Non-*Saccharomyces* Yeast and Acetic Acid Bacteria in Balsamic-Styled Vinegar Production: A Biochemical Process Analysis

By

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DECLARATION

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

20 October 2016

Signed **Date**

ABSTRACT

Grape producers and wine makers in South Africa are currently affected by various challenges, which include anti-alcohol lobbies, climate change, over-production in some vintages and the lack of transformation including empowerment in certain sectors of the industry. Climate change and global warming lead to poor quality wine grapes and as a result, poor quality wine. Therefore, there is a need to channel grapes away from normal wine production and provide an alternative source of income for the industry.

The overall aim of this study was therefore to provide an alternative outlet for overproduced wine grapes by producing balsamic-styled vinegar (BSV) in South Africa. Balsamic vinegar is different from other vinegars because it is a direct product of grape must and not a downstream or by-product of wine production. Balsamic vinegar entails lower production costs when compared to the production of wine due to the low technological process requirements during production; therefore, this could be an opportunity for small business entrepreneurs with low capital start-up. In addition, balsamic vinegar can command a high price, which is a benefit for grape producers.

The primary aim of this investigation was to biochemically analyse a BSV production process in which 5 non-*Saccharomyces* yeast and 15 acetic acid bacteria (AAB) were used for a multicultural alcoholic-acetous (EtOH-AcOH) fermentation process. To achieve this aim, a fermentation process was designed where the data generated was fitted into kinetic models and the proliferation including the population dynamics of the microbial consortia were studied.

EtOH-AcOH fermentation trials using cooked/high-strength and autoclaved/low-strength grape must were inoculated with a defined microbial consortia of non-*Saccharomyces* yeast and AAB using different inoculation strategies (the 0% and 6% inoculation strategy). The 0% inoculation strategy involved the simultaneous inoculation of yeast and AAB at the start of the fermentation when the ethanol concentration was 0% (v/v), while the 6% inoculation involved inoculating yeast only at the start of the fermentation, thus allowing alcoholic fermentation to proceed until 6% (v/v) ethanol concentration was reached subsequent to the inoculation of AAB. The trials were carried out at 22ºC, 28ºC and using a fluctuating temperature (22ºC/28ºC) to evaluate which temperature strategy was suitable. During the EtOH-AcOH fermentation process, the trials were monitored using analytical chemistry, classic microbiology and molecular biology methods. These include substrate consumption, product formation; microbial growth kinetics and polymerase chain reaction to evaluate population dynamics. The final product deemed viable for public consumption was evaluated

for population dynamics using metagenomics to profile the microbial constituency for the viable but non-culturable microorganisms. The experimental data was analysed using fermentation technology and bioprocess engineering concepts. With the use of polymath software, the fermentation data was analysed and fitted into models. The models used are rate equations for consecutive first-order elementary reactions.

The cooked must trials were not successful for both inoculation strategies and temperatures studied. Only alcoholic fermentation was successful while acetous fermentation was not successful. This lack of success was attributed to the high sugar concentration including other chemical parameters of the cooked must and it was hypothesised that this might have led to the AAB entering the viable but nonculturable state. As a result, the EtOH-AcOH fermentation process with the cooked must was deemed unsuitable for kinetic modelling.

The autoclaved must trials showed that the inoculation strategies and temperatures studied have a direct influence on the process. The microbial growth results showed higher biomass concentration at 22°C for both inoculation strategies while yeast showed higher biomass concentration compared to bacteria for all temperatures studied and for both inoculation strategies used. However, the biomass concentration was not considered to represent the entire viable population due to the assumption that some of the microorganisms might have entered the viable but nonculturable state. The 0% inoculation strategy resulted in a faster EtOH-AcOH fermentation period of 42 days while the 6% inoculation strategy took 56 days. Under normal environmental conditions, i.e., under a fluctuating temperature regime, a better performance was observed compared to the fermentation trials at 22°C. The rate of sugar consumption (r_s) was between 2.5 to 3.4 and 4.2 to 5.1 g/L.day for the 0% and 6% inoculation strategies respectively, with the rate of product formation (r_p) being determined to be between 1.2 to 1.5 and 0.9 to 1.2 g/L.day for the 0% and 6% inoculation strategies, respectively.

Furthermore, sensory data showed positive results for the products obtained at 28°C, while the vinegar from the 22°C fermentation temperature was evaluated as being of sub-standard quality; overall, the fluctuating temperature product got the lowest sensory scores. Due to the use of cultivation methods in this study, cultivation dependant molecular biology methods were unsuccessful. Therefore, the 28°C product using the 0% inoculation strategy was analysed for population dynamics using metagenomics. The yeasts results (18S analyses) showed that minimal yeasts were present at the end of the fermentation. The results for the bacterial population (16S analyses) showed that 50.84% *Acetobacter* species were culturable, while 40.18% of unknown bacteria were determined to be non-culturable.

To model the process, consecutive elementary reactions were successfully applied and validated using correlation coefficients (R^2). R^2 values for the sugar consumption model ranged from 0.84 to 0.92 with correlation coefficients of the total acid formation modelled ranging from 0.98 to 0.99.

Overall, at a higher temperature (28ºC), a rapid EtOH-AcOH fermentation of 38 days for the 0% inoculation strategy was achieved, although a slightly elongated fermentation was observed (49 days) for the 6% inoculation strategy. After analysis of all fermentation data, it was therefore concluded that the 0% inoculation strategy is the most suitable strategy and a higher temperature, i.e. 28°C, for both inoculation strategies is the most suitable temperature. The inoculation strategy and temperature were selected based on the length of fermentation and sensory data.

Keywords: Non-*Saccharomyces* yeasts, acetic acid bacteria, microbial consortia, kinetic modelling, proliferation.

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U.F Hutchinson

October. 2016

DEDICATION

This thesis is dedicated to my parents who have taught me that I can achieve anything I set my mind to.

“Do not go gentle into that good night, … Rage, rage against the dying of the light.”

Dylan Thomas

“Science never solves a problem without creating ten more.”

George Bernard Shaw

“In the spirit of science, there really is no such thing as a failed experiment. Any test that yields valid data is a valid test.”

Adam Savage

“You must be the change you wish to see in the world.”

Mahatma Gandhi

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GLOSSARY

List of abbreviations / Nomenclature

Abbreviations	Definition
μ_g	Specific growth yield (day^{-1})
μ_{max}	Maximum growth rate (day^{-1})
AAB	Acetic acid bacteria
ARC	Agricultural Research Council
BSV	Balsamic-Styled Vinegar
cfu	Colony forming units
dP/dt	Product formation rate (g/L.day)
GM	Glucose-Mannitol
K_s	Substrate utilisation constant (day^{-1})
m	Cell maintenance parameter
M_s	Maintenance coefficient (day^{-1})
n	Luedeking-Piret constant
P	Product formation (g/L)
PCR	Polymerase chain reaction
P_m	Maximum productivity (g/L)
P_o	Initial productivity (g/L)
r_p	Product formation rate (g/L.day)
r_s	Substrate utilisation rate (g/L.day)
r_x	Biomass formation rate (g/L.day)
S	Limiting substrate concentration (g/L)
S_{min}	Substrate concentration (g/L)
TBV	Traditional Balsamic Vinegar
X	Microbial biomass concentration (g/L)
X_m	Maximum microbial biomass concentration (g/L)
X_o	Microbial biomass concentration at initial time (g/L)
$Y_{p/s}$	Product yield based on substrate utilisation (g Product/g Substrate)
YPD	Yeast Peptone Dextrose
$Y_{x/s}$	Biomass yield based on substrate utilisation (g Biomass/g Substrate)
α	Growth associated product formation constant (day^{-1})
β	Non-growth associated product formation constant (day^{-1})

CLARIFICATION OF BASIC TERMS AND CONCEPTS

Basic Terms and concepts

- **0% inoculation strategy:** The inoculation of yeast and AAB simultaneously at the start of the fermentation when the ethanol concentration was 0% (v/v),
- **6% inoculation strategy:** The inoculation of yeast only at the start of the fermentation and allowing alcoholic fermentation to proceed until an ethanol concentration of 6% (v/v) or 60 g/L was reached, then inoculating AAB at that point,
- **EtOH-AcOH Fermentation:** Generally, means alcoholic and acetous fermentation, an abbreviation to describe the type of fermentation that occurs during balsamic vinegar fermentation,
- **Fluctuating (FL) temperature trials:** Fermentation trials which were carried out by physically moving fermentation vessels to different incubation rooms with varying temperatures, i.e. 22ºC and 28ºC,
- **Cooked must (High-strength grape must):** Grape must which was cooked in a pressure cooker, then aliquoted in to fermentation vessels,
- **Autoclaved grape must (Low strength grape must):** Grape must which was aliquoted in fermentation vessels and autoclaved prior to inoculation (no cooking procedure involved).

Chapter 1

General Introduction

Chapter 1
General introduction

1.1 Introduction

Grape producers in South Africa are adversely affected by a number of factors including global market trends (Table 1-1) (SAWIS, 2016), over-production and anti-alcohol groups (Anonymous, 2011a). These factors could lead to decreased wine consumption and, in some instances, fewer exports of local wines (SAWIS, 2016). In addition, the South African wine industry is perceived to be lacking in transformation and empowering certain sectors of society (De Waal, 2012; Smith, 2013). Another adverse factor is global warming which could also lead to some viticultural areas being unable to produce premium quality grapes for wines, which are acceptable to consumers (Jones *et al*., 2005; Vink *et al.*, 2012; Mozell & Thach, 2014). With the aforementioned in mind, it is critical that an alternative use for wine grapes be found and that entrepreneurial opportunities be identified. The production of balsamic-styled vinegar (BSV) could serve both purposes. Grapes could be channelled to an alternative high-priced product, with low technological inputs requirements, making it easier to incorporate a low capital start-up business.

Table 1-1: Brief South African wine industry statistics (SAWIS, 2016)

Statistics	
SA's total annual wine grapes harvest	• 81.2% for wine making • 4.5% for brandy • 3% for grape juice concentrate/grape juice
Total SA's exports in mega litres (ML)	• 2013 =517.4 ML • 2014 = 422.7 ML • 2015 = 420.1 ML
Total wine exports decreased by 97.3 ML from 2013 to 2015	

Traditional Balsamic Vinegar (TBV) (*Aceto Balsamico Tradizionale*) is an internationally-regulated name (Italian *Denominazione di Origine Protetta* and the European Union's Protected Designation of Origin) and can only be produced in Reggio Emilia and Modena provinces in Italy (Mattia, 2004; Solieri *et al*., 2006; Wheeler, 2014). It differs from normal wine vinegar in that it is the primary product from grape juice and not a by-product or downstream product of wine production (Oulton & Randal, 2002; Wheeler, 2014). TBV is

made from cooked Trebbiano and/or Lambrusco (*Vitis vinifera*) grape juice by a natural EtOH-AcOH fermentation process in a "*Batteria"* or "*Solera"* styled system of barrels (Solieri *et al*., 2006). The ageing process can take up to 12 years (Solieri *et al.,* 2006; Giudici & Rinaldi, 2007; Solieri & Giudici, 2008; Chinnici *et al*., 2016). Balsamic Vinegar of Modena (*Aceto Balsamico di Modena*) is a more modern product made on an industrial scale that ranges in quality including price and is produced in a shorter period of time (Wheeler, 2014).

TBVs and some BSVs are high-quality products that can command premium prices, i.e. 100 to more than 200 Euros per 100 mL (Oulton & Randal. 2002; Meathead, 2011; Wheeler, 2014). The TBV production process utilises yeasts (specifically non-*Saccharomyces* yeast) in conjunction with AAB for fermentation and the final product is not sweetened with grape must (Oulton & Randal. 2002; Wheeler, 2014). The initial yeast fermentation is critical to the formation of flavour compounds (Mateo *et al*., 1991; Soden *et al*., 2000) and volatile acids other than acetic acid, before the AAB can complete the production process. The nature of vinegar production is such that a wine cellar cannot be used to make vinegar due to the risk of contamination and a separate facility would be required. However, basic equipment (e.g. plastic food grade buckets) could be used for production without the use of cooling. This makes it an ideal venture for a small business with minimal capital outlay.

Before the start of this work, a preliminary study was done, which is described along the following lines: the ARC Infruitec-Nietvoorbij has an extensive non-*Saccharomyces* culture collection that was previously screened for yeasts that produce lower alcohol levels, desirable flavour compounds and have osmophilic traits. The isolation of South African AAB strains and the purchase of reference strains from international culture collections was also completed. In addition, a laboratory-scale protocol for vinegar production with simultaneous and sequential inoculation strategies with various yeast and bacteria combinations was formulated. During this time, the EtOH-AcOH fermentation process was only monitored chemically.

In this investigation, a microbial consortium of non-*Saccharomyces* yeast (n=5) was used in conjunction with AAB (n=15) isolates. The aim was to formulate a "process" and design a fundamental scientific investigation based on bioprocessing engineering concepts. Therefore, several questions needed to be answered, which led to this research.

1.2 Research questions

- Which microorganisms are capable of achieving a complete EtOH-AcOH fermentation for BSV?
- Under what conditions are the microbial consortia effective and at which temperature?
- Which kinetic models will be most suitable to describe the EtOH-AcOH fermentation process of the BSV?
- Which inoculation strategy is suitable for the production of a South African BSV?
- Which EtOH-AcOH fermentation conditions result in a better product in terms of EtOH-AcOH fermentation duration, microbial proliferation and sensory attributes?

1.3 General objectives

The primary objective of this project was to biochemically analyse a BSV production process in which non-*Saccharomyces* yeast and AAB were used for a multicultural EtOH-AcOH fermentation process. The primary objective of the study was achieved with the following aims and objectives:

Phase 1: Aim 1: To prepare the fermentation medium, design an inoculum strategy and to study microbial population proliferation and dynamics using standard microbiology and molecular biology methods.

Objective 1: To develop a method to prepare the fermentation medium for BSV production, using low and high-strength grape must, i.e. autoclaved and cooked must.

Objective 2: To assess the rate of microbial growth for each microorganism in the consortia using spectrophotometric techniques to quantify a suitable inoculum size in order to ensure consistency in the inoculum size for the fermentation.

Objective 3: To monitor population proliferation by the classical plate count method, as to assess microbial performance at various stages during the EtOH-AcOH fermentation process.

Objective 4: To determine proliferation or dominance of individual microbial species at various stages of EtOH-AcOH fermentation process using molecular biology

methods. This is to determine which species carry out the EtOH-AcOH fermentation process.

Phase 2: Aim 2: To apply analytical chemistry methods to measure substrate utilisation including product formation and develop kinetic models for the EtOH-AcOH fermentation process.

Objective 1: To monitor the changes in sugar, alcohol, total acid concentrations and pH during the EtOH-AcOH fermentation period.

Objective 2: To use the data obtained from the analytical chemistry methods to quantify model parameters of product formation, substrate utilisation and yields during the balsamic vinegar EtOH-AcOH fermentation process by the microbial consortia used.

Objective 3: To study and select suitable EtOH-AcOH fermentation conditions for BSV using the overall data set, and determined which reactions describe the process adequately.

Objective 4: To perform sensory analysis (taste and aroma) on the final product obtained. This is for consumer acceptance, in order to assess the viability of the process.

Phase 3: Aim 3: To profile individual microbial species at the end of the EtOH-AcOH fermentation process using metagenomics. To ascertain which species carried out the EtOH-AcOH fermentation only on the selected product.

Objective 1: To metagenomically profile the yeasts and bacterial populations in the selected final product using 18S rDNA for yeasts and 16S rDNA amplicon gene sequencing for bacteria using appropriate primers in order to determine microbiology constituents that facilitated the EtOH-AcOH fermentation.

Objective 2: To classify the microbial distribution as traditional microbiology cannot be used to quantify viable but nonculturable microorganisms.

1.4 Significance of the study

Preliminary trials identified a consortium of non-*Saccharomyces* yeast (n=5) and AAB (n=15) that showed the potential for during balsamic-styled vinegar production. However, the preliminary trials were carried out at one temperature only. In addition, only chemical developments were monitored and the non-*Saccharomyces* yeast and AAB were not investigated as a consortium of 20 organisms but rather an individual non-*Saccharomyces* yeast species in conjunction with only two AAB species or vice versa. Therefore, the significance of this study was to develop a BSV production process for the South African market. This was achieved by investigating a range of temperatures, inoculation strategies and by developing models to describe the process. The aforementioned is critical in avoiding economic losses during the production of BSV, in the case of the EtOH-AcOH fermentation process being well studied and established. Various viticulture industries, albeit even on a small scale, can use excess grapes for the production of BSV and thus mitigate some of the problems encountered by the South African agricultural and wine industry.

1.5 Delineation of the study

This study did not cover or investigate the following aspects:

- The study did not investigate the effect of the inoculated microorganisms on each other, such as the symbiotic relationship between the microorganisms. For example, antimicrobial activity and quorum sensing.
- The study did not investigate yeast protein and other proteins during the fermentation.

OUTLINE OF THE THESIS

The research work presented in this thesis was conducted in the microbiology laboratory, of the Post-Harvest and Wine Technology division, at the Agricultural Research Council; ARC Infruitec-Nietvoorbij (Fruit, Wine and Vine Institute), Western Cape, South Africa. The thesis is presented as a compilation of 7 (Seven) chapters. Each chapter is introduced separately.

Chapter 1: Introduction: General introduction and background to the research project, objectives and the significance of the research.

Chapter 2: Literature review.

Chapter 3: Preparation of fermentation medium, inoculum design, generic microbiology and molecular biology methods with the inclusion of results and discussions.

Chapter 4: Analytical chemistry methods and kinetic modelling for the EtOH-AcOH fermentation process with the inclusion of results and discussions.

Chapter 5: Microbial characterisation on the selected product using a metagenomic approach.

Chapter 6: General discussion, conclusion and recommendations for future research.

Chapter 7: Lists the literature consulted for the study, which was also used to link observations made in the study to other research work.

Chapter 8: Appendices: lists additional information deemed unsuitable for the body of the thesis.

Chapter 2

Literature Review

Chapter 2
Literature review

2.1 A brief overview: what is vinegar?

Vinegar is generally defined as a sour or acidic liquid obtained from a fermentation process (Moncel, 2016a). Additionally, the word vinegar comes from the French word "*vinaigre"* meaning sour wine (Mazza & Murooka, 2009). The presence of acetic acid is basically what defines vinegar (Moncel, 2016a). It is not classified as food but rather a food-flavouring agent (Solieri & Giudici, 2009). Vinegar can be made from just about any carbohydrate source or food product that contains sugar (Solieri & Giudici, 2009; Alleman, 2016). Currently, there are numerous types of vinegars produced in the world and most of these vinegars are made from cheap raw materials. These two factors are the reason why most vinegars are inexpensive. These raw materials can include by-products obtained from food processing, low quality fruit, agricultural surpluses and fruit waste (Solieri & Giudici, 2009; Tesfaye *et al*., 2010). Some other raw materials include honey, cereals, beer, wine, grapes, pears, apples and hydrolysed starches (Mazza & Murooka, 2009; Solieri & Giudici, 2009; Alleman, 2016).

The more expensive vinegars are those made in certain regions, with regional input raw materials and well-defined methods. Examples of these vinegars are oxos vinegar from Greece, sherry vinegar from Spain and the TBV from Reggio Emilia including Modena, Italy (Solieri & Giudici, 2009). Most vinegars are made by means of a two-step fermentation process involving yeast and AAB. However, some vinegars are obtained from non-fermentation processes, such as distillation. Spirit vinegars are obtained from distilled alcohol, which is subsequently oxidised to acetic acid (Solieri & Giudici, 2009).

2.1.1 Balsamic vinegars: the focus of the study

The focus of this study was to develop and analyse a biochemical process, which can be used to produce balsamic-styled vinegar (BSV). Although other vinegars can be produced using excess grape must, a high-value product was determined to be desirable to maximise profits while minimising input costs.

2.1.2 Traditional Balsamic Vinegar

Traditional Balsamic Vinegar (TBV) from Modena and Reggio Emilia provinces in the Emila-Romagna region, Italy (*Aceto balsamico tradizionale di Modena*, ABTM and *Aceto Balsamico Tradizionale di Reggio Emilia* ABTR) is a distinctive product obtained by a traditional production method from grape must (Plessi *et al*., 2006). TBV is the most valuable of all other types of balsamic vinegars and it is only produced in Reggio Emilia and Modena (Fig. 2-1), Italy (Mattia, 2004; Solieri *et al*., 2006). The production of this vinegar is monitored from the start up to completion by a special certification agency, i.e. the Consortium of Balsamic Vinegar of Modena (Wheeler, 2014). TBV is protected by the Italian *Denominazione di Origine Protetta* (DOP) and the European Union's Protected Designation of Origin.

Figure 2-1: Map showing the nine major provinces of Emilia–Romagna Wine region (Italy), Reggio Emilia and Modena are shown circled (adapted from Wineweb.com)

The production of TBV is not a difficult process. However; it requires at least 12 years of ageing (Fig. 2-2) in barrels (Solieri *et al.*, 2006; Giudici, & Rinaldi, 2007; Solieri & Giudici, 2008; Chinnici *et al*., 2016). The minimum time period is one of the reasons the product commands premium prices. The process generally begins with cooking the grape must for several hours until a high sugar concentration is reached and the must is sterile. Cultivars used for TBV are varieties of Lambrusco or Trebbiano. The sterile must is then immediately inoculated with an undefined mother culture leading to an EtOH-AcOH fermentation (De Vero *et al*., 2006).

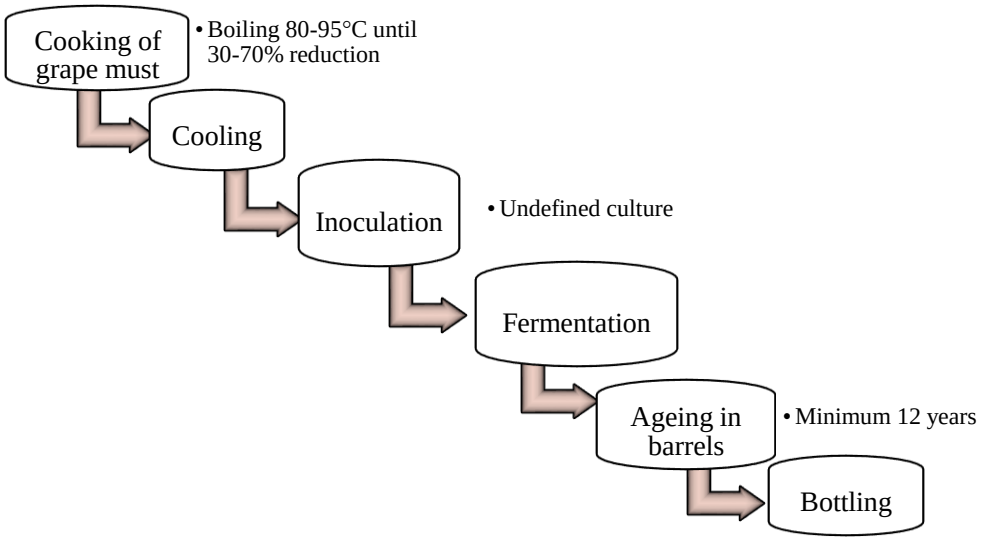


Figure 2-2: An illustration of the production process for Traditional Balsamic Vinegar (TBV).

The production of TBV is a three-stage process. Initially, sugars are converted to ethanol by spontaneous fermentation, followed by ethanol oxidation to acetic acid by AAB (Fig. 2-3) and the last stage is the ageing process for a minimum of 12 years (Solieri & Giudici, 2008), usually in an attic rather than a cellar (Meathead, 2011). The ageing process occurs in a "*Batteria*" (Fig. 2-4), which is similar to the "*Solera*" system of barrels, usually five or more barrels aligned next to each other in a row. The barrels are of different sizes (Fig. 2-4) and made of different woods, such as cherry, oak, mulberry, chestnut and juniper to impart complex flavours to the vinegar (Wheeler, 2014).

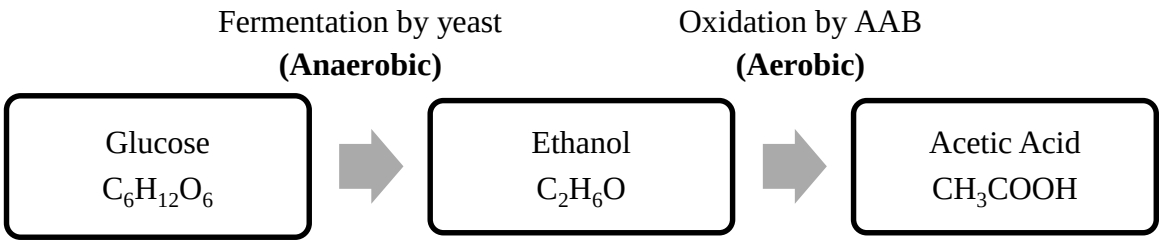


Figure 2-3: A simplified illustration of the EtOH-AcOH fermentation process for balsamic vinegar.

During the ageing process, around 10% of the vinegar evaporates because the opening of the barrels are not closed with stoppers but covered with gauze (Meathead, 2011) or a cloth

(Oulton & Randal, 2002) which is porous enough to allow evaporation (Anonymous, 2011b). Therefore, the vinegar becomes concentrated. The evaporated portion of the vinegar is usually referred to as the "angels' share" (Anonymous, 2011b; Meathead, 2011). The sequence followed is that, annually, roughly 25% of the vinegar is collected and bottled from the smallest barrel (Fig. 2-4) (Meathead, 2011; Wheeler, 2014). Then each barrel is filled up with younger vinegar from the barrel behind it and the largest barrel that is first in the sequence is filled up with fresh cooked grape must. Therefore, the barrels are never empty (Wheeler, 2014).

The collecting and filling process makes it impossible to actually state or determine the exact age of the vinegar, which is why, by law, TBV producers do not include the age of the vinegar on the final product label (Anonymous, 2011b; Meathead, 2011; Wheeler, 2014). It has not been reported that the fundamental process for making TBV has changed significantly over the centuries. Production of BSVs all generally follow the same basic process, with some only diverging in the use of the grape cultivar, seasoning method, the type of wooden barrel and ageing process which is less than 12 years (Alessi, 1996; Wheeler, 2014).

Figure 2-4: *Batteria* system for TBV (Meathead, 2011). (1 gallon = 3.79 litres)

2.1.3 *Condimento Balsamico*

Some balsamic vinegars are called *condimento Balsamico* or condiment grade balsamic vinegars. These vinegars are also produced using the traditional methods used for TBV (Wheeler, 2014; Bertolli, 2016). Therefore, the process is similar with the use of cooked grape must and the *Batteria* (Meathead, 2011). However, the condiment grade balsamic vinegar cannot be called TBV

due to reasons such as not meeting the standard of maturity and not being produced or monitored by the special certification agency (Wheeler, 2014; Bertolli, 2016). The condiment grade balsamic vinegars are also of high quality and can be made outside the demarcated TBV region (Bertolli, 2016). Some TBV producers also produce condiment grade balsamic vinegar if the vinegar turned out not to meet the appropriate standard for TBV or due to ageing the vinegar for fewer years, ranging between three to seven years (Meathead, 2011; Wheeler, 2014; May, 2016).

Additionally, some families make high-quality condiment grade balsamic vinegar but these are not sold in the market (Solazi, 2014). The title 'condiment' is not under a protected designation; therefore, it is often misused and in the near future, the term 'condiment' may be less treasured (Meathead, 2011). Furthermore, some vinegars which are not actually condiment grade balsamic vinegars are found on supermarket shelves with the label 'condiment' (Wheeler, 2014). This makes it difficult to know if they are the genuine condiment grade balsamic vinegar (Meathead, 2011; Bertolli, 2016). However, it is important to emphasise that most of the condiment grade balsamic vinegars are made by TBV producers; that is to say, most of them can be legitimately classified as condiment grade balsamic vinegar. These vinegars are cheaper than TBV but they can still be produced profitably due to their high quality (Wheeler, 2014; May, 2016; Moncel, 2016b). Some three-year-old condiment grade balsamic vinegars can be sold with prices that range from 45 to 90 Euros per 500 mL bottle (Meathead, 2011).

2.1.4 Balsamic vinegar of Modena IGP

Balsamic vinegar of Modena IGP is a type of balsamic vinegar that was produced due to the high demand of TBV by the United States of America (Wheeler, 2014). TBV is exported from Modena to the US resulting in its popularity that demand outstripped supply. As a result, this led to the production of derivative products such as the Balsamic vinegar of Modena IGP. The production of this vinegar is what also led to the implementation of a protected designation (DOP) for TBV (Wheeler, 2014). The IGP designation was also introduced in 2009 and was implemented by the European Union. IGP is an Italian abbreviation meaning "*Indicazione Geografica Protetta*" or PGI meaning "Protected Geographical Information" (Maribel, 2011; Solazi, 2014).

This designation ensures that the vinegar is produced from permitted grape varieties such as Ancellotta, Albana, Lambrusco, Fortana, Montuni, Sangiovese and Trebbiano. It also ensures

that the vinegar is only produced in Modena although the grapes can come from other regions (Buccit, 2012; Wheeler, 2014). In other words, the IGP designation is less strict than the DOP designation (Maribel, 2011; Solazi, 2014). In this way, Modena has been able to meet the high demand. Balsamic vinegar of Modena IGP also involves the cooking of grape must which is allowed to age in large wooden barrels for a minimum of two months. Subsequently, the addition of wine vinegar brings the product to 6% acidity. There is no fermentation stage for this vinegar and up to 50% of wine vinegar can be added (Wheeler, 2014). The final step is the addition of colourants, caramel and some thickening agents to make it resemble TBV. Depending on the quality of the product, this vinegar can sell between 4 to 44 Euros (Wheeler, 2014; May, 2016).

2.1.5 Imitation balsamic vinegar

Imitation balsamic vinegars are of the lowest standard of all balsamic vinegars. While all the previous balsamic vinegars use cooked must, this is not the case with imitation balsamic vinegar (Wheeler, 2014). Some producers refer to these vinegars as industrially-produced balsamic vinegars. They employ the use of cheap ingredients, time-saving technology and the term "balsamic" is primarily used to confuse consumers and for profit making (Buccit, 2012). Fortunately, the integrity of real balsamic vinegar has been protected by the previously-mentioned designations and guidelines. Imitation balsamic vinegars can just be normal spirit vinegar with added colouring and thickening agents. Imitation balsamic vinegars can be made with white vinegar, wine vinegar or cider vinegar.

