Production of biopreservation compounds from non-*Saccharomyces* yeast using a single-stage bioreactor

By

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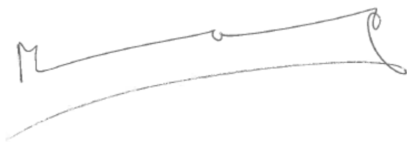
DECLARATION

I, **Maxwell Mewa Ngongang**, declare that the contents of this thesis represent my own unaided work, and that the thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology and the Agricultural Research Council.



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ABSTRACT

Microbial spoilage has been reported in various food products and this has led to increased food, fruit and beverage losses, thereby threatening economic growth, food safety and security. Furthermore, statistics have shown that more than 30% of agricultural produce in developing countries, mostly in Africa, is lost owing to microbial spoilage.

Beverages, food and fruits are predominant contributors to the South African export market. In recent years, contamination of these products resulting in spoilage has been a problem, although partial spoilage control has been achieved using chemical preservatives such as dimethyl dicarbonate, sodium benzoate, potassium sorbate, and sulphur dioxide (SO_2). However, prolonged exposure to these chemical preservatives can cause human health problems such as skin and/or eyesight damage, muscle and stomach pain, cardiovascular disease and the impairment of brain function. To mitigate such health concerns, biologically benign alternatives are deemed suitable, providing the rationale for this study.

Non-*Saccharomyces* yeasts have been found to secrete extracellular compounds with antimicrobial activity, herein referred to as biopreservatives. The first phase of this project was to isolate, identify and characterise microorganisms occurring in grape must and spontaneous fermentation with the potential to produce antimicrobial compounds effective against spoilage organisms such as *Brettanomyces*, *Zygosaccharomyces, Schizsaccharomyces, Kluyeveromyces* and *Candida* species, found in fermented beverages in which the primary ingredients are agricultural produce; and *Botrytis cinerea*, *Colletotrichum acutatum*, *Penicillium expansum* and *Monilinia laxa*, found on fruits.

Several organisms were isolated from spontaneously fermented wine and grape berries. These yeasts were identified using appropriate molecular biology techniques. The yeasts were then screened for antimicrobial activity against *Brettanomyces*, *Zygosaccharomyces, Schizsaccharomyces, Kluyveromyces* and *Candida* species by using seeded agar plates. An isolate, identified as *Candida* sp., was chosen for biopreservative production since it exhibited growth inhibition characteristics against the selected spoilage organisms. Furthermore, the results of the screening showed that the *Candida* sp. selected for biopreservative production managed to inhibit the spoilage of apples (*Malus domestica*) infected by fruit pathogens, *Botrytis cinerea* and *Colletotrichum acutatum*. The crude by-

products of the isolate also showed antimicrobial activity towards *Candida guilliermondii* and four other species of *Brettanomyces* assessed.

Additionally, the crude biopreservatives' efficacy was further evaluated under different pH and temperatures, with results showing that a pH and temperature of 4 and 15℃ respectively are conditions under which the activity of the biopreservatives was at a maximum; however the results obtained at this stage needed to be optimised using an appropriate methodology, such as the Response Surface Methodology (RSM) coupled with the Central Composite Design (CCD). For the application of such an optimisation methodology, the fermentation must be well understood and described by appropriate bioprocess models to determine influential process parameters.

Since the application of mathematical models in biological systems is of importance, as it allows for improving, optimising and predicting the production of products of interest, the process parameters obtained can be used to control fermentation in future. Furthermore, the modelling of bio-product production is key to designing and controlling industrial-scale manufacturing of biological products, such as biopreservation compounds from yeasts, to address the critical need for the use of safer preservation compounds in food, fruits and beverages.

To achieve this aim, the second phase of this study was developed to study the role of different nutrient media, subsequent to the determination of growth and production kinetics, to select suitable production media and to generate mathematical models that would describe the effect of temperature and pH on the production of biopreservation compounds as well as the optimum production condition based on temperature and pH. To model the activity against spoilage organisms, thus biopreservative compound production, *Candida guilliermondii* was selected as the spoilage organism during product-related kinetic parameter evaluation. The biopreservatives were produced under a single-stage batch system using the selected fermenter, i.e. *Candida* sp., with the inoculum being 1×10^6 cells/mL using 150 mL of yeast peptone dextrose (YPD), yeast peptone (YP), yeast dextrose (YD) and peptone dextrose (PD) at 25℃ at a rotary speed of 150 rpm. The concentration of each nutrient medium component, i.e., YPD, YP, YD, and PD, was prepared according to the manufacturer's instructions.

The results showed that the maximum production of biopreservation compounds occurred at 21, 19, 17 and 23 hours at a cell concentration of 1.25, 1.07, 0.90, 0.40×10^9 CFU/mL in YPD, YP, YD and PD respectively, when the maximum specific growth rate was

0.44, 0.38, 0.54, 0.31 h^{-1}. The maximum volumetric zone of clearance, i.e., the volume of clearance per mL of the biopreservation compound used, and the maximum achievable volumetric productivity, i.e., the rate at which the biopreservation agents were produced for all nutrient media, were 1.05 (YPD), 0.19 (YP), 0.77 (YD), 0.96 (PD) L/mL; and 0.086 (YPD), 0.043 (YP), 0.074 (YD), 0.037 (PD) L/mL.h, respectively.

The first section of phase two allowed for the determination of the best fermentation medium among the media tested. It was observed that the YPD medium appeared to be suitable as it yielded the highest volumetric zone of clearance. The information found in the literature demonstrated that temperature and pH are critical parameters to be considered when producing antimicrobial compound from non-*Saccharomyces* yeast, which also concurred with results obtained in this study whereby temperature and pH effects on biopreservation activity and stability were assessed. It was therefore important to investigate the optimum production conditions of the biopreservation compounds, primarily focusing on temperature and pH as independent variables. This was achieved by using 13 statistically designed experimental runs with a CCD on Design-Expert® software. The dependent variable was the biopreservation compound production, observed by the productivity in L/mL. The results obtained showed that the optimum production conditions were 22.5ºC at a pH of 5.

The supernatant containing the biopreservation compounds was fractionated by gel filtration (SEC) and ultrafiltration using an Amicon® ultrafiltration device with a 50kDa MW cut-off size. The SEC results showed that the biopreservative compound size was between 10 and 150 KDa. The ultrafiltration results showed that the filtrate had a 23.3% lower activity than the retentate, suggesting that there might be numerous compounds of interest produced.

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- And lastly, but most importantly, God Almighty, God of our ancestors, for the gift of life and the wisdom to have come this far.

M. Mewa Ngongang

March 2016

DEDICATION

I dedicate this thesis to all men, women and children, who in one way or another, have sacrificed their lives, time, resources or anything else to contribute to the advancement of our beloved continent of Africa to create a better world for all.

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GLOSSARY

Abbreviations/Symbols	**Definition (units)**
°C	Degree Celsius
µ	Specific growth rate (h^{-1})
μ_m	Maximum specific growth rate (h^{-1})
A	Area (cm^2)
A	Coded variable for temperature (ºC)
AAB	Acetic Acid Bacteria
ANOVA	Analysis of variance
ARC	Agricultural Research Council
B	Coded variable for pH
CCD	Central Composite Design
CFU	Colony-Forming Unit
CV	Coefficient of variance
D	Resulting diameter (cm)
D_0	Total diameter (cm)
DI	Disease Incidence
F	Fisher
GC	Gas Chromatography
H	Height (cm)
HPLC	High-Performance Liquid Chromatography
K_S	Substrate saturation constant (g. mL^{-1})
LAB	Lactic Acid Bacteria
LD	Lesion Diameter (mm)
m	Cell maintenance parameter (h^{-1})
MIC	Minimum Inhibitory Concentration (g.L^{-1})
MS	Mass Spectrophotometry
MWCO	Molecular weight cut-off (kDa)
n	Leudeking–Piret constant (L.CFU^{-1} h^{-1})

NCBI	National Center for Biotechnology Information
P	Productivity (L. mL^{-1})
PCR	Polymerase Chain Reaction
PD	Peptone/Dextrose
P_f	Final Productivity (L.mL^{-1})
P_m	Maximum Productivity (L.mL^{-1})
P_o	Initial Productivity (L. mL^{-1})
r	Radius (cm)
R^2	Correlation coefficient/Coefficient of determination
rDNA	Ribosomal Deoxyribonucleic Acid
rpm	Revolutions per minute (rev/min)
r_s	Substrate utilisation rate (g. mL^{-1} h^{-1})
RSM	Response Surface Methodology
r_x	Cellular growth rate (CFU. mL^{-1} h^{-1})
S	Limiting substrate concentration (g mL^{-1})
SEC	Size Exclusion Chromatography
S_o	Initial substrate concentration (g mL^{-1})
t	Time (h)
T	Temperature (ºC)
TLC	Thin-Layer Chromatography
V	Volume (L)
X	Cell concentration (CFU. mL^{-1})
X_f	Final cell concentration (CFU mL^{-1})
X_i	Input variable (Units dependent)
X_j	Input variable (Units dependent)
X_m	Maximum cell concentration (CFU mL^{-1})
X_o	Initial cell concentration (CFU. mL^{-1})
Y	Response variable (L/mL)
YD	Yeast/Dextrose
YP	Yeast/Peptone
$Y_{p/s}$	Productivity yield coefficient L.g^{-1} (Litter volumetric zone of clearance per gram of

	substrate)
YPD	Yeast/Peptone/Dextrose
$Y_{x/s}$	Biomass yield coefficient (CFU/g substrate)
α	Distance of each axial point from the centre in a CCD
β_0	Intercept of response variable (constant)
B_i	Linear coefficient corresponding to factor X_i
B_{ii}	Quadratic coefficient corresponding to factor X_{ii}
B_{ij}	Interactive coefficient corresponding to factors X_i, X_j.
ε	Random error
π	Numerical value of pi (3.14)

Chapter 1

General Introduction

CHAPTER 1
INTRODUCTION

1.1 Introduction

The sustainability of the South African economy is based on several factors, of which the export market of fruit and fermented beverages is the major contributor. The control and maintenance of high-quality and safe products for export, as well as for local consumption, are therefore of utmost importance. It has been reported that more than 30% of food products and beverages in developing countries, including South Africa, is lost owing to microbial spoilage (FAO et al., 2013; Lipinski et al., 2013; Miller & Welch, 2013). To address this problem, chemical preservatives such as sulphur dioxide (SO_2), dimethyl dicarbonate, benzoate, and sorbate salts, among others, are used. These preservatives have been determined to pose health problems such as skin and/or eyesight damage, muscle and stomach ailments, cardiovascular diseases and impairment of brain function. Furthermore, export regulations have been stringent on exporters to reduce and eradicate the use of refined chemicals as preservatives. It is therefore necessary to seek alternative preservatives.

In recent years, yeasts have been investigated for their potential to produce antimicrobial compounds that could be used for food, fruit and beverage preservation. Therefore, the use of biological compounds as biopreservatives seems to be a promising option to eradicate the use of synthetic chemical compounds.

Some yeasts and bacteria have been found to secrete antimicrobial compounds. These compounds are protein based and are lethal to receptive cells of spoilage organisms. These biopreservatives have been determined to have antagonistic effects against spoilage organisms in numerous products, including dairy products. Furthermore, to control microbial spoilage in fermented grape must, for example, few antimicrobial compounds have been identified and purified (Piano et al., 1997; Comitini et al., 2004a; Saravanakumar et al., 2008; Ciani et al., 2010; Mehlomakulu et al., 2014; Oro et al., 2014; Parafati et al., 2015). However, the aforementioned discoveries have not yet addressed the need for using the produced biopreservatives on a large scale in the food and fermented beverage industries. The focus of this study was to investigate the production of antimicrobial compounds, i.e., biopreservation compound production, which could be used by the fermented food/beverage

industries. Since the long-term aim of the project is to develop biopreservative products for the fermented food and fruit industries, it was logical to bioprospect organisms from spontaneous fermentations, in particular focusing on yeasts that facilitate such fermentations.

Although significant progress has been made in discovering the potential that yeasts have as producers of biopreservation agents, some aspects of these discoveries have not been investigated. In this study, productivity kinetics and mathematical modelling of the fermentation process, i.e., the evaluation of fermentation parameters of biopreservation compound production was studied.

1.2 Research questions

From the challenges raised above, the following research questions were of considerable interest:

- Which yeasts are capable of producing compounds which can inhibit the growth of spoilage organisms?
- How effective will the compounds be in controlling beverage and fruit spoilage organisms?
- What are the kinetic and mathematical modelling parameters which can best describe the biopreservation compound-production process using a single-stage bioreactor?

1.3 General objectives

The experiments were divided into two phases to find a suitable isolate which could be used to produce the biopreservation agents (Phase 1), while Phase 2 focused on the production of the compounds interest as shown below.

Phase 1: Aim 1: Isolation, identification and characterisation of yeasts from a South African environment with the ability to produce biopreservation compounds. To achieve this aim, the study focused on the following objectives:

Objective 1: Develop a profile of microorganisms from grape must at different stages of spontaneous wine fermentation.

Objective 2: Assess which molecular biology methods are best suited to differentiate effectively isolates of the same species occurring at different stages of the spontaneous wine fermentation for better identification and subsequent selection of appropriate isolates for further study.

Objective 3: From organisms profiled at different stages of the spontaneous wine fermentation, identify dominant species which exhibit antimicrobial compound secretion, and subsequently.

Objective 4: Select a species that will be used in a fermentation process to produce biopreservatives by using a suitable bioreactor.

Phase 2: Aim 2: To ascertain microbial kinetics, biopreservative production, and stability under varying pH-temperature conditions including application of biopreservatives produced by the species selected from Aim 1 (Objective 4). To achieve this aim, this part of the study focused on the following objectives:

Objective 1: To use the selected fermenter (isolate) in a process to produce biopreservatives using different media (YPD, YP, YD, PD) at room temperature and at a predetermined pH, assessing fermentation process parameters using appropriate kinetic models and the fermentation period at which the biopreservatives exhibit maximum activity.

Objective 2: To obtain crude extracts fermented using the best carbon source determined to be effective in Objective 1 (Aim 2) from the fermentation at an appropriate time, i.e., a time for which the biopreservatives exhibit the highest activity, in order to assess the stability of the biopreservatives when used under different temperature and pH.

Objective 3: Apply the fermentation extracts produced using the best carbon source to determine the effectiveness of the biopreservative produced against spoilage microorganisms growing in grape juice and on fruits to assess efficacy, subsequent to the preliminary determination of the size of the biopreservative compounds.

Aim 3: Optimisation of fermentation conditions for effective biopreservative production using Response Surface Methodology (RSM) and to determine the molecular weight cut-off MWCO size of biopreservation agents using Size Exclusion Chromatography (SEC). To achieve this aim, the primary objectives were the following:

Objective 1: To assess, primarily, the production of the biopreservatives under varying temperature and pH to determine suitable fermentation conditions.

Objective 2: To interpret the experimental data to generate the mathematical model suitable for optimal biopreservation production.

Objective 3: To recover fermentation products, i.e., biopreservation, by fractionating aliquots to determine the size of the biopreservation agents and the activity of each fraction recovered.

1.4 Significance of the study

In this research study, non-*Saccharomyces* yeast, *Candida* sp., was able to control fruit spoilage caused by *Botrytis cinerea* and *Colletotrichum acutatum*. The *Candida* sp. was also found to produce biopreservation compounds active against fermented beverage spoilage organisms, i.e., *Candida guilliermondii* and *Brettanomyces bruxellensis*. Furthermore, the fermentation kinetic parameters, the fitting of experimental data using several models, and the development of a suitable mathematical model from optimisation experiments, allowed for better analysis and understanding of biopreservative production from non-*Saccharomyce*s yeast using a single-stage bioreactor.

1.5 Delineation of the study

In this study, the following aspects were not studied:

- The screening of bacteria and *Saccharomyces cerevisiae* for antimicrobial activity.
- The mechanism of action of the biopreservation compounds produced by the *Candida* sp. was not studied.
- The investigation of whether the inhibition was due to nutrient competition or other factors.
- The elucidation of the biopreservative protein composition.

OUTLINE OF THE THESIS

The research presented in this thesis was conducted at ARC Infruitec/Nietvoorbij (Fruit, Wine and Vine Institute of the Agricultural Research Council), Stellenbosch, South Africa, in collaboration with the Bioresource Engineering Research Group, Department of Biotechnology, Cape Peninsula University of Technology, South Africa.

The thesis comprises seven chapters.

Chapter 1: Gives a brief introduction to the thesis and objectives of the study, and provides an outline of the thesis.

Chapter 2: Contains the literature review.

Chapter 3: Covers the isolation, identification and characterisation of yeasts of interest.

Chapter 4: Involves antimicrobial activity screening, yeast growth and productivity kinetics of the biopreservation compounds.

Chapter 5: Focuses on the general discussion, conclusions and recommendations.

Chapter 6: List bibliographical references consulted for the research, and

Chapter 7: Covers the Appendices, whereby details regarding calculations and other supplementary information, is listed.

Chapter 2

Literature review

CHAPTER 2
LITERATURE REVIEW

2.1 Introduction

Yeasts are widespread in nature and are found in soil, on the surface of vegetables and fruit berries and in the digestive tracts of animals (Ribéreau-Gayon et al., 2006). The microbial community found on grape berries and in grape must comprises bacteria, fungi and yeasts, which are distributed irregularly on grapevines (Ribéreau-Gayon et al., 2006; Bezerra-Bussoli et al., 2013; Wang & Liu, 2013; Ghosh et al., 2015). These microorganisms are beneficial in many biological processes, e.g., they are used in the conversion of glucose to ethanol, malic acid to lactic acid, and in flavour and aroma development in the final products (Jolly et al., 2006; Ribéreau-Gayon et al., 2006).

Encountering undesirable microorganisms at certain stages of any fermentation can result in spoilage and off-flavours. In other words, contamination by unwanted organisms during the production of fermented food products can be economically detrimental, leading to wastage. Several compounds secreted extracellularly by non-*Saccharomyces* yeasts have been identified. These compounds affect the texture, taste and aroma of beverages. However, some of these organisms have been determined to produce antimicrobial compounds (Comitini et al., 2004ab; Saravanakumar et al., 2008; Ciani et al., 2010; Oro et al., 2014; Mehlomakulu et al., 2014; Parafati et al., 2015).

Globalisation of the beverage industry leads to product exchanges between countries through import and export. This can result in some microbial strains becoming dominant in localities in which they do not necessarily exist. Several yeasts and bacteria have been reported to contaminate and cause spoilage in the food and beverage industries. *Dekkera*/*Brettanomyces, Zygosaccharomyces bailii, Candida* and *Hanseniaspora* species were found to be the dominant species causing spoilage in some beverages (Du Toit & Pretorius, 2000; Comitini et al., 2004ab; Mehlomakulu et al., 2014). For example, *Brettanomyces*, specifically, causes spoilage towards the end of red wine fermentation and also during ageing in barrels, while in sweet wine and grape juice the presence of *Zygosaccharomyces bailii* can lead to undesired fermentation (Du

Toit & Pretorius, 2000; Comitini et al., 2004ab; De Ingeniis et al., 2009; Zuehlke et al., 2013). In grape juice, *Pichia guilliermondii* (anamorph: *Candida guilliermondii*) may produce large amounts of volatile phenols, which could result in wine spoilage (Sáez et al., 2010). To mitigate this, preservatives can be used.

2.2 Why do we preserve food?

Food products are a source of nutrients for humans; however food, fruits and beverages are also a suitable milieu for the proliferation of microorganisms (Willey et al., 2008). Food commodities are produced to be able to be consumed within a specific time without deterioration. The food needs to be kept safe and free from spoilage microorganisms, regardless of whether it is perishable, semi-perishable or shelf-stable food, or a beverage. Besides food production for local consumption, there is a need for quality control and maintenance to ensure that the food produced meets global exportation market requirements and has a long shelf life, stability and safety. Generally food products are rated based on stability and safety for consumption, which indicates that the products are free from unwanted microorganisms and certain compounds. The safety requirements of a specific food or beverage, depends on its classification. Therefore, different preservation methods for food and beverages have been developed (Cherrington et al., 1991; Brul & Coote, 1999; Du Toit & Pretorius, 2000; Soliva-Fortuny & Martín-Belloso, 2003; Okafor, 2007; Gould, 2012).

