System design for production of biopreservatives from yeasts for reduction of fruit and beverage spoilage organisms

by

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DECLARATION

I, **Maxwell Mewa Ngongang**, know the meaning of plagiarism and declare that all the work in this thesis is my own unaided work, both in concept and execution, apart from the normal guidance of my supervisors. Furthermore, the thesis represents my own opinions and not necessarily that of the Agricultural Research Council, the National Research foundation of South Africa or that of the Cape Peninsula University of Technology and their sponsors. Furthermore, this thesis has not been submitted for any degree or examination in any other university. The intellectual concepts, theories, methodologies and mathematical derivations and model developments used in this thesis and published in various scientific journals were derived solely by the candidate and first author of the published manuscripts. Where appropriate, the intellectual property of others was acknowledged by using appropriate references.

The contribution of co-authors, for conference and published manuscripts, was in a training capacity, research assistance and supervisory capacity to meet the requirements for the doctoral degree award.

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ABSTRACT

The agro-processing industry is currently facing losses due to microbial spoilage of agricultural produce and associated value-added products such as beverages. Decay and undesired fermentation of fruit and beverages by fungal, yeast and bacterial spoilage organisms are among the major contributors of product losses in the food industry. When looking at the different level of food spoilage, it is common to find different spoilage organisms occurring in the same food item; which usually requires food producers to utilise a mixture of synthetic preservatives for spoilage organism control. Some of the synthetic chemical compounds with growth inhibition properties that have been used in food preservation are sulphur dioxide, benzoic, lactic, sorbic and acetic acid. These compounds act against a variety of spoilage microorganisms. In post-harvest control of fungi, triazoles, hydroanilide fenhexamid, dicarboximides and succinate dehydrogenase are also being used. Some spoilage organisms have been found to be resistant to the use of synthetic chemical preservatives which usually favour the use of higher dosage of preservatives in food. The use of synthetic chemicals as preservative and as postharvest control agents has been found to present serious health risks such as cardiovascular diseases, muscles and stomach pains, eyesight and skin damages and impairment of brain functions. The problem posed by the current use of synthetic chemicals in food put pressure on food producers and exporters to seek alternatives that will allow for the eradication of the use of synthetic chemicals as preservative in beverages and as postharvest control agents on fruits.

Yeasts have been found to have the ability to grow at a faster rate on cheap media and to colonise dried surfaces rapidly. It has also been found that yeasts produce extracellular compounds of proteinaceous and volatile organic nature with growth inhibition properties against spoilage organisms. The current findings lack some engineering concept that could assist in the design of a production system for high scale production of biopreservation compounds from yeasts. The availability of a cost effective production media, the growth and production kinetics data using a cheaply available nutrient sources as well as the biological thermodynamic data are some of the gaps in biopreservation bioprospecting. Although several yeasts have already been studied to have great inhibition properties against fruit fungal pathogens, it was still unclear what was the minimum inoculum dose to be able to have a fungistatic and fungicidal effect on the growth of fruit spoilage organisms. The concept of combination of biopreservatives and the interaction effect of their biopreservation activity against consortia of spoilage organisms has also been lacking.

As an attempt to seek alternatives to the use of synthetic chemicals as preservatives or postharvest control agents, *Candida pyralidae Y1117, Pichia kluyveri Y1125 and Pichia kluyveri Y1164* strains were assessed for antimicrobial activity against spoilage yeasts (*Dekkera bruxellensis, Dekkera anomala, Zygosaccharomyces bailii*) and spoilage fungi (*Botrytis cinerea, Colletotrichum acutatum* and *Rhizopus stolonifer*). As alternative to refined media, a cost effective approach was explored whereby the use of agro-waste, i.e. grape pomace extracts (GPE), as production medium for biopreservation compounds, was

studied. Production kinetics using modified existing models, subsequent to optimization using response surface methodology (RSM) for biopreservation compounds production was studied for the three biocontrol yeasts using GPE broth as the fermentation medium. The evaluation of the interaction study between mixtures of crude biopreservatives against consortia of common spoilage organisms present in beverages was also conducted by producing the crude biopreservation compounds separately from yeasts and then formulating growth inhibition combinations (GICs); GIC 1 (*Candida pyralidae* Y1117 and *Pichia kluyveri* Y1125); GIC 2 (*C. pyralidae* Y1117 and *P. kluyveri* Y1164), GIC 3 (*P. kluyveri* Y1125 and *Pichia kluyveri* Y1164); GIC 4 (*C. pyralidae, P. kluyveri* Y1125 and *P. kluyveri* Y1164). The spoilage organism consortia combinations, i.e. SC1, *D. anomala* and *D. bruxellensis*; SC2 (*D. anomala* and *Z. bailii*); SC3 (*D. bruxellensis* and *Z. bailii*) and SC4 (*D. anomala, D. bruxellensis* and *Z. bailii*) were also prepared. This study also investigated the effect of varying inoculum dose (ID) of *Candida pyralidae* strain Y1117, *Pichia kluyveri* Y1125 and *Pichia kluyveri* Y1164 on the biocontrol of *Botrytis cinerea* by contaminating the headspace of the growth medium with a fungal plug subsequent to biotreatment with different initial inoculum dose of the respective biocontrol yeasts. Finally, grape pomace extracts was used as fermentation medium to study the biological thermodynamics of biopreservation compound production from the three biocontrol yeasts.

The results obtained demonstrated some interesting results. The antagonistic properties of *C. pyralidae* and *P. kluyveri* were observed on cheap solidified medium (grape pomace extracts) as well as on fruits (grapes and apples). These yeasts produced extracellular volatile organic compounds (VOCs) that could be responsible for yeast and fungal growth inhibition. Twenty-five VOCs in the category of alcohols, organic acids and esters were identified by GC-MS. The results of the kinetic study showed that the highest volumetric zone of inhibition (VZI) was 1.24 L contaminated solidified media (CSM) per mL biopreservation compounds used (BCU) when *Candida pyralidae* Y1117 was inoculated in a pH 3-diluted GPE broth (150 g L^{-1}) incubated at 25 °C for 24 h. Similar conditions were applied for *Pichia kluyveri* Y1125 and *P. kluyveri* Y1164, albeit under slightly elongated fermentation periods (up to 28 h), prior to the attainment of a maximum VZI of only 0.72 and 0.76 L CSM mL^{-1} ACU, respectively. The potential biopreservation compounds produced were identified to be isoamyl acetate, isoamyl alcohol, 2-phenyl ethylacetate and 2-phenyl ethanol. The growth inhibition interaction study showed a variation in growth inhibition proficiency depending on the spoilage organisms or the consortia of spoilage organisms being deactivated. It was then suggested that, a food environment contaminated with a consortium of spoilage organisms can be controlled by employing either the crude biopreservation compounds from individual yeast or those of the following yeast combinations, GIC1-4, which showed a better growth inhibition proficiency against SC1-3. The fungistatic and fungicidal effects on the fungal pathogen were dose dependent. The fungistatic characteristics against *Botrytis cinerea* were displayed after 7 days when 10^2-10^5 cells mL^{-1} of *Candida pyralidae* Y1117, *Pichia kluyveri* Y1125 and *Pichia kluyveri* Y1164 were independently used *in-vitro* and *in-vivo*. However, 10^6-10^8 cells mL^{-1} inoculum doses displayed fungicidal

characteristics. Additionally, the fungicidal property of yeasts studied was also confirmed on table grape (in vivo studies) using closed jar method. The biological thermodynamic study showed that, dried biomass molecular weight of 28.9 g/C-mol, 29.163 g/C-mol, and 27.176 g/C-mol were obtained for *Candida pyralidae* strain Y1117, *Pichia kluyveri* Y1125 and *Pichia kluyveri* Y1164 respectively. The results obtained successfully established useful biological thermodynamic data applicable to the design of adequate biopreservatives production system from yeasts using cheaply available nutrients source.

Keywords: Biopreservation compounds, *Candida pyralidae*, *Pichia kluyveri*, Post-harvest biocontrol, Volatile organic compounds, production kinetics, grape pomace; microbial consortia, Fungicidal, Fungistatic, biological stoichiometry and bioenergetics.

DEDICATION

I dedicate this thesis to my parents, papa Ngongang Dieudonné and maman Diemo Epouse Ngongang; and 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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RESEARCH OUTPUTS

The following research outputs represent the contributions by the candidate to scientific knowledge and development during the doctoral candidacy (2017-2019):

Published forming part of the thesis

Mewa-Ngongang, M.; du Plessis, H.W.; Ntwampe, S.K.O.; Chidi, B.S.; Hutchinson, U.F.; Mekuto, L.; Jolly, N.P. The Use of *Candida pyralidae* and *Pichia kluyveri* to Control Spoilage Microorganisms of Raw Fruits Used for Beverage Production. *Foods*, *8*(10), p.454. doi.org/10.3390/foods8100454

Ngongang, M.M.; Ntwampe, S.K.O.; du Plessis, H.W.; Jolly, N.P.; Mekuto, L. Biopreservatives from yeasts with antimicrobial activity against common food, agricultural produce and beverage spoilage organisms. In book Antimicrobial Research: Novel bioknowledge and educational programs, Series Nº 6; Méndez-Vilas, A. In; Formatex Research Center, C/ Zurbarán, 1, 2° - Oficina 1 06002 Badajoz Spain, 2017, pp. 219-228.

Mewa-Ngongang, M., du Plessis, H.W., Ntwampe, S.K., Chidi, B.S., Hutchinson, U.F., Mekuto, L. & Jolly, N.P. 2019. Grape Pomace Extracts as Fermentation Medium for the Production of Potential Biopreservation Compounds. *Foods*, 8(2): 51. doi:10.3390/foods8020051

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M. Mewa-Ngongang, H.W. du Plessis, E. Hlangwani, S.K.O. Ntwampe, B.S. Chidi, U.F. Hutchinson, N.P. Jolly. Activity interaction of crude biopreservation compounds against beverage spoilage consortia. *Fermentation* 2019, 5(3), 53; DOI: 10.3390/fermentation5030053.

LAYOUT OF THESIS

This research study was conducted at the Agricultural Research Council, Infruitec-Nietvoorbij Stellenbosch, South Africa. The references listed at the end of the thesis were listed in accordance with the CPUT Harvard method of referencing.

The thesis is divided into the following chapters:

- **Chapter 1**: This chapter covers the background of the research topic, the problem statement, the motivation for the execution of this study, the hypothesis and research questions that required experimental responses and the delineation of the study.
- **Chapter 2**: This chapter focuses on the literature consulted whereby a detailed explanation of the background is explained with the assistance of published research information. In this chapter, the research gaps and niches are highlighted from the existing available information, with a focused integration of these gaps and niches into the undertaken study.
- **Chapter 3**: This chapter reports on the materials and methods utilised for the accomplishment of the objectives of this study with appropriate references of consulted methodologies.
- **Chapter 4**: This chapter focuses on the screening of biological control yeasts for their ability to inhibit the growth of spoilage yeasts and fungi.
- **Chapter 5**: This chapter focuses on kinetics and optimisation of biopreservation compound production by the biocontrol yeasts using grape pomace extracts as fermentation medium.
- **Chapter 6**: This chapter is centred on the growth inhibition activity of mix crude biopreservatives against beverage spoilage yeasts consortia.
- **Chapter 7**: This chapter focuses on the inoculum dose that can yield a static and/or cidal effect of biocontrol yeast cells against the model spoilage fungus *Botrytis cinerea*.
- **Chapter 8**: This chapter looks at the biological thermodynamics during biopreservation compound production using agrowaste (grape pomace extracts) as fermentation medium.
- **Chapter 9** The summary of this study and future research recommendations are covered in this chapter. This chapter also highlights the scientific advancements that have been achieved in the current study.
- **Chapter 10**: The literature citations used in this study are listed in this chapter, in accordance with the CPUT Havard style of referencing.

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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 time (h)
AAB	Acetic Acid Bacteria
ANOVA	Analysis of variance
ARC	Agricultural Research Council
B	Coded variable for pH
BCU	Biopreservation compounds used (mL)
C	Coded variable for temperature (°C)
CCD	Central Composite Design
CFU	Colony-Forming Unit
CPG	Consumer packaged goods
CSM	Contaminated solidified media
CV	Coefficient of variance
D	Coded variable for total sugar ($g\ L^{-1}$)
D	Resulting diameter (cm)
DI	Disease incidence (%)
D_0	Total diameter (cm)
F	Fisher
FMCG	Fast moving consumer goods
FRI	Fungal radial inhibition (%)
GC	Gas Chromatography
GICs	Growth inhibition combinations
GPEA	Grape pomace extract agar
GPEB	Grape pomace extract broth
H	Height (cm)
K_S	Substrate saturation constant ($g.\ mL^{-1}$)
LD	Lesion Diameter (mm)
m	Cell maintenance parameter (h^{-1})
MID	Minimum inoculum dose ($cells\ mL^{-1}$)

MS	Mass Spectrophotometry
MW	Molecular weight (g C-mol^{-1})
r	Radius (cm)
R^2	Correlation coefficient/Coefficient of determination
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})
SCs	Spoilage combinations
SEC	Size exclusion chromatography
S_o	Initial substrate concentration (g mL^{-1})
t	Time (h)
T	Temperature (^oC)
V	Volume (L)
VOCs	Volitile organic compounds
VZI	Volumatric zone of inhibition (L CSM mL^{-1} BCU)
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)
$Y_{p/s}$	Productivity yield coefficient L.g^{-1} (Litter volumetric zone of clearance per gram of substrate)
YAN	Yeast assimilable nitrogen (mg L^{-1})
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

ε		Random error
	π	Numerical value of pi (3.14)
	ΔG	Gibbs energy (Kj mol^{-1})
	ΔH	Enthalpy changes (Kj mol^{-1})
	ΔS	Antropy changes (Kj mol^{-1})

CHAPTER 1

GENERAL INTRODUCTION

CHAPTER 1

INTRODUCTION

1.1 Introduction

Extending the shelf life and improving the quality of food including agricultural produce post-harvest, and during transportation remains the key concern in the perishable food product industry. Synthetic chemical preservatives and post-harvest control fungicides are commonly used to preserve and extend the shelf life of many food products (Okafor, 2007; FAO, 2013; Lipinski et al., 2013; Miller & Welch, 2013; Mehlomakulu et al., 2014).

The increase in fruit, processed food and beverage contamination has become problematic in the fast-moving consumer goods (FMCG) or consumer packaged goods (CPG) industry (FAO, 2013; Lipinski et al., 2013; Miller & Welch, 2013). Food and fruits produced for local consumption and exportation contribute significantly to the country's economy. However, in recent years, contamination including spoilage of these products by some microbial species resistant to chemical preservatives, result in spoilage; although partial contamination control has been achieved using chemical preservatives such as dimethyl dicarbonate benzoic, lactic, sorbic, acetic acid triazoles, hydroanilide fenhexamid, dicarboximides, succinate dehydrogenase and sulphur dioxide (SO_2) (Sofos & Busta, 1981;Du Toit & Pretorius, 2000; Okafor, 2007; Mehlomakulu et al., 2014).

The utilisation of certain synthetic chemicals as preservatives or fungicides to limit contamination, thus spoilage, is prohibited in beverages and some foods, including fruits produced for export (Benito et al., 2009). The use of synthetic chemicals also presents serious health concerns. This requires food, fruits and beverage producers, including exporters to completely eradicate the use of chemical preservatives from their products and to bioprospect and develop effective, non-toxic and natural compounds, with similar properties to those observed for chemical preservatives.

From a pilot study conducted by Mewa-Ngongang et al. (2017), a strain of *Candida pyralidae* was found to produce compounds with broad antimicrobial activity, thus biopreservation capabilities, against beverage spoilage organisms such as *Candida guilliermondii*, *Dekkera bruxellensis*, *Dekkera anomala* and *Zygosaccharomyces bailli*. This *C. pyralidae* strain was able to control the spoilage of apple fruits caused by *Penicillium expansum* and *Botrytis cinerea*. The discoveries from the pilot study were used to develop a production system and assess the field application of biopreservation compounds produced from other yeasts for the purpose of reducing fruit and beverage spoilage organisms.

1.2 Problem statement

To safeguard the general public nutritional needs, global food production should be increased by 80% in the next four decades in order to be able to feed the world's population (Food & FAO, 2015). So far, a significant quantity of food, fruits and beverages is lost due to microbial spoilage (FAO, 2013; Lipinski et al., 2013; Miller & Welch, 2013). To address this problem, synthetic chemical preservatives such as sulphur dioxide, dimethyl dicarbonate, benzoate, benzoic, lactic, sorbic and acetic acid, triazoles, hydroanilide fenhexamid, dicarboximides and succinate dehydrogenase are used. These chemical preservatives are not sufficient for beverages and postharvest control (Miessner & Stammler, 2010) and they also pose health problem such as skin and/or eyesight damage, muscle and stomach pain, cardiovascular diseases and impairment of brain functions. Therefore, the use of growth inhibition compounds from yeasts referred to as biopreservatives herein seems to be a promising method to eradicate the use of synthetic chemical compounds (Edwards & Seddon, 2001; Comitini, Di Pietro, et al., 2004; Mehlomakulu et al., 2014; Medina-Córdova et al., 2016; Veras et al., 2016).

Some yeasts have been found to secrete biopreservation compounds. These compounds classified as killer toxins and bacteriocins, have broad antagonistic effect on spoilage organisms in food, fruits and beverages. Furthermore, a few biopreservation compounds have been identified and purified (Comitini, et al., 2004ab; Wang et al., 2007; De Ingeniis et al., 2009; Mehlomakulu et al., 2014; Medina-Córdova et al., 2016; Veras et al., 2016). Although many researchers have reported that some partially identified compounds could be used in food and fruits preservation (Comitini et al., 2004b; Wang et al., 2007; Mehlomakulu et al., 2014; Oro et al., 2014), this has not been achieved in fast-moving consumer goods (FMCG) or consumer packaged goods (CPG). The overall advances in the field of biopreservation compound production still lacked knowledge of key aspects such as the use of cheaply available production medium, the kinetics data and optimisation conditions when using renewable agrowaste as fermentation medium; the inoculum dose required for yeast cultures to completely inhibit the growth of fruit spoilage organisms, the activity interactions of crude biopreservatives against spoilage yeast consortia. Besides the aforementioned gaps, there were also a lack of biological stoichiometric and bioenergetics data during biopreservation compound production using grape pomace extracts as fermentation medium.

1.3 Hypothesis

It was hypothesised that, yeasts isolated from Marula and Shiraz juice would demonstrate post-harvest biocontrol potential against fruit spoilage organisms and that those yeasts would also produce biopreservation compounds with growth inhibition activity against beverage spoilage microorganisms.

1.4 Research questions

- What yeast strains isolated from the South African fruit environment (Marula and Shiraz juice) would be able to secrete extracellular compounds with biopreservation and post-harvest control potential against beverage and fruits spoilage organisms?

- Would there be yeasts capable of acting as both post-harvest control agents and producers of biopreservation compounds with broader antimicrobial activity that would appeal to the fast moving consumer goods (FMCG) and consumer packaged goods (CPG) industry?
- What suitable, high yield and cost effective substrates could be used for biopreservation compound production under optimum condition?
- What would be the growth and production kinetics, and the biological stoichiometric equations of the selected yeasts producing the biopreservation compounds?
- How would the functionality of the biopreservation compounds be retained under different storage conditions used for fruit and beverages?
- What would the optimised biopreservation compound production conditions be, when using grape pomace extracts as the fermentation medium?
- How effective would the application of the biopreservation compound and post-harvest biocontrol be, in vitro and in vivo?
- What biological thermodynamic data would be made available in order to understand some engineering aspects of biopreservation compound production using grape pomace extracts as fermentation medium?

1.5 Aims and objectives

The aims and objectives of the study were as follows:

Aim 1: Screen and identify potential yeasts for broad antimicrobial activity against fruit and beverage spoilage organisms.

Objective 1: Screening of yeasts isolated from fruits juices (Marula and Shiraz) for growth inhibition activity against fruit fungal pathogens genera *Botrytis, Colletotrichum* and *Rhizopus* as well as beverages spoilage organisms *Dekkera* and *Zygosaccharomyces* species.

Objective 2: Identification of the three best yeast isolates with broad antimicrobial activity against the selected spoilage organisms.

Aim 2: Study of the growth and production kinetics including optimisation of biopreservative production from the best (n=3) isolates using renewable nutrient sources (grape pomace extracts) in suitable bioreactors.

Objective 1: Growth inhibition compound production kinetics in single stage bioreactors using grape pomace extracts.

Objective 2: Identification of the growth inhibition compounds.

Objective 3: Optimisation of growth inhibition compounds production using grape pomace extracts. Optimisation parameters: fermentation time, pH, temperature and total sugar concentration**.**

Aim 3: The application study of the potential biocontrol agents for fruit and beverage biopreservation: A product from single biopreservatives crude vs product from a mixture of crude biopreservatives.

Objective 1: Biopreservation application of cell free crude biopreservatives samples on beverage spoilage organisms.

Objective 2: Post-harvest application in quantified headspace: Static and cidal concentrations of the biological control agents towards all selected fruit spoilage organisms.

Aim 4: Preliminary biological thermodynamic study

Objective 1: Analysis of fermentation kinetic expressions, i.e. feed (carbon source) concentration, rate equations and yield values and cell density for the biopreservative production.

Objective 2: Study the biological stoichiometry and bioenergetics during biopreservation compound production in grape pomace extracts used as fermentation medium.

1.6 Delineation of the study

This study did not look at:

- The biopreservation compounds production in multi-culture systems.
- The in-depth of the gene responsible for the production of biopreservation compounds.
- The physical design of a new bioreactor but the configuration of existing designs for the production of the biopreservatives.
- The toxicology (in human cell like) of the biopreservatives produced.

1.7 Significance of the research

This study contributed to the following:

- An attempt to minimise the use of artificial chemicals as fungicides and preservatives for the post-harvest control of various fruit and beverage spoilage organisms.
- The establishment of the feasibility of using yeasts or their extracellular products to reduce fruit and beverage spoilage organisms.
- The use engineering concepts to develop a biopreservative production system for beverages and agricultural produce (post-harvest).
- The elucidation of the efficacy of mixture of biopreservatives against consortia of spoilage yeasts.
- The elaboration of biological stoichiometry and bioenergetics models that could serve to simulate and account for material and energy balances during biopreservation compound production by yeasts using grape pomace extract as fermentation medium.

1.8 Expected outcomes, results and contributions to research

In this study, it was expected to generate valuable bioprocess engineering information, i.e. microbial growth and biopreservatives production kinetics from grape pomace extracts, production process optimisation and preliminary microbial thermodynamic data, on yeasts of industrial importance as source of biopreservation compounds and post-harvest biocontrol agents with broad antimicrobial activity, i.e. to control organisms that could spoil food products. This research also served to partially fulfil requirements for a Doctorate of Engineering in Chemical Engineering of the Cape Peninsula University of Technology. Other expected outputs were publication of a book chapter, a peer reviewed conference proceeding and article publications in peer reviewed international journals.

CHAPTER 2

LITERATURE REVIEW

***Published as*: Ngongang, M.M.;** Ntwampe, S.K.O.; du Plessis, H.W.; Jolly, N.P.; Mekuto, L. Biopreservatives from yeasts with antimicrobial activity against common food, agricultural produce and beverage spoilage organisms. In book Antimicrobial Research: Novel bioknowledge and educational programs, Series Nº 6; Méndez-Vilas, A. In; Formatex Research Center, C/ Zurbarán, 1, 2° - Oficina 1 06002 Badajoz Spain, 2017, pp. 219-228.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

As living organisms, spoilage microorganisms also require nutrients for their growth and proliferation (Martorell et al., 2007). Food, fruits and value added beverage products are usually sources of nutrients for wanted and unwanted microorganisms (Willey, 2008). It is critical for the food and beverage industries to ensure that products destined for both local and export markets meet stringent quality and regulatory requirements. These products need to be of high quality and with a considerable shelf-life. It is therefore, vital to maintain their quality characteristics as defined by quality standards and/or legislated requirements (both local and international). This can only be achieved by preservative supplementation or post-harvest control methods, which minimises the alteration of product properties and health related hazards to consumers.

Microbial spoilage has been reported to be one of leading factor responsible for food, fruit and beverage losses, and this impacts negatively on the economy of the producing countries (Liu et al., 2017). Depending on the produce type, producers are currently using synthetic chemicals as mitigation measures for such losses. Benzoic, lactic, sorbic and acetic acids are some of the major organic acids that have been used in food preservation. These compounds act against a variety of spoilage microorganisms including some fruit spoiling fungal pathogens (Sofos & Busta, 1981; Brul & Coote, 1999). In post-harvest control of fungi, triazoles, hydroanilide fenhexamid, dicarboximides and succinate dehydrogenase are used (Miessner & Stammler, 2010; Grzegorczyk et al., 2017). Although the use of chemical preservatives has only partially assisted in reducing product losses associated with microbial spoilage, their use in certain instances can cause the deterioration of human organs such as the heart and lungs, and can lead to respiratory diseases, and allergic reactions (Longnecker et al., 1997; Soderlund et al., 2002).

2.2 Food losses and control methods used

2.2.1 Food, fruits and beverage spoilage organisms

Increasing food, fruits and beverages losses have been reported, owing to microbial spoilage. These losses in return negatively impact on the economy of the producing countries. To mitigate these losses, and depending on the type of produce, producers are currently using synthetic chemicals and some physical treatments such as heat, drying, including storage at low temperature, as control methods. Although these methods have only partially helped to control and reduce food losses, the use of synthetic chemicals poses serious health concerns and besides, some spoilage microorganisms have developed some resistance to the acceptable limit of these chemicals used as preservatives. For example, in fermented beverages, *Brettanomyces bruxellensi*s has been found to be resistant against sulphur dioxide (SO_2) (Mehlomakulu et

al., 2014). The physical treatments applied also affects the taste, aroma and composition of the food products been subjected to these treatments; therefore, reducing the quality and acceptability of the food products by consumers.

Contamination in some food and beverage industries is attributed to *Dekkera bruxellensis, Dekkera anomala, Zygosaccharomyces bailii*, *Hanseniaspora uvarum* and *Candida guilliermondii* yeasts species (Comitini, De, et al., 2004; Comitini, De, et al., 2004; Mehlomakulu et al., 2014). For instance, *Brettanomyces* sp. specifically, causes spoilage in finished wine and during ageing in barrels. Similarly, in sweet wine and grape juice, *Zygosaccharomyces bailii* occurrence can detrimentally lead to an undesired fermentation (Comitini et al., 2004ab; De Ingeniis et al., 2009; Zuehlke et al., 2013). On cereal grains and fruits, spoilage is usually attributed to fungal pathogens belonging to the genera of *Botrytis*, *Rhizopus*, *Colletotrichum, Fusarium, Penicillium, Aspergillus* and *Monilinia* (Jestoi et al., 2004; Williamson et al., 2007; Sharma et al., 2009; Yli-Mattila, 2010; Boutigny et al., 2011).

2.3 Preservation methods and their health effects

Food products destined for local consumption and the export market have to meet specific standards such as taste, quality, shelf life and safety. These standardised food attributes can be compromised if the food is not well handled or processed; which can then lead to the occurrence and proliferation of unwanted microorganisms in products made for human consumption or used as animal feed. To avoid the proliferation of microorganisms in food, chemical compounds with growth inhibiting properties are being used at specific concentrations depending on the targeted spoilage organisms as well as the type of food product being preserved (Du Toit & Pretorius, 2000; Okafor, 2007; Mehlomakulu et al., 2014).

Benzoic, lactic, sorbic and acetic acid are some of the major organic acids that have been used in food preservation. These compounds act against a variety of spoilage microorganisms including some fruit fungal pathogens (Sofos & Busta, 1981; Brul & Coote, 1999). In post-harvest control of fungi, triazoles, hydroanilide fenhexamid, dicarboximides and succinate dehydrogenase are being used (Meissner & stammler, 2010; Grzegorczyk et al., 2017). The use of the aforementioned chemicals as preservatives also has some negative effect on the taste and aroma of the food being preserved. The prolonged exposure to those chemicals even at the lowest dose possible can lead to serious health problems in humans (Benito et al., 2009).

Other food preservation methods include smoking, salting and curing using compounds such as sodium chloride, nitrites and phenolic acids (Brul & Coote, 1999). The consumption of these antimicrobials also poses health related deterioration of human organs such as the heart and lungs, culminating in respiratory diseases as well as some allergic reactions (Benito et al., 2009).

Pasteurisation, cold processing, filtration, the control of water content, ultrasound processing, and irradiation are some of the physical methods used for food preservation (Tiwari et al., 2009; Chemat et al.,

2011; Santos et al., 2012). The drawback of these physical methods is such that, they are not applicable to some important food commodities without deteriorating the food items, such as fruits, vegetables and beverages. Furthermore, among these physical methods, none possess soluble antimicrobial compounds, and therefore, thermophiles, spores, psychrophiles and xerophiles can survive these procedures (Leistner, 1999; Farkas, 2007).

2.4 Bioprospecting: Yeasts and their metabolites as potential alternative to synthetic chemicals

Microorganisms such as yeasts that produce growth inhibition metabolites present a biological alternative for food, fruits and beverage preservation. For instance, *Candida pyralidae*, *Kluyveromyces wickerhamii*, *Kluyveromyces phaffi*, *Tetrapisispora phaffii*, *Candida tropicalis*, *Williopsis mrakii*, *Hanseniaspora uvarum*, *Debaryomyces hansenii*, *Pichia anomala* and *Pichia fermentas* are yeasts that have been reported to produce growth inhibition compounds that acts against spoilage fungi and yeasts (see Table 2.1 and 2.2). Growth inhibition activity can be attributed to the production of extracellular metabolites, proteins, glycoproteins and volatile organic compounds (Mehlomakulu et al., 2014; Oro et al., 2014; Parafati et al., 2015). Some yeasts have competitive growth inhibition characteristics when cultured with other microorganism, using space colonisation and higher nutrients utilisation rate as primary mechanism of inhibition (Kim et al., 1997; Bar-Shimon et al., 2004; Saravanakumar et al., 2008; De Ingeniis et al., 2009; Mendoza et al., 2010; Hatoum et al., 2013). The biochemistry of the antimicrobial agents has been found to be strain dependent (Izgü & Altinbay, 2004; Comitini et al., 2009; Muccilli et al., 2013; Muccilli & Restuccia, 2015).

Biocontrol of fungal pathogens using metabolites produced from yeasts, is achieved by the suppression of β-glucan synthesis or hydrolysis of β-glucan in the cell wall of spoilage organisms. Some control mechanisms inhibit DNA synthesis, which blocks cellular division, thus preventing the proliferation of spoilage organism. Other modes of action include the cleavage of the tRNA as well as interference with the uptake of calcium and the formation of ion-leaking channels on the cytoplasmic membrane culminated in cell deactivation (Klassen & Meinhardt, 2002; Klassen et al., 2008; Brown, 2011). In addition, there are other growth inhibitors such as antifungal hydrolases, bacterial pigments that cause iron depletion in spoilage organisms; antimicrobial peptides, β-lactam antibiotics, and antimicrobial volatile organic compounds (VOC's) (El Ghaouth et al., 2003; Sipiczki, 2006; Parafati et al., 2015), See Table 2.1.

