Development of Innovative Pretreatments for Simultaneous Saccharification and Citric Acid Production from Banana Pseudostem: Bioprocess Optimization and Kinetic Assessment

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By

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PREFACE

The research contained in this thesis/dissertation was completed by the candidate (Milesh Laltha, 216011104) while based in the Discipline of Microbiology, School of Life Sciences, of the College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Pietermaritzburg Campus, South Africa. The research was financially supported by the National Research Foundation (Grant number: 122341).

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

Signed: Professor Evariste Bosco Gueguim Kana (Supervisor)

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Date:

DECLARATION 1: PLAGIARISM

I, Milesh Laltha, declare that:

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

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

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

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

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

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

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

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

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

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DECLARATION 2: PUBLICATIONS AND MANUSCRIPTS

This thesis represents a compilation of a published paper and two manuscripts (under review). Details of contributions to a publication that form part and/or include research presented in this thesis are provided within each publication and manuscript under the Credit Author Statement section. The student (Milesh Laltha) contributed towards experimental work, data collection and manuscript preparation, under the guidance of Professor E.B. Gueguim Kana (Supervisor) and Doctor Y. Sewsynker-Sukai (Co-supervisor).

1. Laltha, M., Sewsynker-Sukai, Y., Meyer, E.L., Gueguim Kana, E.B. 2022. Banana pseudostem as a renewable feedstock for microbial-based value-added products. Bioresource Technology. (Chapter 2 - Under review)

2. Laltha, M., Sewsynker-Sukai, Y., Gueguim Kana, E.B. 2022. Innovative microwave-assisted iodized table salt or paper wastewater pretreatments for enhanced sugar recovery from banana pseudostem. *Biomass Conversion and Biorefinery*, 1-15.
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ABSTRACT

The bioconversion of renewable lignocellulosic biomass into value-added products such as citric acid (CA) has become a research hotspot towards a sustainable economy. Approximately 2 million tons of CA are produced annually with widespread use in the food, chemical, pharmaceutical and textile industries. Nevertheless, the use of lignocellulosic-derived feedstocks for CA production requires expensive, energy- and resource intensive pretreatment and fermentation processes, that result in low product yields. Therefore, the present study explores the valorization of banana pseudostem (BP) for CA production using the simultaneous saccharification and fermentation (SSF) process to alleviate these challenges.

A review on the potential of BP as a feedstock for microbial production of value-added products is discussed together with the pretreatment and bioprocess challenges in addition to the possibilities to overcome these bottlenecks. Subsequently, two novel pretreatments consisting of a: (1) microwave-assisted-iodized table salt (M-ITS) and (2) microwave-assisted-paper wastewater (M-PWW) were modelled and optimized using the response surface methodology (RSM) for the enhancement of sugar recovery from BP. Then, the SSF process with pretreated BP was modelled and optimized using RSM for CA production. Thereafter, the logistic and modified Gompertz models were used to assess the kinetics of *Aspergillus brasiliensis* growth and CA production, respectively.

The pretreatment input parameters for the M-ITS model comprised of salt concentration (1-5%, w/v), microwave power intensity (100-900 W) and pretreatment time (2-10 min). On the other hand, the M-PWW model consisted of substrate solid loading (10-30% w/v), microwave power intensity (100-900 W) and pretreatment time (2-10 min). The output responses were

reducing sugar yield (RSY) and glucose yield (GY) for both models. The optimal pretreatment conditions predicted by the M-ITS model were 2.48% ITS concentration, 800 W (power intensity) and 10 minutes (pretreatment time), corresponding to an experimental RSY (0.515 g/g) and GY (0.433 g/g). Pretreatment and optimization revealed a slightly higher RSY and GY for the M-ITS strategy when compared to the M-PWW method (RSY = 0.498 g/g and GY = 0.413 g/g). The M-PWW model predicted optimal pretreatment conditions of 30% solid loading, 800 W for 8 minutes. Despite the higher sugar yields observed for the M-ITS pretreatment, the M-PWW pretreatment represents a more suitable strategy owing to its complete waste-based approach that generates three times the quantity of pretreated substrate required for subsequent fermentation.

SSF optimization included nitrogen (ammonium nitrate) concentration (0.5-5 w/v), desorbent (acetone) concentration (1-5% v/v) and temperature (30-40 °C) as inputs with CA concentration as the output (g/L). The optimized SSF conditions (0.5% w/v ammonium nitrate, 1% v/v acetone and 35 °C) ($SSF_{optimizedFW}$) gave a maximum CA concentration of 14.408 g/L. For the kinetic experiments, three bioprocesses consisting of the: (1) optimized SSF process with freshwater ($SSF_{optimizedFW}$), (2) $SSF_{optimizedFW}$ process conditions while substituting dairy wastewater in place of freshwater (SSF_{DWW}), and (3) Sabouraud Dextrose Emmon's medium modified by substituting glucose with BP ($SSF_{SDEmodified}$), were assessed. While all three bioprocesses gave the same maximum specific growth rate (μ_{max}) of 0.05 h^{-1}, the $SSF_{SDEmodified}$ process resulted in the highest maximum potential CA concentration (P_m) (13.991 g/L) compared to the SSF_{DWW} (P_m= 13.095 g/L) and $SSF_{optimizedFW}$ (P_m= 12.967 g/L) systems.

The developed pretreatment strategies demonstrated glucose yields >0.40 g/g, shown to be higher than previously established chemical pretreatments. Moreover, the SSF_{DWW} process

displayed a slightly lower P_m value compared to the $SSF_{SDEmodified}$ strategy, and interestingly a higher P_m value than the $SSF_{optimizedFW}$ bioprocess. Major findings from this study paves the way for lignocellulosic bioprocesses by potentially negating the use of costly pretreatment chemicals, fermentation medium constituents and/or fresh water, while achieving a "waste treating waste" approach. Thus, contributing to the water-energy-food nexus in line with global sustainable development goals towards a circular bioeconomy.

Keywords: Banana pseudostem; pretreatment strategies; fermentation; citric acid; kinetics.

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LIST OF ABBREVIATIONS

AFEX	Ammonia fibre explosion
ANOVA	Analysis of Variance
ATCC	American Type Culture Collection
BP	Banana pseudostem
CA	Citric acid
CMC	Carboxymethyl cellulose
CSL	Corn steep liquor
DNA	Deoxyribonucleic acid
DNS	3,5-dinitrosalicyclic acid
DWW	Dairy wastewater
Eq.	Equation
FPU	Filter paper unit
FTIR	Fourier-Transform Infrared spectroscopy
F-value	Fisher-Snedecor distribution value
GLD	Green liquor dregs
GY	Glucose yield
IL	Ionic liquid
ITS	Iodized table salt
LCB	Lignocellulosic biomass
LHW	Liquid hot water
M-	Microwave-assisted
MHP	Microwave heating processes
MI	Microwave irradiation
NAD/H	Nicotinamide adenine dinucleotide/hydrogen
pH	Potential of hydrogen
P_m	Potential maximum citric acid concentration
PPO	Polyphenol oxidase
PSSF	Pre-hydrolysis SSF
PWW	Paper wastewater
p-value	Probability value
$r_{p,m}$	Maximum citric acid production rate

R^2	Coefficient of determination
RSM	Response surface methodology
RSY	Reducing sugar yield
S/L	Solid to liquid ratio
SDE	Sabouraud Dextrose Emmon's
SEM	Scanning electron microscopy
SHF	Separate hydrolysis and fermentation
sp.	Species
SSF	Simultaneous saccharification and fermentation
t	Time
TCA	The citric acid cycle
t_L	Lag time
USD	United States dollar
v	Volume
w	Weight
X_0	Initial cell concentration
X_{max}	Maximum cell concentration
β	Beta
μ_{max}	Maximum specific growth rate

CHAPTER 1

General Introduction

This chapter provides a general outline of the current research and states the aims and objectives to be carried out. Additionally, it elaborates on the development of novel bioprocess strategies, the suitability of utilizing industrial wastes, the generation of citric acid, and optimization and kinetic modelling. Some of these aspects have not been covered in the literature review (Chapter 2). The literature review has been submitted to Bioresource Technology and focuses on innovative pretreatment strategies applied to renewable banana pseudostem for microbial-based value-added products.

1.1. The importance for renewable biobased products

The depletion of sustainable resources has been a fundamental worldwide concern for commercial industries. Over the last three decades, the demand for natural resources has continuously increased due to the exponential population and economic growth rate (Mabhaudhi et al., 2018). The current process for producing versatile commodities such as organic acids and fossil fuels (coal, oil and gas) from finite non-renewable resources are no longer sustainable due to the exhaustion of these sources (Sharma et al., 2021). In addition, these processes result in the accumulation of non-biodegradable and hazardous wastes (toxins and leachates) that are harmful both to human health and to the environment (landfills and water bodies) resulting in pollution and subsequent global warming (Raj et al., 2022). To resolve these problems, carbon-neutral biorefineries are emerging as a promising alternative to support the sustainable production of biobased industrial products such as polymers, chemicals and fuels from renewable lignocellulosic biomass (Singh et al., 2022). Furthermore, for the transition to a complete circular economy, raw materials and wastes from industrial processes are considered ideal for producing essential biobased materials (Ashokkumar et al., 2022). The commercial scale lignocellulosic bioconversion to organic acids is not yet fully established due to several factors that requires optimization to reduce the bioprocessing costs during lignocellulosic pretreatment, enzymatic saccharification and microbial fermentation. To achieve low-cost bioprocesses, efficient microbial consortia capable of producing metabolites in large quantities are employed for the utilization of renewable and cheap lignocellulosic biomass-derived sugars (Ashokkumar et al., 2022).

1.2. The significance of lignocellulosic substrates in biorefineries

In recent times, agro-industrial and crop residual wastes have garnered substantial attention as sustainable raw materials for the generation of marketable products such as organic acids,

without posing ethical issues to food security (Kostas et al., 2021). Renewable lignocellulosic biomass is one of the most abundant and economical resources available with an annual production rate estimated at 181.5 billion tons, while only 8.2 billion tons of biomass is currently utilized (Singh et al., 2022). Lignocellulosic feedstocks comprise of polysaccharides that include cellulose and hemicellulose, which are rich in hexose (glucose and fructose) and pentose (xylose and arabinose), intertwined by fractions of lignin moieties (Hernández Beltrán et al., 2019) (Fig. 1). The valorization and bioconversion of crop residues to value-added organic acids are carried out through a series of established processes that encompass pretreatment, saccharification and fermentation. Pretreatment is implemented to overcome the inherent high degree of recalcitrance, followed by saccharification to fermentable sugars that can be consumed by the microorganisms during the fermentation process (Shimizu et al., 2018). Recently, studies have reported on the utilization of lignocellulosic waste residues and include bamboo (Laltha et al., 2021), corn cobs (David et al., 2020), banana pseudostem (Legodi et al., 2021), sugarcane bagasse (Jugwanth et al., 2020), potato peel waste (Sanusi et al., 2019) and Napier grass (Mohammed et al., 2019), among others. More specifically, banana pseudostem represents an attractive, nonedible, cellulosic feedstock that has shown to be suitable for the production of biofuels and biochemicals. The chemical composition of banana pseudostem is estimated to consist of a high cellulose (42-60%), moderate hemicellulose (19-23%) and a low lignin content (15-17%) (Legodi et al., 2021, Islam et al., 2019). The global annual production of banana pseudostem amounts to approximately 300 million tons (Subagyo and Chafidz, 2018). Post harvesting of the banana fruit leaves behind banana pseudostem that consists of about 75% of the total waste that is generated. This waste is either left to rot at the local dumpsite prior to incineration resulting in the release of unpleasant greenhouse gases such as hydrogen sulphide and ammonia gas in addition to the spread of Panama disease caused by the soil-borne fungus *Fusarium oxysporum* (Costa et al., 2018). Therefore, the utilization of

banana pseudostem will lead to the production of valuable commodities such as citric acid and also overcome problems that are associated with its disposal.

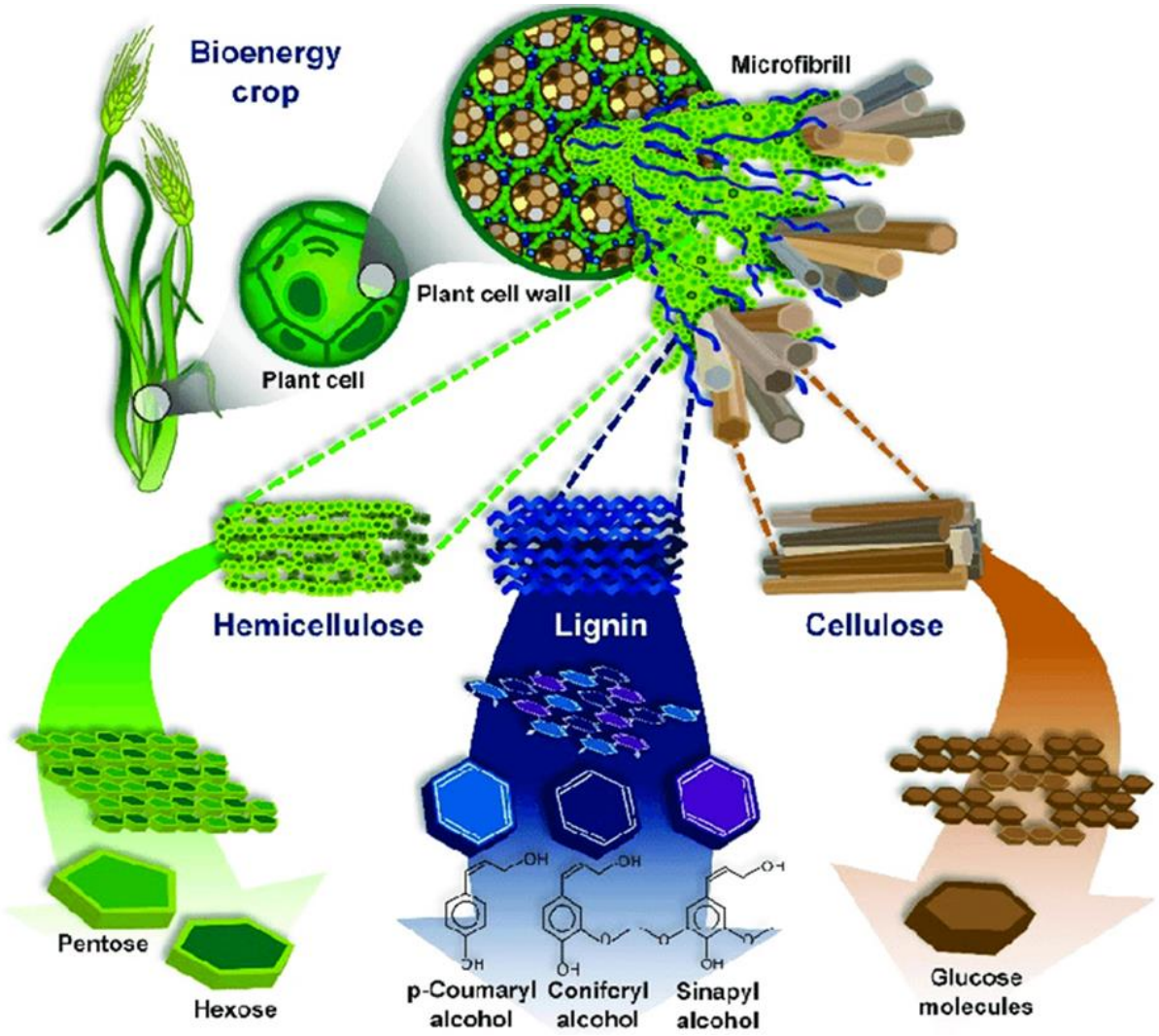

Fig. 1. Structure of lignocellulosic biomass and its biopolymeric constituents (Adapted from Hernández Beltrán et al. 2019).

1.3. The requirement for lignocellulosic pretreatment

Pretreatment is a rate-limiting step that is vital for altering the structure of LCB. By reducing the lignin content, pretreatment enables the access of cellulose and hemicellulose to saccharifying enzymes during enzymatic hydrolysis for enhanced sugar recovery that are required during microbial bioprocesses (Hernández Beltrán et al., 2019). Common

pretreatment strategies that have previously been assessed include alkali, acid, microwave, inorganic salts, steam explosion and ammonia fibre explosion, among others (Yu et al., 2019). From the aforementioned methods, alkaline pretreatments have been sought after attributable to the favourable reaction conditions, low reaction pressures and temperatures, less corrosive chemicals, ability to recover leftover alkali and the use of simplified reactors (Phitsuwan et al., 2016). Nevertheless, these methods are limited by expensive chemical catalysts, requirement for specialized equipment and the utilization of vast amounts of fresh water during pretreatment steps and in between wash cycles (Badiei et al., 2014). Therefore, pretreatment strategies that minimize energy and fresh water usage, do not pose a threat to the environment, utilize cost-effective chemicals that are non-corrosive, minimize the formation of inhibitor compounds and enhance the availability of fermentable sugars for microbial fermentation are imperative (Hernández Beltrán et al., 2019, Baruah et al., 2018). In this regard, our recent study investigated two different alkaline (NaOH and $Na_3PO_4.12H_2O$) pretreatments on bamboo and corn cobs and determined their potential recyclability (Laltha et al., 2021) (Appendix A). Findings from the aforementioned study indicated that the alkaline spent hydrolysate could be recycled up to three times, thereby minimizing the pretreatment costs by reducing the consumption of expensive alkaline chemicals and fresh water. However, sequential pretreatment processes are still faced with the initial chemical and water costs and/or usage, which is considered an imperative bottleneck in the present time (Laltha et al., 2021).

1.4. Bioprocessing of valuable renewable organic acids

Biobased chemicals with three-carbon backbones that include citric acid, lactic acid and itaconic acid have garnered significant attention in academia and industry (Song et al., 2022, Son et al., 2022). This is due to their wide range of applications in the pharmaceutical, manufacturing, agrochemical and textile industries (Behera et al., 2021). Citric acid (CA) also

known as 2-hydroxy-1,2,3-propane tricarboxylic acid is a weak organic acid that has emerged as an important organic acid owing to its palatability, pleasant taste and low toxicity (Ayeni et al., 2019). Moreover, CA is also used as a chelating agent and detergent for metal finishing, cleaning, lubrication, animal feed and as a plasticizer (Show et al., 2015). Approximately 2 million tons of CA is generated on an annual basis while the rate of demand is expected to rise by 5% each year (Ozdal et al., 2019). The global market for CA is expected to exceed $3.2 billion USD by 2023 (Behera et al., 2021). Additionally, the cost of CA production via chemical synthesis is approximately ~$10 USD per kg, whereas for fungal fermentation it has been estimated to be ~50% lower (Ozdal et al., 2019). In industry, CA is mainly produced by the filamentous fungus, *Aspergillus niger* (Adeoye and Lateef, 2022). In addition, bacteria (*Bacillus sp.*, *Brevibacterium sp.,* and *Pseudomonas sp.*) and yeasts (*Candida sp.,* and *Yarrowia sp.*) are capable of producing citric acid (Tong et al., 2019). CA is produced via the citric acid cycle (TCA) through various metabolic pathways (Fig. 2). CA biosynthesis is initiated by the uptake of glucose which undergoes glycolysis to produce two molecules of pyruvate. Pyruvate molecules are oxidized and combined with coenzyme A to form CO_2, Acetyl-CoA and NADH. Acetyl-co-A combines with oxaloacetate to produce citric acid, yielding two molecules of CO_2, Adenosine Triphosphate (ATP) and four-carbon oxaloacetate again (Behera et al., 2021). The expensive and inefficient conversion technologies of biobased organic acid production hinder the commercial establishment of biobased products (Guragain and Vadlani, 2021). In this regard, biobased organic acids can be viewed as economically competitive only if their production costs are made more affordable by utilizing agro-industrial processes (Chan et al., 2022).

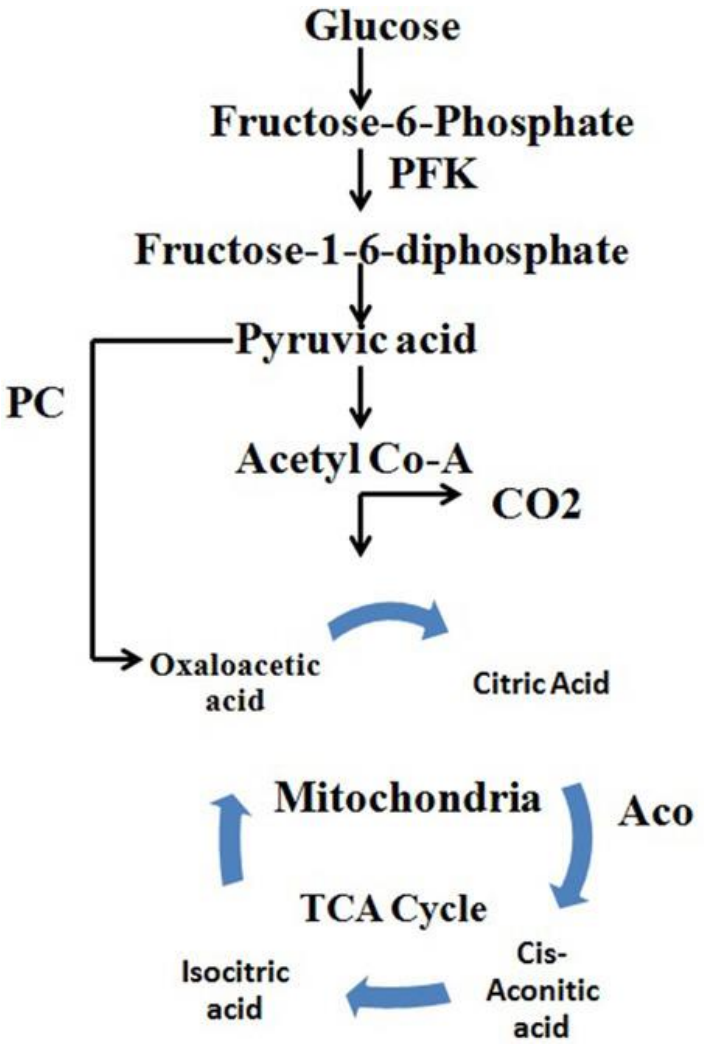

Fig. 2. Schematic representation of the main metabolic reactions involved in the production of citric acid. PFK = phosphofructokinase, PC = pyruvate carboxylase, ACO = aconitase (Adapted from Behera et al., 2021).

1.5. The food, energy and water nexus approach for a circular bioeconomy

Water, energy and food are three of the key pillars upon which humanity exists (Mabhaudhi et al., 2018). Recently, the food, energy and water nexus has attracted significant attention by the political, social, and scientific community (Harwood, 2018). The food, energy and water nexus approach offer several opportunities for improving security while contributing to a green economy (Fernández-Ríos et al., 2021). The approach considers the interactions, synergies and opportunities of the linked securities, to optimize the use of these resources, whereby usage within one sector influences the use and availability in the adjacent sectors (Fernández-Ríos et al., 2021) (Fig. 3).

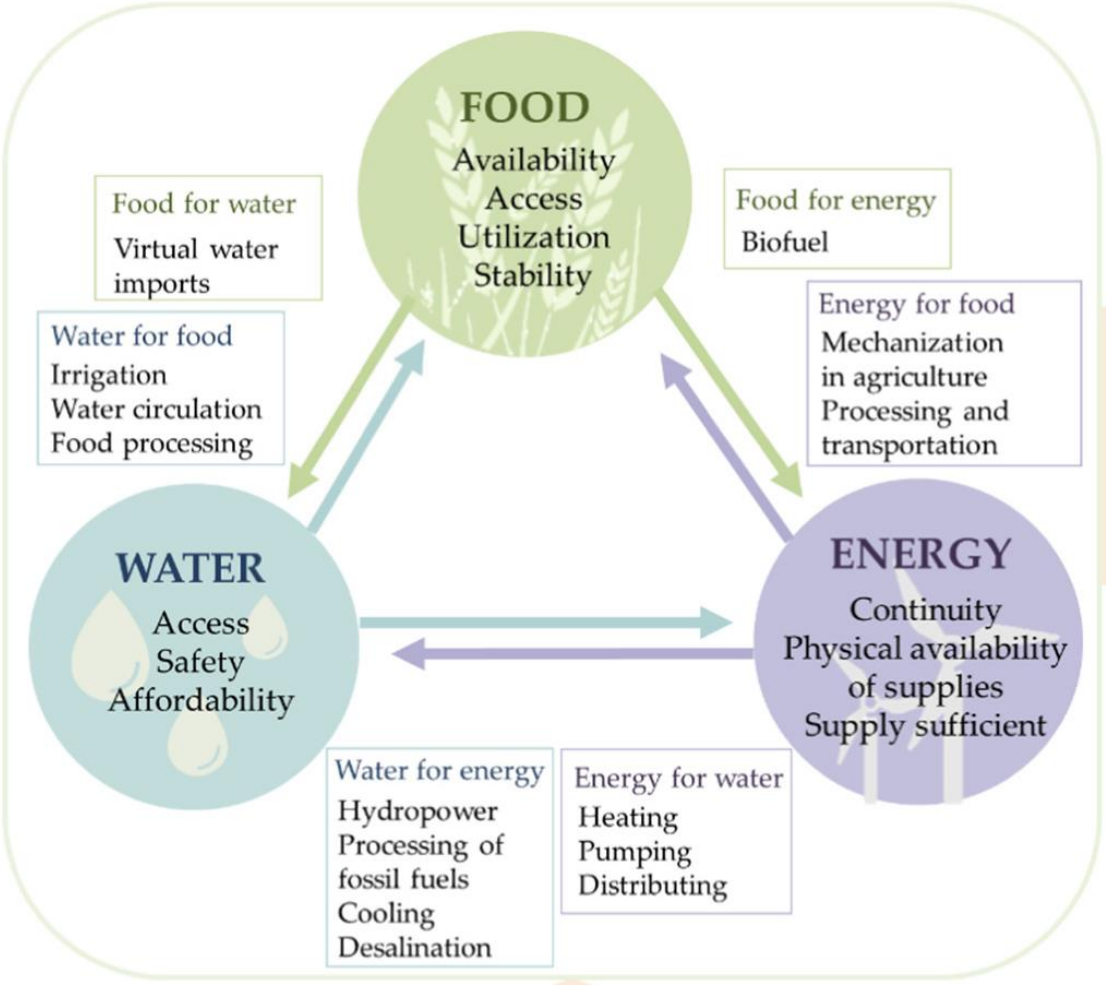

Fig. 3. The interactions and synergy of the food, energy and water nexus (Adapted from Fernández-Ríos et al., 2021).

Water scarcity has been forecasted to intensify within the next few years, resulting in a 60% increase in water demand by 2025 (Fig. 4) (Ghazal and Kazmi, 2014). Water is essential for agricultural and industrial practices, contributing to one of the main costs incurred. Additionally, most forms of energy production are made possible by the incorporation of water. For example, water is required to support livelihoods, fisheries, irrigation, wastewater treatment and food production. Global food production accounts for an estimated 70% of fresh water use, while primary energy production and power generation amount to 10% of water utilization (Fernández-Ríos et al., 2021). Hence, there is a need for efficient alternatives to conventional fresh water.

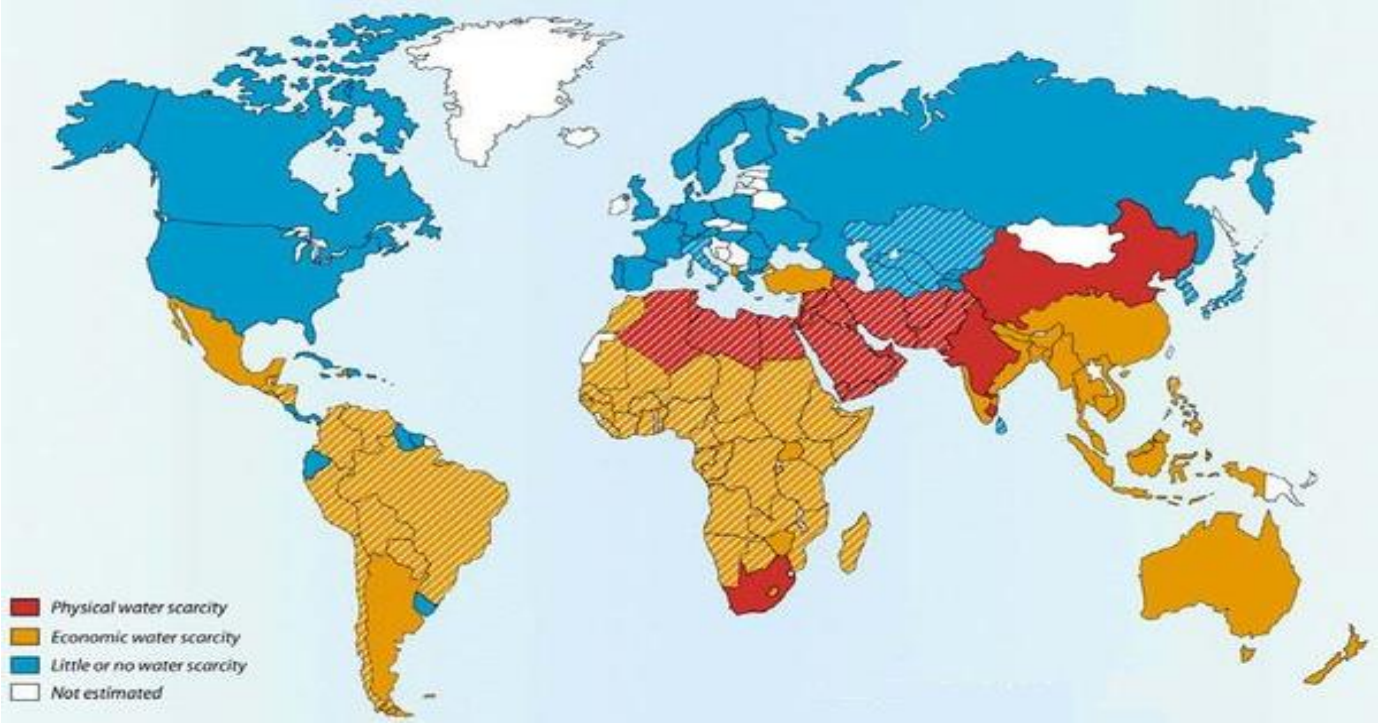

Fig. 4. Projected water scarcity by 2025, indicating "physical water scarcity" occurring in South Africa and in countries coinciding in the equatorial plane (Adapted from Ghazal and Kazmi, 2014).

1.6. Development of innovative bioprocess strategies utilizing industrial wastewater

The Kraft pulp and paper industry is one of the most important industrial sectors in the world (Manasa and Mehta, 2020). Nevertheless, pulp and paper mills are faced with challenges due to energy intensive processes that utilize vast amounts of fresh water in turn generating alkaline paper wastewater (PWW) that has adverse effects on the aquatic environment (Kamali and Khodaparast, 2015). The characteristics of these waste by-products depend on various parameters that include, type of raw material employed, processing technique and property of the desired end-product (Mäkelä et al., 2016). The application of PWW as a chemical and water source during lignocellulosic pretreatment processes may drastically reduce costs and could potentially generate profits that can be channelled towards less invasive biohazardous management, thereby lowering the downstream costs for Kraft industries. Kraft PWW exhibits strong alkali chemicals that lead to the degradation of ester and glycosidic bonds between

hemicellulose and ferulic acid in the cell wall of lignocellulosic biomass, resulting in the swelling of cellulose, hemicellulose solvation, and lignin removal (Costa et al., 2018). PWW serve as a dual-purpose commodity serving as an alternative to fresh water and also for the replacement of expensive lab-derived alkaline reagents (NaOH). In the same vein, the exploitation of cheaper alternatives (iodized table salt) to conventional lab-based chemicals (NaCl) would be economically beneficial for pretreatment processes thereby reducing expenditure for lignocellulosic biorefineries. Recently, inorganic salts have gained significant attention due to their ability to increase cellulose and hemicellulose conversion rates and enhance enzymatic hydrolysis yields (Liu et al., 2009). Inorganic salts function by dissociating Lewis acids in an aqueous solvent to form hydrated complex cations surrounded by water molecules. These water molecules further act as nucleophiles, thus depolymerizing hemicellulose into monosaccharides (Moodley et al., 2020).

In addition to PWW as a pretreatment alternative, another promising waste effluent that can be used in bioprocessing is dairy wastewater (DWW). Water plays a significant role in the dairy industry and is mainly used for cleaning, washing, disinfection, heating and cooling (Slavov, 2017). In recent years, the global production of the dairy industry has increased, resulting in large amounts of discharged wastewater effluent. DWW comprises of abundant nutrients, rich in lactose, proteins, fats, vitamins, and minerals, which can lead to severe eutrophication if disposed of without prior treatment (Kusmayadi et al., 2022). Eutrophication leads to many water quality problems including increased purification costs and the possible sublethal effects of algal toxins on animals and humans (Prazeres et al., 2020). Hence, the application of DWW in bioprocesses could alleviate water security while avoiding its detrimental effects on ecosystems. DWW like PWW serves a dual-purpose in biorefinery systems since this waste can be used as a replacement of fresh water in fermentation reactors and simultaneously

provide nutrients for growth and metabolism of the fermentative microorganism (Son et al., 2022). Furthermore, the utilization of DWW will enable the dairy industry to avoid treatment costs, thus synergistically saving time and capital expenses for both industries. Therefore, the concept of utilizing waste effluent from industry for value-added products will contribute to reducing production costs while alleviating the current water crisis.

1.7. Bioprocess optimization and kinetic modelling

The success of bioconversion processes is dependent on a systemic process that incorporates pretreatment, enzymatic saccharification and fermentation (Fig. 5). The expenditure endured at each of these steps affects the viability of the overall end-product (Devi et al., 2022). Despite the abundance and widespread availability of lignocellulosic biomass, expensive and inefficient bioconversion technologies hinder the commercial establishment of biobased products (Guragain and Vadlani, 2021). Therefore, process integration and optimization for the development of cost-effective and green technologies that valorize lignocellulosic biomass to biofuels and biochemicals is vital for the transition of the current fossil-fuel economy to a sustainable and circular bioeconomy (Guragain and Vadlani, 2021). Modern mathematical techniques are sought after for the optimization of fermentation processes due to their effectiveness, efficiency and robustness. For instance, response surface methodology (RSM) is a collection of statistical and mathematical tools used for the design of experiments (DOE) and optimization of process variables. RSM offers several benefits, for example it reduces the number of experimental trials and takes into consideration the interaction of process parameters (Kumari and Gupta, 2019). RSM has been employed for the optimization of various process parameters that comprise of temperature (Baskar et al., 2018), catalyst concentration (Manmai et al., 2020), solid loading (David et al., 2020), time and microwave power intensity (Laltha et al., 2021). Additional parameters that have been optimized include desorbent (Baskar et al.,

2018) and nitrogen concentration (da Silva et al., 2012). These studies achieved maximum product concentrations for the response variables by optimizing the aforementioned parameters.

Various bioprocess types have emerged that comprise of (1) separate hydrolysis and fermentation (SHF), (2) simultaneous saccharification and fermentation (SSF) (Dahnum et al., 2015) and (3) SSF with pre-hydrolysis (PSSF) (Giang et al., 2019). Recently, the simultaneous saccharification and fermentation (SSF) strategy has attracted significant research among these, owing to its beneficial capabilities that it offers for saving processing time, energy and resources. The SSF method involves the degradation of cellulose by cellulolytic enzymes in combination with the conversion of glucose to fermentable products (Devi et al., 2022). The SSF process is a simpler approach that occurs in a single reactor, thereby saving resources, reducing the risk for contamination and inhibitor compounds.

On the other hand, kinetic models can be utilized to predict microbial growth and product output by quantifying experimental data. The logistic model elaborates on the growth of the microorganism to the rate-limiting substrate over time (Germec et al., 2019). Conversely, the modified Gompertz model estimates the maximum production rate, maximum concentration and the lag phase (Mozhiarasi et al., 2020). Both mathematical and kinetic models maximize product output while reducing chemical expenditure, inhibitor formation and time. Furthermore, the revenue that is sequestered can be channelled towards other bioprocessing steps to enhance productivity and promote a circular bioeconomy.

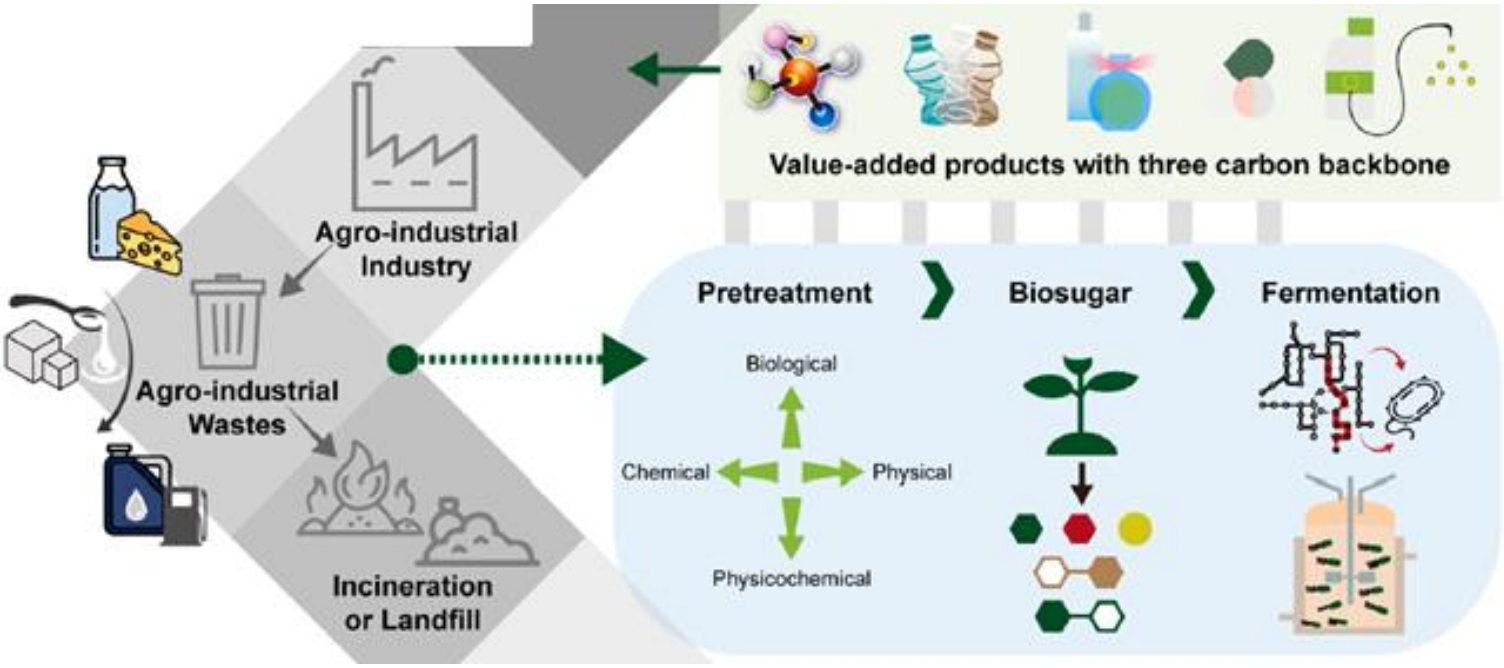

Fig. 5. Circular bioeconomy illustrating the bioconversion of agro-industrial wastes to value-added products (Adapted from Son et al., 2022).

1.8. Research motivation/Problem statement

Establishing a sustainable bioeconomy necessitates overcoming several key challenges encountered at various steps of the lignocellulosic biorefinery value chain. Biorefining is a capital-intensive endeavour that aims to valorize lignocellulosic waste to value-added products (Usmani et al., 2021). However, the bioprocess conversion remains a technical and economic challenge due to various factors that include but is not limited to (1) lignocellulosic substrate availability, structure, and properties, (2) complex pretreatment and saccharification technologies, and (3) high capital and operating expenditures for large-scale industrial implementation (Raj et al., 2022). Citric acid is extensively used in the food and pharmaceutical industry and is one of the largest biotechnological industries for organic acid production. However, a key bottleneck endured during bioprocessing is the availability of fermentable sugars (Massadeh et al., 2022). Therefore, there has been an increasing trend towards the efficient utilization of lignocellulosic biomass as a cost-effective and abundant resource of polysaccharides. A necessary step towards the effective utilization of lignocellulosic biomass

is a pretreatment step that eases the fermentation process subsequently enhancing the downstream product yield (Ashokkumar et al., 2022). However, most conventional pretreatment techniques possess various drawbacks that include high-energy and cost consumption, intermediates and by-product formation that diminish the overall attractiveness of biomass valorization. In addition, the pretreatment step has been estimated to account for nearly 40% of the overall processing cost (Kucharska et al., 2018). These processing costs are mainly attributed to the utilization of freshwater and expensive chemicals in lignocellulosic bioprocesses. Hence, the development of novel, efficient and low-cost pretreatment and fermentation strategies that result in optimum product formation are required. Optimization of key process parameters are essential to determine the interactive effects on the product output yield which saves resources, energy, and time (Odu et al., 2020). On the other hand, employing mathematical kinetic models to assess the microbial growth and product formation is vital for understanding metabolic regulations that occur under varying conditions (Augustine et al., 2015). These findings may contribute to improving the product quality and yield thus enabling industrial-scale implementation. Furthermore, the utilization of agro-industrial wastewater and lignocellulosic biomass in biorefining mitigates environmental pollution and greenhouse gas emissions thus creating a green environment, consequently improving economic growth, and contributing to a sustainable energy system.

1.9. Aims and objectives

The present study aimed to: (1) model and optimize novel and cost-effective pretreatment strategies for the enhancement of reducing sugar from banana pseudostem, (2) optimize key parameters for microbial-based citric acid production by using the simultaneous saccharification and fermentation approach and (3) assess the kinetics of *Aspergillus brasiliensis* growth and product formation under different medium conditions.

To achieve the aforementioned aims, this study is guided by the following objectives:

i. Review banana pseudostem (BP) as a potential lignocellulosic substrate for the generation of microbial-based value-added products. Discuss pretreatment regimes on BP together with their advantages and limitations. Outline the development and utilization of lignocellulosic bioproducts, bioprocess types, kinetic models and present challenges and future prospects.

ii. Model and optimize two novel pretreatments consisting of a: (1) microwave-assisted-iodized table salt (M-ITS) and (2) microwave-assisted-paper wastewater (M-PWW) using the response surface methodology (RSM) for the enhancement of sugar recovery from banana pseudostem.

iii. Evaluate the effectiveness and versatility of the developed M-ITS and M-PWW pretreatment techniques on several lignocellulosic substrates (corn cobs, sorghum leaves, sugarcane bagasse and bamboo).

iv. Model and optimize the simultaneous saccharification and fermentation (SSF) process using the RSM for citric acid production from optimally pretreated BP derived in objective (ii).

v. Utilize the logistic and modified Gompertz models to assess the kinetics of *Aspergillus brasiliensis* growth and citric acid production, respectively, under different medium conditions using the SSF bioprocess type.

2. Outline of thesis structure

This thesis is based on five chapters, the general introduction chapter, literature review chapter and two experimental chapters that are presented in research paper format and a concluding chapter. More specifically, it represents a compilation of one published paper and two manuscripts that have been submitted and are under review, whereby each chapter is an individual entity prepared as per the journals' specifications. Consequently, some repetition between chapters has been unavoidable. The thesis format is based on the template outline provided by the College of Agriculture, Engineering and Science (CAES) of the University of KwaZulu-Natal, South Africa. Each experimental chapter is self-contained, comprising of an introduction, materials and methods, results and discussion, conclusion and references. The representation, evaluation and application of novel, agro-industrial wastes (wastewater and banana pseudostem) for microbial-based value-added products featuring pretreatment and fermentation are pivotal to all chapters.

Chapter 1 provides a general background of the current research and highlights the aims and objectives that were undertaken.

Chapter 2 presents an overview of banana pseudostem as a potential substrate for bioconversion to microbial-based value-added products. Additionally, previous pretreatment regimes and novel strategies applied to BP for the enhancement of sugar recovery and optimum product formation are discussed aligned with their advantages and limitations. The current challenges of lignocellulosic bioprocesses and future innovations for energy efficient and cost-competitive technologies during pretreatment, enzymatic saccharification and fermentation are presented.

Chapter 3 focuses on the modelling and optimization of two novel pretreatments that include: (1) microwave-assisted-iodized table salt (M-ITS) and (2) microwave-assisted-paper wastewater (M-PWW) strategy for the enhancement of sugar recovery from BP. In addition, the efficiency and adaptability of the developed pretreatment models were assessed on various lignocellulosic substrates (corn cobs, sorghum leaves, sugarcane bagasse and bamboo).

Chapter 4 encompasses modelling and optimization of key input parameters such as acetone concentration, ammonium nitrate concentration and temperature in the SSF process for optimal citric acid (CA) production from pretreated BP. Thereafter, kinetic assessment of *Aspergillus brasiliensis* growth and CA production were determined for the optimum conditions using fresh water ($SSF_{optimizedFW}$) or dairy wastewater (SSF_{DWW}) and compared to Sabouraud Dextrose Emmon's medium modified with BP ($SSF_{SDEmodified}$).

Chapter 5 concludes and integrates the key findings from the experimental chapters, highlights the significant conclusions and provides recommendations for future research on lignocellulosic bioprocessing.

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CHAPTER 2

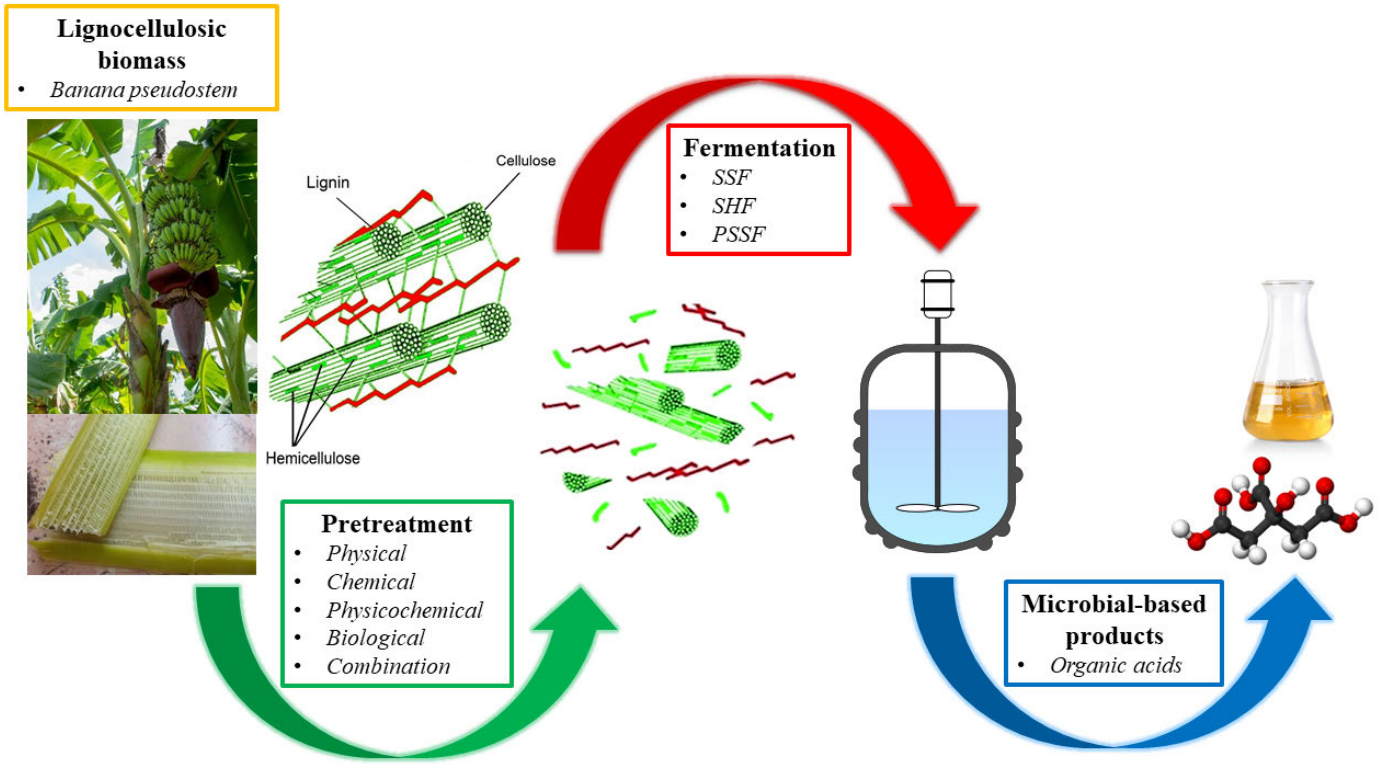
Banana pseudostem as a renewable feedstock for microbial-based value-added products

This chapter has been submitted to the journal *Bioresource Technology* entitled: Banana pseudostem as a renewable feedstock for microbial-based value-added products. (Under review). The manuscript and supplementary material are presented in the following pages.

Highlights

- Reviews the potential of banana pseudostem for microbial-based value-added products.
- Highlights current pretreatment regimes together with their advantages and limitations.
- Investigates novel and cost-effective alternatives to fresh water utilization.
- Comparatively assesses various bioprocess types for optimum product formation.
- Discusses the key challenges and future prospectives of lignocellulosic bioprocesses.

Graphical Abstract



Banana pseudostem as a renewable feedstock for microbial-based value-added products

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Abstract

Bananas have been touted as a vital food crop typically cultivated under tropical climates. Of particular interest is the banana pseudostem (BP) from a biorefinery standpoint, since it is renewable, abundant and a low-cost carbon source for generating a spectrum of high-value bio-based products globally. The production and utilization of lignocellulosic bio-based products offer several advantages to the commercial sector; however, the relatively high cost of production has restricted its mainstream adoption. In light of this, the present review assesses BP as a potential lignocellulosic substrate for the generation of microbial-based value-added products. Previous pretreatment regimes for the enhancement of sugar recovery and optimum product formation are discussed together with their advantages and limitations. Furthermore, the development of different lignocellulosic bioproducts, bioprocess types and the utilization of mathematical kinetic models are outlined. Finally, present challenges and prospects to overcome the bottlenecks associated with the lignocellulosic biorefinery platform are presented.

Keywords: Banana pseudostem; pretreatment; fermentation; bio-based products.

1. Introduction

Lignocellulosic based bioprocesses have emerged as a sustainable approach for the production of various industrial bioproducts (de Paula et al., 2019). Lignocellulosic biomass (LCB) is a naturally occurring complex composite that originates from plant dry matter (Moodley et al., 2020) and are characterized as waste materials. Although considered a waste, LCB is viewed as an ideal feedstock for the production of renewable biofuels and bioproducts, because it does not pose a risk to socio-economic concerns such as food security, deforestation and water shortages. Moreover, LCB is abundant and considered to be cost-effective compared to food-based feedstocks (Sekoai et al., 2019). Current technological advancements focus on exploiting lignocellulosic substrates to produce microbial-based renewable biofuels and other commercial value-added bioproducts (de Paula et al., 2019). The application of LCB aims to offset the fossil fuel demand by replenishing fuel reserves and generating renewable high value bioproducts. LCB represents the world's most economical and highly renewable natural resource with an estimated annual production of 200 billion tons (Hernández Beltrán et al., 2019, Lu et al., 2022). Lignocellulosic feedstocks are primarily composed of three major polymers, namely cellulose (30 to 50%), hemicellulose (15 to 35%) and lignin (10 to 20%) as well as other minor components including heterogeneous phenolic polymers (proteins, extractives and inorganic minerals) (Mussatto and Dragone, 2016, Limayem et al., 2012). Corn cobs (David et al., 2020), banana pseudostem (Islam et al., 2019), sugarcane leaves (Moodley and Gueguim Kana, 2019), and bamboo (Laltha et al., 2021) are some examples of LCB that have previously been assessed. More specifically, banana pseudostem (BP) has emerged as an attractive and non-edible feedstock that exhibits high tensile strength and biodegradability (Shimizu et al., 2018). Additionally, BP has a high cellulosic and low lignin content, thus making it suitable for the production of biofuels and biochemicals. Although LCB such as BP offers several advantages, its recalcitrant nature hinders direct microbial utilization and

represents a major bottleneck that must be overcome. More specifically, the crystallinity of cellulose, encapsulation of cellulose by the lignin-hemicellulose matrix and hydrophobicity of lignin all contribute to the ability of lignocellulose to resist degradation (Kumar et al., 2020, Tayyab et al., 2018). Thus, effective fractionation of the biomass into its main constituents (cellulose, hemicellulose and lignin) is crucial and can be achieved by subjecting the feedstock to a pretreatment stage (Mussatto and Dragone, 2016). Pretreatment is an important step for efficiently converting LCB to biofuel and value-added products (Mussatto and Dragone, 2016). Additionally, this stage is necessary to produce highly digestible solids to enhance the sugar yields obtained during enzymatic hydrolysis (Mussatto and Dragone, 2016). Numerous strategies for biomass pretreatment have been investigated and developed over the years. These include alkali, acid, microwave, inorganic salts, steam explosion and ammonia fibre expansion, among others (Yu et al., 2019). Pretreatment is one of the most energy intensive steps in a lignocellulosic biorefinery and accounts for up to 40% of the total costs of biomass processing towards biofuels or value-added products (Kucharska et al., 2018). This cost can be attributed to the chemicals, water and energy utilized during the pretreatment stage. Furthermore, several studies have reported on the low sugar yields in addition to the degradation of sugars and subsequent formation of inhibitory compounds that hinder the enzymatic and fermentation process (Mussatto and Dragone, 2016, Saha et al., 2019). The development of an economically robust and reliable bioprocess is essential to effectively utilize lignocellulosic materials (Mussatto and Dragone, 2016). Over the last decade, lignocellulosic bioprocessing that incorporates pretreatment and fermentation has become a research hotspot with thousands of studies generated on an annual basis (Usmani et al., 2021). Nevertheless, limitations that continue to plague these systems include the high costs and energy, long processing times, low product yields and detrimental effects on the environment (Baruah et al., 2018). To overcome the aforementioned bottlenecks, it is necessary to tap into various aspects pertaining to

lignocellulosic bioprocesses. For instance, suitable LCB substrates with lower fractions of resistant structures (lignin and hemicellulose), thus inferring a higher digestibility with increased potential to be completely valorized is highly sought and need to be explored (Zhang et al., 2019). Moreover, the development of pretreatment and fermentation strategies that are environmentally friendly, cost-effective and result in high product yields is imperative. Therefore, this review assesses banana pseudostem (BP) as a potential lignocellulosic substrate for the production of microbial-based value-added products. A compilation of previous pretreatment and fermentation studies on BP as a feedstock are presented. In addition, previous pretreatment regimes for improving sugar recovery and optimum product formation are reviewed, along with their benefits and drawbacks. Furthermore, the development of lignocellulosic bioproducts, the use of mathematical kinetic models, and bioprocess types are discussed. Finally, the current challenges and potential prospects for overcoming bottlenecks associated with the lignocellulosic bioprocessing platform are highlighted.

2. Lignocellulosic biomass

Lignocellulosic biomass is an innate macromolecule that is synthesized from sunlight, nutrients and CO_2 sequestration by plants (Fatma et al., 2018). It is regarded as a carbon-neutral resource, since the quantity of CO_2 released during combustion is merely identical to that which the plant absorbs during photosynthesis (Ibarra-Gonzalez and Rong, 2019). There are various groups of raw materials that have been distinguished by their composition, origin and structure such as agricultural residues, woody feedstocks and municipal solid wastes (Limayem et al., 2012). Lignocellulosic substrates are defined as heterogeneous matrices that contain hemicellulose and cellulose bound together by lignin that comprise of cellulose (C_6 sugars) and hemicellulose (C_5 and C_6 sugars) bound together by layers of recalcitrant lignin (Moodley et al., 2020). The lignocellulosic composition varies between substrates and is dependent on the physical

properties and source of the biomass as shown in Table 1. These differentiations may also be attributed to the type of plant species, weather and soil fertility implications (Limayem et al., 2012). Cellulose is the most abundant organic molecule available and is a principal constituent of plants (Wang et al., 2017). The structure of cellulose has been described as a linear homopolymer of glucose ($C_6H_{12}O_6$) units that are linked together in the form of D-anhydro-glucopyranose units through β-(1,4)-glycosidic bonds (Dashtban et al., 2009). Adjacent cellulosic chains are coupled with hydrogen bonds, hydrophobic interactions and Van der Waal's forces. Hemicellulose is the second most abundant component of LCB. Hemicellulose is an amorphous, single chain, branched polysaccharide made up of heterogenous polymers and includes pentose (L-arabinose and D-xylose) as well as hexose (D-glucose, D-mannose and D-galactose) (Kang et al., 2014). The backbone chain of hemicellulose comprises of xylan linkages, namely L-arabinose (10%) and α-xylose (90%) linked together via hydrogen bonding (Kang et al., 2014). The resistant lignin polymer is an amorphous phenolic compound that is made up of three monomeric groups: coniferyl, sinapyl alcohols and *p*-coumaryl (Hernández Beltrán et al., 2019). These alcohols are linked together by ether and carbon-carbon bonds (Stöcker, 2008). Lignin is distributed between the exterior layers of fibres, therefore keeping the polysaccharide fibres together, enhancing the strength and rigidity of the cell wall (Stöcker, 2008). This aromatic biopolymer is covalently bonded to hemicellulose and cellulose, providing the impermeable and recalcitrant characteristic to plant cell walls (Kang et al., 2014, Moodley et al., 2020). The lignin content of LCB is gaining considerable attention due to its inhibitory effect on the hydrolysis and fermentation processes (Kang et al., 2014). Therefore, a pretreatment step is integral to reducing LCB recalcitrance to improve its cellulose digestibility.