Despite the fact that balsamic vinegar is known to have a brown or dark colour, it may be surprising to know that there are white balsamic vinegars under this category (Oulton & Randal. 2002). These vinegars are found on the food market shelves with the label balsamic vinegar and some are labelled 'Made in Italy', but do not have an IGP approval stamp. Imitation balsamic vinegars can be made anywhere throughout the world (Wheeler, 2014), e.g. the United States of America, United Kingdom and Canada (Oulton & Randal. 2002). Under the category of imitation balsamic vinegars, consumers can be misled, as there is a large variety of such vinegars (Buccit, 2012, Solazi, 2014). They are found in bottles of different material, size, style, differentiated price range and claims of vintages (Bertolli, 2016).

The quality, ingredients and the production process of imitation balsamic vinegars can vary widely (Buccit, 2012). However, some of these imitation balsamic vinegars do use cooked grape must, so their quality is higher and almost similar to the balsamic vinegar of Modena IGP (Wheeler, 2014). By international law, it is legal to use the term "balsamic" for all the aforementioned vinegars, as long as the term traditional, IGP or DOP is not included (Oulton & Randal, 2002). Overall, consumers who look for the original balsamic vinegar can always look for the key terms "traditional", "IGP" and "DOP" on the label of the vinegars sold in local supermarkets.

2.2 Non-*Saccharomyces* yeast

Non-*Saccharomyces* yeast is a generic name given by microbiologists to all yeasts apart from *Saccharomyces* yeast (*Saccharomyces cerevisiae* strains). The term non-*Saccharomyces* refers to non-spoilage yeasts, yeasts with positive benefits in wine making. These yeasts are naturally present in wine production, on the skin of grapes and cellar equipment. Non-*Saccharomyces* yeasts were previously considered to be spoilage yeasts and were often denoted as wild yeast. However, some of the non-*Saccharomyces* yeast species were later discovered to have beneficial effects on the final quality of wine (Romano *et al*., 1997; Lambrechts & Pretorius, 2000; Jolly *et al*., 2006; Jolly *et al*., 2014).

Research on non-*Saccharomyces* yeast has gained momentum. These yeasts mostly die during fermentation in wine production. However, strains of *Candida stellata* were identified to be capable of accomplishing a complete fermentation. Due to the fermentation abilities of non-*Saccharomyces* yeasts, recent studies have shown interest in the use of non-*Saccharomyces* yeast to lower alcohol levels in wine (Quirós *et al.,* 2014). Non-*Saccharomyces* yeasts can normally exist in varying intensities, in both inoculated and spontaneous fermentations. These yeasts are known to have an effect on the final taste and aroma of the final product (Muratore *et al*., 2007), due to secondary metabolites that are produced (Mateo *et al*., 1991; Soden *et al*., 2000). Ciani and Maccarelli (1997) demonstrated that, by exposing grapes to air for a long period of time, can result in high numbers of *Candida, Hanseniaspora* and *Metschnikowia* species.

Studies show that some non-*Saccharomyces* yeasts have a low fermentation activity. These studies also show that off-flavour compounds can be formed in excessive quantities by these yeasts

(Di Maio *et al*., 2011). Apiculate yeasts have been mostly found to facilitate the production of unwanted by-products such as ethyl acetate. However, *Torulaspora delbrueckii* and *Candida stellata* have the ability to positively affect the taste and flavour of the end-product (Lambrechts & Pretorius, 2000).

In the case of TBV, several studies have studied the yeast population during TBV production. Solieri and Giudici (2008) report that a variety of non-*Saccharomyces* yeasts are present during TBV fermentation and these yeasts include *Zygosaccharomyces bailii*, *Zygosaccharomyces mellis*, *Zygosaccharomyces rouxii*, *Zygosaccharomyces bisporus*, *Zygosaccharomyces pseudorouxii*, *Zygosaccharomyces lentus*, *Hanseniaspora osmophila*, *Hanseniaspora valbyensis*, *Candida stellata* and *Candida lactis-condensi*. Therefore, it appears that species belonging to the *Zygosaccharomyces* genus dominate. However, Solieri and Giudici (2008) suggest further that the yeast population is broader than what was reported because of the cultivation methods used and therefore the use of culture-independent and metagenomic techniques was recommended. A study by Solieri *et al.* (2006) also shows that *Zygosaccharomyces* yeast dominates during TBV fermentation. However, this study also involves the use of isolation /culture dependant procedures.

2.3 Acetic acid bacteria and their product, acetic acid

Acetic acid bacteria (AAB) are ubiquitous aerobic bacteria (Raspor & Goranovič, 2008) which belong to the *Acetobacteraceae* family (Guillamón & Mas, 2009). These bacteria are mostly found on healthy fruit, rotten fruit or from the nectar of flowers (Bartowsky & Henschke, 2008; Guillamón & Mas, 2009; Sengun & Karabiyikli, 2011). AAB grow well in environments where ethanol is being produced because of the fermentation of sugar to alcohol by yeast (Raspor & Goranovič, 2008; Guillamón & Mas, 2009) which the AAB utilise as the primary carbon source. AAB are considered the major wine spoilage microorganisms in wine production (Bartowsky & Henschke, 2008). However, they are favourable in vinegar and are the primary role players in vinegar production where the final product produced is acetic acid. AAB use ethanol to form acetic acid which is the primary compound in vinegar. The optimum pH for AAB growth has been reported to be between 5.5 to 6.3 (De Ley *et al*., 1984). Subsequently, a study done on palm juice vinegar found that the maximum acetic acid can be produced at pH 5.5 using short EtOH-AcOH fermentation cycles (Ghosh *et al*., 2012). However, since AAB are acid producers, they are

correspondingly acid tolerant and grow well at a pH between 3.0 to 4.0 (Du Toit & Pretorius, 2002). Most AAB that are known and can be used commercially to produce vinegar are members of *Acetobacter, Gluconacetobacter* and *Gluconobacter* (Raspor & Goranovič, 2008).

The strength of vinegar is measured according to the quantity of acetic acid in the final product. However, high levels of acetic acid can produce an aroma that resembles ethyl acetate, which smells like nail polish. Generally, wine vinegars should have 6% acetic acid while other vinegars consist of 4% and 6% acetic acid (Molly, 2011).

2.4 Grape cultivars

Cultivars used for TBV are Trebbiano and Lambrusco grape varieties and the Balsamic Vinegar of Modena IGP permits other varieties such as Ancellotta, Albana, Fortana, Montuni and Sangiovese. Trebbiano is a white grape cultivar known for its high sugar concentration (Solieri *et al*., 2006) while Lambrusco is a red grape cultivar known for its high acidity (Giudici *et al*., 2015). These grapes are usually harvested in September or October in the northern hemisphere when they are very ripe and their sugar concentration is high (Oulton & Randal, 2002). In South Africa, these grape varieties are not grown. White varietals make up 54.6% of the vines in South Africa, with Chenin blanc being among the white varietals constituting 18.0% of the 54.6%. Currently, South Africa has 99,463 hectares of vines which are under cultivation and Chenin blanc is the largest planted variety (SAWIS, 2016). This cultivar may have been one of the first to be grown in South Africa in the year 1655. Chenin blanc wine is also known for its high acidity and ageing potential (Jenster 2008; James, 2013). Therefore, the Chenin blanc grape cultivar could be considered for use in South Africa for the production of BSV- a focus of this study.

2.5 Cooking of the grape must

The fermentation medium used for TBV is cooked grape must. The cooking process may vary to some extent among producers. Subsequent to the grapes being harvested, they are crushed and the juice is filtered (Meathead, 2011). The product is usually referred to as the "*mosto"* (Italian) which means must (Oulton & Randal, 2002). Generally, subsequent to the removal of any coagulated proteins including impurities by skimming, the grape must is boiled in open vessels (Solieri *et al*., 2006; Solieri & Giudici, 2008) or pressurised tanks (Wheeler, 2014). After

skimming, the cooking/boiling continues and at this stage, the temperature is kept between 85 to 90°C. The boiling is performed using a slow process and can take one to two days (Oulton & Randal, 2002). When the grape must reaches a sugar concentration within the range of 35 to 60 °Brix or when half of the initial volume has been lost, the cooking is stopped (Solieri & Giudici, 2008).

The cooked grape must is referred to as "*cotto mosto*" or "*Mosto d'uva cotto*" which means cooked grape must (Oulton & Randal, 2002; Meathead, 2011). For a producer who is less well equipped, the reduction of the grape must to half of its initial volume is a good indication that the sugar concentration is at an acceptable level. Due to the cooking and ageing process, a large volume of grape must is needed, for which 100 litres is required to produce 6 litres of TBV (Oulton & Randal, 2002). A study done by Antonelli *et al.* (2004) demonstrates that by boiling the grape must, the development of pigments by non-enzymatic browning can occur. It was also demonstrated that the dehydration that occurs during the boiling causes the degradation of hexoses, thus resulting in the formation of 5-hydroxymethyl-2-furaldehyde (HMF). Falcone (2010) also reports that polyphenol degradation occurs and new compounds are formed. These are the main chemical reactions known to occur as a result of the cooking of grape must. After the cooking procedure is complete, the grape must is allowed to cool. At this stage, it is sterile and ready for inoculation.

2.6 Inoculation of balsamic vinegar

Generally, the EtOH-AcOH fermentation of TBV is a spontaneous process (Solieri *et al*., 2006). It involves two approaches: the first approach involves transferring the sterile cooked must into previously used and unsterilized barrels and the yeasts and bacteria present in the barrels initiate the EtOH-AcOH fermentation process. This is commonly known, as the contamination of the cooked grape must (Solieri & Giudici, 2008). The second approach is commonly known as back slopping. In this approach, the starter culture is a microbiologically undefined culture commonly known as the seed vinegar. The seed vinegar is obtained from previous fermentations that underwent spontaneous acetification. The seed vinegar is used to inoculate the sterile cooked must (Oulton & Randal, 2002; Solieri *et al*., 2006; Solieri & Giudici, 2008; Meathead, 2011). The back slopping approach is aimed at reducing the risk of fermentation failure and reduction of the acetification period (Solieri & Giudici, 2008).

The making of TBV relies on the EtOH-AcOH fermentation process performed by unknown yeasts and bacteria. This process, as convenient as it seems, can have numerous disadvantages that can result in high cost implications. Another disadvantage is associated with sluggish or stuck fermentations (Mas *et al*., 2007; Tofalo *et al*., 2009), i.e. the failure of the oxidation process due to excessive ethanol production and the imbalance of glucose/fructose which can result in glucose crystallisation (Landi *et al*., 2005). The yeasts used should be osmophilic due to the high sugar environment. The AAB should also be able to tolerate ethanol levels between 6 to 10% (v/v) and retain rapid ethanol oxidation capabilities while being tolerant to high acetic acid concentrations (Gullo & Giudici, 2008). Inoculation with a known mixed culture should be preferred to a spontaneous EtOH-AcOH fermentation to avoid failed fermentations due to contamination by unwanted microorganisms. This requirement is essential, particularly for a controlled bioprocessing system. Furthermore, some advantages of a defined mixed culture include the improvement of quality, safety and efficiency; hence, decreasing fermentation-acetification time, improved stability, improved growth and proliferation of the mixed culture and improved product yield. This will result in decreased production cost and thus an appropriate economical yield (Solieri & Giudici, 2008).

Researchers have suggested technological improvements in TBV making. These include the use of *Zygosaccharomyces bailii* as a starter culture to enhance quality of the fermented cooked must (Giudici *et al*., 1992, as cited in Solieri & Giudici, 2008). Similarly, Solieri and Giudici (2005) emphasised the importance of defining the desired traits required for a mixed culture, implying that the final product quality will be largely influenced by such a culture. In another study, Solieri and Giudici (2008) suggest an increase in the number of microorganisms in the TBV mixed culture, and studies to investigate the metabolic activity of the yeasts used and their effect on TBV. They further suggest the development of a defined mixed culture for inoculation to avoid unpredictable fermentations.

2.7 Microbial growth and proliferation

Fermentation is generally defined as the chemical change of a defined medium by microorganisms under defined conditions (Kango, 2010). The EtOH-AcOH fermentation of balsamic vinegar involves the fermentation of sugar to ethanol by yeast while AAB oxidise the ethanol to acetic acid. In most instances, this process happens simultaneously (Gullo *et al*., 2006).

Microbial growth in fermentation process generally has four phases; the lag phase, exponential phase, stationary phase and decelerating/death phase (Chisti, 1999). The lag phase is the first phase, which begins immediately after microorganisms are inoculated. During this phase, the cells are adapting to the new environment, which results in a very slow growth rate. Yeast growth results in the release of carbon dioxide (CO_2); therefore, the lag phase produces less CO_2. The length of the lag phase depends on the size of the inoculum used and its characteristics. If the inoculum is healthy and active, this phase can last for a couple of hours depending on the type of fermentation. The exponential phase during alcoholic fermentation can be identified by the high production of CO_2. This phase demonstrates that the yeast cells are at an exponential growth stage. The stationary phase is characterised by a constant biomass concentration. In this phase, all fermentable sugars are consumed and the remaining cells settle at the bottom of the fermenter. The decelerating or death phase is characterised by minimal CO_2 production. This is due to the depletion of nutrients. At this stage, limited fermentation is taking place (Holzberg *et al*, 1967; Jones & Greenfield, 1982; Saucedo-Castañeda *et al*., 1994; Stanbury, 2013)

CO_2 release during fermentation can serve as a tool for monitoring microbial growth rate. Other methods of evaluating microbial growth include analytical methods such as spectrophotometer readings to quantify cell density, dry cell weight method, cell counts via microscopic methods and plate counts (colony forming units). Molecular biology methods can assist in evaluating population dynamics (González-Arenzana *et al*., 2013) during a mixed culture EtOH-AcOH fermentation process. All the aforementioned can assist in knowing the growth rate of each species and viability, i.e. their ability to achieve complete fermentation (Kango, 2010; Simpson, 2011).

2.8 Fermentation/biochemical process parameters

One of the important attributes of a fermented product is its chemical composition. The chemical composition of the final product is important, as it verifies whether the product is suitable for the market or not (Hutton, 2002). The chemical composition of each product is significantly influenced by the fermentation parameters (Hutton, 2002; Paziani *et al*., 2005). Fermentation parameters include; temperature, pH, medium composition, inoculum features and more (Almeida *et al*., 2001). A study done by Togarepi *et al*. (2012) has shown that a medium pH of 6 yielded the highest rate of ethanol production. This was because of the better functioning of proteins at that pH (Berg, 2007). A pH of 2 had the lowest carbon dioxide production seemingly because the low pH allows the production of acid rather than alcohol (Jennings, 1995). The work done by Togarepi *et al*. (2012) also showed that a temperature of 30ºC resulted in a high concentration of ethanol being produced, i.e. a high rate of fermentation. Therefore, it is advisable that such parameters be known in order to be controlled to ascertain consistency in the quality of the final product.

2.9 The use of a microbial consortia versus a single culture

A mixed or multi-cultural fermentation which may also be termed a microbial consortium facilitated fermentation is a fermentation process which is carried out by two or more microorganisms (Hesseltine, 1992; Ciani *et al*., 2010). The consortia used in fermentations can consist of unknown species, related species or unrelated species due to the type of fermentation to be achieved (Hesseltine, 1992). Various studies have shown that this type of fermentation has advantages compared to single culture fermentations (Fleet, 2003; Ciani *et al*., 2010; Hoelzle *et al*., 2014). Previously, Macfadyen & Badington (1903) referred to mixed fermentations as mixed infections. The term changed with time and the benefits of mixed fermentations were studied broadly. During microbial consortia facilitated fermentations, the microorganisms in the microbial consortia interact. Four relationships are usually described, namely mutualism, competitivity, parasitism and commensalism (Haruta *et al*., 2009). A mutual relationship is when the microorganisms carry out activities that benefit each other. A competitive relationship is when the microorganisms compete for survival; they may compete for nutrients or carry out activities that may harm other microorganisms in the microbial consortia. A parasitic relationship is when one organism benefits while the other is harmed and/or even perishes; usually, a parasitic relationship is when organism A uses organism B as a host (Anonymous, 2016a). Organism A is a predator

and may replicate within the host/prey organism B (Haruta *et al*., 2009). Commensalism is when both organisms simply survive without having any effect on each other. This is a very rare relationship (Anonymous, 2016a).

Most studies have shown the use of a microbial consortium as an inoculum to be beneficial. For instance, a study, which was done by Driessen *et al.* (1982) on yoghurt production, showed the use of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* as a mixed inoculum to produce 74 mmol (4.44 g/L) of lactic acid. However, when these microorganisms are used separately, they produce less than half of the aforementioned yield. Additionally, the biomass yield of *S. thermophilus* showed an increase when used with *L. bulgaricus*. Fermentations with a microbial consortium include other benefits such as achieving multistep fermentations, improved consumption of substrate and product formation (O'Leary & Woychik, 1976; Hesseltine, 1992; Prpich & Daugulis, 2005; Kedia *et al*., 2007). Other benefits include the production of compounds which complement each other (Chisti, 1999; Irlinger & Mounier, 2009), the production of nutrients that increase the microbial consortia performance (Driessen *et al.*, 1982; Bautista *et al.*, 1966) and the reduction of unwanted compounds (Kaneko *et al*., 2014). As mentioned in the yoghurt study, growth rates may be higher; a mixed culture inoculum can also be easily sustained by unskilled personnel (Hesseltine, 1992). Most importantly, a microbial consortium may produce a better-quality product with improved complexity due to the different compounds produced by the different microorganisms in the microbial consortia (Ciani *et al*., 2010; Comitini *et al*., 2011).

The use of a microbial consortium also has some disadvantages, such as the difficulty to detect contamination and complex growth kinetics, including biochemical tests on individual members within the microbial consortia compared to when a single culture is used (Jannasch & Egli, 1993). Preparing the inoculum for mixed cultures is time consuming compared to a single culture; the maintenance of environmental conditions to benefit the microorganisms in the mixed culture may also be difficult. Furthermore, a mixed culture requires more economical considerations than a single culture (Holzapfel, 2002).

2.10 Yeast consortia: non-*Saccharomyces* yeasts in conjunction with *Saccharomyces* yeasts versus non-*Saccharomyces* yeast only in balsamic vinegar production

During the EtOH-AcOH fermentation of balsamic vinegar, the role that yeast plays is mainly the fermentation of sugar into alcohol. Another important role that yeast has is the production of flavour compounds (Solieri *et al*., 2006). The EtOH-AcOH fermentation process in TBV is performed by an undefined mixed yeast culture. These yeasts can include *Saccharomyces* yeasts and non-*Saccharomyces* yeasts. In wine production, the use of *Saccharomyces cerevisiae* together with non-*Saccharomyces* yeast has been encouraged because it can enhance wine flavour (Jolly *et al*., 2014). However, the choice of non-*Saccharomyces* yeasts only as a defined mixed culture in balsamic vinegar should be preferable due to the following reasons: *Saccharomyces cerevisiae* strains are generally unable to tolerate high sugar concentrations (De Vuyst, 2000) which in balsamic vinegar can be up to or more than 50 °Brix (Solieri & Giudici, 2008). Even if *S. cerevisiae* strains had the ability to tolerate high sugar concentration, they produce high alcohol concentrations (e.g. 12 to 14% v/v) - a problem for AAB when it comes to the oxidation of alcohol (Gullo & Giudici, 2008).

As a result, the use of *Saccharomyces* yeast may not be ideal and may increase the cost of the inoculum. Generally, in winemaking, non-*Saccharomyces* yeasts were discovered to have benefits but they are always used in conjunction with *S. cerevisiae*. This is due to the fact that most non-*Saccharomyces* yeasts have low fermentation rates, thus cannot achieve complete fermentation as desired in wine. Therefore, a collection of desired non-*Saccharomyces* yeast can be used in the making of BSV. Most non-*Saccharomyces* yeasts are osmophilic and can withstand the high sugar concentration of cooked must. Most of the strains can produce up to 6% (v/v) alcohol, which is within the optimum ethanol concentration desired by AAB (Gullo & Giudici, 2008). Furthermore, what is required from the yeasts is the ability to ferment high concentrated sugars and thus contribute to the sensorial quality of the BSV. Therefore, it is reasonable to conclude that, a consortium of non-*Saccharomyces* yeasts only can be used for a fermentation to produce BSV.

2.11 Limitations related to microbial consortia performance

2.11.1 Cooked must: harsh environmental conditions for the growth of a microbial consortia

True balsamic vinegar production always involves the use of cooked grape must. However, due to the loss of water during the cooking process, the grape must become highly concentrated with sugar, with the pH decreasing. The cooked must is considered a suitable medium for the growth of yeast and AAB; however, this environment is not suitable for the growth of microorganisms. The water activity (a_w) of the cooked must is lower than 0.9 (Solieri & Giudici, 2008; Tofalo *et al*., 2009), which is unsuitable for the growth of most yeasts and bacteria, as they require a minimum a_w of 0.90 to 0.97 to grow. However, a few yeasts grow on fruit syrup at a minimum a_w of 0.60 to 0.70 while bacteria grow at a minimum a_w of 0.90 to 1.00 (Chen, 2005). Solieri and Giudici (2008) report that yeasts generally require a minimum a_w of 0.85 to grow. Furthermore, the cooked grape must with high sugar concentration causes hyper-osmotic stress on yeasts such as *S. cerevisiae* because it is non-osmophilic (Hohmann, 2002; Erasmus *et al*., 2003).

D'Amore *et al.* (1991) and Smits and Brul (2005) reported that *Zygosaccharomyces rouxii* survives under high sugar concentration by activating intracellular trehalose and high osmolarity glycerol (HOG) pathways. As for the AAB, sugar tolerance is important because bacterial growth can also be affected by sugar concentration. In higher sugar concentrations, a few strains of AAB can grow while many strains can grow at lower sugar concentrations (Gullo *et al*., 2006). It was also reported that the disadvantage of growing AAB in cooked must is that the high sugar concentration can result in the inhibition of AAB metabolic processes, which can result in low acid concentration being produced (Gullo *et al*., 2006). It is reasonable to hypothesise that although cooked must is desirable for true balsamic vinegar fermentation, an investigation needs to be done to select a microbial consortium that can withstand such a stressful environment.

2.11.2 Microbial tolerance to ethanol

Most AAB have the ability to oxidise ethanol (ethyl alcohol). However, their ability to oxidise ethanol varies among strains and species. Some AAB can tolerate high ethanol concentrations while others cannot withstand high ethanol concentrations (Gullo & Giudici, 2008). Therefore, it is vital to select strains of AAB that have the ability to oxidise all the ethanol produced by the selected yeasts. With a defined yeast consortium, a producer will know how much alcohol

will be produced by the selected yeasts. Although, non-*Saccharomyces* yeasts do not generally produce high ethanol concentrations, there are some strains that produce up to 10% (v/v) ethanol concentration (Di Maio *et al*., 2011). These yeasts include some of the *Zygosaccharomyces* and *Candida* species, which are less affected by high sugar concentrations. Therefore, if a producer knows individual species constituting a consortium, there will be a need to select AAB that can coexist and tolerate such high alcohol concentrations. Additionally, ethanol concentration should not be higher than 10% (v/v) because balsamic vinegar is different from spirit vinegar and should not be too acidic. High alcohol concentration can result in very high acid concentrations, an undesirable characteristic in the case of balsamic vinegar (Gullo & Giudici, 2008).

Environmental factors that affect ethanol oxidation include temperature, pH and oxygen (Drysdale & Fleet, 1988; Du Toit & Pretorius 2002). The EtOH-AcOH fermentation process of TBV is generally an aerobic process, which is suited for AAB, but the dissolved oxygen can also reduce the ability of the yeasts to ferment available sugars. However, the process should be kept aerobic, as most yeasts are not significantly affected by the presence of dissolved oxygen unless they are classified as "Crabtree-negative yeasts". The Crabtree negative yeasts include species of *Z. bailii* (Merico *et al*., 2003) and *Candida* species, which are not affected by aerobic conditions (Soleiri & Guidici, 2008). If oxygen is absent, the AAB growth and thus ethanol oxidation will be negatively affected. Solieri and Giudici (2008) concluded that ethanol concentration should not be considered as a limitation when compared to other environmental conditions such as temperature, pH and oxygen.

2.11.3 Over-oxidation

Over-oxidation is another limitation related to the performance of the microorganisms in a mixed culture. The desired role of AAB is the oxidation of ethanol to form acetic acid. However, AAB can also further oxidise acetic acid to form carbon dioxide (CO_2) and water (H_2O), with the latter being unsuitable with deleterious financial implications. AAB species, which were reported to cause over oxidation, are *Acetobacter* and *Gluconacetobacter* species. The over-oxidation occurs when all the ethanol in the fermented cooked must is depleted and when the dissolved oxygen levels are high. This process is carried out via the tricarboxylic acid cycle (Greenfield & Claus, 1972; also cited in Du Toit and Pretorius, 2002). Other reasons for over-oxidation could be

the population changes or physiological changes of the strains caused by the unavailability of the alcohol substrate (Gullo & Giudici, 2008). When over-oxidation occurs, the bacterial cells undergo three growth phases, the initial being the complete oxidation of ethanol to acetic acid, subsequent to the growth reaching stationary phase. Thereafter, the third growth phase appears, this time with acetic acid being used, resulting in over-oxidation (Mariette *et al*., 1991, Saeki *et al*., 1997). It was reported that there is a high chance of over-oxidation in tropical and temperate countries; therefore, temperature control is required (Saeki *et al*., 1997).

2.11.4 Viable but non-culturable state of microorganisms

A viable but nonculturable (VBNC) state, sometimes referred to as the dormancy state, is a state that some microorganisms enter, mostly bacteria (Oliver, 2005a). The VBNC state is when bacteria do not grow on the solidified growth media used. Previously, when a scientist could not grow bacteria in a defined nutrient medium, they would assume that the bacteria were dead (Oliver, 2000; Oliver, 2010; Li *et al*., 2015). However, the bacteria may have entered the VBNC state while still having the ability to carry out slow or renewed metabolic activity (Oliver, 2000). This phenomenon is problematic mostly in the fermentation industry, because a fermenter might have entered a VBNC state, for which limited control of the organism can be difficult to achieve. Not only is the VBNC state a problem in fermentation systems, but it is also a problem food, bioremediation, medical and other related industries (Oliver, 2005b; Fakruddin *et al*., 2013; Li *et al*., 2015). Fortunately, the VBNC state of bacteria is being studied worldwide and it has been accepted that the culturing of bacteria on biological media is not the only way of testing microbial viability (Colwell, 2009).

A number of methods are used to determine the viability of nonculturable cells (Oliver, 2005b). These methods include testing metabolic activity by studying intracellular hydrolysis of 5-Cyano-2,3-ditolyltetrazolium chloride (CTC) or reduction of 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium (INT) (Rodriguez *et al*., 1992; Breeuwer & Abee, 2000). Staining methods such as acridine orange or diaminophenylindole (DAPI), the substrate responsive assay developed by Kogure *et al.* (1979) reported in Bates & Oliver (2004), Moreno *et al*. (2007) and the BacLight® or propidium iodide method to detect cytoplasmic membrane

(Gunasekera *et al*., 2002). Other useful direct methods include molecular genetic probes and other molecular biology methods (Colwell, 2009), such as metagenomics.

It has been reported that bacteria enter the VBNC state as a survival strategy when responding to stress (Oliver, 2000). Various environmental factors can cause stress to the bacteria and their entry into the VBNC state helps them to survive (Oliver, 2000; Oliver, 2010). These environmental factors include high osmotic concentrations, incubation temperatures that are out of the optimum range for the bacteria, starvation, white light exposure or dissolved oxygen availability (Oliver, 2000). Additionally, there are some processes used in the medical and food industry that have been reported to induce the VBNC state. These processes are meant to eradicate the bacteria but instead cause the bacteria to enter the VBNC state. These processes include antibiotic treatment (Rivers & Steck, 2001; Nowakowska & Oliver, 2013; Pasquaroli *et al*., 2013), pasteurisation (Gunasekera *et al*., 2002; Nicolò & Guglielmino, 2012) and chlorination (Oliver *et al*., 2005; Ozcakir, 2007; Zhang *et al*., 2015).

In the case of balsamic vinegar, many researchers have reported that a large portion of the AAB involved enters the VBNC state. AAB are generally categorised as fastidious microorganisms due to their difficulty to cultivate, although there are numerous biological media available (Sievers *et al*., 1992; Mas *et al*., 2014; De Vero *et al*., 2006). It is reasonable to refer to the cooked grape must as an environment that can induce VBNC. The cooked grape must has high osmotic concentrations, such as high sugar concentration, it has a low water activity and the EtOH-AcOH fermentation process usually occurs in an attic where there is minimal temperature control. This may often be ignored and not be seen as a phenomenon requiring research if the bacteria or yeast enters the VBNC state, because TBV is allowed to ferment and mature for up to 12 years. Slow metabolic activity is not a problem and the microorganisms can enter the VBNC state as long as they can be resuscitated at some point.

It is imperative to use other methods to study population dynamics during the EtOH-AcOH fermentation process that do not include isolation procedures. De Vero *et al.* (2006) involved the application of denaturing gradient gel electrophoresis (DGGE) to study AAB present during the EtOH-AcOH fermentation of balsamic vinegar. The use of DGGE has been shown to be a useful culture-independent method in studying microbial population in the food industry (Cocolin *et al*., 2004; Randazzo *et al*., 2005).

Several studies have demonstrated the entry of bacteria into the VBNC state; however, eukaryotic cells can also enter this dormancy state (Serpaggi *et al*., 2012). Yeasts have also been shown to enter the VBNC state, mostly during wine production. This is commonly induced by the addition of SO_2 in wine to stop fermentation and stabilise the wine (Divol & Lonvaud-Funel, 2005) or to prevent the growth of bacteria including other indigenous yeast (Andorrà *et al*., 2008). As a result, this method induces the yeast to enter the VBNC state. To study the VBNC yeast cells, the use of culture-independent techniques such as DGGE has shown to be useful (Andorrà *et al*., 2008).

