The potential of waste sorghum (*Sorghum bicolor*) leaves for bioethanol process development using *Saccharomyces cerevisiae* BY4743

by

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Preface

The research contained in this dissertation was completed by the candidate while based in the Discipline of Microbiology, School of Life Sciences, of the 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 has not been submitted in any form to another university and, except where the work of others is acknowledged in the text, the results reported are due to investigations by the candidate.

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Signed: Professor E.B. Gueguim Kana (Supervisor)

Date: 09 January 2017

Declaration 1: Plagiarism

I, Daneal Rorke, declare that:

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

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

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

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

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

(vi) this dissertation is primarily a collection of material, prepared by myself, submitted for publication or presented at conferences. In some cases, additional material has been included;

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Declaration 2: Publications

This thesis represents a compilation of manuscripts where each chapter is an individual entity prepared as per the journals' specifications therefore 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 (supervisor) author.

1. Rorke, D. C. S., Suinyuy, T. N. and Gueguim Kana, E. B. Microwave-assisted chemical pre-treatment of waste sorghum leaves: Process optimization and development of an intelligent model for determination of volatile compound fractions. Bioresource Technology 2017. 224: 590-600. (Chapter 3).
2. Kinetics of Bioethanol Production from Waste Sorghum Leaves using *Saccharomyces cerevisiae* BY4743. Submitted to: Fermentation (under review). (Chapter 4).

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Conference proceedings and contributions

1. Rorke, D. and Gueguim Kana, E. B. Volatile Compound Profile Analysis & Modelling for Microwave-assisted Chemical Pre-treatment of Sorghum Leaves. Annual College of Agriculture, Engineering and Science Research & Innovation Day. 29 November 2016, Poster Presentation.

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Abstract

The limitations of first generation biofuels have prompted the quest for alternative energy sources. Approximately 60 million tonnes of sorghum are generated each year, with 90% being lignocellulosic waste, which is an ideal feedstock for biofuel production. The recalcitrance of lignocellulose often demands harsh pre-treatment conditions and results in the generation of fermentation inhibitors, negatively impacting process yields and economics. In this study, an artificially intelligent model to predict the profile of reducing sugars and all major volatile compounds from microwave assisted chemical pre-treatment of waste sorghum leaves (SL) was developed and validated. The pre-treated substrate was assessed for bioethanol production using *Saccharomyces cerevisiae*. Monod and modified Gompertz models were generated and the kinetic coefficients were compared with previous studies on different substrates.

To develop the Artificial Neural Network (ANN) model, a total of 58 pre-treatment process conditions with varying parameters were experimentally assessed for reducing sugar (RS) and volatile compound production. The pre-treatment input variables consisted of acid concentration, alkali concentration, microwave duration, microwave intensity and solid-to-liquid ratio (S:L). Response Surface Methodology (RSM) was used to optimise RS production from microwave assisted acid pre-treatment of sorghum leaves, giving a coefficient of determination (R^2) of 0.76, resulting in an optimal yield of 2.74 g RS/g SL. A multilayer perceptron ANN model was used, with a topology of 5-13-13-21. The model was trained using the backpropagation algorithm to minimise the net error value on validation. The model was validated on experimental data and R^2 values of up to 0.93 were obtained. The developed model was used to predict the profile of inhibitory compounds under various pre-treatment conditions. Some of these inhibitory compounds were: acetic acid (0-186.26 ng/g SL), furfural (0-240.80 ng/g SL), 5-hydroxy methyl furfural (HMF) (0-19.20 ng/g SL) and phenol (0-7.76 ng/g SL). The developed ANN model was further subjected to knowledge extraction. Findings revealed that furfural and phenol generation during substrate pre-treatment exhibited high sensitivity to acid- and alkali concentration and S:L ratio, while phenol production showed high sensitivity to microwave duration and intensity. Furfural generation during pre-treatment of waste SL was majorly dependent on acid concentration and fit a dosage-response relationship model with a 2.5% HCl threshold.

The pre-treated sorghum leaves were enzymatically hydrolysed and subsequently assessed for yeast growth and bioethanol production using *Saccharomyces cerevisiae* BY4743. Kinetic modelling was carried out using the Monod and the modified Gompertz models. Fermentations were carried out with varied initial substrate concentrations (12.5-30.0 g/L). The Monod model fitted well to the experimental data, exhibiting an R^2 of 0.95. The model coefficients of maximum specific growth rate (μ_{max}) and Monod constant (K_s) were 0.176 h^{-1} and 10.11 g/L respectively. Bioethanol production data fitted the modified Gompertz model with an R^2 of 0.98. A bioethanol production lag time of 6.31 hours, maximum ethanol production rate of 0.52 g/L/h and a maximum potential bioethanol concentration of 17.15 g/L were obtained.

These findings demonstrated that waste SL, commonly considered as post-harvest waste, contain sufficient fermentable sugar which can be recovered from appropriate HCl-based pre-treatment, for use as a low cost energy source for biofuel production. The extracted knowledge from the developed ANN model revealed significant non-linearities between the pre-treatment input conditions and generation of volatile compounds from waste SL. This predictive tool reduces analytical costs in bioprocess development through virtual analytical instrumentation. Monod and modified Gompertz coefficients demonstrated the potential of utilising sorghum leaves for bioethanol production, by providing data for early stage knowledge of the production efficiency of bioethanol production from waste SL. The generated kinetic knowledge of *S. cerevisiae* growth on waste SL and bioethanol formation in this study is of high importance for process optimisation and scale up towards the commercialisation of this fuel.

Keywords: sorghum leaves, lignocellulosic pre-treatment, fermentation inhibitors, bioethanol production, kinetic modelling.

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I am sincerely thankful to a number of people in my life, who have supported and believed in me regardless of my doubts.

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List of Abbreviations

SL…	Sorghum Leaves
RS…	Reducing Sugar
S:L…	Solid-to-Liquid
RSM…	Response Surface Methodology
ANN…	Artificial Neural Network
HMF…	5-Hydroxymethyl Furfural
GHG…	Greenhouse Gas
AFEX…	Ammonia Fibre Explosion
HRT…	Hydraulic Retention Time
RPM…	Revolutions per Minute
VVM…	Vessel Volumes per Minute
MESP…	Minimum Ethanol Selling Price
MW…	Microwave
EA…	Ensemble Averaging
DR…	Dose Response
MMF…	Morgan-Mercer-Flodin

Chapter 1
General Introduction

1.1 Fossil fuel depletion and the need for renewable sources

Crude oil is the most important global fuel source, accounting for 36.4% of the world's primary energy consumption. Interestingly, in 2006, it constituted a meagre 17.67% of the remaining fossil fuel reserves (Shafiee and Topal, 2009). The Energy Information Administration predicted an approximate 15% increase in the global energy demand from the year 2012 to the year 2020 (EIA, 2016). Over the last few years, the increasing world population and industrial growth has been the major factor driving the energy sector. Emissions from transportation sources play a significant role in the release of greenhouse gases (GHG) which contribute to the anthropogenic greenhouse gas effect (Uherek et al., 2010). This has led to severe climate change seen in the rising sea levels, melting of the polar ice caps, a rapid decline in biodiversity, as well as extreme weather conditions (Jian-Bin et al., 2012). Furthermore, nitrogen based oxides which are also released, significantly impact air quality, leading to various respiratory issues within populations (Uherek et al., 2010). This has accelerated the research into alternative, renewable energy methods for biofuel production.

Numerous advantages of biofuel development exist, such as an expansion of energy supplies, enhanced energy security, an improvement to the development of rural agriculture as well as reduced GHG emissions (Pradhan and Mbohwa, 2014). In the year 2014, renewable energy provided approximately 19.2% toward the final global energy consumption, further increasing in the year 2015. This suggests that a global energy transition is currently underway (Renewables, 2016). Although biofuels make up only 0.8% of the total renewable energy sector (Renewables, 2016), several countries have implemented policies to encourage the production and use of biofuels. For instance, Thailand has implemented a 15 year plan to increase the bioethanol production capacity to 9 million litres a day by the year 2022 (Ariyajaroenwong et al., 2016). In South Africa, a mandatory blending of 5% biodiesel with diesel and 2-10% blending of bioethanol with gasoline was enforced as of the 1st October 2015 (DoE, 2015). Brazil, which is one of the major bioethanol producing countries at a global scale, has mandated an increase in the biodiesel blend from 7 to 8% by the 23rd of March 2017 (DieselNet, 2016) as

well as a 2% increase from 25 to 27% minimum ethanol blend from the 16th March 2015 (Biofuels Digest, 2016).

1.2 Bioethanol production

Bioethanol is a renewable and environmentally friendly alternative to fossil fuel (Shen et al., 2011) and has swiftly become the most commonly used biofuel for transportation usage as it has a higher octane number and thus burns more efficiently than gasoline (Sarkar et al., 2012). Apart from its renewable and sustainable nature, bioethanol compared to alternate fermentative fuel sources, has high practicality and will contribute towards energy security for the future (Chung and Yang, 2016). A summary of the global production of biofuels in year 2013 is presented in Figure 1. It shows that 87.2 billion litres of bioethanol from a total of 116.6 billion litres of biofuel was produced, with lower contributions from biodiesel (26.3 billion litres) and hydrotreated vegetable oil (HVO) (3.0 billion litres).

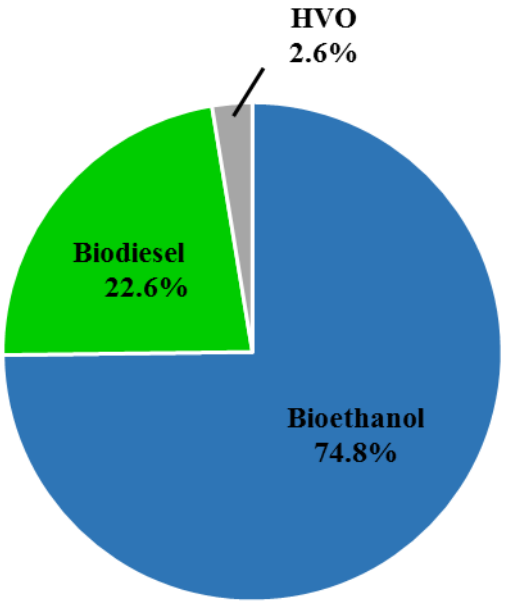

Figure 1: Global biofuel production in 2013 (adapted from Chung and Yang, 2016).

Major leading bioethanol producers include Brazil and the USA with the former sourcing sucrose from sugarcane- and the USA using starch obtained from corn (Sarkar et al., 2012). This highlights the dependency of commercial bioethanol production on first-generation energy sources, which currently places a heavy burden on food security (Sarkar et al., 2012). Research has therefore shifted towards the development of economical and sustainable processes for

future bioethanol production plants. Presently, valuable second generation energy sources such as agricultural crop residues are being sought for fermentative bioethanol production as well as other biofuels (Chandel et al., 2007; Shen et al., 2011; Singh and Bishnoi, 2013; Gabhane et al., 2014; Barcelos et al., 2016). One of the key differences between first- and second- generation bioethanol production is an additional processing step (Figure 2). Lignocellulose is composed of a complex network of cellulose, hemicellulose and lignin in approximate fractions of 25%, 40-50% and 25% respectively (Uju et al., 2016). The recalcitrant nature of lignocellulose warrants a pre-treatment step which requires the use of severe process conditions to break down lignocellulose to release glucose-rich cellulose.

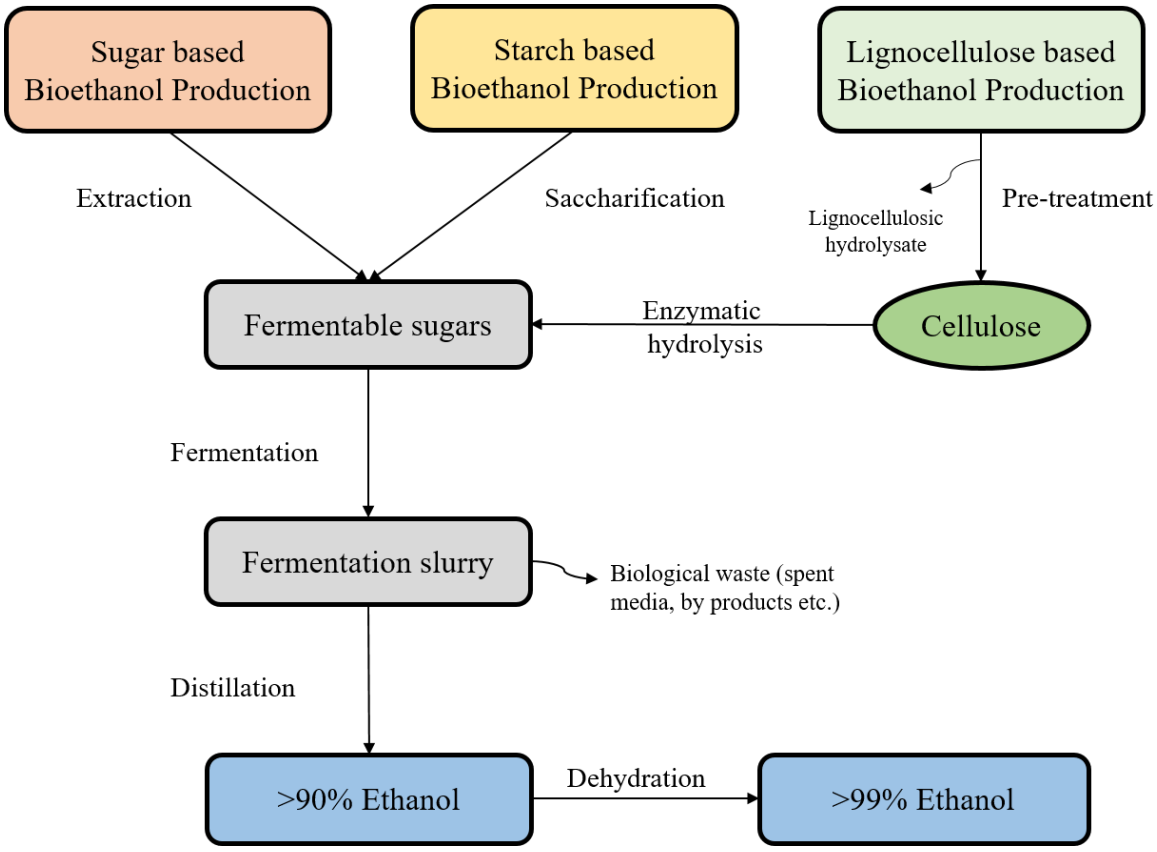

Figure 2: Bioethanol production using renewable energy sources.

Bioethanol from lignocellulosic material is produced by harnessing the ability of microorganisms to convert the glucose released during pre-treatment into bioethanol. The pathway followed under anaerobic (fermentation) conditions for *Saccharomyces cerevisiae* is seen in Figure 3. Numerous factors affect this fermentation process, including the presence of compounds which are inhibitory to fermentation, the affinity of the producing microbe to the

substrate used as well as environmental factors such as pH, operating temperature and initial substrate concentration (Raikar, 2012; Dai et al., 2014).

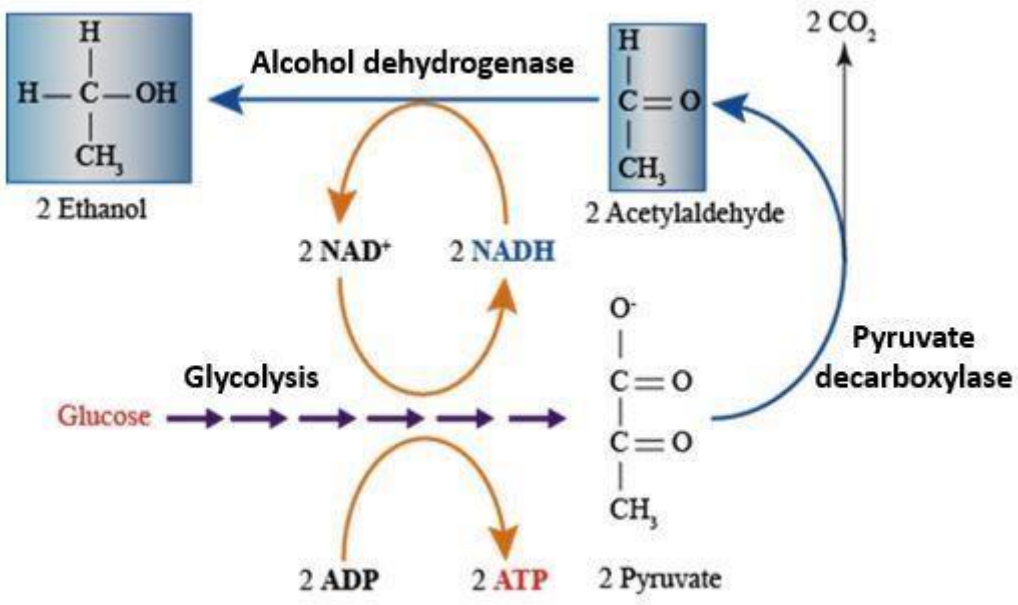

Figure 3: Metabolic pathway for bioethanol production from glucose by *Saccharomyces cerevisiae* under anaerobic conditions.

1.3 Fermentation inhibitors and their effects on fermentation

The harsh process conditions required to degrade the lignocellulose structure often lead to the generation of a number of compounds that have been shown to inhibit the enzymatic hydrolysis step as well as the fermentation process (Kamal et al., 2011).

During acidic pre-treatment, by-products that are formed include weak acids, furan derivatives such as furfural and 5-hydroxymethyl furfural (HMF), and phenylic compounds (Kamal et al., 2011; Jönsson and Martin, 2016). In contrast, alkali pre-treatment preserves the carbohydrate structures to a certain extent, however some degradation may occur, generating carboxylic acids (Jönsson and Martin, 2016). Furfural in particular has been shown to inhibit bioprocesses by the conversion of furfural to furfuryl alcohol by *Saccharomyces cerevisiae*, which subsequently inhibits anaerobic growth of the microorganism (Larsson et al., 1999). Additionally, furfural causes the accumulation of reactive oxygen species within *S. cerevisiae* cells, resulting in damage to vacuole and mitochondrial membranes, among others (Almeida et al., 2007). Furan derivatives therefore hamper or completely inhibit bioethanol production by redirecting the

energy required for product formation to fix cellular damage caused by furans. Additionally, enzymatic hydrolysis is often inhibited and necessary cofactors are needlessly used (Almeida et al., 2007).

Acetic acid has been reported by Soudham et al. (2014) to be generated in large amounts during acid pre-treatment. This becomes detrimental to fermentation as, once it is within the neutral cell environment, it dissociates, leading to a drop in pH which inhibits cell activity (Harmsen et al., 2010). Other acids such as formic and levulinic acid which are furan degradation products, inhibit bioethanol production by causing intracellular accumulation of anions due to this acid dissociation. Microorganisms will then attempt to correct this, leading to the unnecessary use of ATP, therefore less is available for the formation of biomass (Almeida et al., 2007).

The generation of phenolic compounds has also been reported to be more significant during alkali pre-treatment (Jönsson and Martin, 2016). The most significant effect of alkali pre-treatment is the removal of lignin and structural alteration (Jönsson and Martin, 2016), which leads to greater generation of phenolic compounds. Although the overall amount of phenolic compounds generated is much lower than furan derivatives and weak acids, phenolic compounds exert a more toxic effect on bioprocesses (Harmsen et al., 2010). These compounds exhibit antimicrobial properties which leads to the generation of reactive oxygen species, causing a loss in the producing microorganisms' cell membrane integrity, a reduction in cell growth and slower adaptation to sugars present (Harmsen et al., 2010; Almeida et al., 2007).

1.4 Bioprocess Modelling

To optimise the pre-treatment of lignocellulose, modelling tools such as Response Surface Methodology (RSM) and Artificial Neural Networks (ANN) have been employed (Anwar et al., 2012; Nikzad et al., 2015). RSM allows for the identification of many factors and their interactive effects on the process yield (Rorke and Kana, 2016) and has been reported in the modelling and optimisation of various bioprocesses. On the other hand, ANNs are capable of gathering information by detecting patterns and relationships within data and are trained through experience (Agatonovic-Kustrin and Beresford, 2000). ANN can also be used as a predictive tool by acting as a virtual sensor for the estimation of parameters which are costly to monitor (Gonzaga et al., 2009). Bioprocess kinetic modelling enables assessment of the biochemical characteristics of a bioprocess. Monod models are used to describe biomass growth

in terms of the limiting substrate. The modified Gompertz models are used to determine production lag time, maximum product concentration as well as the maximum production rate on a given substrate (Imamoglu and Sukan, 2013; Dodić et al., 2012; Putra et al., 2015). Kinetic modelling allows for increased product yield and productivity and reduced formation of unwanted by-products, to ensure high product quality (Almquist et al., 2014). The bioprocess models can be used for virtual experimentation to reduce time and costs associated with process development. Furthermore, the implementation of these models provides a strong foundation for process design, control and optimisation which will inevitably reduce the challenges faced during scale up (Linville et al., 2013).

1.5 Problem Statement and Justification of Study

Crude oil is one of the main sources for the world energy supply and its complete depletion is anticipated in the next 35 years (Shafiee and Topal, 2009). The dwindling fossil fuel reserves combined with greenhouse gas effects necessitates the broadening of the current energy portfolio to include renewable energy sources for fuels (Cavka and Jönsson, 2013). Despite the advantage of bioethanol as a cost effective alternative, a major challenge facing the transition to bioethanol production is the sourcing of an economical, renewable feedstock that is capable of yielding sufficient amounts of fermentable sugar with less fermentation inhibitors (Gabhane et al., 2014).

Lignocellulosic biomass is a suitable renewable substrate, but its complex structure makes it extremely recalcitrant to microbial degradation. To address this, a pre-treatment step is required to enhance the effect of enzymatic hydrolysis. Several pre-treatment regimes have been reported and these include chemical pre-treatment which makes use of acid or alkali, thermal and microwave, among others. The use of microwave assisted chemical pre-treatment on waste sorghum leaves is scantily reported in literature.

Apart from the abovementioned challenges with regards to the use of lignocellulosic biomass, another limitation is the release of fermentation inhibitor compounds during the severe process conditions required for pre-treatment. These compounds prevent effective bioconversion of fermentable sugars to bioethanol (Cavka and Jönsson, 2013). Some of these by-products have been reported to hamper enzymatic hydrolysis and fermentation processes. The detection and

quantification of these compounds is tedious, yet this knowledge is required to ensure detrimental effects of these compounds are not overlooked.

Therefore, to alleviate concerns regarding food security and fossil fuel depletion, the use of lignocellulosic biomass should be implemented. The generation of fermentation inhibitors should be considered when developing efficient pre-treatment strategies. This can be achieved by the implementation of ANN modelling to capture the complex interactions which link the pre-treatment conditions to fermentable sugar production as well as inhibitor generation. Furthermore, the fermentation process should be assessed in detail. Using kinetic models such as Monod and modified Gompertz models will help to control the process, reduce costs and increase the quality of the bioethanol produced. These findings could therefore contribute to industrial scale productions from lignocellulosic biomass.

1.6 Aims and objectives

This study aimed to model the production of reducing sugar and all volatile compounds from microwave assisted chemical pre-treatment of waste sorghum leaves. Additionally, the kinetic behaviour of *Saccharomyces cerevisiae* growth and bioethanol production from waste sorghum leaves was evaluated.

The following specific objectives were undertaken in order to achieve the abovementioned aims:

i. Modelling and optimisation of microwave assisted acid (HCl) and alkali (NaOH) pretreatment of waste SL for the release of fermentable sugars.
ii. Development of a soft-sensor based on an Artificial Neural Network (ANN) model to predict the volatile compound profile generated during the pre-treatment regime implemented in (i).
iii. Development of Monod kinetic and Modified Gompertz models of *Saccharomyces cerevisiae* BY4743 growth and bioethanol production from waste sorghum leaves.

1.7 Outline of dissertation/thesis

This thesis is comprised of five chapters presented in research paper format. Each experimental chapter is self-contained, containing a literature review, materials and methods, results and discussion, and conclusions. The description, assessment and application of waste sorghum leaves for the production of bioethanol are central to all chapters.

Chapter 2 discusses the use of sorghum as a renewable feedstock for the production of biofuels such as bioethanol and biohydrogen. It examines pre-treatment methodologies for lignocellulosic biomass and the strategies to overcome the negative impacts of pre-treatment on fermentation as well as the potential for producing bioethanol and other products from sorghum leaves.

Chapter 3 focuses on the modelling and optimisation of microwave assisted chemical pretreatment of sorghum leaves for the release of fermentable sugars as well as the profiling of volatile compounds generated during pre-treatment. In addition, a soft-sensor capable of predicting a volatile compound profile under varied process conditions was developed.

In Chapter 4, kinetic modelling of a laboratory scale batch bioethanol fermentation process, using *Saccharomyces cerevisiae* BY4743 was undertaken to determine the dynamics and thus predict the producing microorganism's behaviour based on factors such as the producing microorganism's specific growth rate, affinity to the fermentation substrate and maximum bioethanol production rate.

In conclusion, Chapter 5 integrates the work and states major conclusions derived from this study and provides recommendations for future research.

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Chapter 2

Post-harvest sorghum waste as a renewable feedstock for biofuel production: A mini review.

2.1 Abstract

Lignocellulosic bioethanol production has been highlighted as a promising, renewable replacement for gasoline. Annual sorghum production is estimated at 60 million tonnes. A large fraction (up to 90%) of this is considered as residual lignocellulosic waste. A major setback associated with the pre-treatment of lignocellulose is the generation of compounds which inhibit subsequent enzymatic hydrolysis as well as the fermentation process. In this review, the pre-treatment strategies for post-harvest sorghum lignocellulosic waste are discussed. In addition, the detoxification methods used for the reduction of fermentation inhibitors are reported. Finally, the potential and challenges of using post-harvest sorghum waste as a lignocellulosic substrate for the production of various biofuels are discussed.

Keywords: Sorghum biomass, lignocellulosic pre-treatment, detoxification, bioethanol production, biofuels.