Table 1. Composition of various lignocellulosic biomass feedstocks.

Feedstock	Cellulose (%)	Hemicellulose (%)	Lignin (%)	References
Banana pseudostem	42-60	19-23	15-17	(Islam et al., 2019, Shimizu et al., 2018)
Napier grass	35.0-39.4	19.2-23.4	15.3-19.3	Narinthorn et al. (2019)
Switch grass	40-45	30-35	12	Kang et al. (2014)
Corn cobs	42-45	35-39	14-15	Rabemanolontsoa and Saka (2013)
Bamboo	39.80	19.49	20.81	Rabemanolontsoa and Saka (2013)
Wheat straw	35-39	22-30	12-16	De Bhowmick et al. (2018)
Barley	36-43	24-33	6.3-9.8	De Bhowmick et al. (2018)
Rice husk	25-35	18-21	26-31	Rabemanolontsoa and Saka (2013)
Eucalyptus	52.07	24.51	25.2	Xu et al. (2017)
Sugarcane bagasse	46.1	20.1	20.3	Nosratpour et al. (2018)

2.1. Banana pseudostem as a suitable feedstock for valuable biofuels and biochemicals

Banana (*Musa acuminata*) is a monocotyledonous annual herbaceous plant that belongs to the family Musaceae (Shimizu et al., 2018). It is mainly cultivated in the tropical and subtropical regions of Asia, South America and Africa (Islam et al., 2019). Banana is one of the most essential, perennial fruit crops globally, with an overall yearly production of more than 100 million tons (Costa et al., 2018). In South Africa, approximately 450 000 tons are produced yearly (Panigrahi et al., 2021). After the fruit is harvested, banana lignocellulosic biomass including rachis, foliage, and stems remain (Chen et al., 2017b). This waste is usually crushed and left to decompose on farms to replenish nutrients in the soil (Chen et al., 2017b). However, this disposal method leads to environmental pollution in ground and water bodies resulting in the release of unpleasant greenhouse gases (hydrogen sulphide and ammonia gas) and the

spread of Panama disease caused by the fungus *Fusarium oxysporum* (Chen et al., 2017b, Costa et al., 2018). Therefore, utilizing this lignocellulosic biomass will minimize the aforementioned problems associated with it. For every ton of banana that is harvested, approximately 4 tons of agricultural residues (leaf, pseudostem, rachis, rotten fruit, peel, rhizome) are generated, of which 75% is the pseudostem (Islam et al., 2019). The pseudostem is the cylindrical part of the banana plant, made up of overlapping leaf sheaths that are 20 to 50 cm in diameter (Islam et al., 2019). To date, banana pseudostem has been proposed as a useful biomass for paper production, animal feed production, organic fertilizer, renewable materials, wastewater purification and biofuel production (Chen et al., 2017b). Banana pseudostem contains on average 42-60% cellulose, 19-23% hemicellulose and 15-17% lignin (Islam et al., 2019, Shimizu et al., 2018). Additionally, banana pseudostem also contains approximately 8-10% ash and 3-3.1% extractives (Idrees et al., 2013, da Silva et al., 2019, Begum and Chittibabu, 2015). The fibre of banana pseudostem has a low density and strong moisture absorption ability enabling it to be flexible and increasing the transfer of heat (Li et al., 2010, Subagyo and Chafidz, 2018). Moreover, this agricultural waste displays satisfactory physical properties, including relatively high tensile stiffness and strength, light weight, and biodegradability (Shimizu et al., 2018). The high cellulosic and hemicellulosic content makes it an ideal feedstock for renewable energy production (Narinthorn et al., 2019). Furthermore, it has been used previously to produce valuable bio-based chemicals such as sodium carboxymethyl cellulose (CMC) and polyphenol oxidase (PPO) (Shimizu et al., 2018). Due to the low lignin content, it could serve as a potential substrate for biogas production (Islam et al., 2019). The pseudostem of the banana plant illustrated in Fig. 1. provides and transports nutrients from the soil to the fruits. The pseudostem is the part of the banana plant that resembles a trunk consisting of a soft central core and tightly wrapped by up to 25 leaf sheaths (Subagyo and Chafidz, 2018). These leaf sheaths unwrap from the stem and transform into recognizable

banana leaves after they have matured. Moreover, the height of the banana plant can reach approximately 7.5 metres (Subagyo and Chafidz, 2018). The characteristics of banana pseudostem demonstrated by its high availability, structural composition and previous use in bioprocesses elicits its application for the production of biofuels and other commercial microbial-based value-added products. Recent reports on the utilization of banana pseudostem for the generation of various bioproducts and the respective pretreatment and/or bioprocess parameters are outlined in Table 2. Islam et al. (2019) reported on the production of bioethanol from banana pseudostem using *Saccharomyces cerevisiae.* This author pretreated BP using dilute acid (H_2SO_4) to obtain 1.45 g/L dextrose fermented to 8.28 g/L bioethanol. In the same vein, Mustofa (2018) attained 8.51 g/L of bioethanol from BP using a similar pretreatment. Other authors have also reported on the production of bioethanol from BP using other forms of pretreatment. For instance, Sukmaningtyas (2018), Ingale et al. (2014) and Thakur et al. (2013) pretreated BP using alkali (NaOH) pretreatment and observed 4.32, 17.1 and 3.8 g/L of bioethanol respectively using *Saccharomyces cerevisiae.* Interestingly, in addition to bioethanol, some studies have reported on the production of other valuable bioproducts from BP. For example, Chen et al. (2017b) and Sivanarutselvi et al. (2019) pretreated BP using 8 and 2% w/v NaOH for the attainment of 41.6% lignin removal and 92% delignification, respectively. Bioconversion of these pretreated substrates to biomethane (239.9 mL/g) and biobutanol (10.12 g/L) was achieved using anaerobic digestion and *Clostridium sp*., respectively. Furthermore, Idrees et al. (2013) pretreated BP using sulfuric acid and obtained 38 g/L reducing sugar. These sugars were converted by *Lactobacillus sp.* thus achieving a 92% lactic acid conversion yield. In many of these studies, BP has been pretreated using several pretreatment strategies resulting in various fermentative value-added products. Hence, BP is a versatile lignocellulosic substrate that confers multiple advantages that necessitate exploration more decisively.

Table 2. Studies investigating the potentiality of banana pseudostem for bioproducts corresponding to the various input process conditions.

Fermentative microorganism	Pretreatment parameters	Pretreatment output	Bioprocess parameters	Product output	References
Saccharomyces cerevisiae	Dilute H_2SO_4 (0.25% v/v), 121°C, 30 min and 1 hr	1.45 g/L dextrose	10 g of pretreated substrate, 48 hr, 37 °C, 5 units/mL enzyme, 200 mL of 0.1 M citrate buffer, pH 5, 100 rpm	8.28 g/L bioethanol	Islam et al. (2019)
Lactobacillus acidophilus	1% H_2SO_4, 130 °C, 2.18 hr	38 g/L reducing sugar	Inoculum to solution ratio of 1:20, 10 g/L yeast extract, 2 g/L $(NH_4)2HPO_4$, 0.1 g/L $MgSO_4$, 72 hr	92% lactic acid conversion yield	Idrees et al. (2013)
Anaerobic digest	8% NaOH (w/v), 50 °C, 24 hr	41.6% lignin removal, 23.6% hemicellulose removal	-	239.9 mL/g biomethane	Chen et al. (2017b)
Clostridium sporogenes	2% NaOH (w/v), S/L ratio 1:10, 30 °C, 24 hr	92% delignification and 524 mg/g glucose	10% v/v inoculum, 37 °C, 72 hr, pH 7	10.12 g/L biobutanol	Sivanarutselvi et al. (2019)
Saccharomyces cerevisiae	2 N NaOH, 121 °C, 72 hr	29.8 g/L glucose yield	2 N NaOH , 120 hr, cellulase enzyme 20 FPU/g, 10% v/v inoculum, 37.5 °C	4.32 g/L bioethanol	Sukmaningtyas (2018)
Aspergillus niger	2% w/v H_2SO_4, 121 °C, 20 min	18% delignification, 15% cellulose increase	10% w/v substrate, pH 5, 30 °C, 150 rpm, 6 d	8.51 g/L bioethanol	Mustofa (2018)

Table 2. Continued.

Fermentative microorganism	Pretreatment parameters	Pretreatment output	Bioprocess parameters	Product output	References
Saccharomyces cerevisiae	1 N NaOH, 5g substrate solid loading, room temperature, 18 hr	833 mg/mL reducing sugar	4.1 g sugar, 5% inoculum, 0.5 g/L $(NH_4)2SO_4$, 0.5 g/L KH_2PO_4, 2.5 g/L yeast extract, pH 5.5, 30 °C, 72 hr	17.1 g/L bioethanol	Ingale et al. (2014)
Saccharomyces cerevisiae	1 N NaOH, 50 °C, 48 hr	15.3 g/L reducing sugar	2% v/v inoculum, 0.5 g/L $(NH_4)2SO_4$, 0.5 g/L KH_2PO_4, 2.5 g/L yeast extract, pH 5.5, 30 °C, 48 hr	3.8 g/L bioethanol, 0.35 g/g bioethanol	Thakur et al. (2013)

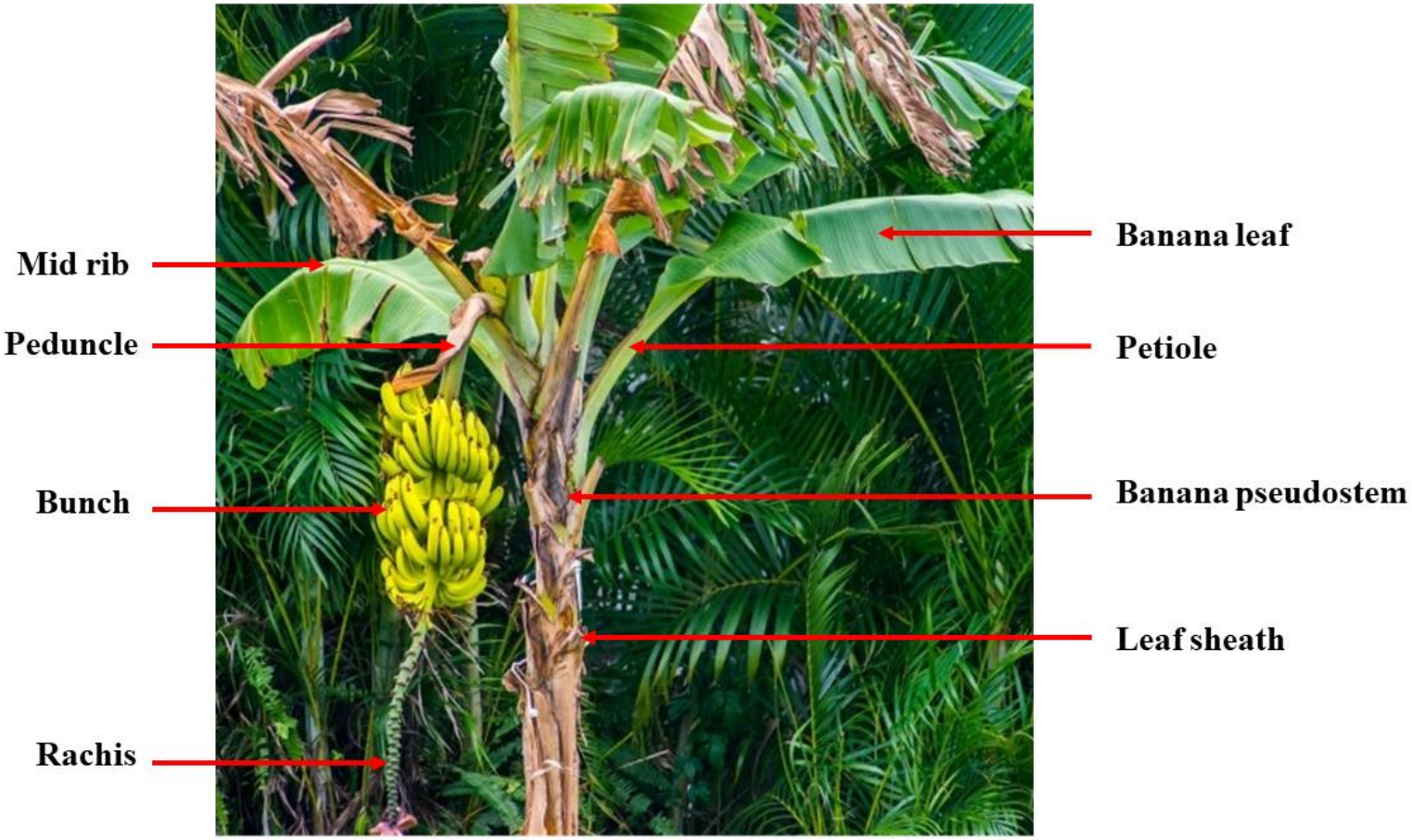

Fig. 1. A banana tree consisting of its several parts including the banana pseudostem (Subagyo and Chafidz, 2018; modified).

2.2. Limitations of lignocellulosic biomass

Even with the immense benefits of LCB substrates, challenges that presently exist render these feedstocks non-viable for large scale application. For one, pretreatment of LCB is necessary but the high: (1) energy consumed, (2) chemical costs and (3) volume of fresh water used during this stage significantly impacts the overall process efficiency and has become the rate-limiting step (Mohammed et al., 2019). This is attributed to the recalcitrance of LCB, which resists degradation into simple sugars such as glucose that are mandatory for microbial consumption towards the generation of biofuel and bio-based products (Narinthorn et al., 2019). Natural factors that contribute to biomass recalcitrance include degree of lignification, the arrangement and density of the vascular bundles, quantity of thick-walled tissue and structural heterogeneity and complexity (Himmel et al., 2007). Additionally, the adjacent

structures of cellulosic and hemicellulosic microfibrils are bound to lignin moieties via hydrogen and covalent bonds, rendering them highly recalcitrant to depolymerization (Zhao et al., 2011). Furthermore, the activity of enzymes that catalyze the substrate into simpler molecules are inhibited, thereby raising production costs (Narinthorn et al., 2019). Therefore, several concerns need to be addressed in order to enhance the digestibility of cellulose. These comprise of the accessible surface region, cellulose crystallinity, lignin resistance and the various characteristics of biomass particles (Zabed et al., 2016). Most often, a pretreatment step involving either physical, chemical, biological or a combination of these are implemented to reduce biomass recalcitrance and enhance lignocellulosic degradation towards enzymatic hydrolysis.

2.3. The significance of lignocellulosic pretreatment

The pretreatment process is an imperative stage that is employed for altering the structure of LCB, making cellulose and hemicellulose available for bioconversion. The main purpose of pretreatment is to disrupt the physical (macrostructure) and chemical (microstructure) barriers of the plant cell wall, to enhance the surface-to-volume ratio, depolymerize and reduce the crystallinity of cellulose (Hernández Beltrán et al., 2019, Tayyab et al., 2018). Pretreatment also reduces the lignin content, thereby enabling the access of cellulose to hydrolytic enzymes. Hence, pretreatment processes inevitably enhance the saccharification efficiency, while lowering production costs (Liu et al., 2017). This stage is regarded as being crucial, both technically and economically, since operating and capital costs could amount to more than 40% of the total processing cost (Bhutto et al., 2017, Mupondwa et al., 2017). Effective pretreatment techniques should aim to minimize energy and fresh water usage, not to pose a threat to the environment by lowering its carbon footprint, utilize cost-effective chemicals that are non-corrosive, minimize the formation of enzymatic and fermentation inhibitor compounds and

enhance the availability of fermentable sugars for microbial fermentation (Brodeur et al., 2011, Chen et al., 2017a).

3. Lignocellulosic pretreatment regimes on Banana pseudostem

The physicochemical and structural complexity of the LCB matrix differs for each substrate (Mussatto and Dragone, 2016). Consequently, the selected pretreatment method is not as equally effective for each type of biomass, therefore it is essential to identify the most efficient pretreatment technology for a particular substrate. The major pretreatment methods that have been studied in recent years comprise of (i) physical pretreatment (mechanical, microwave irradiation and ultrasound), (ii) chemical pretreatment (acid, alkaline, ionic liquids and deep eutectic solvents and inorganic salt), (iii) physicochemical (steam explosion, ammonia fibre explosion, CO_2 explosion and liquid hot water), (iv) biological pretreatment and (v) combination of these strategies. The benefits and limitations of these pretreatment technologies are discussed and highlighted in Table 3. Additionally, the primary pretreatment methods that have been studied on banana pseudostem and similar derivatives of the banana plant in recent years are presented in table 4. Following the pretreatment stage, an enzymatic saccharification step is necessary to depolymerize the cellulose carbohydrate polymer to fermentable sugar (glucose) (Zabed et al., 2016). Cellulase is an enzyme cocktail that comprises mainly of three enzymes that include endoglucanase, cellobiohydrolase and β-glucosidase. At the onset of enzymatic hydrolysis, endoglucanase hydrolyzes amorphous cellulose to a reducing end group. Subsequently, cellobiohydrolase cleaves the glycosidic bonds to form cellobiose. Lastly, cellobiose is hydrolyzed by β-glucosidase to yield glucose in a liquid phase. Cellulase enzymes are highly effective for catalytically converting cellulose to glucose, however, this can only be successfully achieved through efficient pretreatment regimes (Shrotri et al., 2017).

3.1. Physical pretreatment

Physical pretreatment causes damage to the lignocellulosic structure with minimal to no chemical alterations to the different cell wall components (Chundawat et al., 2010). This pretreatment affects the substrate particle size, surface area, crystallinity index and degree of polymerization (Kumar et al., 2020). Physical pretreatment processes circumvent the usage of chemicals and is deemed safe for the environment, since they generate minimal to no toxic compounds. Nevertheless, implementing particle size reduction as an individual pretreatment is not adequate to significantly improve enzymatic digestibility due to the high cost. Despite the cost factor, minimal particle size reduction is required preceding most pretreatments to enhance material handling during processing (Chundawat et al., 2010). Some examples of commonly employed physical pretreatment techniques include mechanical, microwave, and ultrasound and are briefly discussed below.

3.1.1. Mechanical

Various mechanical pretreatment procedures (shredding, chipping, coarse size reduction and milling) have been employed to improve the digestibility of LCB (Tayyab et al., 2018). Mechanical pretreatment decreases cellulose crystallinity, assists in the removal of lignin and hemicellulose, increases the bulk density and surface area, and alters the degrees of polymerization (Tayyab et al., 2018). Additionally, the smaller particle size and larger surface area eradicates the mass and heat transfer limitations. Jiang et al. (2022) applied a high-pressure CO_2-hydrothermal pretreatment to banana pseudostem for value-added porous carbons. Prior to pretreatment, the BP was chopped into 2 × 5 cm pieces and air dried at 30 °C for 6 h to avoid spoilage. Following this, the BP was ground into powder and sieved through a 60-mesh screen, and thereafter dried in an oven at 105 °C for 24 h. These authors observed an improvement in activated carbon yield of 1340%. Likewise, Zhang et al. (2013) applied mechanical

pretreatment to banana stem. The substrate was cut into 1 cm sections, air dried, and stored at -20 °C. After pretreatment, it was determined that banana stem contained 30.07% of cellulose, 10.65% hemicellulose and 17.10% lignin. This pretreatment method has been utilized to facilitate and enhance the efficacy of subsequent biorefinery procedures (Chen et al., 2017a). Furthermore, mechanical pretreatment increases the digestion rate and product yield obtained from LCB (Narinthorn et al., 2019). These strategies produce fewer emissions, but intense energy and capital are required, thus increasing production costs (Chen et al., 2017a).

3.1.2. Microwave irradiation (MI)

Microwave heating technology has garnered immense consideration as a valuable source of power for rapid volumetric heating (Zhu and Chen, 2014). Many studies have extensively used this pretreatment technology for different lignocellulosic materials (Puligundla et al., 2016, Sewsynker-Sukai and Gueguim Kana, 2018, Moodley and Gueguim Kana, 2019). Microwave irradiation (MI) involves two principal mechanisms, ionic conduction and dipolar polarization (Zhu and Chen, 2014). This form of pretreatment functions by heating a material that contains electrical charges such as polar molecules in a liquid or solvent (Zhu and Chen, 2014). During microwave heating, polar water molecules change their orientation due to the rapidly alternating electromagnetic fields (Zhu and Chen, 2014, Singh et al., 2014). Therefore, heat is generated by the friction, rotation and collision of these molecules (Zhu and Chen, 2014). Moreover, MI displays a strong heating capacity, rendering it possible to dissolve the lignin and improve enzymatic susceptibility (Singh et al., 2014). MI exhibits several advantages over the conventional steam heating liquid hot water method. These advantages include: (1) short pretreatment times, (2) lower cost and energy, (3) minimum production of inhibitor compounds, (4) is considered environmentally benign, and (5) employs direct heating that prevents overheating of surfaces (Tayyab et al., 2018, Sewsynker-Sukai and Gueguim Kana,

2018). Furthermore, MI is environmentally safe and can also be conveniently coupled with chemical reactions to speed up the reaction rate, thus reducing the reaction time and energy usage (Nagahata and Takeuchi, 2019). Previous reports on microwave pretreatment have shown enhancements in the cellulose digestibility and/or sugar recovery. For instance, Gabhane et al. (2014) investigated microwave-assisted acid pretreatment on banana waste (pseudostem, leaf and pith) and demonstrated a maximum recovery of reducing sugar of 36.84% under 25 min of reaction time at 700 W. Egwim and Shittu (2015) evaluated the effects of microwave-assisted alkaline pretreatment on banana pseudostem for bioethanol production. A bioethanol yield of 300 mL was attained at an alkaline concentration (2% NaOH) and microwave irradiation (170 W, 10 minutes). Pretreatment studies have indicated that although microwave irradiation is efficient on lab-scale, this heating method has been limited by the disproportionate power distribution, lack of uniformity and reduced penetration efficiency in larger samples, which restricts its industrial scale application (Aguilar-Reynosa et al., 2017).

3.1.3. Ultrasound

Ultrasonic radiation degrades the lignocellulosic material by creating shear forces that disrupt the intricate network structures of LCB. Advantages of ultrasonic pretreatment include the (1) short reaction time, (2) low temperatures, and (3) low concentration of chemicals that are used in combination or for further pretreatment processing. Numerous factors have shown to impact on the efficiency of ultrasound pretreatment and include the sonication power, temperature, duration and ultrasound frequency (Baruah et al., 2018). For instance, Gabhane et al. (2014) investigated the influence of acid ultrasonication for the pretreatment of banana pseudostem. The authors revealed that pretreatment yielded a reducing sugar of 41.98% at a temperature of 50 °C for 60 min. Nevertheless, ultrasonication methods are energy intensive and further

experimentation is imperative to determine the optimum conditions for large scale application (Baruah et al., 2018)

3.2. Chemical pretreatments

Chemical pretreatment utilizes chemical reactions to alter the resistant lignocellulose. Frequently used chemical methods consist of acids, bases, inorganic salts, ionic liquids and organosolvents. The mechanisms of biomass pretreatment and selectivity of lignocellulosic structures differs depending on the chemical that is employed.

3.2.1. Acid

Acid pretreatment encompasses both concentrated and dilute acid that could be used to break down the rigid structure of LCB (Fatma et al., 2018). Sulfuric acid (H_2SO_4), hydrochloric acid (HCl), phosphoric acid (H_3PO_4) and nitric acid (HNO_3) are commonly used acids for pretreatment (Fatma et al., 2018). Concentrated acid (above 30% v/v) targets the polysaccharide chains, mainly hemicelluloses and celluloses (Fatma et al., 2018). Flexible substrate choice and high fermentable sugar yields at low temperatures are the main advantages of acid-catalysed pretreatments (Kumar et al., 2020). Regardless of the low temperature requirement, acid recovery is still a major challenge that is encountered during commercial-scale applications (Fatma et al., 2018). Furthermore, high acid concentrations poses dangers to operating personnel and may result in the corrosion of the bioreactor vessel resulting in high maintenance costs (Hernández Beltrán et al., 2019). Moreover, concentrated acid pretreatment generates inhibitor compounds at a high rate, thereby increasing purification costs (Hernández Beltrán et al., 2019).

Dilute acid pretreatment has been shown to successfully hydrolyze hemicellulose and disrupt the structure of a variety of lignocellulosic feedstocks. Low acid concentrations (0.2 to 2.5% v/v) and temperatures (between 130 °C and 210 °C) are typically employed in industrial applications (Hernández Beltrán et al., 2019). Frequently exploited inorganic acids include (sulfuric, nitric, phosphoric and hydrochloric acid) and organic acids (carboxylic and sulfonic acid) (Chen et al., 2017a). Shimizu et al. (2018) studied the effect of different acid (H_2SO_4) concentrations (5, 10, 15, 20, 25, 30, 35 and 40%) on the chemical composition, cellulose accessibility, and enzymatic digestibility of banana pseudostem. Under the most severe acid pretreatment, hemicellulose was removed entirely, and its percentage decreased to approximately 4.38%. In addition, a glucose yield of 60 to 80% was achieved at a high acid concentration indicating the suitability of banana pseudostem for biofuel production. In the recent time, Legodi et al. (2021) investigated 5% (v/v) H_2SO_4 pretreatment on BP and achieved 48% cellulose amounting to 21.8 g/L glucose after enzymatic hydrolysis (10 FPU/g, 50 °C). Dilute acid pretreatments minimize the amount of degradation products formed while lowering the rate of corrosion in comparison to concentrated acids (Fatma et al., 2018). Additionally, dilute acid improve the pretreatment process efficiency and are feasible for potential large-scale implementation, and can be quickly neutralized by treating with ammonium or lime (Fatma et al., 2018). Nevertheless, the generation of calcium sulfate gypsum creates a disposal problem. Acid recovery is a non-feasible procedure that may result in environmental problems caused by the disposal of it into waste streams (Peral, 2016).

3.2.2. Alkali

Lignin removal from the lignocellulosic biomass is crucial to enhance the reactivity of the remaining polysaccharides. Pretreatment with bases is the most extensively used chemical technique that has shown to be highly efficient for lignin removal from LCB (Kim et al., 2020,

Sewsynker-Sukai et al., 2020). Alkaline pretreatment entails the solvation of lignocellulosic units and the hydrolytic decomposition of lignocellulose, termed saponification (Kucharska et al., 2018). As a result of alkaline pretreatment, cellulose crystallinity decreases, and the maximum specific area of the biomass increases (Kucharska et al., 2018). Consequently, the lignin structure is altered and undergoes unfolding. Popular alkaline reagents include calcium hydroxide ($Ca(OH)_2$), sodium hydroxide (NaOH), ammonia (NH_3), and hydrogen peroxide (H_2O_2) (Phitsuwan et al., 2016). Alkaline reagents interact with lignin by splitting the intermolecular ester bonds between carbohydrate and lignin complexes, thereby modifying the composition of LCB and contributes to the solubilization of lignin (Phitsuwan et al., 2016). Zhang et al. (2013) studied the impact of 6% alkali NaOH pretreatment on banana stem for biogas generation. After pretreatment, it was ascertained that 30.07% of cellulose, 10.65% hemicellulose and 17.10% lignin content was present in banana stem. These authors obtained a maximum biomethane yield of 232.4 mL/g amounting to 21.4%. Likewise, Sukmaningtyas (2018) investigated alkali NaOH (0.5 to 2 N) pretreatment on banana pseudostem in the autoclave for 90 min. The highest sugar content attained after enzymatic hydrolysis (72 h) was 29.8 g/L (2N NaOH solution). The variation of the pretreatment process by increasing NaOH concentration led to a decline of lignin content while increasing the amount of cellulose. The lowest lignin and the highest cellulose content achieved was 11.44% and 51.66%, respectively. Advantages of alkaline-based pretreatment strategies include favourable reaction conditions, low reaction pressures and temperatures, less corrosive chemicals, the potential to recover leftover alkali and the use of simplified reactors (Phitsuwan et al., 2016). Moreover, alkali-based pretreatments have shown to be more effective when used on LCB with low lignin content (herbaceous crops and agricultural residues) compared to hardwood substrates (Baruah et al., 2018). Despite its efficiency, alkaline methods employ chemical catalysts that are

expensive and utilize vast amounts of fresh water during pretreatment steps and in between wash cycles (Badiei et al., 2014).

3.2.3. Ionic liquids and Deep Eutectic Solvents

Ionic liquids (IL) have been described as salts with melting points below 100 °C and are composed of cations and anions (Stark, 2007). Some examples of IL that have been assessed for lignocellulosic pretreatment include imidazolium and pyrrolidinium. The pretreatment mechanism of ionic liquids is thought to be as a result of the dissolution of cellulose (Stark, 2007). ILs possess high solvating properties and can selectively cleave glycosidic bonds in polysaccharides (Xu et al., 2017). Ai et al. (2021) explored the potential of cellulose derived from banana stem as a substitute for polyethylene (PE) film for the long-term preservation of mangos. Cellulose film (23.1 μm) was synthesized from banana stem via pretreatment (1 g substrate, 80 to 90 °C, 200 rpm, 3 to 4 h) with 19 mL of ionic liquid known as 1-Allyl-3-methylimidazolium chloride. Even with the benefits of IL pretreatments, their industrial scale applicability is challenged by the release of inhibitor compounds and high costs (Kucharska et al., 2018). Deep eutectic solvents (DES) that display similar features to ionic liquids, have emerged as low cost and easily synthesized alternatives. Other striking benefits of DES include their low volatility, broad liquid range, low toxicity, high biodegradability and enzyme compatibility.

3.2.4. Inorganic salt

Recently, the utilization of inorganic salt has emerged as an attractive form of pretreatment (Kang et al., 2013). Inorganic salts are gaining attention due to their ability to increase cellulose and hemicellulose conversion rates and hydrolysis yields (Liu et al., 2009). Inorganic salts may be categorized into three different classes and comprise of alkaline earth metal chlorides ($CaCl_2$

and $MgCl_2$), alkaline metal chlorides (KCl and NaCl) and transition metal chlorides (CuCl2, $FeCl_3$ and $FeCl_3$) (Kang et al., 2013). The primary mechanism by which inorganic salts function is the dissociation of Lewis acids in an aqueous solvent to form complex cations. Glycosidic linkages present in LCB are cleaved by the formation of metal cations. The water molecules from the hydrated cation act as nucleophiles that depolymerize hemicellulose into monosaccharides (Moodley et al., 2020). Furthermore, inorganic salts undergo a hydrolysis reaction that releases hydrogen ions into solution, providing a more stable reactive environment (Liu et al., 2009). Madhu et al. (2014) synthesized activated carbon using banana stem. To activate the process, banana stem powder was heated at 700 °C. To determine the nitrite interference, inorganic salts (KCl, $ZnCl_2$) were added to the samples with no significant response. A high porous surface area of 1465 m^2g^{-1} was observed. Advantages of inorganic salts include their cost-effectiveness, recyclability and minimal release of inhibitor compounds (Moodley and Gueguim Kana, 2017, Moodley et al., 2020).

3.3. Physicochemical pretreatments

Physicochemical methods couple physical changes and chemical reactions. The LCB is pretreated under elevated temperature and/or pressure conditions with chemicals, resulting in degradation of the resistant components. Consequently, the easily degradable LCB structures are accessible for subsequent enzymatic saccharification and microbial fermentation processes. Physicochemical strategies consist of steam explosion, ammonia fibre explosion, CO_2 explosion and liquid hot water, which are highlighted below.

3.3.1 Steam explosion

Steam explosion is a frequently used pretreatment method. Temperature and pressure conditions of 160 to 260°C and 0.69 to 4.83 MPa, respectively, are implemented allowing water

molecules to effectively infiltrate the lignocellulosic biomass structure (Baruah et al., 2018). A sudden reduction in the pressure releases water and causes explosive decompression. More specifically, the elevated temperature and pressure conditions degrade glycosidic bonds present within cellulose and hemicellulose and cleaves bonds between hemicellulose and lignin (Cavalaglio et al., 2016). The steam explosion method boasts several advantages for industrial scale application including minimal effects on the environment, short pretreatment time, low chemical usage and reduced energy consumption, making it cost efficient. Santa-Maria et al. (2013) evaluated steam explosion on banana stem and rachis and demonstrated glucan conversion yields of 93 and 77% for stem and rachis, respectively. Steam explosion was carried out in a 100 L reactor with 150 g solids at 190 to 200 °C. Guerrero et al. (2017) optimized acid-catalysed steam explosion of banana pseudostem for fermentable sugar production. A high glucose yield of 91% was attained using 177 °C, 5 min and 2.2% H_2SO_4 (v/v). Nonetheless, longer pretreatment times at elevated temperature may lead to sugar degradation products such as inhibitor compounds that reduces the downstream process efficiency (Carvalho et al., 2018). While steam explosion is an attractive pretreatment technique, limitations that hinder its advancement include low lignin removal and the formation of inhibitory compounds that usually occur under harsh conditions (higher temperatures and/or longer pretreatment times) (Bhutto et al., 2017).

3.3.2. Ammonia fibre explosion

The ammonia fibre explosion (AFEX) technique employs a liquid ammonia to LCB (1:1) ratio at elevated temperature (60-100 °C) and pressure between 0.7 and 2.7 MPa for a specified period of time (5-30 min) with a subsequent rapid release of pressure (Jędrzejczyk et al., 2019). The processing conditions lead to LCB swelling, which increases the substrate surface area, the hemicellulose conversion to sugars, and alteration of the lignin structure. Advantages of

AFEX methods include the high selectivity of ammonia for reaction with lignin, cost-effectiveness of ammonia, which is easily recoverable and recycled, enhancing the industrial feasibility. Numerous factors affect AFEX pretreatment including temperature, pressure, water and ammonia loading. Isibika et al. (2019) evaluated the pretreatment efficiency of concentrated ammonia (24.5% NH_3) and heat (120 °C) on banana peel waste and obtained a biomass conversion ratio (% VS) of 9.6% and 3.5%, respectively. AFEX methods have emerged as efficient techniques for the pretreatment of biomass, however, bioreactor corrosion and highly concentrated ammonia are costly and therefore reduce the economic feasibility of this pretreatment process towards industrial scale (Jędrzejczyk et al., 2019).

3.3.3. CO_2 explosion

Supercritical CO_2 exhibits mass transfer characteristics with both gas-like and liquid-like solvating power, which permits its diffusion across interphases (Lachos-Perez et al., 2017). Supercritical CO_2 infiltrates LCB and fragments hemicellulose and lignin structures upon pressure release and enhancing cellulose accessibility (Capolupo and Faraco, 2016). Benefits of CO_2 explosion pretreatment include the low-cost of CO_2, minimal effect on the environment, lack of inhibitor formation and ease of recovery that justifies its application at an industrial scale. Jiang et al. (2022) investigated a high-pressure CO_2-hydrothermal pretreatment to banana pseudostem for value-added carbons. The pretreatment process resulted in a 1340% yield of activated carbon using a temperature in the range of 120 to 280 °C at 5 MPa, 700 rpm for 5 min.

3.3.4. Liquid hot water (LHW)

Similar to steam explosion, liquid hot water pretreatment employs water at high temperature (140-230°C) and pressure (up to 5 MPa) conditions but in the liquid phase (Baruah et al., 2018,

Jędrzejczyk et al., 2019). Liquid hot water causes partial hemicellulose solubilisation, dissolution of lignin and reduces the biomass structure (Jędrzejczyk et al., 2019). More specifically, liquid hot water cleaves acetyl groups in hemicellulose moieties and also causes the removal of lignin, which exposes the cellulose fibres to enzymatic hydrolysis. Advantages of liquid hot water pretreatment include the formation of little to no toxic compounds and the non-requirement of chemical catalysts. Also, the biomass size does not affect the process because the particles are fragmented using the pretreatment (Bhutto et al., 2017). In a previous report on a liquid hot water pretreatment of banana pseudostem, a cellulose availability of 25% was obtained resulting in 21.8 g/L of glucose (Legodi et al., 2021). This was achieved using a solid loading of 15% w/v, heated using an autoclave at 121 °C, 15 psi for 1 h. Similarly, the study by Santa-Maria et al. (2013) assessed LHW pretreatment on banana rachis and observed an 80% glucose yield in 44 h using 27 FPU/g glucan supplemented with β-glucosidase and xylanase. Even though liquid hot water offers several advantages, it is an energy intensive process requiring large quantities of water (Baruah et al., 2018).

3.4. Biological pretreatment

Biological pretreatments are usually performed by either isolating enzymes from microbes or cultivating the cells directly on the lignocellulosic substrates. Generally, biological pretreatment methods have been shown to be selective, do not require the use of auxiliary chemicals and the energy input is relatively low. Microbial-based or whole cell pretreatment utilizes bacteria or fungi containing enzymes for biological pretreatment. Two different extracellular enzyme types are present within microbes, known as hydrolytic and ligninolytic systems (Wagner et al., 2018). The hydrolytic enzyme system disrupts cellulose and hemicellulose, whereas the ligninolytic type removes lignin macromolecules. White-rot fungi are most commonly used, due to the higher lignin degradation efficiency and enzymatic

digestibility compared to the latter two. Examples of hydrolytic enzymes produced by white-rot fungi include cellulases and xylanases that hydrolyse cellulose and hemicellulose, respectively. Contrary to fungal pretreatment, limited studies are available using bacteria, since only few bacterial strains have demonstrated lignin-degrading ability. Bacteria metabolize and depolymerize lignin releasing high molecular weight metabolites such as organic acids (Chen and Nielsen, 2016). Baig (2005) treated banana pseudostem using a wild strain of *Trichoderma lignorum* to determine the amounts of enzymes produced. The three enzymes that were produced comprised of 0.18 U/mL FPase, 0.35 U/mL CMCase and 0.21 U/mL β-glucosidase. Reaction conditions included an optimum pH that ranged between 5.6 to 5.8 and an optimal temperature of 45 °C. More recently, Islamiyati and Asriany (2020) investigated the nutritive value of banana stem treated using *Trichoderma sp.* The banana stem was inoculated with 2.5, 5 and 7.5% inoculum for 2 weeks. The authors concluded that the higher levels of *Trichoderma sp.* increased the content of crude protein from 4.11 to 4.93% (Islamiyati and Asriany, 2020). Even with the advantage of selectivity when using biological pretreatments, major bottlenecks include the comparatively low sugar recovery due to the low hydrolysis rate and the long incubation times that range between 7 and 60 days (Maurya et al., 2015). Other limitations in fungi and bacterial lignin degradation occur as a result of: (1) strong and stable carbon-carbon and ether bonds that exist within the large lignin polymers that require oxidative systems for degradation and (2) simultaneously degrading lignin and sugar monomers, which leads to carbohydrates losses (Martinez et al., 2004).

3.5. Combined pretreatment strategies

Pretreatment methods have advanced drastically over time, with many studies employing combined methods that incorporate physical, chemical and/or biological strategies for the enhancement of lignocellulosic degradation towards enzymatic hydrolysis. Combined

pretreatment methods are able to effectively penetrate the LCB structure with several different effects on the biomass structure. Costa et al. (2018) determined the potential effect of a combined (acid-organosolv) pretreatment on banana rachis for fermentable sugar production. Glacial acetic acid and acetone were combined and applied to the substrate for 30 min at boiling temperature for the attainment of 16.6% glucose. In addition, the same authors investigated combined electro chemical activated solution using hypochlorous acid (electrolyzed 5 g/L NaCl) and ethanol (96%) and observed a higher glucose content of 48.7% when compared to the previous combined pretreatment. Combined pretreatments are rapidly researched due to the various advantages of effective degradation of the lignocellulosic structures. Nevertheless, the high cost, release of high concentration of inhibitor compounds, energy consumption, and complexity of downstream processing significantly reduce their feasibility (Sewsynker-Sukai and Gueguim Kana, 2018, Moodley et al., 2020). The development of waste-based combined pretreatment methods is highly sought after to improve the industrial feasibility (Sewsynker-Sukai et al., 2020).

Table 3. The mode of action of various pretreatment strategies in combination with their advantages and disadvantages.

Pretreatment classification	Pretreatment method	Mechanism of action	Advantages	Disadvantages	References
Physical	Mechanical	Reduces substrate particle size and increases the substrate surface area and bulk density	Minimum inhibitor formation Eliminates mass and heat transfer limitations Increased surface area No use of chemicals Manage large biomass quantities	High energy requirement High maintenance costs Requires an additional pretreatment stage Not adequate as a single pretreatment	Yusuf and Inambao (2019)
	Microwave irradiation	Uses dielectric polarization that causes molecular collisions to generate thermal energy, leading to breakage of the complex lignocellulosic structures	Short residence time Rapid heat transfer Easy operation and controlled heating Environmentally friendly process Minimum formation of inhibitors Low-cost	Non-uniform heating Low penetration of irradiation energy in bulk materials, restricts its industrial scale application.	Aguilar-Reynosa et al. (2017)
	Ultrasound	Degrades the lignocellulosic material by creating shear forces that disrupt the intricate network structures of LCB	Short reaction time Low temperatures Low concentration of chemicals that are used	Energy intensive Further research is required to determine the optimum conditions for industrial scale application	Baruah et al. (2018), (Contreras-Hernández et al., 2018)
Chemical	Acid	Hydrolyses hemicellulose, thus making cellulose more accessible to enzymatic treatment	Removal of lignin and hemicellulose Dilute acid is more eco-friendly than concentrated acid, generating less inhibitors Flexible substrate choice	Expensive chemicals High quantities of inhibitor compounds High corrosivity of equipment	Hernández Beltrán et al. (2019), Fatma et al. (2018)

Table 3. Continued.

Pretreatment classification	Pretreatment method	Mechanism of action	Advantages	Disadvantages	References
			High fermentable sugar yield at low temperatures	Requires specialized equipment	
	Alkali	Intermolecular saponification of ester bonds present within lignocellulosic biomass, improvement of the internal substrate surface area, reduces cellulose crystallinity and the degree of polymerization	Solubilizes hemicellulose and lignin Decreases polymerization and crystallinity High sugar yield High pretreatment efficiency Low energy usage Minimal inhibitor compound generation	Chemicals are expensive Large amount of fresh water required for washing Downstream recovery of the chemicals is troublesome Further experimentation is imperative	Kucharska et al. (2018), Qing et al. (2016)
	Solvent-dependent	Causes hemicellulose solubilization and lignin removal	Separates three different fractions consisting of dry lignin, an aqueous hemicellulose stream, and pure cellulose fraction. Generates high-quality lignin	Solvent may act as an inhibitor during hydrolysis reactions May be involved in side-reactions, thus, making its removal difficult prior to enzymatic hydrolysis	Kim and Yoo (2021), Hou et al. (2017)
	Ionic liquids	Pretreatment mechanism of ionic liquids is thought to be because of the dissolution of cellulose. The mechanism of action of ionic liquids is not entirely understood	High solvating properties Selectively cleave glycosidic bonds in polysaccharides	Industrial scale applicability is challenged Release of inhibitor compounds High costs	Raj et al. (2018), Xu et al. (2017), Kucharska et al. (2018)

Table 3. Continued.

Pretreatment classification	Pretreatment method	Mechanism of action	Advantages	Disadvantages	References
	Inorganic salt	Generate metal cations that act as Lewis acids and cleave glycosidic linkages within lignocellulosic structures	Solubilizes hemicellulose Less corrosive Efficient at mild temperatures Low production of inhibitors Cost-effectiveness Recyclability	Expensive lab-based chemicals Partial disruption of lignocellulosic structures Low sugar yield	Loow et al. (2015), Liu et al. (2009)
Physico-chemical	Steam explosion	Degrades glycosidic bonds within cellulose and hemicellulose and cleaves bonds between hemicellulose and lignin	Minimal effects on the environment Short pretreatment time Low chemical usage Reduced energy consumption	Low lignin removal The release of inhibitory compounds Occurs under harsh conditions (high temperatures and/or long pretreatment times)	Bhutto et al. (2017), Carvalho et al. (2018)
	Ammonia fibre explosion (AFEX)	Leads to LCB swelling, which increases the substrate surface area, the hemicellulose conversion to sugars, and alteration of the lignin structure	High selectivity of ammonia for reaction with lignin Ammonia is cost-effective Easily recovered and recycled Enhances the industrial feasibility	Corrosive reaction requires specialized equipment Ammonia is expensive Recycling processes is energy intensive costly Ammonia poses safety and environmental concerns	Jędrzejczyk et al. (2019), Flores-Gómez et al. (2018)
	CO_2 explosion	Fragments hemicellulose and lignin structures upon pressure release, and enhancing cellulose accessibility	Low-cost of CO_2 Minimal effect on the environment Lack of inhibitor formation Ease of recovery enhances industrial scale potential	High capital investment due to high-pressure conditions (13.8 MPa)	Yin et al. (2014), Capolupo and Faraco (2016)

Table 3. Continued.

Pretreatment classification	Pretreatment method	Mechanism of action	Advantages	Disadvantages	References
	Liquid hot water	Cleaves acetyl groups in hemicellulose moieties and also causes the removal of lignin, which exposes the cellulose fibres to enzymatic hydrolysis	Little to no toxic compounds Does not require auxiliary catalysts Biomass size does not affect the process because the particles are fragmented using the pretreatment	Energy intensive process attributable to the excessive quantities of water that are required	Bhutto et al. (2017), Baruah et al. (2018)
Biological	Microbial or whole cell	The hydrolytic enzyme system within microorganisms disrupts cellulose and hemicellulose, whereas the ligninolytic type degrades lignin macromolecules	Selective Does not require the use of chemicals Energy input is relatively low No release of inhibitor compounds Easy downstream processing due to the lack of chemical use	Low sugar recovery and long incubation times White-rot fungi presents limitations in lignin degradation Simultaneous degradation of lignin and sugar monomers lead to carbohydrates losses	Wagner et al. (2018), Martinez et al. (2004)
Combined	Physical, chemical and/or biological strategies combined	Effectively penetrate the LCB structure with several different effects on the biomass structure	High pretreatment efficiency High sugar release	High cost Release of high concentration of inhibitor compounds Energy intensive Difficult downstream processing	Sewsynker-Sukai and Gueguim Kana (2018), Moodley et al. (2020)

Table 4. The application of diverse pretreatment strategies on banana pseudostem and similar derivatives of the banana plant.

Pretreatment method	Lignocellulosic substrate	Pretreatment parameters	Output	References
Mechanical	Banana pseudostem	Chopped and dried at 30 °C for 6 h, sieved through 60-mesh screen and oven dried at 105 °C for 24 h	1340% yield of activated carbon	Jiang et al. (2022)
	Banana stem	Cut into 1 cm sections, air dried and stored at -20 °C.	30.07% of cellulose, 10.65% hemicellulose and 17.10% lignin	Zhang et al. (2013)
Microwave irradiation	Banana waste	5% H_2SO_4, 25 min at 700 W	36.84% reducing sugar	Gabhane et al. (2014)
	Banana pseudostem	2% NaOH, 170 W, 10 minutes	300 mL bioethanol yield	Egwim and Shittu (2015)
Ultrasound	Banana pseudostem	50 °C for 60 min	41.98% reducing sugar	Gabhane et al. (2014)
Acid	Banana pseudostem	H_2SO_4 concentrations (5, 10, 15, 20, 25, 30, 35 and 40%)	60 to 80% glucose yield	Shimizu et al. (2018)
	Banana pseudostem	5% (v/v) H_2SO_4, 10 FPU/g, 50 °C	21.8 g/L glucose	Legodi et al. (2021)
Alkali	Banana stem	6% NaOH, 36 °C	232.4 mL/g biomethane yield	Zhang et al. (2013)
	Banana pseudostem	0.5 to 2 N NaOH, 90 min, 121 °C	29.8 g/L sugar yield	Sukmaningtyas (2018)
Ionic liquid	Banana stem	1 g substrate, 19 mL 1-Allyl-3-methylimidazolium chloride, 80 to 90 °C, 200 rpm, 3 to 4 h	23.1 µm translucent cellulose film	Ai et al. (2021)

Table 4. Continued.

Pretreatment method	Lignocellulosic substrate	Pretreatment parameters	Output	References
Inorganic salt	Banana stem	10 g substrate, 0.1 M KCl, $ZnCl_2$, 700 °C, 2 h	High porous surface area of 1465 m^2g^{-1}	Madhu et al. (2014)
Steam explosion	Banana stem	100 L reactor, 150 g solids, 190 to 200 °C	93% glucan conversion yield	Santa-Maria et al. (2013)
	Banana pseudostem	2.2% H_2SO_4, 5 min, 177 °C	91% glucose yield	Guerrero et al. (2017)
Ammonia fibre explosion	Banana peel waste	24.5% NH_3, 120 °C	9.6% and 3.5% biomass conversion yield	Isibika et al. (2019)
CO_2 explosion	Banana pseudostem	120 to 280 °C at 5 MPa, 700 rpm for 5 min	1340% yield of activated carbon	Jiang et al. (2022)
Liquid hot water	Banana pseudostem	Solid loading of 15% w/v, 121 °C, 15 psi for 1 h	21.8 g/L glucose	Legodi et al. (2021)
	Banana rachis	44 h, 27 FPU/g glucan supplemented with β-glucosidase and xylanase	80% glucose yield	Santa-Maria et al. (2013)
Biological pretreatment	Banana pseudostem	*Trichoderma lignorum*, pH 5.6 to 5.8, 45 °C	0.18 U/mL FPase, 0.35 U/mL CMCase and 0.21 U/mL β-glucosidase	Baig (2005)
	Banana stem	*Trichoderma sp.*, 2.5, 5 and 7.5% inoculum, 2 weeks	4.93% crude protein	Islamiyati and Asriany (2020)
Combined pretreatment	Banana rachis	Glacial acetic acid, acetone, 30 min	16.6% glucose	Costa et al. (2018)
	Banana rachis	Hypochlorous acid (electrolyzed 5 g/L NaCl) and ethanol (96%)	48.7% glucose	Costa et al. (2018)

4. Lignocellulosic bioprocess types

A lignocellulosic bioprocess is based on the utilization of lignocellulosic biomass to produce marketable products such as bio-based fuels and chemicals via chemical and biochemical routes (Konwar et al., 2018). Pretreatment is one step that is necessary to improve the accessibility of polysaccharides, making it amenable to enzymatic hydrolysis for subsequent bioprocessing. The fermentation of LCB to valuable bio-based products can be achieved by using different bioprocess types that include separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF) or SSF with a short separate hydrolysis step (PSSF) (Dashtban et al., 2009). In SHF processes, enzymatic hydrolysis and fermentation are performed in separate vessels under specific reaction conditions (Fatma et al., 2018). An advantage of this approach is that specific processes can operate at their optimum setpoints (i.e., optimal temperature ranges between 45 to 50 °C for hydrolysis, whereas it is 30-37°C for fermentation) (Dashtban et al., 2009). However, the main problems associated with SHF are end-product inhibition of enzymes, increased costs, and higher risk of contamination (Dashtban et al., 2009). Major drawbacks of the SHF process may be overcome by the development of the SSF approach in which hydrolysis and fermentation both occur simultaneously in a single vessel (da Silva et al., 2018). The integration of enzymatic hydrolysis and fermentation decreases the number of unit operations, thus saving processing costs and production time. Additionally, the direct conversion of sugars to fermentation products eliminates issues such as sugar accumulation, enzyme inhibition, and the risk for contamination (Öhgren et al., 2007, Mithra et al., 2018). A variation of the SSF configuration is the pre-hydrolysis and simultaneous saccharification and fermentation (PSSF) process. In the PSSF process, the substrate is subjected to a pre-hydrolysis step for short time at an optimum temperature for cellulolytic enzymes (50 °C), following this, the temperature of the bioreactor is lowered for inoculation of the microorganism (Paschos et al., 2020, Carrillo-Nieves et al., 2017). The main

advantage of PSSF over SHF is that it simplifies the process by avoiding the separation of the slurry before fermentation, thus saving processing time and energy. When compared to the SSF configuration, the rate of enzymatic hydrolysis is not reduced by the suboptimal temperature and the production rate is not limited by the low concentration of carbon source (Morales-Martínez et al., 2017). In addition, PSSF is performed at its optimum temperature stimulating the release of sugar and lowering the substrate viscosity (Carrillo-Nieves et al., 2017). In the recent years, the application of SSF processes has been recommended owing to its versatility and ease of applicability in large scale biorefineries. Furthermore, in the SSF process, the enzyme and microorganism are in a synergy in a single step resulting in the immediate conversion of lignocellulosic biomass to valuable products.

4.1. Development of valuable bio-based products

The global demand for organic acid has exceeded the natural supply, hence sustainable biotechnological fermentation processes have become imperative (Odu et al., 2020). Industrially important organic acids include citric acid, lactic acid and itaconic acid (Chen and Nielsen, 2016). Citric acid is one of the most important organic acids with 2 million tons commercially produced annually (Aboyeji et al., 2020). In comparison, approximately 335 000 tons of lactic acid and 80 000 tons of itaconic acid are generated each year (Saha et al., 2019). Citric acid is an essential building block of cellular respiration that is incorporated into the food, beverage, pharmaceutical, chemical, cosmetic and textile industries (Sarkar and Das, 2017, Sawant, 2018). In addition, lactic acid is a precursor for commercially important polylactic acid, polylactide (biopolymer), and ethyl lactate, a bio-based solvent (Konwar et al., 2018). Itaconic acid can be used as a co-monomer of thermoplastics, detergents, acrylic polymers, surfactants and polyester resins (El-Imam and Du, 2014). Within industry, citric acid, lactic acid and itaconic acid are mainly produced by microorganisms such as *Aspergillus*

niger, *Lactobacillus sp*., and *Aspergillus terreus*, respectively (Aboyeji et al., 2020, Idrees et al., 2013, Kolláth et al., 2019). The production pathway also known as the tricarboxylic acid cycle (TCA) generates these microbial metabolites. Several studies have demonstrated the production of these bio-based products from various lignocellulosic substrates (See supplementary material), however, banana pseudostem is an abundant, perennial and renewable lignocellulosic waste, rich in cellulose and low lignin content that renders it feasible for the bioconversion to value-added products (Narinthorn et al., 2019, Shimizu et al., 2018). Additionally, its physicochemical properties allow for low-cost and innovative pretreatment and fermentation technologies that may provide financial sustenance for adjacent sectors. Furthermore, the utilization of BP will combat the implications that are associated with its disposal (airborne pathogens and environmental pollution).

Even with the availability of these studies, a number of experimental conditions must be considered to improve organic acid production by modelling, optimization and kinetic assessment. Due to the complex dynamics of the biorefinery process, there is a dearth of knowledge for understanding the bioprocess and its product output. Mathematical kinetic models are useful for providing knowledge on the metabolic aspects of a fermentation process (Germec et al., 2019). In addition, kinetic models play an essential role in simulating, optimizing, monitoring and predicting the performance of a process under varying parameter conditions (Pramanik et al., 2019). Furthermore, kinetic modelling allows for increased product yield and productivity, to ensure high product quality while reducing the formation of unwanted by-products and saving processing time and costs.

5. Current challenges and future prospectives

5.1. Lignocellulosic biomass selection

The commercial and industrial sectors are seeking novel, cost-effective, and economical biorefinery technologies to keep up with the current adverse market conditions (Raj et al., 2022). In light of this, a cost reduction can be achieved by utilizing less expensive feedstocks such as agro-industrial substrates (lignocellulosic biomass) and their derivatives. However, the biomass recalcitrance of lignocellulosic biomass renders it to be chemically complex in nature and to resist the release of fermentable sugars, thus necessitating pretreatment to overcome these rheological properties and ensure adequate cellulose and hemicellulose availability (Chen and Nielsen, 2016). In addition, the usage of food-based agricultural feedstocks threatens global food security and the economy. Hence, the application of non-food-based feedstocks are highly sought after. Large quantities of banana pseudostem waste end up in land and water bodies, thus occupying space and contributing to adverse environmental impacts when it is incinerated. Banana pseudostem does not coincide with food security and its utilization will minimize the release of detrimental greenhouse gases into the environment. The high cellulosic (42-60%) and hemicellulosic (19-23%) content of banana pseudostem makes it a suitable feedstock for the generation of valuable microbial bio-based products (Islam et al., 2019, Shimizu et al., 2018). Bananas are cultivated in over 135 countries throughout tropical and subtropical climates (Magdama et al., 2020). Furthermore, unlike other LCB substrates from seasonal-dependant crops, bananas are available all year round (Subagyo and Chafidz, 2018). Therefore, the cultivation and harvesting of bananas (along with the BP) is steady with a year-round supply, that is crucial for effective functioning of lignocellulosic bioprocessing operations.