2.12 Reactor conditions and configurations

2.12.1 Batch versus continuous fermentation

Generally, most studies have illustrated the use of a continuous fermentation system to improve yields when compared to the batch fermentation systems (Min-tian *et al*., 2005; Verbelen *et al*., 2006; Xu *et al*., 2006). A batch fermentation system is also referred to as a loop system or a closed system (Montague, 1997; Cinar *et al*., 2003; Anonymous, 2016b). It involves the growth of microorganisms in a closed system where no nutrients are added until the end of the fermentation (Chisti, 1999; Verbelen *et al*., 2006). The microorganisms growing in a batch system multiply until the substrate is exhausted; then microbial growth declines and limited product formation is achieved. The batch reactor is then cleaned and sterilised for a new fermentation cycle (Anonymous, 2016b). In this system, the microbial growth is divided into four phases; lag, exponential growth, stationary and death phase (Chisti, 1999). The product formed, therefore depends on the substrate, which was available initially (Anonymous, 2016b).

The continuous fermentation system was developed to overcome some challenges encountered with the batch system (Chisti, 1999). In this system, there is a constant addition of the substrate with the aim of improving biomass yield and increase product yield. The system usually requires the employment of highly advanced equipment, which can perform automated addition of the substrate (Chisti, 1999). The continuous system prevents the exhaustion of the substrate. When the initial substrate is depleted, more sterile substrate is added such that the substrate concentration remains at acceptable levels (Anonymous, 2016b). Therefore, biomass growth continues and an additional product is harvested (Lynd *et al*., 1989; Min-tian *et al*., 2005; Xu *et*

al., 2006). High substrate concentrations can cause an inhibition in substrate consumption due the regulation of catabolite (Anonymous, 2016b).

Batch and continuous processes have both their advantages and disadvantages. A decision on what process to use is usually based on the product being produced. The advantages of a continuous process include high productivity as there is constant product formation (Verbelen *et al*., 2006). In addition, the automated system is appealing and can result in a high-quality product. The disadvantages include the failure of the fermentation process due to contamination (Chisti, 1999). This can be very disappointing in the case of balsamic vinegar, since the process is long and fermentation failure after many months can cause economic losses. Another disadvantage is that spontaneous mutation can occur for the microorganisms used due to substrate concentration changes. The process is also not very flexible and cannot be used for other production processes without retrofitting (Anonymous, 2016b).

The batch process is versatile, which is an advantage because it can be used for the production of different products. It is also easy to sterilise (Nielsen *et al*., 1995; Kang, 2000). Batch processes can result in the full conversion of the substrate used. Furthermore, the risk of strain mutation is also much lower. The disadvantages of the batch system include high labour cost because skilled personnel are needed to monitor and operate the process; as such, it can also be time consuming because after each fermentation cycle, time is used to empty, sterilise and refill the equipment (Nielsen *et al*., 1995; Kang, 2000; Anonymous, 2016b).

2.12.2 EtOH-AcOH fermentation temperature

Generally, the EtOH-AcOH fermentation of TBV is carried out at ambient (room) temperature. However, the effect of the fluctuating temperature on the microbial consortia has not been well studied. Furthermore, the low temperatures in winter and high temperatures in summer can negatively affect the microbial consortia particularly in a semi-arid country such as South Africa. The temperature at which a microbial growth process is carried out is very important, as it is depended on environmental conditions which can affect the process positively or negatively by causing sluggish fermentations (Solieri & Giudici 2008; Tofalo *et al.*,2009), stuck oxidations (Guillamón & Mas, 2009) or over-oxidations (Gullo & Giudici, 2008). The type of microorganisms used in the process can assist one to decide which conditions to use. In the process

of deciding what temperature to use for the production of balsamic vinegar, one can assess the optimum growth temperature for the yeast and AAB. The temperature, in the opinion of the author, should mostly suit the organisms mostly affected by osmotic pressure due to the high sugar concentration. So one can decide to either operate under conditions, which suit either the yeasts or AAB if their optimum growth temperatures are too different. Solieri and Giudici (2008) reported that AAB are dominant during spring and summer, with the report suggesting that there is a chance of a stuck oxidation when the temperature rises above 40°C.

Generally, the optimum growth temperature for AAB is between 25 and 30°C, meaning AAB can be categorised as mesophilic microorganisms. At very high temperatures, the denaturing of important enzymes can occur thus causing bacterial deactivation (de Ory *et al*., 1998). Bacterial metabolic process deactivation can cause the AAB to be sensitive to the acetic acid being produced. However, some studies have shown that thermotolerant strains of AAB can perform rapid oxidation of ethanol in industrial vinegar at temperatures between 38 to 40°C (Moonmangmee *et al*., 2000). *Acetobacter aceti* was reported not to be able to grow below 8°C (De Ory *et al*., 1998). It must be noted that thermotolerance is a very desirable trait for AAB, mostly for a producer with minimal temperature control capabilities, because there will be a reduction in cost associated with cooling (Adachi *et al*., 2003).

As for the yeasts, they are also mesophilic, which is an advantage for a two-stage EtOH-AcOH fermentation process. Yeast can grow at temperatures between 20 and 30°C. A study done by Fleet (1993) and Rainieri *et al*. (1999) shows that at temperatures above 20°C, *Saccharomyces* yeasts grow well but below 20°C, non-*Saccharomyces* yeasts such as *Hanseniaspora* and *Candida* species grow well. These results cannot be considered for cooked must as their proliferation might be different under such conditions. This study supports the use of non-*Saccharomyces* yeasts and as previously mentioned, *S. cerevisiae* cannot tolerate high sugar concentrations in cooked must. Although the effect of temperature on the proliferation of yeasts has been broadly studied in grape juice (Fleet & Heard, 1993), studies on cooked grape must are insufficient (Solieri & Giudici, 2008).

2.13 Modelling fermentation processes (microbial growth-dependent models)

2.13.1 Kinetic study of microbial growth and stoichiometric parameters

The kinetic study of microbial growth is a very important tool in understanding the relationship between the substrate concentration and microbial specific growth rate in any fermentation process (Kovárová-Kovar & Egli, 1998; Okpokwasili & Nweke, 2006). The empirical model (See Eq. 2.1) which was previously proposed by Monod (1949) has been used successfully in kinetic studies of microbial growth (Ghosh *et al*., 2012). The introduction of a growth-controlling substrate makes Monod's model important when used in conjunction with classical growth models (Mrwebi, 2004). Microbial growth kinetics studies are crucial in understanding the quantity of nutrients needed for a particular cell density or biomass (X). Monod has described the specific microbial growth rate (μ) as determined by the growth limiting substrate concentration (s) (Kovárová-Kovar & Egli, 1998). When the substrate and product are neglected (Liu *et al.*, 2003), cell growth rate can be expressed with the use of Eq. 2.1, which demonstrates that biomass increases with time.

$$\mu X = \frac{dX}{dt} \quad \textbf{Or} \quad \mu = \frac{1}{X}\frac{dX}{dt} \qquad \textbf{Eq. 2.1}$$

If Eq. 2.1 is integrated, it yields Eq. 2.2 below.

$$\mu t = \ln \frac{X}{X_o} \qquad \textbf{Eq. 2.2}$$

Monod's model (Eq. 2.3), which illustrates the relationship between μ and the residual growth-limiting substrate, *S* (Takamatsu *et al*., 1981) can be used with K_s being the substrate utilisation constant which is defined as equivalent to $\mu = 0.5\mu_{max}$.

$$\mu = \mu_{max}\left(\frac{S}{Ks+s}\right) \qquad \textbf{Eq. 2.3}$$

Monod's equation can also be written as:

$$\mu = \frac{\mu_{max} S}{K_S + S} \Rightarrow \frac{dX}{dt} = \frac{\mu_{max} SX}{K_S + S} \quad \textbf{Eq. 2.4}$$

To illustrate the rate of biomass formation, Eq. 2.5 can be used.

$$r_x = \frac{dX}{dt} \quad \textbf{Eq. 2.5}$$

Furthermore, to illustrate the relationship between microbial growth and substrate utilisation, the equation for biomass yield is used (Eq. 2.6).

$$Y_{X/S} = \frac{X_f - X_0}{S_0 - S_f} \quad \textbf{Eq. 2.6}$$

A biomass growth equation that is a representation of the stationary and exponential phase can also be used (See Eq. 2.9). This equation is developed by the integration of Eq. 2.7 to obtain the Riccati equation (Eq. 2.8) (Najafpour, 2015). The Riccati equation expresses *X* as X_0 at t = 0, where *X* is biomass at a specific time. With the assumption that the observed inhibition is second order, Eq. 2.7 will yield Eq. 2.8.

$$\frac{dX}{dt} = K_s . X_m \left(1 - \frac{X}{X_m}\right) \quad \textbf{Eq. 2.7}$$

$$\frac{dX}{dt} = \mu_m \left(1 - \frac{X}{X_m}\right) X \quad \textbf{Eq. 2.8}$$

Furthermore, Eq. 2.9 has advantages, as it can characterise biomass growth at exponential and stationary phases.

$$X = \frac{X_0 X_m e^{\mu_m t}}{X_m - X_0 + X_0 e^{\mu_m t}} = \frac{X_0 e^{\mu_m t}}{1 - \frac{X_0}{X_m}(1 - e^{\mu_m t})} \quad \textbf{Eq. 2.9}$$

2.13.2 Substrate-utilisation kinetics

The kinetic study of substrate utilisation is important, as it allows one to understand the quantity of substrate being utilised during the different phases of biomass growth in any fermentation/acetification system (Gaden, 2000). Eq. 2.10 is a simplified equation, which can be used to illustrate substrate consumption based on biomass growth. This equation (Eq. 2.10) proposed by Monteagudo *et al*. (1997) can be used to express substrate utilisation based on acetic acid production during the acetification process (Ghosh *et al*., 2012). However, such an equation cannot be used for simultaneous formation and consumption of ethanol in a balsamic vinegar fermentation process.

$$-\frac{dS}{dt} = \frac{1}{Y_{X/S}}\frac{dX}{dt} + \frac{1}{Y_{P/S}}\frac{dP}{dt} + m_S X \qquad \textbf{Eq. 2.10}$$

Similarly, an equation that can also be used to express the rate of substrate utilisation can be - Eq. 2.11.

$$r_s = \frac{dS}{dt} \qquad \textbf{Eq. 2.11}$$

2.13.3 Product-formation kinetics

The kinetic study of product formation allows one to associate the product formed to biomass growth, substrate utilisation and the fermentation time (Pazouki *et al*., 2008). To illustrate product yield based on substrate utilisation, Eq. 2.12 can be used.

$$Y_{P/S} = \frac{P_f - P_0}{S_0 - S_f} \qquad \textbf{Eq. 2.12}$$

For which, the product rate can be expressed (Eq. 2.13).

$$r_p = \frac{dP}{dt} \qquad \textbf{Eq. 2.13}$$

Another model that illustrates the product formation rate (Eq. 2.14) which is associated with mixed growth in a fermentation process is the Luedeking-Piret model (Wang, *et al*., 2006).

The equation shows that the product-formation rate is directly influenced by cell concentration and the microbial growth rate (Brandam, 2007; Ghosh *et al*., 2012; Du *et al*., 2014).

$$r_p = \frac{dP}{dt} = \alpha \frac{dX}{dt} + \beta X = (\alpha \mu_{\dot{g}} + \beta) X \qquad \textbf{Eq. 2.14}$$

Whereby α and β are estimated parameters for kinetic expression. This equation is very useful for product-formation data (Brandam *et al*., 2007; Ghosh *et al*., 2012).

2.14 Modelling for fermentation (non-microbial growth dependent equations)

2.14.1 Consecutive elementary reactions

Atkins and De Paula (2006) developed and described models that can be used for reactions that occur with the production and consumption of an intermediate. In the case of balsamic vinegar, the intermediate is ethanol. The rate equations can be used for consecutive elementary reactions. A consecutive reaction described by Atkins and De Paula (2006) is demonstrated in Eq. 2.15, where *A* is the concentration of the initial available substrate, *I* is the intermediate product formed from *A* and *I* decays to form the product *P* (Atkins & De Paula, 2006).

$$\mathbf{A} \xrightarrow{K_a} \mathbf{I} \xrightarrow{K_b} \mathbf{P}$$

Eq. 2.15

In this case, the author chose to use the reaction below which describes the reaction in balsamic vinegar (Eq. 2.16). That is, sugar consumption is used to produce ethanol with ethanol subsequently being used to produce acetic acid.

$$\mathbf{S} \xrightarrow{K_a} \mathbf{E} \xrightarrow{K_b} \mathbf{A}$$

Eq. 2.16

Where *S* is the concentration of the initial available substrate (sugar), *E* is the intermediate product (ethanol) formed from *S* and *E* decays to form *A*, which is the final product (acetic acid).

2.14.2 Substrate and product variations during fermentation

Substrate and product variations during fermentation, particularly for biological reactions in series, requires reactions that can be characterised with the use of rate laws. Eq. 2.17 describes the rate at which *S* decomposes. *E* is formed from *S* at a rate k_a*[S] (see Eq. 2.18) but decays to *A* at a rate k_b*[E] (see Eq. 2.19). All the equations describe the variation of the concentrations of *S*, *E* and *A* over time.

$$\frac{dS}{dt} = -K_a[S] \qquad \textbf{Eq. 2.17}$$

$$\frac{dE}{dt} = K_a[S] - K_b[E] \qquad \textbf{Eq. 2.18}$$

$$\frac{dA}{dt} = K_b[E] \qquad \textbf{Eq. 2.19}$$

Generally, at the start of a fermentation, sugar (*S*) is initially present, so the concentration is $[S]_0$. Eq. 2.17 describes the first-rate law for which it can be modified to Eq. 2.20.

$$[S] = [S]_0 e^{-K_b t} \qquad \textbf{Eq. 2.20}$$

Eq. 2.18 is substituted in Eq. 2.20 to obtain Eq. 2.21.

$$\frac{dE}{dt} + K_b[E] = K_a[S]_0 e^{-K_b t} \qquad \textbf{Eq. 2.21}$$

The differential Eq. 2.21 becomes non-differential when *E* is set at 0. *E* is difficult to quantify as it is the intermediate in balsamic vinegar fermentation and it decays to *A* while being produced. Thus Eq. 2.22 defines a relationship between the formation and sequential utilisation of ethanol in balsamic vinegar fermentation.

$$[E] = \frac{K_a}{K_b - K_a}(e^{-K_a t} - e^{-K_b t})\,[S_0] \qquad \textbf{Eq. 2.22}$$

Additionally, as illustrated by Atkins and De Paula (2006), acetic acid formation, *[A]*, can be described by Eq. 2.23.

$$[A] = \left(1 + \frac{K_a e^{-K_b t} - K_b e^{-K_a t}}{K_b - K_a}\right)[S_0] \qquad \textbf{Eq. 2.23}$$

Overall, during the EtOH-AcOH fermentation batch process, ethanol is produced from fermentable sugars but also thereafter decays to acetic acid. When the reactions are first order, the

time at which *E* will be at its highest concentration can be calculated (Eq. 2.25) by calculating the change in *E* with time (Eq. 2.24).

$$\frac{dE}{dt} = -\frac{K_a\,[S_0]\,\left(K_a e^{-K_a t} - K_b e^{-K_b t}\right)}{K_b - K_a} \qquad \textbf{Eq. 2.24}$$

When the rate is 0, Eq. 2.24 changed to Eq. 2.25.

$$t_{max} = \left(\frac{1}{K_b - K_a}\ln\frac{K_a}{K_b}\right) \qquad \textbf{Eq. 2.25}$$

2.15 Molecular biology methods

2.15.1 Polymerase chain reaction

Before the discovery of the Polymerase chain reaction (PCR), yeasts and bacteria were originally classified based on physiological, morphological and biochemical characteristics (Kurtzman & Fell, 1998; Barnett, 2000). This method was time consuming, but advancement in molecular biology techniques resulted in the development of numerous DNA-based methods for identifying and characterising yeasts (Hierro *et al*., 2004; Charles, 2016). Out of all these techniques, the polymerase chain reaction (PCR) seems to be the best. PCR was invented in 1985 to amplify specific DNA sequences. This technique is well known for its sensitivity, speed and specificity (Newton *et al*., 1997) and it is still being used today. The PCR method permits the differentiation of related species including the identification of bacteria and yeast (De Barros Lopes *et al*., 1998; Charles, 2016). PCR includes the use of primers that select a portion to be amplified in a genome. Billions of copies of the target sequences can result from this method in a short space of time (Anonymous, 2013). The PCR technique is generally seen as a tool for rapid microbial detection, identification and characterisation (Hoff, 2012).

2.15.2 Restriction endonuclease

The restriction fragment length polymorphism (RFLP) method is an enzymatic digestion of PCR amplicons. This method allows one to analyse the internal transcribed spacer (ITS) or 16S ribosomal DNA sequences to identify yeast species and bacterial species respectively (Irobi *et al*., 1999; McEwen *et al*., 2000; Thompson *et al*., 2004; Yang *et al*., 2007). Restriction endonuclease

enzymes are used to cut DNA into smaller fragments by recognising a specific nucleotide sequence and making a cleavage (Philips, 2016; Yang *et al*., 2007). Thousands of restriction enzymes isolated from different microorganisms exist. The first restriction enzyme isolated from *Escherichia coli* strain RY13 was EcoRI (Philips, 2016). With this technique, the selection of suitable restriction enzymes is very important. Wide-ranging experimenting methods have been used for the selection of restriction enzymes (Rachman *et al*., 2004; Singh *et al*., 2006). The restriction endonuclease method allows one to differentiate at species level. Therefore, if the same yeasts or bacterial species are digested with restriction enzymes, the fragments yielded, should be similar (Esteve-Zarzoso *et al*., 1999).

Chapter 3

Preparation of Fermentation Medium and Formulation of an Inoculation Strategy

Chapter 3

Preparation of fermentation medium and formulation of an inoculation strategy

3.1 Introduction

The growth medium used for a fermentation process is essential because it also plays a vital role in determining the success of the process (Almeida *et al*., 2001; Ghosh *et al*., 2012). The fermentation medium for true balsamic vinegar, cooked grape must (Solieri & Giudici, 2008; Wheeler, 2014) requires monitoring changes that occur during the cooking process (Falcone *et al*., 2007). The cooking should be done at a specific temperature and for a specific length of time to achieve the desired chemical changes (Oulton & Randal, 2002). Over-cooking and the consequential loss of a large portion of water, might lead to the formation of a syrup (Damrosch, 2010); thus, a very low water activity which should be avoided. Inoculum design is also an important factor, particularly when a microbial consortium is used. It is, therefore, essential to study the individual growth rates of microorganisms in the microbial consortia used for a defined fermentation system. This will assist in knowing the cell concentration of the individual microorganisms and establishing an appropriate inoculum size to inoculate a fermentation. Inoculum design features are also important for kinetic modelling, particularly when a fermentation process needs to be controlled (Augustin *et al*., 2000). In addition, inoculum size has been reported to have an effect on aroma compounds (Erten *et al*., 2006; Carrau *et al*., 2010) and maximum product formation (Taleghani *et al*., 2016).

Classical microbiology and molecular biology methods are generally employed to study microbial proliferation including population dynamics (Solieri *et al*., 2006; De Vero *et al*., 2006; Vegas *et al*., 2010). A study done on palm wine vinegar employed the dry weight method to evaluate the microbial growth rates (Ghosh *et al*., 2012). Solieri *et al*. (2006) studied yeast populations in TBV by restriction analysis of the 5.8S ribosomal region and the 5.8S ITS region, with De Vero *et al*. (2006) using denaturing gradient gel electrophoresis (DGGE). Furthermore, Vegas *et al*. (2010) identified AAB species during TBV production using Restriction Fragment Length Polymorphism (RFLP)-PCR of 16S rRNA genes with strain typing being done by Enterobacterial Repetitive Intergenic Consensus (ERIC)-PCR including $(GTG)_5$-rep-PCR. As observed in various studies, a combination of numerous methods must be utilised.

3.2 Aims and objectives

The aims and objectives of this part of the study were:

- To develop a method to prepare the fermentation medium for BSV production, defined as low/high-strength (cooked and autoclaved) must.
- To assess the individual rate of microbial growth for each microorganism in the consortia using spectrophotometric techniques to quantify a suitable inoculum size in order to ensure consistency in the inoculum size/concentration for the fermentation.
- To monitor population proliferation by the classical plate count method, in order to assess microbial performance at various stages during the EtOH-AcOH fermentation.

3.3 Materials and methods

Table 3-1: Equipment used for the study

Equipment name and function	Model, manufacturer and country of origin
Double jacketed steam pot (cooking of grape must)	S.W.18, Aluminium Plant & Vessel Co. Ltd (London)
Microscope (cell concentration)	Reichert-Jung Polyvar Met 66 (Austria)
Spectrophotometer (cell density)	Ultrospec 2000 UV/ Visible (Pharmacia Biotech)
pH meter (pH)	Metrohm pH meter 632 (Switzerland)
Density meter (sugar concentration)	Anton Paar Density meter DMA 35 (Austria)
Alcolyzer (alcohol concentration)	Anton Paar Alcolyzer wine M (Austria)
Minititrator (total acid concentration)	Hanna instruments minititrator HI 84502 (South Africa)
PCR Thermal iCycler (amplification of DNA segments)	Swift^{TM} MiniPro Thermal Cylcer SWT-MIP-0.2.2 (South Africa)
Gel electrophoresis power supplier (DNA electrophoresis)	BG-Power 300 (Baygene Biotech Company Limited)
Gel Image analyser (DNA imaging)	BIO-RAD Laboratories- (Segrate, Milan, Italy)

Table 3-2: Chemicals or reagents used for the study

Chemical name and usage in this study	Purchased from / brand name
Yeast Peptone Dextrose (Yeast growth) Glucose Mannitol (AAB growth)	Biolab, Merck, South Africa
Natamax G (inhibit protein synthesis of yeast cells/inhibit yeast growth)	Danisco, Denmark
Chloramphenicol (kill or inhibit the growth of AAB)	AppliChem Panreac NW companies
Buffers (calibrate pH electrodes)	Hanna Instruments, South Africa
Alcohol standards (calibrate alcolyzer instrument)	Labstuff, South Africa
PCR enzymes and ingredients (required for the amplification ITS and 16s gene regions)	Inqaba Biotech, South Africa GeneDirex®, South Africa
Agarose gel (matrix for movement DNA segments during gel electrophoresis)	Seakem® GTG®, Lonza, Rocklands, USA

3.3.1 Preparation of fermentation medium

The fermentation medium used for this investigation was Chenin blanc grape must since it is the most produced grape cultivar in South Africa. Frozen Chenin blanc grape must was collected from the ARC Nietvoorbij Campus Research Cellar in which the grape must was stored in a -10°C freezer (obtained from the 2015 harvest period: January to February). The grape must was kept overnight at 28°C to allow it to thaw. Initial readings for sugar, pH, total acid and alcohol were measured before preparation of the high-strength (cooked) and low-strength (autoclaved) grape must (see Table 3-3).

Table 3-3: Chenin blanc grape must initial readings for sugar, pH, total acid, alcohol

	[Sugar] °Brix	pH	[Alcohol] % (v/v)	[Total acid] g/L
Batch 1: Chenin blanc grape must for cooking	22	3.36	0	9.5
Batch 2: Chenin blanc grape must for autoclaving	21.9	3.35	0	10

3.3.1.1 Cooking of grape must (high-strength grape must)

The grape must which had an initial sugar concentration of 22°Brix was boiled in a double jacketed steam pot (see Appendix A1) until a concentration of 41.95°Brix was achieved with approximately 50% of the volume being lost. The cooked grape must was allowed to cool and then aliquoted into 18 x 3L Erlenmeyer flasks with each Erlenmeyer flask having 2L of the cooked grape must. The Erlenmeyer flasks were stoppered with cotton wool (Appendix A2) and covered with foil prior to autoclaving at 121°C (20 min) to ensure that the cooked grape must was sterile. After autoclaving, the grape must was allowed to cool at ambient temperature prior to inoculation. Before inoculation, the sugar, pH, total acid and alcohol in the grape must were again analysed to monitor changes, which had occurred during the cooking and autoclaving process (Fig. 3-1).

3.3.1.2 Autoclaved grape must (low-strength grape must)

Initially, during the EtOH-AcOH fermentation process using the cooked grape must (high-strength grape must), it was observed that the AcOH fermentation process was slower when compared to results reported in other studies. This was attributed to the low water activity (a_w) and high sugar concentration of the cooked must - a condition unsuitable for some AAB. Thereafter, a decision was taken that a trial should be conducted using only autoclaved grape must, i.e. low-strength grape must. Following the procedure described above for the cooked must, the initial sugar concentration before autoclaving was determined to be 21.9°Brix. The grape must was aliquoted into 18 x 3L Erlenmeyer flasks, with each flask having 2L of grape must. The Erlenmeyer flasks were covered with cotton wool stoppers and foil prior to autoclaving at 121°C (20 min.). The autoclaving was repeated four times, with the Chenin blanc grape must turning dark brown.

After cooling, the chemical composition of the grape must was analysed before inoculation to evaluate any changes that might have occurred during the autoclaving (Fig. 3-1). The sugar concentration after autoclaving the grape must was quantified as 22.5°Brix (Fig. 3-1).

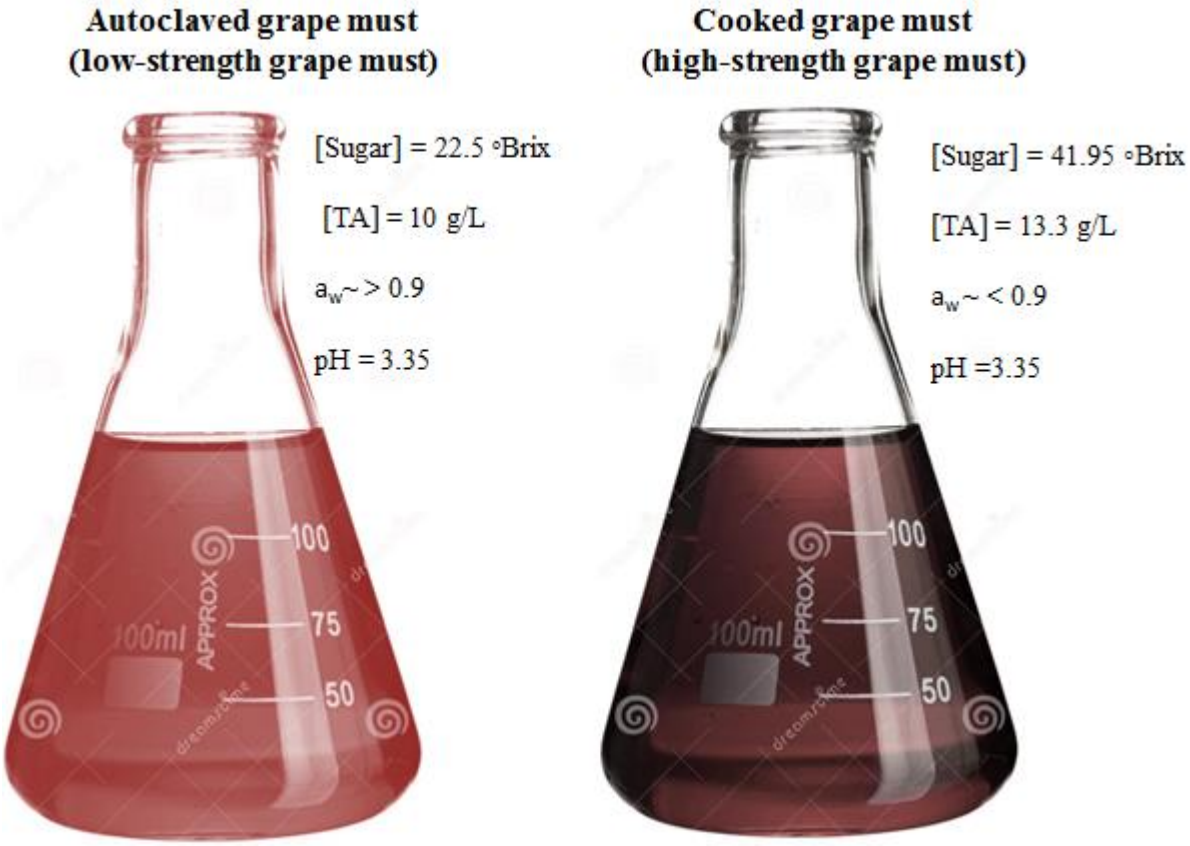


Figure 3-1: The chemical composition determined for the autoclaved and cooked Chenin blanc grape must

3.3.2 Preparation of yeast inoculum

Five cryogenically stored non-*Saccharomyces* yeasts from the culture collection (-80°C) at ARC-Nietvoorbij were used (Table 3-4). The yeasts were selected based on a previous screening investigation based on acid formation on calcium carbonate agar plates, aroma of the final fermented product, osmophilic characteristics and the final concentration of the alcohol produced (data not shown). The yeasts were grown on Yeast Peptone dextrose (YPD) agar plates at a

temperature of 30°C for 96 hours (four days). The yeasts were then inoculated into 150 mL YPD broth in Erlenmeyer flasks and incubated at 30°C for 48 hours, prior to use.

3.3.3 Preparation of acetic acid bacteria inoculum

Fifteen acetic acid bacteria (AAB) were used in this study (Table 3-4). The bacteria were also collected from the ARC-Nietvoorbij culture collection. The bacteria were obtained from previous isolation procedures on various spoiled fruits; namely, grapes and kei apple (*Dovyalis caffra*) while some bacteria were purchased. The bacteria used for this study were selected mostly based on their ethanol oxidation rate when inoculated in diluted wine or sugar utilisation in autoclaved grape juice. To prepare the inoculum, the bacteria were grown for five days on Glucose Mannitol (GM) agar (see Appendix B) plates at 30°C. After growth, the bacteria were harvested and subsequently inoculated in GM broth followed by incubation at 30°C for 96 hours (four days). The list of both non-*Saccharomyces* and AAB used in the study is shown in Table 3-4.