2.3 Preservation methods

2.3.1 Chemical preservatives

The proliferation of unwanted microorganisms in products made for human consumption is a serious concern, because it can either lead to product spoilage or can cause health problems. Compounds with antimicrobial properties are used in food, fruits and beverages to inhibit the growth of unwanted organisms, which could be yeasts, bacteria, bacteriophages or fungi. In wine, for example, sulphur dioxide (SO_2) is the most common chemical preservative used at

different concentrations, depending on the spoilage organism targeted (Du Toit & Pretorius, 2000; Okafor, 2007; Mehlomakulu et al., 2014).

Organic acids have also been used as chemical preservatives in the food industry. These acids are benzoic, lactic, sorbic and acetic acid, which act against a variety of microorganisms. It is important to mention that these acids are also effective against spore germination in some environments (Sofos & Busta, 1981; Brul & Coote, 1999). The mechanism of growth inhibition by organic acids includes the disruption of cell membrane or the suppression of some key biochemical reactions. Furthermore, there can be a build-up of inhibitory anions in products, which can facilitate homeostasis stress. When cells are under homeostasis stress, they tend to attempt to restore the balance of a homeostatic environment; while doing this, the organism uses considerable energy that leads to the depletion of the energy required for growth and some metabolic functions. If the energy required for growth is not available, it then results in cell death (Salmond et al., 1984; Eklund, 1985; Bracey et al., 1998).

The long-term exposure to these artificial preservatives has not yet been addressed. These chemicals can be used in small doses; however some spoilage organisms are resistant to the maximum allowable limit for human consumption, which then requires a higher dose of the preservatives. This then poses health concerns. Furthermore, the use of artificial chemicals as spoilage control tools is deleterious. For example, although some of these compounds can reduce the development of spoilage organisms such as *Brettanomyces bruxellensis* in wine, others such as benzoic acid affect the flavour of the final product. Given that wine is a highly controlled product, the taste and aroma should be kept as natural as possible; therefore the use of compounds such as benzoic acid is restricted in wine (Benito et al., 2009).

Salting, curing and smoking are some other procedures also applied to preserve food products (Brul & Coote, 1999). These procedures rely on antimicrobial compounds, i.e., sodium chloride, nitrites and phenolic acids. As in the case of other chemical preservatives, their consumption poses health problems such as heart and respiratory diseases as well as some allergic reactions (Mehlomakulu et al., 2014).

It is important to note that preservatives are used to target specific spoilage organisms that respond differently at different preservative concentrations. When targeting a specific organism during food preservation, the preservative is used at a specific concentration called the

minimum inhibitory concentration (MIC). The MIC dictates the legislated or permitted level of the compound to be used as a preservative in food. This means if a microorganism becomes resistant to the permitted concentration of a specific chemical compound, preservation by using that chemical compound is limited, because for the compound to be effective, it needs to be at a concentration higher than the legislated level, which poses serious health concerns as previously mentioned.

2.3.2 The use of physical methods

The physical methods of food preservation consist of the use of high temperature through pasteurisation, the control of water content, cold processing, ultrasound processing, filtration and irradiation (Chemat et al., 2011; Santos et al., 2012). Among these physical methods, none of them possesses soluble antimicrobial compounds, and therefore thermophiles, spores, psychrophiles and xerophiles can survive these procedures. It is important to note that these preservation procedures cannot be used for all types of food, fruits and beverages (Leistner, 1999; Farkas, 2007).

2.3.3 Old problem, new solutions

Food preservation methods have advanced and can be grouped as old or modern techniques. Old methods of food preservation consist of drying, refrigerating and fermenting. On the other hand, what can be termed as modern methods include irradiating, freezing, and canning, as well as the use of specific artificial chemicals. The principles of food preservation can be categorised (Leistner, 1999; Gould, 2012). Preservation of food can consist of removing, slowing or inhibiting the growth of unwanted microorganisms. Food preservation can also consist of inactivation of endogenous enzymes, thereby presenting or delaying some unwanted chemical reaction in the food. In recent years, there have been concerns regarding the use of safer, more cost-effective and convenient methods as well as bioactive compounds for food, beverage and fruit preservation. The use of microorganisms and their products has been investigated as a promising alternative to the use of artificial chemicals.

Yeasts secrete proteinaceous compounds and mycotoxins capable of inhibiting the growth of other yeasts (Ozhovan et al., 2001; Marquina et al., 2002; Baeza et al., 2008; Mehlomakulu et al., 2014; Robledo-Leal et al., 2014). Yeasts with microbial growth inhibition characteristics present a significant step in the fermentation industry. Furthermore, these yeasts can also be used for treatment of some infections caused by fungi on the skin. Given that yeasts are easy to handle and manipulate, they can be used in biological processes and mainly as biocontrol agents or sources of antimicrobial compounds (Bar-Shimon et al., 2004; Pimenta et al., 2009; Mehlomakulu et al., 2014).

Some non-*Saccharomyces* yeast produce growth-inhibiting compounds, but not all these compounds are proteinaceous compounds. For example, pulcherrimic acid that is produced by *Metschnikowia pulcherrima* acts as the precursor for pulcherrimin pigment that reduces the iron concentration in a growth medium. Owing to the requirement for the electron transfer reaction and as cofactors to key metabolic enzymes, it is clear that when iron levels are depleted, this results in the growth inhibition of other yeasts (Oro et al., 2014). These findings by Oro et al. (2014) can be used in the control of fruit spoilage organisms such as *Botrytis cinerea*, *Alternaria alternate* and *Penicillium expansum*. This is achieved when ferric ions are deprived of the medium, which then negatively affects the evolution of fungal pathogens.

Furthermore, *Candida pyralidae*, *Candida tropicalis*, *Debaryomyces hansenii*, *Kluyveromyces phaffi*, *Pichia anomala, Kluyveromyces wickerhamii* and *Pichia fermentas* have also shown potential to produce various antimicrobial compounds effective against spoilage fungi, bacteria and yeasts in wine and dairy products (Piano et al., 1997; Comitini et al., 2004ab; Saravanakumar et al., 2008; De Ingeniis et al., 2009; Hatoum et al., 2013; Mehlomakulu et al., 2014; Oro et al., 2014). During a study of the biopreservation activity of yeast and lactic acid bacteria in a wine, Mendoza et al. (2010) found that *C. pulcherrima*, with its low molecular weight metabolites, has the ability to exert inhibitory effects on acidification acetic bacteria.

In addition, *C. tropicalis*, *Debaryomyces hansenii, P. fermentans* and *P. anomala* were reported to have significant inhibitory effects against pathogenic bacteria *Listeria ivanovii* HPB28 (Hatoum et al., 2013), which further emphasised the antimicrobial potential that non-*Saccharomyces* yeasts may have against other microorganisms. This could be of significant importance and contribute to the reduction and/or eradication of synthetic chemicals usage as preservatives in beverages and food products.

Yeast biopreservation compounds have been investigated at genetic and molecular level, and the traits of such compounds have been determined to be directly linked to chromosomal elements (Wickner, 1996; Weiler et al., 2002; Rodríguez-Cousiño et al., 2011).

The investigation of the mode of action of these biopreservatives has also been researched (İzgü et al., 2006; Comitini et al., 2009; Muccilli et al., 2013; Muccilli & Restuccia, 2015). The findings showed that in fungi the reduction mechanism is characterised by the suppression of β-glucan synthesis or hydrolysis of β-glucan in the cell wall of spoilage organisms. Also, the blocking of dividing cells by stopping the synthesis of DNA. The other mode of action found was described by Klassen and Meinhardt (2002), Klassen et al. (2008), and Brown (2011), indicating the cleavage of the tRNA as well as the interference with the uptake of calcium and the formation of ion-leaking channels on the cells' cytoplasmic membrane.

In some cases, the antimicrobial compounds found in yeasts can be classified as killer toxins, which are extracellular proteins or glycoproteins that act on the cell by disrupting the function of the membrane (Comitini et al*.,* 2004ab; Mehlomakulu et al*.,* 2014; Robledo-Leal et al*.,* 2014). Dieuleveux and Guéguen (1998) attributed the antilisterial activity of a *Candida* strain to two organic acids found to be D-3-phenyllactic and D-3-indollactic acid. These acids were found to have stability over a wide pH range as well as resistance to high temperatures up to 120 °C for 20 minutes. In the work conducted by Cavalero and Cooper (2003), it was demonstrated that *C. bombicola* produced extracellular glycolipids called sophorosides that have the ability to inhibit the growth of *Staphylococcus aureus* and *C. albicans*. Mehlomakulu et al. (2014) also found two toxins, named CpKT1 and CpKT2, produced by *C. pyralidae* against *Brettanomyces bruxellensis*. These authors discovered that the toxins were stable at a temperature range between 15 and 25 °C and pH between 3.5 and 4.5, which favours wine-making conditions. These authors also assessed the effect that the sugar and ethanol concentration has on the stability and efficacy of the growth inhibition compounds. They concluded that sugar and ethanol concentration did not have a high significant effect with regard to the efficacy of the toxins. There are great advantages in isolating the antimicrobial compounds that are produced. It is also important to know the stability of specific biopreservation agents or compounds under different conditions, before they can be applied to food products.

Many researchers also attribute the antimicrobial property of some yeasts to nutrient competition in a carbon-rich milieu. The control of *Penicillium expansum* on fruits was achieved

by *Candida sake* and *Candida guilliermondii* as reported by Nunes et al. (2001), and Scherm et al. (2003). On the other hand, some of the activity is also characterised by the ability to secrete cell wall degrading enzymes such as protease, exo-β-1, 3-glucanase and chitinase (Robledo-Leal et al., 2014). Table 2-1 highlights numerous studies conducted to determine biopreservation compound production, compound sizes, their mechanism of action and the target spoilage organisms.

Table 2-1: Selected yeasts, their biopreservation agents, application, mechanism of action and the targeted organism (adapted from Mehlomakulu et al., 2014)

Yeast species	Biopreservative identity, molecular size, temperature and/or pH activity/stability	Application	Mechanism of action	Target yeast
***Candida* (formerly named *Torulopsis glabrata*)**	Unknown	Not determined	Damages the plasma membrane	*S. cerevisiae*
Candida pyralidae	CpKT1 and CpKT2(>50KDa) pH activity: 3.5 – 4.5 Temperature activity: 15 and 25 ºC	Grape juice	Unknown	*Brettanomyces bruxellensis*
Kluyveromyces wickerhamii	Kwkt (72kDa) pH activity: 3.8-4.6 optimum pH: 4.4 optimum temp: 25 ºC	Wine making	β-1,6-glucans receptor	*D. bruxellensis*
Tetrapisispora phaffii	Kpkt(33 kDa) pH activity: 3 – 5 Temperature activity:<40ºC	Wine making	Disrupts the integrity of the cell wall. Also shows β-glucanase activity	*H. uvarum*

Cont. **Table 2-1.**

***Williopsis mrakii* NCYC 500**	K-500(1.8-5.0kDa) pH stability: 2.4 – 4.0 Temperature activity: 30 ºC	Antifungal agent	Membrane permeability	*Candida albicans* and *Sporothrix schenkii*
***Pichia Acacia* (reclassified as *Millerozyma acaciae*)**	PaT (187 kDa: three sub-units of 110, 39 and 38 kDa) pH optimum activity: 7 – 7.5 and 5.3 – 6.6	Not determined	Cell cycle arrest in G1 phase in *S. cerevisiae* cells. Displays chitinase activity	*S. cerevisiae*
Hanseniaspora uvarum	18 kDa pH activity: 3.7 – 3.9	Not determined	β-1,6-glucans receptor	*C. albicans, Sporothrix* sp., *Schenkii* sp., *Heterobasidium* sp., *Postia* sp., *Serpula* sp. and *Fusarium* sp.
***Pichia anomala* DBVPG 3003**	Pikt (8 kDa) pH activity: 4.4 Temperature activity: 25 – 35 °C	Wine making	β-1,6-glucans receptor	*D. bruxellensis*

2.4 Biopreservative compound constituents

The production of antimicrobial compounds by non-*Saccharomyces* yeasts has been investigated following the natural growth environmental conditions in which these yeasts usually occur. The solvent extraction method was used in the work conducted by Stanbury et al. (1995), Ciani et al. (2010), and Hatoum et al. (2013), to extract the antimicrobial compound from the culture broth. After extraction, the proteolytic enzymes Pronase E and Trypsin were used to characterise the nature of the growth-inhibition compound.

Bar-Shimon et al. (2004) also used enzyme assays to specifically assess exo-β-1, 3-glucanase, chitinase and protease activity in the biopreservative extracts used in the study. Partial purification was done and the partially purified protein extract was then analysed using SDS gel electrophoresis. In the same work, the effect of the primary substrate and secondary metabolites, such as the carbon source and organic compounds, was investigated. The purified/identified compounds were tested on damaged fruits. In this study, it was demonstrated that the yeast *C. oleophila* was able to secrete protease, β-1, 3-glucanase and chitinase. Glucose and chitinase were found to be produced maximally at the early stages of growth, whereas the maximum production of protease was observed after a period of six to eight days. In addition to glucose, the production of the growth-inhibition compounds was stimulated by the presence of cell wall fragment action of *Penicillium digitatum* used.

2.5 Biopreservative compound production conditions

Bioactive compounds are produced based on specific conditions. The process can be seen as induced, stimulated by fermentation parameters such as primary substrate requirements, salt content, pH and temperature (Stanbury et al., 1995; Ciani et al., 2010). These parameters can then be used for response surface methodology characteristics of a specific microorganism and can assist in the design and modelling of the biopreservation production system. The other proteomic approach in protein identification is the use of matrix-assisted laser desorption ionisation (MALDI) mass spectrophotometry, which is currently used in numerous research studies to identify the protein constituents on fermentation fractions which exhibit antimicrobial

activity (Dong et al., 2010; Mateos et al., 2012; Niyompanich et al., 2014; Robledo-Leal et al., 2014).

In the study of Robledo-Leal et al. (2014), the biopreservative behaviour within the *C. parapsilosis* complex and the biopreservative activity of *C. metapsilosis* strains were found to occur at 25 °C. Spoilage organisms used were of clinical origin. The growth temperature played a crucial role in biotoxin production, and the optimal production temperatures ranged from 15–20 °C. In the same study, it was also discovered that few biopreservatives were found to still be active at temperatures above 30 °C.

Most of the research done in this field has been quite similar, with differences being that different organisms and environments have been studied. When the antimicrobial compound and/or its activity have been determined, there are still some limitations because there is no detailed breakdown of the molecule; as such, the composition of the antimicrobial compounds cannot be established and studied properly. Hou et al. (2014) were able to establish the total protein composition of the biomolecule they managed to produce and identify. However, in the field of biopreservative production, that kind of approach is still not exploited. Although biopreservatives and other bioactive compounds are identified and partially characterised, usually the estimated size of the protein is given, which still presents a gap in the application of the protein as a biocontrol agent. Whereas if the identified proteinaceous compounds could be fractionated and tested, this could give a clearer understanding of the peptide and polypeptide composition of any proteinaceous biopreservative compound as well as its broader applications.

2.6 Microbial growth kinetics, optimisation and recovery of fermentation products

Cells are fundamental units of life (Mrwebi, 2004; Willey et al., 2008). Several chemical and biochemical reactions usually occur in cells. Therefore, understanding the physiology of cells in biochemical processes is important, as this is used to design fermentation processes where specific designs could be easily controlled and predicted (Doran, 1995; Stanbury et al., 1995; George et al., 2015; Pérez-Través et al., 2015; Weaver et al., 2015). The use of yeast cells has been applied extensively in the history of humankind. They have been used as food producers and food preservatives in beverage and fruit processing. The products from yeast cells

consist of, but not limited to single cells, primary and secondary metabolites such as organic acids, antimicrobial compounds and enzymes. It is therefore necessary to determine and model (mimic) bioreactor performance parameters which influence their production (Mrwebi, 2004).

In biosynthesis, cells consume nutrients to grow and produce more cells and added-value compounds. It is important to monitor and understand the growth kinetics of the primary producer used to produce fermentation compounds along with characteristics of such compounds before the isolate can be used in a large-scale fermentation process. Therefore, quantification of substrate consumption, biomass and product yield in the fermentation medium is essential for such studies, in order to understand process dynamics. Furthermore, different mathematical and process engineering models have been used to quantify such process dynamics (Doran, 1995; Stanbury et al., 1995; Honqi et al., 1998; Wang et al., 2009; Chang et al., 2014).

Models are often classified as segregated and non-segregated (Lobry et al., 1992; García-Ochoa & Casas, 1999; Guardia & Calvo, 2001; Mrwebi, 2004; George et al., 2015). Segregated models in biotechnological processes classify each cell independently; however non-segregated models focus on the plethora of identical cells in the same process at a specific time. In other words, this consideration takes into account the total number of cells as a pure species in a fermentation nutrient medium. Furthermore, it is important to understand metabolic functions and some physiological pathways used by the microorganisms. The models used to understand such phenomena incorporate biochemical, morphological and genetic attributes to understand the cells' physiology (Liao et al., 1988; Kayombo et al., 2003).

Additionally, there are structured and unstructured models. Some of these models focus on the total biomass as identical organisms without focusing on internal cellular functions, i.e., structured models. The unstructured models consider growth kinetics, substrate utilisation and product formation as easily assessable parameters in biotechnological processes (Majewski & Domach, 1990; Coons et al., 1995; Mrwebi, 2004). As highlighted in Table 2-2, and focusing on the scope of the kinetics study in this research, the Monod's model appears suitable to use in this study. This is because other models were derived from the Monod's model, taking into account various fermentation operational conditions and the functioning of cells during the fermentation. Overall, the fermentation parameters sought to describe or determine rate constants can be easily quantified using Monod's model, e.g., the substrate utilisation and the growth-rate constants.

Table 2-2: Unstructured models used in modelling of biotechnological processes (adapted from Mrwebi, 2004)

Model	Description	Representation
Blackman (1905)	In this model, there is an assumption that the rate of substrate utilisation is proportional to substrate concentration when the substrate concentration is low. Whereas the utilisation and growth rate are independent of substrate concentration when the concentration of the substrate is high. Furthermore, when the substrate concentration goes beyond the half saturation constant, the model gives a sharp transition from first order to zero order, meaning that the function is not smooth.	$\mu = \frac{\mu_{max} S}{K}$ if $S<K$ $\mu = \mu_{max}$ if $S \geq K$
Monod (1942)	This is most widely used. This model satisfactorily describes and quantifies parameters such as growth-controlling substrate, which indicates that a specific quantity of substrate can yield a specific quantity of biomass and the required compounds. Furthermore, the microbial culture is defined by a limited growth constant.	$\mu = \frac{\mu_{\max} S}{K_S + S}$
Haldane (1930)	As in other models, and in addition to substrate limitation, the concept of substrate and product inhibition kinetics is looked at in this model.	$\mu = \frac{\mu_{max}}{K_s + S + S^2/K_i}$
Tessier (1942)	This is an experiential model describing the growth rate specifically in the case of continuously supplying a known substrate concentration to a fermentation system. As in the Monod's model, the saturation constant can be determined; however in the Tessier model, the transition to the saturation constant is highly pitched.	$\mu = \mu_{max}(1 - e^{K_T} S)$
Moser (1958) and Contois (1959)	These two models were derived from Monod's equation. As is the case with Monod's model, they can also be applicable in experimental data fitting and analysis of fermentation process parameters.	$\mu = \frac{\mu_{max} S^n}{K_s + S^n}$ $\mu = \frac{\mu_{max} S}{K_s X + S}$

***Cont.* Table 2-2.**

Model	Description	Representation
Logarithmic by Westerhoff et al. (1982)	As proposed in this case, as in the case of Monod's model, this model also explains the scenario of specific growth rate as a continuous function of the concentration of the growth-limiting substrate present; however the transition to saturation concentration is less sharp than in Monod's. This logarithmic model establishes its identity based on the growth rate as the function of a logarithmic single limiting substrate available. Because of these considerations, this model does not depict the maximum specific growth rate resulting from the absence of a saturation constant. In addition, when the amount of substrate is very low, this model would predict a negative growth rate, whereas the generally used kinetic models describing cell growth usually yield a positive growth rate.	$\mu = a + b \ln(S)$ (a and b are parameters of the model)

2.6.1 Kinetics and stoichiometric parameters during production of extracellular compounds

For many process engineering applications, phenomena such as the growth rate or inhibition rate can be described satisfactorily with known and/or quantified parameters such as growth-controlling substrate utilisation rate to indicate that a specific quantity of substrate can yield a specific quantity of biomass and required compounds. Furthermore, the term 'growth-limiting substrate' is also used to indicate that the specific microbial growth rate (μ) is dictated by the actual concentration of a particular substrate as described by the Monod equation (2.1).

$$\mu = \frac{\mu_{\max} S}{K_S + S} \Rightarrow \frac{dX}{dt} = \frac{\mu_{\max} SX}{K_S + S} \tag{2.1}$$

For a specified substrate, μ_{max} and K_S are constants for a given process in relation to the way the organism grows and uses the growth-limiting substrate. It also shows the growth rate of a specific organism under excess limiting substrate concentrations. The fermentation constant K_S specifies how fast the specific growth rate (μ) moves from 0 to maximum specific growth rate (μ_{max}) as the limiting substrate is being utilised (Doran, 1995; Stanbury et al., 1995).