2.4.1 Commercially produced biopreservatives and biocontrol agents

Biopreservatives are not produced nor used on a large scale due to inadequacy of process engineering systems used in their production and application in fast-moving consumer goods (FMCG) or consumer packaged goods (CPG). Many researchers have reported that some partially identified compounds could be used in food, beverage and fruits preservation but that, the use of microorganisms as producers of safe preservation compounds has not been fully applied (Comitini, Di Pietro, et al., 2004; Wang et al., 2007; Mehlomakulu et al., 2014; Oro et al., 2014).

Examples of biocontrol agents acting against post-harvest pathogens that are currently commercialised are based on either one or two strains of yeasts. For example, Shemer ™ (AgroGreen, Asgdod), is a biofungicide formulated using *Metschnikovia fructicola.* Candifruit™ (SIPCAM INAGRA, S.A., Valencia, Spain) using *Candida sake* for pome fruits. Boni-Protect™ (Bio-ferm, Germany) is produced from two strains of *Aureobasidium pullulans*.

Due to uncertainties related to the use of some yeasts in post-harvest spoilage control against spoilage organisms, what of the compounds that they produce? Could they be used in the same manner or could they be applied elsewhere to extend their applications? Can there be yeasts capable of acting as both post-harvest control agents and producers of biopreservation compounds with broader antimicrobial activity thus appeal for the FMCG and CPG industry? These questions must be a focus of the ongoing research effort to mitigate the associated risks of synthetic chemical preservatives.

2.5 Biopreservatives and post-harvest biocontrol using *Candida pyralidae* KU736785 as a model study

2.5.1 Growth/production kinetics, subsequent to process modelling and optimisation

Current research clearly demonstrates the potential that yeasts have as either biocontrol agents and/or producers of antimicrobial compounds. Although few compounds of different characteristics have been identified, many of these compounds are not produced and used at an industrial scale. This could be attributed to production process design and optimisation deficiencies. In a study by Mewa-Ngongang et al., (2017), kinetic modelling and optimisation of biopreservation compound production was assessed using *Candida pyralidae* KU736785 in YPD as fermentation medium, with preliminary screening against fungal pathogen, *Botrytis cinerea* and beverage spoilage organism *Candida guilliermondii*.

The *in-vivo* test was conducted using disinfected apples in which apples were wounded (3 mm deep and 2 mm wide) along the center, with each wound being inoculated with 15 µl of *Candida pyralidae* at a concentration of 1×10^8 cells mL^{-1}, subsequent to the addition of a spore suspension of the spoilage fungus (*B. cinerea*) at 10^5 conidia/mL. The apples were then incubated at 25 ºC for seven days. The ability of *Candida pyralidae* to minimise post-harvest decay on the apple was used as positive results.

For fractionation, different fractions tested were obtained from a size exclusion chromatographic (SEC) system. An isocratic elution with 5 mM sodium citrate at pH 4.5 over 2 column volumes at a flow rate of 1 $mL.min^{-1}$, was used and 25 fractions were obtained. The resin used was Toyopearl HW-55F. All 25 fractions were first tested against *Candida guilliermondii*. Fractions that showed growth inhibiton activity against *Candida guilliermondii* were tested *in-vivo* against *Botrytis cinerea*. The test was conducted as discribed above with the diffrence that 15 µL of the SEC fraction was administered in the wound in replacement of 15 uL of the *Candida pyralidae* culture broth.

The *in-vivo* efficacy test of *C. pyralidae* KU736785 against *B. cinerea* on apple showed that *C. pyralidae* KU736785 completely reduced the disease incidence (DI) (100% DI reduction). Disease incidence was determined by comparing the lesion diameter (LD 27.38 mm) of the negative control with that of the tested samples. This showed that *C. pyralidae* KU736785 was able to minimise decay caused by *B. cinerea* under commercial storage conditions (Figure 2.1). Fractions A8 to A12 showed growth inhibition activity against *Candida guilliermondii* on the plate assay (Figure 2.2a). However, fractions A9 and A12 were also able to considerably reduce the decay caused by *B. cinerea* on apples (Figure 2.2b) The results obtained suggested that a purified crude biopreservative sample from a fermentation broth innoculated with *C. pyralidae* KU736785 under optimum pH and temperature can have a broader spectrum of biopreservation activity, as shown in Figure 2.2 with *B. cinerea* inhibition (A9 and A12). These findings suggested that there could be more value to produce biopreservatives from a single yeast strain that shows growth inhibition activity against atleast one spoilage organism. If the biopreservation activity is also based on the concentration of the antimicrobial compounds present in fermentation broth, an engineering approach to biosystem design and optimisation for production is thus inevitable.

Figure 2-1 Post-harvest control activity of *Candida pyralidae KU736785* in controlling spoilage caused by *Botrytis cinerea*, on *Malus domestica*.

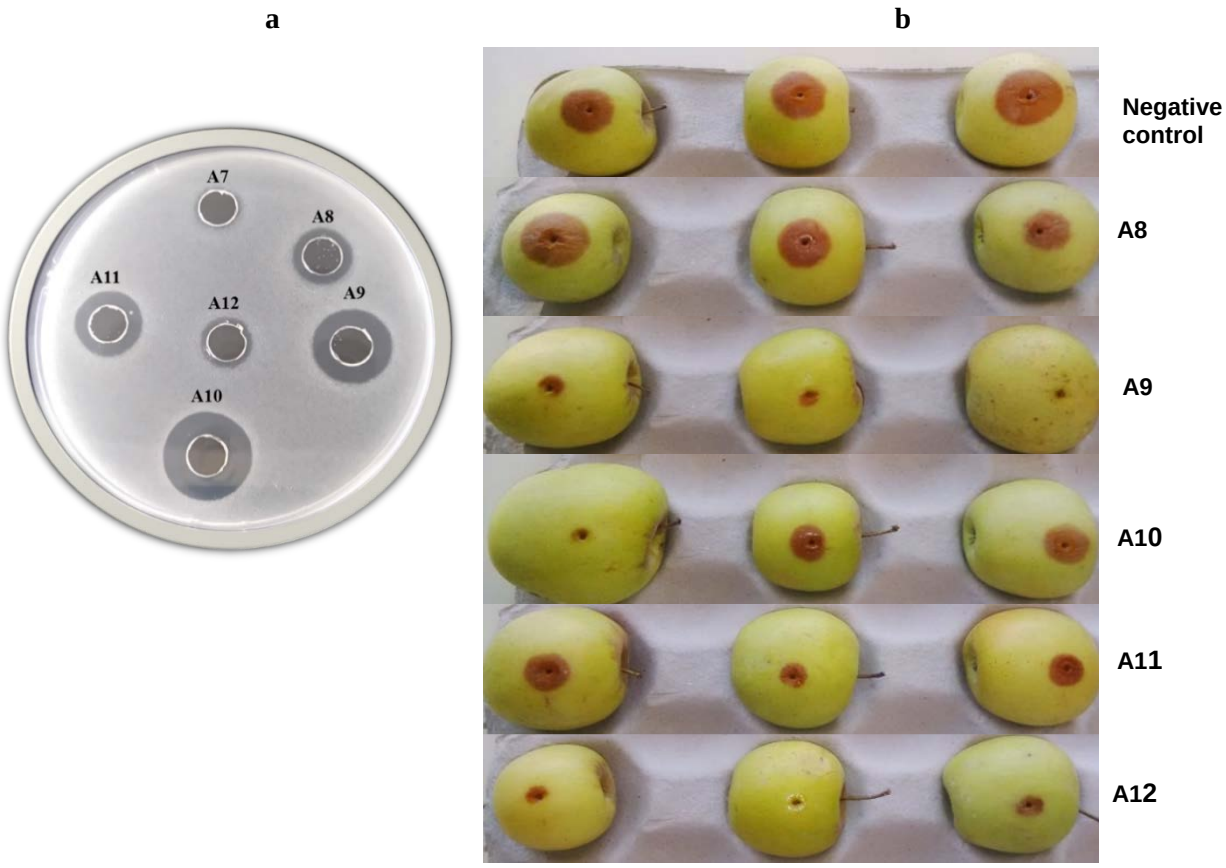

Figure 2-2 (a) = **Size exclusion chromatography fractions A8, A9, A10, A11 and A12 tested against *Candida guilliermondii* (Mewa-Ngongang et al., 2017); (**b) **= Post-harvest control activity against *Botrytis cinerea,* on *Malus domestica*; using compounds produced by *Candida pyralidae* KU736785.**

2.5.2 Engineering approach to bioprocess development and design for biopreservatives and post-harvest control: A focus on control agents from yeasts

In bio-systems engineering, microorganisms utilise nutrients for their physiological activities; in doing so, cellular proliferation occurs, leading to the accumulation of intra and/or extracellular products (Degeest et al., 2001). Generally, primary and secondary metabolites are produced during different fermentation stages. Amino acids, lipids, carbohydrates, nucleic acids and proteins are produced as primary metabolites during cellular growth, while secondary metabolites such as penicillin, cephalosporin, ergotrate and the statins are also produced after the exponential growth phase (Keller et al., 2005).

Process engineering principles are governed by the understanding and elucidation of parameters involved in any production system. In bioprocess engineering systems for example, it is vital to identify, explain and optimise production in order to mitigate input cost and to be able to get the needed operational guidelines of the process. In fermentation technology, key parameters such as substrate requirements and concentration, pH, temperature, salinity and nitrogen source ratio, including disolved oxygen requirements, must be optimised in order to guarantee optimal cell density and extracellular compound production (Ciani

et al., 2010; Stanbury et al., 2013). For optimal production conditions, the development of models describing process kinetics are essential and, also facilitates statistical analyses and process optimisation. Additionally, the engineering aspect of a biological system can also assist in developing processes with low input costs for downstream processes, albeit achieving a high yield of the products of interest. Some aspects such as temperature and pH usually play key roles in fermentation and product stability. The effect of temperature on the growth and stability of extracellular biopreservation compounds has been studied (Robledo-Leal et al., 2014). In the study by Robledo-Leal et al. (2014), biopreservative behaviour within the *C. parapsilosis* complex and the biopreservative activity of *C. metapsilosis* strains occurred at 25 °C. The temperature at which the process was carried out was crucial for the biopreservation compound production and the optimum biopreservation efficacy temperature was reported between 15–20 °C. The effect of temperature on the stability of the biopreservation compound revealed that it was still active at temperatures above 30 °C. A different study showed that, temperature and pH played a significant role on the production and stability of the biopreservation compounds from a *Candida pyralidae* strain (Mewa-Ngongang et al., 2017). The authors also highlighted the importance of pH and temperature on the biopreservation compound production in a single stage bioreactor, and optimal production conditions in that study were at pH 5 and 22.5ºC. In order to fully understand the fermentation parameters involved in such biological systems, the said examples (pH and temperature) are critical for designing appropriate bioprocess systems. Although selected parameters can be optimised, industrial scale process optimisation still remains a challenge, especially during antimicrobial compound production from yeasts. However, the knowledge associated with the growth limiting substrate, the ratio of carbon to nitrogen (C: N) in the medium are among other parameters that can be considered for process scale-up.

To date, synthetic refined media are used for biopreservation compounds production studies. However, the use of such refined media in the biopreservation compounds production is seldom recommended due to high cost. For optimal and sustainable production of biopreservation compounds, the following critical questions must be fully addressed. 1) What other suitable, high yield and cost effective substrates can be used for production? 2) Can other media or substrates be used and optimised for production under optimum bioreactor conditions? 3) Bioreaction vessels such as flasks are commonly used for production of antimicrobial compounds, what is the most suitable bioreactor system for achieving optimal yield

Table 2-1 Examples of antimicrobial compound producing yeasts (Edwards & Seddon, 2001; Comitini, Di Pietro, et al., 2004; Mehlomakulu et al., 2014; Veras et al., 2016)

Yeast species	Antimicrobial compound identity, molecular size, temperature and/or pH activity	Application	Mechanism of action	Target yeast
Candida glabrata **(formerly named *Torulopsis glabrata*)**	n/d	n/d	Damages the plasma membrane	*Saccharomyces cerevisiae*
Candida pyralidae	CpKT1 and CpKT2 (>50KDa); pH: 3.5 – 4.5; temperature: 15 and 25 ºC	Grape juice	n/d	*Brettanomyces bruxellensis*
Kluyveromyces wickerhamii	Kwkt (72kDa); pH: 3.8-4.6; temperature: 25 ºC	Wine making	β-1,6-glucans receptor	*Brettanomyces. bruxellensis*
Tetrapisispora phaffii	Kpkt (33 kDa); pH: 3 - 5; temperature: <40 ºC	Wine making	Disrupts the integrity of the cell wall. Also shows β-glucanase activity	*H. uvarum*
Williopsis mrakii **NCYC 500**	K-500 (1.8-5.0kDa); pH: 2.4 – 4.0 temperature : 30 ºC	Antifungal agent	Membrane permeability	*Candida albicans* and *Sporothrix schenkii*
Pichia Acacia **(reclassified as *Millerozyma acaciae*)**	PaT (187 kDa); pH:7 – 7.5 and 5.3 – 6.6 temperature : n/d	n/d	Cell cycle arrest in G1 phase in *S. cerevisiae* cells. Displays chitinase activity	*S. cerevisiae*
Hanseniaspora uvarum	18 kDa; pH: 3.7 – 3.9 temperature : n/d	n/d	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: 4.4; temperature: 25 – 35 °C	Wine making	β-1,6-glucans receptor	*B. bruxellensis*

n/d-not determined

Table 2-2 Examples of potential biocontrol yeasts assessed against fungal pathogens in post-harvest spoilage control of fruits (El Ghaouth et al., 2003; Scherm et al., 2003; Li et al., 2011; Nally et al., 2012; Calvo-Garrido et al., 2013; Lutz et al., 2013).

Fruit	Fungal pathogen	Biocontrol yeasts
Apple	*Botrytis cinerea*	*Candida saitoana*
Apple	*Penicillium expansum*	*Candida guilliermondii*, and *Saccharomyces cerevisiae M25*
Apple	*Penicillium expansum*	*Rhodotorula. mucilaginosa*
	Botrytis cinerea	
Apple	*Botrytis cinerea*	*Pichia guilliermondii*
Grapes	*Botrytis cinerea*	*Saccharomyces cerevisiae and Schizosaccharomyces pombe*
Grapes	*Botrytis cinerea*	*Candida sake* CPA-1 and Fungicover
Pears	*Penicillium expansum*	*Cryptoccocus albidus NPCC 1248, Pichia membranifaciens*
	Botrytis cinerea	*NPCC 1250, Cryptoccocus victoriae NPCC 1263, NPCC 1259*

2.6 Bioreactor operation and selection

Regardless of whether the product is a primary or a secondary metabolite, a need exists to develop a better system for producing safer and cost effective biopreservatives/biological control agents post-harvest. Recently, a single stage bioreactor was used to produce antimicrobial compounds, from a yeast i.e. *Candida pyralidae* KU736785 (Mewa-Ngongang et al., 2017). The authors reported maximum biopreservation compound production during exponential growth phase. This study also showed that the produced crude biopreservation compound was a primary metabolite, but further downstream processing remained a big challenge, particularly during supernatant recovery stages. Maintaining a high cell density in the exponential growth phase is important and using membrane bioreactor (MBR) technology presents key advantages for the collection of cell free supernatant, and for continuous product recovery. The choice of a reactors' operational mode is important in the modelling and design of the process to be used. Furthermore, the aim of effective bioreactor design is the ability to control key variables, to contain and positively affect the process. For bench-scale experiments, batch, semi-batch and continuous systems are used and each of these processes present advantages and disadvantages. For instance, cell recycling in continuous cultures can be used at steady-state with a continuous feed. The system is capable of elevating productivity, while lowering labour intensivity, but negatively reducing product concentrations. Nevertheless, these processes and parameters can be optimised to achieve maximum yields using statistical design optimisation methods, such as response surface methodology (RSM), with a central composite design (CCD) (Yeh et al., 2006; Cao et al., 2010; Xiao & Lu, 2014).

2.7 Mass and energy balances for biological systems

2.7.1 Stoichiometric of microbial analysis

Mass balances of biological systems represented in microbial growth models can be calculated using the law of conservation of mass. Several methods, such as that of half reactions or regularities can be used to evaluate the stoichiometric requirements of any conversion that takes place during microbial growth and bioproduct formation (Liu et al., 2007; Akinpelu et al., 2018).

During the process of microbial conversion of substrates to extracellular compounds, the catabolic and the anabolic occur. In the catabolic phase, the primary growth controlling substrate such as glucose is broken down and portion of the catabolic product is then used in the anabolic phase for the synthesis of new biomass (Liu et al., 2007). Table 2.3 depicts all the different equations that can be used to fit the above-described processes.

Table 2-3 Microbial growth parameters and the related models to describe the stoichiometric microbial analysis (Liu et al., 2007).

Parameter	Equation
Biological stoichiometry	$\frac{1}{Y_{\frac{X}{S}}} S + Y_{\frac{A}{X}} A + Y_{\frac{N}{X}} NS \rightarrow X + Y_{\frac{P}{X}} P + Y_{\frac{C}{X}} CO_2$
Catabolic reaction	$S + Y_A^{cat} A \rightarrow Y_P^{cat} P + Y_C^{cat} CO_2 \quad (\Delta G_{cat}^0)$
Anabolic reaction	$Y_p^{ana} P + Y_C^{ana} CO_2 + Y_N^{ana} NS \rightarrow X + Y_A^{ana} A \quad (\Delta G_{ana}^0)$

S, *A*, *NS*, *X*, and *P* representing the substrate (energy source), electron acceptor, nitrogen source, dry biomass and reduced electron acceptor respectively.

2.7.2 Energy balances for a biological system

Once stoichiometric requirements of a biological process has been determined, it is imperative to also establish the energy requirements for the system. As substrates are being utilised as electron donors, accounting for the energy requirements means that parameters such as Enthalpy of formation (ΔH_f^O), Gibbs energy (ΔG_f^O) and heat of reaction (ΔH_{RX}^O) are to be considered, with the Gibbs energy being the main driving force (Liu et al., 2007; von Stockar et al., 2008; Battley, 2011; Battley, 2013). In that regard, the values for $\Delta G\ , \Delta H, and\ \Delta S$ could be estimated using the following equation (Akinpelu et al., 2018b):

$$\Delta G = \Delta H - T\Delta S \qquad \textit{Equation 1}$$

With $\Delta G\ , \Delta H, and\ \Delta S$ representing the Gibbs energy, enthalpy and entropy changes respectively.

Practically, this means that, for a bioreaction, experimental values for the formation of cells and the related heat of combustion are to be determined using the following model (Akinpelu et al., 2019).

$$\Delta H_C^{cell}\left(\frac{KJ}{mol}\right) = \Delta H_{cal}^{cell}\left(\frac{KJ}{g}\right) X\ M_X \qquad \textit{Equation 2}$$

With M_X representing the mass of 1 C-mol of the dried biomass.

Once the heat of formation of biomass is obtained, the heat of reaction as the result of the biosynthesis of 1 C-mol of biomass can be calculated using Hess's law as represented by the following equation:

$$\Delta H^{o}_{RX} = \sum \quad n\left(\Delta H_{products}\right) - \sum \quad n(\Delta H_{reactants}) \qquad \textit{Equation 3}$$

Where *n* represents the respective stoichiometric coefficients.

2.8 Summary

Continues food, fruits and beverages losses are experienced at a large scale in industry. The current control methods present many challenges such as health related concerns, while consumers demand natural and safer preservatives. Despites these challenges, the current use of synthetic chemicals as preservatives have not addressed the issue of food losses caused by microbial spoilage. Besides, spoilage organisms have also developed resistance to some of the chemical preservatives. Yeasts have been found to be a potential alternative to chemical preservatives. However, the findings are still superficial in addressing the current need for safer and cost effective preservatives. In this regard, it is demonstrated that an appropriate production system using an engineering approach could advance the development and the use of yeasts, including their extracellular compounds as fruit biocontrol agents and as biopreservatives. The following points formed the research gaps that the current study attempted to address:

- In the literature reviewed, yeasts have been studied as potential biological control agents or as producers of biopreservatives in separate studies with minimal broad efficacy against variety of spoilage organisms, however in this study yeasts that had broader growth inhibition efficacy against both fruit and beverages spoilage organisms were studied.
- There has been minimal research that focused on developing a cheaply available carbon and nitrogen renewable sources that can be explored for the design of an industrial fermentation medium for biopreservatives production from yeasts.
- There have been a lack of appropriate fermentation system and models for the optimum biopreservation compounds production from yeasts, provided that a suitable fermentation medium is developed, from which values could be extracted from both the crude samples and the cell free supernatants.
- The existing literature lacked adapted and appropriate quantitative approach for the development of suitable method for biopreservation compounds activity in order to assess and validate the production system and the efficacy of the biopreservatives produced.
- The effect of biopreservatives from yeasts against the consortia of beverage spoilage organisms has not been studied in the field of biopreservation compounds production from yeasts. Given the fact

that there has been minimal information in this regard, this study also focused on generating some useful data in an attempt to close this gap.

- There has been a lack of data pertaining to the fungistatic and fungicidal effect of biological control yeasts as well as the inoculum dose at which the static and cidal effects are observed.
- Although some biological stoichiometric equations from yeasts have been developed in other bioprocessing systems, there is little information on the biological stoichiometry and bioenergetics during biopreservatives production from yeasts using grape pomace extracts as fermentation medium.

CHAPTER 3

MATERIALS AND METHODS

CHAPTER 3

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3.1 Microorganisms selections

The potential biocontrol yeasts *C. pyralidae* (Y1117, isolated from grape must), *P. kluyveri* (Y1125, isolated from *Sclerocarya birrea* juice), and the beverage spoilage organisms *D. bruxellensis* (ISA 1653), *D. anomala* (MSB/1) and *Z. bailii* (Y0070) were obtained from the ARC Infruitec-Nietvoorbij culture collection (Stellenbosch, South Africa). Furthermore, and on the basis of prevalence as spoilage and disease causing agents in the South African fruit industry, *B. cinerea*, *C. acutatum* and *R. stolonifer* were also obtained from the Post-harvest control laboratory at ARC Infruitec-Nietvoorbij (Stellenbosch South Africa).

Table 3-1 Selected biopreservation compounds producing yeasts based on their ability to inhibit the growth of other microorganisms as well as spoilage organisms prevailing in fruit and beverages spoilage.

Biopreservation Compounds Producer Strains	Beverage Spoilage Strains	Fruit spoilage strains
Candida pyralidae Y1117	*Dekkera anomala*	*Botrytis cinerea*
Pichia kluyveri Y1125	*Dekkera bruxellensis*	*Colletotrichum acutatum*
Pichia kluyveri Y1164	*Zygosaccharomyces bailii*	*Rhizopus stolonifer*

3.2 Grape pomace extracts medium preparation

3.2.1 Grape pomace extract broth

Wet grape pomace from Chenin Blanc berries was obtained from the ARC Infruitec-Nietvoobij research cellar. The extraction method used was similar to the normal white wine juice extraction procedure, but with increased extraction pressure (1 to 2 bar) to allow the recovery of the remaining juice (extract) from the pomace. The juice from the grape pomace was composed of 210 g.L^{-1} total sugar, 185.12 mg L^{-1} YAN and 34.13 mg L^{-1} ammonium.

The resulting juice was racked and the GPE broth obtained and frozen in plastic buckets at −10 °C. Prior to use, the grape pomace extract was thawed and diluted with water to the desired total sugar concentrations expressed in g L^{-1}. After the dilutions, the yeast assimilable nitrogen (YAN) was measured using an enzyme robot (Arena 20XT; Thermo Fisher Scientific, Vantaa, Finland) and found to be sufficient to support the growth of yeasts. The different dilutions were adjusted to the desired pH, using 0.1 M NaOH and immediately autoclaved for 30 min at 120 °C.

3.2.2 Grape pomace extract agar

Grape pomace extract was diluted with sterile distilled water to a sugar concentration of 50 g L^{-1}. As a result of the dilution, the final concentration of the yeast assimilable nitrogen (YAN) was 0.045 g L^{-1} as measured using an enzyme robot (Arena 20XT; Thermo Electron, Finland). The pomace extract was adjusted to pH

5, using 0.1 M NaOH prior to use. Thereafter, bacteriological agar (Biolab, Merck, South Africa) was added at 10 g L^{-1} and autoclaved (United Scientific, Daihan Labtech CO. LTD, India) at 121°C for 20 minutes. After autoclaving, the grape pomace extract agar (GPA) was supplemented with 0.1 g L^{-1} chloramphenicol (Sigma-Aldrich, Germany) to minimise any contamination during the handling of the essays.

3.3 Microorganisms culture conditions and inoculum preparation

3.3.1 Yeasts

Yeasts were cultured on the GPA for 2 days at 28ºC. *C. pyralidae* and *P. kluyveri* cells were prepared by transferring a wire loop full of the culture into a volume (5 mL) of Yeast Peptone Dextrose (YPD) broth (Sigma Aldrich, SA) subsequent to incubation at 28ºC for 24 h. From the 24-h-old yeast cultures, a volume (1 mL) of *C. pyralidae* and *P. kluyveri* containing broth was transferred to 150 mL sterile GPE broth subsequent to incubation at 23ºC and agitated at 150 rpm, using a rotary shaker (LM-53OR, RKC Instrument Inc., Ohta-ku Tokyo, Japan) for 24 h (Mewa-Ngongang et al., 2017). Spoilage yeast suspensions (*D. bruxellensis*, *D. anomala*, and *Z. bailii*) were also prepared using YPD (Sigma Aldrich, SA) broth for 24 h at 28ºC. Cells were recovered by centrifugation at 10000 rpm for 10 minutes. A haemocytometer and a microscope (400X magnification) were used to count the yeast cells.

3.3.2 Fungi

For the preparation of the spore solution, fungal spores were obtained from 14 day old fungal plates incubated at 20ºC. The spores were harvested by gently scraping-off the surface of the agar with sterile distilled rinsing water (n = 3) to attain a 100 mL of a spore solution in 250 mL Scott bottles. Similarly, 5 mm diameter mycelia disks of each fungus were cut from 5-day old plates also grown on GPA at 20ºC. The working fungal spore suspensions of *B. cinerea*, *C. acutatum* and *R. stolonifer* were also prepared. A haemocytometer and a microscope (400X magnification) were also used to count the fungal spores before the mother spore solution was diluted to the desired concentration expressed in spores mL^{-1} (Qin et al., 2015).

3.4 Concept of Volumetric Zone of Inhibition and Calculation

The concept of volumetric zone of inhibition was developed using Equation 4 below:

$$A = \pi r^2 \ (r = D/2); \ V = A.H \qquad \textit{Equation 4}$$

where *H*, *A*, *V* represent the thickness, area and the volume covered by the grape pomace extract agar, respectively. Prior to determining this volume, $A = \pi r^2$ (r = D/2) was used to calculate the area covered by the grape pomace extract (GPE) agar, where *r* and *D* represent the radius, and the diameter of the petri dish, respectively.

The data obtained from the measured zone of inhibition, the thickness of the GPE agar and the diameter of the pierced wells on the GPE agar were used in Equation 4. The diameter zone of inhibition ($D = D_o - d$) measured around the well (Figure 3.1) is a resultant of the volume of 20 µL of the biopreservation compounds used (BCU) which was introduced in the pierced well.

This volume (20 µL) of the inoculated, solidified GPE agar was the concept basis of the volumetric zone of inhibition (VZI). Since 1 cm^3 = 1 mL = 10^{-3} L, the calculated volume ($V = A.H$) units expressed in liters (L) was used for consistency. In this study, the VZI interpretation was based on the fact that 20 µL (0.02 mL) of biopreservation compounds sample was sufficient to inhibit the growth of the spoilage organism inoculated at 1×10^6 CFU.mL^{-1} in a defined volume (L) of the GPE agar plate. The term inhibitory activity (L.mL^{-1}) was then adopted to describe the growth inhibitory effects which are reported as the volume of contaminated (inoculated) solidified media (L CSM) per mL of biopreservation compounds used (mL BCU). The units were presented as L CSM.mL^{-1} BCU.

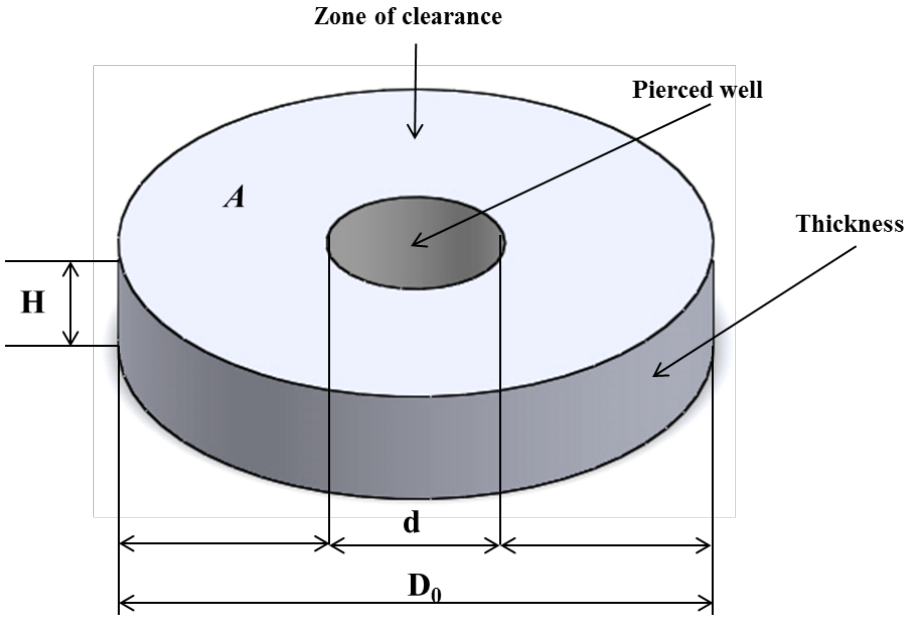

Figure 3-1 Outline of the concept developed to calculate the volumetric zone of inhibition (VZI) (adapted from Mewa-Ngongang et al. (2017).