5.2. Development of cost-effective and/or waste-based pretreatments

A severe challenge of lignocellulosic bioconversion is the pretreatment costs incurred due to the energy, chemicals and fresh water consumption. Numerous pretreatment technologies

require fresh water and organic solvents in at least one of the stages. Conventional pretreatment methods utilize expensive solvents that are volatile and potentially toxic resulting in low sugar yields. Moreover, the disposal of spent pretreatment chemicals is problematic and costly, while commonly used acidic chemicals tend to corrode the bioreactor (Hernández Beltrán et al., 2019). On the other hand, the Kraft paper and pulp industry produces high quantities of waste products that include, mesa lime, green liquor dregs (GLD), paper wastewater (PWW), fibre sludge and fly ash (Mäkitalo et al., 2014), that have emerged as a potential solution to chemical-based pretreatments. Some of these by-products have been employed for lignocellulosic pretreatment and are summarized (See supplementary material). More specifically, the Kraft paper and pulp industry is one of the third largest producers of paper wastewater (PWW) after primary metals and chemical industries (Ashrafi et al., 2015). PWW contains effluent solids, sediments, absorbable organic halides and chlorinated organic compounds that contribute majorly to its high pH and thus alkaline nature (Tobin et al., 2020). Main challenges encountered by the Kraft industry include paper wastewater accumulation and treatment costs that are incurred approximating to a chemical cost of $2.09 USD per cubic meter of wastewater treated. In addition, activated-carbon treatment costs range between $1 to $3 USD/$m^3$ for the treatment of pulp and paper mill wastewater (Mehmood et al., 2019). Recently, Kraft paper wastewater and table salt have garnered significant attention as effective pretreatment technologies instead of commonly used alkaline and inorganic salt, respectively. Unlike lab-based NaCl, iodized table salt is a cheaper alternative. This salt is an ionic complex composed of sodium and chloride ions with the chemical composition of NaCl (Carapeto et al., 2018). Inorganic salts function by dissociating Lewis acids in an aqueous solvent to form hydrated complex cations surrounded by water molecules. These water molecules further act as nucleophiles, thus depolymerizing hemicellulose into monosaccharides (Moodley et al., 2020). In the same vein, the utilization of Kraft paper wastewater to treat lignocellulosic waste

instead of alkaline chemicals such as NaOH provides an economically viable pretreatment option. The alkaline properties of PWW allow it to cleave the ester linkages of lignin and the cross-linkages between hemicellulose and lignin (Saratale et al., 2016). Furthermore, the degradation and removal of lignin moieties increases the solubility of LCB and therefore improves the accessibility of cellulose to enzymatic hydrolysis (Saratale et al., 2016, Gu et al., 2012). The properties of paper wastewater enable it to function as a dual-purpose commodity in pretreatment processes as it exhibits characteristics as an attractive replacement for: (1) fresh water usage within lignocellulosic bioprocessing systems and (2) common and expensive alkaline reagents. Reusing PWW for lignocellulosic pretreatment could potentially generate profits that may then be utilized for biohazardous treatment and management, ultimately reducing the cost of downstream processing for Kraft industries.

Research has shown that exposure of pretreated lignocellulosic polysaccharides to cellulases for enzymatic saccharification processes at a reduced cost is of a great importance (Phitsuwan et al., 2016). However, this can only be effectively achieved through pretreatment strategies that increase the biomass polymers' reactive surface area and porosity for penetration by enzymes. Furthermore, reduced cellulose crystallinity and low lignin content are desirable (Phitsuwan et al., 2016, Raj et al., 2022).

5.3. Lignocellulosic bioprocess optimization

The formation of a valuable product necessitates a specific set of parameter ranges for the optimization of the bioprocess. The addition of a desorbent, enzyme, inoculum, and substrate solid loading may be utilized in the bioprocess but require optimization for optimal fermentation and product formation. For example, in citric acid production, nitrogen is a key requirement, therefore it is essential to determine an alternative to expensive lab-based derivatives by using low-cost nitrogen sources. In addition to nitrogen, desorbents have been

shown to enhance citric acid production by improving the cell permeability to citrate which enables the cell to respond to the diminished intracellular level by increasing citric acid production (Sekoai et al., 2018). Temperature is one of the most influential factors in lignocellulosic citric acid production. This is attributed to the presence of the cellulase-based enzyme and fungal culture. It is therefore advantageous to employ the SSF process to ensure the simultaneous conversion of glucose to a valuable microbial based bioproduct. This process is currently preferred over conventional SHF and PSSF due to the minimization of contamination, energy, time, resources and processing costs. Additionally, the substitution of wastewater as a water source during the SSF bioprocess could prove valuable from an environmental, financial, and social perspective. The dairy sector contributes to a financially stable economy that generates significant amounts of wastewater and may be channelled towards lignocellulosic bioprocesses. Furthermore, mathematical models play a vital role in optimizing, predicting, simulating, and monitoring the performance of a process under varying process conditions (Pramanik et al., 2019). The interactive effects of input parameters and their corresponding output are analyzed using the response surface methodology model. Despite advancements in the process optimization, there needs to be further research attempted to enhance the ability to recover and reuse spent chemicals (paper wastewater) and by-products (dairy wastewater) from the Kraft and dairy industry, respectively. These would be a valuable substitute for large amounts of finite fresh water to eliminate its usage from lignocellulosic bioprocesses and improve the overall cost-efficiency. Moreover, the implementation of mathematical models provides a strong foundation for process design, control and optimization, which may reduce the challenges faced during lignocellulosic bioprocessing (Linville et al., 2013).

6. Conclusion

This review highlights banana pseudostem (BP) as a potential renewable resource for producing valuable microbial by-products. The different pretreatments on BP with its advantages and limitations are discussed. Furthermore, the various bioprocess types and use of mathematical kinetic models are outlined. The challenges faced by lignocellulosic bioprocesses and future perspective on energy efficient and cost-competitive technologies during pretreatment, enzymatic hydrolysis and microbial fermentation has been presented. The development of these technologies could potentially transform the face of these systems, aligned with global sustainable goals regarding the water-food-energy nexus for a circular bioeconomy approach.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version.

Credit authorship contribution statement

Milesh Laltha: Conceptualization, Investigation, Writing - original draft, Writing - review & editing, Visualization, Project administration. **Y. Sewsynker-Sukai:** Conceptualization, Writing – review & editing, Project administration, Supervision. **E.L. Meyer:** Writing – review & editing**. E.B. Gueguim Kana:** Writing – review & editing, Supervision.

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Chapter 2 - Supplementary material

Table S1. Optimal fermentation conditions and concentration of citric acid, itaconic acid and lactic acid production from various studies.

Organic acid	Substrate	Fermentative microorganism	Optimal Parameters	Concentration	References
Citric acid	Banana peel	*A. niger*	30 °C, pH 4, 5% glucose (w/v), 2% zinc (w/v), 0.5% ammonium chloride (w/v), 3% methanol (v/v), 10 days	97.6 g/L	Odu et al. (2020)
	Pineapple waste	*A. niger*	30 °C, 15% sucrose (w/v), 0.25% ammonium nitrate (w/v), 4% methanol (v/v), 7 days	44.1 g/L	Sarkar and Das (2017)
	Sweet potato peel waste	*A. niger*	25 °C , 97.25% carbon source, 1.25% nitrogen (w/v), 7 days, pH 6.5	4.36 g/L	Aboyeji et al. (2020)
	Apple pomace	*A. niger*	32.88 °C, 33.81 g/L Apple pomace, 42.5 g/L of corn steep liquor (CSL), 2.05% methanol (v/v), 33 h, pH 4.54	62-68 g/L	Sekoai et al. (2018)
Itaconic acid	Jatropha seed cake	*A. terreus*	~30 °C, pH 1.5, 5 mL inoculum, 50% substrate, 400 rpm, 9 days	48.7 g/L	Amina et al. (2013)
	Glucose media	*A. terreus*	100 g/L substrate conc., pH 4.5, 120 hr	30.2 g/L	Vassilev et al. (2012)
	Olive and beet waste	*A. terreus*	30 °C, 2 mL inoculum, pH 5.5, 70% humidity	44 g/L	Nikolay et al. (2013)
	Jatropha cake	*A. terreus*	32 °C, 10% inoculum, pH 3, 200 rpm, 120 hr	24.45 g/L	Rao et al. (2006)

Table S1. Continued.

Organic acid	Substrate	Fermentative microorganism	Optimal Parameters	Concentration	References
Lactic acid	Corn stover	*Bacillus coagulans*	10 g/L sodium acetate, 5 g/L levulinic acid, 2 g/L monophenol, 30% solid loading, 24 hr	35.8 g/L	Zou et al. (2021)
	Sugarcane bagasse	*Lactobacillus casei*	37 °C, 10% inoculum (v/v), 35 rpm, 120 hr	21.3 g/L	Oonkhanond et al. (2017)
	Sugarcane bagasse	*Clostridium beijerinckii*	35 °C, 10% inoculum (v/v), 35 rpm, 96 hr, pH 6	13 g/L	Vieira et al. (2021)
	Corn cobs	*Lactobacillus plantarum*	37 °C, 25 mL DWW citrate buffer, 10% pretreated substrate, 25 g/L CSL, 2 mL/L Tween 80, 10% inoculum (v/v)	11.15 g/L	(David et al., 2022)

Table S2. Pretreatment applications of waste by-products obtained from the Kraft paper and pulp industry.

Lignocellulosic substrate	Waste by-product	Pretreatment parameters	Output	References
Oil palm	Black liquor	500 g substrate, 4 bar pressure, 2.5 L pretreatment solution, 60 g NaOH, 160 °C, 40 min	137.9 g/L glucose	Triwahyuni et al. (2015)
Corn stover	Black liquor	80 °C, 2 h, 30 kg black liquor with 0.165 kg NaOH, 3 kg substrate	68-78% glucose yield, 60 g/L ethanol	Chen et al. (2018)
Corn stover	Black liquor	2 g substrate, 30 mL of 2% NaOH, 150 rpm, 80 °C, 2 h	81.53% cellulolytic conversion, 35.41% hemicellulose conversion	Zhou et al. (2019)
Corn stover	Green liquor	8% Alkali, 40% sulfidity, 140 °C	70% pulp yield, 45% lignin removal	Gu et al. (2012)
Poplar	Green liquor	Liquor ratio 1:4, 1.5L/kg green liquor, 100 °C, 90 min	13.81% pentosan extracted	Li et al. (2013)
Rice waste	Green liquor	Liquor ratio 1:1 (10%), 100 °C, 6 h	58.2% delignification, 88% glucan yield, 545 mg/g total sugar	Saratale et al. (2016)
Bamboo	Green liquor	20% titratable alkali, 25% sulfidity, 4 ml/g ratio, 150 °C, 1 h	48.5% maximum sugar yield	Wang et al. (2014)
Corn cobs	Green liquor dregs	80% GLD, 20% S/L, ~50 min	0.42 g/g glucose yield 23.69 g/L ethanol	David et al. (2020)
Paper mill sludge	Green liquor dregs	56% GLD, 44% substrate, 4.5% Tween 80, 60 min, and S:L of 9.5%, 120 °C	16.38 g/L reducing sugar, 3.72 mL/g hydrogen	Rorke et al. (2021)

Table S2. Continued.

Lignocellulosic substrate	Waste by-product	Pretreatment parameters	Output	References
Corn cobs	Green liquor dregs + paper wastewater	49.89% GLD, 118 °C, 5 min	1.53 g/g reducing sugar yield, 0.85 g/g glucose yield	David et al. (2021)

CHAPTER 3

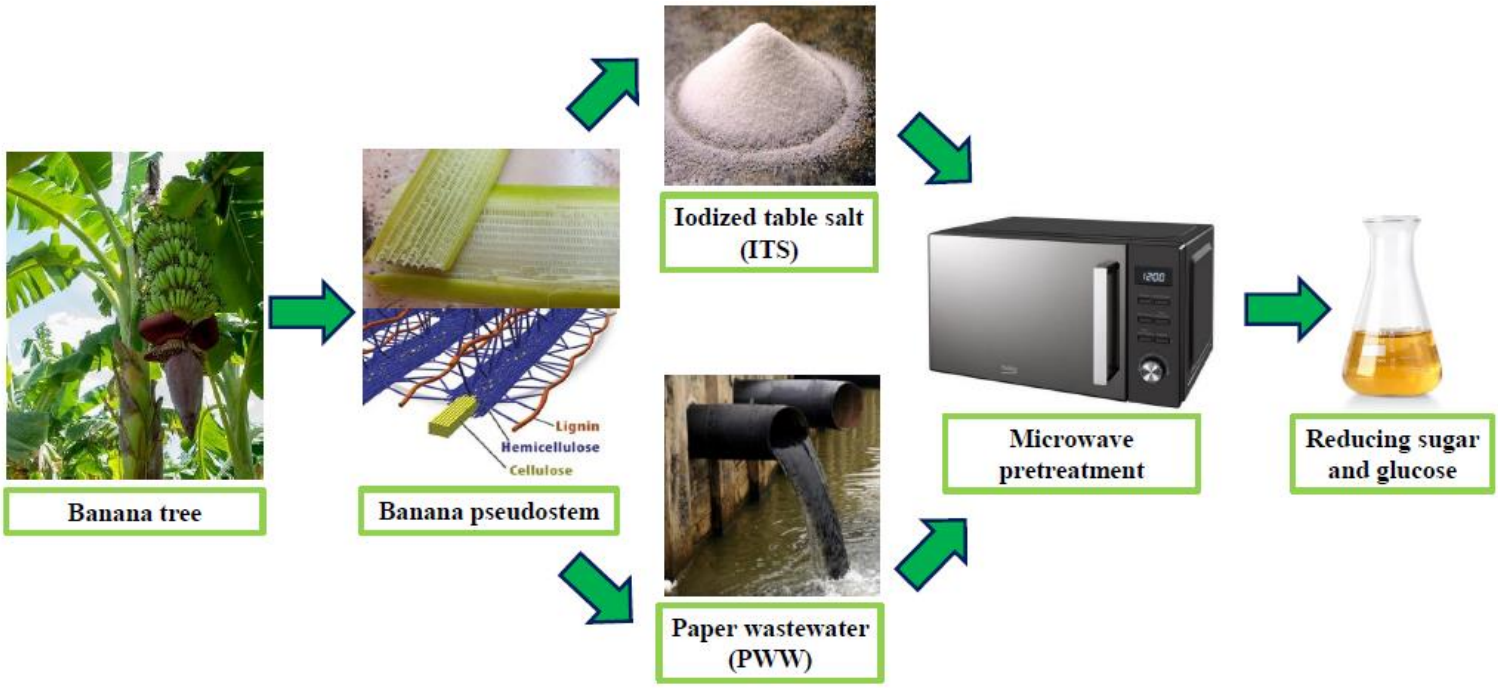
Innovative microwave-assisted iodized table salt or paper wastewater pretreatments for enhanced sugar recovery from banana pseudostem

This chapter has been published in the journal Biomass Conversion and Biorefinery entitled: Innovative microwave-assisted iodized table salt or paper wastewater pretreatments for enhanced sugar recovery from banana pseudostem. *Biomass Conversion and Biorefinery*. DOI: https://doi.org/10.1007/s13399-022-02630-x. The published paper and supplementary material are presented in the following pages.

Highlights

- First report on table salt or paper wastewater for lignocellulosic pretreatment.
- Salt and paper wastewater revealed comparable sugar yields to expensive chemicals.
- Applicability on various lignocellulosic substrates was demonstrated.
- Paves the way towards negating chemicals and/or fresh water in pretreatment.

Graphical Abstract



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ORIGINAL ARTICLE

Innovative microwave-assisted iodized table salt or paper wastewater pretreatments for enhanced sugar recovery from banana pseudostem

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Abstract
Iodized table salt and Kraft paper and pulp wastewater represent potential, cost-effective lignocellulosic pretreatment catalysts. Therefore, to determine their pretreatment applicability, the present study modelled and optimized two novel methods, namely (1) microwave-assisted-iodized table salt (M-ITS) and (2) microwave-assisted-paper wastewater (M-PWW) strategy for the enhancement of sugar recovery from banana pseudostem. Pretreatment optimization revealed a slightly higher reducing sugar (0.515 g/g) and glucose yield (0.433 g/g) for the M-ITS strategy compared to the M-PWW method (reducing sugar = 0.498 g/g and glucose = 0.413 g/g). Although both methods displayed comparable sugar yields to previous pretreatment studies that used expensive chemicals, the M-PWW strategy demonstrated a higher efficiency, since it is completely waste-based and requires fewer pretreatment cycles. Major findings from this study paves the way for lignocellulosic pretreatment by potentially negating the use of costly chemicals and/or fresh water, while achieving a "waste treating waste" approach.

Keywords Pretreatment · Lignocellulosic biomass · Iodized table salt · Paper wastewater · Banana pseudostem

1 Introduction

Lignocellulosic biomass (LCB) represents an attractive, abundant, and cost-effective feedstock for the production of bio-based products [1]. These substrates are characterized as a plant waste that may be harnessed for microbial fermentation, since it is a carbon–neutral and an inedible feedstock that does not interfere with food and feed supplies [2]. LCB is a heterogeneous polymer mainly comprised of 30 to 50% cellulose (C_6 sugars), 15 to 35% hemicellulose (C_5 and C_6 sugars), and 10 to 20% lignin, that are bound by covalent and hydrogen bonds, forming lignin-carbohydrate complexes [3]. Other constituents of LCB that are present in lower quantities include organic extractives (phenols, proteins) and inorganic minerals (ash). Some examples of LCB substrates include banana pseudostem [4], corn cobs [5], bamboo [6], and sugarcane leaves [7]. The banana pseudostem (*Musa sp.*) is a lignocellulosic waste that has recently been recognized for its potential as a renewable and sustainable resource, due to its extraordinary growth rate, high productivity yields, and abundance. Approximately 300 million tonnes of banana pseudostem waste is generated annually after harvesting of the banana fruit [8]. In addition, the high cellulosic (14–31%) and low lignin (6–15%) content makes it a potential feedstock for the microbial production of valuable products [8, 9]. Despite the advantages that LCB offers, hemicellulose and cellulose are strongly held together by the recalcitrant lignin structure via hydrogen and covalent bonding [3]. Furthermore, LCB consists of lignin-carbohydrate bonds such as benzyl ethers, esters, phenyl glycosides, and hemiacetal linkages [3]. Depolymerization of these interlinked constituents has proven to be problematic, since it prevents access of cellulase enzymes to the cellulose polymer and subsequently hinders the enzymatic release of fermentable sugars that can be available for microbial fermentation [10]. Pretreatment of LCB is therefore necessary for the disruption of the resistant network, which ultimately removes lignin and reduces the crystalline structure of cellulose and hemicellulose [8]. Following pretreatment, cellulose is enzymatically hydrolyzed to fermentable sugars such as glucose that are subsequently converted to high-value chemical commodities during

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microbial fermentation [1]. Pretreatment methods comprise physical, chemical, physicochemical, biological, or a combination of these approaches [11]. Examples of common pretreatment methods include solvents or catalysts such as acid and alkali [1] and inorganic salt [7]. Effective pretreatment techniques are required to have low energy requirements, use cost-effective chemicals, minimize the formation of enzymatic and fermentation inhibitor compounds, and achieve high fermentable sugar yields [1]. The development of a pretreatment regime that meets these criteria and overcomes the technoeconomic barriers associated with these systems is imperative for the enhancement of its industrial-scale implementation. Recently, numerous studies have utilized inorganic salt as an effective pretreatment approach [12–14], due to their high catalytic activity, thereby increasing cellulosic and hemicellulosic content, for improved hydrolysis towards sugar release [13, 15]. Additionally, inorganic salts are environmentally friendly and do not corrode pretreatment reactors, thereby avoiding the use of highly specialized systems [7]. In a recent report, Suwannabun et al. [14] compared the effectiveness of six different salts (NaCl, $MgCl_2$, $NaNO_3$, KCl, KNO_3, and $Mg(NO_3)_2$) for the pretreatment of rice straw and observed that 0.9% NaCl resulted in the maximum reducing sugar concentration (16.60 mg/ml). Similarly, Moodley and Gueguim Kana [13] assessed three microwave-assisted inorganic salts, namely $FeCl_3$, $ZnCl_2$, and NaCl on sugarcane leaves and obtained maximum reducing sugar yields of 0.406 g/g, 0.241 g/g, and 0.179 g/g, respectively. Likewise, Kang et al. [12] evaluated five different inorganic salts (NaCl, KCl, $CaCl_2$, $ZnCl_2$, and $FeCl_3$) for the pretreatment of *Miscanthus* straw and observed that $FeCl_3$ resulted in the highest xylan removal of 100% and enzymatic digestibly of 63.4% compared to the other salts.

The primary mechanism by which inorganic salts function is the dissociation of Lewis acids in an aqueous solvent to form complex cations. The Lewis acid accepts an electron from the Lewis base to create a Lewis acid–base adduct. Subsequently, six water molecules are formed around the central metal cation with co-ordinate covalent bonds. Glycosidic linkages present in LCB are then cleaved by the formation of metal cations. Metal ions further assist with bond rupture, as a result of their positive charge and thus pair with negatively charged electrons. The water molecules from the hydrated cation then act as nucleophiles that depolymerize hemicellulose into monosaccharides [16]. Although inorganic salts have emerged as efficient chemical catalysts for lignocellulosic pretreatment, the relatively high costs of laboratory-grade salts coupled with the low sugar recovered, remain major limitations of these processes. To overcome these limitations, cheaper alternative chemicals are currently being sought. Sodium chloride (NaCl) has shown to be a promising, cost-effective chemical pretreatment that is available in two forms: (1) laboratory-grade NaCl and (2) food-grade NaCl. The chemical composition and manufacturing process differentiates these salts. Within industry, food-grade or commonly known as table salt, is mined from inland (underground natural brine streams), coastal saltpans (solar and artificial evaporation), and seawater (salt refining) [17]. Most table salts are supplemented with an essential nutrient such as iodine. Conversely, laboratory-grade salt (halite) is present in the Earth's surface and can be extracted through deep-shaft mining. The mined rock salt is thereafter crushed and used for industrial and other non-food applications that include chemical processes, anti-icing (ice prevention), and de-icing (ice removal) applications on roadways [18]. To date, only the application of laboratory-grade NaCl for lignocellulosic pretreatment has been reported [12, 14], and although it has been deemed cost-effective when compared to other salts ($FeCl_3$, KCl and $MgCl_2$), it is an expensive, purified chemical that is not readily available and is usually supplied in small quantities. On the other hand, its impurified, food-grade equivalent, also known as iodized table salt (ITS), is abundantly available and commonly used as a condiment and preservative of food sources, but has not yet been evaluated for lignocellulosic pretreatment. Interestingly, ITS costs approximately 2% of purified, laboratory-grade NaCl. The average cost per kg of laboratory-grade NaCl ranges between $76.06 and $79.49 USD (Merck, South Africa) compared to less than $1.5 (~ $1.24 to $1.38) for the ITS version (local supermarket, South Africa).

While salt pretreatments such as ITS target hemicellulose solubilization, alkaline-based methods have been deemed the gold standard on the pretreatment front and cause disruptions to the recalcitrant aromatic polymer lignin. Alkaline pretreatment methods utilize chemical catalysts that significantly improve sugar yields, use lower energy input, and have been known to generate a low concentration of inhibitor compounds [19]. Examples of alkaline methods include ammonia recycle percolation, ammonia fiber expansion, aqueous and liquid ammonia, strong alkali (NaOH), and lime ($Ca(OH)_2$) that are expensive and therefore not feasible for use in lignocellulosic pretreatment processes [20]. Recently, cheaper alkaline methods such as $Na_3PO_4.12H_2O$ have been assessed in comparison to NaOH [5, 21]. For instance, our recent study investigated NaOH and $Na_3PO_4.12H_2O$ pretreatments and compared the chemical recyclability of these methods to potentially reduce chemicals and water usage to circumvent costs [21]. Although the alkaline spent liquor hydrolysate from both NaOH and $Na_3PO_4.12H_2O$ could be recycled up to three times, the reduced pretreatment efficiency after successive treatments in addition to the initial water and chemical cost still plague this process [21].

Paper wastewater (PPW), an alkaline industrial waste generated in large quantities by the Kraft paper and pulp industry has emerged as a prospective and economically viable pretreatment approach. Kraft paper and pulp mill industries

discharge an estimated 75 to 225 m^3 effluent (constituting PWW) per ton of paper produced [22]. Although the Kraft industry has a major impact on the global economy, it consumes large amounts of fresh water, a finite resource during the paper-making process [23]. Industrially produced wastewater requires energy and cost intensive treatments prior to its expulsion into the environment, since it is detrimental to the ecosystem and poses a severe threat to plant and aquatic life [23]. Striking characteristics of PWW include high organic loading, in addition to the presence of effluent solids, sediments, absorbable organic halides, and chlorinated organic compounds that contribute majorly to its high pH and thus alkaline nature [24]. Hence, PWW can function as a dual-purpose commodity in pretreatment processes as it exhibits characteristics as an attractive replacement for (1) common and expensive alkaline reagents and (2) freshwater usage within lignocellulosic biorefinery systems. Repurposing PWW for lignocellulosic pretreatment could potentially generate profits that may then be utilized for less invasive biohazardous treatment and management, thereby reducing the downstream processing costs for Kraft industries. Both ITS and PWW display significantly cheaper alternatives to their lab-derived counterparts such as NaCl and NaOH, respectively.

In addition, microwave heating processes (MHP) are emerging as an energy-efficient alternative to conventional heating methods such as autoclave and steam [25]. MHP can be used for rapid volumetric heating and exhibits a shorter reaction time and a higher reaction rate compared to steam [26] and autoclave [3] heat treatment. Despite the significant advancements in lignocellulosic pretreatment, techno-economic challenges associated with chemicals, water, and energy are still major obstacles hindering its commercialization. Conventional lignocellulosic pretreatments with alkaline chemicals are expensive compared to PWW, an alkaline industrial waste that is freely available in large quantities and may potentially achieve a "waste treating waste" approach. Furthermore, food-grade NaCl or ITS is easily accessible, abundant, and a cheaper alternative to lab-grade NaCl. The development of these methods could unlock the potential of lignocellulosic pretreatment by releasing high fermentable sugar that can be channelled towards valuable microbial bio-based compounds.

Therefore, the objectives of this study were to (1) model and optimize two novel pretreatments that include a microwave-assisted-iodized table salt (M-ITS) method and microwave-assisted-paper wastewater (M-PWW) strategy for enhanced sugar recovery from banana pseudostem, (2) determine the individual and interactive effects of (i) salt concentration, microwave power intensity, and pretreatment time (M-ITS) and (ii) substrate solid loading, microwave power intensity, and pretreatment time (M-PWW) on the reducing sugar and glucose yields, and (3) evaluate the effectiveness and versatility of the developed M-ITS and M-PWW pretreatments by applying the optimized conditions on several lignocellulosic substrates (corn cobs, sorghum leaves, sugarcane bagasse, and bamboo) with varying structural compositions.

2 Materials and methods

2.1 Materials

The banana pseudostem substrate used in this study was obtained from Bakerville Heights (Pietermaritzburg, South Africa) (29.5418° S, 30.4077° E). The substrate was dried at 75 °C for 48 h in an oven (Scientific series 2000, South Africa) to reduce the remaining moisture content and was thereafter milled to a particle size that ranged from 1 to 2 mm. The powdered banana pseudostem substrate was stored in an airtight container at room temperature. The lignocellulosic composition (cellulose, hemicellulose, and lignin) of the untreated (native) and optimally pretreated banana pseudostem was analyzed using the detergent fiber technique described by Van Soest and McQueen [27]. The chemical reagents were purchased from Merck (South Africa) where applicable. The iodized table salt (ITS) (Cerebos, South Africa) used in this study was obtained from a local supermarket, while the paper wastewater (PWW) with its chemical composition (Table S1) was kindly provided by Mondi (Richards Bay Mill, South Africa). The Cellic CTec 2 enzyme (160 FPU/mL) that was used for the saccharification stage was generously donated by Novozymes (Novozymes A/S, Denmark). Other lignocellulosic wastes that include corn cobs, sorghum leaves, sugarcane bagasse, and bamboo were procured from local agricultural industries. These substrates were dried, milled, and stored as previously stated above.

2.2 Preliminary screening

For the preliminary screening, laboratory-grade sodium chloride (NaCl) salt and food-grade iodized table salt (ITS) were comparatively assessed for hemicellulose solubilization. Conversely, paper wastewater (PWW) generated from the Kraft paper and pulp industry served as the alkaline waste-based pretreatment method that was used for targeting lignin. Lab-grade NaCl, food-grade ITS, or PWW was coupled with microwave-assisted heating and were used individually in a single-stage to compare their effectiveness and were designated as M-$NaCl_{preliminary}$, M-$ITS_{preliminary}$, and M-$PWW_{preliminary}$ respectively. Additionally, two-stage pretreatments consisting of PWW first, followed by either NaCl or ITS were denoted M-PWW-$NaCl_{preliminary}$ and M-PWW-$ITS_{preliminary}$ respectively. The pretreatment

parameter ranges were selected based on an extensive literature review of related pretreatment studies [12–14]. The preliminary screening experiments were carried out with a standard substrate solid loading of 10% (w/v). The M-$NaCl_{preliminary}$ and M-$ITS_{preliminary}$ pretreatments were performed with a salt concentration of 3% (w/v), microwave power intensity of 500 watts (W), and pretreatment time of 6 min. In addition, the same power intensity and time were employed for the M-$PWW_{preliminary}$ pretreatment. The two-stage pretreatments (M-PWW-$NaCl_{preliminary}$ and M-PWW-$ITS_{preliminary}$) were executed at 500 W in the microwave for 3 min per stage, whereby a salt concentration of 3% (w/v) was employed for the second stage. The pretreated banana pseudostem samples were filtered using a domestic sieve (< 1 mm) and washed several times with deionized water. The washed biomass was subsequently oven-dried at 55 °C overnight prior to the enzymatic hydrolysis stage. Control experiments encompassed (1) distilled water only pretreated banana pseudostem (BP_{water}) at 500 W for 6 min, and (2) untreated/native banana pseudostem ($BP_{untreated}$) by omitting pretreatment.

2.3 Modelling and optimization of pretreatment parameters

The response surface methodology (RSM) was modelled using a three-level Box-Behnken design (Design Expert 7.0, Stat Ease Inc, USA) and generated two separate experimental designs (M-ITS and M-PWW) consisting of 17 pretreatments each. The pretreatment input parameters for the M-ITS model comprised of salt concentration (1–5%, w/v), microwave power intensity (100–900 W), and pretreatment time (2–10 min). On the other hand, the M-PWW model consisted of substrate solid loading (10–30% w/v), microwave power intensity (100–900 W), and pretreatment time (2–10 min). The output responses were reducing sugar yield (RSY) and glucose yield (GY) for both models. The pretreatment parameter ranges for the M-ITS and M-PWW experimental designs were selected according to an extensive literature survey [12–14] in conjunction with data obtained from the preliminary screening. Therefore, four RSM pretreatment models were designated as follows: (1) banana pseudostem pretreated with ITS-reducing sugar yield (M-ITS_{RSY}), (2) banana pseudostem pretreated with ITS-glucose yield (M-ITS_{GY}), (3) banana pseudostem pretreated with PWW-reducing sugar yield (M-PWW_{RSY}), and (4) banana pseudostem pretreated with PWW-glucose yield (M-PWW_{GY}).

2.4 Enzymatic saccharification

The M-ITS or M-PWW pretreated banana pseudostem residue was submerged in sodium citrate buffer (0.05 M, pH 4.8) with a working volume of 10 mL. In addition, standard enzyme loading (10 FPU/g) and solid loading (10%, w/v) were used. Enzymatic hydrolysis was carried out at an agitation and temperature of 120 rpm and 50 °C, respectively, for 72 h in an orbital shaker incubator (FMH Instruments, South Africa). Subsequently, the hydrolysate samples were centrifuged at 10 000 rpm (Microspin 12, Grant-bio) for 5 min to remove any unhydrolyzed biomass. The reducing sugar yield (g/g) and glucose yield (g/g) released after enzymatic saccharification was analyzed using the 3,5-dinitrosalicyclic acid (DNS) method [28] and Megazyme GOPOD assay kits (©Megazyme, Wicklow, Ireland) respectively. For the detection of reducing sugar, the samples collected at the end of the enzymatic hydrolysis stage were centrifuged at 10 000 rpm for 5 min. Samples were thereafter diluted accordingly with distilled water. The diluted sample (3 mL) was pipetted into test tubes prior to the addition of 3 mL of DNS reagent (10 g/L DNS, 2 g/L phenol, 0.5 g/L sodium sulfite, and 10 g/L NaOH). The samples were incubated in a preheated water bath at 90 °C for 15 min. Thereafter, 1 mL of 40% (w/v) sodium potassium tartrate (Rochelle salts) was added to each tube and mixed thoroughly. The absorbance values were recorded at a wavelength of 575 nm. Parallel to this, a glucose (reducing sugar) standard curve (0, 0.2, 0.4, 0.6, 0.8, and 1 g/L glucose) was set up using the above protocol. The absorbance values recorded for the known glucose concentrations were plotted against the glucose concentrations to produce a standard curve. The absorbance values obtained for the experimental runs were substituted into the glucose standard curve equation to determine the reducing sugar concentration (g/L). The reducing sugar yield (g/g) was computed by dividing the reducing sugar concentration by 100 to determine the quantity of reducing sugar produced per gram of substrate.

Glucose was determined using the Megazyme GOPOD assay kit protocols provided by the manufacturer (Megazyme, Ireland). Centrifuged enzymatic hydrolyzed samples were diluted according to the manufacturer's instructions. Diluted sample aliquots (0.1 mL) were thereafter added to test tubes containing 3 mL of GOPOD reagent and mixed thoroughly. These test tubes were incubated at 45 °C for 20 min in a preheated water bath. The glucose standard solution (1.0 g/L) supplied by the manufacturer, and blank (distilled water) were also performed as per the specified protocols. The absorbance values were recorded at a wavelength of 510 nm. The glucose concentration (g/L) was extrapolated by dividing the absorbance values for the experimental samples by the absorbance value obtained for the glucose standard and thereafter multiplying by the dilution factor. The glucose yield (g/g) was computed similar to the reducing sugar yield. The glucose concentration (g/L) obtained was divided by 100 to extrapolate the quantity of glucose produced per gram

of substrate. All experiments were carried out in duplicate according to the RSM design for the iodized table salt (Table 1) and paper wastewater (Table 2) pretreatment models.

2.5 Validation of the optimized pretreatment conditions and assessment on different types of lignocellulosic wastes

The optimized pretreatment conditions for each developed model (M-ITS and M-PWW) were applied on banana pseudostem as validation experiments and designated as $M\text{-}ITS_{optimized}$ and $M\text{-}PWW_{optimized}$ respectively. In addition, other lignocellulosic wastes that include corn cobs, sorghum leaves, sugarcane bagasse, and bamboo were subjected to the optimized M-ITS and M-PWW pretreatment conditions. Each feedstock was then pretreated and enzymatically hydrolyzed following the protocols outlined previously in Sections 2.3 and 2.4 above.

2.6 Analytical techniques

2.6.1 Scanning electron microscopy (SEM)

The banana pseudostem before (untreated) and after (optimized M-ITS or M-PWW) pretreatments were visually analyzed using scanning electron microscopy (SEM). Samples were initially mounted onto aluminum stubs and then sputter coated with four layers of a thin gold film for electron conductivity (Quorum Q150 R ES). Subsequently, the samples were examined at an accelerating voltage of 20 kV with a magnification of ~ 500 × (ZEISS EVO LS 15).

2.6.2 Fourier-Transform Infrared (FTIR) spectroscopy

The chemical structure and functional group changes of the native (untreated) and optimally pretreated (M-ITS or M-PWW) substrate were analyzed by Fourier transform infrared (FTIR) spectroscopy using an Agilent Cary 630 spectrometer. Sample preparation was carried out by mixing the dried samples with spectroscopic grade KBr that was further pressed into pellets for analysis. Data from the analysis were collected using Agilent MicroLab PC 5.1.22 and processed using ResolutionPro 5.0.0.395 software. The resulting FTIR spectra was recorded in the range of 650 and 4000 cm^{-1} at a resolution of 4 cm^{-1} and 30 scans per sample.

3 Results and discussion

3.1 Preliminary screening

Among the various preliminary screening methods evaluated on banana pseudostem, the $M\text{-}ITS_{preliminary}$

Table 1 Reducing sugar and glucose yields observed for the M-ITS pretreatments

Run	Input parameters			Output parameters	
	Salt concentration (%)	Power intensity (W)	Pretreatment time (min)	Reducing sugar yield (g/g)	Glucose yield (g/g)
1	1	100	6	0.107 ± 0.001	0.068
2	3	100	10	0.113 ± 0.001	0.072
3	3	900	10	0.450 ± 0.001	0.389 ± 0.010
4	3	500	6	0.428 ± 0.001	0.331 ± 0.010
5	3	500	6	0.407	0.309
6	1	500	10	0.436 ± 0.001	0.324 ± 0.020
7	5	100	6	0.089 ± 0.006	0.057
8	1	500	2	0.386 ± 0.005	0.256 ± 0.010
9	1	900	6	0.418 ± 0.001	0.334
10	3	100	2	0.119 ± 0.010	0.057
11	5	500	10	0.432 ± 0.003	0.283 ± 0.010
12	3	500	6	0.431 ± 0.003	0.315 ± 0.020
13	3	500	6	0.412	0.295
14	5	900	6	0.433 ± 0.016	0.338 ± 0.030
15	3	900	2	0.411 ± 0.003	0.258 ± 0.020
16	5	500	2	0.371 ± 0.001	0.228
17	3	500	6	0.419 ± 0.007	0.291 ± 0.010

g/g, gram-reducing sugar/gram dry weight substrate or gram glucose/gram dry weight substrate; ± = standard deviation

Table 2 Reducing sugar and glucose yields observed for the M-PWW pretreatments

Run	Input parameters			Output parameters	
	Solid loading (%)	Power intensity (W)	Pretreatment time (min)	Reducing sugar yield (g/g)	Glucose yield (g/g)
1	10	100	6	0.112 ± 0.003	0.079 ± 0.003
2	20	100	10	0.116 ± 0.001	0.075 ± 0.003
3	20	900	10	0.438 ± 0.015	0.295 ± 0.017
4	20	500	6	0.516 ± 0.004	0.231 ± 0.012
5	20	500	6	0.457 ± 0.007	0.286 ± 0.009
6	10	500	10	0.478 ± 0.016	0.292 ± 0.011
7	30	100	6	0.127	0.055 ± 0.001
8	10	500	2	0.435 ± 0.006	0.206 ± 0.018
9	10	900	6	0.524 ± 0.001	0.273 ± 0.011
10	20	100	2	0.135 ± 0.004	0.078 ± 0.006
11	30	500	10	0.469 ± 0.009	0.307 ± 0.013
12	20	500	6	0.477 ± 0.009	0.333 ± 0.008
13	20	500	6	0.473	0.334 ± 0.028
14	30	900	6	0.534 ± 0.013	0.426 ± 0.003
15	20	900	2	0.455 ± 0.008	0.308 ± 0.002
16	30	500	2	0.342 ± 0.003	0.174 ± 0.011
17	20	500	6	0.454 ± 0.012	0.339 ± 0.005

g/g, gram-reducing sugar/gram dry weight substrate or gram glucose/gram dry weight substrate; ± = standard deviation

resulted in the highest reducing sugar yield (RSY) and glucose yield (GY) of 0.424 and 0.298 g/g respectively, followed by the M-$NaCl_{preliminary}$ method (RSY = 0.383 g/g and GY = 0.260 g/g) (Table S2). The M-$PWW_{preliminary}$ pretreatment led to a RSY and GY of 0.366 and 0.266 g/g respectively. On the other hand, the two-stage pretreatment processes demonstrated similar reducing sugar (M-PWW-$NaCl_{preliminary}$ = 0.404 g/g and M-PWW-$ITS_{preliminary}$ = 0.402 g/g) and glucose (M-PWW-$NaCl_{preliminary}$ = 0.245 g/g and M-PWW-$ITS_{preliminary}$ = 0.272 g/g) yields to the single-stage systems. The control experiments displayed significantly lower reducing sugar (BP_{water} = 0.286 g/g and $BP_{untreated}$ = 0.187 g/g) and glucose yields (BP_{water} = 0.182 g/g and $BP_{untreated}$ = 0.107 g/g) compared to the single- and two-stage preliminary pretreatments. Major observations from the preliminary screening revealed that (1) single-stage and two-stage systems exhibited similar RS and GY, and (2) ITS (food-grade NaCl) resulted in a higher RS and GY compared to the commonly used lab-based NaCl for pretreatment. Hence, ITS and PWW pretreatments in single-stage systems were selected for subsequent modelling and optimization using the response surface methodology (RSM) model.

3.2 Effect of the pretreatment input parameters on the output reducing sugar and glucose yields

The output reducing sugar yield (RSY) and glucose yields (GY) obtained for the M-ITS and M-PWW experimental runs are illustrated in Tables 1 and 2 respectively. The M-ITS pretreatment displayed maximum RSY and GY of 0.450 and 0.389 g/g respectively, with 3% salt concentration, 900 W, and 10 min (run 3). The median pretreatment conditions of 3% salt concentration, 500 W, and 6 min exhibited similar RSY and GY as shown for experimental run 4 (RSY = 0.428 g/g and GY = 0.331 g/g), run 5 (RSY = 0.407 g/g and GY = 0.309 g/g), run 12 (RSY = 0.431 g/g and GY = 0.315 g/g), run 13 (RSY = 0.412 g/g and GY = 0.295 g/g), and run 17 (RSY = 0.419 g/g and GY = 0.291 g/g). The lowest RSY and GY for the M-ITS pretreatment were obtained with 5% salt concentration, 100 W, and 6 min (run 7: RSY = 0.089 g/g and GY = 0.057 g/g).

On the other hand, the highest RSY and GY of 0.534 and 0.426 g/g were achieved for the M-PWW pretreatment respectively, with a solid loading of 30%, heated at 900 W for 6 min

(run 14). Additionally, similar RSY and GY were obtained under the median pretreatment conditions (20% solid loading, 500 W, and 6 min) for experimental run 4 (RSY = 0.516 g/g and GY = 0.231 g/g), run 5 (RSY = 0.457 g/g and GY = 0.286 g/g), run 12 (RSY = 0.477 g/g and GY = 0.333 g/g), run 13 (RSY = 0.473 g/g and GY = 0.334 g/g), and run 17 (RSY = 0.454 g/g and GY = 0.339 g/g). Alternatively, experimental run 1 (10% solid loading, 100 W, and 6 min) led to the lowest RSY (run 1 = 0.112 g/g) whereas run 7 (30% solid loading, 100 W for 6 min) resulted in the lowest GY (run 7 = 0.055 g/g).

3.2.1 Interactive effects of the input parameters on the sugar yields for the M-ITS pretreatment

The pretreatment experimental data (Table 1 and 2) were used to develop four polynomial model equations shown in Table 3 that relate the output reducing sugar and glucose yields to the pretreatment input variables. Analysis of variance (ANOVA) was used to assess the fitness of the RSM models (Table 4). The interactions of the pretreatment input parameters on the output reducing sugar and glucose yields are depicted in Fig. 1A–D. The interactive effects of microwave power intensity and salt concentration on the RSY and GY, while pretreatment time was maintained at its median value, are illustrated in Fig. 1A and B for the M-ITS$_{RSY}$ and M-ITS$_{GY}$ models respectively. A high F value for the M-ITS$_{RSY}$ = 182.87 model and a low p value (< 0.05) indicated that this model was statistically significant (Table S3). A simultaneous increase in power intensity and salt concentration from 100 to 700 W and 1 to 3% respectively, increased the RSY from 0.12 to 0.463 g/g (Fig. 1A). Similarly, for the M-ITS$_{GY}$ model, a high F value and low p value of (92.26 and < 0.05) respectively displayed significance of this model (Table S4). The same incremental variations in the power intensity (100–700 W) and salt concentration (1 to 3%) led to an increase in the GY (0.08–0.352 g/g) (Fig. 1B). High R^2 values were achieved for the M-ITS$_{RSY}$ (0.9958) and M-ITS$_{GY}$ (0.9916) model, indicating that these models could account for 99.58% (ITS$_{RSY}$) and 99.16% (ITS$_{GY}$) of the variation in the observed data. From the pretreatment parameters that were investigated, microwave power intensity and pretreatment time were shown to have the most significant effect for the M-ITS$_{RSY}$ ($p < 0.0001$ and $p = 0.0071$) and M-ITS$_{GY}$ ($p < 0.0001$ and $p = 0.0005$) models (Table S3 and Table S4 respectively). The interaction of power intensity and pretreatment time was also shown to have a significant effect ($p = 0.0072$) for the M-ITS$_{GY}$ model (Table S4). This indicates that exposure of the substrate to high microwave power intensities and salt concentration increases the degradation of the recalcitrant lignin constituents [29]. During the microwave heating processes, polar molecules or side-chains of macromolecules rapidly oscillate, due to the incoming electromagnetic waves [30]. Microwave energy is converted to heat by the internal resistance of particles, thus resulting in bond disintegration and reorientation [30]. In addition to microwave power intensity, inorganic salt disrupts the hydrogen bonds and cleaves glycosidic linkages [31]. This reduces cellulose crystallinity, while increasing substrate porosity [15]. Furthermore, water molecules from the hydrated salt participates as nucleophiles, yielding glucose from the substrate [12]. Moreover, during pretreatment, inorganic salt undergoes hydrolysis and releases hydrogen ions as a weak electrolyte into solution, therefore providing a steadier reactive environment [15]. Similar trends on sugarcane leaf waste and *Miscanthus* straw were reported by Moodley and Gueguim Kana [13] and Kang et al. [12] respectively, using inorganic salt pretreatment. Moodley and Gueguim Kana [13] observed a maximum reducing sugar yield of 0.179 g/g after pretreatment with 1.5 M NaCl and 700 W for 8 min. Likewise, Kang et al. [12] observed a high glucan recovery (86.5%) and improved enzymatic digestibility (39.8%) following salt pretreatment (5% NaCl, 200 °C, 15 min). Although the power intensity and salt concentration enhanced the sugar recovery, a further increase in these parameters (> 700 W and > 3%) led to a decrease in the RSY (0.453–0.420 g/g). A similar trend was observed for the GY (0.355–0.33 g/g) when power intensity and salt concentration were simultaneously increased above 700 W and 3% respectively. Water plays a significant role during

Table 3 RSM polynomial model equations relating the input parameters to the output reducing sugar and glucose yields for the M-ITS and M-PWW pretreatments

Model	Equation	Equation number
M-ITS$_{RSY}$	$-0.02 + 1.23\times10^{-3}B + 6.56\times10^{-4}C - 9.07\times10^{-7}B^2$	(1)
M-ITS$_{GY}$	$-0.07 + 7.98\times10^{-4}B + 0.02C + 1.80\times10^{-5}BC - 5.87\times10^{-7}B^2 - 1.26\times10^{-3}C^2$	(2)
M-PWW$_{RSY}$	$-0.03 + 1.39\times10^{-3}B - 9.25\times10^{-7}B^2$	(3)
M-PWW$_{GY}$	$-0.07 + 5.82\times10^{-4}B - 4.76\times10^{-7}B^2$	(4)

M-ITS$_{RSY}$, microwave-assisted iodized table salt pretreatment reducing sugar yield; *M-ITS$_{GY}$*, microwave-assisted iodized table salt pretreatment glucose yield; *M-PWW$_{RSY}$*, microwave-assisted paper wastewater pretreatment reducing sugar yield; *M-PPW$_{GY}$*, microwave-assisted paper wastewater pretreatment glucose yield; *B*, power intensity; *C*, pretreatment time

Table 4 Summary of analysis of variance (ANOVA) for the developed pretreatment models

Model	Sum of squares	df	Mean squares	*F* value	*p* value	R^2
M-ITS$_{RSY}$	0.30	9	0.033	182.87	< 0.0001	0.9958
M-ITS$_{GY}$	0.20	9	0.022	92.26	< 0.0001	0.9916
M-PWW$_{RSY}$	0.37	9	0.042	21.27	0.0003	0.9647
M-PWW$_{GY}$	0.18	9	0.020	7.01	0.0088	0.9002

df, degrees of freedom; *F* value, Fisher-Snedecor distribution value; *p* value, probability value; R^2, coefficient of determination

pretreatment by facilitating heat and irradiation energy. The water content not only affects the dielectric characteristics of LCB, but also determines the efficiency of pretreatment [32]. Furthermore, elevated temperatures that occur under higher power intensities may result in low molecular weight sugars and amino acids that polymerize to form recalcitrant organic compounds via Maillard reactions, also commonly known as caramelization [30], that may have negative implications for further biological reactions such as enzymatic hydrolysis. This finding was in accordance with the study by Sewsynker-Sukai and Gueguim Kana [5], whereby an increase in power intensity above 700 W also showed a 30.8% decrease in the reducing sugar yield from corn cobs. High salt concentrations (> 3%) attract water molecules around their core metal

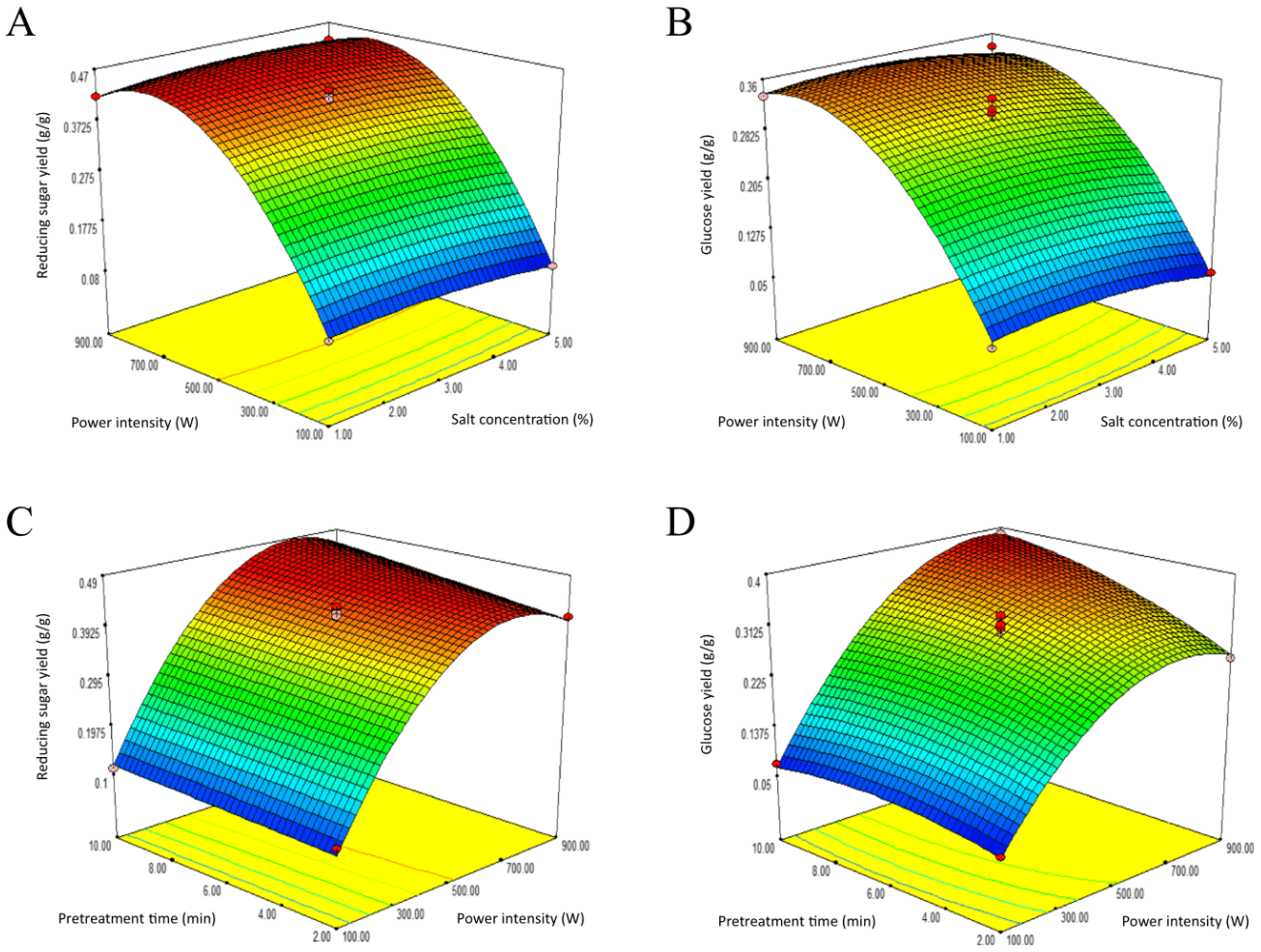

Fig. 1 Response surface graphs illustrating the interactive effects of the various input pretreatment parameters on the output reducing sugar and glucose yields: (**A**) power intensity and salt concentration (M-ITS$_{RSY}$); (**B**) power intensity and salt concentration (M-ITS$_{GY}$); (**C**) pretreatment time and power intensity (M-ITS$_{RSY}$); (**D**) pretreatment time and power intensity (M-ITS$_{GY}$)

cations. This reduces the amount of water that is available to interact with the substrate, which decreases the amount of cellulose in solution, thereby resulting in a low sugar recovery [14]. In a recent study, Suwannabun et al. [14] also observed a decrease in the reducing sugar concentration (3.486 to 2.890 mg/mL) from rice straw when the NaCl concentration was increased (> 5%). The influence of pretreatment time and power intensity on the RSY and GY while the salt concentration was kept at its center point is depicted in Fig. 1C (M-ITS$_{RSY}$) and D (M-ITS$_{GY}$) respectively. A simultaneous increment in pretreatment time (2–10 min) and power intensity (100–700 W) demonstrated an increase in the RSY from 0.11 to 0.484 g/g (M-ITS$_{RSY}$). Similarly, incremental variations in pretreatment time (2–10 min) and power intensity (100–900 W) resulted in an increase in the GY (0.06–0.39 g/g) (Fig. 1D). During microwave heating, polarization influences the dipole–dipole molecules to align in the radiation field. This ultimately leads to the rapid heating of the hydrolysate, thus enhancing the solubility of the substrate [32]. Microwave irradiation has a fast heating transfer rate, thereby reducing pretreatment time and energy consumption in comparison to conventional autoclave heating [32]. The high power intensities and thus high temperatures rapidly generate pressure within the absorbent biopolymers [32], while an increase in exposure time facilitates the accumulation of heat within the solution, leading to the effective disruption of lignin moieties [30]. A similar trend in the reducing sugar yield (0.135–0.232 g/g) was observed by Moodley and Gueguim Kana [13] by simultaneously increasing the $ZnCl_2$ concentration (0–2 M) and power intensity (0–800 W). Nevertheless, further increases in power intensity (> 700 W) led to a decrease in the RSY (0.484 to 0.41 g/g) (Fig. 1C). The lower RSY observed at higher power intensities (> 700 W) may be attributed to heat accumulation, which may result in the evaporation of the solvent (water), thereby limiting the ability of the solute molecules (ITS) to interact and diffuse into the substrate, which reduces the pretreatment efficiency.

