# Optimizing Citric Acid Production through Sugarcane Molasses: Detoxification Strategies and Evaluation of Fermentation Inducer

by

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## Preface

The research contained in this dissertation was completed by the candidate while based in the Discipline of Microbiology, School of Life Sciences of the College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Pietermaritzburg Campus, South Africa.

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

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Signed: Prof Gueguim E.B Kana

Date:

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Signed: Dr Isaac A. Sanusi

Date: 30/08/2024

**Declaration 1: Plagiarism**

I, Bryce Delron Carter Bishop, declare that:

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**Declaration 2: Manuscripts**

This thesis is compiled of two manuscripts, each chapter highlights studies which contribute to the research topic. The first author (student) contributed towards the experimental work, data collection and manuscript preparation under the guidance of the second and third author/supervisor (Isaac A. Sanusi & Gueguim E.B Kana).

1. Bryce D.C. Bishop, Isaac A. Sanusi, Gueguim E. B. Kana. Enhanced substrate suitability of a steam-assisted acid pre-treated sugarcane molasses: Pre-treatment optimization and sequential detoxification strategies (Submitted to the *Journal of Biomass Conversion and Biorefinery*).
2. Bryce D.C. Bishop, Isaac A. Sanusi, Gueguim E. B. Kana. Enhanced Citric acid production from sugarcane molasses via *Aspergillus niger* in submerged fermentation with isopropanol as inducer: Process optimization and kinetic study (To be submitted to the Journal of *Bioresource Technology Reports*).

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**Conference contribution**

1. Bishop, B.D.C., Sanusi, I.A. and Guegium Kana, E.B. Development of a steam-assisted acid pretreatment of sugarcane molasses for citric acid production via *Aspergillus niger* fermentation using an isopropanol desorbent: Process optimization and nano-adsorbent detoxification. Flash presentation, 2 – 3 November 2024, College of Agriculture, Engineering & Science Postgraduate Research & Innovation Symposium, University of KwaZulu-Natal, South Africa.

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## Abstract

Agricultural wastes such as molasses are potential excellent feedstock for renewable and sustainable bio-product production due to the high sugar content. Although, it is relatively cheap with annually global molasses production >50 million tons, sugarcane molasses requires efficient and cost-effective pretreatment as well as detoxification for its utilisation as suitable feedstocks for bio-product. Thus, there is a need to investigate novel strategies for economically feasible sugarcane molasses utilisation. The present study focused on modelling and optimizing fermentable sugar recovery from sugarcane molasses waste using steam-assisted acidic pre-treatment. It investigated the impact of nanoparticle (NP) as an adsorbent for the detoxification of the pre-treated molasses hydrolysate. Then the optimally pre-treated sugarcane molasses hydrolysate was assessed for citric acid production using *Aspergillus niger* in a submerged fermentation process.

The valorisation of waste sugarcane molasses as suitable feedstock for the recovery of fermentable sugar was evaluated. Response surface methodology was used to investigate the effects of sulphuric acid concentration (0 – 1.5%), autoclave duration (5 – 30min) and molasses loading (5 – 20%) on fermentable sugar yield. The developed model showed a high coefficient of determination ($R^2$ = 0.98). The optimized process showed high fermentable yield of 98.14g/L. Inclusion of $Fe_3O_4$ nanoparticle at 0.2% (w/v) reduced 5-Hydroxymethylfurfural (5-HMF) and furfural concentrations by 29.05% and 53.53%, respectively. Likewise, the concentration of metal contents (Ca, Mg, Na and S) was reduced (4.97%, 7.59%, 15.04% and 7.63% respectively). Remarkably, higher metal content detoxification efficiency was obtained when the surface of $Fe_3O_4$ NP was modified. Surface modification, using poly (ethylene glycol)-PEG, Tri sodium citrate–TSC, chitosan–coated and k-Carrageenan–k-C respectively, resulted in the coated $Fe_3O_4$ NPs significantly enhancing the removal of metal contents up to 42.74-fold. The optimally pre-treated sugarcane molasses hydrolysate was assessed for citric acid production using *Aspergillus niger* in a submerged fermentation process. The optimum process set points of molasses loading, isopropanol concentration (fermentation inducer) and phosphate content were investigated for enhanced citric acid. The optimized fermentation model predicted citric acid output of 8.17g/L under the setpoints of 20% (w/v) molasses loading, 0.5% (v/v) isopropanol, and 3% (w/v) potassium dihydrogen phosphate. The validation experiment using these parameters resulted in a maximum citric acid concentration of 7.52g/L compared to 6.24g/L obtained for the control experiment. Moreover, the supplementation of isopropanol as a fermentation inducer led to a 1.21-fold increase in citric

acid production. The innovative and eco-friendly approach can potentially lower the cost of citric acid production. The substantial recovery of reducing sugars and the efficient removal of inhibitory compounds underscore the effectiveness of this pretreatment and detoxification strategy, resulting in high citric acid production. These findings aligned with the goals of a sustainable, cost-effective, and eco-friendly waste-based bioprocess utilisation, advancing the commercial viability of waste-based biorefineries, waste management, and bioproduct production.

**Acknowledgements**

I am forever grateful to those who have supported me along this journey. To my family and friends who believed in me through it all, thank you for being my pillars on which to stand.

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To Avril Turpin, you gave me the opportunity to achieve a dream which I had. My role model that inspired me to reach for greatness. I will forever be grateful for the push you gave me when I needed it most in my life, Thank You truly.

Finally, to myself… it's been a long journey, but this is just the beginning.

**Haruka Mirai.**

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# Chapter One

# General Introduction

## 1.1 Product necessity and market value

Citric acid is an organic acid with a large industrial and global demand (Reena et al., 2022). Although citric acid occurs naturally in fruits, however it can be synthesized chemically to meet global demand. Citric acid is an essential raw material in several industrial sectors (cosmetic, pharmaceutical, food and beverage and detergent manufacturing) due to the acids versatility as an antioxidant, preservative, acidifier, foaming agent, effervescent and a flavour enhancer (Max et al., 2010). Globally, approximately 2.8 million tons of citric acid is produced annually and by 2024, the global market is estimated to reach $3.9 billion USD (Książek, 2023; Reena et al., 2022). Europe, North America, and Asia-Pacific are the largest producers of citric acid. The regions have a combine production and global consumption rate of 73% (Fig. 1.1). Presently, approximately 99% of the citric acid resourced globally is produced via microbial fermentation (Goldberg and Rokem, 2009). Renewable and environmentally friendly microbial production of citric acid is industrially attractive. This approach has the potential to produce citric acid from waste, thereby reducing the overall process cost.

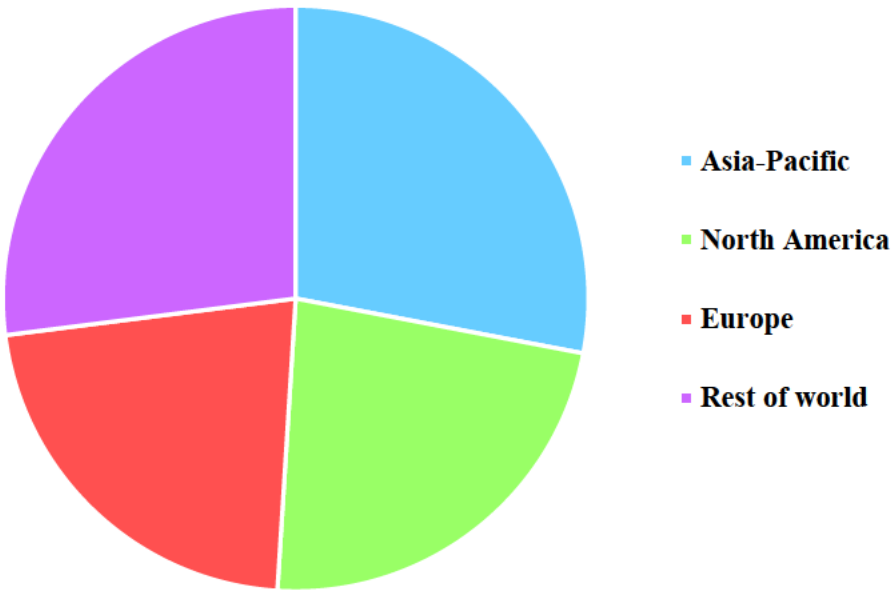


**Fig.1.1: Global citric acid consumption rate (Książek, 2023).**

## 1.2 Citric acid production

*Aspergillus niger* is the microorganism commonly used in commercial production due to its high citric acid yield, although other *Aspergillus* species and yeasts are also utilised to a lesser extent (Papagianni, 2007). Common production strategies include solid-state and surface fermentation, however, 80% of the global citric acid is produced through submerged fermentation (Reena et al., 2022). Citric acid is produced as a primary metabolite in the Tricarboxylic Acid Cycle (TCA cycle) and is a necessity for energy production during cellular metabolism (Kumari, 2018). Commercial production of citric acid has largely shifted from chemical formulation to fermentation strategies, this is accomplished through manipulation of the microorganism's natural production for the release of citric acid. However, maintaining optimal production can prove challenging due to several factors influencing the microbe's biological pathway. This could impact the overall yield of fermentative citric acid production. Nitrogen and phosphorus are examples of pivotal macronutrients necessary to achieve optimal citric acid production. Nitrogen is crucial in fungal cellular metabolism and protein synthesis whilst phosphorus is crucial for ideal mycelial growth and optimal metabolic function (Singh nee' Nigam, 2009; Zhang and Roehr, 2002). Likewise, environmental influences such as operating temperature, aeration, pH, and initial substrate loading are all vital to citric acid fermentation. Hence, process parameter optimal setpoints are necessary to maximize citric acid yield. Also, researchers are constantly seeking methods to further enhance citric acid fermentation at low cost (Thorat and Patil, 2016). These efforts include genetic modification, process modelling and the use of organic catalytic inducers as well as utilisation of agricultural waste for citric acid fermentation (Thorat and Patil, 2016). Inducers are organic solvents (such as ethanol, ethanol, and acetone) which are supplemented into the fermentation media in low concentrations to enhance citric acid yield (Laltha et al., 2022). However, organic inducer at high concentrations could enact irreparable damage to intracellular DNA and organelles (Haq et al., 2003). Hence, the paradox effect of organic inducer on fermentative citric acid is an attractive area of research. Presently, there is a scarcity of information regarding the impact of inducer such as isopropanol on the citric acid bioprocessing of *Aspergillus niger.*

## 1.3 Problem statement and research justification

In order to meet global citric acid demand due to exponential population growth, commercial production of citric acid requires novel approach, therefore, strategies to utilise waste materials as substrates for citric acid fermentation are being investigated. Other efforts as highlighted

above include genetic modification, process modelling and the use of organic catalytic inducers (Thorat and Patil, 2016). In recent years, commercial production has largely shifted to mainly using waste material such as corncobs, sugarcane bagasse, orange peels, grape pomace, brewery waste and sugarcane molasses in citric acid fermentation (Vandenberghe et al., 1999). Although these feedstocks are renewable and relatively inexpensive, organic waste materials largely require pre-treatment steps to convert their complex sugars (polysaccharides and disaccharides) into fermentable sugars (monosaccharides) and subsequent detoxification to remove inhibitory compounds within the hydrolysate which could impede optimal microbial growth and citric acid fermentation (Shazia and Sikander, 2015; Taherzadeh et al., 2000).

South Africa generates an estimated 18.2 million metric tons of sugarcane annually (Jones et al., 2021). The sugar industry is one of South Africa's largest income revenue streams generating an estimated R2 billion from foreign exchange earnings and R6 billion due to global exports, annually (Singels et al., 2015). For the production of commercial sugar, sugarcane is processed through milling and refinement techniques, however, immense quantities of waste products such as bagasse, press mud, cane tops and molasses are also produced. Sugarcane molasses which undergoes maximum sugar extraction and refinement contains high inorganic salt and metal ions content (Blackstrap molasses), therefore it cannot be sold for consumption and is regarded as a waste product (Ali et al., 2002). Nevertheless, molasses consists of approximately 54% sugar content, this high sugar content makes it appropriate as a carbon source for citric acid fermentation processes (Teclu et al., 2009). However, there are challenges associated with utilising sugarcane molasses as fermentation feedstock.

Despite being more economical and environmentally friendly, molasses mostly consists of disaccharide sugars, that requires pre-treatment processes to hydrolyze the feedstock to maximize reducing sugars yield (Acosta-Piantini et al., 2023). There are several pre-treatment strategies such as acid pre-treatment that are commonly implemented commercially for feedstock. The inclusion of an acid such as HCl, $H_2SO_4$, or $HNO_3$ hydrolyzes the α, β-glycosidic linkages within the sucrose disaccharide molecules (Tan-Soetedjo et al., 2017). An alternative strategy is the inclusion of invertase enzymes such as β-fructosidases or α-1,4-glucosidases to cleave the sucrose sugars into glucose and fructose monomers (Toledo et al., 2019). However, the implementation of common pre-treatment methods has unattractive process limitations such as elevated operating costs due to high acid exertion, corrosion of equipment, cost of enzymes, extended operating duration, and the release of fermentation inhibitors (Edwiges et al., 2022).

In order to successfully utilise waste sugarcane molasses as a substrate for citric acid fermentation, novel pre-treatment strategies must be developed to enhance reducing sugar recovery and minimise the formation of inhibitory compounds within the pre-treated hydrolysate. Additionally, investigations into the removal of these inhibitors (organic inhibitors and metal ion contents) using adsorbent such as nanoparticles could be effective in obtaining highly processable pre-treated hydrolysate (Sanusi et al., 2021). Therefore, this research could reduce the operating costs using sugarcane molasses as a substrate, enhance upstream process efficiency, and provide an alternative fermentation inducer for citric acid production (Almquist et al., 2014).

### 1.4 Aims and Objectives

This study aimed to improve citric acid production by utilizing pre-treated sugarcane molasses, effectively removing bioprocess inhibitors, and incorporating a fermentation inducer. To achieve the aims set by the research study, the following objectives were met:

I. Development and optimization of steam-assisted acid pre-treatment of waste sugarcane molasses for the releasing of reducing sugars using response surface methodology.
II. Assessing the potential of nanoparticles as detoxification agent for fermentation inhibitors from pre-treated sugarcane molasses hydrolysate obtained in (I).
III. Optimization of citric acid fermentation from pre-treated and detoxified waste sugarcane molasses hydrolysate (obtained in II) using *Aspergillus niger* and isopropanol as a fermentation inducer.

### 1.5 Thesis outline

This thesis contains five chapters formatted as research papers. Each experimental chapter is comprised of an introduction highlighting known literature as well as motivation for the study, methodologies, experimental results, discussion, and conclusions. Enhancing citric acid bioprocessing and yield at low process cost is central to all chapters.

Chapter 2, a literature review highlighting the potential for waste sugarcane molasses as feedstock for citric acid production. Discussing commercial pre-treatment and detoxification strategies employed, as well as the biochemistry of citric acid produced from microorganisms.

Chapter 3 focuses on the development of a steam-assisted acidic pre-treatment for waste sugarcane molasses. This involves process modelling and optimization to enhance the release of reducing sugars. Furthermore, this chapter explores the kinetics of organic inhibitor (furans) formation during the pre-treatment process and subsequently the removal efficiency of both organic and metallic ion inhibitors using nanoparticle adsorbents.

Chapter 4 assesses the viability of isopropanol as an inducer to enhance *Aspergillus niger's* citric acid production during a batch submerged fermentation. Thereafter, kinetics of citric acid production in the presence of inducers was evaluated.

Chapter 5 outlines the significance of the results, major conclusions obtained from the experimental chapters, and recommendations for future studies.

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# Chapter Two

# Literature Review

## 2.1 Introduction

Citric acid ($C_6H_8O_7$) is in high demand, and this is only expected to increase as the world population rises. Global production was reported to have reached 2.1 million tons in 2016 and presently, global citric acid production is projected to reach 2.9 million tons by 2026 with market value prospect of US$3.9 billion by 2032 (Yoshioka and Kirimura, 2021). Citric acid is utilised in a variety of industries (pharmaceutical, cosmetic, food, chemical) as an antioxidant, emulsifier, anticoagulant, chelator, acidifying agent, a preservative as well as many other useful applications (Ciriminna et al., 2017). Although it is possible to synthesise the acid chemically, but approximately 99% of citric acid produced globally is being produced through microbial fermentation (Goldberg and Rokem, 2009). In the era of exponential population growth and the resultant increase in the consumption of goods, the access to natural resources to satiate the increased demand has begun to dwindle. Scientists are hereby forced to research alternative sources like waste sugarcane molasses in the production of value-added products such as citric acid. Sugarcane molasses, which is produced during the sugar refining process, can serve an excellent substrate for microbial fermentation.

Sugarcane molasses contains approximately 35% sucrose content (Teclu et al., 2009), and only pre-treatment and detoxification processes are required before their usage in fermentation processes (Shazia and Sikander, 2015). There are various pre-treatment techniques utilised in carbohydrate-based feedstock which could enhance the substrate suitability for fermentation by breaking down complex carbohydrates to increase reducing sugar content in the pre-treated hydrolysate. The pre-treatment process is also expected to improve the molasses' enzyme and microbial susceptibility. Some examples of pre-treatment strategies implemented in biomass pre-treatment include acidic, enzymatic and steam-explosion, however there are disadvantages associated with these processes. Some drawbacks associated with pre-treatment processes include equipment damage due to corrosion or steam pressure, high enzyme cost, large chemical burden as well as the formation of inhibitory compounds which can hinder optimal microbial metabolism (Cavka and Jönsson, 2013). Therefore, there is need to mitigate these drawbacks if waste utilisation and commercialization is to be achieved.