3.5 Growth inhibition activity screening against beverage spoilage organisms

C. pyralidae Y1117, *P. kluyveri* Y1125 and *P. kluyveri* Y1164 were previously isolated from grape must and Marula (*Scelerocarya birrea*) juice, then identified using molecular biology techniques and a culture dependent approach (Bezerra-Bussoli et al., 2013). These yeasts were previously screened and some reported for growth inhibition activity against various beverage spoilage yeasts (Mehlomakulu et al., 2014; Mewa-Ngongang et al., 2017). In this work, *Dekkera bruxellensis*, *Zygosaccharomyces bailii* and *Dekkera anomala* were used as spoilage yeasts and seeded in grape pomace extracts agar (GPEA) at the desired

concentration, depending on the assay being carried out. The entire plate assays were carried out using 90 mm petri dishes.

The growth inhibition assay as described by Mehlomakulu et al. (2014) was used. Briefly, direct cell counts with an haemocytometer was used whereby yeast cultures of *C. pyralidae* and *P. kluyveri* were adjusted to a concentration of 10^8 cells mL^{-1} by diluting each culture broth in sterile distilled water (Qin et al., 2015). From these dilutions, 10 µL of each culture were spotted onto GPA plates seeded with *D. bruxellensis, D. anomala* and *Z. bailii* at a concentration of 10^6 cells mL^{-1} each. After each dilution, the cell concentration was verified by direct cell count using a haemocytometer. The seeding procedure consisted of inoculating the yeast in GPA which was kept at 50ºC in a water bath prior to decanting into petri dishes for solidification (Mehlomakulu et al., 2014; Mewa-Ngongang et al., 2017).

A cross-screening procedure was performed whereby the spoilage and growth inhibiting yeasts selected for the study were also screened against each other separately (spoilage yeasts against spoilage yeasts and growth inhibiting yeasts against each other). The grape pomace extracts were tested for growth inhibition activity against any of the spoilage microorganisms selected. They were assessed for growth inhibition activity because of the low pH, which could lead to some stunted microbial growth of some microorganisms used in this study. The growth inhibition activity and quantification was carried out as described above. After 72 h of incubation at 20ºC, the plates, prepared in three replicates per treatment were inspected for zone of inhibition as shown by the formation of a clear zone around the yeasts colonies (Mewa-Ngongang et al., 2017; Mewa-Ngongang et al., 2019).

3.6 Growth inhibition assay of cell free supernatant against beverage spoilage organisms

The growth inhibition assay was adapted from (Mehlomakulu et al., 2014) with some modifications. The GPE agar was used. The growth inhibition assay was prepared by supplementing the GPE broth (150 $g.L^{-1}$) with 2% agar bacteriological (Biolab, Merck, South Africa). A well with a 5-mm diameter was drilled on GPE agar plates using an agar driller. Prior to drilling, the GPE agar plates were seeded with 10^6 cells mL^{-1} of either *Z. bailli*, *D. bruxellensis* or *D. anomala* (Mehlomakulu et al., 2014). In the agar wells, a volume of 20 µL of the crude biopreservation compounds was spotted, subsequent to incubation at 22 °C until clear zones of inhibition were observed around the 5 mm wells. The plates were then assessed for biopreservation activity, which was quantified as described by (Mewa-Ngongang et al., 2017).

3.7 Production of biopreservation compounds using grape pomace extracts

The potential of Chenin Blanc grape pomace extracts as a source of fermentation medium for crude biopreservation compounds production in shake flasks (150 mL in 250 mL Erlenmeyer flasks) was assessed. The grape pomace extracts were diluted to obtain total sugar concentrations of 100, 150 and 200 g L^{-1}, respectively. The pH was adjusted to 5 using 0.1M NaOH. The fermentation media were autoclaved

for 20 minutes at 121ºC, cooled and inoculated with *C. pyralidae* and *P. kluyveri* at an initial concentration of 10^6 cells mL^{-1}.

The flasks were incubated for 24-32 h at 25 °C in a shaking incubator (LM-53OR, RKC® Instrument INC, Ohta-ku Tokyo, Japan) set at 150× rpm. From the initial fermentation time (t = 0 h), samples (2 mL) were taken every 4 h for the duration of the experiment, i.e. 24-32h (n = 6-8). Samples withdrawn every 4 h were centrifuged at 5000× rpm for 5 min and the supernatants were filtered using 0.22 µm sterile nylon membrane filters. Filtered samples (1 mL) were analysed for total sugar and YAN while the remaining aliquots were used for the growth inhibition test and other assays.

3.8 Effect of proteolytic enzymes on the denaturation of the crude biopreservation compounds mixture

To evaluate the nature of the growth inhibiting compounds, it was important to subject the crude samples to protease treatments in order to determine whether the compounds responsible for growth inhibition activity was of a protein nature. The assay used was adapted from (Mehlomakulu et al., 2014). The protease enzymes used were Proteinase K, pepsin and proteases from *Aspergillus saitoi* and *Rhizopus* spp. (Sigma-Aldrich, Darmstadt, Germany). Thereafter, treated crude samples were tested for growth inhibition activity (Mewa-Ngongang et al., 2017) with the positive control being used as the crude sample that was not subjected to any of the treatments. Furthermore, the negative control consisted of just the protease without the crude biopreservative sample.

3.9 pH and temperature activity and stability of the crude biopreservation compounds mixture

The temperature activity study was carried out by spotting three replicates, with 20 µL of the crude sample in a 5 mm diameter well created on GEA seeded with *D. anomala* as a spoilage organism. The plates were incubated at 5, 15, 20, 25, 30, and 40 ºC respectively. Additionally, the pH activity was also determined by spotting 20 µL on GEA adjusted to pH 2.0, 3.0, 4.0 and 5.0 respectively; which is the pH range of many foods and beverages. These GEA plates were then incubated at 25 ºC until the volumetric zone of inhibition (VZI) was observed. Thereafter, the growth inhibition activity quantification was measured. The stability test was also carried out after confirming the temperature and pH optima. The stability test was carried out by storing the crude biopreservation compounds mixtures at different temperatures (-10, 5, 15, 20, 25, 30, and 40 ºC) for 16 weeks. A volume (20 µL) of the crude biopreservation sample stored for that period was thereafter spotted onto the plates prepared at the pH optima, subsequent to incubation at the temperature optima until zone of inhibition was observed.

3.10 Kinetic studies for Production of Potential Biopreservation Compounds from GPE

The production conditions under which the kinetic study was carried out is described in Section 3.7. Key parameters, such as the rates of substrate utilization, biomass formation, specific growth, biopreservation compounds formation, including that based on cell concentration and substrate consumption and biomass yield, were used.

The samples withdrawn at the frequency of 4 hours were tested for growth inhibition activity to determine the time in the fermentation cycle at which maximum growth inhibition activity could be obtained. The level of product formation was assessed by the size of the volumetric zone of inhibition of each sample tested. The same samples were also analysed for sugar, cell concentration and product formation, subsequent to fitting the data in the selected existing models. The total sugar utilization models were used, as described in Table 3.2, in order to assess the efficiency of GPE broth as a fermentation medium. The microbial growth dependency of biopreservation compounds production was determined using the modified Malthus equation (Malthus, 1986), with the specific growth rate of individual yeasts being determined under the defined experimental conditions (Section 3.7).

Since similar growth inhibition trends were observed for all the selected spoilage yeasts (*D. bruxellensis, D. anomala* and *Z. bailii*), *D. bruxellensis* was then selected as the spoilage yeast to be used when testing the withdrawn crude aliquots which would allow to determine the needed fermentation rate constants. Generally, beverage spoilage control entails the prevention of an undesired fermentation during processing or storage; hence, the requirement to use only cell free supernatants as opposed to yeast cells during postharvest control experiments.

Table 3-2 Fermentation parameters studied and models used during production of biopreservation compounds by *Candida pyralidae* Y1117, *Pichia kluyveri* Y1125 and *P. kluyveri* Y1164 in a grape pomace extracts medium

Fermentation parameters	Model/Equation	Description
Substrate utilisation rate ($g\ mL^{-1}\ h^{-1}$)	$r_s = \frac{dS}{dt}$	This describes the speed of substrate depletion during fermentation. In this case, the depletion rate of total sugar and yeast assimilable nitrogen (YAN) can be considered.
Biomass formation rate ($cells\ mL^{-1}\ h^{-1}$)	$r_x = \frac{dX}{dt}$	This describes how fast a specific number of yeast cells are formed during the fermentation period.
Biomass yield ($cells\ g^{-1}$)	$Y_{X/S} = \frac{dX}{dS}$	This estimates how many cells are formed per gram of the substrate utilised.
Specific growth rate (h^{-1})	$\mu = \frac{ln(X_f/X_0)}{t}$	This quantifies the increase in cell concentration during a specific fermentation period regardless of the availability and preference of the growth controlling substrates.
Biopreservation compounds formation rate ($L\ VZI\ mL^{-1}\ BCU\ h^{-1}$)	$r_p = \frac{dP}{dt}$	The observed effects of biopreservation compounds are used to assess the level of production during a specific fermentation time.
Biopreservation compound formation based on cell concentration ($L\ VZI\ cells^{-1}$)	$Y_{P/X} = \frac{dP}{dX}$	Given the fact that the biopreservation compounds are produced as a result of cellular growth, this model helps to explain and quantify the observed effect of biopreservation compounds in relation to the quantity of cells generated during fermentation.
Biopreservation compounds formation based on substrate utilisation ($L\ VZI\ g^{-1}$)	$Y_{P/S} = \frac{dP}{dS}$	Based on how much growth controlling substrate is being utilised during fermentation, this equation correlates the observed effect of biopreservation compounds with the amount of substrate utilised.
Substrate consumption model	$\frac{dS}{dt} = \frac{dX}{Y_{\frac{X}{S}}dt} + \frac{dP}{Y_{\frac{P}{S}}dt} + m_s X$	This model describes the trend of growth controlling substrate utilisation in direct proportion to biomass and product formation, as well as cellular maintenance.

X = Cell concentration ($cells\ mL^{-1}$); X_o = Initial cell concentration ($cells\ mL^{-1}$); X_f = Final cell concentration ($cells\ mL^{-1}$); S = Limiting substrate concentration ($g\ mL^{-1}$); P = Biopreservation compounds formation ($L\ VZI\ mL^{-1}\ BCU$); t = time (h); μ = Specific growth rate (h^{-1}); r_x = Cellular growth rate ($cells\ mL^{-1}\ h^{-1}$); r_P = Volumetric inhibitory activity rate ($L\ CSM\ mL^{-1}\ BCU\ h^{-1}$).

3.11 Response Surface Methodology (RSM) for the Optimization of Biopreservation Compounds Production using GPE Broth as Fermentation Medium

A central composite design (CCD) approach was used and a total of 30 experimental runs for each yeast was generated, using Design-Expert® software version 10.0.0 (Stat-Ease Inc., Minneapolis, MN, USA), to assess the effect of independent variables (fermentation time, pH, temperature and total sugar concentration) on the production of potential biopreservation compounds. The independent variable interactions were determined by fitting the experimental data to a second order polynomial model (Equation 5). Each experiment had three replicates and the mean value of each run was used for data fitting while accounting for variations in the experimental data. Table 3.3 contains the process variables used and their ranges.

The statistical analysis was used to determine the significance of the models generated for each yeast strain. It was carried out by means of analysis of variance (ANOVA) incorporated in the Design-Expert® software version 10.0.0 used. Furthermore, numerical optimization software incorporated in Expert design version 10.0.0 was also used to identify the interactions of independent variables that yielded the highest concentration of potential biopreservation compounds in the crude (Table 3.4).

Table 3-3 Process variables, i.e. time, pH, temperature and total sugar used in the central composite design (CCD) for optimisation of biopreservation compounds production by *Candida pyralidae* Y1117, *Pichia kluyveri* Y1125 and *Pichia. kluyveri* Y1164 using grape pomace extracts as fermentation medium

Factors	Units	Code	Low (-1)	High (+1)
Time	H	A	8	40
pH	-	B	2	7
Temperature	^{o}C	C	15	25
Total sugar	g L^{-1}	D	50	180

$$Y = \beta_0 + \sum \beta_1 X_1 + \sum \beta_{12} X_1 X_2 + \sum \beta_{11} X_1^2 + \varepsilon$$

Equation 5

β_1, β_{12} and β_{11}, are the regression coefficients for the linear, interaction and quadratic effects, respectively. The symbols ε, Y and β_0 are random errors, response variables and the intercept value, respectively. The symbols X_1, X_2 (1,2…n) represent the independent variables. Given that 4 independent variables were used in this optimisation study, the equation above then changed to:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{14} X_1 X_4 + \beta_{23} X_2 X_3 + \beta_{24} X_2 X_4 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{44} X_4^2 + \varepsilon$$

Equation 6

Towards constructing the indices of desirability, five goal possibilities were used and the indices used were none, maximum, minimum, target and within range. The criteria for the selection of the optimum conditions for production of biopreservation compounds are shown in Table 3.4 and the "importance" value of 5 was considered as the maximum desirable goal. The "importance" value assigned to a parameter shows the level of importance or weight that a specific parameter carries towards achieving the set target.

Table 3-4 Criteria for the selection of optimum conditions for production of crude biopreservation compounds (desirability response) in grape pomace extracts as fermentation medium.

Factors	Goal	Lower limit	Upper limit	Lower weight	Upper weight	Importance
Time (h)	In range	4	32	1	1	3
pH	In range	2	7	1	1	3
Temperature (^oC)	In range	15	25	1	1	3
Sugar concentration (g L^{-1})	In range	5	18	1	1	3
Response (L VZI mL^{-1} BCU h^{-1})	Maximise	0.1658	1.2717	1	1	5

3.12 Preparation of crude growth inhibition mixtures and spoilage yeasts consortia

3.12.1 Mixture of biopreservation compounds

Candida pyralidae Y1117, *Pichia kluyveri* Y1125 and *Pichia kluyveri* Y1164 were grown separately in GPEB (75 g L^{-1}) for 24 hours at 25 ^oC in a shaking incubator (LM-53OR, RKC® Instrument INC, Ohta-ku Tokyo, Japan) at 150 rpm. The broths, after fermentation, were centrifuged for 5 minutes at 5000 rpm. The resulting supernatant from each of the yeast culture broth was aliquoted into Eppendorf tubes (2 mL). The mixture of biopreservation crude compounds was prepared as follows: a volume (50 µL) from each of the three cell free supernatants was mixed in a separate Eppendorf tube (2 mL) and stored at 4 ^oC for further use. The different growth inhibitor combinations (GICs) from the cell free supernatants were GIC 1 (*C. pyralidae* Y1117 and *P. kluyveri* Y1125); GIC 2 (*C. pyralidae* Y1117 and *P. kluyveri* Y1164), GIC 3 (*P. kluyveri* Y1125 and *Pichia kluyveri* Y1164); GIC 4 (*C. pyralidae* Y1117, *P. kluyveri* Y1125 and *P. kluyveri* Y1164).

3.12.2 Preparation for consortia of spoilage organisms

Three beverage spoilage yeasts strains, *Dekkera anomala*, *Dekkera bruxellensis* and *Zygosaccharomyces bailii* were grown in GPEB without agitation for 48 hours at 25 ^oC prior to the growth inhibition assays. From the 48-hour old spoilage yeast cultures, a volume (2 mL) of the broth was centrifuged for 5 minutes at 5000 rpm after which the supernatant was discarded, with the resulting pellet from each of the spoilage yeast cultures being washed with sterile distilled water prior to storage for the assays. The mixtures of spoilage yeasts were prepared in different combinations, such that spoilage combination (SC 1) was composed of *D. anomala* and *D. bruxellensis*; SC 2 (*D. anomala* and *Z. bailii*), SC 3 (*D. bruxellensis* and

Z. bailii); SC 4 (*D. anomala*, *D. bruxellensis* and *Z. bailii*). For each consortium, the individual spoilage organism was at a final concentration of 10^3 cells mL^{-1}. The prepared mixtures were then used to seed the GEA plated for growth inhibition assays.

3.13 Growth inhibition study of mixed biopreservation compounds against spoilage organisms' consortia

3.13.1 Effect of cell free supernatants on growth inhibition activity of single spoilage organism

The growth inhibition efficiency of *C. pyralidae* Y1117, *P. kluyveri* Y1125 and *P. kluyveri* Y1164 extracellular metabolites was tested by spotting 20 µL of the cell free supernatant in a 5 mm diameter well on GEA seeded with the spoilage organisms at the concentration of 10^6 cells mL^{-1}. The plates were incubated at 25 ºC until zones of inhibition were observed around the wells in the GEA plates. The growth inhibition activity was quantified as described by (Mewa-Ngongang et al., 2017).

3.13.2 Effect *Effect of cell free supernatant from single yeasts on growth inhibition activity of spoilage organisms' consortia*

The ability of *C. pyralidae* Y1117, *P. kluyveri* Y1125 and *P. kluyveri* Y1164 extracellular metabolites to inhibit the consortia of spoilage yeasts, was assessed by spotting 20 µL of the crude biopreservation samples in the GEA plate wells prepared according to the different spoilage combinations. The plates were incubated at 25 ºC until the zone of inhibition was obtained. The growth inhibition quantification was also assessed by the method developed and reported by (Mewa-Ngongang et al., 2017).

3.13.3 Effect of mixed crude biopreservation compounds on the growth inhibition of spoilage organism consortia

A mixture of crude extracellular compounds from the three producing yeasts was prepared by mixing an equivalent volume of the crude supernatant. The spoilage organisms' consortia was prepared by mixing an equivalent volume of each culture (10^3 cells mL^{-1}) of individual spoilage organisms. From the mixture of the crude supernatant, 20 µL was spotted on the different spoilage organism consortia plates followed by incubation until zones of inhibition were observed, i.e. being quantifiable.

3.14 Effect of *C. pyralidae* and *P. kluyveri* cells on fungal spore germination

A radial inhibition assay was conducted using the agar plate method as described by (Núñez et al. (2015). Yeast (*C. pyralidae* and *P. kluyveri*) cell suspensions (1 x 10^8 cells mL^{-1}) were prepared from the yeast culture broths and a fungal spore suspension of 10^5 spores mL^{-1} was prepared from the mother solution. A volume (100 µL) of yeast was spread-platted on GPA and dried. Thereafter, 10 µL of 10^5 spores mL^{-1} of each fungus was spotted at the centre of the plate with each treatment prepared in three replicates. For the negative control plates, only 10 µL of the spore solution (10^5 spores mL^{-1}) were spotted at the centre of the GPA. The plates were then incubated at 15ºC for 7 days. The fungal radial inhibition (FRI) was calculated using the mathematical expression: $FRI = (D_0\text{-}D_t/D_0) \times 100$, whereby D_0 represented the average diameter

of the fungal colony on the negative control plates and D_t represented the diameter of the fungal colony on the yeast treated plates (Núñez et al., 2015).

3.15 Volumetric Headspace Quantification

The volume of the headspace in the agar plates was calculated by considering the diameter and the height of the closed 90 mm diameter petri dish, using the approach described by (Mewa-Ngongang et al., 2017). The thickness (15 mm) of the empty petri dish was determined and the headspace volume was obtained by accounting for the volume of grape pomace agar (GPA) poured on the plate. The volume of the poured medium was also determined and subtracted from the total. The evaluation of the actual headspace in contact with the fungal pathogen was done to determine the fungistatic and fungicidal effect of the biological control agents when a specific inoculum dose was used in a quantified headspace.

3.16 Effect of volatile organic compounds (VOCs) on fungal growth

The mouth-to-mouth assay method (Medina-Córdova et al., 2016) was repeated twice and used for yeast and fungal growth inhibition assays. Two GPA plates (facing each other) were used, with the bottom plate being spread-platted with a volume (100 µL) of yeast suspension (10^8 cell.mL^{-1}) while the top plate (cover) contained a 5 mm mycelial disk at the centre. The negative control plates were only seeded with a 5 mm diameter mycelial disk (no yeast treatment in the bottom plate). Prior to incubation at 15ºC for 7 days, all plates (in triplicates) were sealed with laboratory film. The VOCs inhibition activity (VOCIA) was calculated as described by the mathematical expression used for FRI.

3.17 *In-vivo* studies: Post-harvest efficacy of *C. pyralidae* and *P. kluyveri* in controlling fruit spoilage

3.17.1 Apple bioassays

Post-harvest biocontrol efficacy assays were performed on apples (*Malus domestica*). Ethanol (70% $^{v}/_{v}$) was sprayed on the fruits and allowed to dry completely before wound infliction. Apples (10 replicates consisting of three apples per replicate) were uniformly wounded with a sterile cork borer (approximately 5 mm diameter and 3 mm deep). After the wound infliction (15 minutes), 15 µL of a yeast inoculum (1 x 10^8 cell.mL^{-1}) was introduced into the wound and then allowed to dry for 30 minutes. Thereafter, 15 µL of the spore suspension (10^5 spores mL^{-1}) was introduced into the wound. Treated fruits were maintained at -0.5 ºC for 4 weeks in a tightly closed container, and then stored at room temperature (±20 ºC) for 7 days, to simulate shipping and shelf life conditions in a commercial setting. The containers in which the fruit were incubated were tightly closed in order to entrap the VOCs in the airspace and to observe the VOCs effect similar to the *in-vitro* plate assay. The biocontrol efficacy was evaluated by comparing the decay diameter of the negative control to those of the treated apples using the FRI. Negative controls were prepared by inoculating fruits with 15µL (10^5 fungal spores mL^{-1}) of *B. cinerea, C. acutatum* and *R.*

stolonifer suspensions under similar maintenance and storage conditions. Positive results were characterised by the absence of fungal development which causes decay on the fruit wound surface.

3.17.2 Grape bioassays

Table grapes (20 replicates consisting of 10 grape berries per replicate) were uniformly inflicted with 3 wounds per spot with a sterile needle (< 1 mm diameter per wound, 1 wound spot per berry) and allowed to dry prior to yeast and fungal treatments. The wounded berries were sprayed with yeast (*C. pyralidae and P. kluyveri*) cell suspension (1 x 10^8 cell.mL^{-1}) until the dried wounds were filled with the yeast suspension, and subsequently allowed to dry again for 30 minutes. The dried berries were then sprayed separately with fungal spore suspension (10^5 spores mL^{-1}). The negative controls (10 berries each) were prepared by spraying the fungal spores on the wounded berries without prior yeast treatment. All grape treatments were also maintained in sealed jars at -0.5 °C for 4 weeks, and then incubated at room temperature (±20 ºC) for 7 days. The antagonistic properties of *C. pyralidae and P. kluyveri* were analysed visually by assessing the berries colour changes and fungal development.

3.18 Identification and quantification of VOCs produced by *C. pyralidae* Y1117, *P. kluyveri* Y1125 and *P. kluyveri* Y1164

Aliquots (10 mL) of cell free supernatant was placed in a 20 mL headspace vial to which NaCl (30% *m*/*v*) was added to facilitate evolution of volatiles into headspace and inhibit enzymatic degradation. Vials were spiked with 100 µL of anisole d8 and 3-octanol as internal standards. Solid-Phase Micro Extraction (SPME) vials were equilibrated for 5 min in the CTC auto sampler incubator (50 °C) at 250× rpm. Subsequently, a 50/30 divinylbenzene/-carboxen/-polydimethylsiloxane (DVB/CAR/PDMS) coated fiber was exposed to the sample headspace for 10 min at 50 °C. After the VOCs' adsorption onto the fiber extraction, desorption of the VOCs from the fiber coating was carried out in the injection port of the gas chromatography-mass spectrometry (GC–MS) for 10 min. The fiber was inserted in a fiber conditioning station for 10 min between samples for cleaning to prevent cross- and carry-over contamination. Chromatographic separation of the VOCs was performed in a Thermo TRACE 1310 gas chromatograph coupled with a Thermo TSQ 8000 mass spectrometer detector. The GC–MS system was equipped with a polar DB-FFAP column (Model number: J&W 122-3263), which is a nitroterephthalic-acid-modified polyethylene glycol (PEG) column of high polarity for the analysis of VOCs, with a nominal length of 60 m; 250-µm internal diameter; and 0.5-µm film thickness. Analyses were conducted using helium as a carrier gas at a flow of 2.9 mL min^{-1}. The injector temperature was maintained at 250 °C. The oven program was as follows: 350 °C for 17 min; and subjected to a final temperature of 240 °C at an increased rate of 12 °C min^{-1} and held for 6 min. The MS was operated in a full scan mode. Both the ion source transfer line temperatures were maintained at 250 °C. Compounds were tentatively identified by comparison with a mass spectral libraries (NIST, version 2.0), subsequent to quantification using the calculated relative abundances.

3.19 Statistical analysis

To determine whether there were significant differences within treatments, analysis of variance (ANOVA) was performed using the SAS software (Version 9.4; SAS Institute Inc, Cary, NC, USA). The significant difference was calculated at the 5% level and $p<0.05$ was considered significant for treatments.

3.20 Biological thermodynamic study during biopreservation compounds production from yeasts using grape pomace extract as fermentation medium

3.20.1 Dried biomass preparation and elemental analysis

The three biocontrol yeasts *C. pyralidae* Y1117, *P. kluyveri* Y1125 and *P. kluyveri* Y1164 were cultured under the optimum growth condition found for each yeast. The inoculation was done as explained under section 3.7. For *C. pyralidae* Y1117 subjected to 150 rpm, a 24 h fermentation period, a temperature of 25 °C, a total sugar concentration of 150 g L^{-1} and a pH of 3 were used as growth conditions; meanwhile the growth conditions for *P. kluyveri* strains were 28 h of fermentation, pH 3, temperature of 25 °C and sugar concentration of 150 g L^{-1}, also subjected to 150 rpm rotational speed of the fermentation flasks. Each yeast was cultured in a three litter Erlenmeyer flask containing 1.5 L growth medium. After the set fermentation period for each batch, the yeast cultures were harvested by centrifugation at 10000 rpm for 10 minutes at 4 ºC. The resulting biomass pallets were washed three times in sterile distilled water and dry in a Duran® vacuum desiccator (DURAN Group GmbH, Germany) the process was repeated three times until a considerable amount of dried biomass was obtained. The drying process was carried out as follows: The wet cells of *C. pyralidae* Y1117, *P. kluyveri* Y1125 and *P. kluyveri* Y1164 were picked using a sterile spatula into sterile petri dishes. The plates were then placed for eight hours in an oven dryer set at 70 ºC and thereafter put in a dissector, placed in a 28 ºC- room for three days. Each plate was weighed twice a day to assess the fluid loss. To remove excess fluid, the plates were transferred to sterile glass beakers and further dried in an oven set at 50 ºC until the weight became constant.

The analysis of carbon, hydrogen, nitrogen and sulphur (CHNS) contents of each dried biomass was performed in three replicates by the Central Analytical facilities (a service laboratory) of the University of Stellenbosch, South Africa. The fraction corresponding to the percentage of oxygen was that of the remaining fraction after the sum of CHNS percentages was subtracted from 100.

3.20.2 Determination of the heat of combustion

An e2k Bomb calorimeter (Digital Data Systems Pty Ltd, South Africa) was used to determine the heat of combustion of the dried biomasses. The principle of general combustion was used, whereby the analyser was set to combust the samples in three replicates in the presence of oxygen to form CO_2, H_2O and N_2. The products of combustion were then separated using a gas chromatograph and then further analysed by a thermal conductivity detector. The peaks obtained, corresponding to each compound (product of combustion) were integrated and then the percentages of C, H, O, N and S were calculated.

Table 3-5 Thermodynamic properties of compounds used at 298.15 K and 1 atm (Battley, 2011)

Substance	Formula	ΔH_f^O (KJ/mol)
Glucose	$C_6H_{12}O_{6(aq)}$	-1263.07
Ammonia	$NH_{3(aq)}$	-80.29
Oxygen	$O_{2(aq)}$	-12.09
Water	$H_2O_{(l)}$	-285.83

3.20.3 Determination of the biological stoichiometric equations

As indicated in the literature (Duboc et al., 1999; Liu et al., 2007; Battley, 2013) microbial metabolism is generally coupled with two stages including the catabolic and the anabolic reactions. Model equations for catabolic, anabolic and metabolic processes were used (see Table 2.3). The biomass yield based on substrate consumption was calculated to determine the number of mole of glucose utilised to form a unit cell. A unit carbon per mole of glucose molecular weight of each yeast was then determined which yielded the equations listed below that would be balanced using the law of conservation of mass based on the balances of the elemental molecular constituents.

C. pyralidae Y1117

$$1.8997C_6H_{12}O_{6(aq)} + aO_{2(aq)} + bNH_{3(aq)} + cSO_{4(aq)}^{2-} + dH_{(aq)}^{+} \rightarrow$$
$$CH_{1.906}N_{0.149}O_{0.805}S_{0.003}(cell) + eCO_{2(aq)} + fH_2O_{(l)}$$

Equation 7

***P. kluyveri* Y1125**

$$1.9293C_6H_{12}O_{6(aq)} + aO_{2(aq)} + bNH_{3(aq)} + cSO_{4(aq)}^{2-} + dH_{(aq)}^{+} \rightarrow$$
$$CH_{1.891}N_{0.132}O_{0.833}S_{0.003}(cell) + eCO_{2(aq)} + fH_2O_{(l)}$$

Equation 8

P. kluyveri Y1164

$$3.043C_6H_{12}O_{6(aq)} + aO_{2(aq)} + bNH_{3(aq)} + cSO_{4(aq)}^{2-} + dH_{(aq)}^{+} \rightarrow$$
$$CH_{1.868}N_{0.146}O_{0.698}S_{0.003}(cell) + eCO_{2(aq)} + fH_2O_{(l)}$$

Equation 9

CHAPTER 4

THE USE OF *CANDIDA PYRALIDAE* AND *PICHIA KLUYVERI* TO CONTROL SPOILAGE MICROORGANISMS OF RAW FRUITS USED FOR BEVERAGE PRODUCTION

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CHAPTER 4

THE USE OF *CANDIDA PYRALIDAE* AND *PICHIA KLUYVERI* TO CONTROL SPOILAGE MICROORGANISMS OF RAW FRUITS USED FOR BEVERAGE PRODUCTION

4.1 Introduction

Fruit and fruit-derived beverages are important nutritional and economically viable commodities. They also provide vitamins and some essential minerals in the human diet. However, fruit losses caused by fungal pathogens pose numerous challenges to the agricultural industry (Parveen et al., 2016; Salman, 2005). Additionally, the shortened shelf life of fruit because of spoilage is a factor that negatively affects the market value and processing of fruit. During post-harvest handling, about 25% of the total harvested fruit is lost due to fungal pathogens (Droby, 2005; El Ghaouth et al., 2004; Singh & Sharma, 2007; Zhu, 2006). *Botrytis cinerea*, *Colletotricum acutatum* and *Rhizopus stolonifer* are primarily responsible for spoilage of produce for about 200 crop species, including apples and table grapes (Sharma et al., 2009; Williamson et al., 2007). The constituents in fruit and derived beverages, i.e. sugar content, macro- and micro-nutrients, provides suitable growth controlling substrates for microbial proliferation and could also render the medium prone to microbial spoilage. Other spoilage microorganisms such as *Dekkera bruxellensis*, *Dekkera anomala* and *Zygosaccharomyces bailii* are known to be responsible for spoilage and product losses in the beverage industry (Comitini, Di Pietro, et al., 2004; Thomas & Davenport, 1985; Zuehlke et al., 2013). To minimise such losses, synthetic chemicals are currently being used for controlling microbial spoilage, but their continued use as post-harvest control agents of fruit or as preservatives, raises human health concerns. As a result, the eradication of these chemicals and the development of safer alternatives is now a priority in fruit and beverage industries, i.e. agro-processing and retail industries (Cheetham, 1997; El Ghaouth et al., 2004; Mostafa et al., 2018; Siroli et al., 2015).