3.2.2 Interactive effects of input parameters on the sugar yields for the M-PWW pretreatment

The pairwise effects of the pretreatment input parameters on the output reducing sugar and glucose yields are depicted in Fig. 2A–D. The interaction between power intensity and solid loading on the RSY and GY, while pretreatment time was maintained at its midpoint in the presence of the PWW, are illustrated in Fig. 2A and B for the M-PWW$_{RSY}$ and M-PWW$_{GY}$ models, respectively. A high F value for the M-PWW$_{RSY}$ = 21.27 and a low p value (< 0.05) indicated that this model was statistically significant (Table S5). An increase in the power intensity (100–900 W), while the solid loading was maintained at 10%, increased the RSY from 0.13 to 0.538 g/g). Likewise, for the M-PWW$_{GY}$ model, a high F value and low p value of 7.01 and < 0.05, respectively displayed significance of this model (Table S6). On the other hand, simultaneous increases in power intensity (100–900 W) and solid loading (10–30%) resulted in an increase in the GY from 0.12 to 0.39 g/g (Fig. 2B). High R^2 values were achieved for the M-PWW$_{RSY}$ (0.9647) and M-PWW$_{GY}$ (0.9002) model, indicating that these models could account for 96.47% (PWW$_{RSY}$) and 90.02% (PWW$_{GY}$) of the variation in the observed data. Moreover, microwave power intensity displayed the highest significant effect for the M-PWW$_{RSY}$ ($p < 0.0001$) and M-PWW$_{GY}$ ($p = 0.0003$) models. Improvements in the RSY and GY may be attributed to the selected pretreatment parameters. For instance, alkaline chemicals present in the PWW such as chloride, sulfate, and chromium originate from sodium hypochlorite, sodium sulfate, and sodium chromate respectively from the Kraft paper and pulp industrial process [22, 23]. These alkaline constituents lead to the expansion of the substrate surface area and further alter and modify the physical properties (crystallinity and porosity) of lignocellulosic biomass. This results in the saponification of intermolecular ester bonds and the solubilization of the amorphous regions (lignin and hemicellulose) [20]. Additionally, high microwave power intensities is associated with rapid collision of anions and cations further enhancing the breakage of hydrogen bonds within the lignocellulosic structural chain [32]. Alagu et al. [33] investigated the pretreatment effect of NaOH concentration (2–4% w/v) on cassava stem and noted an optimum reducing sugar concentration of 43.60 µg/ml with 3.21% NaOH. However, further increments in the solid loading (> 10%) led to a slight decrease in the RSY (0.538–0.505 g/g) (Fig. 2A). The decrease in RSY can be ascribed to the increase in total solid content above its optimal value, since high viscosity of the reaction mixture results in mixing barriers that limit heat and mass transfer efficiencies [34]. Pensri et al. [34] also observed a decrease in the reducing sugar yield (768–612 mg/g) when the Napier grass solid loading was increased from 10 to 20%. The combined effects of pretreatment time and solid loading on the RSY and GY when the power intensity was maintained at its median value is illustrated in Fig. 2C and D respectively. Simultaneous increases in the pretreatment time from 2 to 6 min and solid loading from 10 to 20% increased the RSY from 0.446 to 0.482 g/g (Fig. 2C). Figure 2D displayed a similar trend, whereby incremental variations in these same parameters (pretreatment time: 2 to 6 min and solid loading: 10 to 20%) led to an increase in the GY (0.22 to 0.31 g/g). The higher RSY and GY recorded under pretreatment times of 2 to 6 min may be ascribed to a high heating efficiency of the microwave oven. Short pretreatment times are generally preferred, due to the lower energy usage, reduced inhibitor formation, and increase in number of pretreatment cycles [32]. Moodley and

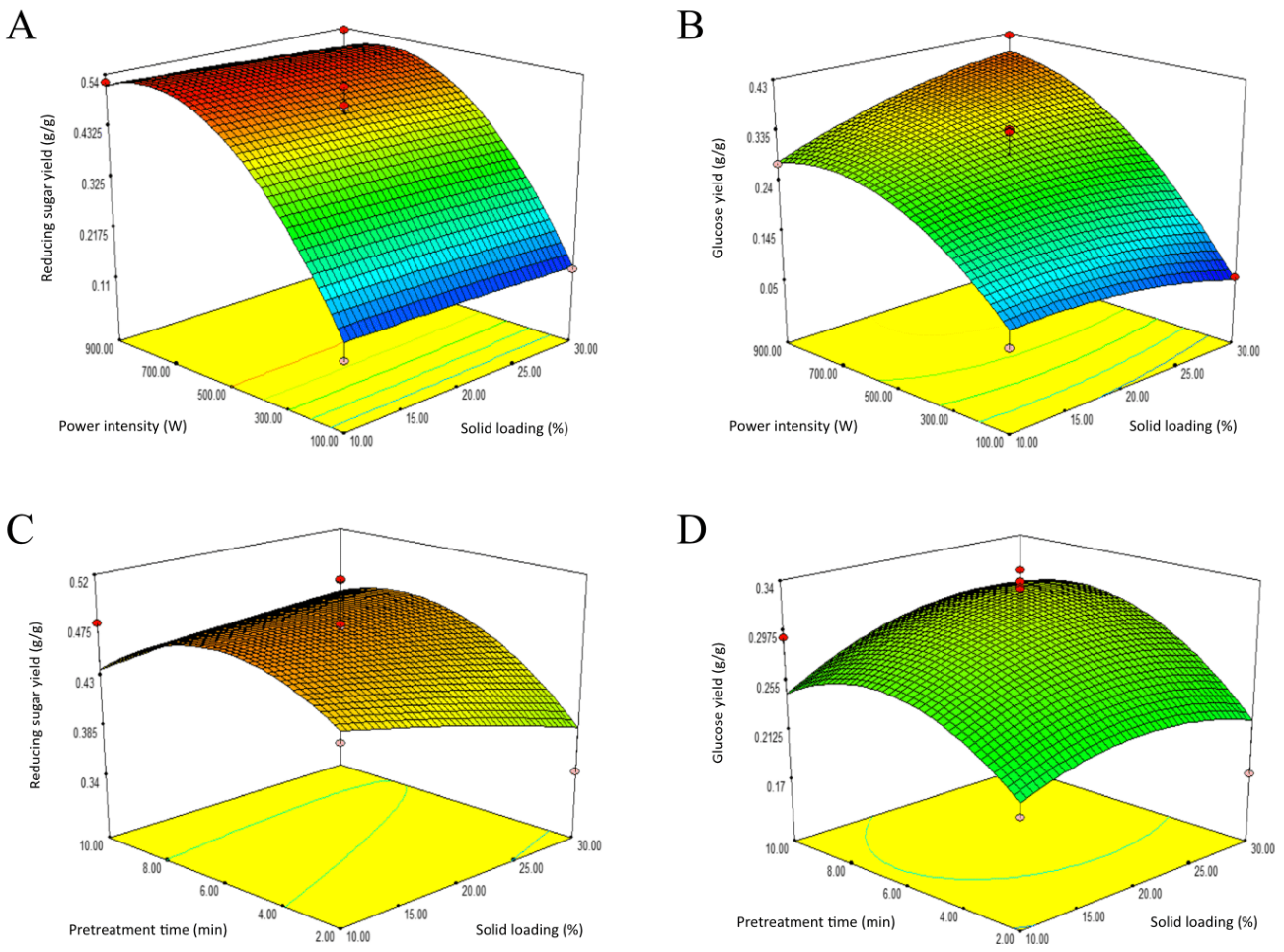

Fig. 2 Response surface graphs illustrating the interactive effects of the various input pretreatment parameters on the output reducing sugar and glucose yields. **A** Power intensity and solid loading ($M\text{-}PWW_{RSY}$). **B** Power intensity and solid loading ($M\text{-}PWW_{GY}$). **C** Pretreatment time and solid loading ($M\text{-}PWW_{RSY}$). **D** Pretreatment time and solid loading ($M\text{-}PWW_{GY}$)

Gueguim Kana [13] also noted that an increase in pretreatment time (2–8 min) increased the RSY (0.10 to 0.185 g/g) from NaCl pretreated sugarcane leaf waste. Apart from the effects of microwave power intensity, pretreatment time, and the biomass solid loading, the high sugar yields can also be attributed to the characteristics of the PWW, that enhance the degradation of lignocellulosic structures. For one, a high pH is vital for delignification by facilitating the oxidation of ethylene double bonds in the side chains of lignin structures, thus enhancing the exposure of cellulose and hemicellulose to enzymes during saccharification [35]. In addition, the presence of strong alkali chemicals such as chloride, sulfate, and chromium within PWW has shown to break down glycosidic and ester bonds between ferulic acid and hemicellulose in the extracellular matrix resulting in cellulose swelling, hemicellulose solvation. and lignin degradation using the lab-derived versions [36]. Despite the positive effect of the aforementioned input parameters, further increases in the pretreatment time (> 6 min) and solid loading (> 20%) negatively influenced the RSY (0.482–0.438 g/g) (Fig. 2C) and GY (0.31–0.25 g/g) (Fig. 2D). Generally, long exposure times to microwave heating results in high evaporation rates and can account for the reduction in the RSY and GY, since water plays a pivotal role by facilitating heat and mass transfer during chemical processes that occur within the pretreatment reactor [32]. A similar trend was observed by Sewsynker-Sukai and Gueguim Kana [37] after investigating the interactive effects of solid loading (7.5–15%) and alkali-salt concentration ($Na_3PO_4.12H_2O$) (5–7.5%) on corn cobs. A high solid loading and low $Na_3PO_4.12H_2O$ concentration decreased the RSY (9.64%). Both pretreatments (M-ITS and M-PWW) demonstrated that microwave power intensity had the most significant pretreatment effect on the output reducing sugar and glucose yields obtained from banana pseudostem. All four pretreatment models displayed a strong fit regarding the correlation between the input pretreatment parameters and the output reducing sugar and glucose yield.

3.3 Experimental validation of the developed pretreatment models

The developed pretreatment models were thereafter validated to achieve maximum RSY and GY. The optimal pretreatment conditions predicted by the M-ITS model were 2.48% ITS concentration, 800 W (power intensity), and 10 min (pretreatment time), corresponding to a RSY and GY of 0.48 g/g and 0.39 g/g respectively (M-$ITS_{optimized}$). Experimental validation of these pretreatment conditions displayed RSY and GY of 0.515 g/g and 0.433 g/g respectively. Deviations of 7.04% and 10.45% were observed between the M-ITS model predicted and experimental yields for RSY and GY respectively. Conversely, the M-PWW model predicted optimal pretreatment conditions of 30% solid loading, 800 W for 8 min that coincided with a RSY and GY of 0.520 g/g and 0.390 g/g respectively (M-$PWW_{optimized}$). Comparable RSY (0.498 g/g) and GY (0.413 g/g) were observed for the experimentally validated pretreatment conditions. Therefore, minor deviations (RSY = 4.32% and GY = 5.73%) were observed between the model predicted and observed yields. The slight variations between the predicted and experimental sugar yields illustrate the high predictability, accuracy, and validity of the developed pretreatment models. Moreover, optimized pretreatment strategies increased the RSY (M-$ITS_{optimized}$ = 2.75-fold and M-$PWW_{optimized}$ = 2.66-fold) and GY (M-$ITS_{optimized}$ = 4.05-fold and M-$PWW_{optimized}$ = 3.86-fold) by several fold compared to $BP_{untreated}$.

3.4 Effects of the M-ITS and M-PWW pretreatments on the lignocellulosic structure of banana pseudostem

3.4.1 Compositional analysis

Changes in lignocellulosic biomass structure may be demonstrated by various analyses that include composition, scanning electron microscopy (SEM), and Fourier transform infrared (FTIR) spectroscopy. The untreated banana pseudostem composition consisted of 5.98% cellulose, 17.54% hemicellulose, and 14.08% lignin. Banana pseudostem pretreated under M-$ITS_{optimized}$ conditions comprised 13.55% cellulose, 16.72% hemicellulose, and 9.73% lignin. This corresponds to a 126.59% cellulose improvement, 4.68% hemicellulose solubilization, and 30.89% lignin removal. The changes in structural composition following the M-$ITS_{optimized}$ pretreatment are due to the dissociation of inorganic salt into complex ions in water that leads to the rupturing of glycosidic linkages and disruption of hydrogen bonds [31]. Similar observations were noted on inorganic salt pretreatment of corn stover [15] and sugarcane leaf waste [13]. In an earlier report, Liu et al. [15] pretreated corn stover with 0.1 M $FeCl_3$ at 140 °C for 5–30 min and observed a hemicellulose removal and cellulose recovery of 91% and 30% respectively. More recently, Moodley and Gueguim Kana [13] reported an 8.9, 48.6, and 71.5% hemicellulose solubilization observed for the optimized microwave-assisted-NaCl, -$ZnCl_2$, and -$FeCl_3$ pretreatments respectively.

Likewise, the M-$PWW_{optimized}$ banana pseudostem exhibited 17.06% cellulose, 13.04% hemicellulose, and 5.9% lignin. The structural changes corresponded to a 185.28% cellulose improvement, 25.66% hemicellulose solubilization, and 58.1% lignin removal. Variation in the biomass structure can be accounted for by the PWW chemical components (chloride, sulfate, and chromium) coupled with microwave heating (power intensity of 800 W). As previously noted, the strong alkali chemicals present in PWW leads to the degradation of ester and glycosidic bonds between hemicellulose and ferulic acid in the cell wall resulting in the cellulose swelling, hemicellulose solvation, and lignin removal [36]. In addition, hemicellulose solubilization and lignin removal lead to an increase in the pore size and accessible surface area of banana pseudostem, thus exposing cellulose for enzymatic saccharification. Shimizu et al. [19] pretreated banana pseudostem with NaOH and recorded a 75.48% increase in cellulose content, 4.38% hemicellulose solubilization, and 7.65% lignin removal. Therefore, the M-$ITS_{optimized}$ and M-$PWW_{optimized}$ pretreatment methods were effective for disruption of the lignocellulosic structures and enhanced the sugar recovery. The M-$PWW_{optimized}$ method resulted in a 25.9%, 22%, and 39.36% higher cellulose improvement, hemicellulose solubilization, and lignin removal respectively compared to the M-$ITS_{optimized}$ pretreatment. Compositional analysis data is further substantiated with SEM and FTIR analyses.

3.4.2 Visual observations using scanning electron microscopy (SEM)

SEM analysis was used to visually evaluate changes that occurred in the cell wall surface morphology of banana pseudostem before and after pretreatment (Fig. 3). It was observed that pretreatment had induced significant physical changes in the biomass. Native (untreated) banana pseudostem exhibited a smooth and rigid surface area with low porosity and an intact substrate structure. The M-$ITS_{optimized}$ and M-$PWW_{optimized}$ samples both demonstrated the disintegration of the lignocellulosic structures. Damaged cell walls with large pores were observed in the optimally pretreated samples. Furthermore, a highly disorganized morphology was characterized by the exposure of fibers and loosening of the fibrous network in these samples (M-$ITS_{optimized}$ and M-$PWW_{optimized}$). Therefore, the banana pseudostem was prone to high levels of fragmentation when pretreated with ITS and PWW. During pretreatment,

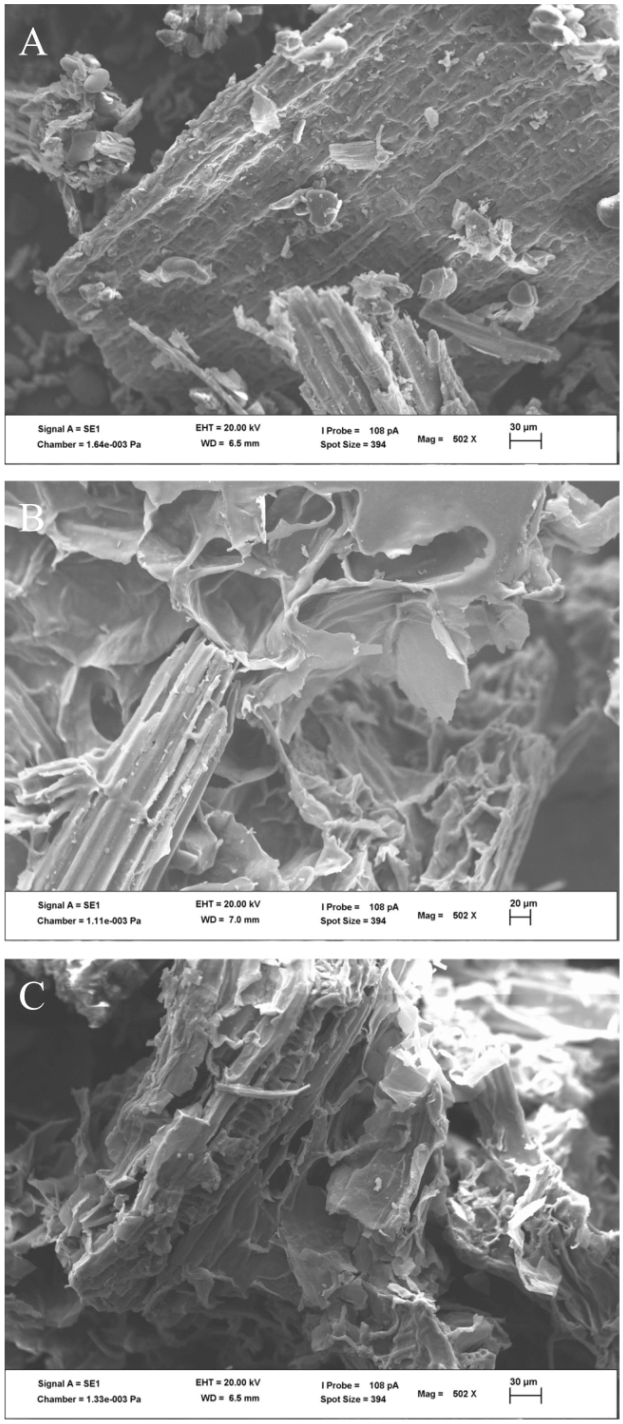

Fig. 3 SEM micrographs (~500×). **A** untreated, **B** M-ITS$_{optimized}$, and **C** M-PWW$_{optimized}$ banana pseudostem

hydrogen bonds are damaged, resulting in the leakage of lignin moieties [31]. Furthermore, an increase in surface area and porosity enhances the availability of cellulose and hemicellulose structures to enzymatic attack during saccharification [29]. Particularly, the use of salts such as ITS lead to hemicellulose solubilization, due to the cleavage of glycosidic linkages, whereas PWW causes disruptions to the recalcitrant aromatic polymer lignin. The SEM results were in accordance with the compositional analysis in addition to previous studies on corn stover [15] and banana pseudostem [4] pretreated with inorganic salt and alkali (NaOH) respectively.

3.4.3 Fourier transform infrared (FTIR) analysis

FTIR spectral analysis was utilized to study the physicochemical properties and conformational variations introduced by the optimized pretreatments on banana pseudostem. The chemical and structural changes of cellulose, hemicellulose, and lignin are represented in the wavelength range of 650 to 4000 cm^{-1}. The absorption spectrum of native and optimized (M-ITS$_{optimized}$ and M-PWW$_{optimized}$) samples displayed distinct peaks at various wavelengths with strong, banding patterns. More specifically, chemical modifications introduced by pretreatment for the M-ITS$_{optimized}$ and M-PWW$_{optimized}$ displayed higher absorption peaks and intensities compared to the native banana pseudostem substrate. For instance, the absorptive peak at 840 cm^{-1} arises from the C–H out of plane bending and implies that the benzene rings of lignin are 1,3,5-trisubstituted [38]. Conversely, the absorptive band at 1000 cm^{-1} is ascribed to the aromatic C–H and C–O distortion and stretching in (alcohols, ethers, or carboxylic acids) of cellulose and hemicellulose [38, 39]. The absorption peak at 1170 cm^{-1} represents the C–O–C asymmetric stretching and vibrations in cellulose and hemicellulose associated with the pyranose ring skeleton [39]. Two absorption peaks were detected at wavelengths of 1315 cm^{-1} and 1490 cm^{-1} and occur in the range between 1300 and 1500 cm^{-1}, illustrating the C–H-bending vibrations of cellulose [40]. On the other hand, the band observed at 1615 cm^{-1} demonstrates in-plane C = C (carbonyl and/or carboxylic) aromatic vibration in the aromatic ring within lignin [40]. A faint band occurred at 2145 cm^{-1} and is indicative of stretching of the triple –C≡C– bond in the alkyne compounds present within lignin. Additionally, C–H stretching and –OH stretching vibrations of intramolecular hydrogen within cellulose were represented by absorptive peaks at 2905 cm^{-1} and 3272 cm^{-1}, respectively [40]. Increases in the intensity at these band positions characteristically indicate the recovery of cellulose from lignocellulosic biomass after pretreatment. Spectral data from the FTIR analysis indicated that the M-ITS$_{optimized}$ and M-PWW$_{optimized}$ pretreatments effectively unravelled lignin and hemicellulose, while altering crystalline cellulose to its amorphous form. The FTIR data was in agreement with the compositional and SEM analysis, in addition to previous reports on inorganic salt (NaClO) [36] and alkali (NaOH) [9] pretreatments.

3.5 Comparative assessment of the optimized M-ITS and M-PWW pretreatment conditions on different lignocellulosic substrates

The variation in the structural composition of different lignocellulosic substrates remains high due to the distribution and proportion of cellulose, hemicellulose, and lignin content. Additionally, the recalcitrant nature of a particular substrate hinders the application of different pretreatment strategies rendering it a research hotspot, since a "one-size-fits-all" approach results in low hydrolysis rates and subsequently negligible fermentation product yields. Therefore, to determine the versatility of the developed pretreatment methods, different lignocellulosic substrates such as corn cobs, sorghum leaves, sugarcane bagasse, and bamboo were subjected to the M-$ITS_{optimized}$ and M-$PWW_{optimized}$ process conditions. Following pretreatment of the different substrates (corn cobs, sorghum leaves, sugarcane bagasse, and bamboo), RSY and GY from these experiments together with the optimized banana pseudostem data were compared to previous pretreatment reports for the corresponding lignocellulosic substrate. The M-$ITS_{optimized}$ and M-$PWW_{optimized}$ pretreatments led to higher sugar yields when compared to previous studies using banana pseudostem. For instance, Idrees et al. [41] utilized an autoclave-assisted dilute acid pretreatment and observed a lower RS concentration of 38 g/L (corresponding to 380 mg/g) in comparison to this study (M-$ITS_{optimized}$). Similarly, Chen et al. [42] employed a strong alkali (8% NaOH) pretreatment on banana pseudostem and observed a lower RSY of 0.34 g/g (340 mg/g) when compared to this study (M-$PWW_{optimized}$). In recent studies, Ayeni et al. [26] and Dolmani and Livleen [43] employed an inorganic salt ($ZnCl_2$) and alkali (NaOH) pretreatment respectively on corn cobs and noted a lower reducing sugar yield of 0.075 g/g (75.54 mg/g) and 0.071 g/g (71.7 mg/g) respectively, in comparison to the present study. Deshavath et al. [44] pretreated sorghum leaves with dilute acid (0.1 M H_2SO_4) and observed a lower GY of 0.052 g/g (52.54 mg/g) compared to the present study (M-$ITS_{optimized}$). Likewise, Manmai et al. [45] pretreated sorghum leaves with strong alkali (2% NaOH) and observed a lower RSY 0.176 g/g (176 mg/g) compared to the present study (M-$PWW_{optimized}$). Interestingly, Tiwari et al. [46] investigated H_2SO_4 and NaOH pretreatments using sugarcane bagasse and observed a higher reducing sugar yield of 0.251 g/g (251 mg/g) and 0.290 g/g (290 mg/g) respectively. Similarly, Wang et al. [47] and Leenakul and Tippayawong [48] pretreated bamboo and reported lower reducing sugar yields of 0.121 (121 mg/g) and 0.085 g/g (85 mg/g) respectively, compared to the M-$ITS_{optimized}$ and M-$PWW_{optimized}$ pretreatment of bamboo. Even though the aforementioned studies [46–48] demonstrated higher RSY compared to the present corresponding substrate, the components that constitute reducing sugar include monosaccharides, disaccharides, and polysaccharides, which are not a direct indication of high fermentable sugars (such as glucose). Besides the reducing sugar yield not being representative of actual fermentable sugars, the use of pure chemicals such as acids and/or alkali in the previous reports by Tiwari et al. [46], Wang et al. [47], and Leenakul and Tippayawong [48] adds to the process costs and negatively impacts on the environment, thereby rendering these unfeasible for lignocellulosic pretreatment.

On the other hand, ITS is cost-effective, abundant, and readily available when compared to its lab-based counterpart (NaCl). The higher RSY and GY achieved in the present study may be attributed to the nucleophilic ability of ITS to depolymerize hemicellulose into monosaccharides, while cleaving glycosidic linkages and hydrogen bonds thereby reducing the crystalline structure of cellulose [12, 16]. Additionally, PWW exhibits a strong alkalic effect that targets the recalcitrant aromatic polymer lignin and facilitates in the saponification of intermolecular ester bonds and oxidation of ethylene double bonds [19, 20, 35]. The present study provides valuable insights for lignocellulosic pretreatment processes, since both ITS and PWW demonstrate high effectiveness compared to their lab-grade equivalents. Despite both pretreatments demonstrating comparable reducing sugar and glucose yields compared to previous pretreatment reports for the corresponding lignocellulosic substrate, the M-$PWW_{optimized}$ is a completely waste-based method that outweighs the M-$ITS_{optimized}$ pretreatment in several aspects. For instance, the M-$PWW_{optimized}$ conditions gave a 30% solid loading, which led to a threefold higher quantity of pretreated banana pseudostem substrate compared to the M-$ITS_{optimized}$ strategy (10% solid loading). Therefore, the M-$PWW_{optimized}$ reduces the number of pretreatment cycles required, by up to 3 times in contrast to the M-$ITS_{optimized}$ pretreatment. This implies that a threefold lower energy and shorter time will be required when using the M-$PWW_{optimized}$ pretreatment instead of the M-$ITS_{optimized}$ method. In addition, repurposing PWW for lignocellulosic pretreatment has the potential to drastically reduce expenditure and negative environmental effects by (1) eliminating hazardous chemicals, (2) reducing wastewater treatment costs, and (3) negating the use of finite resources such as fresh water in lignocellulosic pretreatment systems. Moreover, collaborative efforts between the agricultural, Kraft paper and pulp, and biotechnology sectors may increase profits, while reducing wastes, since the exploitation of PWW and LCB residues as an additional revenue stream can be used to (1) finance downstream wastewater treatment processes for Kraft industries and (2) prevent the burning and/or disposal of LCB that ends up in land and water bodies, thereby minimizing the release of harmful greenhouse gases into the environment and curbing climate change.

4 Conclusion

Two novel microwave-assisted pretreatments using iodized table salt (M-ITS) and paper wastewater (M-PWW) were optimized on banana pseudostem. Although the $M\text{-}PWW_{optimized}$ regime displayed a slightly lower RSY (3.3%) and GY (4.62%) compared to the $M\text{-}ITS_{optimized}$ strategy, it has been concluded to be more effective, since it is a complete waste-based method and requires fewer pretreatment cycles. Furthermore, assessment of the optimized conditions on different lignocellulosic substrates validated the suitability of the developed pretreatment models. This study provides major insights for eliminating chemicals and/or fresh water application in lignocellulosic biorefineries, while generating additional revenue streams for the industrial sector.

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Declarations

Competing interests The authors declare no competing interests.

Disclosure Opinions expressed and conclusions arrived at, are those of the author and are not necessarily attributed to the NRF.

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Chapter 3 - Supplementary material

Table S1. Sample analysis of the major constituents present in paper wastewater

Chemical substance	LC (mg/L)
Arsenic	<0.0025
Boron	0.215
Barium	0.175
Cadmium	<0.0005
Cobalt	<0.002
Chromium	0.5351
Hexavalent Chromium	<0.006
Copper	<0.007
Mercury	<0.001
Manganese	1
Molybdenum	<0.002
Nickel	0.009
Lead	<0.005
Antimony	0.007
Selenium	<0.003
Vanadium	0.0144
Zinc	0.031
Total Dissolved Solids	7522
Chloride	1114.1
Sulphate	448.5
Nitrate	<0.05
Fluoride	<0.3
Carbon and Nitrogen total	<0.06

Footnote: LC = The leachable concentration of a particular element or chemical substance in a waste (mg/L). The chemical substance LC was below the threshold toxic level limit (<LCT1) and classified the paper wastewater as a Type 3 (low-risk) waste (Government Gazette 36784, 2013).

Table S2. Preliminary screening of M-NaCl, M-ITS and M-PWW to determine their pretreatment effectiveness on banana pseudostem

Screening method	Input parameters	Reducing sugar yield (g/g)	Glucose yield (g/g)
$M\text{-}NaCl_{preliminary}$	3% w/v NaCl, 500 W, 6 min	0.383	0.260
$M\text{-}ITS_{preliminary}$	3% w/v ITS, 500 W, 6 min	0.424	0.298
$M\text{-}PWW_{preliminary}$	10% S/L, 500 W, 6 min	0.366	0.266
$M\text{-}PWW\text{-}NaCl_{preliminary}$	10% S/L[a], 500 W[a], 3 min[a], 3% w/v NaCl[b], 500 W[b], 3 min[b]	0.404	0.245
$M\text{-}PWW\text{-}ITS_{preliminary}$	10% S/L[a], 500 W[a], 3 min[a], 3% w/v ITS[b], 500 W[b], 3 min[b]	0.402	0.272
BP_{water}	500 W, 6 min	0.286	0.182
$BP_{untreated}$	-	0.187	0.107

Footnote: M-NaCl = Microwave-assisted-sodium chloride, M-ITS = Microwave-assisted-iodized table salt, M-PWW = Microwave-assisted-paper wastewater, BP_{water} = distilled water pretreated banana pseudostem, $BP_{untreated}$ = untreated/native banana pseudostem, S/L = solid loading, [a] = first-stage pretreatment, [b] = second-stage pretreatment.

Table S3. Analysis of variance of the developed ITS_{RSY} pretreatment model on banana pseudostem

Source	Sum of squares	df	Mean square	F-value	P-value (Probability > F)
Model	0.3004	9	3.34×10^{-2}	182.87	<0.0001 Significant
A-Salt concentration	1×10^{-4}	1	1×10^{-4}	0.3372	0.5796
B-Power intensity	2.063×10^{-1}	1	2.063×10^{-1}	1130.27	<0.0001
C-Pretreatment time	2.6×10^{-3}	1	2.6×10^{-3}	14.08	0.0071
AB	3×10^{-4}	1	3×10^{-4}	1.46	0.2656
AC	0	1	0	0.1376	0.7217
BC	5×10^{-4}	1	5×10^{-4}	2.87	0.1338
A^2	6×10^{-4}	1	6×10^{-4}	3.47	0.1049
B^2	8.886×10^{-2}	1	8.886×10^{-2}	485.32	<0.0001
C^2	3.6×10^{-6}	1	3.6×10^{-6}	0.0197	0.8923
Residual	1.3×10^{-3}	7	2×10^{-4}		
Lack of fit	9×10^{-4}	3	3×10^{-4}	2.93	0.1631 Not significant

Footnote: df = degrees of freedom, F-value = Fisher-Snedecor distribution value, P-value = probability value.

Table S4. Analysis of variance of the developed ITS_{GY} pretreatment model on banana pseudostem

Source	Sum of squares	df	Mean square	F-value	P-value (Probability > F)
Model	0.1967	9	2.19×10^{-2}	92.26	< 0.0001 Significant
A-Salt concentration	7×10^{-4}	1	7×10^{-4}	3.04	0.1246
B-Power intensity	0.1417	1	0.1417	598.37	< 0.0001
C-Pretreatment time	9×10^{-3}	1	9×10^{-3}	38.21	0.0005
AB	1×10^{-4}	1	1×10^{-4}	0.2255	0.6493
AC	0	1	0	0.15	0.71
BC	3.3×10^{-3}	1	3.3×10^{-3}	14.02	0.0072
A^2	1×10^{-3}	1	1×10^{-3}	4.11	0.0822
B^2	3.71×10^{-2}	1	3.71×10^{-2}	156.75	< 0.0001
C^2	1.7×10^{-3}	1	1.7×10^{-3}	7.26	0.0309
Residual	1.7×10^{-3}	7	2×10^{-4}		
Lack of fit	7×10^{-4}	3	2×10^{-4}	0.8769	0.5240 Not significant

Footnote: df = degrees of freedom, F-value = Fisher-Snedecor distribution value, P-value = probability value.

Table S5. Analysis of variance of the developed PWW_{RSY} pretreatment model on banana pseudostem

Source	Sum of squares	df	Mean square	F-value	P-value (Probability > F)
Model	0.3747	9	4.16×10^{-2}	21.27	0.0003 Significant
A-Solid loading	7×10^{-4}	1	7×10^{-4}	0.3724	0.5610
B-Power intensity	0.2669	1	0.2669	136.34	< 0.0001
C-Pretreatment time	2.2×10^{-3}	1	2.2×10^{-3}	1.14	0.3214
AB	4.008×10^{-6}	1	4.008×10^{-6}	2×10^{-3}	0.9652
AC	1.8×10^{-3}	1	1.8×10^{-3}	0.9053	0.3731
BC	2.416×10^{-7}	1	2.416×10^{-7}	1×10^{-4}	0.9914
A^2	0	1	0	1.81×10^{-2}	0.8969
B^2	9.22×10^{-2}	1	9.22×10^{-2}	47.09	0.0002
C^2	7.2×10^{-3}	1	7.2×10^{-3}	3.67	0.0971
Residual	1.37×10^{-2}	7	2×10^{-3}		
Lack of fit	1.12×10^{-2}	3	3.7×10^{-3}	6	0.0580 Not significant

Footnote: df = degrees of freedom, F-value = Fisher-Snedecor distribution value, P-value = probability value.

Table S6. Analysis of variance of the developed PWW_{GY} pretreatment model on banana pseudostem

Source	Sum of squares	df	Mean square	F-value	P-value (Probability > F)
Model	0.1794	9	1.99×10^{-2}	7.01	0.0088 Significant
A-Solid loading	1.6×10^{-3}	1	1.6×10^{-3}	0.5472	0.4835
B-Power intensity	0.1289	1	0.1289	45.35	0.0003
C-Pretreatment time	5.2×10^{-3}	1	5.2×10^{-3}	1.83	0.2186
AB	7.8×10^{-3}	1	7.8×10^{-3}	2.74	0.1418
AC	5×10^{-4}	1	5×10^{-4}	0.1929	0.6737
BC	0	1	0	9.7×10^{-3}	0.9243
A^2	1.8×10^{-3}	1	1.8×10^{-3}	0.6189	0.4572
B^2	2.44×10^{-2}	1	2.44×10^{-2}	8.58	0.0220
C^2	6.5×10^{-3}	1	6.5×10^{-3}	2.29	0.1742
Residual	1.99×10^{-2}	7	2.8×10^{-3}		
Lack of fit	1.12×10^{-2}	3	3.7×10^{-3}	1.73	0.2985 Not significant

Footnote: df = degrees of freedom, F-value = Fisher-Snedecor distribution value, P-value = probability value

Table S7. Validation of optimized conditions for the M-ITS and M-PWW pretreatment on banana pseudostem

Model	Input parameters				Reducing sugar yield (g/g)		Glucose yield (g/g)	
	Salt conc. (%)	Solid loading (%)	Power intensity (W)	Pretreatment time (min)	Predicted	Observed	Predicted	Observed
M-ITS	2.48	-	800	10	0.48	0.515	0.39	0.433
M-PWW	-	30	800	8	0.52	0.498	0.39	0.413

Table S8. Structural composition of the control (untreated) and optimally pretreated banana pseudostem.

Sample	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Native (untreated)	5.98	17.54	14.08
$M\text{-}ITS_{optimized}$	13.55	16.72	9.73
$M\text{-}PWW_{optimized}$	17.06	13.04	5.9

Footnote: $M\text{-}ITS_{optimized}$ = Optimized microwave-assisted-iodized table salt, $M\text{-}PWW_{optimized}$ = Optimized microwave-assisted-paper wastewater.

Table S9. Comparison of the developed pretreatment strategies on various lignocellulosic substrates

Substrate	Heating mechanism	Pretreatment parameters	Reducing sugar yield (g/g)	Glucose yield (g/g)	References
Banana pseudostem	Microwave	2.48% ITS, 800 W, 10 min	0.515	0.433	Present study
Banana pseudostem	Microwave	30% solid loading, 800 W, 8 min	0.498	0.413	Present study
Banana pseudostem	Autoclave	1% H_2SO_4, 130 ºC, 2 h	0.380	ND	Idrees et al. (2013)
Banana pseudostem	Autoclave	8% NaOH (w/w), 100 ºC, 24 h	0.340	ND	Chen et al. (2017)
Corn cobs	Microwave	2.48% ITS, 800 W, 10 min	0.174	0.086	Present study
Corn cobs	Microwave	30% solid loading, 800 W, 8 min	0.238	0.124	Present study
Corn cobs	Water bath	0.1 g $ZnCl_2.4H_2O$/g substrate, 45 ºC, 96 h	0.093	ND	Ayeni et al. (2020)
Corn cobs	Incubator	2% NaOH, 20 ºC, 6 h	0.072	ND	Dolmani and Livleen (2021)
Sorghum leaves	Microwave	2.48% ITS, 800 W, 10 min	0.154	0.096	Present study
Sorghum leaves	Microwave	30% solid loading, 800 W, 8 min	0.211	0.123	Present study
Sorghum leaves	Autoclave	0.1 M H_2SO_4, 121 ºC, 30 min	ND	0.038	Deshavath et al. (2018)
Sorghum leaves	Water bath	2% NaOH, 40 ºC, 3 d	0.032	ND	Manmai et al. (2020)

Table S9. Continued

Substrate	Heating mechanism	Pretreatment parameters	Reducing sugar yield (g/g)	Glucose yield (g/g)	References
Sugarcane bagasse	Microwave	2.48% ITS, 800 W, 10 min	0.093	0.046	Present study
Sugarcane bagasse	Microwave	30% solid loading, 800 W, 8 min	0.117	0.051	Present study
Sugarcane bagasse	Autoclave	5% H_2SO_4, 121 ºC, 30 h	0.251	ND	Tiwari et al. (2020)
Sugarcane bagasse	Autoclave	5% NaOH, 121 ºC, 30 h	0.290	ND	Tiwari et al. (2020)
Bamboo	Microwave	2.48% ITS, 800 W, 10 min	0.058	0.027	Present study
Bamboo	Microwave	30% solid loading, 800 W, 8 min	0.065	0.029	Present study
Bamboo	Autoclave	0.5% OP-10 emulsifier, pH 4, 120 ºC, 40 min	0.121	0.088	Wang et al. (2019)
Bamboo	Autoclave	1.2% H_2SO_4 , 120 ºC, 1 h	0.084	ND	Leenakul and Tippayawong (2010)

Footnote: ND = Not determined.

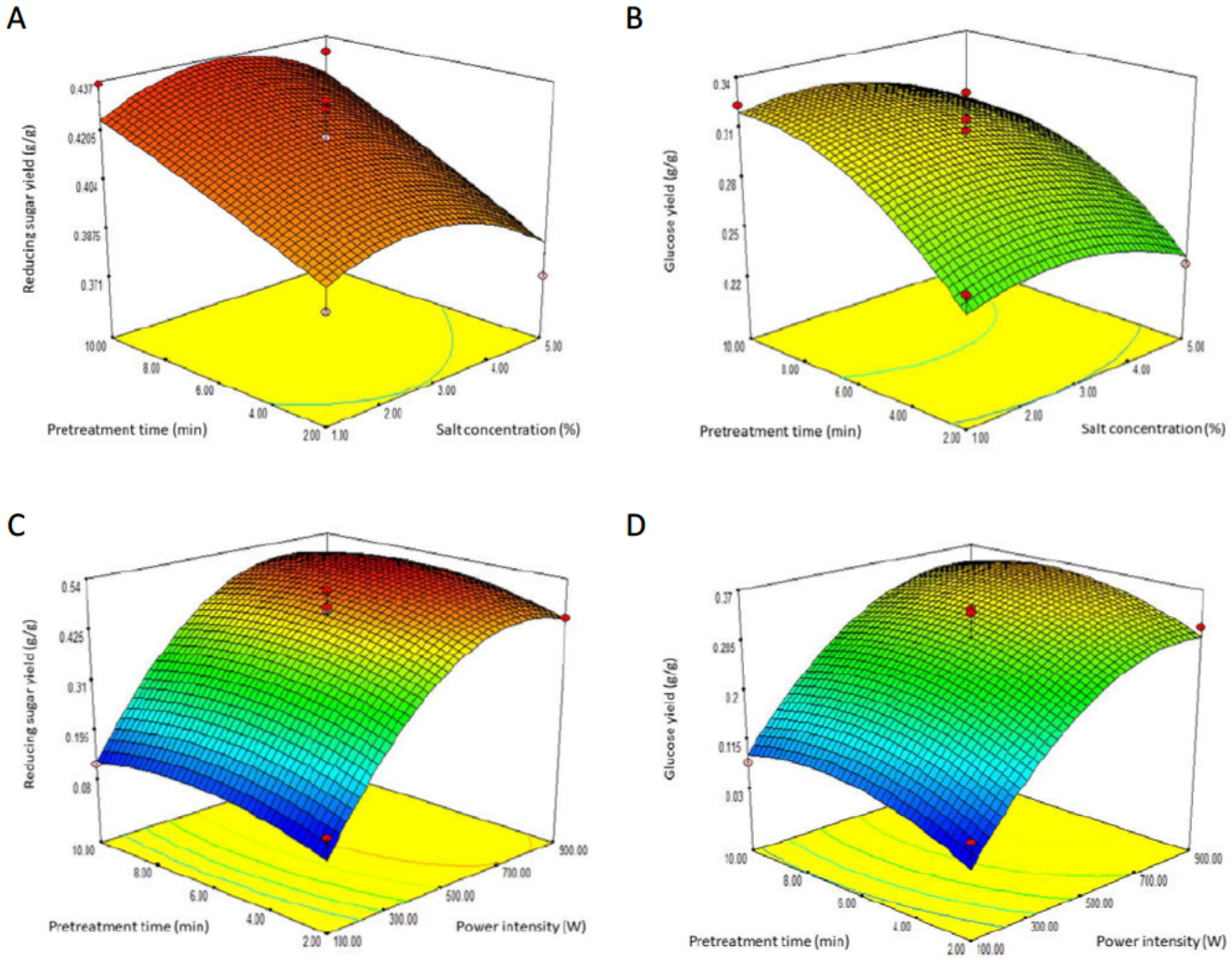

Fig. S1. Response surface graphs illustrating the interactive effects of the various input pretreatment parameters on the output reducing sugar and glucose yields: (A) pretreatment time and salt concentration (M-ITS$_{RSY}$); (B) pretreatment time and salt concentration (M-ITS$_{GY}$); (C) pretreatment time and power intensity (M-PWW$_{RSY}$); (D) pretreatment time and power intensity (M-PWW$_{GY}$).

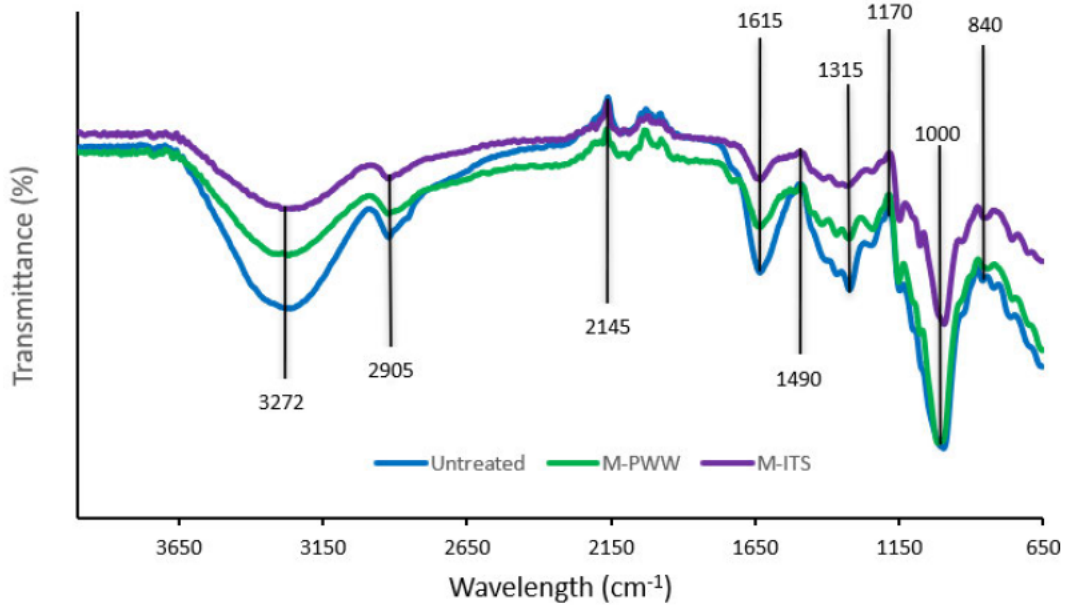

Fig. S2. FTIR spectra of the untreated, M-ITS$_{optimized}$ and M-PWW$_{optimized}$ banana pseudostem.

CHAPTER 4

Simultaneous saccharification and citric acid production from paper wastewater pretreated banana pseudostem: Optimization of fermentation medium formulation and kinetic assessment

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This chapter has been submitted to the journal *Bioresource Technology* with the title: Simultaneous saccharification and citric acid production from paper wastewater pretreated banana pseudostem: Optimization of fermentation medium formulation and kinetic assessment. (Under review). The manuscript and supplementary material are presented in the following pages.

Highlights

- First report on the optimization of citric acid using banana pseudostem
- The optimized process gave a maximum citric acid concentration of 14.408 g/L
- Kinetics were determined on fresh water, wastewater and standard medium
- The wastewater-based bioprocess demonstrated only a 9.11% lower citric acid concentration
- Findings facilitate the development of waste-based lignocellulosic bioprocesses

Graphical Abstract

Simultaneous saccharification and citric acid production from paper wastewater pretreated banana pseudostem: Optimization of fermentation medium formulation and kinetic assessment

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Abstract

This study optimized the simultaneous saccharification and citric acid (CA) production from banana pseudostem (BP). Thereafter, kinetic assessment of *Aspergillus brasiliensis* growth and CA production were determined for the optimum conditions using fresh water ($SSF_{optimizedFW}$) or dairy wastewater (SSF_{DWW}) and compared to Sabouraud Dextrose Emmon's medium modified with BP ($SSF_{SDEmodified}$). The optimized conditions (0.5% ammonium nitrate, 1% acetone and 35 °C) gave a CA concentration of 14.408 g/L. Kinetic assessment revealed the same maximum specific growth rates (μ_{max}) (0.05 h^{-1}) for all three bioprocesses, while the $SSF_{SDEmodified}$ process resulted in the highest maximum potential CA concentration (P_m) (13.991 g/L) in comparison to the SSF_{DWW} (P_m= 13.095 g/L) and $SSF_{optimizedFW}$ (P_m= 12.967 g/L) systems. Findings from this study facilitates the implementation of waste-based lignocellulosic bioprocesses that may eradicate the use of expensive pretreatment chemicals, fermentation medium constituents, and resources, in keeping with the water, energy and food nexus towards developing a circular bioeconomy.

Keywords: citric acid; dairy wastewater; banana pseudostem; simultaneous saccharification and fermentation.

1. Introduction

The bioconversion of renewable lignocellulosic biomass to valuable products such as citric acid (CA) has become a research hotspot towards achieving sustainable global economic development (Yadav et al., 2020). Citric acid is a valuable commercial bioproduct with an existing market and widespread use in the chemical, food, pharmaceutical, beverage, electroplating and cosmetic industries (Sekoai et al., 2018). Numerous microorganisms have been shown to produce CA and comprise *Absidia sp., Botrytis, Acremonium, Eupenicillium, Aspergillus* and *Penicillium sp*. However, approximately 80% of the world's CA is generated mainly by *Aspergillus niger*. *A. niger* has typically been used, due to its exclusive physiological characteristics for industrial CA fermentation. This microbe exhibits polymer degrading enzymes that hydrolyze polymeric substrates, enabling rapid growth and fermentation on several low-cost substrates. In addition, it tolerates extreme acidic environments and outcompetes other microorganisms, thereby reducing the risk for contamination (Behera et al., 2021). *A. niger* shares a phylogenetic relationship with several *Aspergillus* genomes that include *Aspergillus tubingensis, Aspergillus brasiliensis* and *Aspergillus japonicus* (Varga et al., 2007). More specifically, *A. brasiliensis* isolates produce tensidol A and B, naphtho-gamma-pyrones and share this with *A. tubingensis* and *A. niger*. On the contrary, there are also various other unique compounds that *A. brasiliensis* produces, justifying its separation into a separate species from *A. niger* (Varga et al., 2007), while still demonstrating high potential for CA production. Citric acid fermentation occurs via the citric acid cycle (TCA) in microorganisms. During the bioconversion process, the production of organic acids helps to regulate cytoplasmic pH (3 to 3.5) for optimal cell growth (Odu et al., 2020). Approximately 2 million tons of CA is generated annually and its rate of demand is expected to rise by 5% each year (Ozdal et al., 2019, Sekoai et al., 2018). The CA market is constantly under pressure due to expensive substrates and low production yields (Sekoai et al., 2018). Additionally, the cost

of CA production via chemical synthesis is approximately ~$10 USD per kg, whereas for fungal fermentation it has been estimated to be ~50% lower (Ozdal et al., 2019). The global market for CA is expected to surpass $3.2 billion USD by 2023 (Behera et al., 2021). More importantly, the CA global demand has surpassed the natural supply, therefore the incorporation of lignocellulosic-based biotechnological processes that are novel, low-cost, efficient, and environmentally friendly are urgently required. Lignocellulosic biomass is a naturally occurring complex composite that originates from plant dry matter (Moodley et al., 2020), and has emerged as an ideal feedstock for generating renewable bioproducts such as CA. This is attributed to its abundance and cost-effectiveness. In addition, LCB is considered a waste and therefore does not pose any risk to socio-economic concerns such as deforestation, food security and water shortages (Sekoai et al., 2019). Recently, CA has been derived from lignocellulosic substrates that include banana peels (Odu et al., 2020), sweet potato peels (Aboyeji et al., 2020), and sugarcane bagasse (Campanhol et al., 2019). Banana pseudostem with a composition of cellulose (42.35%), hemicellulose (22.63%), lignin (15.36%) represents a novel, cost-effective and abundant substrate for generating CA (Islam et al., 2019). However, lignocellulosic CA production at an industrial scale is prone to several drawbacks that include lack of suitable feedstocks, expensive chemicals and/or nutrients as well as energy intensive processes that often result in low product yields. Therefore, overcoming the aforementioned bottlenecks is imperative to enhance lignocellulosic CA production. For one, finding an alternative to fresh water within lignocellulosic bioprocesses could substantially reduce the burden placed on this finite resource and costs incurred. Taking this into consideration, untreated wastewater from the Kraft and dairy industries represents a sustainable and cost-effective option for the replacement of fresh water and/or chemicals within lignocellulosic bioprocessing systems. The emergence of waste-based technologies for lignocellulosic CA production combined with simultaneous saccharification and fermentation (SSF) processes

could reduce the time, expenditure for operation, energy input, risk of contamination and enhance the overall productivity and product yields. For instance, a Kraft paper wastewater pretreatment has been optimized on BP and demonstrated a high glucose yield (0.413 g/g) (Laltha et al., 2022). Similarly, fermentation medium constituents that are required to satisfy the nutritional needs of microbes are costly. More specifically, the microbial growth of *Aspergillus sp.* typically occurs in the Sabouraud Dextrose Emmon's modification (SDE) media. The media constituents of SDE mainly include peptone and glucose that function as nitrogen and carbon sources, respectively for the microorganism. While the glucose may be supplied by the lignocellulosic substrate, peptone is an expensive constituent estimated at $151 USD per kg, contributing majorly to the entire bioprocessing cost. Therefore, the investigation of a cost-effective nitrogen source is imperative. Ammonium nitrate has an estimated cost of $108 USD per kg and may be employed as a cheaper alternative to peptone, translating to a cost reduction of ~28%. Odu et al. (2020), Sarkar and Das (2017) and Sekoai et al. (2018) previously utilized ammonium-based salts (ammonium nitrate or ammonium sulfate) in the range of 0.1 to 8% w/v and demonstrated its adequacy as a nitrogen source in CA production. Other sources of nitrogen such as urea, corn steep liquor (CSL) and yeast extract are also considered significantly cheaper than peptone and have previously been successfully employed for CA production (Sekoai et al., 2018). In addition, several studies have reported on the improvement of CA yields by the addition of desorbents such as methanol and acetone in the range of 1 to 4% (Sarkar and Das, 2017, Odu et al., 2020). Desorbents have been shown to enhance CA production by improving the cell permeability (Sekoai et al., 2018). In addition to these nutritional components, temperature has a major influence on the cellulase-based enzyme and fungal culture in lignocellulosic CA production. Fungi have been shown to thrive under various temperatures ranging from 25 to 45 °C (Aboyeji et al., 2020), while cellulase-based enzymes require temperatures between 45 to 50 °C for optimal functioning. Exploring a

suitable, intermediate temperature that can provide optimal conditions for both the enzyme and microbial culture functioning is crucial in lignocellulosic bioprocessing. Therefore, optimizing input parameters that comprise of temperature, nitrogen and desorbent concentration may prove valuable for the improvement of CA production. The response surface methodology (RSM) is a mathematical model utilized for the optimization process and determines the interactive effects of input parameters and their corresponding output (Gaitonde et al., 2017). Apart from bioprocess optimization of key parameters, kinetic modelling provides an in-depth understanding of the metabolic nature of a fermentation process (Germec et al., 2019).

Kinetic models play an essential role in predicting, monitoring, optimizing and simulating the performance of a process under varying process conditions (Pramanik et al., 2019). For instance, the logistic model is used to describe the kinetics of microbial biomass growth to the rate-limiting substrate over a certain time period (Germec et al., 2019). Conversely, the modified Gompertz model improves the understanding of kinetic processes relative to the logistic model (Pramanik et al., 2019). The modified Gompertz model estimates the maximum production rate, maximum concentration and the lag phase (Srimachai et al., 2015). Although SDE media has shown to be effective for the cultivation of *Aspergillus sp.*, this medium consists of expensive constituents as highlighted above and uses fresh water, which is a finite resource globally. Ideally, the application of industrial wastes as a water replacement in SSF bioprocesses could alleviate water security. Interestingly, the dairy industry produces high quantities of wastewater (1.575×10^{12} L) that may be harnessed as a substitute for fresh water bioprocesses (Slavov, 2017). Dairy wastewater typically undergoes expensive treatment processes to reduce contaminants before it is expelled into the environment. From an industrial perspective, these treatments are considered an economical burden, due to the high costs and energy as well as the long processing times. Adding to this, is the release of greenhouse gas emissions that has severe negative implications on the environment (Gogoi et al., 2021).

Therefore, using DWW to replace fresh water in lignocellulosic bioprocesses also presents opportunities for commercial industries regarding wastewater disposal and treatment costs. Another striking characteristic of DWW is the presence of trace amounts of carbohydrates, soluble organic compounds, protein and nitrogen that could contribute to promoting the growth of the microbial culture (Kaur, 2021). Even though several studies have reported on the production of CA from different lignocellulosic substrates (Odu et al., 2020, Sarkar and Das, 2017, Aboyeji et al., 2020), there has been a scarcity of information on the effects of temperature, nitrogen concentration (ammonium nitrate) and desorbent concentration (acetone) on CA production using BP. Furthermore, the existing knowledge gap on kinetic data of *A. brasiliensis* growth and CA production with fresh water, wastewater and standard SDE media modified with pretreated banana pseudostem has attracted interest. Therefore, the present study aimed to model and optimize the SSF process using the RSM for CA production from pretreated BP. For the RSM optimization, the effect of acetone concentration, ammonium nitrate concentration and temperature on the CA production was determined. Subsequently, the modified Gompertz and logistic models were employed to ascertain the kinetics of *A. brasiliensis* CA production and growth. For the kinetic experiments, three bioprocesses consisting of the: (1) optimized SSF process with fresh water ($SSF_{optimizedFW}$), (2) $SSF_{optimizedFW}$ process conditions while substituting dairy wastewater in place of fresh water (SSF_{DWW}), and (3) Sabouraud Dextrose Emmon's medium adapted by substituting glucose with BP ($SSF_{SDEmodified}$) were assessed.

2. Materials and methods

2.1. Materials

The banana pseudostem substrate was procured, dried and milled according to Laltha et al. (2022). The chemical reagents utilized were acquired from Merck (South Africa), where suitable. The Cellic CTec2 enzyme (160 FPU/mL) harnessed for the SSF processes was supplied by Novozymes (Novozymes A/S, Denmark). The paper wastewater (pH ~8) together with the safety data sheet containing its chemical composition (Laltha et al., 2022) was provided by a Kraft paper and pulp mill (Mondi, Richards Bay, South Africa). The dairy wastewater employed was obtained from a regional dairy (Fairfield, Howick, South Africa) and its composition has been reported by David et al. (2022).

2.2. Revival and microbial development

The fungal strain employed in this study, *Aspergillus brasiliensis* ATCC16404 was obtained from ATCC® type cultures originating from Microbiologics Incorporated. The Microbiologics KWIK-STIK pack contained a protocol, ampoule of hydrating fluid, qualitative lyophilized microorganism pellet and an inoculating swab. The hydrating fluid was applied to the lyophilized pellet and aseptically swabbed onto SDE (Sabouraud Dextrose Emmon's) agar by using the three-way streak plating technique to facilitate colony isolation. The SDE agar medium formulation comprised of 20 g/L glucose, 20 g/L agar and 10 g/L peptone autoclaved at 121 °C for 15 minutes. The fungal strain was then incubated at 30 °C for 7 days. Preparation of a fungal spore suspension was carried out by the addition of 10 mL of sterilized distilled water that contained non-ionic surfactant (0.1% v/v Tween 80) by covering the fungal growth and using a spatula to scrape the spores into 100 mL of SDE broth (20 g/L glucose and 10 g/L peptone) incubated at 30 °C and 150 rpm for 7 days.