2.2 Introduction

The increasing global demand for energy coupled with diminishing fossil fuel reserves and the associated greenhouse gas (GHG) emissions are driving the need for renewable energy sources (Kurian et al., 2013). First generation bio-products which are produced from food crops such as corn and sugarcane are frequently linked to food insecurity (Kurian et al., 2013), while second generation bio-products which are produced from lignocellulosic biomass are independent of the global food supply. Millions of tonnes of lignocellulosic crop residues are generated from the agricultural sector and show potential to serve as economical feedstocks for bio-production. The bioconversion of lignocellulosic biomass such as agricultural waste, grasses *etc.* into biofuels as well as other value-added products offers a number of environmental benefits (da Silva et al., 2012). Several lignocellulosic materials have been exploited for biofuel production and include sorghum leaves (Rorke and Kana, 2016), sugarcane leaves (Moodley and Kana, 2015) and napier grass, among others. Sorghum biomass, specifically can be utilised for the production of bioethanol and biohydrogen which can be used for transportation and electricity generation, as well as other commercially valuable products such as xylitol (Zegada-Lizarazu and Monti, 2012; Woods, 2001; Sene et al., 2011).

Although lignocellulosic biomass is a potential feedstock for biofuel production, it still presents several challenges. For instance, it is comprised of a resistant matrix of lignin, cellulose and hemicellulose which cannot be directly utilised by microbes during fermentation processes. Therefore, pre-treatment is required in order to unwind these compounds, making the fermentable sugars accessible to the microbes (Taha et al., 2016). Various pre-treatment strategies exist and include chemical, thermal and microwave, among others. Depending on the conditions of pre-treatment, lignocellulosic biomass generates, in addition to the fermentable sugars, various compounds which are inhibitory to microbial metabolisms (Cavka and Jönsson, 2013). Therefore, to enhance process control and enable the reduction of unwanted inhibitory compound generation, while optimising process conditions for maximal fermentable sugar production, bioprocess modelling can be implemented.

Several studies have focused on modelling and optimisation of fermentable sugars and bioethanol production from lignocellulosic biomass. Among the optimisation algorithms used is Response Surface Methodology (RSM) and Artificial Neural Networks (ANNs). RSM is a statistically based experimental modelling method which enables the description of interactive effects among process variables (Bezerra et al., 2008; Wang and Wan, 2009a). A quadratic model is usually developed to illustrate these interactive effects, and is then subsequently used to optimise the process (Wang and Wan, 2009a). Additionally, Artificial Neural Networks (ANNs) as a predictive tool have recently gained much interest. ANN is a data processing system which is a mathematical representation of the neurological functioning of a brain (Vani et al., 2015; Wang and Wan, 2009a). It gathers information by detecting patterns and relationships found in the data. Optimisation of pre-treatment processes using modelling tools allows for maximum fermentable sugar release. Additionally, predictive tools such as ANN may be used to reduce the generation of inhibitory compounds.

This review focuses on the potential of post-harvest sorghum waste as a feedstock for the production of biofuels such as bioethanol and biohydrogen. The pre-treatment strategies employed for the production of fermentable sugars and subsequent detoxification methods used to reduce inhibitors generated during pre-treatment are discussed. Furthermore, the potential and challenges associated with the production of biofuels such as bioethanol and biohydrogen using post-harvest sorghum waste, are reviewed.

2.3 Sorghum Production

Sorghum (*Sorghum bicolor* L. Moench) is an annual energy crop indigenous to Africa (Zegada-lizarazu and Monti, 2012; du Plessis, 2008). It is a fast growing plant which is found in tropical, subtropical and temperate climate zones. Numerous varieties of sorghum cultivars are grown for grain, forage and sugar production, with each cultivar varying between short, leafy plants and tall plants with thick, juicy stalks (Zegada-lizarazu and Monti, 2012). Post-harvest sorghum biomass can be used for both first and second generation biofuel production. Sugar rich stalks can be used for first generation biofuel production, while the leaves or bagasse of grain and forage sorghum are ideal for second generation biofuel production (Zegada-Lizarazu and Monti, 2012).

Sweet sorghum typically consists of cultivars that have been selected for sugar production. The tall stalks of these plants are rich in soluble sugars like sucrose, glucose and fructose (ZegadaLizarazu and Monti, 2012). Grain sorghum is characteristically shorter in height than other types, with the grain being rich in starch. Grain sorghum is often harvested for human consumption as well as animal fodder, however it can also be used as a carbohydrate feedstock for first generation biofuel production. Forage sorghum typically is high in protein and fibre and the biomass is thus harvested for animal fodder. The biomass of all cultivars can be used as a cellulose feedstock for second generation biofuel production (Zegada-Lizarazu and Monti, 2012).

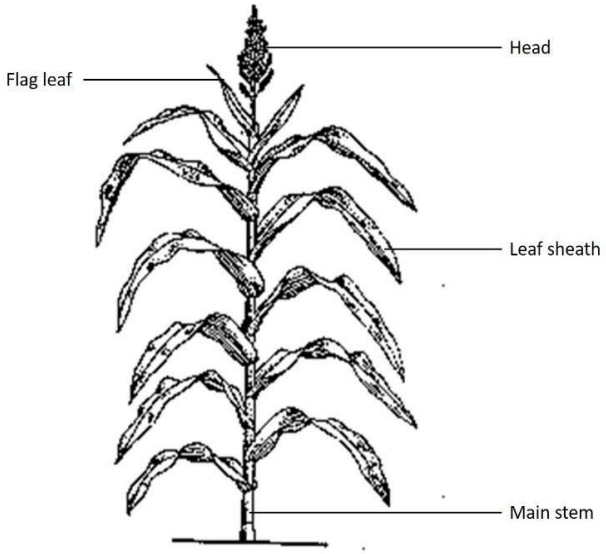

Figure 1: General form of sorghum plant

2.4 Sorghum production in South Africa

Sorghum production in South Africa amounts to approximately 180 000 tons annually (du Plessis, 2008). Nevertheless, this turnover is expected to rise in response to the 2% fuel blending mandate implemented in South Africa (SA). Grain SA, the largest representative of commercial farmers in this country stated that an additional 620 000 tons of sorghum will be required to produce sufficient ethanol in order to meet the 2% fuel blending rate. Additionally, sorghum grain still plays a major role in the food industry since it continues to serve as a staple food for many rural communities (du Plessis, 2008), thus challenging its potential use as a substrate for biofuel production. Therefore post-harvest wastes can easily be used to produce bioenergy in both rural and industrial areas (Woods, 2001).

2.5 Global Sorghum Production

The United States Department of Agriculture (USDA) reported that the global sorghum production during the 2015/2016 period reached 60.16 million tonnes (World Sorghum Production, 2016). This leaves large volumes of remaining biomass, which can be used for the production of biotechnological products of higher value, such as bioethanol, biohydrogen and biobutanol, among others. However, before the lignocellulosic biomass is channelled towards fermentation processes, the biomass must undergo pre-treatment in order to break down its complex structure to enhance saccharification.

2.6 Composition of sorghum leaves

The average sorghum plant takes approximately 14 weeks to undergo five stages of growth, *i.e.* from a seedling to a mature sorghum plant. A study conducted by Firdous and Gilani (2001) showed that an increase in hemicellulose, cellulose and lignin occurs in all parts of the plant throughout these stages. Table 1 shows the hemicellulose content was found to be higher in the leaves (approximately 24%) compared to the stem (20%) or the whole plant (21%). On the other hand, the cellulose and lignin contents were lowest within the leaves (ca. 23% and ca. 4.0 % respectively).

Table 1: Lignocellulosic composition observed in components of various sorghum cultivars. (Adapted from Firdous and Gilani, 2001).

	Average lignocellulosic composition of various sorghum cultivars (%)		
	Hemicellulose	Cellulose	Lignin
Whole sorghum plant	21.06	29.13	4.56
Sorghum stem	20.36	31.88	5.92
Sorghum leaf	24.01	23.49	3.90

Generally, the predominant reducing monosaccharide sugars found in sorghum leaves and stems are glucose and fructose, while the major, non-reducing disaccharide found is sucrose. However, the final sugar content may vary, depending on the type of sorghum that is produced. Sweet sorghum varieties commonly display increased sucrose levels until maturity, while grain sorghum varieties display decreased sugar content in the stems during grain formation (Wall and Blessin, 1970).

An interesting factor to note may be the elevated sugar content observed in the forage parts of sterile sorghum varieties where the setting of the seed is negatively impaired (Webster, 1963). This may prove to be beneficial for second generation biofuel production by potentially releasing more fermentable sugar. A sugar content increase of 3 to 9% was reported by Wall and Blessin (1970) in the leaves two to three weeks after bloom of forage sorghum. Hemicellulose and cellulose contents have also been found to be lower in sweet sorghum when compared to grain, forage and fibre sorghum (Wall and Blessin, 1970), further promoting the use of non-food sorghum cultivars for renewable energy production.

2.7 Suitability of sorghum leaves for fermentable sugar release

The complex matrix of lignocellulose (hemicellulose, cellulose and lignin) has led to the recalcitrant nature of lignocellulosic material. Therefore, it has become common practise to implement a pre-treatment step prior to enzymatic hydrolysis or fermentation, in order to improve the digestibility of the lignocellulose (Cao et al., 2012). This is achieved by the modification and removal of lignin, partial polymerisation and removal of hemicelluloses and the reduction of cellulose crystallinity, as illustrated in Figure 2 (Behera et al., 2014; Mood et al., 2013). Pre-treatment is considered to be one of the most expensive steps within the conversion of lignocellulosic biomass (Singh et al., 2014). Desired characteristics of a successful pre-treatment method include; low energy requirements while maintaining high

performance, minimising sugar loss, reduced chemical usage to avoid the presence of inhibitors during fermentation and the use of low-cost materials (Chiaramonti et al., 2012).

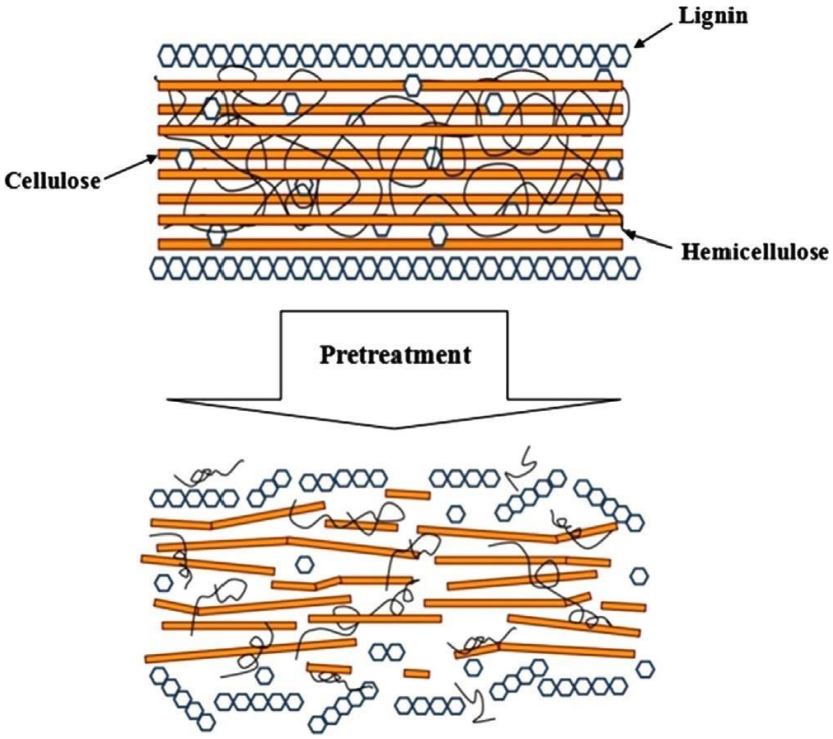

Figure 2: Effect of pre-treatment on lignocellulosic material (Mood et al., 2013).

2.7.1 Physical Pre-treatment

Various mechanical size reduction methods exist such as chipping, shredding, grinding and milling. Size reduction is employed to increase the surface area and decrease the degree of polymerisation and crystallinity of cellulose (Singh et al., 2014). As size reduction is considered as one of the most effective methods of increasing the accessibility of the lignocellulosic material (Chiaramonti et al., 2012; Behera et al., 2014), it is commonly employed prior to other forms of pre-treatment (Mood et al., 2013). However, it is a high energy process and is not considered economically feasible at a larger scale of biofuel production. Therefore, alternative methods are currently being sought (Chiaramonti et al., 2012).

Extrusion is a thermo-physical method which involves mixing, heating and shearing of lignocellulosic material to achieve chemical and physical alteration (Yoo et al., 2011). Some of the advantages of such an approach include high shear and rapid mixing, short residence time, moderate barrel temperatures, no furan type inhibitor [furfural and 5-hydroxy methyl-furfural

(HMF)] formation, easy scale-up as well as the possibility of a continuous operation (Mood et al., 2013). Lastly, extrusion does not produce any effluent, thus reducing disposal costs (Mood et al., 2013).

Microwave irradiation serves as a potential alternative to conventional heating methods which aim to modify the structure of cellulose, partially remove or degrade lignin and hemicellulose and thus enhance the enzymatic susceptibility of the lignocellulosic components (Mood et al., 2013). Chen et al. (2012) reported a glucose concentration of 4.2 g per 10 g dry biomass of sorghum bagasse at 130 °C for 1 hour, using microwave-assisted dilute ammonia pre-treatment. Hu and Wen (2008) also reported that microwave-assisted alkali pre-treatment (0.1 g NaOH/g biomass) of switchgrass at 190 °C for 30 minutes converted the intact structure of biomass to a thinner form.

Freeze pre-treatment is a novel method that has been found to significantly increase the enzymatic digestibility of rice straw. Although this method is more cost intensive, the advantages of lower environmental impact, use of less dangerous chemicals and high efficacy make this a method of significant interest (Mood et al., 2013).

2.7.2 Chemical Pre-treatment

Acid pre-treatment involving the use of sulfuric acid is one of the most common chemical pretreatment methods applied for lignocellulosic biomass (Behera et al., 2014). This pre-treatment may be carried out at low acid concentrations coupled with high temperatures or at high acid concentrations coupled with low temperatures (Mood et al., 2013). Both of these approaches suffer several drawbacks. Using a more concentrated acid may be more economical as the process will be carried out at a lower temperature however, high acid concentrations lead to higher levels of toxicity and corrosion, monosaccharide degradation and the production of fermentation inhibitors such as furfural and HMF (Mood et al., 2013). In addition to this, elevated temperatures may lead to the degradation of produced inhibitors, into unwanted products such as levulinic and formic acids (Mood et al., 2013). Industrially, dilute acid pretreatments (0.5 – 1.5%) are preferred as fewer inhibitors are produced and higher sugar yields are obtained (Singh et al., 2014). Sindhu et al. (2014) reported a yield of 0.319g reducing sugar/g substrate, using 5% sulfuric acid for 30 minutes at 121 °C to pre-treat bamboo.

Another form of chemical pre-treatment involves the use of alkaline agents. Some of the advantages of alkaline pre-treatment include: removing lignin, acetyl groups and different uronic acid substitutions in order to enhance cellulose accessibility for enzymatic saccharification (Mood et al., 2013). Most common alkali reagents are NaOH, KOH and $Ca(OH)_2$ but there are many drawbacks concerning alkali pre-treatment, including; long process durations, lower hemicellulose and cellulose solubilisation and the need for slurry neutralisation (Chiaramonti et al., 2012; Mood et al., 2013). In contrast to acid pre-treatment, alkali pretreatment is operated at lower temperatures. Wu et al. (2011) achieved a saccharification yield of 98.7% when sweet sorghum bagasse was treated with 2.5 M NaOH for 120 minutes at room temperature.

Ionic liquid (IL) pre-treatment involves the use of ionic liquids. ILs are a new class of solvents which possess low melting points (<100 °C), high polarities, high chemical and thermal stability, wide liquid temperature ranges and are non-flammable (Behera et al., 2014). The network formed by hemicellulose, cellulose and lignin is degraded upon the formation of hydrogen bonds between non-hydrated ions of ILs and the hydroxyl proteins of the sugars (Mood et al., 2013). ILs such as 1-allyl-3-methyl imidazonium chloride (AMIMCl) and 1-butyl3-methyl imidazonium chloride (BMIMCl) are found to be very effective as solvents for cellulose dissolution at temperatures below 100 °C (Behera et al., 2014).

Ammonia fibre explosion (AFEX) is a physico-chemical method which involves the exposure of lignocellulosic biomass to liquid ammonia at high temperature and pressure for a specified period of time and then reducing the pressure abruptly (Behera et al., 2014). During the pretreatment process, very little solid material becomes solubilised, minimising the loss of hemicelluloses and lignin. The structure of the lignocellulosic material becomes altered, resulting in an increased capacity to hold water, thus increased digestibility (Kumar et al., 2009). AFEX has several advantages over other pre-treatment methods. AFEX does not result in the formation of toxic materials, it does not require size reduction, results in approximately 99% sugar recovery and it does not necessitate the addition of a nitrogen source during fermentation as the residual ammonia serves as a nitrogen source (Behera et al., 2014).

2.7.3 Biological Pre-treatment

Biological pre-treatment encompasses the use of microorganisms such as brown, white and soft-rot fungi which degrade lignin, hemicelluloses and cellulose. This occurs via the production of enzymes to treat biomass prior to enzymatic hydrolysis (Chiaramonti et al., 2012; Behera et al., 2014), with cellulose being the most difficult to degrade by biological treatment and white-rot fungi being the most effective for lignocellulosic biomass degradation (Chiaramonti et al., 2012). Advantages of biological pre-treatment include its low energy requirements, no generation of toxic compounds (Behera et al., 2014) and the absence of chemical reagents. The main disadvantage is the low process rate (Chiaramonti et al., 2012) as it can take weeks to achieve significant results (Behera et al., 2014). Enzymes commonly used to enhance the saccharification of lignocellulosic biomass include cellulase and hemicellulase however, high costs associated with enzymes limit its commercial application (Zheng et al., 2014).

2.8 Inhibitory compounds from lignocellulosic pre-treatment

The main disadvantage of lignocellulosic pre-treatment is the degradation of sugars and production of unwanted by-products such as fermentation inhibitors (Kamal et al., 2011). Under acidic conditions, fermentation by-products include; weak acids, furan derived compounds and phenylic compounds (Kamal et al., 2011; Jönsson and Martín, 2016) (Figure 3). Under alkaline conditions, carbohydrate structures are better preserved but may undergo some degradation. This leads to the production of carboxylic acids (Jönsson and Martín, 2016). Saponification of the acetyl groups in the material also leads to the production of acetic acid. Some phenolic compounds may also be produced and in certain processes, may be further degraded via oxidation to form carboxylic acids (Jönsson and Martín, 2016).

The presence of acids such as levulinic, formic and acetic acid at concentrations of approximately 100 mM in fermentations with *Saccharomyces cerevisiae* have been described to exhibit an inhibitory effect on the fermentation (Jönsson and Martín, 2016). However, the production of these acids would occur at the expense of sugar release, therefore pre-treatment conditions should allow for the minimisation of weak acid formation.

Phenylic compounds such as phenolic aromatic carboxylic acids and non-phenolic aromatic carboxylic acids originate from lignin or the hydrolysis of esterified phenols. Although these compounds are found in acid hydrolysates at much lower concentrations than weak acids, the

inhibitory effect exerted is frequently much stronger than that exerted by aliphatic carboxylic acids such as acetic and levulinic acid (Jönsson and Martín, 2016). According to Larsson et al (2000), the phenolic aromatic carboxylic acid, ferulic acid, was found to be inhibitory to *S. cerevisiae* at a concentration of 1.0 mM, which is roughly 20 times lower than that of aliphatic carboxylic acids. Lastly, the inhibition of fermentation by aldehydes appears to be similar to that of carboxylic acids, where carbohydrate-derived furan aldehydes may be present in higher concentrations but are not as inhibitory to fermentation, while lignin-derived aromatic aldehydes are present at lower concentrations but possess a much higher toxicity (Jönsson and Martín, 2016).

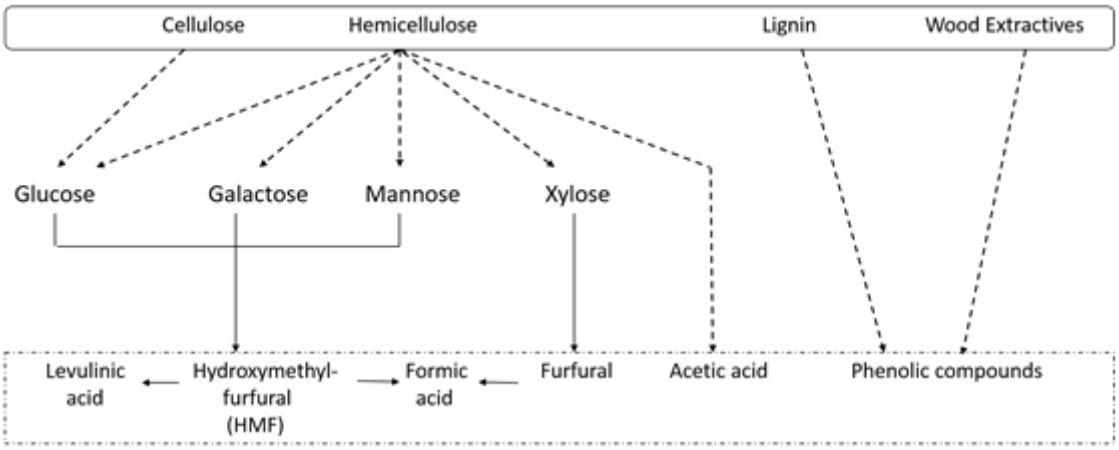

Figure 3: Schematic diagram illustrating the main pathways of inhibitor formation from acid pre-treated lignocellulosic material. Adapted from Almeida et al. (2007).

2.8.1 Hydrolysate Detoxification

Detoxification is carried out to convert fermentation inhibitors to inactive compounds, or to reduce their concentrations (Kamal et al., 2011). Numerous methods of detoxification have been studied, such as the use of chemical additives, i.e. reducing agents (Cavka and Jönsson, 2013) and polymers (Cannella et al., 2014). Other methods such as liquid-solid extraction (Jönsson and Martín, 2016; Kamal et al., 2011), employ the use of ion exchange or treatment with activated charcoal, biological adaptation and many others (Kamal et al., 2011). Of these methods, application of activated charcoal is one of the most widely used. A study conducted by Kamal et al (2011) showed that using 2.5% activated carbon for an adsorption time of 60 minutes achieved a 58% furfural and 78% total phenolic reduction. Therefore, a yield of 0.78

g/g xylose was obtained from sago trunk hydrolysate, which was almost double the yield achieved without a detoxification step (0.307 g/g). In another study by Chandel et al. (2007), sugarcane bagasse acid hydrolysate was treated with anion exchange resin, resulting in a 63.4% reduction of furan-derivatives and 75.8% of total phenolics, while laccase treatment reduced total phenolics by 77.5%. Although extensive research has been carried out in the search for efficient strategies that aid the reduction or removal of well-known inhibitor compounds, the investigation of the profile of various inhibitors generated under different pre-treatment conditions has scarcely been reported.

2.9 Modelling and optimisation of lignocellulosic pre-treatment methods

The optimisation of the pre-treatment process is considered as one of the most important developmental stages of an efficient bioprocess (Saini et al., 2013). Numerous modelling strategies have been used for the development of lignocellulosic pre-treatment processes. The Response Surface Methodology (RSM) has been frequently reported for modelling and optimisation (Li and Xu, 2013; Gabhane et al., 2014; Umagiliyage et al., 2015; Saini et al., 2013; Sindhu et al., 2014). This is largely due to the capability of RSM to account for interactive effects of process parameters on the process output with a lesser number of experimental runs (Rorke and Kana, 2016). RSM generates a polynomial equation which is used to determine optimum process parameter set points (Mandenius and Brundin, 2008).

The Artificial Neural Networks are data-driven modelling tools capable of computing relationships between process parameters and process responses in order to describe the behaviour of a system (Sewsynker et al., 2015). They have an input layer, one or more hidden layers and an output layer. The neurons within the hidden layer contribute to the establishment of complex associations between the parameters which make up the input and output layers (Nagata and Chu, 2003). Due to the marked ability of ANNs to process inaccurate or fuzzy information and describe patterns, they have gained increasing attention as virtual sensors (Gonzaga et al., 2009). Therefore, ANNS show great potential as modelling and predictive analytical tools for bioprocess development.

Additionally, the use of mathematical models to understand, predict and optimise the behaviour of producing microorganisms during fermentation has significantly increased (Almquist et al., 2014). These models are often used to increase product yield and productivity of a bioprocess,

while simultaneously minimising the formation of by-products to produce high quality products (Almquist et al., 2014). Monod kinetic models are commonly used to describe biomass growth and product formation with respect to the limiting substrate (Imamoglu and Sukan, 2013), while the modified Gompertz models are used to determine production lag time, maximum production rate and maximum product concentration on a given substrate (Dodić et al., 2012; Putra et al., 2015). Fermentation kinetics on lignocellulosic biomass therefore provides fundamental information on process characteristics and behaviour, making process control and improvement more efficient.