Table 3-4: Non-*Saccharomyces* yeast and acetic acid bacteria used in the study (with ARC accession numbers)

Non-*Saccharomyces* yeast used	Acetic acid bacteria used
• *Zygosaccharomyces bailii* 48/4 • *Hanseniaspora uvarum/ Kloeckera apiculata* 45/69 • *Candida pulcherrima / Metschnikowia pulcherrima* Y08039 • *Candida zemplinina* Y1020 • *Hanseniaspora guilliermondii* C2-15	• *Acetobacter pasterianus* 171/19 • *Acetobacter pasterianus* 179/12 • *Acetobacter pasterianus* 179/48 • *Acetobacter pasterianus* 179/59 • *Acetobacter pasterianus* 179/64 • *Acetobacter aceti* 172/36A • *Acetobacter aceti* 172/36B • *Acetobacter aceti* 179/68A • *Acetobacter aceti* 87/30 • *Gluconobacter oxydans*179/15 • *Gluconobacter sphaericus* 179/68B • *Gluconacetobacter liquefaciens* 172/43 • Unidentified 179/19 • Unidentified 179/68C • Unidentified 126/34

Isolate identification was not part of this study

3.3.4 Determination of yeasts' inoculum size

Before inoculation into the cooked Chenin blanc and autoclaved grape must, the cell concentration of the five yeasts were individually quantified using a microscope and a Neubauer counting chamber, using a 10x dilution with sterile distilled water which was followed by counting under a microscope. Furthermore, spectrophotometer readings were also done at OD_{600nm} to correlate the spectrophotometer readings with the microscopic cell counts. The inoculum size used was $1x10^4$ cells per mL of fermentation medium.

3.3.5 Determination of acetic acid bacteria inoculum size

The theoretical determination of the AAB inoculum size was done a week prior to inoculation. The bacteria were grown for 96 hours in GM broth at 30°C with the cell concentration quantified using a spectrophotometer at OD_{600nm}. Serial dilutions in saline solution were performed followed by plating on GM agar plates. After incubation at 30°C for 96 hours, colonies were counted. Thereafter, the bacteria were grown in GM broth to prepare inoculum for inoculation with spectrophotometer readings being determined to correlate the results with the previous OD readings on the day of inoculation. The volume inoculated was 2% (v/v) for each of the bacteria used.

3.3.6 Inoculation and fermentation procedures (0% and 6% inoculation strategy)

The diagram below (Fig. 3-2) illustrates the fermentation procedure undertaken to accomplish the objectives in this study.

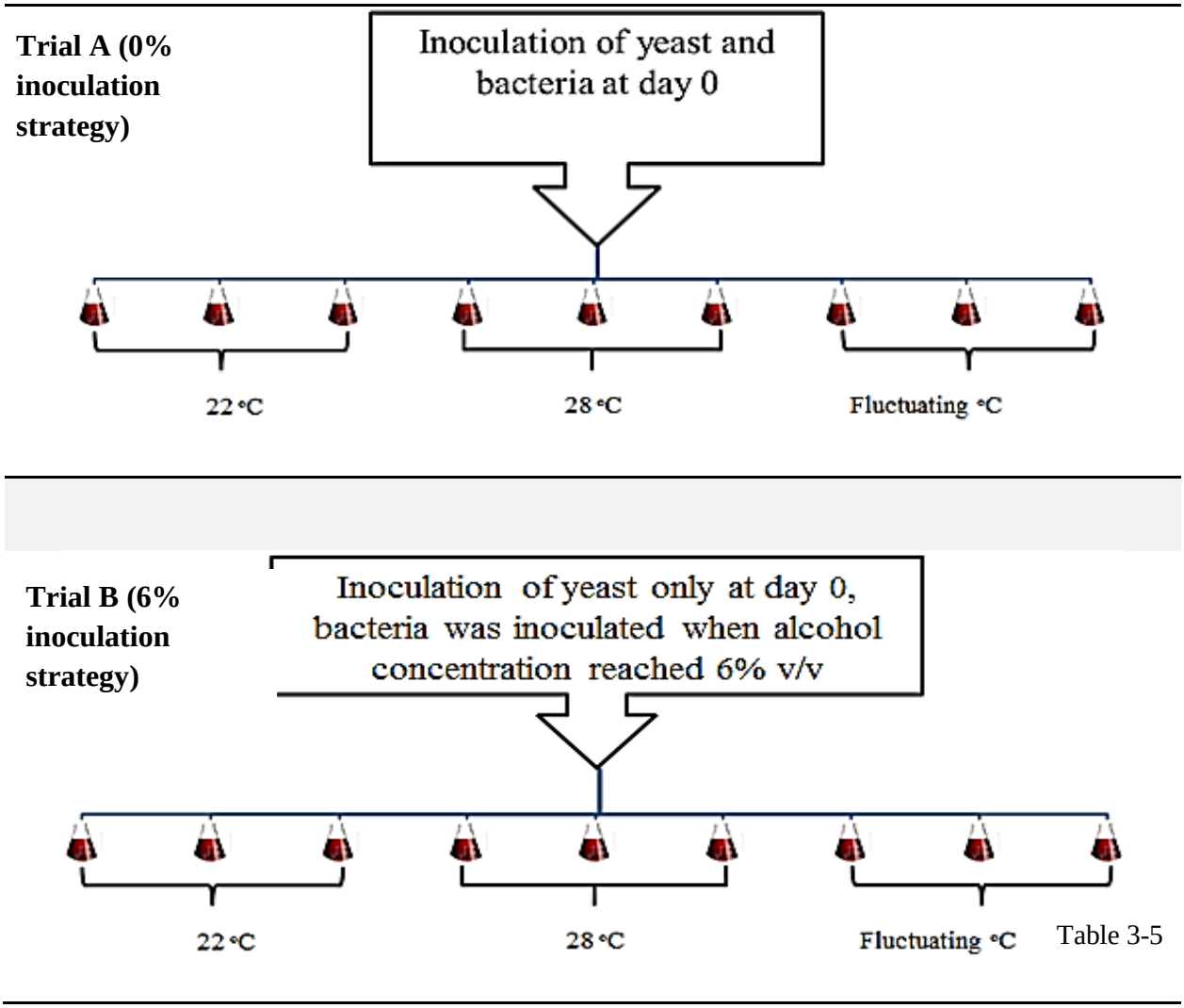


Table 3-5

Figure 3-2: An illustration of the inoculation strategies and trial design used.

3.3.7 EtOH-AcOH fermentation procedure (varying parameters)

Two trials were carried out with the purpose of evaluating the influence of different parameters, i.e. temperature, sugar concentration and inoculation strategy. The fermentation process was performed in 3L Erlenmeyer flasks. Experiments were conducted at temperatures of

22°C, 28°C and a fluctuating temperature (Table 3-5). Fermentation conditions were aerobic with no shaking/stirring. Table 3-6 lists input (evaluated fermenting conditions) and output (quantifiable fermentation results) variables.

Table 3-5: Fluctuating temperature: carried out by physically moving Erlenmeyer flasks to different incubation rooms

	Cooked must trials							
Cooked must trials	Summer		Winter		Summer		Winter	
	1st month 22ºC	2nd month 28ºC	3rd month 15ºC	4th month 10ºC	5th month 22ºC	6th month 28ºC	7th month 15ºC	8th month 10ºC
	Autoclaved must trials							
Autoclaved must trials	1st Week 22ºC	2nd Week 28ºC	3rd Week 22ºC	4th Week 28ºC	5th Week 22ºC	6th Week 28ºC	7th Week 22ºC	8th Week 28ºC

Owing to preliminary studies, it was known that the cooked must trials might be lengthy; therefore, the Erlenmeyer flasks were moved to a different incubation room after a month, with the first two months representing summer temperatures and the 2nd two months representing winter temperatures and so forth. The aim of using different incubation rooms was to evaluate what effect South African summer and winter room temperatures might have in the fermentation process. The autoclaved must trials were only fluctuated between the two temperatures regimes which represented only summer because with autoclaved must, the EtOH-AcOH fermentation process took 2 months to complete. Additionally, there is a high probability that the process would be carried out in summer after the harvest time in South Africa.

Table 3-6: Input and output variables for the study.

List input variables	**List of output variables (response)**
• Inoculation strategy • Temperature • Sugar concentration	• Fermentation time • Microbial growth rates • Product formation • Sensory scores

3.3.8 Sampling

From a preliminary study, it was seen that the fermentation process of BSV is lengthy, therefore sampling was done weekly (seven-day intervals). Sampling was performed using sterile 50 mL polypropylene centrifuge tubes under sterile conditions (Appendix A3).

3.4 Generic Microbiology Methods

3.4.1 Colony forming units/plate counts

Plate counting (Appendix A4) was used to determine the microbial consortia proliferation during the fermentation period. This was done for both yeast and bacteria during the EtOH-AcOH fermentation process. For each sample, serial dilutions were performed using a 1.6% (w/v) saline solution subsequent to plating on agar. For the yeast colony counts, YPD agar plates with the antibiotic chloramphenicol were used. As for the bacterial counts, GM agar plates with natamax (cycloheximide) were used. The plates were grown at 28°C for 48 hours and 96 hours for both yeast and bacteria; respectively.

3.4.2 Yeast: molecular biology methods

3.4.2.1 Yeast Genomic DNA extraction

The extraction of yeast DNA was done using the protocol developed by Lõoke *et al*. (2011), without modification.

3.4.2.2 Polymerase chain reaction mixture: ITS region

The PCR mixture contained the following: 5 µL (10 µM) of each primer, 5 µL (10 to 50 ng) of the genomic DNA, 10 µL of a 5x buffer, 1 µL (2.5 mM) dNTPs, 3.5 µL (25 mM) Magnesium chloride, 0.1 µL (0.5 U) of GoTaq polymerase and 20.4 µL sterile distilled water. The primers that were used in this study were ITS 1 with a sequence of “TCC GTA GGT GAA CCT GCG G” and ITS 4 with a sequence of “TCC TCC GCT TAT TGA TAT GC”.

3.4.2.3 Amplification conditions for PCR: ITS region

A Swift^{TM} MiniPro Thermal iCylcer was used for all PCR reactions. The following settings were used for amplification: 4 min at 94°C followed by 36 cycles of 1 min. at 94°C, 2 min. at 48°C and 20 sec at 72°C and a final elongation step of 4 min. at 72°C.

3.4.2.4 Yeast - Electrophoretic separation and visualisation

The PCR products were separated on 1.5% (w/v) agarose gels and were subjected to 90V for 90 min. in 0.5x TBE (tris-Borate EDTA) buffer.

3.4.3 Bacteria: molecular biology methods

3.4.3.1 Bacteria genomic DNA extraction

The extraction of bacterial DNA was done using the protocol developed by Ausubel *et al*. (1992).

3.4.3.2 Polymerase chain reaction mixture: 16S region

The PCR mixture contained the following: 5 µL (10 µM) of each primer, 10 µL (10 to 50 ng) of the genomic DNA, 5 µL of a 10x buffer, 0.1 µL (0.25 mM) dNTPs, 1.5 µL (10.7 mM) Magnesium chloride, 0.2 µL (1 U) of Taq polymerase and 23.2 µL sterile distilled water. The primers that were used in this study were $16S_F$ (forward primer) and $16S_R$ (reverse primer) with a sequence: $16S_F$: "GCTGGCGGCATGCTTAACACAT" including $16S_R$: "GGAGGTGATCCAGCCGCAGGT".

3.4.3.3 Amplification conditions for PCR: 16S region

The following PCR settings were used for amplification, 5 min for an initial denaturation step at 94°C, followed by 35 cycles of denaturation for 30 sec at 94°C, 30 sec for annealing at 65°C and 1 min for extension at 72°C, for 7 min with the final extension step at 72°C.

3.5 Results and discussion: high-strength grape must

3.5.1 Effect of temperature and inoculation strategy on microbial growth in cooked Chenin blanc must

To study the microbial growth during the EtOH-AcOH fermentation process of BSV using Chenin blanc grape must, plate counts were performed which gave the results observed in Fig. 3-3, 3-4, 3-6 and 3-7. The fundamental growth pattern that was expected for microbial growth in a batch fermentation system follows four phases, where the microbial growth starts with a lag phase, then exponential, stationary and subsequently the death phase (Chisti, 1999). During the EtOH-AcOH fermentation of BSV using cooked and autoclaved must, the lag phase was not observed. When evaluating the effect of temperature on the microbial growth profiles, minimal differences were observed between the way yeast and bacteria grew. This was attributed to the temperature used being within the optimal growth temperature range of the yeast and bacteria.

Fig. 3-3A shows the yeast concentration at different temperatures using the 0% inoculation strategy (yeast and bacteria inoculated at the start of fermentation). It was observed that at the beginning of the fermentation, the fluctuating temperature and 22°C temperature trials showed a higher initial growth rate. However, the growth rate at 22°C remained the highest up to day 42. The fluctuating temperature trials were carried out by physically moving the Erlenmeyer flasks to different incubation rooms, whereby initially, the trials were incubated at 22°C then moved to 28°C after 4 weeks. The effect of the fluctuating temperature resulted in the microbial consortia growing at a similar rate with cultures growing in non-fluctuated temperature, i.e. at 22°C, the growth was similar to the 22°C cultures. When moved to 28°C, the growth rate changed to resemble that of 28°C cultures. The 28°C cultures showed lower cell concentration from when the fermentation was initiated up to 42 days. It is reasonable to hypothesise that there was better yeast growth at a lower temperature (22°C) when using the 0% inoculation strategy.

The 6% inoculation strategy showed a higher cell concentration at the beginning of the fermentation for the fluctuating temperature trials and for the 28°C trials (Fig. 3-3B). The first stages of the 6% inoculation strategy trials had no AAB which may explain the exponential yeast growth observed at the initial stages. Several researchers have addressed the antagonistic effects that AAB have on yeast during fermentation (Sousa *et al*., 2011; Vilela-Moura *et al*., 2011). Sousa *et al*. (2011) and Giudici *et al*. (2015) reported that the presence of acetic acid in the fermentation

medium negatively affects yeast performance and yeast growth, thus causing an inhibitory effect on alcoholic fermentation. AAB were inoculated at day 28, when the ethanol concentration had reached 6% (v/v)/60 g/L. However, prior to the inoculation of the AAB, the yeast cell concentration had already declined (black arrow represents the inoculation of AAB- see Fig. 3-3). At a lower temperature (22°C) yeast proliferation was higher, particularly in the latter stages of the fermentation, i.e. from day 18 to 42, particularly when compared to other temperature regimes used. When comparing yeast proliferation with the use of the two inoculation strategies, it was observed that there was better yeast microbial growth with the 0% inoculation strategy, particularly for the overall fermentation, which required the initial production of ethanol. As seen in Fig. 3-3A, the growth of the yeast decelerated more slowly when compared to the 6% inoculation strategy, where the yeast showed substantial growth in the initial 10 days followed by a rapid decline; an attribute associated with higher alcohol levels. Additionally, after 42 days of fermentation, the yeast culture at 28°C, which included those cultures growing at a fluctuating temperature, were already less than $\log_{10}$ 4 cfu/mL, an observation not encountered with the 0% inoculation strategy. As for the AAB growth profiles, the 0% inoculation strategy (Fig. 3-3D) showed trends that were similar to those observed for the yeast using the 0% inoculation strategy (Fig. 3-3A). The 28°C cultures showed the lowest AAB concentration throughout the entire fermentation process.

The bacteria in the 6% inoculation strategy showed unaccustomed growth patterns for all the temperatures studied (Fig. 3-3D). As expected, there were no AAB colonies prior to inoculation of the AAB. Consequently, at day 28, AAB were inoculated and colonies were observed at three sampling points, day 28, 35 and 42, after which AAB could not be cultivated and it was presumed that the AAB were in a VBNC state as acetic acid was still being formed after day 42 (Fig. 3-5C & F). Plate agar cultivation continued up to the last day of monitoring the trial, which was day 319, but no bacteria colonies grew. Due to chemical developments observed during the EtOH-AcOH fermentation process, sampling continued once a month up to 319 days. Initially, it was not understood as to why the AAB were only observed at the three sampling points. Furthermore, the 6% trial was expected to perform better since the first stages of the fermentation only involved yeast and for which the sugar was fermented to ethanol. As a result, the AAB were inoculated in a fermentation medium with 32°Brix/320 g/L sugar concentration instead of a 41.9°Brix/419 g/L sugar concentration. Additionally, the ethanol concentration formed was 6% (v/v)/60 g/L. This quantity/concentration of ethanol cannot be considered a growth-limiting factor for AAB (Gullo

& Giudici, 2008). This is to say, the environment of the fermentation medium was less harsh to the AAB since the greatest hurdle for AAB growth has been reported to be a high sugar concentration (Gullo *et al*., 2006; Solieri & Giudici, 2008; Tofalo *et al*., 2009).

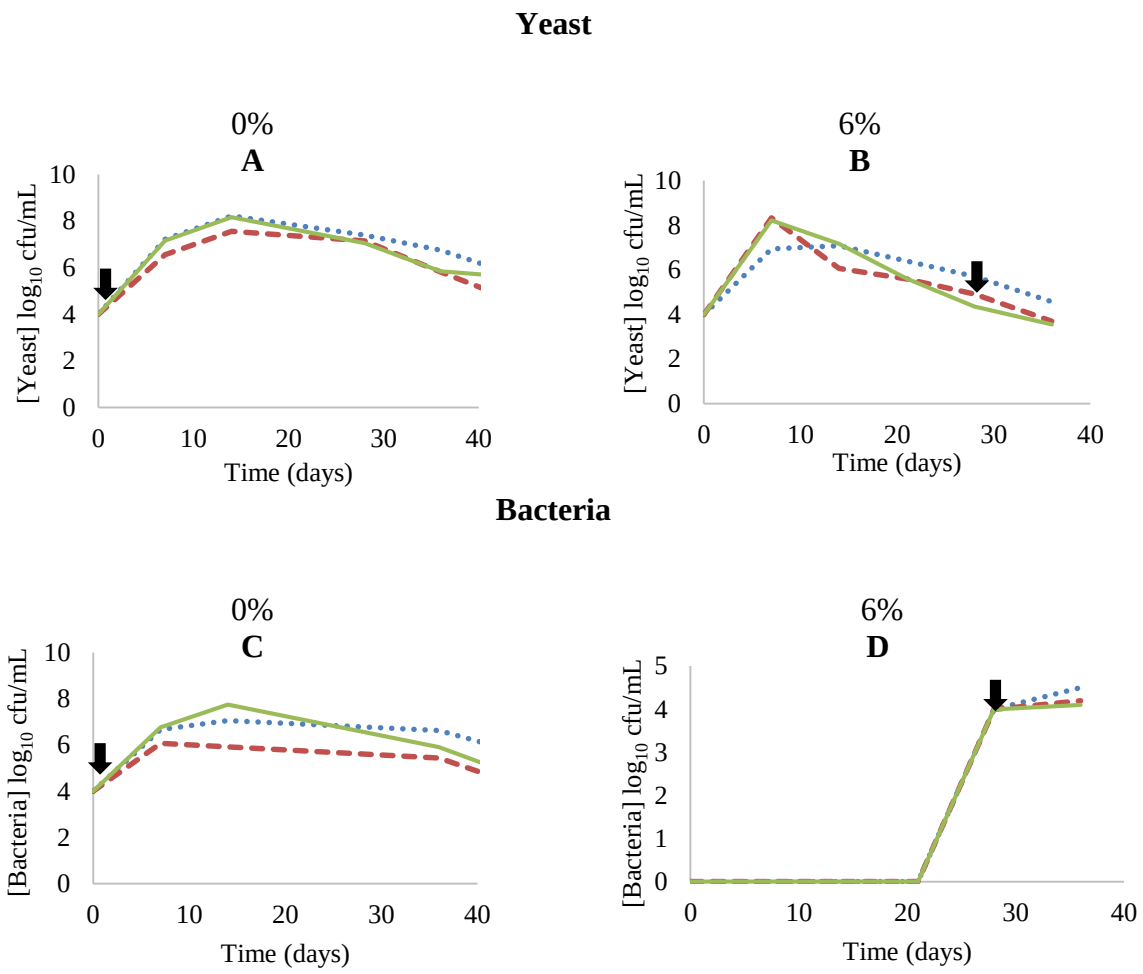


Figure 3-3: Cell concentration – **effect of temperature** (observed during the EtOH-AcOH fermentation process using **cooked Chenin blanc**), ····· 22°C, - - - - 28°C, ——— Fluctuating temperature, ⬇ Inoculation of AAB

3.5.2 Differentiation in yeast and AAB growth under similar temperature and inoculation strategy using cooked grape must

The differences between yeast and bacteria cell concentration for the cooked must under each inoculation strategy and fermentation temperature were evaluated (Fig. 3-4). Using the 0% inoculation strategy (Fig 3-4A, 3-4B & 3-4C), it was observed that yeast proliferation was higher than that observed for the AAB at all temperatures. However, at the beginning of the EtOH-AcOH fermentation (first two weeks), the yeast and bacteria growth patterns were similar (Fig. 3-4A & 3-4B). As the EtOH-AcOH fermentation progressed, the yeast counts continued to be more than those of the AAB. Under a fluctuating temperature regime (Fig. 3-4C), the growth profile for yeast and bacteria showed similar results from the start up period to 42 days. When comparing cultures at 22°C (Fig. 3-4A) and 28°C (Fig. 3-4B), it was observed that the higher temperature (28°C) was the one that showed notable differentiation between the bacterial and yeast growth, a phenomenon not observed at 22°C. This meant that fluctuating the temperature was suitable to sustain both yeast and bacterial growth, particularly when the 0% inoculation strategy is used.

These results obtained with the 0% inoculation strategy should give us an insight into the effect yeast and AAB may have on each other when inoculated simultaneously. Although, the inoculum size for bacteria and yeast were the same, it was difficult to conclude whether or not the AAB or yeast had better growth rates or whether or not they have any effect on each other. Sacchetti (1974), as cited in Giudici *et al*. (2015), reported that the relationship between yeast and AAB is commensal and that alcoholic and acetous fermentation are a simultaneous process. However, Giudici *et al*. (2015) disproved the aforementioned and reported that yeast and AAB do not grow well together. Consequently, if the alcoholic and acetous fermentation are carried out simultaneously, the alcoholic fermentation shows better progress while the acetous fermentation process can take up to a year or more. Giudici *et al*. (2015) reported further that the presence of yeast in the fermentation medium causes the medium to be anaerobic, which negatively influences the growth of AAB. The formation of acetic acid by the AAB can also have negative effects on yeast growth because the presence of acetic acid strongly inhibits the growth of yeast. Sousa *et al*. (2011) also reported that yeasts do not metabolise acetic acid, that the acetic acid can enter the yeast cells in a non-dissociated form. While inside the cells, the acetic acid dissociates.

Additionally, acetic acid causes the pH of the medium to be low (the extracellular pH). As a result, this leads to intracellular acidification, thus negatively affecting cellular metabolism

(Guldfeldt & Arneborg, 1998; Pampulha & Loureiro-Dias, 1989). However, the intracellular acidification phenomenon was reported to occur in *Saccharomyces cerevisiae* strains. It is unknown if the same phenomenon occurs in non-*Saccharomyces* yeast cells, with *Zygosaccharomyces bailii* being reported to be highly resistant to high acetic acid concentrations. In this study, the yeasts used were observed to grow better with the 0% inoculation strategy (Fig. 3-4A, B & C). Therefore, it was assumed that the presence of yeast caused the fermentation medium (cooked grape must) to be anaerobic which inhibited the growth of the AAB. Furthermore, what was reported by Giudici *et al*. (2015) was also observed in the 0% inoculation strategy cultures, whereby alcohol development was successful; reaching the desired 6% (v/v)/60 g/L with the acetic acid formation being sluggish (stuck fermentation) although the process was monitored for 319 days (Fig. 3-5). Overall, the yeasts were not affected by the presence of AAB in this study; this was attributed to the use of non-*Saccharomyces* yeast instead of *Saccharomyces cerevisiae* strains. Another reason could be that there was minimal acetic acid formation in the cooked grape must.

Gullo *et al*. (2006) reported that AAB are the microorganisms which are mostly affected by the high sugar concentration of the cooked grape must during TBV fermentation. Consequently, low acetic acid will be produced particularly if the AAB are not osmotolerant. For this reason, sugar tolerant traits are important when selecting AAB for BSV production. This means that the high sugar concentration in the cooked must (41.95°Brix/419.5 g/L) could have been an inhibitor for AAB growth. The third assumption was that some of the AAB in the microbial consortia may have entered the VBNC state as colony growth on agar plates was only observed up to day 42 when using the cooked must, resulting in a total acid concentration of 14 g/L (Fig. 3-5C). Although plate counts were unsuccessful, an increase in total acid concentration was observed, which reached a maximum of 27 g/L subsequent to fluctuations in total acid concentration assumed to be caused by vaporisation. The total acid concentration observed at day 319 was only 20 g/L, which was considered to be low as balsamic vinegar has an acetic acid concentration which is around 60 g/L.

By using a 6% inoculation strategy, minimal differentiation of yeast and bacteria on each other's growth rates was observed (Fig. 3-4D, E & F). The alcohol concentration reached 6% (v/v)/60 g/L at day 28, a time that was used to inoculate the AAB. However, AAB colonies were only observed at three sampling points after inoculation (day 28, 35 and 42) with minimal colonies

being observed thereafter. Giudici *et al*. (2015) report that during TBV production, the alcoholic and acetous fermentation are separated to prevent the competition between yeasts (alcohol producers) and AAB (acetic acid producers) when inoculated simultaneously. In this study, the 6% inoculation strategy performed better than the 0% inoculation strategy in terms of total acid development (Fig. 3-5F). However, the desired acid concentration (60 g/L) was not reached. It was therefore assumed that the AAB might have been inoculated while the yeast was still actively performing alcoholic fermentation with the fermentation medium being anaerobic at that stage. This might have led to the failure of complete ethanol oxidation (see Fig. 3-5E).

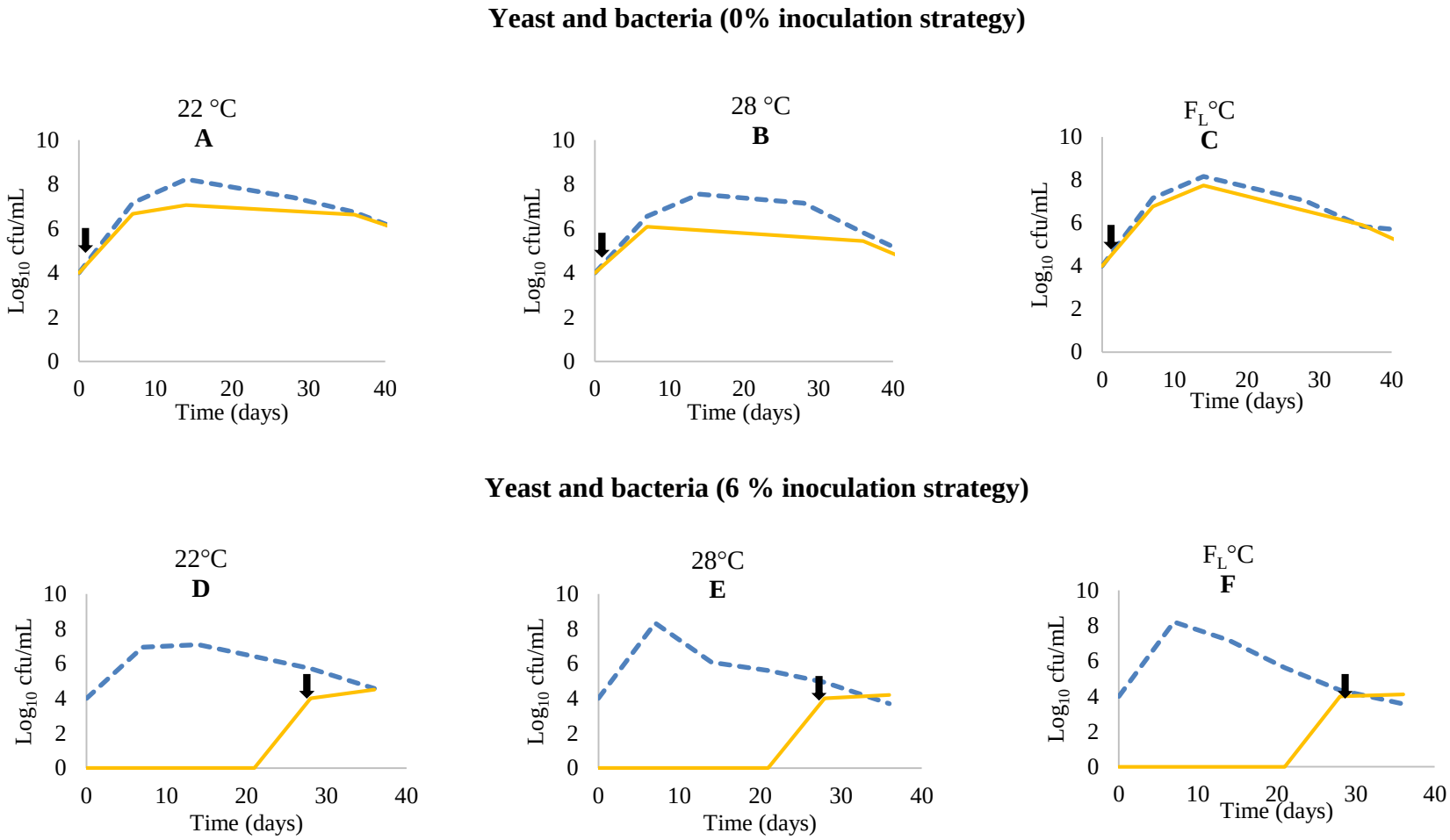


Figure 3-4: Cell concentration – **differentiation between yeast and bacteria under each temperature** (observed during the EtOH-AcOH fermentation process using cooked Chenin blanc). Yeast , Bacteria, Inoculation of AAB

3.5.3 The effect of temperature on sugar, alcohol and total acid consumption/formation using cooked Chenin blanc must

The changes in sugar, alcohol and total acid between the 0% and 6% inoculation strategy trials using cooked grape must showed profiles that were partially similar (Fig. 3-5) to trials using autoclaved must (Fig. 4-3). Fig. 3-5A shows sugar consumption when the 0% inoculation strategy was used. It was observed that there was minimal differentiation among the temperatures studied from day 0 to 120. However, after day 120, some variations were observed among the temperatures studied. At a lower temperature (22°C), a higher sugar consumption rate was observed, followed by trials in which 28°C was used and the fluctuating temperature showed the lowest sugar consumption rate. Fig. 3-5B illustrates ethanol formation including consumption at the 0% inoculation strategy. There were minimal differences observed among all the temperatures studied from day 0 to 80. These observations in some way correlate with the observations made in the sugar consumption profile (Fig. 3-5A), except that ethanol formation/consumption and differences among the temperatures were only observed after day 80 and not after day 120 as seen in the sugar consumption profile (Fig. 3-5A). It was hypothesised that since ethanol was an intermediate (a product and substrate at the same time), ethanol formation rates (Fig. 3-5B) were not proportional nor indicative of sugar consumption rates.