## 2.2 Sugarcane molasses as a feedstock

### 2.2.1 Sugarcane cultivation

Sugarcane is one of the highest cultivated crops with a global production of >1.9 billion tons produced in the 2022 season and is forecasted to increase due to higher global consumption (FAO, 2023). The America's have been established as the world's leading cultivators of sugarcane, producing 973 million tons in 2022 with Asia being second, producing 821 million tons. This is due to Brazil producing up to 38% and India being producing 23% of the global sugarcane market (FAO, 2023). South Africa produced approximately 18.2 million metric tons (2020/2021) of sugarcane making it one of the country's leading agricultural products (Jones et al., 2021). Due to the plant's high sucrose content, it is mainly used in the production of commercial sugar. This process usually results in large quantities of waste materials such as bagasse, press mud, cane tops, dry leaves, and molasses.

### 2.2.2 Composition and advantages of sugarcane molasses in fermentation

Sugarcane molasses is a by-product which is produced after the processes of evaporation, crystallization, and centrifugation of milled sugarcane (Fig. 2.1), with a reported composition consisting of approximately 54% total sugar content (majority being disaccharides) (Teclu et al., 2009). This makes the waste molasse an excellent substrate for fermentation processes. Under less intense refinement conditions, it is possible for sugarcane molasses to be sold as a product to consumers. However, maximum sugar extraction processes (which is implemented by most industrial sugar milling facilities) greatly increases the ash content (approximately 12%) in the molasses making it unsuitable for consumer consumption (Teclu et al., 2009). The molasses obtained could be used as a carbon source in microbial fermentation systems to produce value-added products such as bioethanol, biohydrogen, butanol, acetone, lactic acid, and citric acid. However, to achieve optimal and efficient production of these valuable products using sugarcane molasses, the molasses requires a preliminary pre-treatment process followed by a detoxification process to obtain suitable hydrolysate with fermentable monosaccharides.

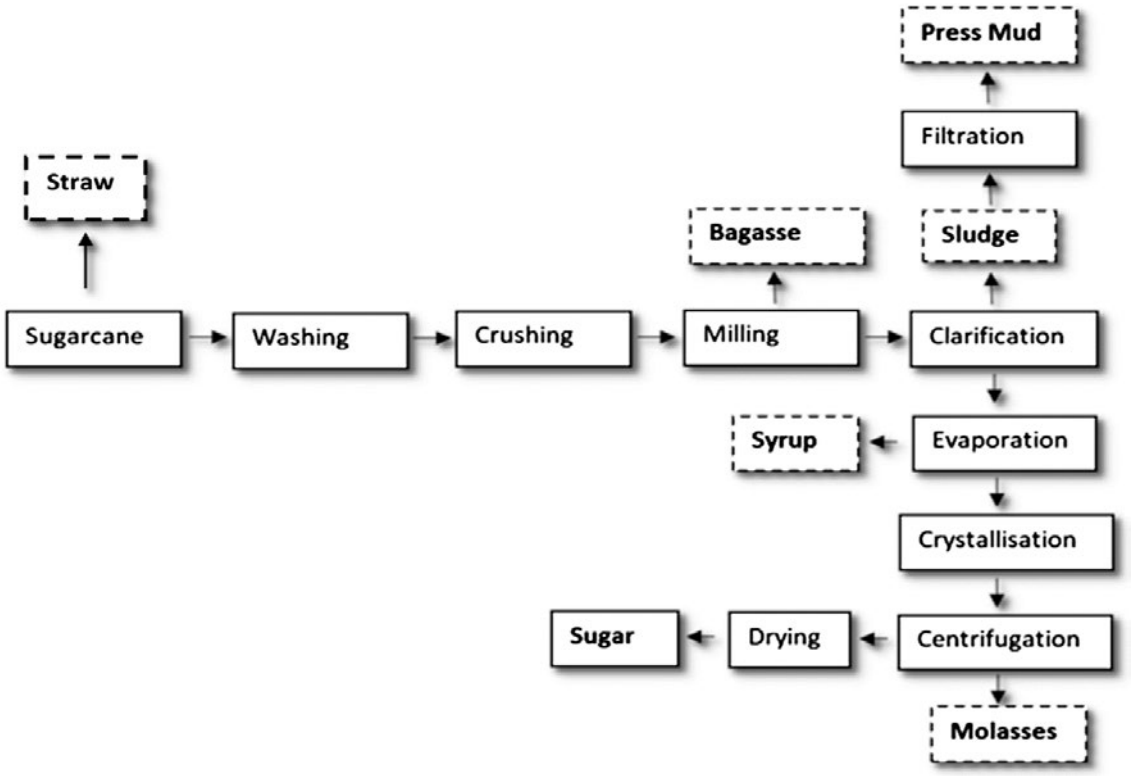


**Fig. 2.1: Sugarcane milling process for sugar production and the generation of waste products (Teixeira et al., 2021).**

## 2.3 Biological and chemical basics of citric acid fermentation

### 2.3.1 Microbial pathways for citric acid production

Citric acid is a primary metabolite that is produced during the Tricarboxylic Acid cycle (TCA cycle) (Fig. 2.2). The enzyme isocitrate dehydrogenase catalyses the reaction of acetyl-CoA whereby the acetyl group is coupled to oxaloacetate to form citrate (Kumari, 2018). The fungus *Aspergillus niger* is used for producing 80% of the citric acid produced through fermentation due to its higher output compared to other microbial species (Laltha et al., 2022). *Aspergillus niger* prefer monosaccharides as a carbon source which they readily metabolize faster than polysaccharides (Show et al., 2015). Glucose is the most suitable monomer as it can be utilised in the glycolytic pathway without any modifications (Veana et al., 2011). Although, in the presence of polysaccharides, enzymes are produced by the microorganisms to catabolize its breakdown, the process prolongs the adaptation phase for the microorganism's growth and metabolic activities.

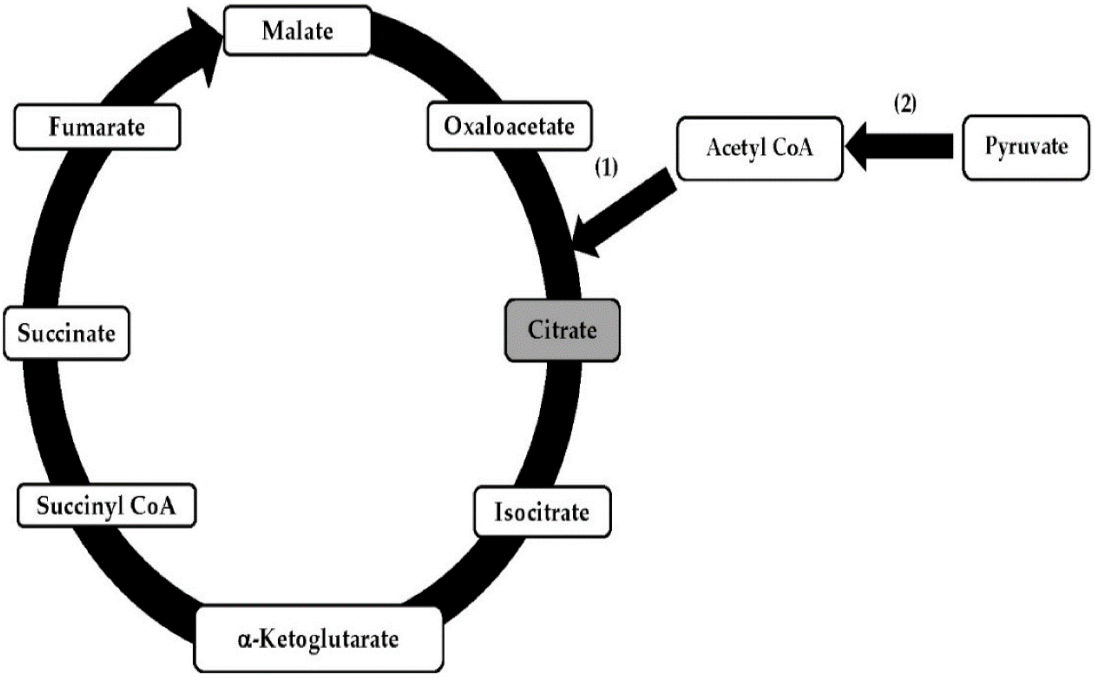


**Fig. 2.2: Schematic overview of the TCA cycle resulting in citric acid synthesis (West, 2023). Key enzymes: (1) citrate synthase; (2) pyruvate carboxylase.**

### 2.3.2 Factors affecting citric acid fermentation efficiency

Macronutrients and micronutrients are required for cell development, growth, and consequently citric acid production (Singh Dhillon et al., 2011). Nitrogen is one of the major macronutrients necessary for the optimal growth of *A. niger* as it plays a key role in protein synthesis and cellular metabolism. For microbial fermentation, ammonium sulphate, ammonium nitrate and urea are the common nitrogen sources used in citric acid bioprocessing. Although, ammonium sulphate is the preferred option as the inorganic salt does not lead to the production of oxalic acid which is inhibitory to citric acid production, ammonium nitrate has been observed to reduce vegetative growth of the fungus (Singh nee' Nigam, 2009). Low vegetative growth could be very detrimental to citric acid production. Nitrogen limitation is key to optimal citric acid yield as nitrogen concentrations above 0.25% lead to an increase in oxalic acid released. These claims were supported by the work of Ali et al. (2002) whereby maximum concentration of citric acid was obtained when nitrogen ($NH_4NO_3$) levels were kept at 0.2%. It was observed that nitrogen concentration >0.2% lower fungal growth and proliferation.

Phosphorus is another macronutrient utilised for optimal mycelial growth and metabolic activities. Phosphorus content induces increased citric acid production whilst non optimal

concentration could adversely affect cellular grow (Zhang and Roehr, 2002). The optimal phosphorus range has been reported to be 0.5 – 5g/L for citric acid production (Show et al., 2015). However, more recent studies reported 0.1 – 0.2% phosphorus content as the optimal range for citric acid production (Nur Hidayat et al., 2019).

Furthermore, organic solvents (known as inducers) have been known to exhibit antimicrobial activity at high concentration, causing cellular damage, denaturing cellular membranes, proteins and enzymes resulting in reduced metabolic activities (Schalck et al., 2021). However, low concentrations of organic solvents inclusion in citric acid bioprocessing have been known to increase citric acid production. One proposed mechanism of action is that the inducer causes increased cell membrane permeability, without damaging the cells internal organelles. Thus, reducing the cell's resistance to mass transfer resulting in high citric acid productivity (Haq et al., 2003). In addition, in the presence of a suitable inducer an increase in pyruvate carboxylase can be observed. Higher expression of pyruvate carboxylase would lead to an increase in the conversion of pyruvate to oxaloacetate within the glycolytic pathway (Fig. 2.2). Oxaloacetate serves as a precursor to citrate within the metabolic cycle, increasing the formation of oxaloacetate would subsequently result in a higher citrate accumulation. Methanol (2 – 6%) is the most utilised inducer in citric acid fermentation as the short chain organic solvent demonstrates lower antimicrobial activity as compared to other longer chain organic solvents (Rodrigues et al., 2009). Other solvents, such as ethanol, have been used to a lesser extent in citric acid fermentations, typically at 3% v/v, due to their increased microbicidal activity (Dhillon et al., 2012). Seldom studies have been conducted to evaluate the viability of organic solvents such as acetone, isopropanol, and chloroform with higher fungicidal activity to enhance citric acid output during microbial fermentation.

Moreover, environmental factors play a key role in microbial growth and product development as they are highly influential in metabolic activities (Show et al., 2015). *Aspergillus niger* is acidophilic in nature and can grow in pH ranging from 1.4 to 9.8 (Laltha et al., 2022). Disparities in reported optimum pH values could be attributed to the different strain of fungus used due to difference in pH tolerance levels. Metabolic by-products released by these microorganisms during cellular metabolism can also influence the pH of the system (del Campo et al., 2006).

Aeration has been noted to also play a critical role in the production of citric acid (Rodrigues et al., 2013). Research has linked the resultant yield of citric acid via fermentation to the

availability of oxygen. For example, increasing aeration from 0.9 to 1.3vvm, resulted in a 1.61-fold in citric acid produced. However, relatively recent studies have observed that higher levels of dissolved carbon dioxide impede optimum citric acid production (Show et al., 2015). Carbon dioxide acts as a competitive inhibitor as it serves as a substrate for pyruvate carboxylase (an enzyme active in the TCA cycle) subsequently reducing citric acid content by impeding optimum metabolic activity (Angumeenal et al., 2003). Therefore, heightened aeration allows for release of dissolve carbon dioxide content within the fermentation medium, resulting in optimal metabolic processes and citric acid accumulation.

Furthermore, temperature could affect the growth rate, activity of enzymes, metabolic activities, and productivity of fermenting fungal consequently, affecting the biosynthesis of citric acid and the overall citric acid yield. Low temperatures hinder enzymatic activity whilst higher temperatures above the optimum values could result in a higher release of toxic by-products such as oxalic acid (Ali et al., 2002).

### 2.3.3 Key microorganisms used in citric acid production

Currently, the global citric acid supply is produced largely through fermentation processes (99%) (Goldberg and Rokem, 2009), *A. niger* being the main microorganism used in the industry (80%) and *Candida* sp. to a lesser extent (Laltha et al., 2022). *Aspergillus niger* is a mesophilic filamentous fungus which can grow in a wide temperature range of 6 - 47°C with an optimum fermentation temperature range of 28 - 32°C (Schuster et al., 2002). Adaptive growth characteristics allowing sustainable biomass proliferation utilising a variety of feedstock, and the physiological ability to secrete citric acid through cytosol and mitochondria organelle. These are some of the factors which make *A. niger* a suitable microorganism for citric acid fermentation processes (Singh Dhillon et al, 2011). In recent years, modified *A. niger* have been developed through mutagenesis (i.e. radiation, chemical agents) and genetic engineering (i.e. CRISPR, DNA recombination). Subsequently, researchers seek to enhance the fungal strains to improve citric acid substrate adaptation and citric acid biosynthesis resulting in higher fermentation yields (Książek. 2023).

## 2.4 Pre-treatment strategies in fermentation

### 2.4.1 Importance of pre-treatment for enhanced substrate utilisation

Sugarcane molasses pre-treatment induces the cleavage of the sucrose disaccharide content within the substrate to produce glucose and fructose monosaccharide sugars (Fig. 2.3) (Shazia

and Sikander, 2015). Subsequently, this allows microorganisms such as *A. niger* to preserve energy spent in generating enzymes (α-amylase, amyloglucosidase, cellulase and β-glucanase) necessary to hydrolyze the disaccharides and the polysaccharides present in the molasses substrate before fermentation. This will inevitably decrease the lag phase of growth of the fermenting microorganism, afterward reducing the fermentation duration and allowing for increased substrate utilisation. The most common approaches used to pre-treat sugarcane molasses, prior to usage as feedstock, are the acidic and enzymatic pre-treatment.

### 2.4.2 Pre-treatment methods for sugarcane molasses

Acid pre-treatment involves the inclusion of an acid to increase the presence of hydrogen ions during the pre-treatment process. In the presence of water, free hydrogen ions hydrolyze the α, β-glycosidic linkage thereby cleaving the sucrose disaccharide to produce α-D-glucose and β-D-fructose monomers (Tan-Soetedjo et al., 2017). Inorganic acids such as HCl, $H_2SO_4$, and $HNO_3$, are the most common acids utilised in the sugarcane molasses pre-treatment processes. For instance, Shazia and Sikander (2015) utilised 5ml of HCl, $H_2SO_4$, and $HNO_3$ respectively (0.5N to 2N concentration) per 15ml of molasses and a heating duration of 1 hour at 90°C for the release of fermentable sugars. Similarly, 3.5% v/v 1N $H_2SO_4$ at 25% molasses sugar level at 100°C for 30 minutes also resulted in the recovery of glucose and fructose sugars (Ali et al., 2002). Although, acidic pre-treatment is more cost-effective compared to enzymatic pre-treatment, the method poses several disadvantages, namely the acidic induced corrosion of operating equipment and the acid-catalyzed formation of several inhibitory compounds within the pre-treated sugarcane molasses hydrolysate.

Enzymatic pre-treatment methods include the use of invertase enzymes such as β-fructosidases and α-1,4-glucosidases to cleave the glycosidic bonds within the sucrose molecule to produce glucose and fructose reducing sugars (Toledo et al., 2019). For example, Vidra et al. (2017) reported on an enzymatic pre-treatment carried out utilising invertase enzyme (100mg/L dosage) at a pH of 4.5, 25°C temperature to obtain hydrolyzed sugarcane molasses. The need for pH adjustment due to enzymes requiring specific pH ranges for optimal performance is another demerit of enzyme-based pre-treatment.

An additional technique used in the pre-treatment of sugarcane molasses is the steam explosion. Steam explosion is a pre-treatment technique which involves submerging the molasses in water under increased temperature and pressure (Shrotri et al., 2017). The system is rapidly decompressed resulting in hydrolysation and depolymerisation of polysaccharides which

breaks the glycosidic linkages for the release of fermentable sugars (Shrotri et al., 2017). The incorporation of the steam and acidic for sugarcane molasses pre-treatment could allow for increased water activity within the viscous molasses inducing higher acid-surface area interaction coupled with pressure decompression, hence, optimal disaccharide hydrolysis.