2.3. Short-term and long-term storage of culture

To ensure continuous longevity of the microorganism, the fungal strain was subcultured every 7 days by transferring a piece of inoculated agar containing mature fungal hyphae and spores onto SDE agar, which was thereafter subjected to incubation at 30 °C for 7 days. The previous growth plate was stored at 4 °C in the refrigerator. For long-term storage, *A. brasiliensis* was inoculated in glycerol stocks (10%, 20% and 50% v/v) and agar slants (double-strength medium) and stored at -80 °C and 4 °C, respectively.

2.4. Pretreatment of banana pseudostem

The powdered BP substrate was subjected to pretreatment using a previously optimized microwave-assisted paper wastewater method (Laltha et al., 2022). The BP substrate at a 30% (w/v) solid loading was submerged in 100 mL of paper wastewater and exposed to microwave heating at 800 W for 8 minutes. The pretreated BP biomass was filtered, washed and dried as stipulated by Laltha et al. (2022), prior to being used in the SSF bioprocess. The composition of the optimally pretreated banana pseudostem was previously determined to contain cellulose (17.06%), hemicellulose (13.04%) and lignin (5.9%) (Laltha et al., 2022).

2.5. Simultaneous saccharification and fermentation (SSF) process

2.5.1. Inoculum development

A. brasiliensis was grown on SDE agar as previously described in section 2.2. The grown suspension was diluted to a final spore concentration of 1×10^6 spores/mL. A Neubauer counting chamber (Neubauer, Germany) was used to determine the spore concentration

2.5.2. Sodium citrate buffer preparation

Sodium citrate buffer (0.05 M) was made up by combining 0.1 M citric acid and 0.1 M sodium citrate in distilled water. The pH was adjusted to 4.8 prior to autoclaving at 121 °C for 15 min.

2.5.3. Preliminary screening

Preliminary screening of three desorbents (3% v/v) (acetone, chloroform and propan-2-ol) and four nitrogen sources (2% w/v) (corn steep liquor, ammonium nitrate, yeast extract and peptone) were utilized to determine their individual effects on CA production. The SSF parameter ranges were designated based on a substantial literature review of related lignocellulosic fermentation studies (Sarkar and Das, 2017, Odu et al., 2020). The pretreated BP with a solid loading of 10% w/v, an inoculum size of 10% (v/v), and 10 FPU/g enzyme loading was added to an Erlenmeyer flask and brought up to a final volume of 50 mL using 0.05 M sodium citrate buffer. The desorbents (3% v/v) (acetone, chloroform or propan-2-ol) and nitrogen sources (2% w/v) (corn steep liquor, ammonium nitrate, yeast extract or peptone) were also added accordingly (See supplementary material). The SSF flasks constituted a working volume of 50 mL, incubated at 35 °C and 150 rpm (Ho and Hood, 2014) for a period of 144 h. Samples (1 mL) were aseptically collected every 24 h and centrifuged at 10 000 rpm for 5 min prior to CA quantification using an acid-base titration method (Ayeni et al., 2019).

2.5.4. RSM experimental design of the SSF process

The response surface methodology (RSM) model (Box-Behnken design) (Design Expert 7.0, Stat Ease Inc, USA) was employed for the optimization. The experimental design consisted of seventeen (17) SSF processes. The fermentation input parameters comprised of desorbent (acetone) concentration (1-5% v/v), nitrogen (ammonium nitrate) concentration (0.5-5% w/v) and temperature (30-40 °C), while the output was CA concentration (g/L). The SSF input parameters with its ranges were chosen based on an extensive literature survey (Sarkar and

Das, 2017, Sekoai et al., 2018, Odu et al., 2020) in combination with data curated from the screening of preliminary experiments. The resultant experimental CA concentration data fitted the RSM polynomial equations to assess the combined input parameter effects for the enhancement and generation of CA.

2.5.5. Citric acid production

The CA experiments were performed in 100 mL Erlenmeyer flasks comprising of a 50 mL working volume. A solid loading of 10% (w/v) of paper wastewater pretreated BP was added to each flask. In addition, 10 FPU/g Cellic CTec2 enzyme, 0.05 M sodium citrate buffer, and 10% v/v inoculum containing 1×10^6 spores/mL *A. brasiliensis* were aseptically added. The respective amounts of acetone (1-5% v/v) and ammonium nitrate (0.5-5% w/v) were added to each fermentation flask and incubated at the desired temperature (30-40 °C) as specified in the RSM experimental design (Table 1). The SSF processes were agitated at 150 rpm for a period of 96 h. Using aseptic techniques, the aliquots (1 mL) were sampled from the SSF flasks every 12-h and stored at -20 °C for further analysis.

2.6. Validation of the optimized SSF bioprocess and kinetic modelling

The variable conditions for optimal CA generation were determined and carried out to validate the developed RSM model. The SSF reaction working volume of 50 mL contained: 10% (w/v) pretreated BP, 1% (v/v) acetone, 0.5% (w/v) ammonium nitrate, with an inoculum size of 10% (v/v) and citrate buffer. This process was designated as $SSF_{optimizedFW}$. In addition, the $SSF_{optimizedFW}$ process was substituted with dairy wastewater in place of fresh water (SSF_{DWW}).

Table 1. Citric acid concentration determined for the SSF process on inputs of acetone, ammonium nitrate concentration and temperature

	Input variables			Output
Run	Acetone (v/v)	Ammonium nitrate (w/v)	Temperature (°C)	Citric acid concentration (g/L)
1	3	0.50	30	1.857 ± 0.121
2	1	0.50	35	14.408 ± 0.594
3	5	2.75	30	0.640 ± 0.005
4	3	2.75	35	9.093 ± 0.126
5	3	2.75	35	9.413 ± 0.162
6	3	0.50	40	1.153 ± 0.052
7	5	2.75	40	0.704 ± 0.033
8	1	2.75	30	2.433 ± 0.128
9	3	5.00	40	1.217 ± 0.063
10	5	0.50	35	6.275 ± 0.178
11	3	2.75	35	8.324 ± 0.059
12	3	2.75	35	5.571 ± 0.115
13	3	2.75	35	9.413 ± 0.231
14	5	5.00	35	6.852 ± 0.220
15	3	5.00	30	2.305 ± 0.098
16	1	5.00	35	12.679 ± 0.106
17	1	2.75	40	0.832 ± 0.143

The SSF_{DWW} process contained: 10% (w/v) pretreated BP, 1% (v/v) acetone, 0.5% (w/v) ammonium nitrate, with an inoculum size of 10% (v/v) and citrate buffer formulated using DWW. The DWW formulated citrate buffer was the same as described in section 2.5.2., with the substitution of DWW instead of fresh water. For comparison, the standard SDE medium was modified by substituting glucose with BP ($SSF_{SDEmodified}$) and consisted of 10% (w/v) pretreated BP, 1% (w/v) peptone, with an inoculum size of 10% (v/v) and citrate buffer. Each SSF process was therefore characterized as follows: (1) optimized SSF process with fresh water ($SSF_{optimizedFW}$), (2) $SSF_{optimizedFW}$ process conditions while substituting dairy wastewater in place of fresh water (SSF_{DWW}), and (3) SDE medium modified by substituting glucose with BP ($SSF_{SDEmodified}$). All SSF experiments were incubated at 150 rpm, 35 °C for 96 h. The

experimental controls were performed parallel to the $SSF_{optimizedFW}$, SSF_{DWW} and $SSF_{SDEmodified}$ and constituted the same conditions with the exception of inoculum, which was omitted. The aforementioned uninoculated controls were used as a reference to determine the initial sugar concentration at a certain time period corresponding to the test SSF experiments ($SSF_{optimizedFW}$, SSF_{DWW} and $SSF_{SDEmodified}$). All of the experiments were carried out in duplicate. Aseptically, sample aliquots (1 mL) were removed from the experimental reaction flask and its equivalent control every 12 h for 96 h. The samples were thereafter analyzed for CA, glucose concentrations and biomass as explained below.

2.7. Analytical methods

2.7.1. Citric acid concentration

SSF processes are dynamic, biological systems and by-products generated during the fermentation may sometimes cause interference when using methods encompassing titration. In the present study, CA concentration was primarily analyzed using the acid-base titration method employed by Ayeni et al. (2019) and thereafter verified by the Megazyme citric acid assay kit, product code (K-CITR) (©Megazyme, Wicklow, Ireland) (See supplementary material). In the acid-base titration analysis, the SSF samples collected from each time point were first centrifuged for 5 min at 10 000 rpm. Subsequently, 100 µL of the supernatant fluid was transferred to a flask containing phenolphthalein indicator (2 to 3 drops). A glass burette was rinsed with distilled water and then filled with the 0.01 M NaOH titrant. The burette was opened to allow few drops of the NaOH (base) titrant to react with the sample (acid) and shaken continuously to observe a change in colour (from colourless to light pink). Excess titrant was washed of the sides of the flask with distilled water. The endpoint of the reaction was determined when the colour change had occurred. Following this, the volume of NaOH utilized was read directly from the burette and substituted into the formula described by Ayeni et al.

(2019) to ascertain the amount of CA present. The corresponding number of moles of alkali NaOH required to neutralize CA, $C_3H_5O(COOH)_3$, was then determined based on the volume and concentration of NaOH solution utilized during the acid-base titration (See supplementary material) (Ayeni et al., 2019). For verification of the acid-base titration method described above, the CA concentration was measured by means of the Megazyme citric acid assay kit, product code (K-CITR) (©Megazyme, Wicklow, Ireland) corresponding to the specific instructions in the manufacturers protocol. The Megazyme citric acid assay kit is an enzyme-based analytical method that is specific. The CA content was analyzed spectrophotometrically using a SpectraMax ABS microplate reader (Molecular Devices, California, USA).

2.7.2. Glucose concentration

The glucose concentration of the control (uninoculated) and experimental kinetic SSF experiments were analyzed using the Megazyme glucose kit (K-GLUC) (©Megazyme, Wicklow, Ireland) and protocols detailed by the manufacturer. To determine the glucose utilization (%), the final glucose concentration (inoculated) was subtracted from the initial glucose concentration as per Eq. (1).

$$\text{Glucose utilization (\%)} = \frac{Initial\ glucose\ concentration\ (g/L) - Final\ gluose\ concentration\ (g/L)}{Initial\ glucose\ concentration\ (g/L)} \times 100 \quad (1)$$

Where the final glucose concentration (g/L) is derived from the experimental SSF flask while the initial glucose concentration (g/L) is attained from the uninoculated control flask at the exact moment in time.

2.7.3. Biomass concentration

The biomass concentration of *A. brasiliensis* was analyzed using the dry cell weight (g/L) as a function of the total spore count (spores/mL). The fungal isolate was grown for 7 days in SDE broth to reach exponential phase (10^6 to 10^7 spores/mL) and diluted accordingly (Vrabl et al., 2019). A 15 mL volume per diluted sample (1, 1/2 ,1/4, 1/8, 1/16 and 1/32) was centrifuged for 5 min at 10 000 rpm. All dilutions were performed in duplicate. Succeeding centrifugation involved discarding of the supernatant and drying of the biomass pellet at 60 °C until the mass was constant. The standard curve was developed by plotting the total spore counts (spores/mL) to the corresponding dry cell weights (biomass concentration in g/L) for each dilution. The spore counts (spores/mL) attained for the kinetic experiments were computed in the equation and extrapolated to ascertain the biomass concentration (g/L).

2.8. Kinetic modelling

2.8.1. The logistic model for A. brasiliensis growth

The logistic model's differential formula, stated in Eq. (2), was integrated to obtain Eq (3). Equation (3) considers the exponential and stationary growth phases. The logistic model Eq. (3) relates biomass (X) to the initial cell concentration (X_0), maximum cell concentration (X_{max}) and maximum specific growth rate (μ_{max}) during the exponential and stationary growth stages of *A. brasiliensis*.

$$\frac{dX}{dt} = \mu_{max}\left(1 - \frac{X}{X_{max}}\right)X \tag{2}$$

$$X = \frac{X_0 \exp(\mu_{max}t)}{1-\left[\left(\frac{X_0}{X_{max}}\right)(1-exp(\mu_{max}t))\right]} \tag{3}$$

2.8.2. The modified Gompertz model for citric acid production

Using the least squares approach, the CA data from the SSF processes ($SSF_{optimizedFW}$, SSF_{DWW} and $SSF_{SDEmodified}$) were fitted to the modified Gompertz model(CurveExpert V1.5.5, MyBiosource, Inc., USA). As indicated in Eq. (4), the modified Gompertz model depicts the link between the CA concentration (P), maximum CA production rate ($r_{p,m}$), potential maximum CA concentration (P_m) and lag time (t_L) from the start of fermentation to the exponential CA generation.

$$P = P_m \,.exp\left\{-exp\left[\frac{r_{p,m}.exp(1)}{P_m}\right].(t_L - t) + 1\right\} \quad (4)$$

3. Results and discussion

3.1. Preliminary screening

Preliminary screening data revealed that the desorbent that gave the highest concentration of 10.886 g/L CA was acetone, followed by propan-2-ol (10.245 g/L) and chloroform (7.044 g/L) (See supplementary material). For the nitrogen source, ammonium nitrate resulted in the highest CA concentration (12.807 g/L), while peptone, corn steep liquor and yeast extract resulted in CA concentrations of 9.605 g/L, 8.324 g/L and 7.684 g/L, respectively. Therefore, acetone and ammonium nitrate were selected as the desorbent and nitrogen source, respectively, for the SSF optimization.

3.2. Development of the RSM model

To assess the fitness of the developed RSM model, Analysis of Variance (ANOVA) was used (See supplementary material). P-values < 0.05 indicate parameter and model significance, whereas high F-values demonstrate that the response trends could be explained using the regression equations (Chaganti et al., 2012). The model had a low P-value of 0.0063, which was < 0.05, indicating model significance. In addition, a high F-value (7.89) was attained for

the SSF model, thus illustrating statistical significance of the model. Of the three key variables that were investigated, acetone concentration ($p = 0.0282$) demonstrated the most significant effect on the CA concentration compared to temperature ($p = 0.5812$) and ammonium nitrate concentration ($p = 0.9146$). Acetone serves as the desorbent and enhances cell permeability of the microorganism for nutrient sequestration and may also repress the enzyme 2-oxoglutarate dehydrogenase, thus increasing CA concentration (Sekoai et al., 2018, Maddox et al., 1986). This desorbent (acetone) is also a ketone that plays a vital role as an alternative metabolic fuel source for eukaryotes (Puchalska and Crawford, 2017). On the other hand, temperature is a key parameter for the optimum functioning of cellulolytic enzymes for the effective saccharification of cellulose to glucose that will be consumed by the microbe. Additionally, temperature also plays a significant role for regulating the internal temperature, growth, secondary metabolite production and activity of fungi such as *A. brasiliensis* (Tudzynski, 2014). The presence of nitrogen such as ammonium nitrate in the fermentation medium is essential for fungal growth since it facilitates cell adaptation to different niches and survival of adverse environmental conditions such as nutrient limitation (Tudzynski, 2014). The coefficient of determination (R^2) value is a statistic that measures the proportion of variation in the output values that can be attributed to the input variables. In general, R^2 values between 0.7 and 1 elicits model significance (Chaganti et al., 2012). The developed SSF model displayed an R^2 value of 0.91, indicating that 91% of the variation in the output variable (CA concentration) could be attributed to the input variables. The polynomial model Eq. (5) was generated for the CA concentration and describes the input parameters in relation to the response variable.

$$\text{CA concentration (g/L)} = 8.36 - 1.99A - 0.080B - 0.42C + 0.58AB + 0.42AC - 0.096BC + 0.61A^2 + 1.09B^2 - 7.82C^2 \quad (5)$$

where, A, B and C correspond to acetone concentration (v/v), ammonium nitrate concentration (w/v) and temperature (°C), respectively.

3.3. Effects of the input parameters on the citric acid concentration

The CA concentrations with corresponding input parameters are portrayed in Table 1. The SSF model displayed a maximum CA concentration of 14.408 g/L for run 2 (1% acetone, 0.5% ammonium nitrate and 35 °C). The median fermentation conditions (3% acetone, 2.75% ammonium nitrate incubated at 35 °C) exhibited similar CA concentration values within the range of 5.571 g/L to 9.413 g/L (run 4, run 5, run 11 run 12 and run 13). The lowest CA concentration recorded (0.640 g/L) was achieved with 5% acetone, 2.75% ammonium nitrate and incubated 30 °C for run 3. The RSM graphs that show the collaborative effects of the different input parameters and the resultant CA concentration is illustrated in Fig. 1A-C. The interactive effect of ammonium nitrate concentration and acetone concentration on the output CA concentration, while temperature was kept at its center point (35 °C) is presented in Fig. 1A. Maximum CA concentration (12.63 g/L) was observed at a minimum acetone (1%) and ammonium nitrate concentration (0.5 %). Further increase in the acetone concentration from 1 to 5%, while ammonium nitrate concentration was maintained at 0.5% led to a decline in the CA concentration from 12.63 to 7.55 g/L. Simultaneous increments in the ammonium nitrate concentration (5%) and acetone concentration (5%) resulted in a decreased CA concentration of 10 g/L. The high CA concentration observed at a low ammonium nitrate concentration may be as a result of reduced vegetative growth of *A. brasiliensis*. On the other hand, the lower CA observed at higher nitrogen concentrations may be due to the shift in the cell metabolism towards fungal growth instead of product formation (Show et al., 2015). *Aspergilli* can utilize a variety of nitrogen sources such as ammonium, peptone and nitrate.

The fungus converts nitrate to ammonium, which is then incorporated into the amino acids glutamate and glutamine, that act as nitrogen donors within the TCA cycle (Krappmann and

Braus, 2005). Furthermore, during CA fermentation, nitrogen limitation is a key requirement to prevent the accumulation of oxalic acid, since this compound has been known to lead to lower CA production. While desorbents such as acetone have shown to enhance fungal growth and CA production, elevated concentrations can be toxic to the cells and may inhibit the fungal cellular metabolic functioning, thus resulting in a lower CA concentration. A similar result was obtained in an earlier study by Roukas and Harvey (1988) when methanol was used as a desorbent. The authors achieved a maximum CA concentration of 4.83 g/L from beet molasses at a methanol concentration of 2%, while concentrations beyond this value led to a decrease in CA production and concluded that methanol's inductive effect was caused by its inhibitory effect on metal ions. Similarly, the study by Sarkar and Das (2017) varied ammonium nitrate (0.1 to 0.5%) and desorbent concentration (0 to 5%) on pineapple waste and obtained an optimal CA concentration of 44.1 g/L by using 15% sucrose, 0.25% ammonium nitrate, 4% methanol and 7 days of incubation at 30 °C.

The impact of temperature and acetone concentration on the CA concentration while the ammonium nitrate concentration was maintained at its median value (2.75%) is represented in Fig. 1B. An increase in temperature (30 to 35 °C) coupled with the lowest acetone concentration (1%) led to an increase in the CA concentration from 4.08 to 10.94 g/L. Further increments in the acetone concentration and temperature > 1% and > 35 °C, respectively resulted in a steep decrease in the CA concentration from 10.94 to 2.1 g/L. Temperature has a dual effect on SSF processes due to its influence on both the enzymatic and microbial metabolic processes. With regards to enzymatic saccharification, temperature plays a key role on the enzymatic activity and saccharification efficiency for sugar release (El-Imam and Du, 2014). Temperature within the range of 45 and 50 °C has been shown to facilitate effective cellulase enzymatic saccharification, however the combined SSF system necessitates a median temperature that stimulates enzyme and microbial activity. Nevertheless, high-performing

cellulase cocktail enzymes such as Cellic CTec2 has been specifically developed to withstand adverse environmental conditions and sustain its activity (Champreda et al., 2019). On the other hand, the temperature parameter also has a profound effect on the microbial growth of fungi. *Aspergillus sp.* are mesophilic microorganisms that exhibit wide growth ranges between 25 to 45 °C (Aboyeji et al., 2020). Hence, to ensure optimal enzyme function and microbial growth, the temperature employed in the SSF process ranged between 30 and 40 °C. In Fig 1B, the highest CA concentration (10.94 g/L) was observed at a temperature of 35 °C. Likewise, Zhang et al. (2018) examined the influence of temperature on the growth of bacteria and fungi and determined that the growth activity increased at 35 °C. A high concentration of CA was observed when a low concentration of desorbent was employed (1%). In the same vein, Moyer (1953) studied the effects of ammonium chloride and methanol concentration for the synthesis of CA from corn meal and concluded that a low desorbent concentration (2%) enhances the citrate synthase activity while decreasing the aconitase activity during CA fermentation. Citrate synthase is a central enzyme involved in sugar oxidation and is also responsible for catalyzing the TCA cycle, whereas aconitase catalyzes the isomerization of citrate to isocitrate within the TCA cycle (Papagianni, 2007, Show et al., 2015). While desorbents improve the activity of enzymes involved in the TCA cycle, they are inhibitory above the threshold levels (> 5%) and could potentially lead to the degradation of these key enzymes and inhibit the growth of microorganisms by drastically altering the pH of the medium. Similarly, Maddox et al. (1986) utilized a low desorbent concentration (1%) and observed a maximum CA concentration of 25 g/L using galactose as a substrate. Furthermore, in the absence of methanol, very little CA was produced (less than 1 g/L). Kareem et al. (2010) evaluated the effect of sucrose, ammonium nitrate and methanol on CA production using fermentation medium consisting of pineapple waste and achieved optimum CA (60.61 g/L) under 15% sucrose, 0.25% ammonium nitrate and 2% methanol when fermented for 5 days at 30 °C. The aforementioned author also noted

that further increases in the methanol concentration (> 3%) led to a diminished fungal growth and lower CA production (Kareem et al., 2010). The pairwise interaction of temperature and ammonium nitrate concentration on the CA concentration when the acetone concentration was retained at its midpoint value (3%) is demonstrated in Fig. 1C. A simultaneous increase in temperature (30 to 35 °C) and ammonium nitrate concentration (0.5 to 5%) resulted in a minor increase in CA concentration from 8.65 to 9.33 g/L. Similar to the interactive effect of temperature and acetone concentration as shown in Fig. 1B, it is interesting to note that increases in the temperature parameter above 35 °C, led to a drastic decline in CA concentration from the optimal value (9.65 g/L) to approximately 2 g/L. Several factors influence fungal growth; however, temperature has shown to have a drastic effect that could potentially dictate the output and quality of the desired end-product. Temperature also influences the growth of hyphae and the activity of extracellular enzymes associated with the TCA cycle. At high temperatures (> 35 °C), hyphal deterioration may occur and result in a critical reduction of CA (Zhang et al., 2018). Nitrogen concentration has been found to have a strong influence on both cellular metabolism and CA production. Owing to the necessity of employing low-cost substrates in order to reduce CA manufacturing costs and make it more ecologically sustainable, glucose derived from pretreated banana pseudostem makes it a valuable carbon source. By increasing the initial glucose concentration, the specific rate of CA production rises (Papagianni et al., 1999), whereas concentrated ammonium nitrate inhibits the growth and metabolism of fungi (Veverka et al., 2007). Veverka et al. (2007) investigated the sensitivity of fungal growth to two different nitrogen sources (urea and ammonium nitrate). It was concluded that ammonium nitrate (0.06 M) had the most notable effect on the growth of *A. niger* with an increase of 1.5-fold greater in comparison to urea (0.19 M). The type of nitrogen source utilized is important due to specific properties that affect the fungal growth and synthesis of CA (Show et al., 2015). For example, the absorption of ammonium by germinating

spores release protons, which lowers the pH of the fermentation medium and results in an improvement in the CA production.

The low pH encountered during the production phase (~2) lowers the risk for contamination by unwanted microorganisms and impedes the development of undesirable organic acids such as oxalic and gluconic acids, thereby impacting on downstream processing by making product recovery easier.

3.4. Validation of the RSM model

Validation of the developed SSF model was carried out by utilizing the optimum process conditions (1% acetone, 0.5% ammonium nitrate and 35 °C). At these setpoints, the model predicted an optimal CA concentration of 12.712 g/L. Experimental validation of these parameters displayed a slightly higher CA concentration of 13.703 g/L, translating to a difference of 7.23% when compared to the models predicted value and was considered negligible. This indicates high accuracy and precision of the developed RSM model with a strong reliability for prediction. The validation parameters established by the RSM model were applied to two other fermentation processes consisting of dairy wastewater in place of fresh water (SSF_{DWW}) and SDE medium adapted by substituting glucose with BP ($SSF_{SDEmodified}$). A comparative assessment between these three SSF processes are detailed under Sections 3.5 and 3.6.

3.5. Comparison of the citric acid production to previous reports

The results obtained from the $SSF_{optimizedFW}$, SSF_{DWW} and $SSF_{SDEmodified}$ bioprocesses were compared to earlier reports on CA production using dairy waste and lignocellulosic biomass (See supplementary material). The $SSF_{optimizedFW}$, SSF_{DWW} and $SSF_{SDEmodified}$ processes achieved CA concentrations of 12.967, 13.095 and 13.991 g/L respectively. The present study revealed higher CA concentrations compared to Aboyeji et al. (2020) (4.36 g/L), Ghanbartabar et al. (2016) (4.45 g/L), and Campanhol et al. (2019) (0.73 g/L) when *A. niger* was utilized for the fermentation of sweet potato peel waste, cheese whey biomass and sugarcane bagasse, respectively. Conversely, Odu et al. (2020), Sarkar and Das (2017) and Ozdal et al. (2019) investigated banana peel, pineapple waste and sugar beet molasse, respectively, for the generation of CA and observed higher CA concentrations when compared to the present study.

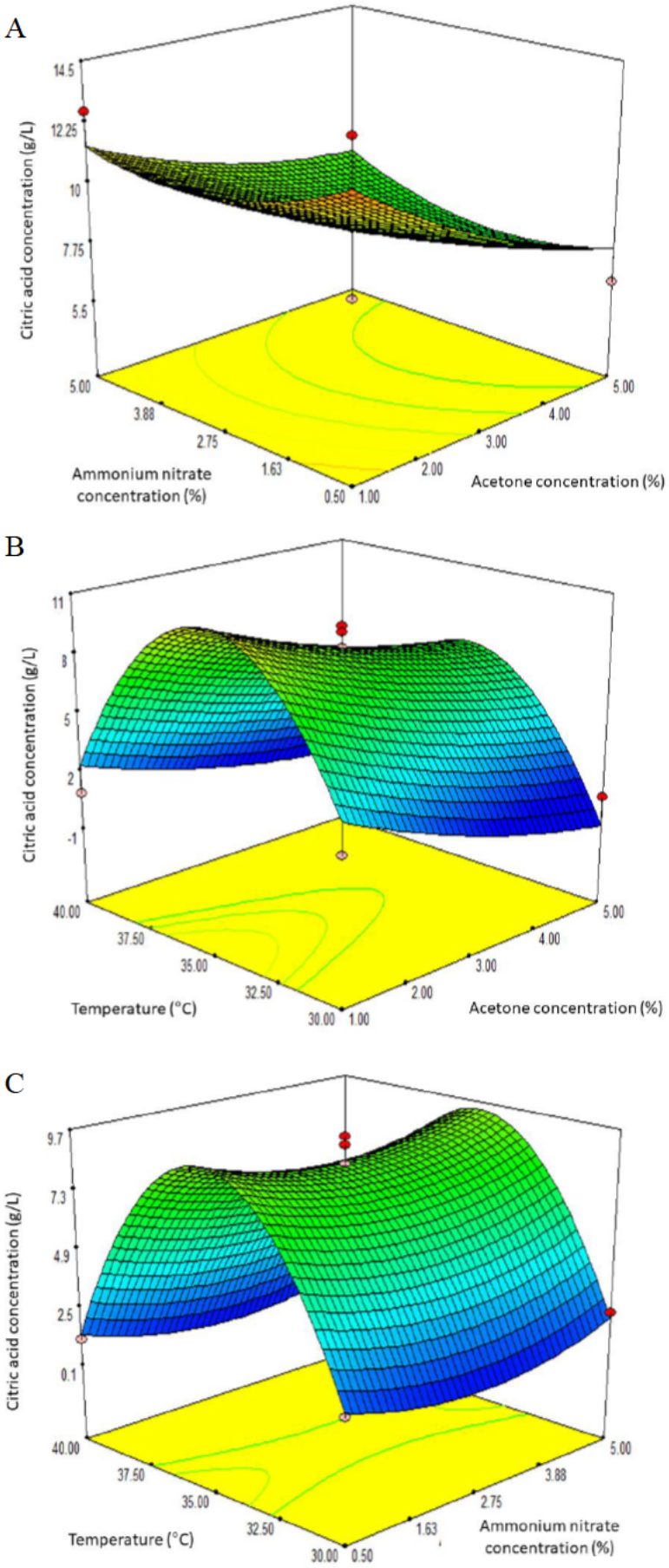


Fig. 1. Three-dimensional response surface graphs showing the interactive effects of the (A) Ammonium nitrate concentration (%) and acetone concentration (%), (B) Temperature (°C)

and acetone concentration (%), and (C) Temperature (°C) and ammonium nitrate concentration (%) on the citric acid concentration.

The variations in this study to previously reported literature may be due to different conditions applied that include: the microbial species (inoculum size, strain), physical parameters (temperature, agitation, pH), the concentration and type of enzyme, desorbent type and concentration, nitrogen source and concentration, substrate and solid loading, in addition to the supplementation of chemical additives. Despite the high concentrations of CA derived from previous reports, the exploitation of fresh water consumption, expensive substrates, chemical supplementation and/or media components render these fermentation processes non-viable and costly for potential industrial scale implementation.

3.6. A. brasiliensis growth kinetics for the $SSF_{optimizedFW}$, SSF_{DWW} and $SSF_{SDEmodified}$ processes

The kinetics of microbial growth and CA production for the SSF bioprocesses ($SSF_{optimizedFW}$, SSF_{DWW} and $SSF_{SDEmodified}$) were determined by utilizing the logistic and modified Gompertz models, respectively. The lag phase, often known as the microorganism's adaption phase occurs when the microbe begins to acclimatize to changes in the new medium environment and for sufficient glucose to be produced by saccharifying enzymes to support the growth of the microorganism (Asaduzzaman, 2007). The lag phase was observed during the first 12 h of fermentation. In addition, the microorganism is metabolically active and synthesizes the required enzymes and metabolites for exponential cell growth (Asaduzzaman, 2007). During the lag phase, the activation of signaling pathways and transcriptional changes occur, leading to lipopolysaccharide biosynthesis and respiration that is required for multiplication and differentiation of the cells (Hamill et al., 2020). A minor lag phase for conidia germination might last several hours, however temperature and stressors can extend this timeframe to days

(Hamill et al., 2020). The lag phase of *A. brasiliensis* has been shown to be within the range of 12 to 24 h in a previous study, where it was cultivated in media containing glucose (Ho and Hood, 2014). Similar lag phase times (5 to 10 h) has been associated with the commonly used and closely related *A. niger* for CA production (Baei et al., 2008). The exponential phase or logarithmic phase transpires when the microbial culture undergoes asexual reproduction at a constant rate through simple cell division, in which one cell separates forming two identical daughter cells after undergoing nuclear division (Ellena et al., 2020, Asaduzzaman, 2007). The exponential phase was recorded between 12 and 84 h for all three SSF processes. In general, the exponential phase is initiated by a particular cell concentration that increases metabolism and initiation of DNA replication (Asaduzzaman, 2007). The enzymatic conversion of cellulose to glucose released by cellulase action occurs almost instantaneously during SSF processes and is utilized by the microbial culture (in this case *A. brasiliensis*) within the TCA cycle for the generation of CA. A high glucose utilization was observed for the $SSF_{optimizedFW}$ (50%), SSF_{DWW} (44%) and $SSF_{SDEmodified}$ (57%) processes (Fig. 2B) and this corresponded to an increase in the fungal growth (1 – 2.5 g/L) (Fig. 2A). The biomass concentration began to stabilize after 84 h for all three SSF bioprocesses. The decline in cell growth above 84 h can be attributed to the decline in Cellic CTec2 enzyme activity that results in a lower saccharification efficiency and thus lower glucose release for the fermentative microorganism. Furthermore, the functioning of enzymes occurs best within a certain designated temperature (45-50 °C) and pH (4.5-5.5) range (Fenila and Shastri, 2016). Therefore, sub-optimal conditions for enzyme functioning under a low temperature (35 °C) and pH (~3.5) required for the initial production of CA, causes a ripple effect on the SSF process, since the enzyme loses its activity and ability to bind to an active site on the substrate. *Aspergillus brasiliensis* growth data obtained from the $SSF_{optimizedFW}$, SSF_{DWW} and $SSF_{SDEmodified}$ processes were inserted into the logistic model and displayed high R^2 values of 0.997, 0.998 and 0.997, respectively. All

three bioprocesses demonstrated the same maximum specific growth rate (μ_{max}) of 0.05 h^{-1}. The maximum specific growth rate is an important kinetic parameter due to its impact on product yield, formation and productivity (Srimachai et al., 2015, Pramanik et al., 2019) . The low μ_{max} values observed may be accounted for by the depletion of nutrients such as glucose or nitrogen for cellular maintenance within the fermentation media. The concentration and availability of nitrogen influences the activity and growth efficiencies of fungi. When nitrogen is scarce within the media, fungi divert their metabolism towards generating more energy for the production of extra-cellular enzymes such as hydrolase and oxidase to sequestrate nitrogen from the surrounding environment, resulting in lower growth efficiencies (Tudzynski, 2014). The maximum (X_{max}) and initial (X_0) biomass cell concentrations are essential indicators for the synthesis of biomass and formation of products (Baei et al., 2008). Kinetic data obtained for the $SSF_{optimizedFW}$, SSF_{DWW} and $SSF_{SDEmodified}$ processes gave X_0 concentration values of 0.19 g/L, 0.15 g/L and 0.21 g/L, respectively. Similar to the X_0, the highest X_{max} was realized for the $SSF_{SDEmodified}$ (2.91 g/L) process followed by the $SSF_{optimizedFW}$ (2.79 g/L) and SSF_{DWW} (2.74 g/L) processes. Extracellular enzymes produced during fermentation are important for the dissolution and absorption of external substances that contribute to the growth of *Aspergillus*. The enzymes secreted during growth are related to the lignocellulosic substrate, while temperature fluctuations influence extracellular enzyme activity (Zhang et al., 2018). The marginally lower X_{max} values reported for the $SSF_{optimizedFW}$ and SSF_{DWW} processes in comparison to the $SSF_{SDEmodified}$ may be attributed to variations in the media composition (nutrients) or metabolic dynamics. The citric acid cycle begins when acetyl CoA transfers its acetyl group to oxaloacetate to create citrate. In the citric acid cycle, carbon dioxide is released in tandem with the reduction of NAD^+ to NADH. However, increased levels of carbon dioxide can be detrimental to the growth of fungal biomass and reduce the concentration of citrate produced (Show et al., 2015). Although peptone and ammonium nitrate are sources of nitrogen,

they vary in the sense that the former is organic, while the latter is inorganic. Furthermore, peptone contains growth factors. A preference for the organic source may indicate a requirement for an amino acid or vitamin present in the organic source. Nevertheless, the addition of amino acids and vitamins to a nutrient medium containing inorganic nitrogen is deemed expensive (Clarke, 2013). Baei et al. (2008) evaluated the potential of *A. niger* for the production of CA from apple pomace under process conditions of 0.1 g/L potassium ferrocyanide, 1.5% (w/v) calcium triphosphate, 2.5 g/L ammonium sulfate and 3 mL methanol, and recorded a lag phase of 5 to 10 h in addition to a μ_{max}, X_0 and X_{max} of 0.015 h^{-1}, 0.04 g/L and 22 g/L, respectively. Discrepancies detected in growth kinetic data between the present study and Baei et al. (2008) may be credited to several aspects such as substrate type, microbial strain, nutrient availability, heat and mass transfer, aeration, agitation, pH, solid and enzyme loading.

3.7. Citric acid kinetics for the $SSF_{optimizedFW}$, SSF_{DWW} and $SSF_{SDEmodified}$ processes

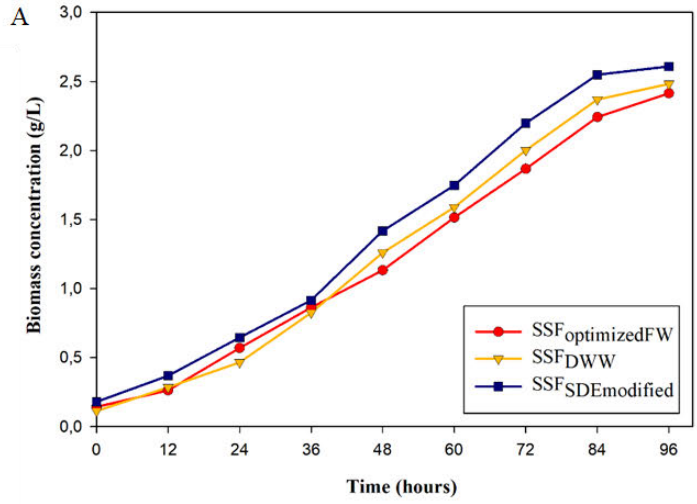
CA production during the $SSF_{optimizedFW}$, SSF_{DWW} and $SSF_{SDEmodified}$ processes, were elucidated over time (Fig. 2C). Similar to an increase in biomass concentration, the exponential CA production occurred from 12 h to 84 h, with a plateau observed from 84 h onwards, indicating the initiation of the stationary phase. A lack of essential resources, such as carbohydrates generally initiates the stationary phase of microbial development. In all three bioprocesses, it was observed that CA increased from the exponential phase and continued into the stationary phase. Citric acid increased from approximately 0.5 to 13 g/L for the $SSF_{optimizedFW}$ process and from 0.6 to 13.1 g/L for the SSF_{DWW} system, while the $SSF_{SDEmodified}$ bioprocess observed the highest CA increase from 0.58 to 14 g/L within the 0 h to 96 h time period (Fig. 2C). Citric acid data obtained for the three SSF processes coincided with the exponential phase of the *A. brasiliensis* cell growth (Fig. 2A) and a high glucose utilization (9.77 to 56.79%) (Fig. 2B).

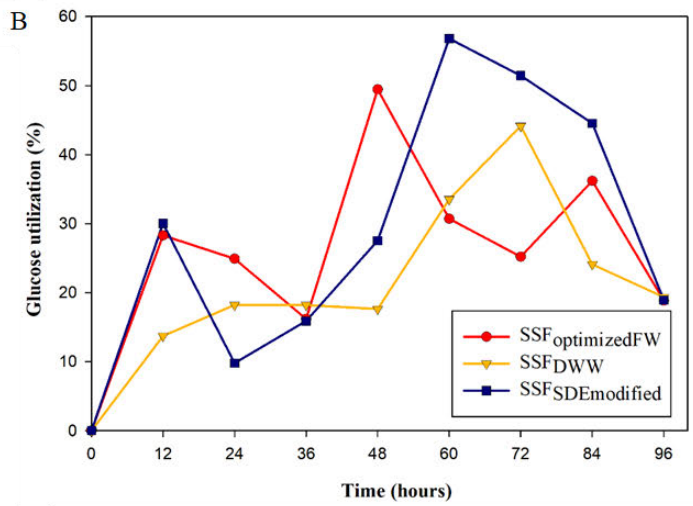
During the exponential phase that occurs within 12 h to 84 h, sufficient time is available for the enzyme to saccharify the BP substrate to release enough glucose to sustain the metabolism of the fungal strain, leading to primary metabolite production of CA. The cellulase-based Cellic CTec2 utilized for enzymatic saccharification is a cocktail of enzymes that act collaboratively on cellulose thus converting it to glucose. Cellic CTec2 exhibits high saccharification efficiencies (β-glucosidase activity) and is less vulnerable to glucose inhibition (Champreda et al., 2019). Furthermore, the buildup of glucose in the fermentation medium enhances the activity of glucose oxidase resulting in improved CA concentrations. The decline in *A. brasiliensis* growth observed after 84 h, overlaps with a lower glucose utilization for the $SSF_{optimizedFW}$ (36.2 to 18.8%), SSF_{DWW} (24.1 to 19.3%) and $SSF_{SDEmodified}$ (44.5 to 18.9%) processes (Fig. 2B). The experimental CA data obtained for the $SSF_{optimizedFW}$, SSF_{DWW} and $SSF_{SDEmodified}$ experiments fitted the modified Gompertz model exhibiting high R^2 values of 0.991, 0.993, and 0.992, respectively. The shortest lag time (t_L) was obtained for the $SSF_{SDEmodified}$ (5.11 h) process followed by the SSF_{DWW} (9.80 h) and $SSF_{optimizedFW}$ (16.47 h) systems. The $SSF_{SDEmodified}$ process consisted of peptone as the nitrogen source, thus the shorter t_L achieved can be attributed to the incorporation of amino acids and vitamins compared to the $SSF_{optimizedFW}$ and SSF_{DWW} systems that utilized ammonium nitrate. Ammonium nitrate has been shown to be approximately 25% cheaper than peptone, however the latter incorporates growth factors that is fundamental for rapid growth of *Aspergillus sp.* and CA metabolism (Clarke, 2013). Furthermore, nitrogen contributes to a change in pH, therefore allowing the microorganism to grow and adapt to the fermentation medium (Tudzynski, 2014). Nitrogen is exploited by *Aspergillus* for the generation of amino acids such as glutamine and glutamate that act as nitrogen donors within the TCA cycle (Krappmann and Braus, 2005). On the other hand, the addition of the acetone desorbent in the $SSF_{optimizedFW}$, and SSF_{DWW} processes, may increase cell permeability to citrate thus increasing CA production via repression of the 2-

oxoglutarate dehydrogenase enzyme compared to the $SSF_{SDEmodified}$ system (Maddox et al., 1986). The availability of initial carbohydrates (sugar polymers) in solution may also enhance the cellulase enzyme activity at the onset of fermentation for the accumulation of monomeric sugars (glucose). The glucose utilization in the first 12 h of fermentation for the $SSF_{optimizedFW}$, SSF_{DWW} and $SSF_{SDEmodified}$ processes corresponded to 28.3%, 13.7% and 30%, respectively. Therefore, the lag phase during CA fermentation is dependent on nutrient and sugar availability. Interestingly, the lag times observed in this study were shorter when compared to data recorded in the previous reports by Dienye et al. (2018) and Odu et al. (2020) that observed lag times of 24 h for CA production. Maximum CA production rates ($r_{p.m}$) were reported for the $SSF_{optimizedFW}$ and SSF_{DWW} (0.17 g/L/h) processes, followed by a slightly lower $r_{p.m}$ for the $SSF_{SDEmodified}$ (0.16 g/L/h) process. These $r_{p.m}$ values indicate that 0.17 g/L and 0.16 g/L of CA can be generated by *A. brasiliensis* per hour. The $r_{p.m}$ observed for the present SSF processes may be linked to growth factors such as nutrients or trace elements. Trace elements (manganese, zinc and iron) have significant effects on the production of CA. Manganese is important for cell functioning, sporulation and for the generation of low-molecular weight secondary metabolites that provide protection from predation, environmental stress, communication, competition for resources and toxicity against other microorganisms (Show et al., 2015), whereas zinc and iron are required for growth and maintenance of the cell. Show et al. (2015) revealed that manganese deficiency negatively influences the anabolism of *A. niger*, resulting in a high intracellular ammonium concentration. Conversely, when sufficient nutrients are available, *Aspergillus sp.* germinate by switching from isotropic to polarized growth, which impacts their stress resistance and regulatory pathways involved in germination (Baltussen et al., 2020). Sugar molecules that trigger fungal germination include glucose, mannose and xylose. A carbon source coupled with either inorganic phosphate, magnesium sulphate or inorganic nitrogen are the minimum requirements for the germination of *A. niger*

(Ijadpanahsaravi et al., 2021). A striking characteristic of industrial wastes such as DWW, is the presence of key nutrients such as Mn (2.56 μg/L), Mg (< 0.63 mg/L), Fe (91 μg/L), Zn (21 μg/L), P (6.96 mg/L) and N (36 mg/L) (David et al., 2022), that may have positively influenced the maximum CA production rate within the SSF_{DWW} process. Another key kinetic parameter is the maximum potential CA concentration (P_m) that provides knowledge on the maximum CA that can be produced using these SSF strategies. The $SSF_{optimizedFW}$ process displayed the highest maximum potential CA concentration (P_m) (29.31) g/L, whereas the SSF_{DWW} and $SSF_{SDEmodified}$ processes exhibited lower P_m values of 18.87 and 21.50 g/L, respectively. High glucose utilization and CA concentrations corresponded with the X_{max} data obtained for the $SSF_{optimizedFW}$ and $SSF_{SDEmodified}$ processes. The lowest P_m value (18.87 g/L) attained from the SSF_{DWW} process may be linked to the lowest X_0 concentration of 0.15 g/L. During CA fermentation, *Aspergillus* obtain their energy through adenosine triphosphate (ATP) via the TCA (the CA) cycle. The addition of certain rate limiting nutrients such as nitrogen and phosphorus from DWW or a rise in pH may have a detrimental effect on the SSF process by inhibiting the activity of the proton pump in the plasma membrane (H^+ATPase), thereby reducing the amount of ATP produced. Also, at a high pH, a change in the rate of glucose uptake occurs, thereby resulting in a decreased biomass concentration and organic acid production (Vrabl et al., 2019). Even with the drawbacks of the developed SSF systems in the present study, these findings may contribute to a more economical and robust operation within lignocellulosic bioprocesses. This is mainly due to the incorporation of DWW as a substitute for fresh water usage, exploitation of banana pseudostem as an alternative to glucose, utilization of ammonium nitrate as a cost-efficient alternative to conventional peptone and by employing acetone with several beneficial properties during SSF induced CA production. The integration of these key elements could facilitate the biotransformation of waste to valuable bioproducts. A synergy between the academic, industrial and commercial sectors to develop

large scale waste-based processes could potentially alleviate the negative environmental effects and economical impacts, which is in line with global sustainable development goals to manage the water, energy and food nexus, and may bring forth the realization of a "waste-to-wealth" circular bioeconomy.





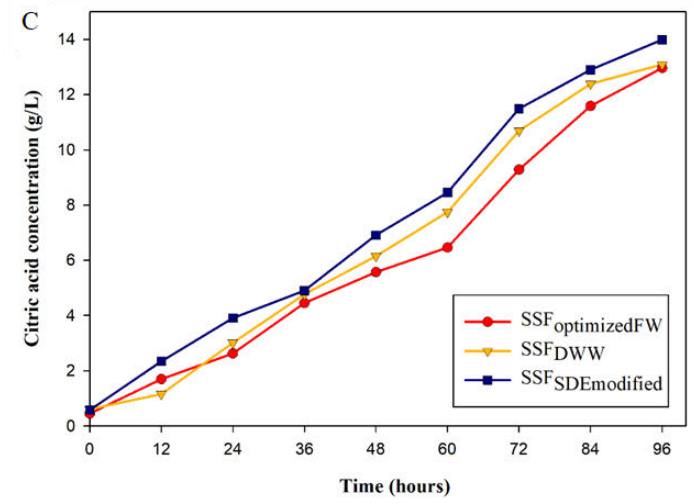


Fig. 2. *Aspergillus brasiliensis* ATCC16404 (A) biomass concentration, (B) glucose utilization and (C) CA concentration, for the $SSF_{optimizedFW}$, SSF_{DWW} and $SSF_{SDEmodified}$ processes.

4. Conclusion

This research presents an innovative optimization of the simultaneous saccharification and fermentation (SSF) bioprocess for citric acid (CA) production. Optimization gave a maximum CA concentration of 14.408 g/L. Afterwards, the kinetics of cell growth and CA production were determined. The $SSF_{SDEmodified}$ system resulted in the highest maximum potential CA concentration (P_m) (13.991 g/L) contrasted to the SSF_{DWW} (P_m= 13.095 g/L) and $SSF_{optimizedFW}$ (P_m= 12.967 g/L) bioprocesses. The findings from this research accelerates the development of waste-based bioprocesses, that could reduce the economical and environmental burden incurred by industries, while it is aligned with the water, energy and food nexus towards a circular bioeconomy.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version.

Credit authorship contribution statement

Milesh Laltha: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Data curation, Writing - original draft, Writing - review & editing, Visualization, Project administration, Funding acquisition. **Y. Sewsynker-Sukai:** Conceptualization, Methodology, Software, Validation, Writing – review & editing, Project administration, Resources, Supervision. **E.B. Gueguim Kana:** Methodology, Software, Validation, Writing – review & editing, Resources, Supervision.

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Chapter 4 - Supplementary material

Table S1. Preliminary screening of various desorbents and nitrogen sources to determine their effectiveness for citric acid production

Screening method	Chemical constituent	Input parameters	Citric acid concentration (g/L)
Desorbent	Acetone	3% (v/v)[a], 10% (w/v)[c], 10% (v/v)[d], 35 °C[e], 150 rpm[f], 144 h[g]	10.886
	Chloroform	3% (v/v)[a], 10% (w/v)[c], 10% (v/v)[d], 35 °C[e], 150 rpm[f], 144 h[g]	7.044
	Propan-2-ol	3% (v/v)[a], 10% (w/v)[c], 10% (v/v)[d], 35 °C[e], 150 rpm[f], 144 h[g]	10.245
Nitrogen source	Ammonium nitrate	2% (w/v)[b], 10% (w/v)[c], 10% (v/v)[d], 35 °C[e], 150 rpm[f], 144 h[g]	12.807
	Corn steep liquor	2% (w/v)[b], 10% (w/v)[c], 10% (v/v)[d], 35 °C[e], 150 rpm[f], 144 h[g]	8.324
	Peptone	2% (w/v)[b], 10% (w/v)[c], 10% (v/v)[d], 35 °C[e], 150 rpm[f], 144 h[g]	9.605
	Yeast extract	2% (w/v)[b], 10% (w/v)[c], 10% (v/v)[d], 35 °C[e], 150 rpm[f], 144 h[g]	7.684

Footnote: [a]=desorbent concentration, [b]=nitrogen concentration, [c]=substrate solid loading, [d]=inoculum size, [e]=temperature, [f]=agitation speed, [g]=time.

Table S2. Citric acid concentration determined for the SSF process using the Megazyme assay kit

Run	Acetone (v/v)	Ammonium nitrate (w/v)	Temperature (°C)	Citric acid concentration (g/L)	
				Megazyme assay	Acid-base titration
1	3	0.50	30	1.615	1.857
2	1	0.50	35	13.219	14.408
3	5	2.75	30	0.631	0.640
4	3	2.75	35	8.842	9.093
5	3	2.75	35	9.090	9.413
6	3	0.50	40	1.048	1.153
7	5	2.75	40	0.639	0.704
8	1	2.75	30	2.177	2.433
9	3	5.00	40	1.090	1.217
10	5	0.50	35	5.920	6.275
11	3	2.75	35	8.206	8.324
12	3	2.75	35	5.341	5.571
13	3	2.75	35	8.950	9.413
14	5	5.00	35	6.412	6.852
15	3	5.00	30	2.110	2.305
16	1	5.00	35	12.467	12.679
17	1	2.75	40	0.546	0.832

Table S3. Analysis of variance (ANOVA) of the developed SSF model for citric acid production

Source	Sum of Squares	Degrees of freedom *(df)*	Mean Square	F-value	p-value (probability > F)
Model	294.34	9	32.7	7.89	0.0063 Significant
A-Acetone	31.52	1	31.52	7.61	0.0282
B-Ammonium nitrate	0.051	1	0.051	0.012	0.9146
C-Temperature	1.39	1	1.39	0.33	0.5812
AB	1.33	1	1.33	0.32	0.5889
AC	0.69	1	0.69	0.17	0.6948
BC	0.037	1	0.037	8.9×10^{-3}	0.9275
A^2	1.54	1	1.54	0.37	0.5611
B^2	4.96	1	4.96	1.20	0.3101
C^2	257.17	1	257.17	62.07	0.0001
Residual	29.00	7	4.14		
Lack of fit	18.47	3	6.16	2.34	0.2150 Not significant
Pure error	10.53	4	2.63		
Cor total	323.34	16			

Footnote: A = Acetone (v/v), B = Ammonium nitrate (w/v) and C = Temperature (°C).

Table S4. Comparative assessment of citric acid derived from lignocellulosic and dairy wastes utilizing *Aspergillus sp*.

Substrate	Fermentative microorganism	Optimal process parameter conditions	Citric acid concentration (g/L)	References
Banana pseudostem ($SSF_{optimizedFW}$)	*Aspergillus brasiliensis* ATCC16404	10% (v/v)[a], 10% (w/v)[b], 1% (v/v) acetone[c], 0.5% (w/v) ammonium nitrate[d], 35 °C[e], 150 rpm[f], 96 h[g]	12.967	This study
Banana pseudostem (SSF_{DWW})	*Aspergillus brasiliensis* ATCC16404	10% (v/v)[a], 10% (w/v)[b], 1% (v/v) acetone[c], 0.5% (w/v) ammonium nitrate[d], 35 °C[e], 150 rpm[f], 96 h[g]	13.095	This study
Banana pseudostem ($SSF_{SDEmodified}$)	*Aspergillus brasiliensis* ATCC16404	10% (v/v)[a], 10% (w/v)[b], 1% (w/v) peptone[d], 35 °C[e], 150 rpm[f], 96 h[g]	13.991	This study
Dairy wastewater	*Aspergillus niger* ATCC9142	150 mL medium mixture[a,b], 30 °C[e], 200 rpm[f], 10 d[g], pH 3[h]	4.50	Kim et al. (2002)
Banana peel	*Aspergillus niger*	10 mL[a], 50 g[b], 3% (v/v) methanol [c], 0.5% (w/v) ammonium chloride[d], 30 °C[e], 10 d[g], pH 4[h], 2% (w/v) zinc[i]	97.60	Odu et al. (2020)
Pineapple waste	*Aspergillus niger*	6 x 10^6 suspension[a], 30 g[b], 4% (v/v) methanol[c], 0.25% w/v ammonium nitrate[d], 30 °C[e], 7 d[g], 15% (w/v) sucrose[i]	44.10	Sarkar and Das (2017)

Table S4. Continued

Substrate	Fermentative microorganism	Optimal process parameter conditions	Citric acid concentration (g/L)	References
Sweet potato peel waste	*Aspergillus niger*	5 d, 6 mm agar spores[a], 90 mL[b], 3% (v/v) methanol[c], 1.25% (w/v) ammonium nitrate[d], 25 °C[e], 7 d[g], pH 6.5[h]	4.36	Aboyeji et al. (2020)
Sugar beet molasse	*Aspergillus niger* EB12	1 mL[a], 150 g/L[b], 4g/L Chicken feather peptone[d], 30 °C[e], 200 rpm[f], 168 h[g], pH 6[h], 0.15 g/L K_2HPO_4[i]	68.80	Ozdal et al. (2019)
Cheese whey biomass	*Aspergillus niger* PTCC5010	50 g/L lactose[b], 0.5 g/L Ammonium iron (II) sulfate[d], 30 °C[e], 130 rpm[f], 6 d[g], pH 4[h], 0.1 g/L $MgSO_4$[i]	4.45	Ghanbartabar et al. (2016)
Apple pomace	*Aspergillus niger*	1 mL[a], 33.81 g/L[b], 2.05% (v/v) methanol[c], 42.5 g/L of corn steep liquor[d], 33 °C[e], 33 h[g], pH 4.54[h]	68.26	Sekoai et al. (2018)
Sugarcane bagasse	*Aspergillus niger* CCT4355	4% (v/v) ethanol[c], 30 °C[e], 144 h[g], 1% (w/v) sucrose[i]	0.73	Campanhol et al. (2019)

Footnote. [a]=inoculum size, [b]=substrate amount, [c]=desorbent concentration, [d]=nitrogen concentration, [e]=temperature, [f]=agitation speed, [g]=time, [h]=pH, [i]= chemical additive.

Table S5. Kinetic parameters of the microbial growth and citric acid production for the $SSF_{optimizedFW}$, SSF_{DWW} and $SSF_{SDEmodified}$ processes

Kinetic parameters	Fermentation process		
	$SSF_{optimizedFW}$	SSF_{DWW}	$SSF_{SDEmodified}$
μ_{max} (h^{-1})	0.05	0.05	0.05
X_o (g/L)	0.19	0.15	0.21
X_{max} (g/L)	2.79	2.74	2.91
P_m (g/L)	29.31	18.87	21.50
$r_{p.m}$ (g/L/h)	0.17	0.17	0.16
t_L (h)	16.47	9.80	5.11

Footnote: μ_{max} = maximum specific growth rate, X_0 = initial cell concentration, X_{max} = maximum cell concentration, P_m = maximum potential citric acid concentration, $r_{p,m}$ = maximum citric acid production rate, t_L=lag time.