It was reasonable to assume that such a variation in temperature does not have a notable impact on the yeast activity to carry out sugar consumption and ethanol formation (alcoholic fermentation). This is to say, a proportional relationship between sugar consumption rate and the ethanol formation rate could be deduced if the process was solely an alcoholic fermentation process and not an EtOH-AcOH fermentation process. However, the varying temperatures had an effect on ethanol oxidation by AAB (see Fig. 3-5C) for which the total acid formation rate showed notable differences among the temperatures studied. In Fig. 3-5C, total acid formation from day 0 to 100 was not desirable. However, for fermentations at 28°C, total acid formation rates from the initiation of the fermentation until day 319 were higher compared to the other temperatures studied. When correlating the total acid formation data to sugar consumption (Fig. 3-5A) and ethanol formation/consumption (Fig. 3-5B), it was observed that a lower temperature (22°C) achieved the highest sugar consumption and therefore, the highest ethanol production. However, that did not relate to the highest total acid formation since the secondary reaction was performed by AAB and not by yeasts. The data implied that a lower temperature supported alcoholic fermentation/yeast

activity while the highest temperature used supported acetous fermentation/AAB activity (ethanol oxidation).

Fig. 3-5D, E & F show the change in chemical profiles observed with the use of the 6% inoculation strategy. The data obtained with the 6% inoculation strategy also showed minimal differentiation among the temperatures studied, a phenomenon observed with the 0% inoculation strategy. Sugar consumption (Fig. 3-5D), showed similar trends among the temperatures from day 0 to 36. AAB were inoculated at day 28, after which, it was observed that a lower fermentation temperature showed the highest sugar consumption. Overall, the alcohol formation/utilisation (Fig. 3-5E) showed that there were similarities in all temperatures studied with differences being observed only after the inoculation of the AAB. The fluctuating temperature trials showed the highest ethanol consumption rate after the inoculation of AAB.

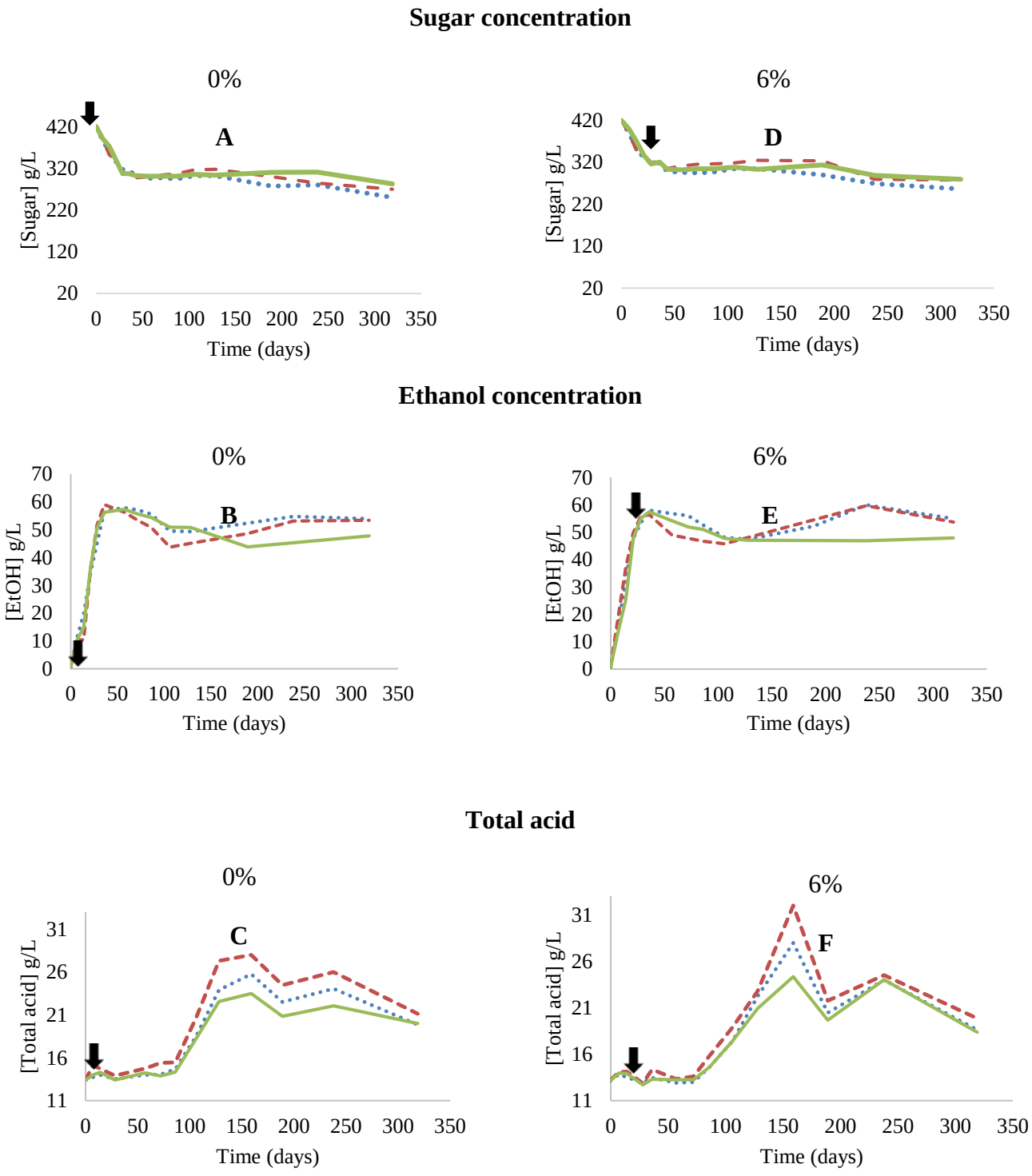


Figure 3-5: An illustration of sugar, alcohol and total acid concentration during the EtOH-AcOH fermentation process (comparing temperatures, ⋯⋯ 22°C, - - - 28°C, ——— fluctuating temperature, ⬇ Inoculation of AAB).- **Cooked grape must**

3.6 Results and discussion: low-strength grape must

3.6.1 Effect of temperature and inoculation strategy on microbial growth in autoclaved Chenin blanc

Microbial growth proliferation evaluation was also done in the autoclaved must (low-strength grape must) trials. As the cooked must trials did not achieve the desired results. In the period of the study based on a pilot study (data not shown), the autoclaved must trials were expected to show a desired fermentation. The microbial growth observed in Fig. 3-6 did not show any correlation between cellular proliferation and the product produced (Fig. 4-1). Studies on fermentation kinetics usually depict that biomass formation is related to substrate consumption including product formation. However, few colonies were observed at numerous sampling points with minimal direct correlation with the chemical developments observed in the fermentations (Fig. 4-1). The cellular concentration was evaluated and ceased when the total acid concentration of approximately 60 g/L was reached. For the 0% inoculation strategy, yeast and bacteria colonies were observed up to day 42 (Fig. 3-6A & C), a sampling point at which the total acid concentration was 60 g/L. As observed with the cooked must trials, at 28°C; lower colony counts for both bacteria and yeast were observed. While the fluctuating temperature trials showed fluctuating cell concentration and the 22°C trials showed the highest cellular counts (Fig. 3-6 A & C).

A different scenario was observed in the 6% inoculation strategy trials (Fig. 3-6B & D). The yeast count data (Fig. 3-6B) did not initially show any significant differences when comparing different temperature regimes up to day 14, which was the day on which AAB were inoculated as the ethanol reached 6% (v/v)/60 g/L. After the inoculation of the AAB, the 22°C trial colony counts showed a slightly increased cellular count compared to the other temperature trials but, thereafter, showed similar cellular counts such as those observed at other temperatures, i.e. from day 30 to 56. As for the bacterial growth using the 6% inoculation strategy, minimal differences were observed among all the temperatures studied (Fig. 3-6D). From day 14, a day that AAB were inoculated, the bacteria cell concentration showed similar trends for all temperatures up to day 56. However, minimal bacteria colonies were observed between day 42 to 56, although acetic acid concentration reached the desired concentration (60 g/L). Overall, by using the 6% inoculation strategy in a trial lasting 56 days the product reached the desired total acid concentration of 60 g/L, which is suitable for commercial BSV. The microbial growth trends observed in Fig. 3-6D are part of the reason some microorganisms were deemed to have been in a VBNC state, thus

nonculturable. A part of this is the reason why some ethanol consumption and total acid formation were still observed after day 42 with minimal colonies being observed in agar plates used.

The VBNC state as observed in the autoclaved must does not seem to be a comprehensible and an adequate assumption, partially due to the continued chemical development observed. There was an exponential rate of total acid formation up until the end of the fermentation (Fig. 4-1C & F). According to Oliver (2000), when microorganisms enter the VBNC, they can carry out slow or renewed metabolic activity, thus product formation. Therefore, slow product formation or change in the exponential rate at which the product is formed was supposed to be observed only up to some point and not be sustained for the entire fermentation (Fig. 4-1C & F). Another argument can be that the non-*Saccharomyces* yeasts used in this process, generally have slow fermentation capabilities (Quirós *et al.*, 2014) and most of them do not achieve a complete fermentation (Di Maro *et al*., 2007; Di Maio *et al*., 2011). Therefore, even when these yeasts are used in low sugar grape must for an alcoholic fermentation process, the fermentation period is long, lasting up to 60 days or more according to preliminary studies performed prior to this investigation (unpublished data). For this reason, non-*Saccharomyces* yeasts are used in conjunction with *Saccharomyces cerevisiae* strains in wine production (Di Maio *et al*., 2011).

Additionally, balsamic vinegar EtOH-AcOH fermentation is normally long and usually takes around 6 weeks to complete (Giudici *et al*., 2015). Another reason for the few colonies observed while monitoring growth rate could simply be due to the biological media used which may not have been adequate for the growth of AAB. Additionally, AAB are referred to as fastidious bacteria due to their difficulty to cultivate on solidified biological growth media (Sievers *et al*., 1992; Mas *et al*., 2014; De Vero *et al*., 2006). When the colony count data (Fig. 3-3 & Fig. 3-6) were correlated with ethanol consumption, including total acid formation for both cooked and autoclaved grape must (Fig. 3-5 & Fig 4-1), it was observed that the trials with a higher temperature (28°C) showed lower colony counts but with an increase in ethanol conversion to acetic acid compared with other trials. Due to this trend, it was reasonable to assume that the VBNC state had been caused by the high acidity produced at a higher temperature. Another assumption can be that substrate consumption and product formation was non-growth dependant, an incongruent phenomenon which is against vinegar fermentation principles, for which acetic acid formation is directly linked to the rate at which AAB grow in a well-defined fermentation system with known conditions (Ghosh *et al*., 2012). It is highly likely that at 28°C, the microorganisms use most of

their energy to form a product and not to grow, while at 22°C, the temperature favours growth rate rather than product formation. In other words, at 22°C, the microorganisms use most of their energy on growing rather than producing a product.

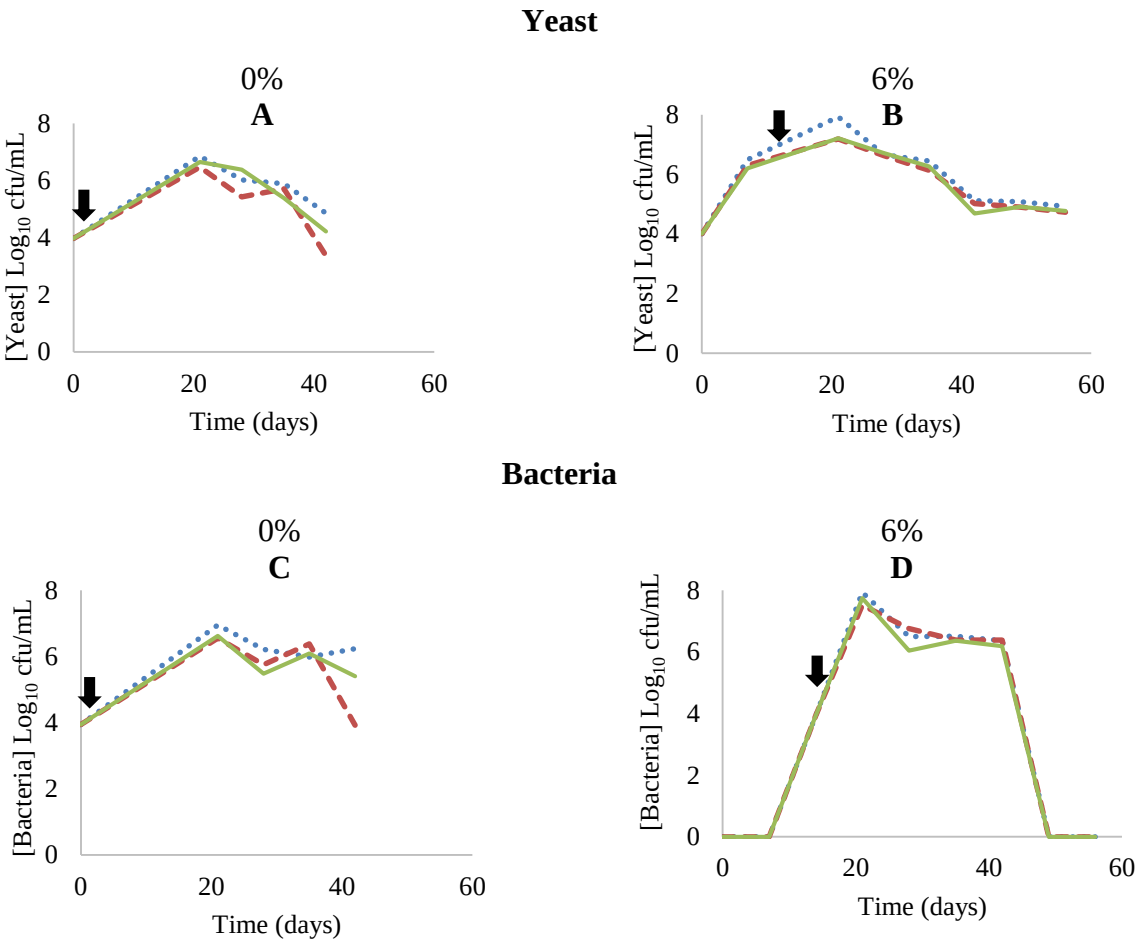


Figure 3-6: Cell concentration– **effect of temperature** (observed during the EtOH-AcOH fermentation process using **autoclaved Chenin blanc**), ····· 22°C, - - - - 28°C, ——— Fluctuating temperature, ⬇ Inoculation of AAB

3.6.2 Differentiation in yeast and AAB growth under similar temperature and inoculation strategy

The microbial growth trend of yeast and bacteria using autoclaved must was different to that observed when using the cooked grape must. With the 0% inoculation strategy, yeast and bacteria colonies were observed until the desired total acid concentration of 60 g/L was reached (Fig. 3-7A, B & C and Fig. 4-1C & F). The yeast and bacteria count showed a proportional relationship with the 0% inoculation strategy under all temperatures studied (Fig. 3-7A, B & C). At 22°C (Fig. 3-7A), with the yeast and bacteria concentration following a similar trend. A minor difference was only observed at the last sampling point, whereby the yeast concentration showed an increase while the bacterial concentration declined. At 28°C (Fig. 3-7B), the yeast and bacteria concentration showed similar growth; a similarity that was also observed when their growth fluctuated. Overall, the AAB concentration was slightly lower than that of yeast between day 21 to 42. Under the fluctuating temperature (Fig. 3-7C), the growth pattern between the yeast and bacteria was also similar up to day 21. Thereafter, the yeast counts showed a decrease, thus becoming lower than those observed for the bacteria used with a subsequent increase to become higher than that initially quantified for the bacteria.

The bacterial concentration, however, simply showed a decrease between day 21 to 42 and there were fewer bacterial counts at day 42 compared to the yeast. The fluctuations in the yeast concentration cannot be necessarily linked to the fluctuating temperature, as yeast concentration also fluctuated at 28°C (Fig. 3-7 B). These results disproved what was recently reported by Giudici *et al*., (2015). In this study, it appears as if the relationship between the yeast and bacteria at the 0% inoculation strategy is proportional (commensal). Not only were the growth trends very similar, the desired and required chemical developments for the fermentation were observed too, i.e. the rate of sugar consumption, ethanol formation, ethanol consumption and total acid formation. Therefore, these results support the observations previously made by Sacchetti (1974). However, it is equally important to mention that Giudici *et al*. (2015) refer to the use of cooked must instead of autoclaved must.

The 6% inoculation strategy showed a degree of proportional relationship between the yeast and bacteria (Fig. 3-7D, E & F). AAB were inoculated at day 14, when the alcohol concentration reached 6% (v/v)/60 g/L. It was observed that after inoculation, the bacterial concentration increased, similar to that of yeast, under all temperatures studied (Fig. 3-7D, E &

F). Generally, a similar scenario was observed for all the temperatures (Fig. 3-7D, E & F) for yeast cell concentration, showing an increase until day 21, which was followed by a decline after the inoculation of the AAB. The presence of AAB may have slightly affected the growth of the yeast due to the production of acetic acid by AAB that had an inhibitory effect on the growth of the yeast (Giudici *et al*., 2015). As the EtOH-AcOH fermentation process progressed there was an increase in acid formation until day 56, with yeast colonies still being observed, while the culturable bacteria growth was observed until day 42, although total acid formation continued until day 56 (Fig. 4-1F).

Overall, it appeared that acetic acid was an inhibitor for yeast growth. It is not clear why minimal AAB colonies were observed after day 42, although it was previously argued that the bacteria were in the VBNC state. For this particular case, a high sugar concentration could not have contributed to the VBNC state, because for this trial, low-strength grape must with an initial sugar concentration of 22.5°Brix/225 g/L was used. However, there are many environmental factors that can lead to the entry of bacteria into the VBNC state and, in this case, it may be difficult to identify a factor which might have been the cause.

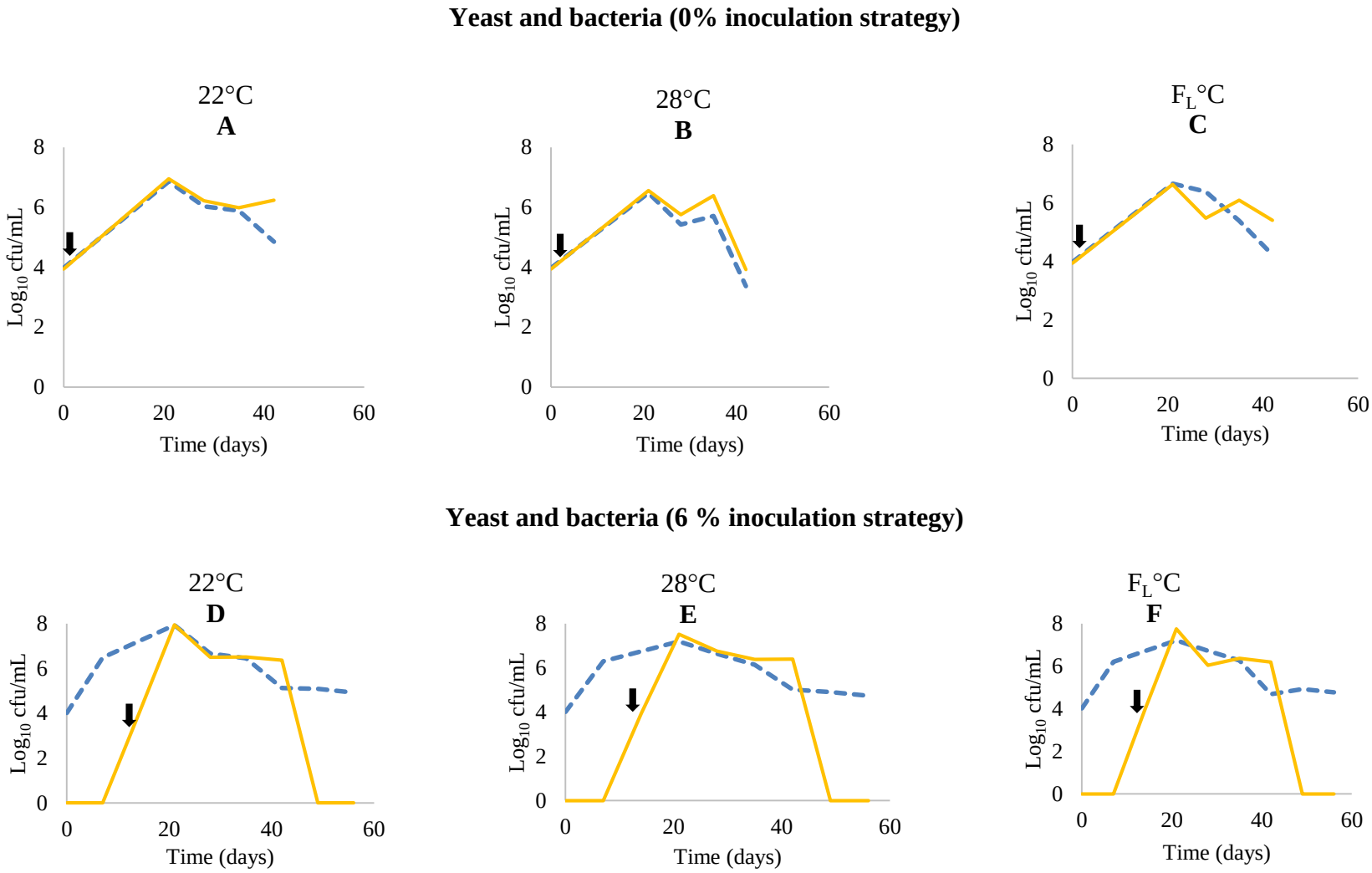


Figure 3-7: Cell concentration – **differentiation between yeast and bacteria under each temperature** (observed during the EtOH-AcOH fermentation process using autoclaved Chenin blanc). Yeast , Bacteria, Inoculation of AAB

3.7 Population dynamics using molecular biology methods

The evaluation of population dynamics during the EtOH-AcOH fermentation process is important so that it is known which yeast and AAB, which constituted the microbial consortia, carried out the entire EtOH-AcOH fermentation process. This is critical in avoiding the use of unnecessary starter cultures and being able to assess the contribution of each organism in the fermentation process. However, due to the direct culture methods, which were initially selected, to study the population dynamics, this question was not completely answered.

In this study, the isolation of yeast and bacteria process using serial dilutions is a traditional microbiology approach that would have led to results that gave a distorted view on which species were actually carrying-out the fermentation process. The selection of colonies, which come from diluted samples, would only result in the growth of the abundant microorganisms and the less abundant would be neglected. Therefore, the conclusions reached would have been biased. Some microorganisms constituting the microbial consortia may have entered the VBNC state and as a result, the use of cultivation procedures would not have resulted in the observation of the nonculturable organisms. Several researchers have suggested the use of culture independent techniques to study population dynamics in TBV (Solieri *et al*., 2006; Solieri and Giudici, 2008).

3.8 Summary: a comparative analysis of microbial growth in cooked and autoclaved grape must for BSV

The cooked and autoclaved must trials gave results that mostly differed, but some similarities were also observed. True balsamic vinegar involves the use of cooked grape must. Therefore, this is basically the reason the study began with the use of cooked grape must. Thereafter, autoclaved grape must trials were performed due to the length of the cooked grape must trials. The cooked grape must trials were monitored for 319 days for both inoculation strategies and differentiated temperature, but the desired chemical development was not observed. Overall, colonies for the bacteria were only observed up to 42 days with minimal colonies being observed after that. Despite this, plating continued up to 319 days due to the chemical developments observed and with the assumption that the fermenters entered the VBNC state and might resuscitate and become culturable.

The autoclaved grape must trials were successful compared to those with cooked must, with the EtOH-AcOH fermentation period being 42 days for the 0% inoculation strategy and 56 days for the 6% inoculation strategy. In addition, yeast and bacteria colonies were observed from the start until the end of the alcoholic acetous fermentation except for the 6% inoculation strategy trial. From the 6% inoculation strategy, colonies were observed on agar for yeast up to 56 days and for the bacteria used, growth was only observed up to 42 days. Some similarities were observed between the cooked must trials and autoclaved must trials with cultures at 22°C showing higher colony counts, followed by cultures growing using the fluctuating temperature regime, with 28°C showing the lowest microbial growth profiles for both the cooked must and autoclaved must. Generally, the trends observed between yeast and bacteria growth were always different. For the cooked must, there was higher yeast proliferation than bacteria while for the autoclaved must trials, minimal differences between both yeast and bacteria were observed.

Chapter 4

Kinetic Modelling for the EtOH-AcOH Fermentation Process

Chapter 4

Kinetic modelling for the EtOH-AcOH fermentation process

4.1 Introduction

In order to understand the behaviour of a fermentation process, kinetic modelling is essential where the process is expressed using mathematical equations. The kinetic models can clearly describe the effect of varying environmental conditions in the fermentation vessel or bioreactor over a certain period. The response of the inoculated microorganisms is also described (Mitchell *et al*., 2004). Furthermore, the development of a kinetic model for a fermentation process can allow one to predict the kinetics of a fermentation process using the kinetic model, i.e. to model parameters such as the simulation of concentration trends for biomass, substrate and product formation. To illustrate the behaviour of a fermentation, the mathematical model should include kinetic features that are commonly measurable during a fermentation process. These features include substrate utilisation, product formation, microbial growth rates and, in certain cases, cell death (Oliveira *et al*., 2016).

Kinetic models differ among batch, fed-batch and continuous fermentation systems. However, there is a possibility of using the observed kinetic parameter behaviour of a batch system to make a reasonable first approximation for fed-batch and a continuous fermentation system (Bonomi *et al*., 1981). For such a study (microbiological system), the most common mathematical equations which are used to describe a fermentation process are described as the prediction of specific growth rates of microorganisms used, using the Monod's equation which is often effectively used. This will generally illustrate that there is a proportional relationship between growth rate and the substrate consumption rate including the product production rate. Similarly, to illustrate the specific rate of product formation, other models can be used, such as the simplified Luedeking and Piret model (Oliveira *et al*., 2016). For the kinetic modelling, only autoclaved must trials data were used.

4.2 Aims and objectives

The aims and objectives of this study were:

- To monitor the changes in sugar, alcohol, total acid and pH during the EtOH-AcOH fermentation period,
- To use the data obtained from the analytical chemistry methods to quantify model parameters of product formation, substrate utilisation and yields among others during the balsamic vinegar EtOH-AcOH fermentation process by the mixed culture,
- To study and select suitable EtOH-AcOH fermentation conditions for BSV using the overall data set and, finally,
- To perform sensory analysis, taste and aroma on the final product obtained. This is for consumer acceptance, in order to assess the viability of the process.

4.3 Materials and methods

4.3.1 Determination of ethanol and sugar concentration

The equipment used for the overall study is listed in Table 3-1, including model type, manufacturing company and country.

Chemical developments during the EtOH-AcOH fermentation were monitored by performing chemical analyses at seven-day sampling time intervals. Alcohol concentration was determined using the alcolyzer instrument (Appendix A5) which measures the alcohol concentration in percent volume of alcohol per volume of the sample. To validate the alcohol concentration at selected sampling intervals, samples were sent to a commercial laboratory, i.e. Koelenhof Winery Laboratory, Stellenbosch, Western Cape, South Africa, where the pycnometric titration procedure was performed. Sugar concentration was determined using a density meter (Appendix A5) which measures the sugar concentration in ^{o}Brix. The alcohol and sugar results were converted to grams per litre (g/L).

4.3.2 Determination of total acid using minititrator and pH using a pH meter

To determine the total acid concentration, the minititrator (Appendix A5) was used, which measures the total acidity of the sample in grams of acid per litre of sample. The determination of pH was also performed using the Metrohm pH meter (Appendix A5).

4.4 Kinetic modelling for fermentation (autoclaved must trials only)

The decision was taken to model only the successful EtOH-AcOH fermentation process, which were the autoclaved must trials. Kinetic modelling for the EtOH-AcOH fermentation process was done using physical chemistry equations for consecutive elementary reactions (Table 4-1), i.e. first order reactions. The modifications of the equations are discussed under the results and discussion section 4.5. Some equations for substrate, product formation rates and yields were also used.

Table 4-1: Equations/ models used for EtOH-AcOH fermentation modelling

Parameters	List of models	Model /Eq. number
Product yield based on substrate utilisation	$Y_{P/S} = \frac{P_f - P_0}{S_0 - S_f}$	**2.12**
Overall reaction for vinegar fermentation (consecutive reaction)	$\mathbf{S} \xrightarrow{K_a} \mathbf{E} \xrightarrow{K_b} \mathbf{A}$	**2.16**
Sugar consumption	$[S] = [S]_0 e^{-\mathrm{K_b t}}$	**2.20**
	$\frac{\mathrm{dS}}{\mathrm{dt}} = -K_a[S]$	**2.17**
Ethanol concentration changes with time	$\frac{\mathrm{dE}}{\mathrm{dt}} = K_a[S] - K_b[E]$	**2.18**
	$\frac{\mathrm{dE}}{\mathrm{dt}} + K_b[E] = K_a[S]_0 e^{-\mathrm{K_b t}}$	**2.21**
	$[E] = \frac{K_a}{K_b - K_a}(e^{-K_a t} - e^{-K_b t})\,[S_0]$	**2.22**
	$\frac{dE}{dt} = -\frac{K_a\,[S_0]\;(K_a e^{-K_a t} - K_b e^{-K_b t})}{K_b - K_a}$	**2.24**

Cont. **Table 4-1**

Time at which ethanol is at its maximum concentration	$t_{max} = \left(\frac{1}{K_b - K_a} \ln \frac{K_a}{K_b}\right)$	**2.25**
Acetic acid formation	$\frac{dA}{dt} = K_b[E]$	**2.19**
	$[A] = \left(1 + \frac{K_a e^{-K_b t} - K_b e^{-K_a t}}{K_b - K_a}\right)[S_0]$	**2.23**

4.5 Sensory evaluation (autoclaved must trials products only)

Sensory evaluation was done on autoclaved must trials products only. The sensory evaluation was done by a panel of 10 trained judges. The end-product obtained from the autoclaved must trials was sweetened with cooked must in a ratio of 1:1. The method followed was similar to the Balsamic Vinegar of Modena IGP. The judges evaluated colour, aroma, viscosity, initial taste, middle taste and after taste, balance of acidity/sweetness and the overall quality of the product. The scores ranged from one to five (Table 4-2).