2.10 Lignocellulosic bioethanol production

One of the alternatives to gasoline is bioethanol produced from agricultural waste as it is both renewable and eco-friendly (Shen et al., 2011). In South Africa, an average bioethanol production cost of 70 US cents/litre was determined (USDA, 2006). This process exploits microbial metabolism to convert simple sugars found in lignocellulosic biomass to bioethanol (Shen et al., 2011). During this process, the following reactions occur:

$$(C_6H_{10}O_5)_n \text{ (starch, cellulose, sugar)} + nH_2O \xrightarrow[\text{Hemicellulases}]{\text{Cellulases}} nC_6H_{12}O_6 \text{ (glucose, fructose)} \quad [1]$$

$$(C_5H_8O_4)_n \text{ (hemicellulose)} + nH_2O \rightarrow nC_5H_{10}O_5 \text{ (xylose, mannose, arabinose, etc.)} \quad [2]$$

$$C_6H_{12}O_6 \rightarrow 2CH_3CH_2OH \text{ (ethanol)} + 2CO_2 \quad [3]$$

$$C_5H_{10}O_5 \rightarrow 5CH_3CH_2OH \text{ (ethanol)} + 5CO_2 \quad [4]$$

To obtain fermentable sugars for bioethanol production, the lignocellulosic biomass must be broken down using one or a combination of the previously mentioned pre-treatment methods. The generation of fermentable sugars, which occurs during lignocellulosic degradation is seen in reactions (1) and (2). Once the lignocellulose has been broken down and fermentable sugars are obtained, it is then fermented at temperatures suited to the producing microorganism to obtain 8-12% bioethanol. The bioethanol is then recovered from the fermentation medium by distillation, de-watered and finally denatured by mixing it with 2-5% gasoline (Guo et al., 2015).

Bioethanol production via fermentation can be carried out using a range of inoculum sources, such as pure and co-cultures (De Bari et al., 2013). Although genetically engineered pure cultures are commonly used for the production of bioethanol, co-cultures of wild microorganisms are fast becoming a more preferred inoculum method. Reasons for this include; simultaneous conversion of mixed sugars, an increased substrate utilisation rate and the ability to consume more than one type of sugar (De Bari et al., 2013). Lignocellulosic material releases numerous sugars such as glucose, xylose, mannose, galactose and arabinose, with xylose being the second most abundant sugar present (Anwar et al., 2014; Li et al., 2015). This release of different types of sugars therefore necessitates the use of microorganisms which are able to metabolise more than one type of sugar.

One of the commonly used yeasts is *Saccharomyces cerevisiae.* However, it has been established that it is incapable of naturally utilising xylose (Li et al., 2015). Therefore, *S. cerevisiae* strains used for bioethanol production from lignocellulose are often engineered to use xylose (Romani et al., 2015; Sakihama et al., 2015). Other commonly used yeast species include *Scheffersomyces stipitis* (De Bari et al., 2013) and the thermo-tolerant *Kluyveromyces marxianus* (Gabardo et al., 2014).

A major challenge encountered during bioethanol process development is striking a balance between optimisation of the process conditions for microbial growth, saccharification and bioethanol production (Lin et al., 2012). Common operational parameters affecting bioethanol production from lignocellulosic materials include pH, temperature, initial substrate concentration and hydraulic retention time (HRT).

pH is considered a key process variable in the production of bioethanol as yeasts grow and produce bioethanol at a slightly acidic pH of between 4 and 5 (Raikar, 2012). Therefore, the fermentation capability of these microorganisms is severely hampered by very acidic (<4), basic (>8) or even neutral (6-7) pH ranges. Raikar (2012) reported an ethyl alcohol % (v/v) of 6.9% at a pH of 4, using grape waste and an increased ethyl alcohol % (v/v) of 7.6% at a pH of 5, which then decreased. Therefore a pH range of 4.0-5.0 may be considered as optimal for bioethanol production by *S. cerevisiae*.

Operating temperature has a significant impact on the fermentation process due to the exothermic nature of bioethanol fermentation (Dai et al., 2014). In a study by Walsh and Martin

(1977), several strains of *S. cerevisiae* exhibited maximum temperature for growth between 37.5 and 39.8 °C and optimum growth between 30.0 and 35.0 °C. A more narrow range of 28°C to 34 °C has been reported by Dai et al (2014) to be optimal for yeast growth as well as bioethanol production.

Initial substrate concentration is crucial to fermentation processes. A study by Dai et al (2014) showed that, during simultaneous saccharification and fermentation (SSF), higher concentrations of sugar and ethanol exhibited synergistic stress on yeast, which may lead to incomplete or "stuck" fermentations. This effect is elucidated in the lower bioethanol concentration obtained by Wang et al (2013) under SSF bioethanol production (Table 2). Additionally, a significant decrease in the bioethanol production rates (from 28.3 to 13.7 and 3.7%) at respective sugar concentrations of 80, 160 and 300 g/L was observed. Therefore an optimal range of 2-6 % is appropriate for a successful fermentation process.

The fermentation process time is chosen to ensure efficiency and to avoid wastage of resources. Studies focusing on the optimisation of bioethanol production employed an HRT of between 48 and 72 hours (Singh and Bishnoi, 2012; Singh and Bishnoi, 2013; Luo et al., 2014). This may be implemented to avoid end product inhibition caused by low ethanol tolerance. A study by Kasavi et al. (2012) which evaluated the ethanol tolerance of industrial *S. cerevisiae* strains showed a significant decrease in growth at ethanol concentrations of approximately 47 g/L and higher.

Table 2 shows reported process conditions and bioethanol concentrations from sorghum substrates. A high concentration of 127,80 g/L bioethanol was obtained by Deesuth et al. (2016) from sweet sorghum juice under very high gravity (VHG) fermentation, while a concentration of 68,00 g/L was reported for the same substrate and fermenting strain under continuous operational conditions (Ariyajaroenwong et al., 2016). This demonstrates that fermentation modes have a significant effect on production and should therefore be assessed prior to process scale up. Additionally, studies by Luo et al. (2014) and Wang et al. (2013) suggested that sweet sorghum bagasse possesses similar fermentation capabilities to sweet sorghum juice. Therefore, although process conditions play an essential role in the optimal production of bioethanol, the energy demand required to release the fermentable sugars from the substrate should meet the required standard to be considered economically feasible.

Table 2: Ethanol production from various sorghum substrates

Substrate	**Fermentation conditions**	**Inoculum**	**Bioethanol conc. (g/L)**	**Reference**
Sweet sorghum juice	30 °C, 200 rpm, pH 4.93, 0.31 vvm, batch, (VHG) fermentation, 72 hr.	*S. cerevisiae* NP 01	127,80	Deesuth et al. (2016)
Sweet sorghum grain	37 °C, 300rpm, pH 4.5, batch, 32 hr.	*S. cerevisiae* JP1	87,00	Barcelos et al. (2016)
Sweet sorghum juice	37 °C, 200 rpm, pH 4.5, batch, 21 hr.	*S. cerevisiae* JP1	72,00	Barcelos et al. (2016)
Sweet sorghum bagasse	30 °C, 200 rpm, pH 6.0, 0.02 vvm, batch, 40 hr	*Schefferomyces stipitis* CBS5774	30,00	Barcelos et al. (2016)
Sweet sorghum juice	35 °C, 150 rpm, pH 5.0, batch, 168 hr	*S. cerevisiae* ATCC 24858	49.48	Luo et al. (2014)
Sweet sorghum juice	30 °C, 0.01 h^{-1}, continuous, immobilised, single-tubular packed bed, 72 hr.	*S. cerevisiae* NP01	68,00	Ariyajaroenwong et al. (2016)
Sweet sorghum bagasse	30 °C, 80 rpm, batch, 24 hr.	*S. cerevisiae*	41.43	Matsakas and Christakopoulos (2013)
Sweet sorghum bagasse	37 °C, 150 rpm, pH 5.0, SSF, 168 hr.	*S. cerevisiae* ATCC 24858	38,00	Wang et al. (2013)

2.10.1 Product extraction

Bioethanol extraction can be carried out by solvent extraction and distillation. Currently, the most common method applied is distillation (Onuki, 2006; Balat et al., 2008). However, it is energy intensive and alternatives are being investigated (Pitt Jr. et al., 1983). A number of distillation techniques can be employed, such as adsorption, azeotropic, diffusion, extractive and membrane distillation (Aditiya et al., 2016). Another method investigated is supercritical fluid extraction, which serves as an alternative to the above-mentioned extraction methods (Rehm and Reed, 1996), though many disadvantages are associated with this. An additional extraction method is *in situ* extraction. As many biotechnological processes are negatively impacted by product inhibition, *in situ* product recovery from the fermentation medium will result in a significant increase in the productivity of fermentation processes (Rehm and Reed, 1996). *In situ* product recovery strategies include adsorption (Ng and Kuek, 2013), distillation, precipitation, electrophoresis, pervaporation, gas stripping, dialysis, reverse osmosis and extraction (Rehm and Reed, 1996).

2.10.2 Techno-economic assessment of bioethanol production from lignocellulosic biomass

Bioethanol has shown great promise as a sustainable alternative to transportation fuel, however its potential is largely dependent on the eventual cost of bioethanol (Chovau et al., 2013). Commercially, low cost, abundantly available feedstocks such as lignocellulosic biomass are ideal (Quintero et al., 2013) as this will result in lowered production costs. This is essential for the introduction of bioethanol as a large-scale transportation fuel (Quintero et al., 2013). Developing a techno-economic model which simulates a commercial scale process plant is therefore critical for process development. It calculates biofuel production costs using a process model and economic model based on experimental data and economic assumptions (Vicari et al., 2012). Techno-economic assessments for the production of bioethanol from various lignocellulosic substrates have been reported (Quintero et al., 2013; Meyer et al., 2013; Macrelli et al., 2012; Sassner et al., 2008). Meyer et al. (2013) achieved a minimum ethanol selling price (MESP) of $2.51/gallon from corn stover, using recombinant *S. cerevisiae*, with a reduced MESP of $2.22/gallon when organic acids are co-produced. In comparison, a lower MESP of $0.97/L was obtained by Macrelli et al. (2012) from sugarcane bagasse and leaves, using a microorganism consortium from a spent sulphite liquor plant. However, a study by Vicari et al. (2012) determined a $0.15/gal uncertainty in MESPs from techno-economic models. Uncertainties in primary measurements such as fermentable sugar yields from pre-

treatment as well as bioethanol yields during fermentation processes have been reported by Vicari et al. (2012) to significantly contribute towards this. This highlights the necessity to accurately model and optimise bioprocesses and quantify products essential to the overall bioprocess.

2.11 Lignocellulosic biohydrogen production

In addition to bioethanol, lignocellulosic biomass such as post-harvest wastes can be used in the production of biohydrogen. It shows great potential as a biofuel, owing to its high energy content of 122kJ/g, recyclability and low carbon emissions when combusted (Wicher et al., 2013). Biohydrogen has also gained interest as it can be obtained from waste and agricultural residues (Han et al., 2015). It can be produced by biological and non-biological methods. Of the biological methods, dark fermentation is the most economical due to its lower energy consumption (Han et al., 2015) as well as its use of moderate environmental conditions (Sagnak et al., 2011).

Anaerobic dark fermentation commonly employs *Clostridium* or *Enterobacter* species. It gives higher fermentation rates and lower process costs in comparison to photo-dependent techniques, as it can use a variety of organic substrates (Show et al., 2011). Other widely used inoculum sources include mixed cultures which are found in anaerobic sludge, municipal waste sludge, compost and soil. Mixed cultures have proven to be more beneficial when a large scale is considered due to simple operation without risk of contamination, they are cost effective and can utilise an array of feedstocks. Dark fermentation follows the Embden-Meyerhof/glycolytic pathway in which hexoses such as glucose are catabolised to form pyruvate, which is further oxidised to form acetyl-CoA (Lee et al., 2011). This reaction requires the reduction of ferrodoxin by ferrodoxin reductase and once reduced, ferrodoxin is oxidised by hydrogenase to regenerate ferrodoxin with the simultaneous release of electrons in the form of molecular hydrogen. This metabolic pathway has been observed in some *Clostridia* species (Lee et al., 2011). The acetyl-CoA is subsequently converted to acetyl phosphate which results in ATP generation and acetate excretion. The accumulation of organic acids leads to a rapid drop in the culture pH which consequently inhibits the production of hydrogen as bacteria are incapable of sustaining themselves at pH levels below 5.0 (Nath and Das, 2004).

Overall, hydrogen production is believed to follow one of the two main metabolic pathways: the acetate pathway (Eq. 5) and the butyrate pathway (Eq. 6) (Barca et al., 2015).

$$C_6H_{12}O_6 + 2H_2O = 2CH_3COOH + 2CO_2 + 4H_2 \quad \Delta G_0 = -206\ kJ \qquad [5]$$

$$C_6H_{12}O_6 = CH_3CH_2CH_2COOH + 2CO_2 + 2H_2 \quad \Delta G_0 = -254\ kJ \qquad [6]$$

Where, in the acetate pathway, 4 moles of hydrogen can be obtained when acetate is the main product of the pathway, or the butyrate pathway where 2 moles of hydrogen can be obtained when butyrate is the main product of the pathway.

Environmental process conditions such as pH, temperature and HRT have been reported to be optimal for biohydrogen production at ranges of 5-7 (Tawfik et al., 2013; Nikhil et al., 2014; Wang and Wan, 2009b), 35 and 37°C (Fan et al., 2006; Moodley and Kana, 2015; Sagnak et al., 2011; Lo et al., 2013) and approximately three days (Show et al., 2011; Liu, 2008) respectively.

Table 3 illustrates the various hydrogen yields reported from sweet sorghum. Although high yields can be achieved, the use of sorghum juice and sorghum grain negatively impact the global food supply, thus post-harvest residues such as sorghum bagasse and sorghum leaves are more suitable for renewable biofuel production.

Table 3: Biohydrogen production from sweet sorghum

Substrate	**Process conditions**	**Inoculum**	**Hydrogen Yield**	**Reference**
Sweet sorghum leaves	37.5 °C, 250 rpm, pH 7.0, 84hr, batch	Anaerobic digested sewage sludge	213.14 mL/g substrate	Rorke and Kana (2016)
Red sorghum grain	30 °C, 100 rpm, pH 7.3, 80 hr	*Escherichia coli* HD701, *Clostridium acetobutylicum* ATCC 824	2.09 mol H2/mol glucose	Morsy (2015)
White sorghum grain	30 °C, 100 rpm, pH 7.3, 80 hr	*Escherichia coli* HD701, *Clostridium acetobutylicum* ATCC 824	3.01 mol H2/mol glucose	Morsy (2015)
Sweet sorghum syrup	30 °C, pH 5.0, 24 hr, continuous	Anaerobic seed sludge	0.68 mol H2/mol hexose	Saraphirom and Reungsang (2011)
Sweet sorghum stalks	35 °C, pH 5.5, 12 hr, continuous	Indigenous microflora of sweet sorghum biomass	0.74 mol H2/mol glucose	Antonopoulou et al. (2011)

2.11.1 Challenges and Future Outlook of Lignocellulosic Biofuel production

Due to the unethical use of first generation energy sources for biofuel production, research has been directed towards the use of lignocellulosic biomass for biofuels. Nevertheless, several factors such as high process costs and low product yields have hindered its use (Balan, 2014). Techno-economic estimates show that second generation biofuels are approximately two to three times more expensive than fossil fuel products. This is largely attributed to low conversion efficiencies during lignocellulosic pre-treatment and enzymatic hydrolysis. Extreme process conditions often lead to the generation of fermentation inhibitors which severely impact the substrate conversion efficiency of the hydrolysate (Jönsson et al., 2013). This by-product generation is heavily dependent on the feedstock used as well as the pre-treatment regime employed, therefore it is essential that useful energy sources are assessed for the possible generation of fermentation inhibitors. Knowledge of the types and quantities of by-products released during pre-treatment will allow for screening and selection of suitable substrates, as well as the appropriate pre-treatment regime, thus enhancing the potential for commercial success. In addition, this knowledge will provide an in-depth perspective for the selection of appropriate substrates and their pre-treatment regimes. This will reduce the resources required for research and development by minimising preliminary experimentation for substrate selection as well as subsequent process design.

Knowledge of the complex dynamics of a biochemical process within a fermentation process is limited. This often leads to numerous fermentation processes being carried out at sub-optimal conditions, leading to low product yields as well as process inefficiency. Therefore, reliable bioprocess models are required during process development. Kinetic models capable of accurately describing a fermentation process will provide invaluable information during biofuel process development. The use of kinetic parameters coupled with mathematical models allows for predictions of key process parameters such as cell concentration, substrate utilisation and production rate within a fermentation process (Ariyajaroenwong et al., 2016). The developed models can be used for virtual experimentation, thus reducing time and costs during bioprocess development. They provide a basis for process design, control and optimisation which can reduce scale-up challenges (Linville et al., 2013). Furthermore, these models enable fine-tuning of the dynamics of fermentation processes for commercial application.

2.12 Conclusion

Fossil fuel depletion coupled with environmental impacts have expedited research towards renewable biofuels. Lignocellulosic sorghum leaf waste is a cheaper feedstock alternative for biofuel production. However, its recalcitrance requires additional processing before microorganisms can metabolise it. Therefore, low-cost pre-treatment strategies that minimise the release of inhibitor compounds are necessary for optimal fermentable sugar recovery. In addition to this, current biofuel production technologies are expensive and result in low energy yields. Fermentative biofuel production from lignocellulose is therefore an attractive approach for renewable energy development since the materials required are low cost, abundant and sustainable. Mathematical optimisation strategies may help overcome pre-treatment challenges by maximising fermentable sugar production whilst reducing inhibitor compounds as well as improve low biofuel yields by use of kinetic modelling. Data generated from these models will provide a comprehensive foundation for scale-up and commercialisation.

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Chapter 3

Microwave-assisted chemical pre-treatment of waste sorghum leaves: Process optimization and development of an intelligent model for determination of volatile compound fractions

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Microwave-assisted chemical pre-treatment of waste sorghum leaves: Process optimization and development of an intelligent model for determination of volatile compound fractions

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HIGHLIGHTS

- Elucidation of 21 volatile compounds from pre-treated lignocellulosic waste.
- Non-linearities observed between process conditions & volatile compound production.
- Dose-response relationship between HCl concentration & furfural production with a 2.5% HCl threshold.
- Application of intelligent model to estimate profile of various volatile compounds.

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ABSTRACT

This study reports the profiling of volatile compounds generated during microwave-assisted chemical pre-treatment of sorghum leaves. Compounds including acetic acid (0–186.26 ng/g SL), furfural (0–240.80 ng/g SL), 5-hydroxymethylfurfural (HMF) (0–19.20 ng/g SL) and phenol (0–7.76 ng/g SL) were detected. The reducing sugar production was optimized. An intelligent model based on Artificial Neural Networks (ANNs) was developed and validated to predict a profile of 21 volatile compounds under novel pre-treatment conditions. This model gave R^2-values of up to 0.93. Knowledge extraction revealed furfural and phenol exhibited high sensitivity to acid- and alkali concentration and S:L ratio, while phenol showed high sensitivity to microwave duration and intensity. Furthermore, furfural production was majorly dependent on acid concentration and fit a dosage-response relationship model with a 2.5% HCl threshold. Significant non-linearities were observed between pre-treatment conditions and the profile of various compounds. This tool reduces analytical costs through virtual analytical instrumentation, improving process economics.

1. Introduction

Rapidly diminishing fossil fuel reserves combined with environmental pollution suggest a need for transition toward renewable and sustainable feedstocks for fuel production and other commercially important products (Cavka and Jönsson, 2013). Sorghum is a fast growing cereal crop which generates large quantities of agricultural waste in the form of sorghum leaves (Zegada-Lizarazu and Monti, 2012). An estimated 64.32 million tons of sorghum has been reported by the United States Department of Agriculture (USDA) to be produced in the 2016/2017 period (World Sorghum Production, 2016), with an estimated 76% of this weight attributed to lignocellulosic waste (Stallcup et al., 1964).

Lignocellulose is composed of three major components - cellulose, hemicellulose and lignin (Kahr et al., 2013). Cellulose and hemicellulose are sugar polymers which can be hydrolyzed to form fermentable sugars (Chaturvedi and Verma, 2013) whereas, lignin serves as a protective layer around hemicellulose and cellulose, significantly reducing accessibility to microbial degradation (Khoo, 2015). This presents a challenge for bio-conversion to fermentable sugars (Cavka and Jönsson, 2013). An appropriate pre-treatment is required to disrupt or break down the lignin barrier in order to recover cellulose and hemicellulose (Anwar et al., 2014). Pre-treatment methods frequently used include dilute or concentrated acids and alkali solutions (Kumar et al., 2009) in conjunction with thermal energy. Extreme process conditions required to partially break down lignocellulose result in formation of unwanted by-products (Cavka and Jönsson, 2013). Some of these by-products have been reported to negatively influence enzymatic

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hydrolysis and fermentation processes (Zha et al., 2012). Fermentation inhibitors include aromatic compounds, furan-derivatives and aliphatic acids, concentrations of which vary, depending on the composition of the lignocellulosic material used and the severity of pre-treatment process conditions (Cavka and Jönsson, 2013).

Some of the inhibitory effects observed during fermentation include a longer lag-phase, slower microbial growth, lower cell density as well as reduced productivity (Zha et al., 2012). Numerous studies have focused on the removal/reduction of common inhibitors in order to enhance microbial fermentation (Jönsson et al., 2013; Palmqvist and Hahn-Hägerdal, 2000a; Liu et al., 2015). However, a complete assessment of the various intermediate volatile compounds which may arise during heat-assisted chemical pre-treatment of lignocellulosic material has not been comprehensively reported.

Challenges associated with pre-treatment often occur during the fermentation or downstream stages of bioprocesses. However, due to the variety and quantity of by-products generated, the identification and quantification of lignocellulosic pre-treatment products is often limited to sugars and commonly reported fermentation inhibitors. This may lead to detrimental effects of unidentified compounds being overlooked. Another major hindrance associated with full assessment of various intermediates has been the high cost of instrumentation required for these analyses (Zhang et al., 2005) as well as extensive sample preparation and extended run times required for some of these methods (Humpula et al., 2011). Consequently, costs associated with direct quantification of large amounts of data can become insurmountable at both research and development (R&D), and production stages due to the lack of instrumentation (Zhang et al., 2005).

Artificial Neural Networks (ANNs) have recently gained increasing interest for modelling of non-linear processes. ANNs gather information by detecting patterns and relationships found in data and are trained through experience (Agatonovic-Kustrin and Beresford, 2000) and can thus model bioprocesses using data obtained from various modelling techniques (Desai et al., 2008). ANNs have been reported in the modelling of bioprocesses by Vats and Negi (2013) and Sewsynker et al. (2015). ANN also has the capability of being used as a virtual sensor (soft-sensor) for the estimation of process parameters which are difficult and costly to monitor (Gonzaga et al., 2009).

Soft-sensors have provided an opportunity for real-time bioprocess monitoring using an indirect approach. In addition to this, real time measurements can be achieved due to quick operating time of soft-sensors. A predictive accuracy of 0.998 has been reported by Herrera and Filho (2013), using a hybrid model comprised of ANN and mass balance to predict product formation rate in bioethanol production, using secondary measurements of pH, turbidity, CO_2 flow rate and temperature. In this study, optimization of microwave assisted acid pre-treatment as well as profiling of volatile compounds generated from waste sorghum leaves (SL) pre-treated under various microwave assisted regimes is carried out. Furthermore, a soft-sensor model for the prediction of a volatile profile from pre-treated lignocellulosic wastes is developed and validated

2. Materials and methods

2.1. Feedstock preparation

Sorghum leaves used in this study were harvested from Ukulinga Research Farm, Pietermaritzburg, South Africa (29°67′E, 30°40′S). Approximately 5–8 sorghum leaves were cut off at the leaf collar of mature (approximately 100–120 days) plants. They were immediately oven dried at 70 °C for 48 h and milled to particle sizes of 1–2 mm using a centrifugal miller (Retsch ZM-1, South Africa). The milled leaves were stored in airtight containers prior to use.

2.2. Experimental design

A four factor Box Behnken design was used for both microwave assisted acid (HCl) and alkali (NaOH) pre-treatments (Table 1). This generated 58 experimental runs with varied input values of acid concentration or alkali concentration, microwave duration, microwave intensity and solid-to-liquid (S:L) ratio.

2.3. Pre-treatment process

Pre-treatment involved weighing out 1.6 g (8% w/v), 2.8 g (14% w/v) or 4.0 g (20% w/v) milled sorghum leaves into 500 ml Erlenmeyer flasks. Leaves were then treated with 20 ml HCl or NaOH at varied concentrations as shown in Table 1. Flasks were covered with a glass plate and microwaved at a varied intensity range of 200–800 W for 2–10 min using a 1000 W capacity Samsung microwave oven (Model: ME9114S1). Thereafter, pre-treated samples were filtered using Munktell filter discs and the liquid phase used for fermentable sugar quantification and volatile profile analysis. Liquid hydrolysate was neutralized using 1.0 M NaOH or 1.0 M HCl and stored at 4 °C until further use. Solid biomass was rinsed using distilled water and analyzed via acid-detergent fiber analysis.

2.4. Analytical methods

2.4.1. Lignocellulosic biomass solubilisation

Lignocellulosic material solubilisation was analyzed using the detergent fiber analysis technique described by Goering and Van Soest (1992) and Wolfrum et al. (2009). This involved subjecting lignocellulosic material to a neutral detergent solution to solubilize and thus separate cell contents from cell wall components. The residual cell wall components were then treated with an acid detergent solution to solubilize and separate hemicellulose. The remaining components (cellulose, lignin and acid insoluble ash) were further treated with 2:1 saturated $KMnO_4$: Lignin buffer solution to solubilize and separate lignin. Ashing was then carried out on the final insoluble residue to separate cellulose and insoluble ash.

2.4.2. Quantification of glucose and reducing sugars

Processed samples were analyzed for glucose content using the YSI 2700 Model Biochemical Analyzer (YSI, USA). The sugar analyzing principle is based on enzyme coupled reactions which produce hydrogen peroxide. This allows for electrochemical detection based on its electrochemical oxidation. Total reducing sugars were quantified by DNS method (Miller, 1959).

2.4.3. Volatile compounds analysis

Volatile compounds from the liquid hydrolysate were analyzed using coupled Varian 3800 gas chromatography (Varian Palo Alto, California, USA) and Varian 1200 mass spectrometry (GC–MS). The GC was equipped with an Alltech EC-WAX column of 30 m × 0.25 mm internal diameter × 0.25 μm film thickness (Alltech Associates Inc., Deerfield, Illinois, USA). Helium was used as the carrier gas at a flow rate of 1 mL/min. From each pre-treated sample, 4 μl was injected into a chromatoprobe trap prepared by cutting glass tubes equaling the size of chromatoprobe quartz microvials (length: 15 mm; inner diameter: 2 mm) and filled with 2 mg of a 50:50 mixture of Tenax TA (Alltech Associates, USA) and graphitized carbon (Carbotrap™, Supelco, USA) and closed on both ends with glass wool. The chromatoprobe traps were placed in a

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Table 1
Codes and levels used for independent input variables for reducing sugar (RS) optimization and intelligent model development.

