	Key finding	Reference
Rice straw	0.1 M FeCl ₃ at 170 °C for 30 min	Increased enzymatic digestibility to 95.1 %	Chen et al., 2015
Corn stover	0.1 M FeCl ₃ at 140 °C for 20 min	91 % hemicellulose removed 89 % recovered sugars	Liu et al., 2009a
Miscanthus straw	5 % ZnCl ₂ at 200 °C for 25 min	Increased enzymatic digestibility to 62.2 %	Kang et al., 2013
Mustard stalk and straw	2 M NaCl at 121 °C for 60 min	Increased enzymatic digestibility to 72 %	Banerjee et al., 2016
Barley straw	7.3 % ZnCl ₂ (acidified) at 67.9 °C for 10.5 min	Increased enzymatic digestibility to 69.3 %	Kim et al., 2014b
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 ₂ CO ₃ at 120 °C for 50 min	71.7 % total sugar recovery	Yang et al., 2012
Bamboo shoot shell	9 % Na ₃ PO ₄ .12H ₂ O and 0.3 g/g H ₂ O ₂ at 80 °C for 2 h	87.7 % delignification 97.1 % reducing sugar yield	Qing et al., 2016b
Corn stover	4 % Na ₃ PO ₄ and 10 % Na ₂ S at 120 °C for 40 min	62.2 % delignification 91.1 % reducing sugar yield	Qing et al., 2016a

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). Relative toxicity of these inhibitor compounds on the bioethanol fermentation process in decreasing order: phenolic compounds>furfural>HMF>acetic acid>extractives (Mussatto and Roberto, 2004). 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 have shown to inhibit the 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 has shown to produce 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 $\text{Na}_3\text{PO}_4 \cdot 12\text{H}_2\text{O}$ 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 °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 °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 ₃ PO ₄ .12H ₂ O, 0.3 g/g H ₂ O 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 ₃ PO ₄ , 10 % Na ₂ S, 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)

ND – Not determined

6. Potential of inorganic salt pretreatment for lignocellulosic bioethanol production

6.1 System configuration

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. Bioethanol can be produced using three system configurations, each with their own advantages and drawbacks: (1) separate hydrolysis and fermentation (SHF), (2) simultaneous saccharification and fermentation (SSF) and, (3) pre-hydrolysis followed by simultaneous saccharification and fermentation (PSSF) (Carrillo-Nieves et al., 2017). The main feature of the SHF strategy is that 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 SSF configuration 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.

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 (Singh 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).

6.3 Process optimization

Process optimization is a key step in the development of economically feasible bioprocesses. Since there are a variety of factors that affect either the sugar or ethanol yield, process optimization allows the determination of optimum values of the input parameters (Chen et al., 2014). Some of the strategies used for bioprocess optimization include: One factor at a time (OVAT) which examines a single factor a time thereby ignoring all other factors and influences (Kalil et al., 2000). Response surface methodology (RSM) allows the extraction of complex interactions through mathematical and statistical techniques. Box-Behnken is a three level factorial RSM design which is an economical method since it uses fewer factors and lack of too high or too low levels (Wang and Wan, 2009).

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. Similarly, there has been a lack of knowledge on microwave-assisted alkalic or metal salt pretreatment with the majority of studies focussing on steam-assisted treatments. 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 (Qing et al., 2016a).

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 (Sindhu et al., 2015). 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 will enhance the sugar recovery from lignocellulosic biomass and at the same time reduce the negative impacts that include reactor corrosion and high costs. Likewise, screening and optimization of microwave-assisted alkalic or metal salt pretreatments could improve degradation of the lignocellulosic matrix and broaden pretreatment knowledge. 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. There is a lack of studies focusing on the kinetics of bioethanol production from alkalic or metal salt pretreated lignocellulosic waste. Knowledge on kinetics is crucial for bioprocess optimization and scale up. 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. In addition, there has been a dearth of knowledge on the impact of different pretreatment regimes on the kinetics of bioethanol production in SHF systems. Studies on the effects of pretreatment on bioethanol production could reduce pretreatment time and costs. Additionally, SHF processes often necessitates separation of the solid residues from the enzymatic hydrolysate by a filtration or centrifugation step that hampers process economics at a large scale. Several studies have indicated that centrifugation for the removal of solid residues is a required step for bioethanol production however, this step has shown to significantly impede the economic feasibility and productivity of SHF processes. Investigation into the comparative effects of filtered and unfiltered enzymatic hydrolysate on process kinetics could provide crucial insight into enhancing the economic and productivity outlook at large scale. On the other hand, 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. Furthermore, *Saccharomyces cerevisiae*, an industrially-known bioethanol producing strain has shown to exhibit changes in growth behaviour under varying oxygen 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. Knowledge on kinetics of cell growth and bioethanol production under microaerophilic and anaerobic conditions are required for enhancement of SSF processes.

Apart from knowledge on kinetics, previous reports on SSF processes have indicated that commercial cellulase-based enzymes are negatively influenced under oxygen deficient conditions. Investigations on the comparative effects of microaerophilic and anaerobic environments on newly developed commercial cellulase cocktails such as Cellic CTec 2 require further exploration in 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 SHF and SSF processes will enhance the process performance and economics for large scale application.

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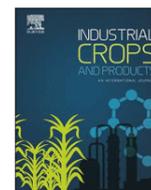
CHAPTER 3

Comparison of a Two-stage and a Combined Single Stage Salt-Acid based Lignocellulosic Pretreatment for Enhancing Enzymatic Saccharification.

This chapter has been published with the title: ‘Comparison of a Two-stage and a Combined Single Stage Salt-Acid based Lignocellulosic Pretreatment for Enhancing Enzymatic Saccharification’ in *Industrial Crops and Products* (2017, 108: 219-224).

Supplementary material associated with this paper can be found at the end of this chapter.

The published paper is presented in the following pages.



Comparison of a two-stage and a combined single stage salt-acid based lignocellulosic pretreatment for enhancing enzymatic saccharification



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ABSTRACT

This study compares the optimization of combined and two-stage salt-acid pretreatment technique for efficient delignification, hemicellulose removal and enhancement of sugar yield from sugarcane leaf waste. The effect of process parameters ZnCl_2 concentration (1 M–5 M), H_2SO_4 concentration (0.10–2.0%, v/v) and solid loading (5–15%, w/v) on the reducing sugar yield were investigated. The developed models showed coefficients of determination (R^2) > 0.94. The two-stage pretreatment under optimal conditions of 3.32 M ZnCl_2 , 1.84% (v/v) H_2SO_4 , and 9.26% (w/v) solid loading gave 0.293 g/g reducing sugar, a 9% improvement compared to the optimized combined pretreatment. SEM and FTIR analysis showed major structural changes in the pretreated biomass. Significant lignin and hemicellulose removal was observed with the two-stage pretreatment. The optimized two-stage pretreatment also showed 66% and 194% yield improvement on sorghum leaves and Napier grass respectively. These results indicate that a two-stage ZnCl_2 - H_2SO_4 pretreatment can significantly enhance enzymatic saccharification of lignocellulosic waste.

1. Introduction

Lignocellulosic biomass is an abundant non-food feedstock that can sustain alternative fuel production thereby alleviating conventional fuel concerns (Behera et al., 2014). However, the major bottleneck in converting lignocellulosic biomass to monomeric sugars lies in the inherent recalcitrance of the material toward enzymatic and microbial degradation (Qing et al., 2016). Native biomass is composed of cellulose (38–50%) and hemicellulose (23–32%) bound together by an impermeable lignin layer (15–25%) preventing effective degradation (Kim et al., 2008; Sindhu et al., 2016a). Thus, the pretreatment of lignocellulosic biomass is a crucial step that disrupts the crystalline structure, removes lignin and hemicellulose allowing the enzymatic conversion of cellulose to glucose.

Pretreatment can be separated into either physical, chemical or biological methods (Taherzadeh and Karimi, 2008). Commonly investigated pretreatments include acid (Bouza et al., 2016; Moodley and Kana, 2015), alkali (Zhu et al., 2016), inorganic salts (Kang et al., 2013), hot water (Kim et al., 2016), microwave (Jin et al., 2016), ionic liquid (Asakawa et al., 2016; Wang et al., 2015) and glycerol (Zhang et al., 2016) among others. A major drawback to most pretreatment technologies are still high cost, toxicity and energy demand. Acids such as hydrochloric, sulfuric, phosphoric and nitric acid are effective in removing lignin and hemicellulose which explains their extensive use

(Zhu et al., 2016). Sulfuric acid is most commonly employed because of its high catabolic activity (Behera et al., 2014). Acid pretreatment, however, tends to produce compounds that are inhibitory to enzymatic activity. In addition, higher process costs can be incurred to acquire corrosion resistant reactors. Thus, lower concentration acids are preferred although it hampers sugar yields (Kim et al., 2016). Conversely, the performance of dilute acid pretreatment could be enhanced when combined with salts (Ramadoss and Muthukumar, 2015). Pretreatment with inorganic salts have recently been reported to increase hemicellulose degradation thus increasing cellulose conversion rates (Kang et al., 2013). Banerjee et al. (2016) reported an 82% saccharification efficiency using NaCl in mustard stalk and straw pretreatment. A combined sodium phosphate and sodium sulfide pretreatment strategy for corn stover was found to yield 91.11% reducing sugar (Qing et al., 2016). However, there are very few reports on lignocellulosic pretreatment using a combination of salt and acid. Li et al. (2014) examined the effect of FeCl_3 and HCl on the crystallinity of cellulose. These authors also studied the effect of ultrasonic waves on HCl- FeCl_3 pretreatment (Li et al., 2015). The combined pretreatment of salt and acid on corn stover and sugarcane baggase have been reported in some studies (Degenstein et al., 2013; Miranda et al., 2015). However, a combination of salt and acid can result in a double replacement reaction which renders the pretreatment ineffective (Helmenstine, 2016). Double replacement reactions or metathesis is the biomolecular process

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where molecules possessing counter ions are exchanged (IUPAC, 1997). For instance, H_2SO_4 co-catalysed with FeCl_2 is routinely employed as a combined salt-pretreatment. In the presence of water, H_2SO_4 can react with FeCl_2 to form HCl and FeSO_4 thus implying HCl as the net pretreatment effect. Therefore, a two-stage salt-acid pretreatment was explored in this study and compared to a combined regime. There is a knowledge gap on the effects of a two stage pretreatment where biomass is pretreated with salt and acid separately. Moreover, knowledge on the interaction of salt and acid on the degradation of lignocellulosic compounds is scanty.

Sugarcane is a major economic crop cultivated globally with an annual yield of 328 Tg. Among its advantages are high biomass yield and sucrose content (Sindhu et al., 2016b). Sugarcane leaves constitute 40% of the plant and are considered waste. These are either burnt in the field or dumped in landfill sites (Nguyen et al., 2010; Smithers, 2014). Native sugarcane leaves contain 44% cellulose and 28% hemicellulose (Moodley and Kana, 2015), indicating its potential as a feedstock for biofuel production. Very few studies have focused on the utilization of sugarcane leaves for biofuel production (Moodley and Kana, 2015; Jutakanoke et al., 2012).

In this study, the efficiency of two-stage and combined pretreatment with ZnCl_2 and H_2SO_4 on the enzymatic saccharification of SLW was modelled, optimized and compared. The optimal operational process conditions of salt concentration, acid concentration and solid loading on reducing sugar yield were elucidated. In addition, structural properties of native and pretreated biomass were investigated by scanning electron microscopy (SEM) and Fourier Transform Infrared analysis (FTIR).

2. Methods

2.1. Materials

Sugarcane leaf wastes (SLW) were collected from a sugarcane plantation located on the North Coast of South Africa (29° 42' 18" S, 31° 02' 44" E). Samples were dried at 60 °C for 72 h and subsequently milled to a particle size of ≤ 1 mm. Native and pretreated sample composition was determined by the NREL method (Sluiter et al., 2008). The commercial cellulase enzyme Cellic CTec 2 was generously provided by Novozymes (Novozymes A/S, Denmark).

2.2. Preliminary screening

Pretreatment was carried out in 100 ml Erlenmeyer flasks with a solid loading of 10% w/v. Varied concentrations of NH_4Cl , NaCl and ZnCl_2 (1 M, 3 M and 5 M) were evaluated with 1.5% (v/v) H_2SO_4 in different combinations and autoclaved at 121 °C for 60 min. Samples were then washed and dried prior to enzymatic hydrolysis. The efficiency of each salt was measured based on the glucose yield.

2.3. Optimization of process parameters affecting salt-acid pretreatment of SLW

Optimization of key process parameters affecting sugar yield from salt-acid pretreatment of SLW was carried out using the Response Surface Box-Behnken design. A total of 17 runs were generated and these were conducted in replicate. The parameters were selected at three levels and these included acid concentration (0.1%, 1.05%, 2.0%, v/v), salt concentration (1 M, 3 M, 5 M) and solid loading (5%, 10%, 15%, w/v). For the two stage pretreatment, SLW was pretreated with salt for 30 min at 121 °C thereafter it was washed with deionized water and dried at 60 °C followed by acid pretreatment for 30 min at 121 °C. For the combined pretreatment, SLW was pretreatment with a salt-acid solution in a single stage at 121 °C for 60 min as set out in the design. Design Expert 7.0 (Stat Ease Inc, USA) was employed for generating the experimental design, statistical analysis and polynomial model

Table 1

Box-Behnken design for optimization of various input parameters affecting salt-acid pretreatment of SLW.

Run Order	H_2SO_4 conc. (% v/v)	ZnCl_2 conc. (M)	Solid loading (% w/v)	Reducing sugar (g/g)	
				Combined	Two stage
1	2.00	5	10	0.205	0.294
2	1.05	1	5	0.0835	0.143
3	1.05	5	15	0.177	0.189
4	0.10	3	5	0.0661	0.105
5	1.05	3	10	0.226	0.256
6	0.10	1	10	0.0297	0.054
7	1.05	5	5	0.116	0.184
8	1.05	1	15	0.111	0.128
9	0.10	3	15	0.115	0.157
10	1.05	3	10	0.24	0.287
11	2.00	3	5	0.102	0.222
12	2.00	1	10	0.148	0.201
13	1.05	3	10	0.25	0.280
14	2.00	3	15	0.144	0.216
15	1.05	3	10	0.238	0.290
16	0.10	5	10	0.102	0.207
17	1.05	3	10	0.277	0.266

development. In addition, response surface plots were generated to illustrate the interactive effect of process parameters on reducing sugar yield. The experimental design is presented in Table 1.

2.4. Enzymatic hydrolysis

Enzymatic hydrolysis of pretreated or untreated SLW was carried out in 100 ml Erlenmeyer flasks by mixing the SLW biomass in 10 ml sodium citrate buffer (pH 4.8, 0.05 M) at a solid and enzyme loading of 10% (w/v) and 10 FPU/g respectively. Hydrolysis was conducted at 50 °C, 120 rpm for 72 h in a shaking waterbath. After enzymatic treatment, the hydrolysate was centrifuged and the supernatant analysed. The total reducing sugar and glucose quantification were obtained using the 3,5-dinitrosalicylic acid method (Miller, 1959) and the YSI 2700 Sugar Analyzer (YSI, USA) respectively.

2.5. Characterization of native and pretreated SLW

2.5.1. Scanning electron analysis

Surface characteristics of pretreated and native SLW was analysed using a ZEISS EVO LS 15 scanning electron microscope. Samples were gold putter coated (Eiko IB-3 Ion Coater) prior to analysis and images were taken at a magnification of $1000 \times$.

2.5.2. FTIR analysis

Fourier transform infrared spectroscopy (FTIR) was performed on pretreated and native SLW using a Perkin Elmer 100 (Waltham, MA, USA). Samples were mixed with spectroscopic grade KBr and pressed into disks. FTIR spectra were recorded between 4000 and 400 cm^{-1} at a resolution of 4 cm^{-1} with an average of 25 scans.

3. Results and discussion

3.1. Composition of native and pretreated SLW

Native SLW was composed of 43.44% cellulose, 30.98% hemicellulose and 9.16% lignin while combined salt-acid pretreated biomass contained 69.62% cellulose, 4.33% hemicellulose and 22.82% lignin. Fibre composition for the two-stage salt-acid pretreated sample was made up of 60.98% cellulose, 4.25% hemicellulose and 19.06% lignin. Thus, hemicellulose solubilization for the combined and two-stage samples were 86% and 90% respectively. Therefore, a 4% enhancement in hemicellulose solubilization using the two-stage pretreatment was

obtained. Inorganic salts play an integral role in hemicellulose removal (Kang et al., 2013). Moreover, the use of acid allows the hydrolysis of hemicellulose and disruption of the lignin structure (Jung and Kim, 2015). Therefore the synergistic effect of salt and acid pretreatment would have a significant effect on hemicellulose dissolution and partial lignin disruption. The formation of HCl through the double replacement reaction between ZnCl_2 and H_2SO_4 could account for the lower hemicellulose solubilization for the combined pretreatment. The increase in cellulose and lignin content after pretreatment can be accounted for by the high solubilization of the hemicellulose fraction during salt and acid pretreatment. A similar observation was reported by Kim et al. (2016) where a 13.8% increase in lignin was observed after dilute acid pretreatment of corn stover. Sindhu et al. (2011) also reported an increase in cellulose from 29.9% to 46.5% in the acidic pretreatment of sugarcane tops.

3.2. Preliminary screening

An initial preliminary screening of the three different salts (NaCl , NH_4Cl and ZnCl_2) revealed that 3 M ZnCl_2 was more effective in releasing sugar (0.0079 g/g) compared to 3 M NH_4Cl (0.064 g/g) and 3 M NaCl (0.057 g/g). This relatively higher efficiency of ZnCl_2 in enhancing enzymatic hydrolysis has previously been observed by Kang et al. (2013), hence it was selected in this study. Pretreatment with H_2SO_4 alone yielded 0.091 g/g of glucose, a 15% improvement from the salt pretreatment. Dilute H_2SO_4 has been demonstrated to effectively pretreat lignocellulosic residues to enhance sugar yields (Sindhu et al., 2016a,b; Kärcher et al., 2016). However, a combined pretreatment of ZnCl_2 and H_2SO_4 yielded 0.11 g/g glucose, a 39% and 21% improvement from salt pretreatment and acid pretreatment respectively. Control experiments yielded a negligible value of 0.048 g/g glucose for water pretreatment and 0.025 g/g for the native sample thus underscoring the effectiveness of the salt-acid pretreatment.

3.3. Model development

Process parameters affecting salt-acid pretreatment of SLW were investigated using the Box-Behnken design. The experimental conditions with the corresponding reducing sugar yields are presented in Table 1. Experimental data was used to develop two polynomial equations that described the relationship between the input parameters and the reducing sugar yield response.

$$\text{Sugar yield (combined model)} = + 0.25 + 0.036A + 0.028B + 0.022C - 3.82 \times 10^{-3} AB - 1.73 \times 10^{-3} AC + 8.38 \times 10^{-3} BC - 0.070 A^2 - 0.055B^2 - 0.069C^2$$

$$\text{Sugar yield (two-stage model)} = + 0.25 + 0.036A + 0.028B + 0.022C - 3.82 \times 10^{-3} AB - 1.73 \times 10^{-3} AC + 8.38 \times 10^{-3} BC - 0.070 A^2 - 0.055B^2 - 0.069C^2$$

where A, B and C represent H_2SO_4 concentration, ZnCl_2 concentration and solid loading respectively.

The suitability of the models were assessed by analysis of variance (ANOVA) and the data is shown in Table 2 and 3. The combined model had an F-value and p-value of 13.53 and 0.0012 respectively, whereas the two-stage model gave an F-value and p-value of 13.87 and 0.0011 respectively, indicating its significance. The suitability of each input coefficient was determined by examining the p-values. Significant parameters are evaluated by having a p-value less than 0.05 (Qing et al., 2016), thus terms A, B and C have a notable effect on the reducing sugar yield for the combined model in the following decreasing order: acid concentration > salt concentration > solid loading. Only terms A and B showed significant effects on the reducing sugar yield for the two-stage model. The co-efficient of determination (R^2) indicates variability in the model response. The combined and two-stage pretreatment models

showed R^2 values of 0.945 and 0.947 thus the model can elucidate 94.5% and 94.7% of variations in the observed data respectively. These statistics showed that the developed model had a good fit in terms of relating the input parameters to the response.

3.4. Effect of different process parameters on combined and two stage salt-acid pretreatment of SLW

The reducing sugar yield from the various experimental runs are shown in Table 1 with the response surface plots illustrated in Fig. 1. Yields ranged from 0.030 to 0.27 g/g and 0.054–0.294 g/g for the combined and two-stage pretreatment respectively thus highlighting the sensitivity of the substrate to the chosen pretreatment conditions. The two stage pretreatment gave higher reducing sugar yields. For instance, 20% more reducing sugar was obtained compared to the combined pretreatment when input parameters were at their median values (run 10). Likewise, when acid and salt concentration were at their lowest value (0.10% and 1 M respectively, run 6), the two-stage pretreatment released 82% (0.054 g/g) more reducing sugar than the combined pretreatment (0.0297 g/g). The effects of salt and acid concentration on the reducing sugar yield when solid loading is maintained at its centre point is shown in Fig. 1A and B. At low concentrations of salt (1.0–2.0 M) and acid (0.10–0.58%, v/v), the reducing sugar yield is relatively low (< 0.02 g/g) for the combined pretreatment (Fig. 1A). The yield increased with an increase in salt and acid concentrations up to values 3 M and 1.05% v/v respectively. This resulted in high sugar yield (0.25 g/g) and a further increase in acid and salt concentration negatively affected the yield. A similar trend was observed for the two-stage pretreatment (Fig. 1B). Lower reducing sugar yields (< 0.05 g/g) were obtained with low concentrations of salt (1.0–2.0 M) and acid (0.10–0.58%, v/v). Unlike the combined pretreatment, highest reducing sugar (0.29 g/g) was observed with salt and acid concentrations up to values 3 M and 2.0% v/v respectively. Therefore a 16% improvement in reducing sugar yield was achieved over the combined pretreatment yield. Ramadoss and Muthukumar (2015) reported a lower glucose yield of 0.153 g/g for the salt and peroxide pretreatment of sugarcane bagasse. Chen et al. (2010) observed that at higher concentrations of salt, monomeric sugars were severely degraded. Acid and salt pretreatment have been shown to effectively delignify biomass and remove hemicellulose respectively thus their combined effect positively enhanced the enzymatic conversion of cellulose to glucose (Kang et al., 2013; Siripong et al., 2016).

The effects of solid loading and salt concentration on the reducing sugar yield when acid concentration is kept at its median value is shown in Fig. 1C and D. For the combined pretreatment (Fig. 1C), an increase in solid loading and salt concentration from 5% to a threshold value of 10% (w/v) and 1 M to 3 M respectively gave a high reducing sugar yield from 0.07 to 0.24 g/g, thereafter a further increase in solid loading (15%, w/v) and salt concentration (5 M) showed a drastic reduction in sugar yield (0.12 g/g). However for the two-stage pretreatment (Fig. 1D), low levels of solid loading (5–7.5%, w/v) and salt concentration (1–2 M) gave a sub-optimal reducing sugar yield (0–0.1 g/g). A simultaneous increase in solid loading and salt concentration up to a threshold value of 11% and 4.5 M respectively resulted in a high reducing sugar yield (0.28 g/g). Further increases in solid loading and salt concentration beyond the threshold values showed an antagonistic effect on the reducing sugar yield. Banerjee et al. (2016) reported a similar non-linear trend with a 72% saccharification efficiency of mustard stalk using 2 M NaCl compared to 32% efficiency with 5 M NaCl . This low saccharification efficiency is attributed to the degradation of sugars at higher salt concentrations (Chen et al., 2010). Moreover, the use of ZnCl_2 has been shown to effectively remove xylan thus allowing the conversion of cellulose to glucose (Kang et al., 2013). Sindhu et al. (2016a,b) reported an optimized reducing sugar yield of 0.43 g/g from acid pretreated chili post harvest residue using a 10% solid loading. Optimal solid loading usually

Table 2
Analysis of variance (ANOVA) of the developed combined pretreatment model.

Source	Sum of squares	df	Mean square	F Value	p-value Prob > F	Remark	
Model	0.081	9	9.00×10^{-3}	13.53	0.0012	Significant	
A-Acid conc.	0.010	1	0.010	15.40	0.0057		
B-Salt conc.	6.48×10^{-3}	1	6.49×10^{-3}	9.76	0.0168		
C-Solid loading	4.02×10^{-3}	1	4.02×10^{-3}	6.05	0.0435		
AB	5.85×10^{-5}	1	5.85×10^{-5}	0.088	0.7753		
AC	1.19×10^{-5}	1	1.19×10^{-5}	0.018	0.8973		
BC	2.81×10^{-4}	1	2.81×10^{-4}	0.42	0.5367		
A ²	0.021	1	0.021	31.09	0.0008		
B ²	0.013	1	0.013	19.13	0.0033		
C ²	0.020	1	0.020	30.47	0.0009		
Residual	4.65×10^{-3}	7	6.65×10^{-4}				Not significant
Lack of fit	3.18×10^{-3}	3	1.06×10^{-3}	2.87	0.1675		
Pure error	1.48×10^{-3}	4	3.69×10^{-4}				
R ²					0.9456		
Cor total	0.086	16					

varies between 5 and 15% (Kim et al., 2008).

3.5. Experimental validation of developed models

According to the model's prediction, maximum reducing sugar yields of 0.256 and 0.297 g/g should be obtained for the combined and two-stage pretreatment under optimal conditions shown in Table 4. Experimental validation carried out in triplicate yielded 0.269 g/g for the combined pretreatment thus inferring a 5% surplus prediction error whereas the two-stage pretreatment yielded 0.293 g/g, showing a 1% deviation from the predicted value. The two-stage pretreatment showed a 9% improvement in the reducing sugar yield compared to the combined pretreatment. Moreover, compared to the controls, the combined pretreatment showed improvements of 27, 33.8 and 186.8% for the acid, salt and water control respectively. However, higher yield improvements of 39, 46 and 211% were observed for the acid control, salt control and water control samples respectively compared to the two-stage pretreatment.

3.6. Structural characterization of native and pretreated SLW

In order to visualize physical changes on the pretreated SLW, scanning electron micrographs were analysed and are presented in supplementary Fig. S1. Native SLW showed a clear and smooth surface with intact fibres. In contrast, the surface coating appears to be unravelled with fibre dismantling for both the combined and two-stage salt-acid sample. In addition, the two-stage sample showed significantly more fragmentation. An identical observation was reported by Zhu et al. (2016) for acid pretreatment of sugarcane bagasse. Acid

pretreated and salt pretreated samples showed significantly less damaged compared to the combined salt-acid pretreatment.

Structural changes of native and pretreated SLW samples were analysed by FTIR spectroscopy and results are shown in Fig. S2. Different bands were monitored to examine the chemical changes that occurred in the lignocellulosic structure after pretreatment. Enhancement of lignin characteristic peaks (1245 cm^{-1} , 1632 cm^{-1} and 1730 cm^{-1} ; Qing et al., 2016) were stronger for the two stage and combined salt-acid sample compared to the native and control samples, indicating the dissolution of lignin. Peaks at 1245 cm^{-1} and 1632 cm^{-1} correspond to the C=O stretching vibration and C=C stretching vibration of the aromatic ring in lignin respectively (Qing et al., 2016). The absorption band at 1505 cm^{-1} represents the aromatic skeletal vibration in lignin (Lai and Idris, 2016). The enhancement of this band for the two-stage pretreatment underscored the effectiveness of this pretreatment type. The acetyl C–O stretching of hemicellulose is shown by the peak at 1241 cm^{-1} in the native sample and is absent after the two-stage and combined salt-acid pretreatment suggesting that the hemicellulose was effectively deacetylated (Zhu et al., 2016). This implies that both the two-stage and combined pretreatment were effective in removing hemicellulose. Peaks at 897 cm^{-1} and 1033 cm^{-1} are related to cellulose and become more distinct after salt-acid pretreatment, further indicating the removal of hemicellulose (Chen et al., 2012). These results show that the two-stage and combined salt-acid pretreatment efficiently removed both lignin and hemicellulose. Inorganic salts such as ZnCl_2 act as Lewis acids and thus dissociate into complex ions in water. These ions diffuse into the wet lignocellulosic matrix and protonate the oxygen on the ether bond between sugar monomers, cleaving the bond (Kamireddy et al., 2013). This effect was observed in

Table 3
Analysis of variance (ANOVA) of the developed two-stage pretreatment model.