Biopreservatives and other antimicrobial compounds have been found to be secreted based on the specific substrate utilised by the producing organisms. This means that the production of extracellular, proteinaceous compounds, in some cases, is usually media dependent (Fredlund et al., 2004; Walker, 2011; Mehlomakulu et al., 2014). The general biosynthesis of extracellular compounds, the microbial growth rate and the cell concentration have a direct relationship with the products formed during fermentation. The effect of secondary metabolites is also a very important aspect to study when quantifying the production of biopreservatives, because it is a requirement to know what the produced compounds become when they remain in the fermentation broth. It is worth noting that in biological processes and in the case of different production systems, even if cells do not grow, they require a metabolisable substrate and micronutrients for their maintenance. The need for cell maintenance consists of keeping the cells from degrading the compounds of interest or keeping the cells from intoxication by the produced compound of interest. During the analysis and modelling, Equations 2.2a, and 2.2b could assist in determining the substrate requirements for cellular maintenance.

$$S = K_S \frac{D}{\mu_{\max} - D} + S_{\min} \tag{2.2a}$$

$$\mu = (\mu_{\max} + m)\frac{S}{K_S + S} - m \tag{2.2b}$$

Other stoichiometric parameters involved in microbial kinetics are yields based on substrates, biomass and bioproduct production during any fermentation process as represented in Table 2-3. The antimicrobial compounds are to be produced and then used under different conditions based on their stability. The production system used in this case was a batch system using a single-stage bioreactor where the parameters to be quantified are as shown in Table 2-3. Even after the fractionation of proteinaceous compounds, these parameters could be fitted in a desired model in order to study production process effects while focusing on the most promising compounds as biopreservation agents.

Table 2-3: Stoichiometric parameters of microbial kinetics (Doran, 1995; Castro Martinez, 2007)

Parameter	Model	Units
Biomass yield	$Y_{X/S} = \frac{X_f - X_0}{S_0 - S_f}$	g biomass/g substrate
Product yield based on biomass formation	$Y_{P/X} = \frac{P_f - P_0}{X_f + X_0}$	g product/g biomass
Product yield based on substrate utilisation	$Y_{P/S} = \frac{P_f - P_0}{S_0 - S_f}$	g product /g substrate
Substrate utilisation rate	$r_s = \frac{dS}{dt}$	g/Lh
Biomass formation rate	$r_x = \frac{dX}{dt}$	g/Lh
Product formation rate	$r_p = \frac{dP}{dt}$	g/Lh
Microbial-specific growth rate	$\mu = \frac{1}{X}\frac{dX}{dt}$	h^{-1}
Microbial death rate	$k' = -\frac{1}{N}\frac{dX}{dt}$	h^{-1}

2.6.2 Response surface methodology (RSM) for biological process optimisation

The development of the best production conditions for biological products under a defined environment is the procedure called optimisation (Larmond, 1977; Hu, 1999; Cao et al., 2010; Galonde et al., 2013). The assessment of optimum production conditions is key to extracellular compounds' production. The use of mathematical modelling is a leading process to achieve optimisation of bioactive compound production (Nwabueze, 2010; He et al., 2012). Several approaches have been used to optimise the biological process to achieve optimum production conditions that are also cost effective. Response Surface Methodology (RSM) using a Central Composite Design (CCD) is one such method that is able to generate an efficient design that may successfully assist in bioprocess optimisation (Annor et al., 2010; Nwabueze, 2010; Xiao & Lu, 2014). The RSM approach was developed in the early 1950s. It was successfully applied in the chemical industries (Wang et al., 2006).

There has been increased use of mathematical modelling coupled with the adaption of RSM for biological process optimisation. Previous research was conducted using RSM and CCD for process optimisation in food and beverage industries where the factors looked at were product quality, sensory properties, nutritional qualities, shelf life, media condition, microbiological quality and product packaging (Ratnam et al., 2005; Iqbal et al., 2009; Sudheer Kumar et al., 2009; Valduga et al., 2009; Nwabueze, 2010). This approach also assists bioprocess engineers to predict, control and solve production challenges in the food and beverage industries by determining and observing some key independent factors that influence the magnitude of any dependent response $f(x)$, where x could be temperature or pH. In this approach, process variables such as temperature and pH are used with an initial aim of bringing a production process speedily and efficiently to the level of optimum production based on the influential variables (Mullen & Ennis, 1979). In essence, in the production of biopreservation compounds, such an approach is sensible as it allows for process analysis and prediction when the operational range of influential factors, such as temperature and pH, is known. Therefore, the system to be adapted for optimum production can result from the use of mathematical models generated from experimental data analysis and fitting. To some extent, larger and complex bioprocesses can be modelled and optimised using the above-mentioned approach.

The adaptation of RSM with CCD for bioprocess optimisation is usually based on actual experimental data. In such cases, depending on the nature of the experiment, a first or second

order response surface model could be appropriate to use. However in biopreservation production, as the case in this research, the second order response surface model would make provision for non-linear terms and could be evaluated for the analysed responses based on process variables. The use of CCD allows for experimental design which gives the efficiency with respect to the number of experimental runs needed (Wang et al., 2006; Montgomery, 2007; Myers et al., 2016).

The successful adaptation of RSM as a mathematical model for process optimisation is governed by several practical steps. These steps consist of the identification of independent variables, design of the statistically orientated experiments or model, followed by the estimation of the coefficients in the statistically designed experiment, the prediction of the response and then the adequacy check of the designed model. Another step consists of multivariate regression analysis as well as the interpretation of the resulting mathematical model, response surface and plots (Ratnam et al., 2005; Wang et al., 2006; Nwabueze, 2010).

2.6.3 Analytical methods for biomolecules separation and purification

For biotechnological processes, biomolecules of different nature and structure are usually found in the same mixture as a result of fermentation (Boyer, 2000). Chromatography is a technique used in industry for the separation and/or identification of components in a mixture. These compounds could be organic acids, enzymes, peptides, polypeptides, polysaccharides or proteins (Boyer, 1993; Boyer, 2000; Volmer et al., 2002; Blay et al., 2011).

The basic principles of chromatography are based on the fact that compounds in a mixture may have different structures and tendencies to adsorb onto a surface, flow through a column or dissolve in a solvent (Ismail & Nielsen, 2010; Guiochon & Trapp, 2012). To address these differences and depending on the molecule or compound of interest, several types of chromatography are used with different operating principles. They are paper chromatography, Thin-Layer Chromatography (TLC), gas chromatography, High-Performance Liquid Chromatography (HPLC), ion-exchange chromatography, and Size Exclusion Chromatography (SEC) which is also known as called gel filtration (Boyer, 2000; Ismail & Nielsen, 2010; Guiochon & Trapp, 2012). It is important to note that the advances made on the analytical

methods using a chromatographic approach have allowed the incorporation of mass spectrophotometry analysis on some of these types of chromatography. Examples are LC-MS, GC-MS or HPLC-MS for high analytical assessment (Bruins et al., 1987; Aebersold & Mann, 2003; Lisec et al., 2006).

All these types of chromatography have the same basic principles. They all require a stationary phase (static part) and a moving part called a mobile phase. The phenomena that actually govern all types of chromatography are based on physical processes such as adsorption, partitioning, ion exchange or molecular exclusion, which can then allow the chromatography techniques to be divided into two types characterised according to how solute compounds interact with the stationary phase. These are partition and adsorption chromatography. Partition chromatography involves the distribution of a solute between two liquid phases, whereas adsorption chromatography consists of the use of ion-exchange resin as a stationary phase and a liquid buffer as the mobile phase (Boyer, 2000; Ismail & Nielsen, 2010; Guiochon & Trapp, 2012).

With the basic principles behind chromatography techniques known, it is clear that the type of biological product in a broth dictates which chromatography method to use for effective separation of target molecules. Partition chromatography is widely applied in the separation and identification of fatty acid, amino acid and carbohydrates. However, for the separation or fractionation of macromolecules, such as nucleic acids and proteins, adsorption techniques are used. Accordingly, the information published in literature on growth inhibition compounds from yeasts indicated that a size exclusion or gel filtration chromatography would be applicable for the fractionation and identification of biopreservation compounds from the non-*Saccharomyces* yeasts.

Chapter 3

Isolation, identification and characterisation of yeasts from a South African environment with the ability to produce biopreservation compounds

CHAPTER 3

ISOLATION, IDENTIFICATION AND CHARACTERISATION OF YEASTS FROM A SOUTH AFRICAN ENVIRONMENT WITH THE ABILITY TO PRODUCE BIOPRESERVATION COMPOUNDS

3.1 Introduction

A spontaneous wine fermentation is a non-inoculated fermentation where the alcoholic fermentation is conducted by naturally occurring yeasts. A spontaneous fermentation is a complex process that involves the interaction between naturally occurring yeasts, bacteria and grape must (Fleet, 2003; Ribéreau-Gayon et al., 2006). The main driving force of a spontaneous wine fermentation is the metabolic activity of *Saccharomyces* and non-*Saccharomyces* yeasts. These yeasts facilitate the fermentation process by converting the sugar to alcohol and carbon dioxide as well as the production of secondary metabolites responsible for the taste and aroma of the final product (Ribéreau-Gayon et al., 2006; Willey et al., 2008). During spontaneous fermentations, apiculate yeasts, *Kloeckera*/*Hanseniaspora* are the predominant species at the beginning, but *Candida, Pichia and Hansenula* species that produce acetic acid and ethyl acetate also occur (Ribéreau-Gayon et al., 2006; Willey et al., 2008). These yeasts usually live in a competitive environment where some yeasts produce antimicrobial compounds to stop the growth of other microorganisms and to make the milieu favourable to themselves (Golubev, 2006; Mendoza et al., 2010). Some yeasts are called spoilage agents because they produce some extracellular compounds that negatively affect the quality and safety of the final product. Although rarely found, *Brettanomyces* species are also found in grape must (Ribéreau-Gayon et al., 2006; Bezerra-Bussoli et al., 2013; Mehlomakulu et al., 2014).

In general, yeasts are the primary fermenters, as they are ethanol tolerant and are usually responsible for the fermentation (Granchi et al., 1999; Bezerra-Bussoli et al., 2013). The term 'non-*Saccharomyces* yeasts' refers to all other yeasts that are not classified as *Saccharomyces.*

Non-*Saccharomyces* yeasts play a role in secondary metabolite production, which contributes to the final aroma and taste of the finished product (Fleet, 2003; Jolly et al., 2006; Ribéreau-Gayon et al., 2006). Furthermore, some of these yeasts have been found to produce proteinaceous compounds with antimicrobial activity (Scherm et al., 2003; Comitini et al., 2004ab; De Ingeniis et al., 2009; Mehlomakulu et al., 2014; Parafati et al., 2015). The occurrence of yeasts during spontaneous fermentation has been found to vary according to endogenous microbial flora, region, and grape variety as well as the fermentation process, therefore making the outcome of fermentation difficult to predict, as this may differ from year to year. Overall, regardless of the cultivar, grape must in combination with spontaneous fermenting grape juice in general, has been found to be the most reliable source for the isolation of non-*Saccharomyces* yeasts. The same yeast species are usually distributed in different types of grape cultivars, therefore rendering the choice of the non-*Saccharomyces* yeast isolation and/or source flexible, as any grape must possess a diverse microbial population that usually resembles the same trend regardless of the cultivar (Granchi et al., 1999; Rementeria et al., 2003; Clemente-Jiménez et al., 2004; Combina et al., 2005; Bezerra-Bussoli et al., 2013; Díaz et al., 2013).

The objective of this part of the study was to isolate, characterise and identify non-*Saccharomyces* yeasts from spontaneous Shiraz fermentation and to assess their ability to produce biopreservation compounds.

3.2 Aims and objectives

The objectives of this part of the research were to:

- develop a profile of microorganism isolation and identification of yeasts from the grape berries and must, and at different stages of spontaneous fermentation;
- assess which molecular biology methods are best suited to effectively differentiate isolates of the same species occurring at different stages of the spontaneous fermentation for better identification and subsequent selection of appropriate isolates for further study;
- identify a species of yeast which exhibits antimicrobial compound secretion from organisms profiled at different stages of the spontaneous wine fermentation; and
- Select a species which will be used in a fermentation process to produce biopreservatives, by using a suitable bioreactor.

3.3 Material and methods

3.3.1 Microbial isolation

Shiraz wine grapes from the Nietvoorbij farm were harvested, crushed and pressed. The must was then aliquoted into three sterile glass bottles (200 mL/bottle) and closed with fermentation locks. The bottles were incubated at 25 °C until the spontaneous fermentations were completed. The fermentation lasted for 15 days. The Shiraz grape must was sampled and analysed for initial sugar content and pH. Samples were also taken during mid-fermentation and at the end of the fermentation. These samples were analysed using an OenoFoss™ (FOSS Analytical A/S, Denmark).

3.3.2 Isolation and preliminary identification

Using samples from the initial grape must, mid- and end-fermentation, a serial dilution was made and 100 µL of the aliquots were plated out on the Wallerstein Laboratory medium (WL) and Lysine media (Biolab, Merck, South Africa). The WL medium was used because it is a differential medium and yeasts can be discerned based on the colony colour, size and morphology. Lysine media were used to specifically select non-*Saccharomyces* yeasts. Yeast colonies were selected and sub-cultured on yeast peptone dextrose agar (YPDA) (Biolab, Merck). Pure yeast colonies were inoculated into YPD broth (Biolab, Merck) and grown at 28 °C while purified cultures were stored at -80 °C in 40% (v/v) glycerol, prior to processing. Thereafter and subsequent to DNA isolation for identification, the yeast isolates were further grown on CHROMagar™ Chromogenic media (CHROMagar Microbiology, France) as precautionary measure to redifferentiate between colonies, based on colour and morphology. Commercial *S. cerevisiae* strain, VIN 13 (Anchor yeast, South Africa), was used as the reference strain.

3.3.3 Genomic DNA isolation and polymerase chain reactions

The genomic DNA was extracted using the protocol described by Hoffman and Winston (1987). The gDNA concentration was determined with a NanoDrop 1000 spectrophotometer (Thermo Scientific, USA). An iCycler (Biorad, South Africa) was used for all PCR reactions. The PCR mixture contained the following: 5 µL of each primer, 1 µL of the genomic DNA, 10 µL of 5x buffer, 1 µL dNTPs, 3.5 µL $MgCl_2$, 0.1 µL of GoTaq polymerase (Anatech, South Africa) or Supertherm polymerase (Separations, South Africa) and 24.4 µL sterile distilled water.

The region between the 18S and 28S rRNA genes was amplified using internal transcribed spacer (ITS) primers, ITS 1 and ITS 4. GoTaq polymerase was used in ITS and microsatellite PCR, while Supertherm polymerase was used for interdelta PCR. The primers that were used in this study are listed in Table 3-1.

Table 3-1: Primers used in this study

Primers	Sequences
CAG_5	CAG CAG CAG CAG CAG
ITS 1	TCC GTA GGT GAA CCT GCG G
ITS 4	TCC TCC GCT TAT TGA TAT GC
$TtRNAS_C$	GCT TCT ATG GCC AAG TTG
Interdelta 12	TCA ACA ATG GAA TCC CAA C
Interdelta 21	CAT CTT AAC ACC GTA TAT GA

3.3.4 Amplification conditions for PCR and the restriction endonuclease digestion of ITS products

The settings used for the PCR for the ITS region amplifications were as follows: 4 min at 94 °C followed by 36 cycles of 1 min at 94 °C; 2 min at 48 °C and 20 sec at 72 °C; and a final elongation step of 4 min at 72 °C. Amplification conditions for microsatellites and for the delta elements were performed using the following programme: 4 min at 95 °C followed by 35 cycles of 30 sec at 95 °C, 30 sec at 46 °C and 90 sec at 72 °C and then a finishing step of 10 min at 72 °C. ITS PCR products were then subsequently digested with C*fo*I, *EcoR*I and *Hae*III enzymes. The restriction mixtures contained: 21 µL of sterile distilled H_2O, 5 µL of ITS product, 3 µL of 10x buffer and 1 µL of the specific enzyme. The mixture was then incubated overnight at 37 °C in a water bath.

3.3.5 Electrophoretic separation and visualisation

The ITS, microsatellite and interdelta products were separated on 1.5% (w/v) agarose (Seakem® GTG®, Lonza, Rocklands, USA) gels at 90 V for 2.5 h in a 0.5x Tris Borate EDTA buffer. All agarose gels contained 15 µL of a 5000x GelRed™ (Biotium) to visualise the PCR and restriction endonuclease products. Generuler 100 bp Plus DNA (Fermentas) was used as a standard marker/ladder on agarose gels. Gels were visualised on a GelDoc imager.

3.4 Results and discussion

3.4.1 Differentiation of yeast isolates obtained

The selected yeast isolates were differentiated by growing them on CHROMagar™ Chromogenic medium plates (Figure 3-1). Yeast isolates found at the beginning of spontaneous fermentation differed in colony colour, shape and morphology. This indicated that there were different yeast species present. The isolates that occurred during the middle and at the end of the fermentation were of the same colour (purple) and possibly from the same species (Figure 3-1b and c), compared with those obtained at the beginning of the fermentation.

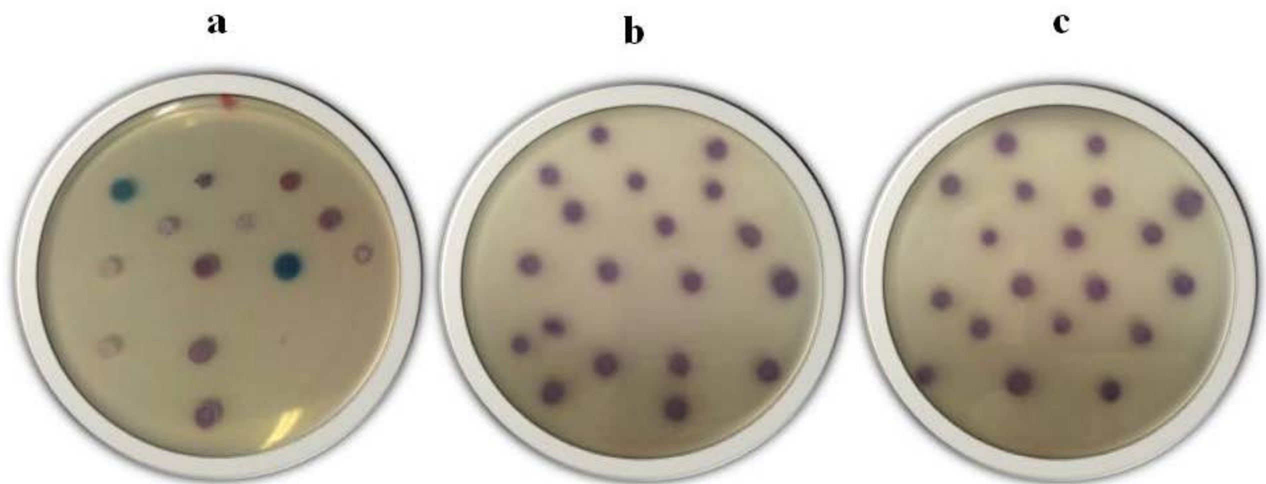


Figure 3-1: Yeast colony appearance on CHROMagar plates. (**a**) Isolates from at the beginning of fermentation. (**b**) Isolates from the middle of fermentation. (**c**) Isolates from the end of fermentation.