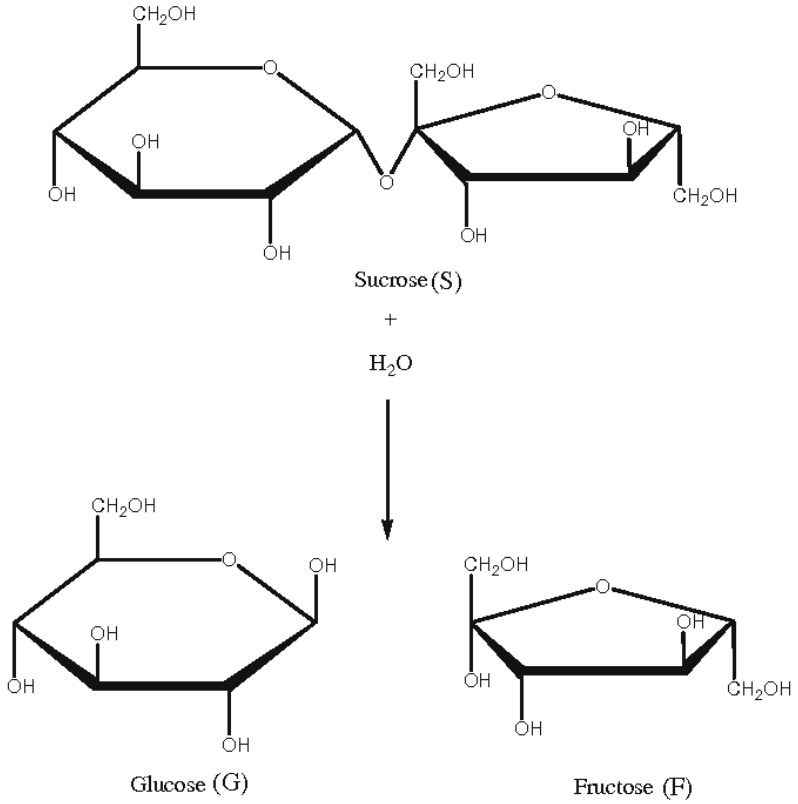


**Fig. 2.3: Hydrolysis of sucrose disaccharide into glucose and fructose monosaccharides (Pito et al., 2009).**

## 2.5 Fermentation inhibitors

### 2.5.1 Sugarcane molasses inhibitor content

Sugarcane molasses contains high ash content consisting of inorganic salts and a range of metal ions such as $Mg^{2+}$, $Mn^{2+}$, $Zn^{2+}$, $Cu^{2+}$, $Pb^{2+}$, $Al^{3+}$, $Cd^{2+}$ and $Fe^{2+}$ (Abdel-Rahman et al., 2016; Ali et al., 2002). The presence of heavy metals has been known to substantially inhibit the biochemical and physical activities of microorganisms during the fermentation process. For example, a study carried out by Tsekova et al. (2000) observed that aconitase, NAD- and NADP-isocitrate dehydrogenase activities were significantly inhibited in the presence of copper ions within the fermentation system. Subsequently, it has been reported that through the pre-treatment of different polysaccharide biomasses such as sugarcane molasses, fermentation inhibitors (such as acetic acid, furfural, and phenol) are released. Specifically,

during the pre-treatment of sugarcane molasses large quantities of fermentation inhibitors, namely 5-hydroxymethylfufural (5-HMF) and furfural, are produced.

### 2.5.2 Mechanisms of inhibition

In the presence of a Lewis acid such as sulphuric acid, fructose monomers can be converted to 5-HMF (Fig. 2.4) through a series of subsequent dehydration reactions, which take place as secondary reaction during the sugarcane molasses pre-treatment (Menegazzo et al., 2018). The existence of 5-HMF within the pre-treated hydrolysate has been reported to have inhibitory effects on microbial growth, in particular the inhibition of pyruvate dehydrogenase and aldehyde dehydrogenase which are key enzymes enabling optimal cellular metabolism (Erkan et al., 2022). Xylose is a 5-carbon sugar present in sugarcane molasses in small quantities, this xylose serves as the prerequisite in the formation of furfural, in the presence of a Bronsted and Lewis acid, during the pre-treatment process. Sulphuric acid (acting as a Lewis acid) catalyses the isomerization of xylose to xylulose, subsequently a dehydration reaction occurs whereby sulphuric acid (now acting as a Bronsted acid) converts xylulose into furfural (Jia et al., 2019) (Fig. 2.5). Through oxidative stress affecting dehydrogenases activity within microbial cells, furfural has been shown to hinder glycolytic activity and the tricarboxylic acid cycle (TCA cycle), inhibiting microbial growth as well as reduced metabolic processes and productivity (Iwaki et al., 2013).

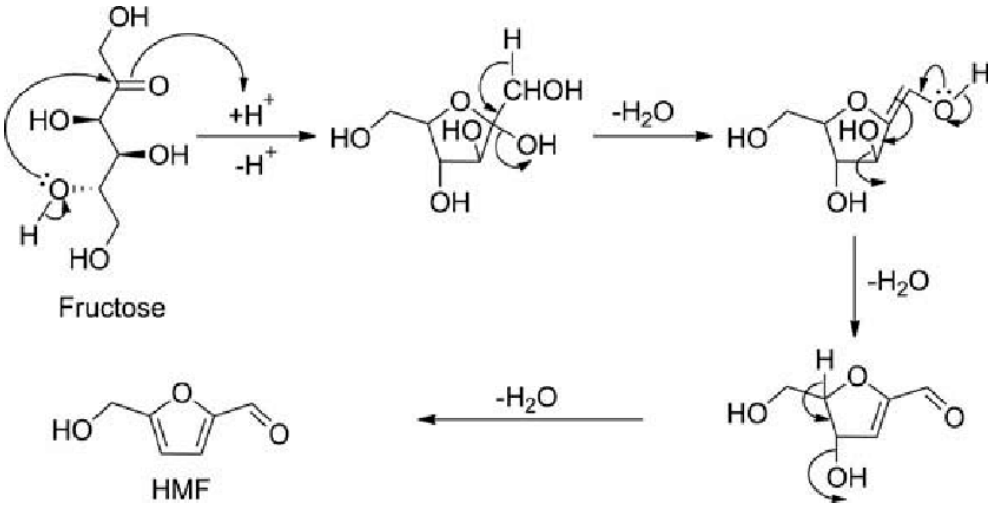


**Fig. 2.4: Formation of 5-Hydroxymethylfurufral from fructose through a series of subsequent dehydration reactions (Bhaumik and Dhepe, 2016).**

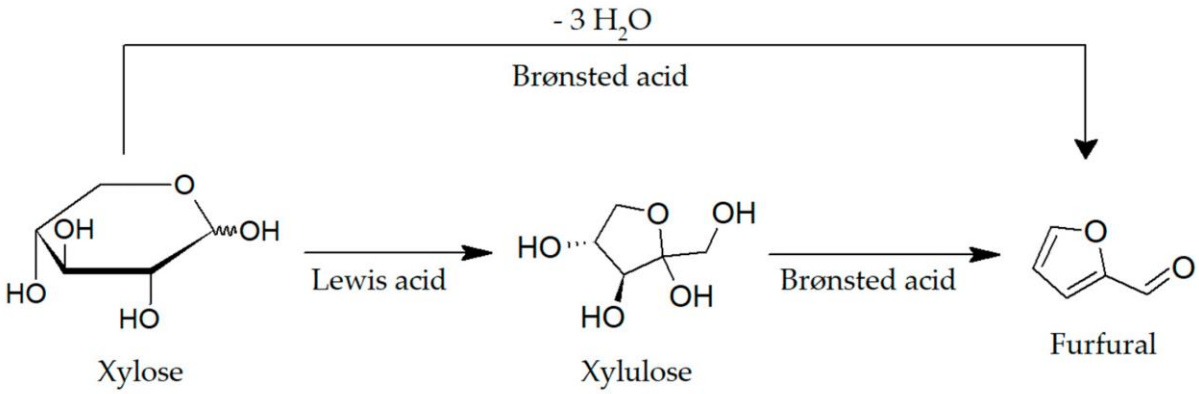


**Fig. 2.5: Formation of furfural from xylose catalyzed by the presence of a Lewis and Bronsted acid (Rusanen et al., 2020).**

## 2.6 Detoxification strategies in fermentation

### 2.6.1 Methods of detoxifying sugarcane molasses

The presence of heavy metals and certain inhibitory compounds within sugarcane molasses is unfavourable for optimal microbial growth and productivity. To mitigate the challenges associated with inhibitory ions and compounds a range of different methods have been employed in the removal or lower their concentration in pre-treated hydrolysate. These techniques include liquid-liquid extraction, heating, evaporation, cation exchange resins, EDTA and potassium cyanide for the removal of these inhibitors. These mechanisms remove metal cations from the pre-treated hydrolysate through bonding with the ions located on the resin structure or by forming a complex with the metal ions inducing a chelating effect allowing the heavy metals to precipitate within the hydrolysate solution (Abdel-Rahman et al., 2016; Sanusi et al., 2021). Also, these strategies lower the concentration of these inhibitors and modification of chemical structure of hazardous inhibitors into less toxic forms (Taherzadeh et al., 2000). However, some of these methods are either expensive, less effective, or environmentally unfriendly. More recently, studies have utilied different adsorbent technologies for inhibitory compound removal from pre-treated hydrolysates. In a study conducted by Candido et al. (2020) using activated charcoal adsorbent, furfural and 5-HMF were reduced in the pre-treated hydrolysate. In addition, certain yeast species have been known to exhibit high removal efficiency in their ability to modify the structure of these inhibitory compounds into molecules that are less toxic (Liu et al., 2004). For example, the main drawbacks of using chemical detoxification are (1) high cost of operation and (2) the aftermath of environmental impact. Therefore, eco-friendly, and cost-effective detoxification processes

such as the use of smart adsorbent are being sought after. Commonly used adsorbents are nanomaterials like nano-filters, nanocomposite, and metallic oxide nanoparticles to potentially remove process inhibitors as these nanomaterials can selectively remove these inhibitors based on their sizes, surface modifications and surface charges (Sanusi et al., 2021).

### 2.6.2 Nanoparticles as a detoxifying agent

The incorporation of nanoparticles in bioprocessing has evidently improved productivity owing to their unique properties such as large surface area and high catalytic activity (Hamawand et al., 2020; Sanusi et al., 2021). The catalytic activities of these nanoparticles are highly dependent on their size, stabilizing agent, and surface area. Nanoparticles can also enhance the removal of inhibitory ions or compounds because of their ability to adsorb these compounds (Fig. 2.6). Despite the potential benefits of nanoparticle inclusion in bioprocessing, there is scarcity of reports on the use of nanoparticles on the detoxification of pre-treated sugarcane molasses. In an applicable study conducted by Shen et al. (2009) the effect of $Fe_3O_4$ nanoparticles resulted in high adsorption of toxic heavy metal ions (cadmium, copper, chromium, iron, and nickel ions) in wastewater remediation. This result showed a drastic decrease in the concentration of the metals within the wastewater. These findings were corroborated by the research conducted by Xin et al., (2012), their study showed that using modified $Fe_3O_4$ nanoparticles as adsorbent in water treatment, 98% removal efficiency for copper, lead, and cadmium ions were obtained.

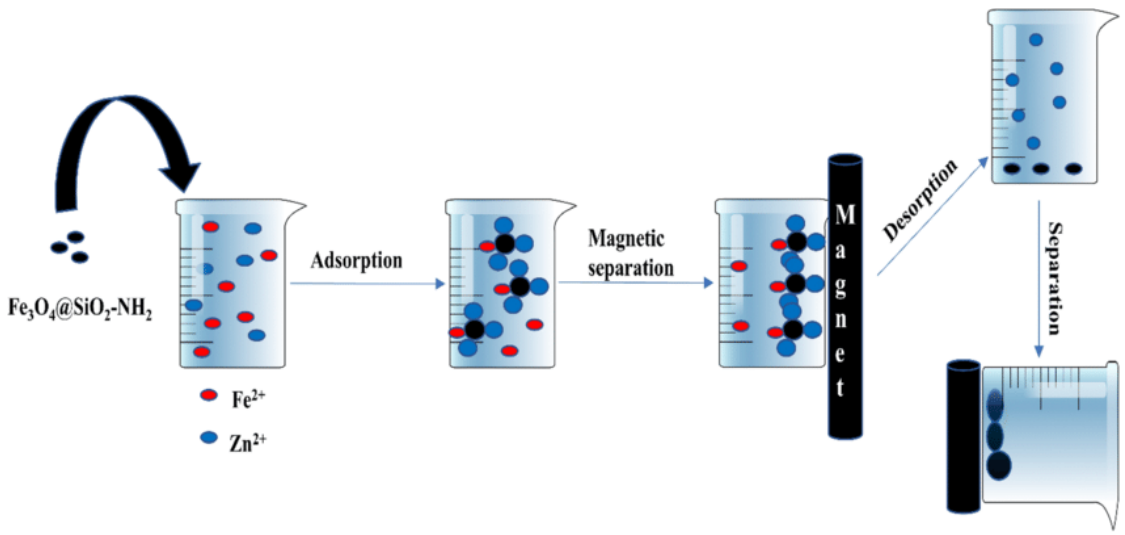


**Fig. 2.6: Mechanism of modified $Fe_3O_4$ nanoparticle adsorption of heavy metals within a solution (Usman et al., 2019).**

## 2.7 Process optimization and kinetics

### 2.7.1 Bioprocess optimization approaches

Several methods that have been utilised in process optimization these include One Variable at a Time (OVAT), Response Surface Methodology (RSM), Artificial Neural Network (ANN), and Genetic Algorithm (GA) (Chohan et al., 2020). RSM is a mathematical-based model that examines the effects of different input parameters with the best feature as the potential to show the interacting effects of input parameters on response variable(s) (Aruwajoye et al., 2020). These outcomes can be represented with three-dimensional or contour plots that help in visualize understanding of the interactions. The RSM could be implemented to optimize the process parameters for a desirable outcome such as improve product quality and yield. Moreover, RSM has the potential of showing both the significant and non-significant linear interactions (Nawaz et al., 2016). RSM is a cost-effective optimization tool that reduces experimental noise, shortens process time, and increases productivity. However, its main drawback is its inability to explain process mechanisms or predict outcomes for conditions outside of the studied range (Zaid et al., 2022). Several studies have reported on the use of RSM to optimize different processes such as bioethanol production, pre-treatment processes, inhibitor removal, waste lubricant degradation, wastewater remediation and citric acid production (Laltha et al., 2022; Sanusi et al., 2022; Eregie et al., 2023). Moreover, ANN exhibits better optimization ability for bioprocessing compared to other optimization tools such as the GA and RSM. ANN has potential to gather information through recognising patterns in data after being trained. Well-developed ANN model could predict reliable outcome from choice process inputs. Therefore, researchers have explored using ANNs in bioprocess systems such as fermentation, this is due to its high accuracy in predicting the non-linear model's outcome by learning from process data (Peng et al., 2014).

### 2.7.2 Kinetic analysis of fermentation processes

Process kinetics is a powerful tool for in-depth assessment and understanding of biological processes, enhancing efficiency, product quality and reducing costs (Almquist et al., 2014). Using these models results in robust process development, design, and control. Commonly used models include the Monod model, modified Gompertz model, and logistic model (Sanusi et al., 2021). Logistic models describe cell growth changes as a function of growth rate, initial biomass, maximum biomass concentration, and time. The modified Gompertz model elucidates the relationship between the lag time, product concentration, and maximum production rate

during bioprocessing. Incorporating process kinetics provides crucial insights into product yield and productivity (Putra et al., 2015).

## 2.8 Environmental and economic aspects

### 2.8.1 Sustainability of using sugarcane molasses as feedstock

Between 2000 and 2020, global sugar consumption rose by 33.4%, reaching 174.5 million tonnes in 2020. During the same period, sugarcane production increased by 33.8%, totalling 125.9 million tonnes by 2019 (Chmielewski, 2021). This growth is expected to continue over the next decade due to population increases and higher consumer demand. Standard milling processes produce an estimated 3 – 7% molasses per 100 tons of sugarcane (Perez and Fujita, 1997). Recent technologies have reduced molasses yield to 2.5 – 3% (Núñez-Caraballo et al., 2019), but this molasses often contains inorganic salts and heavy metals, making it unsuitable for consumption. Given the high ash content, molasses waste is considered unsafe for consumption, rendering it a waste product. As global demand for value-added products rises, it is crucial to utilise all possible avenues to minimise the impact on the food supply. A growing trend in the use of waste biomass, like sugarcane molasses, as feedstock in bioprocessing. Countries such as Pakistan, a leading sugarcane producer, are supporting this trend. Many of its distilleries (21) have switched to using sugarcane molasses as feedstock to reduce gasoline consumption and provide an eco-friendly alternative (Ghani and Gheewala. 2021).

## 2.9 Conclusion

The depletion of the world's natural resources necessitates exploring alternative, renewable and sustainable recourses. Utilising waste resources such as sugarcane molasses opens new possibilities in bioprocessing. However, the need for pre-treatment strategies and the removal of fermentation inhibitors can be costly, time-consuming, and inefficient, limiting large-scale waste-based bioprocessing implementation. Several techniques have been employed to address these challenges and enhance large-scale bioprocessing potential. Innovative approaches, such as using nano-adsorbents for inhibitor removal, are currently under exploration. These novel research outcomes could lead to cost effective, sustainable, and high-yield citric acid bioprocessing.

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## Chapter Three

# Optimization of Steam-Assisted Acid Pre-Treatment for Sugarcane Molasses Waste: Enhancing Reducing Sugar Recovery and Inhibitor Removal

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## Abstract

This study modelled and optimized a steam-assisted acid pre-treatment of sugarcane molasses waste. After this pre-treatment, the efficiency of removing 5-Hydroxymethylfurfural (5-HMF), furfural, and metallic inhibitors from the pre-treated hydrolysate was evaluated using a nano-based adsorbent. A high coefficient of determination ($R^2$ = 0.98) and a maximum reducing sugar concentration of 98.14g/L were achieved under the optimized conditions, which included a substrate loading of 20% (w/v), sulphuric acid concentration of 0.75% (v/v), and an autoclave time of 5 minutes. Furthermore, the application of $Fe_3O_4$ nanoparticle (NP) at a concentration of 0.2% (w/v) led to a reduction in 5-HMF and furfural concentrations by 29.05% and 53.53%, respectively. Additionally, the concentrations of metal contents (Ca, Mg, Na, and S) were decreased by 4.97%, 7.59%, 15.04%, and 7.63%, respectively. Remarkably, surface modification of $Fe_3O_4$ NP using poly (ethylene glycol)-PEG, Tri sodium citrate–TSC, chitosan–coated and k-Carrageenan–k-C enhanced the removal of metal contents up to 42.74-fold. The high efficiencies in reducing sugar recovery and inhibitor removal demonstrate the potential of this pre-treatment and detoxification approach for a sustainable, and eco-friendly molasses biorefinery concept.