Table 4-2: Sensory evaluation scoring values and their meaning

Scoring values	Meaning of scores
1	Unacceptable
2	Acceptable
3	Average
4	Good
5	Excellent

4.6 Results and discussion: low-strength grape must

4.6.1 The effect of temperature on sugar, alcohol and total acid consumption/formation using autoclaved Chenin blanc must

Chemical developments observed when using autoclaved grape must showed expected results. As illustrated in Fig. 4-1A, the sugar consumption during the EtOH-AcOH fermentation process using the 0% inoculation strategy, was observed to be high in the first two weeks of the fermentation process, particularly for fermentations conducted at 28°C, with the second highest sugar consumption being observed for the fluctuating temperature trials. As observed with microbial growth rates, the trials in which the temperature fluctuated also had a fluctuating trend, showing that the fermentation kinetics are directly affected by the fluctuating temperature. At 22°C, the lowest sugar consumption rates were observed. The rate of sugar consumption r_s values for the 22°C, 28°C and fluctuating temperature experiments were 2.57, 3.12 and 3.42 g/L.day, respectively.

Fig. 4-1B illustrates ethanol formation and consumption using the 0% inoculation strategy. The data correlated with the sugar consumption data obtained, with the fluctuating temperature trials showing a higher sugar consumption and, therefore, higher ethanol production. However, the ethanol quantified when using the 0% inoculation strategy did not symbolise the quantity of ethanol that was actually produced during the process. As ethanol is the substrate for AAB that was inoculated simultaneously with the yeast, this makes ethanol production and consumption occur simultaneously. This makes it difficult to measure how much ethanol was produced with the 0% inoculation strategy. Therefore, the r_s/r_p values were not calculated.

Fig. 4-1C showed that the total acid formation at 28°C, i.e. with the highest product formation can easily be modelled. The 22°C and fluctuating temperature curves are comparable. The total acid produced was the final product used in determining model parameters. The total acid profile at 28°C showed that there was higher ethanol formation and thus its consumption. At 28°C (see Fig. 4-1B), a higher ethanol formation within the first few days of fermentation subsequent to its lowering than the profiles obtained in other fermentations was attributed to the high ethanol consumption rate. When the 0% inoculation strategy was used, the 28°C temperature appeared to be the most suitable, as the desired 60 g/L of total acid was formed in less than 40

days. The rate of total acid formation (r_p) values for the 22°C, 28°C and fluctuating temperature were 1.25, 1.56 and 1.36 g/L.day respectively.

In the 6% inoculation strategy (Fig. 4-1D), the sugar was utilised rapidly at 28°C as expected; showing the highest sugar consumption rate, followed by the 22°C trials and, lastly, the fluctuating temperature showing the lowest sugar consumption. The r_s values for the 22°C, 28°C and fluctuating temperature were determined to be 4.64, 5.05 and 4.29 g/L.day, respectively. As observed with the cooked must trials, at some point during the EtOH-AcOH fermentation process, there is an increase in sugar concentration due to the evaporation of water or other volatile constituents in the must. Fig 4-1E shows the ethanol formation and consumption data when the 6% inoculation strategy is used. As seen with the 0% inoculation strategy, the 28°C showed the highest ethanol production rate prior to the inoculation with AAB. The 22°C trials and trials performed at a fluctuated temperature, indicated that the conditions led to similar results. Ethanol production was slightly lower than that observed for the 28°C temperature trial that correlated with the sugar consumption data obtained (Fig. 4-1D). After the inoculation of the AAB, ethanol consumption was high for both the fluctuating and 28°C temperature trials. Fig. 4-1F, showing total acid formation using the 6% inoculation strategy and the fluctuating and 28°C had the highest total acid formation. The r_p values for the 22°C, 28°C and fluctuating temperature were 0.96, 1.16 and 1.17 g/L.day respectively.

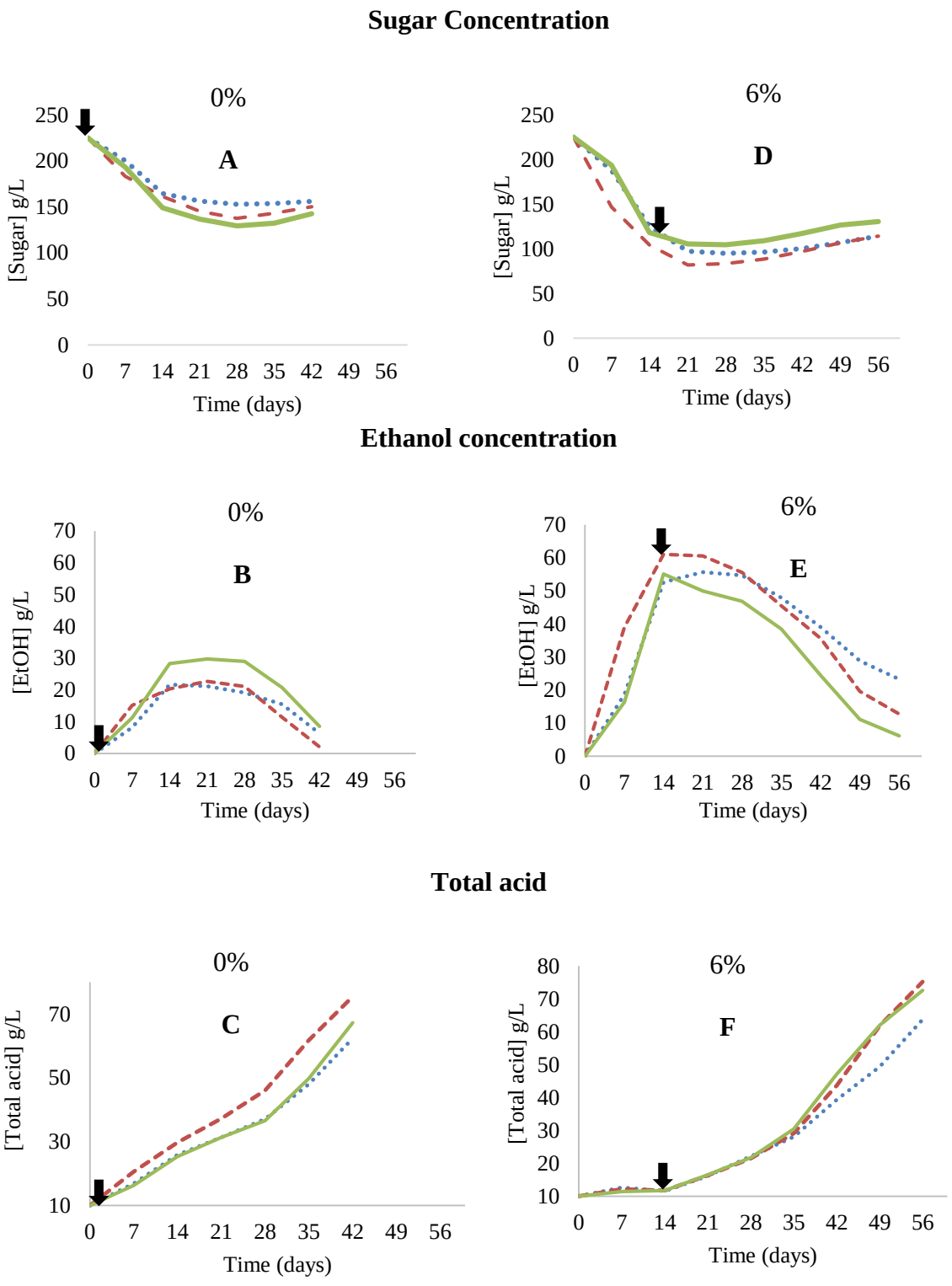


Figure 4-1: An illustration of sugar, alcohol and total acid concentration during the EtOH-AcOH fermentation process (comparing temperatures, ····· 22°C, - - - - 28°C, ——— fluctuating temperature, ⬇ Inoculation of AAB).- **Autoclaved grape must**

4.6.2 The effect of inoculation strategy on sugar, alcohol and total acid consumption/formation using autoclaved grape must

Fig. 4-2 shows the comparison between the 0% and 6% inoculation strategy under the same temperature. Fig. 4-2A, B & C shows the sugar consumption over the period of the EtOH-AcOH fermentation. It was observed that there were lower sugar consumption rates when using the 0% inoculation strategy while higher sugar consumption rates were seen when using the 6% inoculation strategy. The reason for this could be that when yeast and AAB are inoculated simultaneously at day 0, there is simultaneous alcohol consumption and acid formation. The acid formation may have inhibited some of the yeasts reducing their ability to carry out their normal fermentation functions. The 6% inoculation strategy showed that higher sugar consumption was achievable under all temperatures studied (Fig. 4-2A, B & C), a case in which the yeasts carried out the fermentation without the presence of AAB, as result, rapid sugar utilisation was seen, an advantage which can reduce the alcoholic fermentation cycle in batch systems. The r_s was between 2.5 to 3.4 g/L.day and 4.2 to 5.1 g/L.day for the 0% and 6% inoculation strategies, respectively.

Similarly, Fig. 4-2D, E & F shows data for ethanol formation and consumption to acetic acid at all the temperatures when comparing the two inoculation strategies. As expected, the 6% inoculation strategy trials performed better at all temperatures, a strategy in which the AAB were inoculated at day 14 while the yeasts were initially present on their own at the start of the fermentation. After the inoculation of AAB, the alcohol was rapidly metabolised to acetic acid. Furthermore, the trend observed with the use of the 0% inoculation strategy (Fig. 4-2D, E & F) showed a lower alcohol formation, an attribute associated with the presence of AAB. Although this graphical illustration does not fully represent all the ethanol that was being produced, it was reasonable to conclude that lower ethanol concentration was produced due to the competition between yeast and AAB and the low sugar consumption observed. The total acid trends assisted in deciding the most suitable inoculation strategy. Fig. 4-2G, H & I, illustrates the total acid; comparing the two inoculation strategies used. The 0% inoculation strategy resulted in the desired total acid concentration (60 g/L) at 42 days. The process was slower for the 6% inoculation strategy. This meant, although the 0% inoculation strategy results in lower sugar consumption thus lower alcohol formation, it was however, suitable to produce the desired total acid required for the BSV. The AAB appeared to oxidise ethanol rapidly when the ethanol concentration was lower than when it was 6% (v/v) or 60 g/L. The r_p was determined to be between 1.2 to 1.5 and 0.9 to

1.2 g/L.day for the 0% and 6% inoculation strategies, respectively. Furthermore, the higher residual sugar in the fermentation medium (0% inoculation strategy) was deemed beneficial in balsamic vinegar.

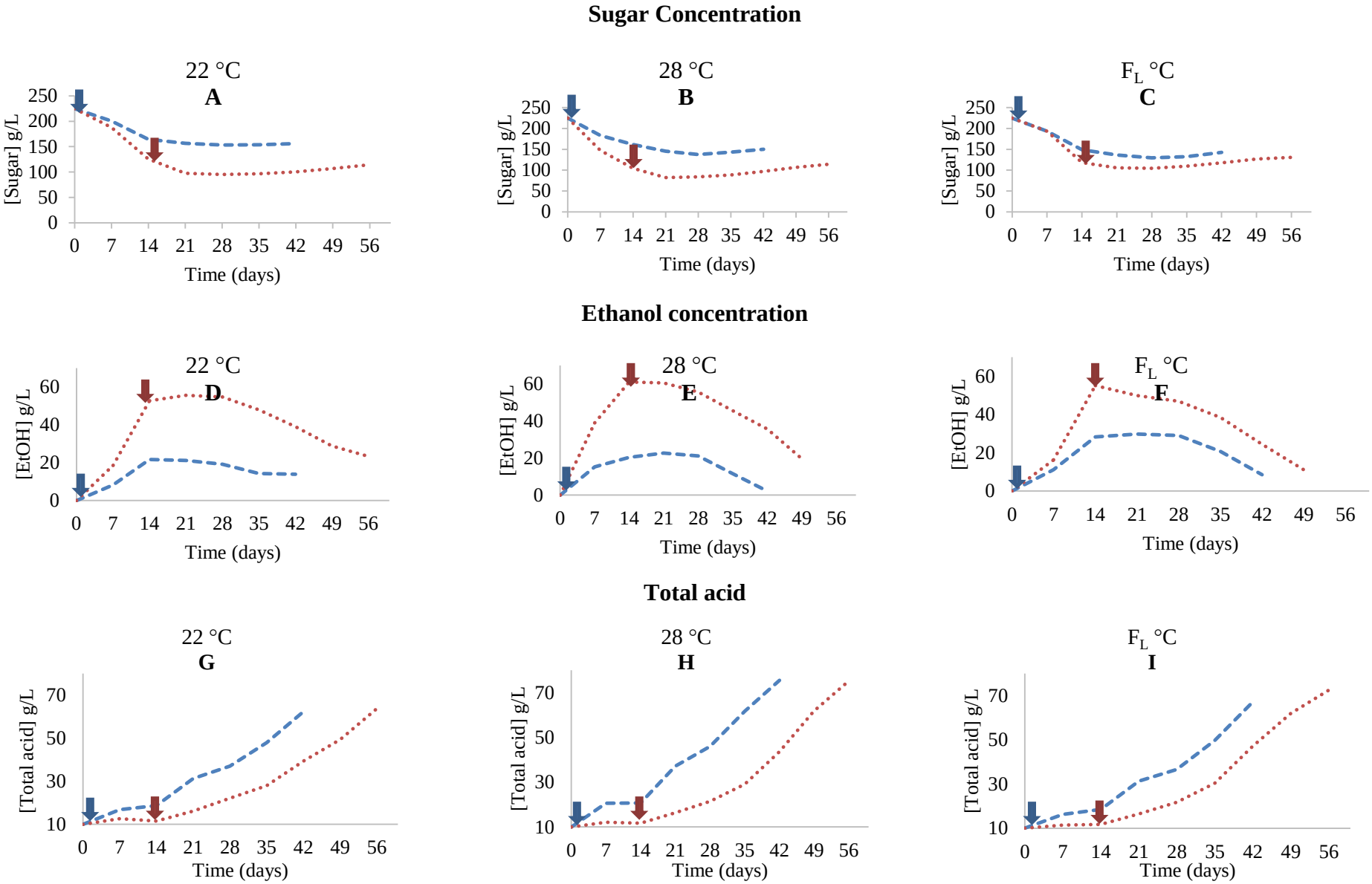


Figure 4-2: An illustration of sugar, ethanol and total acid concentration during the EtOH-AcOH fermentation process (comparing 0% and 6% inoculation strategy)-**Autoclaved grape must** 0% inoculation strategy 6% inoculation strategy, Inoculation of AAB

4.7 Summary: assessment of biochemical kinetic parameters for the low-strength grape must

Differences between cooked and autoclaved must trials were observed in the chemical development of BSV. The important difference was that the acetous fermentation process was not successful with the use of cooked must but was successful with the use of autoclaved must, as the chemical composition of the cooked and autoclaved must varied profoundly. Variations can include sugar concentration, water activity, total acid and polyphenols (Falcone, 2010). However, the sugar concentration was identified as a highly influential parameter which played a vital role in the differences observed between the trials studied. During the investigation, the desired total acid, as previously mentioned, which indicates the strength of the vinegar, quantifiable as the amount of acetic acid formation, was 6% (v/v)/60 g/L acidity. Therefore, the evidence was substantial to conclude that the yeasts used were not significantly affected by varying sugar concentrations or the variations of other chemical parameters studied between the two fermentation media. Comparatively, alcoholic fermentation was slower in cooked must, whereby it took 28 days to reach a 6% (v/v)/60 g/L of alcohol level while it took only 14 days when using the autoclaved must. Hence, it is plausible to state that the alcoholic fermentation activity/rate in autoclaved grape must was doubled. Overall, the acetic acid concentration of 60 g/L was achieved between 42 and 56 days for the autoclaved must trials, when compared with cooked must trials monitored up to 319 days, achieving a final acetic acid concentration of only 20 g/L.

This also meant that the back slopping approach described by Solieri *et al.* (2006) is worth considering for cooked must trials in the production of BSV.

4.8 Results and discussion: kinetic modelling of the EtOH-AcOH fermentation process (only autoclaved must trials)

4.8.1 Modelling for sugar consumption

Assumption:

- Initial reaction rate for sugar consumption (K_a) determines the success of the overall BSV process, such that:

$$\frac{\mathrm{dS}}{\mathrm{dt}} = -K_a[S] \qquad \textbf{Eq. 2.17}$$

To model the sugar consumption, Eq. 2.17 described by Atkins and De Paula (2006) was used. This equation can be used to simulate the decay, i.e. consumption or utilisation of the substrate (sugar). The equation proposed by Monteagudo *et al*. (1997) could not be used to model the sugar consumption since it also includes biomass yield based on substrate utilisation and the maintenance of biomass (See Eq. 2.10). In this study, the biomass was assumed not to represent the entire viable population because of the entry of the microbial consortia into the VBNC state.

$$-\frac{dS}{dt} = \frac{1}{Y_{X/S}}\frac{dX}{dt} + \frac{1}{Y_{P/S}}\frac{dP}{dt} + m_S X \qquad \textbf{Eq. 2.10}$$

Therefore, the simple equation proposed by Atkins and De Paula (2006) does not include any microbial growth or biomass parameter, which makes it applicable for modelling the substrate consumption kinetics during the EtOH-AcOH fermentation of BSV. Using Eq. 2.17, the rate at which the sugar was utilised with time was simulated, using the initial rate of reaction from the experimental data and the initial sugar concentration (*S*). The value used for K_a was determined using the change of sugar concentration between day 0 and 7. The reaction rate values were obtained from the linearised concentration values, whereby the slope (*m*) of the equation was the K_a value. Fig. 4-3 shows the experimental data and the modelled data for each temperature and inoculation strategy, with Fig. 4-3A showing the experimental data and the model for the 22°C trials using the 0% inoculation strategy. The model was deemed acceptable, as the model data gave values that were similar to those observed in the experiments. Fig. 4-4 shows graphical illustrations whereby the experimental data are fitted into the model (Eq. 2.17). The correlation coefficient values (R^2) validated the model, with the R^2 value at 22°C (Fig. 4-4A) being 0.89, which meant that there was a 89% fit. At 28°C (Fig 4-4B), the R^2 value of the model was higher, at 0.92 (92% fit). For the fluctuating temperature trial (Fig. 4-3C), the correlation between the experimental and

modelled data was not as good as the 22°C (Fig. 4-3A) and 28°C (Fig. 4-3B) trial. The reason could be that the fluctuating temperature was not at a steady state, which resulted in the change in reaction rates for the process. The use of the initial reaction rate in this case may not be applicable because after every seven days, the temperature for the fluctuating trial experiments was changed and this might have caused the rate of reaction to change significantly. This made the use of the initial reaction rate for the overall process not applicable. It may assist to use the overall reaction rate or change some of the model parameters to take into account changes in the fermentation conditions. However, the response given by the model (Fig. 4-4C) was still sufficient to simulate the EtOH-AcOH fermentation process for the fluctuating temperature. The R^2 value (Fig 4-4C) was determined to be of 0.92, a 92% fit.

As for the 6% inoculation strategy (Fig. 4-3D, E & F), the observations of the profiles made between the modelled data and experimental data showed limited similarity when compared to graphs for the 0% inoculation strategy (Fig. 4-3A, B & C). When analysing the modelled values, it was observed that the experimental and model data were similar at the initial stages with minimal deviations. The reason for this might have been that initially, the rate of reaction for sugar consumption was determined by the presence of yeast only, for which the AAB were introduced into the system at day 14 which caused alterations to the rate of reaction. Therefore, the initial rate of reaction for the 6% inoculation strategy may not represent the overall process, although the R^2 values for the 22°C, 28°C and the fluctuating temperature were 0.92 (92%), 0.84 (84%) and 0.86 (86%) respectively (Fig. 4-4D, E & F). Accordingly, a better fit was observed only at 22°C.

Table 4-3: Initial reaction rates (K_a) used for modelling sugar consumption for the two inoculation strategies and temperatures

	0% inoculation strategy	**6% inoculation strategy**
Temperature (°C)	K_a (day^{-1})	K_a (day^{-1})
22	0.0166	0.0257
28	0.0296	0.0608
F_L	0.0210	0.0214

Table 4-4: Other important variables used for modelling sugar consumption

	0% inoculation strategy		6% inoculation strategy	
Temperature °C	$[Sugar]_I$ g/L	$[Sugar]_F$ g/L	$[Sugar]_I$ g/L	$[Sugar]_F$ g/L
22		153.00		95.00
28	225	137.67	225	83.67
F_L		129.33		104.67

I- Initial concentration, **F**-Final concentration

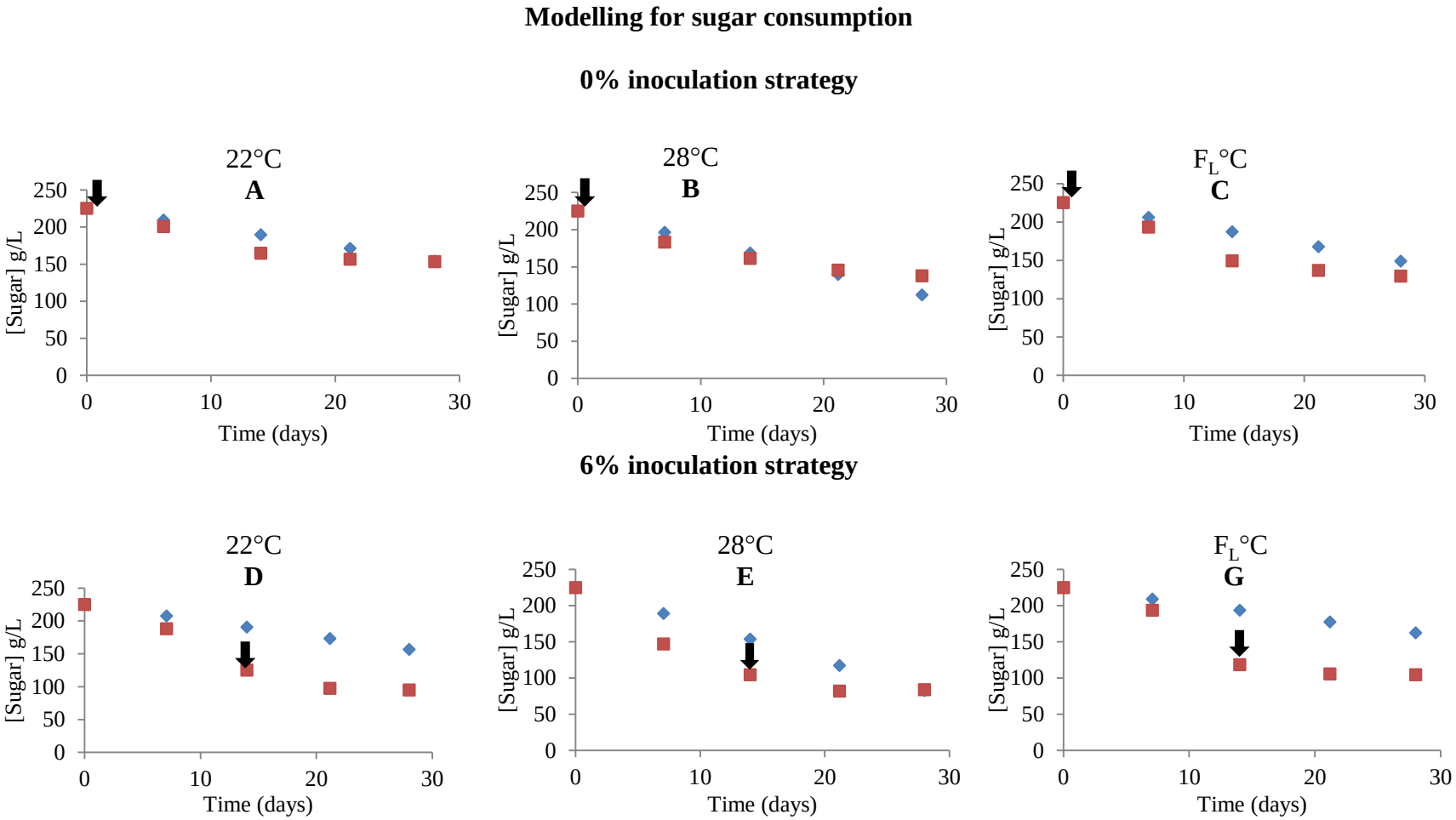
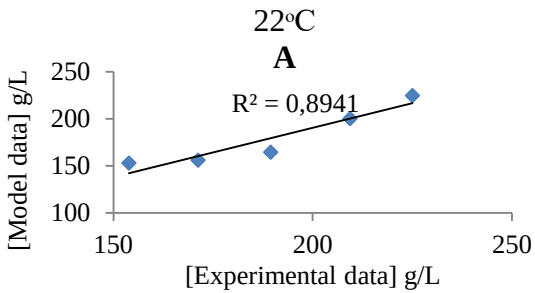
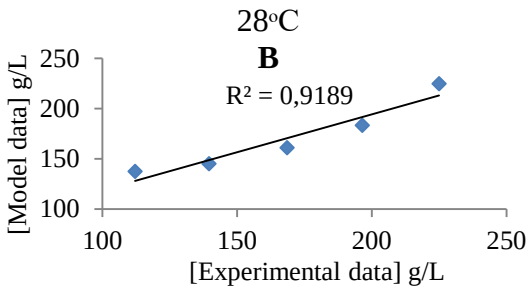


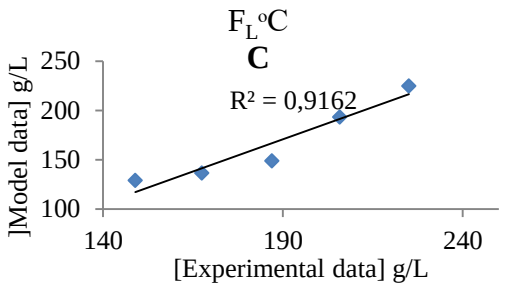
Figure 4-3: Modelling for sugar consumption during the EtOH-AcOH fermentation process using autoclaved Chenin blanc must ◆ Model, ■ Experiment, ⬇ Inoculation of AAB.

Modelling for sugar consumption

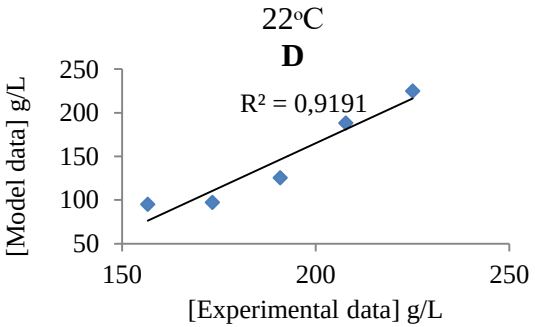
0% inoculation strategy

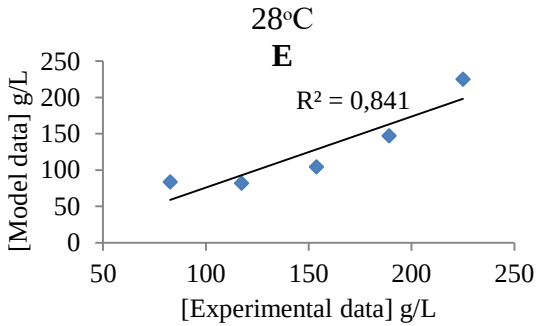






6% inoculation strategy





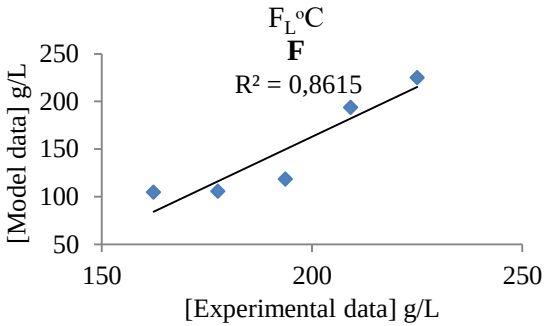


Figure 4-4: Sugar consumption kinetic data fitted into equation 2.17. (Model data versus experimental data).

4.8.2 Modelling for ethanol formation and consumption

Modelling the ethanol formation including consumption was provocative as ethanol was both an intermediate product and substrate in the BSV fermentation system designed. Eq. 2.18 describes a model which accounts for the formation and disappearance of ethanol using fermentable sugar at a rate K_a*S which is further oxidised to acetic acid at a rate K_b*E.

$$\frac{\mathrm{dE}}{\mathrm{dt}} = K_a[S] - K_b[E] \qquad \textbf{Eq. 2.18}$$

The explicit Eq. 2.22 offered a description for the fermentation taking place in the BSV system.

$$[E] = \frac{K_a}{K_b - K_a}(e^{-K_a t} - e^{-K_b t})\,[S_0] \qquad \textbf{Eq. 2.22}$$

The differential form of Eq. 2.18 requires the determination of rate constants K_a and K_b, which are parameters, that can be reused individually in a differential equation solver to model the intermediate formation and disappearance in a sequential manner for cell defined reactions. By using an assumption that the initial rate of reaction value determines the success of the fermentation process, values for both K_a and K_b can be estimated as shown in Table 4-5 for both the 0% and 6% inoculation strategy at all temperatures studied. The initial K_a and K_b values were obtained by linearising 1st order kinetics for ethanol concentration. Fig. 4-5A & B illustrates the point at which the initial K_b values were obtained, shown as K_{b1}. According to Atkins and De Paula (2006), when K_b is way greater or larger than K_a, it means that the intermediate, ethanol, is rapidly being consumed to form acetic acid. However, such an assumption could not be made until a suitable K_b value was identified to simulate ethanol formation including consumption as stated in the literature reviewed. This was also because biological systems behave differently to chemical reactions and as such stoichiometric models should be adapted to model the fermentation data obtained.