3.4.2 Evaluation of PCR methods

3.4.2.1 Amplification of internal transcribed spacer region

After the yeasts were differentiated on CHROMagar, DNA was isolated and the amplification of the ITS region was performed. Results of the ITS amplifications of the isolates from the beginning of fermentation showed that different fragment sizes were obtained for all the samples. This suggested that different yeast species were present at the beginning of the fermentation. The fragments obtained for ITS amplifications of the middle and end samples were all of the same size. This indicated that all the isolates hypothetically belonged to the same species. The yeast isolates were preliminary identified by comparing ITS product sizes with ITS sizes published by

Guillamón et al. (1998) and Granchi et al. (1999). ITS-PCR products of the isolates that were present at the beginning of the fermentation were further sequenced and results are listed in Tables 3-2, 3-3 and 3-4.

3.4.2.2 Restriction endonuclease digestion of ITS products

The yeast isolates were differentiated by digesting the amplified ITS products with *EcoRI, CfoI, HaeIII* as previously mentioned. The ITS restriction products were compared with products obtained from reference strains (data not shown) and the literature reviewed (Guillamón et al., 1998, Granchi et al., 1999) to identify the yeast isolates. The ITS products of the non-*Saccharomyces* were sequenced. Table 3-2, 3-3 and 3-4 show the molecular profiles and the possible identity of the isolates, as well as the colony-forming unit counts obtained for each isolate. Isolates obtained at the beginning of the fermentation were identified as *Hanseniaspora uvarum* (SI 1), *Rhodosporidium babjevae* (SI 2), *Pichia* sp (SI 3), *Saccharomyces cerevisiae* (SI 4), *Candida apicola* (SI 5) and *Candida* sp. (SI 6) (Table 3-2). The isolation of these yeasts at the beginning of fermentation (grape must) is in agreement with what has been reported previously (Granchi et al., 1999; Ribéreau-Gayon et al., 2006; Solieri et al., 2006; Bezerra-Bussoli et al., 2013). Based on colony counts, isolate SI 1 (*H. uvarum*) and SI 3(*Pichia* sp) were respectively the dominant yeasts (1.5 x 10^4 and 1.2 x 10^4 CFU/mL) in the beginning of the fermentation in comparison with the rest of the isolates which were determined to be present at low numbers (Table 3-2).

Table 3-2: Counts (colony forming units/mL) and possible identity of yeasts isolated at the beginning of the spontaneous Shiraz fermentation.

Isolates	Counts (CFU/mL)	ITS fragment size (bp)	*Eco*RI fragment size (bp)	*Cfo*I fragment size (bp)	*Hae*III fragment size (bp)	Possible Identity
SI 1	1.5 x 10^4	750	750	310+110	750	***Hanseniaspora uvarum***
SI 2	7.0 x 10^2	600	510	300+200+100	600	***Rhodosporidium babjevae***
SI 3	1.2 x 10^4	500	500	150+150	325	***Pichia* sp.**
SI 4	1.0 x 10^3	850	480+370	390+360	350+210+190+100	***Saccharomyces cerevisiae***
SI 5	9.0 x 10^3	480	480	200+190	380+80	***Candida apicola***
SI 6	1.2 x 10^3	490	490	250+200	450	***Candida* sp.**

As far as the isolates obtained from the middle and end of the fermentations are concerned, *S. cerevisiae* strains dominated the periods of fermentation (Table 3-3 and 3-4), although present at low numbers at the beginning of the fermentation (Table 3-2). This is in agreement with results reported by Guillamón et al. (1998) in a study in which it was found that *S. cerevisiae* occurred in very low numbers (10% of the initial total counts) at the beginning of the spontaneous fermentation, with the middle and end fermentations being dominated by *S. cerevisiae* (100% of the middle and end counts). In this study, only *S. cerevisiae* strains were isolated during the middle and at the end of the spontaneous fermentation. The non-*Saccharomyces* yeasts that were found in the beginning of the fermentation were either viable but non-culturable, or the cell numbers were too miniscule to detect. It could also be that those non-*Saccharomyces* perished during the fermentation as a result of the high level of alcohol and acetic acid.

Table 3-3: Counts (colony-forming units/mL) and possible identity of yeasts isolated during the middle stage of the spontaneous Shiraz fermentation.

Isolates	Counts (cfu/mL)	ITS fragment size (bp)	*ECo*RI fragment size (bp)	Possible Identity
SI 7	6.4×10^6	850	480+370	***Saccharomyces cerevisiae***
SI 8	2.8×10^6	850	480+370	***Saccharomyces cerevisiae***
SI 9	2.5×10^7	850	480+370	***Saccharomyces cerevisiae***
SI 10	4.4×10^6	850	480+370	***Saccharomyces cerevisiae***
SI 11	4.5×10^6	850	480+370	***Saccharomyces cerevisiae***
SI 12	2.6×10^6	850	480+370	***Saccharomyces cerevisiae***
SI 13	4.0×10^5	850	480+370	***Saccharomyces cerevisiae***
SI 14	2.0×10^6	850	480+370	***Saccharomyces cerevisiae***
SI 15	2.0×10^5	850	480+370	***Saccharomyces cerevisiae***

3.4.2.3. Microsatellite PCR

Microsatellite PCR has in the past been used successfully to differentiate between *S. cerevisiae* yeasts strains (Bowers et al., 1999; Jensen et al., 2009). Thus, only isolates from the

middle and end of fermentation were selected for microsatellite PCR. The microsatellite profiles obtained with $TtRNAS_C$ and CAG_5 appeared to be similar for all the isolates obtained at the middle and end fermentations. Selectively profiled isolate results are shown in Figure 3-2.

Table 3-4: Counts (colony-forming units/mL) and possible identity of yeasts isolated at the end of the spontaneous Shiraz fermentation.

Isolates	Counts (cfu/mL)	ITS fragment size (bp)	*ECo*RI fragment size (bp)	Possible Identity
SI 16	3.0×10^5	850	480+370	***Saccharomyces cerevisiae***
SI 17	1.1×10^7	850	480+370	***Saccharomyces cerevisiae***
SI 18	4.0×10^5	850	480+370	***Saccharomyces cerevisiae***
SI 19	5.0×10^6	850	480+370	***Saccharomyces cerevisiae***
SI 20	5.0×10^6	850	480+370	***Saccharomyces cerevisiae***
SI 21	5.0×10^6	850	480+370	***Saccharomyces cerevisiae***
SI 22	4.4×10^6	850	480+370	***Saccharomyces cerevisiae***
SI 23	4.3×10^6	850	480+370	***Saccharomyces cerevisiae***
SI 24	4.4×10^6	850	480+370	***Saccharomyces cerevisiae***
SI 25	4.3×10^6	850	480+370	***Saccharomyces cerevisiae***

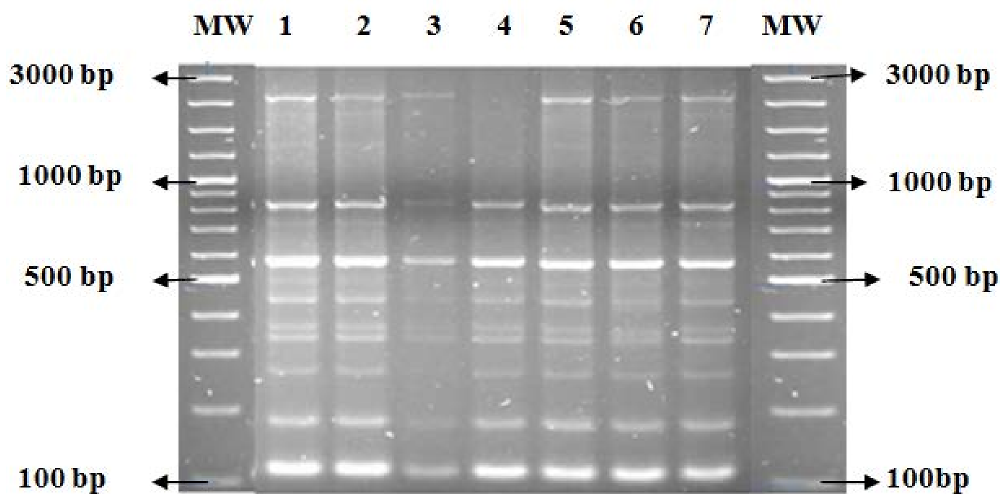


Figure 3-2: The microsatellite profile of six *Saccharomyces cerevisiae* isolates selected from the middle and end of fermentation. MW: molecular weight marker (Generuler 100 bp Plus), lane 1: SI 16, lane 2: SI 17, lane 3: SI 18, lane 4: SI 19, lane 5: SI 20, lane 6: SI 11, lane 7: VIN 13.

3.4.2.4 Interdelta PCR

According to Legras & Karst (2003) and Solieri et al. (2006), the use of delta primers is also an efficient PCR-based method to differentiate between strains of *S. cerevisiae*. In this study, the delta 12 and 21 primers were used to differentiate between the *S. cerevisiae* strains isolated during the middle and end of fermentation. Fourteen different profiles were obtained (Figure 3-3). These results indicated that there were differences in the *S. cerevisiae* strains isolated during the middle and end of fermentation stages. This study also showed that better differentiation between *S. cerevisiae* strains was obtained with delta 12 and 21 primers than with microsatellites, $TtRNAS_C$ and CAG_5. This observation was proven by the fact that isolates SI 20 and SI 11 on the microsatellite gel photo sowed identical profile, however the interdelta gel profile of those two isolates showed significant differences in their gel electrophoresis profile.

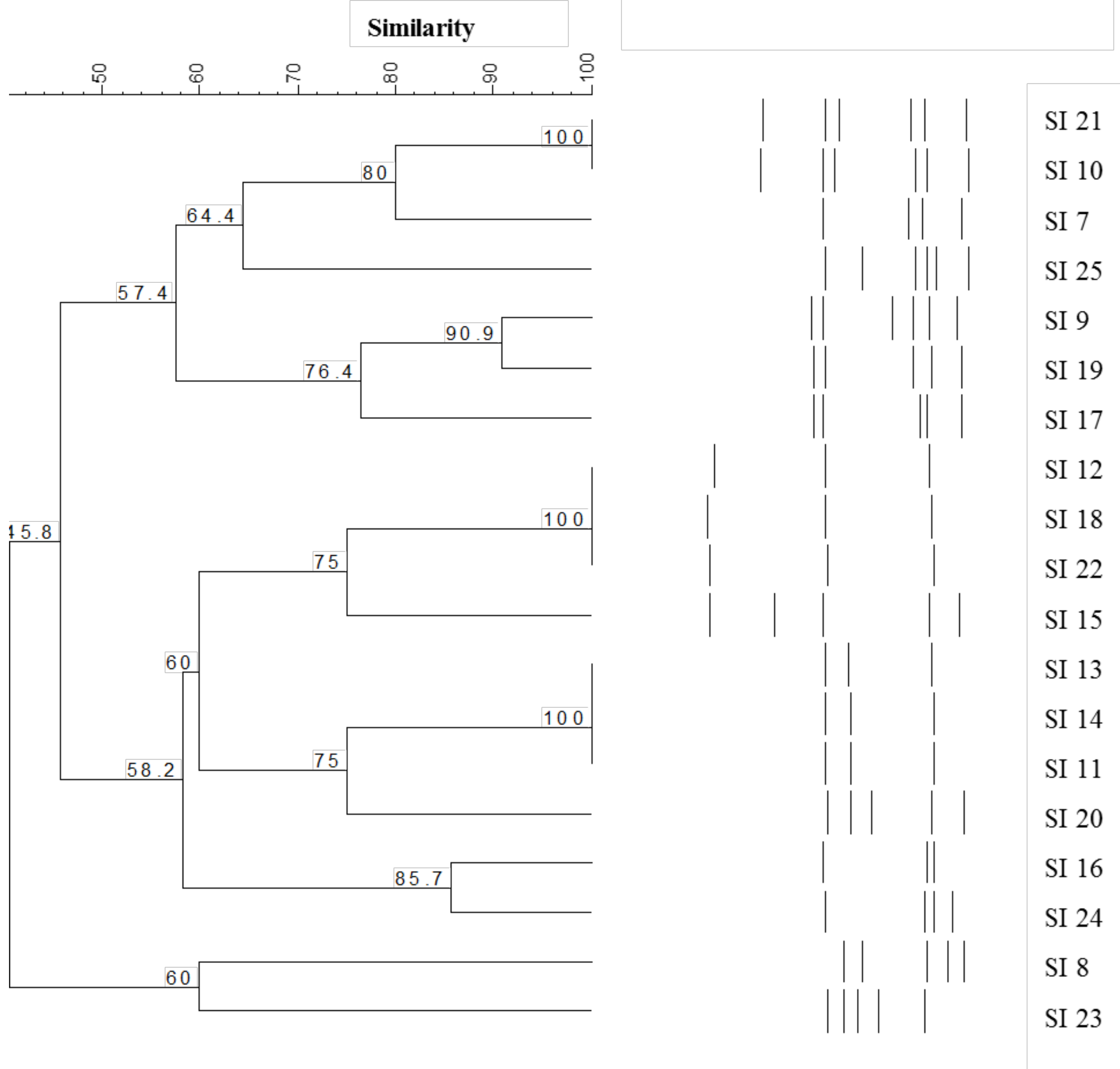


Figure 3-3: Dendogram showing the clustering of different *Saccharomyces cerevisiae* strains obtained by numerical analysis of delta12–21 profiles. Cluster analysis was performed using the unweighted pair-group method with arithmetic mean (UPGMA). Similarities between strains were calculated based on the Dice coefficient.

3.5. Yeast species which exhibited antimicrobial compound secretion

Although numerous microorganisms exhibited more or less antimicrobial compound secretion, it was distinctively determined that aliquots of *S. cerevisiae* isolates which dominated the middle and end of the spontaneous fermentations exhibited a lower antimicrobial activity, particularly when compared with the *Candida* sp. isolated at the beginning of the fermentation. It was presumed that perhaps the rate at which the *S. cerevisiae* isolates thrived was much higher than that of the other species isolated, which in turn resulted in the dominance of the species. Furthermore, environmental conditions at which the spontaneous fermentation took place were deduced to have played a role, thus favouring the proliferation of the *S. cerevisiae* isolates. Although this effect was disconcerting, it was determined that for future studies a thorough assessment of this phenomenon should be conducted. At this stage, although partial screening was conducted, it was clear that a rigorous screening assessment (presented in Chapter 4) is required.

3.6. Summary

It was found that *Hanseniaspora uvarum, Rhodosporidium babjevae, Pichia* sp., *Saccharomyces cerevisiae, Candida apicola, and Candida* sp. were present at the beginning of the spontaneous Shiraz fermentation, but that the middle and end fermentations were completely dominated by *Saccharomyces cerevisiae.* The combination of CHROMagar and ITS results provided sufficient evidence for the evolution of the fermentation. Different *S. cerevisiae* strains occurred in the middle and end fermentations. It was also shown that PCR using delta 12 and 21 primers was a more suitable method than microsatellites to differentiate between *S. cerevisiae* strains. At this stage it was determined that the *Candida* sp. isolated that showed antimicrobial compound secretion with a higher activity should be used in further studies.

Chapter 4

Antimicrobial activity screening, yeast growth and productivity kinetics of the antimicrobial compound by a *Candida* sp.

CHAPTER 4

ANTIMICROBIAL ACTIVITY SCREENING, YEAST GROWTH AND PRODUCTIVITY KINETICS OF THE ANTIMICROBIAL COMPOUND BY A *CANDIDA* SP.

4.1 Introduction

Naturally occurring yeasts and their antimicrobial compounds are used to eliminate spoilage organisms in processed and non-processed food and beverages to extend the shelf life of the products as well as improve their safety and quality. Examples of processed food and beverages referred to are wine, dairy and meat products. Vegetables and fruits are examples of non-processed food that need to be kept, stored safely and be free from unwanted microorganisms. The historical, economic and scientific aspects of non-*Saccharomyces* yeasts, their ease of handling, ease of manipulation and growth, and use in food safety because of their benefits and ability to produce antimicrobial compounds can be natured to be applied to the agricultural and medical sector (Oro et al., 2014; Mucilli & Restuccia, 2015). Some yeasts are known to have simple growth requirements in bioreactors and possess the ability to colonise a variety of environments for a longer period of time and also grow faster in non-expensive substrates compared with other microorganisms (Stiles, 1996; Santos et al., 2004; Chanchaichaovivat et al., 2007; Liu et al., 2013; Muccilli & Restuccia, 2015).

The competitive traits and the antagonistic characteristics of yeasts against other microorganisms can be classified using numerous parameters, e.g., the change in pH as a result of growth and metabolite secretion and nutrient competition (Muccilli & Restuccia, 2015). During their growth and other metabolic activities, some yeasts secrete antimicrobial compounds (Comitini et al., 2004ab; De Ingeniis et al., 2009; Mehlomakulu et al., 2014; Oro et al., 2014; Carocho et al., 2015). For example, killer toxins are protein-based compounds with antimicrobial properties that are produced extracellularly by some yeasts and act on the cell wall of other organisms by disrupting the function of the membrane (Robledo-Leal et al., 2014). Yeasts with

the ability to secrete antimicrobial compounds such as the *Candida* sp. isolated (see Chapter 3) can be of significant importance in the fermentation industry, as these compounds can be used as preservation agents (Mehlomakulu et al., 2014; Robledo-Leal et al., 2014), herein referred to as biopreservation agents.

4.2 Aims and objectives

In this chapter, the objectives were to:

- use the selected fermenter (isolate) in a process to produce biopreservatives with different media (YPD, YP, YD, PD) at room temperature and at a predetermined pH, and assess fermentation process parameters using appropriate kinetic models and the fermentation period at which the biopreservatives exhibit maximum activity;
- obtain crude extracts, fermented using the best carbon source determined from the fermentation at an appropriate time and assess the stability of the biopreservatives when used under different temperature and pH;
- perform an extended screening of the isolate for antimicrobial activity against spoilage organisms belonging to *Candida*, *Brettanomyces* and *Zygosaccharomyces* species and against fruit pathogens belonging to the species of *Botrytis*, *Penicillium*, *Colletotrichum* and *Monilinia*; and
- optimise fermentation conditions for effective biopreservative production using Response Surface Methodology (RSM) subsequent to characterising the biopreservation of compound size.

4.3 Materials and methods

4.3.1 Antimicrobial activity screening

4.3.1.1 Screening against spoilage microorganisms found in beverages

A number of yeast isolates belonging to the genera *Zygosaccharomyces*, *Schizosaccharomyce, Brettanomyces*, *Kluyveromyces* and *Candida*, including spoilage fungi *Botrytis*, *Penicillium*, *Colletotrichum* and *Monilinia*, were selected as spoilage organisms (Table 4-1). A modified version of the seeded plate method by Comitini et al. (2004ab) was used to screen for antimicrobial activity including bacteria, i.e., acetic acid bacteria (AAB) and lactic acid bacteria (LAB). The medium used was white grape juice agar adjusted to pH 4.5, as most fermented products have a pH range in this region. The spoilage organisms were grown prior to the biopreservation assay. A 2.5 mL aliquot of four times strength agar was prepared in test tubes, autoclaved and stored in a water batch at 50 ºC. The filter-sterilised white grape juice was inoculated with the spoilage yeast at a concentration of $1x10^6$ cells/mL. From the inoculated white grape juice, 7.5 mL was pipetted and transferred into the 2.5 mL of a four times strength agar and immediately vortexed. The mixture was then poured into a Petri dish and allowed to solidify. A volume of 5 µL of the fermented *Candida* sp. broth containing the biopreservatives was then spotted in triplicate on the solidified white grape juice agar seeded with spoilage organisms. The plates were then incubated for 48 h at 25 ºC. In this method, the inhibitory effect of the biopreservatives was shown as clear zones surrounding the spoilage microorganisms.

4.3.1.2 Screening against fungal pathogens on fruits

The efficacy test was performed in triplicate on apples by inoculating the fungal spore suspensions and the biopreservation yeast cultures at the concentration of 10^5 spores/mL and 10^8 cells/mL, respectively (Table 4-1).