Source	Sum of squares	df	Mean square	F Value	p-value Prob > F	Remark	
Model	0.076	9	8.40×10^{-3}	13.87	0.0011	Significant	
A-Acid conc.	0.021	1	0.021	34.71	0.0006		
B-Salt conc.	0.015	1	0.015	25.01	0.0016		
C-Solid loading	1.62×10^{-4}	1	1.62×10^{-4}	0.27	0.6209		
AB	9.00×10^{-4}	1	9.00×10^{-4}	1.49	0.2622		
AC	8.41×10^{-4}	1	8.41×10^{-4}	1.39	0.2770		
BC	1.00×10^{-4}	1	1.00×10^{-4}	0.17	0.6966		
A ²	5.58×10^{-3}	1	5.58×10^{-3}	9.22	0.0190		
B ²	0.011	1	0.011	17.67	0.0040		
C ²	0.017	1	0.017	28.85	0.0010		
Residual	4.23×10^{-3}	7	6.05×10^{-4}				Not significant
Lack of fit	3.41×10^{-3}	3	1.14×10^{-3}	5.45	0.0675		
Pure error	8.33×10^{-4}	4	2.08×10^{-4}				
R ²					0.9469		
Cor total	0.080	16					

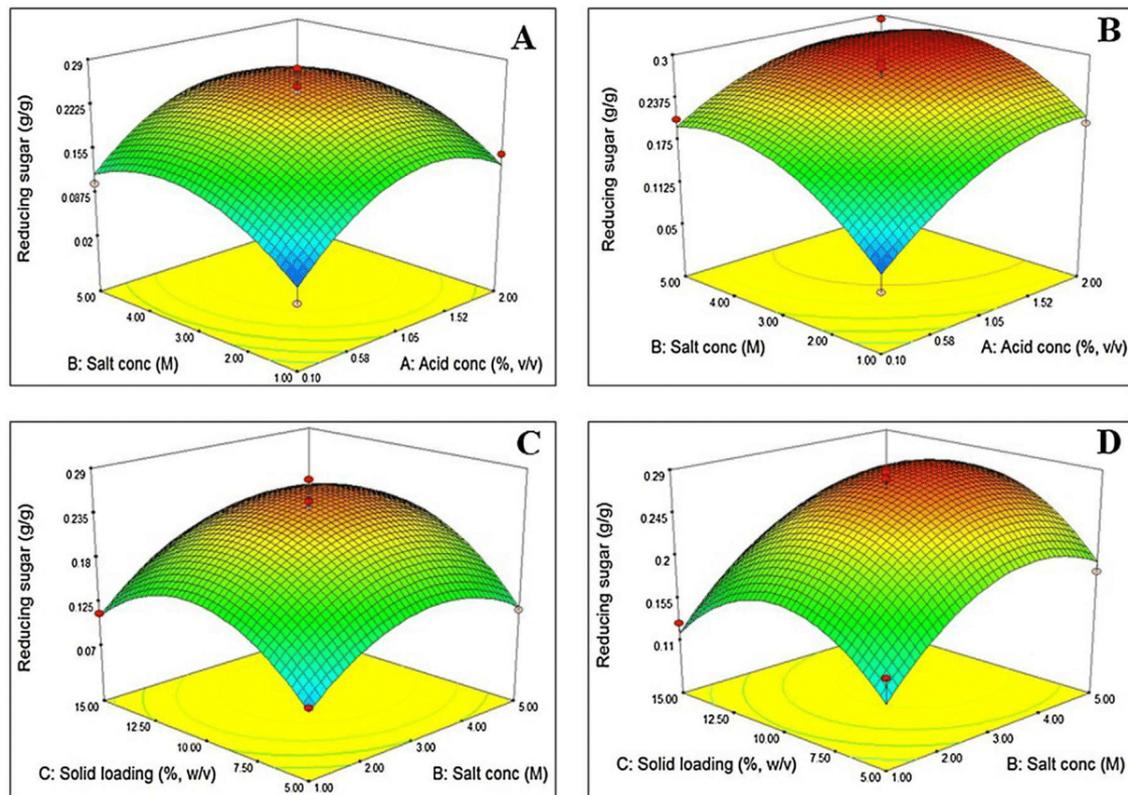


Fig. 1. Response surface plots showing the interaction between salt and acid concentration for (a) combined pretreatment (b) two-stage pretreatment; interaction between solid loading and salt concentration for (c) combined pretreatment and (d) two-stage pretreatment.

Table 4
Validation of optimized conditions for salt-acid pretreatment of SLW.

Run	Acid conc. (% v/v)	Salt conc. (M)	Solid loading (% w/v)	Reducing sugar yield (g/g)	
				Predicted	Observed
Combined model	1.28	3.53	10.87	0.256	0.269
Two-stage model	1.84	3.32	9.26	0.297	0.293
Acid control	1.05	–	10	–	0.211
Salt control	–	3	10	–	0.201
Water control	–	–	10	–	0.094

the total solubilization of hemicellulose using $ZnCl_2$ on *Miscanthus* (Kang et al., 2013). In addition, the catalytic function of metal ions are significantly higher under acidic conditions, suggesting a synergistic relationship between salt and acid activities (Li et al., 2013). In light of the above observations, the combined pretreatment had a reduced effect compared to the two-stage pretreatment. This is accounted for by the double replacement reaction between $ZnCl_2$ and H_2SO_4 to produce HCl when salt is combined with acid in a single stage (Helmenstine, 2016).

3.7. Assessment of the optimized pretreatment regimes on other lignocellulosic residues

The optimized combined and two stage salt-acid pretreatment regimes were further validated on two different lignocellulosic residues namely Napier grass and sorghum leaves and the results are shown in Table 5. A 29% improvement in reducing sugar yield was observed with the combined pretreatment compared to an earlier report on acid pretreated sugarcane leaves (Jutakanoke et al., 2012), and up to 40%

improvement was observed with the two-stage pretreatment. A study employing a combined pretreatment strategy using 6% H_2SO_4 and 100 mM $FeCl_3$ on sugarcane bagasse yielded 0.25 g/g total reducing sugar (Miranda et al., 2015). These authors used a higher concentration of H_2SO_4 compared to the 1.84% used in the current study. This result is similar to the combined $ZnCl_2$ - H_2SO_4 pretreatment (0.269 g/g). However, the two-stage $ZnCl_2$ - H_2SO_4 pretreatment model gave a higher yield of reducing sugar (0.293 g/g). This is a further indication of the effectiveness of a two-stage salt-acid pretreatment.

The combined pretreatment showed increases in reducing sugar yield for sorghum leaves (18%) and Napier grass (8.5%) compared to sugarcane leaves under the same pretreatment conditions whilst higher yield improvements were obtained for sorghum leaves (30%) and Napier grass (41%) with the two-stage pretreatment. In comparison to previous reports, the combined salt-acid technique in a single stage showed a 108% improvement in sugar yield from Napier grass with H_2O_2 pretreatment (Phitsuwan et al., 2016) and a 38% increase in sugar yield from sorghum compared to the two stage acid and alkali pretreatment method reported by Li et al., 2016. In the same trend, considerably higher improvements in sugar yield were obtained with the two-stage salt-acid pretreatment for sorghum leaves (66%) and Napier grass (194%) compared to previous reports.

4. Conclusion

This study evaluated a two-stage and a combined single stage salt-acid pretreatment strategy for enhanced enzymatic saccharification of SLW. It was found that a reducing sugar yield of 0.293 g/g can be obtained under optimal two-stage pretreatment conditions (3.32 M $ZnCl_2$, 1.84% (v/v) H_2SO_4 , and 9.26% (w/v) solid loading). The two-stage pretreatment showed a 9% improvement over the combined salt-acid single stage pretreatment. The two stage pretreatment strategy showed enhanced delignification and hemicellulose removal. Furthermore,

Table 5
Validation of the optimized pretreatment regime on different lignocellulosic residues.

Substrate	Pretreatment conditions	Yield (g/g)	Improvement	Ref
Sugarcane leaves	Combined pretreatment	0.269 ^a	29%	This study
Sugarcane leaves	Two-stage pretreatment	0.293 ^a	40%	This study
Sugarcane leaves	1.5% (w/v) H ₂ SO ₄ , 121 °C 60 min	0.208 ^b	–	Jutakanoke et al., 2012
Sorghum leaves	Combined pretreatment	0.318 ^a	38%	This study
Sorghum leaves	Two-stage pretreatment	0.382 ^a	66%	This study
Sorghum fibre	1% NaOH, 121 °C, 30 min + 1% H ₂ SO ₄ , 121 °C, 30 min	0.23 ^b	–	Li et al., 2016
Napier grass	Combined pretreatment	0.292 ^a	108%	This study
Napier grass	Two-stage pretreatment	0.412 ^a	194%	This study
Napier grass	1% (v/v) H ₂ O ₂ , 37 °C, 24h	0.14 ^b	–	Phitsuwan et al., 2016

^a Reducing sugar yield per gram dry weight.

^b Glucose yield per gram dry weight.

assessment of developed two-stage pretreatment regime on sorghum leaves and Napier grass showed a 66% and 194% yield improvement respectively compared to previous reports. This lignocellulosic pretreatment strategy shows promising potential for second generation biofuels production.

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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.indcrop.2017.06.048>.

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Supplementary material

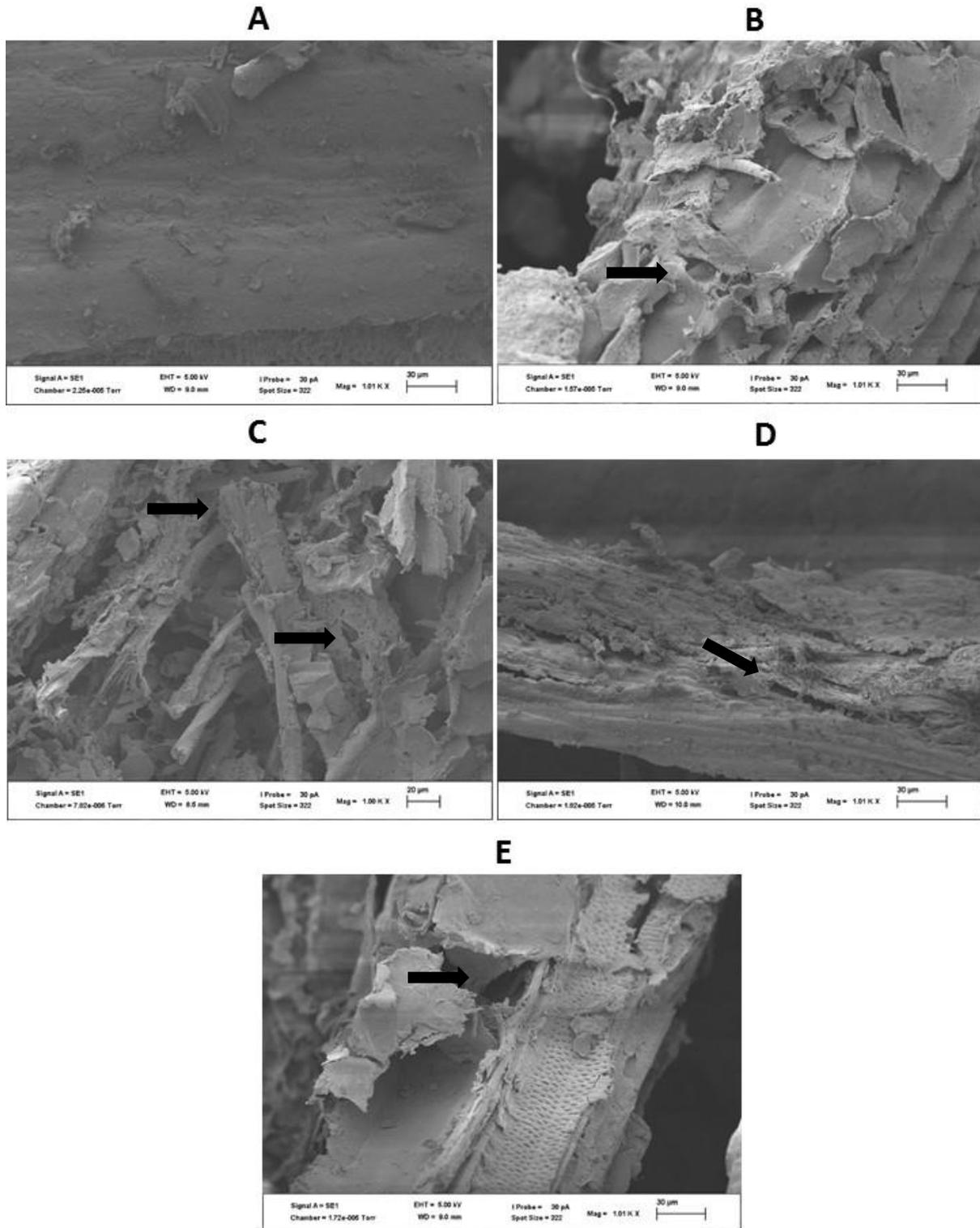


Fig S1. SEM micrographs of (a) native SLW (b) combined single salt-acid pretreated (c) two-stage salt-acid pretreated (d) acid pretreated and (e) salt pretreated

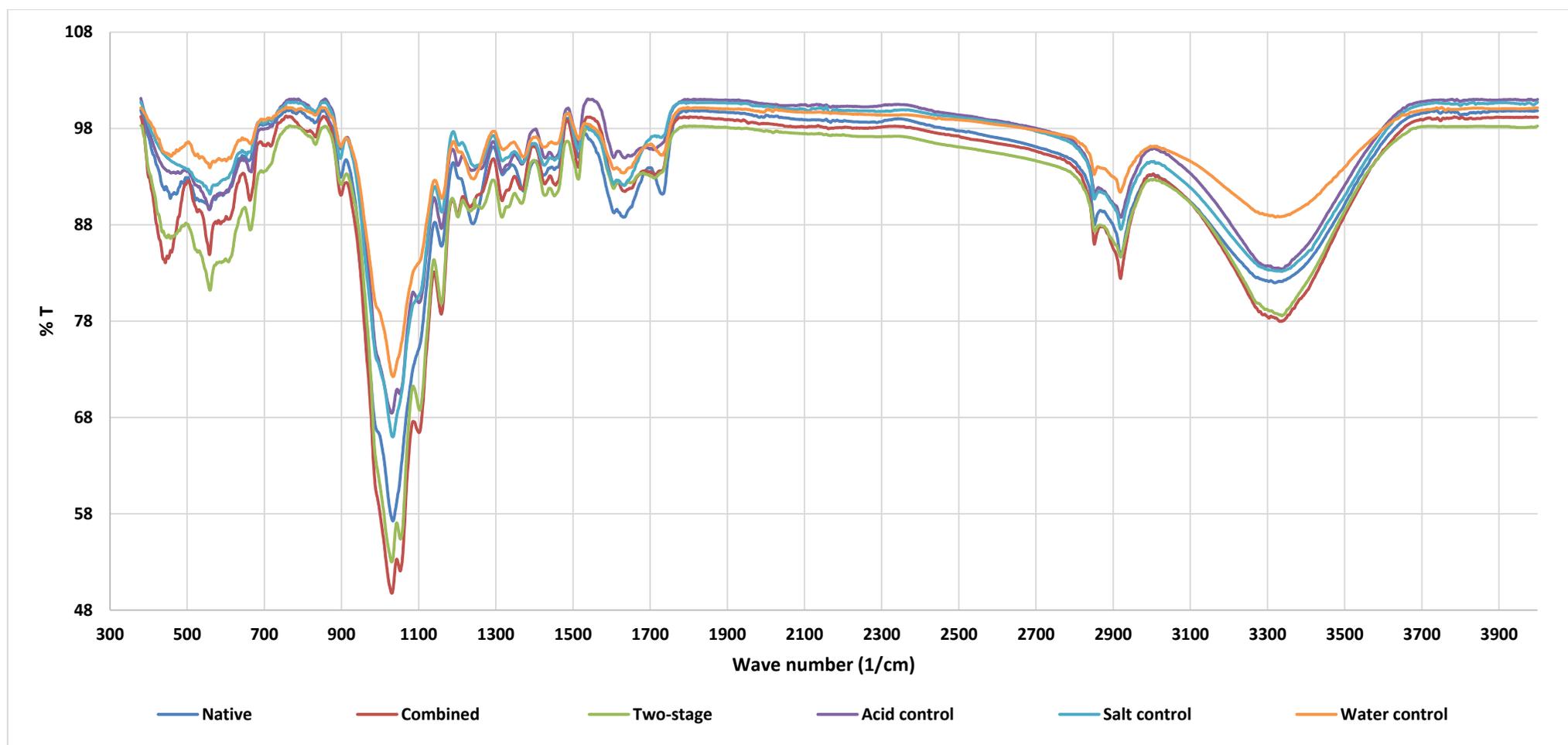


Fig S2. FTIR spectrum of native and pretreated SLW.

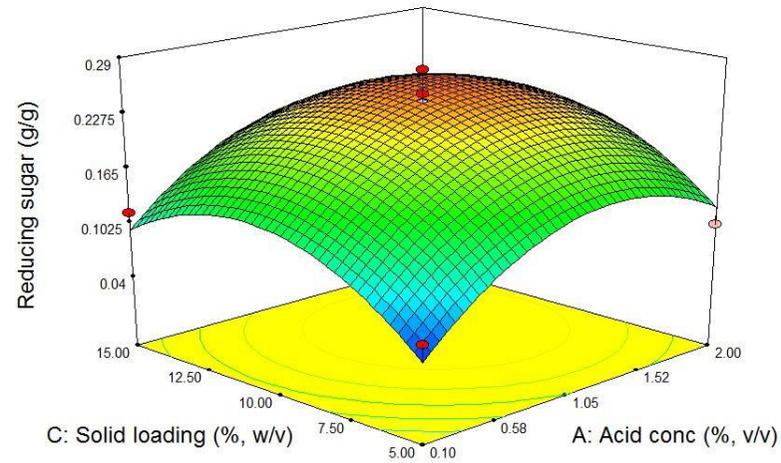


Fig S3. Response surface plot showing the interaction between solid loading and acid concentration for the combined pretreatment.

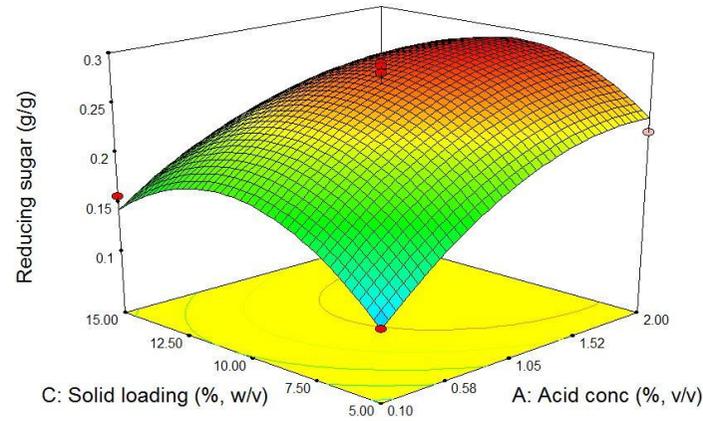


Fig S4. Response surface plot showing the interaction between solid loading and acid concentration for the two-stage pretreatment.

Table S1. Confidence interval for the developed pretreatment regimes

	95% CI Low	95% CI High
Combined pretreatment	0.22	0.27
Two-stage pretreatment	0.25	0.30

CHAPTER 4

Microwave-assisted inorganic salt pretreatment of sugarcane leaf waste: Effect on physiochemical structure and enzymatic saccharification.

This chapter has been published with the title: ‘Microwave-assisted inorganic salt pretreatment of sugarcane leaf waste: Effect on physiochemical structure and enzymatic saccharification.’ in *Bioresource Technology* (2017, 235: 35-42).

Supplementary material associated with this paper can be found at the end of this chapter.

The published paper is presented in the following pages.



Microwave-assisted inorganic salt pretreatment of sugarcane leaf waste: Effect on physiochemical structure and enzymatic saccharification



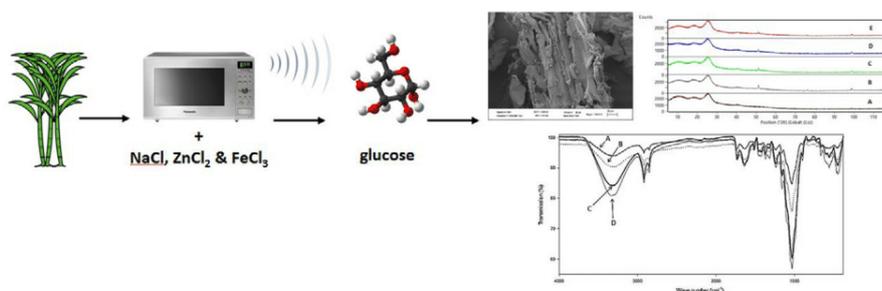
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HIGHLIGHTS

- Report on microwave assisted (MA) inorganic salt pretreatment of biomass.
- Models ($R^2 > 0.80$) were developed and optimized for NaCl, ZnCl₂ and FeCl₃.
- MA-ZnCl₂ and MA-FeCl₃ effectively removed hemicellulose and lignin.
- Maximum reducing sugar yield obtained was 0.406 g/g with the MA-FeCl₃ model.
- This method showed yield improvements on sorghum leaves and Napier grass.

GRAPHICAL ABSTRACT



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ABSTRACT

This paper presents a method to pretreat sugarcane leaf waste using microwave-assisted (MA) inorganic salt to enhance enzymatic saccharification. The effects of process parameters of salt concentration, microwave power intensity and pretreatment time on reducing sugar yield from sugarcane leaf waste were investigated. Pretreatment models based on MA-NaCl, MA-ZnCl₂ and MA-FeCl₃ were developed with high coefficients of determination ($R^2 > 0.8$) and optimized. Maximum reducing sugar yield of 0.406 g/g was obtained with 2 M FeCl₃ at 700 W for 3.5 min. Scanning electron microscopy (SEM), Fourier Transform Infrared analysis (FTIR) and X-ray diffraction (XRD) showed major changes in lignocellulosic structure after MA-FeCl₃ pretreatment with 71.5% hemicellulose solubilization. This regime was further assessed on sorghum leaves and Napier grass under optimal MA-FeCl₃ conditions. A 2-fold and 3.1-fold increase in sugar yield respectively were observed compared to previous reports. This pretreatment was highly effective for enhancing enzymatic saccharification of lignocellulosic biomass.

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1. Introduction

Concerns over energy consumption and environmental pollution has necessitated the production of alternative fuels from lignocellulosic biomass (Ramadoss and Muthukumar, 2015). Lignocellulosic biomass is a heterogeneous polymer consisting mainly of lignin, hemicellulose and cellulose (Kamireddy et al., 2013). Sugarcane is an important agricultural crop grown globally

with an annual yield of 328 Tg (Sindhu et al., 2016a). Sugarcane leaf waste (SLW) constitutes 40% of the total plant and is considered an under-utilized resource since it has no specific use after harvesting (Smithers, 2014). Post-harvest, the leaves are usually dumped in landfill sites or burnt, creating serious environmental concerns (Smithers, 2014). Native SLW is composed of 44% cellulose and 28% hemicellulose, suggesting its potential for biofuel production (Moodley and Kana, 2015).

The conversion of cellulose to glucose poses technical challenges since the physical and chemical association between lignin and the cell wall polymers and the crystallinity of the cellulose

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matrix results in ineffective enzymatic hydrolysis (Diaz et al., 2015). As a result, a pretreatment is a critical step needed to disrupt the complex lignocellulosic structure thereby allowing accessibility of the enzyme to cellulose.

Various pretreatment technologies exist and include the use of acids, alkali, hot water, ionic liquids or salts with each method having merits and demerits (Kamireddy et al., 2013). An effective pretreatment should produce a high sugar yield with low toxic by-products using mild conditions (Lai and Idris, 2016). Inorganic salts have shown higher catalytic activity compared to acids (Yu et al., 2011). Salt pretreatment impacts on the chemical composition of the plant cell wall including the C—O—C and C—H bonds in cellulose (Wei et al., 2011). Metal chloride salts can also be recovered as metal hydroxides and converted back to metal chlorides when treated with conjugate acids (Kamireddy et al., 2013).

Microwave irradiation has been shown to enhance various chemical changes in the lignocellulosic matrix (Zhu et al., 2016). Microwave pretreatment employs an electromagnetic field to the molecular structure of the heated material resulting in physical and chemical reactions (Diaz et al., 2015). Microwave irradiation creates thermal 'hot spots', accelerates the collision of ions with neighbouring molecules and it causes rapid rotation of dipole molecules thereby increasing the temperature. Thus the heating efficiency achieved with microwave is higher than conventional methods (Zhu et al., 2016). For instance, Hu and Wen (2008) reported a 58% increase in sugar yield using microwave to pretreat switchgrass compared to conventional heating. Similarly, Lu et al. (2011) reported a 56% enhancement in glucose yield from rape straw using microwave irradiation. However, there is a knowledge gap on the use of microwave-assisted inorganic salt pretreatment (MAIS) for enhancing enzymatic saccharification of lignocellulosic biomass. Furthermore, metal chloride salts have unique characteristic activities when exposed to an electromagnetic field, owing to their Lewis acid nature. Therefore, mono-, di-, and trivalent salts display different activities, based on their valence in an aqueous solution. There is a dearth of knowledge on microwave-assisted metal chloride salt pretreatment of lignocellulosic biomass.

Therefore, the aim of this study is to determine the effect of three different MAIS pretreatments (NaCl, ZnCl₂ and FeCl₃) for enhanced enzymatic saccharification of SLW. The pretreatment effect on physicochemical structure was further examined using scanning electron microscopy (SEM), Fourier Transform Infrared analysis (FTIR) and X-ray diffraction (XRD). In addition, each pretreatment was modelled using response surface methods to obtain optimal conditions for maximum sugar yield.

2. Methods

2.1. Materials

Sugarcane leaf waste (SLW) was collected from a sugarcane plantation located on the North Coast of South Africa (29° 42' 18" S, 31° 02' 44" E). The leaves were dried at 60 °C for 72 h and milled to a particle size of ≤1 mm and then stored in air tight bags at room temperature until further use. Untreated and pretreated leaf composition was determined by the NREL method (Sluiter et al., 2008). All chemicals were purchased from Sigma-Aldrich, Germany. The commercial cellulase enzyme Cellic CTec 2 was generously provided by Novozymes (Novozymes A/S, Denmark).

2.2. Microwave pretreatment with inorganic salts

Microwave-assisted inorganic salt (MAIS) pretreatment was carried out using a domestic microwave oven (Samsung, ME9114S1). Three inorganic metal chloride salts (NaCl, ZnCl₂ and

FeCl₃) were assessed. The Response Surface Method (Box-Behnken design) was used to examine the singular and interactive effect of process parameters affecting MAIS pretreatment of SLW. These parameters included salt concentration (0, 1, 2 M), power intensity (0, 400, 800 W) and pretreatment time (2, 5, 8 min). Experimental runs which included 0 W were kept at room temperature for the duration stated in the design, without any heating. Five grams of SLW was immersed in 50 ml inorganic salt solution and heated according to the experimental design (Table 1). The solid residue was filtered, washed three times with deionized water and dried prior to enzymatic hydrolysis.

2.3. Enzymatic hydrolysis

Sugarcane leaf waste samples were mixed with 10 ml sodium citrate buffer (pH 4.8, 0.05 M) containing 0.1 ml NaN₃ (2%, w/v) in 50 ml Erlenmeyer flasks with a solid and enzyme loading of 10% (w/v) and 10 FPU/g respectively. The flasks were incubated at 50 °C and 120 rpm for 72 h in a shaking water-bath. Reducing sugar yields were determined by the 3,5-dinitrosalicylic acid method using glucose as the standard (Miller, 1959).

2.4. Analytical methods

Topological characteristics of native and pretreated SLW was analysed using a ZEISS EVO LS 15 scanning electron microscope. Prior to imaging, samples were gold sputter coated (Eiko IB-3 Ion Coater) and images were taken at a magnification of 1000×.

The functional group change of native and pretreated SLW was analysed by Fourier Transform Infrared spectroscopy (FTIR) using a Perkin Elmer 100 (Waltham, MA, USA). Samples were ground with spectroscopic grade KBr and pressed to produce diameter pellets. FTIR spectra were recorded between 380 and 4000 cm⁻¹.

The crystallinity of native and pretreated SLW was examined by XRD analysis using a PANalytical Empyrean diffractometer with a PIXcel detector (Netherlands). Samples were scanned at a range of 1° min⁻¹ from 2θ = 0–120° with a step size of 0.2° using a Co-Kα radiation. Biomass crystallinity (crystallinity index, CrI) was calculated based on the following equation by Segal et al. (1959):

$$\text{CrI}(\%) = [(I_{002} - I_{\text{am}})/I_{002}] \times 100$$

where I_{002} is the intensity of the diffraction from the plane at $2\theta \approx 25.0$ for cellulose and I_{am} is the peak for the amorphous region at about $2\theta \approx 15.1$.

The hydrolysate obtained after MAIS pretreatment was assessed for inhibitors such as acetic acid, furfural and 5-hydroxymethyl furfural. Samples were analysed using coupled Varian 3800 gas chromatography (Varian Palo Alto, California, USA) and Varian 1200 mass spectrometry (GC-MS) following the protocol outlined by Rorke et al. (2016).