Equations for computing CA concentration according to Ayeni et al. (2019):

Moles NaOH required = vol of NaOH required (mL) × conc. of NaOH (moles/L) × 10^{-3} (1)

The amount in moles of citric acid, $C_3H_5O(COOH)_3$, in the titrated sample was determined according to Eq. (2).

$$\text{Moles } C_3H_5O(COOH)_3 = \frac{\text{moles NaOH} \times 1 \text{ mol } C_3H_5O(COOH)_3}{3 \text{ moles NaOH}} \quad (2)$$

The concentration (molarity) of citric acid produced was calculated by Eq. (3).

$$\text{Concentration } C_3H_5O(COOH)_3 = \frac{\text{moles } C_3H_5O(COOH)_3}{volume\ C_3H_5O(COOH)_3} \quad (3)$$

The concentration of $C_3H_5O(COOH)_3$ g/L was determined by Eq. (4) and (5).

$$\text{Molar mass} = \frac{Concentration\ (g/L)}{Concentration\ (mol/L)} \quad (4)$$

Concentration of citric acid (g/L) = Concentration in mol/L × Molar mass (g/mol) (5)

CHAPTER 5

Conclusions and recommendations for further research

5.1. Conclusions

Lignocellulosic bioprocesses are currently challenged by high capital expenditure for water, energy and lab-based chemicals. Apart from these limitations, current bioconversion processes result in low product yields and has significant environmental consequences. Exploring low-cost lignocellulosic bioprocesses for the production of bioproducts will promote a sustainable circular bioeconomy, while overcoming these bottlenecks. Therefore, this study explored the valorization of banana pseudostem (BP) for citric acid (CA) production by developing novel pretreatment strategies and thereafter optimizing the simultaneous saccharification and fermentation (SSF) process to potentially alleviate the abovementioned bottlenecks. Important findings and their significance are summarized as follows:

5.1.1. The present study modelled and optimized two novel methods namely (1) microwave-assisted-iodized table salt (M-ITS) and (2) microwave-assisted-paper wastewater (M-PWW) strategy for the enhancement of sugar recovery from banana pseudostem. Optimization of pretreatment techniques revealed a slightly higher reducing sugar (0.515 g/g) and glucose yield (0.433 g/g) for the M-ITS strategy (2.48% ITS, 800 W and 10 min) compared to the M-PWW method (30% solid loading, 800 W and 8 min) (reducing sugar = 0.498 g/g and glucose = 0.413 g/g). Although the M-$PWW_{optimized}$ regime displayed a slightly lower RSY (3.3%) and GY (4.62%) compared to the M-$ITS_{optimized}$ strategy, it has been concluded to be a more effective approach, since it is waste-based, requires fewer pretreatment cycles and that generates three times the quantity of pretreated substrate required for subsequent fermentation. As a result, the M-$PWW_{optimized}$ method was selected for simultaneous saccharification and fermentation (SSF)

process optimization. The suitability of the developed and optimized pretreatment models were assessed and validated on various lignocellulosic substrates. These findings contribute to eliminating expensive lab-based chemicals and/or fresh water usage, while generating additional revenue.

5.1.2. This study modelled and optimized various fermentation input parameters that comprised of ammonium nitrate concentration, acetone concentration and temperature in the SSF process for optimal citric acid production from pretreated BP. The optimized SSF conditions (0.5% ammonium nitrate, 1% acetone and 35 °C) gave a maximum CA concentration of 14.408 g/L. Thereafter, kinetic assessment of *Aspergillus brasiliensis* growth and CA production were determined for the optimum conditions using fresh water ($SSF_{optimizedFW}$) or dairy wastewater (SSF_{DWW}) and compared to Sabouraud Dextrose Emmon's medium modified with BP ($SSF_{SDEmodified}$). Kinetic assessment revealed the same maximum specific growth rates (μ_{max}) (0.05 h^{-1}) for all three bioprocesses. The $SSF_{SDEmodified}$ system resulted in the highest maximum potential CA concentration (P_m) (13.991 g/L) compared to the SSF_{DWW} (P_m= 13.095 g/L) and $SSF_{optimizedFW}$ (P_m= 12.967 g/L) bioprocesses. Therefore, optimization of a waste-based media formulation approach for CA production can be channelled towards an industrial-scale trajectory. Furthermore, these findings facilitate the implementation of waste-based lignocellulosic bioprocesses for microbial-based value-added products while eradicating costly pretreatment chemicals, fermentation medium constituents and fresh water resources, in line with the food, energy and water nexus towards achieving a circular bioeconomy.

5.2. Recommendations for future studies

Based on the concluding findings from the present study, the following recommendations can be drawn for future research on waste-based citric acid production utilizing banana pseudostem:

5.2.1. In addition to the recycling of expensive chemicals and waste, it may be beneficial to investigate the recyclability of the removed lignin. Lignin is an aromatic rich polymer that is generated as a major by-product in the commercial biorefinery industry. Lignin is usually incinerated in boilers to produce energy and heat for functioning of the biorefinery. Lignin may also be transformed into high value-added chemicals that include vanillin, composite resins, carbon fibers and adhesives, thus reducing the overall biorefinery cost.

5.2.2. Microbial biomass derived from bacteria, yeasts, filamentous fungi or microalgae represents a promising alternative to conventional sources of food and feed. Microorganisms are a beneficial source of vitamin and protein. Protein from the fermentative microorganisms may be converted to amino acids and further used to produce chemicals and precursors for biomaterials.

5.2.3. Following fermentation and extraction of the organic acid, a large volume of effluent is generated known as hydrolysate, representing a challenge for downstream processes. This accumulated waste can be utilized as a feedstock for the generation of valuable biofuels such as biohydrogen and bioethanol. Furthermore, complete valorization of the effluent will contribute to a zero-waste-producing biorefinery. This will significantly reduce costs that are associated with the production, downstream and extraction processes.

5.2.4. Exploiting the existing natural biodiversity of microorganisms through mutagenesis and recombinant DNA technology will promote genetic diversification. Additionaly, this will enhance the industrial feasibility of citric acid production and significantly reduce the expenditure associated with bioprocessing.

5.2.5. Conducting technoeconomic analysis of citric acid production incorporating waste-based strategies will provide valuable data for reducing costs at several steps while contributing to knowledge and investment opportunities.

Appendix A

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Development of microwave-assisted alkaline pretreatment methods for enhanced sugar recovery from bamboo and corn cobs: Process optimization, chemical recyclability and kinetics of bioethanol production

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ABSTRACT

The present study optimized two microwave-assisted alkaline ($Na_3PO_4.12H_2O$ and NaOH) pretreatment methods for the enhancement of sugar recovery from different lignocellulosic substrates (bamboo and corn cobs) with varying structural compositions. Subsequently, the chemical recyclability of the optimized alkaline pretreatments were evaluated on corn cobs. Furthermore, kinetics of microbial cell growth and bioethanol production in simultaneous saccharification and fermentation (SSF) processes were assessed. The optimized $Na_3PO_4.12H_2O$ pretreatment conditions resulted in the maximum reducing sugar yield (0.512 g/g) from corn cobs. Interestingly, the recyclability studies demonstrated that the NaOH-based black liquor resulted in a slightly higher average sugar yield (0.37 g/g) compared to the $Na_3PO_4.12H_2O$ (0.31 g/g) over three pretreatment cycles. Kinetics showed a maximum specific growth rate and maximum potential bioethanol concentration of 0.175 h^{-1} and 16.928 g/L respectively for the optimized NaOH pretreated corn cobs. The findings from this study provide significant knowledge enhancements pertaining to the use of gold standard, well-known NaOH methods, in addition to the emerging $Na_3PO_4.12H_2O$ alkaline catalyst for the pretreatment of lignocellulosic substrates with varying structural compositions. Moreover, recyclability of $Na_3PO_4.12H_2O$ and NaOH black liquor demonstrated the potential to reduce costs associated with the application of chemicals and fresh water in pretreatment systems. Kinetics of cell growth and bioethanol production elucidates noteworthy indices such as the maximum specific growth rate of the cells, lag time and maximum bioethanol production for lignocellulosic process development and scale-up procedures.

1. Introduction

The global energy demand is rapidly escalating due to a sharp increase in the population growth rate (Tagliapietra, 2019). Additionally, the incineration of fossil fuels such as oil, gas and coal contribute to the greenhouse effect and inevitably global warming (Kumari and Singh, 2018). Due to the depletion of fossil fuels and its negative effect on the environment, the search for alternative energy resources that are sustainable, cost-efficient and reduce the carbon footprint is imperative (Kumari and Singh, 2018). Lignocellulosic biomass (LCB) represent excellent feedstocks for the production of biofuels and value-added compounds, since they are cost-effective, abundant and renewable (Sewsynker-Sukai and Gueguim Kana, 2018a).

These substrates (LCB) primarily consist of cellulose (35–50 %), hemicellulose (15–35 %) and lignin (20–25 %) structures (Gu et al., 2019). Examples of LCB include wheat straw (Geng et al., 2014), sugarcane bagasse (Jugwanth et al., 2019), corn cobs (Sewsynker-Sukai and Gueguim Kana, 2018b) and bamboo (Kuttiraja et al., 2013). Bamboo has garnered interest as a promising feedstock for the production of bioethanol, owing to its perennial nature, rapid growth, low management requirements and tolerance to extreme climatic conditions (Chaowana, 2013). Bamboo comprises 30–47 % cellulose, 16–28 % hemicellulose and 14–28 % lignin (Chaowana, 2013; Kuttiraja et al., 2013). Alternatively, corn cobs consist of 38–45 % cellulose, 26–35 % hemicellulose and 8–19 % lignin and represent another promising feedstock for bioethanol production (Kim, 2018). Corn cobs make up about 30 % of maize agricultural wastes with an estimated global annual production of approximately 500 million metric tons (Czajkowski et al., 2019).

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Moreover, the low lignin content present in corn cobs makes it a desirable feedstock for biofuel production (Sewsynker-Sukai and Gueguim Kana, 2018b).

Even with the immense benefits of LCB, its major constituents (cellulose and hemicellulose) are cemented together by layers of recalcitrant lignin moieties that hamper the enzymatic release of fermentable sugars (Gu et al., 2019). Therefore, the conversion of LCB to biofuels and value-added compounds necessitates the disruption of the steric hindrance of lignin and hemicellulose by use of pretreatment regimes (Yu et al., 2019). Pretreatment alters the cell wall structure and chemical composition of LCB (Gu et al., 2019). This in turn increases cellulose accessibility to cellulolytic enzymes, thus enhancing enzymatic hydrolysis (Gu et al., 2019). The pretreated LCB is enzymatically hydrolyzed to fermentable sugars such as glucose, which may be used in microbial fermentation processes (Yu et al., 2019; Vergara et al., 2019). Several methods of lignocellulosic pretreatment have been developed over the years. These include microwave, acid, alkali, inorganic salts, ammonia fibre expansion (AFEX) and steam explosion, among others (Yu et al., 2019). However, none of the aforementioned techniques have suitably addressed the challenges related to the high cost and consumption of energy (Qing et al., 2016b; Vergara et al., 2019). Significant efforts are still being made to develop energy-efficient and environmental friendly pretreatment strategies that produce high sugar yields and are cost-effective (Qing et al., 2016b).

Several previous studies have demonstrated the high efficiency of strong alkaline pretreatments such as sodium hydroxide (NaOH), sodium ethoxide (C_2H_5ONa), calcium hydroxide ($CaOH_2$) and potassium hydroxide (KOH) (Gong et al., 2019). Nevertheless, the high costs of alkaline treatments limit its industrial application (Qing et al., 2016b). Recently, alkalic salts have garnered interest due to their recyclability, cost-effectiveness and high sugar yield. Sodium phosphate dodecahydrate ($Na_3PO_4.12H_2O$), sodium sulphide (Na_2S), and sodium carbonate (Na_2CO_3) are some examples of alkalic salts that have recently appeared, since these mimic strong alkaline catalysts such as NaOH, but provide a cheaper alternative (Gu et al., 2019). The main pretreatment mechanism of alkaline reagents occurs as a result of the cleavage of ester and glycosidic bonds in the cell wall (Qing et al., 2016b). This leads to the alteration and degradation of lignin, in addition to the swelling of cellulose and hemicellulose moieties (Qing et al., 2016b).

Additionally, the chemical hydrolysate that is generated following alkaline pretreatments is commonly referred to as black liquor. The black liquor possesses a high pH (13–14) (Zhu and Theliander, 2015), chemical oxygen demand (COD) and biochemical oxygen demand (BOD) that has shown to be detrimental to the environment (Wang et al., 2010). Interestingly, the alkaline characteristic of black liquor allows it to be recycled and involves the use of spent liquor for successive pretreatments (Vergara et al., 2019). The recycling of black liquor obtained from the fractionation of LCB minimizes fresh water consumption, chemical costs and improves environmental sustainability (Vergara et al., 2019).

Microwave heating process (MHP) has emerged as an efficient alternative to conventional steam-assisted heating for the pretreatment of LCB (Aguilar-Reynosa et al., 2017a). MHP uses microwave energy to vibrate and heat molecules causing them to collide and result in the formation of thermal hot spots. Intensive heat and vibration rupture the components of LCB causing the rearrangement of crystalline cellulose. Advantages of MHP include its faster heat transfer within a short time, high product yields, environmentally benign nature, energy efficiency and cost-effectiveness (Aguilar-Reynosa et al., 2017a).

The comparative optimization of microwave-assisted alkalic salt ($Na_3PO_4.12H_2O$) or a strong base (NaOH) on different lignocellulosic substrates (corn cobs and bamboo) will provide significant insights for the enhancement of sugar recovery. In addition, the potential recyclability of these alkaline solutions will enhance the process economics of the pretreatment for industrial scale implementation. The sugar that is generated from effective lignocellulosic pretreatments can be channelled towards biofuel production or high value commodities. In the recent time, bioethanol has received considerable attention due to several beneficial properties. These may include its high evaporation enthalpy, high-octane number and flammability for combustion generating heat (Waqas et al., 2016). The aforementioned properties of bioethanol also enables its combination with derivatives of hydrocarbons (Waqas et al., 2016). Various fermentation process types exist for bioethanol production. These include separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF) and SSF with a prehydrolysis stage (PSSF).

However, SSF strategies are becoming an attractive approach for bioethanol production (Gonçalves et al., 2016), due to the lower production cost, increased ethanol concentration and ethanol conversion rates by eradicating separate, lengthy hydrolysis steps (Sewsynker-Sukai and Gueguim Kana, 2018b; Gonçalves et al., 2016). The SSF process type also reduces the feedback inhibition of enzymes and the risk for contamination (Mithra et al., 2018). Kinetic modelling approaches may be used for SSF bioprocess development, optimization, scale-up and process control. These models help to reduce undesirable by-products and improve the product yield (Sewsynker-Sukai and Gueguim Kana, 2018a). Some examples of modelling approaches include the logistic and modified Gompertz models. The logistic model describes microbial cell growth whereas the modified Gompertz model predicts maximum bioethanol formation (Sewsynker-Sukai and Gueguim Kana, 2018b).

Therefore, the objectives of the present study were to: (1) develop and assess two different microwave-assisted alkaline-catalyzed pretreatments ($Na_3PO_4.12H_2O$ and NaOH) on bamboo and corn cobs, (2) determine the interactive effects of alkali concentration, microwave power intensity and pretreatment time on the reducing sugar yield, (3) evaluate the potential recyclability of the $Na_3PO_4.12H_2O$ and NaOH black liquor hydrolysate, and (4) assess the kinetics of yeast cell growth and bioethanol production on the pretreated substrate using the logistic and modified Gompertz models respectively.

2. Materials and methods

2.1. Materials

The bamboo and corn cob substrates utilized in this study were obtained from Cedara (Hilton, South Africa) (29°32'25.8"S 30°16'49.8"E) and the Ukulinga research farm (Pietermaritzburg, South Africa) (29°39'45.6"S 30°24'17.9"E) respectively. The substrates were oven dried at 60 °C for 2 days and were thereafter milled to a particle size between 1–2 mm. The powdered bamboo and corn cob substrates were stored in airtight containers at room temperature. The chemicals used in this study were purchased from Merck, South Africa. The enzyme used for saccharification was Cellic CTec 2 (160 FPU/mL) and was kindly provided by Novozymes (Novozymes A/S, Denmark).

2.2. Preliminary screening

Preliminary screening was carried out to determine the influence of a strong base (NaOH) and an alkalic salt ($Na_3PO_4.12H_2O$) on the reducing sugar yield from the bamboo and corn cob substrates. A total working volume of 250 mL was used for the preliminary screening. Bamboo or corn cobs with a solid loading of 10 % (w/v) were submerged in 12.50 % (w/v) of either NaOH or $Na_3PO_4.12H_2O$ alkali solutions and were heated at a power intensity of 500 W for 7.5 min. After the pretreatment process, the substrates were washed rigorously with distilled water and filtered using a domestic sieve (<1 mm). The washed pretreated substrates were thereafter oven dried at 55 °C overnight prior to the enzymatic hydrolysis stage. Control experiments that included: (1) untreated (native) corn cobs or bamboo and, (2) corn cobs or bamboo pretreated and heated with distilled water only (devoid of NaOH or $Na_3PO_4.12H_2O$) were performed.

2.3. Modelling and optimization of pretreatment parameters

The response surface methodology (RSM) model (Box-Behnken design) was used to develop four pretreatment models consisting of 17 experimental runs each. The pretreatment input parameters consisted of alkali concentration (NaOH or $Na_3PO_4.12H_2O$) (5–20 %, w/v), microwave power intensity (100−900 W) and microwave pretreatment time (5−10 min) while the output parameter was the reducing sugar yield (g/g). The input parameter ranges (Table 1) were selected following the preliminary screening in addition to an extensive literature survey (Qing et al., 2016a, b, Boonsombuti et al., 2013). Therefore, four RSM pretreatment models were designated as follows: (1) bamboo pretreated with NaOH ($NaOH_{BB}$), (2) bamboo pretreated with $Na_3PO_4.12H_2O$ ($Na_3PO_4.12H_2O_{BB}$), (3) corn cobs pretreated with NaOH ($NaOH_{CC}$), and (4) corn cobs pretreated with $Na_3PO_4.12H_2O_{BB}$ ($Na_3PO_4.12H_2O_{CC}$). Experimental reducing sugar yields obtained after the enzymatic saccharification stage were used to develop the polynomial models using Design Expert software (Stat-Ease Inc., USA). The general form of the RSM polynomial model equation is shown in Eq. (1):

$$Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_4 x_4 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{33} x_3^2 + \beta_{44} x_4^2 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{14} x_1 x_4 + \beta_{23} x_2 x_3 + \beta_{24} x_2 x_4 + \beta_{34} x_3 x_4 \quad (1)$$

where Y is the reducing sugar yield response, β_0 is the free or offset term. $\beta_1 x_1$ to $\beta_4 x_4$ represents the linear coefficients, $\beta_{11} x_1^2$ to $\beta_{44} x_4^2$ are quadratic coefficients and $\beta_{12} x_1 x_2$ to $\beta_{34} x_3 x_4$ are the interaction coefficients. The significance of the model is assessed by the Analysis of Variance (ANOVA). Process optimization was carried out by solving the polynomial equation using the method of Myers et al. (1995).

2.4. Enzymatic hydrolysis

The alkaline pretreated substrates (corn cobs or bamboo) were submerged in 10 mL of sodium citrate buffer (0.05 M, pH 4.8). In addition, standard enzyme loading (10 FPU/g) and solid loading (10 %, w/v) were used. The enzymatic hydrolysis stage was carried out at a temperature and agitation of 50 °C and 120 rpm respectively, for 72 h in an orbital shaker incubator (FMH Instruments, South Africa). Subsequently, the hydrolysate samples were centrifuged at 10 000 rpm for 5 min (Biofuge pico, Heraeus) and analyzed for reducing sugar yield (g/g) using the 3,5-dinitrosalicyclic acid (DNS) method established by Miller (1959).

2.5. Validation of the developed pretreatment models

Validation pretreatment experiments were carried out using the optimized input conditions as specified by each developed model ($NaOH_{BB}$, $Na_3PO_4.12H_2O_{BB}$, $NaOH_{CC}$ and $Na_3PO_4.12H_2O_{CC}$) for the particular substrate. The chemical pretreatment and enzymatic hydrolysis reactions were performed as previously stated in Sections 2.3 and 2.4 above.

2.6. Potential recyclability of the NaOH and $Na_3PO_4.12H_2O$ black liquor

The black liquor generated after the optimized NaOH and $Na_3PO_4.12H_2O$ (validation experiments) using corn cobs were collected separately and used for the recyclability assessment. The spent black liquor hydrolysate (NaOH or $Na_3PO_4.12H_2O$) was used to pretreat corn cobs with the same heating parameters used for the validation experiments in order to determine the effectiveness of recycling the used chemicals. Recycling of the spent NaOH and $Na_3PO_4.12H_2O$ was carried out over several cycles, until the liquor became highly viscous, turbid and concentrated with soluble solids due to evaporation during the pretreatment process. Thus, rendering the black liquor hydrolysates ineffective for further substrate penetration. The pH of the NaOH or $Na_3PO_4.12H_2O$ spent liquor following each pretreatment cycle was recorded. Enzymatic hydrolysis was carried out on the NaOH and $Na_3PO_4.12H_2O$ spent liquor pretreated corn cobs as previously outlined.

Table 1
Factors and levels employed in the three-factor Box-Behnken design.

InputsUnits		Coded values		
		−1	0	+1
A: Alkaline concentration	% (w/v)	5.00	12.50	20.00
B: Power intensity	W	100	500	900
C: Pretreatment time	min	5	7.5	10

2.7. Ethanol fermentation bioprocess

2.7.1. Development of yeast inoculum

Angel yeast cells were grown on yeast peptone dextrose (YPD) solid media containing 10 g/L yeast extract, 20 g/L glucose, 20 g/L peptone and 20 g/L agar and incubated at 30 °C for 24 h. After incubation, a colony of yeast was inoculated into YPD broth with a composition of 10 g/L yeast extract, 20 g/L glucose, 20 g/L peptone. The inoculated media was incubated at 30 °C and 120 rpm for a period of 18 h, until the exponential growth phase was reached. This culture was then used as the starter culture for the subsequent SSF processes. The Angel yeast cells were quantified by means of a cell counting chamber (Neubauer, Germany).

2.7.2. Simultaneous saccharification and fermentation (SSF) process

Two sets of SSF bioethanol processes were carried out on the optimized NaOH and $Na_3PO_4.12H_2O$ pretreated corn cobs and were designated (1) SSF process with NaOH pretreated corn cobs ($NaOH_{CC(SSF)}$) and (2) SSF process with $Na_3PO_4.12H_2O$ pretreated corn cobs ($Na_3PO_4.12H_2O_{CC(SSF)}$). The SSF processes (50 mL) contained 10 % (w/v) pretreated corn cobs, 40 mL sodium citrate buffer, 5 mL fermentation nutrients (10 g/L peptone, 5 g/L yeast extract), Cellic CTec2 enzyme (10 FPU/g) and 10 % (v/v) yeast inoculum. Control experiments (50 mL) were performed in parallel and consisted of 10 % (w/v) pretreated corn cobs, 45 mL sodium citrate buffer, 5 mL fermentation nutrients (10 g/L peptone, 5 g/L yeast extract) and Cellic CTec2 enzyme (10 FPU/g). The controls (uninoculated) were used to estimate the initial glucose concentration while the inoculated SSF experiments were regarded as the final glucose concentration. The initial and final glucose concentrations were thereafter used to compute the glucose utilization. All the SSF and control experiments were incubated at 35 °C and 120 rpm until the glucose was completely consumed and the ethanol concentration did not increase further. Liquid samples (1 mL) for cell biomass concentration and glucose utilization were extracted in 2 h intervals.

2.8. Analytical methods

2.8.1. Scanning electron microscopy (SEM)

The bamboo and corn cobs substrates (untreated and optimally pretreated) were visually analyzed using scanning electron microscopy (SEM) (ZEISS EVO LS 15). The dried samples were placed on carbon tape and sputter coated with gold for conductivity (Quorum Q150 R ES). The surface morphological changes of the lignocellulosic biomass samples were examined using conventional SEM at a magnification of 500 X (ZEISS EVO LS 15).

2.8.2. Fourier transform infrared spectroscopy (FTIR)

Fourier Transform Infrared (FTIR) spectroscopy was used to determine the chemical structure and functional group changes of the untreated and optimally pretreated substrates (corn cobs and bamboo). The FTIR was carried out using a Perkin Elmer 100 system (Waltham, MA USA). The substrate samples were each mixed with spectroscopic grade potassium bromide (KBr) and pressed into uniform discs. The samples were then scanned and recorded from 400 to 3800 cm^{-1}.

2.8.3. Sugar analysis

Reducing sugar in the preliminary screening, experimental design runs, validation and recyclability processes were analyzed using the DNS method (Miller, 1959). The glucose content for the validation, recyclability and ethanol experiments were determined using Megazyme GOPOD assay kits (Megazyme, Ireland). Glucose utilization (%) was computed using Equation (2) (Srimachai et al., 2015).

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

Where the initial glucose concentration (g/L) is the glucose obtained from the control flask reactor (uninoculated), and the final glucose concentration (g/L) is the glucose obtained from the experimental SSF flask at the same time point.

2.8.4. Biomass concentration

The Angel yeast biomass concentration was quantified by using the dry cell weight (g/L) as a function of the cell count (cells/mL). Angel yeast cells were grown for 16 h in YPD broth consisting of 10 g/L yeast extract, 20 g/L glucose, 20 g/L peptone and was thereafter diluted accordingly (1, 1/2, 1/4, 1/8 and 1/16). A total of 40 mL of the varying dilutions (1, 1/2, 1/4, 1/8 and 1/16) were centrifuged at 5000 rpm for 10 min. The supernatant obtained after centrifugation was discarded and the remaining biomass pellet was dried at 60 °C until a constant mass was obtained. The dry cell weights (or biomass concentration in g/L) and corresponding total cell counts (cells/mL) for each dilution were used to develop the standard calibration curve. Biomass concentrations (g/L) for the $NaOH_{CC(SSF)}$ and $Na_3PO_4.12H_2O_{CC(SSF)}$ processes were then extrapolated from the standard curve by substituting the yeast cell count.

2.8.5. Ethanol concentration

The ethanol concentration was measured every 2 h in the gas phase of the $NaOH_{CC(SSF)}$ and $Na_3PO_4.12H_2O_{CC(SSF)}$ processes using an ethanol vapour sensor (ETH-BTA, Vernier Software and Technology, Beaverton, OR, USA) as previously described by Jugwanth et al. (2019).

2.9. Computation of the kinetic coefficients

2.9.1. The logistic model

The differential formula of the logistic model shown in Eq. (3) was integrated to form Eq. (4). Eq. (4) accounts for the stationary and exponential phases of growth. The logistic model Eq. (4), relates the biomass (X) to the initial cell conc. (X_0), max specific growth rate (μ_{max}) and max cell concentration (X_{max}) at a certain time (t) during the stationary and exponential growth phases of yeast cells.

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

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

2.9.2. The modified Gompertz model

Bioethanol data obtained for the $NaOH_{CC(SSF)}$ and $Na_3PO_4.12H_2O_{CC(SSF)}$ processes were fitted to the modified Gompertz model using the least squares method (CurveExpert V1.5.5). The kinetic parameters such as maximum bioethanol production rate ($r_{p,m}$), lag time (t_L), and the maximum potential bioethanol concentration (P_m) were determined using Eq. (5).

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

The modified Gompertz model Equation (6), denotes P, P_m, $r_{p,m}$, t_L and t as the bioethanol concentration (g/L), potential maximum bioethanol concentration (g/L), maximum bioethanol production rate (g/L/h), lag time (h), and the fermentation time (h) respectively.

3. Results and discussion

3.1. Preliminary screening

The preliminary $Na_3PO_4.12H_2O$ and NaOH pretreatments with 12.50 % (w/v) on bamboo resulted in a reducing sugar yield of 0.11 g/g and 0.25 g/g respectively. However, higher reducing sugar yields of 0.37 g/g ($Na_3PO_4.12H_2O$) and 0.38 g/g (NaOH) were obtained from the corn cob substrate. The preliminary screening using $Na_3PO_4.12H_2O$ and NaOH pretreatments on bamboo and corn cobs indicated the release of fermentable sugars and these processes were subsequently optimized. Control pretreatments (without $Na_3PO_4.12H_2O$ and NaOH) were performed with the native (untreated) and distilled water pretreated substrates (bamboo and corn cobs) and very low yields of fermentable sugars were observed of (0.05 g/g and 0.08 g/g respectively). Similarly, the distilled water pretreated substrates resulted in low sugar yields of 0.06 g/g and 0.09 g/g from bamboo and corn cobs respectively.

3.2. RSM model development and significance

The pretreatment experimental reducing sugar data were used to develop the polynomial model Eqs. (6)–(9) (Table 2). The Analysis of Variance (ANOVA) was used to assess the fitness of the RSM models (Table 3). The high F-values (>4) and low p-values (<0.05) indicated that all four pretreatment models were significant. A p-value <0.05 indicates a significant model (Qing et al., 2016b) and that there is a five percent chance that a 'Model F-value' could occur due to noise (Nasirpour and Mousavi, 2018).

The significance of the pretreatment input parameters and their

Table 2
RSM polynomial model equations relating the input parameters to the output reducing sugar yields for the microwave-assisted alkaline pretreatments.

Model	Equation	Equation number
$Na_3PO_4.12H_2O_{BB}$	$-0.215 + 0.012A + 3.13 \times 10^{-4}B + 0.034C + 2.54 \times 10^{-6}AB - 2.53 \times 10^{-5}AC + 1.41 \times 10^{-6}BC - 4.82 \times 10^{-4}A^2 - 3.02 \times 10^{-7}B^2 - 2.28 \times 10^{-3}C^2$	(6)
$NaOH_{BB}$	$-0.056 + 0.028A + 1.95 \times 10^{-4}B - 5.52 \times 10^{-3}C - 5.17 \times 10^{-6}AB - 6.10 \times 10^{-4}AC + 2.96 \times 10^{-6}BC - 6.05 \times 10^{-4}A^2 - 1.01 \times 10^{-7}B^2 + 8.12 \times 10^{-4}C^2$	(7)
$Na_3PO_4.12H_2O_{CC}$	$+0.463 - 6.98 \times 10^{-3}A + 7.39 \times 10^{-4}B - 0.089C + 2.73 \times 10^{-5}AB + 3.45 \times 10^{-3}AC + 4.48 \times 10^{-5}BC - 8.69 \times 10^{-4}A^2 - 1.05 \times 10^{-6}B^2 + 6.41 \times 10^{-4}C^2$	(8)
$NaOH_{CC}$	$+0.474 + 8.22 \times 10^{-3}A + 1.80 \times 10^{-4}B - 0.035C + 4.61 \times 10^{-7}AB + 4.71 \times 10^{-4}AC - 2.07 \times 10^{-5}BC - 4.53 \times 10^{-4}A^2 - 1.15 \times 10^{-7}B^2 + 2.16 \times 10^{-3}C^2$	(9)

Footnote: A = Alkaline concentration, B = Power intensity, C = Pretreatment time.

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

Model	Sum of squares	df	Mean squares	F-value	p-value	R^2
$Na_3PO_4.12H_2O_{BB}$	0.020	9	2.209×10^{-3}	4.49	0.0302	0.8523
$NaOH_{BB}$	0.027	9	2.990×10^{-3}	31.64	<0.0001	0.9760
$Na_3PO_4.12H_2O_{CC}$	0.42	9	0.046	21.58	0.0003	0.9652
$NaOH_{CC}$	0.019	9	2.143×10^{-3}	4.21	0.0357	0.8439

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

effect on the reducing sugar yields was evaluated by using the corresponding p-values. From all the investigated parameters, the effect of microwave power intensity (0.0303) was shown to be the most significant for the $Na_3PO_4.12H_2O_{BB}$ model whereas both alkaline concentration (<0.0001) and power intensity (0.0006) were significant for the $NaOH_{BB}$ model. For the $Na_3PO_4.12H_2O_{CC}$ model, the power intensity (<0.0001) was shown to be the most significant parameter followed by the alkaline concentration (p = 0.0016). Conversely, the $NaOH_{CC}$ model showed that the power intensity (0.0038) had the most significant effect on the reducing sugar yield followed by pretreatment time (0.0468). Microwave power intensity displayed the greatest effect on the reducing sugar yield for all four pretreatment models. The significance of microwave power intensity can be attributed to the generation of thermal heat zones that accelerate the collision of ions, thus raising the temperature at shorter times with a high heating efficiency during lignocellulosic pretreatment (Sewsynker-Sukai and Gueguim Kana, 2018a, Alvira et al., 2010). Similarly, the influence of pretreatment time may be ascribed to an increase in the temperature, thus enhancing chemical changes in the lignocellulosic matrix (Lin et al., 2015). However, long pretreatment times speed up evaporation that can adversely influence dielectric properties and reduce pretreatment impact (Aguilar-Reynosa et al., 2017a). On the other hand, the significant impact of alkali concentration ($Na_3PO_4.12H_2O$ and NaOH) can be accounted for by the cleavage of ester and glycosidic bonds, altering the structure of lignin, causing cellulose swelling, and partial depolymerization of hemicellulose (Brodeur et al., 2011; Qing et al., 2016b).

To display the developed pretreatment models' reliability, the coefficient of determination (R^2) value was used as a statistical index. R^2 values between 0.70 and 1 represent a model with high accuracy (Jugwanth et al., 2019). High R^2 values were achieved for the $Na_3PO_4.12H_2O_{BB}$ (0.85), $NaOH_{BB}$ (0.98), $Na_3PO_4.12H_2O_{CC}$ (0.97) and $NaOH_{CC}$ (0.84) models. This indicated that the developed models could account for 85 % ($Na_3PO_4.12H_2O_{BB}$), 98 % ($NaOH_{BB}$), 97 % ($Na_3PO_4.12H_2O_{CC}$) and 84 % ($NaOH_{CC}$) of the variation in the observed data. Therefore, all four pretreatment models displayed a good fit regarding the relationship between the pretreatment input parameters and the reducing sugar yield.

3.3. Effect of the pretreatment input parameters on the reducing sugar yield

Table 1 shows the reducing sugar yields (g/g) obtained for each experimental run from all four models. The $Na_3PO_4.12H_2O_{BB}$ model displayed a maximum reducing sugar yield of 0.116 g/g under the median pretreatment conditions of 12.50 % $Na_3PO_4.12H_2O$, 500 W and 7.5 min (run 13). The other median pretreatment runs (12.50 % $Na_3PO_4.12H_2O$, 500 W, 7.5 min) displayed sugar yields of 0.095 g/g (run 1), 0.099 g/g (run 2), 0.107 g/g (run 3) and 0.059 g/g (run 5) for the $Na_3PO_4.12H_2O_{BB}$ model. Conversely, the lowest reducing sugar yield ($Na_3PO_4.12H_2O_{BB}$ = 0.001 g/g) was obtained with 20 % $Na_3PO_4.12H_2O$, 100 W and 7.5 min (run 4). On the other hand, the $NaOH_{BB}$ model displayed a maximum reducing sugar yield of 0.220 g/g with 20 % NaOH, heated at 500 W for 5 min (run 17). The lowest reducing sugar yield ($NaOH_{BB}$ = 0.061 g/g) was obtained with 5.00 % NaOH, 100 W and 7.5 min (run 14). Alternatively, the $NaOH_{BB}$ gave reducing sugar yields of 0.200 g/g (run 1), 0.195 g/g (run 2), 0.200 g/g (run 3), 0.195 g/g (run 5) and 0.208 g/g (run 13) under the median pretreatment conditions (12.50 % NaOH, 500 W, 7.5 min).

For the $Na_3PO_4.12H_2O_{CC}$ model, a maximum reducing sugar yield of 0.463 g/g was observed with 20 % $Na_3PO_4.12H_2O$, heated at 900 W for 7.5 min (run 12). The lowest reducing sugar yield (0.001 g/g) was obtained for run 9 (12.50 % $Na_3PO_4.12H_2O$, 100 W and 10 min). The median pretreatment conditions for the $Na_3PO_4.12H_2O_{CC}$ model (12.50 % $Na_3PO_4.12H_2O$, 500 W, 7.5 min) achieved sugar yields of 0.421 g/g (run 1), 0.359 g/g (run 2), 0.403 g/g (run 3), 0.397 g/g (run 5) and 0.312 g/g (run 13). Conversely, the $NaOH_{CC}$ model displayed a maximum reducing sugar yield of 0.419 g/g for run 2 (12.50 % NaOH, 500 W, 7.5 min) and run 9 (12.50 % NaOH, 100 W, 10 min). The $NaOH_{CC}$ demonstrated the lowest reducing sugar yield (0.290 g/g) for run 8 (5.00 % NaOH, 900 W and 7.5 min). The median pretreatment conditions for the aforementioned model (12.50 % NaOH, 500 W, 7.5 min) resulted in reducing sugar yields of 0.395 g/g (run 1), 0.419 g/g (run 2), 0.362 g/g (run 3), 0.406 g/g (run 5) and 0.372 g/g (run 13).

The interactions between the pretreatment input parameters and the reducing sugar yields are shown in Fig. 1A-H. Fig. 1A illustrates the interactive effects of power intensity and $Na_3PO_4.12H_2O$ concentration on the reducing sugar yield (g/g), while the pretreatment time was maintained at its median ($Na_3PO_4.12H_2O_{BB}$). Simultaneous increases in the $Na_3PO_4.12H_2O$ concentration (5–13 %) and microwave power intensity (100−500 W) increased the reducing sugar yield from 0.001 to 0.11 g/g from bamboo. During microwave heating, polarization influences the dipole-dipole molecules to align. The alignment of these molecules in the radiation field causes rapid heating of the hydrolysate enhancing the solubility of the substrate (Aguilar-Reynosa et al., 2017a). Furthermore, microwave heat accumulates within the material resulting in an improved heat transfer and generates a high-energy yield (Aguilar-Reynosa et al., 2017a). Correspondingly, the increase in sugar yield can be ascribed to the alkaline pretreatment. During pretreatment, alkaline chemicals solubilize amorphous regions (hemicellulose and lignin) resulting in the saponification of intermolecular ester bonds (Kim et al., 2016). Further increases in the $Na_3PO_4.12H_2O$ concentration (>13 %) and power intensity (>500 W) resulted in a decrease in the reducing sugar yield (0.11−0.01 g/g) (Fig. 1A). The decrease in reducing sugars can be due to the elevated temperatures and alkaline conditions that may result in the formation of inhibitory compounds, which are toxic to cellulases, thus reducing the enzyme activity during hydrolysis reactions (Wang et al., 2010). Likewise, Qing et al. (2016b) observed no further increase in sugar yields beyond 11 % $Na_3PO_4.12H_2O$ concentration using bamboo.

The influence of pretreatment time and power intensity on the reducing sugar yield whilst the $Na_3PO_4.12H_2O$ concentration was the median value is depicted in Fig. 1B ($Na_3PO_4.12H_2O_{BB}$). A simultaneous increment in the pretreatment time (5−7 min) and power intensity (100−600 W) increased the reducing sugar yield from 0.01 to 0.10 g/g using bamboo. Microwave irradiation has a fast heat transfer, reduces time by 10-fold and decreases energy consumption (Aguilar-Reynosa et al., 2017a). The high heating efficiency of microwave irradiation can account for the improvement om sugar yield even at lower power intensities and pretreatment times. Alternatively, longer times (7.5−10 min) and a low power intensity (100 W) led to reducing sugar yields <0.014 g/g. Shorter times are generally preferred with higher power intensities during microwave pretreatment since the latter dictates the heating efficiency (Aguilar-Reynosa et al., 2017a). This may be attributed to homogenous heating of the hydrolysate resulting in a higher throughput and bioconversion rate at higher microwave power intensities (Maurya et al., 2015). Moreover, high temperatures due to the elevated power intensities, rapidly generate pressure within the absorbent biopolymers resulting in their rapid expansion (Aguilar-Reynosa

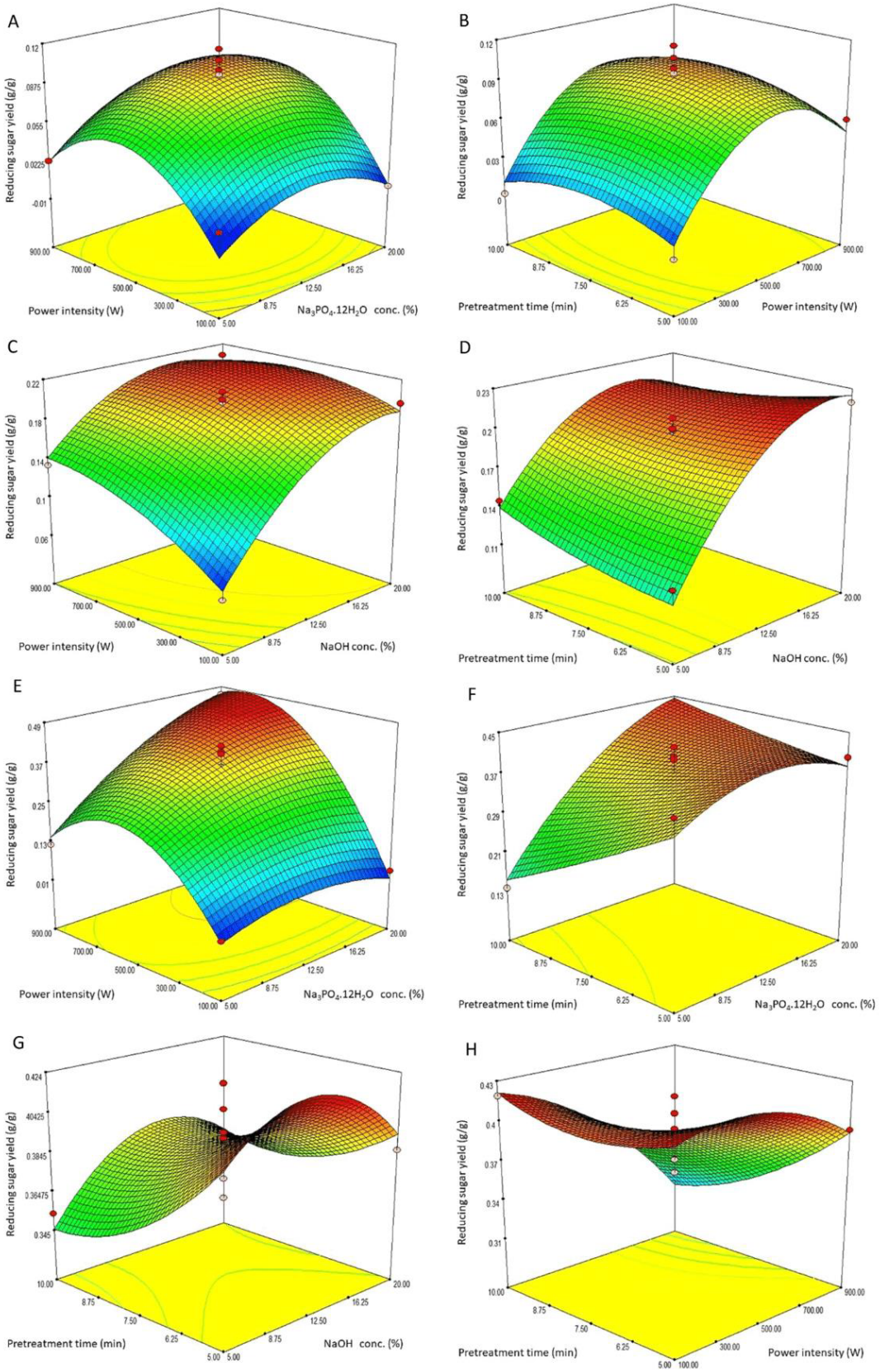

Fig. 1. Response surface graphs illustrating the interactive effects of the various input pretreatment parameters on the output reducing sugar yields: (A) power intensity and $Na_3PO_4.12H_2O$ concentration ($Na_3PO_4.12H_2O_{BB}$); (B) pretreatment time and power intensity ($Na_3PO_4.12H_2O_{BB}$); (C) power intensity and NaOH concentration ($NaOH_{BB}$); (D) pretreatment time and NaOH concentration ($NaOH_{BB}$); (E) power intensity and $Na_3PO_4.12H_2O$ concentration ($Na_3PO_4.12H_2O_{CC}$); (F) pretreatment time and $Na_3PO_4.12H_2O$ concentration ($Na_3PO_4.12H_2O_{CC}$); (G) pretreatment time and NaOH concentration ($NaOH_{CC}$); (H) pretreatment time and power intensity ($NaOH_{CC}$).

Table 4
Experimental runs with the corresponding sugar yields obtained for the alkaline pretreatments on bamboo and corn cobs.

Input parameters				Output			
				Reducing sugar yield from different substrates (g/g)			
Run	Alkaline catalyst concentration (%)	Power intensity (W)	Pretreatment time (min)	$NaOH_{BB}$	$Na_3PO_4.12H_2O_{BB}$	$NaOH_{CC}$	$Na_3PO_4.12H_2O_{CC}$
1	12.50	500	7.50	0.200	0.095	0.395	0.421
2	12.50	500	7.50	0.195	0.099	0.419	0.359
3	12.50	500	7.50	0.200	0.107	0.362	0.403
4	20.00	100	7.50	0.196	0.001	0.401	0.038
5	12.50	500	7.50	0.195	0.059	0.406	0.397
6	12.50	100	5.00	0.168	0.002	0.410	0.108
7	5.00	500	5.00	0.124	0.031	0.416	0.385
8	5.00	900	7.50	0.134	0.023	0.290	0.121
9	12.50	100	10.00	0.169	0.002	0.419	0.001
10	5.00	500	10.00	0.145	0.034	0.354	0.136
11	12.50	900	10.00	0.215	0.066	0.320	0.410
12	20.00	900	7.50	0.207	0.040	0.325	0.463
13	12.50	500	7.50	0.208	0.116	0.372	0.312
14	5.00	100	7.50	0.061	0.014	0.371	0.023
15	20.00	500	10.00	0.195	0.076	0.359	0.412
16	12.50	900	5.00	0.202	0.060	0.394	0.337
17	20.00	500	5.00	0.220	0.074	0.386	0.401

Footnote: g/g = gram reducing sugar/ gram dry weight substrate.

et al., 2017a) (Table 4).

In addition, minimum generation of inhibitors occur at shorter pretreatment times (Maurya et al., 2015). A similar trend was observed by Sewsynker-Sukai and Gueguim Kana (2018a) whereby an increase in power intensity above 700 W did not significantly influence the reducing sugar yield.

Fig. 1C depicts the interaction between power intensity and NaOH concentration on the reducing sugar yield from bamboo while pretreatment time was held at the centre point ($NaOH_{BB}$). Simultaneous increases in the NaOH concentration from 5 to 20 % and power intensity from 100 to 900 W enhanced the reducing sugar yield (0.061 g/g–0.220 g/g). During pretreatment, NaOH disassociates into sodium (Na^+) and hydroxide (OH^-) ions. An increase in OH^- ions increases the rate of hydrolysis resulting in a high sugar yield (Kim et al., 2016). Furthermore, sodium hydroxide cleaves the ether and ester bonds between lignin and hemicellulose and C—C bonds within lignin molecules (Kim et al., 2016). In addition, a high microwave power enhances collisions between anions and cations allowing for fast heating of the intracellular molecules, causing breakages of hydrogen bonds within the structural chain (Aguilar-Reynosa et al., 2017a).

On the other hand, Fig. 1D shows the influence of pretreatment time and NaOH concentration on the reducing sugar yield while power intensity was the median value ($NaOH_{BB}$). Increases in the reducing sugar yield (0.110−0.220 g/g) were observed with a fixed pretreatment time of 5 min and an increase in the NaOH concentration (5–20 %) using bamboo. High alkaline concentrations enhance the degradation of lignocellulosic structures (Qing et al., 2016b). However, an increase in pretreatment time (>5 min) reduced the sugar yields (0.23−0.20 g/g). At longer pretreatment times, abundant heat is generated within the alkaline solution, resulting in a high rate of evaporation (Aguilar-Reynosa et al., 2017a). Dehydrated components within the reaction mixture cannot contend with granule expansion, leading to stress between the biopolymers inevitably causing their structures to collapse and rupture (Aguilar-Reynosa et al., 2017a) and may account for the lower sugar observed at longer pretreatment times. The study by Sewsynker-Sukai and Gueguim Kana (2018a) assessed the effects of pretreatment time and alkali salt concentration on corn cobs and recorded a sugar yield of 0.30 to 0.75 g/g when both parameters were simultaneously increased.

The interaction between power intensity and $Na_3PO_4.12H_2O$ concentration on the reducing sugar yield from corn cobs, while pretreatment time was maintained at its midpoint is illustrated in Fig. 1E ($Na_3PO_4.12H_2O_{CC}$). Simultaneous increments in the $Na_3PO_4.12H_2O$ concentration from 5 to 20 % and power intensity from 100 to 900 W increased the reducing sugar yield (0.001 g/g–0.49 g/g). Alkalic salt has recently been used to enhance enzymatic hydrolysis and sugar yields due to its low corrosivity, ability to alter the lignin structure and increase the substrate porosity (Qing et al., 2016b). In addition, high microwave irradiation creates a magnetic field that facilitates the movement of ions back and forth (oscillation) enhancing substrate penetration (Aguilar-Reynosa et al., 2017a). Similarly, Qing et al. (2016b) reported that higher $Na_3PO_4.12H_2O$ concentration and pretreatment times resulted in a higher reducing sugar yield using bamboo as a substrate.

Fig. 1F relates the interactive effects of pretreatment time and $Na_3PO_4.12H_2O$ concentration on the reducing sugar yield while power intensity was held at the centre point ($Na_3PO_4.12H_2O_{CC}$). The reducing sugar yield increased from 0.15 to 0.45 g/g using corn cobs with simultaneous increases in the pretreatment time (5−10 min) and $Na_3PO_4.12H_2O$ concentration (5–20 %). The high reducing sugar yield can be attributed to the highly concentrated alkaline salt which exhibits a high pH. The high alkaline pH is important for delignification by facilitating the oxidation of ethylene double bonds in the side chains of lignin moieties thus enhancing the exposure of cellulose and hemicellulose structures to enzymes (Geng et al., 2014). Furthermore, an increase in exposure time, facilitates the accumulation of heat within the solution, resulting in the effective disruption of lignin (Pu et al., 2013).

The influence of pretreatment time and NaOH concentration while power intensity was kept at the median value is depicted in Fig. 1G ($NaOH_{CC}$). Pretreatment times ranging from 5 to 6.25 min and NaOH concentrations from 16.25 to 20 % gave the maximum reducing sugar yields of 0.395 to 0.40 g/g using corn cobs. Similar to the effect of $Na_3PO_4.12H_2O$ (Fig. 1F), a high alkaline concentration and pH for the NaOH aids the delignification of the lignocellulosic biomass due to fragmentation of recalcitrant lignin moieties. However, a further increase in the pretreatment time beyond 6.25 min drastically reduced the sugar yield from 0.40 to 0.345 g/g. Reduction in the reducing sugar yields at long exposure times (>6.25 min) may be attributed to the evaporation of water. Water is an important factor during pretreatment, since the moisture content determines the dielectric properties of the material (Aguilar-Reynosa et al., 2017a). Furthermore, water facilitates heating and determines the depth of penetration of the irradiation energy (Aguilar-Reynosa et al., 2017a). The study by Jia-Jia et al. (2017) pretreated bamboo with a power intensity of 440 W for 23 min and observed a decrease in the sugar yield from 0.76 to 0.432 g/g beyond 440 W.

Fig. 1H illustrates the interactive effects between pretreatment time

and power intensity on the reducing sugar yield from corn cobs whilst the NaOH concentration was held at its midpoint ($NaOH_{CC}$). A slight increase in the reducing sugar yield from 0.40 to 0.42 g/g was observed with increases in the pretreatment time from 5 to 10 min and a power intensity of 100 W. Similarly, Lin et al. (2015) studied the interactive effects of NaOH and microwave duration for the pretreatment of water hyacinth and found that an increase in time from 5−10 min increased the reducing sugar yield (0.013−0.028 g/g). Moreover, the present study displayed that power intensity beyond 100 W with NaOH led to a reduction in the sugar yield (0.40−0.35 g/g). High temperatures achieved at increased microwave power intensities coupled with strong alkaline concentrations may negatively affect the substrate by damaging lignocellulosic structures such as cellulose (Wang et al., 2010). As a result, low glucose yields are attained after enzymatic saccharification due to a decrease in cellulose recovery (Wang et al., 2010).

3.4. Experimental validation of the developed models

The four developed pretreatment models were validated and optimized to obtain a maximum reducing sugar yield (Table 5). Under optimal conditions, the $Na_3PO_4.12H_2O_{BB}$, $NaOH_{BB}$, $Na_3PO_4.12H_2O_{CC}$, and $NaOH_{CC}$ models predicted reducing sugar yields of 0.099, 0.220, 0.492 and 0.421 g/g respectively. Experimental sugar yields of 0.086, 0.225, 0.512 and 0.420 were obtained for the $Na_3PO_4.12H_2O_{BB}$, $NaOH_{BB}$, $Na_3PO_4.12H_2O_{CC}$, and $NaOH_{CC}$ optimized pretreatments respectively. Slight deviations of 13 % ($Na_3PO_4.12H_2O_{BB}$), 2.22 % ($NaOH_{BB}$), 3.91 % ($Na_3PO_4.12H_2O_{CC}$), and 0.24 % ($NaOH_{CC}$) were recorded between the predicted and observed reducing sugar yields, illustrating the capability of the developed pretreatment models. In addition, the glucose yields achieved for the optimized pretreated experiments were 0.025 ($Na_3PO_4.12H_2O_{BB}$), 0.123 ($NaOH_{BB}$), 0.171 ($Na_3PO_4.12H_2O_{CC}$) and 0.294 g/g ($NaOH_{CC}$). Furthermore, the native substrate samples resulted in low glucose yields of 0.050 g/g (untreated corn cobs) and 0.022 g/g (untreated bamboo). The reducing sugar yields obtained in this study were compared to other pretreatment reports on bamboo and corn cobs.

The reducing sugar yields obtained in the present study was compared to previous alkaline pretreatment studies on corn cobs and bamboo. The study by Qing et al. (2016b) observed an 11-fold higher sugar yield obtained compared to the present optimized $Na_3PO_4.12H_2O_{BB}$ Likewise, Kuttiraja et al. (2013) recorded a 54 % higher sugar yield in comparison with the current optimized NaOH pretreatment for bamboo Similarly, Sewsynker-Sukai and Gueguim Kana (2018a) and (Boonsombuti et al., 2013) also achieved higher sugar yields from corn cobs. The higher sugar observed for the previous studies can be ascribed to the difference in lignocellulosic composition and pretreatment conditions. For instance, Kuttiraja et al. (2013), Boonsombuti et al. (2013) and Qing et al. (2016a) employed pretreatment times of 30 min, 30 min and 120 min respectively, that was significantly longer than the present study. Generally, a shorter pretreatment time is preferred since it reduces processes costs. Moreover, the studies by Kuttiraja et al. (2013) and Qing et al. (2016a) utilized steam and oven heating respectively for pretreatment. These heating mechanisms are known to be less efficient than microwave heating, thus the use of 20-fold (Qing et al., 2016a) and 5-fold (Kuttiraja et al., 2013) longer pretreatment times.

Furthermore, the present study displayed a significantly higher reducing sugar yield for corn cobs compared to bamboo. Variations in the sugar yields obtained for these substrates may be attributed to differences in the lignocellulosic composition, which has shown to impact the efficiency of enzymatic hydrolysis (Suseela, 2019). The present optimization study demonstrated that $Na_3PO_4.12H_2O$ exhibited a higher pretreatment efficiency than NaOH for the pretreatment of corn cobs with a 21.9 % higher sugar yield. This provides valuable knowledge since $Na_3PO_4.12H_2O$ could potentially be a suitable replacement for expensive NaOH techniques in the near future (Qing et al., 2016a).