**Key words:** Molasses, nanoparticles, inhibitors, pre-treatment, detoxification

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## 3. Introduction

Sugarcane (*Saccharum officinarum*) is a crop utilised to globally produce sugar as the main products. Largely grown in developing countries with warmer climates such as Brazil and India with the production capacity of 768 678 382 and 348 44600 metric tonnes, respectively (Francis et al., 2020). It has been forecasted that sugarcane production will increase by the year 2030 with more production capacity in the developing countries (Oecd/Fao, 2021). The harvested sugarcane is processed through milling and refinement to produce sugar and large amounts of waste products such as molasses, cane tops, sugarcane bagasse, dry leaves, and press mud (Singh et al., 2021). Due to the high production of waste products such as molasses, valorization and recycling are necessary from the economic and environmental point of view.
Sugarcane molasses as one of the by-products of sugar extraction and refinement obtained through the processes of evaporation, crystallization, and centrifugation (Veana et al., 2014). Molasses is composed of approximately 35% sucrose, 20% water content, 19% reducing sugars (mainly glucose and fructose), 5% non-nitrogenous acids, 4.5% nitrogenous compounds, 4% carbohydrates and 12% ash (Teclu et al., 2009). Also, present in sugarcane molasses are inorganic salts that contain magnesium, silver, mercury, lead, manganese, aluminium, iron, and zinc ions that are potential inhibitors (Ali et al., 2002). These compositions differ depending on the sugar extraction and refinement techniques employed by the sugar mill. As a by-product of sugarcane processing, molasses removed during the third stage of the boiling process tends to have high ash and inorganic salt content and is therefore undesirable. On other the hand, this sugarcane molasses can be used as a substrate for the fermentative production of several bioproducts such as lactic acid, citric acid, ethanol, hydrogen, butanol, and acetone. To achieve sugarcane molasses valorization to other bioproducts, a pre-treatment stage is required (Acosta-Piantini et al., 2023). The pre-treatment of the molasses substrate allows for the hydrolysis of sucrose and other complex sugars into the monosaccharides, which are easier for most microorganisms to metabolise to produce valuable products (Acosta-Piantini et al., 2023). There are several techniques implemented to pre-treat cane molasses, the most common types being: acidic and enzymatic pre-treatment (Shazia and Sikander, 2015; Vidra, et al., 2017). Acid pre-treatment involves the addition of an acid to the substrate therefore increasing the presence of hydrogen ions. In the presence of water, free hydrogen ions hydrolyze the α, β-glycosidic linkage and so cleaving to the sucrose disaccharide to produce α-D-glucose and β-D-fructose monomers. HCl, $H_2SO_4$, and $HNO_3$, are the most common mineral acids utilised in the molasses pre-treatment process (Shazia and Sikander, 2015). For instance, Shazia and

Sikander (2015) utilising 5ml acid (0.5N to 2N concentration) per 15 ml of molasses and a heating duration of 1 hour at 90°C and Ali et al. (2002), using 3.5% v/v 1N acid at 25% molasses and heated at 100°C for 30 minutes both studies obtained a pre-treated molasses hydrolysate with higher reducing sugar content. Similarly, enzymatic pre-treatment methods which include the use of invertase enzymes such as β-fructosidases and α-1,4-glucosidases to cleave the glycosidic bonds within the sucrose molecule to produce glucose and fructose reducing sugars (Vidra et al., 2017; Toledo et al., 2019). These enzymes are highly selective for sucrose, some disadvantages of this method include higher operating costs as the price of enzymes are significantly more expensive as compared to acid. Another demerit of using enzyme is the need for pH adjustment due to enzyme pH specificity for optimal performance. Although acidic pre-treatment is cost-effective as compared to enzymatic pre-treatment, the method poses several disadvantages, namely the acidic induced corrosion of operating equipment and the acid-catalysed formation of several inhibitory compounds and the need to adjust pH within the system after pre-treatment (Solarte-Toro et al., 2019; Edwiges et al., 2022).

The pre-treatment of sugarcane molasses can generate by-products that sometimes act as inhibitors in the fermentation process, such as metal ions ($Mg^{2+}$, $Mn^{2+}$, $Zn^{2+}$, $Cu^{2+}$, $Pb^{2+}$, $Al^{3+}$, $Cd^{2+}$, $Fe^{2+}$) (Zohri et al., 2022: Ali et al., 2002). Likewise, other commonly inhibitory compounds in pre-treated molasses include acetic acid, phenol, furfural and 5-Hydroxymethylfurfural (Edwiges et al., 2022). The presence of these inhibitors within the fermentation media can be detrimental to the growth of microorganisms. They inhibit microbial growth through DNA damage via fragmentation, inhibition of enzymes within the glycolytic pathway and the reduction of NADPH available for microorganism metabolism (Wang et al., 2018). The principal formation of 5-Hydroxymethylfurfural from polysaccharides (starch, cellulose) consists of three stages; the hydrolysis of the polysaccharide to glucose monomers catalysed by a Bronsted acid, the isomerization of glucose and fructose monomers catalysed by a Lewis acid and the dehydration of the fructose monomers into 5-Hydroxymethylfurfural catalysed again by a Bronsted acid (Menegazzo et al., 2018). Furfural is typically formed by the dehydration of five carbon-sugars (such as xylose) in the presence of hydrogen ions (Mathew et al., 2018). Specifically, the proposed two stage mechanism initiates with the isomerisation of xylose to xylulose catalysed by a Lewis acid followed by a dehydration reaction catalysed by a Bronsted acid to form furfural (Jia et al., 2019). Due to the large availability of the prerequisite monomers coupled with the acid introduction, the formation of these two fermentation inhibitors is catalysed during the pre-treatment process which

exacerbates their negative effect in undetoxified pre-treated hydrolysate. Hence, the need for an effective technique to lower the inhibitor concentrations within the pre-treated hydrolysate. The presence of heavy metals and certain inhibitory compounds like furfural and 5-hydroxymethylfurfural in pre-treated sugarcane molasses is unfavourable for optimal microbial growth and productivity. Presently, there is scarcity of studies on molasses pretreatment and subsequent detoxification strategies. Hence, there is need for more studies on molasses valorization pretreatment strategies such as sequential molasses pretreatment and detoxification (use matrix such as nanoparticles) since this could significantly increase the desirability of molasses valorization for bioproduct production.

Moreover, sequential nano-based detoxification is promising, since nanoparticle displays high catalytic activity, and reusability potential towards a cost-effective process. Nanoparticles (NPs) have unique properties such as the catalytic potentials, large surface area to volume ratio, absorbent capability, nano-size, and shapes. Therefore, this study aims to enhance the substrate suitability of sugarcane molasses through (1) sugarcane molasses pre-treatment optimization using a steam-assisted acid process for improved reducing sugar recovery, and (2) the sequential detoxification of 5-Hydroxymethylfurfural, furfural, and metal ion inhibitors using nano-sized absorbents.

## 3.1 Materials and Methods

### 3.1.1 Molasses acquisition

The substrate used for this study was sugarcane molasses obtained locally from a sugarcane milling facility located in Kwa-Zulu Natal province, South Africa. The molasses was highly viscous and dark brown in colouration. It was stored in the refrigerator until usage.

### 3.1.2 Response-surface methodology experimental design

The response surface methodology (RSM) is an ideal modelling and optimization technique that has been employed to elucidate the linear and non-linear interactions of process parameters, thereby determining the most appropriate process conditions (Sanusi et al., 2020). In this study, the RSM Box-Behnken design was used to obtained 17 independent experimental runs (Table 1). The input parameters implemented were autoclave time (5 – 30min), molasses loading (5 – 20% w/v), and acid concentration (0 – 1.5% v/v) and the output parameter being reducing sugar concentration (g/L). The ranges of input parameters were curated from literature survey coupled with data from trial experiments (Shazia and Sikander, 2015) The experimental data were subsequentially used to fit a polynomial equation that link the input factors and the

response output. Additionally, analysis of variance (ANOVA) was used to assess the obtained model's data.

Then, the validation experiment was carried out under the predicted optimized conditions obtained from the pre-treatment model. Aliquot sample from the pre-treated molasses hydrolysate was analysed for reducing sugar (g/L) and thereafter, the process inhibitors.

### 3.1.3 Steam-assisted acid pre-treatment of sugarcane molasses

The pre-treatment experiment was set up according to the model design in Table 3.1, using 250ml conical flasks and a working volume of 100ml, dilute acid (0%, 0.75% or 1.5% v/v) and molasses (5, 12.5 or 20% w/v) were transferred to their correlating conical flasks. The conical flasks were then sealed with foil and placed in the autoclave (121 °C) for their set duration (5min, 17.5min or 30min). Once the autoclave period ended, 1ml samples were taken for reducing sugar analysis.

### 3.1.4 Nanoparticle synthesis

Iron (III) oxide ($Fe_3O_4$) NP, iron (II) oxide ($Fe_2O_3$) NP and surface coated NPs were prepared by co-precipitation using previously described protocols (Sanusi et al., 2019). The surface modifications of $Fe_3O_4$ NPs were achieved using the methods described Lakshmanan (2013). Morphology, size, and metal composition of the nanoparticles (already detailed in Sanusi et al. 2019) were obtained with advance microscopy (using transmission electron microscopy (TEM), scanning electron microscopy (SEM)) and spectroscopy techniques (using Fourier Transform Infra-Red spectroscopy (FT-IR)) (Sanusi et al., 2019).

#### 3.1.4.1 Tri sodium citrate–coated $Fe_3O_4$ NP

Tri sodium citrate (TSC) solution was prepared with 0.2g of TSC dissolved in deionised water. Later, the solution was added drop-by-drop to the mixture containing $Fe_3O_4$ NPs with continuous stirring at 90°C. Thereafter, the resultant TSC coated $Fe_3O_4$ NPs charged with citrate groups on their surface was obtained.

#### 3.1.4.2 Poly (ethylene glycol)–coated $Fe_3O_4$ NP

TSC-coated $Fe_3O_4$ NPs was prepared as above. Poly (ethylene glycol) (PEG) solution was added to the TSC coated $Fe_3O_4$ NPs dispersion and stirred at room temperature. Thereafter, the PEG coated $Fe_3O_4$ NPs was washed to remove excess PEG, dry and stored at ambient temperature until further use.

### 3.1.4.3. Chitosan–coated $Fe_3O_4$ NP

Firstly, $Fe_3O_4$ NPs was dispersed in distilled water. Consequently, 0.68g of chitosan was dissolved in acetic acid and added to the $Fe_3O_4$ NPs suspension with a reaction time of 24h. Later, the chitosan coated $Fe_3O_4$ was washed with ethanol followed by distilled water. Finally, chitosan coated $Fe_3O_4$ NPs was stored at 4°C prior to use.

### 3.1.4.4 k-Carrageenan–coated $Fe_3O_4$ NP

TSC-coated $Fe_3O_4$ NPs was prepared as outline in section 2.4.1. k-Carrageenan (k-C) solution was added to the TSC coated $Fe_3O_4$ NPs dispersion and stirred at room temperature. Afterwards, the k-C coated $Fe_3O_4$ NPs was rinsed to remove excess k-C, dry and stored at ambient temperature until further use.

### 3.1.5 Inhibitor release kinetics

The rate of inhibitor release was evaluated by pre-treating molasses under the validated optimal process conditions (1,06% (v/v) sulphuric acid concentration, 6.60% (w/v) substrate loading and 5-minute autoclave duration (this was modified to 1 -3- and 5-minute time intervals)). Specifically, six flasks were prepared, these represented 1 -3- and 5-minute time intervals in duplicate and pre-treatment was conducted accordingly. The rate of 5-Hydroxymethylfurfural and furfural formation were determined using Eq (1) and Eq (2).

$$Concentration\ (M) = \frac{concentration\ (gL^{-1})}{Molar\ mass(gmol^{-1})} \quad \text{(Eq. 1)}$$

$$k\ (Ms^{-1}) = \frac{\Delta concentration\ (M)}{\Delta time(s)} \quad \text{(Eq. 2)}$$

### 3.1.6 Inhibitor detoxification

$Fe_3O_4$ and $Fe_2O_3$ nanoparticles were utilised as absorbents for the detoxification of pre-treated sugarcane molasses hydrolysate. Pre-treated hydrolysate was placed in 250ml conical flasks, $Fe_3O_4$ and $Fe_2O_3$ nanoparticles (0.1% and 0.2% w/v) was added separately, and the flasks were then incubated at 35°C for 30 minutes at a speed of 120rpm (Adebule et al. 2024). The experimental control consisted of pre-treated hydrolysate that contained no nanoparticles and did not undergo incubation. Initial and resultant Furfural, 5-Hydroxymethylfurfural and metal ion inhibitor content were thereafter used to determine the removal efficiencies.

### 3.1.7 Analytical methods

The elemental compositions of both pre-treated and detoxified pre-treated sugarcane molasses were obtained using the established protocol as outlined by Ren et al. (2016).

The reducing sugar concentration quantification was achieved by sample centrifugation at 10 000rpm for 10min and the reducing sugar present in the obtained supernatant was estimated using the 3,5–dinitrosalicylic acid (DNS) method (Miller, 1959).

The concentration ($C_0$=initial concentration and $C_f$=final concentration) and removal efficiencies of the inhibitors (Furfural and 5-Hydroxymethylfurfural) were determined using the protocol described by Adebule et al. (2024) (Eq. 4 and Eq. 5). The percentage removal efficiency (RE) was obtained using Eq (6).

$$HMF\ (mgL^{-1}) = Absorbance_{HMF} - Absorbance_{HMF+SHS} \quad \text{(Eq. 4)}$$

$$HMF\ (mgkg^{-1}) = \frac{[HMF\ (mgL^{-1})] \times V_{sample}}{W_{sample}} \quad \text{(Eq. 5)}$$

$$\%RE = \frac{C_o - C_f}{C_o} \times 100 \quad \text{(Eq. 6)}$$

## 3.2 Results and discussion

### 3.2.1 Model development, input parameter interactions and reducing sugar responses

The pre-treatment data from the model were analysed using Analysis of Variance (ANOVA). The ANOVA (Table 3.2) showed a P-value of <0.0001 (<0.05) and an F-value of 44.43 which indicated the model was significant. The large F-value implied that there is only a 0.01% chance of the value outcome due to noise. The coefficient of determination ($R^2$) value of 0.98 and adjusted $R^2$ value of 0.96 indicated the model fitness and suggested that the model design was reliable in predicting the optimum conditions for the pre-treatment process (Paleologou et al., 2016). The generated polynomial equation (Eq. 7) describes the correlation between reducing sugar concentration response and the coded input parameters as shown below:

$$\boldsymbol{Y = +54.31 + 19.30A - 1.13B + 30.00C - 0.2587AB - 9.98AC - 2.15BC - 19.32A^2 + 1.81B^2 + 3.18C^2} \quad \text{(Eq. 7)}$$

Where Y= Reducing sugar concentration (g/L), A= Sulphuric acid concentration (% v/v), B= Autoclave time (min), and C= Molasses loading (% w/v).

**Table 3.1: Box-Behnken design of input parameters influencing reducing sugar release**

| Run | Input parameters | | | Response |
|---|---|---|---|---|
| | **Sulphuric acid % (v/v)** | **Autoclave time (min)** | Molasses **loading % (w/v)** | **RS (g/L)** |
| **1** | 0.75 | 30 | 5 | 23.46 ± 0.13 |
| **2** | 0 | 5 | 12.5 | 16.50 ± 0.08 |
| **3** | 0.75 | 30 | 20 | 88.39 ± 0.04 |
| **4** | 0.75 | 17.5 | 12.5 | 53.35 ± 0.15 |
| **5** | 0 | 30 | 12.5 | 19.23 ± 0.11 |
| **6** | 0.75 | 17.5 | 12.5 | 54.82 ± 0.13 |
| **7** | 0.75 | 17.5 | 12.5 | 53.93 ± 0.12 |
| **8** | 0.75 | 5 | 5 | 25.91 ± 0.08 |
| **9** | 0.75 | 5 | 20 | 99.45 ± 0.05 |
| **10** | 1.5 | 17.5 | 5 | 22.47 ± 0.16 |
| **11** | 1.5 | 5 | 12.5 | 54.88 ± 0.09 |
| **12** | 1.5 | 17.5 | 20 | 93.21 ± 0.14 |
| **13** | 0.75 | 17.5 | 12.5 | 55.06 ± 0.11 |
| **14** | 0 | 17.5 | 20 | 33.91 ± 0.12 |
| **15** | 1.5 | 30 | 12.5 | 56.58 ± 0.06 |
| **16** | 0.75 | 17.5 | 12.5 | 54.41 ± 0.13 |
| **17** | 0 | 17.5 | 5 | 3.10 ± 0.05 |

**RS = Reducing sugar**

**Table 3.2: ANOVA for a quadratic model representing the reducing sugar response**

| Source | Sum of Squares | df | Mean Square | F-value | p-value | |
|---|---|---|---|---|---|---|
| **Model** | 12204.88 | 9 | 1356.10 | 44.43 | < 0.0001 | Significant |
| A-$H_2SO_4$ | 2980.11 | 1 | 2980.11 | 97.65 | < 0.0001 | |
| B-Autoclave time | 10.29 | 1 | 10.29 | 0.3370 | 0.5798 | |
| C-Molasses loading | 7201.44 | 1 | 7201.44 | 235.97 | < 0.0001 | |
| AB | 0.2678 | 1 | 0.2678 | 0.0088 | 0.9280 | |
| AC | 398.60 | 1 | 398.60 | 13.06 | 0.0086 | |
| BC | 18.52 | 1 | 18.52 | 0.6067 | 0.4615 | |
| $A^2$ | 1572.34 | 1 | 1572.34 | 51.52 | 0.0002 | |
| $B^2$ | 13.74 | 1 | 13.74 | 0.4503 | 0.5237 | |
| $C^2$ | 42.66 | 1 | 42.66 | 1.40 | 0.2757 | |
| **Residual** | 213.63 | 7 | 30.52 | | | |
| Pure Error | 1.90 | 4 | 0.4746 | | | |
| **Cor Total** | 12418.51 | 16 | | | | |

The experimental model, detailed in Table 3.1, showed a range of reducing sugar outputs from 3.10 to 99.45 g/L. Optimal reducing sugar yields were achieved with a 20% w/v acid molasses loading, a 5-minute pretreatment duration, and an acid concentration of 0.75% v/v. The interactions between input parameters and their effects on reducing sugar yields were elucidated using two-dimensional response contour graphs, as illustrated in Fig. 3.1.