Additionally, the rate-limiting step was not identified based on the K_a and K_b values from the experimental data. Although it is known that the rate K_b*E depicts ethanol consumption while

the rate K_a*S depicts ethanol formation; it is unsuitable to utilise such an assertion due to the fact that the evaluation of K_a*S resulted in a model which gave values of ethanol formation that were out of range of the generated fermentation data. Therefore, an attempt was made to utilise only K_b values (Table 4-6; Table 4-7; Table 4-8) for ethanol formation which clearly defined the ethanol formation within a defined range (Fig 4-5A & B).

Table 4-5: Initial reaction rates (K_a and K_{b1}) of the inoculation strategies and temperatures

	0% inoculation strategy		6% inoculation strategy	
Temperature (°C)	K_a (day^{-1})	K_{b1} (day^{-1})	K_a (day^{-1})	K_{b1} (day^{-1})
22	0.0166	0.1279	0.0257	0.0306
28	0.0296	0.2444	0.0608	0.0616
F_L	0.0210	0.1276	0.0214	0.0280

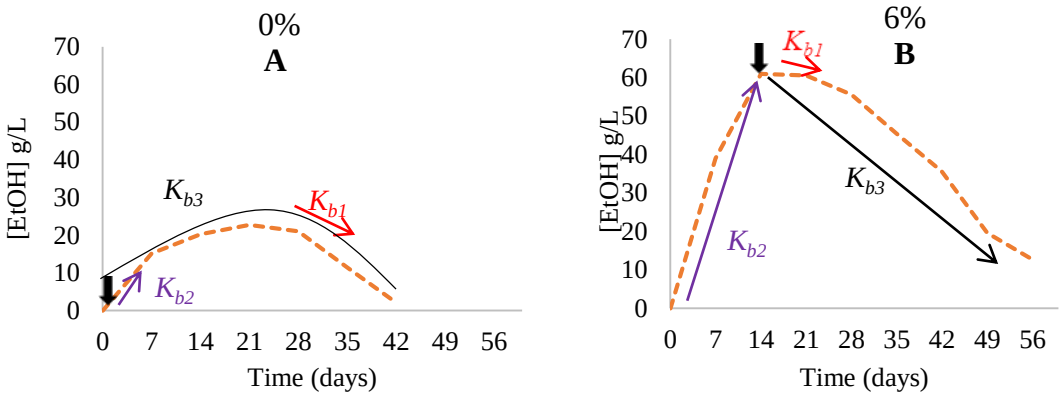


Figure 4-5: Ethanol curve for the 0% inoculation strategy (A), ethanol curve for the 6% inoculation strategy (B) graphs illustrating the possible reaction rates (K_b) which can be used to model for ethanol formation/consumption, ⬇Inoculation of AAB.

Both Fig. 4-5A & B indicate points whereby the K_{b1} values were determined from the experimental data. Table 4-5 also shows the K_{b1} values obtained for both the 0% and 6% inoculation strategy. The observations made in Table 4-5, for the 0% inoculation strategy indicated that the K_{b1} values were mostly greater than the K_a values. This was deemed appropriate since for the 0% inoculation strategy, it was observed that there was a higher total acid formation when compared to the 6% inoculation strategy due to the simultaneous formation and consumption of ethanol. For the 6% inoculation strategy, the ethanol was accumulating in the system prior to the

inoculation of the AAB used for the conversion of ethanol to acetic acid. It is prudent to highlight that the proposed rate equations are for simultaneous reactions, therefore are suitable when the 0% inoculation strategy is used.

Additionally, Eq. 2.25 was used to determine the time (t_{max}) at which ethanol reaches its maximum concentration for the 0% inoculation strategy only. This is important for reactions whereby the intermediate is the desired product. The K_{b1} values (Table 4-5) were used in conjunction with the K_a values to calculate t_{max} (see Table 4-6). The t_{max} values obtained were within a defined range observed for the BSV system with observations being that at a higher temperature (28ºC) ethanol reaches its maximum concentration rapidly compared to the other temperatures studied. When these results were compared to those obtained for the 6% inoculation strategy, the 28ºC BSV fermentation attained a high ethanol concentration while the 22ºC and a fluctuating temperature showed similar trends (Fig. 4-1E). The t_{max} observed for the 0% inoculation strategy, was estimated to be between day 14 and 28, with the calculated t_{max} values obtained using K_{b1} falling within this period, particularly for the 22ºC and fluctuating temperature fermentation. It was also likely that the t_{max} calculated for the fermentations at a higher temperature (28ºC) was correctly estimated using a comparative analysis of the experimental modelled data; however, sampling intervals were 7 days apart, and therefore, it was unknown what the actual ethanol concentration was at day 10. Additionally, the percentage variation was calculated using Eq. 4.1, i.e. calculated using modelled and experimental data using K_{b1} t_{max} and was determined to be as low as 22% and 17% for the 22ºC and fluctuating temperature, respectively, with a higher error observed for the fermentations at 28ºC.

Table 4-6: Time at which ethanol passes through a maximum (calculated using different K_b values –0% inoculation strategy)

Temperature (°C)	Actual/ Experimental t_{max} (day)	K_{b1} t_{max} (day)	K_{b2} t_{max} (day)	K_{b3} t_{max} (day)
22	14[a] to 28[b]	18*	9[D]	33[D]
28	14[a] to 28[b]	10[D]	2[D]	38[D]
F_L	14[a] to 28[b]	17*	9[D]	34[D]

a- minimum actual/Experimental *tmax*, **b**-maximum actual/experimental *tmax* *****- within range; **D**- out of range

$$t_{max} = \left(\frac{1}{K_b - K_a} \ln \frac{K_a}{K_b}\right) \quad \textbf{Eq. 2.25}$$

$$\% \text{ Error} = \left(\frac{|Experimental - Model|}{Model}\right) \text{x } 100 \quad \textbf{Eq. 4.1}$$

It was unreasonable that at a higher temperature (28ºC), ethanol was at its highest concentration at day 2 when K_{b2} (Table 4-7) was used; even for 22ºC and fluctuating temperature fermentations (9 days). In this study, the use of the K_{b3} (Table 4-7) values, i.e., the overall reaction rates was considered impracticable to determine t_{max}. Since the overall reaction rates determined the overall process performance, while the models used required sequential steps of the reaction to be decoupled to estimate the complete formation and disappearance of the intermediate. Additionally, the t_{max} values obtained did not fall within the t_{max} values observed from the experimental data (Table 4-6). Overall, the initial rates offer a better opportunity to describe the process accompanied with the assumption that initially ethanol formation was not affected by the presence of AAB. Additionally, K_{b1} values can be used to successfully describe the process using Eq. 2.24.

$$\frac{dE}{dt} = -\frac{K_a [S_0] \left(K_a e^{-K_a t} - K_b e^{-K_b t}\right)}{K_b - K_a} \quad \textbf{Eq. 2.24}$$

Alternatively, in order to successfully model ethanol formation including consumption, stoichiometric coefficients used in chemical reactions can be considered. Particularly when balanced chemical reactions (Eq. 4.2) are used to illustrate what was not observed in the experimental data. Primarily for the 6% inoculation strategy, in which sequential biological reactions are observed; as the 0% inoculation strategy reactions were classified as simultaneous reactions.

With the consideration of the stoichiometric coefficients, it was observed that the molar ratio of ethanol formation is normally 2:1 (see Eq. 4.2), for which a mole of sugar ($C_6H_{12}O_6$) consumed, generates 2 moles of ethanol (C_2H_5OH). By using such an analogy the reaction rates can therefore be quantified considering the stoichiometric coefficients. This meant, that for the

simulation of ethanol formation, the rate of sugar consumption (K_a) would be directly linked to such coefficients. This provided a feasible and hypothetically conclusive explanation to the high ethanol formation profiles initially observed when K_a*S was used without the consideration of stoichiometric coefficients.

Overall, the balanced molecular reaction for glucose fermentation to ethanol by yeast can be described as:

$$C_6H_{12}O_6 \xrightarrow{\text{Yeast}} 2CO_2 + 2C_2H_5OH \qquad \textbf{Eq. 4.2}$$

While the subsequent reaction to produce acetic acid from ethanol using AAB can be described using a balanced molecular reaction such that:

$$C_2H_5OH + O_2 \xrightarrow{\text{AAB}} CH_3CHO + H_2O \qquad \textbf{Eq. 4.3}$$

For biological systems, Eq. 4.2 & 4.3, although they describe reactions taking place, cannot be used for the direct determination of the fermentation process without modifications.

Table 4-7: Reaction rates for the 0% inoculation strategy

Temperature °C	K_a (day^{-1})	K_{b2} (day^{-1})	K_{b3} (day^{-1})
22	0.0257	0.3006	0.0341
28	0.0608	0.3884	0.0084
F_L	0.0214	0.3451	0.0392

Table 4-8: Reaction rates for the 6% inoculation strategy

Temperature °C	K_a (day^{-1})	K_{b2} (day^{-1})	K_{b3} (day^{-1})
22	0.0257	0.417	0.0024
28	0.0608	0.5234	0.0121
F_L	0.0214	0.3987	0.0140

Table 4-9: Other important variables used for modelling ethanol formation and consumption for both inoculation strategies

	0% inoculation strategy		6% inoculation strategy	
Temperature °C	$[Ethanol]_I$ g/L	$[Ethanol]_F$ g/L	$[Ethanol]_I$ g/L	$[Ethanol]_F$ g/L
22	0	6.33	0^a & 55.63^b	23.27
28		2.07	0^a & 60.00^b	12.73
F_L		8.5	0^a & 57.07^b	9.15

I-Initial concentration, **F**-Final concentration

a- Initial ethanol concentration at the start of fermentation, **b**-Ethanol concentration when AAB was inoculated

4.8.3 Modelling for total acid formation

Modelling the total acid formation requires the modification of Eq. 2.19. Generally, the rate at which total acid is formed is directly proportional to the rate at which ethanol is consumed (K_b*E). However, modelling such a reaction can be impracticable in this context, due to the non-sequential nature of fermentation. Particularly for the 0% inoculation strategy, due to AAB inoculation at the beginning of the EtOH-AcOH fermentation process (Fig. 4-6A). The initial and overall reaction rates (K_{b1}, K_{b2} & K_{b3}) being used to model process dynamics, resulted in the inadequate description of the process with minimum correlation between modelled and experimental data. Thus, the rate K_b observed from the ethanol consumption experimental data was determined to inadequately simulate the profile for total acid formation (see Fig. 4-6B). Challenges were also encountered for the 6% inoculation strategy experiments. As such, the modelling was distinctly separated into two separate sections, i.e. prior and post inoculation of AAB (see Fig. 4-7A).

$$\frac{\mathrm{dA}}{\mathrm{dt}} = K_b[E] \qquad \textbf{Eq. 2.19}$$

This meant that K_b values were used (K_{b2} and K_{b3}) (Fig. 4-7A). Initially, the K_{b2} values obtained were significantly differentiated from actual fermentation data indicating higher acetic acid concentration being formed prior to the inoculation of the AAB. This led to the utilisation of K_{b3} for which the model values were determined to be also unsuitable (Fig. 4-7A). As the total acid profile, although simulated a decrease, may not be a true representative of the actual ethanol consumption due to the presence of yeast. The presence of yeast contributed to the continued

production of alcohol as the K_{b3} values used were small, which resulted in the model data showing a trend lower than that observed for the actual total concentration obtained in the BSV system. Fig. 4-6A & B; Fig. 4-7A & B provides a comparative analogy as to where the K_b calculated from ethanol concentration profiles are used in the total acid graph to illustrate the failure of using K_b values to model total acid formation. Furthermore, the rate at which the sugar is consumed to form ethanol rather than acetic acid cannot be directly used to simulate acetic acid formation. Therefore, the fermentation of sugar to ethanol can be classified as a separate and possibly a partially anaerobic process, which will limit the formation of acetic acid- a strictly aerobic process (see Eq. 4.2).

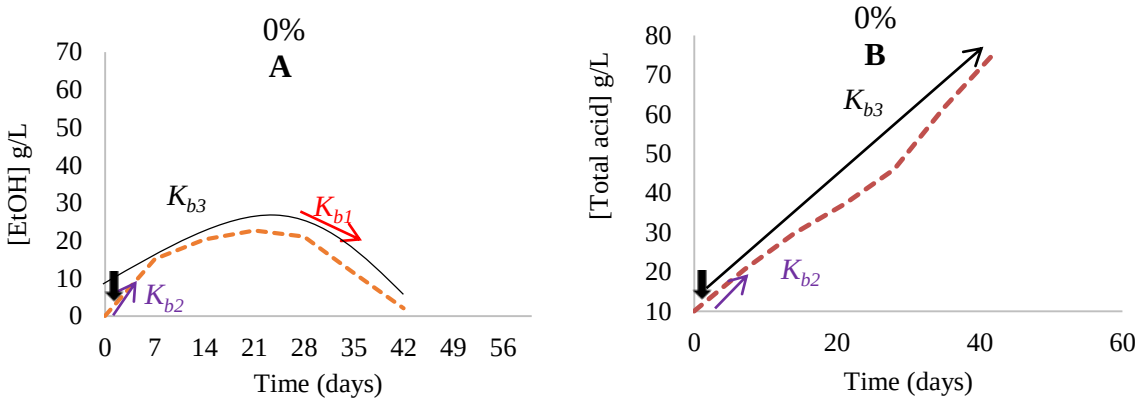


Figure 4-6: Ethanol (A) and total acid (B) graphs illustrating the reaction rates observed when using the 0% inoculation strategy, ⬇ Inoculation of AAB.

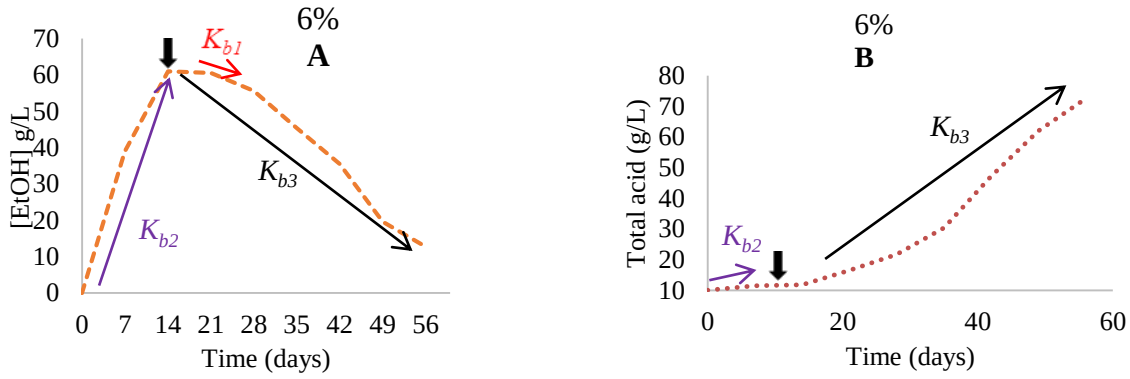
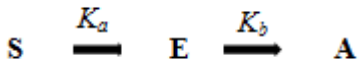


Figure 4-7: Ethanol (A) and total acid (B) graphs illustrating the separate reaction rates observed when using the 6% inoculation strategy, ⬇ Inoculation of AAB.

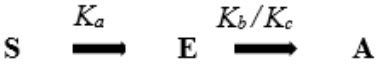
Therefore, Eq. 2.19 was modified. The modified equation has the rate constant, which was independently quantified- K_c (Eq. 4.4). Which meant that the model used to simulate the total acid formation, i.e. the rate at which total acid was formed from the experimental data (K_c*A), was elaborately done to exclude observed ethanol disappearance rates. This approach was used successfully (Fig. 4-8 & Fig. 4-9).

The normal scenario described by Atkins and De Paula (2006) for consecutive reactions is as shown in Eq. 2.16.



Eq. 2.16

The Eq. 2.16 can be decoupled and expressed as Eq. 4.6:



Eq. 4.4

As such, the formation of acetic acid can then be described by modifying Eq. 2.19 to incorporate *Kc*:

$$\frac{\mathrm{dA}}{\mathrm{dt}} = K_c[A] \qquad \textbf{Eq. 2.19}$$

$$[A] = \left(1 + \frac{K_a e^{-K_b t} - K_b e^{-K_a t}}{K_b - K_a}\right)[S_0] \qquad \textbf{Eq. 2.23}$$

The modified Eq. 2.19 was successfully used, i.e., accompanying overall reaction rates (Table 4-10). Fig. 4-8 shows comparative analysis of the modelled and the experimental data for the two inoculation strategies including temperature regimes used. The model and experimental data in all the graphs showed a good correlation, with R^2 values ranging from 0.98 to 0.99; retrospectively, achieving a 98 to 99% fit. Therefore, it was plausible to use the K_c values with the modified Eq. 2.19 to model total acid formation.

Table 4-10: Overall reaction rates (K_c) used for modelling total acid formation

	0% inoculation strategy	6% inoculation strategy
Temperature (°C)	K_c (day^{-1})	K_c (day^{-1})
22	0.0405	0.0337
28	0.0444	0.0377
F_L	0.0424	0.0391

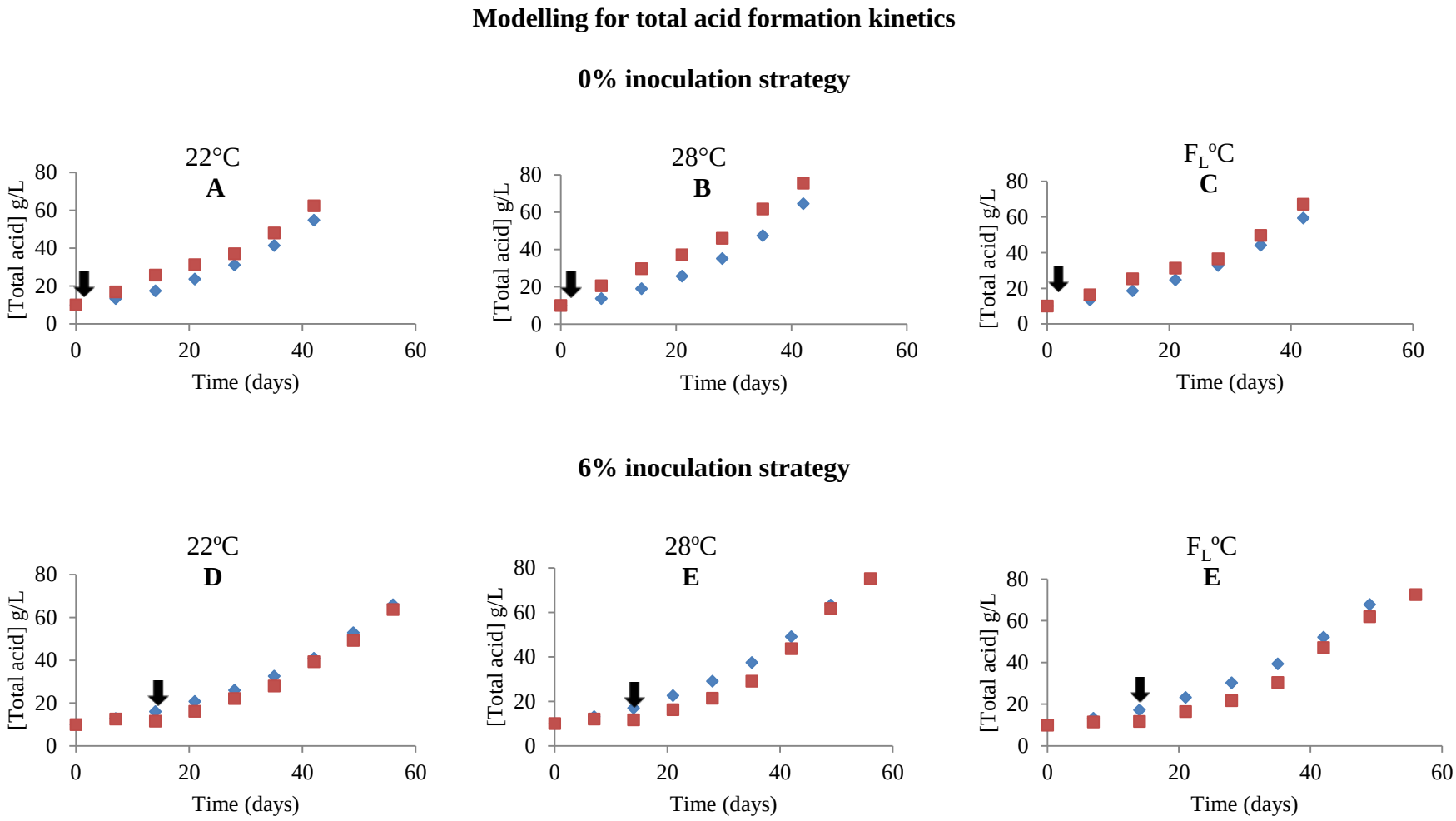
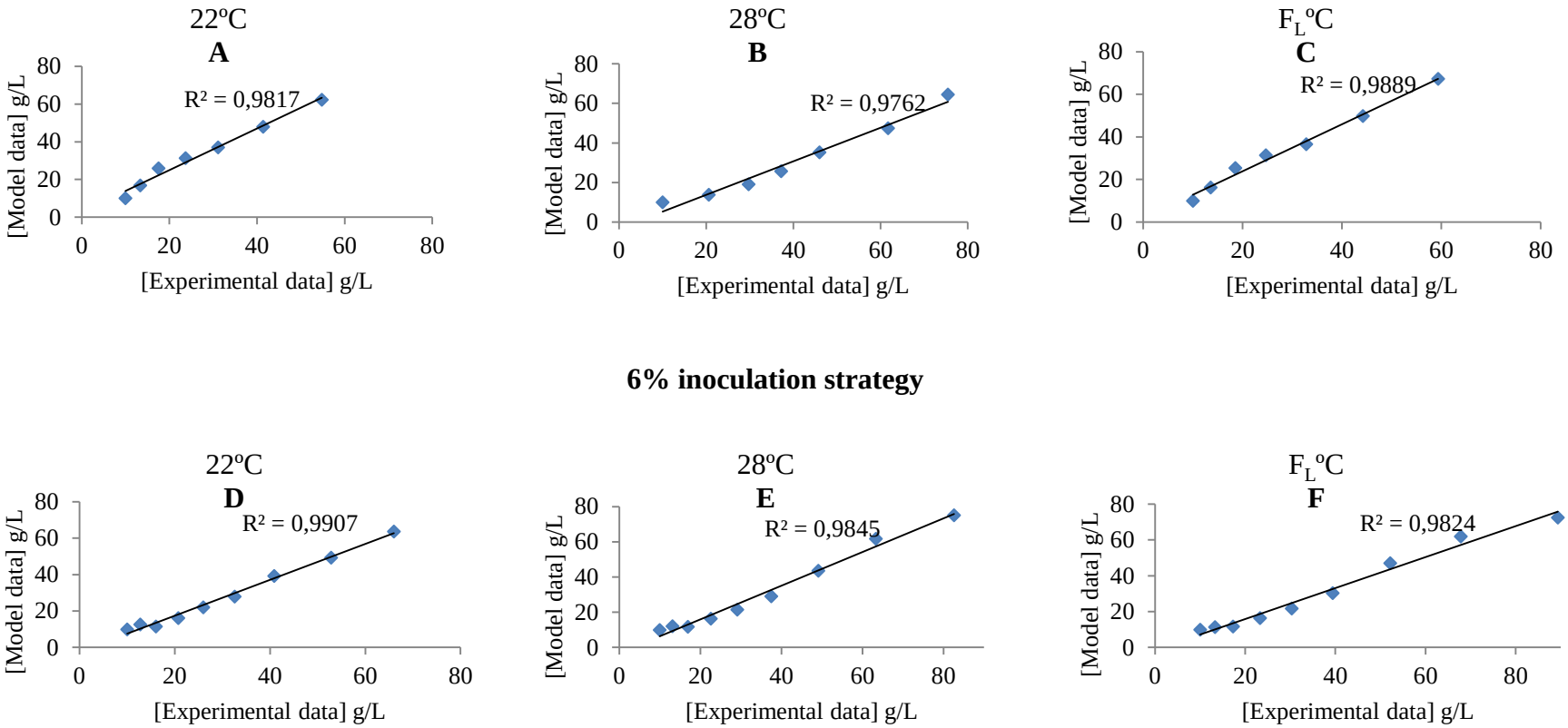


Figure 4-8: Modelling the total acid formation kinetics during the EtOH-AcOH fermentation process using autoclaved grape must ◆ Model, ■ Experiment, ⬇ Inoculation of AAB.

Modelling for total acid formation

0% inoculation strategy



6% inoculation strategy

Figure 4-9: Total acid formation kinetic data fitted into Eq. 2.19 (model data versus experimental data).

4.9 Results and discussion: sensory evaluation of BSV using autoclaved grape must

Sensory attributes are by far the most important attributes in any food product, as they determine consumer acceptance. The final sensory quality of a fermentation product is due to various interactions among chemical components of the fermentation medium and environmental factors (Styger *et al*., 2011). It will not matter whether a certain inoculation strategy or temperature results in a short fermentation cycle and better microbial growth if the end-product is not accepted by consumers. Therefore, it is crucial to evaluate the effect of the inoculation strategy and temperature used on the final product. Temperature has an effect on microbial growth and if a specific temperature is stressful to the microorganisms, this can result in the secretion of off-flavour compounds (Swiegers & Pretorius, 2005; Styger *et al*., 2011). The inoculation strategy can also play a role in the sensory evaluation results such as the 0% inoculation strategy resulting in a competitive relationship between yeast and AAB (Giudici *et al*., 2015). Their antagonistic effects on each other might also result in the secretion of off-flavour compounds.

The general appearance of good balsamic vinegar is described as dense and viscous; it resembles the texture of syrup, should be deep brown in colour and glossy. It is not harsh on the nose like spirit vinegar. It has acetic acid, dried fruit and floral aromas. It is also not sharp on the palate. Unlike other vinegars, it can be tasted on its own. Therefore, there must be a good balance between the sweetness and acidity on the palate. The flavours and taste of balsamic vinegar can be complex, but it is often described to have flavours such as cherries, dates, raisins and caramel (Meathead, 2011; Solazi, 2014).

In this study, a panel of ten judges evaluated the end-product. Sensory evaluation was done only for autoclaved must trials whereby cooked must was added to sweeten the vinegar in a ratio of 1:1. The judges evaluated colour, aroma, viscosity, initial taste, middle taste and after taste, acidity-sweetness balance and the overall quality of the product. The scale ranged from 1 to 5, whereby 1 meant unacceptable and 5 meant excellent. When comparing the 0% and 6% inoculation strategy sensory data (Fig. 4-10A, B & C), some differences were observed. At 22°C (Fig. 4-10A), the colour, aroma and viscosity of the products were rated the same with differences being observed with taste, acidity/sweetness balance and overall quality. Furthermore, the 0% inoculation strategy products at 22°C were given higher scores than the 6% inoculation strategy product for taste, acidity/sweetness balance and overall quality. The taste and overall quality were given scores that were slightly above average with the acidity/sweetness balance being rated as

good. The 28°C BSV for 0% and 6% inoculation strategy gave almost similar results (Fig. 4-10B), with minor differences being observed which meant a great deal in terms of sensory analysis. The minor differences were that a score of three meant average and four meant good, so minor differences should not be ignored. The 6% inoculation strategy products from 28°C were scored at average to less than desirable for most of the sensory attributes while the 0% inoculation strategy products were scored above average and were scored good for taste and acidity/sweetness balance. The fluctuating temperature products for the 0% and 6% inoculation strategy were given similar scores (Fig. 4-10C). Generally, all the sensory attributes were rated average, with only minor differences being observed for aroma and after taste, with the 0% inoculation strategy products being rated slightly higher for these attributes. Overall, the 0% inoculation strategy resulted in an acceptable product based on the sensory evaluation data obtained.

When comparing the different temperature products under each inoculation strategy (Fig. 4-10D & E), some differences were observed for the 0% inoculation strategy, with 22°C and 28°C products being given better scores (Fig. 4-10D). The fluctuating temperature products were scored slightly less and a very lower score was given for the acidity/sweetness balance in this case. As for the different temperature products for the 6% inoculation strategy (Fig. 4-10E), the scores were almost similar under all temperatures studied. The 28°C products were scored slightly higher for acidity and sweetness balance, followed by the fluctuating temperature product and the 22°C products which were scored the least for that attribute. In conclusion, the 0% inoculation strategy products were scored higher for all temperatures with the exception of the fluctuating temperature, which showed minor differences between the 0% and 6% inoculation strategy. As for the temperatures, the 28°C products were scored higher for both inoculation strategies.