As an alternative to synthetic chemicals, the antagonistic mechanisms of yeasts against fruit and beverage spoilage microorganisms have been investigated (Aloui et al., 2015; Cordero-Bueso et al., 2017; Mewa-Ngongang et al., 2017; Sharma et al., 2009). Several antagonistic characteristics are associated with the ability of the yeasts to rapidly reproduce on simple nutrients and to colonise surfaces, while competing for nutrients and space (Bencheqroun et al., 2007; Liu et al., 2013); induction of host resistance for some yeasts (Droby et al., 2002) and the ability of yeasts to produce enzymes such as Laminarinases and chitinases (Fan et al., 2002; Grevesse et al., 2003).

Further antagonistic mechanism can be attributed to the effect of decreasing germ tube length and reduction in fungal spore germination (Zheng et al., 2005) as well as the fungal pathogen growth inhibition by

diffusible volatile organic compounds (VOCs) (Grzegorczyk et al., 2017; Hua et al., 2014; Huang et al., 2011a; Lutz et al., 2013). Species of *C. pyralidae* have previously been studied as producers of killer toxins which can act against beverage spoilage organisms (Mehlomakulu et al., 2014). However, many have not yet being explored as potential biocontrol agents against fruit spoilage organisms. Similarly, *P. kluyveri* has also been determined as an antagonistic yeast for biocontrol applications (Crafack et al., 2013; Gross et al., 2018).

4.2 Objectives

The objectives of this part of the study were:

- To explore whether *C. pyralidae* and *P. kluyveri* strains isolated from South African fruit can act as source of biopreservation compounds against beverage spoilage yeasts.
- To explore the effect of *C. pyralidae* and *P. kluyveri* strains on the growth inhibition of fruit spoilage organisms *in-vitro*.
- To assess the post-harvest biocontrol potential of *C. pyralidae* and *P. kluyveri* cells *in-vivo*.

4.3 Materials and methods

Previously isolated yeasts, *C. pyralidae* and *P. kluyveri* were obtained from the ARC Infruitec-Nietvoorbij culture collection (Stellenbosch, South Africa). A screening procedure was carried out to confirm their growth inhibition potential against beverage spoilage organisms, *D. bruxellensis* (ISA 1653), *D. anomala* (MSB/1) and *Z. bailii* (Y0070) and fruit spoilage organisms *B. cinerea*, *C. acutatum* and *R. stolonifer* as explained in chapter 3.

The potential of grape pomace extracts as growth and fermentation media were explored as decribed in chapter 3. The cell free supernatants, the *C. pyralidae* and *P. kluyveri* cell suspensions were obtained from the yeast culture broths and tested separately. The cell free supernatants were tested on beverage spoilage yeasts using the plate assay where the results were checked by visual assessment of the size of the clear zones around the agar wells. The *C. pyralidae* and *P. kluyveri* cell suspensions were tested against fruit spoilage organisms using the plate assays, i.e. radial inhibition and volatile organic compounds (VOCs) assays on fungal growth subsequent to bioassays on apple fruit and table grapes as explained in chapter 3. Finally, a gas chromatographic analysis of the VOCs was carried out in order to identify the organic compounds that could have been responsible for the growth inhibition activities.

4.4 Results and discussion

4.4.1 Production of biopreservation compounds and their effect on the growth of spoilage yeasts

Figure 4.1 showed that, using GPE as fermentation medium, *C. pyralidae* and *P. kluyveri* were able to produce crude extracellular compounds with growth inhibition activity against *D. bruxellensis*, *D. anomala* and *Z. bailii*, which are common spoilage yeasts in beverages. This was observed by the presence of clear zones of inhibition around the yeast colonies or agar wells. A visual observation of clear zone of inhibition was required at this stage to verify the growth inhibition compound production and the presence of inhibition activity for the yeasts and their cell free supernatants (crude samples). Previous studies on inhibition assays were conducted using costly refined media such as YPD (Mehlomakulu et al., 2014; Mewa-Ngongang et al., 2017). However, the current study successfully managed to use a less expensive GPE medium for antimicrobial compound production. The findings in this work are complementary and in reasonable agreement with the research previously conducted by Mewa-Ngongang et al. (2019), whereby a kinetic study and optimisation of biopreservation compounds production using GPE was carried-out successfully; with findings demonstrating that GPE medium at a total sugar concentration of 150 g L^{-1} was a suitable medium for the production of antimicrobial compounds from *C. pyralidae* and *P. kluyveri* strains.

A sugar-dependent trend was also observed when more growth inhibition and/or a higher volumetric zone of inhibition was promoted for fermentations in which 150 g L^{-1} sugar concentration was used, and allowed to ferment for 24 h (Figure 4.1c). Further observations indicated shorter fermentations and lower substrate concentrations can save operational costs while attaining the desired product formation more rapidly. Although other yeasts strains are known to have growth inhibition properties against beverage (*D. anomala*, *Z. bailii*) and fruit spoilage organisms (*C. acutatum* and *R. stolonifer*), the current study is the first to report on the growth inhibition properties of *C. pyralidae* and *P. kluyveri* against these spoilage organisms. (Mehlomakulu et al., 2014) only reported on the growth inhibition activity of *C. pyralidae* against *D. bruxellensis*. However, the current study additionally identified *P. kluyveri* as an antagonist organism against *D. bruxellensis* (Figure 4.1 (a2). The current study also ascertained that *C. pyralidae* has growth inhibition activities against *Z. bailii* (Figure 4.1b) and *D. anomala* (data not shown); a profile showing *C. pyralidae* as having a much broader biocontrol application.

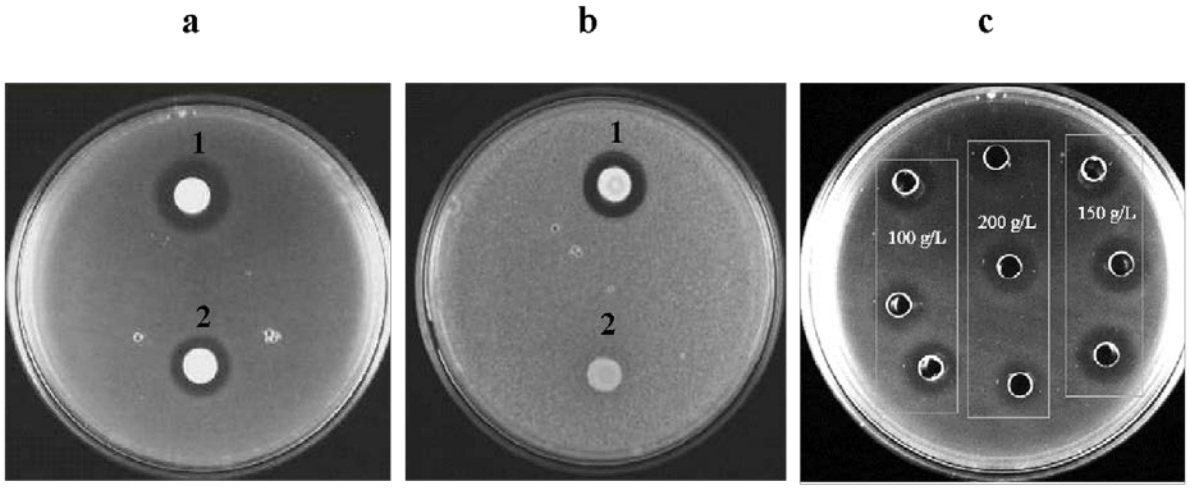


Figure 4-1 (a): Antagonistic activity of *Candida pyralidae* (1) and *Pichia kluyveri* (2) against *Dekkera bruxellensis*. (b): Antagonistic activity of *Candida pyralidae* (1) and *Pichia kluyveri* (2) against *Zygosaccharomyces bailii*. (c): Depiction of the inhibition activity of the biopreservation compounds produced by the antagonistic yeasts *Candida pyralidae* and *Pichia kluyveri* against *Dekkera bruxellensis* after 24 h of fermentation in grape pomace with varying sugar concentration (100, 150 and 200 g L^{-1}).

4.4.2 Effect of *C. pyralidae* and *P. kluyveri* cells on fungal growth

A radial inhibition assay was performed in order to assess the antagonistic effect of *C. pyralidae* and *P. kluyveri* on the germination of *B. cinerea*, *C. acutatum* and *R. stolonifer* spores. A 100% inhibition against the germination of *B. cinerea*, *C. acutatum* and *R. stolonifer* was obtained on the low-cost GPA plates (Figure 4.2A) and validated graphically (Figure 4.2B). It was evident that yeasts can prevent the growth of fungi in different ways, such as the ability to outgrow the fungal pathogens and to rapidly colonise wound surface, thereby reducing fungal development. This study also revealed the post-harvest control potential of these yeast strains against *B. cinerea*, *C. acutatum* and *R. stolonifer*. The antagonistic activity of yeast against fruit fungal pathogens was previously conducted albeit on refined media (Cordero-Bueso et al., 2017; Grzegorczyk et al., 2017; Medina-Córdova et al., 2016), but not on GPA plates as carried out in this work, therefore highlighting the novelty of the current study.

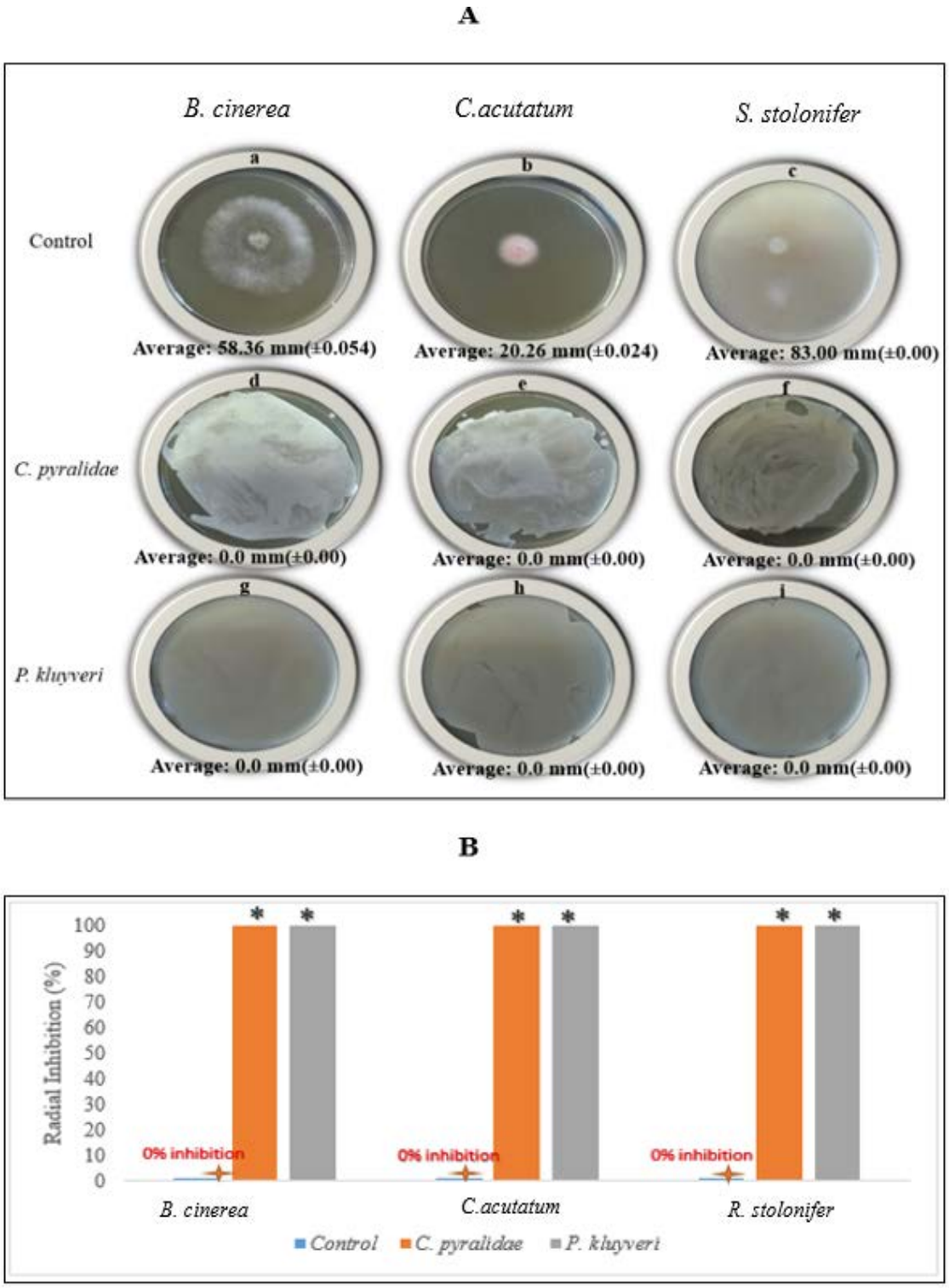
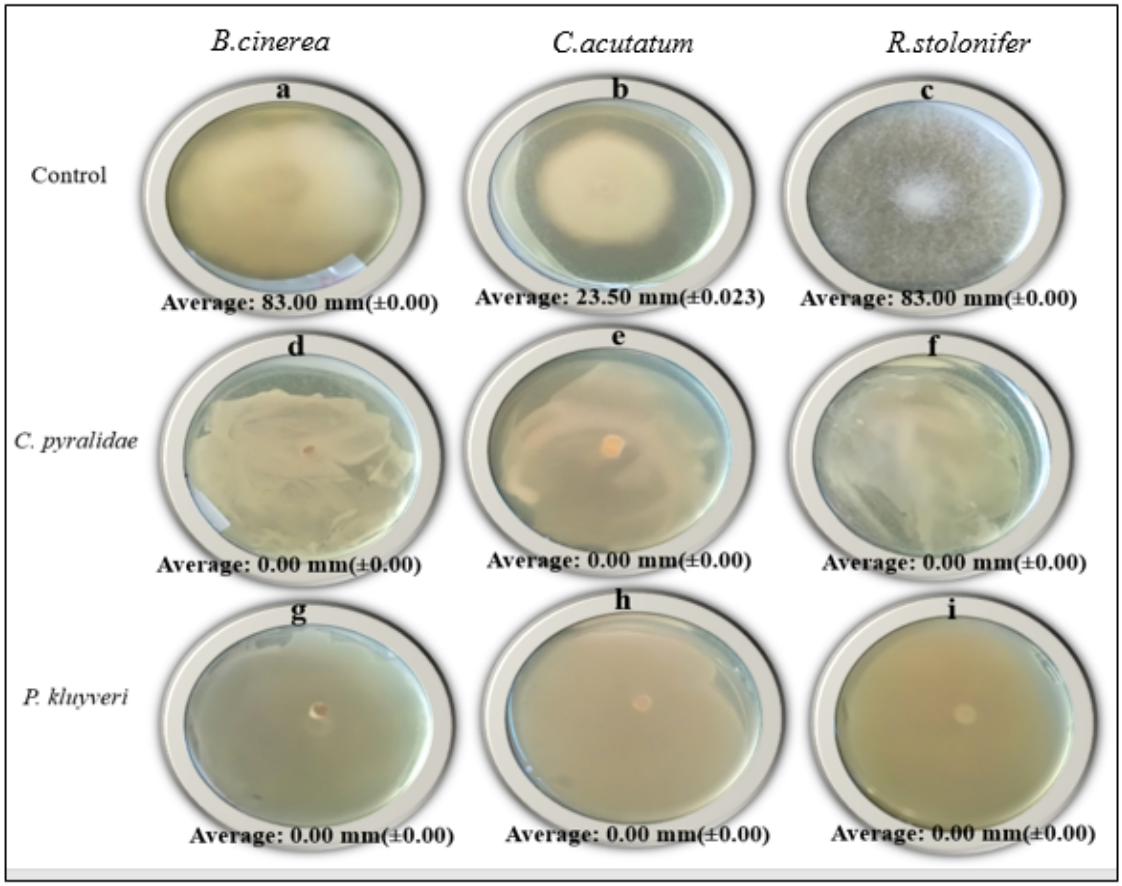


Figure 4-2 The visual (A) and graphical (B) representation the antagonistic effect of *C. pyralidae* cells on *B. cinerea* (A: d), *C. acutatum* (A: e) and *R. stolonifer* (A: f), and the antagonistic effects of *Pichia kluyveri* cells on the growth of *B. cinerea* (A: g), *C. acutatum* (A: h) and *R. stolonifer* (A: i). The negative controls are displayed as *B. cinerea* (A; a), *C. acutatum* (A: b) and *R. stolonifera* (A: c). Values are the average of 1 replicates consisting of three independent treatments ± standard deviation. The asterisk (*) indicates values that differ significantly from the control ($p<0.05$).

4.4.3 Effect of volatile organic compounds (VOCs) on fungal growth

The influence of yeast-producing VOCs on fruit fungal growth was investigated using a mouth to mouth assay. Compared to the negative controls, the visual (Figure 4.3A) and the graphical (Figure 4.3B) representation showed *C. pyralidae* and *P. kluyveri* producing VOCs that have an ability to completely inhibit the growth of *B. cinerea*, *C. acutatum* and *R. stolonifer*. Apart from the abovementioned cell antagonistic properties, the biocontrol mechanisms include the ability to secrete extracellular metabolites (e.g. VOCs) with growth inhibition activity against fungal pathogens (Druvefors et al., 2005; Hua et al., 2014; Huang et al., 2011a). The antagonistic effect of VOCs produced by *Debaryomyces hansenii* against *Mucor circinelloides*, *Aspergillus* sp, *F. proliferatum* and *F. subglutinan* was also confirmed previously using the same mouth to mouth assay technique (Medina-Córdova et al., 2016). The current study further demonstrated the potential of VOCs produced by *C. pyralidae* and *P. kluyveri* to inhibit the growth of *B. cinerea*, *C. acutatum* and *R. stolonifer*.

A



B

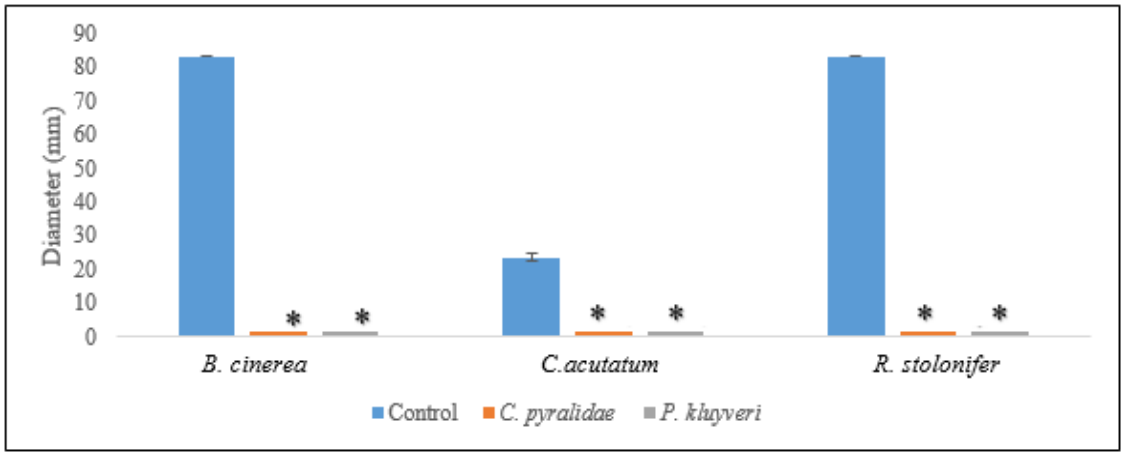


Figure 4-3 The visual (A) and graphical (B) representation the antagonistic effect of volatiles produced by *Candida pyralidae* against *B. cinerea* (A: d), *C. acutatum* (A: e) and *R. stolonifer* (A: f), and the antagonistic effects of *Pichia kluyveri* against the growth of *B. cinerea* (A: d), *C. acutatum* (A: e) and *R. stolonifer* (A: f). The negative controls are displayed as *B. cinerea* (A: a), *C. acutatum* (A: b) and *R. stolonifera* (A: c). Values are the average of 10 replicates consisting of three independent treatments ± standard deviation. The asterisk (*) indicates values that differ significantly from the control ($p<0.05$).

4.4.4 Post-harvest control efficacy of *C. pyralidae* and *P. kluyveri* on fungal growth

4.4.4.1 Apple bioassay (*Malus domestica*)

The evaluation of the efficacy of yeasts in preventing fungal spoilage of apples using bioassays showed a considerable decay reduction (Figure 4.4). For the purpose of visual representation, only three apples per treatment were selected as representatives (Figure 4.4). The apple bioassay demonstrated the ability of *C. pyralidae* and *P. kluyveri* to control spoilage caused by *B. cinerea*, *C. acutatum* and *R. stolonifer*; however, the inhibition responses were yeast and fungal species-dependent. A 100% fungal growth inhibition was observed when both *C. pyralidae* and *P. kluyveri* were tested against *C. acutatum. C. pyralidae*, showing a 43 and 52% growth inhibition of *B. cinerea* and *R. stolonifer*, respectively, while *P. kluyveri* revealed a 38 and 22% growth inhibition of *B. cinerea* and *R. stolonifer*. Fluctuating *in-vivo* post-harvest control responses has been reported for other species including *Sporidiobolus pararoseus* (Huang et al., 2012), *Saccharomyces cerevisiae*, *Wickerhamomyces anomalus, Metschnikowia pulcherrima* and *Aureobasidium pullulans* (Parafati et al., 2015) and *Hanseniaspora uvarum* (Qin et al., 2015). Although *C. pyralidae* and *P. kluyveri* showed greater biocontrol potential on plate assays, the apple bioassay showed a significant decay reduction (Figure 4.4).

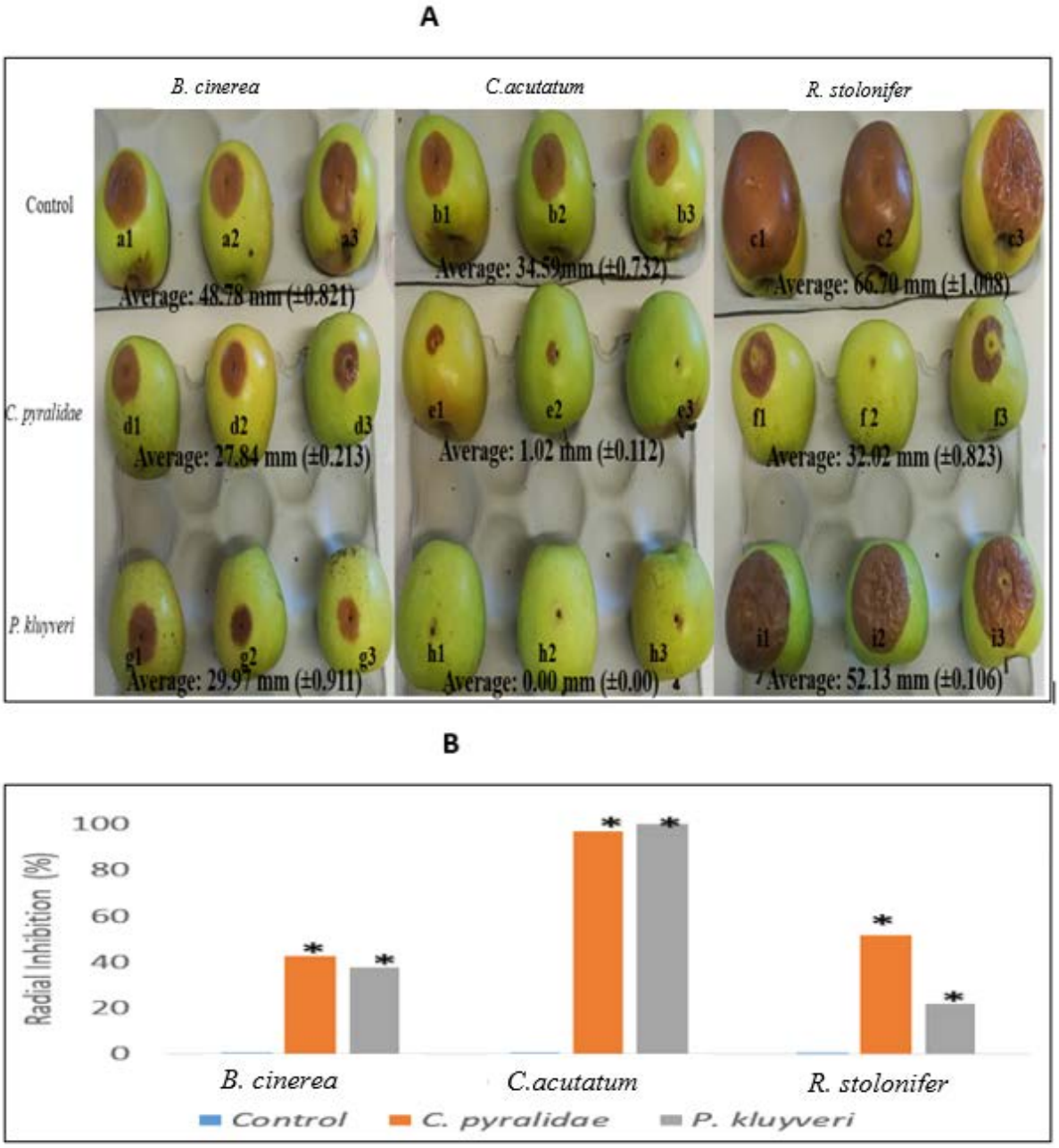


Figure 4-4 The visual (A) and graphical (B) representation of the apple bioassays for C. *pyralidae* against the growth of *B. cinerea* (A: d1-d3), *C. acutatum* (A: e1-e3) and *R. stolonifer* (A: f1-f3), and for *P. kluyveri* against the growth of *B. cinerea* (A: g1-g3), *C. acutatum* (A: h1-h3) and *R. stolonifer* (A: i1-i3). The negative controls are displayed as *B. cinerea* (A: a1-a3), *C. acutatum* (A: b1-b3) and *R. stolonifera* (A: c1-c3). Values are the average of 10 replicates consisting of three apples per replicate ± standard deviation (n = 30). The asterisk (*) indicates values that differ significantly from the control (p<0.05).

4.4.4.2 Grape bioassays

As a result of VOCs, the growth inhibition activity against the fungal spoilage organisms was determined to be effective *in-vitro*. The grape bioassay under a closed airspace was carried out in order to assess the efficacy of the antagonistic effects of VOCs produced by *C. pyralidae* and *P. kluyveri* on table grapes, and to verify the results achieved during the *in-vitro* test (Figure 4.3). Interestingly, a 100% inhibition of *B. cinerea*, *C. acutatum* and *R. stolonifer* growth was observed *in-vivo* (Figure 4.5). Based on these

observations, the biocontrol effect (*in-vivo*) of VOCs from *C. pyralidae* and *P. kluyveri* was confirmed by 100% inhibition or 0% decay on grapes (sealed jar settings). This showed that the VOCs played a vital role in preventing fungal growth. Undoubtedly, the complete inhibition of fungal growth makes *C. pyralidae* and *P. kluyveri* suitable candidates for post-harvest control and confirms the yeast's ability to produce VOCs with antimicrobial properties in sealed/tightly packaged fruit (Huang et al., 2012; Li et al., 2012).

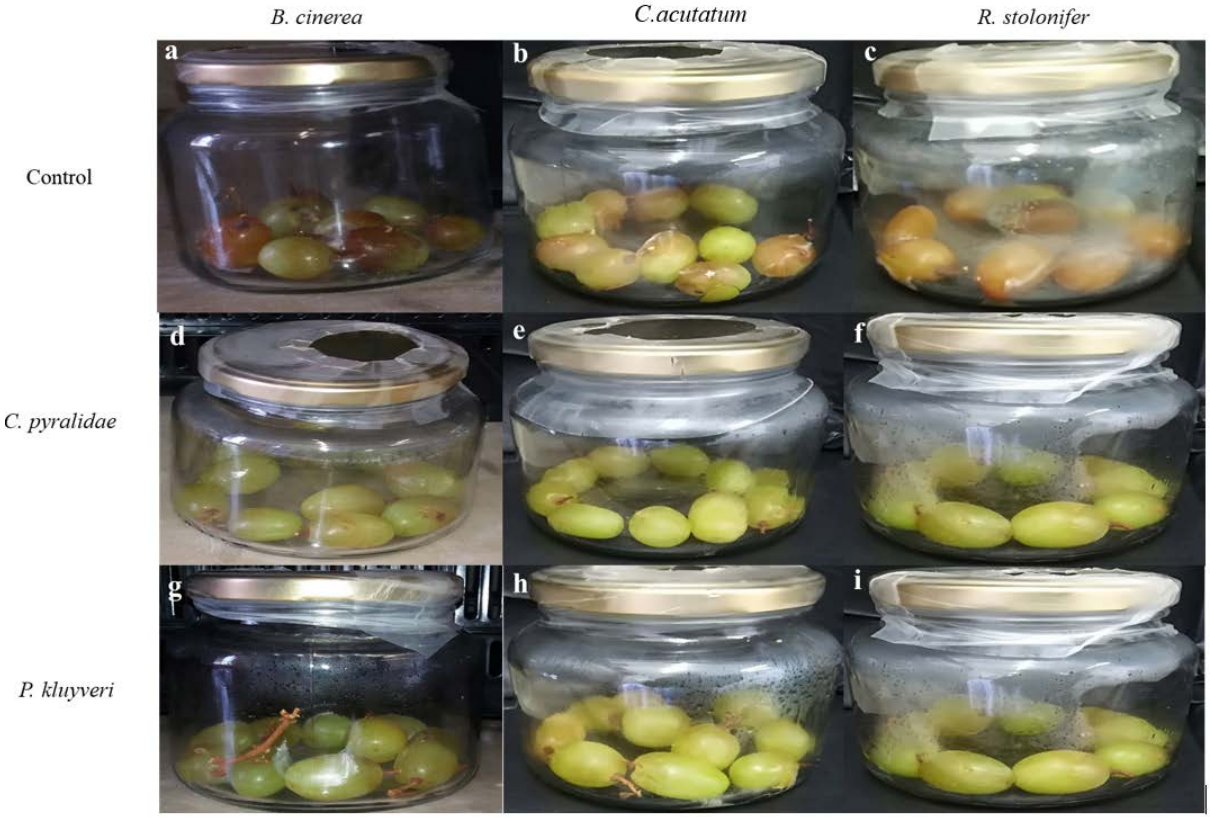


Figure 4-5 The antagonistic effect of volatiles produced by *C. pyralidae* against the growth of *B. cinerea* (d), *C. acutatum* (e) and *R. stolonifer* (f) and, the antagonistic effect of volatiles produced by *P. kluyveri* on *B. cinerea* (g), *C. acutatum* (h) and *R. stolonifera* in sealed jars. Negative controls are shown as *B. cinerea* (a), *C. acutatum* (b) and *R. stolonifer (c)*. Twenty replicates consisting of 10 grapes per replicate were tested (n = 200).