In Fig. 3.1X, the relationship between sulphuric acid concentration (% v/v) and molasses loading (% w/v) on reducing sugar yield was presented. Notably, the reducing sugar content increased from 3 to 32 g/L as the acid concentration was raised from 0 to 1% v/v, while keeping the molasses loading constant at 5% w/v. Further increase in acid concentration (>1% v/v) resulted in slight increase in reducing sugar content from 32 to 33 g/L. However, a substantial increase in reducing sugar content, from 3 to 98 g/L, was observed when both the molasses loading and acid concentration were simultaneously increased from 5% to 20% w/v and 0 to 1% v/v, respectively. This underscores the significant synergistic effect of these two parameters under the experimental conditions evaluated. Additionally, the interaction between acid concentration (% v/v) and autoclave duration (minutes) was shown in Fig. 3.1Y. This revealed that increasing the acid concentration from 0 to 1% v/v, while maintaining a constant autoclave duration, an increase in reducing sugar yield from 16 to 58 g/L was obtained.

Similarly, the interactive impact of molasses loading (% w/v) and autoclave duration (minutes) (Fig. 3.1Z), showed an incremental molasses loading from 5 to 20% w/v with a fixed autoclave duration of 5 minutes resulted in a significant increase in reducing sugar content from 23 to 92g/L. However, simultaneously increasing both parameters (from 5 to 30 minutes and 5 to 20% w/v) resulted in a negligible change in reducing sugar yield. Highlighting a potential threshold beyond which further increases in autoclave time do not correlate with significant sugar yield.

Sulphuric acid concentration and molasses loading were observed to exert the most influencing effects on reducing sugar yield. Although, increase in sulphuric acid above 1% v/v resulted in a decline in the reducing sugar yield possibly caused by acidic sugar degradation (Qian et al., 2005). The thermochemical interaction induced by the presence of sulphuric acid coupled with the autoclave steam allows for the enhanced hydrolysis of glycosidic bonds that exist in disaccharides and polysaccharides within the molasses substrate (Steinbach et al., 2020). This process is catalysed by $H^+$ ions from the sulphuric acid solution (Wijaya et al., 2014). Molasses loading had a linear relationship on the reducing sugar yield, a higher molasses loading would result in higher reducing sugar content. However, sugarcane molasses as a substrate for the release of monosaccharides can be affected by high viscosity at an average density of 1.4g/cm$^3$

(Hassan et al., 2019). Hence, high molasses loading could result in high viscose condition that could affect factors such as heat transfer, the rate at which the solvent penetrates the substrate and the resulting hydrolysate composition. Due to the sensitivity of reducing sugar release to the influence of the input parameters, it is imperative to ensure that the most favourable pre-treatment conditions are implemented for the release of optimum reducing sugar from sugarcane molasses.

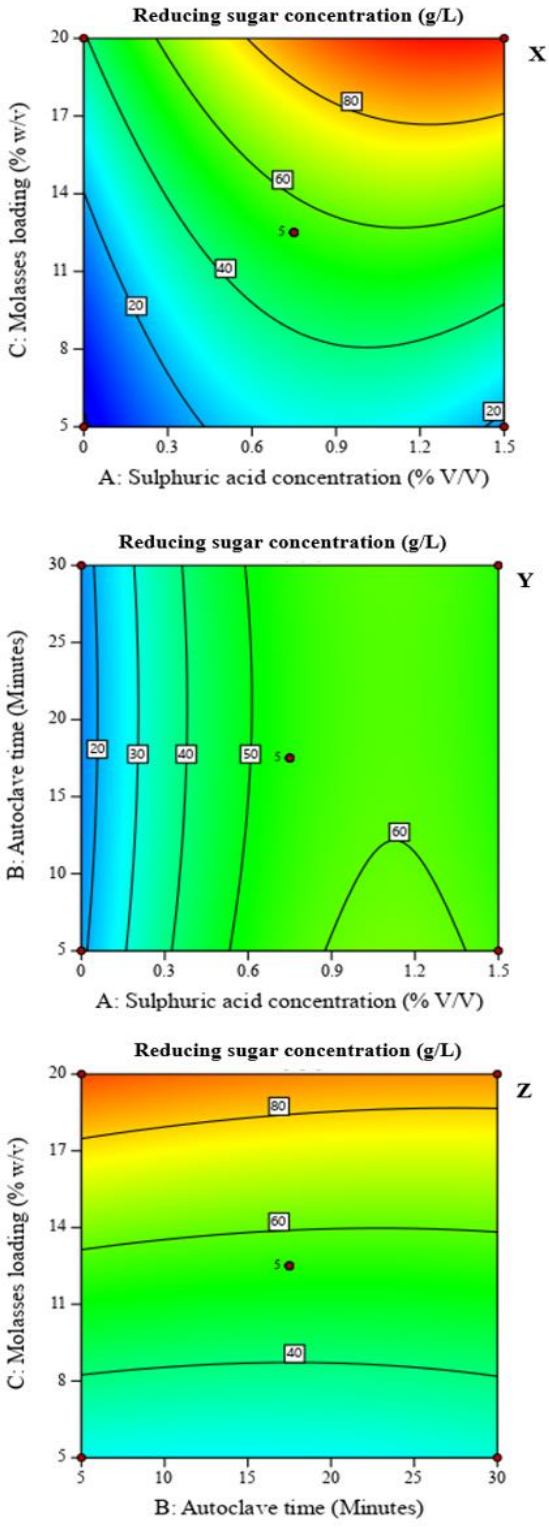


**Fig. 3.1: Two-dimensional response graphs showing the interactions of: X) sulphuric acid concentration (% v/v) and molasses loading (% w/v), Y) sulphuric acid concentration (% v/v) and autoclave time (minutes), Z) autoclave time (minutes) and molasses loading (% w/v) on reducing sugar (g/L) released.**

### 3.2.2 Validation of pre-treatment model

The optimized steam-assisted acid pre-treatment RSM model predicted a reducing sugar yield of 92.59g/L (pre-treatment conditions of 0.75% (v/v) sulphuric acid, 20% (w/v) molasses loading and 5 minutes autoclave duration (Table 3.3). The validation experiment showed a reducing sugar yield of 98.14g/L, resulting in a 5.99% increase compared to model's predicted value. These factors at the optimal set points were the driving forces that synergistically initiate the release of reducing sugar during the pre-treatment regime. The hydrogen ions produced by the acid increased the catalytic rate of the hydrolysis reaction. Secondly, the heat and pressure within the autoclave induces the steam explosion phenomenon that resulted in the cleavage of polymeric bonds through shear-force. The results obtained from the current study are comparable to previous studies on molasses pre-treatment (Shazia and Sikander, 2015; Hawaz et al., 2023). The optimized autoclave duration in the present study is similar to the report of Jayanti et al. (2019) where a duration of 5-10min was implemented to obtain high reducing sugar. This is desirable from the point view of high reducing sugar achievable at lower autoclave duration and the economic benefit of operating the pre-treatment at the same process duration. Moreover, since, pre-treatment processes produce high concentration of fermentation inhibitors such as 5-HMF and furfural using reducing sugars as precursors at extended pre-treatment durations, shorter pre-treatment durations are therefore desirable. On the other hand, the pre-treatment time in the present study was 20-fold and 40-fold lower compared to pre-treatment period implemented by Shazia and Sikander (2015) and Hawaz et al. (2023) respectively. Summarily, this study demonstrated that under the present experimental conditions, an extended autoclave duration has no significant effect on the release of reducing sugar, rather it could increase the formation of inhibitory compounds.

**Table 3.3: Optimum conditions for parameters during sugarcane molasses pre-treatment**

| **Input variables** | | **Predicted optimum levels** |
|---|---|---|
| Sulphuric acid % (v/v) | | 0.75 |
| Autoclave time (min) | | 5 |
| Substrate loading % (w/v) | | 20 |
| **Response** | **Predicted values** | **Observed values** |
| Reducing sugar | 92.592 g/L | 98.14 ± 0.26 g/L |

### 3.2.3 Kinetic analysis of inhibitor release during pre-treatment

The release rates of 5-HMF and furfural during the pre-treatment of sugarcane molasses are illustrated in Fig. 3.2. It was observed that the concentration of 5-HMF within the hydrolysate surged significantly following the pre-treatment process with release rates varying from $3.88\times10^{-6}$M/s to $2.83\times^{-7}$M/s. The peak release rate, marked as point A in the figure, aligns with the initiation of sucrose disaccharide hydrolysis, which subsequently elevates the concentration of reducing sugars one of which is fructose, a direct precursor to 5-HMF.

The heightened release of 5-HMF was observed at high hydrolytic process facilitated by the increase in hexose-reducing sugars, primarily fructose. This process is catalysed by the presence of a Brønsted acid, such as sulphuric acid, which effectively promotes the conversion of hexose sugars into 5-HMF (Menegazzo et al., 2018). Consequently, utilising a lower concentration of Brønsted acid during pre-treatment, as demonstrated in this study, can mitigate the catalysis of hexose-reducing sugars into 5-HMF, potentially reducing the formation of these undesirable byproducts.

Furfural release rates (at points A2, B2 and C2) were $5.30\times10^{-7}$, $3.58\times10^{-7}$ and $4.67\times10^{-7}$M/s respectively. In comparison to the release of 5-HMF, furfural had a lower concentration and slower release rates. Furfural is usually formed by the dehydration of 5-carbon sugars (such as xylose and arabinose) through acid-catalysed reactions. The presence of lower concentration or reaction release rate of furfural can be attributed to the lower concentration of inhibitor's precursor during pre-treatment regime. Due to molasses primarily consisting of sucrose, the hydrolysis of the disaccharide molasses produces mainly glucose and fructose (6-carbon sugars). Hence, higher concentration of 5-HMF compared to furfural in the pre-treated hydrolysate is expected. The presence of inhibitory compounds such as 5-Hydroxymethylfurfural (5-HMF) and furfural in pre-treated hydrolysates poses significant challenges in bioprocessing, especially in the production of biofuels from waste biomass. These inhibitors, often generated during the harsh conditions of biomass pretreatment, can severely affect the efficiency of bioconversion processes. These inhibitors affect microbial bioprocessing by disrupting the normal metabolic functions and enzyme activities within microbial cells. This disruption is primarily due to the creation of imbalances in electrochemical gradients across cell membranes, which are critical for cellular homeostasis and energy production (Jilani and Olson, 2023). The impact of these inhibitors is evident in the delayed exponential growth of fermentative microorganisms, which directly translates to reduced process productivity. In addition to hampering growth, these compounds also interact negatively with saccharifying enzymes and reduce the fermentability of sugars, crucial steps in

biofuel production. This interaction can lead to lower yields of the desired biofuel product, making the process economically unviable. Given their detrimental effects, it is crucial to either prevent the formation of these compounds during pretreatment or to effectively remove them from the hydrolysates before the fermentation process (Adebule et al., 2024). Effective detoxification results in lower concentrations of these inhibitory compounds, thereby enhancing the efficiency of the enzymatic saccharification and the subsequent fermentation steps (Adebule et al., 2024). The successful removal or reduction of these inhibitors is a critical factor for the commercial viability of waste-based biofuel production. Gupta et al. (2017) emphasizes, achieving an optimal balance between effective pretreatment and minimal inhibitor formation or their efficient removal is essential for the development of sustainable and economically feasible biofuel technologies.

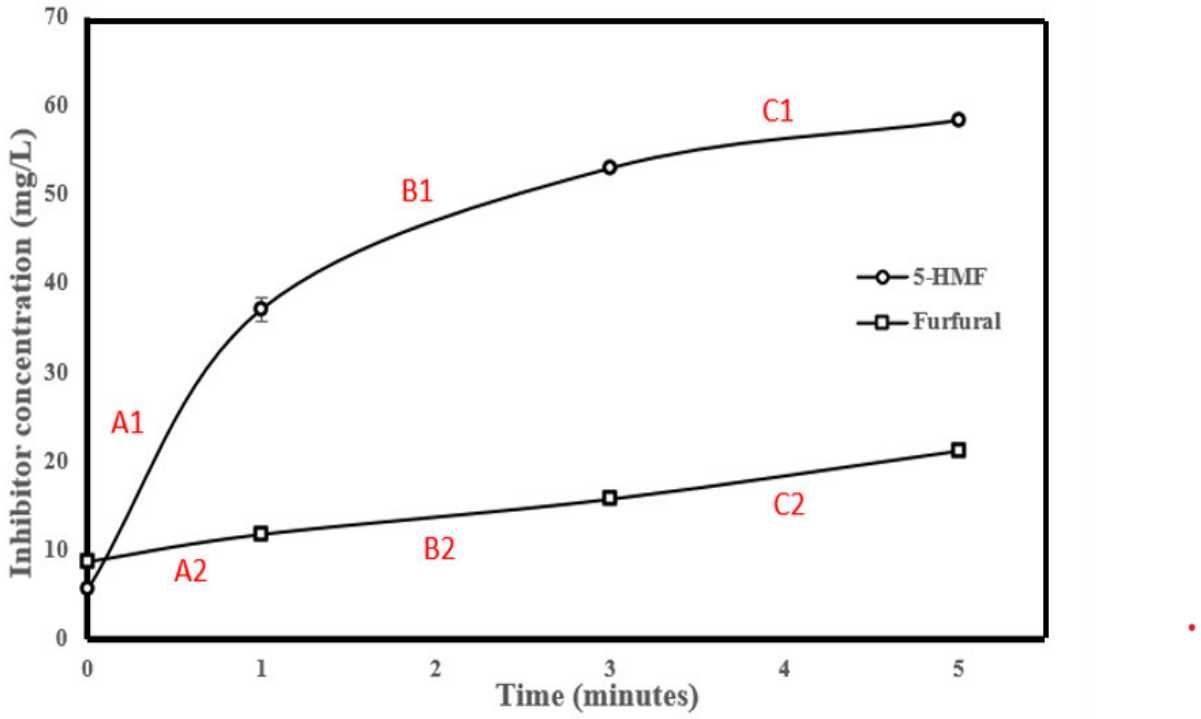


**Fig. 3.2: 5-Hydroxymethylfurfural and furfural release during the steam-assisted acidic pre-treatment of sugarcane molasses.**

### 3.2.4 Inhibitory compound removal

The transmission electron micrographs show the different shapes of the nanoparticles. The nanoparticles had a wide particle size distribution for $F_3O_4$ NP, Tri sodium citrate–coated $Fe_3O_4$ NP, polyethylenimine–coated $Fe_3O_4$ NP, chitosan–coated $Fe_3O_4$ NP and cellulose–coated $Fe_3O_4$ NPs. When nanoparticles such as obtained in the present study are applied at low concentration usually <1wt%, they have been shown to possess desirable catalytic effect (Sanusi et al., 2019). Specifically in this study higher chemical reactivity was observed with

these NP due to their high surface area to volume ratio that provides greater number of attachment reaction sites for the removal of inhibitors and metal ions. The SEM-EDS elemental analysis shows the different metal compositions. Presence of high percentage weight of the core metal is desirable as this determines the primary catalytic functions of each of the nanoparticles even the extent of their activities in chemical reactions. This must have influenced their surface charge, and their detoxification efficiencies as observed in the present report.

The detoxification results are shown in Fig. 3.3, $Fe_3O_4$ and $Fe_2O_3$ nanoparticles produced the desired detoxification effect on 5-HMF and furfural fermentation inhibitors. $Fe_3O_4$ nanoparticles (0.1 and 0.2% w/v) had the most significant removal efficiency of 14.16% and 29.05% reduction in 5-HMF concentration and 37.29% and 53.53% reduction in furfural concentration, respectively when compared to the control sample. Also, the detoxification processes using $Fe_2O_3$ nanoparticle at 0.2% w/v showed 8.32% and 32.42% reduction in 5-HMF and furfural concentrations, respectively. On the other hand, at 0.1% w/v, there was a 23.29% reduction in furfural concentration whilst it was totally ineffective in lowering 5-HMF concentration (a 5.29% increase was rather observed) in the pre-treated molasses.

The removal efficiencies of $Fe_3O_4$ nanoparticles on metal contents within the pre-treated molasses hydrolysate are detailed in Table 3.4. Notably, reductions were observed for calcium (4.97%), magnesium (7.59%), sodium (15.04%), and sulphur (7.63%). These reductions were likely facilitated by the formation of ligand complexes between the metal ions/inhibitors and the nanoparticle surfaces, aiding in the removal process. Furthermore, factors such as metal speciation and the surface charge of also play a role in metal ion/inhibitor removal capabilities (Sanusi et al., 2019). Additionally, the presence of vacant sites on the nanoparticles' surface may enhance the adsorption of metal ions/inhibitors, as noted by Jin et al. (2023). The nanoparticles likely facilitate the removal of these compounds through a radical degradation mechanism, as described by Mani et al. (2018).

The incorporation of nanoparticles into the bioprocessing sequence can alter the pH of the medium, which in turn influences the competitive interactions among various metal ions. This alteration affects the migration patterns of individual metal ions/inhibitors that favour their removal by the nanoparticle adsorbent. Moreover, the electrostatic attractions between the charges of the metal ions and the surface charge of the nano-adsorbent further promote the efficient removal of metal ions/inhibitors (Sanusi et al., 2019).