3.5. Effect of the alkaline pretreatments on the lignocellulosic structure

3.5.1. Compositional analysis

Lignocellulosic structures such as cellulose and hemicellulose polymers are attached to lignin through covalent and hydrogen bonding, thus making these structures highly recalcitrant to both chemical and biological degradation (Zabed et al., 2016). Therefore, unravelling of these structures to release cellulose and hemicellulose while removing lignin is imperative (Nasirpour and Mousavi, 2018). The compositional analysis data was determined for the untreated and optimally pretreated bamboo and corn cob substrates. The native (untreated) bamboo consisted of 54.33 % cellulose, 14.82 % hemicellulose and 25.64 % lignin. Optimally pretreated bamboo with NaOH contained 68.11 % cellulose, 11.57 % hemicellulose and 18.92 % lignin. Thus, a 25.36 % improvement in the cellulose content, 21.93 % hemicellulose solubilization and 26.21 % delignification were observed. Similarly, bamboo optimally pretreated with $Na_3PO_4.12H_2O$ contained 67.78 % cellulose, 12.89 % hemicellulose and 19.22 % lignin. Therefore, a 24.76 % enhancement in the cellulose content, 13.02 % hemicellulose solubilization and 25.04 % delignification were achieved for the optimized $Na_3PO_4.12H_2O$ bamboo sample. Strong alkali chemicals such as NaOH and $Na_3PO_4.12H_2O$ break the glycosidic and ester bonds in the cell wall matrix, therefore causing cellulose swelling and decrystallization, altering the structure of lignin, and partial solvation of hemicellulose (Brodeur et al., 2011). More specifically, NaOH targets specific bonds such as the hydrolysis of the ester bonds between the ferulic acid and hemicellulose causing lignin degradation and lignocellulose particle swelling. The study by Yang et al. (2019) pretreated bamboo with NaOH and achieved a high cellulose and xylan conversion rate of 38.2 % and 39.8 % respectively. On the other hand, alkalic salts such as $Na_3PO_4.12H_2O$ contain nucleophilic species that facilitate the cleavage of phenolic β-aryl ether bonds of lignin resulting in delignification (Gu et al., 2019). Correspondingly, the weak bases enhance the removal of acetyl groups from xylan structures, which improves cellulose digestibility during enzymatic hydrolysis (Gu et al., 2019). Likewise, Qing et al. (2016b) pretreated bamboo shoot shell using $Na_3PO_4.12H_2O$ combined with H_2O_2 and observed an increase in the cellulose content, hemicellulose removal and delignification of 86.1 %, 37.8 % and 85.1 % respectively.

The native (untreated) corn cobs consisted of 40.31 % cellulose, 43.50 % hemicellulose and 9.31 % lignin while the optimized NaOH pretreated corn cobs contained 72.48 % cellulose, 12.73 % hemicellulose and 10.13 % lignin. Therefore, a cellulose improvement and hemicellulose solubilization of 79.81 % and 70.74 % respectively was recorded. You et al. (2019) developed an ultrasound-NaOH pretreatment on corn cobs and achieved a high cellulose improvement and lignin conversion of 47 % and 60 % respectively. Alternatively, the

Table 5
Validation of optimized conditions for the alkaline pretreatment on bamboo and corn cobs.

Model	Input parameters			Reducing sugar yield (g/g)	
	Alkaline catalyst concentration (%)	Power intensity (W)	Pretreatment time (min)	Predicted	Observed
$Na_3PO_4.12H_2O_{BB}$	14	600	7	0.099	0.086 ± 0.001
$NaOH_{BB}$	18	600	6	0.220	0.225 ± 0.0005
$Na_3PO_4.12H_2O_{CC}$	18	600	8	0.492	0.512 ± 0.0025
$NaOH_{CC}$	12	500	5	0.421	0.420 ± 0.0065

optimally pretreated $Na_3PO_4.12H_2O$ corn cobs consisted of 56.08 % cellulose, 30.74 % hemicellulose and 9.91 % lignin. This translated to a cellulose improvement and hemicellulose solubilization of 39.12 % and 29.33 % respectively. A previous study by Qing et al. (2016a) recorded improvements of 74.26 % in the cellulose recovery, 36.24 % hemicellulose removal and 62.20 % delignification of corn stover after using $Na_3PO_4.12H_2O$ based pretreatments. Nevertheless, the present study recorded an increase in the lignin content of 8.81 % and 6.44 % for both the NaOH and $Na_3PO_4.12H_2O$ pretreated corn cobs respectively. The increase in lignin content can be attributed to the formation of Klason lignin. Klason lignin (pseudo-lignin), is a product formed from cellulose and hemicellulose when biomass is pretreated with an acid or base at extreme thermal conditions (Shinde et al., 2018). Moreover, the present study only displayed pseudo-lignin formation from corn cobs whereas the bamboo demonstrated efficient delignification. The monomeric units that make up lignin vary in the cell walls of different lignocellulosic biomasses and can account for the pseudo-lignin formation in the corn cobs without its occurrence for the bamboo substrate. Therefore, the proportion of lignin monomers is an important factor deciding its susceptibility to degradation during chemical pretreatment (Suseela, 2019).

3.5.2. Visual observations using scanning electron microscopy (SEM)

The untreated and optimally pretreated bamboo and corn cobs surfaces were visually analyzed using SEM. The native (untreated) substrates exhibited a smooth and continuous surface area whereas the optimized pretreated substrates demonstrated the disintegration of the lignocellulosic structures. The substrates optimally pretreated with NaOH displayed a high degree of damage to its surface area compared to $Na_3PO_4.12H_2O$ pretreatments. During pretreatment, hydrogen bonds are destroyed resulting in the leakage of lignin moieties (Pu et al., 2013). An increase in porosity and surface area enhances the availability of cellulose and hemicellulose to enzymes during enzymatic hydrolysis (Nasirpour and Mousavi, 2018; Qing et al., 2016a). These results were consistent with previous studies on corn stover (Qing et al., 2016a), bamboo (Qing et al., 2016b), corn cobs (Boonsombuti et al., 2013) and bamboo (Kuttiraja et al., 2013) pretreated with $Na_3PO_4.12H_2O$ (Qing et al., 2016b) and NaOH (Boonsombuti et al., 2013; Kuttiraja et al., 2013). The corn cob substrate displayed a more disorganized morphology characterized by the exposure of fibres and loosening of the fibrous network. This illustrated that the corn cobs were prone to a higher level of fragmentation compared to the bamboo substrate. The high concentration of lignin that is present within plant cell walls hold the fibres of polysaccharides tightly packed together contributing to its recalcitrant nature (Pu et al., 2013) and may account for the more in tact surface structure of the bamboo substrate.

3.5.3. Fourier transform infrared (FTIR) analysis

FTIR spectral analysis was performed and showed the chemical structural changes of cellulose, hemicellulose and lignin in the wavelength range of 400 cm^{-1} to 3800 cm^{-1}. The absorption band peaks were shown to be stronger for the optimized pretreated samples as compared to the native samples for both corn cobs and bamboo. All the pretreated samples displayed a similar banding pattern. For instance, the absorptive band peaks at 900 cm^{-1} corresponds to the breakage of the β-1,4 glycosidic linkages in cellulose. The band peak at 1034 cm^{-1} is ascribed to the aromatic C—H and C—O deformation (Boukir et al., 2019). The absorption peak at 1166 cm^{-1} represents the vibrations of C—O—C associated with the pyranose ring skeleton and C—O—C asymmetric stretching in cellulose and hemicellulose (Boukir et al., 2019). Two absorption bands were detected at 1371 and 1431 cm^{-1} and occur in the range between 1300−1500 cm^{-1} illustrating the C–H-bending vibrations of cellulose (Li et al., 2018). Conversely, the band peaks observed at 1656 cm^{-1} demonstrate in-plane C═C aromatic vibration in the aromatic ring within lignin (Li et al., 2018). Additionally, C—H stretching and O—H stretching vibrations of intramolecular hydrogen within cellulose were indicated by absorptive peaks at 2901 cm^{-1} and 3323 cm^{-1}, respectively (Li et al., 2018). Previous reports on strong alkali (NaOH) and alkalic salt ($Na_3PO_4.12H_2O$) pretreatments on bamboo (Kuttiraja et al., 2013; Qing et al., 2016b) and corn cobs (Boonsombuti et al., 2013; Sewsynker-Sukai and Gueguim Kana, 2018a) respectively displayed similar findings.

3.6. Recyclability of spent alkaline pretreatment liquid

The black liquor obtained after the optimized NaOH and $Na_3PO_4.12H_2O$ pretreatments were collected and subsequently used for the recyclability studies on corn cobs (Fig. 2A-B). Pretreatment cycle 0 corresponds to the optimized experiments that were performed with the original NaOH and $Na_3PO_4.12H_2O$ chemicals and resulted in reducing sugar yields of 0.42 g/g and 0.512 g/g respectively. The first, second and third pretreatment cycles displayed reducing sugar yields of 0.437 g/g, 0.352 g/g and 0.333 g/g for the NaOH black liquor recyclability. Alternatively, the $Na_3PO_4.12H_2O$ black liquor demonstrated sugar yields of 0.425 g/g, 0.302 g/g and 0.191 g/g for the first, second and third pretreatment cycles. The variations observed in the sugar yields between the different pretreatment cycles for the NaOH and $Na_3PO_4.12H_2O$ black liquor recyclability may be linked with the chemical changes that occur in the hydrolysate after each consecutive cycle. For instance, the reducing sugar yield increased in the first cycle (4.05 %) whereas a higher glucose yield (10.33 %) was recorded for the second cycle using the NaOH black liquor. Volatile compounds such as phenolics, aliphatic acids, amines and amides are generally released when ether and covalent bonds in lignin macromolecules are fragmented during alkaline pretreatment (Rorke et al., 2016; Sewsynker-Sukai and Gueguim Kana, 2018a). The improved reducing sugar and glucose yields after recycling the black liquor could be explained by the accumulation of these volatile compounds that may assist with hemicellulose solubilization. The studies by Rorke et al. (2016) and Sewsynker-Sukai and Gueguim Kana (2018a) detected similar volatile compounds when sorghum leaves and corn cobs respectively, were pretreated with microwave-assisted alkaline catalysts.

The first, second and third pretreatment cycles for the NaOH black liquor revealed pH values of 12.84, 12.84 and 12.64 respectively. On the other hand, the $Na_3PO_4.12H_2O$ black liquor recyclability illustrated a sharp decrease in the pH values for the first (pH 11.94), second (pH 11.30) and third (pH 10.83) pretreatment cycles. Alkaline pH has shown to be critical for delignification by facilitating the oxidation of ethylene double bonds in the side chains of lignin phenylpropane units (Geng et al., 2014). The pH change observed for each consecutive recyclability cycle may be attributed to the formation of inhibitor compounds such as formic acid derived from furan derivatives (furfural), carboxylic acids produced from the acetyl groups in hemicelluloses, and lignin-derived phenolics (Kim, 2018). These inhibitors may be acidic and cause a reduction in the pH (Kim, 2018). The study by Mudassar et al. (2014) observed a decrease in pH during second stage pretreatment of sawdust suggesting that alkali was consumed by carboxylic acids and carbon dioxide in the reaction mixture. Likewise, Qing et al. (2016b) also observed a decline in the pH after recyclability of alkalic salt black liquor on bamboo. Mudassar et al. (2014) reported that alkaline liquor exhibits a high performance on lignin removal, enhancing the digestibility of cellulose and hemicellulose.

The present study revealed that both NaOH and $Na_3PO_4.12H_2O$ black liquor could be recycled up to three times. Although NaOH produced a lower reducing sugar yield compared to $Na_3PO_4.12H_2O$ on corn cobs, it released a higher glucose yield before and after recycling. The higher reducing sugar yield may be due to the specificity of the DNS method. DNS is an aromatic compound that is used to detect the presence of free carbonyl groups of reducing sugars (monosaccharides and disaccharides) and is used to quantify α-amylase activity (Keharom et al., 2016). However, the detection range of the DNS method is limited to a specific concentration, and degree of polymerization of the

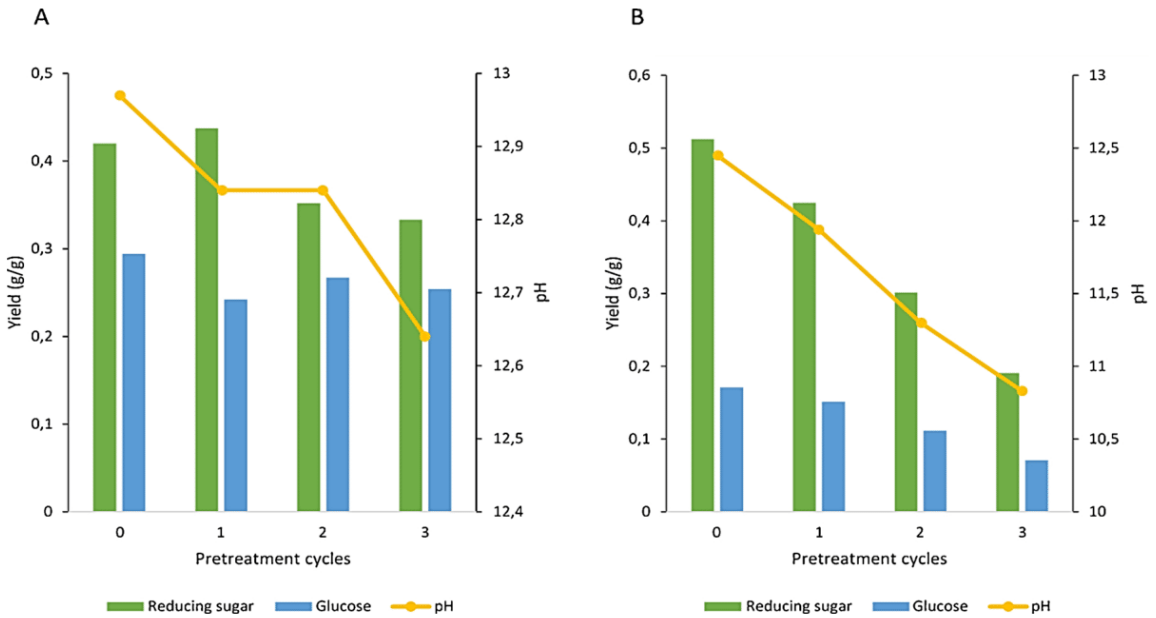

Fig. 2. Chemical recyclability of (A) NaOH and (B) $Na_3PO_4.12H_2O$ for the pretreatment of corn cobs.

carbohydrates (Rivers et al., 1984). Another downfall is that by-products produced as a result of pretreatment processes may interfere with the aromatic compound by supplying reactive reducing groups (aldehydes and ketones) resulting in significant quantitative errors (Rivers et al., 1984). The aforementioned limitations of the DNS method may have led to the overestimation of reducing sugar yields, therefore displaying higher sugar compared to the glucose yields. Even though the DNS method displays these limitations for sugar quantification, it has been extensively used and is a well-established protocol for sugar analysis (Gonçalves et al., 2010).

3.7. Kinetic assessment

3.7.1. Angel yeast cell growth using the logistic model

The growth of Angel yeast over time during the $NaOH_{CC(SSF)}$ and $Na_3PO_4.12H_2O_{CC(SSF)}$ processes are illustrated in Fig. 3A. The lag phase of yeast growth was observed during the first 2 h for both SSF processes. During the lag phase, yeast cells are metabolically active and synthesize proteins needed for growth within the fermentation medium (Ginovart et al., 2018). The $NaOH_{CC(SSF)}$ and $Na_3PO_4.12H_2O_{CC(SSF)}$ processes demonstrated an exponential increase in the Angel yeast cells between 2–18 h. During SSF processes, the enzymatic conversion of cellulose to glucose occurs at a rapid rate. A high glucose utilization was observed by the Angel yeast cells (>70 %) and corresponded with the exponential phase (Fig. 3B). The decline in cell growth above 18 h for the $NaOH_{CC(SSF)}$ and $Na_3PO_4.12H_2O_{CC(SSF)}$ processes can be attributed to the depletion of sugars, a decrease in enzymatic activity, or glucose-induced repression of respiration also known as the Crabtree-effect (De Deken, 1966).

The Angel yeast growth data obtained from the $NaOH_{CC(SSF)}$ and $Na_3PO_4.12H_2O_{CC(SSF)}$ processes fitted the logistic model with high correlation coefficient (R^2) values of 0.973 and 0.988 respectively. The observed kinetic parameters for the $NaOH_{CC(SSF)}$ and $Na_3PO_4.12H_2O_{CC(SSF)}$ processes were compared to previous studies. The $NaOH_{CC(SSF)}$ and $Na_3PO_4.12H_2O_{CC(SSF)}$ processes demonstrated maximum specific growth rate (μ_{max}) values of of 0.175 h^{-1} and 0.049 h^{-1} respectively. Previous kinetic studies on sugarcane bagasse (Jugwanth et al., 2019), potato peel waste (Chohan et al., 2020), and corn cobs (Sewsynker-Sukai and Gueguim Kana, 2018b) produced similar μ_{max} values of 0.150 h^{-1}, 0.195 h^{-1} and 0.216 h^{-1} respectively. The initial (X_0) and maximum (X_{max}) biomass cell concentrations are useful indicators for biomass synthesis and product formation. Kinetic data obtained for the $NaOH_{CC(SSF)}$ and $Na_3PO_4.12H_2O_{CC(SSF)}$ processes gave X_0 values of 0.548 g/L and 0.501 g/L respectively. Also X_{max} values of 1.817 g/L and 5.579 g/L were obtained for the $NaOH_{CC(SSF)}$ and $Na_3PO_4.12H_2O_{CC(SSF)}$ processes respectively. The study by Sewsynker-Sukai and Gueguim Kana (2018b) recorded X_0 and X_{max} values of 0.556 g/L and 3.650 g/L respectively from corn cobs pretreated with $Na_3PO_4.12H_2O$ followed by H_2SO_4. Likewise, Jugwanth et al. (2019) observed X_0 and X_{max} values of 0.160 g/L and 2.580 g/L respectively from sequential $ZnCl_2$-NaOH pretreated sugarcane bagasse. Variables such as yeast strain, substrate type, composition and pretreatment, in addition to fermentation conditions such as pH, temperature, solid and enzyme loading may contribute to the discrepancies observed between the different studies.

3.7.2. Kinetic modelling of bioethanol production using the modified Gompertz model

Bioethanol formation during the $NaOH_{CC(SSF)}$ and $Na_3PO_4.12H_2O_{CC(SSF)}$ processes was recorded (Fig. 3C). Exponential production of bioethanol occurred for both processes from 0 h to 18 h. For the $Na_3PO_4.12H_2O_{CC(SSF)}$ and $NaOH_{CC(SSF)}$ processes, bioethanol increased from 0 to 6.05 g/L and 0–16.71 g/L respectively, with the latter being the highest bioethanol produced in this study after 18 h. Additionally, the highest ethanol produced from both processes after 18 h coincides with the exponential phase of yeast cell growth (Fig. 3A) and a high glucose utilization (>95 %) (Fig. 3B). During the exponential phase, a sufficient amount of glucose was produced and consumed in order to sustain Angel yeast metabolism, resulting in the formation of primary metabolites such as bioethanol. Similar to the low ethanol concentration obtained for the $Na_3PO_4.12H_2O_{CC(SSF)}$ process, Qing et al. (2016b) produced a maximum ethanol concentration of 8.6 g/L from corn stover pretreated with $Na_3PO_4.12H_2O$. The lower ethanol concentration observed for the $Na_3PO_4.12H_2O_{CC(SSF)}$ process compared to the $NaOH_{CC(SSF)}$ system may be ascribed to the reduced glucose generated from the alkalic salt. After 18 h, the ethanol concentration began to decline for both processes, which corresponds to the stationary phase of growth.

Bioethanol production data obtained from the $NaOH_{CC(SSF)}$ and $Na_3PO_4.12H_2O_{CC(SSF)}$ processes were used to fit the modified Gompertz model with high correlation coefficient (R^2) values of 0.96 and 0.933 respectively. The observed bioethanol kinetic parameters in this study were compared to previous studies. A maximum bioethanol concentration (P_m) of 16.928 g/L and 5.286 g/L were obtained for the $NaOH_{CC(SSF)}$ and $Na_3PO_4.12H_2O_{CC(SSF)}$ processes respectively. Similarly,

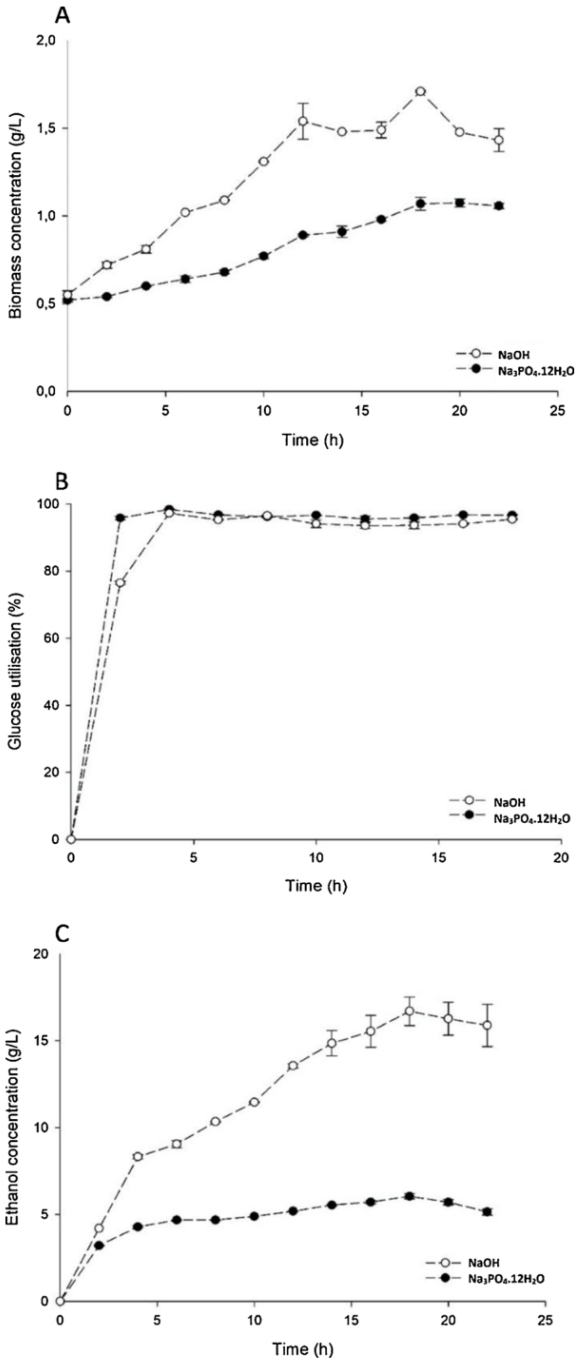

Fig. 3. Angel yeast biomass growth (A), glucose utilisation (B), and bioethanol production (C) during the SSF processes using the optimized pretreated corn cobs.

Aguilar-Reynosa et al. (2017b) comparatively investigated a conduction-convection heating and microwave heating processing pretreatment for bioethanol production using corn stover. The aforementioned authors obtained a maximum bioethanol concentration of 33.8 g/L and a 92 % conversion rate using microwave heating. Previous kinetic studies on acid-pretreated potato peel waste (Chohan et al., 2020), acid-pretreated sorghum leaves (Rorke and Gueguim Kana, 2017), and salt-alkali pretreated sugarcane bagasse (Jugwanth et al., 2019) produced similar P_m values of 15.48 g/L, 17.15 g/L, and 3.12 g/L respectively. The $NaOH_{CC(SSF)}$ and $Na_3PO_4.12H_2O_{CC(SSF)}$ processes demonstrated maximum bioethanol production rate ($r_{p,m}$) values of 1.246 g/L/h and 1.364 g/L/h respectively. This indicated that 1.246 g/L and 1.364 g/L of ethanol could be produced by Angel yeast every 60 min. The studies by Sewsynker-Sukai and Gueguim Kana (2018b) and Chohan et al. (2020) observed similar maximum bioethanol production rates of 2.39 g/L/h and 1.513 g/L/h from corn cobs and potato peel waste respectively. The lag time (t_L) for bioethanol formation was the shortest (0 h) as compared to other studies. Therefore, a shorter time was required for the yeast cells to adapt to the fermentation medium resulting in the production of bioethanol at the onset of the SSF process. Likewise, the studies by Wang et al. (2013) and Jugwanth et al. (2019) reported short lag times of 0 h and 0.97 h respectively. On the other hand, Rorke and Gueguim Kana (2017) and Chohan et al. (2020) encountered extensively longer lag times of 6.31 h and 4.658 h respectively. The kinetic data from the present study demonstrates major knowledge enhancements for the potential of lignocellulosic bioethanol production using SSF processes.

4. Conclusion

From the developed pretreatments on different lignocellulosic substrates, the optimized $Na_3PO_4.12H_2O$ pretreatment on corn cobs resulted in the highest reducing sugar yield (0.512 g/g). On the other hand, recyclability studies showed a 16 % higher average sugar yield for the NaOH-based black liquor compared to the $Na_3PO_4.12H_2O$ hydrolysate over three pretreatment cycles. Interestingly, kinetic data revealed a 72 % and 69 % higher maximum specific growth rate and maximum potential bioethanol concentration respectively for the bioprocess using NaOH pretreated corn cobs. The findings from this study provides valuable insights on newly emerging alkaline chemicals such as $Na_3PO_4.12H_2O$ compared to the well-known NaOH for lignocellulosic pretreatment, hydrolysate recyclability and bioethanol production. While $Na_3PO_4.12H_2O$ represents a cheaper alternative to the gold standard NaOH method, a long trajectory towards achieving competitive lignocellulosic bioprocess yields is foreseeable. Nevertheless, recyclability of the hydrolysate from both chemicals demonstrated the potential to minimize pretreatment costs associated with the usage of chemicals and fresh water in lignocellulosic biorefineries.

CRediT authorship contribution statement

Milesh Laltha: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Data curation, Writing - original draft, Writing - review & editing, Visualization, Project administration, Funding acquisition. **Y. Sewsynker-Sukai:** Conceptualization, Methodology, Software, Validation, Investigation, Data curation, Writing - review & editing, Visualization, Project administration, Resources, Supervision, Funding acquisition. **Gueguim Kana E.B.:** Software, Validation, Data curation, Writing - review & editing, Resources, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.indcrop.2021.114166.

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Appendix A - Supplementary material

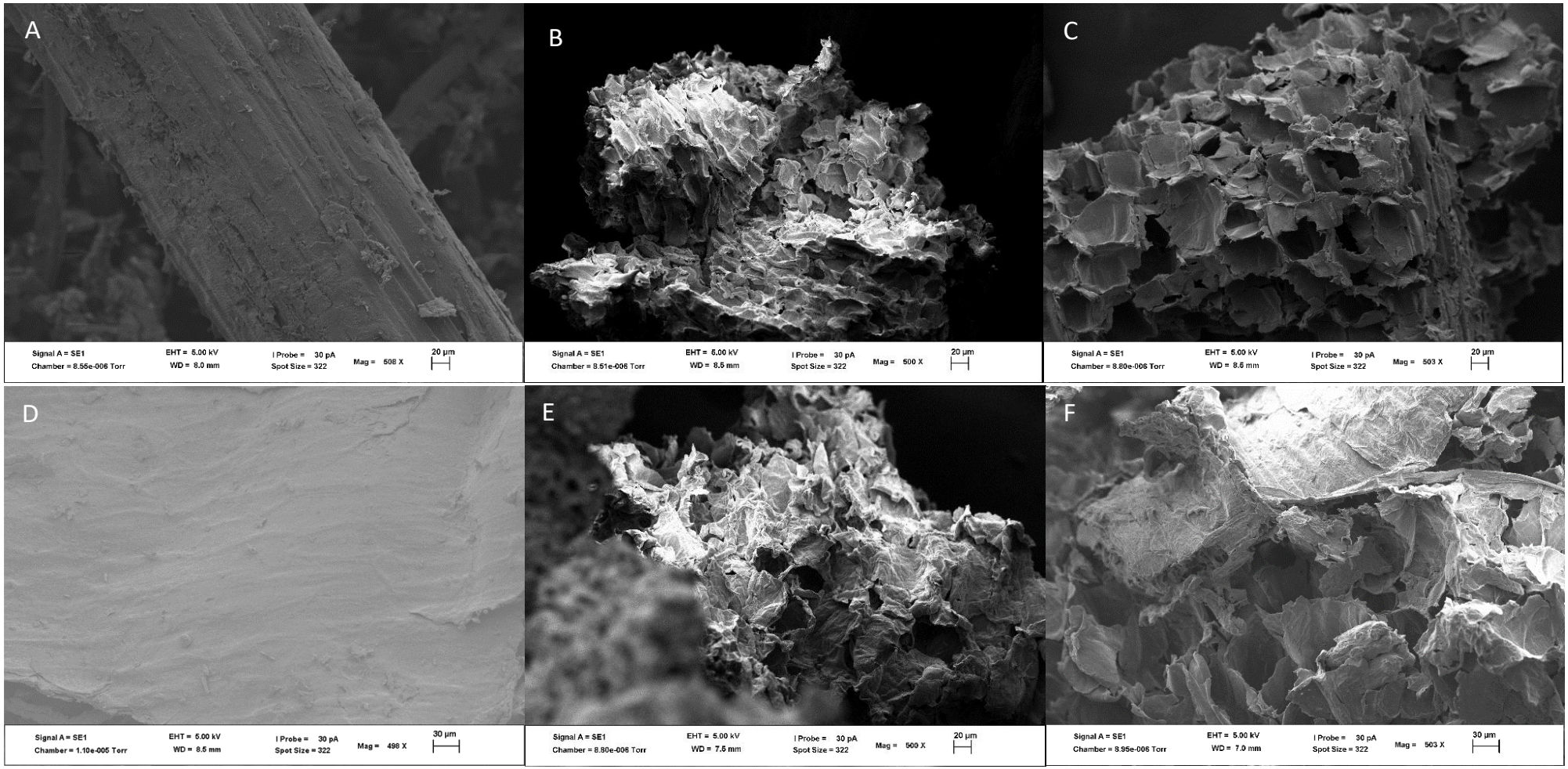

Fig. S1. SEM micrographs (X500): (A) untreated bamboo; (B) optimally pretreated bamboo (NaOH); (C) optimally pretreated bamboo ($Na_3PO_4.12H_2O$); (D) untreated corn cobs; (E) optimally pretreated corn cobs (NaOH); (F) optimally pretreated corn cobs ($Na_3PO_4.12H_2O$).

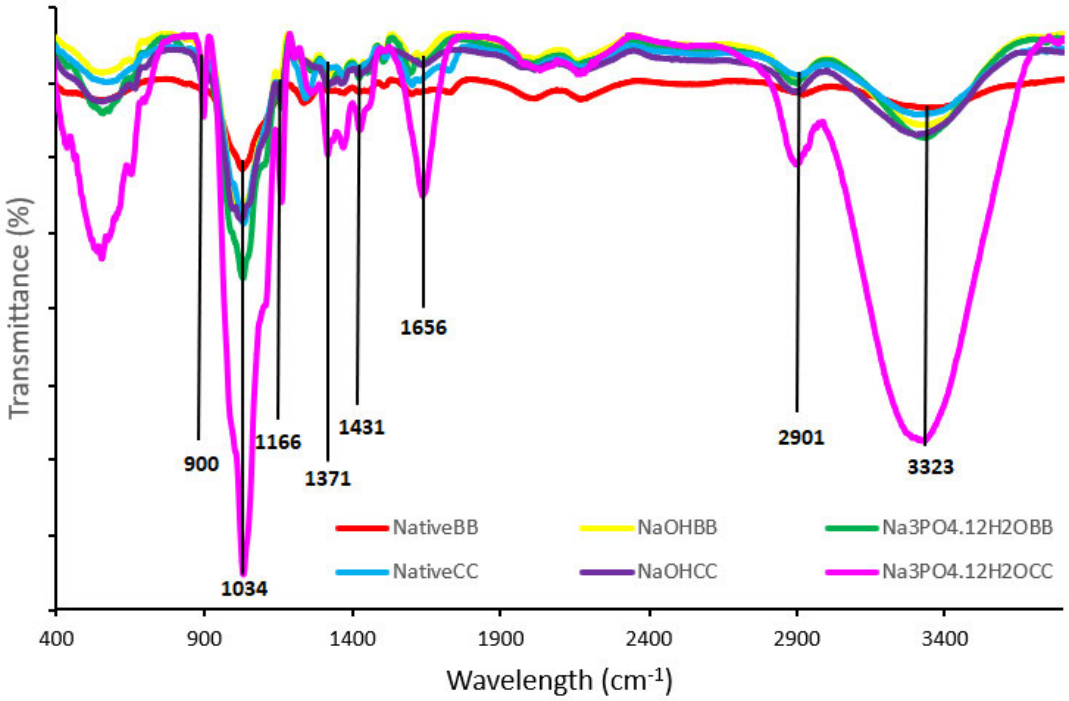

Fig. S2. FTIR spectra of untreated and the optimized NaOH and $Na_3PO_4.12H_2O$ pretreated bamboo and corn cobs.

Table S1. Analysis of variance of the developed model of bamboo pretreated with $Na_3PO_4.12H_2O$[BB]

Source	Sum of squares	df	Mean square	F-value	P-value (Probability > F)
Model	0.020	9	2.209×10^{-3}	4.49	0.0302 Significant
A-Alkaline concentration	9.997×10^{-4}	1	9.997×10^{-4}	2.03	0.1971
B-Power intensity	3.608×10^{-3}	1	3.608×10^{-3}	7.33	0.0303
C-Pretreatment time	1.503×10^{-5}	1	1.503×10^{-5}	0.031	0.8662
AB	2.317×10^{-4}	1	2.317×10^{-4}	0.47	0.5147
AC	9.022×10^{-7}	1	9.022×10^{-7}	1.833×10^{-3}	0.9670
BC	7.983×10^{-6}	1	7.983×10^{-6}	0.016	0.9022
A^2	3.099×10^{-3}	1	3.099×10^{-3}	6.30	0.0404
B^2	9.858×10^{-3}	1	9.858×10^{-3}	20.03	0.0029
C^2	8.531×10^{-4}	1	8.531×10^{-4}	1.73	0.2294
Lack-of-fit	1.563×10^{-3}	3	5.211×10^{-4}	1.11	0.4438 not significant

Footnote: df=degrees of freedom, F-value=Fisher-Snedecor distribution value, P-value=probability value.

Table S2. Analysis of variance of the developed model of bamboo pretreated with $NaOH_{BB}$

Source	Sum of squares	df	Mean square	F-value	P-value (Probability > F)
Model	0.027	9	2.990×10^{-3}	31.64	< 0.0001 Significant
A-Alkaline concentration	0.016	1	0.016	166.52	< 0.0001
B-Power intensity	3.382×10^{-3}	1	3.382×10^{-3}	35.79	0.0006
C-Pretreatment time	1.312×10^{-5}	1	1.312×10^{-5}	0.14	0.7205
AB	9.622×10^{-4}	1	9.622×10^{-4}	10.18	0.0152
AC	5.230×10^{-4}	1	5.230×10^{-4}	5.53	0.0509
BC	3.499×10^{-5}	1	3.499×10^{-5}	0.37	0.5620
A^2	4.874×10^{-3}	1	4.874×10^{-3}	51.59	0.0002
B^2	1.101×10^{-3}	1	1.101×10^{-3}	11.65	0.0112
C^2	1.084×10^{-4}	1	1.084×10^{-4}	1.15	0.3196
Lack-of-fit	5.536×10^{-4}	3	1.845×10^{-4}	6.85	0.0470 significant

Footnote: df=degrees of freedom, F-value=Fisher-Snedecor distribution value, P-value=probability value.

Table S3. Analysis of variance of the developed model of corn cobs pretreated with $Na_3PO_4.12H_2O$CC

Source	Sum of squares	df	Mean square	F-value	P-value (Probability > F)
Model	0.42	9	0.046	21.58	0.0003 Significant
A-Alkaline concentration	0.053	1	0.053	24.68	0.0016
B-Power intensity	0.17	1	0.17	78.63	< 0.0001
C-Pretreatment time	9.282×10^{-3}	1	9.282×10^{-3}	4.33	0.0759
AB	0.027	1	0.027	12.50	0.0095
AC	0.017	1	0.017	7.83	0.0266
BC	8.030×10^{-3}	1	8.030×10^{-3}	3.75	0.0941
A^2	0.010	1	0.010	4.70	0.0669
B^2	0.12	1	0.12	55.77	0.0001
C^2	6.760×10^{-5}	1	6.760×10^{-5}	0.032	0.8640
Lack-of-fit	7.370×10^{-3}	3	2.457×10^{-3}	1.29	0.3926 not significant

Footnote: df=degrees of freedom, F-value=Fisher-Snedecor distribution value, P-value=probability value.

Table S4. Analysis of variance of the developed model of corn cobs pretreated with $NaOH_{CC}$

Source	Sum of squares	df	Mean square	F-value	P-value (Probability > F)
Model	0.019	9	2.143×10^{-3}	4.21	0.0357 Significant
A-Alkaline concentration	2.040×10^{-4}	1	2.040×10^{-4}	0.40	0.5470
B-Power intensity	9.197×10^{-3}	1	9.197×10^{-3}	18.05	0.0038
C-Pretreatment time	2.961×10^{-3}	1	2.961×10^{-3}	5.81	0.0468
AB	7.647×10^{-6}	1	7.647×10^{-6}	0.015	0.9059
AC	3.124×10^{-4}	1	3.124×10^{-4}	0.61	0.4593
BC	1.721×10^{-3}	1	1.721×10^{-3}	3.38	0.1087
A^2	2.729×10^{-3}	1	2.729×10^{-3}	5.36	0.0539
B^2	1.420×10^{-3}	1	1.420×10^{-3}	2.79	0.1390
C^2	7.669×10^{-4}	1	7.669×10^{-4}	1.50	0.2596
Lack-of-fit	1.321×10^{-3}	3	4.404×10^{-4}	0.78	0.5617 not significant

Footnote: df=degrees of freedom, F-value=Fisher-Snedecor distribution value, P-value=probability value.

Table S5. Comparison of reducing sugar yields from bamboo and corn cobs reported for various studies with present study

Heating mechanism	Feedstock	Pretreatment conditions	RSY (g/g)	References
Microwave	Bamboo	14% $Na_3PO_4.12H_2O$, 10% SL, 600 W for 7 min	0.086	This study
Microwave	Bamboo	18% NaOH, 10% SL, 600 W for 6 min	0.225	This study
Microwave	Corn cobs	18% $Na_3PO_4.12H_2O$, 10% SL, 600 W for 8 min	0.512	This study
Microwave	Corn cobs	12% NaOH, 10% SL, 500 W for 5 min	0.420	This study
Microwave	Corn cobs	11.55% $Na_3PO_4.12H_2O$, 10% SL, 700W for 6 min	0.760	Sewsynker-Sukai and Gueguim Kana (2018a)
Microwave	Corn cobs	2% NaOH, 6.7% SL, 100 °C for 30 min	0.680	Boonsombuti *et al.* (2013)
Steam	Bamboo	2% NaOH, 2% SL, 180 °C, 150 rpm and 30 min	0.764	Kuttiraja *et al.* (2013)
Oven	Bamboo	9% $Na_3PO_4.12H_2O$ and 0.3 g/g H_2O_2, SL of 1/20 (w/w) at 80 °C for 120 min	0.971	Qing *et al.* (2016b)

Footnote: SL = Solid loading, RSY = Reducing sugar yield.

Table S6. Structural composition of the control (untreated) and optimally pretreated lignocellulosic substrates

Sample	Cellulose (%)	Hemicellulose (%)	Lignin (%)
$Native_{BB}$	54.33	14.82	25.64
$NaOH_{BB}$	68.11	11.57	18.92
$Na_3PO_4.12H_2O_{BB}$	67.78	12.89	19.22
$Native_{CC}$	40.31	43.50	9.31
$NaOH_{CC}$	72.48	12.73	10.13
$Na_3PO_4.12H_2O_{CC}$	56.08	30.74	9.91

Footnote: $_{BB}$ = Bamboo, $_{CC}$ = Corn cobs.

Table S7. Comparison of kinetic coefficients obtained from the logistic model to previous studies

Substrate	μ_{max} (h^{-1})	X_0 (g/L)	X_{max} (g/L)	Reference
Corn cobs	0.175	0.548	1.817	This study ($NaOH_{CC(SSF)}$)
Corn cobs	0.049	0.501	5.579	This study ($Na_3PO_4.12H_2O_{CC(SSF)}$)
Corn cobs	0.216	0.556	3.650	Sewsynker-Sukai and Gueguim Kana (2018b)
Potato peel waste	0.195	0.441	2.428	Chohan *et al.* (2020)
Sugarcane bagasse	0.150	0.160	2.580	Jugwanth *et al.* (2019)

Footnote: μ_{max} = maximum specific growth rate; X_0 = initial cell concentration; X_{max} = maximum cell concentration.

Table S8. Comparison of the ethanol kinetic coefficients obtained using the modified Gompertz model to previous studies

Substrate	P_m (g/L)	$r_{p,m}$ (g/L/h)	t_L (h)	Reference
Corn cobs	16.928	1.246	0	This study ($NaOH_{CC(SSF)}$)
Corn cobs	5.286	1.364	0	This study ($Na_3PO_4.12H_2O_{CC(SSF)}$)
Corn cobs	42.24	2.39	1.98	Sewsynker-Sukai and Gueguim Kana (2018b)
Potato peel waste	15.475	1.513	4.658	Chohan *et al.* (2020)
Sorghum leaves	17.15	0.52	6.31	Rorke and Gueguim Kana, (2017)
Sugarcane bagasse	3.12	0.29	0.97	Jugwanth *et al*. (2019)
Sorghum bagasse	16.64	0.51	0	Wang *et al.* (2013)

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

Appendix B

Chapter 4 of the thesis has been published during the examination period in the journal Bioresource Technology (BITE) entitled: Simultaneous saccharification and citric acid production from paper wastewater pretreated banana pseudostem: Optimization of fermentation medium formulation and kinetic assessment.

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Simultaneous saccharification and citric acid production from paper wastewater pretreated banana pseudostem: Optimization of fermentation medium formulation and kinetic assessment

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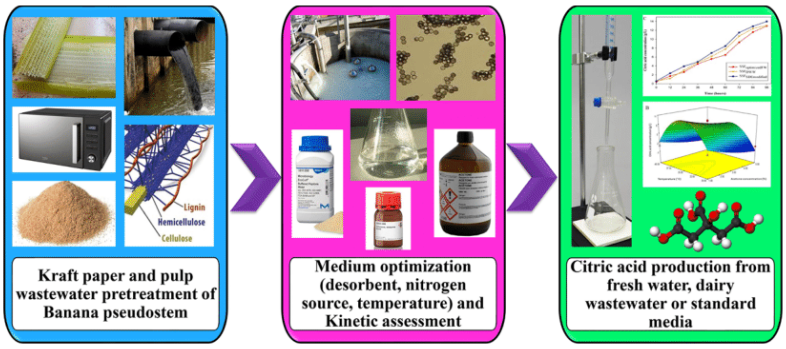
HIGHLIGHTS

- First report on the optimization of citric acid (CA) using banana pseudostem.
- The optimized process gave a maximum CA concentration of 14.408 g/L.
- Kinetics were determined on fresh water, wastewater and standard medium.
- The wastewater-based bioprocess demon strated only a 9.11% lower CA concen tration.
- Findings facilitate the development of waste-based lignocellulosic bioprocesses.

GRAPHICAL ABSTRACT



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ABSTRACT

This study optimized the simultaneous saccharification and citric acid (CA) production from banana pseudostem (BP). Thereafter, kinetic assessment of *Aspergillus brasiliensis* growth and CA production were determined for the optimum conditions using fresh water ($SSF_{optimizedFW}$) or dairy wastewater (SSF_{DWW}) and compared to Sabouraud Dextrose Emmon's medium modified with BP ($SSF_{SDEmodified}$). The optimized conditions gave a CA concentration of 14.408 g/L. Kinetic assessment revealed the same maximum specific growth rates (μ_{max}) (0.05 h^{-1}) for all three bioprocesses, while the $SSF_{SDEmodified}$ process resulted in the highest maximum potential CA concentration (P_m) (13.991 g/L) in comparison to the SSF_{DWW} (P_m = 13.095 g/L) and $SSF_{optimizedFW}$ (P_m = 12.967 g/L) systems. Findings from this study facilitates the implementation of waste-based lignocellulosic bioprocesses that may eradicate the use of expensive pretreatment chemicals, fermentation medium constituents, and resources, in keeping with the water, energy and food nexus towards developing a circular bioeconomy.

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1. Introduction

The bioconversion of renewable lignocellulosic biomass to valuable products such as citric acid (CA) has become a research hotspot towards achieving sustainable global economic development (Yadav et al., 2020). CA is a valuable commercial bioproduct with an existing market and widespread use in the chemical, food, pharmaceutical and cosmetic industries (Sekoai et al., 2018). Approximately 80 % of the world's CA is generated mainly by *Aspergillus niger*. This microbe has typically been used, due to its exclusive physiological characteristics for industrial CA fermentation. *A. niger* shares a phylogenetic relationship with *Aspergillus brasiliensis,* (Varga et al., 2007), however, *A. brasiliensis* produces various other unique compounds that justify its separation into a separate species from *A. niger* (Varga et al., 2007), while still demonstrating high potential for CA production via the citric acid cycle (TCA). Around 2 million tons of CA is generated annually and its rate of demand is expected to rise by 5 % each year (Ozdal and Kurbanoglu, 2019, Sekoai et al., 2018). The CA market is constantly under pressure due to expensive substrates and low production yields (Sekoai et al., 2018). Additionally, the cost of CA production via chemical synthesis is approximately ~$10 USD per kg, whereas for fungal fermentation it has been estimated to be ~ 50 % lower (Ozdal and Kurbanoglu, 2019). The global market for CA is expected to surpass $3.2 billion USD by 2023 (Behera et al., 2021). More importantly, the CA global demand has surpassed the natural supply, therefore the incorporation of lignocellulosic-based bioprocesses that are novel, low-cost, efficient, and environmentally friendly are urgently required. Lignocellulosic biomass is a naturally occurring complex composite that originates from plant dry matter (Moodley et al., 2020), and has emerged as an ideal feedstock for generating renewable bioproducts such as CA due to its abundance and cost-effectiveness. In addition, LCB is considered a waste and therefore does not pose any risk to socio-economic concerns such as deforestation, food security and water shortages (Sekoai et al., 2019). CA has been derived from lignocellulosic substrates that include banana peels (Odu et al., 2020), sweet potato peels (Aboyeji et al., 2020), and sugarcane bagasse (Campanhol et al., 2019). Banana pseudostem with a composition of cellulose (42.35 %), hemicellulose (22.63 %) and lignin (15.36 %) represents a novel substrate for generating CA (Islam et al., 2019). However, the development of lignocellulosic CA production is prone to several drawbacks that include lack of suitable feedstocks, expensive chemicals and/or nutrients as well as energy intensive processes that often result in low product yields. Therefore, overcoming the aforementioned bottlenecks is imperative to enhance lignocellulosic CA production. For one, finding an alternative to fresh water within lignocellulosic bioprocesses could substantially reduce the burden placed on this finite resource and costs incurred. Taking this into consideration, untreated wastewater from the Kraft and dairy industries represents a sustainable and cost-effective option for the replacement of fresh water and/or chemicals within lignocellulosic bioprocessing systems. The emergence of waste-based technologies for lignocellulosic CA production combined with simultaneous saccharification and fermentation (SSF) processes could reduce the time, expenditure for operation, energy input, risk of contamination and enhance the overall productivity and product yields. Fermentation medium constituents that are required to satisfy the nutritional needs of microbes are costly. The microbial growth of *Aspergillus* sp. typically occurs in the Sabouraud Dextrose Emmon's modification (SDE) media, which constitutes peptone and glucose that function as nitrogen and carbon sources, respectively. While glucose may be supplied by the LCB, peptone is an expensive constituent estimated at $151 USD per kg, contributing majorly to the entire bioprocessing cost. Therefore, the investigation of a cost-effective nitrogen source is imperative. Ammonium nitrate has an estimated cost of $108 USD per kg and may be employed as a cheaper alternative to peptone, translating to a cost reduction of ~ 28 %. Odu et al. (2020), Sarkar and Das (2017) and Sekoai et al. (2018) previously utilized ammonium-based salts (ammonium nitrate or ammonium sulfate) in the range of 0.1 to 8 % w/v and demonstrated its adequacy as a nitrogen source in CA production. Other sources of nitrogen for CA production include urea, corn steep liquor (CSL) and yeast extract that are also considered significantly cheaper than peptone (Sekoai et al., 2018). Moreover, several studies have reported on the improvement of CA yields by the addition of desorbents such as methanol and acetone in the range of 1 to 4 % (Sarkar and Das, 2017, Odu et al., 2020). Desorbents have shown to enhance CA production by improving the cell permeability (Sekoai et al., 2018). In addition to these nutritional components, temperature influences the cellulase-based enzyme and fungal culture in lignocellulosic CA production. Fungi such as *A. brasiliensis* have shown to thrive under various temperatures ranging from 25 to 45 °C (Aboyeji et al., 2020), while cellulase-based enzymes require temperatures between 45 and 50 °C for optimal functioning. Exploring a suitable, intermediate temperature that provide optimal conditions for the enzyme and microbial culture functioning is crucial in lignocellulosic bioprocessing. Optimizing input parameters that comprise of temperature, nitrogen and desorbent concentration may prove valuable for CA production. The response surface methodology (RSM) is a mathematical model that may be used for the optimization process and determines the interactive effects of input parameters and their corresponding output (Gaitonde et al., 2017). Apart from bioprocess optimization, kinetic modelling provides an in-depth understanding of the metabolic nature of a fermentation process (Germec et al., 2019) and plays an essential role in predicting, monitoring, optimizing and simulating the performance of a process (Pramanik et al., 2019). The logistic model is used to describe the kinetics of microbial biomass growth to the rate-limiting substrate over a certain time period (Germec et al., 2019). Conversely, the modified Gompertz model estimates the maximum production rate, maximum concentration and the lag phase (Srimachai et al., 2015). Although SDE media has shown to be effective for the cultivation of *Aspergillus* sp., this medium consists of expensive constituents and uses fresh water, which is a finite resource. Ideally, the application of industrial wastes as a water replacement in SSF bioprocesses could alleviate water security. Interestingly, the dairy industry produces 1.575×10^{12} L of wastewater that may be harnessed as a substitute for fresh water (Slavov, 2017). Dairy wastewater undergoes expensive treatment processes to reduce contaminants before it is expelled into the environment. From an industrial perspective, these treatments are considered an economical burden, due to the long processing times, high costs and energy. Adding to this, is the release of greenhouse gas emissions that has severe negative implications on the environment (Gogoi et al., 2021). Therefore, using DWW in lignocellulosic bioprocesses also presents opportunities for commercial industries regarding disposal and treatment costs. Another striking characteristic of DWW is the presence of trace amounts of carbohydrates, soluble organic compounds, protein and nitrogen that could contribute to promoting the growth of the microbial culture (Kaur, 2021). Even though several studies have reported on the production of CA from different lignocellulosic substrates (Odu et al., 2020, Sarkar and Das, 2017, Aboyeji et al., 2020), there has been a scarcity of information on the effects of temperature, ammonium nitrate and acetone on CA production using BP. Furthermore, the existing knowledge gap on kinetic data of *A. brasiliensis* growth and CA production with fresh water, DWW and standard SDE media has piqued interest. Therefore, the present study aimed to model and optimize the SSF process using the RSM for CA production from pretreated BP. For the RSM optimization, the effect of acetone concentration, ammonium nitrate concentration and temperature on the CA production was determined. Subsequently, the modified Gompertz and logistic models were employed to evaluate the kinetics of *A. brasiliensis* growth and CA production respectively. For the kinetic experiments, three bioprocesses consisting of the: (1) optimized SSF process with fresh water ($SSF_{optimizedFW}$), (2) $SSF_{optimizedFW}$ process conditions while substituting dairy wastewater in place of fresh water (SSF_{DWW}), and (3) SDE medium adapted by substituting glucose with BP ($SSF_{SDEmodified}$) were assessed.

2. Materials and methods

2.1. Materials

The banana pseudostem substrate used in this study was obtained from Bakerville Heights (Pietermaritzburg, South Africa) (29.5418° S, 30.4077° E). The substrate was dried at 75 °C for 48 h in an oven (Scientific series 2000, South Africa) to reduce the remaining moisture content and was thereafter milled to a particle size that ranged from 1 to 2 mm. The chemical reagents utilized were acquired from Merck (South Africa), where suitable. The Cellic CTec2 enzyme (160 FPU/mL) harnessed for the SSF processes was supplied by Novozymes (Novozymes A/S, Denmark). The paper wastewater (pH ~ 8) together with the safety data sheet containing its chemical composition (Laltha et al., 2022) was provided by a Kraft paper and pulp mill (Mondi, Richards Bay, South Africa). The dairy wastewater employed was obtained from a regional dairy (Fairfield, Howick, South Africa) and its composition has been reported by David et al. (2022).

2.2. Revival and microbial development

The fungal strain employed in this study, *Aspergillus brasiliensis* ATCC16404 was obtained from ATCC® type cultures originating from Microbiologics Incorporated. The Microbiologics KWIK-STIK pack contained a protocol, ampoule of hydrating fluid, qualitative lyophilized microorganism pellet and an inoculating swab. The hydrating fluid was applied to the lyophilized pellet and aseptically swabbed onto SDE (Sabouraud Dextrose Emmon's) agar by using the three-way streak plating technique to facilitate colony isolation. The SDE agar medium formulation comprised of 20 g/L glucose, 20 g/L agar and 10 g/L peptone autoclaved at 121 °C for 15 min. The fungal strain was then incubated at 30 °C for 7 days. Preparation of a fungal spore suspension was carried out by the addition of 10 mL of sterilized distilled water that contained non-ionic surfactant (0.1 % v/v Tween 80) by covering the fungal growth and using a spatula to scrape the spores into 100 mL of SDE broth (20 g/L glucose and 10 g/L peptone) incubated at 30 °C and 150 rpm for 7 days.

2.3. Short-term and long-term storage of culture

To ensure continuous longevity of the microorganism, the fungal strain was subcultured every 7 days by transferring a piece of inoculated agar containing mature fungal hyphae and spores onto SDE agar, which was thereafter subjected to incubation at 30 °C for 7 days. The previous growth plate was stored at 4 °C in the refrigerator. For long-term storage, *A. brasiliensis* was inoculated in glycerol stocks (10 %, 20 % and 50 % v/v) and agar slants (double-strength medium) and stored at −80 °C and 4 °C, respectively.

2.4. Pretreatment of banana pseudostem

The powdered BP substrate was subjected to pretreatment using a previously optimized microwave-assisted paper wastewater method (Laltha et al., 2022). The BP substrate at a 30 % (w/v) solid loading was submerged in 100 mL of paper wastewater and exposed to microwave heating at 800 W for 8 min. The pretreated banana pseudostem was filtered using a domestic sieve (<1 mm) and washed several times with deionized water, oven-dried at 55 °C overnight prior to being used in the SSF bioprocess. The lignocellulosic composition (cellulose, hemicellulose, and lignin) of the untreated (native) and optimally pretreated banana pseudostem was analyzed using the detergent fiber technique described by Van Soest and McQueen, 1973. The composition of the optimally pretreated banana pseudostem was previously determined and consisted of cellulose (17.06 %), hemicellulose (13.04 %) and lignin (5.9 %) (Laltha et al., 2022).

2.5. Simultaneous saccharification and fermentation (SSF) process

2.5.1. Inoculum development

A. brasiliensis was grown on SDE agar as previously described in section 2.2. The grown suspension was diluted to a final spore concentration of 1×10^6 spores/mL. A Neubauer counting chamber (Neubauer, Germany) was used to determine the spore concentration.

2.5.2. Sodium citrate buffer preparation

Sodium citrate buffer (0.05 M) was made up by combining 0.1 M citric acid and 0.1 M sodium citrate in distilled water. The pH was adjusted to 4.8 prior to autoclaving at 121 °C for 15 min.

2.5.3. Preliminary screening

Preliminary screening of three desorbents (3 % v/v) (acetone, chloroform and propan-2-ol) and four nitrogen sources (2 % w/v) (corn steep liquor, ammonium nitrate, yeast extract and peptone) were performed to determine their individual effects on CA production. The SSF parameter ranges were designated based on an extensive literature review of related lignocellulosic fermentation studies (Sarkar and Das, 2017, Odu et al., 2020). The pretreated BP with a solid loading of 10 % w/v, an inoculum size of 10 % (v/v), and 10 FPU/g enzyme loading was added to an Erlenmeyer flask and brought up to a final volume of 50 mL using 0.05 M sodium citrate buffer. The desorbents (3 % v/v) (acetone, chloroform or propan-2-ol) and nitrogen sources (2 % w/v) (corn steep liquor, ammonium nitrate, yeast extract or peptone) were also added accordingly (See supplementary material). The SSF flasks constituted a working volume of 50 mL, incubated at 35 °C and 150 rpm (Ho and Hood, 2014) for a period of 144 h. Samples (1 mL) were aseptically collected every 24 h and centrifuged at 10 000 rpm for 5 min prior to CA quantification using an acid-base titration method (Ayeni et al., 2019).