4.4.5 The identification of the VOCs produced by *C. pyralidae* and *P. kluyveri*

Based on our findings, a follow-up experiment was necessary to identify the potential VOCs. As a result, GC-MS analysis identified twenty-five VOCs produced by each yeast. These VOCs were isobutyl acetate, Isobutanol, Ethyl acetate, Isoamyl acetate, Limonene, Isoamyl alcohol, Ethyl caproate, Hexyl acetate, Acetoin, 4-Hexen-1-ol, acetate, Hexanol, Nonanal, Ethyl caprylate, Acetic acid, trans-1-Phenyl-1-butene, Furfuryl acetate, 2-Methyl-3-thiolanone, 4-Methylbenzaldehyde, Isobutyric acid, 3-(Methylthio) propylacetate, Ethyl dec-9-enoate, Phenethyl acetate, 2,5-Dimethylbenzaldehyde and 2-Phenylethanol. Most of the identified compounds were esters, alcohols and fatty acids that are widely used in the food, beverages, pharmaceutical and the cosmetic industries (Cheetham, 1997). In the context of natural biological control, the antagonistic action mechanisms of some yeasts have been linked to the production

of some VOCs in the category of alcohols, organic acids and esters (Grzegorczyk et al., 2017; Hua et al., 2014; Huang et al., 2011a) and some of the identified VOCs are currently used as biocontrol agents in the food and beverage industries. Some of the commonly known growth inhibition compounds are 2-phenylethanol (Druvefors et al., 2005; Fredlund et al., 2004), ethyl acetate (Hua et al., 2014) and acetic acid (Huang et al., 2011a).

Although the antagonistic effect of *C. pyralidae* and *P. kluyveri* is also related to the production of VOCs, it is not yet clear which of the aforementioned compounds, or combinations thereof, may be responsible for the growth inhibition activity. Hence, more research about these compounds still needs to be conducted.

4.5 Summary

The crude samples collected from fermentation broths inoculated with *C. pyralidae* and *P. kluyveri* showed a broader growth inhibition activity against beverage spoilage organisms *D. bruxellensis, D. anomala* and *Z. bailii*. Additionally, the cell suspensions of *C. pyralidae* and *P. kluyveri* showed growth inhibition properties against fungal growth (*B. cinerea, C. acutatum and R. stolonifera*) through a VOCs mechanism. The study also showed that cost-effective renewable bioresources such as GPE can be used as effective nutritional sources for the growth of biocontrol yeasts and production of biopreservation compounds. This study was the first to report on a broader growth inhibition activity of *C. pyralidae* and *P. kluyveri* therefore extending the interest on their extracellular metabolites and cell suspensions as potential alternative to synthetic chemicals as preservatives in beverages and as biological control agents on fruits post-harvest. Furthermore, a cost effective medium used as both yeasts growth and fermentation medium drew significant attention, therefore giving rise to exploring grape pomace extracts as fermentation medium for the production of potential biopreservation compounds, by studying the kinetics and process optimization which is presented in the next chapter.

CHAPTER 5

GRAPE POMACE EXTRACTS AS FERMENTATION MEDIUM FOR THE PRODUCTION OF POTENTIAL BIOPRESERVATION COMPOUNDS

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CHAPTER 5

GRAPE POMACE EXTRACTS AS FERMENTATION MEDIUM FOR THE PRODUCTION OF POTENTIAL BIOPRESERVATION COMPOUNDS

5.1 Introduction

The loss of fruit and beverages due to microbial spoilage impacts negatively on the economy of the producing countries (Salman, 2005; Parveen et al., 2016). In developing countries, such losses are severe due to poor conservation and transportation facilities available to producers (Abdullah et al., 2016). Some of the common microorganisms that are associated with fruit and beverage spoilage are *Botrytis*, *Colletotricum*, *Rhizopus*, *Dekkera*, *Zygosaccharomyces*, *Pichia* and *Hanseniaspora* species (Du Toit & Pretorius, 2000; Comitini, Di Pietro, et al., 2004; Sáez et al., 2010). The current methods for the preservation of beverages and fruits are mainly based on synthetic chemicals. However, due to the serious health concerns associated with the use of synthetic chemicals (Droby, 2005), there is an urgent need for their replacement with less harmful preservatives, which are a healthier alternative for humans and cost effective. Recently, yeasts were identified as potential producers of biopreservation compounds and potential biocontrol agents against several spoilage organisms (Comitini, Di Pietro, et al., 2004; Comitini, De, et al., 2004; Mehlomakulu et al., 2014; Parveen et al., 2016; Grzegorczyk et al., 2017). These investigations lacked an industrial engineering approach for sustainable production of these biopreservation compounds, and used expensive refined media that is not cost-effective (Ngongang et al., 2017). However, yeasts also have the ability to produce useful metabolites while growing in inexpensive media (Chanchaichaovivat et al., 2007; Liu et al., 2013; Muccilli & Restuccia, 2015).

Grape pomace extract (GPE) is an inexpensive potential raw material that could be used for the production of value-added products like biopreservation compounds. South Africa has a thriving wine industry (Lategan et al., 2017) and large quantities of grape pomace are generated, essentially as a waste product. The chemical composition of GPE could make a suitable alternative medium for the growth of various microorganisms, since it contains fermentable reducible sugars such as glucose, fructose and yeast-assimilable nitrogen (YAN) that can fulfil the nutritional requirements for microbial growth of producers of biopreservatives.

During microbial growth, the relationship between growth rates and the production kinetics of extracellular metabolites, is key in assessing the general physiological requirements of yeasts, including substrate utilization and extracellular metabolites formation rates. The process for the production of biopreservation compounds needs to be optimized for higher product yield and minimal cost of production. Optimization

approaches such as response surface methodology (RSM) and Box-Behnken have been used previously (He et al., 2012; Galonde et al., 2013; Uzoh et al., 2014). However, based on the number of experimental runs required when developing suitable response surface models, the central composite design is the most preferred design with regard to response surface methodology (Nwabueze, 2010; Demirel & Kayan, 2012; Uzoh et al., 2014).

As supported by the literature, the nature of the growth inhibition by yeasts have been attributed to its higher growth rate, which makes them competitive for nutrient and space (Liu et al., 2013). Another attribute is the production of extracellular compounds such as killer toxins and volatile organic compounds (VOCs) (Comitini, De, et al., 2004; Liu et al., 2013; Mehlomakulu et al., 2014). Since the current literature has demonstrated the potential of yeasts as producers of VOCs with growth inhibition properties mostly at the screening level without much production using renewable bioresources, this part of the research investigated the use of wild yeasts as producers of biopreservation compounds in GPE medium.

5.2 Objectives

- To study the fermentation process through the estimation of kinetic parameters during crude biopreservation compounds production by *C. pyralidae* and *P. kluyveri* using grape pomace extracts broth (GPEB), a cheap, readily available raw material as fermentation medium.
- To optimize the crude biopreservation compound production process in GPEB.

5.3 Materials and methods

Growth inhibition properties of *C. pyralidae* Y1117, *P. kluyveri* Y1125 and *P. kluyveri* Y1164 was studied against spoilage yeasts *D. bruxellensis*, *D. anomala* and *Z. baillii* using place assay as described in chapter 3. Prior to kinetic studies of crude biopreservation compound production, preliminary production process was carried out whereby, a concentration of 10^6 cells mL^{-1} of *C. pyralidae* Y1117, *P. kluyveri* Y1125 and *P. kluyveri* Y1164 was inoculated separately (in triplicate) in 250 mL Erlenmeyer flasks, containing 150 mL of the autoclaved GPEB at a total sugar concentration of 100, 150 and 200 g L^{-1}. The flasks were incubated for 32 h at 25 °C in a shaking incubator (LM-53OR, RKC® Instrument INC, Ohta-ku Tokyo, Japan) set at 150 rpm. For the purpose of kinetics study, modified existing models were used (see table 3.2). Samples were withdrawn every 4 h, centrifuged at 5000 rpm for 5 min and the supernatants were filtered using 0.22 µm sterile nylon membrane filters. Filtered samples (1 mL) were analyzed for total residual sugar and YAN while the remaining aliquots were used for the growth inhibition assay as describe in chapter 3.

Subsequent to biopreservation compound production process optimisation using RSM-CCD, a kinetic study was conducted by fitting the experimental data in modified existing models (Table 3.2). The RSM-CCD approach was used. The ranges of the independent and the dependent variables used are listed in Table 3.3. Furthermore, the criteria used for the selection of optimum conditions are highlighted in Table 3.4.

5.4 Results and discussion

5.4.1 Growth Inhibition Assay on Beverage Spoilage Yeasts

Candida pyralidae Y1117 showed growth inhibition activity against all three beverage spoilage organisms (*D. bruxellensis*, *D. anomala* and *Z. baillii*), while the two *P. kluyveri* strains only showed inhibition activity against *D. bruxellensis* and *D. anomala* (Figure 5.1). Mehlomakulu et al. (2014) reported growth inhibition activity of *C. pyralidae* against *D. bruxellensis*, while this study showed the inhibition activity of *C. pyralidae* against *Z. baillii* and *D. anomala* as well (Figure 5.1). According to the literature reviewed some growth inhibition activities are associated with killer toxins produced by yeasts, including *Candida pyralidae* (Mehlomakulu et al., 2014). However, this study paid a special attention to the VOCs as well as the quantification aspects of the overall growth inhibition activity obtained using cheaply available raw material as fermentation medium.

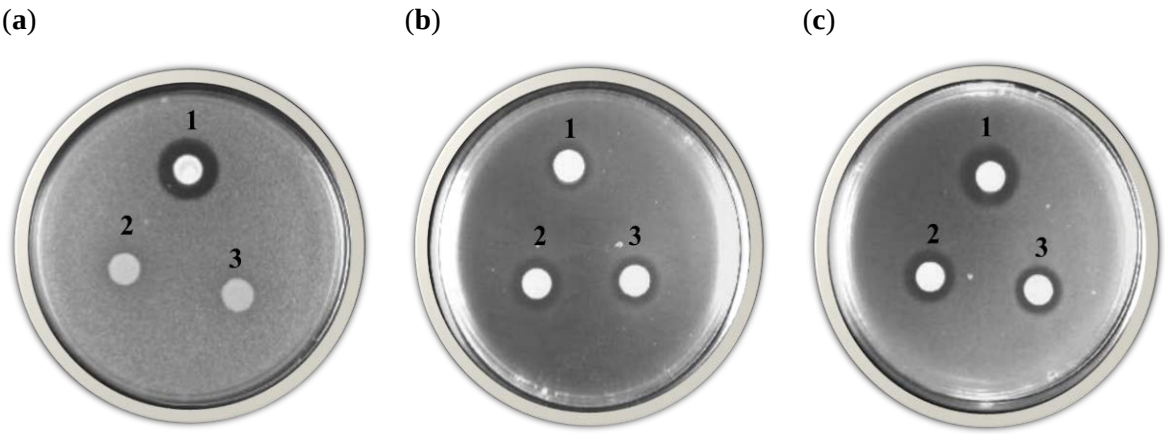

Figure 5-1 Growth inhibition activity of *Candida pyralidae* Y1117 (1), *Pichia kluyveri* Y1125 (2) and *P. kluyveri* Y1164 (3) against *Zygosaccharomyces baillii* (a), *Dekkera bruxellensis* (b) and *Dekkera anomala* (c) on grape pomace extracts agar medium.

5.4.2 Fermentation Kinetics of Potential Biopreservation Compounds Produced in GPE Broth

The highest growth inhibition activity for *C. pyralidae* (0.797 L CSM mL^{-1} BCU) was obtained at 150 g L^{-1} after 24 h of fermentation, 20 to 28 h for *P. kluyveri* Y1164 (0.412 L CSM mL^{-1} BCU) and 28 h for *P. kluyveri* Y1125 (0.373 L CSM mL^{-1} BCU) (Figure 5.2). Based on the current observations, the potential of diluted residual GPE as a cheap raw material for the production of possible biopreservation compounds from yeasts was tentatively shown.

C. pyralidae Y1117 had the lowest substrate utilization rate (0.333 g L^{-1} h^{-1}) compared to *P. kluyveri* Y1125 and *P. kluyveri* Y1164 (Table 5.1). In general, the substrate utilization rate and biomass yield were inversely proportional to the rate of biopreservation compounds formation. In addition, minor differences in specific growth rate for *C. pyralidae* Y1117 (0.196 h^{-1}), *P. kluyveri* Y1125 (0.202 h^{-1}) and *P. kluyveri* Y1164 (0.190 h^{-1}) were observed. The findings in Table 5.1 are also indicative of a direct relationship between the formation rate of biopreservation compounds and biomass yield. Overall, the substrate utilization model showed that cellular growth and production of biopreservation compounds were directly linked to sugar utilization rates for all the yeasts. Similarly, Mewa-Ngongang et al. (2017) previously established a direct relationship between biopreservation compounds formation and substrate utilization rate. However, in this study, *C. pyralidae* yielded more biopreservation compounds, while utilizing less substrate, a trait that can be exploited at industrial scale.

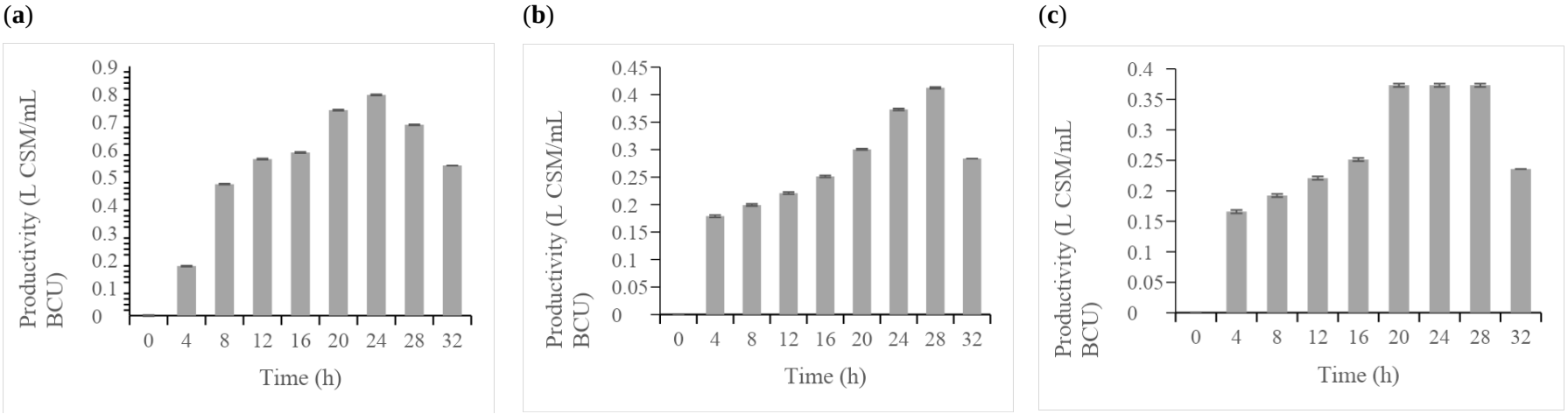

Figure 5-2 Biopreservation compound production in a grape pomace extracts medium by *Candida pyralidae* Y1117 (a**), *Pichia kluyveri* Y1125 (**b**) and *P. kluyveri* Y1164 (**c**) in a single stage bioreactor at the total sugar concentration of 150 g L^{-1}.**

Table 5-1 Kinetics of biopreservation compound production by *Candida pyralidae* Y1117, *Pichia kluyveri* Y1125 and *P. kluyveri* Y1164 using grape pomace extracts broth (total sugar concentration of 150 g L^{-1}) as fermentation medium.

Fermentation Parameters	Model	Antimicrobial Compound Producing Yeasts		
		C. pyralidae Y1117	*P. kluyveri* Y1125	*P. kluyveri* Y1164
Substrate (total sugar) utilization rate (g L^{-1} h^{-1})	$r_s = \frac{dS}{dt}$	0.333	1.912	1.947
Biomass formation rate (× 10^7 cells mL^{-1} h^{-1})	$r_x = \frac{dX}{dt}$	4.542	5.208	3.917
Biomass yield (× 10^8 cells g^{-1})	$Y_{X/S} = \frac{dX}{dS}$	1.365	0.272	0.201
Specific growth rate (h^{-1})	$\mu = \frac{ln(X_f/X_0)}{t}$	0.196	0.202	0.190
Biopreservation compound formation rate (× 10^3 L VZI mL^{-1} BCU h^{-1})	$r_p = \frac{dP}{dt}$	33.209	15.547	15.547
Biopreservation compound formation based on cell concentration (× 10^{-12} L VZI $cells^{-1}$)	$Y_{P/X} = \frac{dP}{dX}$	73.121	29.850	39.694
Biopreservation compound formation based on substrate (total sugar) utilization (× 10^{-3} L VZI g^{-1})	$Y_{P/S} = \frac{dP}{dS}$	99.840	8.130	7.985
Total sugar utilization rate (g L^{-1} h^{-1}) proportional to cellular growth and formation of biopreservation compounds	$\frac{dS}{dt} = \frac{1}{2}\left(\frac{dX}{Y_{\frac{X}{S}}dt} + \frac{dP}{Y_{\frac{P}{S}}dt}\right)$	0.333	1.912	1.947

5.4.3 Response Surface, Model Validation and Optimum Conditions for the Production of Biopreservation Compounds

The interactive effect of the four independent parameters (fermentation time, pH, temperature and total sugar concentration) on production of biopreservation compounds is represented in a 3D plot (Figure 5.4). An interdependence of fermentation time, temperature and sugar concentration on the production of biopreservation compounds was observed. It was noted that the optimal conditions for production of biopreservation compounds were both pH and temperature dependent. Therefore, optimal production was only obtained at pH lower than 5 and temperature between 15 and 25 °C for all three yeasts strains studied (Figure 5.4a-1,b-1,c-1). These conditions typify mild and low cost conditions, as these bioreactor conditions would be easily maintained.

Previously, Mewa-Ngongang et al., (2017) showed that temperature and pH had a significant effect on cellular growth subsequent to the production of biopreservation compounds. The optimal production time for the biopreservation compounds ranged between 16 and 24 h for *C. pyralidae* Y1117, and between 16 to 28 h for *P. kluyveri* Y1125 and Y1164 (Figure 5.4a-2, b-2, c-2). Variations in sugar concentration of the production medium had an insignificant effect on biopreservation compound production. However, a combinatorial effect of fermentation period and sugar concentrations of the GPE broth on production of biopreservation compounds was noted when both conditions (viz. 'prolonged fermentations in higher sugar must' and 'shorter fermentations in lower sugar must') were responsible for higher growth inhibition activity (Figure 5.4a-3, b-3, c-3). It was then economically realistic to consider the use of lower sugar containing broth (diluted GPE broth to 150 g L^{-1}), which still showed the highest growth inhibition activity rapidly (±24 h) for *C. pyralidae* Y1117 (Figure 5.4a-3). Contrarily, *P. kluyveri* Y1125 (Figure 5.4b-3) and Y1164 (Figure 5.4c-3) seemed to have higher physiological requirements that were characterized by longer fermentation periods and higher sugar requirements. The significance of fermentation parameters such as pH, temperature and carbon source for production of biopreservation compounds was also highlighted for *Lactobacillus* and *Saccharomyces* species (Cheigh et al., 2002; Messens & De Vuyst, 2002; Narendranath & Power, 2005). In this study, the determination of the optimum conditions for production of biopreservation compounds in a GPE medium was done based on the desirability function. Insignificant variations in biopreservation compound production were noted when both 'low sugar concentration-short fermentation time' and 'high sugar concentration-prolonged fermentation time' settings were compared for all strains.

Table 5-2 ANOVA (analysis of variance) for the response surface quadratic model, with A, B, C and D coded for Time (h), pH, Temperature (°C) and Total sugar (g L^{-1}), respectively

Candida pyralidae KU736785

Source	Sum of square	df	Mean square	F Value	Prob > F	
Model	2.45	14	0.18	200.56	<0.0001	significant
A-Time	0.049	1	0.049	56.17	<0.0001	
B-pH	0.12	1	0.12	138.39	<0.0001	
C-Temp	2.128E-003	1	2.128E-003	2.44	0.1393	
D-Sugar	0.050	1	0.050	57.12	<0.0001	
AB	4.096E-003	1	4.096E-003	4.69	0.0468	
AC	6.404E-003	1	6.404E-003	7.33	0.0162	
AD	1.894E-004	1	1.894E-004	0.22	0.6481	
BC	0.050	1	0.050	56.98	<0.0001	
BD	0.039	1	0.039	44.28	<0.0001	
CD	0.021	1	0.021	24.40	0.0002	
A2	0.012	1	0.012	13.89	0.0020	
B2	0.035	1	0.035	39.89	<0.0001	
C2	0.071	1	0.071	81.05	<0.0001	
D2	3.287E-003	1	3.287E-003	3.76	0.0714	
Residual	0.013	15	8.733E-004			
Lack of Fi	0.013	10	1.310E-003			
Pure Erro	0.000	5	0.000			
Cor Total	2.47	29				
Std. Dev.	0.030		R-Squared	0.9947		
Mean	0.80		Adj R-Squared	0.9897		
C.V. %	3.69		Pred R-Squared	0.9721		
PRESS	0.069		Adeq Precision	51.553		

Pichia kluyveri MG212504

Source	Sum of Squares	df	Mean Square	F Value	Prob > F	
Model	0.71	14	0.051	96.05	<0.0001	significant
A-Time	0.015	1	0.015	27.63	<0.0001	
B-pH	0.042	1	0.042	80.03	<0.0001	
C-Temp	5.517E-003	1	5.517E-003	10.39	0.0057	
D-Sugar	0.024	1	0.024	46.02	<0.0001	
AB	9.417E-003	1	9.417E-003	17.73	0.0008	
AC	8.698E-003	1	8.698E-003	16.38	0.0011	
AD	0.014	1	0.014	25.93	0.0001	
BC	0.019	1	0.019	36.36	<0.0001	
BD	8.374E-004	1	8.374E-004	1.58	0.2284	
CD	0.011	1	0.011	21.50	0.0003	
A2	9.211E-003	1	9.211E-003	17.35	0.0008	
B2	1.279E-003	1	1.279E-003	2.41	0.1416	
C2	6.142E-003	1	6.142E-003	11.57	0.0040	
D2	0.042	1	0.042	78.58	<0.0001	
Residual	7.966E-003	15	5.311E-004			
Lack of Fit	7.966E-003	11	7.242E-004			
Pure Error	0.000	4	0.000			
Cor Total	0.72	29				
Std. Dev.	0.023		R-Squared	0.9890		
Mean	0.36		Adj R-Squared	0.9787		
C.V. %	6.32		Pred R-Squared	0.8385		
PRESS	0.12		Adeq Precision	37.755		

Pichia kluyveri MG600139

Source	Sum of Squares	df	Mean Square	F Value	Prob > F	
Model	0.99	14	0.071	736.89	<0.0001	significant
A-Time	0.027	1	0.027	279.68	<0.0001	
B-pH	0.22	1	0.22	2255.21	<0.0001	
C-Temp	0.013	1	0.013	134.91	<0.0001	
D-Sugar	0.021	1	0.021	214.31	<0.0001	
AB	0.019	1	0.019	203.25	<0.0001	
AC	5.156E-003	1	5.156E-003	53.88	<0.0001	
AD	1.668E-003	1	1.668E-003	17.43	0.0008	
BC	5.314E-003	1	5.314E-003	55.52	<0.0001	
BD	0.012	1	0.012	121.08	<0.0001	
CD	1.038E-003	1	1.038E-003	10.85	0.0049	
A2	0.052	1	0.052	539.54	<0.0001	
B2	0.027	1	0.027	279.59	<0.0001	
C2	1.315E-003	1	1.315E-003	13.74	0.0021	
D2	1.642E-005	1	1.642E-005	0.17	0.6845	
Residual	1.436E-003	15	9.570E-005			
Lack of Fit	1.436E-003	12	1.196E-004			
Pure Error	0.000	3	0.000			
Cor Total	0.99	29				
Std. Dev.	9.783E-003		R-Squared	0.9985		
Mean	0.32		Adj R-Squared	0.9972		
C.V. %	3.05		Pred R-Squared	0.9927		
PRESS	7.232E-003		Adeq Precision	95.431		

Three different quadratic models (Y_1, Y_2 and Y_3) were generated to explain the production of biopreservation compounds in GPE medium. Based on the analysis of variance for each model, it was found that all models were significant with a probability value of <0.05. The lack of fit value 0.013, 0.080 and 0.014 corresponding to *C. pyralidae* Y1117, *P. kluyveri* Y1125 and *P. kluyveri* Y1164, respectively, implied that the models developed were significant (Table 5.2). From the ANOVA analyses, the significance of the models was observed by the values of the lack of fit obtained, which confirmed a good predictability of the models. It was observed that the predicted regression coefficient of 0.9721, 0.8385 and 0.9927 was in reasonable agreement with the adjusted regression coefficient of 0.9897, 0.9787 and 0.9972 for *C. pyralidae* Y1117, *P. kluyveri* Y1125 and *P. kluyveri* Y1164, respectively, which further confirmed a good predictability of the models to observed experimental data.

Table 5.3 clearly illustrates the comparison of the experimental and the predicted response values of each model developed for the production of potential biopreservation compounds by each yeast. In addition, studentized and normal percentage probability data were also generated to confirm any response transformation. Overall, normality challenges were not encountered. The predicted response for production of biopreservation compounds was carried out using numerous models (Equations 10, 11, and 12). It is clearly visible from the studentized residuals and actual versus predicted plots that the predicted and the measured (actual) VZI values were comparable. This can further be explained by the alignments of the points close to the slope of the graphs corresponding to each yeast (Figure 5.3).

$$Y_1 = 0.69 + 0.17A - 0.18B + 0.018C + 0.11D - 0.064AB + 0.12AC - 0.014AD - 0.32BC + 0.48BD + 0.21CD - 0.26A^2 + 0.15B^2 + 0.15C^2 - 0.11D^2$$

Equation 10

$$Y_2 = 0.43 + 0.083A - 0.15B + 0.018C + 0.065D - 0.13AB - 0.11AC + 0.081AD - 0.076BC - 0.044BD + 0.073CD - 0.11A^2 + 0.039B^2 + 0.0385C^2 - 0.16D^2$$

Equation 11

$$Y_3 = 0.34 + 0.10A - 0.25B - 0.031C + 0.062D - 0.10AB - 0.047AC + 0.041AD + 0.037BC - 0.092BD - 0.015CD - 0.21A^2 + 0.20B^2 + 0.020C^2 - 0.00495D^2 \quad (6)$$

Equation 12

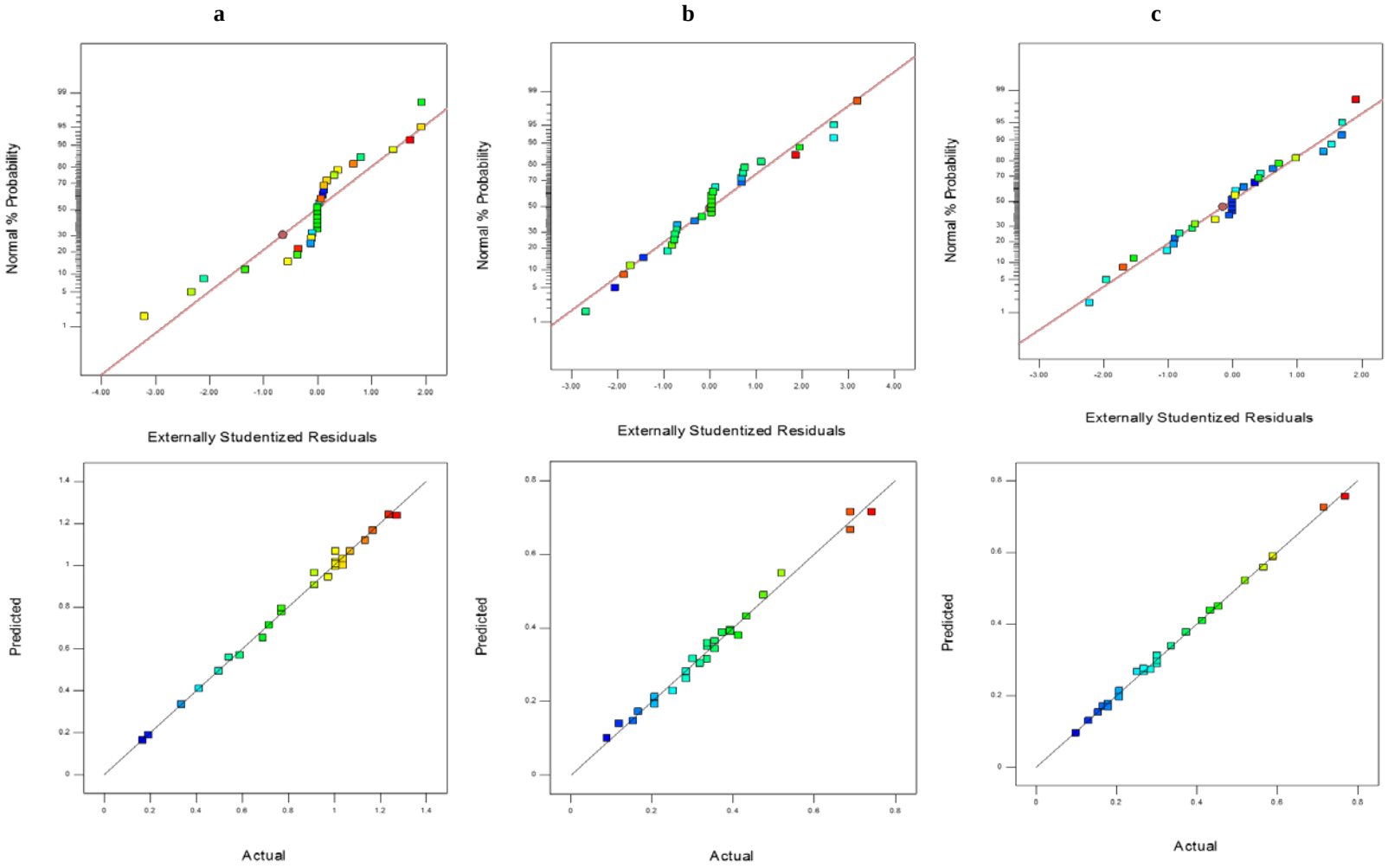

Figure 5-3 The external studentized residuals versus the normal percentage probability and the actual versus the predicted response plots for production of biopreservation compounds by *Candida pyralidae* Y1117 (a**), *Pichia kluyveri* Y1125 (**b**) *and P. kluyveri* Y1164 (**c**) in a grape pomace extracts medium.**

Table 5.3: Predicted and observed experimental productivity (L VZI mL^{-1} BCU) runs generated for *Candida pyralidae* Y1117 (a), *Pichia kluyveri* Y1125 (b) and *P. kluyveri* Y1164 (c) by the central composite design (CCD).