Independent variables	Symbols	Code		
		−1	0	1
Acid concentration (% v/v)	A	0	2.00	4.00
Alkali concentration (% v/v)	A[a]	0	1.17	2.35
Microwave duration (min)	B	2.00	6.00	10.00
Microwave intensity (W)	C	200	500	800
S:L ratio (% w/v)	D	8.00	14.00	20.00

[a] Separate model used without acid concentration as an input.

Varian 1079 injector by means of a chromatoprobe fitting and thermally desorbed. The temperature of the injector was 40 °C and was held for 2 min with a 20:1 split ratio and then increased to 200 °C. It was then held at 200 °C min^{-1} in splitless mode for thermal desorption. Compound detection was delayed for 6 min. After a 3 min hold at 40 °C, the GC oven was ramped up to 240 °C at 10 °C/min and held there for 12 min. Compound identification was carried out using the NIST05 mass spectral library and comparisons with retention times of chemical standards, as well as comparisons between calculated Kovats retention indices and those published in the literature. Clean chromatoprobe traps were run in GC–MS as controls to identify background contamination. Compounds present at higher or similar percentages in the blanks were considered as contaminants and excluded from the analysis. For quantification of compounds, known amounts of standards (97–99.5% purity) of dominant compounds obtained from Sigma Aldrich Inc. GmbH, Germany were injected into cartridges and thermally desorbed under identical conditions to the samples and the peak area of compounds in the samples were compared with those of the standards and used to calculate the total amount of compound per gram of sorghum leaves (Suinyuy et al., 2013).

2.4.4. Optimization of reducing sugar using Response Surface Methodology (RSM)

The experimental total reducing sugar yields were used to fit a polynomial model equation, relating the input parameters to the yield of total RS using Design Expert software (Stat-Ease Inc., USA). A general form of the model is shown in Eq. (1), where Y represents the process response which, in this case is total RS release, α_0 is the free or offset term, α_1, α_2, α_3 and α_4 are the linear coefficients, α_{11}^2, α_{22}^2, α_{33}^2 and α_{44}^2 are the quadratic coefficients and α_{12}, α_{13}, α_{14}, α_{23} and α_{24} are the interaction coefficients.

$$Y = \alpha_0 + \alpha_1 x_1 + \alpha_2 x_2 + \alpha_3 x_3 + \alpha_4 x_4 + \alpha_{11} x_1^2 + \alpha_{22} x_2^2 + \alpha_{33} x_3^2 + \alpha_{44} x_4^2 + \alpha_{12} x_1 x_2 + \alpha_{13} x_1 x_3 + \alpha_{14} x_1 x_4 + \alpha_{23} x_2 x_3 + \alpha_{24} x_2 x_4 \quad (1)$$

2.5. Artificial intelligent model development to predict a profile of volatile compounds

An Ensemble Neural Network was used to develop an intelligent model to predict the type and fraction of volatile compounds from microwave assisted pre-treatment of SL. The committee consisted of 3 multilayer perceptron Artificial Neural Networks. The topology of each committee member consisted of 1 input layer of 5 neurons, 2 hidden layers comprised of 13 neurons each and 1 output layer of 21 neurons (5-13-13-21).

The inputs included acid concentration, alkali concentration, microwave duration, microwave intensity and S:L ratio while the outputs were (in g/l) total reducing sugar and glucose and (in%) furfural, 1-hydroxy-2-propanone, 5-methyl furfural, acetic acid, formic acid, 2-phenyl acetaldehyde, citrannic anhydride, guaiacol, phenol, 4-ethyl guaiacol, 4-ethyl phenol, 4-vinyl guaiacol, pyrranone, levulinic acid, dihydrobenzofuran, benzoic acid, HMF, phenyl acetic acid and 4-hydroxy benzaldehyde. Subsequently the experimental data set was divided into 75% for training and 25% for validation. A logistic transfer function was employed for the hidden layer. This layer had two main purposes; addition of weighted inputs as well as the linked bias (2) and shift input data to a non-linear form (3) (Desai et al., 2008).

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

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

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

The experimental data were normalized according to the following equation:

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

where e_i is the normalized data and E_{min} and E_{max} denote the minimum and maximum values.

2.5.1. ANN training and Validation

The network was trained using a back propagation algorithm with the goal of achieving a minimum net error on the validation data set while preventing overtraining or memorization. A net error value on the validation data set of 0.026 was achieved after 2500 training epochs. The accuracy of the intelligent model was assessed using regression analysis on predicted and observed process outputs and coefficients of determination (R^2) were calculated for each model output, illustrating the model's ability to accurately predict fractions of volatile compounds generated.

2.5.2. Sensitivity analysis and knowledge discovery

Sensitivity analysis was used to determine the sensitivity of the model to changes in input parameter values (Sewsynker et al., 2015). Sensitivity studies were carried out to determine the rate and direction of output change when each input was varied from its minimum to maximum values, while remaining inputs were kept at their median value. To extract the functional relationship between process inputs and outputs from the developed model, mathematical equations illustrating the various functional relationships were derived using curve fitting.

3. Results and discussion

3.1. Sorghum leaf composition

Fiber analysis indicated that raw, untreated SL contain cellulose, hemicellulose and lignin at fractions of 30.73, 32.72 and 3.56% respectively (Table 2). This is in line with previously analyzed samples of SL which contained 28.56, 29.18 and 3.94% of cellulose,

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Table 2
Fiber composition of untreated microwave assisted chemically pre-treated sorghum leaves.

Sample	Cellulose (%)	Hemicellulose (%)	Hemicellulose solubilisation (%)	Lignin (%)
Untreated	30,73	32,72	0	3,56
Acid treatment	44,77	25,12	23	10,98
Alkali treatment	39,95	31,51	3,7	17,76

hemicellulose and lignin respectively (Rorke and Kana, 2016). A very low reduction in hemicellulose content was observed for both treatments (23 and 3.7% hemicellulose solubilisation for acid and alkali treatments respectively) when compared to HCl pre-treatment using a water bath for heating at 100 °C, which resulted in 77% hemicellulose solubilisation (Rorke and Kana, 2016).

3.2. Volatile compound profile

The profile of groups of volatile compounds obtained under various combinations of microwave assisted acid pre-treatment conditions is shown in Fig. 1. Major groups of compounds found were aldehydes, aliphatic acids and ketones, as well as lower fractions of alcohols, lactones and aromatic compounds (benzenoids and phenolics). Commonly reported volatile compounds from pre-treatment of lignocellulose include; furfural, HMF, formic acid, levulinic acid, acetic acid and phenolic compounds (Palmqvist and Hahn-Hägerdal, 2000a; Jönsson et al., 2013), (Cavka and Jönsson, 2013; Chandel et al., 2007; Soudham et al., 2014; Dussán et al., 2014). Table 3 illustrates the relative volatile compound distribution ranges observed under varying pre-treatment conditions. The largest volatile fraction observed was the aldehydes (up to 70%), of which furfural makes up a large portion (up to 68%), corresponding to a yield of 240.80 ng/g SL (Table 3). Furfural is a product of xylose degradation, which occurs at high temperature and pressure during pre-treatment (Palmqvist and Hahn-Hägerdal, 2000a).

In addition to furfural was HMF, which is similarly formed upon hexose degradation (Larsson et al., 1999). Also, these furan derivatives are produced in larger quantities as process conditions become more severe, with higher exposure time to acidic conditions or temperatures (Harmsen et al., 2010). This suggests a higher acid concentration will lead to greater generation of furan-derivatives. Furfural has been reported to be inhibitory to bioprocesses. For example, *Saccharomyces cerevisiae* metabolizes furfural under aerobic, oxygen-limited and anaerobic conditions to produce furfuryl alcohol (Palmqvist and Hahn-Hägerdal, 2000a). The formation of furfuryl alcohol impedes ethanol production as it inhibits anaerobic growth of *S. cerevisiae* (Palmqvist and Hahn-Hägerdal, 2000a). Moreover, furfural causes reactive oxygen species to accumulate within *S. cerevisiae* cells, as well as damage to vacuole and mitochondrial membranes, chromatin and actin (Almeida et al., 2007). Similarly, HMF is metabolized by *S. cerevisiae*, producing 5-hydroxymethyl furfuryl alcohol, however this occurs at a lower rate than that of furfural (Palmqvist and Hahn-Hägerdal, 2000a), causing a longer lag phase in microbial growth. Therefore, furan derivatives inhibit or hamper ethanol production by; redirecting energy used for ethanol production to fix damage caused by furans; enzymatic inhibition or use of necessary cofactors (Almeida et al., 2007).

The second largest fraction of the volatile profile is attributed to aliphatic acids, amounting to a maximum of 80%, with acetic acid being the most prominent (up to 48%, corresponding yield of 186.26 ng/g SL) due to the release of acetate upon hemicellulose hydrolysis (Larsson et al., 1999). Once within the relatively neutral cell environment, acetic acid dissociates, leading to a drop in pH which ultimately inhibits cell activity (Harmsen et al., 2010). It is therefore imperative that acid is neutralized before fermentation. Other aliphatic acids formed were, among others, formic acid (<12%), levulinic acid (<18%) and hexanoic acid (<15%), corresponding to yields of 15.50, 21.20 and 6.70 ng/g SL respectively. Formation of formic acid occurs due to further degradation of HMF and furfural while levulinic acid is formed upon the degradation of HMF only (Larsson et al., 1999). Larsson et al. (1999) reported that these acids are able to inhibit ethanol production by reduction of biomass formation as well as ethanol yields. This occurs via intracellular accumulation of anions due to acid dissociation, which cells will attempt to correct by using a proton pump to remove protons from the cell (Almeida et al., 2007). This process inevitably utilizes ATP, therefore less is available for biomass formation.

Phenolic compounds such as phenol, 4-hydrozybenzaldehyde and guaiacol are formed due to partial degradation of lignin (Palmqvist and Hahn-Hägerdal, 2000a; Jönsson and Martín, 2016). Although the fraction of phenolic compounds present in

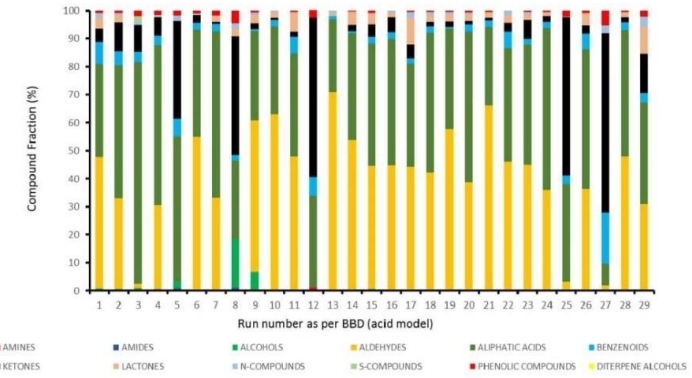

Fig. 1. Profile of volatile compound groups obtained under different pre-treatment conditions using microwave assisted HCl pre-treatment.

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Table 3
Relative amount (%) and corresponding yields of the profile of volatile compounds from microwave assisted acid pre-treatment of sorghum leaves.

Compounds[a]	Relative compound distribution range (%)		Corresponding yield (ng/g)	
	Acid pretreatment	Alkali pretreatment	Acid pretreatment	Alkali pretreatment
Amines				
Trimethylamine	0–1.25	0–63.69	0–0.62	0–2.93
Amides				
Acetic acid amide	0	0–10.33	0	0–2.09
2-Propenamide	0–1.15	0	0–0.96	0
Alcohols				
2,3-butanediol	0–15.79	0	0–19.08	0
Benzyl alcohol	0–0.60	0	0–0.87	0
Furfuryl alcohol	1.34–2.24	0–9.03	0–2.32	0–0.20
Aldehydes				
Furfural	0–67.70	0	0–240.80	0
5-Methyl-furfural	0–4.78	0	0–10.54	0
5-Hydroxymethylfurfural (HMF)	0–19.72	0	0–19.20	0
Aliphatic acids				
Acetic acid	0–47.61	0–39.22	0–186.26	0–18.46
Formic acid	1.84–11.97	0–6.79	0.85–15.50	0–2.46
Hexanoic acid	0–14.88	0–10.31	0–6.70	0–3.73
Heptanoic acid	0–8.28	0–4.66	0–4.03	0–1.69
4-Oxo-pentanoic acid	0–17.66	0	0–21.20	0
Hexadecanoic acid	0–2.33	0	0–2.40	0
Propanoic acid	0–2.33	0	0–0.99	0
Butanoic acid	0–2.22	0	0–0.95	0
Isovaleric acid	0	0–3.06	0	0–1.44
Pentanoic acid	0.4.60	0–2.29	0–4.96	0–0.83
2-Oxo-propanoic acid	0–2.35	0	0–6.30	0
Tetradecanoic acid	0–0.51	0	0–0.75	0
Benzenoids				
Benzeneacetaldehyde	0–7.99	0–9.85	0–11.82	0–3.57
Benzoic acid	0–4.30	0	0–9.48	0
Benzeneacetic acid	0–5.22	0–1.24	0–4.74	0–0.45
Ketones				
1-Hydroxy-2-propanone	0–4.54	0–46.42	0–2.42	0–8.78
Isomaltol	0–20.16	0	0–8.99	0
5,6-Dihydro-2-pyranone	0–3.41	0	0–2.91	0
Ethanone	0–2.43	0–4.45	0–2.30	0–2.09
Levoglucosenone	0–5.20	0	0–12.27	0
Furyl hydroxymethyl ketone	0–4.21	0	0–3.81	0
Pyrranone	0–58.33	0–15.67	0–28.81	0–5.67
2-Cyclopentene-1,4-dione	0	0–0.91	0	0–0.33
2,4-Dimethyl-1,3-Cyclopentanedione	0	0–2.72	0	0–1.33
2-Pyrrolidone	0	0–3.01	0	0–1.42
2,5-Dimethyl-4-hydroxy-3-furanone	0–1.86	0–0.96	0–2.50	0–0.35
Lactones				
5-Methyl-2-furanone	0–0.66	0	0–1.46	0
3-Methyl-2,5-furanone		0–9.84	0	0–18.88
Nitrogen-containing compounds				
1H-pyrrole-2-carboxaldehyde	0–3.39	0	0–4.03	0
Indole	0	0–4.64	0	0–0.58
Dihydrobenzofuran	0.-2.80	0.60–23.54	0–1.67	0.03–4.54
Sulphur-containing compounds				
Dimethyl sulfoxide	0–2.22	0–2.71	0–0.95	0–0.58
Phenolic compounds				
2-Methoxy-4-vinylphenol	0–1.95	0–23.06	0–0.94	0–4.17
4-Hydroxy-benzaldehyde	0–3.46	0–3.81	0	0–1.79
Guaiacol	0	0–22.21	-2.760	0–10.84
Phenol	0–2.11	0–37.32	0–2.60	0–7.76
4-Ethyl-2-methoxy-phenol	0	0–10.11	0	0–2.29
4-Ethyl phenol	0	0–20.66	0	0–4.03
Diterpene alcohols				
Phytol	0	0–33.04	0	0–9.57

[a] Compounds listed according to compound class.

samples was relatively low (less than 6%), phenolic compounds have shown to be the more toxic group of fermentation inhibitors (Harmsen et al., 2010). This may be due to the antimicrobial properties exhibited by phenolic compounds (Adeboye et al., 2014), where reactive oxygen species are generated, causing a loss of the fermenting organism's cell membrane integrity, reducing cell growth and adaptation to the sugars present (Harmsen et al., 2010; Almeida et al., 2007). Additionally, solubilized phenolic com-

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pounds are known to impede enzymatic saccharification of lignocellulose (Jönsson and Martín, 2016). Whereas, weakly acidic phenolic compounds can transport protons back across mitochondrial membranes, leading to destruction of cellular electrochemical gradients (Almeida et al., 2007). Due to the heterogeneity of phenolic compounds, inhibition mechanisms discussed in this study are not exhaustive.

The volatiles profile for samples pre-treated at varying microwave assisted alkali process conditions is shown in Fig. 2, with the largest fraction observed being phenolic compounds (<87%). A similar observation has been reported by Jönsson and Martín (2016), as the most significant effect of alkali pre-treatment is the removal of lignin from lignocellulosic biomass as well as structural alteration. This leads to greater accessibility to cellulose during enzymatic hydrolysis (Chaturvedi and Verma, 2013) as well as greater generation of phenolic compounds. Similarly, a fraction of aliphatic acids (up to 62%) was observed in alkali pre-treatment compared to a fraction of (<78%) seen in acidic pre-treatment. This pattern has also been reported by Jönsson and Martín in 2016, as alkali pre-treatment is known to hydrolyze hemicellulose (Chaturvedi and Verma, 2013), but at a lower rate than that of acid hydrolysis. As previously mentioned, the effect of aliphatic acids such as acetic, formic and levulinic acid can be detrimental to microbial growth. However, in the case of *S. cerevisiae*, low internal pH caused by the presence of these acids within the cell has been reported to enhance thermotolerance by inducing certain heat shock genes (Palmqvist and Hahn-Hägerdal, 2000b). The marked presence of diterpene alcohols of up to 33% (9.57 ng/g SL) may be explained by the occurrence of phytol, which attaches to plant chlorophyll as a side chain (Zerbe and Bohlmann, 2015) during chlorophyll production. As lignocellulosic biomass was used in this study, the marked presence of phytol may be due to the chlorophyll found in sorghum leaves.

3.3. Maximization of reducing sugars release using a regression pre-treatment model

Experimental results seen in Table 4 showed that higher concentrations of glucose as well as total RS were obtained using microwave assisted acid pre-treatment. Thus, microwave assisted acid pre-treatment was selected for further optimization.

The developed polynomial model is represented in Eq. (5) where A: acid concentration (% v/v) B: microwave duration (min) C: microwave intensity (W) and D: S:L ratio (% w/v).

$$\begin{aligned}\text{Total reducing sugar} = {} & +5.82 + 1.55A + 0.31B - 0.84C \\ & + 0.58D + 0.15AB - 0.12AC \\ & - 1.29AD - 2.71BC - 0.65BD \\ & - 0.43CD - 1.04A^2 - 0.40B^2 \\ & - 0.44C^2 - 0.090D^2 \end{aligned} \quad (5)$$

Model fitness was assessed using Analysis of Variance (ANOVA). A co-efficient of determination (R^2) of 1 is an indication of a good fit (Rorke and Kana, 2016). The RSM model gave an R^2 value of 0.76, thus the model can account for 76% of the variation observed in the experimental data. An F-value of 3.19 in conjunction with a low P-value of <0.05 shows the significance of the model.

3.3.1. Optimal RS generation from sorghum leaves using Response Surface Methodology (RSM)

Fig. 3a shows the interactive effect of S:L ratio and acid concentration on RS yield. A S:L ratio (< 15%) and low acid concentration (<1%) gave a RS recovery of 3 g/l. The yield increased to about 7 g/l when low S:L ratio (8–11%) was employed with an acid concentration of 4%. The interactive effect of microwave intensity and duration (Fig. 3b) shows an increase in RS yield from 1 g/l to almost 9 g/l when microwave duration was increased from 2 to 10 min at 200 W intensity. However, a decrease in sugar yield was observed with longer irradiation time (about 10 min) and an increase in microwave intensity from 200 W to 800 W. This suggests that prolonged periods of exposure to significant levels of irradiation may damage the plant cell structure (Li et al., 2014). Optimum set-points of 3.83% HCl, 2 min microwave duration, 600 W microwave intensity and 16.66% S:L ratio were predicted by the RSM model with a RS yield of 6.0 g/l. An average observed RS yield of 9.13 g/l was obtained from the validation experiments.

3.4. Artificial Neural Network Based model predicting the fractions of volatile compounds from microwave-assisted chemical pre-treatment

3.4.1. ANN committee model assessment

A committee of ANN models was developed to predict the relative fractions of 21 volatile compounds from microwave-assisted

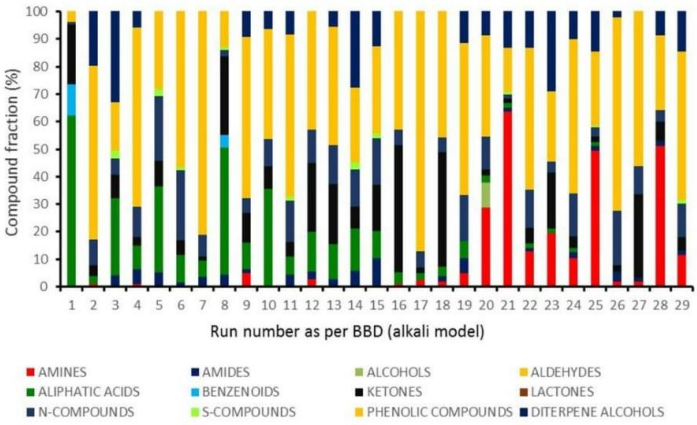

Fig. 2. Profile of volatile compound groups obtained under different pre-treatment conditions using microwave assisted NaOH pre-treatment.

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Table 4
Reducing sugar and glucose yields observed for microwave-assisted acid and alkali pre-treatment of sorghum leaves.

Input level code					Acid pre-treatment		Alkali pre-treatment	
Acid Conc.	Alkali Conc.[a]	MW duration	MW intensity	S:L ratio	Total RS (g/l)	Glucose (g/l)	Total RS (g/l)	Glucose (g/l)
−1	−1	−1	0	0	12.61	1.25	2.75	1.24
+1	+1	−1	0	0	19.30	2.34	1.77	0.15
−1	−1	+1	0	0	15.39	0.24	2.22	0.20
+1	+1	+1	0	0	25.10	0.91	3.28	0.02
0	0	0	−1	−1	46.67	1.36	0.35	0.04
0	0	0	+1	−1	21.07	0.70	0.63	0.06
0	0	0	−1	+1	28.51	2.62	1.46	0.07
0	0	0	+1	+1	17.66	1.02	3.23	0.29
−1	−1	0	0	−1	8.45	0.96	0.96	0.82
+1	+1	0	0	−1	38.35	2.44	1.54	0.06
−1	−1	0	0	+1	27.50	2.12	5.52	1.94
+1	+1	0	0	+1	31.28	1.57	2.67	0.24
0	0	−1	−1	0	23.46	1.52	0.58	0.23
0	0	+1	−1	0	38.35	1.74	1.66	0.16
0	0	−1	+1	0	78.46	3.12	0.61	0.15
0	0	+1	+1	0	5.30	0.30	6.56	0.23
−1	−1	0	−1	0	14.88	0.96	3.94	1.16
+1	+1	0	−1	0	37.34	3.06	1.31	0.12
−1	−1	0	+1	0	8.70	0.08	1.29	0.13
+1	+1	0	+1	0	28.76	0.83	5.12	0.16
0	0	−1	0	−1	12.99	1.10	1.26	0.04
0	0	+1	0	−1	31.28	0.61	0.98	0.04
0	0	−1	0	+1	60.04	3.09	1.71	0.19
0	0	+1	0	+1	35.32	1.05	5.90	0.09
0	0	0	0	0	46.42	2.29	1.92	0.24
0	0	0	0	0	38.09	2.13	1.72	0.19
0	0	0	0	0	48.18	2.46	1.69	0.09
0	0	0	0	0	68.62	2.06	1.51	0.22
0	0	0	0	0	44.40	2.89	1.41	0.18

[a] Separate model used without acid concentration as an input, MW: Microwave.

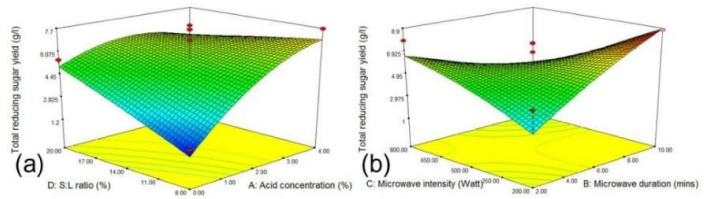

Fig. 3. 3-D Response Surface plots of the microwave assisted acid pre-treatment model, showing the interactive effects of variable input parameters on the release of total reducing sugar.

acid and alkali pre-treated sorghum leaves. Compounds exhibiting fractions above 1.0% across the majority of experiments were selected for model outputs and included; reducing sugar, glucose, furfural, 1-hydroxy-2-propanone, 5-methyl furfural, acetic acid, formic acid, 2-phenyl acetaldehyde, citrannic anhydride, guaiacol, phenol, 4-ethyl guaiacol, 4-ethyl phenol, 4-vinyl guaiacol, pyrranone, levulinic acid, dihydrobenzofuran, benzoic acid, 5-hydroxy methyl furfural, phenyl acetic acid and 4-hydroxy benzaldehyde (Fig. 4). The strategy of using an ensemble of neural networks rather than a single multilayer perceptron was to enhance the network prediction performance by using ensemble averaging (EA).