3. Results and discussion

3.1. Model development

Experimental conditions and the corresponding reducing sugar yields from each of the examined salts are shown in Table 1. The experimental data were used to generate three polynomial models for MA-NaCl, MA-ZnCl₂ and MA-FeCl₃ pretreatment with the following equations relating the yield of reducing sugar to the input parameters:

$$\begin{aligned} \text{MA-NaCl} = & +0.17 + 0.017A + 2.00 \times 10^{-3}B + 4.63 \times 10^{-3}C \\ & + 2.50 \times 10^{-4}AB - 5.00 \times 10^{-3}AC - 2.50 \times 10^{-4}BC \\ & - 0.012A^2 - 1.25 \times 10^{-3}B^2 + 3.50 \times 10^{-3}C^2 \end{aligned}$$

Table 1
Box-Behnken design of various input parameters affecting the reducing sugar yield from microwave-assisted inorganic salt pretreatment of SLW.

Run Order	Salt conc. (M)	Power (Watts)	Pretreatment time (min)	Reducing sugar yield (g/g ^a)		
				NaCl	ZnCl ₂	FeCl ₃
1	1.0	800	2.0	0.172	0.157	0.204
2	1.0	400	5.0	0.171	0.164	0.237
3	0.0	800	5.0	0.153	0.147	0.150
4	0.0	400	2.0	0.127	0.124	0.140
5	2.0	0	5.0	0.163	0.160	0.153
6	1.0	400	5.0	0.171	0.165	0.240
7	1.0	800	8.0	0.170	0.171	0.017
8	1.0	400	5.0	0.173	0.163	0.236
9	1.0	0	8.0	0.177	0.148	0.160
10	0.0	0	5.0	0.139	0.150	0.157
11	1.0	400	5.0	0.175	0.143	0.238
12	2.0	400	8.0	0.189	0.224	0.430
13	2.0	800	5.0	0.178	0.232	0.401
14	2.0	400	2.0	0.179	0.199	0.354
15	1.0	0	2.0	0.178	0.167	0.124
16	1.0	400	5.0	0.170	0.166	0.243
17	0.0	400	8.0	0.157	0.160	0.163

^a g reducing sugar/g dry weight SLW.

$$\text{MA-ZnCl}_2 = +0.16 + 0.029A + 0.010B + 7.00 \times 10^{-3}C + 0.019AB - 2.75 \times 10^{-3}AC + 8.25 \times 10^{-3}BC + 0.014A^2 - 1.98 \times 10^{-3}B^2 + 2.53 \times 10^{-3}C^2$$

$$\text{MA-FeCl}_3 = +0.24 + 0.091A + 0.022B - 6.5 \times 10^{-3}C + 0.064AB + 0.013AC - 0.056BC + 0.061A^2 - 0.085B^2 - 0.028C^2$$

where A, B and C represent the salt concentration, power intensity and pretreatment time respectively. In the above equations, positive regression coefficients suggest a synergistic effect while negative coefficients indicate an antagonistic effect (Qing et al., 2016).

The adequacy of the developed models were examined by analysis of variance (ANOVA) and the results are presented in Table 2. The model F-values of 4.03, 5.34 and 4.36 for MA-NaCl, MA-ZnCl₂ and MA-FeCl₃ respectively suggests that these models were significant. In addition, the significance of each model was also evaluated based on the 'p-value', since a value <0.05 denotes significance (Qing et al., 2016). The MA-NaCl, MA-ZnCl₂ and MA-FeCl₃ models showed 'p-values' of 0.0397, 0.0190 and 0.0392 respectively. The developed models gave coefficients of determination (R²) of 0.84, 0.87 and 0.85 for MA-NaCl, MA-ZnCl₂ and MA-FeCl₃ respectively. These values suggest that 84, 87 and 85% of the variation in observed data respectively can be explained by the MA-NaCl, MA-ZnCl₂ and MA-FeCl₃ model respectively.

The significance of the input parameters on the reducing sugar yield was assessed using the 'p-value' and results are shown in Tables S1–S3. It was determined that salt concentration was the most significant parameter among all models (0.0016, 0.0009 and 0.0040 for MA-NaCl, MA-ZnCl₂ and MA-FeCl₃) whereas the least significant parameter was power intensity for MA-NaCl (0.5670) and pretreatment time for the MA-ZnCl₂ (0.2255) and MA-FeCl₃ (0.7727) models.

3.2. Model validation

Based on the developed models, an optimization was performed to maximize the reducing sugar yield, as shown in Table 3. According to the model's prediction, under optimal conditions, a reducing sugar yield of 0.184, 0.235 and 0.419 g/g should be obtained for the MA-NaCl, MA-ZnCl₂ and MA-FeCl₃ models respectively.

Experimental validation of these optima gave reducing sugar yields of 0.179, 0.241 and 0.406 g/g for the MA-NaCl, MA-ZnCl₂ and MA-FeCl₃ pretreatment regimes respectively thus showing a negligible difference between the predicted and observed values.

3.3. Effect of MA-NaCl pretreatment on SLW digestibility

The reducing sugar yield from the different experimental runs are shown in Table 1. Maximum reducing sugar yield (0.189 g/g) was obtained using pretreatment conditions of 2 M, 400 W and 8.0 min for NaCl concentration, power intensity and pretreatment time respectively. The lowest yield (0.129 g/g) was obtained with milder pretreatment conditions of 0 M, 400 W and 2.0 min for NaCl concentration, power intensity and pretreatment time respectively thus the presence of NaCl positively affected the reducing sugar yield. The combined effect of NaCl concentration and power intensity on the reducing sugar yield when pretreatment time is maintained at its centre point is shown in Fig 1A. Low reducing sugar yields (<0.139 g/g) were obtained with pretreatment at low concentrations of NaCl (<0.5 M) and microwave intensity in the range 0 to 800 W. An increase in NaCl concentration from 0.5 M to 2 M, increased the reducing sugar yield from 0.139 g/g to 0.179 g/g. These observations further suggest that power intensity has a marginal effect on the reducing sugar yield. Banerjee et al. (2016) reported a 72% saccharification efficiency with mustard stalk and straw using 2 M NaCl and autoclave heating. These authors also reported that further increases in NaCl concentration beyond

Table 2
Analysis of Variance (ANOVA) of developed polynomial models for MA-NaCl, MA-ZnCl₂ and MA-FeCl₃ based pretreatments.

Model	Sum of squares	df	Mean squares	F-value	P-value	R ²
MA-NaCl	3.22 × 10 ⁻³	9	3.58 × 10 ⁻⁴	4.03	0.0397	0.84
MA-ZnCl ₂	1.10 × 10 ⁻²	9	1.19 × 10 ⁻³	5.34	0.0190	0.87
MA-FeCl ₃	1.50 × 10 ⁻¹	9	1.60 × 10 ⁻²	4.36	0.0325	0.85

df: degrees of freedom, F-value: Fisher-Snedecor distribution value, P-value: probability value, R²: coefficient of determination.

Table 3
Validation of optimized conditions for MA-salt pretreatment of SLW.

Model	Input parameters			Predicted RS (g/g)	Observed RS (g/g)
	Salt conc. (M)	Power (Watts)	Pretreatment time (min)		
MA-NaCl	1.5	700	8.0	0.184	0.179
MA-ZnCl ₂	2.0	800	6.0	0.235	0.241
MA-FeCl ₃	2.0	700	3.5	0.419	0.406

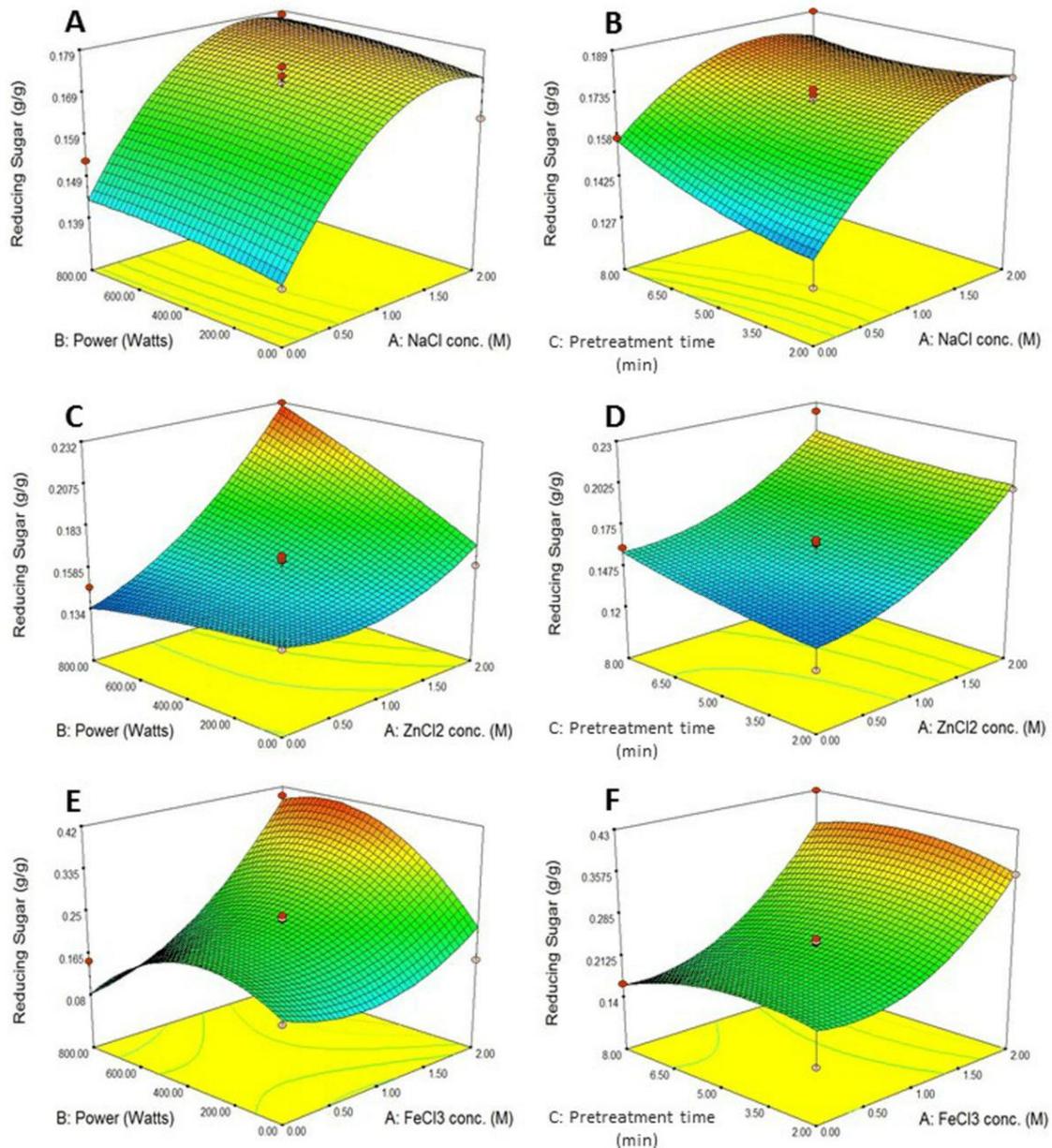


Fig. 1. Response surface plots showing the interactive effect of the various input parameters affecting MAIS pretreatment of sugarcane leaf waste (A) interaction between NaCl concentration and power intensity (B) NaCl concentration and pretreatment time (C) ZnCl₂ concentration and power intensity (D) ZnCl₂ concentration and pretreatment time (E) FeCl₃ concentration and power intensity and (F) FeCl₃ concentration and pretreatment time.

1 M, did not increase lignin removal thus suggesting a further increase in hemicellulose removal. The interactive effect of pretreatment time and NaCl concentration on the reducing sugar yield when power intensity is kept at its median value is illustrated in Fig 1B. Low pretreatment time (2.0–3.5 min) and NaCl concentration (0–0.5 M) resulted in low reducing sugar yields (0.10 g/g). A

simultaneous increase in pretreatment time and NaCl concentration from 2.0 to 8.0 min and 0 to 1.75 M respectively resulted in an increase in reducing sugar yield from 0.10 to 0.185 g/g. Similar results were reported by Lin et al. (2015) where an increase in reducing sugar from 0.013 to 0.028 g/g TVS was observed when microwave pretreatment time was increased from 5.0 to

10.0 min with alkali pretreatment. Longer microwave pretreatment durations (> 10 min) result in the degradation of monomeric sugars (Lin et al., 2015).

3.4. Effect of MA-ZnCl₂ pretreatment on SLW digestibility

The reducing sugar yield with MA-ZnCl₂ pretreatment ranged from 0.124 to 0.232 g/g (Table 1). The lowest reducing sugar yield (0.124 g/g) was observed with 0 M ZnCl₂, 400 W power intensity and 2.0 min pretreatment time whereas the highest yield (0.232 g/g) was obtained using 2 M ZnCl₂, 800 W power intensity and 5.0 min pretreatment time. This suggested that the presence of ZnCl₂ enhanced the enzymatic saccharification of SLW. The interactive effect of ZnCl₂ concentration and power intensity on the reducing sugar yield when pretreatment time is maintained at its centre point is shown in Fig 1C. At a low concentration of ZnCl₂ (0–1 M) and high power intensity (600–800 W), the reducing sugar yield was 0.135 g/g. A simultaneous increase in ZnCl₂ concentration from 0 to 2 M and power intensity from 0 W to 800 W resulted in an increase in reducing sugar from 0.135 to 0.232 g/g. A similar trend was reported by Kang et al. (2013) where an increase in ZnCl₂ from 0.5 to 5.0% with heating at 185 °C for 15 min resulted in a 20% increase in enzymatic digestibility. The effect of ZnCl₂ concentration and pretreatment time on reducing sugar yield when power intensity is retained at its median value is shown in Fig 1D. Low reducing sugars yields (0.12 g/g) were obtained with low concentrations of ZnCl₂ (<0.5 M) and low pretreatment time (2.0 min). However, a higher reducing sugar yield was obtained (0.20 g/g) using high concentrations of ZnCl₂ (2.0 M) and high pretreatment time (8.0 min). Kim et al. (2014) reported a similar result with maximum sugar recovery from barley straw using concentrated ZnCl₂ (10%) at 170 °C for 5 min. ZnCl₂ has been shown to be an effective swelling chemical reagent thus facilitating biomass fractionation and enhancing enzymatic digestibility (Alfred, 1964).

3.5. Effect of MA-FeCl₃ pretreatment on SLW digestibility

The reducing sugar yield from the experimental runs ranged from 0.017 to 0.43 g/g (Table 1). The significant difference in the yields show the sensitivity of the reducing sugar yield to the considered input parameters. An extremely low yield of reducing sugar (0.017 g/g) was obtained with 1 M FeCl₃, 800 W power intensity and 8 min pretreatment time. High power intensity and pretreatment time accelerates water evaporation consequently limiting the pretreatment effect (Jin et al., 2016). Maximum reducing sugar (0.43 g/g) was observed with 2 M FeCl₃, 400 W power intensity and 8 min pretreatment time. This suggested that reducing sugar yield is higher with a higher concentration of FeCl₃, high pretreatment time and medium microwave power intensity. The combined effect of FeCl₃ concentration and power intensity on the reducing sugar yield when pretreatment time is maintained at its centre point is shown in Fig 1E. A low reducing sugar yield (0.08 g/g) was observed at a low concentration of FeCl₃ (0 M) and high power intensity (800 W) whereas a high reducing sugar yield (0.42 g/g) was obtained with high power intensity (800 W) and salt concentration (2 M). A simultaneous increase in FeCl₃ concentration and power intensity from 0 to 2 M and 0 to 800 W respectively resulted in an increase in reducing sugar from 0.10 to 0.42 g/g. Kang et al. (2013) reported a 25.7% increase in enzymatic digestibility of *Miscanthus* when FeCl₃ concentration was increased from 0.2 to 1.0% using an oil bath at 185 °C. The interactive effect of FeCl₃ concentration and pretreatment time on reducing sugar yield when power intensity is kept at its median point is illustrated in Fig 1F. At low levels of pretreatment time (2.0 min) and FeCl₃ concentration (0 M), the reducing sugar yield was low

(0.14 g/g) whereas at low levels of pretreatment time (2.0 min) and high levels of FeCl₃ concentration (2 M), the reducing sugar yield was high (0.36 g/g). Similarly, high pretreatment time (8.0 min) and low FeCl₃ concentration (0 M) resulted in low reducing sugar (0.16 g/g) however, high pretreatment time (8.0 min) and high FeCl₃ concentration (2 M) gave high reducing sugar (0.37 g/g). This indicated that pretreatment time had little effect on the reducing sugar yield whereas FeCl₃ concentration had a significant effect. A similar trend was observed by Liu et al. (2009), where an increase in pretreatment time from 5 to 15 min with FeCl₃ at 160 °C, resulted in a negligible difference in glucose recovery from corn stover. Lu and Zhou (2011) reported a lower reducing sugar concentration of 6.62 g/L using 0.14 M FeCl₃ at 800 W for 12 min.

3.6. Comparison of effects of various MAIS pretreatments

Overall, results indicated that MA-NaCl was the least effective pretreatment whereas MA-FeCl₃ was the most effective. The highest reducing sugar yields obtained for MA-NaCl, MA-ZnCl₂ and MA-FeCl₃ pretreatments were 0.189, 0.232 and 0.43 g/g (Table 1) respectively thus illustrating the sensitivity of reducing sugar yield to different inorganic salts. The reducing sugar yield increased 0.3- and 2.2-fold for ZnCl₂ and FeCl₃ respectively compared to the NaCl pretreatment. These results are comparable to those reported by Kang et al. (2013) where FeCl₃ the most effective salt and NaCl was the least effective. The presence of lignin hinders enzymatic hydrolysis since the enzyme is adsorbed into the lignin, forming an enzyme-lignin complex thereby rendering the enzyme inactive. Metal chloride salts such as ZnCl₂ and FeCl₃ form metal cations that eliminate the formation of enzyme-lignin complexes by forming lignin-metal complexes thus enhancing enzymatic hydrolysis (Kamireddy et al., 2013). The high reducing sugar observed with MA-FeCl₃ could be attributed to its ability to form a strong Fe³⁺ cation. Since NaCl is a monovalent alkali metal chloride, its ability to form lignin-metal complexes is weak. In addition, metal chlorides act as Lewis acids in aqueous solution and dissociate into complex ions. These ions diffuse into the wet lignocellulosic matrix where they protonate an oxygen molecule of the ether bond between sugar monomers, cleaving the bond leading to the degradation of hemicellulose (Mamman et al., 2008). The effectiveness of inorganic salts increases with the valence of the metal cation i.e. Fe³⁺ > Zn²⁺ > Na⁺ (Kang et al., 2013; Liu et al., 2009). The optimized MA-FeCl₃ model required the lowest pretreatment time (3.5 min; Table 3) and yielded the highest reducing sugar (0.406 g/g) compared to the optimized MA-NaCl and MA-ZnCl₂ pretreatment models which required 8.0 and 6.0 min to yield 0.179 and 0.241 g/g respectively. This is because FeCl₃ forms the strongest cation compared to ZnCl₂ and NaCl, therefore increased acceleration between molecules occur thus inducing significant internal resistance between molecules causing an increase in temperature (Anwar et al., 2015). Overall, these results showed that optimum pretreatment conditions differ with the salt type used.

3.7. Characterization of native and pretreated SLW

Native SLW consisted of 9.39% lignin, 44.78% cellulose and 27.38% hemicellulose. Hemicellulose solubilization was observed to be 8.9, 48.6 and 71.5% for MA-NaCl, MA-ZnCl₂ and MA-FeCl₃ pretreatment respectively thus indicating MA-FeCl₃ to be the most effective at degrading hemicellulose. An identical observation was reported by Liu et al. (2009) where hemicellulose degradation in corn stover increased 11-fold when pretreated with FeCl₃ at 180 °C compared to the native sample. An increase in lignin and cellulose was observed after pretreatment. Similar increases were reported by Kim et al. (2016) and Kang et al. (2013) after chemical pretreatment. A 16.6% increase in cellulose was recorded by Sindhu

et al. (2011) after the acidic pretreatment of sugarcane tops. The increase in lignin and cellulose after pretreatment is a phenomenon linked to the high solubilization of the hemicellulose fraction. Inorganic salts play a key role in the breakage of ether bonds between xylan polymers thus resulting in substantial hemicellulose solubilization (Kamireddy et al., 2013).

3.8. Scanning electron microscopy

Topographical changes in SLW that were induced by MAIS pretreatment were analysed by scanning electron microscopy. The native sample showed a contiguous and smooth surface since the fibres were all intact. However, after pretreatment, loosening of fibres and cellular distortion was observed, suggesting the occurrence of structural changes. More pronounced structural changes were observed in the MA-ZnCl₂ and MA-FeCl₃ pretreated sample compared to the MA-NaCl sample. The surface area of the MA-FeCl₃ and MA-ZnCl₂ pretreated samples showed cavitation and the formation of cracks. This result is consistent with those reported in microwave-alkali pretreatment by Lai and Idris (2016). The fragile surface area is attributed to the weakening of the cell wall during delignification (Ramadoss and Muthukumar, 2015). The disorganized fractionated surface appearance observed with the MA-FeCl₃ sample suggest it was most effective in altering the lignocellulosic structure, which is in line with the high hemicellulose solubilization and high reducing sugar yield obtained. Thus it is well evidenced that MAIS pretreatment caused structural damage which allowed for enhanced enzymatic saccharification. The disruption to the SLW surface structure is corroborated by the modifications in structural components shown by the FTIR analysis.

3.9. FTIR spectra analysis

The spectral data showed the presence of lignin, hemicellulose and cellulose which are common moieties among lignocellulosic biomass. There were significant differences in the banding patterns from the pretreated biomass compared to the native biomass, indicating structural modifications after microwave-assisted inorganic salt pretreatment. Strong absorbance bands at 1730 cm⁻¹ and 1515 cm⁻¹ corresponding to the stretching of the carbonyl group and the vibration of the aromatic ring in lignin showed reductions in intensity indicating the disruption of lignin. These bands were notably enhanced after MA-FeCl₃ pretreatment compared to MA-NaCl and MA-ZnCl₂, thereby providing further evidence that FeCl₃ was the most effective pretreatment. Similar attenuation bands were reported by Lin et al. (2015) using microwave-alkali to pretreatment hyacinth. In addition, typical lignin band at 1240 cm⁻¹ (Diaz et al., 2015), corresponding to the guaiacyl unit were observed. The presence of guaiacyl in lignin inhibits fibre swelling consequently hampering enzymatic accessibility (Lai and Idris, 2016). Therefore, the disappearance of this band revealed that the lignin structure had been modified. Similar banding trends were observed by Lai and Idris (2016) and Diaz et al. (2015). MA-FeCl₃ was shown to be the most effective delignifying agent followed by ZnCl₂ and then NaCl. The peaks at 1126 cm⁻¹ and 1712 cm⁻¹ represent the C—O—C stretching vibration in hemicellulose and cellulose and the C=O stretching vibration of the acetyl group in hemicellulose respectively. A reduction in these signals imply the breakdown of hemicellulose either by the release of sugars or the deacetylation of hemicellulose (Diaz et al., 2015; Zhu et al., 2016). These bands were strongest for the MA-NaCl sample whereas the weakest band was observed for MA-FeCl₃. This was an indication that MA-FeCl₃ was more effective in removing hemicellulose than MA-ZnCl₂ and MA-NaCl which is in agreement with the high hemicellulose solubilization reported earlier. Banerjee

et al. (2016) observed similar banding signals in the salt pretreatment of mustard stalk and straw. The pronounced peaks at 1030 cm⁻¹ correspond to the C—O—C vibration of the pyranose ring in cellulose and becomes more prominent after MA-FeCl₃ pretreatment compared to MA-NaCl and MA-ZnCl₂. The enhancement of bands between 1000 and 1100 cm⁻¹ for the pretreated samples indicates an increase in cellulose recovery (Binod et al., 2012). An identical observation was also reported by Ramadoss and Muthukumar (2015). These data indicate that microwave-assisted inorganic salt pretreatment effectively removes hemicellulose and lignin thus enhancing enzymatic saccharification.

3.10. Biomass crystallinity (XRD diffraction)

Major diffraction peaks of the cellulose crystallographic plane were identified at $2\theta = 25^\circ$, signifying a highly organized crystalline region whereas the peaks at $2\theta = 15^\circ$ indicated the less organised amorphous region (Lin et al., 2015). Crystalline cellulose is highly recalcitrant due to the intermolecular hydrogen bonding between the chains, thus inhibiting degradation (Sun et al., 2010). The crystallinity index (CrI) expresses the ratio of crystalline cellulose to the amorphous region, often lignin and hemicellulose. Since biomass crystallinity significantly affects the enzymatic digestibility of cellulose, CrI is a suitable indicator to estimate the effect of chemical pretreatment on biomass crystallinity. A high CrI indicates a low crystalline structure and a high crystalline structure is represented by a low CrI (Jin et al., 2016; Lai and Idris, 2016). The CrI and crystalline size of native and pretreated SLW are shown in Table 4. The highest CrI (31.26%) was observed for the MA-FeCl₃ pretreated sample while the lowest CrI (24.23%) was recorded for the MA-NaCl sample. The CrI for MA-NaCl, MA-ZnCl₂ and MA-FeCl₃ pretreated SLW increased by 5.25, 6.59 and 10.81% respectively compared to the native sample. The increase in CrI is attributed to the removal of lignin and hemicellulose in the amorphous region (Sindhu et al., 2016b). An identical observation was earlier reported by Jin et al. (2016) in which microwave-assisted alkali pretreated catalpa sawdust showed a higher CrI (39.4%) than the native biomass (32.3%). Li et al. (2015) also reported increases in the CrI after ultrasonic-assisted HCl-FeCl₃ pretreatment of cellulose. The crystalline size of the native biomass was found to be higher (3.1 nm) compared to the samples pretreated with MA-NaCl (2.9 nm), MA-ZnCl₂ (2.9 nm) and MA-FeCl₃ (2.8 nm) which further indicated a decrease in crystallinity after pretreatment. Similar results were earlier reported by Sindhu et al. (2016b).

Table 4
Crystallinity index and crystalline size of native and pretreated SLW.

Sample	Crystallinity index (%)	Crystalline size (nm)
Native	20.45	3.1
NaCl pretreated	25.70	2.9
ZnCl ₂ pretreated	27.04	2.9
FeCl ₃ pretreated	31.26	2.8

Table 5
Inhibitor concentrations from native and MAIS pretreated SLW.

Sample	Inhibitor concentration (µg/g)		
	Acetic acid	Furfural	HMF
Water	4.9×10^{-3}	8.2×10^{-4}	4.6×10^{-4}
MA-NaCl	4.5×10^{-3}	1.1×10^{-4}	3.2×10^{-4}
MA-ZnCl ₂	7.8×10^{-3}	2.3×10^{-2}	2.1×10^{-3}
MA-FeCl ₃	9.3×10^{-1}	2.5×10^{-1}	ND

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

Table 6

Validation of the optimized pretreatment regime on different lignocellulosic residues.

Substrate	Pretreatment conditions	Yield (g/g)	Improvement	Refs.
Sugarcane leaves	2.0 M FeCl ₃ , 700 W, 3.5 min	0.406 ^α	1.9-fold	This study
Sugarcane leaves	1.5% (w/v) H ₂ SO ₄ , 121 °C 60 min	0.208 ^β	–	Jutakanoke et al. (2012)
Sorghum leaves	2.0 M FeCl ₃ , 700 W, 3.5 min	0.462 ^α	2.0-fold	This study
Sorghum fibre	1% NaOH, 121 °C, 30 min + 1% H ₂ SO ₄ , 121 °C, 30 min	0.23 ^β	–	Li et al. (2016)
Napier grass	2.0 M FeCl ₃ , 700 W, 3.5 min	0.443 ^α	3.1-fold	This study
Napier grass	1% (v/v) H ₂ O ₂ , 37 °C, 24 h	0.14 ^β	–	Phitsuwan et al. (2016)

^α Reducing sugar yield per gram dry weight.^β Glucose yield per gram dry weight.

MA-FeCl₃ was the most effective salt while MA-NaCl was the least effective salt.