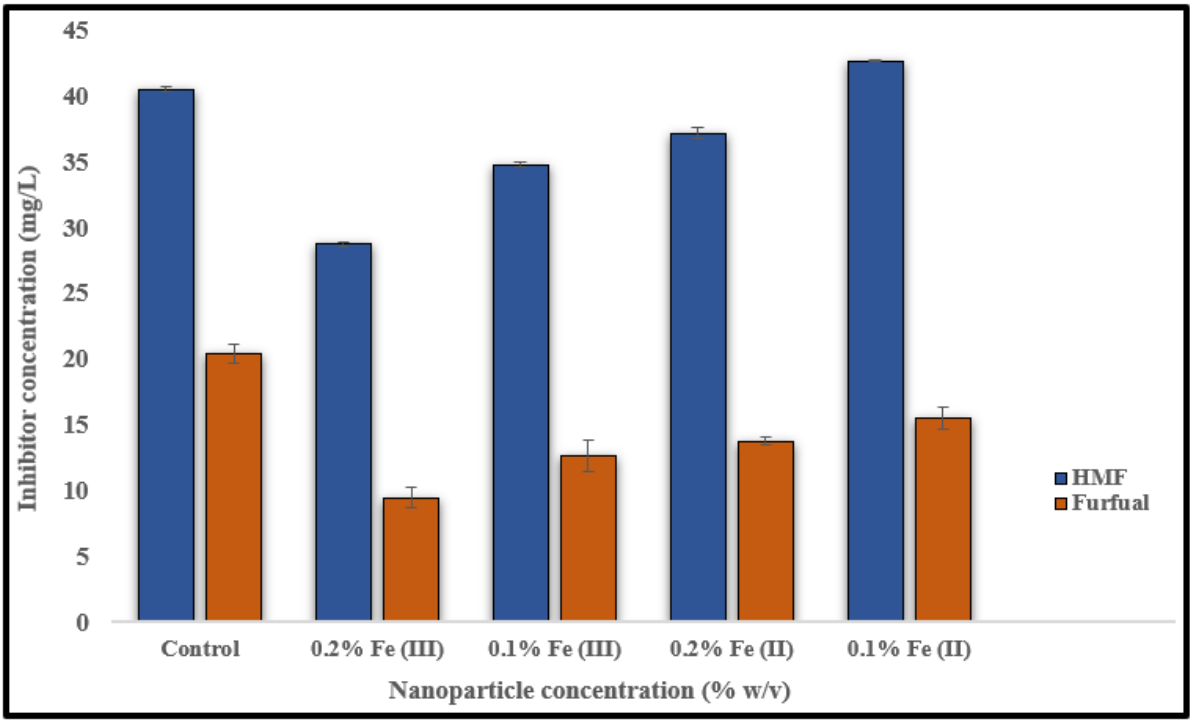


**Fig. 3.3: Effect of different nanoparticles on inhibitory compound detoxification.**

### 3.2.5 Effect of adsorbent surface modification on metal removal

$Fe_3O_4$ NP without coating or surface modification was assessed for the detoxification of metal inhibitors before and after treatment of sugarcane molasses hydrolysate (Table 3.4). The uncoated $Fe_3O_4$ NP was barely effective in lowering the concentration of main inhibitors except for calcium (1.05-fold), magnesium (1.08-fold), sodium (1.18-fold), and sulphur (1.08-fold). Conversely, uncapped $Fe_3O_4$ NP was not as effective in removing metal inhibitors in the pre-treated molasses hydrolysate. This might be due to the very low concentration of some of the metal inhibitors in the pre-treated molasses, varied metallic chemical properties and different complexity potentials. In another approach, using surface modified $Fe_3O_4$ NP to improve the removal efficiency of $Fe_3O_4$ NP, poly (ethylene glycol)–coated $Fe_3O_4$ NP, Tri sodium citrate–coated $Fe_3O_4$ NP, chitosan–coated $Fe_3O_4$ NP and k-Carrageenan–coated $Fe_3O_4$ NP were tested for the removal of selected metal inhibitors (zinc, silver, tin, titanium, thallium, vanadium, and zirconium) with high inhibitory potential in the pre-treated molasses. The initial concentration of these metal inhibitors was determined in the pre-treated molasses hydrolysate (Table 3.4) and the initial concentration of zinc, silver, tin, titanium, thallium, vanadium, and zirconium were found to be approximately <50, <0.24, <0.49, <0.73, <0.49, <0.49 and <0.49 in

hydrolysate. In the detoxified molasses (DM), using nano-adsorbents ($Fe_3O_4$ DM, KC-$Fe_3O_4$ DM, PEG-$Fe_3O_4$ DM, TSC-$Fe_3O_4$ DM and Chitosan-$Fe_3O_4$ DM), the metal inhibitor concentrations were lower appreciably in 30 minutes. Of the modified $Fe_3O_4$ nanoparticle surfaces, chitosan coated $Fe_3O_4$ nano-adsorbent was the most efficient in the removal of metal inhibitors in the pre-treated molasses.

Remarkably, using chitosan coated $Fe_3O_4$ nano-adsorbent, zinc ion concentration (mg/kg) was lower 42.74-fold. It was also better in lowering the concentration of zinc ion concentration compared to KC-$Fe_3O_4$ (1.18-fold), PEG-$Fe_3O_4$ (1.21=fold), and TSC-$Fe_3O_4$ (1.07-fold). Moreover, chitosan coated $Fe_3O_4$ NP significantly lower (2.40-fold) the concentration of silver ion in the pre-treated molasses. It was also more efficient (1.1-fold) in reducing silver ion in the pre-treated molasses in comparison to KC-$Fe_3O_4$ NP adsorbent. Similarly, tin ion concentration was reduced by 2.45-fold in the pre-treated molasses using chitosan coated $Fe_3O_4$ NP. When this was compared with TSC-$Fe_3O_4$, chitosan coated $Fe_3O_4$ NP was better (1.10-fold) in lowering tin ion concentration (mg/kg). furthermore, the coating of $Fe_3O_4$ NP with chitosan improved (1.59) the removal of titanium ion in the pre-treated molasses hydrolysate and was equally better (1.09=fold) in removing titanium ion in the pre-treated sample compared to $Fe_3O_4$ NP coated with TSC. On the other hand, the coating of $Fe_3O_4$ NP with poly (ethylene glycol), Tri sodium citrate, chitosan and k-Carrageenan desirably reduced (2.45-fold) the concentration of thallium, vanadium, and zirconium ions. For this reason, it could be concluded that the different surface modifications' interaction with the coated $Fe_3O_4$ enhances the removal of the various metallic inhibitors, thereby forming different $Fe_3O_4$-based complexes, depending on the metal ion. Specifically, the interactions of these coating agents with the metal inhibitors are strongly related to the individual chemical nature of the coating agent. PEG capped $Fe_3O_4$ nanoparticle has low isoelectric potential (IEP), with precipitation potential as well as binding capacity. Chitosan is a cationic biopolymer with $–NH_2$ groups. Chitosan has specific properties like selectivity and hydrophilicity together with biocompatibility. TSC provides a highly negatively charged with multiple carboxylic acid ligand surface. While K-Carrageenan is an anionic polysaccharide with one sulphate group per disaccharide. For these reasons, it could be speculated that the different interactions with the surfaces enhance the metal inhibitor removal efficiency, forming different complexes. In addition, nanoparticles have large surface-to-volume ratio with tendency to form complexes with other compounds that also promote their binding efficiencies (Lakshmanan, 2013). At certain chemical conditions the potential of nanoparticle to form complexes towards stability such as it is obtained in this study increases. Hence, these properties promote complexation and adsorption on the surfaces of these NPs.

Also, from this perspective, the capped $Fe_3O_4$ NP has the potential for the removal of several of the metal inhibitors. Therefore, the surface modification of $Fe_3O_4$ NP plays a major role in effective removal of the metal inhibitors from the pre-treated molasses. Earlier studies have also reported that the presence of functional group on nanoparticle enhances the adsorption of metals such zinc (Lakshmanan, 2013). Interestingly, on the other hand, the usage of low $Fe_3O_4$ NP concentration (0.2% w/v) for the hydrolysate detoxification process shows the practical feasibility of the process. The use of $Fe_3O_4$ nanoparticles for hydrolysate detoxification addresses a critical challenge in bioprocessing, paving the way for more efficient and economically viable biofuel production: offering a comprehensive solution for enhancing the sustainability of biofuel production from sugarcane molasses.

**Table 3.4: Efficiency of $Fe_3O_4$ NP in metal ion removal from pre-treated molasses**

| Metal content | Concentration (mg/kg) | | | | | |
|---|---|---|---|---|---|---|
| | PM | $Fe_3O_4$ DM | KC-$Fe_3O_4$ DM | PEG-$Fe_3O_4$ DM | TSC-$Fe_3O_4$ DM | Chitosan-$Fe_3O_4$ DM |
| Calcium | 925 | 879 | 863 | 856 | 899 | 902 |
| Magnesium | 975 | 901 | 641 | 634 | 658 | 650 |
| Sodium | 113 | 96 | 80 | 87 | 83 | 98 |
| Zinc | <50 | NE | 1.38 | 1.42 | 1.25 | 1.17 |
| Sulphur | 2294 | 2119 | NE | NE | NE | NE |
| Potassium | 8167 | NE | 7816 | 7070 | NE | 7605 |
| Silver | <0.24 | NE | 0.11 | <0.1 | <0.1 | <0.1 |
| Tin | <0.49 | NE | NE | NE | 0.22 | <0.2 |
| Titanium | <0.73 | NE | NE | 0.38 | 0.50 | 0.46 |
| Thallium | <0.49 | NE | <0.2 | <0.2 | <0.2 | <0.2 |
| Vanadium | <0.49 | NE | <0.2 | <0.2 | <0.2 | <0.2 |
| Zirconium | <0.49 | NE | <0.2 | <0.2 | <0.2 | <0.2 |

**NE= Not Effective, DM=Detoxified Molasses, PM=Pre-treated Molasses**

### 3.3 Conclusion

This study successfully optimized the pre-treatment of sugarcane molasses to enhance the release of reducing sugars, achieving a notable concentration of 98.14g/L. Additionally, the detoxification of the pre-treated sugarcane molasses hydrolysate was effectively carried out using $Fe_3O_4$ NP. The nano-adsorbent demonstrated significant efficiency in removing key inhibitors such as 5-Hydroxymethylfurfural (5-HMF), furfural, and various metal ion inhibitors. Moreover, surface modification (PEG, TSC, Chitosan, and k-C) of $Fe_3O_4$ NP significantly enhanced the removal of zinc (42.74-fold), silver (2.40-fold), tin (2.45-fold), titanium (1.59-fold), thallium (2.45-fold), vanadium (2.45-fold), and zirconium (2.45-fold) metal ions. These findings underscore the viability of sugarcane molasses as a promising feedstock for the production of bioproducts. More importantly, the study introduces a novel detoxification strategy which significantly enhances the quality of the pre-treated hydrolysate, making it more conducive for fermentation processes. The implications of these results are profound, providing a twofold advantage (optimized pre-treatment and efficient detoxification) in bioconversion processing.

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## Chapter Four

# Enhanced Citric acid production from sugarcane molasses via *Aspergillus niger* in submerged fermentation with isopropanol as inducer: Process optimization and kinetic study

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## Abstract

This study aimed to optimize the production of citric acid by employing the microorganism *Aspergillus niger* in submerged fermentation, with sugarcane molasses as the substrate. The research also explored the effectiveness of isopropanol as a fermentation inducer and investigated its impact on microbial growth kinetics. The optimization of the process through experimentation was achieved using a Box-Behnken response-surface methodology (RSM) model with a coefficient of determination ($R^2$) of 0.96. Under the optimized conditions of 20% molasses loading, 0.5% isopropanol concentration, and 3% phosphate content, the fermentation resulted in a maximum citric acid concentration of 7.52g/L. Moreover, the inclusion of isopropanol significantly increased citric acid production by 1.21-fold. The results showed that optimizing the process and leveraging the impact of the isopropanol inducer, a notable enhancement in citric acid production can be achieved. This demonstrates the potential of isopropanol as a catalytic inducer for citric acid fermentation systems using sugar cane molasses waste: a viable and suitable feedstock to replace food-based substrate towards reducing the threat on food security.

**Keywords:** Citric acid, molasses, kinetics, fermentation, inducer, isopropanol

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## 4.1 Introduction

As the planet's natural resources begin to dwindle whilst product demand escalates due to the rising population, researchers look to waste material, such as biomass, as a source for sustainable living (Eloffy et al., 2022). The conversion of biomass waste into valuable commodities has become a crucial research focus to enhance economic sustainability. Approximately, 19.03 million metric tons of sugarcane were produced in South Africa between the years 2018 - 2019, it is estimated that 3 - 7% of molasses is generated per 100 tons of sugarcane under normal milling conditions (Perez and Fujita, 1997), under advanced processing the figure can decrease to 2.5 – 3% (Núñez-Caraballo et al., 2019). Blackstrap molasses, which has undergone advanced sugar extraction and refinement, contains high ash content which has been reported to include inorganic salts and heavy metal ions such as lead, copper, nickel, cadmium, zinc, aluminium, magnesium, and manganese making it unsuitable for retail consumption (Ali, 2002). However, cane molasses contains high sugar content which can be utilised as a carbon source in fermentation processes. Citric acid, characterized as a mild acid, finds diverse applications across industries, including food, chemical, cosmetic, and pharmaceutical, where it serves as an exceptional antioxidant and preservative (Ciriminna, et al., 2017). Nearly 99% of the global citric acid supply is derived from microbial fermentation, with a significant 80% contribution from the fungus *Aspergillus niger* (Goldberg and Rokem, 2009; Laltha et al., 2022). The metabolic prowess of *A. niger*, illustrated through the tricarboxylic acid cycle (Yigitoglu, 1992), enables it to efficiently transform reducing sugars, such as glucose, into notably larger quantities of citric acid. This distinctive capability sets *A. niger* apart from other microbial species, establishing it as a particularly proficient producer within the metabolic pathway. Because of its ability to withstand high sugar concentrations, *A. niger* can utilise sugarcane molasses as a substrate in fermentation for citric acid production. Theoretically, the incorporation of an inducer and the application of machine learning algorithms could enhance the maximum citric acid production during fermentation.

Moreover, organic solvents that are introduced into fermentation media to promote increased product formation are known as inducers. It is theorised that the use of an inducer increases cell permeability (Haq et al., 2003, Samartsev et al., 2023) whereby reducing the mass transfer resistance of the cells membrane without inflicting internal damage to organelles or causing the cell to lyse. Studies have also shown that the presence of an inducer represses the enzyme 2-oxoglutarate dehydrogenase (Maddox et al., 1986), whilst favouring the expression of pyruvate carboxylase which catalysis the conversion of pyruvate into oxaloacetate (a precursor for citrate in the citric acid cycle). Although the expression of pyruvate carboxylase

subsequently increases citric acid formation, the repression of 2-oxoglutarate could prove to affect the energy supply in the cell as the enzyme catalyses the conversion of alpha-ketoglutarate to succinyl-CoA as well as coenzymes which are used in cellular energy transfer (NADH and FADH2), thus further research on product formation and cell growth under inducer conditions must be done (Kanti et al., 2019). Additionally, inducers have been linked to the stimulation of mycelial pellet formation, an advantageous phenomenon in fermentation processes. This stimulation, reported by Haq et al. (2003), increases the surface area of the fungi, inhibits spore formation, and creates and environment conducive to optimal fermentation conditions. The study undertaken by Moyer (1952) observed that the inducer's presence in the fermentation media increased *A. niger's* tolerance for minerals/trace elements, therefore, enhancing the viability of waste products such as blackstrap molasses, which is high in inorganic salt content, as a carbon source for citric acid production. Methanol, typically employed at concentrations ranging from 2% to 6% v/v, has been the most used organic solvent as inducer for citric acid fermentation. This preference was attributed to its characteristics as a short-chain solvent, coupled with its lower microbicidal activity (Rodrigues et al., 2009). Studies have also been conducted utilising ethanol with the optimal concentration being deduced at 3% v/v (Dhillon et al., 2012). Longer chain organic solvents such as acetone have been studied, due to its stronger anti-microbial activity it was noted that lower concentration (1% v/v) were ideal to enhance citric acid production (Laltha et al., 2022). Isopropanol, a three-carbon compound, has undergone testing as an inducer in conjunction with methanol and ethanol. However, in a study conducted by Moyer in 1952, isopropanol was introduced at higher concentrations (3% v/v), leading to a hindrance in the fermentation process. Notably, isopropanol exhibits potent fungicidal activity akin to acetone. To address this issue, researchers proposed a reduction in the solvent concentration supplemented to the fermentation system, ranging from 0.01% to 2% v/v. Therefore, the primary objective of this study was to determine the optimal threshold for alcohol supplementation in the fermentation system, aiming to induce the optimal release of citric acid. This process involves thinning the cellular membrane to enhance permeability to primary metabolites while preserving membrane integrity and preventing damage to intracellular components, thereby avoiding potential growth inhibition. The incorporation of an organic inducer and optimizing its application could enhance high citric acid production.

Moreover, phosphorous is a macronutrient, similar to nitrogen, utilised in several metabolic pathways within the microbial cell, therefore its presence in the fermentation media could enhance cell proliferation and product formation. The optimum concentration of phosphorous

for *A. niger* growth is 2% w/v (Nur Hidayat et al., 2019), whilst a report by Zhang and Roehr (2002) suggested that optimum citric acid levels are produced in a phosphorous-limiting environment at 1% w/v. Previous studies have examined the interaction between the nitrogen sources and the inducers during citric acid fermentation (Latha et al., 2022), whilst research on the impact of phosphorous inducer and their catalytic interaction for improving citric acid production is scanty.