The positive impact of EA on ANN model's prediction accuracy has been detailed by Piotrowski et al. (2016). Upon training with 75% of experimental data, the accuracy of the developed committee model was assessed by using it to predict the relative fraction of volatile compounds from 15 experimental runs of microwave assisted acid and alkali pre-treatments which were not previously exposed to the committee. Output values for the 21 compounds gave varied coefficients of up to 0.93. Higher co-efficients of determination (>0.7) were obtained for furfural, formic acid, guaiacol, phenol, 4-ethyl guaiacol, 4-vinyl guaiacol, levulinic acid, dihydrobenzofuran and HMF (Fig. 4), suggesting a higher reproducibility and accuracy in the prediction of the concentration of these compounds by the developed model when subjected to novel pre-treatment conditions. Fig. 5 shows the predicted versus observed values where most of the data points are located along the diagonal line. The relative higher prediction accuracy of the ANN model on furan derivatives, aliphatic acids and phenol is of significant importance as this shows potential for virtual analytical assessment. This knowledge will enhance the design of suitable pre-treatment and detoxification regimes with minimization of inhibitory side-products (Larsson et al., 1999), boosting the economic viability of SL and other lignocellulosic substrates for the production of biofuels and biomaterials. The ANN predictive accuracy on glucose, 1-hydroxy-2-propanone, 5-methyl furfural, acetic acid, 4-ethyl phenol, benzoic acid, phenyl acetic acid and 4-hydroxy benzaldehyde gave coefficient values between 0.42 and 0.69. The model predictive efficiency on this set of volatile compounds was lesser compared to the previous set as a result of possible interactive interferences of some intermediate volatile

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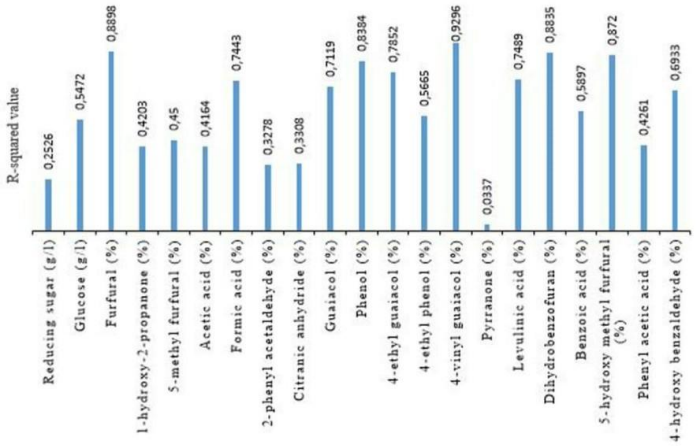

Fig. 4. Chart showing the various R^2 values obtained for each microwave assisted pre-treatment process output using ANN.

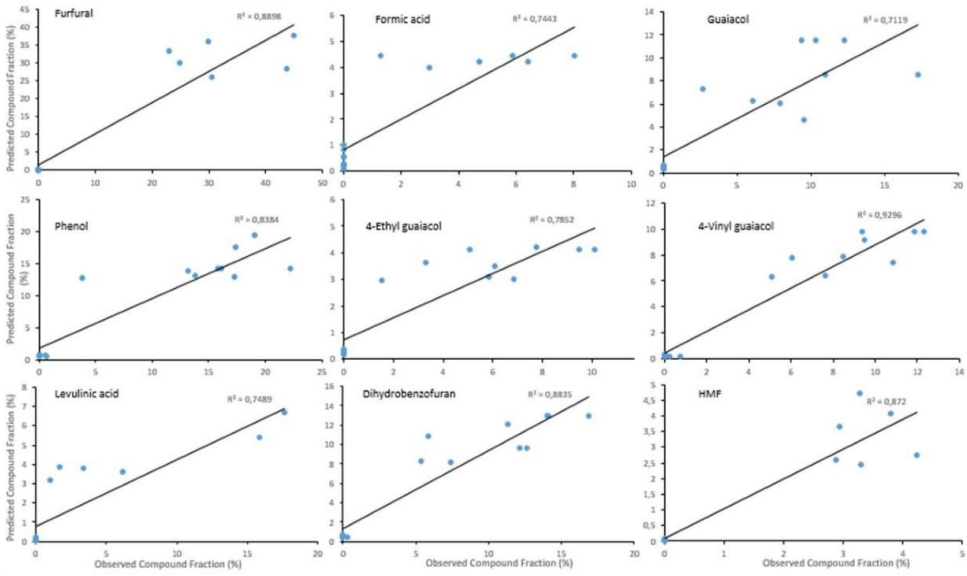

Fig. 5. Regression plots showing the average predicted versus observed compound fraction values which exhibit R^2 values above 0.7.

compounds impacting the detection accuracy. However the model could still account for about half of the variations in experimental data. The negative impact of outliers on ANN based model development has been reported by Khamis et al. (2005). Compounds exhibiting coefficients of determination below 0.4 were not considered significant enough to be estimated using the developed model. These included; reducing sugar, citrannic anhydride, 2-phenyl acetaldehyde and pyrranone.

3.4.2. Impact of input changes on process outputs

Sensitivity studies focused on the impact that process input variation had on the evolution of commonly reported inhibitor compounds. Thus, a high sensitivity to an input implies that the concentration of the inhibitor will be highly affected with little variation on the process input and vice versa as described by Sewsynker et al. (2015). The effects of varying input parameter values within the ranges used, on process outputs of furfural, formic

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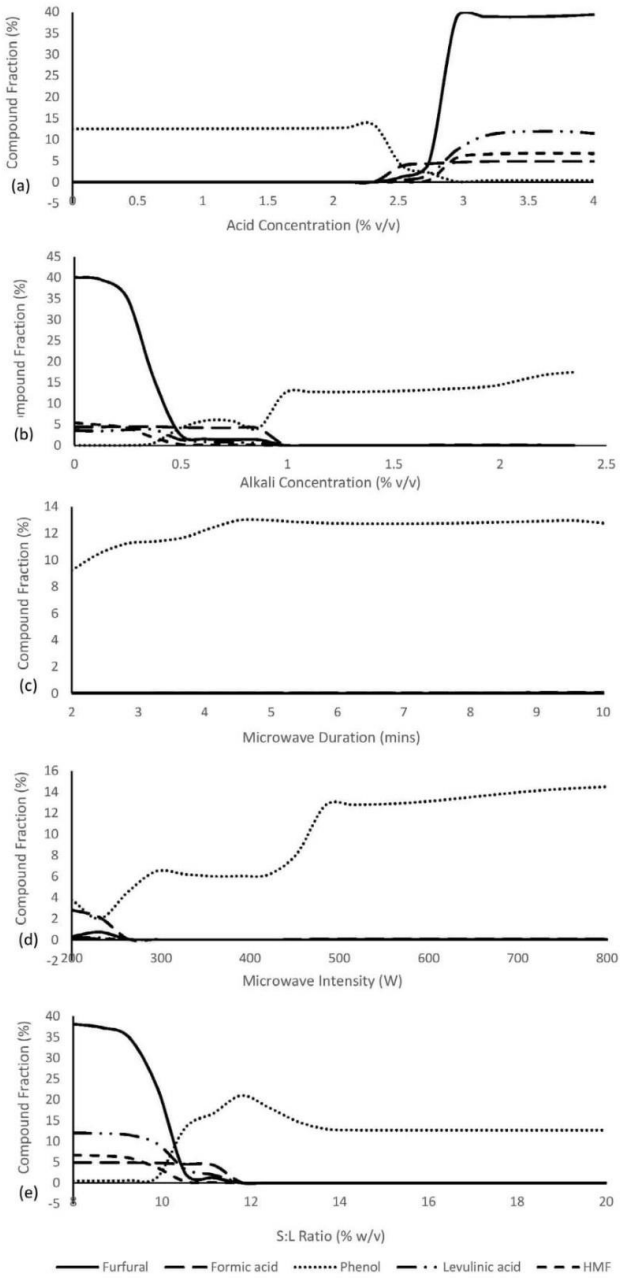

Fig. 6. Impact of variations in input parameters acid concentration (a), alkali concentration (b), microwave duration (c), microwave intensity (d) and S:L ratio (e) on process output (furfural, formic acid, phenol, levulinic acid and HMF).

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Table 5
Model equations illustrating the direction and rate of change of selected volatile compound outputs when input parameters were varied within their boundaries.

Eq.	Process input/output	Model equation form	Equation type	Fitted model	R^2 value
(a)	Acid conc.: Furfural	$y = \theta x^{\eta}/\kappa^{\eta} + x^{\eta}$	DR-Hill	$y = 39.02x^{86.22}/2.80^{86.22} + x^{86.22}$	0.99
(b)	Acid conc.: Phenol	$y = \gamma + (1-\gamma)\Gamma(\alpha, \beta x)$	DR-gamma	$y = 1.27x10^{1} + (1 - 1.27x10^{1})\Gamma(7.19x10^{3}, 2.87x10^{3})$	0.99
(c)	Alkali conc.: Furfural	$y = a/(1 + e^{b-cx})$	Ratkowsky	$y = 4.02x10^{1}/1 + e^{-6.40+8.59x}$	0.99
(d)	Alkali conc.: Phenol	$y = \theta x^{\eta}/\kappa^{\eta} + x^{\eta}$	DR-Hill	$y = 17.44x^{2.52}/1.93^{2.52} + x^{2.52}$	0.94
(e)	MW duration: Furfural	$y = ab + cx^{d}/b + x^{d}$	MMF	$y = 2.25x10^{-2}(1.00x10^{-8}) + 2.84x10^{-2}x^{-1.56x10^{1}}/1.00x10^{-8} + x^{-1.56x10^{1}}$	0.72
(f)	MW duration: Phenol	$y = a - be^{-cx^{d}}$	Sigmoidal	$y = 1.28x10^{1} - 6.59e^{-1.61x10^{-1}x^{2.00}}$	0.97
(g)	MW intensity: Furfural	$y = 1/(a + bx + cx^{2})$	Reciprocal quadratic	$y = 1/3.74x10^{2} - 3.41x + 7.76x10^{-3}x^{2}$	0.99
(h)	MW intensity: Phenol	$y = \theta x^{\eta}/\kappa^{\eta} + x^{\eta}$	Dr-Hill	$y = 8.87x^{2.01x10^{1}}/4.57x10^{2^{2.01x10^{1}}} + x^{2.01x10^{1}}$	0.94
(i)	S:L ratio: Furfural	$y = a/(1 + e^{b-cx})$	Ratkowsky	$y = 3.72x10^{1}/1 + e^{-4.56x10^{1}+4.56x}$	0.99
(j)	S:L ratio: Phenol	$y = \theta x^{\eta}/\kappa^{\eta} + x^{\eta}$	DR-Hill	$y = 1.40x10^{1}x^{8.41x10^{1}}/1.02x10^{1^{8.41x10^{1}}} + x^{8.41x10^{1}}$	0.86

DR: Dose-Response, MMF: Morgan-Mercer-Flodin, MW: Microwave.

acid, phenol, levulinic acid and HMF are shown in Fig. 6a–e. Sensitivity analysis indicated that an acid concentration above 2% resulted in a linear increase in furfural, formic acid, levulinic acid and HMF (Fig 6a). A drastic increase in furfural from approximately 2% to almost 40% (144 ng/g SL) was observed when acid concentration was increased from 2.5 to 3% illustrating a high sensitivity within this region. This revealed that furfural production was largely dependent on acid concentration and fit a dosage-response kind of relationship (Table 5a) with a threshold concentration at about 2.5% HCl. By implication, dilute acid pre-treatment regimes will generate lesser amounts of inhibitor products such as furfural than that of concentrated acid due to less severe process conditions as suggested by Behera et al. (2014). However, an inverse relationship was observed for phenol, where a further increase in acid concentration from 2% to 4% led to a decrease in phenol from approximately 12% (ca. 5.00 ng/g SL) to 0 (Fig. 6a). The direction and rate of change of phenol production under acid pre-treatment was best illustrated by a dose response gamma type relationship (Table 5b). In contrast to this, alkaline pre-treatment at a concentration of approximately 1% resulted in complete reduction of all compounds except phenol (Fig. 6b). An increase in alkali concentration from 1% to 2.35% triggered a rise in phenol concentration from 5% (0.27 ng/g SL) to approximately 18% (0.74 ng/g SL) (Fig. 6b). The relationship was best fitted using a dose-response type equation (Table 5d). Alkali pre-treatment has been shown to partially degrade lignin, resulting in generation of phenolic compounds such as phenol (Palmqvist and Hahn-Hägerdal, 2000a; Jönsson and Martín, 2016). Fig. 6c illustrates low sensitivity of all non-phenolic compounds to a change in microwave duration, as all of these compounds remained near 0%. However, a linear increase in phenol from 9% (1.26 ng/g SL) to 13% (2.60 ng/g SL) was observed when microwave duration was increased from 2 to 5 min, exhibiting a sigmoidal type relationship seen in Table 5f. A similar low sensitivity was observed for non-phenolic compounds when microwave intensity was increased from 200 W to 800 W (Fig. 6d), while a non-linear increase in phenol was observed, which was best fitted by a dose-response type equation (Table 5h). These data therefore suggest that generation of furan derived compounds such as furfural and HMF is largely dependent on acid concentration. This is in line with observations made by Jönsson and Martín (2016), suggesting that acidic conditions result in the formation of furan derivatives which are often further degraded due to instability in an acidic medium or other process conditions.

The impact of S:L input on the production of non-phenolic compounds showed that an increase in S:L ratio from 8 to 12% led to a linear decrease (Fig. 6e) and was best illustrated by a ratkowsky type equation (Table 5i). Furthermore, a non-linear increase in phenol from 0 to 20% (ca. 6.0 ng/g SL) was observed when S:L ratio was increased from 10 to 12%, illustrating a relatively high sensitivity of phenol generation to small changes in S:L ratio. A further increase in S:L ratio to 13% led to a sharp decrease in phenol from 20% to approximately 12% which then plateaued at 12% (ca. 2.40 ng/g SL) even with increasing S:L ratio to 20%. This non-linear relationship between S:L ratio and phenol production was best illustrated using a Dosage Response model equation (Table 5j). This type of response may be due to lower S:L ratio allowing for increased accessibility of either alkali or acid solutions to the lignocellulosic material, resulting in increased lignocellulosic degradation. Vargas Betancur and Pereira (2010) reported an optimum S:L ratio of 1:2.8 (g:ml) for high xylose release and low inhibitor generation from sugar cane bagasse. The developed Artificial Neural Network models have been deposited into the Repository of Intelligent Models (REDIM, 2016) with accession numbers (PRHE000249 and PRAI000402).

4. Conclusion

Optimization of reducing sugar release from microwave-assisted acid pre-treatment resulted in an optimal release of 9.13 g/l. The developed intelligent model gave R^2-values of up to 0.93 and an average R^2-value of 0.59, illustrating that it could accurately estimate concentrations of various volatile compounds. It is therefore an efficient virtual analytical tool which lowers process economics. Sensitivity analysis showed high sensitivity of furfural to acid and alkali concentration and S:L ratio. Significant non-linearities were observed between pre-treatment conditions and the profile of volatile compounds. This knowledge will enhance the design of lignocellulosic pre-treatment regimes for production of biofuels and biomaterials.

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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.2016.10.048.

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Chapter 3 - Appendix

Data not shown as per journal specifications are included as supporting information.

Table 1: Analysis of Variance (ANOVA) for total reducing sugar model from microwave assisted acid pre-treatment

Model output	**Sum of squares**	**df**	**Mean squares**	**F value**	**P-value**	**R^2**	**Adjusted R^2**	**Adeq. precision**
Total reducing sugar	88.99	14	6.36	3.19	0.019	0.76	0.52	7.469

df: degrees of freedom, F-value: Fisher-Snedecor distribution value, P-value: probability value, R^2: coefficient of determination.

Table 2: Predicted and observed reducing sugar and glucose yields for microwave-assisted acid and alkali pre-treatment of sorghum leaves.

Input level code					Acid Pre-treatment				Alkali Pre-treatment			
Acid Conc.	**Alkali Conc.***	**MW Duration**	**MW Intensity**	**S:L Ratio**	**Total obs. RS (g/l)**	**Total pred. RS (g/l)**	**Obs. Glucose (g/l)**	**Pred. Glucose (g/l)**	**Total obs. RS (g/l)**	**Total pred. RS (g/l)**	**Obs. Glucose (g/l)**	**Pred. Glucose (g/l)**
-1	-1	-1	0	0	12.61	20.31	1.25	1.40	2.75	2.53	1.24	1.20
+1	+1	-1	0	0	19.30	34.23	2.34	2.53	1.77	1.35	0.15	0
-1	-1	+1	0	0	15.39	9.44	0.24	0.35	2.22	3.50	0.20	0.53
+1	+1	+1	0	0	25.10	37.96	0.91	2.28	3.28	4.35	0.02	0.20
0	0	0	-1	-1	46.67	35.64	1.36	1.41	0.35	0	0.04	0.07
0	0	0	+1	-1	21.07	23.39	0.70	1.02	0.63	1.58	0.06	0
0	0	0	-1	+1	28.51	35.18	2.62	2.61	1.46	2.58	0.07	0.24
0	0	0	+1	+1	17.66	37.68	1.02	1.27	3.23	5.90	0.29	0.25
-1	-1	0	0	-1	8.45	5.08	0.96	0.18	0.96	0.34	0.82	0.45
+1	+1	0	0	-1	38.35	33.57	2.44	2.12	1.54	2.40	0.06	0.36
-1	-1	0	0	+1	27.50	25.05	2.12	1.91	5.52	5.63	1.94	1.19
+1	+1	0	0	+1	31.28	27.43	1.57	1.82	2.67	4.26	0.24	0.16
0	0	-1	-1	0	23.46	17.89	1.52	1.71	0.58	1.77	0.23	0.25
0	0	+1	-1	0	38.35	52.55	1.74	1.97	1.66	1.33	0.16	0

0	0	-1	+1	0	78.46	57.03	3.12	2.36	0.61	0.70	0.15	0.70
0	0	+1	+1	0	5.30	3.65	0.30	0	6.56	5.12	0.23	0
-1	-1	0	-1	0	14.88	22.04	0.96	0.97	3.94	3.38	1.16	1.00
+1	+1	0	-1	0	37.34	39.42	3.06	2.47	1.31	0.49	0.12	0
-1	-1	0	+1	0	8.70	0	0.08	0.02	1.29	2.72	0.13	0.37
+1	+1	0	+1	0	28.76	11.34	0.83	0.16	5.12	6.30	0.16	0.35
0	0	-1	0	-1	12.99	24.49	1.10	1.45	1.26	1.05	0.04	0.11
0	0	+1	0	-1	31.28	36.64	0.61	0.97	0.98	0.81	0.04	0
0	0	-1	0	+1	60.04	52.91	3.09	2.95	1.71	1.28	0.19	0.45
0	0	+1	0	+1	35.32	35.51	1.05	1.78	5.90	5.51	0.09	0.19
0	0	0	0	0	46.42	44.89	2.29	1.93	1.92	2.26	0.24	0.11
0	0	0	0	0	38.09	44.89	2.13	1.93	1.72	2.26	0.19	0.11
0	0	0	0	0	48.18	44.89	2.46	1.93	1.69	2.26	0.09	0.11
0	0	0	0	0	68.62	44.89	2.06	1.93	1.51	2.26	0.22	0.11
0	0	0	0	0	44.40	44.89	2.89	1.93	1.41	2.26	0.18	0.11

*: Separate model used without acid concentration as an input, MW: Microwave

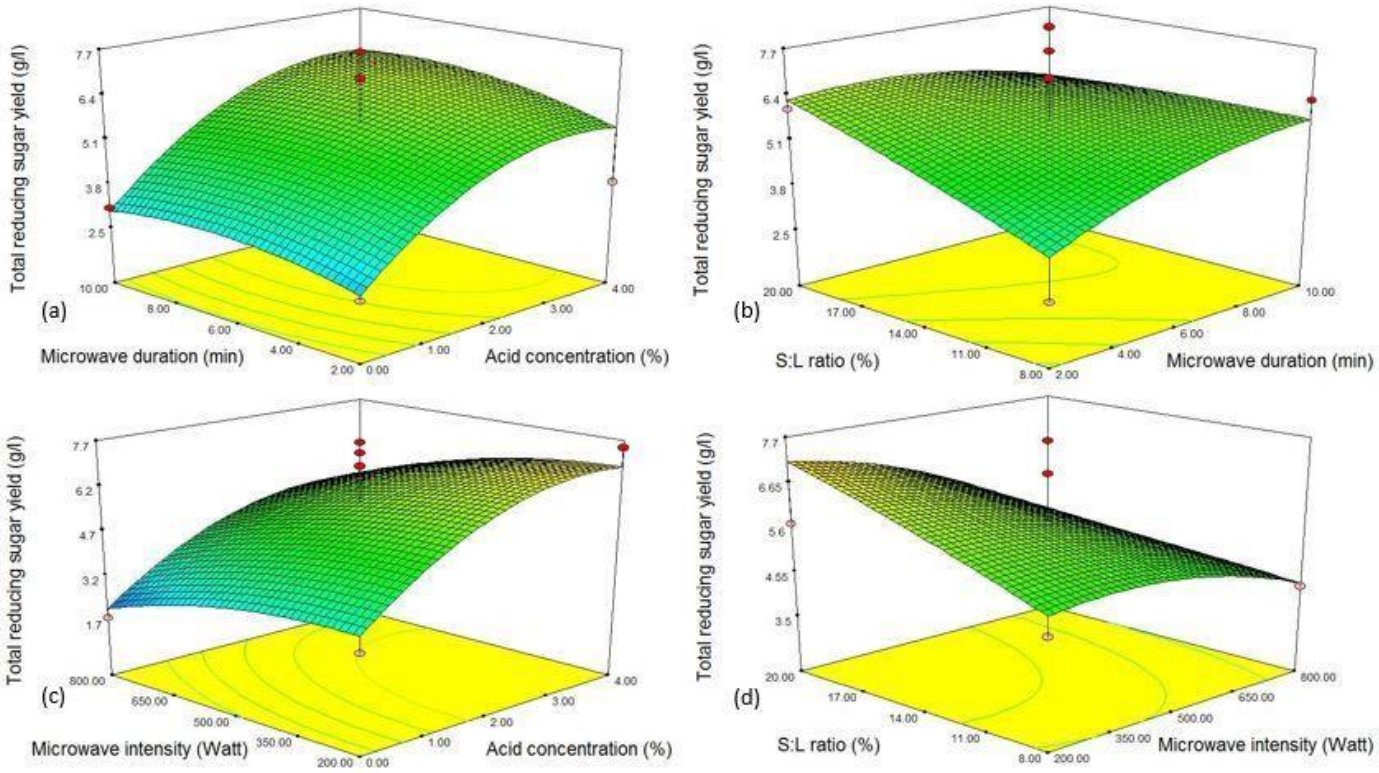

Figure 1: 3-D Response Surface plots of the microwave-assisted acid pre-treated model, showing the interactive effects of the process variables on the release of total reducing sugar.

Chapter 4

Kinetics of Bioethanol Production from Waste Sorghum Leaves using *Saccharomyces cerevisiae* BY4743

This chapter has been submitted to Fermentation with the title: Kinetics of bioethanol production from waste sorghum leaves using *Saccharomyces cerevisiae* BY4743.

The manuscript is presented in the following pages:

Kinetics of Bioethanol Production from Waste Sorghum Leaves using *Saccharomyces cerevisiae* BY4743

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Abstract

Kinetic models for bioethanol production from waste sorghum leaves by *Saccharomyces cerevisiae* BY4743 are presented. Fermentation processes were carried out at varied initial glucose concentrations (12.5-30.0 g/L). Experimental data on cell growth and substrate utilisation fitted the Monod kinetic model with a coefficient of determination (R^2) of 0.95. A maximum specific growth rate (μ_{max}) and Monod constant (K_S) of 0.176 h^{-1} and 10.11 g/L, respectively were obtained. The bioethanol production data fitted the modified Gompertz model with an R^2 value of 0.98. A maximum bioethanol production rate ($r_{p,m}$) of 0.52 g/L/h, maximum potential bioethanol concentration (P_m) of 17.15 g/L and a bioethanol production lag time (t_L) of 6.31 hours were observed. The obtained Monod and modified Gompertz coefficients indicated that waste sorghum leaves can serve as an efficient substrate for bioethanol production. These models with high accuracy are suitable for the scale up development of bioethanol production from lignocellulosic feedstocks such as sorghum leaves.

Keywords: Monod equation, Modified Gompertz equation, Bioethanol, Sorghum leaves.

1. Introduction

Ideal crops for commercial bioethanol production in South Africa include maize, grain sorghum and sugar cane [1], however, in order to completely utilise these materials, post-harvest field wastes should be employed for biofuel production. Sweet sorghum (*Sorghum bicolor* (L) Moench) for one, yields significant amounts of biomass (leaves and pressed stalks) and sugar (found in stalk) [2]. Bioconversion of lignocellulosic material to renewable fuels is currently

receiving much interest since it does not impact food security [2]. Several studies on the enhancement of fermentable sugar release from lignocellulosic substrates have been reported [3-5]. However a significant knowledge gap exists on the kinetic assessment of the pre-treated lignocellulosic substrates for biofuel production.

Bioethanol is one such fuel which exhibits several advantages over conventional fossil fuels. This includes its renewable nature, ease of storage, higher oxygen content, higher octane number, it is free of sulfur, contributes less to global warming as well as air pollution [6-7]. In recent times, the application of bioethanol as a fuel replacement has become more appealing [7]. Globally, efforts are being made to further expedite the use of renewable fuel sources as an alternative. These efforts are being challenged by a significant increase in the cost of production [8]. This suggests that further modelling and optimisation studies are required for the development of biofuel from lignocellulosic substrates.

Kinetic modelling refers to a mathematical description of the changes in the properties of a system in which biochemical reactions take place [9]. These models assist in the design of a production process by representing the complex biochemistry of cells. Kinetic models can be used to understand, predict and evaluate the effects of altering the components of a fermentation process [10]. Most commonly, these models are used to increase yield and productivity as well as minimise the formation of undesired by-products, ensuring the product is of high quality [10]. Models capable of describing the kinetics of microbial growth, substrate utilisation and product formation play a fundamental role in process optimisation and control [11] by providing a basis for process design, control and scale-up [12].

Monod kinetics models are commonly used to describe biomass growth and product formation with respect to the limiting substrate [13], while the modified Gompertz models are used to determine production lag time, maximum production rate and maximum product concentration on a given substrate [6,14]. Very few studies have reported on bioethanol fermentation kinetics using lignocellulosic biomass as a feedstock [12, 2 and 13]. These studies include feedstocks such as populus hydrolysate [12], sweet sorghum stalks [2] and rice hulls [13]. Despite this, there is a scarcity of knowledge on the fermentation kinetics of this fuel using waste sorghum leaves.