***Candida pyralidae* Y1117**								***Pichia kluyveri* Y1125**								***Pichia kluyveri* Y1164**							
Run	**Factors**				**(L VZI/mL BCU)**			**Run**	**Factors**				**(L VZI/mL BCU)**			**Run**	**Factors**				**(L VZI/mL BCU)**	
	A (h)	B	C (oC)	D g/L	Predicted	Actual			A (h)	B	C (oC)	D g/L	Predicted	Actual			A (h)	B	C (oC)	D g/L	Predicted	Actual
1	8	4,5	20	5	0,165	0,166		1	8	6	15	15	0,147	0,153		1	8	4.5	20	11.25	0,130	0.12977
2	12	7	20	11,25	0,496	0,497		2	8	4,5	20	5	0,140	0,119		2	8	4.5	20	11.25	0,130	0.12977
3	28	4,5	20	11,25	0,715	0,715		3	12	3	15	7,5	0,262	0,284		3	8	4.5	20	11.25	0,130	0.12977
4	28	4,5	20	11,25	0,715	0,715		4	12	4,5	20	18	0,213	0,206		4	8	4.5	20	11.25	0,130	0.12977
5	28	3	15	7,5	0,995	1,005		5	12	4,5	20	5	0,172	0,166		5	8	4.5	20	5	0,096	0.098125
6	16	3	25	7,5	1,017	1,005		6	16	4,5	20	5	0,194	0,206		6	8	6	25	7.5	0,175	0.178833
7	36	3	15	7,5	0,907	0,913		7	20	6	15	15	0,315	0,336		7	8	7	20	11.25	0,171	0.165831
8	28	4,5	20	11,25	0,715	0,715		8	20	7	20	11,25	0,316	0,301		8	12	3	25	15	0,438	0.432731
9	28	3	25	7,5	1,242	1,237		9	24	4,5	20	11,25	0,432	0,433		9	12	6	15	7.5	0,178	0.178833
10	36	6	15	7,5	0,655	0,689		10	24	4,5	20	11,25	0,432	0,433		10	12	6	25	15	0,196	0.206308
11	24	3	25	15	1,238	1,272		11	24	4,5	20	11,25	0,432	0,433		11	16	6	25	7.5	0,267	0.2512
12	32	3	15	7,5	0,964	0,913		12	24	4,5	20	11,25	0,432	0,433		12	20	6	15	15	0,289	0.300508
13	40	6	25	7,5	0,191	0,192		13	24	6	15	15	0,350	0,336		13	20	6	25	15	0,267	0.267145
14	28	6	15	7,5	0,778	0,769		14	24	4,5	20	11,25	0,432	0,433		14	20	3	25	7.5	0,450	0.453583
15	24	6	15	15	1,033	1,036		15	24	6	15	7,5	0,365	0,354		15	20	7	20	11.25	0,297	0.300508
16	28	4,5	20	11,25	0,715	0,715		16	24	6	25	7,5	0,229	0,251		16	24	6	15	15	0,313	0.300508
17	20	3	25	7,5	1,118	1,134		17	24	7	20	11,25	0,304	0,318		17	24	6	25	7.5	0,274	0.283581
18	8	4,5	20	18	0,413	0,412		18	24	3	15	15	0,489	0,475		18	24	3	15	15	0,726	0.715331
19	28	6	15	15	1,007	1,005		19	28	4,5	20	18	0,380	0,412		19	28	3	15	7.5	0,589	0.588995
20	20	6	15	7,5	0,795	0,769		20	28	3	25	15	0,715	0,742		20	28	7	20	11.25	0,275	0.267145
21	20	3	25	15	1,166	1,168		21	28	6	25	15	0,358	0,336		21	28	3	15	15	0,756	0.7693

22	24	2	20	11,25	1,067	1,069		22	28	7	20	11,25	0,281	0,284		22	32	4.5	20	18	0,409	0.41237
23	36	7	20	11,25	0,561	0,542		23	28	3	15	15	0,549	0,519		23	32	3	25	15	0,591	0.588995
24	28	4,5	20	11,25	0,715	0,715		24	32	4,5	20	18	0,395	0,393		24	32	6	25	15	0,214	0.206308
25	12	3	25	15	0,945	0,974		25	32	6	15	15	0,388	0,373		25	36	4.5	20	18	0,378	0.37312
26	28	4,5	20	11,25	0,715	0,715		26	32	3	25	15	0,689	0,689		26	36	3	25	15	0,558	0.5652
27	24	3	15	7,5	1,000	1,036		27	36	6	15	15	0,391	0,393		27	36	6	25	15	0,154	0.15332
28	16	3	25	15	1,069	1,005		28	36	6	15	7,5	0,344	0,354		28	36	7	20	11.25	0,169	0.178833
29	16	7	20	11,25	0,572	0,589		29	40	4,5	20	5	0,100	0,089		29	40	3	15	7.5	0,522	0.519081
30	8	3	15	15	0,337	0,336		30	40	3	15	15	0,667	0,689		30	40	3	25	7.5	0,339	0.335833

In the current study, the majority of independent variables were significant terms in the models. The *C. pyralidae* model showed that temperature has less of an effect on biopreservation compound production. The linear effect of time and sugar concentration (including the quadratic effect of sugar) was also insignificant. These observations showed the ability of *C. pyralidae* to produce biopreservation compounds regardless of changes in temperature and sugar concentration. For *P. kluyveri* Y1125, most model terms were significant, except for the linear effect of pH and sugar concentration. However, only the quadratic effect of sugar was insignificant for *P. kluyveri* Y1164 (Table 5.2).

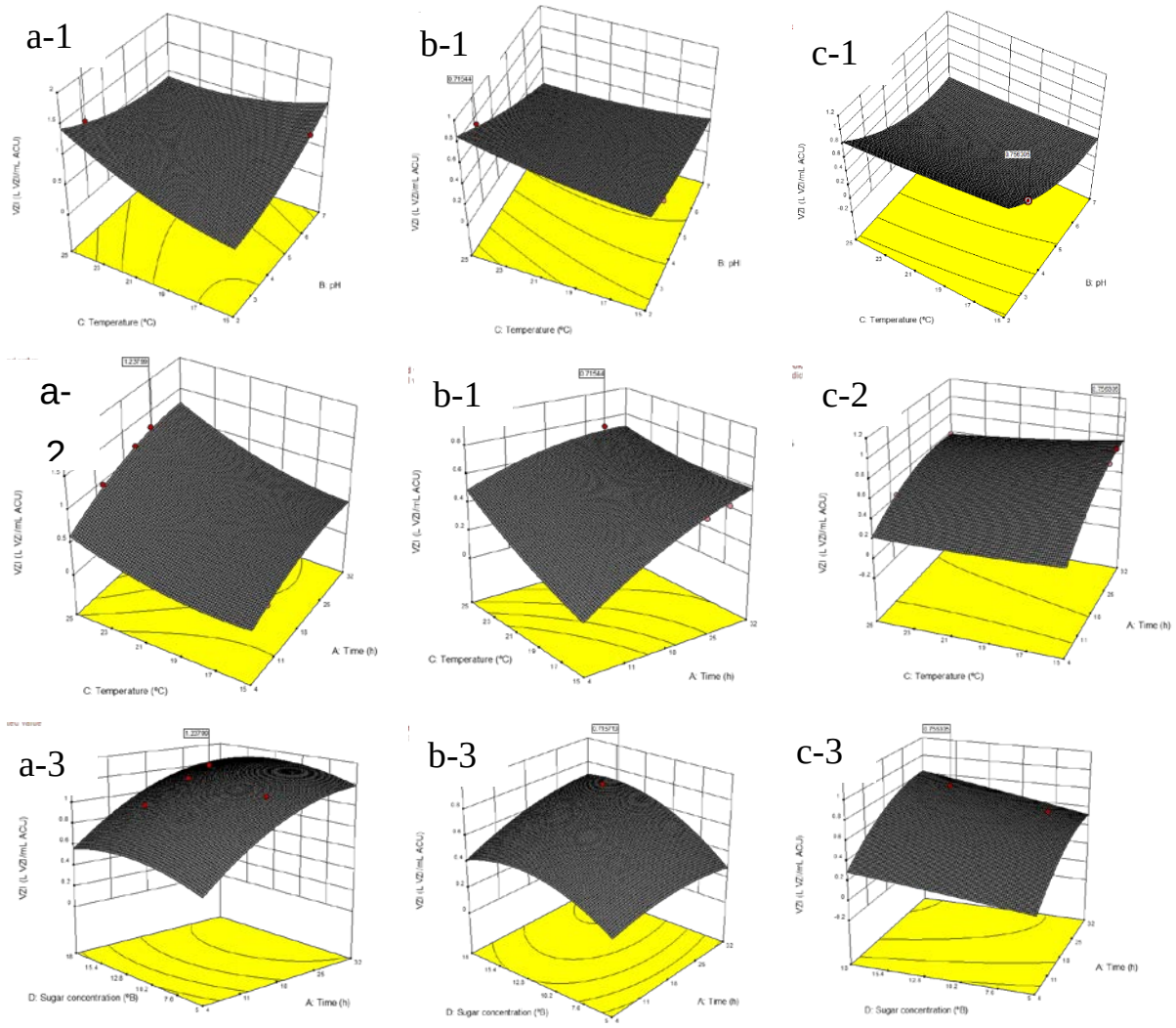

Figure 5-4 The combined effect of fermentation time and pH (1), time and incubation temperature (2), time and sugar concentration (3) on production of potential biopreservation compounds by *Candida pyralidae* Y1117 (a**), *Pichia kluyveri* Y1125 (**b**) *and P. kluyveri* Y1164 (**c**). An example: the description, (a-1), represents a = *C. pyralidae* Y1117 and (1) = fermentation time and pH effects.**

Based on the criteria and the boundaries selected for achieving maximum production of biopreservation compounds, a 24 h fermentation period, a temperature of 25 °C, a total sugar concentration of 150 g L^{-1} and a pH of 3 were identified as the optimum conditions for production of potential biopreservation compounds for *C. pyralidae* Y1117 (Figure 5.5a). The optimum production conditions for *P. kluyveri*

strains were 28 h of fermentation, pH 3, temperature of 25 °C and sugar concentration of 150 g L^{-1} (Figure 5.5b, c). These optimization findings were confirmed by the results of the growth inhibition assay (Figure 5.6). Growth inhibition activity against *D. bruxellensis* before (Figure 5.6a) and after (Figure 5.6b) the optimization study was compared and larger inhibition zones were observed after optimization.

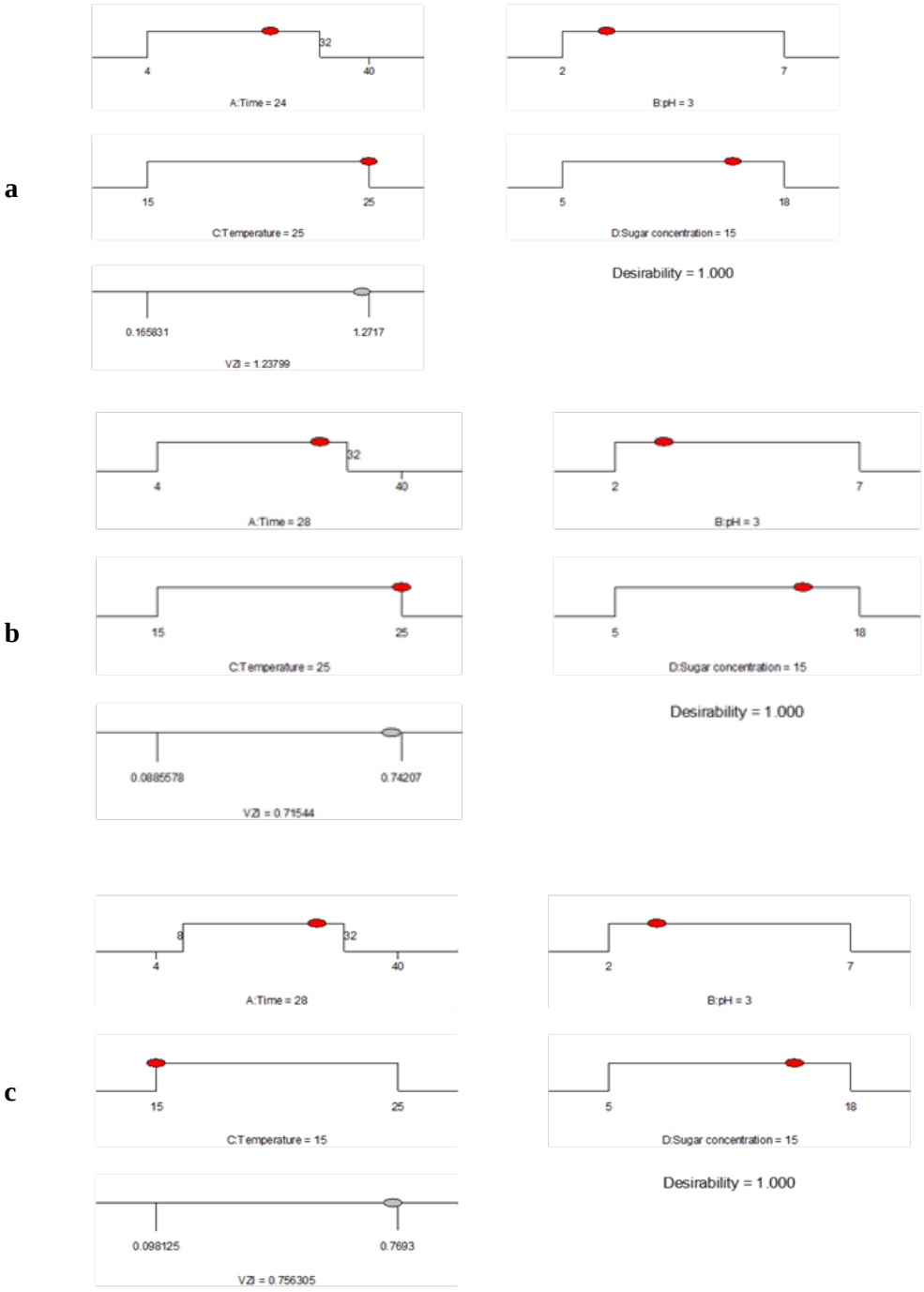

Figure 5-5 Ramp diagram and desirability values for optimal conditions for biopreservation compounds production under the conditions studied (time, pH, temperature and sugar concentration). a, b, c = optimal production conditions for *Candida pyralidae* Y1117, *Pichia kluyveri* Y1125 and *P. kluyveri* Y1164, respectively

(**a**)

1. *C. pyralidae* Y1117 = 0.797 L VZI.mL^{-1} BCU h^{-1}

2. *P. kluyveri* Y1125 = 0.412 L VZI.mL^{-1} BCU h^{-1}

3. *P. kluyveri* Y1164 = 0.373 L VZI.mL^{-1} BCU h^{-1}

(**b**)

1. *C. pyralidae* Y1117 = 1.238 L VZI mL^{-1} BCU h^{-1}

2. *P. kluyveri* Y1125 = 0.715 L VZI mL^{-1} BCU h^{-1}

3. *P. kluyveri* Y1164 = 0.756 L VZI mL^{-1} BCUh^{-1}

Figure 5-6 Growth inhibition assay plates showing the inhibition activity against *D. bruxellensis* before (a) and after (b) optimization of biopreservation compounds production by *Candida pyralidae* Y1117 (1), *Pichia kluyveri* Y1125 (2) and *P. kluyveri* Y1164 (3).

5.4.4 Identification and Quantification of VOCs

Many VOCs were detected, but only eight were at a quantification limit. The compounds quantified were isoamyl acetate, butyric acid, isoamyl alcohol, 2-phenyl ethylacetate, hexanoic acid, 2-phenyl ethanol, octanoic acid and decanoic acid. Some of these compounds have been found to have growth inhibition effect on selected fungal pathogens (Masoud et al., 2005; Huang et al., 2011a). VOCs with inhibition activity, isoamyl acetate, 2-phenyl ethylacetate, isoamyl alcohol and 2-phenyl ethanol (Masoud et al., 2005) were considered to be responsible for growth inhibition activity in this study. The yeasts used were able to produce these compounds at different concentrations (Table 5.4). Only *C. pyralidae* produced isoamyl acetate below the detection limit. The findings of the current work were in agreement with the current literature reviewed. The exploration of GPE as fermentation medium for the production of biopreservation compounds showed that this growth medium actually allowed a production of isoamyl acetate, butyric acid, isoamyl alcohol, 2-phenyl ethylacetate, hexanoic acid, 2-phenyl ethanol, octanoic acid and decanoic acid at a higher concentration than that reported by (Masoud et al., 2005) when synthetic refined media was used. “What other suitable, high yield and cost effective substrates can be used for production of biopreservation compounds?” is one of the questions raised in the current literature reviewed that can be answered through the findings of the research reported herein as diluted GPE was found to yield higher quantities of biopreservation compounds. The result obtained in this work further extends the possible use

of wild yeasts as a source of biopreservation compounds while using agro-waste (grape pomace extracts) as fermentation medium.

Table 5-4 List of quantified volatile organic compounds (VOCs) identified to be produced by *Candida pyralidae* Y1117, *Pichia kluyveri* Y1125 and *P. kluyveri* Y1164 when grape pomace extract is used as fermentation medium.

	VOCs and Concentrations (mg/L)		
Compound	***C.pyralidae* Y1117**	***P. kluyveri* Y1125**	***P. kluyveri* Y1164**
Isoamyl acetate	not detected	16.51	17.73
Isoamyl alcohol	1.73	1.74	1.89
Butyric acid	1.24	1.25	1.25
2-Phenyl ethylacetate	1.47	1.97	1.99
Hexanoic acid	0.93	0.93	0.93
2-Phenyl ethanol	1.61	1.66	1.68
Octanoic acid	1.32	1.32	1.32
Decanoic acid	1.44	1.44	1.44

5.5 Summary

The use of cheap, readily available agricultural waste (i.e., grape pomace extracts) for the production of potential biopreservation compounds by yeasts presents a cost-effective and realistic alternative to synthetic chemical preservatives was explored. Yeasts have proven to be a potential source of biopreservation compounds with inhibition properties against various spoilage organisms. *C. pyralidae* Y1117 showed growth inhibition activity against *Z. baillii*, *D. bruxellensis* and *D. anomala*, while the two *P. kluyveri* strains only showed inhibition activity against *D. bruxellensis* and *D. anomala.* The production process was optimized for *C. pyralidae* Y1117, *P. kluyveri* Y1125 and *P. kluyveri* Y1164 and the biopreservation compounds produced identified and quantified. The models developed for the production of biopreservation compounds under the optimum conditions for each yeast were shown to be appropriate and statistically sound to mathematically explain the production process for potential biopreservation compounds, using GPE as fermentation medium. With the evidence of broad antagonistic potential of biopreservation compounds from these yeasts towards many species of spoilage yeasts on one hand, and the fact that microbial spoilage is usually a random phenomenon in another hand, it was imperative in the next phase to study the effect of mixed crude biopreservation compounds against spoilage yeast consortia.

CHAPTER 6

ACTIVITY INTERACTIONS OF CRUDE BIOPRESERVATIVES AGAINST SPOILAGE YEAST CONSORTIA

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CHAPTER 6:

ACTIVITY INTERACTIONS OF CRUDE BIOPRESERVATIVES AGAINST SPOILAGE YEAST CONSORTIA

6.1 Introduction

Protection of food and beverages against microbiological spoilage is essential for maintaining an adequate food supply to growing world populations. Yeast species such as *Dekkera bruxellensis*, *Dekkera anomala*, *Zygosaccharomyces bailii*, *Zygosaccharomyces fermentati*, *Zygosaccharomyces florentinus*, *Zygosaccharomyces microellipsoides*, *Zygosaccharomyces rouxii*, *Hanseniaspora uvarum*, *Candida guilliermondii*, and even *Saccharomyces cerevisiae* have been reported to cause spoilage in alcoholic and non-alcoholic beverages (Comitini, De, et al., 2004; Doyle, 2009; Mehlomakulu et al., 2014). To curb microbial spoilage, synthetic chemicals and/or physical methods can be used (Doyle, 2009; Ruiz-Capillas & Jiménez-Colmenero, 2009; Tiwari et al., 2009; Chemat et al., 2011; Santos et al., 2012). Physical methods include pasteurization, cold processing, filtration, the control of water content, ultrasound processing, and irradiation. However, some of these physical methods can have a negative effect on the quality of food items, such as fruits, vegetables, and beverages (Doyle, 2009; Tiwari et al., 2009; Chemat et al., 2011; Santos et al., 2012). Furthermore, thermophiles, spores, psychrophiles, and xerophiles can sometimes survive these methods (Leistner, 1999; Farkas et al., 2007).

Chemical preservatives such as SO_2, dimethyl dicarbonate, benzoate, benzoic, lactic, sorbic and acetic acid, triazoles, hydroanilide fenhexamid, dicarboximides, and succinate dehydrogenase sorbate are commonly used to extend the shelf life and to improve the safety of food. However, in some cases, more than one chemical preservative is used to prevent spoilage from a consortium of spoilage microorganisms. This can lead to the presence of undesirably high levels of chemical preservatives in food. Increasingly, consumer fears about potential preservative toxicity and antimicrobial-resistant pathogens in food have led to strict regulations for the use of preservatives on fruit and in fruit-derived beverages. Some regulations also advocate the use of safer alternative preservatives (Ross et al., 2002; Droby, 2005; Carocho et al., 2015).

The desire for a safe alternative to synthetic chemicals has led to the investigation of benign microorganisms, specifically yeasts and bacteria, with growth inhibition properties against spoilage microorganisms (Comitini, De, et al., 2004; De Ingeniis et al., 2009; Mehlomakulu et al., 2014; Abbey et al., 2019) From bioprospecting studies, some microorganism-derived biocontrol agents, such as killer toxins, have been identified (Ciani & Fatichenti, 2001; Comitini, De, et al., 2004; Mehlomakulu et al., 2014). Volatile organic compounds (VOCs) such as ethyl acetate, phenylethyl alcohol, 1,3,5,7-cyclooctatetraene, isobutyl acetate, 2-phenyl ethyl acetate, ethyl propionate, isoamyl acetate, and isoamyl

alcohol have also been linked to growth inhibition properties (Ciani & Fatichenti, 2001; Huang et al., 2011b; Mehlomakulu et al., 2014; Qin et al., 2017; Mewa-Ngongang et al., 2019; Contarino et al., 2019).

In previous studies (Mewa-Ngongang et al., 2017; Mewa-Ngongang et al., 2018.; Mewa-Ngongang et al., 2019), yeast-derived crude biopreservatives from *C. pyralidae* Y1117, *P. kluyveri* Y1125, and *P. kluyveri* Y1164 were individually tested against selected spoilage fungi and yeasts. However, the growth inhibition efficiency of combining the different crude biopreservatives has not been investigated.

6.2 Objectives

The objectives of this study were:

- To study the effect of pH, temperature and proteolytic enzymes on the denaturation of crude biopreservation compounds from *C. pyralidae* Y1117, *P. kluyveri* Y1125 and *P. kluyveri* Y1164
- To test the growth inhibition activity of crude biopreservation compounds from *C. pyralidae* Y1117, *P. kluyveri* Y1125 and *P. kluyveri* Y1164 against consortia of spoilage organisms *D. anomala, D. bruxellensis* and *Z. bailii*.
- To test the growth inhibition activity of mix crude biopreservation compounds against single and consortia of spoilage organisms.

6.3 Materials and methods

As detailed in chapter 3, the crude biopreservation compounds were produced separately from *C. pyralidae* Y1117, *P. kluyveri* Y1125 and *P. kluyveri* Y1164. To evaluate the possible nature of the biopreservation compounds present in the crudes, the protein denaturation approach was used where the crude biopreservatives samples were subjected to protease enzymes i.e. Proteinase K, pepsin and proteases from *Aspergillus saitoi* and *Rhizopus* spp. (Sigma-Aldrich, Darmstadt, Germany). The effect of pH and temperature on the denaturation and stability of the crude biopreservatives was also studied.

A cross-screening procedure was performed whereby the selected growth inhibiting yeasts were also screened against each other. The grape pomace extract was also tested for growth inhibition activity against the selected spoilage yeasts (*Dekkera anomala*, *Dekkera bruxellensis*, and *Zygosaccharomyces bailii*. Growth inhibition activity and quantification was carried out as highlighted in chapter three, described by Mewa-Ngongang et al. (2017); Mewa-Ngongang et al. (2019).

For the activity interaction of crude biopreservation compounds against beverage spoilage consortia, different growth inhibition combinations (GICs) and spoilage organism consortia combinations were prepared. The growth inhibition combinations that resulted from the three yeasts were: GIC 1, composed of *Candida pyralidae* Y1117 and *Pichia kluyveri* Y1125; GIC 2, constituted by *C. pyralidae* Y1117 and *P. kluyveri* Y1164, GIC 3, by *P. kluyveri* Y1125 and *Pichia kluyveri* Y1164; GIC 4, by *C. pyralidae, P.*

kluyveri Y1125 and *P. kluyveri* Y1164. The spoilage organism consortia combinations were: SC1, *D. anomala* and *D. bruxellensis*; SC2, *D. anomala* and *Z. bailii*; SC3, *D. bruxellensis* and *Z. bailii* and SC4, *D. anomala*, *D. bruxellensis* and *Z. bailii* were also prepared as described in chapter 3.

6.4 Results and discussion

6.4.1 Growth inhibition activity screening and crude biopreservation compounds production

The growth inhibition activity of *C. pyralidae* Y1117, *P. kluyveri* Y1125, and *P. kluyveri* Y1164 was screened against the yeasts listed in Table 6.1, which included *D. anomala* (2), *D. bruxellensis* (2), *Brettanomyces lambicus* (4), *C. magnoliae*, *C. guilliermondii*, *Z. baillii* (3), *Z. bisporus* (2), *Z. cidri*, *Z. fermentati*, *Z. florentinus*, *Z. microellipsoides*, and *Z. rouxii* (2). A zone of inhibition around the sensitive yeast colony indicates growth inhibition as depicted in Figure 6.1. In this study, in order to limit uncertainties in the origin of the growth inhibition activities, biopreservative-producing yeasts were screened against the spoilage yeasts and were cross-screened among themselves to see whether they displayed inhibition activity against each other (Table 6.1). The results showed that the biopreservative-producing yeasts exhibited no growth inhibition activity against each other. The GPE did not have any growth inhibition effect on any of the yeasts studied.

Table 6-1 Screening for growth inhibition activity, including the cross screening of biocontrol yeasts against each other on grape pomace extract. All yeasts used in the study.

		Biological control yeasts			
		Candida pyralidae Y1117	*Pichia kluyveri* Y1125	*P. kluyveri* Y1164	Grape Pomace extract
Spoilage yeasts	*Dekkera anomala* C96V37	-	+	+	-
	D. anomala C96V38	+	+	+	-
	D. bruxellensis C96V33	+	+	+	-
	D. bruxellensis C96V30	-	+	+	-
	Brettanomyces lambicus Y0106	-	-	-	-
	B. lambicus Y0175	+	+	-	-
	B. lambicus Y0191	+	+	-	-
	B. lambicus Y0167	+	+	-	-
	C. magnoliae Y1061	-	-	-	-
	C. guilliermondii Y0848	+	-	-	-
	Zygosaccharomyces bailii Y0070	+	-	-	-
	Z. bailii Y0891	+	-	-	-
	Z. bailii Y0186	+	-	-	-
	Z. rouxii Y0115	-	-	-	-
	Z. rouxii Y0111	-	-	-	-
	Z. microellipsoides Y0159	-	-	-	-
	Z. cidri Y0169	-	+	+	-
	Z. florentinus Y0277	-	-	-	-
	Z. fermentati Y0182	-	-	-	-
	Z. bisporus Y0288	-	-	-	-
	Z. bisporus Y0113	-	-	-	-
Biological control yeasts	*C. pyralidae* Y1117	-	-	-	-
	P. kluyveri Y1125	-	-	-	-
	P. kluyveri Y1164	-	-	-	-

Thereafter, grape pomace juice extract broth was used as the fermentation medium for the production of crude biopreservatives. This was confirmed by the zone of inhibition observed on the plates when 20 µL of the crude samples from each yeast culture was spotted in a 5 mm well on the GEA plates against spoilage organisms. The VZI was not quantified in this section because only visual inspections were required for further experiments.

Figure 6-1 Growth inhibition activity of *Candida pyralidae* Y1117 (1), *Pichia kluyveri* Y1125 (2), and *Pichia kluyveri* Y1164 (3) against *Dekkera anomala* on grape pomace extract agar. One plate out of all the different treatments was selected for a visual representation of growth inhibition activity.

6.4.2 Effect of Proteolytic Enzymes on the Denaturation of the Crude Biopreservatives

The denaturation ability of crude biopreservatives was investigated using a proteolytic enzymes treatment approach. Minimal denaturation of the crude biopreservatives was observed (data not shown). This suggests that the crude biopreservatives responsible for growth inhibition activity are not proteinaceous compounds produced during fermentation. A few *Candida* species, including *Candida pyralidae*, have been found to secrete non-proteinaceous type killer toxins that have growth inhibition activity (Mehlomakulu et al., 2014). This study therefore suggests that these yeasts could offer different types of metabolites for growth inhibition, against a variety of spoilage organisms, than those of a proteomic nature, which is in agreement with other reports (Chen et al., 2000; Ciani & Fatichenti, 2001). We previously reported (Mewa-Ngongang et al., 2019) that the growth inhibition activity of the crude biopreservatives produced by *C. pyralidae* Y1117, *P. kluyveri* Y1125, and *Pichia kluyveri* Y1164 was possibly due to volatile organic compounds, i.e., isoamyl acetate, isoamyl alcohol, 2-phenyl ethylacetate, and 2-phenyl ethanol. However, the focus of this study was on investigating the growth inhibition activity of the crude biopreservatives, which can potentially be used as pre- and post-harvest biocontrol agents.