Response-surface methodology (RSM) is a mathematical and statistical approach for analysing multiple experimental parameters and their interactions to determine optimal conditions. Implementing RSM allows researchers to identify the most impactful parameters, evaluate model significance through $R^2$ values, and visualise synergistic interactions via 2-dimentional contour or 3-Dimentional response surface graphs (Yun et al., 2018).

The aim of this study is to optimize citric acid production from pre-treated waste sugarcane molasses using response surface methodology (RSM). It analyses the interactions of the substrate loading, phosphate content, and isopropanol concentration as an inducer. Specifically, this study seeks to determine the optimal conditions for citric acid production using a Box-Behnken RSM model, evaluate the impact of varying isopropanol concentrations on *Aspergillus niger's* growth kinetics and citric acid production, and investigate the role of phosphorus in the fermentation media and its interaction with isopropanol.

## 4.2 Material and Method

### 4.2.1 Molasses acquisition

Sugarcane molasses was the substrate utilised for this experiment. The dark brown, highly viscous cane molasses was sourced from a local sugarcane mill situated in the province of KwaZulu Natal, South Africa.

### 4.2.2 Response-surface methodology experimental model design

The input parameters consist of molasses loading (15 – 25% w/v), potassium dihydrogen phosphate content (1 -3% w/v) and inducer concentration (0.5 – 1.5% v/v) and the output parameter being citric acid concentration (g/L). The ranges of the input parameters were obtained from literature as well as data curated from preliminary experiments. For instance, previous studies have utilised a range of sugarcane molasses substrate loading varying from 15% (Shazia and Sikander, 2015) to 25% (Ali et al., 2002) prompting the use of the selected range in the optimization model. A Box-Behnken response surface methodology design consisting of three-factors was implemented to generate a fermentation experimental model

consisting of seventeen independent runs (Table 4.1). The concentrations of citric acid obtained after the experimental inducer-influenced fermentation processes were used to fit a polynomial equation (Eq. 1). This equation evaluates the synergistic impacts of the model's input parameters on citric acid yield, using Design Expert Software. Additionally, a comparative validation experimental was conducted using the predicted optimized fermentation conditions against a controlled experiment (0% (v/v) inducer).

$$Y = +6.82 + 0.88A - 0.2013B + 0.6013C - 0.28AB - 0.36AC - 0.7625BC - 0.8293A^2 - 0.0267B^2 - 0.1868C^2 \quad \text{(Eq. 1)}$$

Where: Y= Citric acid concentration (g/L), A = Molasses loading (% w/v), B = Isopropanol concentration (% v/v) and C = Potassium dihydrogen phosphate content (% w/v)

### 4.2.3 Inoculum preparation

*Aspergillus niger* was used in this fermentation study, initially grown on Potato Dextrose Agar (PDA) plates, which were incubated at 30°C for 7 days to ensure exponential growth and optimal spore formation. Then matured fungal spores were subsequently suspended by disrupting the surface area of the PDA plates aseptically using a sterilised spatula to obtain spore suspension of $1.54\times10^7$spores/ml. The concentration of the fungal spore suspension was determined using a Neubauer counting chamber, thereafter the final spore concentration was adjusted to $1.15\times10^6$spores/ml by diluting with sterilized distilled water in preparation for the fermentation process.

### 4.2.4 Pre-treatment and detoxification of sugarcane molasses

Based on the Box-Behnken RSM experimental design (17 runs), 500ml's of the sugarcane molasses substrate was prepared at three different concentrations. The sugarcane molasses, at substrate loading 15, 20 and 25% (w/v), was weighed and placed in a 1000ml glass laboratory bottle. The volume was adjusted with dilute sulphuric acid (0.75%). The glass bottles containing molasses and acid solution were sealed with foil autoclaved 121°C for 5 minutes to induce the pre-treatment process. After autoclaving, 0.2% (w/v) $Fe_3O_4$ nanoparticles were added to the pre-treated hydrolysate. The glass bottles were then incubated at 35°C for 30 minutes at a shaking speed of 120rpm to induce detoxification. Filtration of the substrate and separation of the nanoparticles were achieved using 125mm pore-sized filter paper and a strong magnet, making the solution ready for fermentation.

### 4.2.5 Reducing sugar analysis

The reducing sugar concentration quantification was achieved by centrifuging 1ml samples of the pre-treated sugarcane molasses substrate at 10 000rpm for 10min thereafter the reducing sugar content present in the supernatant was estimated using the 3,5–dinitrosalicylic acid (DNS) method (Miller, 1959).

### 4.2.6 Citric acid fermentation process

The fermentation process was carried out in batch model using 100ml Erlenmeyer flasks with a working volume of 50ml under process parameters specified by the response-surface methodology model design. Ammonium sulphate was added to the fermentation media at 0.1% as to induce a nitrogen-limiting environment. The pH of the media was adjusted to 6 and the inoculum was aseptically added at 10% v/v ($1.15\times10^6$spores/ml). Thereafter the fermentation flasks were incubated at 30°C for 120 hours at an agitation of 120rpm. Aliquot samples (1ml) were taken every 24 hours and stored at 0°C for citric acid content (g/L) and biomass analysis (g/L).

### 4.2.7 Citric acid determination

The citric acid content was determined post-fermentation using the acid-base titration method outlined by Ayeni et al. (2019). Aliquots (1ml) of previously collected fermentation samples at various time points were centrifuged at 8000rpm for 10 minutes. Following centrifugation, 100µl of the supernatant was transferred to a 25ml conical flask, and 2 drops of phenolphthalein indicator were added. The supernatant was then titrated against 0.005M NaOH until a colour change indicated the endpoint. The concentration of citric acid was calculated by determining the number of moles of NaOH needed to neutralize the citric acid, based on the concentration and volume of NaOH used in the titration.

### 4.2.8 Biomass concentration

A standard curve was established to quantify the biomass concentration of *Aspergillus niger* by correlating the dry weight (g/L) to the fungal spore count (spores/ml). Initially, a fungal spore isolate was cultivated for 7 days to ensure optimal growth resulting in a concentration of $10^7$spores/ml. Dilutions were prepared following the method outlined by Laltha et al. (2022), and dry weight of the fungal pellet at each dilution was measured to construct a standard curve relating dry weight to spore counts (spores/ml). During kinetic analysis of the fermentation processes, biomass concentrations (g/L) were determined by extrapolating the fungal counts at different time points to the standard curve equation.

### 4.2.9 *Aspergillus niger* growth kinetics using logistic model

The obtained biomass data fit into a logistic model (Eq. 1). This model represents the kinetics of *Aspergillus niger* growth both at the log phase and the stationary phase.

$$X = \frac{X_0 \cdot \exp(\mu_{max} \cdot t)}{1 - \left(\frac{X_0}{X_{\max}}\right) \cdot (1 - \exp(\mu_{max} \cdot t))} \quad \text{(Eq. 1)}$$

where X=biomass concentration (g/L), $X_0$=initial cell concentration, $X_{max}$= maximum cell concentration obtainable, $\mu_{max}$=maximum specific growth rate of *Aspergillus niger* at specific time (t) point during the growth phases.

**Table 4.1: Box-Behnken design of input parameters influencing citric acid production**

| Run | Input parameters | | | Response |
|---|---|---|---|---|
| | A: Molasses loading % (w/v) | B: Isopropanol % (v/v) | C: $KH_2PO_4$ % (w/v) | Citric acid (g/L) |
| 1 | 25 | 1.5 | 2 | 6.08 ± 0.00 |
| 2 | 20 | 1 | 2 | 7.04 ± 0.45 |
| 3 | 20 | 1 | 2 | 6.56 ± 0.23 |
| 4 | 15 | 1 | 1 | 4.00 ± 0.23 |
| 5 | 20 | 1.5 | 1 | 6.72 ± 0.45 |
| 6 | 15 | 1.5 | 2 | 4.96 ± 0.23 |
| 7 | 15 | 1 | 3 | 5.76 ± 0.45 |
| 8 | 20 | 1 | 2 | 6.72 ± 0.91 |
| 9 | 20 | 0.5 | 3 | 8.01 ± 0.00 |
| 10 | 25 | 1 | 3 | 6.88 ± 0.23 |
| 11 | 25 | 1 | 1 | 6.56 ± 0.23 |
| 12 | 20 | 1.5 | 3 | 6.56 ± 0.23 |
| 13 | 15 | 0.5 | 2 | 5.28 ± 0.23 |
| 14 | 20 | 0.5 | 1 | 5.12 ± 0.45 |
| 15 | 20 | 1 | 2 | 7.04 ± 0.91 |
| 16 | 25 | 0.5 | 2 | 7.52 ± 0.23 |
| 17 | 20 | 1 | 2 | 6.72 ± 0.45 |

**$KH_2PO_4$ = Potassium Dihydrogen Phosphate**

### 4.3. Results and discussion

The observed experimental responses from the design are shown in Table 4.1. Experimental observations in Table 4.1 showed that highest concentration of citric acid (8.01 ± 0.00g/L) was obtained at lowest isopropanol using high molasses loading and potassium dihydrogen phosphate content. On the other hand, lowest citric acid (4.00 ± 0.23g/L) was recorded for molasses loading of 15%, at mid set point for isopropanol and potassium dihydrogen phosphate. Showing the sensitivity of the citric acid production to the input parameters. Polynomial equation developed for the model is presented in Eq. (2). The generated polynomial equation describes the correlation between the coded input variables and the citric acid concentration response.

Moreover, the model fitness was assessed using Analysis of Variance (ANOVA). The analysis of variance (ANOVA) presented in Table 4.2 displayed a p-value of 0.0005 (<0.05) indicating the model significance. Also, the F-value of 17.32 was obtained which implied that the model was significant as well as a lack of fit value of 3.77 suggesting that the lack of fit was not significant. The coefficient of determination ($R^2$) and adjusted $R^2$ values of 0.96 and 0.90 respectively, emphasized the model's fitness and suggested that the model design was reliable in predicting the optimum parameters for the fermentation process.

$$\boldsymbol{Y = +6.82 + 0.88A - 0.2013B + 0.6013C - 0.28AB - 0.36AC - 0.7625BC - 0.8293A^2 - 0.0267B^2 - 0.1868C^2} \quad \text{(Eq. 2)}$$

Where: Y= Citric acid concentration (g/L), A = Molasses loading (% w/v), B = Isopropanol concentration (% v/v) and C = Potassium dihydrogen phosphate content (% w/v)

#### 4.3.1 Synergistic effects of the input parameters on citric acid concentration

The 2D response surface graphs representing the synergistic impact of the input parameters on the citric acid produced are represented in Fig. 4.1. In Fig. 4.1X, it is observed that at low molasses loading (15%), a higher isopropanol inducer concentration (1.5%) favours a higher citric acid production. However, increasing molasses loading from 15 – 20% whilst simultaneously reducing isopropanol concentration from 1.5 – 0.5% resulted in a significant increase in citric acid produced (6.08 to 8.01g/L). Further increase in molasses loading (>20%) resulted in slight reduction in citric acid concentration from 8.01 to 7.04g/L. The results observed in the 2D graph shown in Fig. 4.1X shows that molasses loading of 20% (w/v) is sufficient to obtain optimal citric acid production as further increase molasses loading (>20%)

could result in substrate inhibition (Brautaset and Ellingsen, 2011). Substrate inhibition could occur at high solid loading due to the high substrate viscosity resulting in water efflux from the cells (osmotic stress) or inefficient mass transfer leading to negative effect on cellular activities. Ultimately substrate inhibition lower product formation and productivity (Brautaset and Ellingsen, 2011).

The synergistic impact of the phosphate concentrating and molasses loading on citric acid is shown in Fig. 4.1Y. Increasing the phosphate concentration from 1 – 3% whilst simultaneously increasing the substrate loading from 15 – 20% resulted in an increase in citric acid produced from 4 to 7.52g/L. A further increase in molasses loading (>20%) at 3% phosphate concentration led to a decrease in citric acid concentration (7.52 to 6.88g/L). Phosphorus is a macronutrient required in several cellular metabolic pathways, therefore the presence of phosphorus within a growth medium allows for increased metabolic activities within the microorganism. The reported optimum phosphorous content for *Aspergillus niger* is 2% (Nur Hidyat, 2019), however, a study by Zhang and Roehr (2002) suggests that increased phosphorous content supports fungal biomass production whilst enhanced citric acid formation is observed under phosphorous-limiting condition (1%). Possible discrepancies in the results as compared to the previously reported study could be the consequence of different inducers used in the fermentation systems.

Moreover, the interactive effect of phosphate content and isopropanol concentration is illustrated in Fig. 4.1Z. At low phosphate concentrations (1%), increasing the inducer concentration from 0.5 - 1.5% led to a significant increase in citric acid concentration (5.12 to 7.04g/L). Conversely, at a low inducer concentration (0.5%), increasing phosphate content (1 – 3%) resulted in optimal citric acid production (5.12 – 8.01g/L). This inverse relationship is further demonstrated as simultaneous increases in both parameters (>2% phosphate content and >1% inducer concentration) led to a drastic reduction in citric acid formation. There is limited literature elucidating the interactive effects of phosphorus and inducers on fungal cell metabolism, as explored in this study.

Few studies have established optimal inducer concentrations, with 3% ethanol and 3 - 4% for methanol (Kanti et al., 2019; Laltha et al., 2022). Methanol is commonly used as the primary inducer in citric acid fermentation, with ethanol utilised to a lesser extent. However, isopropanol, with a three-carbon chain, exhibits greater antimicrobial activity compared to other solvents like methanol and ethanol, showing high viability as an inducer for improved citric acid metabolism. Previous studies using higher concentrations of isopropanol (3%) were deemed ineffective. However, at an optimal substrate loading of 20% w/v, reducing the

isopropanol concentration from 1.5% to 0.5% increased citric acid formation from 7.04 to 8.01g/L. This observation aligns with a study by Laltha et al. (2022), where acetone was investigated as a potential inducer in citric acid fermentation. The study suggested that a low acetone concentration of 1% was optimal for citric acid fermentation, effectively negating the microbicidal potential of the acetone solvent while enhancing citric acid production.

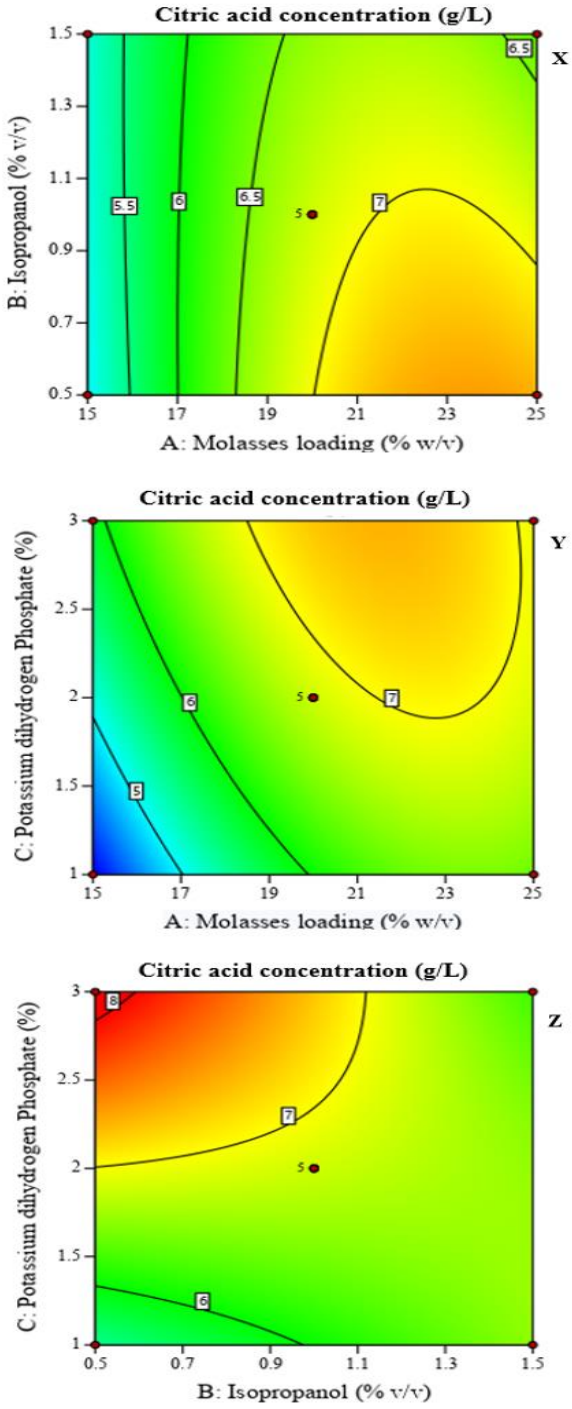


**Fig. 4.1: Two-dimensional response-surface graphs of the synergistic interactions of isopropanol concentration (% v/v), molasses loading (% w/v) and potassium dihydrogen phosphate content (% w/v) on citric acid production (g/L).**

**Table 4.2: Analysis of variance for the citric acid production model**

| Source | Sum of Squares | df | Mean Square | F-value | p-value | |
|---|---|---|---|---|---|---|
| **Model** | 15.72 | 9 | 1.75 | 17.32 | 0.0005 | Significant |
| A-Molasses loading | 6.20 | 1 | 6.20 | 61.43 | 0.0001 | |
| B-Isopropanol | 0.3240 | 1 | 0.3240 | 3.21 | 0.1162 | |
| C-$KH_2PO_4$ | 2.89 | 1 | 2.89 | 28.68 | 0.0011 | |
| AB | 0.3136 | 1 | 0.3136 | 3.11 | 0.1212 | |
| AC | 0.5184 | 1 | 0.5184 | 5.14 | 0.0577 | |
| BC | 2.33 | 1 | 2.33 | 23.06 | 0.0020 | |
| $A^2$ | 2.90 | 1 | 2.90 | 28.71 | 0.0011 | |
| $B^2$ | 0.0030 | 1 | 0.0030 | 0.0299 | 0.8677 | |
| $C^2$ | 0.1468 | 1 | 0.1468 | 1.46 | 0.2667 | |
| **Residual** | 0.7059 | 7 | 0.1008 | | | |
| Lack of Fit | 0.5216 | 3 | 0.1739 | 3.77 | 0.1161 | Not significant |
| Pure Error | 0.1843 | 4 | 0.0461 | | | |
| **Cor Total** | 16.42 | 16 | | | | |

**$KH_2PO_4$ = Potassium Dihydrogen Phosphate**

### 4.3.2 Validation of fermentation model

The optimized fermentation model predicted citric acid output of 8.17g/L under the setpoints of 20% (w/v) molasses loading, 0.5% (v/v) isopropanol, and 3% (w/v) potassium dihydrogen phosphate (Table 4.3). The validation experiment produced a citric acid concentration of 7.52g/L compared to 6.24g/L obtained for the control experiment. Consequently, the optimized process using low concentration of isopropanol inducer, 1.21-fold enhancement in the citric acid production over the control experiment underscoring the impact of the isopropanol inclusion in the fermentation medium. Maximum citric acid production was associated with the optimized process conditions employed, and the inducer presence. Organic solvents such as isopropanol have been known to promote citric acid formation with the potential inducer increases cell permeability (Haq et al., 2003, Samartsev et al., 2023). This increases mass transfer permeability of the cell's membrane without internal damage to organelles. In additional, isopropanol inducer represses the enzyme 2-oxoglutarate dehydrogenase in favour of pyruvate carboxylase that catalysis the conversion of pyruvate into oxaloacetate. Oxaloacetate is a precursor for citrate in the citric acid cycle subsequently increasing citric acid formation. Also, inducers have been linked to the stimulation of mycelial pellet. Mycelial pellet increases the surface area of the cell as well as creates conducive environment for enhanced citric acid fermentation production.