Knowledge from fermentation kinetic studies on waste sorghum leaves will provide fundamental information on process characteristics and behaviour. Furthermore, decisions involving process control and improvement can be made with relative ease when a bioprocess is fully understood, advancing its commercial application. In this study, the Monod and modified Gompertz models were used to assess the kinetic behaviour of a bioethanol fermentation process (in batch system) using waste sorghum leaves.

2. Materials and Methods

2.1 Feedstock Preparation and Pre-treatment

Sorghum leaves used in this study were harvested from Ukulinga Research Farm, Pietermaritzburg, South Africa (29°67′E, 30°40′S). Approximately 5-8 sorghum leaves were cut off at the leaf collar of mature (approximately 100-120 days) plants. They were immediately oven dried at 70 °C for 48 hours and milled to particle sizes of 1-2 mm using a centrifugal miller (Retsch ZM-1, South Africa). Milled leaves were treated under previously optimised conditions [15] i.e. a 3.83% (v/v) HCl solution at a solid-to-liquid (S:L) ratio of 16.66% for 2 minutes at 600 W in a 1000 W capacity microwave oven (Samsung, Model: ME9114S1).

2.2 Enzymatic Hydrolysis

Pre-treated biomass was rinsed with distilled water until a pH of 4.0 was achieved. The biomass was then oven dried at 60 °C for 24 hours and enzymatically hydrolysed using powdered cellulase enzyme, Onozuka R-10 (Merck). This was carried out under optimal conditions of pH 4.0-5.0 and temperature of 40-50°C as specified by Gabhane et al. [16] in 500 ml Erlenmeyer flasks. A solid loading rate of 20 g dry biomass in 200 ml 0.05 M citrate buffer with an enzyme loading rate of 50mg/g of dry biomass was employed. The pH during enzymatic hydrolysis was 4.8 and the temperature was maintained at 50 °C using a waterbath (Gesellschaft für Labortechnik mbH D 3006, Burgwedel) at 120 rpm for 72 hours. The hydrolysate was filtered and the filtrate analysed for glucose concentration using a 2.50 g/L dextrose standard.

2.3 Fermentation Medium Formulation

A mineral salt solution (pH adjusted to 4.5 using 1.0M HCl) containing (in g/L); yeast extract, 1.0; $(NH_4)_2SO_4$, 2.0 and $MgSO_4$, 1.0 was autoclaved at 121 °C for 15 minutes. Filter sterilised enzymatic hydrolysate was then added to the mineral salts and initial glucose concentrations of 12.5-30.0 g/L were obtained by diluting or, where needed, supplementing with pure glucose.

Glucose concentrations of between 15.0 and 20.0 g/L obtained during enzymatic hydrolysis determined the range used for subsequent fermentation.

2.4 Microorganism and Inoculum Preparation

S. cerevisiae BY4743 used in this study was obtained from the Department of Genetics, University of KwaZulu-Natal., Pietermaritzburg, South Africa. A single flask containing 100 mL Yeast-Peptone-Dextrose (YPD) medium was inoculated with a single colony and grown at 150 rpm, 30 °C for 16 hours until exponential growth phase was reached. This culture was inoculated (10%) into prepared fermentation medium (working volume of 100 mL) containing an initial glucose concentration of 12.5 g/L. The culture was then grown under the same conditions as previously described and then used as a starter culture for subsequent fermentation processes**.**

2.5 Fermentation process and Analytical Methods

Fermentation processes were carried out in sterilised 250 ml flasks with a working volume of 100 mL. Aliquots of 10 mL (10% inoculum) containing $0.94x10^6$ cells/mL *S. cerevisiae* were aseptically added to the fermentation flasks and the cultures were incubated at 30 °C, at 120 rpm for a minimum of 24 hours or until glucose concentrations were depleted to ensure that microbial metabolism of glucose took place. Fermentations were aseptically sampled every two hours and assessed for biomass concentration, sugar content and bioethanol content.

The sugar content of filtered enzymatic hydrolysate and fermentation media was determined using a YSI 2700 Model Biochemical Analyser (YSI, USA). Ethanol content was determined in the gas phase of the fermentation process using an ethanol vapour sensor (ETH-BTA, Vernier Software and Technology, USA). The absorbance of culture broths was measured using a spectrophotometer (UV-Vis Spectrophotometer, UVmini-1240, Shimadzu) at 650 nm. Cell biomass quantification was achieved by using absorbance as a function of the concentration of yeast cells. A standard curve was prepared by determining the dry weights and corresponding absorbance values of yeast biomass at varied dilutions of a 24 hour *S. cerevisiae* culture grown in fermentation media containing 12.5 g/L glucose. Dry weights were determined by centrifuging 5 ml of each dilution (1:1, 1:2, 1:4, 1:8 and 1:10) for 10 minutes at 5000 rpm. The supernatant was removed and the remaining biomass was dried at 60 °C until a constant mass was obtained.

2.6 Calculations of kinetic model constants

The average specific growth rates (μ) of duplicate fermentation processes were calculated using Equation 1. The specific growth rate values (μ) and the substrate concentration data were subsequently used to estimate the maximum specific growth rate (μ_{max}) and Monod constant (K_S) using a Lineweaver-Burk plot.

$$\mu = \frac{lnX_2 - lnX_1}{t_L - t_0} \quad (1)$$

The linear form of this equation is as follows:

$$\frac{1}{\mu} = \frac{1}{\mu_{max}} + \frac{K_S}{\mu_{max}}\left(\frac{1}{S}\right) \quad (2)$$

In addition, experimental data on bioethanol production over time were used to fit the modified Gompertz model (Equation 3) which showed the lag time, maximum bioethanol production rate, and the potential maximum product concentration.

$$P = P_m \,.\, exp\left\{-exp\left[\frac{r_{p,m}.exp(1)}{P_m}\right].(t_L - t) + 1\right\} \quad (3)$$

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

Sugar utilisation, ethanol yield, ethanol productivity and fermentation efficiency were calculated using the following Equations 4, 5, 6 and 7 respectively [17]:

$$Sugar\ utilisation\ (\%) = \frac{Original\ sugar\ content - Residual\ sugar\ content}{Original\ sugar\ content}\ x\ 100 \quad (4)$$

$$Ethanol\ yield\ \left[\frac{g(ethanol)}{g(glucose)}\right] = \frac{Maximum\ ethanol\ concentration\ (g/L)}{Utilised\ glucose\ (g/L)} \quad (5)$$

$$Ethanol\ productivity\ (g/L/h) = \frac{Maximum\ ethanol\ concentration\ (g/L)}{Fermentation\ time\ (hr)} \quad (6)$$

$$Fermentation\ efficiency\ (\%) = \frac{Actual\ ethanol\ yield\ (g/L)}{Theoretical\ ethanol\ yield\ (g/L)}\ x\ 100 \quad (7)$$

3 Results and Discussion

3.1 Monod kinetic model of *Saccharomyces cerevisiae* on waste sorghum leaves

Cell biomass, bioethanol production and glucose consumption were monitored throughout the fermentation process. The correlation between absorbance and dry weight of yeast biomass was determined by linear regression, which gave a correlation coefficient (r) of 0.96. The specific growth rate (μ) values obtained were 0.096, 0.104, 0.114, 0.122 and 0.123 h^{-1} at initial substrate concentrations of 12.5, 13.3, 19.4, 21.8 and 23.1 g/L respectively (Figure 1). In comparison, Echegaray et al. [18] obtained a range of specific growth rates between 0.019 and 0.240 h^{-1} using diluted sugarcane molasses as a substrate (170 – 270 g/L total reducing sugar range) under anaerobic cultivation of *S. cerevisiae*. In addition, an increase in μ values from 0.096 to 0.123 h^{-1} was observed when the initial glucose concentration increased from 12.5 to 23.0 g/L. A similar trend was reported by Laopaiboon et al. [19] whereby an increase in glucose concentration from 10 to 150 g/L resulted in an increase of μ value from 0.43 to 0.49 h^{-1}. These findings suggest that the specific growth rate of a culture increases with increasing substrate concentration, until substrate saturation is reached [19].

Data on the specific growth rate (μ) values and initial substrate concentrations were used to estimate K_S and μ_{max} (Figure 2). A maximum specific growth rate (μ_{max}) value of 0.176 h^{-1} was obtained, which is the maximum growth rate of *S. cerevisiae* under the specified conditions. This was close to the value of 0.169 h^{-1} previously reported by Dodić et al [14] using *S. cerevisiae* cells grown on sugar beet raw juice. As cell growth rate is largely dependent on substrate concentration, it is expected that a higher initial sugar concentration will result in higher Monod coefficients [20]. The K_S value obtained (10.11 g/L) was in line with values previously reported from several studies on lignocellulosic substrates (Table 1). Using citrus pulp waste as a substrate, a K_S value of 10.690 g/L was reported by Raposo et al. [21], while Srimachai et al. [17] obtained a K_S value of 10.210 g/L using oil palm frond juice. These observations imply that *S. cerevisiae* has a similar affinity ($1/K_S$) to sorghum leaves as oil palm frond juice, glucose and citrus waste pulp. In contrast to this, Ariyajaroenwong et al. [2] reported a Monod constant (K_S) of 47.510 g/L when using sweet sorghum juice as a substrate. This decreased affinity may be due to the presence of more than one type of sugar in sweet sorghum juice [2]. Singh and Sharma [22] reported a K_S value of 3.700 g/L using glucose, which is much lower than the range observed in previous studies, however this corresponds to a higher affinity constant, which is expected as glucose is metabolised with ease.

Variations in K_S values (from 3.7 to 213.6 g/L) can be attributed to substrate type and concentration, strains of yeast employed or the fermentation process itself [2]. These data demonstrate that the suitability of waste sorghum leaves as a substrate for *S. cerevisiae* growth is similar to that of sugar beet raw juice and oil palm frond juice. Furthermore, the fermentation volume size may impact the K_S value. This is illustrated by the vast differences in substrate affinity for glucose obtained by Shafaghat et al. [23] using a working volume of less than 250 mL and Ahmad et al. [7] with a working volume of 8 L. The differences observed between the aforementioned studies may be attributed to additional process challenges encountered in large volume such as poor agitation, low mass transfer and inhomogeneity.

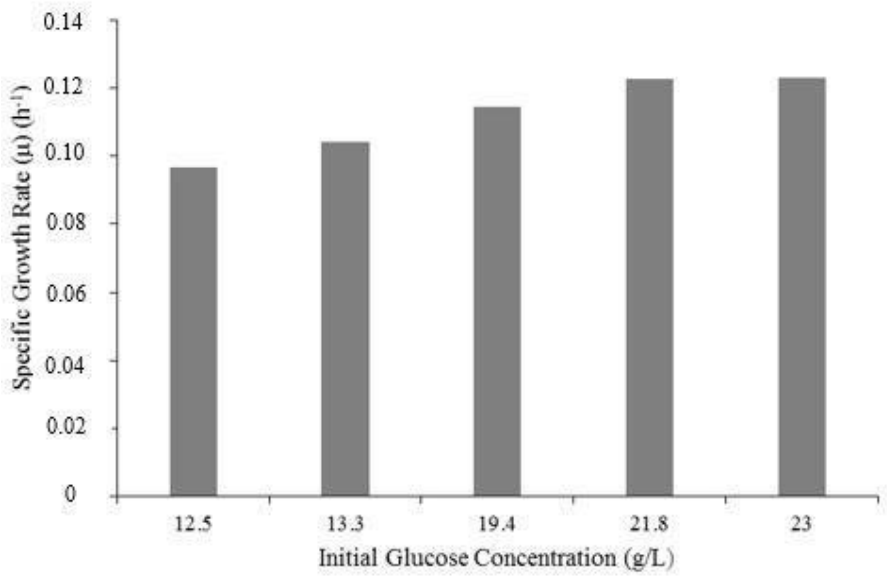

Figure 1: Specific growth rates (μ) of *S. cerevisiae* BY4743 at varied initial glucose concentrations.

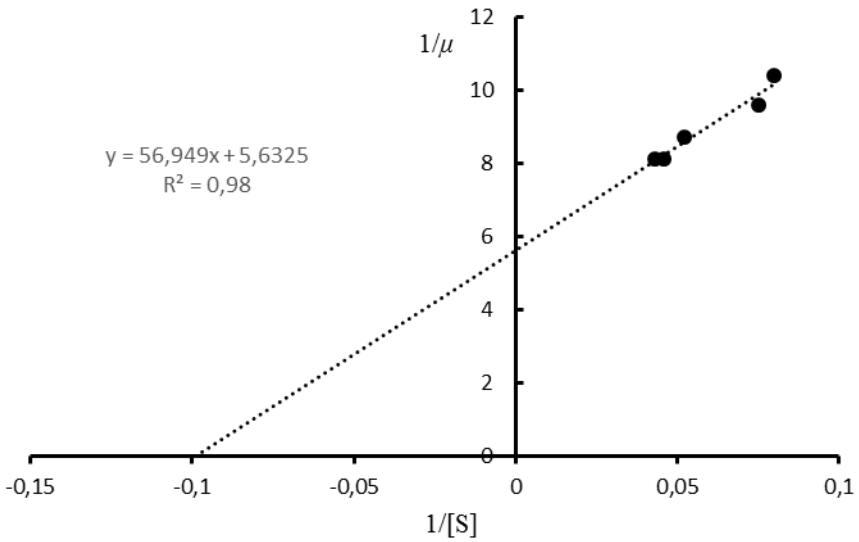

Figure 2: Lineweaver-Burk plot used to estimate Monod constants for batch ethanol production from waste SL.

Table 1: Comparison of the obtained Monod model coefficients with previous studies

Substrate	**μ_{max} (h^{-1})**	**K_S (g/L)**	**Reference**
Sorghum leaves	0.176	10.110	This study
Oil Palm Frond Juice (10-20 years)	0.150	10.210	Srimachai et al. [17]
Sugar beet raw juice	0.169	ND	Dodić et al. [14]
Sweet sorghum juice	0.313	47.510	Ariyajaroenwong et al. [2]
Glucose	0.291	ND	Govindaswamy et al. [24]
Banana peels	1.500	25.000	Manikandan et al. [25]
Glucose	0.084	213.60	Ahmad et al. [7]
Glucose	0.650	11.390	Shafaghat et al. [23]
Citrus waste pulp	0.350	10.690	Raposo et al. [21]
Glucose	0.133	3.700	Singh and Sharma [22]

ND: Not determined

3.2 Bioethanol production

The bioethanol production trend of *S. cerevisiae* cultivated on fermentation medium prepared from sorghum leaves is shown in Figure 3. A rapid depletion of glucose was observed from 0 to 32 hours. A lag phase in bioethanol production of 6 hours was obtained. This corresponds to cell adaptation and synthesis of key nutrients required for biomass or product (bioethanol) formation [14]. Ardestani and Shafiei [26] reported exponential growth of *S. cerevisiae* after 7 hours of incubation. A rapid increase in ethanol concentration was observed from 6 to 28 hours corresponding to the exponential stage (Figure 4). This is expected as ethanol is a primary metabolite and is therefore produced during exponential phase of cell growth. A similar observation has been reported by Lin et al. [27] where a steady increase in ethanol was observed over a duration of 48 hours at 30 and 40 °C. An average ethanol yield of 0.49 g-ethanol/g-glucose was obtained, corresponding to a 96% fermentation efficiency during this period. Fermentation efficiencies between 72.78 and 78.43% have been reported by Srimachai et al. [17] using oil palm frond juice as a substrate, whilst ethanol yields between 0.40 and 0.49 g/g have been obtained from sugar beet raw juice [14]. Waste sorghum leaves show excellent potential for lignocellulosic bioethanol production. A productivity of 0.345 g/L/h was observed in this study. Ethanol productivities on other lignocellulosic substrates in the range of 0.25 to 1.01 g/L/h have been reported [28-31], further pointing to the relative higher potential of waste sorghum leaves for bioethanol production.

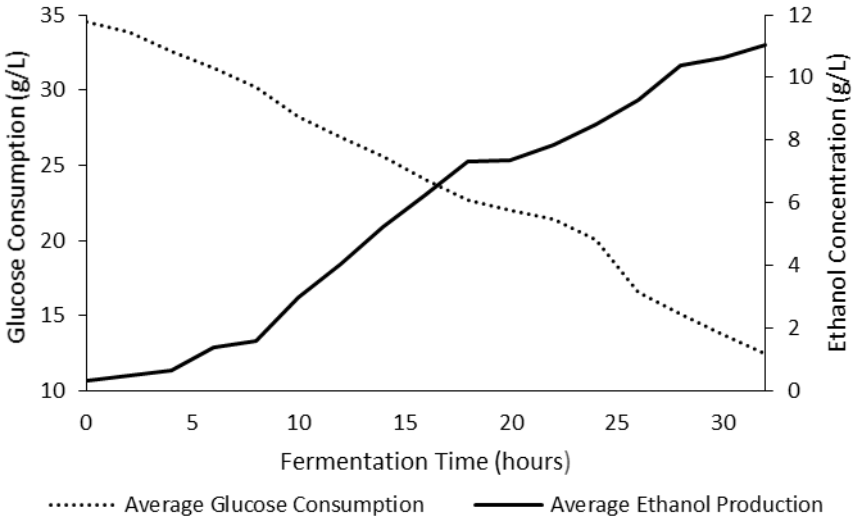

Figure 3: Average glucose utilisation and ethanol formation during batch fermentation by *S. cerevisiae* BY4743.

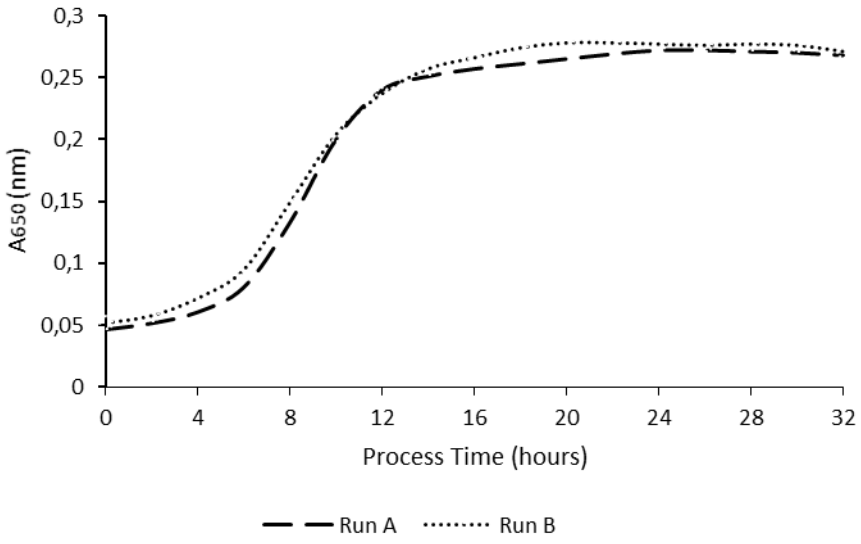

Figure 4: Growth curve of *Saccharomyces cerevisiae* BY4743 during batch ethanol production from waste SL.

Experimental data were fitted to the modified Gompertz model and kinetic coefficients were determined (Equation 7).

$$P = 17.15exp\left\{-exp\left[\frac{0.52\ exp(1)}{17.15}\right].(6.31 - t) + 1\right\} \quad (7)$$

The fitted regression curve (Figure 4) exhibited an R^2 value of 0.98 and a correlation coefficient (r) of 0.99, suggesting that this model is able to efficiently describe bioethanol production during fermentation of sorghum leaf wastes. The Gompertz coefficients for maximum potential bioethanol concentration (P_m), maximum bioethanol production rate ($r_{p,m}$) and lag time were 17.15 g/L, 0.52 g/L/h and 6.31 hours respectively from waste sorghum leaves. Very few studies have reported a lag time of longer than one hour [14]. This suggests that a duration of at least 6 hours was required for yeast cells to adapt to fermentation medium derived from waste sorghum leaves. Additionally, the maximum potential bioethanol concentration of 17.15 g/l, which corresponds to 2.17% (v/v) illustrates that the impact of ethanol concentration within the medium may have a slight effect on the specific growth rate of *S. cerevisiae*. This is supported by an earlier study by Dinh et al. [32], which showed that a higher initial ethanol concentration within fermentation media resulted in an increase in the time required for cells to reach the optimal bioethanol production rate as well as a reduction in the maximum ethanol concentration.

Table 2 shows a comparison of the Gompertz coefficients obtained from this study using sorghum leaves and those reported from oil palm frond juice and sugar beet raw juice. From sorghum leaves, a higher maximum potential bioethanol concentration was achieved. In addition to this, an observed bioethanol production rate of 0.52 g/L/hr was two times of that achieved by Srimachai et al [17] from oil palm frond juice. This illustrates the higher potential of waste sorghum leaves to accommodate a higher production rate.

Table 2: Comparison of modified Gompertz model parameters with previous studies

Substrate	P_m **(g/L)**	$r_{p,m}$ **(g/L/hr)**	t_L **(hr)**	**Reference**
Sorghum Leaves	17.15	0.52	6.31	This study
Oil Palm Frond Juice (10-20 years)	3.79	0.08	0.77	Srimachai et al. [17]
Oil Palm Frond Juice (3-4 years)	11.50	0.24	0.12	Srimachai et al. [17]
Sugar beet raw juice	73.31	4.39	1.04	Dodić et al. [14]

4 Conclusion

This study developed two kinetic models to describe the growth of *S. cerevisiae* BY4743 on pre-treated waste sorghum leaves for bioethanol production. Experimental data fitted the Monod and modified Gompertz model with high accuracy and gave R^2 values of 0.95 and 0.98, respectively. From the Monod model, a maximum specific growth rate and Monod constant of 0.176 h^{-1} and 10.11 g/L was obtained, respectively. These findings show that waste sorghum leaves have a higher relative potential for bioethanol production by accommodating for a higher production rate, thus higher productivity than other lignocellulosic substrates. Furthermore, a maximum yield of 0.49 g-ethanol/g-glucose was achieved after 32 hours of fermentation. The generated kinetic knowledge of *S. cerevisiae* growth on sorghum leaves and bioethanol formation in this study is of high importance for process optimisation and scale up towards commercialisation of this fuel.

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Chapter 5
Conclusions and Recommendations for future studies

5.1 Conclusions

This study examined the development of a bioethanol production process from waste sorghum leaves. A profile of the fermentation inhibitors generated during microwave assisted chemical pre-treatment was developed and the production of fermentable sugar was modelled. Furthermore, a soft-sensor capable of predicting the volatile compound profile under varied process conditions was developed using Artificial Neural Network (ANN). Additionally, the kinetic model of *Saccharomyces cerevisiae* growth and bioethanol production from waste sorghum leaves was assessed. Major findings and their significance are summarised as follows:

Chapter 3:

i. In addition to reducing sugars, microwave assisted chemical pre-treatment of waste sorghum leaves revealed the presence of 21 volatile compounds. Among these compounds were fermentation inhibitors such as acetic acid, furfural, HMF, phenol, levulinic acid and formic acid. Acidic pre-treatment led to an increased generation of furfural and HMF while phenol generation was associated with alkali pre-treatment. These results demonstrate that an initial profile analysis of compounds generated during pre-treatment is a critical step for the selection of a suitable lignocellulosic pre-treatment and detoxification strategy for a novel substrate. Furthermore, knowledge on the viability of various substrates can be determined based on the amount of inhibitory compounds formed during pre-treatment.

ii. Acid pre-treatment was optimal for reducing sugar release, with an optimum of 9.13 g/L, while alkali pre-treatment gave an optimum of 2.50 g/L. Therefore, microwave assisted HCl pre-treatment was more efficient for lignocellulosic degradation and sugar release from waste sorghum leaves. This indicates that waste sorghum leaves, which are usually left in the field after harvest or disposed of by burning, contain sufficient fermentable sugar which is recoverable through

an appropriate acid pre-treatment, illustrating its potential as a low cost feedstock for bioethanol production.

iii. The prediction of furfural, formic acid, guaiacol, phenol, 4-ethyl guaiacol, 4vinyl guaiacol, levulinic acid, dihydrobenzofuran and HMF generation by the ANN soft-sensor gave coefficients of determination between 0.71 and 0.93, illustrating adequate predictive accuracy. These findings indicate that ANN can be successfully implemented as a soft-sensor for the prediction of inhibitor compounds generated from chemical pre-treatment of waste sorghum leaves. This predictive tool will enhance the design of lignocellulosic pre-treatment regimes to optimise for the generation of fermentable sugars while reducing fermentation inhibitors.

iv. Sensitivity analysis showed high sensitivity of furfural and phenol to increased acid concentration, with furfural generation occurring until a 2.5% HCl threshold. Phenol exhibited a mild sensitivity to an increase in the concentration of NaOH and a significant dependency to increasing microwave duration and intensity. Knowledge of the functional relationships between the pre-treatment operational conditions and fermentation inhibitor generation is paramount to the design of lignocellulosic pre-treatment regimes and the impact on the overall fermentation process economics.

Chapter 4:

i. The Monod model, with an R^2 of 0.95, gave a maximum specific growth rate (μ_{max}) of 0.176 h^{-1} and Monod substrate saturation constant (K_S) of 10.11 g/L. This data show that waste sorghum leaves are well suited as a substrate for bioethanol production by *Saccharomyces cerevisiae.* Furthermore, a relatively high affinity for waste sorghum leaves was observed, which may significantly enhance the growth of *S. cerevisiae* biomass on waste sorghum leaves, for bioethanol production.

ii. The modified Gompertz model with R^2 of 0.98 showed a maximum bioethanol production rate ($r_{p,m}$) of 0.52 g/L/h, maximum potential bioethanol concentration (P_m) of 17.15 g/L and a bioethanol production lag time (t_L) of 6.31 hours. In addition, a maximum yield of 0.49 g-ethanol/g-glucose was obtained, corresponding to a 96% conversion efficiency in 32 hours of fermentation. The generated kinetic knowledge of *S. cerevisiae* growth on sorghum leaves and bioethanol formation in this study is of high importance for process optimisation and scale up towards commercialisation of this fuel.