Table 4-1: Spoilage organisms used

Spoilage Yeasts*	Spoilage Fungi*	Spoilage Bacteria*
Zygosaccharomyces bailii [7]	*Botrytis cinerea*[1]	LAB[3]
Zygosaccharomyces rouxii [2]	*Penicillium expansum*[1]	AAB[8]
Schizosaccharomyces pombe [4]	*Colletotrichum acutatum*[1]	-
Candida guilliermondii[2]	*Monilinia lax*[1]	-
Brettanomyces bruxellensis[4]	-	-
Kluyveromyces thermotolerans[4]	-	-

***(number of isolates)**

The apples were then incubated at 25 ºC for seven days, which is the standard and recommended duration for commercial fruit on the shelf in a commercial setting. Positive results were observed by the absence of fungal development on the fruit skin, therefore eliminating the disease-causing pathogens on the fruits. The fungal pathogens used were *Botrytis cinerea*, *Penicillium expansum*, *Colletotrichum acutatum* and *Monilinia laxa*. YieldPlus (Anchor Bio-Technologies) was used as a reference for the efficacy test.

4.3.2 Antimicrobial compound: Production and activity tests under different pH and temperature

4.3.2.1 Productivity study

The productivity was done using a single-stage bioreactor with all nutrients present using different substrates. *Candida* sp. cells were picked, using a sterilised wire loop, from the freshly grown culture and inoculated into 5 mL YPD and incubated overnight at 25 ºC. From the overnight culture, 100 µL was inoculated in 50 mL sterile YPD broth (pH 4.5). The culture was incubated at 25 ºC overnight and agitated at 150 rpm. Four bioreactors were set up with each reactor containing 150 mL of YPD, YP, YD and PD (pH 4.5). The inoculum concentration was 1×10^6 cells/mL for each of the four bioreactors. Agitation was set at 150 rpm and the operating temperature of the fermenters was 25 ºC. Samples were taken before and after inoculation. After

9 h of fermentation, samples were taken every 2 h until the stationary growth phase was reached. Even when growth reached the stationary phase, the last sample was taken 36 h after the stationary phase had been reached. During growth, the samples were plated out to assess cell viability. A volume of 1 mL was withdrawn during each sampling cycle from all four bioreactors and was centrifuged at 5000 rpm for three minutes and then filtered using a 0.45 µm sterile syringe filter. All samples were stored at 4 ºC for further use.

After the fermentation was complete, the supernatant collected at each sampling period was spotted in triplicate on white grape juice agar plates into which cylindrical wells had been pierced, and seeded with one of the spoilage organisms, i.e., *Candida guilliermondii,* as the selected spoilage yeast grown overnight at 25 ºC. After 48 h, the plates were inspected for zones of clearance as the result of growth inhibition activity around the wells.

4.3.2.2 Activity test at different pH

The crude extract from the best fermenting broth, filtered through a 0.45 µm sterile syringe filter, was evaluated at different pH. The pH variations were 2, 3, 4, 5, 6, 7, 8, 9 and 10. This was performed by seeding the spoilage organism *Candida guilliermondii* in white grape juice adjusted to each of the above-mentioned pH using 0.1M NaOH or 0.1M HCl for pH adjustment. A volume of 50 mL white grape juice was aliquotted in 9x100 mL glass bottles. Each of the nine glass bottles containing white grape juice was adjusted to the specific pH. The adjusted white grape juice was then filter sterilised through a 0.45 µm sterile syringe and transferred to sterile glass bottles. The seeded agar plate assay was used to test the activity of the crude extract at various pH using the above prepared white grape juice.

The seeding procedure was as follows: a four times strength agar was used of which 2.5 mL was autoclaved for 20 minutes at 120 ºC in a test tube and kept in a water bath at 50 ºC. The sterile white grape juices was also put in the water bath to bring the temperature closer to the limit at which the agar would not set faster when the juice and agar were mixed. A corresponding broth volume of the spoilage organism was used to obtain a final concentration of 1x 10^6 cells/mL subsequent to centrifugation at 5000 rpm for 3 min. The pellet obtained was then

dissolved in the 50 mL of white grape juice. From the inoculated white grape juice, 7.5 mL was transferred into the test tube containing 2.5 mL liquid agar with the mixture being vortexed and poured into the Petri dishes and allowed to set. After all the plates for different pH were prepared with the aid of the agar well piercer (8.5 mm diameter), five holes were drilled on each of the solidified white grape juice agar. A volume of 20 µL of the biopreservation supernatant was then filled in the pierced area on the white grape juice agar plate. Every test was prepared in triplicate and incubated at 25 ºC for 48 h.

4.3.2.3 Activity test at different temperatures

The biopreservation assay was performed as described previously (Section 4.3.2.2). The pH of the white grape juice was adjusted to 4.5. In the cylindrical pierced agar plates, 20 µL of the crude extract was filled into the wells and the plates seeded with the spoilage organism incubated at different temperatures (4, 10, 15, 20, 25, 30 and 40 °C), with positive results shown by formation of clear zones as previously described.

4.3.2.4 Stability of the crude extracts under storage at different temperatures

A volume of 2 mL of the crude extract was stored at various temperatures (4, 10, 15, 20, 25, 30 and 40 ºC) for 2 to 14 days and tested for stability. This was performed as described previously using the seeded method in white grape juice agar plates (Section 4.3.2.2). A volume of 20 uL of the crude extract from each temperature was pipetted into the pierced cylindrical wells in the plates and incubated at 25 ºC for 48 h.

4.3.3 Kinetic study of biopreservative production

In kinetic studies of biological systems, the microbial population is one the first parameters quantified to assess whether the product is growth or non-growth associated. In this study, the relationship described by the following Malthus equation was used (Eq. 4.1).

$$\frac{dx}{dt} = ux \tag{4.1}$$

with *X*, being the cell concentration in colony-forming units/mL (CFU/mL), quantified as an increase over time regardless of the substrate present and preferences. The integration and rearrangement of the above equation yielded the following (Eq. 4.2):

$$\ln \frac{x}{x_0} = ut \tag{4.2}$$

It is also important to assess various structured as well as non-structured models for the purpose of determining rate constants and the prediction of performance in batch systems. Overall, non-structured kinetic models are rated highly and are much easier to manipulate to obtain kinetic parameters (Shuler & Kargi, 2002). They are also useful in conditions where several experimental conditions have been employed such as in this study, e.g., substrate manipulation and productivity predictions. Monod's model, which is an unstructured model along with the Malthus equation, can be used in the description of microbial growth kinetics – Eq. 4.3 (Fujikawa, 2010; Ghosh et al., 2012; Weaver et al., 2015; George et al., 2015).

$$\mu = \mu_m \left(\frac{S}{K_s + S} \right) \tag{4.3}$$

where K_s is the substrate utilisation constant applicable when:

$$\mu = \frac{1}{2}\mu_m \quad (4.4)$$

This model describes microbial growth profile in combination with a decrease in substrate concentration and vice versa. Therefore, a more descriptive model can result from combining the Malthus equation with Eq. 4.3, while taking into account the number of cells that perish during the process as shown in Eq. 4.5.

$$\frac{dX}{dt} = \mu_m \left(\frac{S}{K_s + S}\right) X - K_d X \quad (4.5)$$

where K_d is the cell death rate constant. Knowing that a portion of the available substrate would be converted to biomass, it can be computed that the maximum cell concentration is proportional to the sum of the inoculum size and the yield coefficient multiplied by the substrate concentration. This can be mathematically expressed as Eq. 4.6:

$$X = \left(X_0 + Y_{\frac{X}{S}}\right) S \quad (4.6)$$

The concept of substrate consumption, usually proportional to biomass generation and product formation, as well as to cell maintenance, was applied in this study. Eq. 4.8 was generated to describe the experimental observations.

$$\frac{dS}{dt} = \frac{dX}{Y_{X/S} dt} + \frac{dP}{Y_{P/S} dt} + m_s X \quad (4.7)$$

It is worth noting that the above equation describes the case of single substrate consumption, normally for growth, product formation and cellular maintenance.

Similarly, the productivity (P) determination aspect during this process was evaluated using a modified Luedeking–Piret model whereby the parameters were assessed based on the experimental data and conditions. The integrated equation yielded the following (Eq. 4.8).

$$P(t) - P_0 - n\left(\frac{X_{max}}{\mu_{max}}\right)\ln\left[1 - \left(\frac{X_0}{X_{\max}}\right)\left(1.0 - e^{\mu_{max}t}\right)\right] = m[X(t) - X_0] \qquad (4.8)$$

It is known that in using the Luedeking–Piret model, under stationary conditions, $dX/dt = 0$, while $X = X_{max}$.

4.3.4 Mathematical modelling coupled with response surface methodology (RSM)

According to the data previously reported on antimicrobial compounds from non-*Saccharomyces* yeasts, it was of importance to investigate the optimum production condition of the biopreservation secreted by the *Candida* sp. studied, using RSM, which is a modelling technique applicable in evaluating interaction between experimental factors such as temperature and pH which are not only controllable but also critical for biopreservation compound production from non-*Saccharomyces* yeasts (Nwabueze, 2010; Parafati et al., 2015). It is worth noting that the process of optimisation was conducted following distinctive steps which consisted of firstly conducting statistically designed experiments, secondly estimating the mathematical model coefficient, and finally predicting the response to be obtained (Box & Hunter, 1957; Nwabueze, 2010). In this research, the above-mentioned steps were carried out using the central composite design (CCD) approach. Design-Expert® software version 10.0.0 (Stat-Ease Inc., Minneapolis, USA) was used to generate 13 experimental runs. The independent variables used were temperature coded as (A) and pH coded as (B) (see Table 4-1 and 4-2).

Table 4-2: Process variable used in the CCD for optimisation of biopreservation production using temperature and pH as controllable experimental factors

Variables	Code	High level (+1)	Medium level (0)	Low level (-1)
Temperature	A	30	22.5	15
pH	B	6	5	4

α=1.41 and represents the axial point with coded level of 1.41

Table 4-3: The CCD using temperature (A) and pH (B) as process variables

Run number	A	B	Productivity (L/mL)
1	0	0	1.05
2	0	0	1.05
3	-1	-1	0.85
4	+1	-1	0.80
5	0	0	1.05
6	0	-α	1.08
7	0	0	1.05
8	0	+ α	0
9	+1	+1	0
10	- α	0	0
11	0	0	1.05
12	+ α	0	0
13	-1	+1	0

The fermentation period for each run was 21 h, using the previously determined best-performing media (Section 4.3.2.1). Each run was carried out in three independent replicates. The samples drawn after each fermentation run were tested to evaluate the actual best production conditions. To test these samples for biopreservation activity, the growth inhibition assay was done following the procedure described in Section 4.3.2.2. The response considered was the volumetric zone of clearance Y (productivity in L/mL). Design-Expert® software version (10.0.0 Stat-Ease Inc, Minneapolis, USA) was used to analyse and calculate the second-order polynomial coefficients. A representation of the system is displayed in Eq. 4.9.

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j + \varepsilon \quad (4.9)$$

where Y, $\beta_0, \beta_{i,}$ β_{ii}, and β_{ij} are response variables, intercept of the response variable, and coefficient corresponding to the factor $X_i, X_j (i, j = 1, 2, ... n)$. The input variables that influence the response Y are X_i and X_j; the random error is represented by ε.

4.3.5 Characterisation of biopreservation compounds

The preliminary characterisation of the antimicrobial compounds was conducted by Size Exclusion Chromatography (SEC). The choice of this method was based on the principles that govern SEC. This process involves compound separation by virtue of molecular size of the compounds of interest. This method has been extensively used in industry for the separation of biomolecules such as nucleic acids, enzymes, polysaccharides and proteins (Bruins et al., 1987; Boyer, 1993; Volmer et al., 2002; Guiochon & Trapp, 2012).

In this work, an isocratic elution (single solvent) with 5 mM sodium citrate pH 4.5 over 2 column volumes at a flow rate of 1 mL per min was used. A volume of 2 mL of the crude biopreservative samples per run was injected and a volume of 2 mL was collected in each run. The resin used was TOYOPEARL HW-55F. The chromatography system was the Bio-Rad NGC Quest™, controlled by the software ChromLab V3.1. Furthermore, ultra-centrifugation using a 10 kDa and 50 kDa filter was used to narrow the molecular weight size range. The crude extract, the retentate and the filtrate from the Amicon filters were tested individually to determine which fraction showed growth inhibition activity.

4.4 Results and discussion

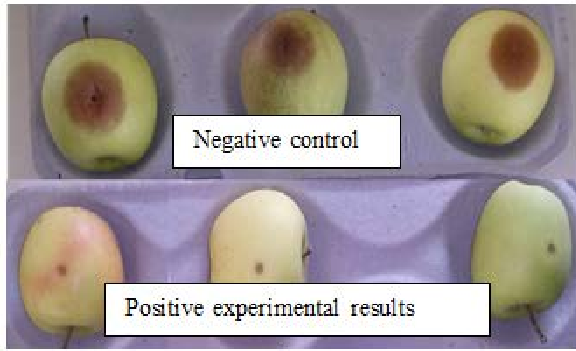
4.4.1 Antimicrobial activity screening against spoilage microorganisms

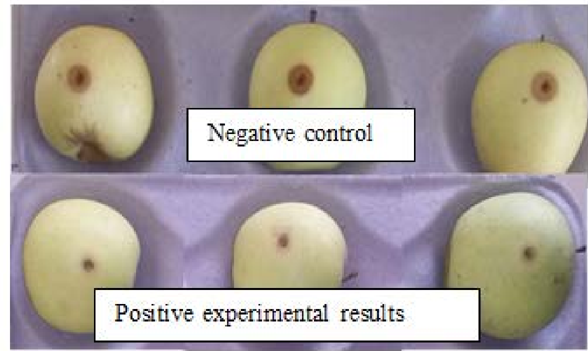
The antimicrobial activity was tested against selected spoilage organisms and fungal pathogens belonging to the genera *Zygosaccharomyces*, *Brettanomyces*, *Candida,* and *Kluyveromyces.* Furthermore, strains of *Botrytis cinerea*, *Penicillium expansum*, *Colletotrichum acutatum* and *Monilinia laxa* were also used to assess the efficacy of the biopreservatives

produced. This strategy is suitable for widespread usage of the biopreservative agent produced. The aliquots used were found to have a growth-inhibiting effect activity against all the spoilage yeasts and the acetic acid bacteria (AAB) strains tested. The producer of biopreservatives in this study also had activity against *Candida guilliermondii* and *Brettanomyces bruxellensis.* Interestingly, the *Candida* sp. used did not show any growth inhibition activity against *Saccharomyces cerevisiae*, making it a potential biocontrol agent in processed food and beverages where *Saccharomyces cerevisiae* is the main role player, for example, in wine making.

4.4.2 Antimicrobial activity screening against spoilage fungi on fruits

The in-vitro efficacy test of *Candida* sp. against *Botrytis cinerea*, *Penicillium expansum*, *Colletotrichum acutatum* and *Monilinia laxa* on apples showed that *Candida* sp. isolate could completely reduce the disease-causing incidence (100% DI reduction) against *B. cinerea* and *C. acutatum.* Comparing the lesion diameter (LD) of the negative control with that of the tested samples, a zero LD was observed with *Botrytis cinerea* and *Colletotrichum acutatum*. The negative control showed an LD of 27.38, 11.23 and 25.33 mm for *B. cinerea, C. acutatum* and *P. expansum,* respectively (Figure 4-1). This shows that the *Candida* sp. was able to control the development of *B. cinerea* and *C. acutatum* under commercial storage conditions but could not halt the DI caused by *P. expansum*. It was observed that the *M. laxa,* used as a negative control, did not infect the apples; therefore negative results were obtained for the control of *M. laxa* with the species of *Candida* studied for biopreservative efficacy on fruit.





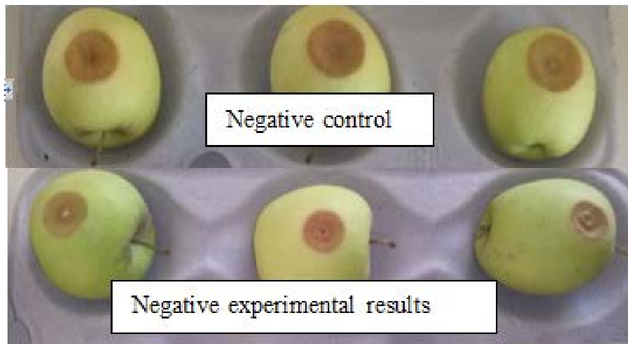


Figure 4-1: Biopreservation activity of *Candida* sp. in controlling spoilage caused by *Botrytis cinerea, Colletotrichum acutatum* and *Penicillium expansum* on *Malus domestica*.

4.4.3 Productivity studies

4.4.3.1 Period of maximum antimicrobial activity

Yeast Peptone dextrose (YPD; Biolab; Merck, South Africa), yeast peptone (YP), yeast dextrose (YD) and peptone dextrose (PD) broth were used in production media. Bioreactors containing YPD (yeast extract, 10g/L; peptone, 20g/L and dextrose 20g/L), YP (yeast extract, 10g/L and peptone, 20g/L), YD (yeast extract, 10g/L and dextrose, 20g/L) and PD (peptone, 20g/L dextrose 20g/L), respectively, were used. These media contained peptone as a source of nitrogen, vitamins and minerals. Yeast extract provided B-complex vitamins, which stimulate cell growth. Dextrose was the carbohydrate and growth-limiting substrate, and its use was quantified. Results showed that the maximum biopreservation production, thus activity, occurred at different times of fermentation for conditions used in this experiment. The media that yielded biopreservatives with a high activity was YPD after 21 hours of fermentation (see Figure 4-2); however in the PD medium, compared with YPD, there was minimal differentiation. The fermentation period required to produce biopreservatives was longer compared with the period observed in YPD. In large-scale production, although the use of PD as the production medium could be applicable and cheaper, the drawback will be the idle time during fermentation.

In other words, it will not be suitable to use the PD medium since would allow the bioreactors to run for a longer period to achieve the desired production and will result in high operational costs. It is important to mention that the crude samples that were withdrawn after 35 h in reactors containing YPD and YP did not show any growth-inhibition activity on the spoilage organisms. Results suggest that biopreservative activity and stability are compromised when YPD and YP are used as production media. It was observed that the maximum inhibition occurred at the logarithmic growth phase of the *Candida* sp. growth. This occurred at an OD_{600} of 12.45; 8.4; 11.35 and 10.08 in YPD, YP, YD, PD, respectively, corresponding to maximum cell concentration of 1.25x 10^9, 1.07x 10^9, 0.90x 10^9 and 0.40x 10^9 CFU/mL (Figure 4-4 and Table 4-4). The crude samples were also tested against the *Candida* sp. used and it was found that the yeast was resistant to its own antimicrobial compound.

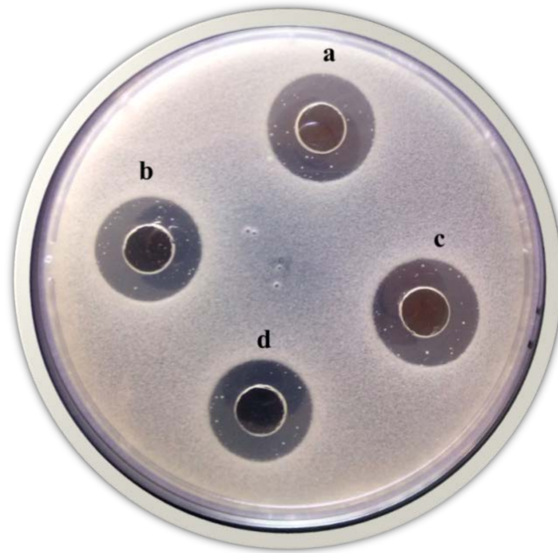


Figure 4-2: Growth inhibition patterns in YPD observed during the antimicrobial compound production: a, b, c and d = inhibition zone of crude sample after 21, 25, 27 and 29 hours of fermentation, respectively.