The positive impact of the isopropanol inducer on citric acid fermentation in this study aligns with previous literature findings (Table 4.4). For example, Roukas and Kotzekidou (2020) reported a 1.09-fold increase in citric acid concentration when using a 3% v/v methanol inducer. Similarly, Thorat and Patil (2016) observed a 1.18-fold increase in citric acid production with the supplementation of 3% methanol in fermentation, albeit lower than the results obtained in this study. However, Dhillon et al. (2013) and Rodrigues et al. (2009) reported higher increases in citric acid concentration, with 1.81-fold and 1.46-fold increments, respectively, using 3% ethanol and 4% methanol for citric acid fermentation. These studies indicated higher citric acid production at higher inducer concentrations, making those approaches less favourable compared to the findings of this study. The variation in citric acid production observed among the different studies can be attributed to the differences in *A. niger* strains, substrates, and the operating parameters (temperature, pH, duration, and dissolved oxygen) implemented.

**Table 4.3: Optimum conditions for fermentation parameters during citric acid production**

| **Input variables** | | **Predicted optimum level** |
|---|---|---|
| Molasses loading % (w/v) | | 20 |
| Isopropanol % (v/v) | | 0.5 |
| Potassium dihydrogen phosphate % (w/v) | | 3 |
| **Response** | **Predicted value** | **Observed value** |
| Citric acid concentration | 8.17 g/L | 7.52 ± 0.23 g/L |

**Table 4.4: Comparison of citric acid production with other inducer-based fermentation**

| **Substrate** | **Process conditions** | **Inducer % (v/v)** | **Citric acid increase** | **Reference** |
|---|---|---|---|---|
| Sugarcane molasses | $6.0^{a}, 30°C^{b}, 20\ \%wt^{c}$ | Isopropanol 0.5% | 1.21-fold | This study |
| Apple pomace sludge | $3.5^{a}, 30\ °C^{b}, 25\ \%wt^{c}$ | Ethanol 3% | 1.81-fold | Dhillon et al., 2013 |
| Mahua flowers | $5.0^{a}, 30\ °C^{b}, 10\ \%wt^{c}$ | Methanol 3% | 1.18-fold | Thorat and Patil. 2016 |
| Citric pulp | $5.5, 30\ °C^{b}, NA\ \%wt^{c}$ | Methanol 4% | 1.46-fold | Rodrigues et al., 2009 |
| Dried pomegranate peel | $8.0^{a}$,$25°C^{b}$, $10\ \%wt^{c}$ | Methanol 3% | 1.09-fold | Roukas and Kotzekidou, 2020 |
| Brewery spent liquid | $3.5^{a}, 30\ °C^{b}, NA\ \%wt^{c}$ | Ethanol 3% | 1.43-fold | Dhillon et al., 2012 |

$\mathbf{1}^{a}$ pH

$\mathbf{1}^{b}$ temperature

$\mathbf{1}^{c}$ substrate solid loading

NA- not available

### 4.3.3 *Aspergillus niger* growth kinetics

Fig. 4.2 shows the growth profile of *A. niger* during the citric acid fermentation period under the optimized process conditions. A short lag phase of 24h was observed, thereafter, a prolong exponential phase from 24 till 96h. During the lag phase necessary enzymes and metabolites are synthesizes to adapt to the new environment. Afterwards, *A. niger* enters its exponential phase of growth, indicated by a subsequent increase in biomass concentration (g/L). During the exponential phase, primary metabolites such as citric acid are produced, playing a crucial role in energy production within the tricarboxylic acid (TCA) cycle. Therefore, the increased proliferation of *A. niger* during the exponential phase in the current study is desirable.

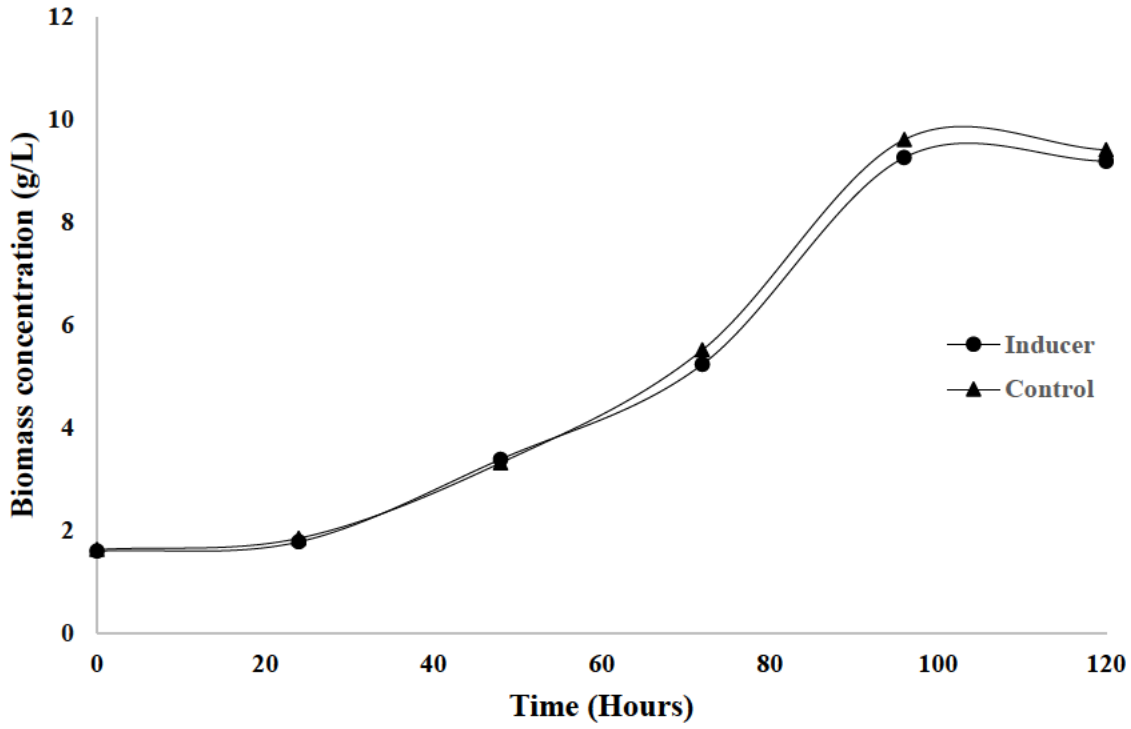


**Fig. 4.2: Growth curve denoting biomass concentration (g/L) of Aspergillus niger under the isopropanol inducer influenced optimized fermentation process conditions (hours).**

The experimental data (Fig. 4.2) from the biomass concentration (g/L) over the fermentation period were used to fit the logistic model with correlation coefficients ($R^2$) >0.96 (Table 4.5). This indicate the model is capably of describing *A. niger* growth under the growth conditions. Considerably lower maximum cell concentration ($X_{max}$=11.28g/L) and maximum specific growth rate ($\mu_{max}$=0.036h$^{-1}$) were attained with the inducer in-cooperated process in comparison with the control experiment (Table 4.5). An indicates that isopropanol

supplementation favoured citric acid formation rather than biomass accumulation. Although, lower $X_{max}$ and $\mu_{max}$ were observed in the inducer-supplemented experiment compared to the control, a higher citric acid concentration was obtained for the inducer-supplemented process., thus suggesting that the presence of the isopropanol inducer mainly enhanced *A. niger* conversion of glucose to citric acid. Inoculum development with isopropanol inducer could improve cell growth rate towards boosting citric acid formation (Rodrigues et al., 2009; Dhillon et al., 2013; Roukas and Kotzekidou, 2020).

**Table 5: Aspergillus niger fermentation growth kinetics using the logistic model**

| Model coefficient | Inducer | Control |
|---|---|---|
| Specific growth $\mu$ ($h^{-1}$) | 0.017 | 0.017 |
| Growth constant $Kg$ ($h^{-1}$) | 0.017 | 0.017 |
| $X_0$ (g/L) | 0.853 | 0.81 |
| $X_{max}$ (g/L) | 11.28 | 11.39 |
| $\mu_{max}$ ($h^{-1}$) | 0.036 | 0.038 |
| $R^2$ | 0.96 | 0.96 |

$X_0$ – initial biomass concentration, $X_{max}$ – maximum biomass concentration, $\mu_{max}$ – maximum specific growth rate, $R^2$ – coefficient of determination

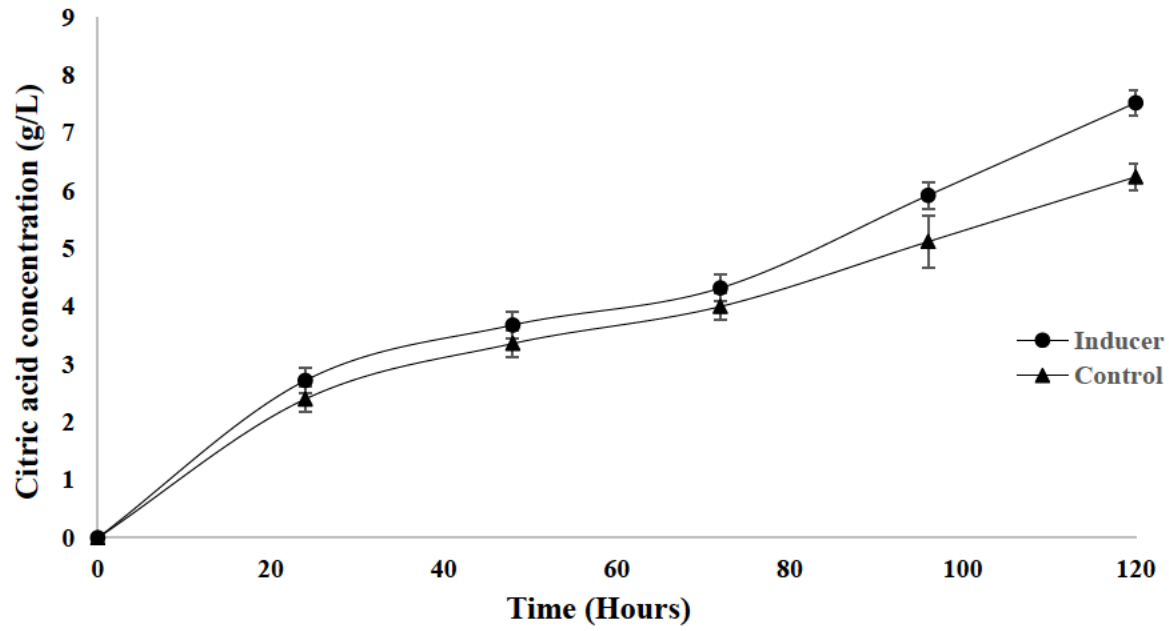


**Fig. 4.3: Kinetic analysis of citric acid (g/L) produced by Aspergillus niger under the isopropanol inducer influenced optimized fermentation process conditions (hours).**

## 4.4. Conclusion

This study successfully optimized isopropanol as an inducer for citric acid production by *Aspergillus niger*. The optimized parameters identified were molasses loading of 20% (w/v), isopropanol concentration of 0.5% (v/v), and potassium dihydrogen phosphate concentration of 3% (w/v), resulting in a maximum citric acid concentration of 7.52g/L. The addition of isopropanol led to a 1.21-fold improvement in citric acid production. This research presents a novel method for enhancing citric acid production, efficiently utilising sugarcane molasses as feedstock and establishing isopropanol as a suitable inducer. Furthermore, the study underscores the viability of using isopropanol as an inducer to improve citric acid production and offers promising prospects for enhancing industrial citric acid fermentation process. Future research should explore the effects of different inducers on *Aspergillus niger* to identify the most effective inducer for citric acid fermentation.

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# Chapter Five

## Conclusion and recommendations for future research

### 5.1 Conclusion

The depletion of the worlds natural resources has prompted researchers to explore waste material as renewable substrates for bioprocessing. Agricultural wastes, such as sugarcane molasses, are renewable and sustainable feedstocks for bio-product production due to their high sugar content. With global molasses production exceeding 50 million tons, efficient valorization of sugarcane molasses necessitates cost-effective pre-treatment and detoxification steps. This study developed an economically feasible pre-treatment and detoxification methods to valorize waste sugarcane molasses. The research focused on modelling and optimizing fermentable sugar recovery from sugarcane molasses waste using steam-assisted acidic pre-treatment. Additionally, it investigated the impact of nanoparticles (NP) as adsorbents for the removal of organic and metal inhibitors in pre-treated sugarcane molasses hydrolysate. Subsequently, citric acid production was conducted using the pre-treated and detoxified waste sugarcane molasses as feedstock, along with a suitable inducer for enhanced citric acid fermentation. The major findings and their significance are summarized below.

The steam-assisted acidic pre-treatment regime was developed to enhance the release of fermentable sugars from waste sugarcane molasses. A high co-efficient of determination ($R^2$) of 0.98 and maximum reducing sugar concentration of 98.14g/L were achieved, representing a 10-fold increase in fermentable sugars content in the pre-treated molasses hydrolysate. During the pre-treatment process, the kinetics of 5-HMF and furfural formation were evaluated. A substantial release of 5-HMF was observed during pre-treatment compared to furfural, highlighting the necessity of a detoxification step. Detoxification of the pre-treated hydrolysate with $Fe_3O_4$ nanoparticles at 0.2% (w/v) substantially reduced furfural and 5-HMF concentrations by 53.53% and 29.05%, and respectively. Additionally, the detoxification process significantly reduced the metal content within the pre-treated hydrolysate (Ca, Mg, Na and S) to a desirable extent. Remarkably, surface modification of $Fe_3O_4$ nanoparticles (using PEG, TSC, Chitosan, and k-C) enhanced the removal of metal content within the pre-treated hydrolysate up to 42.74-fold. The high fermentable sugar yield in waste sugarcane molasses, coupled with an effective detoxification process, made molasses a suitable feedstock for bioprocessing. Furthermore, the fermentation process utilising *Aspergillus niger* to produce citric acid from the pre-treated hydrolysate was optimized.

The combined effect of molasses loading, isopropanol concentration, and potassium dihydrogen phosphate on citric acid production was modelled and optimized using the Response Surface Methodology (RSM). Optimization of the model's input parameters produced an $R^2$ value of 0.96 and a maximum citric acid concentration of 7.52g/L. Assessment of isopropanol's inducer viability indicated a 1.21-fold increase in citric acid production. Analysis of *Aspergillus niger* growth kinetics using the logistic model revealed that isopropanol inclusion had a minimal impact on the maximum specific growth rate ($0.036h^{-1}$ for the inducer, compared to $0.038h^{-1}$ for the control) and biomass concentration (11.28g/L for the inducer, compared to 11.39g/L for the control), but a significant impact on citric acid metabolism. The combination of effective pre-treatment, detoxification, and optimized fermentation strategies, which resulted in enhanced citric acid production, underscores the suitability of waste sugarcane molasses as a bioprocessing feedstock. These findings provide insights into advancing the viability of waste valorization, waste management, and eco-friendly biorefinery processes.

### 5.2 Recommendations

Based on the findings of this study, the following recommendations for future research are suggested to enhance the production of citric acid from waste sugarcane molasses:

I. The steam-assisted acid pre-treatment for the waste sugarcane molasses detailed in this study was successful. However, a comparative study assessing the viability of a microwave-assisted pre-treatment strategy and its subsequent effects on reducing sugar release and inhibitor formation could highlight key areas for improvement.

II. The nano-adsorbent detoxification using $Fe_3O_4$ nanoparticles was insufficient in removing certain organic fermentation inhibitors (acetic acid and phenols) present in the pre-treated hydrolysate at lower concentrations. Future research should explore the effect of control parameters on nanoparticle productivity during the detoxification process. Alternatively, studies could evaluate the effectiveness of different nanoparticles in removing organic inhibitors, potentially presenting a two stage or co-detoxification method.

III. The optimization of citric acid fermentation supplemented with isopropanol to enhance *Aspergillus niger* citric acid metabolism was successful. However, future studies should explore utilising Artificial Neural Networks and large language models, in combination with a broader profile of yeast and *Aspergillus* species, to improve the accuracy of the

predictive model outputs. This approach could also provide additional insights into the viability of inducer inclusion across a wide range of citric acid fermentation process.