5.2 Recommendations for future studies

Based on the findings of this study, the following recommendations can be made for future research on bioethanol process development from waste sorghum leaves:

5.2.1 Taking into consideration the marked generation of fermentation inhibitors during microwave-assisted acid pre-treatment, a two-stage bioprocess mode may be adapted in which bioethanol production is followed by a biohydrogen stage (Appendix). In this case, the lowered sensitivity of mixed cultures to fermentation inhibitors, pre-treatment hydrolysate can be directed to biohydrogen production, while enzymatic hydrolysate is directed toward subsequent bioethanol production. This will assist in achieving a higher substrate conversion efficiency when using lignocellulosic substrates such as waste sorghum leaves.

5.2.2 The implementation of ANN as a virtual sensor for compounds which are difficult or costly to monitor in real time will significantly lower the cost of process development. Additionally, this will enable the monitoring of several process parameters during fermentation processes.

5.2.3 Kinetic assessment of *S. cerevisiae* growth and bioethanol production on various lignocellulosic substrates will pave the way for a more comprehensive comparison of the productivities of the assessed microorganisms. Selection of the microorganism demonstrating the best capability to produce high yields of

bioethanol can therefore be carried out at smaller scales to reduce process development costs.

5.2.4 Improving the capability of *S. cerevisiae* by using immobilisation techniques and metabolic engineering for improved bioethanol yields from lignocellulosic biomass will enhance the industrial feasibility of lignocellulosic bioethanol production. This will significantly reduce costs associated with the production and extraction processes.

Appendix A

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Biohydrogen process development on waste sorghum (*Sorghum bicolor*) leaves: Optimization of saccharification, hydrogen production and preliminary scale up

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ABSTRACT

Waste Sorghum Leaves (WSL) are considered as agricultural waste residue and a potential feedstock for biofuel production. This study investigates the optimum recovery of xylose and glucose from WSL using six Response Surface-based models. Furthermore, the optimum physico–chemical set-points for hydrogen production from these fermentable sugars are determined and a preliminary scale up is assessed.

Models for saccharification were based on HCl (HCl-model), H_2SO_4 (H_2SO_4-model) and HNO_3 (HNO_3-model) pre-treatments subjected to input variables of acid concentration, heating time, solid to liquid ratio and acid exposure lag time in the ranges of 1–6%, 70–240 min, 30–50% and 0–24 h respectively. The models gave high coefficients of determination of up to 0.93. The HCl-model showed the highest recovery of xylose and glucose, with yields of 54.05 g/L and 15.98 g/L respectively, corresponding to 77% hemicellulose solubilisation and a shorter pre-treatment time in comparison to the other two acids.

Optimization of physico–chemical variables for biohydrogen production gave set-points of 50% inoculum concentration, process time of 83 h, 11 min with an initial pH of 7. Process scale up in a 13 L bioreactor resulted in a peak hydrogen fraction of 43.75% and a volumetric hydrogen yield of 213.14 ml/g of fermentable sugar (FS), which is comprised of xylose and glucose. These findings illustrate the potential of sorghum leaves which are generally considered as agricultural waste for large scale production of fermentative hydrogen.

Introduction

Fossil fuels play a vital role in the global energy requirement and account for more than 80% of the primary energy consumption. Fossil fuel production profiles suggest a global peak production before 2025 and a projected decline thereafter [15]. The heavy dependence on fossil fuel-based energy and increased emissions in greenhouse gas have resulted in worldwide awareness of socio-economic impacts that may arise. This search for alternative, renewable energy sources has thus become an urgent issue [11].

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Bio-hydrogen is a promising source of renewable energy. It is a clean, inexhaustible fuel and possesses a high energy content of 122 kJ/g [22]. It can be used to generate electricity in fuel cells [3] and it is considered to be environmentally friendly as its combustion produces water, thus it is the only carbon free fuel available [22,11]. First generation biofuel production makes use of food crops as an energy source and is therefore associated with land displacement and environmental deterioration due to deforestation, competition with the global food supply-resulting in higher food prices, loss of biodiversity and increased greenhouse gas emissions caused by land displacement [16]. Second generation biofuels using wastes are proving to be the most promising energy sources. These wastes include organic and animal waste, wastewater, agricultural and industrial residues and energy crops-all of which can be used for the production of biofuels [1].

Sorghum which is a fast growing energy crop, is a promising feedstock source for biofuel production [35]. Moreover, sorghum leaves are generally considered as agricultural waste upon harvesting [24] and thus could potentially overcome the drawbacks associated with first generation biofuel production. Sorghum is indigenous to Africa and it grows well in dry, arid soil as well as shallow and heavy clay soils, which allows for lower production costs and a larger potential farming area. Sorghum has low nutrient requirements and a shorter growth period when compared to other biomass feedstocks such as sugarcane and sugar beet [20,35]. The amount of energy generated from sorghum varies depending on the part of the plant used in the saccharification process as well as in the actual fermentation process itself. Woods (2001) [37] reported a yield of 60 tons of fresh sweet sorghum biomass from a hectare of land, comprising 46 tons of fresh weight stems, which can potentially produce 3000 L/hectare of ethanol, equating to 12.6 GJ of electricity. Prior to using lignocellulosic material for microbial fermentation, a pre-treatment stage is required. This has proven to be the most crucial and costly process in the production of renewable energy [16].

Sorghum lignocellulosic material is made up of cellulose, hemicellulose and lignin which form an intricate structure that is extremely resistant to decomposition and they account for approximately 42.03 ± 3.38%, 24.53 ± 4.45% and 9.89 ± 2.35% of the lignocellulosic material respectively [24]. These constituents can therefore potentially be exploited for biofuel production. Cellulose, the main constituent of lignocellulose, is a polysaccharide made up of a linear chain of D-glucose molecules and is insoluble in water and many organic solvents due to hydrogen bonds that link the cellulose fibres to one another. Hemicelluloses are heterogeneous branched polymers that contain 5-C and 6-C carbohydrates like xylose, arabinose, mannose, glucose and galactose and/or urgonic acids. Hemicellulose is thus easier to hydrolyse due to its amorphous, branched structure. Removing it will increase the accessibility and thus the digestibility of cellulose. It is also more sensitive to operating process conditions and can lead to the production of unwanted products like furfurals and hydroxymethyl furfurals which can inhibit the fermentation process of biofuel production [9]. Lignin is an aromatic polymer which cross-links with hemicelluloses to provide structural strength and prevention of microbial degradation [16].

To optimally release fermentable sugars from lignocellulosic material, an appropriate and cost effective pre-treatment is required. Previous studies by Sathesh-Prabu and Murugesan, [24]; Suresh et al. [28] and Siwarasak et al. [27] employed physical and chemical pre-treatment prior to enzymatic hydrolysis to facilitate its effect on the saccharification of lignocellulosic material. Enzymatic hydrolysis and the use of enzymes at a commercial scale has proven to be one of the main contributing factors to the high costs associated with pre-treatment [28], therefore novel pre-treatment strategies need to be optimized to make the pre-treatment stage economically viable. Physical techniques like milling and chemical techniques that employ acid or alkali have been used to enhance fibre hydrolysis [9]. Combining pre-treatment techniques may possibly result in higher fermentable sugars being released [17].

Response Surface Methodology (RSM) is a modelling and optimization tool where many factors and their interactive effects on the process response can be identified with a smaller number of experimental runs. RSM has been reported in the optimization of various bioprocesses including the optimization of ethanol production from sweet sorghum juice [13], biohydrogen production from sugarcane molasses [31] and biohydrogen production using nickel nanoparticles [18].

There is a dearth of knowledge in public domains modelling the recovery of fermentable sugars from WSL, coupled with biohydrogen production at bench scale. This study focuses on the modelling and optimization of xylose and glucose recovery from waste sorghum leaves using three acid pre-treatments; hydrochloric acid (HCl), sulphuric acid (H_2SO_4) and nitric acid (HNO_3), which are subjected to variations in acid concentration, heating time, solid to liquid (S:L) ratio and acid exposure lag time. Furthermore, the optimum physico–chemical bioprocess setpoints for hydrogen biofuel production from these substrates are investigated followed by a preliminary assessment at bench scale.

Materials and methods

Sorghum leaves feedstock and experimental design

The sorghum leaves used in this study were harvested from the Ukulinga Research Farm, University of KwaZulu-Natal in Pietermaritzburg (29°67′E, 30°40′S), South Africa. Once the plants were fully grown and flowering (approximately 90 days), 5–8 sorghum leaves were cut off at the leaf collar and immediately dried at 80 °C for 48–72 h and further milled to reduce particle sizes to 1–2 mm using a centrifugal miller (Retsch ZM-1, South Africa). The milled leaves were stored in airtight containers prior to use.

A four-factor Box-Behnken design was used to generate 87 experimental runs (29 for each acid type), shown in Table 1, with varied inputs of acid concentration, heating time, S:L ratio and acid exposure lag time in the ranges of 1–6%, 70–240 min, 30–50% (w/v) and 0–24 h respectively. The acid types include HCl, H_2SO_4 and HNO_3. An additional process parameter of acid exposure lag time, which involves exposing the milled leaves to the acid solution at varied durations prior

Table 1 – Pre-treatment parameter limits implemented for the optimisation of sorghum lignocellulose pre-treatment.

Run	A: Acid (%)	B: Heating time (mins)	C: S:L ratio (%)	D: Acid exposure lag time (hours)
1	1.00	155.00	30.00	12.00
2	6.00	155.00	40.00	24.00
3	3.50	155.00	40.00	12.00
4	3.50	240.00	40.00	24.00
5	1.00	155.00	40.00	24.00
6	6.00	240.00	40.00	12.00
7	3.50	70.00	40.00	24.00
8	6.00	155.00	30.00	12.00
9	6.00	155.00	50.00	12.00
10	3.50	155.00	50.00	0.00
11	3.50	155.00	30.00	24.00
12	3.50	70.00	30.00	12.00
13	3.50	155.00	40.00	12.00
14	6.00	70.00	40.00	12.00
15	3.50	155.00	40.00	12.00
16	1.00	155.00	40.00	0.00
17	1.00	155.00	50.00	12.00
18	3.50	240.00	50.00	12.00
19	6.00	155.00	40.00	0.00
20	3.50	70.00	40.00	0.00
21	3.50	240.00	40.00	0.00
22	3.50	70.00	50.00	12.00
23	1.00	70.00	40.00	12.00
24	3.50	155.00	40.00	12.00
25	3.50	155.00	50.00	24.00
26	3.50	155.00	30.00	0.00
27	3.50	240.00	30.00	12.00
28	1.00	240.00	40.00	12.00
29	3.50	155.00	40.00	12.00

to heating was included. The idea was to assess the impact of acid exposure time prior to heating on sugar release pattern.

Pre-treatment process

Milled sorghum leaves at weights of 3 g (30%), 4 g (40%) or 5 g (50%) were placed in 25 ml Erlenmeyer flasks and treated with 10 ml acid solution of varied concentrations (1.0, 3.5 or 6.0% (v/v)) as depicted in Table 1. Prior to heating, an acid exposure lag time (0–24 h) was introduced, where the flasks were sealed and the leaves left in the acid at room temperature to facilitate additional hydrolysis. The flasks were subsequently heated at 100 °C using a water bath (Gesellschaft für Labortechnik mbH D 3006, Burgwedel) at varied heating time periods as specified in Table 1. Heating timing was recorded once the temperature of the substrate had reached 100 °C. The pre-treatment samples were then filtered and the liquid phase was used for fermentable sugar quantification. The solid phase was rinsed with distilled water and analysed using acid-detergent fibre analysis, seen in Table 2. All 87 experimental runs were carried out in duplicate.

Optimization of biohydrogen production

Inoculum development and fermentation process optimization

Anaerobic digested sewage sludge from the Darville Wastewater treatment plant (Pietermaritzburg, South Africa), was used as the source of inoculum in this study. The sludge was heat treated at 121 °C for 10 min. According to Yin et al. [34]; this technique is highly effective in deactivating the non-spore forming hydrogen consuming organisms that are incapable of survival at high temperatures while maintaining hydrogen producing, spore-forming microorganisms such as the endospore-forming *Clostridia* [25].

For process optimization, the selected physico–chemical parameters were inoculum concentration, fermentation time and initial pH within the ranges of 30–50%, 24–96 h and 4–7 respectively, generating 17 experiments with varied input conditions using Design Expert (Stat Ease, Inc.). The pH of the pre-treated sorghum leaves was adjusted to 7 using 1 M NaOH in preparation for hydrogen production. 50 ml of unfiltered, optimally pre-treated leaves were fed into 250 ml fermentation vessels with appropriate volumes of a sterile mineral salt mix containing (in g/L): NH_4CL 0.5, KH_2PO_4 0.5, K_2HPO_4 0.5, $NaHCO_3$ 4.0, $FeCl.2H_2O$ 0.15, $MgCl.6H_2O$ 0.085, $ZnSO4.7H_2O$ 0.01, $MnCl_2.4H_2O$ 0.03, H_3BO_3 0.03, $CaCl_2.6H_2O$ 0.01, $Na_2MoO_2.2H_2O$ 0.03 [17]. Heat-treated anaerobic sludge at concentrations of 10%, 30% or 50% (working volume of 200 ml) as described in Table 6 was added to the fermentation vessels. The initial pH was adjusted to either 4.0, 5.5 or 7.0 with 1 M NaOH and flushed with N_2 gas for 30 s, allowing for anaerobic conditions to prevail. The fermentation processes were carried out in a shaking water bath at a temperature of 37.5 °C and 230 rpm agitation for specified fermentation periods.

Development and validation of the pre-treatment models

Xylose and glucose yield data were used to fit 6 polynomial model equations for the three acid types, relating the monomeric sugar yields (xylose and glucose) to the input parameters. A general form of the model is shown in Equation (1), where Y represents the process response which, in this case is xylose or glucose yield, α_0 is the free or offset term called the intercept, α_1, α_2, α_3 and α_4 are the linear coefficients, $\alpha_{11}{}^2$, $\alpha_{22}{}^2$,

Table 2 – Composition of untreated and acid pre-treated sorghum leaves.

Sample	Cellulose (%)	Hemicellulose (%)	Hemicellulose solubilisation (%)	Lignin (%)
Untreated	28.56	29.18	0	3.94
HCl-treatment	48.08	6.71	77	13.69
H_2SO_4-treatment	49.50	6.35	78	13.46
HNO_3-treatment	38.08	7.48	74	14.58

$\alpha_{33}{}^2$ and $\alpha_{44}{}^2$ are the quadratic coefficients and α_{12}, α_{13}, α_{14}, α_{23} and α_{24} are the interaction coefficients.

$$Y = \alpha_0 + \alpha_1 x_1 + \alpha_2 x_2 + \alpha_3 x_3 + \alpha_4 x_4 + \alpha_{11} x_1^2 + \alpha_{22} x_2^2 + \alpha_{33} x_3^2 + \alpha_{44} x_4^2 + \alpha_{12} x_1 x_2 + \alpha_{13} x_1 x_3 + \alpha_{14} x_1 x_4 + \alpha_{23} x_2 x_3 + \alpha_{24} x_2 x_4 \quad (1)$$

For the modelling and optimization of biohydrogen production with the considered physico–chemical variables, yield data obtained from the fermentation experiments were used to fit another polynomial model relating the hydrogen yield to the input parameters. A general form of the model is seen in Equation (2) where Y is the yield of hydrogen in ml/g FS.

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

The significances of the developed models were assessed by the Analysis of Variance (ANOVA) using Design Expert Software (Stat Ease, Inc.) (Tables 4 and 6). The optimum set-point values for xylose and glucose release as well as hydrogen production were obtained by solving the polynomial equations using the method of Myers and Montgomery [19]. These set-points were validated experimentally in triplicate.

Analytical methods

The pre-treated samples were analysed for xylose and glucose content using the YSI 2700 Model Biochemical Analyser (YSI, USA). The sugar analysing principle is based on enzyme coupled reactions producing hydrogen peroxide which is electrochemically oxidized, allowing for electrochemical detection. The solubilisation of lignocellulosic material was analysed using detergent fibre analysis technique described by Goering and van Soest [8] and Wolfrum et al. [33]. Biogas volumes were recorded using the water displacement method [31]. The hydrogen fraction of the evolving biogas was measured using a hydrogen-specific biogas sensor (BCP-H_2) (Bluesens, Germany). The cumulative volume of biohydrogen produced was calculated according to Equation (3), where $V_{H,i}$ and $V_{H,i-1}$ are cumulative hydrogen gas volumes at the current (i) and previous (i−1) time intervals, $V_{G,i,}$ and $V_{G,i-1}$ the total biogas volumes in the current and previous time intervals, $C_{H,i}$ and $C_{H,i-1}$ the fraction of hydrogen gas in the headspace of the fermentation vessel in the current and previous time intervals, and V_H the total volume of headspace in the fermentation vessel [21].

$$V_{H,i} = V_{H,i-1} + C_{H,i}\,(V_{G,i} - V_{G,i-1}) + V_H\,(C_{H,i} - C_{H,i-1}) \quad (3)$$

Process scale-up

Bench scale experimental setup

A bench scale fermentation process was carried out in a 13 L bioreactor (Labfors-INFORS HT, Switzerland) at a working volume of 8 L and the optimized physico–chemical set-points were used. Two litres of optimally pre-treated sorghum leaves at xylose and glucose concentrations of 10.19 and 4.01 g/L respectively and two litres of sterilised mineral salts were fed into the sterile bioreactor containing an inoculum of four litres of heat-treated anaerobic sewage sludge. The bioreactor was flushed with N_2 gas for 3 min prior to fermentation, creating an anaerobic environment. The fermentation temperature, agitation and process time set-points were 37.5 °C, 250 rpm and 84 h respectively.

Process monitoring

The biogas produced was continuously monitored at an interval of 1 min to determine the fraction of hydrogen produced in real time. This was achieved by using a BCP-H_2 sensor (Bluesens, Germany). The sensor has a measuring range of 0–100% hydrogen and employs the thermal conductivity measurement principle. The evolving biogas volume was monitored using a milligas counter (MGC, Bluesens, Germany). The culture broth was sampled every 7 h for sugar and pH analysis.

Results and discussion

Sorghum leaf composition

Fibre analysis showed that native sorghum leaves contain cellulose, hemicellulose and lignin at 28.56, 29.18 and 3.94% respectively (Table 2). A reported sorghum bagasse composition of 41.20, 24.50 and 4.80% cellulose, hemicellulose and lignin respectively [9] illustrates the variation in composition of sorghum leaves and sorghum bagasse. With regards to sorghum leaves, the more easily accessible hemicellulose portion makes up a larger fraction (29.18%) of the leaf composition when compared to sorghum bagasse (24–25%), allowing for a larger release of fermentable sugar from sorghum leaves compared to sorghum bagasse. The cellulose fraction of 41.20% is also significantly larger in sorghum bagasse making it more resistant to acid degradation [12]. Similarly, there is a lower fraction of lignin in sorghum leaves compared to sorghum bagasse. The low lignin content of the sorghum leaves can be explained by ADL (acid detergent lignin) method estimating a lower lignin content than what is actually present. Quantification of lignin can be carried out using two methods; Klason lignin and acid detergent lignin [10]. The acid detergent method is only capable of quantifying lignin residue that remains after solubilisation in acid. Jung et al. [10] estimated Klason lignin of grasses to be approximately 2–4 times larger than that of acid detergent lignin, illustrating a more complete quantification of lignin as Klason lignin accounts for both acid soluble and insoluble lignin. The variation observed in the composition of sorghum lignocellulosic biomass can be attributed to genetic and environmental factors as well as farming conditions and harvesting time [9], suggesting that sorghum bagasse composition is dependent on growth conditions and the cultivar type used.

Pre-treatments using the three respective acids resulted in an unexpected increase in cellulose and lignin. This can be

attributed to the reduced solubility of cellulose which occurred during acid pre-treatment as the pre-treatment caused cleaving of the hemicellulose-lignin bonds responsible for the recalcitrant character of lignocellulosic material. This leaves the acid-insoluble cellulose residue in a less reduced state of depolymerisation than before [12]. The accumulation of condensed degraded polysaccharides during pre-treatment may also partially play a role in the increase in lignin observed [23]. H_2SO_4-based pre-treatment gave the highest level of hemicellulose solubilisation of 78%, with HCl achieving a similar level of hemicellulose solubilisation of 77%. HNO_3-based pre-treatment showed the lowest solubilisation.

Sugar release from pre-treatment

Modelling of xylose and glucose release using acid-heat pre-treatments

The pre-treatment process responses seen in Table 3 were used to generate two polynomial model equations (Equations (4)–(9)) for each acid-based model. The xylose and glucose yields were related to the input variables A: acid concentration, B: heating time, C: S:L ratio and D: acid exposure lag time, for HCl, H_2SO_4 and HNO_3 respectively.

HCl-based model equations

$$\text{Xylose} = +35.63 + 22.26A + 2.83B + 1.94C + 0.58D - 0.23AB + 2.61AC + 1.39AD + 2.69BC + 2.76BD + 3.85CD - 12.22A^2 - 2.16B^2 - 1.34C^2 + 4.56D^2 \quad (4)$$

$$\text{Glucose} = +10.69 + 5.66A + 1.31B + 0.75C + 0.12D + 0.69AB + 0.93AC + 0.36AD + 0.43BC + 0.84BD + 1.01CD - 0.57A^2 - 0.48B^2 - 0.43C^2 + 1.79D^2 \quad (5)$$

H_2SO_4-based model equations

$$\text{Xylose} = +24.54 + 18.18A + 2.02B - 5.64C - 0.54D + 1.55AB - 2.44AC + 0.33AD + 0.062BC - 3.43BD - 0.18CD - 5.84A^2 + 1.48B^2 - 3.58C^2 + 1.51D^2 \quad (6)$$

$$\text{Glucose} = +8.00 + 3.74A + 0.42B - 0.79C - 0.087D + 1.08AB - 0.71AC + 0.15AD - 0.39BC - 0.86BD - 0.083CD - 9.583E{-}004A^2 + 0.40B^2 - 0.86C^2 + 0.21D^2 \quad (7)$$

Table 3 – Four factor Box-Behnken design used for pre-treatment of sorghum leaves. The input parameters are: acid concentration, heating time, S:L ratio and acid exposure lag time.

					HCl		H_2SO_4		HNO_3	
Run	A: Acid (%)	B: Heating time (mins)	C: S:L ratio (%)	D: Acid exposure lag time (hours)	Response 1: Xylose (g/L)	Response 2: Glucose (g/L)	Response 1: Xylose (g/L)	Response 2: Glucose (g/L)	Response 1: Xylose (g/L)	Response 2: Glucose (g/L)
1	1.00	155.00	30.00	12.00	2.10	4.50	0.36	3.82	0	3.72
2	6.00	155.00	40.00	24.00	52.02	18.42	42.22	13.37	12.94	6.81
3	3.50	155.00	40.00	12.00	20.64	5.73	28.51	8.41	17.97	6.45
4	3.50	240.00	40.00	24.00	48.06	15.49	22.06	6.64	49.06	15.95
5	1.00	155.00	40.00	24.00	0.86	5.18	0.26	4.35	0.09	4.81
6	6.00	240.00	40.00	12.00	45.43	16.70	44.68	14.44	12.37	5.92
7	3.50	70.00	40.00	24.00	27.34	7.92	27.25	8.87	24.90	8.54
8	6.00	155.00	30.00	12.00	36.96	12.74	32.00	10.23	17.27	5.56
9	6.00	155.00	50.00	12.00	45.49	16.02	21.96	7.77	12.79	7.19
10	3.50	155.00	50.00	0.00	38.05	11.78	14.57	5.91	17.11	6.33
11	3.50	155.00	30.00	24.00	35.37	11.15	31.93	8.97	33.87	8.43
12	3.50	70.00	30.00	12.00	32.29	8.62	25.33	7.16	21.54	6.47
13	3.50	155.00	40.00	12.00	36.99	10.76	24.69	8.36	18.74	6.36
14	6.00	70.00	40.00	12.00	45.60	14.75	37.91	11.07	8.24	5.54
15	3.50	155.00	40.00	12.00	42.36	13.40	24.84	8.26	13.54	5.82
16	1.00	155.00	40.00	0.00	5.00	6.03	0.69	4.63	0.48	4.31
17	1.00	155.00	50.00	12.00	0.20	4.07	0.10	4.22	0.31	4.06
18	3.50	240.00	50.00	12.00	35.65	11.73	21.51	8.40	18.05	7.68
19	6.00	155.00	40.00	0.00	50.61	17.86	41.34	13.06	10.32	5.76
20	3.50	70.00	40.00	0.00	31.78	9.48	23.10	7.62	19.80	7.19
21	3.50	240.00	40.00	0.00	41.45	13.69	31.63	8.82	31.46	9.76
22	3.50	70.00	50.00	12.00	28.79	9.45	14.59	7.36	6.58	6.11
23	1.00	70.00	40.00	12.00	0.02	4.79	0	4.52	0	4.41
24	3.50	155.00	40.00	12.00	44.70	13.31	16.73	6.12	19.15	6.97
25	3.50	155.00	50.00	24.00	49.52	14.54	13.49	5.67	5.83	4.40
26	3.50	155.00	30.00	0.00	39.32	12.42	32.31	8.87	24.72	6.90
27	3.50	240.00	30.00	12.00	28.39	9.18	31.99	9.78	28.60	7.90
28	1.00	240.00	40.00	12.00	0.78	3.97	0.57	3.56	0	3.95
29	3.50	155.00	40.00	12.00	33.45	10.26	29.94	8.87	26.08	8.01

HNO_3-based model equations

$$\text{Xylose} = +19.09 + 6.09A + 4.87B - 5.44C + 1.90D + 1.03AB - 1.20AC + 0.75AD + 1.10BC + 3.12BD - 5.11CD - 15.04A^2 + 3.18B^2 - 1.06C^2 + 4.43D^2 \quad (8)$$

$$\text{Glucose} = +6.72 + 0.97A + 1.07B - 0.28C + 0.72D + 0.21AB + 0.30AC + 0.14AD + 0.035BC + 1.21BD - 0.87CD - 2.17A^2 + 1.24B^2 - 0.58C^2 + 1.21D^2 \quad (9)$$

The fitness of these models was assessed using Analysis of Variance (ANOVA), presented in Table 4. The coefficient of determination (R^2) is a measure of variance that falls between 0 and 1, with 1 indicating the model's ability to navigate the design accurately and 0 indicating a total inability [31]. The R^2 values for the HCl- and H_2SO_4-based models for xylose and glucose were well above 0.8 (0.88 and 0.93 respectively), indicating that these models could account for 88 and 93% of the variation observed in the data. The F-values for xylose and glucose of these two acid models were relatively high (>7) showing the significance of these models. The low P-values observed for sugar outputs in both of the acid models further illustrated the significance of these models.