4.4.3.2 Activity of antimicrobial compound under different pH and temperature including stability test

For application purposes, it was of great importance to test the activity of the crude samples under various pH and temperature to ascertain whether these parameters govern its efficacy. The outcome of the activity assessments would advise on the possible types of food where this antimicrobial compound could be applied. The temperature and pH variation usually occurred in broad range for various antimicrobial compounds; as reported in the work conducted by Chen et al; 2000, Mehlomakulu et al; 2014, Villalba et al; 2016, the highest activity of the antimicrobial compounds was observed at pH between 3.5 and 5.4. Meanwhile the activity of those antimicrobial compounds based on temperature was observed till 30 ºC. In the present work, the antimicrobial compound activity test under different pH showed that the compound was active between pH of 2.0 to 6.0, and that the maximum activity was observed at pH 4 with the maximum zone clearance of 11.36 mm (Figure 4-3). The spoilage organisms could grow at a pH above 6.0 and the biopreservation compounds were not able to inhibit their growth at such pH. This finding is also in agreement with the findings reported in the literature on similar work (Mehlomakulu et al; 2014). As far as the activity of the antimicrobial compounds at different temperatures was concerned, it was demonstrated that the plates incubated at 4, 10, 15, 20, 25

and 30 showed sufficient activity, although maximum activity was observed at 15 ℃ (Figure 4-3). Furthermore, the stability of the crude extract was performed and the results showed that the crude extract stored at 4 ℃ still had growth inhibition activity after 12 weeks, with a reduced activity of 30%.

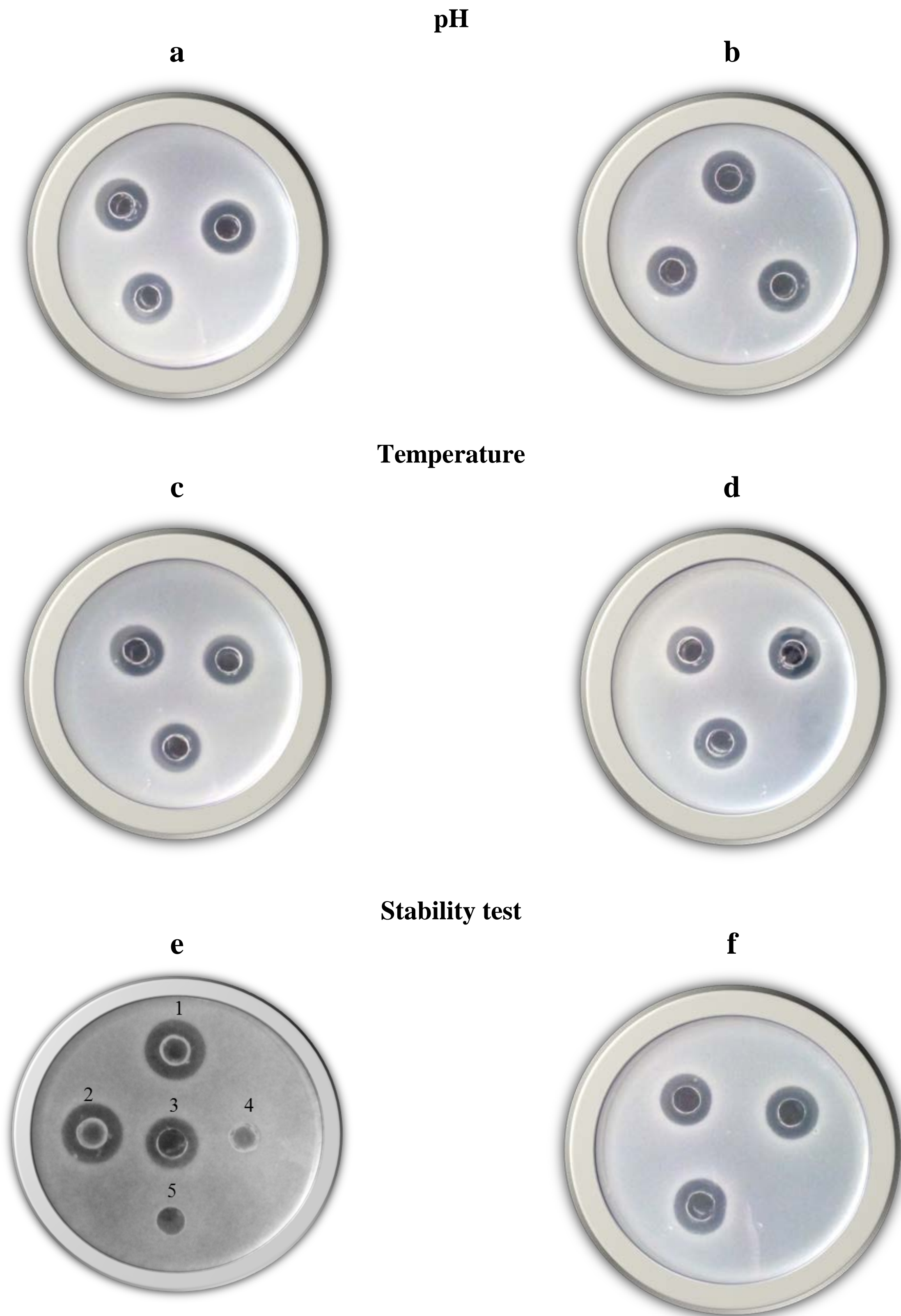


Figure 4-3: Inhibition zone of clearance observed during pH and temperature activity test: a (activity at pH 4.5); b (activity at pH6); c (activity at 15 ºC); d (activity at 25 ºC); $e_{1,\ 2,3,4,5}$ (stability at 15, 20, 25 30 and 40 ºC respectively); f (stability up to 12 weeks at 4 ºC).

4.4.4 Kinetics study and modelling

For fermentation systems, it is of utmost importance to understand the kinetic parameter involved in the process, especially when the extracellular product is dependent on the rate of cell growth, as is the case in this research. Growth kinetics was studied during the biopreservation compound production by the *Candida* sp. used. It was observed that the microbial growth was hyperbolic and could be described by the Malthus equation (Eq. 4.2).

The experimental data were fitted to the Malthus-derived hyperbolic function and it was observed that the production in all four bioreactors occurred at different specific and maximum specific growth rates as depicted in Table 4-5. The experimental data were further analysed to determine the other fermentation parameters related to the growth kinetics.

4.4.4.1 Biopreservative productivity kinetics

The productivity kinetic parameters were quantified using appropriate models. The maximum antimicrobial activity was observed during the exponential growth phase as shown by the highest cell concentration achieved as displayed in Table 4-4 and Figure 4-4, indicating phenomena which are growth related. This means that to fit the kinetics data to the model (Eq. 4.8), the constant *n* in the model could be safely discarded. The new rearranged equation yielded the following (Eq. 4.10):

$$\mathrm{P(t)} - P_0 = m[X(t) - X_0] \tag{4.10}$$

A plot of $\mathrm{P(t)}$ versus $[X(t) - X_0] + P_0$ gave a linear trend with slope m, which was the achievable rate of biopreservative product formation in each of the bioreactors (see Table 4-4 and Figure 4-6). Overall, the highest rate of product formation was observed in the bioreactor containing the YPD medium. The volumetric productivity obtained during the experiment in each of the bioreactors is displayed in Table 4-4 and Figure 4-5 (See Appendix B, Figure 7-2 for the concept used to calculate the volumetric productivity).

Following further experimental data analyses, it was observed that Eq. 4.7 could be used to further demonstrate that the substrate utilisation rate was directly proportional to cell growth and product formation. Since the maximum production was observed during the exponential growth phase, it was unnecessary to consider substrate consumption for cell maintenance. Thus maintenance was assumed negligible based on these research findings, which resulted in -Eq. 4.11. It is pertinent to indicate that this equation also verified the individual calculations obtained for substrate depletion rate in all four bioreactors with a similar trend observed.

$$\frac{dS}{dt} = \frac{1}{2}\left(\frac{dX}{Y_{X/S}\,dt} + \frac{dP}{Y_{P/S}\,dt}\right) \tag{4.11}$$

Where, $\frac{1}{2}$ symbolises the proportionality of cell growth and product formation based on substrate utilisation rate.

4.4.4.2 Antimicrobial compound productivity in different media

In some previous research it was reported that the ability of some yeasts to produce antimicrobial compound is media dependent. In this study, the production of the biopreservative was successful in YPD, YP, YD and PD media, which suggested that the production was not media dependent since the compounds of interest were successfully produced in all four media used, although the activity of the compounds was reduced in the YP medium. The activity shown by the zone of clearance diameter (D_0), after 9 hr of fermentation, ranged from 11.46 mm to a maximum of 16.38; 9.28 to 13.91 mm; 10.90 to 14.25 mm; 11.23 to 15.61 mm in YPD, YP, YD and PD media respectively. These results were achieved using a maximum fermentation time of 32 hr. Although the antimicrobial compounds were extracellular, it was observed that in other media assessed, except for the YPD medium, the cell concentration did not have a noticeable direct correlation on clearing zones achieved for aliquots obtained from each bioreactor. In other words, the maximum inhibition activity was not observed at the highest cell density in the medium composed of YP; however, it was observed that the compounds were produced during the exponential growth phase of the isolate used. Another observation was that when the fermentation was not discontinued, the samples withdrawn after 32 hr showed reduced activity.

Table 4-4: Maximum cell concentration, volumetric productivity, and achievable rate of productivity obtained during the fermentation

Media	Parameter			
	Maximum cell concentration ($x10^9$ CFU/mL)	Maximum volumetric productivity (L/mL)	Maximum achievable rate of productivity, m (L/mL.hr)	Time to maximum productivity (hr)
YPD	1.25	1.05	0.086/R^2=0.99	21
YP	1.07	0.19	0.043/R^2=0.96	19
YD	0.90	0.77	0.074/R^2=0.97	17
PD	0.40	0.96	0.037/R^2=0.99	23

In this study, several stoichiometric parameters for microbial growth and productivity kinetics were studied and compared. The stoichiometric parameters applicable to the production of biopreservation compounds were; specific and maximum specific growth rate, biomass yield, volumetric productivity based on cell growth and substrate utilisation, rate of volumetric productivity as well as the Monod's saturation constant. These parameters were studied in all the production media and the results are displayed in Table 4-5. It was found that YPD medium yielded the highest cell formation rate ($0.06x10^9$ CFU/mL.hr) and the maximum volumetric productivity rate (0.18 L/mL.hr). The highest volumetric productivity based on cell growth and substrate utilisation was also observed in YPD ($0.43x10^{-9}$ L/CFU and 86.76 L/g respectively), making YPD medium the best production medium compared to the other media used (See Appendix B for productivity calculations).

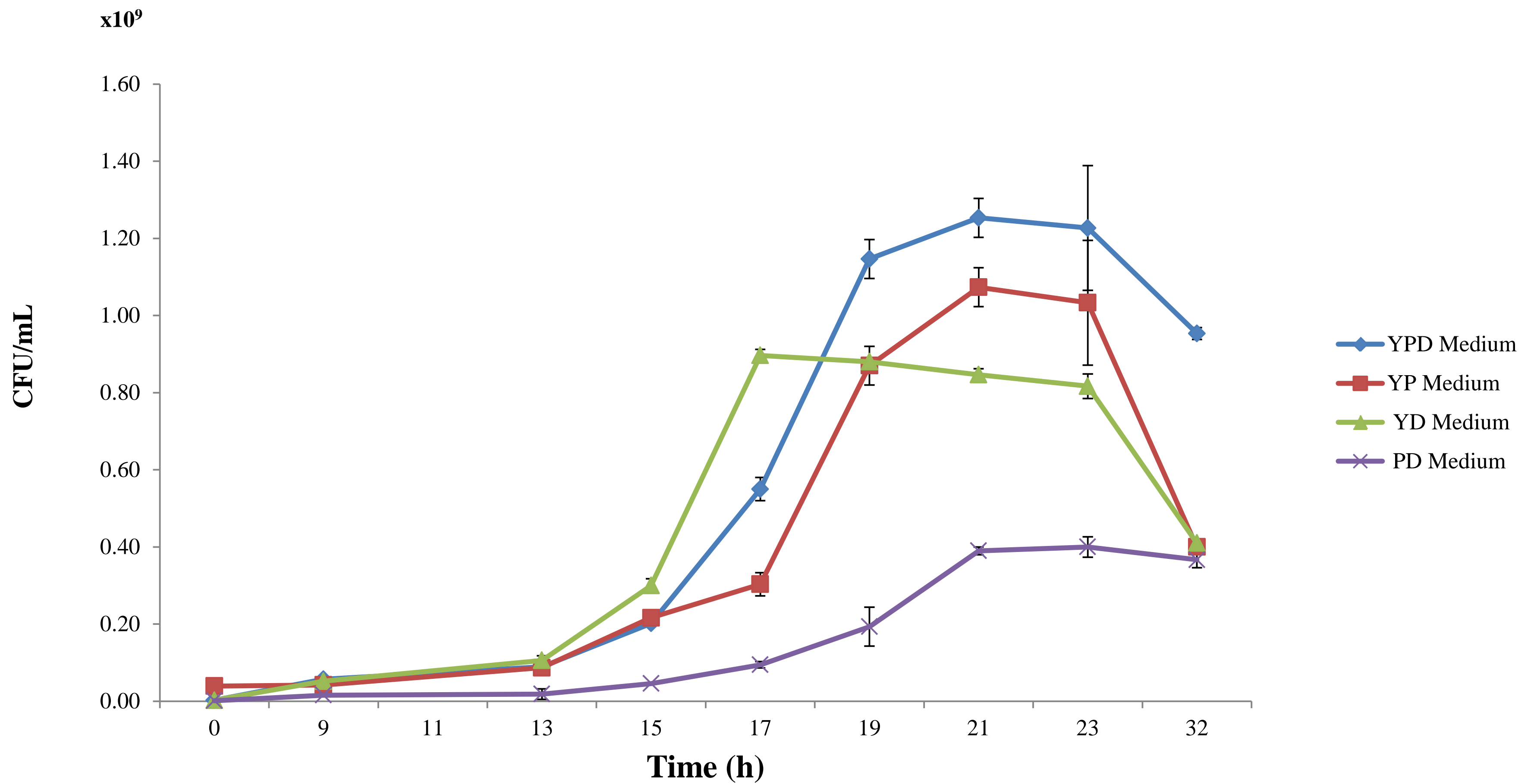
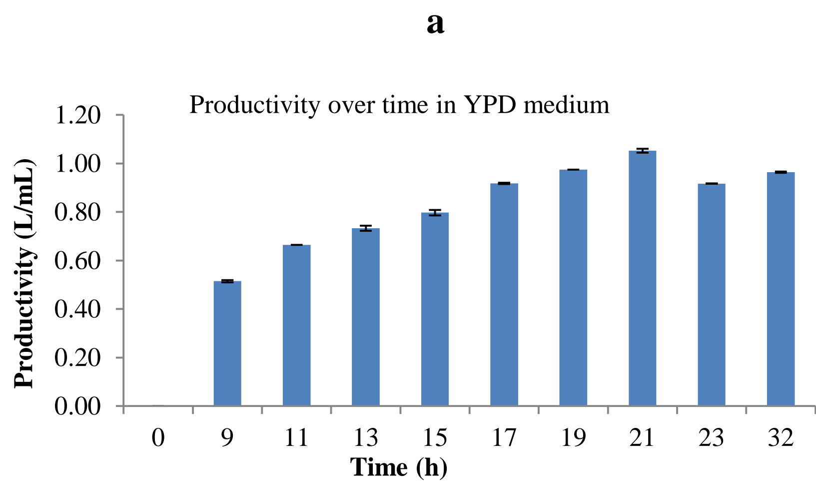


Figure 4-4: Cell counts in colony-forming units (CFU/mL) of *Candida* sp. in YPD, YP, YD and PD media.

a



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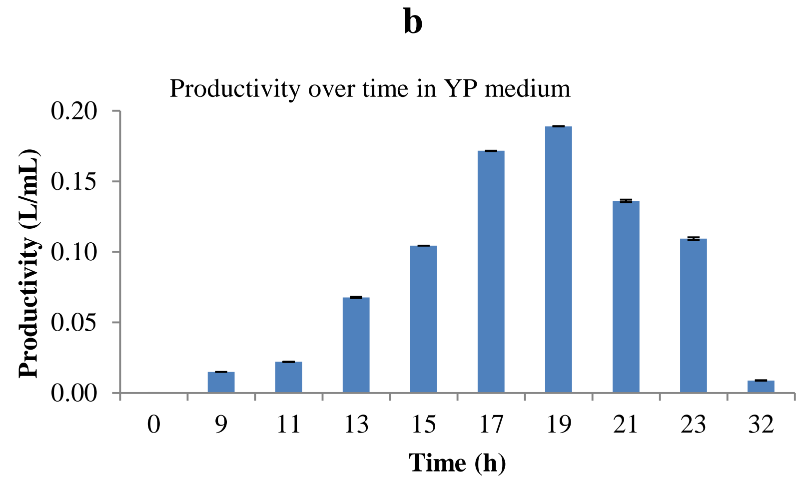

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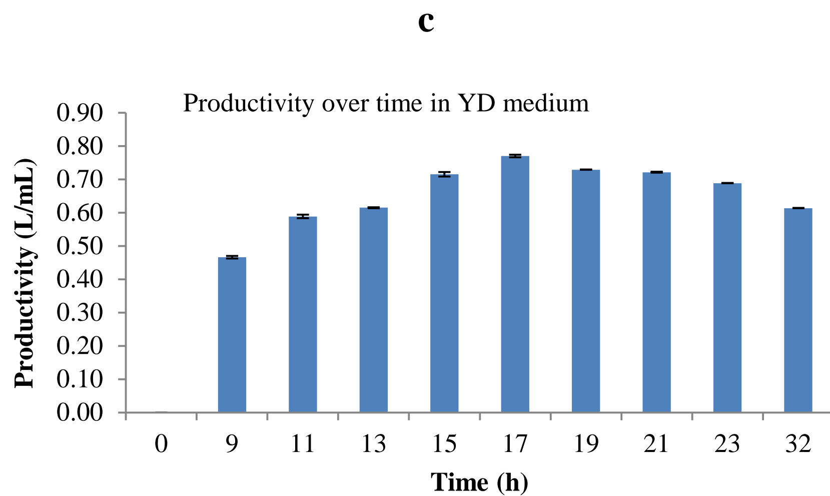


d

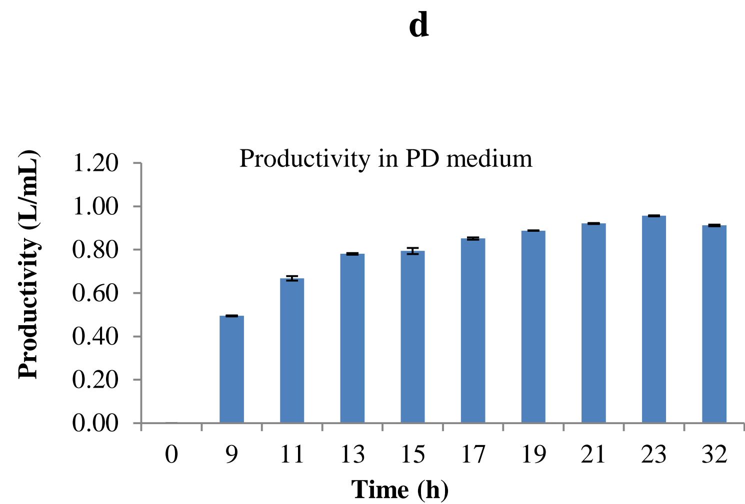
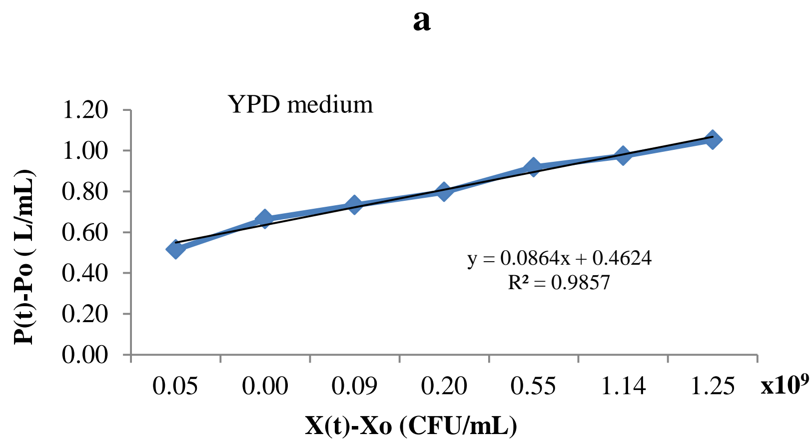
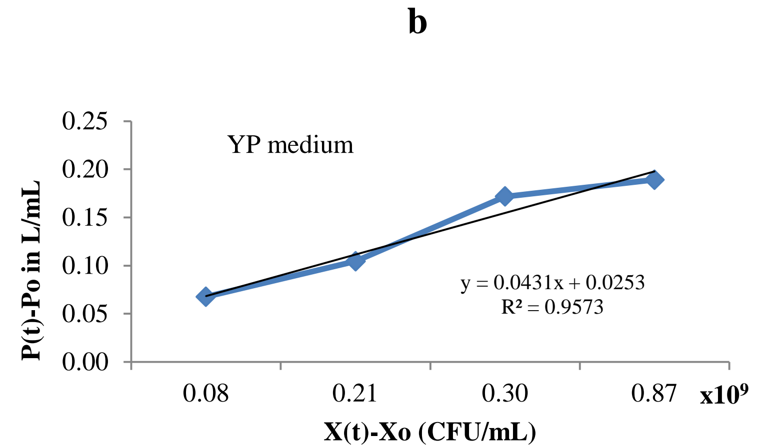
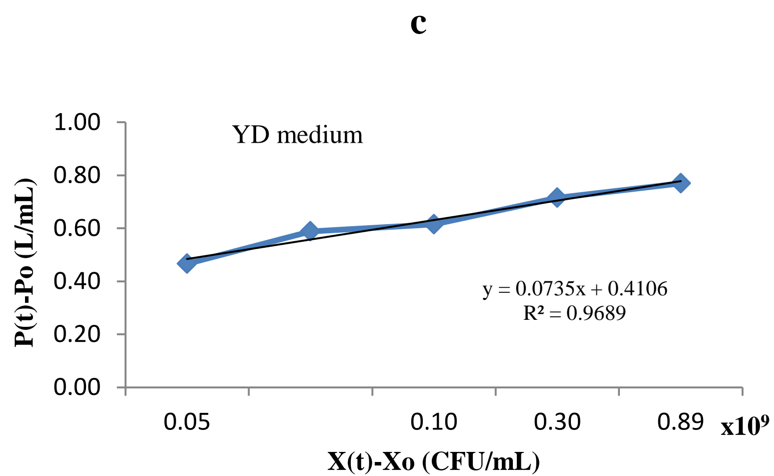