3.11. Inhibitor profile analysis

The inhibitor profile of the hydrolysate is presented in Table 5. These include compounds such as acetic acid, furfural and 5-hydroxymethyl furfural (HMF), which may inhibit microbial growth and metabolism (Sindhu et al., 2016b). The highest concentration of acetic acid was found in the MA-FeCl₃ sample whereas the lowest concentration was found in the MA-NaCl sample. Acetic acid is mainly formed as a result of the breakage between acetyl linkages in plant biomass (Kamireddy et al., 2013). The concentration of furfural was found higher in samples pretreated with MA-FeCl₃ compared to MA-ZnCl₂ and MA-NaCl. This could be due to the trivalent Fe³⁺ cation being more active in the presence of a carboxylic acid such as acetic acid compared to the weaker divalent Zn²⁺ or monovalent Na⁺ cations which have weaker Lewis acid activities (Leshkov and Davis, 2011). Samples pretreated with MA-ZnCl₂ showed a higher release of HMF compared to MA-NaCl whereas no HMF was detected with MA-FeCl₃. HMF is formed due to the dehydration of fructose molecules however, in the presence of a water solvent, HMF can be rehydrated into levulinic acid (Yang et al., 2012). This could explain the relatively low yields of HMF.

3.12. Comparison of reducing sugar yield with different lignocellulosic residues

The optimized MA-FeCl₃ pretreatment was carried out on Napier grass and sorghum leaves and the results are presented in Table 6. A 1.9-fold improvement in sugar yield was observed compared to a previous report on acid pretreated sugarcane leaf waste using conventional heating (Jutakanoke et al., 2012). The developed MA-FeCl₃ pretreatment was also validated on two other lignocellulosic substrates namely Napier grass and sorghum leaves which are two commonly considered lignocellulosic feedstocks. In comparison to SLW, Napier grass and sorghum leaves showed a 9.1 and 13.8% improvement respectively. Furthermore, by comparison to an earlier report on sorghum leaves using a two stage acid and alkali pretreatment (Li et al., 2016), the current study showed a 2-fold increase in the sugar yield using MA-FeCl₃ pretreatment. Similarly, a 3.1-fold increase in sugar yield was obtained compared to a previous report on the H₂O₂ pretreatment of Napier grass (Phitsuwan et al., 2016). Overall, these data suggest that the developed pretreatment model (MA-FeCl₃) can successfully be applied to different lignocellulosic feedstocks with enhanced sugar yields.

4. Conclusion

A novel microwave-assisted inorganic salt pretreatment strategy of SLW was evaluated in this study. Significant models (R² >0.80) were developed and optimized for NaCl, ZnCl₂ and FeCl₃ pretreatments. Maximum reducing sugar yield (0.406 g/g) was

obtained with MA-FeCl₃ under optimal conditions of 2 M FeCl₃ at 700 W for 3.5 min. Major structural changes of pretreated samples were observed after SEM, FTIR and XRD analysis with FeCl₃ showing significant modifications. Pretreatment of sorghum leaves and Napier grass under optimal MA-FeCl₃ conditions showed enhanced sugar yields compared to previous reports.

Acknowledgements

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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.2017.03.031>.

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Supplementary material

Table S1. ANOVA of the MA-NaCl model

Source	Sum of squares	df	Mean square	F Value	p-value Prob > F
Model	3.22×10^{-3}	9	3.58×10^{-4}	4.03	0.0397
A- Salt conc.	2.21×10^{-3}	1	2.21×10^{-3}	24.93	0.0016
B- Power	3.20×10^{-5}	1	3.20×10^{-5}	0.36	0.5670
C- Time	1.71×10^{-4}	1	1.71×10^{-4}	1.93	0.2074
AB	2.50×10^{-7}	1	2.50×10^{-7}	2.82×10^{-3}	0.9591
AC	1.00×10^{-4}	1	1.00×10^{-4}	1.13	0.3235
BC	2.50×10^{-7}	1	2.50×10^{-7}	2.82×10^{-3}	0.9591
A ²	6.58×10^{-4}	1	6.58×10^{-4}	7.42	0.0296
B ²	6.58×10^{-6}	1	6.58×10^{-6}	0.074	0.7932
C ²	5.16×10^{-5}	1	5.16×10^{-5}	0.58	0.4706
Cor total	3.84×10^{-3}	16			

Table S2. ANOVA of the MA-ZnCl₂ model

Source	Sum of squares	df	Mean square	F Value	p-value Prob > F
Model	1.10×10^{-2}	9	1.19×10^{-3}	5.34	0.0190
A- Salt conc.	6.85×10^{-3}	1	6.85×10^{-3}	30.84	0.0009
B- Power	8.41×10^{-4}	1	8.41×10^{-4}	3.79	0.0927
C- Time	3.92×10^{-4}	1	3.92×10^{-4}	1.77	0.2255
AB	1.41×10^{-3}	1	1.41×10^{-3}	6.34	0.0400
AC	3.03×10^{-5}	1	3.03×10^{-5}	0.14	0.7229
BC	2.72×10^{-4}	1	2.72×10^{-4}	1.23	0.3046
A ²	8.28×10^{-4}	1	8.28×10^{-4}	3.73	0.0947
B ²	1.64×10^{-5}	1	1.64×10^{-5}	0.074	0.7934
C ²	2.68×10^{-5}	1	2.68×10^{-5}	0.12	0.7382
Cor total	1.20×10^{-2}	16			

Table S3. ANOVA of the MA-FeCl₃ model

Source	Sum of squares	df	Mean square	F Value	p-value Prob > F
Model	1.50×10^{-1}	9	1.60×10^{-2}	4.36	0.0325
A- Salt conc.	6.60×10^{-2}	1	6.60×10^{-2}	17.67	0.0040
B- Power	3.96×10^{-3}	1	3.96×10^{-3}	1.06	0.3382
C- Time	3.38×10^{-4}	1	3.38×10^{-4}	0.090	0.7727
AB	1.60×10^{-2}	1	1.60×10^{-2}	4.34	0.0758
AC	7.02×10^{-4}	1	7.02×10^{-4}	0.19	0.6782
BC	1.20×10^{-2}	1	1.20×10^{-2}	3.32	0.1114
A ²	1.60×10^{-2}	1	1.60×10^{-2}	4.18	0.0803
B ²	3.00×10^{-2}	1	3.00×10^{-2}	8.02	0.0253
C ²	3.31×10^{-3}	1	3.31×10^{-3}	0.88	0.3789
Cor total	1.70×10^{-1}	16			

Table S4. Composition of native and pretreated SLW.

Sample	Composition (%)		
	Lignin	Cellulose	Hemicellulose
Native	9.39	44.78	27.38
MA-NaCl	15.67	44.67	24.95
MA-ZnCl ₂	16.94	51.33	15.68
MA-FeCl ₃	15.74	61.88	7.81

Table S5. Confidence interval for the developed pretreatment regimes

	95% CI Low	95% CI High
NaCl pretreatment	0.16	0.18
ZnCl₂ pretreatment	0.14	0.18
FeCl₃ pretreatment	0.17	0.30

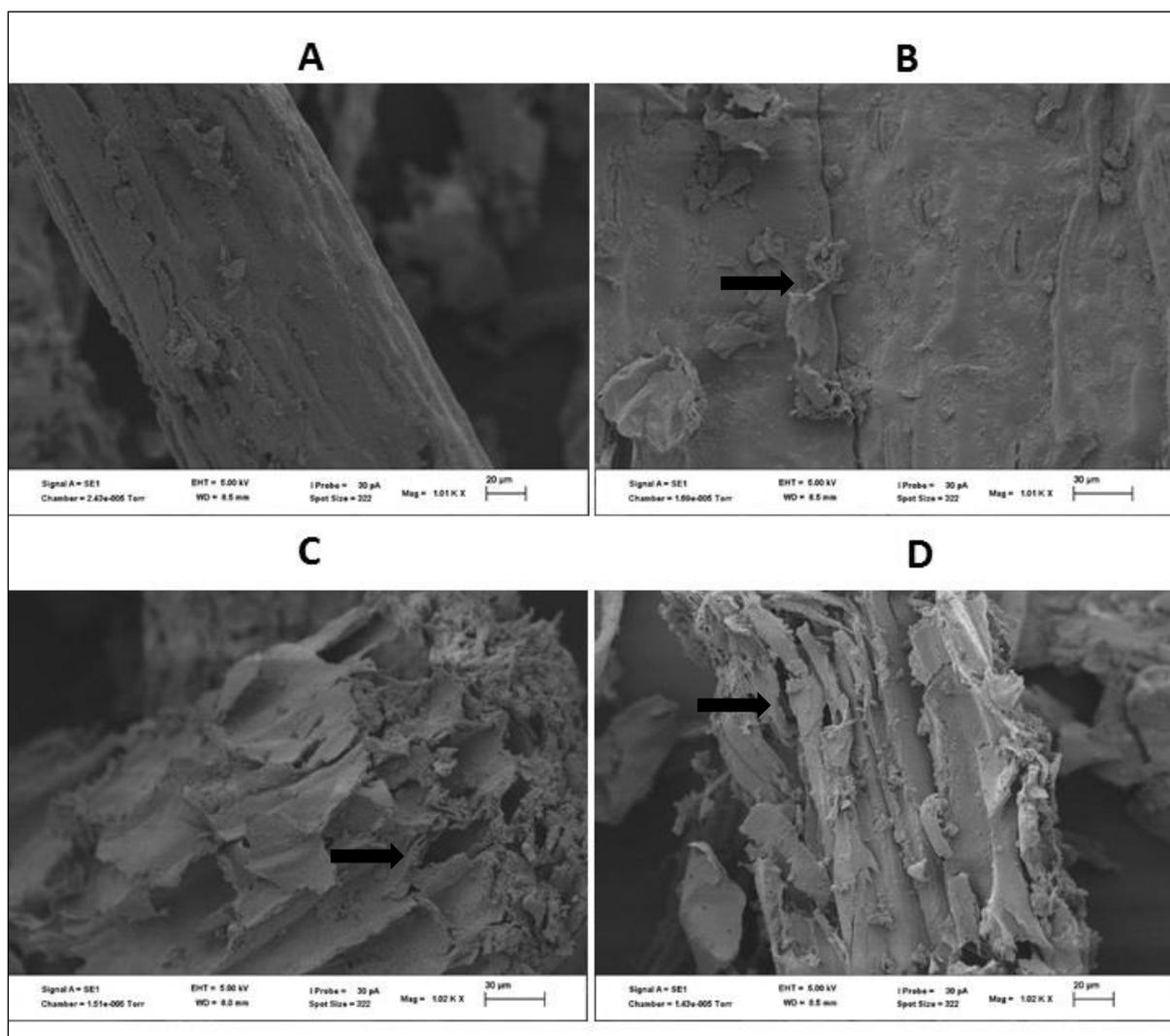


Fig S1. SEM micrographs of (A) native SLW (B) MA-NaCl pretreated SLW (C) MA-ZnCl₂ pretreated SLW and (D) MA-FeCl₃ pretreated SLW

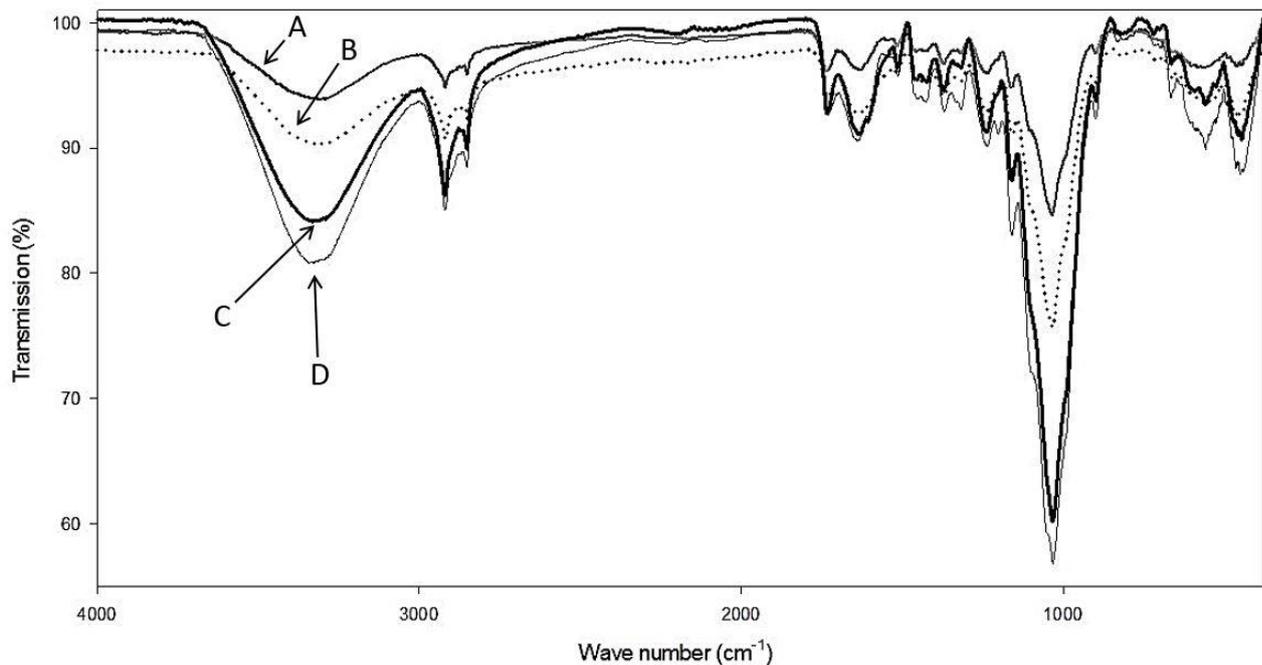


Fig S2. FTIR spectra of SLW samples: native (A); SLW pretreated with NaCl (B); SLW pretreated with ZnCl₂ (C); and SLW pretreated with FeCl₃ (D)

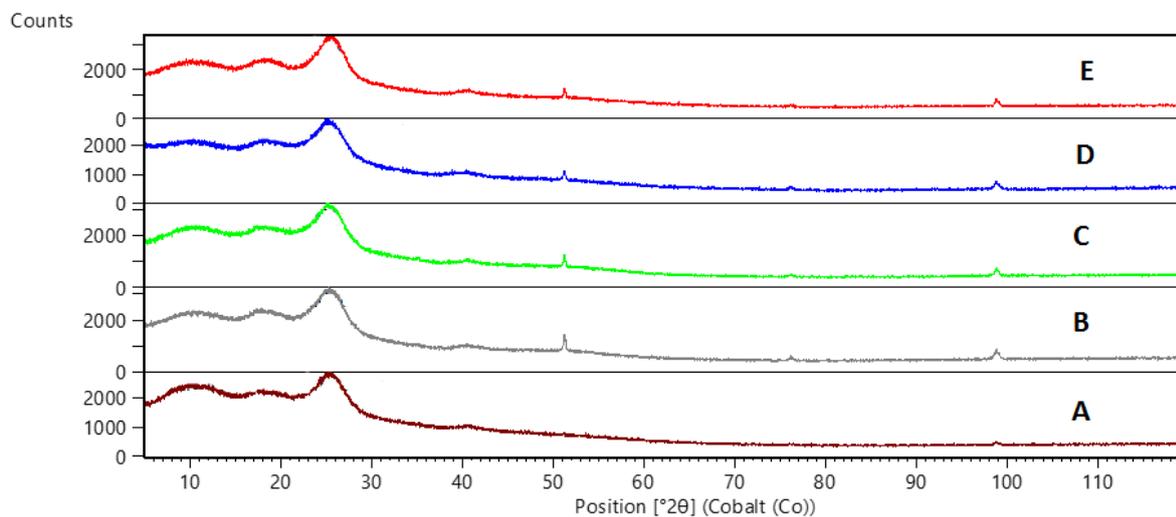


Fig S3. Diffractograms of the SLW samples; Native (A), water (B), MA-NaCl (C), MA-ZnCl₂ (D) and MA-FeCl₃ (E)

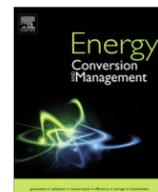
CHAPTER 5

Development of a steam or microwave-assisted sequential salt-alkali pretreatment for lignocellulosic waste: Effect on delignification and enzymatic hydrolysis.

This chapter has been published with the title: ‘Development of a steam or microwave-assisted sequential salt-alkali pretreatment for lignocellulosic waste: Effect on delignification and enzymatic hydrolysis.’ in Energy Conversion and Management (2017, 148: 801-808.).

Supplementary material associated with this paper can be found at the end of this chapter.

The published paper is presented in the following pages.



Development of a steam or microwave-assisted sequential salt-alkali pretreatment for lignocellulosic waste: Effect on delignification and enzymatic hydrolysis



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ABSTRACT

This study compares two different sequential pretreatments for sugarcane leaf waste (SLW): steam salt-alkali (SSA) and microwave salt-alkali (MSA). The two pretreatment types were modelled, optimized and validated with $R^2 > 0.97$. Reducing sugar yields of 1.21 g/g were obtained with optimized SSA pretreatment using 1.73 M $ZnCl_2$, 1.36 M NaOH and 9.69% solid loading, and 1.17 g/g with optimized MSA pretreatment using 1.67 M $ZnCl_2$, 1.52 M NaOH at 400 W for 10 min. A lower pretreatment time (10 min) was required for the MSA model (83% lower). The structure of pretreated SLW was assessed using scanning electron microscopy (SEM) and Fourier Transform Infrared analysis (FTIR). The optimized SSA and MSA models showed lignin removal of 80.5 and 73% respectively. The MSA pretreatment was further examined on sorghum leaves and Napier grass and showed yield improvements of 1.9- and 2.8-fold compared to recent reports. The developed pretreatment methods demonstrated high efficiency at enhancing enzymatic hydrolysis on various lignocellulosic substrates.

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1. Introduction

Lignocellulosic biomass represents an extraordinarily large amount of bio-resources suitable for the production of many bio-based products such as fuels and chemicals [20]. Sugarcane is one such biomass, with annual yields reaching 328 Tg [35]. Sugarcane leaf waste (SLW) makes up approximately 40% of the plants total biomass, equating to 131 Tg. Since SLW has no specific use post-harvest, it is either burnt or dumped in landfill sites [38]. Lignocellulose, such as SLW, is composed mainly of the carbohydrates glucan and xylan in the cellulose and hemicellulose layer respectively, indicating its feedstock potential [18]. These polymers are encrusted within a recalcitrant lignin layer. Due to the integral structural complexity of lignocellulose, the conversion of these polymers into monomeric sugars pose significant challenges [20]. The presence of lignin severely constrains enzymatic hydrolysis by irreversibly binding to cellulases [5]. Moreover, enzymatic hydrolysis is restricted by accessibility of the enzyme to cellulose, thus a robust pretreatment is required to delignify and disrupt the lignocellulosic matrix [43]. Some of the main criteria for an effective pretreatment is: (a) minimal energy demand, (b) low-cost, (c) minimal by-products [41].

Numerous pretreatment technologies have been investigated. These include the use of organic acids [26,34], alkali [40], ionic liquids [4], cold alkali extraction [9], microwave-assisted alkaline glycerol [10], microwave-alkali [21], inorganic salt [17], alkalic salt [30], alkaline peroxide [2] and sequential (two stage) dilute acid-alkali [22], among others.

Alkali pretreatment has emerged as one of the most effective pretreatments, given its low polluting, non-corrosive nature that requires less intensive conditions compared to other technologies. Alkali's, such as sodium hydroxide, primarily function to remove lignin through fibre swelling [18]. Inorganic salts have also garnered much attention since they are non-polluting, have very low toxicity and show high catalytic activity compared to acids [44]. Inorganic salts such as metal chlorides interact mainly with the ether linkages in xylan, leading to the removal of hemicellulose [16]. Therefore, the synergistic effect of sequential salt-alkali pretreatment could increase the degradation of lignocellulose and enhance enzymatic saccharification. Metal chloride salts in combination with steam, dilute acid, ferric oxide and ionic liquids have been investigated [33], however, there is a dearth of knowledge on the impact of sequential salt-alkali pretreatment on the enzymatic saccharification and delignification of lignocellulosic biomass.

Microwave irradiation has also attracted considerable interest over conventional heating since it causes different chemical changes in lignocellulosic biomass [45]. Microwave heating causes

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fibre swelling and fragmentation which reportedly enhances enzymatic saccharification. In addition, microwave irradiation breaks down the complex lignocellulosic structure through dielectric polarization causing molecular collisions [10,45]. Microwave irradiation also offers significant advantages over conventional heating such as: (a) short reaction times, (b) fast heat transfer, (c) energy efficiency, (d) direct heating, and (e) it is considered environmentally friendly [3]. However, there are no reports on microwave-assisted sequential salt-alkali pretreatment of lignocellulosic biomass.

The aim of this study was to develop a steam salt-alkali (SSA) and a microwave-assisted salt-alkali (MSA) pretreatment for SLW. In addition, the delignification and enzymatic saccharification efficiency of these two pretreatments were compared. Furthermore, Fourier Transform Infrared Spectroscopy (FTIR) and scanning electron microscopy (SEM) were used to determine physicochemical changes in the pretreated SLW structure.

2. Methods

2.1. Materials

Sugarcane leaf waste (SLW) was collected from a sugarcane plantation located on the North Coast of South Africa (29°42' 18"S, 31°02' 44"E). The leaves were dried at 60 °C for 72 h post-collection and milled to a particle size ≤ 1 mm. SLW fibre composition was determined by the NREL method [37]. The commercial cellulase enzyme preparation, Cellic CTec 2, was generously provided by Novozymes (Novozymes A/S, Denmark).

2.2. Preliminary screening

Pretreatments were carried out in 100 ml Erlenmeyer flasks with a solid loading of 10% w/v. Various inorganic metal chloride salts (NH_4Cl , NaCl and ZnCl_2) and alkali ($\text{Ca}(\text{OH})_2$ or NaOH) were evaluated at a 1 M concentration at 121 °C for 60 min. Samples were then washed and dried prior to enzymatic hydrolysis. The efficiency of each salt and alkali was measured based on the reducing sugar yield. Thereafter, the best salt and alkali was further examined in two different sequential combinations (salt-alkali and alkali-salt) to determine the best sequence for the pretreatment.

2.3. Steam salt-alkali (SSA) pretreatment

The process parameters affecting the reducing sugar yield from steam salt-alkali pretreatment were optimized using the Box-Behnken design (Design Expert 7.0, Stat Ease Inc, USA). The chosen parameters were selected at three levels and included salt concentration (0.1, 1.05, 2.0 M), alkali concentration (0.1, 1.05, 2.0 M) and solid loading (5%, 10%, 15%, w/v). At the first stage, SLW was pretreated with ZnCl_2 for 30 min at 121 °C followed by washing with three volumes of deionized water, drying at 60 °C and then pretreated with NaOH at 121 °C for 30 min at the second stage followed by washing with deionized water to a neutral pH and drying at 60 °C overnight. The concentration of salt and alkali are specified in the experimental design (Table 1). The controls were pretreated with either 1.05 M salt or alkali at 121 °C for 60 min. The SLW biomass was washed three times with deionized water and dried at 60 °C subsequent to enzymatic hydrolysis.

2.4. Microwave-assisted salt-alkali (MSA) pretreatment

Microwave-assisted salt-alkali pretreatment of SLW was also optimized using the Box-Behnken design (Design Expert 7.0, Stat

Ease Inc, USA). The considered input parameters were salt concentration (0.1, 1.05, 2.0 M), alkali concentration (0.1, 1.05, 2.0 M) and time (2, 5, 8 min). Power intensity and solid loading were maintained at 400 W and 10% (w/v) respectively as per Binod et al. [6], Jin et al. [14] and Sindhu et al. [36]. SLW biomass (10 g) was first pretreated with ZnCl_2 at durations specified in the experimental design (Table 2). After washing with three volumes of deionized water and drying, the samples were pretreated with NaOH for a period of time as specified in the experimental design. Pretreated samples were then washed to a neutral pH with deionized water and dried at 60 °C overnight prior to enzymatic hydrolysis. For the controls, SLW was pretreated with 1.05 M of either salt or alkali at 400 W for 10 min.

2.5. Enzymatic hydrolysis

Enzymatic hydrolysis of SLW was performed in 100 ml Erlenmeyer flasks. SLW biomass was immersed in 10 ml sodium citrate buffer (pH 4.8, 0.05 M) using a solid and enzyme loading of 10% (w/v) and 10 FPU/g respectively. Saccharification was carried out at 50 °C, 120 rpm for 72 h in a shaking waterbath. Following the enzymatic treatment, the hydrolysate was centrifuged and the supernatant analysed. The total reducing sugar was obtained using a glucose standard with the 3,5-dinitrosalicylic acid method [25].

2.6. Assessment of the optimized pretreatment strategy on other lignocellulosic residues

Sorghum leaves and Napier grass were dried at 60 °C for 72 h post-collection and milled to a particle size ≤ 1 mm. Each feedstock was then pretreated following the protocol outlined in Sections 2.4 and 2.5 using the optimum conditions.

2.7. Characterization of native and pretreated SLW

Changes in the surface morphology of native and pretreated SLW biomass was observed using a ZEISS EVO LS 15 scanning electron microscope. Prior to analysis, samples were gold sputter coated with an Eiko IB-3 Ion Coater. All images were taken at 1000 \times magnification.

Fourier transform infrared spectroscopy (FTIR) was performed using a Perkin Elmer 100 (Waltham, MA, USA). The spectrum was recorded between 380 and 4000 cm^{-1} at a resolution of 25 scans per sample. Sample discs were prepared by mixing the dried sample with spectroscopic grade KBr which was pressed into discs for analysis.

3. Results and discussion

3.1. Preliminary screening

Among the three different salts evaluated at the initial screening stage, ZnCl_2 was found to yield the highest reducing sugar (0.14 g/g) followed by NaCl (0.10 g/g) and NH_4Cl (0.09 g/g). NaOH was found to be the most effective alkali, yielding 0.95 g/g reducing sugar while $\text{Ca}(\text{OH})_2$ gave 0.65 g/g. ZnCl_2 and NaOH have been separately reported for enhancing enzymatic hydrolysis due to their strong solubilization properties [17,18]. Therefore, ZnCl_2 and NaOH were chosen for the sequential pretreatment. In addition, the sequential pretreatment with alkali first followed by salt gave a yield of 1.02 g/g whereas pretreatment with salt first followed by alkali yielded 1.08 g/g, thus the sequence of salt-alkali was chosen for further optimization.

Table 1

Box-Behnken design for optimization of various input parameters affecting steam salt-alkali pretreatment of SLW.

Run	Salt conc. (M)	Alkali conc. (M)	Solid loading (% w/v)	Reducing sugar (g/g)
1	1.05	1.05	10	1.15
2	0.10	0.10	10	0.649
3	2.00	0.10	10	0.589
4	0.10	1.05	5	1.17
5	2.00	2.00	10	1.22
6	1.05	1.05	10	1.16
7	1.05	1.05	10	1.16
8	0.10	1.05	15	1.12
9	2.00	1.05	5	1.18
10	1.05	1.05	10	1.10
11	1.05	2.00	15	1.12
12	1.05	0.10	15	0.42
13	1.05	1.05	10	1.11
14	1.05	2.00	5	0.921
15	0.10	2.00	10	0.985
16	2.00	1.05	15	1.09
17	1.05	0.10	5	0.745

Table 2

Box-Behnken design for optimization of various input parameters affecting microwave salt-alkali pretreatment of SLW.

Run	Salt conc. (M)	Alkali conc. (M)	Time (min)	Reducing sugar (g/g)
1	1.05	1.05	5	1.11
2	0.10	0.10	5	0.35
3	2.00	0.10	5	0.463
4	0.10	1.05	2	0.72
5	2.00	2.00	5	1.15
6	1.05	1.05	5	1.06
7	1.05	1.05	5	1.07
8	0.10	1.05	8	1.08
9	2.00	1.05	2	0.963
10	1.05	1.05	5	1.17
11	1.05	2.00	8	1.10
12	1.05	0.10	8	0.57
13	1.05	1.05	5	1.08
14	1.05	2.00	2	0.97
15	0.10	2.00	5	0.862
16	2.00	1.05	8	1.06
17	1.05	0.10	2	0.532

3.2. Model development

Key parameters affecting the reducing sugar yield from SLW pretreated with either steam or microwave-assisted sequential salt-alkali were investigated using the Box-Behnken Design. Reducing sugar yields obtained from Table 1 (SSA) and Table 2 (MSA) were used to generate the following polynomial equations using the coded factors:

$$\begin{aligned} \text{Sugar yield (SSA)} = & +1.14 + 0.23A + 0.019B - 0.033C \\ & + 0.074AB + 0.13AC - 0.010BC \\ & - 0.31A^2 + 0.032B^2 - 0.028C^2 \end{aligned} \quad (1)$$

$$\begin{aligned} \text{Sugar yield (MSA)} = & +1.10 + 0.27A + 0.078B + 0.078C \\ & + 0.044AB + 0.023AC - 0.066BC \\ & - 0.28A^2 - 0.11B^2 - 0.028C^2 \end{aligned} \quad (2)$$

where A and B represent alkali concentration and salt concentration respectively with C representing solid loading and time for the SSA model and MSA model respectively.