Linear effect of input variables on monomeric sugar release

The high sensitivity of fermentable sugar release to the acid pre-treatment type is illustrated by the high variability in sugar yields under various acidic treatments. As observed in Table 3, the xylose and glucose yields varied from 0.02 to 52.02 g/L and 3.97–18.42 g/L respectively for the HCl-based model, 0–44.68 g/L and 3.56–14.44 g/L respectively for the H_2SO_4-based model and 0–49.06 g/L and 3.72–15.95 g/L respectively for the HNO_3-based model. This sensitivity has been observed in the studies of Moodley and Kana [17] with xylose and glucose yields varying between 1 and 10 g/L depending on acid type and concentration.

At low acid concentrations (<1%), all pre-treatments exhibited low yields (>6.03 g/L) for both xylose and glucose, while a marked increase in sugar recovery was observed with acid pre-treatments at high concentration (>6%) with xylose and glucose yields as high as 52.02 and 18.42 g/L respectively when using HCl. A ratio of 2:3 g/L xylose per g/L glucose was observed across all acid pre-treatments. Besides the concentration, the acid type had a relevant impact on the sugar yield. A concentration of 6% H_2SO_4 gave xylose and glucose yields of 44.68 and 14.44 g/L respectively while the same concentration of HNO_3 resulted in lower xylose and glucose yields (12.94 and 6.81 g/L respectively) which is in contrast to the higher yields observed at a concentration of 3.5% HNO_3 (49.06 and 15.95 g/L of xylose and glucose respectively). Sindhu et al. [26] reported higher fermentable sugar release from bamboo pre-treated with 5% H_2SO_4 for 30 min compared to 2% HCl. These observations highlight the sensitivity of fermentable sugar recovery on acid type, concentration and nature of the substrate.

Pre-treatment carried out at various heating times between 70 and 240 min gave different yields for all three acid pre-treatment experiments. For the HCl-based model, xylose and glucose yields increased from an average of 27.63 and 9.16 g/L to a maximum of 50.61 and 17.86 g/L respectively as the pre-treatment time was increased from 70 to 155 min. An increase beyond this pre-treatment time showed no marked improvement on the release of xylose and glucose. For the H_2SO_4-based model, xylose and glucose yields increased from an average of 21.36 and 7.76 g/L to a maximum of 44.68 and 14.44 g/L respectively when pre-treatment time was increased from 70 to 240 min. The HNO_3-based model gave maximum xylose and glucose yields of 49.06 and 15.95 g/L respectively when pre-treatment heating time was increased to 240 min. This is in line with the studies by Mafuleka and Kana [14] who reported a similar pattern of sugar release as a function of pre-treatment time.

As observed in Table 3, a solid to liquid ratio of 40% resulted in higher yields (48.06 g/L xylose and 15.49 g/L glucose) whereas S:L ratios of 50% and 30% showed a low yield of fermentable sugars for all three acid-based models. This could be linked to the higher accessibility of the substrate to the acid at low S:L ratios. An optimal S:L ratio of approximately 36% has been reported by Vargas Betancur and Pereira [29] using sugarcane bagasse.

Experimental data showed that an acid exposure lag time of 24 h enhanced the recovery of fermentable sugars by increasing the yields of xylose and glucose by 2.79 and 3.14%, 2.11 and 2.34% and 25.39 and 18.23% for HCl, H_2SO_4 and HNO_3 respectively. These observations highlighted the importance of including a lag time between the acid treatment and the onset of the heating phase for the three acid models investigated. There is a dearth of information in public repositories on investigations of acidic pre-treatment of lignocellulosic feedstocks with an acid exposure lag time.

Interactive effect of pre-treatment variables on sugar release

The interactive effects of the pre-treatment input variables on the sugar release pattern for the HCl-based model are shown

Table 4 – Analysis of Variance (ANOVA) for xylose and glucose models for HCl, H_2SO_4 and HNO_3.

Acid-based model	Model output	Sum of squares	df	Mean squares	F-value	P-value	R^2
HCl	Xylose	7540.95	14	538.64	12.77	<0.0001	0.93
	Glucose	457.20	14	32.66	7.47	<0.001	0.88
H_2SO_4	Xylose	4851.04	14	346.50	13.58	<0.0001	0.93
	Glucose	195.25	14	13.95	7.19	<0.001	0.88
HNO_3	Xylose	3276.79	14	234.06	4.75	0.0031	0.83
	Glucose	106.17	14	7.58	2.20	0.0763	0.69

df: degrees of freedom, F-value: Fisher-Snedecor distribution value, P-value: probability value, R^2: coefficient of determination.

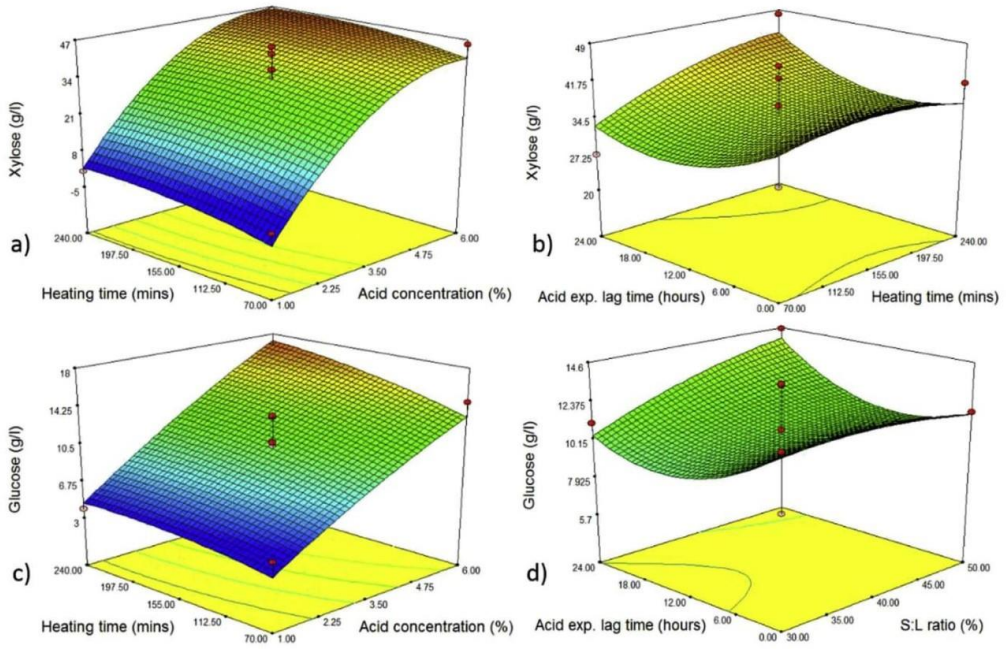

Fig. 1 – 3-D Response Surface plots of the HCl-based model, showing the interactive effects of variable input parameters on the release of fermentable sugars.

in Fig. 1a–d. It is observed that when the acid concentration and the heating time were simultaneously increased from 1 to 6% and 70–180 min respectively, the recovery of xylose improved to a maximum yield of approximately 47 g/L (Fig. 1a). Furthermore, the interaction of these input parameters on glucose release showed that at high set-point values (>above 18 h) of acid exposure lag time and 155 min heating time, higher yields were obtained. Similar observations on the improvement of sugar recovery at high acid concentration and process time have been reported by Mafuleka and Kana [14]. However, given the cost of these operational variables, a suitable techno-economic analysis is required to determine the most suitable operational set-points. As reported by da Costa Sousa et al. [5]; the more severe a pre-treatment regime is, the less economically feasible the process becomes. This is due to the costs associated with the treatment process of the effluents and ensuring that all components of the process are maximally utilised. Therefore, although sugar release increases with acid concentration, the treatment of subsequent waste products becomes more costly.

The interaction of lag time and heating time (Fig. 1b) showed a peak yield of xylose of approximately 48 g/L, with an acid exposure lag time of about 20 h and heating time of 155 min. A similar pattern was observed for glucose recovery with an optimal yield of 14 g/L at an acid exposure lag time of 20 h and a S:L ratio of between 40 and 50% (Fig. 1d). These observations indicate that a higher lag time value is preferable to enhance the sugar release. However a reduced yield was observed with an acid exposure lag time of between 12 and 18 h for both sugars. This suggests a non-linear effect or a complex interaction of this input parameter on sugar release. Such a phenomenon may be explained by the exposure to acid resulting in bond formation between the lignin and hemicellulose fractions [10], which requires further heating to cleave.

The interactive effects of the input variables for the H_2SO_4 model are shown in Fig. 2a–d. It is observed that when S:L ratio was decreased from 50 to 30%, a significant increase in xylose yield was observed to a maximum of almost 28 g/L. A similar pattern is shown in Fig. 2d, where an optimal glucose yield of 8 g/L is achieved at a low S:L ratio. This yield increased to 32 g/L with a simultaneous increase in the heating time from 70 to 240 min (Fig. 2a). A further increase in sugar recovery to almost 42 g/L is observed when acid concentration is increased from 1 to 6%. In contrast to this, studies carried out by Wang et al. [30] using lime, showed that a simultaneous increase in S:L ratio and lime concentration was most beneficial for sugar recovery. The interaction of heating time and acid concentration (Fig. 2c) showed a peak glucose yield of 14 g/L when heating time and acid concentration were simultaneously increased from 70 to 200 min and 1–6% respectively. Furthermore, the interactive effect of S:L ratio and acid concentration (Fig. 2b) showed a sharper increase in xylose yield from 0 to 42 g/L when an increasing acid concentration from 1 to 6% was used at a low S:L ratio of (30%), in comparison to a high S:L ratio.

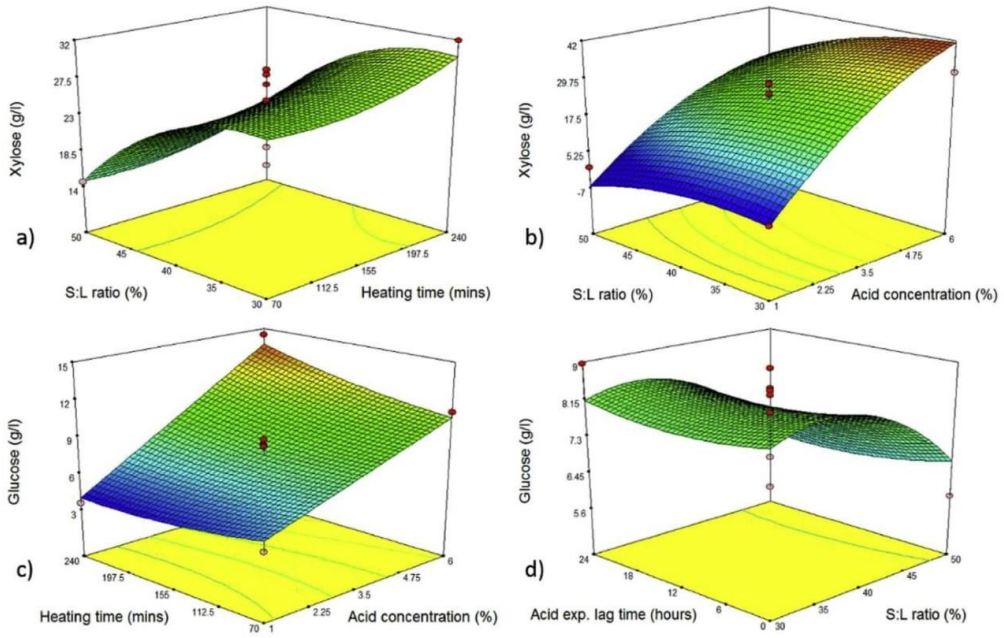

Fig. 2 – 3-D Response Surface plots of the H_2SO_4-based model, showing the interactive effects of the variable input parameters on the release of fermentable sugars.

The interactive effects of the HNO_3 pre-treatment are shown in Fig. 3a–d. It was observed that when acid concentration and heating time were simultaneously increased from 1 to 3.5% and 70–240 min respectively, the recovery of both xylose (Fig. 3a) and glucose (Fig. 3c) was improved from 4 and 6 g/L to a maximum yield of 28 and 9 g/L respectively. In addition to this, an optimal xylose yield of 35 g/L was obtained when acid exposure lag time was increased from 0 to 24 h, concomitantly with a low S:L ratio of 30% (Fig. 3b). The interaction of S:L ratio and heating time (Fig. 3d) showed a peak yield of glucose of 9 g/L with a S:L ratio of approximately 40% and an increase in heating time from 70 to 240 min. These observations indicate that an acid concentration of 3.5% is preferable to enhance sugar release. Above this concentration threshold, there is a low yield of xylose and glucose.

Validation of developed models

Experimental validation was carried out in triplicate for all models using their predicted optimum set-points. Fig. 4 shows the predicted and observed xylose and glucose yields with the highest observed yields of xylose and glucose of 54.05 and 15.98 g/L respectively obtained using the HCl-based pre-treatment. HCl-based pre-treatment was found to be optimal for fermentable sugar release from sorghum leaves, which correlates with the findings by Demirbas [6] stating that HCl has an increased ability to permeate lignocellulosic material more easily in comparison to other acids. Optimum set-points for acid concentration, heating time, S:L ratio and acid exposure lag time were 5.95% HCl, 176 min, 49.7% and 18 h and 15 min respectively.

Optimization of biohydrogen production

Model assessment for hydrogen production

A polynomial model equation (Equation (10)) which relates the process output (Hydrogen yield) to the variable input parameters of inoculum concentration (A), fermentation time (B) and initial pH (C) was generated using the experimental data in Table 5.

$$\text{Hydrogen yield} = +0.90 + 2.28A + 0.58B + 10.70C - 0.16AB + 5.70AC + 1.61BC + 2.43A^2 - 1.56B^2 + 9.37C^2 \quad (10)$$

The fitness of this model was assessed using Analysis of Variance (ANOVA), shown in Table 6. The coefficient of determination (R^2) for this model (0.91) shows that the model could account for 91% of variance observed in the data. The relatively low P-value observed further illustrated the significance of the model and thus, it can be used to navigate the optimization space. P-value of below 0.0500 indicated that the model was significant. The F-value of 7.85 also indicated that the model was significant and there was less than 1% chance that this value was due to noise.

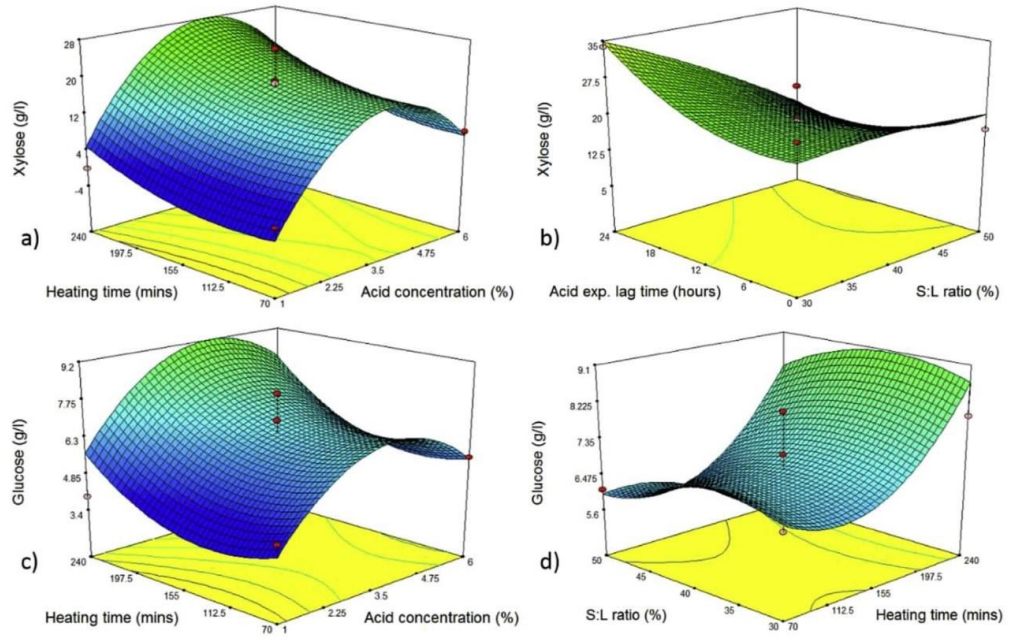

Fig. 3 – 3-D Response Surface plots of the HNO_3-based model, showing the interactive effects of variable input parameters on the release of fermentable sugars.

Linear and interactive effect of input variables on hydrogen production

The yields of hydrogen are shown in Table 5. It was observed that the production of hydrogen was extremely dependent on the considered input variables with yields varying between 0 and 36.8 ml/g FS.

Hydrogen yield fluctuated as inoculum concentration was increased. This may be due to the use of mixed microbial inoculum source [7]. Although heat pre-treatment is known to enrich for hydrogen producing *Clostridia* [32], other endospore-forming hydrogen consumers may still be present, leading to lower yields of hydrogen. In this study, the highest hydrogen yield was observed at 50% inoculum concentration. The fermentation time of 60 h gave a maximum hydrogen yield of 36.80 ml/g FS. Beyond this fermentation time, there was no further increase in hydrogen production.

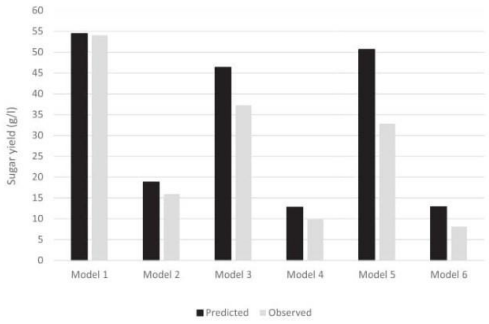

Fig. 4 – Predicted and observed sugar yields for HCl pre-treatment (model 1 = xylose, model 2 = glucose), H_2SO_4 pre-treatment (model 3 = xylose, model 4 = glucose) and HNO_3 pre-treatment (model 5 = xylose, model 6 = glucose) respectively.

Fig. 5a showed that a maximum yield of hydrogen (36.8 ml/g FS) was achieved with an increase in inoculum concentration and increase of pH up to 7. A sharp increase in hydrogen yield was observed at higher inoculum concentration. This may be due to the high population of hydrogen producing bacteria and the buffering capacity of the fermentation medium [32]. As shown in Fig. 5b, more hydrogen was obtained with an initial pH of 7 and process time of 60 h.

Validation of hydrogen production model

Validation experiments gave a hydrogen yield of 47.30 ml/g FS, against a predicted value of 32 ml/g FS using 50% inoculum concentration, 83 h of fermentation time and a pH of 7, which was 47% higher than the predicted response value.

Bench scale experimentation

As shown in Fig. 6a, hydrogen gas production was initiated after a lag phase of 17 h and reached a peak fraction of 44% after 60 h, followed by a decline in hydrogen fraction corresponding to the depletion of fermentable sugars. A longer lag

Table 5 – Three factor Box-Behnken design used to model and optimize hydrogen yields on variables of inoculum concentration, fermentation time and initial pH.

Run	A: Inoculum concentration (%)	B: Fermentation duration (hours)	C: Initial pH	Output: Hydrogen yield (ml/g FS)
1	50.00	60.00	7.00	36.80
2	10.00	60.00	4.00	0
3	30.00	96.00	4.00	0
4	50.00	60.00	4.00	0
5	30.00	24.00	7.00	14.19
6	30.00	60.00	5.50	0
7	10.00	96.00	5.50	2.61
8	30.00	60.00	5.50	4.41
9	50.00	24.00	5.50	1.24
10	10.00	24.00	5.50	3.21
11	10.00	60.00	7.00	13.98
12	30.00	24.00	4.00	0
13	30.00	96.00	7.00	20.64
14	30.00	60.00	5.50	0
15	50.00	96.00	5.50	0
16	30.00	60.00	5.50	0
17	30.00	60.00	5.50	0.07

Table 6 – Analysis of Variance (ANOVA) of Hydrogen production Model.

Model output	Sum of squares	df	Mean squares	F-value	P-value	R^2
Hydrogen yield (ml/g FS)	1510.29	9	167.81	7.85	<0.01	0.91

df: degrees of freedom, F-value: Fisher-Snedecor distribution value, P-value: probability value, R^2: coefficient of determination.

phase of 30 h has been reported by Zheng et al. [36] when using untreated sludge as an inoculum source. A cumulative volume of 2952 ml H_2 was obtained, corresponding to a hydrogen yield of 213.14 ml/g FS. The initial sugar concentration decreased from 14.2 to 8.61 g/L during the lag phase, which may be explained by rapid cell formation.

As shown in Fig. 6b, cumulative volume of hydrogen began to increase at the 17th hour and an exponential increase was observed for 20 h and plateaued thereafter. A decrease in pH from 7.50 to 6.12 coincided with the initiation of hydrogen production. Similar results were reported by Sekoai and Kana [25]; suggesting that the drop in pH may be due to the production of volatile fatty acids (VFAs) during hydrogen production. The pH remained relatively constant throughout the rest of the fermentation time, from the 30th hour to the 67th hour.

Fig. 7 shows the phase contrast microscopy carried out on the bench scale process effluent after 68 h of fermentation. Both rod-shaped microorganisms and rod-shaped cells with endospores were observed. The cells were approximately 3–3.3 μm in length, which corresponds to sizes of *Clostridial* species reported by Bergey et al. [2]. These microorganisms have previously been reported in hydrogen production [4,7]. They are considered as major hydrogen producers found in heat-treated sewage sludge [7].

Conclusion

Of the pre-treatment models assessed, HCl pre-treatment was most efficient, as a 77% hemicellulose solubilisation

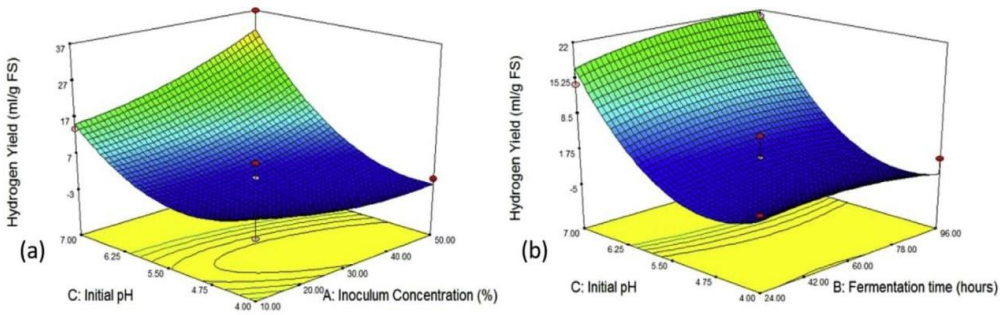

Fig. 5 – 3-D Response Surface Plots of a Hydrogen Production model, showing the interactive effects between the variable process inputs on production of hydrogen.

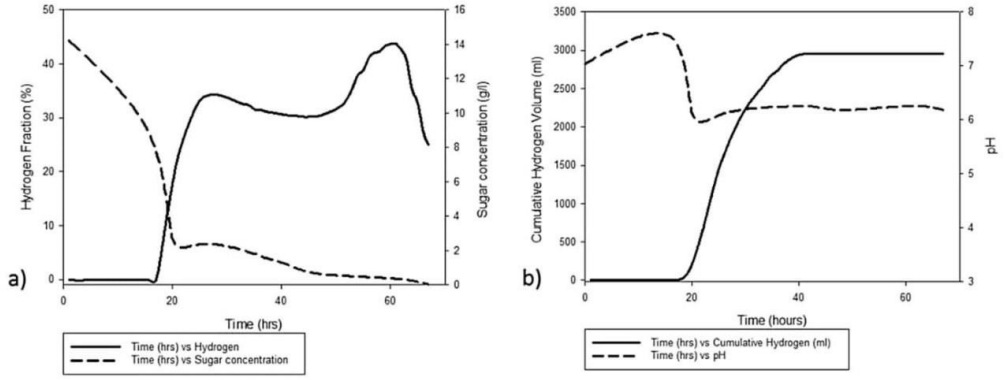

Fig. 6 – Plots of the time course of (a) hydrogen production (%) and total sugar consumption and (b) cumulative hydrogen volume (ml) and the change in pH.

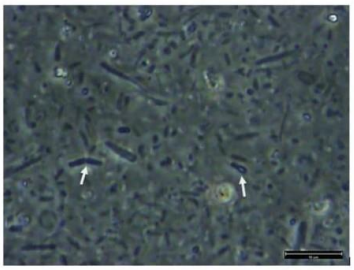

Fig. 7 – Phase contrast microscopy of the bench scale process effluent sample, showing the presence of presumptive rod-shaped endospore forming bacteria.

was achieved using 5.95% HCl for a lag time of 18 h and heat application at 100 °C for 176 min with a S:L ratio of 49.7%. HCl pre-treatment allowed for a reduced heating time of 176 min compared to H_2SO_4 and HNO_3 which required 240 min. This reduces the energy input requirement for pre-treatment. Xylose and glucose recovered were subsequently used for the optimization of hydrogen production. A yield of 47.30 ml H_2/g FS was obtained under optimized conditions. The feasibility of this process at bench scale was assessed. A peak hydrogen fraction of 43.75% with a yield of 213.14 ml H_2/g FS. Findings presented in this study highlight the feasibility of using waste sorghum leaves as an excellent feedstock for biofuel or biomaterial production.

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