6.4.3 pH, temperature activity and stability of the biopreservation compounds

The effect of pH and temperature on activity was tested. Observations of *C. pyralidae* indicate that the best pH to retain the highest growth inhibition activity was pH 3.0; however, for the two *P. kluyveri* strains, the suitable pH level was 2.0, while a pH of 4.0 and 5.0 showed extremely reduced growth inhibition activity for these yeasts (Figure 6.2). Similar results were observed against all three spoilage yeasts. Comparing these results to some findings reported in the literature, these pH values are within the range previously reported for other yeasts (Chen et al., 2000; Ciani & Fatichenti, 2001; Mehlomakulu et al., 2014). The stability of crude biopreservatives at the pH optima observed was investigated further.

For the stability test of the crude samples at different pH levels, it was noted that *P. kluyveri* samples were stable at a pH of 2.0 for 16 weeks and *C. pyralidae* samples was stable at a pH of 3.0 for the same period. Furthermore, temperature variation resulted in stability assessments for all the crude samples, which were found to be stable between - 10, 5, and 10 °C, respectively, where the activity was retained for more than 16 weeks. The pH and temperature assessments with regard to retaining growth inhibition activity of the crude biopreservatives were performed under different pH and temperature conditions that are usually used for consumer-packaged goods. The optimal activity and stability of the crude biopreservatives were obtained at a lower pH (2 and 3), which falls within the pH ranges of most beverages. These findings further expand and elucidate the in-situ application of crude biopreservatives in other types of food and beverage items with the same pH and storage temperature conditions.

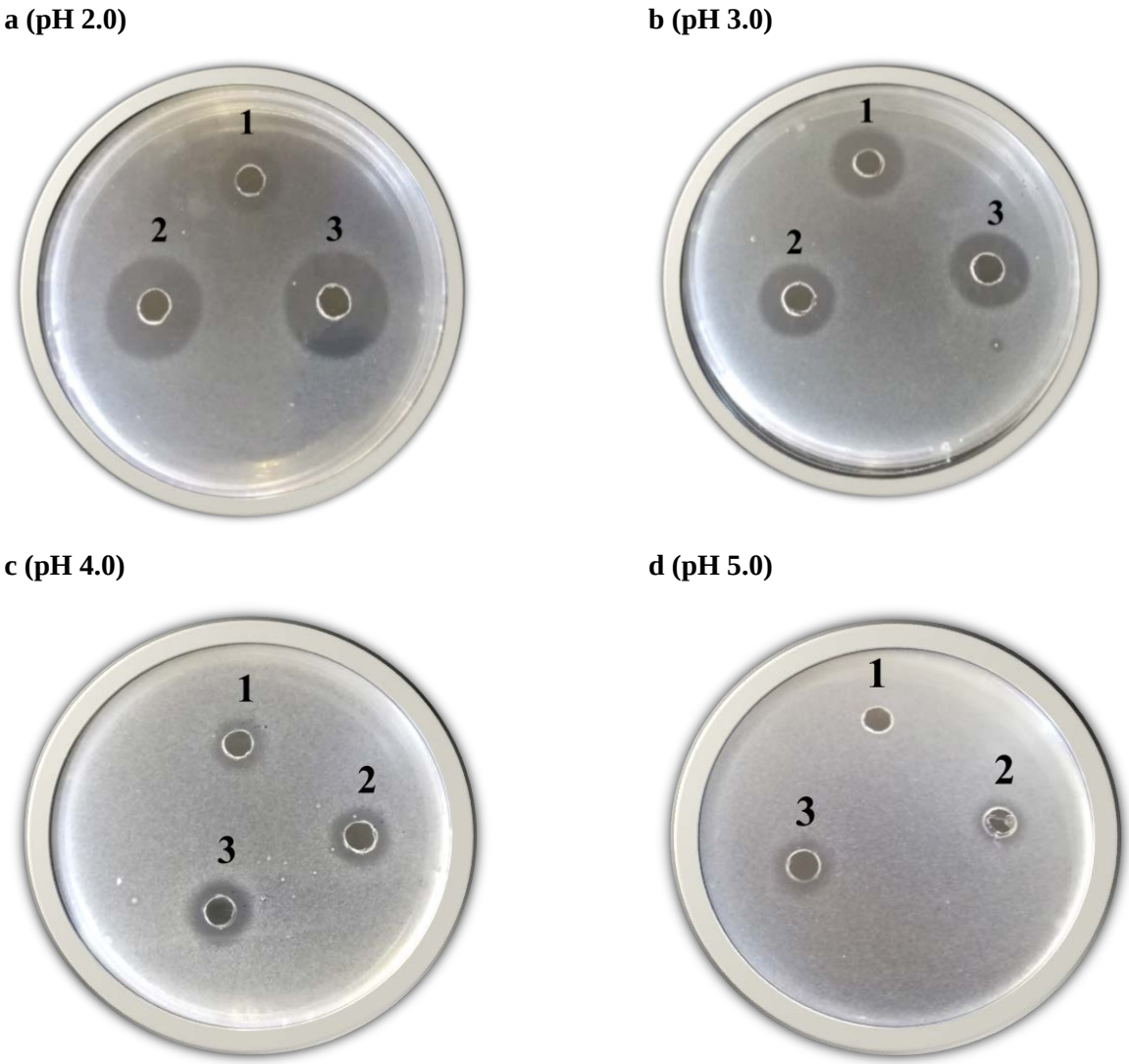

Figure 6-2 Growth inhibition activity of the crude biopreservatives from *Candida pyralidae* Y1117 (1), *Pichia kluyveri* Y1125 (2), and *Pichia kluyveri* Y1164 (3) against *Dekkera anomala* on grape pomace extract agar at different pH levels: (a**) pH 2.0, (**b**) pH 3.0, (**c**) pH 4.0, and (**d**) pH 5.0.**

6.4.4 Growth inhibition interaction study

6.4.4.1 Effect of single isolates and their cell free supernatants on growth inhibition activity of single spoilage

The quantification results of the effect of single crude versus single spoilage showed that *C. pyralidae* did not inhibit the growth of *D. anomala*, but inhibited the growth of *D. bruxellensis* and *Z. bailii* (Figure 3a). Meanwhile, the *P. kluyveri* strains inhibited the growth of *D. anomala* and *D. bruxellensis* with minimal growth inhibition activity against *Z. bailli*. Growth inhibition activity (visually indicated VZI) of the three biopreservation yeasts against *D. bruxellensis* is shown in Figure 3b.

The results obtained were in agreement with what usually occurs industrially during food preservation – microbial spoilage may still occur even though a specific food environment contains a preservation agent. In this case, the occurrence of spoilage organisms could be attributed to the resistance of spoilage organisms to the synthetic preservation agent used. Furthermore, a specific preservation agent could be used in targeting specific spoilage organisms; however, it may happen that a different spoilage organism contaminates the manufactured product. When this phenomenon occurs, the efficacy of the preservation agent is retained, but the biocontrol efficiency may seem to be lost due to the incorrect microbial target or microbial resistance to the crude biopreservatives used by the spoilage organism. Based on these reasons, more than one preservation agent can be used in the same food/beverage environment; hence the preference to use a mixture of preservation agents. In combating beverage spoilage suspected to occur due to the presence of *D. anomala*, *D. bruxellensis*, and *Z. bailli*, a combination of crude biopreservatives from *Candida pyralidae* Y1117 and *Pichia kluyveri* Y1125 or *Pichia kluyveri* Y1164 is recommended. Furthermore, the high efficacy observed for *P. kluyveri* against *D. anomala* and *D. bruxellensis* means that either *P. kluyveri* Y1125 or Y 1164 could be used in combating spoilage for either single or mixed spoilage organisms.

a **b**

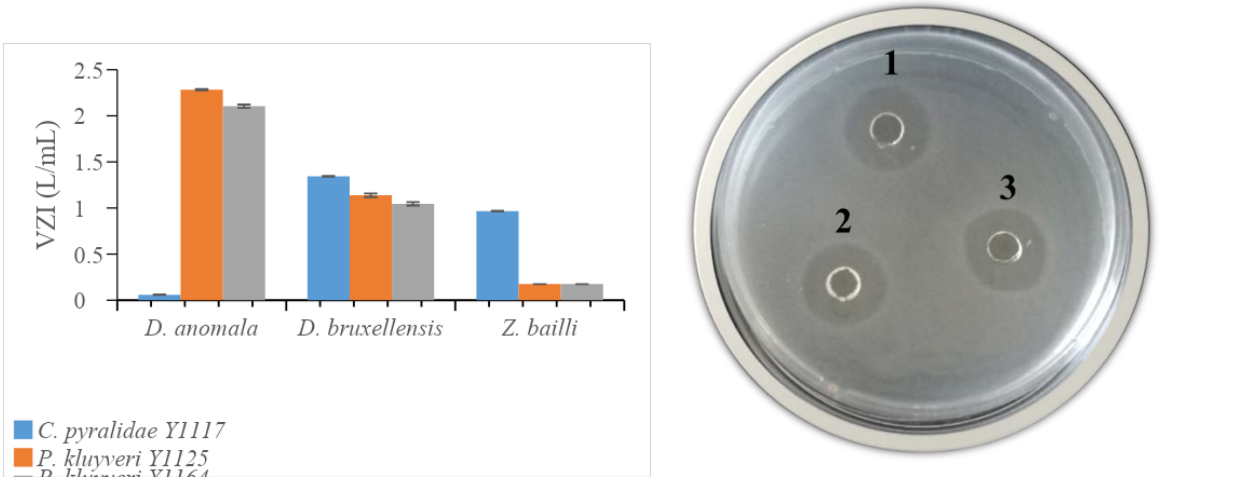

Figure 6-3 (a) Values of the volumetric zone of inhibition obtained from the studied effect of single isolates and their cell free supernatants on growth inhibition activity of single spoilage yeast. (b) an example of a plate showing the volumetric zone of inhibition obtained for *Candida pyralidae* Y1117 (b1), *Pichia kluyveri* Y1125 (b2) and *Pichia kluyveri* Y1164 (b3) against *Dekkera. bruxellensis.*

6.4.4.2 Effect of biopreservation compounds from single yeasts on growth inhibition activity against consortia of spoilage organisms

Since there was a broad-spectrum growth inhibition activity from single yeasts, the efficacy of the biopreservation extracts against the consortia of spoilage yeasts was assessed. All crude biopreservation samples showed growth inhibition activity against the spoilage yeast consortia, SC1-SC4, albeit, at different

efficacy levels (Figure 6.4a), proving the hypothesis that crude biopreservatives from a single yeast can act against a consortium of spoilage organisms. However, the highest growth inhibition was observed with *C. pyralidae* and *P. kluyveri* Y1164 against SC1 and SC3 (Figure 6.4b). Figure 4b shows the clear zones of inhibition obtained. This observation suggested that a beverage that is contaminated with a mixture of *D. anomala* and *D. bruxellensis* including a mixture of *D. bruxellensis* and *Z. bailli*, can be preserved using biopreservative from either *C. pyralidae* or *P. kluyveri* Y1164. Although the biopreservatives from *P. kluyveri* Y1125 can also be used in all spoilage organism combinations, a higher dose might be required compared to the quantity used if *C. pyralidae* or *P. kluyveri* Y1164 are to be used as fermenters of the crude biopreservatives of interest. Overall, it can be mentioned that each of the biopreservative crude samples was able to inhibit the growth of *D. anomala*, *D. bruxellensis*, and *Z. bailli* simultaneously under the same medium and conditions. Previously, *C. pyralidae* yielded a relatively lower growth inhibition activity (VZI) of 1.05 L mL^{-1} when Yeast Peptone Dextrose (YPD) was used as a fermentation media by (Mewa-Ngongang et al., 2017). This study suggests that utilizing a combination of crude biopreservatives from different yeasts could improve the inhibition activity.

a **b**

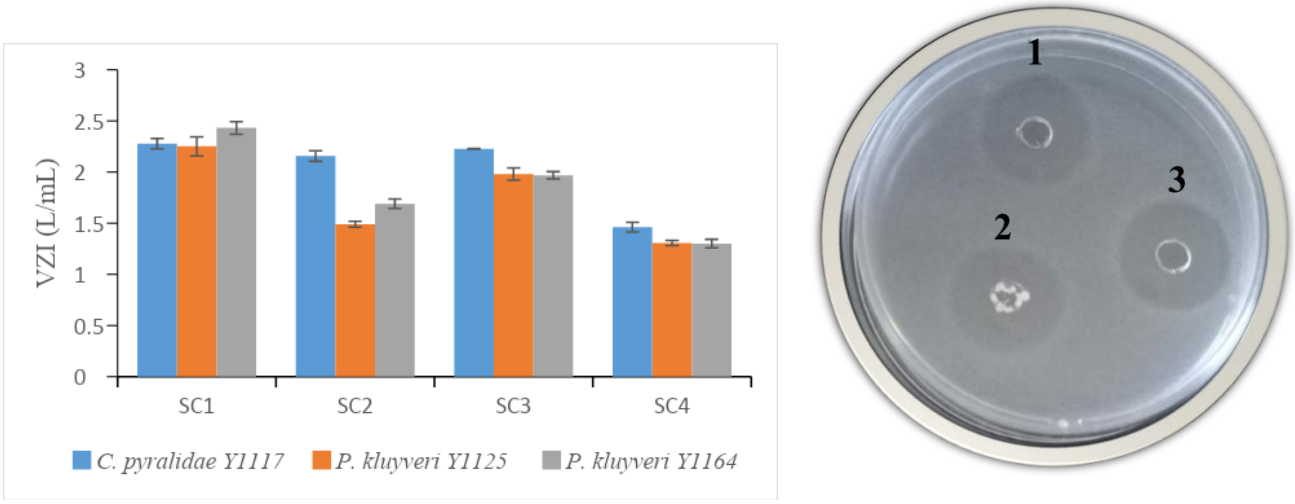

Figure 6-4 (a) values of the volumetric zone of inhibition obtained from the studied Effect of biopreservation compounds from single yeasts on growth inhibition activity of mixed spoilage. (b) is an example of a plate showing the volumetric zone of inhibition obtained for *Candida pyralidae* Y1117 (b1), *Pichia kluyveri* Y1125 (b2) and *Pichia kluyveri* Y1164 (b3).

6.4.4.3 Effect of Crude Biopreservative Mixtures on the Growth Inhibition of Single Spoilage Yeasts

A further interest in assessing the efficacy of mixed crude biopreservatives and their ability to inhibit single spoilage yeast was developed. It was observed that the spoilage yeast consortia were a lot more sensitive to the mixed crude biopreservative mixtures. It was found that, in all the growth inhibition combinations (GIC) studied, *D. anomala* was the most sensitive spoilage yeast, symbolized by a bigger VZI of 2.5 L mL^{-1} (Figure 6.5a), while *D. bruxellensis*, and *Z. bailli* had the least sensitivity, with a VZI of 0.17 L mL^{-1} for

GIC3 (Figure 6.5a, b). A VZI of 2.5 L mL^{-1} suggests that 1 mL of the crude biopreservatives inhibits the growth of *D. anomala* in a 2.5 L of beverage contaminated at a cell concentration of 10^6 cells mL^{-1}. In addition, the least sensitive, i.e., with a VZI of 0.17 L mL^{-1}, meant that the same volume (1 mL) of crude biopreservatives can only inhibit the growth of *D. bruxellensis* and *Z. bailli* in a 0.17 L volume of beverage contaminated with 10^6 cells mL^{-1}. The model of the current study therefore showed that crude volumes are dependent on the sensitivity of the spoilage organism towards the biopreservation agents. Although different levels of growth inhibition were observed, it was interesting to note that, if the mixed crude biopreservatives from all yeasts are used, the growth inhibition of spoilage will occur under a minimal biopreservative dosage. When processing food or beverages, the addition of preservatives should always be minimal, while considering an optimum efficacy. This is employed to limit the negative impact of large quantities of the crude biopreservatives in the final product that could affect the taste, texture, and possibly the appearance of the final product. However, based on our observations, a mixture of crude biopreservatives can also be used to target a single spoilage organism.

A **b**

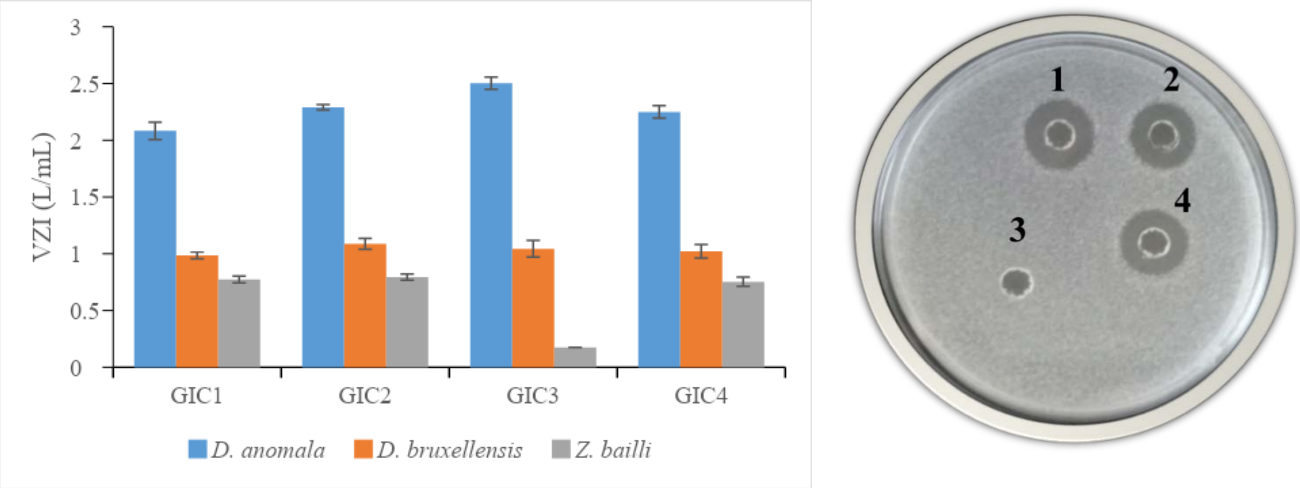

Figure 6-5 (a) values of the volumetric zone of inhibition obtained from the studied effect of mixed biopreservation compounds on the growth inhibition of single spoilage. (b) an example of a plate showing the volumetric zone of inhibition obtained from the GIC1 (b1), GIC2 (b2), GIC3 (b3) and GIC4 (b4) when *Zygosaccharomyces bailli* was used.

6.4.4.4 Effect of Crude Biopreservative Mixtures on the Growth of Spoilage Yeast Consortia

In food spoilage, it is reasonable to hypothesise that different spoilage organisms can be within the same beverage. The efficacy of a mixture of crude biopreservation compounds was assessed against consortia of spoilage yeasts. Therefore, it was observed that all growth inhibition combinations showed growth inhibition activity at different levels depending on the spoilage combination being assessed. The highest

growth inhibition activity (VZI) was observed on the GIC2 (2.27 L mL^{-1}) and GIC4 (2.21 L mL^{-1}) against SC3 with all GICs against SC4, showing the lowest growth inhibition activity of 1.55, 1.41, 1.34, 1.36 corresponding to GIC1-GIC4 respectively (Figure 6.6a). This observation could be due to compounds interactions in the crude biopreservation mixtures.

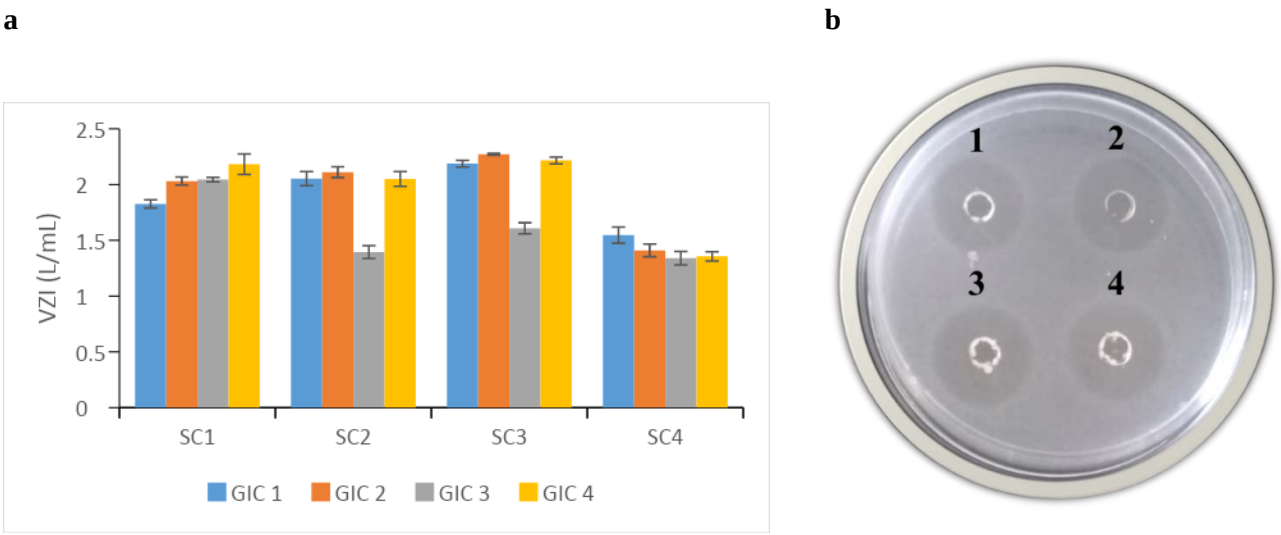

Figure 6-6 (a) Values of the volumetric zone of inhibition (VZI) obtained from growth inhibition combinations (GICs), i.e., GIC1, *C. pyralidae* Y1117 and *P. kluyveri* Y1125 (1); GIC2, *C. pyralidae* Y1117 and *P. kluyveri* Y1164 (2); GIC3, *P. kluyveri* Y1125 and *Pichia kluyveri* Y1164 (3); and GIC4, *C. pyralidae* Y1117, *P. kluyveri* Y1125, and *P. kluyveri* Y1164 (4) against spoilage yeast consortia (SC), i.e., SC1 (*D. anomala* and *D. bruxellensis*), SC2 (*D. anomala* and *Z. bailii*), SC3 (*D. bruxellensis* and *Z. bailii*), and SC4 (*D. anomala*, *D. bruxellensis*, and *Z. bailii*). (b) An example of a plate showing the VZI obtained from the GICs against SCs. The values presented are the means of three replicates with the standard deviation ranging between 0.004 and 0.09.

In a review by Kumar and Jagadeesh (Kumar & Jagadeesh, 2016), several microbial consortia against phytopathogens were highlighted. The microbial consortia reported thus far against *Botrytis*, *Colletotrichum*, *Rhizoctoniasolani*, and *Pyriculariaoryzae* species were composed of two or more species of yeasts and/or bacteria acting on wounded fruits (Calvo et al., 2003; Conway et al., 2005; Lucas et al., 2009; Yobo et al., 2010; Kumar & Jagadeesh, 2016). Comparing the results obtained from this work with those reported on phytopathogens, it could be suggested that microbial consortia cannot only be composed of two strains of microorganisms. Furthermore, this study also suggests that crude biopreservatives from yeasts, without supplementation by chemical preservatives, could be effective against beverage spoilage yeasts, even when they are in a consortium.

6.5 Summary

Subsequent to finding the conditions where the crude biopreservatives were stable, the activity of the individual, as well as GICs, was dependent on the composition of the SCs. Our findings showed the potential of GICs from *C. pyralidae* Y1117, *P. kluyveri* Y1125, and *P. kluyveri* Y1164, as an alternative to synthetic chemicals as preservatives in food, including beverages susceptible to contamination by *D. bruxellensis*, *Z. baillii*, and *D. anomala*. Future research should focus on testing the reported GICs against other spoilage microorganisms and toxicological studies should be performed to assess the impact of these GICs on human health, as well as the microorganisms associated with the human gastrointestinal tract.

With the interesting interplay among the GICs studied against the spoilage combinations SCs, the question would be, how effective would this be against spoilage fungi? Given the fact that combating fruit spoilage by fungal spoilage organisms include any technique that would allow stopping fungal development, it was important to explore the use of the biological control yeasts cultures for postharvest preservation experiments. The next part of the work consisted of studying the postharvest biocontrol efficacy of *Candida pyralidae* and *Pichia kluyveri in-vitro* and *in-vivo*.

CHAPTER 7

FUNGISTATIC AND FUNGICIDAL PROPERTIES OF *CANDIDA PYRALIDAE* Y1117, *PICHIA KLUYVERI* Y1125 AND *PICHIA KLUYVERI* Y1164 ON THE BIOCONTROL OF *BOTRYTIS CINEREA*

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CHAPTER 7

FUNGISTATIC AND FUNGICIDAL PROPERTIES OF *CANDIDA PYRALIDAE* Y1117, *PICHIA KLUYVERI* Y1125 AND *PICHIA KLUYVERI* Y1164 ON THE BIOCONTROL OF *BOTRYTIS CINEREA*

7.1 Introduction

Fruit spoilage caused by fungal pathogens is an agricultural and post-harvest challenge (Salman, 2005; Parveen et al., 2016). A significant quantity of fruit is lost annually during post-harvest processing and transportation (Droby, 2005; Zhu, 2006; Singh & Sharma, 2007). Generally, *Botrytis cinerea* is one of the major fungal spoilers of table grapes (Williamson et al., 2007; Sharma et al., 2009). Currently, synthetic chemicals with fungicidal properties have been used in order to reduce microbial spoilage of grapes and other fruits. It has also been widely reported that the currently used synthetic chemicals pose serious health concerns that necessitates an alternative to synthetic preservatives for fruit producers and processors (Cheetham, 1997). The use of yeasts as biological control agents is a better alternative to the use of synthetic chemicals because of the ability to compete for nutrients, space, and to grow at a faster rate than fungal pathogens (Comitini, De, et al., 2004; Mehlomakulu et al., 2014; Grzegorczyk et al., 2017; Nadai et al., 2018). The inhibitory effect of yeasts has also been attributed to the production of volatile organic compounds (VOCs) (Huang et al., 2011a; Parafati et al., 2015). Although yeasts are known to inhibit fungal growth by releasing volatile organic compounds, their fungistatic and the fungicidal effects at varying inoculum doses has not been reported for the postharvest biocontrol of *Botrytis cinerea* using *Candida pyralidae* Y1117, *Pichia kluyveri* Y1125 and *Pichia kluyveri* Y1164 as biocontrol yeasts.

7.2 Objective

The objectives of this part of the study were to:

- Assess the fungistatic and fungicidal activity of the yeasts *Candida pyralidae* strain Y1117, *Pichia kluyveri* strain Y1125 and *Pichia kluyveri* strain Y1164 against *Botrytis cinerea in-vitro* and *in-vivo*.
- To determine the minimum inoculum dose (MID) of yeasts required to completely inhibit *Botrytis cinerea* in a quantified contaminated headspace, while confirming these properties on table grapes.

7.3 Materials and methods

Prior to the *in-vitro* and *in-vivo* test, the volume of the headspace of the 90 mm petri dish plate and the jars used were determined as described in chapter 3. The invitro test was carried out using the mouth-to-mouth plate assay while the in vivo test on table grapes was conducted using the jar method. Serial dilution technique was used to prepare all the different biocontrol yeast inoculum concentrations that ranged from 10^1 to 10^8 Cells mL^{-1}. The treatments approach, the incubation conditions and assessment of results used are described in chapter three.

7.4 Results and discussion

7.4.1 Headspace Quantification

Biocontrol studies have been done previously but none focused on the impact of headspace on fungal inhibition. In this study, the headspace volume in which the growth inhibition occurred was quantified. The volume covered by the GPA poured onto the petri dish was found to be 12.7 mL. The actual headspace in which the efficacy of the VOCs produced by the biological control took place was 111 mL compared to 400 mL in vivo (total volume of headspace in the jar used).

7.4.2 Efficacy of the VOCs *in-vitro*

The evaluation of the effect of VOCs in a closed and quantifiable headspace was carried out. *Candida pyralidae* strain Y1117, *Pichia kluyveri* Y1125 and *Pichia kluyveri* Y1164 inhibited the growth of *Botrytis cinerea* at different inoculum dose. The 10^1 cell mL-1 treatments were mostly similar to the negative control (Figure 7.1 Aa, Ba and Ca) whereas the inoculum dose of 10^2 - 10^5 cells mL^{-1} showed the fungistatic characteristics (figure 1 A, B, Cb,c,d,e). The confirmation was also made when fungal growth was observed after opening the plates. Fungal growth inhibition was also displayed in 10^6 -10^8 cells mL^{-1} plates (figure 1 A, B, Cf,g,h,). However, no fungal growth was observed after opening the plates. This was a clear indication that higher inoculum doses completely inhibit fungal growth whereas lower inoculum doses temporarily suppress growth until VOCs escape from the container.

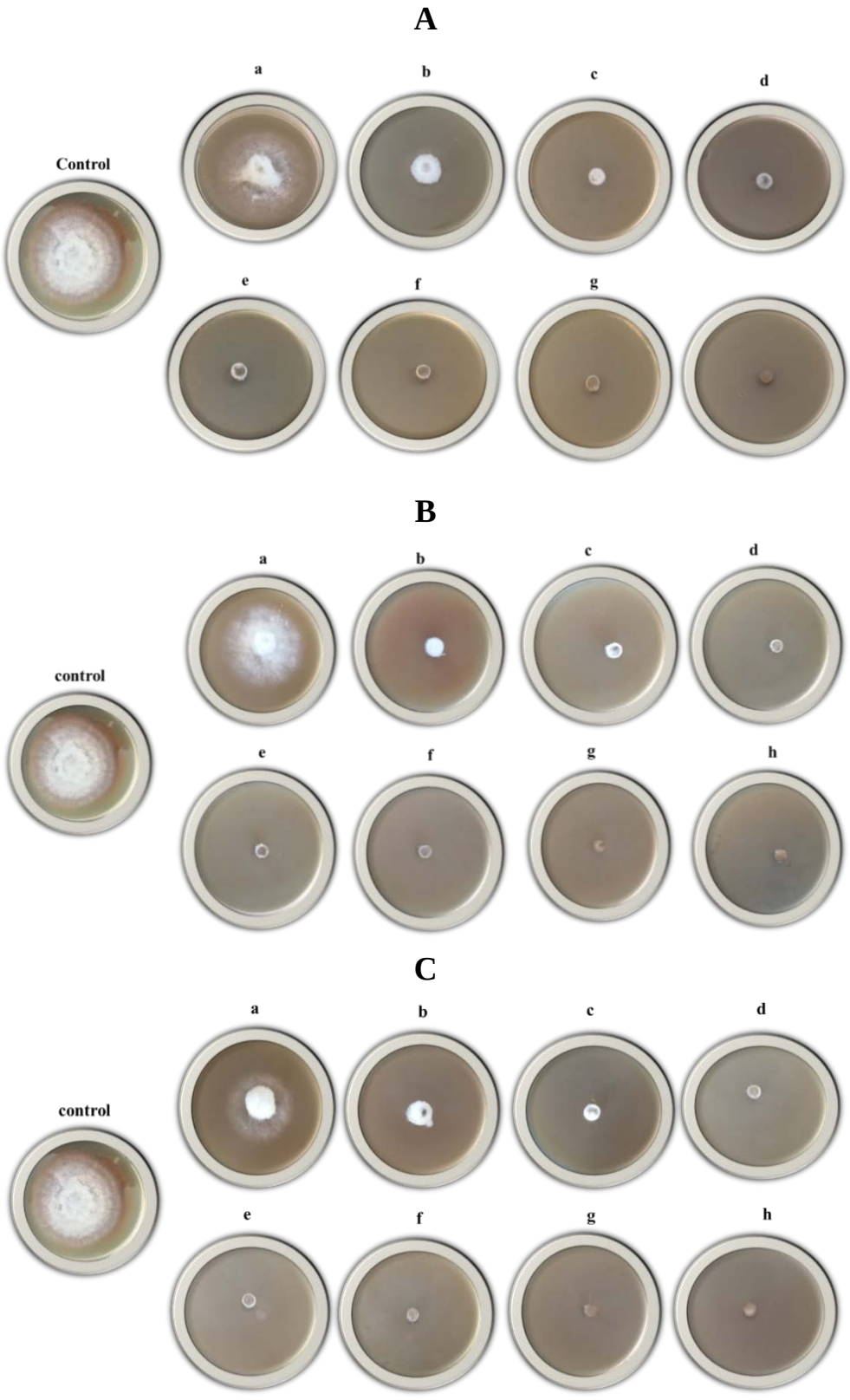

Figure 7-1 Representative agar plates showing the *in-vitro* fungistatic and fungicidal effect of *Candida pyralidae* Y1117 (A), *Pichia kluyveri* Y1125 (B) and *P. kluyveri* Y1164 (C) on the biocontrol of Botrytis cinerea. (a) represents the plates spread with initial inoculum of 10^1 cells mL^{-1} ; b, c d and e depict the initial inoculum of 10^2 , 10^3 , 10^4 and 10^5 cells mL^{-1} respectively. f, g, h correspond to the initial inoculum of 10^6 , 10^7 and 10^8 cells mL^{-1} . Assays were conducted in triplicates.