The suitability of the developed models were assessed by Analysis of Variance (ANOVA) and results are presented in Tables 3 and 4 for the SSA and MSA models respectively. The SSA and MSA polynomial models were found to be significant since both the P values were <0.0001 and were smaller than the F-values (79.42 and 29.98 for SSA and MSA respectively), thereby suggesting that the model

equations sufficiently described the responses. In addition, there is only a 0.01% chance that model F-values this large for both models could occur due to noise. Furthermore, the coefficient of determination for the SSA and MSA models were 0.99 and 0.97 respectively, indicating the models could account for 99% and 97% of variations in the observed data respectively. The lack of fit values of 2.33 (SSA) and 3.57 (MSA) impute that the models are not significant relative to the pure error. Among all examined variables, the effects of alkali concentration and solid loading for the SSA model and alkali concentration, salt concentration and time for the MSA model, were found to be significant, owing to their p-values <0.05.

3.3. Effect of process parameters on SSA and MSA pretreatment of SLW

The reducing sugar yield from the experimental runs ranged from 0.42 to 1.22 g/g (Table 1) and 0.35 to 1.17 g/g (Table 2) for the SSA and MSA pretreatments respectively. The observed sugar yield greater than 1 per gram of substrate is explained by the 25% mass gain when a water molecule reacts with each sugar unit during cellulose and hemicellulose hydrolysis as observed by Wyman et al. [42]. In addition, hydrothermal pretreatment has been shown to affect the average molecular weight of cellulose [12]. Furthermore, 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

Table 3
Analysis of variance (ANOVA) of the developed SSA pretreatment model.

Source	Sum of squares	df	Mean square	F Value	p-value Prob > F	Remark
Model	0.93	9	0.10	79.42	<0.0001	Significant
A-Alkali conc.	0.42	1	0.42	325.43	<0.0001	
B-Salt conc.	3.00×10^{-3}	1	3.00×10^{-3}	2.30	0.1730	
C-Solid loading	8.84×10^{-3}	1	8.84×10^{-3}	6.78	0.0352	
AB	0.022	1	0.022	16.68	0.0047	
AC	0.069	1	0.069	52.61	0.0002	
BC	4.00×10^{-4}	1	4.00×10^{-4}	0.31	0.5970	
A ²	0.40	1	0.40	303.92	<0.0001	
B ²	4.21×10^{-3}	1	4.21×10^{-3}	3.23	0.1155	
C ²	3.21×10^{-3}	1	3.21×10^{-3}	2.46	0.1616	
Residual	9.13×10^{-3}	7	1.31×10^{-3}			
Lack of fit	5.81×10^{-3}	3	1.94×10^{-3}	2.33	0.2153	Not significant
Pure error	3.32×10^{-3}	4	8.30×10^{-4}			
R ²					0.9903	
Cor total	0.94	16				

Table 4
Analysis of variance (ANOVA) of the developed MSA pretreatment model.

Source	Sum of squares	df	Mean square	F Value	p-value Prob > F	Remark
Model	1.12	9	0.12	29.98	<0.0001	Significant
A-Alkali conc.	0.59	1	0.59	141.93	<0.0001	
B-Salt conc.	0.049	1	0.049	11.77	0.0110	
C-Time	0.049	1	0.049	11.81	0.0109	
AB	7.66×10^{-3}	1	7.66×10^{-3}	1.85	0.2158	
AC	2.12×10^{-3}	1	2.12×10^{-3}	0.51	0.4976	
BC	0.017	1	0.017	4.18	0.0801	
A ²	0.32	1	0.32	78.26	<0.0001	
B ²	0.055	1	0.055	13.35	0.0081	
C ²	3.24×10^{-3}	1	3.24×10^{-3}	0.78	0.4053	
Residual	0.029	7	4.14×10^{-3}			
Lack of fit	0.021	3	7.02×10^{-3}	3.57	0.1256	Not significant
Pure error	7.88×10^{-3}	4	1.97×10^{-3}			
R ²					0.9747	
Cor total	1.14	16				

[32]. Higher reducing sugar yields were observed for the steam salt-alkali pretreatment compared to the microwave salt-alkali pretreatment. High alkali (2.0 M) and salt concentration (2.0 M) gave the highest reducing sugar yield (1.22 g/g, run 5) with the SSA model whereas for the MSA model, the median concentrations for alkali and salt (1.05 M) showed the highest reducing sugar yield (1.17 g/g, run 10). The lowest sugar yield (0.42 g/g) for the SSA pretreatment was observed with 1.05 M alkali, 0.10 M salt and 15% solid loading while 0.37 g/g was obtained with 0.10 M alkali, 0.10 M salt and 5 min for the MSA pretreatment.

The interactive effect of the input parameters on the reducing sugar yield are shown in Fig. 1. The effect of salt and alkali concentration on sugar yield for the SSA model is shown in Fig. 1A. Low reducing sugar yields (0.58 g/g) were observed at high salt (2 M) and low alkali concentration (0.10 M). A simultaneous increase in salt and alkali concentration from 0.10 to 2.0 M and 0.10 to 1.52 M respectively led to an increase in the reducing sugar yield from 0.60 to 1.27 g/g. A further increase in alkali concentration beyond this threshold, from 1.52 to 2.0 M, decreased the reducing sugar yield from 1.27 to 1.22 g/g. A similar trend was observed by Jin et al. [15] where an increase in alkali (Ca(OH)₂) concentration from 0.25 to 1.75% (w/v) with catalpa sawdust, increased the reducing sugar yield from 125 mg/g to 225 mg/g and a further increase in alkali beyond this threshold to 2.25% (w/v), decreased the reducing sugar yield from 225 mg/g to 200 mg/g. Du et al. [11] reported a lower reducing sugar yield of 0.547 g/g using 0.75 M NaOH (3.0% (w/v)) to pretreat cotton stalk at 125 °C for 40 min. This low yield can be attributed to the extremely low lignin removal efficiency (3% removal) of the pretreatment as well as the low content of cellulose (39.85%) in cotton stalks. The investigation

of surfactant assisted base pretreatment of pine foliage gave a reducing sugar yield of 0.477 g/g under the following pretreatment conditions: 0.5 M (2% (w/v)) NaOH, 1% (w/v) PEG 6000, 121 °C [28]. Native pine foliage contains significantly more lignin (32.04%) than cellulose (30.91%) and hemicellulose (22.4%) thus requiring a robust pretreatment for removing lignin and increasing cellulose. These authors observed a slight increase in cellulose from 30.91 to 43.5% in pretreated pine foliage thereby resulting in the slight increase in reducing sugar yield. Furthermore, since conventional methods heat only the surface of the material, longer heating durations would be sometimes required to achieve effective enzymatic saccharification. Moreover, the current pretreatment achieves a higher reducing sugar yield compared to these studies while employing similar pretreatment conditions.

The interactive effect of solid loading and alkali concentration on reducing sugar yield when salt concentration is kept at its centre point for the SSA model, is shown in Fig. 1B. At a high solid loading (15%, w/v) and low alkali concentration (0.10 M), the reducing sugar yield was extremely low (0.42 g/g) whereas at low solid loading (5%, w/v) and alkali concentration (0.10 M), the reducing sugar yield increased to 0.745 g/g thereby indicating that a high solid loading coupled with a low alkali concentration hampers the reducing sugar yield. High reducing sugar yield (1.19 g/g) was observed with a simultaneous increase in solid loading and alkali concentration from 5 to 12% (w/v) and 0.10 to 1.30 M respectively. Further increases in solid loading (15%, w/v) and alkali concentration (2.0 M) slightly decreased the reducing sugar yield to 1.12 g/g. Higher solid loadings could decrease the surface area accessibility for the alkalic agent. A similar trend was observed by Raghavi et al. [31] with the pretreatment of sugarcane trash where the

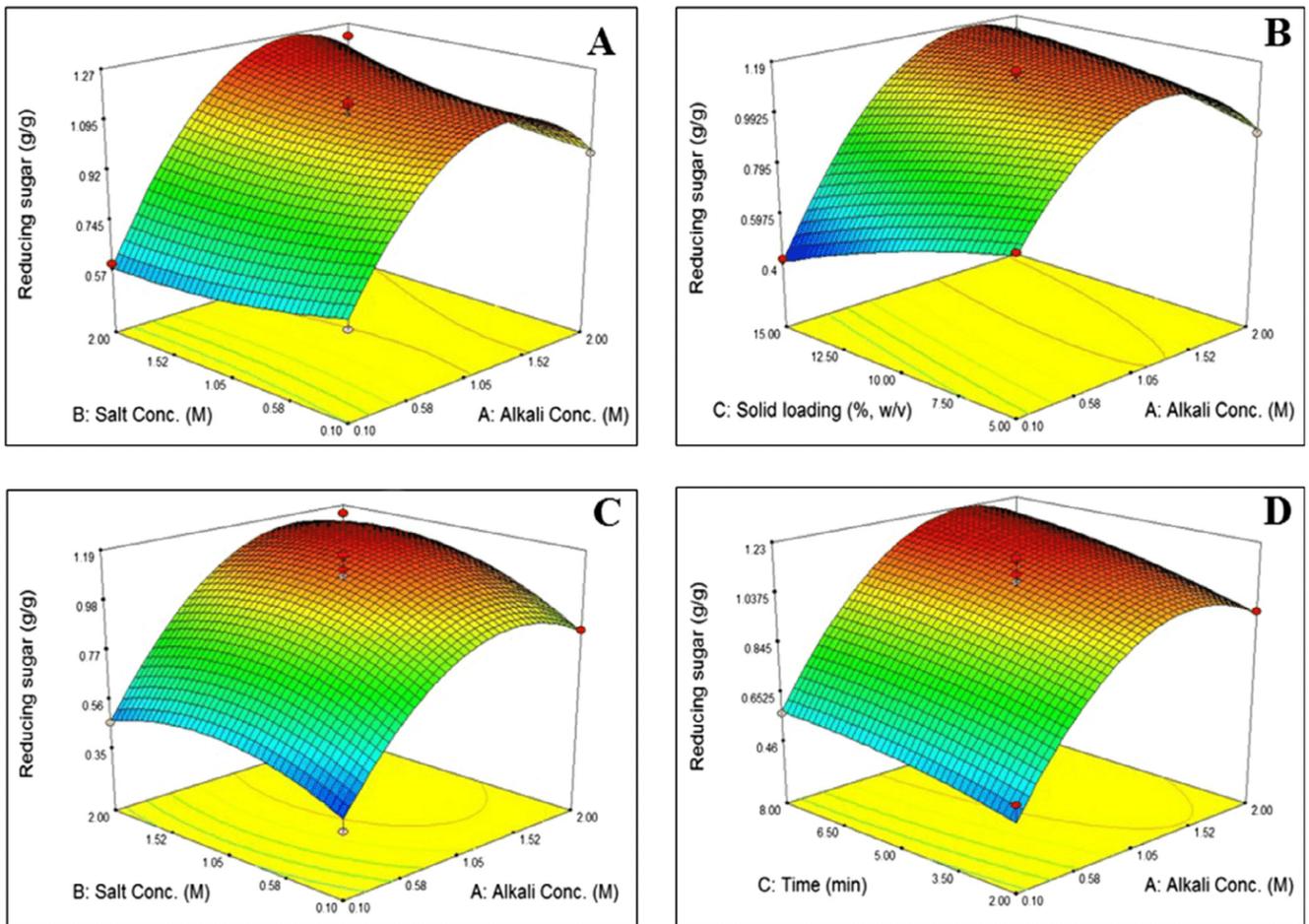


Fig. 1. Response surface plots showing the interaction between input variables on reducing sugar yield between (a) salt and alkali concentration for SSA pretreatment; (b) solid loading and alkali concentration for SSA pretreatment; (c) salt and alkali concentration for MSA pretreatment and (d) time and alkali concentration for MSA pretreatment.

reducing sugar yield decreased from >0.70 to <0.50 g/g when the solid loading was increased from 10 to 15% while maintaining 1% FeCl_3 , 3.5% NaOH and 4.5% crude glycerol at 121°C for 60 min.

The effect of salt and alkali concentration on the reducing sugar yield for the MSA model is shown in Fig. 1C. At low concentrations of salt (0.10 M) and alkali (0.10 M), the lowest reducing sugar yield was observed (0.40 g/g). An increase in salt from 0.10 to 2.0 M while maintaining alkali at 0.10 M, resulted in a minor increase in reducing sugar from 0.40 to 0.46 g/g. However, maintaining salt at 0.10 M and increasing alkali from 0.10 to 2.0 M, doubled the reducing sugar yield from 0.40 to 0.86 g/g. This suggested alkali concentration had a more pronounced effect on reducing sugar yield compared to salt concentration. Higher alkali concentrations have been reported to achieve complete mercerization of cellulose, resulting in the effective conversion of cellulose to glucose [23]. A simultaneous increase in salt and acid concentration from 0.10 to 1.60 M and 0.10 to 1.52 M respectively gave the highest reducing sugar yield (1.19 g/g). This high yield could be attributed to the effective delignification and hemicellulose removal of SLW with alkali and salt respectively. Akhtar et al. [1] reported the combination of microwave-assisted alkali-acid pretreatment of rice straw to yield 0.246 g/g reducing sugar. Low lignin removal (50.6%) and low cellulose recovery (40%) could account for this low yield. Another study focusing on the effect of microwave-assisted lime pretreatment of sweet sorghum bagasse, employed a low concentration of lime (1%, w/v) with a low pretreatment time and high

power (4 min and 1000 W respectively) and obtained 0.24 g/g reducing sugar [8]. This low yield can be ascribed to the low concentration of lime since our previous study reported a reducing sugar yield of 0.462 g/g with microwave-assisted inorganic salt pretreatment of sorghum leaves under milder microwave conditions of 700 W for 3.5 min [27].

The influence of time and alkali concentration on reducing sugar yield is shown in Fig. 1D. The curvature of the graph suggested time had little effect on the reducing sugar yield whereas alkali concentration had a significant effect. For instance, varying time between 2 and 8 min at 0.10 M alkali, the reducing sugar remains relatively unchanged at 0.50 g/g whereas at 1.05 M alkali, the reducing sugar remains around 1.01 g/g. Maximum reducing sugar (1.23 g/g) was observed when time and alkali concentration were within the range of 6 to 8 min and 1.40 to 1.52 M respectively. Binod et al. [6] reported on microwave-assisted chemical pretreatment of sugarcane bagasse and observed 0.80 g/g reducing sugar with a sequential alkali-acid method at 300 W power intensity for 7 min. The current MSA pretreatment regime has shown that salt-alkali is more effective at enhancing enzymatic hydrolysis compared to the commonly reported alkali-acid pretreatment. Optimal conditions for microwave-assisted alkali pretreatment of sawdust was reported to yield 0.40 g/g with 2.25% (w/v) $\text{Ca}(\text{OH})_2$ at 400 W for 6 min [14]. The relatively low content of cellulose (56%) after pretreatment can be a significant contributor to this low yield.

3.4. Validation of developed model

The SSA model predicted a reducing sugar yield of 1.22 g/g with 1.36 M alkali, 1.73 M salt and 9.69% solid loading whereas the MSA model predicted a yield of 1.19 g/g with 1.52 M alkali, 1.67 M salt for 5 min, as shown in Table 5, thus only a 2.45% difference. Experimental validation carried out in triplicate gave reducing sugar yields of 1.21 ± 0.038 and 1.17 ± 0.031 g/g for the SSA and MSA models respectively. The predicted and observed yields showed a negligible difference between 0.8 and 1.7%. The SSA model showed yield improvements of 84% over the salt control (0.19 g/g) and 10.7% compared to the alkali control (1.08 g/g). Yield improvements of 82 and 13.7% were observed for the salt (0.21 g/g) and alkali (1.01 g/g) controls respectively compared to the MSA model.

3.5. Comparison of SSA and MSA pretreatment models

A higher reducing sugar yield was observed for the optimized SSA model (1.21 g/g) compared to the MSA model (1.17), however, the difference between the two models was not significant (3.3%). The MSA pretreatment required a significantly lower pretreatment time (10 min) compared to the 60 min required for the SSA pretreatment. In addition, the optimized MSA model required 4% lower salt concentration (1.67 M) compared to the SSA model (1.73 M). Microwave heating penetrates lignocellulosic biomass and directly vibrates the molecules causing an increase in temperature, shattering the lignocellulosic structure. Furthermore, this vibration breaks inter- and intra-molecular hydrogen bonds, leading to enhanced accessibility of enzyme to cellulose [3]. This rationale accounts for the low pretreatment time and high reducing sugar yield observed with the MSA model. Conventional steam heating has been reported to only increase the temperature of the surface of the heated material, thus requiring a higher heating time [3]. Lai and Idris [21] compared steam and microwave-assisted alkali pretreatment of oil palm trunk. These authors reported a high efficiency of enhanced enzymatic hydrolysis with microwave-alkali (18 g/L glucose) compared to steam-alkali (16 g/L glucose). However, extremely high pretreatment times were used for the microwave-alkali method (60 min) compared to the steam-alkali (15 min). Since the optimized MSA pretreatment requires a significantly lower pretreatment time, at a large scale it will have a lower energy consumption thereby saving costs.

3.6. Composition of native and pretreated SLW

The composition of native and pretreated SLW biomass is shown in Table S1. The lignin, cellulose and hemicellulose content in the native SLW biomass was 9.16, 43.44 and 30.98% respectively. The SSA sample showed significant lignin removal (80.5%) while the MSA sample showed 73% lignin removal. The steam and microwave NaOH control also showed 41.9 and 37.8% lignin removal respectively. Alkali interacts predominantly with lignin resulting in efficient lignin removal. Sodium hydroxide breaches the link between lignin and hemicellulose, particularly the ether and ester bonds [18]. Efficient hemicellulose removal was also observed for the SSA (51.9%) and MSA (62%) samples. This is consistent with

the mechanistic action of NaOH as well as the integral role metal chloride salts play in hemicellulose removal [17,18]. Owing to the Lewis acid character of metal chloride salts, they dissociate into complex ions in water and assist in rupturing the glucosidic linkages of sugar chains in hemicellulose [33]. No lignin removal was observed with the salt control samples. This was expected since inorganic salt does not interact with lignin [17]. There were similar improvements in cellulose content after SSA (88%) and MSA (87%) pretreatment whereas lower recoveries were seen with the different controls (steam alkali (75%), microwave alkali (80%), steam salt (0%) and microwave salt (36%)). Previous studies have reported lower cellulose improvements. For instance, Akhtar et al. [1] reported a 17.53% increase in cellulose after microwave-alkali-acid pretreatment of rice straw. A lower cellulose increase (5.41%) was reported by Jin et al. [14] after microwave assisted alkali pretreatment of catalpa sawdust whereas a lower improvement of 2.45% was reported by Tao et al. [39] with microwave-assisted acid pretreatment of *Tiarrhena lutarioparia*. Therefore, the current SSA and MSA pretreatment methods can effectively increase the cellulose content per gram of dry matter thus enhancing the reducing sugar yield.

3.7. Structural characterization of native and pretreated SLW

The topographical changes that were induced by the SSA and MSA pretreatments are shown in Fig. S1. The micrographs depict major structural differences between untreated and pretreated SLW biomass. The untreated sample showed a smooth, highly ordered surface with minimal aberrations. The SSA and MSA pretreatments appear to have disrupted the structural integrity of the sample with a clear increase in surface fractionation and roughness. The SSA sample showed a higher degree of structural damage with dismantling of the vascular fibres and partial delocalization which led to an appreciable recovery of reducing sugar. However, the MSA sample showed the development of major cavities and pores. Similar observations in surface structure were previously reported in different studies using microwave-crude glycerol [13], hydrogen peroxide assisted CuCl_2 [24] and high pressure alkali pretreatment [11]. The steam-alkali control also appeared to have significant surface damaged with the characteristic fibre detachment associated with alkali pretreatment [7], compared to the microwave-alkali control. In addition, the microwave-salt control showed minimal surface damage whereas the steam-salt control showed minor cavitation with the development of pores.

The fingerprint of the FTIR spectra for the untreated and pretreated SLW biomass was analysed to detect chemical changes and results are shown in Fig. S2. The characteristic broad band associated with the O–H stretching of the hydrogen bonds of cellulose at 3330 cm^{-1} and the C–H stretching within the methylene of cellulose increased markedly after SSA pretreatment compared to the native sample. Furthermore, the C–O–H stretching vibration of the primary and secondary alcohols of cellulose at 1030 cm^{-1} was more pronounced after SSA pretreatment. These results indicate an increase in cellulose, owing to the removal of hemicellulose [22]. The removal of hemicellulose was supported by the dissolution of the band at 1730 cm^{-1} (aldehyde C=O

Table 5

Validation of optimized conditions for salt-alkali pretreatment of SLW.

Run	Alkali conc. (M)	Salt conc. (M)	Time (min)	Solid loading (% w/v)	Reducing sugar yield (g/g)	
					Predicted	Observed
SSA model	1.36	1.73	–	9.69	1.22	1.21
MSA model	1.52	1.67	5	–	1.19	1.17

Table 6

Validation of the optimized pretreatment regime on different lignocellulosic residues.

Substrate	Pretreatment conditions	Yield (g/g)	Improvement	Ref
Sugarcane leaves	1.67 M ZnCl ₂ at 400 W for 5 min, 1.52 M NaOH at 400 W for 5 min	1.17 ^a	62%	This study
Sugarcane trash	6% glycerol, 1% FeCl ₃ , 1.25 M NaOH, at 121 °C for 60 min	0.721 ^a	NA	[31]
Sorghum leaves	1.67 M ZnCl ₂ at 400 W for 5 min, 1.52 M NaOH at 400 W for 5 min	1.29 ^a	193%	This study
Sorghum biomass	1.25 M NaOH at 100 °C for 60 min	0.44 ^a	NA	[19]
Napier grass	1.67 M ZnCl ₂ at 400 W for 5 min, 1.52 M NaOH at 400 W for 5 min	1.35 ^a	275%	This study
Napier grass	0.5 M NaOH at 121 °C for 60 min	0.36 ^b	NA	[29]

^a Reducing sugar yield per gram dry weight NA- Not applicable^b Glucose yield per gram dry weight

stretching in hemicellulose) after SSA and MSA pretreatment. In addition, the discerned band at 900 cm⁻¹, assigned to the glycosidic linkage in hemicellulose, became more pronounced after MSA pretreatment compared to the SSA sample. The main activity of salt and alkali in pretreatment is to remove hemicellulose and lignin respectively, thus their combined effect in turn increased the cellulose content [17,31]. Lignin characteristic peaks at 1270 cm⁻¹ (aromatic ring vibration of guaiacyl lignin) and 1600 cm⁻¹ (aromatic ring vibration and C=O stretching of lignin) were significantly enhanced after SSA and MSA pretreatment compared to the native sample, suggesting the removal the lignin. The presence of guaiacyl in lignin prevents fibre swelling and ultimately hinders enzyme accessibility [21], therefore the removal of this functional group enhanced enzymatic hydrolysis. Similar results were reported with alkali pretreated oil palm trunk [21] and microwave-alkali pretreated rice straw [1].

3.8. Assessment of the optimized pretreatment strategy on other lignocellulosic residues

The MSA model required a significantly lower pretreatment time thus it was chosen for the assessment on other lignocellulosic residues. A comparison of the optimized MSA pretreatment model with other pretreatment methods is shown in Table 6. SLW pretreated under optimized MSA conditions in this study showed a higher reducing sugar yield (1.17 g/g) compared to a previous report (0.721 g/g) by Raghavi et al. [31]. These authors employed a combination of alkali, salt and surfactant for pretreatment of sugarcane trash. The optimum MSA pretreatment conditions was also assessed on sorghum leaves and Napier grass, and showed improvements of 10 and 15% respectively, compared to the yield obtained for SLW. Koradiya et al. [19] pretreated sorghum biomass with 1.25 M NaOH and obtained 0.44 g/g reducing sugar, thus the MSA model showed a 1.9-fold increase in reducing sugar yield with 1.29 g/g. MSA pretreatment of Napier grass exhibited a yield of 1.35 g/g reducing sugar, a 2.8-fold enhancement over the value reported by Phitsuwan et al. [29]. The results from the current study suggest that the developed MSA pretreatment model can successfully be applied to a range of lignocellulosic feedstocks to obtain high yields of reducing sugar. In addition, the developed MSA regime was shown to be more effective at enhancing enzymatic hydrolysis compared to recently reported pretreatment methods (Table 6).

4. Conclusion

In this study, two different sequential pretreatments were examined- SSA and MSA. These pretreatments were modelled, optimized and validated with R² > 0.97. Reducing sugar yields of 1.21 and 1.17 g/g were obtained with the SSA and MSA pretreatments respectively. Major structural changes were observed with the pretreated biomass after SEM and FTIR analysis. High delignification was also achieved (80.5 and 73% for SSA and MSA respectively). The developed pretreatment methods demonstrated high

efficiency at enhancing enzymatic hydrolysis on Napier grass and sorghum leaves. MSA pretreatment emerged with low pretreatment time, and high reducing sugar yields compared to various reported lignocellulosic pretreatment strategies.

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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.2017.06.056>.

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Supplementary material

Table S1 – Lignin, cellulose and hemicellulose content of native and pretreated SLW biomass.

Pretreatment type	Lignin	Cellulose	Hemicellulose
Native	9.16	43.44	30.98
SSA	1.79	81.70	14.91
MSA	2.49	81.42	11.84
S-alkali control	5.32	76.26	17.16
M-alkali control	5.70	78.25	12.05
S-salt control	14.49	43.20	15.21
M-salt control	12.23	59.02	16.41

M- microwave-assisted

S- Steam

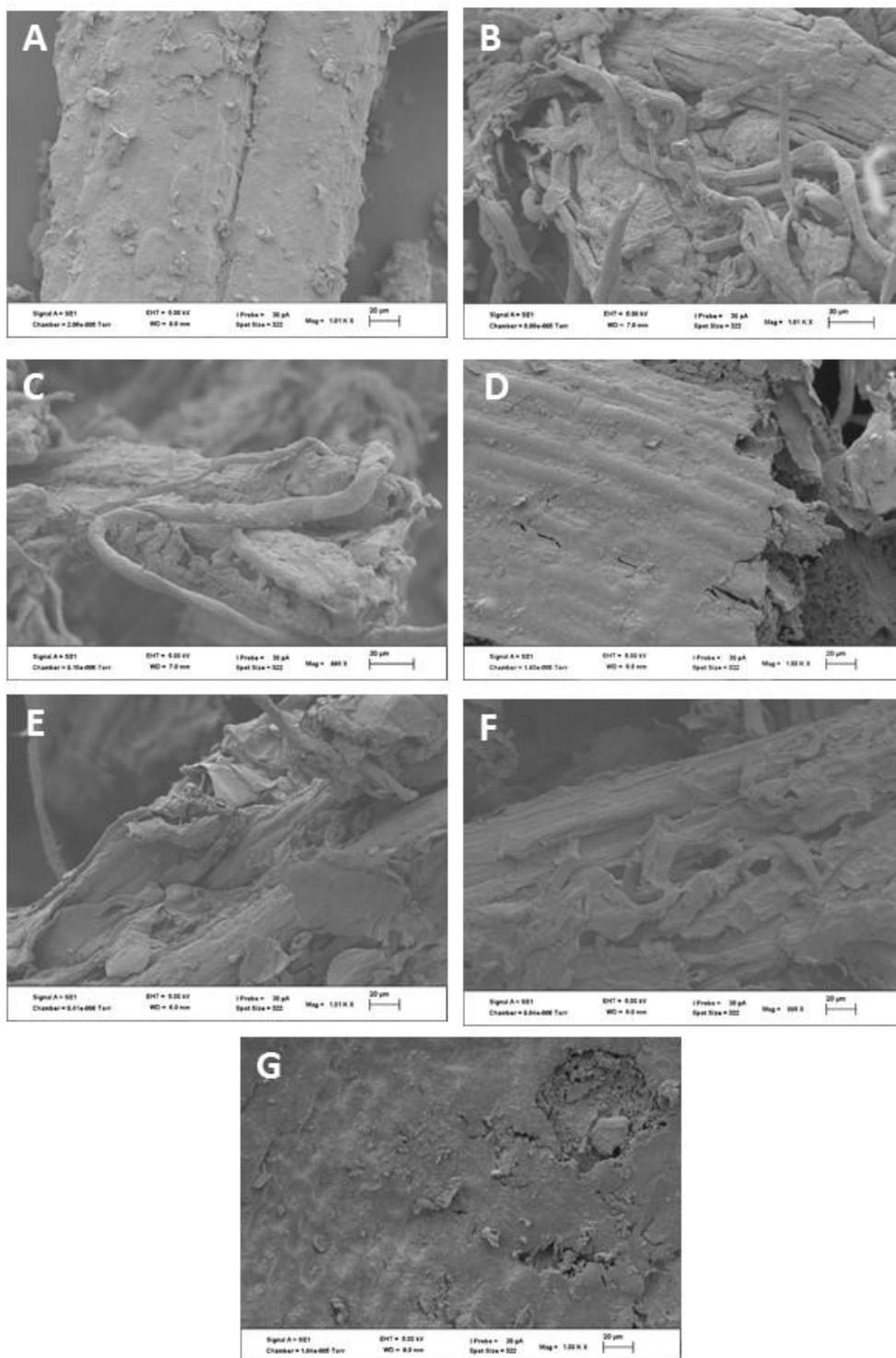


Fig S1. SEM micrographs of (A) native SLW; (B) SSA pretreated SLW; (C) Steam alkali control; (D) Steam salt control; (E) MSA pretreated SLW; (F) Microwave alkali control; (G) Microwave salt control

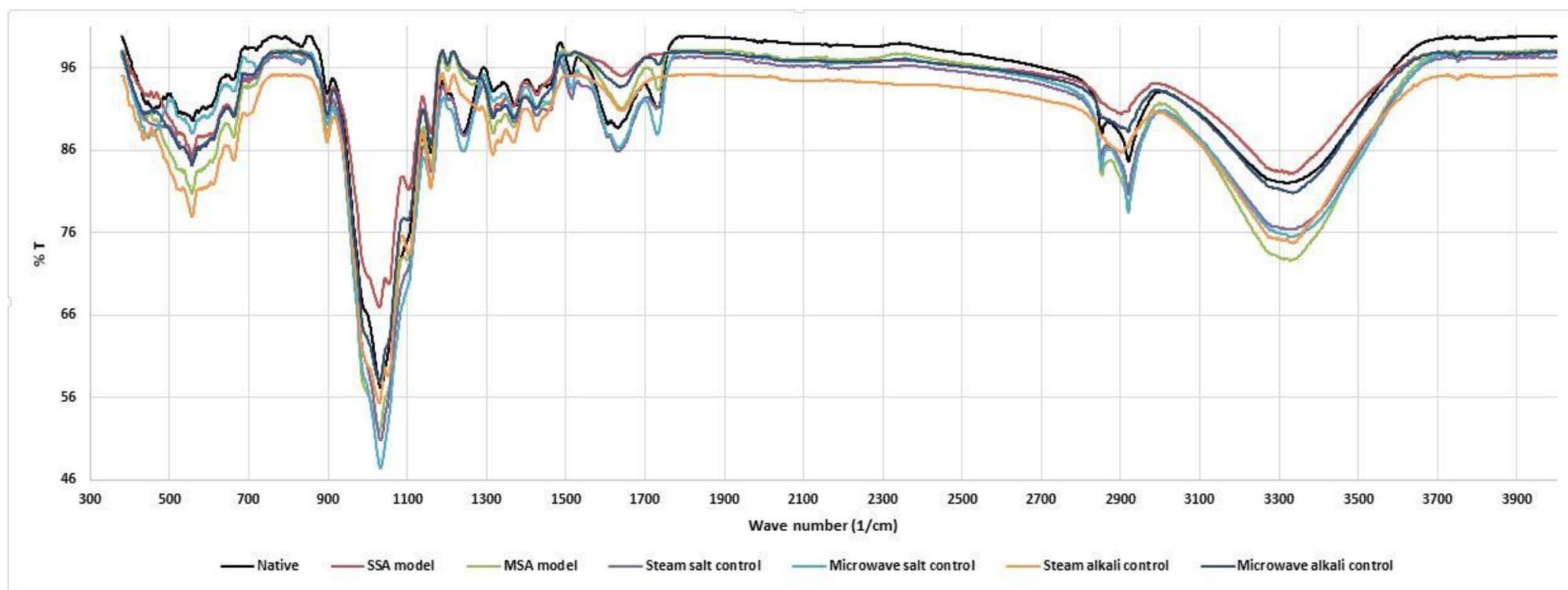


Fig S2. FTIR spectra for the native and pretreated SLW

CHAPTER 6

Artificial neural network tools for predicting sugar yields from inorganic salt-based pretreatment of lignocellulosic biomass.