2.5.4. RSM experimental design of the SSF process

The response surface methodology (RSM) model (Box-Behnken design) (Design Expert 7.0, Stat Ease Inc, USA) was employed for the optimization. The experimental design consisted of seventeen (17) SSF processes. The fermentation input parameters comprised of desorbent (acetone) concentration (1–5 % v/v), nitrogen (ammonium nitrate) concentration (0.5–5 % w/v) and temperature (30–40 °C), while the output was CA concentration (g/L). The SSF input parameters with its ranges were chosen based on an extensive literature survey (Sarkar and Das, 2017, Sekoai et al., 2018, Odu et al., 2020) in combination with data curated from the screening of preliminary experiments. The resultant experimental CA concentration data fitted the RSM polynomial equations to assess the combined input parameter effects for the enhancement and generation of CA.

2.5.5. Citric acid production

The CA experiments were performed in 100 mL Erlenmeyer flasks comprising of a 50 mL working volume. A solid loading of 10 % (w/v) of paper wastewater pretreated BP was added to each flask. In addition, 10 FPU/g Cellic CTec2 enzyme, 0.05 M sodium citrate buffer, and 10 % v/v inoculum containing 1×10^6 spores/mL *A. brasiliensis* were aseptically added. The respective amounts of acetone (1–5 % v/v) and ammonium nitrate (0.5–5 % w/v) were added to each fermentation flask and incubated at the desired temperature (30–40 °C) as specified in the RSM experimental design (Table 1). The SSF processes were agitated at 150 rpm for a period of 96 h. Using aseptic techniques, the aliquots (1 mL) were sampled from the SSF flasks every 12-hour and stored at −20 °C for further analysis.

2.6. Validation of the optimized SSF bioprocess and kinetic modelling

The variable conditions for optimal CA generation were determined and carried out to validate the developed RSM model. The SSF reaction working volume of 50 mL contained: 10 % (w/v) pretreated BP, 1 % (v/

Table 1
Citric acid concentration for the SSF process on inputs of acetone, ammonium nitrate concentration and temperature.

	Input variables			Output
Run	Acetone (v/v)	Ammonium nitrate (w/v)	Temperature (°C)	Citric acid concentration (g/L)
1	3	0.50	30	1.857 ± 0.121
2	1	0.50	35	14.408 ± 0.594
3	5	2.75	30	0.640 ± 0.005
4	3	2.75	35	9.093 ± 0.126
5	3	2.75	35	9.413 ± 0.162
6	3	0.50	40	1.153 ± 0.052
7	5	2.75	40	0.704 ± 0.033
8	1	2.75	30	2.433 ± 0.128
9	3	5.00	40	1.217 ± 0.063
10	5	0.50	35	6.275 ± 0.178
11	3	2.75	35	8.324 ± 0.059
12	3	2.75	35	5.571 ± 0.115
13	3	2.75	35	9.413 ± 0.231
14	5	5.00	35	6.852 ± 0.220
15	3	5.00	30	2.305 ± 0.098
16	1	5.00	35	12.679 ± 0.106
17	1	2.75	40	0.832 ± 0.143

v) acetone, 0.5 % (w/v) ammonium nitrate, with an inoculum size of 10 % (v/v) and citrate buffer. This process was designated as $SSF_{optimizedFW}$. In addition, the $SSF_{optimizedFW}$ process was substituted with dairy wastewater in place of fresh water (SSF_{DWW}). The SSF_{DWW} process contained: 10 % (w/v) pretreated BP, 1 % (v/v) acetone, 0.5 % (w/v) ammonium nitrate, with an inoculum size of 10 % (v/v) and citrate buffer formulated using DWW. The DWW formulated citrate buffer was the same as described in section 2.5.2., with the substitution of DWW instead of fresh water. For comparison, the standard SDE medium was modified by substituting glucose with BP ($SSF_{SDEmodified}$) and consisted of 10 % (w/v) pretreated BP, 1 % (w/v) peptone, with an inoculum size of 10 % (v/v) and citrate buffer. Each SSF process was therefore characterized as follows: (1) optimized SSF process with fresh water ($SSF_{optimizedFW}$), (2) $SSF_{optimizedFW}$ process conditions while substituting dairy wastewater in place of fresh water (SSF_{DWW}), and (3) SDE medium modified by substituting glucose with BP ($SSF_{SDEmodified}$). All SSF experiments were incubated at 150 rpm, 35 °C for 96 h. The experimental controls were performed parallel to the $SSF_{optimizedFW}$, SSF_{DWW} and $SSF_{SDEmodified}$ and constituted the same conditions with the exception of inoculum, which was omitted. The aforementioned uninoculated controls were used as a reference to determine the initial sugar concentration at a certain time period corresponding to the test SSF experiments ($SSF_{optimizedFW}$, SSF_{DWW} and $SSF_{SDEmodified}$). All of the experiments were carried out in duplicate. Aseptically, sample aliquots (1 mL) were removed from the experimental reaction flask and its equivalent control every 12 h for 96 h. The samples were thereafter analyzed for CA, glucose concentrations and biomass as explained below.

2.7. Analytical methods

2.7.1. Citric acid concentration

SSF processes are dynamic, biological systems and by-products generated during the fermentation may sometimes cause interference when using methods encompassing titration. In the present study, CA concentration was primarily analyzed using the acid-base titration method employed by Ayeni et al. (2019) and thereafter verified by the Megazyme citric acid assay kit, product code (K-CITR) (©Megazyme, Wicklow, Ireland) (See supplementary material). In the acid-base titration analysis, the SSF samples collected from each time point were first centrifuged for 5 min at 10 000 rpm. Subsequently, 100 μL of the supernatant fluid was transferred to a flask containing phenolphthalein indicator (2 to 3 drops). A glass burette was rinsed with distilled water and then filled with the 0.01 M NaOH titrant. The burette was opened to allow few drops of the NaOH (base) titrant to react with the sample (acid) and shaken continuously to observe a change in colour (from colourless to light pink). Excess titrant was washed of the sides of the flask with distilled water. The endpoint of the reaction was determined when the colour change had occurred. Following this, the volume of NaOH utilized was read directly from the burette and substituted into the formula described by Ayeni et al. (2019) to ascertain the amount of CA present. The corresponding number of moles of alkali NaOH required to neutralize CA, $C_3H_5O(COOH)_3$, was then determined based on the volume and concentration of NaOH solution utilized during the acid-base titration (See supplementary material) (Ayeni et al., 2019). For verification of the acid-base titration method described above, the CA concentration was measured by means of the Megazyme citric acid assay kit, product code (K-CITR) (©Megazyme, Wicklow, Ireland) corresponding to the specific instructions in the manufacturers protocol. The Megazyme citric acid assay kit is an enzyme-based analytical method that is specific. The CA content was analyzed spectrophotometrically using a SpectraMax ABS microplate reader (Molecular Devices, California, USA).

2.7.2. Glucose concentration

The glucose concentration of the control (uninoculated) and experimental kinetic SSF experiments were analyzed using the Megazyme glucose kit (K-GLUC) (©Megazyme, Wicklow, Ireland) and protocols detailed by the manufacturer. To determine the glucose utilization (%), the final glucose concentration (inoculated) was subtracted from the initial glucose concentration as per Eq. (1).

$$\text{Glucose utilization}\,(\%) = \frac{\textit{Initial glucose concentration}\,(g/L) - \textit{Final gluose concentration}\,(g/L)}{\textit{Initial glucose concentration}\,(g/L)} \times 100 \quad (1)$$

where the final glucose concentration (g/L) is derived from the experimental SSF flask while the initial glucose concentration (g/L) is attained from the uninoculated control flask at the same moment in time.

2.7.3. Biomass concentration

The biomass concentration of *A. brasiliensis* was analyzed using the dry cell weight (g/L) as a function of the total spore count (spores/mL). The fungal isolate was grown for 7 days in SDE broth to reach exponential phase (10^6 to 10^7 spores/mL) and diluted accordingly (Vrabl et al., 2019). A 15 mL volume per diluted sample (1, 1/2,1/4, 1/8, 1/16 and 1/32) was centrifuged for 5 min at 10 000 rpm. All dilutions were performed in duplicate. Succeeding centrifugation involved discarding of the supernatant and drying of the biomass pellet at 60 °C until the mass was constant. The standard curve was developed by plotting the total spore counts (spores/mL) to the corresponding dry cell weights (biomass concentration in g/L) for each dilution. The spore counts (spores/mL) attained for the kinetic experiments were computed in the equation and extrapolated to ascertain the biomass concentration (g/L).

2.8. Kinetic modelling

2.8.1. The logistic model for A. brasiliensis growth

The logistic model's differential formula, stated in Eq. (2), was integrated to obtain Eq (3). Equation (3) considers the exponential and stationary growth phases. The logistic model Eq. (3) relates biomass (X) to the initial cell concentration (X_0), maximum cell concentration (X_{max}) and maximum specific growth rate (μ_{max}) during the exponential and stationary growth stages of *A. brasiliensis*.

$$\frac{dX}{dt} = \mu_{max}\left(1 - \frac{X}{X_{max}}\right)X \tag{2}$$

$$X = \frac{X_0 \exp(\mu_{max} t)}{1 - \left[\left(\frac{X_0}{X_{max}}\right)(1 - exp(\mu_{max} t))\right]} \tag{3}$$

2.8.2. The modified Gompertz model for citric acid production

Using the least squares approach, the CA data from the SSF processes ($SSF_{optimizedFW}$, SSF_{DWW} and $SSF_{SDEmodified}$) were fitted to the modified Gompertz model(CurveExpert V1.5.5, MyBiosource, Inc., USA). As indicated in Eq. (4), the modified Gompertz model depicts the link between the CA concentration (P), maximum CA production rate ($r_{p,m}$), potential maximum CA concentration (P_m) and lag time (t_L) from the start of fermentation to the exponential CA generation.

$$P = P_m.exp\left\{ - exp\left[\frac{r_{p,m}.exp(1)}{P_m}\right].(t_L - t) + 1\right\} \tag{4}$$

3. Results and discussion

3.1. Preliminary screening

Preliminary screening data revealed that the desorbent that gave the highest concentration of 10.886 g/L CA was acetone, followed by propan-2-ol (10.245 g/L) and chloroform (7.044 g/L) (See supplementary material). For the nitrogen source, ammonium nitrate resulted in the highest CA concentration (12.807 g/L), while peptone, corn steep liquor and yeast extract resulted in CA concentrations of 9.605 g/L, 8.324 g/L and 7.684 g/L respectively. Therefore, acetone and ammonium nitrate were selected as the desorbent and nitrogen source respectively, for the SSF optimization.

3.2. Development of the RSM model

To assess the fitness of the developed RSM model, Analysis of Variance (ANOVA) was used (See supplementary material). P-values < 0.05 indicate parameter and model significance, whereas high F-values demonstrate that the response trends could be explained using the regression equations (Chaganti et al., 2012). The model had a low P-value of 0.0063, which was < 0.05, indicating model significance. In addition, a high F-value (7.89) was attained for the SSF model, thus illustrating statistical significance of the model. Of the three key variables that were investigated, acetone concentration ($p = 0.0282$) demonstrated the most significant effect on the CA concentration compared to temperature ($p = 0.5812$) and ammonium nitrate concentration ($p = 0.9146$). Acetone serves as the desorbent and enhances cell permeability of the microorganism for nutrient sequestration and may also repress the enzyme 2-oxoglutarate dehydrogenase, thus increasing CA concentration (Sekoai et al., 2018, Maddox et al., 1986). This desorbent (acetone) is also a ketone that plays a vital role as an alternative metabolic fuel source for eukaryotes (Puchalska and Crawford, 2017). On the other hand, temperature is a key parameter for the optimum functioning of cellulolytic enzymes for the effective saccharification of cellulose to glucose that will be consumed by the microbe. Additionally, temperature also plays a significant role for regulating the internal temperature, growth, secondary metabolite production and activity of fungi such as *A. brasiliensis* (Tudzynski, 2014). The presence of nitrogen such as ammonium nitrate in the fermentation medium is essential for fungal growth since it facilitates cell adaptation to different niches and survival of adverse environmental conditions such as nutrient limitation (Tudzynski, 2014). The coefficient of determination (R^2) value is a statistic that measures the proportion of variation in the output values that can be attributed to the input variables. In general, R^2 values between 0.7 and 1 elicits model significance (Chaganti et al., 2012). The developed SSF model displayed an R^2 value of 0.91, indicating that 91 % of the variation in the output variable (CA concentration) could be attributed to the input variables. The polynomial model Eq. (5) was generated for the CA concentration and describes the input parameters in relation to the response variable.

$$\begin{aligned}\text{CA concentration (g/L)} = 8.36 &- 1.99A - 0.080B - 0.42C + 0.58AB \\ &+ 0.42AC - 0.096BC + 0.61A^2 + 1.09B^2 \\ &- 7.82C^2\end{aligned}$$

where, A, B and C correspond to acetone concentration (v/v), ammonium nitrate concentration (w/v) and temperature (°C), respectively.

3.3. Effects of the input parameters on the citric acid concentration

The CA concentrations with corresponding input parameters are portrayed in Table 1. The SSF model displayed a maximum CA concentration of 14.408 g/L for run 2 (1 % acetone, 0.5 % ammonium nitrate and 35 °C). The median fermentation conditions (3 % acetone, 2.75 % ammonium nitrate incubated at 35 °C) exhibited similar CA concentration values within the range of 5.571 g/L to 9.413 g/L (run 4, run 5, run 11 run 12 and run 13). The lowest CA concentration recorded (0.640 g/L) was achieved with 5 % acetone, 2.75 % ammonium nitrate and incubated 30 °C for run 3. The RSM graphs that show the collaborative effects of the different input parameters and the resultant CA concentration is illustrated in Fig. 1A-C. The interactive effect of ammonium nitrate concentration and acetone concentration on the output CA concentration, while temperature was kept at its center point (35 °C) is presented in Fig. 1A. Maximum CA concentration (12.63 g/L) was observed at a minimum acetone (1 %) and ammonium nitrate concentration (0.5 %). Further increase in the acetone concentration from 1 to 5 %, while ammonium nitrate concentration was maintained at 0.5 % led to a decline in the CA concentration from 12.63 to 7.55 g/L. Simultaneous increments in the ammonium nitrate concentration (5 %) and acetone concentration (5 %) resulted in a decreased CA concentration of 10 g/L. The high CA concentration observed at a low ammonium nitrate concentration may be as a result of reduced vegetative growth of *A. brasiliensis*. On the other hand, the lower CA observed at higher nitrogen concentrations may be due to the shift in the cell metabolism towards fungal growth instead of product formation (Show et al., 2015). *Aspergilli* can utilize a variety of nitrogen sources such as ammonium, peptone and nitrate. The fungus converts nitrate to ammonium, which is then incorporated into the amino acids glutamate and glutamine, that act as nitrogen donors within the TCA cycle (Krappmann and Braus, 2005). Furthermore, during CA fermentation, nitrogen limitation is a key requirement to prevent the accumulation of oxalic acid, since this compound has been known to lead to lower CA production. While desorbents such as acetone have shown to enhance fungal growth and CA production, elevated concentrations can be toxic to the cells and may inhibit the fungal cellular metabolic functioning, thus resulting in a lower CA concentration. A similar result was obtained in an earlier study by Roukas and Harvey (1988) when methanol was used as a desorbent. The authors achieved a maximum CA concentration of 4.83 g/L from beet molasses at a methanol concentration of 2 %, while concentrations beyond this value led to a decrease in CA production and concluded that methanol's inductive effect was caused by its inhibitory effect on metal ions. Similarly, the study by Sarkar and Das (2017)

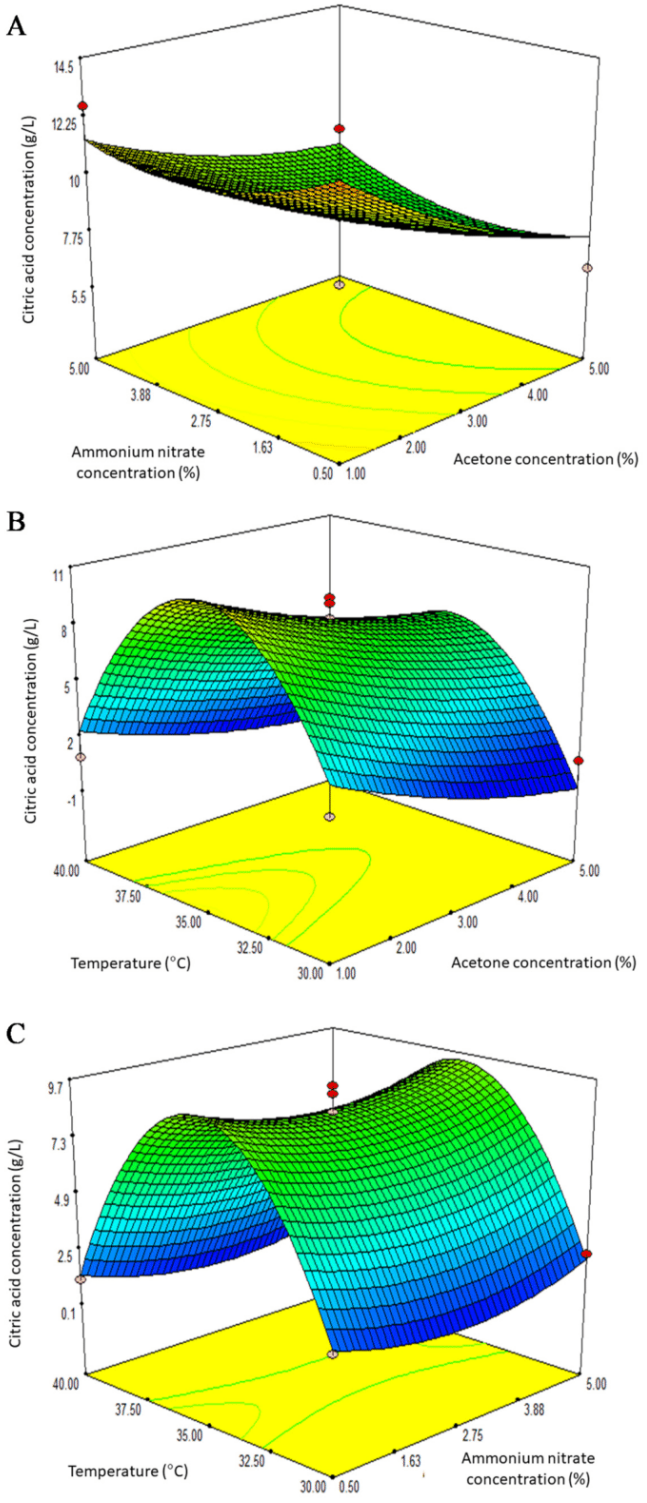

Fig. 1. Three-dimensional response surface graphs showing the interactive effects of the (A) Ammonium nitrate concentration (%) and acetone concentration (%), (B) Temperature (°C) and acetone concentration (%), and (C) Temperature (°C) and ammonium nitrate concentration (%) on the citric acid concentration.

varied ammonium nitrate (0.1 to 0.5 %) and desorbent concentration (0 to 5 %) on pineapple waste and obtained an optimal CA concentration of 44.1 g/L by using 15 % sucrose, 0.25 % ammonium nitrate, 4 % methanol and 7 days of incubation at 30 °C.

The impact of temperature and acetone concentration on the CA concentration while the ammonium nitrate concentration was maintained at its median value (2.75 %) is represented in Fig. 1B. An increase in temperature (30 to 35 °C) coupled with the lowest acetone concentration (1 %) led to an increase in the CA concentration from 4.08 to 10.94 g/L. Further increments in the acetone concentration and temperature > 1 % and > 35 °C respectively resulted in a steep decrease in the CA concentration from 10.94 to 2.1 g/L. Temperature has a dual effect on SSF processes due to its influence on both the enzymatic and microbial metabolic processes. With regards to enzymatic saccharification, temperature plays a key role on the enzymatic activity and saccharification efficiency for sugar release (El-Imam and Du, 2014). Temperature within the range of 45 and 50 °C has been shown to facilitate effective cellulase enzymatic saccharification, however the combined SSF system necessitates a median temperature that stimulates enzyme and microbial activity. Nevertheless, high-performing cellulase cocktail enzymes such as Cellic CTec2 has been specifically developed to withstand adverse environmental conditions and sustain its activity (Champreda et al., 2019). On the other hand, the temperature parameter also has a profound effect on the microbial growth of fungi. *Aspergillus* sp. are mesophilic microorganisms that exhibit wide growth ranges between 25 and 45 °C (Aboyeji et al., 2020). Hence, to ensure optimal enzyme function and microbial growth, the temperature employed in the SSF process ranged between 30 and 40 °C. In Fig. 1B, the highest CA concentration (10.94 g/L) was observed at a temperature of 35 °C. Likewise, Zhang et al. (2018) examined the influence of temperature on the growth of bacteria and fungi and determined that the growth activity increased at 35 °C. A high concentration of CA was observed when a low concentration of desorbent was employed (1 %). In the same vein, Moyer (1953) studied the effects of ammonium chloride and methanol concentration for the synthesis of CA from corn meal and concluded that a low desorbent concentration (2 %) enhances the citrate synthase activity while decreasing the aconitase activity during CA fermentation. Citrate synthase is a central enzyme involved in sugar oxidation and is also responsible for catalyzing the TCA cycle, whereas aconitase catalyzes the isomerization of citrate to isocitrate within the TCA cycle (Papagianni, 2007, Show et al., 2015). While desorbents improve the activity of enzymes involved in the TCA cycle, they are inhibitory above the threshold levels (>5%) and could potentially lead to the degradation of these key enzymes and inhibit the growth of microorganisms by drastically altering the pH of the medium. Maddox et al. (1986) observed a similar observation with a low desorbent concentration (1 %) and observed a maximum CA concentration of 25 g/L using galactose as a substrate. Furthermore, in the absence of methanol, very little CA was produced (<1 g/L). Kareem et al. (2010) evaluated the effect of sucrose, ammonium nitrate and methanol on CA production using fermentation medium consisting of pineapple waste and achieved optimum CA (60.61 g/L) under 15 % sucrose, 0.25 % ammonium nitrate and 2 % methanol when fermented for 5 days at 30 °C. The aforementioned author also noted that further increases in the methanol concentration (>3%) led to a diminished fungal growth and lower CA production (Kareem et al., 2010). The pairwise interaction of temperature and ammonium nitrate concentration on the CA concentration when the acetone concentration was retained at its midpoint value (3 %) is demonstrated in Fig. 1C. A simultaneous increase in temperature (30 to 35 °C) and ammonium nitrate concentration (0.5 to 5 %) resulted in a minor increase in CA concentration from 8.65 to 9.33 g/L. Similar to the interactive effect of temperature and acetone concentration as shown in Fig. 1B, it is interesting to note that increases in the temperature parameter above 35 °C, led to a drastic decline in CA concentration from the optimal value (9.65 g/L) to approximately 2 g/L. Several factors influence fungal growth; however, temperature has shown to have a drastic effect that could potentially dictate the output and quality of the desired end-product. Temperature also influences the growth of hyphae and the activity of extracellular enzymes associated with the TCA cycle. At high temperatures (>35 °C), hyphal deterioration may occur and result in a critical reduction of CA (Zhang et al., 2018). Nitrogen

concentration has been found to have a strong influence on both cellular metabolism and CA production. Owing to the necessity of employing low-cost substrates in order to reduce CA manufacturing costs and make it more ecologically sustainable, glucose derived from pretreated banana pseudostem makes it a valuable carbon source. By increasing the initial glucose concentration, the specific rate of CA production rises (Papagianni et al., 1999), whereas concentrated ammonium nitrate inhibits the growth and metabolism of fungi (Veverka et al., 2007). Veverka et al. (2007) investigated the sensitivity of fungal growth to two different nitrogen sources (urea and ammonium nitrate). It was concluded that ammonium nitrate (0.06 M) had the most notable effect on the growth of *A. niger* with an increase of 1.5-fold greater in comparison to urea (0.19 M). The type of nitrogen source utilized is important due to specific properties that affect the fungal growth and synthesis of CA (Show et al., 2015). For example, the absorption of ammonium by germinating spores release protons, which lowers the pH of the fermentation medium and results in an improvement in the CA production.

The low pH encountered during the production phase (~2) lowers the risk for contamination by unwanted microorganisms and impedes the development of undesirable organic acids such as oxalic and gluconic acids, thereby impacting on downstream processing by making product recovery easier.

3.4. Validation of the RSM model

Validation of the developed SSF model was carried out by utilizing the optimum process conditions (1 % acetone, 0.5 % ammonium nitrate and 35 °C). At these setpoints, the model predicted an optimal CA concentration of 12.712 g/L. Experimental validation of these parameters displayed a slightly higher CA concentration of 13.703 g/L, translating to a difference of 7.23 % when compared to the models predicted value and was considered negligible. This indicates high accuracy and precision of the developed RSM model with a strong reliability for prediction. The validation parameters established by the RSM model were applied to two other fermentation processes consisting of dairy wastewater in place of fresh water (SSF_{DWW}) and SDE medium adapted by substituting glucose with BP ($SSF_{SDEmodified}$). A comparative assessment between these three SSF processes are detailed under Sections 3.5 and 3.6.

3.5. Comparison of the citric acid production to previous reports

The results obtained from the $SSF_{optimizedFW}$, SSF_{DWW} and $SSF_{SDEmodified}$ bioprocesses were compared to earlier reports on CA production using dairy waste and lignocellulosic biomass (See supplementary material). The $SSF_{optimizedFW}$, SSF_{DWW} and $SSF_{SDEmodified}$ processes achieved CA concentrations of 12.967, 13.095 and 13.991 g/L respectively. The present study revealed comparable CA concentrations compared to Aboyeji et al. (2020) (4.36 g/L), Ghanbartabar et al. (2016) (4.45 g/L), and Campanhol et al. (2019) (0.73 g/L) when *A. niger* was utilized for the fermentation of sweet potato peel waste, cheese whey biomass and sugarcane bagasse respectively. Conversely, Odu et al. (2020), Sarkar and Das (2017) and Ozdal and Kurbanoglu (2019) investigated banana peel, pineapple waste and sugar beet molasse respectively, for the generation of CA and observed higher CA concentrations when compared to the present study. The variations in this study to previously reported literature may be due to different conditions applied that include: the microbial species (inoculum size, strain), physical parameters (temperature, agitation, pH), the concentration and type of enzyme, desorbent type and concentration, nitrogen source and concentration, substrate and solid loading, in addition to the supplementation of chemical additives. Despite the comparable concentrations of CA derived from previous reports, the exploitation of fresh water consumption, expensive substrates, chemical supplementation and/or media components render these fermentation processes non-viable and costly for potential industrial scale implementation.

3.6. *A. brasiliensis* growth kinetics for the $SSF_{optimizedFW}$, SSF_{DWW} and $SSF_{SDEmodified}$ processes

The kinetics of microbial growth and CA production for the SSF bioprocesses ($SSF_{optimizedFW}$, SSF_{DWW} and $SSF_{SDEmodified}$) were determined by utilizing the logistic and modified Gompertz models respectively. The lag phase, often known as the microorganism's adaption phase occurs when the microbe begins to acclimatize to changes in the new medium environment and for sufficient glucose to be produced by saccharifying enzymes to support the growth of the microorganism (Asaduzzaman, 2007). The lag phase was observed during the first 12 h of fermentation. In addition, the microorganism is metabolically active and synthesizes the required enzymes and metabolites for exponential cell growth (Asaduzzaman, 2007). During the lag phase, the activation of signaling pathways and transcriptional changes occur, leading to lipopolysaccharide biosynthesis and respiration that is required for multiplication and differentiation of the cells (Hamill et al., 2020). A minor lag phase for conidia germination might last several hours, however temperature and stressors can extend this timeframe to days (Hamill et al., 2020). The lag phase of *A. brasiliensis* has been shown to be within the range of 12 to 24 h in a previous study, where it was cultivated in media containing glucose (Ho and Hood, 2014). Similar lag phase times (5 to 10 h) has been associated with the commonly used and closely related *A. niger* for CA production (Baei et al., 2008). The exponential phase or logarithmic phase transpires when the microbial culture undergoes asexual reproduction at a constant rate through simple cell division, in which one cell separates forming two identical daughter cells after undergoing nuclear division (Ellena et al., 2020, Asaduzzaman, 2007). The exponential phase was recorded between 12 and 84 h for all three SSF processes. In general, the exponential phase is initiated by a particular cell concentration that increases metabolism and initiation of DNA replication (Asaduzzaman, 2007). The enzymatic conversion of cellulose to glucose released by cellulase action occurs almost instantaneously during SSF processes and is utilized by the microbial culture (in this case *A. brasiliensis*) within the TCA cycle for the generation of CA. A high glucose utilization (GU) (Fig. 2B) and glucose consumption (GC) (See supplementary material) was observed for the $SSF_{optimizedFW}$ (GU = 50 %, GC = 9 g/L), SSF_{DWW} (GU = 44 %, GC = 8 g/L) and $SSF_{SDEmodified}$ (GU = 57 %, GC = 14 g/L) processes and this corresponded to an increase in the fungal growth (1–2.5 g/L) (Fig. 2A). The biomass concentration began to stabilize after 84 h for all three SSF bioprocesses. The decline in cell growth above 84 h can be attributed to the decline in Cellic CTec2 enzyme activity that results in a lower saccharification efficiency and thus lower glucose release for the fermentative microorganism. Furthermore, the functioning of enzymes occurs best within a certain designated temperature (45–50 °C) and pH (4.5–5.5) range (Fenila and Shastri, 2016). Therefore, sub-optimal conditions for enzyme functioning under a low temperature (35 °C) and pH (~3.5) required for the initial production of CA, causes a ripple effect on the SSF process, since the enzyme loses its activity and ability to bind to an active site on the substrate. *Aspergillus brasiliensis* growth data obtained from the $SSF_{optimizedFW}$, SSF_{DWW} and $SSF_{SDEmodified}$ processes were inserted into the logistic model and displayed high R^2 values of 0.997, 0.998 and 0.997 respectively. All three bioprocesses demonstrated the same maximum specific growth rate (μ_{max}) of 0.05 h^{-1}. The maximum specific growth rate is an important kinetic parameter due to its impact on product yield, formation and productivity (Srimachai et al., 2015, Pramanik et al., 2019). The low μ_{max} values observed may be accounted for by the depletion of nutrients such as glucose or nitrogen for cellular maintenance within the fermentation media. The concentration and availability of nitrogen influences the activity and growth efficiencies of fungi. When nitrogen is scarce within the media, fungi divert their metabolism towards generating more energy for the production of extra-cellular enzymes such as hydrolase and oxidase to

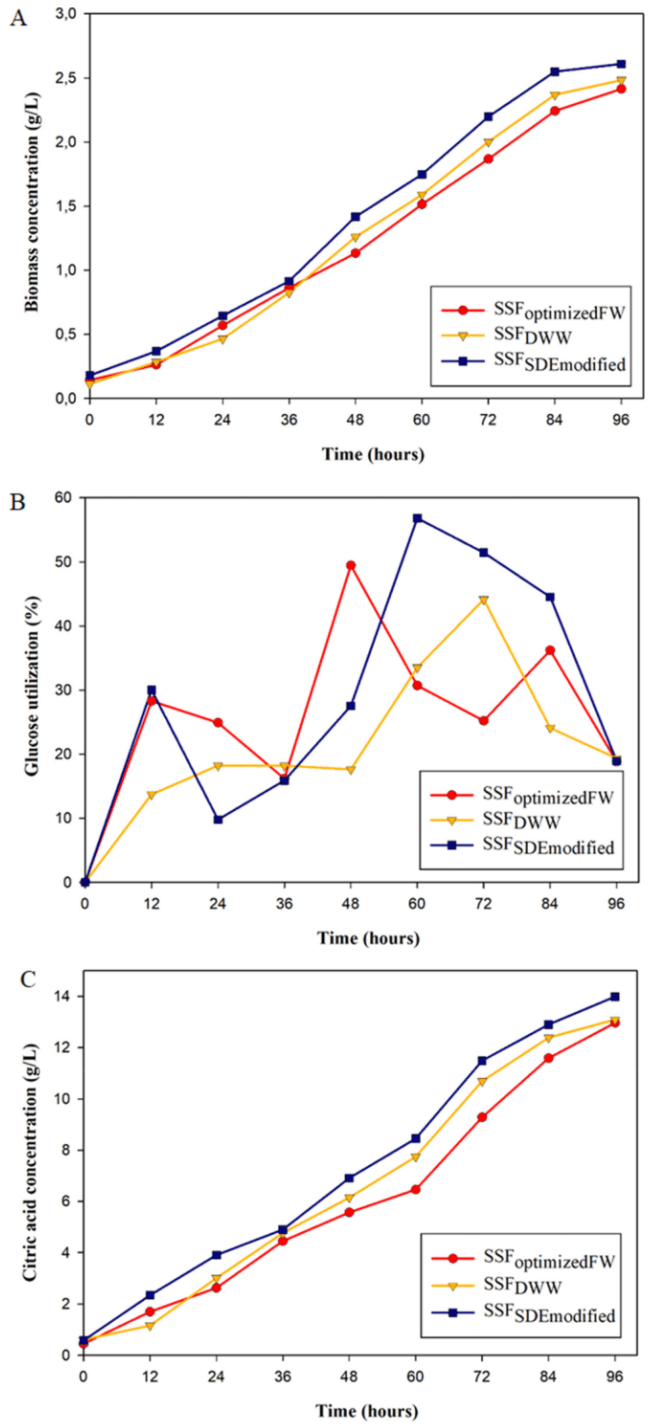

Fig. 2. *Aspergillus brasiliensis* ATCC16404 biomass concentration (A), glucose utilization (B) and CA concentration (C), for the $SSF_{optimizedFW}$, SSF_{DWW} and $SSF_{SDEmodified}$ processes.

sequestrate nitrogen from the surrounding environment, resulting in lower growth efficiencies (Tudzynski, 2014). The maximum (X_{max}) and initial (X_0) biomass cell concentrations are essential indicators for the synthesis of biomass and formation of products (Baei et al., 2008). Kinetic data obtained for the $SSF_{optimizedFW}$, SSF_{DWW} and $SSF_{SDEmodified}$ processes gave X_0 concentration values of 0.19 g/L, 0.15 g/L and 0.21 g/L respectively. Similar to the X_0, the highest X_{max} was realized for the $SSF_{SDEmodified}$ (2.91 g/L) process followed by the $SSF_{optimizedFW}$ (2.79 g/L) and SSF_{DWW} (2.74 g/L) processes. Extracellular enzymes produced during fermentation are important for the dissolution and absorption of external substances that contribute to the growth of *Aspergillus*. The enzymes secreted during growth are related to the lignocellulosic substrate, while temperature fluctuations influence extracellular enzyme activity (Zhang et al., 2018). The marginally lower X_{max} values reported for the $SSF_{optimizedFW}$ and SSF_{DWW} processes in comparison to the $SSF_{SDEmodified}$ may be attributed to variations in the media composition (nutrients) or metabolic dynamics. The citric acid cycle begins when acetyl CoA transfers its acetyl group to oxaloacetate to create citrate. In the citric acid cycle, carbon dioxide is released in tandem with the reduction of NAD^+ to NADH. However, increased levels of carbon dioxide can be detrimental to the growth of fungal biomass and reduce the concentration of citrate produced (Show et al., 2015). Peptone is an organic source that contains growth factors such as amino acid or vitamins, while ammonium nitrate is inorganic. However, the addition of amino acids and vitamins to a nutrient medium containing inorganic nitrogen is deemed expensive (Clarke, 2013). Baei et al. (2008) evaluated the potential of *A. niger* for the production of CA from apple pomace under process conditions of 0.1 g/L potassium ferrocyanide, 1.5 % (w/v) calcium triphosphate, 2.5 g/L ammonium sulfate and 3 mL methanol and recorded a lag phase of 5 to 10 h in addition to a μ_{max}, X_0 and X_{max} of 0.015 h^{-1}, 0.04 g/L and 22 g/L, respectively. Discrepancies detected in growth kinetic data between the present study and Baei et al. (2008) may be ascribed to variations in substrate type, microbial strain, nutrient availability, heat and mass transfer, aeration, agitation, pH, solid and enzyme loading.

3.7. Citric acid production kinetics for the $SSF_{optimizedFW}$, SSF_{DWW} and $SSF_{SDEmodified}$ processes

CA production during the $SSF_{optimizedFW}$, SSF_{DWW} and $SSF_{SDEmodified}$ processes, were elucidated over time (Fig. 2C). Similar to an increase in biomass concentration (Fig. 2A), a steady increase in the CA production occurred from 0 to 96 h (Fig. 2C), no further increments in CA were recorded from 96 h (data not shown), indicating the initiation of the end of the fermentation process. A lack of essential resources, such as carbohydrates generally initiates the stationary phase of microbial growth. In all three bioprocesses, it was observed that CA increased from the exponential phase of microbial growth and continued into the stationary phase. Citric acid increased from approximately 0.5 to 13 g/L for the $SSF_{optimizedFW}$ process and from 0.6 to 13.1 g/L for the SSF_{DWW} system, while the $SSF_{SDEmodified}$ bioprocess observed the highest CA increase from 0.58 to 14 g/L (Fig. 2C). Citric acid data obtained for the three SSF processes coincided with the exponential phase of *A. brasiliensis* cell growth (Fig. 2A). The highest glucose utilization (GU) and glucose consumption (GC) values for the $SSF_{optimizedFW}$ (GU = 50 %, GC = 9 g/L at 48 h), SSF_{DWW} (GU = 44 %, GC = 8 g/L at 72 h) and $SSF_{SDEmodified}$ (GU = 57 %, GC = 14 g/L at 60 h) processes were observed from 48 to 72 h (Fig. 2B). The period before maximum CA production (0 h to 36 h) allows for adequate time, that is imperative for the enzyme to saccharify the BP substrate to release sufficient glucose that is required to sustain the metabolism of the fungal strain, leading to primary metabolite production of CA. The cellulase-based Cellic CTec2 utilized for the SSF process is a cocktail of enzymes that act collaboratively on cellulose thus converting it to glucose and exhibits high saccharification efficiencies coupled with a reduced vulnerability to glucose inhibition (Champreda et al., 2019). The decline in *A. brasiliensis* growth observed after 84 h, overlaps with a lower glucose utilization and glucose consumption for the $SSF_{optimizedFW}$ (GU = 36.2 to 18.8 %; GC = 4.5 to 2.7 g/L), SSF_{DWW} (GU = 24.1 to 19.3 %; GC = 4.2 to 2.8 g/L) and $SSF_{SDEmodified}$ (GU = 44.5 to 18.9 %; GC = 5.4 to 2.2 g/L) processes (Fig. 2B). The experimental CA data obtained for the $SSF_{optimizedFW}$, SSF_{DWW} and $SSF_{SDEmodified}$ experiments fitted the modified Gompertz model exhibiting high R^2 values of 0.991, 0.993, and 0.992 respectively. The shortest lag time (t_L) was obtained for the $SSF_{SDEmodified}$ (5.11 h) process followed by the SSF_{DWW} (9.80 h) and $SSF_{optimizedFW}$ (16.47 h) systems. The $SSF_{SDEmodified}$ process consisted of peptone as the nitrogen source, thus the shorter t_L achieved

can be attributed to the incorporation of amino acids and vitamins compared to the $SSF_{optimizedFW}$ and SSF_{DWW} systems that utilized ammonium nitrate. Ammonium nitrate has been shown to be approximately 25 % cheaper than peptone, however the latter incorporates growth factors that is fundamental for rapid growth of *Aspergillus* sp. and CA metabolism (Clarke, 2013). Nitrogen is exploited by *Aspergillus* for the generation of amino acids such as glutamine and glutamate that act as nitrogen donors within the TCA cycle (Krappmann and Braus, 2005). On the other hand, the addition of the desorbent in the $SSF_{optimizedFW}$, and SSF_{DWW} processes, may increase cell permeability to citrate thus increasing CA production via repression of the 2-oxoglutarate dehydrogenase enzyme compared to the $SSF_{SDEmodified}$ system (Maddox et al., 1986). The availability of initial carbohydrates (sugar polymers) in solution may also enhance the cellulase enzyme activity at the onset of fermentation for the accumulation of monomeric sugars (glucose). The glucose utilization in the first 12 h of fermentation for the $SSF_{optimizedFW}$, SSF_{DWW} and $SSF_{SDEmodified}$ processes corresponded to 28.3 %, 13.7 % and 30 % respectively. Therefore, the lag phase during CA fermentation is dependent on nutrient and sugar availability. Interestingly, the lag times observed in this study were shorter when compared to data recorded in the previous reports by Dienye et al. (2018) and Odu et al. (2020) that both observed lag times of 24 h for CA production. Maximum CA production rates ($r_{p.m}$) were reported for the $SSF_{optimizedFW}$ (0.17 g/L/h) and SSF_{DWW} (0.17 g/L/h) processes, followed by a slightly lower $r_{p.m}$ for the $SSF_{SDEmodified}$ (0.16 g/L/h) process. The $r_{p.m}$ observed for the present SSF processes may be linked to growth factors such as nutrients or trace elements. Trace elements (manganese, zinc and iron) have significant effects on the production of CA. Manganese is important for cell functioning, sporulation and for the generation of low-molecular weight secondary metabolites that provide protection from predation, environmental stress, communication, competition for resources and toxicity against other microorganisms (Show et al., 2015), whereas zinc and iron are required for growth and maintenance of the cell. Show et al. (2015) revealed that manganese deficiency negatively influences the anabolism of *A. niger*, resulting in a high intracellular ammonium concentration. Conversely, when sufficient nutrients are available, *Aspergillus* sp. germinate by switching from isotropic to polarized growth, which impacts their stress resistance and regulatory pathways involved in germination (Baltussen et al., 2020). Sugar molecules that trigger fungal germination include glucose, mannose and xylose. A carbon source coupled with either inorganic phosphate, magnesium sulphate or inorganic nitrogen are the minimum requirements for the germination of *A. niger* (Ijadpanahsaravi et al., 2021). A striking characteristic of industrial wastes such as DWW, is the presence of key nutrients such as Mn (2.56 µg/L), Mg (<0.63 mg/L), Fe (91 µg/L), Zn (21 µg/L), P (6.96 mg/L) and N (36 mg/L) (David et al., 2022), that may have positively influenced the maximum CA production rate within the SSF_{DWW} process. Another key kinetic parameter is the maximum potential CA concentration (P_m) that provides knowledge on the maximum CA that can be produced using these SSF strategies. The $SSF_{optimizedFW}$ process displayed the highest maximum potential CA concentration (P_m) (29.31) g/L, whereas the SSF_{DWW} and $SSF_{SDEmodified}$ processes exhibited lower P_m values of 18.87 and 21.50 g/L respectively. 5778501400048000High glucose utilization and CA concentrations corresponded with the X_{max} data obtained for the $SSF_{optimizedFW}$ and $SSF_{SDEmodified}$ processes. The lowest P_m value (18.87 g/L) attained from the SSF_{DWW} process may be linked to the lowest X_0 concentration of 0.15 g/L. During CA fermentation, *Aspergillus* obtain their energy through adenosine triphosphate (ATP) via the TCA (the CA) cycle. The addition of certain rate limiting nutrients such as nitrogen and phosphorus from DWW or a rise in pH may have a detrimental effect on the SSF process by inhibiting the activity of the proton pump in the plasma membrane (H^+ATPase), thereby reducing the amount of ATP produced. Also, at a high pH, a change in the rate of glucose uptake occurs, thereby resulting in a decreased biomass concentration and organic acid production (Vrabl et al., 2019). Even with the drawbacks of the developed SSF systems in the present study, these findings may contribute to a more economical and robust operation within lignocellulosic bioprocesses. This is mainly due to the incorporation of DWW as a substitute for fresh water usage, exploitation of banana pseudostem as an alternative to glucose, utilization of ammonium nitrate as a cost-efficient alternative to conventional peptone and by employing acetone with several beneficial properties during SSF induced CA production. The integration of these key elements could facilitate the biotransformation of waste to valuable bioproducts. A synergy between the academic, industrial and commercial sectors to develop large scale waste-based processes could potentially alleviate the negative environmental effects and economical impacts, which is in line with global sustainable development goals to manage the water, energy and food nexus, and may bring forth the realization of a "waste-to-wealth" circular bioeconomy.

4. Conclusion

This research presents the optimization of citric acid (CA) production using simultaneous saccharification and fermentation (SSF). Optimization gave a maximum CA concentration of 14.408 g/L. Subsequently, the kinetics of cell growth and CA production were determined. The $SSF_{SDEmodified}$ system resulted in the highest maximum potential CA concentration (P_m) (13.991 g/L) contrasted to the SSF_{DWW} ($P_m = 13.095$ g/L) and $SSF_{optimizedFW}$ ($P_m = 12.967$ g/L) bioprocesses. The findings from this research accelerates the development of waste-based bioprocesses that could reduce the economical and environmental burden sustained by commercial industries, while still aligned with the water-energy-food nexus towards a circular bioeconomy.

CRediT authorship contribution statement

Milesh Laltha: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization, Project administration, Funding acquisition. **Y. Sewsynker-Sukai:** Conceptualization, Methodology, Software, Validation, Writing – review & editing, Project administration, Resources, Supervision, Funding acquisition. **E.B. Gueguim Kana:** Methodology, Software, Validation, Writing – review & editing, Resources, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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Supplementary material

Table S1. Preliminary screening of various desorbents and nitrogen sources to determine their effectiveness for citric acid production.

Screening method	Chemical constituent	Input parameters	Citric acid concentration (g/L)
Desorbent	Acetone	3% (v/v)[a], 10% (w/v)[c], 10% (v/v)[d], 35 °C[e], 150 rpm[f], 144 hr[g]	10.886
	Chloroform	3% (v/v)[a], 10% (w/v)[c], 10% (v/v)[d], 35 °C[e], 150 rpm[f], 144 hr[g]	7.044
	Propan-2-ol	3% (v/v)[a], 10% (w/v)[c], 10% (v/v)[d], 35 °C[e], 150 rpm[f], 144 hr[g]	10.245
Nitrogen source	Ammonium nitrate	2% (w/v)[b], 10% (w/v)[c], 10% (v/v)[d], 35 °C[e], 150 rpm[f], 144 hr[g]	12.807
	Corn steep liquor	2% (w/v)[b], 10% (w/v)[c], 10% (v/v)[d], 35 °C[e], 150 rpm[f], 144 hr[g]	8.324
	Peptone	2% (w/v)[b], 10% (w/v)[c], 10% (v/v)[d], 35 °C[e], 150 rpm[f], 144 hr[g]	9.605
	Yeast extract	2% (w/v)[b], 10% (w/v)[c], 10% (v/v)[d], 35 °C[e], 150 rpm[f], 144 hr[g]	7.684

Footnote: [a]=desorbent concentration, [b]=nitrogen concentration, [c]=substrate solid loading, [d]=inoculum size, [e]=temperature, [f]=agitation speed, [g]=time.

Table S2. Citric acid concentration determined for the SSF process using the Megazyme assay kit.

Run	Acetone (v/v)	Ammonium nitrate (w/v)	Temperature (°C)	Citric acid concentration (g/L)	
				Megazyme assay	Acid-base titration
1	3	0.50	30	1.615	1.857
2	1	0.50	35	13.219	14.408
3	5	2.75	30	0.631	0.640
4	3	2.75	35	8.842	9.093
5	3	2.75	35	9.090	9.413
6	3	0.50	40	1.048	1.153
7	5	2.75	40	0.639	0.704
8	1	2.75	30	2.177	2.433
9	3	5.00	40	1.090	1.217
10	5	0.50	35	5.920	6.275
11	3	2.75	35	8.206	8.324
12	3	2.75	35	5.341	5.571
13	3	2.75	35	8.950	9.413
14	5	5.00	35	6.412	6.852
15	3	5.00	30	2.110	2.305
16	1	5.00	35	12.467	12.679
17	1	2.75	40	0.546	0.832

Table S3. Analysis of variance (ANOVA) of the developed SSF model for citric acid production.

Source	Sum of Squares	Degrees of freedom *(df)*	Mean Square	F-value	p-value (probability > F)
Model	294.34	9	32.7	7.89	0.0063 Significant
A-Acetone	31.52	1	31.52	7.61	0.0282
B-Ammonium nitrate	0.051	1	0.051	0.012	0.9146
C-Temperature	1.39	1	1.39	0.33	0.5812
AB	1.33	1	1.33	0.32	0.5889
AC	0.69	1	0.69	0.17	0.6948
BC	0.037	1	0.037	8.9×10^{-3}	0.9275
A^2	1.54	1	1.54	0.37	0.5611
B^2	4.96	1	4.96	1.20	0.3101
C^2	257.17	1	257.17	62.07	0.0001
Residual	29.00	7	4.14		
Lack of fit	18.47	3	6.16	2.34	0.2150 Not significant
Pure error	10.53	4	2.63		
Cor total	323.34	16			

Footnote: A = Acetone (v/v), B = Ammonium nitrate (w/v) and C = Temperature (°C).

Table S4. Comparative assessment of citric acid derived from lignocellulosic and dairy wastes utilizing *Aspergillus sp*.

Substrate	Fermentative microorganism	Optimal process parameter conditions	Citric acid concentration (g/L)	References
Banana pseudostem ($SSF_{optimizedFW}$)	*Aspergillus brasiliensis* ATCC16404	10% (v/v)[a], 10% (w/v)[b], 1% (v/v) acetone[c], 0.5% (w/v) ammonium nitrate[d], 35 °C[e], 150 rpm[f], 96 hr[g]	12.967	This study
Banana pseudostem (SSF_{DWW})	*Aspergillus brasiliensis* ATCC16404	10% (v/v)[a], 10% (w/v)[b], 1% (v/v) acetone[c], 0.5% (w/v) ammonium nitrate[d], 35 °C[e], 150 rpm[f], 96 hr[g]	13.095	This study
Banana pseudostem ($SSF_{SDEmodified}$)	*Aspergillus brasiliensis* ATCC16404	10% (v/v)[a], 10% (w/v)[b], 1% (w/v) peptone[d], 35 °C[e], 150 rpm[f], 96 hr[g]	13.991	This study
Dairy wastewater	*Aspergillus niger* ATCC9142	150 mL medium mixture[a,b], 30 °C[e], 200 rpm[f], 10 d[g], pH 3[h]	4.50	Kim et al. (2002)
Banana peel	*Aspergillus niger*	10 mL[a], 50 g[b], 3% (v/v) methanol [c], 0.5% (w/v) ammonium chloride[d], 30 °C[e], 10 d[g], pH 4[h], 2% (w/v) zinc[i]	97.60	Odu et al. (2020)
Pineapple waste	*Aspergillus niger*	6 x 10^6 suspension[a], 30 g[b], 4% (v/v) methanol[c], 0.25% w/v ammonium nitrate[d], 30 °C[e], 7 d[g], 15% (w/v) sucrose[i]	44.10	Sarkar and Das (2017)

Substrate	Fermentative microorganism	Optimal process parameter conditions	Citric acid concentration (g/L)	References
Sweet potato peel waste	*Aspergillus niger*	5 d, 6 mm agar spores[a], 90 mL[b], 3% (v/v) methanol[c], 1.25% (w/v) ammonium nitrate[d], 25 °C[e], 7 d[g], pH 6.5[h]	4.36	Aboyeji et al. (2020)
Sugar beet molasse	*Aspergillus niger* EB12	1 mL[a], 150 g/L[b], 4g/L Chicken feather peptone[d], 30 °C[e], 200 rpm[f], 168 hr[g], pH 6[h], 0.15 g/L K_2HPO_4[i]	68.80	Ozdal et al. (2019)
Cheese whey biomass	*Aspergillus niger* PTCC5010	50 g/L lactose[b], 0.5 g/L Ammonium iron (II) sulfate[d], 30 °C[e], 130 rpm[f], 6 d[g], pH 4[h], 0.1 g/L $MgSO_4$[i]	4.45	Ghanbartabar et al. (2016)
Apple pomace	*Aspergillus niger*	1 mL[a], 33.81 g/L[b], 2.05% (v/v) methanol[c], 42.5 g/L of corn steep liquor[d], 33 °C[e], 33 hr[g], pH 4.54[h]	68.26	Sekoai et al. (2018)
Sugarcane bagasse	*Aspergillus niger* CCT4355	4% (v/v) ethanol[c], 30 °C[e], 144 hr[g], 1% (w/v) sucrose[i]	0.73	Campanhol et al. (2019)

Footnote. [a]=inoculum size, [b]=substrate amount, [c]=desorbent concentration, [d]=nitrogen concentration, [e]=temperature, [f]=agitation speed, [g]=time, [h]=pH, [i]= chemical additive.

Table S5. Kinetic parameters of the microbial growth and citric acid production for the $SSF_{optimizedFW}$, SSF_{DWW} and $SSF_{SDEmodified}$ processes.

Kinetic parameters	Fermentation process		
	$SSF_{optimizedFW}$	SSF_{DWW}	$SSF_{SDEmodified}$
μ_{max} (h^{-1})	0.05	0.05	0.05
X_o (g/L)	0.19	0.15	0.21
X_{max} (g/L)	2.79	2.74	2.91
P_m (g/L)	29.31	18.87	21.50
$r_{p.m}$ (g/L/h)	0.17	0.17	0.16
t_L (h)	16.47	9.80	5.11

Footnote: μ_{max} = maximum specific growth rate, X_0 = initial cell concentration, X_{max} = maximum cell concentration, P_m = maximum potential citric acid concentration, $r_{p,m}$ = maximum citric acid production rate, t_L=lag time.

Moles NaOH required = vol of NaOH required (mL) × conc. of NaOH (moles/L) × 10^{-3} (1)

The amount in moles of citric acid, $C_3H_5O(COOH)_3$, in the titrated sample was determined according to Eq. (2).

$$\text{Moles } C_3H_5O(COOH)_3 = \frac{\text{moles NaOH} \times 1 \text{ mol } C_3H_5O(COOH)_3}{3 \text{ moles NaOH}} \quad (2)$$

The concentration (molarity) of citric acid produced was calculated by Eq. (3).

$$\text{Concentration } C_3H_5O(COOH)_3 = \frac{\text{moles } C_3H_5O(COOH)_3}{volume\ C_3H_5O(COOH)_3} \quad (3)$$

The concentration of $C_3H_5O(COOH)_3$ g/L was determined by Eq. (4) and (5).

$$\text{Molar mass} = \frac{Concentration\ (g/L)}{Concentration\ (mol/L)} \quad (4)$$

Concentration of citric acid (g/L) = Concentration in mol/L × Molar mass (g/mol) (5)

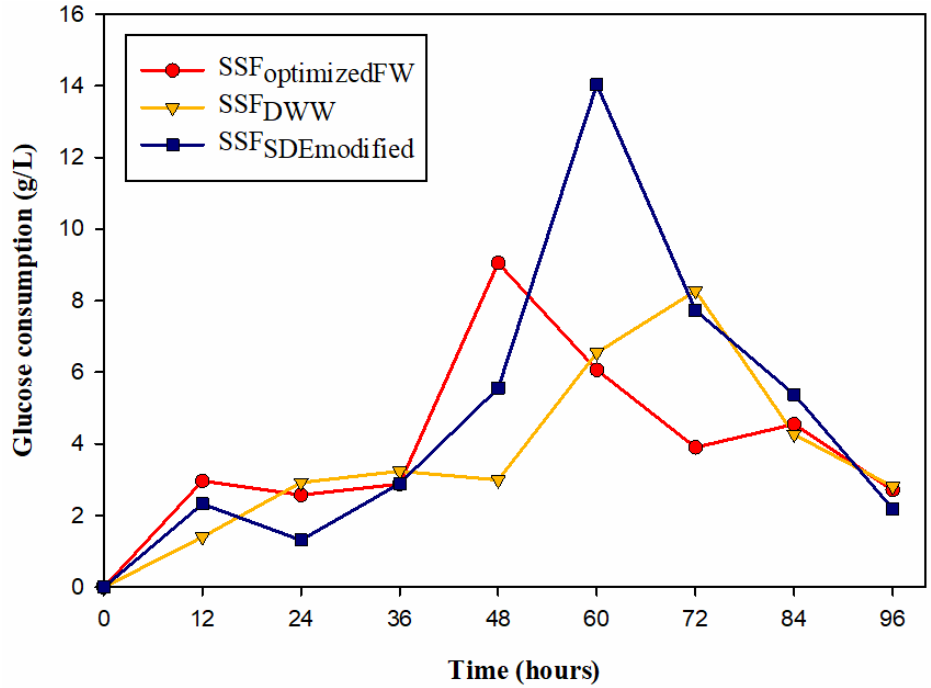

Fig. S1. *Aspergillus brasiliensis* ATCC16404 glucose consumption over time for the $SSF_{optimizedFW}$, SSF_{DWW} and $SSF_{SDEmodified}$ processes.