Figure 4-5: a, b, c, and d displaying productivity using the volumetric zone of clearance per volume of broth used in YPD, YP, YD and PD, respectively from *Candida* sp. against *Candida guilliermondii*







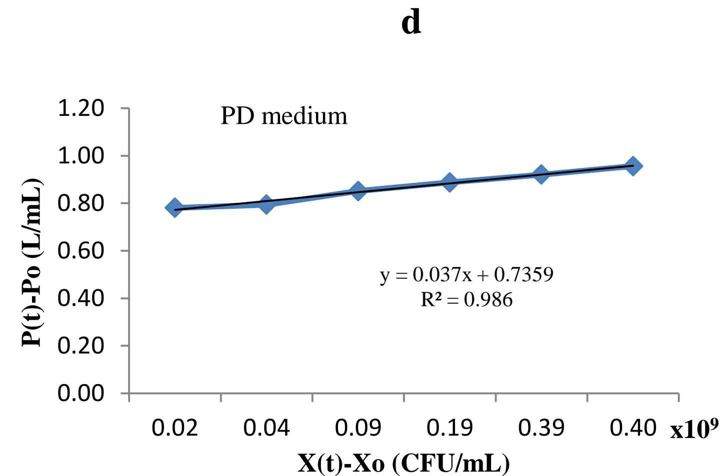
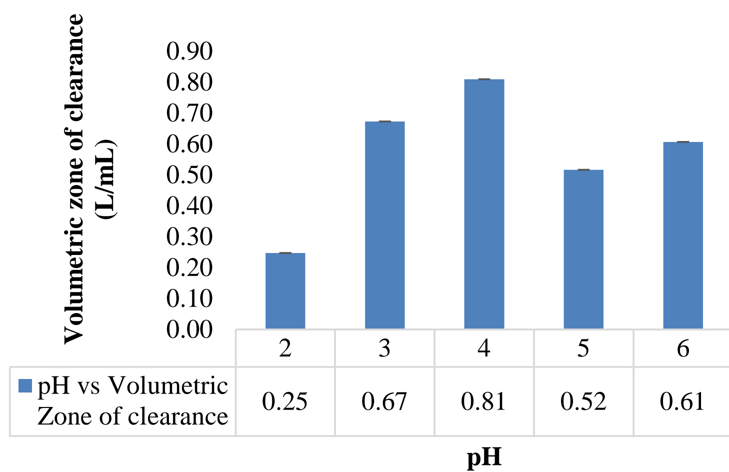


Figure 4-6: a, b, c, and d showing a linear trendline using experimental data in the modified Luedeking–Piret model for YPD, YP, YD and PD respectively from *Candida* sp. over time

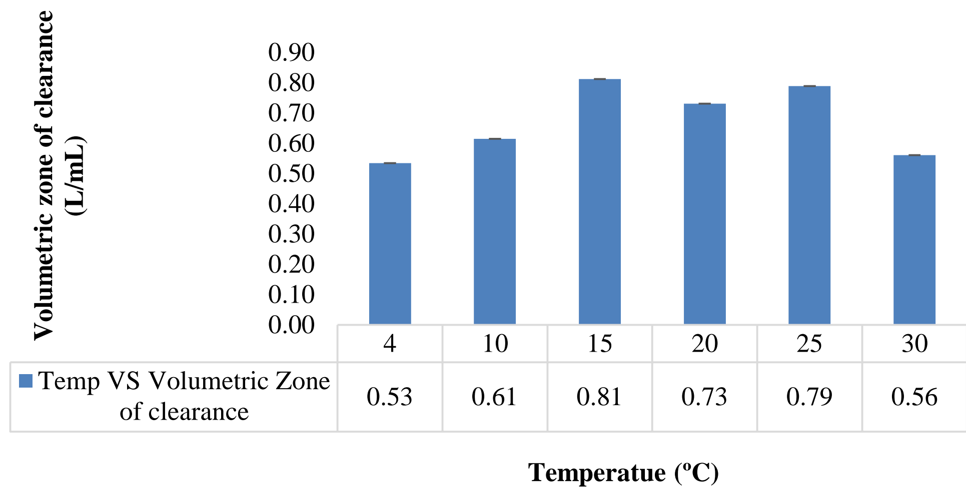
Table 4-5: Fermentation parameters obtained during the biopreservation compound production

Parameter (units)	Model	Values			
		YPD	YP	YD	PD
Biomass yield ($x10^9$ CFU/g)	$Y_{X/S} = \frac{dX}{dS}$	103.07**	152.15**	82.02**	34.96**
Volumetric productivity ($x10^{-9}$ L/CFU) based on cell growth	$Y_{P/S} = \frac{dP}{dX}$	0.43*	0.22*	0.86*	2.40*
Volumetric productivity (L/g) based on substrate utilisation	$Y_{P/S} = \frac{dP}{dS}$	86.76	33.17	70.65	83.90
Substrate utilisation rate ($x10^{-4}$ g/mL.hr)	$r_s = \frac{dS}{dt}$	$5.8^{\#}$	$3.0^{\#}$	$6.4^{\#}$	$5.0^{\#}$
Substrate saturation constant g/mL	$K_s = \frac{(\mu_{max} - \mu)S}{\mu}$	0.02	0.01	0.02	0.02
Biomass formation rate ($x10^9$CFU/ml.hr	$r_x = \frac{dX}{dt}$	0.06**	0.05**	0.05**	0.02**
Volumetric productivity rate (L/ml.hr)	$r_p = \frac{dP}{dt}$	0.18	0.01	0.05	0.04
Specific growth rate (1/hr)	$\mu = \frac{ln(X_f/X_0)}{t}$	0.27	0.26	0.25	0.17
Maximum specific growth rate (1/hr)	$\mu_{max} = \frac{ln(X_f/X_0)}{t}$	0.44	0.38	0.54	0.31

*** ($x10^{-9}$); $^{\#}$($x10^{-4}$) **($x10^{9}$)**



	2	3	4	5	6
pH vs Volumetric Zone of clearance	0.25	0.67	0.81	0.52	0.61



	4	10	15	20	25	30
Temp VS Volumetric Zone of clearance	0.53	0.61	0.81	0.73	0.79	0.56

Figure 4-7: Depiction of the activity test of the biopreservation compounds under different pH and temperatures

4.4.4.3 Optimisation using Response Surface Methodology

The effect of temperature and pH on the biopreservation compounds production was investigated. CCD was used to evaluate what effect temperature and pH would have on the production of the biopreservation compounds in a single-stage bioreactor as well as the optimum production conditions based on the selected independent variables, i.e., temperature (A) and pH (B). This was achieved with a total of 13 runs. The summary of the experimental design is displayed in Appendix C, Table 7-1. The response variable quantified was the biopreservation compounds productivity (Y) expressed in L/mL. The fitness of the model and the reduced quadratic model that explain the biopreservation compounds production, i.e., based on the reduction of the statistical model, sum of squares and lack-of-fit tests, are depicted in Table 4-6. On the one hand, the results obtained showed that the temperature used during the experiment had minimal impact on the productivity compared with the pH, which showed that it significantly impacted the production of the biopreservation compounds. The limited contributory effect of temperature confirmed the fact that the biopreservation compounds would be produced under different fermenter growth temperatures. In other words, whenever there is sufficient growth of *Candiada* sp., there will be biopreservation compounds production; however, these compounds would only be active and stable if the pH is favourable. From the data obtained, temperature had a P-value of 0.82, whereas the P-value for the pH was found to be less than 0.0001. The generated model is shown in Eq. 4.12. It explains the significant effect of each of the input variables. The F-value and correlation coefficient (R^2) for the model were found to be 102.20 and 0.99, respectively, which demonstrates the significance of the mathematical model as well as the good correlation between the values obtained during the experiments and the predicted values. Furthermore, the precision ratio, shown by the coefficient of variance (CV), usually indicates the degree of precision under which the experimental observations are compared – the higher the CV, the lower the precision and reliability of the model (Montgomery, 2007; Nwabueze, 2010; Khuri & Mukhopadhyay, 2010; Myers et al., 2016). In this research work, the model developed had a CV of 0.13 that also demonstrated good precision and reliability of the developed model. Other parameters evaluated include the analysis of variance (ANOVA) for the response surface quadratic model (see Table 4-6).

The probability assessment using the residual plot yielded a linear trendline, meaning that the normality assumption was satisfactory, therefore confirming the adequacy of the model developed (Figure 4-8). Table 4-7 shows the predicted and actual experimental values based on the quadratic model generated in this study. The relationship between the response and experimental behaviour of each variable (temperature and pH) was assessed by generating a 3-D response surface graph (Figure 4-9). Therefore, the optimum production conditions for the biopreservation compounds production was found to be at 22.5 ºC and a pH of 5. The productivity resulting from the biopreservation compounds produced using CCD was satisfactory, thus Eq. 4.12 can be reduced to Eq. 4.13. This was because the analysis of variance conducted demonstrated that A (temperature), AB (product of temperature and pH) had a P-value of 0.82 and 0.74 respectively. This meant that A and AB parameters in the model did not have a significant influence on the prediction for the model developed, therefore Eq. 4.12 could also be represented as:

$$Y = 1.05 - 0.0066A - 0.40B + 0.013AB - 0.49A^2 - 0.22B^2 \quad (4.12)$$

where A, B are coded values for temperature and pH respectively.

$$Y = 1.05 - 0.40B - 0.49A^2 - 0.22B^2 \quad (4.13)$$

Table 4-6: Analysis of variance (ANOVA) for temperature (A) and pH (B)

Source	Sum of Squares	*df*	Mean square	*F* value	Prob > F
Model	3.11	5	0.62	102.20	< 0.0001
A-Temperature	0.00035	1	0.00035	0.057	0.8174
B-pH	1.27	1	1.27	208.50	< 0.0001
AB	0.00070	1	0.00070	0.11	0.7445
A^2	1.67	1	1.67	274.38	< 0.0001
B^2	0.33	1	0.33	54.78	0.0001
Residual	0.043	7	0.0061	-	-
Lack of Fit	0.043	3	0.014	-	-
R^2=0.99					

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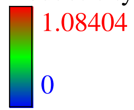

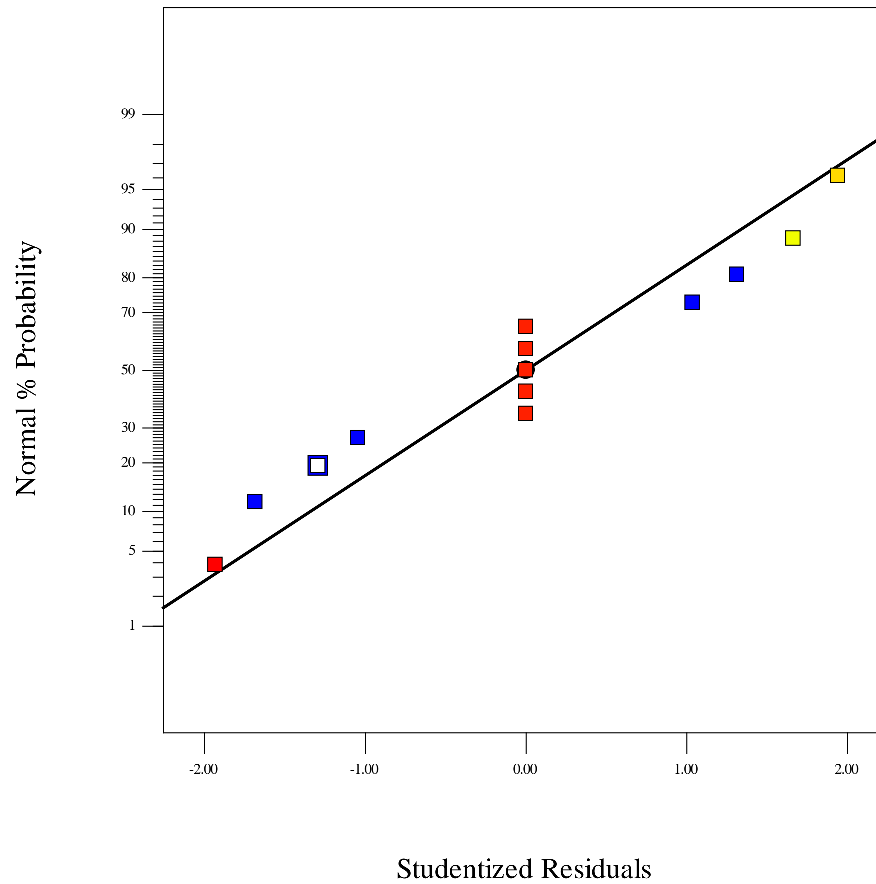

Figure 4-8: Plot of the normal probability versus studentised residuals

Table 4-7: Predicted and observed productivity response obtained using CCD.

Run number	Predicted productivity (L/mL)	Actual productivity (L/mL)
1	1.05	1.05
2	1.05	1.05
3	0.76	0.85
4	0.72	0.80
5	1.05	1.05
6	1.17	1.08
7	1.05	1.05
8	0.05	0
9	-0.04	0
10	0.08	0
11	1.05	1.05
12	-0.06	0
13	0	0

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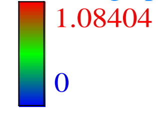
Productivity (L/mL)
Design points above predicted value



X1 = A: Temperature
X2 = B: pH

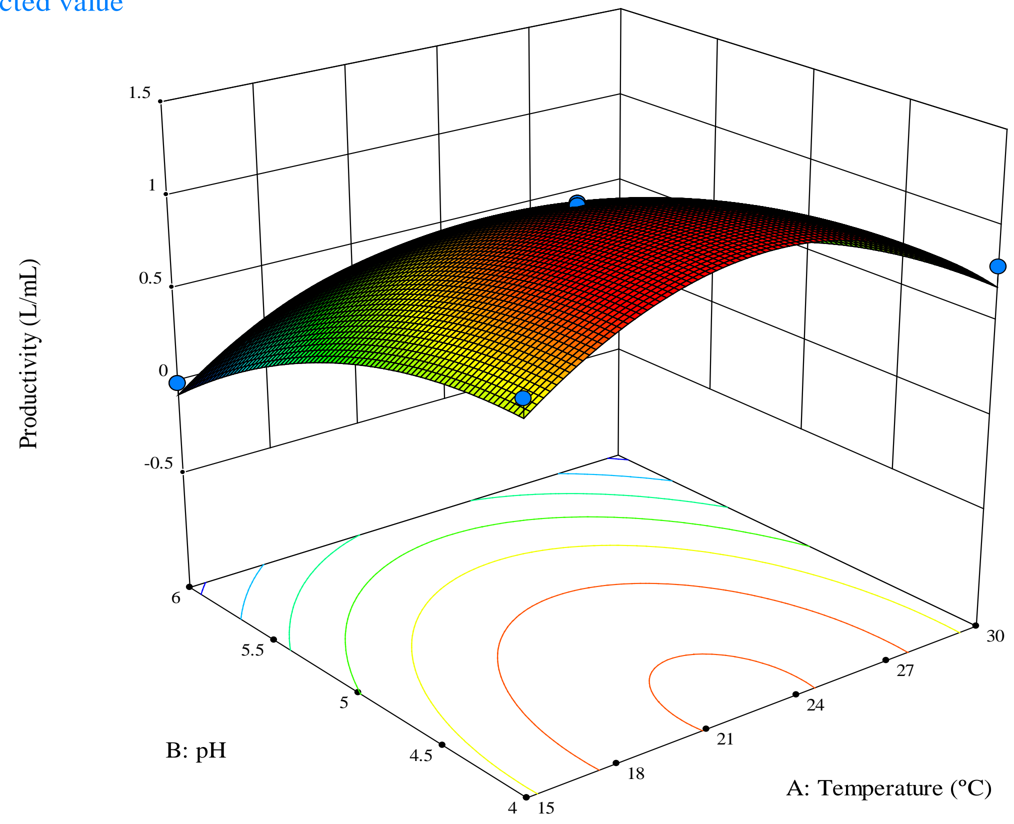


Figure 4-9: Response of temperature (A) and pH (B) on productivity (L/mL)

4.4.5 Characterisation of the antimicrobial compounds

The gel filtration chromatography of the crude biopreservative samples allowed for the collection of 25 fractions of 2 mL each (See Figure 7-3). The results showed that fraction A10 showed the highest activity observed by a higher zone of clearance (D_0, Figure 4-10 also see Figure 7-2) of 10.5 mm, followed by fractions A9, A11, A8 and A12, with zone clearance diameter of 7.83, 7, 3.83 and 2.83 mm, respectively. The sizes of these biomolecules varied between 30 and 40 µm, corresponding to the molecular weight range of 5 and 150 kDa. To narrow the range of the molecular weight of the compounds, the same supernatant containing the biopreservation compounds was further separated by ultrafiltration using Amicon ultrafiltration tubes with 10 and 50 kDa MWCO size. It was found that the retentate of the 10 kDa tubes, which is the portion that was retained by the filters, showed antimicrobial activity, indicating that the biopreservation compounds were bigger than 10 kDa (Figure 4-11a). For the 50 kDa MWCO ultrafiltration tubes, the biopreservative activity was observed in both the retentate and the filtrate. However, it was found that the filtrate had 23.3% less activity than the retentate (see Fig. 4-11b). This confirmed the observation from prior (initial) experiments in which 10 kDa ultrafiltration tubes were used, i.e., that the size of the compounds of interest were larger than 10 kDa. Overall observations were that the crude extract contained more than one biopreservation compound. Further studies are therefore required.