This chapter has been submitted to Biofuel Research Journal with the title: Artificial neural network tools for predicting sugar yields from inorganic salt-based pretreatment of lignocellulosic biomass.

The manuscript is presented in the following pages.

Artificial neural network tools for predicting sugar yields from inorganic salt-based pretreatment of lignocellulosic biomass.

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Abstract

Pretreatment of lignocellulosic biomass has been identified as a major bottleneck in the bioprocess development of biofuels and bioproducts. In addition, the initial screening and selection of pretreatment input parameters and ranges is a laborious process that incurs costs before an appropriate regime is identified. This study developed two artificial neural network (ANN) tools for predicting sugar yields from inorganic salt-based pretreatment of lignocellulosic biomass. Pretreatment data from 90 experimental runs with 8 different input conditions were used to develop a microwave-based and a steam-based model. Both models showed high coefficients of determination (R^2) of 0.97. Knowledge extraction from the ANN models using curve fitting revealed that the reducing sugar yield for both models were highly dependent on salt and alkali concentration, exhibiting a sigmoidal and dose response relationship respectively for the steam model, and a regression and sigmoidal relationship respectively for the microwave model. These models may be employed as initial screening tools in lignocellulosic bioprocesses, thereby potentially enhancing the economics and productivity of lignocellulosic-based bioprocesses.

Keywords: Pretreatment, Salt, Sugarcane leaves, Lignocellulosic biomass

1. Introduction

Lignocellulosic biomass has become a prime source for the development of biofuels and bioproducts due to its abundant and renewable nature. More specifically, agricultural crops and their residues are regenerated on an annual basis and are thus considered inexhaustible (Kapdan and Kargi, 2006). More than 70% of lignocellulosic biomass is composed of the biopolymers hemicellulose, cellulose and lignin (Chen, 2014). Hemicellulose (24 – 40%) is a branched polymer of five and six carbon sugars (Ayeni et al., 2015), while cellulose (40 – 55%) is a linear polymer of β -1, 4 glucose units (Garcia-Maraver et al, 2013). Lignin however, is a non-carbohydrate polymer consisting mainly of phenylpropane units and makes up 15 – 25% of lignocellulosic biomass (Ayeni et al., 2015; Garcia-Maraver et al., 2013; Kim et al., 2008). The complex arrangement of these molecules offer remarkable rigidity and recalcitrance towards enzymatic hydrolysis. Furthermore, commonly employed microorganisms in biofuel production processes such as *Saccharomyces cerevisiae* and *Pichia stipitis* are incapable of accessing and degrading the cellulosic or hemicellulosic fractions within the lignocellulosic matrix thus a pretreatment step is required prior to bioconversion

Pretreatment is an integral process in a biorefinery system where lignocellulose is converted into liquid fuel (Bhutto et al., 2017). Various pretreatment methodologies exist which aim to reduce the obstinacy of the lignocellulosic structure thereby enhancing subsequent enzymatic saccharification and microbial conversion. Some of these methods include acid, alkali, inorganic salt, organosolv, ionic liquids and microwave irradiation among others (Zabed et al., 2017; Kang et al., 2013). Inorganic salt pretreatment is currently emerging as an effective pretreatment, owing to its high catalytic activity, low cost and low toxicity (Moodley and Gueguim Kana, 2017a). Several studies have investigated the catalytic effect of salt in combination with acid, alkali or surfactant to enhance enzymatic hydrolysis (Raghavi et al., 2016; Kim et al., 2014; Moodley and Gueguim Kana, 2017a; Moodley and Gueguim Kana, 2017c).

Microwave irradiation is gaining significant interest as an alternative heating source for lignocellulosic pretreatment (Aguilar-Reynosa et al., 2017). Microwave pretreatment causes disruptions to the lignocellulosic material on a molecular level, resulting in fibre swelling and fragmentation (Diaz et al., 2015). In addition, microwave heating has many advantages compared to conventional steam heating, such as faster heating, shorter reaction times and energy efficiency (Aguilar-Reynosa et al., 2017).

In most cases, modelling and optimization processes are conducted to determine the best pretreatment conditions to release maximum sugar (Sindhu et al., 2016). However, the modelling of pretreatment does incur high cost and time due to the large number of experimental runs performed. Furthermore, extensive preliminary screenings are often carried out in order to determine an appropriate navigation space prior to the optimization process. The development of an intelligent predictive tool could significantly enhance the workflow by negating many of the laborious tasks prior to the bioconversion of lignocellulose to biofuels.

Artificial Neural Network (ANN) models have been employed in various industries to accurately establish relationships between the input and output variables of non-linear processes (da Silva Bispo, 2017; Rorke et al., 2017). ANNs are capable of identifying patterns found in data and are trained through experience, allowing for the modelling of processes by using data obtained from various modelling techniques (Desai et al., 2008). This enables the use of ANN as a virtual experimentation tool for real-time estimation of process parameters which are not easily monitored (Gonzaga et al., 2009).

The aim of this study was to develop two artificial intelligent models (microwave-based and steam-based) for predicting reducing sugar yield using data from inorganic salt-based pretreated lignocellulosic waste. In addition, the functional relationships between the input parameters and the reducing sugar yield are investigated.

2. Methods

2.1 Experimental data used for ANN model development

The experimental data used for model development were obtained from our previous studies on pretreatment of sugarcane leaf waste (SLW). These pretreatments were based on: (a) a combined single stage and a two-stage sequential salt-acid (Moodley and Gueguim Kana, 2017a), (b) microwave assisted ZnCl_2 (Moodley and Gueguim Kana, 2017b) and (c) microwave-assisted and steam-assisted sequential salt-alkali (Moodley and Gueguim Kana, 2017c). A total of 90 experimental runs were used for the development of the ANN predictor models. Data from only ZnCl_2 -based runs were selected since ZnCl_2 is significantly more cost effective compared to FeCl_3 , and it was shown to effectively enhance enzymatic saccharification. The acid and alkali employed were H_2SO_4 and NaOH respectively.

2.2 Artificial intelligent model development to predict sugar yield

Artificial Neural Networks were used to develop two models to predict the yield of reducing sugar from inorganic salt-based steam-assisted and microwave-assisted pretreatment of SLW after enzymatic hydrolysis. The topology of the steam-based ANN model comprised of 1 input layer of 5 neurons, 2 hidden layers of 13 neurons each and 1 output layer with 1 neuron (5-13-13-1) while the microwave model had an ANN topology consisting of 1 input layer of 4 neurons, 2 hidden layers of 13 neurons each and 1 output layer with 1 neuron (4-13-13-1). The hidden layer employed a logistic transfer function, which served two purposes: (a) weight addition to inputs and linked bias and (b) shift data to a non-linear form (Desai et al., 2008).

The input parameters and ranges for the microwave model included salt concentration (0 - 2 M), alkali concentration (0 - 2 M), power intensity (0 - 800 W) and heating time (0 - 16 min). In the case of the sequential two-stage study, heating time was combined to give a total heating time (Moodley and Gueguim Kana, 2017c). The input parameters for the steam model consisted of salt concentration (0 - 5 M), acid concentration (0 – 2 %, v/v), alkali concentration (0 – 2 M), solid loading (5 and 15 %, w/v) and stage (1 or 2) where 1 corresponds to a combined single stage and 2 corresponds to a sequential two stage pretreatment. The output for both models was reducing sugar yield (g/g). For each model, the experimental data set was divided into training subset (75%) and validation subset (25%).

2.2.1 ANN training and validation

The back propagation algorithm was employed to train the models with the target to obtain a minimum net error on the validation data set while simultaneously preventing memorization. Model accuracy was examined on validation data through regression analysis on the predicted versus experimental process output. Coefficients of determination (R^2) were computed for each model.

2.2.2 Impact of pretreatment input variation on reducing sugar analysis

Sensitivity analysis was conducted to determine the effect of variations in the input parameters on the model output (Rorke et al., 2017). This was achieved by varying each input parameter between its minimum and maximum value, while all other inputs were maintained at their median values. Mathematical equations describing the functional relationships between pretreatment inputs and the sugar yield output were extracted from each model using curve fitting.

3. Results and discussion

3.1 ANN model assessment

The developed ANN models were assessed on the prediction of reducing sugar generation, using the validation data set. These models have been deposited into the Repository of Intelligent Models (REDIM, 2017) with accession numbers (PRZW001370 and PRHK001087). Both the steam-based and microwave-based models gave coefficients of determination (R^2) of 0.97 as shown in the graphs of predicted versus observed in Figures 1 and 2, large majority of the data points can be seen congregating along the predictive trend line, indicating higher accuracy at predicting the reducing sugar yield under new process conditions.

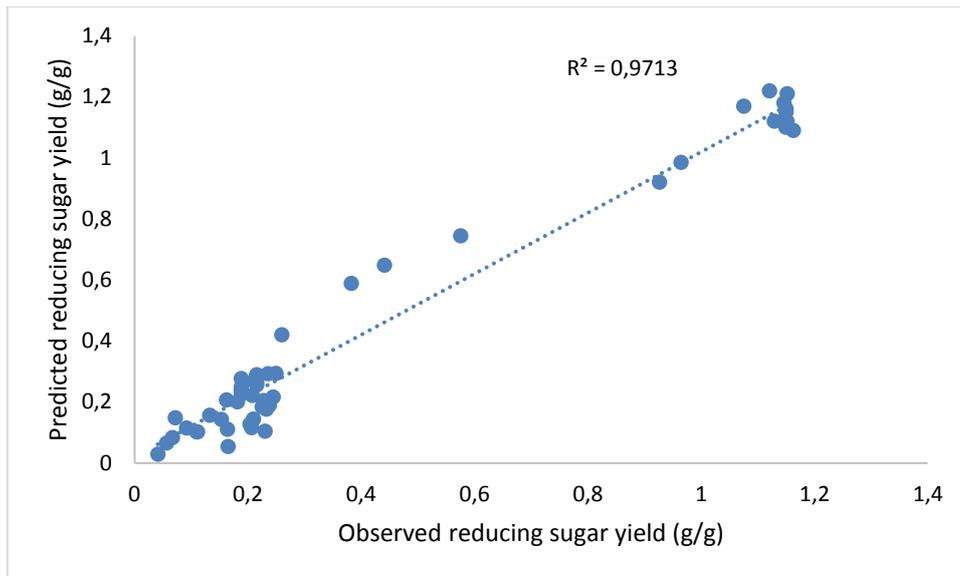


Fig 1. Regression plot showing observed versus predicted reducing sugar yield for the steam-based model (g/g)

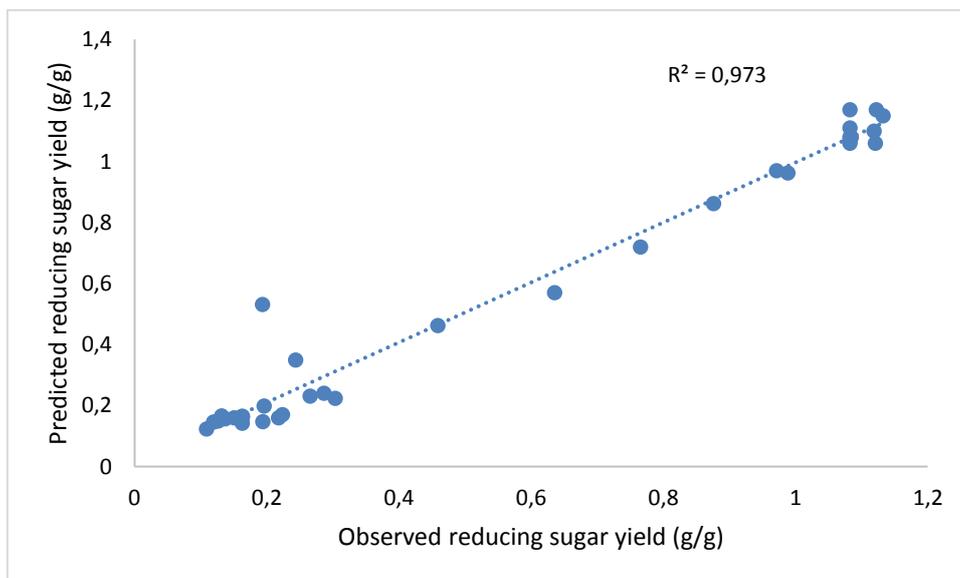


Fig 2. Regression plot showing observed versus predicted reducing sugar yield for the microwave-based model (g/g)

3.2 Effect of changes in input parameters on sugar yield

Mathematical relationships derived from curve fitting illustrate the functional relationships between input parameters and reducing sugar yield (Table 1). For the steam model, an increase in salt concentration from 1 to 5 M was shown to enhance the reducing sugar yield from 0.07 to 0.25 g/g (Fig 3A), and this association fit a sigmoidal type of relationship (Table 1, Eq e). The microwave model exhibited a hyperbolic relationship between salt concentration and reducing sugar yield where an increase from 0 to 1.25 M gave an increase in reducing sugar from 0.92 to approximately 1.08 g/g while a further increase in salt concentration to 2 M decreased the sugar yield to 1.04 g/g (Fig 3B). This observation is further corroborated by our previous study (Moodley and Kana, 2017c) where the median values of salt concentration resulted in optimal reducing sugar yields. A similar trend was observed by Banerjee et al. (2016) where an increase in NaCl concentration from 1 to 2 M caused a 10 % decrease in saccharification efficiency. The lower yield could be attributed to the degradation of sugars at high salt concentrations (Chen et al., 2010). With regards to the steam model, lower acid concentrations between 0 and 0.5 % were found to release maximum sugar ranging from 0.80 to 1.16 g/g (Fig 3C) and this interaction was well illustrated by a Dose response relationship (Table 1, Eq f). This sugar release trend can be accounted for by the presumptive release of fewer inhibitory compounds owing to the relatively mild process conditions employed thus increasing the reducing sugar yield (Jung and Kim, 2015). An increase in microwave power intensity from 0 to 800 W resulted in a 4 % decrease in sugar yield, from 1.08 to 1.04 g/g (Fig 3D). This was an indication that microwave power intensity had a negligible effect on the reducing sugar yield. Furthermore, it suggested that other input parameters played a more significant role in enhancing reducing sugar yield. With the steam-based model, an increase in solid loading from 5 to 15% resulted in an increase in reducing sugar yield from 0.16 to 0.23 g/g (Fig 3E). The interaction between solid loading and sugar yield fit a Dose response type of functional relationship (Table 1, Eq h). Solid loading in the range of 10 - 15 % have been reported to yield maximum reducing sugar (Raghavi et al., 2016). Similarly, our previous work showed a trend of high reducing sugar (1.12 g/g) with 15 % solid loading and 1.52 M alkali concentration (Moodley and Gueguim Kana, 2017c). The heating time in the microwave model was a significant factor since an increase from 2 to 16 min enhanced the reducing sugar yield from 0.87 to 1.17 g/g (Fig 3F). The interaction between heating time and reducing sugar yield was best illustrated by the modified Gompertz model (Table 1, Eq d). Binod et al. (2012) reported a similar trend in a sequential microwave-assisted alkali-acid pretreatment, where a

15 min heating time gave the highest reducing sugar yield of 0.9 g/g. High alkali concentrations (1.5 – 2.0 M) gave the most noticeable increase in reducing sugar from 0.4 to 0.8 g/g with the steam model. This association was best described by a Dose response relationship (Table 1, Eq g). In contrast, lower alkali concentrations (0 – 0.9 M) gave the highest increase in reducing sugar from 0.2 to 1.02 g/g with the microwave model (Fig 3G) and this interaction best fit the sigmoidal equation (Table 1, Eq b). The role of alkali in pretreatment is primarily delignification with some hemicellulose solubilization. Alkali pretreatment combined with high temperature usually achieves optimal results. Microwave irradiation is also a more efficient heating tool and achieves a higher temperature in a shorter time (Aguilar-Reynosa et al., 2017). Therefore, the lower alkali concentration coupled with microwave heating could account for the higher sugar yield compared to the steam model.

Table 1. Model equations describing the effect of fractional changes in the input parameters on the process output.

Eq.	Input	Model Equation Form	Equation Type	Fitted Model	R ² value
Microwave					
(a)	Salt Conc.	$y = \frac{a + bx}{1 + cx + dx^2}$	Regression	$y = \frac{0.925 + 0.647x}{1 + 0.409x + 0.068x^2}$	0.99
(b)	Alkali Conc.	$y = \frac{a}{(1 + e^{b-cx})^{1/d}}$	Sigmoidal	$y = \frac{1.076}{(1 + e^{2.075-5.703x})^{1/1.423}}$	0.99
(c)	Microwave Power	$y = \frac{a + bx}{1 + cx + dx^2}$	Regression	$y = \frac{1.082 - 0.0004x}{1 - 0.0003x}$	0.99
(d)	Time	$y = p \exp(-\exp(\frac{r \exp(1)}{p})(L - x) + 1))$	Modified Gompertz	$y = 1.151 \exp(-\exp(\frac{0.072 \exp(1)}{1.151})(-12.010 - x) + 1))$	0.99
Steam					
(e)	Salt Conc.	$y = \frac{a}{(1 + be^{-cx})}$	Sigmoidal	$y = \frac{0.269}{(1 + 2.298e^{-0.781x})}$	0.99
(f)	Acid Conc.	$y = \alpha + \frac{\theta x^\eta}{\kappa^\eta + x^\eta}$	Dose Response	$y = 1.113 + \frac{-1.033x^{3.621}}{0.597^{3.621} + x^{3.621}}$	0.99
(g)	Alkali Conc.	$y = \alpha + \frac{\theta x^\eta}{\kappa^\eta + x^\eta}$	Dose Response	$y = 0.205 + \frac{0.619x^{12.484}}{1.532^{12.484} + x^{12.484}}$	0.99
(h)	Solid loading	$y = \alpha + \frac{\theta x^\eta}{\kappa^\eta + x^\eta}$	Dose Response	$y = 0.163 + \frac{0.118x^{1.936}}{13.799^{1.936} + x^{1.936}}$	0.99

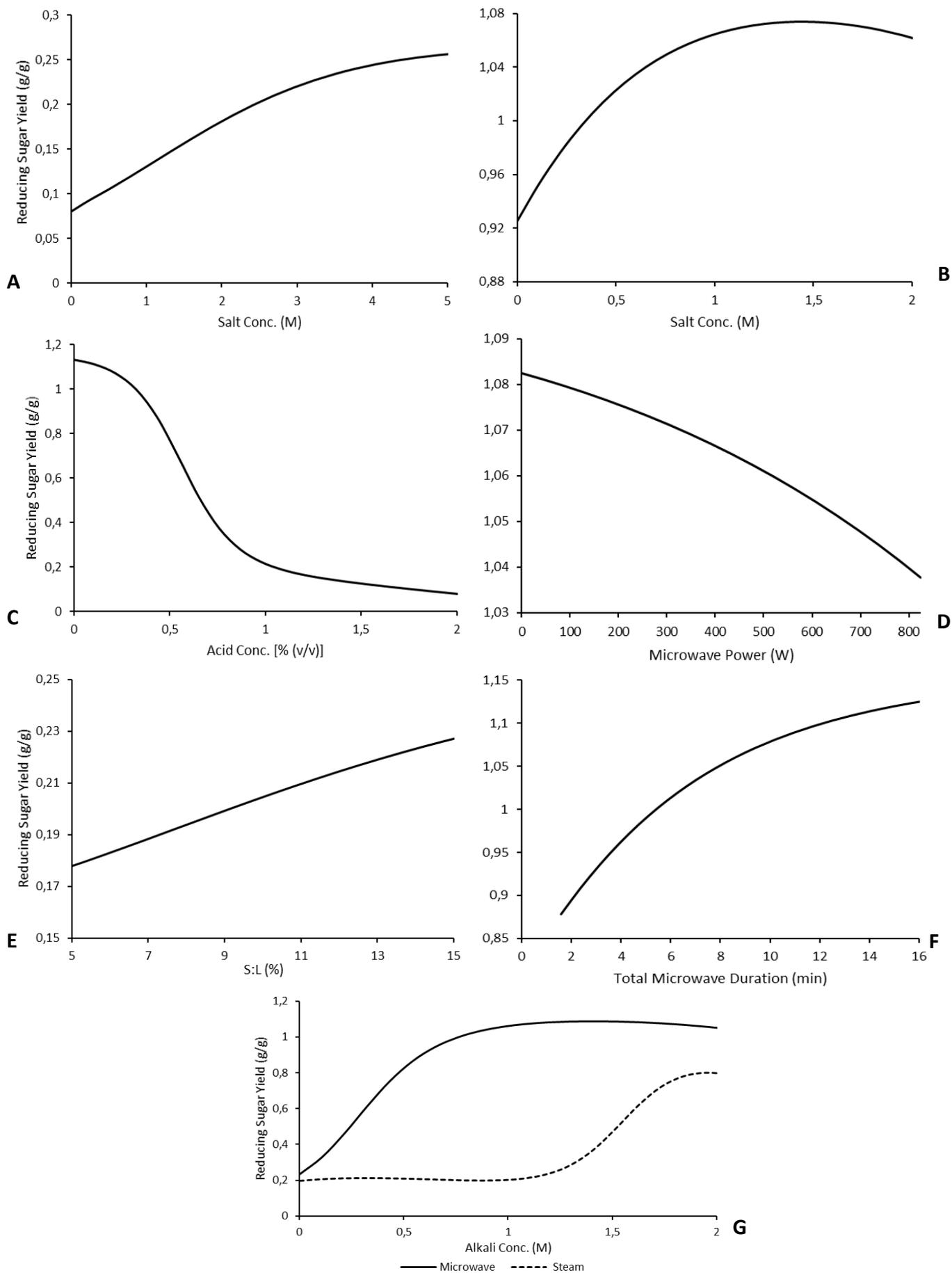


Fig 3. Effect of fractional changes in input parameters on reducing sugar yield for the steam-based model (A, C, E, G) and microwave-based model (B, D, F, G).

4. Conclusion

This study developed a steam-based and a microwave-based ANN models to predict reducing sugar yields. The models were based on sugar yields from the enzymatic hydrolysis of inorganic salt pretreated lignocellulosic waste. Pretreatment data from five models included 90 experimental runs and were data-mined for model development. Both the steam- and microwave-based models were considered statistically adequate since they showed coefficients of determination (R^2) of 0.97. Knowledge extraction revealed reducing sugar yield for both models were highly sensitive to alkali and salt concentration. Interactions between acid concentration, salt concentration and reducing sugar formation showed a dose response and sigmoidal-type of relationship respectively for the steam model. The salt concentration and alkali concentration exhibited a regression and sigmoidal relationship with sugar yield for the microwave model. These models are therefore efficient virtual predicting tools for the screening of pretreatment parameters towards enhancing enzymatic digestibility, potentially improving the economics and productivity of lignocellulosic-based fuels and products.

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CHAPTER 7

Bioethanol production from sugarcane leaf waste using *Saccharomyces cerevisiae* BY4743: Effect of various optimized pretreatments and fermentation conditions on process kinetics.

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Bioethanol production from sugarcane leaf waste using *Saccharomyces cerevisiae* BY4743: Effect of various optimized pretreatments and fermentation conditions on process kinetics.

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Abstract

This study examines the kinetics of *S. cerevisiae* BY4743 growth and bioethanol production from sugarcane leaf waste (SLW), using two different optimized pretreatment regimes; under two fermentation modes: steam salt-alkali filtered enzymatic hydrolysate (SSA-F), steam salt-alkali unfiltered (SSA-U), microwave salt-alkali filtered (MSA-F) and microwave salt-alkali unfiltered (MSA-U). The kinetic coefficients were determined by fitting the Monod, logistic and modified Gompertz models to the experimental data with high coefficients of determination ($R^2 > 0.97$). A maximum specific growth rate (μ_{max}) of 0.153 h^{-1} was obtained under SSA-F and SSA-U whereas, 0.150 h^{-1} was observed with MSA-F and MSA-U. SSA-U gave a potential maximum bioethanol concentration (P_m) of 31.06 g/L compared to 30.49 , 23.26 and 21.79 g/L for SSA-F, MSA-F and MSA-U respectively. No significant difference was observed in the μ_{max} and P_m for the filtered and unfiltered enzymatic hydrolysate for both SSA and MSA pretreatments, thus potentially reducing a unit operation. These findings provide significant insights for process scale up.

Keywords: Lignocellulosic bioethanol, Microwave pretreatment, Sugarcane, Fermentation kinetics, Inorganic salt pretreatment

Nomenclature

SSA-F	Steam salt-alkali filtered enzymatic hydrolysate
SSA-U	Steam salt-alkali unfiltered enzymatic hydrolysate
MSA-F	Microwave salt-alkali filtered enzymatic hydrolysate
MSA-U	Microwave salt-alkali unfiltered enzymatic hydrolysate
X	Cell concentration, g/L
X_0	Initial cell concentration, g/L
X_{max}	Maximum cell concentration, g/L
μ_{max}	Maximum specific growth rate
P	Ethanol concentration, g/L
P_{max}	Maximum potential ethanol concentration, g/L
$r_{p,m}$	Maximum ethanol production rate, g/L.h
t	Fermentation time, h
t_L	Lag phase, h
S	Substrate concentration, g/L
K_S	Monod constant, g/L

1. Introduction

Global energy demand is currently met by fossil fuels with more than 80% of the total energy market comprising of these conventional sources. The transport sector alone accounts for 60% of the total usage (Zabed et al., 2016). However, the finite supply of these fossil fuels and their contribution to greenhouse gas emission upon combustion are major challenges. It is therefore necessary to obtain an alternative source of energy (Chng et al., 2017).