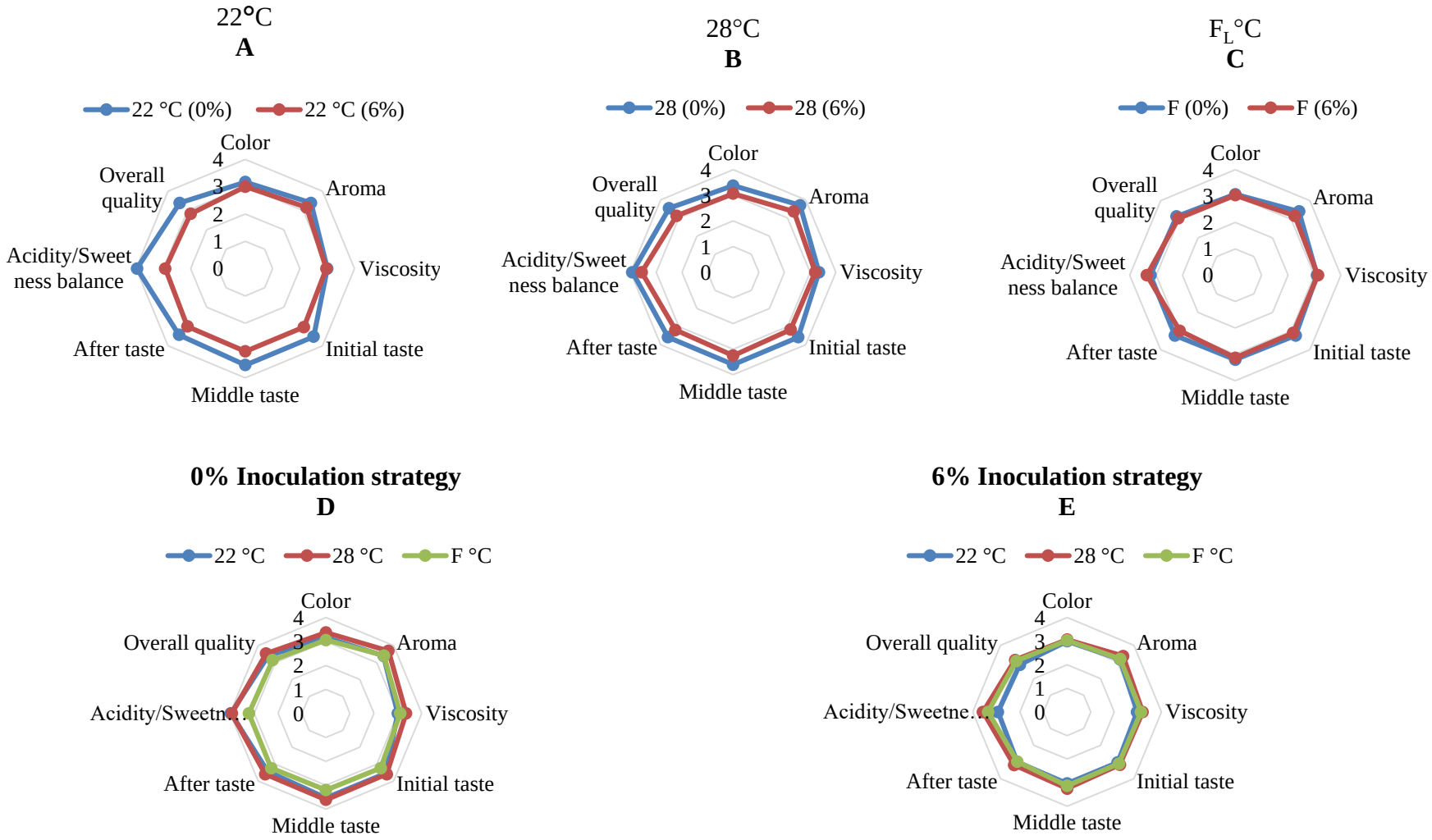


Figure 4-10: Radar graphs depicting sensory results comparing the two different inoculation strategies and temperatures used.

Chapter 5

Population dynamics on selected product using a metagenomics approach

Chapter 5

Microbial characterisation on selected product using a metagenomic approach

5.1 Introduction

The colonisation of fermentation systems by microbial species has been mostly reported using culture-dependent techniques. This approach misrepresents the microbial diversity with the fermentation process since most microorganisms are unable to grow in laboratory conditions.

Researchers have reported that more than 99% of microorganisms cannot be grown cultured in the laboratory although they are viable (Schloss & Handelsman, 2005). This led to limitations in the understanding of microbial colonisation in food products until the discovery of the metagenomic approach in 2004 (Chistoserdova, 2014). Metagenomics is a culture independent method of analysing a diverse microbial community from genetic material extracted directly from a sample (Schloss & Handelsman, 2005; Frank & Pace, 2008; Swanson *et al*., 2011).

Metagenomics has provided a solution to some of the challenges faced by researchers with respect to bioenergy, biotechnology, biomedical sciences, bioremediation, agriculture, earth sciences, life sciences and microbial forensics (Handelsman *et al*., 2007). To discover the diverse microbial community of a defined environment. DNA can be directly extracted from the sample using the most reliable DNA extraction methods prior to analysis using metagenomics (Thomas *et al*., 2012; Natarajan, 2013). Furthermore, longer read lengths are desirable because they are reliable for the identification of the organisms, specifically new organisms. However, the DNA extracted is often too fragmented; hence, making it difficult to obtain libraries with longer read lengths (Natarajan, 2013). The platforms used for metagenomics are different. Previously, the classical Sanger sequencing technology was used; arguably, this technology is still of high standard due to the low error output. Although currently, metagenomics usually employs next generation sequencing (NGS) applicable using the 454/Roche and the Illumina/Solexa systems (Thomas *et al*., 2012; Natarajan, 2013).

The 454/Roche system makes use of emulsion polymerase chain reaction (ePCR). The ePCR allows the amplification of random DNA fragments that are fixed on microscopic

beads. Furthermore, the 454/Roche technology enables the production of 600-800 bp read lengths, with most reads being interpreted significantly. The Illumina/Solexa technology approach employs the immobilization of random DNA fragments on a surface. Thereafter, solid-surface PCR amplification is performed, which results in clusters of the same DNA fragments (Thomas *et al*., 2012). Overall, due to the nature of the fermentation being studied, in which VBNC state was observed, it was vital to assess the microbial ecology of the product identified as being of suitable quality, as discussed in Chapter 4.

5.2 Aims and objectives

- To identify the microbial composition subsequent to the EtOH-AcOH fermentation process using metagenomics. To ascertain which species were present throughout the EtOH-AcOH fermentation process.

5.3 Materials and methods

For the metagenomics approach, the 28°C-0% inoculation strategy-day 42 product, was sampled and DNA was directly extracted from the sample using a ZR Fungal/Bacterial DNA Kit (Zymo Research, California, USA). The DNA was PCR-amplified using the universal primer pairs 341F 5'-CCTACGGGNGGCWGCAG-3' and 785R 5'-GACTACHVGGGTATCTAATCC-3' targeting the V3 and V4 of the 16S rRNA genes (bacteria) whereas the 18S rRNA gene was amplified using the 566F 5'-CAGCAGCCGCGGTAATTCC-3' and 1200R 5'-CCCGTGTTGAGTCAAATTAAGC-3' primer pairs, for detection of moulds. The PCR-amplicons were sequenced at Inqaba Biotechnical industries (South Africa), a commercial Next-Generation Sequencing (NGS) service provider. Briefly, the amplicons obtained were gel purified, repaired and Illumina® specific adapter sequences were ligated to each amplicon.

Thereafter, the samples were individually indexed followed by a purification step. The amplicons were then sequenced on an Illumina® MiSeq-2000 platform, using a MiSeq v3 (600 cycle) kit. 20Mb of data, i.e. 2x300bp long paired end reads were produced for each sample. The readings were used in the Basic Local Alignment Search Tool (BLAST) while the resulting file was saved. The top hit for every BLAST result (i.e. genus and species name) was counted and a record was kept of how many times each species appeared as a hit. The data was then used for taxonomic classification.

5.4 Results and discussion

5.4.1 Yeast population (18S analyses)

It was essential to analyse the yeast population in the final product. Normally, primary alcoholic fermentation takes a period of three to seven days to complete (Mendes-Ferreira *et al*., 2004); however, non-*Saccharomyces* yeast have slower growth rates, which leads to sluggish fermentations (Quirós *et al.*, 2014). Therefore, it was important to qualitatively assess the non-*Saccharomyces* yeasts in the final product. Such data would also elucidate the microbial profile of the EtOH-AcOH fermentation process. Studies have reported that some of the organisms used in the inoculum are resistant to high acetic acid concentrations (Guldfeldt & Arneborg, 1998; Pampulha & Loureiro-Dias, 1989) therefore; some of the yeasts were expected to occur at the end of the fermentation.

The 18S analyses with reference to kingdom classification (Fig. 5-1A) showed that 46.65% of the population was unknown, while 41.68% was fungi. The phylum classification results (Fig. 5-1B) showed that 66.13% of the population is unknown, while 12.98% is Ascomycota, with Glomeromycota being 9.32%. Ascomycota and Glomeromycota share a common ancestor, i.e. Eumycota, with reclassification resulting to 22.3% Eumycota. The class classification results (Fig. 5-1C) showed that 66.14% of the population is unknown, 12.48% is Sordariomycetes and 9.32% is Glomeromycetes, with the order classification results (Fig. 5-1D) showing that 66.14% of the population is unknown, while 12.47% is Hypocreales and 9.32% being Diversisporales. Overall, the family classification results (Fig. 5-1E) showed that 66.14% of the population is unknown, 12.46% is Nectriaceae, while 7.41% is Gigasporaceae. Additionally, the family Nectriaceae, belonging to the Ascomycota phylum, has spore forming *Fusarium* sp., which can be found on the surface of grapes; and is known to cause wilt in grape vines, i.e. Fusarium wilt, and secondly, Mycorrhizal fungi, members of the Gigasporaceae are widely prevalent in grapevines, explaining their notable presence in the product assessed. This suggested that spores were not deactivated by the employed sterilization technique, resulting in the germination of the fungal during fermentation. Generally, fungal spores are not always deactivated by methods such as autoclaving (Tournas, 1994).

Although, a large proportion of the population was indicated as being largely unknown, under the kingdom classification, 41.68% was attributed to be fungi; tentatively, yeast does fall

under the fungi kingdom. Additionally, the phylum classification results showed that the fungi reported under kingdom classification constituted 12.98% Ascomycota and 9.32% Glomeromycota, i.e. 22.3% Eumycota. The yeasts initially inoculated were *Zygosaccharomyces bailii, Candida zemplinina, Metschnikowia pulcherrima, Hanseniaspora guilliermondii* and *Kloeckera apiculata, which* belong to the Ascomycota phylum. The blast output results (Fig. 5-1F) showed that 20.2% of an unknown population was deemed culturable – which was not observed during experimentation, 19.36% unculturable fungus, 14.63% uncultured marine type organisms, and 10.04% unculturable eukaryotes. This meant that 64.23% of the population was unculturable, which supported the observation of a VBNC state. Furthermore, the presence of marine organisms was attributed to the coastal geographical location of the vineyards.

Additionally, the grape micro-flora, which might be a source of the identified organisms, can be influenced by various factors, such as the grape cultivar, location of the vineyard and other vineyard related practices (Barata *et al*., 2012). Some soil derived microorganisms can also colonize grape berries through root endophytes. Soil dust carries some other microorganisms, which can easily colonise the grape surface via rain splashing. During harvesting, people can also transfer other microorganisms, which are normally not expected to be found on the grapes. In addition, neighbouring plants can also transfer other microorganisms via aerial or insect transportation (Barata *et al*., 2012).

Overall, a fermentation sample can contain a diversified microbial population. Similarly, a metagenomics study done in *Potopoto*, which is a maize dough used for the weaning of babies detected that there was *Escherichia coli* and *Bacillus cereus* present in the maize dough (Abriouel *et al*., 2006). Similarly, another study done on an African fermented cereal detected the presence of *Clostridium perfringens* and *Bacillus cereus* (Oguntoyinbo *et al*., 2011). These studies showed that metagenomics is important in evaluating food safety and population dynamics of the final product. Overall, it was clear that the microbial ecology of the final product was complex with the yeast inoculated constituted a significantly lower portion in the final product. This was considered reasonable considering the quantity of acetic acid that was present at that stage (60 g/L), a condition unsuitable for most organisms.

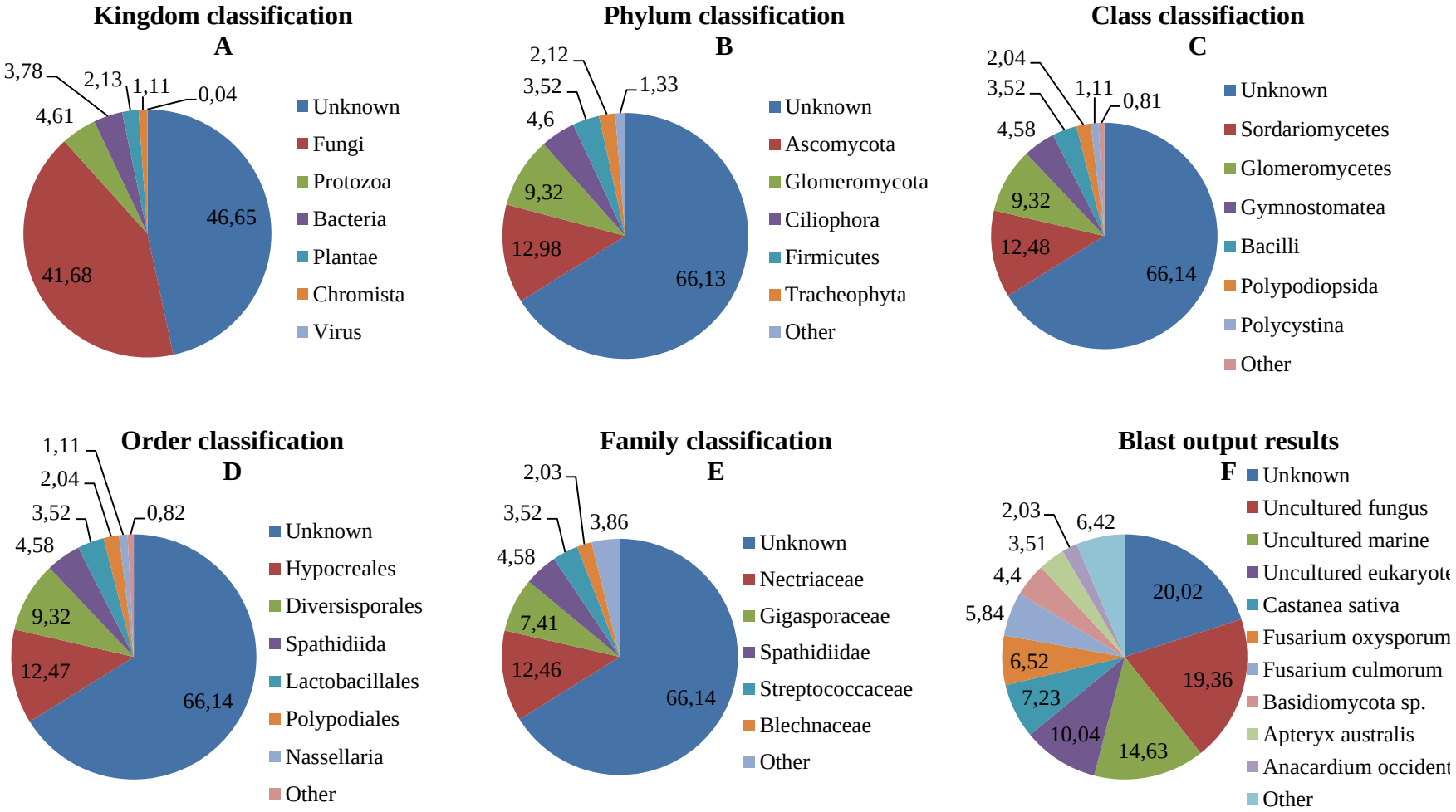


Figure 5-1: 18S analyses (yeast population) of the 0% inoculation strategy- 28ºC product.

5.4.2 Bacteria population (16S analyses)

The literature reviewed indicated that AAB are known to enter the VBNC state during the EtOH-AcOH fermentation process of balsamic vinegar. Therefore, this part of the study was to confirm the initial assumptions made, that the fewer colonies observed on agar plates was due to the VBNC state. As indicated previously, the 0% inoculation strategy (28°C) product was analysed using metagenomics.

The results obtained with respect to kingdom classification (Fig. 5-2A) showed that 98.16% of the population belongs to the bacteria kingdom. The phylum classification results (Fig. 5-2B) showed that 55.96% are Proteobacteria while 39.48% were unknown. These results were considered reasonable, because AAB in the phylum taxonomic category are classified as Proteobacteria. Additionally, the class classification results (Fig. 5-2C) showed that 52.24% of the population is Alphaproteobacteria while 39.50% was unknown while the order classification results (Fig. 5-2D) indicated that 51.17% of the population was Rhodospirillales while 39.48% was unknown. The Rhodospirillales are an order of Proteobacteria, with two families: the Acetobacteraceae and the Rhodospirillaceae. As observed with the 18S rRNA data, a major source of some Rhodospirillaceae is seawater, thus the outcome was seen in the 16S analyses.

The family classification results showed that 51.17% of the population belongs to the *Acetobacteraceae* family while 39.63% was unknown. The results were reasonable, because 98% of the population is bacteria, 56% Proteobacteria, 52% Alphaproteobacteria, 51% Rhodospirillales, with 51% Acetobacteraceae- a scientific classification of AAB. The reason behind 40% of the population being unknown in all the classes assessed is unclear; however, the unknown population is clearly bacteria since 98% of the population is classified under the bacteria kingdom.

The blast output results (Fig. 5-2F), also showed that 50.84% are culturable *Acetobacter* species while 40.18% bacteria in the sample were identified as being VBNC. These results suggest that the assumptions initially made were reasonable, if 40.18% of the bacterial population is unculturable, then this provides an explanation to the fewer colonies observed on agar plates. Hypothetically, one might say, if 40.18% of the population is unculturable, then the rest of the culturable population should have been observed on agar plates; however, the concentration of the

culturable AAB might have been low, and their fastidious characteristics might have been of paramount influence. Overall, AAB consist of 26 genera, among these, *Acetobacter* being one of them. It is clear that AAB belonging to the *Acetobacter* species were present throughout the fermentation process, although, *Gluconobacter* and *Gluconacetobacter* species were also inoculated.

A study done on TBV detected the following AAB, *Gluconacetobacter europaeus* was dominant, with 25 different strains, *Gluconacetobacter xylinus* (1 strain), *Gluconacetobacter hansenii* (1 strain), *Acetobacter malorum* (7 strains), *Acetobacter aceti* (1 strains)and *Acetobacter pasteurianus* (2 strains) (De Vero *et al*., 2006 as quoted in Gullo & Giudici 2008). As indicated in the literature reviewed, Most AAB that are known and can be used commercially to produce vinegar are members of *Acetobacter*, *Gluconacetobacter* and *Gluconobacter* (Raspor & Goranovič, 2008). Overall, the study done by De Vero *et al*., (2006) showed the domination of *Gluconacetobacter* species while this investigation showed the dominance of the *Acetobacter* species.

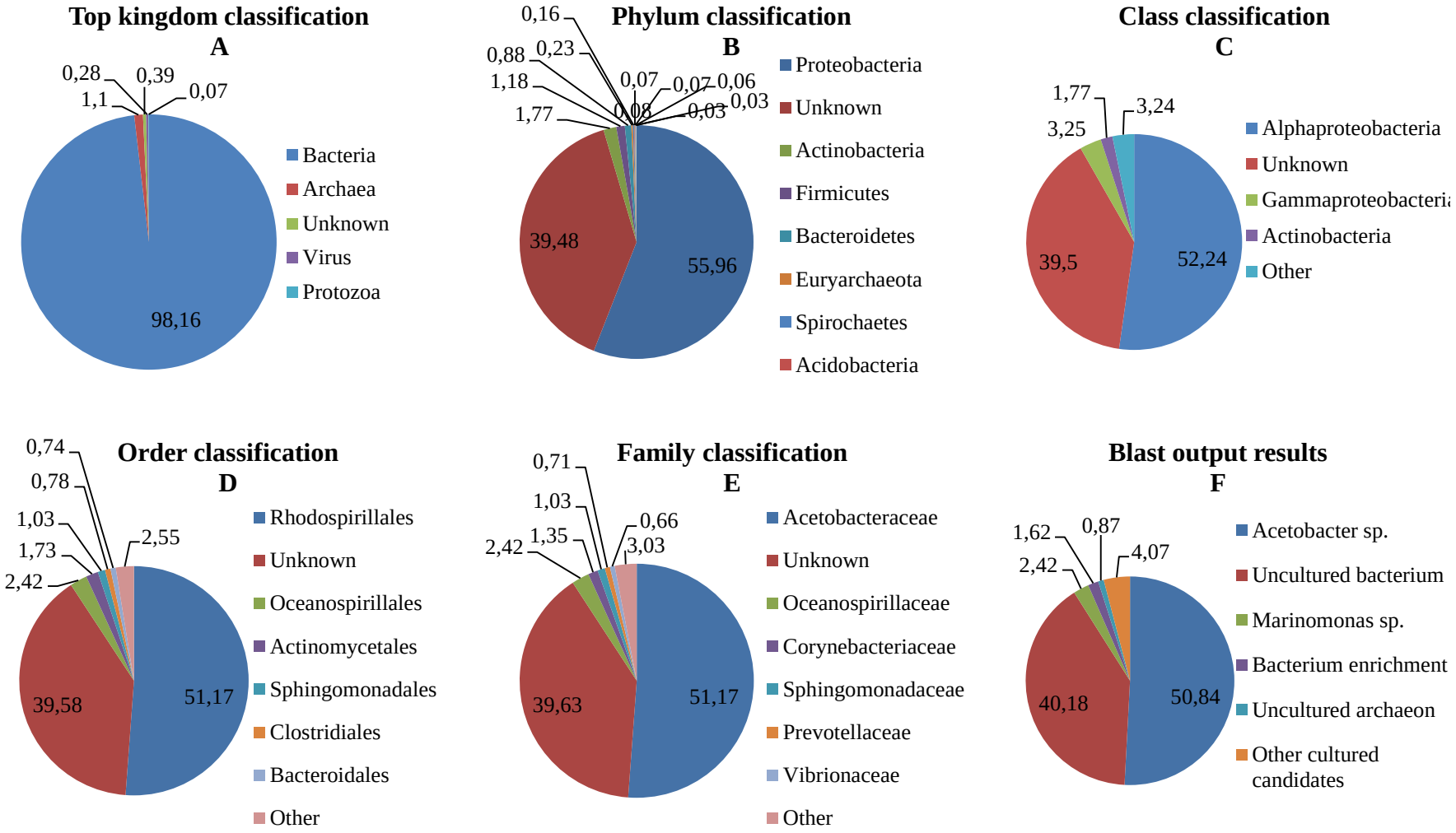


Figure 5-2: 16S analyses (bacterial population) of the 0% inoculation strategy- 28ºC product.

Chapter 6

General Discussion, Conclusions and Recommendations

Chapter 6

General discussion, conclusion and recommendations

6.1 General discussion

The primary aim of this investigation was to analyse a BSV production process biochemically in which non-*Saccharomyces* yeast and AAB were used for a multicultural EtOH-AcOH fermentation process. Based on the results obtained, it can be concluded that the primary aim of the study was achieved. The EtOH-AcOH fermentation process of balsamic vinegar is a complex process, which is influenced by a number of factors. Despite the popularity of balsamic vinegar and its growing economic importance, research such as in this study, has not been done. Many studies research problems that are quite similar, with research focusing on chemical development trends and the mathematical modelling of such a fermentation for process control purposes has not been done. This is understandable because, considering the *Batteria* system used and the ageing period required, the modelling of such a lengthy spontaneous process may not be useful for TBV. However, this research is necessary for consistency for BSV's fermentations that are carried out using the method investigated in this study.

Microbial growth profiles, chemical development trends, rates of reactions, including the modelling of the EtOH-AcOH fermentation process, were done. The use of cooked must (high-strength grape must) was deemed unsuccessful using the two inoculation strategies. Therefore, the results obtained were determined to be inconclusive in terms of the cooked must trials, as to whether the EtOH-AcOH fermentation should be carried out simultaneously or not. However, the 6% inoculation strategy was observed to perform better than the 0% inoculation strategy. Additionally, the alcoholic fermentation was more advantageous with appropriate outcomes being observed for both inoculation strategies. The failure of the acetous fermentation process using cooked must was explained using several hypotheses. These assumptions were: with the entry of the AAB into the VBNC state, the high sugar concentration was deemed to be an inhibiting factor to the microbial growth and activity of the AAB. Additionally, the alcoholic fermentation caused the medium to be anaerobic, a result that inhibited the activity of the AAB. Furthermore, it was

assured that such an environment facilitated the dominance of the yeast used, which, in turn, resulted in the medium being unsuitable for the AAB used.

The use of the autoclaved must (low-strength grape must) was successful and proved that the high sugar content in the cooked grape must have had a negative impact on the fermentation. In low-strength grape must, yeast microbial growth was observed from the initiation of the fermentation and up to the conclusion of the fermentation for the 0% and 6% inoculation strategy. AAB microbial growth was also observed from the start up to the conclusion of the fermentation for the 0% and for the 6% inoculation. AAB microbial growth was observed up to day 42 even though the process was concluded at 56 days. Although both inoculation strategies were successful in terms of chemical developments, a lower temperature (22°C) always showed higher yeast and bacteria concentration, a phenomenon which was similar for both inoculation strategies used. However, at 22°C lower, product formation was observed when compared to fermentations at 28°C which showed the highest product formation with lowest biomass concentration. This finding led to the conclusion that the product formation was non-growth dependant. To describe these results adequately, physical chemical reaction equations were used to model the process. The inclusion of microbial growth rates to model the process would have led to complications due to the non-growth dependence of product formation, which would have made the modelling/ simulation of the process unviable.

Modelling was only done for the autoclaved must. Modelling was successful for sugar consumption kinetics where the Eq. 2.17 was used and validated with R^2 values that ranged between 0.84 and 0.92. The 6% inoculation strategy trials gave lower R^2 values as the initial rates of reaction were used to describe the process. Therefore, the initial reaction rates from the 6% inoculation strategy trials were hypothetically determined not to describe the overall process adequately due to the inoculation of the AAB at the later stages of the fermentation, which can cause alterations on the reaction rates. However, the lower R^2 values obtained when modelling the 6% inoculation strategy trials were not essential as the 0% inoculation strategy was selected as the most adequate inoculation strategy. As for ethanol consumption/formation, modelling this intermediate product/substrate was challenging. However, the modification of appropriate models led to the successful description of the ethanol formation including consumption. Modelling for total acid consumption also required the modification of rate equations and the final equation used was Eq. 2.19. The model used resulted in R^2 values ranging between 0.98 and 0.99.

The sensory results showed an accomplished product. The results did not contradict or oppose the results obtained from the chemical developments. Based on the chemical developments data, the 0% inoculation strategy and 28°C temperature gave the shortest EtOH-AcOH fermentation period of only 38 days. Therefore, the 0% inoculation strategy-28°C product was analysed for population dynamics using a metagenomics approach. The yeast and bacteria population was analysed using 18S and 16S universal primers respectively. The 18S results showed that minimal yeasts were present at the end of the fermentation process. The results for the 16S analyses showed that 50.84% of the population is culturable *Acetobacter* species, while 40.18% of the population is unculturable bacteria. Overall, the results suggested that *Acetobacter* species carried out the oxidation process to the end.

6.2 Conclusion

A conclusion can be drawn on the basis of the fermentation length and sensory data on the autoclaved must trials only. Sensory data was the key response and would overrule all other output variables obtained from the process. The selected inoculation strategy was the 0% inoculation strategy, which resulted in a shorter fermentation cycle compared to the 6% inoculation strategy. With the use of the 0% inoculation strategy, yeast and AAB were observed to coexist with proportionality according to microbial growth-rate results. The 28°C temperature was observed to be more suitable on the basis of fermentation length and sensory data. Overall, end-products obtained from the 0% inoculation strategy using the 28°C temperature were scored the highest for sensory attributes. The models selected for the study were used successfully with the use of reaction rates. This will allow data simulation for future processes. Additionally, metagenomics results showed that minimal yeasts were present at the end of the fermentation. The bacteria population showed that 50.84% of *Acetobacter* species carried out the ethanol oxidation process up to the end while 40.18% were unknown unculturable bacteria.

6.3 Recommendations

- It is recommended that more AAB be isolated and their acetification capabilities be evaluated for use with high-strength (high-sugar) grape must. In other words, AAB with appropriate osmophilic characters need to be identified.
- A study needs to be done which looks at the effect of water activity and osmotic pressure on individual species constituting the microbial consortia to understand the susceptibility to high sugar concentrations further.
- The process developed for this study needs to be optimised using cooked must to evaluate a wide range of input variables such as pH, temperature, sugar concentration and water activity, in order to evaluate where the best response is attained.

Chapter 7

Literature Consulted

Chapter 7

Literature consulted

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Chapter 7

Appendices

Chapter 8

Appendices

8.1 Appendix A: Experimental/investigation photographs

8.1.1 A1: Boiling of Chenin blanc grape must

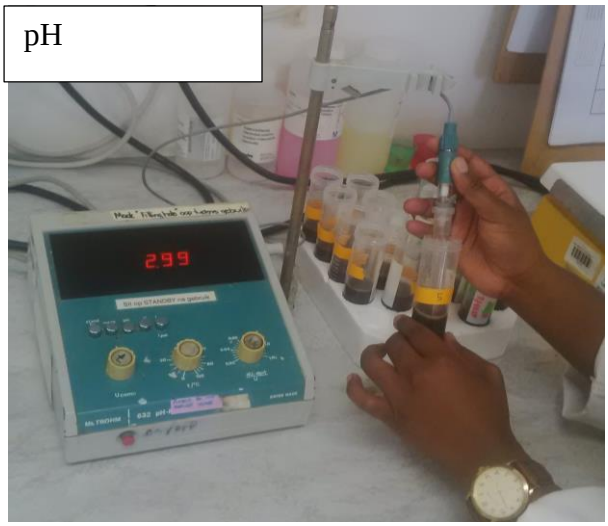
8.1.2 A2: Fermentation trials (Erlenmeyers with cotton wool stoppers)

8.1.3 A3: Sampling procedure under laminar flow

8.1.4 A4: Colony counts

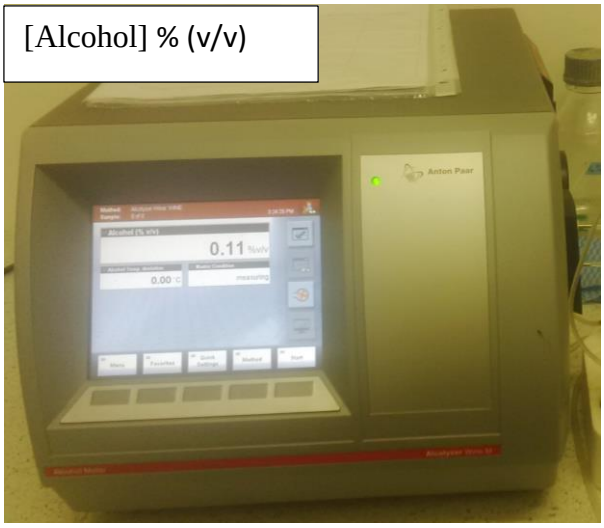
8.1.5 A5: Analytical chemistry methods

pH



[Sugar] °Brix

[Alcohol] % (v/v)



[Total acid] g/L

8.2 Appendix B: Ingredients for GM agar

Ingredients	Volume (g per 500 mL) / concentration	Concentration % (w/v)
Glucose	4	0.8
Mannitol	8.5	1.7
Peptone	1.5	0.3
Yeast extract	2.5	0.5
Agar	7	1.4

8.3 Appendix C: Diagram illustrating overall methodology for the study