Figure 4-10: The size exclusion chromatography crude fractions that showed biopreservation activity

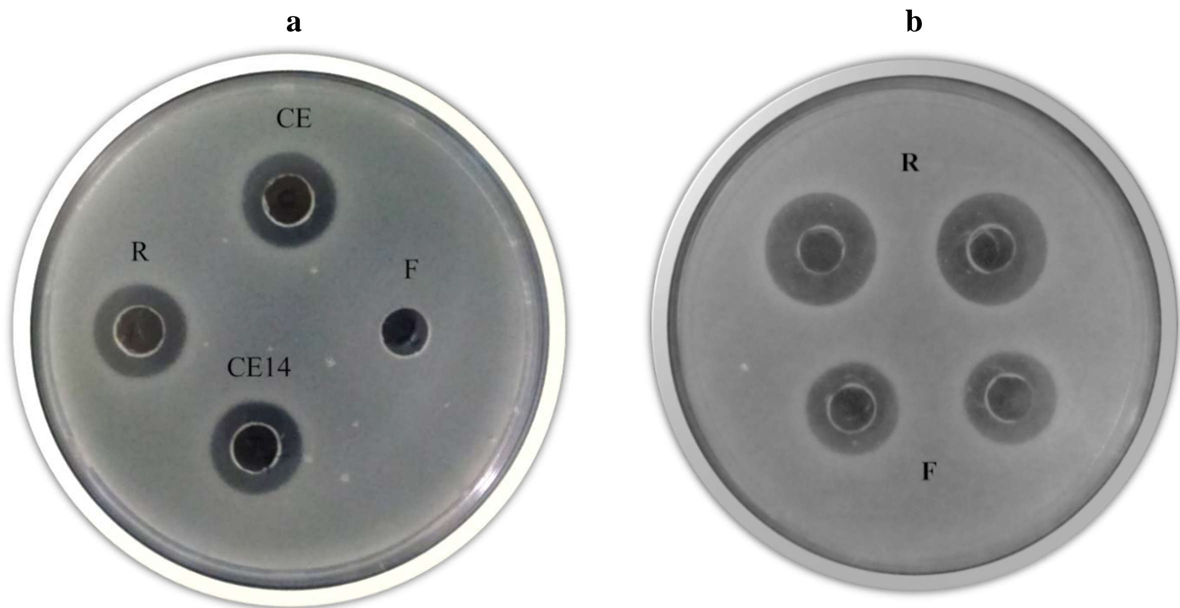


Figure 4-11: Growth inhibition activity of the filtrate and retentate after filtration through (a) 10 kDa and (b) 50 kDa Amicon ultrafiltration tube

4.5 Summary

The *Candida* sp. selected as the fermenter exhibited growth-inhibition activity against numerous strains, including *Brettanomyces bruxellensis*, and *Candida guilliermondii*, among others. The isolate was also able to control completely apple fruit spoilage caused by *Botrytis cinerea* and *Colletotrichum acutatum*. The production kinetics of the crude extract showed that YPD was the best production medium and that the maximum growth inhibition activity was observed during the cellular exponential growth phase of the isolate. The pH and temperature activity studies revealed that the antimicrobial compounds are stable at slightly acidic pH and that they can be stored for longer under refrigeration conditions (4 °C), making the compound suitable for various fermented foods and beverages which are normally kept at 4 °C.

For kinetic studies, it was observed that some fermentation parameters can be estimated using rate constants by fitting fermentation data to suitable models. It was also observed that the growth kinetics of the process carried out in this research fitted the hyperbolic relationship described by the Malthus equation. It was also found that the biopreservation compounds' productivity was growth associated and thus directly proportional to the cellular growth whereby the maximum activity was observed during the exponential growth phase. The antimicrobial compound-producing isolate used was found to have simple growth requirements in accordance with the information found in literature regarding non-*Saccharomyces* yeasts. The rate of substrate utilisation was found to be directly proportional to the cell growth and biopreservation compounds production, justifying the use of Monod's model, i.e., an unstructured model used in this study. These findings were modelled, which led to the development of equations used to explain and represent the fermentation kinetics parameters based on substrate utilisation and maximum achievable rate of biopreservative product formation.

A model was also developed using RSM, which adequately described the fermentation studied. Overall, there was more than one bioactive fraction, with an average MWCO size of 50 kDa being the size of the biopreservatives with a high efficacy against spoilage organisms.

Chapter 5

General discussion, conclusions and recommendations

CHAPTER 5

GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 General discussion

The microbial ecology of yeasts is significantly diverse. During spontaneous fermentations, alcohol-resistant organisms tend to dominate such fermentations. In this work, varieties of yeasts were prevalent in the grape must prior to spontaneous fermentation. It was found that *Saccharomyces cerevisiae* dominated the latter stages of the spontaneous fermentation. To differentiate strains for such fermentations, the use of microsatellite primers could not assist in differentiating between the *Saccharomyces cerevisiae* strains. The electrophoretic gel photographs showed that the *Saccharomyces cerevisiae* strains could not be differentiated using this technique; however the analysis of the delta elements using delta 12 and 21 primers was a better way of differentiation between *Saccharomyces cerevisiae* strains. As far as the differentiation between the non-*Saccharomyces* strains is concerned, it still remains a challenge, since specific primers designed for such a purpose are currently unavailable. Therefore a research study should be undertaken to design such primers. Overall, sequencing remains the best method of identification and differentiation, although not readily affordable.

According to the reported ITS and restriction digest sizes by Granchi et al. (1999) and Solieri et al. (2006), as well as the sequence analysis of the ITS region of the biopreservation compounds producing isolate, it was found that the isolate was *Candida* sp. The NCBI BLAST using the ITS sequence data showed that the non-*Saccharomyces* yeast used in this study could only be grouped with the sequence available in the NCBI database at genus level, and therefore could not be grouped with any isolates at species level in the database. An accession number allocated to this isolate was KU736785.

The antimicrobial efficacy assessments of this *Candida* sp. against fungal pathogens in apple (*M. domestica*) fruits showed that this yeast, fermented in a culture suspension grown to

$1x10^8$ cells/mL, would completely inhibit the growth of pathogens *Botrytis cinerea* and *Colletotrichum acutatum*.

The antimicrobial activity of the biopreservative produced from the non-*Saccharomyces* yeast, *Candida* sp. against the genera of *Brettanomyces* and *Candida* was also studied. Furthermore, the productivity kinetics showed that the maximum antimicrobial activity was achieved during the exponential growth phase of the fermenter after 21, 19, 17 and 23 h in YPD, YP, YD and PD growth medium, respectively. The crude broth samples also showed that the *Bretanomyces lambicus* and *Candida guilliermondii* could be controlled by the antimicrobial compound produced by the species of *Candida* isolated.

The activity of the antimicrobial compound at different pH and temperatures as well as the stability at different storage temperatures was investigated with the results showing that the 0.45µm filtered crude samples could retain their activity under various pH and temperature. The storage stability test demonstrated that the crude biopreservative sample could still demonstrate antimicrobial activity even when stored at a lower temperature (4 ºC) for several weeks. Overall, at low temperature (4 ºC), the crude samples retained their antimicrobial activity up to 12 weeks.

The use of kinetic studies and mathematical modelling when studying non-*Saccharomyces* yeast as producers of antimicrobial compounds usable in food and beverage preservation is of importance for several reasons. For instance, in the case of the use of cell suspension to control spoilage, it is imperative to understand the requirements and kinetics of the biocontrol agent to be able to predict and account for the substrate to be utilised by the organism. In fermented foods and beverages, the initial substrate for the entire process is usually known, therefore it is important to account for the quantities utilised by the growth-inhibiting organism so that any deviation and product formation can be accounted for and corrected when necessary during the production process. In other words, downstream process requirements could be assessed and improved efficiently when the fermentation kinetics as well as the factors affecting the fermentation process is known. The mathematical modelling of the biopreservation production process was conducted and a quadratic equation was developed to predict the biopreservative production under optimised conditions. The developed model was found to be of high significance in explaining the fermentation parameters and conditions adopted in the process studied. The use of RSM and CCD was also found to be a suitable approach in

bioprocess engineering modelling applicable in the biopreservation compound production in a single-stage bioreactor as demonstrated in this study.

The growth requirements, when the end-product of interest is extracellular, are of great importance (Doran, 1995; Stanbury et al., 1995). During the biopreservative production study, under different media and composition, it was found that the species of *Candida* used to produce antimicrobial compounds had very simple growth requirements. It was also found that it could still grow and secrete the antimicrobial compound under limited nutrient conditions.

In the YPD medium, the maximum activity as a result of the biopreservation compounds was observed after 21 h of fermentation, corresponding to the maximum cell concentration achieved. In the other bioreactors, the same scenario was observed but at different fermentation times. It was discovered that in the media containing yeast extract and peptone, the crude extracts obtained beyond 32 h of fermentation showed minimal antimicrobial activity, whereas in YD and PD media, the crude sample still showed antimicrobial activity beyond 32 h, with reduced activity. This suggested the noticeable effect of the combination of yeast extract and peptone on the biopreservative stability post fermentation. Overall, the extracts from YD and PD media showed a higher antimicrobial activity when compared with YP cultures.

The media composition variation by doubling the PD to 6 g/L of peptone and dextrose did not show a significant variation. Similarly, using half of the nutrient composition (1.5 g/L) resulted in almost the same activity as when using 3 g/L and 6 g/L; however it was observed that the maximum antimicrobial activity was reached earlier during fermentation in media containing less of the growth-limiting substrate, which suggested that the fermenter produces these compounds under nutrient-limited conditions – an antagonistic act which is used by organisms to reduce the prevalence of other organisms under nutrient-limited conditions. These findings proved the simplicity of growth requirements of this species of yeast, which is also in agreement with the information published in literature regarding non-*Saccharomyces* yeasts.

An attempt to characterise the antimicrobial compound was made with findings demonstrating that the antimicrobial activity observed could be the result of more than one bioactive compound with a size range approximated at 50 kDa MWCO.

5.2 Conclusions

During this research work, different *Saccharomyces* and non-*Saccharomyces* were successfully isolated and identified from grape must during spontaneous fermentation. A species of *Candida* sp. was found to produce an antimicrobial compound active against spoilage organisms found in fermented beverages and fruits. It was concluded that the crude antimicrobial compound found was active at various pH and temperatures and could be stored at low temperatures to retain its efficacy.

The experimental data obtained could be used in mathematical models with modifications and rearrangements of previously developed models such as the Malthus, Monod, and Luedeking–Piret models. These models were determined to be suitable for modelling processes of the antimicrobial compound production as observed in this research study. It was finally concluded that the antimicrobial compound was growth associated, media independent and directly proportional to the rate of substrate utilisation and cellular growth.

The mathematical model generated from the response surface methodology experiments was successfully proved to be appropriate and statistically significant in explaining the biopreservation compounds production as observed in this research study.

5.3 Recommendations

The aim of the present study was to produce antimicrobial compounds from non-*Saccharomyces* yeast using a single-stage bioreactor. The first phase consisted of isolation and identification of non-*Saccharomyces* yeast with the ability to produce biopreservation compounds. The second phase involved the production, kinetic study, optimisation and modelling of the process. After the findings during the course of this research work, it is recommended that:

- for non-*Saccharomyces* yeast identification, specific differentiating PCR primers be used to differentiate between non-*Saccharomyces* yeasts strains found in this study;
- the crude samples be assessed at different concentrations on pathogenic fruit fungi to determine whether the antimicrobial activity on fruits is pathogen-concentration dependent;

- the absence of antimicrobial activity after a certain period of fermentation be investigated by studying substrate inhibition effects; and
- the crude sample fractions from the SEC be investigated to identify the individual constituents in the biopreservatives, which will allow researchers to understand the biological action of the biocontrol agents.

Chapter 6

References

CHAPTER 6
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Chapter 7

Appendices

CHAPTER 7

APPENDICES

7.1 Appendix A: Sequence of the internal transcribed spacer (ITS) region used for the elucidation of the identification of the biopreservative-producing isolates

The PCR product resulted from the ITS region of the *Candida* sp. was sequenced. The sequence below was obtained and blasted in the NCBI database.

```
  1 GGGGCTTTTG ACATTATATC CACGTACTGA TAACAGAGCC
 41 TCGGCGGCTT GCCGCCAAGG ACCAAACTAA ACAAACTTAA
 81 TTTGTCAAGA AACTAATAAT CAAAACTTTC AACAACGGAT
121 CTCTTGGTTC TCGCATCATG AAAAACGCGC CGAACTGGGA
161 TAAGTAATAT GAGTTGCATA CTTGGTGAAT CATCCAATTT
201 TTGAACGCAC ATTGCGCCTT GTGGCTTTCC ACATGGCATG
241 CCGGTTTGAG CGTCTTCCAC CCCCAAACCC CCGGGTTTGG
281 AAAGGATTGA TCAGACGTCT CCTGAAAATT TATGGAATTT
321 TGGGTCGGGG ATTGCCCGCC AGAAGCTTAC TAACTAAATT
361 TTGACCCCAA ATCAGGAAGG ATTACCCGCT AAACTTACTC
401 ATATCAGTAA CCGAAGGAAA
```

Figure 7-1: The ITS sequence of the *Candida* sp. studied.

7.2 Appendix B: Antimicrobial compound productivity calculations

7.2.1 B1: Volumetric zone of clearance concept development and calculations

The agar plate previously seeded with the sensitive organism possesses a thickness of 0.2 cm represented as *H* (similar to height of a cylinder).

The zone of clearance was circular, including the pierced hole where the volume of 0.02mL of the biopreservative was spotted.

The diameter (d) of the pieced hole (8.5 mm) was subtracted from the total zone of clearance (D_0) calculated.

The resulting diameter (D) corresponded to the diameter zone of clearance generated by the volume of 0.02 mL of the biopreservation compounds spotted in the pierced well.

The volumetric zone of clearance was then calculated as follows:

Area of the circular surface of clearance (A) x Height (H)

$A = \pi r^2$ (r =D/2)

$V = A.H$

This represented the volumetric zone of clearance (cm^3), but the symbol mL or L was used for consistency. The interpretation of the volumetric zone of clearance was that 0.02mL of the biopreservative sample was able to inhibit the growth of the spoilage organism present in that specific volume at a concentration of $1x10^6$ Cells/mL.

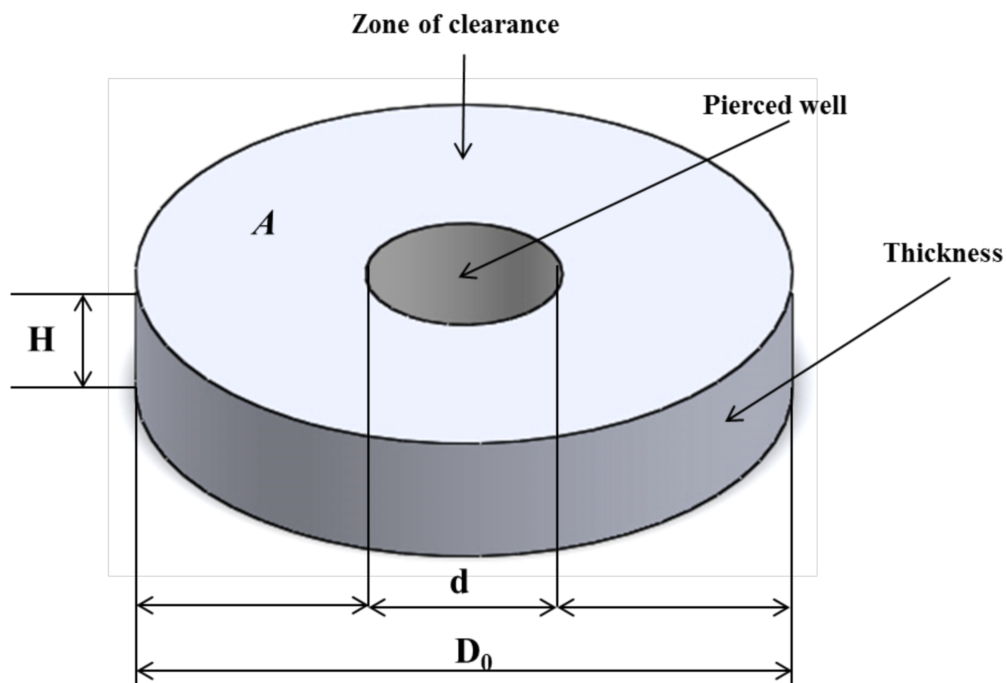

Figure 7-2: Sketch of the concept developed to calculate the volumetric zone of clearance.

7.2.2 B2: Volumetric productivity rate calculations

The volumetric productivity rate was calculated based on what volumetric zone of clearance 0.02mL of the biopreservative would yield at specific times during fermentation. This approach led to the units of volumetric rate of productivity to be L/mL.hr. In other terms, this could be interpreted as the homogeneous volume (L) of food or beverage that 1 mL of the biopreservative could control.

7.3 Appendix C: Central composite design summary used for optimisation of biopreservative production.

Table 7-1: Summary of the CCD used in this study

Design Summary					
File Version	10.0.0.3				
Study Type	Response Surface	**Subtype**	Randomised		
Design Type	Central Composite	**Runs**	13		
Design Model	Quadratic	**Blocks**	No Blocks	**Build Time (min)**	62.00

Factor	Name	Units	Type	Subtype	Minimum	Maximum	Coded	Values	Mean	Std Dev.
A	Temperature	C	Numeric	Continuous	11.8934	33.1066	-1.000=15	1.000=30	22.5	6.12372
B	pH		Numeric	Continuous	3.58579	6.41421	-1.000=4	1.000=6	5	0.816497

Response	Name	Units	Obs	Analysis	Minimum	Maximum	Mean	Std Dev.	Ratio	Trans	Model
R1	Productivity	L/mL	13	Polynomial	0	1.08404	0.614747	0.512528	N/A	None	Quadratic

7.4 Appendix D: Size exclusion chromatography profile

The biopreservative crude sample was produced using the optimised condition based on temperature and pH (22.5ºC, pH 5). A total volume of 100 mL was loaded into the column and 25 fractions were collected as depicted in Figure 7-3 below.

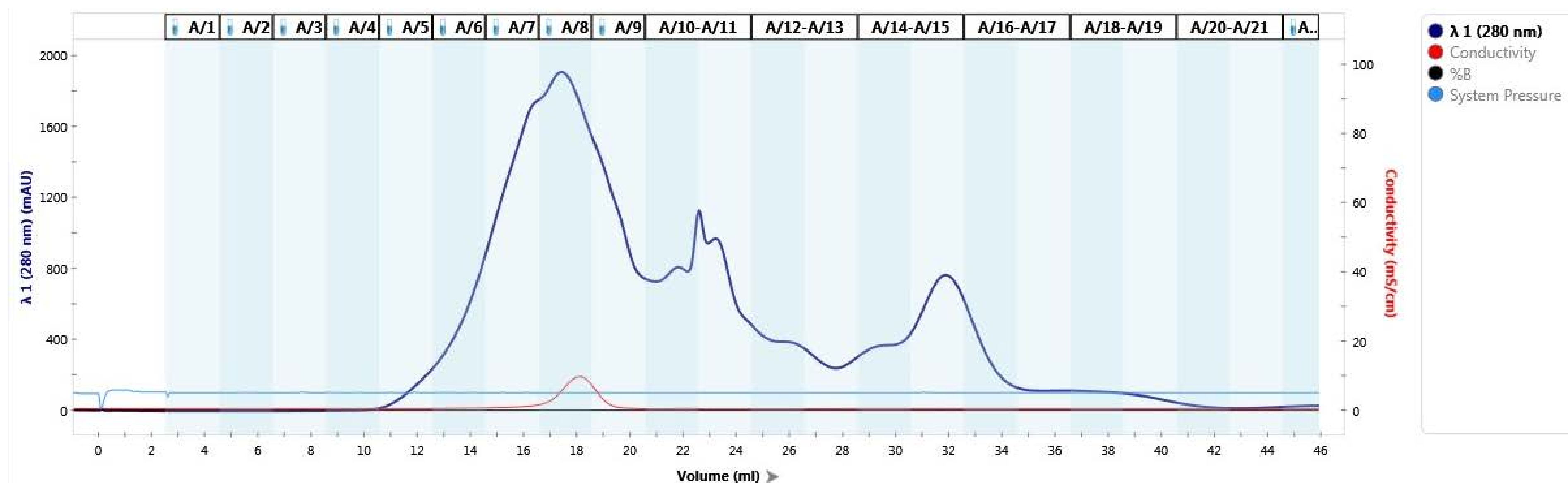

Figure 7-3: Chromatogram obtained during the size exclusion chromatography on the biopreservation compounds.