Lignocellulosic biomass is considered an important feedstock for biofuel production in mitigating fossil fuel dependence and its related greenhouse gas emissions (Akthar et al., 2016; Franko et al., 2016). Agricultural wastes, such as sugarcane leaves are currently a major problem for agriculture from an environmental standpoint, thus its conversion to biofuels is highly advantageous (Dominguez-Bocanegra et al., 2015). Second generation bioethanol is one such fuel and is considered clean, affordable and sustainable with the inherent capacity to replace conventional fuel (Mansouri et al., 2016). In contrast, first generation bioethanol utilizes edible feedstocks thereby contributing to the food versus fuel debate (Bhatia et al., 2017).

Microorganisms such as *Saccharomyces cerevisiae* are often employed in the production of bioethanol, thus playing a key role in the fermentation process. However, due to the recalcitrant properties of lignocellulosic biomass, these microorganisms are unable to hydrolyse or access the glucose polymer, cellulose. Furthermore, enzymatic hydrolysis is also hampered due to the complexities in the lignocellulosic structure (Zabed et al., 2016). For this reason, the biomass has to undergo an effective pretreatment prior to fermentation (Sakimoto et al., 2017). Our previous work established a steam and microwave-assisted sequential salt-alkali pretreatment (SSA and MSA respectively) which effectively enhanced enzymatic hydrolysis (Moodley and Gueguim Kana, 2017a). However, the effect of steam and microwave pretreatment could significantly impact on the process kinetics and ultimately the scale-up efficiency and productivity. Currently, there is a scarcity of studies comparing the effect of steam and microwave pretreatment of sugarcane leaves on bioethanol production kinetics using *Saccharomyces cerevisiae* BY4743.

Two main fermentation modes have been frequently reported for bioethanol production, separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) (Carrillo-Nieves et al., 2017). In the SHF process, the pretreated material is hydrolysed to simple sugars and subsequently undergoes fermentation. A major advantage

of this process is that it allows independent optimization of the enzymatic and fermentation phase to maximize sugar and ethanol yield respectively (Carrillo-Nieves et al., 2017). However, there is high cost to separating the solid and liquid fractions of the hydrolysate, particularly at large scale (Aden and Foust, 2009). Several studies have highlighted the SSF system as a potential solution. In this strategy, the hydrolysis and fermentation occur in the same reactor, thus negating the need for a separation stage. This technique also has significant drawbacks such as specialised equipment requirements, high concentration of inhibitor formation and non-reusability of the yeast due to lignin separation (Carrillo-Nieves et al., 2017). However, the main drawback is the different optimum temperatures required for the enzyme (usually cellulase) and the fermenting microorganism, usually 50 and 30°C respectively (Olofsson et al., 2008). Ultimately, preference is given to the microorganism resulting in a sub-optimal saccharification process. Another potential solution, is to remove the separation stage from the SHF process. There is a dearth of knowledge on the effect of filtered and unfiltered enzymatic hydrolysate on fermentation process kinetics. Moreover, the solid waste residue from the SHF process effluent could be an attractive additional revenue stream for animal feed since the plant material has been delignified to enhance digestibility. Furthermore, there could be an increase in protein content due to the yeast cell biomass (Zadrazil and Puniya, 1995).

With increasing interest in the commercial applications of batch bioethanol processes, several kinetics models have been developed which describe microbial growth, product formation and substrate consumption (Phukoetphim et al., 2017). These models are extremely useful in the process development of bioethanol production, since they assist in predicting fermentation performance in response to changes in various factors (Manikandan et al., 2008). This study employs the Monod, logistic and modified Gompertz models to comparatively describe the microbial growth and bioethanol production from pretreated SLW.

The aim of this study was to therefore examine the kinetics of SHF bioethanol fermentation from two previously optimized pretreatment techniques of sugarcane leaf waste, under two fermentation modes using *Saccharomyces cerevisiae* BY4743. These include SSA filtered enzymatic hydrolysate (SSA-F), SSA unfiltered enzymatic hydrolysate (SSA-U), MSA filtered enzymatic hydrolysate (MSA-F) and MSA unfiltered enzymatic hydrolysate (MSA-U). In addition, the potential of the fermentation effluent as animal feed was also explored.

2. Methods

2.1 Feedstock and pretreatment

Sugarcane leaf waste (SLW) was harvested from a sugarcane plantation located in the North Coast of South Africa (29° 42' 18" S, 31° 02' 44" E). Prior to pretreatment, the leaves were dried at 60°C for 72 h and milled to particle sizes ≤ 1 mm. The substrate pretreatment protocols have been described in our previous study (Moodley and Gueguim Kana, 2017a). Briefly, for the steam salt-alkali method (SSA), SLW was first treated with 1.73 M ZnCl₂ for 30 min at 121 °C followed by 1.36 M NaOH at 121 °C for 30 min. For the microwave-assisted salt-alkali (MSA), SLW was pretreated with 1.67 M ZnCl₂ at 400 W for 5 min in the first stage followed by 1.52 M NaOH in the second stage. All pretreated samples were washed thoroughly with deionized water until a neutral pH was reached. Enzymatic hydrolysis was performed in sodium citrate buffer (pH 4.8, 0.05 M) using a solid and enzyme loading of 10 % (w/v) and 10 FPU/g respectively. The commercial cellulase enzyme preparation, Cellic CTec 2, was generously provided by Novozymes (Novozymes A/S, Denmark). Saccharification was achieved at 50 °C for 72 h at 120 rpm in a shaking incubator.

2.2 Microorganism and inoculum development

Saccharomyces cerevisiae BY4743 used in this study was obtained from the Department of Genetics, University of KwaZulu-Natal, Pietermaritzburg, South Africa. The culture was aseptically maintained on a double strength YPD slant (20 g/L yeast extract, 40 g/L peptone and 40g/L dextrose). Prior to fermentation, the stock culture was streaked onto YPD media (10 g/L yeast extract, 20 g/L peptone and 20g/L dextrose) and incubated at 30°C for 24 h thereafter a single colony was inoculated into YPD broth and incubated at 30 °C overnight in a shaking incubator at 120 rpm.

2.3 Batch fermentation

2.3.1 Fermentation medium

Bioethanol production was investigated with two pretreatment types under two different fermentation modes. These included: steam salt-alkali filtered enzymatic hydrolysate (SSA-F), steam salt-alkali unfiltered enzymatic hydrolysate (SSA-U), microwave salt-alkali filtered

enzymatic hydrolysate (MSA-F) and microwave salt-alkali unfiltered enzymatic hydrolysate (MSA-U). For experiments examining the effects of unfiltered enzymatic hydrolysate on process kinetics, the enzymatic hydrolysate did not undergo a filtering process to remove the solid residues. Pretreated SLW was added to give an initial glucose concentration of 60 g/L in the fermentation media. Additional nutrients consisted of yeast extract 5 g/L, peptone 5 g/L, KH_2PO_4 2g/L, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 1g/L and $(\text{NH}_4)_2\text{SO}_4$ 1g/L.

2.3.2 Fermentation conditions

Bioethanol fermentation was performed in 100 ml Erlenmeyer flasks in duplicate. A 10 % (v/v) inoculation was used with an initial cell count of between 10^6 and 10^8 cells/ml. The pH of the fermentation medium was adjusted to 4.5 and fermentation was carried out at 30 °C with an agitation of 120 rpm for approximately 24 h or until ethanol production ceased. Aliquots from duplicate runs were withdrawn for sugar, ethanol and biomass analysis every 2 h.

2.4 Analytical methods

Total reducing sugar was quantified using a glucose standard with the 3,5-dinitrosalicylic acid method (Miller, 1959) and glucose was measured using a glucose kit assay (Megazyme). The yeast biomass concentration in the fermentation broth was determined using a pre-established correlation dependence on biomass dry weight as a function of cell count (Phukoetphim et al., 2017). The concentration of bioethanol was determined using a Vernier Ethanol sensor interfaced with the Vernier LabQuest monitor (ETH-BTA, Vernier Software and Technology, USA). The sensor employs a metal oxide semiconductor to detect ethanol. In the measuring principle, ethanol is consumed in a combustion reaction with the metal oxide, thus reducing the internal resistance of the sensor element. The change in resistance is converted to a response voltage corresponding to ethanol concentration. The sensor was calibrated and tested with known concentrations of ethanol prior to analysis. Crude protein, ash and fat content in the fermentation effluent was analysed using previously established protocols (Whitaker and Granum, 1980; Horwitz, 1980).

2.5 Kinetic models and calculation of kinetic parameters

The kinetic studies of the four fermentation types (SSA-F, SSA-U, MSA-F and MSA-U) were investigated. The growth kinetics was described using Monod's equation with the following assumptions: (a) the broth culture in the flask was homogenous, (b) yeast cells did not become non-viable and (c) the mixing speed of 120 rpm was in excess of the needs for the fermentation process to provide adequate mass transfer. The Monod equation describes the relationship between cell growth rate and substrate concentration. To obtain the Monod kinetic parameters K_S and μ_{max} , five experiments with varying initial glucose concentration (10, 20, 40, 50, 70 g/L) were conducted in duplicate. The processes were sampled every 2 h, and sugar consumption and cell growth were monitored. The specific growth rates (μ) were estimated using experimental data obtained during the exponential phase by linear regression from the slope of natural log of biomass vs time (Eq. 1):

$$\mu = \frac{\ln(X_2 - X_1)}{t_2 - t_1} \quad (1)$$

The maximum specific growth rate (μ_{max}) and Monod constant (K_S) were subsequently estimated using the non-linear least squares method (Englezos and Kalogerakus, 2001).

$$\mu = \frac{\mu_{max}S}{K_S + S} \quad (2)$$

In addition to the Monod kinetic model, the logistic model is increasingly being used to describe microbial growth systems. A term considering inhibition of growth by ethanol concentration was not included, since the maximum ethanol concentration obtained in this study is far below the 15% threshold which inhibits yeast cells (Bai et al., 2008). The differential form of the logistic equation (3) is shown below:

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

where X_{max} is the maximum yeast cell concentration (g/L) and μ_{max} is the maximum specific cell growth rate (h^{-1}). With the following boundary conditions: $t = 0, \therefore X = X_0$, a sigmoidal variation of X is given as a function of t . Equation 3 can then be integrated to give the logistic equation 4 which describes the exponential and stationary phase. The experimental data was used to fit this equation.

$$X = \frac{X_0 \cdot \exp(\mu_{max} \cdot t)}{1 - (X_0/X_{max}) \cdot (1 - \exp(\mu_{max} \cdot t))} \quad (4)$$

The above logistic model does not predict the death phase of microorganisms after the stationary phase (Phukoetphim et al., 2017).

The modified Gompertz model was adopted to describe the kinetics of bioethanol formation (Eq. 5). This model defines the change in ethanol concentration during the course of fermentation. Experimental data was used to fit the modified Gompertz equation using the least squares method (CurveExpert V1.5.5):

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

where P is the bioethanol concentration (g/L), P_m is the potential maximum bioethanol concentration (g/L), $r_{p,m}$ is the maximum bioethanol production rate (g/L h) and t_L is the lag phase (h).

The sugar utilization, ethanol (EtOH) productivity and fermentation efficiency were calculated using equations 6, 7 and 8 respectively:

$$\text{Sugar utilization (\%)} = \frac{\text{Amount of Initial sugar} - \text{final sugar}}{\text{Amount of initial sugar}} \times 100 \quad (6)$$

$$\text{Ethanol productivity (g/L.h)} = \frac{\text{Maximum ethanol concentration (g/L)}}{\text{Fermentation time (h)}} \quad (7)$$

$$\text{Fermentation efficiency (\%)} = \frac{\text{Experimental ethanol yield (g/L)}}{\text{Theoretical ethanol yield (g/L)}} \times 100 \quad (8)$$

3. Results and discussion

3.1 Growth kinetics of *S. cerevisiae* BY4743 on pretreated SLW

The change in biomass concentration over time under the four different fermentation conditions is shown in Fig 1. A shorter lag time of 4 h was observed under SSA-F and SSA-U whereas, slightly longer lag times of 6 h were observed under MSA-F and MSA-U. This can be attributed to the presence of trace amounts of certain inhibitory products in the MSA enzymatic hydrolysate. Microwave-assisted pretreatment has been reported to have a higher severity factor; thus producing a higher concentration of inhibitory compounds compared to steam pretreatment (Aguilar-Reynosa et al., 2017). In addition, there may be production of inhibitors during enzymatic hydrolysis due to the hydrothermal breakdown of the lignocellulosic components owing to the process temperature and duration. Some of the reported inhibitors from microwave-assisted metal chloride pretreatment of SLW include acetic acid, furfural and hydroxymethyl furfural (HMF) (Moodley and Gueguim Kana, 2017b). Acetic acid increases the intracellular pH of *S. cerevisiae* leading to an increase in the lag phase and decrease in the growth rate (Pampulha and Loureiro-Dias, 2000). Similarly, furfural and HMF can synergistically affect the growth rate of *S. cerevisiae* by affecting glycolytic activity, causing oxidative stress and reducing the activity of various dehydrogenases (Iwaki et al., 2013).

The logistic models were in agreement with the experimental values, showing high coefficients of determination ($R^2 > 0.97$). The estimated values for the kinetic parameters were found to be in close range with the empirical values. These values and the developed models for each fermentation type are summarized in Table 1. Slightly higher X_{max} values were obtained under SSA-F and SSA-U fermentation conditions (4.70 and 4.56 g/L respectively) compared to 4.36 and 4.35 g/L for MSA-F and MSA-U respectively, further suggesting the steam pretreated substrate was more favourable and promoted cell growth. Phukoetphim et al. (2017) reported a similar maximum cell concentration (X_{max}) of 5.145 g/L from sweet sorghum juice whereas, Dodic et al. (2012) reported a value of 8.381 g/L from sugar beet juice. These varying X_{max} values could be accounted for by differences in yeast strain, substrate and working volume. The maximum specific growth rate (μ_{max}) obtained from the logistic model under SSA-F and SSA-U conditions were 0.24 and 0.26 h⁻¹ respectively whereas, MSA-F and MSA-U were 0.28 and 0.29 h⁻¹ respectively. This was an indication that filtered and unfiltered enzymatic hydrolysate had a negligible effect on the maximum specific growth rate. This further implies the non-requirement of a separation stage, thus enhancing process economics and productivity at a large scale. However, the μ_{max} values obtained from the Monod model were 0.153 h⁻¹ for SSA-F and SSA-U, and 0.150 h⁻¹ for MSA-F and MSA-U. The difference in the μ_{max} values between model types (logistic and Monod) can be ascribed to the intrinsic parameters and boundaries employed by each model. For instance, the logistic model considers the biomass concentration from the lag phase to stationary phase, disregarding the substrate utilization whereas, Monod considers both the biomass concentration (however only in exponential phase) and the rate limiting substrate (Kargi, 2008). Differences in μ_{max} values from the logistic and Monod models have been previously reported. Manikandan and Viruthagiri (2009) observed a μ_{max} of 0.307 and 0.095 h⁻¹ using the Monod and logistic model respectively for ethanol production from wheat flour. Likewise, the Monod and logistic model gave μ_{max} values of 0.65 and 0.45 h⁻¹ using glucose for ethanol production (Shafaghat et al., 2009). The maximum specific growth rates obtained in the present study are within range of previous studies. Srimachai et al. (2015) reported a μ_{max} of 0.15 h⁻¹ from oil palm frond juice and a μ_{max} of 0.27 h⁻¹ was reported from sweet sorghum juice (Phukoetphim et al., 2017). The obtained μ_{max} values are highly desirable, particularly for commercial scale up since growth rates > 0.025 h⁻¹ have been shown to linearly increase the fermentative capacity of *Saccharomyces* species. Furthermore, higher growth rates may trigger respirofermentative metabolism, thus resulting in an increase in fermentative capacity (Hoek et al., 1998). Moreover, the μ_{max} values are within

range of previous pilot scale studies. For example, a μ_{max} of 0.34 h^{-1} was reported in the production of ethanol from molasses at 300 000 L (Arshad et al., 2017).

Monod constants (K_S) of 4.91 g/L for SSA-F and SSA-U, and 5.61 g/L for MSA-F and MSA-U were obtained. A lower K_S value indicates the microorganism's inherent affinity to the substrate since its reciprocal describes the cells affinity to the substrate type. The higher K_S value obtained under MSA-F and MSA-U conditions could be explained by the presumptive presence of inhibitory compounds in the fermentation medium. Overall, *S. cerevisiae* showed a higher substrate affinity with the steam (0.20 g/L^{-1}) and microwave (0.17 g/L^{-1}) pretreated SLW compared to previous studies on sweet sorghum juice (0.021 g/L^{-1} , Ariyajaroenwong et al., 2016) and sorghum leaves (0.10 g/L^{-1} , Rorke and Gueguim Kana, 2017). The difference in K_S is affected by substrate type and concentration, and yeast strain and concentration (Felix et al., 2014).

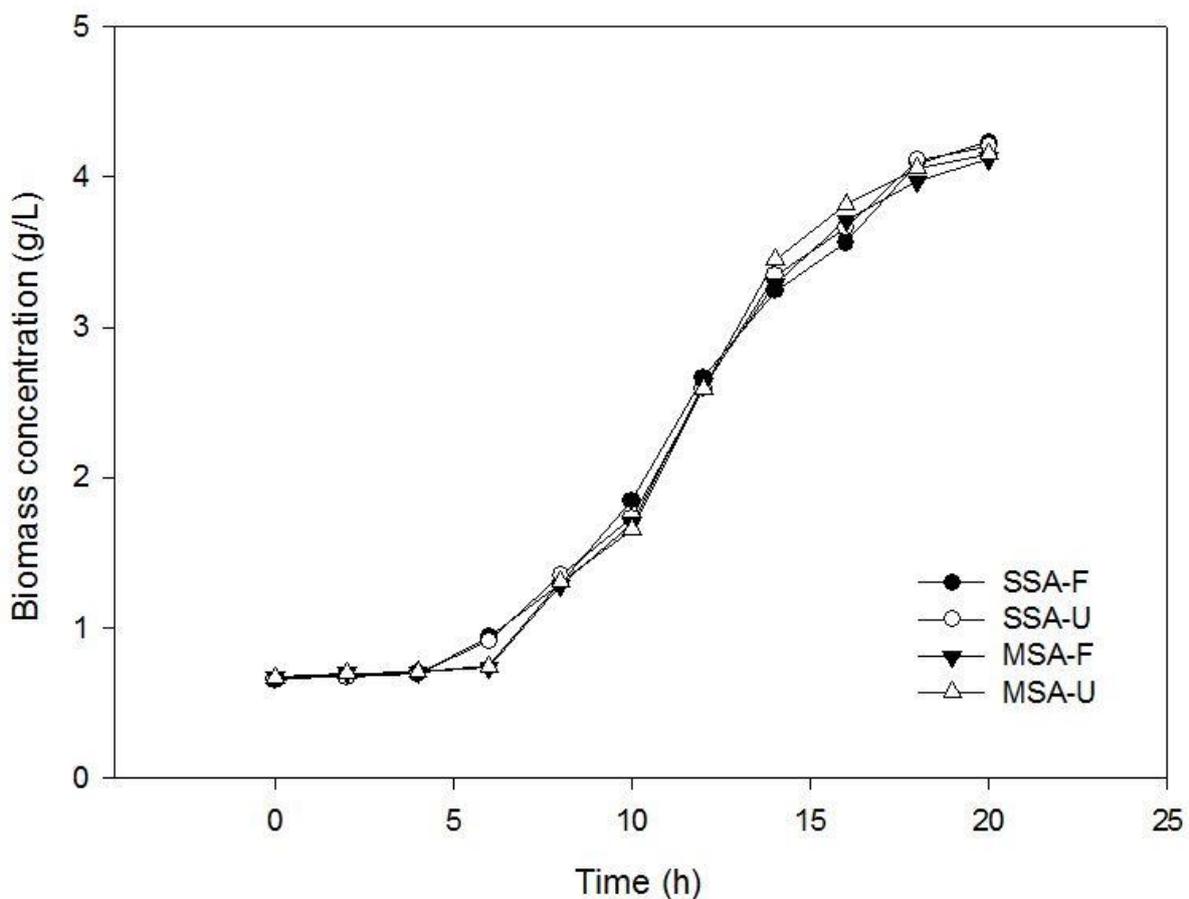


Fig 1. Time course of biomass concentration under the four examined fermentation conditions.

Table 1: The logistic models describing cell growth under different fermentation condition

Fermentation conditions	X_0 (g/L)		X_{max} (g/L)		μ_{max} (h ⁻¹)		Logistic equation	R^2
	Pred	Exp	Pred	Exp	Pred	Exp		
SSA-F	0.27	0.26	4.70	4.41	0.24	0.24	$X = \frac{0.27 \cdot \exp(0.24 \cdot t)}{1 - (0.27/4.70) \cdot (1 - \exp(0.24 \cdot t))}$	0.98
SSA-U	0.23	0.20	4.56	4.54	0.26	0.24	$X = \frac{0.23 \cdot \exp(0.26 \cdot t)}{1 - (0.23/4.56) \cdot (1 - \exp(0.26 \cdot t))}$	0.98
MSA-F	0.20	0.16	4.36	4.27	0.28	0.26	$X = \frac{0.20 \cdot \exp(0.28 \cdot t)}{1 - (0.20/4.36) \cdot (1 - \exp(0.28 \cdot t))}$	0.98
MSA-U	0.18	0.14	4.35	3.15	0.29	0.27	$X = \frac{0.18 \cdot \exp(0.29 \cdot t)}{1 - (0.18/4.35) \cdot (1 - \exp(0.29 \cdot t))}$	0.97

Pred – Predicted

Exp – Experimental

Table 2. The effect of different fermentation conditions and substrate type on Monod kinetic parameters

Substrate	Kinetic parameter		Reference
	μ_{\max} (h ⁻¹)	Ks (g/L)	
SLW (SSA-F and SSA-U)	0.153	4.19	This study
SLW (MSA-F and MSA-U)	0.150	5.61	This study
Sorghum leaves	0.176	10.11	Rorke and Gueguim Kana, 2017
Sweet sorghum juice	0.119	2.08	Thangprompan et al., 2013
Sweet sorghum juice	0.313	47.51	Ariyajaroenwong et al., 2016
Glucose	0.133	3.7	Singh and Sharma, 2015
Oil palm frond juice	0.15	10.21	Srimachai et al., 2015

3.2 Kinetics of bioethanol fermentation from pretreated SLW

The experimental profiles for bioethanol production and glucose consumption with *S. cerevisiae* from SSA-F and MSA-F are shown in Fig 2. Ethanol production commenced almost immediately from the initial hours of fermentation and increased gradually until it peaked at 18 h into the process. Under SSA-F conditions, a considerably higher ethanol concentration (28.47 g/L) was achieved compared to MSA (23.01 g/L). This higher ethanol production under SSA-F compared to MSA-F conditions is substantiated by the higher maximum specific growth rate and substrate affinity observed in the Monod models. Li et al. (2016) reported a significantly lower ethanol concentration (17.5 g/L) from acid pretreated corn leaves. Similarly, a lower ethanol concentration (4.71 g/L) from acid pretreated sugarcane leaves was reported by Jutakanoke et al. (2012). Under SSA-F conditions, *S. cerevisiae* showed a higher glucose consumption rate of 4.5 g/L.h from 0 to 6 h into the fermentation whereas a lower glucose consumption rate of 4.0 g/L.h was observed with MSA-F between 0 and 8 h of fermentation.

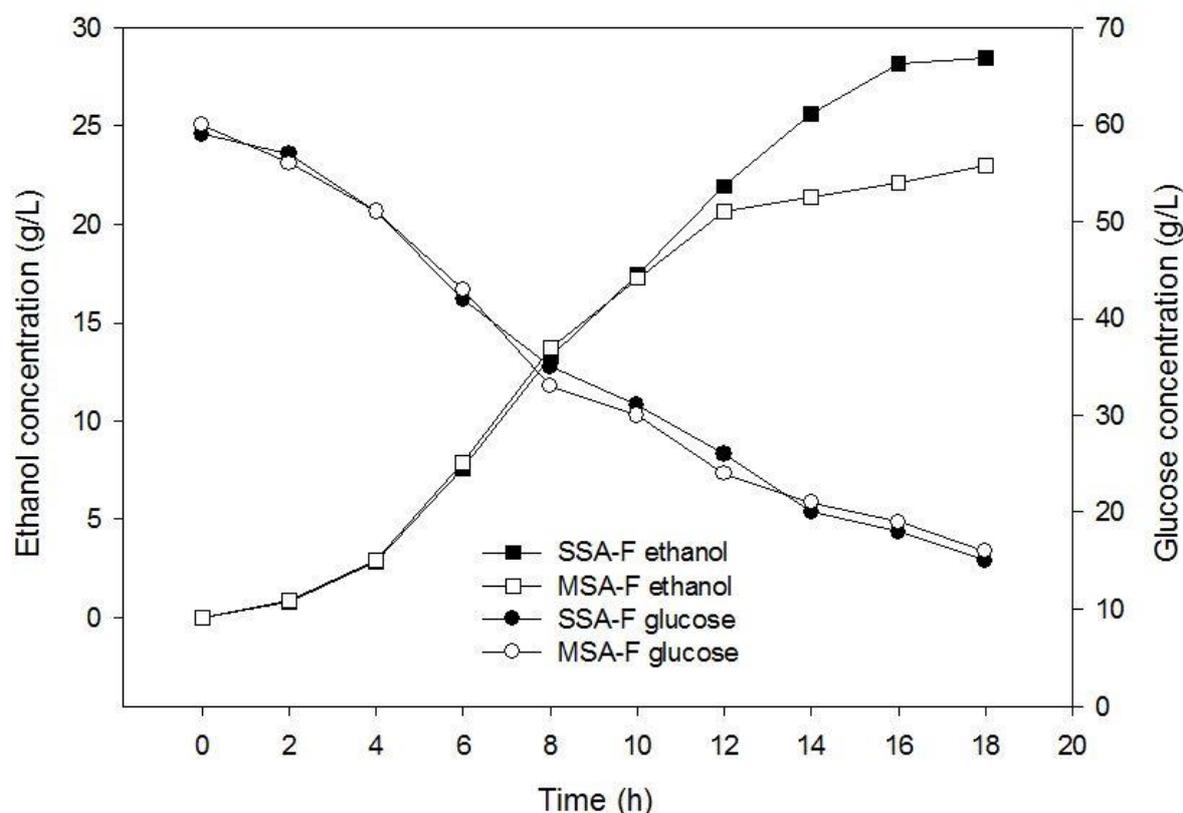


Fig 2. Times course of bioethanol production and glucose consumption from SLW under SSA-F and MSA-F fermentation conditions.

The profile of ethanol production and glucose consumption from SLW with SSA-U and MSA-U is shown in Fig 3. The ethanol production and sugar consumption trends, showed similarities to the SSA-F and MSA-F conditions presented in Fig 2. The ethanol production commenced 2 h into the fermentation and peaked at 18 h with 28.81 g/L (SSA-U) and 16 h with 22.72 g/L (MSA-U). Similarly, *S. cerevisiae* reached a maximum glucose consumption rate of 5g/L.h during the first 6 h of fermentation with SSA-U whereas, a glucose consumption rate of 4.5 g/L.h was obtained during the first 7 h of fermentation with MSA-U. The lower ethanol concentration observed under MSA-U conditions could be ascribed to the decline in pH from 4.54 to 3.90 compared to the SSA-U process with a relatively stable pH slightly decreasing from 4.55 to 4.23 (Fig. 4). The decline in pH could be attributed to the generation of acetic acid from the hydrothermal breakdown of the hemicellulosic acetyl groups present in the pretreated sugarcane leaf waste biomass (Jonsson and Martin, 2016). Furthermore, the severity of microwave pretreatment would infer a higher concentration of acetic acid release compared to steam pretreatment. In addition, the presumptive presence of furfural in the enzymatic hydrolysate could be a contributor to the decline in pH since *S. cerevisiae* has been shown to metabolize furfural compounds into furoic acid and furfuryl alcohol (Horvath et al., 2003). The decline in pH from the 11th h of fermentation (MSA-U) also coincided with the decrease in glucose consumption and ethanol production rates from 2.5 to 1 g/L.h and 2.3 to 0 g/L.h respectively. Furthermore, *S. cerevisiae* is known to produce ethanol optimally at pH of 4.5. A pH beyond this range affects the activity of plasma membrane-bound proteins and includes both enzymes and transport proteins (Narendranath and Power, 2005). The pH of the SSA-F and SSA-U experiments showed a relatively slower drift remaining close to the optimum value of 4.5 compared to MSA-F and MSA-U with the final pH values below 4. This could account for the lower ethanol concentration and glucose consumption.