7.4.3 Efficacy of the VOCs *In-vivo*

To confirm the fungicidal effect observed *in-vitro*, the *in-vivo* test was carried-out using a grape bioassay. The fungicidal effect of *Candida pyralidae* strain Y1117, *Pichia kluyveri* Y1125 and *Pichia kluyveri* Y1164 was observed as a resultant of VOCs released (figure 7.2). Compared to the negative controls, fungal growth was still completely inhibited after leaving the jars for five weeks for all tested yeast (figure 7.2). The growth inhibition of *Botrytis cinerea* by VOCs from yeasts have been reported in literature (Parafati et al., 2015; Nally et al., 2015; Qin et al., 2015; Qin et al., 2017) and comparing those findings to the results obtained in this work, it can be noted that the method used in this work and the VOCs produced by *Candida pyralidae* Y1117, *Pichia kluyveri* Y1125 and *Pichia kluyveri* Y1164 completely inhibited the growth of *Botrytis cinerea* on table grapes. Furthermore, the findings from this work clearly demonstrated the fungicidal potential of *Candida pyralidae* Y1117, *Pichia kluyveri* Y1125 and *Pichia kluyveri* Y1164 for the biocontrol of *Botrytis cinerea*. The minimum inoculum dose (MID) strategy proved beneficial in exploring the interactive relationship between yeasts and fruit fungal pathogens. For all yeasts, the MID of 10^6 cells mL^{-1} was sufficient to display fungicidal activity against *Botrytis cinerea*. Since a more preventative approach against *Botrytis cinerea* was adopted in this study, the inoculum dose and headspace findings could be used to model and optimise biofungicide activities of yeasts under commercial transportation and storage conditions.

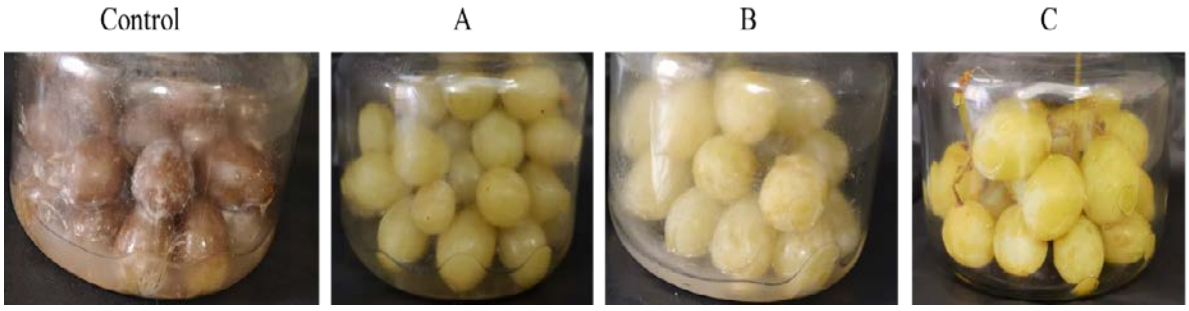

Figure 7-2 Representative jars showing the fungicidal effects of *Candida pyralidae* Y1117 (A), *Pichia kluyveri* Y1125 (B) and *P. kluyveri* Y1164 (C) on the biocontrol of *Botrytis cinerea*. Only one jar per treatment was selected as a representative.

7.5 Summary

This study demonstrated the fungistatic and fungicidal potential of the biocontrol yeasts. The VOCs produced by *C. pyralidae* Y1117, *P. kluyveri* Y1125 and *P. kluyveri* Y1164 when trapped in a closed environment were found to be responsible for the fungicidal effect on the growth of *B. cinerea* both *in-vitro* and *in-vivo*. The use of yeast with biocontrol activity presents a potential alternative to synthetic chemicals currently used as fungicides on fruit and other fruit derived beverages. Thus far, it has been demonstrated that the biocontrol yeasts studied and their extracellular metabolites have the potential to be used as biopreservatives and biofungicides. For system design and biocontrol yeasts production scale up, it is

imperative to study some engineering aspects i.e. the biological stoichiometry and bioenergetics of the process that could allow a production system in which both the yeast cells and their extracellular compounds could be generated and used as biopreservatives (against spoilage yeasts) and postharvest control (against spoilage fungi).

CHAPTER 8

BIOLOGICAL STOICHIOMETRY AND BIOENERGETICS OF YEASTS DURING BIOPRESERVATION COMPOUNDS PRODUCTION

Paper not submitted

CHAPTER 8

BIOLOGICAL STOICHIOMETRY AND BIOENERGETICS OF YEASTS DURING BIOPRESERVATION COMPOUNDS PRODUCTION

8.1 Introduction

The potential of yeasts as biological control agents and as producers of biopreservation compounds has been established by several independent studies (Ciani & Fatichenti, 2001; Gürakan et al., 2007; Nally et al., 2015; Nadai et al., 2018; Abbey et al., 2019). Those previous studies have been carried out in in refine media at a laboratory scale with less engineering aspects for system designs towards high scale production. Recently, grape pomace extract was explored as cheaply available fermentation medium for the production of biopreservation compounds from yeasts (Mewa-Ngongang et al., 2019). Microbial growth and production kinetics with optimisation were successfully studied (Mewa-Ngongang et al., 2019).

The feasibility of a bioprocess engineering system and the related physiological conditions under which bioreactions can occur would only be possible by studying the biothermodynamic of the intended process, mostly when considering industrial scale production. This has not yet been applied extensively in biological processes (von Stockar et al., 2008). In addition, yield parameters can also be estimated during fermentation in order to assess the material and energy requirements in relation to the economic viability of any intended biological process (Duboc et al., 1999; Liu et al., 2007; Heijnen, 2010).

For the purpose of establishing significant data set for a system design for biopreservation compound production using renewable carbon source, GPE as fermentation medium, the biothermodynamic study in this regard was therefore imperative. In the current literature, the stoichiometric analysis of microbial growth and related yields have been studied for some biological processes (Akinpelu et al., 2018b).

The efficiency of microorganisms towards extracellular compounds production could be determined by the stoichiometric coefficients and bioenergetic analysis which can better assist in elucidating the feasibility of a bioprocess system being designed.

8.2 Objectives

The objectives of this part of the work were to:

- Generate the biological stoichiometric equations and bioenergetics data during biopreservation compounds production from yeasts using grape pomace extract as fermentation medium.
- Provide a set of requirements for the design of a system for efficient prediction and industrial scale production of biopreservation compounds from the selected biopreservatives producing yeasts.

8.3 Materials and methods

After studying the optimum condition for biopreservation compound production from all the three biopreservatives producing yeasts, each yeast was grown under the optimum conditions found in Chapter 5, harvested by centrifugation, and dried for further analysis as described in chapter 3. The dried samples were analysed for carbon, hydrogen, oxygen, nitrogen and sulphur content. The percentage of oxygen was obtained by subtracting the total percentages of CHNS from 100. Subsequent to the determination of energy requirements during the conversion of the growth controlling substrates to biomass and the related by-products (biopreservatives), the stoichiometric equations corresponding to each yeast was determined. The thermodynamic parameters as highlighted were determined using a bomb colorimeter and the mathematical models as described in chapter 3.

8.4 Results and discussion

8.4.1 Elemental analysis of the dried biomass

Table 8.1 shows the results of the elemental analysis corresponding to *C. pyralidae* Y1117, *P. kluyveri* Y1125 and *P. kluyveri* Y1164. The elements considered were C, H, N, O and S only. As previous studies (Duboc et al., 1999; Battley, 2011) demonstrated, the contribution of other ions are negligible when constructing the empirical formula, because their presence has an effect only on the composition in relation to that of the oxygen fraction of the biomass. It was also observed that similar trends were obtained for all three biopreservatives producing yeasts, mainly the percentages of the elements analysed, the average molecular weight and the unit carbon values (Table 8.1). The strain *P. kluyveri* Y1164 was the yeast with the highest carbon and hydrogen content (44.149 and 6.871% respectively), meanwhile *C. pyralidae* Y1117 and *P. kluyveri* Y1125 had a carbon content of 41.435 and 41.157 % and a hydrogen contents of 6.582 and 6.484 % respectively. The nitrogen content of the dried biomasses was quite similar to each other 7.200, 6.333 and 7.530 corresponding to *C. pyralidae* Y1117, *P. kluyveri* Y1125 and *P. kluyveri* Y1164 respectively. The same observations were made for the sulphur and oxygen content of all the three dried yeasts as well as on average MW and Unit-carbon mole (Table 8.1). these result are comparable to what was observed by (Battley, 2011; Akinpelu et al., 2018) in different studies.

Table 8-1 Elemental analysis, average molecular weight and unit carbon mole of *C. pyralidae* Y1117, *P. kluyveri* Y1125 and *P. kluyveri* Y1164 during biopreservation compound production using grape pomace extracts (GPE) as fermentation medium. The values presented in the table are the average on three replicates with

		[C]	[H]	[N]	[O]	[S]
C.	%	41,435	6,582	7,200	44,501	0,282
pyralidae	Average MW	3,453	6,582	0,514	2,781	0,009
Y1117	Unit-carbon	1,000	1,906	0,149	0,805	0,003
P.	%	41,157	6,484	6,333	45,713	0,313
kluyveri	Average MW	3,430	6,484	0,452	2,857	0,010
Y1125	Unit-carbon	1,000	1,891	0,132	0,833	0,003
P.	%	44,149	6,871	7,530	41,104	0,346
kluyveri	Average MW	3,679	6,871	0,538	2,569	0,011
Y1164	Unit-carbon	1,000	1,868	0,146	0,698	0,003

8.4.2 Determination of the biological stoichiometric equations

The empirical and the elemental formula of *C pyralidae* Y1117, *P. kluyveri* Y1125 and *P. kluyveri* Y1164 are listed in Table 8.2. The MW of a unit carbon mole of each yeast was calculated from each of the elemental formula. It was found that the MW of *C pyralidae* Y1117, *P. kluyveri* Y1125 and *P. kluyveri* Y1164 were found to be 28.968, 29.163 and 27,176 g C-mol^{-1} respectively, which is in reasonable agreement with what has been reported elsewhere (Akinpelu et al., 2018).

The similarities of these findings with that of the published literature could be attributed to the use of renewable macro and micronutrient rich sources (Akinpelu et al., 2018) and grape pomace extracts medium as the case in this study.

Table 8-2 Empirical, elemental formula and molecular weight of unit carbon mole of *C. pyralidae* Y1117, *P. kluyveri* Y1125 and *P. kluyveri* Y1164 during biopreservation compound production using grape pomace extracts (GPE) as fermentation medium.

	C. pyralidae Y1117	*P. kluyveri* Y1125	*P. kluyveri* Y1164
Empirical formula	$C_{3.454}H_{6.585}N_{0.514}O_{2.78}S_{0.009}$	$C_{3.453}H_{6.484}N_{0.452}O_{2.857}S_{0.010}$	$C_{3.679}H_{6.871}N_{0.538}O_{2.569}S_{0.011}$
Elemental formula	$CH_{1.906}N_{0.149}O_{0.805}S_{0.003}$	$CH_{1.891}N_{0.132}O_{0.833}S_{0.003}$	$CH_{1.868}N_{0.146}O_{0.698}S_{0.003}$
MW of unit carbon (g/C-mol)	28.968	29,163	27,176

Since the unit carbon molecular weight for *C. pyralidae* Y1117, *P. kluyveri* Y1125 and *P. kluyveri* Y1164 were found, the biological stoichiometric equations were derived and balanced by determining the stoichiometric coefficients (a-f) using the conservation of mass approach (Table 8.3). The models used to represent and describe *C pyralidae* Y1117, *P. kluyveri* Y1125 and *P. kluyveri* Y1164 metabolism per unit carbon mol during biopreservation compounds production using GPE as fermentation medium are displayed in Table 8.4. The model equations were classified into three steps corresponding to the catabolic, anabolic and metabolic reactions. Comparing these models to those reported in literature, it was interesting to find some similarities although these equations were generated from different types of biological processes. However, some of the processes were carried out using glucose and ammonia as energy sources for the microbial growth. It was also observed that the three metabolic reaction models corresponding to the three biopreservatives producing yeasts were distinct from each other, which also justified the different metabolic observation during biopreservation compound production processes. During the biopreservation compound production, it was observed that *P. kluyveri* Y1164 utilised more glucose than *C pyralidae* Y1117 and *P. kluyveri* Y1125. The VZI output as the result of the biopreservation compounds concentration in the crude was found to be lower for *P. kluyveri* Y1164 than those of *C pyralidae* Y1117 and *P. kluyveri* Y1125. The biological stoichiometric results showed an interesting interplay in this regards where, according to the growth model generated for *P. kluyveri* Y1164, it was observed that more oxygen was used in comparison to other yeasts, less biomass was generated with more water formation in *P. kluyveri* Y1164's the product of metabolism which can explain the VZI variation observed. Because this part of the study aimed at generating useful data for system design for biopreservation compound production using GPE as fermentation medium, these model (Table 8.4) could be used in that regards to simulate a mass and energy balance relationship for an adequate bioprocess design.

Table 8-3 Microbial growth equation of *C pyralidae* Y1117, *P. kluyveri* Y1125 and *P. kluyveri* Y1164 during biopreservation compound production using grape pomace extracts as fermentation medium, derived and balanced by determining the stoichiometric coefficients (a-f) using the conservation of mass.

***C. pyralidae* Y1117**
$1.929C_6H_{12}O_{6(aq)} + 10.431O_{2(aq)} + 0.149NH_{3(aq)} + 0.003SO_{4(aq)}^{2-} + 0.006H_{(aq)}^{+} \rightarrow$ $CH_{1.906}N_{0.149}O_{0.805}S_{0.003}(cell) + 10.398CO_{2(aq)} + 10.672H_2O_{(l)}$
***P. kluyveri* Y1125**
$1.900C_6H_{12}O_{6(aq)} + 10.608O_{2(aq)} + 0.132NH_{3(aq)} + 0.003SO_{4(aq)}^{2-} + 0.006H_{(aq)}^{+} \rightarrow$ $CH_{1.891}N_{0.132}O_{0.833}S_{0.003}(cell) + 10.576CO_{2(aq)} + 10.831H_2O_{(l)}$
***P. kluyveri* Y1164**
$3.043C_6H_{12}O_{6(aq)} + 17.249O_{2(aq)} + 0.146NH_{3(aq)} + 0.003SO_{4(aq)}^{2-} + 0.006H_{(aq)}^{+} \rightarrow$ $CH_{1.868}N_{0.146}O_{0.698}S_{0.003}(cell) + 17.258CO_{2(aq)} + 17.543H_2O_{(l)}$

Table 8-4 Microbial growth equations for biopreservation compound production per unit glucose by *C. pyralidae* Y1117, *P. kluyveri* Y1125 and *P. kluyveri* Y1164 using grape pomace extracts as fermentation medium

***C. pyralidae* Y1117**
Catabolism: $C_6H_{12}O_{6(aq)} + 6O_{2(aq)} \rightarrow 6H_2O_{(l)} + 6CO_{2(aq)}$
Anabolism: $0.383H_2O_{(l)} + 0.526CO_{2(aq)} + 0.078NH_{3(aq)} + 0.002SO_{4(aq)}^{2-} + 0.003H_{(aq)}^{+} \rightarrow 0.526CH_{1.906}N_{0.149}O_{0.805}S_{0.003}(cell) + 0.509O_2$
Metabolism: $C_6H_{12}O_{6(aq)} + 0.078NH_{3(aq)} + 5.491O_{2(aq)} + 0.002SO_4^{2-} + 0.003H^{+} \rightarrow 5.419CO_{2(aq)} + 5.617H_2O_{(l)} + 0.526CH_{1.906}N_{0.149}O_{0.805}S_{0.003}(cell)$
***P. kluyveri* Y1125**
Catabolism: $C_6H_{12}O_{6(aq)} + 6O_{2(aq)} \rightarrow 6H_2O_{(l)} + 6CO_{2(aq)}$
Anabolism: $0.386H_2O_{(l)} + 0.519CO_{2(aq)} + 0.068NH_{3(aq)} + 0.001SO_{4(aq)}^{2-} + 0.003H_{(aq)}^{+} \rightarrow 0.518CH_{1.891}N_{0.132}O_{0.833}S_{0.003}(cell) + 0.502O_2$
Metabolism: $C_6H_{12}O_{6(aq)} + 0.068NH_{3(aq)} + 5.498O_{2(aq)} + 0.001SO_4^{2-} + 0.003H^{+} \rightarrow 5.481CO_{2(aq)} + 5.614H_2O_{(l)} + 0.518CH_{1.891}N_{0.132}O_{0.833}S_{0.003}(cell)$
***P. kluyveri* Y1164**
Catabolism: $C_6H_{12}O_{6(aq)} + 6O_{2(aq)} \rightarrow 6H_2O_{(l)} + 6CO_{2(aq)}$
Anabolism: $0.235H_2O_{(l)} + 0.329CO_{2(aq)} + 0.048NH_{3(aq)} + 0.001SO_{4(aq)}^{2-} + 0.002H_{(aq)}^{+} \rightarrow 0.329CH_{1.868}N_{0.146}O_{0.698}S_{0.003}(cell) + 0.332O_2$
Metabolism: $C_6H_{12}O_{6(aq)} + 0.048NH_{3(aq)} + 5.668O_{2(aq)} + 0.001SO_4^{2-} + 0.002H^{+} \rightarrow 5.671CO_{2(aq)} + 5.765H_2O_{(l)} + 0.329CH_{1.868}N_{0.146}O_{0.698}S_{0.003}(cell)$

8.4.3 Bioenergetics parameters determination

The combustion equations used to determine the bioenergetic parameters for each of the biocontrol yeasts are listed in Table 8.5.

Table 8-5 The combustion equations used for the calculation of energy requirements during biopreservation compound production by *C. pyralidae* Y1117, *P. kluyveri* Y1125 and *P. kluyveri* Y1164 using grape pomace extracts as fermentation medium.

***C. pyralidae* Y1117**
$CH_{1.906}N_{0.149}O_{0.805}S_{0.003}(cell) + 1.475O_2 \rightarrow CO_{2(g)} + 0.95H_2O_{(l)} + 0.0745N_{2(g)} + 0.003\ H_2S_{(g)}$
***P. kluyveri* Y1125**
$CH_{1.891}N_{0.132}O_{0.833}S_{0.003}(cell) + 1.055O_2 \rightarrow CO_{2(g)} + 0.942H_2O_{(l)} + 0.066N_{2(g)} + 0.003\ H_2S_{(g)}$
***P. kluyveri* Y1164**
$CH_{1.868}N_{0.146}O_{0.698}S_{0.003}(cell) + 1.116O_2 \rightarrow CO_{2(g)} + 0.931H_2O_{(l)} + 0.066N_{2(g)} + 0.003\ H_2S_{(g)}$

Table 8.6 shows the thermodynamic parameters obtained during biopreservation compound production by the biocontrol yeasts in GPE broth as fermentation medium. As analysed in a bomb calorimeter, the heat of combustion $\left(\Delta H_c^{cell}\right)$ for the dried *C. pyralidae* Y1117, *P. kluyveri* Y1125 and *P. kluyveri* Y1164 were found to be 12.29, 17.98 and 17.09 kJ/g. However, the experimental enthalpy of combustion for the three biocontrol yeasts was found to be -471.89 kJ/C-mol for *C pyralidae* Y1117, -524.35 kJ/C-mol for *P. kluyveri* Y1125 and -464.44 kJ/ C-mol for *P. kluyveri* Y1164. The variations in bioenergetics was calculated (Table 8.6) using the values obtained from the bomb calorimeter and the thermodynamic properties found in literature and listed in Table 3.5. prior to the calculation of the above mentioned bioenergetic variations, Thornton's rule and an approach similar to this work (Thornton, 1917; Akinpelu et al., 2018) was used as described in the following equation.

$$\Delta H_c^o = -107.9\ \frac{kj}{eq} X(eq.transferred\ to\ oxygen\ during\ bomb\ calorimetric\ combustion)$$

Equation 13

The thermodynamic parameters studied for the biopreservation compound production in GPE as fermentation medium were enthalpy of biomass formation ΔH_f^{cell}, heat of reaction ΔH_{RX}^O and the entropy changes ΔS_{RX}^O. The results obtained for the above-mentioned parameters are presented in Table 8.6. The highest exothermic reaction was observed for *P. kluyveri* Y1164 with -195.23 kJ C-mol^{-1} heat of formation, and then followed by *C. pyralidae* Y1117, with a ΔH_f^{cell} value of -193.22 kJ C-mol^{-1}. The ΔH_f^{cell} for *P. kluyveri* Y1125 was the lowest with -138.46 kJ C-mol^{-1}. It is worth mentioning that a higher exothermic value translated to higher values for yeast growth during fermentation which could also explained the yield values obtained in chapter 5. The negative enthalpy of reaction ΔH_{RX}^O observed for all the three biopreservation compound producing yeasts demonstrated that the biomass formation was hypothetically spontaneous for the system developed. The change in antropy ΔS_{RX}^O values obtained were found to be -0.45, -0.21 and -0.53 kJ K^{-1} C-mol for *C. pyralidae* Y1117, *P. kluyveri* Y1125 and *P. kluyveri* Y1164 respectively. These values were found to be weak, translating to an entropically driven process. Based on previous studies

on bioenergetics, the values obtained for the bioenergetic parameters studied in this work are in reasonable agreement with what has been reported (Duboc et al., 1999; von Stockar et al., 2008; Battley, 2013; Akinpelu et al., 2018).

Table 8-6 Thermodynamic parameters during biopreservation compound production by *C. pyralidae* Y1117, *P. kluyveri* Y1125 and *P. kluyveri* Y1164 using grape pomace extracts as fermentation medium at 298.15 K and 1 atm.

Yeasts	ΔH_f^{cell} (kJ C-mol^{-1})	ΔH_{RX}^{O} (kJ C-mol^{-1})	$-\Delta G_{RX}^{O}$ (kJ C-mol^{-1})	ΔS_{RX}^{O} (kJ K^{-1} C-mol^{-1})
C pyralidae Y1117	-193.22	-5006.75	-5141.07	-0.45
P. kluyveri Y1125	-138.463	-5160.48	-5223.87	-0.21
P. kluyveri Y1164	-195.23	-8283.54	-8441.26	-0.53

8.5 Summary

For an adequate design of a system for the production of biopreservation compounds by yeasts in a bioreactor system, important engineering aspects are always needed. This has been successfully addressed by the outcome of this work. The biological stoichiometric models have been effectively developed which could indeed allow to account for mass and part of the energy balances required for industrial scale production of biopreservation compounds using cheaply available renewable agrowaste as raw material. The findings of this work also play in the favour of the prediction (mass and energy balance) as well as the feasibility of metabolic reactions during industrial scale production of biopreservatives and biocontrol yeasts. This work is the first to report on biological stoichiometry and bioenergetics during biopreservation compounds production by yeasts using grape pomace extract as fermentation medium.

CHAPTER 9

SUMMARY AND CONCLUSION

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9.1 Summary and conclusions

General safe food production is greatly needed in the society at all times. There are several challenges of sustainably producing high quality of food. When food commodities are produced, they need to be kept for some times at their highest quality possible. Regarding fruits and beverages in particular, there are different methods of preservation that are used to keep the quality and shelf life as good as possible. Agricultural produce such as fruit need to be preserved against fungal contamination at all stages of processing from harvest until it reaches the consumers. As far as beverages are concerned, the spoilage organisms that usually occur are yeasts and bacteria that usually cause undesired fermentation during processing, therefore leading to spoilage. *Botrytis cinerea, Colletotrichum acutatum and Rhizopus stolonifer* are some of the fungal species causing fruit spoilage with *Botrytis cinerea* being one of the most dangerous fungal spoilage organisms in more than 200 crops. In beverages, three spoilage yeasts, *Dekkera bruxellensis, Dekkera anomala* and *Zygosaccharomyces bailii*, are spoilage organisms used in this research work. The physiological requirements of microorganisms in general and all spoilage organisms selected for this work in particular make fruit such as apples and table grapes as well as their derived beverages a suitable source of nutrients for microbial growth and proliferation. This then means, for high quality and security of the agricultural produce, undesired microorganisms should be kept away from the fruit and beverages during harvest, processing, packaging and transportation until they reach consumers in good condition. It is worth to recall that all fruit and beverages have different spoilage mechanisms. Beverages are spoiled when undesired fermentation occur while fruit spoilage occur when there is fungal development occurring on fruit through the wound surface. Fruit and beverage industries currently suffer great product and capital losses due to microbial spoilage. To minimise those losses, there have been extensive used of synthetic chemical with antimicrobial properties against spoilage organisms. Some spoilage organisms are found to be resistant to the chemical preservative which favours the use of higher dose in an attempt to inhibit the resistant spoilage organisms. Not only the fact that a high dose could be used, different synthetic chemicals as preservatives in the same food environment could also be used because the same food environment could be contaminated with more than one spoilage organism at the same time. Consumers are prone to ingest a significant amount of those chemical preservatives when consuming different types of fruit and beverages on a daily basis. A prolong exposure to these chemicals poses serious health thread such as heart, muscles, skin and eye problems. These health problems attributed to the use of synthetic chemical preservatives in fruit and beverages has led to stringent regulations for producers and exporter to eradicate the use of

synthetic chemicals as beverage preservatives and as postharvest fungicides on fruits. This call has led to a search for alternative approach to the use of synthetic chemicals as preservative and fungicides.

In an attempt to eradicate the use of synthetic chemicals in fruit and beverages as preservatives or postharvest control agents, some bioprospecting approaches have been explored that involve the use of plan extracts, essential oils, bacteria and yeasts metabolites with growth inhibition properties against spoilage organisms. Different mode growth inhibition has been identified when using yeasts as either biological control agents or producers of growth inhibiting compounds. In fruits postharvest control, the higher growth rate of yeasts compared to that of the spoilage fungi is one of the antagonistic mechanism used. The production of killer toxins and enzymes by some yeasts are also part of the antagonistic mechanisms used to combat spoilage caused by yeasts and fungi. The effect of volatile organic compounds (VOCs) from yeasts on the inhibition of spoilage fungi is also another important mode of action to inhibit the growth of spoilage organisms. It is based on the aforementioned modes of action that it is preferable to use yeast cell cultures as biocontrol agents against fungal spoilage organisms and yeast extracellular metabolites for the control of yeasts spoilage in beverages. Prior to this work, the abovementioned findings had not yet addressed the critical needs of alternatives to the use of synthetic chemicals as preservatives. In the literature consulted, many gaps were found with regards to the bioprospecting approach consisting of using yeasts and their extracellular metabolites as alternatives to the use of synthetic chemicals for fruit and beverage preservation, i.e. use of cost ineffective media for screening and production, lack of engineering aspects applicable to production of biopreservatives at industrial scale. In the current literature, it was found that the research conducted on yeasts as potential biological control agents was carried out using refined media formulated from yeast and potato extracts, Peptone, Dextrose which is not a cost effective practice. From the gaps found in the literature, several questions were raised and answered by the current study.

1) What suitable, high yield and cost effective substrates could be used for biopreservatives production? This study successfully answered this concern as it was found that agro-waste such as grape pomace extracts, a cheaply available nutrient source presented a rich source of macro and micro-nutrients that could support the growth and proliferation of biological control yeasts while promoting the production of VOCs that could be used for beverage biopreservation.

2) Could the cheaply available source of substrates be used and optimised for biopreservation compound production under optimum bioreactor conditions? Indeed, this question was also successfully answered as a higher yield optimum production condition was found when grape pomace extracts was used as production medium.

3) The third question regarding a suitable bioreactor system for achieving optimal yield was also answered whereby all the fermentation conditions for higher biopreservation compound production was established. Furthermore, a suitable method for biopreservation compounds activity quantification to assess and validate the production system and the efficacy of the biopreservation compounds produced was also successfully elaborated. The lack of engineering data found in the literature such as the availability of biological stoichiometric and bioenergetics data for a system design for the production of biopreservatives from yeasts has been generated and can now be used to describe, explain and simulate a desired bioprocess engineering design to produce the needed biopreservatives and biocontrol agents at an industrial scale.

9.2 Recommendations for future work

A system design for production of biopreservatives from yeasts for reduction of fruit and beverage spoilage organisms have been achieved with some aspects that still required investigations. It is recommended that the volatile organic compounds identified be tested as single agent and as a mixture using a multifactorial design in order to have a clear cut of which compound or combination of compounds are responsible for the growth inhibition observed. Although the VOCs identified in this work are usually found in some commercialised beverages, it is recommended that the toxicology of the biopreservatives be studied. The formulation of biological control agents from the three yeasts in a powder form should be carried out and tested on other agricultural commodities in a field trial and also on human pathogens prior to exploring the commercialisation aspects.

CHAPTER 10

REFERENCES

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