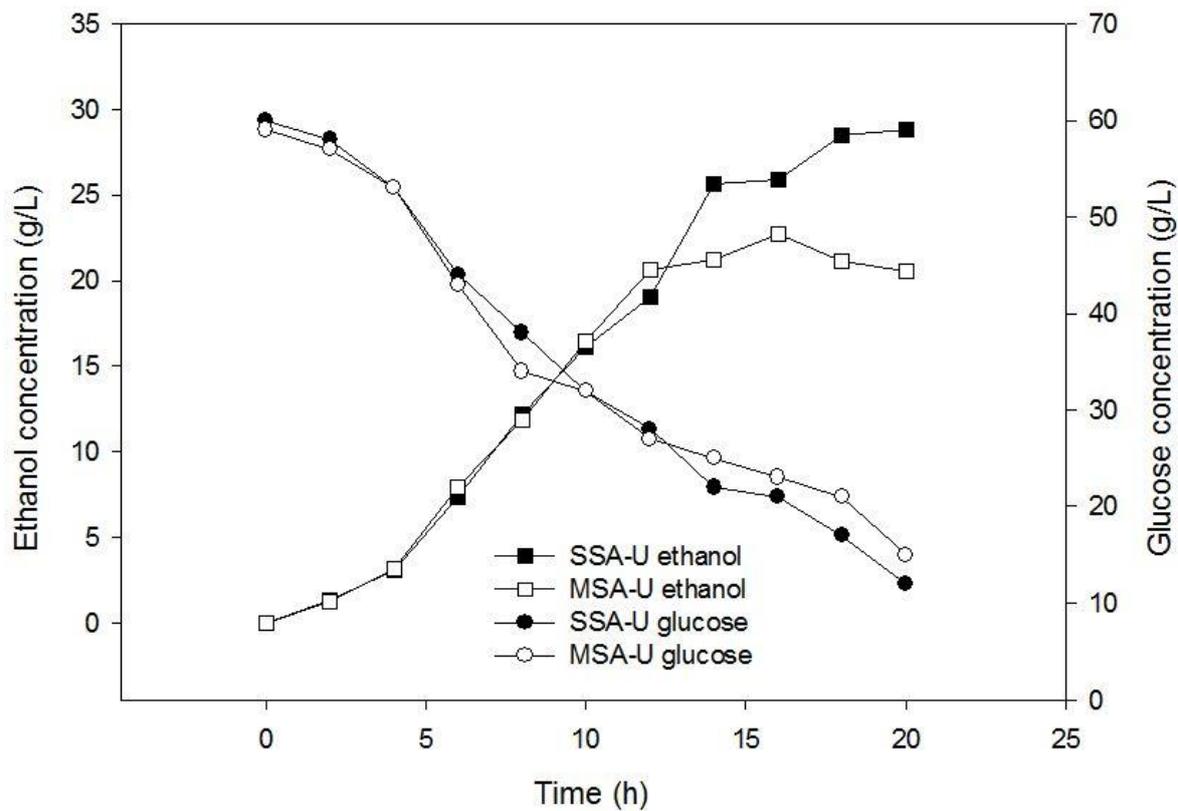


Fig 3. Times course of bioethanol production and glucose consumption from SLW under SSA-U and MSA-U fermentation conditions.

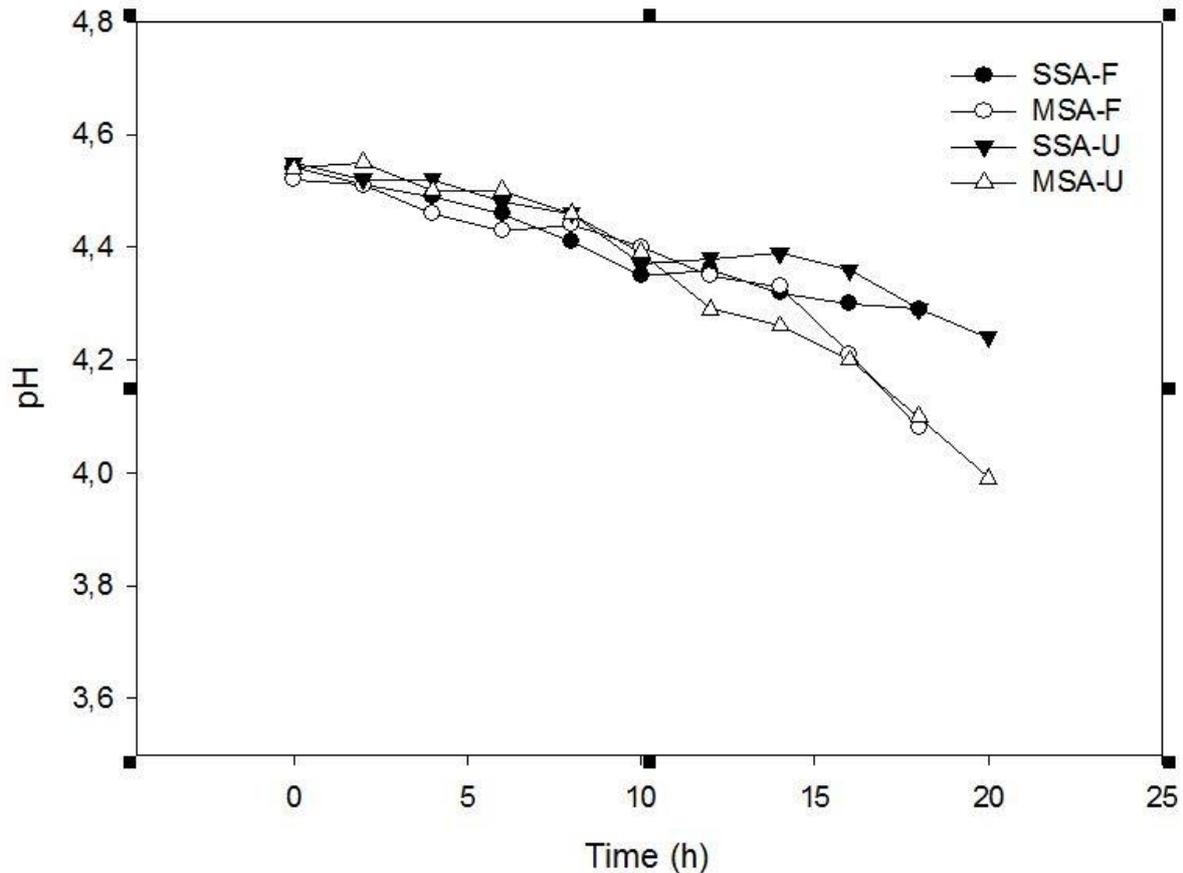


Fig 4. Time course of pH evolution during ethanol fermentation from SLW under SSA-F, SSA-U, MSA-F and MSA-U conditions.

The modified Gompertz model fit the experimental data well under the four fermentation conditions (SSA-F, SSA-U, MSA-F, MSA-U) with high coefficients of determination (R^2) > 0.99 (Table 3). The potential maximum bioethanol concentration (P_m) ranged from 31.06 g/L (SSA-U) to 21.79 g/L (MSA-U). The high P_m value obtained under SSA-U can be attributed to its higher μ_{max} and $1/K_s$ values compared to MSA-U thus inferring the *S. cerevisiae* cells had a higher affinity for the steam pretreated substrate. In addition, taking into account the aforementioned factors that contributed to the decline in pH, the higher P_m obtained for SSA-U was expected. The undissociated form of weak lipophilic acids such as acetic acid induces acidification of the cell cytoplasm by accumulating inside the cells. This leads to a decrease in cell metabolic activity (Pampulha and Loureiro-Dias, 1989).

Yan et al. (2013) reported a higher P_m (104 g/L) from food waste using *S. cerevisiae* HO58 whereas, a lower P_m (17.15 g/L) was recorded from sorghum leaves using *S. cerevisiae* BY4743 (Rorke and Gueguim Kana, 2017). The differences in P_m can be attributed to different sugar concentrations and yeast strains employed. Compared to the reported maximum ethanol production rate ($r_{p,m}$) of 2.09 g/L.hr obtained from sweet sorghum juice and 0.08 g/L.hr reported from oil palm frond (Srimachai et al., 2015), the $r_{p,m}$ obtained in this study between 2.44 and 2.85 g/L.h, are desirable since higher production rates are preferred at large scale. Likewise, a shorter lag time is favoured thereby implying the yeast cells have acclimated to the fermentation conditions. The lag time (t_L) for bioethanol production in this study ranged from 3.14 to 3.39 h thereby indicating no significant variation between the SSA-F, SSA-U, MSA-F and MSA-U experiments. Higher lag times have been reported by Rorke and Gueguim Kana (2017) from sorghum leaves (6.31 h) and Yan et al. (2013) from enzymatically pretreated food waste (6.41 h). A similar lag time of 3.07 h was observed by Phukoetphim et al. (2017) from sweet sorghum juice whereas a low lag time (1.04 h) was reported from sugar beet raw juice (Dodic et al., 2012). Lag time can be affected by factors such as working volume, inoculum type and size, and substrate type and concentrations.

The SSA-F and SSA-U experiments gave a similar fermentation efficiency (92.86 and 93.97 % respectively) and ethanol productivity (1.095 and 1.11 g/L h respectively), as shown in Table 4. Sugar utilization followed a similar trend with 86.67 % and 83.33 % for SSA-F and SSA-U respectively. MSA-F and MSA-U gave lower fermentation efficiencies of 75.05 and 74.10 % respectively and ethanol productivities of 0.885 and 0.874 g/L h respectively. Therefore, no significant difference was observed between filtered and unfiltered enzymatic hydrolysate, indicating that the presence of sugarcane leaf biomass did not hinder bioethanol production. In fact, under SSA-U conditions, ethanol production was slightly higher. Reported ethanol production from oil palm frond juice and sugarcane juice showed a lower fermentation efficiency compared to SSA but a higher efficiency compared to MSA (Table 4; Srimachai et al., 2015; Ramos et al., 2013).

The unfiltered enzymatic hydrolysate results (SSA-U and MSA-U) are comparable to previous studies where the enzymatic hydrolysate was filtered prior to fermentation. For instance, Mishra et al. (2016) observed an ethanol concentration of 29 g/L from filtered enzymatic hydrolysate of acid pretreated rice straw. A maximum ethanol concentration of 2.95 g/L was reported from the filtered enzymatic hydrolysate of alkali pretreated hazelnut shells (Hosgun et al., 2017). This is an indication that unfiltered enzymatic hydrolysate gave similar ethanol

concentrations to filtered enzymatic hydrolysate in a separate hydrolysis and fermentation (SHF) system. The separation of the solid biomass requires an additional unit operation that can contribute to about 5 % of the annual operating costs; thus impacting on the process economics at large scale (Aden and Foust, 2009). In addition, the process time for centrifugation or filtering reduces the productivity of the process and impacts the number of batch runs annually. Some studies have employed the simultaneous saccharification and fermentation (SSF) system to circumvent the need for a filtering stage however, previous reports on SSF have given significantly lower ethanol concentrations compared to the concentrations obtained in this study. For example, a SSF system using steam exploded acorn produced 1.97 g/L ethanol (Sasaki et al., 2014) while acid pretreated *Saccharina japonica* gave 6.65 g/L ethanol (Lee et al., 2013). A slight higher ethanol concentration of 13.6 g/L was reported from *Arundo donax* (Mutturi and Liden, 2013). Although these lower yields could be attributed to many factors such as yeast strain and substrate, the SHF system does offer some attractive features. This includes the ability to optimize the saccharification and fermentation process separately, thereby improving the respective product yields.

Table 3. Comparison of the kinetic values in the modified Gompertz model from SLW and other lignocellulosic biomass

Substrate	Modified Gompertz model				References
	P_m (g/L)	$r_{p,m}$ (g/L.hr)	t_L (h)	R^2	
SLW (SSA-F)	30.49	2.81	3.39	0.99	This study
SLW (SSA-U)	31.06	2.44	3.14	0.99	This study
SLW (MSA-F)	23.26	2.85	3.17	0.99	This study
SLW (MSA-U)	21.79	2.79	3.22	0.99	This study
Sorghum	17.15	0.52	6.31	0.98	Rorke and Gueguim Kana (2017)
Sugar beet raw juice	73.31	4.39	1.04	0.99	Dodic et al. (2012)
Sweet sorghum juice	60.04	2.09	3.07	0.99	Phukoetphim et al. (2017)
Food waste	104	2.22	6.41	0.99	Yan et al. (2013)
Oil palm frond juice	3.79	0.08	0.77	-	Srimachai et al. (2015)

Table 4. Comparison of bioethanol production from SLW and other reported lignocellulosic biomass

Substrate	Sugar utilization (%)	Max ethanol production (g/L)	Ethanol productivity (g/L hr)	Fermentation efficiency (%)	Reference
SLW (SSA-F)	86.67	28.47	1.095	92.86	This study
SLW (SSA-U)	83.33	28.81	1.11	93.97	This study
SLW (MSA-F)	78.33	23.01	0.885	75.05	This study
SLW (MSA-U)	76.27	22.72	0.874	74.10	This study
Oil palm frond juice	94.05	11.50	0.12	76.52	Srimachai et al, 2015
Sugarcane juice	98.00	67.00	0.93	78.43	Ramos et al., 2013
Sweet sorghum juice	100	72.43	1.01	94.60	Wu et al., 2010

3.3 Feed analysis

Effluent from the SSA-U process (ethanol concentration of 28.81 g/L and final biomass concentration of 4.56 g/L) underwent compositional and nutritional analysis to determine its potential as animal feed (Table 5). The solid biomass from the SSA-U effluent was shown to contain 6.0 % crude protein. Protein content values of between 1.6 and 26 % are commonly reported in feedstock compositions and therefore the obtained protein content of 6.0% fell within this range (NRC, 2001). Other common animal feed such as wheat and corn cobs have reported a protein content of 4.8 and 3.0 % respectively (NRC, 2001). The high protein content from the SSA-U biomass can be accounted for by the nitrogen rich yeast biomass. Furthermore, the SSA-U process gave a fat content of 2.57 %, which was well within the reported range of 0.1 to 19.3 % from other lignocellulosic biomass (NRC, 2001). Cotton seeds and wheat have previously been reported to contain a similar fat content, 2.5 and 1.9 % respectively. Since fat provides more than twice the energy compared to carbohydrates and proteins, it is an essential component in animal feed (Gurr, 1984). A major bottleneck with many animal feeds is the low digestibility due to the high lignin content (Zadrazil and Puniya, 1995). The SSA pretreatment of SLW caused significant (80.5%) delignification, thereby enhancing the digestibility (Moodley and Gueguim Kana, 2017a). The effluent can be supplemented with additional nutrients, depending on the specific requirements. Developing a suitable methodology for the use of this waste-stream for animal feeding could enhance the environmental and economic outlook of this process since no waste treatment and disposal will be required.

Table 5. Comparison of the feed analysis for the SSA-U solid residues and other common animal feed

Substrate	Ash	Fat	CP	Ca	Mg	K	Na	P	Zn	Cu	Mn	Fe	Ref
	%								mg/kg				
SLW (SSA-U)	6.27	2.57	6.0	0.11	0.09	0.33	0.60	0.29	33	4	30	132	This study
Wheat	7.6	1.9	4.8	0.31	0.14	1.55	0.12	0.10	ND	ND	ND	ND	NRC, 2001
Corn cob	2.2	0.6	3.0	0.10	0.06	0.90	0.04	0.06	ND	ND	ND	ND	NRC, 2001
Cotton seeds	2.8	2.5	6.2	0.18	0.17	1.16	0.02	0.12	ND	ND	ND	ND	NRC, 2001

CP- Crude protein

ND – Not determined

4. Conclusion

In this study, three empirical models, i.e. Monod, logistic and modified Gompertz, were employed to describe *S. cerevisiae* BY4743 growth and ethanol production from pretreated SLW under SSA-F, SSA-U, MSA-F and MSA-U fermentation conditions. All models fit the experimental data well with high coefficients of determination $R^2 > 0.98$, indicating their potential application for large scale operations. Steam salt-alkali pretreated SLW produced 25 % more bioethanol compared to microwave salt-alkali. Furthermore, no difference was observed between filtered and unfiltered enzymatic hydrolysate experiments for both pretreatments. These findings provide crucial insights into enhancing the cost, productivity and environmental outlook for scale up processes.

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Supplementary material

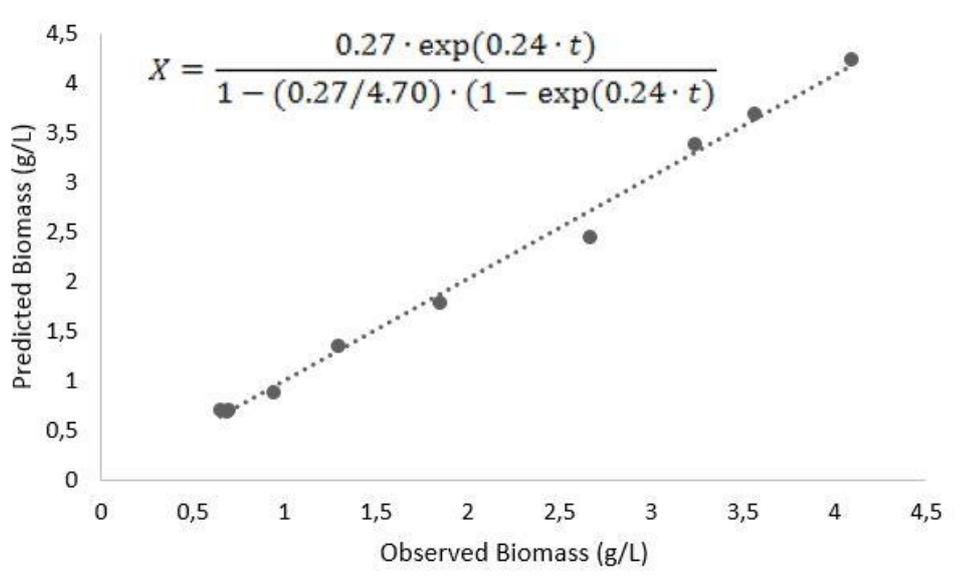


Fig S1. Scatter plot of observed vs predicted biomass yield under SSA-F conditions using the Logistic model.

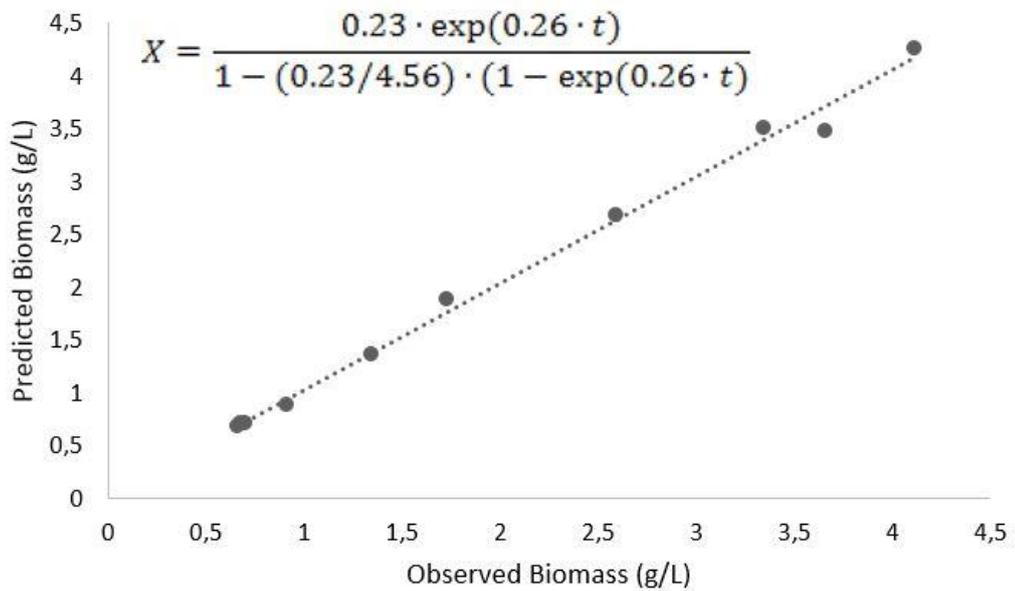


Fig S2. Scatter plot of observed vs predicted biomass yield under SSA-U conditions using the Logistic model.

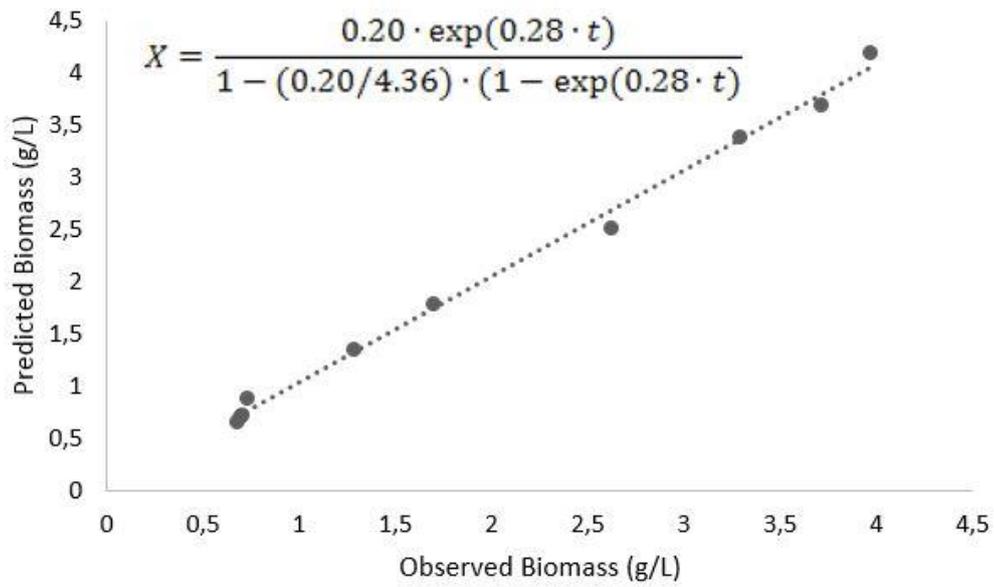


Fig S3. Scatter plot of observed vs predicted biomass yield under MSA-F conditions using the Logistic model.

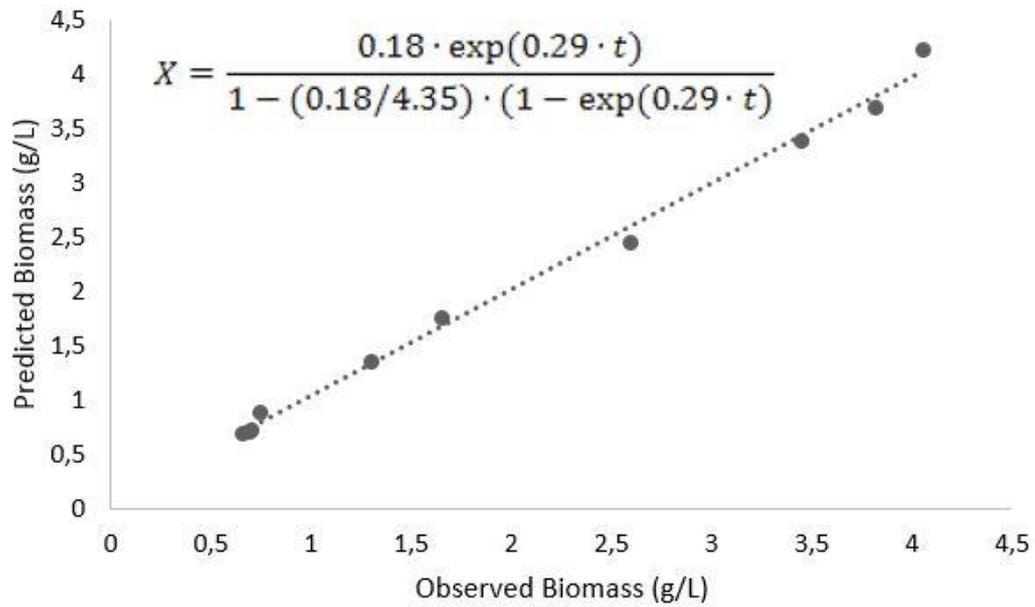


Fig S4. Scatter plot of observed vs predicted biomass yield under MSA-U conditions using the Logistic model.

CHAPTER 8

Conclusions and Recommendations for future work

The implementation of a feasible lignocellulosic bioethanol production process will contribute significantly towards achieving a carbon-neutral bio-economy. This is beneficial for a sustainable energy supply coupled with a reduction in the carbon footprint and environmental pollution. In order to improve lignocellulosic bioethanol production, an efficient and effective pretreatment is required to disrupt the lignocellulosic matrix and yield high sugar. Additionally, the kinetic knowledge on the effect of pretreatment and unfiltered enzymatic hydrolysate on fermentation may provide further insight into enhancing the bioethanol production process. This study focused on developing strategies towards achieving these goals. It was established that:

- A two-stage salt acid lignocellulosic pretreatment regime gave a reducing sugar yield of 0.293 g/g using 3.32 M ZnCl_2 at 121°C for 30 min followed by 1.84 % (v/v) H_2SO_4 at 121°C for 30 min with a 9.26 % (w/v) solid loading. The two-stage regime showed a 9 % yield improvement over the more commonly employed combined single stage technique. This regime also showed a 90 % hemicellulose solubilization and a 1.9 fold yield improvement compared to previous reports. These findings indicated that a two stage ZnCl_2 - H_2SO_4 pretreatment can significantly enhance enzymatic saccharification of lignocellulosic waste.
- Microwave-assisted inorganic salt pretreatment produced 0.406 g/g reducing sugar under pretreatment conditions of 2 M FeCl_3 at 700 W for 3.5 min. This regime showed up to 71.5 % hemicellulose removal efficiency and a 3.1 fold improvement in sugar yield compared to previous reports using similar substrates. These results highlighted that microwave-assisted inorganic salt pretreatment can achieve a considerably high sugar yield in a shorter heating time compared to conventional heating methods.
- A sequential two-stage salt-alkali pretreatment targets hemicellulose removal and delignification of lignocellulosic waste thereby enhancing sugar recovery. Under optimal conditions, the steam salt-alkali pretreatment model yielded 1.21 g/g reducing sugar using 1.73 M ZnCl_2 , 1.36 M NaOH and 9.69 % (w/v) solid loading whereas the

microwave-assisted salt-alkali pretreatment model gave 1.17 g/g sugar from 1.67 M ZnCl₂, 1.52 M NaOH at 400 W for 10 min. The microwave model achieved a high sugar yield in a substantially shorter heating time (83 % lower) compared to the steam model.

- The developed predictive models based on Artificial Neural Networks efficiently predict the sugar yield from inorganic salt-based pretreatments. The steam- and microwave-based models gave high coefficients of determination (R^2) of 0.97. This indicated both models ability to accurately predict on new inputs. These tools can significantly reduce pretreatment development time and cost.
- Lignocellulosic bioethanol production is enhanced when an appropriate pretreatment regime is employed. Bioethanol concentrations of 28.47 and 23.01 g/L were obtained using steam salt-alkali and microwave salt-alkali pretreatments respectively. Microwave heating was shown to negatively affect the lag time (t_L , 3.17 h) and the potential maximum bioethanol concentration (P_m , 23.26 g/L) compared to steam heating which gave a t_L and P_m of 3.39 h and 30.49 g/L respectively. The maximum growth rate (μ_{max}) of *Saccharomyces cerevisiae* BY4743 was 0.150 h⁻¹ and 0.153 h⁻¹ for the microwave and steam pretreated substrate respectively. This was an indication that microwave pretreatment impeded the growth of *S. cerevisiae* BY4743. Furthermore, no significant difference was observed in the μ_{max} and P_m for the filtered and unfiltered enzymatic hydrolysate for both SSA and MSA pretreatments, potentially reducing a unit operation thus enhancing process economics and productivity.

Recommendations for future studies

- In order to enhance bioethanol production yields, metabolically engineered yeast strains should be examined. In addition, yeast strains capable of metabolizing both five- and six-ring sugars may be investigated to enhance the bioconversion efficiency of lignocellulosic waste to ethanol.

- The bioethanol yield may be improved by optimizing key parameters affecting the fermentation process. These include pH, temperature, agitation, substrate concentration and inoculum concentration, among others.
- The process effluent of bioethanol production from lignocellulosic biomass could further be optimized to produce excellent grade animal feed and thus create an additional revenue stream from this bioprocess. Some parameters to be considered are lignin, protein, fat and sugar content.
- Integration of bioethanol production with other renewable energy sources such as biohydrogen or biogas in a biorefinery concept could further enhance substrate conversion efficiency and energy recovery from sugarcane leaf waste and improve the process economics.